PSEUDO-DYNAMIC COMBINATORIAL LIBRARIES

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A thesis submitted to the Faculty of Graduate Studies and Research
of McGill University in partial fulfillment of the requirements of the degree of
Doctor of Philosophy

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Montréal, Québec, Canada
May 2003

"For those who know they are only human, but try not to be anything less."

-Lloyd Alexander

ABSTRACT

A new method of drug discovery, the pseudo-dynamic combinatorial library, is described. The technique involves expanding on the dynamic combinatorial library method, where a reversible synthetic reaction is carried out in the presence of a target. In the pseudo-dynamic combinatorial library, this reversible reaction is separated into two irreversible steps. The independent control of the two steps leads to superior selectivity in the screening process. A model system based on peptidic inhibitors of carbonic anhydrase was developed and studied.

The synthesis of a series of new carbonic anhydrase inhibitors is described. Amino acids with sulfonamide and sulfamide functional groups, which are known to bind to carbonic anhydrase, were prepared.

The synthesis of a series of active esters attached to solid support are described. The tetrafluorophenyl esters are shown to be ideal for amide bond formation in aqueous media. The properties of this class of active esters were investigated.

Initial studies on a static library screening method are reported. The design and results of a series of dynamic combinatorial library experiments are described.

RÉSUMÉ

Une nouvelle méthode pour la découverte de composés bio-actifs et une bibliothèque pseudo-dynamique, est décrite. Cette méthode est une amélioration de la bibliothèque dynamique où une réaction réversible a lieu en présence de l'enzyme ciblée. La méthode pseudo-dynamique utilise deux réactions irréversible au lieu dùne réaction réversible. En optimizant ces deux réactions il est possible d'avoir une séléctivité supérieure à celle de la bibliothèque dynamique. Une système composé de l'anhydrase carbonique et de ces inhibiteurs sont décrites.

La préparation d'une série de nouveaux inhibiteurs de l'anhydrase carbonique est expliquée. Ceux-ci sont des acides aminées avec des groupes fonctionnels sulfonamide et sulfamide.

La préparation d'une série d'éstères activés attachés sur support solide est décrite. Les éstères tétratfluorophényl sont de bons réactif de départ pour la synthèse de liens amide dans l'eau. Leur réactivité est étudiée.

Une bibliothèque statique a aussi été préparée à titre d'example. Le développement de quelques bibliothèques dynamiques sont discutés.

ACKNOWLEDGEMENTS

I would like to thank my research supervisor, Professor James Gleason (a.k.a. Jim) for his invaluable guidance and advice. I learned a lot and if given the chance to do it all over I wouldn't change a thing.

I also wish to thank Professor Romas Kazlauskas, with whom we collaborated on this project. His advice was always sound and his door was always open.

This project would not have been successful without Jeremy Cheeseman's essential contributions. Thanks Jer.

I would like to thank Jonathan Croteau and Rong Hua Shu for their contributions in the early stages of the project.

My time at McGill would not have been the same without my labmates. Thank you to Ljubomir Isakovic, Evelyn Martins, Régis Dolaine, Christopher Rescorla, Jeffrey Manthorpe, Ngiap Kie Lim, Andrew Sawayama, Alain Ajamian, Diane Burke, Wolfgang Felzmann, David Sud, James Ashenhurst, Mike Yamashita, Sally Rodrigo, Tim Cernak, Nicolas Eghbali and Ciaran O'Dahlaigh.

Thank you to Diane for proofreading the original manuscript and Alain for help with the abstract and submitting my thesis for me.

Nadim Saade's patience and guidance with the LC-MS was much appreciated.

I would like to thank Drs. Fred Morin and Zhicheng Xia for providing training in and assistance with NMR spectroscopy.

I would like to thank Renée Charron, Chantal Marotte, Fay Nurse, Paulette Henault, Carol Brown and Sandra Aerssen for smoothing out the many administrative bumps on the road in grad school.

Work can be very dull and often discouraging, that's why you need friends and beer. Thank-you to all the friends I made in the Otto Maass.

Financial assistance from McGill University and the department of chemistry in the form of Alma Mater Travel Grants, Robert Zamboni Prize in chemistry, and the Pall dissertation fellowship is gratefully acknowledged.

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I Amplification of Screening Sensitivity through Selective Destruction: Theory and Screening of a Library of Carbonic Anhydrase Inhibitors *Journal of the American Chemical Society* **2002**, *124*, 5692-5701 © 2002 American Chemical Society 148

ABBREVIATIONS

AA	amino acid
Abu	aminobuteric acid
Ac	acetyl
AChE	acetylcholine esterase
Ala	alanine
Anal.	analysis
Aq	aqueous
Ar	aryl
Ara	arabinose
Arg	arginine
Asp	aspartic acid
Asn	asparagine
Bicine	*N, N*-bis(2-hydroxyethyl)glycine
Bn	benzyl
BOC	*tert*-butoxycarbonyl
BSA	bovine serum albumine
Bu	butyl
Bz	benzoyl
°C	degree Celsius
CA	carbonic anhydrase
ca.	approximately
Calcd	calculated
cat.	catalyst/catalytic
Cbz	benzyloxycarbonyl
Con A	concanavalin A
CSI	chlorosulfonylisocyanate
Cys_{SA}	β–sulfonamidoalanine
d	doublet
dansyl	5-dimethylamino-1-napthalenesulfonyl

DCL	dynamic combinatorial library
dd	doublet of doublets
ddd	doublet of doublet of doublets
de	diastereomeric excess
DIC	diisopropyl carbodiimide
DMAP	4-(dimethylamino)pyridine
DMF	*N,N*–dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dr	diastereomeric ratio
DTT	dithiothreitol
EDC•HCl	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EtOC	ethoxycarbonyl
ee	enantiomeric excess
equiv.	equivalents
ESI	electrospray ionization
Et	ethyl
fM	fentomolar
g	gram(s)
Gal	galactose
GC	gas chromatography
Glc	glucose
Gly	glycine
h	hour(s)
HMPA	hexamethylphosphoramide
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HOPt	*N*-hydroxyphthalimide
HOSu	*N*-hydroxysuccinimide
HPLC	high performance liquid chromatography

Hz	hertz
i	iso
IC	inhibition concentration
J	coupling constant
K_a	affinity constant
K_D	dissociation constant
K_i	inhibition constant
K_s	equilibrium constant of synthesis
L	litre
Leu	leucine
Lys_{SA}	lysine-ε–sulfamide
m	meta
m	milli, multiplet
M	moles per litre, metal
Man	mannose
mCPBA	*meta*-chloroperoxybenzoic acid
Me	methyl
MIC	minimum inhibitory concentration
mL	millilitre
mmol	millimole
mol	mole
MS	mass spectrometry
m/z	mass to charge ratio
n	normal
NANA	*N*-acetylneuraminic acid
ND	not determined
NHSSu	*N*-hydroxysulfosuccinamide
nM	nanomolar
NMR	nuclear magnetic resonance
p	para
PEG	polyethylene glycol

PFP	pentafluorophenol
pH	-log[H^+]
Ph	phenyl
PhMe	toluene
Phe	phenylalanine
Phe_{SA}	4-sulfonamidophenylalanine
pKa	-log(Ka)
Pr	propyl
Pro	proline
psi	pounds per square inch
RNA	ribonucleic acid
rt	room temperature
s	secondary
s	singlet
SM	starting material
STP	4-sulfo-2,3,5,6-tetrafluorophenol
t	tertiary
T	target
t	triplet
tert	tertiary
tetra-FP	tetrafluorophenol
TEA	triethylamine
tri-FP	trifluorophenol
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Tyr	tyrosine
UV	ultraviolet
μg	microgram
μM	micromolar
v/v	volume by volume comparison
Val	Valine

w/w	weight by weight comparison
WGA	wheat germ agglutinin
Xyl	xylose

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DECLARATION OF CONTENT

The research project described in this thesis was carried out as a collaboration between the research groups of professors James L. Gleason and Romas J. Kazlauskas of the department of chemistry of McGill university. The inhibition constant measurements cited in chapter two (Table 2-1) were performed by Jeremy D. Cheeseman under the supervision of professor R. J. Kazluaskas. All experiments described in chapter four were carried out in equal part by the author and Jeremy D. Cheeseman.

All other work described in this thesis was carried out by the author.

CHAPTER ONE

INTRODUCTION TO DYNAMIC COMBINATORIAL LIBRARIES

1.1 COMBINATORIAL CHEMISTRY

Combinatorial chemistry has evolved to become one of the most widespread tools for drug discovery.[1] The ability to synthesize very large numbers of compounds in a minimum amount of time has allowed access to millions of compounds for screening against potential therapeutic candidates. This method has also been used in the discovery of new catalysts and materials. The two most common styles of combinatorial synthesis are parallel synthesis and mix and split synthesis[1a] (also referred to as split and pool synthesis).

The general scheme of a mix and split synthesis, illustrated in Figure 1-1, shows a common starting material, represented by the triangle, reacting with reagents A, B, and C in separate vessels to afford 3 compounds. These compounds are then mixed together and divided into equal portions (three in this case) and allowed to react again with reagents A, B, and C to form nine new compounds. This process can be continued for as many cycles as desired. If the number of reagents (A, B, C) is defined as "n" and the number of cycles is defined as "m", the total number of compounds produced is n^m. In the example of Figure 1-1, n = 3 and m = 3. Therefore 27 compounds were prepared as mixtures in three different vessels, each containing a nine-compound library. These mixtures can then be screened against a target to identify "hits". Vessels containing "hit" compounds can have their contents resynthesized as discreet entities allowing for identification of the "hits" and for further studies.

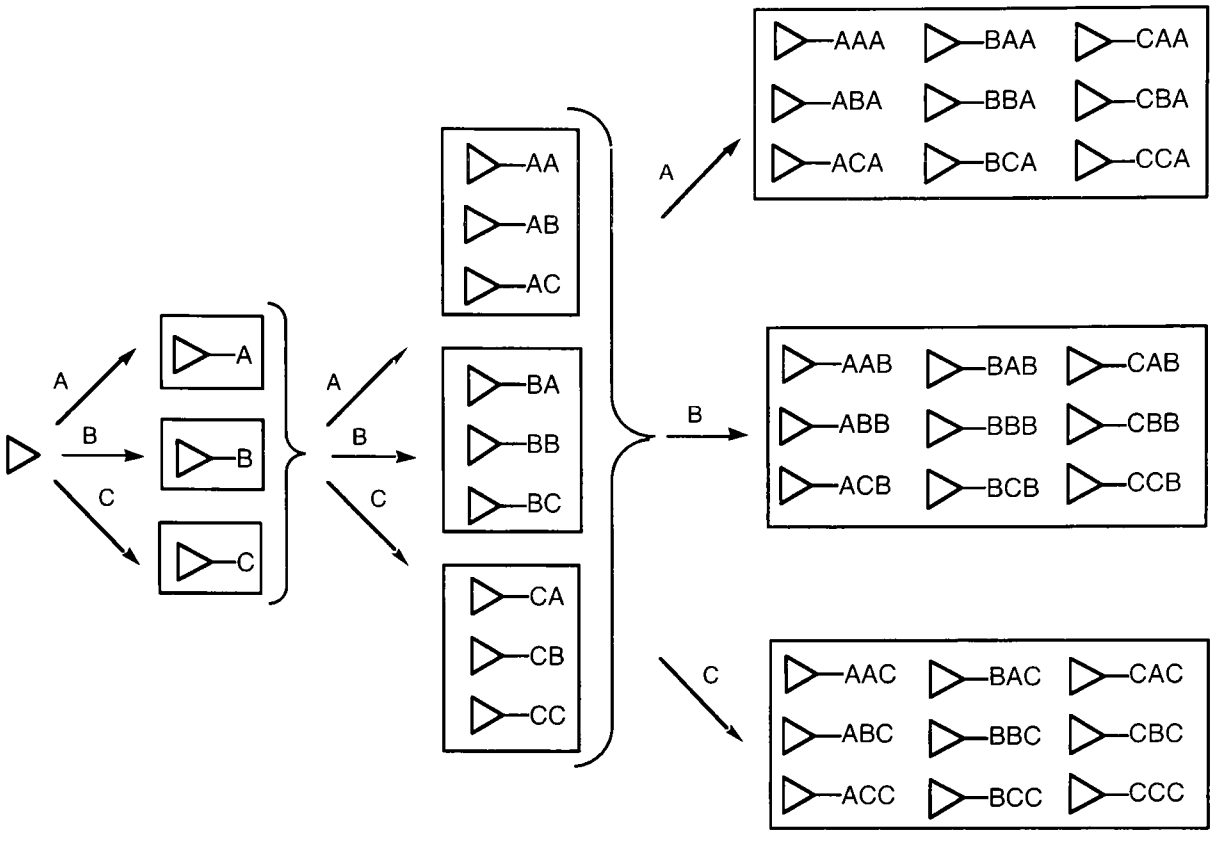


Figure 1-1: A general mix and split synthesis.

In the most common mode of mix and split synthesis, the common starting material is a polystyrene resin which supports the new molecule being formed. The individual beads can be separated and their contents screened against the target. In general, each bead will only have one compound on it, thus eliminating the tedious process of screening mixtures and resynthesis of the potential "hit" compounds for unequivocal identification. Unfortunately, since the amount of material on the average polystyrene bead is very small (micromolar amounts), it can often be difficult to fully characterize a "hit" via conventional means. Many well designed, but often complex, methods have been developed to identify the synthetic path which led to the product on a given bead.[1a]

A general parallel synthesis using a nine-well plate is illustrated below in Figure 1-2. A matrix is setup with reagents A, B and C. The nine possible reactions are carried out, each in a separate well to afford nine different compounds. This method provides access to a smaller number of compounds per reaction compared to the mix and split method, but offers the advantage of having each compound discreetly separated and its synthetic process identified without the use of tagging or another secondary identification method. The general example illustrated is a smaller version of a common parallel synthesis on a spatial array (e.g. a 96-well plate).

	A	B	C
A	AA	BA	CA
B	AB	BB	CA
C	AC	BC	CC

Figure 1-2: General scheme of a parallel synthesis.

While the path to an individual product is unambiguous, it is more difficult to produce large numbers of compounds when compared to a mix and split synthesis. The use of automation techniques has made parallel synthesis a useful tool for generating diversity.

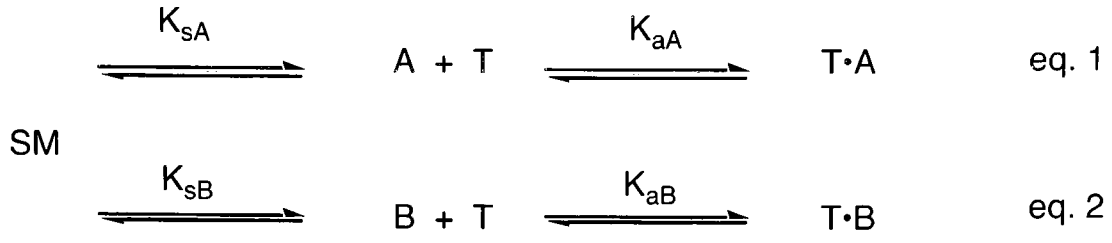
Although both of the above methods have been used successfully to generate large libraries of compounds, these methods of drug discovery have certain drawbacks. Both methods require separate screening steps after the syntheses are complete and these screening processes must be carried out with each compound or the contents of a vessel containing a mixture of compounds. Analysis of a library generated via a mix and split

methodology often involves the screening of these mixtures, which may lead to false positives, and as previously mentioned the characterization of the products is often not a trivial task.

1.2 DYNAMIC COMBINATORIAL LIBRARIES

Dynamic combinatorial chemistry represent a new theory in the field of combinatorial chemistry.[2] The basic concept of a dynamic combinatorial library (DCL) involves carrying out a reversible synthetic reaction in the presence of a target, such that the event of binding of a "hit" compound to the target will remove it from the synthetic equilibrium. This increases the amount of that compound that is synthesized in the reversible reaction (Le Chatelier's principle). Such a process should allow for the best binder in the library to be synthesized in the highest proportion relative to the poorer binders. This phenomenon offers two major advantages; 1) the synthesis and screening processes are integrated into one step, and 2) the identification and characterization of the best inhibitor from a mixture will be much easier than in a conventional system since it should be the most abundant compound in the library.

The concept of a DCL can be defined mathematically. Consider a reversible synthesis of A (eq. 1) and B (eq. 2), in the presence of a target (T), from a common starting material (SM), with synthesis equilibrium K_{sA} and K_{sB} (defined by eq. 3), which can bind to the target (T) with binding affinities K_{aA} and K_{aB} (defined by eq. 4).



eq. 1

eq. 2

$$K_{sA} = [A] / [SM] \qquad \text{eq. 3}$$

$$K_{aA} = [T \cdot A] / [T]\,[A] \qquad \text{eq.4}$$

Solving eq. 1 for the total amount of A ($[A]_T$ as defined in eq. 5), both that which is free and bound to the target gives eq. 6.

$$[A]_T = [A] + [T \cdot A] \qquad \text{eq. 5}$$

$$[A]_T = K_{sA}[SM](K_{aA}[T] + 1) \qquad \text{eq. 6}$$

The ratio of $[A]_T$ and $[B]_T$ can be found by dividing eq. 6 by the analogous equation for B to give eq. 7.

$$[A]_T / [B]_T = K_{sA}[SM](K_{aA}[T] + 1) / K_{sB}[SM](K_{aB}[T] + 1) \qquad \text{eq. 7}$$

If the assumption is made that both inhibitors are tight binders and the concentration of target is relatively high then $K_{aA}[T] >> 1$. This assumption leads to eq. 8, where the ratio of the products of the DCL are dependent on their equilibrium of synthesis and their affinity constants.

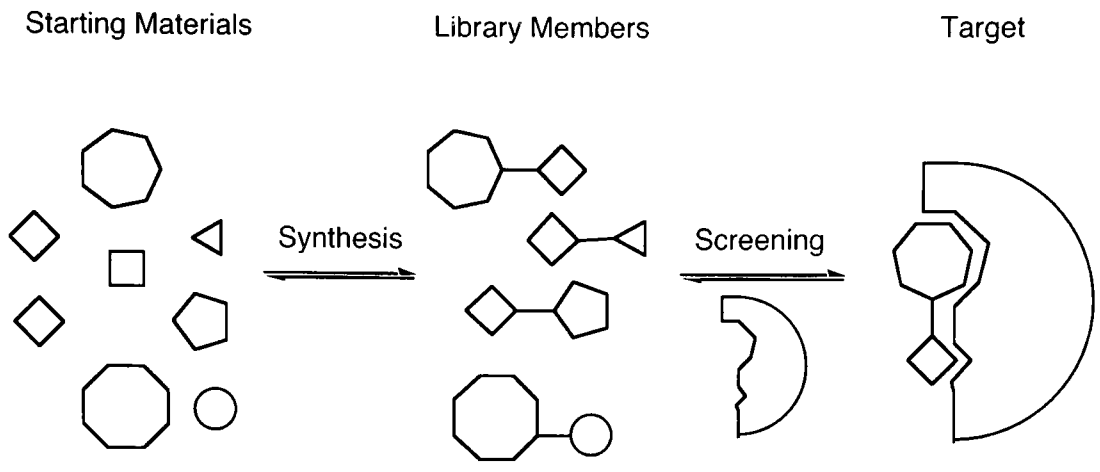
$$[A]_T / [B]_T = K_{sA}K_{aA} / K_{sB}K_{aB} \qquad \text{eq. 8}$$

In a synthesis of two similar products (A and B) via a reversible reaction, the equilibrium constants of the synthesis would be quite close to each other. Thus the major factor controlling the ratio of the products will be the ratio of their affinity constants.[3] This theory can be applied to a larger system with many products (A, B, C,...N) to afford eq. 9.

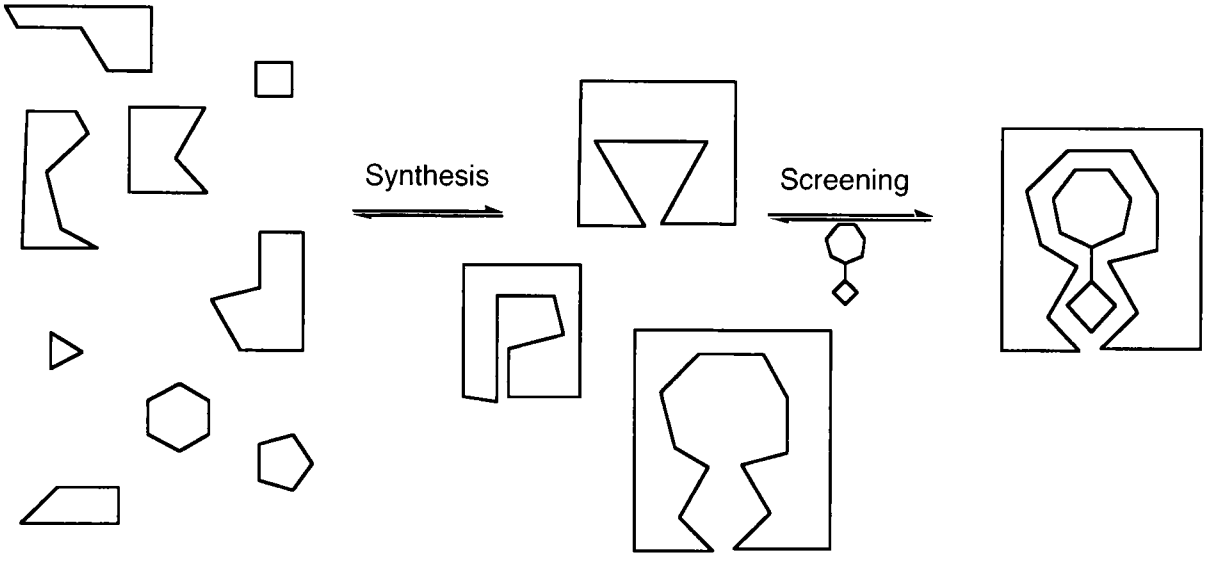
$$[A]_T / [B]_T + [C]_T + ...[N]_T = K_{sA}K_{aA} / K_{sB}K_{aB} + K_{sC}K_{aC} + ... K_{sN}K_{aN} \qquad \text{eq. 9}$$

Clearly as the number of components in the library increases, the difference in concentration between the inhibitors will decrease.

There are two common motifs for generation of a DCL. The first is generation of a library of small compounds whose synthesis is influenced by binding to a large target such as a biomolecule (often referred to as substrate casting[2e]). The second style is generation of a library of macrocyclic compounds whose synthesis is influenced by coordination around a small target (often referred to as receptor molding[2e]). Both the molding and casting types of DCLs are under thermodynamic control. The compound that has the highest affinity for the target will be removed from the synthesis equilibrium and thus formed in the greatest amount (Le Chatelier's principle). A general representation of these two modes of DCLs is illustrated in Figure 1-3. Conceptually these two types of libraries are the same, only the size of the target and the type of molecule in the library are different.



A DCL of small molecules in the presence of a large target.



A DCL of large molecules in the presence of a small target

Figure 1-3. The two common types of DCLs.

An alternate approach, called target-accelerated synthesis or virtual combinatorial libraries, involves screening via an irreversible reaction in the presence of the target where formation of products only takes place in the presence of the target[4,2e]. These methods are related to DCLs, but are distinctly different (Figure 1-4). Since the reaction in question is not reversible, amplification of the best binder by removing it from the

synthetic equilibrium will not occur. The selectivity of the process is solely based on binding of the starting materials to the target such that their proximity allows them to react with each other.

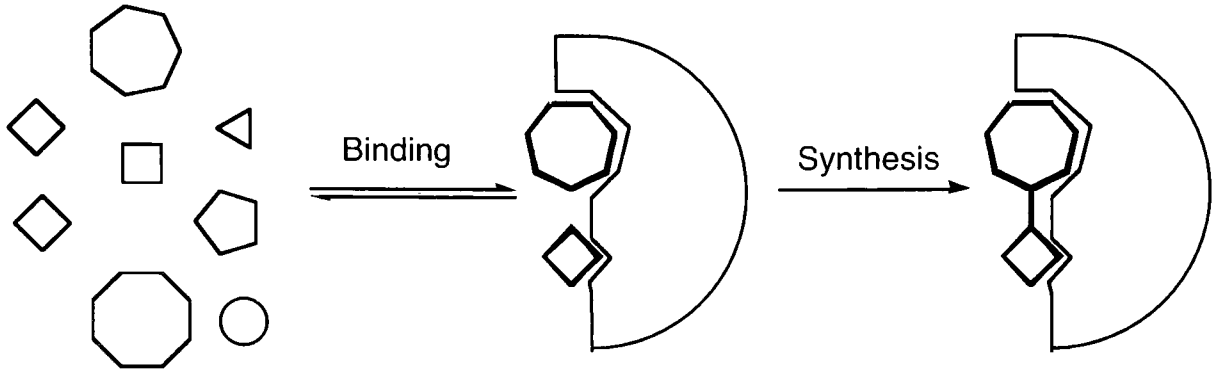


Figure 1-4. General scheme of a target accelerated synthesis.

Target accelerated synthesis relies on the formation of the best binder to the target being catalyzed by the target itself. This compound is clearly the kinetic product of the reaction. The scope of these systems is limited by the ability of the chosen target to catalyze a particular synthetic reaction. Most systems rely on reactions whose rates are raised significantly by increasing the proximity of two moieties.

The current work in the field of DCLs will be reviewed by type of bond formation regardless of the type of target. Some examples of target accelerated synthesis will be presented after the DCLs in a separate section.

1.2.1 DISPROPORTIONATION OF DISULFIDES

Disulfides can readily disproportionate in aqueous media under very mild conditions. This makes this reversible bond formation an ideal candidate for generation of a dynamic library and numerous groups have investigated this possibility. The first

reported example was disclosed by W. Clark Still and co-workers in 1998.[5] Still's library was based on the ability of a disulfide-linked pair of amide macrocycles to co-ordinate to small peptides attached to a polystyrene support (Figure 1-5). The macrocycle was tagged with a dansyl unit for ease of product concentration measurement.

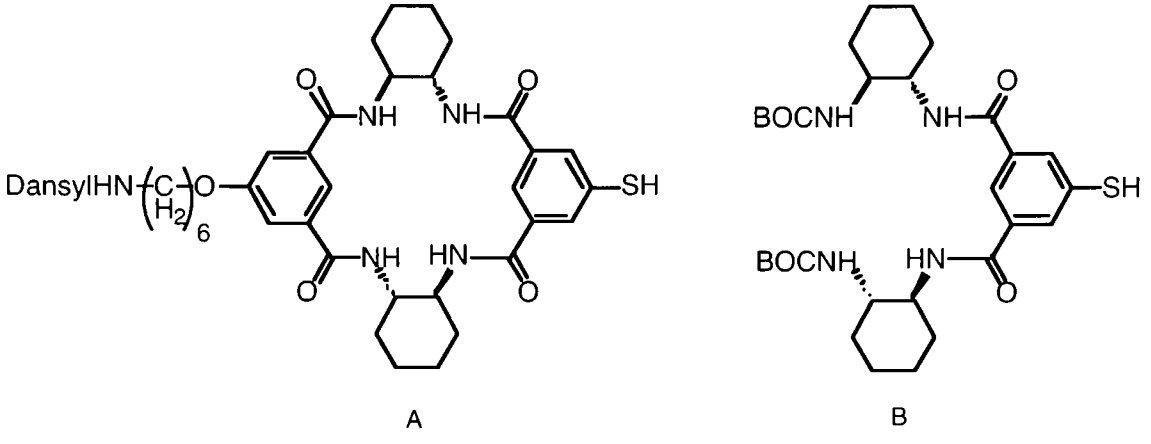


Figure 1-5. Thiols A and B of Still's disulfide library.

In a simple example, a mixture of three disulfides was prepared (A-SS-A, B-SS-B, and A-SS-B). In the absence of a peptide target, the ratios of the disulfides were roughly 1:1 (A-SS-A + B-SS-B : A-SS-B). Upon incubation of the mixture for 36 h in $CHCl_3$ with a small peptide, Ac-D-Pro-L-Val-D-Val, linked to a polystyrene resin via the C-terminus, the ratio of homodimers to heterodimers was shifted towards the former (85 : 15), and of these homodimers, 88% of the A-SS-A formed was bound to the solid supported peptide. The other homodimer, B-SS-B, was in solution. It was determined independently that the tripeptide did not bind to either A-SS-B or B-SS-B.

Sanders and co-workers have recently delved into the field of disulfide libraries in aqueous media.[6] Their initial work focussed on the generation of a library of poly-disulfide macrocycles from a series of dithiols. Analysis of a library generated from four dithiols, which could potentially generate 119 compounds, revealed that 56 of the

possible 66 unique masses were detected by ESI-MS (only products up to tetradisulfides were considered). Subsequent studies using various mixtures of disulfides as starting components led Sanders to conclude that generation of this class of library was under thermodynamic control.

In a subsequent publication,[7] Sanders and co-workers applied their disulfide library methodology to the casting of macrocycles around quaternary ammonium salts (Figure 1-6). Generation of the disulfide library from three dithiols (Figure 1-7) in the absence of the target with dithiothreitol (DTT) as a reducing agent in water at pH 8, provided a mixture containing 36 library members (determined by HPLC and ESI-MS). Two of these products were considered to be the major components (>10 fold more than the others). When the library was incubated with 2-methylisoquinolinium iodide, one of the minor components increased significantly at the expense of one of the major products. An even larger amplification of a different minor product was observed when N-methylmorphinium iodide was used as the target. Sanders used a carboxylate functionality in each of their dithiol units so that ionic coordination to the quaternary ammonium salts would induce macrocycle formation. This and all of Sanders subsequent work in this field, are examples of a DCL of large molecules that assemble around a small target. No product ratios were cited in the publication, but HPLC traces were included to illustrate the change in the composition of the library.

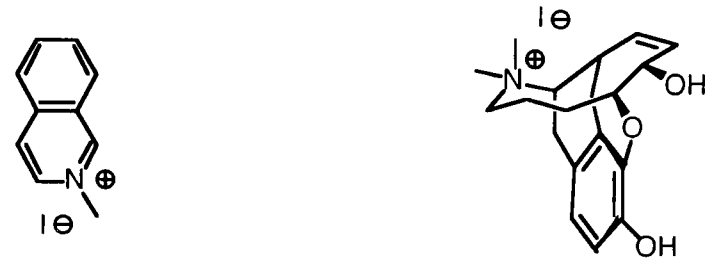


Figure 1-6. Ammonium salts used a s targets in Sanders' disulfide library.

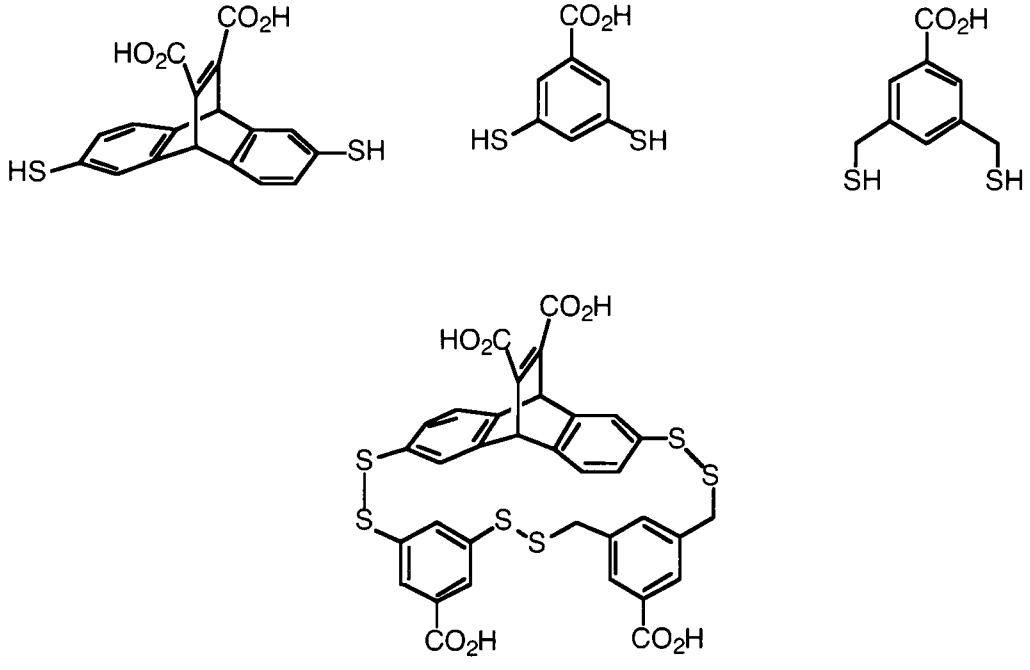
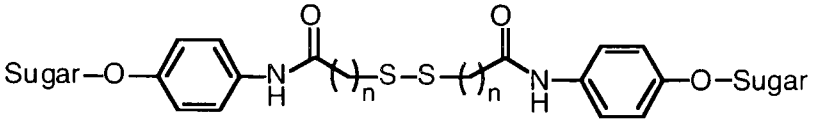


Figure 1-7. The three dithiols used to generate Sanders disulfide library and a sample library member.

It is worthy of note that while introduction of a target drastically altered the composition of the library compared to one generated in the absence of a target, other products were still present in the altered libraries with similar UV absorption intensities to the compound which was amplified. In short, the library does amplify the best binder, but not with absolute selectivity. This is consistent with the theory described earlier in the chapter governing the relationship between the ratio of the products and the ratios of their binding affinities.

The most impressive example to date of a dynamic library is a disulfide-based system developed by Jean-Marie Lehn and Olof Ramström.[8] Concanavalin A (Con A), a plant lectin known to bind mannose-rich carbohydrates, was chosen as the target of this library. A general dimeric class of binders was prepared with sugars on the termini connected via a disulfide-containing linker (Figure 1-8).



Sugars = D-mannose (Man), D-galactose (Gal), D-glucose,
L- arabinose (Ara), D-xylose (Xyl)

n = 3 for Man and Gal
n = 2 for Gal, Glc, Ara and Xyl

Figure 1-8. General structure of Lehn's Con A binders.

Four homodimers (Man, n = 3, Ara, n = 2, Xyl, n = 2, and Gal, n = 2) were incubated with dithiothreitol (as a disulfide exchange initiator), and Con A, which had been immobilized on sepharose, in water buffered to pH 7.4 for a period of two weeks. Analogous libraries were prepared without Con A present to ensure all possible products were generated. At the end of the experiment, the immobilized Con A was removed from the solution and denatured. Analysis by HPLC revealed that only four of the possible 10 products were bound to Con A. These were the mannose homodimer, and the three mannose–containing hetereodimers. The homodimer, as expected, was the major product, present in a two-fold greater concentration than any of the heterodimers. In the library prepared without the target, the ratio of the mannose homodimer to the greatest heterodimer present was 1.5: 1.0.

This methodology was then applied to a slightly larger library (6 homodisulfides as starting materials, resulting in 21 possible products) and similar results to those described above were obtained. Only the mannose-containing dimers were found bound to the Con A, and the homodimer was the product present in the highest concentration. Lehn's system represents a clear example of a DCL where a biopolymer is the target, and there are no significant biases to the synthesis process. The only drawback of this system is that if the target was not immobilized, it would be difficult to identify the best binder due to its small amplification in a relatively large (21 member) library. This is again consistent with the proposed theory (Section 1.2).

1.2.2 HYRAZONE FORMATION

Formation of hydrazones from aldehydes and hydrazines is a reversible reaction which has been exploited extensively in the field of DCLs. Jeremy K. M. Sanders and co-workers at Cambridge University have studied a pair of different acylhydrazone libraries for the formation of macrocycles. In both cases an amino acid unit is used to link acyl hydrazine and aldehyde functionalities (Figure 1-9).

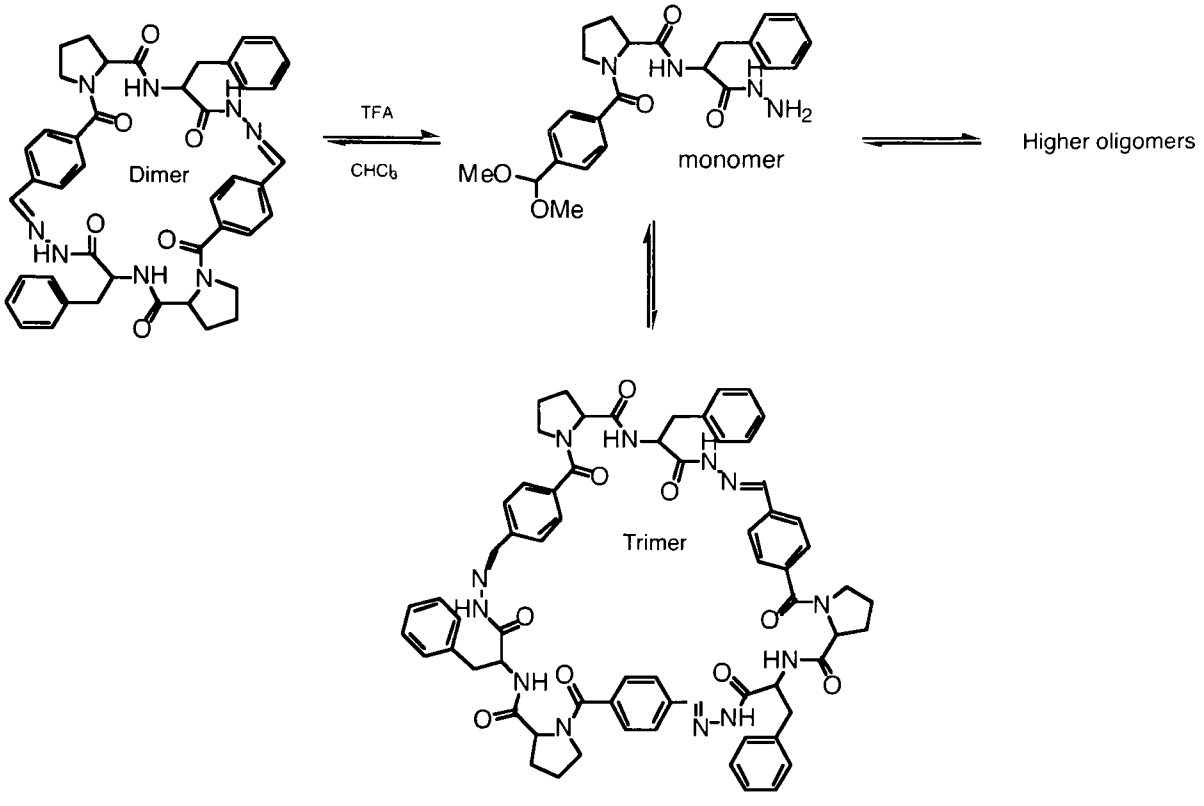


Figure 1-9. General form of Sanders' first acylhydrazone library.

Sanders first library monomer was shown by MS to form polycondensation products ranging from dimers to undecamers, though not all products were cyclic.[9] The library was then generated in the presence of 18-crown-6, which forms a complex with the free hydrazide residues.[10] The presence of the crown ether minimized the amount of cyclic products by removing the acyclic products from the synthetic equilibrium. Subsequent treatment of the $CHCl_3$ solution in question with excess KBr (relative to the crown ether) shifted the equilibrium of the library products back to their initial state. Finally, various salts were used as targets for this library.[11] In the absence of salts, the macrocyclic tetramer was the major product, but upon addition of NaI, the trimer became the major component. In the presence of LiI the trimer accounted for 98% of the products detected by HPLC due to the chelation of Li^+ to the hydrazone nitrogens.

In a very similar system, Sanders and co-workers screened a slightly different library of macrocyclic acylhydrazones against a pair of quaternary ammonium salts[12] (Figure 1-10). In the absence of a target, 88% of the macrocycles are in the dimeric form, 1% in the trimeric form. Upon addition of 2 equivalents of N-methylquinuclidinium iodide, fourteen cyclic products were observed by HPLC and ESI-MS, and the ratio was shifted to 41% dimer and 56% trimer. When acetylcholine chloride was used as the target, only 12% of the dimer was observed, with the trimer, at 88%, being the major component by far. The selectivity between the dimer and trimer was due to the size and shape of the target. Coordination of the electron-rich hydrazone nitrogens to the quaternary ammonium salt was favored in the dimer for the relatively flat N-methylquinuclidinium cases. For the more tetrahedron-shaped acetylcholine, a larger macrocycle, the trimer, was the most stable for surrounding the larger target.

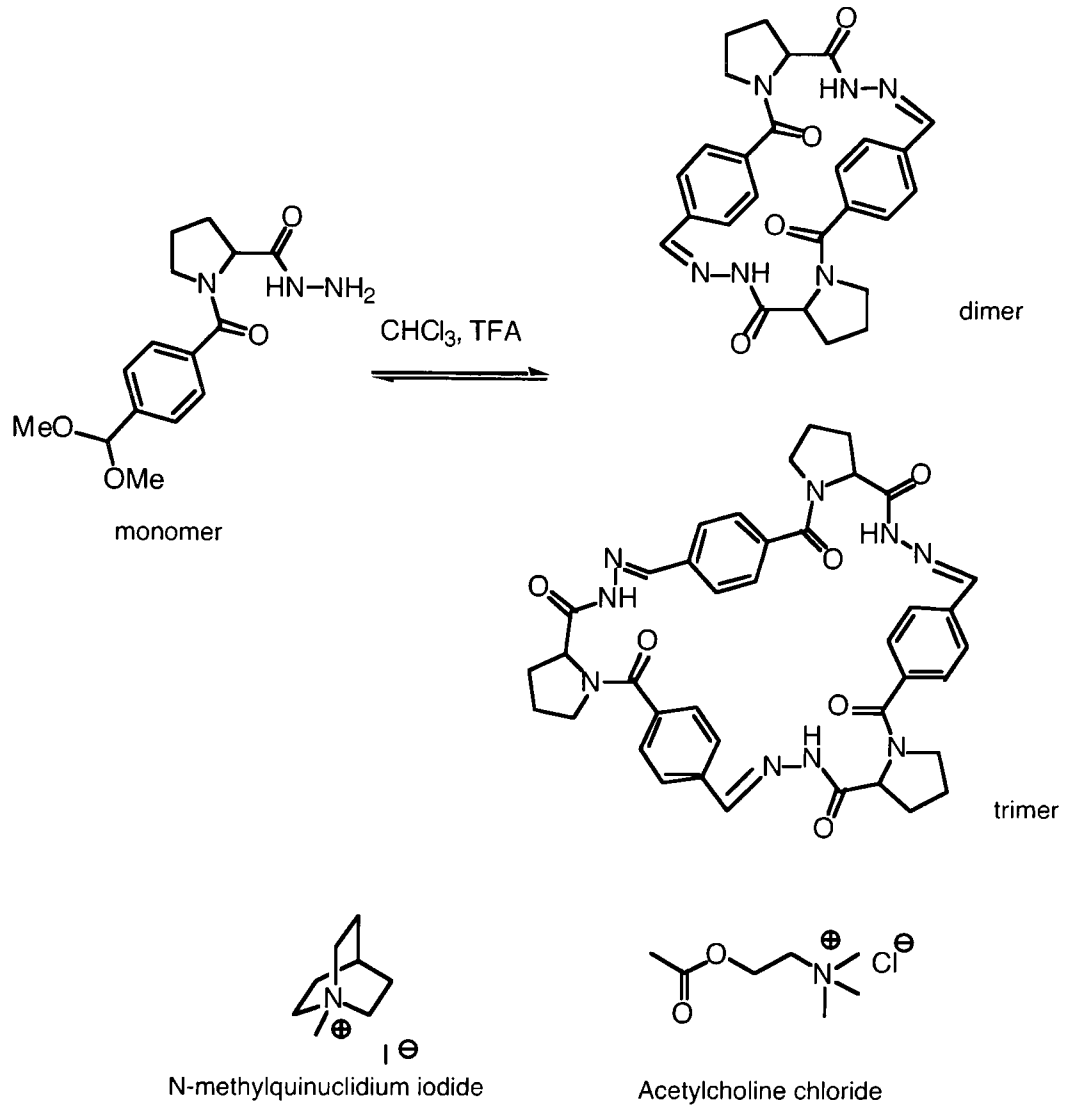


Figure 1-10. Sanders' second acylhydrazone library, screened against quaternary ammonium salts.

In a subsequent full paper,[13] this library was screened against five other quaternary ammonium salts with the trimer being the most common major product in all cases. As well, the library was generated in the presence of Amberlyst-27 ion-exchange resin, whose functional terminus is a benzyltrimethylammonium chloride unit.[14] The library members disappeared from the $CHCl_3$ solution over a 24-hour period. When the resin was washed with chloroform only a small amount of the dimeric species was in the

eluent. Washing of the resin with methanol afforded the trimer. This was the only product that had been tightly bound to the resin.

In another example of synthesis of a large molecule to bind a smaller one, Lehn and co-workers prepared a library of dihydrazones as potential binders of dibutyl barbiturate.[15] A solution of 5,5-dimethyl-1,3-cyclohexanedione and two equivalents of 2-hydrazinopyridine in $CDCl_3$ was allowed to equilibrate to form a mixture of the mono- and di- hydrazones as mixtures of *cis* / *trans* isomers (Figure 1-11). The composition of the mixture was monitored by ^1H NMR as 0.25 equivalent portions of dibutyl barbiturate were added. After a full equivalent of the target had been added the spectra had resolved to indicate that only one compound was present. The expected *cis*, *cis* dihydrazone was clearly the best binder to the target. A crystal structure of the target bound to the dihydrazone in question was also obtained.

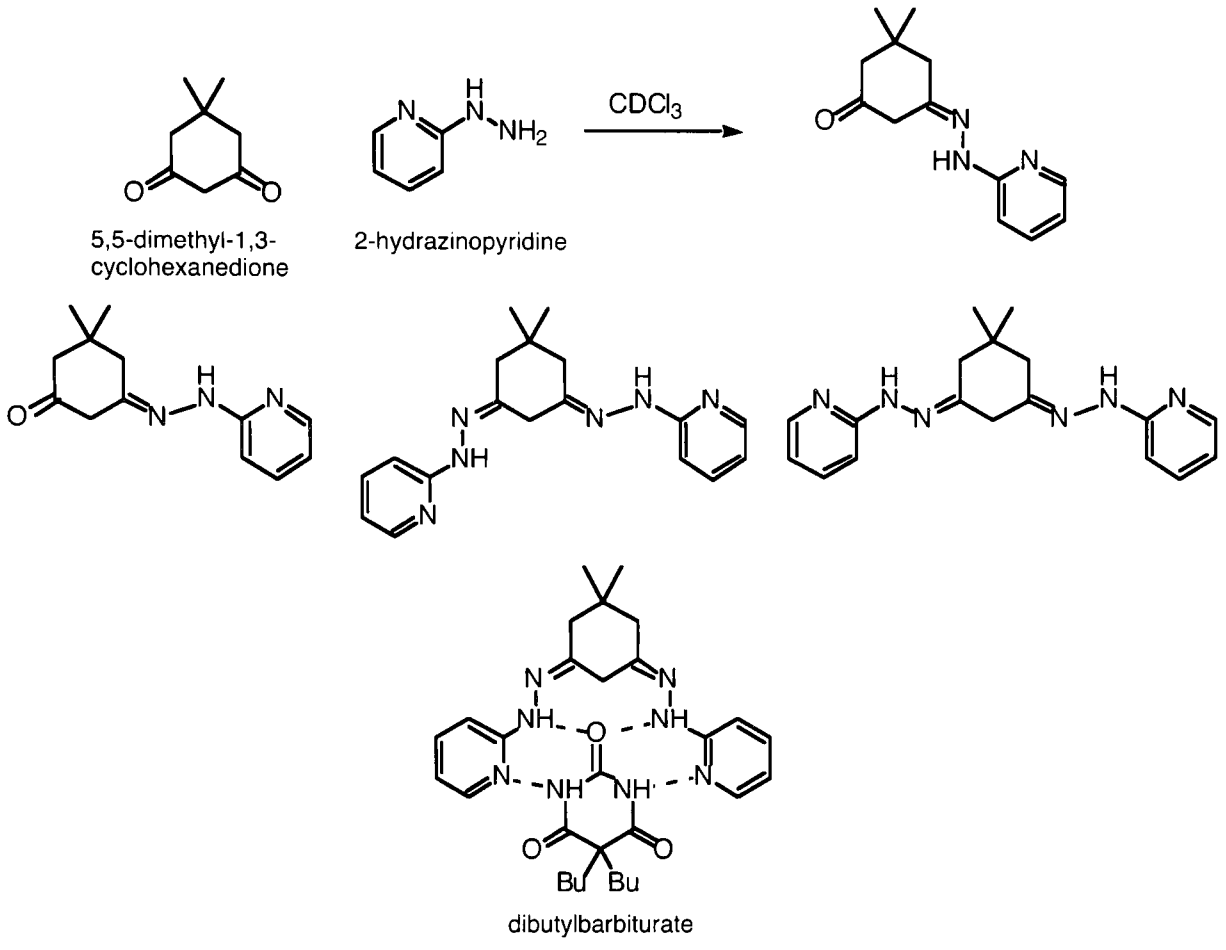


Figure 1-11. Hydrazones and target of Lehn's barbiturate library

1.2.3 NON-COVALENT INTERACTIONS

An interesting twist on the use of imines in DCLs is described in the work of Miller and co-workers where Schiff-base-metal complexes are generated and screened against DNA immobilized on a solid support.[16,17] A series of imines derived from salicylaldehyde were prepared (six in total) and incubated with $ZnCl_2$ in an aqueous DMSO solution. The resultant mixture could form 36 bis(salicylaltaminato) zinc complexes (Figure 1-12).

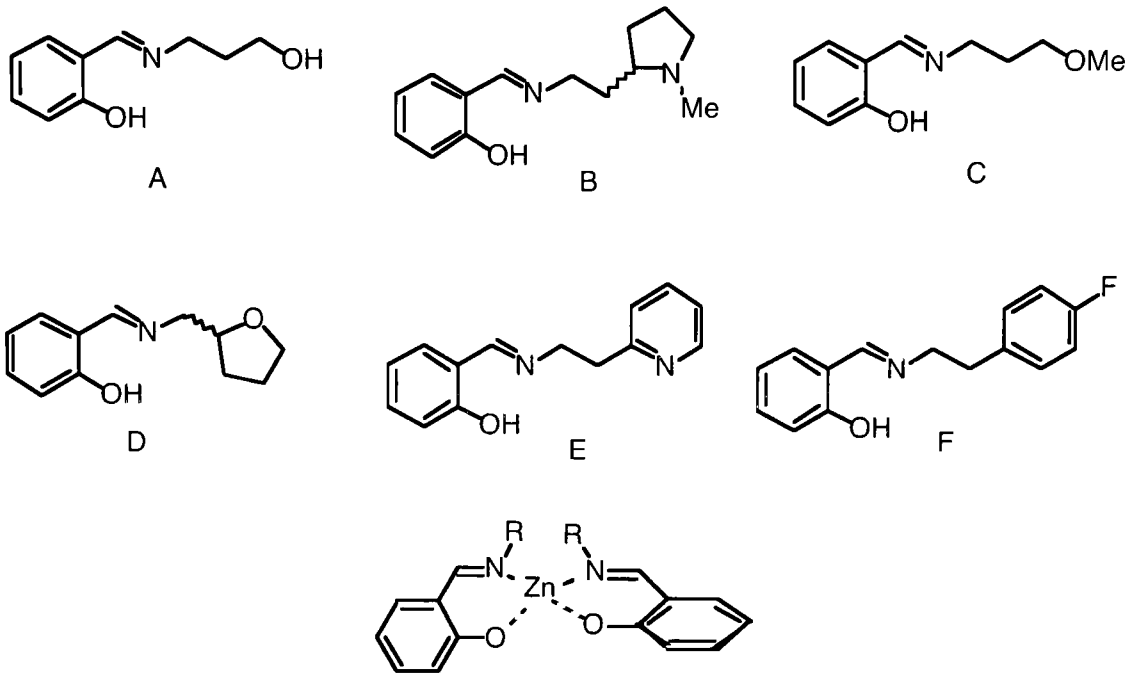


Figure 1-12. Miller's Schiff bases and the general form of the Schiff-base-zinc complexes.

This mixture of 36 zinc complexes was then eluted down a column of polythymidine-oligo immobilized on cellulose which had been previously treated with a solution of homopolymeric adenosine oligo to form an immobilized DNA duplex. The affinity of a given zinc complex to the DNA duplex was determined by measuring the amount of each Schiff base that eluted from the column. Significantly lower amounts of **B** and **D** were obtained relative to a standard prepared and eluted without zinc. Subsequent studies showed that the dimeric zinc complex of **D** was retained on the cellulose, thus leading to the identification of the **B**-Zn-**B** complex as the best binder. Resynthesis and screening of **B** showed it to have a K_D of 1.1 μM for the same DNA duplex, far lower than any of the other complexes which were resynthesized and screened as individual compounds. This method provides interesting results, but has the same

drawback as Lehn's, the target must be immobilized for successful library analysis. Quantification of the bound library members by residual monomer analysis would prove difficult in a larger system with multiple non-dimeric products.

In subsequent work, Miller and co-workers generated a similar library using salicylamide complexes of copper as potential binders to DNA and RNA hairpins.[18] A series of six salicylamides were prepared from salicylic acid and six amino acids (Figure 1-13). A dialysis bag containing an RNA or DNA hairpin was then immersed into an equimolar aqueous solution of these components with excess $CuCl_2$. A dialysis bag with a buffer solution was used as the standard for normalization of the results. After an incubation period of 12 h, the contents of the dialysis bag were denatured into a fresh buffer solution, lyophilized and analyzed for ligand concentration by HPLC.

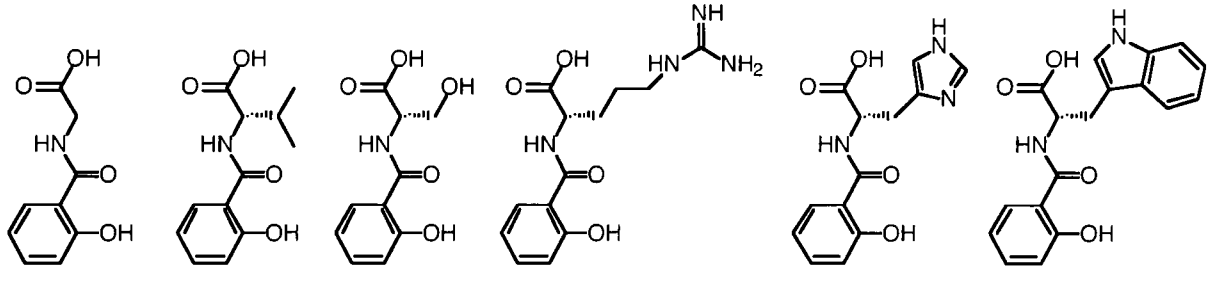


Figure 1-13. Members of Miller's salicylamide library.

The copper complex-dimer formed with the histidine containing salicylamide was observed in a 25 % higher concentration than in the standard, and was later found to have a K_D of 152 nM. This increase in concentration was well in excess of the other salicylamide ligands which showed little or no change from the standard. None of the ligands had affinity for the RNA hairpin in the absence of copper. The same complex was the only one to exhibit an increase in concentration in the presence of the DNA

hairpin, although its K_D was 300 times larger than for the RNA hairpin. No evidence was provided to indicate that this was in fact the best binder of the library members.

Influencing the equilibrium of the conformations of flexible molecules provides access to DCLs of limited structural diversity. In early publications in the field, Eliseev and co-workers explored the conformations of anionic species with the ability to bind to arginine.[19,20] A representative example of this method involves a solution of a bis(α, β–unsaturated) dicarboxylate in ethanol (Figure 1-14) being pumped through an apparatus which exposes the solution to UV light to effect *cis-trans* isomerization of the double bond. At the other end of the "pipe" the solution passes through a column containing arginine on silica (Figure 1-15).

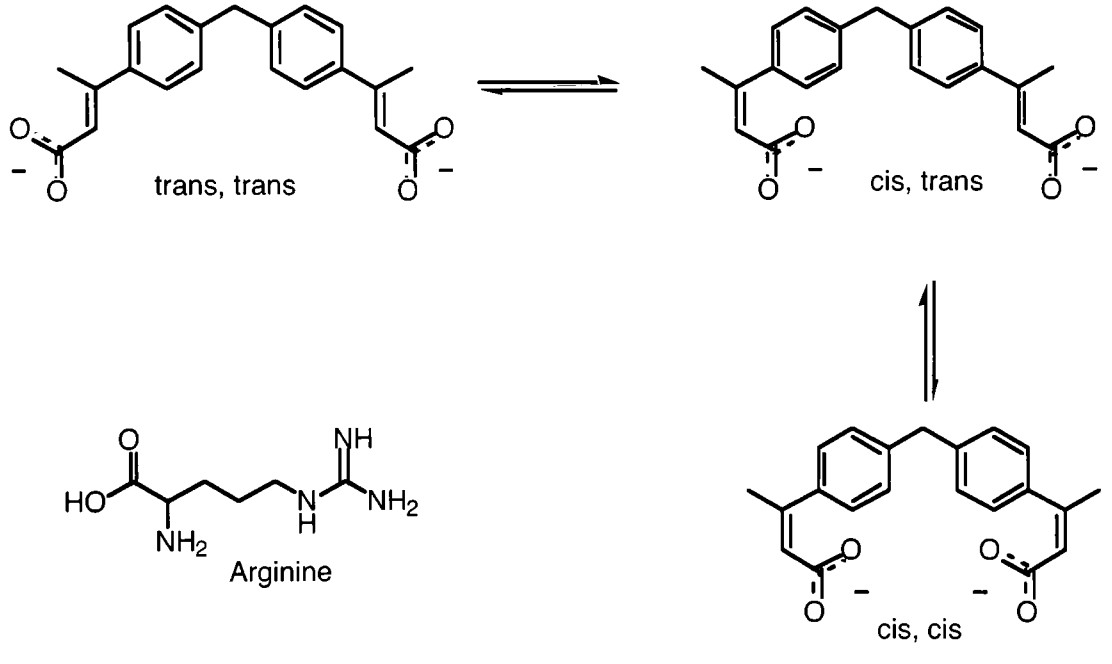


Figure 1-14. The dicarboxylates in Eliseev's arginine binding DCL.

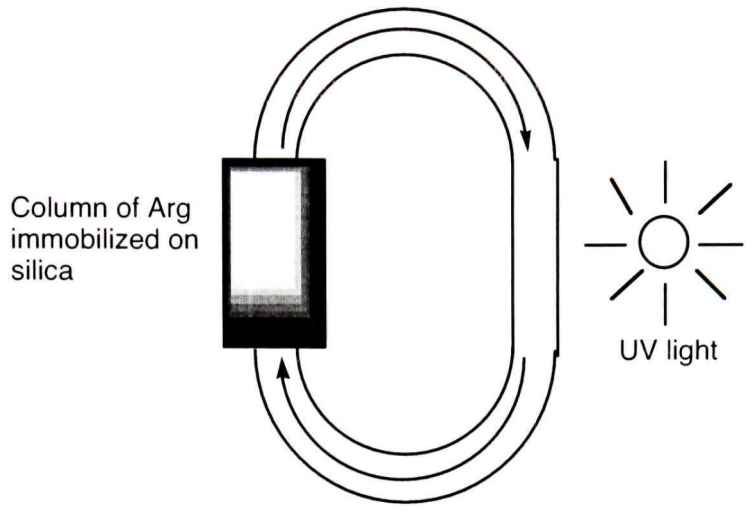


Figure 1-15. Cartoon of Eliseev's continuous flow apparatus.

Clearly the *cis, cis* isomer should be the strongest binder to the guanidine unit of arginine. The initial product ratio in solution was (*cis, cis* / *cis, trans* / *trans, trans*) 3 / 28 / 69. After the solution had been pumped through the system roughly 30 times (over an eight hour period) the concentration of dicarboxylate in solution had fallen by 89%. The isomer ratio in this solution was 48 / 29 / 23. The carboxylates bound to the affinity column were in a ratio of 85 / 13 / 2. For comparison purposes, the binding constant ratios of *cis, cis* to *cis, trans* were determined for methylguanidine by NMR titration. The ratio of these constants was only 2.4 : 1 in ethanol, indicating a likely significant amplification by the DCL process in question.

1.2.4 AMIDE FORMATION

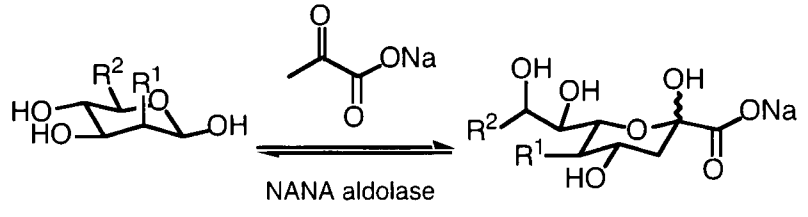
Amides are a common motif in small molecule enzyme inhibitors due to their stability and ability to mimic natural peptide recognition elements. The use of the amide bond in DCLs has not be thoroughly explored to date. A protease catalyzed synthesis of

polypeptides would appear to be an obvious candidate for generation of a DCL, but only one group has reported progress in this field. Venton and co-workers[21] used thermolysin to catalyze synthesis and hydrolysis of polypeptides in the presence of biopolymer targets. In one of their examples, 3E7 monoclonal antibody (endorphin-binding antibody) was used as a target where the starting peptidic components were Tyr-Gly-Gly and Phe-Leu. Pentapeptide Tyr-Gly-Gly-Phe-Leu is known to bind to this monoclonal antibody with a K_A of 7.1 nM. Various experiments were carried out in the absence of the target to establish that the synthetic process would generate a variety of polypeptides. Generation of the library in the presence of the target afforded Tyr-Gly-Gly-Phe-Leu as the major product over others that were not all characterized.

The second section of this work involved generating broader diversity in the libraries by using bovine serum albumin (BSA) hydrolases as the source of amino acids, thus providing all of the amino acids in various polypeptide motifs as starting materials. For this library, fibrinogen was used as the target, since various small peptides are known to inhibit fibrinogen monomer polymerization. For reasons that were not clearly explained, the BSA hydrolases were spiked with Gly-Pro-Arg, a tripeptide known to inhibit fibrinogen polymerization with an IC_{50} of 0.15 mM. The major products that were detected were independently synthesized and their IC_{50}s measured; Gly-Pro-Arg-Leu, IC_{50} = 1.5 mM, Gly-Pro-Arg-Phe, IC_{50} = 0.15 mM, and hexapeptide Asp-Lys-Pro-Asp-Asn-Phe, IC_{50} = 1.2 mM. No significantly stronger binders than the Gly-Pro-Arg which was present initially were generated from this library, thus making the success of this particular method difficult to evaluate.

1.2.5 ALDOL REACTIONS

The only other example to date of a DCL mediated by an enzyme is described in the work of Flitsch, Turner and co-workers[22] where N-acetylneuraminic acid aldolase (NANA aldolase) was used to catalyze both the formation and destruction of a small library of sialic acid analogues. Their target, wheat germ agglutinin (WGA), is known to bind sialic acid with K_a values in the millimolar range. Their system involved generating sialic acid and two related sugars (Figure 1-16) from sodium pyruvate and three starting components by incubating these with NANA aldolase in the presence of WGA for 160 h.



i) R^1= NHAc, R^2= CH_2OH
ii) R^1= OH, R^2= CH_2OH
iii) R^1= OH, R^2= H

iv) R^1= NHAc, R^2= CH_2OH
v) R^1= OH, R^2= CH_2OH
vi) R^1= OH, R^2= H

Figure 1-16. Flitsch and Turner's aldol-based dynamic library.

This three-membered library exhibited a meaningful amplification of product **iv**, sialic acid, with its concentration amplified by 70% relative to **vi**, and 150% relative to **v**, when compared to a control library generated with no WGA present. Though the library is small, it represents the only example to date of a DCL based on the aldol reaction and is one of the few that takes advantage of the reversible nature of enzyme catalyzed synthesis.

1.2.6 IMINE FORMATION

The concept of the DCL (at the time referred to a virtual combinatorial library) was first reported by Jean-Marie Lehn at the Pasteur Institute in France.[23] The target in Lehn's library was carbonic anhydrase (CA), a readily available, well-characterized zinc metallo-enzyme. The reversible synthetic process used was imine formation from a mixture of three aromatic aldehydes and four primary amines (Figure 1-17). One of the aldehydes was substituted at the para position with a sulfonamide, which is a functionality known to promote binding to CA.

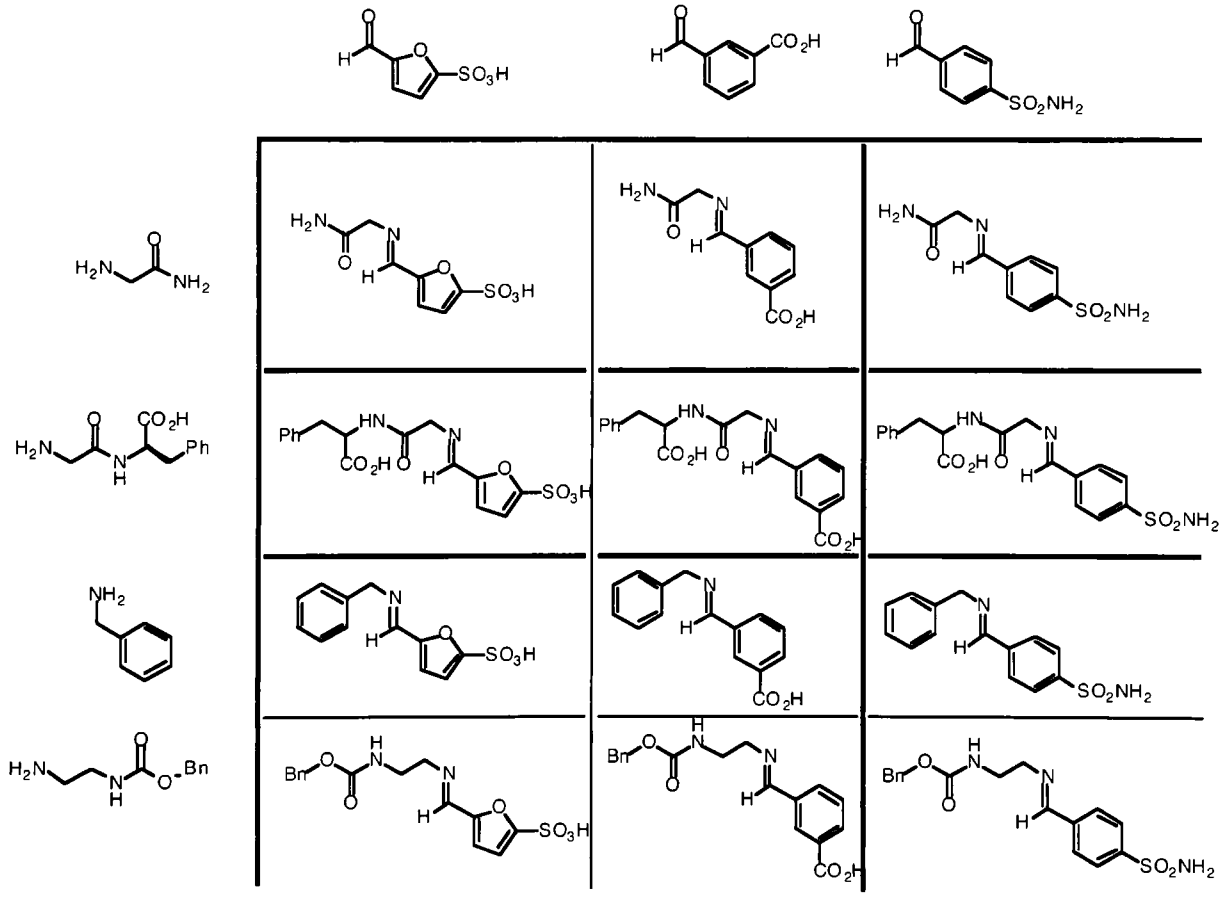
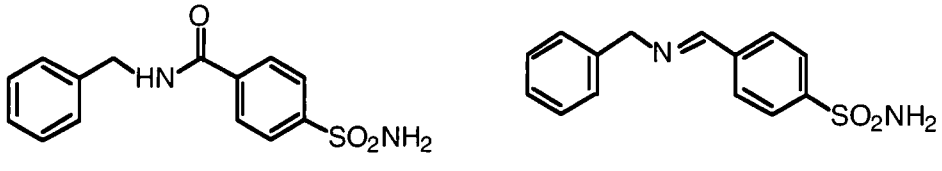


Figure 1-17. The member's of Lehn's imine library.

Lehn's library of amines (15 equiv) and aldehydes (1.0 equiv) was allowed to incubate in phosphate buffer at pH 6 in the presence of excess CA, relative to the aldehydes, and an unstated amount of $NaBH(CN)_3$ for two weeks. The enzyme was then thermally denatured and the resulting mixture of amines was analyzed by high-performance liquid chromatography (HPLC) and compared to a library generated in a similar manner, but with no CA present. The comparison of the two libraries indicated a roughly 2-fold increase in the concentration of the product arising from the condensation of benzylamine and p-sulfonamidobenzaldehyde.

Unfortunately, the imines that were generated were not stable enough for independent determination of binding affinity and the amine products did not bind due the protonation of the amine at pH 6. The proposal of the aforementioned product as the best inhibitor was made by a structural comparison to a known compound, 4-sulfamoylbenzoic acid benzylamide (Figure 1-18). Furthermore, the interplay of the reducing agent on the bound and unbound imines was not investigated. Imines that were formed outside of the binding pocket would also have been reduced, thus the composition of the library of amines that was analyzed may not accurately reflect the distribution of the imines bound to CA.



4-sulfamoylbenzoic acid benzylamide
K_d = 1.1nM

Lehn's best inhibitor

Figure 1-18. The best inhibitor of Lehn's imine library and the known analogue.

In a similar system, Eliseev and colleagues at Therascope AG generated a library of amines in the presence of neuraminidase, an enzyme involved in the propagation of the

influenza virus.[24] Eliseev proposed that incubation of a diamine with a series of aldehydes in the presence of neuraminidase extracellular domain in a water / DMSO solution allows for the formation a series of imines and aminals. After a ten minute period, the library was treated with a solution of Bu_4NBH_3CN, to allow formation of a wide variety of amine products from the imine and aminal precursors. These should only be generated when the amines and aldehydes interact with the target (Figure 1-19).

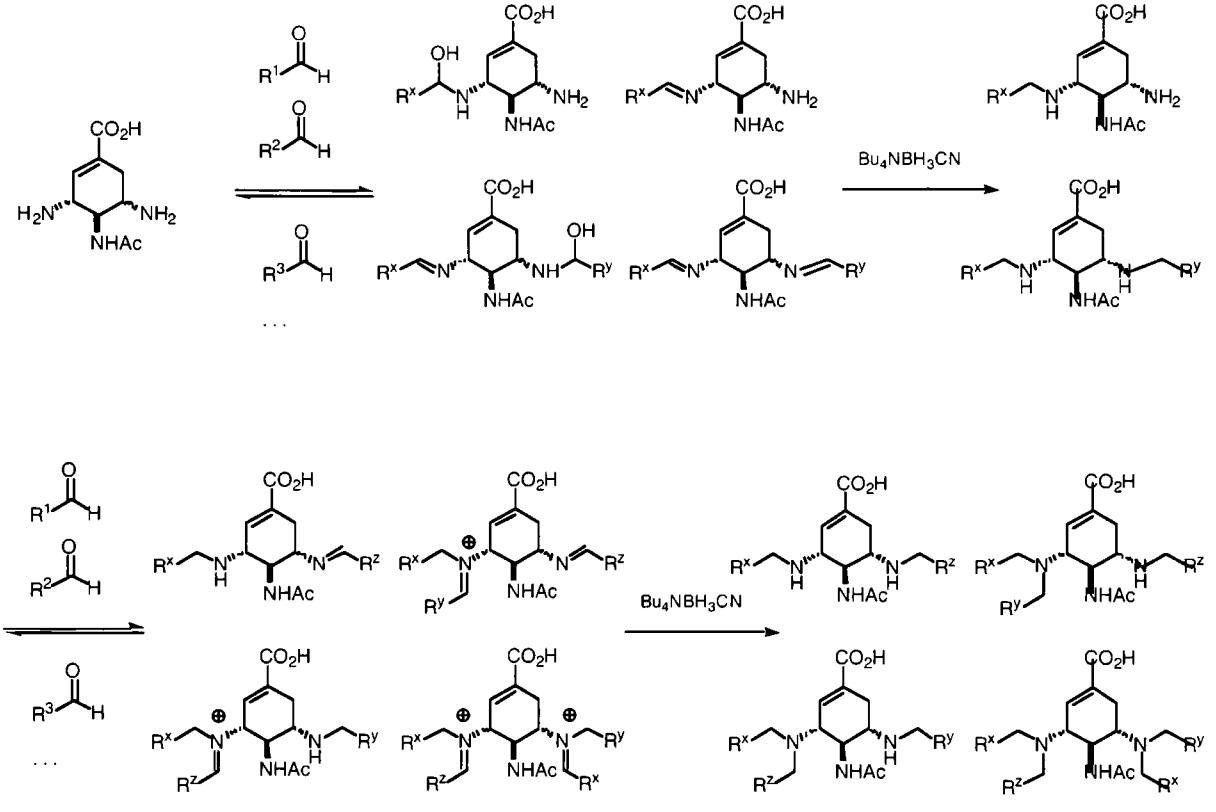
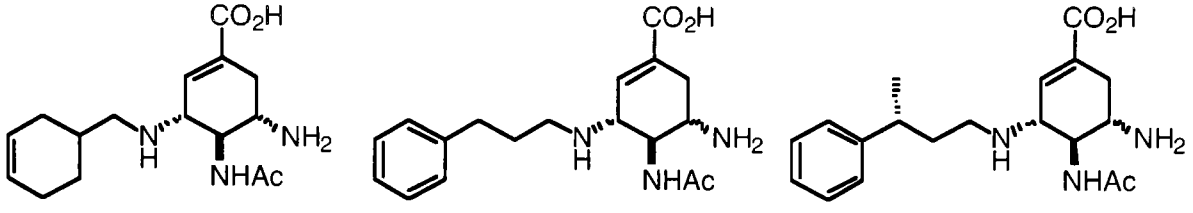


Figure 1-19. Synthesis of Eliseev's amine-neuraminidase inhibitors.

Generation of Eliseev's library with ten aldehydes, which they propose could generate 3115 different compounds, and in the absence of the target afforded no amine products (all analyzed by HPLC-MS). When the experiment was carried out in the presence of the target, three amines were detected (Figure 1-20). Inhibition constants were not provided for all of the possible library members making it difficult to accurately assess the results. The authors did admit that "...the diversity of the transient library is

difficult to assess...". The presence of many of the intermediate imines and aminals could not be proven, and when a static library was prepared (no target present, high concentration of reagents) not all of the di-tertiary amines were detected, thus making their claim of a library that could contain over 3000 compounds suspect. From the initial results, a second-generation library, with only one amine functionality on the cyclohexenyl starting material, was explored. This library afforded compounds with K_is in the nanomolar range.



K_i = 2.16 μM K_i = 1.64 μM K_i = 22.7 μM

Figure 1-20. The three products detected in Eliseev's potential 3115 member DCL.

None of the poly-alkylated amine products were detected in the library analysis. This makes it difficult to be confident that these products were ever formed during the DCL experiment, since the simple compounds were in fact the best inhibitors. This result again draws into question the actual diversity of the library. As in the previous example by Lehn, the interplay between the reduction process and the binding of the imines to the target has not been extensively investigated although in this case no products were formed in the absence of the neuraminidase.

These two similar methods of imine forming DCLs fall into a gray area between DCL and target accelerated synthesis. The use of an irreversible process means there is no truly reversible synthetic reaction occurring continuously in the presence of the target. Eliseev's lack of product formation in the absence of a target implies this may be in fact a

target accelerated synthesis of an imine which is then "trapped" as the amine. This would imply that the nature of the bond formed (imine or amine) is of no consequence, and only the position of the two (or more) starting components on the target and their proximity to each other determined the product that was favored in the library process. These results are related to those described below.

1.3 TARGET ACCELERATED SYNTHESIS

A clear example of a target accelerated synthesis is presented in the "Click Chemistry" method of Sharpless and co-workers.[4] Their work focuses on a target accelerated synthesis of acetylcholine esterase (AChE) inhibitors. The system was designed such that two units known to bind to AChE were attached to alkyl chains of varying lengths (Figure 1-21). At the other end of each chain either an azide or an acetylene, were present. These functionalities (azides and acetylenes) are known to undergo a 1,3 dipolar cycloaddition under forcing conditions (80°C, neat, 6 days). Fourty-nine different pairs of these azide and acetylene containing units were incubated in a solution of AChE for one week. Only one of these pairs underwent the cycloaddition to form a product. The reasoning for this one reaction occurring was that only this pair had the optimal chain length between the two binding functionalities to bring the azide and acetylene termini in close proximity to undergo the cycloaddition. Subsequent resynthesis and study of the "hit" compound, showed it to have a K_d of 99 fM.

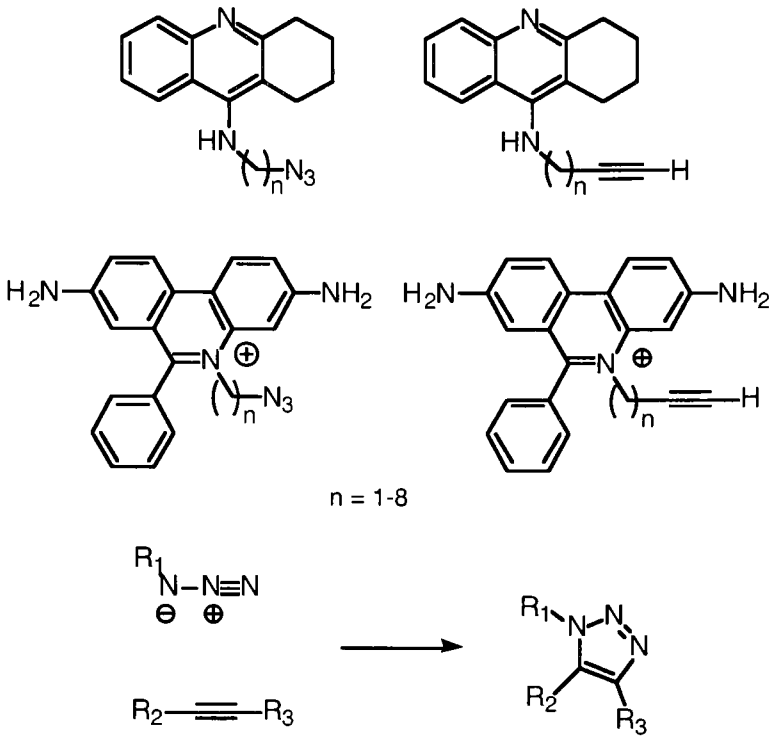
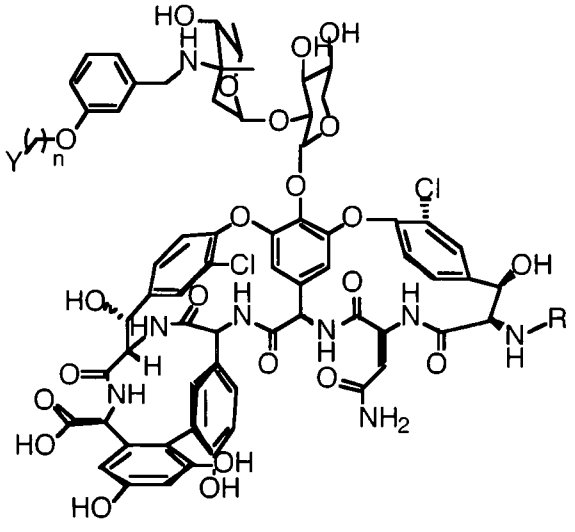


Figure 1-21. Sharpless' starting components and general cycloaddition of the target accelerated synthesis.

Another recent example is the work of Nicolaou and co-workers on the generation of analogues of the glycopeptide antibiotic vancomycin.[25] Many potent glycopeptide antibiotics are known to be dimers. Vancomycin, however has a very low dimerization constant, no significant amount has ever been detected. This led Nicolaou to propose that a vancomycin dimer may be effective in treating vancomycin–resistant bacteria. Drawing from their previous total synthesis of vancomycin, the group developed a series of vancomycin derivatives having alkyl "arms" of various chain lengths. As well, some variations on the amino acid at the R position were incorporated (Figure 1-22). At the terminus of the linking "arms" two different functionalities were installed such that two separate target-accelerated synthesis schemes could be carried out in aqueous media. In one case, a terminal alkene was used such that the dimerization could be carried out by

olefin metathesis (A, Figure 1-23). In a second strategy (B, Figure 1-22), thioacetates were prepared for use in oxidative formation of a disulfide bond.



A
Y = $CH{=}CH_2$
R = H, β–Ala, L-Asn, D-Leu-NMe, γ-Abu, L-Arg, L-Phe.
n = 1,2,3,7.

B
Y = SAc
R = D-Leu-NMe
n = 2,3,4,7,8.

Figure 1-22. Nicolaou's monomers for target–accelerated synthesis of vancomycin analogue dimers.

The target for each of these target-accelerated syntheses was a known vancomycin recognition element, the tripeptide Ac_2-L-Lys-D-Ala-D-Ala. For the synthesis via olefin metathesis, the starting components were stirred in 95:5 water : chloroform with the metathesis catalyst $[(PCy_3)_2Ru(CHPh)Cl_2$ and trimethyldocecal ammonium bromide as a phase-transfer catalyst. The compound present in the highest concentration (by HPLC) was the homodimer where R = Leu-NMe, n = 2. Subsequent resynthesis and screening of the compound against a vancomycin resistant strain afforded an MIC of 2 μg / mL (MIC for vancomycin > 100 μg / mL. This compound was in fact the most potent of the 10 library members that were resynthesized and screened.

The disulfide library was generated by treating the thioacetates shown in Figure 1-22 with sodium hydroxide and allowing the thiol to react in water under an air atmosphere with the same target as above. Fewer results from this library were reported, but some examples of these compounds were also resynthesized to afford compounds with MICs in the μg / mL range.

1.4 PSEUDO-DYNAMIC LIBRARIES

Although DCLs and target accelerated synthesis have established that it is possible to incorporate the synthesis and screening aspects of a drug discovery process, the methodology still requires further improvement before it can be considered a practical process. The two major drawbacks of the dynamic library process are the number of compounds to be identified and the amount of each product obtained.

The vast majority of the DCLs described in this chapter have numerous compounds present at the end of the process. This necessitates identification of all the products, resynthesis and screening to unambiguously determine which is in fact the best binder of the group. This first problem leads directly into the second; the amount of product obtained from DCL is often not enough for complete characterization. Authors rarely report actual yields of products from their DCLs. Most DCL analysis is based on mass spectrometry techniques, since only micrograms of material are needed for accurate results. Unfortunately, in larger and more complex systems, mass spectrometry is not always sufficient for unequivocal characterization. In many cases the use of conventional NMR spectroscopy would be ideal.

To address these shortcomings, we proposed the concept of a pseudo-dynamic library (pseudo-DCL). A pseudo-DCL is a case where the forward and reverse reactions of the traditional reversible synthetic reaction used in a DCL are separated into two irreversible reactions. This will permit fine-tuning of the system by turning the synthetic process on and off. Controlling the synthesis allows a kinetic component, an irreversible "destruction" of the products, to act and achieve superior selectivity in the library screening process. Ideally, non-binding (or weakly bound) products of the synthetic library can effectively be removed from the analysis process, leaving only the best binder along with easily identifiable starting components at the end of the library experiment. Recycling of the products from this destructive reaction would then give this process the opportunity for repeated synthesis and screening cycles, leading to the amplification of the best binder, such that it could possibly be isolated and fully characterized. In short, a pseudo-dynamic library could lead to the generation and isolation of one compound that is a strong binder to a given biological target in milligram quantities from a one-pot synthesis and screening process.

To probe the proposed method, a system was designed for the generation of a pseudo-dynamic library of carbonic anhydrase (CA) inhibitors. The bond at the crux of this library would be an amide, which would be generated from the coupling of an amine with an activated carboxylic acid. The reverse reaction, hydrolysis of the amide bond, was envisioned to be accomplished using a protease. The use of the protease necessitated that the inhibitors resemble peptides, such that substrate recognition by the protease would not be a major issue.

The three components of the proposed pseudo-dynamic library; synthesis, binding to the target, and destruction, would be physically separated from each other to prevent unwanted interactions, such as the protease acting on the target or premature hydrolysis of the inhibitors. A three-chambered vessel (with the three chambers separated by a permeable dialysis membrane (Figure 1-23)), was designed to meet these requirements, To prevent the activated carboxylic acids from passing through the membrane, it would be necessary to attach them to a solid support.

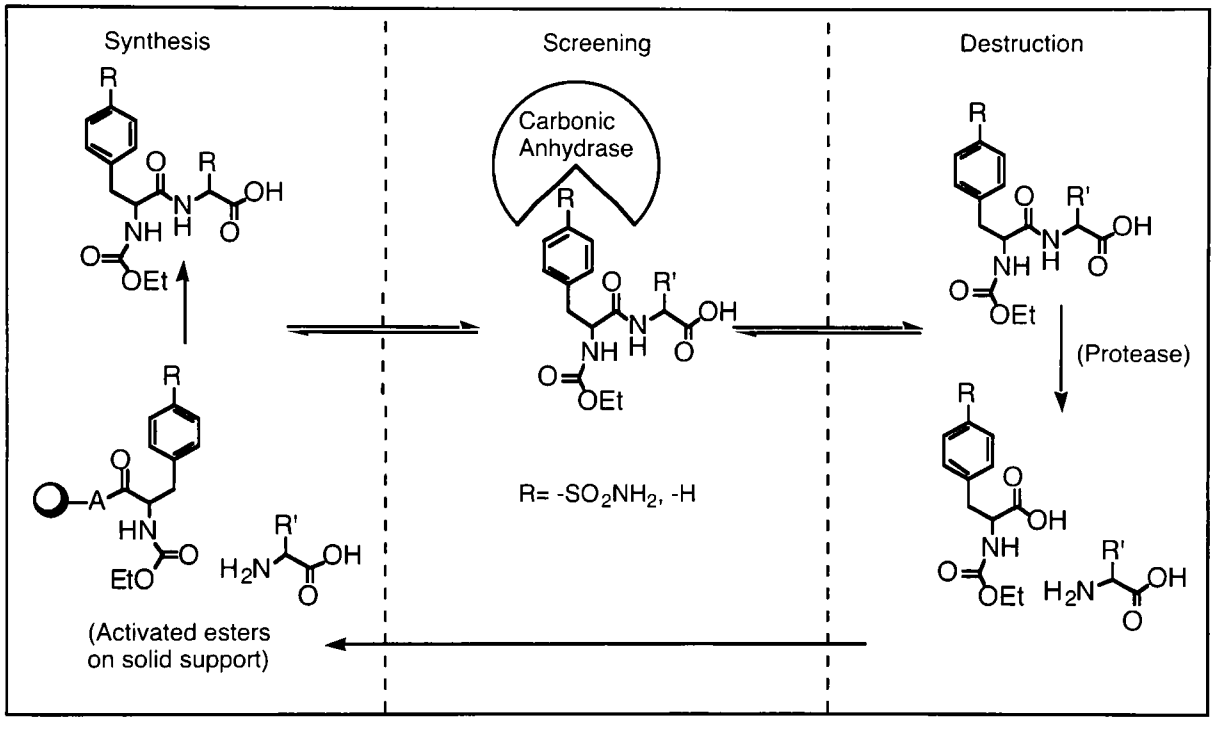


Figure 1-23. The general scheme for the pseudo-dynamic library.

Progress towards the generation of a pseudo-dynamic library involved research into three distinct aspects. Preparation of potential CA inhibitors with a peptide motif will be described in Chapter 2. Development of an appropriate active ester on solid support for effective peptide coupling in aqueous media will be summarized in Chapter 3. Examination of the interplay between the screening and destruction chambers and

combining synthesis and screening into a complete pseudo-dynamic library will be discussed in Chapter 4.

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CHAPTER TWO

SYNTHESIS OF CARBONIC ANHYDRASE INHIBITORS

2.1 CARBONIC ANHYDRASE

Carbonic Anhydrase (CA) is a zinc-containing enzyme which was first isolated and purified from erythrocytes in 1933.[1] Subsequent members of this enzyme family have been isolated from various sources. They fall into three classes: the α-class which are isolated as dimers, tetramers and octamers from mammalian sources; the β-class which are monomeric and isolated from plant, algae and bacteria; and the γ-class of which only one example has been isolated to date, a trimer from a *Methanosarcina thermophila*.[2] In all cases, CA catalyzes the reversible hydration of carbon dioxide via a two-step mechanism (Fig 2-1).[3]

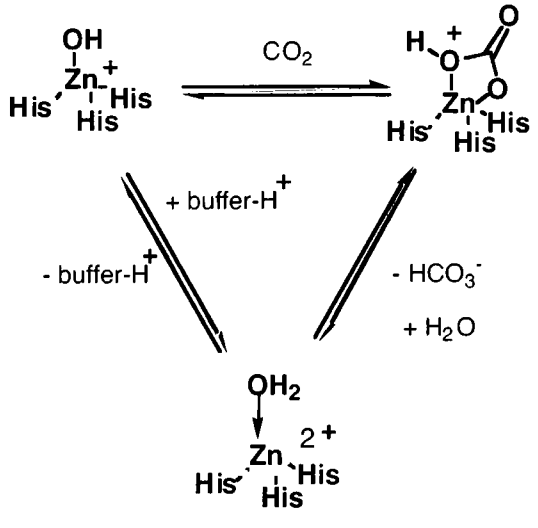


Figure 2-1. Catalytic cycle of the reversible hydration of CO_2.

In the α-class there are at least seven different isoenzymes. CA I is found mainly in red blood cells; CA II is found in red blood cells and in some neurons; CA III occurs mainly in the cytoplasm of muscle cells; CA IV, the most efficient of the isoenzymes in

carbonic acid dehydration, is found in lung, kidney brain and eye tissues; CA V is found in mitochondria; CA VI is found in saliva; and CA VII is present in the salivary glands.[4]

CA II deficiency is characterized by renal tubular acidosis, osteoporosis and mental retardation[5] A significant decline in CA concentration in the brain has been observed in Alzheimer's disease patients.[4] The most common role for CA as a therapeutic target is in the regulation of intraoccular pressure in glaucoma patients. CA II regulates pH in the cilary tissues, and thereby, controls the formation of the aqueous humor.[6] The use of orally available and topical CA inhibitors reduces water flow in the eye via a reduction in aqueous humor formation.

2.2 CARBONIC ANHYDRASE INHIBITORS

The most common structural motif among CA inhibitors is an aryl sulfonamide. There are currently six clinically used CA inhibitors, each of which possess an aryl sulfonamide unit (Fig. 2-2).[3] It is now well accepted that inhibition occurs through competitive binding of the sulfonamide nitrogen to zinc, which prevents binding of hydroxide ion, and subsequently, CO_2.

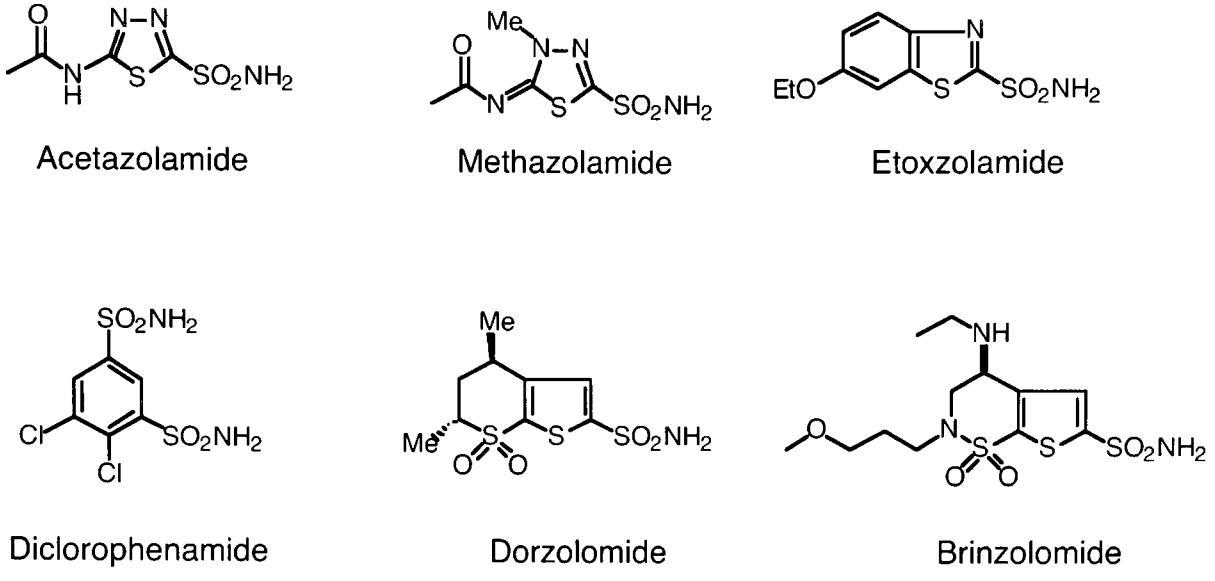


Figure 2-2. Some CA inhibitors that are in clinical use.

Another functional group known to bind strongly to zinc-containing enzymes is the hydroxamic acid. It has been recently established by co-crystallization of a simple hydroxamic acid with human CA II that the binding mode is monodentate through the nitrogen, not bidentate via the OH and carbonyl oxygen as often proposed.[7] The carbonyl oxygen and the OH of the hydroxamic acid appear to form H-bonds with a proximate threonine residue (Fig. 2-3). The additional bonding between zinc and fluorine in fluorinated hydroxamic acids was also established.

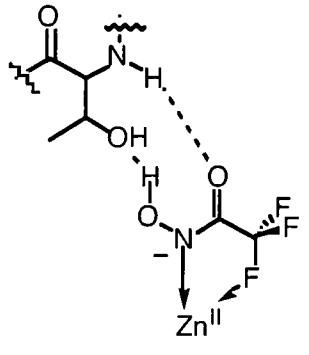


Figure 2-3. A representation of the binding mode observed in Christianson and co-workers crystal structures.

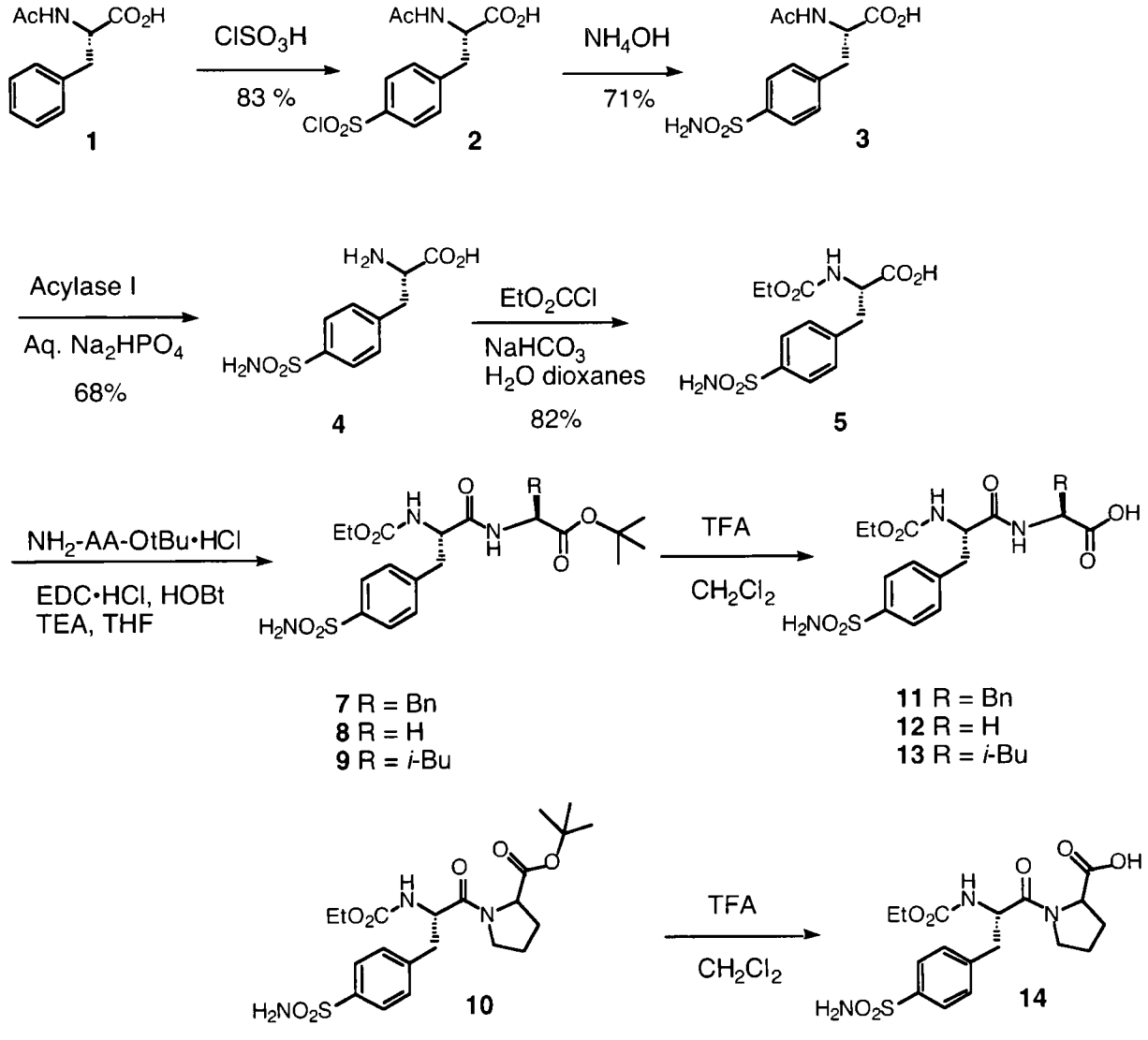
2.3 SYNTHESIS OF PEPTIDIC CARBONIC ANHYDRASE INHIBITORS

In an effort to develop a series of carbonic anhydrase inhibitors on a peptide scaffold, we endeavored to derivatize amino acids with functionalities containing a nitrogen atom whose protons exhibit a fairly low pKa. Sulfonamides, sulfamides, sulfamates and hydroxamic acids were the most likely candidate classes. Dipeptides incorporating these modified amino acids were also prepared for evaluation since they would constitute members of our proposed pseudo-DCL.

2.3.1 4-SULFONAMIDOPHENYLALANINE

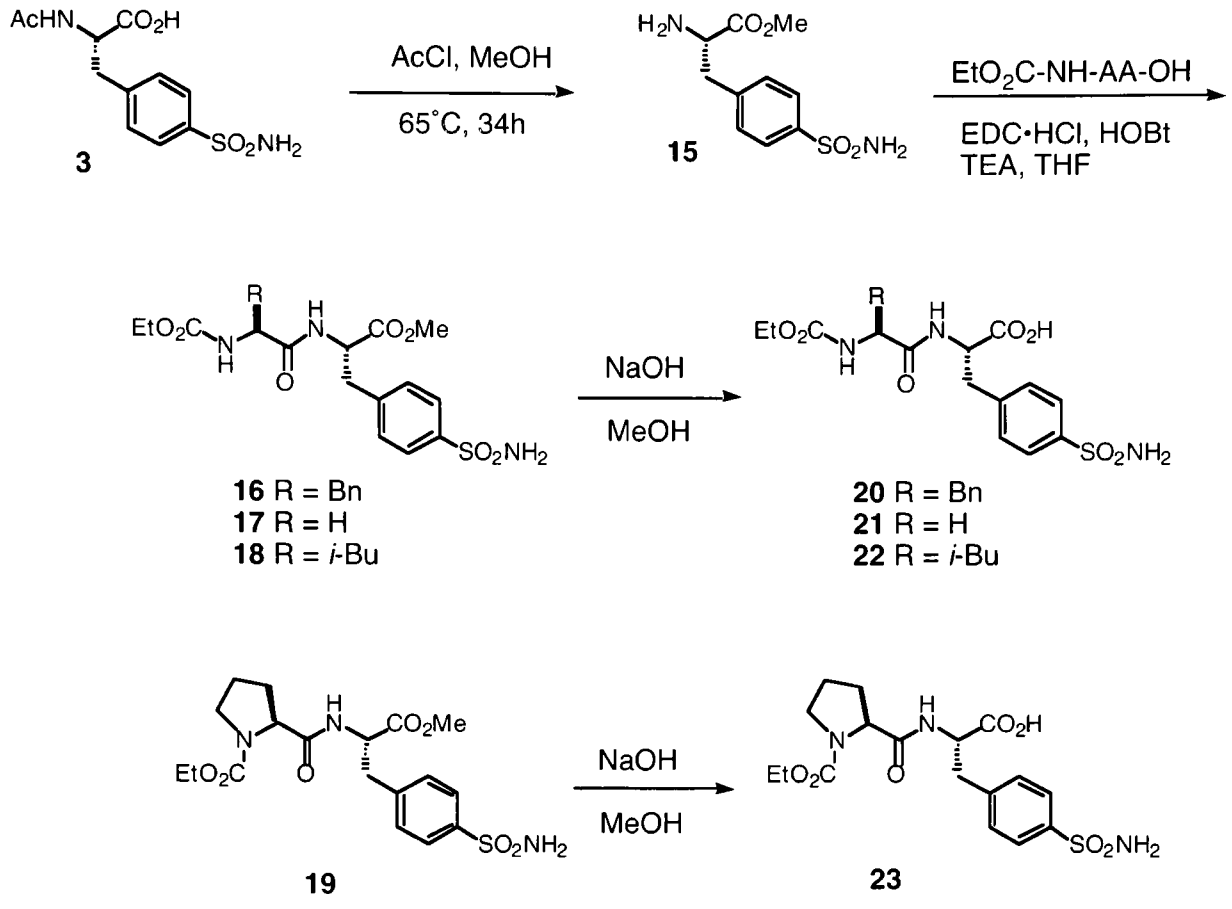
4-Sulfonamidophenylalanine was chosen as the initial sulfonamide–containing amino acid for our pseudo-DCL. It was initially prepared by Escher and co-workers.[8] However, we modified their procedure in order to produce large quantities.[9] In this modified procedure (L)-*N*-Acetylphenylalanine (**1**) was chlorosulfonylated with neat chlorosulfonic acid, and the resultant sulfonylchloride **2** was treated with concentrated NH_4OH to afford *N*-acetyl(4-sulfonamido)phenylalanine (**3**) in 59% yield. Acylase I from hog kidney was used for mild deprotection of the α-amine.[10] Although the deprotection could never be pushed to completion (most likely due to oxidation of the enzyme), the unreacted starting material was easily recovered for resubmission to the hydrolysis. Purification by ion-exchange chromatography and recrystallization from water afforded analytically pure 4-sulfonamidophenylalanine ($Phe_{SA,}$ **4**) in 77% yield based on recovered starting material.

Blocking of the α-amine in **4** with ethyl chloroformate under Schotten-Baumann conditions afforded **5**, a suitable electrophilic coupling partner for library synthesis. Four dipeptides (**11-14**) were prepared from this unit and the amino esters of Phe, Gly, Leu and Pro via conventional peptide chemistry.



Scheme 2-1. Synthesis of Phe_{SA} and its dipeptides.

A series of dipeptides with Phe_{SA} at the P1' position were also synthesized. These were prepared by Fischer esterification of **3** in methanol with concomitant amide hydrolysis. The resultant amine (**15**) was coupled with the ethyl carbamates of Phe, Gly, Leu and Pro to afford dipeptides **20-23**.

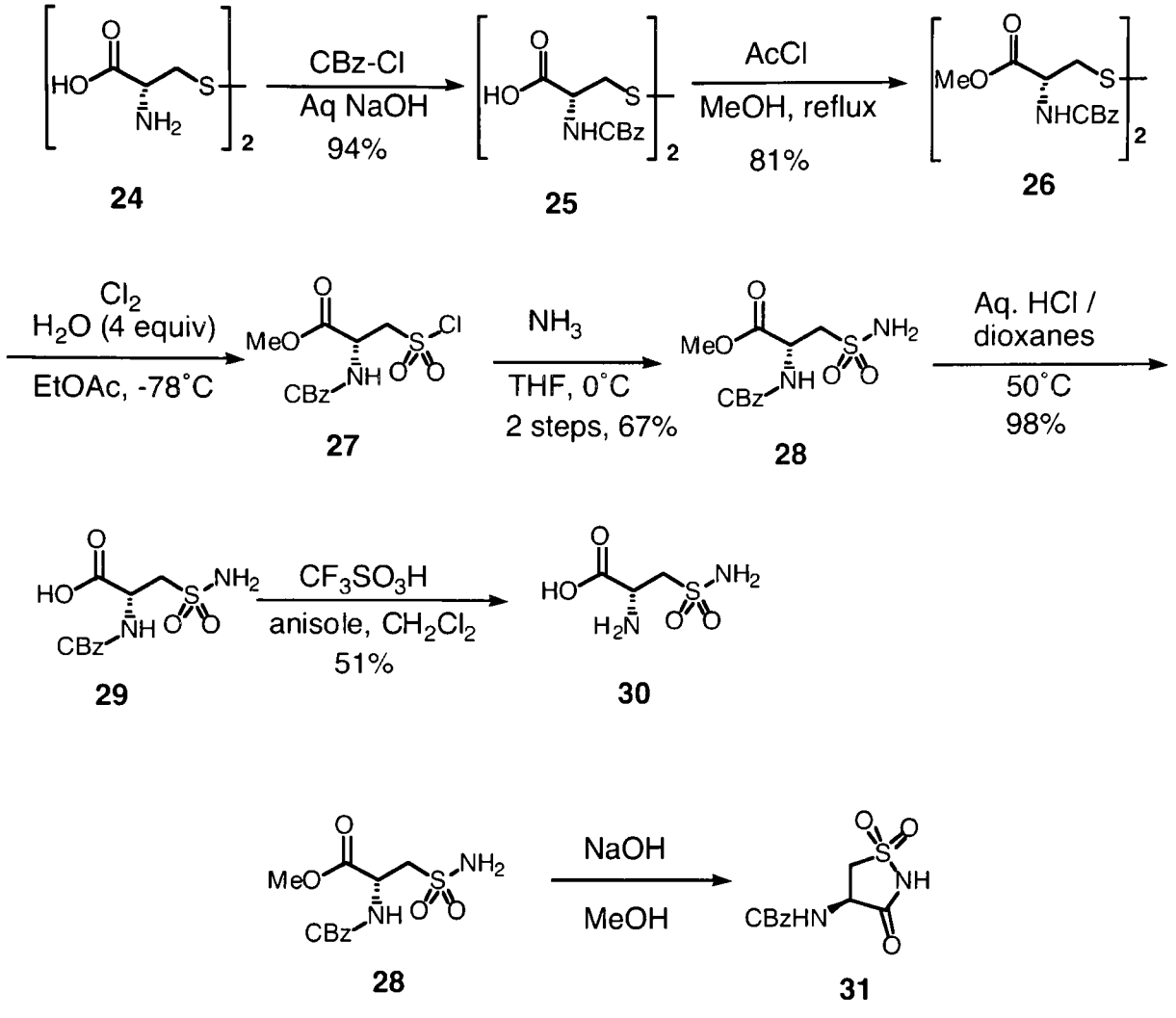


Scheme 2-2. Synthesis of dipeptides with Phe_{SA} at the C-terminus.

2.3.2 β-SULFONAMIDOALANINE

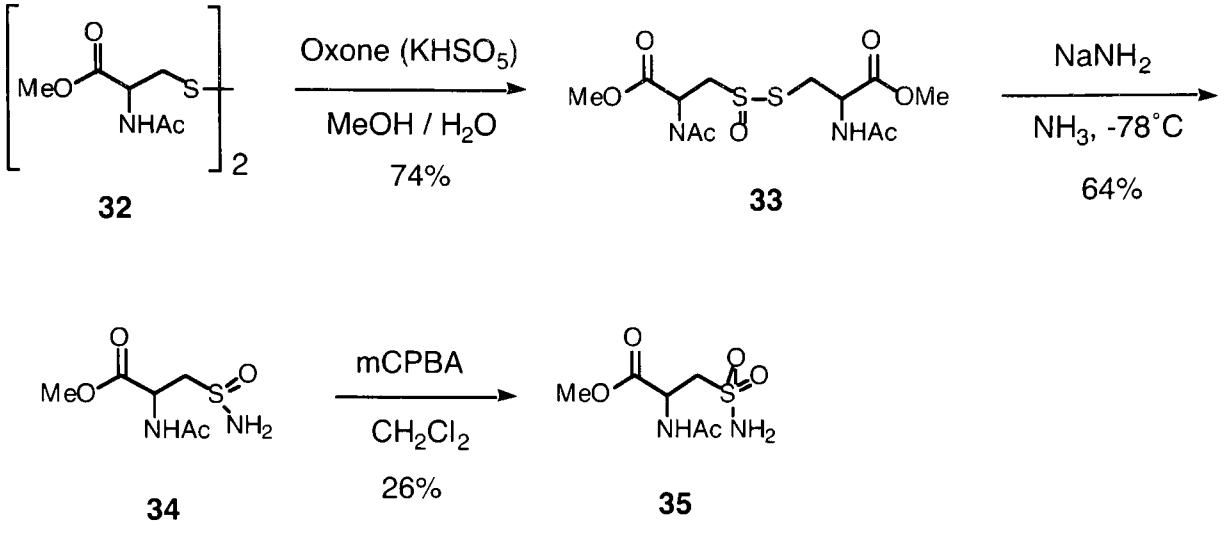
The sulfonamide derived from cysteine (**30**, Cys_{SA}) was originally prepared in 1959.[11] The reasonable yields reported by the authors for the oxidation of the disulfide, cystine, with chlorine in water as solvent were not reproducible in our hands nor by others.[12,13] The use of a stoichiometric amount of water proved to be essential, as described by Chen and co-workers,[11,14] but their rigorous and awkward control of the amount of chlorine was unnecessary for our purposes.

Our procedure (Scheme 2-2) entailed a conventional CBz protection of cystine followed by Fischer esterification to afford a fully protected disulfide **26** in 81% overall yield. Oxidation of this disulfide proved successful when it was dissolved in ethyl acetate with four equivalents of water, cooled to –78°C and treated with an excess of chlorine gas. Subsequent treatment of the resulting sulfonyl chloride with ammonia in THF afforded the fully protected sulfonamide **28** in 67% yield over the two steps. Saponification of the methyl ester afforded only the cyclized product **31**, while acid hydrolysis provided the desired carboxylic acid **29.** After attempting various sets of palladium-catalyzed hydrogenation conditions, it became apparent that coordination of the sulfonamide to the palladium source was deactivating the catalyst. Removal of the CBz group under ionizing conditions proved successful to afford the desired amino acid **30**.



Scheme 2-3. Synthesis of Cys_{SA}.

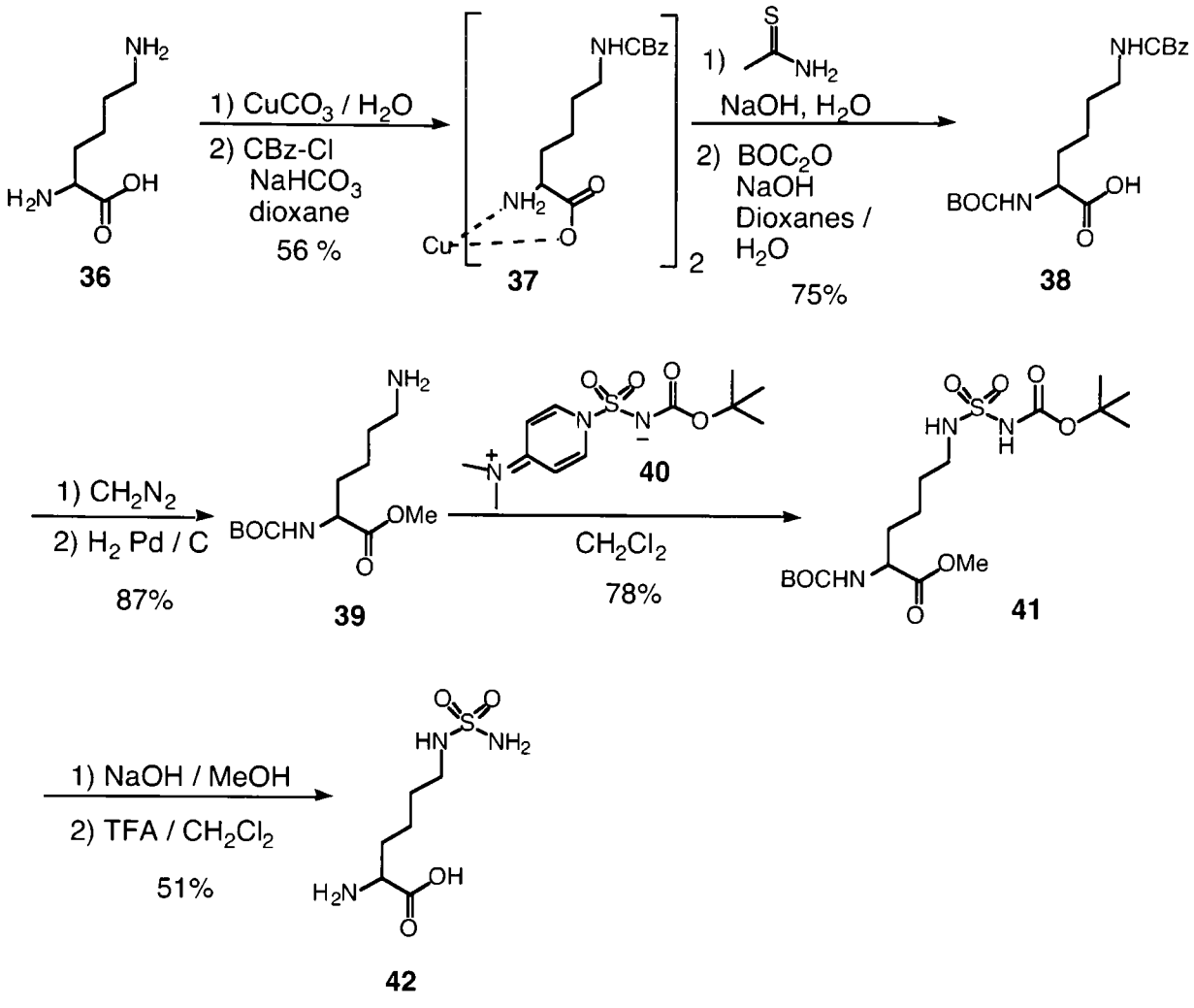
An alternative procedure employing a step-wise oxidation of disulfide **32** was also explored. Oxidation with one equivalent of Oxone afforded the oxysulfide **33** in good yield,[15] but the subsequent addition of sodium amide, and final oxidation with *m*-chlorobenzoic acid[16] were poor yielding (Scheme 2-4). The chlorine oxidation procedure provided facile access to larger quantities of material.



Scheme 2-4. Alternate preparation of Cys_{SA}.

2.3.3 **LYSINE-ε-SULFAMIDE**

Sulfamides have not been extensively investigated as CA inhibitors, but do possess the appropriate acidic nitrogen that could make it a strong binder to zinc. The sulfamide derived from the functionalization of lysine (**42**, Lys_{SA}) was a previously unreported compound (Scheme 2-5). Selective side chain protection of lysine was carried out by a modification of known procedures via a copper complex.[17,18] Subsequent BOC protection and esterification with diazomethane afforded the fully protected amino acid. Selective side chain deprotection by hydrogenation afforded amine **39**. Treatment of **39** with sulfamoylating agent **40**, which was prepared as previously described,[19] afforded **41**, the fully-protected analogue of the desired compound. Deprotection provided access to Lys_{SA}, **42**, in acceptable yield.

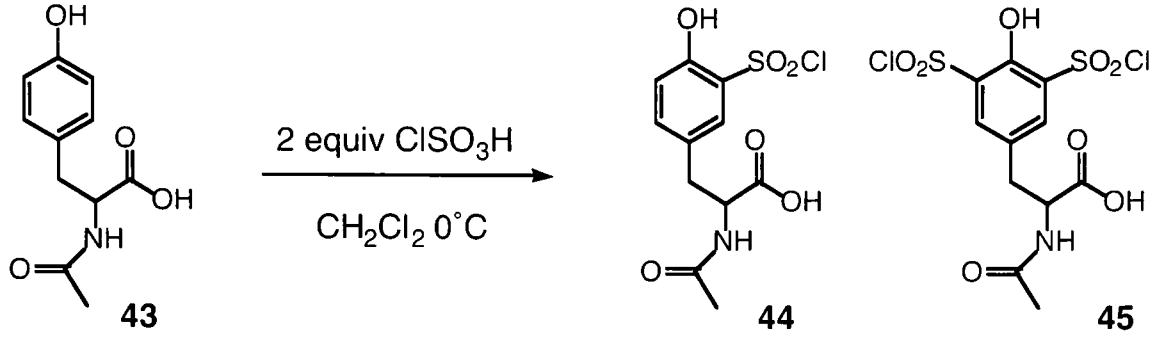


Scheme 2-5. Synthesis of Lys_{SA}.

2.3.4 FUNCTIONALIZATION OF OTHER AMINO ACIDS

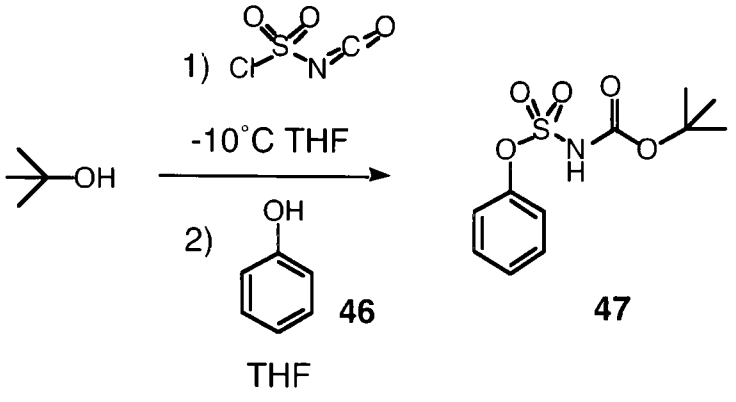
Numerous attempts were made at the chlorosulfonylation of other aromatic amino acids, but without much success. Chlorosulfonylation of N_{α}-acetyltryptophan afforded many undesired sulfonylated products, even with only a stoichiometric amount of cholorosulfonic acid in CH_2Cl_2. Protection of the indole nitrogen as the formamide, in an effort to decrease the reactivity of the aromatic ring towards electrophilic aromatic substitution was fruitless.

Chlorosulfonylation of *N*-acetyltyrosine, **43**, was more promising. Mixtures of the mono- and dichlorosulfonylated products (**44** and **45**, respectively) were obtained form cholorosulfonylation using the conditions described above for tryptophan. This route was abandoned when access to only the mono-chlorosulfonylated product exclusively was not forthcoming (Scheme 2-6).

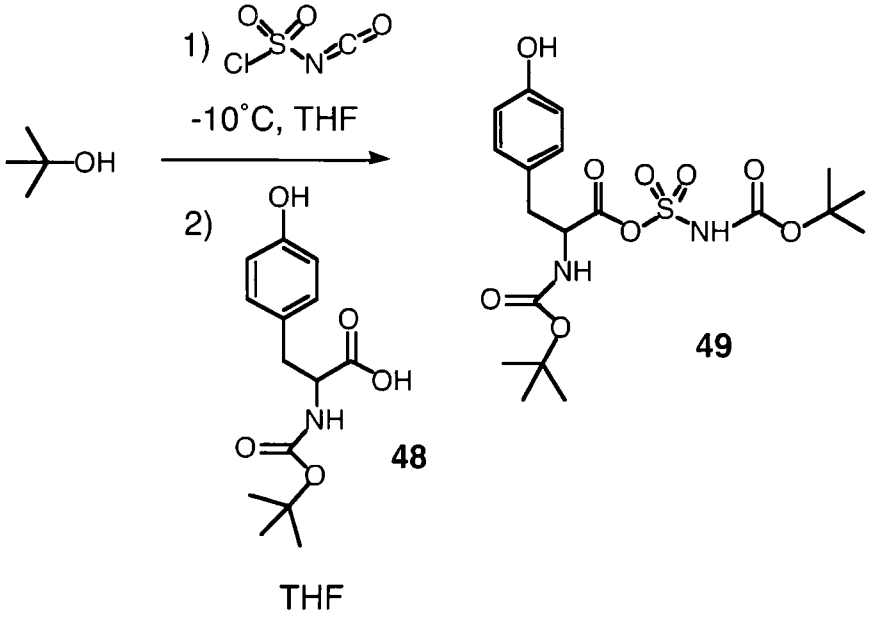


Scheme 2-6. Attempted chlorosulfonylation of tyrosine derivative.

Stanfield and co-workers reported the use chlorosulfonylisocyanate in the synthesis of BOC protected sulfamates from phenols.[20] Sulfamates were another relatively unexplored class of potential zinc binders. Although the method was reproducible with phenol (**46**) (Scheme 2-7) the reaction with *N*-BOC-tyrosine (**48**) provided an unexpected sulfamoylanhydride product **49** (Scheme 2-8). Treatment of *N*-BOC-tyrosine methyl ester with CSI in the same manner as above afforded multiple products. Investigation of his reaction is still ongoing.



Scheme 2-7. Preparation of BOC protected phenolic sulfamates by Stanfield and co-workers' procedure.



Scheme 2-8. Attempted sulfamoylation of *N*-BOC-tyrosine.

2.4 INHIBITION CONSTANTS

To verify the results of initial screening and pseudo-DCL experiments, inhibition constants (K_i) were measured for a series of the above compounds via a competitive hydrolysis assay for *p*-nitrophenylacetate (Table 2-1).[9,21] All K_i's were in the micromolar

range, with enough variety for purposes of our studies. Analogous dipeptides containing no sulfonamide had K_i's greater than 1.0 mM at both pHs.[9] The sulfonamides studied were better binders to CA at higher pH due to the higher proportion of deprotonated sulfonamide under the more basic conditions.

Table 2-1. Inhibition constant of potential library members.

Compound	K_i at pH 7.5 (μM)	K_i at pH 9.0 (μM)
Phe_{SA} (4)	13 ± 1.6	8.0 ± 2
EtOC-Phe_{SA}-OH (5)	12 ± 1.4	13 ± 2
EtOC-Phe_{SA}-Phe-OH (11)	1.2 ± 0.2	0.45 ± 0.09
EtOC-Phe_{SA}-Gly-OH (12)	2.5 ± 0.5	0.75 ± 0.1
EtOC-Phe_{SA}-Leu-OH (13)	4.4 ± 0.7	1.3 ± 0.3
EtOC-Phe_{SA}-Pro-OH (14)	9.4 ± 1.6	6.6 ± 0.2
EtOC-Phe-Phe_{SA}-OH (20)	ND	8.7 ± 0.09
EtOC-Gly-Phe_{SA}-OH (21)	ND	5.6 ± 0.08
EtOC-Leu-Phe_{SA}-OH (22)	ND	2.5 ± 0.03
EtOC-Pro-Phe_{SA}-OH (23)	ND	1.1 ± 0.02

2.5 CONCLUSIONS

The successful syntheses of three amino acids with CA binding elements, Phe_{sa}, Cys_{sa} and Lys_{sa}, was executed. A series of dipeptides with the Phe_{sa} unit at either the P1 or P1' positions were all modest inhibitors of CA, with K_i's in the micromolar range.

Progress was made towards the sulfamoylation of tyrosine and chlorosulfonylation of tyrosine and tryptophan.

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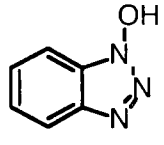
CHAPTER THREE

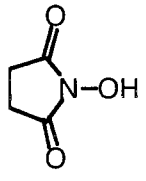
THE STUDY OF SOLID-SUPPORTED ACTIVE ESTERS IN AQUEOUS MEDIA

3.1 ACTIVE ESTERS IN PEPTIDE SYNTHESIS

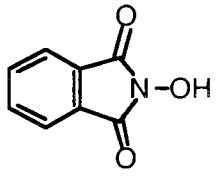
Active esters have become a staple of modern peptide synthesis. Their role in minimizing racemization and *N*-acylurea formation have made their use a standard method in the field.[1] The most common types of alcohols used to form active esters (Fig. 3-1) have made their way into everyday organic synthesis, not only for the formation of amides, but for use with a variety of nucleophiles.[2]



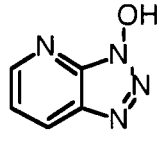
1-Hydroxybenzotriazole
(HOBt)



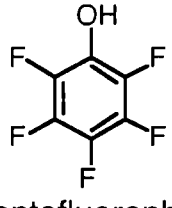
N-Hydroxysuccinamide
(HOSu)



N-Hydroxyphthalimide
(HOPt)



1-Hydroxy-7-azobenzotriazole
(HOAt)



Pentafluorophenol
(PFP)

Figure 3-1. Alcohols most commonly used to form active esters.

As shown in Figure 3-2, active esters are most commonly formed through activation of a carboxylic acid with a carbodiimide followed by addition of an appropriate alcohol, such as HOBt. The alcohol attacks the carbonyl carbon forming a urea and the desired active ester. An amine can then attack the ester, displacing the alcohol, to form the desired amide.

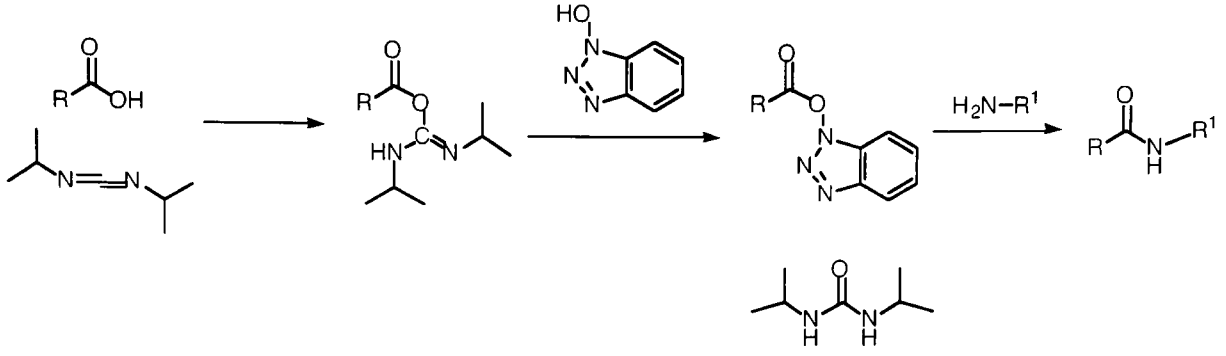
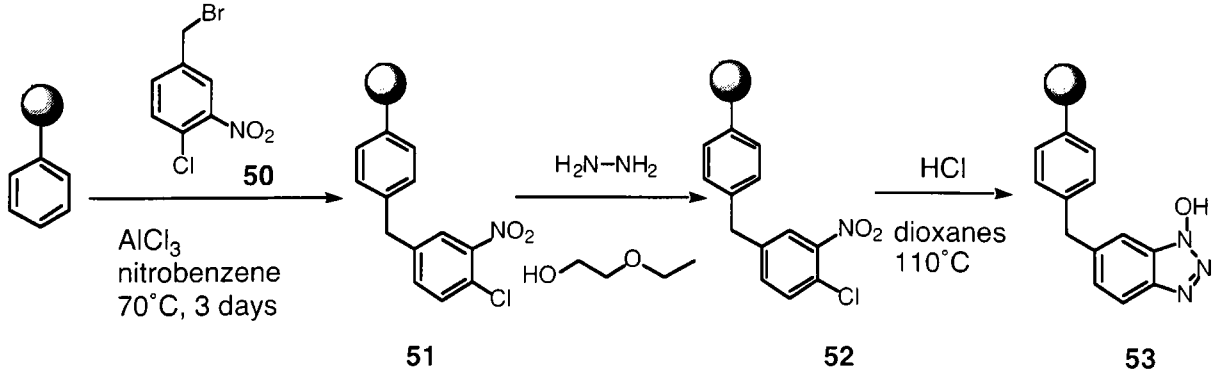


Figure 3-2. A general amide formation via an active ester.

3.2 ACTIVE ESTERS ON SOLID SUPPORT

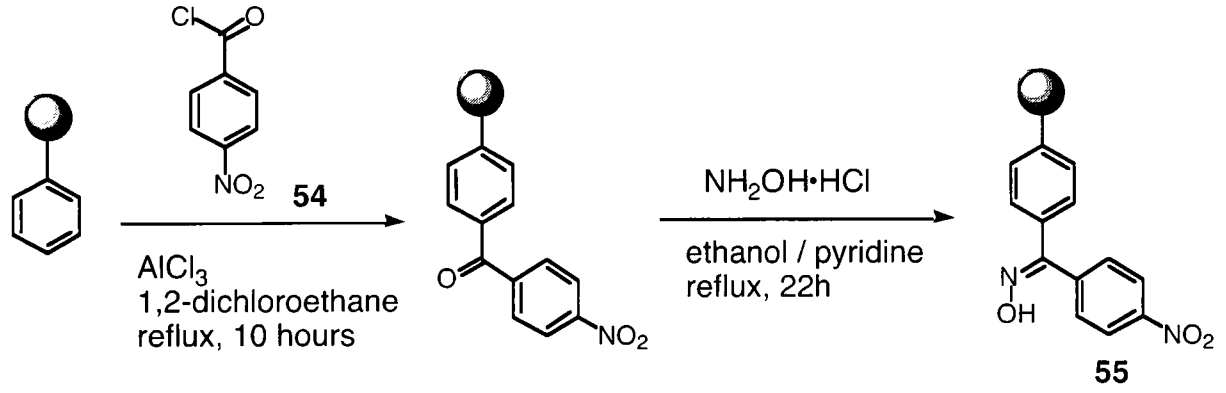
Various active ester precursors have been attached to solid supports for ease of preparation, isolation and storage of active esters. The first reported example was by Marshall and Lerner where phenol was attached to poystyrene beads via a sulfide linkage at the 4-position.[3] After these esters were formed, the sulfide was the oxidized to the sulfone, thus allowing for a subsequent reaction with an amine to form an amide.

In 1975 Patchornik *et. al.* attached HOBt to a polystyrene support by Friedel-Crafts alkylation of macroporous polystyrene beads with *p*-chloro-*m*-nitrobenzyl bromide **50** (Scheme 3-1).[4] Nucleophilic aromatic substitution with hydrazine and cyclization of the aryl hydrazine with HCl formed the hydroxytriazole **53**.



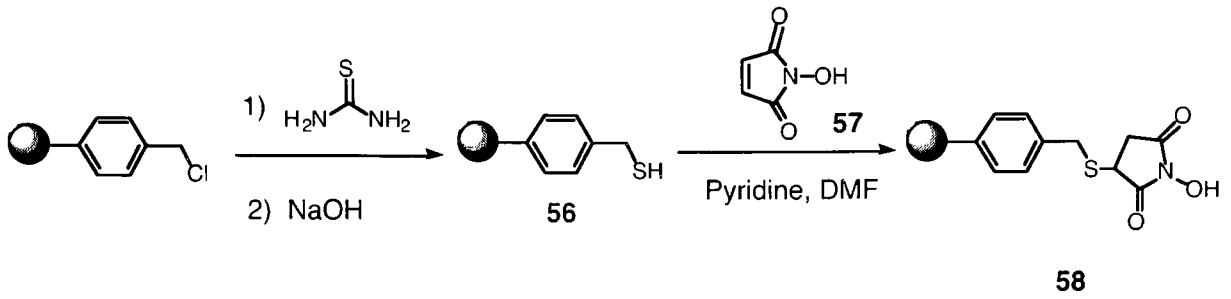
Scheme 3-1. Patchornick's synthesis of polymer-bound HOBt.

Kaiser oximes are the only class of solid supported active esters to see extensive use in polypeptide synthesis.[5,6] Kaiser's method of using electron-deficient aryl oximes to link the C-terminus of an eventual polypeptide to a solid support was quite ingenious since only a strong nucleophile such as hydrazine or a primary amine is capable of removing the peptide from the support. These active esters were sufficiently acid stable for use in a conventional BOC-style synthesis.[5] Kaiser oxime resin is prepared via Friedel-Crafts acylation of a polystyrene resin with p-nitro-benzoyl chloride **54**, and subsequent condensation with hydroxylamine to form the desired oxime-functionalized resin **55** (Scheme 3-2).



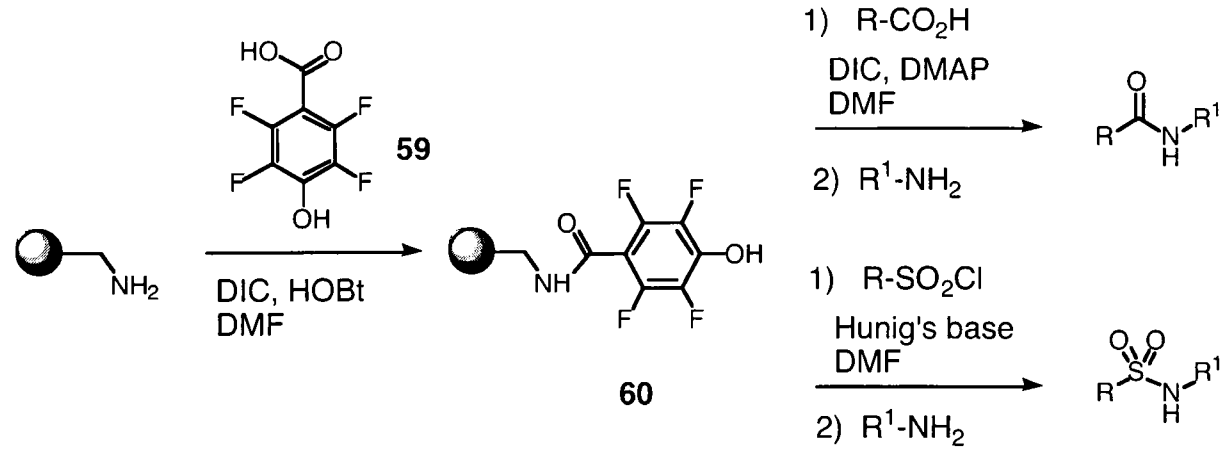
Scheme 3-2. Synthesis of Kaiser's oxime.

N-hydroxysuccinamide (HOSu) has been attached to solid support via a simple procedure starting from *N*-hydroxymaleimide (**57**).[7] Micheal addition of a thiol-functionalized Merrifield resin, **56**, to **57** afforded the desired *N*-hydroxysuccinamide on a solid support **58** (Scheme 3-3). Active esters were prepared from a variety of carboxylic acids and coupled to some simple primary and secondary amines in $CHCl_3$ / DMF. All couplings proceeded in good to excellent yield.



Scheme 3-3. Synthesis of polymer-supported HOSu.

A phenol analogous to pentafluorophenol has also been attached to a solid support.[8] Commercially available 4-hydroxy-2,3,5,6-tetrafluorobenzoic acid, **59**, was coupled to an amino-functionalized polystyrene resin using diisopropylcarbodiimide (DIC) (Scheme 3-4). Active esters and activated sulfonates were prepared and allowed to react with amines to form amides and sulfonamides, respectively. The intermediate active esters can be stored in a fridge in the presence of a desiccant with very little loss of activity over several months.



Scheme 3-4. Preparation and use of Salvino's tetrafluorophenol derived active esters.

3.3 ACTIVE ESTERS IN AQUEOUS MEDIA

Alcohols, such as those in Figure 3-1, have been shown to participate in solution-phase peptide coupling even when they are carried out in aqueous media. Nozaki showed that the number of equivalents of the activating alcohol present had a significant effect on the overall yield of a peptide coupling in water and in water / DMF mixtures.[9] As well, the amount of racemization observed in these aqueous couplings was drastically diminished when an active ester was employed. In an example where CBz-Gly-Phe-OH was coupled in aqueous solution with H_2N-Val-OMe with EDC and TEA, 29 % of the tripeptide contained the unnatural (R) enantiomer of phenylalanine. When 0.1 equiv of HOBt was used in the coupling only 2.8% of the tripeptide contained the unnatural phenylalanine enantiomer. When one equivalent of HOBt was used only 1.1% of this undesired product was obtained. These results were a clear indication that HOBt active esters were in fact formed in aqueous media and hydrolyzed at a slow enough rate that they had the chance to react with an amine.

A further study indicated that when amide bond formation was carried out in an alcoholic solvent, the amount of coupling additive had a significant effect.[10] As the number of equivalents of an additive such as HOBt were increased in an EDC-mediated coupling, the product ratio of amide to ester decreased. A representative example was a case where CBz-Ala-OH was coupled to piperidine in methanol. When 0.05 equivalents of HOBt were used the yield of amide and methyl ester was 74% and 21%, respectively. When 2.0 equivalents of HOBt were present the yields were 4% and 95%, respectively.

This was presumably due to protonation of the amine by the excess HOBt, thus leaving only the alcohol and the HOBt present as nucleophiles.

N-Hydroxysulfosuccinimide (NHSSu, Figure 3-3) was first introduced as an alcohol for active ester formation by Stavros and co-workers as a method of preparing a membrane-impermeable crosslinker.[11] The coupling of radio-labeled glycine and a small protein, hemocyanin, with EDC was carried out in the presence and absence of NHSSu.[12] Significant amounts of product were only observed when NHSSu was used. Stavros also measured the relative rates of reactivity of alkyl and aryl NHSSu esters under a variety of conditions.[13] In general, the NHSSu class of active esters have not seen much use beyond the field of protein derivatization, in part due to the high cost of the reagent.

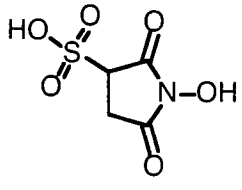


Figure 3-3. *N*-hydroxysulfosuccinamide (NHSSu).

Recently, a new class of water soluble active esters derived from pentafluorophenol have been studied.[14] 4-sulfo-2,3,5,6-tetrafluorophenyl esters were prepared in moderate to good yields via carbodiimide or mixed anhydride coupling of the phenol (STP, Figure 3-4) to a carboxylic acid followed by purification by silica gel chromatography. These active esters were described as coupling effectively with amines, but to date, their reactivity in water has not been studied.

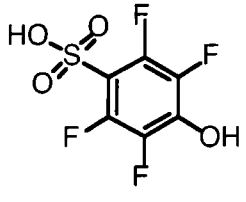


Figure 3-4. 4-sulfo-2,3,5,6-tetrafluorophenol (STP)

3.4 SYNTHESIS OF ACTIVE ESTERS ON TENTAGEL

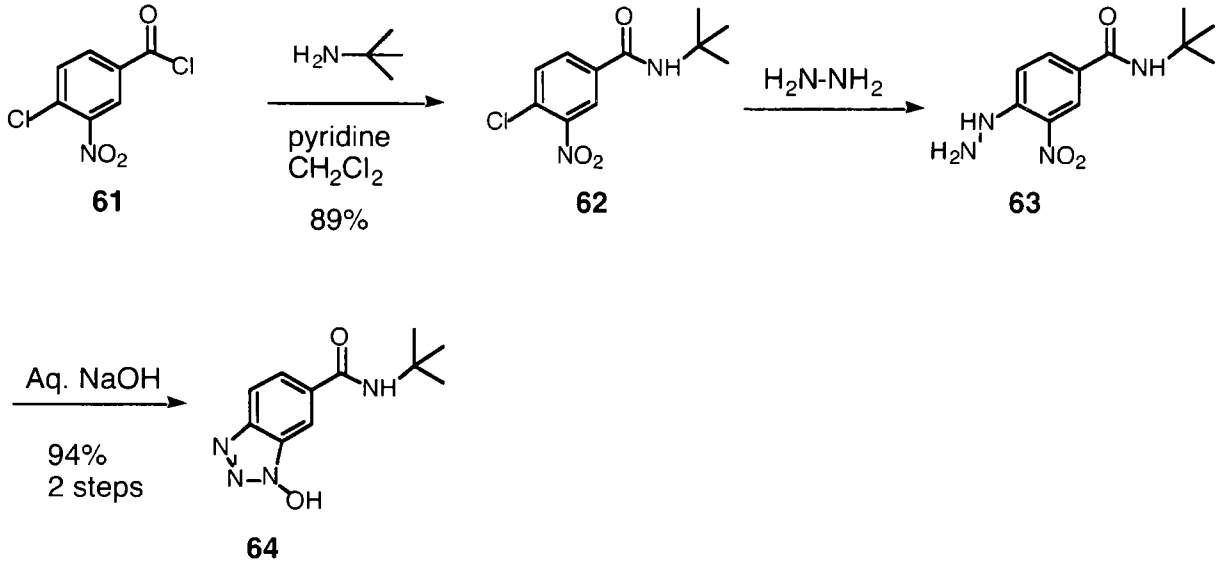
A practical amide bond formation method for our pseudo-dynamic library process had to meet certain criteria. The alcohol used to form the active ester must be attached to a solid support that will swell in water or have a high number of accessible active sites in a rigid form. The support in question must be large enough as not to permeate through a given dialysis membrane. The active ester must afford a good ratio of coupled product (amide) to hydrolyzed active ester (carboxylic acid) at a pH appropriate for the generation and screening of a pseudo-DCL. The active ester should have comparable reactivity with a variety of amines and/or amino acids. Ideally, the reagent should be either inexpensive and amenable to large-scale synthesis, or recyclable or both.

TentaGel is a polystyrene resin functionalized with polyethylene glycol (PEG) chains, which exhibits excellent swelling properties in water and other polar solvents. Various functionalities at the terminus of the PEG chain are commercially available. We prepared several solid supported active esters to probe their reactivity and determine which would be ideal for use in the synthesis process of our pseudo-DCLs.

3.4.1 1-HYDROXYBENZOTRIAZOLE

Based on the promising results published by Nozaki,[8] HOBt was the first class of active ester investigated. A solution phase synthesis of an HOBt unit attached to a *tert*-butyl amine was carried out. It was envisioned that after optimization of the yield in

solution, this procedure could be used with an amino-functionalized resin. Amide bond formation with acid chloride **61** and *tert*-butyl amine proceeded in high yield (Scheme 3-5). Nucleophilic aromatic substitution of anhydrous hydrazine (neat) and condensation under alkaline conditions to form the hydroxytriazole **64**, occurred in high yield. Use of Patchornick's acidic conditions for triazole formation (as illustrated in Scheme 3-1) afforded a very low yield of the desired product (**64**).

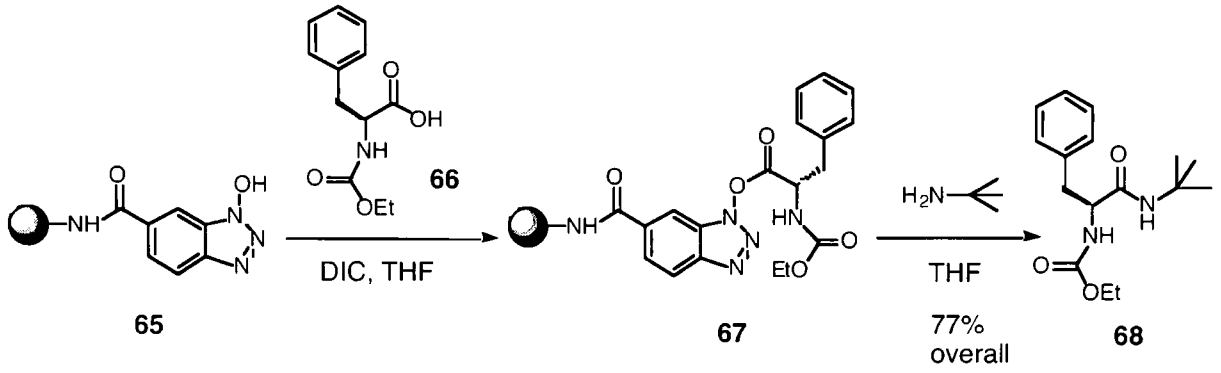


Scheme 3-5. Solution-phase synthesis of an amide-linked HOBt.

The above synthesis was applied to an amine resin with only a minor modification. TentaGel resin does not swell significantly in hydrazine and this resulted in a low yield of HOBt on the resin. The use of a 3:1 mixture of DMF and hydrazine resulted in adequate swelling of the resin thus providing a quite effective synthesis of **65**.

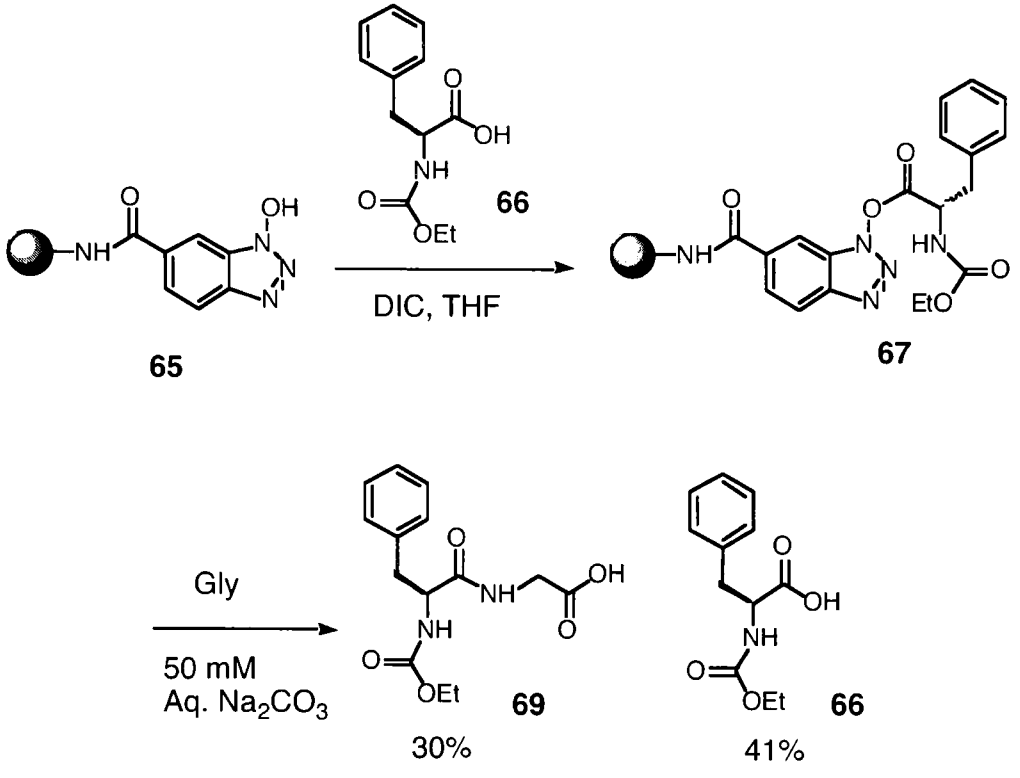
Quantification of this and other resins was done via a simple amide bond formation. The resin was swollen in a THF solution containing 10 equivalents (relative to the maximum possible loading) of EtOC-Phe-OH **66** and 10 equivalents of DIC were then added. The mixture was gently shaken for a period of at least 16 h and then

thoroughly washed. The resin was then swollen again in THF and 20 equivalents of *tert*-butylamine were added and the mixture was shaken for 16 h (Scheme 3-6). The amide **68** was isolated and purified. The amount of isolated product was assumed to be equal to the loading of active esters on the resin. This method was used for quantification of the loading of active esters for all resins discussed herein.



Scheme 3-6. Standard peptide coupling for quantification of **65**.

To ascertain the efficiency of these solid supported active esters for coupling in aqueous media, a portion of resin, functionalized with an ester as above, was swollen with a 0.1 M aqueous solution of glycine, (> 10 equivalents) buffered to pH 10 with 50 mM Na_2CO_3, and shaken for 16 h (Scheme 3-7). In the case of active ester **66**, hydrolysis was the major product in 41% yield. The desired amide **68** accounted for only 30% of the isolated product. The mass-balance for this reaction is only 71%. Not all of the active esters were available for the coupling reaction due to the roughly one-third smaller volume of the resin when swollen in water as opposed to THF. Subsequent swelling of a used portion of resin in THF and treatment with excess *tert*-butylamine afforded the amide **68** in 25% yield, this brings the mass-balance to 96% based on the loading measured initially by a coupling in THF.[15]

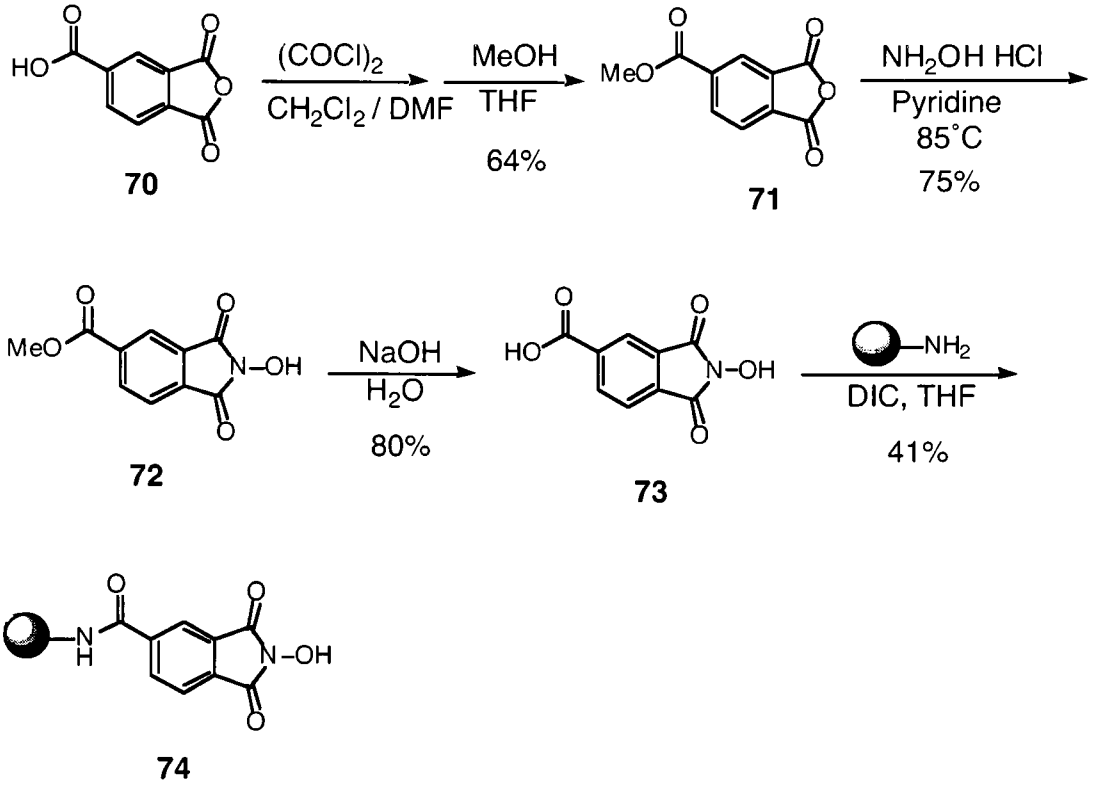


Scheme 3-7. Coupling with a solid supported HOBt active ester in aqueous media.

3.4.2 *N*-HYDROXYPHTHALIMIDE

The poor coupling to hydrolysis ratio obtained with the HOBt active esters led us to investigate a less reactive class, those derived from *N*-hydroxyphthalimide. Synthesis of the phthalimide unit **73** with a carboxylate linker was executed as follows (Scheme 3-8). Starting from tricarboxylic acid anhydride **70**, the carboxylate was protected as the methyl ester **71**, via the acid chloride. Treatment of this anhydride with hydroxylamine hydrochloride afforded the *N*-hydroxyphthalimide **72** in good yield after chromatography. Saponification of the methyl ester provided the desired phthalimide unit ready for coupling to the resin which was accomplished with a DIC mediated coupling. The loading of the resin was 41% (determined as described above) and was not optimized due

to the poor performance of this active ester in aqueous media. Upon attempted reaction of the EtOC-Phe-OH active ester of **74** with glycine as above, no coupling product was obtained. Furthermore, the batch of resin was no longer able to effect coupling in THF. Nucleophilic attack of glycine on one of the imide carbonyls may be a likely explanation of this problem.



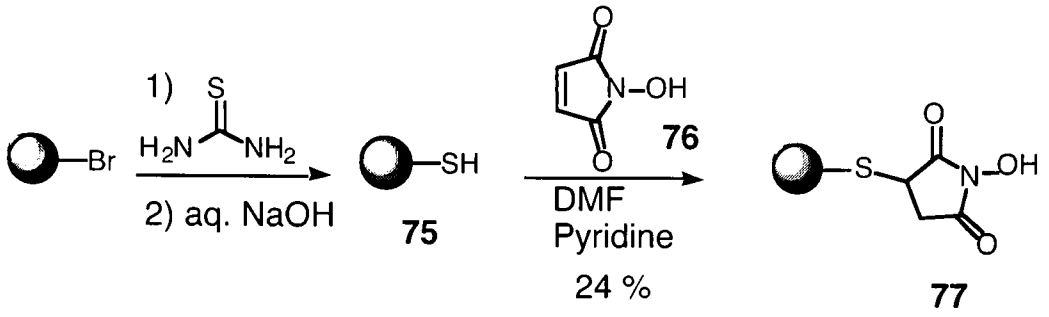
70 **71** **72** **73** **74**

Scheme 3-8. Synthesis of *N*-hydroxyphthalimide linked to a solid support.

3.4.3 *N*-HYDROXYSUCCINAMIDE

N-Hydroxysuccinamide active esters were proposed as an alternative to the phthalimides in an effort to find a more stable species. A thiol-functionalized TentaGel resin was prepared from the commercially available bromide by reaction with thiourea.[16,17] The succinimide unit was attached via Micheal addition of the thiol **73** to *N*-

hydroxymalimide **74**. This is a variation of a known procedure for attachment of HOSu to polystyrene resins (Scheme 3-9).[6]



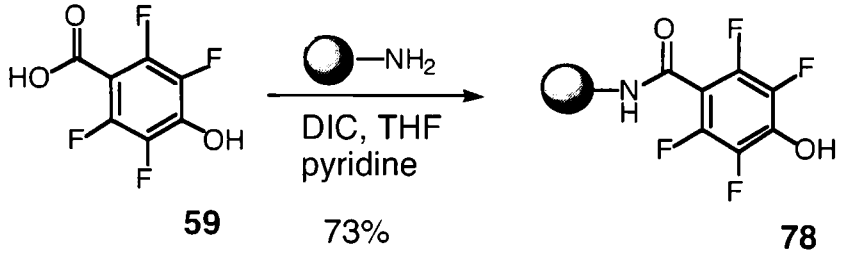
Scheme 3-9. Synthesis of the HOSu functionalized resin.

Although the loading of this resin was low, it was sufficient to determine the reactivity of these active esters in water. Upon executing the coupling described in Scheme 3-7 with this resin, the amide **69** was obtained in 51% yield while only 14 % of the hydrolysis product **66** was obtained. The less than ideal coupling to hydrolysis ratio and the prohibitive cost of *N*-hydroxymalimide led us to undertake further investigations.[14]

3.4.4 TETRAFLUOROPHENOL

Esters of pentafluorophenol are known to be easily isolated and in many cases, are stable to silica gel chromatography.[18,19] The reactivity of these active esters in aqueous media had not yet been explored. As previously mentioned (Scheme 3-4), Salvino and co-workers had prepared an analogous class of active esters on a polystyrene support.[7] A tetrafluorophenol unit linked to a TentaGel support was easily prepared (Scheme 3-10) with some minor variations on Salvino's procedure.[14] The DIC mediated coupling between the carboxylic acid **56** and amino-TentaGel was carried out in THF with 20

equiv of pyridine by shaking the mixture gently for 16 h, thoroughly washing the resin and repeating the acylation procedure to ensure complete functionalization of the resin. The resin was then treated with a large excess of *tert*-butylamine in THF to cleave any oligomeric products to afford a resin that was ready for use. The use of acylation catalysts such as HOBt and DMAP, as described by Salvino, did not improve the yield. Pyridine proved to be an adquate acyl group activator for the preparation of **78**.



59 73% **78**

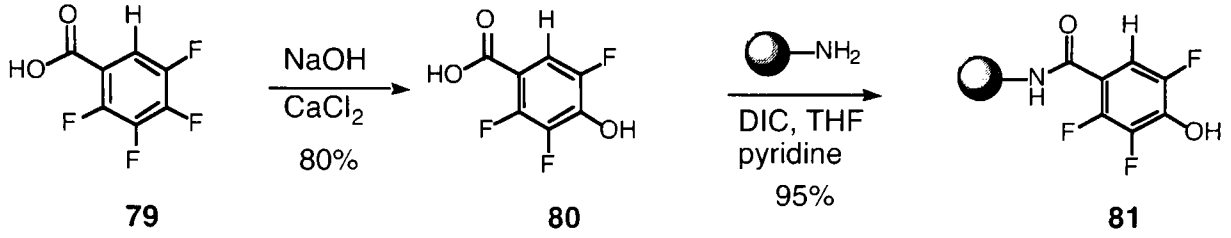
Scheme 3-10. Synthesis of tetrafluorophenol linked to TentaGel.

Coupling of the tetrafluorophenyl active ester of EtOC-Phe-OH with glycine in water as previously described afforded a 62% yield of the amide **69**, and only 3% of the hydrolyzed active ester **66**. These results clearly indicated that this method had the potential to be the mode of amide synthesis in the pseudo-DCL system.

3.4.5 TRIFLUOROPHENOL

In an effort to improve the coupling to hydrolysis ratio even further, an even less electrophilic class of ester was explored. The trifluorophenol class of active esters was proposed to be a less reactive species than the tetrafluoro class. Nucleophilic aromatic substitution of a hydroxide ion on 2,3,4,5 tetrafluorobenzoic acid (**79**) at reflux afforded the desired phenol **80** in high yield (Scheme 3-11).[20] The coupling of this phenol to the

amino-TentaGel proceeded in excellent yield. Unfortunately, the standard coupling with glycine in aqueous media afforded poorer results than the tetrafluorophenol resin. A 63% yield of the amide was obtained, but 13% of the hydrolyzed ester was also observed. No further efforts to lessen the electrophilicity of the esters were undertaken.



79 **80** **81**

Scheme 3-11. Synthesis of trifluorophenyl esters on solid support.

3.4.6 COMPARISON OF THE ACTIVE ESTERS

Each of the different types of active esters prepared were subjected to a coupling under a common set of conditions in aqueous media (Scheme 3-7). The results are summarized below in Table 3-1. The best coupling to hydrolysis ratio was obtained with the tetrafluorophenol active ester (**78**). This was a critical component in determining which class of active esters to use in the synthesis process of our proposed pseudo-DCL. A high coupling to hydrolysis ratio would help minimize the amount of hydrolyzed active ester, such as **66**, which will build up in the system. As well, a high-yielding dipeptide synthesis would allow the best inhibitor to build up in a more rapid fashion.

Table 3-1. Comparison of the reactivity in water of the active esters prepared.

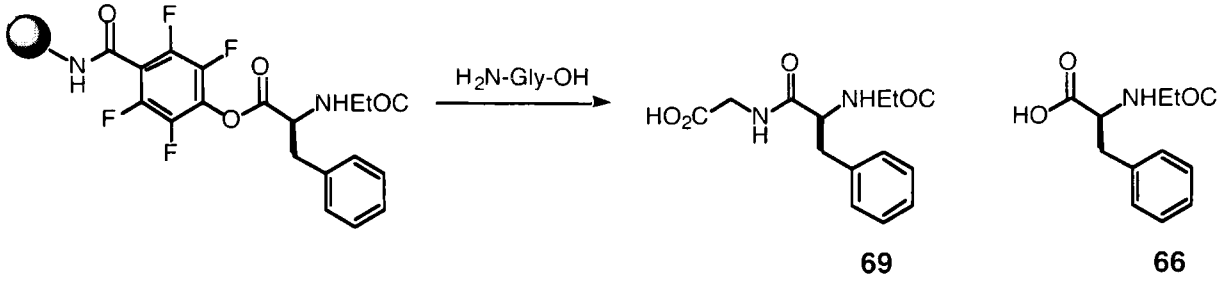
Active ester	EtOC-Phe-Gly-OH (**69**)	EtOC-Phe-OH (**66**)
HOBt (**65**)	30 %	41 %
HOPt (**74**)	0 %	ND
HOSu (**77**)	51 %	14 %
Tetra-FP (**78**)	62 %	3%
Tri-FP (**81**)	63 %	13 %

3.5 REACTIVITY OF TETRAFLUOROPHENOL ACTIVE ESTERS

With the above results in hand, we proceeded to further probe the reactivity of the tetrafluorophenol class of active esters on TentaGel support.[13] The effect of pH on the coupling reaction was important to explore since, ideally, the pseudo-DCL should be generated at a pH as close to physiological conditions as possible. Clearly, for effective amide bond formation, the pH of the solution must be close to the pKa of the amine to allow a significant amount of the amine to be deprotonated such that it can act as an effective nucleophile.

To explore these properties, the simple coupling of the resin-bound tetrafluorophenyl ester of EtOC-Phe-OH (Scheme 3-12) was carried out at several basic pHs. At pH 8.5 and 9.0, bicine (*N, N*-bis(2-hydroxyethyl)glycine, 50 mM) was used to buffer the solutions. At pH 9.5 and 10, the glycine itself served as the buffer. As shown

in Table 3-2, as the pH of the solution was increased, the coupling to hydrolysis ratio, as well as the overall yield, clearly improved.



69 66

Scheme 3-12. General coupling used to probe pH and concentration dependence.

Table 3-2. pH dependence of coupling in Scheme 3-12.

Entry	[Glycine] (M)	pH	EtOC-Phe-Gly-OH (**69**)	EtOC-Phe-OH (**66**)
1	0.10	8.5	48%	8%
2	0.10	9.0	52%	4%
3	0.10	9.5	61%	4%
4	0.10	10.0	63%	3%

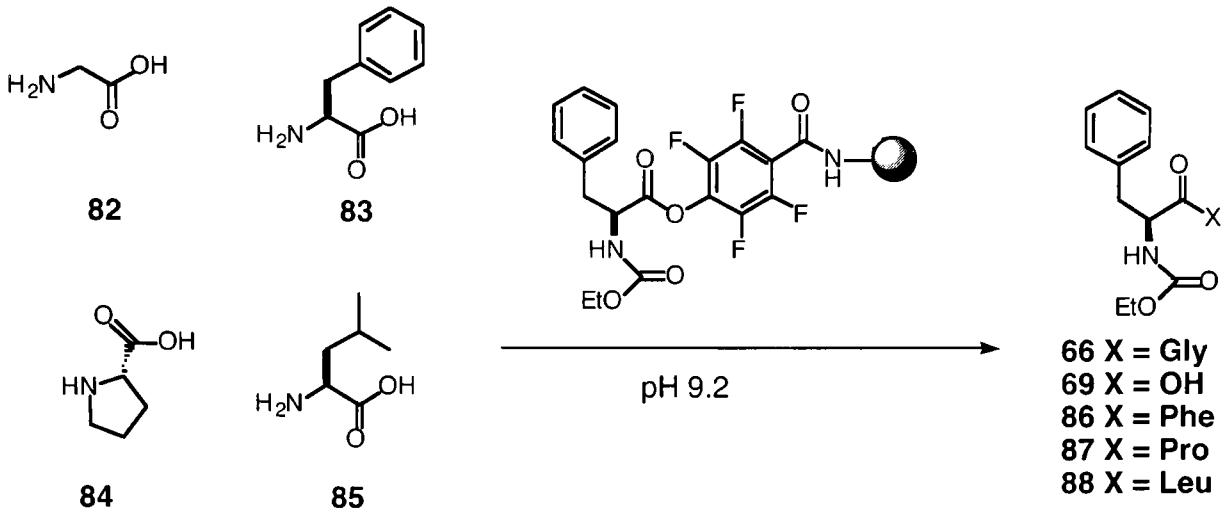
Concentration of the nucleophile could play a significant role in the library design. Ideally, the minimum concentration of nucleophile for effective coupling should be used. High concentrations (such as saturation of the solution) may lead to unwanted interactions such as binding of the nucleophile to the target or to protease-catalyzed peptide synthesis. From a fundamental point of view, it was important to establish that the nucleophiles were in fact being recycled in the pseudo-DCL. A large excess of nucleophiles would allow for continuous dipeptide synthesis without re-use of the nucleophilic coupling partner. A series of couplings were conducted at pH 10 using

several concentrations of glycine which were varied over two orders of magnitude. These results are summerized in Table 3-3. At pH 10 the coupling to hydrolysis ratio is still acceptable even at low concentrations (entry 7). However, at pH 9.0 the ratio is quite poor at 0.05 M (entry 6). This may be the lower limit for concentration at this pH.

Table 3-3. Concentration dependence of coupling in Scheme 3-12 at pH 9.0 and 10.

Entry	[Glycine] (M)	pH	EtOC-Phe-Gly-OH (**69**)	EtOC-Phe-OH (**66**)
1	1.0 (120 equiv)	10	69%	2%
2	1.0 (120 equiv)	9.0	72%	5%
3	0.10 (12 equiv)	10	62%	3%
4	0.10 (12 equiv)	9.0	66%	11%
5	0.05 (6.0 equiv)	10	55%	4%
6	0.05 (6.0 equiv)	9.0	57%	21%
7	0.01 (1.2 equiv)	10	50%	10%

In order to successfully generate a library of dipeptides, the reactivity of these active esters with different amino acids acting as nucleophiles had to be examined. A static library was generated by allowing an equimolar solution of four amino acids (glycine, phenylalanine, leucine and proline) to react with a single active ester in aqueous biscine adjusted to pH 9.2 as illustrated in Scheme 3-13. For simplicity, only amino acids with unreactive sidechains were used. The library was generated at three different concentrations so that any drastic differences in nucleophilicity could be observed.



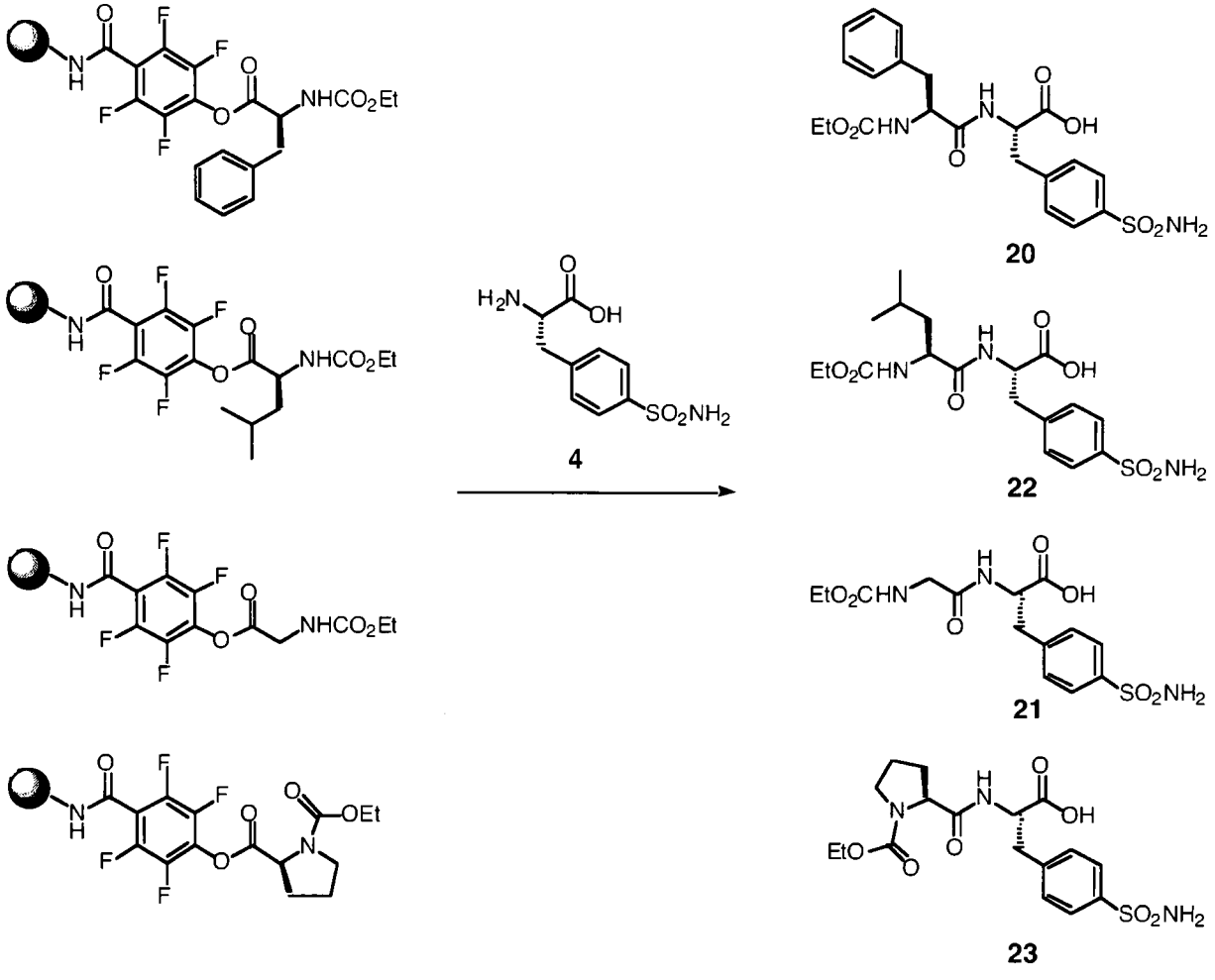
Scheme 3-13. Generation of a static library.

The results of these experiments were quite informative (Table 3-4). Clearly, glycine is a far better nucleophile than the other three amino acids studied. This is most likely due to the lack of any steric hindrance due to the lack of a side chain at the α-position. The trend in nucleophilicity of the other three amino acids appears to be related to the pKa's of their respective amines. Phenylalanine is the second best nucleophile (pKa of α-NH_3^+ 9.2) followed by leucine (pKa of α-NH_3^+ 9.7), and proline which is the most basic amine of the series (pKa of α-NH_3^+ 10.6).[21] Glycine breaks this trend (pKa of α-NH_3^+ 9.8) as mentioned previously. Thus the reaction rate is related to the concentration of free amine. It is also worthy of note that at the highest concentration (100 mM, 3.5 equiv) there was not a significantly larger proportion of the product formed through the reaction of the best nucleophile (Gly) when compared to the lower concentration (50 mM, 1.8 equiv). In fact the disparity in the concentrations of the dipeptides was lessened by the drop in nucleophile concentration. This may prove useful in controlling the distribution of products in the synthesis of a pseudo-DCL to ensure that roughly equal amounts of the inhibitors will be generated in the synthesis process.

Table 3-4. Distribution of products in a static library synthesis.

X =	Gly (**69**)	Phe (**86**)	Pro (**87**)	Leu (**88**)	OH (**66**)
100 mM (3.5 equiv)	46%	27%	9.6%	12%	5.4%
50 mM (1.8 equiv)	47%	24%	9.0%	12%	9.0%
10 mM (0.35 equiv)	21%	18%	7.8%	7.2%	46%

In the course of our pseudo-DCL investigations, the reactivity of a single nucleophile with a variety of active esters became important. A large difference in reactivity between active esters could lead to an overwhelming production of a given product, thus creating a bias that the pseudo-DCL may not be able to overcome. A static library of four compounds was prepared from the coupling of the active esters prepared from EtOC-Phe-OH, EtOC-Gly-OH, EtOC-Leu-OH, and EtOC-Pro-OH with a single nucleophile, 4-sulfonamidophenylalanine, (**4**), as illustrated in Scheme 3-14.



Scheme 3-14. Synthesis of a static library with four different electrophiles.

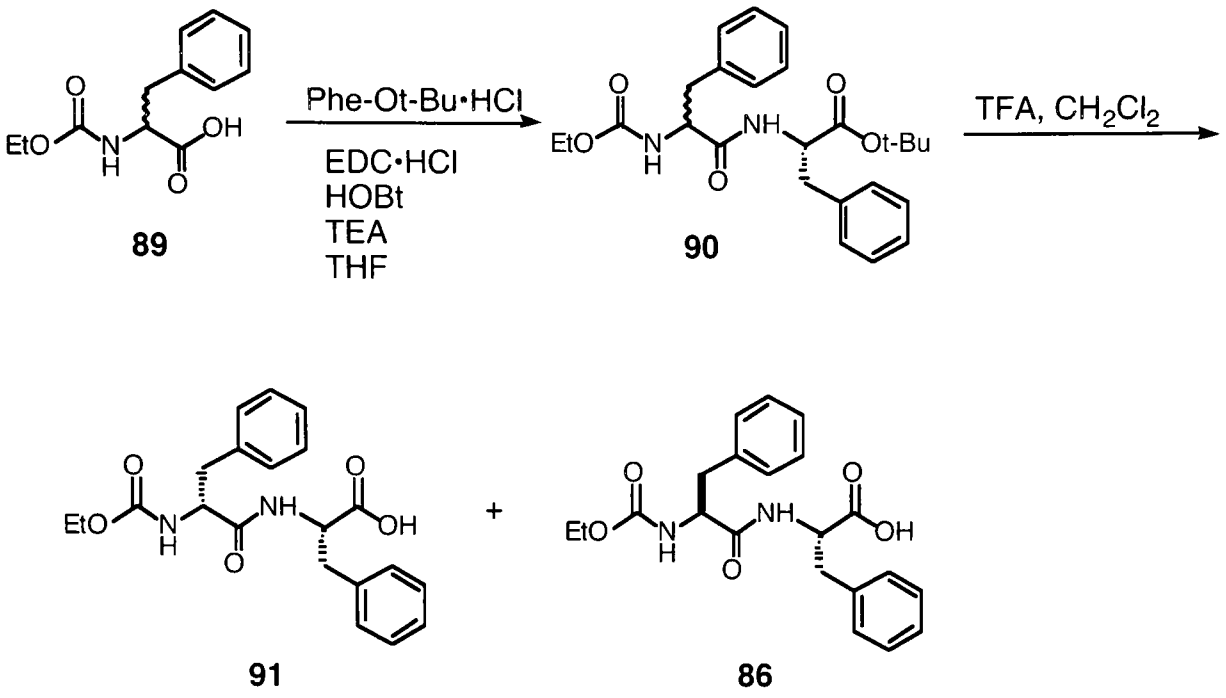
The library was generated at four different concentrations of nucleophile (10, 5, 4, 3, and 1 mM) at pH 9.0, with the nucleophile in excess in every case. The relative amounts of each of the four products present after four hours is summarized in Table 3-5. These results indicate that there is little effect on the distribution of products with respect to the total concentration. The active ester of EtOC-Gly-OH was the best electrophile in every case, while the other three dipeptides were present in comparable amounts to each other. It should be mentioned that the total amount of product obtained was consistent in all cases except for when the concentration was 1 mM. In the latter case, less than half of

the total product was observed relative to the other cases at the four hour mark. Further sampling indicated that the reaction had not yet gone to completion.

Table 3-5. Distribution of products from the library described in Scheme 3-14.

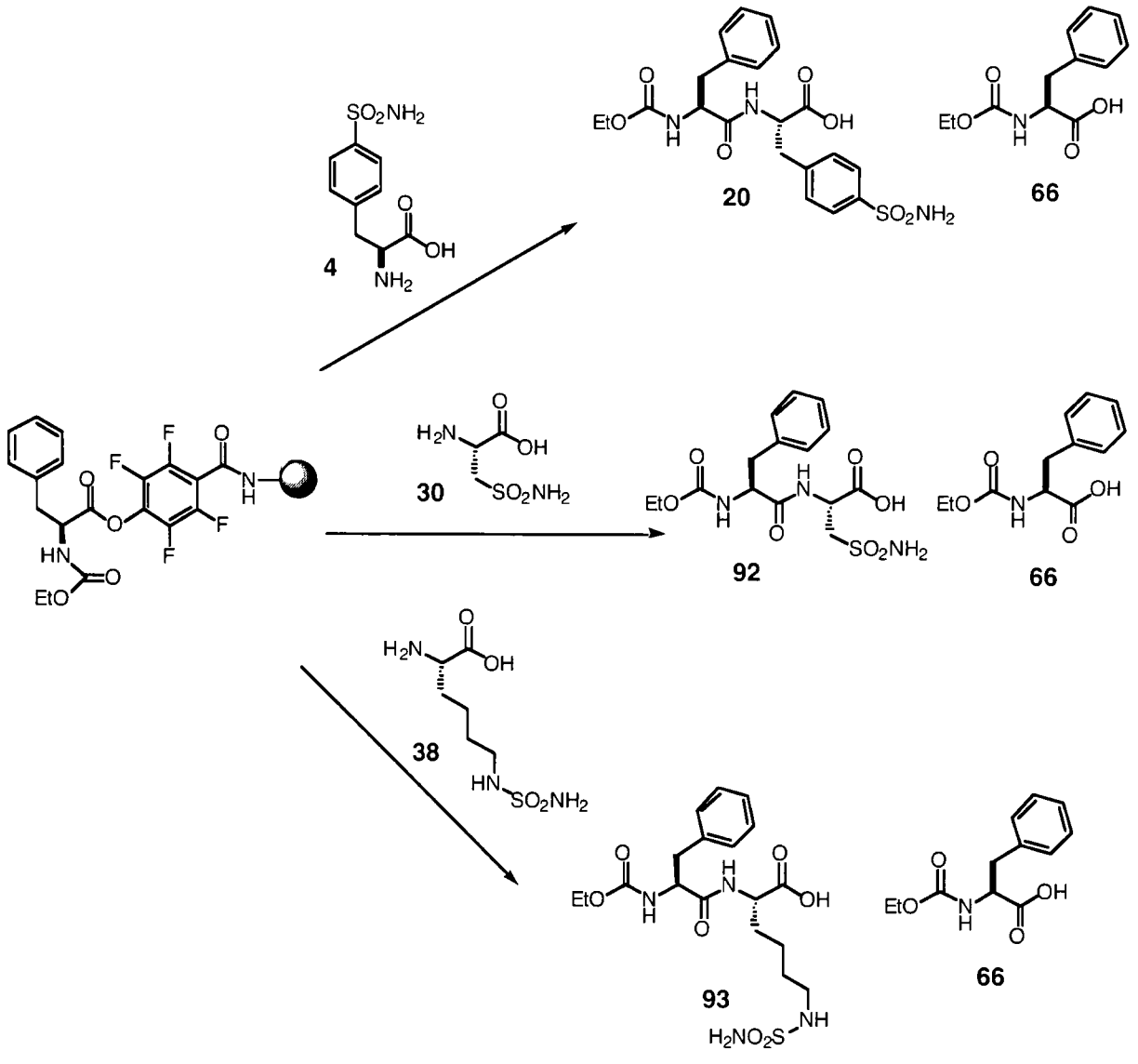
Concentration (mM)	EtOC-Gly-Phe_{sa}-OH (**21**)	EtOC-Pro-Phe_{sa}-OH (**23**)	EtOC-Phe-Phe_{sa}-OH (**20**)	EtOC-Leu-Phe_{sa}-OH (**22**)
10 (16 equiv)	44 %	25 %	17 %	14 %
5 (8.0 equiv)	46 %	21 %	18 %	14 %
4 (6.4 equiv)	44 %	23 %	18 %	15 %
3 (4.8 equiv)	44 %	22 %	18 %	16 %
1 (1.6 equiv)	52 %	18 %	18 %	12 %

It was important to ensure that racemization during the coupling procedure was minimal. In the pseudo-DCL process, the protease would only recognize the natural enantiomer of the amino acid at P1. A standard of two diastereomers of an EtOC-Phe-Phe-OH dipeptide (**86** and **91)** were prepared by solution phase chemistry (Scheme 3-15) These diastereomers were easily separated by reverse-phase HPLC. Integration of the two peaks obtained from a conventional coupling of (S)-EtOC-Phe-OH active ester and (S)-Phe-OH revealed only a 2.2% yield of the racemized product **91** (95.6% de).



Scheme 3-15. Preparation of a pair of diastereomers for the racemiztion investigation.

In an effort to increase the diversity of the library, it was necessary to establish that more complex nucleophiles would couple cleanly with these active esters. Specifically, it was important to establish that the sulfonamides and sulfamide described in Chapter 2 would not undergo unwanted reactions on their side chains (Scheme 3-16). In each case a 10 mM solution of the amino acid in 30 mM aqueous biscine was prepared and the pH of the solutions were adjusted to 9.0. This solution was used to swell a 1.5 equiv portion of resin, and the mixture was shaken for 16 h. All three nucleophiles coupled cleanly to give only two peaks on each individual HPLC trace, corresponding to the coupled product **20** (and presumably **92** and **93**)along with the hydrolyzed active ester **66**.



Scheme 3-16. Test coupling of amino acids **4, 30** and **38**.

3.6 CONCLUSION

After surveying a series of active esters attached to a TentaGel solid support, the tetrafluorophenol class proved most useful for our purposes. These active esters exhibit an acceptable ratio of coupled product to hydrolyzed active ester during amide bond formation in aqueous media. The coupling to hydrolysis ratio improved as the pH was

raised. The coupling was effective even at low concentrations of nucleophile (10 mM). The active ester exhibited sufficiently comparable reactivity with different amino acid nucleophiles for our purposes. The reactivity of potentially nucleophilic sulfonamide, sulfamide and hydroxamic acid functionalities was established under the conditions for pseudo-DCL generation.

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CHAPTER FOUR

STATIC SCREENING AND PSEUDO-DYNAMIC COMBINATORIAL LIBRARIES

4.1 STATIC LIBRARY SCREENING

As described in Chapter One, the pseudo-DCL is composed of three elements; synthesis of inhibitors, a binding event and a destructive process. The development of the general class of inhibitors was completed (Chapter Two) and their binding affinities measured. The synthesis of these inhibitors via a peptide coupling in aqueous media was accomplished using the tetrafluorophenol active esters (Chapter Three). The next step in the development of this pseudo-DCL was an initial study of the interplay between the binding of the inhibitors to the target and the destruction of unbound inhibitors. Numerous experiments were undertaken with a two-chambered vessel, where CA was in one chamber and Pronase in the other separated by a dialysis membrane as illustrated in Figure 4-1.[1]

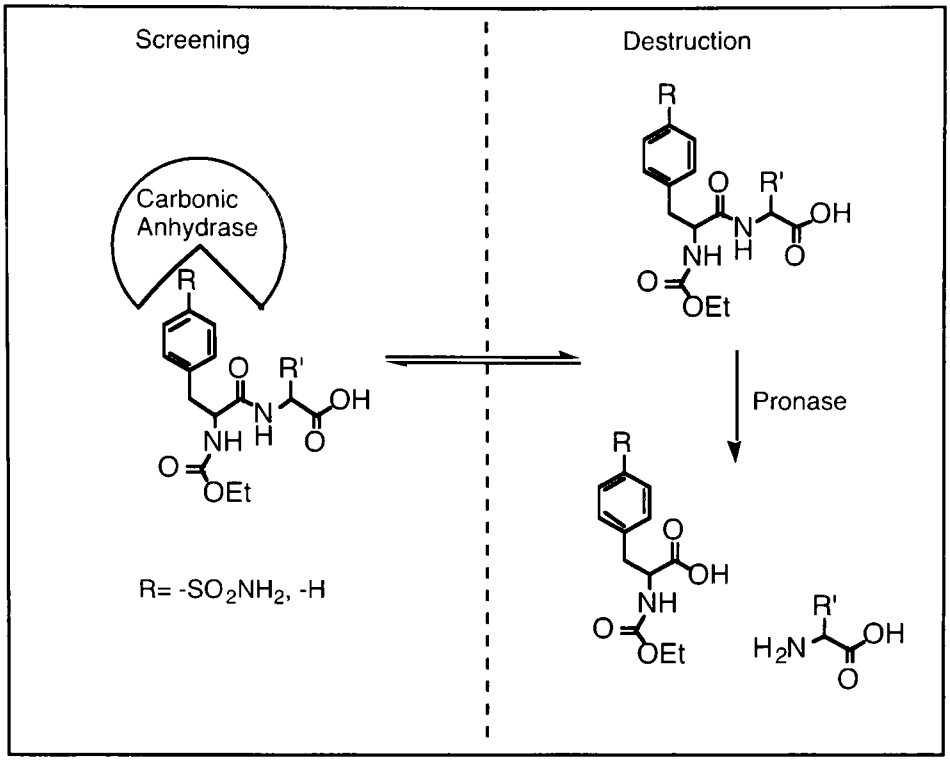


Figure 4-1. General form of the two-chambered screening reaction.

This method was used for the screening of a static library of dipeptidic CA inhibitors. In a setup described in Figure 4-1, a solution with 4.3 μmol of each of five dipeptides **11-14** and **86** (Figure 4-2) buffered at pH 7.5 was placed in the screening chamber with 25.6 μmol of CA. The concentration of these dipeptides in the CA chamber was measured by HPLC while the vessel was gently shaken over a period of eight days. Analysis of the results (Figure 4-3) indicates that the dipeptides leave the CA chamber over time in an order determined by their inhibition constants (Table 2-1). The dipeptide with no sulfonamide **86** (K_i >1000 μM) was the first compound to be completely removed, and did so in less than 10 h. Compound **14** (K_i = 9.4 μM) was the next compound removed from the system, followed by **13** (K_i = 4.4 μM). At the end of

the experiment, only the two best inhibitors, **12** (K_i = 2.5 μM) and **11** (K_i = 1.2 μM) remained, and the better inhibitor, **11**, was present in the higher concentration.

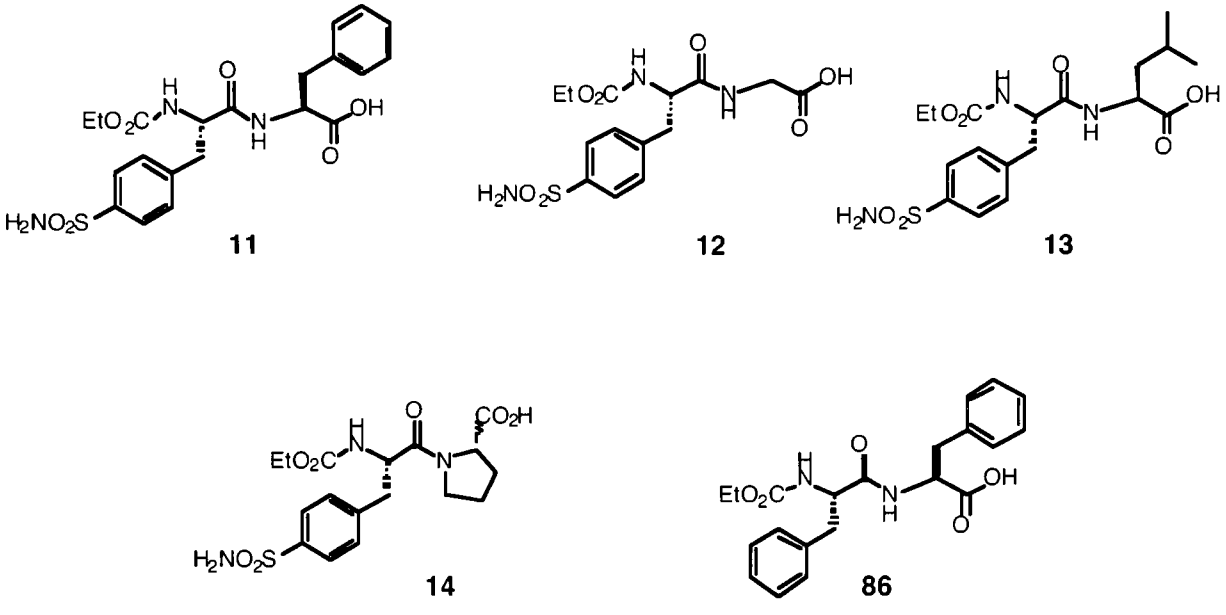


Figure 4-2. Components of the static library.

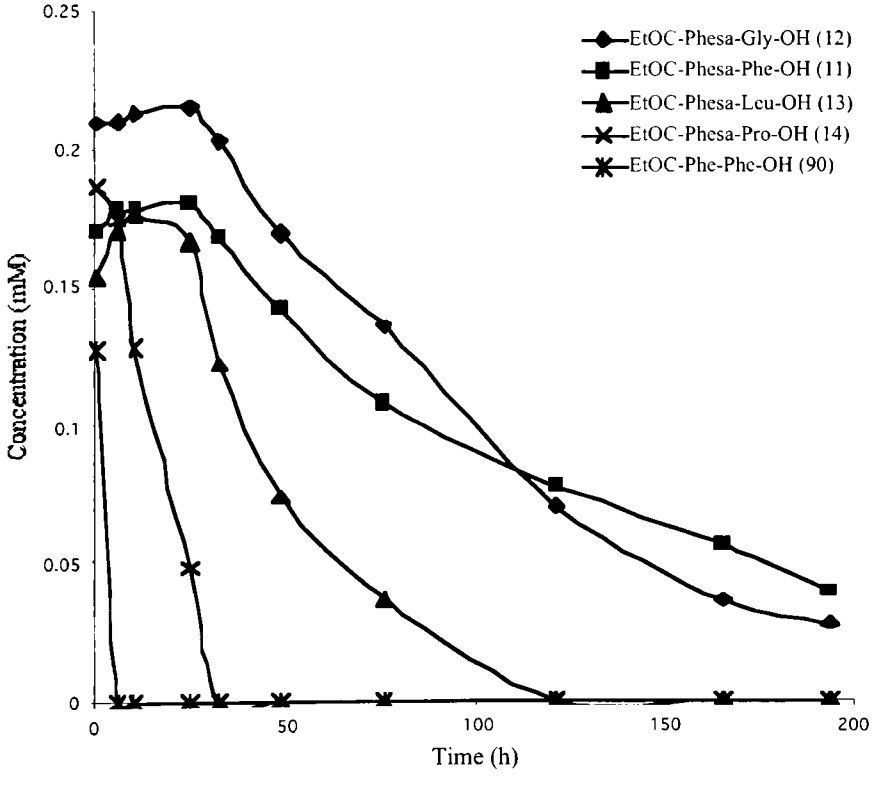


Figure 4-3. Concentrations of **11-14** and **86** in the static screening experiment.

The results of this static screening experiment can be explained by a mathematical relationship. A thorough discussion of this theory can be found in Appendix 1. Essentially, this theory states that inhibitors with lower K_is will be present in a lower concentration in the solution, free of CA, than the poorer binders. Correspondingly, the poorer binders are present in a higher concentration and thus will be more prone to diffuse into the destruction chamber. A large excess of Pronase is present in the destruction chamber to ensure that the hydrolysis of the inhibitors that diffuse across the membrane is virtually instantaneous. Thus, diffusion of the inhibitors into the destruction chamber is the rate-limiting step. The rates of diffusion of all the inhibitors are roughly equal. This screening process comprises two-thirds of the aforementioned three-chambered DCL experiment. It is expected that the differential hydrolysis of inhibitors observed in this simple experiment will form the basis for the selectivity in the full DCL.

When considering the addition of an independent synthesis process to this screening method, it is important to note that in spite of all attempts at accuracy in the measurement of the initial amounts of each inhibitor, the concentration of **12** was slightly higher than the others at time = 0 h. Despite the fact that the second-best inhibitor was present in a higher concentration initially, at the end of the experiment the best inhibitor, **11**, was the most abundant. This indicated that the system would be able to tolerate different initial concentrations of inhibitors and still be an effective screening process. The ability of this static system to adapt to differing concentrations of inhibitors indicated that a pseudo-DCL based on a similar destruction (or recycling) system would be able to compensate for biases in the synthesis process to a certain extent. Such biases were

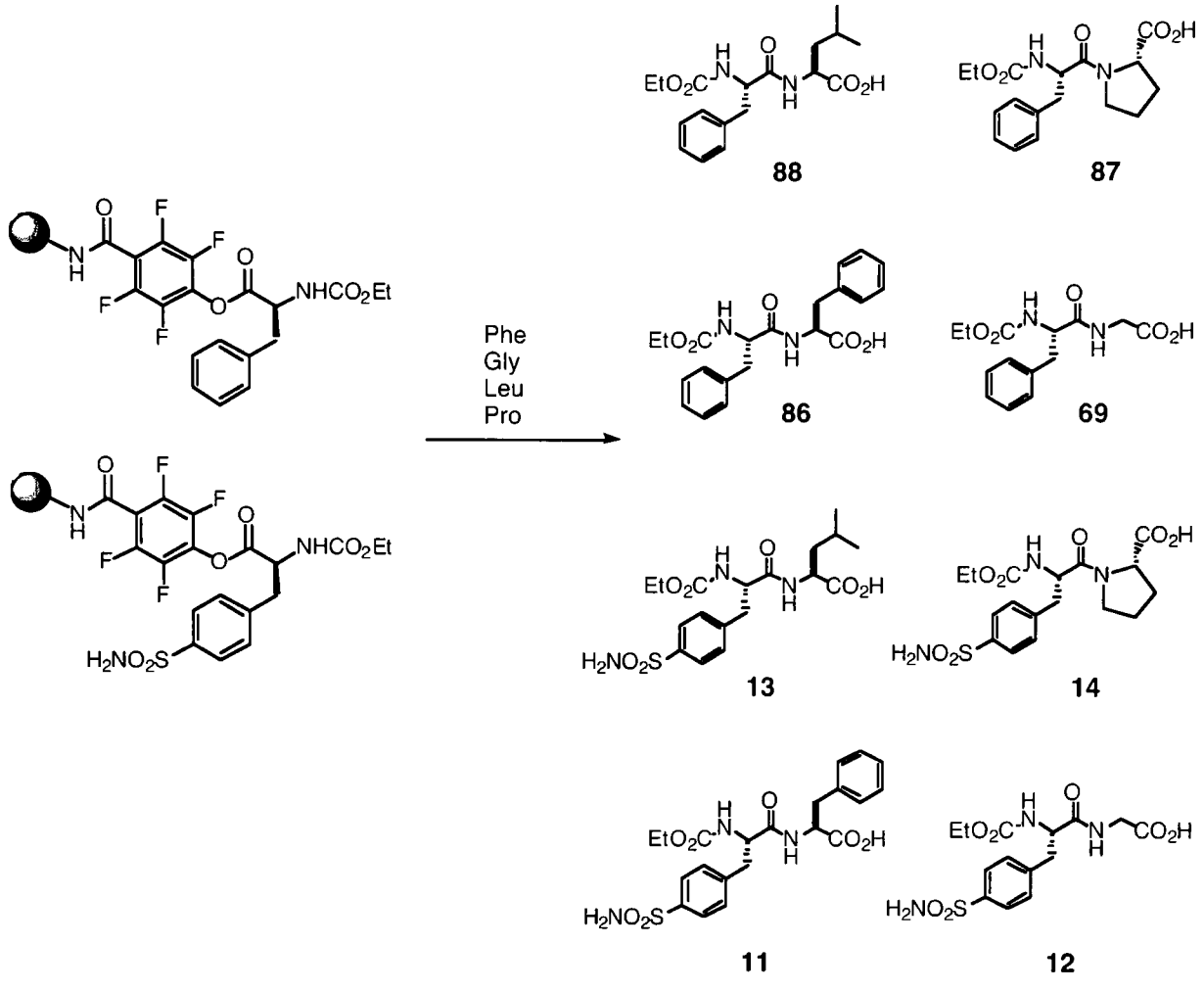
observed in the static library synthesis illustrated in Figure 3-13 and Table 3-3 where, coincidentally, the product from the coupling of glycine, **69,** was the most abundant.

4.2 PSEUDO-DCLS

The major drawback of the static screening process described above is the small amount of the inhibitor(s) present once the desired selectivity is obtained. Approximately 1.0 μmol (0.46 mg) of **11**, the best inhibitor, was present at the end of the 200 h screening process. This amount is more than sufficient to identify a known compound, but may not be enough for full characterization of a new, unknown compound. It was hoped that the repeated synthesis of the inhibitors by addition of new active esters (as described in Chapter 3) at regular intervals in the pseudo-DCL would result in higher concentrations of the best inhibitors compared to the concentrations obtained in the static screening process. As well, the small difference in concentration between the two best inhibitors at the end of the static screening process should be amplified by the repeated synthesis and screening cycles of the pseudo-DCL.

4.2.1 PSEUDO-DCLS WITH SULFONAMIDES AS ACTIVE ESTERS

The initial libraries were generated from two active esters, those derived from EtOC-Phe-OH (**66**) and EtOC-Phe_{sa}-OH (**5**) and four amino acids as nucleophiles; Phe, Gly, Leu and Pro to form compounds **11-14** as well as the analogous dipeptides with no sulfonamide functionality **90**, **93-95** (Scheme 4-1).



Scheme 4-1. Synthesis portion of the first generation pseudo-DCL.

In this initial study, a three-chambered vessel was set up as illustrated in Figure 1-24. A plastic dish with a snap-shut lid was used for the CA chamber. Dialysis bags, which were to be immersed in the CA chamber, served as the synthesis and destruction chambers. This setup is illustrated in Figure 4-4. A 5.0 mM stock solution of the four amino acids was prepared in aqueous Bicine and adjusted to pH 9.0. In the synthesis chamber, an approximately 160 μmol (ca. 5.7 equiv) portion of resin composed of equal portions of the active esters of EtOC-Phe-OH (**66**) and EtOC-Phe$_{sa}$-OH (**5**) were swollen with 20 mL of the amino acid stock solution. A solution of CA (28.0 μmol, 1.0 equiv) in

the stock solution (20 mL) was added to the screening chamber. Pronase, dissolved in the stock solution (20 mL) was added to the destruction chamber. There were 250 μmol (8.9 equiv) of the amino acids present in the entire system.

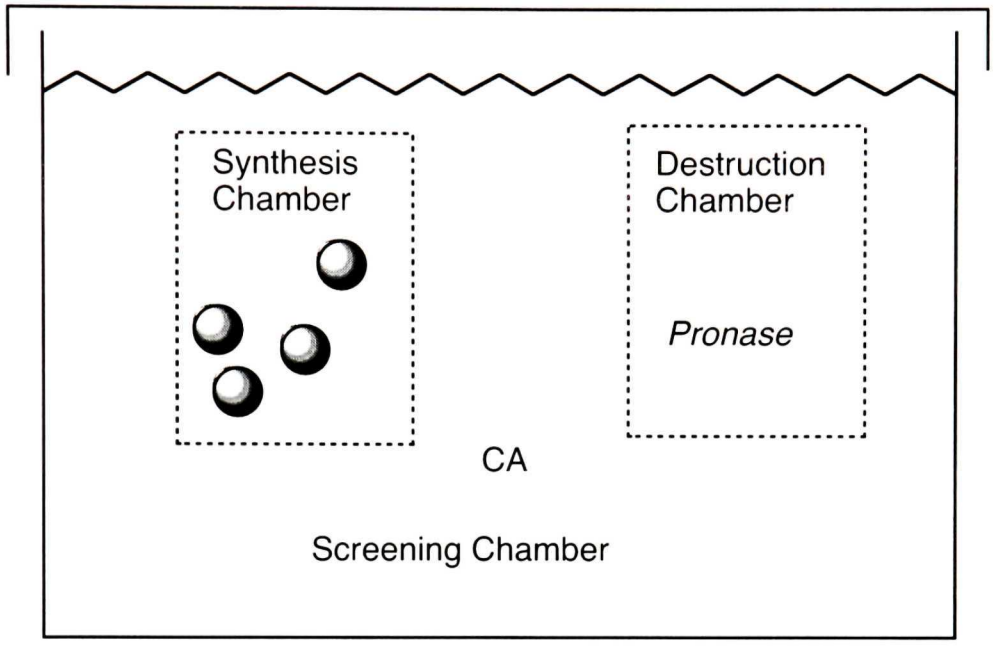


Figure 4-4. Illustration of the three-chambered reaction vessel.

The reaction vessel was shaken for 24 h, at which time the spent active ester resin was replaced with another 160 μmol portion. The concentration of the dipeptides in the screening chamber was monitored at two-hour intervals by HPLC over a 48 hour period. Only three compounds were present in detectable concentration after the first four hours of the experiment. The changes in concentration in the screening chamber over time of **11**, **12**, and **13** are illustrated in Figure 4-5.

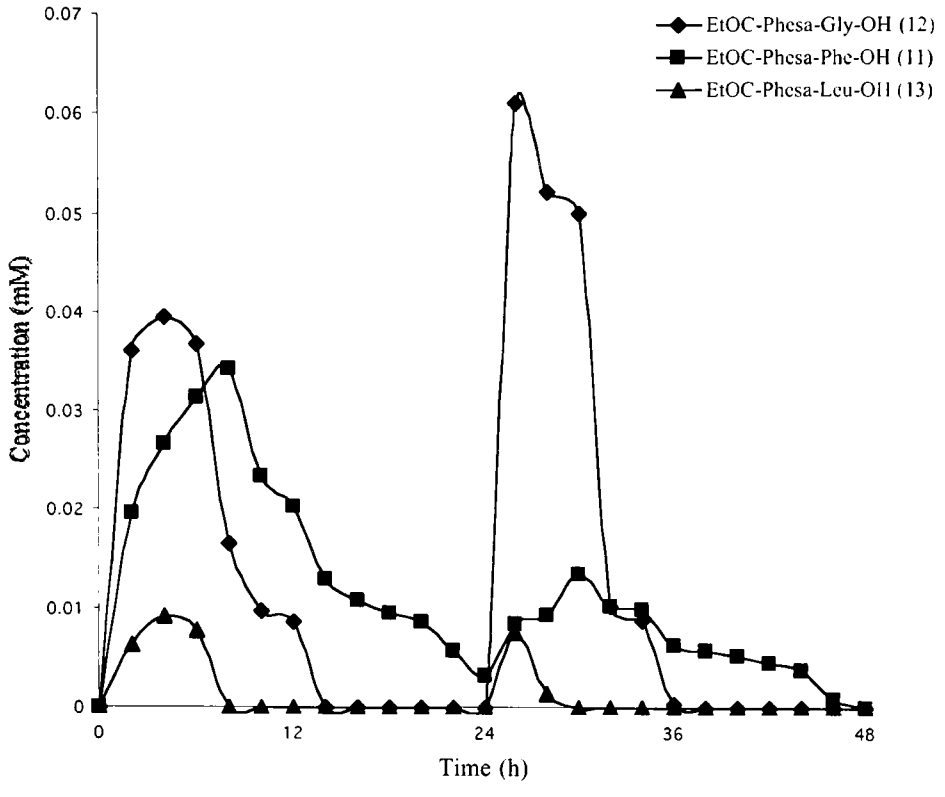


Figure 4-5. Results of the first pseudo-DCL experiment.

Many interesting observations were made in this experiment. First, only the three best inhibitors in the system were detectable throughout most of the experiment, a clear indication that the screening method was somewhat successful. The concentration of **12** was very high after the synthesis process of each cycle is believed to be over (t = 2-6 h and t = 26-30 h). This was not surprising given the results of the static library generation studies which clearly indicated that glycine was by far the best nucleophile of the four amino acids used (Scheme 3-13 and Table 3-3). Despite the fact that **12** is the second-best inhibitor in the system, it was removed from the screening chamber at a much higher rate than the best inhibitor **11**. For two periods in the experiment (t = 16-24 h and t = 36-44 h) only the best inhibitor, dipeptide **11**, was present, albeit in rather low concentrations (at t = 24 h; 3.2 μM, ca. 0.2 % in Figure 4-3). This pseudo-DCL method had succeeded

in identifying the best inhibitor, but no amplification, or increase in its concentration, had occurred. Essentially, the best inhibitor had been identified early in the first cycle of the experiment, and the long period of time before the next synthetic cycle served only to lower the concentration of **11** in the screening chamber even further.

In an effort to increase the concentration of the best inhibitor, the experiment was repeated only with smaller portions of the resin being added at more frequent intervals. In a setup identical to the previous one, 80.0 μmol active ester portions were added at 12 h intervals (4 times) over a 48 h period (Figure 4-6).

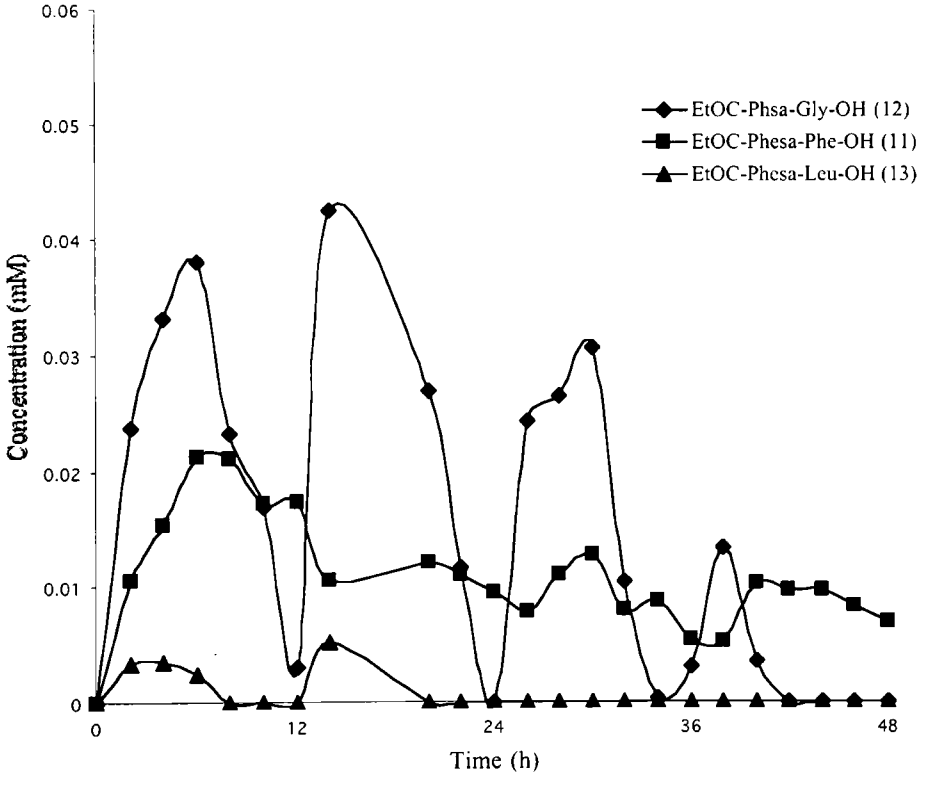


Figure 4-6. Results of the second pseudo-DCL experiment.

This method offered no significant improvement in the selectivity of the screening or in increasing the overall concentration of the inhibitors. The repetitive pattern from the first experiment was apparent with four clear cycles. EtOC-Phe_{sa}-Gly-OH (**12**) was

present in a high concentration initially in each cycle. As in the previous experiment, in the latter stages of each cycle, only **11**, the best inhibitor is present for a period of time. At t = 24 h, the concentration of **11** was 9.6 μM (0.19 μmol, 0.7 %) This method provided no meaningful improvement in concentration of the best inhibitor as compared to the first pseudo-DCL experiment.

An alternative approach to increase the concentration of the inhibitors in the library was entitled the "Dip-Dip" experiment. It was proposed that the presence of Pronase during the early stages of the pseudo-DCL experiment was not conducive to maximizing the concentration of the best, and indeed all inhibitors, in the screening chamber. An experiment was designed to test this hypothesis where the destruction chamber (a dialysis bag) was added to a system identical to the first pseudo-DCL described after a six-hour period had elapsed. The Pronase was allowed to act on the system for 18 hours. At the 24 hour mark, the Pronase-containing chamber was removed and the resin replaced as previously described. After another six hour delay, a new destruction chamber was added until the end of the second cycle (t = 48 h). The concentrations **11**, **12**, and **13** versus time are plotted in Figure 4-7.

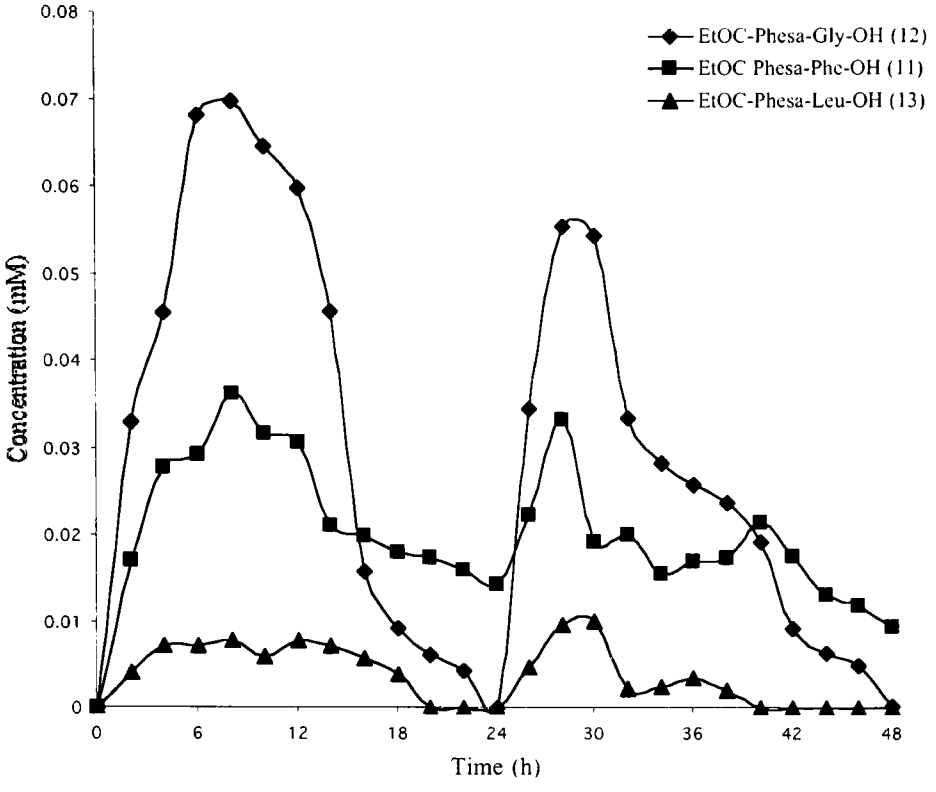


Figure 4-7. Results of the "Dip-Dip" experiment.

The "Dip-Dip" method did increase the overall amount of the three best inhibitors present in the screening chamber when compared to the first pseudo-DCL. The concentration of **11** was also slightly higher at the time when it was the only dipeptide present (at t = 24 h; 14 μM, 1%, in Figure 4-7 and 3.2 μM, ca. 0.2 % in Figure 4-3). Unfortunately, the increase in concentration was not as large as was expected (i.e. the value is still on the order of 1 %). Clearly there is no amplification in this system due to the second cycle of the experiment, since the concentration of **11** was slightly less at t = 48 h than it was at t = 24 h.

This trend of decreasing yield over time is apparent in all three pseudo-DCLs described above. This decrease in concentration of the dipeptidic inhibitors in the screening chamber can be attributed to an increase in concentration of EtOC-Phe_{sa}-OH

(**5**) over the course of the experiment. The amino acids, which act as nucleophiles in the library, are recycled during each cycle of the experiment. The electrophilic portions, EtOC-Phe-OH **65** and EtOC-Phe_{sa}-OH **5**, however, will build up over the successive cycles. EtOC-Phe_{sa}-OH, **5**, is an inhibitor of CA (K_i = 13 at pH 9.0) and there is no destructive process or pressure in the system to prevent it from binding to CA. As its concentration increases over the cycles, it will begin to occupy more and more of the CA binding sites.

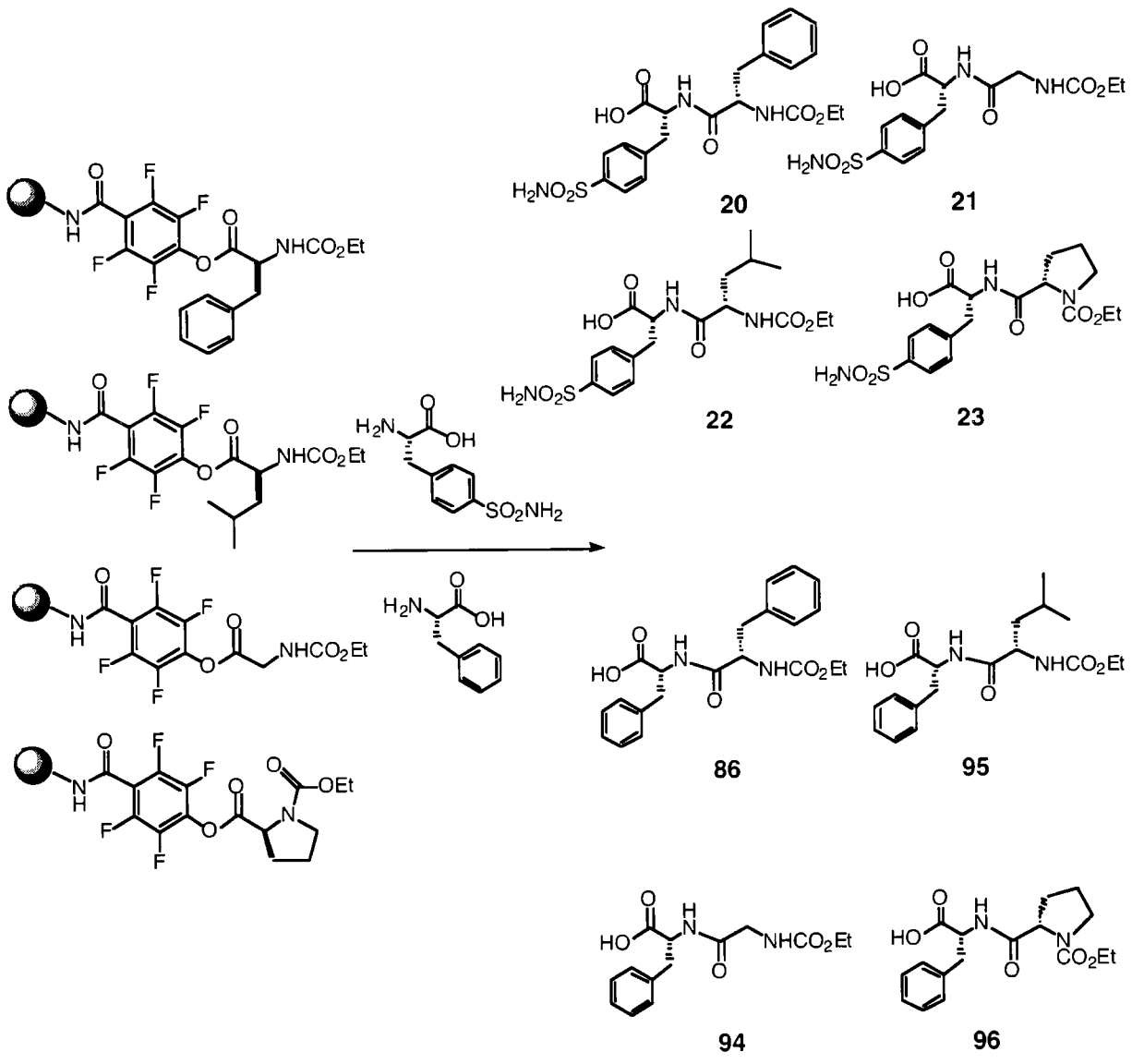
Consider a specific example: At t = 48 h in the second pseudo-DCL experiment (illustrated in Figure 4-5) there were 0.138 μmol of **11** remaining. There were 160 μmol of **5** added to the system in the form of active esters over the four cycles, but only 0.138 μmol remain in the dipeptide form (**11**). Therefore, 159.9 μmol of **5** were present in solution. The ratio of **5**:**11** was approximately 1150:1. This was far in excess of the ratio of the inhibition constants of **5** and **11** (29:1) so **5** would occupy the vast majority of the binding sites on the 28 μmol of CA present.

4.2.2 PSEUDO-DCLS WITH SULFONAMIDES AS NUCLEOPHILES

The potential for the build-up of weak inhibitors such as **5** was a fundamental flaw in the design of the first-generation pseudo-DCLs. Clearly, an important constraint in these systems will be that all potential binding units (i.e. sulfonamides, sulfamides, hydroxamic acids, etc.) must be the recycled component. Maintaining a constant concentration of all potential inhibitors, be they either as an amino acid or as a dipeptide, should assure that the concentration of the inhibitors generated in the pseudo-DCL would

increase over repeated cycles of synthesis and destruction until an upper limit is reached. This plateau may be due to the system reaching a "steady-state" type of equilibrium, where the rate of synthesis and binding of the inhibitors has equaled that of the off-rate and the destruction process.

In an effort to meet these constraints, a new eight-membered library was generated from a variation on the first-generation system. The four amino acids that were nucleophiles in the previous system were *N*-blocked as their ethyl carbamates and used as the active esters. The nucleophiles were phenylalanine and 4'-sulfonamidophenylalanine (**4**). This library will generate eight different dipeptidic CA inhibitors (Scheme 4-2) and allow for the recycling of the sulfonamide-containing unit. The *N*-EtOC protected amino acids will build up as more cycles are performed, but they should be innocuous (Figure 4-8). The first experiment of the second-generation pseudo-DCL involved 181 μmol (6.5 equiv) each of Phe_{SA} (**4**) and phenylalanine, approximately 90 μmol (ca. 3.2 equiv, total) of an equal mixture of the four active esters in each of four cycles (360 μmol, 13 equiv, total) and 28.0 μmol (1.0 equiv) of CA, all arranged in the conventional three-chambered vessel illustrated in Figure 4-8.



Scheme 4-2. Synthesis portion of the second-generation library

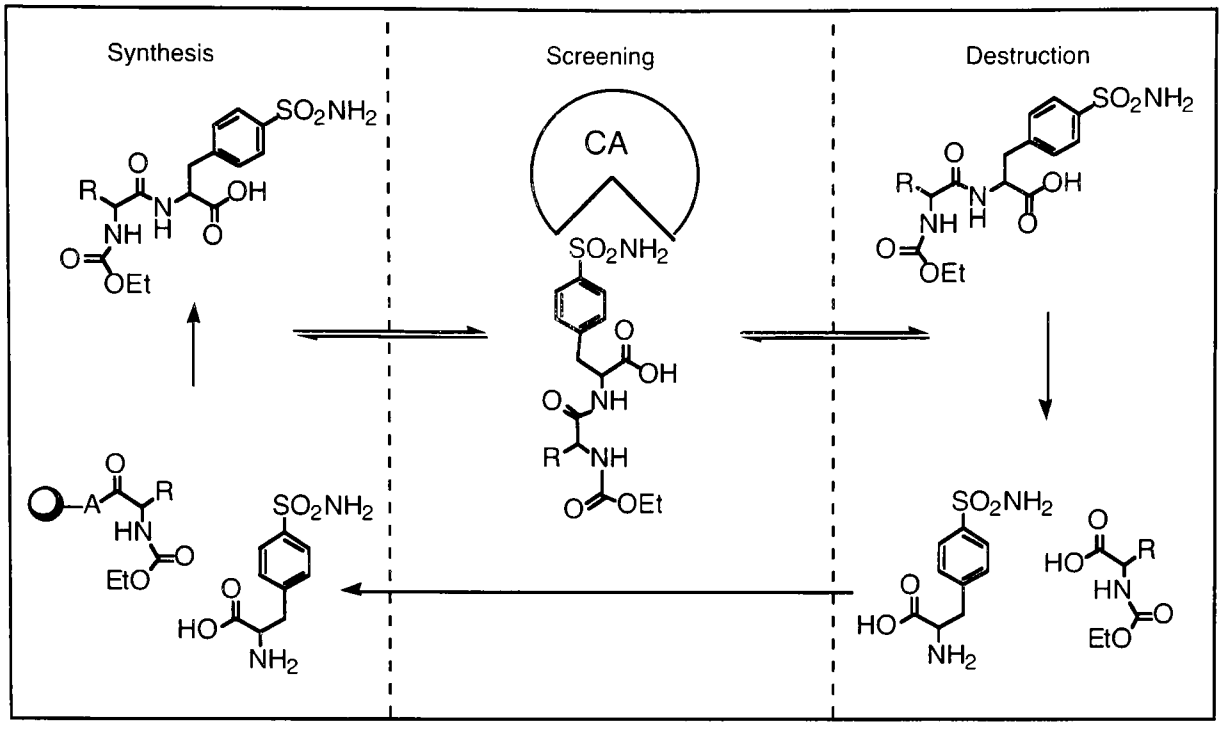


Figure 4-8. General scheme of the second-generation pseudo-DCL.

Four 12 h cycles were carried out and the concentrations of the four sulfonamide-containing dipeptides **20-23** were plotted versus time (Figure 4-9). Clearly there was a significant improvement in yield over the first-generation method. At t = 48 h there were 4.2 μmol of **23** (K_i = 1.1 μM) present, 1.4 μmol of **22** (K_i = 2.5 μM) and 0.41μmol of **21** (K_i = 5.6 μM). These three inhibitors were occupying 21% (6.01 μmol) of the 28 μmol of theoretical active sites on the CA present in the screening chamber. The molar ratio of the three inhibitors, 10 : 3.4 : 1.0 (**21** : **22** : **23**) is greater than the ratios of their inhibition constants (5.1 : 2.3 : 1.0). This pseudo-DCL method has clearly increased the yield of the best inhibitor compared to the first generation system and, overall, the yield is increasing beyond that obtained in the first cycle. As well, the ratio of inhibitors was greater than the ratio of their K_is.

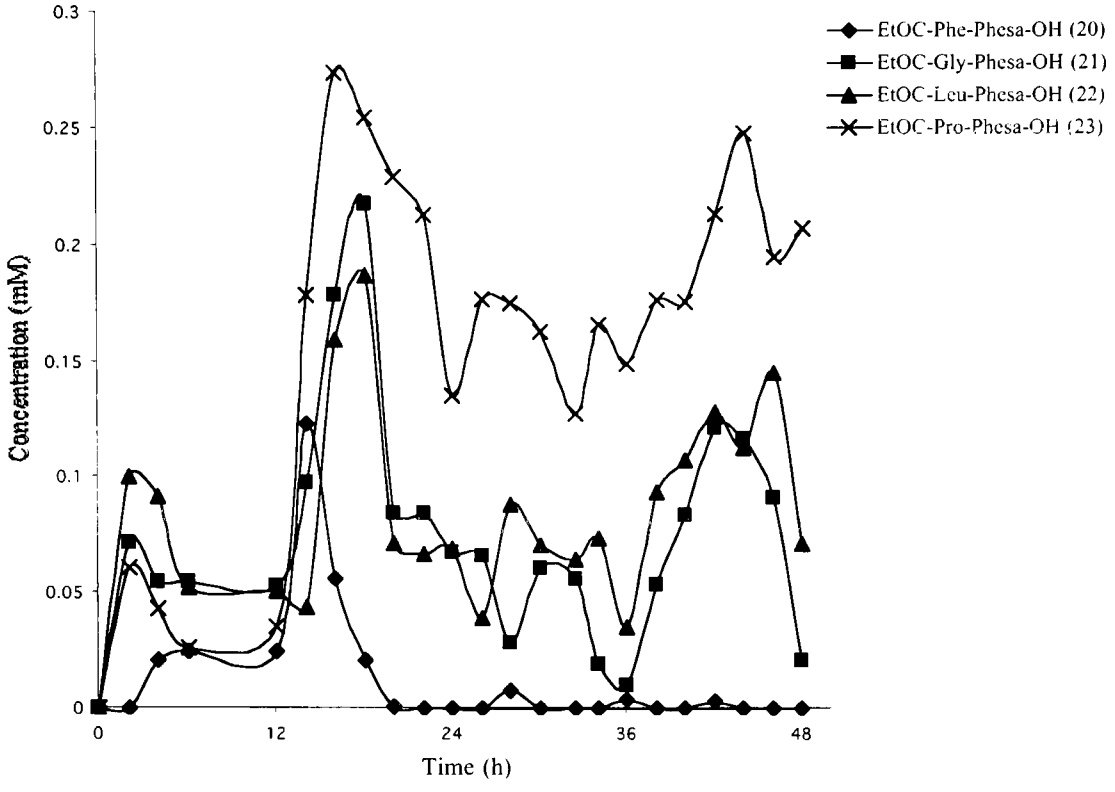


Figure 4-9. Results of the initial second generation pseudo-DCL.

There was clearly room for improvement in the system. Although the observed selectivity was above the ratio of the K_is, it was far from absolute. Because three products from an eight-membered library (3 / 8 of the compounds generated in the library) were "hits", this could not be considered a screening method with the potential to be applied to larger libraries. Fortunately, many parameters in the system could be altered to improve both the selectivity and yield.

The first parameter to be investigated was the length of the cycle time. If the cycle time was shortened, the destructive process would have less time to act on the strongest inhibitors, leaving more of them present when the next synthetic cycle begins. This may increase the concentration of dipeptides in the screening chamber and, ideally,

increase the overall yield of all the inhibitors, leading to an increase in the yield of the best inhibitor.

To explore this theory, an experiment was setup with an eight hour cycle time as opposed to the 12 hour cycles of the previous experiments. Six cycles were carried out with same amounts of active esters, and CA as in the previous experiment, but with 270 μmol (9.6 equiv) of each nucleophile. The reaction was monitored every four hours by HPLC (Figure 4-10).

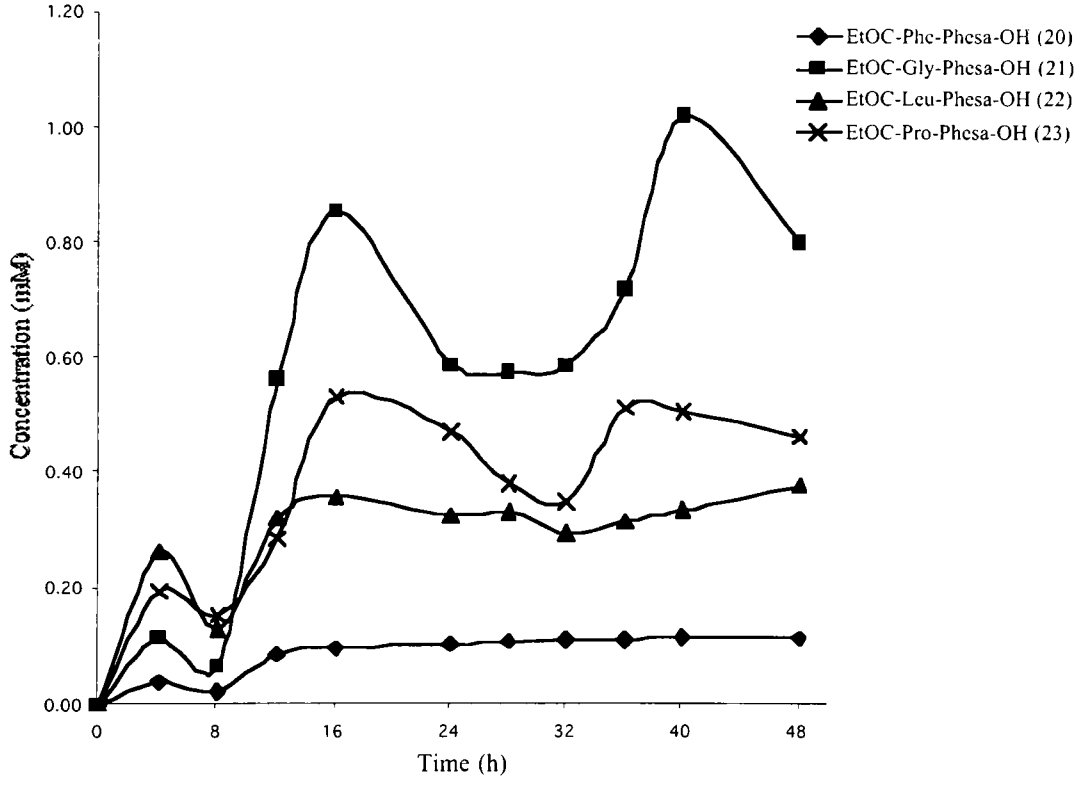


Figure 4-10. Results of the eight hour cycle experiment.

The concentration of all of the dipeptides in the screening chamber increased significantly. At a late point in the experiment (t = 40 h) the amounts of each dipeptide present were: EtOC-Phe$_{sa}$-Gly-OH, (**21**), 20.5 μmol (73%); EtOC-Phe$_{sa}$-Pro-OH, (**23**), 10.2 μmol (36%); EtOC-Phe$_{sa}$-Leu-OH, (**22**), 6.80 μmol (24%); EtOC-Phe$_{sa}$-Phe-OH,

(**20**), 2.39 μmol (8.5%). These results were disappointing. The amount of **21** was not consistent with the general trend relating the relative amounts of each inhibitor present with their inhibition constants as observed in the other experiments. Furthermore, the total amount of all four inhibitors in the screening chamber, 39.9 μmol, was greater than the amount of CA present, 28 μmol, so therefore not all of the inhibitors in the screening chamber were bound to CA. The high concentration of the glycine-containing dipeptide **21** was consistent with its active ester being the best electrophile of the series, as observed in the static library generation experiments (Scheme 3-14 and 3-5). These results indicated that the higher frequency of synthesis was overwhelming the destructive process, allowing for unbound dipeptides to remain in the screening chamber, which would lead to inaccurate results in the analysis of the pseudo-DCL. Clearly shortening the length of the cycles was not conducive to good selectivity or accuracy in these pseudo-DCLs.

It was therefore postulated that extending the cycle time, to a period longer than 12 hours, would give better selectivity between the best inhibitors in the DCL. One potential drawback of the longer cycle time was that the destructive process could continue to act even when there was only one inhibitor, the best one, left in the screening chamber. This would lower the amount of this inhibitor present at the end of each cycle and thus lessen the amount of this product obtained by having numerous cycles.

With all other parameters kept the same, a pseudo-DCL experiment with a 16 hour cycle time was performed. Four cycles were carried out and the results are illustrated in Figure 4-11.

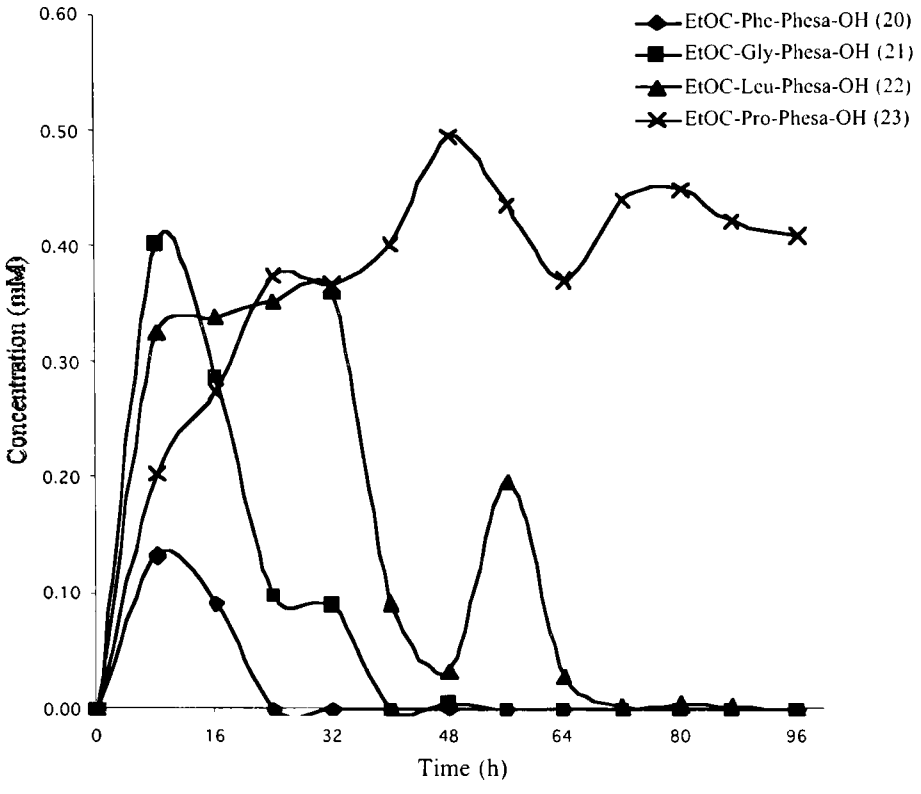


Figure 4-11. Result of the Pseudo-DCL with a 16 h cycle time.

The results of the 16 hour cycle experiment were a major improvement over previous attempts. The amount of EtOC-Pro-Phe_{sa}-OH (**23**) present at t = 72 h was 8.84 μmol. This inhibitor, **23**, occupies 31% of the theoretical binding sites on the CA in the screening chamber. The second best inhibitor, EtOC-Leu-Phe_{sa}-OH (**22**) was the only other dipeptide present, and only 0.0625 μmol (0.22%) of this dipeptide was detected. At the next measurement, t = 80 h, this dipeptide was no longer detectable. The ratio of the two best inhibitors at t = 72 h was 140:1 even though the ratio of their inhibition constants at pH 9.0 was only 2.27:1. The selectivity and amplification of the best inhibitor are both excellent. It was unlikely that the selectivity could be improved in a meaningful way by further optimization of the cycle time for this library. The yield of

the best inhibitor, 31 % (8.84 μmol, 3.65 mg), would be sufficient for full spectroscopic characterization, after purification by HPLC.

The only possible area for improvement in this pseudo-DCL was the yield. The use of large quantities of the biological target, in these cases 840 mg of CA, was necessary for ongoing sampling from the screening chamber. Access to these amountsof receptor would rarely be possible with most desirable targets. For this method to be useful on small amounts of target, the yield of the best inhibitor, relative to the amount of target, needed to be improved.

In the experiment described above there was in fact a significant amount of Phe_{SA} (**4**), a modest CA inhibitor, present at the end of the experiment. Initially, 270 μmol of **4** was added to the library. At t = 72 h, 8.90 μmol of the Phe_{SA} was contained in dipeptides **22** and **23**. This left 261 μmol of free Phe_{SA} available to be bound to CA. The ratio of the K_is of EtOC-Pro-Phe_{SA}-OH **(23)** and Phe_{SA} **(4)** at pH 9.0 was 7.3:1.0. Therefore it would only require a concentration of Phe_{SA} 7.3 times greater than that of EtOC-Pro-Phe_{SA}-OH for them to be equally competitive for a given binding site. When the ratio of **4** to **23** present in the library is weighted to account for this factor, the ratio of the amounts of the two inhibitors bound to the target would be 4.0:1.0 (for **4** : **23**) . The ratio of the moles of CA occupied by the dipeptides at t = 72 h to the CA which is unoccupied by dipeptides was 1.0:2.2. Given the assumptions made in this rough calculation and the associated uncertainty in the inhibition constants, the similarity of these two ratios seemed to suggest a plausible explanation for the low yield of the best inhibitor in the previous pseudo-DCL experiment.

In an effort to probe this possibility, an experiment was devised such that a smaller amount of the nucleophiles was used. Therefore, 100 μmol (3.6 equiv) of phenylalanine and 100 μmol (3.6 equiv) of Phe_{SA} (**4**) were used with 28 μmol (1.0 equiv) of CA. The portions of the four active esters (92 μmol, 3.3 equiv) used previously were added in each of the six 16-hour cycles of the experiment (552 μmol total, 20 equiv). The choice of 200 μmol as the total amount of nucleophile present was based on the fact that it provided a 4 mM concentration (50 mL total volume of the three chambers) of the nucleophiles throughout the system. The previous static library generation experiment had shown that this concentration of nucleophiles would still provide adequate dipeptide synthesis (Table 3-4). The results of this pseudo-DCL are illustrated in Figure 4-12.

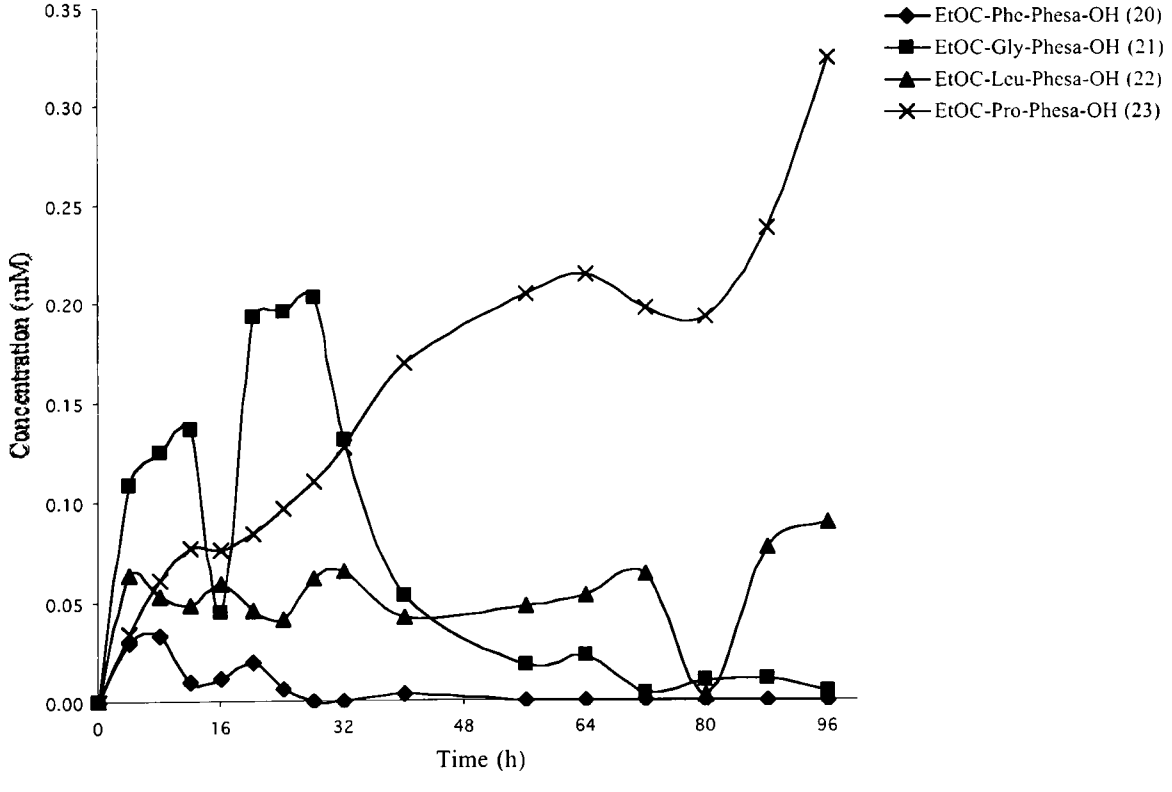


Figure 4-12. Results of the pseudo-DCL with 100 μmol of each nucleophile.

Clearly decreasing the concentration of the nucleophiles did not have an effect on the total amount of the inhibitors present after six cycles were complete. There was 6.52 μmol (23 %) of EtOC-Pro-Phe$_{SA}$-OH (**23**) present along with 1.81 μmol (6.5 %) of EtOC-Leu-Phe$_{SA}$-OH (**22**), 0.091 μmol (0.33 %) of EtOC-Gly-Phe$_{SA}$-OH (**21**) and no EtOC-Phe-Phe$_{SA}$-OH (**20**) at t = 96 h. The total yield of the three inhibitors found in the screening chamber was 30 % (8.42 μmol), not a significant change from the experiment where the concentration of nucleophile was 1.8 times higher. 23% of the active sites of the CA were occupied by the best inhibitor **23**. The selectivity of this pseudo-DCL appeared to be increasing with each additional cycle based on the decreasing amount of the poorer inhibitors such as EtOC-Gly-Phe$_{SA}$-OH (**21**) over time. As well, the concentration of the best inhibitor did not seem to have reached a plateau as it had in the other experiments. In an effort to explore this particular set of pseudo-DCL conditions further, the previous experiment was repeated and nine 16-hour cycles were carried out. These results are illustrated in Figure 4-13. The first three cycles were not monitored as frequently as the remaining cycles since they would have been the same as in the previous experiment.

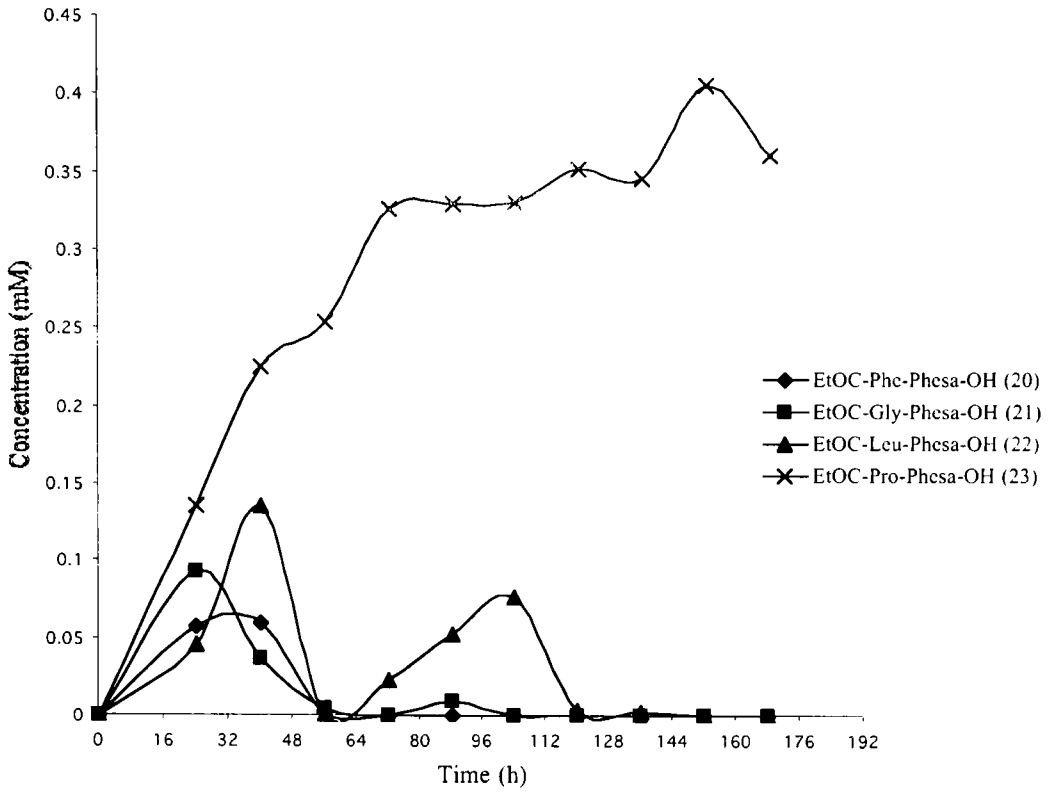


Figure 4-13. Nine cycle experiment analogous to that in 4-10.

As Figure 4-12 illustrates, additional cycles significantly improved the selectivity in this pseudo-DCL. At t = 136 h there were 6.98 μmol of EtOC-Pro-Phe_{sa}-OH (**23**), and 0.035 μmol of EtOC-Leu-Phe_{sa}-OH (**22**). These two inhibitors accounted for 25% of the theoretical CA active sites (24.9 and 0.1 %, respectively). After the 136 hour mark, only the best inhibitor, **23**, was detected. The amount of **23** present rose to a maximum level of 8.18 μmol (29%) during the last cycle. The results from this set of conditions did not exhibit any drastic improvement over those illustrated in Figure 4-11. The yield of the best inhibitor was somewhat lower, and nine cycles were needed to obtain the selectivity achieved in only six cycles in the previous experiment.

In an effort to improve the amount of the best inhibitor present in the screening chamber when the selectivity appeared optimal, our attention focused on improving the

synthesis portion of the pseudo-DCL. In the previous experiment, approximately 92 μmol of active esters were present at the beginning of each cycle. This constituted a significant deficit (3.3 equiv) relative to the amount of nucleophile present (7.1 equiv total). Thus the yield of the first cycle could be increased since in the first cycle, not all of the nucleophiles participated in the coupling. The use of excess active ester in every cycle would maximize the yield of dipeptides in each of these cycles regardless of the amount of nucleophile available to participate.

A new experiment was devised identical to the previous one (illustrated in Figure 4-13) except for an increase in the amount of active esters used. Approximately 277 μmol (9.9 equiv) of active esters were used in each cycle. This constituted 1.39 equivalents relative to the amount of nucleophile present initially, and would represent an even larger excess in each of the subsequent cycles. Fifteen 16 h cycles were carried out and the results are illustrated in Figure 4-14.

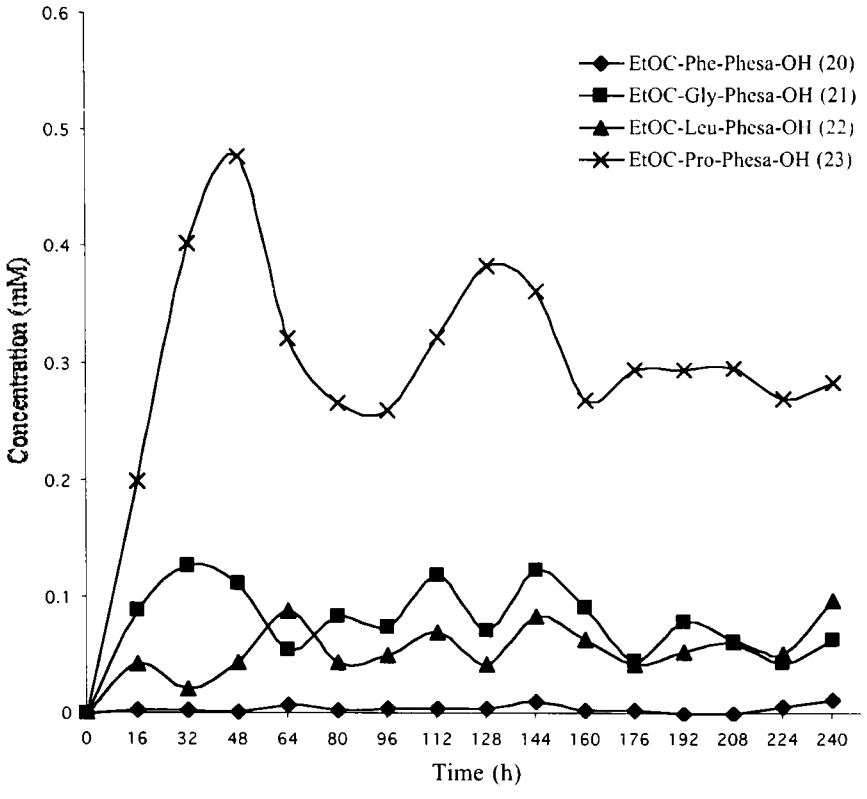


Figure 4-14. Pseudo-DCL where the equivalents of active ester were increased.

As expected, there was a greater amount of dipeptides present in the early cycles of this trial than in the previous two trials. The total amount of inhibitors present in this experiment at t = 32 h was 11.1 μmol, corresponding to 40 % of the CA active sites, while at t = 32 h in the earlier trial illustrated in Figure 4-11, only 5.90 μmol (21%) of the inhibitors were present. Unfortunately, at the end of the experiment (t = 240 h) all four sulfonamide-containing dipeptides were present: EtOC-Phe_{sa}-Pro-OH, (**23**), 5.70 μmol; EtOC-Phe_{sa}-Gly-OH, (**21**), 1.97 μmol; EtOC-Phe_{sa}-Leu-OH, (**22**), 1.30 μmol; EtOC-Phe_{sa}-Phe-OH, (**20**), 0.241 μmol. The total of the four inhibitors was 9.22 μmol, corresponding to 33% of the CA active sites. Not only did the total amount of inhibitors synthesized not increase, the selectivity of the pseudo-DCL process dropped significantly. Due to the increase in the amount of product produced in the synthesis

process it is probably required to re-optimize the cycle time used in the experiment. It is not clear how the system would respond to a longer cycle time in an effort to increase the yield of the best inhibitor as well as the selectivity. Optimization of both the yield and selectivity may be mutually exclusive since the screening process involves an inherent decrease in yield of product via the destructive process necessary to obtain selectivity.

4.3 CONCLUSIONS

Our foray into the field of DCLs has proven successful. The pseudo-DCLs that have been prepared have consistently produced the best inhibitor in a significantly higher concentration than all others. Under optimized conditions, the best inhibitor was the only one detected by HPLC. This corresponds to an amplification of over 100-fold greater than the ratio of the inhibition constants of the compounds.

Many fundamental aspects of the pseudo-DCL were established which will aid in the design and optimization of future pseudo-DCLs. The concentration of any inhibitors used as starting materials in the synthesis process must be kept to a minimum. Ideally, they should be present in a constant concentration and not allowed to build up over time, as in our first-generation system. The optimization of selectivity (i.e. to have only one product of the library remaining) can be accomplished, but increasing the yield of this one product above 30 % has proven difficult. This may be due to the "synthesis in bursts" that occurs upon addition of new portions of resin to the pseudo-DCL. A steady, continuous synthesis process may prove to be advantageous, but difficult to execute.

Quantitative yield of an inhibitor relative to the target and screening such that only one inhibitor is present in a given system may prove to be mutually exclusive.

The system has demonstrated itself to be capable of absorbing modest biases in initial concentration of the inhibitors produced such that the library members need not be generated in equal portions. This eliminates the need of a "perfect" synthetic process which should allow for a wider structural variety of components to be used as starting materials.

4.4 FUTURE WORK

There are many aspects of this pseudo-DCL system that still need to be investigated. Some further attempts should be made at increasing the amount of the best inhibitor in a given library, but this need not be the only, or even main, focus of further fundamental studies. The tolerance of the system for biases, both in the form of differing rates of synthesis and different rates of destruction (hydrolysis) should be explored. Pronase is known to accept a wide variety of substrates, but its limits with respect to 1) recognition of non-peptidic compounds and 2) rates of hydrolysis of different compounds should be established. Once the recognition limits of Pronase are determined, screening for a complementary protease could be undertaken to allow for further broadening of the system via the addition of a second destruction chamber.

Once the limits imposed by these factors are known, guidelines for the minimum relative rate of synthesis that would be required for a compound that is an excellent substrate for a protease(s) can be determined. As well the reverse can be established. The maximum relative rate of synthesis for a poor protease substrate can be determined

such that the system will not be overwhelmed by a build-up of this inhibitor. These general limits can be used for measuring the scope or generality of the pseudo-DCL process. Ideally, a mathematical relationship that explains these limits should be established.

The current eight-membered CA-Pronase pseudo-DCL is an excellent model for initial attempts at miniaturization of the system. A 10 to 100-fold decrease in the amount of target (CA) used in the pseudo-DCL (presumably in a much smaller vessel) will most likely necessitate a re-optimization of the cycle time due to the likely decrease in surface area of the dialysis membrane. The use of active esters on a soluble support in place of those on solid supports may be required for this miniaturization.

Expansion of the current library from eight members to a more useful size should be investigated. A stepwise increase in size would be the most prudent way to go about this. The effect of a larger number of nucleophiles which could also act as inhibitors (such as those described in chapter two) must be investigated. If a given nucleophile is "too good" an inhibitor by itself, will it react in the synthesis process or will the majority of it simply remain bound to the target? How can an occurrence of this phenomenon be identified in the midst of a large library experiment? These are questions that should be answered. Expansion of the library will necessitate the development of a reliable method of analysis for unknown compounds. A stable isotope elution assay on an HPLC-MS would be an option.

Once the current system is well understood, other amide-based libraries can be screened in the presence of new targets. The development of pseudo-DCLs based on other bonds are currently being explored in the group.

[1] Cheeseman, J. D.; Corbett, A. D.; Shu, R.; Croteau, J.; Gleason, J. L.; Kazlauskas, R. J.; *J. Am. Chem. Soc.* **2002**, *124*, 5692-5701.

CONTRIBUTIONS TO KNOWLEDGE

1. A series of new CA inhibitors were synthesized.

2. Many of the common types of active esters used in peptide synthesis were prepared on a TentaGel solid support and their reactivity in water was studied. The tetrafluorophenol class of active esters were found to react with amines in basic aqueous media to form amides with very little appearance of the analogous carboxylic acid.

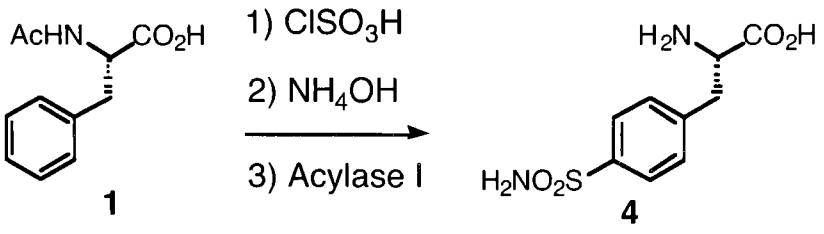
3. The concept of the pseudo-DCL was proposed and and the initial designs have exhibited a marked increase in selectivity and yield of a given inhibitor when compared to the DCLs found in the literature.

CHAPTER 5

EXPERIMENTAL SECTION

General experimental: All chemicals and enzymes were purchased from Sigma-Aldrich Canada with the following exceptions. Acylase I from hog kidney was obtained from Fluka. Amino and bromo NovaSyn Tentagel resins were obtained from Caledon-NovaBiochem. All chemical were used without further purification with the following exceptions. Tetrahydrofuran and diethyl ether were distilled from sodium benzophenone ketyl. Toluene, methylene chloride and triethylamine were distilled from calcium hydride. All amino acids used were natural L-enantiomers unless otherwise noted. All solid phase synthesis, with the exception of the library experiments, were carried out in coarse-fritted peptide synthesis vessels obtained from Chemglass. Chromatography was conducted using 200-400 mesh silica gel from Silicycle. NMR spectra were recorded at 270, 300, or 400 MHz for ^{1}H and 67.5, 75, and 100 MHz for ^{13}C. Elemental analyses were perfromed by Quantatative Technologies Inc, Whitehouse, NJ, USA. High-resolution mass spectra were performed by The Université de Sherbrooke, Sherbrooke, QC, Canada.

In all cases where yields were determined by HPLC, molar absorptivity values were determined with solutions of known concentration.



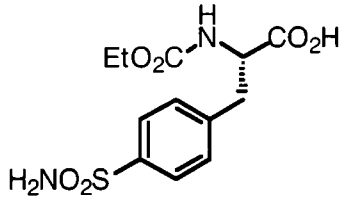
1 4

4'-Sulfonamidophenylalanine (4). *N*-Acetylphenylalanine (37.7 g, 178 mmol, 1.0 equiv) was added in portions over a 1 h period to neat chlorosulfonic acid (110 mL, 1.65 mol, 9.5 equiv) at –10 °C. The resulting yellow solution was stirred at –10 °C for 2.5 h, at 25 °C for 2.5 h, and then heated to 60 °C until gas evolution had ceased (ca. 12 h). The resulting orange solution was cooled to 0 °C and poured carefully onto 750 mL of ice (Caution: exotherm!). The resulting mixture was extracted with ethyl acetate (3 x 1 L) and the combined organic layers were dried over Na_2SO_4, filtered and concentrated in vacuo to afford the sulfonyl chloride (45.1 g, 83%) as an orange solid which was used immediately without further purification. ^{1}H NMR ($(CD_3)_2SO$) δ 8.26-8.21 (d, 1H, J = 8.5 Hz), 7.55 (d, 2H, J = 6.9 Hz), 7.22 (d, 2H, J = 6.8 Hz), 4.49-4.34 (m, 1H), 3.13-3.00 (dd, 1H, J = 14.4 and 6.8 Hz), 2.92-2.79 (dd, 1H, J = 11.0 and 10.2 Hz), 1.80 (s, 3H).

The sulfonyl chloride **2** was dissolved in 28% aq. NH_4OH (240 mL) and the resulting solution was heated at reflux for 3 h. After cooling to 0 °C, the solution was acidified to pH 1 by addition of 3 M H_2SO_4 (ca. 200 mL) and extracted with ethyl acetate (3 x 200 mL). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuo to afford the sulfonamide **3** (29.9 g, 71%) as a white solid. The *N*-acetyl sulfonamide **3** could not be purified to homogeneity by either chromatography or recrystallization. ^{1}H NMR ($(CD_3)_2SO$) δ 8.29-8.24 (d, 1H, J = 8.5 Hz), 7.77 (d, 2H, J =

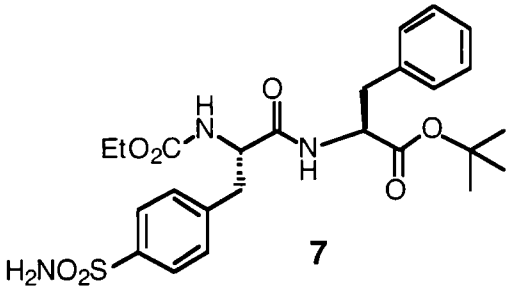
3.9 Hz), 7.45 (d, 2H, J = 6.9 Hz), 7.33 (s, 2H), 4.53-4.41 (m, 1H), 3.20-3.09 (dd, 1H, J = 14.2 and 6.8 Hz), 3.01-2.87 (dd, 1H, J = 11.2 and 10.1 Hz), 1.80 (s, 3H).

A suspension of the sulfonamide **3** (20.0 g, 69.9 mmol, 1.0 equiv) in distilled water (300 mL) was adjusted to pH 5.00 with LiOH (900 mg). A 0.25 M solution of Na_2HPO_4 (85 mL) was used to raise the pH to 7.50. Acylase I from hog kidney (200 mg, 17.8U/mg, 3560U) was added as an aqueous solution (12 mL) and the resulting solution was stirred at 21°C for 70 h. The solution was then acidified to pH 1.0 with 3M H_2SO_4 and extracted with ethyl acetate (3 x 500 mL), the organic layer was then dried with anhydrous Na_2SO_4 and concentrated in vacuo to afford 2.28g (11.4%) of the sulfonamide starting material **(3)**. The aqueous layer was neutralized with 2 M NaOH and concentrated. The solution was then applied to an Amberlite 120(plus) acidic ion exchange column (Cl form). The column was rinsed with water until the eluent was at pH 6.0 and the it was rinsed with 0.50 M NH_4OH solution until the eluent became basic. The basic wash was concentrated *in vacuo* and recrystallized from water to afford provided 4'-sulfonamidophenylalanine **(4)** as a white solid (11.60 g, 68%). ^{1}H NMR (D_2O / DCl) δ 7.62 (d, 2H, J = 8.1 Hz), 7.26 (d, 2H, J = 8.1 Hz), 4.14 (t, 1H, J = 6.8 Hz), 3.19-3.12 (dd, 1H, J = 14.6 and 5.7 Hz), 3.08-3.01 (dd, 1H, J = 14.4 and 6.9 Hz). ^{13}C NMR (D_2O / DCl) δ 170.73, 140.451, 139.49, 130.38, 126.55, 53.49, 35.19. FABMS in sat NaCl *m/z* 267 (M+ Na, $C_9H_{12}N_2O_4SNa$ requires 267.)



5

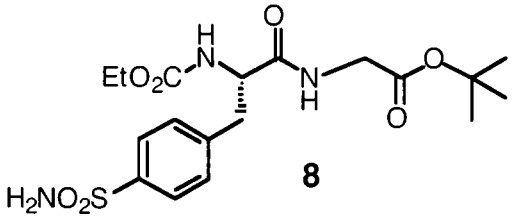
N-Ethoxycarbonyl-4'-sulfonamidophenylalanine (5). Ethyl chloroformate (398 μL, 4.17 mmol, 1.1 equiv) was added to a two phase mixture of 4'-sulfonamidophenylalanine **(4)** (925 mg, 3.73 mmol, 1 equiv) in 1,4-dioxane (25 mL) and sat. $NaHCO_3$ solution (25 mL) at 0 °C and the resulting solution was stirred for 6 h at 0 °C. The mixture was extracted with ethyl acetate (100 mL), the aqueous layer was acidified to pH 1 by addition of 2 M HCl (ca. 20 mL) and then extracted with ethyl acetate (3 x 50 mL). Latter organic extracts were combined, dried over Na_2SO_4, filtered and concentrated in vacuo to afford the ethyl carbamate **(5)** (879 mg, 83%) as an analytically pure oil. ^1H NMR ($(CD_3)_2CO$) δ 7.85 (d, 2H, J = 7.1 Hz), 7.51 (d, 2H, J = 6.9 Hz), 6.54 (s, 2H), 6.45 (d, 1H, J = 6.7 Hz), 4.62-4.45 (m, 1H), 4.01-3.97 (q, 2H, J = 2.4 Hz), 3.41-3.28 (dd, 1H, J = 11.3 and 4.0 Hz), 3.16-3.05 (dd, 1H, J = 10.4 and 7.8 Hz), 1.13 (t, 3H, J = 6.0 Hz). HR-CIMS (m/z): [MH+] calculated for $C_{12}H_{17}N_2O_6S$, 317.0807; found, 317.0817.



7

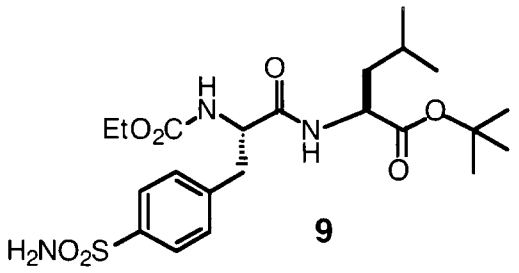
EtO_2C-(4'-SO_2NH_2)Phe-Phe-O-t-butyl (7). Prepared as **8** to afford to afford 173 mg (72%). ^1H NMR ($CDCl_3$) δ 7.80 (d, 2H, J = 7.8 Hz), 7.33-7.24 (m, 5H), 7.09 (d, 2H, J = 6.0 Hz), 6.41 (d, 1H, J = 5.7 Hz), 5.19 (d, 1H, J = 6.1 Hz), 4.96 (s, 2H), 4.75-4.61 (m, 1H), 4.52-4.38 (m, 1H), 4.10-4.03 (q, 2H, J = 6.9 Hz), 3.20-2.96 (m, 4H), 1.39 (s, 9H), 1.21 (t, 3H, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$) δ 170.34, 170.11, 142.21, 140.84, 136.03,

130.38, 129.65, 128.67, 127.31, 126.96, 82.93, 77.44, 61.76, 53.86, 38.40, 38.21, 28.16, 14.69. Analysis calculated for $C_{25}H_{33}N_3O_7S$ C, 57.79; H, 6.40; N, 8.09. Found: C, 57.71; H, 6.34; N, 7.96.



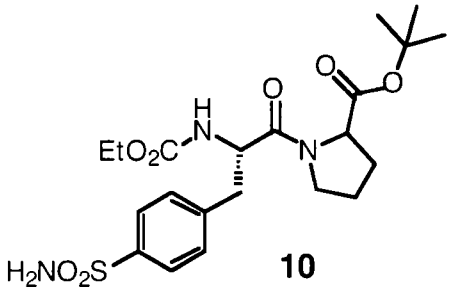
8

EtO_2C-(4'-SO_2NH_2)Phe-Gly-O-t-butyl (8). EDC•HCl (136 mg, 0.711 mmol, 1.1 equiv), HOBT (87.3 mg, 0.646 mmol, 1.0 equiv) and triethylamine (269 μL, 1.94 mmol, 3.0 equiv) were added to a solution of **5** (204 mg, 0.646 mmol, 1.0 equiv) in THF (3 mL) at 0 °C. Glycine t-butyl ester•HCl (119 mg, 0.711 mmol, 1.1 equiv) was added and the resulting solution was allowed to warm to 21 °C while stirred for 13 h, at which point the bulk of the THF was removed by concentration in vacuo. The residue was dissolved in ethyl acetate (45 mL) and extracted with 0.1 M HCl (3 x 25 mL) and sat. $NaHCO_3$ solution (3 x 25 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The solid residue was purified by mixed solvent recrystallization (ethyl acetate/hexanes) to afford 193 mg (70%) of **8**. ^{1}H NMR ($(CD_3)_2CO$) δ 7.82 (d, 2H, J = 7.5 Hz), 7.63 (s, 1H), 7.50 (d, 2H, J = 7.5 Hz), 6.52 (s, 2H), 6.40 (d, 1H, J = 7.5 Hz), 4.50 (m, 1H), 4.00-3.88 (m, 4H), 3.40 (dd, 1H, J = 14.1 and 4.2 Hz), 3.02 (dd, 1H, J = 13.5 and 9.9 Hz), 1.45 (s, 9H), 1.12 (t, 3H, J = 6.9 Hz). ^{13}C NMR ($(CD_3)_2CO$) δ 171.48, 168.93, 156.33, 142.70, 130.06, 126.18, 81.04, 60.48, 55.92, 41.79, 37.83, 27.52, 14.22. Analysis calculated for $C_{18}H_{27}N_3O_7S$ C, 50.34; H, 6.34; N, 9.78. Found: C, 50.33; H, 6.35; N, 9.73.



9

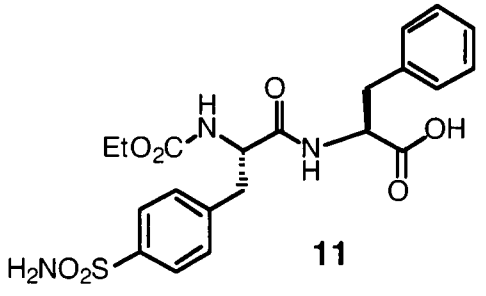
EtO_2C-(4'-SO_2NH_2)Phe-Leu-O-t-butyl (9). Prepared as **8** to afford 227 mg (78%). ^{1}H NMR ($(CD_3)_2CO$) δ 7.81 (d, 2H, J = 8.4 Hz), 7.55 (m, 2H), 7.48 (d, 2H, J = 8.1 Hz), 6.51 (m, 1H), 6.31 (m, 1H), 4.50 (m, 1H), 4.39 (m, 1H), 4.00-3.95, (q, 2H, J = 5.7), 3.32-3.26 (dd, 1H, J = 13.8 and 3.9 Hz), 3.05-2.97 (dd, 1H, J = 13.6 and 9.6 Hz), 1.75-1.64 (m, 2H), 1.61-1.56 (m, 2H), 1.45 (s, 9H), 1.12 (t, 3H, J = 7.4 Hz), 0.94-0.90 (m, 7H). ^{13}C NMR ($(CD_3)_2CO$) δ 171.8, 171.0, 156.4, 142.5, 130.1, 126.2, 81.0, 60.5, 55.7, 51.6, 41.1, 37.8, 27.5, 24.8, 22.5, 21.3, 19.5, 14.2. Analysis calculated for $C_{22}H_{35}N_3O_7S$ C, 54.42; H, 7.26; N, 8.65. Found: C, 54.28; H, 1.27; N, 8.46.



10

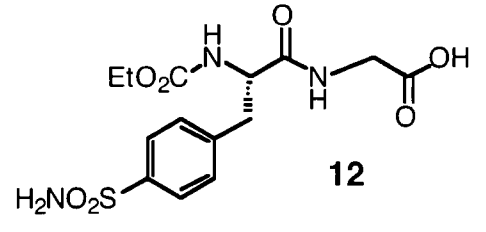
EtO_2C-(4'-SO_2NH_2)Phe-Pro-O-t-butyl (10). Prepared as **8** to afford 312 mg (67%). ^{1}H NMR ($(CD_3)_2SO$) δ 7.69 (d, 2H, J = 7.6 Hz), 7.48 (d, 2H, J = 7.0 Hz),7.36 (d, 2H, J = 7.2 Hz), 7.28 (s, 2H), 4.36 (m 1H), 4.19 (m, 1H), 3.85 (t, 2H, J = 6.7 Hz), 3,65 (m, 2H), 2.97-2.94 (dd 1H, J = 11.7 and 4.2 Hz), 2.82-2.74 (dd 1H, J = 11.1 and 9.2 Hz), 2.14 (m, 1H), 1.90 (m, 1H), 1.77 (m, 1H), 1.35 (s, 9H), 1.04 (t, 3H, J = 7.3 Hz). ^{13}C NMR ($(CD_3)_2SO$)δ 171.6, 170.4, 156.8, 143.0, 142.7, 130.5, 126.1, 81.0, 60.5, 60.1, 54.7, 47.1,

36.4, 29.2, 28.3, 25.3, 15.2. Analysis calculated for $C_{18}H_{27}N_3O_7S$ C, 50.34; H, 6.34; N, 9.78. Found: C, 50.33; H, 6.35; N, 9.73.



11

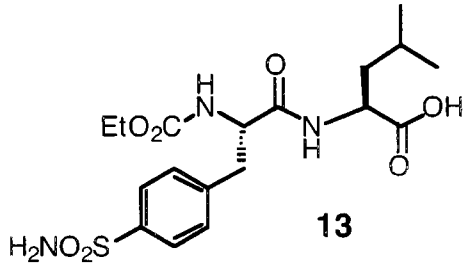
EtO_2C-(4'-SO_2NH_2)Phe-Phe-OH (11). Prepared as **12** to afford 270 mg (68 %). ^1H NMR (CD_3OD) δ 8.19 (d, 1H, J = 9.3 Hz), 7.80 (d, 2H, J = 8.4 Hz), 7.38 (d, 2H, J = 9.3 Hz), 7.27-7.21 (m, 5H), 7.07 (d, 1H, J = 8.7 Hz), 4.68-4.63 (m, 1H), 4.40-4.35 (q, 2H, J =6.1 Hz), 3.24-3.18 (dd, 1H, J =13.9 and 5.2 Hz), 3.18-3.11 (dd, 1H, J =14.3 and 5.5 Hz), 3.04-2.97 (dd, 1H, J = 13.9 and 8.2 Hz), 2.88-2.80 (dd, 1H, J = 13.9 and 9.7 Hz), 1.18-1.14 (t, 3H, J = 6.9 Hz). ^{13}C NMR (CD_3CD) δ 173.1, 172.3, 157.2, 142.3, 137.0, 129.8, 129.2, 128.3, 126.6, 126.0, 60.9, 55.9, 53.9, 37.6, 37.2, 13.7. HR-CIMS (m/z): [MH+] calculated for $C_{21}H_{26}N_3O_7S$, 464.1491; found, 464.1501.



12

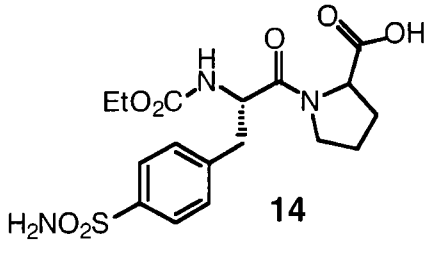
EtO_2C-(4'-SO_2NH_2)Phe-Gly-OH (12). TFA (7 mL) was added to a solution of **8** (175 mg, 0.409 mmol, 1.0 equiv) in CH_2Cl_2 (8 mL) and the solution was stirred for 25 min at 21°C under an atmosphere of argon. The solvents were removed in vacuo, and the residue was purified by recrystallization from acetone to afford 121 mg (79 %) of **12**. ^1H NMR (CD_3OD) δ 8.55 (s, 1H), 7.83 (d, 2H, J = 7.2 Hz), 7.46 (d, 2H, J = 7.2 Hz), 4.45-4.42 (m,

1H), 4.02-3.98 (q, 2H, J =6.8), 3.95-3.92 (m, 1H), 3.32-3.25 (m, 2H), 2.97-2.89 (dd, 1H, J = 13.5 and 9.9 Hz), 1.18-1.14 (t, 3H, J = 6.8). ^{13}C NMR (CD_3CD) δ 173.0, 171.8, 157.3, 148.7, 142.4, 137.6, 129.8, 126.0, 60.9, 56.0, 37.7, 13.7. HR-CIMS (m/z): [MH+] calculated for $C_{14}H_{20}N_3O_7S$, 374.1022; found, 374.1030.



13

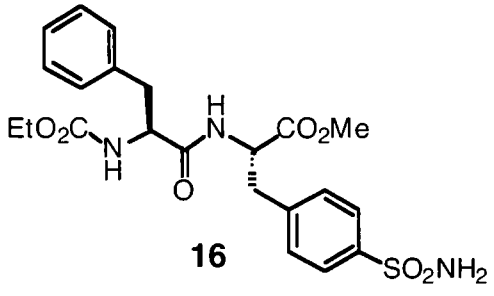
EtO_2C-(4'-SO_2NH_2)Phe-Leu-OH (13). Prepared as **12** to afford 174 mg (80 %). ^{1}H NMR (CD_3OD) δ 7.82 (d, 2H, J = 8.4 Hz), 7.68 (d, 1H, J = 8.1 Hz), 7.45 (d, 2H, J = 8.1 Hz), 4.47-4.42 (m, 2H), 4.01-3.95 (q, 2H, J = 6.4 Hz), 3.31-3.29 (m, 1H), 3.25-3.3.19 (dd, 1H, J = 13.9 and 4.9 Hz), 2.95-2.87 (dd, 1H, J = 13.9 and 9.7 Hz), 1.71-1.62 (m, 2H), 1.18-1.13 (t, 3H, J = 7.1 Hz), 0.97-0.91 (m, 6H). ^{13}C NMR (CD_3OD) δ 174.6, 172.7, 157.2, 142.3, 129.8, 126.7, 126.0, 60.9, 55.8, 50.9, 40.4, 37.7, 24.8, 22.2, 20.6, 13.7. HR-CIMS (m/z): [MH+] calculated for $C_{18}H_{28}N_3O_7S$, 430.1648; found, 430.1654.



14

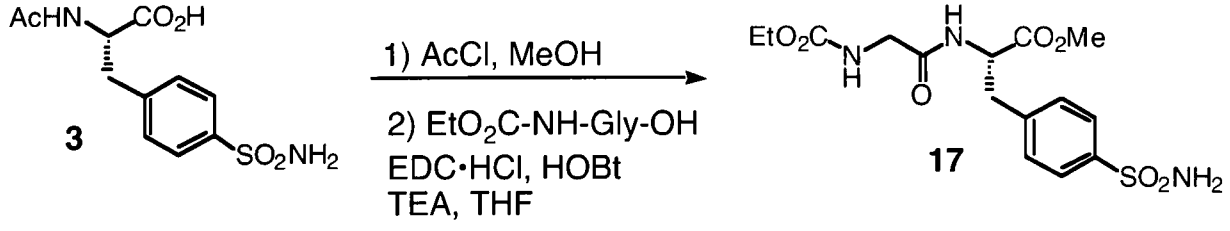
EtO_2C-(4'-SO_2NH_2)Phe-Pro-OH (14). Prepared as **12** except recrystallized from iso-propanol / hexanes to afford 94.5 mg (55 %). ^{1}H NMR (CD_3OD) δ 7.83 (d, 2H, J = 8.1 Hz), 7.50 (d, 2H, J = 8.4 Hz), 7.25 (d, 1H, J = 3.9 Hz), 4.66-4.61 (dd, 1H, J = 8.8 and 5.5 Hz), 4.47-4.43 (dd, 1H, J = 8.4 and 3.9 Hz), 4.02-3.95 (q, 2H, J = 7.2 Hz), 3.80-3.75 (m, 1H), 3.56-3.51 (m, 1H), 3.32-3.29 (m, 1H), 3.21-3.14 (dd, 1H, J = 13.9 and 5.2 Hz),

2.96-2.89 (dd, 1H, J = 13.8 and 8.7 Hz), 2.27-2.21 (m, 1H), 2.05-1.96 (m, 2H), 1.19-1.16 (t, 3H, J =7.2 Hz. ^{13}C NMR (CD_3CD) δ 174.0, 171.1, 157.3, 141.9, 130.1, 129.1, 128.2, 126.0, 60.8, 59.4, 53.9, 37.0, 29.0, 24.6, 13.7. HR-CIMS (m/z): [MH+] calculated for $C_{17}H_{24}N_3O_7S$, 414.1335; found, 414.1325.



16

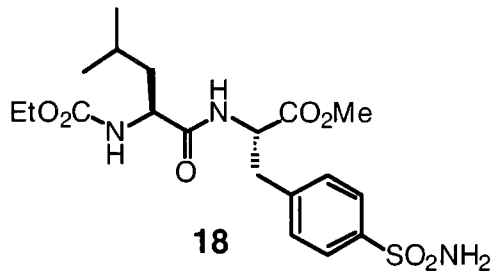
EtO_2C-Phe-(4'-SO_2NH_2)Phe-OMe (16). Prepared as **17** to afford 706 mg (67%). ^{1}H NMR ($(CD_3)_2SO$) δ 8.50 (d, 1H, J = 7.8 Hz), 7.70 (d, 2H, J = 8.4 Hz), 7.39 (d, 2H, J = 8.1 Hz), 7.30 (s, 2H), 7.26-7.20 (m, 5H), 7.19-7.14 (m, 1H), 4.54-4.48 (m, 1H), 4.24-4.18 (m, 1H), 3.89-3.82 (m, 2H), 3.59 (s, 3H), 3.14-3.08 (dd, 1H, J = 13.7 and 5.0 Hz), 3.05-2.98 (dd, 1H, J = 13.5 and 8.4 Hz), 2.93-2.87 (dd, 1H, J = 14.1 and 3.9 Hz), 2.70-2.62 (dd, 1H, J = 14.1 and 10.5 Hz), 1.09-1.04 (t, 3H, J = 7.1 Hz). ^{13}C NMR ($(CD_3)_2SO$) δ 172.6, 172.1, 156.5, 143.1, 141.9, 138.7, 130.3, 129.8, 128.7, 129.9, 126.2, 60.5, 56.6, 54.1, 52.8, 38.0, 37.0, 15.4.



3 **17**

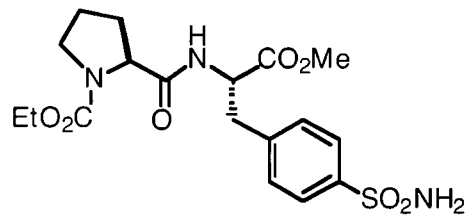
EtO_2C-Gly-(4'-SO_2NH_2)Phe-OMe (17). Acetyl chloride (1.03 mL, 14.5 mmol. 1.0 equiv) was added to a solution of N-acetyl-4-sulfonamidophenylalanine (4.16 g, 14.5 mmol, 1.0 equiv) in methanol (250 mL) and the resulting solution was stirred at room temperature for 2 h then refluxed at 65°C for 34 h. The solution was concentrated in vacuo to afford a 3.5:1 mixture of the desired 4-sulfonamidophenylalanine methyl ester **(15)** and N-acetyl-4-sulfonamidophenylalanine methyl ester and was used without further purification.

EDC•HCl (813 mg, 4.24 mmol, 1.5 equiv), HOBt (382 mg, 2.83 mmol, 1.0 equiv), and TEA (1.97 mL, 14.1 mmol, 5.0 equiv) were added to a solution of EtO_2C-Gly-OH in 5:1 THF : DMF (12 mL) at 0°C. 4-sulfonamidophenylalanine methyl ester **(15)** (731 mg, 2.83 mmol, 1.0 equiv) was added as a solution in THF (5 mL) and the resulting solution was allowed to warm to 21°C while stirring for 16 h. The bulk of the solvent was removed in vacuo and the residue was taken up in ethyl acetate (100 mL) and extracted with 0.1 M HCl (3 x 30 mL) and sat. $NaHCO_3$ solution (3 x 30 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was recrystallized from 4:1 ethyl acetate/hexanes to afford 781 mg (71%) of **17**. ^1H NMR ($(CD_3)_2SO$) δ 8.34 (d, 1H, J = 7.5 Hz), 7.70 (d, 2H, J = 8.1 Hz), 7.37 (d, 2H, J = 7.8 Hz), 7.31 (s, broad, 2H), 7.23 (t, 1H, J = 11.4 Hz), 4.55-4.53 (m, 1H), 4.00-3.93 (q, 2H, J = 7.1 Hz), 3.60 (s, 3H), 3.58-3.53 (m, 2H), 3.13-3.06 (dd, 1H, J = 14.4 and 5.3 Hz), 3.01-2.94 (dd, 1H, J = 14.5 and 9.0 Hz), 1.18-1.12 (t, 3H, J = 7.0 Hz). ^{13}C NMR ($(CD_3)_2SO$) δ 172.2*, 172.1, 170.0, 169.9*, 157.2*, 157.1, 144.7*, 143.0, 141.9, 138.8*, 133.2*, 130.3, 129.5*, 126.8*, 126.2, 124.6, 60.7, 60.6*, 54.2*, 54.0, 52.8, 52.8*, 43.9, 37.3*, 37.1, 15.5 (* indicates minor rotomer).



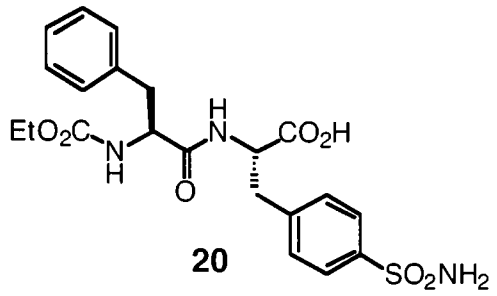
18

EtO_2C-Leu-(4'-SO_2NH_2)Phe-OMe (18). Prepared as **17** to afford 192 mg (90%). %). ^{1}H NMR ($(CD_3)_2SO$) ∂ 8.32 (d, 1H, J = 7.6 Hz), 7.68 (d, 2H, J = 8.8 Hz), 7.38 (d, 2H, J = 8.4 Hz), 7.30 (s, 2H), 7.15 (d, 1H, J = 8.0 Hz), 4.51-4.45 (m, 1H), 4.04-3.93 (m, 3H), 3.58 (s, 3H), 3.12-3.08 (dd, 1H, J = 13.8 and 5.4 Hz), 3.04-2.98 (dd, 1H, J = 14.0 and 8.8 Hz), 1.57-1.52 (m, 1H), 1.40-1.32 (m, 2H), 1.16-1.13 (t, 3H, J = 7.0 Hz), 0.86-0.79 (m, 6H). ^{13}C NMR ($(CD_3)_2SO$) ∂ 173.2, 172.1, 156.5, 143.0, 141.9, 130.3, 1262, 60.6, 53.9, 53.5, 52.8, 36.9, 25.0, 23.8, 22.4, 15.5.



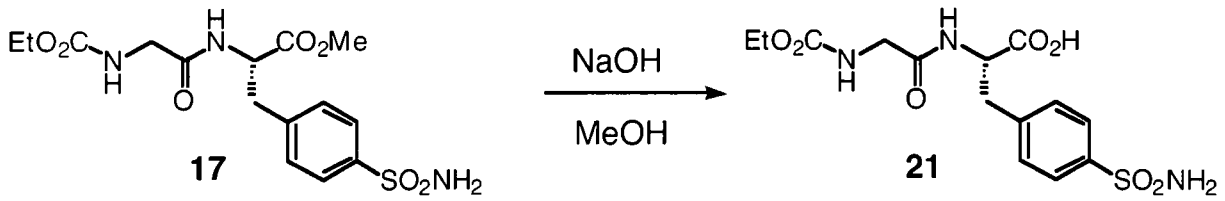
19

EtO_2C-Pro-(4'-SO_2NH_2)Phe-OMe (19). Prepared as **17** to afford 330 mg (38%). ^{1}H NMR ($(CD_3)_2SO$) δ 8.37-8.31 (m, 1H), 7.69 (d, 2H, J = 8.1 Hz), 7.39 (d, 2H, J = 8.1 Hz), 7.31 (s, 2H), 4.60-4.46 (m, 1H), 4.17-3.94 (m, 2H), 3.89-3.79 (m, 1H), 3.61 (s, 3H), 3.37-3.22 (m, 2H), 3.18-2.96 (m, 2H), 2.05-1.98 (m, 1H), 1.72-1.60 (m, 3H), 1.15-.1.10 (t, 3H, J = 7.1 Hz). ^{13}C NMR ($(CD_3)_2SO$) δ 172.9, 172.8*, 172.2, 172.1*, 154.9*, 154.6, 143.1, 142.3, 142.0*, 130.3*, 130.2, 126.2*, 126.1, 61.2*, 61.1, 60.3*, 60.0, 54.0*, 53.5, 52.8, 52.7*, 47.6, 47.2*, 36.8*, 36.7, 31.7, 30.6*, 24.5*, 23.7, 15.5*, 15.3 (* indicates minor rotomer).



20

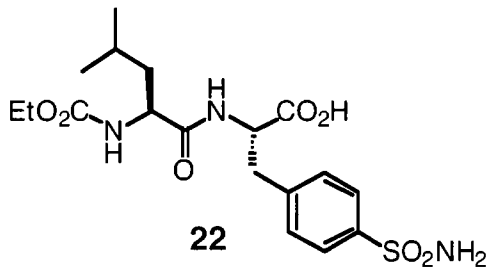
EtO_2C-Phe-(4'-SO_2NH_2)Phe-OH (20). Prepared as **21** to afford 162 mg (84%). ^1H NMR ($(CD_3)_2SO$) ∂ 8.32 (d, 1H, J = 7.6 Hz), 7.70 (d, 2H, J = 8.0 Hz), 7.40 (d, 2H, J = 8.0 Hz), 7.30 (s, 2H), 7.27-7.16 (m, 5H), 7.15-7.14 (m, 1H), 4.48-4.45 (m, 1H), 4.23-4.19 (m, 1H), 3.87-3.84 (m, 2H), 3.16-3.11 (dd, 1H, J = 13.6 and 5.2 Hz), 3.03-2.97 (dd, 1H, J = 13.4 and 9.0 Hz), 2.94-2.91 (m, 1H), 2.69-2.63 (m, 1H), 1.08-1.05 (t, 3H, J = 6.8 Hz). ^{13}C NMR ($(CD_3)_2SO$) ∂ 173.1, 172.5, 156.6, 142.9, 142.2, 138.7, 130.4, 129.8, 128.7, 126.9, 126.2, 60.6, 56.6, 53.9, 38.0, 37.1, 15.4. HR-CIMS (*m/z*): [MH^+] calculated for $C_{21}H_{26}N_3O_7S$, 464.1491; found, 464.1483.



17 21

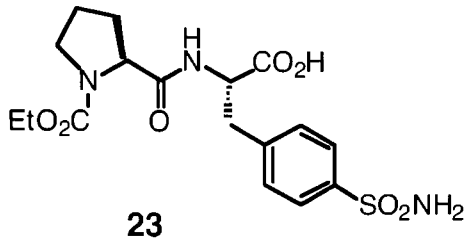
EtO_2C-Gly-(4'-SO_2NH_2)Phe-OH (21). 2 M NaOH (702 µL, 1.40 mmol, 4.0 equiv) was added to a solution of **17** (136 mg, 0.351 mmol, 1.0 equiv) in methanol (8.0 mL) and allowed to stir at 21°C for 12 h. 2 M HCl (702 µL, 1.40 mmol, 4.0 equiv) was added and the methanol was removed in vacuo. The aqueous solution was then acidified to pH 1 with 2 M HCl (1.0 mL) and then extracted with ethyl acetate (3 x 30 mL). The

organic layer was dried over Na_2SO_4, filtered and concetrated in vacuo to afford 129 mg of **21** (99%) as a white solid. ^{1}H NMR ($(CD_3)_2SO$) ∂ 7.93 (d, 1H, J = 8.0 Hz), 7.71 (d, 2H, J = 8.4 Hz), 7.36 (d, 2H, J = 8.0 Hz), 7.15 (s, 2H), 7.0 (s (broad), 1H), 4.52-4.46 (m, 1H), 3.98 (q, 2H, J = 6.4 Hz), 3.63-3.51 (m, 2H), 3.15-3.10 (m, 1H), 3.01-2.96 (dd, 1H, J = 13.8 and 8.6 Hz), 1.17-1.13 (t, 3H, J = 7.0). ^{13}C NMR ($(CD_3)_2SO$) ∂ 173.1, 169.8, 157.2, 143.0, 142.3, 130.3, 126.2, 60.7, 53.9, 43.9, 37.2, 15.5. HR-CIMS (*m/z*): [M^+] calculated for $C_{14}H_{19}N_3O_7S$, 373.0944; found, 373.0949.



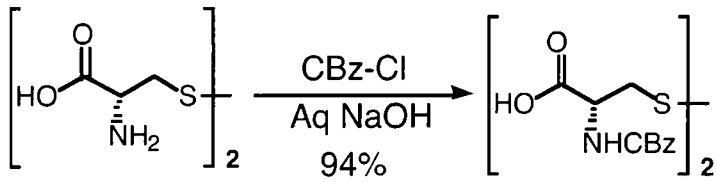
22

EtO_2C-Leu-(4'-SO_2NH_2)Phe-OH (22). Prepared as **21** to afford 727 mg (58%). ^{1}H NMR ($(CD_3)_2SO$) ∂ 8.10 (d, 1H, J = 8.1 Hz), 7.68 (d, 2H, J = 8.4 Hz), 7.37 (d, 2H, J = 7.8 Hz), 7.29 (s, 2H), 7.16 (d, 1H, J = 8.9 Hz), 4.18-4.41 (m, 1H), 3.99-3.92 (m, 3H), 3.14-3.09 (m, 1H), 3.01-2.94 (m, 1H), 1.62-1.52 (m, 1H), 1.40-1.32 (m, 2H), 1.16-1.12 (t, 3H, J = 6.9 Hz), 0.86-0.81 (m, 6H). ^{13}C NMR ($(CD_3)_2SO$) ∂ 173.1, 156.6, 142.9, 142.3, 130.3, 126.1, 60.6, 53.7, 37.0, 24.9, 23.9, 22.3, 15.5. HR-CIMS (*m/z*): [MH^+] calculated for $C_{18}H_{28}N_3O_7S$, 430.1648; found, 430.1654.



23

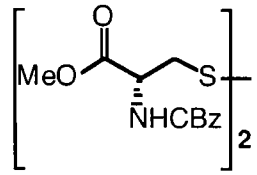
EtO_2C-Pro-(4'-SO_2NH_2)Phe-OH (23). Prepared as **21** to afford 278 mg (91%). ^1H NMR ($(CD_3)_2SO$) ∂ 8.19-8.15 (m, 1H), 7.69 (d, 2H, J = 7.6 Hz), 7.41-7.38 (m, 2H), 7.30 (s, 2H), 4.50-4.40 (m, 1H), 4.18-4.06 (m, 1H), 4.01-3.95 (m, 1H), 3.83-3.79 (q, 2H, J = 6.0 Hz), 3.31-3.24 (m, 2H), 3.17-3.09 (m, 2H), 3.01-2.95 (m, 2H), 2.03-1.98 (m, 1H), 1.71-1.62 (m, 2H), 1.15-1.10 (t, 2H, J = 7.0 Hz). ^{13}C NMR ($(CD_3)_2SO$) ∂ 173.3, 173.1*, 172.7, 172.6*, 154.9*, 154.6, 142.9, 142.7*, 130.4*, 130.1, 126.0, 61.2*, 61.1, 60.3*, 60.0, 53.9*, 53.6, 47.6, 47.2*, 36.9, 31.7, 30.5*, 24.5*, 23.7, 15.5*, 15.3 (* indicates minor rotomer). HR-CIMS (*m/z*): [M^+] calculated for $C_{17}H_{23}N_3O_7S$, 413.1257; found, 413.1266.



24 **25**

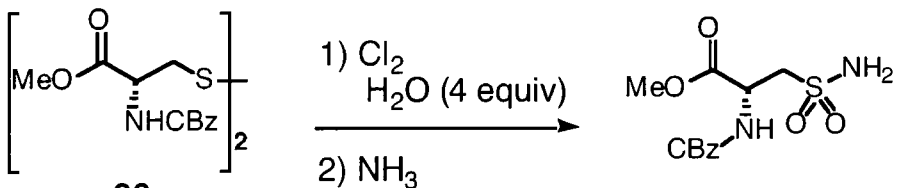
(CBz-Cys-OH)$_2$ (25). Benzoyl chloroformate (12.4 mL, 63.6 mmol, 2.2 equiv) was added to a solution of cystine (6.95 g, 28.9 mmol, 1.0 equiv), in 2 M NaOH (80 mL) at 0°C in 2 mL portion over a period of an hour while stirring vigourously. The pH of the solution was adjusted with 2 M NaOH as needed to maintain pH 12 to litmus. The solution was allowed to stirr for 30 min and the aqueous layer was washed with ether (3 x 100 mL), then the aqueous layer was acidified to pH 1 with 2 M HCl. The solution was stored for 16 h at 4°C and the white solid which formed was isolated by filtration and

lyopholized for 16 h to afford **(25)** (13.2 g, 94%) as a white solid. ^{1}H NMR ($(CD_3)_2SO$) ∂ 7.68 (d, 2H, J = 8.0 Hz), 7.34-7.29 (m, 10H), 5.02 (s, 4H), 4.27-4.25 (m, 2H), 2.92-2.86 (m, 4H).



26

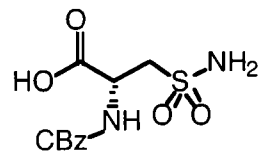
(CBz-Cys-OMe)$_2$ (26). Acetyl chloride (429 µL, 6.03 mmol, 1.0 equiv) was added to a solution of **(25)** (3.07 g, 6.03 mmol, 1.0 equiv) in methanol (80 mL) and the solution was refluxed at 65°C for 13 h. The solution was concentrated in vacuo and the residue taken up in ethyl acetate (100 mL). The organic solution was washed with sat. $NaHCO_3$ solution (3 x 30 mL), brine (1 x 30 mL), and 0.1 M HCl (3 x 30 mL) then dried over Na_2SO_4, filtered, and concentrated in vacuo to afford **26** (2.69 g, 86%). ^{1}H NMR ($CDCl_3$) δ 7.35-7.30 (m, 10H), 5.69 (d, 2H, J = 7.50 Hz), 5.11 (s, 4H), 4.69-4.65 (m, 2H), 3.75 (s, 6H), 3.18-3.15 (m, 4H). ^{13}C NMR ($CDCl_3$) δ 170.9, 155.8, 136.2, 128.7, 128.5, 128.4, 67.5, 53.6, 53.1, 41.5.



26 **28**

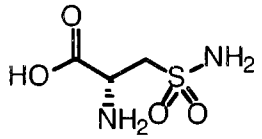
CBz-Cys(SO_2NH_2)-OMe (28). Distilled water (1.21 mL, 67.5 mmol, 4.0 equiv) was added to a solution of **26** (8.82 g, 16.9 mmol, 1.0 equiv) in ethyl acetate (180 mL) and the solution was cooled to –78°C. Chlorine gas (Caution, strong oxidant) was gently bubbled through the solution for 40 min, and the resultant yellow solution was warmed to

21°C. The solution was purged with argon, then concentrated in vacuo to afford a white solid which was dissolved in toluene and concentrated in vacuo to remove residual chlorine. The residue was dissolved in THF (150 mL) and treated with a stream of ammonia at 0°C for 40 min. The solution was concentrated in vacuo and eluted on a column of silica with ethyl acetate to afford **28** (4.93 g, 46%). ^{1}H NMR ($(CD_3)_2SO$) δ 7.86 (d, 1H, J = 7.6 Hz), 7.36-7.29 (m, 5H), 7.03 (s, 2H), 5.04 (s, 2H), 4.56-4.51 (m, 1H), 3.65 (s, 3H), 3.51-3.47 (dd, 1H, J = 14.0 and 4.0 Hz), 3.42-3.36 (dd, 1H, J = 14.2 and 8.6 Hz). ^{13}C NMR ($(CD_3)_2SO$) δ 171.0, 156.2, 137.4, 129.0, 128.5, 128.3, 66.5, 55.7, 53.3, 51.0.



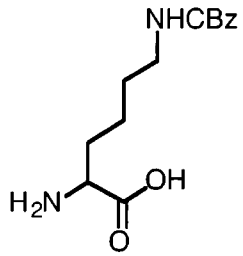
29

CBz-Cys(SO_2NH_2)-OH (29). 2 M HCl (125 mL, 250 mmol, 16 equiv), was added to a suspention of **28** (4.93 g, 15.6 mmol, 1.0 equiv), in 1,4 dioxanes (125 mL) and the solution was heated to 55°C for 16 h. The solution was extracted with ethyl acetate (3 x 100 mL) and the organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo to afford **(29)** (4.71 g, 98%). ^{1}H NMR ($(CD_3)_2SO$) ∂ 7.72 (d, 1H, J = 8.0 Hz), 7.34-7.29 (m, 5H), 6.98 (s, 2H), 5.04 (s, 2H), 4.44-4.20 (m, 1H), 3.51-3.47 (m, 1H), 3.41-3.38 (m, 1H). ^{13}C NMR ($(CD_3)_2SO$) ∂ 172.0, 156.3, 137.4, 129.0, 128.5, 128.3, 66.3, 55.8, 51.0.



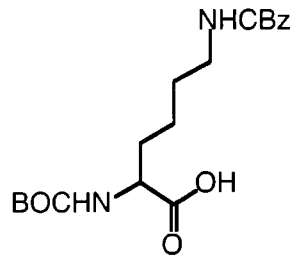
30

H_2N-Cys(SO_2NH_2)-OH (30). Anisole (2.95 mL, 27.1 mmol, 2 equiv) and triflic acid (4.80 mL, 54.2 mmol, 4.0 equiv) were added to a solution of **29** (4.10 g, 13.7 mmol, 1.0 equiv) in methelene chloride (120 mL) to afford a red solution which was stirred at 21°C for for 20 h. The solution was saturated with ether (300 mL) and let stand for 2 h. The resulting oil was isolated and and crystallized from ethyl acetate / hexanes to afford **30** (973 mg, 51%) as an off-white solid.. ^{1}H NMR ($(CD_3)_2SO$) δ 7.32 (s, 2H), 4.34 (s, broad, 1H), 3.55-3.36 (m, 2H). ^{13}C NMR ($(CD_3)_2SO$) δ 169.1, 54.5, 49.3.



H_2N-Lys(N_ε-CBz)-OH. $CuCO_3$ (59.0 g, 267 mmol, 1.2 equiv) was added over a 15 minute period to a solution of lysine hydrochloride (40.6 g, 222 mmol, 1.0 equiv) at reflux in distilled H_2O (400 mL) and allowed to stir at reflux for 1.5 h. The warm solution was filtered through a coarse fluted filter paper and the solids were washed with hot water (4 x 100 mL). 1,4 dioxanes (600 mL) was added to the aqueous solution at 0°C. Benzoyl chloroformate (41.3 mL, 289 mmol, 1.3 equiv) was added to the biphasic solution and allowed to stir for 16 h while the temperature was raised to 21°C. The blue solid that formed was then isolated by vaccum filtration and washed with H_2O (3 x 200

mL) and ether (3x 200mL). Thioacetamide (20.0 g, 267 mmol, 1.2 equiv) was added to a suspention of the blue solid in distilled H_2O (800 mL). 2 M NaOH (40 mL) was added to raise the pH of the aqueous solution to 9.0. The solution immediately became dark brown and was allowed to stirr for 21 h. 2 M HCl (55 mL) was added to lower the pH to 1 and the insoluble materials were removed by filtration. The filtrate was netralized with solid Na_2SO_4 and the precipitate that formed was isolated by filtration, washed with distilled H_2O (3 x 100 mL) and ether (3 x 100 mL) then dried on the funnel to afford the side chain protected lysine (35.1 g, 56%) as a beige solid. ^{1}H NMR (D_2O) ∂ 7.10-7.00 (m, 5H), 4.74 (s, 2H), 3.74-3.69 (t, 1H, J = 6.3 Hz), 2.80-2.75 (t, 2H, J = 6.6 Hz) 1.65-1.54 (m, 2H), 1.24-1.04 (m, 4H). ^{13}C NMR (D_2O) ∂ 171.7, 158.3, 136.4, 128.7, 128.3, 127.6, 66.9, 52.7, 40.0, 29.4, 28.4, 21.5.

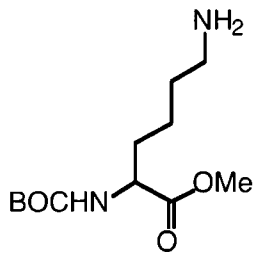


38

***N_α*-BOC-Lys(*N_ε*-CBz)-OH (38).** Di-tert-butylpyrocarbonate (48.5 g, 222 mmol, 1.7 equiv) was added to a solution of H_2N-Lys(*N_ε*-CBz)-OH (35.0 g, 125 mmol, 1.0 equiv) in an equivolume mixture of 2 M NaOH and 1,4 dioxanes (1600 mL) at 0°C and allowed to stir for 19 h while slowly warmed to 21°C. The solution was filtered through coarse filter paper and extracted with ethyl acetate (2 x 400 mL). The aqueous layer was ajusted to pH 7 with 2 M HCl and to pH 1 with 0.5 M aqueous potassium hydrogen sulfate. The solution was then extracted with ethyl acetate (4 x 500 mL) and the organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo to afford **38** (35.0 g,

75%) as a clear brown oil. ^{1}H NMR ($(CD_3)_2SO$) ∂ 7.37-7.20 (m, 5H), 7.24-7.20 (t, 1H, J = 5.6 Hz), 7.00 (d, 1H, J = 8.1 Hz), 5.00 (s, 2H), 3.85-3.79 (m, 1H), 3.00-2.94 (m, 2H), 1.63-1.53 (m, 2H), 1.38 (m, 13H). ^{13}C NMR $(CD_3)_2SO$) ∂ 174.8, 156.7, 156.2, 137.9, 129.0, 128.4, 78.7, 65.9, 54.2, 32.1, 31.2, 29.8, 29.0, 28.8, 23.8.

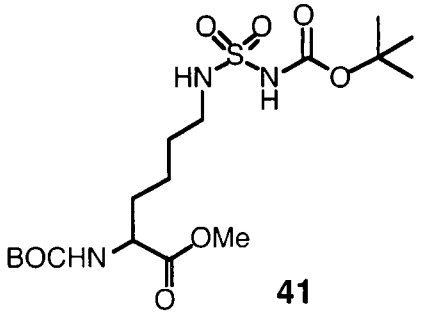
N_α-BOC-Lys(N_ε-CBz)-OMe. Diazomethane (prepared from Diazald), as a solution in ether, (163 mL, ca. 41 mmol, 1.0 equiv) was added to a solution of **38** (17.5 g, 45.9 mmol, 1.1 equiv) in THF (400 mL) in 15 mL portions over 20 min. The solution was allowed to stir at 21°C for 30 min, then concentrated in vacuo. The residue was taken up in ethyl acetate (200 mL) and washed with a sat. aqueous $NaHCO_3$ solution (3 x 100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo to afford the fully protected lysine N_α-BOC-Lys(N_ε-CBz)-OMe (13.6 g. 75%). The aqueous layer was acidified with 2 M HCl and extracted with ethyl acetate (3 x 80 mL), dried over Na_2SO_4, filtered and concentrated in vacuo to afford recovered starting material **38** (3.80 g, 22%). ^{1}H NMR ($(CD_3)_2SO$) ∂ 7.37-7.26 (m, 5H), 7.23-7.18 (m, 2H), 4.99 (s, 2H), 3.91-3.89 (m, 1H), 3.60 (s, 3H), 2.99-2.93 (m, 2H), 1.62-1.53 (m, 2H), 1.44-1.24 (m, 13H). ^{13}C NMR ($CDCl_3$) ∂ 173.4, 156.6, 155.6, 136.7, 128.7, 128.3, 128.3, 80.2, 66.9, 53.5, 52.7, 41.0, 32.7, 29.7, 28.7, 22.8.



39

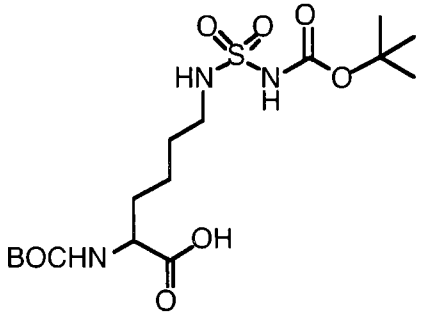
N_α-BOC-Lys($NH_{2\varepsilon}$)-OMe (39). Palladium hydroxide on carbon (20 % by weight), Degussa type ,wet, (2.42 g, 3.45 mmol, 0.10 equiv), was added to a solution of

N_α-BOC-Lys(N_ε-CBz)-OMe in methanol (125 mL) and the mixture was placed under an atmosphere of hydrogen for 4 h. The solution was filtered through a bed of Celite and the bed was washed with methanol (100 mL). The combined methanol solutions were concentrated in vacuo to afford **(39)** (12.2 g, 90%) as a clear oil. ^{1}H NMR ($(CD_3)_2SO$) δ 7.25-7.23 (s, broad, 1H), 3.93-3.85 (m, 1H), 3.60 (s, 3H), 2.50-2.48 (m, 2H), 1.58-1.52 (m, 2H), 1.42-1.32 (m, 13H). ^{13}C NMR ($(CD_3)_2SO$) δ 173.8, 156.2, 78.9, 54.4, 52.5, 41.8, 32.9, 31.4, 29.1, 23.7.

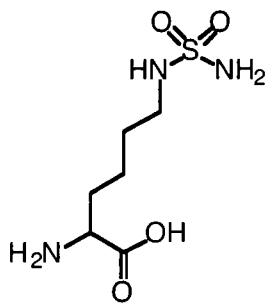


41

N_α-BOC-Lys(N_ε-SO_2NH-BOC)-OMe (41). *N*-(tert-Butoxycarbonyl)-*N*-[4-(dimethylazaniumylidene)-1,4-dihydropyridin-1-ylsulfonyl]azanide **(40)**[1] (10.2 g, 33.8 mmol, 1.1 equiv) was added to a suspention of **39** (7.99 g, 30.7 mmol, 1.0 equiv) in CH_2Cl_2 (125 mL) and allow to stir for 16 h. The solution was concentrated in vacuo and was purified by flash chromatography on silica gel eluting with 1:1 ethyl acetate / hexanes to afford **41** (6.64 g, 49 %) as a clear oil and recoverd starting material **39** (2.93 g, 37 %). ^{1}H NMR ($CDCl_3$) δ 7.64 (s, 1H), 5.31-5.27 (t, 1H, J = 6.0 Hz), 5.10 (d, 1H, J = 8.4 Hz), 4.33-4.26 (m, 1H), 3.73 (s, 3H), 3.09-3.02 (m, 2H), 1.83-1.75 (m, 2H), 1.70-1.55 (m, 4H) 1.52-1.13 (m, 18H). ^{13}C NMR ($CDCl_3$) δ 173.3, 155.6, 150.5, 83.9, 80.3, 53.4, 52.7, 43.8, 32.6, 28.7, 28.4, 22.7, 14.6. HR-CIMS (*m/z*): [MH^+] calculated for $C_{17}H_{34}N_3O_8S$, 440.2066; found 440.2069.



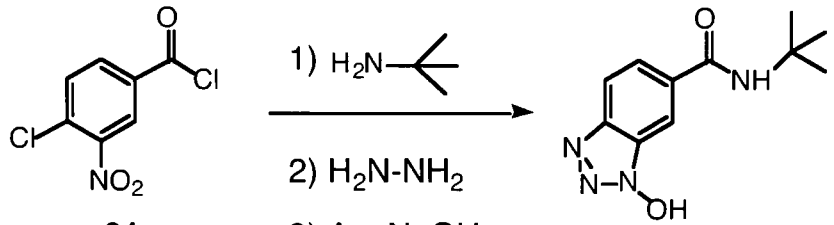
N_α-BOC-Lys(N_ε-SO_2NH-BOC)-OH. 2 M NaOH (44.4 mL, 88.7 mmol, 6.0 equiv), was added to a solution of **(41)** (6.50 g, 14.8 mmol, 1.0 equiv) in methanol (150 mL) and was allowed to stir for 16 h. 2 M HCl (44.4 mL) was added to neutralize the solution which was concentrated in vacuo to ca. 30 mL. The remaining aqueous solution was diluted with ethyl acetate (100 mL) and 0.5 M HCl (20 mL) and extracted with ethyl acetate (3 x 100 mL). The organic layers were dried over Na_2SO_4, filtered and concentrated in vacuo to afford N_α-BOC-Lys(N_ε-SO_2NH-BOC)-OH (5.56 g, 88%) as a clear oil. ^1H NMR ($(CD_3)_2SO$) ∂ 12.30 (s, 1H), 10.74 (s, 1H), 7.53-7.49 (t, 1H, J = 6.0 Hz), 7.02 (d, 1H, J = 7.8 Hz), 3.80-3.76 (m, 1H), 2.86-2.80 (m, 2H), 1.63-1.48 (m, 2H), 1.42-1.31 (m, 22 H). ^{13}C NMR ($(CD_3)_2SO$) ∂ 174.8, 156.2, 151.2, 81.8, 78.7, 54.2, 43.3, 32.2, 31.1, 29.1, 28.6, 23.7.



42

H_2N-Lys(N_ε-SO_2NH_2)-OH (42). TFA (20 mL) was added to a solution of N_α-BOC-Lys(N_ε-SO_2NH-BOC)-OH (5.56 g, 13.1 mmol, 1.0 equiv) in DCM (60 mL) and

was stirred under an atmosphere of argon for 3.0 h. The solution was concentrated in vacuo and the residue was recrystallized from methanol / ether to afford **(42)** (1.84 g, 58%) as a white solid. ^{1}H NMR ($(CD_3)_2SO$) ∂ 8.30-7.95 (s, broad, 1H), 6.46 (s, 2H), 3.77 (s, broad, 1H), 2.85-2.81 (m, 2H), 1.82-1.70 (m, 2H), 1.51-1.25 (m, 4H). ^{13}C NMR ($(CD_3)_2SO$) ∂ 171.7, 53.0, 43.0, 30.7, 29.3, 22.7. HR-CIMS (*m/z*): [MH^+] calculated for $C_6H_{16}N_3O_4S$, 226.0861; found 226.0858.

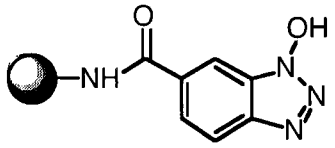


61 **64**

7-*N*-*tert*-butylacetamido-1-hydroxybenzotriazole (64). *tert*-Butylamine (0.467 mL, 4.44 mmol, 1.1 equiv) was added to a solution of **61** (888 mg, 4.04 mmol, 1.0 equiv) and pyridine (0.358 mL, 4.44 mmol, 1.1 equiv) in CH_2Cl_2 (25 mL) and allowed to stir at 21°C for 16 h. The solution was concentrated in vacuo and the residue was taken up in ethyl acetate (20 mL) and washed with saturated $NaHCO_3$ solution (3 x 10 mL) and the washed with 0.1 M HCl (3 x 10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo to afford amide **62** (910 mg, 89 %).

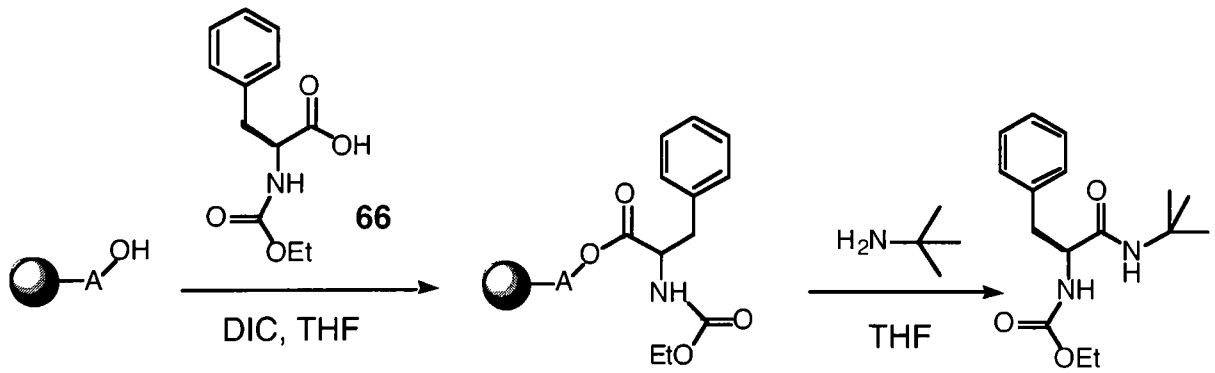
Amide **62** (383 mg, 1.49 mmol, 1.0 equiv) was dissolved in anhydrous hydrazine (5 mL) and stirred at 21°C for 4 h. The solution was concentrated in vacuo and the residue was dissolved in 2 M NaOH (10 mL) and stirred for 16 h. The solution was washed with ethyl acetate (1 x 15 mL) then acidified to pH 1 with 2M HCl (25 mL). The solution was extracted with ethyl acetate (3 x 20 mL) and the organic layer was dried

over Na_2SO_4, filtered and concnetrated in vacuo to afford **64** (329 mg, 94 %). ^1H NMR (CD_3OD) ∂ 8.13 (s, 1H) 7.94-7.92 (m, 1H), 7.85-7.82 (m, 1H) 1.49 (s, 9H).



65

HOBt attached to amino TentaGel (65). Amino-functionalied Tentagel (1.0 g, 0.44 mmol, 1.0 equiv) was swollen with CH_2Cl_2 (12 ml) and pyridine (364 μL, 4.40 mmol, 10 equiv). 4-chloro-3-nitrobenzoylchloride (968 mg, 4.40 mmol, 10 equiv) was added and the mixture was gently shaken for 16 h. The resin was washed with CH_2Cl_2 (3 x 30 mL), DMF (3 x 30 mL), and CH_2Cl_2 (3 x 30 mL). The resin was swollen with a solution of anhydrous hydrazine (4.0 mL, 127 mmol, 290 equiv) in DMF (12 mL) and shaken for 16 h. The resin was washed with DMF (4 x 30 mL) and H_2O (3 x 30 mL). The resin was then swollen with 1 M NaOH (30 mL) and shaken for 20 h. The resin washed with H_2O (2 x 30 mL), 1 M NaOH (2 x 30 mL), H_2O (2 x 30 mL), DMF (1 x 30 mL) and ether (1 x 30 mL).

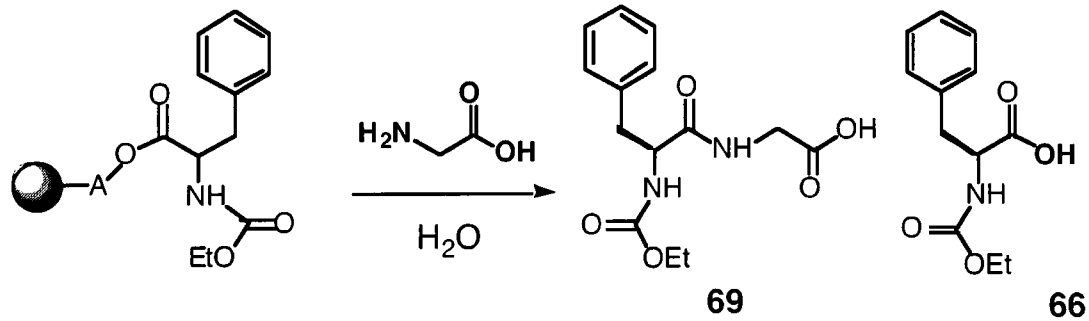


68

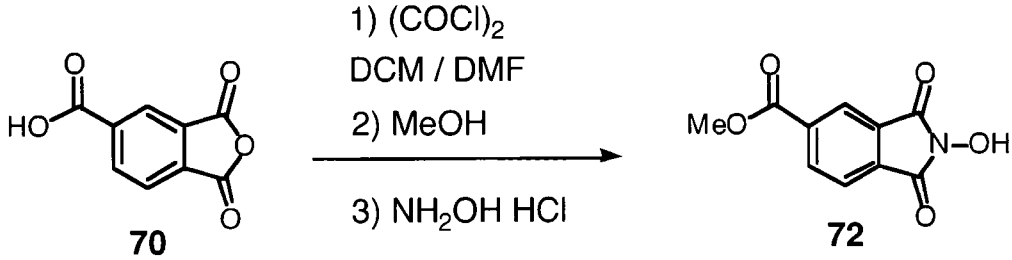
General method for quantification of all resins used to form active esters. The resin in question (0.44 mmol/g maximum for amino-functionalized TentaGel, 1.0

equiv) was swollen in 10 mL of distilled THF and **66** was added as a 0.5 M THF solution (8.8 mL, 4.40 mmol, 10 equiv) along with DIC (689 μL, 4.40 mmol, 10 equiv) and the mixture was gently shaken for 16 h. The resin was then washed with THF (2 x 30 mL), DMF (2 x 30 mL) and THF (1 x 30 mL). The resin was then swollen with 30 mL of THF and shaken for 3 h (longer time periodes were used for larger batches of resin). The resin was washed again with THF (2 x 30 mL), DMF (2 x 30 mL) and THF (1 x 30 mL) and dried in vacuo.

The resin was swollen in THF (10 mL) and *tert*-butyl amine (925 μL, 8.80 mmol, 20 equiv) was added. The mixture was shaken for 16 h then washed with THF (3 x 30 mL). The solvent was concentrated in vacuo and the residue was taken up in ethyl acetate (10 mL) and washed with 0.1 M HCl (2 x 10 mL) and sat. $NaHCO_3$ solution (2 x 10 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford **68** (99 mg, 77 %) as a foamy white solid. ^{1}H NMR ($(CD_3)_2SO$) ∂ 7.44 (s, 1H), 7.24-7.15 (m, 5H), 7.04 (d, 1H, J = 9.0 Hz), 4.18-4.10 (m, 1H), 3.91-3.85 (m, 2H), 2.88-2.82 (dd, 1H, J = 4.8 and 13.5 Hz), 2.74-2.66 (m, 1H), 1.24-1.20 (s, 9H), 1.08 (t, 2H, J = 6.9 Hz). ^{13}C NMR ($(CD_3)_2SO$) ∂ 171.3, 156.4, 138.7, 130.0, 128.6, 126.8, 60.4, 56.9, 50.9, 31.3, 29.2, 15.4.



General method for all couplings in aqueous media. An active ester on solid support was prepared as in the general method described above (e.g. 0.22 mmol of active ester, 1.0 equiv). The resin was swollen with a solution of glycine (75.1 mg, 1.0 mmol, 4.5 equiv) in 10 mL of 50 mM aqueous biscine for pH 8.0-9.0 or 50 mM aqueous Na_2CO_3 for pH 10.0 which was adjusted to the appropriate pH with 2 M KOH. The mixture was shaken for 16 h and the resin was washed with H_2O (3 x 50 mL). The aqueous solution was acidified to pH 1 with 2 M HCl (15 mL) and extracted with ethyl acetate (3 x 50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was dissolved in 2 mL of methanol and the amount of each product was measured by HPLC using a Phenomenex C-8 column eluting with 30/70/0.1 MeOH/H_2O/TFA at 0.80 mL/min for 15 min and then 50/50/0.1 at 0.80 mL/min for 40 min. The peak areas monitored were **69**: 25.9 min, **66**: 32.6 min, all at 220 nm.

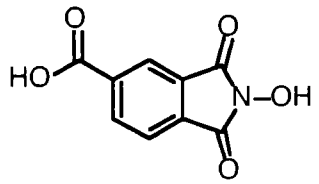


70 **72**

4-Carbomethoxy-*N*-hydroxyphthalimide (72), Oxallyl chloride (1.15 mL, 13.2 mmol, 1.25 equiv) was added to a solution of **70** (2.18 g, 10.5 mmol, 1.0 equiv) in a 1:1 mixture of CH_2Cl_2 and DMF (16 mL) at 0°C. The solution was warmed slowly to 21°C while stirring under an atmosphere of argon for 16 h. The solution was concentrated in vacuo and the residue was dissolved in 25 mL of THF and cooled to 0°C. Methanol (469 μL, 11.6 mmol, 1.10 equiv) was added and the solution was warmed slowly to 21°C while stirring for 16 h. The solution was concnetrated in vacuo and the residue was taken

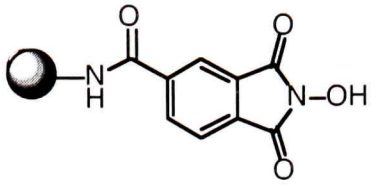
up in ethyl acetate (20 mL) and washed with a saturated $NaHCO_3$ solution (3 x 10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo to afford **71**.

Hydroxylamine hydrochloride (1.09 g, 15.7 mmol, 1.5 equiv) was added to a solution of **71** in pyridine (60 mL) and stirred for 2 h at 21°C then heated to 85°C for 18 h. The solution was concnetrated in vacuo and the residue was taken up in ethyl acetate (30 mL) and washed with 0.1 M HCl (3 x 10 mL). The solution was concnetrated in vacuo and the residue was purified by silica gel chromatogrphy, eluting with ethyl acetate to afford **72** (1.11 g, 48 % over 2 steps). ^{1}H NMR (CD_3OD) ∂ 8.45-8.42 (dd, 1H, J = 1.8 and J = 7.8 Hz), 8.37-8.36 (m, 1H), 7.94 (d, 1H, J = 7.8 Hz), 4.89 (s, 3H). ^{13}C NMR (CD_3OD) ∂ 165.3, 163.9, 135.8, 135.4, 133.0, 129.8, 123.4, 123.1, 52.1.



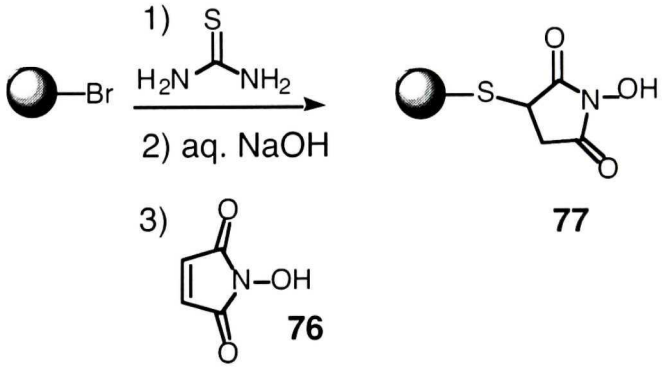
73

4-Carboxy-*N*-hydroxyphthalimide (73). 2 M NaOH (10 mL, 10 mmol, 4.0 equiv) was added to a solution of **72** (565 mg, 2.51 mmol, 1.0 equiv) in H_2O (10 mL) and stirred for 10 h. The solution was acidified to pH 1 with 2 M HCl (20 mL) and washed with ethyl acetate (3 x 30 mL). The organic layer was dried over Na_2SO_4 and concnetrated in vacuo to afford **73** (415 mg, 80 %). ^{1}H NMR ($(CD_3)_2SO$) ∂ 8.82 (d, 1H, J = 1.5 Hz), 8.10-8.07 (dd, 1H, J = 8.0 and 1.6 Hz), 7.73-7.70 (dd, 1H, J = 8.0 and 1.6 Hz). ^{13}C NMR ($(CD_3)_2SO$) ∂ 169.1, 168.1, 166.7, 138.1, 133.1, 132.8, 132.5, 130.0, 129.3.



74

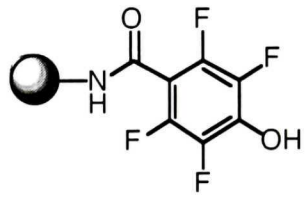
***N*-Hydroxyphthalimide attached to amino TentaGel (74).** Amino-functionalied Tentagel (646 mg, 0.284 mmol, 1.0 equiv) was swollen with THF (5 ml). **73** (200 mg, 0.966 mmol, 3.4 equiv) and DIC (156 μL, 0.994 mmol, 3.50 equiv) were added and the mixture was shaken for 20 h. The resin was washed with THF (2 x 30 mL), DMF (2 x 30 mL) and THF (1 x 30 mL). The loading of **73** units on the resin was quantified as described above in the general method for quantification of all resins used to form active esters to afford **68** (34.6 mg, 41 %). Repeated attempts at coupling in aqueous media as described above in the general method for all couplings in aqueous media afford none of the desired **69**.



HOSu attached to amino TentaGel (77). Bromo NovaSyn TentaGel (904 mg, 0.244 mmol, 1.0 equiv) was swollen with a solution of thiourea (186 mg, 2.44 mmol, 10 equiv) in ethanol (6 mL). The mixture was shaken vigourously at 60°C for 20 h. The resin was washed with ethanol (2 x 30 mL), THF (2 x 30 mL) and H_2O (2 x 30 mL) then

dried on a coarse frit. The resin was then suspended in a solution of 15 % aqueous NaOH at 95°C for 16 h, with occaisional shaking. The resin was washed with 2 M NaOH (2 x 30 mL), H_2O (2 x 30 mL) and 0.1 M HCl (2 x 30 mL). *N*-Hydroxymaleimide (138 mg, 1.22 mmol, 5.0 equiv) and pyridine (197 μL, 2.44 mmol, 10.0 equiv) were added to a suspention of the resin in DMF (6 mL) and shaken for 16 h. The mixture was then shaken at 55°C for 4.5 h, allowed to cool, and then the resin was washed with DMF (2 x 30 mL), H_2O (2 x 30 mL), methanol (2 x 30 mL) DMF (2 x 30 mL) and methanol (2 x 30 mL).

The loading of HOSu units on the resin was quantified as described above in the general method for quantification of all resins used to form active esters to afford **68** (17 mg, 24 %). Coupling in aqueous media at pH 10 as described above in the general method for all couplings in aqueous media afforded **69** (51 %) and **66** (14 %).

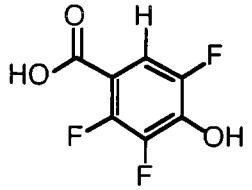


78

Tetrafluorophenol attached to amino TentaGel (78). 2,3,5,6-Tetrafluoro-4-hydroxy-benzoic acid **(59)** (925 mg, 4.40 mmol, 5.0 equiv) was added to amino-functionalied Tentagel (2.0 g, 0.88 mmol, 1.0 equiv) suspended in THF (30 mL). DIC (690 μL 4.40 mmol, 5 equiv) and pyridine (1.52 mL, 17.6 mmol, 20 equiv) were added and the mixture was shaken for 16 h. The resin was then washed with THF (2 x 50 mL) DMF (2 x 50 mL) and THF (2 x 50 mL). The resin was then swollen in 30 mL of THF

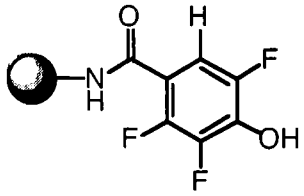
and shaken for 2 h when the acylation procedure described above was repeated. The resin was then swollen with THF (30 mL) and *tert*-butylamine was added (1.85 mL, 17.6 mmol, 20.0 equiv) and the mixture was shaken for 16 h.

The loading of tetrafluorophenol units on the resin was quantified as described above in the general method for quantification of all resins used to form active esters to afford **68** (188 mg, 73 %). Coupling in aqueous media at pH 10, as described above in the general method for all couplings in aqueous media, afforded **69** (62 %) and **66** (3 %).



80

4-Hydroxy-2,3,5-trifluorobenzoic acid (80). 2,3,4,5,-Tetrafluorobenzoic acid (**79**), (1.48 g, 7.63 mmol, 1.0 equiv) was diisolved in 2 M NaOH (75 mL) and heated to relux (100°C) for 16 h. The solution was cooled and $CaCl_2$ (3.36 g, 22.9 mmol, 3.0 equiv) was added and the solution was stirred for 2 h. The solution was acidified to pH 1 with 2 M HCl (100 mL) and extracted with ethyl acetate (4 x 100 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford **80** (1.09 g 78 %). The spectroscopic data was consistant with that found in the literature[2] ^{1}H NMR ($(CD_3)_2SO$) ∂ 7.47-7.42 (ddd, 1H, J = 11.2, 6.6 and 1.8 Hz).



81

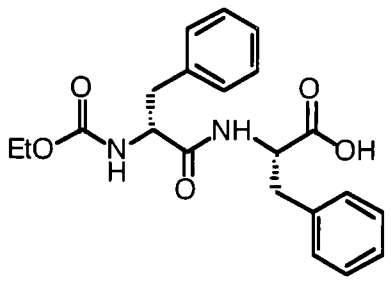
Trifluorophenol attached to amino TentaGel (81). Amino TentaGel resin (210 mg, 0.0924 mmol, 1.0 equiv) was swollen with a solution of **80** (120 mg, 0.577 mmol, 6.2 equiv) in THF (5 mL). DIC (90 μL, 0.577 mmol, 6.2 equiv) was added and the mixture was gently shaken for 18 h. The resin was washed with THF (2 x 30 mL), DMF (2 x 30 mL), THF (1 x 30 mL) then the resin was then resbmitted to the coupling procedure as described above. The resin was swollen with 20 ml of THF and *tert*-butylamine (1.0 mL, 9.20 mmol, 100 equiv) was added and the mixture was shaken for 16 h. The resin was washed with THF (4 x 30 mL).

The loading of triflorouphenol units on the resin was quantified as described above in the general method for quantification of all resins used to form active esters to afford **68** (28 mg, >95 %). Coupling in aqueous media at pH 10, as described above in the general method for all couplings in aqueous media, afforded **69** (63 %) and **66** (13 %).

Couplings at various pHs and concentrations were carried out as described above in general method for all couplings in aqueous media. Couplings to generate static libraries of **66**, **69**, and **86-88**,were carried out in the same manner with analysis by HPLC using a Phenomenex C-8 column eluting with 30/70/0.1 MeOH/H_2O/TFA at 0.80 mL/min for 15 min and then 50/50/0.1 at 0.80 mL/min for 70 min. The peak areas

monitored were **69**: 25.9 min, **66**: 32.6 min, **87**: 35.6 min, **88**: 59.1 min, **86**: 63.2 min,. all at 220 nm.

The static libraries of **20-23** were prepared in a manner analogous to those already described with analysis by HPLC using a Phenomenex C-8 column eluting with 20/80/0.1 MeOH/H_2O/TFA to 100/0/0.1 MeOH/H_2O/TFA over 120 min at 0.8 mL/min. The peak areas monitored were : **21**: 11.3 min, **23**: 18.5 min, **22**: 39.8 min, **20**: 43.5 min all at 220 nm.



91

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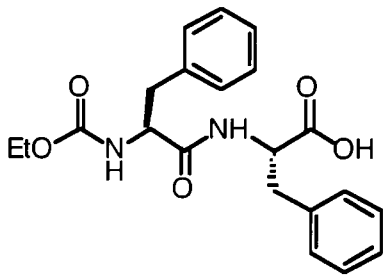

86

Racemization study. A coupling was carried as previously described with the active ester of EtOC-phenylalanine **(66)** and phenylalanine. The resultant mixture was analyzed by HPLC using a Phenomenex C-8 column eluting with 50/50/0.1 MeOH/H_2O/TFA at 0.80 mL/min for 90 min. The peak areas monitored were **91**: 47.9 min, **86**: 63.5 min, all at 220 nm. A mixture of the two diastereomers (**91** and **86**) was prepared from EtOC-(dl)-phenylalanine by conventional solution-phase peptide chemistry for the purposes of establishing separtion conditions.

Test couplings with nucleophiles 4, 30, and 38. All couplings were carried out at pH 9.0 as described in the general method for all couplings in aqueous media and analyzed by HPLC using a Phenomenex C-8 column eluting with 30/70/0.1 MeOH/H_2O/TFA for 5 min and then a steady gradient to 100/0/0.1 over 120 min all at

0.80 mL/min. The peak areas monitored were **92**: 29.0 min, **93**: 31.2 min, **66**: 42.6 min all at 220 nm.. Analysis for the product from the coupling with **4** was carried as described above in the static library synthesis.

The static screening experiment whose results are illustrated in Figure 4-3 was carried as described in the literature.[3]

General procedure for first-generation DCLs (whose results are illustrated in Figures 4-5, 4-6 and 4-7). A 500 mL stock solution was prepared with phenylalanine, glycine, leucine and proline (2.50 mmol of each amino acid), Bicine (15 mmol) and penicllin (0.05 g). The pH of the solution was adjusted to 9.0 with 1 M NaOH.

CA (840 mg, 28 μmol, 1.0 equiv) was dissolved in 20 mL of the stock solution and placed in a 100 mL plastic container. Pronase (500 mg, ca. 24 μmol, ca. 0.86 equiv) was dissolved in 20 mL of the stock solution and placed in a dialysis bag (molecular weight cut-off 1 kDa) and immersed in the container.

Active esters of **66** and **5** were prepared as described above and equal amounts of each were used in each resin portion added to the library. Portions of the resin (900 mg, ca. 0.28 mmol, 10 equiv, for 4-5 and 4-6, 450 mg, ca. 0.14 mmol, 5.0 equiv, for 4-6) were swollen in 10 mL of the stock solution and placed in a dialysis bag (molecular weight cut-off 12 kDa) and immersed in the container. The container was shaken on an orbital shaker at 60 rpm and the resin replaced at 24 h intervals for figures 4-5 and 4-7 and 12 h intervals for 4-6, with the solution in the dialysis bag recovered and used to swell the new resin.

Samples from the CA solution (200 μL) were taken at the intervals indicated in the graphs. These samples were heated to 80°C for 5 min, centrifuged for 10 min and

filtered through a 0.22 μm sterile filter. These samples were analyzed by HPLC using a Phenomenex C-8 column eluting at 0.8 mL/min with 0/100/0.1 to 30/70/0.1 MeOH/H_2O/TFA over 30 min, from 30/70/0.1 to 37/63/0.1 MeOH/H_2O/TFA over 40 min, then from 37/63/0.1 to 62/38/0.1 MeOH/H_2O/TFA over 20 min. The peaks monitored were **12**: 25.2 min (6.64×10^{14} Abs/mol), **13**: 57.4 min (7.82×10^{14} Abs/mol), **11**: 58.6 min (1.03×10^{15} abs/mol), all at 220 nm.

In the experiment whose results were illustrated in Figure 4-7, the dialysis bag containing Pronase was added six hours after the experiment was started. It was removed at the 24 h mark and a new dialysis bag containing Pronase was inserted at the 30 h mark.

General procedure for second-generation DCLs (whose results are illustrated in Figures 4-9, 4-10, 4-11, 4-12, 4-13 and 4-14). For the first of the second-generation experiments (Figure 4-9): A 50 mL stock solution was prepared with **4** and phenylalanine (181 μmol, 6.5 equiv, of each amino acid), Bicine (1.5 mmol) and penicllin (5 mg). The pH of the solution was adjusted to 9.0 with 1 M NaOH.

CA (840 mg, 28 μmol, 1.0 equiv) was dissolved in 20 mL of the stock solution and placed in a 100 mL plastic container. Pronase (500 mg, ca. 24 μmol, 0.86 equiv) was dissolved in 20 mL of the stock solution and placed in a dialysis bag (molecular weight cut-off 1 kDa) and immersed in the container.

Active esters of EtOC-Phe-OH, EtOC-Gly-OH, EtOC-Leu-OH, and EtOC-Pro-OH were prepared as described above and equal amounts of each were used in each individual resin portion added to the library. Portions of the resin (500 mg, ca. 90 μmol, ca. 3.2 equiv) were swollen in 10 mL of the stock solution and placed in a dialysis bag (molecular weight cut-off 12 kDa) and immersed in the container. The container was

shaken on an orbital shaker for 48 h and the resin replaced at 12 h intervals, with the solution in the dialysis bag recovered and used to swell the new resin.

Samples from the CA solution (200 μL) were taken at the intervals indicated in the graphs. These samples were heated to 80°C for 5 min, centrifuged for 10 min and filtered through a 0.22 μm sterile filter. These samples were analyzed by HPLC using a Phenomenex C-8 column eluting with 20/80/0.1 to 100/0/0.1 MeOH/H_2O/TFA over 120 min at 0.8 mL/min. The peaks monitored were **21**: 11.3 min (9.87×10^{12} abs/mol), **23**: 18.5 min (7.27×10^{12} abs/mol), **22**: 39.8 min (1.10×10^{13} abs/mol), **20**: 43.5 min (1.47×10^{13} abs/mol).

The experiment whose results are illustarted in Figure 4-10 was carrried as described except 270 μmol, 9.6 equiv of each nucleophile was used and the resin was replaced at 8 h intervals. The experiment was carried out for 48 h.

The experiment whose results are illustarted in Figure 4-11 was carrried as described except 270 μmol, 9.6 equiv of each nucleophile was used and the resin was replaced at 16 h intervals. The experiment was carried out for 100 h.

The experiment whose results are illustarted in Figure 4-12 was carrried as described except 100 μmol, 3.6 equiv of each nucleophile was used and the resin was replaced at 16 h intervals. The experiment was carried out for 100 h.

The experiment whose results are illustarted in Figure 4-13 was carried out as described except 100 μmol, 3.6 equiv of each nucleophile was used and the resin was replaced at 16 h intervals. The experiment was caried out for 165 h.

The experiment whose results are illustarted in Figure 4-14 was carried out as described except 100 μmol, 3.6 equiv of each nucleophile was used and 277 μmol, 9.9

equiv portions of the resin were used and it was replaced at 16 h intervals. The experiment was carried out for 240 h.

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[3] Cheeseman, J. D.; Corbett, A. D.; Shu, R.; Croteau, J.; Gleason, J. L.; Kazlauskas, R. J.; *J. Am. Chem. Soc.* **2002**, *124*, 5692-5701.

APPENDIX ONE

J|A|C|S
ARTICLES
Published on Web 04/26/2002

Amplification of Screening Sensitivity through Selective Destruction: Theory and Screening of a Library of Carbonic Anhydrase Inhibitors

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Received September 17, 2001

Abstract: A new method for identifying enzyme inhibitors is to conduct their synthesis in the presence of the targeted enzyme. Good inhibitors form in larger amounts than poorer ones because the binding either speeds up synthesis (target-accelerated synthesis) or shifts the synthesis equilibrium (dynamic combinatorial libraries). Several groups have successfully demonstrated this approach with simple systems, but application to larger libraries is challenging because of the need to accurately measure the amount of each inhibitor. In this report, we dramatically simplify this analysis by adding a reaction that destroys the unbound inhibitors. This works similar to a kinetic resolution, with the best inhibitor being the last one remaining. We demonstrate this method for a static library of several sulfonamide inhibitors of carbonic anhydrase. Four sulfonamide-containing dipeptides, EtOC-Phe$_{sa}$-Phe (**4a**), EtOC-Phe$_{sa}$-Gly (**4b**), EtOC-Phe$_{sa}$-Leu (**4c**) and EtOC-Phe$_{sa}$-Pro (**4d**), were prepared and their inhibition constants measured. These inhibitors migrated to the carbonic anhydrase compartment of a two-compartment vessel. Although higher concentrations of the better inhibitors were observed in the carbonic anhydrase compartment, the concentration differences were small (1.83:1.71:1.54:1.46:1 for **4a:4b:4c:4d:5**, where **5** is a noninhibiting dipeptide EtOC-Phe-Phe). Addition of a protease rapidly cleaved the weaker inhibitors (**4d** and **5**). Intermediate inhibitor **4c** was cleaved at a slower rate, and at the end of the reaction, only **4a** and **4b** remained. In a separate experiment, the ratio of **4a** to **4b** was found to increase over time to a final ratio of nearly 4:1. This is greater than the ratio of their inhibition constants (approximately 2:1). The theoretical model predicts that these ratios would increase even further as the destruction proceeds. This removal of poorer inhibitors simplifies identification of the best inhibitor in a complex mixture.

Introduction

Synthesis using combinatorial chemistry allows testing of hundreds of thousands of drug candidates using high throughput screening techniques. Although this rapid pace has revolutionized drug development, the search for faster and more efficient testing methods continues. One promising method is in situ screening of mixtures such as in dynamic combinatorial libraries.[1] Dynamic combinatorial libraries are equilibrating mixtures of organic molecules. Equilibration in the presence of a therapeutic target increases the equilibrium amounts of those library members that bind tightly to that target. The difference in library composition with and without a stoichiometric amount of target identifies the best inhibitors.

Dynamic libraries are still in the developmental stage, and only a few examples have been reported.[2] For example, Ramström and Lehn[3] created a dynamic library of 10 disaccharides by disulfide exchange starting from a mixture of monosaccharide thiols. The library was screened against concanavalin A, which binds mannose-rich oligosaccharides. A mannoside homodimer was the strongest binder in the library. When the disulfide exchange was carried out in the presence of concanavalin A, the amount of mannoside homodimer present increased by 40%. This increase in the amount of mannoside homodimer identifies it as the best-binding disaccharide. Analysis of a larger 21-member library was more difficult because HPLC did not resolve each member. Nevertheless, the mannoside homodimer was clearly favored in this library as well.

To make a real impact on drug discovery, methods must be developed to screen dynamic combinatorial libraries with thousands of members. This screening is complicated because

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(2) For examples aimed towards biological targets, see: (a) Huc, I.; Lehn, J.-M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2106−2110. (b) Nicolaou, K. C.; Hughes, R.; Cho, S. Y.; Winssinger, N.; Smethurst, C.; Labischinski, H.; Endermann, R. *Angew. Chem., Int. Ed.* **2000**, *39*, 3823−3828. (c) Karan, C.; Miller, B. L. *J. Am. Chem. Soc.* **2001**, *123*, 7455−7456. (d) Bunyapaiboonsri, T.; Ramström, O.; Lohmann, S.; Lehn, J.-M.; Peng, L.; Goeldner, M. *ChemBioChem* **2001**, *2*, 438−444. (e) Nguyen, R.; Huc, I. *Angew. Chem., Int. Ed.* **2001**, *40*, 1774−1776.

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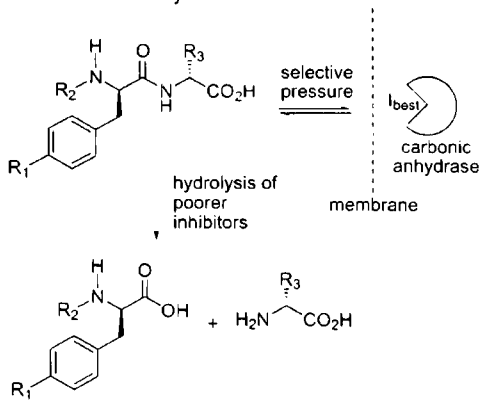
10.1021/ja017099+ CCC: $22.00

Scheme 1. Aryl Sulfonamide-Based Dipeptide Libraries as Inhibitors of Carbonic Anhydrase[a]



[a] Strong binding inhibitors will be bound to carbonic anhydrase and protected. Weaker inhibitors will be hydrolyzed by a protease.

it is often difficult to measure the concentration of each library member in the absence and presence of a target. Further, the libraries will likely contain not one but many good inhibitors because many library members have similar structures and thus similar binding constants. In these cases, adding the target increases the concentration of many library members, rather than a single member, and makes analysis very difficult or impossible. Eliseev and Nelen[4] estimated that a dynamic library combined with an affinity column containing the target would yield one major compound (>50%) only if K_{strong}/K_{weak} was at least n, where n is the number of members of the library. Thus, for one member to predominate in a library of 1000 members, that member would have to bind >1000 times stronger than the others, an unlikely possibility. This inability to distinguish between inhibitors of similar binding constants is a major limitation of the current dynamic combinatorial libraries.

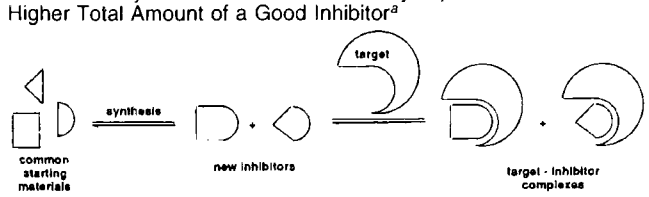
In this report, we propose a screening method that enhances the ability to detect the best inhibitor in a mixture of similar inhibitors. The key to the method is an irreversible destruction reaction that destroys the unbound and weakly bound inhibitors, similar to a kinetic resolution. The best inhibitor is the last one remaining. We demonstrate that this method works for a static library and discuss its potential application to a dynamic system.

Our library targets carbonic anhydrase and consists of dipeptides with an N-terminal 4′-sulfonamidophenylalanine (**1**, Phe_{sa}).[5] These dipeptides can either bind to carbonic anhydrase or be cleaved by a protease, Scheme 1. This cleavage increases the ratio of the strongest binder relative to weaker binders. Importantly, the ratio may increase to values significantly greater than the ratio of the binding constants, thus overcoming the limitation identified by Eliseev and Nelen and making it easy to identify the best inhibitor in the mixture.

Theory

Finding the Best Inhibitor by Shifting the Equilibrium To Make More of the Better Inhibitors. Most dynamic combinatorial library experiments contain two equilibria: an equilibrium for the synthesis of inhibitors and an equilibrium for binding of the inhibitors to the target, Scheme 2. The binding equilibrium removes the good inhibitors from the synthesis equilibrium, and to reestablish equilibrium, the synthesis produces more of the good inhibitors than it would in the absence of target. First, we show that the ratio of good inhibitor to a poor inhibitor depends linearly on the ratio of the binding constants.

Scheme 2. Dynamic Combinatorial Library Equilibria Yield a Higher Total Amount of a Good Inhibitor[a]



[a] Binding of the inhibitor to a target removes it from the synthesis equilibrium so that the synthesis produces more of the good inhibitor.

Consider a common starting material, SM, in equilibrium with two inhibitors, A and B, which can each bind reversibly to a target, T, to form complexes T·A and T·B

$$\text{SM} \underset{}{\overset{K_{sA}}{\rightleftharpoons}} A + T \overset{K_{aA}}{\rightleftharpoons} T\cdot A \qquad (1)$$

$$\text{SM} \overset{K_{sB}}{\rightleftharpoons} B + T \overset{K_{aB}}{\rightleftharpoons} T\cdot B \qquad (2)$$

If $[A_T]$ is the total of bound and unbound forms of A ($[A_T] = [A] + [T\cdot A]$), it can be shown that under typical conditions (e.g., tight binding and an excess of target at a high concentration), the equilibrium ratio of the total amounts of the two inhibitors, $[A_T]$ and $[B_T]$, depends linearly on their relative association constants (eq 3, see Supporting Information for a derivation).[6]

$$\frac{[A_T]}{[B_T]} = \frac{K_{sA} \times K_{aA}}{K_{sB} \times K_{aB}} \qquad (3)$$

For the ideal case where there is one good inhibitor in a pool of noninhibitors, these equilibria indeed will yield the good inhibitor. For example, assuming the rates of synthesis are equal, a mixture of two inhibitors differing in their inhibition constants by a factor of 10 will give a 1:1 mixture in the absence of target (50 mol % of better inhibitor) but gives a 1:10 mixture in the presence of target (91 mol % of better inhibitor). Similarly, a mixture of 1000 inhibitors would yield 0.1 mol % of each inhibitor in the absence of target, but in the presence of target, the poorer inhibitors would decrease to 0.09 mol % each, while the one good inhibitor would increase to 0.9 mol %. This very simple example is already a difficult analysis problem. The more common situation where many inhibitors with similar binding constants are present may become difficult or impossible to analyze.

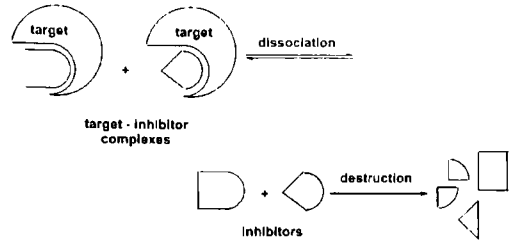
Finding the Best Inhibitor by Destruction of Poorer Inhibitors. One way to enhance the concentration differences between inhibitors with similar binding constants is to add an irreversible reaction that removes the unbound poorer inhibitor.

(4) (a) Eliseev, A. V.; Nelen, M. I. *J. Am. Chem. Soc.* **1997**, *119*, 1147−1148. (b) Eliseev, A. V.; Nelen, M. I. *Chem. Eur. J.***1998**, *4*, 825−834.

(5) Glaucoma patients often take carbonic anhydrase inhibitors to reduce the pressure in the eye. All commercial inhibitors contain a sulfonamide moiety. We chose carbonic anhydrase as a test case for inhibitor design and screening methods.

(6) If the binding is not tight or the target is not in excess at high concentration, then the concentrations of the inhibitors will be more similar than those discussed in the text above, and it will be even harder to distinguish which is the better inhibitor.

Scheme 3. Destruction of Inhibitors[a]



[a] The free concentration of the poorer inhibitor is higher, thus it is destroyed more readily. This destruction reaction exponentially increases the relative concentration of the good inhibitor similar to a kinetic resolution.

This situation is similar to a kinetic resolution of enantiomers.[7] As the destruction reaction winnows away the poorer inhibitors, the relative concentration of the best inhibitor increases exponentially (Scheme 3). The analysis below is similar to that for kinetic resolutions.[7]

Consider two inhibitors, A and B, that compete for a target, T, and are also destroyed by an irreversible reaction to yield P and Q with rates of k_{2A} and k_{2B}.

$$T{\cdot}A \overset{K_{dA}}{\rightleftharpoons} T + A \xrightarrow{k_{2A}} P \tag{4}$$

$$T{\cdot}B \overset{K_{dB}}{\rightleftharpoons} T + B \xrightarrow{k_{2B}} Q \tag{5}$$

The rate of disappearance of inhibitor A is

$$\frac{d[A]}{dt} = -k_{2A}[A] \tag{6}$$

If $[A_T]$ is the total concentration of bound and unbound forms of inhibitor A, it can be shown that

$$[A_T] = [A]\left(1 + \frac{[T]}{K_{dA}}\right) \tag{7}$$

Upon solving for [A] and substituting into eq 6, the rate of disappearance of A is given by

$$\frac{d[A]}{dt} = -\frac{k_{2A}K_{dA}[A_T]}{K_{dA} + [T]} \tag{8}$$

When the target is in excess of the inhibitor, the concentration of the free target, T, will be much larger than the dissociation constant, K_{dA}, so $[T] \gg K_{dA}$, therefore, eq 8 simplifies to

$$\frac{d[A]}{dt} = -\frac{k_{2A}K_{dA}[A_T]}{[T]} \tag{9}$$

A similar equation is obtained for inhibitor B. The ratio of their partial reaction rates is

$$\frac{d[A]}{d[B]} = \frac{K_{dA}}{K_{dB}} \times \frac{k_{2A}}{k_{2B}} \times \frac{[A_T]}{[B_T]} = \frac{1}{S} \times \frac{[A_T]}{[B_T]},$$

$$\text{where } S = \frac{K_{dB}}{K_{dA}} \times \frac{k_{2B}}{k_{2A}} \tag{10}$$

This equation shows that the relative rate of disappearance of the two inhibitors depends linearly on their total concentration, their relative binding ability, and their relative rate of destruction. For simplicity, we define S as the product of the relative binding abilities and relative rates of destruction of the two inhibitors. If the rates of destruction of the two inhibitors are equal, then S is the ratio of the dissociation constants and will be greater than one if A is a stronger inhibitor of the target than B.

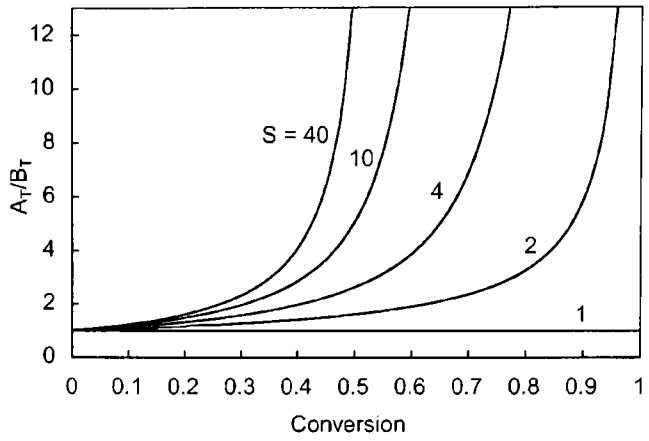


Figure 1. Predicted ratio of the total (bound and unbound) concentrations of two hypothetical inhibitors, A and B, as a function of the degree of conversion for given values of S. The degree of conversion is the fraction of the total amount of inhibitors that have been destroyed. The calculated lines follow eqs 11 and 12 where the initial total concentration of the inhibitors is one. This graph shows that the ratio of the two inhibitors can be much larger than the value of S, even for values of S as low as 2.

Upon integration of eq 10, one finds that the ratio of the total amounts of the two inhibitors varies exponentially with S (eq 11), where $[A_T]_0$ represents the initial total concentration of inhibitor A. This exponential relationship enhances the ability to detect small differences as the destruction reaction progresses.

$$\frac{\ln([B_T]/[B_T]_0)}{\ln([A_T]/[A_T]_0)} = S \tag{11}$$

By measuring the relative concentration of the two inhibitors during the destruction reaction, the value of S can be determined using eq 11. Alternatively, eq 12 below, which expresses $[A_T]$, $[B_T]$, $[A_T]_0$, and $[B_T]_0$ in terms of the conversion, C, and the ratio of the total concentrations of the two inhibitors, can be used to determine S.

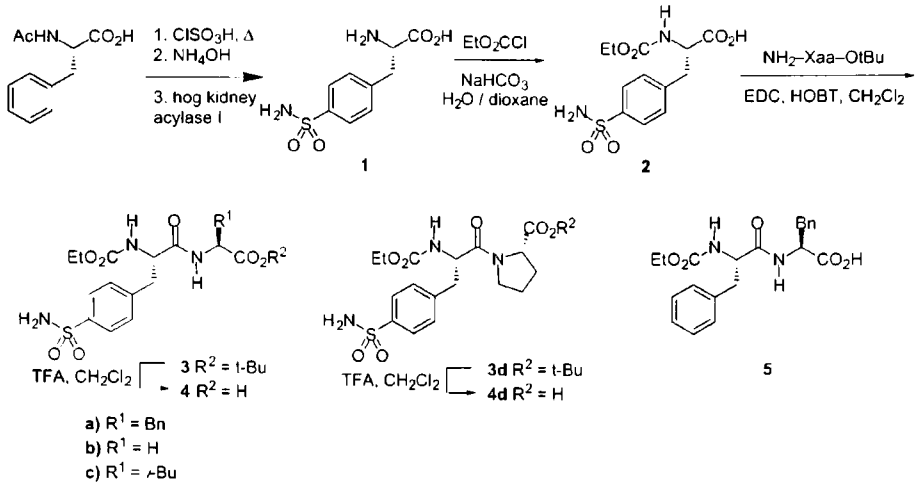
$$\frac{\ln[(1 - C)(2/(1 + R))]}{\ln[(1 - C)(2R/(1 + R))]} = S$$

$$\text{where } C = 1 - \frac{[A_T] + [B_T]}{[A_T]_0 + [B_T]_0} \quad \text{and } R = \frac{[A_T]}{[B_T]} \tag{12}$$

These predictions are shown graphically for several values of S in Figure 1. If the rates of destruction of the two inhibitors are equal, then S is the ratio of the dissociation constants. As the destruction reaction proceeds (conversion increases from 0 to 1), the ratio of the total amounts of the two inhibitors, $[A_T]/[B_T]$, varies when $S \neq 1$. When S is large (e.g., 40), the relative concentration of the good inhibitor increases steeply near 50% conversion. When S is small (e.g., 2), the relative concentration of the good inhibitor increases steeply near 90% conversion.

(7) The analysis below follows closely the mathematical treatment for kinetic resolutions. For examples, see: Martin, V. S.; Woodard, S. S.; Katsuki, T.; Yamada, Y.; Ikeda, M.; Sharpless, K. B. *J. Am. Chem. Soc.* **1981**, *103*, 6237–6240; Chen, C. S., Fujimoto, Y., Girdaukas, G., Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299; Kagan, H. B., Fiaud, J. C. *Top. Stereochem.* **1988**, *18*, 249–330.

Scheme 4. Preparation of 4′-Sulfonamidophenylalanine Dipeptides



In either case, the ratio of the total amounts of the two inhibitors, $[A_T]/[B_T]$, can be much larger than the value of S.

Results

Preparation of 4′-Sulfonamidophenylalanine Dipeptides. (*S*)-4′-Sulfonamidophenylalanine (**1** or Phe_{sa}) was prepared from (*S*)-*N*-acetylphenylalanine by a modification of the procedure described by Escher et al.[8] Thus, chlorosulfonylation of *N*-acetylphenylalanine in chlorosulfonic acid at 60 °C followed by ammonolysis afforded *N*-acetyl-4′-sulfonamidophenylalanine. Direct purification of this intermediate proved difficult. Therefore, it was deacetylated using hog kidney acylase I,[9] and the resulting free amino acid **1** was purified by ion-exchange chromatography and recrystallization. Using this procedure, **1** was prepared as an analytically pure solid in 40% yield from *N*-acetyl-Phe. The α-amino group was selectively blocked using ethyl chloroformate under standard Schotten–Baumann conditions. The requisite dipeptides were then prepared by coupling **2** with *tert*-butyl amino acid esters using EDC/HOBT,[10] followed by trifluoroacetic acid-mediated deprotection of the ester function to afford dipeptides **4a–d** (Scheme 4). No acylation of the sulfonamide nitrogen was observed under either the Schotten–Baumann or peptide-coupling conditions. Dipeptide EtOC-Phe-Phe (**5**), which does not contain a sulfonamide group and serves as a control, was prepared by standard methods.

Inhibition of Carbonic Anhydrase. Sulfonamides **1** and **2** as well as sulfonamide dipeptides **4a–d** all inhibited the carbonic anhydrase-catalyzed hydrolysis of 4-nitrophenyl acetate (pNPA). The inhibition was competitive and Lineweaver–Burk plots revealed similar inhibition constants, which varied by only a factor of 10 (Table 1). The parent amino acid **1** (Phe_{sa}) was the poorest sulfonamide inhibitor ($K_I = 13\ \mu M$), while dipeptides **4a** (EtOC-Phe_{sa}-Phe) and **4b** (EtOC-Phe_{sa}-Gly) were the best sulfonamide inhibitors (K_I = 1.2 and 2.5 μM, respectively). Dipeptides **4c** (EtOC-Phe_{sa}-Leu) and **4d** (EtOC-Phe_{sa}-Pro) showed slightly higher inhibition constants (4.4 and 9.4 μM, respectively). Other simple sulfonamides also inhibit carbonic anhydrase with similar inhibition constants.[11] As expected, the dipeptide lacking a sulfonamide group, **5**, did not inhibit carbonic anhydrase.

Table 1. Inhibition of Carbonic Anhydrase by Sulfonamides **1** and **2**, Sulfonamide Dipeptides **4a–d**, and Dipeptide **5**

compound	K_I (μM)[a]
Phe_{sa} (**1**)	13 ± 1.6
EtOC-Phe_{sa} (**2**)	12 ± 1.4
EtOC-Phe_{sa}-Phe (**4a**)	1.2 ± 0.2
EtOC-Phe_{sa}-Gly (**4b**)	2.5 ± 0.5
EtOC-Phe_{sa}-Leu (**4c**)	4.4 ± 0.7
EtOC-Phe_{sa}-Pro (**4d**)	9.4 ± 1.6
EtOC-Phe-Phe (**5**)	≫1000[b]

[a] Competetive inhibition constants for the carbonic anhydrase-catalyzed hydrolysis of *p*-nitrophenyl actetate (*p*NPA) at 25 °C in phosphate buffer (10 mM pH 7.5). A typical procedure was to add carbonic anhydrase solution (100 μL, 0.05 mg/mL) containing inhibitor (0.0–100 μM in most cases) to an acetonitrile solution of *p*NPA (5.0 μL, 2.0–32 mM) and follow the release of *p*-nitrophenoxide spectrophotometrically at 404 nM. [b] No inhibition detected at an inhibitor concentration of 1 mM.

Selective Extraction of Inhibitors by Carbonic Anhydrase. First, we demonstrated that a strongly binding inhibitor concentrates into the carbonic anhydrase-containing compartment of a two-compartment vessel (cf. Figure 4 without Pronase). The two compartments were created by suspending a dialysis bag containing a solution of bovine carbonic anhydrase[12] (0.34 mM) in a solution of phosphate buffer. The dialysis membrane (12-kDa cutoff) separated the two compartments so that small molecules such as the sulfonamide dipeptides could diffuse freely across the membrane, while carbonic anhydrase (30 kDa) could not. Both compartments initially contained a mixture of 0.16 mM sulfonamide dipeptide **4a** and 0.19 mM noninhibitor dipeptide **5**. Over several hours the total sulfonamide concentration increased in the inside compartment containing carbonic

(8) Escher, E.; Bernier, M.; Parent, P. *Helv. Chim. Acta* **1983**, *66*, 1355–1365.

(9) Researchers often use hog kidney acylase to resolve enantiomers of *N*-acetyl amino acids. Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 6354–6364; Roberts, S. M., Ed. *Preparative Biotransformations*; Wiley: Chichester 1992–1998; Module 1:14. In our case, this intermediate was already enantiomerically pure. We used hog kidney acylase to cleave the acetyl group under milder conditions than those required by chemical cleavage methods.

(10) EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HOBT = 1-hydroxybenzotriazole.

(11) For example, Nguyen and Huc investigated a simple sulfonamides with inhibition constants of ~0.1–1 μM (Nguyen, R.; Huc, I. *Angew. Chem., Int. Ed.* **2001**, *40*, 1774–1776), while Doyon et al. investigated other simple sulfonamides with inhibition constants of ~0.001 μM (Doyon, J. B.; Hansen, E. A. M.; Kim, C.-Y.; Chang, J. S.; Christianson, D. W.; Madder, R. D.; Voet, J. G.; Baird, T. A., Jr.; Fierke, C. A.; Jain, A. *Org. Lett.* **2000**, *2*, 1189–1192).

(12) These experiments required stoichiometric amounts of carbonic anhydrase. We used an inexpensive mixture of isozymes from bovine sources. Although material was not pure carbonic anhydrase, we calculated the concentrations assuming it was pure. Thus, the true concentration will be less than the number given.

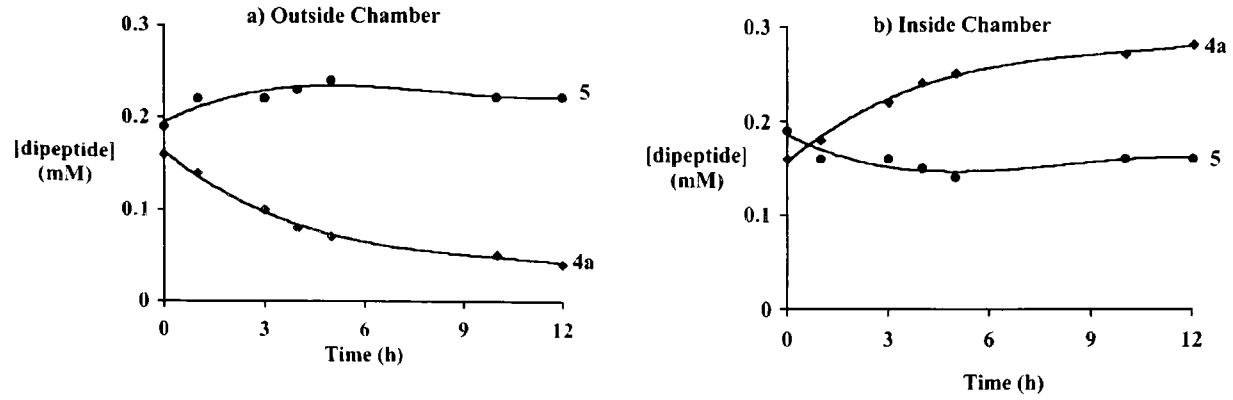


Figure 2.[13] Selective concentration of the sulfonamide **4a** over noninhibitor **5** into the carbonic-anhydrase-containing compartment of a two-compartment vessel. One compartment contained carbonic anhydrase (0.34 mM), while both compartments (20 mL each) initially contained equal concentrations of sulfonamide **4a** (0.16 mM) and noninhibitor **5** (0.19 mM). The sulfonamide diffused freely across the dialysis membrane and concentrated in the carbonic-anhydrase-containing compartment as shown. In contrast, the concentrations of noninhibitor **5** remained similar in both compartments. After 12 h, the concentration of sulfonamide **4a** in the outside compartment decreased to 0.04 mM and increased in the inside compartment to 0.28 mM (total of free and carbonic anhydrase-bound). The final ratio of **4a** to **5** in the carbonic anhydrase chamber was 1.75:1.

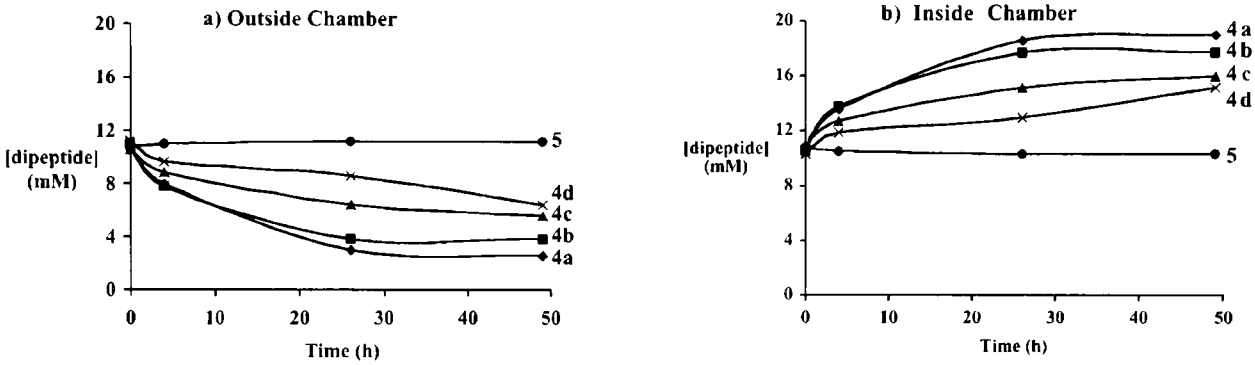


Figure 3.[13] Selective concentration of the sulfonamides **4a-4d** over noninhibitor **5** into the carbonic-anhydrase-containing compartment of a two-compartment vessel separated by a dialysis membrane. One compartment contained carbonic anhydrase (0.485 mM), while both compartments (20 mL each) initially contained equal concentrations of sulfonamides **4a-4d** and noninhibitor **5** (~0.11 mM each). The sulfonamides diffused freely across the dialysis membrane and concentrated in the carbonic-anhydrase-containing compartment. In contrast, the concentration of noninhibitor **5** increased slightly in the outer compartment.

anhydrase and decreased in the outside compartment (Figure 2). Alternatively, the concentrations of the noninhibitor **5** remained similar in both compartments. This result showed that tight binding to a target could concentrate a good inhibitor into one compartment of a two-compartment reaction vessel.

In a similar experiment using a mixture of inhibitors, we could further detect differences in relative inhibition constants. A more tightly binding inhibitor concentrated in the carbonic anhydrase compartment to a greater extent than a less tightly binding inhibitor. Starting with an equimolar mixture of sulfonamide dipeptides **4a**–**d** and the noninhibitor **5** in both compartments, the sulfonamide dipeptides concentrated into the carbonic anhydrase compartment, Figure 3. The fraction of dipeptide in the carbonic anhydrase compartment varied: 88, 82, 74, 70, and 48% for **4a**, **4b**, **4c**, **4d**, and the noninhibitor **5**, respectively (or a ratio of 1.83:1.71:1.54:1.46:1 for **4a:4b:4c:4d:5**). The order of highest to lowest concentration in the carbonic anhydrase chamber reflects the order of the binding constants of the inhibitors.

These results show that is possible to distinguish between inhibitors, but the differences in concentration are small, especially among inhibitors of similar strength. Even comparing the best inhibitor (**4a**) with a noninhibitor (**5**) gives a concentration differing by less than a factor of 2. To enhance this difference in concentration, we explored the use of proteases to destroy the poorer inhibitors.

Screening of Proteases. We screened 22 commercially available proteases to identify those that could hydrolyze the dipeptide EtOC-Phe$_{sa}$-Phe (**4a**). All proteases showed some activity. Using 0.1 mg of protease and 2 μmol (2 mM) dipeptide **4a**, the five most active proteases (α-chymotypsin, protease from *Streptomyces casepitosus*, proteinase K, Pronase from *Streptomyces griseus*, and protease from *Bacillus thermoproteolyticus rokko*) cleaved all of the dipeptide within 24 h, while two moderately active proteases (protease N "Amano", protease from *Bacillus polymyxa*) cleaved all of the dipeptide within 48 h. The remaining proteases cleaved less than half of the dipeptide after 72 h. We chose one of the most active yet inexpensive enzymes, Pronase from *S. griseus*, for subsequent experiments. Pronase was found to cleave all five dipeptides (**4a**–**d** and **5**), although the glycine and proline dipeptides (**4b** and **4d**) were cleaved more slowly (80–90% hydrolysis within 24 h). To ensure high cleavage rates, larger amounts of Pronase were used in the competitive degradation experiments described below.

Selective Protection of Inhibitors by Carbonic Anhydrase from Hydrolysis. We compared the ability of carbonic anhydrase to protect sulfonamide inhibitor **4a** from hydrolysis while allowing a noninhibitor, **5**, to be hydrolyzed. An experiment

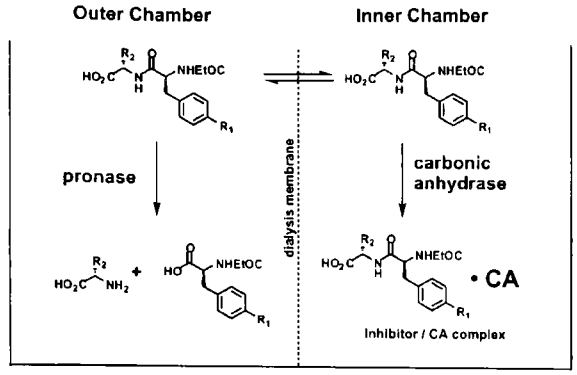


Figure 4. Reaction design for the selective destruction experiments. The dipeptides can diffuse across the dialysis membrane into either chamber. One chamber contains carbonic anhydrase, the other contains Pronase. Dipeptides in the Pronase chamber are rapidly cleaved to their constituent pieces. Carbonic anhydrase prevents strong binding dipeptides from diffusing across the membrane and thus slows their hydrolysis.

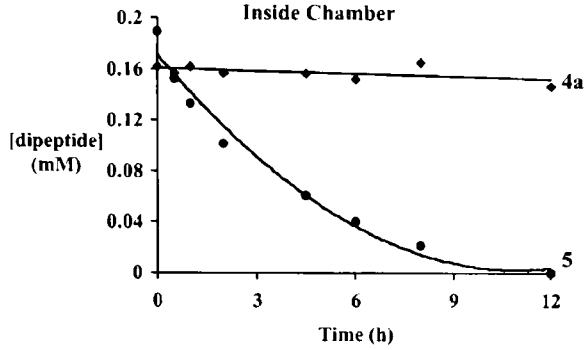


Figure 5.[13] Selective protection from hydrolysis of sulfonamide **4a** over noninhibitor **5** by carbonic anhydrase. A vessel containing two compartments of equal volume (20 mL each) separated by a dialysis membrane was filled with a solution of. sulfonamide **4a** (0.16 mM) and noninhibitor **5** (0.19 mM). The inside compartment contained carbonic anhydrase (0.34 mM), while the outside compartment contained Pronase. The protease rapidly cleaved the dipeptides in the outside compartment to the corresponding amino acids (data not shown). The noninhibitor **5** diffused freely across the dialysis membrane and was cleaved by the protease. In contrast, the inhibitor **4a** bound to carbonic anhydrase in the inside compartment and was not consumed at a significant rate. After 6 h, the concentration of sulfonamide **4a** in the inside compartment decreased by only 6% (0.15 mM), while the concentration of noninhibitor **5** decreased to 0.041 mM during the same time period (ratio = 3.7:1).

similar to that described above, except with Pronase added to the outer chamber was set up (Figure 4). On the one hand, in the Pronase-containing chamber, both dipeptides were rapidly cleaved to the constituent pieces within 15 min. On the other hand, the inside compartment showed a steady decrease in the concentration of noninhibitor **5** over 12 h (Figure 5), while the concentration of sulfonamide **4a** remained nearly constant (a decrease of 9% over 12 h).[14] After even just 6 h, the ratio, **4a**:**5**, in the inside compartment was 3.7:1 and continued to increase to greater than 20:1 after 12 h. By comparison, the experiment which does not contain Pronase had a final ratio of **4a** to **5** of 1.75:1.

In a simlar experiment, dipeptides **4a** and **4b**, which have very similar binding constants, were exposed to carbonic anhydrase and Pronase. In this experiment, the dipeptides were

(13) Lines drawn in all figures (except Figure 1 and Figure 9) are for illustration purposes only. They do not represent theoretical lines of any sort.
(14) Both **4a** and **5** diffused through the membrane at identical rates with a half-life of about 3 h. (Data not shown.)

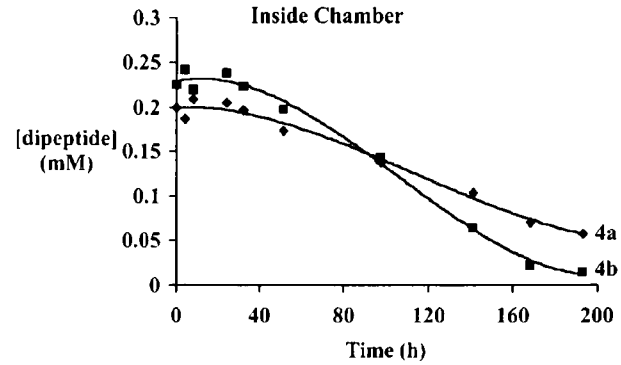


Figure 6.[13] Selective protection from hydrolysis of dipeptide **4a** over **4b** by carbonic anhydrase. A reaction vessel was separated into two compartments (20 mL each) by a dialysis membrane. The inside compartment contained carbonic anhydrase (13.6 mol), dipeptide **4a** (4.3 mol) and dipeptide **4b** (4.3 mol) in 20 mL of buffer. The outer compartment contained Pronase (5 mg) dissolved in 20 mL of buffer. The time course of the reaction in the carbonic anhydrase chamber is shown in the figure. At 83% conversion (193 h) the ratio of **4a** to **4b** was 3.8:1.

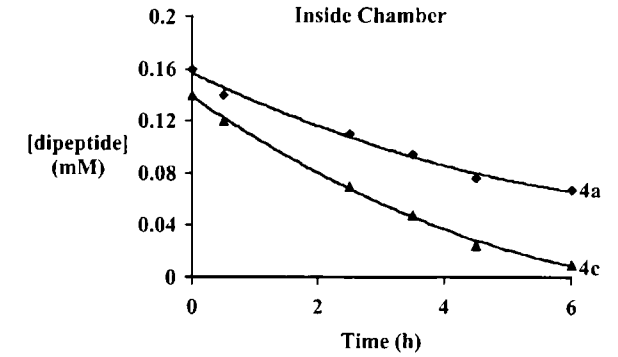


Figure 7.[13] Selective protection from hydrolysis of dipeptide **4a** over **4c** by carbonic anhydrase. A reaction vessel was separated into two compartments (20 mL each) by a dialysis membrane. The inside compartment contained carbonic anhydrase (0.34 mM), while the outside compartment contained Pronase (4 mg). Both compartments initially contained similar concentrations of dipeptide **4a** (0.16 mM) and dipeptide **4c** (0.14 mM). The protease rapidly cleaved the dipeptides in the outside compartment to the corresponding amino acids (data not shown). The time course of the reaction in the carbonic anhydrase chamber is shown in the figure. After 6 h, 93% of **4c** inside the CA chamber had been hydrolyzed, while only 58% of **4a** had hydrolyzed. A control experiment which did not contain carbonic anhydrase showed an equal rate of hydrolysis for the two dipeptides in the chamber not containing Pronase.

placed only in the carbonic anhydrase chamber, and an excess of carbonic anhydrase was used (1.6:1 ratio of CA to dipeptides) so that the conditions adhered rigorously to those of the theory described above. As expected, due to the excess of target and tight binding of both dipeptides, the hydrolysis of **4a** and **4b** was slow. However, as in the first reaction, the weaker binder, **4b**, was consumed at a higher rate (Figure 6). After 193 h, 83% of the total dipeptides had been hydrolyzed, and the ratio of **4a** to **4b** was 3.8:1. This final ratio is in excess of the ratio of the independently determined binding constants of the dipeptides (2.1:1).

In a related experiment, we compared two sulfonamide dipeptides **4a** and **4c** which also have similar inhibition constants (Figure 7). In this experiment, both the inside and outside chambers initially contained equal concentrations of the dipeptides, and the total concentration of dipeptides was in excess (2.1:1 ratio of dipeptides to CA). The result was a much faster hydrolysis of both dipeptides in the carbonic anhydrase chamber. This faster rate reflects the rapid release of 1 equiv of Phe_{SA}

(2) from the Pronase chamber. Although **2** is a weaker binder than either **4a** or **4c**, enough of it was produced such that it could displace a small amount of **4a** and **4c** from the carbonic anhydrase binding pocket, thus accelerating their hydrolysis by Pronase. However, the net result was still the same. After 6 h, 93% of **4c** was hydrolyzed after 6 h, but only 58% of **4a** was hydrolyzed. Thus, the ratio of concentrations was 6:1, which is much larger than the 1.6:1 ratio observed in a control experiment which did not contain Pronase and larger than the 3.7:1 ratio of their binding constants.

Finally, an experiment containing all five dipeptides (**4a**–**d** and **5**) was conducted using an excess of carbonic anhydrase (ratio of CA to dipeptides is 1.2:1). The experiment was consistent both with the theory and with the prior results. Dipeptide **5** was cleaved rapidly while dipeptides **4a**–**d** disappeared at rates which corresponded to their binding constants (Figure 8).

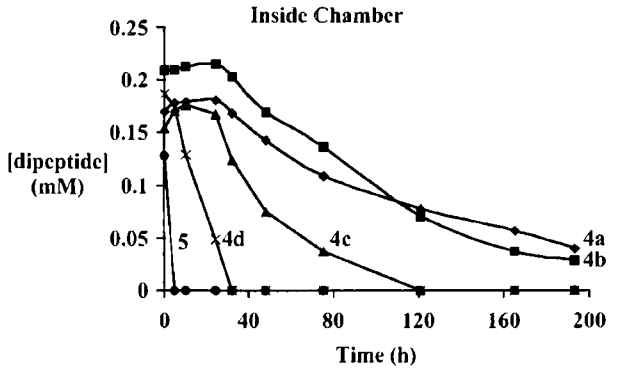


Figure 8.[13] Selective protection from hydrolysis of dipeptides by carbonic anhydrase. A reaction vessel separated into two compartments (20 mL each) by a dialysis membrane was set up. The inside compartment contained carbonic anhydrase (25.6 mol) and dipeptides **4a**–**d** and **5** (4.3 mol each) in 20 mL of buffer. The outer compartment contained Pronase (5 mg) dissolved in 20 mL of buffer. The time course of the reaction in the carbonic anhydrase chamber is shown in the figure.

Discussion

As expected, the four sulfonamide dipeptides **4a**–**d** all inhibit carbonic anhydrase competitively with similar inhibition constants (within a factor of 10 of each other). Classical kinetics using initial rates easily identified these differences, but these classical methods are slow and require the separate measurement of each inhibitor. This becomes laborious for libraries containing thousands of members.

To rapidly identify the best inhibitor, we used competitive binding to carbonic anhydrase in one compartment of a two-compartment cell. The inhibitors concentrated into the carbonic anhydrase compartment of a two-compartment cell. Higher concentrations of the better inhibitors were observed in the carbonic anhydrase compartment, but the concentration differences were small (1.83:1.71:1.54:1.46:1 for **4a:4b:4c:4d:5**). If the mixture contained 1000 dipeptides, this competitive experiment would not identify the best inhibitor because it would be difficult to separate all the dipeptides, and the differences in concentration with and without target would be small.

Although this experiment does not include a dipeptide-synthesis step and thus is not a dynamic library, the diffusion across the membrane mimics a synthesis step in a dynamic library in that both are equilibrium processes. For the diffusion process, in the absence of a target, each compartment should contain equal amounts of each inhibitor. In the presence of the target, the carbonic anhydrase chamber contains more of the tight-binding inhibitors. Thus, the equilibrium for the diffusion process has shifted.

A nonselective destruction of the library members should enhance differences in the relative concentrations of the members bound to the target. The poor binding members are destroyed at a rate higher than that for the strong-binding members, and as the degradation progresses, the ratio improves exponentially in favor of the latter. This was observed in our library, where dipeptide hydrolysis by Pronase was used as the destruction process. In a competition experiment between a strong and weak binder (**4a** vs **5**), the ratio of **4a** to **5** in the carbonic anhydrase chamber increased from 1.75:1 in the absence of Pronase to 3.7:1 in the presence of Pronase (at 45% conversion). Furthermore, this ratio continued to increase to >20:1 as the reaction progressed. A second experiment with two species with very similar K_i's (**4a** vs **4b**) had a final ratio of 3.8:1 when the ratio of the binding constants was 2.1:1. As shown in Figure 9a, these results follow the theoretical model closely. Similar results were obtained for an experiment containing two inhibitors (**4a** and **4c**) where an excess of a weaker binder, Phe_{SA} (**2**), was generated in the reaction mixture. The presence of **2** accelerated the rate of cleavage of **4a** and **4c**, but as can be seen in Figure 9b, the ratio of dipeptides during the course of the reaction still followed the theoretical model. At 70% conversion, the ratio of **4a** to **4c** was 6:1, which is much larger than the 1.6:1 ratio observed in a reaction not containing Pronase. In all cases, the model indicates that the ratios should continue to increase if the reactions are carried out for even longer periods. In experiments with a large number of library members, this increase will be critical in allowing the tightest binding species to be easily identified.[15]

One potential limitation of this screening method is selectivity in the destruction reaction. For example, dipeptide **4c** is cleaved by Pronase at a much slower rate than that for dipeptide **4a**. In such a case, S from eqs 10–12 will not be equal to the ratio of the binding constants, and thus the degradation reaction will not follow the theoretical curves of Figure 1. To accommodate this situation, we used a large amount of protease, and more importantly, we employed a dialysis membrane to separate the target–inhibitor complexes from the protease. In this setup, the rate-limiting step in the destruction reaction is not the protease-catalyzed cleavage but diffusion across the dialysis membrane. Unlike the protease-catalyzed cleavage, the rate of diffusion does not vary significantly with the structure of the inhibitor, and the result is that the destruction reaction follows the theoretical curve. Although Pronase accepts a wide variety of peptides, substrate specificity of the enzyme may become problematic if highly diverse libraries are studied. A dipeptide which is not cleaved by Pronase would be retained in the reaction mixture even if it did not bind to carbonic anhydrase. One way to alleviate this problem would be to use a mixture of enzymes with a wide range of specificities. Alternatively, it is important to note that the degradation reaction is not limited to enzymic processes. Other chemical degradation methods can be envisioned, depending upon the type of library being studied. For

(15) The reaction mixture will contain the products of the degradation reactions. However, in most cases, this method will be applied to combinatorial libraries, and as such, the degradation products will often be the common starting materials used to make the library members. Thus, only a limited number of degradation products will be produced.

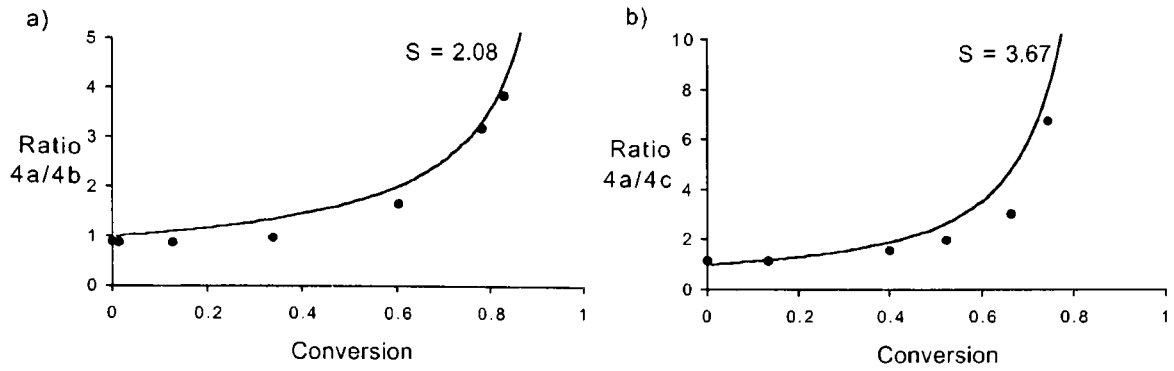


Figure 9. The graph shows theoretical and experimental ratios for the screening experiments. Theoretical lines are shown as smooth lines. The *S* values correspond to the ratios of the experimentally determined binding constants. The data points show the experimentally determined ratios at different conversions for a) **4a/4b** (cf. Figure 6) and b) **4a/4c** (cf. Figure 7).

example, a library based on disulfide exchange could be degraded by adding a reducing agent (e.g., a phosphine) to cleave any unbound disulfides. Alternatively, physical methods for removal of unbound inhibitors (e.g., adsorption to a solid phase, extraction) should accomplish the same effect as a chemical degradation.

Another potential limitation of this screening method, and indeed for methods based on the dynamic combinatorial library technique, is the need for stoichiometric amounts of the target. The initial experiments reported here used large amounts of carbonic anhydrase (100−500 mg/experiment) as we expect to apply it to a dynamic library process where the best inhibitor will actually be isolated and characterized. However, for purely analytical screening purposes, the methods can easily be scaled down using smaller compartments, assuming that more sensitive analytical tools are used (e.g., mass spectroscopy). These modifications could reduce the amount of target needed to <0.1 mg/experiment, an amount that is easily accessible for targets that have been cloned and overexpressed.

In conclusion, we have developed a method for screening mixtures of compounds against a therapeutic target which readily identifies the best binder in a library. The method works by selectively degrading the poorer inhibitors with an enzyme. This results in a significant improvement in the ability to distinguish between inhibitors which have very close binding constants. We plan to extend this method to dynamic libraries with the goal of improving the enhancement observed in synthesis of good inhibitors in the presence of a therapeutic target.

Experimental Section

General Experimental. *p*-Nitrophenyl acetate (*p*NPA), carbonic anhydrase (CA, from bovine erythrocytes, a mixture of isozymes, C-3934) and proteases were purchased from Sigma unless otherwise noted and used without further purification. HPLC analyses were conducted using a Phenomenex-C_8 reversed phase HPLC column (10 mm × 250 mm) with detection at 220 nm, unless otherwise noted. Elemental analyses were obtained from Quantitative Technologies Inc., Whitehouse, NJ. High-resolution mass spectra were obtained from Université de Sherbrooke, Sherbrooke, QC.

4′-Sulfonamidophenylalanine (1). *N*-Acetylphenylalanine (37.7 g, 178 mmol, 1 equiv) was added in portions over a 1-h period to neat chlorosulfonic acid (110 mL, 1.65 mol, 9.5 equiv) at −10 °C. The resulting yellow solution was stirred at −10 °C for 2.5 h, at 25 °C for 2.5 h, and then heated to 60 °C until gas evolution had ceased (approximately 12 h). The resulting orange solution was cooled to 0 °C and poured carefully onto 750 mL of ice (Caution: exotherm!). The resulting mixture was extracted with ethyl acetate (3 × 1 L), and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the sulfonyl chloride (45.1 g, 83%) as an orange solid which was used immediately without further purification. ^{1}H NMR ($(CD_3)_2SO$) δ 8.26−8.21 (d, 1H, *J* = 8.5 Hz), 7.55 (d, 2H, *J* = 6.9 Hz), 7.22 (d, 2H, *J* = 6.8 Hz), 4.49−4.34 (m, 1H), 3.13−3.00 (dd, 1H, *J* = 14.4 and 6.8 Hz), 2.92−2.79 (dd, 1H, *J* = 11.0 and 10.2 Hz), 1.80 (s, 3H).

The sulfonyl chloride was dissolved in 28% NH_4OH (240 mL), and the resulting solution was heated at reflux for 3 h. After cooling to 0 °C, the solution was acidified to pH 1 by addition of 3 M H_2SO_4 (ca. 200 mL) and extracted with ethyl acetate (3 × 500 mL). The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the sulfonamide (29.9 g, 71%) as a white solid. The *N*-acetyl sulfonamide could not be purified to homogeneity by either chromatography or recrystallization. ^{1}H NMR ($(CD_3)_2SO$) δ 8.29−8.24 (d, 1H, *J* = 8.5 Hz), 7.77 (d, 2H, *J* = 3.9 Hz), 7.45 (d, 2H, *J* = 6.9 Hz), 7.33 (s, 2H), 4.53−4.41 (m, 1H), 3.20−3.09 (dd, 1H, *J* = 14.2 and 6.8 Hz), 3.01−2.87 (dd, 1H, *J* = 11.2 and 10.1 Hz), 1.80 (s, 3H).

A suspension of the sulfonamide (20.0 g, 69.9 mmol, 1 equiv) in distilled water (300 mL) was adjusted to pH 5.00 with LiOH (900 mg). A 0.25 M solution of Na_2HPO_4 (85 mL) was used to raise the pH to 7.50. Acylase I from hog kidney (200 mg, 17.8 U/mg, 3560 U) was added as an aqueous solution (12 mL), and the resulting solution was stirred at 21 °C for 70 h. The solution was then acidified to pH 1.0 with 3 M H_2SO_4 and extracted with ethyl acetate (3 × 500 mL); the organic layer was then dried with anhydrous sodium sulfate and concentrated in vacuo to afford 2.28 g (11%) of the sulfonamide starting material. The aqueous layer was neutralized with 2 M NaOH and concentrated. The solution was then applied to an Amberlite 120(plus) acidic ion-exchange column. The column was rinsed with water until the eluent was at pH 6.0, and then it was rinsed with 0.50 M NH_4OH solution until the eluent became basic. The basic wash was concentrated in vacuo and recrystallized from water to afford the provided 4′-sulfonamidophenylalanine as a white solid (11.60 g, 68%).^{1}H NMR (D_2O/DCl) δ 7.62 (d, 2H, *J* = 8.1 Hz), 7.26 (d, 2H, *J* = 8.1 Hz), 4.14 (t, 1H, *J* = 6.8 Hz), 3.19−3.12 (dd, 1H, *J* = 14.6 and 5.7 Hz),3.08−3.01 (dd, 1H, *J* = 14.4 and 6.9 Hz). ^{13}C NMR (D_2O/DCl) δ 170.73, 140.451, 139.49, 130.38, 126.55, 53.49, 35.19. FABMS in saturated NaCl *m/z* 267 (M + Na, $C_9H_{12}N_2O_4SNa$ requires 267.)

***N*-Ethoxycarbonyl-4′-sulfonamidophenylalanine (2).** Ethyl chloroformate (398 μL, 4.17 mmol, 1.10 equiv) was added to a two-phase mixture of 4′-sulfonamidophenylalanine (925 mg, 3.73 mmol, 1 equiv) in 1,4-dioxane (25 mL) and saturated $NaHCO_3$ solution (25 mL) at 0 °C, and the resulting solution was stirred for 6 h at 0 °C. The mixture was extracted with ethyl acetate (100 mL), and the aqueous layer was acidified to pH 1 by addition of 2 M HCl (ca. 20 mL) and then extracted with ethyl acetate (3 × 50 mL). Latter organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the

ethyl carbamate (879 mg, 83%) as an analytically pure oil. ^{1}H NMR ($(CD_3)_2CO$) δ 7.85 (d, 2H, J = 7.1 Hz), 7.51 (d, 2H, J = 6.9 Hz), 6.54 (s, 2H), 6.45 (d, 1H, J = 6.7 Hz), 4.62−4.45 (m, 1H), 4.01−3.97 (q, 2H, J = 2.4 Hz), 3.41−3.28 (dd, 1H, J = 11.3 and 4.0 Hz), 3.16−3.05 (dd, 1H, J = 10.4 and 7.8 Hz), 1.13 (t, 3H, J = 6.0 Hz). HR-CIMS (*m/z*): [MH^+] calculated for $C_{12}H_{17}N_2O_6S$, 317.0807; found, 317.0817.

EtO_2C-(4′-SO_2NH_2)Phe-Gly-*O-tert*-butyl (3b). EDC·HCl (136 mg, 0.711 mmol, 1.10 equiv), HOBT (87.3 mg, 0.646 mmol, 1.00 equiv), and triethylamine (269 μL, 1.94 mmol, 3.00 equiv) were added to a solution of **2** (204 mg, 0.646 mmol, 1 equiv) in THF (3 mL) at 0 °C. Glycine *tert*-butyl ester·HCl (119 mg, 0.711 mmol, 1.10 equiv) was added, and the resulting solution was allowed to warm to 21 °C while stirring for 13 h, at which point the bulk of the THF was removed by concentration in vacuo. The residue was dissolved in ethyl acetate (45 mL) and extracted with 0.1 M HCl (3 × 25 mL) and saturated $NaHCO_3$ solution (3 × 25 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The solid residue was purified by mixed solvent recrystallization (ethyl acetate/hexanes) to afford 193 mg (70%) of **3b**. ^{1}H NMR ($(CD_3)_2CO$) δ 7.82 (d, 2H, J = 7.5 Hz), 7.63 (s, 1H), 7.50 (d, 2H, J = 7.5 Hz), 6.52 (s, 2H), 6.40 (d, 1H, J = 7.5 Hz), 4.50 (m, 1H), 4.00−3.88 (m, 4H), 3.40 (dd, 1H, J = 14.1 and 4.2 Hz), 3.02 (dd, 1H, J = 13.5 and 9.9 Hz), 1.45 (s, 9H), 1.12 (t, 3H, J = 6.9 Hz). ^{13}C NMR ($(CD_3)_2CO$) δ 171.48, 168.93, 156.33, 142.70, 130.06, 126.18, 81.04, 60.48, 55.92, 41.79, 37.83, 27.52, 14.22. Analysis calculated for $C_{18}H_{27}N_3O_7S$ C, 50.34; H, 6.34; N, 9.78. Found: C, 50.33; H, 6.35; N, 9.73.

EtO_2C-(4′-SO_2NH_2)Phe-Gly-OH (4b). TFA (7 mL) was added to a solution of **3b** (175 mg, 0.409 mmol, 1 equiv) in CH_2Cl_2 (8 mL), and the solution was stirred for 25 min at 21 °C under an atmosphere of argon. The solvents were removed in vacuo, and the residue was purified by recrystallization from acetone to afford 121 mg (79%) of **4b**. ^{1}H NMR (CD_3OD) δ 8.55 (s, 1H), 7.83 (d, 2H, J = 7.2 Hz), 7.46 (d, 2H, J = 7.2 Hz), 4.45−4.42 (m, 1H), 4.02−3.98 (q, 2H, J = 6.8), 3.95−3.92 (m, 1H), 3.32−3.25 (m, 2H), 2.97−2.89 (dd, 1H, J = 13.5 and 9.9 Hz), 1.18−1.14 (t, 3H, J = 6.8). ^{13}C NMR (CD_3CD) δ 173.0, 171.8, 157.3, 148.7, 142.4, 137.6, 129.8, 126.0, 60.9, 56.0, 37.7, 13.7. HR−CIMS (*m/z*): [MH^-] calculated for $C_{14}H_{20}N_3O_7S$, 374.1022; found, 374.1030.

Measurement of Inhibition Constants. Kinetic constants for carbonic anhydrase (CA) were measured according to Pocker and Stone using *p*-nitrophenyl acetate (*p*NPA) as the substrate.[16] The CA-catalyzed hydrolysis of *p*NPA was followed spectrophotometrically at 25 °C in a 96-well microplate spectrophotometer by monitoring the appearance of *p*-nitrophenolate at 404 nm. The values of K_m and V_{max} were determined by measuring the hydrolysis rate as a function of the *p*NPA concentration. To determine the inhibition constants, the values of K_m and V_{max} were redetermined in the presence of varying amounts of inhibitor. Since the values of K_m for pNPA increased in the presence of the inhibitor, but the values of V_{max} remained unchanged, we concluded that the inhibition is competitive. The concentration of inhibitor that increased the K_m for pNPA by a factor of 2 is the inhibition constant. A typical procedure was to add CA solution (100.0 μL) with inhibitor to acetonitrile solution of *p*NPA (5.0 μL). In the assay solution, the concentration of inhibitor ranges from 0.0 to 6.0 μM, while the concentration of *p*NPA ranged from 0.2 to 2.5 mM. The microplate was shaken for 5 s before the first reading and for 3 s between readings.

Selective Concentration of EtOC-Phe_{sa}-Phe (4a) over EtOC-Phe-Phe, (5) into a Compartment Containing Carbonic Anhydrase. A solution of **4a** (2.9 mg, 6.3 μmol) and **5** (2.9 mg, 7.5 μmol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.20 g, approx. 6.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). This dialysis bag was suspended in the second portion, and the reaction vessel was shaken gently (200 rpm) at 30 °C. Aliquots were removed periodically from each compartment, heated to 80 °C until a white precipitate formed (~5 min), and centrifuged, and the supernatant was filtered through a 0.22-μm pore filter. The amount of dipeptides was measured by HPLC using a Zorbax C8 column and 40/60/0.1 water/methanol/trifluoroacetic acid at 0.40 mL/min. After 12 h 88% of **4a** (retention time 11.4 min) had accumulated inside the dialysis bag while only 42% of **5** (retention time 25.5 min) was found inside the bag.

Selective Concentration Of EtOC-Phe_{sa}-Phe (4a) from a Mixture of EtOC-Phe_{sa}-Leu (4c), EtOC-Phe_{sa}-Gly (4b), and EtOC-Phe-Phe (5) by Carbonic Anhydrase. Dipeptides **4a** (2.0 mg, 4.3 μmol), **4b** (1.6 mg, 4.3 μmol), **4c** (1.9 mg, 4. μmol) **4d** (1.8 mg, 4.3 μmol), and **5** (1.7 mg, 4.3 μmol) were dissolved in 40 mL of 10 mM KH_2PO_4 buffer, pH 7.5 containing 0.1 mg/mL penicillin G (to avoid bacterial growth). Carbonic anhydrase (CA) (0.29 g, 9.7 μmol, 0.45 equiv) was dissolved in 20 mL of this solution and placed in a dialysis bag (the bag was washed in ddH_2O for 1 h, rinsed in EtOH once, and then washed again with ddH_2O). The bag was suspended in the remaining 20 mL of inhibitor solution in a 100-mL container and shaken at 60 rpm on a three-dimensional orbital shaker at room temperature for 49 h. Samples (1 mL) were taken periodically from inside and outside the dialysis bag, heated in an 80 °C water bath for 5 min, and then centrifuged for 10 min. The supernatant was filtered through a 0.22-μM sterile filter. The supernatant (700 μL) was added to MeOH (300 μL) to form the HPLC sample (30% MeOH, 70% aqueous). The sample was run on a Phenomenex C8 reverse phase column under the following conditions: 0−15 min 30% MeOH, 70% H_2O, 15−60 min 37% MeOH 63% H_2O, 60−90 min 62% MeOH, 38% H_2O. The peak areas were monitored: Phe_{sa}Gly: 7.9 min, PhesaPro: 17.6 min, Phe_{sa}Leu: 54.5 min, Phe_{sa}Phe: 60.0 min, PhePhe: 69.5 min. The percentages are accurate to ±2%. All nonsterile apparatus used was autoclaved prior to use to avoid bacterial growth.

Screening of Proteases for the Hydrolysis of EtOC-Phe-Phe Dipeptide (4a). The protease to be screened (0.1 mg) was added to a solution of **4a** (1.0 mg, 2.2 mol) in 0.01 M aqueous phosphate buffer (pH 7.5). The solution was kept at 30 °C, and aliquots were removed periodically, worked up as above, and analyzed by HPLC.

Selective Protection of Inhibitors from Hydrolysis by Carbonic Anhydrase. A solution of **4a** (3.0 mg, 6.5 μmol) and **5** (2.8 mg, 7.3 μmol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.20 g, approx 6.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). Pronase from *S. griseus* (Sigma P-5147, 4 mg) was dissolved in the second portion, and the dialysis bag was then suspended in the resulting solution. The reaction vessel was then shaken gently (200 rpm) at 30 °C, and aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC. After 30 min, neither substrate was detectable in the solution outside the dialysis bag. Inside the dialysis bag, 78% of **5** had hydrolyzed, while only 6% of **4a** had hydrolyzed after 6 h. In a control experiment containing no carbonic anhydrase, inside the dialysis bag, 76% of **4a** and 80% of **5** had hydrolyzed after 6 h.

Selective Binding of EtOC-Phe_{sa}-Phe (4a) over EtOC-Phe_{sa}-Leu (4c). A solution of **4a** (3.3 mg 7.1 mol) and **4c** (3.6 mg, 8.4 mol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.20 g, approx 6.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). This dialysis bag was suspended in the second portion, and the reaction vessel was shaken gently (200 rpm) at 30 °C. Aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC using a Zorbax C8 column. After 12 h 98% of **4a** had accumulated inside the dialysis bag, while only 60% of **4c** was found inside the bag.

Hydrolysis of EtOC-Phe_{sa}-Gly (4b) and EtOC-Phe_{sa}-Phe (4a) in the Presence of Carbonic Anhydrase. Phe_{sa}Phe **4a** (2.0 mg, 4.3 μmol)

(16) Pocker, Y.; Stone, J. T. *Biochemistry* **1968**, *7*, 3021−3031.

and Phe$_{sa}$Gly **4b** (1.6 mg, 4.3 μmol) were dissolved in 20 mL of 10 mM KH_2PO_4 buffer, pH 7.5. Carbonic anhydrase (CA) (0.4090 g, 13.6 μmol, 1.60 equiv) was dissolved in this solution and placed in a dialysis bag (the bag was washed in ddH_2O for 1 h, rinsed in EtOH once, and then washed again with ddH_2O). The bag was suspended in 20 mL of the phosphate buffer containing Pronase from *S. griseus* (5.0 mg, 0.01 equiv) in a 150-mL beaker and shaken at 150 rpm at 30 °C for 313 h. Samples (1 mL) were taken periodically from inside, worked up as above, and analyzed by HPLC. After 193 h, only 71% of **4a** had hydrolyzed, while 93% of **4b** had hydrolyzed.

Hydrolysis of EtOC-Phe$_{sa}$-Leu (4c) and EtOC-Phe$_{sa}$-Phe (4a) in the Absence of Carbonic Anhydrase. A solution of **4a** (2.9 mg, 6.3 mol) and **4c** (2.4 mg, 5.6 mol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. The first portion was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). Pronase from *S. griseus* (Sigma P-5147, 4 mg) was dissolved in the second portion, and the dialysis bag was then suspended in the resulting solution. The reaction vessel was then shaken gently (200 rpm) at 30 °C, and aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC using a Zorbax C8 column. After 30 min, neither substrate was detectable in the solution outside the dialysis bag. After 8 h, 86% of **4a** and 88% of **4c** inside the dialysis bag had hydrolyzed.

Hydrolysis of EtOC-Phe$_{sa}$-Leu (4c) and EtOC-Phe$_{sa}$-Phe (4a) in the Presence of Carbonic Anhydrase. A solution of **4a** (2.9 mg, 6.3 mol) and **4c** (2.4 mg, 5.6 mol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.14 g, approx 4.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). Pronase from *S. griseus* (Sigma P-5147, 4 mg) was dissolved in the second portion, and the dialysis bag was then suspended in the resulting solution. The reaction vessel was then shaken gently (200 rpm) at 30 °C, and aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC. After 6 h, 93% of **4c** had hydrolyzed, while only 58% of **4a** was hydrolyzed.

Hydrolysis of EtOC-Phe$_{sa}$-Phe (4a), EtOC-Phe$_{sa}$-Gly (4b) EtOC-Phe$_{sa}$-Leu (4c), EtOC-Phe$_{sa}$-Pro (4d) and EtOC-Phe-Phe (5), in the Presence of Carbonic Anhydrase. Phe$_{sa}$Phe **4a** (2.0 mg, 4.3 μmol), Phe$_{sa}$Gly **4b** (1.6 mg, 4.3 μmol), Phe$_{sa}$Leu **4c** (1.9 mg, 4.3 μmol), Phe$_{sa}$-Pro **4d** (1.8 mg, 4.3 μmol), and PhePhe **5** (1.7 mg, 4.3 μmol) were dissolved in 20 mL of 10 mM KH_2PO_4 buffer, pH 7.5. Carbonic anhydrase (CA) (0.7670 g, 25.6 μmol, 1.20 equiv) was dissolved in this solution and placed in a dialysis bag (the bag was washed in ddH_2O for 1 h, rinsed in EtOH once, and then washed again with ddH_2O). The bag was suspended in 20 mL of the phosphate buffer containing Pronase from *S. griseus* (4.9 mg, 0.01 equiv) in a 150-mL beaker and shaken at 150 rpm at 30 °C for 193 h. Samples (1 mL) were taken, worked up as above, and analyzed by HPLC. Data for this experiment is shown in Figure 8.

Acknowledgment. We thank Merck Frosst Inc., BioChem Pharma Inc., Boehringer Ingelheim Inc., AstraZeneca Inc., and FCAR for generous funding of this research. J.D.C. thanks NSERC for a postgraduate fellowship. The reviewers are acknowledged for helpful comments and suggestions.

Supporting Information Available: A derivation of eq 3 and characterization data for **3a,c,d** and **4a,c,d** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA017099+