1994

Sensitive detection and study of catalytic species for bimolecular reactions

James William Lane
Iowa State University

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Sensitive detection and study of catalytic species for bimolecular reactions

Lane, James William, Ph.D.
Iowa State University, 1994
Sensitive detection and study of catalytic species for bimolecular reactions

by

James William Lane

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfilment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Chemistry
Major: Organic Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work
Signature was redacted for privacy.

For the Major Department
Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1994
DEDICATION

To Malú, Mom, Dad, Steve, Dave and Grandma
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<tr>
<td>ABTS</td>
<td>3-ethyl-2-azino benzthiazoline sulfonate</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>bs</td>
<td>broad singlet (NMR)</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BBS</td>
<td>10 mM borate buffer, 150 mM NaCl</td>
</tr>
<tr>
<td>CBZ</td>
<td>carbobenzyloxy</td>
</tr>
<tr>
<td>CT</td>
<td>(\alpha)-chymotrypsin</td>
</tr>
<tr>
<td>d</td>
<td>doublet (NMR)</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets (NMR)</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-dimethylanminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DNP</td>
<td>dinitrophenyl</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eq.</td>
<td>equivalent(s)</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>HOBt</td>
<td>N-hydroxybenzotriazole hydrate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>i-PrOH</td>
<td>isopropanol</td>
</tr>
<tr>
<td>IR</td>
<td>infrared (spectroscopy)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LPH</td>
<td>limulus polyphemus hemolymph hemocyanin</td>
</tr>
<tr>
<td>m</td>
<td>multiplet (NMR)</td>
</tr>
<tr>
<td>Me</td>
<td>methanol</td>
</tr>
<tr>
<td>m.p.</td>
<td>melting point</td>
</tr>
<tr>
<td>Ms</td>
<td>methanesulfonyl (mesyl)</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>10 mM phosphate buffer, 150 mM NaCl</td>
</tr>
<tr>
<td>Ph&lt;sub&gt;3&lt;/sub&gt;P</td>
<td>triphenylphosphine</td>
</tr>
<tr>
<td>q</td>
<td>quartet (NMR)</td>
</tr>
<tr>
<td>Rf</td>
<td>retention factor</td>
</tr>
<tr>
<td>s</td>
<td>singlet (NMR)</td>
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<tr>
<td>sat'd</td>
<td>saturated</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>t</td>
<td>triplet (NMR)</td>
</tr>
<tr>
<td>t-Boc</td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td>TEG</td>
<td>tetraethylene glycol</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMSI</td>
<td>trimethylsilyl iodide</td>
</tr>
<tr>
<td>TyrOMe</td>
<td>tyrosine methyl ester</td>
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</table>
WSC N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (water soluble carbodiimide)
GENERAL INTRODUCTION

Monoclonal antibodies are used extensively in various fields of biology and medicine. Some important applications include the investigation of cellular mechanisms, the isolation of interferons, cancer research, clinical diagnosis and gene product analysis. The generation of monoclonal antibodies with specific catalytic functions is an emerging technology which combines the high specificities of antibodies with chemical catalysis. A broad range of chemical transformations have been successfully catalyzed by monoclonal antibodies. The rationale is to immunize mice with predicted transition state analogs in the hope that molecules which bind the analog preferentially will function analogously to known enzymic mechanisms when presented with substrate. By current estimates, the immune system of mammals, humans included, has the potential to generate at least 100 million different antibodies, each with a distinctive binding site. This capacity exists to protect the body from disease-causing microorganisms and toxins.

However, current methods for generating monoclonal antibodies, which are entirely dependent on hybridoma technology, do not provide adequate survey of the natural immunological repertoire and limits the number of catalysts which may be obtained. Successful expression of recombinant antibody molecules in bacterial systems has recently been accomplished. These prokaryotic systems satisfy all the criteria for assembly of functional antibody fragments. Libraries can be constructed and screened for a desired antigen binding activity in less than two weeks. This efficiency allows for the rapid isolation of rare catalytic antibodies in a form suitable for genetic manipulation and propagation. However, these techniques are limited by the ability to efficiently screen for potential catalysts. Efficient screening is required for rapid development of catalytic antibodies. An assay which is sensitive
enough to detect catalysis at very low concentrations of catalyst would clearly have great
value in the development of improved catalysts by modification of any type of existing
catalyst, or even for screening of natural enzymatic activities.

Explanation of the dissertation organization

This dissertation will be divided into three papers. Each paper contains a separate
research topic and includes an introduction, a results and discussion section, an experimental
section describing preparation of key compounds, and a conclusion. The numbering of tables,
figures, and structure is independent for each section. In the first paper, methods and
considerations for the generation of catalytic antibodies for peptide ligation will be discussed.
The second paper will describe the development of a sensitive assay for the detection of
coupling catalysts and the synthesis of useful biological linkers. Finally, the third paper will be
devoted to the discussion of a collaborative project between myself, under the guidance of Dr.
Alan Schwabacher and researchers in professor Jacob Petrich's group involving the use of
biotinylated 7-azatryptophan as a fluorescent probe of protein structure and dynamics.
PAPER I

STUDIES ON ANTIBODY CATALYSIS OF PEPTIDE LIGATION
INTRODUCTION

With the preparation of the first monoclonal antibodies, the doors to a vast number of biological applications were opened. This advance occurred when Kohler and Milstein\(^1\) demonstrated that it was possible to generate in vitro monoclonal antibodies; antibody molecules consisting of a single distinct molecular structure. The generation of large amounts of homogeneous antibodies of desired specificity reproducibly has greatly expanded the role of antibodies in biology and medicine. Monoclonal antibodies have since been used increasingly in medicine and biology as therapeutic and imaging agents. Recently, chemists have begun to utilize and study these receptors as both synthetic aids and to gain a deeper understanding of how nature's catalyst, the enzyme, can achieve its remarkable catalytic efficiency and selectivity.

Antibodies, or immunoglobulins, are a class of protein and are the product of the immune system in vertebrates. They are produced through a series of cellular processes in response to a natural or synthetic molecule ranging in size from a few hundred amu to very large macromolecules such as proteins. Recently, x-ray crystallographic studies have provided structures of antibody molecules and have revealed the nature of antibody-antigen recognition.\(^2\) Antibodies are large proteins with molecular weights of about 150,000 for immunoglobulin G and they consist of four polypeptide chains: two identical heavy chains and two identical light chains. The combining site consists of approximately 110 amino acids of the heavy and light chains and is known as the variable region. Within this region are six loops, three on the heavy chain and three on the light chain. These loop segments are referred to as the hypervariable or complementarity determining regions and display a high degree of sequence variability which provides the basis for the diversity in the antibodies produced and for the specificity exhibited by a particular antibody molecule.
Antibodies bind molecules with dissociation constants ranging from $10^{-4}$ to $10^{-14}$ M.\textsuperscript{3} The specificity of antibodies for their ligands can exceed that of the enzyme for their substrates. Pauling stated almost 50 years ago that the fundamental difference between enzymes and antibodies is that enzymes selectively bind transition states while antibodies selectively bind ground states.\textsuperscript{4} This high selectivity is mandated by the environment in which they function: in living organisms. Without the exhibited high specificity, disastrous consequences would result as has been observed in the case of autoimmune disease.

Their main function is to protect the organism from invading and potentially harmful molecules such as bacteria, viruses, and parasites. Their high affinity and selectivity are essential in order to distinguish foreign material from the organism's own tissue. Antibodies achieve these essential traits in the same way that enzymes and other proteins do: through the appropriate positioning of amino acid functional groups to form a uniquely defined three-dimensional binding site. These amino acid functional groups act to stabilize the antibody-hapten complex through a large number of non-covalent interactions consisting of hydrogen bonding, van der Waals and electrostatic attractions, and hydrophobic interactions. The many appropriately positioned interactions result in complexes with binding strengths rivaling those
of covalent bonds. A good example is the avidin-biotin complex which has a dissociation constant of $10^{-15}$ M. This corresponds to a diffusion controlled on rate giving a complex with a half-life of two years!

The concept of utilizing antibodies for the catalysis of chemical reactions, a task which is performed by enzymes, was born from Linus Pauling's postulate that enzymes achieve their catalysis, in part, through the preferential binding and stabilization of the transition-state in a chemical transformation. Indeed, many good inhibitors of enzymes have structures which resemble the transition-state for the reaction they catalyze. This being the case, it was reasonable to conclude that if an antibody could be elicited which could tightly bind one of these inhibitors or any other transition-state analog for that manner, it would also be capable of catalyzing the corresponding chemical reaction in a similar fashion. This theory was first brought to fruition in 1986 with the isolation of antibodies that could selectively catalyze the hydrolysis of carbonates and esters.

Since that time, a number of other catalytic antibodies have been developed exhibiting a variety of enzyme-like mechanisms in addition to just transition-state stabilization. This mechanistic diversity arises from both the diversity of the immune response itself where $10^6$-$10^7$ different antibody molecules can be produced in response to an antigen and from the general strategies used to elicit antibodies. These strategies include the use of antibodies to stabilize negatively or positively charged transition states, the use of antibodies as entropic traps, and the generation of catalytic antibodies with catalytic groups or cofactors in their combining sites.

One of the greatest advantages in the use of catalytic antibodies over enzymes is that they can be "custom made" to catalyze a desired reaction for which there is no known enzyme catalyst. From the sheer number of antibody molecules produced, catalysts can be obtained which catalyze the same reaction but with different selectivities or through different
mechanisms. Another advantage is that when using monoclonal antibodies derived from immortal hybridoma cells, catalytic antibodies can be obtained easily in gram quantities. Attempts to produce polyclonal catalytic antibodies have been made, however, they are much less desirable as it is more difficult to detect small amounts of enzyme contaminants and to determine the amount and source of the catalyst present. Also, since the mixture is likely to contain non-catalytic antibodies which bind substrate, study of reaction rates is more difficult as well.

Antibody Production

The most common procedure for the production of catalytic antibodies begins with the selection of an appropriate transition-state analog. An antibody elicited against such a molecule should lower the free energy of activation for the corresponding reaction by preferentially stabilizing the transition-state relative to reactants and products. Since transition-states are unstable high energy species, stable analogs must be used. When a reaction proceeds through a tetrahedral intermediate or transition-state, as in the case of hydrolysis or aminolysis reactions, a phosphorus atom can be substituted for the central carbon giving a stable analog. For other transition-state analogs more imaginative approaches are required which incorporate the required geometries, bond lengths, and bond polarizations present in the transition state. It should be kept in mind, however, that the analog may not necessarily need to be an exact model of the transition-state. This is an advantage of the immune system diversity where many similar binding sites with subtle variations are produced.

Once an appropriate transition-state analog has been synthesized, it must then be conjugated to a carrier protein to give the desired antigen. A carrier protein is required in order to have a structure large enough for the immune system to recognize. Usually keyhole limpet hemocyanin (KLH) or limulus polyphemus hemolymph (LPH) are used as carrier
proteins because they elicit a strong immunogenic response in mammals. The transition-state analog is coupled through covalent linkages to amino acid side chains in the protein. For best results there should be a spacer of 6-8 angstroms in length between the hapten and the protein.

A preparation of the antigen in an equal mixture of buffer and complete Freund's adjuvant is then injected into mice. After 3-4 weeks the mice are bled and the antibodies contained in the serum are tested by enzyme linked immunosorbent assay for their ability to bind the hapten. The mice are given boost injections until antibodies showing strong binding to hapten are obtained. After a final boost, the spleen cells are taken and fused with myeloma cells in polyethylene glycol to form an immortal line of antibody producing hybridoma cells. The hybridoma cells are then cloned by a series of dilution and regrowth stages. After each cycle, the hybridoma supernatant must be checked to pinpoint those cells producing antibodies with the desired hapten affinity. Once a hybridoma has been cloned, it can then be injected into mice promoting the formation of a tumor which produces the desired antibody. One of the advantages to the use of catalytic antibodies is that the structural framework of all antibodies is conserved. Therefore, the desired antibodies can be purified through standard means which includes dialysis followed by ion exchange chromatography.

To date, antibodies elicited to transition-state analogs have been demonstrated to catalyze a variety of reactions ranging from hydrolysates of carbonate, ester, and amide substrates to the Diels-Alder reactions and Claisen rearrangements. Following are some representative examples of catalytic antibody production and the strategies used to obtain them.

The first examples of catalytic antibodies specific for transition-state analogs were elicited against tetrahedral, negatively charged phosphate and phosphonate transition-state analogs for the hydrolysis of carbonates and esters.
A number of factors were considered in the design of this first generation of catalytic antibodies. First, these reactions have been an object of interest to physical organic chemists for decades and are among the most thoroughly understood organic reactions. Second, the steric and electronic nature of the transition-state for these reactions is sufficiently different from substrate so that it should be possible to generate antibodies that selectively stabilize the high-energy transition-state. The features of these transition-state species are mimicked well by relatively stable phosphates and phosphonates, which were already known to be potent inhibitors of some acyl-transfer enzymes and as a result, were presumed to be good transition-state analogs. The use of these "mechanism based" haptens was expected to induce antibody binding sites that are sterically and electronically complementary to the transition-state configuration. Antibodies specific for these haptens acted as catalysts with rate accelerations on the order of 10^3 to 10^4 over the uncatalyzed reactions. The antibodies followed classical Michaelis-Menten kinetics, displayed substrate specificity, and bound the transition-state analog considerably more tightly than substrate, consistent with the notion of transition-state stabilization.

Schultz and coworkers have produced monoclonal antibodies which specifically bind the tetrahedral nitrophenyl phosphonate transition-state analogs 2 and 4, which catalyze the aqueous hydrolysis of the corresponding carbonates 1 and 3, respectively, with kinetics consistent with the Michaelis-Menten rate expression. The antibody-catalyzed hydrolytic reactions displayed substrate specificity and were competitively inhibited by the corresponding phosphate or phosphonate transition-state analog. Preferential binding and stabilization of the transition-state arising from the attack of water on antibody-complexed substrate 1 and 3 should result in a decrease of the free energy of activation for the reactions. In each case, the inhibition constant (Ki) is substantially lower than the corresponding Km of the substrate, suggesting that transition-state stabilization plays a role in catalysis.
Since these early experiments, more than twenty acyl transfer reactions have been catalyzed. The fact that these antibodies can carry out stereospecific hydrolyses of unactivated esters with an enantiomeric excess greater than 100 to 1 points to commercial applications of catalytic antibodies. These results are significant because at present there exists no general method for generating stereospecific esterolytic catalysts.

As an example of their specificity and potential utility in developing therapeutic agents, antibodies against transition-state analog 5 were prepared to obtain catalysts for the hydrolysis
of the tripeptide ester 6 under basic conditions. The antibody-catalyzed hydrolyses were 50 to 
300 fold faster than reaction with hydroxide ion. With relatively large transition-state analogs 
such as tripeptide 5, the tetrahedral phosphonate probably contributes proportionally less to 
the overall binding energy of the hapten to the antibody and, consequently, the rate 
acceleration observed is less than those of antibodies raised to smaller transition-state analogs. 
Interestingly, all 18 antibodies (out of the 25 that bound the hapten) found to accelerate the 
hydrolysis of ester 6 were selective for the D-phenylalanine-containing diastereomer although 
a mixture of diastereomers was used to generate the antibodies. This is a phenomenon 
commonly observed in catalytic antibodies and is due to preferential stimulation of cells 
producing these D-phenylalanine specific antibodies. The antibodies also show a high degree 
of substrate specificity.

![Chemical structure of compounds 5 and 6]

While these examples certainly illustrate the potential application of antibodies for 
selective catalysis and provide interesting mechanistic insights into how enzymes perform their 
functions in vivo, they do not illustrate the synthetic capabilities of the catalytic antibody. 

To the synthetic chemist, reactions in which two molecules are joined together are of
much more interest than the reverse reaction. Often it is necessary to carry out these conversions under mild conditions and with high selectivity and specificity. Catalysts which can fulfill these requirements are of great utility. Enzymes, which are among the most efficient catalysts known would be good candidates, however they have selectivities for particular substrates which presumably have been developed over thousands of years of evolution and are limited in the types of reactions which they can catalyze. Although they are increasingly being used in synthesis, the synthetic substrates must resemble the natural substrate for the enzyme. With the advent of the catalytic antibody, this limitation was overcome as custom-made enzymes can now be produced for a wide variety of reactions. Only in recent years has it been discovered that antibodies and enzymes, which are geared toward reactions in aqueous solution, can also catalyze reactions in organic solvents through such techniques as solubilization in reverse micelles and immobilization on surfaces. These advances, along with the aforementioned properties, have made catalytic antibodies very attractive candidates as catalysts in organic synthesis.

One particularly good example of the potential of catalytic antibodies in synthesis is the antibody catalyzed Claisen rearrangement of chorismic acid to prephenate. This

\[ \text{7} \rightarrow \text{8} \rightarrow \text{9} \]
thermal 3,3-sigmatropic rearrangement proceeds stereospecifically and occurs through an asymmetric chairlike transition-state in which the carbon-oxygen bond is substantially broken while carbon-carbon bond formation has not proceeded to any appreciable extent. There is a naturally occurring enzyme, chorismate mutase, for this transformation and Paul Bartlett had previously shown that the oxabicyclic derivative 9 was a potent inhibitor of the enzyme with a dissociation constant of $1.5 \times 10^{-7}$ M.\textsuperscript{12} Hilvert and coworkers (and simultaneously but independently Schultz and coworkers) reasoned that if an antibody were formed against the inhibitor 9, it may catalyze the conversion through transition-state stabilization. This is indeed what they observed.\textsuperscript{11a,b} One of the eight antibodies which bound the hapten was found to catalyze the Claisen rearrangement of 7 to 8, with initial rates consistent with Michaelis-Menton kinetics. The antibody catalyzed reaction was found to be accelerated by a factor of $10^4$ over that of the uncatalyzed unimolecular rearrangement ($k_{\text{cat}}/k_{\text{uncat}} = 1 \times 10^4$ at 10° C and pH 7.0). This is comparable to the rate of the enzyme catalyzed process which gave a $k_{\text{cat}}/k_{\text{uncat}}$ value of $10^6$. The antibody was also stereoselective catalyzing the conversion of only the (-)-isomer of chorismate. Because the conversion of chorismate to prephenate is an essential step in the biological synthesis of aromatic amino acids in bacteria, this system offers the possibility of applying random mutagenesis and selection to the evolution of antibodies with improved catalytic efficiency. Hilvert has shown that by replacing the gene encoding for the natural enzyme with one encoding for the antibody and allowing mutagenesis and selection to take place better catalysts were obtained. Bacterial cells with poorer performing catalytic antibodies died while those with improved activity thrived.

Recently, Hilvert and coworkers have determined the crystal structure for one of their catalytic antibodies exhibiting chorismate mutase activity.\textsuperscript{13} The antibody (1F7) was crystallized as a complex with the transition state model 9 and its crystal structure was determined to 3Å resolution and compared with the structure of a naturally occurring
chorismate mutase from *Bacillus subtilis* also complexed to 9. This comparison provides an excellent opportunity for the determination of the factors are involved in antibody catalyzed reactions and of how they relate to the enzyme analogs. The types of contacts between antibody and 9 are similar to those observed for other antibody hapten interactions and were consistent with the types of interactions one would expect to observe from antibodies elicited against 9 such as an ion pair between Arg\textsuperscript{H95} and the C-11 carboxylate. Comparison with the structure of chorismate mutase illustrated some differences which may explain the higher rate accelerations by the enzyme and suggest ways of improving the catalytic efficiency of the antibody.

The transition state for the conversion of chorismate to prephenate is a pseudodiaxial, highly polarized species and it was observed that the enzyme possessed two positively charged Arg residues in an area of the binding site near the C-11 carboxylate and a negatively charged Glu residue near the hydroxyl group at C-4. This arrangement effectively creates an electrostatic environment complimentary to the polarized transition state and may be one of the key factors in its stabilization. Also, it is conceivable that one or both of these side chains may participate directly in the reaction. These comparisons led to some suggestions for designing a transition state analog for the reaction that may lead to the formation of an antibody binding site which more closely resembles that of the enzyme. For instance if the C-4 hydroxyl group is replaced by an ammonium ion it may elicit the production of a negatively charged Glu residue as is present in the enzyme. Examples such as this clearly provide a strong case for the use of antibodies both as catalysts and as a means of further studying enzyme catalysis.

Another example of the use of antibodies to act as entropic traps involves the study of an antibody-catalyzed Diels-Alder reaction. This reaction, which has long been one of the most powerful transformations in synthetic organic chemistry, is a reaction between a diene
and an alkene giving rise to a cyclohexene product. The transition state for this reaction involves a highly ordered cyclic array of interacting orbitals in which carbon-carbon bonds are broken and formed in a single concerted mechanistic event. As a result, an unfavorable entropy of activation on the order of -30 to -40 eu is generally observed. The reaction product is not an appropriate hapten for generating catalytic antibodies since severe product inhibition would be expected to result. Therefore, the design of haptens that would lead to catalytic antibodies for this bimolecular reaction must not only make use of proximity effects but must also provide a mechanism for eliminating product inhibition. Hilvert and coworkers were successful in designing a hapten which satisfies both criteria.\textsuperscript{14} They reasoned that a stable analog of the bicyclic adduct could elicit an antibody combining site with the proper shape for promoting the target reaction. As the final product does not closely resemble the transition state of the reaction, product inhibition would be minimized, allowing multiple turnover of the catalyst.

The particular reaction chosen was that of tetrachlorothiophene dioxide with N-ethylemaleimide. They prepared five monoclonal antibodies that bound the hapten 11 which was a stable transition state analog of the bicyclic adduct 10. Of these antibodies, one catalyzed the formation of the initial Diels-Alder adduct which then spontaneously extrudes sulfur dioxide to give the secondary dihydrophthalimide product which is bound weakly by the antibody due its large structural differences relative to the transition state. This experiment, although requiring a somewhat unusual diene, provided the first report of a catalytic antibody for a useful chemical transformation and demonstrated the feasibility of utilizing antibodies in organic synthesis.
\[
\text{16: } \text{Cl}_2\text{C}_2\text{O}_2 + \text{N}_2\text{C}_2\text{H}_5 \rightarrow \text{Cl}_2\text{C}_2\text{O}_2\text{N}_2\text{C}_2\text{H}_5
\]

\[
\text{10: }
\begin{align*}
&\text{Cl}_2\text{C}_2\text{O}_2\text{N}_2\text{C}_2\text{H}_5 \\
&\text{Cl}_2\text{C}_2\text{O}_2\text{N}_2\text{C}_2\text{H}_5
\end{align*}
\]

\[
\text{11: } \text{Cl}_2\text{C}_2\text{O}_2\text{N}_2\text{C}_2\text{H}_5 + \text{SO}_2
\]
RESULTS AND DISCUSSION

It is evident through the examples cited that antibody catalysts have been applied to a number of useful chemical transformations. However, although there are several examples of antibody-catalyzed amide bond formation\(^{15-17}\), no catalytic antibody has yet been produced which is capable of catalyzing peptide bond formation between specific amino acids - a reaction which would be extremely useful for peptide ligation.

A common practice for the production of peptide chains is the use of the Merrifield peptide synthesis. In this procedure, peptide chains are built up one amino acid at a time by a series of coupling and deprotection steps. For shorter peptides, this method works very well as the entire process can be automated to produce the desired peptide. The procedure cannot however, be easily applied to the preparation of peptide chains longer than about twenty amino acid units due to the requirement for extremely high integrity in the coupling and deprotection steps. For example, even if every step proceeded with 99.9 percent completion, after twenty cycles there would be a 4 percent impurity, which for purposes of accurate characterization is unacceptable. The best strategy then, is to form the shorter peptide fragments using the Merrifield procedure and then to couple these fragments by another method. A coupling catalyst with high selectivity and efficiency would be ideal for this second method and we envisioned that a catalytic antibody would be very well suited to this role. As a result, we set out to produce a catalytic antibody with the desired peptide ligase ability and with specificity for a peptide containing an N-terminal histidine residue.

To achieve our goal, we designed and synthesized the phosphoramidate 17 as a transition-state analog for the desired reaction. In an aminolysis reaction between an amine
and an ester, a tetrahedral intermediate is formed and, based on the Hammond postulate, the transition-state for this reaction should have a similar structure. The overall reaction and intermediates are shown in Figure 1 below. By substituting phosphorus for the carbonyl carbon, a stable, tetrahedral model of the transition-state can be formed. The bonds being broken and formed are expected to be elongated in the transition-state and the analog

![Figure 1. Proposed coupling reaction and transition state analog](image)
is also a good model in this respect as the phosphorus-oxygen and phosphorus-nitrogen bonds are slightly longer than the corresponding carbon-based bonds. The dinitrophenyl group on histidine is useful both as a protecting group for the imidazole nitrogen and as a means for eliciting a stronger antigenic response. The major difference between the phosphoramidate 17 and the actual transition-state is that the latter is expected to have some zwitterionic character whereas 17 is neutral. Since both the substrate and the product contain planar sp² carbon, the tetrahedral transition-state is expected to be bound preferentially in an antibody catalyzing the desired reaction. Although the analog is most closely correlated with aminolysis of a carbonate, catalytic antibodies for ester aminolysis are expected to be obtained due to the diversity of the immune response and the antibodies obtained. The free carboxylate on one of the phenyl groups serves as a connecting point to the carrier protein.

Synthesis of the hapten 17 involved a convergent synthesis beginning with m-hydroxybenzoic acid and l-Boc-histidine. Benzyl m-hydroxybenzoate 12 was formed by treatment of the starting acid with one equivalent of KOH to produce the potassium salt which was then treated with benzyl chloride. The crude product was recrystallized from ether/hexanes to give the pure product in 72% yield. The bis(m-benzyloxycarbonylphenyl) chlorophosphate 13 was prepared from two equivalents of benzyl m-hydroxybenzoate and one equivalent of phosphorus oxychloride in tetrahydrofuran by addition of an excess of pyridine. l-Boc-histidine was treated with dinitrofluorobenzene in a one to one mixture of tetrahydrofuran and water containing sodium bicarbonate to obtain the Nim-protected dinitrophenyl derivative 14 which could be recrystallized from isopropanol in 89% yield. Refluxing this product in ethanol containing thionyl chloride caused simultaneous deprotection of the alpha amino group and formation of the ethyl ester. The ethyl ester (15) could then be purified further through recrystallization in methanol and tetrahydrofuran with a yield of 62%.
The chlorophosphate, prepared in situ, was then introduced to a solution of the histidine ethyl ester in tetrahydrofuran and triethylamine to form the phosphoramidate 16 which could be purified on a silica gel column to obtain the product as a yellow oil in 52% yield. Finally, deprotection of the carboxylates with trimethylsilyl iodide in deuterochloroform afforded the diacid 17 which could then be purified by HPLC using a C18 column.
A sample of hapten was conjugated to KLH in order to obtain the desired antigen 17b. This was done through activation of the carboxylate function by formation of its NHS ester in acetonitrile followed by addition to a solution of KLH in pH 8.4 BBS. To purify the conjugate, a sephadex G-50 size exclusion column was used. 1.5 ml fractions were collected.
using pH 7.4 PBS as eluent. Fraction 3 contained the largest protein concentration which was determined by BCA assay to be 1.5 mg/ml.

The extent of conjugation was determined by either TNBS assay of free amines on the protein before and after coupling and/or by thiolysis of the dinitrophenyl group of the hapten by 0.4 M mercaptoacetic acid. The thiolysis reaction was carried out in pH 7.0 phosphate buffer under conditions worked out by D.V. Jewell who was an undergraduate student working under my direction. The thiolysis product mixture was extracted into ethyl acetate after acidification of the aqueous layer with sulfuric acid. A standard using t-Boc-(DNP)-histidine was treated in the same manner. The residue was dissolved in HPLC eluent and components were then quantified by HPLC using an isocratic eluent of 1:1 acetonitrile : 0.1% TFA/H₂O and monitoring at 340 nm. Quantitation of the peak eluting between 5.0 and 5.4 minutes, which corresponds to the dinitrophenyl mercaptoacetic acid, indicated that three to four haptens per protein were present.

The procedures for conjugation of hapten to protein were the same when using BSA or LPH except that the hapten was in DMF solution when conjugated to BSA and the LPH conjugate was purified on a sephadex G-25 size exclusion column.

A sample of the antigen was then given to the hybridoma service, headed by Dr. Richard Van Deusen, at Iowa State University. The hybridoma service proceeded to immunize mice with our hapten following techniques similar to those described in the introduction. Serum samples were then received from their laboratory approximately every four weeks. These samples were then screened by enzyme-linked immunosorbent assay for their ability to bind hapten. For this purpose, the hapten was conjugated to BSA protein in order to distinguish between samples binding hapten as opposed to the carrier protein. Specificity for the entire hapten as opposed to just the dinitrophenyl or dinitrophenylhistidine group was also checked by making BSA conjugates of these latter compounds.
ELISA of serum from mouse L indicated that it contained antibodies recognizing hapten. This sample gave a titer of 640 when using hapten as ligand and only 160 with the dinitrophenyl group as a ligand. This is significant since the dinitrophenyl group is such a strong antigenic determinant. The fact that binding was stronger to the whole hapten molecule rather than to its pieces indicates that antibodies recognizing other structural features such as the tetrahedral phosphorus are present. The antibody-producing spleen cells from this mouse were fused with myeloma cells to prepare hybridoma cells which would proliferate in the cell culture medium while both the unfused myeloma and spleen cells would die. As described in the introduction, the hybridomas were expanded in vitro and samples of the hybridoma supernatant were tested for their ability to bind hapten over other components of the antigen. Wells containing cells producing these antibodies were cloned by dilution in the Van Deusen lab until a purified line of hybridoma cell was obtained.

Upon initial screening, samples labelled 2E5, 3E8, 3E10, 5C5, 7G4, 8F2, 10D2, and 6C8 indicated that they could contain potential candidates for transition-state specific antibodies. Through the dilutions, however, many of the samples which had looked promising were evidently overgrown by non-antibody producing cells. The results are shown in Table 1. One sample which survived, labelled 3E10, had a hapten titer of 64, a dinitrophenyl titer of 8, and showed no binding to KLH. The cloned hybridoma cells, labelled 3E10:E3, were injected into mice to initiate an antibody-producing tumor. The ascites fluid from this tumor, which contained the antibody of interest, was taken for analysis.

Any catalysis exhibited by this antibody was looked for both by the hydrolysis of substrates and by a catalytic assay described below. In a typical hydrolysis assay, a sample of the catalyst was introduced to a solution of dinitrophenyl histidine ethyl ester and bis(m-carboxyphenyl) carbonate 18 in pH 8.0 BBS. The extent of formation of m-hydroxybenzoate
Table 1.
Results of immunization of mouse L with Hapten.

**Mouse L Serum:**

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<tr>
<th></th>
<th>KLH</th>
<th>Hapten</th>
<th>BSA</th>
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<tr>
<td>640</td>
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<td>160</td>
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**Fusion Supernatant:**

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<th>Hapten</th>
<th>DNP</th>
<th>After Selection A410</th>
<th>Hapten</th>
<th>DNP</th>
<th>KLH</th>
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</thead>
<tbody>
<tr>
<td>2E5</td>
<td>1.033</td>
<td>8</td>
<td>64</td>
<td>0</td>
<td>1.033</td>
<td>8</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>3E8</td>
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<td></td>
<td>0</td>
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<td></td>
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</tr>
<tr>
<td>3E10</td>
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<td>256</td>
<td>64</td>
<td>1.233</td>
<td>64</td>
<td>8</td>
<td>0</td>
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</tr>
<tr>
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<td>64</td>
<td>16</td>
<td>0</td>
<td>1.305</td>
<td>64</td>
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<td>0</td>
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was then monitored by HPLC. Following this procedure, no increase in production of \( m \)-hydroxybenzoate was observed over the background hydrolysis in which no catalyst was added.

The steps required to maintain, clone, and purify large amounts of a particular antibody are costly in terms of both time and money. Oftentimes it is found that the best catalytic antibodies are not necessarily those that bind the hapten with the highest affinity. In some cases potential catalysts for a desired reaction may be overlooked simply because they did not bind the expected transition-state analog tightly enough. These antibodies may achieve catalysis through a mechanism other than simple transition-state stabilization and in many cases lead to interesting insights into the way enzymes have come to perform their catalytic
roles. Therefore, instead of the standard protocol of screening antibody samples for their ability to bind hapten, it would also be desirable to screen these antibodies directly for catalytic activity. Preferably this catalytic screening would be carried out as early as possible after hybridoma cells had been prepared. Hilvert and coworkers have done this in identifying catalytic antibodies for the decarboxylation of benzisoxazole-3-carboxylate ions to yield 2-cyano-5-nitrophenol which could easily be detected by UV-Vis spectroscopy. The problem to date is that no general assay exists for this purpose. A major reason for this is that antibody catalysts produced in hybridoma supernatant are present in very small amounts and this requires an extremely sensitive assay. Once antibody-rich ascites fluid is obtained, standard
analysis techniques such as HPLC or NMR can be used to determine their reaction rates but such techniques are not applicable to the screening of hybridoma supernatant.

To address this problem, we had envisioned an assay which is similar in practice and sensitivity to the enzyme linked immunosorbent assay and can be of general utility in finding coupling catalysts for a variety of reactions. In order to detect the antibodies with peptide ligase ability, as was the goal of our attempt to obtain catalytic antibodies, we covalently attached one substrate for our antibody (the dinitrophenyl histidine moiety) to BSA. This substrate could then be fixed to a hydrophobic surface by binding of the BSA to which it was attached. The second substrate which is an m-carboxyphenyl ester was then labelled with biotin through an amino caproic acid spacer arm.

To detect catalytic activity, a solution of the biotin-labelled m-carboxyphenyl ester and the hybridoma supernatant sample were placed in a microtiter plate well containing the surface bound dinitrophenyl histidine substrate. Amide bond formation would result in the linkage of the biotin tag to the surface. After rinsing away excess reagents, the biotin molecule could be detected through visualization using streptavidin-horseradish peroxidase and 3-ethyl-2-azino benzthiazoline sulfonate (ABTS). Streptavidin binds biotin very tightly with a dissociation constant of $10^{-15}$ M. The attached peroxidase can then be used to convert the colorless ABTS into its intensely green colored oxidation product. By monitoring absorbance at 415 nm the extent of reaction can then be quantitated.

Carrying out this assay on our supernatant samples, we found one candidate, 5C5, to show some absorbance above the control reaction containing no catalyst. Although these results were observed by both myself and my assistant David Jewell, they were unfortunately not reproduced in subsequent trials and it could therefore not be known with certainty whether 5C5 was actually catalytic.
One problem with applying this assay to the detection of catalysis in our antibody samples was that we were dealing with two unknowns. It was difficult therefore to work out appropriate conditions for the assay when it was uncertain that there was in fact a catalyst to detect. Another problem was that the solubility of the phenyl ester substrate in water was limited, which could have contributed to the variability in results observed. A more soluble version of a substrate would be desirable as well. At any rate, it was decided that working out assay conditions using a known catalyst would be highly desirable and the results obtained will be addressed in the following chapter.
CONCLUSIONS

In this chapter, the considerations and methods required to produce catalytic antibodies have been discussed. These methods were followed closely in our attempts to elicit catalytic antibodies for an as yet unreported but useful peptide coupling reaction. Despite our efforts, catalytic antibodies were not obtained. The success of isolating catalytic antibodies hinges on the production and subsequent characterization of a large number of hapten-specific antibodies. For reasons which are difficult to determine, an unusually low number of hapten-specific antibodies were obtained after the immunizations of eight mice by the hybridoma facility. Several possible explanations for this low number of antibodies were discussed in the preceding section. This is especially unusual in light of the fact that a dinitrophenyl group, which is known to be a strong antigen, was incorporated into the hapten structure.

As for the hapten-specific antibodies which were detected in supernatant, many were overgrown before they could be cloned and purified. This was partly because of the lag time between sampling in one laboratory, screening in our laboratory, and cloning in the first. A faster turnaround is an important aspect of success in such projects. Since the catalytic assay did not indicate the presence of any catalysts, it was difficult to determine whether we had a working assay. For this reason, we turned to a study of a known catalyst in order to develop the assay as described in the following chapter. The one antibody that was obtained through ascites fluid did not show catalytic activity when screened by the methods described.

Future attempts to form catalytic antibodies of this type may involve the use of combinatorial antibody libraries which must be screened to locate hapten-specific antibodies. In these instances, as well as in the conventional immunological methods, it will be important to quickly and sensitively locate the antibodies which exhibit catalytic properties. For this
reason, concentration was focused on the development of an assay for the general and sensitive detection of catalytic species as will be described in the following chapter.
EXPERIMENTAL

General methods

All infrared spectra were obtained on an IBM IR98 FT-IR spectrophotometer and all proton and carbon nuclear magnetic resonance spectra were obtained on a Nicolet NT-300 NMR spectrometer. Chemical shifts are reported relative to tetramethylsilane (TMS) as an internal standard and the solvent is deuterochloroform unless otherwise noted. Coupling constants (J) are reported in Hz. High resolution mass spectral analyses were performed on a Kratos MS-50 spectrometer. Low resolution mass spectra were obtained from a Finnegan 4023 mass spectrometer. Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected. Thin-layer chromatography (TLC) was performed on commercially prepared E. Merck silica gel 60F glass plates (0.25 mm) and flash chromatography was carried out as described by Still with the use of E. Merck Kieselgel 60 silica gel (230-400 mesh).

All of the chemicals used were directly obtained from commercial sources unless otherwise noted. Acetonitrile used for reactions was freshly distilled from P_{4}O_{10}. Methylene chloride and triethylamine were distilled from CaH_{2} and pyridine was also distilled from CaH_{2} except for use in ninhydrin assays in which case it was distilled from ninhydrin. THF was distilled from potassium metal and benzophenone.

Benzyl m-hydroxybenzoate (12)

To a solution of m-hydroxybenzoic acid (394 mg, 2.852 mmol) in 4 ml of EtOH was added KOH (168 mg, 2.995 mmol). The KOH dissolved, and after about 2 minutes, product precipitated. Solvent was removed by rotary evaporation, and then by azeotropic toluene
evaporation (2 X 5 ml). This crude potassium m-hydroxybenzoate was dissolved in DMF (1.8 ml), and treated with benzyl chloride (348 mg, 3.023 mmol) and KI (23.6 mg, 0.143 mmol) at 85-95°C for 1.5 hr, until starting material was consumed. (TLC 8% CH₂CN / CH₂Cl₂ UV, PMA: product Rf= .50, BnCl .88, salt 0). The reaction mixture was cooled to room temperature, diluted with Et₂O, and washed three times with H₂O and aqueous NaHCO₃. After drying over MgSO₄, solvent was removed on a rotary evaporator to give an oil which crystallized upon standing overnight on the vacuum pump. Recrystallization from ether/hexanes gave pure benzyl m-hydroxybenzoate as reported in the literature (MP 69-70°C, 423 mg, 65%) (lit.²⁰ MP = 70°C). ¹H NMR (300 MHz, CDCl₃) δ = 5.34 (s, 2 H), 6.36 variable (s, 1H), 7.01-7.66 (m, 9H). FT-IR (cm⁻¹, CDCl₃) 3380(br); 3033; 2954; 1716; 1589; 1455; 1289; 1222; 754.

bis (m-(benzyloxycarbonyl)phenyl) chlorophosphate (13)

A solution of triethylamine (1.9ml, 14 mmol) in 10 ml of THF was added dropwise with stirring to a 0°C solution of benzyl m-hydroxybenzoate (1.29g, 5.66 mmol) and freshly distilled phosphorus oxychloride (0.259 ml, 2.83 mmol) in 8 ml THF. The reaction mixture was allowed to warm to room temperature, and stirred for an additional 1 1/2 hours. The triethylamine hydrochloride salt was allowed to settle. Best results were obtained when the chlorophosphate reagent was not isolated, but instead measured volumetrically and transferred by syringe for subsequent reaction. Evaporation of solvent gave crude product. ¹H NMR (300 MHz, CDCl₃) δ = 5.38 (s, 2H), 7.43-7.37 (m, 6H), 7.49-7.54 (m, 1H), 7.96 (t, J = 1.5 Hz, 1H), 8.04 (t, J = 2.7 Hz, 1H).
N$_{\alpha}$-t-Boc-N$_{\text{Im}}$-DNP histidine (14)

N$_{\alpha}$-t-Boc histidine (892.5 mg, 3.496 mmol) obtained from Sigma was dissolved in 10 ml nanopure water along with 673.1 mg (7.991 mmol) sodium bicarbonate. Meanwhile, 2,4-dinitrofluorobenzene (0.502 ml, 3.995 mmol) was dissolved in 10 ml THF in a separate container. After the histidine solution had been chilled to 0° C in an ice bath, the dinitrofluorobenzene/THF solution was added with stirring. The resulting solution was placed in a refrigerated chromatography cabinet maintained at 4° C and stirred for 6 1/2 hours.

The solution was then washed with 60 ml ether, acidified to pH 2 with NaHSO$_4$ and extracted with ethyl acetate (3 x 40 ml). The ethyl acetate solution was dried with sodium sulfate, filtered and solvent removed under vacuum to give a yellow oil. Carbon tetrachloride (15 ml) was added and evaporated off to remove other solvents. After removing solvent, a yellow-orange foam remained.

The foam was then dissolved in refluxing isopropanol and cooled to room temperature after which time a large amount of solid product had recrystallized. The solid was broken up and mixed with carbon tetrachloride and filtered using carbon tetrachloride as the wash. After being placed on the vacuum pump for 6 hours, a $^1$H NMR spectrum was taken which showed the product was present along with some isopropanol (9.1% by weight). The yield subtracting out the weight of isopropanol was 1.3096 g (3.11 mmol) which corresponds to a 89% yield.

TLC Rf = 0.8 (eluent = 15:3:2.5 MEK:AcOH:H$_2$O) visualized by UV. FT-IR (CDCl$_3$, cm$^{-1}$): 3370, 3110, 2955, 1711, 1608, 1544, 1500 $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 1.45 (s, 9H), 3.12-3.43 (m, 2H), 4.47-4.60 (m, 1H), 5.51 (d, $J$ = 5.97 Hz, 1H), 6.89 (s, 1H), 7.78 (d, $J$ = 8.69 Hz, 1H), 7.88 (s, 1H), 8.60 (dd, $J_1$ = 2.46 Hz, $J_2$ = 8.73 Hz, 1H), 8.87 (d, $J$ = 2.46 Hz, 1H). M.P. = 98-102° C. Data were consistent with that previously reported$^{21}$. 
Ethanol (12 ml) was chilled in an ice bath in a dry flask, and 0.178 ml (2.483 mmol) thionyl chloride was added with stirring. Next, 451 mg (1.080 mmol) of $N_{\alpha}$-$\beta$-Boc-$N_{\beta}$-DNP histidine, which had been dried under vacuum, was added with stirring. The solution was refluxed and the solid dissolved. Refluxing was continued for 5 hours after which time a large amount of solid had precipitated from the refluxing solution. The mixture was filtered through a coarse filter glass funnel to collect the solid weighing 276.9 mg. Yield 66% (0.710 mmol). The product can be purified further through recrystallization from methanol and tetrahydrofuran in a ratio of 3 to 1. TLC: $R_f = 0.6$ on silica gel using MEK:AcOH:H$_2$O in a ratio 15:3:2.5 as eluent and UV to visualize. MP = 210-212$^\circ$C. $^1$H NMR (300 MHz, CD$_3$OD) $\delta = 1.31$ (t, J = 7.1 Hz, 3H), 3.40-3.49 (m, 2H), 4.26-4.40 (m, 2H), 4.46 (t, J = 5.4 Hz, 1H), 7.81 (s, 1H), 8.15 (d, J = 8.71 Hz, 1H), 8.78 (dd, $J_1 = 2.43$ Hz, $J_2 = 8.74$ Hz, 1H), 9.11 (d, J = 2.46 Hz, 1H) 9.18 (s, 1H).

**Phosphoramidate (16)**

Dry toluene (4 ml) and trimethylsilyl chloride (100 ul, 0.788 mmol)) were added to $N_{\beta}$-DNP histidine ethyl ester (108.8 mg, 0.2813 mmol) and, after stirring for 35 min., were evaporated off. Another 4 ml toluene was then added and removed under vacuum. A THF solution (5.0 ml) of the chlorophosphate (0.0469M, 0.233 mmol) was then added with stirring, followed by 0.25 ml triethylamine. After 2.5 hrs., an intense spot of $R_f$ 0.33 was observed by TLC on silica gel with ethyl acetate as eluent. After 12 hrs., the reaction mixture was filtered on a coarse filtered glass funnel, washing with THF. A clear yellow filtrate was collected and solvent was removed under vacuum. The yellow oil remaining was flashed using ethyl acetate as eluent to give the desired phosphoramidate in 52% yield (88.5 mg). $^1$H NMR (300 MHz, CDCl$_3$) $\delta = 8.76$(d, 1H, $J = 2.5$), 8.48(dd, 1H, $J = 2.5, 8.7$), 7.90-7.78 (m, 3H), 7.50-7.35(m, 16H), 6.79 (s, 1H).
5.32 (d, 4H, J = 6.0), 4.59 (t, 1H, J = 9), 4.49-4.39 (m, 1H), 4.11 (q, 2H, J = 7.2), 3.18-2.96 (abx, 2H, J = 4.6, 5.8, 14.6), 1.18 (t, 3H, J = 7.2). Mass Spectrum: DCI-NH₃ M⁺ = 850, m/e = 849.75 (calculated for C₄₂H₃₆N₅O₁₃P).

**TMSI cleavage of benzyl esters to yield 17**

The phosphoramidate (22.4 mg, 0.0264 mmol) was dissolved in 1.0 mls CDCl₃ which had been previously stored over 4A molecular sieves. The resulting solution was transferred to a dry NMR tube, which was then flushed with nitrogen and sealed with a septum.

After taking an initial NMR spectrum, trimethylsilyl iodide (21.8 ul, 0.153 mmol) was added, and the solution vortexed and placed in an Abderhalden drying flask heated to 56 C.

After 22 hrs., the reaction was judged to be complete and the contents of the NMR tube was poured into 4 mls ammonium bicarbonate buffer (25 mM). The aqueous solution was extracted 3 times with equal volumes of ethyl acetate. The resulting aqueous solution was lyophilized.

The residue was dissolved in 3 ml nanopure water and covered with 3 ml ethyl acetate. The aqueous layer was acidified with one drop 1M HCl which caused the yellow colored product to be extracted into the organic layer. The aqueous layer was extracted with an additional 3 ml ethyl acetate and the extracts were then combined, dried with sodium sulfate, filtered and ethyl acetate removed under vacuum to give the product in quantitative yield. Rf = 0.6, 1:1 0.1% TFA:CH₃CN, C-18. ¹H NMR (300 MHz, d₆-acetone) δ = 8.92 (d, 1H, J = 2.5), 8.68 (dd, 1H, J = 2.5, 8.7), 8.05-7.86 (m, 6H), 7.63-7.45 (m, 4H), 7.13 (s, 1H), 5.70 (t, 1H, J = 9), 4.52-4.39 (m, 1H), 4.08 (q, 2H, J = 7.2), 3.15 (bs, 2H), 1.18 (t, 3H, J = 7.2).

HPLC: r.t. = 12.5 min. on Alltech C-18 econosphere 4.8 x 250 mm; f.r. = 1 ml/min.; 50% CH₃CN 50% H₂O with 0.1% TFA.
Conjugation of hapten to protein

10 mg of 17 were placed in a 2 ml vial and dissolved in 0.5 ml distilled acetonitrile. Next, 8.6 mg water soluble carbodiimide and 5.2 mg N-hydroxysuccinimide were added and the reaction mixture was stirred for 2 hours. The solvent was then removed under vacuum. 1.0 ml of a 4 mg/ml KLH solution in pH 7.4 PBS was added to a clean and dry 2 dram vial. Next, 300 ul of a 300 mM pH 8.4 BBS solution was added followed by 103 ul of a 5.6 mg/ml solution of the hapten as the NHS ester.

The final solution, which was 64 mM borate and 8% acetone, turned slightly cloudy upon the addition of the acetone solution.

The solution was vortexed for 3 hours at 4°C.

To purify the conjugate, a sephadex G-50 size exclusion column (150 mm x 15 mm) was used. 1.5 ml fractions were collected using pH 7.4 PBS as eluent. Fraction 3 contained the largest protein concentration which was determined by BCA assay to be 1.5 mg/ml.

The extent of conjugation was determined by either TNBS assay of free amines on the protein before and after coupling and/or by thiolysis of the dinitrophenyl group of the hapten by mercaptoacetic acid. The thiolysis reaction was carried out in pH 7.0 phosphate buffer under conditions worked out by D.V. Jewell who was an undergraduate student working under my direction.

Thiolysis procedure

Sodium phosphate (116.1 mg, 0.814 mmol) was dissolved in 965 ul nanopure water and the container then purged with nitrogen. Mercaptoacetic acid (25 ul, 0.467 mmol) was then added to the aqueous solution and, after mixing well, the solution was transferred to a quartz cuvette. The cuvette was then placed in a thermostated cuvette holder set at 35°C and allowed to equilibrate for five minutes. After this period, 10 ul a 9.0 mM solution of the DNP
derivative to be analyzed (in nanopure water) was added and spectra were taken every three minutes. The reaction was judged to be complete after 18 minutes and the pH at this time was found to be 6.6.

The thiolysis product mixture was extracted into ethyl acetate after acidification of the aqueous layer with sulfuric acid and, after drying with sodium sulfate, the solvent was removed under vacuum. A standard using t-Boc-(DNP)-histidine was treated in the same manner. The residue was dissolved in HPLC eluent to make it 5.5 mg/ml and components were then quantified by HPLC using an isocratic eluent of 1:1 acetonitrile : 0.1% TFA/H₂O and a flow rate of 1.0 ml/minute and monitoring at 340 nm. An Alltech econosphere C-18 column (length = 250 mm i.d. = 4.6 mm) was used for the analysis. Quantitation of the peak eluting between 5.0 and 5.4 minutes, which corresponds to the dinitrophenyl mercaptoacetic acid, indicated that three to four haptens per protein were present.

The procedures for conjugation of hapten to protein were the same when using BSA or LPH except that the hapten was in DMF solution when conjugated to BSA and the LPH conjugate was purified on a sephadex G-25 size exclusion column.

**bis (m-carboxyphenyl) carbonate (18)**

Triphosgene (66.0 mg, 0.2224 mmol) was weighed into a dry flask under nitrogen. Benzyl m-hydroxybenzoate (225.6 mg, 0.9884 mmol) was weighed into a separate flask, traces of water were removed by azeotrope with toluene, and a solution of the phenol in 2.0 ml CH₂Cl₂ was prepared (0.49 M). Three equivalents of the phenol (0.6672 mmol) was added to the solid triphosgene followed by 0.16 ml pyridine (1.978 mmol). TLC after 30 min. indicated that some of the product carbonate had formed but also showed that unreacted phenol was still present. The remainder of the phenol solution was added after observing this.

After 5 hours, starting phenol was still observed by TLC. To test whether the phenol
seen could be from hydrolysis of the chloroformate, the reaction mixture was cospotted with ammonia saturated methanol. Phenol was still observed but there was also a new spot at Rf 0.1. This test was not conclusive. After stirring overnight, 0.200 ml water was added to hydrolyze any remaining phosgene and to convert chloroformate to carbonate. Bubbling was observed immediately but TLC indicated that phenol was still present. After stirring with water for 45 min., solvent was removed on the vacuum pump using a KOH trap after checking for phosgene with indicator paper.

EtOAc was added to the crude residue and was extracted three times with pH 2-3 0.02M sodium bisulfate. After drying over sodium sulfate, solvent was removed under vacuum and proton NMR and IR were taken that the desired bis (m-(benzyloxy-carbonyl)phenyl) carbonate had been formed quite cleanly. The product had an Rf virtually identical to that of benzyl m-hydroxybenzoate using EtOAc/Hexanes as eluent however it was found that product and starting material could be separated using 40:60 CCl₄;CH₂Cl₂ as eluent. The mixture was flashed in this eluent to give 176.2 mg (70% yield) of carbonate. FT-IR (CDCl₃, cm⁻¹) 3067, 3034, 2955, 1780, 1719, 1589, 1445. ¹H NMR (300 MHz, CDCl₃) δ = 5.36 (s, 4 H), 7.24-7.49 (m, 18(14) H) 7.96-8.02 (m, 4 H).

The (m-(benzyloxycarbonyl)phenyl) carbonate was dissolved in 5 ml EtOAc and the benzyl groups were removed by hydrogenolysis using 10% Pd/C (51.1 mg) as catalyst. After stirring overnight, starting material was gone and a white solid had precipitated. The mixture was filtered through celite and the solid rinsed with 15 ml CH₃CN, The solvent from the combined rinsings was removed in vacuo to give 80 mg (0.2645 mmol, 72% yield) white solid. FT-IR (CH₃CN, cm⁻¹) 3336, 3033, 1781, 1564. ¹H NMR (300 MHz, CH₃CN) δ = 7.23-7.51 (m, 5(4) H) 8.01-8.08 (m, 4 H).
Immunoassays

After obtaining antibody-containing hybridoma supernatant samples for Dr. Van Deusen's laboratory, they were screened for their ability to bind the hapten used to elicit antibodies. This was done using established ELISA procedures\textsuperscript{23a,b} where the hapten is linked to BSA which is, in turn, adsorbed to a polystyrene. Antibody which binds the hapten can then be detected by addition of goat-anti-mouse antibodies conjugated to horseradish peroxidase followed by addition of ABTS.

Carbonate Coupling

A solution of 100 mM bis(m-carboxyphenyl) carbonate was prepared by dissolving the solid in pH 8.0 BBS. Next, $N_{im}$-DNP-histidine ethyl ester was dissolved in pH 8.0 BBS to make a solution which was 300 mM in $N_{im}$-DNP-histidine ethyl ester. The two solutions were mixed (0.5ml of each) and either 100 ul PBS or 100 ul of the antibody sample was added. The test tubes were vortexed and the the reaction solution sampled at 30, 60, 90, 120 and 240 minutes.

100 ul of the reaction solution was diluted with 400 ul water, acidified to pH 2 with 100 ul 0.1 M NaHSO$_4$, and then extracted with 4 x 1.0 ml ether. The ether extracts were dried by passing through a pipet containing Na$_2$SO$_4$ and the solvent was then removed under vacuum. The residue was then dissolved in HPLC eluent to make it 2 mg/ml and analyzed by HPLC. For HPLC analysis, an isocratic eluent of 1:1 acetonitrile : 0.1% TFA/H$_2$O and a flow rate of 1.0 ml/minute was used. The amount of $m$-hydroxybenzoate (retention time 3.0-3.1 min.) was determined by monitoring at 252 nm. An Alltech econosphere C-18 column (length = 250 mm i.d. = 4.6 mm) was used for the analysis.
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PAPER II

SENSITIVE DETECTION OF CATALYTIC SPECIES

WITHOUT CHROMOPHORIC SUBSTRATES
INTRODUCTION

With the steady progression in the development of catalytic antibodies as useful models of enzymic reactions and as synthetic tools, it has become increasingly important to streamline the production of the desired antibody. To this end, general rules or factors important for the successful generation of catalytic antibodies need to be outlined. This area includes factors important in the structure and design of the hapten. Another area that needs to be developed further, is the rapid and accurate screening of the potential catalyst pool for the desired catalytic activity and specificity.\textsuperscript{1-2} A typical sample from a spleen cell fusion will produce hybridoma cells generating as many as $10^8$ different antibody structures.\textsuperscript{3} From this large pool, it is necessary to find the few candidates for catalysis as quickly as possible. In addition, many non-antibody producing hybridoma cells are also present and these cells tend to multiply more rapidly than those which do produce antibody.\textsuperscript{4} As a result, hybridoma cells producing potential catalysts may be starved out in the cell culture medium unless they are identified and isolated in a short period of time. The concentration in hybridoma supernatant is typically very low making screening by HPLC analysis of reaction product impractical. An assay therefore, is needed which is very sensitive, rapid, and specific for catalysts of the desired reaction. It has been observed in more than one case that in screening for antibodies that bind the hapten, many good catalysts can be overlooked. These catalysts may provide rate accelerations through methods other than simply preferential binding of the transition state structure.\textsuperscript{5a-d} The best approach may involve augmenting "mechanism-based" hapten design with direct genetic screens for catalytic activity. The generation of protein and polynucleotide structures from combinatorial libraries looks to be a promising general method for the rapid production of catalytic species.\textsuperscript{6-10} In this method an even greater number of structures may
be produced.

To date, most assays that have been used to screen directly for catalytic activity involved antibody-catalyzed hydrolysis reactions in which a chromophoric group was generated.\(^1\)\(^1\) Although this method was fairly sensitive it could not be used as a general assay since it places a limitation on the hapten design as the chromogenic moiety must be incorporated. In the past couple years, researchers have begun to focus more attention on the development of a sensitive and general catalytic assay and some noteworthy advances have been made.

Recently, Peter Schultz and coworkers have developed a chromogenic assay for screening large antibody libraries.\(^1\) The assay is useful in that it may lend itself to the screening of bacterial colonies or plaques generated by mutagenesis or combinatorial strategies. The assay was developed by using the catalytic antibody 48G7 as a model.\(^1\)\(^2\) This antibody catalyzes the hydrolysis of \(p\)-nitrophenyl esters and carbonates. A chromogenic substrate 2 was designed, which consists of 3-hydroxyindole (4) linked to the \(p\)-nitrophenyl carbonate moiety via 4-hydroxybutyric acid (Scheme I). It was envisioned that hydrolysis of the carbonate to \(p\)-nitrophenol and indolylbutyric acid (3) should be followed by rapid intramolecular cyclization via attack by the generated hydroxyl group to give butyrolactone and 3-hydroxyindole. Under the reaction conditions, 3-hydroxyindole undergoes oxidative dimerization to form the chromophore indigo, which rapidly precipitates to indicate the location where the hydrolysis took place. The indigo, with an extinction coefficient of 29,700 can then be sensitively detected.

The rate of hydrolysis of substrate 2 was assayed by monitoring nitrophenyl release spectrophotometrically and found to be comparable to the hydrolysis of other substrates, such as 4-nitrophenyl butyl carbonate 5, which do not contain the hydroxyindole moiety.
Scheme I. Generation of indigo chromophore

Antibody catalyzed hydrolysis of 2 resulted in the formation of 0.3 equivalents of indigo per equivalent of p-nitrophenol. Indigo formation was found to be dependent upon antibody catalytic activity. In the absence of antibody, no indigo formation was detected. The antibody-dependent indigo formation was inhibited by the nitrophenyl phosphonate transition-state analog 1 to which the antibody was generated. In addition, the benzyl ether of indoylbutyric acid 3 did not form detectable indigo when incubated with the antibody, indicating that the formation of indigo is not simply due to the antibody catalyzed hydrolysis of the indoyl ester. The fact that the indigo chromophore precipitates from solution enhances the sensitivity of this assay as other chromophores such as p-nitrophenol are diluted by rapid diffusion. The assay has also been applied to the detection of an antibody (7D4) which catalyzes the hydrolysis of alkylphenyl carbonates, a reaction in which no visible chromophore
is produced. In this latter case, the 5-bromo-4-chloro-indolyl substrate 6 was needed.

In general, the authors have introduced a clever idea for the detection of catalytic antibodies. The assay seems to be quite sensitive and directly applicable to the screening of bacterial colonies on agar plates and provides a simple qualitative assay of catalytic activity rather than hapten binding. The production of an insoluble dye or indicator is essential in order to localize the positions in which colonies are producing antibodies of the desired activity and specificity. These efforts will undoubtedly bring more attention to the importance of developing sensitive screening assays and will lead to more generally applicable assays.

Although the assay is useful and may be applicable to the screening of catalytic antibodies produced in bacterial colonies or plaques, it appears to be limited to the detection of esterolytic reactions. High background from the non-specific hydrolysis of the labile carbonate or ester may be misleading. In addition, it requires that the indolylbutyric acid moiety be present at the reacting center which may limit the number of reactions for which one can screen. One can envision that in some cases antibodies could directly hydrolyze the indolyl ester given the large variety of antibodies produced. Although the authors do not mention why the 5-bromo-4-chloro-indolyl substrate 6 was needed when studying the second antibody (7D4), it may be to prevent hydrolysis of the indolyl ester by making it less accessible.

In a recent article, Green and coworkers have introduced a catalytic assay based on existing ELISA methodology. The idea behind their assay is to have the antibody substrate bound to a surface. The surface is then exposed to a solution containing the antibody sample and if it is catalytic, it will accelerate the formation of the desired product. Anti-product antibodies from rabbit, which were previously prepared, are then bound to locations in which product had been produced. Finally, the surface is exposed to an enzyme-labeled second antibody which binds the mouse antibody and the enzyme catalyzes the formation of a colored
product indicating that the desired product was formed.

They tested the feasibility of this strategy by studying the enzyme- or base-catalyzed hydrolysis of esters 7a-d and imide 11. The BSA conjugates of these substrates were absorbed to microtiter plates then treated with either pH 10.9 sodium carbonate solution or pancreatic lipase, an enzyme which can catalyze the hydrolysis of esters. Formation of the resulting acid product 9 was then determined by conventional ELISA using the rabbit anti-9 antibodies

\[
\text{H}_2\text{O} \quad \text{Substrate (7, } X = \text{O}; 8, X = \text{NH)} \rightarrow \text{Product (9)}
\]

followed by peroxidase-linked, anti-rabbit immunoglobulin antibodies. No signal was seen for the amide analogs of 7a-d which are not substrates. The rates of hydrolysis of esters 7a-d in the presence of various concentrations of lipase reached a maximum at relatively low enzyme concentration. They attribute this to the small amount of substrate which is absorbed to the microtiter plate.

The researchers then applied the assay to the screening of antibody supernatant for catalysis. To generate catalytic antibodies, they used a phosphonate hapten 10 which mimicked the transition-state for the desired hydrolysis reaction. After fusing the immunized mice spleen cells with an immortal myeloma cell line, the resulting 1570 hybrid clones were
assayed for their ability to induce the hydrolysis of ester 7a and amide 8. Of the dozens of clones that bound hapten 10, only two were found to give a positive signal in the assay, indicating that catalysis had taken place. The catalytic antibodies isolated showed remarkable specificity as they were specific for the p-nitrobenzyl ester 7a while 7b-d were not accepted as substrates.

This assay developed by Green and coworkers is of utility in that it allows for the screening of large numbers of hybridoma supernatant samples using existing ELISA methodology which is already very familiar to researchers in this area. The assay also seems to be quite specific in screening for antibodies capable of catalyzing the desired reaction. The signal observed, however, was not much above that observed for hydrolysis in pH 8.25 TBS solution. Again, as in the preceding example, the question of general applicability arises although it is not nearly as limited as the former. The system requires the use of a product-
specific antibody which must be produced through costly and time-consuming immunological methods. It may be possible to lower this cost through use of polyclonal antibodies however, questions of selectivity still remain. As a result, this could be a limitation if the detection of a number of different products is desired. In that case, many new product-specific antibodies are required. The reaction screened for, as in the last example, is an ester hydrolysis reaction and is somewhat limited in this regard although it is possible that the acid moiety could be used as a label for a second substrate in a coupling reaction. If the detailed study of a single reaction is desired, this assay has a certain advantage in that it poses no limitation on the substrate or product structure (other than the ability of the immune system to recognize it).

The Green assay could be applied to reactions in which two molecules are coupled together. These reactions are of much greater interest from both a synthetic and mechanistic point of view and a general assay for the detection of catalysts for this type of reaction would be invaluable. The assay, as was the goal of those mentioned above, should be general, inexpensive, rapid, and easily applied to the handling of large numbers of samples. In the following sections, I will discuss the development of such an assay which does not impose any restrictions on the reaction center and is operationally quite similar to the ELISA already employed to detect antibody binding to substrate.
RESULTS AND DISCUSSION

In the development of antibody catalysts and other protein catalysts from combinatorial libraries it is becoming increasingly important to be able to screen the large number of potential catalysts produced. In order to effectively screen the large pool of candidates generated from current methods, a very sensitive and preferably simple assay must be developed. The ELISA, which is used to detect antibody binding to hapten molecules, is a very sensitive assay for detecting dilute amounts of antibody. It is also convenient to perform as a simple and general protocol has been developed. It was a logical extension then, to model our desired assay for detecting catalysts of bimolecular reactions after this procedure.

The general idea for our assay was to have one substrate for a catalyst bound to a surface which could be manipulated and washed. The second substrate would then be bound to a tag which could easily and sensitively be detected.

As a tag, we chose biotin since it is bound by the protein avidin with a dissociation constant of $10^{-15}$ M. When a solution of the labelled substrate and the catalyst is exposed to the surface bound substrate, a covalent attachment between the two substrates is formed resulting in the tag being irreversibly bound to the surface. Any unreacted labelled substrate could be simply rinsed away. Biotin on the surface could then be detected using an enzyme amplification procedure involving streptavidin linked to the enzyme horseradish peroxidase. Exposing the modified surface to a solution of this protein conjugate results in the horseradish peroxidase (HRP) being bound at those sites where a reaction has taken place. As the final
step in this sequence, the surface is rinsed free of unbound HRP and then exposed to a chromogenic substrate for the peroxidase such as 3-ethyl-2-azino-benzthiazoline sulfonate (ABTS). As a result, a colored spot is observed at those locations on the surface where a reaction has taken place.

For practical purposes, it becomes apparent that the assay's success depends on the fidelity of all the steps along the way to the final color development. Specifically, it requires that all of the rinsings completely remove unbound reagents and that the reagents used to visualize the biotin tag are all functioning at maximum sensitivity. An additional consideration in this assay is that the detection of a bimolecular reaction is desired and one of the substrates by definition must be essentially stationary. Thus, the rate is going to be affected by how rapidly the soluble substrate and catalyst can diffuse to the surface. Also, since a bulky protein catalyst is being studied, it becomes all the more important that the substrate is easily accessible so that steric hindrance between the surface and the catalyst is not a problem. For this reason, a long, flexible linker is needed to force the surface bound substrate into the bulk solution.

The advantages of this assay procedure lie in its sensitivity and in its generality. By use of the commercially available streptavidin-horseradish peroxidase and ABTS developing system, a very sensitive signal can be generated. This is a result of the amplification that takes place as the peroxidase enzyme converts many ABTS substrate molecules to their corresponding intensely colored oxidation product. In most cases, the results can be observed visually as a green color and can be measured quantitatively with the use of a spectrophotometer. ABTS is a convenient substrate as it is commercially available and forms a distinct water soluble dye. However, other visualization sources such as chemiluminescent substrates are also available and in some cases may provide even greater sensitivity. The assay has generality in that there are no chemical requirements at the reacting center. No
chromophoric substrate need be produced by the reaction assayed. The substrate molecules must be tethered to either a surface or to a biotin tag however, as the position of these tethers is not important, much greater flexibility is achieved. As was already mentioned, existing catalytic assays have been used to detect hydrolysis reactions generating a chromophoric leaving group. Although this type of assay has been used with success, it is confined to the study of hydrolyses involving chromogenic substrates. Another disadvantage is that sensitivity may be lost due to background hydrolysis of the active ester. Until now, no assay has adequately addressed the problem of screening for catalysts of bimolecular coupling reactions. The development of catalyst of this type is likely to be important in a number of synthetic applications. As one further advantage, we envisioned that since the assay is operationally quite similar to the ELISA, it should be possible to perform screening of various catalyst samples in parallel.

**Assay Development**

To develop a working assay, we chose to study the reaction involving solvent damaged α-chymotrypsin catalyzed aminolysis of amino acid esters. Wong has previously reported that the serine protease α-chymotrypsin can act to catalyze irreversible amide bond formation in aqueous media containing greater than 55% organic cosolvent. The most suitable substrates are the methyl esters of hydrophobic amino acids as acyl donor and alanine or other relatively unhindered amino acids as acyl acceptor.
Under the reaction conditions, chymotrypsin is a useful, although not particularly stable catalyst for this transformation. For this reason we considered it to be a relevant benchmark to gauge our assay for its potential to detect protein catalysts, which initially may be only moderately efficient catalysts. Therefore, to determine the sensitivity of our assay, we studied the chymotrypsin-catalyzed coupling of an N-acylated tyrosine methyl ester with an alanine amide in 60:40 acetonitrile:water.

The first step in the assay development was to synthesize the desired modified amino acid substrates. Since the aminolysis reaction requires high concentrations of the acyl acceptor, it was necessary to have alanine as our soluble substrate so that the concentration could be easily controlled.
Therefore, alanine would have the biotin tag. Initially, an aminocaproyl ethylene diamine linker was used as a spacer between alanine and the biotin tag to form 15. The \( p \)-methylsulfinylphenyl ester of aminocaproic acid, which had been previously prepared, was aminolyzed by treatment with ethylene diamine. Acylation of the free amine 14 with biotin NHS ester followed by removal of the \( t \)-butoxycarbonyl protecting group afforded the desired reagent. Initial experiments were performed using this substrate, however it exhibited poor solubility in the solvent mixture used for the assay and it soon became obvious that a more soluble derivative would be required.
For this reason, I turned to the use of the much more soluble tetraethylene glycol derived linkers reported by Bednarski and coworkers. Along the way, I have developed new procedures for the preparation of linkers complimentary to those of Bednarski\textsuperscript{16} and I will discuss this further in the next section. The synthetic sequence leading to the desired substrate is shown. This synthesis began again with the preparation of the active \textit{t}-Boc alanine N-hydroxysuccinimide ester 16 as described in the literature\textsuperscript{17}. \textit{t}-Boc-alanine NHS ester 16 was then used to acylate the amino azide 17 using pyridine and methylene chloride as solvent. The amino azide, obtained from extraction, contained triphenylphosphine oxide as a byproduct. However, the ratio of triphenylphosphine oxide to amino azide could be calculated by NMR integration and the acylation product could be purified through silica gel chromatography to give pure 18 in 68% yield.
The azide function was converted to the corresponding amine through reduction with triphenylphosphine and was subsequently acylated by biotin NHS ester. The protected form of the substrate could then be purified through flash chromatography and stored in this form. Removal of the t-Boc group by HCl saturated THF affords the desired substrate as the hydrochloride salt of 13.

Biotin NHS ester 20b was prepared by the literature procedure\(^\text{14}\) which involved drying both biotin and N-hydroxysuccinimide overnight over P\(_2\)O\(_5\) in vacuo. Biotin was then reacted with carbonyldiimidazole at 80\(^\circ\)C in DMF and the intermediate biotinylimidazolide 20a, which precipitated from solution, was then treated with NHS to form the product. The crude product could be recrystallized from refluxing isopropanol to give the pure product in 78% yield.
The acyl donor, which was chosen as tyrosine methyl ester based on the observations by Wong\textsuperscript{13}, was modified for coupling to a support by reaction with glutaric anhydride. The resulting hemiglutaryl tyrosine methyl ester 21 could then be activated through various means such as formation of the NHS ester 22, and then coupled to a support containing a nucleophile.
Our first choice of a support for the acyl donor was the protein bovine serum albumin (BSA) adsorbed to polystyrene. (Interestingly another support, polyallyl amine was also tried in early experiments but the results showed that the support itself caused the conversion of ABTS to its green oxidation product. This could possibly be due to the presence of metals present in the polymer sample.) This is the standard protein that we and many other researchers have used as the support for hapten conjugates in ELISA screenings. The substrate molecule can be covalently linked through amide bonds to the lysine side chains in the protein.

A quantitative determination of the number of free amino groups before and after coupling indicates the number of substrates bound per protein. The protein can then be irreversibly adsorbed to the wells of a polystyrene microtiter plate and, after blocking any remaining polystyrene surface by exposure to more BSA, the well is exposed to a solution of catalyst and acyl acceptor. After the reaction has taken place, the biotin label is covalently bound to protein and is thus fixed to the surface, with the weakest connection being that of the interaction of protein with the polystyrene surface. It then becomes necessary to rinse away excess reagents without rinsing off the absorbed protein. Tween 20, a non-ionic surfactant is a good choice for this purpose as it does not denature proteins. After these rinsings, the biotin label is then detected using the standard streptavidin-HRP / ABTS detection system and absorbances are read at 415nm.
To test the feasibility and detection limits of this approach, a model experiment was run in which BSA absorbed to the wells of a microtiter plate, was exposed to a buffered solution of biotin and varying amounts of \(N\)-ethyl-\(N\)'-dimethylaminopropyl carbodiimide hydrochloride (WSC), a reagent known to couple carboxylic acids to amino groups in proteins. Under the conditions assayed, it was found that coupling could be clearly detected down to WSC concentrations of 15 \(\mu\)M at pH 6.2 and 30 \(\mu\)M at pH 7.4. This indicated that a moderately good catalyst could be detected at low levels and that the strategy was worth pursuing.

![Chemical structure](image)

When the coupling assay was initially tried under the described conditions, what was observed was that both chymotrypsin and biotin-labelled alanine were required to give the strongest signal. Various controls were performed, however, which indicated that just chymotrypsin alone gave a signal half as strong as that when biotin-labelled alanine was also present. This result was difficult to explain but could be due to the chymotrypsin causing some of the polystyrene surface to be exposed so that in the subsequent incubation with streptavidin-HRP, some may have been non-specifically absorbed to the surface giving rise to the signal. It did seem that a portion of the signal (approximately half) could be attributed to the coupling reaction. To increase the accessibility of the tyrosine methyl ester, a linker, 1-methylcarboxy-1,3,6,9-tetraoxa-11-azidoundecane, was inserted between the BSA and the substrate through WSC-mediated coupling followed by reduction of the azido function with tris(carboxyethyl) phosphonium hydrochloride. The linker however, did not appear to make a difference when compared side by side with the substrate not containing the linker. A subsequent experiment in which chymotrypsin concentration was serially diluted showed an
approximate dependence of observed signal on chymotrypsin concentration. In this experiment the background signal with chymotrypsin alone was not observed. Up to this point however, no experiment was run in which the acyl donor, tyrosine methyl ester, was absent.

The next experiment addressed this question. In addition to absorbing BSA-tyrosine methyl ester to the surface, a row of wells, to which unmodified BSA had been absorbed, was used as a control. Both the modified and unmodified BSA were exposed to a solution of acyl acceptor, chymotrypsin, or both. From this experiment, it was found that although the controls in which chymotrypsin or biotin-labelled alanine had been omitted were negative, an equally strong signal was observed for both the reactions with and without tyrosine methyl ester. Therefore, both chymotrypsin and biotin-labelled alanine are required for a positive result but tyrosine methyl ester is not. One possible explanation for this is that the chymotrypsin retains some of its amidase activity forming acyl enzyme with the BSA, which is then aminolyzed by biotin-labelled alanine. Decreasing the reaction time, which had been several hours, to several minutes may help as the ester should react more rapidly than the amide. Also, up until this point a one to one mixture of acetonitrile and water had been used. Using a higher percentage of organic cosolvent would help in that case. The problem with using too high a concentration of organic solvent is that this decreases the solubility and stability of the enzyme.

It would be nice to know what component of the signal was due to reaction with tyrosine methyl ester, that is, is there any enhancement of signal due to the recognition and greater reactivity of tyrosine methyl ester substrate with chymotrypsin. In order to have a chance at seeing this effect, besides making the changes described in the preceding paragraph, it is also necessary to lower the concentration of catalyst to see better selectivity. This would also give limits of detection for the assay. To this end, rows containing BSA-TyrOMe and BSA were absorbed to the surface, then a solution of biotin-labelled alanine and
varying amounts of chymotrypsin in 60:40 acetonitrile:water were placed into the wells. After developing, it was found that chymotrypsin could clearly be detected down to 0.1 uM in the presence of BSA containing tyrosine methyl ester and to 1 uM when BSA without tyrosine methyl ester was present. At a fixed chymotrypsin concentration of 6 uM, biotin-labelled alanine down to 3.6 mM could be detected. These results, shown in Figure 1, indicate an approximately ten fold difference in sensitivity attributable to the presence of the substrate acyl donor on the surface.

It was decide that it would be necessary to eliminate the signal from the presumed background reaction of chymotrypsin with BSA. For this reason, the feasibility of using other supports was studied. The use of nitrocellulose as a solid support for ELISA had been reported previously so this was looked at first. As an initial test, biotin was coupled to nitrocellulose via biotin NHS ester and this was then exposed to the reaction conditions. It was found however, that the nitrocellulose paper was not compatible with the 60%
acetonitrile cosolvent used in the assay. For this reason, the use of chromatography paper as a solid support was then examined.

Lebl and coworkers had reported the use of Whatman chromatography paper as a solid support for peptide synthesis so it seemed reasonable that it could also be used for the purposes of our assay. To this end, surface hydroxyl groups on the paper were acylated with azido acid 23 as outlined in the scheme below. The azido group was then reduced to the amine using stannous chloride and finally, the free amine was acylated with the NHS ester of hemiglutaryl tyrosine methyl ester. Between coupling steps, the paper was extensively rinsed with a variety of solvents. Coupling was confirmed by reflectance IR and by quantitative ninhydrin assay. It was also found that it was important to wash the paper with aqueous base after the reduction in order for successful coupling in the next step.

\[
\text{Cellulose—OH} \xrightarrow{\text{DCC, HOBr, DMAP}} \text{Cellulose—O(\text{NHS-ester of hemiglutaryl tyrosine methyl ester})}
\]

When this new sample of acyl donor was tested in the assay, successful results were obtained. The paper sample was cut into a small disk and placed in the bottom of a microtiter plate well. It was then exposed to a 60% acetonitrile solution containing biotin-labeled alanine and chymotrypsin for two minutes as described above. Controls were also run in which one or
more of the required elements was omitted. After developing, it was found that the two wells containing the catalyst and the two substrates gave a signal much stronger than the others. Paper without ester substrate is ineffective, and biotin tethered to L-Boc-alanine, without the nucleophilic amine, also leads to no signal above background. Background, in this case, was the signal obtained from incubating derivatized paper with only chymotrypsin.

Once the desired response was obtained, the next step was to find the optimal conditions and limitations for the assay. On varying conditions, it was found that pre-blocking the paper with a solution of Tween 20 was important to decrease the amount of background signal. Optimal amounts of hydrogen peroxide and ABTS in the developer were also determined as well as the appropriate dilution for the streptavidin-HRP conjugate. By varying the volume of reaction solution, it was found that as little as 5 ul of solution could be used with a clear signal still obtained. Next, the effect of reaction time was examined by allowing the reaction to go for twenty or two minutes. Wong's studies indicated that longer reaction times could result in some cleavage of peptides formed. The results of this experiment, as shown in Figure 2, indicated that a reaction time of twenty minutes gave a better signal as illustrated by the upper line in the figure. Controls in which a chymotrypsin catalyst was boiled to eliminate activity gave much lower signal. Various cosolvents were also screened for their efficacy in coupling and we have found several that appear to be equivalent to the acetonitrile system identified by Wong et al13. Although duplicate experiments reveal a fairly large scatter in some cases, it does provide a good illustration of the potential application of the assay.

A titration of chymotrypsin concentration was also performed in order to find the sensitivity limits of our assay. Chymotrypsin concentrations were varied from 38 to 0.25 nM and it was found that a clearly visible signal was detectable down to 1 nM. Considering that 5 ul of solution were used, this corresponds to the detection of only 5 femtomoles of catalyst. The absorbance increases linearly with increasing chymotrypsin concentration in the range
between 0 and 10 nM however, at higher concentrations, absorbance was observed to level off. This leveling off of absorbance occurred concomitantly with the development of a purple color on the paper which could be an oxidation byproduct from the enzyme reaction. The results are illustrated in Figure 3.
An attempt was also made to link hemiglutaryl tyrosine methyl ester to paper through a tetraethylene glycol diamine linker. The linker itself can be covalently attached to the paper through a urethane linkage providing a more stable bond to paper. This modification also had an advantage in that it would require fewer chemical steps to attach substrate to paper. After rinsing with TFA and triethylamine as described in the experimental section, the paper could be activated by treatment with carbonyl diimidazole to form 30. Treatment of this intermediate with commercially available tetraethylene glycol diamine resulted in the desired amine tethered to paper 31 as confirmed by reflectance IR and quantitative ninhydrin assay. Hemiglutaryl tyrosine methyl ester could then be attached as described above. Unfortunately, when this sample was used in the assay, no signal was observed. It was noted that this linker was slightly shorter than the azido acid 23 used previously and this may be the reason for loss of signal in the assay. If this is the case, the problem can be overcome by subsequent acylation of the amine 31 with azido acid 23 followed by reduction of the azide and coupling of hemiglutaryl tyrosine methyl ester.
Preparation of useful tetraethylene glycol derived linkers

In many biological assays it is necessary to couple relatively small molecules to proteins or other polymers for purposes of labeling of the protein or immobilization of the molecule. In doing so, it is often desirable to use a linker, or spacer arm, between the two substituents so that the small molecule is more accessible. Linkers have been widely used to attach a variety of small sized tags or labels to a larger polymer such as a protein. Such linkers have found use in medicine, as in radiolabeling of organ targeting drugs as well as in the study of biological pathways and metabolism of particular compounds. It is essential that the linkers used do not interfere with the property being studied and therefore should usually be long, flexible and readily soluble in a variety of solvents. The reagent should also contain terminal functional groups which can easily react to form covalent linkages with the two molecules of interest. Based on the observation of Bednarski et. al. that tetraethylene glycol derivatives displayed these desirable properties as linkers, we looked into this application in more detail which led to the development of useful new linkers, and improved preparation of known linkers for the labeling of a variety of compounds.

As mentioned above, Bednarski had prepared and used the azido alcohol TEG derivative 27 as a linker. However, the side products from the reaction, namely the starting TEG 26 and the diazido TEG 28 were discarded. Although the starting material is inexpensive it would still be desirable to make more efficient use of it as well as the diazide.

In the linkers which were developed as part of this thesis, both the azido alcohol 27 and diazide 28 TEG derivatives are used as precursors. The azido alcohol leads to a useful azido acid derivative in one step while the diazide can be converted in one step and in good yield to the complimentary amino azide linker reported previously by Bednarski in three steps.
from azido alcohol. The two starting materials, as described by Bednarski, can be efficiently separated from one another by flash chromatography.

The obtained azido alcohol can be converted to the acid azide 23 by alkylation with chloro or bromoacetic acid in the presence of potassium hydroxide. A number of reactions conditions have been examined in order to find the appropriate conditions to drive this reaction to completion. Initially, chloroacetic acid was employed as the alkylating agent, KOH as the base, and DMF was used in a minimal amount as solvent in order to stir the reaction mixture. While reasonable yields were obtained, the reaction did not go to completion after five days even under reduced pressure (to remove water, driving the deprotonation of alcohol). It was found that running the reaction between 45 - 55° C helped to accelerate the reaction while heating at higher temperatures tended to lead to decomposition of the azide. Since using chloroacetic acid as alkylating agent necessarily requires the production of one mole of water, the potassium salt was formed prior to the alkylation reaction and this caused the reaction to proceed essentially to completion after four days. By further changing the alkylating agent to the more reactive potassium bromoacetate, the reaction time was lessened to two days at 45° C.
It was also postulated that if a stronger base was used, the reaction may be accelerated further. Sodium hydride is often used as base in the alkylation of alcohols however, in this case the reagent would be a bad choice as it could act to reduce the azido group present in the molecule\(^9\). Potassium \(t\)-butoxide seemed to be a good alternative as it is more basic than hydroxide and is also hindered so that it is not likely to cause the formation of side product by displacement. This latter qualification was important since it was desired to purify the acid azide by extraction so that the process could be easily scaled up. The consumption of azido alcohol was essentially complete within one day, however, although \(t\)-butoxyacetic acid did not appear to form as a side product, proton NMR of the product after workup indicated the presence of other impurities, and the use of \(t\)-butoxide was abandoned.

Since a large excess of both potassium hydroxide and bromoacetate was required to ensure that the reaction went to completion in a reasonable amount of time, it was necessary to separate the product acid azide from these reagents. Potassium hydroxide can be readily extracted into the aqueous phase but bromoacetic acid was observed to be a potential contaminant. However, the hydrolysis product of bromoacetic acid, glycolic acid, is quite soluble in water and it was found that by adding water and stirring for an additional eight hours the remaining bromoacetic acid could be converted to the water soluble glycolic acid.

One nice aspect of this reaction is that if the starting azido alcohol is pure, any unreacted azido alcohol can be recovered in high purity through extraction of the basic aqueous phase.
with methylene chloride. The desired pure azido acid, as determined by proton NMR and TLC, can be made from tetraethylene glycol without the need for chromatography.

The azido acid thus obtained can be thought of as an N-protected amino acid derivative. Once the acid function has been coupled to a desired compound, the amino group can be introduced through mild reduction with triphenylphosphine\textsuperscript{20-22} or stannous chloride\textsuperscript{23-24}, or by catalytic hydrogenation\textsuperscript{25-27}, thiol reduction\textsuperscript{28-29}, or NaBH\textsubscript{4} \textsuperscript{30-31} reduction. The amine can then participate as a nucleophile to form a covalent attachment to a second substrate or, if desired, form an interaction through ion-pairing. Another desirable feature of the techniques developed here is that through the same chemistry, a number of linkers may be easily formed with varying chain lengths simply by choosing the starting polyethylene glycol of the desired formula weight.

The second useful linker, the amino azide TEG derivative 17, can be obtained from the symmetrical diazide through preferential reduction of only one azido group. Initially, this was attempted through palladium catalyzed hydrogenation in the presence of cation exchange resin. The idea here was to allow the reduction to take place on one of the azido groups at the palladium surface to form the amino azide which would then diffuse away into the bulk solution under rapid stirring. When the free amine of the amino azide encountered the cation exchange resin, it would be protonated and bound inside by ion-pairing and, as a result, protected from the palladium catalyst so that a second reduction could not take place. Once all of the starting diazide had been consumed, the resin could be rinsed first with methanol followed by ammonia saturated methanol to elute the product. Removal of solvent would then afford the desired desymmetrized amino azide as the major product. Methanol was found to be the best solvent for allowing the rapid adsorption of amine into the resin.

Unfortunately, under the conditions described above, decomposition of the starting material or one of the formed intermediates was observed. By thin layer chromatography,
three separate ninhydrin positive spots were observed. A weaker, carboxylic acid based cation exchange resin was also tried however similar results were observed. An explanation for the observed decomposition concerns the acidity inside the resin. Once the amino azide had been bound to the cation exchange resin, the remaining azido group was exposed to a highly acidic environment due to the large concentration of acid in a relatively non-polar medium. Decomposition under such conditions could occur through protonation of nitrogen in the azide leading to denitrogenation to form and intermediate imine. Hydrolysis of the imine would then lead to formation of the TEG derived aldehyde and ammonia. This mechanism is outlined in the scheme below.

Because of the problem cited above, an alternative route to the selective reduction was examined. It was well known that triphenylphosphine is capable of effecting the mild reduction of alkyl azides. Since the diazide is readily extracted from aqueous solution into ethyl acetate, and triphenylphosphine is clearly more soluble in organic solvents than in water, it followed that a selective reduction may be achieved by use of a biphasic organic/water system.

The idea here is described as follows. The diazide and the reducing agent are dissolved in a water immiscible organic solvent over an acidic aqueous solution. Under rapid stirring, as
one azide group is reduced to the amine, the amino azide formed (or possibly the intermediate phosponimine) will be rapidly protonated and extracted into the aqueous layer. Since the triphenylphosphine reducing agent is confined to the organic phase, reduction of the second azide will not take place. Instead, diazide remaining in the organic phase will continue to be reduced to the amino azide leading to preferential formation of the latter.

Initial conditions studied involved the use of ethyl acetate as organic phase and 0.1 M sodium bisulfate as the aqueous phase. Tributylphosphine was chosen initially, however, it was found that the partition coefficient of triphenylphosphine for the organic phase was better than that of tributylphosphine. Besides this, tributylphosphine oxide was seen to remain in the aqueous phase to a large extent and it was hoped that the triphenylphosphine oxide formed would be more organic soluble. As will be described however, triphenylphosphine oxide has a remarkable tendency to associate with a number of hydrogen bond donors.

Best conditions for the selective reduction were obtained using ethyl ether as the organic phase and 0.25 M H₃PO₄ as the aqueous phase. For the large scale preparation, a solution of the triphenylphosphine in ether was added to a stirring mixture of the diazide to ensure mixing of the reducing agent prior to reaction. The reaction was found to be complete, using 0.85 equivalents triphenylphosphine, after ten to twelve hours. The workup involved ether extraction of the acidic solution to remove remaining diazide and triphenylphosphine oxide.
oxide, followed by addition of sodium hydroxide to make the aqueous solution 4M in hydroxide. Subsequent extractions using methylene chloride afforded the amino azide along with, surprisingly, triphenylphosphine oxide in a ratio of approximately ten to one. Evidently, the high polarity of the phosphorus-oxygen double bond allows the byproduct to remain soluble in water despite the three phenyl groups. Some association of the phosphine oxide with protonated amine may also be responsible.

The ratio of amino azide to diamine was determined by HPLC analysis of the CBZ-protected product mixture. Prior to extraction, the basic aqueous layer containing amine was treated with benzyl chloroformate until no amine could be visualized using ninhydrin indicator. The product mixture was then extracted into methylene chloride and analyzed by HPLC to determine the yield of each product. The results indicated that the amino azide was formed preferentially in a ratio of greater than fifty to one. TLC analysis of the amine obtained after extraction was consistent with this observation as only one spot corresponding to amino azide could be seen using ninhydrin indicator.

By following the methods developed above, it was possible to carry out the synthesis of these useful linkers on a large scale. Thus, 50 g of tetraethylene glycol were treated with 1.6 equivalents of mesyl chloride followed by sodium azide under the conditions outlined by Bednarski to obtain 49 g (85% yield) of a 70:30 mixture of diazide and azido alcohol. Alkylation of this mixture with potassium bromoacetate afforded the azido acid in 90% yield after extraction. The recovered diazide was purified by flash chromatography and converted to the amino azide in 79% yield after extraction.

It has been demonstrated that the versatile TEG derived azido acid and amino azide linkers can be prepared easily on a large scale. Bednarski has demonstrated the utility of TEG derived linkers for labeling and conjugation and these linkers have proved valuable in my own
work involving the fixation to surfaces or labeling of substrates. It is my hope that these procedures will be of use to others desiring linkers to serve similar functions.
CONCLUSIONS

An assay has been developed which can be of great general utility in the production of novel enzyme and antibody catalysts for bimolecular reactions. The usefulness of this assay has been illustrated by using solvent-damaged chymotrypsin as a good model for the types of catalytic species which one would expect to find in the initial stages of catalyst production. The assay has the following advantages over existing assays:

- A chromophoric group need not be produced by the reaction
  Requiring the use of a chromogenic substrate puts serious limitations on the number and type of reactions which may be studied.

- Active esters are not used
  The assay detects coupling of substrates not generation of a chromophore in the reaction step. In this way, one would not be misled by hydrolysis of the active ester.

- Many assays may be carried out in parallel
  As the assay is carried out in a 96 well microtiter plate, many reactions may be conveniently performed at once.

- The assay has general applicability
  The only requirement is that one substrate be attached to a surface and the other to a biotin tag.

The assay can be of great utility in the development of catalytic antibody technology where rapid and efficient screening for potential catalysts is essential. With increasing advances in the area of combinatorial antibody libraries, which produce even larger repertoires of structures, the need for such a screening assay is increased still more.
Besides screening to find new catalysts, the assay will also be of great utility in the evaluation and study of existing catalysts. The assay, which is rapid, sensitive and easily performed in parallel could be used to study structure-reactivity relationships for these catalysts leading to a better understanding of the catalyst mechanism. In this way, optimal reaction conditions for a particular catalyst would be more easily determined. Currently, efforts are under way to apply the assay to the screening of antibodies which could be catalysts for a Diels-Alder reaction. Future work could involve minaturization of the assay to allow screening of catalysts with varying specificity on a single sheet of paper and the use of more sensitive detection methods such as chemiluminescence.

Also, as a spin off of the assay development, methods have been worked out for the production of new bifunctional linkers. These linkers afford the desirable properties of increased solubility and accessibility in substrates or reagents to which they are attached and can be of great use in the labeling or conjugation of a variety of small molecules and proteins.
EXPERIMENTAL

BSA / Hemiglutaryl Tyrosine Methyl Ester Conjugate.

The NHS ester (22) of hemiglutaryl tyrosine methyl ester (21) (12.8 mg, 0.0312 mmol) was dissolved in 200 ul DMF. The resulting solution was added to 2.5 ml of a solution consisting of 10 mg/ml BSA in pH 8.4 borate buffered saline solution. Upon addition of the DMF solution to the aqueous protein solution, a small amount of white solid, presumably denatured protein, was observed to precipitate from solution. The mixture was vortexed at room temperature for 4 hours. The supernatant was then taken and the protein conjugate purified on a sephadex G25 size exclusion column. TNBS assay indicated that there were 8-10 hemiglutaryl tyrosine methyl esters per BSA.

Cellulose Conjugated to Acid-Azide (24).

The method of Lebl and coworkers\textsuperscript{16} was used for coupling to cellulose. A 5 cm\textsuperscript{2} piece of Whatman #1 Chromatography paper was placed in a petri dish and soaked in 4 ml 25\% TFA/CH\textsubscript{2}Cl\textsubscript{2} (30 min.), 3 x 4 ml CH\textsubscript{2}Cl\textsubscript{2} (5 min.), 2 x 5 ml 10\% Et\textsubscript{3}N/CH\textsubscript{2}Cl\textsubscript{2} (30 min.), and 2 x 3 ml CH\textsubscript{2}Cl\textsubscript{2}. The carrier was then pressed between filter paper to dry.

The paper was then treated with 4 ml of 0.1 M azido acid (23)/ DCC / HOBt / 0.03 M DMAP in DMF. The mixture was allowed to vortex overnight after which time a white precipitate (DCU) had formed. The solution above the paper was pipeted off and the paper was then rinsed with 2 x 4.5 ml DMF, 4 x 4.5 ml EtOH (dissolved DCU), and 3 x 4.5 mls CH\textsubscript{2}Cl\textsubscript{2}. The paper was then dried between filter paper.
Reflectance IR showed the presence of an azide band at 2111 cm⁻¹ and a CO band at 1750 cm⁻¹ indicative of an ester linkage.

**Cellulose Conjugated to TyrOMe Through TEG Linker (25).**

The azido-acid derivatized paper (24) prepared as described above was placed in a petri dish and 5 ml of a 1.3 mmol/ml SnCl₂ suspension in MeOH was added. The dish was vortexed for 2 hours. The paper was then rinsed with 5 x 4.5 ml MeOH, 3 x 4.5 ml 25% sat'd NaHCO₃, 4 x 4.5 ml EtOH, and 3 x 4.5 ml CH₂Cl₂, then dried between filter paper.

Reflectance IR of the paper showed a CO stretching band at 1749 cm⁻¹ however, no band at 2100 cm⁻¹, indicative of azide, was observed. Quantitative ninhydrin assay indicated that 60-62 nmol amine were present per each 4.8 mm diameter paper disk assayed.

Esterified cellulose with the free amine was prepared as described above. A 40 mM solution of hemiglutaryl TyrOMe NHS ester (22) in DMF (3 ml) was added to the paper, followed by 1 drop of distilled triethylamine and the paper vortexed overnight. The DMF solution was pipeted off and the paper was rinsed with 3 x 4.5 ml EtOH and 3 x 4.5 ml CH₂Cl₂.

After drying, a reflectance IR was taken which showed bands at 1742 (ester), 1660 and 1560 cm⁻¹ (amide). Coupling was judged to be complete by ninhydrin assay.

**Paper Linked to Tetraethylene Glycol Diamine (31).**

A procedure analogous to that of Bethell and coworkers was followed. Whatman chromatography paper, which had been washed as described in the preceding procedure was dried under vacuum overnight using KOH as desiccant. Next, a solution of 341.1 mg (2.1036
mmol) CDI in 6.5 mls acetonitrile (freshly distilled from P2O5) was added to 0.3554 g of the cellulose sample (one 5 x 5 cm sheet each of paper and dialysis tubing) contained in a petri dish. The dish was placed in a desiccator, which was flushed with nitrogen, and agitated occasionally for 45 min. The acetonitrile solution was then removed and the paper washed with 4 x 5 mls freshly distilled acetonitrile.

After the rinsings, 10 ml of an aqueous solution containing 5 ml tetraethylene glycol diamine (Texaco) was added to the petri dish containing cellulose. The dish was then vortexed for 16 hrs.

After this time, the solution was pipeted off and the paper was rinsed with 4 x 10 mls water, 4 x 10 mls 1M NaCl and again with 4 x 10 mls water. After 3 x 10 mls rinses with EtOH, the paper was dried under vacuum.

As a control, a clean paper sample, which had not been treated with CDI, was allowed to sit overnight under the an identical tetraethylene glycol diamine solution and rinsed in an identical manner. Using the control as a blank, reflectance IR was consistent with the presence of a urethane functional group (1716 and 1533 cm⁻¹). Quantitative ninhydrin assay indicated that 52 nmol amine/mm² paper was present.

Hemiglutaryl-L-tyrosine methyl ester (21)

L-Tyrosine methyl ester hydrochloride (153 mg, 0.714 mmol) from Aldrich was suspended in 2.5 ml methylene chloride. The suspension turned to a clear liquid immediately after distilled triethylamine (141.7 mg, 1.4 mmol) was added. After the addition of a solution of glutaric anhydride (62.3 mg, 0.55 mmol) in 1.0 ml methylene chloride, the reaction mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc, and then acidified with saturated NaHSO₄ to pH 2. Upon dilution with 1.0 ml water, the aqueous layer was removed and the organic layer washed once more with 0.1 M NaHSO₄ and once with
saturated NaCl. The EtOAc was dried over anhydrous sodium sulfate, filtered, and solvent was removed under vacuum to give a white solid mixture of 30:1 molar ratio of product to glutaric acid as determined by NMR. The adjusted yield was 61%. The NMR spectrum was consistent with the desired product except for the glutaric acid contaminant. The crude product was used directly for the preparation of the NHS ester. However, an analytical sample was obtained by flash chromatography. Rf = 0.35 (85:10:5 CHCl₃/CH₃OH/HOAc); ¹H NMR (300 MHz, CD₃CN) δ = 1.8 (quintet, J = 7.0 Hz, 2H), 2.2 (t, J = 7.0 Hz, 4H), 2.9 (ABX, J_gem = 14 Hz, J_vic_Ha = 6 Hz, J_vic_Hb = 7 Hz, 2H), 3.6 (s, 3H), 4.6 (bm, 1H), 6.7 (d, J = 8 Hz, 2H), 6.9 (d, J = 8 Hz, 2H).

L-Tyrosine methyl ester hemiglutaramide NHS ester (22)

Hemiglutaryl-L-tyrosine methyl ester (21) (60mg, 0.19 mmol) was suspended in 3.5 ml acetonitrile. The suspension became clear immediately after the addition of N-hydroxy-succinimide (66 mg, 0.57 mmol). It then turned to a suspension again after the addition of a solution of dicyclohexylcarbodiimide (126mg, 0.61 mmol) in 0.2 ml methylene chloride. The mixture was stirred at R.T. for 18 hours. After evaporation of the solvent, the residue was taken up in ethyl acetate and filtered to remove insoluble dicyclohexylurea and NHS. After removing solvent, 51 mg (64% yield) of white solid was obtained. Rf = 0.30 (6:1 EtOAc/hexanes); m.p. = 47-48 C; ¹H NMR (300 MHz, CD₃OD) δ (ppm) = 1.99 (m, 2H), 2.29 (t, J = 7.2 Hz, 2H), 2.50 (t, J = 7.2 Hz, 2H), 2.83 (s, 4H), 2.90 (dd, J₁ = 7.8 Hz, J₂ = 5.3 Hz, 1H), 3.11 (dd, J₁ = 13.8 Hz, J₂ = 5.3 Hz, 1H), 3.74 (s, 3H), 4.86 (dd, J₁ = 13.2 Hz, J₂ = 7.8 Hz, 1H), 5.87 (bs, 1H), 6.44 (d, J = 8.1 Hz, 1H), 6.72 (d, J = 8.4 Hz, 2H), 6.96 (d, J = 8.1 Hz, 2H); IR (neat) 3400, 3302, 1811, 1782, 1734, 1663, 1516, 1205, 1072 cm⁻¹.
N-(Boc-alanyl)-1-amino-11-azido-3,6,9-trioxaundecane (18).

1-amino-11-azido-3,6,9-trioxaundecane (17) (164.3 mg, 0.7528 mmol) was dissolved in pyridine (0.80 ml) and added to a dry flask containing t-Boc alanine NHS ester (215.5 mg, 0.7526 mmol). The solution was stirred under nitrogen. After an hour, TLC showed that t-Boc alanine NHS ester was still present. After 3 hrs., the pyridine solvent was removed under vacuum and the crude product flashed on a 20 mm x 8 in. silica gel column using 5% isopropanol in methylene chloride as eluent. The product was collected in 8 ml fractions from 16-24 and, after removing solvent, a clear oil remained. 160.0 mg was collected but contained isopropanol in a 1:1 molar ratio as determined by NMR. The yield of product was 139.3 mg (48%). TLC: Rf = 0.3 (5% isopropanol in methylene chloride), IR: 2107.4, 1709.7, 1668.0, 1555.5 cm\(^{-1}\), \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta = 6.6\) (b.s., 1H), 5.2 (b.s., 1H), 4.18 (b.t., 1H), 3.73-3.61 (m, 10H), 3.58 (t, \(J = 5.4\) Hz, 2H), 3.48 (t, \(J = 5.4\) Hz, 2H), 3.41 (t, \(J = 4.9\) Hz, 2H), 1.46 (s, 9H), 1.36 (d, \(J = 6.8\) Hz, 3H).

N-Boc-alanyl-N'-biotinoyl-1,11-diamino-3,6,9-trioxaundecane (19).

N-(Boc-alanyl)-1-amino-11-azido-3,6,9-trioxaundecane (18) (499.6 mg, 1.283 mmol), dissolved in 4.9 mls EtOAc, was reduced to the free amine by the addition of 360.6 mg (1.375 mmol) triphenylphosphine and 50 ul water. Starting material was consumed after 4 hrs. as judged by TLC, which showed the appearance of a ninhydrin positive spot. The crude product, after removal of solvent, was used directly in the coupling with biotin NHS ester. Rf = 0.40 (Rf = 0.80 for Ph\(_3\)PO biproduct) (5% NH\(_4\)OH in EtOH). IR of the crude reaction mixture was consistent with the desired product and showed disappearance of the azide band at 2106 cm\(^{-1}\). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta = 1.36\) (d, \(J = 7.01\) Hz, 3H), 1.44 (s, 9H), 2.14 (bs, 1H), 2.89(t, \(J = 4.85\) Hz, 2H), 3.56(t, \(J = 4.9\) Hz, 2H), 3.65(m, 12H), 4.22(bs, 1H), 5.41(bs, 1H), 7.3-7.5(m, Ph\(_3\)PO byproduct).

The amine obtained (466.4 mg, 1.283 mmol) and biotin NHS ester (441.3 mg, 1.293
mmol) were placed in a flask and dissolved in 4.0 mls distilled pyridine to give a clear solution. The solution was stirred under nitrogen overnight, after which time TLC showed that starting amine was completely gone. The crude mixture was flashed using a 30 mm silica gel column and 400 ml 1:1 CH$_3$CN:15% MeOH/CHCl$_3$ followed by 400 ml 15% MeOH/CHCl$_3$. The desired product was collected in 76 % yield (323.4 mg) and was judged pure by NMR. TLC: Rf = 0.3 (15% MeOH/CHCl$_3$), $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ = 7.2 (b.s.), 7.0 (b.t.), 6.8 (b.s.), 6.0 (b.s.), 5.6 (b.d), 4.5 (AB q., J = 4.84, 2.65, 4.88), 4.3 (AB q., J = 4.58, 2.26, 5.26), 4.2 (b.t.), 3.9 - 3.5 (m), 3.4 (t, J = 4.8), 3.2 (q, J = 7.7, 7.8), 2.9 (d of d, J = 4.86, 8.04, 4.62), 2.2 (t, J = 7.7), 1.9-1.6 (m), 1.5 (s), 1.4 (d, J = 7.0).

N-alanyl-N'-biotinoyl-1,11-diamino-3,6,9-trioxaundecane hydrochloride (13·HCl)

19 (97.3 mg, 0.16 mmol) was dissolved in THF (3 ml) and HCl was then bubbled through the solution for 40 minutes. Evaporation of the solvent provided 13 as its hydrochloride salt in the form of a white foam. (84 mg, 100% yield). TLC: Rf = 0.5 (10% NH$_4$OH in EtOH), $^1$H NMR (300 MHz, CD$_3$OD): $\delta$ = 1.51 (d, J = 7.0 Hz, 3H), 1.6-1.8 (m, 6H), 2.26 (t, J = 7.3 Hz, 2H), 2.74 (d, J = 12.9 Hz, 1H), 2.97 (dd, J$_1$ = 12.9 Hz, J$_2$ = 4.89 Hz, 1H), 3.2-3.3 (m, 1H), 3.42 (t, J = 5.6 Hz, 2H), 3.55 (t, J = 5.4 Hz, 2H), 3.59 (2t's, J = 5.6 Hz, 4H), 3.62-3.70 (m, 8H), 3.96 (q, J = 6.9 Hz, 1H), 4.40 (dd, J$_1$ = 7.8 Hz, J$_2$ = 4.5 Hz, 1H), 4.58 (dd, J$_1$ = 7.8 Hz, J$_2$ = 4.9 Hz, 1H), Mass Spectrum (Electrospray, 1:1 H$_2$O:MeOH, positive ion) m/e = 490.3 (calculated 490.6) (M$^+$-Cl$^-$), 512.9 (calculated 512.6) (M$^+$-HCl + Na).

tert-Butyloxycarbonyl-L-alanine NHS ester (16)

A solution of t-Boc-L-alanine (3.78 g, 20 mmol) and N-hydroxysuccinimide (2.3 g, 20 mmol) in 25 ml THF was cooled in an ice water bath the dicyclohexylcarbodiimide (4.5 g, 22
mmol) dissolved in 10 ml THF was added slowly with stirring over a five minute period under nitrogen. Some white precipitate was formed after all of the DCC solution was added. The mixture was allowed to stand overnight at 0°C. The precipitated N,N'-dicyclohexylurea was removed by filtration and the solvent removed under vacuum. The crude solid was purified further through recrystallization from refluxing isopropanol to afford the product as a white solid (4.25 g, 74% yield). m.p. = 165-166°C (Literature m.p. = 167°C) \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta = 1.46 \text{ (s, 9H), } 1.57 \text{ (d, } J = 7.2 \text{ Hz, 3H), } 2.84 \text{ (s, 4H), } 4.7 \text{ (bs, 1H), } 5.07 \text{ (bs, 1H).}

D-Biotin NHS ester (20b)

D-(+)-Biotin and NHS were dried overnight over \(\text{P}_2\text{O}_5\) in vacuo. Biotin (118 mg, 0.48 mmol) was dissolved in 3 ml DMF in a dried flask by gently heating at 80°C. A solution of N,N'-carbonyldiimidazole (78 mg, 0.48 mmol) in 0.7 ml DMF was then introduced to the flask. The mixture was stirred under nitrogen at 80°C for 30 minutes until CO\(_2\) evolution ceased and then at room temperature for 2.5 hours. The intermediate, biotinylimidazolide, came out of the solution as a flocculent white precipitate which was converted to the N-hydroxysuccinimide ester by the addition of NHS (55.6 mg, 0.48 mmol) in 0.6 ml DMF. The mixture was then stirred at room temperature under nitrogen overnight. After removal of DMF under vacuum, recrystallization of the crude product from refluxing isopropanol provided the product as a white solid (115.5 mg, 73% yield). Rf = 0.46 (1:1 CHCl\(_3\)/i-PrOH); m.p. = 199-200°C (Literature m.p. = 210°C) \(^1\)H NMR (300 MHz, CD\(_3\)OD) \(\delta = 1.58 \text{ (m, 3H), } 1.78 \text{ (m, 3H), } 2.66 \text{ (t, } J = 7.2 \text{ Hz, 2H), } 2.70 \text{ (d, } J = 14.1 \text{ Hz, 1H), } 2.83 \text{ (s, 4H), } 2.93 \text{ (dd, } J_1 = 12.9 \text{ Hz, } J_2 = 4.8 \text{ Hz, 1H), } 3.22 \text{ (m, 1H), } 4.32 \text{ (dd, } J_1 = 7.8 \text{ Hz, } J_2 = 4.5 \text{ Hz, 1H), } 4.49 \text{ (dd, } J_1 = 7.8 \text{ Hz, } J_2 = 4.8 \text{ Hz, 1H).}
Azido acid (23)

A mixture of azido alcohol (27) and diazide (28) (10.9916 g), determined to be 27.8% by weight azido alcohol (3.0891 g, 0.01409 mmol) by NMR, and potassium bromoacetate (5.62 g, 31.74 mmol) were weighed into a dry flask. Next, 6.0 ml DMF, which had been dried by storage over 4A molecular sieves, was added and stirring initiated. Powdered KOH was weighed out quickly and transferred to the flask under nitrogen and stirring was continued for two days at 45°C. After this time, 6 ml nanopure water was added and stirring was continued for an additional 12 hours at 45°C.

The solvent was then removed under vacuum and the residue rinsed into a separatory funnel with 100 ml water and 50 ml methylene chloride. The layers were separated and the aqueous layer was extracted twice more with 50 ml methylene chloride (each time). The aqueous layer was then acidified with solid NaSO₄ until the pH had been lowered to 2 and extracted thrice with 50-60 ml methylene chloride (each time). The methylene chloride extracts were dried over anhydrous sodium sulfate, filtered, and solvent removed to give a yellow oil. To ensure that all of the DMF had been removed, CCl₄ was added and evaporated off three to four times. After placing the sample on the vacuum pump overnight, 3.5057 g remained which corresponds to 90% yield based on azido alcohol. ¹H NMR looked quite clean with no visible contaminant peaks. TLC showed one spot using 85:10:5 CHCl₃:MeOH:AcOH as eluent. ¹H NMR (300 MHz, CDCl₃): δ = 4.15 ppm (2 H, s), 3.7 ppm (14 H, m), 3.4 ppm (2 H, t, 6 Hz). FTIR 3399, 2909, 2107, 1748, 1123, 755 cm⁻¹. TLC(silica gel): Rf = 0.25 using 85:10:5 CHCl₃:MeOH:AcOH.

Amino azide (17)

The diazide (28) (3.0196 g, 12.36 mmol) was placed in a 250 ml flask and dissolved in 55 ml ether. Next, 0.25 M H₃PO₄ was added. Triphenylphosphine (2.8196 g, 10.75 mmol)
was placed in a 25 ml addition funnel and dissolved in 20 ml ether. Rapid stirring was initiated and the solution of \( \text{Ph}_3\text{P} \) was added dropwise over a 10 minute period. The system was sealed with a septum and balloon to prevent excessive evaporation of ether. During the course of the reaction, the balloon was observed to inflate, presumably due to nitrogen gas being liberated. After stirring for 24 hours, no triphenylphosphine was visible by TLC.

The layers were then transferred to a separatory funnel using an additional 10 ml water and ether to rinse the flask and the ether layer removed. The aqueous layer was extracted twice more with 60 ml ether (each time). Next, the solution was covered with 10-15 ml ether and 12.8 g powdered \( \text{NaOH} \) was added. The addition generates heat and the solid initially dissolves, however, on cooling it precipitates from solution as a flocculant white solid. This precipitate did not interfere significantly with the separations and the aqueous layer was extracted thrice with ether (50-60 ml each time) and twice with 40 ml methylene chloride (each time). The combined organic extracts were dried over anhydrous \( \text{Na}_2\text{SO}_4 \), filtered, and solvent removed to give a clear oil. The product amino azide (1.8420 g, 8.44 mmol) was obtained in 79% yield based on \( \text{Ph}_3\text{P} \) and 68% yield based on starting diazide. \(^1\text{H} \) NMR indicated that the product was quite pure except for the usual contaminant of \( \text{Ph}_3\text{PO} \) (9:1). TLC showed one very heavy spot and another barely visible by ninhydrin. When spotted more lightly, the lower spot could not be seen. This observation was consistent with the previously determined >50:1 selectivity. The >50:1 selectivity was determined by HPLC. After making the aqueous solution containing amine 4M in \( \text{NaOH} \), 100 ul benzylchloroformate was added. Two more 100 ul aliquots were added every 8 hours until amine was no longer visible by TLC using ninhydrin to visualize. The solution was extracted four times with distilled \( \text{CH}_2\text{Cl}_2 \), dried over \( \text{Na}_2\text{SO}_4 \), and solvent removed to give an oil. A 2-3 mg/ml solution was then prepared by dissolving the sample in HPLC eluent (1:1 \( \text{CH}_3\text{CN}(0.05\% \text{TFA}):\text{H}_2\text{O}(0.05\% \text{TFA}) \)) and the samples were then analyzed by HPLC on a C-18 usorb-mv 4.6 x 100 mm
column, flow rate = 0.750 ml/min., λ = 254 nm. Quantitation of the Cbz derivatives of the amino azide and diamine were performed by injecting 150 ul sample solution on a 20 ul sample loop. Amounts of amino azide and diamine Cbz derivatives eluting at 3.8 and 4.9 minutes respectively were determined using response factors of the standard compounds. Response factors for the amino azide and diamine Cbz derivatives were 1.048 x 10^8 area/umol and 1.624 x 10^8 area/umol respectively. 1H NMR (300 MHz, CDCl₃): δ = 1.9v (br. s, 2H), 2.89 (t, J = 5.2 Hz, 2H), 3.40 (t, J = 5.0 Hz, 2H), 3.50 (t, J = 5.2 Hz, 2H), 3.55-3.80 (m, 10H); FTIR = 3415, 2912, 2106; TLC Rf = 0.55 using 10:90 NH₄OH : ethanol as eluent.

Coupling assay procedure

1) Cut paper sample (25 or underivatized paper) into disks using a #2 cork borer and place in a full well microtiter plate.

2) Pretreat both derivatized and underivatized paper disks by incubating overnight in 100 ul 0.1% Tween 20 in pH 7.4 PBS.

3) Pipet off Tween 20 solution and add reagents.
   a) Prepare a 72 mM stock solution of biotin-labelled alanine (13-HCl) in 65% CH₃CN/ Na₂CO₃ (aq.) as follows:
      - Transfer 15.0 mg (29.1 umol) 13-HCl to a tared vial.
      - Add 100 ul H₂O, 200 ul CH₃CN, and 7 ul saturated Na₂CO₃ (to make pH 8-9).
      - Add 35 ul H₂O and 63 ul CH₃CN.

      The solution at this point should be a single phase. If two phases are present, the salt concentration is probably too high. Add more water until one phase is obtained.

   b) Prepare a 420 uM chymotrypsin solution in PBS by dissolving 5.32 mg (210 umol) chymotrypsin in 0.500 mls PBS.
Transfer 50 ul of the 13-HCl stock solution to each of five test tubes.

Add 100 ul of chymotrypsin stock solution to a test tube containing 100 ul PBS. Mix by pipetting up and down 15 times. Transfer 50 ul of this solution into the next test tube which contains 150 ul PBS, mix, and repeat three more times. Five solutions are obtained with concentrations of 210, 52.5, 13.1, 3.28, and 0.82 uM.

Add 5 ul of the diluted chymotrypsin solution to a test tube containing 50 ul 13-HCl stock solution and mix. Five reagent solutions are obtained with [13-HCl] = 65.4 mM and [chymotrypsin] = 19.1, 4.80, 1.19, 0.298, 0.0746 uM. The CH3CN % = 59 %. These solutions should be prepared just before use as chymotrypsin is unstable under the conditions. Preparation time from first solution to fifth solution was approx. 3 min. 30 sec..

Place 5 ul of 19.1 uM CT in B2, D2, F2, G2, G3, D11, and B11.

Place 5 ul of 4.80 " B3, D3, F3.

Place 5 ul of 1.19 " B4, D4, F4.

Place 5 ul of 0.298 " B5, D5, F5.

Place 5 ul of 0.075 " B6, D6, F6.

Place 5 ul of 72 mM 13-HCl in 65% CH3CN in B8 and B9.

Place 5 ul of 210 uM CT in D9, F8, and F9.

The paper absorbs most of the liquid. The plate was vortexed for 2 min. and 30 sec. after all additions were complete.

Rinse with 1% SDS in nanopure H2O being careful not to cross contaminate in early rinses. Rinse five times, transfer paper, then rinse five more times with SDS and three times with nanopure H2O.

Block with 200 ul 3% BSA in PBS for 1 hr. 15 min. at 37 C. Pipet off.

Add 50 ul streptavidin-HRP diluted 1:6000 in 3% BSA/PBS. Incubate 30 min. at 37 C and pipet off.
7) Rinse twice with PBS, nine times with 0.1% Tween 20 in PBS, and three times with nanopure H₂O.

8) Add 100 μl ABTS developer (2.4 mg ABTS + 6.0 mls citrate + 3.6 H₂O₂).
Develope for 20 min. and read absorbance at 405 nm subtracting 630 nm.

CT = Chymotrypsin (Sigma), A = biotin-labelled alanine (JWL-4-075),
T = tyrosine derivatized paper (JWL-4-127), P = underivatized paper,
D = dialysis tubing.
REFERENCES


PAPER III

USE OF BIOTINYLATED 7-AZATRYPTOPHAN
AS A PROBE OF SMALL MOLECULE-PROTEIN INTERACTIONS
INTRODUCTION

The utility of 7-azatryptophan as an alternative to tryptophan for optically probing protein structure and dynamics is demonstrated by investigating the complex of egg-white avidin and biotinylated 7-azatryptophan. We report the synthesis of biotinylated 7-azatryptophan and optical measurements of its complexation with avidin. Although there are four biotin binding sites, the emission from the 7-azatryptophan tagged to biotin decays according to a single exponential, whereas the tryptophyl emission from avidin requires at least three exponentials in order to be adequately fit. Fluorescence depolarization measurements of the complex probed by emission from 7-azatryptophan reveal both rapid (~50 ps) and much longer-lived decay. The former component is attributable to the local motion of the probe with respect to the protein; the latter component represents overall protein tumbling.

The Petrich group has proposed the nonnatural amino acid, 7-azatryptophan, as an alternative to tryptophan as an optical probe of protein structure and dynamics. The merits of 7-azatryptophan lie in its intrinsic single exponential fluorescence decay in water as compared to the nonexponential decay exhibited by tryptophan as well as in its spectroscopic distinguishability with respect to tryptophan in both absorption and emission. This allows measurement of rapid motions of the 7-azatryptophan side chain in the presence of other tryptophan residues. Furthermore, 7-azatryptophan can be incorporated into bacterial protein and is amenable to peptide synthesis. An important application of 7-azatryptophan is the incorporation of it into small peptides, its binding to cofactors, and subsequent investigation of the dynamics of these smaller, tagged molecules bound to the target protein of interest. In this article, we demonstrate the feasibility of this
approach by studying biotinylated 7-azatryptophan (inset of Figure 1) bound to avidin.

Avidin is a tetrameric protein found in avian egg white. Each subunit contains 128 residues of which 4 are tryptophan. Avidin is believed to function as an antibacterial agent through its ability to reduce the free concentration of biotin. The dissociation constant of the avidin-biotin complex is about $10^{-15}$ M$^{19-24}$. The essentially irreversible binding afforded by this complex and the ready modification of the carboxylate group of biotin have permitted the study of the interactions of several biotin adducts with avidin. Examples are the interactions of biotin lipids$^{21}$ and biotinylated asparagine-oligosaccharides$^{22}$ with avidin and streptavidin. Recently, x-ray structures of egg-white avidin and its complex with biotin have appeared$^{24}$. Biotin is shown to bind in a β-barrel constructed from eight antiparallel β strands. The tryptophan residues 70 and 97 form part of the avidin binding site and are anchored through hydrogen bonds to other residues, thus stabilizing the binding site. The bicyclic ureido ring of biotin forms hydrogen bonds with Asn-12, Ser-16, Tyr-33, Thr-35, Asn-118, and possibly Thr-77$^{24}$.

We have prepared a biotin-7-azatryptophan adduct in order to demonstrate further the spectroscopic distinguishability of 7-azatryptophan from tryptophan and to investigate the mobility of the 7-azatryptophan moiety in the complex.

The research description that follows was a collaboration between myself, under the guidance of Dr. Alan Schwabacher, and researches in Dr. Jacob Petrich's group namely, R. Rich and F. Gai. They had previously studied the use of 7-azatryptophan as a fluorescent probe and it was my role in this collaboration to synthesize the key compound for the fluorescence studies which was biotin-labeled 7-azatryptophan methyl ester. I was chosen for this project due to my familiarity with the properties and characterization of biotinylated derivatives. As very sensitive fluorescence measurements would be used to study the binding of biotin-labeled 7-azatryptophan to avidin, it was necessary to prepare a very pure sample of
this derivative. Any underivatized 7-azatryptophan contaminating the sample would remain in
solution at any concentration of avidin leading to a different fluorescence spectrum and
conflicting results. The sample which I prepared was twice recrystallized and well
characterized as described below.
RESULTS AND DISCUSSION

Biotinylated 7-azatryptophan methyl ester 1 was synthesized beginning with d,l-7-azatryptophan. The methyl ester was prepared by dissolving the amino acid in methanol and subsequent addition of thionyl chloride. The crude ester could then be purified through recrystallization from methanol and ether to afford the dihydrochloride salt in 74% yield. The 7-azatryptophan methyl ester was then treated with biotin NHS ester (prepared as described in the previous chapter) in pyridine to acylate the α-amino group and form the desired product 2 which was then twice recrystallized from chloroform and methanol in 49% yield. Elemental analysis and $^1$H NMR were both consistent with a crystal form incorporating one mole each of chloroform and methanol.

Figure 1 displays the fluorescence spectrum of avidin, the biotin-7-azatryptophan adduct, and the complex of the biotin-7-azatryptophan adduct with avidin. Figure 2 demonstrates that with an excitation wavelength of 310 nm, 7-azatryptophan is detected...
predominantly in the complex.

Figure 3 presents the fluorescence anisotropy decay of the biotinylated 7-azatryptophan; Figure 4, of avidin alone. Figure 5 presents the fluorescence anisotropy decay of the complex of biotinylated 7-azatryptophan with avidin. Even in the presence of 16 tryptophan residues, the 7-azatryptophan is detected unambiguously. The fluorescence anisotropy decay of the avidin-biotin complex that is detected by means of the 7-azatryptophan chromophore is clearly different from that observed from avidin itself (detected by means of the tryptophyl residues).

The fluorescence lifetime of biotinylated 7-azatryptophan is dominated by emission from 7-azatryptophan and is well described by the function: \( K(t) = 0.96 \exp(-t/639 \text{ ps}) + 0.04 \exp(-t/2550 \text{ ps}) \). The residual 4% of this fluorescence decay is attributed to biotin itself, which is characterized by a fluorescence lifetime with a long component of about 3 ns.

The complex of avidin and biotinylated 7-azatryptophan is fit well to a double-exponential fluorescence decay. The dominant component is again attributed to 7-azatryptophan and has a time constant of 610 ps. Because the Petrich group had chosen a 3-ns full-scale time base to investigate the rapid dynamics, they consequently have not made a very precise determination of either the magnitude or the duration of the longer-lived component, which arises mostly from tryptophan itself but contains a small contribution from biotin. Fitting the data collected on a full scale of 3 ns indicates that about 85% of the emission collected arises from 7-azatryptophan.

Using the steady-state absorption and emission spectra, the Petrich group could then calculate the amount of longer-lived component that ought to be expected in the time-resolved measurements. We assume that \( \varepsilon_{\text{avidin}}(310 \text{ nm}) = 1600 \text{ cm}^{-1} \text{ M}^{-1} \) since \( \varepsilon_{\text{Trp}}(310 \text{ nm}) = 100 \text{ cm}^{-1} \text{ M}^{-1} \) and there are 16 tryptophans in the protein. We also know that \( \varepsilon_{\text{7-azatrp}}(310 \text{ nm}) = 1100 \text{ cm}^{-1} \text{ M}^{-1} \). If the steady-state fluorescence spectra of avidin itself and the complex
of avidin and biotinylated 7-azatryptophan are scaled according to their fluorescence quantum yields (or their average fluorescence lifetimes) and the extinction coefficients at 310 nm, and if these scaled spectra are integrated starting at 400 nm, we find that about 7% is expected to arise from tryptophan itself, in good agreement with the estimate from the fluorescence lifetime measurements.

Detection of emission wavelengths redder than 400 nm was chosen to accelerate the data collection time and to ensure discrimination against emission from tryptophan itself. Of course, the "homogeneity" of the 7-azatryptophan signal could have been improved by selecting a emission wavelength at even lower energies as a cutoff. The important conclusion to be drawn from these experiments is that even in the presence of a small amount of emission from a substance that is not 7-azatryptophan, the interpretation of our results will not be hindered because we know the identity of this substance.

The steady-state absorption and fluorescence properties of 7-azatryptophan are sufficiently different from those of tryptophan that selective excitation and detection may be effected. The absorption maximum of 7-azatryptophan is red shifted by 10 nm with respect to that of tryptophan. There is also a significant red shift of about 50 nm of the maximum of the fluorescence spectrum of 7-azatryptophan with respect to that of tryptophan. We have measured the fluorescence decays of mixtures of tryptophan and 7-azatryptophan. Only when the ratio of tryptophan to 7-azatryptophan is as great as 10:1 does the tryptophyl emission become detectable. Furthermore, as opposed to aqueous tryptophan at pH 7, the fluorescence decay of 7-azatryptophan is single exponential. This result holds across the emission band and over the pH range we have studied, from 4 to 13. The monoexponential fluorescence decay in itself indicates the enormous preference for using 7-azatryptophan instead of tryptophan as a fluorescent probe.

Recently, there have been reports suggesting that 5-hydroxytryptophan is a useful
biological probe as well. While in some cases 5-hydroxytryptophan may prove useful (if relatively long excitation wavelengths, ~320 nm, are employed), we have demonstrated that because its fluorescence spectrum and lifetime are similar to those of tryptophyl chromophores, it can be more difficult to distinguish from tryptophan than is 7-azatryptophan. This is due in large part to the 3.8-ns lifetime of 5-hydroxytryptophan, which is similar to that of the long component of tryptophan. In mixtures of 5-hydroxytryptophan and the tryptophyl chromophore, NATA (N-acetyl tryptophanamide), in a ratio as high as 1/10, the presence of 5-hydroxytryptophan cannot be discriminated from the mixture (ex = 305 nm; em > 335 nm). On the other hand, when the ratio of 7-azatryptophan to NATA is as low as 1/40, the 7-azatryptophan is easily detected.

The spectroscopic distinguishability of 7-azatryptophan in the presence of avidin is demonstrated clearly in all of the Figures. As is noted above and in the caption to Figure 5, about 85% of the emission of the complex of biotinylated 7-azatryptophan with avidin is attributable to 7-azatryptophan. More importantly, the emission attributable to 7-azatryptophan decays according to a single exponential even though there are four biotin binding sites in avidin. On the other hand, as indicated in the caption to Figure 4, the fluorescence decay of avidin itself requires at least three exponentials in order to be adequately fit. This is not surprising considering that there are 16 tryptophans present and taking into account the intrinsic nonexponential fluorescence decay of tryptophan.

The fluorescence anisotropy decay of biotinylated 7-azatryptophan in complex with avidin is fit well to two exponentially decaying components, the second of which is very long lived: $r(t) = r_1(0)\exp(-t/\tau_1) + r_2(0)\exp(-t/\tau_2) = 0.08\exp(-t/47 \text{ ps}) + 0.15$ (Figure 5). That the fluorescence anisotropy is fit to two exponentials indicates that we are probing the rapid librational motion of the 7-azatryptophan probe with respect to avidin as well as the overall tumbling motion of the avidin itself. For probes attached to globular proteins, the order
parameter, $S^2$, is a model independent measure of the extent to which restricted motion can occur\textsuperscript{26}. $S^2 = [r(1)/r(0)] \exp(t/\tau_{r}) = r(0+)/r_{\text{eff}}(0)$. $\tau_r$ and $r(0+)$ are determined by the fit of the long-time behavior of the anisotropy decay (the overall protein reorientation or tumbling) to a single exponential and are equivalent to $\tau_2$ and $r_2(0)$, respectively. $r_{\text{eff}}(0)$ is the initial value of the anisotropy less those nonmotional factors contributing to the anisotropy decay\textsuperscript{27}. In the treatment of the data, $r_{\text{eff}}(0) = r_1(0) + r_2(0)$. $S^2$ gives an indication of the magnitude of the depolarizing motions that are present in addition to the overall protein reorientation. Thus a value of $S^2 < 1$ implies local motion of the chromophore with respect to the body of the protein, and $S^2 = 1$ implies a rigid chromophore that undergoes depolarization only by means of overall protein motion. The order parameter can be related to a hypothetical cone semiangle, $\theta_0$, within which the transition dipole moment can diffuse\textsuperscript{26,28}: $S = 1/2 \cos\theta_0 (1 + \cos\theta_0)$. In this example, $\theta_0 = 57^\circ$. The large value for the cone semiangle indicates that while the biotin itself is firmly attached to the avidin, the 7-azatryptophan tag lies either in a very mobile part of the protein or actually remains exterior to the protein. The latter of these possibilities is more likely given the large contribution of the rapid component of the anisotropy decay and the similarity of the fluorescence spectrum of 7-azatryptophan in the complex (Figures 1 and 2) to 7-azatryptophan in water\textsuperscript{5,10}. If 7-azatryptophan were buried in the protein interior, not only would the rapid component be less pronounced (or absent) but its fluorescence spectrum would be expected to resemble more closely what is observed in pure alcohols. In alcohols, a second maximum is observed at lower energies. This second band arises from excited-state tautomerization\textsuperscript{2,5,8,31}, which does not occur to any significant extent either in pure water\textsuperscript{8} or in the complex studied here.
CONCLUSIONS

1. Fluorescence anisotropy measurements of the complex of biotinylated 7-azatryptophan with biotin demonstrate that 7-azatryptophan is a powerful probe of small molecule-protein interactions owing to its spectroscopic distinguishability with respect to tryptophan and to its intrinsic single-exponential fluorescence decay.

2. Biotinylated 7-azatryptophan binds tightly to avidin (as demonstrated by chromatography), but the 7-azatryptophan moiety enjoys substantial mobility and can be modelled as diffusing in a cone with a half angle of 57°.

3. The four avidin binding sites provide equivalent environments as indicated by the fluorescence lifetime and anisotropy decay of the bound biotinylated 7-azatryptophan.
EXPERIMENTAL

dl-7-azatryptophan methyl ester dihydrochloride (1)

dl-7-azatryptophan (229.4 mg, 1.028 mmol) was placed in a dry flask and suspended in
4.5 mls methanol. Thionyl chloride (980 ul, 13.6 mmol) was added slowly, the flask was
sealed and the resulting solution was stirred. After about 1/2 hour, a large amount of white
precipitate came out of solution. An additional 5 mls methanol was added and the solution
was heated briefly. This dissolved the solid. Stirring was continued for 12 hours and after this
time the reaction was judged to be complete by TLC. Solvent was removed under vacuum and
the residue was recrystallized from methanol and ether. 1st crop = 220.3 mg, 74% yield.
White crystals. M.P. = 214-216°C ¹H NMR (300 MHz, CD₃OD): δ = 8.82 (d, J = 7.8 Hz,
1H); 8.50 (d, J = 5.8 Hz, 1H); 7.75 (s, 1H); 7.66 (dd, J₁ = 6.1 Hz, J₂ = 7.7 Hz, 1H); 4.49 (t, J
= 6.4 Hz, 1 H); 3.83 (s, 3H); 3.64-3.49 (m, 2H) Rf on TLC: 0.66 on silica gel using 1:9
NH₄OH:ethanol as eluent and ninhydrin or UV to visualize. Fluoresces at 354 nm.

N-α-Biotinoyl-DL-7-azatryptophan methyl ester (2)

Biotin N-hydroxysuccinimide ester (100 mg, 0.302 mmol) and D,L-7-azatryptophan
methyl ester dihydrochloride (71.2 mg, 0.245 mmol) in 3.0 mL pyridine (distilled from
ninhydrin) were stirred under N₂ for 19 hr. The pyridine was removed under vacuum at 40°C
and the residue was suspended in approximately 30 mL of ethyl acetate and extracted three
times with 50% saturated aqueous NaHCO₃, and once with saturated NaCl. The ethyl acetate
layer was then dried over Na₂SO₄, decanted, and the solvent was removed to yield 90 mg
(83% yield) of product as a white solid. An analytical sample was obtained by recrystallization
from methanol and chloroform (53 mg, 49%, MP = 220-222°C). ¹H NMR(300 MHz,
CD₃OD): δ = 8.07 (d, 4.78 Hz, 1 H); 7.92 (d, 7.87 Hz, 1 H); 7.81 (s, 1H); 7.01 (dd, 4.84
Hz, 7.83 Hz, 1 H); 4.66 (m, 1 H); 4.39 (m, 1 H); 4.12 (m, 1 H); 3.59 (s, 3 H); 3.09-2.97 (m, 2 H); 2.82 (dd, 4.89 Hz, 12.72 Hz, 1 H); 2.59 (d, 12.73 Hz, 1 H); 2.09 (t, 6.93 Hz, 2 H); 1.62-1.39 (m, 6 H). IR (KBr): 1735, 1699, 1536, 1655 cm$^{-1}$. Anal: calcd for $C_{21}H_{27}N_5O_4S(CHCl_3)_{1}(CH_3OH)_{1}$, C 46.28%, H 5.40%, N 11.73%; found, C 46.54%, H 5.39%, N 11.55%.

Initially, proton NMR in CD$_3$OD indicated that both methanol and chloroform were present in a 1:1 ratio with 2. However, after removal of the deuterated solvent under vacuum and redissolving in CD$_3$OD, proton NMR indicated that methanol and chloroform were no longer present. This observation is consistent with methanol and chloroform being incorporated into the crystal structure of 2.

Samples used in fluorescence studies were dissolved in a 95/5 H$_2$O/methanol mixture. The small amount of methanol was necessary to dissolve the biotinylated 7-azatryptophan. No degradation of avidin was observed at this concentration of methanol. Affinity-purified, egg-white avidin was obtained from Sigma and used without further purification. Since avidin has four biotin binding sites, the complex was prepared in the ratio of 4 biotinylated 7-azatryptophan molecules to 1 avidin molecule. The concentration of avidin was determined spectrophotometrically using $e(282$ nm) = 24,000 cm$^{-1}$ M$^{-1}$. Binding of the biotin-7-azatryptophan complex to avidin was verified by chromatography.

Spectroscopic measurements

Fluorescence lifetimes, K(t), and fluorescence anisotropy decays, r(t), were obtained using the time-correlated, single-photon counting technique$^{5,6,9}$. Parallel and perpendicular emission intensities for measurements of anisotropy decays were collected alternately with a rotating analyzer polarizer in order to obviate scaling procedures$^6$. The full-scale time base for all lifetime and anisotropy measurements was 3 ns in order to measure accurately the rapid
component of the anisotropy decay. Consequently, long-time depolarizing events (which are not the primary concern of this work) were not fully characterized. All measurements of the 4:1 biotinylated-7-azatryptophan:avidin complex were performed with $\lambda_{ex} = 310$ nm and $\lambda_{em} > 400$ nm to minimize the detection of emission from tryptophan residues within the protein. All other measurements were performed with $\lambda_{ex} = 285$ nm and $\lambda_{em} > 320$nm.
FIGURE CAPTIONS

Figure 1.

Fluorescence spectra of (a) avidin ($\lambda_{em \max} = 339 \text{ nm}$); (b) biotinylated-7-azatryptophan ($\lambda_{em \max} = 390 \text{ nm}$), whose structure is displayed in the inset; and (c) the complex of avidin and the biotinylated 7-azatryptophan. $\lambda_{ex} = 310 \text{ nm}$.

Figure 2.

Fluorescence spectra of the complex of avidin and biotinylated 7-azatryptophan (1 per binding site) as a function of excitation wavelength. Note that at $\lambda_{ex} = 310 \text{ nm}$ essentially only emission from 7-azatryptophan is observed whereas at bluer excitation wavelengths the contribution from tryptophan in avidin is more pronounced.

Figure 3.

Parallel and perpendicular fluorescence intensity profiles from which the anisotropy decay is calculated [6] for biotinylated 7-azatryptophan: $\lambda_{ex} = 285 \text{ nm}$, $\lambda_{em} > 320 \text{ nm}$, $20^\circ \text{C}$. The fluorescence anisotropy decay was fit to a single exponential: $r(t) = 0.09\exp(-t/60 \text{ ps})$, $\chi^2 = 1.50$. Displayed above the polarized fluorescence profiles are the residuals for the parallel and the perpendicular emission, respectively. The fluorescence lifetime of this compound was fit to the function: $K(t) = 0.96\exp(-t/639 \text{ ps}) + 0.04\exp(-t/2550 \text{ ps})$, $\chi^2 = 1.27$. The residual contribution of long-lived component is due to biotin itself, which is weakly fluorescent.

Biotin has an average fluorescence lifetime of 1.5 ns (and a long-lived component of about 3 ns) in a 95/5 water/methanol mixture. The values of the limiting anisotropies, $r(0)$, reported here and in Figures 4 and 5 are consistent with steady-state measurements obtained in glasses.
[6,29,30]. The upper set of residuals corresponds to emission polarized parallel to the excitation source; the lower, perpendicular to the excitation source.

Figure 4.
Parallel and perpendicular fluorescence intensity profiles from which the anisotropy decay is calculated for avidin: $\lambda_{ex} = 285$ nm, $\lambda_{em} > 320$ nm, 20°C. The fluorescence anisotropy decay was fit to a single exponential: $r(t) = 0.11 \exp(-t/3.97 \text{ ns})$, $\chi^2 = 1.25$. The fluorescence lifetime of avidin was fit to the function: $K(t) = 0.43 \exp(-t/53 \text{ ps}) + 0.37 \exp(-t/498 \text{ ps}) + 0.20 \exp(-t/2360 \text{ ps})$, $\chi^2 = 1.16$. The upper set of residuals corresponds to emission polarized parallel to the excitation source; the lower, perpendicular to the excitation source.

Figure 5.
Parallel and perpendicular fluorescence intensity profiles from which the anisotropy decay is calculated for the complex of avidin and biotinylated 7-azatryptophan (1 per binding site), $\lambda_{ex} = 310$ nm, $\lambda_{em} > 400$ nm. As indicated in the Results section, more than 80% of the emission comprising the anisotropy decay is due to the 7-azatryptophan chromophore. The anisotropy decay is well described by two components: $r(t) = 0.08 \exp(-t/47 \text{ ps}) + 0.15$; $\chi^2 = 1.30$. The second component of the anisotropy decay reflects the overall tumbling of the protein itself and is too long-lived to be accurately determined on a 3-ns time scale, on which it appears to be infinite. The upper set of residuals corresponds to emission polarized parallel to the excitation source; the lower, perpendicular to the excitation source.
Figure 1.
Solid : ex=310nm
Dotted : ex=300nm
Dashed : ex=285nm

Figure 2.
Figure 4.

Intensity (counts)

Time (ns)

550
REFERENCES


GENERAL SUMMARY

In this thesis, the development of a general assay for the detection of coupling catalysts at low concentration has been discussed. In operation, the assay is quite similar to the ELISA used to detect antibody binding, and as a result, should be quite easy to carry out using existing materials and techniques. Such an assay is timely in light of the continuing advances in antibody production through combinatorial approaches. Production of catalytic antibodies has been limited by the ability to efficiently screen the large pool of potential catalysts directly for catalytic activity and it is hoped that the assay developed here will be of great utility in streamlining this process.

Although our attempts to obtain a catalytic antibody for peptide ligation was unsuccessful, the potential for the assay has been demonstrated by the detection of peptide coupling catalyzed by solvent-damaged α-chymotrypsin at extremely low levels. The activity of the enzyme under these conditions is comparable to that observed for first generation catalytic antibodies and, as a result, this system provides a good model.

One factor contributing to the high sensitivity of the assay is the extremely large association constant between avidin and biotin. This property has also been taken advantage of by attaching biotin to 7-azatryptophan the latter of which serves as a fluorescent probe owing to its unique excitation and emission spectra. The biotin tag causes 7-azatryptophan to be held in close proximity to the protein avidin and by monitoring changes in the emission spectrum of 7-azatryptophan, information has been obtained about the motional constraints felt by small molecules near the surfaces of proteins.

Connectors capable of linking two molecules of interest together are in great demand by researchers in a variety of fields who wish to perform labeling studies or studies involving molecules tethered to surfaces. Through my research, I have also worked out methods for the
large-scale production of bifunctional linkers which have been found useful for the purposes described above. The linkers are complementary in nature, one being an amino acid analog while the other is a monoprotected diamine. The linkers have the desirable properties of being long and flexible and have been found to increase the solubility of the conjugates formed relative to the corresponding hydrocarbon analogs. It is my hope that these linkers will find continued use in a variety of applications.
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