NOVEL METHODS IN CHROMATOGRAPHIC SEPARATIONS:

NEW CHIRAL STATIONARY PHASES AND

MOLECULAR TAGGING

By

Jordan Danielle Haney Blodgett

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry

December, 2004

Nashville, Tennessee

Approved:

Professor David M. Hercules

Professor Prasad L. Polavarapu

Professor Ned A. Porter

Professor David W. Wright

ACKNOWLEDGEMENTS

This work would not have been possible without the support from the National Institutes of Health, and the Vanderbilt Discovery Grant. I would also like to acknowledge the trust that my advisor Dr. Tingyu Li gave me in order to work independently on such interesting projects both while he was at Vanderbilt, and thereafter. He has been a true mentor, and I have been lucky to have worked for him. I would also like to acknowledge the Vanderbilt Department of Chemistry under the leadership of both Dr. David Hercules and Dr. Ned Porter for the endless support that I received when circumstances became difficult. Without such nurturing surroundings, my accomplishments would have not been possible. I am grateful to Dr. Ned A. Porter for sharing his group with me, and for giving me a forum to share my research. I am also grateful to the Porter group for making me feel at home. I am grateful to Dr. Brian O. Bachmann and his group for sharing his laboratory and group with me. Their support has made my success possible. I am also grateful to the various graduate students who shared their labs and expertise with me. Lisa Sullivan and Brett Meredith truly made the silyl chloride protective group happen, and they were always available for the exchange of knowledge and for friendship. I also acknowledge my wonderful husband for always being there with patience and understanding when I needed it. He shares all my successes.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	degrees Celsius (degrees Centigrade)
α	separation factor
γAbu, Abu	4-aminobutyric
μm	micron, micrometer
μmol	micromole; micromolar
μl	microliter
AA, Aa	amino acid
Ac	acetyl
AcOH	acetic acid
Ac_2O	acetic anhydride
Ala	alanine
AMPS	aminomethylated polystyrene
APS	3-aminopropylsilica gel
Asp	asparagine
Boc	*t*-butoxycarbonyl
Bz	benzoyl
Bn	benzyl
cm	centimeter
DCM	dichloromethane; methylene chloride
DIC	diisoproylcarbodiimide

DIPEA	*N,N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DNB, Dnb	3,5-dinitrobenzoyl
DNB-OH	3,5-dinitrobenzoic acid
EOF	electroosmotic flow
EtOH	ethanol
Fmoc	9-fluorenylmethoxycarbonyl
g	gram(s)
Gly	glycine
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetra methyluronium hexafluorophosphate
HCl	hydrochloric acid
^{1}H NMR	proton nuclear magentic resonance spectroscopy
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IPA	isopropyl alcohol, 2-propanol
k'	retention factor, in HPLC
LA	lithocholic acid
LABE	lithocholic acid benzyl ester
LABEME	lithocholic acid benzyl ester methyl ether
LC	liquid chromatography

Leu	leucine
M	molar, molarity
MeOH	methanol
mg	milligram(s)
mm	millimeter(s)
MW	molecular weight
ml	milliliter
Mtt	methyltrityl
nm	nanometer
NMM	*N*-methylmorpholine
Phe	phenylalanine
Pro	proline
psi	pounds per square inch
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
T	temperature
t_r	retention time
TBAI	tetra-butyl ammonium iodide
*t*bu	*tert*-butyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Trp	tryptophan

Tyr	tyrosine
UV	ultraviolet

CHAPTER I

PARALLEL COMBINATORIAL LIBRARY SCREENING TO DETERMINE SELECTORS FOR CHIRAL LIQUID CHROMATOGRAPHY

Introduction

The importance of the handedness of molecules is of great interest in chemistry. Biological systems depend on their enzymes and receptors to interact with and use chiral molecules selectively in different manners depending on their individual handedness. It is for this reason that it is necessary to study each enantiomer individually for many applications. This necessity creates the demand for methods that can efficiently separate and analyze the enantiomers of chiral compounds. An important example of the industrial importance of chiral separations is the pharmaceutical industry's dependence on their ability to study the biological effects of each enantiomer of potential drugs for clinical trials. Chiral drug sales of single enantiomer chiral drugs rose more than 13% in the year 2000 making it a $133 billion industry. 40% of all drug sales in 2000 were of single enantiomer prescriptions. The rise in single enantiomer drug formulations is due in part to the increased capabilities in chiral synthesis and chiral separations. [1] Of the possible methods for obtaining enantiomerically pure compounds, chromatographic techniques are most commonly employed for both analytical and preparative chiral separations.

High-performance liquid chromatography (HPLC) using chiral stationary phases (CSPs) in the separation of mixtures of enantiomers has found widespread use in many applications. Many types of enantioselective stationary phases have been utilized for chiral separations[2] including those based on cyclodextrins[3], macrocyclic antibodies[4], polysaccharides[5, 6], and the brush-type Pirkle style columns[7]. The brush-type Pirkle style CSPs will be the focus of the following discussions, and are typically peptide-based.[8, 9] Development of an effective brush-type CSP for a given analyte has proven to be a difficult task if approached empirically. It is for this reason that combinatorial techniques are becoming popular tools for the fast and effective development of CSPs.

Combinatorial techniques are characterized by three main requirements: (1) the method must generate a large number of compounds in a relatively short amount of time; (2) the method must encompass a high-throughput screening method that in turn determines the activities of the member of the library; (3) and the method must include deconvolution steps in order to determine the exact structure of the library member responsible for the activity found during screening.[10] There are several approaches to attain all three of the aforementioned requirements for combinatorial techniques. There is the one-bead one-selector library approach, the mixture library approach, and the parallel library approach. The latter two can be on bead or in solution. The one-bead one-selector approach involves the pool and split synthesis strategy. In this strategy, a solid support is divided into portions and each portion undergoes

some reaction. These portions are pooled and mixed, then divided into portions again to undergo the second reaction. This sequence can be repeated as many times as necessary to incorporate the required modules. The advantage of this approach is the possibility to impart an extraordinary amount of diversity into the final library. [11, 12] The library can then be screened and subsequently deconvoluted. One possible drawback for this technique is the complicated deconvolution steps that must follow screening in order to elucidate which library member exhibits the favorable response. Often this type of library is encoded or tagged in order to expedite the deconvolution process.

The mixture library approach involves the synthesis of many library members and the subsequent pooling of the members for screening. The mixture library can be in solution [13, 14], or immobilized onto a support [15]. Regardless of which screening approach is utilized, there are several deconvolution steps that follow. To deconvolute mixture libraries one generally synthesizes library sub-sets and screens these sub-sets. After the sub-set containing the "active" member is identified, sub-sets of that subset must be made and screened. When the necessary iterations of this procedure are completed, the active library member will be identified. The advantage of this approach is that it is possible to screen very large libraries in this manner. The disadvantages are that many deconvolution steps are required, and in synthesizing the many subsets needed the researcher expends much time and unnecessary reagent resources.

The parallel library approach has become a very popular combinatorial approach due to its straightforward design and deconvolution requirements. [10, 16-20] These libraries are synthesized in parallel, meaning the members are not pooled and split but instead remain in a spatially addressable array for the entire synthesis and screening procedures. This can be easily accomplished in a 96-well plate array. The deconvolution in order to identify the member responsible for the desired response simply requires matching its position in the array to the template used in designing the synthesis. The key advantages to this approach are the ease of ascertaining the members' identities and the simple synthesis design. The disadvantage to this approach is that it is much more difficult to achieve libraries comprised of the extremely large numbers of members that are routinely found in mixture libraries.

The Li group has effectively demonstrated the feasibility of using both mixture and parallel libraries in order to develop new brush-type chiral stationary phases for use in chiral HPLC. The mixture library approach will be discussed first. An all L-(1-naphthyl)leucine and an all D-(1-naphthyl)leucine stationary phase was synthesized and packed into a column for screening a 16 member small peptide mixture library of all L stereochemistry and all D stereochemistry in solution using HPLC. The resulting chromatograms showed differences in the retention times for the library members, indicating that one or more of the library members must interact differently with the two stationary phases. Several sub-libraries were then synthesized, deconvoluting the library and elucidating the

members that are possible chiral selectors. This approach takes advantage of the principle of reciprocity described by Pirkle, Welch, and Lamm. [7, 19, 21] It has been shown that if a single enantiomer of a chiral selector shows specific affinity for one enantiomer of a compound then the reciprocal will most likely be true as well. Through the principle of reciprocity the library "hits" are shown upon immobilization onto silica gel and their subsequent use as an HPLC stationary phase to successfully separate the (1-naphthyl)leucine analyte. The Li group later demonstrated an alternative approach to this mode of screening for mixture libraries. Some problems with the previous screening method include the need to synthesize two enantiomerically pure libraries of opposite chiral orientation, which can be difficult depending on the availability of each enantiomer. Also typical chiral columns exhibit poor column efficiency thus limiting the possible library size for the previous screening method. The newer screening method involves the immobilization of the two enantiomers of the racemic analyte of interest separately and the synthesis of one library of enantiomerically pure possible chiral selectors. An equal amount of the mixture library is allowed to equilibrate with equal amounts of the stationary phases under identical conditions. The library members that were adsorbed onto each stationary phase are evaluated using HPLC and any differences in intensity of peaks at the same retention time indicate the presence of an effective chiral selector. [13]

Li and Wang first demonstrated the feasibility of a screening method involving a parallel combinatorial library with a model study. [17] A 16-member

library was synthesized using Fmoc solid phase peptide chemistry on aminomethylated polystyrene resin using the Hi-top manual synthesizer system. For synthesis the resin was added to the wells of a 96-well filter plate. This made the subsequent washings after each synthesis step much easier as the solvent flows through a membrane at the bottom of each well into a waste reservoir. The library consisted of two modules attached to the resin by a 4-aminobutyric acid linker. Module 1 was one of four aromatic acid end groups and module 2 was one of four amino acids, including glycine as an achiral control. The resin was then equilibrated with the racemic analyte N-(1-naphthyl)leucine ester. The ellipticity of the supernatant was measured using circular dichroism (CD). DNB-L-Leu and DNB-L-Ala were indicated as good potential chiral selectors by this method. These two selectors were synthesized and attached to aminopropyl silica gel (APS). The racemic N-(1-naphthyl)leucine ester was found to be resolved well by each selector: DNB-L-Leu gave a separation factor of 12 and DNB-L-Ala gave a separation factor of 4.7. The advantages of this method over those previously described in the literature is that solid phase peptide synthesis is expected to be much more reliable on resin than on silica gel. Some limitations are that CD is not applicable with analytes without an appreciable CD signal. Another concern is that this technique may miss some selectors with lower separation factors, but we are primarily interested in large separation factors.

The Li group then developed a 200-member library on the premise already proven by the parallel library model study. [13] This dipeptide library was

comprised of three modules attached to APS by 4-aminobutyric acid as in the model study. The end-group module was either a 3,5-dinitrobenzoyl group or an acetyl group. Modules 2 and 3 consisted of one of 10 amino acids. The analyte is also the same N-(1-naphthyl)leucine ester. The amino acids chosen for modules 2 and 3 were chosen based on the possible sources of interaction of the analyte molecule which contains an electron rich aromatic ring along with hydrogen bond donor and acceptor groups. The synthesis and screening methods were the same as those used in the model study discussed above except that the equilibration time was shortened from 24 hrs to ~3hrs by incorporating chloroform into the solvent system. It was determined that library members possessing the DNB end group were much more successful as chiral selectors than those with the acetyl end group. This indicates that the π-π interactions between the selector and analyte are indeed important for enantioselective recognition. Other possible interactions include steric repulsion and hydrogen bonding. Seven CSPs were synthesized and packed into columns for HPLC analysis of the target analyte and gave separation factors varying from 1.4 to 18 using chloroform-hexane mobile phases. The 200-member library study showed that the combinatorial approach for development of new CSPs indeed produced valid selectors for the analyte screened. The 200-member library was also screened to develop a chiral selector for 2,2,2-triflouroanthrylethanol (TFAE), and *tert*-butyl-(2-methyl)naphthyl phosphine oxide (TBNPO), but with very little success.

In an effort to improve chiral separations, we expanded upon what was learned in the 16-member model study and the 200-member library study in order to develop a new library designed to separate TFAE and TBNPO. The structures for the analytes are shown in Figure 1-1. A new 81-member library was designed with special attention being given to the choices for the modules used in order to develop chiral selectors that offer better selectivity for these analytes. The template for the new library involves three modules just like in the 200-member library and is shown in Figure 1-2 to be a dipeptide library including two amino acid modules and a DNB end group. The amino acid modules can be one each of nine different amino acids and include several that were not included in the 200-member library. D-asparagine (D-Asn), trans-4-hydroxy-L-proline (Hyp), and L-histidine (His) are the new amino acids used and were chosen due to their increased capacity to participate in hydrogen bonding. In addition, D-Asn adds the element of the reversed stereochemistry.

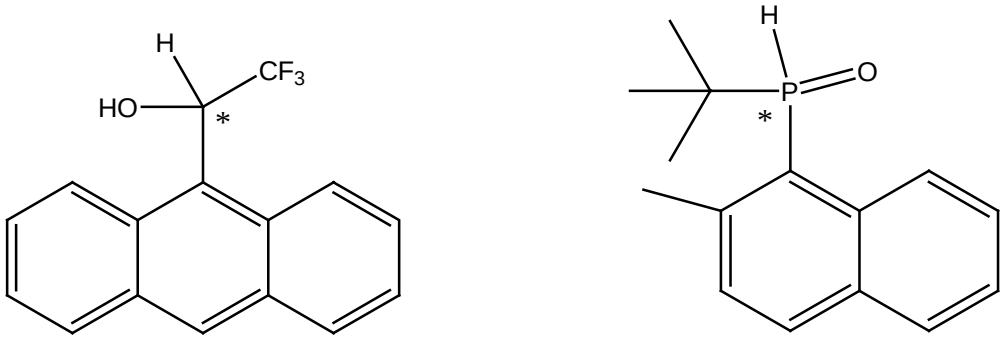


Figure 1-1. Structures of the racemic analytes TFAE and TBNPO.

Experimental

Chemicals and Supplies

R-TFAE and *S*-TFAE were used as received from Aldrich (Milwaukee, WI, USA). Racemic TBNPO was obtained as a gift. All Fmoc-protected amino acids and solid phase synthesis resins were from Novabiochem (San Diego, CA, USA). The 96-well filter plates were from Whatman Polyfiltronics (Clifton, NJ, USA), and the 96-well collection plates were from Sun International (Wilmington, NC, USA). Both types of plates had a well volume of 2 ml and were constructed of polypropylene. The Combiclamp apparatus used to facilitate library synthesis as well as library incubation with the racemic analytes of interest was from Whatman Polyfiltronics. To aid in rinsing the resin between synthetic steps, an aluminum vacuum device from Affymax (Santa Clara, CA, USA) was used. To assist with recovery of the analyte solution from the filter plate after incubation, a filtration module from Affymax was employed.

DNB-81 Template:

DNB-[Module2]-[Module1]-Abu-AMPS

Modules 1 and 2 are among the following amino acids:

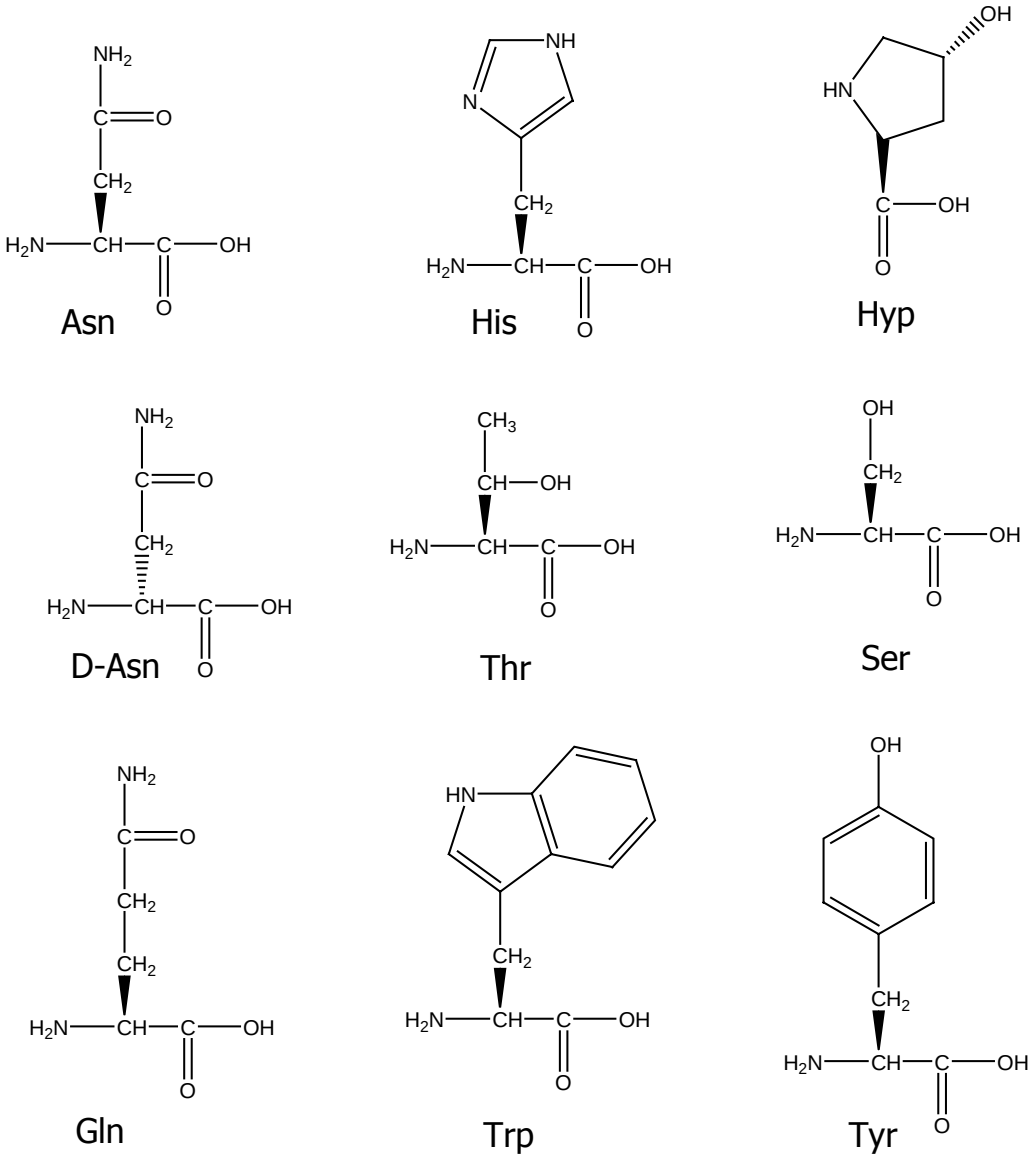


Figure 1-2. Template and amino acid modules of DNB-81 library.

Instrumentation

All chromatographic analyses were completed on a Beckman (Fullerton, California, USA) analytical system equipped with System Gold software and a monochromatic UV detector. All hardware for preparing the HPLC columns was from Isolation Technologies (Hopedale, Massachusetts, USA), and an Alltech column packer. Atlantic Microlab, Inc.(Norcross, Georgia, USA) provided all elemental analysis. All stationary phases were packed into empty HPLC columns (4.6 mm i.d. x 5 cm) using an ethanolic slurry.

Calculations

Separation factors (α values) were determined using Formula (1).

$$\alpha = k'_2/k'_1 \qquad (1)$$

Retention factors (*k'*) were calculated according to Formula (2). For all the chiral stationary phases, the void volume (t_0) was estimated to be 0.42 min for a flow rate of 1.2

$$k' = (t_r - t_0)/t_0 \qquad (2)$$

ml/min, and .250 min for a flow rate of 2.0 ml/min using 1,3,5-tri-t-butylbenzene as probe analyte.

Synthesis of the DNB-81 Library

The synthesis procedure for this library follows the one used to produce the 200-member library and is shown in Figure 1-3.[15] After swelling in DCM for

30 minutes, AMPS resin (7.0 g, 0.36 mmol loading) was treated with Fmoc-γAbu-OH (4 equiv), DIC (4 equiv) and DIPEA (4 equiv) in 1:4 DMF/DCM for 4 hours. Once the reaction was complete, the resin was washed with DMF, IPA, and DCM (2 x 50 ml each) and allowed to dry thoroughly at room temperature. The resin was then divided into 80 mg portions and placed into 81 individual wells of a 96-well filter plate. The Fmoc-Abu-AMPS resin was then deprotected with 20% piperidine/DMF for 30 minutes, followed by rinsing with DMF, IPA, and DCM (2 x 1 ml per well).

The next step was incorporation of module 1, the first amino acid module. After placing the filter plate into the Combiclamp, a 0.5 ml aliquot of the Fmoc-protected amino acid (3 equiv) dissolved in DMF was placed in each well, followed by DIPEA (4 equiv) and a 0.5 ml aliquot of a mixture of PyBOP (2 equiv) and HOBt (2 equiv) in DMF. As 9 different amino acids comprise this module, each amino acid was reacted with resin in 9 wells. After shaking for 2 hours, the resin was rinsed as described previously. About 5 mg resin was removed from several different wells and tested using ninhydrin solution in order to determine complete surface coverage. If complete surface coverage was not obtained, the reaction was repeated as necessary.

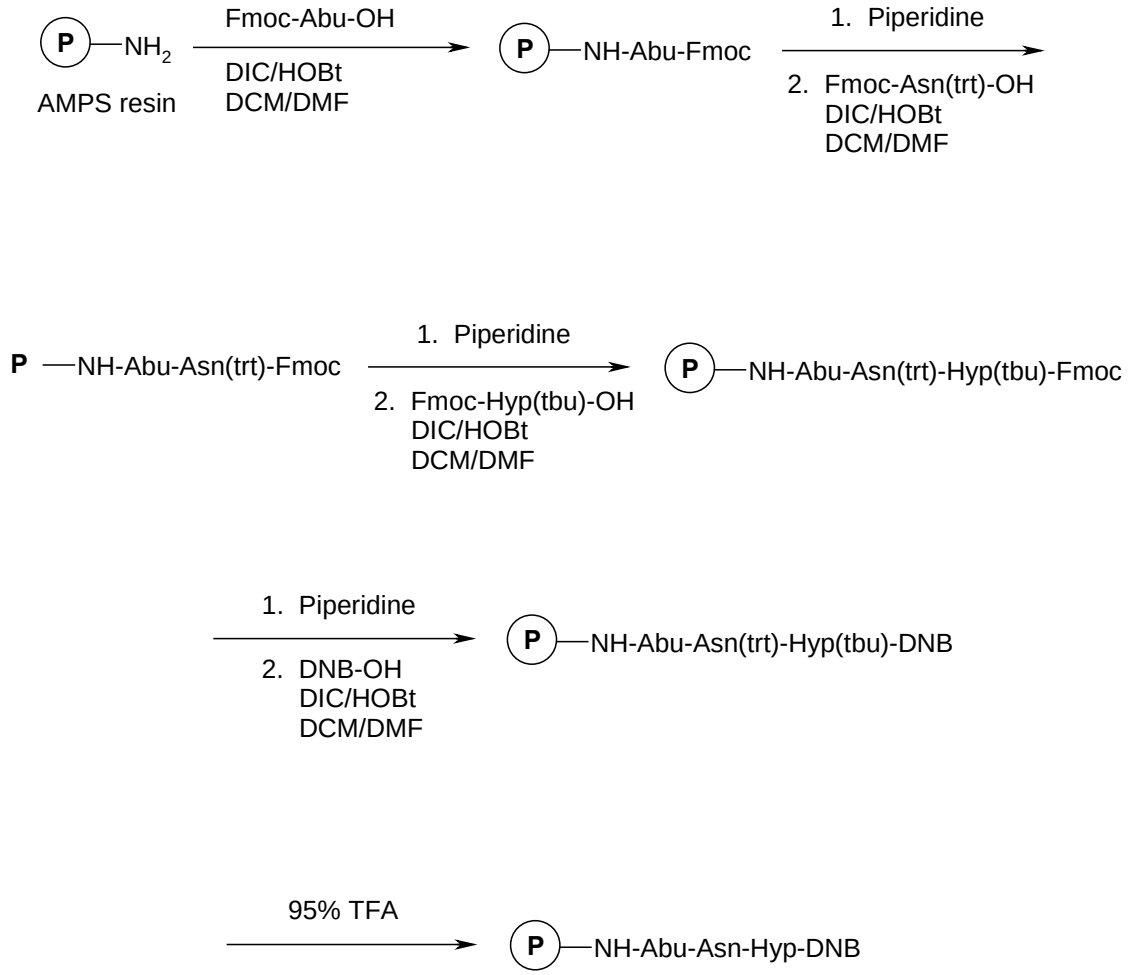


Figure 1-3. Example synthesis scheme for preparation of DNB-81 library. AMPS-Abu-Asn-Hyp-DNB is shown as a representative for the other members.

Once module 1 was successfully attached to the resin, deprotection of the Fmoc groups from these amino acids was accomplished using 20% piperidine/DMF as described previously. Next, incorporation of module 2, consisting of the same 9 amino acids, was completed. This step was done in the same manner as module 1; however, addition of the amino acid was varied in order to produce 81 unique library members. As before, ninhydrin testing of a few of the wells was carried out to ensure complete surface modification.

After removal of the Fmoc groups from module 2, the final link involved addition of 3,5-dinitrobenzoic acid (DNB-OH). DNB-OH (3 equiv) was placed in each well as a 0.5 ml aliquot in DMF, followed by DIPEA (4 equiv) and a 0.5 ml aliquot of a mixture of PyBOP (2 equiv) and HOBt (2 equiv) in DMF. After shaking for 2 hours, the library members were rinsed as before. This step was repeated twice more to ensure complete incorporation of the DNB functionality to the surface of the resin. Finally, in order to remove any protecting groups from the amino acid side chains, a solution of 95% TFA, 2.5% H_2O, and 2.5% triisopropylsilane was introduced. After shaking for 1 hour, the library members were rinsed as described earlier. At this stage, the library was ready for incubation and screening using the racemic analyte of interest.

General procedure for selector synthesis

The general protocol used to prepare each selector in larger quantities in order to prepare the appropriate chiral stationary phases is outlined in Figure 1-4. The only variation among the selectors is the choice of module at each step.

DNB-D-Asn(trt)-Thr(tBu)-Abu-OH: ^{1}H NMR (DMSO-d_6) δ 0.85-0.88 (d, 3H), 0.9 (s, 9H), 1.1-1.2 (m, 2H), 1.4-1.6 (t, 2H), 2.0-2.2 (m, 2H), 2.9 (br, 1H), 3.8 (m, 1H), 4.0 (m, 1H), 4.9 (m, 1H), 7.0 (s, 15H), 7.5 (t, 1H), 7.6 (d, 1H), 7.8 (s, 1H), 8.5 (s, 1H), 8.8-9.0 (m, 3H), 9.4 (br, 1H).

DNB-D-Asn(trt)-Thr(tBu)-NH-$(CH_2)_{10}$-OH: ^{1}H NMR (DMSO-d_6) δ 1.0 (s, 12H), 1.2 (m, 14H), 1.4-1.5 (br, 6H), 2.1 (t, 2H), 3.9 (m, 1H), 4.1 (br, 2H), 4.8 (br, 2H), 7.0-7.2 (m, 15H), 8.0 (m, 2H), 8.9 (s, 1H), 9.0 (m, 1H), 9.2 (m, 2H).

Synthesis of Fmoc-Abu-Rink Acid Resin

Rink Acid resin (0.6 mmol/g loading) was swollen with DCM containing 0.1% v/v DIPEA (30 ml for 30 min). After solvent removal, the resin was modified with Fmoc-γAbu-OH (12 mmol) using DIC (12 mmol), DMAP (1 mmol), and NMM (6 mmol) in 20% DMF/DCM (30 ml). After the mixture was agitated for 5 hours, the resin was collected by filtration and washed using DMF, IPA, and 0.1% DIPEA/DCM (2 x 20 ml each). The surface concentration of Fmoc-γAbu groups on the resin was found to be approximately 0.57 mmol/g, as determined by UV monitoring of the Fmoc chromophore after cleavage. This reaction was repeated as necessary to make all selectors of interest. Fmoc-Ahx-rink acid resin and Fmoc-Aoc-rink acid resin were synthesized in exactly the same fashion. The synthesis of the resin using other linkers was done similarly.

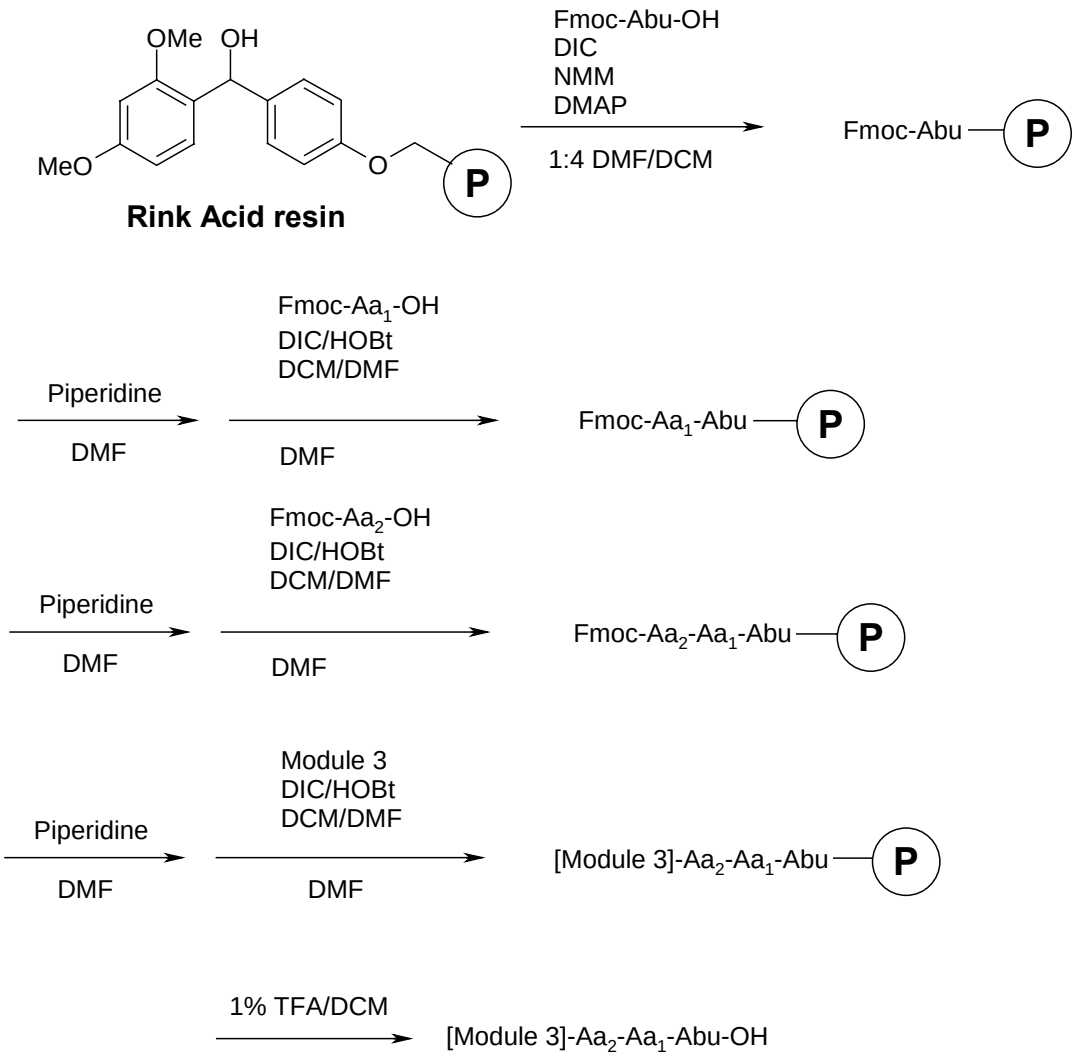


Figure 1-4. Selector synthesis on acid labile rink acid resin for subsequent attachment to APS.

Synthesis of Selector [Module3]-Aa_2-Aa_1-Abu-OH

The first step of this reaction involved treating Fmoc-γAbu-O-Rink acid resin with DMF (30 ml for 15 minutes) to induce swelling. After solvent removal, the resin was treated with 20% v/v piperidine in DMF for 30 minutes for removal

of surface Fmoc groups. The deprotected H_2N-γAbu-O-Rink acid resin was rinsed with DMF, IPA, and 0.1% DIPEA/DCM (2 x 20 ml each). Qualitative confirmation of the presence of free amino groups was completed using ~5 mg resin and ~100 μl ninhydrin solution (0.5 g in 100 ml EtOH) followed by heating for 1 min at 90°C. A resin color change from white to deep blue indicated surface free amino groups.

After Fmoc removal, Fmoc-Aa_1-OH (3 equiv) was coupled to the resin using PyBOP (2 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in DMF (15 ml). Following agitation for 2 hours, the resin was collected and washed with DMF, IPA, and 0.1% DIPEA/DCM (2 x 20 ml each). A negative ninhydrin test confirmed that all free amino groups had been modified.

After Fmoc-Aa_1-Abu-O-Rink acid resin was treated with 20% piperidine in DMF to remove surface Fmoc groups, Fmoc-Aa_2-OH (3 equiv) was coupled to the resin using PyBOP (2 equiv), HOBt (2 equiv), and DIPEA (4 equiv) in DMF (15 ml). After shaking the mixture for 2 hours, the resin was collected and washed with DMF, IPA, and 0.1% DIPEA/DCM (2 x 20 ml each). As with the attachment of Fmoc-Aa_1-OH, a negative ninhydrin test confirmed that all free amino groups had been modified. Other selectors that were immobilized onto silica gel were synthesized on rink acid resin in exactly the same manner with the obvious changes in reagents for different modules.

Preparation of acid-washed silica gel stationary phase

HPLC grade silica gel from Alltech was subjected to an acid washing procedure to remove impurities before further chromatographic use. To accomplish this, a mixture of silica gel (10 g), concentrated sulfuric acid (40 ml), and concentrated nitric acid (10 ml) was refluxed overnight. After cooling the mixture, the silica gel was collected by vacuum filtration and rinsed with water (20 x 100ml), methanol (3 x 50ml), and dichloromethane (2 x 50ml), followed by placement in the oven at 150°C for 24 hours. The silica gel was subsequently stored in a desiccator at room temperature until further use.

To prepare a normal phase column, acid-washed HPLC grade silica gel was placed in a stainless steel column (4.6 mm i.d. x 5 cm) using a slurry packing method. After cleaning all column hardware by sonication in methanol for five minutes, one end of the column was assembled while the other end was attached to the slurry chamber of the column packer. Combining the silica gel (0.7 g) in ethanol (27 ml) followed by sonication for ten minutes produced a slurry that was immediately poured into the slurry chamber. After attaching the pressurizing solvent line to the slurry chamber, the nitrogen pressure was increased so that a pressure of 7000 psi was achieved. This pressure was maintained until approximately 75 ml of solvent was collected. Once the pressure was reduced, the column was removed and assessed for its chromatographic ability. The void volume for this column was determined to be 0.5 minutes using tri-t-butylbenzene (TTBB) as a void volume marker.[14]

Synthesis of 3-aminopropylsilica gel (APS)

10 g of high-purity silica gel (surface area 220 m^2/g, pore size 80 Å, particle size 5 μm) was placed in a round-bottom flask suspended in 40 ml toluene, the mixture was refluxed for 1 hour to remove surface-adsorbed water. The reaction was stopped temporarily to introduce ~8.0 ml 3-aminopropyltriethoxysilane and allowed to continue overnight. The silica was collected by filtration, rinsed with toluene, EtOH, and DCM (2 x 30 ml each), and placed in the oven at 60°C overnight. A desicator served to store the aminopropylsilica gel (APS) for long periods. Elemental analysis indicated C = 2.51%, H = 0.65%, N = 0.78%; the surface amino coverage based on %N was found to be 0.55 mmol/g.

Prior to the attachment of a selector to a column, the APS was subjected to a washing procedure to remove physisorbed silane from the surface. To accomplish this, 1.0 g APS was first treated with H_2O (1 x 20 ml), 0.1 M aqueous HCl (1 x 10 ml), then H_2O again (to neutrality) to remove nonbonded species from the surface. To deprotonate the remaining aminopropyl groups, the stationary phase was then washed with MeOH (2 x 20 ml), 10% TEA/MeOH (1 x 10 ml), MeOH again (to neutrality), and DCM (2 x 20 ml). Elemental analysis of the newly washed phase indicated C = 2.28%, H = 0.55%, N = 0.67%.

Synthesis of the chiral stationary phases using each selector

All selectors were attached to 3-aminopropylsilica gel (APS) using the same general procedure, as shown in Figure 1-5. The selector (1.5 equiv) was dissolved in 10 ml DMF, to which was added PyBOP (2 equiv), HOBt (1 equiv), DIPEA (3 equiv) and APS (typically 1.0 g). The mixture was stirred at room temperature for 4 h, after which the silica gel was collected by filtration and rinsed with DMF, IPA, and DCM (2 x 30 ml each). In order to mask any remaining free aminopropyl groups, Ac_2O (4 equiv) and DIPEA (4 equiv) in DMF (5 ml) were stirred with the silica gel at room temperature for 1 h. Afterwards, the silica was rinsed as described previously. A ninhydrin test showed there to be no detectable free amino groups present. Finally, in order to deprotect any side chains, a mixture of 95% TFA, 2.5% triisopropylsilane, and 2.5% water were added to the silica gel with stirring for 1 h. The silica was retrieved by filtration and rinsed with DCM, DMF, IPA, and MeOH (2 x 30 ml each), and the resulting stationary phase was allowed to dry at room temperature.

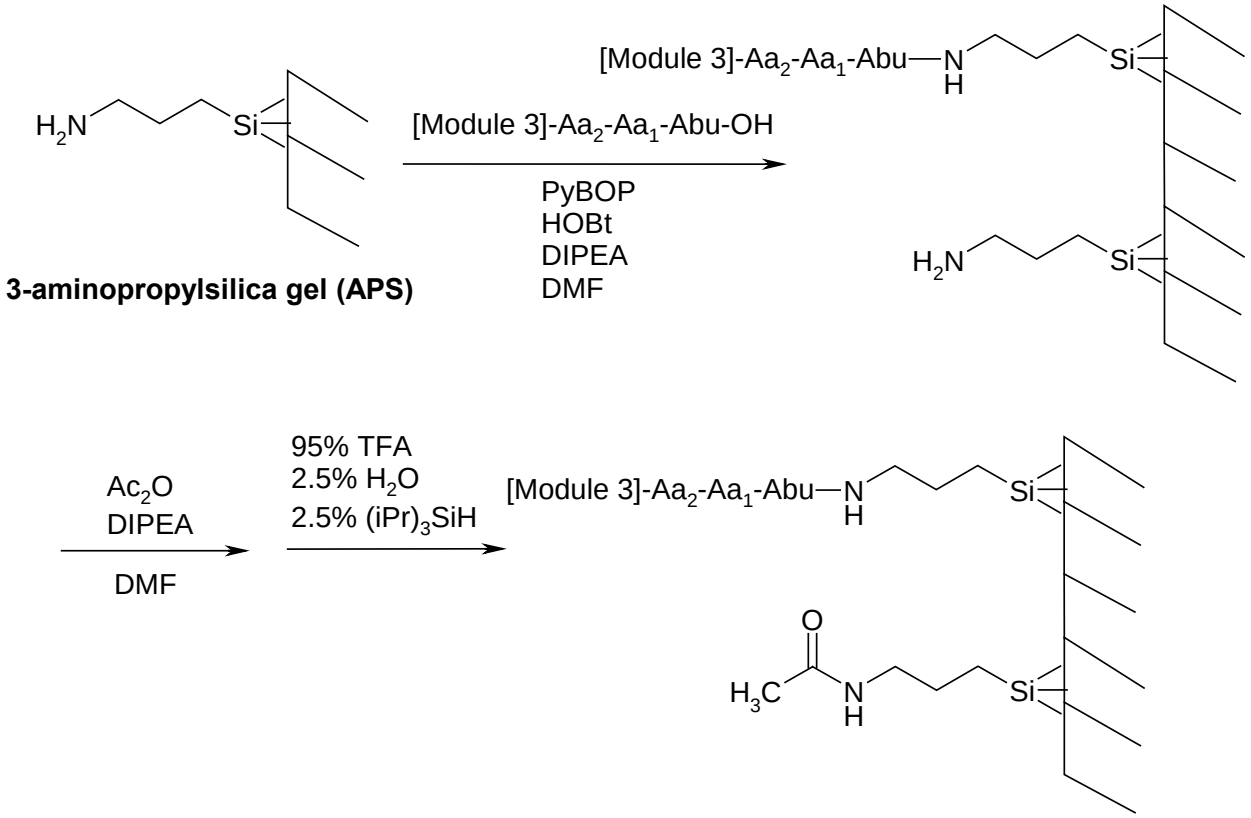


Figure 1-5. Immobilization of chiral selector on aminopropyl silica gel.

Synthesis of Fmoc-Aminooctanoic Acid (Aoc)

1 equiv of NaOH dissolved in a 1:1 solution of H_2O/THF, and 1.5 equiv of aminooctanoic acid is added to this solution. Then 1 equiv of Fmoc-OSu is added and the mixture is allowed to stir for 2 h. The mixture is then adjusted to a pH of 1 using concentrated HCl, achieving a phase separation followed by three ethyl acetate extractions. The product should partition into the ethyl acetate layer as the starting material stays behind in the aqueous layer. After the ethyl acetate containing the Fmoc octanoic acid is dried, the product can be collected via vacuum filtration. Synthesis of Fmoc-aminoundecanoic acid was done similarly.

Fmoc-aminooctanoic acid: ^{1}H NMR (DMSO-d_6) δ 1.2 (s, 6H), 1.5 (m, 4H), 2.1 (t, 2H), 2.9 (t, 2H), 4.1 (m, 1H), 4.2 (m, 2H), 7.35 (m, 2H), 7.4 (m, 2H), 7.7 (m, 2H), 7.9 (m, 2H).

Fmoc-aminoundecanoic acid: ^{1}H NMR (DMSO-d_6) δ 1.2 (s, 12H), 1.5 (m, 4H), 2.1 (t, 2H), 2.9 (t, 2H), 4.1 (m, 1H), 4.2 (m, 2H), 7.35 (m, 2H), 7.4 (m, 2H), 7.7 (m, 2H), 7.9 (m, 2H).

Results

Screening of DNB-81 Library with TFAE

The 81-member library (DNB-81) was then screened for possible chiral selectors with TFAE. As with previous libraries, DNB-81 was equilibrated with 0.8ml of a 0.2 mg/ml solution of racemic TFAE in both 1:4 IPA/heptane and 1:4 $CHCl_3$/heptane for 24 and 6h respectively. After the incubation was complete the supernatant was collected using a vacuum manifold. The filtrate was allowed to evaporate and was subsequently reconstituted in equal amounts of the solvent system to be used as the mobile phase in the HPLC screening. The reconstituted filtrate was then assessed using chiral HPLC with a DNB-Leu-Abu-APS column as this column was found to separate the TFAE enantiomers reasonably well and in a short period of time. A sample chromatogram is given in Figure 1-6. The racemates are not baseline separated, but since we are only looking for significant differences in the peaks corresponding to the enantiomers the screening method is adequate. The analyte is examined with a flow rate of 1.2ml/min at 254nm with 15% IPA/hexane and 20% $CHCl_3$/hexane mobile phases. The results from the screening are shown in Table 1 and the numbers correspond to the ratio of the differences in peak height of the two enantiomers. when the IPA/heptane solvent system was used for equilibration, no promising leads were discovered. When we switched equilibration solvents from 1:4 IPA/heptane to 1:4 $CHCl_3$/heptane more interesting results were found. Table 2

shows the screening results. The $CHCl_3$/heptane solvent system proves to be a better incubation solvent for this library and we surmise that there are several reasons for this. First, $CHCl_3$ is known to be a better solvent to induce resin swelling which in turn allows for greater accessibility of selector sites for the

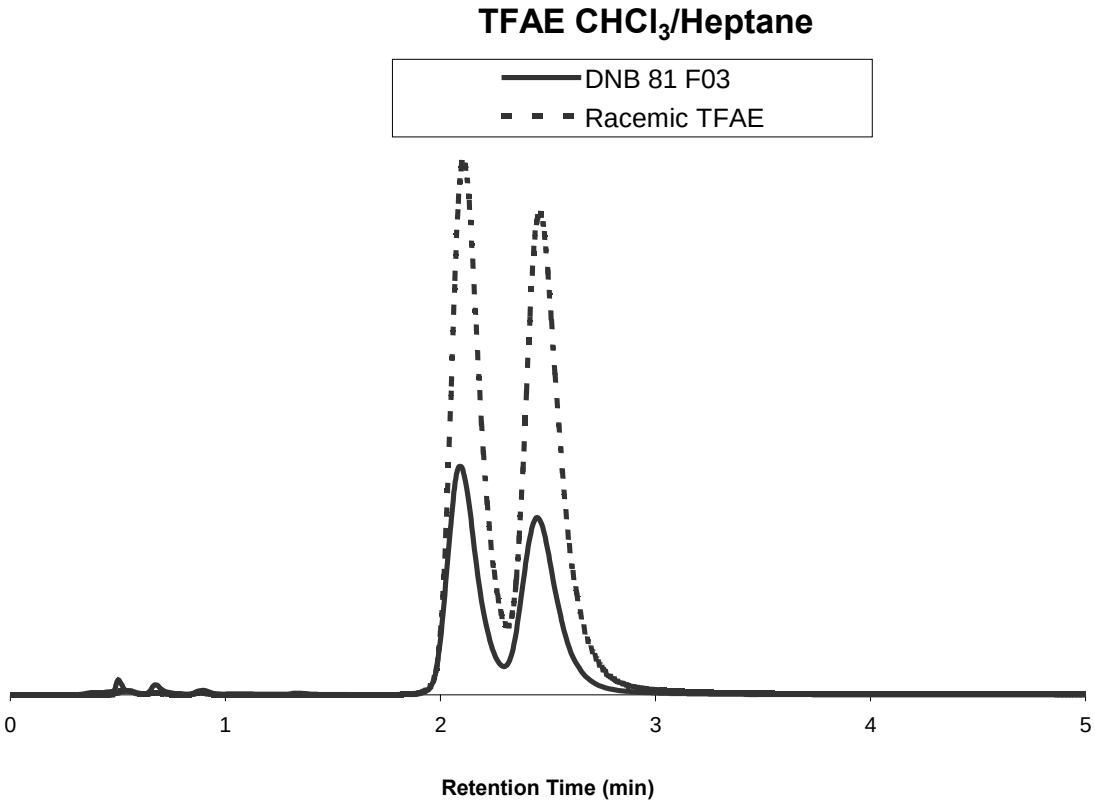


Figure 1-6. Sample chromatogram of TFAE from well f03 equilibration of DNB-81 library graphed over the racemic test.

analyte.[22] Also the library was designed to utilize hydrogen bonding for its enantioselective characteristics. IPA could disrupt the potential hydrogen bonding

between the selectors and the analyte due to its own hydrogen bonding capabilities.

Two"hits" from the library were synthesized according to Figure 1-4 and immobilized onto silica gel according to Figure 1-5 then assessed via HPLC. This fact that the "hits" identified for TFAE were based on modules not included in the 200-member library design indicates that the 81-member library is indeed an improved library design. DNB-D-Asn-Thr-Abu-AMPS gave a 16% difference in peak height and DNB-Hyp-Thr-Abu-AMPS gave a 20% difference in peak height ratio during screening. The two columns that were made and assessed were: DNB-D-Asn-Thr-Abu-APS (CSP 1) and DNB-Hyp-Thr-Abu-APS (CSP 2). The structures of CSP 1 and 2 are in Figures 1-7 and 1-8.

Assessment of TFAE with Selectors Identified from the DNB-81 Library

Table 3 shows the separation factors given by the chiral separation of TFAE by the two CSPs developed from the screening of the 81-member library using both the $CHCl_3$/hexane and the IPA/hexane solvent systems. DNB-Hyp-Thr-Abu-APS gave moderate separation in both IPA/hexane (α=1.22) and $CHCl_3$/hexane (α=1.31) mobile phases, but DNB-D-Asn-Thr-Abu-APS was not able to resolve the TFAE enantiomers in either solvent system. The poor performance by CSP 1 in comparison to CSP 2 is unfortunate, but can be explained, as it was a weaker library "hit" with a smaller difference in peak height ratio than CSP 2.

Table 1-1. Peak height ratios for TFAE in 1:4 IPA/heptane after 24 hr equilibration with DNB-81 library. Reference ratio:1.177

His-His 1.186	**His-D-Asn** 1.163	**His-Hyp** 1.210	**His-Trp** 1.168	**His-Ser** 1.212	**His-Thr** 1.171	**His-Asn** 1.177	**His-Gln** 1.179	**His-Tyr** 1.185
D-Asn His 1.181	**D-Asn D-Asn** 1.178	**D-Asn Hyp** 1.178	**D-Asn Trp** 1.193	**D-Asn Ser** 1.185	**D-Asn Thr** 1.181	**D-Asn Asn** 1.193	**D-Asn Gln** 1.174	**D-Asn Tyr** 1.187
Hyp-His 1.176	**Hyp D-Asn** 1.179	**Hyp-Hyp** 1.182	**Hyp-Trp** 1.167	**Hyp-Ser** 1.196	**Hyp-Thr** 1.168	**Hyp-Asn** 1.177	**Hyp-Gln** 1.179	**Hyp-Tyr** 1.194
Trp-His 1.204	**Trp D-Asn** 1.093	**Trp-Hyp** 1.171	**Trp-Trp** 1.175	**Trp-Ser** 1.182	**Trp-Thr** 1.161	**Trp-Asn** 1.175	**Trp-Gln** 1.181	**Trp-Tyr** 1.156
Ser-His 1.190	**Ser D-Asn** 1.175	**Ser-Hyp** 1.170	**Ser-Trp** 1.182	**Ser-Ser** 1.188	**Ser-Thr** 1.179	**Ser-Asn** 1.148	**Ser-Gln** 1.181	**Ser-Tyr** 1.185
Thr-His 1.184	**Thr D-Asn** 1.175	**Thr-Hyp** 1.196	**Thr-Trp** 1.188	**Thr-Ser** 1.180	**Thr-Thr** 1.193	**Thr-Asn** 1.174	**Thr-Gln** 1.162	**Thr-Tyr** 1.174
Asn-His 1.181	**Asn D-Asn** 1.114	**Asn-Hyp** 1.173	**Asn-Trp** 1.160	**Asn-Ser** 1.175	**Asn-Thr** 1.185	**Asn-Asn** 1.180	**Asn - Gln** 1.168	**Asn-Tyr** 1.161
Gln-His 1.177	**Gln D-Asn** 1.150	**Gln-Hyp** 1.192	**Gln-Trp** 1.141	**Gln-Ser** 1.171	**Gln-Thr** 1.187	**Gln-Asn** 1.178	**Gln-Gln** 1.184	**Gln-Tyr** 1.187
Tyr-His 1.188	**Tyr-D-Asn** 1.168	**Tyr-Hyp** 1.183	**Tyr-Trp** 1.176	**Tyr-Ser** 1.182	**Tyr-Thr** 1.181	**Tyr-Asn** 1.168	**Tyr-Gln** 1.175	**Tyr-Tyr** 1.199

Table 1-2. Peak height ratios for TFAE in 1:4 $CHCl_3$/heptane after 6 hr equilibration with DNB-81. Reference ratio:1.104

His-His 1.126	**His-D-Asn** 1.073	**His-Hyp** **1.298**	**His-Trp** 1.172	**His-Ser** 1.202	**His-Thr** 1.148	**His-Asn** 1.100	**His-Gln** 1.131	**His-Tyr** 1.180
D-Asn His 1.141	**D-Asn D-Asn** 1.072	**D-Asn Hyp** 1.220	**D-Asn Trp** 1.149	**D-Asn Ser** 1.198	**D-Asn Thr** 1.132	**D-Asn Asn** 1.143	**D-Asn Gln** 1.133	**D-Asn Tyr** 1.182
Hyp-His 1.129	**Hyp D-Asn** 1.045	**Hyp-Hyp** 1.167	**Hyp-Trp** 1.167	**Hyp-Ser** 1.165	**Hyp-Thr** 1.133	**Hyp-Asn** 1.149	**Hyp-Gln** 1.140	**Hyp-Tyr** 1.191
Trp-His 1.149	**Trp D-Asn** 1.008	**Trp-Hyp** 1.236	**Trp-Trp** 1.169	**Trp-Ser** 1.195	**Trp-Thr** 1.170	**Trp-Asn** 1.080	**Trp-Gln** 1.123	**Trp-Tyr** 1.183
Ser-His 1.117	**Ser D-Asn** 1.063	**Ser-Hyp** 1.265	**Ser-Trp** 1.218	**Ser-Ser** 1.203	**Ser-Thr** 1.186	**Ser-Asn** 1.052	**Ser-Gln** 1.141	**Ser-Tyr** 1.184
Thr-His 1.148	**Thr D-Asn** **1.285**	**Thr-Hyp** **1.328**	**Thr-Trp** 1.214	**Thr-Ser** 1.214	**Thr-Thr** 1.207	**Thr-Asn** 1.127	**Thr-Gln** 1.125	**Thr-Tyr** 1.136
Asn-His 1.127	**Asn D-Asn** 1.029	**Asn-Hyp** 1.162	**Asn-Trp** 1.105	**Asn-Ser** 1.185	**Asn-Thr** 1.153	**Asn-Asn** 1.118	**Asn - Gln** 1.114	**Asn-Tyr** 1.159
Gln-His 1.152	**Gln D-Asn** 1.018	**Gln-Hyp** 1.143	**Gln-Trp** 1.131	**Gln-Ser** 1.149	**Gln-Thr** 1.182	**Gln-Asn** 1.134	**Gln-Gln** 1.074	**Gln-Tyr** 1.138
Tyr-His 1.155	**Tyr-D-Asn** 1.062	**Tyr-Hyp** 1.197	**Tyr-Trp** 1.173	**Tyr-Ser** 1.193	**Tyr-Thr** 1.159	**Tyr-Asn** 1.136	**Tyr-Gln** 1.123	**Tyr-Tyr** 1.233

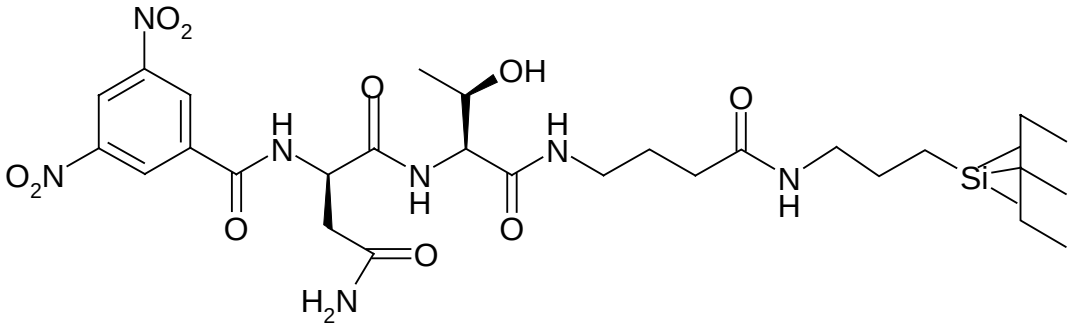


Figure 1-7. CSP 1 as developed from DNB-81 screening with TFAE. DNB- D-Asn- Thr-Abu-APS.

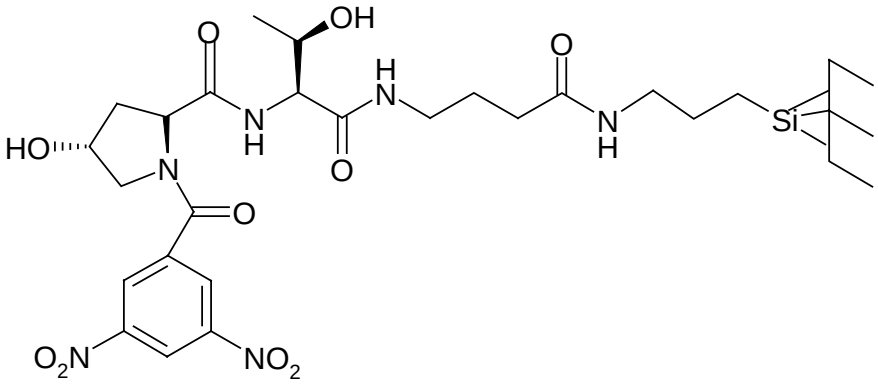


Figure 1-8. CSP 2 as developed from DNB-81 screening with TFAE. DNB- Hyp-Thr-Abu-APS.

Table 1-3. Chiral resolution of chiral selectors developed from DNB-81 library screening with TFAE.

Chiral Stationary Phase	Mobile Phase	Separation Factor (α)
DNB-D-Asn-Thr-Abu-APS	1:4 $CHCl_3$/heptane	1.00
	1:4 IPA/heptane	1.00
DNB-Hyp-Thr-Abu-APS	1:4 $CHCl_3$/heptane	1.31
	1:4 IPA/heptane	1.22

Screening of DNB-81 Library with TBNPO

The screening of DNB-81 with TBNPO was similar to that of TFAE except 0.8 ml of 0.3mg/ml TBNPO solution in both 1:4 IPA/heptane and 1:4 $CHCl_3$/heptane. The same equilibration times were used as with TFAE: 24hrs with 1:4 IPA/heptane and 6hrs with 1:4 $CHCl_3$/heptane, and the supernatant was collected via filtration and reconstituted as before. HPLC was also used in this case for screening, but the screening was done at 230nm rather than 254nm as with TFAE due to the fact that TBNPO did not give an adequate signal at 254nm. Also the mobile phase necessary for timely elution was 15% $CHCl_3$/hexane. The same chiral column was used as before for assessment of the library. A sample chromatogram including the racemic standard is given in Figure 1-9.

As with TFAE, the TBNPO equilibrations in IPA/heptane (shown in Table 1-4) produced no CSP leads, but when screened in $CHCl_3$/heptane the library (shown in Table 1-5) produced several leads. As before the leads all contain amino acid components that were not included in the 200-member library. Each lead contains a D or L-Asn adjacent to the DNB group. One of the lead members was also a hit when screening TFAE: DNB-D-Asn-Thr-Abu-AMPS. This is not surprising as TFAE and TBNPO have some of the same molecular features that we are taking advantage of for possible chiral recognition.

The DNB-D-Asn-Thr-Abu-APS selector was synthesized and assessed using two solvent systems analogous to the solvent systems used for screening. The results show that there is no separation using IPA/hexane, whereas with $CHCl_3$/hexane provided a moderate separation factor of 1.30. This further supports our theory on the influence of solvent choice on the efficacy of the CSP due to the disruption of hydrogen bonding by protic solvents. In the case of the $CHCl_3$/hexane mobile phase, however, it would be expected that the potential separation factor of TBNPO using CSP1 would be much higher considering the large difference in peak height ratio seen during screening. It is possible that the difference between the two supports used in screening and in the chromatography could be the problem. The screening support is a flexible, hydrophobic polymeric resin whereas the silica gel support for the

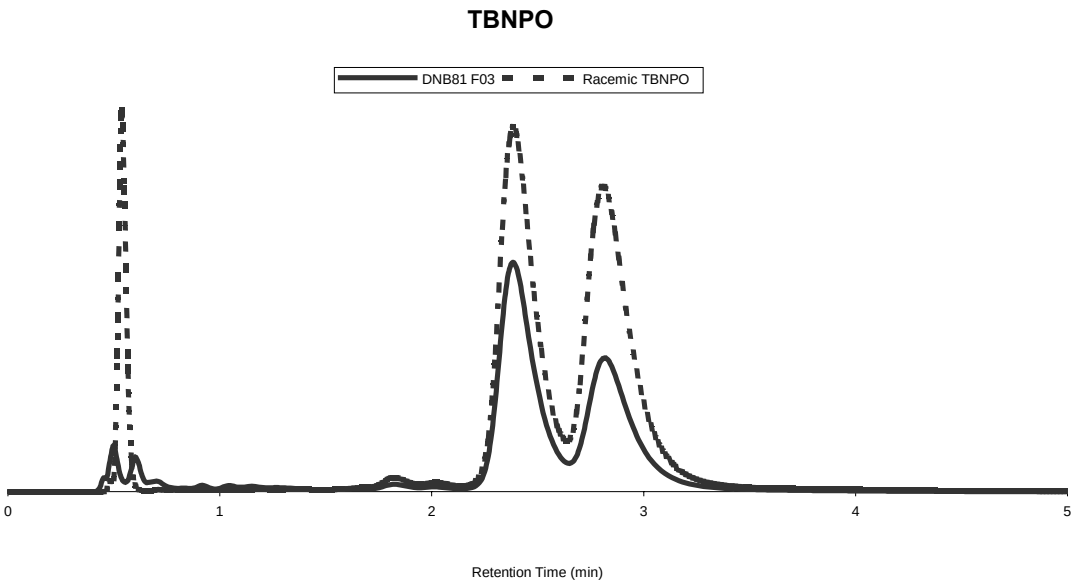


Figure 1-9. Sample chromatogram of TBNPO from well f03 equilibration of DNB-81 library graphed over the racemic test.

Table 1-4. Peak height ratios for TBNPO in 1:4 IPA/heptane after 24 hr equilibration with DNB-81. Reference ratio:1.190.

His-His 1.181	**His-D-Asn** 1.186	**His-Hyp** 1.178	**His-Trp** 1.184	**His-Ser** 1.199	**His-Thr** 1.206	**His-Asn** 1.168	**His-Gln** 1.175	**His-Tyr** 1.165
D-Asn His 1.198	**D-Asn D-Asn** 1.221	**D-Asn Hyp** 1.190	**D-Asn Trp** 1.188	**D-Asn Ser** 1.184	**D-Asn Thr** 1.194	**D-Asn Asn** 1.162	**D-Asn Gln** 1.184	**D-Asn Tyr** 1.177
Hyp-His 1.188	**Hyp D-Asn** 1.197	**Hyp-Hyp** 1.192	**Hyp-Trp** 1.200	**Hyp-Ser** 1.186	**Hyp-Thr** 1.197	**Hyp-Asn** 1.184	**Hyp-Gln** 1.183	**Hyp-Tyr** 1.179
Trp-His 1.192	**Trp D-Asn** 1.223	**Trp-Hyp** 1.184	**Trp-Trp** 1.173	**Trp-Ser** 1.184	**Trp-Thr** 1.184	**Trp-Asn** 1.156	**Trp-Gln** 1.178	**Trp-Tyr** 1.159
Ser-His 1.189	**Ser D-Asn** 1.203	**Ser-Hyp** 1.196	**Ser-Trp** 1.195	**Ser-Ser** 1.189	**Ser-Thr** 1.193	**Ser-Asn** 1.162	**Ser-Gln** 1.186	**Ser-Tyr** 1.174
Thr-His 1.192	**Thr D-Asn** 1.233	**Thr-Hyp** 1.189	**Thr-Trp** 1.186	**Thr-Ser** 1.180	**Thr-Thr** 1.196	**Thr-Asn** 1.158	**Thr-Gln** 1.177	**Thr-Tyr** 1.169
Asn-His 1.194	**Asn D-Asn** 1.206	**Asn-Hyp** 1.190	**Asn-Trp** 1.169	**Asn-Ser** 1.189	**Asn-Thr** 1.187	**Asn-Asn** 1.153	**Asn - Gln** 1.184	**Asn-Tyr** 1.170
Gln-His 1.182	**Gln D-Asn** 1.198	**Gln-Hyp** 1.189	**Gln-Trp** 1.222	**Gln-Ser** 1.200	**Gln-Thr** 1.168	**Gln-Asn** 1.167	**Gln-Gln** 1.178	**Gln-Tyr** 1.185
Tyr-His 1.156	**Tyr-D-Asn** 1.212	**Tyr-Hyp** 1.169	**Tyr-Trp** 1.177	**Tyr-Ser** 1.185	**Tyr-Thr** 1.201	**Tyr-Asn** 1.150	**Tyr-Gln** 1.155	**Tyr-Tyr** 1.169

Table 1-5. Peak height ratios for TBNPO in 1:4 $CHCl_3$/heptane after 6 hr equilibration with DNB-81. Reference ratio:1.193.

His-His 1.168	**His-D-Asn** 1.292	**His-Hyp** 1.135	**His-Trp** 1.191	**His-Ser** 1.218	**His-Thr** 1.279	**His-Asn** 1.121	**His-Gln** 1.152	**His-Tyr** 1.137
D-Asn His 1.135	**D-Asn D-Asn** ***1.489***	**D-Asn Hyp** 1.145	**D-Asn Trp** 1.145	**D-Asn Ser** 1.203	**D-Asn Thr** 1.371	**D-Asn Asn** 1.065	**D-Asn Gln** 1.224	**D-Asn Tyr** 1.131
Hyp-His 1.157	**Hyp D-Asn** 1.212	**Hyp-Hyp** 1.149	**Hyp-Trp** 1.209	**Hyp-Ser** 1.230	**Hyp-Thr** 1.334	**Hyp-Asn** 1.081	**Hyp-Gln** 1.177	**Hyp-Tyr** 1.173
Trp-His 1.155	**Trp D-Asn** ***1.506***	**Trp-Hyp** 1.128	**Trp-Trp** 1.177	**Trp-Ser** 1.269	**Trp-Thr** 1.300	**Trp-Asn** ***0.968***	**Trp-Gln** 1.151	**Trp-Tyr** 1.143
Ser-His 1.128	**Ser D-Asn** 1.380	**Ser-Hyp** 1.142	**Ser-Trp** 1.122	**Ser-Ser** 1.221	**Ser-Thr** 1.308	**Ser-Asn** 1.047	**Ser-Gln** 1.162	**Ser-Tyr** 1.128
Thr-His 1.158	**Thr D-Asn** ***1.718***	**Thr-Hyp** 1.135	**Thr-Trp** 1.152	**Thr-Ser** 1.197	**Thr-Thr** 1.269	**Thr-Asn** 1.014	**Thr-Gln** 1.151	**Thr-Tyr** 1.096
Asn-His 1.162	**Asn D-Asn** 1.329	**Asn-Hyp** 1.142	**Asn-Trp** 1.129	**Asn-Ser** 1.303	**Asn-Thr** 1.162	**Asn-Asn** ***0.976***	**Asn - Gln** 1.091	**Asn-Tyr** 0.997
Gln-His 1.200	**Gln D-Asn** 1.326	**Gln-Hyp** 1.155	**Gln-Trp** 1.219	**Gln-Ser** 1.259	**Gln-Thr** 1.303	**Gln-Asn** 1.085	**Gln-Gln** 1.166	**Gln-Tyr** 1.201
Tyr-His 1.131	**Tyr-D-Asn** 1.240	**Tyr-Hyp** 1.124	**Tyr-Trp** 1.154	**Tyr-Ser** 1.252	**Tyr-Thr** 1.365	**Tyr-Asn** 1.021	**Tyr-Gln** 1.115	**Tyr-Tyr** 1.155

chromatography is a rigid, hydrophilic support. In order to obtain better correlation between the two, we tried the screening and chromatography using longer tethers[23] between the selector and the APS. By utilizing a longer tether, the impact of the support is diminished. A long linker for the chromatographic support could better mimic the hydrophobic screening support and improve correlation. Linkers of four different lengths were examined and the results are shown in Table 1-6. It was shown that during screening the better separation appears to occur on the CSP with shorter linkers, while the CSP tethered by the longer linkers gives better chromatographic separation. This interesting trend establishes our suspicion that the difference in the solid support has an influence on the performance of the library during screening versus its chromatographic performance.

In order to fully address this problem with library correlation, we decided to re-synthesize DNB-81 using one of the longer linkers. We chose to use aminohexanoic acid as the linker as it is commercially available and seems to be the intermediate performer in both the screening and the chromatography. We are hoping that this will give us the best correlation for screenings to follow. The library members in DNB-81-2 are identical to those in DNB-81 except for the linker, and the synthesis was carried out using diisopropyl carbodiidide (DIC) as the coupling reagent. Selected members were screened on a preliminary basis using a DNB-Leu-Tyr-Abu-APS column with 15% IPA/hexane as the mobile phase and a flow rate of 2.00 ml/min. The results in Table 1-6 show that the length of

the linker is moderately important, but the more important factor is how many times the selectors are coupled to the aminopropyl silica gel surface. It had been previously thought that the length of the linker was very important until the coupling experiments were done. CSP 1 gives a slightly less impressive screening result than when it was screened with a shorter linker in DNB-81, but these results do correlate better with the chromatography observed.

Table 1-6. Chiral separation of TBNPO on CSP 1 with various linker lengths.

CSP	Separation Factor (α)	Screening % Difference in Peak Height
DNB-D-Asn-Thr-NH$(CH_2)_{\mathbf{3}}$CO-APS	1.30 (2xcoupling)	51%
DNB-D-Asn-Thr-NH$(CH_2)_{\mathbf{5}}$CO-APS	1.90 (2xcoupling), 2.97(3xcoupling)	44%
DNB-D-Asn-Thr-NH$(CH_2)_{\mathbf{7}}$CO-APS	1.30(2xcoupling)	27%
DNB-D-Asn-Thr-NH$(CH_2)_{\mathbf{10}}$CO-APS	3.30(3xcoupling)	12%

Two other selectors in DNB-81 were also synthesized and immobilized onto silica gel as a result of the TBNPO screening. DNB-Asn-Asn-Abu-AMPS and DNB-Trp-D-Asn-Abu-AMPS were the library hits, but they were synthesized as DNB-Asn-Asn-NH$(CH_2)_{10}$CO-APS (CSP 3 shown in Figure 1-10) and DNB-D-Asn-Trp-NH$(CH_2)_{10}$CO-APS (CSP 4 shown in Figure 1-11) in light of our findings on

the effects of tether lengths. CSP 3 gave a separation factor of 1.73 and CSP 4 astoundingly gave no separation. CSP 1 and CSP 3, the two selectors that gave

Table1-7. Peak Area ratios for TBNPO in 1:4 $CHCl_3$/heptane after 6 hr equilibration with DNB-81. Reference ratio: 0.990.

Library Member	**Peak Area Ratio**
His-Ser	1.019
His-Gln	0.988
D-Asn-D-Asn	1.309 **(32%)**
D-Asn-Tyr	0.875
Hyp-Ser	1.022
Trp-D-Asn	1.040
Trp-Asn	0.887
Ser-Tyr	0.962
Thr-D-Asn	1.257 **(27%)**
Thr-Asn	0.839 **(15%)**
Asn-Asn	0.844 **(15%)**
Gln-His	0.980

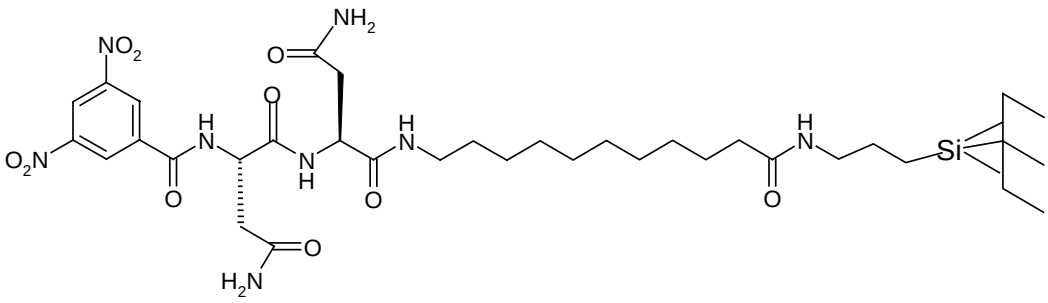


Figure 1-10. CSP 3: DNB-Asn-Asn-NH$(CH_2)_{10}$-APS.

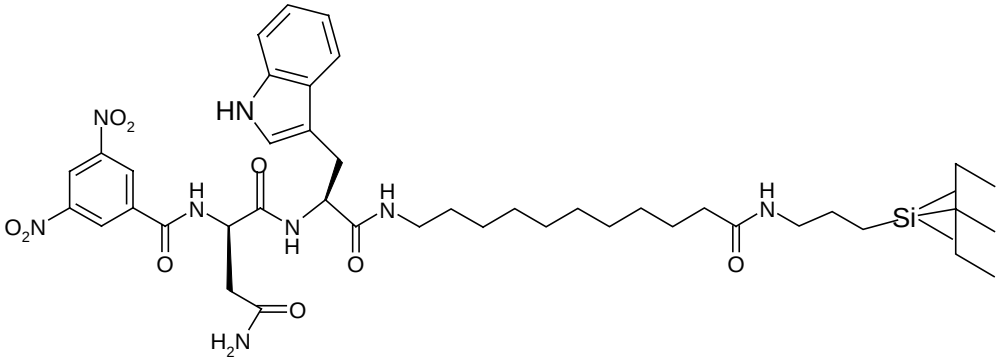


Figure 1-11. CSP 4: DNB-Trp-D-Asn-NH$(CH_2)_{10}$-APS.

separation for TBNPO were also immobilized onto a polymer chromatographic support[24]. The results are given in Table 1-8. The polymer chromatographic support was shown to give superior separation with comparison to the silica gel-based selector. A possible reason for this could be the lack of non-specific interactions with the analyte with the polymer supported CSPs.

Table 1-8. Results from selector assessment on polymer support.

Selector	**α**
Polymer-Abu-Thr-D-Asn-DNB	2.0
Polymer-Abu-Asn-Asn-DNB	1.24
Polymer-$C_{11}H_{20}$-Thr-D-Asn-DNB	3.3
Polymer-$C_{11}H_{20}$-Asn-Asn-DNB	3.2

However, CSP 3 gave an interesting result. CSP 3 gave a library screening result of 19% difference in peak height, but with a reversed selectivity of one enantiomer over another. It has been shown that this selector indeed follows our predictions of a reversed elution order. It would also be expected that if DNB-D-Asn-D-Asn-Abu-AMPS were synthesized and assessed it would give the same separation factor as CSP 3, but the opposite elution order as its screening results showed an equal and opposite difference in peak height ratio as that of CSP 3.

Since the long linker approach seemed to give more successful results with the TBNPO analyte, the next step was to reproduce past screenings using

TFAE and the longer linker library approach. The 81-member library had been synthesized with longer linkers already, but there were some members that were included in the original 200-member library that were missing from the 81-member library. This gives rise to the 45-member library that is comprised of the excluded members. Table 1-9 shows the template for the library. This library was screened for TFAE and several selectors were identified as "hits". The screening results from the most important members are shown in Table 1-10. Three CSPs were synthesized and assessed as a result of this screening: Support-$C_{11}H_{20}$-Leu-Pro-DNB (CSP 5), Support-$C_{11}H_{20}$-Pro-Pro-DNB (CSP 6), and APS-$C_{11}H_{20}$-Thr-Pro-DNB (CSP 7). The structures for CSP 5, 6, and 7 are given in Figures 1-12, 1-13, and 1-14 respectively. The common theme for the "hits" is that that all have a proline module nearest the DNB group. The selectors were immobilized onto aminopropyl silica gel as well as onto a polymer chromatographic support. The results from the column assessment are given in Table1-11. Interestingly the polymer supported CSPs performed worse than the silica gel supported CSPs for TFAE whereas for TBNPO the opposite was found to be true. More investigation would be necessary to make an informed hypothesis as to why this is the case. Unfortunately a significant selector did not result from the TFAE screening of the 45-member library with a long linker.

Table 1-9. 45 Member Library Template.
AMPS-Ahx-Aa1-Aa2-DNB

Leu-Leu	**Leu - Phe**	**Leu - Pro**	**Leu - Trp**	**Leu - Ser**	**Leu - Thr**	**Leu - Asn**	**Leu - Gln**	**Leu - Tyr**	**Tyr-Leu**
Phe-Leu	**Phe Phe**	**Phe Pro**	**Phe Trp**	**Phe Ser**	**Phe Thr**	**Phe Asn**	**Phe Gln**	**Phe Tyr**	**Tyr-Phe**
Pro-Leu	**Pro-Phe**	**Pro - Pro**	**Pro - Trp**	**Pro - Ser**	**Pro - Thr**	**Pro - Asn**	**Pro - Gln**	**Pro - Tyr**	**Tyr-Pro**
Trp-Leu	**Trp Phe**	**Trp-Pro**							
Ser-Leu	**Ser Phe**	**Ser-Pro**							
Thr-Leu	**Thr Phe**	**Thr-Pro**							
Asn-Leu	**Asn Phe**	**Asn-Pro**							
Gln-Leu	**Gln Phe**	**Gln-Pro**							

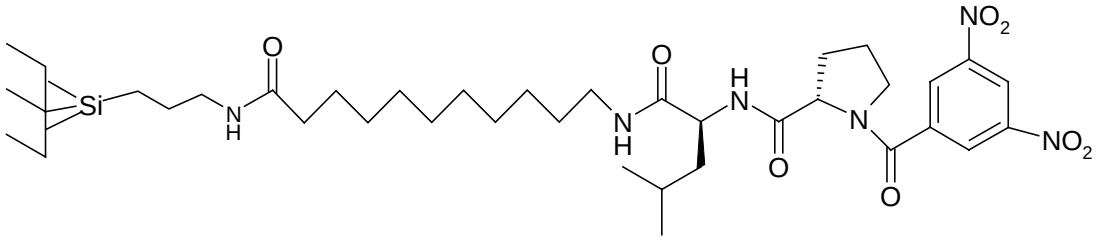


Figure 1-12. CSP 5: DNB-Pro-Leu-NH$(CH_2)_{10}$-APS.

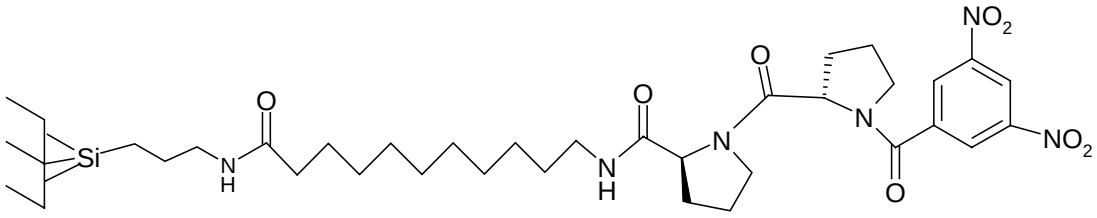


Figure 1-13. CSP 6: DNB-Pro-Pro-NH$(CH_2)_{10}$-APS.

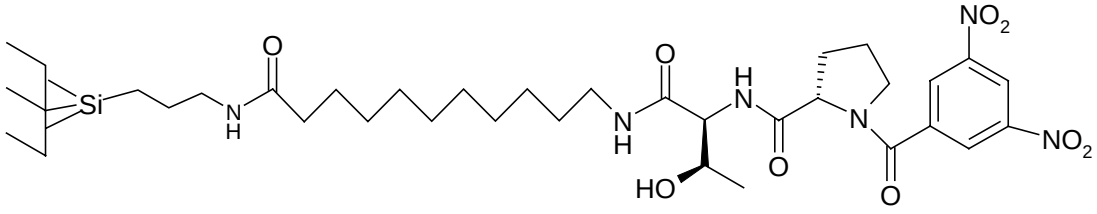


Figure 1-14. CSP 7: DNB-Pro-Thr-NH$(CH_2)_{10}$-APS.

Table 1-10. Results from TFAE screening for 45-member library.

Member	Ratio	% Difference
Standard	1.300	NA
Leu-Pro	1.555	20
Pro-Pro	1.485	14
Thr-Pro	1.491	15

Table 1-11. Results from column assessment for 45-member library screening results.

Selector	APS (α)	Polymer(α)
Thr-Pro	1.38	1.19
Leu-Pro	1.37	1.32
Pro-Pro	1.37	1.28

Though the TBNPO screening did successfully identify a successful chiral selector[25] for the analyte, the screening was not without its pitfalls. The first screening produced the most selective results, and each subsequent screening produced slightly different results. In the case of the TFAE screening of the 45-member library, the same troubles with irreproducibility were experienced. In order to determine the source of the irreproducibility we synthesized a new 144-

member library that encompassed all of the most successful members from previous screenings and a few new modules as well. This new library was synthesized according to previous protocol on AMPS resin as well as on chromatography grade silica gel. The silica gel synthesis procedure utilized the same peptide coupling strategies, but in order to ensure good coverage, each module was coupled twice and the selectors were end-capped with acetic anhydride before cleaving the side-chain protecting groups and screening. This procedure will likely result in a non-uniform surface, but if a "hit" is identified the CSP will be prepared in the same manner so it should not be a concern. The screening of the silica gel library showed no "hits" for TFAE, but the results were reproducible. Since the silica gel was so difficult to filter in the filter plates, the supernatant was withdrawn from the top of each well. This makes sense as with filtering the supernatant from the chromatographic support one is actually executing a mini-chromatography experiment and this could skew results and lead to irreproducibility. Also under the slight vacuum of the manifold one of the solvents in the solvent system could evaporate faster or filter faster leading to irreproducibility in screening. Since this procedure seemed to give more reproducible results it was used in the screening of the resin-based library. This screening showed some "hits", but they were not all reproducible. Since several of the "hits" were columns that were previously made by the group for TFAE screening of the 45-member library and they did not efficiently separate TFAE, it can be surmised that the fidelity of the screening results are questionable.

The next approach for determining the source of the irreproducibility was to perform selected screenings in glass vials assuming that the only variable left to explore was the actual 96-well filter plates. The library members that were "hits" in the TBNPO screenings for the 81-member library were removed from their filter plate and equilibrated with TBNPO in glass vials. We were able to get reproducible results in this manner, but at this point we no longer saw any selectivity.

Summary

In conclusion it has been shown that through creative application of combinatorial synthesis and screening techniques it is possible to expedite the process of developing new chiral stationary phases for chiral HPLC, but not without difficulty. Effective new chiral stationary phases were developed for the TBNPO analyte as a result of this project, and the technique should provide for the development of more new stationary phases in the future. The challenge is to determine where the reproducibility and correlation issues lie and develop new methods that circumvent these problems.

References

(1) Stinson, S. C. *Chemical and Engineering News* **2001**, *79*, 79.

(2) Subramanian, G. *Chiral Separation Techniques: A Practical Approach*; Wiley: Weinheim, 2001.

(3) Armstrong, D. W.; Ward, T. J.; Armstrong, R. D.; Beesley, T. E. *Tetrahedron Letters* **1997**, *38*, 6171-6172.

(4) Berthod, A.; Liu, Y.; Bagwill, C.; Armstrong, D. W. *Journal of Chromatography* **1996**, *731*, 123-137.

(5) Oguni, K.; Oda, H.; Ichida, A. *J. Chromatogr., A* **1995**, *694*, 91-100.

(6) Kawaguchi, Y. T., M.; Nakae, M.; Funazo, K.; Shono, T. *Analytical Chemistry* **1983**, *55*, 1852-1857.

(7) Welch, C. J. *J. Chromatogr., A* **1994**, *666*, 3-26.

(8) Haginaka, J. *Journal of Chromatography, A* **2001**, *906*, 253-273.

(9) Pirkle, W. H.; Welch, C. J.; Hyun, M. H. *J. Chromatogr.* **1992**, *607*, 126-130.

(10) Lam, K. S. *Anti Cancer Drug Design* **1997**, *12*, 145-167.

(11) Maclean, D.; Baldwin, J. J.; Ivanov, V. T.; Kato, Y.; Shaw, A.; Schenider, P.; Gordon, E. M. *Journal of Combinatorial Chemistry* **2000**, 562-578.

(12) Weingarten, M. D.; Sekanina, K.; Still, W. C. *Journal of the American Chemical Society* **1998**, *120*, 9112-9113.

(13) Bluhm, L. H.; Wang, Y.; Li, T. *Analytical Chemistry* **2000**, *72*, 5201-5205.

(14) Wu, Y.; Wang, Y.; Yang, A.; Li, T. *Analytical Chemistry* **1999**, *71*, 1688-1691.

(15) Murer, P.; Lewandowski, K.; Svec, F.; Frechet, J. M. *Analytical Chemistry* **1999**, *71*, 1278-1284.

(16) Lewandowski, K.; Murer, P.; Svec, F.; Frechet, J. M. *Journal of Combinatorial Chemistry* **1999**, 105-112.

(17) Wang, Y.; Li, T. *Analytical Chemistry* **1999**, *71*, 4178-4182.

(18) Wang, Y.; Bluhm, L. H.; Li, T. *Analytical Chemistry* **2000**, *72*, 5459-5465.

(19) Welch, C. J.; Protopopova, M. N.; Bhat, G. *Enantiomer* **1998**, *3*, 471-476.

(20) Welch, C. J.; Bhat, G.; Protopopova, M. N. *Journal of Combinatorial Chemistry* **1999**, *1*, 364-367.

(21) Pirkle, W. H.; Welch, C. J.; Lamm, B. *J. Org. Chem.* **1992**, *57*, 3854-3860.

(22) Rana, S.; White, P.; Bradley, M. *Journal of Combinatorial Chemistry* **2001**, *3*, 9-15.

(23) Pirkle, W. H.; Hyan, M. H. *Journal of Chromatography* **1985**, *328*, 1-9.

(24) Lewandowski, K.; Murer, P.; Svec, F.; Frechet, J. M. J. *Chemical Communications* **1998**, 2237-2238.

(25) Blodgett, J. W., Yan; Li, Tingyu; Polarvarapu, Prasad L.; Drabowicz, Jozef; Pietrusiewicz, K. Michal; and Zygo, Krystyna *Analytical Chemistry* **2002**, *74*, 5212-5216.

(26) Merrifield, R. B. *Journal of the American Chemical Society* **1963**, 2149-2154.

(27) Bodansky, M. *Peptide Chemistry: A Practical Approach*, Second ed.; Springer-Verlag: Berlin, 1993.

(28) Fields, G. B., Ed. *Solid-Phase Peptide Synthesis*; Academic Press: New York, 1997.

(29) Beaucage, S. L. I., Radhakrishnan, P. *Tetrahedron* **1992**, *48*, 2223-2311.

(30) Ellman, J. A. *Acc. Chem. Res.* **1996**, *29*.

(31) Hermkens, P. H. H. O., H.C.J.; Rees, D. *Tetrahedron* **1997**, *53*.

(32) Vaino, A. R. J., Kim D. *Journal of Combinatorial Chemistry* **2000**, *2*, 579-596.

(33) Krull, I. S. S., M.E.; Bourque, A.J.; Zhou, F.X.; Yu, J.; Strong, R. *Journal of Chromatography, B* **1994**, *659*, 19-50.

(34) Moran, E. J. S., S.; Cargill, J.F.; Shahbaz, M.M.; Lio, A.M.; Mjalli, A.M.M.; Armstrong, R.W. *Journal of the American Chemical Society* **1995**, *117*, 10787.

(35) Nicolaou, K. C. X., X-Y; Parandoosh, Z.; Senyei, A>; Nova, M.P. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2289.

(36) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *Journal of the American Chemical Society* **1980**, *102*, 5463-5470.

(37) Flynn, D. *Med. Res. Rev.* **1999**, *19*, 408-431.

(38) Kaldor, S. S., M.; Fritz, J.; Dressman, B.; Hahn, P. *Tetrahedron Letters* **1996**, *37*, 7193.

(39) Flynn, D. C., J.; Devraj, R.; Hockerman, S.; Parlow, J.; South, M.; Woodard, S. *Journal of the American Chemical Society* **1997**, *119*, 4874.

(40) Booth, R. H., J. *Journal of the American Chemical Society* **1997**, *119*.

(41) Dickerson, T. R., N.; Janda, K.D. *Chem. Rev.* **2002**, *102*, 3325-3343.

(42) Bayer, E. M., M. *Nature* **1972**, *237*, 512-513.

(43) Gravert, D. J. J., K.D. *Chem. Rev.* **1997**, *38*, 489.

(44) Haag, R. S., Al.; Hebel, A.; Roller, S. *Journal of Combinatorial Chemistry* **2002**, *4*, 112-119.

(45) Han, H. J., K.D. *Tetrahedron Letters* **1997**, *38*, 1527.

(46) Harned, A. M., S.; Flynn, D.; Hanson, P.R. *Organic Letters* **2003**, *5*, 15-18.

(47) Toy, P. H. J. K. D. *Acc. Chem. Res.* **2000**, *33*, 546-554.

(48) Porco, J. *Combinatorial Chemistry and High Throughput Screening* **2000**, *3*, 93-102.

(49) Studer, A. H., Sabine; Ferritto, Rafael; Kim, Sun-Young; Jeger, Patrick; Wipf, Peter; Curran, Dennis P. *Science* **1997**, *275*, 823-826.

(50) Curran, D. P. O., Y. *Tetrahedron* **2001**, *57*, 5243-5253.

(51) Curran, D. P. F., T. *Organic Letters* **2002**, *4*, 2233-2235.

(52) Luo, Z. Z., Q.; Oderaotoshi, Y.; Curran, D.P. *Science* **2001**, *291*, 1766-1769.

(53) Zhang, L. L., Z.; Chn, C.H-T.; Curran, D.P. *Journal of the American Chemical Society* **2002**, *124*, 10443-10450.

(54) Zhang, L. C., D.P.; Chn, C.H-T. *Tetrahedron* **2002**, *58*, 3871-3875.

(55) Zhang, L. R., A.; Curran, D.P. *Journal of the American Chemical Society* **2002**, *124*, 5774-5781.

(56) Zhang, W. *Tetrahedron* **2003**, *59*, 4475-4489.

(57) Zhang, W. *Chem. Rev.* **2004**, *104*.

(58) Zhang, Q. L., Z.; Curran, D.P. *J. Org. Chem.* **2000**, *65*, 8866-8873.

(59) Curran, D. P. H., S.; He, M *J. Org. Chem.* **1997**, *62*, 6714-6715.

(60) Curran, D. P., Luo, Z. *Journal of the American Chemical Society* **1999**, *121*, 9069-9072.

(61) Curran, D. P. *Synlett* **2001**, 1488-1496.

(62) Matsugi, M. C., D.P. *Organic Letters* **2004**, *6*, 2717-2720.

(63) Dandapani, S. N., J; Curran, D.P. *Tetrahedron Letters* **2004**, *45*, 6653-6656.

(64) Blodgett, J. L., Tingyu *Tetrahedron Letters* **2004**, *45*, 6649-6652.

(65) Jones, J. *Amino Acid and Peptide Synthesis*; Oxford Science Publications: New York, 1992.

(66) Danishefsky, S. J. M., K.F.; Randolph, J.T.; Ruggeri, R. B. *Science* **1993**, *260*.

(67) Araki, T. T., Shinji; Tanaka, Minoru *Analytica Chimica Acta* **2000**, *410*, 37-45.

(68) Armstrong, D. W. D., Wade; Alak, Ala; Hinze, Willie L.; Riehl, Terrence E.; Bui, Khanh H. *Analytical Chemistry* **1985**, *57*, 234-237.

(69) Breslow, R. Z., B. *Journal of the American Chemical Society* **1996**, *118*.

(70) Cabrer, P. A.-P., E.; Meijide, F.; Seijas, J.A.; Nunez, E.R.; Tato, J.V. *Langmuir* **1999**, *15*, 5489-5495.

(71) Debowski, J. J., J.; and Sybilska, D. *Journal of Chromatography* **1983**, *282*, 83-88.

(72) Debowski, J. S., D. *J. Chromatogr.* **1986**, *353*, 409-416.

(73) Feng, Y.-Q. X., Ming-jie; Da, Shi-Lu *Analytica Chimica Acta* **2000**, *403*, 187-195.

(74) Fujimara, K. K., M.; Takayanagi, H.; Ando, T *Journal of Liquid Chromatography* **1986**, 607-620.

(75) Gazdag, M. S., G.; Huszár, L. *J. Chromatogr.* **1986**, *351*, 128-135.

(76) Haginaka, J. W., J. *Analytical Chemistry* **1990**, *62*, 997-1000.

(77) Han, S. M. H., Yoon I.; Armstrong, Daniel W. *J. Chromatogr.* **1988**, *441*, 376-381.

(78) Hargitai, T. K., Y.; Okamoto, Y. *J. Chromatogr.* **1993**, *628*, 11-22.

(79) Hinze, W. L. R., T.E.; Armstrong, D.W.; DeMond, W.; Alak, A.; Ward, T. *Analytical Chemistry* **1985**, *57*, 237-242.

(80) Italia, A. S., M.;Ventura, P. *J. Chromatogr.* **1990**, *503*, 266-271.

(81) Li, S. P., W.C. *J. Chromatogr.* **1992**, *625*, 109-120.

(82) Maguire, J. H. *J. Chromatogr.* **1987**, *387*, 453-458.

(83) Matsuzawa, H. M., K. *Synlett* **2002**, *10*, 1607-1612.

(84) Nowakowski, R. C., Philippe J.P.; Coleman, ANthony W.; Villard, Emmanuelle; Guiochon, Georges *Analytical Chemistry* **1995**, *67*.

(85) Shimada, K. K., Yoshihiro; and Mitamura, Kuniko *Journal of Chromatographic Science* **1990**, *28*, 331-335.

(86) Tan, Z. J. Z., X.X.; and Brown, G.R. *Langmuir* **1994**, *10*, 1034-1039.

(87) Yang, Z. B., Ronald *Tetrahedron Letters* **1997**, *38*, 6171-6172.

(88) Zhang, L. W., Y.; Chen, L; Hg, S. *Tetrahedron Letters* **1999**, *40*, 1815-1818.

(89) Zukowski, J. S., D.; Bojarski, J. *J. Chromatogr.* **1986**, *364*, 225-232.

(90) Matsui, Y. N., T.; Fujita, T. *Top. Curr. Chem* **1985**, *128*, 61-89.

(91) Greene, T. W. W., Peter G. M. *Protective Groups in Organic Synthesis*, 3rd ed.; Wiley & Sons, Inc.: New York, NY, 1999.

CHAPTER II

MOLECULAR TAGGING TO FACILITATE SEPARATIONS: AN OVERVIEW

Introduction: Classical Separation Methods

Classically, organic synthesis has involved a twofold strategy of the synthetic step followed by the purification step. In the past these two steps have been completely separate endeavors, and often the purification steps require more time, energy, and creativity in order to accomplish satisfactory results. The organic chemist's choices for purification methods included recrystallization, extraction, or chromatography. These methods are time-consuming, and while they work well for reactions with good yields that produce only one product, they are not sufficient for less than perfect reaction schemes. As the demand for more high-throughput synthetic techniques and larger libraries of compounds for screening grew, the demand for high-throughput techniques for their purification and process automation also grew. Many techniques have been developed due to this new demand including synthesis on polymer supports, both soluble and insoluble; phase trafficking reagents, both soluble and insoluble; and molecular tagging strategies. These techniques, along with their advantages and disadvantages will be introduced in this chapter.

Insoluble Polymer Supports

The field of combinatorial chemistry, especially at its inception, has depended greatly on the development of techniques for synthesis on insoluble polymer supports termed solid-phase synthesis. This technique was first used for peptide synthesis[26-28], but has expanded over time to be used for oligonucleotide[29] and small organic molecule synthesis[30-33] as well. Solid phase synthesis is implemented by first attaching the first set of reagents or modules to the insoluble polymer bead of choice. Then subsequent organic transformations are executed in order to produce a desired product. The only purification between each transformation that is needed is the washing of the polymer beads to remove excess reagents. After the necessary transformations are complete, the product can then be cleaved from the polymer bead, producing pure compound that needs little or no further purification. Solid-phase synthesis has many advantages over classical synthesis-purification strategies. Because the excess reagents can be easily washed away, large excesses of reagent can be used in order to accomplish better yields. Due to the fact that the technique is amenable to encoding[34, 35], solid-phase synthesis is an extremely useful technique for generating large libraries of compounds. This technique alleviates the need for the time-consuming classical purification techniques discussed previously.

Solid-phase synthesis is a powerful tool, however it has its disadvantages as well. The reagents must be at least bifunctional in order to utilize on-bead

synthesis techniques. Often reactions must be re-optimized to work on beads due to the heterogeneous nature of the reaction mixture. Solvent choices[22, 32, 36] are also limited as the reaction must be executed in a solvent that also promotes swelling of the polymer resin, and some organic reactions are less than ideal in such solvents. Because the product remains polymer-bound throughout the entire process, it is impossible to purify and characterize intermediates, and conventional analytical techniques such as NMR, MS, and IR are difficult to get information from until after cleavage. It is also very difficult to optimize conditions such that each reaction works well for each functionality involved for each library member, so that ultimately the library is compromised due to contamination from deletion product formation. However in spite of the many difficulties with executing solid-phase synthesis, the technique has found widespread use and has revolutionized combinatorial chemistry techniques. In cases where the difficulties are too cumbersome, other techniques have since been developed.[37]

Alternatively, chemoselective phase trafficking reagents have been developed for what has been termed polymer-assisted solution-phase synthesis (PASP). This technique involves specific polymer-bound reagents that are designed to capture very specific reaction components or by-products after the reaction is complete. This is advantageous as the reaction can proceed in a homogeneous atmosphere, and there is still a capability to separate out the product. Another advantage is that the products can remain in solution, or can

be phase switched, according to the polymer reagent design, and the incomplete reaction intermediates can be purified at each step.[38-40]

Soluble Polymer Supports

One alternative to solid-phase reactions is reactions on soluble polymer supports. These soluble polymer supports are an attractive alternative as the reaction mixture is homogeneous during the course of the reaction, but the polymer support can be drawn out of solution at will when the product needs to be isolated. Common polymers for this practice include polyethylene glycols (PEGs) and non-cross-linked polystyrene among others.[37, 41-47] The soluble polymer-bound products can be recovered in a variety of ways including precipitation where the resulting solid polymer-bound product can then be filtered. In this case, sometimes impurities can coprecipitate and lead to a mixture. Sometimes the product can be recrystallized to avoid this occurrence. Membrane filtration, centrifugation, and gel permeation chromatography are other methods that have been used to separate soluble polymer-bound products from unbound contaminants[41]. However, these techniques do not purify away the unwanted polymer-bound products. This purification problem is still relegated to the classical separation techniques discussed previously.

Chemoselective phase trafficking reagents have also been developed for use in solution as well. For the most part, such reagents will be functionalized in a way as to facilitate their removal after the reaction is complete, for instance in

the case of water soluble carbodiimides for which the urea by-products can be extracted away in water or Mitsunobu reagents with masked hydrophilic groups that can be unmasked at will and extracted away.[37]

Affinity Tagging Systems

Affinity tagging is yet another way to facilitate the integration of chemical synthesis and purification. This technique involves the chemical tagging of reagents or reactants traditionally, but also intermediates or products in the case of phase switching strategies. These chemical tags enable the chemoselective removal of the tagged compound from the reaction mixture, but they also facilitate the ability to take advantage of all of the favorable reaction and characterization features of classical organic synthesis due to the fact that they are single molecules instead of polymers as in the case of solid-phase synthesis. Such advantages include solution phase reaction conditions, facile identification and characterization by spectroscopic instrumentation, analytical, and chromatographic techniques. These chemoselective tags have been developed in a variety of ways including fluorous technologies as well as some non-fluorous strategies such as chemoselective reagents for the Mitsunobu reaction and Swern oxidations, heterocyclic nitrogen derivatives that facilitate selective precipitation, as well as crown ether tagging strategies.[48] The fluorous technologies have received far more interest and have enjoyed greater success

and widespread use in the literature. It is for this reason that the fluorous tags will be discussed in more detail.

Fluorous synthesis is a technique that has enjoyed widespread use as a chemoselective way to integrate synthesis with purification. Instead of exploiting the insolubility of polymer beads in liquids as with solid-phase synthesis, fluorous techniques exploit the immiscibility of fluorocarbon solvents in both aqueous and organic solvents. There are three main approaches to using fluorous techniques. There is fluorous synthesis using fluorous tagged reaction substrates, fluorous phase-switching, and fluorous multi-phase reactions[49]. The fluorous synthesis technique involves using a fluorine rich protective group that after the reaction is complete, will partition the product into the fluorous phase during a three phase extraction process.[48, 49] This strategy has been utilized for many types of synthesis with great success.[49-57] Organic-fluorous phase switching is another way to take advantage of the unique properties of fluorine-rich compounds in order to facilitate purification. In this strategy, the reaction of certain functional groups on a molecule with a fluorous protective group produces the phase switch into the fluorous phase. This process is similar to a "capture" event. For example, in a Grignard reaction of an aldehyde, the alcohol intermediate can be trapped by a cleavable fluorous protective group reagent and extracted from the reaction mixture. The protective group can then be cleaved away leaving behind the pure product.[48, 49] There are many examples of similar uses for the fluorous phase – switching technique. Lastly, fluorous tags can be used in order to accomplish the

easy purification of multi-component reactions such as the Ugi and Biginelli reactions. These reactions are so important as they can be used to create a tremendous amount of diversity when use to generate a combinatorial library of compounds, but they are of limited value with no high-throughput way in which to purify them. These types of libraries have been effectively demonstrated on the solid-phase in the past, and Curran has also developed a successful fluorous method as well.[49] Fluorous liquid-liquid extractions as well as fluorous solid-phase extractions have been developed as successful ways to retrieve the fluorous tagged compounds. A newer "light" fluorous technique using fluorous reverse phase silica gel has also been developed that behaves more like chromatography than extraction and alleviates some of the difficulties of working with the heavy fluorous compound such as reagent solubility and reactivity in organic solvents for the reaction.[54, 56-62]

Another mode of affinity tagging has been introduced in the literature recently by our group as well as the Curran group involving the inclusion complex formation of a chemical tag with beta-cyclodextrin immobilized onto silica gel. The Curran group has introduced a separation tag based on the adamantyl functional group, which displays a high binding affinity for beta-cyclodextrin. The Mitsunobu reaction is a reaction of particular interest for chemoselective tagging strategies because of the difficulties encountered with separating its products from reagents and by-products. Two new Mitsunobu reagents tagged with the adamantyl group were used to effect Mitsunobu

transformations, and the dicarbonyl hydrazides were successfully and preferentially removed by a beta-cyclodextrin stationary phase and HPLC.[63] In the preceding journal issue and in Chapter III that follows, a similar tagging strategy based on 4-*tert*-bytulphenol is introduced as a simple and effective tagging strategy for solution phase synthesis. The method is demonstrated with peptide synthesis, but could conceivably be used for practically any type of chemistry due to its simplicity.[64]

References

(1) Merrifield, R. B. *Journal of the American Chemical Society* **1963**, 2149-2154.

(2) Bodansky, M. *Peptide Chemistry: A Practical Approach*, Second ed.; Springer-Verlag: Berlin, 1993.

(3) Fields, G. B., Ed. *Solid-Phase Peptide Synthesis*; Academic Press: New York, 1997.

(4) Beaucage, S. L. I., Radhakrishnan, P. *Tetrahedron* **1992**, *48*, 2223-2311.

(5) Ellman, J. A. *Acc. Chem. Res.* **1996**, *29*.

(6) Hermkens, P. H. H. O., H.C.J.; Rees, D. *Tetrahedron* **1997**, *53*.

(7) Vaino, A. R. J., Kim D. *Journal of Combinatorial Chemistry* **2000**, *2*, 579-596.

(8) Krull, I. S. S., M.E.; Bourque, A.J.; Zhou, F.X.; Yu, J.; Strong, R. *Journal of Chromatography, B* **1994**, *659*, 19-50.

(9) Moran, E. J. S., S.; Cargill, J.F.; Shahbaz, M.M.; Lio, A.M.; Mjalli, A.M.M.; Armstrong, R.W. *Journal of the American Chemical Society* **1995**, *117*, 10787.

(10) Nicolaou, K. C. X., X-Y; Parandoosh, Z.; Senyei, A>; Nova, M.P. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2289.

(11) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *Journal of the American Chemical Society* **1980**, *102*, 5463-5470.

(12) Rana, S.; White, P.; Bradley, M. *Journal of Combinatorial Chemistry* **2001**, *3*, 9-15.

(13) Flynn, D. *Med. Res. Rev.* **1999**, *19*, 408-431.

(14) Kaldor, S. S., M.; Fritz, J.; Dressman, B.; Hahn, P. *Tetrahedron Letters* **1996**, *37*, 7193.

(15) Flynn, D. C., J.; Devraj, R.; Hockerman, S.; Parlow, J.; South, M.; Woodard, S. *Journal of the American Chemical Society* **1997**, *119*, 4874.

(16) Booth, R. H., J. *Journal of the American Chemical Society* **1997**, *119*.

(17) Dickerson, T. R., N.; Janda, K.D. *Chem. Rev.* **2002**, *102*, 3325-3343.

(18) Bayer, E. M., M. *Nature* **1972**, *237*, 512-513.

(19) Gravert, D. J. J., K.D. *Chem. Rev.* **1997**, *38*, 489.

(20) Haag, R. S., Al.; Hebel, A.; Roller, S. *Journal of Combinatorial Chemistry* **2002**, *4*, 112-119.

(21) Han, H. J., K.D. *Tetrahedron Letters* **1997**, *38*, 1527.

(22) Harned, A. M., S.; Flynn, D.; Hanson, P.R. *Organic Letters* **2003**, *5*, 15-18.

(23) Toy, P. H. J. K. D. *Acc. Chem. Res.* **2000**, *33*, 546-554.

(24) Porco, J. *Combinatorial Chemistry and High Throughput Screening* **2000**, *3*, 93-102.

(25) Studer, A. H., Sabine; Ferritto, Rafael; Kim, Sun-Young; Jeger, Patrick; Wipf, Peter; Curran, Dennis P. *Science* **1997**, *275*, 823-826.

(26) Curran, D. P. O., Y. *Tetrahedron* **2001**, *57*, 5243-5253.

(27) Curran, D. P. F., T. *Organic Letters* **2002**, *4*, 2233-2235.

(28) Luo, Z. Z., Q.; Oderaotoshi, Y.; Curran, D.P. *Science* **2001**, *291*, 1766-1769.

(29) Zhang, L. L., Z.; Chn, C.H-T.; Curran, D.P. *Journal of the American Chemical Society* **2002**, *124*, 10443-10450.

(30) Zhang, L. C., D.P.; Chn, C.H-T. *Tetrahedron* **2002**, *58*, 3871-3875.

(31) Zhang, L. R., A.; Curran, D.P. *Journal of the American Chemical Society* **2002**, *124*, 5774-5781.

(32) Zhang, W. *Tetrahedron* **2003**, *59*, 4475-4489.

(33) Zhang, W. *Chem. Rev.* **2004**, *104*.

(34) Zhang, Q. L., Z.; Curran, D.P. *J. Org. Chem.* **2000**, *65*, 8866-8873.

(35) Curran, D. P. H., S.; He, M *J. Org. Chem.* **1997**, *62*, 6714-6715.

(36) Curran, D. P., Luo, Z. *Journal of the American Chemical Society* **1999**, *121*, 9069-9072.

(37) Curran, D. P. *Synlett* **2001**, 1488-1496.

(38) Matsugi, M. C., D.P. *Organic Letters* **2004**, *6*, 2717-2720.

(39) Dandapani, S. N., J; Curran, D.P. *Tetrahedron Letters* **2004**, *45*, 6653-6656.

(40) Blodgett, J. L., Tingyu *Tetrahedron Letters* **2004**, *45*, 6649-6652.

CHAPTER III

DEVELOPMENT OF A SIMPLE AND EFFECTIVE TAGGING SYSTEM FOR SOLUTION PHASE ORGANIC SYNTHESIS BASED ON 4-*tert*-BUTYLPHENOL

Introduction

Since the practical synthesis of organic compounds is limited not only by yield of specific reactions but also by the ability to isolate product efficiently, various strategies to facilitate product isolation have been developed in the past. Among them, syntheses using insoluble polymeric support (solid phase synthesis)[26-28, 65] are widely used for peptide and oligo-nucleotide synthesis[29]. Solid phase synthesis has been investigated for the preparation of other types of compounds such as polysaccharides[66] and other small organic molecules[31, 38] in connection with high throughput synthesis. While it proves to be an effective strategy, it has its limitations. For example, many organic reactions need to be re-optimized for solid phase synthesis and some do not work well on solid support. Reactions on solid support are also more difficult to monitor. Moreover, it is impossible to purify reaction intermediates. Another strategy to facilitate product isolation involves solution phase synthesis onto a soluble tag. In this strategy, a facile isolation process is made possible due to certain properties of the tag. The ideal tag should also be inert in the reaction conditions. Examples of this tag strategy include synthesis using soluble polymers, which have relatively low loading capacity due to the size of the tag. More recently, small fluorous tags that have significant affinity towards fluorous silica gel have attracted significant

attention in organic chemistry.[49] We would like to report a new tagging system based on the interaction of beta-cyclodextrin and the 4-t-butylphenyl group.

Beta-cyclodextrin, a cyclic oligosaccharide, has been well studied in host-guest chemistry.[3, 6, 63, 67-89] It is known to bind 4-*tert*-butylphenol with an association constant on the order of 10^4 M^{-1}in water.[87, 90] Therefore, a stationary phase comprised of immobilized beta-cyclodextrin may provide an efficient mechanism of separating compounds containing either the lithocholic acid group or the 4-t-butylphenyl group from compounds that do not have such a functional group. Since 4-t-butylphenyl is also inert in many organic reactions, it could prove to be a useful tag for solution phase synthesis and will be discussed first.

For the successful application of tag directed synthesis, it is important that functional groups attached to the tag do not influence its separation mechanism. In this particular case, since the intended isolation mechanism of this tag involves chromatographic separation using a cyclodextrin column, it is important that the retention time of tagged compounds do not differ significantly from others. Therefore, chromatographic behavior of a number of compounds was studied. They vary significantly in their overall polarity.

Experimental

General Supplies and Equipment

Amino acid derivatives were purchased from NovaBiochem (San Diego,

CA). All other chemicals and solvents were purchased from either Aldrich (Milwaukee, WI), Fluka (Ronkonkoma, NY), or Fisher Scientific (Pittsburgh, PA). Beta-cyclodextrin bonded HPLC column was purchased from Advanced Separation Technologies Inc. (Whippany, NJ). Selecto silica gel (32-63 μm) from Fisher Scientific was used for flash chromatographic purification of target compounds. Thin-layer chromatography was completed using EM silica gel 60 F-254 TLC plates (0.25 mm) (Emerck KGaA, 64271 Darmstadt, Germany). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded with a Bruker 300 MHz instrument. HPLC analyses were conducted using a Beckman analytical gradient system (System Gold).

Preparation of the First Generation 4-t-butylphenyl Tag

In a flame-dried flask under argon gas, 187mg potassium hydroxide was dissolved in 3ml anhydrous dimethylformamide. Next 100mg of 4-t-butylphenol was added and dissolved, followed by the addition of .95ml t-butylchloroacetate. The mixture was stirred and followed by TLC. Upon completion, the mixture was quenched with methylene chloride and extracted three times with copious amounts of water. The methylene chloride layers were dried with sodium sulfate and evaporation of the solvent yielded a white solid. The crude product was purified by flash chromatography using 2.5% ethyl acetate in hexane mobile phase. The t-butyl protecting group was then removed by stirring the product in 95% trifluoroacetic acid in methylene chloride for 2h. The mixture was then dried

by vacuum to give the deprotected acid.

Characterization of tert-butyl 2-(4-tert)-butylphenoxy acetate: ^{1}H NMR ($CDCl_3$) δ 1.36(s, 9H), 1.48 (s, 9H), 4.55 (s, 2H), 6.8-6.9(d, 2H), 7.25-7.35 (d, 2H)

Characterization of 2-(4-tert)-butylphenoxy acetic acid: ^{1}H NMR ($CDCl_3$) δ 1.36(s, 9H), 4.55 (s, 2H), 6.8-6.9(d, 2H), 7.25-7.35 (d, 2H)

Preparation of Histidine tagged with the 1st Generation 4-t-butylphenyl Tag

In a flame-dried flask under argon gas, 50mg of 4-*tert*-butylphenoxy acetic acid was dissolved in anhydrous methylene chloride. Then .2 ml of oxalyl chloride was added and let stir. Next 1 drop of anhydrous dimethylformamide was added and let react 2h. Upon completion, the reaction mixture was evaporated to dryness by vacuum. In another flame-dried flask under argon gas, 160mg His-OMe in .3ml diisopropylethylamine and a small amount of anhydrous methylene chloride were added and let stir. Then the acid chloride was dissolved in anhydrous methylene chloride and added to the His-OMe in solution and let react 4h. Upon completion, the reaction was quenched with copious amounts of water and extracted three times with methylene chloride. The methylene chloride layers were dried with sodium sulfate and evaporation of the solvent yielded a white solid. The crude product was purified by preparative TLC with a 10% methanol in methylene chloride mobile phase.

Characterization of tagged Histidine: ^{1}H NMR ($CDCl_3$) δ 1.3(s, 9H), 3.0-3.3 (m, 2H), 3.6-3.7 (s, 3H), 4.4-4.5(s, 2H), 4.85-5.0 (s, 1H), 6.8 (s, 1H), 6.8-6.9 (d, 2H), 7.2-7.3 (d, 2H), 7.5 (s, 1H), 7.9-8.2 (b, 1H)

Characterization of tagged Tyrosine: ^{1}H NMR ($CDCl_3$) δ 1.28(s, 18H), 1.3 (s, 9H), 3.0-3.2 (m, 2H), 4.5(s, 2H), 4.8-4.9 (m, 1H), 6.75-6.9 (m, 4H), 7.0-7.1 (d, 2H), 7.3-7.4 (d, 2H)

Coupling to 2nd Generation 4-t-butylphenyl Tag

To a flame-dried flask under argon gas, 2.6g Fmoc-Ser-OH and 890mg cesium carbonate were dissolved in 6ml of dimethylformamide. Then .5ml 4-*tert*-butylbenzyl bromide was added and let react. Upon completion, the mixture was quenched with methylene chloride and extracted with 5% citric acid in water to ensure that the Fmoc-Ser-OH stayed in the mixture in order to test the tagging procedure for separation. The methylene chloride layers were dried with sodium sulfate and the solvent was evaporated leaving a clear, colorless oil.

Characterization of 2nd generation tagged serine: ^{1}H NMR ($CDCl_3$) δ 1.0(s, 9H), 1.23 (s, 9H), 3.5, 3.75 (2d, 1H), 4.1-4.2(m, 1H), 4.2-4.4 (m, 2H), 4.5 (d, 1H), 5.0-5.2 (q, 2H), 5.65 (d, 1H), 7.1-7.4 (m, 8H), 7.5-7.6 (d, 2H), 7.7-7.8 (d, 2H)

Characterization of 2nd generation tagged glycine: ^{1}H NMR ($CDCl_3$) δ 1.3(s, 9H), 1.46 (s, 9H), 3.9-4.0 (d, 2H), 5.0(b, 1H), 5.24 (s, 2H), 7.2-7.3 (d, 2H), 7.4-7.5 (d, 2H)

4-tert-Butylphenyl Tagged Coupling Reaction

240mg Boc-Phe-OH was added to 100mg of deprotected, tagged glycine dissolved in 20%dimethylformamide in methylene chloride. To this mixture, .142ml diisopropylcarbodiimide was added and let react for 5h. Upon completion, the reaction mixture was extracted with water and the methylene chloride layers were dried with sodium sulfate. The methylene chloride was then evaporated leaving a white solid mixture. The mixture was purified by HPLC using a beta-cyclodextrin column under reversed phase conditions.

Cleavage of 4-tert-Butylphenyl Tag

To 100mg of the tagged glycine and 10-15mg of palladium hydroxide, 20wt. % Pd on carbon, 5 ml ethanol were added and let stir to dissolve. Next, hydrogen gas was bubbled into the reaction mixture and followed by TLC. After about 3h the hydrogenolysis was complete, and the reaction mixture was evaporated to dryness via vacuum.

Preparation of silyl chloride tagging reagent

To a flame dried flask under argon, 16mmol (2ml) dichlorodimethylsilane were added at 0°C. Then, using an addition funnel under argon, 8mmol (4.0ml) *tert*-butylbenzyl magnesium bromide 2.0M in ether were added slowly dropwise over 20 min with stirring. The reaction was then allowed to gradually warm up to room temperature and react overnight under argon. The product was worked up in the drybox. The workup involved first pumping off the ether and excess dichlorodimethylsilane. Next dry hexane was added to the mixture, dissolving the product and leaving the MgBrCl salts behind. The salts were then filtered off and the hexane was pumped off leaving behind white crystalline product. The product was stored in a drybox freezer under nitrogen at -26°C.

Characterization of silyl chloride tag: ^{1}H NMR (C_6D_6) δ 0.60(s, 6H), 1.3 (s, 9H), 7.35-7.4 (d, 2H), 7.6-7.7 (d, 2H). ^{13}C NMR (C_6D_6) δ 2.0, 31.3, 34.7, 125.3, 132.7, 133.5, 153.4. Anal. calcd for $C_{12}H_{19}ClSi$: C, 63.54; H, 8.44; Cl, 15.63. Found: C, 1.43; H, 7.92; Cl, 13.99.

Preparation of the carbamate tagged compounds

To a flame dried flask 0.26mmol Tyr(OtBu)-OtBu, 0.26mmol (222mg) cesium carbonate, and 0.26mmol (252mg) tetrabutylammonium iodide were added and dissolved in 10ml anhydrous dimethylformamide. Then carbon dioxide gas was bubbled through the solution for 45min. Next 0.26mmol (0.125ml) benzyl

bromide were added and the reaction was allowed to react with CO_2 bubbling for 8h. The reaction mixture was then poured into water and extracted three times with ethyl acetate, three times with water, and three times with brine. The ethyl acetate layer was then dried with sodium sulfate and the ethyl acetate was removed by rotary evaporation. The product was purified by preparative thin layer chromatography using a 10% ethyl acetate/hexane mobile phase.

Characterization of carbamate tagged tyrosine: ^{1}H NMR ($CDCl_3$) δ 1.2-1.29 (m, 24H), 2.8-3 (m, 2H), 4.2 (t, 1H), 4.9 (d, 2H), 6.8 (d, 2H), 7.0 (d, 2H), 7.15 (d, 2H), 7.3 (d, 2H).

Preparation of the thiol tagged compound

In a flame dried flask 2.7mmol (890mg) cesium carbonate, 5.0mmol (0.56ml) thiophenol were dissolved in 3ml anhydrous dimethylformamide and let stir for 30min. Then 2.7mmol (0.5ml) 4-*tert*-butylbenzyl bromide were added and let react. The reaction was quenched with water and extracted with hexane. The product was purified by preparative thin layer chromatography using a 10% ethyl acetate/hexane mobile phase.

Characterization of thiol tagged thiophenol: ^{1}H NMR (MeOD) δ 1.29 (s, 9H), 4.1 (s, 2H), 7.1-7.4 (m, 9H).

Representative experimental preparation of the water soluble tags

In a flame dried flask under argon, 5.0mmol (7000mg) 4-*tert*-butylphenol, 15.0mmol (1.660g) potassium iodide, and 15.0mmol (1.380g) potassium carbonate were dissolved in 10ml anhydrous dimethylformamide and let stir for 15min at 80°C. Then 2.0mmol (0.34ml) 2-[2-(2-chloroethoxy)ethoxy]ethanol were added and let react overnight. The reaction mixture was quenched with water and extracted three times with ether. The ether layers were then combined, dried with sodium sulfate, and then evaporated by rotary evaporation. The product was purified by flash chromatography. The excess 4-*tert*-butylphenol is eluted by 10% ethyl acetate/hexane, and then the product can then be eluted with ethyl acetate.

Characterization of (2-(4-tert-butylphenoxy) ethanol tag: ^{1}H NMR ($CDCl_3$) δ 1.3 (s, 9H), 2.11 (br, 1H), 3.9 (t, 2H), 4.1 (t, 2H), 6.9 (d, 2H), 7.35 (d, 2H).

Characterization of (2-(2-(4-tert-butylphenoxy)ethoxy) ethanol tag: ^{1}H NMR ($CDCl_3$) δ 1.0 (s, 9H), 3.3 (m, 2H), 3.4 (m, 6H), 3.55 (m, 2H), 3.85 (m, 2H), 6.6 (d, 2H), 7.0 (d, 2H). ^{13}C NMR (C_6D_6) δ 31.8, 36.8, 61.9, 67.6, 70.0, 70.5, 70.7, 71.0, 72.9, 114.4, 126.4, 143.8, 156.8.

Characterization of 2-(2-(2-(4-tert-butylphenoxy)ethoxy)ethoxy)ethanol) tag: ^{1}H NMR ($CDCl_3$) δ 1.3 (s, 9H), 2.11 (br, 1H), 3.9 (t, 2H), 4.1 (t, 2H), 6.9 (d, 2H), 7.35 (d, 2H).

Preparation of tripeptide

1.38g (0.0024mol) Boc-Phe-OH was added to .35g (0.0012mol) of deprotected, 4-*tert*-butylbenzyl tagged glycine dissolved in dimethylformamide. To this mixture, .805ml (0.0024mol) diisopropylcarbodiimide was added and let react for 4h. Upon completion, the reaction mixture was extracted with water and the methylene chloride layers were dried with sodium sulfate. The methylene chloride was then evaporated leaving a white solid mixture. The mixture was purified by filtration using flash-grade silica gel with immobilized beta-cyclodextrin packing material in a fritted funnel. The funnel was packed with 15.0g of the flash-grade silica gel with immobilized beta-cyclodextrin, and then the sample was applied to the packing in a solution of 40% methanol in water. The impurities were then eluted by passing 300.0ml 40% methanol in water through the funnel, and then the pure product was eluted by washing the support with 300.0ml methanol. The Boc protecting group was then removed by stirring in about 10ml of a 95% trifluoroacetic acid solution in methylene chloride for 2h. The trifluoroacetic acid and methylene chloride were removed by azeotroping with benzene and evaporating by rotary evaporation. The process was repeated to couple Boc-Leu-OH. After the Boc group was removed from the Leu, the 4-*tert*-butylbenzyl tag was removed by hydrogenolysis.

Characterization of tripeptide: ^{1}H NMR (MeOD) δ 1.0 (d, 6H), 1.7-1.85 (m, 3H), 2.9 (m, 1H), 3.1 (m, 1H), 3.5 (m, 1H), 4.15 (s, 2H), 5.1 (m, 1H), 7.1-7.3 (m, 5H).

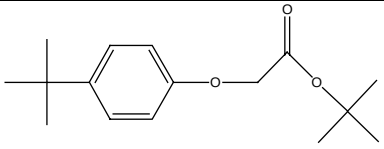
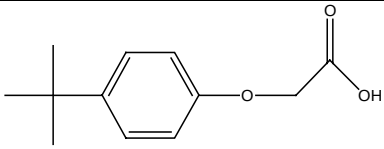
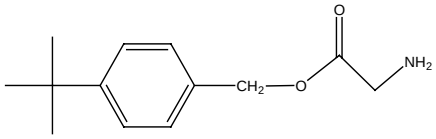
Results

In order to determine the potential efficacy of a tagging system based on the 4-t-butylphenol moiety, the retention times of several compounds with structural similarities to 4-t-butylphenol were evaluated with a beta-cyclodextrin column under reversed-phase conditions (Table 3-1). As compared to benzoic acid and benzyl alcohol, which do not contain the 4-*tert*-butylphenol moiety, the compounds that do contain the tag functional group are retained much longer. Also the retention time is not greatly affected by which type of functional group that is attached to it. For example, Figure 3-1 shows the Boc protected 4-*tert*-butylphenoxyacetic acid (I) has a retention time that is similar to that of the more polar acid analog (II). In addition, the amine analog (III) has a retention time similar to that of 4-*tert*-butylphenol itself. It should be noted that there are two reported retention times for the amine analogue, one in the presence of TFA and one without TFA. This distinction is important because in the presence of TFA, the amine is charged. It is for that reason that in the presence of TFA the amine analogue elutes faster. It is also important to point out that the charged amine analogue still retains much longer than the compounds that do not contain the tag moiety.

In another experiment many types of compounds were also analyzed by HPLC (Table 3-4). These compounds include amino acids and other analytes that were readily available containing many different functional groups, and therefore many different polarities. These compounds were analyzed using a faster gradient

in order to expedite the study, and the compounds studied were only retained for a fraction of the time that 4-*tert*-butylphenol was retained. Included in this list is the end-capped version of the *tert*-butyl protected tyrosine (Figure 3-4). This experiment is important because the *tert*-butyl protected side chain is similar in structure to the tagging moiety. Because the similar functional group did not produce the same effect as the tag itself, the tag is shown to be especially effective. These results suggest that the 4-*tert*-butylphenyl tag has potential to be an efficient and universal tag for solution-phase organic synthesis.

Table 3-1. Retention times of initial test compounds.

Compound	**Retention Time (min)[a]**
Benzyl Alcohol	5
Benzoic Acid	15
4-*tert*-Butylphenol	91
 I	78
 II	70
 III	40, 90[b]

[a] Mobile phase gradient from 0% methanol to 100% methanol in 0.5%TFA/water in 3h. [b] Mobile phase gradient from 0% methanol to 100% methanol in water in 3h.

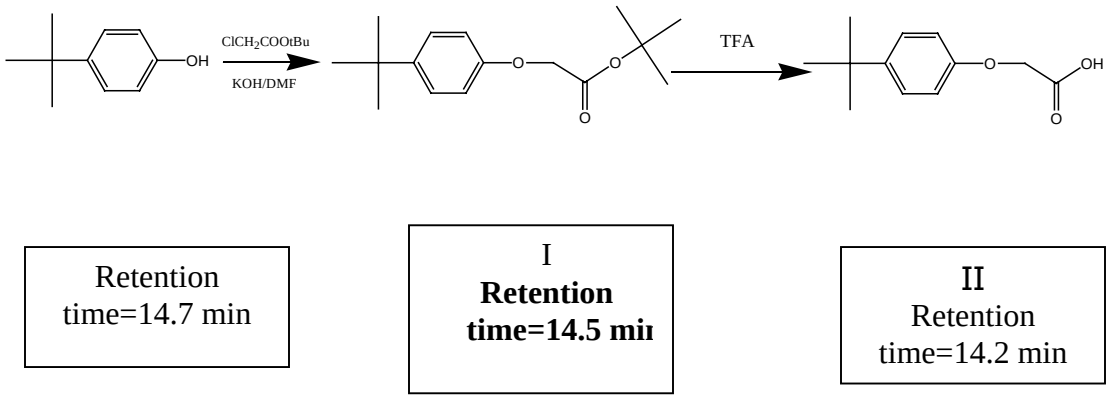


Figure 3-1. Tag viability test[a]

[a] Mobile phase gradient from 50% methanol to 100% methanol in 0.5%TFA/water in 1h.

Correlation of binding affinity to chromatographic retention times

In order to move forward with the development of the 4-*tert*-butylphenyl tag, it was necessary to do some experiments that would correlate the chromatographic retention times that looked promising with the actual binding affinities of compounds to ensure reliability of results. This was accomplished by Determining the chromatographic retention times of various compounds for which the binding affinities are known[90], and then studying how well they correlate. The retention time-binding affinity relationship was examined for a group of *para*-phenolic compounds as well as for a group of *meta*-phenolic compounds. The results are shown in Figures 3-2 and 3-3, with raw data in Tables 3-2 and 3-3.

The chromatographic data does indeed correlate well with the data in the literature for binding of the various phenolic compounds with beta-cyclodextrin.

1st Generation tag based on 4-*tert*-*Butylphenol*

The first generation tag developed based on the 4-*tert*-butylphenol moiety is compound II shown in Figure 3-1. In order to determine the potential of a tag, its efficacy must be demonstrated with useful chemical reagents. Amino acid reagents were chosen to demonstrate the tagging system due to the ready availability of a wide variety of side-chain functional groups and the widespread interest in peptide synthesis. Histidine and tyrosine residues were tagged

Figure 3-2. Comparison of retention time and binding affinity of *para*-phenolic compounds.

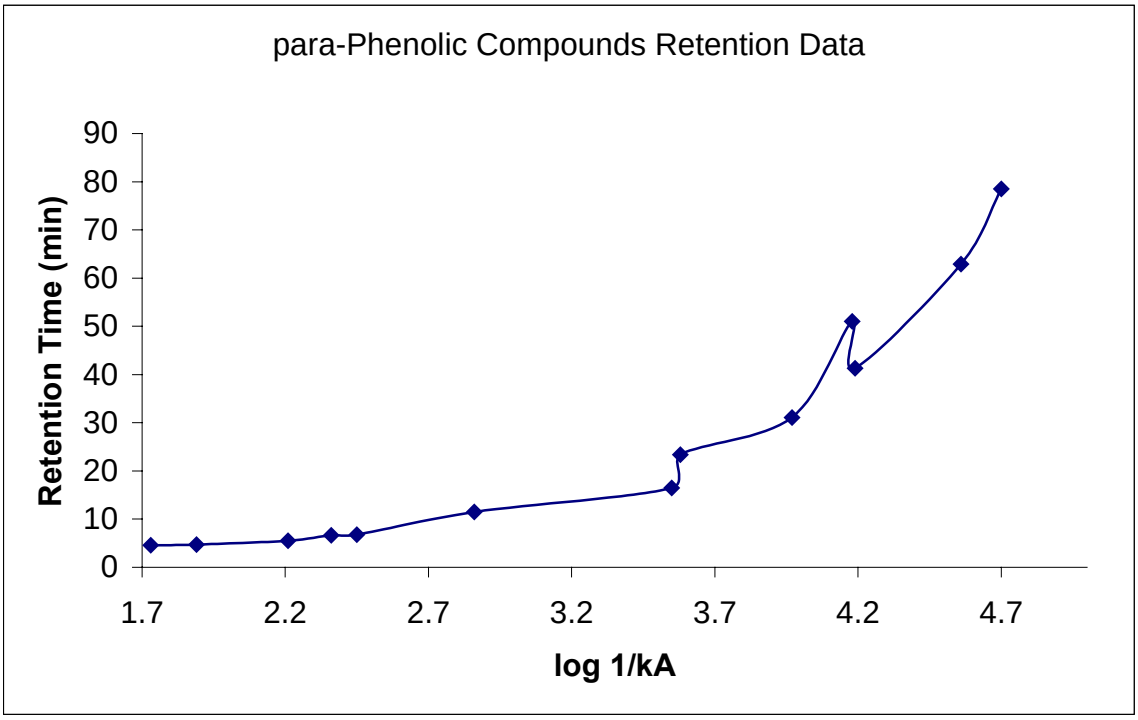


Table 3-2. *para*-Phenolic compound retention data.

***p*-X-PhOH (X)**	**Retention time (min)[a]**	**Log1/kD from ref.**
F	4.567	1.73
H	4.700	1.89
MeO	5.517	2.21
Me	6.650	2.36
Cl	6.767	2.45
Et	11.500	2.86
n-Pr	16.500	3.55
iso-Pr	23.367	3.58
n-Bu	31.093	3.97
sec-Bu	51.017	4.18
n-Amyl	41.333	4.19
tert-Bu	62.883	4.56
tert-Amyl	78.517	4.70

[a]3h gradient from 100% water (0.5%TFA) to 100% methanol.

Figure 3-3. Comparison of retention time and binding affinity of *meta*-phenolic compounds.

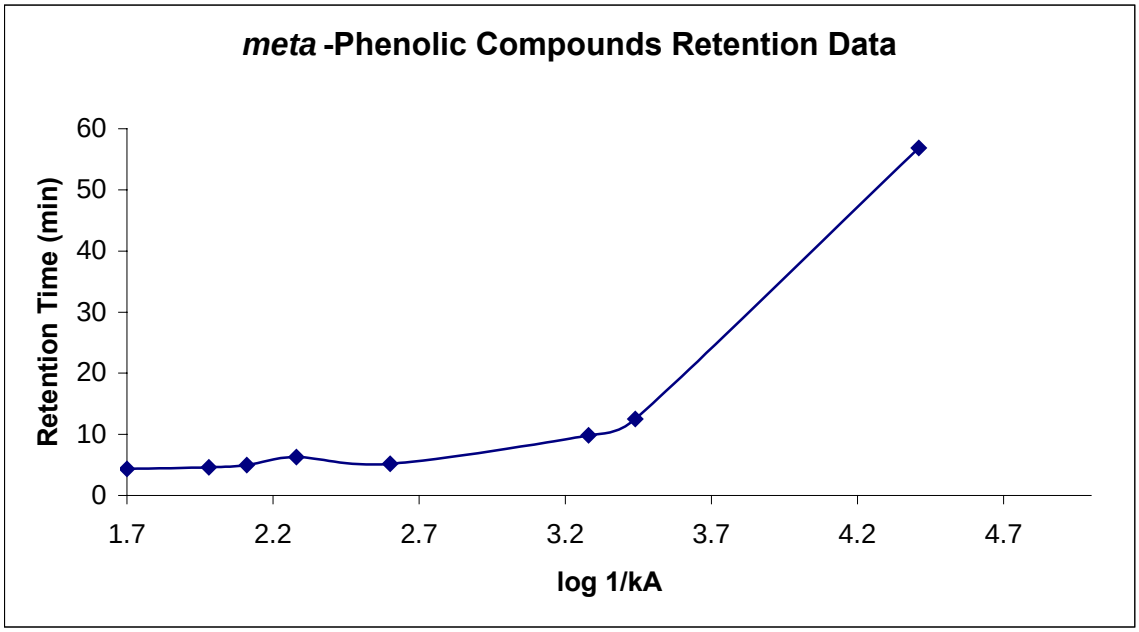


Table 3-3. *meta*-Phenolic compound retention data.

***m*-X-PhOH (X)**	**Retention time (min)[a]**	**Log1/kD from ref.**
F	4.367	1.7
Me	4.600	1.98
MeO	4.983	2.11
Cl	6.283	2.28
Et	5.183	2.60
n-Pr	9.833	3.28
iso-Pr	12.533	3.44
t-butyl	56.850	4.41

[a]3h gradient from 100% water (0.5%TFA) to 100% methanol.

Table 3-4. Retention times of various analytes.

Tyr(tBu)-OtBu	5.1
Fmoc-Phe-OH	5.3
Fmoc-Ile-OH	4.1
Fmoc-Tyr(tBu)-OH	6.5
Fmoc-His(trt)-OH	4.7
Fmoc-Lys(Mtt)-OH	4.4
Fmoc-Arg(Mtt)-OH	4.1
2,2,2-triflouroanthrylethanol	4.2
Binaphthol	6.3
Phenethyl alcohol	4.1
Methyl Mandelate	3.3
Warfarin	6.1
Hexobarbitol	3.5
Benzoin	4.1
Benzyl alcohol	4.2
Ac-Tyr(tBu)-OtBu	5.1

[a] Mobile phase gradient from 50% methanol to 100% methanol in 0.5% TFA/water in 1h. Dead volume=3.1 min.

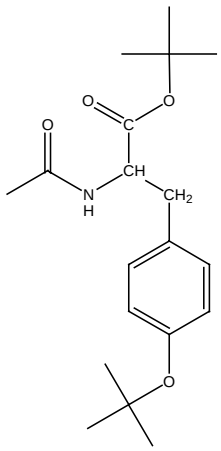


Figure 3-4. End-capped tyrosine**.**

according to the procedure in Figure 3-5 and tested chromatographically for their affinity for the beta-cyclodextrin stationary phase. As a control, H-Tyr(tbu)-Otbu was evaluated chromatographically, and the untagged reagent was not effectively retained by the beta-cyclodextrin stationary phase. The complementary experiment was not completed for the untagged H-His-OMe as it cannot be detected by a UV detector at 254nm without further derivitization. Both tagged reagents were retained for a considerable time on the column, thus indicating that the tags indeed are effective at inducing retention and thus useful for purification purposes (Table 3-5). It should be noted that there are two retention times reported for the tagged histidine compound, one in the presence of TFA and one without TFA. This data shows that charge influences the retention time of the tagged compounds to some extent however, even the charged moiety retains considerably longer than the control compounds studied. The two main drawbacks of this tag which make a second generation tag necessary are that

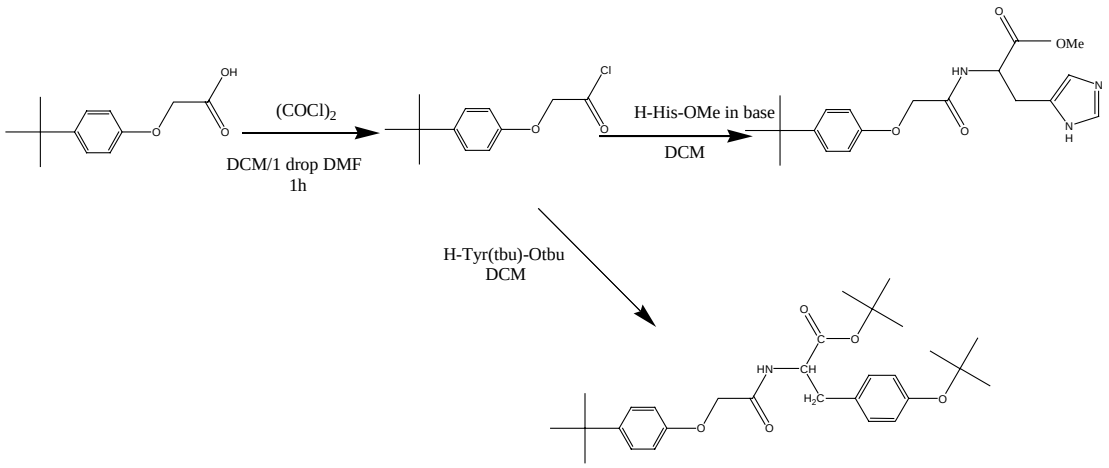
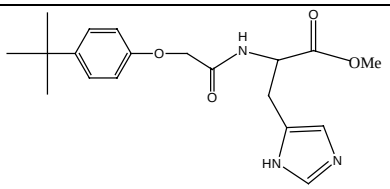
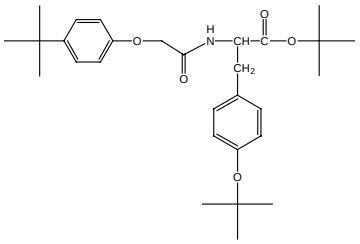


Figure 3-5. 1st generation tagging system.

Table 3-5. 1st Generation tagged reagents.

Compound	Retention Time (min)[a]
	**54, 80[b]**
	**88**

[a] Mobile phase gradient from 0% methanol to 100% methanol in 0.5%TFA/water in 3h. [b] Mobile phase gradient from 0% methanol to 100% methanol in water in 3h.

easy recovery of the reagent is not possible, and the direction of peptide synthesis is N to C, opposite from customarily used C to N method. The second-generation tag aims to resolve both of the drawbacks.

2nd Generation tag based on 4-tert-Butylphenol

A second-generation tag was developed in order to resolve the drawbacks involved with the first generation tag. 4-(*tert*-Butyl)benzyl bromide was used as the tagging reagent, and as a result the ester linkage should be easily cleaved by catalytic hydrogenolysis. Also by forming the ester, the subsequent peptide synthesis can be performed according to the customary C to N method. Fmoc-Ser(tbu)-OH was first tagged according to the conditions in Figure 3-6. Excess amino acid was used and the product plus excess amino acid were purposely taken as a mixture and evaluated chromatographically by HPLC. It was determined that up to approximately 50% methanol, the tagged compound is efficiently retained by the beta-cyclodextrin stationary phase even when injected in large amounts. Subsequently, a protocol was developed and tested for separation of the coupling reaction mixture. A 30mg aliquot of reaction mixture was successfully separated using an analytical scale HPLC column. The mixture was eluted at 40% methanol until all impurities were eluted, and then a gradient was employed up to 100% methanol to elute the product. The recovery yield of the product was over 95%. This experiment demonstrates the potential utility of the 4-t-butylphenyl tagging strategy for solution phase synthesis workup.

The next test to prove the utility of the 4-*tert*-butylphenyl tagging strategy involves using the tag in an actual coupling reaction. A series of compounds based on tagged glycine were evaluated chromatographically. First the tagged, Boc-protected glycine unit was evaluated chromatographically. Then the Boc group was cleaved and the tag was again evaluated. Next the deprotected glycine unit was coupled to a Boc-protected phenylalanine unit as shown in Figure 3-6. The reaction mixture was then purified by HPLC using the beta-cyclodextrin column. In the 3h gradient elution experiment all of the impurity peaks were observed early in the chromatogram, whereas the tagged product was eluted much later, thus the reaction impurities were easily removed leaving the pure product. This experiment shows that the tagging motif indeed works in a real synthesis situation thus proving the utility of the tagging system. The purification technique was then streamlined by quickly eluting the impurities by adjusting the mobile phase to 40% methanol in .5%TFA/water, then moving to a methanol mobile phase to elute the product.

The tagged glycine was also coupled to Boc-protected tyrosine to test the tagging efficiency with additional functional groups. Scheme 3-3 shows the coupling strategy to be identical to that used with phenylalanine. The results indicate that even though the tryptophan has additional functional groups that influence the polarity of the amino acid, the 4-*tert*-butylphenyl tag is not greatly influenced. As with the purification of the phenylalanine coupling reaction mixture, the separation was effected by quickly eluting the impurities by

adjusting the mobile phase to 40% methanol in .5%TFA/water, then moving to a methanol mobile phase to elute the product.

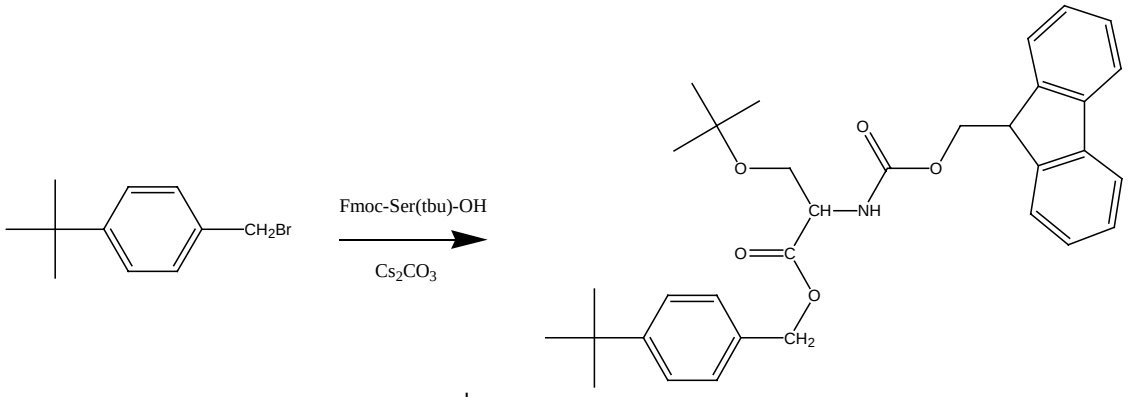


Figure 3-6. 2nd Generation tagging strategy.

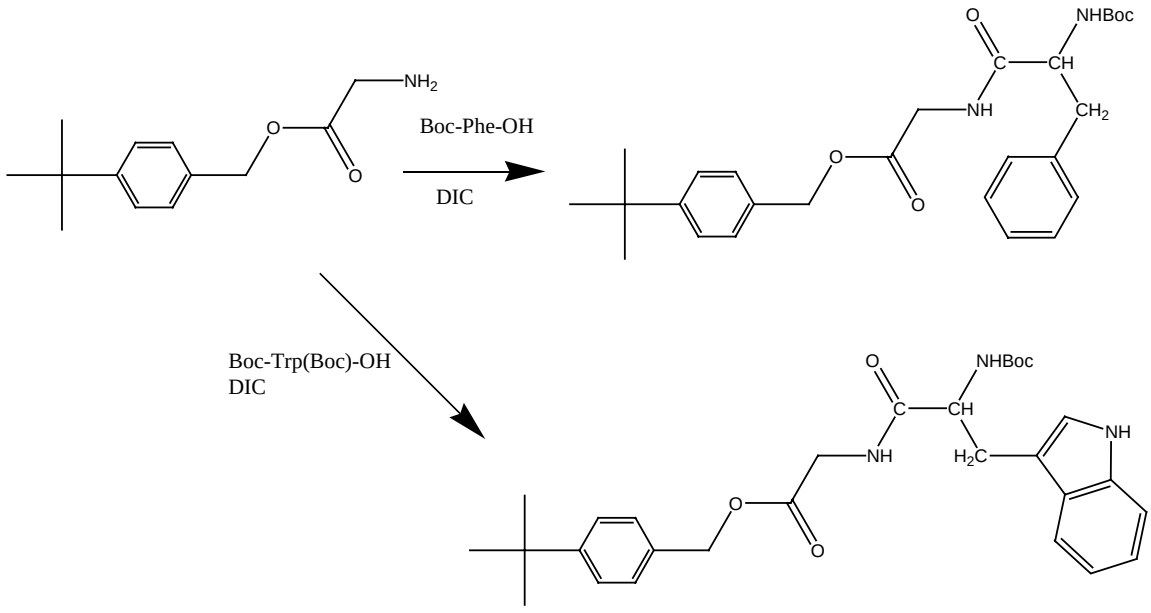
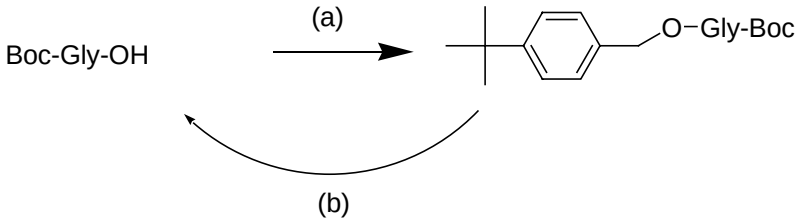


Figure 3-7. Tagged coupling reactions.

Tag manipulation

A concern with any tagging system is the ease with which one can manipulate the tag. In this tagging strategy the tag has been shown to be easily applied, and the method of choice for cleavage of the tag is catalytic hydrogenolysis using palladium on carbon and hydrogen gas (Figure 3-8). The catalytic hydrogenolysis to recover glycine from the 4-*tert*-butylphenyl tagged form was effected in as little 3h. Cleavage was clean and after workup, Boc-glycine was recovered in quantitative yield.



(a) Cs_2CO_3, 4-*(tert*-butyl)benzyl bromide
(b) H_2, Pd/C

Figure 3-8. Ease of tag manipulation.

Protective group "toolbox"

In order for the 4-*tert*-butylphenyl tagging strategy to truly become practical for routine use in solution phase organic synthesis, there must be convenient methods for adding the 4-*tert*-butylphenyl group to a variety of functional groups as useful reagents will have ever-changing functional groups available for tag attachment. It is for this reason that a protective group "toolbox" was developed.

Silyl chloride reagents are the most popular protective groups for the protection of alcohols in organic synthesis[91]; therefore a silyl chloride reagent was developed based on the 4-*tert*-butylphenyl group as a convenient tag for reagents with alcoholic functional groups. The (4-*tert*-butylphenyl)chlorodimethylsilane reagent was made by a Grignard reaction according to the synthetic scheme in Figure 3-9. The reagent was then used to protect benzyl alcohol as a test reagent (Figure 3-10). The resulting tagged compound was evaluated chromatographically as previous tagged compounds using the commercial beta-cyclodextrin column and the three-hour gradient. The tagged benzyl alcohol retained for 113 min, which is longer than any other compounds studied and proves the silyl chloride protective group effective as a tag for purification of solution phase synthesis products.

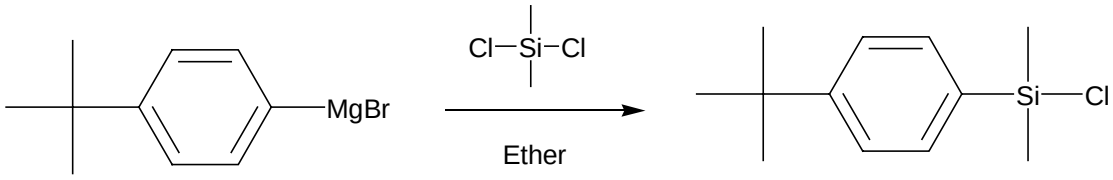


Figure 3-9. Silyl chloride synthesis.

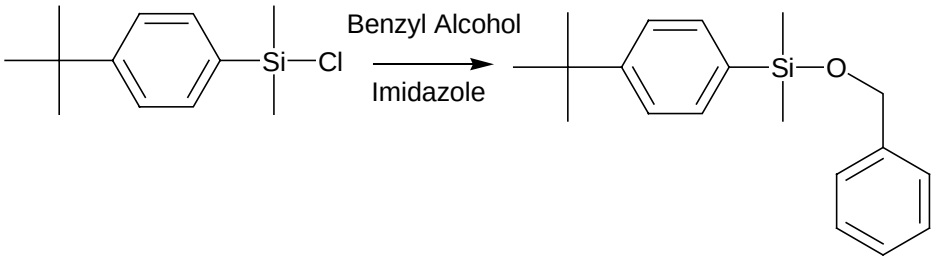


Figure 3-10. Protection of benzyl alcohol with silyl chloride.

Another functional group that is important to develop a tag for is the amine group. This was accomplished conveniently by forming the 4-*tert*-butylbenzyl carbamates from the amines on the reagent molecules (Figure 3-11). Both H-Tyr(tbu)-Otbu and H-His-OMe were tagged by this method and evaluated chromatographically. The resulting tagged compounds were retained for 87min and 54min respectively. These results show that the carbamate protective group strategy is a successful means using the amine functional group of a potential reagent as a site for the convenient attachment of the 4-*tert*-butylbenzyl group,

thus facilitating the simple purification of the products of subsequent solution phjase organic reactions. It is also important to note that the resulting 4-*tert*-butylbenzyl carbamate group is easily cleaved by hydrogenolysis.

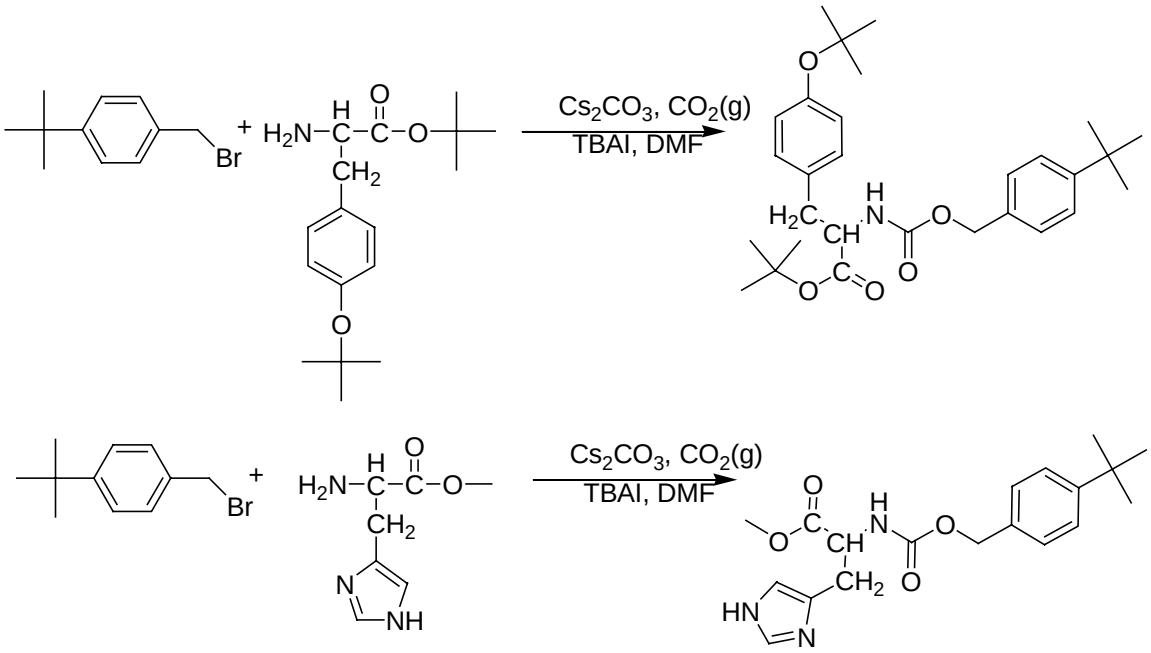


Figure 3-11. Application of carbamate protective group.

In addition to acids, amines, and alcohols; thiols are also important functional groups in organic synthesis. As a proof of principle attempt, the thiol group of thiophenol was protected by a 4-*tert*-butylbenzyl group according to Figure 3-12 and evaluated chromatographically in order to assess the potential for this type of reagent tagging strategy. The resulting tagged thiol was retained on the beta-cyclodextrin column for almost 92min. This experiment shows the

potential for thiol tagging as a means to affect easier separation of products, however the development of a disulfide tag would be more useful as it would be easier to manipulate.

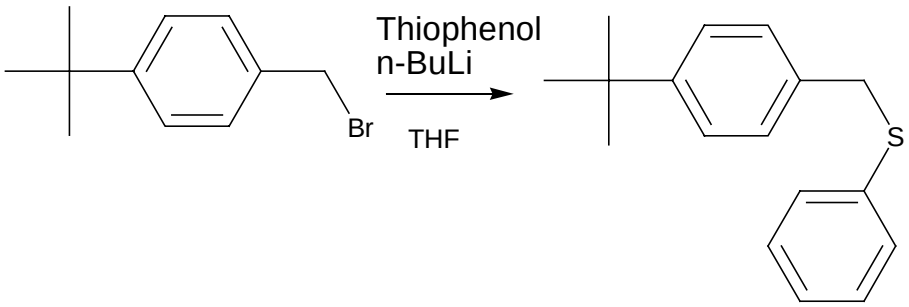


Figure 3-12. Protection of thiol group.

Development of a water-soluble tag

A major concern with the current tagging strategy is the fact that the 4-*tert*-butylphenyl group is hydrophobic and therefore will not be soluble in water. This is important because the conditions used to perform the separation involve a water-based mobile phase. If the 4-*tert*-butylphenyl tagged reagents are not soluble enough in the water/methanol mobile phase that they are applied in, they will precipitate instead of including into the beta-cyclodextrin cavity and impede effective separation of tagged compounds from untagged compounds. It is for this reason that more water-soluble tags were developed.

Ethylene glycol based moieties have been used effectively in the past in order to induce water solubility for many types of compounds. In the case of the tagging strategy that has been described, the most advantageous way to introduce these ethylene glycol groups is through a linker. The mono-, di-, and tri- ethylene glycol linkers were made according to Figure 3-13 and evaluated chromatographically in the same manner as the previously described tagged compounds. The retention times of these tags were compromised a bit due to their greatly increased polarity, but not to the detriment of their utility. Another thing to point out is that the three tags are retained for approximately the same length of time, indicating that the robustness of the 4-*tert*-butylphenyl group. The monoethylene glycolated structure was retained for 54min, whereas the di- and triethylene glycolated tags (2-(2-(4-*tert*-butylphenoxy)ethoxy) ethanol and 2-(2-(2-(4-*tert*-butylphenoxy)ethoxy)ethoxy)ethanol) were retained for 61 and 58min respectively. These tags are still retained well enough to effect successful separation strategies for organic solution phase synthesis. They are also successful in inducing increased water solubility for the 4-*tert*-butylphenyl tag. Before adding these linkers, tagged compounds were not soluble in the 40% water/methanol mobile phase used to apply the reaction mixture and elute the impurities. After adding the tri-ethylene glycol linkage, the solubility was increased dramatically such that a 0.1M solution of the tag could be produced.

The tri-ethylene glycolated tag was then used to tag a glycine residue in order to assess its potential as a tag and in order to compare its performance to

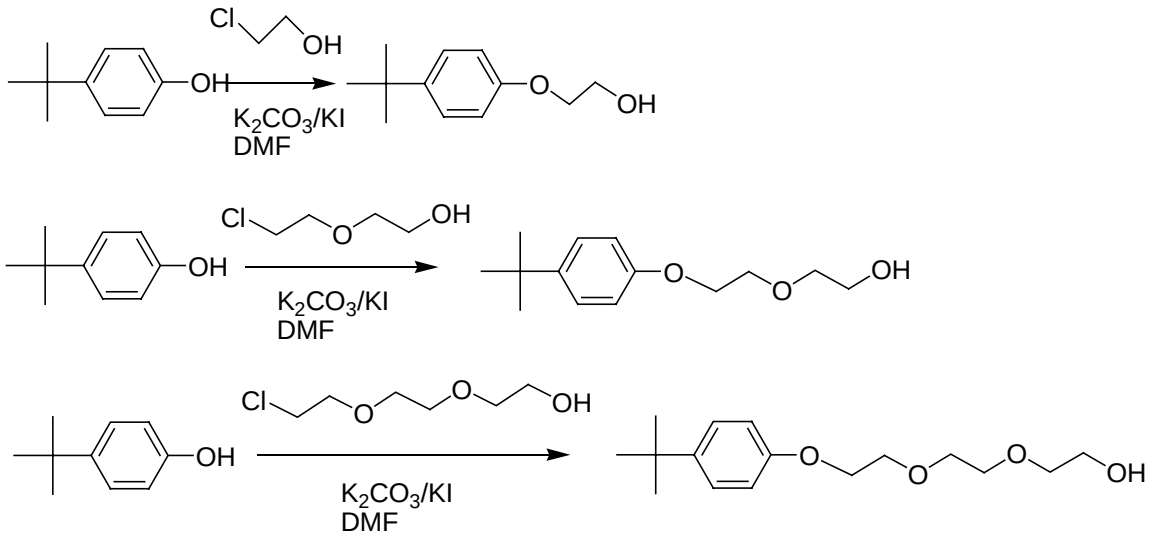


Figure 3-13. Attachment of ethylene glycol based linkers.

that of the 4-*tert*-butylphenyl tag. The tag was first converted to the chloride, and then reacted with the Boc-Gly-OH to give the tagged glycine according to Figure 3-14. The 2-(2-(2-(4-*tert*-butylphenoxy)ethoxy)ethoxy)ethanol tagged glycine was retained on the beta-cyclodextrin column for 80min which is the same amount of time that the 4-*tert*-butylphenyl tagged glycine was retained. This experiment shows that the increased water solubility of the 2-(2-(2-(4-*tert*-butylphenoxy)ethoxy)ethoxy)ethanol tag does not come at the price of good retention of tagged compounds and that the tag shows promise as a tool for easy purification of solution phase organic reaction products.

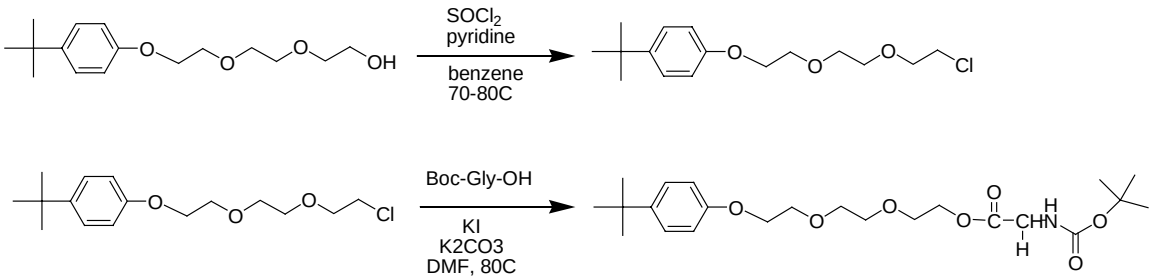


Figure 3-14. Attachment of 2-(2-(2-(4-*tert*-butylphenoxy)ethoxy)ethoxy)ethanol tag to Boc-Gly-OH.

Synthesis of a tripeptide

The solution phase synthesis of a tripeptide was also completed depending solely on the 4-tert-butylphenyl tagged separation strategy. The coupling reactions were executed without re-optimization according to well-known peptide coupling procedures, and the workup steps involved simple extraction followed by filtration of the sample through flash-grade silica gel with beta-cyclodextrin immobilized on the surface. The Gly-Phe-Leu tripeptide was successfully synthesized in solution using this strategy and was recovered in 91% overall yield. Figure 3-15 shows the synthetic scheme in detail.

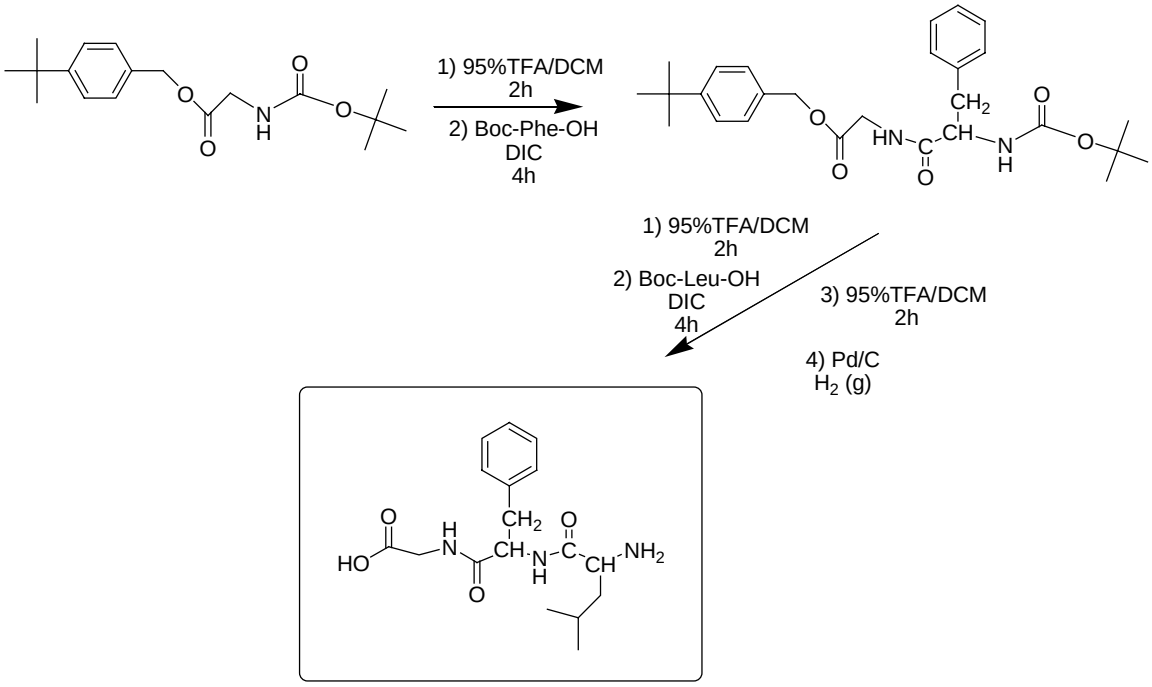


Figure 3-15. Tripeptide synthesis.

Summary

The solution-phase synthesis tagging system described here has been shown to be an effective method for easy purification of products from the crude reaction mixture.[64] This strategy is particularly attractive, as the tag is not greatly affected by the functional groups present on the reagent, and the tag can be easily attached as well as detached from the product upon recovery from the mixture. Also there is little need for mobile phase optimization as with traditional flash chromatography making for faster, easier separations. With future work, this strategy can be adapted for larger, preparative scale separations that function similarly to filtrations.

References

(1) Merrifield, R. B. *Journal of the American Chemical Society* **1963**, 2149-2154.

(2) Bodansky, M. *Peptide Chemistry: A Practical Approach*, Second ed.; Springer-Verlag: Berlin, 1993.

(3) Fields, G. B., Ed. *Solid-Phase Peptide Synthesis*; Academic Press: New York, 1997.

(4) Jones, J. *Amino Acid and Peptide Synthesis*; Oxford Science Publications: New York, 1992.

(5) Beaucage, S. L. I., Radhakrishnan, P. *Tetrahedron* **1992**, *48*, 2223-2311.

(6) Danishefsky, S. J. M., K.F.; Randolph, J.T.; Ruggeri, R. B. *Science* **1993**, *260*.

(7) Hermkens, P. H. H. O., H.C.J.; Rees, D. *Tetrahedron* **1997**, *53*.

(8) Kaldor, S. S., M.; Fritz, J.; Dressman, B.; Hahn, P. *Tetrahedron Letters* **1996**, *37*, 7193.

(9) Studer, A. H., Sabine; Ferritto, Rafael; Kim, Sun-Young; Jeger, Patrick; Wipf, Peter; Curran, Dennis P. *Science* **1997**, *275*, 823-826.

(10) Araki, T. T., Shinji; Tanaka, Minoru *Analytica Chimica Acta* **2000**, *410*, 37-45.

(11) Armstrong, D. W. D., Wade; Alak, Ala; Hinze, Willie L.; Riehl, Terrence E.; Bui, Khanh H. *Analytical Chemistry* **1985**, *57*, 234-237.

(12) Armstrong, D. W.; Ward, T. J.; Armstrong, R. D.; Beesley, T. E. *Tetrahedron Letters* **1997**, *38*, 6171-6172.

(13) Breslow, R. Z., B. *Journal of the American Chemical Society* **1996**, *118*.

(14) Cabrer, P. A.-P., E.; Meijide, F.; Seijas, J.A.; Nunez, E.R.; Tato, J.V. *Langmuir* **1999**, *15*, 5489-5495.

(15) Dandapani, S. N., J; Curran, D.P. *Tetrahedron Letters* **2004**, *45*, 6653-6656.

(16) Debowski, J. J., J.; and Sybilska, D. *Journal of Chromatography* **1983**, *282*, 83-88.

(17) Debowski, J. S., D. *J. Chromatogr.* **1986**, *353*, 409-416.

(18) Feng, Y.-Q. X., Ming-jie; Da, Shi-Lu *Analytica Chimica Acta* **2000**, *403*, 187-195.

(19) Fujimara, K. K., M.; Takayanagi, H.; Ando, T *Journal of Liquid Chromatography* **1986**, 607-620.

(20) Gazdag, M. S., G.; Huszár, L. *J. Chromatogr.* **1986**, *351*, 128-135.

(21) Haginaka, J. W., J. *Analytical Chemistry* **1990**, *62*, 997-1000.

(22) Han, S. M. H., Yoon I.; Armstrong, Daniel W. *J. Chromatogr.* **1988**, *441*, 376-381.

(23) Hargitai, T. K., Y.; Okamoto, Y. *J. Chromatogr.* **1993**, *628*, 11-22.

(24) Hinze, W. L. R., T.E.; Armstrong, D.W.; DeMond, W.; Alak, A.; Ward, T. *Analytical Chemistry* **1985**, *57*, 237-242.

(25) Italia, A. S., M.;Ventura, P. *J. Chromatogr.* **1990**, *503*, 266-271.

(26) Kawaguchi, Y. T., M.; Nakae, M.; Funazo, K.; Shono, T. *Analytical Chemistry* **1983**, *55*, 1852-1857.

(27) Li, S. P., W.C. *J. Chromatogr.* **1992**, *625*, 109-120.

(28) Maguire, J. H. *J. Chromatogr.* **1987**, *387*, 453-458.

(29) Matsuzawa, H. M., K. *Synlett* **2002**, *10*, 1607-1612.

(30) Nowakowski, R. C., Philippe J.P.; Coleman, ANthony W.; Villard, Emmanuelle; Guiochon, Georges *Analytical Chemistry* **1995**, *67*.

(31) Shimada, K. K., Yoshihiro; and Mitamura, Kuniko *Journal of Chromatographic Science* **1990**, *28*, 331-335.

(32) Tan, Z. J. Z., X.X.; and Brown, G.R. *Langmuir* **1994**, *10*, 1034-1039.

(33) Yang, Z. B., Ronald *Tetrahedron Letters* **1997**, *38*, 6171-6172.

(34) Zhang, L. W., Y.; Chen, L; Hg, S. *Tetrahedron Letters* **1999**, *40*, 1815-1818.

(35) Zukowski, J. S., D.; Bojarski, J. *J. Chromatogr.* **1986**, *364*, 225-232.

(36) Matsui, Y. N., T.; Fujita, T. *Top. Curr. Chem* **1985**, *128*, 61-89.

(37) Greene, T. W. W., Peter G. M. *Protective Groups in Organic Synthesis*, 3rd ed.; Wiley & Sons, Inc.: New York, NY, 1999.

(38) Blodgett, J. L., Tingyu *Tetrahedron Letters* **2004**, *45*, 6649-6652.

CHAPTER IV

EVALUATION OF LITHOCHOLIC ACID AS A POSSIBLE TAGGING SYSTEM FOR SOLUTION PHASE ORGANIC SYNTHESIS

Introduction

Beta-cyclodextrin is known to bind lithocholic acid with an association constant on the order of 10^6 M^{-1}in water.[70, 85-87, 92] Therefore, just as was shown with 4-*tert*-butylphenolic tags[64] in the previous chapter, a stationary phase comprised of immobilized beta-cyclodextrin should facilitate the efficient separation of compounds containing the lithocholic acid group from compounds that do not have such a functional group.

While the development of the 4-*tert*-butylphenolic tags was a more straightforward task, there are several considerations that must be evaluated in the case of lithocholic acid. By evaluating the structure of lithocholic acid (Figure 4-1), one immediately notices the lack of an ultraviolet (UV) chromophore by which the tag or tagged compounds could easily be visualized. It is also apparent that there are several functional groups that could interfere with organic reactions, and therefore must be either masked, or used as the attachment site. This chapter will deal with these issues and evaluate the utility of lithocholic acid as a tagging strategy for solution phase organic synthesis.

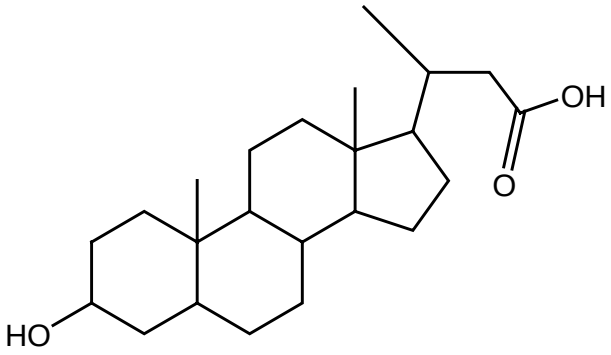


Figure 4-1. Structure of lithocholic acid.

Experimental

General Supplies and Equipment

Amino acid derivatives were purchased from NovaBiochem (San Diego, CA). All other chemicals and solvents were purchased from either Aldrich (Milwaukee, WI), Fluka (Ronkonkoma, NY), or Fisher Scientific (Pittsburgh, PA). Beta-cyclodextrin bonded HPLC column was purchased from Advanced Separation Technologies Inc. (Whippany, NJ). Selecto silica gel (32-63 μm) from Fisher Scientific was used for flash chromatographic purification of target compounds. Thin-layer chromatography was completed using EM silica gel 60 F-254 TLC plates (0.25 mm) (Emerck KGaA, 64271 Darmstadt, Germany). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded with a Bruker 300 MHz instrument. HPLC analyses were conducted using a Beckman analytical gradient system (System Gold).

Preparation of Benzyl Ester

To a flame dried flask 13.0mmol (5.0g) lithocholic acid and 13.0mmol (4.335g) cesium carbonate were added and dissolved in anhydrous dimethylformamide. Next 16.0mmol (3.0ml) benzyl bromide were added and let stir. When the reaction was finished according to thin layer chromatography, the reaction was quenched with water and worked up by extracting with methylene chloride five times. The methylene chloride layers were combined, dried with sodium sulfate, and concentrated by rotary evaporation. The pure product was recovered by recrystallization to give an 80% yield.

Characterization of lithocholic acid benzyl ester: ^{1}H NMR (MeOD) δ 0.57(s, 3H), 0.87 (s, 6H), 0.9-1.4 (m, 20H), 1.5-2.0 (m, 9H), 2.1-2.4 (m, 2H), 3.4-3.5 (m, 1H), 5.1 (d, 2H), 7.3 (s, 5H)

Preparation of methoxy benzyl ester

To a flame dried flask 9.5mmol (4.15g) lithocholic acid benzyl ester and 18.0mmol (3.735g) 2,6-di-*tert*-butyl-4-methyl pyridine were added and dissolved in anhydrous methylene chloride under argon. Next 18.0mmol (2.0ml) methyl trifluoromethanesulfonate were added and let stir. When the reaction was finished according to thin layer chromatography, the reaction was quenched with dilute hydrochloric acid and worked up by extracting with methylene chloride five times. The methylene chloride layers were combined, dried with sodium sulfate,

and concentrated by rotary evaporation. The pure product was recovered by flash chromatography in 5%ethyl acetate mobile phase.

Characterization of methoxy lithocholic acid benzyl ester: ^{1}H NMR (MeOD) δ 0.57(s, 3H), 0.87 (s, 6H), 0.9-1.4 (m, 20H), 1.5-2.0 (m, 9H), 2.1-2.4 (m, 2H), 3.2 (s, 3H), 3.4-3.5 (m, 1H), 5.1 (d, 2H), 7.3 (s, 5H)

Preparation of the 2nd generation lithocholic acid tag

To a flame dried flask under argon 5.3mmol (2.0g) lithocholic methoxy lithocholic acid were added and dissolved in 10.0ml anhydrous methylene chloride. Next 26.5mmol (2.0ml) oxalyl chloride were added and let stir. Then 2-3 drops anhydrous dimethylformamide were added and let react for 2h. After the reaction was complete, the reaction mixture was immediately evaporated to dryness under vacuum, leaving the methoxy lithocholic acid chloride. Anther flame dried flask under argon was charged with 5.0ml ammonium and cooled in an ice bath. The methoxy lithocholic acid chloride was then dissolved in anhydrous methylene chloride and added slowly to the cold ammonium on ice and let react 15min. Then the reaction mixture was removed from the ice bath and let warm up to room temperature and continued to react. When the reaction was complete, the reaction was worked up by separating the two phases and extracting the water layer with methylene chloride three times. The methylene chloride layers were combined, dried with sodium sulfate, and then evaporated

off by rotary evaporation leaving the amide product behind. The amide was then dissolved in tetrahydrofuran, with a small amount of methylene chloride added for solubility, and then cooled in an ice bath. Next, 0.5g lithium aluminum hydride were added slowly with stirring. The mixture was then allowed to warm up to room temperature slowly. Next the mixture was heated to a reflux under argon. After 3h the reaction was complete by thin layer chromatography. The reaction was worked up by quenching with copious amounts of water, followed by subsequent extractions with diethyl ether. The ether layers were then combined and dried with sodium sulfate and evaporated off under reduced pressure.

Characterization of amide: ($CDCl_3$) δ 0.53(s, 3H), 0.95 (s, 6H), 0.9-1.4 (m, 16H), 1.5-2.0 (m, 8H), 2.1-2.4 (m, 2H), 3.0-3.1 (m, 1H),3.2 (s, 3H), 7.1 (s, 2H)..

Characterization of amine: ($CDCl_3$) δ 0.53(s, 3H), 0.95 (s, 6H), 0.9-1.4 (m, 16H), 1.5-2.0 (m, 8H), 2.1-2.4 (m, 2H), 2.65 (m, 2H), 3.0-3.1 (m, 1H),3.2 (s, 3H).

Coupling to 2nd Generation Lithocholic Acid Tag

To a flame dried flask under argon 0.17mmol (.06g) amine and 0.33 (0.129g) mmol Fmoc-Phe-OH, 0.33 (0.117g) mmol Fmoc-Ile-OH, 0.33 (0.175g) mmol Fmoc-Trp(Boc)-OH, 0.33 (0.207g) mmol Fmoc-Lys(Mtt)-OH, 0.33 (0.152g) mmol Fmoc-Tyr(tBu)-OH, or 0.33 (0.103g) mmol Fmoc-His(trt)-OH were added and dissolved in methylene chloride. Next 0.19mmol (30µl)

diisopropylcarbodiimide were added and let stir and react overnight. When the reaction was complete, the reaction was worked up by quenching with water and extracting with methylene chloride three times. The methylene chloride layers were combined, dried with sodium sulfate, and then evaporated off by rotary evaporation. The pure product was recovered by preparative thin layer chromatography using a 2.5% methanol in methylene chloride mobile phase.

Characterization of amine-Phe-Fmoc: ($CDCl_3$) δ 0.62(s, 3H), 0.86 (s, 3H), 0.95 (s, 6H), 1.0-2.0 (m, 20H), 3.0-3.3 (m, 6H), 3.4 (s, 3H), 4.2 (t, 1H), 4.25-4.5 (m, 3H), 5.35-5.4 (b, 1H), 5.45-5.5 (b, 1H), 7.15-7.4 (m, 7H), 7.4-7.5 (m, 2H), 7.6-7.7(m, 2H), 7.8-7.9(m, 2H).

Characterization of amine-His(trt)-Fmoc: ($CDCl_3$) δ 0.62(s, 3H), 0.86 (s, 3H), 0.95 (s, 6H), 1.0-2.0 (m, 20H), 3.0-3.3 (m, 6H), 3.4 (s, 3H), 4.1 (m, 1H), 4.2 (m, 2H), 4.5 (m, 1H), 6.9-7.1(m, 8H), 7.15-7.35 (m, 157H), 7.5 (m, 2H), 7.6 (m, 2H).

Characterization of amine-Tyr(tBu)-Fmoc: ($CDCl_3$) δ 0.61(s, 3H), 0.85 (s, 3H), 0.9(s, 3H), 1.0-2.0(m, 33H), 2.8-3.2 (m, 6H), 3.4 (s, 3H), 4.0 (m, 1H), 4.2(t, 1H), 4.3(m, 2H),6.8 (t, 2H), 7.2(d, 2H), 7.3(m, 2H), 7.4(m, 2H), 7.6(d, 2H), 7.8(d, 2H).

Characterization of amine-Trp(Boc)-Fmoc: ($CDCl_3$) δ 0.51(s, 3H), 0.73 (s, 3H),

0.85(s, 3H), 1.0-2.0(m, 20H), 1.60 (s, 9H), 3.3 (s, 3H), 4.1 (t, 1H), 4.3(d, 3H), 5.5(b, 2H),7.1-7.35 (m, 8H), 7.4(d, 2H), 7.6(d, 1H), 7.65(d, 2H), 7.7(d, 2H), 7.9(s, 1H), 8.1(d, 1H).

Characterization of amine-Lys(Mtt)-Fmoc: ($CDCl_3$) δ 0.50(s, 3H), 0.85 (s, 6H), 0.9-2.0(m, 36H), 3.0-3.2(m, 2H), 3.3 (s, 3H), 3.85 (t, 1H), 4.15(t, 1H), 4.35(m, 2H), 5.35 (d, 1H), 5.3(b, 1H), 7.2 (m, 2H), 7.35(m, 2H), 7.5(m, 2H), 7.7(d, 2H).

Results

The first issue that must be explored in order to evaluate the possible utility of a tagging system based on lithocholic acid is to examine whether blocking the reactive functional groups on the molecule will in any way affect its ability to form strong inclusion complexes with beta-cyclodextrin. This was done systematically by first protecting the acid group as a benzyl ester as shown in Figure 4-2. This accomplished three goals; the reactive acid group was blocked so that it could not participate in any reactions while acting as a tag. This determines whether the acid group could be the possible site for attachment to reagents, and this provides a UV chromophore for easy visualization. After this transformation was complete, the lithocholic acid benzyl ester (LABE) was evaluated chromatographically using a commercially available beta-cyclodextrin HPLC column. The chromatographic conditions were as follows: gradient elution from 50% methanol in 0.5%TFA/water to 100% methanol in 1 h, detected by UV

absorbance at 254nm. The benzyl ester retained for 25 min under these conditions. Because the parent lithocholic acid molecule does not have a UV chromophore, it was not feasible to compare the ester to the acid, however it was possible to compare the retention time of the ester to that of compounds that form strong inclusion complexes with beta-cyclodextrin such as 4-*tert*-butylphenol, and to that of compounds which do not form strong inclusion complexes with beta-cyclodextrin such as benzyl alcohol and benzoic acid. The results, shown in Table 1,indicate that the lithocholic acid benzyl ester does indeed form a strong inclusion complex with beta-cyclodextrin as it is retained almost twice as long as the previously studied tag 4-*tert*-butylphenol. This correlates well with the fact that lithocholic acid has a much larger binding affinity for beta-cyclodextrin than 4-*tert*-butylphenol. This result also indicates that the acid group is not essential for strong inclusion of lithocholic acid into the beta-cyclodextrin cavity.

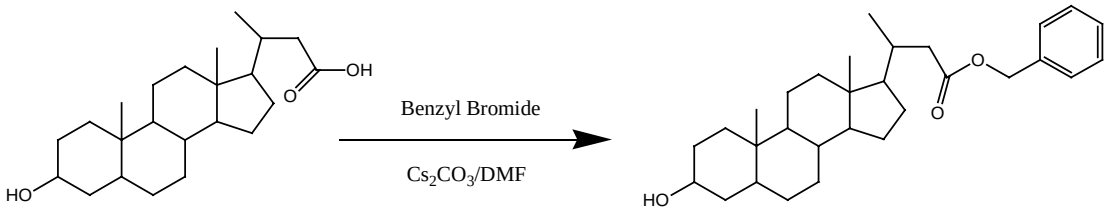


Figure 4-2. Protection of acid group with a benzyl group.

Table 4-1. Comparison of retention times of lithocholic acid analogues with various compounds on beta-cyclodextrin column.

Analyte	**Retention time** (min)	**Binding Constant K (M^{-1})**
Lithocholic acid	NA	10^{6}
LABE	25	NA
LABEME	29	NA
4-*tert*-Butylphenol	14.7	10^{4}
Benzyl alcohol	4.2	NA
Benzoic acid	3.5	NA

Next the alcohol group of the benzyl ester was protected according to the method shown in Figure 4-3 with a methyl ether group in order to determine whether the alcoholic functional group is an important contributor to the inclusion complex formation of lithocholic acid with beta-cyclodextrin. This is important as the alcohol group is a reactive functional group that could disrupt efficient tagging strategies by participating in the reaction intended for the tagged compound or by creating the possibility of unwanted side-reactions. The resulting methyl ether (LABEME) was then evaluated chromatographically in the same manner as the

benzyl ester. The results as shown in Table 4-1 indicate that the alcohol group is not essential for inclusion complex formation, and the blocking of the alcohol functional group actually increases the retention time and thus enhances binding with beta-cyclodextrin. The retention time of the methyl ether is 29 min where the retention time of the benzyl ester with an unprotected alcohol functional group is 25 min. The results of these preliminary experiments indicate that the lithocholic acid structure shows promise as a tag to facilitate ease of product recovery for solution phase synthesis.

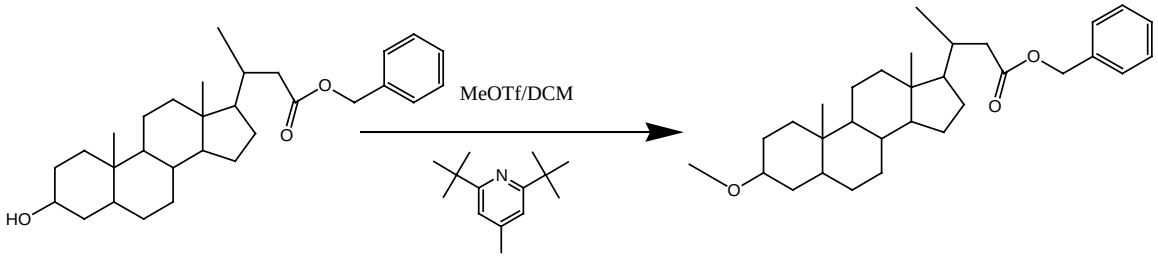


Figure 4-3. Protection of alcohol group with a methyl group.

1st Generation tag based on lithocholic acid

The first generation lithocholic acid-based tag was then developed as the preliminary experiments proved promising. The tagging of amino acids is a convenient and rigorous method to test the efficacy of the tagging strategy, as there is a wide range of functional groups in the form of side chains available with a wide range of polarities. Because the inclusion complex formation with beta-

cyclodextrin depends not only on how closely the analyte fits the cavity, but also on the hydrophobicity of the analyte. It is important to test the ability of potential tags to produce good retention even when they are used to tag polar reagents in order for the tag to be successful. The tyrosine and histidine amino acids were chosen as they are examples of both an alcohol and an amino polar functional group and will give good indication of how tolerable the tagging strategy will be to polar reagents.

The fully protected lithocholic acid (LABEME) was deprotected at the acid site in order to utilize the acid functionality as an attachment site. The acid was then converted into the acid chloride and coupled to both H-Tyr(tBu)-OtBu and H-His-OMe as shown in Figure 4-4. The tagged amino acids, both in their protected and deprotected forms, were then evaluated chromatographically by using both the 1h gradient described previously, as well as a 3h gradient from 0% methanol in 0.5%TFA/water to 100% methanol. The 3h gradient was used in order to highlight more subtle differences in analyte retention as well as to facilitate better analyte retention as the inclusion complex formation is driven in part by the hydrophobic effect. By loading the analytes onto the column while the mobile phase is comprised mainly of water, inclusion complex formation is encouraged.

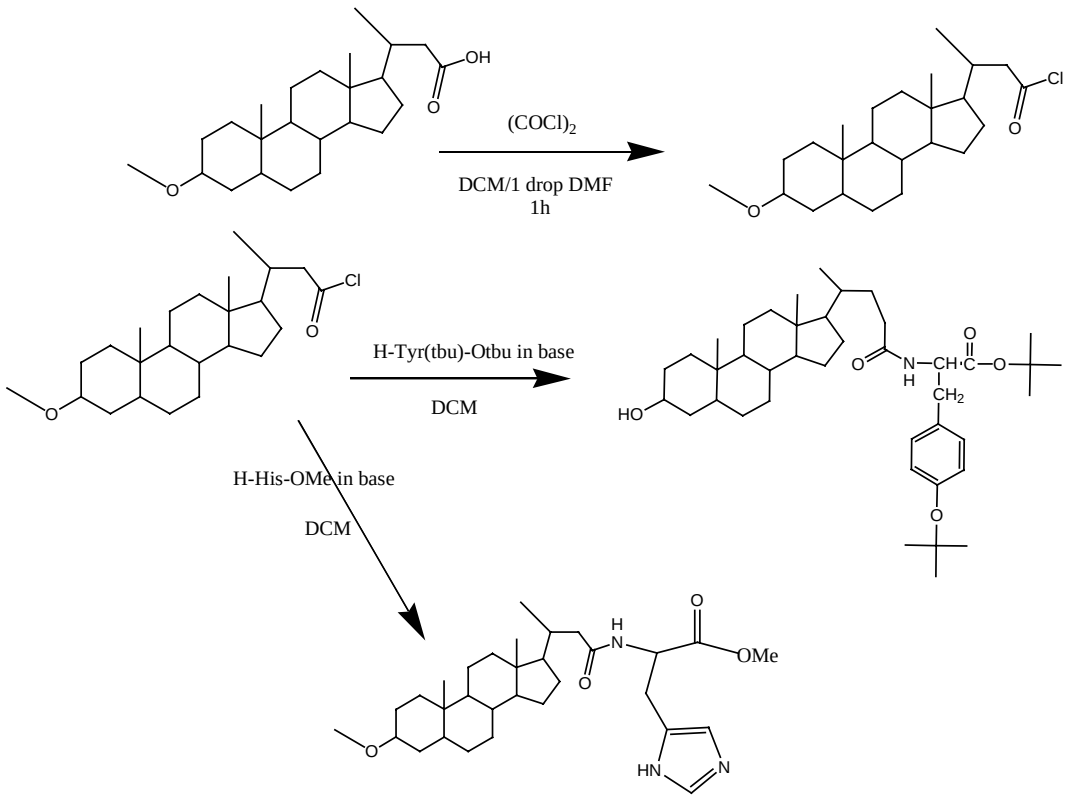


Figure 4-4. 1st Generation lithocholic acid tag used to tag tyrosine and histidine.

Table 4-2. Comparison of retention times of lithocholic acid tagged tyrosine and histidine with various compounds on beta-cyclodextrin column.

Analyte	**Retention Time 1h gradient (min)**	**Retention Time 3h gradient (min)**
Benzoic Acid	3.5	14.8
4-*tert*-Butylphenol	14.7	91
LABE	20	121
LA-Tyr-(*t*Bu)-O*t*Bu	20	130
LA-Tyr-OH	17	110
LA-His-OMe	14	94
LA-His-OH	20	98

The results in Table 4-2 show that the tagged amino acids are retained for a significantly long time, thus indicating that their presence does not greatly influence the ability of lithocholic acid to include into the beta-cyclodextrin cavity and form a strong complex. The protected tagged tyrosine and histidine are retained for 130 and 94 min respectively, but the functional group deprotected

tagged tyrosine and histidine are retained for an impressive 110 and 98 min respectively. When the amino acids are deprotected leaving their very polar side-chains free to form interactions, the affinity of lithocholic acid for the beta-cyclodextrin cavity is still strong enough to complex strongly. The 1st generation tag based on lithocholic acid shows promise, but a new linkage method would be more convenient as the direction of peptide synthesis is customarily C to N as opposed to the N to C method demonstrated here. It is for this reason that there are few choices of commercially available amino acids with convenient protective group strategies for the 1st generation tag, however it could be useful as a tag for reagents used for other chemistries. The second-generation tag aims to resolve this drawback.

2nd Generation tag based on lithocholic acid

In order to facilitate the customary C to N direction of peptide synthesis and develop another choice for reagent attachment, the second-generation tag must have an amine functional group linker. This was accomplished by first converting the acid to the acid chloride, then to the amide, followed by the reduction of the amide to give the amine (Figure 4-5). The amine was then coupled to various amino acids (Figure 4-6) using traditional peptide coupling chemistry and evaluated chromatographically. The results are shown in Table 4-3.

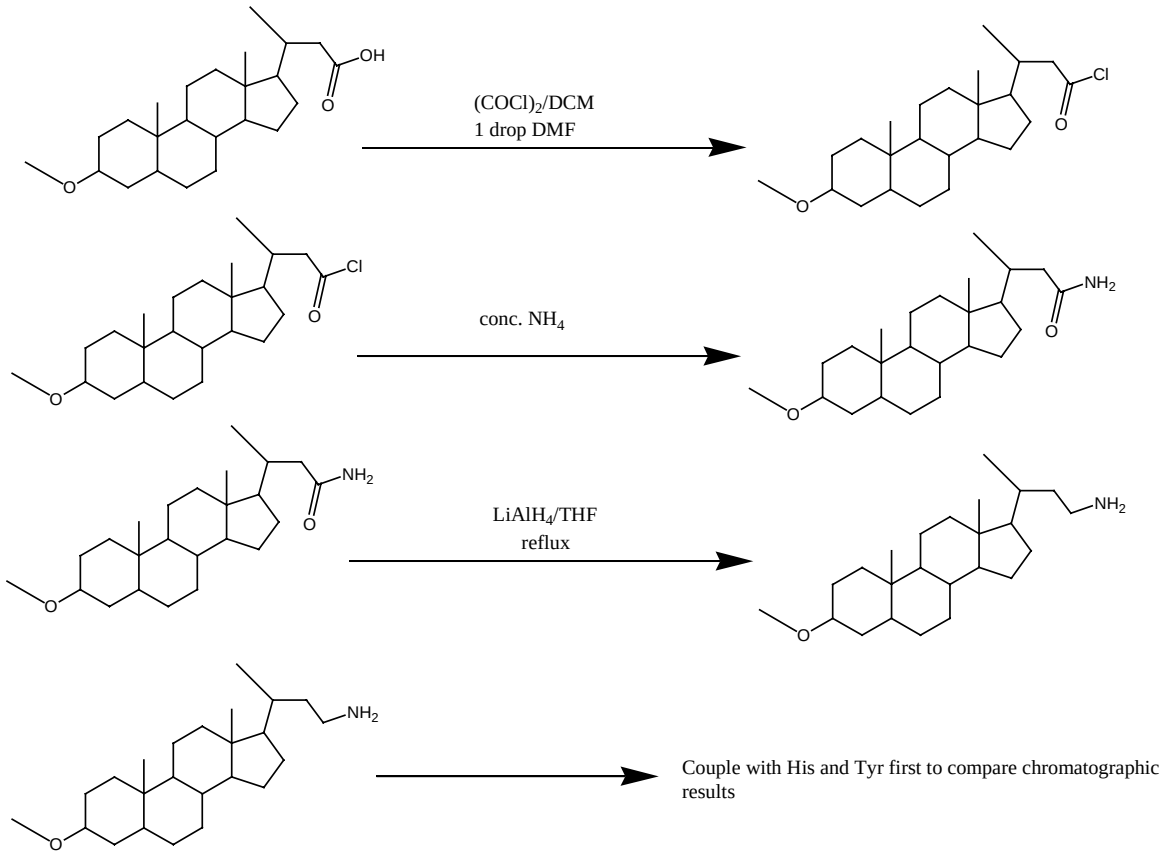


Figure 4-5. Synthesis of 2nd generation lithocholic acid tag.

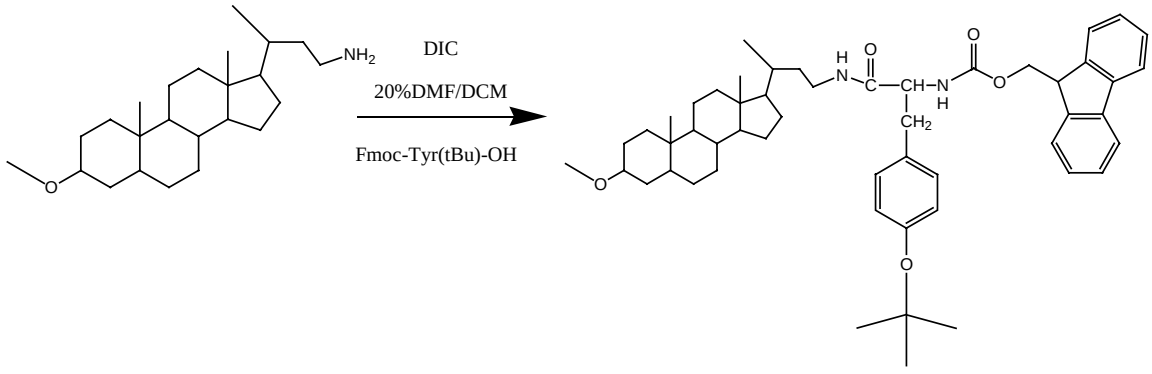


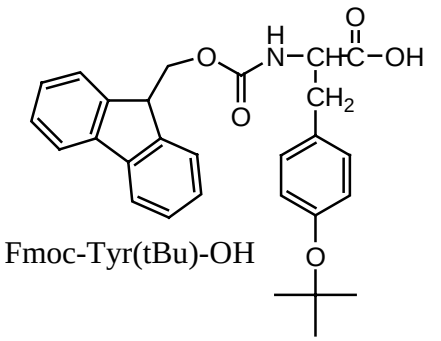
Figure 4-6. Representative coupling reaction of 2nd generation lithocholic acid tag and amino acids.

Table 4-3. Comparison of retention times of various amino acids tagged with 2nd generation lithocholic acid tag on beta-cyclodextrin column.

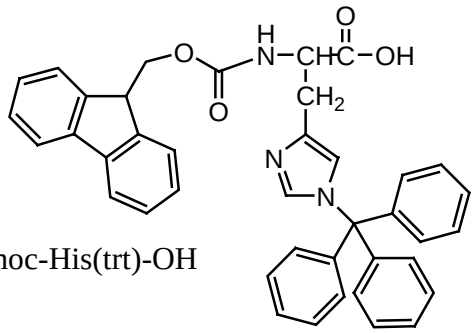
Amino Acid	t_R (min)
Fmoc-Tyr(*t*Bu)-OH	146
Fmoc-Tyr-OH	122
Fmoc-His(trt)-OH	130
Fmoc-His-OH	96
Fmoc-Lys(Mtt)-OH	108
Fmoc-Lys-OH	96
Fmoc-Trp(boc)-OH	142
Fmoc-Trp-OH	126
Fmoc-Ile-OH	140
Fmoc-Phe-OH	131

The chromatographic data from the 2nd generation lithocholic acid tagged amino acids shows that the tag performance is impressive. Six different amino acids with various side-chain functional groups and various polarities were studied in both their protected from and their deprotected form where applicable. The

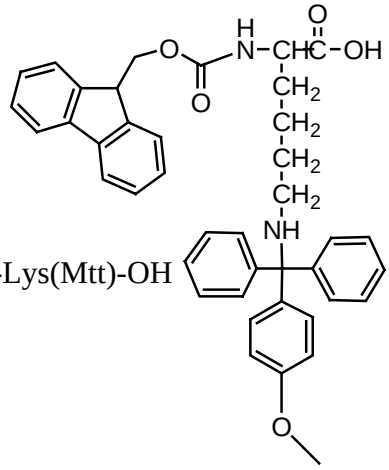
amino acid structures are shown in figure 4-7. It is also worth noting that in Table 3-2 it was shown that these same amino acids and various aromatic compounds were studied chromatographically as control compounds and were eluted at the column dead volume. The breadth of conditions explored by this experiment highlights the remarkable ability of the lithocholic acid portion of the molecule to include into the beta-cyclodextrin cavity and form a stable complex, and therefore perform well as a tag to facilitate the separation of products in solution phase synthesis.



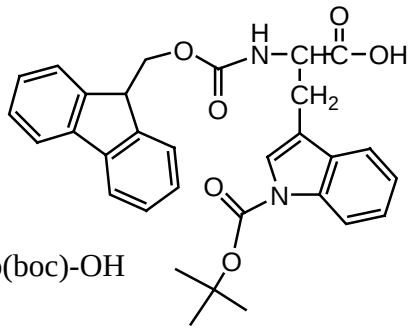
Fmoc-Tyr(tBu)-OH



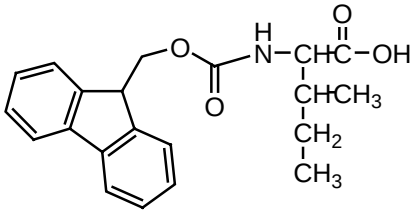
Fmoc-His(trt)-OH



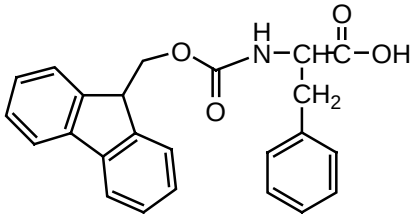
Fmoc-Lys(Mtt)-OH



Fmoc-Trp(boc)-OH



Fmoc-Ile-OH



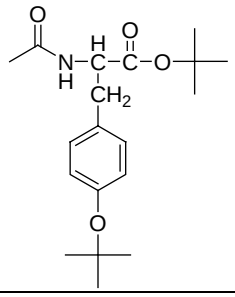
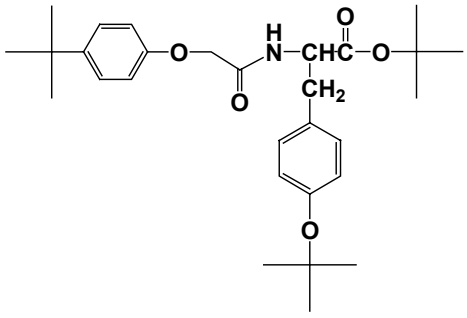
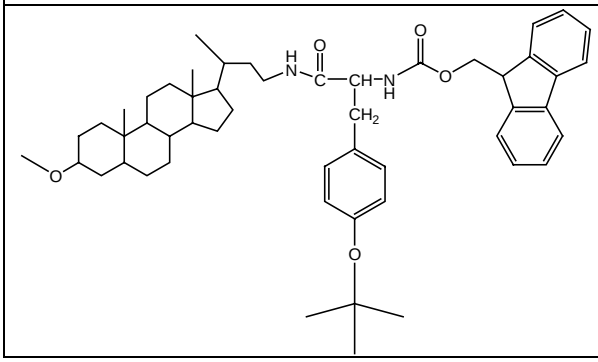
Fmoc-Phe-OH

Figure 4-7. Structures of amino acids that were evaluated chromatographically.

Lithocholic acid vs.4-t-butyl-phenol as a Tag

A comparison of the performance of the tags based on 4-*tert*-butylphenol to those based on lithocholic acid yields a very complicated answer as to which tag is best. Table 4-4 shows the percent organic mobile phase component that is required to elute a representative sample of an untagged compound, a compound tagged with the 4-*tert*-butylphenol tag, and a compound tagged with the lithocholic acid tag. This gives some indication of the relative affinity of each tag for the beta-cyclodextrin stationary phase, and therefore how tolerant the separation method is to reagent compounds that may also have a moderate affinity as well. This also gives some indication of how versatile each tagging system will be. The representative reagent compound is tyrosine. It is clear that lithocholic acid tag is retained the best on the beta-cyclodextrin stationary phase, followed by the 4-*tert*-butylphenol tag, and the untagged compound is easily eluted by very small amounts of organic mobile phase component. This experiment shows that a tagged compound using either tag can easily and quickly be separated from an untagged compound, and also a 4-*tert*-butylphenol tagged compound can be expected to easy to separate from a lithocholic acid tagged compound as well.

Table 4-4. Percent organic mobile phase component required to elute analytes.

Compound	**% Acetonitrile**	**% Methanol**
	15	15
	45	50
	65	70

Summary

This project confirms that lithocholic acid can be used as a potential tag for solution phase synthesis to facilitate the purification of synthesis products. On the other hand, at this point the 4-*tert*-butylphenyl tag is more convenient as it is

easily attached to the reagent, as well as cleaved from easily cleavable. In addition, the 4-*tert*-butylphenyl tag has an inherent UV chromophore in order to facilitate easy visualization. Another advantage that the 4-*tert*-butylphenyl tag has is that it is inert to most organic reactions without any derivitizations, whereas the lithocholic acid has an extra functional group that must be blocked for use in many organic reactions. In most cases the 4-*tert*-butylphenyl tag would be most viable at this point, but with future work the lithocholic acid tag could become much more useful as it has very impressive retention capabilities on the beta-cyclodextrin stationary phases.

References

(1) Stinson, S. C. *Chemical and Engineering News* **2001**, *79*, 79.

(2) Subramanian, G. *Chiral Separation Techniques: A Practical Approach*; Wiley: Weinheim, 2001.

(3) Armstrong, D. W.; Ward, T. J.; Armstrong, R. D.; Beesley, T. E. *Tetrahedron Letters* **1997**, *38*, 6171-6172.

(4) Berthod, A.; Liu, Y.; Bagwill, C.; Armstrong, D. W. *Journal of Chromatography* **1996**, *731*, 123-137.

(5) Oguni, K.; Oda, H.; Ichida, A. *J. Chromatogr., A* **1995**, *694*, 91-100.

(6) Kawaguchi, Y. T., M.; Nakae, M.; Funazo, K.; Shono, T. *Analytical Chemistry* **1983**, *55*, 1852-1857.

(7) Welch, C. J. *J. Chromatogr., A* **1994**, *666*, 3-26.

(8) Haginaka, J. *Journal of Chromatography, A* **2001**, *906*, 253-273.

(9) Pirkle, W. H.; Welch, C. J.; Hyun, M. H. *J. Chromatogr.* **1992**, *607*, 126-130.

(10) Lam, K. S. *Anti Cancer Drug Design* **1997**, *12*, 145-167.

(11) Maclean, D.; Baldwin, J. J.; Ivanov, V. T.; Kato, Y.; Shaw, A.; Schenider, P.; Gordon, E. M. *Journal of Combinatorial Chemistry* **2000**, 562-578.

(12) Weingarten, M. D.; Sekanina, K.; Still, W. C. *Journal of the American Chemical Society* **1998**, *120*, 9112-9113.

(13) Bluhm, L. H.; Wang, Y.; Li, T. *Analytical Chemistry* **2000**, *72*, 5201-5205.

(14) Wu, Y.; Wang, Y.; Yang, A.; Li, T. *Analytical Chemistry* **1999**, *71*, 1688-1691.

(15) Murer, P.; Lewandowski, K.; Svec, F.; Frechet, J. M. *Analytical Chemistry* **1999**, *71*, 1278-1284.

(16) Lewandowski, K.; Murer, P.; Svec, F.; Frechet, J. M. *Journal of Combinatorial Chemistry* **1999**, 105-112.

(17) Wang, Y.; Li, T. *Analytical Chemistry* **1999**, *71*, 4178-4182.

(18) Wang, Y.; Bluhm, L. H.; Li, T. *Analytical Chemistry* **2000**, *72*, 5459-5465.

(19) Welch, C. J.; Protopopova, M. N.; Bhat, G. *Enantiomer* **1998**, *3*, 471-476.

(20) Welch, C. J.; Bhat, G.; Protopopova, M. N. *Journal of Combinatorial Chemistry* **1999**, *1*, 364-367.

(21) Pirkle, W. H.; Welch, C. J.; Lamm, B. *J. Org. Chem.* **1992**, *57*, 3854-3860.

(22) Rana, S.; White, P.; Bradley, M. *Journal of Combinatorial Chemistry* **2001**, *3*, 9-15.

(23) Pirkle, W. H.; Hyan, M. H. *Journal of Chromatography* **1985**, *328*, 1-9.

(24) Lewandowski, K.; Murer, P.; Svec, F.; Frechet, J. M. J. *Chemical Communications* **1998**, 2237-2238.

(25) Blodgett, J. W., Yan; Li, Tingyu; Polarvarapu, Prasad L.; Drabowicz, Jozef; Pietrusiewicz, K. Michal; and Zygo, Krystyna *Analytical Chemistry* **2002**, *74*, 5212-5216.

(26) Merrifield, R. B. *Journal of the American Chemical Society* **1963**, 2149-2154.

(27) Bodansky, M. *Peptide Chemistry: A Practical Approach*, Second ed.; Springer-Verlag: Berlin, 1993.

(28) Fields, G. B., Ed. *Solid-Phase Peptide Synthesis*; Academic Press: New York, 1997.

(29) Beaucage, S. L. I., Radhakrishnan, P. *Tetrahedron* **1992**, *48*, 2223-2311.

(30) Ellman, J. A. *Acc. Chem. Res.* **1996**, *29*.

(31) Hermkens, P. H. H. O., H.C.J.; Rees, D. *Tetrahedron* **1997**, *53*.

(32) Vaino, A. R. J., Kim D. *Journal of Combinatorial Chemistry* **2000**, *2*, 579-596.

(33) Krull, I. S. S., M.E.; Bourque, A.J.; Zhou, F.X.; Yu, J.; Strong, R. *Journal of Chromatography, B* **1994**, *659*, 19-50.

(34) Moran, E. J. S., S.; Cargill, J.F.; Shahbaz, M.M.; Lio, A.M.; Mjalli, A.M.M.; Armstrong, R.W. *Journal of the American Chemical Society* **1995**, *117*, 10787.

(35) Nicolaou, K. C. X., X-Y; Parandoosh, Z.; Senyei, A>; Nova, M.P. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2289.

(36) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *Journal of the American Chemical Society* **1980**, *102*, 5463-5470.

(37) Flynn, D. *Med. Res. Rev.* **1999**, *19*, 408-431.

(38) Kaldor, S. S., M.; Fritz, J.; Dressman, B.; Hahn, P. *Tetrahedron Letters* **1996**, *37*, 7193.

(39) Flynn, D. C., J.; Devraj, R.; Hockerman, S.; Parlow, J.; South, M.; Woodard, S. *Journal of the American Chemical Society* **1997**, *119*, 4874.

(40) Booth, R. H., J. *Journal of the American Chemical Society* **1997**, *119*.

(41) Dickerson, T. R., N.; Janda, K.D. *Chem. Rev.* **2002**, *102*, 3325-3343.

(42) Bayer, E. M., M. *Nature* **1972**, *237*, 512-513.

(43) Gravert, D. J. J., K.D. *Chem. Rev.* **1997**, *38*, 489.

(44) Haag, R. S., Al.; Hebel, A.; Roller, S. *Journal of Combinatorial Chemistry* **2002**, *4*, 112-119.

(45) Han, H. J., K.D. *Tetrahedron Letters* **1997**, *38*, 1527.

(46) Harned, A. M., S.; Flynn, D.; Hanson, P.R. *Organic Letters* **2003**, *5*, 15-18.

(47) Toy, P. H. J. K. D. *Acc. Chem. Res.* **2000**, *33*, 546-554.

(48) Porco, J. *Combinatorial Chemistry and High Throughput Screening* **2000**, *3*, 93-102.

(49) Studer, A. H., Sabine; Ferritto, Rafael; Kim, Sun-Young; Jeger, Patrick; Wipf, Peter; Curran, Dennis P. *Science* **1997**, *275*, 823-826.

(50) Curran, D. P. O., Y. *Tetrahedron* **2001**, *57*, 5243-5253.

(51) Curran, D. P. F., T. *Organic Letters* **2002**, *4*, 2233-2235.

(52) Luo, Z. Z., Q.; Oderaotoshi, Y.; Curran, D.P. *Science* **2001**, *291*, 1766-1769.

(53) Zhang, L. L., Z.; Chn, C.H-T.; Curran, D.P. *Journal of the American Chemical Society* **2002**, *124*, 10443-10450.

(54) Zhang, L. C., D.P.; Chn, C.H-T. *Tetrahedron* **2002**, *58*, 3871-3875.

(55) Zhang, L. R., A.; Curran, D.P. *Journal of the American Chemical Society* **2002**, *124*, 5774-5781.

(56) Zhang, W. *Tetrahedron* **2003**, *59*, 4475-4489.

(57) Zhang, W. *Chem. Rev.* **2004**, *104*.

(58) Zhang, Q. L., Z.; Curran, D.P. *J. Org. Chem.* **2000**, *65*, 8866-8873.

(59) Curran, D. P. H., S.; He, M *J. Org. Chem.* **1997**, *62*, 6714-6715.

(60) Curran, D. P., Luo, Z. *Journal of the American Chemical Society* **1999**, *121*, 9069-9072.

(61) Curran, D. P. *Synlett* **2001**, 1488-1496.

(62) Matsugi, M. C., D.P. *Organic Letters* **2004**, *6*, 2717-2720.

(63) Dandapani, S. N., J; Curran, D.P. *Tetrahedron Letters* **2004**, *45*, 6653-6656.

(64) Blodgett, J. L., Tingyu *Tetrahedron Letters* **2004**, *45*, 6649-6652.

(65) Jones, J. *Amino Acid and Peptide Synthesis*; Oxford Science Publications: New York, 1992.

(66) Danishefsky, S. J. M., K.F.; Randolph, J.T.; Ruggeri, R. B. *Science* **1993**, *260*.

(67) Araki, T. T., Shinji; Tanaka, Minoru *Analytica Chimica Acta* **2000**, *410*, 37-45.

(68) Armstrong, D. W. D., Wade; Alak, Ala; Hinze, Willie L.; Riehl, Terrence E.; Bui, Khanh H. *Analytical Chemistry* **1985**, *57*, 234-237.

(69) Breslow, R. Z., B. *Journal of the American Chemical Society* **1996**, *118*.

(70) Cabrer, P. A.-P., E.; Meijide, F.; Seijas, J.A.; Nunez, E.R.; Tato, J.V. *Langmuir* **1999**, *15*, 5489-5495.

(71) Debowski, J. J., J.; and Sybilska, D. *Journal of Chromatography* **1983**, *282*, 83-88.

(72) Debowski, J. S., D. *J. Chromatogr.* **1986**, *353*, 409-416.

(73) Feng, Y.-Q. X., Ming-jie; Da, Shi-Lu *Analytica Chimica Acta* **2000**, *403*, 187-195.

(74) Fujimara, K. K., M.; Takayanagi, H.; Ando, T *Journal of Liquid Chromatography* **1986**, 607-620.

(75) Gazdag, M. S., G.; Huszár, L. *J. Chromatogr.* **1986**, *351*, 128-135.

(76) Haginaka, J. W., J. *Analytical Chemistry* **1990**, *62*, 997-1000.

(77) Han, S. M. H., Yoon I.; Armstrong, Daniel W. *J. Chromatogr.* **1988**, *441*, 376-381.

(78) Hargitai, T. K., Y.; Okamoto, Y. *J. Chromatogr.* **1993**, *628*, 11-22.

(79) Hinze, W. L. R., T.E.; Armstrong, D.W.; DeMond, W.; Alak, A.; Ward, T. *Analytical Chemistry* **1985**, *57*, 237-242.

(80) Italia, A. S., M.;Ventura, P. *J. Chromatogr.* **1990**, *503*, 266-271.

(81) Li, S. P., W.C. *J. Chromatogr.* **1992**, *625*, 109-120.

(82) Maguire, J. H. *J. Chromatogr.* **1987**, *387*, 453-458.

(83) Matsuzawa, H. M., K. *Synlett* **2002**, *10*, 1607-1612.

(84) Nowakowski, R. C., Philippe J.P.; Coleman, ANthony W.; Villard, Emmanuelle; Guiochon, Georges *Analytical Chemistry* **1995**, *67*.

(85) Shimada, K. K., Yoshihiro; and Mitamura, Kuniko *Journal of Chromatographic Science* **1990**, *28*, 331-335.

(86) Tan, Z. J. Z., X.X.; and Brown, G.R. *Langmuir* **1994**, *10*, 1034-1039.

(87) Yang, Z. B., Ronald *Tetrahedron Letters* **1997**, *38*, 6171-6172.

(88) Zhang, L. W., Y.; Chen, L; Hg, S. *Tetrahedron Letters* **1999**, *40*, 1815-1818.

(89) Zukowski, J. S., D.; Bojarski, J. *J. Chromatogr.* **1986**, *364*, 225-232.

(90) Matsui, Y. N., T.; Fujita, T. *Top. Curr. Chem* **1985**, *128*, 61-89.

(91) Greene, T. W. W., Peter G. M. *Protective Groups in Organic Synthesis*, 3rd ed.; Wiley & Sons, Inc.: New York, NY, 1999.

(92) Ollila, F. P., O.; Forss, S.; Johnson, M.; Slotte, J.P. *Langmuir* **2001**, *17*, 7107-7111.

(93) Schneider, H.-J. H., F.; Rudiger, V. *Chem. Rev.* **1998**, *98*, 1775-1785.

(94) Rekharsky, M. V. I., Y. *Chem. Rev.* **1998**, *98*.

(95) Connors, K. A. *Chem. Rev.* **1997**, *97*, 1325-1357.

(96) Hedges, A. R. *Chem. Rev.* **1998**, *98*, 2035-2044.

(97) Uekama, K. H., F.; Irie, T. *Chem. Rev.* **1998**, *98*, 2045-2076.

(98) Fujimura, K. K., M.; Takayanagi, H.; Ando, T *Journal of Liquid Chromatography* **1986**, 607-620.

(99) Deng, Q. K., S.; Xu, Z. *American Laboratory* **1999**, 43-47.

(100) Li, T. *Jounal of Chromatography A* **2004**, *1035*, 151-152.

CHAPTER V

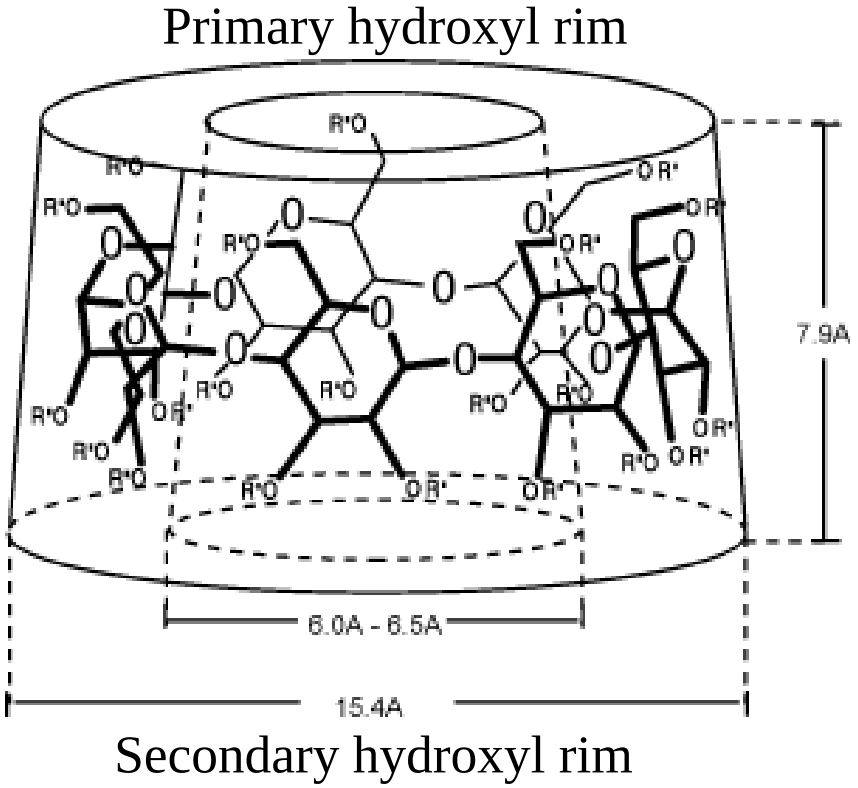
DEVELOPMENT AND EVALUATION OF IMMOBILIZED BETA-CYCLODEXTRIN STATIONARY PHASES FOR USE IN A TAGGING SYSTEM FOR SOLUTION PHASE ORGANIC SYNTHESIS

Introduction

Cyclodextrins are a product of the partial degradation of starch followed by the enzymatic coupling of the cleaved glucose units into macrocyclic polymers that take on a toroidal shape. The most common sizes of these macrocycles formed are of 6,7, or 8 glucose units in undistorted chair conformations corresponding to alpha, beta, and gamma-cyclodextrins. Beta-cyclodextrin will be of primary concern, and therefore will be discussed in greater detail. Beta-cyclodextrin consists of 7 D-(+)-glucopyranose units that are connected by α-(1,4) linkages forming a toroidal , truncated cone shaped structure shown in Figure 5-1. The interior cavity of the beta-cyclodextrin structure is comprised of the glucoside oxygens and methylene hydrogens, lending it a hydrophobic character. The exterior is a hydrophilic surface due to hydroxyl groups. As a result of these unique properties, beta-cyclodextrin can include apolar or hydrophobic molecules of appropriate size and configuration into its cavity to form inclusion complexes of varying stabilities[93, 94] and at the same time are water soluble. The stability of these complexes is related to how well they fit into the cavity as well as the strength of the interactions that they encounter within the cavity. The attractive forces attributable to the formation of these inclusion

complexes include Van der Waals, dipole-dipole, hydrogen bonding, and hydrophobic interactions[95].

Figure 5-1. Structure of beta-cyclodextrin.



Beta-cyclodextrin has many interesting commercial uses[96] due to its unique ability to include hydrophobic molecules, and yet remain water-soluble. Beta-cyclodextrin is used in industry to stabilize and deliver flavors and fragrances, absorb or mask odors and flavors, deliver therapeutics[97], remove

cholesterol from foods, and also in separations. While all of the applications are interesting and useful, separations is the area that will be discussed further.

Cyclodextrins, and specifically beta-cyclodextrin has been widely used for inclusion complex chromatography as well as for chiral chromatography.[3, 67, 68, 71-74, 76, 78-80, 82, 84, 85, 89] There have been several methodologies adapted for use of cyclodextrins in separation science. One strategy is to use cyclodextrins in solution. The soluble cyclodextrin can be added to the mobile phase[75] during the chromatography. It then includes the analyte[77], and since the cyclodextrin should show very little affinity for the reversed phase stationary phase, the compound will then elute much faster than when not included. If the analyte forms a stable complex with the cyclodextrin, then it should easily be separated from the mixture. Another approach involves immobilizing cyclodextrin directly onto silica gel, typically by using some sort of linker molecule. This stationary phase may then be packed into a column. There are a wide variety of strategies for executing this immobilization[6, 67, 81, 88, 98, 99], but the most successful seem to utilize approaches that do not leave any unreacted spacer arms on the surface. A popular way to achieve this, and the one that will be discussed further utilizes 3-glycidoxypropyltrimethoxysilane as a linker to connect the beta-cylcodextrin to the silica gel by first reacting the silane with the beta-cyclodextrin at the epoxide end, and subsequently immobilizing the entire assembly via a siloxane linkage[73]. This leaves the surface consisting entirely of the beta-cyclodextrin units. Another approach involves the chemical modification of the hydroxyl groups on the

cyclodextrin molecules to give the cavity a slightly different shape and size, changing the types of molecules that will include and bind in the cavity. The strategies previously mentioned have long been successfully used in order effect separation of organic molecules, popularly aromatic organic molecules, and also to separate enantiomers for chiral separations due to the 35 stereogenic centers found in the beta-cyclodextrin structure. However, there have been no simple strategies developed to effect a tagging system for solution phase organic synthesis workup.

In the initial studies to develop a simple tagging system for solution phase organic synthesis, it was satisfactory to simply use a commercially available beta-cyclodextrin column and quickly assess the effectiveness of our tags by high-performance liquid chromatography. As the efficacy of this tagging strategy based on 4-*tert*-butylphenol[64] has been established, it is now important to know the nature of the linkages used to immobilize the beta-cyclodextrin and to further study its loading and capacity. It is for this reason that the HPLC and flash grade supports were synthesized.

Experimental

General Supplies and Equipment

Amino acid derivatives were purchased from NovaBiochem (San Diego, CA). All other chemicals and solvents were purchased from Aldrich (Milwaukee,

WI), Fluka (Ronkonkoma, NY), or Fisher Scientific (Pittsburgh, PA). Beta-cyclodextrin bonded HPLC column was purchased from Advanced Separation Technologies Inc. (Whippany, NJ). Selecto silica gel (32-63 μm) from Fisher Scientific was used for flash chromatographic grade silica gel. Thin-layer chromatography was completed using EM silica gel 60 F-254 TLC plates (0.25 mm) (Emerck KGaA, 64271 Darmstadt, Germany). HPLC analyses were conducted using a Beckman analytical gradient system (System Gold). All hardware for preparing the HPLC columns was from Isolation Technologies (Hopedale, Massachusetts, USA), and an Alltech column packer. Atlantic Microlab, Inc.(Norcross, Georgia, USA) provided all elemental analysis. All stationary phases were packed into empty HPLC columns (4.6 mm i.d. x 5 cm) using an ethanolic slurry.

Calculations

The ligand surface density or ligand coverage of the beta-cyclodextrin on silica gel supports were calculated as follows according to a literature method[100]: In the case of the flash grade silica gel for example, based on carbon percentage (determined by elemental analysis) 14.07%, in every 100g of beta-cyclodextrin silica gel there are 14.07g/50 (there are 50 carbons in the ligand)/12 (molar mass of C)=0.0235 moles of beta-cyclodextrin ligand on 100g flash grade silica gel (MW=1340). According to the method for immobilization, for every mole of ligand attached, one mole of proton is lost. This means that 100g silica gel

contains 100-0.0235 x 1340 + 0.0235 x 1=68.4865g of silica gel. Therefore, the surface coverage of ligand is 0.0235/(68.4865g x 500m^2/g)=6.86 x 10^{-7} mol/m^2, or 3.43 x 10^{-4} mol/g, or 0.34 mmol/g. This abbreviated method is sufficient as there was no end-capping of the surface after derivitizations to skew the elemental analysis results.

Preparation of HPLC and Flash Grade Silica Gel

In the case of HPLC grade silica gel (5 μm, 500m^2/g,) from Alltech was used. For the flash grade support, Selecto silica gel (32-63 μm, 220m^2/g,) from Fisher Scientific was used. First the appropriate silica gel was subjected to an acid washing procedure to remove impurities before further chromatographic use. To accomplish this, a mixture of silica gel (10 g), concentrated sulfuric acid (40 ml), and concentrated nitric acid (10 ml) was refluxed overnight. After cooling the mixture, the silica gel was collected by vacuum filtration and rinsed with water (20 x 100ml), methanol (3 x 50ml), and dichloromethane (2 x 50ml), followed by placement in the oven at 110°C for 12 hours. The silica gel was subsequently stored in a desiccator at room temperature until further use.

Next 12.0 Beta-cyclodextrin was dissolved in 200ml dry dimethylformamide. Then 1.2g sodium hydride was added and let stir and react for 30 min at room temperature. The beta-cyclodextrin alkoxide was recovered in solution by centrifugation of the reaction mixture and washing the solid by-product with dry dimethylformamide. The dimethylformamide fractions were

collected and combined. The filtrate was then allowed to react with 3.0ml 3-glycidoxypropyltrimethoxysilane under argon gas at 85-90° C overnight. Then 10.0g acid-washed silica gel was added to the reaction mixture and let stir at 110° C for 24h, after which the silica gel was collected by filtration and rinsed with DMF, H_2O, methanol, acetone, and DCM (2 x 30 ml each). The supported beta-cyclodextrin was then dried in an oven at 115° C for 3h. The silica gel was subsequently stored in a desiccator at room temperature until further use.

Results

Evaluation of HPLC Grade Silica Gel

After immobilization of the beta-cyclodextrin units onto the silica gel as described in the experimental section[73] (Figure 5-2), the loading was determined by elemental analysis. Table 5-1 shows the results of two elemental analyses on the beta-cyclodextrin immobilized onto HPLC grade silica gel. The literature preparation that was followed gave a 13% carbon content where beta-cyclodextrin units are the primary source of carbon as the carbon content added by the linker is negligible in comparison to the bulk added as beta-cyclodextrin.

Table 5-1. Elemental analysis results for immobilization of beta-cyclodextrin onto HPLC grade silica gel. EA1 and EA2 are two repeat analyses of the same batch of material. The literature value is from the literature preparation cited in the experimental procedure.

Element	**EA1**	**EA2**	**Literature**
C	16.44	16.30	13
H	13.00	2.96	2.50
N	0.27	0.27	0.00

The literature value was prepared on comparable silica gel, but the surface coverage achieved in these experiments was higher at more than 16%. The ligand density shown in Table 5-2 as calculated from the elemental analysis data is 4.33×10^{-4} mol/g as compared to the literature value at 3.06×10^{-4} mol/g. The commercial column is reported to have an even lower loading of 3×10^{-6} mol/g. At first glance this data suggests that the prepared HPLC grade column is superior to the commercial column as intuitively one might be tempted to think that a higher surface loading always leads to better column performance. It is important to consider that the commercially available beta-cyclodextrin HPLC columns are optimized and marketed for chiral separations, and such applications are very different from these tagging applications. Alternatively, after careful consideration, it could also be reasoned that a higher surface loading could be wasteful in a sense if the functionality as in this case is so large that only some

of the beta-cyclodextrin cavities would be accessible in the case of significantly higher surface loading. It is for this reason that the loading capacity of the stationary phase was studied using 4-*tert*-butylphenol.

The column capacity reported is defined as the amount of 4-*tert*-butylphenol that can be loaded onto the column without experiencing compound elution at the dead volume, thus indicating column overloading. The column capacity as shown in Table 5-3 was determined to be 15mg 4-*tert*-butylphenol as compared to 8 mg on the commercial column. This data indicates that indeed the prepared HPLC grade column does perform better than the commercial column for this application. It is also worth pointing out that the prepared column is 5 times shorter in length than the commercial column, and thus contains less stationary phase making its improved performance even more significant.

Table 5-2. Ligand density calculated from elemental analysis data. The literature value is from the literature preparation cited in the experimental procedure, and the commercial column data is from Advanced Separations Technologies,Inc.

Support	**Ligand Density (mol/g)**
HPLC grade	4.33×10^{-4}
Flash grade	3.43×10^{-4}
Literature prep value	3.06×10^{-4}
Commercial column	4.60×10^{-5}

Table 5-3. Column capacity as measured using 4-*tert*-butylphenol injections.

Commercial Column (250 x 4.6mm)	**Flash Grade Column (50 x 4.6mm)**	**HPLC grade Column (50 x 4.6mm)**
8mg	10mg	15mg

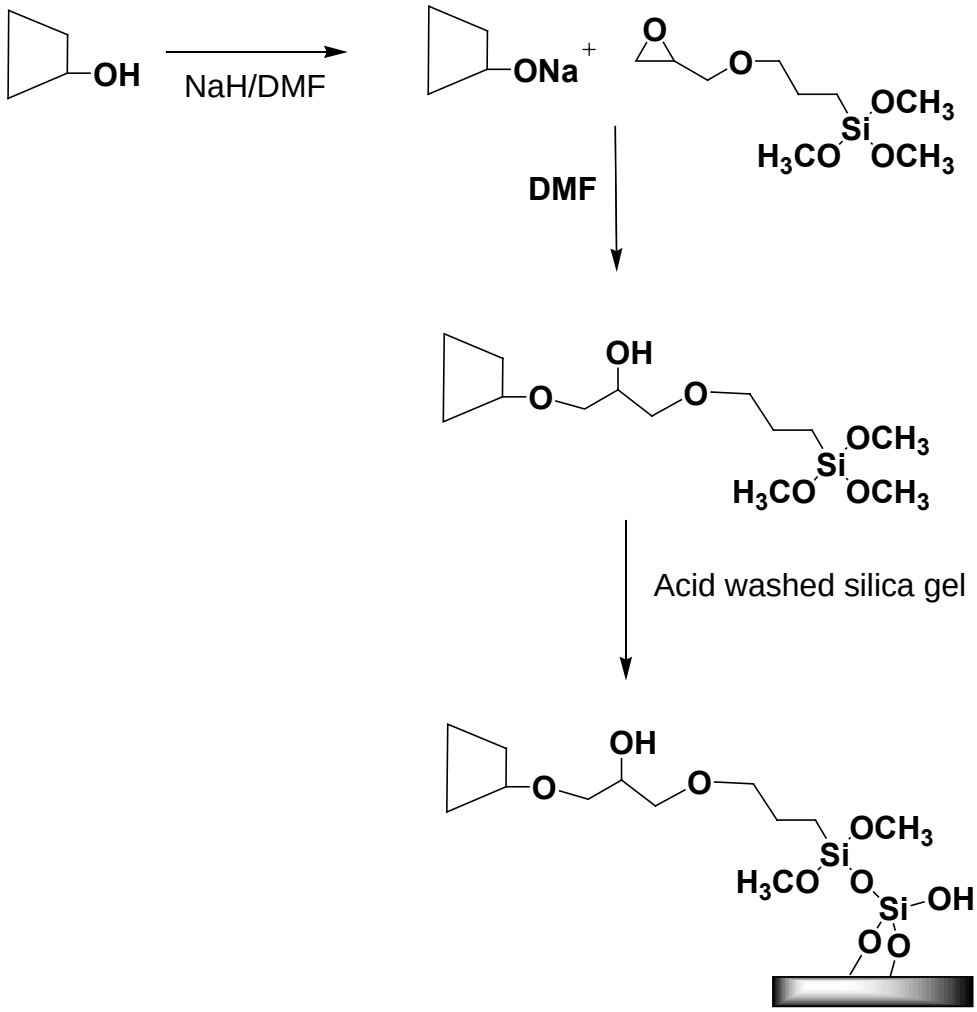
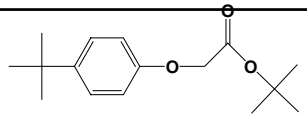
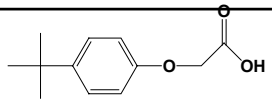
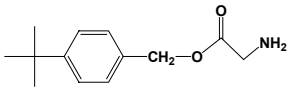


Figure 5-2. Immobilization of beta-cyclodextrin on silica gel support.

The HPLC grade column was then tested for retention time with the various compounds that have been previously studied using the commercial column. Table 5-4 shows the results of the column performance test as compared to that of the commercial column. The prepared column retains almost all of the test compounds longer than the commercial column, and again it is noteworthy that it is 5 times shorter than the commercial column making the increased retention even more impressive. Remarkably, the only case showing decreased retention time is with benzyl alcohol. This is important because benzyl alcohol is the one compound that does not show any significant retention on the beta-cyclodextrin stationary phase, and this data supports that as it is the only compound that is more poorly retained on the prepared HPLC grade column than the commercial column. The prepared column exhibits a longer retention for compounds that show an affinity for beta-cyclodextrin and a shorter retention for compounds that do not show significant affinity for beta-cyclodextrin. This characteristic is especially useful given the nature of the separation technique described for solution phase organic synthesis. This stationary phase can effect a better separation, as any compound that displays the tagging functionality will be strongly retained while untagged compounds will easily be washed away.

Table 5-4. Column performance with various analytes.

Compound t_R(min)	**In-house HPLC** t_R(min)	**Commercial** t_R(min)
Benzoic acid	23	15
Benzyl alcohol	4	5
	113	78
	97	70
	64[a],98[b]	40[a],90[b]
2-*tert*-Butylphenol	86	54
3-*tert*-Butylphenol	98	76
4-*tert*-Butylphenol	100	91

Evaluation of Flash Grade Silica Gel

Table 5-5 shows the results of two elemental analyses on the beta-cyclodextrin immobilized onto flash grade silica gel. The literature preparation that was followed gave a 13% carbon content and the flash grade preparation gave a carbon loading of 14%. The ligand density shown in Table 5-2 as calculated from the elemental analysis data is 3.43×10^{-4} mol/g as compared to the literature value at 3.06×10^{-4} mol/g. The commercial column is reported to have an even lower loading of 4.6×10^{-5} mol/g. The column capacity as shown in

Table 5-3 was determined to be 10mg 4-*tert*-butylphenol as compared to 15mg on the HPLC grade column and 8 mg on the commercial column.

Practical Application of Flash grade Support

In order to test the utility of the flash grade supported beta-cyclodextrin, an Fmoc-Ser(tBu)-OH was tagged according to Figure 3-6 just as described in Chapter III for the HPLC purification experiment. Excess amino acid was also used in this case as well. By applying the reaction mixture to the solid support

Table 5-5. Elemental analysis results for immobilization of beta-cyclodextrin onto flash grade silica gel. EA1 and EA2 are two repeat analyses of the same batch of material. The literature value is from the literature preparation cited in the experimental procedure.

Element	**EA1**	**EA2**	**Literature**
C	14.03	14.07	13
H	2.67	2.72	2.50
N	0.32	0.31	0.00

packed in a fritted funnel as a plug of stationary phase, and then first eluting the impurities with 40% methanol/water mobile phase that was determined appropriate for this technique in the HPLC experiments the tagged Fmoc-Ser(tBu)-OH was then recovered pure and in quantitative yield by eluting with

100% methanol. Remarkably, this purification process required a mere 5 minutes to execute where a traditional flash column would have required much more time to both determine the appropriate conditions for and to execute. This tagging strategy using 4-*tert*-butylphenol requires essentially no optimization of mobile phase even with more complex crude reaction mixtures.

Summary

The immobilization of beta-cyclodextrin guest molecules for use as a sorbent is advantageous for the development of a new affinity tagging method as it gives information about the surface chemistry of the support, and can help optimize the separation capabilities of the method. A method for the production of beta-cyclodextrin immobilized onto two types of silica gel as a solid support to facilitate the separation of affinity tagged compounds was successfully demonstrated. This method produced supports with improved ligand density over available supports as well as improved loading capacity for the application of affinity tagging separations. The supports also demonstrated improved separation performance for compounds with affinity for beta-cyclodextrin over those without an affinity for beta-cyclodextrin. The supports were proven successful for both HPLC applications as well as for flash chromatographic and filtration applications.

References

(1) Schneider, H.-J. H., F.; Rudiger, V. *Chem. Rev.* **1998**, *98*, 1775-1785.

(2) Rekharsky, M. V. I., Y. *Chem. Rev.* **1998**, *98*.

(3) Connors, K. A. *Chem. Rev.* **1997**, *97*, 1325-1357.

(4) Hedges, A. R. *Chem. Rev.* **1998**, *98*, 2035-2044.

(5) Uekama, K. H., F.; Irie, T. *Chem. Rev.* **1998**, *98*, 2045-2076.

(6) Shimada, K. K., Yoshihiro; and Mitamura, Kuniko *Journal of Chromatographic Science* **1990**, *28*, 331-335.

(7) Feng, Y.-Q. X., Ming-jie; Da, Shi-Lu *Analytica Chimica Acta* **2000**, *403*, 187-195.

(8) Araki, T. T., Shinji; Tanaka, Minoru *Analytica Chimica Acta* **2000**, *410*, 37-45.

(9) Nowakowski, R. C., Philippe J.P.; Coleman, ANthony W.; Villard, Emmanuelle; Guiochon, Georges *Analytical Chemistry* **1995**, *67*.

(10) Maguire, J. H. *J. Chromatogr.* **1987**, *387*, 453-458.

(11) Debowski, J. J., J.; and Sybilska, D. *Journal of Chromatography* **1983**, *282*, 83-88.

(12) Debowski, J. S., D. *J. Chromatogr.* **1986**, *353*, 409-416.

(13) Zukowski, J. S., D.; Bojarski, J. *J. Chromatogr.* **1986**, *364*, 225-232.

(14) Fujimara, K. K., M.; Takayanagi, H.; Ando, T *Journal of Liquid Chromatography* **1986**, 607-620.

(15) Haginaka, J. W., J. *Analytical Chemistry* **1990**, *62*, 997-1000.

(16) Hargitai, T. K., Y.; Okamoto, Y. *J. Chromatogr.* **1993**, *628*, 11-22.

(17) Armstrong, D. W. D., Wade; Alak, Ala; Hinze, Willie L.; Riehl, Terrence E.; Bui, Khanh H. *Analytical Chemistry* **1985**, *57*, 234-237.

(18) Armstrong, D. W.; Ward, T. J.; Armstrong, R. D.; Beesley, T. E. *Tetrahedron Letters* **1997**, *38*, 6171-6172.

(19) Hinze, W. L. R., T.E.; Armstrong, D.W.; DeMond, W.; Alak, A.; Ward, T. *Analytical Chemistry* **1985**, *57*, 237-242.

(20) Italia, A. S., M.;Ventura, P. *J. Chromatogr.* **1990**, *503*, 266-271.

(21) Gazdag, M. S., G.; Huszár, L. *J. Chromatogr.* **1986**, *351*, 128-135.

(22) Han, S. M. H., Yoon I.; Armstrong, Daniel W. *J. Chromatogr.* **1988**, *441*, 376-381.

(23) Kawaguchi, Y. T., M.; Nakae, M.; Funazo, K.; Shono, T. *Analytical Chemistry* **1983**, *55*, 1852-1857.

(24) Li, S. P., W.C. *J. Chromatogr.* **1992**, *625*, 109-120.

(25) Zhang, L. W., Y.; Chen, L; Hg, S. *Tetrahedron Letters* **1999**, *40*, 1815-1818.

(26) Fujimura, K. K., M.; Takayanagi, H.; Ando, T *Journal of Liquid Chromatography* **1986**, 607-620.

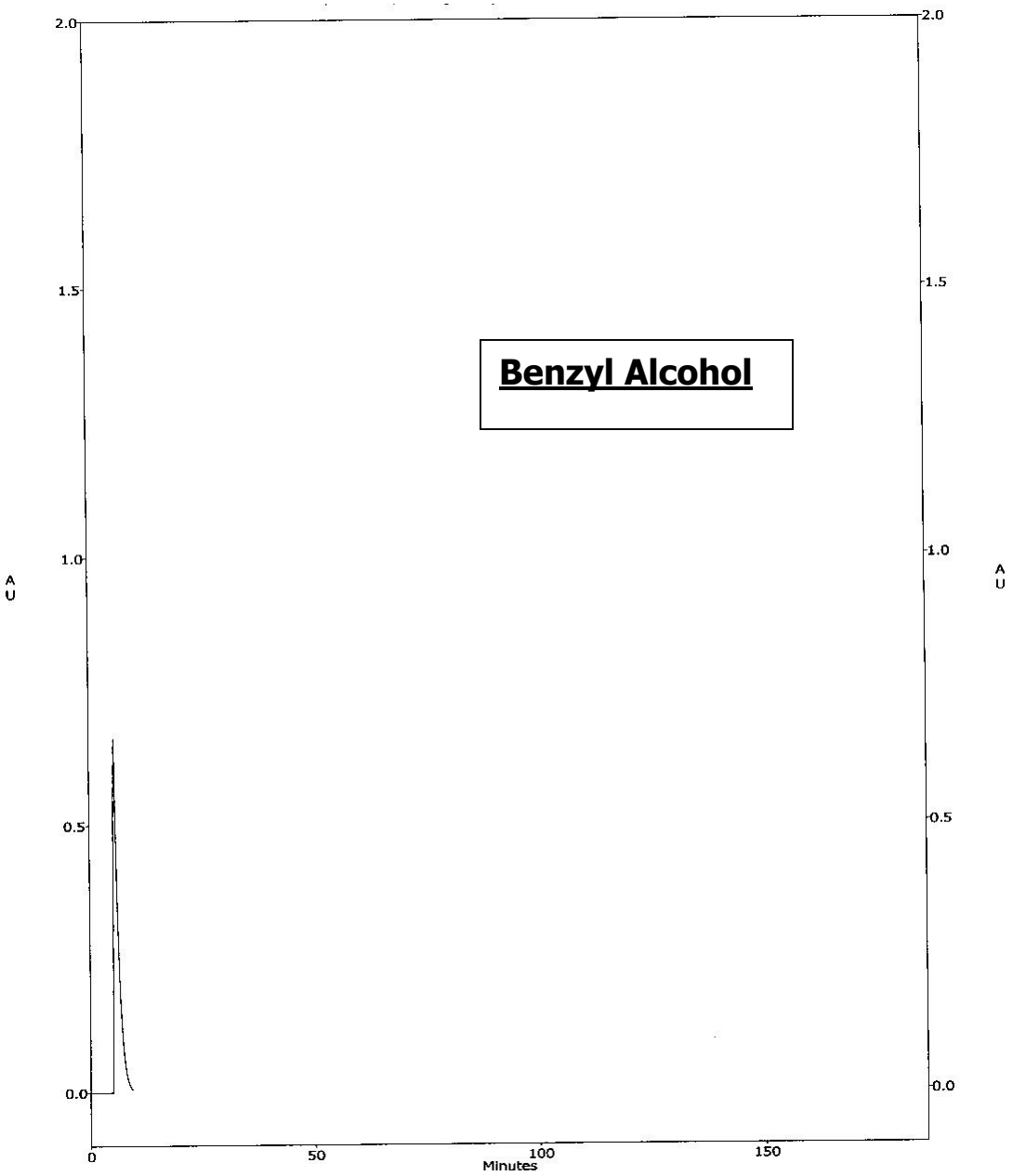
(27) Deng, Q. K., S.; Xu, Z. *American Laboratory* **1999**, 43-47.

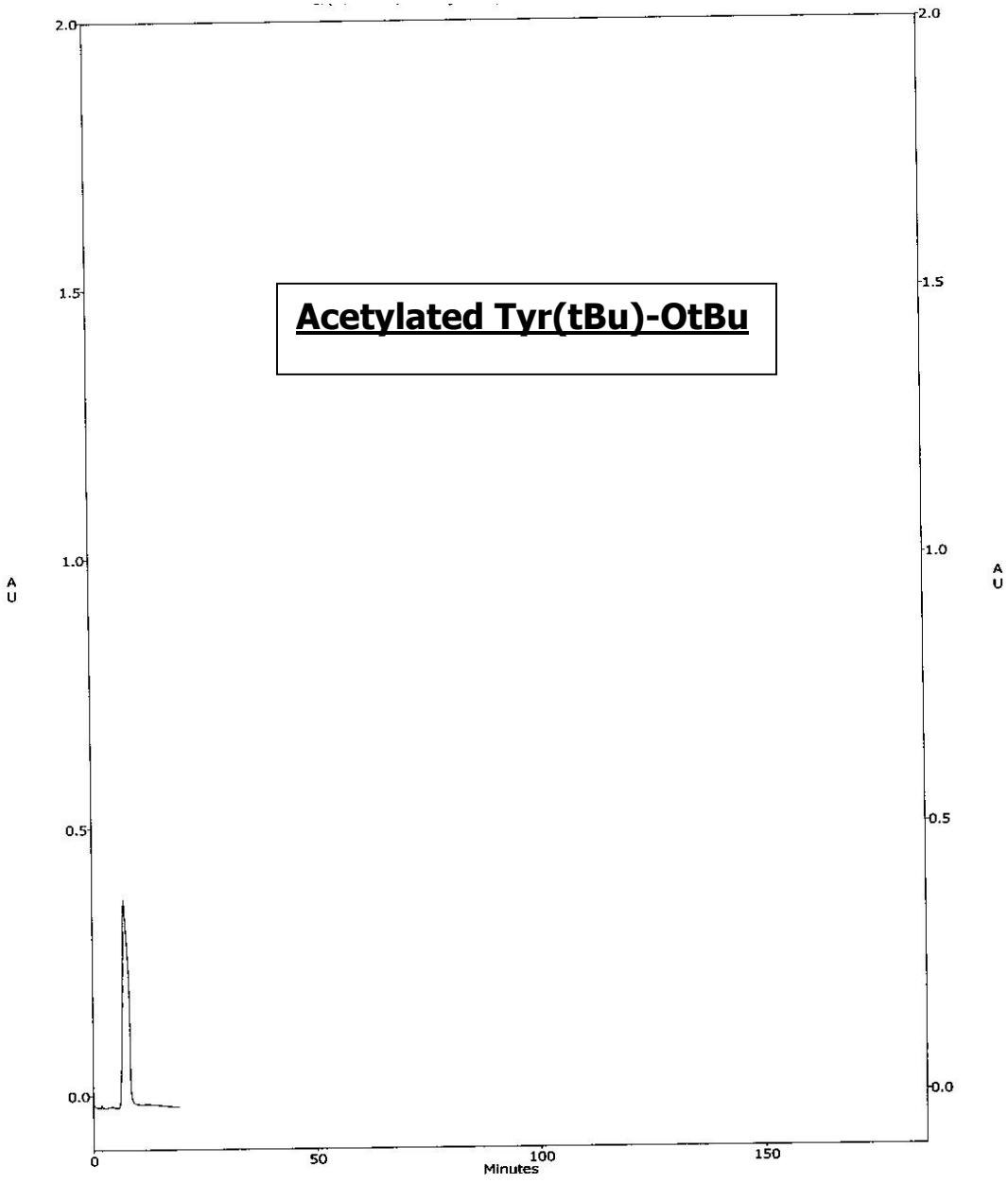
(28) Blodgett, J. L., Tingyu *Tetrahedron Letters* **2004**, *45*, 6649-6652.

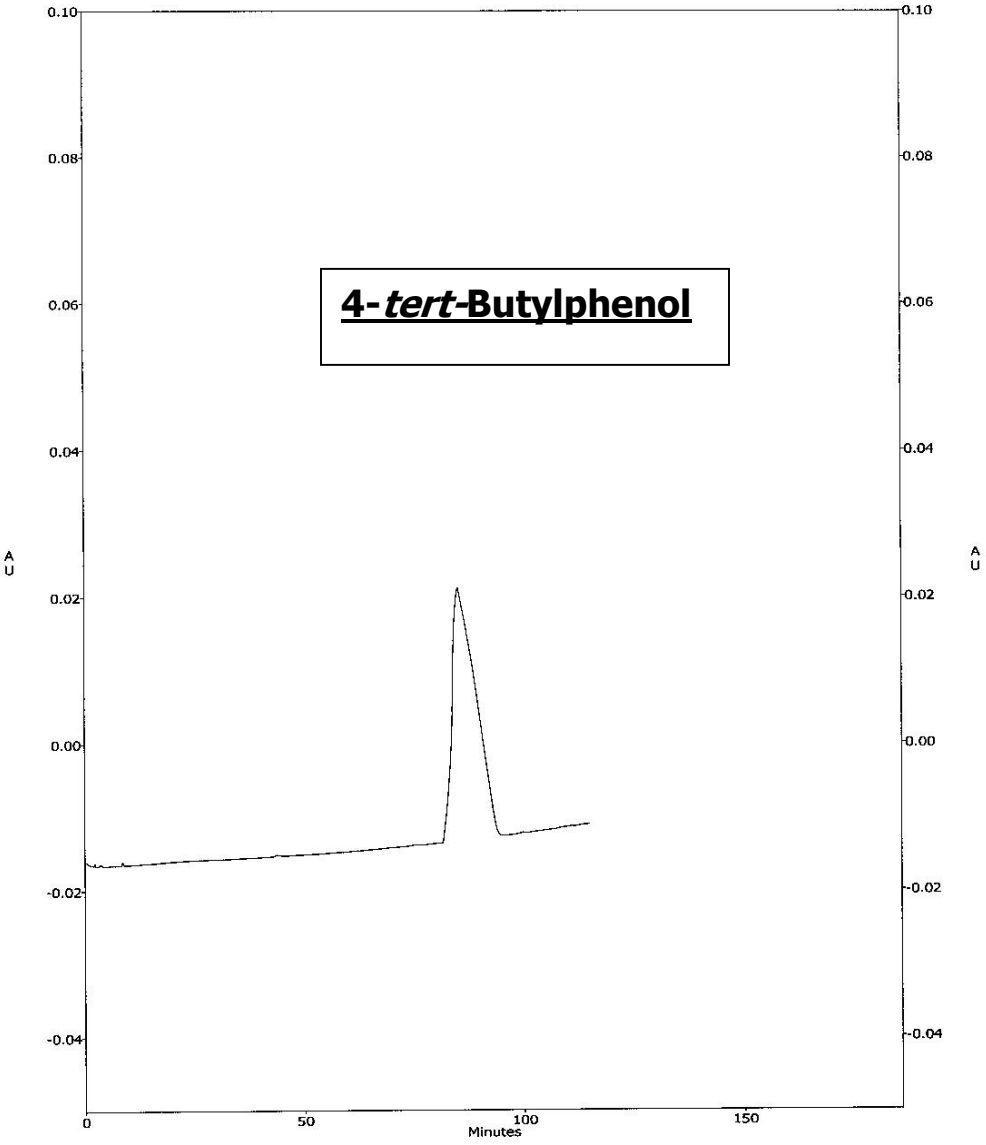
(29) Li, T. *Jounal of Chromatography A* **2004**, *1035*, 151-152.

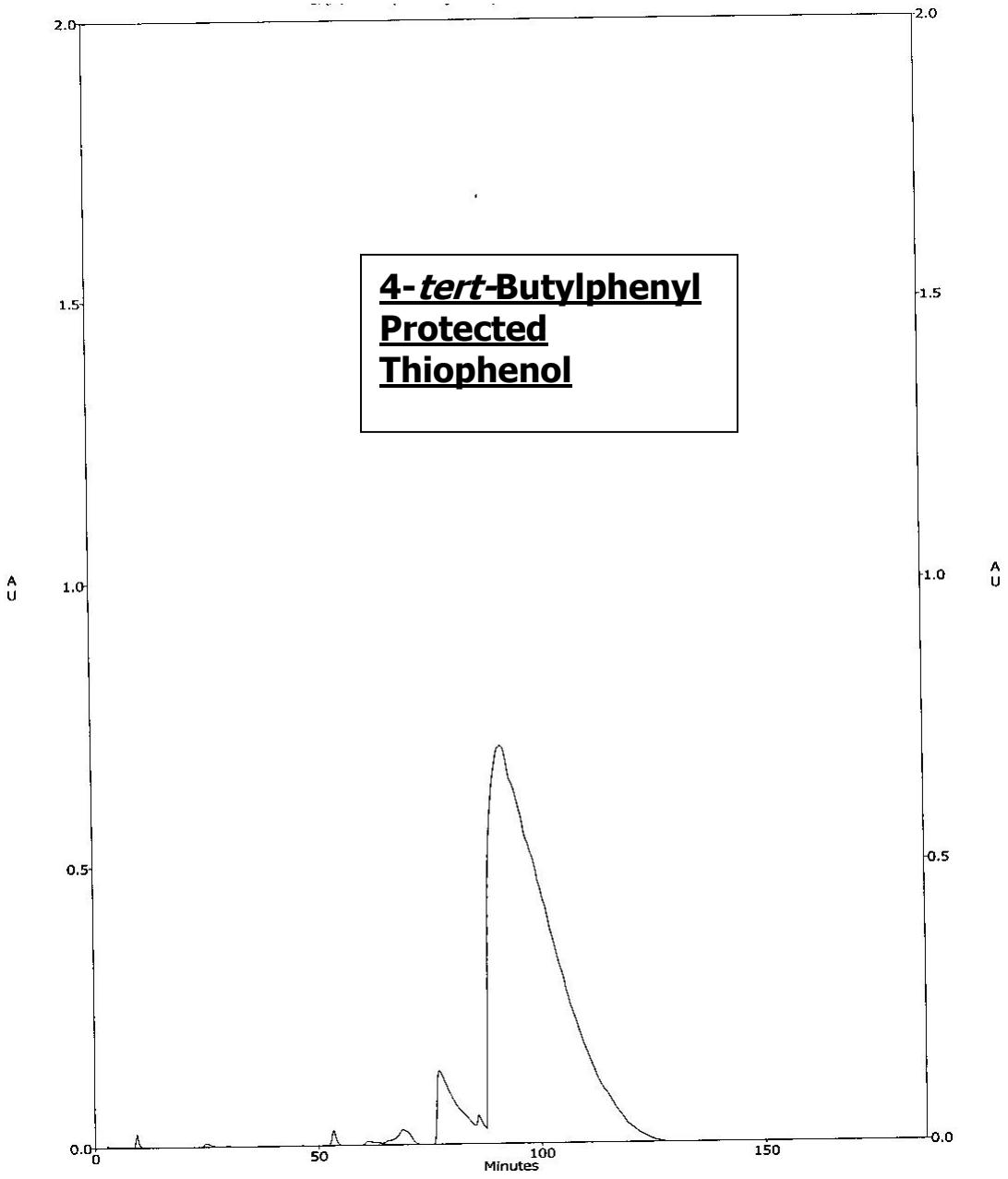
APPENDIX:

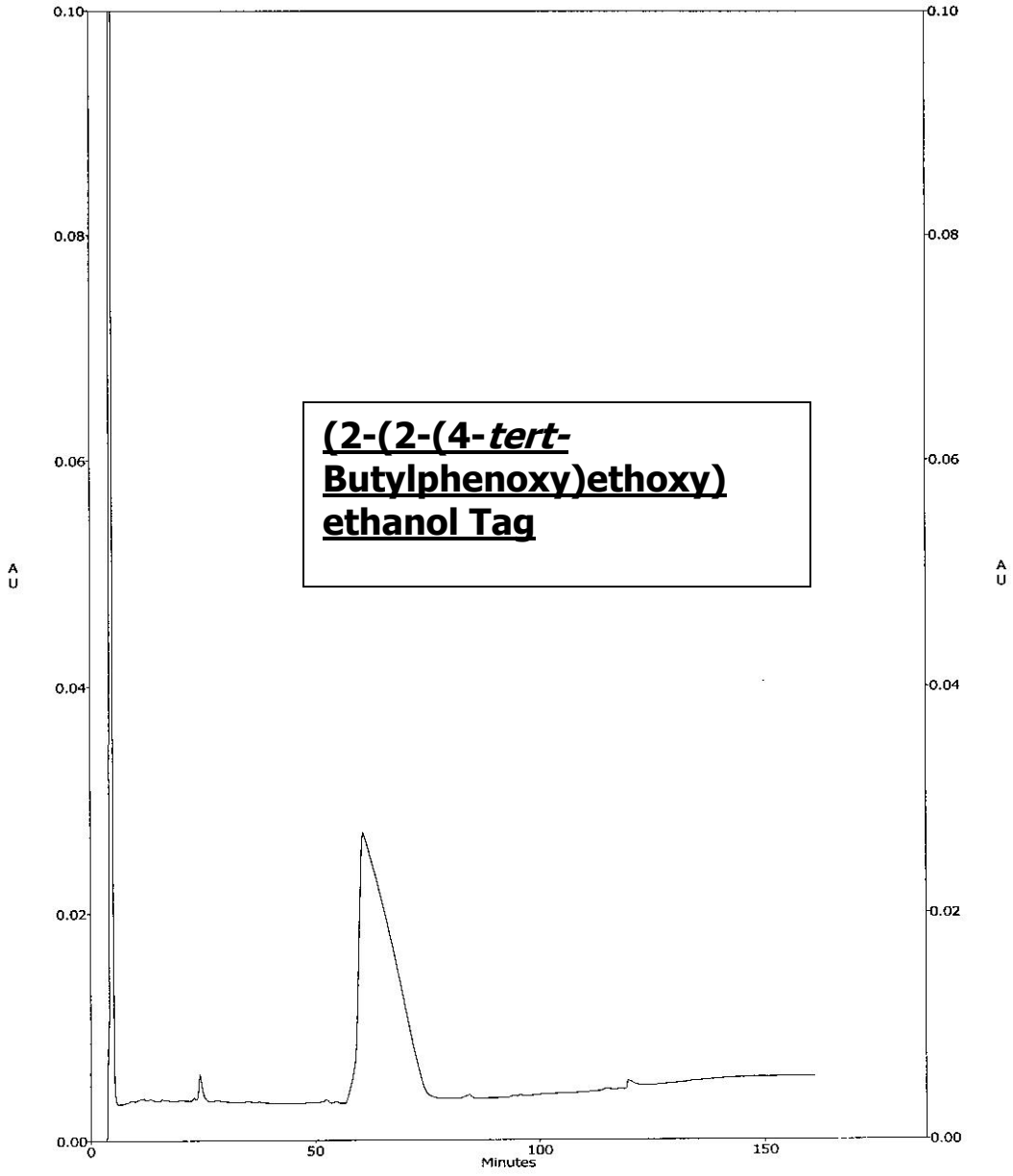
SELECTED REPRESENTATIVE CHROMATOGRAMS




Acetylated Tyr(tBu)-OtBu
AU
2.0
1.5
1.0
0.5
0.0
0
50
100
150
Minutes


4-tert-Butylphenol
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
AU
0
50
100
150
Minutes


4-*tert*-Butylphenyl Protected Thiophenol
AU
2.0
1.5
1.0
0.5
0.0
0
50
100
150
Minutes


(2-(2-(4-*tert*-Butylphenoxy)ethoxy) ethanol Tag
0.10
0.08
0.06
0.04
0.02
0.00
AU
0
50
100
150
Minutes