ANTIBODY-CATALYZED FORMATION OF A 14-MEMBERED RING LACTONE

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ABSTRACT

A number of macrolide antibiotics have a 14-membered ring lactone skeleton. Besides the stereochemical challenges associated with the syntheses of these macrocyclic compounds, the difficulty in controlling the ring forming step has provided the basis for many synthetic organic methodology studies. Attempts to use enzymes in aqueous and organic solvents to catalyze medium to large ring lactonizations has met with limited success, particularly with secondary alcohols. We report monoclonal antibody F123, raised against transition state analogue **50**, which catalyzed the intramolecular transesterification of hydroxyester **57** to give the 14-membered ring lactone **19**. The reaction of antibody F123 with substrate **57** displayed enzyme-like

Michaelis-Menten kinetics. The kinetic parameters of this antibody reaction were determined by two methods, namely a multiwell method and the spectrophotometric cuvette method. Analysis of the reaction of F123 with **57** via the multiwell method yielded a K_m of $250 \pm 10 \mu\text{M}$, V_{max} of $0.62 \pm 0.01 \mu\text{mol/min mg}$ and a k_{cat} of 1.1 min^{-1} . The spectrophotometric cuvette method yielded a K_m of $330 \pm 50 \mu\text{M}$, V_{max} of $1.4 \pm 0.1 \mu\text{mol/min mg}$ and a k_{cat} of 2.2 min^{-1} . The results obtained from these two methods were found to be in good agreement once the delay times in determining initial rates, characteristic of the multiwell method, were accounted for. In both methods, the observed rates were

corrected for the background hydrolysis in buffer. Substrate specificity and competitive inhibition by the hapten derivative **60** ($K_i = 2.9 \pm 0.4 \mu\text{M}$) demonstrated that the catalytic activity was associated with binding in the antibody-combining site. Finally, the lactone product was isolated from pooled antibody-catalyzed reactions by ether extraction and identified using gas chromatography-mass spectroscopy by comparison with an authentic lactone sample.

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ABBREVIATIONS

Ac	acetyl
Anal	analysis
Bn	benzyl
bp.....	boiling point
br	broad
BSA	bovine serum albumin
Bu	butyl
cc.....	cubic centimeter
C region.....	constant region
Calcd	calculated
Cbz	(benzyloxy) carbonyl
CDR.....	complementarity determining regions
D region.....	diversity region
d.....	doublet
Da	Dalton
DCC.....	1,3-dicyclohexylcarbodiimide
DCI	desorption chemical ionization
DHP	3,4-dihydro-2H-pyran
DEAD.....	diethyl azodicarboxylate
DMAP	4,4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	dimethyl sulphoxide
ee.....	enantiomeric excess
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
Et.....	ethyl

FCS	fetal calf serum
GC	gas chromatography
H	proton
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectroscopy
<i>i</i> -Pr	isopropyl
Ig	immunoglobulin
IR	infrared
J	coupling constant
J region	joining region
k_{cat}	catalytic rate constant
KLH	keyhole limpet hemocyanin
K_m	Michaelis constant
LAH	lithium aluminium hydride
lit	literature
LRMS	low-resolution mass spectroscopy
m	multiplet
M	molar
M+	molecular ion
m/z	mass-to-charge ratio
MAb	monoclonal antibody
Me	methyl
Mes	4-morpholineethanesulfonic acid
MHz	megahertz
mp	melting point
n	normal
PNP	p-nitrophenol
p-TsOH	p-toluenesulfonic acid

s..... singlet

t..... triplet

Tris ..... tris(hydroxymethyl)aminomethane

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CHAPTER I. INTRODUCTION TO MACROLIDES

1.1 MACROLIDE ANTIBIOTICS

Macrolide antibiotics, a class of natural products largely isolated from microorganisms, have been characterized extensively with regard to their structure and function. Ever since the isolation and structure elucidation of this general class of naturally-occurring macrocyclic antibiotics starting in the 1950's, syntheses of these large ring compounds have posed a challenge to chemists. The first of these macrocyclic antibiotics to be isolated was pikromycin, in 1950.¹

Pikromycin

Woodward² originally suggested that the name "macrolide" apply only to those compounds containing a macrocyclic lactone ring such as pikromycin or erythromycin A. Today, however, that definition has been expanded to include a wider range of macrocyclic compounds, for instance, polyene macrolides such

Erythromycin A

FK-506

Amphotericin B

Cyclosporin A

Figure 1. Examples of some macrolides.

as amphotericin B, lactams such as FK-506, and cyclic peptides such as cyclosporin A (Figure 1). The production, isolation, and biological activity of the macrolide class of compounds has been reviewed elsewhere.³⁻¹²

Although the macrolides have been studied for several years, they still remain of interest to researchers for a number of reasons. Some macrolides have been shown to function as insect pheromones, while many others are clinically important. Erythromycin, for example, used in the treatment of Legionnaires' disease,^{13,14} is a commonly prescribed antibiotic.¹⁵⁻¹⁷ The macrolides FK-506 and cyclosporin A are immunosuppressants, while others have shown anticancer activity.¹⁸⁻²¹ And lastly, the complex structure and stereochemistry associated with these compounds presents a significant challenge to synthetic chemists. Since the introduction of penicillin as a medicine half a century ago, more than 80 antibiotics have been developed as human medicines, agrochemicals and veterinary medicines.²² The development of ampicillin in 1961 by chemical modification of penicillin lead to the evolution of semisynthetic antibiotics through the considerable efforts of organic chemists. Clearly, the stereochemical complexity of the macrocyclic compounds attracts the interest of synthetic chemists, however, their clinical importance necessitates a search for general, efficient methods for their preparation. Some reviews have been published which focus on the efforts towards the synthesis of these compounds.²³⁻²⁷

The majority of synthetic approaches used to build these macrocyclic compounds rely on first constructing a complex acyclic precursor and then cyclizing this precursor late in the synthesis. An alternative approach that has been the focus of our research group for some time is the macrocyclic conformational approach. In this route, the macrocyclic ring is formed early in the synthesis and the conformation of the ring is used to introduce the various substituents with regiochemical and stereochemical control. Efforts using this conformational approach have been published by our research group²⁸⁻³⁴ and others.³⁵⁻³⁸

1.2 LACTONIZATION METHODS

1.2.1 Chemical Methods

Lactone formation becomes significantly slower in going from 5- and 6-membered rings to large ring sizes (Table 1).³⁹ The difficulty in carrying out the ring-forming step has lead to the development of a number of methodologies to effect large ring lactonization.⁴⁰

The direct cyclization of ω -hydroxy acids in the presence of mineral or Lewis acids at high temperature and in dilute solution is not recommended because other functional groups present in the molecule often are destroyed under such conditions. Research in this area began to focus on methods to activate the

reacting groups of the open-chain ω -hydroxy acids. Activation of the carboxylic acid, involving such derivatives as active esters, mixed anhydrides or thiol esters, is a common cyclization strategy. Alternatively, one could enlist an approach which activates the hydroxyl end of the ω -hydroxy acid, for example using the Mitsunobu method.⁴¹⁻⁴³ The remainder of this section will detail examples of both approaches.

Table 1. Relative rate constants at 0°C for the following reaction (taken from ref 44);

Ring size	Relative rate
3	21.7
4	5.4×10^3
5	1.5×10^6
6	1.7×10^4
7	97.3
8	1.00
9	1.12
10	3.35
11	8.51
12	10.6
13	32.2
14	41.9
15	45.1
16	52.0
18	51.2
23	60.4

In 1974 Corey and Nicolau⁴⁵ reported a lactonization method which involved activation of the carboxyl moiety of ω -hydroxy acids using triphenylphosphine and 2,2'-dipyridyl disulfide in xylene at room temperature to form 2-pyridinethiol

esters (Scheme 1a). Without isolation, the slow addition of these activated intermediates to refluxing xylene to achieve high-dilution resulted in lactone formation. Mukaiyama et al.⁴⁶ followed with an activation methodology which involved slow addition of the ω -hydroxy acid to an excess of 2-chloro-1-methylpyridinium iodide and triethylamine in refluxing acetonitrile or dichloromethane to afford lactones. A third approach, reported by Yamaguchi

Scheme 1. Lactonization strategies which proceed through activated carboxyl intermediates; (a) Corey method (ref 45); (b) Mukaiyama method (ref 46); (c) Yamaguchi method (ref 47).

and co-workers in 1979,⁴⁷ involved the reaction of the ω -hydroxy acid in THF with one equivalent of 2,4,6-trichlorobenzoyl chloride. After removal of the triethylammonium chloride, the filtrate was diluted with a large volume of toluene and slowly added to a solution of refluxing toluene containing 3-6 equivalents of 4-dimethylaminopyridine to effect lactonization.

Lactonization methods of ω -hydroxy acids via activation at the hydroxyl end are less common. The Mitsunobu method is a simple one-pot approach which uses a mixture of triphenylphosphine (Ph_3P) and diethyl azodicarboxylate (DEAD) as dehydrating agent in the synthesis of lactones under mild, neutral conditions (Equation 1).⁴¹⁻⁴³ It has been suggested that the key intermediate of this method is the alkoxyphosphonium salt that undergoes nucleophilic displacement by the carboxylate anion with inversion of stereochemistry, releasing the very stable by-product triphenylphosphine oxide ($\text{Ph}_3\text{P}=\text{O}$). Smith et al.⁴⁸ demonstrated the use of the Mitsunobu protocol, in the total synthesis of the latrunculins (Equation 2), to effect a macrocyclization of compound **1** with inversion of configuration at the secondary hydroxyl position giving compound **2**.

These chemical approaches require that at least an equimolar ratio of reagent to hydroxy acid be used. A catalytic reagent would be of value. An added bonus would occur if the cyclizing reagent (or catalyst) was enantioselective.

1.2.2 Enzymatic Methods

Macrolactonization strategies involving the use of enzymes to effect stereoselective catalytic cyclizations have also been investigated. In 1984, Gatfield⁴⁹ was the first to report the intramolecular esterification of 15-hydroxypentadecanoic acid catalyzed by *Mucor miehei* lipase to obtain pentadecanolide. Yamada and co-workers⁵⁰ reported that *Pseudomonas* sp. and porcine pancreas lipases mediate macrolactonization of methyl esters of ω-hydroxy acids C₁₂-C₁₆, producing mono- and dilactones. Subsequently others have also reported the use of lipases in macrolactonizations.⁵¹⁻⁵³ For example, Guo et al. found the direction of the reaction and the yield of mono-, di-, and trilactones from 16-hydroxyhexadecanoic acid depended on the lipase used.⁵³

Attempts to use enzymes in aqueous and organic solvents to catalyze medium to large ring lactonization of secondary alcohols have met with only limited success.^{54,55}

1.2.3 Antibody Methods

Since the first successful reports by Lerner⁵⁶ and Schultz⁵⁷ on the use of antibodies to catalyze chemical transformations, a large number of antibody-catalyzed reactions have been reported (Table 3, Chapter 2). Two reports to date describe the use of catalytic antibodies to effect 5- and 6-membered ring lactone cyclizations.^{58,59} These two examples illustrate the successful use of antibodies to act as chiral catalysts in enantioselective lactonizations. The difficulties associated with the macrolactonization reaction mentioned earlier (section 1.2) pose a challenge in the syntheses of macrolide antibiotics. We believe the use of catalytic antibodies may provide a key for the formation of 12-16 membered ring lactones (macrolides), and in addition, may function enantioselectively and catalytically as in the small ring examples. Chapter 2 of this thesis will examine the general use of antibodies as catalysts in a range of organic transformations.

CHAPTER II. INTRODUCTION TO CATALYTIC ANTIBODIES

2.1 HISTORICAL ASPECTS

The vast repertoire of antibodies expressed by the immune system enables it to generate a response to virtually any foreign molecule. Scientists have been able to exploit this diversity, and use the immune response to study various chemical processes. The first reports on the use of antibodies to catalyze chemical transformations appeared in 1986,^{56,57} when two research groups independently published their results on acyl transfer reactions. However, the idea of harnessing the adaptive ability of the immune system to produce chemically useful catalysts was first eluded to by Linus Pauling in 1946.⁶⁰ It was, in fact, Pauling's recognition of the similarity of antibodies and enzymes with respect to complementarity and the nature of catalysis that is acknowledged as one of the most important contributions to the field of catalytic antibodies. Despite a lack of details about protein structure, Pauling concluded that enzymes were similar to antibodies in that they both are complementary to the species they bind. The fundamental difference between these two types of proteins is that an antibody binds a stable species (hapten), while an enzyme binds the activated complex of the substrate in an enzymatic reaction. Pauling also pointed out that non-covalent factors, such as strain and ground state destabilization, are important for enzymatic catalysis.

It was approximately 20 years later that Jencks wrote "if complementarity between the active site and the transition state contributes significantly to enzymatic catalysis, it should be possible to synthesize an enzyme by constructing such an active site. One way to do this is to prepare an antibody to a haptenic group which resembles the transition state of a given reaction. The combining site of such antibodies should be complementary to the transition state and should cause an acceleration by forcing bound substrates to resemble the transition state and should cause an acceleration by forcing bound substrates to resemble the transition state."⁶¹ The stabilization gained in the binding associated with the antibody combining site is illustrated in the free energy diagram below (Figure 2).

Figure 2. Free energy diagram of an antibody-catalyzed reaction compared to the corresponding uncatalyzed reaction.

The basis of this approach to the generation of catalytic antibodies requires a stable transition state analogue (a hapten) that is similar in size, shape, and charge distribution to the putative transition state of the reaction of interest. Furthermore, small molecules are generally not recognized by the immune system, and require coupling to a high molecular weight carrier protein before immunization, typically in mice or rabbits. Booster injections of the hapten-protein conjugate (antigen) are required at regular intervals, following the primary immune response to the initial immunization, until the antigen-antibody binding interaction has reached a plateau. That is to say, the primary immune response to the antigen is mounted by the animal's existing "naive" antibody repertoire, which upon repeated exposure to antigen, evolves through mutations to a more complimentary binding association between antigen and antibody. At this point, a blood sample is taken from the host animal and the antibodies are partially purified, and screened for binding and catalytic activity. Early attempts to test the transition state hypothesis were disappointing because the researchers used this polyclonal sera, i.e., a mixture of many antibodies. Later work using monoclonal antibodies,⁶² the development for which Köhler and Milstein⁶³ were awarded the Nobel prize in Physiology and Medicine in 1984, lead to the initial successful demonstrations of catalytic antibodies by the research groups of Lerner⁵⁶ and Schultz.⁵⁷

2.2 ANTIBODY STRUCTURE

Antibodies are large Y-shaped molecules (molecular weight ≈ 150 kDa) that belong to a group of proteins called immunoglobins. The antibody molecule consists of four polypeptide chains - two identical heavy chains (50 kDa each) and two identical light chains (25 kDa each) held together by covalent disulfide linkages between the two heavy chains and between the heavy and light chains (Figure 3). The light chains are composed of two distinct structural domains known as the variable (V_L) and constant (C_L) regions. The heavy chains contain

Figure 3. The schematic structure of immunoglobins (antibodies), (taken from ref 64).

four domains: V_H , C_{H1} , C_{H2} , and C_{H3} , each of which is composed of approximately 110 amino acids.

Enzymatic digestion of the antibody molecule with pepsin gives rise to three fragments; two of which are identical (Fab fragments) and contain the variable region where antigen binding occurs, the third fragment is readily crystallizable (Fc) and is responsible for immune regulation (i.e. effector functions). In the uncleaved antibody, the Fab and Fc fragments meet at a region called the hinge, which allows lateral and rotational movement of the molecule.

Antibodies are divided into five classes, IgG, IgM, IgA, IgE, and IgD. Each antibody class, or isotype, is distinguished by the type of heavy chain found in the molecule. The heavy chains of IgG molecules are known as γ -chains, IgMs have μ -chains, IgAs have α -chains, IgEs have ϵ -chains, and IgDs have δ -chains. The differences in their heavy chains allows each isotype to function in different immune responses, and at different stages of the developing immune response. As for the light chains, there are only two different types, κ and λ . Recall that in the Y-shape antibody molecule, the two light polypeptide chains each associate with one heavy chain (Figure 3). Although there exist five classes of heavy chains and two of light, each antibody molecule will have only one type of light chain and one type of heavy chain. Furthermore, different classes of antibodies may vary in the number of Y-like units that join to form the complete

immunoglobulin (Table 2). The IgG class along with the IgE and IgD antibodies are monomeric (only one Y unit), while the IgA class can form either monomeric, dimeric, or trimeric complexes, and finally, the IgM antibodies are pentameric (containing five Y units each). These Y units are joined at the base of the Fc regions via short polypeptide chains in the oligomers. IgM antibodies then, being pentamers, have 10 identical antigen binding sites, while monomeric IgG antibodies have two identical binding sites. In addition, the differences in the heavy chains between the antibody classes accounts for the different molecular weights of the various monomers. IgG monomers, for example, have a molecular weight of approximately 150 kDa, while IgM monomers are approximately 175 kDa.

Table 2. Characteristics of the various Antibody classes (taken from ref 65).

Characteristics	IgG	IgM	IgA	IgE	IgD
Heavy Chain	γ	μ	α	ϵ	δ
Light Chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Molecular Formula	$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$	$(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$	$(\alpha_2\kappa_2)_n^*$ or $(\alpha_2\lambda_2)_n$	$\epsilon_2\kappa_2$ or $\epsilon_2\lambda_2$	$\delta_2\kappa_2$ or $\delta_2\lambda_2$
Y Structure					
Valency	2	10	2, 4, or 6	2	2
Concentration in Serum	8–16 mg/ml	0.5–2 mg/ml	1–4 mg/ml	10–400 ng/ml	0–0.4 mg/ml
Function	Secondary response	Primary response	Protects mucous membranes	Protects against parasites (?)	?

*n = 1, 2, or 3.

The variable or Fab region of an antibody consists of one V_H and one V_L chain, to form one antigen binding site. The diversity of the variable region provides the basis for the large repertoire of binding sites that constitutes an effective immune response. However, the diversity within the variable region is limited to only those regions where contact with the antigen occurs, known as hypervariable regions. The light and heavy chains have three hypervariable regions each, and these form the majority of contact residues for the binding of the antigen to the antibody. Because they are the actual contact points with the antigen, they are referred to as the *complementarity determining regions* or CDRs. The nature of these structural details, including the secondary and tertiary structure of antibodies and the features of the binding site, have come from X-ray crystallographic studies of variable fragments, some of which contain the complexed antigen.^{66,67} In the case of small organic molecules, the binding occurs in clefts with volumes on the order of $600 \text{ \AA}^3$.⁶⁸ The clefts exclude water and antigen binding occurs via van der Waals forces, hydrophobic and electrostatic interactions, and hydrogen bonding, in the same manner as observed for substrate and inhibitor binding to enzymes.

2.3 THE IMMUNE SYSTEM / RESPONSE

The principal function of the immune system is to protect animals from foreign molecules and organisms. This highly efficient defense mechanism utilizes

proteins and cells which circulate throughout the body to perform this function. There are many different mechanisms which act in this system, all of which can be divided into two broad categories - nonadaptive and adaptive immunity.

Nonadaptive immunity is mediated by cells that respond nonspecifically to foreign molecules. This response does not improve with repeated exposure. Examples of nonadaptive immunity are phagocytosis by macrophages, secretion of lysozyme by lacrimal cells, and cell lysis by natural killer cells. Conversely, *adaptive immunity* is directed at specific molecules and is enhanced by repeated exposure. Adaptive immunity is mediated by cells called lymphocytes, which have two main functions, namely the synthesis of cell-surface receptors or secretion of proteins that bind specifically to foreign molecules. These secreted proteins are known as antibodies. The terms "antigen" and "immunogen" are used to describe different properties of molecules in the context of the immune system. An antigen is any molecule that will bind to an antibody, while an immunogen is defined as a molecule that induces an immune response. Furthermore, a "hapten" is a small molecule which by itself cannot act as an immunogen, but when coupled to a high molecular weight carrier protein, the complex is capable of eliciting an immune response.

The immune system contains more than 10^9 lymphocyte cells which travel throughout the blood and lymphatic system, enabling them to respond promptly

to foreign molecules. The lymphocytes are found in higher concentrations in the lymphoid organs, namely the lymph nodes and spleen. It is in these organs that the immunogens tend to accumulate, thus, becoming the focus of the immune response.

A number of different lymphocytes have been identified within the immune system. Three basic types of lymphocytes have been found to be associated with the cellular functions of the immune system, namely B cells, killer or cytotoxic T cells, and helper T cells (Figure 4). B lymphocytes, which originate in the bone marrow, secrete antibodies and carry a bound form of the same antibody on their cell surface, where it acts as a receptor for antigens. T lymphocytes, originate in the thymus, and carry out either a regulatory role (helper T cells) or an antigen attacking role (cytotoxic T cells) through their surface antigen receptors, known as T-cell receptors. While they share similar structural features, the surface antibodies on B cells and the T-cell receptors are encoded by separate gene families; and their expression is cell-type specific. In addition, the surface antibodies on B cells can bind to soluble antigens, while the T-cell receptors recognize antigens only when displayed on the surface of other cells. Once an antigen is bound to the surface antibody on a B cell, for instance, the helper T cells play an essential regulatory role in governing the subsequent response of B cells and cytotoxic T cells during an immune response. The molecular basis for the specificity of the immune response is controlled by a

simple mechanism - all the surface antigen receptors on a single lymphocyte cell, whether a B- or T-lymphocyte, are identical and recognize one and only one antigen.

Figure 4. Overview of the immune system.

While the immune system is challenged constantly by a large number of foreign molecules, it has the ability to distinguish these invaders from natural components of the body. By constantly eliminating lymphocytes that bind to and attack self molecules, an organism is said to have tolerance. Should this self-preserving mechanism, called clonal deletion, break down and the organism mounts a response against itself, a range of disorders known collectively as autoimmune diseases may result (e.g. rheumatoid arthritis, diabetes, Graves' disease). Clonal deletion occurs in the thymus during the development of the immune system by a poorly understood process. Another important feature of

the immune system is immunological memory. Upon first exposure to a foreign antigen, an animal's immune response is slow. This slow response may be insufficient to prevent disease if the antigen is pathogenic. On subsequent exposures to the same antigen, the response is stronger and more rapid due to this immunological memory. The memory is specific for each antigen and can last as long as the animal lives.

2.4 ANTIBODY PRODUCTION IN VIVO

Antibody molecules are produced by white blood cells called B cells (bone marrow derived cells committed to producing antibodies). With no prediction of the shapes of foreign molecules to be encountered during the lifetime of an organism, nature has evolved a "catch-all" defense mechanism which is strengthened by a positive feedback system. The production of naive antigen receptors occurs in the absence of antigen. A diverse repertoire of B cells each with a unique antigen receptor, more than 10^7 in number, is produced continuously and ready for interaction with antigen. Each individual B cell or colony of identical B cells ("clones") makes numerous antibody molecules. The naive repertoire is expressed on the surface of the B cells, with each individual cell expressing many molecules of a unique antibody. Initially, when an antigen or foreign material is encountered, it binds to a limited number of B cells which express an antibody with some affinity for that invader. These cells are

stimulated and divide. This involves a complicated series of events, and ultimately is responsible for the differentiation of B cells into plasma cells and memory cells. Plasma cells are short-lived cells found primarily in the lymphoid organs that specialize in secreting large amounts of antibodies. Memory cells, however, are long-lived cells that remain in circulation and do not secrete antibodies. Rather, memory cells retain cell-surface antibodies and are primed to respond to any subsequent exposure to the antigen.

The differentiation of B cells has two important processes associated with it; *affinity maturation*, a process that involves point mutations in the immunoglobulin (Ig) V genes followed by the selective proliferation of B cells expressing antibodies with increased affinity for antigen; and *class switching*, a process that involves rearrangement of the chromosomal DNA encoding for the heavy chain polypeptide.

Early in an immune response, division of B cells results in hypermutation of the antibody genes, leading to the production of many variants of the initial clones selected. The antigen in turn binds to and stimulates most effectively those clones having the highest affinity for the antigen. Affinity maturation is effectively a Darwinian process, involving variation resulting from mutations, followed by selection of cells driven by the requirement for high antigen-affinity

for cell survival. Rounds of mutation and selection thus act to single-out the highest affinity clones; this is the basis of affinity maturation.

After the affinity maturation process, class switching follows which leads to the production of antibodies with different specific functions. This process involves rearrangement of the chromosomal DNA encoding for the heavy chain polypeptide. The result of this heavy chain shuffling allows the same variable region to be expressed with different constant regions. It is the heavy chain that dictates which class (isotype) the antibody belongs to, and these different classes have different functions. During a typical immune response, class switching is responsible for the replacement of IgM antibodies, characteristic of the early immune response, by IgG antibodies. As well, because the affinity maturation has taken place, IgG molecules typically have higher affinities for the antigen than the short-lived IgMs.

The level of antibodies in the system peaks about 7-10 days after first contact with the antigen and the secreted antibodies enhance the rate of clearance of the foreign molecule. If no further antigen is present after this time, the number of plasma cells will decrease but the helper T-cells and memory cells remain in circulation. Upon re-exposure to the antigen, the secondary response will occur. This secondary response involves a similar sequence of events as in the primary response; however, there are some differences. Due to the high number of

helper T-cells and memory cells already in the system, the response is faster. Also, since the antibodies have already undergone affinity maturation, they have a higher affinity for the antigen making the response stronger. This immunological memory can remain with the animal for its entire lifetime, leaving the immune system primed for any further contact with the antigen.

2.5 DIVERSITY OF THE IMMUNE RESPONSE

The naive immune repertoire consists of some 10^7 - 10^9 different antibody molecules before any exposure to a foreign material or antigen. This large repertoire is expected to be able to recognize, albeit with moderate affinity, virtually any foreign material. The interpretation of the diversity of this extraordinary system has been the subject of several reviews.⁶⁹⁻⁷² This next section will look at the four main sources of this diversity, namely recombination, junctional diversity, mutation, and association of heavy and light chains.

2.5.1 Recombination

Recombination essentially involves the rearrangement of the germ-line DNA (the nonfunctional gene) of both the heavy and light chains to produce mature B-cell DNA (the functional gene). The coding sequences for the variable and constant regions of the germ-line DNA are separated by several hundred kilobase pairs, and as a result the gene is silent in these cells. During B cell differentiation,

these intervening regions are excised and the variable and constant regions are joined, producing a functional B cell gene. The diversity in the recombination step arises from the number of different associations possible between the variable and constant regions of the germ-line DNA. An example which illustrates this diversity involves cells synthesizing functional κ chains. In this case, there are approximately 200 different κ variable regions and only one constant region. During the recombination process, one of the variable regions is selected basically at random, and is joined to the constant region to produce a functional gene (Figure 5).

Figure 5. Kappa (κ) rearrangements in germ line and B-lymphocytes (taken from ref 65).

2.5.2 Junctional Diversity

It became clear however, after further studies of the fine structure of the recombination process between the variable and constant regions, that

recombination alone does not account for all the variation detected in the immune repertoire. A third segment, known as the J region for joining, was discovered to exist between the variable and constant regions. Recombination, then, brings the randomly selected variable region together with one of the four functional J regions making a V-J fragment, which on association with the constant region produces a functional κ gene. In addition, the recombination events between the variable and J regions do not always occur at exactly the same nucleotides, and this allows for further diversity in the V-J junction.

Figure 6. Heavy chain VDJ joining (taken from ref 65).

Heavy chains have, in addition to the variable, J, and constant regions, a fourth coding region referred to as the D, or diversity, region. Thus, two recombination

events are required for the production of a functional heavy chain gene, the first joins the D region to a J-constant region, and a second joins a variable region to the D region (Figure 6).

2.5.3 Mutation

Mutations arising from a complex process referred to as affinity maturation lead to fine-tuning of the binding site, thus creating a better fit for antigen-antibody interaction. These mutations occur during an immune response to a foreign molecule or antigen, and the mechanisms that produce these mutations are not known.

2.5.4 Association Of Heavy And Light Chains

The genetic events described above provide heterogeneity in both heavy and light chains. The complete antibody molecule then requires joining of a heavy and a light chain. Once recombination has lead to the generation of functional heavy and light chain genes, a poorly understood process, called allelic exclusion, prevents further recombination, fixing the antigen binding site for the life of the cell. This explains why B lymphocytes secrete antibodies with only one type of antigen binding site, and why antibodies have only one type of light chain. The type, or class, of heavy chain produced by a particular B lymphocyte is determined by the process of *class switching* described earlier. Because the

different classes and subclasses determine the functional roles of antibodies, class switching is an important mechanism for controlling where and how the antigen binding site will be used in the immune response.

2.6 ANTIBODY GENERATION

The method used to produce catalytic monoclonal antibodies is the same as that used to produce monoclonal antibodies used for other purposes.⁶³ The synthesis of the hapten is often the most difficult and time demanding part to the production of catalytic monoclonal antibodies. Once the appropriate hapten has been made, it is then coupled to a carrier protein such as keyhole limpet hemocyanin (KLH) for immunization. The coupling strategies used must be compatible with the hapten design, and produce a conjugate that is stable under physiological conditions. The hapten is coupled to the carrier protein via a spacer arm, which typically is on the order of 6 Å,⁷³⁻⁷⁶ so as to avoid any steric interference from the carrier. The spacer arm usually has a carboxy or an amine terminus to allow amide bond formation between the hapten and corresponding amino or carboxy groups on the surface of the carrier protein.

2.7 HYBRIDOMA TECHNOLOGY

The basis of the hybridoma technique involves combining the nuclei of normal antibody-producing cells with those of their malignant counterparts with retention

of both types of function (Figure 7). In this way, an immortal hybridoma cell line that secretes an antigen-specific monoclonal antibody is obtained. This monumental advancement in immunology made possible the isolation and

Figure 7. IMMUNE RESPONSE is initiated (a) when an antigen molecule carrying several antigenic determinants enters the body of an animal. The immune system responds: lines of B-lymphocytes proliferate, each secreting an immunoglobulin molecule that fits a single antigenic determinant (or part of it). Conventional antiserum contains a mixture of these antibodies. Monoclonal antibodies are derived by fusing lymphocytes from the mouse spleen with malignant myeloma cells (b). Individual hybrid cells are cloned, and each of the clones secretes a monoclonal antibody that specifically binds a single antigenic determinant on the antibody molecule (from ref 77).

production of large quantities of monoclonal antibodies that would specifically bind virtually any molecule.⁶³

2.8 SCREENING OF HYBRIDOMAS FOR HAPTEN BINDING

Following the hybridization of the mouse spleen cells using the hybridoma technology, the stable clones are screened for binding activity against the same hapten to which they were raised. Currently, because there is no general assay available for directly screening the library of hybridomas for catalytic activity, clones must be selected for further studies based on their ability to bind to hapten. The assumption is made that strong hapten binding translates to catalytic activity.

The two binding assays that will be discussed in this section are, first, the enzyme-linked immunosorbent assay (ELISA), and second, the particle concentration fluorescence immuno-assay (PCFIA).

2.8.1 The ELISA Assay

The ELISA assay is widely used to study hapten binding of catalytic antibodies. With this assay, the hapten molecule is coupled to a carrier protein different from the protein used for the immunization protocol. KLH is often chosen as the immunization carrier protein, while bovine serum albumin (BSA) is frequently

chosen as the conjugating protein for binding assays. This ensures that antibodies are selected for binding specifically to the hapten, rather than the

Figure 8. ELISA assay for screening mouse antibodies (figure provided by Gay Yuyitung).

carrier protein. In the ELISA assay (Figure 8), the BSA-hapten conjugate is affinity bound to the wells of a polystyrene microtiter plate via nonspecific interactions, and the excess conjugate is washed away (Step 1). Any free binding sites in the polystyrene wells are blocked with the protein albumin. In the next step, the antibody-containing serum or hybridoma supernatant of interest is incubated with the bound hapten-protein conjugate, and unbound portions are washed away (Step 2). An enzyme-linked antibody (or secondary antibody) that binds to immunoglobulin bound to the plate (i.e. binding to antibody having specific affinity for the hapten) is added, and after an appropriate incubation time unbound secondary antibody is then washed away (Step 3). A substrate is added to each well and it is subsequently processed by the enzyme coupled to the secondary antibody, producing a chromogenic product (*) that is detected and quantified spectroscopically (Step 4).

2.8.2 The PCFIA Assay

The PCFIA assay, commonly referred to as the Pandex binding assay, can be used in place of the traditional ELISA assay for the initial screening of hybridomas. Because this technique uses fluorescence detection, it is more sensitive than ELISA. The PCFIA assay (see Figure 9) uses hapten-protein conjugate-coated carboxyl polystyrene particles in 96-well filter assay plates, and a goat anti-mouse fluorescein isothiocyanate (FITC) secondary antibody for detection. The filter assay plates allow loading and incubating of buffered

solutions in the wells, followed by suction filtration and washing of excess reagents through the bottoms of the wells. Once the hapten-protein conjugate-coated carboxyl polystyrene particles (hapten-beads) are made, these are added to the 96-well filter assay plates (Step 1). The antibody-containing serum or hybridoma supernatant of interest is incubated with the hapten-beads, and unbound immunoglobulins are washed away (Step 2). Next, the fluorescent labelled secondary antibody is added and binds to antibodies recognizing the hapten. The excess FITC labelled secondary antibody is washed away prior to reading the wells for a fluorescent signal, indicating specific hapten binding (Step 3).

Once hapten-specific hybridomas are selected based on the binding assay results, large quantities of monoclonal antibodies can be obtained by expanding the cells in tissue culture and harvesting the antibody secreted into the supernatant, or more commonly, the cells can be propagated *in vivo* in other mice to produce tumors which secrete a high concentration of antibody (ascites fluid). The characterization of binding and reaction kinetics of the monoclonal antibody with various substrates requires large quantities of antibody.

Figure 9. PCFIA assay for screening mouse antibodies (figure provided by Gay Yuyitung).

2.9 ASCITES PRODUCTION

Ascites fluid is an intraperitoneal fluid that forms as a result of an intraperitoneal tumor produced by injection of a hybridoma cell line into the mouse. Hybridoma cells are injected intraperitoneally into a mouse. The cells grow to a high density

and thus produce a high titer solution of the monoclonal antibody, typically between 0.5 and 10.0 mg/mL.⁶⁵

2.10 ENZYMATIC KINETICS AND RELEVANCE TO SYNTHESIS

Enzymatic reactions can be broken down into two components: substrate binding and catalysis. Most enzymes are highly selective in binding their substrates, and particularly selective in binding the transition state of the reaction they catalyze, which explains the catalytic specificity of enzymes. In 1902, Brown⁷⁸ proposed that the substrate dependence of enzyme-catalyzed reaction rates could best be described qualitatively by Equation 3 below. In the absence of inhibition, the free enzyme (E) first complexes with the substrate (S) to form one distinct enzyme-substrate complex (ES), with a rate constant k_1 . Once formed, the ES complex, according to Equation 3, has two possible fates. It can dissociate to E and S, with a rate constant k_{-1} , or it can be converted to the free enzyme and product (P), with a rate constant k_2 .

This qualitative picture of enzyme reactions was subsequently put into a mathematical framework first by Henri⁷⁹ in 1903 and later by Michaelis and Menten⁸⁰ in 1913. The basic rate equation derived below is commonly referred to as the Michaelis-Menten equation, although, some writers have recently taken

to referring to the equation as the Henri-Michaelis-Menten equation. For the situation where the initial step in formation of the ES complex is not rate-limiting, a general expression that relates the rate of reaction (v) to the concentrations of substrate and enzyme and the rates of the individual steps can be derived, starting with the general rate expression,

$$v = \frac{d[P]}{dt} = k_2 [ES]. \quad (4)$$

From Equation 3, the net change in concentration of the ES complex with time ($d[ES]/dt$) can be expressed as follows:

$$\frac{d[ES]}{dt} = k_1 [E][S] - k_{-1} [ES] - k_2 [ES]. \quad (5)$$

Equation 3 can represent steady-state conditions. In a *steady state*, the concentrations of intermediates remain constant while the concentrations of starting materials and products are changing. Hence, the rate of formation of ES must equal its rate of consumption over most of the course of the reaction; that is,

$$\frac{d[ES]}{dt} = 0. \quad (6)$$

According to Equation 5, therefore,

$$k_1 [E][S] = (k_{-1} + k_2) [ES]. \quad (7)$$

By rearranging Equation 7,

$$[ES] = \frac{[E][S]}{(k_{-1} + k_2) / k_1} \quad (8)$$

Equation 8 can be simplified by combining the rate constants into a new constant, K_m , referred to as the Michaelis constant:

$$K_m = \frac{(k_{-1} + k_2)}{k_1} \quad (9)$$

and substituting it into Equation 8, which then becomes

$$[ES] = \frac{[E][S]}{K_m} \quad (10)$$

Therefore, K_m is a ratio of the rate constants for the breakdown of the ES complex, $k_{-1} + k_2$, over the rate constant for the formation of ES, k_1 . The magnitude of K_m is routinely used as a measure of the inverse relative affinity an enzyme has for a particular substrate. The significance of K_m will later be discussed in more detail.

Experimentally, substrate concentrations are typically very large compared to the enzyme concentration (e.g. mM in substrate and nM in enzyme). Under these conditions, the concentration of uncombined substrate, $[S]$, is essentially equal to the total substrate concentration. The concentration of uncombined enzyme, $[E]$, is equal to the total enzyme concentration, $[E_T]$, minus the concentration of the ES complex.

$$[E] = [E_T] - [ES] \quad (11)$$

By substituting this expression for $[E]$ in Equation 10, we get

$$[ES] = \frac{([E_T] - [ES])[S]}{K_m} \quad (12)$$

Solving Equation 12 for $[ES]$ now gives

$$[ES] = [E_T] \frac{[S]/K_m}{1 + [S]/K_m} \quad (13)$$

or

$$[ES] = \frac{[E_T][S]}{[S] + K_m} \quad (14)$$

On substituting this expression for $[ES]$ into Equation 4, the initial rate of the reaction can then be expressed in terms of the experimentally measurable quantities $[E_T]$ and $[S]$, as shown below in the Michaelis-Menten equation:

$$v = \frac{k_{cat} [E_T][S]}{[S] + K_m} \quad (15)$$

where a rate constant k_{cat} is commonly written as k_2 and often referred to as the catalytic constant of the enzyme. k_{cat} is also known as the turnover number of the enzyme because it is the number of reaction processes (turnovers) that each enzyme molecule catalyzes per unit time.

The effect of substrate concentration on the rate of the enzyme-catalyzed reaction is shown in Figure 10. At low substrate concentrations or high K_m 's, where $[S] \ll K_m$, the initial rate of the reaction is proportional to the substrate concentration, and Equation 15 simplifies to:

$$v = \frac{k_{\text{cat}} [E_T][S]}{K_m} \quad (16)$$

Conversely, at saturating substrate concentrations, where $[S] \gg K_m$ (so that $[S]/([S] + K_m)$ approaches 1), the initial rate of the reaction becomes independent of the substrate concentration, and approaches a constant maximum rate, V_{max} :

$$v = k_{\text{cat}} [E_T] = V_{\text{max}} \quad (17)$$

Therefore, the parameter K_m represents the concentration of substrate at which the initial rate of the reaction is equal to one-half of the maximal rate, i.e. $v = V_{\text{max}}/2$, as shown in Figure 10. In addition, from Equation 3, when $k_{-1} \gg k_2$, Equation 9 simplifies to give $K_m \approx k_{-1}/k_1$, and the value of K_m gives an inverse measure of the stability of the enzyme-substrate complex (ES). An enzyme with a low K_m has a high affinity for a substrate.

The Michaelis constant, K_m , and the maximal rate, V_{max} , can be readily determined from rates of reaction measured at different substrate concentrations for enzyme systems that operate under steady state conditions as in Equation 3.

Figure 10. The effect of substrate concentration on the rate of the enzyme-catalyzed reaction.

Traditionally, the values of K_m and $V_{\max}$ were obtained by transforming the Michaelis-Menten equation, Equation 15, into a straight line plot by substituting in the expression $k_{\text{cat}}[E_T] = V_{\max}$, then taking the reciprocal of both sides to give:

$$\frac{1}{v} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} \quad (18)$$

A plot of $1/v$ versus $1/[S]$, called a *Lineweaver-Burk plot*, yields a straight line with an intercept of $1/V_{\max}$ and a slope of $K_m/V_{\max}$ as shown in Figure 11. More commonly today, these values are determined by fitting the rate data to a weighted nonlinear regression of the Michaelis-Menten equation using a computer program, such as Grafit™.

Figure 11. A double-reciprocal plot, called a Lineweaver-Burk plot, of enzyme kinetics: $1/v$ is plotted as a function of $1/[S]$.

The quantity k_{cat}/K_m , often referred to as the enzyme's catalytic efficiency, is also referred to as the enzyme's specificity constant. When the substrate concentration is much greater than K_m , the rate of catalysis is equal to k_2 (or k_{cat}) which equals the turnover number, as described above in Equation 16. Thus, when $[S] \ll K_m$, the enzymatic rate depends on the value of k_2/K_m , or k_{cat}/K_m , and on $[S]$. It should be noted that this ratio depends on k_1 , k_{-1} , and k_2 , as can be shown by substituting for K_m :

$$\frac{k_{\text{cat}}}{K_m} = \frac{k_2 k_1}{(k_{-1} + k_2)} < k_1 \quad (19)$$

Therefore, the ultimate limit on the value of k_{cat}/K_m is set by k_1 , the rate of formation of the ES complex. This rate of formation of the ES complex cannot be faster than the diffusion-controlled encounter of an enzyme and its substrate. Hence, the upper limit on k_{cat}/K_m is between 10^8 and $10^9 \text{ M}^{-1} \text{ s}^{-1}$, the diffusion-controlled limit for small molecules with macromolecules such as enzymes.⁸¹

While knowledge of the kinetic constants for the enzymatic reaction used in a synthetic organic chemistry application is not required, obtaining and understanding the kinetic constants enables maximum efficiency of the use of the enzyme. For instance, if the substrate concentration is too low, a certain fraction of enzyme active sites are vacant at equilibrium, whereas, in some cases, if substrate concentrations are too high, the enzyme may be deactivated (as the character of the solvent becomes markedly different from that of water). The Michaelis constant K_m is therefore valuable in determining the minimum concentration of substrate which must be maintained in solution to achieve efficient use of the enzyme.

Without kinetic analyses of the enzyme system employed, often the catalytic activity of the enzyme observed under synthetic conditions is lower than the value obtainable under ideal (assay or $V_{\max}$) conditions. This discrepancy in rates may have several origins, a few being nonspecific deactivation of the enzyme by high or inappropriate concentrations of substrates, products, buffers, ions (pH), or co-solvents.

2.11 ENZYMES AND CATALYTIC ANTIBODIES IN ORGANIC SOLVENTS

Water is a poor solvent for the majority of reactions in organic chemistry because most organic compounds are insoluble in this medium. In addition, side-reactions such as hydrolysis, polymerization, racemization and decomposition are common in the presence of water. These limitations were avoided long ago by the use of organic solvents for nearly all organic chemical reactions. Conversely, biocatalysts have mainly been used in aqueous solutions since they generally display their highest catalytic power in water, and may be destroyed in organic solvents. However, this commonly held perception is far too general, and has likely impeded investigations into the utility of enzymes in nonaqueous media in the past. Review articles are becoming available dealing with the developing field of enzyme-catalyzed reactions in organic solvents.⁸² While the optimal amount of exogenous water to be added to the organic solvent

is enzyme dependent, there is a general consensus that some water is required for the catalytic function of the enzyme.⁸³

Although activities are generally lower in an organic environment, many other advantages can be accrued by using biocatalysts in organic solvents. Some advantages of biocatalysts over chemical catalysts include: enzymes are very efficient catalysts, typically employed in a mole fraction of 10^{-3} - 10^{-4} % whereas most chemical catalysts are generally used in a mole fraction of 0.1-1%; enzymes are environmentally acceptable unlike heavy metals often employed in chemical catalysts; enzymes act under mild conditions; enzymes can catalyze a broad range of chemical transformations; and, enzymes often exhibit a high substrate tolerance by accepting a variety of unnatural substances and often they are not limited to a purely aqueous environment.

Both enzymes and catalytic antibodies have been successfully used in organic solvents under a variety of conditions, including: (i) the enzyme^{84,85} or antibody⁸⁶⁻⁹¹ dissolved in a *monophasic aqueous-organic solution* consisting largely of water and a water-miscible organic co-solvent (such as dimethyl sulfoxide, dimethyl formamide, acetonitrile) or surfactant (such as Triton X-100) to facilitate substrate solubility; (ii) the enzyme^{92,93} or antibody⁹⁴ dissolved in a *biphasic aqueous-organic solution* consisting of two macroscopic phases; (iii) the enzyme^{95,96} or antibody⁹⁷ suspended in a *monophasic organic solution* (with < 2%

water content); (iv) the enzyme⁹⁸⁻¹⁰³ or antibody¹⁰⁴⁻¹⁰⁶ immobilized onto solid supports or in reverse micelles; and, (v) at least one example of a lipid-coated antibody in water-miscible organic solvents.¹⁰⁷

Such successful demonstrations of biocatalysts in organic solvents continue to increase, providing organic chemists with alternate and often unique solutions to challenging synthetic transformations.

2.12 DESIGN OF TRANSITION STATE ANALOGUES

The approaches used to generate catalytic antibodies have been based largely on principles of enzymatic catalysis such as transition state stabilization,¹⁰⁸ general acid-base catalysis, nucleophilic and electrophilic catalysis, as well as strain and proximity effects.^{61,109} Clearly, the success of catalytic antibodies has and will continue to depend on the clever design of haptens or transition state analogues. This is the fundamental requirement when one sets out to study catalytic antibodies. A number of functional groups, some examples being sulphones, phosphonates and phosphoramidates, quaternary ammonium ions, 1,3-diketones and secondary alcohols, have proved successful in mimicking the reactive centers in a transition state. Phosphonates have been most widely used, undoubtedly due to their proven utility as transition state inhibitors¹⁰⁸ for a variety of enzymes including a number of proteases and esterases. The dipole

of the P=O bond of phosphonate analogues reflects the developing negative charge on the carbonyl oxygen in the transition state of transesterification reactions (figure 12). In addition, antibodies complementary to the tetrahedral phosphonate haptens should have considerably lower affinity for the trigonal products, thereby reducing product inhibition. The efficiency of enzymes is the result of employing several of the above mentioned catalytic mechanisms simultaneously, to achieve large rate enhancements. In order to realize similar rate accelerations with antibodies, it may require the simultaneous application of more than one of these strategies for introducing catalytic activity into antibodies as well. There have been a few reports of hydrolytic antibodies that appear to utilize active site acid-base chemistry in addition to the expected transition state stabilization.^{86,87,110,111}

The great diversity offered by the immune system, coupled with well designed transition state analogues, has allowed chemists to exploit the immune system to study the antibody catalysis of a number of diverse reactions.

2.13 REACTION TYPES CATALYZED BY ANTIBODIES

The ability of enzymes to use binding energies to orient a substrate into a reactive conformation or to bring two molecules together in the proper orientation

Figure 12. The putative transition state and the corresponding phosphonate transition state analogue for the displacement reaction of an ester.

for a reaction to occur represents an effective way to compensate for the entropy losses that may accompany such a chemical transformation. Mechanistic investigations of antibody-catalyzed reactions involving structural modifications to substrates and X-ray crystal structure studies have shown that antibodies utilize binding energies analogously to enzymes to effect chemical catalysis.

While all antibodies share a common structural motif, the shape of the binding site can vary extensively, from deep pockets, clefts and grooves to flatter surfaces, complimenting the antigen against which it was raised. This versatility, essential for the ability of these antibodies to maintain surveillance against foreign molecules *in vivo*, also accounts for the ability of these antibodies to distinguish subtle changes in substrate molecules, and is responsible for their

ability to catalyze different types of reactions. The field of catalytic antibodies has been extensively reviewed elsewhere, and since the first successful reports, has flourished to encompass an impressive array of antibody-catalyzed reactions. Table 3 below outlines many of these antibody-catalyzed reactions.

Table 3. Reactions Catalyzed by Antibodies.

Reaction Type	Reference
Ester, carbonate hydrolysis	56,57
Lactonization of γ,δ -unsaturated acids	58
Enantioselective lactonization	59
Amide bond formation/hydrolysis	112-114
Redox	115,116
β -Elimination	117
Porphyrin-mediated metal chelation	118
Protecting group removal	119,120
Transesterification	86
Enantiofacial protonation	121
Cis-trans isomerization of alkenes	122
Glycosidic bond hydrolysis	123
Stereoelectronically disfavoured cyclization	88
Diels-Alder reactions	124-126
Regio- and enantioselective reduction	127
Michael addition	128
Aldol condensation	129-131

Two successful antibody-catalyzed reactions listed above, and a third attempt, not in the above list, are of particular relevance to this project and will be discussed in detail. They are:

- (1) Lactonization of γ,δ -unsaturated acids;
- (2) Enantioselective lactonization;
- (3) Intramolecular acyl migration.

2.13.1 Lactonization of γ,δ -unsaturated acids

In 1995 Kitazume and Takeda described the use of antibodies to catalyze the cyclization of unsaturated carboxylic acids to 5- and 6-membered ring lactones.⁵⁸ The authors employed hapten-conjugates **3** and **4**, cyclic ethers bearing a sulfoxide group, to elicit antibodies to catalyze the lactonization of the γ,δ -unsaturated acid **5**. Two diastereomers of the KLH-hapten conjugate **3** (the *cis* and *trans* isomers; referring to the relative stereochemistry of the trifluoromethyl and sulfoxide containing side chains) were used to immunize BALB/c mice.

The antibodies induced from hapten **3**, derived from the *trans*- γ -lactone, acted to promote exclusively exocyclic ring closure of the γ,δ -unsaturated acid **5** to the *trans*- γ -lactone **6** with a k_{cat} of 0.86 min^{-1} , a K_m of $190 \text{ }\mu\text{M}$, and a selectivity of $> 97\%$ de (58% yield). The antibodies raised against the *cis* isomer of hapten **3**

catalyzed the formation of the cis- γ -lactone isomer of **6**, from reaction with the same substrate **5**, with a k_{cat} of 0.64 min^{-1} , a K_m of $170 \text{ }\mu\text{M}$, and a selectivity of $> 96\%$ de (61% yield). In addition, antibodies were elicited to the trans KLH-hapten conjugate **4**, and were found to promote cyclization of **5** to form the δ -lactone **7** with a k_{cat} of 0.81 min^{-1} , a K_m of $180 \text{ }\mu\text{M}$, and a selectivity of $> 94\%$ ee (δ -lactone **7** : γ -lactone **6** = 93:7), and the antibody induced from the cis isomer of **4** acted to form the δ -lactone **7** (δ -lactone : γ -lactone = 94:6) with a comparable enantioselectivity.

2.13.2 Enantioselective lactonization

In 1987, Napper, *et al.* reported the enantioselective lactonization of the corresponding racemic 5-hydroxy ester **8** to lactone **9** by a catalytic monoclonal antibody.⁵⁹ Antibodies were raised against the cyclic phosphonate hapten **10**.

This hapten is a transition state mimic for the six-membered ring cyclization. Lactonization of **8** was catalyzed until 50% of the initially added substrate was consumed. Upon addition of a second aliquot of substrate, the antibody again converted 50% of the freshly added substrate. Although the hapten and the substrate were racemic, the monoclonal antibody, being chiral, showed selectivity for the (R)-(-)-enantiomer of **8**. The monoclonal antibody (MAB) displayed enzyme-like Michaelis-Menten kinetics with a k_{cat} of 0.5 min^{-1} , a K_m of $76 \text{ }\mu\text{M}$ and a $k_{\text{cat}}/k_{\text{uncat}}$ of 167, which afforded the 6-membered ring lactone **9** in an enantiomeric excess of 94 percent.

2.13.3 Intramolecular acyl migration

Antibodies were raised against the cyclic phosphonate **13** in an attempt to obtain antibodies capable of catalyzing an intramolecular acyl migration reaction.¹³²

Antibodies specific for phosphonate **13** were expected to bind substrate **11** with the hydroxyl group in close proximity to the carbonate, resulting in a rapid acyl migration to give **12**. Monoclonal antibodies raised against hapten **13** did not catalyze the desired acyl migration of **11** to **12**, but simply hydrolyzed the substrate, giving **14**, CO₂, and ROH. The author speculated that the hapten-protein conjugate **15** may have hydrolyzed during the course of the

immunization, yielding **16**, a hapten now resembling the phosphonic acid transition state analogues employed to obtain antibodies which catalyze hydrolytic reactions.

If hydrolysis of the hapten did occur during the course of immunization, endocyclic cleavage would have had to occur (as illustrated in *path a* above) for the hapten to remain intact with the carrier protein, and lead to the production of

hydrolytic antibodies. Exocyclic cleavage would have resulted in the hapten molecule being cleaved from the carrier protein, and such small molecules alone usually do not induce an immune response.

The antibody-catalyzed lactonization by Napper, *et al.*, mentioned above, provided the impetus for our investigation of catalytic antibodies in the synthesis of macrocyclic lactones. As outlined in Chapter 1, investigators have searched for a general high-yielding method to prepare these macrocyclic compounds. An antibody capable of catalyzing such a macrolactonization step, with high enantioselectivity, would be a welcome and interesting achievement in the field of macrolide chemistry.

CHAPTER III. RESULTS AND DISCUSSION

3.1 SYNTHESIS OF MACROCYCLIC PHOSPHONATE 20

Based on the success by Napper et al.,⁵⁹ our initial goal towards the generation of a catalytic antibody for macrocyclic lactonization was the synthesis of the macrocyclic phosphonate hapten **20**. Phosphonate **20** is a stable analogue of the putative transition state for the lactonization of hydroxy ester **18** to macrolactone **19**.

The synthesis of compound **20** was intended as an introductory study for the investigation of the stability and spectral characteristics of macrocyclic phosphonates. Earlier attempts in our laboratory toward the synthesis of the

macrocyclic methyl phosphonate **22** from **21** via a direct displacement reaction (Scheme 2) carried out under high dilution conditions resulted in low and irreproducible yields of the desired product.¹³³ To our knowledge, this was the first synthesis of such a macrocyclic phosphonate. However, this is not a practical method to prepare large quantities of these compounds, therefore we turned our attention to an alternate approach.

Scheme 2. Synthesis of macrocyclic phosphonate **22**.

In this alternate approach, the final steps in the synthesis of phosphonate **20** were envisioned to proceed via an intramolecular Wittig reaction using the acyclic precursor **23**, followed by hydrogenation of the vinyl phosphonate.

Carbon-carbon bond formation can be carried out under near-neutral conditions using a stabilized Wittig reagent. This methodology was successfully used by Wilson¹³⁴ to yield the 12-membered ring α,β -unsaturated ketone **25** from **24**.

Other reports^{135,136} demonstrate intramolecular cyclization reactions of aldehyde phosphonates for the construction of macrocyclic α,β -unsaturated lactones of 13 and more carbon atoms with varying degrees of substitution. The 14-membered α,β -unsaturated lactone **27** was obtained by the intramolecular condensation of the phosphonate **26** in a 40-50% yield. Such examples from the literature suggested that our strategy for the synthesis of compound **20** was reasonable.

Before embarking on a synthesis of the cyclic phosphonate **20**, however, we decided to carry out a model study using phosphonate **30**. Direct displacement of the iodide of compound **28** with triphenylphosphine in an attempt to produce intermediate **29** was not successful. A report by Moffatt and coworkers¹³⁷

detailed the synthesis of a diphenylphosphonate analogue of the Wittig reagent **30**, and in the same report, discussed the difficulties associated with the displacement route from **28** to **29**. Using the Moffatt method, the stabilized Wittig reagent **32** was synthesized in good yield. Commercially available chloromethylphosphonic dichloride was reacted with 2 equivalents of phenol to produce **31** in 90% yield. Subsequently, quaternization of triphenylphosphine with **31** at 175 °C and deprotonation lead to **32** in 77% yield.

Aldehyde **33** was also prepared (Scheme 6) for subsequent conversion to compound **34**, which would be followed by coupling with **32** to produce the stabilized Wittig **35** as in Scheme 3 below. However, with **33** and **32** in hand, a Wittig coupling of these two compounds was carried out, and the vinyl phosphonates **36a** and **36b** were produced in a moderate yield of 52% with a

Scheme 3. Proposed synthetic sequence for compound **35**.

4:1 ratio of trans to cis isomers, respectively, based on the integration of the 400 MHz ^1H NMR of the crude mixture.

This synthesis of **36** inspired an alternate route towards the synthesis of macrocyclic phosphonate **20**. With compound **36** in hand, all that remained was hydrogenation of the double bond followed by cleavage of the THP ether giving rise to **37**, and finally, a viable means of condensing the phosphonate moiety with the secondary hydroxyl of **37** to give **38**.

Furthermore, we anticipated that the intended model compound **38** could be further modified at the phosphonate center with a linker molecule to enable

coupling to a carrier protein, and the resulting conjugate **39** might be used as an antigen for the production of a lactonizing catalytic antibody. One concern we had regarding the use of such a conjugate was the hydrolysis problem encountered by Jacobs with a similar 7-membered ring phosphonate mentioned in Chapter 1.¹³² However, we felt our 14-membered ring hapten would not undergo hydrolysis as readily as the 7-membered ring phosphonate employed by Jacobs, since 14-membered ring macrocycles in general possess less ring strain than their 7-membered ring counterparts.

To achieve cyclization of the hydroxy phosphonate ester **37** to give **38**, we turned our attention to the Mitsunobu reaction. Over the past two decades, the Mitsunobu reaction has found extensive use in organic synthesis, particularly for the inversion of alcohols via an esterification/hydrolysis procedure.⁴² The

Mitsunobu reaction has also been proven a mild coupling reaction for the preparation of mixed phosphonates from phosphonic acid monoesters and primary or secondary alcohols (Equation 3).¹³⁸ It has been used with carboxylic

acids, phenols, phosphates, and phosphonic acids as the nucleophilic component. Thus we hoped the Mitsunobu reaction would prove successful in the transformation of the monophosphonic acid of **37** into the cyclic phosphonate **38**. The precedent by Smith et al. in employing the Mitsunobu protocol in the key lactone forming step in the total synthesis of the latrunculins (14-membered ring lactone compounds; Equation 2)⁴⁸ suggested that our strategy was reasonable. A proposed mechanism for the Mitsunobu reaction of benzoic acid and a secondary alcohol¹³⁹ is shown in Scheme 4 below.

While a variety of acids have been employed in the Mitsunobu reaction, benzoic acid has been the most widely used. Subsequently, it has been found that the strength of the acid used has an effect on the relative reactivity of the phosphonium adduct. Thus, for a successful Mitsunobu esterification to occur, the alcohol must react faster (path A) than the carboxylate (path B) with the phosphonium adduct as shown in Scheme 5. If the conjugate acid of the

Scheme 4. A proposed mechanism for the Mitsunobu reaction (from ref 139).

carboxylate is a relatively strong acid, then the carboxylate will be stable and a poor nucleophile, and path A is favoured. If, however, the conjugate acid is a weak acid, its conjugate base may be sufficiently nucleophilic to compete with the alcohol, resulting in path B being favoured and thus lowering the yield of the desired product.

Scheme 5. Possible pathways in the Mitsunobu reaction (from ref 139).

With the new synthetic strategy involving an intramolecular Mitsunobu reaction decided, the synthesis of phosphonate **38** (Scheme 6) was attempted, beginning with the Grignard reaction of commercially available undecylenic aldehyde and methylmagnesium bromide to give the hydroxy alkene **40** in 90% yield. The hydroxyl group of **40** was protected as the THP ether **41** in 84% yield. This addition of the THP group created a second chiral centre in **41**, and therefore, two diastereomers of **41** were isolated. The two diastereomers were obtained in a 1:1 ratio as seen in the ^1H NMR spectrum of **41**, which reveals two methyl doublets of equal intensity and two methine multiplets, corresponding to the methine proton of the THP group, also of equal intensity. The two diastereomers of **41** were not separated, but treated together in the following sequences. Hydroboration of **41** followed by oxidation gave **42** in 96% yield. The alcohol **42** was subsequently oxidized under the Swern conditions to give the aldehyde **33** in 87% yield. Aldehyde **33** was then reacted with the stabilized Wittig reagent **32** in refluxing toluene to give the vinyl phosphonate **36** in a moderate yield of 52% with a 4:1 ratio of trans to cis isomers based on the integration of the 400 MHz ^1H NMR of the crude mixture. The alkene **36** was then reduced using H_2 and 10% Pd/C in EtOAc to give the saturated diphenyl phosphonate **43** in 99% yield. Phosphonate **43** was subsequently reacted with p-toluenesulphonic acid to liberate the secondary hydroxyl of compound **37** in 86% yield. Removal of the THP group from **43** eliminated one of the two chiral centres in the molecule, reflected in the ^1H NMR spectrum of **37** which contains only one methyl doublet.

The hydrolysis of a single "OR" group of a symmetric phosphonate such as **37** is readily achieved under refluxing aqueous sodium or potassium hydroxide, while the hydrolysis of both "OR" groups can be achieved in refluxing hydrochloric acid.¹⁴⁰ Therefore, phosphonic acid monoester **44** was produced in 76% yield by the base hydrolysis of **37**.

The key step in the synthesis of the macrocyclic hapten phosphonate **38** was the cyclization of **44** to give the 14-membered ring phosphonate **38**. The hydroxy phosphonic acid monoester **44** was reacted with DEAD and triphenylphosphine in benzene under high dilution conditions to produce the cyclic phosphonates **38a** and **38b** in an 82% yield and a 5:1 ratio of diastereomers based on the ¹H NMR of the crude. For each pair of 14-membered ring phosphonate diastereomers referred to in this thesis, the distinction between isomer 'a' and isomer 'b' of a pair of diastereomers was made based on their relative R_F-values from thin-layer chromatography (TLC) performed on Merck silica gel F₂₅₄ pre-coated aluminium sheets. With each pair of 14-membered ring phosphonate diastereomers studied, the 'a' isomer has a higher R_F value (i.e. travelled faster on the TLC plate) than its corresponding 'b' diastereomer.

Scheme 6. Synthesis of macrocyclic phosphonates **38a** and **38b**.

In addition to the two 14-membered ring diastereomers **38a** and **38b**, a cyclic dimer side product **39** was isolated as rod-shaped crystals on purification of **38a**

Figure 13. Stereoview of an ORTEP representation of dimeric cyclic phosphonate, **39**, showing 33% probability ellipsoids. Hydrogen atoms have been given arbitrary thermal parameters for clarity.

and **38b**. The structure of dimer **39** was determined by single-crystal X-ray diffraction (Figure 13). Dimer **39**, a centrosymmetric 28-membered ring

compound with two parallel chains bridged at both ends by the phosphonate group, contains four stereogenic centres with R^*R^* stereochemistry on one bridge, and the mirror image S^*S^* stereochemistry at the opposite bridge. Dale has reported that cycloalkanes above twenty carbons have the collapsed shape of two parallel straight chains bridged at both ends¹⁴¹ as in dimer **39**. This collapsed shape is likely a result of a sufficiently large interior "hole" which allows for stabilizing hydrophobic interactions across the ring. Groth reported the X-ray structure of cyclohexaicosane, which was also found to have a centre of symmetry.¹⁴² Dimer **39** has a centre of symmetry as a result of the R^*R^* and S^*S^* stereochemistry at the bridge ends.

In an attempt to explain why dimer **39** has the R^*R^* relative stereochemistry, rather than S^*R^* , molecular models of the transition states leading to the R^*R^* and S^*R^* products were examined. The transition state leading to the R^*R^* stereochemistry, illustrated by **A** below, proceeds with lower steric interactions than transition state **B** which would result in the S^*R^* stereochemistry. In **B** there is a steric interaction between the methyl group on the secondary alcohol and the phosphonate oxygen that is not found in model **A**. If the **A** transition state leading to the R^*R^* stereochemistry is extended to the monomers **38a** and **38b**, it is reasonable to suggest that the major monomer **38a** might also exhibit the R^*R^* relative stereochemistry.

The bimolecular condensation of two molecules of **44** via the Mitsunobu methodology resulted in the R^*R^* stereochemistry, and it follows that closure of the opposite end of the acyclic dimer would be governed by the same steric control, resulting in either the SS or RR stereochemistry for the second phosphonate bridge. Then the question to answer is why the only dimer molecule isolated on cyclization of **44** was the $\text{R}^*\text{R}^*\text{S}^*\text{S}^*$ dimer **39**? Molecular models of the $\text{R}^*\text{R}^*\text{S}^*\text{S}^*$ and $\text{R}^*\text{R}^*\text{R}^*\text{R}^*$ diastereomers of dimer **39** were constructed. The $\text{R}^*\text{R}^*\text{S}^*\text{S}^*$ model was constructed according to the X-ray structure obtained for dimer **39**. The $\text{R}^*\text{R}^*\text{R}^*\text{R}^*$ model was constructed from the same conformation as **39**, where the second R^*R^* bridge was created by

inverting the stereochemistry at the S*S* bridge. This R*R*R*R* model retained one R*R* bridge common to **39**, where the aryloxy moiety attached to the phosphorus atom occupies an equatorial position, projecting away from the ring while the doubly-bonded oxygen atom projects into the ring (as in Figure 13). However, the R*R* stereochemistry at the opposite bridge of the R*R*R*R* model forces the methyl and aryloxy moieties to project in toward the ring, as illustrated below. MACROMODEL calculations¹⁴³ on the R*R*S*S* and R*R*R*R* dimers using the MM2 force field¹⁴⁴ gave a lower energy conformation for the R*R*S*S* dimer, 92.40 kJ/mol, compared to 109.23 kJ/mol for the R*R*R*R* dimer. These calculations suggest that the R*R*S*S* dimer is of lower energy, and may provide a rationale for why only this dimer was isolated on cyclization of compound **44**.

In the ¹H NMR spectrum of the macrocyclic phosphonates, we observed a downfield shift of the C-13 methine signal relative to that of the open-chain

precursor **44**. In the synthesis of simple macrocyclic lactones, we also used the downfield shift of this methine proton in the cyclized product relative to the methine signal of the open chain hydroxy acid precursor to establish whether cyclization has taken place. Similarly, for the cyclic phosphonates, the electron withdrawing nature of the phosphonate moiety deshields the methine proton in the cyclic compound relative to the open chain precursor (Table 4), giving rise to the observed downfield shift. In addition, the C-1 methyl signal of the cyclic phosphonates **38a** and **38b** shifts downfield in the ^1H NMR spectrum as is observed for the 13-tetradecanolide lactones.

Table 4. Comparison of the ^1H NMR chemical shift data for the methine and methyl signals of the open chain hydroxy acid **44** with those of the macrocyclic phosphonates **38a** and **38b**.

Compound	Methine multiplet (ppm)	Methyl doublet (ppm)
44	3.78	1.17
38a	4.75	1.22
38b	4.65	1.43

3.2 ANTIBODY PRODUCTION

3.2.1 Preparation of Antigen KLH-50

As stated earlier in Section 2.3, small hapten molecules must be coupled to a carrier protein prior to immunization, in order to elicit an immune response. This

coupling is achieved by means of a spacer molecule. A 5-carbon unit as linker has been shown to be the optimal length for a spacer, or linker, in a number of studies.⁷³⁻⁷⁶ For this purpose, the linker molecule **48** was synthesized in a 35% yield by reaction of 5-amino-5-pentanol with benzyloxy chloride (Cbz-Cl).

Of the two cyclic phenyl phosphonate diastereomers, **38a** and **38b**, only the minor diastereomer **38b** was coupled to a carrier protein (KLH) for immunization in mice for antibody production. Displacement of the phenoxy moiety of **38b** by the linker molecule **48** produced **49a** in a 59% yield. Finally, hydrogenolysis of **49a** in ethanol with hydrogen and 10% Pd/C afforded **50** in 85% yield.

Amine **50** was coupled to carboxy residues of the carrier protein keyhole limpet hemocyanine (KLH) using the water soluble coupling reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). The coupling takes place between the amino group of **50** and carboxyl residues on the carrier protein.

An attempt to generate the diastereomeric hapten of **49a**, namely **49b**, from phosphonate **38a** using the same reaction scale and conditions that produced **49a** from **38b** was attempted. However, isolation of the expected product by flash chromatography was not successful due to a poor yield. A subsequent attempt to isolate and characterize **49b** from the reaction of **38a** with **48** after doubling the scale of the reaction resulted in a 14% yield of **49b**.

Nucleophilic displacement reactions at phosphorus have been investigated.¹⁴⁵⁻¹⁴⁷ Green and Hudson carried out a series of nucleophilic displacement reactions on phosphorus.¹⁴⁸ The stereochemical outcomes of these displacement

reactions have been interpreted as proceeding with predominant inversion of the configuration at the phosphorus atom. Wadsworth reports mixed stereochemical results upon displacement at a phosphorus center, and the effect of added salt on the stereochemistry of nucleophilic displacements at phosphorus in phosphate esters and their analogues.¹⁴⁹

The nucleophilic reactions involving displacement of the phenoxy moieties of cyclic phosphonates **38a** and **38b** with the linker molecule **48** are believed to have undergone inversion at the phosphorus centers. In both of the experiments, displacement of the phenoxy groups of compounds **38a** and **38b** with linker **48** produced clean diastereomeric products. In addition, the faster moving 'a' isomer of **38** (by TLC) on reaction with **48** generated the slower moving isomer, **49b**, while the reaction of **38b** with **48** generated exclusively the faster moving isomer, **49a**. These results are suggestive of inversion at the phosphorus centers of **38a** and **38b** on displacement with **48**. Further evidence to support inversion at the phosphorus centers of **38a** and **38b** on displacement with **48** is found in the analysis of the NMR data of these compounds as outlined below in Section 3.7.

3.2.2 Antibody Raised Against Antigen KLH-50

Balb/c mice (6-8 week old females) were immunized with the KLH-50 conjugate. After the last injection, the mice were sacrificed and the spleens harvested. The mortal antibody-secreting spleen cells were fused with immortal myeloma cells to afford immortal antibody-producing hybrids (hybridomas), and these were plated into 96-well culture plates in HAT selection media (media containing hypoxanthine, aminopterin and thymidine). Spleen-myeloma hybrids are selected as follows: the fusion partners (myelomas; those used in this experimental are P3-X63-Ag8-653, or clone 653) are deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT- or HPRT-) and cannot utilize exogenous hypoxanthine in the synthesis of purine nucleotides. The presence of aminopterin, a dihydrofolate analogue and competitive inhibitor of dihydrofolate reductase ($K_i < 10^{-9}$ M),⁶² blocks the *de novo* synthesis of nucleotides. Thus in the presence of HAT selection media, unfused myelomas do not survive. Since spleen cells do not grow well in tissue culture, they die in about a week. Hybridomas are HGPRT+ (donated by the spleen cell) and can utilize exogenous hypoxanthine and thymidine in their salvage pathway and survive. Cell lines obtained in this fashion can be maintained in continuous culture or frozen in liquid nitrogen indefinitely.

3.3 BINDING ASSAYS

The particle concentration fluorescence immunoassay (PCFIA) technique¹⁵⁰ was used in place of the traditional enzyme-linked immunosorbant assay (ELISA) for the initial screening of hybridomas. The PCFIA assays were carried out using hapten-protein conjugate-coated carboxyl polystyrene particles, 96 well filter assay plates, a goat antimouse fluorescein isothiocyanate (FITC) secondary antibody for detection at 385 nm wavelength, and a PCFIA buffer (2% fetal calf serum (FCS), 0.2% sodium azide, and 1x PBS, filter-sterilized through a 0.22 μ m filter).

A typical PCFIA assay involved the screening of supernatants from the hybridoma cells for hapten affinity using BSA-50 conjugate-coated carboxyl polystyrene particles as a solid phase. The BSA-50 conjugate was produced under the same conditions as the KLH-50 conjugate, then the BSA-50 conjugate was coupled through amino residues on the BSA with the carboxylate of the carboxy polystyrene particles using EDC as in Scheme 7. Once the supernatants from the hybridoma cells have incubated with the BSA-50 conjugate-coated carboxyl polystyrene particles, a FITC secondary antibody was used for detection (at 385 nm wavelength) of hapten-specific antibody binding. Positives from these screenings were subcloned by further limiting dilution onto 96-well microtiter plates to ensure we had stable monoclonal cell lines. A

second PCFIA binding assay was then carried out to verify that the hybridomas were still secreting anti-hapten antibodies.

Scheme 7. Coupling of the BSA-50 conjugate with the carboxy polystyrene particles (PSP).

3.4 ANTIBODY F123 ISOTYPING

Using a standard isotyping assay, our monoclonal antibody F123 was found to be an IgM class antibody. IgM class antibodies, as described in Chapter 1, are expressed in the primary immune response.

Typical immunization protocols, such as the one we followed, include a large initial dose (challenge) of antigen, followed by booster injections containing a lower dose of antigen. Our protocol called for 100 μ g of the immunoconjugate KLH-50 per mouse on immunization, and 50 μ g per mouse for each booster injection. The time delay between the administration of the immunoconjugate into the mouse and the triggering of the primary response is not clear, nor is it clear at which stage of B cell differentiation, from stem cell to mature B cell,

commitment to an ultimate antibody specificity occurs. Further refinement to the antibody specificity, by such mechanisms as affinity maturation and class switching (evolution, for example, from an IgM to an IgG class antibody), occurs under conditions in which the immune system is continuously challenged with the immuno-conjugate. A sufficiently stable hapten-protein conjugate is required to remain intact *in vivo* between booster injections to provide a constant challenge to the immune system of the mouse. One might speculate that if a sufficiently large immunizing dose of the KLH-50 conjugate was administered causing an initial immune response, while the amount of antigen in subsequent booster injections was not sufficiently large to allow sufficient lifetime of the conjugate for further refinement mechanisms to occur, fusion of these naive spleenocytes with immortal cells would result in IgM secreting hybridomas. Success in obtaining a lactone-forming catalytic antibody depended on the lifetime of the phosphonate immuno-conjugate, KLH-50, in the mouse. If KLH-50 was susceptible to hydrolysis in the mouse, exocyclic (path a) rather than endocyclic (path b) cleavage (Scheme 8) should prevail since the 14-membered ring does not suffer from the ring strain in the seven membered ring phosphonate. In addition, endocyclic cleavage would result in the intact conjugate KLH-51, a hapten now resembling the phosphonic acid transition state analogues employed to obtain antibodies which catalyze hydrolytic reactions. However, as will be described later, we obtained a lactone-forming IgM antibody, one which was not found to have hydrolytic activity.

Scheme 8. Exocyclic (path a) versus endocyclic (path b) hydrolysis of immuno-conjugate KLH-50 in mouse serum.

3.5 ASCITES PRODUCTION

Monoclonal antibodies selected for kinetic analysis, based on the binding assay results, were prepared by injecting a million hybridoma cells into pristane-primed Balb/c mice (6-8 week old females). The resulting ascites fluid was collected, and monoclonal antibodies were purified from ascites fluid by sheep-antimouse (SAM) affinity chromatography. The antibody samples were dialyzed into PBS buffer (8.2 mM NaHPO_4 , 137 mM NaCl , 2.7 mM KCl , 1.5 mM H_2PO_4 , pH 7.4) for

activity assays. Antibody concentration was determined by the absorbance at 280 nm.

3.6 SYNTHESIS OF SUBSTRATES AND HAPTEN DERIVATIVES

3.6.1 Synthesis of Substrate 55

Gong, *et al.*⁸⁹ reported that a chromogenic substrate containing a 3-hydroxyindole moiety (see Scheme 9) might be useful in a direct catalytic assay. The indoyl strategy was suggested to be a facile chromogenic assay that might lend itself to the screening of large antibody libraries. The insoluble indigo product obtained upon carbonate cleavage affords high detection sensitivity since the indigo accumulates at the site of reaction, rather than diffusing through the reaction medium as is the case with soluble chromogenic species such as the p-nitrophenolate anion.

The synthesis of **55**, shown in Scheme 10, began with the hydrolysis of 3-indoyl acetate in a deoxygenated solution of refluxing 2N NaOH. The anhydride **52**, in deoxygenated EtOH was then cannulated into this reaction solution at 0 °C. Upon

Scheme 9. Chromogenic assay reported by Gong, *et al.* (taken from ref 89).

isolation of crude indole **53** by ether extraction, followed by hydrogenolysis of the crude with H_2 gas and Pd/C in EtOAc, the resulting crude isolate was coupled to the hydroxy acid **54**^{*} with 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 yielding the target hydroxy ester **55**.

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* Prepared by D. Clyne.

Scheme 10. Synthesis of indoyl substrate **55**.

Anhydride **52** was prepared by the DCC coupling of carboxylic acid **56***.

Substrate **55** was found to be insoluble in the PBS buffer. A variety of co-solvents, including MeOH, DMF, CH₃CN and DMSO, were used in an attempt to solublize **55**. Of the co-solvents used, a PBS solution containing 10% DMSO was found to be the most effective, however, complete solubility of **55** was only achieved by employing 0.5% Triton X-100 with the 10% DMSO. Solutions were

* Prepared by J. P. Ounsworth.

determined to be homogeneous by examination under an inverted microscope (10x magnification).

Reaction of the indoyl substrate **55** with monoclonal antibody F123 did indeed lead to the expected insoluble byproduct, namely indigo, while none was observed in the blank experiment, however, the indigo could neither be easily nor conveniently quantified on the assay scale used.

Due to the low solubility of substrate **55** in the reaction buffer, along with the problem associated with quantifying the extent of the reaction (i.e. the amount of indigo formed), we elected to switch to the shorter, more soluble p-nitrophenyl ester substrate **57**. In addition, the soluble p-nitrophenolate anion, resulting from reaction of **57** with the catalytic antibody, is a convenient chromophore for acquiring kinetic data spectrophotometrically.

Substrate **57**, used in the catalytic assays for the monoclonal antibodies, differs from **55** in that **55** mimicks more closely the linker-arm portion of hapten molecule **50**. However, it should not be surprising that the antibodies are tolerant to this substrate variability, since others^{89,151,152} suggest that antibody

binding specificity is relatively insensitive to elements of the hapten and substrate close to the conjugation site. Also, antibodies specific for the steroid androstenedione were screened⁷⁵ for specificity using derivatives of this steroid. This work showed that the antibodies were much less sensitive to changes in the ligand which were near the position of linker attachment on the steroid, and that the specificity was greatly affected by changes farthest from the position of attachment to the linker. This evidence supports the idea that the portion of the hapten which extends away from the carrier protein provides the most accessible epitopes for antibodies.

3.6.2 Synthesis of Substrate 57

The hydroxy p-nitrophenyl ester substrate **57** was obtained by reaction of 13-hydroxytetradecanoic acid (**54**) with p-nitrophenol in CH_2Cl_2 in the presence of DCC and DMAP under a N_2 atmosphere to produce the desired substrate in 87% yield.

3.6.3 Synthesis of Substrate 58

Myristic acid, or tetradecanoic acid, was reacted with p-nitrophenol as below to produce p-nitrophenyl tetradecanoate (**58**) in 88% yield.

3.6.4 Synthesis of Hapten Derivatives 60a and 60b

The synthesis of the p-nitrophenylphosphonate hapten derivatives **60a** and **60b** began with the hydrolysis of phenyl phosphonate **38b** using refluxing 3N NaOH-THF produced the phosphonic acid derivative **59** in a 73% yield. Conversion of **59** to the acid chloride with oxalyl chloride, followed by coupling of this phosphoryl chloride intermediate with p-nitrophenol (PNP) gave a 1.2:1 ratio of diastereomers **60a** and **60b** in 69% yield.

3.6.5 Synthesis of Six-membered Ring Phenyl Phosphonates, **61a** and **61b**

The six-membered ring phenyl phosphonates, **61a** and **61b**, were synthesized as an offshoot of the 14-membered ring project, and as such, the full characterization of the intermediates that led to compounds **61a** and **61b** is not complete. However, the targets, **61a** and **61b**, have been fully characterized, and the stereochemistry of these two phenyl phosphonates has been firmly established, and this proved useful in elucidating the stereochemistry of the 14-membered ring analogues, **38a** and **38b**, discussed in Section 3.7. The synthesis of **61a** and **61b** (Scheme 11) began with the benzylation of the primary hydroxyl group of 1,3-butanediol to give **62** in 58% yield. The secondary hydroxyl of **62** was then protected as the THP ether **63** in 76% yield. This addition of the THP group created a second chiral center in **63**, and therefore, two diastereomers of **63** were obtained. The two diastereomers were obtained in a 1:1 ratio as seen in the ^1H NMR spectrum of **63**, which reveals two methyl doublets of equal intensity and two methine multiplets, corresponding to the

methine proton of the THP group. Hydrogenolysis of the benzyl moiety of compound **63** gave alcohol **64** in an isolated yield of 85%. The ^1H NMR spectrum of the flash chromatography-purified diastereomers of compound **64** reveals a diastereomeric ratio of approximately 1:4, rather than a 1:1 as would be expected, based on the relative intensities of the diagnostic methyl and methine signals. A portion of one of the diastereomers of **64** was accidentally discarded during purification resulting in this 1:4 ratio. This was of no consequence, however, since the THP group was subsequently removed, destroying one of the two stereogenic centres in the molecule. The 1:4 ratio of isomers of alcohol **64** were then oxidized under the Swern conditions to give the aldehyde **65** in 96% yield. Aldehyde **65** was subsequently reacted with the stabilized Wittig reagent **32** to give the trans vinyl phosphonate **66** in a moderate yield of 55%. Phosphonate **66** was then reacted with p-toluenesulphonic acid to liberate the secondary hydroxyl compound **67** in 70% yield. Removal of the THP group from **66** eliminated one of the two stereogenic centres in the molecule giving a single diastereomer, reflected in the ^1H NMR spectrum of **67** which contains only one methyl doublet. The alkene **67** was then reduced to give the saturated diphenyl phosphonate **68** in 93% yield. Finally, the hydroxy diphenyl phosphonate **68** was reacted with n-butyllithium in THF at $-78\text{ }^\circ\text{C}$ to produce the cyclic phosphonates **61a** and **61b** in a 96% yield and a 6:1 ratio of diastereomers based on the ^1H NMR spectrum of the crude product.

Scheme 11. Synthesis of cyclic phosphonates **61a** and **61b**.

3.7 INVESTIGATION INTO THE RELATIVE STEREOCHEMISTRY OF CYCLIC PHOSPHONATES **38a** AND **38b**

Table 5 below lists the ^1H NMR chemical shifts of the methine proton and the methyl protons for the 14-membered ring phosphonates **38a**, **38b**, **60a**, **60b**, **49a**, **49b** and **50**, as well as those for the 28-membered ring dimer **39** and the six-membered ring phosphonates **61a** and **61b**. In addition, Table 5 lists the ^{31}P NMR signals and TLC R_f values for each compound. We first looked for a correlation between the ^1H and ^{31}P NMR spectra of the 28-membered macrocyclic phosphonate dimer **39** and those of the cyclic monomeric diastereomers, **38a** and **38b**. Since we only had a crystal structure for the dimer

39, we hoped that the ^1H and ^{31}P NMR spectra of the 28-membered macrocyclic phosphonate dimer **39**, together with the structural information from the X-ray structure of **39** would allow us to infer structural information about monomers **38a** and **38b**, and ultimately the hapten molecule **50**, to which antibody F123 was raised.

The chemical shifts of the C-1 methyl doublets for both isomer **38a** and the dimer **39** are at higher field than the methylene envelope, while that of isomer **38b** is at lower field than this envelope (see Spectral Appendix). In the case of compound **50**, the methyl doublet lies just within the highfield region of the methylene envelope, that is, to the "isomer a side". This result is consistent with inversion at phosphorus, since **50** was produced on substitution of the phenol moiety of diastereomer **38b** with the linker **48**, giving rise to **49a** which upon hydrogenolysis of the Cbz moiety of **49a** yielded compound **50**. Compound **49b** was synthesized by displacement of the phenol moiety of **38a** with linker **48** giving rise to compound **49b** exclusively.

Diastereomers **60a** and **60b** were produced by conversion of the cyclic phosphonic acid **59** into two phosphoryl chloride intermediates (as observed by

38a,38b R = Ph
 60a,60b R = PNP
 49a,49b R = (CH₂)₅NHCbz
 50 R = (CH₂)₅NH₂

39

61a + 61b

Table 5. Key ¹H and ³¹P NMR chemical shifts (δ values) and TLC R_f-values for the cyclic phosphonates.

Compound	δ ¹ H for CH ₃ protons (ppm)	δ ¹ H for methine proton (ppm)	δ ³¹ P (ppm)	TLC R _f values [†]
38a	1.22	4.75	29.6	0.71
38b	1.43	4.65	27.8	0.57
60a	1.23	4.77	30.4	0.66
60b	1.43	4.65	28.5	0.56
49a	1.30	4.59	32.5	0.18
49b	1.35	4.43	31.3	0.12
50	1.32	4.95	33.1	0
39	1.15	4.66	29.0	0.62
61a	1.35	4.51	20.5	0.21
61b	1.36	4.68	23.4	0.10

[†] Obtained using a 1:1 mixture of petroleum ether and ethyl acetate.

TLC) followed by substitution with p-nitrophenol. The ^1H and ^{31}P NMR chemical shifts (δ values) for compounds **49a** and **49b** as well as for diastereomers **60a** and **60b** are consistent with the pattern observed with **38a** and **38b**. That is, within each pair of 14-membered ring phosphonate diastereomers listed in Table 5, the ^1H NMR chemical shift of the C-1 methyl doublet is at higher field, while the shift of the methine proton is at lower field for all the **a** isomers compared with those for the corresponding **b** isomers. The ^1H NMR chemical shift of the C-1 methyl doublet for dimer **39** is consistent with those of the **a** isomers of the 14-membered ring phosphonates. The stereoview of the ORTEP representation of the macrocyclic dimer **39** (Figure 13) reveals that the doubly-bonded oxygen on the phosphorus atom is 'syn' to the C-13 methine proton, and the C-1 methyl group is 'syn' to the oxygen of the phenol moiety as illustrated in Figure 14. We are suggesting, therefore, that the **a** isomers of the 14-membered ring phosphonates listed in Table 5 have the R^*,R^* relative stereochemistry between the C-1 methyl and the phosphorus atom consistent with that of compound **39**. In addition, we believe that the C-1 methyl group and the OR group on phosphorus are mainly in a conformation in which both are equatorial in the **a** isomer as found in the X-ray structure of **39** (Figure 14). Support for the equatorial assignment of the OR groups on the phosphorus atoms of the 14-membered ring phosphonates listed in Table 5 comes from the ^{31}P NMR chemical shift data. It has been observed that the ^{31}P NMR signal for the equatorial P-OR isomer is approximately 2 ppm lower field than the

Figure 14. Relative stereochemistry about the phosphorus atom and the C-1 carbon atom of dimer **39**.

corresponding axial isomer in the cyclic phosphonates of hexopyranoses¹⁵³⁻¹⁵⁵ and in other six-membered ring phosphonates.^{156,157} This trend supports our correlation between compounds **39** and **38a**, since the ³¹P NMR shift for isomer **38a** is 2 ppm downfield from that of isomer **38b**.

A final piece of evidence to support the equatorial assignment for the OPh group in compound **38a** comes from a comparison of **38a** and **38b** with the six-membered ring analogues, **61a** and **61b**. The stereochemical assignments (as shown below) for compounds **61a** and **61b** were determined on the basis of ¹H NMR NOE (nuclear overhauser effects) difference experiments. Irradiation of the methine signal at 4.51 ppm of phosphonate **61a** resulted in enhancement of the phenyl signal between 7.4-7.2 ppm, whereas, irradiation of the corresponding methine signal for **61b** failed to produce an enhanced signal in the phenyl region. This NOE data is consistent with the stereochemical assignments for compounds **61a** and **61b**.

61a **61b**

In addition, the ^{31}P NMR shifts for compounds **61a** and **61b**, listed in Table 5, are in agreement with the trends described above for similar six-membered ring phosphonates. That is, the ^{31}P signal for phosphonate **61b**, with the OPh group in the equatorial position, is at lower field than the corresponding axial isomer, **61a**. With the stereochemistry of compounds **61a** and **61b** firmly established, a correlation was made between these compounds and the 14-membered ring analogues, **38a** and **38b**. Considering the structures for compounds **61a** and **61b** along with ^1H NMR data listed in Table 5, a 1,3-diaxial (or syn) relationship between the methine proton and the doubly-bonded oxygen attached to the phosphorus atom (as in **61b**) results in a shift in the methine signal to lower field compared to the higher field methine signal when these two groups are anti to one another (as in **61a**). The methine signal for compound **38a** (at 4.75 ppm) is at lower field than that of compound **38b** (at 4.65 ppm), which supports our assignment of the OPh group being equatorial (and hence the doubly-bonded oxygen attached to the phosphorus atom in a 1,3-diaxial relationship with the methine proton) in phosphonate **38a**.

3.8 INITIAL INVESTIGATION FOR REACTIVITY OF MAB'S WITH SUBSTRATE 57.

The concentrations of antibody stock solutions used in this study, obtained from immunoaffinity purification of ascites preparations, were determined using $n = 1.4$ and a molecular weight of 175 kDa for IgM monomer.

Clones that showed the strongest binding to our hapten molecule were chosen for catalytic screening. Analyses were performed using 96-well microtiter plates and a microtiter plate reader (Bio-Tek Instruments) equipped with the Delta Soft II computer program (referred to later as the 'multiwell method'). A report by Masamune and co-workers⁹⁰ also uses a microtiter plate reader for kinetic analysis of their catalytic monoclonal antibodies. Furthermore, Copeland states that many workers now perform absorption measurements with 96-well microtiter plate readers.¹⁵⁸ All assays reported in this thesis by the microtiter method were performed at least in duplicate.

Three monoclonal antibodies, F123, F125 and F150 were selected, based on binding assays, for a reactivity test against substrate **57** (Figure 15). These clones were assayed spectrophotometrically (405 nm) at 37 °C for 4-nitrophenolate anion release from substrate **57** on reaction with the antibody. Only monoclonal F123 showed significant reactivity with **57**, and was selected for further study. An initial test of the catalytic potential of monoclonal F123 was

carried out, involving three independent experiments on one microtiter plate. The first experiment was a reaction of substrate **57** ($88\ \mu\text{M}$) with antibody F123

Figure 15. Monoclonal antibodies F123 (O), F125 ($\square$) and F150 ($\bullet$), each at a concentration of $2\ \mu\text{M}$, were assayed for activity with substrate **57** ($200\ \mu\text{M}$) in PBS buffer (pH 7.4) containing 10% DMSO and 0.5% Triton X-100 at $37\ ^\circ\text{C}$.

($0.88\ \mu\text{M}$). The second experiment involved reaction of **57** ($88\ \mu\text{M}$) with antibody F123 ($0.44\ \mu\text{M}$). From the slopes (rates) of these two experiments (see Figure 16), we see that halving the antibody concentration resulted in a halving of the reaction rate (see Table 6). Finally, the third experiment involved reaction of **57** ($88\ \mu\text{M}$) in the presence of 0.25 equivalents ($0.22\ \mu\text{M}$) of hapten analogue **60** (a one-to-one mixture of both diastereomers) with antibody F123 ($0.88\ \mu\text{M}$). Here we expected to see inhibition of the p-nitrophenol release in the presence of the hapten derivative **60**. From the slopes in Table 6, it is clear that compound **60**

inhibits the antibody activity, demonstrating that antibody activity is associated with binding in the antibody-combining site.

Figure 16. Initial experiments with antibody F123, substrate **57**, and hapten derivative **60** using PBS buffer (pH 7.4) containing 10% DMSO and 0.5% Triton X-100 at 37 °C. Reactions were run; (i) (●) 88 μ M in **57** and 0.88 μ M in F123, (ii) (○) 88 μ M in **57** and 0.44 μ M in F123, (iii) (□) 88 μ M in **57**, 0.88 μ M in F123 and 0.22 μ M of **60** (1:1 ratio of diastereomers).

The findings of these three experiments were encouraging, and suggested that monoclonal F123 was behaving as a catalyst, and that the catalytic activity of F123 is associated with binding in the antibody-combining site. The next step was to prove that antibody F123 is indeed catalysing the cyclization of **57** to lactone **19**, and to then carry out a kinetic analysis of the reaction between

antibody F123 and substrate **57** to determine the Michaelis-Menten parameters K_m , V_{max} and k_{cat} .

Table 6. Results of initial F123 antibody experiments.

Experiment	Slope ($\Delta OD/min$)	Correlation Coefficient
(i)	0.0101	0.9995
(ii)	0.0056	0.9983
(iii)	0.0008	0.9935

3.9 ISOLATION OF F123-CYCLIZED MACROLACTONE **19**

Confirmation that F123 catalyzed the cyclization of substrate **57** to lactone **19** was achieved by isolation of **19** through ether extractions from pooled catalytic experiments. Several small-scale catalytic reactions of antibody F123 with substrate **57** in PBS buffer, pH 7.4, containing 10% DMSO and 0.5% Triton X-100 at 37 °C (ranging from 100–400 μM in **57**, and each having a final volume of 250 μL) were pooled for ether extractions.

Initial identification of the antibody generated lactone was achieved by GC analyses. GC analysis of an independently synthesized sample of lactone **19** revealed a broad peak with a retention time of approximately 17 minutes, as seen in trace (a) of Figure 17 below. The GC trace of the ether extracts from the

pooled antibody experiments also contained a broad peak at approximately 17 minutes, Figure 17 trace (b). The GC run for the ether extracts from the antibody experiment spiked with lactone **19**, GC trace (c), showed an increase in the peak

Figure 17. GC chromatograms of: (a) an independently synthesized sample of lactone **19**; (b) ether extracts from the pooled F123 catalyzed cyclization reactions of substrate **57**; (c) a spiked GC run of the ether extracts from the antibody experiment with an authentic sample of **19**.

corresponding to lactone **19** having a retention time of approximately 17 minutes, confirming the presence of lactone **19** in the pooled antibody experiments.

Furthermore, the unambiguous identification of the antibody generated lactone was obtained by GCMS analysis. The high resolution mass spectrum of the

GCMS lactone peak from the ether extracts was consistent with that of the independently synthesized lactone sample. As expected, the molecular ion peak for lactone **19** at m/z 226 was observed in both mass spectra. In addition, the anticipated fragment at m/z 208 was present in both mass spectra.

Finally, a large scale background experiment with **57** (250 μ M) in the reaction buffer (containing no antibody) was conducted at 37 °C for five days. GCMS analysis of ether extracts from this background reaction revealed no detectable lactone.

3.10 SPECIFICITY OF MONOCLONAL F123

To illustrate that the activity of antibody F123 with **57** is not due to an esterolytic contaminant of the serum, an affinity purified control MAb raised against an unrelated hapten was run in parallel with F123. The control MAb showed no activity with substrate **57** (see Figure 18).

3.11 REACTION OF P-NITROPHENYL TETRADECANOATE WITH F123

Substrate **58**, which lacks the secondary hydroxyl necessary for lactonization, was tested with catalytic antibody F123 and showed no activity. This also provided strong evidence that MAb F123 was not simply behaving as an

esterase by hydrolyzing the p-nitrophenol ester moiety of substrate **57**, but rather, acted by carrying out the desired macrolactonization (Figure 19).

Figure 18. The activity of monoclonal F123 (O) is compared to that of a control monoclonal antibody, HIL-20 (●), using substrate **57** in both of the experiments. Each experiment was run 200 μ M in **57** and 2 μ M in antibody, carried out in PBS buffer (pH 7.4) containing 10% DMSO and 0.5% Triton X-100.

Figure 19. Comparison of antibody F123 activity with hydroxy ester substrate **57** (O); with ester **58** (●). In each case, antibody was 0.46 μM ; substrates were 100 μM ; carried out in PBS buffer (pH 7.4) containing 10% DMSO and 0.5% Triton X-100; total reaction volume was 225 μL .

3.12 INHIBITION ASSAYS

The F123 antibody catalyzed reaction of substrate **57** to lactone **19** was analyzed for inhibition by an independently synthesized sample of lactone **19**. Lactonization of **57** by F123 was not inhibited in the presence of one molar equivalent of **19** (relative to substrate **57**) as shown in Figure 20 below.

Figure 20. Antibody F123 activity with hydroxy ester substrate **57** (O); with hydroxy ester substrate **57** in the presence of an equimolar amount of lactone **19** (●). Experiments were carried out in PBS buffer (pH 7.4) with 10% DMSO; 0.46 μM in antibody F123; 200 μM in substrate; total reaction volume was 250 μL .

Continuing from the initial reactivity studies of antibody F123 described in Section 3.8, inhibition assays with a one-to-one mixture of the 4-nitrophenyl phosphonate diastereomers, **60a** and **60b**, were performed in the presence of substrate **57** and MAb F123. As Figure 21 shows, the mixture of **60a** and **60b** competitively inhibited the F123 antibody-catalyzed reaction of **57**. Each data point in Figure 21 represents an average of two reaction runs, which agree within 10%.

Figure 21. Competitive inhibition of the F123-catalyzed macrolactonization of substrate **57** by a one-to-one mixture of 4-nitrophenyl phosphonate diastereomers, **60a** and **60b**. The reactions were performed in PBS buffer (pH 7.4) containing 10% DMSO and 0.5% Triton X-100. The concentrations of the mixture of **60a** and **60b** (1:1) were: (O) 0 μM ; (●) 2 μM ; and (□) 5 μM .

By fitting the data from Figure 21 with the inhibitor concentrations to the computer program GraFit™, a value of $K_i = 2.9 \pm 0.4 \text{ } \mu\text{M}$ was determined. Because of the chiral nature of enzymes and antibodies, we believe that this inhibition constant of $2.9 \pm 0.4 \text{ } \mu\text{M}$ represents an upper limit (where a smaller K_i value is indicative of a more potent inhibitor), and the true K_i is expected to be one-quarter this value since only one of the four stereoisomers in the mixture of **60a** and **60b** diastereomers is believed to be responsible for the inhibition observed. Further inhibition studies using the four stereoisomers of **60**

separately are necessary to refine this inhibition constant. Nonetheless, the competitive inhibition of F123 with this mixture of 4-nitrophenyl phosphonate diastereomers, **60a** and **60b**, demonstrates that the catalytic activity is associated with binding in the antibody-combining site, as expected.

3.13 DETERMINATION OF K_m , V_{max} AND k_{cat} FOR ANTIBODY F123 WITH SUBSTRATE **57**

Approximate values of K_m and V_{max} may be determined for substrates by measuring initial reaction rates using three different (and wide-ranging) concentrations of the substrate. Once approximate values are obtained, accurate values of K_m and V_{max} are then determined by measuring initial reaction rates using 5-8 different concentrations of the substrate, concentrations that typically range between 0.3-5 times the approximated K_m value.

For substrate **57**, the experiment to determine approximate K_m and V_{max} values was carried out with concentrations of 40, 100, 200, and 400 μM of **57** in the reaction buffer consisting of 10% DMSO and 0.5% Triton X-100 in PBS buffer, pH 7.4. The approximate K_m was found to be 250 μM for substrate **57**. With a solubility limit of 400 μM for **57**, and an approximate K_m of 250 μM , this meant that in order to achieve a more accurate value of K_m , we would need to increase the solubility of **57** in the reaction buffer in order to bracket the approximate K_m by 0.3-5 times. This led to a solubility study of substrate **57**.

3.13.1 Determination of the Solubility Limit of Substrate **57** and the pH of the Reaction Solution

In an effort to attain a higher substrate solubility limit, the effect of varying the Triton X-100 concentration in the reaction buffer mixture was investigated. Solubility limits for substrate **57** in the reaction buffer containing 0%, 0.25%, 0.5% and 1.0% Triton X-100 were determined by examination of solutions under a microscope (10x magnification). As shown in Table 7, the solubility limit of **57** increases with increasing concentration of Triton X-100 in the reaction buffer used in this study. Conversely, it was found that the pH of the reaction solution decreased on increasing the concentration of Triton X-100.

Table 7. Solubility Study of Substrate **57** with Triton X-100.

Triton X-100 Content (%)	Solubility Limit of 57 (μM)	pH
0	100	7.55
0.25	200	7.58
0.50	400	7.35
1.00	800	6.95

However, it was found that a Triton X-100 content of 1% or more in the reaction solution not only increased the solubility limit of **57** and decreased the pH of the solution, it appeared also to inhibit the antibody activity. To probe this apparent solubility/reactivity problem, catalytic experiments were carried out in the presence of 0%, 0.25%, 0.5% and 1% Triton X-100 (with 10% DMSO, 200 μ M in

substrate **57**, in PBS buffer, pH 7.4). It was observed that antibody activity appeared to decrease with increasing Triton X-100 content (see Figure 22 below). The pH of the four test buffers (containing 0%, 0.25%, 0.5% and 1.0%

Figure 22. Antibody F123 reaction with substrate **57** compared to the background hydrolysis of **57** in the reaction buffer alone.

Triton X-100) was determined, and found to be 7.55; 7.58; 7.35 and 6.95, respectively (Table 7). At this point, we believed that the apparent loss in antibody activity may be attributed to one of the following two factors, namely the increase in the surfactant concentration, Triton X-100, or the drop in pH of the reaction buffer as a result of increasing the surfactant concentration. A decrease in pH of the reaction buffer (on increasing the Triton X-100 concentration) would result in a shift in the equilibrium shown below toward the protonated form, p-nitrophenol, which would explain a decrease in the

absorbance reading. Alternatively, the antibody may simply lose activity as the pH is reduced.

To determine if this pH depression was the cause of the apparent loss in antibody activity, the buffer strength of the PBS buffer used in the reaction solution was increased from 8 mM sodium phosphate to 500 mM sodium phosphate. The higher buffer capacity was sufficient to suppress the drop in pH, (pH was measured and found to be 7.45 for the 500 mM sodium phosphate solution, compared to pH 6.95 for the 8 mM sodium phosphate solution), however, catalysis (as measured by the rate of release of p-nitrophenolate anion above background) was not observed (see Figure 23). This finding suggests that the increased concentration of Triton X-100 does not affect the position of equilibrium between the p-nitrophenolate anion and the protonated form, but rather, in some way inhibits antibody activity when the concentration of Triton X-100 is 1% or higher. Further experiments are required to investigate the nature of this effect. Perhaps we have reached or exceeded the critical micelle concentration (cmc) of the surfactant for our buffer system, or perhaps substrate molecules are being partitioned within the micelle wall, and in effect, the Triton

X-100 is competing with the antibody for substrate binding. If this is the case, the loss of substrate molecules to micelles would make it impossible to know the actual substrate concentration in our reaction mixture. Furthermore, at 1% Triton X-100, perhaps the surfactant binds sufficient substrate such that none is available to the antibody, and we simply observe background hydrolysis, as in the background samples.

Figure 23. Comparison of the F123 reaction with substrate **57** and background hydrolysis of **57** using 10% DMSO and 1.0% Triton X-100 in 8 mM PBS buffer (pH 6.95); and F123 and **57** in a 10% DMSO with 1.0% Triton X-100 in 500 mM PBS buffer (pH 7.45).

3.13.2 Determination of K_m , V_{max} and k_{cat} for Monoclonal F123 with Substrate **57**

K_m , V_{max} and k_{cat} determinations were performed on antibody F123 using two independent methods. The first method will be referred to as the 96-well multiwell method, employing a microtiter plate reader (Bio-Tek Instruments)

equipped with the Delta Soft II computer program. The second method will be referred to as the spectrophotometer cuvette method.

The *molar absorptivity* (ϵ) for the p-nitrophenolate anion in the reaction buffer was determined as follows. Measuring the absorbance ($\lambda_{\text{max}} = 405 \text{ nm}$) of a 0.1 mM solution of p-nitrophenolate anion in the reaction buffer and applying Beer's Law, $A = \epsilon cl$ (where A is the absorbance, c is the solute concentration expressed in moles per liter, and l is the pathlength in centimeters of the solution being measured), ϵ for the p-nitrophenolate anion was found to be 10,830 L/mol·cm.

While the pathlength of the 1 mL quartz cuvettes used with the Unicam UV/VIS spectrophotometer, employed in the spectrophotometer cuvette method, is known to be 1 cm, the pathlength of the 250 μL volumes of reaction buffer in the 96-well microtiter plates employed in the multiwell method had to be determined.

The absorbance of a 0.1 M solution of p-nitrophenolate in the reaction buffer was measured using the Unicam spectrometer ($\lambda_{\text{max}} = 405 \text{ nm}$) having a pathlength of 1 cm. This result was used to determine the pathlength of the 250 μL reaction volume in the multiwell experiments by measuring the absorbance of a 250 μL aliquot of this solution in a microtiter plate, and applying Beer's Law. The pathlength of the 250 μL volume of reaction buffer in the microtiter plate was found to be 0.64 cm.

3.13.2.1 Determination of K_m , V_{max} and k_{cat} for Monoclonal F123 with 57 via the Multiwell Method

Assays were performed in 96-well microtiter plates, and the reaction buffer consisted of PBS containing 10% DMSO and 0.5% Triton X-100 (for substrate solubility), at pH 7.4. Without Triton X-100, the solubility limit of substrate 57 is 200 μM , and with 0.5% Triton X-100, this limit is increased to 400 μM . Final reaction volumes used were 250 μL , and the pathlength of the ELISA reader was determined above to be 0.64 cm. Rates were determined by measuring 4-nitrophenolate release at 405 nm ($\epsilon = 10,830 \text{ L/mol}\cdot\text{cm}$) and defined as mOD/min. The antibody concentration used was 0.23 μM (based on IgM monomer molecular weight of 175 kDa). Kinetic parameters were obtained using the method of initial rates and fitted to a Lineweaver-Burk analysis to obtain the Michaelis-Menten parameters K_m , V_{max} and k_{cat} for substrate 57 (Figure 24). It should be noted that prior to reading the microtiter plates by the ELISA reader, mixing of the reaction buffer with a micro-pipette, until homogenous, caused the formation of bubbles at the liquid surface of the reaction solutions. Since the ELISA reader measures absorbance down through the top of each well, plate reading was delayed (approximately 10 minutes) until the bubbles had collapsed, after which time, the initial rates were measured. Since the antibody rate is expected to decrease with time, one might expect the actual initial rates would be greater if the reactions were monitored immediately after mixing, without a 10 minute delay time.

Figure 24. A Lineweaver-Burk analysis of the F123-catalyzed macrolactonization of hydroxy ester substrate **57** using the multiwell method.

At an antibody concentration of $0.23 \mu\text{M}$, the multiwell method yielded a V_{max} of $0.62 \pm 0.01 \mu\text{mol/min mg}$ and a K_m of $250 \pm 10 \mu\text{M}$. The k_{cat} for this experiment was calculated to be 1.1 min^{-1} .

3.13.2.2 Determination of K_m , V_{max} and k_{cat} for Monoclonal F123 with **57** via the Spectrophotometer Cuvette Method

Analyses for K_m , V_{max} and k_{cat} determinations were also performed using a Unicam UV/VIS spectrophotometer in 1 mL quartz cuvettes with 1 cm pathlength (600 μL reaction volume) to compare techniques. Rates were determined by measuring 4-nitrophenolate release at 400 nm ($\epsilon = 10,730 \text{ L/mol}\cdot\text{cm}$), and the

same reaction buffer as in the multiwell method was used in these measurements. Kinetic parameters were obtained using the method of initial rates and fitted to a Lineweaver-Burk analysis to obtain the Michaelis-Menten parameters K_m , V_{max} and k_{cat} for substrate **57** (Figure 25). The antibody concentration used was 0.25 μM (based on IgM monomer molecular weight of 175 kDa). With this approach, unlike the multiwell method, the absorption can be read immediately after mixing, to obtain more accurate initial rates. Once all reagents and buffers were added to the cuvettes, they were covered with Parafilm, and inverted several times to ensure thorough mixing, then inserted into the spectrophotometer. A delay time of 1.5 minutes was programmed into the spectrophotometer prior to reading the cuvettes to obtain the initial rates. This delay time of 1.5 minutes was to ensure equilibration of the reaction solution temperature to that of the spectrophotometer, 37 °C, prior to reading the absorbances. The time required for mixing, together with the 1.5 minute delay, meant that the total delay time prior to recording the initial rates with this approach was approximately 2 minutes, compared to the 10 minute delay time for the multiwell method.

The Unicam UV/VIS spectrometer experiments gave a V_{max} of 1.4 ± 0.1 $\mu\text{mol}/\text{min mg}$ and a K_m of 330 ± 50 μM from Figure 25. Since the antibody concentration was 0.25 μM for this experiment, the k_{cat} was calculated to be 2.2 min^{-1} by this approach. The K_m value obtained by this approach is in agreement

with that of the multiwell method, within experimental error. Of the two methods used,

Figure 25. A Lineweaver-Burk analysis of the F123-catalyzed macrolactonization of hydroxy ester substrate **57** using the spectrophotometer cuvette method.

however, the correlation proved better with the multiwell method. The k_{cat} values differ, however, by a factor of two. As we anticipated, the difference in delay times for the two approaches resulted in a difference in k_{cat} values. This can be accounted for by taking into consideration the delay time required, in the multiwell method, prior to beginning reading the multiwell experiments. At 10 minutes, it is expected that the rates would have diminished somewhat from the true initial rates. It is reasonable to assume, therefore, that this delay in the measurement of initial rates for the multiwell experiments would result in a lower

$V_{\max}$, and ultimately a lower k_{cat} for the multiwell method. The K_m values are, however, in good experimental agreement, as one would expect, since K_m determination is relatively insensitive to this time delay problem.

Finally, the catalytic efficiency, or k_{cat}/K_m , of antibody F123 is compared below in Table 8 with that of the antibody reported by Napper, *et al.*⁵⁹ which catalyzes the cyclization of a six-membered ring lactone (as described in Section 2.12.2).

Table 8. A comparison of the catalytic efficiency (k_{cat}/K_m) of antibody F123 with that of the antibody reported by Napper, *et al.*⁵⁹

Ring Size	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
14 ^a	0.018	2.50×10^{-4}	72
14 ^b	0.037	3.30×10^{-4}	112
6 ^c	0.008	7.6×10^{-5}	105

^a Data obtained by the multiwell method. ^b Data obtained by the spectrophotometer cuvette method. ^c Data obtained from ref 59.

As shown in Table 8, the antibody-catalyzed reaction for the formation of the six-membered ring lactone, **9**, has a lower k_{cat} value compared to the two values of k_{cat} for the antibody-catalyzed reaction leading to formation of the 14-membered ring lactone, **19**. However, the lower K_m value in the six-membered ring data set of 7.6×10^{-5} M compared with those of the 14-membered ring data sets results in comparable values for the catalytic efficiencies for these two antibodies. k_{cat}/K_m values for other catalytic antibodies reported in the literature¹⁵⁹ range from $3.6 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ for a decarboxylating

catalytic antibody to as high as $5.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for an antibody that catalyzes a proton transfer.

3.14 SUMMARY AND CONCLUSIONS

The macrocyclic phosphonate, **38**, was synthesized in nine steps with a modest overall yield of 17%. However, since only 20 mg of **38** was required for this project, obtaining the target was of greater importance than the optimization of its yield, which can be accomplished at a later date. The key step in the synthesis of **38** was the use of the Mitsunobu reaction in the challenging ring-closing step, which proceeded in an 82% yield giving a 5:1 ratio of the two possible diastereomers.

The successful coupling of the macrocyclic transition state analogue **38** to the carrier protein KLH, and subsequent production of monoclonal antibodies against the transition state analogue was evident by the positive results obtained in binding assays. Eight monoclonal antibodies showed binding above background to the BSA-transition state analogue conjugate in the PCFIA binding assay.

Ascites fluid was produced in Balb/c mice from the hybridomas of the three strongest binders, then affinity purified using a SAM column in preparation for

catalytic testing. Of these three monoclonals, only F123 showed significant catalytic activity with substrate **57** as measured by release of the p-nitrophenolate anion.

Isotyping of the hybridoma supernatant revealed monoclonal F123 to be an IgM antibody. It is not clear why F123 is of the IgM class, however, this may suggest that the immuno-conjugate KLH-**50** was stable in the mouse long enough to elicit a primary immune response, but not long enough to allow for class switching to occur.

Pooled catalytic experiments revealed that F123 was catalyzing the production of the macrocyclic lactone **19**. Ether extracts of the pooled reaction solutions were compared with that of an independently synthesized sample of authentic lactone by GC/MS analysis. Conversely, a large scale background experiment with **57** carried out at 37 °C for five days, followed by extraction with ether and GCMS analysis of the ether extracts revealed no detectable lactone.

Comparison of the reaction of F123 with substrate **57** run in parallel with an affinity purified monoclonal antibody raised against an unrelated antigen with **57** confirmed, that the release of p-nitrophenolate anion was not the result of an esterase contaminant; that the release of p-nitrophenolate anion occurred only with F123; and that F123 functions as a specific catalyst. Further evidence of

the specific activity of F123 was established by the lack of activity of F123 with p-nitrophenyl tetradecanoate (**59**).

The reactivity of F123 with substrate **57** in the presence of one equivalent of lactone product **19** showed no product inhibition.

Hapten derivative **60** (in a one-to-one ratio of both diastereomers) proved to be a potent competitive inhibitor of the F123 catalysis with **57** (where the inhibition constant, K_i , for **60** was found to be $2.9 \pm 0.4 \mu\text{M}$), demonstrating that the catalytic activity is associated with binding in the antibody-combining site.

The results of the solubility study of substrate **57** in the reaction buffer revealed that the maximum concentration of **57** in this buffer system to be $400 \mu\text{M}$. An attempt was made to increase the concentration of **57** by increasing the concentration of the Triton X-100. However, it appeared that an increase in Triton X-100 concentration resulted in inhibition of the antibody activity. Hence, $400 \mu\text{M}$ **57** was accepted as the upper solubility limit for this study.

Antibody F123 displayed saturation kinetics, and the Michaelis-Menten kinetic parameters of K_m , $V_{\max}$ and k_{cat} for F123 with substrate **57** were obtained using the method of initial rates and fitted to Lineweaver-Burk analyses using two independent methods. The multiwell method yielded a K_m of $250 \pm 10 \mu\text{M}$, $V_{\max}$

of 0.62 ± 0.01 $\mu\text{mol}/\text{min mg}$ and $k_{\text{cat}} = 1.1 \text{ min}^{-1}$, while the spectrophotometer cuvette method yielded a K_m of $330 \pm 50 \mu\text{M}$, V_{max} of $1.4 \pm 0.1 \mu\text{mol}/\text{min mg}$ and $k_{\text{cat}} = 2.2 \text{ min}^{-1}$. These two methods were found to be in good agreement with each other once we accounted for the time delay inherent in the multiwell method when determining initial rates.

In conclusion, while this work opens the door to the antibody-catalyzed formation of macrocyclic lactones, F123 is not yet viewed as a practical catalyst for preparative scale production of macrocyclic lactone **19**. Much work remains in the full characterization of the activity of antibody F123 as a catalyst for this application.

In general, the chemical approaches towards the syntheses of macrocyclic lactones remain superior in terms of yields, however, biocatalytic methods offer promise as a result of the asymmetric control they often provide, an important factor to synthetic chemists.

3.15 SUGGESTIONS FOR FUTURE WORK

The work described in this thesis is the first step towards the characterization of a monoclonal antibody raised against a macrocyclic phosphonate, capable of catalyzing the formation of a 14-membered ring lactone.

Future directions for this project potentially include:

- 1) Investigations into the use of organic solvents to raise the solubility limit of substrate **57**.
- 2) Testing antibody F123 for stereospecific cyclization, as demonstrated in the work by Napper, *et al.* involving the stereospecific cyclization of a six-membered ring lactone by a catalytic antibody, by reacting F123 with enantiomerically pure isomers of substrate **57**, namely **69** and **70**.

- 3) Varying the structure of the substrate molecule to further the study of the specificity of F123. Some suggestions for substrate modifications include compounds **71**, **72** and **73**. Compounds **71**, **57** and **72** would allow a comparison between the rates of lactonization involving a primary, secondary and a tertiary hydroxyl group. Compound **73** would probe the effect of a double bond, placed at various positions along the open chain molecule, on the rate of lactonization.

Finally, the rates of lactonization for different ring sizes can be probed by varying the chain lengths of the substrate molecules.

4) Production of a more efficient catalyst might be possible by modifying the existing hapten molecule. Two new hapten designs are suggested below, each of which offers qualities not possessed by the hapten used in this study, namely **50**. Perhaps a hapten molecule such as **74** with a 14-membered ring PNP phosphonate and a linker arm extending from a remote junction, rather than through the phosphonate center as in **50**, would serve as a more appropriate transition state analogue for eliciting catalytic antibodies to carry out macrolactonization reactions for two reasons. First, the PNP phosphonate moiety incorporates a nitroaryl ring, believed to be desirable for induction of a strong immune response.¹⁶⁰ Secondly, linking the carrier protein through a more remote position from the phosphonate center should allow greater exposure of the phosphonate center as the key antibody epitope. The second proposed hapten design, **75**, may not resemble the presumed transition state for the reaction as well as **50** or **74**, however, it may provide more stability than the

other potential haptens. This increased stability may lead to the production of a higher affinity IgG class antibody, rather than the IgM class obtained.

- 5) Enzymatic cleavage of the antibody molecule with pepsin, and isolation of the resulting Fab fragments by affinity chromatography followed by a kinetic analysis of the Fab portion, which contains the antibody combining site, for the lactonization reaction to obtain the Michaelis-Menten constants, and compare them to those of the native antibody.
- 6) The Fab fragment might allow NMR and MS studies of the antibody-substrate, and antibody-hapten complexes.
- 7) Crystallization of the Fab-hapten complex may be possible, and would allow sequencing of the antibody combining site. Knowing the amino acid sequence of the combining site would open the door to mutagenesis experiments for the purpose of probing the catalytic mechanism of the reaction.

CHAPTER IV EXPERIMENTAL

4.1 BIOLOGICAL METHODS

Deionized water was used in the preparation of all buffers.

Phosphate buffered saline (PBS), available as a 10x solution, was diluted to give the 1x PBS (8.2 mM NaHPO₄, 137 mM NaCl, 2.7 mM KCl, 1.5 mM H₂PO₄, pH 7.4).

4.1.1 Monoclonal Antibody Purification

Monoclonal antibodies were purified from ascites fluid first by, filter-sterilization through a 0.22 µm filter, followed by immunoaffinity purification using a sheep-antimouse (SAM) affinity column.

The ascites fluid was diluted in half using 1x PBS before 0.80 µm filtration, followed by 0.45 µm, and finally 0.22 µm filtration. The ascites were then loaded onto a SAM affinity column and washed with four bed volumes of 1x PBS. Antibodies were eluted from the SAM column with five bed-volumes of 0.1 M glycine (pH 2.5) and collected in 1.5 mL centrifuge tubes. Eluates were neutralized by the addition of 50 µL of saturated Tris buffer to each tube containing purified antibody. The antibody samples were dialyzed against 1X

PBS buffer, pH 7.4, and the concentration was determined by measuring the absorbance at 280 nm (where the OD reading/1.4 gives the concentration in mg/mL) prior to kinetic assays.

4.1.2 Preparation of Immuno- and Binding-conjugates of 50

Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) were supplied by the Sigma Chemical Co.

4.1.2.1 KLH-50 Hapten Conjugate

To a solution of KLH (15 mg) and **50** (10.0 mg, 28.8 μ mol) in 0.5 mL of 0.1 M MES buffer, pH 4.5, and 4.5 mL of water was added 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) (25.0 mg, 0.131 mmol). The mixture was stirred at room temperature overnight. The coupled KLH-**50** conjugate was then dialyzed against PBS buffer, pH 7.4.

4.1.2.2 BSA-49 Hapten Conjugate-Coated Carboxyl Polystyrene Particles

Into a 30 mL siliconized Corex glass centrifuge tube hapten **50** (1.0 mg, 2.9 μ mol) and BSA (1 mg) dissolved in 8 mL of 0.1M MES buffer, pH 4.5, were added, followed by 1 mL of a 5% (w/v) Fluoricon carboxyl polystyrene assay particle suspension and EDC (5.0 mg, 26 μ mol). The mixture was incubated at room

temperature for 24 hours. The BSA-50 crosslinked particles were then centrifuged (6000 rpm, 22 °C, 20 min, Sorval HB4 rotor) and the resulting pellet washed 2x in 20 mL of PCFIA buffer, pH 7.4 (1x PBS pH 7.4, 2% FCS and 0.2% NaN_3). Finally, the particles were resuspended in a 40 mL volume of PCFIA buffer, pH 7.4, to produce a final concentration of 0.125% (w/v) particles and 25 $\mu\text{g/mL}$ protein.

4.1.3 Kinetic Assays

4.1.3.1 Multiwell Method

The catalytic reactions were performed in 96 well microtiter plates, and monitored in a microtiter plate reader (Bio-Tek Instruments) equipped with the Delta Soft II computer program. All reactions were performed in duplicates.

Typical reactions were 250 μL in total volume, and all contained final concentrations (v/v) of 10% DMSO, 0.5% Triton X-100 and 89.5% 1x PBS buffer, pH 7.4. Catalyzed reactions were performed at 37 °C in the presence of 0.23 μM monoclonal antibody (based on IgM monomer molecular weight of 175 kDa) and were initiated by the addition of various amounts (50–400 μM) of substrate from a DMSO stock solution. After addition of reagents, substrate, and antibody to the wells of the microtiter plate, a multichannel micropipette was used to mix contents until homogeneous. This mixing created bubbles at the surface of the reaction wells due to the Triton X-100, and a 10 minute delay was required for all

bubbles to collapse prior to reading the plates to obtain initial rate data. The rates were determined by measuring the initial change in absorbance, due to 4-nitrophenolate release, at 405 nm ($\epsilon = 10,830 \text{ L/mol}\cdot\text{cm}$). The background hydrolysis rate of the substrate (in the absence of antibody) was also measured each time.

For antibody F123, the antibody-catalyzed reaction rates with four different substrate concentrations from 50 to 400 μM were measured in the presence of 0.23 μM of the antibody concentration. The Michaelis-Menten parameters were determined by standard methods.

4.1.3.2 Spectrophotometer Cuvette Method

Analysis for V_{max} and K_{m} determinations were also performed using a Unicam UV/VIS spectrophotometer in 1 mL quartz cuvettes with 1 cm pathlength to compare this technique with the multiwell method.

Typical reactions were 600 μL in total volume, and all contained final concentrations (v/v) of 10% DMSO, 0.5% Triton X-100 and 89.5% 1x PBS buffer, pH 7.4. Catalyzed reactions were performed at 37 °C in the presence of 0.25 μM monoclonal antibody (based on IgM monomer molecular weight of 175 kDa) and were initiated by the addition of various amounts (50–400 μM) of substrate from a DMSO stock solution. The top of the cuvette was covered with Parafilm and the cell shaken several times prior to placement in the cell holder, and observation

at the desired wavelength was obtained after a one-minute delay time. The rates were determined by measuring the initial change in absorbance, due to 4-nitrophenolate release, at 400 nm ($\epsilon = 10,730$ L/mol·cm). The background hydrolysis rate of the substrate (in the absence of antibody) was also measured each time. The Michaelis-Menten parameters were determined by standard methods.

4.1.4 General Immunological Techniques.

4.1.4.1 Immunizations.

Balb/c mice (6-8 week old females) were immunized with the KLH conjugate of hapten **50** (KLH-**50**) according to the following schedule: day 1, 100 μ g of KLH-**50** emulsified in complete Freund's adjuvant, subcutaneous injection; days 19, 50 and 117, administered booster immunizations of 50 μ g of KLH-**50** emulsified in incomplete Freund's adjuvant, subcutaneous injection; day 127 the mice received an intraperitoneal booster immunization of 15 μ g of KLH-**50** in saline (100 μ L). Four days after this last booster injection, the mice were sacrificed and the spleens harvested.

4.1.4.2 Test Bleeds.

The mouse was first warmed to dilate its blood vessels by shining a heat lamp directly into the open cage (from a distance of approximately 18 inches) until the

tail veins became visible (usually between 5-10 minutes). The mouse was removed and placed in a mouse restraint. The tail was wiped with 70% ethanol to enhance vasodilation of the tail veins. One of the tail veins was then pierced with a 26-gauge needle. Blood from the puncture hole was collected with a sterile heparinized capillary tube, and transferred to a microcentrifuge tube. The microcentrifuge tube was placed in a 37 °C water bath until the blood clotted (approximately 20 minutes). The blood was then centrifuged at 600 g to pellet the clotted cells and separate the serum. The serum was then drawn off and transferred to a new tube and stored at -20 °C until needed.

4.1.5 Polyethylene Glycol (PEG) Fusion

The procedures described here are derived from a protocol prepared by Ms. Helen Merkens in Prof. Hermann Ziltener's lab at The Biomedical Research Centre entitled *Fusion Protocol for Mouse Spleen Cell Hybrids for Monoclonal Antibody Production*.

Table 9. Media and components used in hybridoma production.

Media	Components
DMEM	1 M Hepes buffer 0.37% HCO ₃ ⁻ 2 mM L-Glutamine
HT Medium	1x10 ⁻⁴ M hypoxanthine 1.6x10 ⁻⁵ M thymidine

HAT Medium	1×10^{-4} M hypoxanthine 4×10^{-7} M aminopterin 1.6×10^{-5} M thymidine 1 M NaOH
Lysing Solution	0.17 M NH_4Cl 0.017 M Tris-HCl, pH 7.2-7.4
Fusion Medium	DMEM High Glucose or RPMI 1640 20% FBS 5×10^{-4} M 2 β -mercaptoethanol 2% 10x 3T3 conditioned medium 2 mM 1-glutamine
Freeze Medium	90% FCS 10% DMSO
PCFIA Buffer	2% FCS 0.2% sodium azide 1x PBS

4.1.5.1 Cell Counting

To a 10 μL aliquot of a thoroughly suspended cell sample in a microfusion tube is added 10 μL of a 0.2% (w/v) solution of trypan blue, a cell staining dye, in aqueous 580 mM NaCl solution and the resulting suspension thoroughly mixed. A 10 μL volume of the cell suspension is slowly pipetted into the sample groove of a clean hemacytometer. The hemacytometer is placed on a inverted phase microscope and the cells in the four counting grids are counted, divided by four, and multiplied by 1×10^4 . This number is then multiplied by two to account for the trypan blue dilution to afford the number of cells/mL.

4.1.5.2 Myelomas

As the myeloma fusion partner, we used P3-X63-Ag8-653 (clone 653). The cells must be in the log phase (2×10^5 viable cells/mL) at the time of fusion.

Day 1. The clone 653 is expanded into 4 x 30 mL of DMEM-20% heat inactivated FCS at approximately 10^3 cells/mL.

Day 4. The cells are counted and each dish is split into four new dishes and brought up to a volume of 30 mL each with DMEM-20% heat inactivated FCS at approximately 10^5 cells/mL.

Day 5. The cell count is approximately 2×10^5 live cells per mL, and 2×10^7 live cells with a 99% viability are required per fusion.

4.1.5.3 PEG Solution for Cell Fusion

A 50% (w/w) solution of polyethylene glycol (Merck PEG 4000) was prepared by autoclaving 10 gram of polyethylene glycol at 121 °C for 10 minutes. The liquid was cooled to about 56 °C and 10 mL of warm DMEM was added. Finally, the mixture was neutralized with 7.5% HCO_3^- and stored at room temperature.

4.1.5.4 Harvesting Spleenocytes

All dissecting instruments are sterilized in 70% ethanol prior to use.

The mouse was anesthetized with carbon dioxide and killed by cervical dislocation, then wetted with 70% ethanol. A small incision was made through the skin of the abdomen. The skin was then stretched and torn to expose the peritoneal cavity along the left side of the mouse. An incision was made through the muscle of the peritoneal wall and the spleen carefully removed. Tweezers were used to extract the spleen and gently remove any connective tissue. The spleen was quickly transferred into a 10 mL sterile tube containing medium.

A spleen cell suspension was prepared by gently mashing the spleen through a fine mesh screen using the rubber end of a 10 cc syringe plunger into a Petri dish containing 2% FCS-Hanks* solution, pH 7.4. The cell suspension was then transferred into a 10 mL centrifuge tube, and the cells centrifuged at 300 g for 5 minutes. The supernatant was aspirated off and the cell pellet is resuspended in 5 mL of red blood cell lysing solution at 37 °C for 7 minutes. The lysing buffer was neutralized by adding 5 mL of 2% FCS-Hanks solution and the cell suspension then centrifuged at 300 g for 5 minutes. The cell pellet was resuspended in 10 mL of 2% FCS-Hanks solution and a 1 in 20 dilution was made from which the number of viable cells was counted. This procedure results

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* See ref 161 for a list of components contained in Hanks buffer.

in $> 1 \times 10^8$ cells of approximately 95% viability. 10^8 live spleen cells are used per fusion.

4.1.5.5 Harvesting Myelomas

Healthy myelomas (in log phase, phase bright with smooth cell membrane, less than 2% dead by trypan blue staining) were pelleted by centrifugation at 300 g for 5 minutes, decanted, resuspended in 30 mL of complete DMEM and then transferred to a 50 mL conical tube. The cells were again pelleted by centrifugation, washed twice with 50 mL of serum-free DMEM, and resuspended in 10 mL of DMEM. An aliquot was diluted 1:10 (v/v) and then the cells were counted by the method described above.

4.1.5.6 Fusion

Myeloma and spleen cells are combined in a ratio of 1:5 in a 50 mL Falcon tube, centrifuged at 300 g for 5 minutes, and the supernatant was removed. The cell pellet was then resuspended in serum-free medium. This wash step was repeated three times. It is essential to remove all serum from the cell surface since this inhibits the fusion process. After the last wash the medium was removed until there was only a meniscus of liquid over the cell pellet. The tube bottom was knocked on a hard surface to liquify the pellet. Prior to the fusion, the PEG and serum-free RPMI* solutions were warmed to 37 °C. Next, 0.8 mL

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* See ref 161 for a list of components contained in RPMI buffer.

of the PEG solution was added dropwise to the pellet over 60 seconds while gently tapping the tube. The mixture was allowed to stand for one minute before 1 mL of serum-free RPMI solution, pH 7.4, was added dropwise over 60 seconds, followed by an additional 20 mL over a 5 minute period. The cells were then centrifuged for 5 minutes at 300 g, and the supernatant was aspirated off. Finally, the pellet was resuspended in 40 mL of fusion medium.

4.1.5.7 Transferring Fused Cells to the Growth Plates

The cell suspension was plated into four 96-well flat bottomed plates, each well received 100 μ L or 5×10^4 cells, and the final volume of each well made up to 200 μ L with fusion medium. On days 1, 2, 3, 7, 10 and 14, approximately half of the medium in each well was removed using a sterile pasteur pipette and suction, and replaced with 100 μ L of fresh fusion medium. After approximately 7-8 days, colonies of hybridomas were visible.

4.1.5.8 Selection and Expansion of Hybridomas

The particle concentration fluorescence immunoassay (PCFIA) technique was used in place of the traditional enzyme-linked immunosorbant assay (ELISA) for the initial screening of hybridomas. The PCFIA assays were carried out using hapten-protein conjugate-coated carboxyl polystyrene particles, 96 well filter assay plates, a goat antimouse fluorescein isothiocyanate (FITC) secondary

antibody for detection at 535 nm wavelength (excitation at 485 nm), and a PCFIA buffer (filtered through a 0.22 μm filter), pH 7.4.

Supernatants from the hybridoma cells were screened for hapten affinity using the PCFIA immunoassay with BSA-50 conjugate-coated carboxyl polystyrene particles as the solid phase.

Cells from cultures displaying anti-hapten antibodies were subcloned by limited dilution in 96-well plates as described below.

4.1.5.9 Cloning Cell Lines

Any hybridoma cells from cultures displaying anti-hapten activity were cloned by limited dilution. The cells from a positive well (100 μL of cell suspension) were transferred and titrated 1:2 dilution step down the first column of a fresh 96-well growth plate that contained 100 μL of media per well. The column was then also titrated across the plate and each well made up to a final volume of 100 μL with additional media. The cells were allowed to grow for approximately 10 days, supernatants from wells with hybridomas were assayed, and then hapten-specific colonies were expanded as described above. Copies of these clones were expanded in 2 mL tissue culture dishes and frozen in liquid nitrogen. To prepare the clones for freezing, they were expanded in growth media. Once a cell density of $1\text{-}2 \times 10^5$ cells/mL was reached the cells were

centrifuged at 300 g for 5 minutes and supernatant was removed. The cell pellet was resuspended in freeze medium at a concentration of 2×10^6 cells/mL and transferred to a 1 mL freezing vial. The cells were then cooled at a rate of $1^\circ\text{C}/\text{minute}$ until they were frozen at a temperature -195°C . The frozen cells were then transferred into the liquid nitrogen storage tank. Once the hybridomas have been cloned and copies frozen in liquid nitrogen, they can be assayed for catalytic activity. Large quantities of monoclonal antibodies were produced in pristane-primed Balb/c mice as described below.

4.1.5.10   Ascites Production

Prior to ascites production, the Balb/c mice (6-8 week old females) were primed by injection of 200 μL of pristane (2,6,10,14-tetramethylpentadecane) into the interperitoneal cavity. Pristane acts as an irritant causing macrophages to secrete cytokines thus creating a good environment for growing hybridomas. Approximately 10-14 days after pristane priming, each mouse was injected with 1×10^6 hybridoma cells in 0.5 mL of PBS into the peritoneal cavity using a 22 gauge needle. After 10-14 days the liquid tumors (ascites fluid) were ready to harvest. The mice were anesthetized with carbon dioxide and killed by cervical dislocation. A small incision was made in the skin above the abdomen and the skin pulled back to expose the abdomen muscles. Using tweezers, the muscles were pinched and held upwards like a tent, and a small incision was made through the muscles just large enough to drain the ascites fluid using a

disposable Liquepette. The fluid was centrifuged to pellet any cells and the oily pristane layer on top was removed. The ascites fluid was decanted into a new tube and stored at -20 °C.

4.2 GENERAL CHEMICAL METHODS

Unless otherwise stated, all reactions were performed under a N₂ atmosphere using flame-dried glassware. Cold temperature baths were prepared as follows: -78 °C (dry ice-acetone), -20 °C (dry ice-CCl₄), and 0 °C (ice-water).

Anhydrous reagents and solvents were purified and prepared according to literature procedures.¹⁶² Chromatographic solvents and reagents were used as received unless noted otherwise. The low boiling fraction (35-60 °C) of petroleum ether was used. All reagents were supplied by the Aldrich Chemical Co. and unless otherwise stated were used without further purification. *n*-Butyllithium (*n*-BuLi) was standardized by titration against 2,2-diphenylacetic acid in THF at room temperature to the appearance of a faint yellow colour.

All reactions were monitored by thin layer chromatography (TLC) and were judged to be completed when the starting material was consumed as determined by TLC. In the description of reaction work-ups, washing with brine refers to a saturated solution of NaCl, drying of the organic phase was accomplished with

MgSO₄ and removal of solvent *in vacuo* or concentration of solvent refers to the use of a rotary evaporator using a water aspirator and heating using a water bath.

Preparative flash chromatography¹⁶³ was performed using 230-400 mesh ASTM silica gel supplied by E. Merck Co. As an indicator of purity, all compounds were purified such that they showed a single spot by TLC.

Melting points (mp) were determined using a Mel-Temp III (Laboratory Devices) melting point apparatus and are uncorrected.

Infrared (IR) spectra were recorded on a Bomem Michelson 100 FT-IR spectrometer using internal calibration. IR spectra were recorded on the neat liquid or as a chloroform solution using NaCl solution cells.

Except where noted, proton nuclear magnetic resonance spectra (¹H NMR) were recorded in deuteriochloroform solutions on a Bruker AC-200 (200 MHz) or Bruker WH-400 (400 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale versus chloroform (δ 7.24 ppm) as an internal standard. Signal multiplicity, spin-spin coupling constants (where possible) and integration ratios are indicated in parentheses. Phosphorus nuclear magnetic resonance spectra (³¹P NMR) with proton decoupling were recorded in

deuteriochloroform solutions on a Bruker AC-200 (81.75 MHz) spectrometer. Chemical shifts are given in parts per million (ppm) on the δ scale versus 85% phosphoric acid as an external standard.

Low- and high-resolution electron-impact (EI) mass spectral analyses were performed on a Kratos-AEI model MS 50 mass spectrometer. An ionization energy of 70 eV was used in all measurements. Low-resolution desorption chemical ionization (DCI) mass spectral analyses, using CH_4 or NH_3 reagent gas as indicated, were recorded on a Delsi Nermag R10-10C mass spectrometer. High-resolution DCI mass spectral analyses, using CH_4 or NH_3 reagent gas as indicated, were recorded on a Kratos MS 80 RFA mass spectrometer.

Kinetic analyses using ultraviolet-visible (UV-Vis) spectroscopy were performed on a UV-4 Unicam spectrophotometer.

Thin-layer chromatography was performed on Merck silica gel 60 F₂₅₄ pre-coated aluminium sheets. Visualization was achieved by irradiation with ultraviolet light at 254 nm and/or by spraying with anisaldehyde reagent (a solution of 1 mL anisaldehyde, 5 mL conc. H_2SO_4 and 10 mL glacial acetic acid in 90 mL MeOH) followed by heating.

Microanalyses were carried out at the microanalytical laboratory of the University of British Columbia Chemistry Department using a Carlo Erba Elemental Analyzer 1106. Samples for microanalysis were purified by column chromatography using the solvent system indicated for each compound.

4.3 CHEMICAL METHODS

11-(Tetrahydropyranyloxy)-dodecanal (**33**).

To a stirred solution of oxalyl chloride (1.01 mL, 11.5 mmol) in 75 mL of dry CH_2Cl_2 under N_2 at -78°C was added DMSO (1.80 mL, 25.2 mmol) dissolved in 5 mL CH_2Cl_2 via a stainless steel cannula. After 10 min. of stirring, compound **42** (3.00 g, 3.50 mmol) in 25 mL of CH_2Cl_2 was added to the activated DMSO via a stainless steel cannula. Finally, after stirring an additional 10 min., Et_3N (7.31 mL, 52.5 mmol) was syringed in, and the reaction mixture was allowed to warm to room temperature while maintaining stirring. After 3 h, the solution was diluted with 100 mL of Et_2O followed by 100 mL of water. The aqueous layer was further extracted with 3 x 50 mL of Et_2O and the combined Et_2O extracts were dried over anhydrous MgSO_4 , filtered and concentrated under reduced pressure to a yellow oil. The crude product was then purified by flash chromatography using 4:1 petroleum ether and ethyl acetate to afford 2.59 g (87%) of **33** as a colourless oil.

IR (neat): 2927, 2854, 2728, 1725, 1458, 1362, 1322, 1124, 1075, 901,
867 cm^{-1} ;

^1H NMR (200 MHz, CDCl_3) δ : 9.74 (t, $J = 1.7$ Hz, 1H), 4.68 (m, 0.5H), 4.62

(m, 0.5H), 3.88 (m, 1H), 3.75 (m, 0.5H), 3.68 (m, 0.5H), 3.46 (m, 1H),
 2.38 (m, 2H), 1.22-1.90 (m, 22H), 1.18 (d, $J = 6.2$ Hz, 1.5H), 1.07
 (d, $J = 6.2$ Hz, 1.5H);

LRMS (DCI, NH_3) m/z (relative intensity): 302 ($M^+ + 18$, 12), 285 ($M^+ + 1$, 3),
 218 (30), 201 (15);

HRMS (DCI, CH_4) calcd. for $\text{C}_{17}\text{H}_{32}\text{O}_3$: 284.2351, found 284.2409.

1-Diphenylphosphinyl-12-(tetrahydropyranyloxy)-1-tridecene (36a and 36b).

The Wittig reagent **32** (2.15 g, 4.23 mmol) and aldehyde **33** (1.2 g, 4.2 mmol) were dissolved in 17 mL of toluene and refluxed under N_2 for 3 days. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography using 5:1 petroleum ether and ethyl acetate to afford 1.12 g (52%) of a colourless oil which was a mixture of cis and trans isomers **36a** and **36b**. A small quantity of the crude product was repurified by flash chromatography using 5:1 petroleum ether and ethyl acetate and pure **36a**, the cis isomer, and pure **36b**, the trans isomer, were isolated separately for analysis.

Cis isomer **36a**:

IR (neat): 2929, 2855, 1623, 1593, 1490, 1459, 1377, 1270, 1192,

1163, 1129, 1074, 1025, 998, 931, 763 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.15-7.38 (m, 10H), 6.59 (ddt, $J = 7.8, 13.0, 57.4$ Hz, 1H), 5.76 (dd, $J = 13.0, 21.8$ Hz, 1H), 4.74 (m, 0.5H), 4.67 (m, 0.5H), 3.94 (m, 1H), 3.81 (m, 0.5H), 3.74 (m, 0.5H), 3.52 (m, 1H), 2.27 (dt, $J = 7.8, 7.8$ Hz, 2H), 1.85 (m, 1H), 1.72 (m, 1H), 1.24-1.68 (m, 20H), 1.23 (d, $J = 6.2$ Hz, 1.5H), 1.13 (d, $J = 6.2$ Hz, 1.5H);

^{31}P NMR (81 MHz, CDCl_3) δ : 9.7;

LRMS (EI) m/z (relative intensity): 514 (0.2), 429 (6), 415 (20), 414 (54), 413 (100), 94 (28), 85 (31);

HRMS calcd. for $\text{C}_{30}\text{H}_{43}\text{O}_5\text{P}$: 514.2848, found 514.2835;

Anal. Calcd. for $\text{C}_{30}\text{H}_{43}\text{O}_5\text{P}$: C, 70.02; H, 8.42. Found: C, 69.93; H, 8.34.

Trans isomer **36b**:

IR (neat): 2929, 2854, 1628, 1593, 1490, 1455, 1374, 1273, 1198,

1164, 1128, 1075, 1025, 996, 932, 869, 826, 767 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.10-7.38 (m, 10H), 7.20 (ddt, $J = 6.6, 17.0, 25.4$ Hz, 1H), 5.88 (dd, $J = 17.0, 23.4$ Hz, 1H), 4.74 (m, 0.5H), 4.67 (m, 0.5H), 3.94 (m, 1H), 3.81 (m, 0.5H), 3.74 (m, 0.5H), 3.52 (m, 1H), 2.27 (dt, $J = 6.6, 7.0$ Hz, 2H), 1.85 (m, 1H), 1.72 (m, 1H), 1.24-1.68 (m, 20H), 1.23 (d, $J = 6.2$ Hz, 1.5H), 1.13 (d, $J = 6.2$ Hz, 1.5H);

^{31}P NMR (81 MHz, CDCl_3) δ : 11.9;

LRMS (EI) m/z (relative intensity): 514 (0.2), 429 (6), 415 (20), 414 (54), 413 (100), 94 (28), 85 (31);

HRMS calcd. for $\text{C}_{30}\text{H}_{43}\text{O}_5\text{P}$: 514.2848, found 514.2852;

Anal. Calcd. for $\text{C}_{30}\text{H}_{43}\text{O}_5\text{P}$: C, 70.02; H, 8.42. Found: C, 69.91; H, 8.30.

12-Hydroxy-1-diphenylphosphinyl-tridecane (37).

p-TsOH (0.65 g, 3.4 mmol) was added to a stirred solution of compound **43** (8.75 g, 17.0 mmol) in 200 mL of MeOH at room temperature. After 18 h, the MeOH was removed under reduced pressure and the resulting oil was dissolved in Et_2O , washed with 2 x 50 mL of water followed by 2 x 50 mL of brine, dried over anhydrous MgSO_4 , filtered and concentrated under reduced pressure. The crude oil was purified by flash chromatography using 1:1 petroleum ether and ethyl acetate to afford 6.31 g (86%) of alcohol **37** as a colourless oil.

IR (neat): 3424, 3066, 2925, 2854, 1592, 1490, 1460, 1265, 1214, 1192,

1163, 1071, 1025, 1007, 934, 802, 764 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.11-7.32 (m, 10H), 3.76 (m, 1H), 2.04 (m, 2H),

1.77 (m, 2H), 1.20-1.50 (m, 18H), 1.15 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 25.9;

LRMS (EI) m/z (relative intensity): 432 (1), 431 (4), 415 (7), 414 (19);

HRMS calcd. for $\text{C}_{25}\text{H}_{37}\text{O}_4\text{P}$: 432.2429, found 432.2398;

Anal. Calcd. for $\text{C}_{25}\text{H}_{37}\text{O}_4\text{P}$: C, 69.41; H, 8.63. Found: C, 69.62; H, 8.72.

1-Phenyl-1-oxo-1-phospho-13-tetradecanolide (38a and 38b) and Dimer

39.

38a + 38b

39

To a stirred solution of compound **44** (125 mg, 0.351 mmol) in 150 mL of dry PhH at room temperature under N_2 was added Ph_3P (368 mg, 1.40 mmol) followed by DEAD (221 μL , 1.40 mmol). After 2 h, the solvent was removed under reduced pressure and the resulting crude mixture was purified by flash chromatography using 2:1 hexane and ethyl acetate to afford the separated diastereomers; **38a** and **38b**, in a 5:1 ratio based on the recovered yields, and a combined yield of 97.1 mg (82%). In addition, 6 mg of the dimeric side product, **39**, was isolated as a colourless, crystalline solid. The R_f values of

the three cyclic products, **38a**, **38b** and **39**, were found to be 0.71, 0.57 and 0.62 respectively by thin-layer chromatography using a 1:1 mixture of petroleum ether and ethyl acetate.

Diastereomer **38a** (faster moving by TLC):

IR (neat): 2927, 2859, 1593, 1490, 1458, 1380, 1253, 1210, 1163, 1071, 991, 920, 766 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.05-7.38 (m, 5H), 4.75 (m, 1H), 1.24-1.88 (m, 22H), 1.22 (d, $J = 6.3$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 29.6;

LRMS (EI) m/z (relative intensity): 338 (52), 323 (5), 227 (16), 199 (9), 185 (38), 172 (69), 94 (100), 93 (31), 77 (17), 43 (39);

HRMS calcd. for $\text{C}_{19}\text{H}_{31}\text{O}_3\text{P}$: 338.2011, found 338.2002;

Anal. Calcd. for $\text{C}_{19}\text{H}_{31}\text{O}_3\text{P}$: C, 67.42; H, 9.24. Found: C, 67.20; H, 9.20.

Diastereomer **38b** (slower moving by TLC):

mp: 97-99 $^{\circ}\text{C}$

IR (CDCl_3): 2932, 2860, 1593, 1492, 1456, 1223, 1011 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.05-7.38 (m, 5H), 4.65 (m, 1H), 1.93 (m, 2H), 1.20-1.73 (m, 20H), 1.43 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 27.8;

LRMS (EI) m/z (relative intensity): 338 (26), 323 (2), 227 (11), 199 (7), 185

(30), 172 (59), 94 (100), 93 (3), 77 (20), 43 (75);

HRMS calcd. for $C_{19}H_{31}O_3P$: 338.2011, found 338.2009;

Anal. Calcd. for $C_{19}H_{31}O_3P$: C, 67.42; H, 9.24. Found: C, 67.15; H, 9.39.

Dimer 39:

mp: 103-105°C

IR ($CDCl_3$): 2930, 2856, 1593, 1491, 1229, 1012 cm^{-1} ;

^1H NMR (400 MHz, $CDCl_3$) δ : 7.10-7.33 (m, 10H), 4.66 (m, 2H), 1.84 (m, 4H),
1.68 (m, 4H), 1.56 (m, 4H), 1.20-1.45 (m, 40H), 1.15 (d, $J = 6.2$ Hz,
6H);

^{31}P NMR (81 MHz, $CDCl_3$) δ : 29.0;

LRMS (EI) m/z (relative intensity): 676 (1), 661 (1), 583 (100), 339 (19),

338 (10), 245 (7), 227 (12), 199 (6), 185 (15), 172 (21), 94 (27), 77 (5);

HRMS calcd. for $C_{38}H_{62}O_6P_2$: 676.4022, found 676.4002;

Anal. Calcd. for $C_{38}H_{62}O_6P_2$: C, 67.42; H, 9.24. Found: C, 67.51; H, 9.28.

11-Dodecen-2-ol (40).

To a stirred solution of commercially available 10-undecenal (8.1 g, 48 mmol) in 250 mL of THF under N_2 at 0 °C was slowly added 3 M methylmagnesium bromide (18 mL, 53 mmol). After 0.5 h the reaction was quenched with

saturated aqueous NH_4Cl , diluted with Et_2O , and the resulting precipitate was filtered and discarded. The filtrate was washed with 2 x 100 mL of brine, dried over anhydrous MgSO_4 , filtered and the solvent removed under reduced pressure to give 13.6 g of crude oil. The residue was purified by flash chromatography using 4:1 petroleum ether and ethyl acetate to afford 7.96 g (90%) of **40** as a colourless oil.

IR (neat): 3351, 3077, 2922, 2855, 1641, 1457, 1373, 1122, 994, 910, 842, 722 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 5.78 (ddt, $J = 6.7, 10.4, 17.3$ Hz, 1H), 4.94 (d, $J = 10.4, 17.1$ Hz, 2H), 3.75 (m, 1H), 2.02 (dt, $J = 6.3, 6.7$ Hz, 2H), 1.63 (br. s, 1H), 1.20-1.50 (m, 14H), 1.16 (d, $J = 6.2$ Hz, 3H);

LRMS (EI) m/z (relative intensity): 184 (0.4), 166 (2), 151 (1), 141 (2), 138 (3), 124 (10), 109 (10), 96 (29), 82 (51), 69 (40), 45 (100), 41 (62);

HRMS calcd. for $\text{C}_{12}\text{H}_{24}\text{O}$: 184.1827, found 184.1832;

Anal. Calcd. for $\text{C}_{12}\text{H}_{24}\text{O}$: C, 78.20; H, 13.12. Found: C, 78.40; H, 12.99.

11-(Tetrahydropyranyloxy)-1-dodecene (41).

To a stirred solution of **40** (10 g, 54 mmol) in 300 mL of CH_2Cl_2 was added DHP (5.5 mL, 65 mmol) and p-TsOH (0.52 g, 2.7 mmol), and the mixture was stirred at room temperature under N_2 for 2 h. The solvent was then removed and the residue was diluted with Et_2O . The ether layer was washed with 2 x 25 mL of saturated NaHCO_3 , 1 x 25 mL water and 1 x 50 mL of brine, and dried over anhydrous MgSO_4 and filtered. The solvent was removed under reduced pressure and the crude material (15.7 g of oil) was purified by flash chromatography using 10:1 petroleum ether and ethyl acetate to afford 12.2 g (84%) of **41** as a colourless oil.

IR (neat): 3076, 2929, 2855, 1727, 1641, 1453, 1375, 1352, 1321, 1260,

1200, 1126, 1076, 1026, 996, 911, 869, 812, 734 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 5.78 (ddt, $J = 6.7, 10.4, 17.1$ Hz, 1H), 4.93

(dd, $J = 10.4, 17.1$ Hz, 2H), 4.70 (m, 0.5H), 4.59 (m, 0.5H), 3.89

(m, 1H), 3.76 (m, 0.5H), 3.68 (m, 0.5H), 3.47 (m, 1H), 2.02 (dt,

$J = 6.3, 6.7$ Hz, 2H), 1.22-1.90 (m, 20H), 1.07 (d, $J = 6.2$ Hz, 1.5H),

1.19 (d, $J = 6.2$ Hz, 1.5H);

LRMS (EI) m/z (relative intensity): 268 (0.3), 182 (1), 167 (2), 129 (25), 101

(43), 97 (28), 85 (100), 83 (36), 69 (40), 55 (73), 41 (67);

HRMS calcd. for $\text{C}_{17}\text{H}_{32}\text{O}_2$: 268.2402, found 268.2404;

Anal. Calcd. for $\text{C}_{17}\text{H}_{32}\text{O}_2$: C, 76.06; H, 12.02. Found: C, 76.23; H, 11.95.

11-(Tetrahydropyranyloxy)-1-dodecanol (42).

To compound **41** (1.00 g, 3.73 mmol) in 5 mL of dry THF at 0 °C under N₂ was added 1 M BH₃.THF (3.0 mL, 3.0 mmol) dropwise via syringe. After 1 h, the reaction was quenched with 1.0 mL of water, and oxidized by the addition of 1.2 mL of 3N NaOH and 1.2 mL of 30% H₂O₂. After 15 minutes of vigorous stirring, the mixture was extracted with 3 x 50 mL of Et₂O. The combined ether extracts were dried over MgSO₄, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using 4:1 petroleum ether and ethyl acetate to afford 1.02 g (96%) of **42** as a colourless oil.

IR (neat): 3391, 2928, 2855, 1455, 1375, 1339, 1321, 1260, 1200, 1184,

1126, 1076, 1026, 995, 941, 904, 869, 811, 722 cm⁻¹;

¹H NMR (400 MHz, CDCl₃) δ: 4.70 (m, 0.5H), 4.59 (m, 0.5H), 3.89 (m, 1H),

3.76 (m, 0.5H), 3.68 (m, 0.5H), 3.62 (dt, J = 5.1, 6.4 Hz, 2H), 3.47

(m, 1H), 1.81 (m, 1H), 1.68 (m, 1H), 1.53 (m, 6H), 1.18-1.43 (m, 16H),

1.19 (d, J = 6.2 Hz, 1.5H), 1.08 (d, J = 6.2 Hz, 1.5H);

LRMS (DCI, NH₃) *m/z* (relative intensity): 304 (M⁺ + 18, 100), 287 (M⁺ + 1,

53), 286 (M⁺, 5), 262 (6);

HRMS (DCI, CH₄) calcd. for C₁₇H₃₄O₃: 286.2508, found 286.2499;

Anal. Calcd. for C₁₇H₃₄O₃: C, 71.27; H, 11.97. Found: C, 71.04; H, 11.91.

1-Diphenylphosphinyl-12-(tetrahydropyranyloxy)-tridecane (43).

A spatula tip of Pd-C (10%) in 250 mL of EtOAc was saturated with H₂ for 15 h before compound **36** (9.00 g, 17.5 mmol) in 5 mL of EtOAc was added via a steel cannula. This mixture was allowed to stir for an additional 3 days under H₂ gas at a pressure slightly greater than one atmosphere. The reaction mixture was filtered through Celite and concentrated under reduced pressure to afford 8.95 g (99%) of **43** as a colourless oil.

IR (neat): 2929, 2854, 1739, 1593, 1491, 1461, 1373, 1272, 1200, 1163, 1127, 1074, 1026, 931, 870, 808, 764 cm⁻¹;

¹H NMR (400 MHz, CDCl₃) δ: 7.11-7.32 (m, 10H), 4.68 (m, 0.5H), 4.62 (m, 0.5H), 3.88 (m, 1H), 3.72 (m, 1H), 3.47 (m, 1H), 2.04 (m, 2H), 1.22-1.90 (m, 26H), 1.08 (d, J = 6.2 Hz, 1.5H), 1.19 (d, J = 6.2 Hz, 1.5H);

³¹P NMR (81 MHz, CDCl₃) δ: 26.0;

LRMS (EI) *m/z* (relative intensity): 516 (0.4), 472 (0.2), 416 (42), 415 (68),

388 (30), 359 (3), 345 (5), 303 (6), 289 (4), 261 (22), 248 (29), 172 (11), 141 (10), 94 (81), 85 (61), 78 (98), 77 (46), 55 (100);

HRMS calcd. for $C_{30}H_{45}O_5P$: 516.3005, found 516.3007;

Anal. Calcd. for $C_{30}H_{45}O_5P$: C, 69.73; H, 8.78. Found: C, 69.94; H, 8.90.

Phenyl 12-Hydroxytridecane Phosphonic acid (44).

Compound **37** (199 mg, 0.461 mmol) was dissolved in 5 mL of THF and 20 mL of 3M KOH, and refluxed for 1 h. The THF was removed under reduced pressure and the remaining aqueous solution was acidified with 4N HCl, then extracted with Et_2O . The combined Et_2O extracts were dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure to afford 125 mg (76%) of **44** as a white solid.

mp: 72-73°C

IR ($CDCl_3$): 3614, 2929, 2856, 1593, 1492, 1456, 1213, 1010, 984 cm^{-1} ;

^1H NMR (400 MHz, $CDCl_3$) δ : 7.08-7.30 (m, 5H), 6.82 (br s, 2H), 3.78 (m, 1H), 1.80 (m, 2H), 1.62 (m, 2H), 1.20-1.50 (m, 18H), 1.17 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, $CDCl_3$) δ : 32.7.

LRMS (EI) m/z (relative intensity): 356 (0.8), 355 (3), 341 (8), 339 (2),

338 (5), 312(17), 227 (12), 199 (12), 185 (49), 172 (79), 157 (2), 110

(4), 94 (100), 77 (22);

HRMS calcd. for $C_{19}H_{33}O_4P$: 356.2116, found 356.2108;

Anal. Calcd. for $C_{19}H_{33}O_4P$: C, 64.01; H, 9.34. Found: C, 64.22; H, 9.45.

N-Cbz-5-amino-1-pentanol (48).

To a stirred solution of commercially available 5-amino-1-pentanol (1.00 g, 9.69 mmol) in 30 mL of aqueous Na_2CO_3 at 0 °C was added benzyl chloroformate (Cbz-Cl) (0.96 mL, 11 mmol) drop wise. The reaction mixture was stirred and allowed to warm to room temperature. After one hour the solution was diluted with EtOAc (50 mL) and the organic layer was extracted. The aqueous layer was extracted twice more with EtOAc (75 mL). The combined organic extracts were washed with 1M HCl (50 mL), saturated aqueous $NaHCO_3$ (50 mL), and finally with water (2 x 50 mL). The mixture was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography using 2:1 petroleum ether and ethyl acetate to afforded 0.78 g (34%) of **48** as a white

solid. In addition, 1.69 g of colourless oil, thought to be the di-protected product, was isolated and subsequently hydrolyzed in 1M NaOH under reflux conditions. This reaction mixture was neutralized with 1M HCl, extracted with EtOAc, dried over anhydrous MgSO_4 , filtered and the solvent was removed under reduced pressure to yield an additional 1.08 g of compound **48**. The combined yield of **48** was 81%.

mp: 43-45 °C;

IR (CDCl_3): 3692, 3620, 3453, 2939, 2862, 1718, 1516, 1232 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.33 (m, 5H), 5.08 (s, 2H), 4.72 (br s, 1H),

3.62 (t, $J = 6.4$ Hz, 2H), 3.19 (q, $J = 6.5$ Hz, 2H), 1.65-1.30 (m, 6H);

LRMS (DCI, NH_3) m/z (relative intensity): 255 ($\text{M}^+ + 18$, 10), 238 ($\text{M}^+ + 1$, 75),

237 (M^+ , 3), 220 (1), 194 (99), 146 (2), 108 (62), 102 (15), 91 (100);

HRMS calcd. for $\text{C}_{13}\text{H}_{19}\text{NO}_3$: 237.1365, found 237.1373;

Anal. Calcd. for $\text{C}_{13}\text{H}_{19}\text{NO}_3$: C, 65.78; H, 8.07; N, 5.91. Found: C, 65.86;

H, 8.23; N 5.93.

Cbz linked 1-oxo-1-phospho-13-tetradecanolide (49a). **49a**

The alcohol **48** ($\text{HO}(\text{CH}_2)_5\text{NHCbz}$) (26.0 mg, 108 μmol) was dissolved in 4.0 mL of THF at -78°C under a N_2 atmosphere and treated with 1.2 M $n\text{BuLi}$ (98.0 μL , 118 μmol). The alkoxide was allowed to form for 10 min before this mixture was cannulated into a stirred solution of **38b** (16.6 mg, 49.0 μmol) in 8.0 mL of THF at -78°C under a N_2 atmosphere. This mixture was allowed to warm slowly to room temperature, and was stirred overnight. The THF was then removed under reduced pressure, and the crude oil purified by column chromatography (2% MeOH in CH_2Cl_2) to afford 13.8 mg (59%) of **49a**, a colourless oil.

IR (CDCl_3): 3453, 2935, 2861, 1715, 1516, 1451, 1233, 1016 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.34 (m, 5H), 5.07 (s, 2H), 4.86 (br s, 1H),

4.59 (ddq, $J = 5.3, 6.2, 6.5$ Hz, 1H), 3.95 (m, 2H), 3.17 (dt, $J = 6.4,$

6.5 Hz, 2H), 1.20-1.80 (m, 28H), 1.30 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 32.5;

LRMS (DCI, NH_3) m/z (relative intensity): 482 ($M^+ + 1$, 100), 374 (3), 284 (4),

255 (14), 238 (30), 194 (31), 91 (10);

HRMS calcd. for $\text{C}_{26}\text{H}_{44}\text{NO}_5\text{P}$: 481.2957, found 482.3032 ($M^+ + 1$);

Anal. Calcd. for $\text{C}_{26}\text{H}_{44}\text{NO}_5\text{P}$: C, 64.83; H, 9.21; N, 2.91. Found: C, 64.59; H,

9.10; N, 3.00.

Cbz linked 1-oxo-1-phospho-13-tetradecanolide (49b).

49b

Alcohol **48** ($\text{HO}(\text{CH}_2)_5\text{NHCbz}$) (59.0 mg, 230 μmol) was dissolved in 15.0 mL of THF at -78°C under a N_2 atmosphere and treated with 1.4 M $n\text{BuLi}$ (176 μL , 250 μmol). The alkoxide was allowed to form for 10 min before this mixture was cannulated into a stirred solution of **38a** (69.0 mg, 283 μmol) in 10.0 mL of THF at -78°C under a N_2 atmosphere. This mixture was allowed to warm slowly to room temperature, and was stirred overnight. The THF was then removed under reduced pressure, and the crude oil purified by column chromatography (2% MeOH in CH_2Cl_2) to afford 18.5 mg (14%) of **49b**, a colourless oil.

IR (neat): 3293, 3064, 3033, 2927, 2859, 1711, 1533, 1456, 1242, 1013 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.33 (m, 5H), 5.07 (s, 2H), 4.84 (br s, 1H),

4.43 (ddq, $J = 2.7, 3.7, 6.3$, 1H), 4.01 (m, 2H), 3.18 (dt, $J = 6.4$,

6.6 Hz, 2H), 1.20-1.80 (m, 28H), 1.35 (d, $J = 6.3$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 31.3;

LRMS (EI) m/z (relative intensity): 481 (15), 374 (55), 346 (20), 263 (100),

219 (10), 193 (7), 91 (69);

HRMS calcd. for $\text{C}_{26}\text{H}_{44}\text{NO}_5\text{P}$: 481.2957, found 481.2951.

1-O-linker-1-oxo-1-phospho-13-tetradecanolide (50).

A spatula tip of Pd-C (10%) stirring in 15 mL of EtOH was saturated with H_2 for 12 h before a solution of compound **49a** (13.8 mg, 28.6 μmol) in 5 mL of EtOH was added via a steel cannula. This mixture was allowed to stir for an additional five hours under H_2 pressure of one atmosphere. The reaction

mixture was filtered through Celite and concentrated under reduced pressure to afford 8.5 mg (85%) of **50** as a white solid.

IR (CDCl₃): 3692, 3632, 3181, 2934, 2861, 1603, 1265 1209, 1008 cm⁻¹;

¹H NMR (400 MHz, CDCl₃) δ: 8.37 (br s, 2 H), 4.61 (m, 1 H), 4.05 (m, 1H), 3.95 (m, 1H), 3.01 (br s, 2 H), 1.20-1.90 (m, 28 H), 1.32 (d, J = 6.2 Hz, 3H);

³¹P NMR (81 MHz, CDCl₃) δ: 33.1;

LRMS (DCI, NH₃) *m/z* (relative intensity): 348 (M⁺ + 1, 93), 345 (7), 344 (30), 86 (21);

HRMS (DCI, CH₄ + NH₃) calcd. for C₁₈H₃₈NO₃P: 347.2589, found 347.2593.

4-Benzoxabutanoic anhydride (**52**).

4-Benzoxabutanoic acid (**56**) (2.0 g, 10 mmoles) was dissolved in 50 mL of anhydrous Et₂O with DCC (1.1 g, 5.33 mmoles) at room temperature under N₂. After 3.5 hours of stirring, the DCU (dicyclohexylurea) formed during the reaction was filtered off by aspiration, and the ether was removed under reduced pressure to give a quantitative yield of anhydride **52** as a colourless

oil. The crude **52** obtained was carried on to the next step without further purification.

IR (CDCl₃): 3031, 2932, 2857, 1814, 1719, 1643, 1496, 1452, 1274, 1101, 1067, 741 cm⁻¹;

¹H NMR (200 MHz, CDCl₃) δ : 7.33 (m, 5H), 4.49 (s, 2H), 3.50 (t, J = 6.2 Hz, 2H), 2.57 (t, J = 6.2 Hz, 2H), 1.95 (quint, J = 6.2 Hz, 2 H);

LRMS (DCI, NH₃) *m/z* (relative intensity): 388 (M⁺ + 18, 70), 195 (9), 193 (3), 178 (11), 177 (100), 108 (26), 91 (23);

HRMS (DCI, CH₄) calcd. for C₁₂H₂₂O₅: (M⁺ + 1) 371.1856, found (M⁺ + 1) 371.1874.

Indoyl 4-benzoxybutanoate (53).

To a N₂ purged solution of 2N NaOH (5 mL) in a 3-neck round-bottom flask, fitted with a condenser, was added 3-indolyl acetate (135 mg, 0.77 mmoles) via a stainless steel cannula. The mixture was heated to reflux until the 3-indolyl acetate had completely dissolved. The reaction mixture was then cooled to 0 °C, then anhydride **52** was added via cannula. After one hour of

stirring at 0 °C the mixture was transferred to a separatory funnel containing Et₂O and water. The layers were separated, and the ether layer was dried over MgSO₄, filtered, and the solvent removed under reduced pressure to yield 102 mg (43%) of **53** as a dark red-burgundy oil.

IR (CDCl₃): 3478, 3065, 2959, 2931, 2868, 1747, 1223, 1156, 1128 cm⁻¹;

¹H NMR (200 MHz, CDCl₃) δ: 7.84 (br s, 1H), 7.60-7.08 (m, 10H), 4.54 (s, 2H), 3.62 (t, J = 6.2 Hz, 2H), 2.77 (t, J = 6.2 Hz, 2H), 2.10 (m, J = 6.2 Hz, 2H);

LRMS (EI) *m/z* (relative intensity): 309 (0.4), 177 (4), 132 (40), 107 (36), 91 (100), 77 (7);

HRMS calcd. for C₁₉H₁₉NO₃: (M⁺ + 1) 310.1443, found (M⁺ + 1) 310.1445.

(Indoyl butanoate) 13-hydroxytetradecanoate (**55**).

Crude **53** (143 mg, 0.461 mmoles) was hydrogenolyzed overnight under an atmosphere of N₂ (slightly greater than one atmosphere of pressure) with a spatula tip of 10% Pd/C in 30 mL of EtOH. The mixture was filtered through

Celite, and the EtOH removed under reduced pressure, and the crude product was reacted with the hydroxy acid **54** (134 mg, 0.548 mmoles) using DCC (113 mg, 0.548 mmoles) and a spatula tip of DMAP (11 mg, 0.091 mmoles) in CH₂Cl₂. After 2 hours of stirring, the reaction mixture was filtered by aspiration to remove the DHU formed, and the CH₂Cl₂ was evaporated under reduced pressure. The resulting crude red oil was purified on silica using a 2:1 mixture of petroleum ether and EtOAc to give 78 mg (38%) of the substrate **55**.

IR (CDCl₃): 3395, 2924, 2852, 1730, 1458, 1164, 740 cm⁻¹;

¹H NMR (200 MHz, CDCl₃) δ: 8.02 (br s, 1H), 7.58 (m, 5H), 4.21 (t,

J = 6.2 Hz, 2H), 3.78 (m, 1H), 2.73 (t, J = 6.2 Hz, 2H), 2.30 (t, J = 6.2

Hz, 2H), 2.14 (t, J = 6.2 Hz, 2H), 1.60 (t, J = 6.2 Hz, 2H), 1.40 (m, 2H),

1.25 (m, 16 H), 1.17 (d, J = 6.0 Hz, 3H);

LRMS (EI) *m/z* (relative intensity): 445 (0.7), 226 (4), 184 (0.5), 141 (24),

133 (100), 86 (1);

HRMS calcd. for C₂₆H₃₉NO₅: (M⁺) 445.2828, found (M⁺) 445.2829;

p-Nitrophenyl 13-Hydroxytetradecanoate (57).

To a stirred solution of **54** (100 mg, 0.41 mmol) in 20 mL of CH_2Cl_2 was added p-nitrophenol (68 mg, 0.49 mmol), DCC (102 mg, 0.38 mmol) and DMAP (10 mg, 82 μmol). The mixture was allowed to stir at room temperature under N_2 overnight. The solvent was then removed under reduced pressure, and the crude material was purified by flash chromatography using 4:1 petroleum ether and ethyl acetate to afford 130 mg (87%) of **57** as a white solid. A small quantity of p-nitrophenol was found to be present in the purified sample of **57** as seen by the multiplets at 8.15 and 6.88 ppm in their ^1H NMR spectra which correspond to the aromatic protons of p-nitrophenol.

mp: 69-72°C;

IR (CDCl_3): 3613, 2930, 2856, 1762, 1616, 1594, 1492, 1348, 1208 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 8.25 (m, 2H), 7.25 (m, 2H), 3.78 (m, $J = 6.2$ Hz, 1H), 2.57 (t, $J = 7.5$ Hz, 2H), 1.74 (quint, $J = 7.4$ Hz, 2H), 1.21-1.63 (m, 19H), 1.18 (d, $J = 6.2$ Hz, 3H);

LRMS (DCI, NH_3) m/z (relative intensity): 383 ($\text{M}^+ + 18$, 85), 366 ($\text{M}^+ + 1$, 4), 365 (3), 348 (77), 227 (10), 226 (22), 225 (100), 143 (15), 125 (10), 123 (35), 110 (12), 109 (16), 100 (13), 99 (25), 98 (18);

HRMS (DCI, NH_3) calcd. for $\text{C}_{20}\text{H}_{32}\text{NO}_5$: ($\text{M}^+ + 1$) 366.2281, found ($\text{M}^+ + 1$) 366.2271;

Anal. Calcd. for $C_{20}H_{31}NO_5$: C, 65.71; H, 8.55; N, 3.83. Found: C, 66.00;

H, 8.49; N, 3.84.

p-Nitrophenyl Tetradecanoate (58).

To a stirred solution of commercially available myristic acid (0.50 g, 2.2 mmoles) in 50 mL of CH_2Cl_2 was added p-nitrophenol (0.37 g, 2.6 mmoles), DCC (0.70 g, 2.6 mmoles) and DMAP (54 mg, 0.44 mmoles). The mixture was allowed to stir at room temperature under N_2 overnight. The solvent was then removed under reduced pressure, and the crude material was purified by flash chromatography using 20:1 petroleum ether and ethyl acetate to afford 0.67 g (88%) of **58** as a white solid.

mp: 52-54°C;

IR ($CDCl_3$): 2931, 2856, 1762, 1616, 1593, 1492, 1457, 1348, 1207 cm^{-1} ;

^1H NMR (400 MHz, $CDCl_3$) δ : 8.25 (m, 2H), 7.25 (m, 2H), 2.57 (t, $J = 7.5$ Hz,

2H), 1.73 (quint, $J = 7.5$ Hz, 2H), 1.45-1.15 (m, 23H);

LRMS (EI) m/z (relative intensity): 349 (0.1), 319 (0.6), 239 (4), 211 (100),

151 (0.8), 137 (3), 123 (4), 109 (27), 85 (16), 71 (28), 57 (45), 43 (38);

HRMS calcd. for $C_{20}H_{31}NO_4$: 349.2253, found 349.2251;

Anal. Calcd. for $C_{20}H_{31}NO_4$: C, 68.72; H, 8.95. Found: C, 68.89; H, 8.90.

1-Oxo-1-phosphonic acid-13-tetradecanolide (59).

59

Compound **38b** (62 mg, 0.18 mg) was dissolved in 20 mL of THF and 10 mL of 1.5M NaOH and the resulting solution was refluxed for 18 h. The THF was removed and the residual solution was acidified with 4N HCl then extracted with Et_2O . The combined ether extracts were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to afford 35 mg (73%) of **59** as a white solid. This compound was used in subsequent steps without further purification.

mp: 65-68 °C;

IR ($CDCl_3$): 3160, 2932, 2861, 1701, 1452, 1203, 1007 cm^{-1} ;

^1H NMR (400 MHz, $CDCl_3$) δ : 4.54 (m, 1H), 3.75 (br s, 1H), 1.77 (m, 2H), 1.58 (m, 4H), 1.20-1.48 (m, 16H), 1.36 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 35.2;

LRMS (DCI, NH_3) m/z (relative intensity): 280 ($\text{M}^+ + 18$, 4), 263 ($\text{M}^+ + 1$, 100),
262 (9), 179 (25), 165 (35), 151 (48), 137 (25), 109 (14), 96 (16);

HRMS (DCI, CH_4) calcd. for $\text{C}_{13}\text{H}_{27}\text{O}_3\text{P}$: 262.1698, found 262.1627;

Anal. Calcd. for $\text{C}_{13}\text{H}_{27}\text{O}_3\text{P}$: C, 59.50; H, 10.38. Found: C, 59.58; H, 10.47.

1-p-Nitrophenyl-1-oxo-1-phospho-13-tetradecanolide (60a and 60b).

60a + 60b

Acid **59** (90.0 mg, 344 μmol) was dissolved in 40 mL of CH_2Cl_2 at 0 $^\circ\text{C}$ under a N_2 atmosphere. Then $(\text{COCl})_2$ (120 μL , 1.37 mmol) was added, and the mixture was allowed to stir for 20 hr. The solvent, as well as excess $(\text{COCl})_2$, were removed under reduced pressure. The resulting acid chloride was redissolved in 40 mL of CH_2Cl_2 , then p-nitrophenol (150 mg, 1.08 mmol) and Et_3N (100 μL , 717 μmol) were added, and the mixture was refluxed overnight. The reaction mixture was then washed with 3 x 25 mL of NaHCO_3 , followed by 3 x 25 mL of water. The CH_2Cl_2 layer was dried over MgSO_4 , filtered and the solvent removed under reduced pressure to afford 130 mg of crude

product. This crude material was purified by column chromatography using 3:1 petroleum ether and ethyl acetate to afford the separated diastereomers; **60a** and **60b**, in a 1.2:1 ratio based on the isolated yields and a combined yield of 91.0 mg (69%). A small quantity of p-nitrophenol was found to be present in both of the purified diastereomers as seen by the multiplets at 8.15 and 6.88 ppm in their ^1H NMR spectra which correspond to the aromatic protons of p-nitrophenol.

Diastereomer **60a**:

IR (neat): 3459, 3113, 3080, 2929, 2859, 1611, 1591, 1522, 1492, 1458,

1403, 1380, 1346, 1292, 1238, 1162, 1110, 995, 911, 860, 751 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 8.22 (m, 2H), 7.34 (m, 2H), 4.77 (m, 1H), 1.95

(m, 2H), 1.20-1.84 (m, 20H), 1.23 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 30.4;

LRMS (EI) m/z (relative intensity): 383 (43), 367 (20), 366 (77), 353 (21), 349

(35), 340 (17), 337 (4), 322 (17), 286 (18), 258 (25), 246 (8), 245 (56),

244 (16), 230 (44), 217 (100), 201 (10), 189 (16), 129 (28), 97 (11), 83

(21), 69 (43), 55 (97);

HRMS calcd. for $\text{C}_{19}\text{H}_{30}\text{NO}_5\text{P}$: 383.1862, found 383.1859.

Diastereomer **60b**:

IR (neat): 3450, 3113, 3080, 2929, 2859, 1611, 1591, 1522, 1491, 1459,

1345, 1235, 1163, 1110, 1004, 910, 859, 753 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 8.22 (m, 2H), 7.36 (m, 2H), 4.65 (m, 1H), 2.20 (m, 2H), 1.20-1.84 (m, 20H), 1.43 (d, $J = 6.3$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 28.5;

LRMS (EI) m/z (relative intensity): 383 (9), 367 (2), 366 (9), 353 (2), 349 (3), 340 (2), 337 (0.2), 322 (2), 286 (3), 258 (5), 246 (2), 245 (11), 244 (4), 230 (11), 217 (22), 201 (2), 189 (3), 129 (2), 109 (29), 97 (5), 83 (12), 81 (24), 69 (37), 55 (100);

HRMS calcd. for $\text{C}_{19}\text{H}_{30}\text{NO}_5\text{P}$: 383.1862, found 383.1861.

6-Methyl-2-oxo-2-phenoxy-1,2-oxaphosphorinane (61a and 61b).

61a + 61b

Compound **68** (495 mg, 1.55 mmol) was dissolved in 30 mL of dry THF and stirred under N_2 , then cooled to -78 $^{\circ}\text{C}$ with a dry-ice acetone bath. 1.2 M $n\text{-BuLi}$ (94 μL , 1.1 mmol) was then added via syringe, and after stirring at -78 $^{\circ}\text{C}$ for 2.5 h, the dry-ice acetone bath was removed. The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography using 1:1 petroleum ether and ethyl

acetate to afford 0.286 mg (83%) of **61a** and 0.57 mg (17%) of **61b** as colourless oils.

Isomer **61a**:

IR (neat): 2946, 1592, 1491, 1455, 1384, 1302, 1255, 1209, 1161, 1079, 1049, 1032, 980, 921, 839, 789, 765 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.11-7.34 (m, 5H), 4.51 (m, 1H), 2.02-2.20 (m, 2H), 1.94 (m, 1H), 1.68-1.84 (m, 2H), 1.51 (m, 1H), 1.35 (dd, $J = 2.3, 6.3$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 20.5;

LRMS (EI) m/z (relative intensity): 226 (29), 211 (6), 185 (23), 172 (25), 144 (24), 115 (9), 94 (100), 77 (18);

HRMS calcd. for $\text{C}_{11}\text{H}_{15}\text{O}_3\text{P}$: 226.0759, found 226.0756;

Isomer **61b**:

IR (neat): 2943, 1593, 1491, 1383, 1291, 1250, 1210, 1153, 1026, 951, 783 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.11-7.34 (m, 5H), 4.68 (m, 1H), 1.96-2.22 (m, 3H), 1.71-1.86 (m, 2H), 1.45-1.54 (m, 1H), 1.36 (dd, $J = 2.3, 6.3$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 23.4;

LRMS (EI) m/z (relative intensity): 226 (41), 211 (7), 185 (27), 172 (30),

144 (26), 115 (9), 94 (100), 77 (18);

HRMS calcd. for $C_{11}H_{15}O_3P$: 226.0759, found 226.0759;

Anal. Calcd. for $C_{11}H_{15}O_3P$: C, 58.39; H, 6.69. Found: C, 58.07; H, 6.71.

1-Benzyloxy-3-butanol (62).

1,3-Butanediol (5.0 g, 56 mmol) was dissolved in 125 mL of dry THF, and cooled to 0 °C under N_2 prior to the addition of a 60% dispersion of NaH in mineral oil (2.7 g, 67 mmol) over a 10 minute period. Upon addition of the NaH, the mixture was stirred for 10 minutes, then Bu_4NI (1.0 g, 2.8 mmol) was added, followed by the dropwise addition of benzyl bromide (8.0 mL, 67 mmol). The ice-water bath was removed, and the reaction mixture was stirred at room temperature for 2.5 h before diluting the mixture with Et_2O , and quenching with water, followed by 1 M HCl. The mixture was then extracted with Et_2O , and the combined Et_2O extracts were dried over anhydrous $MgSO_4$, filtered and solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography using 1:1 petroleum ether and ethyl acetate to afford 5.8 g (58%) of **62** as a colourless oil.

^1H NMR (200 MHz, CDCl_3) δ : 7.32 (m, 5H), 4.52 (s, 2H), 4.00 (m, 1H), 3.65 (m, 2H), 2.63 (s, 1H), 1.73 (m, 2H), 1.18 (d, $J = 6.2$ Hz, 3H);

LRMS (EI) m/z (relative intensity): 180 (2), 161 (11), 120 (15), 108 (11), 107 (46), 92 (16), 91 (100), 79 (16), 65 (12).

1-Benzoyloxy-3-(Tetrahydropyranyloxy) butane (63).

To a stirred solution of **62** (260 mg, 1.44 mmol) in 5 mL of CH_2Cl_2 was added DHP (158 μL , 1.73 mmol) and p -TsOH (14 mg, 0.072 mmol), and the mixture was stirred at room temperature under N_2 for 2 h. The solvent was then removed and the residue was diluted with Et_2O . The ether layer was washed with 2 x 25 mL of saturated NaHCO_3 , 1 x 25 mL water and 1 x 50 mL of brine, dried over anhydrous MgSO_4 and filtered. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography using 5:1 petroleum ether and ethyl acetate to afford 288 mg (76%) of **63** as a colourless oil.

^1H NMR (200 MHz, CDCl_3) δ : 7.30 (m, 5H), 4.70 (m, 0.4H), 4.55 (m, 0.6H), 4.49 (m, 2H), 3.75–4.08 (m, 2H), 3.62 (m, 1H), 3.48 (m, 2H), 1.60–2.00 (m, 4H), 1.40–1.60 (m, 4H), 1.24 (d, $J = 6.2$ Hz, 1.5H), 1.11 (d,

$J = 6.2 \text{ Hz, } 1.5\text{H}$);

LRMS (DCI, NH_3) m/z (relative intensity): 282 ($\text{M}^+ + 18$, 4), 265 ($\text{M}^+ + 1$, 4),

181 (66), 179 (16), 91 (88), 85 (100);

HRMS (DCI, CH_4) calcd. for $\text{C}_{16}\text{H}_{24}\text{O}_3$: 264.1725, found 264.1648.

3-(Tetrahydropyranyloxy) butan-1-ol (64).

A spatula tip of Pd-C (10%) stirring in 20 mL of EtOAc was saturated with H_2 for 12 h before a solution of compound **63** (230 mg, 0.871 mmol) in 5 mL of EtOH was added via a steel cannula. This mixture was allowed to stir for an additional five hours under H_2 pressure of one atmosphere. The reaction mixture was filtered through Celite and concentrated under reduced pressure to give 146 mg of crude product, which was purified by flash chromatography using 1:1 petroleum ether and ethyl acetate to afford 129 mg (85%) of **64** as a colourless oil.

IR (neat): 3408, 2918, 1447, 1376, 1322, 1260, 1201, 1128, 1027, 1001,

907, 868, 811 cm^{-1} ;

^1H NMR (200 MHz, CDCl_3) δ : 4.68 (m, 0.2H), 4.55 (m, 0.8H), 3.40–4.13

(m, 6H), 2.35 (br s, 1H), 1.28 (d, $J = 6.2$ Hz, 0.6H), 1.15 (d,

$J = 6.2$ Hz, 1.4H);

LRMS (DCI, NH_3) m/z (relative intensity): 192 ($\text{M}^+ + 18$, 1), 175 ($\text{M}^+ + 1$,

4), 173 (2), 108 (6), 101 (13), 86 (10), 85 (100), 55 (18).

3-(Tetrahydropyranyloxy) butanal (65).

65

To a stirred solution of oxalyl chloride (75 μL , 0.86 mmol) in 10 mL of dry CH_2Cl_2 under N_2 at -78°C was added DMSO (102 μL , 1.44 mmol) dissolved in 2 mL CH_2Cl_2 via a stainless steel cannula. After 10 min. of stirring, compound **64** (102 mg, 0.575 mmol) in 5 mL of CH_2Cl_2 was added to the activated DMSO via a stainless steel cannula. Finally, after stirring an additional 10 min., Et_3N (410 μL , 2.95 mmol) was syringed in, and the reaction mixture was allowed to warm to room temperature while maintaining stirring. After 3 h, the solution was diluted with 50 mL of Et_2O followed by 50 mL of water. The aqueous layer was further extracted with 3 x 50 mL of Et_2O and the combined Et_2O extracts were dried over anhydrous MgSO_4 , filtered and concentrated under reduced pressure to a yellow oil. The crude product

was then purified by flash chromatography using 4:1 petroleum ether and ethyl acetate to afford 95 mg (96%) of **65** as a colourless oil.

^1H NMR (200 MHz, CDCl_3) δ : 9.80 (m, 1H), 4.68 (m, 0.8H), 4.55 (m, 0.2H), 4.18–4.43 (m, 1H), 3.63–3.99 (m, 2H), 3.48 (m, 1H), 2.55 (m, 2H), 1.40–1.85 (m, 8H), 1.26 (d, $J = 6.2$ Hz, 0.6H), 1.20 (d, $J = 6.2$ Hz, 2.4H).

1-Diphenylphosphinyl-4-(tetrahydropyranyloxy)-1-pentene (66).

The Wittig reagent **32** (1.9 g, 3.8 mmol) and aldehyde **65** (0.43 g, 2.5 mmol) were dissolved in 40 mL of toluene and refluxed under N_2 for 3 days. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography using 5:1 petroleum ether and ethyl acetate to afford 0.55 g (55%) of **66** as a colourless oil.

IR (neat): 2941, 2870, 1629, 1592, 1489, 1455, 1379, 1341, 1271, 1214, 1192, 1163, 1127, 1074, 1026, 994, 934, 767 cm^{-1} ;

^1H NMR (200 MHz, CDCl_3) δ : 7.10–7.38 (m, 10H), 7.00 (m, 1H), 5.95 (m, 1H), 4.68 (m, 0.5H), 4.55 (m, 0.5H), 3.73–4.00 (m, 2H), 3.35–3.60

(m, 1H), 2.30-2.55 (m, 2H), 1.35-1.90 (m, 6H), 1.18 (d, $J = 6.2$ Hz, 1.5H), 1.08 (d, $J = 6.2$ Hz, 1.5H);

^{31}P NMR (81 MHz, CDCl_3) δ : 10.8;

LRMS (EI) m/z (relative intensity): 402 (1), 318 (28), 301 (22), 275 (26),

274 (100), 259 (17), 181 (13), 162 (10), 141 (13), 133 (15), 118 (18),

117 (96), 116 (65), 115 (47), 94 (85), 77 (87);

HRMS calcd. for $\text{C}_{22}\text{H}_{27}\text{O}_5\text{P}$: 402.1596, found 402.1605.

4-Hydroxy-1-diphenylphosphinyl-1-pentene (67).

p-TsOH (26 mg, 0.14 mmol) was added to a stirred solution of compound **66** (0.55 g, 1.4 mmol) in 20 mL of MeOH at room temperature. After 5 h, the MeOH was removed under reduced pressure and the resulting oil was dissolved in Et_2O , washed with 2 x 20 mL of water followed by 2 x 20 mL of brine, dried over anhydrous MgSO_4 , filtered and concentrated under reduced pressure. The crude oil was purified by flash chromatography using 1:1 petroleum ether and ethyl acetate to afford 0.31 g (70%) of alcohol **67** as a colourless oil.

IR (neat): 3403, 2971, 1629, 1592, 1489, 1456, 1198, 1164, 1120, 1072,

1025, 937, 814, 767 cm^{-1} ;

^1H NMR (400 MHz, CDCl_3) δ : 7.12-7.35 (m, 10H), 6.94 (ddt, $J = 7.2, 17.1,$

23.1 Hz, 1H), 5.94 (ddt, $J = 1.4, 17.1, 23.1$ Hz, 1H), 3.88 (m, 1H), 2.37

(dt, $J = 1.4, 7.7$ Hz, 2H), 1.61 (br s, 1H), 1.14 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 10.8;

LRMS (EI) m/z (relative intensity): 318 (16), 274 (48), 259 (8), 181 (6),

133 (7), 117 (830), 94 (82), 77 (100);

HRMS calcd. for $\text{C}_{17}\text{H}_{19}\text{O}_4\text{P}$: 318.1021, found 318.1014;

Anal. Calcd. for $\text{C}_{17}\text{H}_{19}\text{O}_4\text{P}$: C, 64.13; H, 6.02. Found: C, 64.03; H, 6.11.

1-Diphenylphosphinyl pentan-4-ol (68).

A spatula tip of Pd-C (10%) in 20 mL of EtOAc was saturated with H_2 for 16 h before compound **67** (0.31 g, 0.97 mmol) in 3 mL of EtOAc was added via a steel cannula. This mixture was allowed to stir for an additional 2 days under H_2 gas at a pressure slightly greater than one atmosphere. The reaction mixture was filtered through Celite and concentrated under reduced pressure to afford 0.29 g (93%) of **68** as a colourless oil.

IR (neat): 3411, 2940, 1592, 1490, 1456, 1374, 1202, 1164, 1126, 1069,

1025, 1007, 935, 763 cm^{-1} ;

^1H NMR (300 MHz, CDCl_3) δ : 7.13-7.37 (m, 10H), 3.84 (m, 1H), 2.02-2.22 (m, 2H), 1.76-2.02 (m, 3H), 1.61 (dt, $J = 6.4, 7.5$ Hz, 2H), 1.21 (d, $J = 6.2$ Hz, 3H);

^{31}P NMR (81 MHz, CDCl_3) δ : 25.6;

LRMS (EI) m/z (relative intensity): 320 (1), 305 (10), 227 (100), 185 (15), 172 (25), 140 (25), 95 (57), 94 (96), 91 (75), 77 (86).

REFERENCES

- 1) Brockmann, H.; Henkel, W. *Naturwissenschaften* **1950**, 37, 138.
- 2) Woodward, R. B. *Angew. Chem.* **1957**, 69, 50.
- 3) Morin, R.; Gorman, M. *Kirk-Othmer Encycl. Chem. Technol.* 2nd ed. **1967**, 12, 632.
- 4) Vanek, Z.; Majer, J. *Antibiotics (N.Y.)* **1967**, 2, 154.
- 5) Omura, S.; Nakagawa, A. *J. Antibiot.* **1971**, 28, 401.
- 6) Vazquez, D. *Antibiotics (N.Y.)* **1975**, 3, 459.
- 7) Majer, J. *J. Chromatogr. Lib.* **1978**, 15, 273.
- 8) Mallams, A. *Kirk-Othmer Encycl. Chem. Technol.* 3rd ed. **1978**, 2, 937.
- 9) Korzybski, T.; Kowszyk-Gindifer, Z.; Kurylowicz, W. *Antibiotics: Origin, Nature, and Properties*; American Society for Microbiology: Washington, DC, 1978; Vol. 1.
- 10) Berdy, J. *CRC Handbook of Antibiotic Compounds*; CRC: Boca Raton, FL, 1980; Vol. 2.
- 11) Hata, T.; Omura, S. *Drug Action and Drug Resistance in Bacteria*; University of Tokyo: Tokyo, 1971; Vol. 1.
- 12) Oleinick, N. L. *Antibiotics (N.Y.)* **1975**, 3, 396.
- 13) Edelstein, P. H.; Meyer, R. D. *Antimicrob. Agents Chemother.* **1980**, 18, 403.
- 14) Edelstein, P. H.; Pasiecznik, K. A.; Yasui, V. K.; Meyer, R. D. *Antimicrob. Agents Chemother.* **1982**, 22, 90.
- 15) Gribble, M. J.; Chow, A. W. *Med. Clin. North Am.* **1982**, 66, 79.
- 16) Smith, H. *Antibiotics in Clinical Practice*; 3rd ed.; University Park Press: Baltimore, MD, 1977.

- 17) Vannuffel, P.; Di Giambattista, M.; Morgan, E. A.; Cocito, C. *J. Biol. Chem.* **1992**, 267, 8377.
- 18) Omura, S.; Nakagawa, A.; Shibata, K.; Sano, H. *Tetrahedron Lett.* **1982**, 23, 4713.
- 19) Omura, S.; Nakagawa, A.; Tanaka, Y. *Trends in Antibiotic Research*; Japan Antibiotics Research Foundation: Tokyo, 1982.
- 20) Komiyama, K.; Edanami, K.; Yamamoto, H.; Umezawa, I. *J. Antibiot.* **1982**, 35, 703.
- 21) Umezawa, I.; Takeshima, H.; Komiyama, K.; Koh, Y.; Yamamoto, H.; Kawaguchi, M. *J. Antibiot.* **1981**, 34, 259.
- 22) Glasby, J. S. *Encyclopaedia of Antibiotics*, 2nd ed.; John Wiley & Sons: New York, 1979.
- 23) Krohn, K.; Kirst, H.A.; Maag, H. *Antibiotics and Antiviral Compounds: Chemical Synthesis and Modification*; VHC Verlagsgesellschaft mbH, Weinheim (Federal Republic of Germany) and VHC Publishers Inc., New York, NY (USA), 1993.
- 24) Lukacs, G. *Recent Progress in the Chemical Synthesis of Antibiotics and Related Microbial Products Vol. 2*; Springer-Verlag: New York, NY, 1993.
- 25) Lukacs, G. *Recent Progress in the Chemical Synthesis of Antibiotics*; Springer-Verlag: New York, NY, 1990.
- 26) Omura, S. *Macrolide Antibiotics: Chemistry, Biology and Practice*; Academic: Orlando, FL, 1984.
- 27) Paterson, I.; Mansuri, M. M. *Tetrahedron* **1985**, 41, 3569.
- 28) Spracklin, D. K.; Weiler, L. *J. Chem. Soc., Chem. Commun.* **1992**, 1347.
- 29) Graham, R. J.; Weiler, L. *Tetrahedron Lett.* **1991**, 32, 1027.
- 30) Keller, T. H.; Neeland, E. G.; Rettig, S.; Trotter, J.; Weiler, L. *J. Am. Chem. Soc.* **1988**, 110, 7858.
- 31) Keller, T. H.; Weiler, L. *Tetrahedron Lett.* **1990**, 31, 6307.
- 32) Keller, T. H.; Weiler, L. *J. Am. Chem. Soc.* **1990**, 112, 450.

- 33) Ferreira, J. T. B.; Neeland, E. G.; Ounsworth, J. P.; Weiler, L. *Can. J. Chem.* **1987**, 65, 2314.
- 34) Neeland, E. G.; Ounsworth, J. P.; Sims, R. J.; Weiler, L. *Tetrahedron Lett.* **1987**, 28, 35.
- 35) Vedejs, E.; Dolphin, J. M.; Mastalerz, H. *J. Am. Chem. Soc.* **1983**, 105, 127.
- 36) Still, W. C.; Novak, V. J. *J. Am. Chem. Soc.* **1984**, 106, 1148.
- 37) Baker, W. R.; Clark, J. D.; Stephens, R. L.; Kim, K. H. *J. Org. Chem.* **1988**, 53, 2340.
- 38) Evans, D. A.; Carreira, E. M. *Tetrahedron Lett.* **1990**, 31, 4703.
- 39) Galli, C.; Illuminati, G.; Mandolini, L. *J. Am. Chem. Soc.* **1973**, 95, 8374.
- 40) Bartra, M.; Urpi, F.; Vilarrosa, J. *Recent Progress in the Chemical Synthesis of Antibiotics and Related Microbial Products*; Springer-Verlag: New York, NY, 1993; Vol. 2.
- 41) Castro, B. R. *Org. React.* **1982**, 29, 1.
- 42) Mitsunobu, O. *Synthesis* **1981**, 1.
- 43) Kurihara, T.; Nakajima, Y.; Mitsunobu, O. *Tetrahedron Lett.* **1976**, 2455.
- 44) March, J. *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*; 3 ed.; John Wiley & Sons: New York, NY, 1985, p 186.
- 45) Corey, E. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1974**, 96, 5614.
- 46) Mukaiyama, T.; Usui, M.; Saigo, K. *Chem. Lett.* **1976**, 49.
- 47) Inanaga, J.; Hirata, K.; Saeki, H.; Katsuki, T.; Yamaguchi, M. *Bull. Chem. Soc. Jpn.* **1979**, 52, 1989.
- 48) Smith, A. B., III; Leahy, J. W.; Noda, I.; Remiszewski, S. W.; Liverton, N. J.; Zibuck, R. *J. Am. Chem. Soc.* **1992**, 114, 2995.
- 49) Gatfield, I. L. *Ann. NY Acad. Sci.* **1984**, 434, 569.
- 50) Makita, A.; Nihira, T.; Yamada, Y. *Tetrahedron Lett.* **1987**, 28, 805.

- 51) Antczak, U.; Gora, J.; Antczak, T.; Galas, E. *Enzyme Microb. Technol.* **1991**, 13, 589.
- 52) O'Hagan, D.; Zaidi, N. A. *J. Chem. Soc. Pl* **1993**, 2389.
- 53) Guo, Z.-W.; Ngooi, T. K.; Scilimati, A.; Fulling, G.; Sih, C. J. *Tetrahedron Lett.* **1988**, 29, 5583.
- 54) Mori, K.; Tomioka, H. *Liebigs Ann. Chem.* **1992**, 1011.
- 55) Lobell, M.; Schneider, M. P. *Tetrahedron Asym.* **1993**, 4, 1027.
- 56) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, 234, 1566.
- 57) Jacobs, J. W.; Pollack, S. J.; Schultz, P. G. *Science* **1986**, 234, 1570.
- 58) Kitazume, T.; Takeda, M. *J. Chem. Soc., Chem. Commun.* **1995**, 39.
- 59) Napper, A. D.; Benkovic, S. J.; Tramontano, A.; Lerner, R. A. *Science* **1987**, 237, 1041.
- 60) Pauling, L. *Chem. Eng. News* **1946**, 24, 1375.
- 61) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, NY, 1969.
- 62) Goding, J. W. *Monoclonal Antibodies: Principles and Practices*; 2nd Ed. ed.; Academic: Orlando, FL, 1986.
- 63) Kohler, G.; Milstein, C. *Nature (London)* **1975**, 256, 495.
- 64) Schultz, P.G. *Angew. Chem. Int. Ed. Eng.* **1989**, 28, 1283.
- 65) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1988.
- 66) Davies, D. R.; Padlan, E. A.; Sheriff, S. *Annu. Rev. Biochem.* **1990**, 59, 439.
- 67) Haynes, M. R.; Stura, E. A.; Hilvert, D.; Wilson, I. A. *Science* **1994**, 263, 646.
- 68) Getzoff, E. D.; Tainer, J. A.; Lerner, R. A.; Geysen, H. M. *Adv. Immunol.* **1988**, 43, 1.

- 69) Winter, G.; Milstein, C. *Nature* **1991**, 349, 293.
- 70) Davis, M. M.; Bjorkman, P. J. *Nature (London)* **1988**, 334, 395.
- 71) Honjo, T.; Habu, S. *Annu. Rev. Biochem.* **1985**, 54, 803.
- 72) Tonegawa, S. *Nature (London)* **1983**, 302, 575.
- 73) Schechter, I. *Nature (London)* **1970**, 228, 639.
- 74) Lowe, C. R.; Harvey, M. J.; Craven, D. B.; Dean, P. D. G. *Biochem. J.* **1973**, 133, 499.
- 75) Milewich, L.; Gomez-Sanchez, C.; MacDonald, P. C.; Siiteri, P. K. *J. Ster. Biochem.* **1975**, 6, 1381.
- 76) Humphries, G. M. K.; McConnel, H. M. *Biophys. J.* **1976**, 16, 275.
- 77) Milstein, C. *Sci. Am.* **1980**, 243, 66.
- 78) Brown, A. J. *J. Chem. Soc.* **1902**, 81, 373.
- 79) Henri, V. *Lois Générales de l' action des diastases*, Hermann, Paris, 1903.
- 80) Michaelis, L., Menten, M. L. *Biochem. Z.* **1913**, 49, 333.
- 81) Walsh, C. *Enzymatic Reaction Mechanisms*; W.H. Freeman: San Francisco, 1979, p 34.
- 82) (a) Otera, J. *Chem. Rev.* **1993**, 93, 1449; (b) Chen, C. S.; Sih, C. J. *Angew. Chem. Int. Ed. Engl.* **1989**, 28, 695.
- 83) Klibanov, A. M. *CHEMTECH.* **1986**, 354.
- 84) Zaks, A.; Kilbanov, A. M. *Science* **1984**, 224, 1249.
- 85) Cotterill, I. C.; Sutherland, A. G.; Roberts, S. M.; Grobbauer, R.; Spreitz, J.; Faber, K. J. *Chem. Soc., Perkin Trans. I* **1991**, 1365.
- 86) Ashley, J. A.; Benkovic, S. J.; Janda, K. D.; Wirsching, P.; Lerner, R. A. *Science* **1991**, 252, 680.
- 87) Tramontano, A.; Ammann, A. A.; Lerner, R. A. *J. Am. Chem. Soc.* **1988**, 110, 2282.

- 88) Janda, K. D.; Shevlin, C. G.; Lerner, R. A. *Science* **1993**, 259, 490.
- 89) Gong, B.; Lesley, S. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, 114, 1486.
- 90) Tsumuraya, T.; Suga, H.; Meguro, S.; Tsunakawa, A.; Masamune, S. *J. Am. Chem. Soc.* **1995**, 117, 11390.
- 91) Jacobsen, J. R.; Prudent, J. R.; Kochersperger, L.; Yonkovich, S.; Schultz, P. G. *Science* **1992**, 256, 365.
- 92) Lilly, M. D. *J. Chem. Technol. Biotechnol.* **1982**, 32, 162.
- 93) Antonini, E.; Carrea, G.; Cremonesi, P. *Enzyme Microb. Technol.* **1981**, 3, 291.
- 94) Shevlin, C.G.; Hilton, S.; Janda, K.D. *Bioorg. Med. Chem. Lett.* **1994**, 4, 297.
- 95) Zaks, A.; Kilbanov, A. M. *J. Biol. Chem.* **1988**, 263, 8017.
- 96) Zaks, A.; Kilbanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, 82, 3192.
- 97) Ashley, J. A.; Janda, K. D. *J. Org. Chem.* **1992**, 57, 6691.
- 98) Cambou, B.; Kilbanov, A. M. *J. Am. Chem. Soc.* **1984**, 106, 2687.
- 99) Guibe-Jampel, E.; Rousseau, G. *Tetrahedron Lett.* **1987**, 28, 3563.
- 100) Hsu, S.- H.; Wu, S.- S.; Wang, Y.- F.; Wong, C.- H. *Tetrahedron Lett.* **1990**, 31, 6403.
- 101) Luisi, P. L. *Angew. Chem. Int. Ed. Engl.* **1985**, 24, 439.
- 102) Meier, P.; Luisi, P. L. *Solid Phase Biochem.* **1980**, 5, 269.
- 103) Barbaric, S.; Luisi, P. L. *J. Am. Chem. Soc.* **1981**, 103, 4239.
- 104) Janda, K. D.; Ashley, J. A.; Jones, T. M.; McLeod, D. A.; Schloeder, D. M.; Weinhouse, M. I. *J. Am. Chem. Soc.* **1990**, 112, 8886.
- 105) Spitznagel, T. M.; Jacobs, J. W.; Clark, D. S. *Enzyme Microb. Technol.* **1993**, 15, 916.

- 106) Durfor, C. N.; Bolin, R. J.; Sugasawara, R. J.; Massey, R. J.; Jacobs, J. W.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 8713.
- 107) Okahata, Y.; Yamaguchi, M.; Tanaka, F.; Fujii, I. *Tetrahedron* **1995**, *51*, 7673.
- 108) Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271-306.
- 109) Fersht, A. *Enzyme Structure and Mechanism*; Freeman: New York, 1985.
- 110) Adams, J. A.; Benkovic, S. J.; Borders, J., C.L.; Janda, K. D.; Lerner, R. A. *Science* **1990**, *250*, 1135.
- 111) Martin, M. T.; Napper, A. D.; Schultz, P. G.; Rees, A. R. *Biochemistry* **1991**, *30*, 9757.
- 112) Janda, K. D.; Lerner, R. A.; Tramontano, A. *J. Am. Chem. Soc.* **1988**, *110*, 4835.
- 113) Benkovic, S. J.; Napper, A. D.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5355.
- 114) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1188.
- 115) Janjic, N.; Tramontano, A. *J. Am. Chem. Soc.* **1989**, *111*, 9109.
- 116) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Angew. Chem. Int. Ed. Eng.* **1988**, *27*, 1172.
- 117) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Nature (London)* **1989**, *338*, 269.
- 118) Cochran, A.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 9414.
- 119) Hilton, S.; Li, T.; Janda, K. D. *J. Am. Chem. Soc.* **1995**, *117*, 2123.
- 120) Iverson, B. L.; Cameron, K. E.; Jahangiri, G. K.; Pasternak, D. S. *J. Am. Chem. Soc.* **1990**, *112*, 5320.
- 121) Fujii, I.; Lerner, R. A.; Janda, K. D. *J. Am. Chem. Soc.* **1991**, *113*, 8528.
- 122) Jackson, D. Y.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2319.
- 123) Reymond, J.-L.; Janda, K. D.; Lerner, R. A. *Angew. Chem. Int. Ed. Eng.* **1991**, *30*, 1711.

- 124) Hilvert, D. *Acc. Chem. Res.* **1993**, 26, 552.
- 125) Jackson, D. Y.; Liang, M. N.; Bartlett, P. A.; Schultz, P. G. *Angew. Chem. Int. Ed. Engl.* **1992**, 31, 182.
- 126) Bence, L. M.; Irvine, J. I.; Stimson, W. H.; Tedford, M. C.; Suckling, C. J. *J. Chem. Soc. Perkin Trans. 1* **1993**, 1925.
- 127) Hsieh, L. C.; Yonkovich, S.; Kochersperger, L.; Schultz, P. G. *Science* **1993**, 260, 337.
- 128) Cook, C. E.; Allen, D. A.; Miller, D. B.; Whisnant, C. C. *J. Am. Chem. Soc.* **1995**, 117, 7269.
- 129) Wagner, J.; Lerner, R. A.; Barbas, C. F. I. *Science* **1995**, 270, 1797.
- 130) Koch, T.; Reymond, J.-L.; Lerner, R. A. *J. Am. Chem. Soc.* **1995**, 117, 9383.
- 131) Reymond, J.-L.; Chen, Y. *Tetrahedron Lett.* **1995**, 36, 2575.
- 132) Jacobs, J. W. Ph.D. Thesis, University of California, Berkeley, 1990.
- 133) Spracklin, D. K. Ph.D. Thesis, University of British Columbia, 1994.
- 134) From reference 69 in Masamune, S.; Bates, G.S.; Corcoran, J.W. *Angew. Chem., Int. Ed. Engl.* **1977**, 16, 585.
- 135) Roush, W. R. *J. Org. Chem.* **1979**, 44, 4010.
- 136) Stork, G.; Nakamura, E. *J. Org. Chem.* **1979**, 44, 4011.
- 137) Jones, G. H.; Hamamura, E. K.; Moffatt, J. G. *Tetrahedron Lett.* **1968**, 5731.
- 138) Campbell, D. A. *J. Org. Chem.* **1992**, 57, 6331.
- 139) Hughes, D. L.; Reamer, R. A. *J. Org. Chem.* **1996**, 61, 2967.
- 140) Jacobs, J.; Schultz, P. G. *J. Am. Chem. Soc.* **1987**, 109, 2174.
- 141) Dale, J. *J. Chem. Soc.* **1963**, 85, 93.
- 142) Groth, P. *Acta Chem. Scand. A* **1979**, 33, 199.

- 143) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comp. Chem.* **1990**, *11*, 440.
- 144) Burkert, U.; Allinger, N. L. *ACS Monograph 177* American Chemical Society: Washington, DC, 1982.
- 145) McEwen, W.E. *Topics in Phosphorus Chemistry Vol. 2*; John Wiley & Sons: New York, NY, 1965.
- 146) Wadsworth, Jr., W. S.; Larsen, S.; Horten, H. L. *J. Org. Chem.* **1973**, *38*, 256.
- 147) Thatcher, G.R.J.; Kluger, R. *Adv. Phys. Org. Chem.* **1989**, *25*, 99.
- 148) Green, M.; Hudson, R. F. *Proc. Chem. Soc.* **1959**, 227.
- 149) Wadsworth, Jr., W. S. *J. Org. Chem.* **1973**, *38*, 2921.
- 150) Babcock, J.; Watts, J.; Aebersold, R.; Ziltener, H. *Anal. Biochem.* **1991**, *196*, 245.
- 151) Nakayama, G. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 780.
- 152) Lavey, B. J.; Janda, K. D. *J. Org. Chem.* **1996**, *61*, 7633.
- 153) (a) Darrow, J. W.; Drueckhammer, D. G. *J. Org. Chem.* **1994**, *59*, 2976;
(b) Darrow, J. W.; Drueckhammer, D. G. *Bioorg. Med. Chem.* **1996**, *4*, 1341.
- 154) Hanessian, S.; Galéotti, N.; Rosen, P.; Oliva, G.; Babu, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2763.
- 155) Harvey, T. C.; Simiand, C.; Weiler, L.; Withers, S. G. *J. Org. Chem.*, in press.
- 156) Gorenstein, D. G. *Chem. Rev.* **1987**, *87*, 1047.
- 157) Chang, J. W.; Gorenstein, D. G. *Tetrahedron* **1987**, *43*, 5187.
- 158) Copeland, R. A. *Enzymes: A Practical Introduction To Structure, Mechanism, And Data Analysis*; VCH Publishers Inc., New York, NY (USA), 1996, p 135.
- 159) Neil, T.R., *Nat. Prod. Rep.* **1996**, *13*, 479.

- 160) Lavey, B. J.; Janda, K. D. *J. Org. Chem.* **1996**, 61, 7633.
- 161) Morton, H. J. *In Vitro* **1970**, 6, 89.
- 162) Perrin, D.D.; Armarego, W.L.F. *Purification of Laboratory Chemicals*; 3rd ed.; Permagon: New York, NY, 1988.
- 163) Still, W.C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, 43, 2923.

SPECTRAL APPENDIX

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- 17) Vannuffel, P.; Di Giambattista, M.; Morgan, E. A.; Cocito, C. *J. Biol. Chem.* **1992**, *267*, 8377.
- 18) Omura, S.; Nakagawa, A.; Shibata, K.; Sano, H. *Tetrahedron Lett.* **1982**, *23*, 4713.
- 19) Omura, S.; Nakagawa, A.; Tanaka, Y. *Trends in Antibiotic Research*; Japan Antibiotics Research Foundation: Tokyo, 1982.
- 20) Komiyama, K.; Edanami, K.; Yamamoto, H.; Umezawa, I. *J. Antibiot.* **1982**, *35*, 703.
- 21) Umezawa, I.; Takeshima, H.; Komiyama, K.; Koh, Y.; Yamamoto, H.; Kawaguchi, M. *J. Antibiot.* **1981**, *34*, 259.
- 22) Glasby, J. S. *Encyclopaedia of Antibiotics*, 2nd ed.; John Wiley & Sons: New York, 1979.
- 23) Krohn, K.; Kirst, H.A.; Maag, H. *Antibiotics and Antiviral Compounds: Chemical Synthesis and Modification*; VHC Verlagsgesellschaft mbH, Weinheim (Federal Republic of Germany) and VHC Publishers Inc., New York, NY (USA), 1993.
- 24) Lukacs, G. *Recent Progress in the Chemical Synthesis of Antibiotics and Related Microbial Products Vol. 2*; Springer-Verlag: New York, NY, 1993.
- 25) Lukacs, G. *Recent Progress in the Chemical Synthesis of Antibiotics*; Springer-Verlag: New York, NY, 1990.
- 26) Omura, S. *Macrolide Antibiotics: Chemistry, Biology and Practice*; Academic: Orlando, FL, 1984.
- 27) Paterson, I.; Mansuri, M. M. *Tetrahedron* **1985**, *41*, 3569.
- 28) Spracklin, D. K.; Weiler, L. *J. Chem. Soc., Chem. Commun.* **1992**, 1347.
- 29) Graham, R. J.; Weiler, L. *Tetrahedron Lett.* **1991**, *32*, 1027.
- 30) Keller, T. H.; Neeland, E. G.; Rettig, S.; Trotter, J.; Weiler, L. *J. Am. Chem. Soc.* **1988**, *110*, 7858.
- 31) Keller, T. H.; Weiler, L. *Tetrahedron Lett.* **1990**, *31*, 6307.
- 32) Keller, T. H.; Weiler, L. *J. Am. Chem. Soc.* **1990**, *112*, 450.

- 33) Ferreira, J. T. B.; Neeland, E. G.; Ounsworth, J. P.; Weiler, L. *Can. J. Chem.* **1987**, *65*, 2314.
- 34) Neeland, E. G.; Ounsworth, J. P.; Sims, R. J.; Weiler, L. *Tetrahedron Lett.* **1987**, *28*, 35.
- 35) Vedejs, E.; Dolphin, J. M.; Mastalerz, H. *J. Am. Chem. Soc.* **1983**, *105*, 127.
- 36) Still, W. C.; Novak, V. J. *J. Am. Chem. Soc.* **1984**, *106*, 1148.
- 37) Baker, W. R.; Clark, J. D.; Stephens, R. L.; Kim, K. H. *J. Org. Chem.* **1988**, *53*, 2340.
- 38) Evans, D. A.; Carreira, E. M. *Tetrahedron Lett.* **1990**, *31*, 4703.
- 39) Galli, C.; Illuminati, G.; Mandolini, L. *J. Am. Chem. Soc.* **1973**, *95*, 8374.
- 40) Bartra, M.; Urpi, F.; Vilarrasa, J. *Recent Progress in the Chemical Synthesis of Antibiotics and Related Microbial Products*; Springer-Verlag: New York, NY, 1993; Vol. 2.
- 41) Castro, B. R. *Org. React.* **1982**, *29*, 1.
- 42) Mitsunobu, O. *Synthesis* **1981**, 1.
- 43) Kurihara, T.; Nakajima, Y.; Mitsunobu, O. *Tetrahedron Lett.* **1976**, 2455.
- 44) March, J. *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*; 3 ed.; John Wiley & Sons: New York, NY, 1985, p 186.
- 45) Corey, E. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1974**, *96*, 5614.
- 46) Mukaiyama, T.; Usui, M.; Saigo, K. *Chem. Lett.* **1976**, 49.
- 47) Inanaga, J.; Hirata, K.; Saeki, H.; Katsuki, T.; Yamaguchi, M. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 1989.
- 48) Smith, A. B., III; Leahy, J. W.; Noda, I.; Remiszewski, S. W.; Liverton, N. J.; Zibuck, R. *J. Am. Chem. Soc.* **1992**, *114*, 2995.
- 49) Gatfield, I. L. *Ann. NY Acad. Sci.* **1984**, *434*, 569.
- 50) Makita, A.; Nihira, T.; Yamada, Y. *Tetrahedron Lett.* **1987**, *28*, 805.

- 51) Antczak, U.; Gora, J.; Antczak, T.; Galas, E. *Enzyme Microb. Technol.* **1991**, *13*, 589.
- 52) O'Hagan, D.; Zaidi, N. A. *J. Chem. Soc. Pl* **1993**, 2389.
- 53) Guo, Z.-W.; Ngooi, T. K.; Scilimati, A.; Fulling, G.; Sih, C. J. *Tetrahedron Lett.* **1988**, *29*, 5583.
- 54) Mori, K.; Tomioka, H. *Liebigs Ann. Chem.* **1992**, 1011.
- 55) Lobell, M.; Schneider, M. P. *Tetrahedron Asym.* **1993**, *4*, 1027.
- 56) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566.
- 57) Jacobs, J. W.; Pollack, S. J.; Schultz, P. G. *Science* **1986**, *234*, 1570.
- 58) Kitazume, T.; Takeda, M. *J. Chem. Soc., Chem. Commun.* **1995**, 39.
- 59) Napper, A. D.; Benkovic, S. J.; Tramontano, A.; Lerner, R. A. *Science* **1987**, *237*, 1041.
- 60) Pauling, L. *Chem. Eng. News* **1946**, *24*, 1375.
- 61) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, NY, 1969.
- 62) Goding, J. W. *Monoclonal Antibodies: Principles and Practices*; 2nd Ed. ed.; Academic: Orlando, Fl, 1986.
- 63) Kohler, G.; Milstein, C. *Nature (London)* **1975**, *256*, 495.
- 64) Schultz, P.G. *Angew. Chem. Int. Ed. Eng.* **1989**, *28*, 1283.
- 65) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1988.
- 66) Davies, D. R.; Padlan, E. A.; Sheriff, S. *Annu. Rev. Biochem.* **1990**, *59*, 439.
- 67) Haynes, M. R.; Stura, E. A.; Hilvert, D.; Wilson, I. A. *Science* **1994**, *263*, 646.
- 68) Getzoff, E. D.; Tainer, J. A.; Lerner, R. A.; Geysen, H. M. *Adv. Immunol.* **1988**, *43*, 1.

- 69) Winter, G.; Milstein, C. *Nature* **1991**, 349, 293.
- 70) Davis, M. M.; Bjorkman, P. J. *Nature (London)* **1988**, 334, 395.
- 71) Honjo, T.; Habu, S. *Annu. Rev. Biochem.* **1985**, 54, 803.
- 72) Tonegawa, S. *Nature (London)* **1983**, 302, 575.
- 73) Schechter, I. *Nature (London)* **1970**, 228, 639.
- 74) Lowe, C. R.; Harvey, M. J.; Craven, D. B.; Dean, P. D. G. *Biochem. J.* **1973**, 133, 499.
- 75) Milewich, L.; Gomez-Sanchez, C.; MacDonald, P. C.; Siiteri, P. K. *J. Ster. Biochem.* **1975**, 6, 1381.
- 76) Humphries, G. M. K.; McConnel, H. M. *Biophys. J.* **1976**, 16, 275.
- 77) Milstein, C. *Sci. Am.* **1980**, 243, 66.
- 78) Brown, A. J. *J. Chem. Soc.* **1902**, 81, 373.
- 79) Henri, V. *Lois Générales de l' action des diastases*, Hermann, Paris, 1903.
- 80) Michaelis, L., Menten, M. L. *Biochem. Z.* **1913**, 49, 333.
- 81) Walsh, C. *Enzymatic Reaction Mechanisms*; W.H. Freeman: San Francisco, 1979, p 34.
- 82) (a) Otera, J. *Chem. Rev.* **1993**, 93, 1449; (b) Chen, C. S.; Sih, C. J. *Angew. Chem. Int. Ed. Engl.* **1989**, 28, 695.
- 83) Klibanov, A. M. *CHEMTECH.* **1986**, 354.
- 84) Zaks, A.; Kilbanov, A. M. *Science* **1984**, 224, 1249.
- 85) Cotterill, I. C.; Sutherland, A. G.; Roberts, S. M.; Grobbauer, R.; Spreitz, J.; Faber, K. J. *Chem. Soc., Perkin Trans. I* **1991**, 1365.
- 86) Ashley, J. A.; Benkovic, S. J.; Janda, K. D.; Wirsching, P.; Lerner, R. A. *Science* **1991**, 252, 680.
- 87) Tramontano, A.; Ammann, A. A.; Lerner, R. A. *J. Am. Chem. Soc.* **1988**, 110, 2282.

- 88) Janda, K. D.; Shevlin, C. G.; Lerner, R. A. *Science* **1993**, 259, 490.
- 89) Gong, B.; Lesley, S. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, 114, 1486.
- 90) Tsumuraya, T.; Suga, H.; Meguro, S.; Tsunakawa, A.; Masamune, S. *J. Am. Chem. Soc.* **1995**, 117, 11390.
- 91) Jacobsen, J. R.; Prudent, J. R.; Kochersperger, L.; Yonkovich, S.; Schultz, P. G. *Science* **1992**, 256, 365.
- 92) Lilly, M. D. *J. Chem. Technol. Biotechnol.* **1982**, 32, 162.
- 93) Antonini, E.; Carrea, G.; Cremonesi, P. *Enzyme Microb. Technol.* **1981**, 3, 291.
- 94) Shevlin, C.G.; Hilton, S.; Janda, K.D. *Bioorg. Med. Chem. Lett.* **1994**, 4, 297.
- 95) Zaks, A.; Kilbanov, A. M. *J. Biol. Chem.* **1988**, 263, 8017.
- 96) Zaks, A.; Kilbanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, 82, 3192.
- 97) Ashley, J. A.; Janda, K. D. *J. Org. Chem.* **1992**, 57, 6691.
- 98) Cambou, B.; Kilbanov, A. M. *J. Am. Chem. Soc.* **1984**, 106, 2687.
- 99) Guibe-Jampel, E.; Rousseau, G. *Tetrahedron Lett.* **1987**, 28, 3563.
- 100) Hsu, S.- H.; Wu, S.- S.; Wang, Y.- F.; Wong, C.- H. *Tetrahedron Lett.* **1990**, 31, 6403.
- 101) Luisi, P. L. *Angew. Chem. Int. Ed. Engl.* **1985**, 24, 439.
- 102) Meier, P.; Luisi, P. L. *Solid Phase Biochem.* **1980**, 5, 269.
- 103) Barbaric, S.; Luisi, P. L. *J. Am. Chem. Soc.* **1981**, 103, 4239.
- 104) Janda, K. D.; Ashley, J. A.; Jones, T. M.; McLeod, D. A.; Schloeder, D. M.; Weinhouse, M. I. *J. Am. Chem. Soc.* **1990**, 112, 8886.
- 105) Spitznagel, T. M.; Jacobs, J. W.; Clark, D. S. *Enzyme Microb. Technol.* **1993**, 15, 916.

- 106) Durfor, C. N.; Bolin, R. J.; Sugasawara, R. J.; Massey, R. J.; Jacobs, J. W.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 8713.
- 107) Okahata, Y.; Yamaguchi, M.; Tanaka, F.; Fujii, I. *Tetrahedron* **1995**, *51*, 7673.
- 108) Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271-306.
- 109) Fersht, A. *Enzyme Structure and Mechanism*; Freeman: New York, 1985.
- 110) Adams, J. A.; Benkovic, S. J.; Borders, J., C.L.; Janda, K. D.; Lerner, R. A. *Science* **1990**, *250*, 1135.
- 111) Martin, M. T.; Napper, A. D.; Schultz, P. G.; Rees, A. R. *Biochemistry* **1991**, *30*, 9757.
- 112) Janda, K. D.; Lerner, R. A.; Tramontano, A. *J. Am. Chem. Soc.* **1988**, *110*, 4835.
- 113) Benkovic, S. J.; Napper, A. D.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5355.
- 114) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1188.
- 115) Janjic, N.; Tramontano, A. *J. Am. Chem. Soc.* **1989**, *111*, 9109.
- 116) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Angew. Chem. Int. Ed. Eng.* **1988**, *27*, 1172.
- 117) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Nature (London)* **1989**, *338*, 269.
- 118) Cochran, A.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 9414.
- 119) Hilton, S.; Li, T.; Janda, K. D. *J. Am. Chem. Soc.* **1995**, *117*, 2123.
- 120) Iverson, B. L.; Cameron, K. E.; Jahangiri, G. K.; Pasternak, D. S. *J. Am. Chem. Soc.* **1990**, *112*, 5320.
- 121) Fujii, I.; Lerner, R. A.; Janda, K. D. *J. Am. Chem. Soc.* **1991**, *113*, 8528.
- 122) Jackson, D. Y.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2319.
- 123) Reymond, J.-L.; Janda, K. D.; Lerner, R. A. *Angew. Chem. Int. Ed. Eng.* **1991**, *30*, 1711.

- 124) Hilvert, D. *Acc. Chem. Res.* **1993**, 26, 552.
- 125) Jackson, D. Y.; Liang, M. N.; Bartlett, P. A.; Schultz, P. G. *Angew. Chem. Int. Ed. Engl.* **1992**, 31, 182.
- 126) Bence, L. M.; Irvine, J. I.; Stimson, W. H.; Tedford, M. C.; Suckling, C. J. *J. Chem. Soc. Perkin Trans. 1* **1993**, 1925.
- 127) Hsieh, L. C.; Yonkovich, S.; Kochersperger, L.; Schultz, P. G. *Science* **1993**, 260, 337.
- 128) Cook, C. E.; Allen, D. A.; Miller, D. B.; Whisnant, C. C. *J. Am. Chem. Soc.* **1995**, 117, 7269.
- 129) Wagner, J.; Lerner, R. A.; Barbas, C. F. I. *Science* **1995**, 270, 1797.
- 130) Koch, T.; Reymond, J.-L.; Lerner, R. A. *J. Am. Chem. Soc.* **1995**, 117, 9383.
- 131) Reymond, J.-L.; Chen, Y. *Tetrahedron Lett.* **1995**, 36, 2575.
- 132) Jacobs, J. W. Ph.D. Thesis, University of California, Berkeley, 1990.
- 133) Spracklin, D. K. Ph.D. Thesis, University of British Columbia, 1994.
- 134) From reference 69 in Masamune, S.; Bates, G.S.; Corcoran, J.W. *Angew. Chem., Int. Ed. Engl.* **1977**, 16, 585.
- 135) Roush, W. R. *J. Org. Chem.* **1979**, 44, 4010.
- 136) Stork, G.; Nakamura, E. *J. Org. Chem.* **1979**, 44, 4011.
- 137) Jones, G. H.; Hamamura, E. K.; Moffatt, J. G. *Tetrahedron Lett.* **1968**, 5731.
- 138) Campbell, D. A. *J. Org. Chem.* **1992**, 57, 6331.
- 139) Hughes, D. L.; Reamer, R. A. *J. Org. Chem.* **1996**, 61, 2967.
- 140) Jacobs, J.; Schultz, P. G. *J. Am. Chem. Soc.* **1987**, 109, 2174.
- 141) Dale, J. *J. Chem. Soc.* **1963**, 85, 93.
- 142) Groth, P. *Acta Chem. Scand. A* **1979**, 33, 199.

- 143) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comp. Chem.* **1990**, *11*, 440.
- 144) Burkert, U.; Allinger, N. L. *ACS Monograph 177* American Chemical Society: Washington, DC, 1982.
- 145) McEwen, W.E. *Topics in Phosphorus Chemistry Vol. 2*; John Wiley & Sons: New York, NY, 1965.
- 146) Wadsworth, Jr., W. S.; Larsen, S.; Horten, H. L. *J. Org. Chem.* **1973**, *38*, 256.
- 147) Thatcher, G.R.J.; Kluger, R. *Adv. Phys. Org. Chem.* **1989**, *25*, 99.
- 148) Green, M.; Hudson, R. F. *Proc. Chem. Soc.* **1959**, 227.
- 149) Wadsworth, Jr., W. S. *J. Org. Chem.* **1973**, *38*, 2921.
- 150) Babcock, J.; Watts, J.; Aebersold, R.; Ziltener, H. *Anal. Biochem.* **1991**, *196*, 245.
- 151) Nakayama, G. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 780.
- 152) Lavey, B. J.; Janda, K. D. *J. Org. Chem.* **1996**, *61*, 7633.
- 153) (a) Darrow, J. W.; Drueckhammer, D. G. *J. Org. Chem.* **1994**, *59*, 2976;
(b) Darrow, J. W.; Drueckhammer, D. G. *Bioorg. Med. Chem.* **1996**, *4*, 1341.
- 154) Hanessian, S.; Galéotti, N.; Rosen, P.; Oliva, G.; Babu, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2763.
- 155) Harvey, T. C.; Simiand, C.; Weiler, L.; Withers, S. G. *J. Org. Chem.*, in press.
- 156) Gorenstein, D. G. *Chem. Rev.* **1987**, *87*, 1047.
- 157) Chang, J. W.; Gorenstein, D. G. *Tetrahedron* **1987**, *43*, 5187.
- 158) Copeland, R. A. *Enzymes: A Practical Introduction To Structure, Mechanism, And Data Analysis*; VCH Publishers Inc., New York, NY (USA), 1996, p 135.
- 159) Neil, T.R., *Nat. Prod. Rep.* **1996**, *13*, 479.

- 160) Lavey, B. J.; Janda, K. D. *J. Org. Chem.* **1996**, 61, 7633.
- 161) Morton, H. J. *In Vitro* **1970**, 6, 89.
- 162) Perrin, D.D.; Armarego, W.L.F. *Purification of Laboratory Chemicals*; 3rd ed.; Permagon: New York, NY, 1988.
- 163) Still, W.C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, 43, 2923.

SPECTRAL APPENDIX

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