Acyl Transfer to Lysine - An Investigation of Acyl Transfer Across Large Rings

by

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A novel acyl transfer side reaction involving rapid acyl transfer to the ε amine of lysine across large rings (19 - 34 members) has been explored by kinetic and product ratio studies on a series of model peptides. The design of model thiol capture peptides included features that allow selective deprotection of the cysteine and lysine amines as well as variation in the distance of the ε amine from the phenolic ester. Three goals were addressed by this thesis: 1) evaluation of the distance constraints of lysine acyl transfer reaction, 2) determination of the basis for its high reactivity relative to cysteine and, 3) development of conditions favoring formation of the cysteine acyl transfer product. Two types of experiments were used for the analysis of the lysine acyl transfer reaction: 1) a competition using peptides where both the cysteine and lysine amines are free to react and 2) kinetic studies where the rates of cysteine and lysine reactions are studied individually.

The cysteine:lysine acyl transfer ratio was found to be quite sensitive to the number of equivalents of DIEA added to the reaction. This sensitivity indicated that minimizing the lysine acyl transfer by careful control of peptide:DIEA stoichiometry was not the best solution and that alternative methods should be sought.

The individual rates of cysteine and lysine acyl transfer were also determined. The rate of lysine acyl transfer was found to vary as a function of distance, decreasing from 500 to 15 times the rate of cysteine acyl transfer as the number of intervening alanines between the cysteine and lysine amine increased from 1 to 5. Calculations based on the change in the rate of lysine acyl transfer indicated that under conditions of maximum velocity, acyl transfer to lysine across a 43 membered ring should occur at the same rate as acyl transfer to cysteine across a 12 membered ring. Furthermore, a separation between cysteine and lysine corresponding to 12 amino acids would be necessary to result in 95% acyl transfer to cysteine regardless of the amount of base added to the reaction.

Determination of the effective molarity of the lysine amines in the models showed that for all the models the effective molarity was significantly lower than the cysteine amine effective molarity. This indicated that the source high reactivity of ε amine of lysine is not due
to a conformation that placed the ε amine closer than the cysteine α amine to the phenolic ester but is due to the higher intrinsic nucleophilicity of the lysine amine.

The effectiveness of an alternative buffer in DMSO, sodium dichloroacetate, in suppressing both lysine acyl transfer and disulfide interchange was demonstrated. This has resulted in the ability to increase the concentration of the acyl transfer reaction 100 fold, improving its effectiveness as a preparative method for the synthesis of large peptides. In addition, investigation into the action of sodium dichloroacetate has been undertaken. From the studies performed we have shown that the mode of action is consistent with sodium dichloroacetate's behavior as a base. The potential application of sodium dichloroacetate to peptide couplings in other dipolar aprotic solvents is discussed.

Thesis Supervisor: Daniel S. Kemp
Title: Professor of Chemistry
Well once again my friend, we find that Science is a two headed beast. One head is nice - it gives us aspirin and other modern conveniences. But the other head of Science is bad!!! Oh, beware the other head of Science, Arthur! *It bites and it can really ruin a good day off!!!*

The Tick
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Abbreviations:
DMF - dimethylformamide
CHA - cyclohexylamine
DMSO - dimethylsulfoxide
DMAP - 4-dimethylaminopyridine
DIEA - diisopropylethylamine
EDCI - 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
HFIP - hexafluoroisopropanol
TFA - trifluoroacetic acid
HOBt - 1-hydroxybenzotriazole hydrate
Dbf - dibenzofuran
ScmCl - methoxycarbonylsulfenyl chloride
Acm - acetamidomethyl
pNA - paranitroanilide
Z - benzyloxy carbonyl
Boc - tert-butyl oxy carbonyl
Fmoc - 9-fluorenylmethoxy carbonyl.
Introduction
Introduction

Proteins and nucleic acids play a central role in biochemistry. Together they control the thousands of reactions necessary to sustain living cells. In order to probe the diverse actions mediated by these molecules, reliable methodologies for their manipulation and alteration must be available. The study of nucleic acids has been greatly facilitated by the existence of enzymes that allow selective modification in vitro. Especially important are the ligases, enzymes which catalyze the formation of a phosphodiester bond between two nucleic acid fragments. This ligation is completely specific, eliminating the need for protection of other potentially reactive functional groups present. As a result, chimeric nucleic acids can be rapidly built up from fragments obtained from chemical or biological sources.

Analogous enzymes that catalyze the specific formation of a peptide bond have not been found in nature. As a result, the synthesis of proteins in vitro has required the protection of all potentially reactive groups other than those involved in the formation of the desired amide bond. Difficulties associated with the maximal protection schemes (discussed in detail below) have spurred attempts to develop chemical “ligases”. A method developed in this lab, acyl transfer by prior thiol capture, has been generally successful in ligating unprotected peptides. Certain side chain functionalities however, have proved troublesome. In this thesis, a side reaction involving preferential amide bond formation with the ε amine of lysine is investigated and methods for its suppression developed. Prior to discussing the lysine acyl transfer reaction, the current methodologies available for peptide synthesis must be briefly discussed.
The purpose of this introduction is not to provide a review of protein synthesis. Rather, it is aimed at presenting the problems associated with the standard peptide synthesis techniques that spurred the development of the acyl transfer by prior thiol capture methodology. Peptides and protein synthesis can be divided into two classes on the basis of their sources - either biological or chemical. Biological synthesis includes isolation of proteins from organisms in which they naturally exist, as well as cloning and overproduction in host organisms (14). The details of this methodology lies outside of the scope of this thesis - for our purposes it is sufficient to say that biological synthesis can produce natural peptides and proteins\(^1\) of any size and sequence. Peptides containing unnatural amino acids or non peptidic sequences however, are unobtainable from biological sources, requiring alternative methods for their production.

Chemical methods are not limited by the requirement for natural amino acids and linkages. Unnatural amino acids, amino acids in the D configuration, non peptide fragments can all be accommodated by chemical peptide synthesis. Unlike biological synthesis however, chemical methodologies are limited in the size of the protein that can be made. The existing methods have been shown to be adequate for the routine synthesis of peptides containing up to 50 amino acids. Although larger peptides have been synthesized, the accumulation of impurities due to incomplete acylation and deprotection, and the associated problems involved in purification makes successful chemical synthesis of large peptides extremely difficult. In the following section, we will discuss in detail the strengths and weaknesses of the two standard

\(^1\) Natural proteins are proteins containing only the 20 naturally occurring amino acids in the L configuration, linked by amide bonds through the \(\alpha\) amine.
chemical synthesis methods, solid and solution phase coupling, elucidating the issues that necessitated the development of an alternative method, acyl transfer by prior thiol capture.

**Solution Phase Peptide Synthesis**

Solution phase peptide coupling represents the classical form of peptide synthesis and has a history dating back to the turn of the century (34). The general sequence, outlined in Scheme 1, relies on maximal protection of all but the amine and carboxylic acid functionalities involved in forming the amide bond. After coupling, the peptide is isolated, carefully purified, and deprotected, unmasking the amine or carboxylic acid in preparation for the next cycle. The peptide fragments are then ligated in a highly convergent manner to yield the final polypeptide. This convergent synthetic scheme greatly facilitates the purification of the product from the starting peptides as they differ greatly in size and physical properties.

![Scheme 1 - Solution Phase Peptide Synthesis](image-url)

Scheme 1 - Solution Phase Peptide Synthesis
Despite the advantage that accrues from the ability to isolate and purify the product after each coupling, solution phase coupling suffers from a problem that is intrinsic to the amide bond forming step (56). Amide bond formation is a bimolecular reaction involving the nucleophilic attack of the nitrogen of a peptide amine on the electrophilic carbon of an activated acyl derivative (Scheme 2, equation 1).

\[ \text{RCO-X} + \text{NH}_2\text{R'} \rightarrow \text{RCO-NHR'} + \text{HX} \]

Rate of amide formation = \( k \ [\text{RCOX}][\text{R'NH}_2] \)

Rate of intramolecular side reactions = \( k' \ [\text{RCOX}] \)

Scheme 2

As the peptide fragments being coupled increase in size, the number of moles of amine and carboxylic acid represented by gram quantities of peptides decrease drastically. This, coupled with the low solubility of fully protected peptides (even in solvents like DMSO, DMF, and HFIP) results in the high dilution of the reactive components. The resulting decrease in the rate of the acylation reaction, (increasing the half life from minutes to days) allows side reactions like solvolysis and racemization (which are pseudo first order, equation 2) to compete successfully. Racemization is especially a problem, as it results in products of the proper chemical weight and similar physical properties, but with the wrong configuration, causing reduced biological activity. Attempts to increase the electrophilic character of the carbonyl carbon and hence the rate of the acylation reaction, by changing to a better leaving group, have not been uniformly successful. Such a change not only increases the rate of the
bimolecular reaction but also the rate of the unimolecular reaction, as both rates rely on the lability of the activated ester.

The second problem associated with chemical synthesis stems from a reliance on protecting groups to assure that only the desired amide bond is formed. Side chain protecting groups must be stable enough to last through multiple cycles of deprotection of the amino terminal amines. After the synthesis of the peptide is completed however, the protecting groups must be removed quantitatively. Complete deprotection often requires harsh conditions resulting in a decreased yield of the product. Furthermore, even extremely high yields per step cannot assure complete deprotection in large peptides. To illustrate the difficulties associated with deprotection, let us consider a peptide containing 20 side chain protecting groups. If the yield per deprotection step is 99%, the yield of fully deprotected peptide will be only 81.7%. The rest of the product will consist of peptides retaining various numbers of protection groups, with the majority of the peptides containing 1 or 2 protecting groups. For short peptides, the physical and chemical differences between the product and the impurities are large enough for purification to be achieved by modern methods although the process may be lengthy and tedious. For larger peptides however, that these impurities will differ only slightly in physical and chemical properties from the desired product, making purification almost impossible.

The solution phase synthesis of the 124 amino acid residue protein RNase A by Fuji and Yajima (43-48) clearly shows the effects of both inefficient coupling methods and the difficulties associated with quantitative removal of protective groups. The coupling of the larger fragments associated with the final steps of the synthesis required the use of
DMSO/HMPA as a solvent with 30 fold excesses of coupling agent and coupling times of 72 hours. Removal of the 33 internal protecting groups from the product resulted in a yield of RNase A of only 4 % after purification. It is clear that the limitations imposed by the obligatory maximal protection of all functional groups, the low coupling yields and increased potential for racemization associated with the ligation of two large peptide fragments drastically limits the ability to synthesize large peptides by solution phase coupling. Alternative methods must be developed.

**Solid Phase Peptide Synthesis**

Solid phase peptide synthesis, was developed by Merrifield in 1959 (75, 76) to avoid the solubility problems associated with the protected peptides in solution phase couplings. The method involves linking a protected amino acid to an insoluble polymer resin through its carboxyl group. Elongation of the peptide occurs through a cycle involving deprotection of the N-terminal amine, and acylation with an excess of activated amino acid ester (Scheme 3).
Purification of the reagents used for deprotection and acylation is achieved by simple filtration and washing - the peptide is retained on the solid matrix while the impurities are washed away. Unlike solution phase synthesis, the final product is synthesized in a sequential rather than convergent manner.

The ease with which the peptide can be purified from the reagents used in the synthesis is the major advantage of the solid phase method, allowing for easy automation of the technique. Like solution phase synthesis, the chemistry of the amide bond formation and deprotection however, result in limitations inherent to the methodology. The growing peptide chain is covalently linked to the peptide resin and cannot be removed and purified, so that purity of the final product relies on the quantitative coupling and deprotection of each amino acid added to the growing peptide chain. The problem is analogous to that associated with removal of side chain protecting groups discussed above. If we consider a yield 99.5% for each amino acid added to the growing chain, the purity of the final product of a 20 amino acid synthesis before removal of the internal protecting groups is 90.5%. The majority of impurities will consist of truncated peptides with one missing amino acid. As discussed above, for these short peptides it is likely that purification can be achieved using modern separation methods. For larger peptides, impurities caused by incomplete couplings increase dramatically. If we consider the same yield per step as above, a 124 amino acid peptide will consist of only 54% desired product, with 33.4% of the impurities consisting of peptides with 1 missing amino acid. The difficulties associated with removal of the internal protecting groups discussed above further exacerbate the problem.
The solid phase synthesis of the 140 amino acid protein Interleukin-3 by Kent (25) dramatically illustrates the limitations of the synthesis of large proteins by the solid phase methodology. After removal of the internal protecting groups only 41% of the material had the target sequence. The remaining 59% of the material consisted of truncated peptides with a single internal amino acid missing. The greatest level of purity that could be achieved, by taking a narrow cut from the chromatography of the material, had less than 30% of the activity of IL-3 isolated from natural sources, indicating that the sample was still significantly contaminated. It is clear from this illustration and our discussion above that, like solution phase synthesis, solid phase synthesis is adequate for the routine synthesis of short peptides (<50 amino acids) but is incapable of synthesizing large peptides with an acceptable level of purity.

Chemical Ligation of Unprotected Peptides

From our discussion, it is clear that traditional methods of peptide synthesis suffer from limitations imposed by the need for maximal protection of groups not involved in the formation of the desired amide bond. Because of these limitations, much recent work in peptide synthesis has focused on ligation strategies that selectively form amide bonds in the absence of side chain protection. A few of these methods are reviewed briefly below.

Taking advantage of the ability of proteases to catalyze both hydrolysis and aminolysis, Wells has developed an enzymatic method for ligating unprotected peptides. Using mutagenesis and recombinant expression technologies, a variant of subtilisin (a serine protease) termed subligase has been developed (1). Mutations in the catalytic residues act to enhance
the aminolysis to hydrolysis ratio of peptide esters, favoring formation of the peptide bond. Using subtiligase, milligram quantities of RNase A containing unnatural amino acids have been synthesized by the stepwise ligation of six unprotected peptide esters (51). The average yield per ligation was 70%, and the final product, after refolding was found to be greater than 98% pure by SDS Page.

Chemical strategies for ligation of unprotected peptides have also been developed. Some of these methods generate peptide bond analogs (61) or unnatural amino acids (73). Generation of a normal peptide linkage between unprotected fragments however has recently been demonstrated by Kent (Scheme 4, ref. 29). The method involves the nucleophilic attack of a thioester by the sulfur of an N-terminal cysteine. Spontaneous rearrangement of the ligation product generates the peptide linkage.

![Scheme 4 - reproduced from Kent (ref. 29)](image)

The technique has been successfully applied to the preparation of human interleukin 8 (IL-8), a 72 amino acid peptide, by the coupling of two fragments 33 and 38 residues in length. The
formation of the amide bond occurred quite cleanly as evidenced by the mass spectrometry of
the purified peptide, which showed the presence of a single species with the expected mass for
the product. In both the enzymatic and chemical cases described here, the advantage of
ligation of unprotected peptides is quite clear. The peptide fragments can be synthesized
chemically and deprotected at a stage where they are small enough that the chemical and
physical properties of the deprotected compound allow its isolation. Ligation yields the target
molecule in final form without the need for further manipulation, avoiding the impurities
produced by a final global deprotection.

**Thiol Capture, An Alternative Peptide Synthesis Methodology**

Although it appears from the literature that ligation methodologies are a recent
phenomenon, investigation into methods for the ligation of unprotected peptides has been
going on for some time. For the past two decades, research in this laboratory has focused on
the development of an alternative strategy for the chemical ligation of unprotected peptides.
The method, acyl transfer by prior thiol capture replaces the weak affinity of the amine for the
activated acyl carbon with a highly efficient capture step that links the two peptides to be
ligated (Scheme 5). The actual acyl transfer forming the amide bond occurs intramolecularly
across the linker. The linker (or template) holds the two reactive ends of the fragments in
proximity, eliminating the need for the acyl derivative to be highly activated - the enthalpic
activation of traditional coupling agents is replaced by entropic activation. Theoretically, if the
acyl carbon and amine are close enough, the need for internal protection would be eliminated, as effective competition from reactive side chains would be impossible.

Scheme 5

The intramolecular acyl transfer step also decreases the impurities associated with traditional peptide synthesis. Conversion of the amide bond forming step to a unimolecular reaction allows it to compete more effectively with the formation of impurities. Furthermore, the rate of the unimolecular acyl transfer is unaffected by concentration so that racemization, which involves cleavage of the ester fragment as a necessary first step, can be eliminated by high dilution. Once the ester is cleaved, the acylation step leading to the racemized peptide becomes bimolecular and the low concentration would drastically decrease the rate.

A number of studies in this lab demonstrated the feasibility of intramolecular O, N acyl transfer across medium size rings (55, 62, 72, 91). The efficiency of different linkers at
facilitating acyl transfer was determined by measuring the effective molarity\(^2\) (abbreviated EM), which indicates the proximity of the two reactive groups. Initial attempts focused on amine capture, with an EM of 18 seen for the xanthenyl system (Figure 1), showing that acyl transfer across medium size rings (12 atoms) could occur with high efficiency.

![12-Ring Acyl Transfer](image)

Figure 1

Amine capture however, did not meet the requirements for a high efficiency capture step. It was necessary to come up with an alternative strategy for linking the two peptide fragment prior to acyl transfer.

The high nucleophilicity of sulfur and its affinity for activated thiols suggested a possible solution. The two peptides could be linked by the formation of an asymmetrical disulfide between the ester linked template and an n-terminal cysteine residue. In order for this scheme to be successful, two criteria had to be satisfied: 1) a sulfur template had to be discovered that demonstrated an acceptable EM for acyl transfer to the cysteine amine and 2) a method for the quantitative and rapid formation of an asymmetrical disulfide bond had to be developed. In order to meet criterion 1, design of a template based on the structure of the

\(^2\) Effective molarity is the ratio of the rate of an intramolecular reaction to the corresponding intermolecular reaction and corresponds to the concentration of an external nucleophile that is required to achieve a rate acceleration equal to that seen in the intramolecular reaction.
acyl transfer transition state (as determined from model studies) was undertaken. It was observed that the O, S distance of the transition state (5.27 angstroms) matched almost exactly the O,S distance of 4-hydroxy-6-mercaptodibenzofuran (Figure 2, ref. 44). Synthesis and testing of the compound as an acyl transfer template showed that it gave an effective molarity of 5 M, and a half life of approximately 2 hours in DMSO for acyl transfer to cysteine.

![Figure 2](image)

Once a viable acyl transfer template had been obtained, it was necessary to develop a method for formation of the asymmetrical disulfide bond. Successful formation of asymmetrical disulfides had been demonstrated by Brois (16) using the carboxymethylsulferyl group, which activated sulfur towards nucleophilic attack by a free thiol. Galakatos (44) showed that at low concentration ($10^{-3}$ M) the formation of a disulfide between the free thiol of the template and an Scm functionalized cysteine residue occurred rapidly ($t_{1/2} = 4$ minutes) and
cleanly in 60% HFIP-40%H₂O in the presence of both free and fully protected amines (Scheme 6).

Scheme 6

The reaction of histidine with ScmCl under conditions where the histidine residues were not fully protonated, was the notable exception to the specific activation of sulfur in deprotected peptides (20). By running the reactions under acidic conditions however, this side reaction could be minimized.

With both the acyl transfer template and a high affinity linkage method in hand, the next hurdle to be overcome was the generation of peptide fragments functionalized as template esters at their carboxy terminus. As discussed above, solid phase, although inadequate for the synthesis of large proteins, provides a rapid and simple route to the medium size peptides. Kemp, Galakatos and Fotouhi (38, 39, 57) designed a new type of resin to allow peptides, functionalized at their C terminus as 4-acyloxy-6-mercaptopdibenzo-furan esters, to be synthesized by solid phase methodology (Figure 3). S-(4-acetoxy-6-mercaptdibenzo-furanthio)-Cbz-Cys-OH was covalently linked to aminomethyl polystyrene, Deprotection of the phenol of dibenzo-furan yielded the solid phase resin. Peptides were synthesized by standard DCC coupling from the phenol of the dibenzo-furan template. After
completion of the synthesis, treatment with triethylphosphine released the peptide functionalized as the template ester with a free thiol ready for thiol capture.

Figure 3

The specificity with which disulfides can be cleaved by triethylphosphine, coupled with the ability to rapidly and cleanly generate asymmetrical disulfides, allowed the possibility of avoiding the slow build up of impurities normally associated with solid phase synthesis. If impurities were generated during the solid phase synthesis, the peptide could be cleaved from the resin, purified and reattached by thiol capture to fresh resin containing covalently linked Scm derivatized cysteine. Furthermore, the ability to cleave the peptide from the resin enabled the monitoring of both deprotection and acylation steps, by treating small samples of resin with triethylphosphine and analyzing the released peptide by HPLC.
During the acyl transfer step, a major competing reaction was found to be disulfide interchange, catalyzed by free thiol left over from the capture step. Disulfide interchange results in scrambling of the asymmetric disulfides, yielding all possible disulfide combinations. Disulfide interchange can occur with either the thiol capture material (in which case it can no longer acyl transfer) or after the acyl transfer has been completed. In an extensive study of disulfide interchange by Kemp and Fotouhi (58), it was found that the addition of several mole percents of silver nitrate could result in a drastic decrease in the level of the scrambling, as the salt scavenges the catalytic free thiols which promote the disulfide interchange.

As discussed above, the impurities associated with the final deprotection of the side chains make synthesis of large peptides by fragment condensation unworkable. For the prior thiol capture methodology to be successful, therefore, the ability to ligate fully deprotected peptides had to be developed. The relatively small size of the peptides used in thiol capture would allow them to be purified extensively, as the desired products would differ greatly both chemically and physically from impurities containing residual protecting groups. Furthermore, the thiol capture and acyl transfer of fully deprotected peptides would avoid the solubility problems observed in classical solution phase condensations with maximally protected peptides. Work by Fotouhi and Carey demonstrated the feasibility of the Scm activation, thiol capture and acyl transfer of large unprotected peptide fragments. In a synthesis of the 29 residue C-terminal fragment of bovine pancreatic trypsin inhibitor (BPTI), Fotouhi was able to Scm activate a completely deprotected 20 amino acid fragment corresponding to residues 38-58 of BPTI (38, 39). Thiol capture of a peptide corresponding to residues 30-37 and acyl transfer under proper buffering conditions yielded the C-terminal fragment. In the presence of
excess base however, an extremely rapid acyl transfer to the ε amine of a lysine proximal to the n-terminal cysteine was observed. The observation represented a significant failure of the general methodology given the goal of specific ligation of peptides in the absence of side chain protection. Precise control of the DIEA:thiol capture peptide could minimize this side reaction, although it was realized that this could prove difficult as a general method for suppression of lysine acyl transfer. Study and elimination of this reaction is the focus of the work presented in this thesis. Further attempts at the coupling of unprotected peptides by Carey (20) resulted in the successful synthesis of repressor of primer protein (ROP) by two separate couplings of unprotected fragments. These results indicate that, with the exception of lysine, the acyl transfer mediated by dibenzofuran is sufficient to promote acyl transfer to cysteine in the absence of protecting groups.

Scheme 7 illustrates the overall strategy for acyl transfer by prior thiol capture as it now stands. Peptide 1 is synthesized from the free phenol of the dibenzofuran template, linked to solid phase resin by a disulfide. Treatment of the resin linked peptide with triethylphosphine results in the release of peptide 1 covalently attached to the dibenzofuran template. Peptide 2, containing a cysteine on the amino terminus (activated toward disulfide formation) is “captured” by 1 forming an asymmetrical disulfide 3. The source of peptide 2 can be any synthetic method and the thiol capture can occur with either fully protected or deprotected peptides. After thiol capture, the product can be purified by preparative HPLC prior to acyl transfer.

3 The lysine acyl transfer is discussed in depth in Chapter 1
4 In subsequent discussions, the formation of the disulfide will always be referred to as the thiol capture step and the product as the thiol capture material.
\[
\text{Resin} \quad \begin{array}{c}
\text{O} \\
\text{H}
\end{array} \\
\text{R--S-S--OH}
\]

\[
\text{R--S-S--OH} \quad \text{Peptide}_1
\]

\[
\begin{array}{c}
\text{S-S} \\
\text{OH}
\end{array} \\
\text{Peptide}_2
\]

\[
\begin{array}{c}
\text{O} \\
\text{S-Scm}
\end{array} \\
\text{Peptide}_1
\]

\[
\begin{array}{c}
\text{O} \\
\text{S-S}
\end{array} \\
\text{Peptide}_2
\]

\[
\begin{array}{c}
\text{O} \\
\text{Et}_3\text{P}
\end{array} \\
\text{Acyl Transfer Product 4}
\]

\[
\begin{array}{c}
\text{O} \\
\text{SH}
\end{array} \\
\text{Peptide}_5
\]
Protection of the cysteine amine of the thiol capture material as a TFA salt allows reaction between the aryl ester and the amine to be prevented until selectively triggered with base. The resulting O to N acyl transfer of 3 across a 12 membered ring, from aryl ester to cysteine amine, forms the peptide bond, yielding 4. After acyl transfer is complete cleavage of the disulfide bond linking the dibenzofuran template to the acyl transfer product liberates the peptide 5 which can be purified by preparative HPLC. Provided the newly formed peptide has an amino terminal cysteine, the cycle can start again.

The work of the past 15 years has demonstrated the feasibility of the acyl transfer by prior thiol capture strategy. In the chapters that follow, the acyl transfer reaction to lysine will be discussed further. The parameters of the acylation will be determined and the source of the reactivity of the lysine amine investigated. Finally methods for its suppression are studied and the development of an organic buffer system that minimizes the lysine acyl transfer reaction is presented.
Chapter 1 - Design and Synthesis of The Model Thiol Capture Peptides
Introduction

From the discussion of synthetic methodology presented in the introduction, it is clear that the ability to couple large unprotected fragments is vital to the synthesis of large proteins. Thiol capture, by taking advantage of the tendency of sulfur to form disulfides, enables unprotected peptide fragments to be linked in a favorable relationship for peptide bond formation by acyl transfer. The dibenzofuran spacer holds the cysteine in proximity to the phenolic ester, thereby eliminating acylation of other reactive groups in the peptide backbone.

Practical studies have shown that, for the most part, this strategy is successful in eliminating side reactions. In this thesis however, a novel side reaction involving rapid acyl transfer to the \(\varepsilon\) amine of lysine across large rings (19 - 34 members) will be explored and methods for its suppression will be developed. Investigations of the possible source of the high reactivity of lysine will be presented. This chapter discusses the background and the design of the system used to study the lysine acyl transfer reaction.

Acyl Transfer to Lysine - Background

Acyl transfer to the \(\varepsilon\) amino group of lysine was first observed when thiol capture was applied to synthesis of the C-terminal half of the small protein BPTI. During the acyl transfer of 6 in the presence of two equivalents of DIEA, Dr. Nader Fotouhi observed two products, 7 and 8 in a 1:3 ratio with retention times of 7.9 and 9.2 minutes respectively (Figure I-1). A kinetic study of the coupling indicated a half life of 2 minutes which was quite surprising, as earlier rate studies had indicated that the half life of glycine acyl transfer was 2 hours. Initial
Boc-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-\( \text{O} \)


\( \text{S DNP} \)

\( \text{S DNP} \)

<table>
<thead>
<tr>
<th>( \text{6} )</th>
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<tbody>
<tr>
<td>Acyl Transfer</td>
</tr>
<tr>
<td>DMSO, 2 eq. DIEA</td>
</tr>
<tr>
<td>AgNO(_3), 14 hrs</td>
</tr>
</tbody>
</table>

2 products

\( \text{7} \) \( \text{8} \) (1:3)

HPLC Retention time 7.9 min 9.2 min

Figure 1-1
model studies by Dr. Fotouhi ruled out the possibilities of an inductive effect or catalysis by the nearby guanidino moiety of arginine as the cause of the rate induction.

A careful study in which acyl transfers of 6 were carried out with varying quantities of DIEA indicated that in the presence of one equivalent of DIEA, 8 was obtained as the sole isolated product. When the number of equivalents of DIEA were increased to 10, 7 was the predominant product obtained. Acyl transfers in which intermediate amounts of DIEA were used gave mixtures of the products.

To identify 7 and 8, the products were purified. Treatment of the purified products released 4-hydroxy-6-mercaptodibenzofuran, indicating that the products were due to acyl transfer. Additionally both products had the correct amino acid composition and mass (as determined by FAB mass spectrometry) expected for the acyl transfer product. Edman sequencing however revealed the structures to be 7 and 8.

![Figure 1-2](image-url)
Product 8 is generated by normal acyl transfer to cysteine. Product 7, obtained at high base concentration, is generated by acyl transfer to the ε amine of lysine 41\textsuperscript{1}, across a 25 membered ring. No trace of acyl transfer to lysine 46 was observed. Production of 7 by an intermolecular acyl transfer was discounted, as the low concentration of the thiol capture material (1×10\textsuperscript{-4} M) would make this reaction extremely slow.

The pKa data presented in Appendix A for the functional groups in the peptide can be used to explain the reactions observed with differing amounts of DIEA. The thiol capture material 6 contains a number of acidic groups present in the protonated form due to the isolation of the thiol capture material from mildly acidic conditions. The addition of one equivalent of DIEA would be expected to first deprotonate one of the three carboxylic acids which are the most acidic, with pKa values in DMSO of 5 to 6. The expected pKa of the cysteine α ammonium in DMSO is 6-7 so that a buffer system between the carboxylic acids and the cysteine amine could be set up allowing acyl transfer to take place. The ε ammonium ion of lysine, with an expected pKa in DMSO of 10-11, would most likely not be deprotonated under these conditions. With the addition of 10 equivalents of DIEA however, the lysine ammonium ion would be deprotonated as well.

The dibenzofuran template has been designed to maximize the proximity of the cysteine amine to the phenolic ester. Studies have indicated that the effective molarity of the cysteine amine is 5 M. As discussed in Appendix B, this is the concentration of an external nucleophile of the same strength that would have to be achieved in order to see a rate equal to the rate

\textsuperscript{1} Amino acid sequence numbers correspond to the sequence numbers of BPTI with the amino terminus as 1.
observed for the intramolecular acyl transfer. Dr. Fotouhi hypothesized that efficient acyl transfer to lysine competes with that to cysteine acyl transfer because the peptide assumes a conformation in DMSO that places lysine close to the phenolic ester (Figure I-3).

Based on the assumption of equal nucleophilicity of the competing amines toward the phenyl ester, Dr. Fotouhi calculated an effective molarity of the ε amine of lysine 41 on the order of 150 M. He suggested that the formation of a stable 25 membered loop could be due to the formation of an anti-parallel sheet in which the 25 membered ring forms a turn. However an NOE study of the thiol capture material in DMSO showed no evidence for a stable ring or sheet conformation.

Although the absence of NOE evidence was suggestive, only direct measurement of effective molarities could definitively rule out the possibility of a high effective molarity for lysine 41. However, the large difference between the pKa of the cysteine α ammonium and the lysine ε ammonium moieties (2-3 pKa units in DMSO), suggested a more likely possibility for
the source of rapid acyl transfer to lysine. As discussed in appendix A, Bordwell has shown that for structurally related compounds, nucleophilicity is related to basicity in DMSO as in protic solvents. The greater reactivity observed for lysine 41 could be due to its inherently greater nucleophilicity. If this hypothesis was true however, the lack of any evidence for acyl transfer to lysine 46 was somewhat confusing and had to be accounted for.

Since one of the most important features of the acyl transfer methodology is its potential for ligating peptide fragments bearing little or no side chain protection, the observation of acyl transfer to lysine was a significant negative result. It was important to explore the problem more fully and to determine the distance constraints of lysine acyl transfer so that we would know what positions of lysine relative to the aryl ester were likely to cause problems. In addition, a method to suppress this reaction had to be developed. Without simple methods for precisely determining the amount of protein and TFA present in the sample, control of lysine acyl transfer by careful measurement of DIEA would be impractical. The hygroscopic properties of proteins eliminated the possibilities of determining the amount of protein by mass. Other avenues had to be explored.

The rapid rate of lysine acyl transfer across a 25 membered ring was interesting in its own right. Although a great number of studies have been performed on the cyclization reactions for other polymer types, only a few studies on protein cyclizations exist. Acyl transfer to lysine could provide a good system for studying these sorts of reactions. Given the necessity of eliminating the lysine acyl transfer reaction and the potential to use lysine acyl transfer to probe reactions across large rings, we decided that a detailed study of rationally designed model systems was in order. The same models used to develop methods suppressing
acyl transfer to lysine could also be used to probe the effect of ring size on protein cyclization reactions.

**Initial Design of Model peptides**

Addressing the questions posed in the preceding section concerning the intramolecular O,N acyl transfer reaction to the lysine $\varepsilon$-amine required a structural redefinition of the complex framework of peptide $\mathbf{6}$. This peptide is unsuitable for detailed kinetic study since it is synthesizable with difficulty and inconvenient to analyze and models were required which permitted study of the acyl transfer reaction in a more tractable context. These could not be devised without preliminary experimentation to test the feasibility of both the synthetic protocols and the analyses planned for measuring product ratios and reaction rates. Results of planning and preliminary experiments are reported in this chapter.

Three goals were to be accomplished by this study - 1) evaluation of the distance constraints of lysine acyl transfer reaction, 2) determination of the basis for its high reactivity relative to cysteine and, 3) development of conditions favoring formation of the cysteine acyl transfer product. Two types of experiments could be used to accomplish these goals. The first is a study of the relative rates of intramolecular acyl transfer to cysteine and lysine in the same peptide, as a function of acyl transfer conditions and separation. This study would model the original case in which the lysine acyl transfer was observed, and the experiments would be directed at finding experimental conditions that maximize the ratio of the yield of the cysteine acyl transfer product, relative to that of lysine. The second is a kinetic study of the individual lysine and cysteine acyl transfer reactions, in systems that allow only one of these reactions to occur. The effect of conditions and separation on the rate of the individual reactions would
help to clarify the results of the competition experiments. The results of the lysine kinetic measurements, in conjunction with kinetics of a model bimolecular reaction, would allow assessment of effective molarity.

Inspection of the three general formulas of Fig. I-4 reveals our design plan. These embody the minimum features consonant with the required study. Model peptides 2 and 3 would allow the assignment of the rate constants for O,N transfer to the N-terminal cysteine amine and to the side chain lysine e-amine groups, respectively. The variation of the length of the spacing peptide $X_{nn}$ permits study of the effect of Cys-Lys separation on these rate constants. Though its study was expected to be more difficult, the model peptide 1 was designed to permit direct measurement of the cysteine:lysine product ratio. The rate constant for loss of starting material, should be correlatable with the rate constants seen for peptides 2 and 3.

These structures also reveal key chemical features shared by any study of acyl transfer by dibenzofuran templated thiol capture. The S-S bond and the phenyl ester shared by these structures are labile in the presence of a variety of nucleophilic reagents. Such reagents must therefore be avoided in selecting appropriate side chain and chain terminal protective groups. R1, R2, and R3 and R4 are as yet unspecified terminal blocking groups that, owing to their separation from the reaction centers, are not expected to affect the rate constants significantly. Nonetheless they must be selected carefully for their capacity to facilitate synthesis and analysis. Finally, because of its unique capacity to catalyze the O,N-acyl transfer reactions of phenyl esters, the choice of solvent for the reaction was restricted to DMSO.
Amino-Terminal Sequence

R1-Xxx-Xxx-Xxx-O

Peptide Spacer

Cys-Xxx<sub>n</sub>-Lys-R2

Model Peptides 1 - Both Cysteine α amine and lysine ε amine unprotected (TFA salts)

Model Peptides 2 - Cysteine α amine protected and Lysine ε amine as TFA salt

Model Peptides 3 - Cysteine α amine as TFA salt and Lysine ε amine protected

R1-R4 are undefined protecting groups,
Xxx is an undefined amino acid

Figure I-4
The C-terminal Peptide Sequence

In Fig. I-4 the nature of the sequences of peptides at the acyl and amine sites are left unspecified. Even with restriction to the twenty natural amino acids, a very large number of choices were possible. For medium-sized or large peptides that adopt well-defined secondary structure in solution, amino acid composition and sequence strongly influence overall conformation, which in turn is expected to control the effective through-backbone length of the spacing element or C-peptide shown in the figure. For short peptides of the size appropriate for this study however, experimental examples of well-defined, highly populated conformations are rare, particularly in aggressively polar solvents like DMSO (as discussed in Appendix B). It is therefore likely that the effective length of the C-terminal spacing peptides of Fig I-4 would be relatively insensitive to sequence and choice of amino acid. Moreover, we felt that for this pilot study, it was important to select a simple amino acid sequence, excluding natural amino acid residues that have unusual conformational properties or that introduce other complications. These include the following. Owing to its pyrrolidine ring constraint, proline is conformationally highly restricted, as is the unnatural amino acid α-aminoisobutyric acid. Due to the restraining effect of β-branching at the junction of the side chain and the peptide backbone, threonine, valine, and isoleucine are also unusually constrained. On the other hand the conformations of peptides containing glycine are unusually permissive, as noted in the Ramachandran diagram of Fig. I-5. Glutamic acid, glutamine, aspartic acid, asparagine, and serine are capable of hydrogen bond formation between side-chain functions and backbone amides. Phenylalanine, tryptophan, and tyrosine bear large hydrophobic residues that
Conformational map for the glycyl α carbon. Normally allowed conformations are outlined by a solid line and partially allowed conformations are outlined by a thick dashed line. The thin dashed lines across $\psi = 0^\circ$ represent regions where the contact distances are only slightly smaller than the extreme limits. [From Ramachandran, G. N., and Sasisekharan, V., *Adv. Protein Chem.* 23, 333 (1968)]

Plot of main chain dihedral angles $\phi$ and $\psi$ experimentally determined for the glycines in 20 high resolution protein structures. [From Richardson, J.S., *Adv. Protein Chem.* 34, 174 (1981)]

Plot of main chain dihedral angles $\phi$ and $\psi$ experimentally determined for approximately 1000 nonglycine residues in eight proteins whose structures have been refined at high resolution (chosen to be representative of all categories of tertiary structure). [From Richardson, J.S., *Adv. Protein Chem.* 34, 174 (1981)]
potentially introduce conformational effects that are best avoided, and the positive charge of arginine and potential nucleophilicity of histidine both complicate synthesis, owing to the need for additional, independently removable protective groups, and potentially complicate interpretation of the kinetic results. For obvious reasons, cysteine and lysine residues were inappropriate choices for the spacing element. The remaining natural amino acids were alanine, leucine, and methionine, and of these, alanine is the natural choice.

Alanine is the simplest of the amino acids that displays a normal Ramachandran conformational bias at the $\phi, \psi$ dihedral angles of the peptide backbone (Figure I-6). Alanine residues have been found with high probability in both sheet and helical conformations within natural proteins, but short alanine containing peptides have been shown in our laboratories to be unstructured in DMSO, and their synthesis and analysis were expected to pose few problems. If a single structural archetype was to be chosen for this pathfinding investigation, polyalanine appeared to be the appropriate choice. Although polyalanines longer than 5 residues can be difficult to synthesize, smaller derivatives are usually accessible, For considerations of solubility and synthetic ease, the maximum size of the C-terminal spacer peptide was limited to five alanines, since it was felt that the length range of $\text{Ala}_n \text{n} = 0-5$ would reveal the most significant features of the dependence of the reactivity of the e-lysine amino function with increasing separation from the dibenzofuran active ester. It was expected that the practical needs for blocking of the side chain amine of lysine during the acyl transfer step of thiol capture could also be explored with this small spacer library.
Conformational map for the Alanine α carbon (Cα present). Normally allowed conformations are outlined by a solid line and partially allowed conformations are outlined by a thick dashed line. The thin dashed lines across $\psi = 0^\circ$ represent a region where the contact distances are only slightly smaller than the extreme limits. [From Ramachandran, G. N., and Sasisekharan, V., *Adv. Protein Chem.* 23, 328 (1968)]

Plot of main chain dihedral angles $\phi$ and $\psi$ experimentally determined for approximately 1000 nonglycine residues superimposed on the conformational map of Alanine.

Figure I-6
Protection of the Cysteine and Lysine Amines

The transformation chemistry that the model peptides of 1-4 must undergo is shown schematically in Scheme I-1, and this chemistry provides constraints that dictate the choice of protective groups. The reactions of this figure are divided into three compartments---1) solid phase synthesis of the N-α-blocked N-terminal peptide linked by an active ester to the dibenzofuranthiol; 2) synthesis of the C-terminal spacer peptide Q2-Cys(A)-Ala-Lys(Q1)-R2, which bears three removable protective groups at Cys N-α (Q2), Lys N-ε (Q1), and Cys-β-S (A = Acm or Trityl); 3) preparation of the latter peptide for the thiol capture step and effecting of that step to generate the starting materials of 1-4. Special features of this scheme include the requirement for models 2 and 3 that the cysteine and lysine amine protecting groups have orthogonal deprotection conditions, allowing one to be selectively removed prior to acyl transfer and the other during work up to facilitate the characterization of products. The presence of the unusually vulnerable Scm group at cysteine sulfur for a key intermediate was also an important consideration. Scm is introduced by treating an acetamidomethyl or trityl protected sulfur with methoxycarbonylsulfonyl chloride in HFIP:H₂O. Although, the Scm group can be introduced in the presence of free amines, the reaction proceeds with more reliability and fewer impurities with protected amines. As either option was available with the short model peptides, deprotection after Scm activation was chosen for reasons of synthetic ease.

The lability of Scm dictated the choice of deprotection conditions and hence of protecting groups. Scm decomposes in the presence of base, but is stable to anhydrous acid.
Consequently, TFA labile Boc group was the natural choice for the amine protecting group to be removed prior to acyl transfer. Z was chosen as the “permanent” amine protecting group.

**Synthesis of N-Terminal Dibenzo furyl containing Peptide**

\[
\text{R-S-S-OH} \quad \rightarrow \quad \text{Solid Phase} \quad \rightarrow \quad \text{Peptide} \quad \xrightarrow{\text{Et}_3\text{P}} \quad \text{HS} \quad \text{O} \quad \text{Peptide} \quad \text{O} \quad \text{Peptide}
\]

**Synthesis of C-terminal Cysteine Peptide**

Solid or Solution PhaseSynthesis

\[
\text{Q}_1 \quad \rightarrow \quad \text{R}_2\text{-Lys-Ala}_{n}\text{-Cys-Q}_2
\]

Where Q can represent the permanent protecting groups indicated in Figure 1-4 or removable protecting groups that will unmask the reacting amine. A represents sulfur protecting groups that will removed with Scm activation.

**Scm Activation, Deprotection**

For this diagram we will consider deprotection of the Cysteine amine only.

**Thiol Capture**

\[
\text{HS} \quad \text{O} \quad \text{Peptide}_1 \quad \xrightarrow{\text{S-Scm}} \quad \text{Peptide}_2 \quad \text{Cys}^+\text{NH}_3 \quad \text{TFA}^-
\]

\[
\text{Peptide}_1 \quad \xrightarrow{\text{Thiol Capture}} \quad \text{Thiol Capture Material}
\]

Scheme I-1
due to its high acid stability. Figure I-8 shows the models of Figure I-4 with the protecting groups in place.

**The N-terminal Peptide Sequence**

Only the amino acid linked to the phenolic ester takes part in acyl transfer, so that the requirements for the N-terminal sequence were fairly lax. Although it would have been possible to use models with only one amino acid linked to dibenzofuran, we decided that a peptide would model real acyl transfer conditions much more closely. Previous research had shown that the rate of acyl transfer was dependent on the identity of the amino acid linked to the phenolic ester. A more rapid rate would enable kinetics studies to be followed through several half lives before complications due to side reactions made interpretation difficult. As the rate of acyl transfer to glycine is among the fastest of all the amino acids, it was chosen to form the ester with dibenzofuran.

The choice of the other amino acids was much more flexible. For the same reasons as above, amino acids with reactive side chains were avoided. Alanine and phenylalanine were chosen as representative amino acids with no reactive functionality and alanine was arbitrarily chosen as the N-terminal amino acid, as the particular order was of no significance. For the same reason as discussed above, Z was chosen as the N-terminal protection for alanine, allowing deprotection after acyl transfer for purposes of characterization.
The para Nitroanilide Chromaphore

In the past, the strongest UV chromophore present in the thiol capture peptides was the dibenzofuran spacer. As discussed in the introduction, disulfide interchange during a protracted acyl transfer step can sometimes generate byproducts, necessitating treatment with triethylphosphine to simplify the product mixture, facilitating analysis of the acyl transfer reaction. While the treatment greatly simplifies the HPLC traces by cleaving all disulfides it has the unfortunate effect of removing the dibenzofuran chromophore, greatly decreasing the UV absorbance of the peptide. Furthermore, the cysteine-containing peptides as designed above contained no strong UV chromophore. Incorporation of a permanent chromophore would greatly facilitate the synthesis of the cysteine fragment and provide a valuable spectroscopic handle for following the model peptide through all the stages of thiol capture and acyl transfer process.

The chromophore had to meet several criteria. It had to be easily introduced into the cysteine peptide at an early stage (preferably as a commercially functionalized amino acid). As the chromophore was to be used to monitor the synthesis of the cysteine containing fragment, it had to be stable to the conditions of peptide coupling and amine deprotection. It had to have a high extinction coefficient, preferably at a long wavelength, so that peptide couplings, deprotections and acyl transfers could be monitored by HPLC, without interference from coupling agents or solvents. The magnitude should be comparable to that of dibenzofuran, so that during thiol capture the consumption of the cysteine(Scm) fragment could also be observed, an additional test that the thiol capture was proceeding properly. Finally, the
chromophore had to be stable to treatment with trialkyphosphines, as it would used for
detecting the peptide once dibenzofuran had been removed.

A search of the commercially available functionalized peptides revealed a C-terminal p-
nitroanilide as a reasonable candidate. The parent aniline has an extinction coefficient of
1.54x10^4 L.mol^{-1} cm^{-1} at 373 nm. Correspondingly when measured, the p-nitroanilide was
found to have a peak at 330 nm with an extinction coefficient of roughly 1.4x10^4 L.mol^{-1} cm^{-1}
in DMSO (Figure I-7).

![UV spectra of Lys-pNA in DMSO](image)

**Figure I-7**

Lysine p-nitroanilide was commercially available with the e-amine protected by either Z
or Boc, allowing selectivity in its deprotection. The chromophore could be introduced during
the first coupling and the peptide could be synthesized by normal acylation and deprotection of the amino terminus. Although the aryl amide might be somewhat more labile than an alkyl amide, it was likely that the linkage would be stable under the conditions for peptides synthesis, acyl transfer and deprotection. Its presence on the carboxy terminus excluded solid phase synthesis and limited the synthesis options to solution phase couplings, but it was felt that the potential advantages of the UV tag would outweigh any synthetic difficulties.

Figure 1-8 shows final model peptide designs with the features that we have discussed in this section. The rest of the chapter will be devoted to characterization techniques.

**Synthesis of the Model Peptides**

The syntheses of the cysteine fragments of the model peptides are outlined in Schemes I-2 through I-17. The choice of pNA as the chromophore and Boc and Z as the temporary and permanent protecting groups, respectively, determined the choice of amine protecting group used during the synthesis. Fmoc was chosen, as its deprotection conditions are orthogonal to those of all the other protecting groups and would have no effect on pNA. Although the sequence of reactions was designed to reuse as many fragments as possible, in a few cases fragments were synthesized using Z protection as well as Fmoc protection (FmocAla₂OtBu, ZAla₂OtBu; FmocAla₃OtBu, ZAla₃OtBu). The laboratory stocks contained relatively large

---

2 For the sake of convenience, thiol capture materials will be referred to by the sequence of the cysteine containing peptide as the sequence of the acyloxy peptide is always Z-Ala-Phe-Oly. Therefore Model Peptide Series 1 with both the cysteine and lysine amines free as TFA salts will be referred to as CAnK thiol capture peptides. Model Series 2 with the lysine amine as a TFA salt and the cysteine amine protected with a Z group will be ZCAnK thiol capture peptide and Model Peptide Series 3 with cysteine amine present as a TFA salt and the lysine amine protected with a Z group will be referred to as the CAnK(Z) thiol capture peptide where n is the number of intervening alanines.
quantities of Z-AlaOH while FmocAlaOH had to be purchased. An attempt was made to avoid unnecessary expense by reserving Fmoc fragments for cases where Boc or pNA were present, and using Z derived fragments in the other cases. For the same reason a choice was made to use Boc protection for alanine in cases where the ε amine of lysine was protected by Z.

**Figure 1-8**
Scheme I-2

HO S-S-R

Boc-Gly-\(\gamma\)-O, CH\(_2\)Cl\(_2\), DIEA, DMAP
1 hour

\(50\%\) TFA in CH\(_2\)Cl\(_2\)
20 min.

Boc-Phe-Gly-\(\gamma\)-O

Boc-Phe-Gly-\(\gamma\)-O, CH\(_2\)Cl\(_2\), DIEA
1 hour

\(50\%\) TFA in CH\(_2\)Cl\(_2\)
20 min.

Z-Ala-Phe-Gly-SH

Z-Ala-Phe-Gly-\(\gamma\)-O, CH\(_2\)Cl\(_2\), DIEA
1 hour

\(\text{Et}_3\)P, CH\(_2\)Cl\(_2\), HFIP

R-S-S-OH

\(\equiv\)

Resin

R-S-S-OH

\(\equiv\)

Resin
Scheme I-3
H-Lys(Boc)-pNA + Boc-Cys(Acm)OH $\xrightarrow{\text{HOBt, EDCI, THF}}$ Boc-Cys(Acm)Lys(Boc)-pNA
1) ScmCl, CH$_2$Cl$_2$:CH$_3$OH
$4^\circ$C, 1 h
2) 50% TFA/50%CH$_2$Cl$_2$
$4^\circ$C, 30 min
TFA•H-Cys(Scm)Lys(H•TFA)-pNA

Scheme I-4
Fmoc-AlaOH + H-Lys(Boc)-pNA $\xrightarrow{\text{HOBt, EDCI, CH$_2$Cl$_2$}}$ Fmoc-Ala-Lys(Boc)-pNA
$4^\circ$C, 2 h
Fmoc-Ala$_2$OH + H-Lys(Boc)-pNA $\xrightarrow{\text{HOBt, EDCI, THF}}$ Fmoc-Ala$_2$-Lys(Boc)-pNA
$4^\circ$C, 3 h

Scheme I-5
Fmoc-AlaOH + H-AlaOtBu $\xrightarrow{\text{HOBt, EDCI, THF}}$ Fmoc-Ala$_2$OH + H-Lys(Boc)-pNA
$4^\circ$C, 2 h
TFA•H-Cys(Scm)-Ala$_2$-Lys(H•TFA)-pNA

Scheme I-6
Fmoc-Ala$_2$OH + H-AlaOtBu $\xrightarrow{\text{HOBt, EDCI, THF}}$ Fmoc-Ala$_3$OH + H-Lys(Boc)-pNA
$4^\circ$C, 25°C overnight
TFA•H-Cys(Scm)-Ala$_3$-Lys(H•TFA)-pNA
Scheme I-7

\[ \text{Fmoc-Ala}_2 \text{OH} + \text{H-Ala}_2 \text{-Lys(Boc)-pNA} \xrightarrow{\text{HOBT, EDCI, DMF, THF}} \text{Fmoc-Ala}_4 \text{-Lys(Boc)-pNA} \]

\[ 4 - 25^\circ \text{C, overnight} \]

\[ \xrightarrow{30\% \text{ piperidine/DMF}} \]

\[ 25^\circ \text{C, 10 min} \]

\[ \text{H-Ala}_4 \text{-Lys(Boc)-pNA} + \text{Boc-Cys(Acm)} \text{-OH} \xrightarrow{\text{HOBT, EDCI, DMF}} \]

\[ 4 - 25^\circ \text{C, overnight} \]

\[ 1) \text{ScmCl, CH}_2 \text{Cl}_2 : \text{CH}_3 \text{OH} \]

\[ 4^\circ \text{C, 1 h} \]

\[ \text{Boc-Cys(Acm)-Ala}_4 \text{-Lys(Boc)-pNA} \xrightarrow{2) 50\% \text{TFA/50\%CH}_2 \text{Cl}_2} \]

\[ 4^\circ \text{C, 30 min} \]

\[ \text{TFA}\text{*H-Cys(Scm)-Ala}_4 \text{-Lys(H*TFA)-pNA} \]

Scheme I-8

\[ \text{Fmoc-Ala}_2 \text{OH} + \text{H-Ala}_2 \text{-Lys(Boc)-pNA} \xrightarrow{\text{HOBT, EDCI, DMF, THF}} \text{Fmoc-Ala}_5 \text{-Lys(Boc)-pNA} \]

\[ 4 - 25^\circ \text{C, overnight} \]

\[ \xrightarrow{30\% \text{ piperidine/DMF}} \]

\[ 25^\circ \text{C, 10 min} \]

\[ \text{H-Ala}_5 \text{-Lys(Boc)-pNA} + \text{Boc-Cys(Acm)} \text{-OH} \xrightarrow{\text{HOBT, EDCI, DMF}} \]

\[ 4 - 25^\circ \text{C, overnight} \]

\[ 1) \text{ScmCl, CH}_2 \text{Cl}_2 : \text{CH}_3 \text{OH} \]

\[ 4^\circ \text{C, 1 h} \]

\[ \text{Boc-Cys(Acm)-Ala}_5 \text{-Lys(Boc)-pNA} \xrightarrow{2) 50\% \text{TFA/50\%CH}_2 \text{Cl}_2} \]

\[ 4^\circ \text{C, 30 min} \]

\[ \text{TFA}\text{*H-Cys(Scm)-Ala}_5 \text{-Lys(H*TFA)-pNA} \]

Scheme I-9

\[ \text{TFA}\text{*H-Lys(Z)-pNA} + \text{Boc-Cys(Acm)} \text{-OH} \xrightarrow{\text{HOBT, EDCI, THF, DIEA}} \text{Boc-Cys(Acm)Lys(Z)-pNA} \]

\[ 4 - 25^\circ \text{C, 2 h} \]

\[ 1) \text{ScmCl, CH}_2 \text{Cl}_2 : \text{CH}_3 \text{OH} \]

\[ 4^\circ \text{C, 1 h} \]

\[ \xrightarrow{2) 50\% \text{TFA/50\%CH}_2 \text{Cl}_2} \]

\[ 4^\circ \text{C, 30 min} \]

\[ \text{TFA}\text{*H-Cys(Scm)Lys(Z)-pNA} \]

Scheme I-10

\[ \text{TFA}\text{*H-Lys(Z)-pNA} + \text{Boc-Ala OH} \xrightarrow{\text{HOBT, EDCI, CH}_2 \text{Cl}_2, \text{DIEA}} \text{Boc-Ala-Lys(Z)-pNA} \]

\[ 50\% \text{TFA/50\%CH}_2 \text{Cl}_2 \]

\[ 4 - 25^\circ \text{C, 2 h} \]

\[ \xrightarrow{4^\circ \text{C, 30 min}} \]

\[ \text{TFA}\text{*H-Ala-Lys(Z)-pNA} + \text{Boc-Cys(Acm)} \text{-OH} \xrightarrow{\text{HOBT, EDCI, THF, DIEA}} \text{Boc-Cys(Acm)-Ala-Lys(Z)-pNA} \]

\[ 4 - 25^\circ \text{C, 2 h} \]

\[ 1) \text{ScmCl, CH}_2 \text{Cl}_2 : \text{CH}_3 \text{OH} \]

\[ 4^\circ \text{C, 1 h} \]

\[ \xrightarrow{2) 50\% \text{TFA/50\%CH}_2 \text{Cl}_2} \]

\[ 4^\circ \text{C, 30 min} \]

\[ \text{TFA}\text{*H-Cys(Scm)-Ala-Lys(Z)-pNA} \]
Scheme I-11

\[
\text{TFA}^{*}\text{H-Lys(Z)-pNA} + \text{Boc-Ala}_2\text{OH} \xrightarrow{\text{HOBT, EDCI, THF, DIEA}} \text{Boc-Ala}_2\text{Lys(Z)-pNA} \quad \text{4°C, 30 min}
\]

\[
\text{TFA}^{*}\text{H-Ala}_2\text{-Lys(Z)-pNA} + \text{Boc-Cys(Acm)OH} \xrightarrow{\text{HOBT, EDCI, THF, DIEA}} \text{Boc-Cys(Acm)-Ala}_2\text{-Lys(Z)-pNA} \quad \text{4 - 25°C, overnight}
\]

\[
\text{1) ScmCl, CH}_2\text{Cl}_2:\text{CH}_3\text{OH} \quad \text{4°C, 1 h}
\]

\[
\text{TFA}^{*}\text{H-Cys(Scm)-Ala-Lys(Z)-pNA}
\]

\[
\text{2) 50%TFA/50%CH}_2\text{Cl}_2 \quad \text{4°C, 30 min}
\]

Scheme I-12

\[
\text{H-Lys(Boc)-pNA} + \text{Z-Cys(Trit)OH} \xrightarrow{\text{HOBT, EDCI, THF}} \text{Z-Cys(Trit)Lys(Boc)-pNA} \quad \text{4°C, 2 h}
\]

\[
\text{1) ScmCl, CH}_2\text{Cl}_2:\text{CH}_3\text{OH} \quad \text{4°C, 1 h}
\]

\[
\text{Z-Cys(Scm)Lys(H*TFA)-pNA}
\]

\[
\text{2) 50%TFA/50%CH}_2\text{Cl}_2 \quad \text{4°C, 30 min}
\]

Scheme I-13

\[
\text{H-Ala-Lys(Boc)-pNA} + \text{Z-Cys(Trit)OH} \xrightarrow{\text{HOBT, EDCI, CH}_2\text{Cl}_2} \text{Z-Cys(Trit)-Ala-Lys(Boc)-pNA} \quad \text{4°C, 2 h}
\]

\[
\text{1) ScmCl, CH}_2\text{Cl}_2:\text{CH}_3\text{OH} \quad \text{4°C, 1 h}
\]

\[
\text{Z-Cys(Scm)-Ala-Lys(H*TFA)-pNA}
\]

\[
\text{2) 50%TFA/50%CH}_2\text{Cl}_2 \quad \text{4°C, 30 min}
\]

Scheme I-14

\[
\text{H-Ala}_2\text{-Lys(Boc)-pNA} + \text{Z-Cys(Trit)OH} \xrightarrow{\text{HOBT, EDCI, THF}} \text{Z-Cys(Trit)-Ala}_2\text{-Lys(Boc)-pNA} \quad \text{4°C, 2 h}
\]

\[
\text{1) ScmCl, CH}_2\text{Cl}_2:\text{CH}_3\text{OH} \quad \text{4°C, 1 h}
\]

\[
\text{Z-Cys(Scm)-Ala}_2\text{-Lys(H*TFA)-pNA}
\]

\[
\text{2) 50%TFA/50%CH}_2\text{Cl}_2 \quad \text{4°C, 30 min}
\]
Scheme I-15

Z-Cys(Acm)OH + HCl*H-AlaOtBu $\xrightarrow{\text{HOBT, EDCI, DIEA, THF}}$ Z-Cys(Acm)-AlaOtBu

\[4 \text{ - } 25^\circ C, \text{ overnight} \quad \xrightarrow{\text{50%TFA/50%CH}_2\text{Cl}_2} \quad 4^\circ C, 30 \text{ min}\]

Z-Cys(Acm)-AlaOH + H-Ala-Lys(Boc)-pNA $\xrightarrow{\text{HOBT, EDCI, DMF:THF}}$ Z-Cys(Acm)-Ala-Lys(Boc)-pNA

1) ScmCl, CH$_2$Cl$_2$:CH$_3$OH
\[4^\circ C, 1 \text{ h}\]
2) 50%TFA/50%CH$_2$Cl$_2$
\[4^\circ C, 30 \text{ min}\]

Scheme I-16

Z-Cys(Acm)OH + HCl*H-Ala$_2$OtBu $\xrightarrow{\text{HOBT, EDCI, DIEA, THF}}$ Z-Cys(Acm)-Ala$_2$OtBu

\[4 \text{ - } 25^\circ C, \text{ overnight} \quad \xrightarrow{\text{50%TFA/50%CH}_2\text{Cl}_2} \quad 4^\circ C, 30 \text{ min}\]

Z-Cys(Acm)-Ala$_2$OH + H-Ala$_2$-Lys(Boc)-pNA $\xrightarrow{\text{HOBT, EDCI, DMF:THF}}$ Z-Cys(Acm)-Ala$_2$-Lys(Boc)-pNA

1) ScmCl, CH$_2$Cl$_2$:CH$_3$OH
\[4^\circ C, 1 \text{ h}\]
2) 50%TFA/50%CH$_2$Cl$_2$
\[4^\circ C, 30 \text{ min}\]

Scheme I-17

Z-Ala$_2$OH + HCl*AlaOtBu $\xrightarrow{\text{HOBT, EDCI, DIEA, CH}_2\text{Cl}_2}$ Z-Ala$_2$OtBu

\[4 \text{ - } 25^\circ C, \text{ overnight} \quad \xrightarrow{\text{50 atm H}_2, \text{Pd/C}} \quad \text{ethanol, 24 h}\]

H-Ala$_3$OtBu + Z-Cys(Acm)OH $\xrightarrow{\text{HOBT, EDCI, THF}}$ Z-Cys(Acm)-Ala$_3$OtBu

\[4 \text{ - } 25^\circ C, \text{ overnight} \quad \xrightarrow{\text{50%TFA/50%CH}_2\text{Cl}_2} \quad 4^\circ C, 30 \text{ min}\]

Z-Cys(Acm)-Ala$_3$OH + H-Ala$_2$-Lys(Boc)-pNA $\xrightarrow{\text{HOBT, EDCI, DMF:THF}}$ Z-Cys(Acm)-Ala$_3$-Lys(Boc)-pNA

1) ScmCl, CH$_2$Cl$_2$:CH$_3$OH
\[4^\circ C, 1 \text{ h}\]
2) 50%TFA/50%CH$_2$Cl$_2$
\[4^\circ C, 30 \text{ min}\]
Testing of the Model Peptides

To test the performance of the model design, a trial peptide, Z-Ala-Phe-Gly-O-Dbf-S-(TFA.H)Cys-Ala-Lys(Z)-pNA was taken through a typical sequence of reactions. The peptide couplings and thiol capture were monitored at 313 nm, and HPLC traces indicated that the pNA performed perfectly as a chromophore, allowing the peptides to be followed easily. The thiol capture product was purified by preparative HPLC and taken through a typical acyl transfer reaction. Although the HPLC contained a number of peaks presumably due to disulfide interchange, deprotection with HBr/Acetic acid and cleavage of the peptide linker disulfide with triethylphosphine yielded a single product, presumably Ala-Phe-Gly-Cys-Ala-Lys-pNA. After purification by prep HPLC, the product was characterized by fragmentation mass spectrometry, amino acid analysis and peptide sequencing. The characterization data are summarized in Figure I-9. The masses detected confirmed the sequence of the peptide and showed that the pNA had been retained throughout the entire synthesis, acyl transfer and deprotection sequences. The results from sequencing and amino acid analysis indicated that the Z protected ε amine of lysine did not participate in acyl transfer, and both Z groups were easily removed by HBr/acetic acid. The sequence of the product was identical to that detected by fragmentation mass spectrometry. Lysine detection in both the sequencing and amino acid analysis indicated that the pNA did not interfere with the characterization of the product by amino acid analysis or peptide sequencing. It was clear from these initial experiments that the peptide design was consistent with the chemistry of the thiol capture/acyl transfer methodology and the methods of characterization.
Mass Spectroscopy Data

C-terminal ions of the peptide

\[ \text{ALA} \rightarrow \text{PHE} \rightarrow \text{GLY} \rightarrow \text{CYS} \rightarrow \text{ALA} \rightarrow \text{LYS} \rightarrow \text{pNA} \]

N-terminal ions of the peptide

<table>
<thead>
<tr>
<th>No.</th>
<th>Amino Acid</th>
<th>( a ) ion</th>
<th>( b ) ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala (A)</td>
<td>44.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Phe (F)</td>
<td>191.12</td>
<td>219.11</td>
</tr>
<tr>
<td>3</td>
<td>Gly (G)</td>
<td>248.14</td>
<td>276.13</td>
</tr>
<tr>
<td>4</td>
<td>Cys (C)</td>
<td>348.14</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ala (A)</td>
<td>422.19</td>
<td>450.18</td>
</tr>
<tr>
<td>6</td>
<td>Lys (K)</td>
<td>550.28</td>
<td></td>
</tr>
</tbody>
</table>

N-terminal ions of the peptide Detected

<table>
<thead>
<tr>
<th>No.</th>
<th>Amino Acid</th>
<th>( y ) ion</th>
<th>( v ) ion</th>
<th>( w ) ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lys (K)</td>
<td>267.15</td>
<td></td>
<td></td>
</tr>
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<td>2</td>
<td>Ala (A)</td>
<td>322.15</td>
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<td></td>
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<td>3</td>
<td>Cys (C)</td>
<td>392.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Gly (G)</td>
<td>498.21</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>Phe (F)</td>
<td>645.28</td>
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</tbody>
</table>

Internal Fragment ions of the Peptide Detected

<table>
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<th>No.</th>
<th>Fragment</th>
<th>Mass</th>
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<tr>
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<td>2</td>
<td>CA</td>
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<tr>
<td>3</td>
<td>FG</td>
<td>205.10</td>
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<tr>
<td>4</td>
<td>FCC</td>
<td>308.11</td>
</tr>
<tr>
<td>5</td>
<td>FGCA</td>
<td>379.14</td>
</tr>
</tbody>
</table>


Sequencing Data

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<tr>
<th>Cycle</th>
<th>Assignment</th>
<th>Picomoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala</td>
<td>517.1</td>
</tr>
<tr>
<td>2</td>
<td>Phe</td>
<td>456.5</td>
</tr>
<tr>
<td>3</td>
<td>Gly</td>
<td>346.4</td>
</tr>
<tr>
<td>4</td>
<td>[Cys]</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ala</td>
<td>202.4</td>
</tr>
<tr>
<td>6</td>
<td>Lys</td>
<td>69.6</td>
</tr>
</tbody>
</table>

Amino Acid Analysis Data

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>picomoles</th>
<th>molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>437</td>
<td>1.0</td>
</tr>
<tr>
<td>Phe</td>
<td>419</td>
<td>1.0</td>
</tr>
<tr>
<td>Lys</td>
<td>468</td>
<td>1.1</td>
</tr>
<tr>
<td>Ala</td>
<td>875</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Note- Cysteine not protected -
44 picomoles cysteic acid detected

Figure I-9
HPLC as a Diagnostic Tool

In order to study the production of cysteine and lysine acyl transfer products under different conditions of acyl transfer, a rapid and simple assay method had to be developed. Past work on thiol capture had relied on HPLC as the primary diagnostic tool and had shown that the thiol capture material and acyl transfer products were well behaved under typical HPLC conditions. Unlike the other techniques available for the study of acyl transfer, HPLC allowed the identification of each of the species present by their retention times. Peak shapes showed little tendency to tail, allowing accurate calculation of starting material and product ratios from the integration of peak areas. Finally, the high extinction coefficient of the dibenzofuran linker and in our case, the C-terminal p-nitroanilide, made the technique quite sensitive, allowing the various species to be detected easily at the dilute concentrations typical of acyl transfer reactions.

Figure I-10
The use of HPLC peak areas to determine the relative amounts of thiol capture and acyl transfer materials present in the reaction mixture required that the spectral qualities of these species be known. The structure of pNA was unaffected by the acyl transfer and was therefore a constant. During the reaction however, dibenzofuran was converted from an ester to a phenol. The effect of this reaction on its UV absorbance had to be determined. Figure I-10 shows the UV spectra of the dibenzofuran template in model thiol capture and acyl transfer compounds. The thiol capture material has a sharp UV maximum at 314 which shifts to 320 in the acyl transfer product. Fortuitously however, UV absorbance of the product\(^3\) at 314 nm is identical to the thiol capture material, with an extinction coefficient of $6.96 \times 10^3$ L mol\(^{-1}\) cm\(^{-1}\). This allowed the use of peak areas at this wavelength to be used directly in the calculation of starting material and product ratios.

**Identification of the HPLC Peaks**

In order to study the phenomenon of acyl transfer to lysine using HPLC as the primary investigative method, it was also necessary to positively identify all the HPLC peaks (Figure I-11) of species formed during the acyl transfer step. The HPLC retention time of the thiol capture peptide was identified by the injection of pure thiol capture product, after its identity had been confirmed by plasma desorption mass spectrometry. Tentative identification of the acyl transfer products as being due to cysteine or lysine acyl transfer were based on whether the peak appeared under conditions of high or low base concentration.

---

\(^3\) The absorbance in the acyl transfer product is identical, regardless of whether reaction with the cysteine or lysine amine has occurred as the disulfide linked phenol is produced in either case.
Product of Acyl Transfer To Cysteine → 28.7
Product of Acyl Transfer To Lysine → 52.1

Peaks due to DMSO and AgNO₃

Impurities Due to Disulfide exchange

Product of Acyl Transfer To Cysteine → 48.1
Product of Acyl Transfer To Lysine → 62.1

Acyl Transfer with 0.7 equivalents DICE
Acyl Transfer with 1.5 equivalents DICE

Figure II-11
As discussed in Appendix A, the pKa of the ε amine of lysine is 3 units higher than that of the α amine of cysteine. The cysteine amine would therefore be expected to be deprotonated and undergo acyl transfer at low base concentration, where the lysine amine is still protonated. Peaks observed as the major product under conditions of low base concentration are likely to be due to acyl transfer to cysteine while those observed as the major product at high concentration are likely to be due to acyl transfer to lysine. Additionally, relative retention times could also be used for initial assignment. In the original acyl transfer observed by Dr. Fotouhi during the synthesis of BPTI, the lysine acyl transfer product was observed to have a shorter retention time than the cysteine product. Similarly, in the model studies performed for this thesis, it was found that in all acyl transfers, the peak observed at high base concentration had a retention time that was approximately one minute shorter (10.5 - 10.8 minutes) than the peak observed at low base concentration (11.5 - 12 minutes; HPLC conditions - 40% CH₃CN-60% 0.1% TFAaq to 100% CH₃CN over 10 minutes). Proof of structure however, required that the peaks be purified by preparative HPLC and characterized by a method that determined the peptide sequence directly. Once this had occurred, HPLC retention times could be used for identification.

Characterization of the Acyl Transfer Products

The two acyl transfer products were isomers, differing only in sequence. Of the methods available for characterization of the peptides only two, fragmentation mass spectrometry and peptide sequencing, are capable of determining sequence. Fragmentation mass spectrometry determines peptide sequence by detecting peptide fragments with masses
corresponding to portions of the expected sequence. Some of the characteristic fragment types produced are indicated in Figure I-12. The structural requirements are rather lax, provided characteristic fragments exist, which in the case of this study were fragments containing either Gly-Lys or Gly-Cys.

Fragments produced from protonated linear peptides by cleavage independent of R.

Figure I-12

Peptide sequencing involves repeated cycles of cleavage of the N-terminal amino acid from a peptide chain via the Edman reaction and its identification by HPLC (Figure I-13). The structural requirements are somewhat more stringent than for fragmentation mass spectrometry.
Peptide Sequencing by Edman Degradation

Phenyl isothiocyanate (PITC)

Anhydrous acid

Thiazolinone derivative

PTH-amino acid - identified by HPLC

Figure I-13
Sequencing requires a free amino terminus and a normal peptide backbone, and the cycle of reactions stops when an abnormal linkage (such as the amide formed by acyl transfer to the ε amine of lysine) is encountered.

In order to use either fragmentation mass spectrometry or peptide sequencing, the samples must be extremely pure. Any contaminating peptides will yield sequences also, making interpretation of the results difficult or impossible. The only method available that could yield the level of purity necessary was preparative HPLC. As discussed above, the total amount of peptide in an acyl transfer was quite small - the presence of the pNA group was necessary to allow the peptide elution from the HPLC column to be monitored. It was hoped that the amide linkage of the paranitroanilide would cleave under normal Edman conditions allowing the lysine residue to be identified. In final testing of the models this turned out to be the case, as can be seen from the data presented below.

Blocking the α amine of alanine in the model peptides was necessary to prevent side reactions from nucleophilic attack during acyl transfer. By using Z as the protecting group, the acyl transfer products could be deprotected exposing the free amino terminus necessary for sequencing. Let us examine the expected peptide sequences produced for the products produced by acyl transfer to lysine and cysteine. Let us use Z-A-F-G-O-Dbf-S-S-(TFA.H)CA₂K(H.TFA)-pNA as the model for this hypothetical discussion as it was the thiol capture material actually used to identify the HPLC peaks as the products of cysteine or lysine acyl transfer (Figure I-14).
Z-Cys-Ala-Ala-Lys-pNA

1) Acyl Transfer to Cysteine
2) Et₃P

Z-Ala-Phe-Gly-Cys-Ala-Ala-Lys-pNA

HBr/Acetic Acid

H₃N⁺-Cys-Ala-Ala-Lys-pNA

Figure 1-14
Acyl transfer to cysteine generates a normal peptide bond. After deprotection and purification, sequencing of the product would yield (for 100 picomoles of peptide):

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino Acid</th>
<th>picomoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Phe</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Gly</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>Ala</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Ala</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>Lys</td>
<td>100</td>
</tr>
</tbody>
</table>

Theoretically, the quantity of amino acid detected should reflect its stoichiometry in the peptide. In practice, the quantity of amino acid detected drops slightly with each cycle. Cysteine, unless protected is usually destroyed during sequencing, yielding a blank run. Finally, although lysine could be detected in the actual peptides, the levels were much lower, indicating the cleavage of the pNA was more difficult.

The product of acyl transfer to lysine would yield, after deprotection a peptide with two amino termini. Sequencing would yield (for 100 picomoles of peptide):

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala (100 picomoles)</td>
</tr>
<tr>
<td>2</td>
<td>Phe (100 picomoles) and Ala (100 picomoles)</td>
</tr>
<tr>
<td>3</td>
<td>Ala (100 picomoles)</td>
</tr>
<tr>
<td>4</td>
<td>---</td>
</tr>
</tbody>
</table>

If the Z group is not removed after acyl transfer, sequencing yields a different result. The product due to cysteine acyl transfer would not yield any sequence as there is no free
amino terminus, although mass spectrometry would show the proper mass. The product due
to acyl transfer to lysine would yield (for 100 picomoles of peptide)

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>Ala (100 picomoles)</td>
</tr>
<tr>
<td>3</td>
<td>Ala (100 picomoles)</td>
</tr>
</tbody>
</table>

with nothing detected in subsequent cycles.

In practice, this second method was used to identify the peaks. For purposes of cost,
sequencing was only performed on one model: Z-A-F-G-O-Dbf-S-S-(TFA.H)CA₂K(H.TFA)-pNA . Acyl transfers were run under conditions of low and high DIEA concentration and the
products purified by preparative HPLC. The major product at low DIEA concentration had a
retention time of 11.6 minutes (HPLC conditions - 40% CH₃CN-60% 0.1% TFAaq to 100%
CH₃CN over 10 minutes) and was tentatively identified as the cysteine acyl transfer product.
The major product at high base concentration had a retention time of 10.7 minutes (under
identical HPLC conditions) and was tentatively identified as the product of acyl transfer to
lysine. Although no amino acids should have been detected for the cysteine acyl transfer
product, threshold quantities corresponding to the expected sequence were observed. In the
case of the putative lysine acyl transfer product, the sequencing data is reproduced below.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>Ala (13.4 picomoles)</td>
</tr>
<tr>
<td>3</td>
<td>Ala (10.9 picomoles) Phe (1.0 picomoles)</td>
</tr>
<tr>
<td>4</td>
<td>----</td>
</tr>
</tbody>
</table>

Nothing was observed in cycle 4 or subsequent cycles
The detection of amino acids from the Z protected amino terminus implies that a small amount of deprotection occurred during sequencing. Thioanisole has been observed to accelerate deprotection of Z groups in TFA, and it is possible that the b-mercaptoethanol present in the TFA cleavage mixture could have the same effect (64). As with Dr. Fotouhi's original BPTI example, the more rapidly eluting HPLC peak observed as the major product at high base concentration was shown by sequencing to be due to lysine acyl transfer, while the slower HPLC peak observed at low base concentration was the cysteine acyl transfer product. Correlation of the sequences with the retention times and base concentration for two structurally different products enabled HPLC retention times to be used as identification for all subsequent model studies.

Identification of Disulfide Interchange Impurities

The tendency for the starting materials and products to undergo disulfide interchange was of some concern. The level of interchange was less important for the reactions that studied the competition between the cysteine and lysine amine (model 1 in Figure 4), as reactions with high levels of disulfide interchange could be analyzed after treatment with triethylphosphine. As long as disulfide interchange did not consume too much starting material (easily determined by the lack of observable Z-A-F-G-O-Dbf-SH after triethylphosphine treatment), the product ratio was unlikely to have been affected.

For the kinetic measurement of acyl transfer however, it was critical that the products of disulfide interchange be maintained at low levels. Depletion of the starting materials and products by interchange would affect their rate of change. Even more importantly, many
analysis techniques rely heavily on the amount of a given species at the infinity point. Disulfide interchange would change the amount detected, resulting in large errors in the kinetic constants. The use of HPLC however, allows the detection of impurities directly. Any reactions showing high levels of impurities could be discarded.

Once the HPLC peaks due to thiol capture peptide and the acyl transfer products had been identified, other peaks were assumed to be due to impurities produced by disulfide interchange. An extensive study performed by Dr. Fotouhi had identified disulfide interchange as the major source of impurities during acyl transfer. HPLC purification of the thiol capture peptide was found to drastically reduce the amount of disulfide interchange and the addition of a few mole percent of AgNO₃ as thiol capture scavengers further decreased the side reaction. Under typical acyl transfer conditions, the levels were usually acceptable, as can be seen in the HPLC traces (Figure I-11). That peaks indicated were produced by disulfide interchange was supported by my observation that treatment of the reaction mixture with excess triethylphosphine resulted in HPLC traces only containing peaks due to the 4-hydroxy-6-mercaptodibenzofuran template and the free thiols of the acyl transfer products. In a few cases of high disulfide interchange, additional peaks due to Z-Ala-Phe-Gly-O-Dbf-SH and TFA.HCys(SH)-Ala₄-Lys(H.TFA)-pNA were also identified after treatment with triethylphosphine due to disulfide interchange that occurred before acyl transfer.

**Identification of Other Peaks**

Peaks due to the residual absorbance of DMSO and AgNO₃, and its oxidation products were also identified by the HPLC analysis of a solution of these compounds after 20 hours
under typical acyl transfer conditions. The compounds yielded a number of small overlapping
peaks with a retention time faster than 4.5 minutes under the normal HPLC conditions. Once
each of the peaks had been assigned HPLC retention times were used for identification in the
experiments presented in the following chapters.

Summary

In this chapter, a review of previous work on lysine acyl transfer has been presented. The goals of the thesis have been stated. They are: 1) evaluation of the distance constraints of lysine acyl transfer reaction, 2) determination of the basis for its high reactivity relative to cysteine and, 3) development of conditions favoring formation of the cysteine acyl transfer product. The design of the models to be used to study the lysine acyl transfer phenomenon, their synthesis and testing has been presented. Finally, HPLC as a diagnostic tool has been discussed and the steps taken to identify the peaks outlined.
Chapter 2 - Cysteine and Lysine Acyl Transfer With DIEA
In Chapter 1, the design, synthesis and testing of the models to be used in the investigation of lysine acyl transfer was presented and the HPLC assay to be used in the analysis discussed. In this chapter, the results of experiments using diisopropylethylamine (DIEA) as a base are presented. Two types of experiments are used for the analysis of the lysine acyl transfer reaction: 1) a competition using peptides where both the cysteine and lysine amines are free to react and 2) kinetic studies where the rates of cysteine and lysine reactions are studied individually. Together, the results of these reactions allow the parameters of the lysine acyl transfer reaction with DIEA to be determined. Before discussing the experiments however a brief review of kinetics must be presented.

The observed cysteine:lysine product ratio arises from an acid:base neutralization, followed by a kinetic competition between the cysteine and lysine amines for the acyl ester. Let us consider the kinetic equations for reactions involving acid base equilibria (Scheme II-1) as well as for a single reactant going irreversibly to multiple products (Scheme II-2).

Scheme II-1  The Kinetics of Reactions Involving Acid Base Equilibria:

\[
AH^+ + B \rightleftharpoons A + BH^+ \quad \text{eqn. II-1}
\]

\[
A \rightarrow P \quad \text{eqn. II-2}
\]

For a reaction involving an acid base neutralization in the first step, where the reactive intermediate \( A \) is in rapid equilibrium with its progenitor \( AH^+ \), and decays slowly to product, the rate of formation of product is:

\[
\frac{d[P]}{dt} = k_3[A] \quad \text{eqn. II-3}
\]
where \([A]\) represents the concentration of reactive intermediate at equilibrium, and \(k_3\), the rate constant of the reaction. The rate constant, \(k_3\) is measured under conditions of maximum velocity, where all the \(AH^+\) has been converted to A. For clarity, \(k_3\) will be referred to as the intrinsic rate constant, to distinguish it from the apparent rate constants measured under conditions where \(AH^+\) is only partially deprotonated.

Scheme II-2 The Kinetics of One Reactant Going To Multiple Products

For a single reaction that produces more than one product the sequence:

\[
\begin{align*}
A & \stackrel{k_1}{\longrightarrow} B \quad \text{eqn. II-4} \\
A & \stackrel{k_2}{\longrightarrow} C \quad \text{eqn. II-5}
\end{align*}
\]

has a velocity

\[
\frac{d[A]}{dt} = -k_1[A] - k_2[A] \quad \text{eqn. II-6}
\]

The loss of reactant is simple first order and

\[
[A] = ae^{-(k_1+k_2)t} \quad \text{eqn. II-7}
\]

The rates of formation of products are:

\[
\begin{align*}
\frac{d[B]}{dt} &= k_1[A] \\
\frac{d[B]}{dt} &= k_1 ae^{-(k_1+k_2)t} \\
\frac{d[C]}{dt} &= k_2[A] \\
\frac{d[C]}{dt} &= k_2 ae^{-(k_1+k_2)t}
\end{align*}
\]

These can be integrated to give

\[
\begin{align*}
[B] &= \frac{k_1a}{k_1 + k_2} \left(1 - e^{-(k_1+k_2)t}\right) \quad \text{eqn. II-8} \\
[C] &= \frac{k_2a}{k_1 + k_2} \left(1 - e^{-(k_1+k_2)t}\right) \quad \text{eqn. II-9}
\end{align*}
\]
Therefore the ratio of the products formed is in proportion to their individual rate constants (measured under the same conditions)

\[
\frac{[B]}{[C]} = \frac{k_1}{k_2}
\]

\textit{eqn. II - 10}

**Determination of the Sensitivity of The Cysteine:Lysine Acyl Transfer Ratio to the Molar Equivalents of DIEA**

The first set of experiments performed probed the sensitivity of the acyl transfer reaction to the number of equivalents of DIEA added\(^1\). Model peptides with both cysteine and lysine amines initially present as TFA salts and variable numbers of intervening alanines (CA\textit{nK} thiol capture peptides) were used. By observing the sharpness of the transition between cysteine and lysine acyl transfer for each model as more base was added, we could determine whether acyl transfer to lysine could be eliminated by careful control of the amount of base. The effect of increasing the distance of the lysine from the aryl ester\(^2\) (determined by a comparison between the models) on the fraction of acyl transfer to lysine observed at high concentrations of DIEA, might determine where a cut off point exists, beyond which acyl transfer to lysine ceases to be a problem.

**Extinction Coefficients of The Thiol Capture Peptide**

Before determining the percent of acyl transfer to cysteine and lysine as a function of added DIEA, it was necessary to measure the concentration of thiol capture material

\(^1\) For convenience, these experiments are referred to as acyl transfer titrations.
\(^2\) caused by increasing the number of alanines between cysteine and lysine.
accurately. Simple measurement of the mass of material added to a given volume of DMSO
was inadequate, as TFA salts of peptides are hygroscopic and a significant proportion of the
mass could be due to water. By determining the extinction coefficient of the thiol capture
material however, the amount of material present in a given volume could be accurately
determined, regardless of the presence of absorbed water. As rule of thumb, the extinction
coefficient of a compound is the sum of the extinction coefficients of its component
chromophores, so long as the chromophores are electronically isolated from one another. As
stated in Chapter 1, the extinction coefficient at 313 nm of dibenzofuran in the thiol capture
material is $6.96 \times 10^3$ L mol$^{-1}$ cm$^{-1}$ while the extinction coefficient of the p-nitroanilide is
$1.13 \times 10^4$ L mol$^{-1}$ cm$^{-1}$. The overall extinction coefficient of the thiol capture model peptides
therefore, was $1.84 \times 10^4$ L mol$^{-1}$ cm$^{-1}$.

To show that the calculated extinction coefficient could accurately determine
concentrations, concentrations were also determined by mass for a series of scrupulously
purified and dried thiol capture peptides. On average the two values showed a difference of
less than 5%, indicating that the extinction coefficient method was accurate.

The Acyl Transfer Titrations

The sensitivity of the cysteine:lysine acyl transfer ratio to the number of added DIEA
equivalents was assessed by performing acyl transfers at $10^{-4}$ M in DMSO using HPLC purified
CA$_2$K thiol capture peptides, with incremental amounts of DIEA. The concentration of the
thiol capture peptide stock solutions were determined spectrophotometrically, while stocks of
DIEA were made by volumetric dilution. The volumes of peptide and DIEA in the acyl
transfers were measured by Ranin Pipetman. After 20 hours at room temperature, the
products of the acyl transfer titrations were analyzed by HPLC and percent of acyl transfer to
cysteine and lysine and the percent of disulfide interchange calculated from the HPLC peak
areas\(^3\).

The extent of the reaction reflected the number of equivalents of added DIEA (Figure
II-1). Thiol capture material was observed in the HPLC traces for acyl transfers performed
with less than 1 equivalent of base. Small amounts of cysteine acyl transfer (10-20\%) were
observed in the absence of any added base, although in a few cases abnormally high levels of
acyl transfer were seen\(^4\). Although the level of disulfide interchange was generally low for
most of the acyl transfer titrations, in the few cases where high levels of disulfide interchange

\(^3\) See Calculations under section entitled Acyl Transfer Titration of Model Peptides with DIEA

\(^4\) Table II-1 presents the results of a number of acyl transfers run in the absence of base. Only acyl transfer to
cysteine was observed. At 10\(^4\) M, the amount of acyl transfer after 20 h was low, generally less than 20\%,
while at 10\(^-5\) M, an average of over 50\% acyl transfer was observed.

<table>
<thead>
<tr>
<th>Thiol Capture Material</th>
<th>Concentration</th>
<th>Percent Acyl Transfer in 20 h</th>
<th>Percent Acyl Transfer to Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>10(^4) M</td>
<td>8</td>
<td>95</td>
</tr>
<tr>
<td>CAAK</td>
<td>10(^4) M</td>
<td>5</td>
<td>88</td>
</tr>
<tr>
<td>CAAK</td>
<td>10(^-5) M</td>
<td>7</td>
<td>87</td>
</tr>
<tr>
<td>CAAA K</td>
<td>10(^4) M</td>
<td>13</td>
<td>95</td>
</tr>
<tr>
<td>CAAA K</td>
<td>10(^-5) M</td>
<td>15</td>
<td>90</td>
</tr>
<tr>
<td>CK</td>
<td>10(^-5) M</td>
<td>55</td>
<td>94</td>
</tr>
<tr>
<td>CAAK</td>
<td>10(^-5) M</td>
<td>47</td>
<td>88</td>
</tr>
<tr>
<td>CAAA K</td>
<td>10(^-5) M</td>
<td>51</td>
<td>92</td>
</tr>
</tbody>
</table>

The percent of observed acyl transfer roughly correlates with the percent ionization of the cysteine
amine at high dilution. If we consider the pKa of the cysteine ammonium ion to be 6.9, simple equilibrium
calculations indicate that at 10\(^4\) M, 3.5\% will be present as the free amine, increasing to 11.2 \% at 10\(^-5\) M. In
addition, DMSO is 1-2 pKa units more basic than water, so that the percentage of free amine could be
somewhat higher in this solvent. It is likely that the level of acyl transfer observed is due to auto-ionization of
ammonium ion in DMSO.

Abnormally high levels of acyl transfer in the absence of base (data not shown) however, indicated that
at least some lots of commercially available spectrophotometric grade DMSO may contain small amounts of
basic impurities. In an acyl transfer of CAK in the absence of base 34\% acyl transfer product was observed.
In addition, the percent acyl transfer to cysteine observed for this sample was 52%, similar to that observed with DIEA.

In order to understand the source of the abnormally high acyl transfer, a control, using the same CAAAK sample that had undergone 15% acyl transfer in the absence of DIEA, was performed using this lot of DMSO. This time, 57% acyl transfer product was observed, indicating that the result was due to the solvent and not the sample. The results were attributed to basic impurities present in small amounts in the DMSO. Once this had been observed, acyl transfers in the absence of base were run routinely to determine the level of basic contaminants, and lots showing abnormally high levels were discarded.
occurred, a relationship between the disulfide interchange level and the amount of added DIEA was observed (Figure II-2). In these cases, less than 10% disulfide interchange was seen below 1 equivalent added DIEA, rising sharply to approximately 40% above 1.5 equivalents. The relationship between disulfide interchange and base is considered in more detail in Chapter 3.

Figures II-3 through II-8 present the results of the acyl transfer titrations for the CK through CA3K thiol capture materials. The graphs indicate that the percent of acyl transfer to cysteine\(^5\) is quite sensitive to the stoichiometry of DIEA. Below one equivalent, approximately 80% cysteine acyl transfer product was observed decreasing rapidly to around 60% with 1.5 equivalents and 40% with 2. Above 2 equivalents, the amount of cysteine acyl transfer appeared to level out at 20% regardless of the number of intervening alanines between cysteine and lysine. The apparent insensitivity of the lysine acyl transfer reaction to distance from the acyl ester was somewhat surprising especially as no acyl transfer to lysine where the lysine is separated from cysteine by 7 amino acids was reported by Dr. Fotouhi for the original BPTI example.

The rate of acyl transfer differed with the product formed. At less than 1 equivalent of DIEA where acyl transfer to cysteine was the primary reaction, a half life of approximately one hour was observed. In presence of excess DIEA, where the lysine acyl transfer product was the

\(^5\) The percent acyl transfer to cysteine (\(\text{A}_{\text{Cy}}\)) is normalized to the total amount of acyl transfer and is calculated by dividing the peak area due to cysteine acyl transfer (C) by the sum of the peak areas due to cysteine (C) and lysine acyl transfer (K). \(\text{A}_{\text{Cy}} = \frac{\text{C}}{\text{C} + \text{K}} \times 100\). As the extent of disulfide interchange could be quite high above 1.5 equivalents DIEA, peak areas in these cases were calculated after treatment with triethylphosphine.
primary species formed, the rate of the reaction increased dramatically, with half lives on the order of minutes. These results were consistent with the observations made by Dr Fotouhi.

Figure II-3

Percent A cyl Transfer to Cysteine

Equivalents of DIEA added

Figure II-4

Percent A cyl Transfer to Cysteine

Equivalents of DIEA added
Figure II-5

Equivalents of DIEA Added

0 1 2 3 4 5 6

Percent Acyl Transfer to Cysteine

Figure II-6

Equivalents of DIEA Added

0 1 2 3 4

Percent Acyl Transfer To Cysteine
Figure II-7
CA4K

Figure II-8
CA5K
The observed change from cysteine to lysine acyl transfer with increasing base can be explained by considering the relative pKa's of the cysteine and lysine amines. As discussed Appendix A, the expected pKa of the cysteine α amine in DMSO is 6.9 ± 0.7 while the expected pKa of the ε amine of lysine is 10.4 ± 0.7. With less than one equivalent of DIEA (expected pKa of approximately 9.0 in DMSO) the more acidic cysteine amine will be deprotonated first and so will undergo acyl transfer almost exclusively. With excess DIEA, however, both the cysteine and lysine amines will be deprotonated and available for acyl transfer. At this point, the observed ratio of cysteine to lysine acyl transfer product will depend on the intrinsic rates of the two reactions and the relative percentage of free amine as discussed above.

The CAK thiol capture material was a notable exception to the trend observed in the acyl transfer titrations above. Even with less than one equivalent of DIEA, 50% acyl transfer to lysine was observed. Two possible hypotheses could be advanced for this phenomenon. The first was that the pKa of the ε amine of lysine in the CAK thiol capture material was abnormally low. If so, a significant proportion of the lysine amine would be free with less than one equivalent of DIEA, allowing it to compete for the acyl ester. The rate of a reaction involving an equilibrium however, depends on the intrinsic rate constant as well as the concentration of the reacting species. The observed result could be due to an exceptionally fast intrinsic rate for the lysine acyl transfer of CAK (relative to the other lysine acyl transfers). In this case the percentage of lysine acyl transfer product could be much higher, even though the concentration of free amine at equilibrium was identical to that of the other models.

Results presented in Chapter 3, in which the fraction of free amine [NH₂]/A₀ with sodium

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6 The amount of deprotonation will depend on the exact values of the pKa's of DIEA and Lysine, neither of which has been measured in DMSO.
dichloroacetate is determined, indicate that there is no significant difference between the pKa of the lysine ammonium of CAK and the other models. It is likely, therefore that the higher level of lysine acyl transfer observed for CAK is due to a faster intrinsic rate of acyl transfer. Kinetic studies comparing the rate of lysine acyl transfer for the models are presented below.

Although the results of the titrations indicated that stoichiometric control of DIEA could be used to minimize acyl transfer to lysine, the sensitivity of the cysteine:lysine acyl transfer ratio to the amount of added base indicated that was not the best solution. Acyl transfers run with less than one equivalent yielded primarily cysteine acyl transfer product, although at the expense of incomplete reaction. Acyl transfers run with only slightly more than one equivalent of DIEA resulted in significant amounts of acyl transfer to lysine.

The high extinction coefficient of the dibenzofuran linker in the thiol capture material enabled accurate determination of peptide concentrations as dilute as $1 \times 10^{-5}$ M. Unfortunately, the amount of DIEA necessary to exactly deprotonate the cysteine ammonium ion did not depend only on peptide concentration. HPLC purification of the thiol capture material was the final step prior to acyl transfer. TFA, present as an HPLC solvent component, is known to tenaciously associate with peptides and could have been present in stoichiometric excess. In order to add the proper amount of DIEA, it was necessary to know the amount of excess TFA as well as the peptide concentration.

The acyl transfer by prior thiol capture method was designed for the synthesis of large proteins, so that any method of quantitation has to be quite sensitive. The high molecular weights of proteins mean that milligram quantities of a peptide can represent a vanishingly small number of moles. Attempts were made to determine the level of excess TFA by $^{19}$F
NMR. Unfortunately, the sensitivity of the technique was low, requiring hundreds of scans for each measurement and making the technique time consuming to use. Although concentrated samples of the relatively low molecular weight models used in this study could be prepared, for large proteins molar concentrations high enough for easy observation and accurate quantitation by NMR would represent extremely large gram quantities of peptides. Thus $^19F$ NMR could not be used as a general technique to determine excess TFA.

Titrations like those performed above could indicate where the equivalence point lay, maximizing both yield and cysteine:lysine product ratio. Unfortunately, this would require sacrifice of laboriously synthesized compound, an alternative especially unpleasant during the final couplings of a synthesis, where the yield of product had already been diminished by obligatory losses associated with synthesis and purification. Given the potential difficulties described above, it was clear that careful control of the molar ratio of peptide and DIEA was not the best solution for suppressing lysine acyl transfer, and an alternative method had to be sought. It was decided that a weaker base might be more successful in limiting acyl transfer to lysine. Chapter 3 presents the results of successful experiments using sodium dichloroacetate as an acyl transfer base.

**Introduction to the Kinetic Studies of the Acyl Transfer**

**Studies of the Distance Dependence of the Lysine Acyl Transfer Reaction**

The above experiments failed to show a distance dependence for the lysine acyl transfer reaction. Even with five intervening alanines, acyl transfer to lysine with excess DIEA was only 80%, quite surprising given that in this case, the lysine and cysteine acyl transfers
were occurring across 34 and 12 membered rings, respectively. Failure could result from an extremely fast lysine reaction that decreased only slowly with the number of intervening alanines. The length of the spacer could be too short to decrease the lysine acyl transfer reaction to the point at which competition by the cysteine amine for the phenolic ester could be observed in the product ratios. Measurement of the individual lysine acyl transfer rates (using the ZCAEnK model peptide series) however, could directly show the distance dependence for the lysine acyl transfer reaction. Comparison with the rate of cysteine acyl transfer (measured with the CAEnK(Z) model peptides) would enable calculation, via eqn. II-10, of the distance that must separate the phenolic ester and the lysine amine before cysteine could effectively compete. This would enable us to determine when special conditions or protection must be used to avoid the lysine acyl transfer reaction.

Studies of the Source of The High Reactivity of Lysine

In Chapter 1, two hypothesis for the source of the high reactivity of lysine were advanced. The rate of acyl transfer could be due to an average conformation that placed the amine of lysine closer than the cysteine amine to the dibenzofuran ester. This proximity would be reflected in a higher effective molarity for the lysine amine. The second hypothesis proposed that lysine's higher reactivity was due to the greater inherent nucleophilicity of the ε amine. The higher reactivity would allow lysine to compete successfully for the acyl ester even if it were in a much less favorable position. The measurement of the rates of lysine acyl transfer, coupled with a kinetic study of a model bimolecular reaction, would allow the effective molarity of lysine to be calculated. If the effective molarity were higher than 5 M, it would indicate that the ε-amine was in a favorable position relative to cysteine. If significantly
lower effective molarities were observed however, this would indicate that the source of product ratio observed in the acyl transfer titrations at high base concentration was due to the greater inherent nucleophilicity of the lysine ε amine.

**Kinetic Studies of Cysteine Acyl Transfer**

The kinetic studies of cysteine acyl transfer were performed using HPLC-purified preparations of CAK(Z) and CAAK(Z) thiol capture peptides. The concentrations of the reactions were $10^{-5}$ M. The peptides were dissolved in DMSO, and the reaction was initiated by the addition of DIEA. Aliquots were taken at specific time intervals, quenched with TFA and frozen in liquid N$_2$ until analysis by HPLC. As mentioned in Chapter 1, the level of disulfide interchange was of some concern as it would affect the amount of the acyl transfer product at the infinity point, causing an error in the calculated rate constant. The use of HPLC allowed the amount of disulfide interchange to be assessed directly. Reactions showing high levels of disulfide interchange were not analyzed.

![Figure II-9: Cysteine Acyl Transfer Kinetics Time Course for CAK(Z)](image-url)
Figure II-9 shows a typical time course for the acyl transfer reactions. The rates were calculated from the HPLC peak areas of the acyl transfer product by graphing $\ln\left(\frac{\lambda_0 - \lambda_\infty}{\lambda - \lambda_\infty}\right)$ vs time\(^7\). The slope of the graphs yielded the rate constants, listed in Table II-2.

<table>
<thead>
<tr>
<th>Thiol Capture</th>
<th>Concentration</th>
<th>Equivalents</th>
<th>Rate Constant</th>
<th>Half Life</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAK</td>
<td>$10^{-5}$ M</td>
<td>1</td>
<td>$1.3 \times 10^{-2}$ min(^{-1})</td>
<td>52 min</td>
</tr>
<tr>
<td>CAK</td>
<td>$10^{-5}$ M</td>
<td>2.5</td>
<td>$1.6 \times 10^{-2}$ min(^{-1})</td>
<td>46 min</td>
</tr>
<tr>
<td>CAAK</td>
<td>$10^{-5}$ M</td>
<td>1</td>
<td>$1.1 \times 10^{-2}$ min(^{-1})</td>
<td>63 min</td>
</tr>
</tbody>
</table>

The two-unit difference in pKa between cysteine and DIEA makes it likely that the cysteine ammonium ion was completely deprotonated with 1 equivalent of DIEA. The somewhat lower value for the half life determined with 2.5 equivalents of DIEA reflects a slightly higher level of disulfide interchange, although the results were not bad enough to warrant discarding the run. The average half life for cysteine acyl transfer to glycine was determined to be 55 minutes, in close agreement with the rate observed in the acyl transfer titrations and the earlier model studies performed by Dr. Fotouhi.

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\(^7\) In order to analyze first order reactions it is not necessary to measure actual concentrations; any property that is proportional to the concentration of the reactant or product will do as well. Let us consider $\lambda$ to be some property (absorption coefficient, HPLC peak area) that varies linearly with the extent of reaction. We denote the initial ($t=0$) value as $\lambda_0$ and the value at the end of the reaction ($t \to \infty$) as $\lambda_\infty$. Then the concentration of reactant C is $C(t)=p(\lambda - \lambda_\infty)$ and $C_0=p(\lambda_0 - \lambda_\infty)$ where $p$ is a proportionality constant. As a result

$$\ln\frac{C_0}{C} = kt$$ becomes

$$\ln\left(\frac{\lambda_0 - \lambda_\infty}{\lambda - \lambda_\infty}\right) = kt$$
Kinetic Studies of Lysine Acyl Transfer

Development of the Spectrophotometric Assay

The rate of lysine acyl transfer observed in the acyl transfer titrations indicated that the reaction was much too fast to be followed by a discontinuous assay, in which aliquots were taken from the acyl transfer reaction, quenched and analyzed by HPLC. A new method by which the reaction could be continuously monitored was preferable. Personal experience with enzyme kinetic studies had shown the utility of UV monitoring of kinetic reactions. To utilize such a method, a wavelength which showed the difference between the thiol capture material and acyl transfer product was needed. Figure II-10 shows the UV spectra of dibenzofuran in the model thiol capture and acyl transfer compounds.

![UV spectra of Thiol Capture Material in DMSO](image1)

![UV spectra of Acyl Transfer Product in DMSO](image2)

Figure II-10
The thiol capture material has a sharp UV maximum at 314 nm, due to the presence of the phenolic ester. In the acyl transfer product, this peak shifts to 320 reflecting the free phenol. As a result, the change in the absorbance at 320 could potentially be used to monitor the extent of the lysine acyl transfer product.

It was also necessary to prove that the observed change in absorbance during acyl transfer corresponded to the starting concentration of thiol capture material in the cuvette. The change in molar absorbance expected for the complete conversion of thiol capture material to acyl transfer product was determined from the difference in the extinction coefficients of the thiol capture and acyl transfer models at 320 nm and found to be $3.8 \times 10^{-3} \text{ L mol}^{-1} \text{ cm}^{-1}$.

Kinetics were run with excess DIEA and HPLC purified thiol capture peptide. The starting concentration was determined from the absorbance at 313 nm. At the end of the run, the concentration was separately determined from the observed change in absorbance at 320 nm and compared to the starting concentration. The two results showed less than a 5% difference indicating that the change in absorbance could be used to follow the reaction.

It has been mentioned several times that disulfides formed by disulfide interchange are the major impurity associated with acyl transfer. Although their level can usually be minimized by HPLC purification of the thiol capture material, there are a few cases where this does not suffice. Disulfide interchange results in large errors in the kinetic constants by effecting the amount of product at the infinity point. The advantage of using HPLC as a assay method is that the level of impurities can be observed directly and runs showing high levels of disulfide interchange discarded. This advantage is lost when switching to a spectrophotometric
To use the assay with confidence, no disulfide interchange should occur under the conditions of the kinetic runs. Several kinetic runs for each model were assayed by HPLC at the end point. In all cases, the acyl transfer product peak was greater than 90% of the total peak area and disulfide interchange less than 10%.

Due to the rapid rate of the lysine reactions, it was necessary that the starting point of the reaction be determined accurately. Initiation of the reaction with DIEA while acquiring data made this possible as is indicated below. Figure II-11 shows the change in absorbance over time for a typical lysine acyl transfer kinetic run.

The thiol capture peptide stock solution was placed in a 1 cm cuvette, sealed with a rubber stopper and stirred at 700 revolutions per minute. Prior to the addition of DIEA, absorbance
data were acquired at time intervals corresponding to 0.1 half lives, for 5-10 half lives, yielding a baseline. With the data still being acquired, the stopper was removed, the DIEA added, and the stopper replaced. The total volume change upon the addition of base was 1-2% depending on the volume of base added. This slight dilution produces a small negative spike, (indicated by the arrow in Figure II-11). The presence of this spike allowed the starting point to be determined quite accurately. The relatively large size of the stir bar and the small volume of the cuvette meant that mixing was quite rapid, producing a negligible lag time. For this reason, the negative spike was equated to $t=0$ seconds for the purpose of analysis. The analysis also required that the absorbance at $t=\infty$ be determined. Absorbance data was taken for 20 half lives, until a flat plateau had been reached. The total change in absorbance was determined from the bottom of the negative starting spike to the flat plateau. Like the cysteine kinetics, the results were analyzed by graphing $\ln\left(\frac{A_0 - A_\infty}{A - A_\infty}\right)$ versus $t$.

Finally, as the intrinsic rate of lysine acyl transfer was desired, the amount of DIEA necessary to completely deprotonated the lysine ε ammonium ion had to be determined. From our discussion in Appendix A, we estimated that the lysine amine is more basic than DIEA. As the exact pKa values were unknown however, the concentration of DIEA necessary to achieve Vmax had to be determined experimentally. This was performed by running kinetics with increasing levels of DIEA, until the rate of reaction leveled off. Since the pKa of the lysine amine should not change from model to model, the amount of DIEA necessary to fully deprotonate the amine would remain the same over the entire set of models. Although complete titrations were not performed for each model, enough kinetics data at high base concentration was available to determine at which point added base no longer resulted in
a rate increase. Above 25 equivalents of DIEA, no further change in the lysine acyl transfer rate of a given model, relative to the maximum rate measured for that model, was observed. This is shown graphically in Figure II-12 for a number of the models. The data clearly indicate that above 25 equivalents of DIEA, the lysine amine was fully deprotonated and lysine acyl transfer occurred at maximum velocity.

Figure II-12
Acyl Transfer as a Function of DIEA
Table II-3 lists the average results of the lysine kinetic runs. The results clearly show a distance dependence for the rate of lysine acyl transfer. The fastest acyl transfer occurred with the CAK peptides and decreased as the number of intervening alanines increased, indicating a decrease in the proximity of the lysine ε amine to the phenolic ester. The exception to this trend was the CK acyl transfer peptide which, although the closest, had an acyl transfer rate comparable to that of CA3K. The anomalous result is most likely due to an average conformation that places the amine in an unfavorable position relative to the ester or to strain in the cyclic transition state. The distance dependence is discussed further in the section presenting the results of the effective molarity determinations. In all cases the rate of acyl transfer to lysine was significantly faster than to cysteine, ranging from 500 times for CAK to 20 times for CA3K. It is clear from these results that the apparent lack of distance dependence to lysine acyl transfer observed in the acyl transfer titrations was merely an artifact of the extremely high rate of reaction. Even with five intervening alanines, the rate of lysine acyl transfer was fast enough to compete successfully for the glycine ester.

Although the kinetic equations presented above showed that product ratios could be calculated from a ratio of rate constants, a cautionary note must be sounded concerning the calculation of cysteine:lysine acyl transfer product ratios from the kinetic studies and their comparison with the product ratios observed in the acyl transfer titrations. The kinetic studies presented below were run under conditions of maximal velocity with
The results of the lysine acyl transfer kinetic runs were also consistent with the observation of anomalous levels of acyl transfer to lysine with less than 1 equivalent of DIEA. The acyl transfer to lysine observed with the CAK model is the fastest seen for any of the peptides. It is likely therefore that the high level of lysine acyl transfer observed at low DIEA concentrations merely reflects its unusual reactivity and not a significantly different level of free amine.

![Graph showing change in lysine acyl transfer rate constant with intervening alanines between cysteine and lysine.](image)

\[ y = -1.2343 - 0.85291x \]
\[ R^2 = 0.995 \]

both cysteine and lysine amines fully deprotonated. In the acyl transfer titrations however, while enough DIEA was used to ensure that cysteine was completely deprotonated, the higher pKa of lysine makes it likely that a significant proportion of the lysine amine was still protonated. The kinetic relationships presented above show that for reactions involving acid base equilibria, the observed rate depends on both the intrinsic rate and the concentration of reacting species. The rate of lysine acyl transfer in the titrations therefore, was slower (relative to that of cysteine) than would be expected from a comparison of the rates measured at maximal velocity. As a result, the proportion of cysteine acyl transfer observed in the titrations was higher than would be predicted from a comparison of the rate constants measured under conditions of maximal velocity. Product ratio's determined from kinetic constants would represent the end point of the acyl transfer titration where enough DIEA had been added to ensure that both cysteine and lysine were fully deprotonated.
Variation in the Rate Constant for Acyl Transfer to Lysine with the Number of Alanines between Cysteine and Lysine.

From Graph
\[ \ln(\text{rate constant in units of s}^{-1}) = -1.2343 - 0.85291(\text{number of Alanines}) \]

1) How many Alanines must be present before there is 95% acyl transfer to Cysteine?

\[ \frac{[\text{Acyl Transfer to Cysteine}]}{[\text{Acyl Transfer to Lysine}]} = \frac{\text{rate constant for acyl transfer to Cysteine}}{\text{rate constant for acyl transfer to Lysine}} \]

\[ [95] = \text{rate constant for Cysteine} \]

[ 5 ] rate constant for Lysine

Calculate the Lysine rate constant based on Cysteine rate constant and then calculate the number of Alanines from eqn.

<table>
<thead>
<tr>
<th>Half Life</th>
<th>Rate Constant Cys</th>
<th>Rate Constant Lys</th>
<th># of Alanines</th>
</tr>
</thead>
<tbody>
<tr>
<td>52 min</td>
<td>2.23 x 10^{-4} s^{-1}</td>
<td>1.2 x 10^{-5} s^{-1}</td>
<td>11.9</td>
</tr>
</tbody>
</table>

So there must be approximately 12 alanines separating Cysteine and Lysine before there is 95% acyl transfer to Cysteine.

Even if the two outside values for the cysteine acyl transfer rate constant are used in this calculation the results change very little.

<table>
<thead>
<tr>
<th>Half Life</th>
<th>Rate Constant Cys</th>
<th>Rate Constant Lys</th>
<th># of Alanines</th>
</tr>
</thead>
<tbody>
<tr>
<td>63 min</td>
<td>1.78 x 10^{-4} s^{-1}</td>
<td>9.4 x 10^{-6} s^{-1}</td>
<td>12.1</td>
</tr>
<tr>
<td>44 min</td>
<td>2.71 x 10^{-4} s^{-1}</td>
<td>1.4 x 10^{-5} s^{-1}</td>
<td>11.6</td>
</tr>
</tbody>
</table>

2) What is the expected percent of Acyl Transfer to Lysine at position 46 in the presence of a Lysine at position 41 in the synthesis of BPTI?

Lysine at position 41 - 2 intervening residues
Lysine at position 46 - 7 intervening residues

rate constant for acyl transfer of Lysine at position 41 is the same as the Ala 2 case, avg = 5.56 x 10^{-2} s^{-1}

Calculate the rate constant for Lysine at position 46 = 7.43 x 10^{-4} s^{-1}

\[ \frac{[\text{Acyl Transfer to Lys} 41]}{[\text{Acyl Transfer to Lys} 46]} = \frac{\text{rate constant for acyl transfer to Lys 41}}{\text{rate constant for acyl transfer to Lysine 46}} \]

\[ [\text{Acyl Transfer to Lys} 41] = 5.56 x 10^{-2} s^{-1} \]

\[ [\text{Acyl Transfer to Lys} 46] = 7.43 x 10^{-4} s^{-1} \]

\[ [\text{Lys} 41]:[\text{Lys} 46] = 75:1 \]

Acyl transfer to lysine 46 would be 1.3% of the acyl transfer to lysine with the result that acyl Transfer to Lys 46 would not be likely to be observed by HPLC.
We are particularly interested in how far lysine must be from the phenolic ester before lysine acyl transfer no longer presents a problem. Using the rate constants for cysteine and lysine acyl transfer this can easily be calculated. Figure II-13 presents a graph of the natural log of the lysine rate constant as a function of the number of alanines between cysteine and lysine. Calculations, (presented in Figure II-14) from this graph indicate that 12 intervening alanines must be present before 95% acyl transfer to cysteine is observed at high pH. As the calculations are based on the rate constants at maximal velocity for both cysteine and lysine, this value really represents a maximum value. Above 12 amino acids, the cysteine acyl transfer product will essentially be the only product no matter how much DIEA is added. As the amount of DIEA necessary to fully deprotonate lysine is so much greater than that needed for cysteine however, it is likely that under standard conditions fewer than 12 amino acids would be necessary to ensure the primary product results from acyl transfer to cysteine.

Using the same method, the point at which the rate of acyl transfer to cysteine and lysine would be equal can be calculated (calculations not shown in Figure 14). The results indicate that under conditions of maximum velocity, acyl transfer to cysteine across a 12 membered ring would occur at the same rate as acyl transfer to lysine across a 43 membered ring!

The relationship between the rate of lysine acyl transfer and number of amino acids in the spacer can also be used to determine why no acyl transfer was observed to lysine 46 in the original BPTI case. As both lysines are likely to have the same pKa, the ratio of rate constants predicts the ratio of products no matter how much DIEA is present, as the fraction of reacting species is the same for both. As can be seen from the calculation in Figure II-14,

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9 For the construction of this graph all the rate constants were used rather than just the average values.
approximately 1.3% acyl transfer to lysine 46 should have occurred. This level is so small that it is likely that the peak produced was overlooked in the original HPLC monitoring of the reaction.

**Effective Molarity of the ε Amine of Lysine in The Model Peptides**

Confirmation that the source of lysine’s high reactivity was not caused by a high concentration of the amine relative to the phenolic ester required a method for determining their proximity. Although computational methods based on the conformational statistics of polymers exist for calculating the distance between the ends of a polymer (discussed in Appendix B), these methods show poor correlation with experimentally measured values for short polymers. Because of the small size of the thiol capture model peptides, the proximity of the reactive groups had to be determined experimentally, by measuring the effective molarity of the lysine ε amine. Effective molarity is the ratio of the rate constant of an intramolecular reaction to the rate constant for a model bimolecular reaction with the same mechanism:

\[
\frac{k_{\text{intra}}}{k_{\text{bimolecular}}} = \frac{s^{-1}}{M^{-1}s^{-1}} = EM
\]

 eqn. II-11

The quotient can be thought of as the concentration of a neighboring group relative to the reactive center. If the effective molarity of the lysine amine was significantly less than the EM of the cysteine amine (5 M), the source of lysine’s high reactivity would have to be attributed to a greater inherent nucleophilicity of the ε amine, rather than a favorable proximity to the lysine amine.

The choice of the proper model reaction is crucial to the accuracy of the results. The reaction chosen was the nucleophilic attack of the ester of Z-Gly-O-Dbf by the ε amine of
Z-Lys-benzylamide. The reactions were run under pseudo-first order conditions and monitored by the same spectrophotometric assay used for the lysine kinetics above. In addition to the kinetic runs, controls were performed with Z-Gly-O-Dbf and either DIEA or Z-Lys-benzylamide alone to show that the observed rate for these reactions was negligible. From the results of the kinetic runs, a rate constant $k_{\text{bimolecular}} = 0.12 \text{ M}^{-1}\text{s}^{-1}$ was calculated for the reaction.

Dividing the rate constants listed in Table II-3 by the bimolecular rate constant yielded the effective molarities for the ε amine in the model peptides, listed in Table II-4.

<table>
<thead>
<tr>
<th>Thiol Capture Material</th>
<th>Concentration</th>
<th>Average Rate Constant</th>
<th>EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCK</td>
<td>$10^{-5}$ M</td>
<td>$3.1 \times 10^{-2}\text{s}^{-1}$</td>
<td>0.25 M</td>
</tr>
<tr>
<td>ZCAK</td>
<td>$10^{-5}$ M</td>
<td>$0.1142\text{s}^{-1}$</td>
<td>0.92 M</td>
</tr>
<tr>
<td>ZCA$_2$K</td>
<td>$10^{-5}$ M</td>
<td>$5.6 \times 10^{-2}\text{s}^{-1}$</td>
<td>0.46 M</td>
</tr>
<tr>
<td>ZCA$_3$K</td>
<td>$10^{-6}$ M</td>
<td>$2.4 \times 10^{-2}\text{s}^{-1}$</td>
<td>0.20 M</td>
</tr>
<tr>
<td>ZCA$_4$K</td>
<td>$10^{-5}$ M</td>
<td>$9.1 \times 10^{-3}\text{s}^{-1}$</td>
<td>0.076 M</td>
</tr>
<tr>
<td>ZCA$_5$K</td>
<td>$10^{-5}$ M</td>
<td>$4.1 \times 10^{-3}\text{s}^{-1}$</td>
<td>0.034 M</td>
</tr>
</tbody>
</table>

As expected, the EM values show the same relationship to the number of alanines in the spacer as the intramolecular rate constants. The observed decrease in the lysine effective molarity from ZCAK to ZCA$_3$K resembles the trend observed in Appendix B associated with an increase in the number of rotatable bonds in a linear molecule. The net result of increasing the number of alanines is that the conformational space and conformational entropy of the thiol capture peptide increases as well. In the cyclic transition state of the acyl transfer, bond rotation is lost, along with much of the conformational entropy$^{10}$. The formation of the acyl transfer product is disfavored by the magnitude of the conformational entropy lost, which in

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$^{10}$ The flexibility of large rings partially compensates for the effects of ring closure by allowing partial rotation around bonds.
turn depends on the number of rotatable bonds and hence the number of alanines in the spacer. As the number of alanines in the spacer increases, the rate of the lysine acyl transfer will decrease, resulting in lower effective molarities.

The exception to trend observed above is the ZCK peptide, which has a significantly lower EM than ZCAK, despite the fact that it has fewer rotatable bonds. In this case the effect resembles that observed for the cyclization of small rings. The slower rate can be attributed to an unfavorable configuration that must be assumed for ring closure. Although, the 19 membered ring formed in the transition state is far larger than the strained lactone rings described in Appendix B, the trans-planar amide bonds could be expected to change the ring size needed to form an unstrained ring. The effect could be considered analogous to that of introducing trans double bonds into cycloalkanes. Furthermore, the planar amide bonds would act to prevent the relief of strain by partial rotation around bonds. Despite its large size therefore, ring strain could be associated with the 19 membered ring formed in the transition state of ZCK lysine acyl transfer.

It must be stressed that these explanations are conjectural and are based on similarities observed for other cyclization reactions. To determine the true source of the change in effective molarity observed for the models, the change in enthalpy and entropy of the lysine acyl transfer must be determined as a function of distance.
Regardless of the position of the lysine amine along the peptide chain, the effective molarity was significantly lower than the EM of 5 M observed for cysteine. From these results, therefore, it can be definitively stated that the exceptional reactivity of the lysine ε amine towards the phenolic ester of dibenzofuran is not due to a conformation that places it closer than the α amine of cysteine. Rather, the reactivity is due to a greater inherent nucleophilicity as predicted by its significantly higher pKa.

**Summary**

In this chapter acyl transfer with DIEA was investigated. The cysteine:lysine acyl transfer ratio was found to be quite sensitive to the number of equivalents of DIEA added to the reaction. This sensitivity indicated that minimizing the lysine acyl transfer by careful control of peptide:DIEA stoichiometry was not the best solution and that alternative methods should be sought.

The individual rates of cysteine and lysine acyl transfer were also determined. The change in the rate of lysine acyl transfer indicated that a separation between cysteine and lysine corresponding to 12 amino acids would result in 95% acyl transfer to cysteine regardless of the amount of base added to the reaction. Determination of the effective molarity of the lysine amines indicated that the source of their high reactivity is not due to a conformation that placed the ε amine closer than the cysteine α amine to the phenolic ester but is due to the higher intrinsic nucleophilicity of the lysine amine.
Chapter 3 - Cysteine and Lysine Acyl Transfer With Sodium Dichloroacetate
Introduction

In Chapter 2, our attempts at controlling acyl transfer to lysine by careful control of added DIEA were presented. Studies of the dependence of the cysteine: lysine acyl transfer ratio on the molar ratio of added base showed that the ratio was quite sensitive to the amount of DIEA added to the reaction. Addition of exactly 1 equivalent resulted in 90% acyl transfer to cysteine, but with 1.5 equivalents the level of cysteine acyl transfer dropped to 50-60%. Successful application of this method required the ability to determine the molar quantity of peptide and excess TFA accurately. The aim of the acyl transfer by prior thiol capture method is the synthesis of large proteins, so that methods of quantitation had to be quite sensitive. The high molecular weights of proteins mean that milligram quantities of a peptide can represent a vanishingly small number of moles. The extinction coefficient of dibenzofuran linker was large enough to enable the quantitation of small amounts of thiol capture peptide enabling accurate determination of concentrations as dilute as $1 \times 10^{-5}$ M.

A process for rapid and easy quantitation of excess TFA proved elusive. Attempts were made to determine the amount of excess TFA by $^{19}$F NMR. As the sensitivity of the technique was low, hundreds of scans for each measurement were required, making the technique time consuming to use. Although concentrated samples of the models used in this study could be made, for large proteins solubility, even in DMSO, would limit the concentration. It was clear that careful control of the molar ratio of peptide and DIEA was not the best solution for suppressing lysine acyl transfer, and an alternative had to be sought.
Criteria for Choosing Alternative Bases

The obvious alternative to precise control of the acid base ratio is the use of a buffering additive that discriminates between deprotonation of the lysine and cysteine ammonium ions. The high dielectric constant of water and its capacity for strongly solvating both cations and anions allows aqueous proton transfer reactions to be modeled very accurately by simple mass action expressions. As a result, buffering in water is so easily achieved that its application to a particular case of proton transfer is usually relegated to the experimental section of main stream chemical publications. Unfortunately, proton transfer processes in less polar solvents are usually more complex, less well understood and poorly controllable. For these cases, effective buffering can be difficult to achieve.

As was noted in the introduction to this thesis, owing to its unique capacity to facilitate the $\text{O, N acyl transfer step}$, DMSO is the obligatory solvent for this reaction. Though not well recognized by the broader chemical community, proton transfer reactions in DMSO have an extensive literature, and DMSO appears to be unique among the dipolar aprotic solvents in rivaling water for the predictability and simplicity of its proton transfer equilibria. The part of the literature that is directly pertinent to this thesis is analyzed in Appendix A, where it is shown that pKa values for a wide variety of organic acids and bases have been measured in DMSO. The data presented there in also indicate that stabilization of oxyanions through homo and heteroconjugation, which complicates both buffer design and the interpretation of pKa behavior in most aprotic solvents, is relatively unimportant in DMSO at the low concentrations generally used for acyl transfer. As is evident from the previous chapters, the conjugate acids most pertinent to this thesis are the cysteine and lysine
ammonium ions. As discussed in Appendix A, the pKa (in DMSO) of the \( \alpha \) ammonium ion of cysteine is lower than the \( \varepsilon \) ammonium of lysine (estimated as 6.9±0.7 and 10.4 ±0.7, respectively). Selective deprotonation of the \( \alpha \) ammonium ion of cysteine might be possible by choosing a base whose conjugate acid had the same pKa. The difference of three orders of magnitude between the acidity of cysteine and lysine ammonium ions means the \( \alpha \) ammonium ion of cysteine could be deprotonated completely in the presence of a fully protonated \( \varepsilon \) amine of lysine.

Acyl transfers are run at high dilution to control competing bimolecular reactions, notably disulfide interchange. Any base chosen would have to be able to work efficiently under these conditions while retaining its selectivity for the cysteine \( \alpha \) ammonium ion. Potential bases would have to be evaluated for their compatibility with the chemistry and techniques of acyl transfer. As a number of equivalents might be necessary for deprotonation of the cysteine amine in thiol capture materials with acidic groups in the side chain, the base should be UV invisible so as not to interfere with HPLC monitoring of the acyl transfer.

Evidence presented in Chapter 2 showed that regardless of conditions of high dilution and the presence of AgNO\(_3\), under certain conditions, disulfide interchange seemed to be catalyzed by excess DIEA. Purification by preparative HPLC decreased the level of disulfide interchange, but this was a long process requiring a multiple injections due to low solubility of the thiol capture material in HPLC solvents. Avoidance of this tedious step would greatly increase the convenience of the thiol capture methodology. Potential bases could also be
evaluated for their ability to suppress disulfide interchange, with the hope that one of the bases successful in suppressing lysine acyl transfer would also limit this side reaction.

**Initial Screening of Alternative Bases**

As our estimate of the cysteine α ammonium pKa in DMSO had yielded a range of 6.9 ± 0.7, bases whose conjugate acid pKa's bracketed this region had to be tested. The existence in the literature of a large body of measured pKa's in DMSO greatly simplified the choice of bases for initial screening. Four bases were initially chosen - sodium salycilate and the sodium salts of mono, di, and trichloroacetic acid. The pKa values in DMSO are listed in Table III-1.

<table>
<thead>
<tr>
<th>Base</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroacetic acid</td>
<td>8.2*</td>
</tr>
<tr>
<td>Dichloroacetic acid</td>
<td>6.4</td>
</tr>
<tr>
<td>Trichloroacetic acid</td>
<td>4.6*</td>
</tr>
<tr>
<td>Salicylic Acid</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*estimated from pKa values of acetic, chloroacetic, and dichloroacetic acid in DMF and the relationship between pKa's in DMF and DMSO

The bases were evaluated by the acyl transfer titration method described in Chapter 2 - CK\(^1\) thiol capture material (10\(^{-4}\) M in DMSO) containing both cysteine and lysine present as TFA salts was allowed to undergo acyl transfer in the presence of 1, 3 and 5 equivalents of the test base. After 24 hours, the cysteine lysine acyl transfer product ratio was evaluated by HPLC. The results of the initial screening are presented in Figure III-1. Sodium chloroacetate was too strong a base, showing a relationship between acyl transfer to lysine and equivalents of added base similar to that observed for DIEA. Sodium trichloroacetate appeared to undergo a side

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\(^1\) For convenience, thiol capture materials and acyl transfer products are referred to by the sequence of the cysteine containing fragment as the peptide linked by the dibenzofuran ester always has the same sequence Z-A-F-G.
reaction, suggested by the appearance of HPLC peaks with retention times that did not correspond to those expected for acyl transfer to either cysteine or lysine. Both sodium dichloroacetate and sodium salicylate however, showed no tendency to promote acyl transfer to lysine. In both cases, greater than 90% acyl transfer to cysteine (normalized) was observed, even in the presence of 5 equivalents of the bases.

**Figure III-1: Acyl Transfer With Alternative Bases**

Examination of the HPLC traces showed significant differences in the level of disulfide interchange as well. Figure III-2 presents the level of disulfide interchange as a function of the equivalents of added base for sodium mono and dichloroacetate and sodium salicylate. Data from acyl transfers of the same material, in the presence of varying equivalents of DIEA, is
presented for the purpose of comparison. Once again, sodium chloroacetate appeared to
behave much in the same way as DIEA, with the level of disulfide interchange increasing in
proportion to the amount of base added. By contrast, the levels of disulfide interchange
observed with both sodium dichloroacetate and sodium salicylate were significantly lower and
appeared to be independent of the amount of base present.

![Figure III-2: Disulfide Interchange Produced by Different Bases](image)

Both sodium salicylate and sodium dichloroacetate appeared to be successful in limiting
disulfide interchange and acyl transfer to lysine. As mentioned above, it was preferable that the
base be UV invisible, as HPLC was used to monitor the progress of the acyl transfer reaction.
While sodium dichloroacetate has no absorbance in the 260-320 nm range, this is not the case
for sodium salicylate, and as a result it was eliminated from further testing. All subsequent tests were performed using sodium dichloroacetate as the base.

**Acyt Transfer At High Concentration**

Disulfide interchange is a bimolecular reaction involving nucleophilic attack on a disulfide by the thiolate anion. An extensive study on disulfide interchange in thiol capture materials, performed by Dr. Fotouhi, showed that disulfide interchange could be controlled by running the acyl transfer reactions at high dilution (10^{-4} M). The dilute conditions however, mean that large amounts of DMSO were required for even moderate scale acyl transfers. Recovery of the acyl transfer product involved long evaporation steps at elevated temperatures, due to the high boiling point of DMSO (45°C at 0.1 mm Hg - evaporation occurs at the rate of 100-150 mL/hour).

The decrease in the level of disulfide interchange associated with using sodium dichloroacetate as a base immediately suggested the possibility of performing acyl transfers at significantly higher concentrations. Figures III-3 and III-4 present the results of acyl transfers performed at 10^{-2} and 10^{-3} M. Acyl transfers performed with DIEA as a base are presented for comparison. As with the dilute acyl transfers performed earlier, the primary reaction was cysteine acyl transfer (> 90%, normalized, Figure III-3). The levels of disulfide interchange observed in the concentrated reactions were less than 13 %, even in the presence of 5 equivalents of sodium dichloroacetate, comparing quite favorably with the levels observed for acyl transfers run at 10^{-4} M in the presence of DIEA, using thiol capture material that had been scrupulously purified by preparative HPLC (Figure III-4).
Figure III-3: Acyl Transfer at High Concentrations

Figure III-4: Disulfide Interchange Produced by Different Bases
The ability to increase the concentration 100 fold with no loss in purity greatly simplified work up of the acyl transfer products, as most reactions could be purified without evaporation by direct injection on the preparative HPLC. Using this method an acyl transfer of the CAAK thiol capture material (5.3 mg, 3.9x10^{-3} mmol) was run in 200 μL DMSO with 5 equivalents sodium dichloroacetate. The same reaction under the old conditions would have required 25 mL DMSO. After acyl transfer, the reaction was diluted with 800 μl of 0.1 % TFA and injected directly on the preparative HPLC. The yield of cysteine acyl transfer product after purification was 74% (3.9 mg, 2.9 x10^{-3} mmol) which compares quite favorably with the theoretical maximum (77% calculated by including 10 % acyl transfer to lysine and 13 % disulfide interchange). Further investigation of the ability of sodium dichloroacetate to suppress disulfide interchange is presented later in this chapter along with possible explanations based on the disulfide interchange mechanism.

**NMR Studies of the Deprotonation of Cysteine and Lysine by Sodium Dichloroacetate**

As discussed above, the observed cysteine:lysine product ratio was most likely due to the ability of sodium dichloroacetate to differentiate between the ammonium ions of cysteine and lysine. The degree to which the cysteine and lysine ammonium ions are deprotonated can be directly determined from NMR studies. The ^1^H NMR chemical shift of C-H functions α to an ammonium ions is shifted downfield relative to its chemical shift in the presence of the free amines, due to the deshielding effect of the adjacent positive charge. By determining the C-H chemical shift in the presence of a fully protonated and free amine, as well as in the presence
of a given amount of base, the fraction of free amine caused by the amount of base, $[\text{NH}_2]/A_0$, can be calculated from equation III-1:

$$\left[\text{NH}_2\right] = \delta \text{ C-H of fully protonated amine} - \delta \text{ C-H in the presence of x equivalents of base} \quad \text{eqn. III-1}$$

$$A_0 = \delta \text{ C-H of fully protonated amine} - \delta \text{ C-H of fully deprotonated amine}$$

where $A_0$ represents the starting concentration of protonated amine before the addition of base.

Table III-2 - $^1$H NMR Studies of the Deprotonation of Cysteine and Lysine Ammonium Ions With Sodium Dichloroacetate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Equivalents Sodium Dichloroacetate</th>
<th>Chemical Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl*Cys(S-Benzyl)OMe</td>
<td>0</td>
<td>4.319</td>
</tr>
<tr>
<td>HCl*Cys(S-Benzyl)OMe</td>
<td>1</td>
<td>4.204</td>
</tr>
<tr>
<td>HCl*Cys(S-Benzyl)OMe</td>
<td>3</td>
<td>4.135</td>
</tr>
<tr>
<td>HCl*Cys(S-Benzyl)OMe</td>
<td>5</td>
<td>4.113</td>
</tr>
<tr>
<td>HCl*Cys(S-Benzyl)OMe</td>
<td>10</td>
<td>4.077</td>
</tr>
<tr>
<td>HCl*Cys(S-Benzyl)OMe NaHCO$_3$</td>
<td></td>
<td>3.502*</td>
</tr>
<tr>
<td>AcLys(eNH$_2$*HCl)OMe</td>
<td>0</td>
<td>2.745</td>
</tr>
<tr>
<td>AcLys(eNH$_2$*HCl)OMe</td>
<td>1</td>
<td>2.745</td>
</tr>
<tr>
<td>AcLys(eNH$_2$*HCl)OMe</td>
<td>3</td>
<td>2.745</td>
</tr>
<tr>
<td>AcLys(eNH$_2$*HCl)OMe</td>
<td>5</td>
<td>2.745</td>
</tr>
</tbody>
</table>

*Chemical shift of fully deprotonated ammonium ion

Figure III-5 and III-6 show the results of titrations of DMSO d6 solutions (0.016 M) of HCl salts of H-Cys(S-benzyl)-OMe and Ac-Lys-OMe, with sodium dichloroacetate. No perceptible change in the lysine C-H chemical shift was observed over the entire range of base concentrations measured. The chemical shift of the cysteine C-H however showed a significant change. Application of equation III-1 to the data shows that the ammonium ion is 14% deprotonated in the presence of 1 equivalent of sodium dichloroacetate, increasing to 30% in the presence of 10 equivalents (results listed in Table III-2). The results support the assertion

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2 This method assumes that the change in $[\text{NH}_2]/A_0$ is linear with the change in chemical shift.
NMR of HCl*Cys(S-Benzyl)OMe in DMSO
In the Presence of Sodium Dichloroacetate

Chemical shift of the alpha hydrogen in the presence of a fully deprotonated amine.

Equivalents of Added Sodium Dichloroacetate

Figure III-5

NMR of AcLys(HCl)OMe in DMSO
In the Presence of Sodium Dichloroacetate

Chemical Shift of the epsilon CH2 Protons of Lysine

Equivalents of Added Sodium Dichloroacetate

Figure III-6
that the source of the lysine acyl transfer suppression lies in the selective deprotonation of the cysteine α ammonium ion.

**Calculation of the pKa of the Cysteine α Ammonium Ion From the NMR Data by The Application of Mass Action Relationships**

The relative pKa of the protonated amine of cysteine can be calculated from the NMR data in conjunction with the literature value for the pKa of dichloroacetic acid by using mass action relationships. The derivation of the mass action relationships are presented in Scheme III-1.

**Scheme III-1**

\[
\begin{align*}
\text{Mass Action Expressions} \\
-\text{NH}_3^+ & \xrightarrow{k_1} -\text{NH}_2^- + H^+ \quad \text{eqn. III-2} \\
-\text{CO}_2\text{H}^- & \xrightarrow{k_2} -\text{CO}_2^- + H^+ \quad \text{eqn. III-3}
\end{align*}
\]

\[
\begin{align*}
[-\text{NH}_3^+] + [\text{NH}_2^-] &= A_0 \quad \text{eqn. III-4} \\
[\text{CO}_2\text{H}] + [\text{CO}_2^-] &= B_0 \quad \text{eqn. III-5} \\
-\text{NH}_3^+ + H^+ &= \left(\left[-\text{CO}_2^-\right] - \left[\text{Na}^+\right]\right) + \text{TFA}^- \quad \text{eqn. III-6}
\end{align*}
\]
We want $[\text{NH}_2]/A_0$ as a function of $A_0$

$$k_1 = \frac{[-\text{NH}_2][\text{H}^+]}{[-\text{NH}_3^+]}$$  \hspace{1cm} \text{eqn. III - 7}

$$k_1[-\text{NH}_3^+] = [-\text{NH}_2][\text{H}^+]$$

$$+ k_1[-\text{NH}_3^+] + k_1[-\text{NH}_3^+]$$

$$k_1A_0 = [-\text{NH}_2](\text{[H}^+] + k_1)$$  \hspace{1cm} \text{eqn. III - 8}

$$\frac{[-\text{NH}_2]}{A_0} = \frac{k_1}{(\text{[H}^+] + k_1)}$$  \hspace{1cm} \text{eqn. III - 9}

in the same way

$$\frac{[-\text{CO}_2^-]}{B_0} = \frac{k_2}{(\text{[H}^+] + k_2)}$$  \hspace{1cm} \text{eqn. III - 10}

$$\text{H}^+ = [-\text{CO}_2^-] - [\text{Na}^+] + [\text{TFA}^-] - [-\text{NH}_3^+]$$  \hspace{1cm} \text{eqn. III - 11}

Let us consider the case where $A_0 = B_0$

$$[\text{Na}^+] = [\text{TFA}^-]$$

Then $\text{H}^+ = [-\text{CO}_2^-] - [-\text{NH}_3^+]$  \hspace{1cm} \text{eqn. III - 12}

$$\text{H}^+ = A_0 \left( \frac{k_2}{(\text{[H}^+] + k_2)} \right) - A_0 \left( \frac{\text{H}^+}{(\text{[H}^+] + k_1)} \right)$$  \hspace{1cm} \text{eqn. III - 13}

$$\text{H}^+ = (\text{[H}^+] + k_2)(\text{[H}^+] + k_1) = A_0k_2(\text{[H}^+] + k_2) - A_0\text{H}^+(\text{[H}^+] + k_1)$$  \hspace{1cm} \text{eqn. III - 14}

$$\text{H}^+ = \text{H}^+ (k_1 + k_2 + A_0) + \text{H}^+(k_1k_2) - A_0k_1k_2$$  \hspace{1cm} \text{eqn. III - 15}
$A_0$ is the starting concentration of the amine salt and $k_2$ is the dissociation constant for dichloroacetic acid. Values of $k_1$, the dissociation constant for the cysteine amine, are chosen and equation III-15 is solved for $H^+$. Substitution into equation III-9 yields values for $[\text{NH}_2]/A_0$. The best choice of $k_1$ is the value which gives the closest match to $[\text{NH}_2]/A_0$ determined from the chemical shifts. The results of the calculation show that at $10^{-2} \, \text{M}$, the relative pKa of the cysteine amine is 7.9, which is in good agreement with the range calculated from model studies in Appendix A (6.2 to 7.6).

The NMR measurements were made at relatively high concentration (0.01 M) and it is likely that $[\text{NH}_2]/A_0$ reflects the effects of homo and heteroconjugation. These effects have been observed for acetic acid in DMSO (as discussed in Appendix A) and it is not unreasonable to expect them for dichloroacetic acid also. The mass action expressions used above do not take these effects into account, and it is likely that the predicted pKa of the cysteine ammonium ion is somewhat in error. Deprotonation of the ammonium ion of cysteine by sodium dichloroacetate produces dichloroacetic acid. As discussed in Appendix A, homoconjugation between dichloroacetic acid and its anion would stabilize the anion, decreasing its basicity. Similarly, heteroconjugation between the protonated cysteine amine and the dichloroacetate anion would stabilize both species. The net result of these two processes would be to decrease the relative basicity of sodium dichloroacetate and increase the relative basicity of the cysteine amine, resulting in a predicted pKa that was too high. At low concentrations the pKa of cysteine is likely to be closer to the pKa of dichloroacetic acid.
Acyl Transfers in The Presence of Excess TFA

For the work presented in this thesis, HPLC purification of the thiol capture material was the final step prior to acyl transfer. As discussed in Chapter 2, TFA, present as an HPLC solvent component, is known to tenaciously associate with peptides and could have been present in stoichiometric excess. Neutralization of any excess TFA was necessary for acyl transfers to proceed to completion, so it was necessary to test the buffering capacity of sodium dichloroacetate in the presence of excess TFA. Acyl transfers were performed in the presence of 10 equivalents of sodium dichloroacetate, with increasing amounts of excess TFA. The reactions were allowed to run for 24 hours, and the extent of the acyl transfer reaction was determined by HPLC. Figure III-7 presents the results.
While sodium dichloroacetate was capable of effectively scavenging 5 equivalents of excess TFA at $10^{-4}$ M, at $10^{-2}$ M only 1 equivalent could be tolerated without a decrease in the yield of acyl transfer product. NMR titrations at $10^{-2}$ M in the presence of 10 equivalents sodium dichloroacetate showed that the addition of 1 equivalent of TFA resulted in a decrease in deprotonation from 30% to 10%. At 5 equivalents excess TFA, the observed chemical shift of the C-H function $\alpha$ to the cysteine ammonium ion was identical to that of the HCl salt in the absence of any base. The results clearly showed that sodium dichloroacetate is incapable of tolerating large excesses of acid at high concentrations.

The methods involved in preparing the thiol capture material for the acyl transfers presented in this thesis, make it less likely that TFA would ever be present in great stoichiometric excess. After HPLC purification, the solvents were removed in vacuo and the residue triturated with ether. The resulting white solid was then subjected to multiple cycles of sonication in ether followed by centrifugation. Finally the powder was dried overnight under high vacuum. The claim that these manipulations were effective in removing most of the contaminating TFA is supported by the observation that all of the acyl transfers performed at $10^{-2}$ M with sodium dichloroacetate went to completion. In practical synthesis therefore, it is unlikely that contaminating TFA would cause a problem.

**The Kinetics of Cysteine and Lysine Acyl Transfer with Sodium Dichloroacetate**

So far, discussion of the effects of sodium dichloroacetate has focused on its ability to deprotonate cysteine in the presence of lysine. The degree of acyl transfer to cysteine and lysine observed however, is due to kinetic competition between the cysteine and lysine amines.
As with DIEA in Chapter 2, measurements of the individual rates of acyl transfer to cysteine and lysine in the presence of sodium dichloroacetate was necessary to confirm that the observed product ratios were consistent with the rates.

**Cysteine Acyl Transfer Kinetics**

Figures III-8 through III-11 present kinetics studies of acyl transfer to the CAAK thiol capture material, performed under a variety of conditions and the kinetic constants for the overall formation of acyl transfer products and the rate of acyl transfer to cysteine are listed in Table III-3. Given the product ratio observed with sodium dichloroacetate as a base, it is not surprising that the rate constants for overall acyl transfer and acyl transfer to cysteine were virtually identical.

<table>
<thead>
<tr>
<th>Conc. of Thiol Capture</th>
<th>Equivalents</th>
<th>Equivalents</th>
<th>Overall Rate</th>
<th>Cysteine Acyl Transfer Rate</th>
<th>Half Life of Cysteine Acyl Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sodium</td>
<td>TFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dichloroacetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-2} M</td>
<td>5</td>
<td>0</td>
<td>3.9x10^{-3} min^{-1}</td>
<td>3.6x10^{-3} min^{-1}</td>
<td>190 min</td>
</tr>
<tr>
<td>10^{-2} M</td>
<td>10</td>
<td>5</td>
<td>3.9x10^{-4} min^{-1}</td>
<td>3.8x10^{-4} min^{-1}</td>
<td>30 hours</td>
</tr>
<tr>
<td>10^{-4} M</td>
<td>5</td>
<td>0</td>
<td>1.1x10^{-2} min^{-1}</td>
<td>1.0x10^{-2} min^{-1}</td>
<td>69 min</td>
</tr>
<tr>
<td>10^{-4} M</td>
<td>10</td>
<td>5</td>
<td>2.5x10^{-3} min^{-1}</td>
<td>2.4x10^{-3} min^{-1}</td>
<td>290 min</td>
</tr>
</tbody>
</table>

The results of the kinetic studies show that at 10^{-4} M, the rate of acyl transfer to cysteine in the presence of 5 equivalents sodium dichloroacetate (t_{1/2} = 69 min.) was close to the maximum rate observed with for acyl transfer in the presence of DIEA (t_{1/2} = 52 min.). At 10^{-2} M however, the rate of cysteine acyl transfer was decreased 3.7 fold (t_{1/2} = 194 min).
Figure III-8

5 Equivalents Dichloroacetate
10-2 M Conc.

Thiol Capture Material
△ Acyl Transfer Product
□ Disulfide Interchange

Figure III-9

10 Equivalents Dichloroacetate
5 Equivalents TFA
10-2 M Conc.

Thiol Capture Material
△ Acyl Transfer Product
□ Disulfide Interchange
Figure III-10

5 Equivalents Dichloroacetate
10-4 M Conc.

Thiol Capture Material
Acyl Transfer Product
Disulfide Interchange

Time In Minutes

Figure III-11

10 Equivalents Dichloroacetate
5 Equivalents TFA
10-4 M Conc.

Thiol Capture Material
Acyl Transfer Products
Disulfide Interchange

Time in Minutes
Calculation of $[NH_2]/A_0$ from Kinetic Data

The fraction of free amine, $[NH_2]/A_0$, can be calculated from the ratio of the kinetic constants determined in the presence of 5 equivalents of dichloroacetate to the intrinsic rate constant, $k_3$, measured in Chapter 2. As we have already measured this value by NMR at $10^{-2}$ M, correspondence between the two acts validates the kinetic method. Once the results have been show to be consistent, the method can be extended to measure the extent of deprotonation under conditions that could not be observed by NMR, such as the deprotonation of the cysteine amine by sodium dichloroacetate at $10^{-4}$ M and the deprotonation of the lysine amine. The derivation of the relationship between $[NH_2]/A_0$ and the kinetic constants is presented below. From Chapter 2 we know that the rate of product formation is the product of the intrinsic rate constant and the concentration of reactive species

$$\frac{dP}{dt} = k_3[NH_2] \text{ eqn III-16}$$

At Vmax, the ammonium ion is completely deprotonated so that $[NH_2] = [A_0]$, where $[A_0]$ is the starting concentration of ammonium ion before the addition of base. Therefore:

$$\frac{dP_{Vmax}}{dt} = k_3[A_0] \text{ eqn. III-17}$$

Now let us consider the case of acyl transfer at $10^{-2}$ M in the presence of sodium dichloroacetate. The rate of the reaction is given in equation III-16. The concentration of free amine can be rewritten as the fraction of deprotonation multiplied by the starting concentration of ammonium ion:

$$[NH_2] = \left(\frac{[NH_2]}{A_0}\right)[A_0] \text{ so that } \frac{dP_{sodium\ dichloroacetate}}{dt} = k_3\left(\frac{[NH_2]}{A_0}\right)[A_0] \text{ eqn III-18}$$
The term \( k_3 \left( \frac{[\text{NH}_2]}{A_0} \right) \) can be gathered into a new constant, \( k_{\text{exp}} \) so that

\[
\frac{dP_{\text{sodium dichloroacetate}}}{dt} = k_{\text{exp}} [A_0] \quad \text{eqn. III-19}
\]

The constant \( k_{\text{exp}} \) is the constant measured experimentally for the rate of production of the cysteine acyl transfer product in the presence of sodium dichloroacetate. The ratio of eqn. III-19 and III-17 is:

\[
\frac{dP_{\text{dichloroacetate}}}{dP_{\text{Vmax}}} = \frac{k_{\text{exp}} A_0}{k_3 A_0} = \frac{[\text{NH}_2]}{[\text{NH}_2]} \quad \text{eqn. III-20}
\]

Therefore \([\text{NH}_2]/[A_0]\) equal to the kinetic constant measured with sodium dichloroacetate as a base, \( k_{\text{exp}} \), divided by the kinetic constant measured under Vmax conditions, \( k_3 \)

\[
\frac{k_{\text{exp}}}{k_3} = \frac{[\text{NH}_2]}{A_0} \quad \text{eqn. III-21}
\]

From the NMR experiments we determined that at \( 10^{-2} \text{ M} \) in the presence of 5 equivalents sodium dichloroacetate, \([\text{NH}_2]/A_0\) for the cysteine α ammonium ion was 0.25.

From the relevant kinetic constants for cysteine acyl transfer, listed in Table III-3 and the value of \( 1.34 \times 10^{-2} \text{ min}^{-1} \) for the rate constant for cysteine acyl transfer at Vmax, \([\text{NH}_2]/A_0\) is calculated from equation III-21 to be 0.24. Applying the same calculation to the kinetic data for acyl transfer at \( 10^{-4} \text{ M} \) with 5 equivalents of sodium dichloroacetate yields a value for \([\text{NH}_2]/A_0\) of 0.70.

The calculations show that the deprotonation of the cysteine ammonium ion is significantly greater at \( 10^{-4} \text{ M} \) than at \( 10^{-2} \text{ M} \), under identical conditions. The increased
ionization cannot be explained simply by the effects of dilution on the ionization of weak acids. If we use the pKa derived above (7.9), the percent deprotonation of the cysteine α ammonium ion (in the absence of any added base) would be 0.1% at 10^{-2}\, M and would only increase to 1% at 10^{-4}\, M. If we consider that the actual pKa for the cysteine ammonium ion could be as low as 6, then the deprotonation would be 1% at 10^{-2}\, M, increasing to 10% at 10^{-4}\, M. These values are too small to account for the change in [NH₂]/A₀ calculated above. Homo and heteroconjugation may also be involved, increasing the relative basicity of the cysteine amine and decreasing the basicity of the dichloroacetate anion as mentioned above. These effects are concentration dependent and will be significantly less at 10^{-4}\, M, partially accounting for the greater extent of deprotonation. The homo and heteroconjugation constants have not been measured for dichloroacetic acid or TFA salts in DMSO and as a result, it is difficult to assess the relative contributions of each of these processes to the increase in deprotonation observed with dilution.

**Lysine Acyl Transfer Kinetics**

Table III-4 lists the kinetic constants for lysine acyl transfer in the presence of sodium dichloroacetate using the ZCAnK thiol capture models. The rate of the acyl transfer reactions were measured at 10^{-3}\, M, by the spectrophotometric assay described in Chapter 2, so that the results could be compared directly to rate in the presence of DIEA (constants also listed). Acyl transfers were allowed to run for approximately 30 minutes, after which the end point absorbance was determined by the addition of 10 equivalents of DIEA, causing complete turnover to acyl transfer product within seconds (Figure III-12)
The observed rate of acyl transfer to lysine under Vmax conditions (Chapter 2) ranged from a maximum of 500 to a minimum of 15 times the rate of acyl transfer to cysteine (depending on the number of intervening alanines in the spacer). The velocity of the lysine reaction in the presence of sodium dichloroacetate was 100-200 times slower than the maximal velocities observed with DIEA, with half lives ranging from 11 to 121 minutes depending on the concentration of dichloroacetate. The exact value of the kinetic constants for some of the longer reactions (indicated by * ) is somewhat uncertain, as the reaction were followed for less than 1/3 of a half life. Regardless of this uncertainty, the results are quite clear - sodium dichloroacetate drastically decreases the rate of acyl transfer to lysine while decreasing the rate of acyl transfer to cysteine only slightly.
Table III-4 - Kinetic Constants for Acyl Transfer of ZCA\textsubscript{n}K with Sodium Dichloroacetate and DIEA at 10\textsuperscript{-3} M

Sodium Dichloroacetate

<table>
<thead>
<tr>
<th>Thiol Capture</th>
<th>Sodium Dichloroacetate</th>
<th>Lysine Acyl Transfer Rate Constant</th>
<th>Half Life of Lysine Acyl Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCK</td>
<td>6.8</td>
<td>1.32 x10\textsuperscript{-2} min\textsuperscript{-1}</td>
<td>57 min</td>
</tr>
<tr>
<td>ZCK</td>
<td>13.6</td>
<td>1.86 x10\textsuperscript{-2} min\textsuperscript{-1}</td>
<td>37 min</td>
</tr>
<tr>
<td>ZCAK</td>
<td>10.3</td>
<td>4.04 x10\textsuperscript{-2} min\textsuperscript{-1}</td>
<td>17 min</td>
</tr>
<tr>
<td>ZCAK</td>
<td>18.6</td>
<td>6.0 x10\textsuperscript{-2} min\textsuperscript{-1}</td>
<td>11.5 min</td>
</tr>
<tr>
<td>ZCAAK</td>
<td>7.8</td>
<td>2.22 x10\textsuperscript{-2} min\textsuperscript{-1}</td>
<td>31 min</td>
</tr>
<tr>
<td>ZCAAK</td>
<td>15.4</td>
<td>3.6 x10\textsuperscript{-2} min\textsuperscript{-1}</td>
<td>19 min</td>
</tr>
<tr>
<td>ZCA\textsubscript{3}K</td>
<td>6.3</td>
<td>5.76 x10\textsuperscript{-3} min\textsuperscript{-1}</td>
<td>121 min*</td>
</tr>
<tr>
<td>ZCA\textsubscript{3}K</td>
<td>12.6</td>
<td>1.08 x10\textsuperscript{-2} min\textsuperscript{-1}</td>
<td>62 min</td>
</tr>
<tr>
<td>ZCA\textsubscript{4}K</td>
<td>13.5</td>
<td>7.2 x10\textsuperscript{-3} min\textsuperscript{-1}</td>
<td>96 min*</td>
</tr>
</tbody>
</table>

DIEA

<table>
<thead>
<tr>
<th>Thiol Capture</th>
<th>Lysine Acyl Transfer Rate Constant</th>
<th>Half Life of Lysine Acyl Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCK</td>
<td>3.10 x10\textsuperscript{-2} s\textsuperscript{-1}</td>
<td>22 s</td>
</tr>
<tr>
<td>ZCAK</td>
<td>0.114 s\textsuperscript{-1}</td>
<td>6.1 s</td>
</tr>
<tr>
<td>ZCAAK</td>
<td>5.56 x10\textsuperscript{-2} s\textsuperscript{-1}</td>
<td>12.5 s</td>
</tr>
<tr>
<td>ZCA\textsubscript{3}K</td>
<td>2.37 x10\textsuperscript{-2} s\textsuperscript{-1}</td>
<td>29 s</td>
</tr>
<tr>
<td>ZCA\textsubscript{4}K</td>
<td>9.1 x10\textsuperscript{-3} s\textsuperscript{-1}</td>
<td>76 s</td>
</tr>
</tbody>
</table>

Calculation of [\text{NH}_2]/\text{Ao} for Lysine in the presence of Sodium Dichloroacetate

The drastic decrease in rate is attributed to the decrease in the deprotonation of the \(\varepsilon\) ammonium in the presence of sodium dichloroacetate, relative to DIEA. As was done for cysteine above, [\text{NH}_2]/\text{Ao} can be calculated from the ratio of the lysine acyl transfer rate constants.
Table III-5: Fraction of Lysine ε Amine Deprotonated by Sodium Dichloroacetate - Calculated From Kinetic Constants

<table>
<thead>
<tr>
<th>Thiol Capture Material</th>
<th>Equivalents</th>
<th>[NH₂]/A₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Dichloroacetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZCK</td>
<td>6.8</td>
<td>7.0 x10⁻³</td>
</tr>
<tr>
<td>ZCK</td>
<td>13.6</td>
<td>9.7 x10⁻³</td>
</tr>
<tr>
<td>ZCAK</td>
<td>10.3</td>
<td>5.8 x10⁻³</td>
</tr>
<tr>
<td>ZCAK</td>
<td>18.6</td>
<td>8.6 x10⁻³</td>
</tr>
<tr>
<td>ZCAAK</td>
<td>7.8</td>
<td>6.6 x10⁻³</td>
</tr>
<tr>
<td>ZCAAK</td>
<td>15.4</td>
<td>1.1 x10⁻²</td>
</tr>
<tr>
<td>ZCA₃K</td>
<td>6.3</td>
<td>4.0 x10⁻³</td>
</tr>
<tr>
<td>ZCA₃K</td>
<td>12.6</td>
<td>7.6 x10⁻³</td>
</tr>
<tr>
<td>ZCA₄K</td>
<td>13.5</td>
<td>1.3 x10⁻²</td>
</tr>
</tbody>
</table>

The results (listed in Table III-5) show that [NH₂]/A₀ ranged from 5x10⁻³ to 1x10⁻² (0.5 to 1% deprotonation) even with as many as 19 equivalents of sodium dichloroacetate. The magnitudes of [NH₂]/A₀ are consistent with the insensitivity of the lysine ε CH₂ chemical shift to the amount of added dichloroacetate, as the calculated degree of deprotonation is much too small to observe by NMR. If the pKa of lysine is considered to be independent of position in the polypeptide chain, it should be possible to show a relationship between [NH₂]/A₀ and the amount of sodium dichloroacetate added to the acyl transfer using the data in Table III-5 (Figure III-13). Although a general trend is clear, the graph shows a large degree of scatter, most likely due to uncertainties in the kinetic constants. The DIEA kinetic constants for some of the fastest lysine acyl transfers are likely to be somewhat in error as the measurements were made at the practical limits of the technique. Similarly, some of the slower sodium dichloroacetate reactions were only followed for a fraction of a half-life and are likely to be

3 The fraction of lysine ammonium ion deprotonation of ZCAK with sodium dichloroacetate is not markedly different from the other peptides supporting our assertion that the degree of lysine acyl transfer seen at for CAK in the presence of less than one equivalent DIEA is due to its faster rate.
Relationship of Fraction of Free Amine, [NH2]/Ao, to the Equivalents of Sodium Dichloroacetate Added

\[ y = 3.0290e-3 + 4.4127e-4x \]
\[ R^2 = 0.461 \]

Figure III-13

slightly in error as well. The fraction of deprotonation is the ratio of both kinetic constants and the errors might not cancel out. If these points are ignored however, the fraction of amine deprotonated appears to be a roughly linear function of the amount of sodium dichloroacetate added.

Regardless of the scatter, the magnitude of [NH2]/Ao is much lower for the lysine amine than that observed for the cysteine amine. The next question that must be answered is: Are the levels of lysine deprotonation calculated from the kinetic measurements low enough to account for the observed cysteine:lysine acyl transfer product ratio. This is addressed in the next section.
Prediction of the Acyl Transfer Product Ratio from the Kinetic Constants Measured for the Individual Cysteine and Lysine Acyl Transfers

As stated previously, the observed cysteine lysine ratio is the product of a competition of the cysteine and lysine amines for the glycyl ester. For a single starting material going to multiple products, the observed product ratio at the end of the reaction is equal to the ratio of the rate constants. The cysteine/lysine acyl transfer product ratio has been measured for the CK and CAAK thiol capture materials at both $10^{-2}$ and $10^{-4}$ M, with 5 equivalent sodium dichloroacetate. A comparison of the observed product ratio with that predicted from the kinetics data for the individual cysteine and lysine reactions should indicate whether the suppression of the lysine acyl transfer reaction by dichloroacetate is consistent with changes in the individual rate constants.

The kinetics of lysine acyl transfer with sodium dichloroacetate were measured under conditions that differed from the cysteine kinetics. In order for the product ratio to be calculated, the values of the lysine rate constants at $10^{-2}$ and $10^{-4}$ M, in the presence of 5 equivalents sodium dichloroacetate, have to be extrapolated from the kinetic data. The experimentally determined rate constant depends on both $k_3$, rate constant at $V_{max}$, and $[\text{NH}_2]/A_0$. As we saw for the cysteine ammonium ion, $[\text{NH}_2]/A_0$ can be drastically changed with dilution. Due to the high pKa of lysine (estimated at 10.4 ±0.7) however, dilution in the absence of base should have little effect on degree of deprotonation. If a pKa value as low as 9 is assumed for the protonated $\varepsilon$ amine, in the absence of base $[\text{NH}_2]/A_0$ is only $1 \times 10^{-7}$ ($1 \times 10^{-4}$ %) at $10^{-5}$ M. This value is significantly lower than the lysine deprotonation caused by sodium dichloroacetate (Table III-5). The fraction of free amine will decrease at higher concentrations,
so deprotonation due to dilution alone can be assumed to be essentially zero over the range of interest. Evidence supporting this comes from the studies of acyl transfer in the absence of base (discussed in Chapter 2) which showed that at $10^{-4}$ and $10^{-3}$ M, only the cysteine acyl transfer product was observed. We can assume therefore, that the degree of deprotonation of lysine is only due to the number of equivalents of sodium dichloroacetate present.

What is the extent of deprotonation of the lysine ε amines of ZCK and ZCAAK in the presence of 5 equivalents of sodium dichloroacetate? Extrapolation from their data in Table III-5 leads to values of $[\text{NH}_2]/A_0$ of $6.4 \times 10^{-3}$ and $5.1 \times 10^{-3}$ for CK and for CAAK respectively, which are in good agreement with the value of $5.2 \times 10^{-3}$, obtained by linear regression of the entire data set from Table III-5 (Figure III-13). The corresponding rate constants for lysine acyl transfer with 5 equivalents sodium dichloroacetate are $1.2 \times 10^{-2}$ min$^{-1}$ (ZCK) and $1.7 \times 10^{-2}$ min$^{-1}$ (ZCAAK).

From Chapter 2 we know that the ratio of products is the ratio of the individual rate constants determined under the same conditions (equation III-23). The results from the application of to the rate constants for cysteine and lysine acyl transfer are listed in Table III-6 along with the experimentally derived cysteine/lysine product ratios.

\[
\frac{\text{Acyl Transfer to Cysteine}}{\text{Acyl Transfer to Lysine}} = \frac{\text{rate constant for acyl transfer to Cysteine}}{\text{rate constant for acyl transfer to Lysine}} \quad \text{eqn III-23}
\]

---

4 This extrapolation assumes that for very small changes in deprotonation, the shape of the titration curve can be approximated by a linear function. In this case, the entire range of $[\text{NH}_2]/A_0$ is $4.0 \times 10^{-3}$ to $1.3 \times 10^{-2}$. The assumption is supported by the general trend shown in Figure III-13 regardless of the scatter.
The results of Table III-6 clearly show that the decrease in the rate of lysine acyl transfer in the presence of sodium dichloroacetate is not enough to account for the observed product ratios. The kinetic constants predict that acylation of the lysine amine should be the major product. The inconsistent prediction could be due to an error in the calculation of the degree of deprotonation - lysine could be significantly more protonated at 5 equivalents sodium dichloroacetate. In order to account for the observed rate however, \([\text{NH}_2]/A_0\) for CAAK would have to be \(9 \times 10^4\), 6 fold less than the estimated value. Doubling the sodium dichloroacetate concentration from 7.8 to 15.4 equivalents only results in a factor of 1.6 increase in \([\text{NH}_2]/A_0\), so it is unlikely that a decrease in base concentration from 7.8 to 5 equivalents could cause the necessary 6 fold decrease in free amine.

The kinetic studies of lysine acyl transfer in the presence of sodium dichloroacetate were run at \(10^{-3}\) M. As we showed earlier, homo and heteroconjugation would decrease the relative basicity of sodium dichloroacetate at high concentration and increase basicity at low concentration. In our discussion of homo and heteroconjugation in Appendix A, we showed

---

5 The difference in the ratios observed for CK and CAAK are consistent with the differences in the lysine rate constants at \(V_{\text{max}}\). If one assumes a constant degree of lysine deprotonation, the CK cysteine:lysine ratio should be larger than CAAK as \(k_3\) for CAAK is larger than for CK (5.56\(\times 10^{-2}\) s\(^{-1}\) as opposed to 3.10\(\times 10^{-2}\) s\(^{-1}\)).
that the effects are small at low concentration. It is unlikely that at $10^{-4}$ M these effects are large enough to significantly decrease the basicity of dichloroacetate, (relative to $10^{-5}$ M) and therefore cannot account for the discrepancy between the observed and the calculated product ratios at this concentration. The presence of homoconjugation effects at $10^{-2}$ M however, cannot be discounted and might account for the increase in the cysteine : lysine acyl transfer ratio (relative to $10^{-4}$ M ) observed above.

A possible resolution to the discrepancy between the predicted and observed acyl transfer product ratios lies in sodium dichloroacetate’s inability to tolerate excess acid. As we observed with the $^1$H NMR studies and the cysteine acyl transfer kinetics, the addition of acid results in a drastic decrease of free amine and the rate of acyl transfer. The presence of the acidic cysteine ammonium ion could act in the same way, decreasing the fraction of free amine produced by sodium dichloroacetate. The lysine kinetic studies were performed using thiol capture peptides that contained Z blocking groups on the cysteine amines. The absence of the acidic cysteine amine possibly resulted in greater deprotonation (relative to the unblocked peptides used in the product ratio studies) and hence the acyl transfer rate of the lysine in these models was faster than that of the models with free cysteine amines. As a result, the amount of acyl transfer to lysine predicted from the kinetic constants was too high.

To test this hypothesis, kinetic studies of lysine acyl transfer reaction were run under the same conditions as above, with the addition of small amounts of TFA. As TFA is more acidic than a protonated amine, the studies cannot prove our hypothesis although the results, presented in Table III-7 however, are quite suggestive. The presence of TFA decreased the
rate of lysine acyl transfer approximately 4 fold, a decrease comparable with the 5 to 6 fold
decrease in [NH₂]/Ao necessary to account for the observed cysteine:lysine ratio.

Table III-7 - Kinetic Constants for Acyl Transfer of ZCanK with Sodium Dichloroacetate and
TFA at 10⁻⁵ M
Sodium Dichloroacetate

<table>
<thead>
<tr>
<th>Thiol Capture Material</th>
<th>Equivalents Sodium</th>
<th>Equivalents TFA</th>
<th>Lysine Acyl Transfer Rate Constant</th>
<th>Half Life of Lysine Acyl Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZCK</td>
<td>6.8</td>
<td>0</td>
<td>1.32 x 10⁻² min⁻¹</td>
<td>57 min</td>
</tr>
<tr>
<td>ZCK</td>
<td>6.8</td>
<td>1.4</td>
<td>2.89 x 10⁻³ min⁻¹</td>
<td>240 min</td>
</tr>
<tr>
<td>ZCAK</td>
<td>10.3</td>
<td>0</td>
<td>4.04 x 10⁻² min⁻¹</td>
<td>17 min</td>
</tr>
<tr>
<td>ZCAK</td>
<td>10.3</td>
<td>2.0</td>
<td>6.0 x 10³ min⁻¹</td>
<td>113 min</td>
</tr>
<tr>
<td>ZCAAk</td>
<td>7.8</td>
<td>0</td>
<td>2.22 x 10⁻² min⁻¹</td>
<td>31 min</td>
</tr>
<tr>
<td>ZCAAk</td>
<td>7.8</td>
<td>1.57</td>
<td>3.0 x 10⁻³ min⁻¹</td>
<td>232 min</td>
</tr>
</tbody>
</table>

Further tests, performed with added tetralkyl ammonium salts could better elucidate the effect
of sodium dichloroacetate on the protonation states of the cysteine and lysine amines. These
initial results however, allow the formulation of a refined model by which sodium
dichloroacetate works to suppress lysine acyl transfer. Sodium dichloroacetate is capable of
only minimal deprotonation of the lysine ε ammonium ion, depressing the lysine acyl transfer
reaction relative to that of cysteine. This however is not enough. It is sodium
dichloroacetate’s very weakness as a buffer that makes it successful at suppressing lysine acyl
transfer. After the cysteine ammonium ion is deprotonated, the buffering capability of sodium
dichloroacetate is weakened so that the minimal level of lysine deprotonation is decreased even
further. This further depresses the rate of lysine acyl transfer, allowing cysteine to compete
successfully for the phenolic ester.
Although the use of sodium dichloroacetate as a buffering agent was aimed at suppressing a side reaction specific to dibenzofuran templated acyl transfer, there may be application to other peptide synthesis methodologies as well. As indicated in Appendix A, similar pKa values are observed for compounds dissolved in DMF and DMSO. This suggests that the ability of dichloroacetate to discriminate between peptide derived ammonium salts will be retained in DMF and perhaps DMAc, solvents used much more widely in peptide synthesis (60). This is especially attractive for semisynthesis methodologies, where unprotected peptide amine components are derived from biological sources and are likely to contain a number of reactive nucleophiles in the side chains.

Caution must be used in choosing the method of activation of the carboxylic acid of the acylating moiety. The popular in situ activation protocols using carbodiimides are precluded as dichloroacetate will be converted to an acylating agent and will compete with the activated peptide for the amine nucleophile. However, dichloroacetate may be successfully applied to acylations with peptides containing preformed activated esters such as paranitrophenol ester, pentafluorophenyl ester, and succinimide esters. Experiments aimed at determining its compatibility with peptide synthesis protocols and its ability to discriminate between N-terminal and side chain amine nucleophiles are planned for the future.

**Disulfide Interchange**

In addition to suppressing the lysine acyl transfer reaction, the change of base to sodium dichloroacetate also appeared to eliminate the disulfide interchange associated with DIEA. As discussed above, this allowed the concentration of acyl transfer to be increased 100
fold, increasing its utility as a method for the preparation of large proteins. In order to clarify this phenomenon, further studies of disulfide interchange were undertaken. Figure III-14 shows the results of acyl transfers performed with thiol capture materials purified by either HPLC or isopropanol precipitation, with either DIEA or sodium dichloroacetate as the acyl transfer base. The degree of disulfide interchange for each acyl transfer was assessed by HPLC, as discussed in Chapter 2. The lowest level of disulfide interchange was observed with the HPLC purified material regardless of whether DIEA or sodium dichloroacetate was used as a base. Acyl transfers performed with materials purified by isopropanol precipitation had somewhat higher levels of interchange. In these cases, the use of sodium dichloroacetate resulted in significantly cleaner reactions, with approximately 20% disulfide interchange regardless of acyl transfer concentration. Reactions using DIEA were the least clean with disulfide interchange levels of 35% and 55% at $10^{-4}$ and $10^{-2}$ M respectively.

Figure III-14: Disulfide Interchange As a Function of Base Type, Purification Method and Concentration

- ▲ DIEA; Isopropanol ppt, 10-2 M
- ▼ DIEA; Isopropanol ppt, 10-4 M
- ● Dichloroacetate, Isopropanol ppt, 10-4 M
- ▲ Dichloroacetate, Isopropanol ppt, 10-2 M
- + DIEA 2x HPLC Purified, 10-4 M
- ○ Dichloroacetate; HPLC Purified, 10-2 M
Thiol-disulfide interchange is an SN2 reaction involving the attack of a thiolate anion on a disulfide (Singh and Whitesides - Scheme III-3).

\[ \text{RSH} \leftrightarrow \text{RS}^- + \text{H}^+ \]
\[ \text{RS}^- + \text{R'SSR'} \leftrightarrow \text{RSSR'} + \text{R'S}^- \]
\[ \text{H}^+ + \text{R'S}^- \leftrightarrow \text{R'SH} \]

Scheme III-3

The reaction is second order overall, first order in disulfide and in thiolate anion. It has been observed that the thiol R-SH is not an active nucleophile - acidification stops the interchange reaction. Trace levels of thiol however, catalyze the reaction, presumably by providing a source of initial thiolate anion. Given the results of the study above and the mechanism presented in Scheme III-3 it is possible to construct a model to explain the effects of changing bases on the level of disulfide interchange observed. The pKa of an alkyl thiol in DMSO is approximately 15 (ref. 10), increased relative to water by the incapacity of DMSO to stabilize anions through hydrogen bonding. As stated above, the pKa of conjugate acids of dichloroacetate and DIEA are 6.4 and 9. The difference in the observed degree of disulfide interchange can possibly be explained by the difference in the degree of deprotonation of trace levels of thiol present in the acyl transfer mixture. Lower levels of thiolate anion result in a slower disulfide interchange reaction in the same way that decreasing the concentration of free amine decreased the acyl transfer rate. DIEA is a stronger base than sodium dichloroacetate and should therefore deprotonate the thiol to a greater extent, resulting in the higher level of disulfide interchange observed. The relationship between the amount of disulfide interchange and the number of equivalents of added DIEA is also explained by this hypothesis - as more
DIEA is added, the deprotonation of the thiol increases thereby increasing the rate and hence the level of disulfide interchange.

The source of the catalytic thiol is most likely contaminating Scm activated cysteine peptide. Thiol captures are usually run with excess Scm peptide (usually 1.05 - 1.1 equivalents) and it has been shown that in basic solvents such as DMF and DMSO, the functionality undergoes slow decomposition. HPLC purification after thiol capture effectively removes contaminating Scm peptide and eliminates the source of catalytic thiol, so that acyl transfers with this material show no difference in disulfide interchange regardless of base type and number of equivalents. Isopropanol precipitation is less effective in fully removing the contaminating Scm peptide resulting in the higher levels of disulfide interchange observed.

Summary

In conclusion, we have demonstrated the effectiveness of an alternative base, sodium dichloroacetate, in suppressing both lysine acyl transfer and disulfide interchange. This has resulted in the ability to increase the concentration of the acyl transfer reaction 100 fold, improving its effectiveness as a preparative method for the synthesis of large peptides. In addition, investigation into the action of sodium dichloroacetate has been undertaken. From the studies performed we have shown that the mode of action is consistent with sodium dichloroacetate's behavior as a compound capable of acting as a buffer in DMSO.
Appendix A - The Physical Organic Chemistry of DMSO Solutions
Appendix A - The Physical Organic Chemistry of DMSO Solutions

The nature of a solvent can strongly influence the properties of dissolved solute. This thesis is concerned with solvent effect on ionizations of weak acids in dipolar aprotic media. Solvation changes are least likely to influence the properties of relatively nonpolar solutes. Even in this limiting case, however, important and controversial effects can arise as evidenced by the abundant literature on the energetics of hydrophobicity in water. At the other extreme, the properties of ionic molecules are maximally sensitive to solvent changes, since the high electric fields in the vicinity of ions induce large complementary stabilizing interactions with neighboring solvent molecules and these interactions are quite solvent specific. Ionization reactions in which neutral, acidic molecules dissociate to form ions thus are particularly sensitive to solvent changes. These reactions underlie much of the work described in this thesis. In order to understand the ramifications of solvent solute interactions for acyl transfer in DMSO, an introduction to the effect of DMSO on solute properties is presented here.

In this thesis all studies of acyl transfer were performed in DMSO, and an understanding of the physical organic chemistry is necessary in order to interpret the experimental results. The first section of this appendix will present the properties of DMSO and its effect on solute-solute interactions (ion pairing and hydrogen bonding). The effects of DMSO on the pKa's of acids and bases dissolved in it will also be explored, and a simple method for qualitatively estimating pKas in DMSO from aqueous pKa data will be presented. The relationship between reactivity and pKa will also be discussed briefly. Each of these topics will be discussed in reference to the thiol capture model peptides.
**DMSO**

Dimethyl sulfoxide (DMSO, Figure A.1) is a solvent with a number of unique properties. Physically, it is an almost odorless, colorless liquid with a high boiling point (189°C), melting point (18.5°C), dielectric constant and dipole moment (46.6 and 3.9 at 25°C, respectively). Miscible in all proportions with water, it is also miscible with a wide range of organic liquids including ethanol, acetone, ether, benzene, and chloroform although it does not mix with aliphatic hydrocarbons. Similarly it is capable of dissolving a wide range of solids, from ionic salts through large non-polar aromatic compounds, to biological polymers like proteins (71). Few other organic solvents share this property.

![Chemical Structure of DMSO](image)

**Figure A.1**

The structure of DMSO, with the charge separation indicated, is shown in Figure A2.1. The negative oxygen end of the molecule is exposed allowing cations to be strongly stabilized. Free energies of transfer indicate that DMSO solvates cations more strongly than water (18). A review by Parker (81) lists the cation solvating potentials of a number of solvents in the following order: DMSO, DMAc > DMF, SO₂, H₂O > acetone, sulfolane > methanol > acetonitrile. DMSO has the strongest cation solvating ability of the common organic solvents.

Anions are only weakly stabilized in DMSO (48). In protic solvents, anions are strongly stabilized by accepting a hydrogen bond from the solvent. DMSO cannot act as a hydrogen bond donor because it lacks O-H or N-H bonds and therefore cannot stabilize anions via this interaction. By virtue of the unpaired electrons on oxygen however,
DMSO can act as a hydrogen bond acceptor. Hydrogen bond enthalpies for the interaction of p-fluorophenol have been measured for 65 polar aprotic bases (5). The data indicate that the ability of DMSO to accept a hydrogen bond is among the strongest of any organic solvent. Comparisons with water, the prototypical hydrogen bonding solvent, are difficult since water can both donate and accept hydrogen bonds. Experimental separation of these abilities is impossible. Ethers can be used to model hydrogen bond acceptance by the oxygen of water. Based on the hydrogen bond enthalpies for a number of ethers, including diethyl ether, THF and dioxane, water appears to be a somewhat weaker hydrogen bond acceptor than DMSO (5). This interpretation must be treated with great caution, given the great structural difference between water and the ether models. DMSO also appears to be a much stronger Brønsted base than water. In acetonitrile, the pKa of \( \text{H}_3\text{O}^+ \) is found to be 2.5-4 while that of protonated DMSO is 5.8.(66).

As many organic reactions involve anionic reagents and charge delocalized transition states, the ability of DMSO to solvate the positive counterion while leaving the lone pairs of the anion relatively free to react as a nucleophile, can result in dramatic rate enhancements relative to same reaction in protic solvents (74, 81). The ability of DMSO to dissolve both ionic and non-polar compounds makes it a solvent of almost unique versatility for organic synthesis. This has been of great advantage for thiol capture reactions which contain nonpolar organic bases (DIEA), inorganic salts (AgNO₃), and peptides with varying degrees of deprotection. As discussed in Chapter 2, its ability to dissolve a wide range of salts was of greatly aided our development of an organic buffer system.
**Ion Pairing effects in DMSO**

Salts in solution can exist as fully dissociated ions in which each ion is surrounded by several layers of solvent (Figure A2.2). In solvents of low to moderate polarity however ions in solution can be associated to varying degrees. The existence of two types of ion pairs has been demonstrated; the contact ion pair, $M^+X^-$, in which there is no solvent between the ions; and the solvent separated ion pair $M^+|X^-$ in which a molecule of solvent lies between the ions (Figure A2.2, 48).

![Diagram showing fully dissociated ions, contact ion pair, and solvent separated ion pair.](image)

Figure A.2

A number of other ionic aggregation species have also been demonstrated. In solvents of very low polarity, ion triplets, ion pair dimers and salt polymers have also been demonstrated. In benzene for example, the ratio of the average molecular weight relative to the molecular weight of the salt monomer, has been shown to be 2.4 to 3.2, as determined by freezing point depression (48).
A number of methods exist for evaluating the degree of ion pairing in a solvent. The preponderance of evidence has come from studies of the conductance of salts in different solutions. Conductance measures the current carrying capacity of, and hence the number of ions in, a solution. When the electrostatic interaction between ions is large, the association of the anions and cations leads to the formation of neutral ion pairs which do not contribute to the conductance in solution. The result of ion pairing is that the conductance of a salt is smaller than that predicted by equations representing the behavior of completely dissociated electrolytes.\(^1\) (28).

Table A.1 Solvent Dielectric Constants

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric Constant at 25.0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>78.3</td>
</tr>
<tr>
<td>DMSO</td>
<td>46.6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>42.5</td>
</tr>
<tr>
<td>DMF</td>
<td>36.7</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>37.5</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>9.2</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>6.2</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>6.0</td>
</tr>
</tbody>
</table>


Table A.1 lists the dielectric constants\(^2\) of several typical solvents. The degree of ion pairing of quaternary ammonium salts in a number of organic solvents has been

1) The conductance of a completely dissociated solution is represented by the Onsager limiting law, the corrected form of which is \( \Lambda = \Lambda^0 - S\sqrt{c} + Ec \ln c + J_1c - J_2c^{3/2} \) where \( \Lambda^0 \) represents the molar conductance at infinite dilution, \( c \) represents concentration and \( S, E, J_1 \) and \( J_2 \) are related to a number of physical properties including the viscosity and dielectric constant of the solvent and the charge of the electrolyte. The conductance equation is able to represent the behavior of completely dissociated electrolytes. When the electrostatic interactions between ions is large the conductance is smaller than that predicted by the equation. An extrapolation of experimentally derived \( \Lambda \) to infinite dilution yields \( \Lambda^0 \) which differ from the predicted values, the difference increasing with increasing association constant \( K_A \).

2) When a charged particle is immersed in a solvent the strength of the electric field surrounding that particle is affected. The dielectric constant, \( \varepsilon \), is a measure of the strength of this field relative to the field strength of the same particle in a vacuum and is related to solvent polarity (74)
measured(68). The association constant $K_A$ has been shown to be inversely related to solvent dielectric constant. Plots of $\log K_A$ versus $1/e$ are linear over an $\varepsilon$ range of 5 to 35. The results indicate that ions are likely to be dissociated in solvents with high dielectric constants, while in solvents with low to moderate dielectric constants, ion pairing may be extensive. Because reactivity of an ion may be drastically reduced by the proximity of a counter ion, solvent polarity plays an important role in ionic reactions. Examples of reactions where ion pairing effects can be important are nucleophilic substitution with anion nucleophiles, E2 eliminations, and reactions involving carbocations or carbanions (74).

Ion pairing also affects the position of equilibrium and therefore can change the apparent pKa values of acids (65). Consider the ionization of an acid HA

$$HA \rightleftharpoons [H^+A^-] \rightleftharpoons H^+ + A^- \quad \text{eqn.A.1}$$

where $[H^+A^-]$ is an ion pair. The ionization constant of the acid can be written as

$$K_i = \frac{[H^+A^-]}{[HA]} \quad \text{eqn.A.2}$$

Likewise the dissociation constant of the ion pair can be written as

$$K_{di} = \frac{[H^+]A^-}{[H^+A^-]} \quad \text{eqn. A.3}$$

It can be shown that overall dissociation constant of the acid is

$$K_o = \frac{[H^+]A^-}{[H^+A^-] + [HA]} \quad \text{eqn. A 4}$$

Notice that if ion pairing is small $K_o$ reverts to the constant for a normal acid dissociation

$$HA \rightleftharpoons H^+ + A^- \quad \text{eqn.A.5}$$

$$K_d = \frac{[H^+]A^-}{[HA]} \quad \text{eqn. A.6}$$

Equation A.6 defines the equilibrium constant used in the determination of pKa, and represents the limit of equation A.4 as the concentration of ion pairs approaches zero. A comparison of equations A.4 and A.6 indicates that the effect of ion pairing is to decrease the overall dissociation constant $K_o$ relative to $K_d$, raising the pKa and causing the acid
to appear to be weaker.

As indicated in Table A.1, DMSO, with few exceptions has one of the highest dielectric constants of the common organic solvents. Based on the relationship between dielectric constant and degree of ion pairing discussed above, it is likely that ion pairing would be small in DMSO. The conductance of a number of electrolytes in DMSO have been measured for solvent concentrations less than $10^{-3}$ M (89). For each of the salts, the equivalent conductance was plotted against the square root of the concentration. The slopes of the experimental data showed excellent agreement with theoretical values predicted from the Onsager equation (average % deviation = 5 %), indicating that the salts are completely dissociated in DMSO. A conductance study of quaternary ammonium halides in DMSO has also been performed (4). A comparison of the experimental and theoretical slopes of Onsager’s equation shows an average deviation of less than 2%. Finally, a vibrational spectroscopy study (Raman and IR) of LiSCN in DMSO indicates that at 0.1 M, the salt is completely dissociated (22). The evidence from these studies shows that at low to moderate concentrations, salts in DMSO are completely dissociated. The effects of ion pairing on reactivity and pKa can therefore be ignored.

**Hydrogen Bonding in DMSO - Homo and Heteroconjugation**

In addition to stabilization through electrostatic interactions, resulting in ion pairs, there is an additional mode by which ions can be stabilized in solution. Ions can be stabilized through hydrogen bonding. Dipolar aprotic solvents are unable to act as hydrogen bond donors although some, such as DMSO, DMF and acetonitrile, are hydrogen bond acceptors. In the absence of stabilization from the solvent, anions in...
solution can be stabilized by hydrogen bonding to other species present in solution. In the case where the anion is hydrogen bonded to its parent acid (A$^-$--HA), the interaction is termed homoconjugation (66). If the interaction is with another hydrogen bond donor (A$^-$--HR), it is termed heteroconjugation. The net result of homo and heteroconjugation is that, at high concentrations, there is an increase in apparent acidity, as the interactions stabilize the anion thereby enhancing acid dissociation. As solvents like DMSO are strong hydrogen bond acceptors themselves, the anion being stabilized must compete with the solvent for the hydrogen bond donor. A highly polar solvent is likely to be an aggressive hydrogen bond acceptor, and therefore conjugation is likely to play a lesser role in the stabilization of anions in these solvents.

In addition to stabilization of anions via homoconjugation, there is evidence that amines can also be stabilized by hydrogen bonding. In acetonitrile, potentiometric evidence indicates that primary, secondary and tertiary amines are capable of forming homoconjugates BH$^+$--B (27,48). As above, solvent molecules that are strong hydrogen bond acceptors would compete with the neutral amine for the ammonium ion. It is likely, therefore that amine homoconjugates play only a small role in the stabilization of amines in strongly hydrogen bonding solvents like DMSO and DMF.

Table A.2 and Table A.3 list the homoconjugation and heteroconjugation constants of oxygen acids in various polar aprotic solvents (66, 67). As expected from considerations of solvent polarity, the homo and heteroconjugation constants measured in DMSO are smaller than those in acetonitrile and DMF (67). The homo and heteroconjugation constants in DMSO are of approximately the same order of magnitude and are 10-1000x and 8-100x smaller respectively, than those measured in acetonitrile.

One can estimate the effects of ion pairing for carboxylic acids in DMSO. The homoconjugation constant $K_{HA_2^-} = ([A^-•••HA]/[A^-][HA])$ of acetic acid has been measured to be 30 (Kolthoff, Chantooni, and Bhowmik 1968). In a 1:1 mixture of acetic
acid:acetate ion (0.01M each), 20% of the acetate ion is present as the homoconjugate. If the concentrations are decreased to 0.001 M each, less than 3% of acetate is present as the homoconjugate. The same analysis can be performed for heteroconjugation, with similar results.

Table A.2 Homoconjugation Constants $K_{\text{HA}^-}$ of Oxygen Acids in DMSO and Acetonitrile$^a$

<table>
<thead>
<tr>
<th>Acid</th>
<th>DMSO</th>
<th>Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylic</td>
<td>30</td>
<td>2000</td>
</tr>
<tr>
<td>3,5-Dinitrobenzoic</td>
<td>23</td>
<td>10,000</td>
</tr>
<tr>
<td>3,5-Dinitrophenol</td>
<td>30</td>
<td>44,000</td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td>40</td>
<td>5000</td>
</tr>
<tr>
<td>Benzoic</td>
<td>60</td>
<td>4000</td>
</tr>
<tr>
<td>Acetic</td>
<td>30</td>
<td>4700</td>
</tr>
</tbody>
</table>


Table A.3 Heteroconjugation Constants $K_{\text{HR}A^-}$ of Substituted Benzoates (A-) with p-Bromophenol (HR) in DMSO, DMF, and Acetonitrile$^a$

<table>
<thead>
<tr>
<th>Benzoates</th>
<th>DMSO</th>
<th>DMF</th>
<th>Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsubstituted</td>
<td>47</td>
<td>240</td>
<td>3550</td>
</tr>
<tr>
<td>m-Bromo</td>
<td>26</td>
<td>129</td>
<td>1820</td>
</tr>
<tr>
<td>p-Nitro</td>
<td>17</td>
<td>83</td>
<td>1100</td>
</tr>
<tr>
<td>3-Nitro-4-Chloro</td>
<td>13</td>
<td>76</td>
<td>950</td>
</tr>
<tr>
<td>3,5-Dinitro</td>
<td>5</td>
<td>37</td>
<td>363</td>
</tr>
<tr>
<td>o-Hydroxy</td>
<td>2</td>
<td>11</td>
<td>132</td>
</tr>
</tbody>
</table>


As predicted from a consideration of the hydrogen bonding properties of DMSO, only a small fraction of the anions are stabilized through hydrogen bonding to other species in solution. One can conclude from this analysis that at the concentrations usually used for acyl transfer in this thesis (< 5x10^{-4} M), homo and heteroconjugation, like ion pairing, can be ignored.
Solvent effects on pKa

As noted above, the nature of the solvent has a significant effect on equilibria. For example, in the case of acid dissociation (eqn. A.5), the ability of the solvent to solvate the proton, the conjugate base and the undissociated acid will determine the extent to which it is ionized, and thus its pKa in that solvent (10). The measurement of pKa values in nonaqueous solvents has usually involved the use of indicators. This method yields only relative pKa's as they are measured relative to the ionization of the indicators. As the indicators themselves are organic bases, their ionization equilibria are also perturbed by the change in solvent, making comparison between solvents difficult. In the case of a few solvents such as DMSO and DMF however, a method for measuring pKa potentiometrically with a glass electrode has been developed (86). This has allowed the determination of "absolute" acidities making possible direct comparisons with the pKa's in the gas phase and water ("absolute in the sense that the equilibrium constants are referred to a standard state in the solvent in which they are measured" (10, 87).

The effect on pKa upon changing solvent from water to DMSO can be analyzed in terms of stabilization of the species in solution. The pKa change can be related to the free energy of transfer ($\Delta G_{tr}$) from one solvent to the other, for each of the species involved

$$\Delta pKa = \frac{[ \delta \Delta G_{tr \ sol.2-sol.1}(H^+) + \delta \Delta G_{tr \ sol.2-sol.1}(A^-) - \delta \Delta G_{tr \ sol.2-sol.1}(HA)]}{2.303 \ RT};$$

eqn. A.7

(18). All other interaction being equal, if solvent 2 stabilizes the undissociated acid relative to solvent 1, the pKa in solvent 2 would be higher (apparent acidity decreased). If solvent 2 stabilizes the conjugate base relative to solvent 1, the pKa in solvent 2 would be lower (apparent acidity increased).

Representative pKa's for neutral oxygen acids are tabulated in Table A.4. As no published values in DMSO exist for several compounds of interest for this thesis, values determined in DMF (which in general are only slightly higher than DMSO) are also
given. Values in water are tabulated for comparison. It is important to note that the values listed in parenthesis were determined using organic indicators and are likely to be too low by 1 unit. Table A.5 lists pKa values for protonated amines in DMSO, and water.

### Table A.4 - pKa Values for Uncharged Oxygen Acids in Water, DMF and DMSO

<table>
<thead>
<tr>
<th>Acid</th>
<th>Water</th>
<th>DMF</th>
<th>DMSO</th>
<th>ΔpKaDMSO-Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picric</td>
<td>0.4(^d)</td>
<td>-----</td>
<td>-1.0(^a)(-1.9)(^c)</td>
<td>-1.2</td>
</tr>
<tr>
<td>4-Chloro-2,6-dinitrophenol</td>
<td>3.0(^a)</td>
<td>-----</td>
<td>3.0(^a)</td>
<td>0.6</td>
</tr>
<tr>
<td>Salicylic</td>
<td>3.0(^a)</td>
<td>8.2(^a)</td>
<td>6.7(^a)</td>
<td>3.7</td>
</tr>
<tr>
<td>Benzoic</td>
<td>4.2(^a)</td>
<td>12.3(^a)(10.2)(^c)</td>
<td>11.1(^a)(10.0)(^c)</td>
<td>6.9</td>
</tr>
<tr>
<td>Acetic</td>
<td>4.8(^c)</td>
<td>(11.1)(^c)</td>
<td>12.3(^a)(11.4)(^c)</td>
<td>8.5</td>
</tr>
<tr>
<td>Trifluoroacetic</td>
<td>0.5(^d)</td>
<td>-----</td>
<td>3.45(^b)</td>
<td>2.9</td>
</tr>
<tr>
<td>Chloroacetic</td>
<td>2.9(^d)</td>
<td>(9.0)(^c)</td>
<td>-----</td>
<td>---</td>
</tr>
<tr>
<td>Dichloroacetic</td>
<td>1.3(^d)</td>
<td>(7.2)(^c)</td>
<td>6.4(^b)</td>
<td>5.1</td>
</tr>
</tbody>
</table>

DMSO and DMF values in parenthesis were measured using organic indicators and are too low by approximately 1 unit. All other pKa's were measured potentiometrically with a glass electrode standardized in the solvent.


### Table A.5 - pKa's of Protonated Amines in Water and DMSO\(^a\)

<table>
<thead>
<tr>
<th>Acid</th>
<th>Water</th>
<th>DMSO</th>
<th>ΔpKa(_{DMSO-Water})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyamine</td>
<td>10.6</td>
<td>11.0</td>
<td>0.4</td>
</tr>
<tr>
<td>n-Butylamine</td>
<td>10.6</td>
<td>11.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>11.0</td>
<td>10.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>Di-n-butylamine</td>
<td>11.3</td>
<td>10.0</td>
<td>-1.3</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>10.7</td>
<td>9.0</td>
<td>-1.7</td>
</tr>
<tr>
<td>Tri-n-butylamine</td>
<td>10.9</td>
<td>8.4</td>
<td>-2.5</td>
</tr>
</tbody>
</table>

All pKa's were determined potentiometrically.

Using equation A.7 the difference between the pKa's of carboxylic acids in water and DMSO can be explained by stabilization. Both water and DMSO are strong hydrogen bond acceptors and as such, would be expected to stabilize the undissociated oxygen acid and the proton similarly. Ionization forms oxyanions which in water are stabilized by donation of hydrogen bonds from water to the anion. In contrast, DMSO lacks the ability to donate hydrogen bonds and cannot stabilize the oxyanion. The net result is that the oxyanion is stabilized in water relative to DMSO which from equation A.7 should result in a decrease in the apparent acidity (increase in pKa) of oxygen acids. The change in pKa can be quite dramatic, as shown in the cases of acetic and benzoic acids (ΔpKa of 7.8 and 6.9, respectively). There are several notable exceptions to this trend. Strong acids such as picric acid, trifluoroacetic acid and 4-Chloro-2,6-dinitrophenol form highly delocalized anions upon dissociation, and are not greatly stabilized by hydrogen bond donation from water. The consequent loss of this mode of stabilization in DMSO therefore does not result in a great decrease in acidity. In salicylic acid, the oxyanion is stabilized by an intramolecular hydrogen bond, so that it too shows a smaller change in acidity upon changing the solvent from water to DMSO.

The change from water to DMSO has little effect on the pKa of protonated alkyl amines. The effect ranges from +0.5 to -2.5 units, with an average of 1.1. There seems to be a correlation with the number of alkyl groups attached to the nitrogen, although it is difficult to generalize from the small number of compounds in each class measured. N-alkyl amines seem to be slightly more basic in DMSO than in water, while di and tri-alkylamines are less basic. Like the case of the oxygen acids, this effect can be analyzed in terms of stabilization. Let us consider each species separately. In both DMSO and water the protonated n-alkyl amine is stabilized by donating three hydrogen bonds to the solvent. Di and tri-alkyl amines can form two and one hydrogen bond respectively. When we consider the neutral amine however, the situation changes. Water can donate a hydrogen bond to the neutral amine via its lone pair. As a result water can still form the
same number of hydrogen bonds to water as the protonated amines. DMSO can only accept hydrogen bonds however, so that the number of hydrogen bonds formed by the n, di and tri-alkyl amines are two, one and zero respectively. As a result, the conjugate base of the acid in water is stabilized relative to DMSO, which according to equation A.7, should increase its acidity. This stabilization is opposed to some degree by the greater hydrogen bonding strength of DMSO (which would stabilize the protonated amine relative to water) and by the greater ability of DMSO to solubilize the nonpolar side chains of the amines. Overall, the stabilization in water and DMSO should balance, so that there should be little change in the pKa upon changing solvents from water to DMSO.

The correspondence of the observed pKa's with the predictions from equation 7 indicate that the change in pKa is a function of interactions with the solvents. By analyzing the effect of each solvent on the undissociated acid, the proton and the conjugate base, a qualitative estimate of the direction and magnitude of the change in pKa can be obtained.

**Amino Acid pKa's in DMSO**

Amino acid pKa's can be analyzed in the same manner as the acids and bases discussed above. Table A.6 lists the dissociation constants of amino acids and peptides in DMSO and water (46;49, 82). As can be seen from the ΔpKa's, the amine and carboxylic acid functional groups behave very similarly to their oxygen and protonated amine counterparts. The carboxylic acid is significantly less acidic in DMSO with an average

4) Consider the solubilities of the n-alkyl groups in water and DMSO. When a nonpolar group in placed in water, the water molecules orient around it, creating a clathrate or ice like structure which is more structured than bulk water. The formation of the clathrate results in a net decrease in entropy (-ΔS) and an increase in free energy of solvation (+ΔG). DMSO is much more miscible with nonpolar solvents and is better able to solvate the alkyl groups. Thus the addition of alkyl groups stabilizes the neutral amine in DMSO relative to water, which results in a decrease in the free energy of transfer of the conjugate base from water to DMSO.
increase in pKa of 5.3 units. The pKa's of the amines do not change significantly, with a
range of -1.1 to +0.6 pKa units. The average of the absolute values of ΔpKa is 0.7 units.
Although the pKa of the amino terminus in a peptide has never been measured in DMSO,
an estimation from the values in water suggests that in DMSO the pKa would range from
6.9 to 8.3.

Table A.6 Dissociation Constants of Amino Acids and Peptides in DMSO and Water

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>pKa COOH</th>
<th>pka NH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>DMSO</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.3(^a)</td>
<td>7.5(^c)</td>
</tr>
<tr>
<td>Glycine ethyl ester</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Serine</td>
<td>2.2(^a)</td>
<td>7.2(^c)</td>
</tr>
<tr>
<td>Serine methyl ester</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.8(^a)</td>
<td>6.8(^c)</td>
</tr>
<tr>
<td>Phenylalanine methyl ester</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pKa COOH</th>
<th>pka NH</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(^3)N(^+)-CH(_2)CONH(_2)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Hexaglycine</td>
<td>3.0(^a)</td>
<td>---</td>
</tr>
<tr>
<td>Tetraalanine</td>
<td>3.4(^a)</td>
<td>---</td>
</tr>
</tbody>
</table>

pKa's in DMSO were measured spectrophotometrically by the method of Matthews,

1965.

Upon changing solvent, the variations observed in the pKa's of different functional
groups, can result in anomalous types of ionization in multi-functional compounds (18,
66). For example, amino acids in aqueous solution exist as dipolar ions (a) rather than as
uncharged molecules (b) (Figure A2.2).

(a) H\(^3\)N\(^+\)-CH(R)-COO\(^-\) (b) H\(_2\)N-CH(R)-COOH

Figure A2.3

The ratio of dipolar ion to uncharged molecule for α amino acids in water has been
determined to be $10^4$-$10^5$ (49). In DMSO the pKa of the carboxylic acid function has increased by approximately 5 pKa units while the pKa of the amine is essentially unchanged. As a consequence, there has been a drastic shift in the ratio of zwitterion to neutral molecule in DMSO, ranging from 2-40 in favor of the zwitterion (49). This shift in equilibrium provides a dramatic demonstration of the effect that changing solvent can have on acid base equilibrium.

**PKa's of the Functional Groups in the Thiol Capture Peptide**

The pKa (DMSO) of the n-terminal cysteine amine in the thiol capture material can be estimated from the available data for cystine peptides in water. In the peptide diglycyl-L-cystine$^5$ the pKa's for the glycine n-terminal amines have been measured as 7.94 (46). This value is very similar to the literature values for the n-terminal amines of tetraalanine and hexaglycine (Table A.6). In the cases L-cystinyl diglycine$^5$ and L-cystinyl-diglycine$^5$ however, the pKa values observed for the cysteine n-terminal amines range from 6.01-6.95 (46). The 1-2 unit decrease in the pKa of the n-terminal amine of cystine peptides cannot be attributed to the proximity of the two amines as this would also be observed in diglycyl-L-cystine. The effect has been attributed to the presence of a disulfide close to the amino terminus (46). The asymmetrical disulfide of the thiol capture material should also affect the pKa of the n-terminal cysteine amine in a similar manner, lowering the pKa by at least 1 unit. The effect of the disulfide, coupled with the observation that pKa's do not change much from water to DMSO ($\pm$ 0.7 units), yields an expected pKa range in DMSO of 6.2 to 7.6 for the N-terminal amine of the

5) diglycyl-L-cystine = $\text{H}_3\text{N}^+\cdot\text{Gly-Cys-OH}$  \hspace{1cm} L-cystinyl-diglycine = $\text{H}_3\text{N}^+\cdot\text{Cys-Gly-OH}$

$\text{H}_3\text{N}^+\cdot\text{Gly-Cys-OH}$ \hspace{1cm} $\text{H}_3\text{N}^+\cdot\text{Cys-Gly-OH}$

5) L-cystinyl-diglycine = $\text{H}_3\text{N}^+\cdot\text{Cys-Gly-Gly-OH}$

$\text{H}_3\text{N}^+\cdot\text{Cys-Gly-Gly-OH}$
thiol capture material (based on a value of 6.9 in water).

The pKa of the ε-amine of lysine in the thiol capture peptide can also be similarly estimated. Peptides in which the lysine is not in the n-terminal position yield an average of 10.4 ±0.2 for the pKa of the ε-amine. Application of the same ±0.7 units for the change in pKa of amines in DMSO relative to water yields a range of 9.7 to 11.1. This value corresponds well with the values of n-butyl and n-ethylamine in DMSO and with the value of 10.8 determined for the lysine ε-amine of the free amino acid in DMSO.

From the pKa values for protonated trialkyl amines in DMSO listed in Table A-5, the pKa of DIEA in DMSO can be estimated. Given the values observed for triethylamine and tri-n-butylamine, 9.0 and 8.4 respectively, it is likely that the pKa of protonated DIEA lies within this range.

The Use of pKa Data

In nucleophilic substitution, the nucleophile shares its unshared electron pair with the electrophile, displacing the leaving group. In acid base reactions, a Lewis base donates its lone pair to the acid. As both reactions involve donation of the unshared electrons, nucleophilicity should be correlated with basicity and hence pKa. Most of the early studies on the relationship between basicity and nucleophilicity were performed in protic solvents such as water. Due to the leveling effect of water, only a few nucleophiles could be studied (74). Although a general correlation could be shown, accurate predictions of nucleophilicity could not be made from the basicity of the compounds in water..

The measurement of pKa's in DMSO has allowed a far greater range of acidities to be measured than can be observed in water. The expanded range has allowed the study of a variety of nucleophile types and enabled an assessment of the effects that structural variations in organic molecules have on their electronic and steric properties (10). Among the many structural variations studied are α electron withdrawing groups, α heteroatoms, sp hybridization at carbon, ion pairing, and steric inhibition of resonance (a
full list is provided in ref. 10). Hammett and Taft relationships between families of acids can be produced, allowing the pKa to be estimated for a compound for which there is no experimental data (10).

Reaction rates for a number of reactions involving electrophiles and conjugate bases have been found in DMSO to correlate with basicity (10, 11, 12). Under conditions where steric and solvent effects are constant, the rate of the reaction (log $k_{obsd}$) of an electrophile with a conjugate base, when plotted against the pKa of the parent acid, yields a linear relationship. Linear Brønsted plots have been obtained for a number of reactions including $S_N2$, $S_N2^\prime$, $E2$, $S_NAr$, $H^+$, and $e^-$. These Brønsted plots indicate that the nucleophilicity of the bases depends on their basicity, as measured by pKa (10). The slope of the plots, $\beta_{NU}$, represents the sensitivity of the reaction to changes in the basicity and is believed to be related to electrostatic interactions in the ion-molecule complex (12). The Brønsted plots, coupled with the ability to estimate pKa's in DMSO from the Hammett and Taft relationships, allows the prediction of reaction rates for new combinations of nucleophiles and electrophiles. The difference between cysteine and lysine in the models is most likely due to their difference in pKa.

**Summary**

It is clear that there are a great number of complications that arise in interpreting acid-base equilibria in polar aprotic solvents like DMSO. The modes of stabilization available to the species in solution along with the bulk properties of the solvent must be considered. Careful analysis of the interaction of each species with the solvent, coupled with information from model compounds however, allows a relatively accurate assessment of the direction and magnitude of the change in pKa.

The evidence presented in this appendix allows the behavior of the thiol capture peptides in DMSO to be predicted. DMSO as a solvent for thiol capture has several
advantages. It is capable of solvating a wide variety of compounds from inorganic salts to proteins, and salts dissolved in it do not associate as ion pairs nor do they form homo or heteroconjugates. The low level of ion pairing observed in DMSO indicates that association is unlikely to be a complicating factor at the dilute concentrations at which acyl transfers are usually run. The TFA salts of the peptides are likely to be completely dissociated at $10^{-4}$ M.

As the first step in an acyl transfer is the deprotonation of the amine salt, an understanding of acid base properties in DMSO is necessary. There is an immense body of measured pKa's allowing accurate estimation of pKa's for new compounds. Of importance to this thesis, the pKa of carboxylic acids is greatly increased in DMSO, relative to water, while the pKa of amines is essentially unchanged. Given the behavior of amines in DMSO, it is likely that the pKa difference between the lysine $\varepsilon$ amine and the cysteine $\alpha$ amine observed in water is retained in DMSO. Attempts to selectively deprotonate the cysteine amine based on this pKa difference are presented in Chapter 2.

The effect of changing from water to DMSO on the pKa values of carboxylic acids is used in the development of an organic buffer system for acyl transfer. In DMSO the pKa of dichloroacetic acid is similar to the pKa of the ammonium ion of cysteine. This allows sodium dichloroacetate to be used as a buffer that selectively deprotonates the cysteine ammonium ion. Experiments studying the use of sodium dichloroacetate as a buffer are discussed further in Chapter 3.

Finally, pKa data has been related to reactivity in DMSO. The nucleophilicity of bases in DMSO is related to their basicity, as measured by the pKa of the conjugate acid. The difference in reactivity between the cysteine and lysine amines in the thiol capture model peptides can be therefore attributed to the difference in their basicity. This is discussed further in Chapter 2.
Appendix B - Effective Molarity and Polymer Conformation
Appendix B - Effective Molarity and Polymer Conformation

Introduction

In the body of this thesis, a novel acyl transfer to the ε amine of lysine was studied. The distance dependence on the rate of lysine acyl transfer was determined and methods for limiting this reaction developed. Acyl transfer to lysine involves a reaction between two groups attached by a flexible peptide tether. Methods for estimating the distance between the lysine ε amine and the phenolic ester are necessary for understanding the observed rate of acyl transfer. The background for utilizing effective molarity to estimate reactant proximity is presented in this appendix. The relationship between the effective molarity and conformational entropy and bond strain is also discussed.

Acyl transfer to lysine involves the reaction of two groups separated by a flexible tether. The ability to calculate the effective molarity and relate it to the experimentally determined value would be useful. Polymer chemists have developed statistical methods for determining dimensions of interest in solution. A brief discussion of polymer conformational statistics and their relation to macrocyclization theory follows. This theory allows the computational determination of effective molarity for the two ends of a polymer. As special problems are associated with short polymer conformations, a comparison of calculated and experimentally determined conformational distributions is presented. The failure of polymer theory to predict experimental results is cited as the major reason not to apply this statistical method to the short polymers used in this thesis to study lysine acyl transfer.

In order to interpret the relationship between the lysine effective molarities and the distance between the ε amine and phenolic ester, it is vital to understand the structure of the thiol capture peptide in solution must be understood. The conformational equilibria of linear polypeptide chains between solvent exposed and compact, internally hydrogen
bonded states depends strongly on their interaction with solvent. The gross conformation of macromolecules such as peptides are strongly affected by solvent changes. As all acyl transfer reactions studied in this thesis take place in DMSO, it is this solvent that is of primary interest. Finally, this appendix briefly reviews the forces stabilizing the folded and unfolded protein and the available information on poly-peptide structure in DMSO.

**Entropy of Molecules**

The concept of effective molarity was first proposed to account for the rate accelerations observed for enzymatic and intramolecular reactions relative to their bimolecular counterparts. It is developed from a consideration of the differences in entropy changes between intra and intermolecular reactions.

Before discussing the source of the rate accelerations observed in intramolecular and enzymatic reactions, a brief review of entropy is necessary. The basic formula for the statistical interpretation of entropy is

\[
S = k \ln W \quad \text{eqn. B.1}
\]

where \( W \) represents the statistical probability of the state whose entropy is \( S \) (Noggle). Qualitatively, \( W \) is the number of distinct ways a system can be arranged. As a highly ordered system has fewer distinct states than a disordered system, entropy, as a function of \( W \), can be thought of as a measure of the degree of disorder of a system.

A non linear, polyatomic molecule of \( n \) atoms requires \( 3n \) coordinates to describe its location in space. Similarly there are \( 3n \) degrees of freedom which are separable into 3 degrees of translational freedom, 3 degrees of rotational freedom and \( 3n-6 \) degrees of vibrational or conformational freedom (79) Each of these degrees of freedom has an associated entropy. The total entropy of a molecule is the sum of the entropic contributions of the various modes of motion;

\[
S = S_{\text{trans}} + S_{\text{rot}} + S_{\text{conform}}. \quad \text{eqn. B.2}
\]
In the gas phase the translational entropy of a molecule makes the highest contribution to the overall entropy, about 40 kJ/mol at 25°C\(^1\) (Table B.1, ref. 33, 80). The translational entropy shows only a slight positive dependence on mass - a ten fold mass increase results in only a 2 fold increase in entropy. The magnitude of the translational entropy is also proportional to the average volume accessible to the molecule. At high concentrations, the average volume available to the molecule decreases, with a corresponding decrease in translational entropy (33). Therefore the rotational entropy of a molecule can approach the magnitude\(^2\) of the translational entropy at high concentrations (Table B.1, 33). Rotational entropy relatively independent of both concentration and mass (33).

Conformational entropy is a function of the number of ways the nuclei in a molecule can orient themselves relative to one another. Steric interactions with other bonds and nuclei in the molecule are quite important as they can limit the conformations available to the molecule. In all but the simplest molecules, the total conformational entropy of a

\(^{1}\)The translational entropy can be calculated from the Sakur -Tetrode equation

\[
S_{\text{trans}} = R \ln(V) + \frac{3}{2} \ln(T) + \frac{3}{2} \ln(M)
\]

\text{eqn. B.3}

where \(V\) is the average volume occupied by the molecule, \(T\) is the temperature, \(M\) is the molecular mass and \(R\) is the gas constant (79).

\(^{2}\)The rotational entropy can be calculated from the symmetry number and moment of inertia of the molecule

\[
S_{\text{rot}} = R \ln \left( \frac{T}{(\sigma \theta_r^2)} \right) + R
\]

\text{eqn. B.4}

\[
\theta_r = \frac{\hbar^2}{(8\pi^2 I k)}
\]

\text{eqn. B.5}

where \(T\) is the temperature, \(\sigma\) is the symmetry number of the molecule, \(I\) is the moment of inertia and \(\theta_r\) is the rotational characteristic temperature (79). The symmetry number of a molecule is defined as "the number of indistinguishable positions in space that can be reached by rigid rotations" (79). Symmetry numbers and hence rotational entropies depend most strongly on molecular geometry and only slightly on mass.
molecule is much too difficult to calculate explicitly, since a great number of interactions must be considered. Individual values for different vibrational modes and for the rotation around a bond can be calculated, however, and representative values are listed in Table B.1 for the gas phase. The values for the entropy of individual vibrations and rotations are quite small compared to translational and rotational entropy of the molecule as a whole but their total contribution depends on the number of bonds, and in aggregate can be extremely large. For example, evidence suggests that the conformational entropy of the denatured state is the major opposition to protein folding. The effects of conformational entropy on macromolecular structure will be discussed later in this appendix.

Table B.1 - Typical Entropy Contributions From Translations, Rotations and Vibrations at 298 K in the Gas Phase

<table>
<thead>
<tr>
<th>Motion</th>
<th>$S^0$ (J/deg/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three degrees of Translational Freedom</td>
<td>120 - 150</td>
</tr>
<tr>
<td>Three degrees of Rotational Freedom</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>44</td>
</tr>
<tr>
<td>n-Propane</td>
<td>90</td>
</tr>
<tr>
<td>endo-Dicyclopentadiene</td>
<td>114</td>
</tr>
<tr>
<td>Internal Rotation</td>
<td>13-21</td>
</tr>
<tr>
<td>Vibrations (cm$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.4</td>
</tr>
<tr>
<td>800</td>
<td>0.8</td>
</tr>
<tr>
<td>400</td>
<td>4.2</td>
</tr>
<tr>
<td>200</td>
<td>9.2</td>
</tr>
<tr>
<td>100</td>
<td>14.2</td>
</tr>
</tbody>
</table>

1) Molecular weight 20 -200, standard state 1 M

Source of the Rate Acceleration in Intramolecular Processes

Enzymes, through their ability to bind substrates, act as agents to transform intermolecular reactions into intramolecular ones in biological systems. The binding
affinity comes from a number of complementary interactions between substrate and enzyme, including hydrogen bonding, hydrophobicity, van der Waals and electrostatic interactions. To determine the source of the rate accelerations observed in intramolecular and enzymatic reactions, Jencks and Page (80) constructed an artificial model, in which the intermolecular dimerization in the gas phase of two molecules is compared with its intramolecular counterpart, where the two molecules are linked by a flexible tether. A number of simplifying assumptions were made in the construction of this model. The effects of internal rotation, vibration and strain associated with the flexible tether were ignored - the tether simply holds the molecules in the proper orientation for bond formation. Furthermore, the effects of molecular interactions were ignored by using entropies measured in the gas phase. Finally, as the same bonds are being broken and formed in both cases, the change in enthalpy was assumed to be the same for the intra and intermolecular reactions.

In Tables B.1 and B.2 the changes in translational, rotational, and vibrational entropy are listed for the hypothetical intra and intermolecular reactions.

Table B.2 - Entropy Changes Associated with the Rates of Intra and Intermolecular Reactions

<table>
<thead>
<tr>
<th></th>
<th>Intermolecular</th>
<th>Intramolecular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A + B → A-B</td>
<td>A B → A-B</td>
</tr>
<tr>
<td>Number of Atoms</td>
<td>(m) (m) (2m)</td>
<td>(n) (n)</td>
</tr>
<tr>
<td>Degrees of Freedom</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translational</td>
<td>(3 + 3) (3)</td>
<td>(3) (3)</td>
</tr>
<tr>
<td>Rotational</td>
<td>(3 + 3) (3)</td>
<td>(3) (3)</td>
</tr>
<tr>
<td>Vibrational</td>
<td>(3m-6 + 3m-6) (6m-6)</td>
<td>(3n-6) (3n-6)</td>
</tr>
</tbody>
</table>


In the intermolecular reaction, 3 degrees each of translational and rotational freedom is
lost, and 6 degrees of vibrational freedom are gained while in the intramolecular process there is no net change in the number of degrees of freedom. From Table B.1, the overall entropy loss in the intermolecular reaction, relative to its intramolecular counterpart can be calculated. The entropy change associated with the loss of 3 degrees each of translational and rotational freedom ranges from 50 -90 kJ/mol. The entropy associated with the gain of 6 degrees of vibrational freedom can compensate for this to only a small extent as the individual magnitudes are small (4 - 6 kJ/mol, ref. 80). At 25°C and 1 M concentration, the overall entropy loss involved in the bimolecular reaction, relative to its intramolecular counterpart, is 50 -60 kJ/mol (80).

The effect of this entropy loss on the relative rate of the intra and intermolecular reactions can be calculated using activated complex theory (33, 80). In the intermolecular reaction the entropy loss has occurred in the formation of a unimolecular activated complex. The rate constant \( k \) is related to the entropy change from starting material to transition state

\[
k = C T e^{-\Delta S/R}
\]
eqn. B-6

where where \( C \) is a constant\(^3\). \( \Delta S \) corresponds to the entropy loss associated with the bimolecular reaction, relative to its intramolecular counterpart. In other words, the intramolecular reaction is favored entropically over the bimolecular reaction by the magnitude of \( \Delta S \). Substituting 55 Kcal/mol into equation B-6 yields a rate acceleration for the intramolecular reaction of approximately \( 6 \times 10^9 \) (33,80).

The rate acceleration associated with changing an intermolecular reaction to an intramolecular provides an intellectual framework for understanding the role of an enzyme in catalyzing a bimolecular reaction. During an enzymatic reaction, the substrates are bound to the enzyme active site by favorable interactions with the protein.

\(^3\) \( C = k_b T h C_0^6 \) where \( k_b \) is Boltzmann’s constant, \( h \) is Planck’s constant and \( C_0^6 \) is a factor for standard state concentration
The chemical reaction occurs and as both reactants are bound to the active site, the reaction proceeds as if it were intramolecular. After reaction, the product is released. In a sense, an enzyme transforms an intermolecular reaction to an intramolecular one. Applying the results of their model to the action of enzymes, Page and Jencks concluded that "translational and overall rotational motions provide the important entropic driving force for enzymatic and intramolecular rate accelerations".

The extension of the above analysis to enzymatic and intramolecular reactions in solution must be treated with caution. Jenck's equations and values for different types of entropy apply to molecules in the gas phase. As mentioned in Appendix A, the properties of a molecule in solution are strongly influenced by its interaction with the solvent. No theory exists for the direct calculation of entropies in solution and so the magnitude of their contribution to the total entropy of the molecule is unknown. Similarly, a molecule bound at the active site of an enzyme interacts with the functional groups present in a number of ways. The effect of hydrogen bonding, van der Waals forces, electrostatic interactions and the desolvation that occurs when a substrate binds are difficult to predict, so that a difference in rate, relative to a model bimolecular reaction, cannot be absolutely attributed to entropy. (63, 80).

**The Effect of Internal Rotations**

The conformational entropy of a molecule is comprised of both vibrational entropy and the entropy associated with internal rotations. In Page and Jenck's analysis presented above, the effect of internal rotations were ignored in calculating the entropy driven acceleration of intramolecular reactions. In this section the effect of rotatable bonds on reaction rate will be discussed.

What is the magnitude of the entropy associated with bond rotation? Page and Jencks (80) studied a series of hypothetical reactions forming rings of size $C_3 - C_8$ (Table B.3).
They observed that the magnitude of the entropy loss was roughly proportional to the number of bonds in rings up to C\textsubscript{6}. In larger rings, however the entropy loss upon cyclization seemed to level out. The leveling effect was attributed to the greater flexibility of the larger rings which allowed partial rotation around bonds, compensating somewhat for the effects of ring closure (80). After correction for these low frequency motions in the rings, they found that the entropy loss ranged from 3.7-4.9 e.u. (an average of 4.5 e.u.) per each internal rotation frozen out.

Table B.3 - Entropy Changes Accompanying Cyclization at 298 K\textsuperscript{1}

<table>
<thead>
<tr>
<th>System</th>
<th>$\Delta S^\circ$ (Cal deg$^{-1}$ mol$^{-1}$)</th>
<th>$\Delta S^\circ$/ (no. int. rotors)</th>
<th>$\Delta S^\circ$corr./ (no. int. rotors)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\begin{align*} &amp; \rightarrow \quad \Delta \end{align*} \begin{align*} &amp; \rightarrow \quad \square \end{align*} \begin{align*} &amp; \rightarrow \quad \pentagon \end{align*} \begin{align*} &amp; \rightarrow \quad \octagon \end{align*} \begin{align*} &amp; \rightarrow \quad \octagon \end{align*} \begin{align*} &amp; \rightarrow \quad \octagon \end{align*}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td>3.85</td>
<td>3.85</td>
</tr>
<tr>
<td></td>
<td>10.9</td>
<td>3.63</td>
<td>4.90</td>
</tr>
<tr>
<td></td>
<td>13.3</td>
<td>3.32</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>21.2</td>
<td>4.25</td>
<td>4.25</td>
</tr>
<tr>
<td></td>
<td>19.8</td>
<td>3.30</td>
<td>3.72</td>
</tr>
<tr>
<td></td>
<td>19.0</td>
<td>2.71</td>
<td>3.91</td>
</tr>
</tbody>
</table>


The effects of onformational entropy on ring closure have been explored for a number of ring forming reactions by Mandolini (75). Figure B.1 plots the entropy change $\theta \Delta S$ as a function of the number of rotors in the starting materials. The factor
\[ \theta \Delta S = \Delta S_{\text{intramolecular}} - \Delta S_{\text{intermolecular}} \]
eqn. B.9

may also be written as

\[ \theta \Delta S = \Delta S_{\text{conf.}} - (\Delta S_{\text{trans}} + \Delta S_{\text{rot}}) \]
eqn. B.10

The value \((\Delta S_{\text{trans}} + \Delta S_{\text{rot}})\) represents the change in entropy upon converting a bimolecular reaction to a unimolecular one in the absence of any rotatable bonds. The change in conformational entropy upon cyclization for an intramolecular reaction is represented by \(\Delta S_{\text{conf.}}\). \(\theta \Delta S\) therefore represents the decrease in the magnitude of
conformational entropy upon cyclization normalized for the corresponding model bimolecular reaction. This normalization allows different reaction types to be compared.

The data is remarkably consistent despite the range of reaction types measured. The slope of the line is -4.0 e.u. per rotor, which corresponds well with the value of -4.5 e.u. determined by Page and Jencks. The y intercept of the line measures the change in entropy for an ideal gas phase cyclization with no rotatable bonds. It therefore represents the only a change in rotational and translational entropy and is analogous to the difference between an intra and an intermolecular reaction in the Page and Jencks model. The value of the intercept, 33 e.u., yields an effective molarity of $10^{7.2}$ M, which corresponds well with the value of $10^8$ M determined by Page and Jencks.

How does the entropy associated with bond rotation affect the rate of intramolecular reactions? Table B-4 presents the relative rates for the cyclization of Br(CH$_2$)$_{n-1}$NH$_2$ in 30% isopropanol at 73°C.

<table>
<thead>
<tr>
<th>Ring Size $n$</th>
<th>6</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Rate</td>
<td>3000</td>
<td>47</td>
<td>1</td>
</tr>
</tbody>
</table>


The results show that the relative rate of cyclization drops off rapidly with the number of bonds in the ring. Using the of arguments of the Page and Jencks analysis, the results in Table B-4 can be analyzed. In the linear precursor, rotation around bonds contributes to the conformational entropy. A linear molecule of $n$ atoms will have $n-1$ bonds contributing to the entropy of internal rotation. In the formation of the ring these internal rotations are frozen out (Figure B.2). All other contributions to the overall entropy being equal, the formation of a ring is disfavored by the magnitude of the conformational entropy lost, which in turn depends on the number bonds. The following trend is
observed for a homologous series of polymers that differ from one another by the number of rotatable bonds. As the number of possible internal rotations increases, the rate of the ring forming reaction decreases.

**Strain Effects**

Linear molecules in solution exist as a constellation of interconverting minimum energy structures. Cyclization can result in unfavorable steric interactions and strained bond and torsional angles due to the structural constraints involved in forming the bond that closes the ring. This is illustrated in Figures B.3 and B.4 (75) which present the change in enthalpy (as compared to a model bimolecular reaction) and the relative rate of lactone formation in solution as a function of ring size. The high $\Delta H$ and low rate associated with the formation of small rings ($\leq 4$ members), is caused the geometry required to close the ring, deforming the bond angles and causing strain. Cyclization leading to a 5 membered ring shows a maximum rate and minimal enthalpy change, reflecting the formation of bond angles close to the optimal tetrahedral value. As ring sizes is increased still further however, the $\Delta H$ associated with lactone formation increases once more and the rate of reaction drops, indicating strain caused by gauche,
eclipsed and transannular interactions. The strain reaches a maximum for the formation

Figure B.3 - ΔH profile for the formation of lactones and for the related intermolecular reaction.

Figure B.4 - Reactivity profile for the formation of lactones in DMSO at 50°C
of rings with 8 or 9 members, after which it begins to drop again. The decrease in ring strain is caused by the increasing flexibility of larger rings, allowing partial bond rotation which enables the molecule to assume conformations that minimize the steric strain. For very large rings, ΔH approaches the value observed for the bimolecular reaction, implying that the strain associated with ring closure is virtually eliminated by the conformational flexibility of the ring. This is reflected in the rate, with little change observed as the ring size is increased from 13 to 23 members.

**Effective Molarity**

The advantage of converting an intermolecular reaction to an intramolecular one can be calculated from rate studies. By measuring the first order rate constant of an intramolecular reaction and dividing it by the second order rate constant of a model bimolecular reaction, a constant having the units of concentration is obtained

\[
\frac{k_{\text{intra}}}{k_{\text{inter}}} = \frac{t^{-1}}{t^{-1} M^{-1}} = M
\]

This quotient is called the effective molarity (EM; 63, 75) and can be thought of as the concentration of a neighboring group relative to the reactive center. It can also be thought of as the concentration of external reactant that must be added to the bimolecular system to cause a rate acceleration equivalent to that observed in the unimolecular reaction (63, 80). Effective molarity can also be determined from equilibrium studies, by determining the ratio of equilibrium constants for an intramolecular reaction and its bimolecular analog.

As discussed above, the rate of an intramolecular reaction is affected by the number of rotatable bonds and the presence of strain. For a series of ring forming reactions, the intramolecular reaction reaction rate decreases with an increase in the number of possible bond rotations in the starting material. As the rate of the intermolecular analog is a
constant, this rate decrease is reflected in a decrease in the effective molarity. The observed decrease in EM can be thought of as a decrease in the proximity of the two ends to one another. As the number of rotatable bonds increases, the number of unreactive rotamer distributions that the molecule can assume will increase as well, resulting in the decrease in EM. Ring strain also decreases the rate of the intramolecular reaction, resulting in a lower EM. Strain associated with the conformation that must be assumed for ring closure is unfavorable. As above, the fraction of "productive conformations" and therefore the EM, is decreased.

Choosing the proper model reactions for measurements of effective molarity can be problematic. Conversion of a bimolecular reaction to a unimolecular one can be accompanied by changes in solvation, hydrogen bonding, strain and even mechanism (63). For example, the hydrolysis of substituted phenyl succinates and glutarates occurs by intramolecular nucleophilic attack of the carboxylate anion on the acyl group to expel phenolate anion and form the cyclic anhydride (Figure B.5). Hydrolysis occurs in a subsequent step. The corresponding bimolecular reaction of phenyl acetates with acetate however, proceeds by general base catalysis rather than by nucleophilic attack by the carboxylate group (19). In many cases the remedy is to study a number of bimolecular models and choose the best based on all the available information (63). This approach has been used by Illuminati et al in the study of reactions forming large rings (45, 69).

\[ \text{C-OR} \xrightarrow{+} \text{C-OI} \]

\[ \text{O} \]

\[ \text{C-OH} \]

\[ \text{H}_2\text{O} \]

Figure B.5 - Hydrolysis of substituted phenyl succinates

In spite of the uncertainties involved in translating gas phase entropies to the liquid state,
and the difficulties in determining the proper bimolecular model, Page and Jenck's analysis has shown quite a good correspondence with experimental observations. Effective molarities in solution have been measured for a large number of systems and values as high as $10^8$ and higher have been observed (63). The results of these measurements have generally supported the analysis presented above, namely that the rate acceleration observed in intramolecular and enzymatic reactions is due to the conservation of translational and rotational entropy during reaction.

The Conformational Statistics of Polymers

The acyl transfer step involves a reaction between two functional groups located at the end points of a polymer. Understanding the orientation of the reacting groups is necessary to interpret the reaction rates and product ratios observed experimentally. In the absence of structure, the number of conformations accessible to a polymer in solution is enormous. Calculation of distinct conformations would be a difficult task. To simplify the problem, polymer chemists have developed statistical methods for determining the dimensions of interest in solution. In his classic work, Flory developed mathematical methods for calculating the probability distribution of the distance between the two ends of a polymer (35, 36).

Figure B.6 - Polymer chain represented as a random walk of fifty steps. reproduced from Flory, P.J., "Principles of Polymer Chemistry"

He showed that the conformation of the freely jointed polymer chain resembles the path
described by a freely diffusing molecule in solution where bonds represent the paths between collisions and the number of elements represent the number of collisions (Figure B.6). The probability, $W(r)$, of finding the two ends within a given distance of each other is a Gaussian distribution (Figure B.7) of the form:

$$W(r) = A r^2 e^{-B r^2}$$  

where $r$ represents the distance between the ends and $A$ and $B$ are constants. Subsequent extension of this model to polymer chains with various constraints on bond and dihedral angles showed that the form of the distribution was unchanged\(^{35, 36}\). The constraints could be accounted for by assumptions that allowed the real polymer chain to be approximated by an equivalent freely jointed chain.

![Figure B.7 - Radial distribution function $W(r)$](image)

**The Properties of Real Polymers**

In developing the equations to describe polymer conformation, polymer atoms were treated as point masses and bonds as vectors \(^{90}\). Real polymers however, differ from their models as their component atoms have a finite volume. The volume of the atoms in the real polymer requires that certain conformations accessible to the dimensionless
model are forbidden, as they would result in a polymer atom occupying space already occupied by other atoms (90). Long range interactions between substituents distant in chain sequence but near in space are primarily responsible for the effect of "excluded volume" on chain dimensions (35, 36). In addition to their dependence on the volume of the atoms, the long range effects depend heavily on solvent and on temperature.

Excluded volume causes real polymers to sample only a limited subset of the conformations available to the model, and therefore the distribution is skewed towards extended conformations (35, 36, 90). In compact conformations, much of the internal volume is occupied by the atoms, whereas extended conformations contain much more free space. As a result, the probability of an angularly allowed compact conformation being impossible due to interactions that place two segments in the same space, is higher than for an extended conformation (35, 36, 90). No methods for evaluating these effects computationally exist and correction factors must be determined experimentally.

**Macrocyclization Theory.**

Work by Flory and Mutter has shown that the equations describing polymer conformation in solution can be extended to the formation of rings from linear molecules (37). Let us consider a cyclization

\[
A-M_x-B \rightleftharpoons c-M_x+A-B \quad \text{eqn. B.10}
\]

where -Mx- is a chain molecule of x repeating units and c-Mx is the corresponding cyclic compound. The corresponding bimolecular reaction between two acyclic species is

\[
A-M_x-B + A-M_y-B \rightleftharpoons A-M_{(x+y)}-B + A-B \quad \text{eqn. B.11}
\]

The mechanism of bond formation in both processes is considered to be identical. The cyclization constant, Kx, is the ratio of the equilibrium constants governing the two
processes

\[ K_x = \frac{K_{\text{cyclization}}}{K_{\text{bimolecular}}} \quad \text{eqn. B.12} \]

and is equivalent to the effective molarity defined above.

In order for the cyclization of a linear molecule to occur, several conditions must be met: 1) the two reacting moieties must be situated at a distance from one another equal to the length of the forming bond; 2) the direction of the vector corresponding to the nascent bond must make acceptable bond and dihedral angles with adjacent bonds; and 3) the conformation necessary to form the bond must not result in torsional angles with unacceptable levels of strain. Together, these three conditions control the fraction of conformations that are capable of cyclizing (37).

As stated above, \( W(r) \), represents the probability of finding the two ends of a polymer within a given distance of each other. \( W(0) \), represents portion of the distribution \( W(r) \) where the two ends are separated by the length of the forming bond. The probabilities that the conformations assumed during cyclization satisfy the bond and torsional angle requirements are given by the factors \( \Gamma_0(1) \) and \( \langle \Phi_0 \rangle \), respectively. These values are calculated theoretically by treating the polymer bonds as vectors subject to angular constraints. The effective molarity for the two ends of a polymer in a cyclization is therefore given by:

\[ K_x = W(0)2\Gamma_0(1)\langle \Phi_0 \rangle / \sigma_{\text{cx}}N_A \quad \text{eqn. B.13} \]

where \( \sigma_{\text{cx}} \) is the symmetry number of the ring and \( N_A \) is Avogadro's number.

Calculation of \( \Gamma_0(1) \), \( \langle \Phi_0 \rangle \) and \( \sigma_{\text{cx}} \) is quite difficult and a number of simplifying assumptions are routinely used in evaluating equation B.13. For polymers of sufficient length and "flexibility", the directions of the bonds linking the reactive moieties to the polymer should be uncorrelated with one another, allowing the proper bond and torsional angles for bond formation to be assumed. In this case, both \( 2\Gamma_0(1) \) and \( \langle \Phi_0 \rangle = 1 \), as all reactive conformation are capable of meeting the angular restrictions (37). The
symmetry number is defined as the number of indistinguishable positions in space that can be reached by rigid rotations. As a result, it will be related to the geometry of the molecule and the number of rotatable bonds. For a homologous series of polymers, the symmetry number is be considered to be proportional to the number of rotatable bonds, n.

Using these simplifying assumptions, equation B.13 becomes:

\[ EM = K_x = \frac{W(0)}{Cn N_A} \]  

where \( C \) is a constant for a given homologous series of polymers (37).

Equation B.14 expresses the limiting form of the relationship between polymer conformation for very large n. The approximations underlying this relationship are the following: 1) the assumption that \( W(r) \) is Gaussian (from the polymer equations), and 2) the neglect of angle correlations. Other assumptions implicit in the treatment are that the symmetry number is proportional to the number of rotatable bonds and that the volume of the molecules in the chain can be ignored. All these approximations must fail as the size of the polymer decreases.

**Relationship between Effective Molarity and Macrocyclization Theory**

As discussed above, effective molarity is an experimentally defined value, determined from the ratio of the rate or equilibrium constants for a unimolecular reaction and its bimolecular counterpart. The macrocyclization constant \( K_x \), which is identical to \( EM \), is determined from a theoretical treatment of polymer conformation as a collection of vectors and is related to the probability that the two ends of a polymer are close enough to form a bond. Macrocyclization theory therefore, enables the effective molarity of long polymers to be determined theoretically.

Each of the terms \( W(0) \), \( 2\Gamma_0(1) \) and \( \langle \Phi_0 \rangle \), in equation B.13 can be related the effects of rotatable bonds and strain discussed above, allowing these effects to be treated separately. The computational study of a homologous series of polymers allows the
change in the relative magnitude of these effects to be studied as a function of polymer size, clarifying the structural constraints that determine effective molarity.

**Short Polymers in Solution**

The Gaussian distribution of the distance between the ends of a polymer was developed from a theoretical treatment of polymers as a collection of vectors subject to certain geometrical constraints and is based on the assumption of infinitely long polymer chains. Is the model valid for short polymers? The technique of fluorescence energy transfer allows the experimental determination of the probability distribution for the distance between the ends of a polymer by measuring the time dependent transfer of fluorescence energy from a chromophore attached to one end of a chain to a chromophore attached to the other end. The results have indicated that even for quite short polymers in solution, the distribution is indeed Gaussian (Figure B.8, ref. 47).

![Figure B.8](image-url)

Figure B.8 - The distribution function of the distances between donor and acceptor for the series of oligopeptides I, n = 4 - 9 determined by fluorescence energy transfer. Reproduced from Haas, E., Wilchek, M., Katchalski-Katzir, E. and Steinberg, Z., *Proc. Nat. Acad. Sci. USA*, 72, p.1807 (1975)

Implicit in the theoretical treatment of polymer conformation is the assumption that the polymer chains are infinite in length. This assumption enables the calculation of polymer conformation and $K_x$ from the equations given above. How well do the polymer
equations describe the conformations of short polymers? Figure B.9 shows the
conformation of two short polymers as determined by fluorescence energy transfer and
the theoretical distribution (70). The model used for the prediction is the rotational
isomeric state model of Flory$^4$, in which the probability of each conformation is
weighted by the conformation's internal energy. In order for the model to be used, a
number of simplifying assumptions must be made. The number of rotational positions
limited to the low energy minima, which in the case of an alkyl chain are trans, gauche +
and gauche - ; for a chain of $n$ links, this is equal to $3^{n-1}$ distinct conformations. The
total energy of a conformation is calculated as the sum of contributions from each bond;
these contributions depend only on the rotational position of a bond and the one
preceding it. Long range interaction, such as the 1-4 hydrogen bonds in an $\alpha$ helical
section of a protein are ignored (70). In Figure B.9 a reasonable fit was observed for the
largest of the polymers (28 rotatable bonds), but large deviations were observed for the
smaller polymers with 13, and 18 rotatable bonds respectively. The deviation is
especially large for short contact distances, corresponding to the portion of the
conformational distribution where the two ends are separated by the distance of a forming
bond, $W(0)$. Overall the failure of this model to predict the conformation distributions of
short molecules is clear.

The poor correlation of the theoretical values for short polymers can be attributed to
the simplifying assumptions used in developing the polymer equations. The volume of
atoms cannot be ignored as steric interactions can severely limit the number of acceptable
conformations. Furthermore, limiting the rotational positions to the three low energy
minima as well as ignoring long range interactions, steps necessary to make the RIS

$^4$ Of all the models currently available, this has been shown to best predict the
experimental results of medium and large polymers.
Figure B.9 - Comparison of the experimental distance distributions determined by fluorescence energy transfer (-----) and the rotational isomeric state model calculationsa (——) for TCD, TUD, and TU2D.

a) The rotational isomeric state model of Flory weighs the distance distribution by the Boltzmann energies for each conformation to calculate the probability of the conformations that lead to each end to end distance interval.

model computationally accessible, causes further error. As macrocyclization theory relies on the polymer models to determine $W(0)$, the inability of the polymer models to accurately predict the conformation of short polymers limits its use in effective molarity calculations. For short polymers like the thiol capture peptides, effective molarity must be determined experimentally.

**Protein and peptide structure in DMSO**

Implicit in our discussions of the effects of bond rotation on effective molarity and our discussion of polymer statistics, is the concept that polymers exist in solution as a constellation of interconverting conformations. Proteins however, are somewhat unique among polymers, as in some solvents a single well defined structure is observed. Although examples of well defined, highly populated conformations are rare for small peptides of the size studied in this thesis, a few examples have been observed for peptides containing artificial templates (3, 8, 59). In order to interpret the effective molarities observed for the thiol capture peptides in terms of the effects of bond rotation and strain, an appreciation for the likely thiol capture peptide structure in DMSO must be gained. The following section summarizes what is known about the structure of peptides and proteins in DMSO.

DMSO is one of the few organic solvents that can solubilize proteins and peptides. Evidence has shown that the dissolution is often accompanied by denaturation although there is some evidence for structure. Before discussing the evidence for and against structure in DMSO, the forces stabilizing and opposing protein structure should be briefly mentioned. A large body of research suggests that proteins in water are stabilized by a number of forces, including hydrogen bonding, van der Waals interactions, electrostatics, and hydrophobic interactions (2, 26, 31). The magnitude of contribution of each of these
effects however is a matter of contention and active research.

The hydrophobic effect was introduced in 1959 by Kauzman (54) in an insightful review that was all the more remarkable given the lack of structural data at the time. As briefly discussed in appendix A, the hydrophobic effect is based on the observation that when a nonpolar group is placed in water, the water assumes a highly ordered ice like (clatherate) structure around it. This structuring of water is accompanied by a loss of entropy. Unfolded proteins have nonpolar side chains exposed to water while in folded proteins, the side chains are shielded from solvent. Folded structure, therefore is stabilized by the gain in entropy of water that occurs upon burial of the nonpolar side chains in the protein core (54, 31).

That hydrogen bonding plays a role in the organization of protein structure is clear from secondary structure. In order to determine whether hydrogen bonding is involved in stabilizing the folded structures of proteins is less clear (2, 26, 31). In order to answer this question it is necessary to determine whether hydrogen bonds between the N-H and C=O groups in the folded protein are stronger than hydrogen bonds that would occur between these groups and water in the unfolded state. In short, does hydrogen bonding stabilize the folded or unfolded state. A number of model studies have been performed with small models in water and the results are not clear one way or the other. To complicate things, the large number of hydrogen bonds in a protein means that even a small error in estimating their strength would cause large errors in estimation of their effect on protein stability (31).

A similar difficulty occurs in estimating the effects of van der Waals interactions play in stabilizing protein structure (31). The packing efficiency of proteins has been determined to compare with that of organic crystals (packing densities of 0.72-0.77, ref.26). It is clear that the number of interactions is quite high. As with hydrogen bonds estimation of their stabilizing effect is impossible due to the level of uncertainty in their
A number of studies have indicated that the major force opposing protein folding is the conformational entropy of the unfolded chain. As discussed above for small molecules, internal vibrations and rotations are present in an unconstrained linear peptide chain. Folding of the protein would result in the loss of the conformational entropy, just as it is lost upon cyclization of small molecules.

Cross-linking the peptide by disulfides or chemical methods has provided an important test of the effect of conformational entropy on protein stability. Addition of a single cross link has been shown to increase the denaturation temperature of proteins by 25-30°C (2). By addition of a cross-link, the number of conformations accessible to the peptide is drastically reduced, decreasing the magnitude of entropy loss upon folding and thereby favoring the folded state.

Studies of protein structure in DMSO have indicated that it is a strong denaturant. The wide range of solubility of organic liquids indicates that the magnitude of the hydrophobic effect might be somewhat smaller in DMSO. DMSO is also able to form strong hydrogen bonds and can compete with the carbonyl of the peptide backbone for hydrogen bonds to the backbone amide protons. The demonstration of homo and heteroconjugation in DMSO however, shows that other hydrogen bond accepters can successfully compete for hydrogen bonds. It is therefore possible that under certain conditions, hydrogen bonded structure may exist in proteins and peptides dissolved in DMSO.

**Peptide Structure in DMSO**

NMR and molecular dynamics studies have been performed on a number of small peptides, including oxytocin, bradykinin, and analogues of somatostatin and enkephaline. Evidence suggests that in these highly structured and conformationally constrained
molecules, some structure is present in DMSO.

In the case of oxytocin\textsuperscript{5} (6), the structure obtained from the application of NOE restraints to molecular dynamic simulations yielded a solution structure that was very similar to the crystal structure of deamino-oxytocin. The analysis indicated that in DMSO the peptide contained two $\beta$-turn conformations, covering residues Tyr$^2$-Asn$^5$ (in the ring) and Cys$^6$-Gly$^9$ (tail region). Similarly, 2D NMR studies of the hexa\textsuperscript{6} and octapeptide\textsuperscript{7} ((Sandoz 201-456 and SMS 201-995, respectively) cyclic analogues of somatostatin (92, 93) have indicated that both adopt a conformation in DMSO that is less flexible than in water. N-H-CaH coupling constants, NOE effects and temperature coefficients of the amide protons indicate that the conformation contains a $\beta$II/ turn involving Phe$^3$-Trp$^4$-Lys$^5$. In the case of SMS 201-995 the turn is further stabilized by an additional hydrogen bond involving D-Phe$^1$ and Thr$^8$(ol).

Further evidence from linear peptides supports the case for $\beta$-structure in DMSO. Both the nonapeptide bradykinin\textsuperscript{8}(94) and 5 analogues of enkephalin\textsuperscript{9} (21) have been studied by NMR and were found to adopt $\beta$-bend conformations. In additions, work on linear peptide analogues containing epindolidione (Blanchard and Arico-Muendel thesis) in the Kemp labs provides strong evidence for the presence of sheet structure in DMSO. The peptides were designed to model both parallel and antiparallel sheets. Fourteen parallel sheet analogues (3) and more that 20 antiparallel sheet (8) analogues were

5 Sequence Cys$^1$-Tyr$^2$-Ile$^3$-Gln$^4$-Asn$^5$-Cys$^6$-Pro$^7$-Leu$^8$-Gly$^9$-NH$_2$

6 Sequence Cys$^2$-Phe$^3$-D-Trp$^4$-Lys$^5$-Thr$^6$-Cys$^7$-NH$_2$

7 Sequence D-Phe$^1$-Cys$^2$-Phe$^3$-D-Trp$^4$-Lys$^5$-Thr$^6$-Cys$^7$-Thr$^8$(ol)

8 Sequence Arg$^1$-Pro$^2$-Pro$^3$-Gly$^4$-Phe$^5$-Ser$^6$-Pro$^7$-Phe$^8$-Arg$^9$

9 Sequence Tyr-X-Gly-Phe-Y where $X = \text{D-Ala}$ or DNle and $Y = \text{D/L Leu}$ or $\text{D/L Nle}$
examined in DMSO and found to adopt, to a some degree, sheet structure, based on evidence from $^1$H NMR NOE, chemical shift, amide temperature dependence and N-H-CαH coupling constants (3).

In an attempt to understand peptide structure in DMSO, parameters allowing molecular dynamics simulations with DMSO have been developed (78). The solvent model has been used in a molecular dynamics simulation involving a model cyclic hexapeptide cyclo[-D-Ala-Phe-Val-Lys-Trp-Phe-]. The simulated structure found is similar to that observed by NMR, with two β-turns. Hydrogen bonds are formed between the solvent and the backbone amides and lysine side chain. In addition, intramolecular hydrogen bonds are observed between Val N-Hand Phe$_6$O and Phe$_6$ N-H and Lys O (with and without the application of NOE restraints). These bonds are especially interesting as it has been asserted that conformations of cyclic peptides are primarily stabilized by strong hydrogen bonds to the solvent. The modeling suggests that N-H O=C hydrogen bonds can even persist in a strong hydrogen bond accepting solvent like DMSO and may play a role in stabilization of the β-structure observed by NMR in other small peptides.

Although the evidence presented above suggests that β structure can exist in DMSO, caution must be used in extending these results to other small peptides. The peptides studied above are highly structured under most conditions, due to the presence of cyclic regions, D amino acids or structure nucleating templates. The one exception is bradykinin. Until more representative peptides have been studied in DMSO, the evidence for structure must be treated cautiously.

**Protein Structure in DMSO**

Unlike the small peptides discussed above, little evidence for protein structure in DMSO exists. The action of DMSO on proteins has been studied with a number of
techniques, including NMR, photo-CIDNP, and FT-IR. FT-IR studies in DMSO generally support the evidence for its denaturing effects. An early IR study of myoglobin (52) observed that denaturation occurs with increasing DMSO concentration. In pure DMSO, a band at 1621 cm\(^{-1}\) was observed. This band has been attributed by some investigators to the absorptions arising from the solvent accessible edges of \(\beta\)-sheets. The band has also been observed to accompany protein denaturation and aggregation, indicating that it may represent intermolecular hydrogen bonding resulting in the formation of \(\beta\)-sheets. The observation of this band indicates that in pure DMSO, myoglobin conformation may be a combination of \(\beta\)-structure and random chains.

A study of representative \(\alpha\)-helix (myoglobin), \(\beta\)-sheet (concanavalin A) and disordered (\(\alpha\)-casein) proteins has shown a regular progression of events associated with increasing DMSO concentration (51). At low concentrations (< 0.33 mole fraction), there seems to be no gross change in the structure of myoglobin and concanavalin A, although the thermal stability of these proteins is markedly reduced. Between 0.33 and 0.5 mole fraction DMSO, the spectra show the appearance of the 1621 cm\(^{-1}\) band. Above 0.5 mole fraction, the spectra indicate that DMSO may disrupt not only intramolecular but intermolecular hydrogen bonds as well. At 0.95 mole fraction and in pure DMSO, the 1621 cm\(^{-1}\) band is only a weak shoulder and a strong new band at 1662 cm\(^{-1}\) (characteristic of free C=O groups) appears. The authors interpret this as indicating that in pure DMSO all amide N-H groups are hydrogen bonded to solvent, leaving the C=O groups free. They suggest that in pure DMSO, the proteins are completely unfolded with all amides hydrogen bonded to solvent.

The protein \(\alpha\)-casein in DMSO behaves somewhat differently, as characterized by the absence of the 1621 cm\(^{-1}\) band. Instead a smooth transition to the 1662 cm\(^{-1}\) band is observed with increasing DMSO concentration. The authors suggest that unstructured proteins are primarily stabilized by hydrogen bonds between the backbone C=O and
water. The smooth transition with increasing DMSO concentration has been interpreted as a direct competition of S=O with C=O for hydrogen bonds to water.

In an FT-IR study of the Ca$^{2+}$ ATPase of sarcoplasmic reticulum in 70% DMSO(aq)(17), the authors also observe the presence of \(\beta\)-structure as characterized by the appearance of a band at 1638 cm\(^{-1}\), customarily associated with \(\beta\)-structure. They caution against using 1621 cm\(^{-1}\) as evidence however, suggesting that it is probably due in part to COOH vibrations.

NMR has been used to assess the rates of hydrogen isotope exchange in proteins dissolved in organic solvents (30). When fully deuterated BPTI was dissolved in DMSO containing 1% water, protected amides were found to exchange with solvent completely within 24 hours. In contrast, protected protons were not found to exchange after incubation in H\(_2\)O (pH3, 37\(^{\circ}\)C), or glycerol (known to stabilize protein structure) for 24 hours. Given that certain amides in BPTI do not show appreciable hydrogen exchange even after 2 months at 36\(^{\circ}\)C in D\(_2\)O (Wagner, G. and Wuthrich, K, J.Mol. Biol.160:343-361) the complete exchange in DMSO strongly suggests denaturation of the protein.

The denaturing effect of DMSO has been observed directly by NMR. Thermal and chemical (DMSO) denaturation of hen egg white lysozyme was been studied by Dobson et al (32). They observed that, based on \(\alpha\)C-H chemical shifts, disulfide reduced, DMSO denatured protein approached a random coil more closely than any other denatured state measured.

No evidence for \(\beta\)-structure was found in the chemically denatured lysozyme. A perturbation of the methyl resonance of Thr-51 indicates a close hydrophobic interaction with its side chain and the aromatic ring of Tyr-53. Both these residues are part of the \(\beta\)-sheet in the native conformation and could be evidence for structure in the denatured state. The \(\alpha\)CH chemical shifts however, do not support this. The authors suggest that
even in a random coil, an extended β-structure would be a relatively favored conformation and could result in the proximity of Tyr-53 and Thr-51.

The conclusion, that DMSO is one of the most aggressive denaturants, has been supported by a photochemically induced dynamic nuclear polarization study (photo-CIDNP) study on denatured hen egg white lysozyme (15). Photo-CIDNP uses a photoexcited dye to generate nuclear spin polarizations (detected as enhancements in the NMR spectrum) in tryptophan, tyrosine and histidine. The dye reacts only with accessible residues, thus providing a measure of the extent of unfolding of a protein.

DMSO denatured lysozyme, which was either fully reduced or with intact disulfides, was found to have a spectrum that closely resembled a random coil (modeled from a spectrum of a mixture of amino acids with the same proportions as lysozyme). By contrast, thermal and urea denaturation resulted in spectra which differed substantially from both the native protein and the random coil model. This was interpreted to indicate that under these conditions, a number of hydrophobic interactions were still present (15).

Overall, the evidence suggests that DMSO is a strongly denaturing solvent, although some structure may persist in a few circumstances. The evidence for structure in proteins is weak, and any structure present seems to be in water/DMSO mixtures rather than pure DMSO. DMSO/water mixtures however, behave very differently than either DMSO or water alone and it may not be possible to extend observations made in the mixture to pure DMSO. Although it is difficult to compare proteins and peptides, one could possibly attribute the lack of structure in proteins to the conformational entropy of the polypeptide chain. As discussed above, conformational entropy of the unfolded protein is considered to be one of the major effects opposing protein folding. As the concentration of DMSO increases there is a progressive loss of tertiary structure. This denaturation would be expected to increase protein chain flexibility with an accompanying increase in conformational entropy. As flexibility increases with increasing denaturation, the
entropic opposition would be expected to increase also until in pure DMSO the opposition due to entropy is high enough that even secondary structure is disrupted. The structurally constrained peptides have far fewer conformational degrees of freedom and as a result the entropic opposition would be less, allowing structure to persist even in pure DMSO. Given the evidence presented above, it is unlikely that the thiol capture peptide is as highly structured as the small peptides studied above.

Summary

Many observed rate accelerations can be accounted for by entropy changes. A model based on the comparison between unimolecular and bimolecular reactions attributes this rate acceleration to the differences in entropy changes associated with the reaction modes. The reaction of two molecules to form one results in the loss of both translational and rotational entropy. In the corresponding unimolecular reaction however this loss does not occur and consequently, unimolecular reactions are faster than their bimolecular counterparts. The model ignores the effect of bond rotation and strain on the rate of unimolecular reactions, both of which act to oppose cyclization. The effects of bond rotation are entropic and depend on the number of bonds, while strain effects are due to the geometry and size of the ring being formed.

The effects of converting a bimolecular reaction to a unimolecular one can be determined experimentally by a comparison of their rate or equilibrium constants. The effective molarity provides information on the proximity of the reacting groups. Studies of polymer conformation have led to a theoretical model which predicts the effective molarity computationally for medium to large polymers. For short polymers however, the simplifying assumptions that make the computation possible are not valid, with the result that effective molarity for these molecules must be determined experimentally.

In Chapter 2, the effective molarity of a series of thiol capture peptides is measured,
in order to determine the relative proximity of the lysine ε amine to the glycine phenolic ester. In order to interpret the experimental results in the light of bond rotation and strain discussed above, an appreciation of the structure of the thiol capture peptide in DMSO must be obtained. Given the evidence presented in this appendix, it is likely that, in DMSO, the thiol capture peptides resemble a random coil.
Experimental Section
Experimental Section

High resolution $^1$H NMR spectra were obtained on a Varian XL-300 instrument. Chemical shifts are reported in ppm downfield from $(CH_3)_4Si$ and splitting patterns are designated as s, singlet; d, doublet; t, triplet; dd, doublet of doublets; q, quartet; m, multiplet; b, broad. Plasma desorption mass spectra were recorded on an Applied Biosystems Biopolymer Mass Analyzer (model Bio-Ion 20). UV spectra were measured on a Hewlett-Packard Diode Array Spectrophotometer model 8452A. Amino acid sequencing was performed by the MIT Biopolymers Laboratory, Cambridge MA.

Analytical thin layer chromatography was performed on glass precoated silica gel 60 plates. Compounds were visualized by UV absorption (254 nm), or 1% ninhydrin in a 9:1 ethanol - TFA solution. Flash chromatography was performed on silica gel 60 (230-400 mesh).

Analytical HPLC was performed on a Waters system consisting of two Model 501 pumps, a Model 660 solvent programmer, a Model U6K injector, a Model 441 absorbance detector, a Model 746 data module, and a Vydac Protein and Peptide reverse phase C$_{18}$ column. Preparative HPLC was performed on a system including a Waters Associates Model 590 pump fitted with preparative heads, a Rheodyne injector, Autochrome OPG/S prepump mixer, a Waters Associates Model 450 variable wavelength detector, and a Vydac 218TP1022 reverse phase C$_{18}$ column.

Abbreviations:

DMF, dimethylformamide; CHA, cyclohexylamine; DMSO, dimethylsulfoxide; DMAP, 4-dimethylaminopyridine; DIEA, diisopropylethylamine; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HFIP, hexafluoroisopropanol, TFA, trifluoroacetic acid; HOBt, 1-hydroxybenzotriazole hydrate; Dbf, dibenzofuran;
ScmCl, methoxycarbonylsulfenyl chloride; Acm, acetamidomethyl; pNA, paranitroanilide; Z, benzyloxy carbonyl; Boc, tert-butyloxy carbonyl; Fmoc, 9-fluorenylmethoxy carbonyl.

Solvents and Reagents

All protected amino acids were of the L configuration and were purchased from Bachem Bioscience or synthesised from the free amino acid in this lab. All solvents and reagents are of reagent grade unless otherwise specified. ScmCl was prepared from COCl₂S (Aldrich) by a literature procedure, purified by fractional distillation and stored at -20°C in a sealed round bottom flask under argon. Burdick and Jackson DMF was used without purification for all couplings. DMSO (anhydrous, Aldrich) was used without further purification for acyl transfers. THF was freshly distilled from sodium benzophenone ketyl. HFIP, triethylphosphine, EDCI, and HOBt were purchased from Aldrich and used without further purification. TFA (sequanal grade) and DIEA were purchased from Pierce.

Preparation of Z-Cys(Acm) OH

To a solution of HCl·HCys(Acm)OH (5g, 0.022 mol) in 150 mL saturated NaHCO₃ at 4°C, was added 20 g solid NaHCO₃ followed by the dropwise addition of benzyl chloroformate (10 mL, 0.069 mol). The solution was stirred for 1 hour and then placed in a refrigerator at 4°C overnight. The NaHCO₃ was brought into solution by the addition of distilled water (500 mL) and the aqueous phase was washed 4 times with a total of 500 mL CH₂Cl₂ to remove excess benzyl chloroformate. The aqueous phase was acidified to pH 1 by the addition of concentrated HCl and extracted 3x with a total of 500 mL CH₂Cl₂. The organic extracts were pooled and washed with water (1x), with brine (1x) and dried with MgSO₄. The CH₂Cl₂ was removed in vacuo and the resulting oil foamed
under high vacuum. TLC (17:2:1 CHCl₃:CH₃OH:Acetic Acid) indicated that the product was pure. Yield (2.51 g, 7.7 mmol, 35%).

Synthesis of Models for Thiol capture and Acyl transfer products.

Z-L-Cys(4-acetoxydibenzofuranyl-6-thia)-OH (a spectroscopic model for the thiol capture product) was synthesized by the method presented in Fotouhi, Galakatos, and Kemp (1989). Z-L-Cys (4-hydroxydibenzofuranyl-6-thia)OH-CHA (a spectroscopic model for the acyl transfer product) was produced by the same method. To a solution of Z-L-Cys(Scm)-OH-CHA (0.71 g, 1.60 mmol) in HFIP-CH₂Cl₂-H₂O (5:3:1) was added 4-hydroxy-6-mercaptodibenzo[6]furan (0.34 g, 1.57 mmol). The reaction was stirred for 2 hours at room temperature under N₂. The solvent was removed and the resulting oil taken up in toluene to which was added cyclohexylamine (191 μl, 1.66 mmol) resulting in a solid. Precipitation from toluene - hexanes (2x) yielded the Z-L-Cys(4-hydroxydibenzofuranyl-6-thia)-OH-CHA pure by NMR (>99%). Yield (0.55 g, 0.97 mmol, 63%).

Z-L-Cys(4-acetoxydibenzofuranyl-6-thia)-OH -¹H NMR (300 MHz, 80% CDCl₃-20 % deuterated DMSO): δ 7.88 (1 H, d), 7.81 (1 H, d), 7.70 (1 H, d), 7.39-7.21 (8 H, m), 5.96 (1 H, d), 5.07 (2 H, q), 4.66 (1H, q), 3.48 (1 H, dd), 3.37 (1 H, dd), 2.46 (3 H, s)

Z-L-Cys(4-hydroxydibenzofuranyl-6-thia)-OH-CHA -¹H NMR (300 MHz, deuterated DMSO), δ 8.8 (3 H, s, b), 8.05 (1 H, d), 7.75 (1 H, d), 7.54 (1 H, d), 7.42 - 7.19 (7 H, m), 7.12 (1 H, d), 6.80 (1 H, d), 5.0 (2 H, s), 4.13 (1 H, m), 3.51 3.46 (1 H, dd), 3.12 (1 H, q), 2.91 (1 H, m), 1.85 (2 H, m), 1.69 (2 H, m), 1.57 (1 H, d), 1.3 - 1.1 (5 H, m).

Preparation of Z-Gly-4-hydroxydibenzofuranyl ester (Z-Gly-O-Dbf)

A solution of 4-hydroxydibenzofuran (1.6 g, 8.7 mmol), Z-Gly-OH (1.88 g, 9.0 mmol), DMAP (1.22 g, 10.0 mmol); and EDCI (2.03 g, 10.6 mmol) in 20 mL CH₂Cl₂ was stirred at room temperature for 2 hours. The reaction was poured into 200 mL
CH₂Cl₂ and washed with water (3x). The CH₂Cl₂ was dried with MgSO₄ and the solvent removed in vacuo. The product was further purified by flash chromatography (17:2:1 CHCl₃:CH₃OH:Acetic Acid), the solvents removed in vacuo and the residue triturated with 1:1 diethyl ether: petroleum ether (1x) and 1:1 hexane:CH₂Cl₂ (1x). The product was dried under high vacuum overnight yielding a white powder (0.83 g, 2.2 mmol, 25%). TLC (17:2:1 CHCl₃:CH₃OH:Acetic Acid) showed a single spot and ¹H NMR showed only the peaks expected for the product.

Z-Gly-4-hydroxydibenzofuranyl ester - ¹H NMR (300MHz CDCl₃) δ 7.95 (1 H, d), 7.84 (1 H, d), 7.19 - 7.66 (8 H, m), 5.39 (1 H, s, b), 5.17 (2 H, s), 4.45 (2 H, d).

Preparation of Z-Lys(H·TFA)-benzylamide

A solution of Z-Lys(Boc)OH (5.0 g, 13 mmol), benzylamine (1.42 mL, 13 mmol), HOBt (1.99 g, 14.7 mmol), and EDCI (2.5 g, 13 mmol) in 25 mL CH₂Cl₂ at 4°C under N₂ was stirred for 2 hours. The solution was then poured into 500 mL CH₂Cl₂ and subjected to the following washes: 0.5 M citrate buffer pH 3.5 (1x), water (1x), saturated NaHCO₃ (1x), water (1x), and brine (1x). The CH₂Cl₂ was dried with MgSO₄, evaporated and sonicated in diethyl ether. The solid was pelleted with centrifgation, dried under high vaccuum and dissolved in 50% TFA in CH₂Cl₂ under N₂ at 4°C. The solution was stirred for 1 hour at 4°C and the solvents removed in vacuo. The resulting oil was triturated in diethyl ether with sonication and and the solid recovered by centrifugation in a clinical centrifuge. The cycle of sonication in diethyl ether and centrifugation was repeated 5x, after which the resulting powder was dried overnight under high vacuum. NMR showed the product to be pure - overall yield for 2 steps (2.39 g, 4.94 mmol, 39%).

Z-Lys(H·TFA)-benzylamide - ¹H NMR (300 MHz, deuterated DMSO) δ 8.48 (1 H, t), 7.71-7.87 (3 H, s, b), 7.18-7.51 (11 H, m), 5.03 (2 H, s), 4.28 (2 H, d), 4.01 (1 H, m), 2.74 (2 H, s, b), 1.45 - 1.72 (4 H, m, b), 1.32 (2 H, m, b).
Preparation of Z-Ala-Phe-Gly-O-Dbf-SH

The acylation method is described below and follows Fotouhi, Galakatos, and Kemp (1989). Scale of synthesis was 1.82 mmol. Starting from the C-terminus amino acids are listed in the order of acylation. Purity is reported from the HPLC traces: Boc-Gly (96%), Boc-Phe (96%), Z-Ala (92%).

Method of Acylation: A weighed sample of preswelled (CH$_2$Cl$_2$) 4-hydroxydibenzofuran-6-thia-functionalized polystyrene (prepared as described in Fotouhi, Galakatos, and Kemp 1989) was placed in the reaction vessel and subjected to the following washes and chemical steps: (1) CH$_2$Cl$_2$ (4x1 min); (2) Acylation - 3 equivalents DIEA and 4 equivalents amino acid anhydride (1 x 15-30 min) prepared as described in Fotouhi, Galakatos, and Kemp 1989; (3) CH$_2$Cl$_2$ (6 x 1 min); (4) Deprotection - 50% TFA in CH$_2$Cl$_2$ (1 x 1 min, 1 x 20 min); (6) CH$_2$Cl$_2$ (6 x 1min).

Capping was not used after the acylation step due to the shortness of the peptide. After step 3 the completeness of acylation was assayed by triethyl phosphine cleavage and HPLC (70% CH$_3$CN - 30% 0.1% TFA aq.). Likewise completeness of deprotection was assayed after step 6.

General method for release of Z-Ala-Phe-Gly-O-Dbf-SH from resin: The resin bound peptide Z-A-F-G-O-Dbf-SH was released from the resin as needed for thiol capture by the following method. A weighed amount of resin was washed in the reaction vessel with CH$_2$Cl$_2$-HFIP (4:1, 2 x 1 min) and then swelled in CH$_2$Cl$_2$-HFIP (4:1) containing triethyl phosphine (1.2 equivalents). After 15 minutes the solvent was filtered under positive nitrogen pressure and collected in a 100 mL flask. The resin was washed with CH$_2$Cl$_2$ (4:1, 3 x 1 min) and the washes and filtrates evaporated. The resulting oil was triturated with hexanes - diethyl ether 1:1 with sonication, yielding a white suspension. The solid was pelleted by centrifugation in a table top clinical centrifuge and the liquid
discarded. The trituration was repeated 3 times, and then the pellet dried briefly under high vacuum. Typical yields for release from resin -75 - 81% after trituration.

Z-Ala-Phe-Gly-O-Dbf-SH - $^1$H NMR (300 MHz, deuterated DMSO) $\delta$ 8.06 (2 H, t), 7.97 (1 H, d), 7.54 (1 H, d), 7.17-7.46 (15 H, m), 5.73 (1 H, s), 5.01 (2 H, d), 4.65 (1 H, q), 4.35 (1 H, d), 4.07 (1 H, t), 3.10 (1 H, dd), 2.85 (1 H, dd), 1.13 (3 H, d)

Mass spectrum [MH$^+$] calculated 625.6 [MH$^+$] observed 626.2

**Standard Methods for Solution Phase Synthesis of the Cysteine Model Peptides**

Work Up: Standard procedures are described below and will be referred to in the text by letter. Other purification procedures will be described in the body of the text where relevant

**Method A** - after the completion of the coupling, the peptide solution was dissolved in CH$_2$Cl$_2$ and then subjected to the following washes: 0.5 M citrate buffer pH 3.5 (3x), 5% NaHCO$_3$ (3x), water (1-2x), Brine (1x). The CH$_2$Cl$_2$ was dried with MgSO$_4$, the solvent removed in vacuo and the resulting residue dried overnight under high vacuum.

**Method B** - (used in cases where Fmoc was present) - Identical to Method A with the elimination of the 5% NaHCO$_3$ washes.

**Method C** - after completion of the coupling the reaction solvents were removed in vacuo and the product was suspended in 0.5 M citrate buffer, pH 3.5 with sonication followed by centrifugation in a clinical centrifuge. The cycle was repeated 3x with the citrate buffer, followed by 1x with distilled water, 3x with 5% NaHCO$_3$, and 3x with distilled water. The resulting solid was dried overnight under high vacuum.

**Method D** - (used in the case of insoluble products containing Fmoc protecting groups) - Identical to C with the elimination of the 5% NaHCO$_3$ steps.

**HPLC:** HPLC was used to follow the progress of reactions and to determine the purity of the products. The changes in retention times relative to the expected change in polarity
for a given transformation was used as an initial method of characterizing the products of
a reaction and indicated that the reaction had proceeded properly. Standard HPLC
conditions are tabulated below and will be referred to in the text by letter.

**Assay**  
**Solvent System**  
A  40% CH₃CN-60% 0.1% TFA aq. to 100% CH₃CN over 10 minutes  
B  40% CH₃CN-60% 0.1% TFA aq. to 100% CH₃CN over 20 minutes  
C  30% CH₃CN-70% 0.1% TFA aq. to 100% CH₃CN over 10 minutes  
D  50% CH₃CN-50% 0.1% TFA aq. to 100% CH₃CN over 10 minutes  
E  10% CH₃CN-90% 0.1% TFA aq. to 100% CH₃CN over 10 minutes  
F  60% CH₃CN-40% 0.1% TFA aq. to 100% CH₃CN over 10 minutes  
G  25% CH₃CN-75% 0.1% TFA aq. to 75% CH₃CN-25% 0.1% TFA aq. over
10 minutes  
H  25% CH₃CN-75% 0.1% TFAaq. to 100% CH₃CN over 10 minutes  
I  55% CH₃CN-45% 0.1% TFA aq.  
J  20% CH₃CN-80% 0.1% TFA aq. to 100% CH₃CN over 20 minutes

**Deprotection:** Standard methods used for the removal of Boc, Fmoc and tBu are
described below:

**Boc** - The peptide was dissolved in 50% TFA:50% CH₂Cl₂ containing 1 equivalent of
anisole under N₂ and stirred for 30 minutes at 4°C. The solvent was removed in vacuo
and the residue sonicated in diethyl ether. Centrifugation at top speed in a clinical
centrifuge was used to pellet the resulting powder, the diethyl ether was removed and the
process repeated 4-6 times. The peptide was dried under high vacuum and used without
additional purification unless otherwise noted.

**Fmoc** - The peptide was dissolved in 30 % piperidine in DMF under N₂ and stirred for
10 minutes. The solvents were evaporated on a rotary evaporator under high vacuum and
the residue was evaporated 3x from toluene under high vacuum to remove traces of the
deprotection solvents. The resulting residue was sonicated in diethyl ether with
centrifugation (4x) to remove the dibenzofulvalene. The resulting powder was dried
under high vacuum and used without further purification unless otherwise noted.
tBu - The peptide was dissolved in neat TFA under N₂ and stirred for 1 hour at room temperature. The solvents were evaporated under high vacuum with a rotary evaporator and diethyl ether was added to the residue. The peptide was triturated with sonication and centrifugation (3x) to remove the TFA. The resulting powder was dried under high vacuum and used without further purification unless otherwise noted.

Scm derivatization - The standard procedure developed by Nader Fotouhi for the derivatization of fully protected peptides was used (described below) with the substitution of DIEA for diethylaniline. The peptide was dissolved in 1:1 CH₂Cl₂:CH₃OH to the concentration of 1-2 mg/mL and cooled to 4°C. DIEA (1eq.) was added followed by freshly distilled ScmCl (2 eq.) and the solution was stirred for 1 hour at 4°C under N₂. The solvent was then evaporated and the residue triturated with diethyl ether (3x) and dried briefly under high vacuum. In all cases the peptide was immediately deprotected by the standard Boc deprotection method.

Yields - Yields were determined for the purified compound as crude yields would reflect the weight of contaminating coupling agents and unreacted fragments. As the goal of the model peptide synthesis was to obtain enough material to study, most of the reactions were only run once, using a standard procedure. Reaction temperatures, solvents and purification procedures were not optimized, which was reflected in the yields.

Preparation of TFA·H-Cys(Scm)Lys(H·TFA)-pNA
Boc-Cys(Acm)-Lys(Boc)-pNA

A solution of Boc-Cys(Acm)-OH (0.52 g, 1.8 mmol), H-Lys(Boc)-pNA (0.59 g, 1.61 mmol) and HOBt (0.24 g, 1.77 mmol) and EDCI (0.36 g, 1.88 mmol) in THF (20 mL) at 4°C under N₂ was stirred for 1 hour and then allowed to warm up to room temperature.
with stirring for an additional hour. The THF was removed in vacuo and the resulting thick oil was worked up by method A. The crude product was further purified by flash chromatography (9:1 CHCl₃:CH₃OH ). Yield (0.81 g, 1.25 mmol, 76%). Purity by HPLC - 86% (λ, 214 nm; retention time - 5.3 min).

Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Lys(Boc)-pNA (0.45 g, 0.70 mmol) was reacted with ScmCl and Boc deprotected by the standard protocols yielding TFA•H-Cys(Scm)Lys(•TFA)-pNA as a powder (0.27 g, 0.39 mmol, 57%). Purity by HPLC - 99% (λ,214 nm; retention time - 9.1 min.)

TFA•H-Cys(Scm)Lys(•TFA)-pNA - ¹H NMR (300 MHz, D₂O) δ 8.23 (2 H, d), 7.65 (2 H, d), 4.57 (1 H, t), 4.37 (1 H, t), 3.82 (3 H, s), 3.42 (2 H, m), 3.01 (2 H, t), 1.93 (2 H, m), 1.74 (2 H, m), 1.50 (2 H, m).

Preparation of TFA•H-Cys(Scm)-Lys(Z)-pNA

Boc-Cys(Acm)-Lys(Z)- pNA

To a solution of Boc-Cys(Acm)-OH (0.60 g, 2.05 mmol ),TFA•H-Lys(Z)- pNA (1.0g, 1.94 mmol), DIEA (0.350 mL, 2.0 mmol) and HOBt (0.28 g, 2.1 mmol) in THF (20 mL) at 40°C under nitrogen was added EDCI (0.40 g, 2.0 mmol). The reaction was stirred at 40°C for 1 hour and then allowed to warm up to room temperature with stirring for 1 hour. The THF was removed in vacuo and the residue worked up by method A. Further purification was by flash chromatography (9:1 CHCl₃:CH₃OH ) yielding a white powder (1.15 g, 1.70 mmol, 87%). Purity by HPLC - 99% (λ, 313; retention time - 7.33 min).

Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Lys(Z)-pNA (1.15 g, 1.70 mmol) was reacted with ScmCl and Boc
deprotected by the standard protocols yielding TFA·H-Cys(Scm)-Lys(Z)-pNA as a powder (0.731 g, 1.03 mmol, 61%). Purity by HPLC - 94% (D, 313 nm; retention time - 4.39 min).

TFA·H-Cys(Scm)-Lys(Z)-pNA - $^1$H NMR (300 MHz, CD$_3$OD) δ 8.19 (2 H, d), 7.80 (2 H, d), 7.31 (5 H, s), 5.02 (2 H, s), 4.52 (1 H, q), 4.18 (1 H, q), 3.91 (3 H, s), 3.45 (1 H, dd), 3.21 - 3.04 (3 H, m), 1.98 - 1.71 (2 H, m), 1.64 - 1.40 (4 H, m).

Mass spectrum [MH$^+$] calculated 593.6 [MH$^+$] observed 593.7

Preparation of Z-Cys(Scm)-Lys(H'TFA)-pNA

Z-Cys(sTrit)-Lys(Boc)-pNA

A solution of Z-Cys(Trit)OH (1.34 g, 2.7 mmol), H-Lys(Boc)-pNA (0.95 g, 2.6 mmol), HOBt (0.36 g, 2.7 mmol), and EDCI (0.52 g, 2.7 mmol) in 50 mL THF at 4°C under N$_2$. The reaction was stirred for 2 hours and worked up by method A, yielding a white foam (2.05 g, 2.41 mmol, 92%). Purity by HPLC - 96% (F, 313 nm; retention time - 14.04 minutes.).

Scm derivatization and TFA deprotection

Z-Cys(sTrit)-Lys(Boc)-pNA (2.05 g, 2.41 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding Z-Cys(Scm)-Lys(H'TFA)-pNA as a powder (0.51 g, 0.71 mmol, 29%). Purity by HPLC - 97% (E, 313 nm; retention time - 13.66 minutes.).

Z-Cys(Scm)-Lys(H'TFA)-pNA - $^1$H NMR (300 MHz, CD$_3$OD) δ 10.73 (1 H, s, b), 8.49 (1 H, d), 8.23 (2 H, d), 7.78 - 7.70 (6 H, m), 7.34 (5 H, m), 5.07 (2 H, d), 4.38 (2 H, m), 3.87 (3 H, s), 3.21 (1 H, dd), 3.02 (1 H, dd), 2.79 (2 H, q), 1.80 (2 H, m), 1.54 (2 H, m), 1.35 (2 H, m).

Mass spectrum [MH$^+$] calculated 593.6 [MH$^+$] observed 594.1
Preparation of TFA·H-Cys(Scm)Ala-Lys(Z)-pNA

Boc-Ala-Lys(Z)- pNA

To a solution of Boc-Ala-OH (0.154 g, 0.814 mmol), TFA·H-Lys(Z)- pNA (0.38 g, 0.74 mmol), DIEA (128 µl, 0.74 mmol), and HOBt (0.11 g, 0.81 mmol) in CH₂Cl₂ (20 mL) at 4°C under nitrogen was added EDCI (0.17 g, 0.89 mmol). The reaction was stirred at 4°C for 1 hour and then allowed to warm up to room temperature with stirring for an additional hour. The THF was removed in vacuo and the residue worked up by method A. The product was taken directly to the next step without further purification.

Boc-Cys(Acm)-Ala-Lys(Z)- pNA

Boc-Ala-Lys(Z)- pNA (0.74 mmol, theoretical yield) was deprotected by the standard Boc protocol and the crude product dissolved in THF (10 mL) at 4°C under nitrogen. To the solution was added, in the order listed, Boc-Cys(Acm)-OH (0.238 g, 0.814 mmol), HOBt (0.11 g, 0.814 mmol), DIEA (128 µL, 0.74 mmol), and EDCI (0.172 g, 0.90 mmol). The reaction was stirred at 4°C for 1 hour and then allowed to warm up to room temperature with stirring for an additional hour. The THF was removed in vacuo and the resulting thick oil worked up by method A. The crude product was further purified by preparative TLC (9:1 CHCl₃:CH₃OH). Overall yield from TFA·H-Lys(Z)- pNA (0.33 g, 0.44 mmol, 60%). Purity by HPLC - 90% (D, 313; retention time - 6.7 minutes).

Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Ala-Lys(Z)-pNA (0.33 g, 0.443 mmol) was reacted with ScmCl and Boc deprotected by the standard protocols yielding TFA·HCys(Scm)Ala-Lys(Z)-pNA as a powder (0.228 g, 0.292 mmol, 66%). Purity by HPLC 96% (D, 313 nm; retention time - 3.97 minutes.)

Mass spectrum [MH⁺] calculated 665.6 [MH⁺] observed 665.1
Preparation of TFA-H-Cys(Scm)-Ala-Lys(H*TFA)-pNA

Fmoc Ala-Lys(Boc)-pNA

To a solution of Fmoc-Ala-OH (2.0 g, 6.4 mmol), H-Lys(Boc)-pNA (2.0 g, 5.4 mmol), HOBt (0.81 g, 6.0 mmol) in 100 mL CH₂Cl₂ at 4°C was added EDCI (1.2 g, 6.2 mmol). The solution was stirred for 2 hours at 4°C and monitored by TLC (9:1 CHCl₃:CH₃OH). The reaction was worked up by method B, yielding a powder (3.0 g, 0.45 mmol, 83%). NMR showed only the peaks expected for the peptide and the product was used without further purification.

Fmoc Ala-Lys(Boc)-pNA -¹H NMR (300MHz, deuterated DMSO) δ 8.21 (3 H, m), 7.88 (4 H, t), 7.73 (2 H, t), 7.58 (1 H, d), 7.42 - 7.33 (5 H, m), 6.76 (1 H, t), 4.40 (1 H, q), 4.32 - 4.11 (4 H, m), 2.90 (2 H, q), 1.81 - 1.59 (2 H, m), 1.50 - 1.24 (16 H, m)

Mass spectrum [MH⁺] calculated 682.7 [MH⁺] observed 682.3

Boc-Cys(Acm)-Ala-Lys(Boc)-pNA

Fmoc-Ala-Lys (Boc) -pNA (1.5 g, 2.3 mmol) was deprotected using the standard Fmoc protocol. To a solution of H-Ala-Lys(Boc)-pNA (2.27 mmol, estimated from starting material), Boc-Cys(Acm)-OH (0.73 g, 2.5 mmol), and HOBt (0.39 g, 2.9 mmol) in 50 mL THF at 4°C under nitrogen was added EDCI (0.48 g, 2.5 mmol). The reaction was stirred for 1 hour at 4°C and then allowed to warm up to room temperature and stirred for an additional hour. The THF was removed in vacuo and the resulting thick oil worked up by method A. The products were further purified by flash chromatography (9:1 CHCl₃:CH₃OH) yielding a white foam after evaporation of the chromatograph solvents (1.0 g, 1.5 mmol, 64%). Purity by HPLC - 98% (I, 214; retention time - 4.84 minutes)
Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Ala-Lys(Boc)-pNA (0.12g, 0.17 mmol) was reacted with ScmCl and Boc deprotected by the standard protocols yielding TFA·H-Cys(Acm)-Ala-Lys(H·TFA)-pNA as a powder (0.05 g, 0.07 mmol, 40%). Purity by HPLC - 97% pure (E,313 nm; retention time - 8.68 minutes.). Peptide characterized after thiol capture.

Preparation of Z-Cys(Scm)-Ala-Lys(H·TFA)-pNA

Z-Cys(sTrit)-Ala-Lys(Boc)-pNA

To a solution of H-Ala-Lys(Boc)-pNA (0.41 mmol, from standard Fmoc protection of Fmoc-Ala-Lys(Boc)-pNA) in 20 mL CH₂Cl₂ at 40°C under nitrogen was added Z-Cys(sTrit)OH (0.20 g, 0.41 mmol), HOBt (0.07 g, 0.52 mmol) and EDCI (0.09 g, 0.47 mmol). The reaction was allowed to stir at 40°C and the progress was followed by TLC (17:2:1 CHCl₃:CH₃OH:Acetic Acid). After the TLC indicated no remaining starting material, the solution was poured into CH₂Cl₂ and worked up by method B. The product was further purified by preparative thin layer chromatography (17:2:1 CHCl₃:CH₃OH:Acetic Acid), yielding a white foam (0.15 g, 0.16 mmol, 40% yield). Purity by HPLC - 91% (F, 313 nm; retention time - 13.64 minutes.)

Scm derivatization and TFA deprotection

Z-Cys(sTrit)-Ala-Lys(Boc)-pNA (0.15g, 0.16 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding Z-Cys(Scm)-Ala-Lys(H·TFA)-pNA as a powder (0.06 g, 0.077 mmol, 47%). Purity by HPLC - 92% (E, 313 nm; retention time - 13.62 minutes).

Mass spectrum  [MH⁺] calculated 665.6  [MH⁺] observed 665.4
Preparation of TFA·H-Cys(Scm)-Ala₂-Lys(H·TFA)-pNA

Fmoc-Ala₂-OH

A solution of H-Ala-OtBu·HCl (6.69 g, 36.8 mmol), DIEA (6.4 mL, 36.7 mmol), Fmoc-Ala-OH (11.51 g, 37.0 mmol), HOBt (5.66 g, 37.0 mmol), and EDCI (7.1 g, 37.0 mmol) in 100 mL THF, at 4°C under N₂, was stirred for 2 hours, and monitored by TLC (9:1 CHCl₃: CH₃OH). When the TLC indicated completion, the THF was evaporated and the reaction worked up by method B. The crude residue was deprotected by the standard tBu procedure and placed under high vacuum overnight yielding a white powder (9.25 g, 24.2 mmol, 66%). Purity by HPLC > 95% (C, 214; retention time - 11.55 minutes).

Fmoc-Ala-Ala-OH ¹H NMR (300 MHz, CD₃OD) δ 7.78 (2 H, d), 7.65 (2 H, m), 7.41-7.26 (4 H, m), 4.45-4.29 (3 H, m), 4.25-4.12 (2H, m), 1.41 (3 H, d), 1.32 (3H, d).

Mass spectrum [MH⁺] calculated 382.3 [MH⁺] observed 384.1

Fmoc-Ala₂-Lys(Boc)-pNA

To a solution of Fmoc-Ala₂OH (2.7 g, 7.0 mmol), H-Lys(Boc)-pNA (2.5 g, 6.8 mmol), HOBt (1.1 g, 7.9 mmol) in 50 mL THF at 4°C was added EDCI (1.3 g, 7.0 mmol). The solution was stirred for 3 hours at 4°C, under a nitrogen atmosphere and monitored by TLC (9:1 CHCl₃: CH₃OH). The THF was removed in vacuo and the residue purified by method B. Crude Yield (4.66 g, mmol, %). The crude product was purified by flash chromatography (9:1 CHCl₃:CH₃OH) yielding pure peptide (4.05 g, 5.5 mmol, 82%). Peptide was 99% pure by HPLC (D, 214; retention time - 12.04 minutes).

H-Ala₂Lys(Boc)-pNA

A solution of Fmoc-Ala₂-Lys(Boc)-pNA (1.86 g, 2.55 mmol) was deprotected by the
standard Fmoc protocol, yielding H-Ala2-Lys(Boc)-pNA as a powder (0.87g, 1.71 mmol, 67%). Purity by HPLC > 95% (A, 214; retention time - 4.32 minutes).

Mass spectrum [MNa+] calculated 531.5 [MNa+] observed 531.1

Boc-Cys(Acm)-Ala2-Lys(Boc)-pNA

To a solution of H-Ala2-Lys(Boc)-pNA (0.44g, 0.85 mmol), Boc-Cys(Acm)-OH (0.30 g, 0.95 mmol), and HOBt (0.15 g, 1.1 mmol), in 20 mL THF at 4°C under N₂ was added EDCI (0.18 g, 0.95 mmol). The reaction was stirred for 2 hours at 4°C and then allowed to warm up to room temperature and stirred for an additional hour. The THF was removed in vacuo and the resulting thick oil was worked up by method A, yielding a white foam (0.602 g, 0.769 mmol, 90%).

Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Ala2-Lys(Boc)-pNA (0.60 g, 0.77 mmol) was reacted with ScmCl and Boc deprotected by the standard protocols yielding TFA-H-Cys(Scm)-Ala2-Lys(HTFA)-pNA as a powder (0.44 g, 0.56 mmol, 73%). Purity by HPLC - 72% (E, 313 nm; retention time - 8.66 minutes.).

TFA·H-Cys(Scm)-Ala2-Lys(HTFA)-pNA - ¹H NMR (300 MHz, D₂O) δ 8.26 (2 H, d), 7.70 (2 H, d), 4.22 - 4.41 (4 H, m), 3.95 (3 H, s), 3.41 (1 H, dd), 3.28 (1 H, dd), 3.01 (2 H, t), 1.91 (2H, m), 1.73 (2 H, m), 1.54 (2 H, m), 1.42 (3 H, d), 1.39 (3 H, d).

Preparation of Z-Cys(Scm)-Ala2-Lys(H*TFA)-pNA

Z-Cys(sTrit)-Ala2-Lys(Boc)-pNA

To a solution of H-Ala2-Lys(Boc)-pNA (0.43 g, 0.85 mmol) in 20 mL THF at 4°C under nitrogen was added Z-Cys(sTrit)OH (0.51 g, 0.95 mmol), HOBt (0.15 g, 0.95 mmol), and EDCI (0.18 g, 0.95 mmol). Reaction was stirred at 4°C for 2 hours during which it became a thick suspension. The product was evaporated to an oil and purified
by method D yielding a white powder (0.74 g, 0.75 mmol, 88%) Purity by HPLC - 87% (F, 313 nm; retention time - 13.13 minutes).

Scm derivatization and TFA deprotection

Z-Cys(sTrit)-Ala₂-Lys(Boc)-pNA (0.74 g, 0.75 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding Z-Cys(Scm)-Ala₂-Lys(H*TFA)-pNA as a powder (0.33 g, 0.34 mmol, 44%). Purity by HPLC - 70% (E, 313 nm; retention time - 13.52 minutes).

Z-Cys(Scm)-Ala₂-Lys(H*TFA)-pNA - ¹H NMR (300 MHz, deuterated DMSO) δ 10.65 (1 H, s), 8.31 - 8.12 (5 H, m), 8.04 (1 H, s), 7.92 - 7.65 (6 H, m), 7.39 (4 H, s), 5.09 (2 H, s), 4.30 (4 H, m), 3.84 (3 H, s), 3.15 (1 H, dd), 2.95 (1 H, dd), 2.78 (2 H, s, b), 1.80 (2 H, m, b), 1.60 (2 H, m), 1.41 (2 H, m), 1.24 (6 H, m).

Mass spectrum [MH⁺] calculated 736.6 [MH⁺] observed 736.4

Preparation of TFA*Cys(Scm)-Ala₂-Lys(Z)-pNA

Boc-Ala₂-Lys(Z)-pNA

A solution of Boc-Ala₂OH (0.52 g, 2.0 mmol), TFA·H-Lys(Z)-pNA (1.0 g, 1.95 mmol), HOBt (0.30 g, 2.2 mmol), DIEA (400 μL, 2.3 mmol) and EDCI (0.40 g, 2.1 mmol) in 50 mL THF at 4°C under N₂, was stirred for 2 hours. The solution was concentrated by rotary evaporator to an oil and worked up by method B yielding a white foam (0.96 g, 1.5 mmol, 76%). Product was taken on without further purification.

Boc-Cys(Acm)-Ala₂-Lys(Z)-pNA

Boc-Ala₂-Lys(Z)-pNA (0.96 g, 1.5 mmol) was deprotected by the standard Boc procedure, yielding a cream powder (0.86 g, 1.30 mmol, 87%). The product was dissolved in 50 mL THF at 4°C, to which was added Boc-Cys(Acm)OH (0.50 g, 1.7 mmol), HOBt(0.30 g, 2.2 mmol), DIEA (290 μL, 1.6 mmol) and EDCI (0.33 g, 1.7
mmol). The solution was stirred for 2 hours under N₂ and allowed to warm to room temperature overnight. The solvent was removed in vacuo and the resulting oil worked up by method B yielding a white foam (1.04 g, 1.27 mmol, 98%). Purity by HPLC - 93 % (D, 313 nm; retention time - 6.12 minutes).

Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Ala₂-Lys(Z)-pNA (1.04 g, 1.27 mmol) was activated with ScmCl and Boc deprotected by the standard yieldings yielding TFA·Cys(Scm)-Ala₂-Lys(Z)-pNA as powder (0.501 g, 0.589 mmol, 46%). Purity by HPLC - 94 % (D, 313 nm; retention time - 3.87 minutes.).

TFA·Cys(Scm)-Ala₂-Lys(Z)-pNA - ¹H NMR (300 MHz, CD₃OD) δ 8.17 (2 H, d), 7.81 (2 H, d), 7.32 (5 H, s), 5.05 (2 H, s), 4.51 - 4.29 (3 H, m, b), 4.10 (1 H, q), 3.92 (3 H, s), 3.42 (1 H, dd), 3.13 (3 H, m), 1.96 - 1.71 (2 H, m, b), 1.55 (2 H, m), 1.39 (8 H, m).

Mass spectrum [MH⁺] calculated 736.6 [MH⁺] observed 736.2

Preparation of TFA·Cys(Scm)-Ala₃-Lys(H·TFA)-pNA

Fmoc-Ala₃OH

To a solution of Fmoc-Ala₂-OH (0.50 g, 1.3 mmol), HCl·HAlaOtBu (0.22 g, 1.2 mmol), DIEA (200 µl, 1.2 mmol) and HOBt (0.19 g, 1.4 mmol) in 10 mL 2:1 DMF:THF at 40°C under N₂ was added EDCI (0.26 g, 1.4 mmol). The reaction was stirred for 2 hours at 40°C and allowed to warm up to room temperature overnight. Work up was by method D, yielding a white solid (0.502 g, 0.984 mmol, 76%). TLC (9:1 CHCl₃:CH₃OH) showed a single spot. The product was taken up in 20 mL TFA and stirred for 2 hours at room temperature under N₂. The TFA was removed in vacuo and the resulting oil triturated (with centrifugation) with diethyl ether (3x) and dried overnight to yield a white solid (0.391 g, 0.86 mmol, 88%). Purity by HPLC - 91% (A,
Fmoc-Ala3OH - $^1$H NMR (300 MHz, deuterated DMSO) $\delta$ 8.11 (1 H, d), 7.97 (1 H, d), 7.89 (2 H, d), 7.73 (2 H, t), 7.55 (1 H, d), 7.45 - 7.31 (4 H, m), 4.31 - 4.17 (5 H, m), 4.07 (1 H, t), 1.28 - 1.20 (9 H, m).

Mass spectrum [MNa$^+$] calculated 476.3 [MNa$^+$] observed 476.1

Fmoc-Ala3-Lys(Boc)-pNA

To a solution of Fmoc-Ala3OH (1.23 g, 2.7 mmol), H-Lys(Boc)-pNA (1.0 g, 2.7 mmol), and HOBt (0.405 g, 3.0 mmol), in 50 mL THF at 4°C under N$_2$ was added EDCI (0.58 g, 3.0 mmol). The reaction was stirred for 2 hours at 4°C. After 1 hour a white solid precipitated. The reaction was allowed to warm up to room temperature overnight, with continued stirring. Work up was by method D, yielding a white solid (1.37 g, 1.71 mmol, 63%). HPLC showed 98% purity (B, 214 nm; retention time - 16.36 minutes).

Boc-Cys(Acm)-Ala3-Lys(Boc)-pNA

Fmoc-Ala3-Lys(Boc)-pNA (1.37 g, 1.71 mmol) was deprotected by the standard Fmoc protocol, yielding H-Ala3-Lys(Boc)-pNA as a white powder (0.45 g, 0.78 mmol, 46%). The product was dissolved in 10 mL 1:1THF:DMF at 4°C to which was added BocCys(Acm)OH (0.45 g, 1.55 mmol), HOBt (0.21 g, 1.55 mmol), and EDCI (0.31 g, 1.6 mmol). The solution was stirred overnight under N$_2$, slowly coming to room temperature. The solvents were then removed in vacuo and the resulting oil was worked up by method C yielding a white solid. Flash chromatography (9:1 CHCl$_3$:CH$_3$OH) yielded the pure product as a white powder (0.272 g, 0.319 mmol, 42%). HPLC showed > 95% purity (A, 313 nm; retention time - 10.59 minutes)

H-Ala3-Lys(Boc)-pNA - $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 8.19 (2 H, d), 7.89 (2 H, d), 4.48 - 4.28 (3 H, m, b), 3.66 (1 H, q), 3.05 (2 H, q), 3.96 - 1.72 (2 H, m, b), 1.57 - 1.21
Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Ala3-Lys(Boc)-pNA (0.272 g, 0.319 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding TFA•Cys(Scm)-Ala3-Lys(H•TFA)-pNA as a cream powder (0.175 g, 0.194 mmol, 61%). Purity by HPLC - 82% (E, 313 nm; retention time - 8.82 min.).

TFA•Cys(Scm)-Ala3-Lys(H•TFA)-pNA - 1H NMR (300 MHz, CD3OD) δ 8.25 (2 H, d), 7.88 (2 H, d), 4.55 - 4.26 (4 H, m), 4.11 (1 H, q), 3.97 (3 H, s), 3.43 (1 H, dd), 2.93 (2 H, t), 2.09 - 1.80 (2 H, m, b), 1.71 (2 H, m), 1.54 (2 H, m), 1.40 (9 H, m).

Preparation of Z-Cys(Scm)-Ala3-Lys(H•TFA)-pNA

Z-Cys(Acm)-Ala-OH

A solution of Z-Cys(Acm)OH (1.8 g, 5.5 mmol), HCl-AlaOtBu (1.0 g, 5.5 mmol), HOBt (0.74 g, 5.5 mmol), EDCI (1.0 g, 5.5 mmol), and DIEA (974 μL, 5.5 mmol) in 50 mL THF under N2 was stirred overnight starting at 4°C with slow warming to room temperature. The solution was worked up by method A yielding an impure product (50% by HPLC, A, 214; retention time - 8.11 minutes). A second round of extractions yielded pure product (1.19 g, 2.6 mmol, 47%). TFA deprotection under standard tBu deprotection conditions gave Z-Cys(Acm)-AlaOH (0.33 g, 0.830 mmol, 32%). HPLC indicated that the product was pure enough to be used in the next coupling (>80%, A, 214; retention time - 4.01 minutes).

Z-Cys(Acm)-Ala3-Lys(Boc)-pNA

A solution of Z-Cys(Acm)-Ala OH (0.33 g, 0.83 mmol), H-Ala2-Lys(Boc)-pNA (0.42 g, 0.825 mmol), HOBt (0.11 g, 0.83 mmol) and EDCI (0.32 g, 1.7 mmol) in 10 mL 1:1 DMF:THF under N2 was stirred overnight at 4°C with slow warming to room temperature. The solvents were removed in vacuo and the resultant oil was worked up by...
method C. Flash chromatography (9:1 CHCl₃:CH₃OH) gave the product as a white solid (0.252 g, 0.284 mmol, 34%). Purity by HPLC - 78% (A, 214; retention time - 11.14 minutes).

Scm derivatization and TFA deprotection

Z-Cys(Acm)-Ala₃-Lys(Boc)-pNA (0.25 g, 0.28 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding Z-Cys(Scm)-Ala₃-Lys(H⁺TFA)-pNA as a powder (0.14 g, 0.15 mmol, 54%). Purity by HPLC - 91% (E, 313 nm; retention time - 13.62 minutes). Peptide characterized after thiol capture.

Preparation of TFA⁺Cys(Scm)-Ala₄-Lys(H⁺TFA)-pNA

Fmoc-Ala₄-Lys(Boc)-pNA

To a solution of Fmoc-Ala₂OH (0.5 g, 1.3 mmol), H-Ala₂-Lys(Boc)-pNA (0.61 g, 1.2 mmol), and HOBT (0.19 g, 1.4 mmol), in in 10 mL 2:1 DMF:THF at 4°C under N₂ was added EDCI (0.26 g, 1.4 mmol). The reaction was stirred for 2 hours at 4°C and then allowed to warm up to room temperature and stirred overnight. The resulting viscous oil was worked up by method D, yielding a white solid (0.996 g, 1.14 mmol, 88%). TLC (17:2:1 CHCl₃:CH₃OH:acetic acid) showed a single spot. (Note - further purification in one preparation was performed by precipitation of the compound from boiling ethanol).

Fmoc-Ala₄-Lys(Boc)-pNA -¹H NMR (300 MHz, deuterated DMSO) δ 8.22 (2 H, d), 7.83 - 8.12 (9 H, m), 7.71 (2 H, t), 7.56 (1 H, d), 7.42 (2 H, t), 7.31 (2 H, t), 6.75 (2 H, t), 4.40 - 4.15 (7 H, m), 4.08 (1 H, t), 2.87 (2 H, q), 1.70 (2 H, m), 1.33 (13 H, s, b), 1.22 (12 H, m).

Mass spectrum [MH⁺] calculated 895.7 [MH⁺] observed 895.7

Boc-Cys(Acm)-Ala₄-Lys(Boc)-pNA
Fmoc-Ala₄-Lys(Boc)-pNA (0.292 g, 0.335 mmol) was deprotected by the standard Fmoc protocol and dried under high vacuum for 48 hours. HPLC showed the product to be 99% pure (A, 313 nm; retention time - 4.22 minutes). The entire product was used in the next coupling. A solution containing the H-Ala₄Lys(Boc)-pNA (0.335 mmol), BocCys(Acm)OH (0.489 g, 1.675 mmol), HOBt (0.226 g, 1.675 mmol) and EDCI (0.322 g, 1.675 mmol) in 5 mL DMF under N₂ was stirred overnight starting at 4°C and slowly warming up to room temperature. The resulting thick oil was concentrated by rotary evaporator to remove the DMF and triturated with diethyl ether to a powder. After trituration the reaction was worked up by method C. Final purification was achieved by precipitation from boiling ethanol yielding pure peptide (0.13 g, 0.138 mmol, 41%). Purity by HPLC - 90% (D, 313 nm; retention time - 5.94 minutes)

Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Ala₄-Lys(Boc)-pNA (0.13 g, 0.14 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding TFA'Cys(Scm)-Ala₄-Lys(H±TFA)-pNA as a powder (0.10 g, 0.10 mmol, 73%). Purity by HPLC 76% (E, 313 nm; retention time - 8.85 minutes). Peptide characterized after thiol capture.

Preparation of Z-Cys(Scm)-Ala₄-Lys(H±TFA)-pNA

Z-Cys(Acm)-Ala₂OH

A solution of Z-Cys(Acm)OH (1.8 g, 5.5 mmol), HCl•HAla₂OtBu (1.0 g, 3.9 mmol), HOBt (0.74 g, 5.5 mmol), DIEA (0.68 mL, 3.9 mmol), and EDCI (1.0 g, 5.5 mmol) in 50 mL THF under N₂ was stirred overnight with slow warming from 4°C to room temperature. The solution was worked up by method A yielding a white solid (1.30 g, 2.5 mmol, 64%). HPLC indicated that the product contained significant impurities (A, 214 nm; retention time - 6.58 minutes). As the TFA deprotection often resulted in a
significant improvement in purity, it was decided to proceed directly to the next step. The tBu group was removed by the standard method yielding a powder after trituration (0.78 g, 1.7 mmol, 68%). HPLC showed the peptide to be > 70% pure (A, 214 nm; retention time - 3.96 minutes) with a number of small associated impurities (each < 3%). The peptide was judged pure enough to be used in the next coupling.

Z-Cys(Acm)-Ala4-Lys(Boc)-pNA

A solution of Z-Cys-Ala2-OH (0.78 g, 1.7 mmol), H-Ala2-Lys(Boc)-pNA (0.84 g, 1.7 mmol), HOBt (0.22 g, 1.6 mmol) and EDCI (0.64 g, 3.3 mmol) in 50 mL 1:1 DMF:THF under N2, was stirred overnight with slow warming from 4°C to room temperature. The resulting thick oil was evaporated to remove the solvents and the residue worked up by method C. Further purification was attempted by precipitation from boiling ethanol (0.81 g, 0.84 mmol, 49%). Purity by HPLC - 71% (A, 313nm; retention time - 10.75 minutes) with a number of small baseline impurities. 1H NMR (DMSO-d6) showed only the peaks expected for the peptide, indicating that the purity was somewhat greater.

Z-Cys(Acm)-Ala4-Lys(Boc)-pNA - 1H NMR (300 MHz, deuterated DMSO) δ 8.52 (1 H, t), 8.22 (2 H, d), 8.12 - 7.91 (6 H, m), 7.86 (2 H, d), 7.56 (1 H, d), 7.33 (6 H, m), 6.77 (1 H, t), 5.04 (2 H, s), 4.26 (8 H, m), 2.85 (3 H, m), 2.65 (1 H, dd), 1.85 (3 H, s), 1.8 - 1.6 (2 H, m), 1.35 (13 H, s, b), 1.22 (12 H, m).

Scm derivatization and TFA deprotection

Z-Cys(Acm)-Ala4-Lys(Boc)-pNA (0.81 g, 0.84 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding Z-Cys(Scm)-Ala4-Lys(H'TFA)-pNA as a powder (0.30 g, 0.30 mmol, 36 %). Purity by HPLC - 89 % (E, 313 nm; retention time - 13.63 minutes). Peptide characterized after thiol capture.
Preparation of TFA-H-Cys(Scm)-Ala₅-Lys(H•TFA)-pNA

Fmoc-Ala₅-Lys(Boc)-pNA

A solution of Fmoc-Ala₃OH (0.89 g, 1.96 mmol), H-Ala₂-Lys(Boc)-pNA (1.0 g, 1.96 mmol), HOBt (0.27 g, 1.96 mmol), and EDCI (0.75 g, 39 mmol) in 1:1 THF:DMF under a nitrogen atmosphere, was stirred overnight with slow warming from 4°C to room temperature. A gel formed in the flask which was chopped up with a spatula and sonicated in 0.5 M citrate buffer, pH 3.5 until a suspension was formed. The suspension was centrifuged to form a pellet which was resuspended in citrate buffer. The standard work up by method C was then followed. The peptides were then precipitated at -20°C from hot ethanol, yielding a white powder (1.02 g, 1.08 mmol, 55%) ¹H NMR (DMSO-d₆) showed only the peaks expected for the peptide and the product was taken on to the next step.

Fmoc-Ala₅-Lys(Boc)-pNA - ¹H NMR (300 MHz, deuterated DMSO) δ 8.24 (2 H, d), 8.15 - 7.80 (10 H, m), 7.75 (2 H, t), 7.57 (1 H, d), 7.42 (2 H, t), 7.27 (2H, t), 6.78 (1 H, t), 4.23 (8 H, m), 4.03 (1 H, t), 2.88 (2 H, q), 1.70 (2 H, m, b), 1.35 (13 H, s, b), 1.21 (15 H, m, b).

Boc-Cys(Acm)-Ala₅-Lys(Boc)-pNA

Fmoc-Ala₅-Lys(Boc)-pNA (1.02 g, 1.08 mmol) was deprotected by the standard Fmoc protocol and dried under high vacuum for 48 hours. HPLC showed that the product was 98% pure (A, 313 nm; retention time - 4.15 minutes). The entire product was used in the next coupling. A solution containing the H-Ala₅-Lys(Boc)-pNA (1.08 mmol); BocCys(Acm)OH (1.57 g, 5.4 mmol), HOBt (0.729 g, 5.4 mol) and EDCI (1.04 g, 5.4 mmol) in 15 mL THF under N₂ was stirred overnight with slow warming from 40°C to room temperature. The resulting thick oil was evaporated under high vacuum to remove the DMF and then triturated to a powder with diethyl ether. The powder was
worked up by method C. Final purification was achieved by precipitation at -20°C from boiling ethanol, yielding a white powder (0.493 g, 0.495 mmol, 46% yield). Purity by HPLC - 86% (A, 313 nm; retention time - 12.36 minutes)

Scm derivatization and TFA deprotection

Boc-Cys(Acm)-Ala₅-Lys(Boc)-pNA (0.494 g, 0.496 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding TFA·H-Cys(Scm)-Ala₅-Lys(H·TFA)-pNA as a powder (0.330 g, 0.316 mmol, 64%). Purity by HPLC - 86% (E, 313 nm; retention time - 8.94 minutes).

TFA·H-Cys(Scm)-Ala₅-Lys(H·TFA)-pNA - ¹H NMR (300 MHz, deuterated DMSO) δ, 8.80 (1 H, d), 8.50 - 7.70 (17 H, m), 4.28 (7 H, m), 3.90 (3 H, s), 3.25 (1 H, dd), 3.10 (1 H, dd), 2.80 (2 H, s, b), 1.80 (2 H, m), 1.60 (2 H, m), 1.40 (2 H, m), 1.22 (15 H, m).

Mass spectrum [MH⁺] calculated 815.5 [MH⁺] observed 815.6

Preparation of Z-Cys(Scm)-Ala₅-Lys(H·TFA)-pNA

Z-Ala₃OtBu

A solution containing Z-Ala₂OH (1.7 g, 5.63 mmol), HCl·H-AlaOtBu (1.02 g, 5.63 mmol), HOBt (0.84 g, 6.25 mmol), EDCI (1.2 g, 6.25 mmol) and DIEA (0.98 mL, 5.6 mmol) in 30 mL CH₂Cl₂ under N₂ was stirred overnight with slow warming from 4°C to room temperature. The solution was worked up by method A and the crude product recrystallized from ethanol (1.3 g, 3.2 mmol, 57%). Purity by HPLC - 96% (C, 214 nm; retention time - 11.56 minutes).

Z-Cys(Acm)-Ala₃OH

H-Ala₃OtBu was prepared by hydrogenation of Z-Ala₃OtBu (1.3 g, 3.2 mmol) in ethanol (50 mL) under 50 atmospheres H₂ in the presence of a catalytic amount of 10%
Pd on carbon. Hydrogenation was allowed to proceed for 24 hours at room temperature, after which the solution was filtered through Celite 521 (Aldrich). The Celite was washed several times with ethanol which was added to the filtrate. The ethanol was removed in vacuo and the resulting thick oil was used directly in the coupling. A solution of H-Ala3OtBu (3.2 mmol), Z-Cys(Acm)OH (1.8 g, 5.6 mmol), HOBt (0.84 g, 6.25 mmol), and EDCI (1.2 g, 6.25 mmol) in 50 mL THF under N₂ was stirred overnight with slow warming from 4°C to room temperature. The solution was poured into 500 mL CH₂Cl₂ and purified by method A. Crude yield (0.45 g, 0.73 mmol, 23%). HPLC indicated significant impurities (A, 214 nm; retention time - 5.71 minutes). The tBu protecting group was removed from the impure material under standard conditions, yielding a powder after trituration (0.187 g, 0.346 mmol, 47%). HPLC showed the peptide to be 70% pure with a number of small baseline impurities (A, 313 nm; retention time - 3.64 minutes).

Z-Cys(Acm)-Ala5-Lys(Boc)-pNA

A solution of Z-Cys-Ala3OH (0.187 g, 0.346 mmol), H-Ala2-Lys(Boc)-pNA (0.351 g, 0.692 mmol), HOBt (0.10 g, 0.74 mmol) and EDCI (0.134 g, 0.70 mmol) in 25 mL 1:1 DMF:THF under N₂, was stirred overnight with slow warming from 4°C to room temperature. The resulting thick oil was evaporated to remove the solvents and the residue worked up by method C. Further purification was achieved by precipitation from boiling ethanol yielding a white powder (0.162 g, 0.157 mmol, 45%). Purity by HPLC - 77% (A, 313 nm; retention time - 13.2 minutes).

Scm derivatization and TFA deprotection

Z-Cys(Acm)-Ala5-Lys(Boc)-pNA (0.162 g, 0.157 mmol) was activated with ScmCl and Boc deprotected by the standard protocols yielding Z-Cys(Scm)-Ala5-Lys(H-TFA)-
pNA as a powder (0.121 g, 0.113 mmol, 72%). Purity by HPLC - 90% pure (E, 313 nm; retention time - 13.60 minutes). Peptide characterized after thiol capture.

**Thiol capture of Scm peptides**

The thiol capture method of Fotouhi, Galakatos, and Kemp (1989) was used. The scale of the thiol capture was approximately 0.2 mmol for all peptides. The procedure suggests the use of 1.0 to 1.05 equivalents of the Scm peptide, therefore the weights of peptide used were adjusted based on the purity of the Scm material as indicated by HPLC. Due to the insolubility of the Z-Ala-Phe-Gly-O-Dbf-SH peptide the thiol captures were run in HFIP-CH$_2$Cl$_2$-H$_2$O (5:3:1). The thiol captures were performed for 30 minutes and monitored by HPLC (E, 313 nm). HPLC indicated no remaining Z-Ala-Phe-Gly-O-Dbf-SH. A fraction of the crude thiol capture material was purified before acyl transfer by preparative HPLC (G, 313 nm) yielding pure thiol capture product (>99% by HPLC). HPLC retention times (A, 313) within a class of thiol capture peptides were only slightly affected by the number of alanines and are tabulated below.

<table>
<thead>
<tr>
<th>Thiol Capture Peptide Class</th>
<th>HPLC retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA<em>H)CA$_n$K(H</em>TFA)-pNA</td>
<td>8.5-9.5 minutes</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA*H)CA$_n$K(Z)-pNA</td>
<td>10.5-11.5 minutes</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CA$_n$K(H*TFA)-pNA</td>
<td>10.5-11.5 minutes</td>
</tr>
</tbody>
</table>

Plasma desorption mass spectroscopy was used to confirm that the proper asymmetric disulfides had been produced.

<table>
<thead>
<tr>
<th>Thiol Capture peptide</th>
<th>[MH$^+$] calc.</th>
<th>[MH$^+$] observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA<em>H)CK(H</em>TFA)-pNA</td>
<td>993.0</td>
<td>994.0</td>
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<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA<em>H)CA$_n$K(H</em>TFA)-pNA</td>
<td>1064.1</td>
<td>1064.6</td>
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<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA<em>H)CA$_2$K(H</em>TFA)-pNA</td>
<td>1134.2</td>
<td>1134.0</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA<em>H)CA$_3$K(H</em>TFA)-pNA</td>
<td>1206.3</td>
<td>1205.1</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA<em>H)CA$_4$K(H</em>TFA)-pNA</td>
<td>1277.3</td>
<td>1276.9</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA<em>H)CA$_5$K(H</em>TFA)-pNA</td>
<td>1348.4</td>
<td>1347.5</td>
</tr>
</tbody>
</table>
Thiol Capture peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>[MH(^+)] calc.</th>
<th>[MH(^+)] observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA·H)CK(Z)-pNA</td>
<td>1126.9</td>
<td>1127.6</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA·H)CAK(Z)-pNA</td>
<td>1198.0</td>
<td>1198.1</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA·H)CA₂K(Z)-pNA</td>
<td>1269.1</td>
<td>1270.1</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CK(H·TFA)-pNA</td>
<td>1126.9</td>
<td>1127.7</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CAK(H·TFA)-pNA</td>
<td>1198.0</td>
<td>1198.2</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CA₂K(H·TFA)-pNA</td>
<td>1269.1</td>
<td>1271.8</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CA₃K(H·TFA)-pNA</td>
<td>1340.2</td>
<td>1341.2</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CA₄K(H·TFA)-pNA</td>
<td>1411.3</td>
<td>1412.2</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CA₅K(H·TFA)-pNA</td>
<td>1482.3</td>
<td>1483.6</td>
</tr>
</tbody>
</table>

For the purposes of simplification for the rest of this experimental, thiol capture materials will be referred to by the following abbreviations:

<table>
<thead>
<tr>
<th>Thiol Capture Peptide Class</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA·H)CAₙK(H·TFA)-pNA</td>
<td>CAₙK thiol capture material</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(TFA·H)CAₙK(Z)-pNA</td>
<td>CAₙK(Z) thiol capture material</td>
</tr>
<tr>
<td>Z-A-F-G-O-Dbf-S-S-(Z)CAₙK(H·TFA)-pNA</td>
<td>ZCAₙK thiol capture material</td>
</tr>
</tbody>
</table>

Likewise acyl transfer products will be referred to by the abbreviation followed by acyl transfer material.

**Determination of Extinction coefficients**

Extinction coefficients at 313 nm in DMSO were determined for the functional groups in the thiol capture material and for the acyl transfer product using the following model compounds. All spectra were measured using a Hewlett Packard Diode Array Spectrophotometer model 8452A. All masses were weighed on an analytical balance and all volumes measured with volumetric glassware. For the para-nitroanilide - H-Lys(Boc)-pNA (extinction coefficient =1.13x10\(^4\) L mol\(^{-1}\) cm\(^{-1}\) ). For the disulfide linked 4-acyloxydibenzofuran-6-thia function of the thiol capture material - Z-L-Cys(4-acetoxydibenzofuranyl -6-thia)-OH (extinction coefficient = 6.96x10\(^3\) L mol\(^{-1}\) cm\(^{-1}\) ). For the disulfide linked 4-hydroxydibenzofuran-6-thia function of the acyl transfer
product - Z-L-Cys(4-hydroxydibenzofuranyl-6-thia)-OH·CHA (extinction coefficient = 6.95 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1})$. Extinction coefficients for both phenylalanine and the Z group were not determined as both benzene and toluene have negligible absorbance at 313 nm. The extinction coefficient for the thiol capture material and the acyl transfer product was taken to be the sum of the extinction coefficients $= 1.83 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$.

Experiments with a variety of carefully dried thiol capture peptides showed maximum error from the concentration determined by weight of peptide versus the concentration determined by extinction coefficient of less than 5%.

Extinction coefficients were also measured at 320 nm in DMSO for both Z-L-Cys(4-acetoxydibenzofuranyl-6-thia)-OH (extinction coefficient = 4.03 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1})$ and Z-L-Cys(4-hydroxydibenzofuranyl-6-thia)-OH·CHA (extinction coefficient = 7.83 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1})$. The difference in extinction coefficient corresponds to the change in absorbance upon release of the phenol during formation of the amide bond and was used as the basis for the development of a spectroscopic assay for acyl transfer.

Extinction coefficients were also measured at 316 nm in DMSO for 4-hydroxydibenzofuran (2.44 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1})$ and ZGly-O-Dbf (92.6 \text{ L mol}^{-1} \text{ cm}^{-1}).$

**Acyl Transfer Titration of Model Peptides with DIEA**

Stock solutions of HPLC purified thiol capture peptides (CAnK) containing the TFA salts of the cysteine and lysine amines were prepared by dissolving weighed amounts of material in DMSO to the approximate concentration of $4 \times 10^{-4}$ M. Exact concentrations were determined spectrophotometrically using a Hewlett Packard Diode Array Spectrophotometer model 8452A. The absorbance of a 1 in 10 volumetric dilution of the stock solutions were measured and the concentration of the stock calculated using the extinction coefficient $1.83 \times 10^4 \text{ liter/mol·cm}$ determined from model studies. Stock solutions of DIEA in DMSO ($1.15 \times 10^{-3}$ M, and $5.74 \times 10^{-4}$ M) were prepared by
volumetric dilution. Acyl transfer reactions were set up containing thiol capture material, (concentration - 4x10^{-4} M), 0.5 equivalents of AgNO_3 and varying equivalents of DIEA. All measurements were performed using volumetric glassware and each reaction was performed in duplicate. Constant volume was not maintained so the final concentration of thiol capture material in the acyl transfer reactions varied between 2x10^{-4} and 4x10^{-4} M, depending on the volume of base added. Acyl transfers were allowed to run 20 hours at room temperature in the dark and then were stored at -20°C until analysis by HPLC (A, 313 nm).

Identification of the HPLC peaks:

The HPLC retention time (A, 313 nm) of the thiol capture peptide was identified by the injection of pure thiol capture product. Initial identifications of the HPLC peaks as the product of acyl transfer to the ε amine of cysteine or to the ϵ amine of lysine of the thiol capture peptide were based on whether the peak was produced in the presence of one or excess equivalents of DIEA. The putative lysine acyl transfer products were produced with excess DIEA and had an HPLC retention time of approximately 10.8 minutes. The putative cysteine acyl transfer products were produced with less than 1 equivalent of DIEA and had a retention time of approximately 11.5 minutes (A). In all cases the retention time of peak produced by 2 or more equivalents of DIEA was 0.7-1 minute shorter. The initial identifications were confirmed in one case by peptide sequencing after purification of the CA2K acyl transfer peptides. Acyl transfer reactions were pooled by major product and purified by preparative HPLC (G, 313 nm). The HPLC solvents were removed in vacuo and the acyl transfer products identified by plasma desorption mass spectroscopy ([MH^+] calculated 1134.2, observed 1135) and by analytical HPLC identification of the release of the 4-hydroxy-6-mercaptodibenzofuran template upon treatment with triethylphosphine. The pure acyl transfer products were treated with triethylphosphine to remove the 4-hydroxy-6-mercaptodibenzofuran template and
repurified by preparative HPLC. Sequencing of the purified CA2K acyl transfer products confirmed the initial identification of HPLC peaks as the product of acyl transfer to either lysine or cysteine.

Identification of acyl transfer impurities - Once the HPLC peaks due to thiol capture peptide and the acyl transfer products had been identified, other peaks were assumed to be due to impurities produced by disulfide interchange. That these peaks were due to impurities produced by disulfide interchange was supported by the observation that treatment of the reaction mixture with excess triethylphosphine resulted in HPLC traces only containing peaks due to the 4-hydroxy-6-mercaptodibenzofuran template and the free thiols of the acyl transfer products. In a few cases of high disulfide interchange, additional peaks due to Z-Ala-Phe-Gly-O-Dbf-SH and TFA·H-Cys(SH)-Alaₙ-Lys(H·TFA)-pNA were also identified after treatment with triethylphosphine due to disulfide interchange that occurred before acyl transfer. Peaks due to the residual absorbance of dimethylsulfoxide and AgNO₃, and its oxidation products were also identified by the HPLC analysis of a solution of these compounds after 20 hours under typical acyl transfer conditions. The compounds yielded a number of small overlapping peaks with a retention time faster than 4.5 minutes under the normal HPLC conditions (A, 313 nm). Other small baseline peaks not identified were probably the products of hydrolysis of the glycine phenolic ester. Once each of the peaks had been assigned HPLC retention times were used for identification in all subsequent acyl transfers with other thiol capture peptides.

Calculations: As the thiol capture peptide and the acyl transfer peptide both have the same extinction coefficient at 313nm, it was possible to use the integrations of the HPLC in order to obtain the amounts of each of the material. Likewise, as both sides of the disulfide had chromophores, disulfide interchange would result in the loss of two assymetrical disulfides with the gain of two symetrical disulfides with the same total
absorbance. Therefore, HPLC integrations could also be directly used to determine the amount of impurities.

Total Absorbance (T): calculated by subtracting the area of all peaks with a retention time shorter than 4.5 minutes (due to AgNO₃, its oxidation products, and dimethylsulfoxide) from the total absorbance given by the HPLC. This yielded the new total absorbance (T) and was used in all subsequent calculations.

Percent Starting Material (SM): calculated by dividing the peak area due to thiol capture peptide (S) by the total absorbance (T). \( SM = \frac{S}{T} \times 100 \)

Percent Acyl Transfer (A): calculated by dividing the sum of the peak areas due to Cysteine (C) and Lysine (K) acyl transfer by the total absorbance. \( A = \frac{(C+K)}{T} \times 100 \).

Percent Acyl Transfer to Cysteine (Normalized) (A_{cys}): calculated by dividing peak area due to Cysteine acyl transfer by the sum of the peak areas due to Cysteine (C) and Lysine (K) acyl transfer. \( A_{cys} = \frac{C}{(C+K)} \times 100 \)

Percent Impurities (I): the area due to impurities was calculated by subtracting the sum of the peak areas due to thiol capture peptide and acyl transfer products from the total area. Dividing the area due to impurities by the total area yields the percent impurities. \( I = \frac{(T-(S+C+K))}{T} \times 100 \)

These calculation were used in all subsequent studies.

**Kinetics of Acyl Transfer to Cysteine**
Acyl Transfer Kinetics-CAK(Z)

Three stocks of HPLC purified thiol capture peptide (CAK(Z)) were prepared by dissolving a weighed amount of material in DMSO. Three kinetics runs were set up:
Run 1: 5x10^{-5} M thiol capture peptide in DMSO, 0.1 equivalents AgNO_3, 0.5 equivalents DIEA; Run 2: 4x10^{-5} M thiol capture peptide in DMSO, 0.1 equivalents AgNO_3, 1 equivalent DIEA; Run 3: 4.5x10^{-5} M thiol capture peptide in DMSO, 0.1 equivalents AgNO_3, 2.5 equivalents DIEA. All volumes were measured with a Ranin Pipetman. Reactions were run at 24°C. Aliquots were taken at specific times, quenched with 50-fold excess TFA in and stored in liquid nitrogen until analysed by HPLC (A, 313 nm). HPLC peaks were identified as thiol capture peptide or acyl transfer product (after triethyl phosphine treatment) by fragmentation mass spectroscopy of both compounds and by sequencing of the acyl transfer product after removal of the two Z groups and treatment with triethylphosphine.

CAK(Z) thiol capture material [MH^+] calculated 1198.02 [MH^+] observed 1198.4
CAK(Z) acyl transfer material [MH^+] calculated 716.3 [MH^+] observed 716.3

Acyl Transfer Kinetics-CA_2K(Z)

A stock solution of HPLC purified thiol capture peptide (CA_2K(Z)) containing the TFA salt of the cysteine amine was prepared by dissolving a weighed amount of material (0.0076 g, 5.4 x10^{-6} mol) in DMSO (200 mL) to the approximate concentration of 2.8x10^{-5} M. Exact concentration was determined spectrophotometrically as described above and found to be 2.6x10^{-5} M. A stock solution of DIEA in DMSO (5.75 x10^{-3} M) was prepared by volumetric dilution. An acyl transfer was set up containing 5.6x10^{-6} mol thiol capture peptide, 0.1 equivalents AgNO_3, and 1 equivalent DIEA, Final concentration of thiol capture peptide 2.6 x10^{-5} M. All volumes were measured with a Ranin Pipetman. Reactions were run at 24°C. Aliquots were taken at specific times, quenched with 50-fold excess TFA in and stored in liquid nitrogen until analysed by
Identification of the HPLC peaks as thiol capture peptide or acyl transfer product were based on HPLC retention times.

**Acyl Transfer to Lysine Kinetics**

**General procedure for UV kinetics experiments**

Stock solutions of HPLC purified thiol capture peptides(ZCA_nK) containing the TFA salt of the lysine amine were prepared by dissolving a weighed amount of material in DMSO to the approximate concentration of $2 \times 10^{-4}$ M containing 0.1 eq. AgNO_3. Volumetric dilution was used to prepare two solutions of $2 \times 10^{-5}$ M and $4 \times 10^{-5}$ M. Exact concentrations were determined spectrophotometrically as described above. Stock solutions of DIEA ($5.74 \times 10^{-2}$ M), Cl_2CHCOO^-Na^+ ($2.87 \times 10^{-2}$ M), and TFA ($2.87 \times 10^{-2}$ M), in DMSO were prepared by volumetric dilution. All kinetics runs were performed at 24°C on a Hewlett Packard Diode Array Spectrophotometer model 8452A running HP 89531A MS-DOS UV-Vis Operating Software and fitted with an air driven magnetic stirrer.

**General procedure -** Three mL of the thiol capture peptide stock solution was placed in a 1 cm cuvette sealed with a rubber stopper and stirred at 700 revolution/minute. If the kinetics were being run in the presence of TFA, it was also added at this time. Absorbance data was acquired at time intervals corresponding to 0.1 half lives (in seconds) at 320 nm for 5-10 half lives, yielding a baseline. With the data still being acquired, the stopper was removed, 5-40 equivalents of the appropriate base stock added with a Ranin Pipetman, and the stopper replaced. The volume change upon addition of base was 1-2% depending on the equivalents of base added. This slight dilution produced a small negative spike which was equated to $T=0$ seconds for the purpose of analysis. Absorbance was taken for 20 half lives for kinetic runs where DIEA was the base and for 1 half life with Cl_2CHCOO^-Na^+ after which 20 equivalents of DIEA were added to
rapidly convert all material to products (to determine the final absorbance). The kinetic runs are tabulated below.

<table>
<thead>
<tr>
<th>Run</th>
<th>Peptide</th>
<th>Conc.</th>
<th>Base (equivalents)</th>
<th>TFA (equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ZCK</td>
<td>4.4x10^-5 M</td>
<td>DIEA (13 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>2</td>
<td>ZCK</td>
<td>4.4x10^-5 M</td>
<td>DIEA (25 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>3</td>
<td>ZCK</td>
<td>2.1x10^-5 M</td>
<td>DIEA (27 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>4</td>
<td>ZCK</td>
<td>2.1x10^-5 M</td>
<td>DIEA (27 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>5</td>
<td>ZCK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(7.0 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>6</td>
<td>ZCK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(14 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>7</td>
<td>ZCK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(7.0 eq)</td>
<td>1.4 eq</td>
</tr>
<tr>
<td>8</td>
<td>ZCAK</td>
<td>2.8x10^-5 M</td>
<td>DIEA (19 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>9</td>
<td>ZCAK</td>
<td>2.8x10^-5 M</td>
<td>DIEA (39 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>10</td>
<td>ZCAK</td>
<td>2.8x10^-5 M</td>
<td>DIEA (39 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>11</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(10 eq)</td>
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<tr>
<td>12</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(19 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>13</td>
<td>ZCAK</td>
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<td>Cl_2CHCOO^-Na^+(10 eq)</td>
<td>2.0 eq</td>
</tr>
<tr>
<td>14</td>
<td>ZCAK</td>
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<td>DIEA (15 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>15</td>
<td>ZCAK</td>
<td>3.7x10^-5 M</td>
<td>DIEA (30 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>16</td>
<td>ZCAK</td>
<td>3.7x10^-5 M</td>
<td>DIEA (30 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>17</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (34 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>18</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (34 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>19</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(8.0 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>20</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(15 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>21</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(8.0 eq)</td>
<td>1.6 eq</td>
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<tr>
<td>22</td>
<td>ZCAK</td>
<td>3.8x10^-5 M</td>
<td>DIEA (12 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>23</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (14 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>24</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (14 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>25</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (28 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>26</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (28 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>27</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(6 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>28</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(13 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>29</td>
<td>ZCAK</td>
<td>3.3x10^-5 M</td>
<td>DIEA (14 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>30</td>
<td>ZCAK</td>
<td>2.0x10^-5 M</td>
<td>DIEA (16 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>31</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (16 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>32</td>
<td>ZCAK</td>
<td>1.7x10^-5 M</td>
<td>DIEA (25 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>33</td>
<td>ZCAK</td>
<td>2.1x10^-5 M</td>
<td>Cl_2CHCOO^-Na^+(13 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>34</td>
<td>ZCAK</td>
<td>2.4x10^-5 M</td>
<td>DIEA (20 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>35</td>
<td>ZCAK</td>
<td>2.4x10^-5 M</td>
<td>DIEA (20 eq)</td>
<td>0.0 eq</td>
</tr>
<tr>
<td>36</td>
<td>ZCAK</td>
<td>2.4x10^-5 M</td>
<td>DIEA (39 eq)</td>
<td>0.0 eq</td>
</tr>
</tbody>
</table>
The assumption that the change in absorbance observed in the assay was only due to acyl transfer was confirmed by HPLC (A,313 nm) of the reactions at the apparent end point of the assays. Only the peaks expected for the acyl transfer product were present. In addition the products of the UV assays for ZCK, ZCA3K and ZCA5K were purified and characterized by mass spectroscopy after treatment with triethylphosphine. Only masses consistent with acyl transfer were observed.

ZCK acyl transfer peptide [MNa+] observed: 937.3 [MNa+] calculated: 
other masses observed: 1301.2 - ZCK acyl transfer product triethylphosphine adduct

ZCA3K acyl transfer peptide [MH+] observed: 1127.7 [MH+] calculated: 
other masses observed: 1148.6 - [MNa+]; 1212.3 - intact Dbf disulfide, loss of a Z group; 1243.3 - ZCA3K acyl transfer product triethyl phosphine adduct

ZCA5K acyl transfer peptide [MNa+] observed: 1290.9 [MNa+] calculated:

Acyl Transfer to Lysine - Bimolecular Kinetics

Bimolecular kinetics were performed using the spectrophotometric assay described above with a few modifications. Stock solutions of Z-GlyO-Dbf (2.79x10^-2 M) and Z-Lys(H*TFA)-benzylamide (0.118 M) were prepared by dissolving a weighed amount of material in DMSO. DIEA was used neat. The temperature of the kinetic reactions was 24°C and the wavelength used was 316 nm. Reactions containing Z-GlyO-Dbf and DIEA were set up in the same manner as for the unimolecular kinetic runs. After a baseline had been taken for 1/4 half life, the ZLys(H*TFA)-benzylamide was added to the cuvette. Reactions were followed for 1/2 to 1 half life. The end point absorbance was calculated based on the difference between the extinction coefficients of HO-Dbf and Z-GlyODbf, assuming that at T=infinity all the Z-GlyO-Dbf would have been converted to HO-Dbf. In addition to the kinetics runs, control runs were performed with Z-GlyO-Dbf and either DIEA or Z-Lys(H*TFA)-benzylamide alone to show that these rates were
negligible. The kinetics runs are tabulated below.

<table>
<thead>
<tr>
<th>Run</th>
<th>Gly conc.</th>
<th>Lys conc.</th>
<th>DIEA conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4x10^{-4} M</td>
<td>2.3x10^{-3} M</td>
<td>2.8x10^{-2} M</td>
</tr>
<tr>
<td>2</td>
<td>2.4x10^{-4} M</td>
<td>2.3x10^{-3} M</td>
<td>5.6x10^{-2} M</td>
</tr>
<tr>
<td>3</td>
<td>4.7x10^{-4} M</td>
<td>4.5x10^{-3} M</td>
<td>0.108 M</td>
</tr>
<tr>
<td>4</td>
<td>4.7x10^{-4} M</td>
<td>0.0 M</td>
<td>0.108 M</td>
</tr>
<tr>
<td>5</td>
<td>4.7x10^{-4} M</td>
<td>4.5x10^{-3} M</td>
<td>0.0 M</td>
</tr>
</tbody>
</table>

**Acyl Transfer with Alternative Bases**

A stock solution of HPLC purified thiol capture material (CK) containing the TFA salt of the cysteine and lysine amines was prepared by dissolving a weighed amount of material (0.0125 g, 1.0x10^{-5} mol) in DMSO (25 mL) to the approximate concentration of 4x10^{-4} M. Exact concentration was determined spectrophotometrically as described above and found to be 3.53x10^{-4} M. Stock solutions of DIEA (5.74x10^{-4} M and 1.15x10^{-3} M) and of the test bases ClCH_2COO^-Na^+, Cl_2CHCOO^-Na^+, Cl_3CCOO^-Na^+, F_3CCOO^-Na^+, and sodium salicylate (2.0x10^{-2} M), in DMSO were prepared with volumetric glassware. Acyl transfer reactions were set up containing 3.53x10^{-7} moles thiol capture material, 0.5 equivalents of AgNO_3 and 0, 1.0, 2.5, or 5.0 equivalents of each of the test bases. Volume of added base caused the final concentration of thiol capture peptide in the acyl transfer reactions to vary from 3.2 to 3.5x10^{-4} M depending on the volume of base added. All volume measurements were performed with volumetric glassware and all reactions were run in duplicate. Acyl transfers were allowed to run 20 hours at room temperature in the dark and then were stored at -20°C until analysis by HPLC (A, 313 nm). Identification of the HPLC peaks were based on HPLC retention times. All measurements were performed using volumetric glassware and each reaction was performed in duplicate. Acyl transfer products were pooled by major product and purified by preparative HPLC (G, 313 nm) and the HPLC solvents were removed in vacuo. Confirmation that acyl transfer had occurred was confirmed by plasma desorption.
mass spectroscopy of the acyl transfer product, by HPLC identification of the release of the 4-hydroxy-6-mercaptopdibenzofuran template upon treatment with triethylphosphine, and by plasma desorption mass spectroscopy of the acyl transfer peptide after the release of the 4-hydroxy-6-mercaptopdibenzofuran template. CK acyl transfer products - \([\text{MH}^+]\) calculated 992.8, observed 993. CK acyl transfer peptide after the release of the 4-hydroxy-6-mercaptopdibenzofuran template - \([\text{MH}^+]\) calculated 777.7, observed 779.

**Acyl transfer with Alternative Bases - Comparison of Dichloroacetate and DIEA with CA\(_2\)K Thiol Capture Material**

A stock solution of HPLC purified thiol capture material (CA\(_2\)K) was prepared by dissolving a weighed amount of material (0.02 g, 1.5x10\(^{-5}\) mol) in DMSO (50 mL) to the approximate concentration of 3x10\(^{-4}\) M. Exact concentration was determined spectrophotometrically as described above and found to be 3.3x10\(^{-4}\) M. Stock solutions of DIEA (2.3x10\(^{-3}\) M) and Cl\(_2\)CHCOO\(^-\)Na\(^+\) (3.0x10\(^{-3}\) M) were prepared with volumetric glassware. Acyl transfer reactions were set up containing 3.3x10\(^{-7}\) moles of thiol capture material, 0.5 equivalents of AgNO\(_3\) and 0, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0 equivalents of either DIEA or Cl\(_2\)CHCOO\(^-\)Na\(^+\). All volume measurements were performed using Ranin Pipetman and each reaction was performed in duplicate. Constant volume was not maintained so the final concentration of thiol capture peptide in the acyl transfer reactions varied between 2x10\(^{-4}\) and 4x10\(^{-4}\) M, depending on the volume of base added. Acyl transfers were allowed to run 20 hours at room temperature in the dark and then were stored at -20\(^{\circ}\)C until analysis by HPLC (A, 313 nm). Identification of the HPLC peaks were based on HPLC retention times.

**Acyl Transfer at High Concentration With Cl\(_2\)CHCOO\(^-\)Na\(^+\)**

A stock solution of HPLC purified thiol capture peptide (CA\(_2\)K) was prepared by
dissolving a weighed amount of material (0.014 g, 1.0x10^{-5} mol) in DMSO (700 μL) to the concentration of 1.5x10^{-2} M. The stock solution also contained 1.5x10^{-3} M AgNO₃. A 1 in 10 dilution with a Ranin Pipetman was used to prepare a thiol capture peptide stock solution containing 1.5x10^{-3} M peptide, 1.5x10^{-4} M AgNO₃. Stock solutions of Cl₂CHCOO⁻Na⁺ (0.15 M and 1.5x10^{-2} M) in DMSO were prepared with volumetric glassware. Acyl transfer reactions were set up at the concentrations of 1x10^{-2} M (1.5x10^{-6} moles) thiol capture peptide and 1x10^{-3} M (1.5x10^{-7} moles) thiol capture peptide, 0.1 equivalents of AgNO₃ and 0, 1.5, or 3.0 equivalents of Cl₂CHCOO⁻Na⁺. Volumes were controlled so that the final concentration was constant at 1x10^{-2} M or 1x10^{-3} M thiol capture peptide regardless of the amount of base added. All volume measurements were performed using a Ranin Pipetman and each reaction was performed in duplicate. Acyl transfers were allowed to run 6 hours at room temperature in the dark and then were stored at -20°C until analysis by HPLC (A, 313 nm). Any samples with remaining starting material were allowed to react for an additional 24 hours at room temperature. Identification of the HPLC peaks were based on HPLC retention times. Acyl transfer products were pooled by major product and purified by preparative HPLC (G, 313 nm) and the HPLC solvents were removed in vacuo. Confirmation that acyl transfer had occurred was confirmed by plasma desorption mass spectroscopy of the acyl transfer product. CA₂K acyl transfer product - [MH⁺] calculated 1134.2, observed 1135. CA₂K acyl transfer peptide after the release of the 4-hydroxy-6-mercaptodibenzofuran template - [MH⁺] calculated 920.0, observed 921.

**Acyl Transfer at High Concentration With Cl₂CHCOO⁻ Na⁺ #2 - Yield**

A stock solution of HPLC purified thiol capture peptide (CA₂K) was prepared by dissolving a weighed amount of material (5.3 mg; 3.9 x10^{-3}) mmol in DMSO to the
concentration of $1.5 \times 10^{-2}$ M. The stock solution also contained $1.5 \times 10^{-3}$ M $\text{AgNO}_3$. A stock solution of $\text{Cl}_2\text{CHCOO}^-\text{Na}^+$ (0.15 M) was prepared by dissolving a weighed amount of the compound in DMSO in a volumetric flask. An acyl transfer reaction was set up at the concentration of $1 \times 10^{-2}$ M ($1.5 \times 10^{-6}$ moles) thiol capture peptide, 0.1 equivalents of $\text{AgNO}_3$ and equivalents of $\text{Cl}_2\text{CHCOO}^-\text{Na}^+$. The volumes was controlled so that the final concentration was constant at $1 \times 10^{-2}$ M. The acyl transfer was allowed to run 20 hours at room temperature in the dark and analyzed for completeness by HPLC (A, 313 nm). Identification of the HPLC peaks were based on HPLC retention times. The peak due to acyl transfer to cysteine was purified by preparative HPLC (G, 313 nm) and the HPLC solvents were removed in vacuo, azeotroped 2x from HFIP, and triturated with diethyl ether. Sonication in diethyl ether followed by centrifugation (3x) removed traces of HFIP and TFA after which the pellet was dried overnight under high vacuum. Purity of product was assayed by HPLC (A, 313 nm, <99%). Yield (3.9 mg, 73.6 %)

**Acyl Transfer with Impure Materials**

Thiol capture material (CA$_2$K) was purified by isopropanol precipitation yielding material slightly contaminated (<2% by HPLC, A, 313 nm) with starting materials from the thiol capture reaction. A stock solution of the impure thiol capture peptide was prepared by dissolving a weighed amount of material in DMSO to the concentration of $1.5 \times 10^{-2}$ M. The stock solution also contained $1.5 \times 10^{-3}$ M $\text{AgNO}_3$. Stock solutions of $\text{Cl}_2\text{CHCOO}^-\text{Na}^+$ (0.15 M) and DIEA (0.15 M) in DMSO were also prepared using volumetric glassware. Acylation transfer reactions were set up containing $1.5 \times 10^{-6}$ moles thiol capture peptide, 0.1 equivalents of $\text{AgNO}_3$ and 0, 1.5, 3.0, or 5.0 equivalents of $\text{Cl}_2\text{CHCOO}^-\text{Na}^+$ or DIEA. Volumes were adjusted so that the final volumes of the acyl transfer reactions were either $1 \times 10^{-2}$ M or $1 \times 10^{-4}$ M thiol capture peptide regardless of the amount of base added. All volume measurements were performed using a Ranin
Pipetman or volumetric pipets and each reaction was performed in duplicate. A series of parallel reactions were also performed with no AgNO\textsubscript{3} present. Acyl transfers were allowed to run 20 hours at room temperature in the dark and then were stored at -20°C until analysis by HPLC (A, 313 nm). Identification of the HPLC peaks were based on HPLC retention times. HPLC peaks not due to starting material or acyl transfer products were considered to be impurities caused by hydrolysis of the glycine phenolic ester or by disulfide interchange.

**Acyl Transfer Kinetics with Sodium Dichloroacetate**

A stock solution of HPLC purified thiol capture peptide (CA\textsubscript{2}K) was prepared by dissolving a weighed amount of material in DMSO to the concentration of 1.5x10\textsuperscript{-2} M. The stock solution also contained 1.5x10\textsuperscript{-3} M AgNO\textsubscript{3}. Stock solutions of Cl\textsubscript{2}CHCOO\textsuperscript{-}Na\textsuperscript{+} (0.30 M, 0.03 M.) and TFA (0.30 M, 0.03 M) were prepared using volumetric glassware. Four kinetics runs were set up: Run1: 3x10\textsuperscript{-6} mol thiol capture peptide, 0.1 equivalents AgNO\textsubscript{3}, 5 equivalents Cl\textsubscript{2}CHCOO\textsuperscript{-}Na\textsuperscript{+}, final concentration of thiol capture peptide 1x10\textsuperscript{-2} M; Run 2: 3x10\textsuperscript{-6} mol thiol capture peptide, 0.1 equivalents AgNO\textsubscript{3}, 10 equivalents Cl\textsubscript{2}CHCOO\textsuperscript{-}Na\textsuperscript{+}, 5 equivalents TFA, final concentration of thiol capture peptide 1x10\textsuperscript{-2} M; Run 3: 3x10\textsuperscript{-7} mol thiol capture peptide, 0.1 equivalents AgNO\textsubscript{3}, 5 equivalents Cl\textsubscript{2}CHCOO\textsuperscript{-}Na\textsuperscript{+}, final concentration of thiol capture peptide 1x10\textsuperscript{-4} M; Run 4: 3x10\textsuperscript{-7} mol thiol capture peptide, 0.1 equivalents AgNO\textsubscript{3}, 10 equivalents Cl\textsubscript{2}CHCOO\textsuperscript{-}Na\textsuperscript{+}, 5 equivalents TFA, final concentration of thiol capture peptide 1x10\textsuperscript{-4} M. All volumes were measured with a Ranin Pipetman. Reactions were run at 24°C. Aliquots were taken at specific times, quenched with 50-fold excess TFA in and stored in liquid nitrogen until analysed by HPLC (A, 313 nm). Identification of the HPLC peaks were based on HPLC retention times.
Acyl Transfer in the presence of Trifluoroacetic acid

A stock solution of HPLC purified thiol capture peptide (CA2K) was prepared by dissolving a weighed amount of material (0.010 g, 7.3x10^{-6} mol) in DMSO (250 µL) to the concentration of 3.0x10^{-2} M. The stock solution also contained 3.0x10^{-3} M AgNO3. Stock solutions of Cl2CHCOO^+Na+ (0.30 M) and TFA, (0.15 M, 0.075 M) in DMSO were prepared using volumetric glassware. Acyl transfer reactions were set up containing 7.5x10^{-7} moles thiol capture peptide, 0.1 equivalents of AgNO3, 10 equivalents of Cl2CHCOO^+Na+, and 1, 2, 3, or 4 equivalents of TFA. All volume measurements were made with a Ranin Pipetman. Acyl transfers were allowed to run 24 hours at room temperature in the dark and then were quenched with 50-fold excess TFA in water and stored in liquid nitrogen until analysis by HPLC (A, 313 nm). Identification of the HPLC peaks as thiol capture peptide or acyl transfer products were based on HPLC retention times.

NMR studies of Cl2CHCOO^+Na+

1H NMR studies of HCl·Cys (Sbenzyl)OMe and AcLys(H·HCl)OMe at 1.6 x10^{-2} M in deuterated dimethylsulfoxide were undertaken in the presence of varying equivalents of Cl2CHCOO^+Na+ and TFA. 1H NMR of HCl·Cys (Sbenzyl)OMe after extraction with saturated sodium bicarbonate and AcLys(H·HCl)OMe after extraction with NaOH were performed as controls. The studies are tabulated below.

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<th>Compound</th>
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