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Enantiomer analysis using electrospray ionization mass spectrometry

Chengli Zu

ENANTIOMER ANALYSIS USING ELECTROSPRAY IONIZATION MASS SPECTROMETRY

By

Cheng-Li Zu

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IONIZATION MASS SPECTROMETRY

By

Cheng-Li Zu

Approved:

Michael E. Koscho Assistant Professor of Chemistry (Major-advisor)	Keith T. Mead Professor of Chemistry Department Head (Committee Member)
Alicia M. Beatty Assistant Professor of Chemistry (Committee Member)	Andrzej Sygula Associate Professor of Chemistry (Committee Member)
Gloria Thomas Assistant Professor (Committee Member)	Stephen C. Foster Associate Professor of Chemistry (Graduate Coordinate of the Department of Chemistry)

Philip B. Oldham
Dean of the College of Arts and Science

Name: Cheng-Li Zu

Date of Degree: May 5, 2007

Institution: Mississippi State University

Major Field: Organic Chemistry

Major Professor: Dr. Michael E. Koscho

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Candidate for Degree of Doctor of Philosophy

The design, synthesis and evaluation of chiral selectors that allow the determination of enantiomeric composition using electrospray ionization mass spectrometry are detailed herein. The enantiomers of the chiral selector were mass labeled at a distant site from the chiral recognition sites of the molecules. The mass-labeled enantiomers were mixed in a one-to-one ratio to form a quasi-racemate. Chiral recognition can be observed by comparing relative abundances of the pseudo-diastereomeric selector-analyte complexes in the mass spectrum. The observed sense of chiral recognition with mass spectrometry was consistent with that observed chromatographically using a corresponding chiral stationary phase in every case. The complex intensity fraction (CIF, intensity of one selector-analyte complex divided by the sum of the intensities for both selector-analyte complexes) is linear with the enantiomeric composition. The slope of this line is an indication of the extent of the enantioselectivity: the larger the slope, the more significant the enantioselectivity. In addition, this line can be used as a calibration curve for the

quantitative determination of enantiomeric composition of the same analyte with unkown enantiomeric composition.

Amide derivatives of DNB-amino acids were first used as pseudo-enantiomeric chiral selectors in the presence of added lithium chloride. The enantioselectivity values were smaller than those observed on chiral HPLC using the corresponding chiral stationary phase. The use of deprotonated DNB-amino acids as chiral selectors provides higher enantioselectivities, but with low ion abundances. Tertiary amine appended analogues of the chiral stationary phase DNB-Leucine were prepared. The amine was appended to provide a site for ready ionization (through protonation). The performance of chiral selectors of this type was compared to the original chiral selectors that lack this functional group.

Chiral recognition was also observed in a reciprocal sense using proline-derived pseudo-enantiomeric chiral selectors and analytes similar to DNB-amino acid esters or amides. Optimization of the electrospray ionization conditions provided similar enantioselectivities to those from chiral HPLC.

Libraries of tertiary amine appended derivatives of DNB-dipeptides, which were prepared through combinatorial peptide synthesis, were screened using electrospray ionization mass spectrometry. The use of electrospray ionization mass spectrometry as a discovery tool for new chiral selectors is discussed.

DEDICATION

I dedicate this work to my parents, Zu ZhongXian and Liu AiRong; my wife, Hu XueMei; my daughter, Zu XinYue, and my son, Zu XinQi.

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Committee Members: Dr. Keith T. Mead, Dr. Alicia M. Beatty, Dr. Gloria Thomas, and Dr. Andrzej Sygula

Graduate Coordinator, Dr. Stephen C. Foster

Mr. William E. Holmes, Director of Mass Spectrometry and Advanced Instrumentation, Mississippi State Chemical Laboratory

Mr. Bart van den Berg, Graduate student of the School of Veterinary, Mississippi State University

Dr. Bobby N. Brewer, Postdoctoral researcher in the group.

Beibei Wang, Group colleague

Jonathan Woolfolk, Group colleague.

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LIST OF ABBREVIATIONS

ESI	electrospray ionization
MS	mass spectrometry
e.e	enantiomer excess
CIF	complex intensity fraction
DIC	diisopropylcarbodiimide
HOSu	*N*-hydroxysuccinimide
HBTU	*O*-Benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluoro-phosphate
THF	tetrahydrofuran
ACN	acetonitrile
Boc	tert-butyloxycarbonyl
DNB	3,5-dinitrobenzoyl
EEDQ	ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
ttbb	1, 3, 5-*tri*-*t*-butylbenzene
CSP	chiral stationary phase
DIPEA	*N*,*N'*-Diisopropylethylamine
m/z	mass to charge ratio
rac	racemic

CHAPTER I

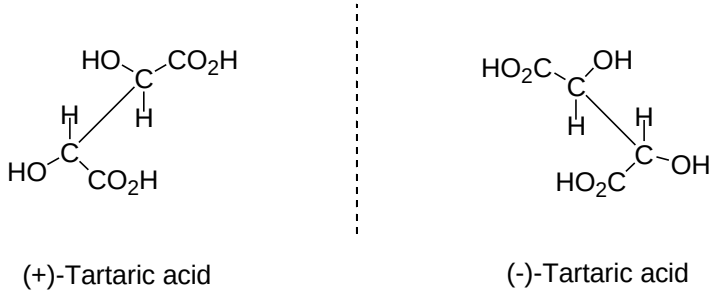
INTRODUCTION AND BACKGROUND

1.1 HISTORICAL PERSPECTIVE

Studies of stereochemistry stemmed from the discovery of plane-polarized light by the French physicist Malus in 1809. In 1815, another French scientist, Biot[1, 2] found that the plane of polarization of polarized light can be rotated by some organic substances, including both liquids (turpentine) and solutions of solids (like sucrose, camphor, and tartaric acid). In 1848, Louis Pasteur noticed that there were two types of crystals formed from the solution of the racemic (non-rotating) mixture of sodium ammonium salts of tartaric acid. He was able to separate the two types of crystals by the use of a lens and a pair of tweezers. When he re-dissolved the two types of crystals, he found that both solutions rotated the plane of polarization of polarized light, but one solution rotated to the right [molecules in this solution correspond to *dextro*-tartaric acid, or (+)-tartaric acid], while the other rotated to the left [*levo*-tartaric acid, or (-)-tartaric acid]. He postulated that (+)-tartaric acid and (-)-tartaric acid are mirror images of each other. In fact, these two types of tartaric acids are enantiomers.

In 1874, the Dutch scientist, van't Hoff and the French scientist Le Bel independently proposed a theory, which successfully explained the above phenomenon. It was proposed that molecules have different three-dimensional (3D) arrangements. The central carbon in

tartaric acids are asymmetric. Each of these carbons is directly linked to four substituents that are arranged tetrahedrally around it. This linkage makes two non-superimposable arrangements possible (Figure 1-1).[2]



(+)-Tartaric acid (-)-Tartaric acid

Figure 1 - 1. Enantiomers of tartaric acid.

1.2 TERMS AND DEFINITIONS

Enantiomers are defined as stereoisomers that are mirror image and non-superimposable with each other. Enantiomers are also termed as "chiral" molecules (from Greek "cheir", which means "hand"). Enantiomers usually are contingent on the existence of one or more stereogenic centers. A stereogenic center corresponds to the asymmetric atom bearing different ligands, *e.g*. sulfur atom, phosphorus atom and carbon atom. Switching opposing ligands about a stereogenic center in an enantiomer will give the other enantiomer of the molecule. In the case of a tetrahedral carbon atom, the center of chirality exists as long as the four substituents on the carbon are not equal. Overall, the condition for enantiomers is that they are non-superimposable mirror images (or they lack any improper axes of rotation, i.e. S_n symmetry elements). Diastereomers are

stereoisomers that are not enantiomers. Both enantiomers and diastereomers are stereoisomers. Unlike enantiomers, diastereomers do not bear a mirror image relation to each other.

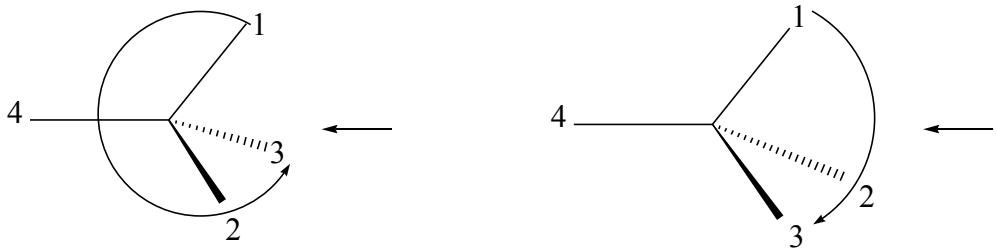
1.3 DESCRIPTION OF CHIRAL MOLECULES

Two enantiomers of a substance can be assigned to either (+)- or (-)- according to the property of optical rotation. In order to characterize the 3D arrangements of enantiomers without considering optical rotation, symbols (so-called descriptors) are introduced to differentiate two enantiomers. The Cahn-Ingold-Prelog designation (*R* / *S*) system is most commonly used, while the planar projection (Fisher) system is limited for the designation of amino acids and carbohydrates. Only in cases where there is a single stereogenic element do these describe absolute configuration; technically these describe configuration of stereogenic elements in a molecule.

1.3.1 Cahn-Ingold-Prelog designation (R / S designations)

In this system, letters "*R*" and "*S*" are used as descriptors ["*S*" is from Latin, "sinister" meaning the left or counterclockwise and "*R*" is from Latin, "rectus", meaning the right or clockwise] (Figure 1-2). A priority rule is following Cahn-Ingold-Prelog convention. The atom of lowest atomic number is considered the lowest priority number, while the highest priority is assigned to the atom of the highest atomic number. When a tie is encountered for substituents containing the same atom, the rule is applied by comparing the atoms attached to the first atom. The same procedure is repeated until the priorities of the substituents are assigned. As one is looking down the bond from the

chiral center toward the lowest priority atom, the other three ligands will be on the rim of a circle. Going along the circle from ligand 1 to ligand 2 to ligand 3 will allow one to determine whether the chirality of the molecule is clockwise (*R*) or counter-clockwise (*S*).[3]



(S) *(R)*

Figure 1 - 2. The convention for naming enantiomers as (*S*) or (*R*).

1.3.2 Planar Projection

This system was commonly used before 1960s to describe the configuration at stereogenic center. Currently, it is used only for amino acids and carbohydrates. The descriptors are "*D*" and "*L*". Fischer proposed that a chiral molecule can be drawn with two vertical cross lines. The cross point represents the chiral carbon center, and four ligands lie in the ends of lines. All horizontal lines are assumed to come forward to observers, while all vertical lines backward to observers. The example is given in Figure1-3, where if the OH group attached to the carbon next to the bottom CH_2OH group is on the right side, the molecule is designated as (*D*); if the OH group is on the left side, the molecule is designated as (*L*).[3]

D-glyceraldehyde *L*-glyceraldehyde

Figure 1 - 3. Fischer projection of *D* / *L* glyceraldehydes.

1.4 EFFECTS OF ENANTIOMERS ON THE HUMAN HEALTH

Since most biological molecules (*e.g.*, proteins, polysaccharides, and nucleic acids) are chiral, enantiomers of a chiral drug will have different effects, sometimes even leading to tragic consequences. The unfortunate birth defects which resulted from one of the enantiomers of thalidomide is a notorious example.[4] This drug was developed and sold as a racemic mixture to treat the morning sickness. However, it caused some serious fetal deformities. It was recognized later that the (*S*)-enantiomer of thalidomide is teratogenic, while (*R*)-enantiomer is sedative (Figure 1-4).

The thalidomide accident has evoked a large interest in the research of chiral chemistry. The chiral drug market is increasing rapidly. It is expected that revenues from chiral technology will near $ 15 billion by 2009.[5] The United States Food and Drug Administration (FDA) recommended that the physiological activity of each isomer of all new drugs should be well addressed. Therefore, the ability to produce single-enantiomer compounds, and to analyze the enantiomeric composition of chiral compounds is of paramount importance.

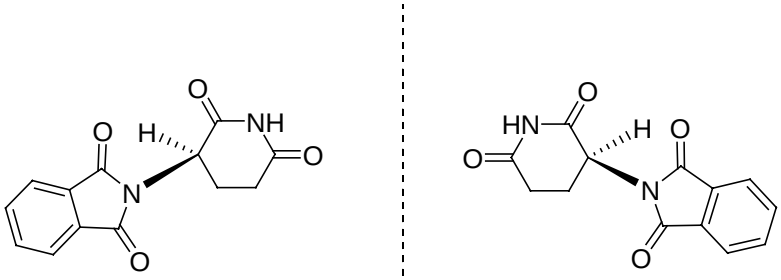


Figure 1 - 4. Enantiomers of Thalidomide.

1.5 METHODS TO DETERMINE THE ENANTIOMERIC COMPOSITION

There are a variety of instruments and techniques designed to measure enantiomeric purity of a chiral substance, such as polarimetry, NMR, chiral chromatography (including LC, GC, and SFC), capillary electrophoresis (CE), chiral chemosensors, and mass spectrometry.

1.5.1 Polarimetry

This is the oldest technique in determining the enantiomer purity of a chemical substance. Polarimetry takes advantage of the property of enantiomer in optical rotation. Measurement of optical rotation of compounds either in crystal form or in solution provides optical activity of a sample. The plane of polarization of polarized light can be rotated. The absolute rotation angle is dependent both on the nature of the substance as well as its concentration. It is defined that the clockwise rotation of the polarized light by the sample is (+), and the anticlockwise (-). In order to standardize the measurement for

the same substance differing in concentration, a specific rotation of a substance [α], was expressed as,

$$[\alpha] = \frac{\alpha}{l \bullet c}$$

where, α is the measured rotation;
l is the cell length (dm);
c is the concentration of sample (g/ml).
The optical purity is given by,

$$optical\ purity = [\frac{(\alpha)}{\alpha_{\max}}] \times 100$$

The application of polarimetry in determining the enantiomeric composition is affected by several factors: (1) The existence of impurities; (2) wavelength of polarized light used; (3) the nature of the solvent used; (4) concentration of the solution; (5) temperature; and (6) in some cases, optical rotation is not linear with the concentration.[2]

1.5.2 NMR

Chiral Derivatizing Agents (CDA)

Enantiomers cannot be discriminated in an achiral environment by NMR, since the resonances of enantiotopic nuclei are isochronous. However, it is possible to distinguish a pair of diastereomers by NMR because of the nonequivalence of diastereotopic nuclei in diastereomers. The first reported CDA is (*R*)-(-)-O-methylmandeloyl chloride, which can readily react with enantiomers of amines or alcohols to form a pair of diastereomers.[6] The integration ratio of any easily identifiable diastereotopic nuclei in two diastereomers serves as the ratio of enantiomers of the original analyte (See Figure 1-5).

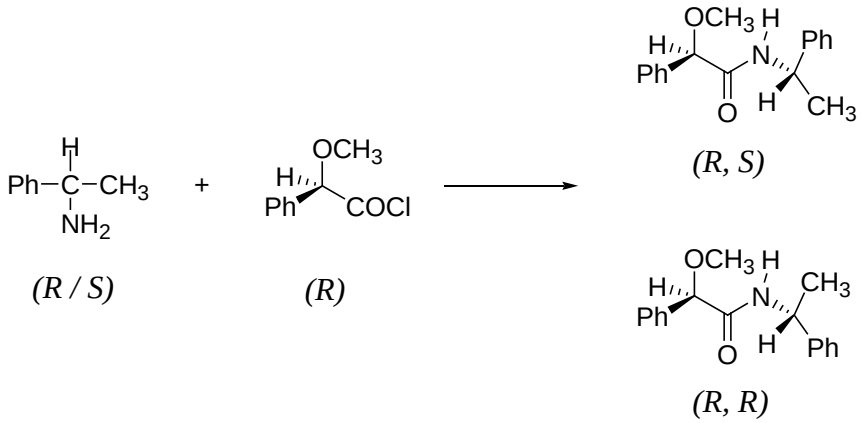
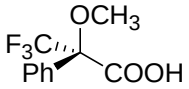


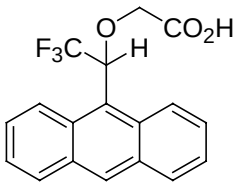
Figure 1 - 5. Derivatizing enantiomers of phenylethylamine with (*R*)-(-)-*O*-methylmandeloyl chloride.

Caution should be practiced for racemization may occur during the formation of diastereomers. It was observed that there is a disadvantage of using (*R*)-(-)-*O*-methylmandeloyl chloride as CDA owing to its liability to epimerization of the α-hydrogen.[7] This problem was overcome by replacing the α-hydrogen with a CF_3 group as is shown in the structure of Mosher's reagent (MTPA reagent, see Figure 1-6).[8] The incorporation of fluorine atom provides an alternative measure by means of ^{19}F NMR spectroscopy. In a similar way, phosphorous CDAs were developed for the analysis of nonracemic samples of primary and secondary alcohols by ^{31}P NMR spectroscopy.[9]

Another application is the use of ATEA (Figure 1-6) in the determination of enantiomer composition of amines. This was developed by Pirkle and Simmons in 1981.[10] It was designed to reduce conformational mobility by incorporating a larger anthrocene group to engender greater aniso-effects so that the differences in chemical shifts of diastereomers are more significant.



MTPA: Mosher's reagent



ATEA

Figure 1 - 6. CDA reagents.

The CDA-NMR technique affords not only an approach for quantitative analysis of enantiomeric composition, but also a method in determining the absolute configuration of a chiral molecule. Some limitations are: (1) the incomplete derivatization reaction would lead to errors; (2) Possible epimerization occurs in some cases; (3) the nonracemic CDA must be enantiomerically pure. These shortcomings can be solved by carefully selecting an appropriate CDA to carry out the derivatization reaction.[2]

Chiral Solvating Agents (CSA)

In 1966, Pirkle demonstrated the possibility of discriminating enantiomers by monitoring the ^{19}F NMR spectrum of racemic 2,2,2-trifluoro-1-phenylethanol (TFPE) in (-)-α-methylbenzylamine (PEA), based on the prediction of anisochrony in NMR spectroscopy with the use of nonracemic chiral solvents.[11, 12] It was proposed that the diastereomeric complexes were formed between PEA and TFPE through π-π interaction and hydrogen bonding. Different NMR spectra were observed for the diastereomeric complexes. It was recognized later that a nonracemic CSA in an achiral solvent can provide enough discrimination ability for enantiomers of the analyte.

A large number of CSAs have been developed, and their applications to the analysis of a wide scope of analytes have been demonstrated.[13] Enantiopure 2,2,2-trifluoro-1-(anthryl)-ethanol, TFAE is one of the most popular CSAs.[14] Some structures of CSA are shown in Figure 1-7.

The limitation of using a CSA is that the observed chemical shift differences are quite small. This problem can be overcome by using higher resolution spectrometers, lowering the temperature thereby increasing solvate formation as long as solubility is not a serious issue, or adding chemical shift reagents.[2] A greater advantage of this method is that the absolute enantiomeric pure CSA is not required since it involves formation of transient diastereomers or dynamic diastereomer systems and the observed anisochrony is simply attenuated.

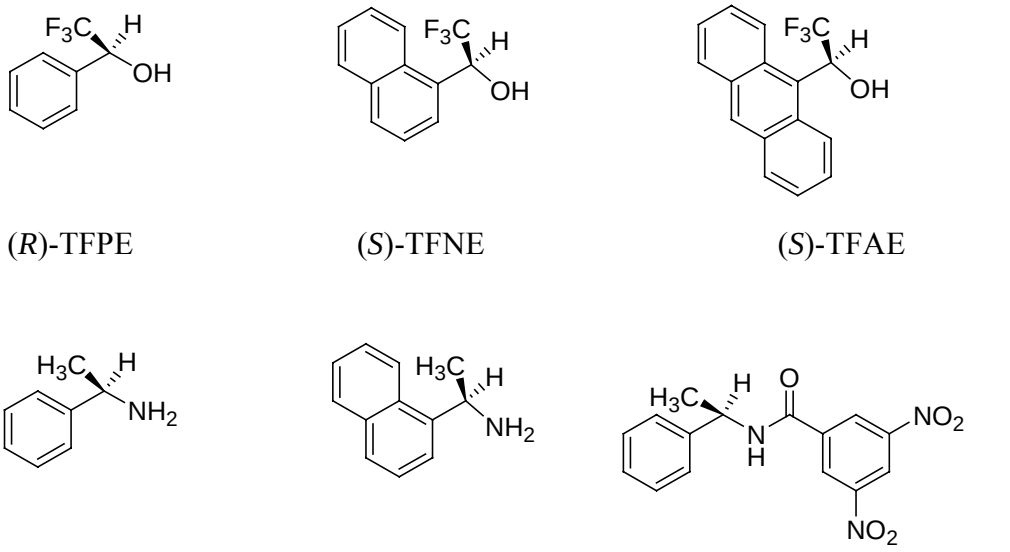


Figure 1 - 7. Chiral solvating agents.

Chiral Lanthanide Shift Reagents (CLSR)

Chiral lanthanide shift reagent reagents for the determination of enantiomeric composition was first demonstrated by Whitesides and Lewis.[15] Lanthanides have long been known as chemical shift agents useful in enhancing spectral dispersion and leading to spectral simplification.[16, 17] When a lanthanide ion is complexed with a chiral ligand, the formed lanthanide complex may exhibit discrimination to enantiomers. The mechanism of chiral lanthanide shift reagents is that a weak complex between chiral lanthanide shift reagent and organic substrate is formed, thereby causing a large difference in the magnetic susceptibility tensors for the seven-coordinate complex. Besides the early work of Whitesides, several other chiral lanthanide shift reagents were developed, many of which shows good capability in differentiating enantiomers.[13]

1.5.3 Chromatography

HPLC analysis with chiral solvents / additives

The formation of transient diastereomeric complexes can be induced by the addition of a chiral additive, which is added to nonpolar mobile phase.[18] In 1976, Pirkle and Sikkenga demonstrated the partial resolution of a racemic sulfoxide by LC on silica gel, using CCl_4 with added TFAE as mobile phase.[19] β-cyclodextrin, a cyclic carbohydrate is another important chiral additive for the mobile phase, which is capable of resolving different types of analytes.[20] Enantiomerically pure amino acids can also be used as chiral solvent additives together with transition metal ions such as Cu^{2+}, and Zn^{2+}. Har and Gil-Av in 1979 demonstrated the resolution of amino acids using Cu^{2+}-proline additives in

the mobile phase. The proline is combined to the enantiomer through the Cu^{2+} ion to form diastereomeric complexes with differential stability, which leads to the enantiomer resolution.[21]

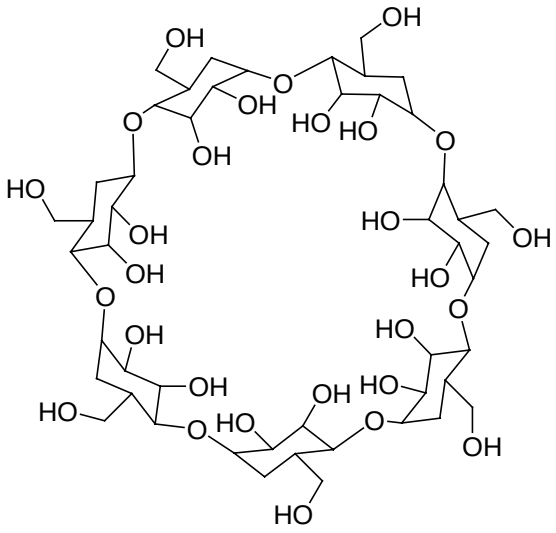
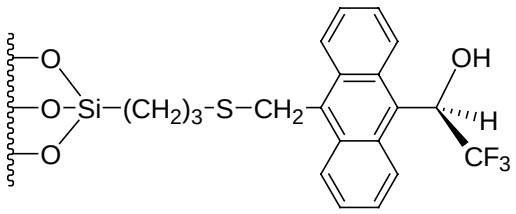


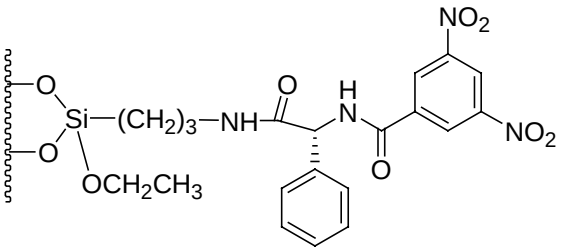
Figure 1 - 8. The structure of β-cyclodextrin.

Chromatography based on chiral stationary phases (CSP)

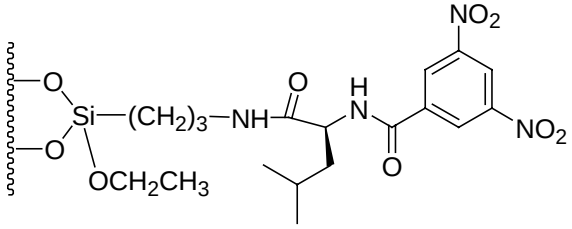
A pure or enantiomerically enriched compound is anchored to the surface of a solid support (*e.g.* Silica gel) to form a CSP. The energy difference in the association and dissociation of enantiomers with the CSP effects a separation.[22, 23] The interaction forces between analytes and CSP include $\pi-\pi$ interaction, H-bonding, inclusion complexation, dipole-dipole moment, and hydrophobic interaction.[24]



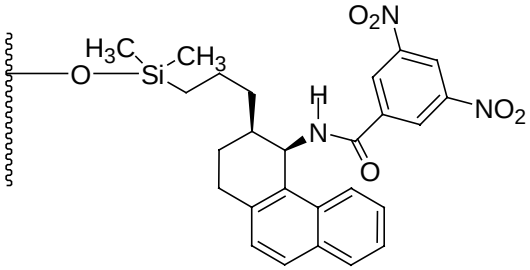
(*R*)-TFAE CSP



(*R*)-DNB-Phenylglycine CSP



(*S*)-DNB-Leucine CSP



(*S*, *S*)-Whelk-O1

Figure 1 - 9. Some Pirkle type CSPs.

The early trials of this technique in 1960's were centered on preparative resolution, but not very successful, partially owing to the efficiency of chiral selectors and the relatively low resolution of instrumental chromatographic techniques.[25] Pirkle and co-workers adapted NMR chiral solvating agents to applications of enantiomer separation by HPLC.[26] For example, the analogous (10-methyl-TFAE) of TFAE was bonded to γ-mercaptopropyl silanized silica to form a CSP, which is capable of resolving the enantiomers of π-acidic sulfoxides and some other π-acidic racemates (Figure 1-9).[27]

This and other so-called "Pirkle type" CSPs were designed based on the principle of the three-point recognition model. As is shown in Figure 1-10, these interactions are: (1) the interaction between π-base and π-acid; (2) a hydrogen bond between the hydrogen

donor in the analyte and the hydrogen acceptor in the selector; and (3) a second hydrogen bond between the hydrogen donor in the selector and the hydrogen acceptor in the analyte.[28-30]

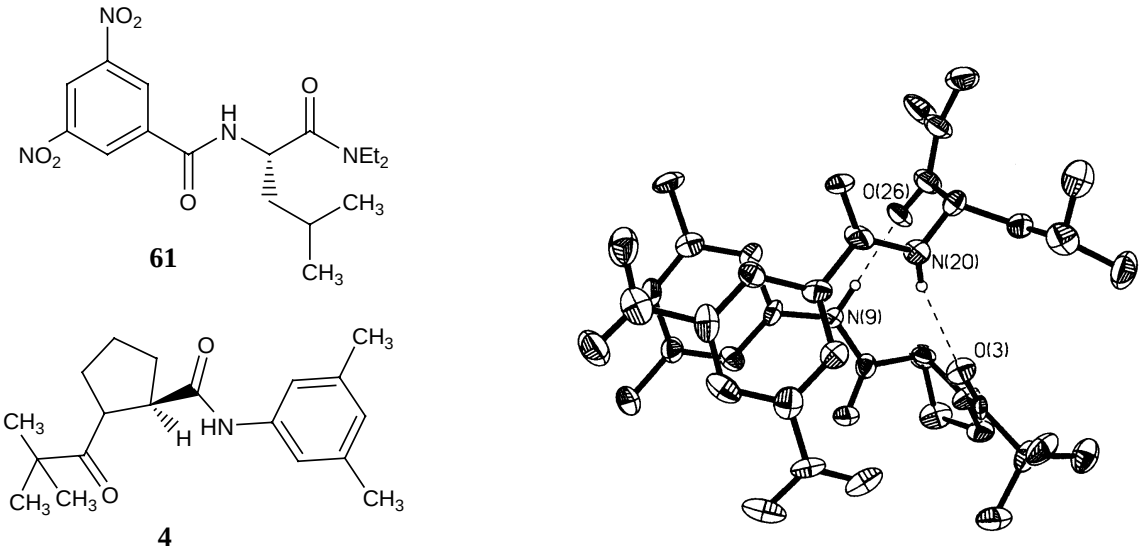


Figure 1 - 10. Complex structure from a 1 : 1 co-crystal between (*S*)-**61** and (*S*)-**4**.

Further development based on reciprocal protocol afforded a series of π-acidic chiral selectors such as DNB-phenylglycine, and DNB-leucine CSPs, useful in the separation of π-basic compounds.[31] Pirkle and coworkers also developed a CSP bearing both π-acidic and π-basic groups, Whelk-O1, which has broad application in a variety of analytes (Figure 1-9).[32]

In addition to the Pirkle type CSPs, other different types of CSPs have been developed since 1980.[24] Currently, there are over 200 different CSPs commercially available, but most of them can be simply categorized into four types:[24]

(1) polymeric: mainly including naturally occurring and synthetic polymers. Examples are ester or carbamate derivatives of cellulose and amylose, anchored onto silica gel. These types of CSPS are useful in separating a wide scope of chiral compounds without derivatization. They might be the most successful CSPs.

(2) macrocyclic: Including α, β, and γ-cyclodextrins and their derivatives, glycopeptides (macrocyclic antibiotics), and chiral crown ethers. All of these types of CSPs have been commercially available. β-cyclodextrin CSPs are the most useful ones, which are widely used to the analysis of chiral amines, amino alcohols, and binaphthyl derived crown ethers by HPLC.

(3) Ligand exchange: including hydroxyproline, and penicillamine *etc*.

(4) Brush type (Pirkle) CSPs as addressed before.

Even though there are a number of CSPs available, few detailed separation mechanisms are clearly interpreted. A successful separation of an enantiomeric mixture usually depends on the empirical experience and "trial and error" strategy. Pirkle-type CSPs are exceptional since the separation can be predicted at most times due to the relatively simple interactions between analytes and chiral selectors.

Similarly, chiral stationary phases have been adapted to the application of chiral analysis by other techniques, such as gas chromatography (GC)[23, 33] and capillary electrophoresis (CE).[23, 34] The fundamentals are not much different for a successful chiral separation between these techniques, although selectors may be tailored to suffice the specific features of each technique. Less time is required for analysis of a sample with both GC and CE. The success rate, however, is not as significant as is obtained with HPLC. Super-fluid chromatography (SFC) is another chromatographic technique with

which a chiral separation or analysis can be conducted. The column used for HPLC can be directly used on SFC to accomplish the analysis.[35]

1.5.4 Chiral Recognition Using Molecular Chemosenors

Chemosensors are considered to be molecular devices that signal the presence of matter or energy. They have found wide applications in sensing substances including anions, cations, and neutral molecules. [36, 37] A chemosensor is constructed by incorporating a binding site, a chromophore or fluorophore, and with / without a spacer in terms of mechanism of communication between the two sites. Chiral recognition using chemosensors takes advantage of the real-time sensing feature of sensors so that it is possible for this technique to be used in high throughput chiral assays.

A few successes have been obtained in the development of chiral chemosensors in recent years.[38] An example was reported by Pu and co-workers who demonstrated chiral bis-binaphthyl-based fluorescent sensors for the enantioselective recognition of α-hydroxycarboxylic acids (Figure 1-11). The benzene solution of compound (*S*, *S*)-**62** shows greater fluorescence enhancement (2.87 fold) as (*S*)-mandelic acid is added, while lower enhancement (1.75 fold) as (*R*)-mandelic acid is added. The nitrogen atom serves to quench the fluorescence of the binaphthyl chromophores by a photoninduced electron transfer (PET) process. Complexation to an acid switches off this process, causing the fluorescence enhancement. The information of differential complexation of each enantiomer to the sensor is reflected with the relative fluorescence enhancement.[39]

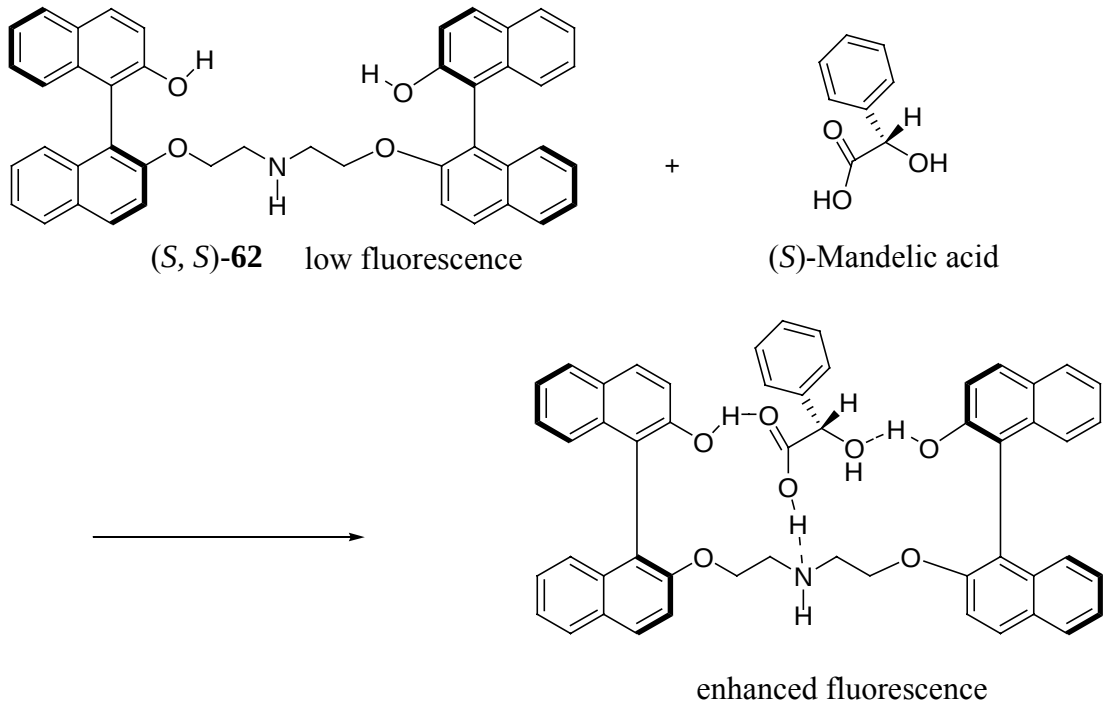


Figure 1 - 11. A proposed structure for complex (*S*, *S*)-**62** + (*S*)-mandelic acid.

Another development is the adaptation of reaction array method into chiral analysis with chemosensors. The enantiomeric composition information of the sample was converted into a ratio of fluorescent intensities observed by a CCD camera. This method enables the determination of the enantiomeric composition of structurally diverse *R*-amino acids with a single set of probes so that it provides a foundation for reaction microarrays as a general method for high-throughput e.e. (enantiomeric excess) analysis.[40]

The chiral recognition with chemosensors affords an alternative for enantiomer analysis, but it still has a long way to go before becoming a widely applicable method. More chemosensors are waiting to be designed and prepared for different types of chiral samples.

1.5.5 Mass Spectrometry (MS) Methods

Most chiral technologies require an asymmetric environment for chiral recognition and the determination of enantiomeric composition. Since there is not a chiral environment within the mass spectrometer, it was not traditionally considered to be a tool for chiral recognition. Indeed, both enantiomers of an analyte will afford identical mass spectra under the same experimental conditions. This apparent difficulty has recently been overcome in a few ways.[41-44] Owing to the merits, such as its broad analyte scope, tolerance to impurities, high sensitivity, fewer amount of samples needed, and potential for high throughput analysis, MS has become an attractive approach in chiral recognition and quantifying enantiomeric composition.

The ability to produce and maintain ionized molecules / complexes is a prerequisite for the enantiomer analysis by mass spectrometry. The appearance of soft ionization techniques makes this possible. The common used ionization sources are atmospheric pressure ionization (API), including electrospray ionization (ESI), and air pressure chemical ionization (APCI), and matrix assisted laser disorption ionization (MALDI).[45]

Electrospray ionization (ESI)

ESI is among the most popular ionization techniques equipped with mass spectrometer for chiral recognition. Malcom Dole and co-workers first demonstrated the utilization of electrospray ionization of intact chemical species and invented the technique of ESI in the 1970's.[46, 47] A progress was made by Fenn and co-workers in the late 1980's,[48] when they successfully developed the ESI technique to observe multiple

charged protein ions as well as ionized complexes. This research earned Fenn a share of the 2002 Nobel Prize for chemistry.[49]

Figure 1-12 shows the general scheme of ESI. The analyte solution is introduced to the source through the electrospray capillary that is applied a high potential difference (typically in the range from 2.5 to 4 kV). This potential helps the formation of charged droplets, which are repelled out of the capillary needle. The charged droplets fly toward the source cone on the counter electrode. During the traversing between the needle tip and the cone, the droplets lose its solvent such that the droplet shrinks repeatedly until it becomes a naked ion. This is a very mild ionization approach so that little energy is retained by the analyte upon ionization. The molecular ions rarely dissociate under the optimized condition. Additionally, the complexes formed via non-covalent bonds can be maintained and observed in the mass spectrometry spectrum.[50]

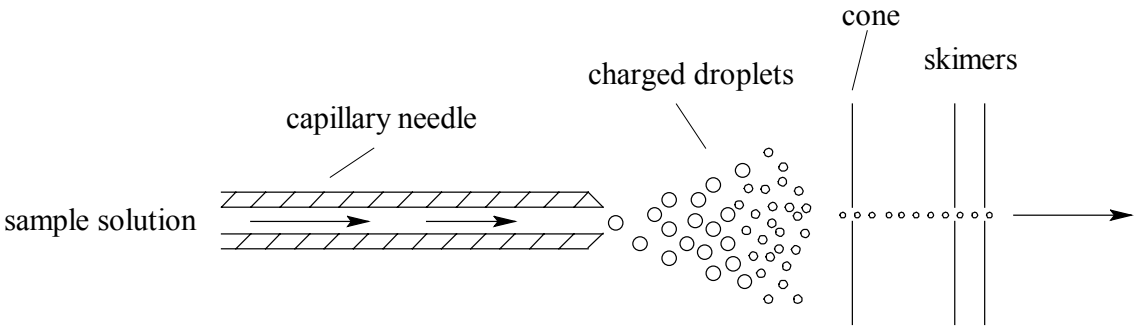


Figure 1 - 12. The diagram of electrospray ionization.

Mass analyzers

Quadrupole mass analyzer was invented in 1950s by Paul,[51] and is commonly used in many mass spectrometers. The triplequadrupole setup is a tandem technique, allowing

collisional dissociation studies performed by MS/MS.[52] The first quadrupole is used to select an ion or ion complex of interest, the second quadrupole is operated for the purpose of collisional decomposition of the selected ion, and the third quadrupole is used to scan the product ions released.

The original principle of ion trap (IT) mass analyzer is also mainly attributed to Paul.[51] There are several different configuration of IT, but the basic theory is similar: The ionized molecules are injected into and stored in the IT. The helium gas at low pressure inside the IT helps dampen the kinetic energy of the ions. Then, mass analysis can be performed, and ions of specific *m/z* can be excited for multi-stage MS experiment.

Time of Flight (TOF) Mass Analyzer. A time of flight mass spectrometer measures the mass-dependent time it takes ions of different masses to move from the ion source to the detector. The flight time of ions is dependent on the *m/z* ratio.[53-55]

Ion Cyclotron Resonance (ICR). Ions move in a circular path in the magnetic field after being excited by an external radio frequency. This motion is called cyclotron resonance that is a property of ions with its certain *m/z*. As packets of resonant ions pass close to the receiver plates in the ICR cell, a signal can be recorded and digitized.[56]

Enantiomer analysis based on host-guest or selector-analyte association

The information of chiral recognition can be collected by applying either single stage MS or tandem MS technique. The very first adaptation of MS into this area was reported by Fales and Wright using CI-MS in 1977.[57] The equal amount of the d_6-labelled dialkyl tartrate and the unlabelled dialkyl tartrate affords the mass spectrum in which the hetero-dimer peak is much lower than expected. This indicates that enantiomers prefer the

homochiral association to the heterochiral association. Since then, interest in the development of methods for the chiral recognition and the determination of enantiomers purity by MS, has increased dramatically. A variety of ionization methods have been adapted to the mass spectrometric chiral recognition with a number of host-guest systems, such as CI,[57] FAB,[58-63] MALDI,[64] and ESI.[58, 65-69] The majority of the earlier studies was concentrated on the observation of chiral recognition. Recent efforts, however, have been made to the development of quantitative enantiomer assays using MS.[63, 70, 71]

In the single stage MS experiment, the chiral selector-analyte (or host-guest) complexes are ionized as a whole entity in the ionization source, and then were transferred to the mass analyzer for a mass scan. In order to observe the significant complex abundances, conditions have to be finely tuned to maintain the stability of complexes during the analysis. In the earlier reports, the analysis was performed for at least two solutions, with each solution containing one enantiomer of the analyte together with the chiral selector. In order to collect the reliable result, a stock solution of an internal standard is made and added to each solution. The ratio of the selector-analyte (or host-guest) complexes from two measurements is an indication of chiral recognition.[59]

In the absence of an internal standard, however, it is feasible to normalize the target complex intensity to other ion intensities, such as that of the free selector (host), free analyte (guest), or a combination.[66, 72] Consistency should be controlled for comparison of similar systems. It is convenient in sample preparation while the accuracy is lower compared to enantiomer labeled method.

In other cases, the mixture of *pseudo*-enantiomeric host molecules (like Fales' experiment mentioned above[57]) can be used for determination of enantiomeric composition of the guest molecule. Isotopic mass labeling technique is applied in order to differentiate the peaks of two diastereomeric complexes in the mass spectrum. The isotopic influence can be ignored as long as the labeling sites are not involved in the chiral recognition. The relative peak abundance is the reflection of the corresponding enantiomer purity of analyte. Sawada and coworkers have utilized deuterium-labeled techniques (with chiral crown ethers,[61, 67, 73] and synthesized saccharides[74, 75] as chiral hosts) to differentiate a variety of chiral molecules. Recently, they applied a so-called dual label technique in the chiral analysis by MS.[71] Information of chiral recognition was extricated from one mass spectrum. The disadvantage of this method is also obvious: It requires extra preparation steps in labeling both the host and the guest.

Enantiomer analysis based on guest-exchange ion/molecule reactions (IMR)

Generally, a host-guest complex is formed in solution and brought to the gas phase by the ionization source. An exchange reaction occurs between the complex and a third non-chiral agent upon its being introduced into the mass spectrometer using a variable leak valve under low pressure. The rates of the reactions are dependent on the stability of host-guest complexes, which is controlled by the intrinsic feature of enantiomers.[41, 43]

Lebrilla and co-workers reported the differentiating ability of permethylated β-cyclodextrin toward enantiomers of amino acids by IMR with FTICR-MS.[76] The *n*-propylamine is used to replace the amino acid in the complex by means of proton exchange. The ratio of rate constants between enantiomers is 1.6 with alanine, 3.1 with

valine, and 0.8 with phenylalanine. This method is applicable for quantitatively determining enantiomeric composition.

A second type of host molecules studied in a similar scheme are the chiral crown ethers, demonstrated by Dearden and co-workers.[77-80] They are useful in discriminating enantiomers of chiral amine guests. Unlike the cyclodextrin type of hosts, chiral crown ethers have been shown to favor enthalpically driven selectivity mechanism. This is attributed to the formation of multi-hydrogen bonds.

In addition to the methods mentioned above, Finn's group took advantage of the concept of kinetic resolution of enantiomers using derivatizing reagents to determine the enantiomers purity quantitatively using ESI-MS. [81, 82] A mass-labeled enantiomer was mixed with its regular antipode at 1:1 ratio as a pair of *pseudo*-enantiomeric derivatizing reagents, which were utilized to react with the analyte. The mixture of the derivatives was analyzed by ESI-MS. The relative peak abundance of the *pseudo*-diastereomers can be related back to the enantiomeric composition of the analyte.

Enantiomer analysis based on dissociation of complexes (Tandem MS method)

Chiral recognition and the determination of enantiomeric composition using tandem MS involve the dissociation of the trimeric or dimeric complexes. The ionized complex formed in the first stage MS is isolated and guided into the second stage mass, where it undergoes the collision-induced dissociation (CID) or exchanges with another reagent to form a new species.[42, 52, 83]

The fundamental basis for CID of complexes is the isolation and the fragmentation of heterodimeric or heterotrimeric diastereomeric ions. The observable enantioselectivity

can be passed on to the branching ratio between the daughter ions and the non-dissociated complex ions. Quantitative analysis of enantiomeric composition also can be performed using the calibration curve constructed by using enantiomeric mixtures of known *e.e.*%.[84]

Wan and coworkers developed a discriminating system for 19 common amino acids using ESI-ITMS^2 with *N*—Boc-blocked phenylalanine, proline, and O-benzyl-serine as chiral references. The protonated trimers were dissociated by CID experiment to form protonated dimmers, the branching ratio can be related back to its *e.e.*% in solution.[84-86]

More recently, Gronert *et al* carried out the CID of complex systems containing a chiral dication and a chiral anion. CID of these complexes leads to the transfer of a proton or an alkyl cation to the chiral anion leading to a singly-charged dication. The abundance of product ions from each diastereomeric salt pair is different, therefore, a chiral recognition can be observed by MS.[87]

Lindner's group utilized cinchona alkaloid-type chiral selectors for the chiral recognition of dipeptides. The CID of [selector-analyte+Na]$^+$ complexes affords the information of enantiomer discrimination of dipeptides.[88]

A MS method involving the discrimination of diastereomeric *N*-acetylhexosamine monosaccharides was reported by Leary *et al*.[89-93] Firstly, the saccharide was derivatized to form the metal complex with Co^{3+} before infusing into ESI for ionization. Different product ion spectra were generated upon CID. They can be used to identify the stereochemistry of *N*-acetylhexosamines from a hydrolyzed oligosaccharide. A quantitative study based on this method has been done to predict the precise amount of each diastereomer present in an unknown mixture.

Another MS method similar to those mentioned above is categorized as kinetic method using CID technique. It was first developed by Cooks and coworkers.[94-110] The trimeric diastereomeric complexes, formed from transition metal ion, chiral references and chiral analyte enantiomer, are dissociated by CID. The process of dissociation is kinetic. The trimeric competitively loses either analyte or the reference molecule. The branching ratios for dissociation of the complexes containing the *R*- and *S*-enantiomers of the analyte correspond to the degree of chiral recognition. The metal ion used here is to provide high-order metal ion-bound cluster ions. The idea was borrowed from ligand-exchange chromatography that requires creating diastereomeric complexes by binding the chiral analyte to a chirally ligated metal center.[21] Different groups have reported a variety of chiral recognition systems using various chiral references and transition metal ions.

1.6 SUMMARY

The "classical" methods, such as NMR, and chromatography techniques for chiral assay, are useful in terms of accuracy and generality, regardless of the speed. Mass spectrometry is an attractive method considering the speed, especially for high throughput analysis on a large numbers of chiral analytes. This method would definitely be a boon to the development of catalytic enantioselective reactions, particularly by combinatorial asymmetric catalysis, whereby libraries of potential asymmetric catalysts are produced in parallel and each catalyst is screened for its ability to produce a product of high enantiomeric purity.[81, 111-113] Typically, the size of combinatorial catalyst libraries has not been limited by the number of potential catalysts that one can prepare, but rather by the time needed to evaluate large libraries using contemporary methods for enantiomer

analysis. Such a method could also be used for the discovery and optimization of chiral selectors by combinatorial methods.[114-120] Instead of measuring the enantiomeric composition, one would instead be using mass spectrometry to directly measure the relative binding of analyte enantiomers to potential chiral selectors. In addition to the potential for rapid analysis, other attributes such as a high tolerance to impurities, broad analyte scope, and high sensitivity are beneficial for the determination of the enantiomeric composition of samples originating from a variety of sources, particularly samples of biological origin.

In this dissertation, the adaptation of soluble analogues of Pirkle-type CSPs for use in electrospray ionization mass spectrometry experiments that demonstrate chiral recognition and allow quantification of enantiomeric composition is presented. Mass labeled mixtures of *pseudo*-enantiomeric selectors were used to perform enantiomer analysis by MS for analytes separable chromatographically on the corresponding CSPs. Efforts in improving the extent of enantioselectivity and the accuracy will be discussed subsequently. The scope and limitations of this method will also be addressed. Lastly, preliminary experiments of the synthesis and screening of a dipeptide library is demonstrated.

CHAPTER II

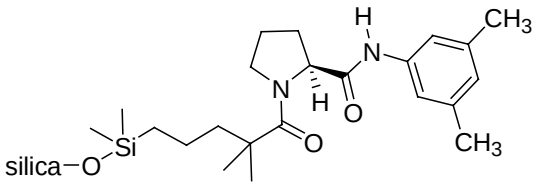
ENANTIOMER ANALYSIS USING AMIDE DERIVATIVES OF 3,5-DINITROBENZOYL-LEUCINE AND 3,5-DINITROBENZOYL - PHENYLGLYCINE AS CHIRAL SELECTORS

2.1 INTRODUCTION

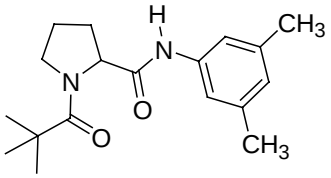
The Pirkle type CSPs are widely used for the chromatographic analysis of amino acids, chiral amines, and chiral alcohols *etc*.[27, 121, 122] It normally takes minutes to measure the enantiomeric composition by HPLC. It is a desire to develop new methods for enantiomer analysis that allow rapid analysis, especially in the case of screening a large number of chiral compounds. Considering its potential for rapid analysis, mass spectrometry seems to be well-suited for the development of new and rapid enantiomer assays.[42]

Herein is discussed the adaptation of the soluble analogues of Pirkle type CSPs[19, 27-31] as chiral selectors for enantiomer analysis by mass spectrometry. The analyte is *N*-pivaloyl-2-(3,5-dimethylanilide)proline **4**, the enantiomers of which are resolvable chromatographically on the analogues CSP. The *pseudo*-enantiomeric chiral selectors (where each enantiomer is mass-labeled at a remote site) were used in pairs [i.e., the amide derivatives of 3,5-dinitrobenzoyl (DNB) leucine, (*S*)-**6** / (*R*)-**7** and DNB-phenylglycine, (*R*)-**8** / (*S*)-**9**]. Solutions containing the analyte and the chiral selectors in

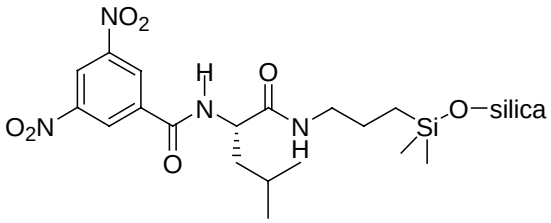
the presense of lithium chloride were introduced into the electrospray ionization (ESI) source for ionization. The selector-analyte complexes formed in solution can be transferred to the gas phase, and observed by MS. The abundances of *pseudo*-diastereomeric selector-analyte complexation ions are proportional to the concentrations of *pseudo*-diastereomeric selector-analyte complexes in solution. Since each enantiomer of the chiral selectors preferentially binds one enantiomer of the analyte, the relative peak height of *pseudo*-diastereomeric selector-analyte complexation ions can be related back to the enantiomeric composition of the analyte in solution.



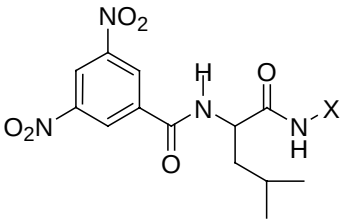
(*S*)-**CSP 1**



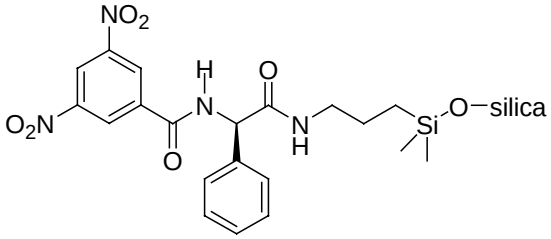
4



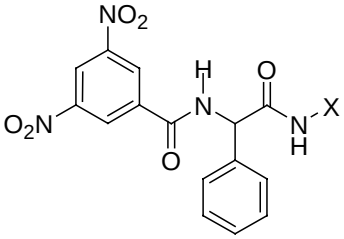
(*S*)-**CSP 2**



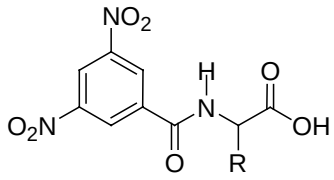
5 X = n-Propyl
6 X = n-Butyl
7 X = n-Pentyl



(*R*)-**CSP 3**



8 X = n-Butyl
9 X = n-Pentyl



10: R = i-propyl
11: R = i-butyl
12: R = Phenyl

Figure 2 - 1. Structures of Pirkle-type chiral stationary phases and their soluble analogs.

2.2 EXPERIMENTAL

General

The chemicals, reagents, and solvents used throughout the dissertation were obtained either from Aldrich or Fisher. Tetrahydrofuran (THF) was dried over potassium and freshly distilled before use. Dichloromethane was dried over CaH_2 and freshly distilled before use. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AMX-300 spectrometer operating at 300 MHz for ^1H and 75 MHz for ^{13}C. HPLC pump was from Alcott (Alcott 760), and the UV detector was from Varian (Varian 9050). All mass spectra were obtained on a Micromass QuattromicroTM (Beverly, MA) Triple Quadruple Mass Spectrometer equipped with Electrospray Ionization Source.

Synthesis of N-pivaloyl-2-(3,5-dimethylanilide)proline

To a 2 M NaOH aqueous (aq.) solution (174 ml) in a round bottom (r.b) flask was added 87 mmol of (*S*)-proline. The dissolved proline basic solution was cooled in an ice-water bath before 87 mmol of pivaloyl chloride was added dropwise. The solution was maintained basic during the addition. The mixture was allowed to stir for another 0.5 hour at 0 ^0C, and then warmed to room temperature (r.t) and stirred for another 3 hours. 150 ml of ethyl ether was added to the reaction mixture, partitioned and the ethyl ether layer was discarded. The aq. layer was acidified with concentrated HCl to pH~2, then extracted with dichloromethane (150 ml × 2). The combined dichloromethane extract was dried over $MgSO_4$, filtered, and evaporated *in vacuo* to afford colorless oil. To the oil was added ~50 ml hexanes to precipitate a white solid. The solid was collected, washed with

hexanes, and dried in air to afford 13.07 gram of white solid. (75.2%). (*R*)-*N*-pivaloylproline and (*rac*)-*N*-pivaloylproline were made in the same manner. ESI-MS (*m/*z, 200, $[M+H]^+$) approved the target molecules.

To a flame dried 100 mL r.b flask cooled under dry N_2, was added 2.0 mmol of *N*-pivaloylproline, followed by 10 mL dichloromethane. 2.0 mmol EEDQ was added and allowed to dissolve before the addition of 2.0 mmol 3,5-dimethyl aniline. The mixture was stirred at r.t for 2 hours, and then diluted with 100 mL EtOAc and washed with 2 M HCl (100 mL × 2), saturated $NaHCO_3$, and dried over $MgSO_4$. The solvent was removed *in vacuo* to provide 505 mg (yield, 84%) white solid.

4: ^{1}H NMR: $CDCl_3$, 300 MHz, δ, 9.23 (br. s, 1H), 7.13 (s, 2H), 6.67 (s, 1H), 4.82 (d, *J* = 6.0 Hz, 1H), 3.80-3.72 (m, 2H), 2.38-2.16 (m, 8H), 2.00-1.79 (m, 2H), 1.30 (s, 9H). ^{13}C NMR: $CDCl_3$, 75 MHz, δ, 178.4, 169.8, 138.4, 138.2, 125.4, 117.4, 62.4, 48.3, 39.3, 27.6, 26.1, 25.9, 21.3.

General procedure for the synthesis of amide derivatives of N-(3,5-dinitrobenzoyl)amino acids

An oven dried r.b flask was cooled by flushing dry nitrogen gas. To it were added 30 mmol 3,5-dinitrobenzoyl chloride and 30 mmol of an amino acid, followed by the addition of 90 mL of dry THF. Finally, 6.3 mL (90 mmol) propylene oxide was added in one portion via syringe. The suspension was stirred overnight. The unreacted amino acid was filtered through cotton. The filtrate was evaporated *in vacuo*. The brown residue was recrystallized with acetone / hexane to provide the DNB-amino acid.

10: white solid, yield, 75%. ^{1}H NMR: acetone-d_6, 300 MHz, δ, 9.12 (s, 2H), 9.10 (s, 1H), 8.54 (d, J = 6.7 Hz, 1H), 4.68 (m, 1H), 2.35 (octet, J = 6.5 Hz, 1H), 1.08 (d, J = 6.8 Hz, 6H).

11: light yellow solid, yield, 81%. ^{1}H NMR: acetone-d_6, 300 MHz, δ, 9.12 (s, 2H), 9.10 (s, 1H), 8.71 (d, J = 7.1, 1H), 4.81-4.78 (m, 1H), 1.89-1.77 (m, 3H), 0.99 (d, J = 6.0 Hz, 6H).

12: white solid, yield, 61%. ^{1}H NMR: acetone-d_6, 300 MHz, δ, 9.15 (s, 2H), 9.09 (s, 1H), 7.59-7.41 (m, 5H), 5.81 (m, 1H).

To the oven dried r.b flask was added the obtained DNB-amino acid (1.0 mmol) and EEDQ (1.0 mmol), followed by the addition of dry CH_2Cl_2. The mixture was sonicated to dissolve all solids before adding the corresponding amine. After addition of amine, the mixture was allowed to stir for about half an hour at r.t. The reaction mixture was washed with 1 M HCl (30 mL × 2), 5% $NaHCO_3$ (30 mL × 2), and then washed with water (30 mL × 2). The organic solution was dried over $MgSO_4$ and evaporated *in vacuo* to provide the final product.

(*S*)-**6**: light yellow solid, yield, 74%. ^{1}H NMR: $CDCl_3$, 300 MHz, δ, 9.09 (s, 1H), 8.95 (s, 2H), 8.62 (d, J = 7.5 Hz, 1H), 6.39 (brs, 1H), 4.71 (m, 1H), 3.45-3.34 (m, 1H), 3.21-3.15 (m, 1H), 1.92-1.29 (m, 7H), 1.00-0.90 (m, 9H). ^{13}C NMR: $CDCl_3$, 75 MHz, δ, 172.2, 162.6, 148.5, 137.1, 127.4, 121.1, 53.2, 41.6, 39.6, 31.4, 25.0, 22.9, 22.1, 20.0, 13.7.

(*R*)-**7**: light yellow solid, yield, 59%, ^{1}H NMR: $CDCl_3$, 300 MHz, δ, 9.07-8.98 (m, 4H), 6.77 (brs, 1H), 4.76 (m, 1H), 3.41-3.32 (m, 1H), 3.15-3.08 (m, 1H), 1.94-1.67 (m,

3H), 1.51 (m, 3H), 1.29 (m, 4H), 0.99-0.88 (m, 9H). ^{13}C NMR: $CDCl_3$, 75 MHz, δ, 172.5, 162.7, 148.5, 137.0, 127.5, 121.0, 53.3, 41.3, 39.8, 29.0, 25.0, 22.9, 22.3, 22.0, 13.9.

(*R*)-**8**: light yellow solid, yield, 80%. ^{1}H NMR: acetone-d_6, 300 MHz, δ, 9.14 (s, 2H), 9.08 (s, 1H), 9.00 (d, J = 6.0 Hz, 1H), 7.67 (brs, 1H), 7.58-7.37 (m, 5H), 5.79 (s, 1H), 3.24 (m, 2H), 1.48-1.24 (m, 4H), 0.86 (t, J = 7.1 Hz, 3H). ^{13}C NMR: acetone-d_6, 75 MHz, δ, 171.1, 164.1, 150.6, 140.3, 139.4, 130.4, 129.9, 129.7, 129.6, 122.7, 59.9, 40.9, 33.3, 21.6, 15.0.

(*S*)-**9**: light yellow solid, yield, 82%. ^{1}H NMR: acetone-d_6, 300 MHz, δ, 9.15 (s, 2H), 9.09 (s, 1H), 7.66 (brs, 1H), 7.58-7.36 (m, 5H), 5.77 (s, 1H), 3.22 (m, 2H), 1.49-1.25 (m, 6H), 0.84 (t, J = 6.3 Hz, 3H). ^{13}C NMR: acetone-d_6, 75 MHz, δ, (peaks for two carbonyls were not observed in the NMR spectrum, due to the low concentration of the sample), 150.4, 140.3, 139.4, 130.4, 129.9, 129.7, 129.6, 122.7, 59.8, 41.0, 27.2, 23.9, 15.2.

The NMR spectra of the above compounds agree with the liturature.[121-125]

Preparation of (*S*)-*CSP* ***2***

5.0 mmol (*S*)-**11** and 5.0 mml EEDQ were added into 200 mL dry dichloromethane, and sonicated to dissolve all solid. The solution was pumped into amino column at ~0.5 ml / min. The chiral selector coated column was washed with dichloromethane, THF, and, methanol subsequently until no significant absorption was recorded by UV-detector.[28]

Preparation of (*R*)-*CSP* ***3***

(*R*)-**12** was used as chiral selector in this case. Other conditions were the same as before.[28]

Preparation of solutions

2 mM stock solution of (*R*)-**4** was made by dissolving 0.02 mmol (*R*)-**4** in 10 mL methanol. Stock solutions (*rac*)-**4**, and (*S*)-**4** were made in the same way. Differing amounts of these three solutions were mixed to afford eleven stock solutions of analyte **4**, each having the desired enantiomeric composition. The enantiomeric composition of each stock solution was also measured by HPLC (see below for HPLC conditions). Equal amount of **6** and **7** (0.2 mmol each) were combined and dissolved in 10 mL methanol to afford the stock solution of *pseudo*-enantiomeric selectors (20 mM each); also, the stock solution of selectors **8** and **9** was prepared in the same way. The stock solution of lithium chloride (100 mM) was prepared by dissolving 10 mmol lithium chloride in 100 mL water. End solutions were made by combining the metered amount of the corresponding stock solution of analyte, the stock solution of *pseudo*-enantiomeric selectors, and the stock solution of lithium chloride, and diluting with methanol and acetone to afford a final concentration of 2.5 mM for chiral selectors **6** and **7**, 0.25 mM for analyte **4** and 25 mM for lithium chloride in methanol / water / acetone (1:1:2). Likewise, solutions with a final concentration of 1.0 mM for chiral selectors **8** and **9**, 0.1 mM for analyte **4** and 10 mM for lithium chloride in methanol / water / tetrahyrofuran (2:1:2) were prepared.

HPLC

HPLC condition: 20 μL of each stock solution of analyte **4** was injected into HPLC system, respectively. The mobile phase was 90% hexanes / 10% isopropyl alcohol at a flow rate of 2 mL / min. The peak was monitored with UV-detector at λ = 254 nm. Each solution was measured three times, and the average value of three tests was used as the enantiomeric composition of analyte **4** in end solutions. The void time was determined by recording the retention time of 1,3,5-*tri*-*t*-butylbenzene (ttbb) at the same condition. The separation factor (α_{HPLC}) was calculated according to eq. 2-1~3:

$$\alpha_{HPLC} = \frac{k_2}{k_1} \quad .....eq.2-1$$

$$k_1 = \frac{t_1}{t_1 - t_0} \quad .....eq.2-2$$

$$k_2 = \frac{t_2}{t_2 - t_0} \quad .....eq.2-3$$

where, k_1 and k_2 are capacity factors of two peaks in the chromatogram, respectively; t_1 and t_2 are retention time of enantiomers; and t_0 is void time.

Mass spectrometry

Solutions were infused with a syringe pump into the ESI source at a rate of 5 μL / min for the solutions with selectors **6** and **7**, and 8 μL / min for the solutions with selectors **8** and **9**. Spectrometer conditions were as follows: capillary, 3.5 kV; RF lens, 0.5 V; source temperature, 80 ^{0}C; desolvation temperature, 350 ^{0}C; cone gas flow, 70 L / h; desolvation gas flow, 757 L / h. For each experiment, data were collected for

approximately 2 min, each full scan requiring ~0.7 s, with all the scans averaged to afford the final spectrum.

2.3 Results And Discussion

2.3.1 Chiral recognition using (*S*)-*6* and (*R*)-*7 as chiral selectors*

The soluble analogues used in this study were the amide derivatives of DNB-leucine **6** / **7**, DNB-phenylglycine **8** / **9** and the 3,5-dimethyl anilide of *N*-pivaloyl proline **4**. The enantiomers of analyte **4** afforded chromatographic separation factors of 3.41 and 3.74 on CSPs **2**, and **3**, respectively[122-126] (Figure 2-2, and 2-3). The elution order of analyte enantiomers relies on their difference in interacting with the solid phase. In both cases, the more retained enantiomer has the same stereochemical designation as the chiral selector, *i.e*., (*S*)-**4** is more retained on (*S*)-CSP **2**, and (*R*)-**4** is more retained on (*R*)-CSP **3**. In a reciprocal way, the enantiomers of soluble analogues of CSPs **2** and **3** were resolved on CSP **1**. For example, it was reported that the enantiomers of the n-propyl amide of DNB-leucine **5** afforded a separation factor of 26.6 on CSP **1**.[125] It had been confirmed by X-ray crystallography that a diastereomeric complex can be formed between the Diethyl amide of (*S*)-DNB-Leu and (*S*)-**4** (*c.f* Figure 1-10). Owing to this ability of forming diastereomeric complexes and chiral recognition in solution, it was proposed that the complex could be maintained and the chiral recognition could be observed in gas phase. Therefore, the analyte **4** and DNB-amino acid derivatives, *i.e*. **6**, **7**, **8**, and **9** were selected as the initial analyte-selector combinations for the mass spectral enantiomer analysis with electrospray as ionization source.

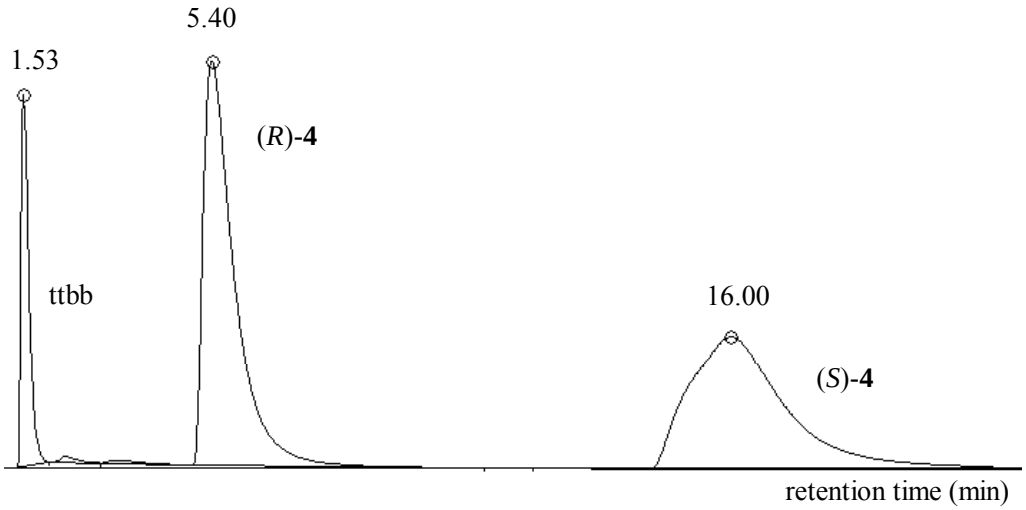


Figure 2 - 2. Chromatogram of racemic **4** on (*S*)-CSP **2**. mobile phase: 90% hexanes / 10% isopropyl alcohol. Flow rate: 2 mL / min. Wavelength: 254 nm. (ttbb was injected together with **4**, and its retention time was used as void time).

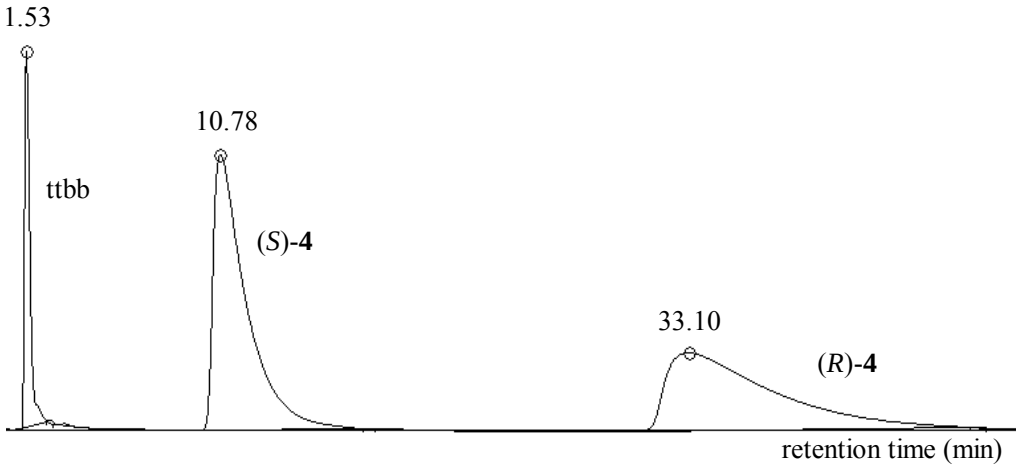


Figure 2 - 3. Chromatogram of racemic **4** on (*R*)-CSP **3**. Mobile phase: 90% hexanes / 10% isopropyl alcohol. Flow rate: 2 mL / min. Wavelength: 254 nm. (ttbb was injected together with **4**, and its retention time was used as void time).

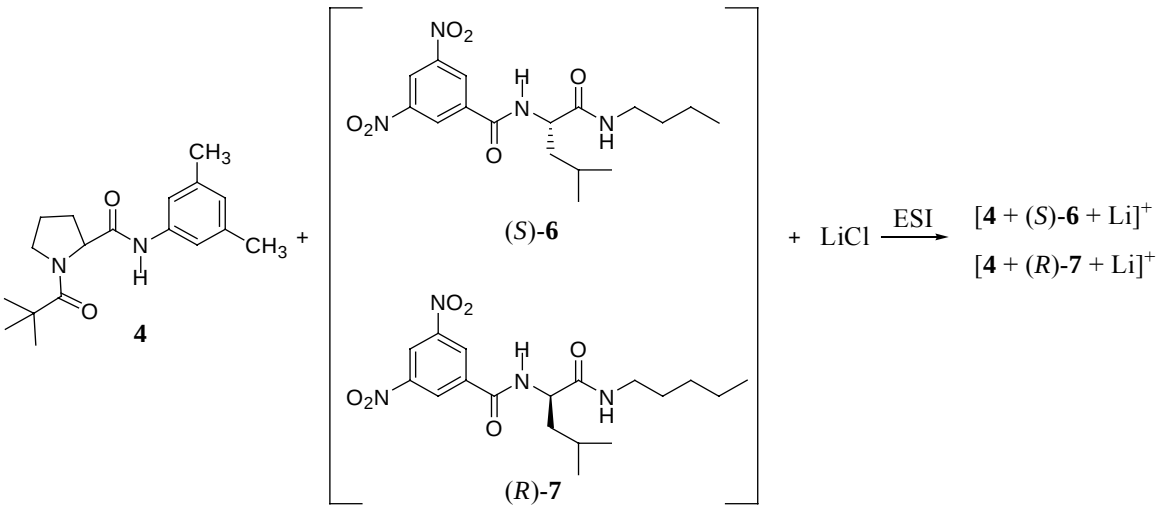


Figure 2 - 4. ESI-MS experiment of the solution containing (*S*)-**6**, (*R*)-**7** and **4** with added lithium chloride in 1:1 methanol / water.

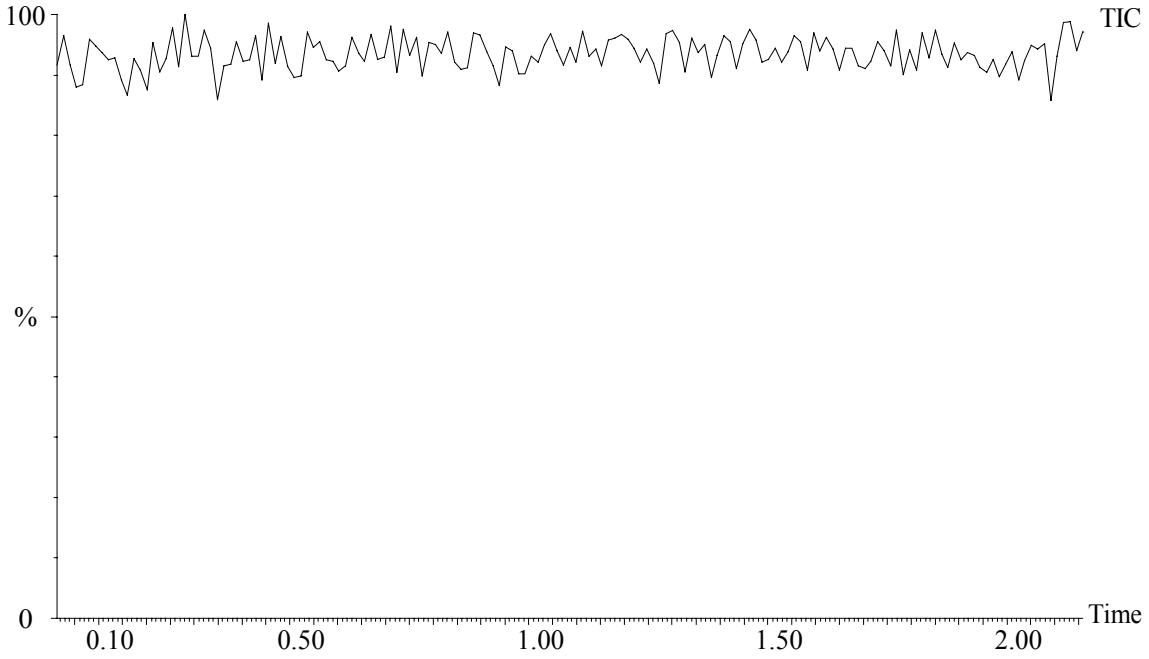


Figure 2 - 5. A typical total ion count chromatogram of a solution infused through a syringe pump.

A solution containing an equal amount of the butyl amide of (*S*)-DNB-leucine **6** and the pentyl amide of (*R*)-DNB-leucine **7** was prepared and used as the *pseudo*-enantiomeric chiral selectors. The difference in length of side chain is used in order to have peaks of two *pseudo*-diastereomeric complexes presented at differing *m/z* in the mass spectrum. It is assumed that the length of the side chain would not significantly affect the sense or the extent of the selectivity of the chiral selectors. In addition, their ionization efficiency is assumed to be equal. Each solution containing selectors **6** and **7** and the analyte **4** was directly infused into mass spectrometer at a flow rate of 5 μL / min with a syringe pump (see Figure 2-4). A typical total ion count chromatogram (TIC) is shown in Figure 2-5. The mass spectrum was extracted by averaging ~1 min of TIC. Substantial selector-analyte complexes were observed in the mass spectrum with the addition of lithium chloride (the Li^+ adducts).

Figure 2-6 shows the mass spectrum of a solution of the chiral selectors (*S*)-**6** and (*R*)-**7** with racemic-**4** and lithium chloride. The lithiated monomeric ions were observed at *m/z* 309 [**4**+Li^+], 387 [**6**+Li^+] and 401 [**7**+Li^+]. The solvated lithiated monomeric ions were also observed: *m/z* 367 [**4**+acetone+Li^+], 445 [**6**+acetone+Li^+], and 459 [**7**+acetone+Li^+]. The lithiated homo-dimeric ions were observed at *m/z* 611 [$\mathbf{4}_2$+Li^+], 767 [$\mathbf{6}_2$+Li^+] and 795 [$\mathbf{7}_2$+Li^+], while the lithiated hetero-dimers were observed at *m/z* 689 [**4**+**6**+Li^+], 703 [**4**+**7**+Li^+] and 781 [**6**+**7**+Li^+]. The analyte-selector hetero-dimers *m/z* 689 and 703 are supposed to afford information about chiral recognition.

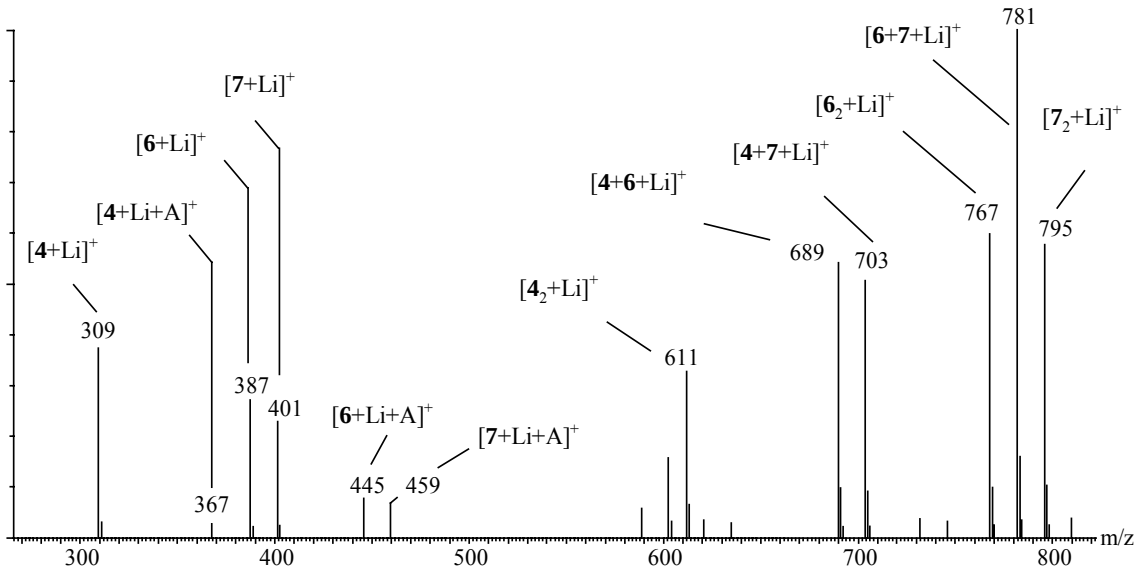


Figure 2 - 6. Mass spectrum of a solution of *pseudo*-enantiomeric selectors **6** and **7** (2.5 mM) and analyte **4** (0.25 mM) with added lithium chloride (25 mM) in methanol / water / acetone (1 : 1 : 2). Note: A = acetone.

The mass spectra of the other ten solutions with the differing enantiomeric composition of **4** were recorded under the same conditions. It was observed that the relative intensity of the peaks at *m/z* 689 and 703 varied accordingly with enantiomeric composition of analyte **4** in solution. In order to compare the chiral recognition clearly the partial spectra containing *m/z* 689 and 703 only are presented in Figure 2-7 for solutions highly enriched in both enantiomers of analyte **4**, as well as the racemate. The sense of chiral recognition is consistent with what is observed chromatographically. The homo-dimeric analyte-selector complexes are more stable than hetero-dimeric analyte-selectors ions, as is can be seen from the relative intensities of 689 and 703 in each case: When the sample is enriched in the (*S*)-**4**, the larger peak is *m/z* 689, which is the complex between the (*S*)-**6** and the analyte (Figure 2-7-a); while when enriched with the (*R*)-**4**, *m/z* 703 is higher, which is corresponding to the (*R*)-**7** and the analyte (Figure 2-7-

c); while when racemic with **4**, the two peaks are almost in equal height (Figure 2-7-b). The other peaks in the spectrum remained relatively invariant with enantiomeric compositions of the sample.

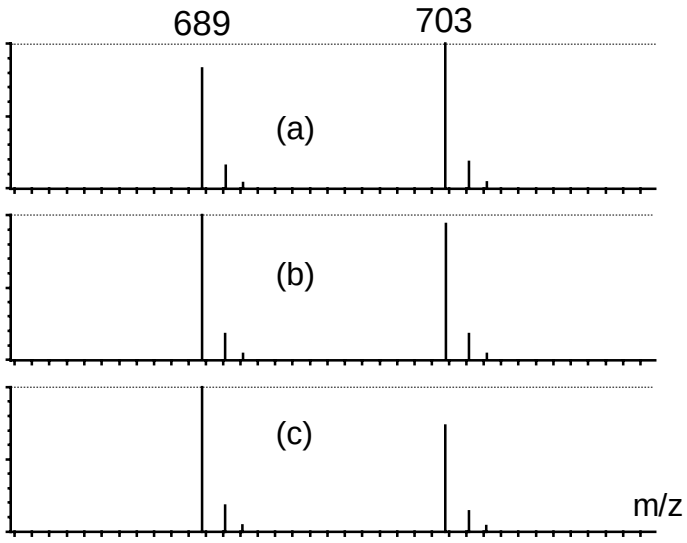


Figure 2 - 7. Partial mass spectra of *pseudo*-enantiomeric selectors (*S*)-**6** and (*R*)-**7** (2.5 mM) and analyte **4** (0.25 mM) with added lithium chloride (25 mM) in methanol / water / acetone (1 : 1 : 2). Spectrum: (a) 89.8% of (*R*)-**4**; (b) racemic; (c) 9.1% of (*S*)-**4**.

Owing to the observation above, one is able to give an affirmative conclusion: the chiral recognition using *pseudo*-enantiomeric chiral selectors can be observed through the ESI-mass spectrometry. With the enriched (*S*)-**4** in the solution, the intensity ratio between the peak at *m/z* 689 and the peak at *m/z* 703 is 0.71; while with the enriched (*R*)-**4** is 1.27.The overall selectivity, given by the ratio of peak ratios for two enriched samples is 1.79. It was considered that the ratio of peak ratios should be larger than the value 1.79, provided that the pure (*R*)-**4** and pure (*S*)-**4** are used.

Enantioselectivity

A plot of complex intensity fraction (CIF, intensity of one selector-analyte complex divided by the sum of the intensities for both selector-analyte complexes) of the selector-analyte complexes *vs* the mole fraction of the (*R*)-**4** in the sample (as determined by chiral HPLC) for eleven different enantiomeric compositions is presented in Figure 2-8. The plot affords a straight line with a correlation coefficient of 0.993 and a slope of 0.1451. This plot can be used for subsequent enantiomer analysis. One can simply measure the CIF of the selector-analyte complexes in the mass spectrum and apply this value to the calibration line to determine the enantiomeric composition on X-axis.

Figure 2 - 8. Plot of the complexes intensity fraction (CIF) of peaks at *m/z* 703 and 689 in the ESI-MS vs the mole fraction of (*R*)-**4** in the solution, using *pseudo*-enantiomeric chiral selectors **6** and **7** (slope = 0.1451, intercept = 0.4142, r^2 = 0.993).

The slope of this plot can also be used to indicate the degree of enantioselectivity: The larger the slope of this plot is, the greater the enantioselectivity. In this study, it was presumed that the transfer efficiency of each complex from solution to the gas phase has no significant difference one another so that the relative amount of each complex in solution is reported in the mass spectrum. This makes possible relating the relative abundance of diastereomeric ions observed in the gas phase through the mass spectrometry back to the chiral recognition in solution.

$$\alpha = \frac{[\Psi_R \bullet A_R]}{[\Psi_R \bullet A_S]} = \frac{[\Psi_S \bullet A_S]}{[\Psi_S \bullet A_R]} = \frac{[\Psi_R \bullet A_R]}{[\Psi_S \bullet A_R]} = \frac{[\Psi_S \bullet A_S]}{[\Psi_R \bullet A_S]} \quad \text{.....} eq.2-4$$

In the above equations, Ψ_R and Ψ_S represent the *pseudo*-enantiomeric chiral selectors, and A_R and A_S are the analyte enantiomers. The enantioselectivity in solution, α, is defined as any of the ratios of concentrations shown in eq 2-5, providing that the *pseudo*-enantiomeric chiral selectors are acting as enantiomers, and $[\Psi_R] = [\Psi_S]$, which can be guaranteed by carefully preparing the stock solution of the *pseudo*-enantiomeric chiral selectors.

The complexes are likely ionized via the coordination of Li^+ with the functional groups of chiral entities. During the electrospray ionization process, lithium adducts of the *pseudo*-diastereomeric complexes will be transferred to the gas phase, where the relative concentration of ions is recorded by the mass spectrometry. Another assumption is given here, that each of the four possible complexes will have equal transfer coefficient, t_x. Similarly to the definition of the enantioselectivity in solution, α, the

enantioselectivity in gas phase observed through the mass spectrometry is given as α_{MS}, which is equal to any of the ratios of the ion counts of the four complex ions. The definition equation is given in eq 2-5, where the ratios of the transfer coefficients will be unity. Since the condition required by the electrospray ionization is much harsher than that in solution, more disruption for the four major equilibria may exist. It is considered, however, that the relative abundance of the complex ions would not change as long as the condition is kept constant.

$$\alpha = \frac{t_{RR}\left[\Psi_R \bullet \mathrm{A}_R + Li\right]^+}{t_{RS}\left[\Psi_R \bullet \mathrm{A}_S + Li\right]^+} = \frac{t_{SS}\left[\Psi_S \bullet \mathrm{A}_S + Li\right]^+}{t_{SR}\left[\Psi_S \bullet \mathrm{A}_R + Li\right]^+} = \frac{t_{RR}\left[\Psi_R \bullet \mathrm{A}_R + Li\right]^+}{t_{SR}\left[\Psi_S \bullet \mathrm{A}_R + Li\right]^+} = \frac{t_{SS}\left[\Psi_S \bullet \mathrm{A}_S + Li\right]^+}{t_{RS}\left[\Psi_R \bullet \mathrm{A}_S + Li\right]^+} \quad .....eq.2-5$$

In the mass spectrum, the ion counts of each diastereomeric complex are the reflection of one chiral selector with both analyte enantiomers, *i.e*. $[\Psi_R+A_R+Li]^+$ / $[\Psi_R+A_S+Li]^+$, and $[\Psi_S+A_R+Li]^+$ / $[\Psi_S+A_S+Li]^+$. The CIF is given by sum of ion counts of both analyte enantiomers with one chiral selector divided by the sum of ion counts of all selector-analyte complexes, from which one hopes to elucidate the information of the enantioselectivity of chiral selectors.

$$CIF = \frac{\left[\Psi_R \bullet \mathrm{A}_R + Li\right]^+ + \left[\Psi_R \bullet \mathrm{A}_S + H\right]^+}{\left[\Psi_R \bullet \mathrm{A}_R + Li\right]^+ + \left[\Psi_S \bullet \mathrm{A}_S + Li\right]^+ + \left[\Psi_S \bullet \mathrm{A}_R + Li\right]^+ + \left[\Psi_S \bullet \mathrm{A}_S + Li\right]^+} \quad .....eq.2-6$$

A relationship between the CIF and the enantiomeric composition of the analyte can be built through an appropriate mathematical expression, which will provide information

of the enantioselectivity since both the CIF and the enantiomeric composition of the analyte are known values. A series of substitutions and rearrangement of eq. 2-6 afford such an equation as shown in eq. 2-7, where α is the enantioselectivity by MS and X_R is mole fraction of the (*R*)-analyte. This equation affords a linear curve by plotting the CIF *vs* X_R.

$$CIF = \left(\frac{\alpha - 1}{\alpha + 1}\right) X_R + \left(\frac{1}{\alpha + 1}\right) \quad \text{.....} eq.2-7$$

Furthermore, the linearity of the plot will not be affected as long as the differences* remain constant throughout, though there probably are differences in the transfer coefficients of the complexes, differences in enantioselectivity of the *pseudo*-enantiomeric chiral selectors or differences in the concentrations of chiral selectors. These should manifest themselves in the intercept of the plot. In fact, owing to this, scarcely do the α_{MS} values determined from the slope and the intercept of this plot agree. In the cases where the analyte is pure enantiomer ($X_R = 1$, and $X_R = 0$), the CIF values will be $\alpha_{MS}/(\alpha_{MS} + 1)$ and $1/(\alpha_{MS} + 1)$, respectively. The ratio of these CIF values is α_{MS}, which can be used as an additional measure of the enantioselectivity. Practically, it is more convenient to use the slope of the calibration plot than the intercept because only measuring the CIF at two different enantiomeric compositions will satisfy in constructing the plot. Otherwise, in order to determine the CIF values for both pure enantiomers, one needs access to both pure analyte enantiomers, which is usually a burden. Actually, as

* The differences could be ionization efficiency, extent of chiral recognition of each selector, tether effects and solvents…

can be seen in this study, pure enantiomers were not used for the construction of the plot. According to the equation 2-7, the α_{MS} for chiral selectors **6** and **7** is equal to 1.34. Also, all enantioselectivities reported in this dissertation were calculated from the slope of the calibration plots.

Quantitative determination of enantiomeric composition

In order for this method to be used for quantitative enantiomer assays, further analysis was done by using the leave-one-out cross validation technique. In this method, each data point can be considered to be for "an unknown" sample, while the remaining data points were fit to a calibration line, which was then used to determine the enantiomeric composition of "the unknown". The results of this analysis for all 11 samples are shown in Table 2-1. As can be seen from Table 2-1, the enantiomeric composition determinations by MS are in good agreement with the values obtained by chiral chromatography. The average absolute difference between these measurements is 1.5 with a standard deviation of 1.6.

Solutions with an enantiomeric composition of (*R*)-**4** of 89.8%, 49.2%, and 9.1% (as determined by HPLC), were further analyzed by performing nine injections for each one. The CIF was applied to the above calibration curve to afford average values of 89.9 ± 2.2%, 48.5 ± 2.3%, and 7.5 ± 7.8% respectively, at the 95% confidence level.

Table 2 - 1. Enantiomeric composition of analyte **4** by mass spectrometry using chiral selectors **6** and **7** compared to the enantiomeric composition as measured by chiral chromatography.

Average % (*R*)-**4** by HPLC	% (*R*)-**4** by MS	Difference[b,c]
89.8	88.8	-1.0
80.9	78.8	-2.1
73.6	74.2	0.6
65.8	65.6	-0.2
57.8	57.2	-0.6
49.2	48.7	-0.5
40.5	45.1	4.6
32.8	35.5	2.7
25.1	24.8	-0.3
17.7	19.2	1.5
9.1	2.3	-6.8

[a] HPLC condition: Column: (*S, S*)-whelk-O1, Mobile phase: 60% hexanes / 38% isopropyl alcohol / 2% MeOH. Flow rate: 2 mL / min.
[b] The absolute difference average is 1.9.
[c] The standard deviation is 2.09.

2.3.2 Chiral recognition using (*R*)-*8 and* (*S*)-*9 as chiral selectors*

The ESI-mass spectrum of a solution of the butyl amide of (*R*)-DNB-phenylglycine **8** and the pentyl amide of (*S*)-DNB-phenylglycine **9**, with added lithium chloride, afforded the monomeric and dimeric species as before with the DNB-leucine analogues (Figure 2-9).

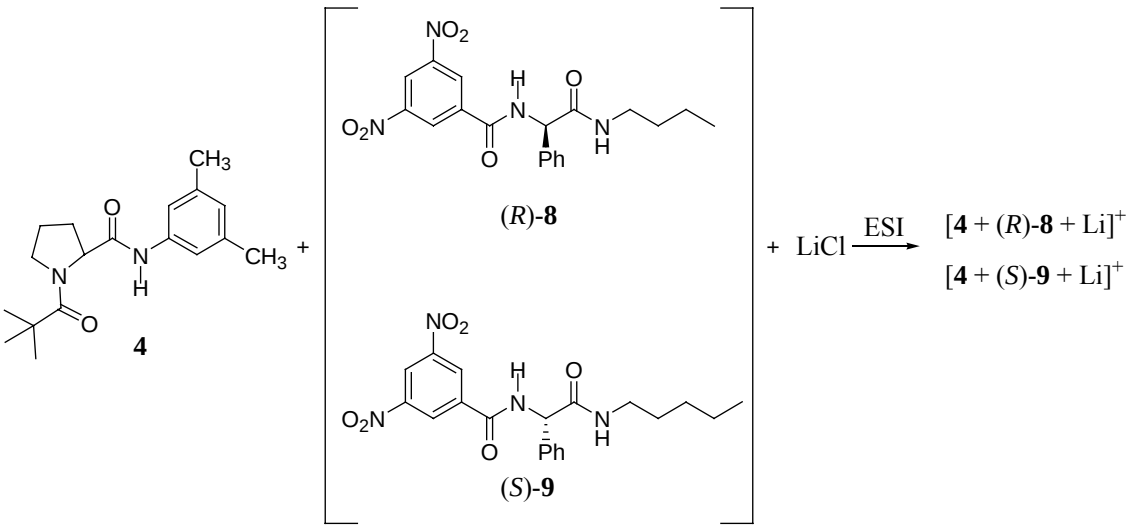


Figure 2 - 9. ESI-MS experiment of solutions containing (*R*)-**8** / (*S*)-**9** and analyte **4** in the presence of lithium chloride.

Figure 2-10 is the full spectrum of the solution containing *pseudo*-enantiomeric selectors and racemic **4** as well as lithium chloride. The monomeric ions were observed at *m/z* 309 [**4**+Li^+], 407 [**8**+Li^+], and 421 [**9**+Li^+]. The solvated peaks were also observed at *m/z* 381 [**4**+THF+Li^+], 479 [**8**+THF+Li^+], and 493 [**9**+THF+Li^+]. The lithiated homo-dimeric ions were observed at *m/z* 611 [$\mathbf{4}_2$+Li^+], 807 [$\mathbf{8}_2$+Li^+] and 835 [$\mathbf{9}_2$+Li^+], while the hetero-dimers were observed at *m/z* 709 [**4**+**8**+Li^+], 723 [**4**+**9**+Li^+] and 821 [**8**+**9**+Li^+].

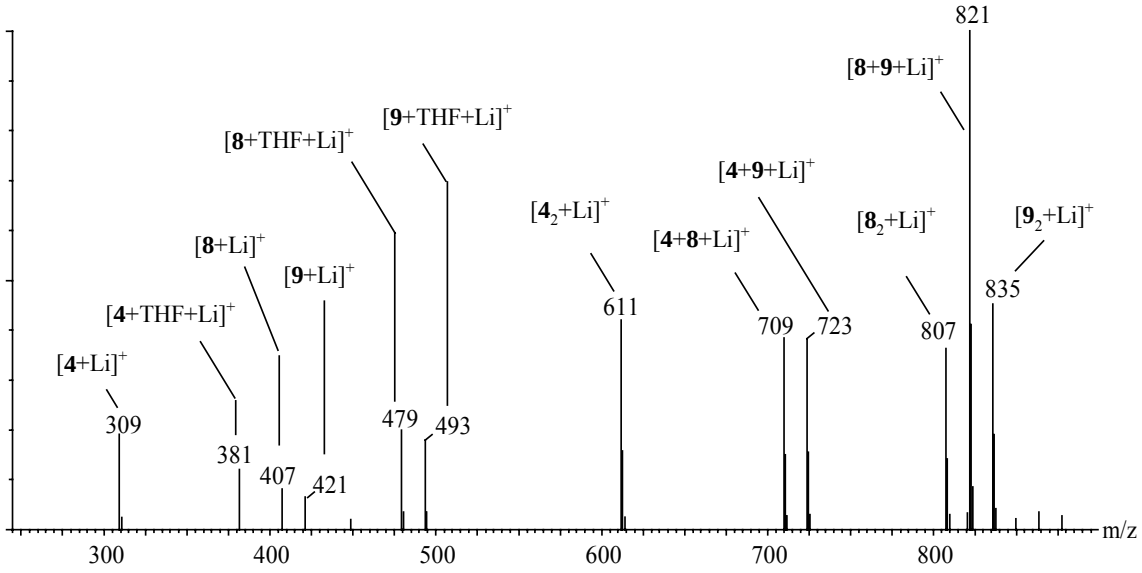


Figure 2 - 10. Mass spectrum of a solution of *pseudo*-enantiomeric selectors (*R*)-**8** and (*S*)-**9** (1.0 mM) and analyte **4** (0.1 mM) with added lithium chloride (10 mM) in methanol / water / tetrahydrofuran (2 : 1 : 2).

A regular change of the dimeric ions between each *pseudo*-enantiomer of DNB-phenylglycine and analyte **4** were observed with the change of the enantiomeric composition of **4**. Figure 2-11 presents the partial spectra of solutions with the enantiomeric composition of **4** varying. As the amount of (*R*)-**4** was increased in the sample, the relative intensity of the peak at *m/z* 709 increased (with respect to the peak at *m/z* 723). Likewise, as the amount of (*S*)-**4** in the sample increased, the relative intensity of the peak at *m/z* 723 increased (with respect to the peak at *m/z* 709). This clearly demonstrates that the sense of chiral recognition in these samples is the same as is observed chromatographically. The (*R*)-selector has a higher affinity for (*R*)-**4**, therefore when the sample is enriched in (*R*)-**4**, the intensity of the lithiated (*R*)-**8** + **4** ion (*m/z* 709) in the mass spectrum will be increased. Likewise, an increase in (*S*)-**4** would increase the intensity of the lithiated (*S*)-**9** + **4** ion (*m/z* 723).

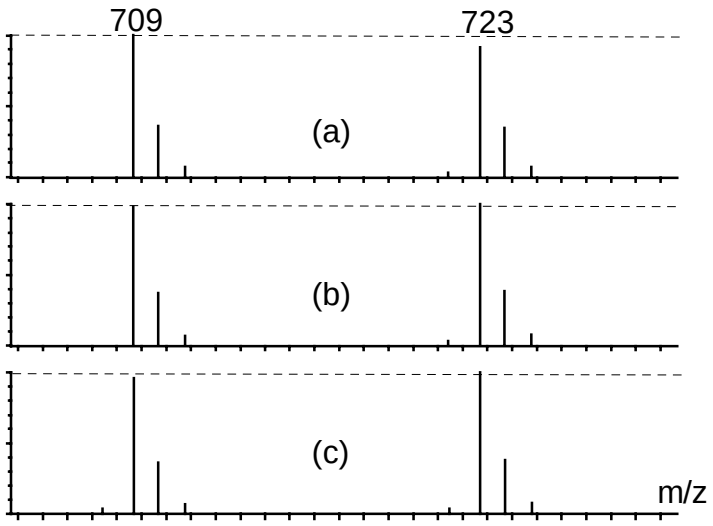


Figure 2 - 11. Partial mass spectra of *pseudo*-enantiomeric selectors (*R*)-**8** and (*S*)-**9** (1.0 mM) and analyte **4** (0.1 mM) with added lithium chloride (10 mM) in methanol / water / THF (2 : 1 : 2). Spectrum: (a) 89.8% of (*R*)-**4**; (b) racemic; (c) 9.1% of (*R*)-**4**.

Enantioselectivity

A plot of CIF *vs* the enantiomeric composition of analyte **4** afforded a straight line with a correlation coeffient of 0.996 and a slope of 0.0595 (Figure 2-12). The α_{MS} was calculated to be equal to 1.12. As before, this illustrates the potential use of this type of *pseudo*-enantiomeric chiral selectors for enantiomeric composition determinations by MS.

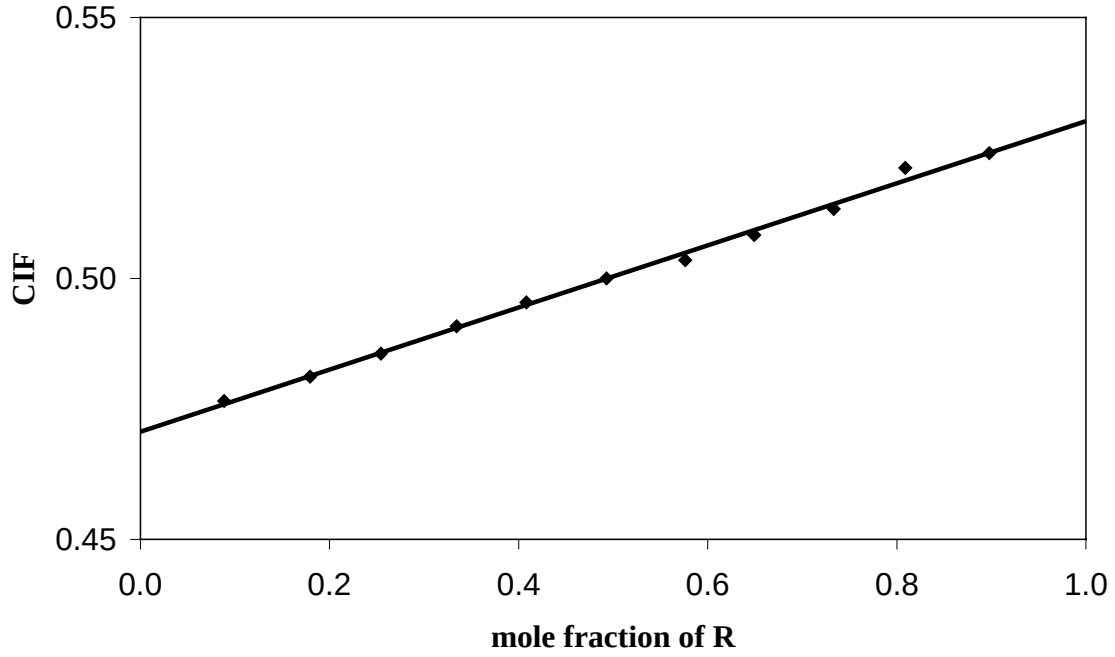


Figure 2 - 12. Plot of the complexes intensity fraction (CIF) of peaks at *m/z* 709 and 723 in the ESI-MS *vs* the mole fraction of (*R*)-**4** in the solution, using *pseudo*-enantiomeric chiral selectors **8** and **9** (slope = 0.0595, intercept = 0.4706, r^2 = 0.996).

Quantitative determination of enantiomeric composition

These data were also analyzed using the leave-one-out cross validation techniques. The results of this analysis for all 11 samples are presented in Table 2-2. As can be seen from Table 2-2, the enantiomeric composition determinations by MS are in good agreement with the values obtained by chiral chromatography. The largest absolute difference is 5.3% and the average absolute difference between these measurements is 1.4 with a standard deviation of 1.6.

Table 2 - 2. Enantiomeric composition of analyte **4** by mass spectrometry using chiral selectors **8** and **9** compared to the enantiomeric composition as measured by chiral chromatography.

% (*R*)-**4** by HPLC	% (*R*)-**4** by MS	Difference
89.8	89.6	0.2
80.9	86.2	-5.3
73.6	71.3	2.0
65.8	63.0	1.9
57.8	54.9	2.9
49.2	49.4	-0.2
40.5	41.6	-0.8
32.8	33.9	-0.5
25.1	25.1	0.4
17.7	18.2	-0.2
9.1	10.3	-1.5

Similarly to what was done for chiral selectors **6** / **7** system, the comparison of results between the MS and the HPLC were made at three different enantiomeric compositions of (*R*)-**4** (89.8%, 57.6%, and 9.1% as determined by HPLC). The CIF was applied to the above calibration curve to afford average values of 88.8 ± 6.8%, 55.2 ± 9.6%, and 8.6 ± 5.1% respectively, at the 95% confidence level.

2.4 SUMMARY

These preliminary efforts have clearly established that chiral recognition was observed in the ESI-MS using *pseudo*-enantiomeric chiral selectors. A calibration line can be constructed by plotting the CIF *vs* the mole fraction of (*R*)-analyte, which will not only be used as a calibration curve for quantitative determination of the unknowns, but also afford a convenient approach to determine the ability of the enantioselectivity without using the pure enantiomers. Additionally, in the mass spectrum, the relative

abundance of selector-analyte peaks is over 50% relative to the base peak, which is beneficial for reproducibility of the measurement

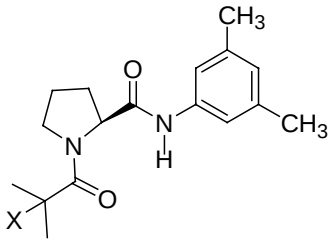
The enantioselectivity of the DNB-leucine derived chiral selectors is higher than the selectivity observed for the DNB-phenylglycine derived chiral selectors, as demonstrated by the α_{MS} (1.33 *vs* 1.12) for analyte **4**, even though the chromatographic separation factors for the enantiomers of analyte **4** are very similar on both CSPs **2** and **3**. A number of factors account for this, each of which will be investigated in subsequent studies. Firstly, the concentrations of the chiral selectors are different (**6** / **7**, 2.5 mM; **8** / **9**, 1.0 mM), although the ratio of selector to analyte is maintained for each (10:1). Secondly, the solvent used for each system is different, mainly for solubility reasons (methanol/water/acetone = 1:1:2 for **6** / **7**, and methanol/water/tetrahydrofuran = 2: 1 : 2 for **8** / **9**). Thirdly, it is not clear at this point as to the role that Li^+ plays in affecting chiral recognition between chiral selector and analyte, although Li^+ is definitely required for efficient ionization. The primary interactions between the chiral selectors and analyte **4** which are responsible for chiral recognition, as has been demonstrated through a number of studies,[121-125] are (1) a π-stacking interaction between the π-acidic dinitro aromatic ring of the selector and the π-basic dimethylanilide ring of the analyte; (2) a hydrogen bond between the DNB amide proton of the selector and the pivaloyl carbonyl of the analyte; and (3) a hydrogen bond between the C-terminal carbonyl of the selector and the anilide proton of the analyte. Given that the chromatographic separation factors are larger than the selectivities observed by ESI-MS, it is likely that the presence of Li^+ in solution may actually interfere with some of these requisite interactions, particularly the hydrogen bonding interactions. Moreover, the solvent system used in chromatography

and in mass spectrometry is different, which may introduce some discrepancies of the enantioselectivity. Also, it is not clear if the ESI-mass spectrometry condition will affect the enantioselectivity, which is necessary to be investigated in the subsequent studies.

CHAPTER III

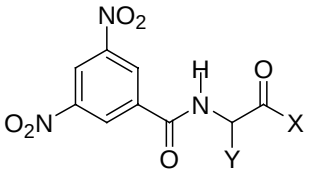
ENANTIOMER ANALYSIS USING DEPROTONATED 3,5-DINITROBENZOYL AMINO ACIDS AS CHIRAL SELECTORS

3.1 INTRODUCTION



CSP **1**: X = -$(CH_2)_3Si(CH_3)_2O$-silica

4: X = CH3



CSP **5**: Y = i-Pr, X = -$NH(CH_2)_3Si(CH_3)_2O$-silica
CSP **2**: Y = i-Bu, X = -$NH(CH_2)_3Si(CH_3)_2O$-silica
CSP **3**: Y = Ph, X = -$NH(CH_2)_3Si(CH_3)_2O$-silica
10: Y = i-Pr, X = OH
11: Y = i-Bu, X = OH
12: Y = Ph, X = OH

Figure 3 - 1. Structures of Pirkle-type chiral stationary phase and soluble analogs.

The observation of chiral recognition between analyte-**4** and *pseudo*-enantiomeric chiral selectors was demonstrated in Chapter 2. The presence of lithium ion might, however, have deleterious effects on the extent of enantioselectivity. It was thought that lithium ion likely interferes with the requisite selector-analyte hydrogen bonds needed for effective chiral recognition. In the experiment discussed in this chapter, the conjugate bases of various DNB-amino acids were used as chiral selectors, effectively removing the

additional complication. In this arrangement the hydrogen bond accepting ability of the carboxylate of the DNB-amino acid should not be adversely affected as compared to the corresponding DNB-amino acid CSP. Using the carboxylate also allows ready detection of the selectors, and any complexes thereof, in the negative ion mode. Still, mass-labeled *pseudo*-enantiomeric chiral selectors were used so that the selector-analyte complexes can be differentiated in the mass spectrum. In order to accomplish this, mixtures of two different DNB-amino acids were used as the chiral selectors, where the two selectors have opposite absolute stereochemistry, therefore opposite affinity for the analyte enantiomers.

3.2 EXPERIMENTAL

Synthesis

The syntheses of selectors and analyte are presented in Chapter 2.

Preparation of solutions

The same stock solutions of analyte-**4** from Chapter 2 were used in this experiment. Equal amount of (*S*)-**10** and (*R*)-**11** (0.2 mmol each) were combined and dissolved in 10 mL methanol to afford the stock solution of *pseudo*-enantiomeric selectors (20 mM each). Similarly, stock solutions of (*S*)-**10** / (*R*)-**12**, and (*R*)-**11** / (*S*)-**12** were prepared. The stock solution of sodium hydroxide was prepared by dissolving 5 mmol sodium hydroxide in 50 mL water. Metered amounts of stock solutions of the chiral selectors, the analyte, and sodium hydroxide were combined and diluted to afford final concentration

of 5.0 mM for chiral selectors, 5.0 mM for analyte **4**, and 10 mM for sodium hydroxide in methanol / water (1 : 1).

HPLC

See Chapter 2.

Mass spectrometry

The ESI-MS was set up to run in the negative ion mode for all solutions. Solutions were flow injected into the ESI source through a 10-μL injection loop with a mobile phase of methanol / water (1 : 1) at a flow rate of 0.2 mL / min. ESI-MS conditions are: capillary voltage, 2.0 kV; cone voltage, 17 V; extractor voltage, 1.0 V; RF lens, 0.5 V; source temperature, 80^0C; desolvation temperature, 350^0C; cone gas flow, 70 L/h; desolvation gas flow, 757 L/h. Data from three separate injections were averaged for the calibration data.

3.3 RESULTS AND DISCUSSION

The chiral selectors and analyte used in this chapter are soluble analogues of CSPs **1**, **2**, **3**, and **5**. (Figure 3-1).[122, 123, 125, 126] Three pairwise combinations of DNB-amino acids, (*S*)-**10** / (*R*)-**11**; (*S*)-**10** / (*R*)-**12**, and (*R*)-**11** / (*S*)-**12** were used as *pseudo*-enantiomeric chiral selectors. The analyte, **4**, was from Chapter 2.

3.3.1 Chiral recognition using (S)-10 and (R)-11 as chiral selectors

A mixture of the DNB-valine **10** and DNB-leucine **11** was used as the chiral selectors. Solutions that were nominally 5 mM in both (*S*)-**10** and (*R*)-**11**, 10 mM in sodium hydroxide and 5 mM in analyte **4** were prepared. The enantiomeric composition of analyte **4** was varied. A one-to-one mixture of methanol / water was used as solvent throughout. The solution was flow injected through a 10 μL loop, and swept into the electrospray unit with mobile solvent (1 : 1 methanol / water) at a flow rate of 200 μL / min. The scan time for a full negative-ion spectrum was set to be 0.7 s. After injection of the sample, the total ion count chromatogram was recorded and all spectra were averaged together for which there was a significant ion count observed (Figure 3-2). Typically, the total ion count would return to baseline shortly after injection (10~20 s).

Figure 3-3 presents the mass spectrum of one of these solutions, where analyte **4** is racemic. The monomeric anions are observed at *m/z* 301 [**4**-H]$^-$, 310 [**10**-H]$^-$, and 324 [**11**-H]$^-$. The homodimeric anions are observed at *m/z* 621 [**10**$_2$-H]$^-$, 643 [**10**$_2$+Na–2H]$^-$, 649 [**11**$_2$–H]$^-$, and 671 [**11**$_2$+Na–2H]$^-$, while the hetero-dimeric anions are observed at *m/z* 612 [**4**+**10**–H]$^-$, 626 [**4**+11–H]$^-$, 635 [**10**+**11**–H]$^-$, and 657 [**10**+**11**+Na–2H]$^-$.

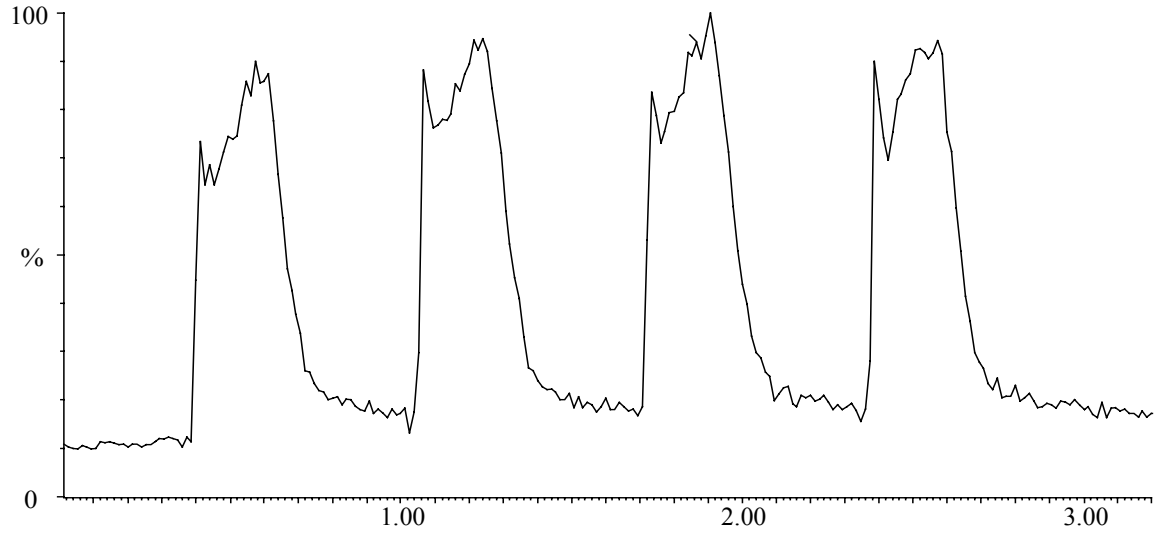


Figure 3 - 2. Total ion count chromatogram of four consecutive injections.

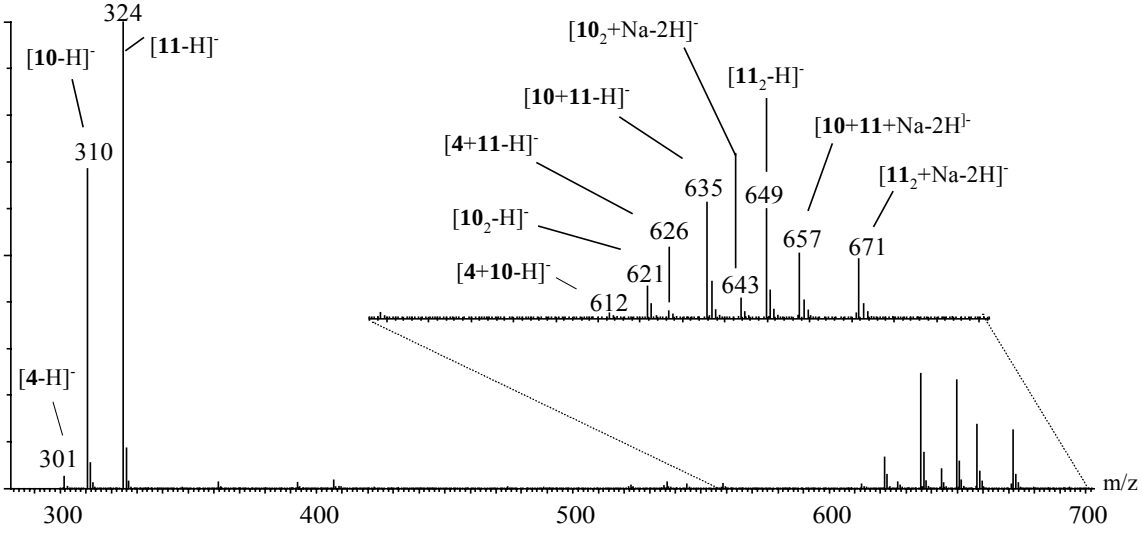


Figure 3 - 3. Mass spectrum of a solution of *pseudo*-enantiomeric chiral selectors (*S*)-**10** and (*R*)-**11** (5.0 mM each) and racemic analyte **4** (5.0 mM) with added sodium hydroxide (10 mM) in methanol / water (1 : 1).

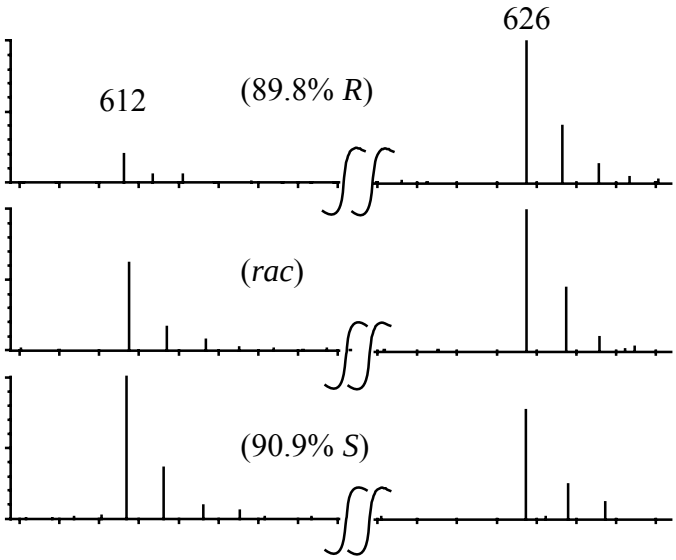


Figure 3 - 4. Partial mass spectra of *pseudo*-enantiomeric selectors (*S*)-**10** and (*R*)-**11** (5.0 mM each) and analyte **4** (5.0 mM) with added sodium hydroxide (10 mM) in methanol / water (1 : 1) (omitting the larger intensity homo-dimeric ion at *m/z* 621 for clarity).

The relative peak intensities of the selector-analyte complex ions, *m/z* 612, and 626, are much smaller than other ions (i.e. monomer and dimmer ions of **10**, **11**) in the mass spectrum. It is, however, the relative intensity of the ions that is of importance, and this can readily be determined. The partial portions of the mass spectra were presented in figure 3-4. For clarity, only the selector-analyte complex ions, for solutions highly enriched in both enantiomers of analyte **4**, as well as the racemate were showed. As is shown in the spectra, the relative intensities of the selector-analyte peaks in the mass spectrum are varying regularly with the enantiomeric composition of analyte-**4** in solution. When the sample is enriched in the (*R*)-enantiomer of analyte **4**, higher peak intensity is observed for the complex between the (*R*)-selector and the analyte (*m/z* 626),

and when enriched with the (*S*)-enantiomer of analyte **4**, higher peak intensity is observed for the complex between the (*S*)-selector and the analyte (*m/z* 612).

3.3.2 Chiral recognition using (S)-10 and (R)-12 as chiral selectors

The one to one mixture of the DNB derivatives of valine, (*S*)-**10** and phenylglycine, (*R*)-**12** is another pair of *pseudo*-enantiomeric chiral selectors used in this study. Figure 3-5 presents the mass spectrum of the solution containing racemic analyte-**4**. The monomeric anions are observed at *m/z* 301 [**4**-H]$^-$, 310 [**10**-H]$^-$, and 344 [**12**-H]$^-$. The homo-dimeric anions are observed at *m/z* 621 [$\mathbf{10}_2$-H]$^-$, 643 [$\mathbf{10}_2$+Na–2H]$^-$, 689 [$\mathbf{12}_2$–H]$^-$, and 711 [$\mathbf{12}_2$+Na–2H]$^-$, while the hetero-dimeric anions are observed at *m/z* 612 [**4**+**10**–H]$^-$, 646 [**4**+**12**–H]$^-$, 655 [**10**+**12**–H]$^-$, and 677 [**10**+**12**+Na–2H]$^-$.

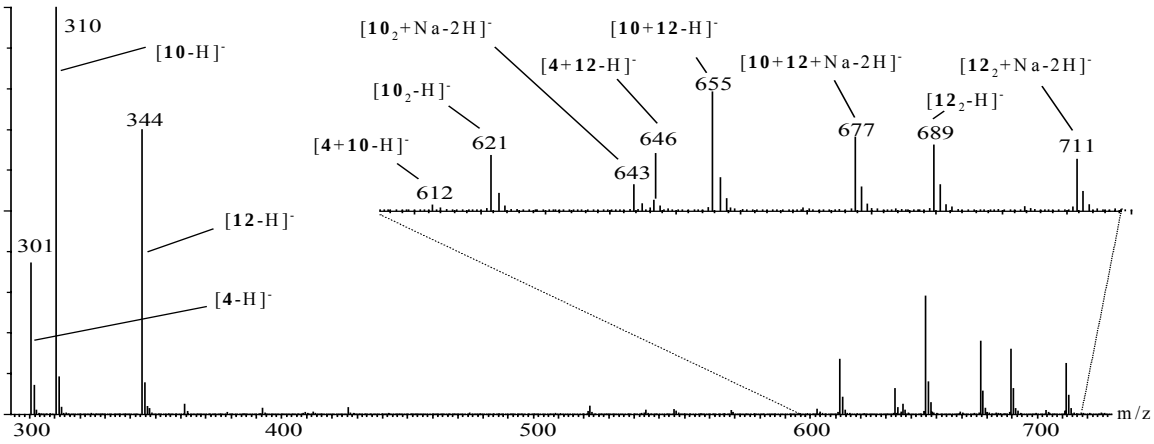


Figure 3 - 5. Mass spectrum of a solution of *pseudo*-enantiomeric chiral selectors (*S*)-**10** and (*R*)-**12** (5.0 mM each) and racemic analyte **4** (5.0 mM) with added sodium hydroxide (10 mM) in methanol / water (1 : 1).

The relevant portions of the negative ion mass spectra containing the selector-analyte complex ions are shown in Figure 3-6 (omitting the larger intensities homo-dimeric

anions [**10**$_2$–H] at *m/z* 621 and [**10**$_2$+Na–2H]$^-$ at *m/z* 643 for clarity). Apparently, the relative peak intensities reflect the enantiomeric composition of analyte **4** in solution. For the solution containing enriched (*S*)-**4**, the peak at *m/z* 612 is higher (compared to the peak at *m/z* 646), which represents the complex (*S*)-**6** + **4**; while for the solution containing enriched (*R*)-**4**, the peak at *m/z* 646 is higher (compared to the peak at *m/z* 612), which represents the complex (*R*)-**12** + **4**,

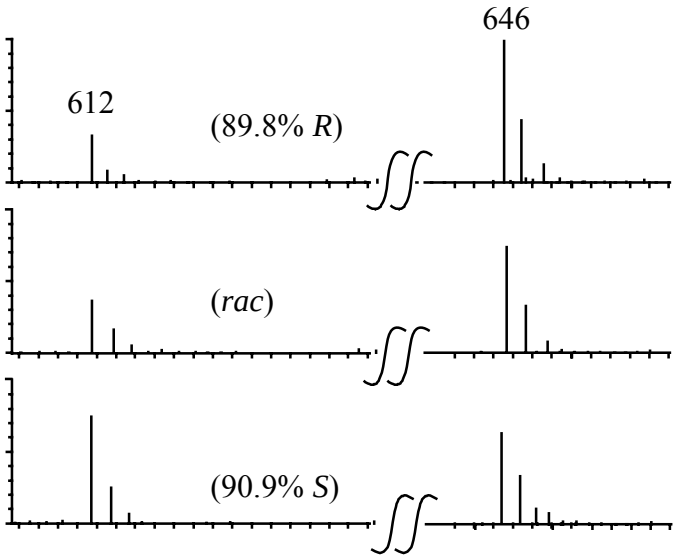


Figure 3 - 6. Partial mass spectra of *pseudo*-enantiomeric selectors (*S*)-**10** and (*R*)-**12** (5.0 mM each) and analyte **4** (5.0 mM) with added sodium hydroxide (10 mM) in methanol / water (1 : 1) (omitting the larger intensity homo-dimeric ions at *m/z* 621, 643 for clarity).

3.3.3 Chiral recognition using (R)-11 and (S)-12 as chiral selectors

Finally, the third pairwise combination of the three chiral selectors, a one to one mixture of the DNB-derivatives of leucine, (*R*)-**11**, and phenylglycine (*S*)-**12**, were used as the chiral selectors. Figure 3-7 presents the full spectrum for the solution containing

the chiral selectors (*R*)-**11**, and (*S*)-**12**, and the racemic analyte **4**. The monomeric anions are observed at *m/z* 301 [**4**-H]$^-$, 324 [**11**-H]$^-$, and 344 [**12**-H]$^-$. The homo-dimeric anions are observed at *m/z* 649 [$\mathbf{11}_2$-H]$^-$, 671 [$\mathbf{11}_2$+Na–2H]$^-$, 689 [$\mathbf{12}_2$–H]$^-$, and 711 [$\mathbf{12}_2$+Na–2H]$^-$, while the hetero-dimeric anions are observed at *m/z* 626 [**4**+**11**–H]$^-$, 646 [**4**+**12**–H]$^-$, 669 [**11**+**12**–H]$^-$, and 691 [**11**+**12**+Na–2H]$^-$.

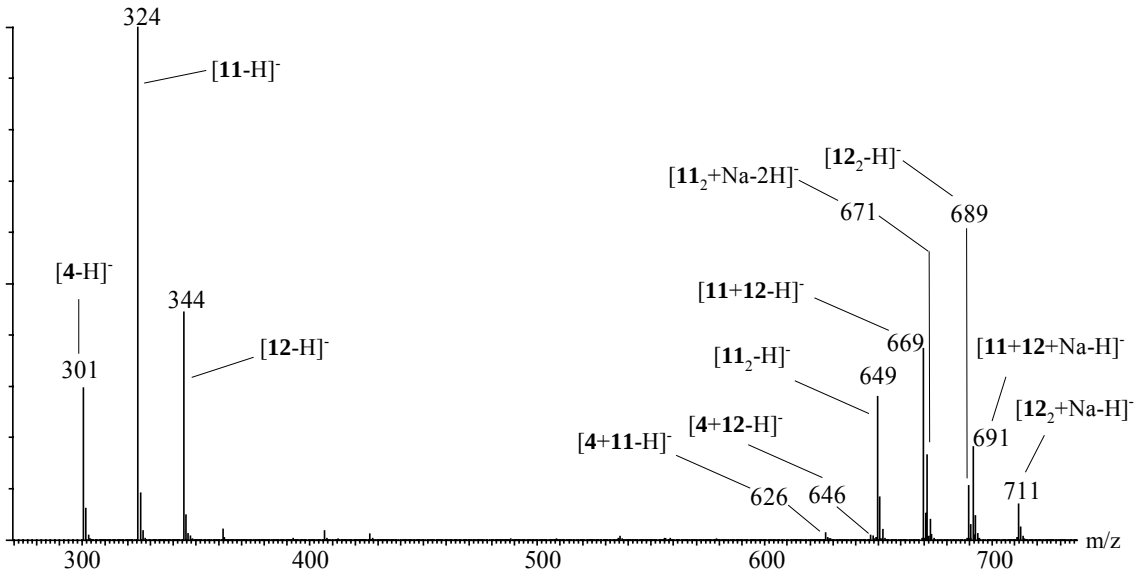


Figure 3 - 7. Mass spectrum of a solution of selectors (*R*)-**11** and (*S*)-**12** (5.0 mM each) and racemic analyte **4** (5.0 mM) with NaOH (10 mM) in methanol / water (1 : 1).

Figure 3-8 presents the relevant portions of the negative ion mass spectra containing the selector-analyte complexation ions. When the sample is enriched in the (*R*)-enantiomer of analyte **4**, the relative intensity of the complex between (*R*)-**11** and analyte-**4** (*m/z* 626) in the mass spectrum is greater (with respect to the peak at *m/z* 646), and when the (*S*)-enantiomer enriched, the relative intensity of the complex between (*S*)-**12** and analyte-**4** (*m/z* 646) is increased (with respect to the peak at *m/z* 626).

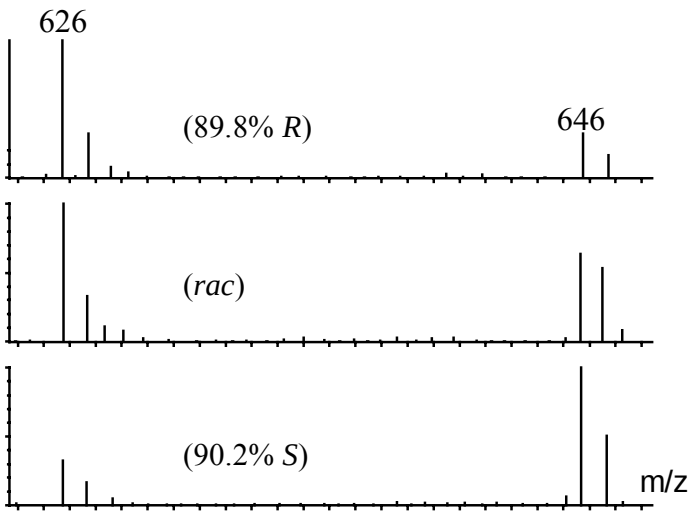


Figure 3 - 8. Partial mass spectra of *pseudo*-enantiomeric selectors (*R*)-**11** and (*S*)-**12** (5.0 mM each) and analyte **4** (5.0 mM) with NaOH (10 mM) in methanol / water (1 : 1).

3.3.4 Enantioselectivities

In each case, it is obvious that chiral recognition between the DNB-amino acid chiral selectors and analyte **4** is being observed in the negative-ion ESI mass spectrum. In addition, the sense of chiral recognition is consistent with what is observed chromatographically. In every case, as the amount of the (*R*)-enantiomer of analyte **4** is increased in the sample, the relative intensity of the complex ion between **4** and the (*R*)-selector is increased. Likewise, an increase in the amount of the (*S*)-**4** affords an increase in the relative intensity of the complex ion between **4** and the (*S*)-selector. This is consistent with the homochiral [*i.e.*, (*S*)-selector + (*S*)-analyte, or (*R*)-selector + (*R*)-analyte] diastereomeric complex being the more stable, which is what is predicted from the chromatographic elution order of analyte **4** on the DNB-amino acid derived CSPs.

For each set of data, a plot of the CIF versus the mole fraction of (*R*)-**4** is given in Figure 3-9. In all cases the plot is linear with a high correlation coefficient [0.998 for (*S*)-**10** / (*R*)-**11**, 0.993 for (*S*)-**10** / (*R*)-**12**, and 0.997 for (*R*)-**11** / (*S*)-**12**), respectively. According to equation 2-7, the α_{MS} for analyte **4** obtained using chiral selectors (*S*)-**10** / (*R*)-**11** is 3.01; and with chiral selectors (*S*)-**10** / (*R*)-**12** it is 2.55; and with chiral selectors (*R*)-**11** / (*S*)-**12**) it is 3.51. As the substituent at α-position of the DNB-amino acid differs, the extent of the enantioselectivity chiral selector might be different. The α_{MS} can be considered to be the average value of the two chiral selectors. At this point, it is not hard to find the α_{MS} value for each chiral selector. If α_{val}, α_{leu}, and α_{pg} are used to represent α_{MS} for selectors **10**, **11**, and **12,** respectively, three equations can be written as below:

$$(\alpha_{val} + \alpha_{leu})/2 = 3.01 \quad .....eq.3-1$$
$$(\alpha_{val} + \alpha_{pg})/2 = 2.55 \quad .....eq.3-2$$
$$(\alpha_{leu} + \alpha_{pg})/2 = 3.51 \quad .....eq.3-3$$

Solving these equations affords the values as: $\alpha_{val} = 2.05$, $\alpha_{leu} = 3.97$, and $\alpha_{pg} = 3.03$. These values are in good agreement with the selectivities obtained chromatographically, where analyte **4** affords a separation factor (α_{HPLC}) of 3.41 on (*S*)-CSP **2**, and a separation factor of 3.74 on (*R*)-CSP **3**. The enantioselectivities are improved significantly as compared to the data shown in Chapter 2. As was expected, removal of lithium ion increased the observed enantioselectivity greatly

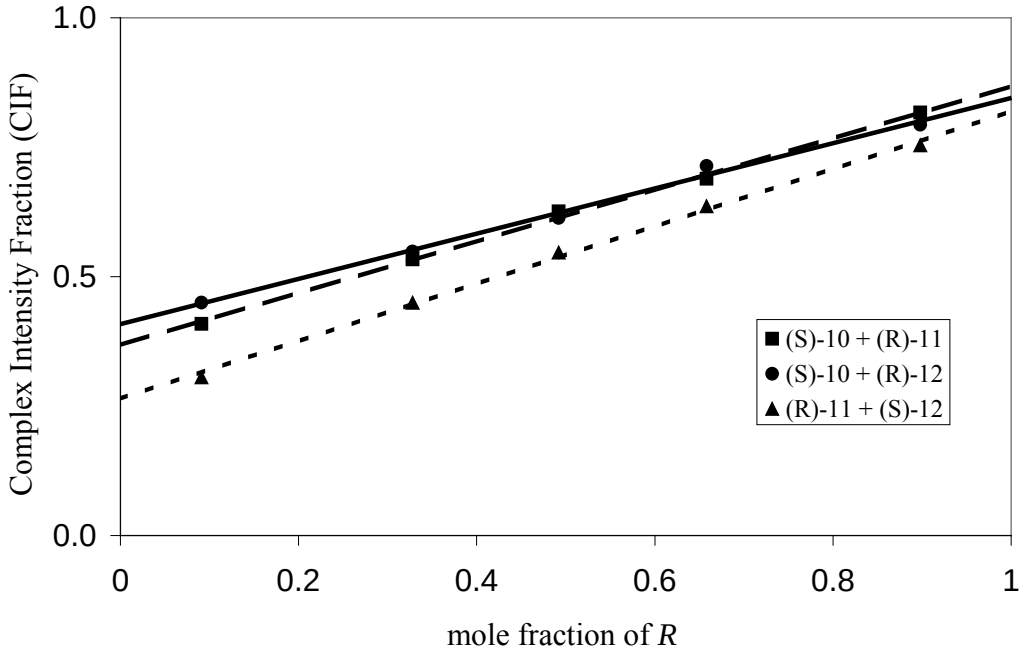


Figure 3 - 9. Plot of the complex intensity fraction (CIF) for the selector-analyte complexes in the ESI-MS *vs* the mole fraction of (*R*)-**4** in the solution, using *pseudo*-enantiomeric chiral selectors (*S*)-**10** and (*R*)-**11**; (*S*)-**10** and (*R*)-**12**; and (*R*)-**11** and (*S*)-**12**.

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3.3.5 Quantitative determination of enantiomeric composition

Similarly to the methods adopted in Chapter 2, the data were further analyzed using leave-one-out technique. The results of this analysis for all samples are shown in Table 3-1. The enantiomeric composition determinations by MS are in good agreement with the values obtained by chiral chromatography. Using chiral selectors (*S*)-**10** and (*R*)-**11**, the average absolute difference between these measurements is 2.0 with a standard deviation of 1.4; with (*S*)-**10** and (*R*)-**12**, the average absolute difference is 3.5 with a standard deviation of 2.7; and with (*R*)-**11** and (*S*)-**12**, the average absolute difference is 2.7 with a standard deviation of 1.7.

Table 3 - 1. Calculated % (*R*)-**4** by mass spectrometry according to leave-one-out method compared to the enantiomeric composition measured by chiral chromatography.

% (*R*)-**4** by HPLC	% (*R*)-**4** by MS		
	Chiral selectors (*S*)-**10** and (*R*)-**11**	Chiral selectors (*S*)-**10** and (*R*)-**12**	Chiral selectors (*R*)-**11** and (*S*)-**12**
89.8	89.4	83.1	85.2
65.8	63.3	71.6	67.4
49.2	52.5	48.5	51.2
32.8	33.5	33.8	33.6
9.1	6.0	5.6	4.6

Three "unknown" samples were also made and tested to determine the enantiomeric composition by this MS method. In order to assure that the ratio of the chiral selectors remained constant, stock solutions for each pair of chiral selectors were used throughout. The required amount of analyte was added to an aliquot of each stock solution, along with a metered amount of sodium hydroxide, in order to prepare the solution for mass spectral analysis. Acquisition of the mass spectrum, followed by application of the observed CIF of the selector-analyte complex ions to the calibration curve readily affords a measurement of the enantiomeric composition of the analyte. Nine replicate samples were analyzed at three different enantiomeric compositions of (*R*)-**4** and applied to the calibration curve for each set of chiral selectors (Table 3-2). While the values obtained by MS are in good agreement with the values obtained by HPLC, the precision of each measurement is lower than what is obtained by HPLC, and even worse than the enantiomer analysis performed in the positive MS as shown in Chapter 2, which is most likely due to the low absolute intensities of the selector-analyte complexes in each of the mass spectra. However, in many applications, such as screening asymmetric combinatorial catalysts, one would be willing to trade precision for analysis time.

Samples that afford a large enantiomeric excess could then be re-analyzed using slower and more accurate methods.

Table 3 - 2. Enantiomeric composition of analyte **4** measured by mass spectrometry (with the 95% confidence level) compared to the enantiomeric composition as measured by chiral chromatography.

% (*R*)-**4** by HPLC	Average % (*R*)-**4** by MS		
	Chiral selectors (*S*)-**10** and (*R*)-**11**	Chiral selectors (*S*)-**10** and (*R*)-**12**	Chiral selectors (*R*)-**11** and (*S*)-**12**
89.8	90.3±2.4	93.1±3.6	88.9±1.0
49.2	61.8±3.4	56.6±3.0	49.7±4.3
9.1	7.8±3.4	11.8±3.5	9.3±3.6

3.4 SUMMARY

The use of the conjugate base of DNB-amino acids as chiral selectors for the determination of enantiomeric composition by ESI-MS is demonstrated. Solutions containing two different DNB-amino acids, sodium hydroxide, and analyte afforded deprotonated selector-analyte complexes in the negative ion electrospray ionization mass spectrometry. The relative peak intensities can be related back to the enantiomeric composition of the analyte. Quantitative determination of enantiomeric composition also was performed with each pairwise of chiral selectors. The use of deprotonated DNB-amino acids afforded much larger selectivities than has previously been observed using this method, though the precision is decreased. Additionally, preparation of amide derivatives of DNB-amino acids was avoided by directly using DNB-amino acids as the chiral selectors in the negative-ion ESI mass spectrometry.

CHAPTER IV

ENANTIOMER ANALYSIS USING TERTIARY AMINE APPENDED DERIVATIVES OF 3,5-DINITROBENZOYL-LEUCINE AS CHIRAL SELECTORS

4.1 INTRODUCTION

In the previous chapters, he use of soluble analogues of Pirkle-type CSPs as chiral selectors for enantiomer analysis by ESI-mass spectrometry has been demonstrated. In each case, a solution of *pseudo*-enantiomeric chiral selectors, when mixed with a chiral analyte whose enantiomers are separable on the corresponding CSP, was shown to afford selector-analyte complexes in the mass spectrum. The relative peak intensities of the complexes are dependent on the enantiomeric composition of the analyte.

For example, mass-labeled amide derivatives of DNB-leucine, (*S*)-**6** / (*R*)-**7,** when mixed with analyte **4** and excess lithium chloride afforded two peaks in the mass spectrum that corresponding to the following complexes: $[\mathbf{4}+\mathbf{6}+Li]^+$ and $[\mathbf{4}+\mathbf{7}+Li]^+$. It was found that a plot of the CIF in the mass spectrum versus the enantiomeric composition of the analyte is linear. Similar results were obtained using amide derivatives of DNB-phenylglycine, (*R*)-**8** / (*S*)-**9** as the *pseudo*-enantiomeric chiral selectors. In both cases, the enantioselectivities were rather low, compared to the enantioselectivity obtained by HPLC using either of the *N*-(3,5-dinitrobenzoyl)amino

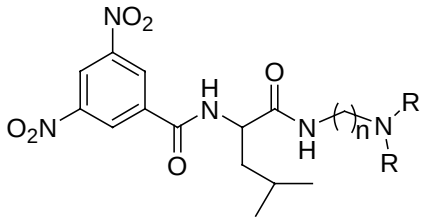
acid CSPs. It was presumed that the diminished enantioselectivity is due to interference of the requisite selector-analyte hydrogen bonds needed for effective chiral recognition by the lithium cations.

Chiral recognition was also observed when using mixtures of deprotonated *N*-(3,5-dinitrobenzoyl) amino acids (binary combinations of **10**, **11**, and **12**) as chiral selectors. In these cases, the enantioselectivities were comparable to the separation factors obtained on the corresponding CSPs, though the abundances of selector-analyte complexes were much lower compared to the rest peaks in the mass spectrum.

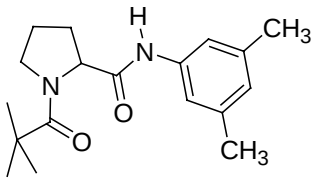
The determination of enantiomeric composition of the analyte was done using calibration curves. In both instances, the reproducibility was found to be ~ ±0.05 for the calculated mole fraction of either enantiomer. In the first instance, with Li^+ as additive for ionization, the poor reproducibility was considered primarily to be a low selectivity where small differences in measured relative peak intensities will result in significant change in the calculated mole fraction of analyte. In the second instance, with the conjugate bases of DNB-amino acids as chiral selectors, the low reproducibility was considered to be a small absolute value of the intensities of the selector-analyte complexes in the ESI-mass spectrum. In fact, the ion counts were ~2 orders of magnitude less for the complexes derived from the deprotonated selectors compared to the lithiated selectors. The variability was therefore a manifestation of a decreased signal with respect to experimental noise.

In this Chapter, the use of tertiary amine appended derivatives of *N*-(3,5-dinitrobenzoyl) leucine, **13** and **14** as *pseudo*-enantiomeric chiral selectors is discussed. These chiral selectors were designed to overcome the problems shown above, via a

separation of the requisite chiral recognition sites from the ionization site. The tertiary amine group is appended to DNB-leucine through an alkyl chain. Addition of an acid to the solution to be assayed should protonate the tertiary amine, affording high ion counts for the selector in the electrospray ionization mass spectrum. Since the ionization site is removed from the sites needed for chiral recognition, its interference with the formation of selector-analyte complexes should be minimized. In order for this method to be a viable analytical method, it would be useful to measure the enantiomeric composition of the analyte at a variety of concentrations, even without knowing the analyte's concentration. Also, it will be helpful to use differing solvent compositions to accommodate samples of many types. Additionally, the scope and limitations of this new chiral selector system will be discussed, including the effect of analyte concentration and a survey of solvent effects, using protonated *pseudo*-enantiomeric chiral selectors, (*S*)-**13** and (*R*)-**14**, for enantiomeric analysis by electrospray ionization mass spectrometry.



(*S*)-**13**: n = 2, R = Et
(*R*)-**14**: n = 3, R = Et



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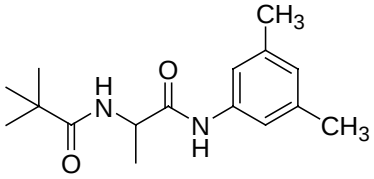

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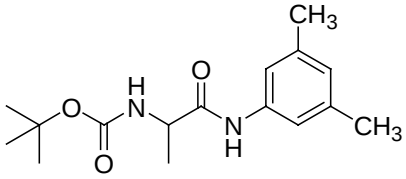

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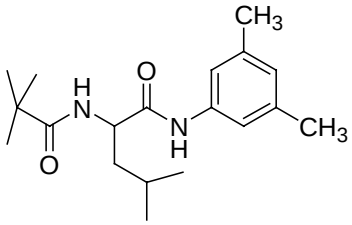
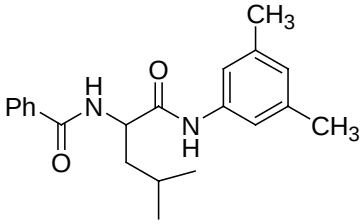
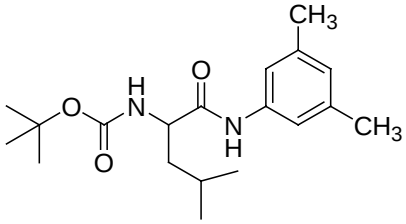


17



18



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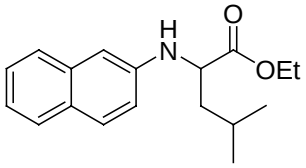

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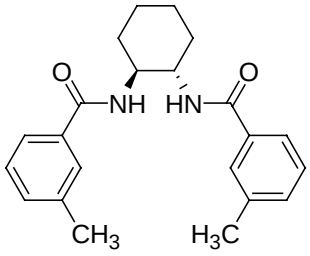

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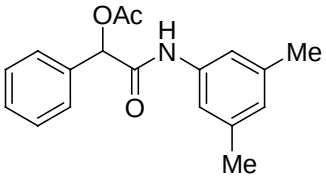

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Figure 4 - 1. Structures of Selectors and Analytes.

4.2 EXPERIMENTAL

General procedure for the synthesis of (*S*)-***13*** *and* (*R*)-***14***

To an oven dried round bottom flask was added *N*-(3,5-dinitrobenzoyl)leucine (2 mmol) and *N*-hydroxysuccinimide (2mmol) under a nitrogen atmosphere. THF (10mL) was then added, and the flask was cooled in an ice-water bath. Di-isopropylcarbodiimide (2 mmol) was added slowly via syringe. After the addition, the reaction was allowed to stir for 2.5 hours, after which the reaction mixture was filtered to remove the precipitated urea by-product. Removal of the solvent *in vacuo* afforded a pale yellow solid (the succinimide ester). This solid was then dissolved in acetonitrile (10 mL), cooled in an ice-water bath, and added slowly via syringe to a solution of the appropriate *N*,*N*-diethyl-α,ω-diamine (4 mmol) dissolved in acetonitrile (10 mL) at 0 °C (the succinimide ester of (*S*)-*N*-(3,5-dinitrobenzoyl)leucine was added to *N*,*N*-diethyl-1,2-diaminoethane, and the (*R*)-enantiomer was added to *N*,*N*-diethyl-1,3-diaminopropane). After stirring for twenty minutes, the solvent was evaporated under reduced pressure. Purification by column chromatography, eluting with 20% methanol / 80% ethylacetate, provided the product.

(*S*)-**13**: yellow solid, 31% yield. m.p. 141-143 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.14 (t, *J* = 2.0 Hz, 1H), 8.97 (d, *J* = 2.0 Hz, 2H), 7.91 (d, *J* = 6.1 Hz, 1H), 6.68 (m, 1H), 4.68 (m, 1H), 3.36 (m, 2H), 2.57 (m, 6H), 1.75 (m, 3H,), 1.04 (m, 6H), 1.00 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.1, 162.5, 148.4, 137.2, 127.8, 53.5, 51.9, 47.2, 41.3, 35.9, 25.0, 22.9, 21.7, 9.9. ESI-MS: *m/z,* 424 $[M+H]^+$.

(*R*)-**14**: brown oil, 42% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.23 (d, *J* = 2.0 Hz, 2H), 9.06 (t, *J* = 1.9 Hz, 1H), 8.53 (d, *J* = 6.0 Hz, 1H), 8.31 (m, 1H), 4.73 (m, 1H), 3.54

(m, 1H), 3.34 (m, 1H), 3.11 (m, 6H), 2.08 (m, 3H), 1.82 (m, 2H), 1.38 (m, 6H), 0.98 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.5, 162.8, 148.0, 137.1, 128.1, 120.5, 54.0, 49.4, 46.4, 40.5, 36.2, 24.9, 23.4, 22.8, 21.3, 8.4. ESI-MS: *m/z,* 438 $[M+H]^+$.

General procedure for the synthesis of the 3,5-dimethylanilides

1 mmol of the chiral acid and 1 mmol of EEDQ were dissolved in dichloromethane in an oven dried round bottom flask under a nitrogen atmosphere. The solution was cooled in an ice-water bath, followed by addition of 1.5 mmol of 3,5-dimethylaniline via syringe. The reaction mixture was allowed to stir for two hours at 0 °C, diluted with ethyl acetate, washed with 2 M HCl, 5% $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$. Removal of the solvent by rotary evaporation afforded the product.

15: white solid, 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.42 (br.s, 1H), 7.31 (s, 2H), 6.83 (s, 1H), 6.75 (s, 1H), 4.87 (m, 1H), 2.28 (s, 6H), 1.51 (d, J = 6.6 Hz, 3H), 1.27 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.6, 170.9, 138.4, 137.9, 125.8, 117.8, 49.5, 38.7, 27.5, 21.2, 19.2. ESI-MS: *m/z,* 277 $[M+H]^+$.

16: white solid, 88% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.56 (br.s, 1H), 7.14 (s, 2H), 6.71 (s, 1H), 5.32 (m, 1H), 4.39 (m, 1H), 2.24 (s, 6H), 1.46 (s, 9H), 1.41 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.1, 156.0, 138.4, 137.6, 125.9, 117.6, 80.4, 50.9, 28.3, 21.2, 18.0. ESI-MS: *m/z,* 293 $[M+H]^+$.

17: white solid, 78% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.07 (br.s, 1H), 7.13 (s, 2H), 6.68 (s, 1H), 6.39 (d, J = 7.7 Hz, 1H), 4.72 (m, 1H), 2.21 (s, 6H), 1.72 (m, 3H), 1.21 (s, 9H), 0.95 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 179.2, 170.7, 138.4, 137.8, 125.8, 117.7, 52.6, 40.8, 38.7, 27.4, 24.9, 22.9, 22.3, 21.2. ESI-MS: *m/z*, 319 $[M+H]^+$.

18: white solid, 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.15 (br.s, 1H), 7.80 (d, *J* = 7.7 Hz, 2H), 7.49 (t, *J* = 7.0 Hz, 1H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.14 (m, 3H), 6.68 (s, 1H), 5.06 (m, 1H), 2.17 (s, 6H), 1.87 (m, 3H), 1.00 (m, 6H). 13C NMR (75 MHz, $CDCl_3$): δ 170.4, 167.9, 138.5, 137.7, 133.7, 131.8, 128.6, 127.1, 126.0, 117.8, 53.1, 41.2, 25.0, 22.9, 22.5, 21.2. ESIMS: *m/z,* 339 $[M+H]^+$.

19: white solid, 56% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.51 (br.s, 1H), 7.14 (s, 2H), 6.70 (s, 1H), 5.14 (m, 1H), 4.35 (m, 1H), 2.24 (s, 6H), 1.74 (m, 3H), 1.45 (s, 9H), 0.95 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.9, 156.2, 138.5, 137.7, 125.9, 117.6, 41.0, 28.3, 24.8, 22.9, 21.2, 22.0. ESI-MS: *m/z,* 335 $[M+H]^+$.

22: brown oil, 91% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.80 (br.s, 1H), 7.49 (m, 2H), 7.38 (m, 3H), 7.16 (m, 2H), 6.76 (m, 1H), 6.17 (m, 1H), 2.27 (s, 6H), 2.24 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.1, 166.2, 138.8, 136.7, 135.3, 128.8, 127.4, 126.6, 117.7. ESI-MS: *m/z,* 298 $[M+H]^+$.

Analytes **20**[127], **21**[128] were made following the published procedure.

20: ESI-MS, *m/z*, 246 $[M+H]^+$.

21: ESI-MS, *m/z*, 351 $[M+H]^+$.

HPLC

Accurate determination of enantiomeric composition of all analytes was performed on (*S, S*)-Whelk-O1 chiral column. Mobile phase: hexanes / 2-propanol / methanol (60:38:2) at 2 mL / min. The capacity factor and separation factor values for all analytes were calculated according to the separation results obtained on (*S*)-CSP **2** with hexane / 2-propanol (90 : 10) as mobile phase at 1.2 ml / min.

Preparation of solutions

Solutions of analyte **4** were prepared in methanol in the same way as that presented in Chapter 2. An equal amount of (*S*)-**13** and (*R*)-**14** (0.05 mmol each) were combined and dissolved in 10 mL methanol to afford the stock solution of *pseudo*-enantiomeric selectors (5 mM for each). The stock solution of ammonium chloride (100 mM) was made in water. End solutions were made by combining the metered amount of the corresponding stock solution of analyte, the stock solution of *pseudo*-enantiomeric selectors, and the stock solution of ammonium chloride, and diluting with appropriate solvents to afford a final concentration needed for every specific use.

Likewise, solutions for all other analytes were made in the same manner, but only three solutions for each analyte were made for the construction of the plot.

Mass spectrometry

Solutions were flow injected into the electrospray ionization source through a 10-μL injection loop with mobile phase running at a flow rate of 200 μL/min. The full positive ion spectrum was recorded every 0.7 s. All scans for which a significant total ion count was observed, were averaged together to afford the final spectrum. Spectrometer conditions are as follows: capillary, 3.0 V; cone voltage, 12 V; extractor voltage, 1.0 V; RF lens, 0.5 V; source temperature, 80 ^{0}C; desolvation temperature, 325 ^{0}C; cone gas flow, 62 L / h; desolvation gas flow, 608 L / h. For data collected by direct infusion with a syringe pump, scans collected over a 2-min period were averaged together to afford the final spectrum.

4.3 RESULTS AND DISCUSSION

Chiral recognition

The chiral selectors **13** and **14** were designed to (1) remove the ionization site from the sites required for chiral recognition and (2) act as "enantiomers" where both can be detected in a single mass spectrometric experiment (Figure 4-1). Chiral analytes similar to *N*-pivaloyl-2-(3,5-dimethylanilide)proline, **4**, whose enantiomers are separable on the N-(3,5-dinitrobenzoyl)leucine CSP, **5**, were chosen for this study. These are analytes **15**~**22** (Figure 4-1). The tertiary amine appended side chain is considered to do neither detriment nor benefit to the chiral recognition, but is indispensable for ready ionization of the selector-analyte complexes derived thereof via protonation.

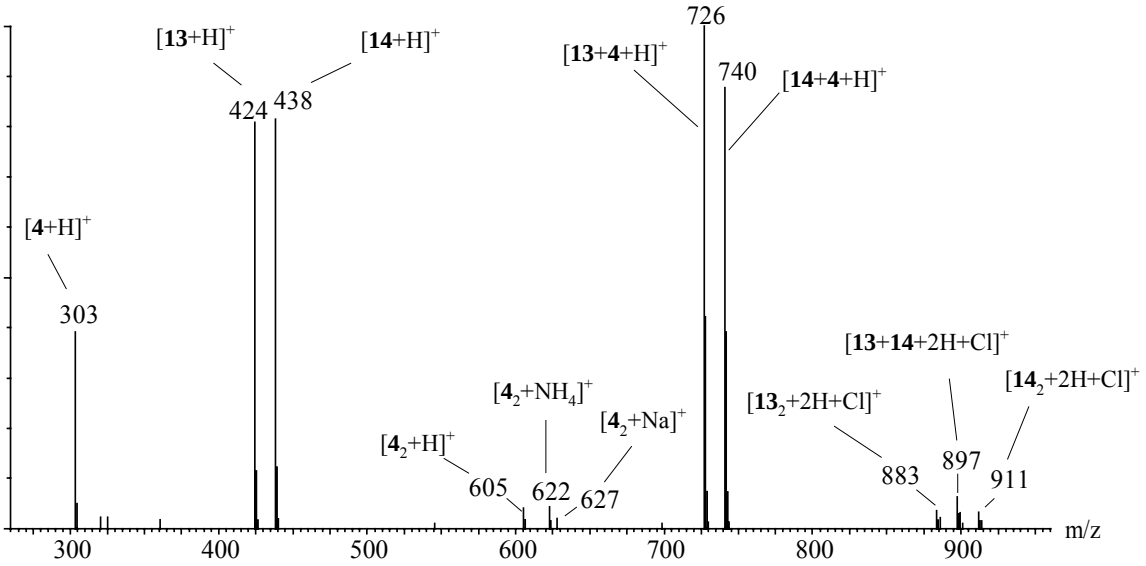


Figure 4 - 2. Mass spectrum of a solution of *pseudo*-enantiomeric selectors **13** and **14** (1.0 mM) and analyte **4** (1.0 mM) with added ammonium chloride (10 mM) in methanol / water (1 : 1).

The difference between selectors **13** and **14** arises from (1) the configuration of chiral center ("*S*" for **13** and "*R*" for **14**); (2) the length of side chain (two carbons for **13** and three carbons for **14**). A one to one mixture of (*S*)-**13** and (*R*)-**14** in solvent affords a pair of *pseudo*-enantiomeric chiral selectors, which present different affinity to enantiomers of the analyte. Electrospray ionization mass spectrometry of solutions containing the chiral selectors, the analyte, and ammonium chloride in methanol / water provides substantial protonated selector-analyte complexes in the mass spectrum for each analyte. The samples were introduced into a 10 μL sample loop, and then swept into the electrospray unit with solvent (1:1 methanol / water) pumped at 200 μL / min. The full positive ion spectrum was recorded every 0.7 s. All scans for which a noticeable total ion count was observed were averaged together to afford the final spectrum.

Figure 4-2 shows the mass spectrum for the solution containing racemic analyte **4** and the chiral selectors, (*S*)-**13** / (*R*)-**14**. The protonated monomeric ions are observed at *m/z* 303 $[\mathbf{4}+H]^+$, 424 $[\mathbf{13}+H]^+$, and 438 $[\mathbf{14}+H]^+$, respectively; Analyte dimmers and selector dimers are observed at *m/z* 605 $[\mathbf{4}_2+H]^+$, 622 $[\mathbf{4}_2+NH_4]^+$, 627 $[\mathbf{4}_2+Na]^+$, 883 $[\mathbf{13}_2+2H+Cl]^+$, 897 $[\mathbf{13}+\mathbf{14}+2H+Cl]^+$ and 911 $[\mathbf{14}_2+2H+Cl]^+$; The selector-analyte-complexes, which, in this instance, are the largest peaks in the mass spectrum, are observed at *m/z* 726 $[\mathbf{13}+\mathbf{4}+H]^+$ and 740 $[\mathbf{14}+\mathbf{4}+H]^+$.

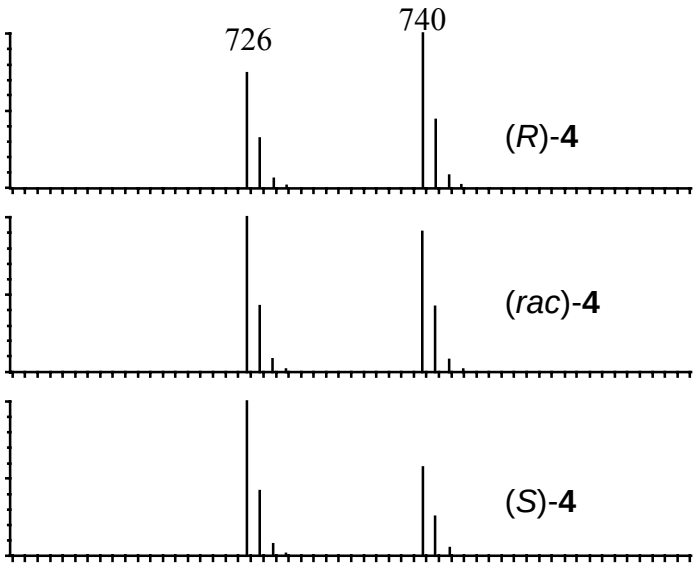


Figure 4 - 3. Partial mass spectra of *pseudo*-enantiomeric selectors selectors (*S*)-**13** and (*R*)-**14** (1.0 mM) and analyte **4** (1.0 mM) with added ammonium chloride (10 mM) in methanol / water (1 : 1). Spectrum: (a) 89.8% of (*R*)-**4**; (b) racemic; (c) 9.1% of (*R*)-**4**.

Figure 4-3 presents three partial spectra for the protonated selector-analyte complexes at *m/z* 726 and 740 obtained at enriched (*R*)-enantiomer, racemic, and enriched (*S*)-enantiomer of analyte **4**, respectively. The relative peak intensities of the selector-analyte complexes vary regularly with the enantiomeric composition of the analyte, clearly demonstrating chiral recognition. When the sample is enriched with (*R*)-**4**, the peak at *m/z* 740 for the complex between (*R*)-**14** and **4** is greater (compared to *m/z* 726); For the sample enriched with (*S*)-**4**, the peak at *m/z* 726 for the complex between (*S*)-**13** and **4** is greater (compared to *m/z* 740). The sense of the chiral recognition is consistent with what is observed on the corresponding (*S*)-CSP **2**, whereby (*S*)-enantiomer of analyte **4** is more retained than (*R*)-enantiomer of analyte **4**. Moreover, each of the remaining analytes, **15**-

22, is discriminated by selectors **13** and **14** with the ESI-MS, and the sense of chiral recognition is consistent with that of chromatographic chiral recognition in each case.

A plot of CIF *vs* the mole fraction of (*R*)-**4** is presented in Figure 4-4. The plot is linear with a slope of 0.261 and a correlation coefficient of 0.998. The enantioselectivity by mass spectrometry, α_{MS}, is calculated to be 1.71. This plot is useful for subsequent enantiomer analysis.

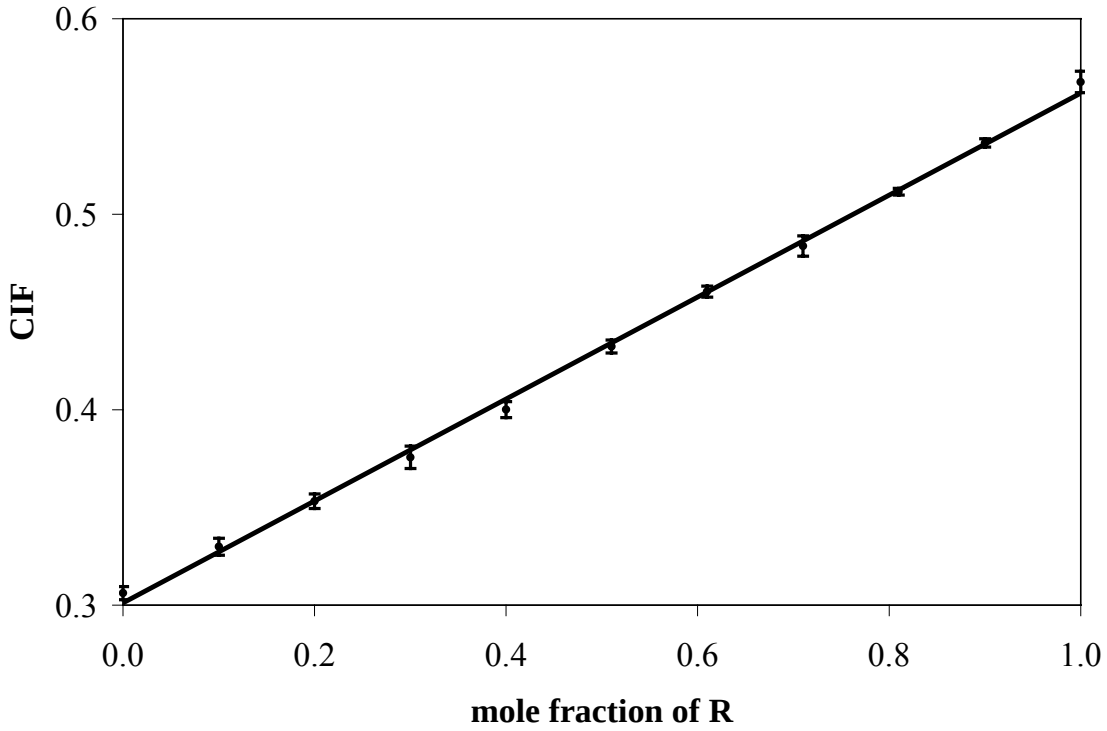


Figure 4 - 4. Plot of the complexes intensity fraction (CIF) of peaks at *m/z* 726 and 740 in the ESI-MS *vs* the mole fraction of (*R*)-**4** in the solution, using *pseudo*-enantiomeric chiral selectors **13** and **14** with added ammonium chloride (10 mM) in methonal/water. (slope = 0.261, intercept = 0.301, r^2 = 0.998).

Effects of selector / analyte concentration on enantioselectivity

In order for this method to be practicable for quantitative enantiomer assays, the results of any assay should be independent of the absolute concentrations of the analyte as much as possible. One can readily control the concentrations of the selectors and additives and the solvent composition by preparing a stock solution, where this same solution is used for the construction of the calibration curve and for subsequent enantiomer assays. Ideally, one would like to add a sample of the analyte to a small aliquot of this stock solution and record the electrospray ionization mass spectrum, without measuring the amount of analyte.

Table 4-1 shows the enantioselectivities obtained at a variety of different concentrations of analyte **4**. In each case, the concentrations of the chiral selectors, ammonium chloride, and solvent composition were kept constant. The α_{MS} values are relatively invariant as long as the chiral selectors are in excess of the analyte (entry 1-5). The α_{MS} values for the entries at a selector/analyte ratio of 20 : 1, 10 : 1, 5 : 1, and 1 : 1 are all within 5% of the average α_{MS} (1.67). As the concentration of the analyte is decreased, the intensity of the selector-analyte complexes in the mass spectrum also decreases, compared to the intensity of the monomeric ions. Since the measured enantioselectivity only depends on the relative amounts of the *pseudo*-diastereomeric complexes formed in solution, the ratio of the complexes should not change in the limit of low analyte concentration. In essence, each *pseudo*-enantiomer (via the *pseudo*-diastereomeric selector-analyte complexes) is acting as an internal standard for the other. Increasing the analyte concentration beyond the concentration of the chiral selectors results in saturation, where further increases in the analyte concentration will further

reduce the observed enantioselectivity. The onset of this diminution of enantioselectivity was observed at a 2 : 1 analyte/selector ratio for analyte **4**.

Table 4 - 1. Observed enantioselectivity as a function of the concentration of analyte **4** at a constant concentration of *pseudo*-enantiomeric chiral selectors *(S)*-**13** and *(R)*-**14**.

Entry	Ratio[a]	Concentration (mM)		α_{MS}[b] (σ)[c]
		(S)-**13**, *(R)*-**14**	**4**	
1	20:1	1	0.05	1.61 (0.03)
2	10:1	1	0.1	1.66 (0.04)
3	5:1	1	0.2	1.64 (0.02)
4	2:1	1	0.5	1.75 (0.02)
5	1:1	1	1	1.71 (0.01)
6	1:2	1	2	1.54 (0.02)
7	1:5	1	5	1.42 (0.02)
8	1:10	1	10	1.33 (0.02)

[a] Concentration of NH_4Cl is 10 mM in all cases.
[b] Observed MS enantioselectivity.
[c] σ is the standard deviation obtained from the calibration curve for three replicate measurements at three different enantiomeric compositions.

Table 4-2 presents the observed enantioselectivities at a number of different absolute concentrations of selectors, analyte **4**, and ammonium chloride. The relative concentrations of all the components were kept constant throughput. The concentration of analyte **4** was as high as 2000 μM and as low as 1.6 μM. Relatively little variation of the α_{MS} values was observed throughout this concentration range. All but one of these values are within 3% of the average α_{MS} (1.67) for this data set (entry 1-8), and this value also agrees very well with the average α_{MS} value for the data in Table 4-1. Again, since it is

the ratio of *pseudo*-diastereomeric selector-analyte complexes that determines the selectivity, the relative amounts of these complexes should not be affected by dilution. At lower concentrations, the intensities of the selector-analyte complexes in the mass spectra are diminished, which limits the extent to which the solutions can be diluted by the signal-to-noise ratio that one can obtain experimentally. This is exemplified in entry 9, Table 4-2, where the concentration of analyte **4** is down to 1.6 μM, and the α_{MS} is 1.45 with a standard deviation of 0.082. The analyte concentration does not represent an actual limit of detection since the mass spectrometry conditions were not optimized.

These two data sets have important implications for the use of this method for quantitative enantiomer assays: (1) as long as the concentrations of the selectors are in excess of the analyte, the calibration line and subsequent enantiomer assays can be done at any concentration of the selectors and analyte, and (2) valid data can readily be obtained over at least 2 orders of magnitude of analyte concentration (15.6 μM-2.0 mM for **4**). Solubility will likely be the upper concentration bound, while the magnitude of the signal (compared to the experimental noise) from the selector-analyte complexes will be the lower bound.

Table 4 - 2. Observed enantioselectivity as a function of the concentration of *pseudo*-enantiomeric chiral selectors *(S)*-**13** / *(R)*-**14** and analyte **4** at a constant selector/analyte ratio[a,b]

Entry	*(S)*-**13**/*(R)*-**14,** μM	**4**, μM	α_{MS}[c] (σ)[d]
1	4000	2000	1.75(0.024)
2	2000	1000	1.72(0.008)
3	1000	500	1.67(0.004)
4	500	250	1.70(0.012)
5	250	125	1.66(0.010)
6	125	62.5	1.62(0.012)
7	62.5	31.3	1.66(0.010)
8	31.3	15.6	1.61(0.033)
9	3.1	1.6	1.45(0.082)

[a] Concentration of NH_4Cl is 10 folds [**4**] in all cases.
[b] Solvent is methanol/water (1 : 1) in all cases.
[c] Observed MS enantioselectivity.
[d] σ is the standard deviation obtained from the calibration curve for three replicate measurements at three different enantiomeric compositions.

Quantitative Enantiomer Analysis

To test the validity of this method to accurately determine enantiomeric composition independent of analyte concentration, the enantiomeric composition of five different samples was determined at three different concentrations, using a calibration line that was constructed at a concentration different from each of these. The calibration curve was constructed from three replicate measurements of the CIF values in the electrospray ionization mass spectrum, where the sample was introduced by flow injection, at 11 different enantiomeric compositions. In each case the concentrations of the selectors were 1.0 mM, the concentration of ammonium chloride was 10 mM, and the analyte concentration was 200 μM. This one calibration curve was used for all subsequent enantiomer determinations. The analyte concentrations of the assayed solutions span 1

order of magnitude (50-500 μM). To estimate the precision of this method, each solution was injected a total of nine times, the average and standard deviation of the calibrated enantiomeric composition determinations are shown in Table 4-3. As can be seen from the data, accurate enantiomeric composition values are obtained over this data range, with observed standard deviations that are less than 1.0% of the composition of either enantiomer, for the nine replicate analyses for each sample.

Table 4 - 3. Determination of the enantiomeric composition of five different samples of analyte **4** by mass spectrometry at three different concentrations using a single calibration line[a,b]

	mole fraction of *(R)*-**4**	
[**4**], μM	HPLC[c,d]	MS (σ)[e]
500	0.976	0.979 (0.009)
500	0.774	0.782 (0.009)
500	0.497 [f]	0.502 (0.008)
500	0.240	0.240 (0.009)
500	0.044	0.041 (0.009)
100	0.976	0.960 (0.009)
100	0.774	0.782 (0.009)
100	0.497 [f]	0.482 (0.008)
100	0.240	0.239 (0.009)
100	0.044	0.056 (0.009)
50	0.976	0.996 (0.009)
50	0.774	0.779 (0.009)
50	0.497 [f]	0.495 (0.008)
50	0.240	0.235 (0.009)
50	0.044	0.075 (0.009)

[a] [**13**] = [**14**] =1.0 mM**,** and [NH_4Cl] = 10 mM in methanol/water (1 : 1).
[b] Calibration curve constructed at [**4**] = 200 μM.
[c] (*S, S*)-Whelk-O1, hexanes / 2-propanol/methanol (60: 38 : 2) at 2 mL / min.
[d] Repeated injections afforded standard deviations of < 0.003 in all cases.
[e] σ is the standard deviation obtained from the calibration curve for nine replicate measurements.
[f] Racemic.

Solvent Effects

Electrospray ionization mass spectra of solutions of chiral selectors (*S*)-**13** and (*R*)-**14** and analyte **4** with added acid, in a variety of solvents, were obtained in order to determine the scope of solvents for which effective chiral recognition would be observed. The observed mass spectrometric enantioselectivities for a number of these are presented in Table 4-4. In addition to the solvent combinations shown, hexanes, toluene, THF, dichloromethane, acetonitrile, methanol, ethanol, and 2-propanol were each tested individually as solvents. For solubility purposes, acetic acid was used instead of ammonium chloride as the protonation source. In each case, either no selector-analyte complexes were observed in the mass spectrum, or when the complexes were observed, chiral recognition was minimal. It is apparent from these data that water is a necessary component of the solutions to be assayed if effective chiral recognition is to be observed.

Entries 1-5 demonstrate the effect that the amount of water in the solvent (using binary water/methanol solvent compositions) has on the extent of the observed enantioselectivity. The highest α_{MS} value was observed at a composition of 10% water, while the enantioselectivity decreased regularly with an increase in water composition.

Effects of the type of acid additives were simply compared between the use of acetic acid and the use of ammonium chloride. Comparing entries 3, 6, and 12, or entries 2 and 7, clearly demonstrates that using acetic acid as the proton source affords higher enantioselectivities than ammonium chloride. Effects of acid additives also will be discussed in more detail in Chapter 5.

Table 4 - 4. Observed enantioselectivity of *pseudo*-enantiomeric chiral selectors (*S*)-**13** and (*R*)-**15** for analyte **4**.

entry	solvent	[(*S*)-**13** / (*R*)-**14**] mM	[**4**] mM	Acid	α_{MS}[a]
1[b]	MeOH/H_2O (9:1)	0.25	0.125	NH_4Cl (1.25 mM)	1.98
2[b]	MeOH/H_2O (3:1)	0.25	0.125	NH_4Cl (1.25 mM)	1.83
3[b]	MeOH/H_2O (1:1)	0.25	0.125	NH_4Cl (1.25 mM)	1.70
4[b]	MeOH/H_2O (1:3)	0.25	0.125	NH_4Cl (1.25 mM)	1.66
5[b]	MeOH/H_2O (1:9)	0.25	0.125	NH_4Cl (1.25 mM)	1.60
6[c]	MeOH/H_2O (1:1)	1.0	0.5	HOAc (2.0 mM)	2.04
7[c]	MeOH/H_2O (3:1)	1.0	0.5	HOAc (2.0 mM)	2.15
8[c]	ACN/MeOH/H_2O (2:1:1)	1.0	0.5	HOAc (2.0 mM)	2.14
9[c]	THF/MeOH/H_2O (2:1:1)	1.0	0.5	HOAc (2.0 mM)	2.23
10[c]	DCM/MeOH/H_2O (1:6:3)	1.0	0.5	HOAc (2.0 mM)	2.12
11[c]	Tol/MeOH/H_2O (1:8:1)	1.0	0.5	HOAc (2.0 mM)	2.08
12[c]	MeOH/H_2O (1:1)	1.0	0.5	NH_4Cl (5.0 mM)	1.75
13[b]	ACN/MeOH/H_2O (1:5:5)	1.0	0.5	NH_4Cl (5.0 mM)	1.79
14[b]	THF/MeOH/H_2O (1:5:5)	1.0	0.5	NH_4Cl (5.0 mM)	1.77

[a] Observed MS enantioselectivity.
[b] Flow injection of a 10-μL sample at 200 μL/min.
[c] Syringe pump infusion at 20 μL/min.

Comparing the enantioselectivities obtained for entries 7-11, it is evident that solvent compositions containing substantial amounts of organic solvents other than methanol, such as acetonitrile, THF, dichloromethane, or toluene, can be used. This has important implications for the use of this method in quantitative enantiomer assays, namely, that the analyte does not have to be dissolved in the same solvent as the selector and acid stock solution. One can either construct a calibration line using the appropriate final solvent composition or consider the additional solvent as interference and use the calibration curve constructed without this interference. To determine the extent that a small amount of an additional solvent effects the enantioselectivity, a small amount (0.1 mL) of acetonitrile, THF, dichloromethane, and toluene was each added to solutions of chiral selectors, analyte, and ammonium chloride in 1 : 1 water/methanol (1.0 mL). For the additions of dichloromethane and toluene, a two-phase mixture was formed, which added the partitioning between the two phases as an additional complication. Though, for the single-phase solutions obtained on addition of a small amount of acetonitrile or THF, as is evident from entries 12-14, the effect of this addition on the observed enantioselectivity is small.

Comparison of enantioselectivities of a scope of analytes by mass spectrometry and chiral HPLC

In addition to being used as a method for quantitative enantiomeric composition determinations, this method should also be useful for the discovery and optimization of chiral selectors.[58-64] Enantioselectivities of analytes **4**, **15**-**22**, as measured by electrospray ionization mass spectrometry using chiral selectors (*S*)-**13** and (*R*)-**14**, and by HPLC using the (*S*)-DNB-leucine CSP, under both normal-phase and reversed-phase conditions are presented in Table 4-5. To facilitate comparisons among the data, the HPLC conditions (as indicated in Table 4-5) were not optimized for any of the analytes; rather they were held constant throughout for both the normal-phase and the reversed-phase conditions. Additionally, for the solutions assayed by mass spectrometry, the same solvent system and concentrations were used for all of the analytes. A linear relationship between the CIF and the enantiomeric composition of the analyte was observed in all cases. The sense of observed chiral recognition was consistent between these two methods in all cases. The analyte enantiomer selectively retained on the (*S*)-DNB-leucine CSP, **2** complexes to the (*S*)-chiral selector to a greater extent in every case.

Table 4 - 5. Comparison of the observed enantioselectivities of analytes **4**, **15-22** by HPLC on (*S*)-N-(3, 5-dinitrobenzoyl)leucine[a] derived CSP and the enantioselectivity obtained by mass spectrometry using *pseudo*-enantiomeric chiral selectors (*S*)-**13** and (*R*)-**14**.

analyte	normal phase[b]		reversed phase[c]		more retained	α_{MS}[ef] (σ)[g]	higher affinity for (*S*)-**25**
	k_1	α[d]	k_1	α[d]			
4	5.12	2.97	1.71	1.80	(*S*)	1.67 (0.02)	(*S*)
15	1.15	1.42	1.11	1.30	(*S*)	1.47 (0.02)	(*S*)
16	1.39	1.14	0.90	1.12	(*S*)	1.28 (0.02)	(*S*)
17	0.65	1.80	1.62	1.36	(*S*)	1.75 (0.03)	(*S*)
18	1.37	1.56	2.38	1.36	(*S*)	1.53 (0.02)	(*S*)
19	0.69	1.20	1.32	1.15	(*S*)	1.40 (0.03)	(*S*)
20	2.77	3.47	5.18	1.88	(*S*)	2.01 (0.08)	(*S*)
21	1.29	1.58	1.14	1.11	(*R*, *R*)	1.16 (0.02)	(*R*, *R*)
22	5.45	1.21	1.15	1.08	(*S*)	1.29 (0.02)	(*S*)

[a] See Experimental Section for column details.
[b] Mobile phase: hexanes/2-propanol (90:10), 2 mL/min.
[c] Mobile phase: methanol/water (75:25), 1.2 mL/min. *d* Chromatographic separation factor (ratio of retention factors for the analyte enantiomers).
[e] [**13**] = [**14**] = 1.0 mM, [analyte] = 0.50 mM, and [NH_4Cl] = 5.0 mM in methanol/water (1:1).
[f] Observed MS enantioselectivity.
[g] σ is the standard deviation derived from the calibration line.

Figure 4-5 presents plots of α_{HPLC} in both normal phase and reversed phase *vs* α_{MS}, respectively. The correlation coefficient is 0.67 with the normal phase data, while it is 0.77 with the reversed phase data. A similar correlation has recently been reported by Lindner and coworkers using cinchona alkaloid chiral selectors.[66] Often, there are differences between selector-analyte solution equilibria and separation factors obtained chromatographically, primarily due to "tether effects". Even so, this will undoubtedly be a valuable method for the discovery and optimization of chiral selectors, particularly by combinatorial methods if one considers the alacrity with which one can obtain the

enantioselectivity for a given analyte. The practice of the discovery of the new chiral selectors prepared through solid combinatorial synthesis and the preliminary data will be presented in Chapter 6.

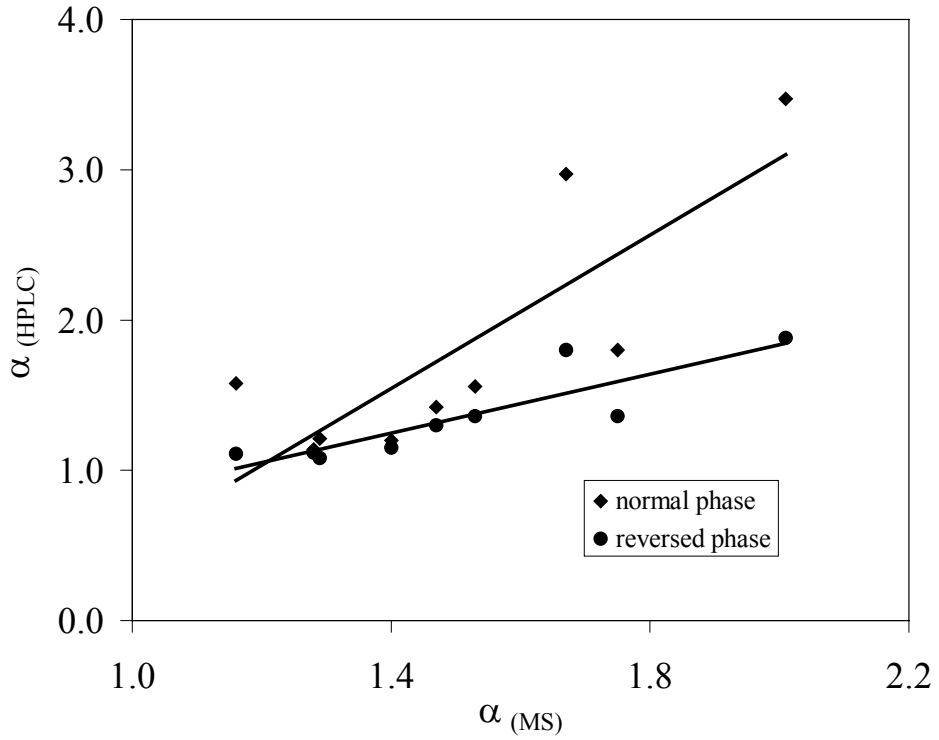


Figure 4 - 5. Plots of α_{HPLC} *vs* α_{MS} with a correlation coefficient of 0.67 for the normal phase data and 0.77 for the reversed phase data.

4.4 SUMMARY

Observations of chiral recognition in the electrospray ionization mass spectra, using tertiary amine appended derivatives of *N*-(3,5-dinitrobenzoyl)leucine as chiral selectors, have been demonstrated for a number of chiral analytes. A plot of complex intensity fraction versus mole fraction of the (*R*)-enantiomer of analyte is linear. This relationship provides a measurement of the extent of enantioselectivity and allows quantitative

determination of the enantiomeric composition. The results are independent of analyte concentration, provided that saturation of the association between the selector and analyte is not observed. Water is needed for a successful chiral recognition, given that a homogeneous phase is formed when mixing with organic solvent.

The chiral selectors used in this study were derived from the well-established chiral stationary-phase DNB-leucine. Given the correlation between the enantioselectivities observed chromatographically and by mass spectrometry, one would expect the scope of analytes that one can assay by this method should be comparable to the scope of analytes that can be enantioresolved on the corresponding chiral stationary phase. Given the large number of known chiral stationary phases, and the scope of chiral analytes for each, adaptation of these myriad chiral selectors into appropriate mass spectrometric chiral selectors would expand the set of chiral analytes that can be assayed by this method nearly congruously with the set of analytes that can be assayed by enantioselective chromatography. Additionally, it is expected that this method will be a useful tool for the discovery of new chiral selectors, particularly by combinatorial methods, which can then in turn be used for the preparation of new chiral stationary phases.

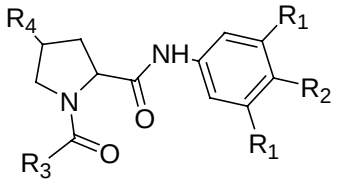
CHAPTER V

ENANTIOMER ANALYSIS USING PROLINE (AND HYDROXYPROLINE) DERIVATIVES AS CHIRAL SELECTORS

5.1 INTRODUCTION

The proline-based Pirkle type CSPs, such as CSP **23**, were reported to well separate enantiomers of derivatives of *N*-(3,5-dinitrobenzoyl) amino acids.[125] The goal of the work in this chapter is to perform enantiomer analysis of this type of chiral analytes using ESI-mass spectrometry. For this purpose, soluble analogues of CSP **23**, (*i.e*. anilide derivatives of *N*-pivaloylproline, **4** and **24**), were first used as *pseudo*-enantiomeric chiral selectors. Lithium chloride was used as additives for ionization in the intial experiment. Subsequently, tertiary amine appended *trans*-4-hydroxy-proline derivatives were designed, synthesized, and evaluated for enantiomer analysis of DNB-amino acid esters and amides. The factors affecting the extent of enantioselectivity were exhaustively investigated.

Chiral selectors



(*S*)-**CSP 23**: $R_1 = CH_3$, $R_2 = R_4 = H$,

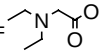
$R_3 = -(CH_2)_{10}Si(CH_3)_2$-silica

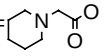
(*S*)-**24**: $R_1 = R_4 = H$, $R_2 = CH_3$, $R_3 =$ t-Bu

(*R*)-**4**: $R_1 = CH_3$, $R_2 = R_4 = H$, $R_3 =$ t-Bu

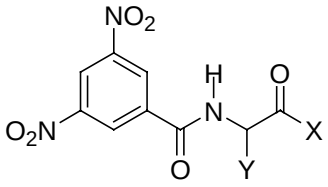
(*2S*, *4R*)-**25**: $R_1 = H$, $R_2 = CH_3$, $R_3 =$ t-Bu, $R_4 = OH$

(*2R*, *4S*)-**26**: $R_1 = CH_3$, $R_2 = H$, $R_3 =$ t-Bu, $R_4 = OH$

(*2S*, *4R*)-**27**: $R_1 = CH_3$, $R_2 = H$, $R_3 =$ t-Bu, $R_4 =$ 

(*2R*, *4S*)-**28**: $R_1 = CH_3$, $R_2 = H$, $R_3 =$ t-Bu, $R_4 =$ 

Chiral analytes



X =	NHBu	NEt_2	OEt	OH
Y = $CH_2CH(CH_3)_2$	**6**	**29**	**30**	**11**
Y = $CH(CH_3)_2$	**31**	**32**	**33**	**10**
Y = CH_2Ph	**34**	**35**	**36**	**40**
Y = CH_3	**37**	**38**	**39**	**41**

Figure 5 - 1. Structures of the chiral stationary phase, the chiral selectors, and the chiral analytes.

5.2 EXPERIMENTAL

Synthesis of N-pivaloyl-L-proline-4-methylanilide

The procedure is the same as that for **4** in Chapter 2.

(*S*)-**24**. ^{1}H NMR: $CDCl_3$. δ 9.33 (br.s, 1H), 7.36 (d, *J* = 8.0 Hz, 2H), 7.01 (d, *J* = 7.8 Hz, 2H), 4.80 (m, 1H), 3.76-3.72 (m, 2H), 2.35-1.85 (m, 6H), 1.30 (s, 9H). ^{13}C NMR: $CDCl_3$. δ 178.2, 169.8, 135.8, 133.1, 129.1, 119.6, 62.4, 48.3, 39.2, 27.5, 26.2, 25.9, 20.7. ESI-MS: *m/z* 289, $[M+H]^+$.

Preparation of cis-4-hydroxy-D-proline

See the literature.[129-131]

General procedure for the preparation of N-pivaloyl-4-hyroxyproline

To a sodium hydroxide aqueous solution (2 M, 76 ml) in a 250 mL round bottom flask was added hydroxyproline (38 mmol) at room temperature. The solution was lowered to ice-water bath, followed by the addition of pivaloyl chloride (76 mmol) dropwise via an additional funnel. The mixture was allowed to stir at 0^0C overnight. The reaction mixture was washed with 30 mL EtOAc and the organic wash was discarded. The aqueous solution was acidified with concentrated HCl to pH ~ 1, and extracted with EtOAc exhaustively. The combined organic layer was dried over $MgSO_4$. Removal of the solvent *in vacuo* provided a white solid that was further recrystallized in acetone and hexane.

trans-N-pivaloyl-hydroxy-4-proline: ^{1}H NMR: $CDCl_3$. δ 6.99 (br s, 2H), 4.55~4.47 (m, 2H), 3.95~3.74 (m, 2H), 2.31~2.05 (m, 2H), 1.27 (s, 9H); ESI-MS: *m/z* 216 $[M+H]^+$, 238 $[M+Na]^+$, 431 $[M_2+H]^+$, 453 $[M_2+Na]^+$, 667 $[M_3+Na]^+$.

General procedure for the preparation of anilide derivatives of N-pivaloyl-4-hydroxyproline

To an oven dried 50 ml round bottom flask was added *N*-pivaloyl-4-hydroxyproline (5.6 mmol), and then 30 mL dry THF. DIPEA (6.7 mmol) was added to the solution, followed by the addition of HBTU (5.6 mmol). 8 ml of anhydrous DMF was added to help dissolve HBTU. Lastly, aniline (20.1 mmol) was added to provide the target molecule.

(*2S, 4R*)-**25**: ^{1}H NMR: acetone-d_6, δ 9.26 (br.s, 1H), 7.53 (d, J = 8.2 Hz, 1H), 7.09 (d, J = 7.9 Hz, 1H), 4.67 (t, J = 7.8 Hz, 1H), 4.55 (br.s, 1H), 3.88-3.76 (m, 2H), 2.95-2.92 (m, 3H), 2.26 (s, 3H), 1.24 (s, 9H). ^{13}C NMR: acetone-d_6, δ 177.5, 171.5, 137.8, 133.2, 129.9, 120.0, 71.2, 62.3, 57.4, 39.3, 37.0, 27.9, 20.8.

(*2S, 4R*)-**26**: ^{1}H NMR: $CDCl_3$. δ 9.12 (br. s, 1H), 7.16 (s, 2H), 6.72 (s, 1H), 4.94 (t, 1H), 4.68 (m, 1H), 3.87 (m, 1H), 3.70 (m, 1H), 2.66 (m, 1H), 2.26 (s, 6H), 1.98 (m, 1H), 1.62 (m, 1H), 1.30 (s, 9H); ^{13}C NMR: $CDCl_3$. δ 181.0, 176.4, 144.1, 142.8, 129.9, 122.0, 74.8, 66.2, 61.5, 43.1, 41.5, 32.3, 26.2.

(*2R, 4R*)-**26**: ^{1}H NMR: $CDCl_3$. δ 9.58 (br s, 1H), 7.07 (s, 2H), 6.65 (s, 1H), 4.82 (m, 1H), 4.49 (m, 1H), 4.01~3,97 (m, 1H), 3.79~3.74 (m, 1H), 2.27~2.14 (m, 9H), 1.29 (s, 9H); ^{13}C NMR: $CDCl_3$. δ 178.6, 172.0, 138.4, 137.4, 126.1, 117.9, 72.1, 63.0, 58.6, 39.2, 34.6, 27.4, 21.3.

Preparation of trans-N-pivaloyl-4-hydroxy-D-proline-(3,5-dimethyl)anilide

Methanesulfonyl chloride (6.0 mmol) was added to a suspension of (*2R, 4R*)-**26** (1.5 mmol) in 15 ml THF containing DIPEA (9 mmol) at 0^0C. The solution was stirred at this temperature for 3 hours. The solid was filtered, and the excess solvent was removed *in vacuo* to provide a brown yellow residue. Flash chromatography with 50 % hexanes / 50% EtOAc as mobile phase gave a colorless oil (740 mg, quantitative yield). The stereocenter, where the mesolate group was attached, was inversed by the reaction with tetrabutylammonium acetate (2.8 mmol) in toluene (20 mL) under reflux overnight. The reaction mixture was cooled to room temperature, diluted with 50 ml ethyl acetate, and washed with water twice. The organic layer was dried over $MgSO_4$, and removed *in vacuo* to provide a brown gum. Purification with flash chromatography (from 20 % EtOAc in hexanes to 30 % EtOAc) produced a white solid (0.29 g, 53%). This solid was hydrolyzed in EtONa / EtOH solution at room temperature for half an hour. An aliquot of solid NH_4Cl was added to destroy the excess EtONa. The solid was filtered by vacuo filtration. The filtrate was dried *in vacuo*. The white residue was further purified by flash chromatography (50 % EtOAc / 50% hexanes) to give 0.18 g white solid, 72%.

(*2R, 4S*)-**26**. ^{1}H NMR: $CDCl_3$. δ 9.09 (br s, 1H), 7.16 (s, 2H), 6.73 (s, 1H), 4.94 (dd, J_1 = 9.0 Hz, J_2 = 6.0 Hz, 1H), 4.69 (br s, 1H), 3.85 (m, 1H), 3.69 (m, 1H), 2.75~2.67 (m, 1H), 2.28 (s, 6H), 1.99~1.91 (m, 1H), 1.30 (s, 9H).

Preparation of trans-N-pivaloyl-4-(N,N-diethylamino acetyloxy)-L-proline-(3,5-dimethyl)anilide

(*2S, 4R*)-**26** (0.5 mmol), was dissolved in 5 ml dry THF in a flame dried r.b flask under N_2, followed by the addition of DIPEA (2.2 mmol). α-chloroacetylchloride (2.0 mmol) was added dropwise via syringe. The reaction mixture turned black immediately upon the first drop of α-chloroacetylchloride. The mixture was stirred for 3 hours, and then diluted with 50 ml of EtOAc, washed with 2 M NaOH, 2 M HCl, and water, consequently. The organic solution was dried over $MgSO_4$, and the solvent was removed *in vacuo* to give a brown gum. Purification by flash chromatography (from 30% EtOAc to 40% EtOAc in hexanes) gave a light yellow oil (0.15 g, 75% percent yield). $R_f = 0.15$ (30% EtOAc in hexanes).

α-chloroacetate of **26**: ^{1}H NMR: $CDCl_3$. δ 9.32 (br s, 1H), 7.09 (s, 2H), 6.65 (s, 1H), 5.50 (m, 1H), 4.94 (t, $J = 6.0$ Hz, 1H), 4.13~4.03 (m, 3H), 3.87 (dd, $J_1 = 12.0$ Hz, $J_2 = 3.0$ Hz, 1H), 2.77~2.68 (m, 1H), 2.21 (s, 6H), 2.16 (m, 1H), 1.28 (s, 9H). ^{13}C NMR: $CDCl_3$. δ 178.2, 168.9, 166.7, 138.3, 137.8, 125.6, 117.4, 75.8, 60.4, 53.1, 40.5, 38.8, 31.7, 27.4, 21.2.

The above oil (0.12 mmol) was dissolved in 5 ml acetone. Excess of diethyl amine was added and stirred at room temperature for two days. The solvent was removed *in vacuo.* The residue was purified by flash chromatography with EtOAc to provide 105 mg of yellow gum (48%).

(*2S, 4R)*-**27**: ^{1}H NMR: $CDCl_3$. δ 9.13 (br s, 1H), 7.13 (s, 2H), 6.70 (s, 1H), 5.42 (m, 1H), 4.94 (t, $J = 7.2$ Hz, 1H), 4.08 (m, 1H), 3.74 (dd, $J_1 = 12.0$ Hz, $J_2 = 3.6$ Hz, 1H), 3.30~2.29 (m, 2H), 2.85~2.76 (m, 1H), 2.64 (q, J = 7.6 Hz, 4H), 2.26 (s, 6H), 2.18~2.00

(m, 1H), 1.07 (s, 9H), 1.05 (t, J = 6.9 Hz). ^{13}C NMR: $CDCl_3$. δ 178.5, 171.1, 166.6, 138.5, 137.8, 125.8, 117.5, 73.9, 60.4, 53.9, 53.4, 47.6, 38.9, 31.4, 27.5, 21.3, 12.3. ESI-MS: 432 $[M+H]^+$.

Preparation of trans-N-pivaloyl-4-(piperidinylacetyloxy)-D-proline-(3,5-dimethyl)anilide

(*2R, 4S*)-**26** was used as the starting material. All the procedure was the same to the preparation of *(2S, 4R)*-**27,** except the last step, where the piperidine was used instead of diethyl amine. White gum, 72 mg, yield 53%.

(*2R, 4S*)-**28**: ^{1}H NMR: $CDCl_3$. δ 9.21 (br s, 1H), 7.11 (s, 2H), 6.67 (s, 1H), 5.43 (m, 1H), 4.94 (t, J = 7.2 Hz, 1H), 4.11~4.04 (m, 1H), 3.80 (dd, J1 = 12.0 Hz, J2 = 3.0, 1H), 3.17~3.15 (m, 2H), 2.80~2.71 (m, 1H), 2.49 (m, 4H), 2.24~2.17 (s, 8H), 1.62~1.60 (m, 4H), 1.45~1.44 (m, 2H), 1.27 (s, 9H). ^{13}C NMR: $CDCl_3$. δ 178.3, 170.1, 168.8, 138.3, 137.8, 125.6, 117.4, 73.9, 60.4, 59.9, 54.1, 53.4, 38.8, 31.5, 27.4, 25.7, 23.7, 21.2. ESI-MS: 444 $[M+H]^+$.

HPLC

The chiral stationary phase, **CSP 23** (4.6 x 250 mm column), was available from previous studies.[123] The operation conditions are given in the data tables.

Mass spectrometry

Solutions were introduced either by flow injection (10 μL sample loop, mobile phase flow rate: 200 μL), or by direct infusion. The full positive ion spectrum was recorded

every 0.7 seconds. For flow injection, all scans for which a significant total ion count was observed were averaged together to afford the final spectrum. For data collected by direct infusion with a syringe pump (8 μL / min), scans collected over a one-minute period were averaged together to afford the final spectrum. Spectrometer conditions are as follows: extractor voltage, 1.0 V; RF lens, 0.5 V; source temperature, 80 °C; cone gas flow, 61 liter / h; desolvation gas flow, 409 liter / h. The cone voltage, capillary voltage, and desolvation temperature are given in the data tables.

5.3 Results And Discussion

5.3.1 Using anilide derivatives of proline, (S)-24 and (R)-4 as chiral selectors

Chiral recognition

Observations of chiral recognition of derivatives of DNB-amino acids, using ESI-MS, have been demonstrated in previous chapters. Herein, the author sets out to determine whether chiral recognition would be observed in a reciprocal sense, i.e. using *pseudo*-enantiomeric chiral selectors derived from **4**, and analytes similar to **6**. The *pseudo*-enantiomeric chiral selectors, (*R*)-**4** / (*S*)-**24**, were prepared in such a way that the mass difference was resulted from substitution on the aromatic ring, which is likely to have very little effect on chiral recognition.

Figure 5-2 presents the ESI-mass spectrum of a solution containing the *pseudo*-enantiomeric chiral selectors, (*S*)-**24** and (*R*)-**4**, and racemic analyte **6**, with added lithium chloride in methanol / water (1 : 1). The lithiated selectors and analyte are observed at *m/z* 295 [**24**+Li]$^+$, 309 [**4**+Li]$^+$, and 387 [**6**+Li]$^+$, along with the methanol adducts at *m/z*

327 [**24**+Li+MeOH]$^+$, 341 [**4**+Li+MeOH]$^+$, and 419 [**6**+Li+MeOH]$^+$, respectively. The selector dimers are observed at *m/z* 583 [**24**$_2$+Li]$^+$, 597 [**24**+**4**+Li]$^+$, and 611 [**4**$_2$+Li]$^+$. The selector-analyte complexes are observed at *m/z* 675 [**24**+**6**+Li]$^+$ and 689 [**4**+**6**+Li]$^+$.

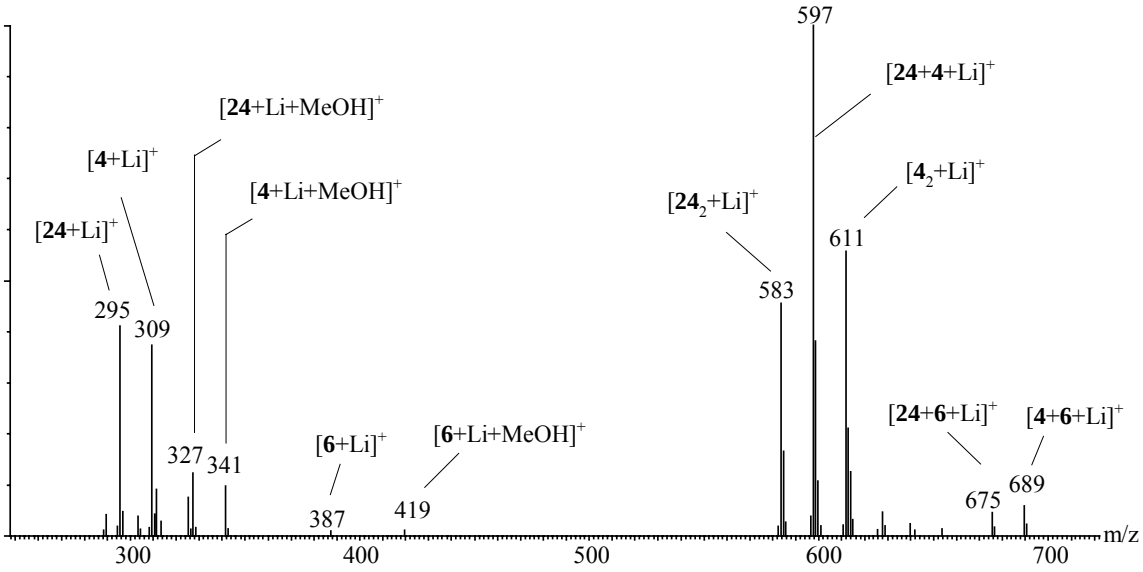


Figure 5 - 2. Electrospray ionization mass spectrum of a solution containing *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4** (1.0 mM), racemic analyte **6** (0.50 mM), and lithium chloride (5.0 mM) in methanol / water (1 : 1).

Figure 5-3 presents the portions of mass spectra showing the selector-analyte complexes for solutions containing analyte (*R*)-**6**, (*rac*)-**6**, and (*S*)-**6**, respectively. The relative intensities of the selector-analyte complexes change regularly with the enantiomeric composition of analyte **6**. It is apparent from the figure that (*R*)-**6** has a greater affinity with the (*R*)-enantiomer of the chiral selector, and that (*S*)-**6** preferentially binds to the (*S*)-enantiomer of the chiral selector. The sense of chiral recognition agrees with what is observed chromatographically on the corresponding CSP. The elution order for analyte **6**, using (*S*)-**CSP 23**, is (*R*) then (*S*).

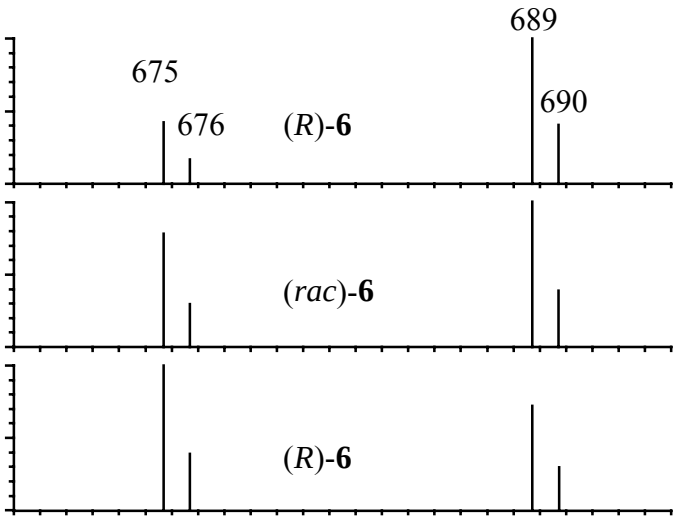


Figure 5 - 3. Electrospray ionization mass spectra of solutions containing *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4** (1.0 mM), analyte **6** (0.50 mM), and lithium chloride (5.0 mM) in methanol / water (1 : 1). Enantiomeric composition of analyte **6** is noted in the figure.

Analyte survey and comparison of enantioselectivities with added lithium chloride by MS and by chiral HPLC

By determining the CIF value at (a minimum of) two differing analyte enantiomeric compositions, the α_{MS} value can be evaluated from a plot of CIF versus the mole fraction of the (*R*)-enantiomer of analyte. Table 5-1 presents the α_{MS} values obtained for *N*-(3,5-dinitrobenzoyl)amino acid derivatives (**6**, **29**-**38**), using *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4**, with added lithium chloride. Also presented in Table 5-1 are the chromatographic separation factors (α_{HPLC}) for these same analytes, using **CSP 23** under reverse-phase conditions. It is apparent, for the majority of analytes that the α_{HPLC} value is much greater than the observed α_{MS} value, using these experimental conditions.

Table 5 - 1. Comparison of the chromatographic separation factors (α_{HPLC}) for the enantiomers of analytes **6, 29** – **38** on **CSP 23**[a], and observed mass spectrometric enantioselectivities (α_{MS}) using *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4** with added lithium chloride for ionization.

analyte	k_1[b]	α_{HPLC}[c]	α_{MS}[d]
6	2.79	3.53	1.69
29	2.31	4.80	1.28
30	2.99	1.63	1.58
31	2.14	2.77	1.53
32	1.95	2.71	1.07
33	2.14	1.39	1.42
34	3.18	2.86	1.28
35	2.79	3.23	1.07
36	3.31	1.41	1.09
37	1.72	1.93	1.54
38	1.45	3.25	1.21

[a] eluent: CH_3CN / H_2O (60 : 40), 1.0 mL / min
[b] retention factor for the first eluted enantiomer
[c] ratio of the retention factors for the analyte enantiomers
[d] [**24**] = [**4**] = 1.0 mM; [analyte] = 0.5 mM; [LiCl] = 5.0 mM; MeOH / H_2O (1 : 1); desolvation temp 325 ^{0}C, cone voltage 15 V.

Optimization of enantioselectivity

Given the disparity between the enantioselectivities observed by MS and HPLC, it was reasoned that the enantioselectivity was being diminished during the MS experiment, and that the actual solution-state enantioselectivity was likely much closer to the α_{HPLC} value than the α_{MS} value. One of the reasons was considered to be the interference by the added ions. This has been investigated before. The effect that the instrumental parameters, desolvation temperature and cone voltage as well as the additive have on the magnitude of the observed α_{MS} value is discussed here.

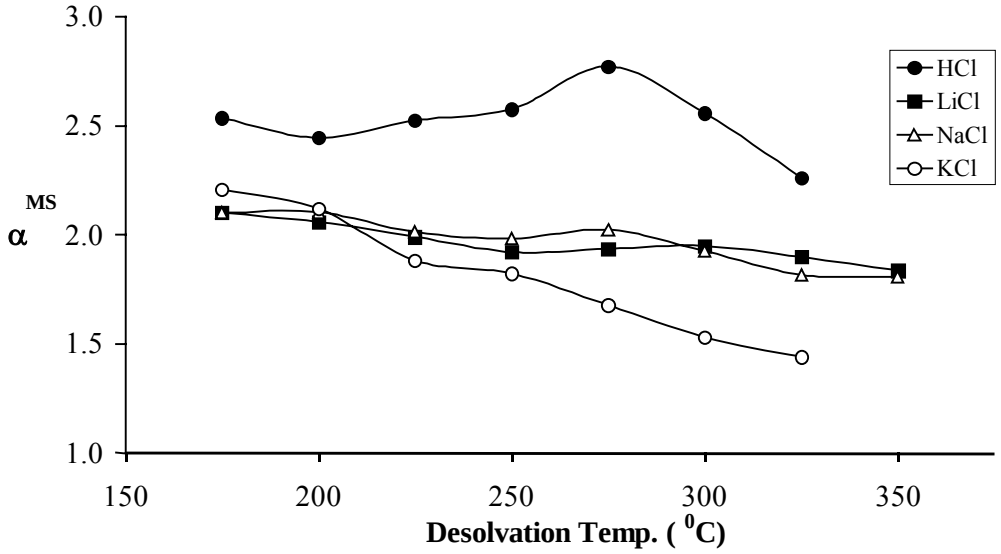


Figure 5 - 4. Enantioselectivity (α_{MS}) as a function of desolvation temperature and additive: [**24**] = [**4**] = 250 μM, [**6**] = 125 μM, [additive] = 5.0 mM, cone = 8 V.

Figure 5-4 presents enantioselectivity data using *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4**, analyte **6**, with added hydrogen chloride, lithium chloride, sodium chloride, or potassium chloride, as a function of desolvation temperature. Ionized selector-analyte complexes were too small to allow a determination of enantioselectivity above 325 °C with hydrogen chloride or potassium chloride as the additive. It should be noted that the data discussed previously were collected at a desolvation temperature of 350 °C. It is apparent from the figure that the α_{MS} value generally increases with decreasing desolvation temperature. Additionally, the additives afford the following order: HCl > LiCl ~ NaCl > KCl for the α_{MS} values.

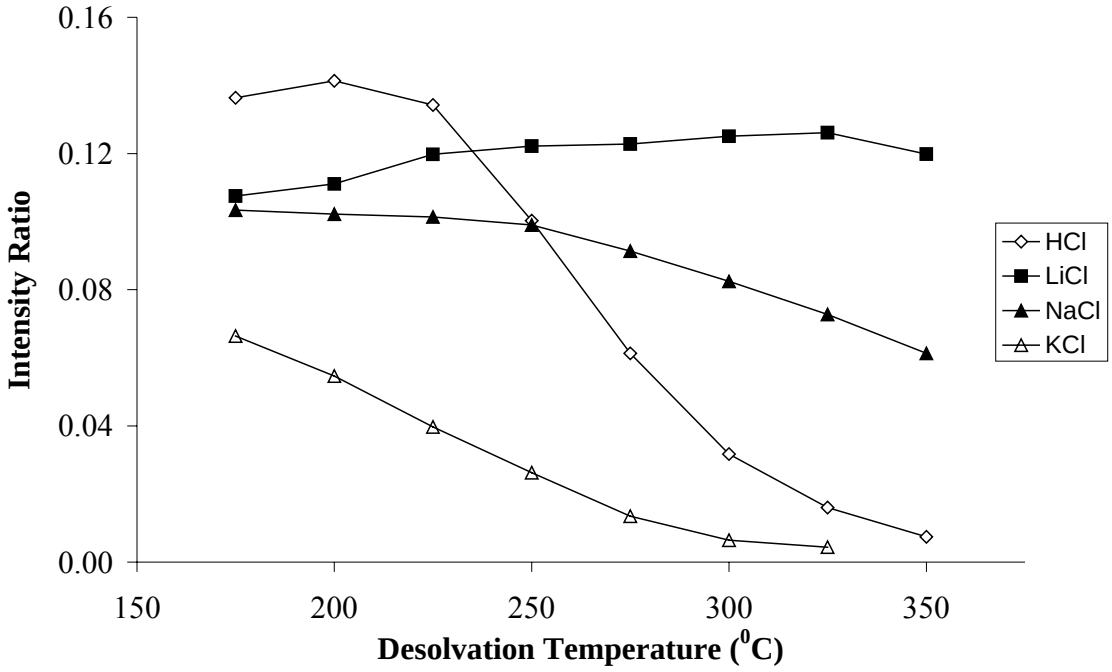


Figure 5 - 5. Ratio of the sum of the selector-analyte ion counts and the sum of the monomer ion counts as a function of desolvation temperature and additive: [**24**] = [**4**] = 250 μM, [**6**] = 125 μM, [additive] = 5.0 mM, cone = 8 V.

In addition to the effect that the desolvation temperature and additive have on enantioselectivity, which is related to the relative intensities of the selector-analyte complexes, one must also consider the effect these parameters have on the absolute intensity of the bimolecular complexes. Figure 5-5 presents the relative intensities of the selector-analyte complexes compared to the intensities of the monomeric species (*i.e.* intensity ratio in the figure). With the exception of the lithium complexes, the relative intensities of the selector-analyte complexes are increased as the desolvation temperature is decreased. Though as the desolvation temperature is decreased below 200 °C the ion counts of all species are decreased substantially.

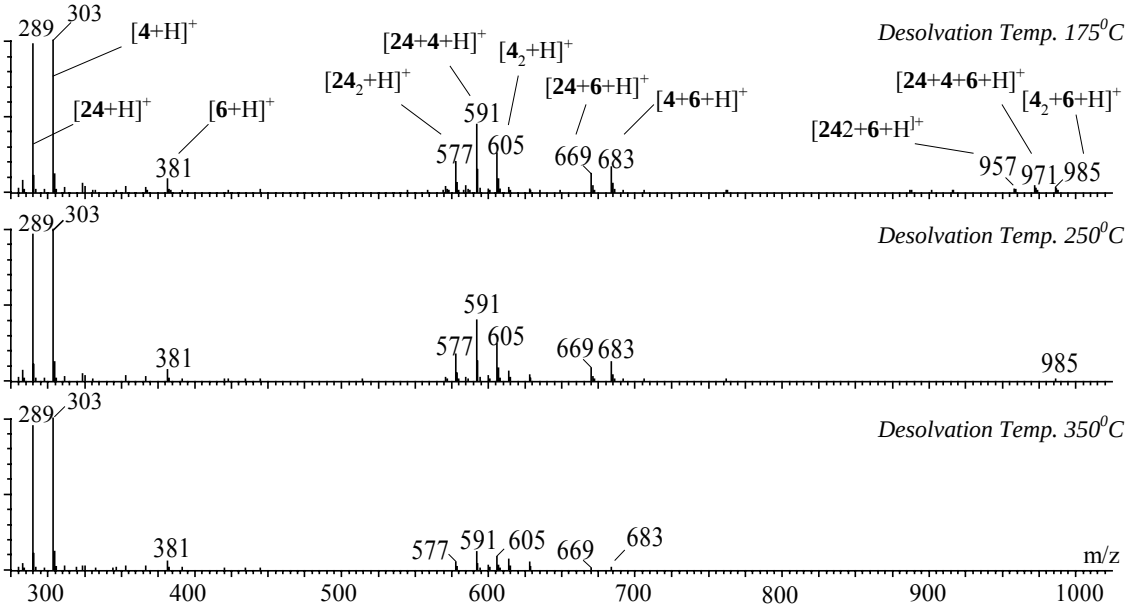


Figure 5 - 6. Comparison of electrospray ionization mass spectra for the solution containing *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4** (250 μM), (*rac*)-**6** (125 μM), and hydrogen chloride (5.0 mM) in acetonitrile / water (1 : 1), measured at different desolvation temperature. The temperature is noted in the figure.

Figure 5-6 presents three mass spectra for the same solution containing racemic **6**, which was measured at 175 ^{0}C, 250 ^{0}C, and 350 ^{0}C, respectively. The intensities of the protonated monomer ions of selectors and analyte at *m/z* 289, 303, and 381, are relatively invariant, while the intensities of the selector-analyte complexes at *m/z* 669 [**24**+**6**+H]$^+$ and 683 [**4**+**6**+H]$^+$ are decreased with the increase of the temperature. The intensities of the protonated selector dimers at *m/z* 577 [**24**$_2$+H]$^+$, 591 [**24**+**4**+H]$^+$, and 605 [**4**$_2$+H]$^+$ are also smaller at higher temperature. The trimer ions observed at *m/z* 957 [**24**$_2$+**6**+H]$^+$, 971 [**24**+**4**+**6**+H]$^+$, and 985 [**4**$_2$+**6**+H]$^+$ at the desolvation temperature 175 ^{0}C are not observed any longer at 350 ^{0}C.

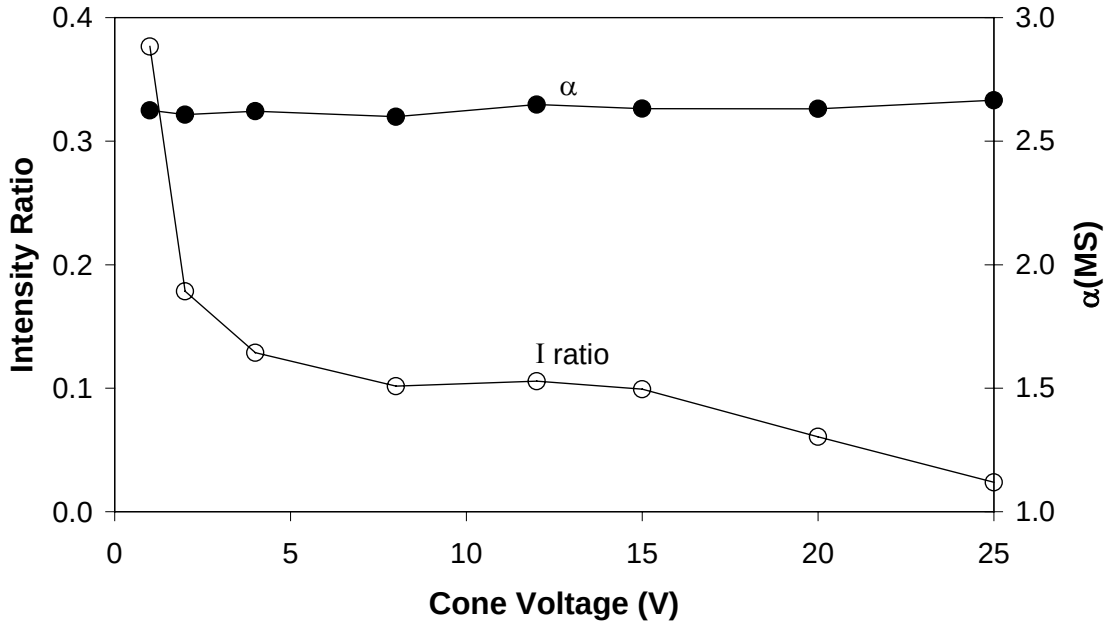


Figure 5 - 7. Ratio of the sum of the selector-analyte ion counts and the sum of the monomer ion counts (open-circles) as a function of cone voltage; observed mass spectrometric enantioselectivity (closed-circles) as a function of cone voltage: [**24**] = [**4**] = 250 μM, [**6**] = 125 μM, [HCl] = 5.0 mM, desolvation temperature = 275 °C.

The final parameter investigated was the cone voltage. As can be seen from Figure 5-7, this parameter has almost no effect on the enantioselectivity, though it does have a major effect on the absolute intensity of the selector-analyte complexes. As the cone voltage increases, the relative intensity of the selector-analyte complexes (compared to the intensities of the monomers) decreases. Although the absolute ion counts for all increase with increasing cone voltage, such that at cone voltages less than 5 V the ion counts were too low to be of practical utility.

Based on our optimization experiments, hydrogen chloride was used as the additive, the desolvation temperature was set to 200 °C, and the cone voltage was set to 8 V. The spectrum at 200 °C is similar to that at 175 °C as is shown in Figure 5-6.

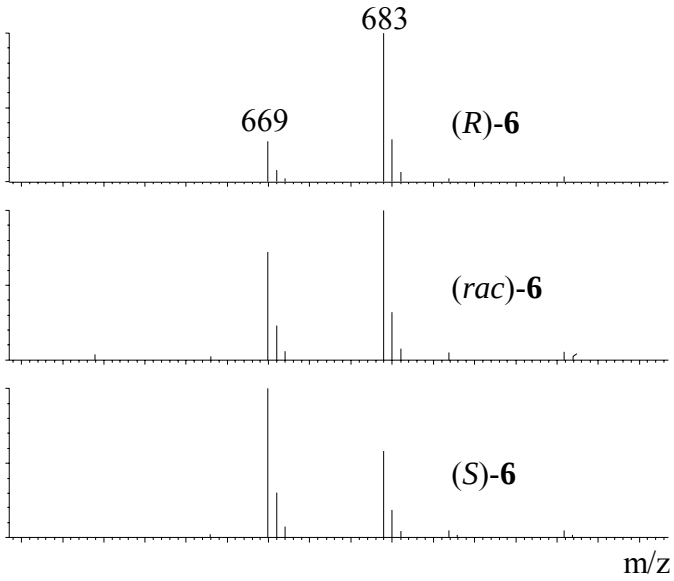


Figure 5 - 8. Partial mass spectra of *pseudo*-enantiomeric selectors (*S*)-**24** and (*R*)-**4** (1.0 mM) and analyte **6** (0.5 mM) with added hydrogen chloride (5 mM) in acetonitrile / water (1 : 1).

Figure 5-8 presents the portion of the mass spectrum showing the protonated selector-analyte complexes that are observed at three different enantiomeric compositions of analyte **6**. As is expected, the relative intensity of the selector-analyte complexes changes regularly with the enantiomeric composition of analyte **6**, and that the sense of chiral recognition is consistent with the data shown previously and the chromatographic data. The α_{MS} is 2.56, which is substantially increased from the initial conditions (*cf*. Fig. 5-3), though it is still less than the enantioselectivity observed by chiral HPLC (Table 5-1).

Analyte survey with the optimized ESI-MS conditions

The MS enantioselectivities, using *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4** and our optimized conditions, for all of our test analytes, is presented in Table 5-2.

It should be noted that for the ester derivatives, **33**, **36**, and **39**, no protonated selector-analyte complexes were observed in the mass spectrum. In every case where the selector-analyte complexes were observed in the ESI-mass spectrum, the α_{MS} value for the protonated complexes were greater than the α_{MS} values observed for the lithiated complexes, and in many cases (especially for the *N*-butyl amide derivatives) the α_{HPLC} values are comparable to the α_{MS} values (*cf*. Tables 5-1 and 5-2). The similarity between the enantioselectivities observed by these two methods portends the use of ESI-MS as a screening tool for chiral selector discovery.

Table 5 - 2. Observed mass spectrometric enantioselectivities (α_{MS}) using *pseudo*-enantiomeric chiral selectors (*S*)-**24** and (*R*)-**4** with added hydrogen chloride for ionization.

Analyte	α_{MS}[a]
6	2.56
29	1.59
31	2.08
32	1.12
34	1.59
35	1.16
37	2.00
38	1.41

[a] [**24**] = [**4**] = 250 μM; [analyte] = 125 μM; [HCl] = 5.0 mM; CH_3CN / H_2O (1:1); desolvation temp 200 °C, cone voltage 8 V, syringe pump 8 μL / min.

Enantiomeric composition determinations

In order to test the validity of this method to accurately determine enantiomeric composition independent of analyte concentration, the enantiomeric composition of five different samples was determined at four different concentrations, using calibration lines that were constructed at concentrations different from each of these. The calibration lines

were constructed by fitting a plot of the CIF value *vs* mole fraction of (*R*)-**6** to a straight line. In each case three enantiomeric compositions of **6** were used to construct the plot: (*R*)-**6**, *rac*-**6**, and (*S*)-**6**. The concentrations of the selectors were 250 μM and the concentration of hydrogen chloride was 5.0 mM throughout, using acetonitrile and water as the solvents (1 : 1). Two calibration lines were constructed, one with an analyte concentration of 125 μM, the other with an analyte concentration of 12.5 μM. Both lines are shown in Figure 5-9. It can be seen that the slope is relatively invariant, though the analyte concentrations have ten folds difference.

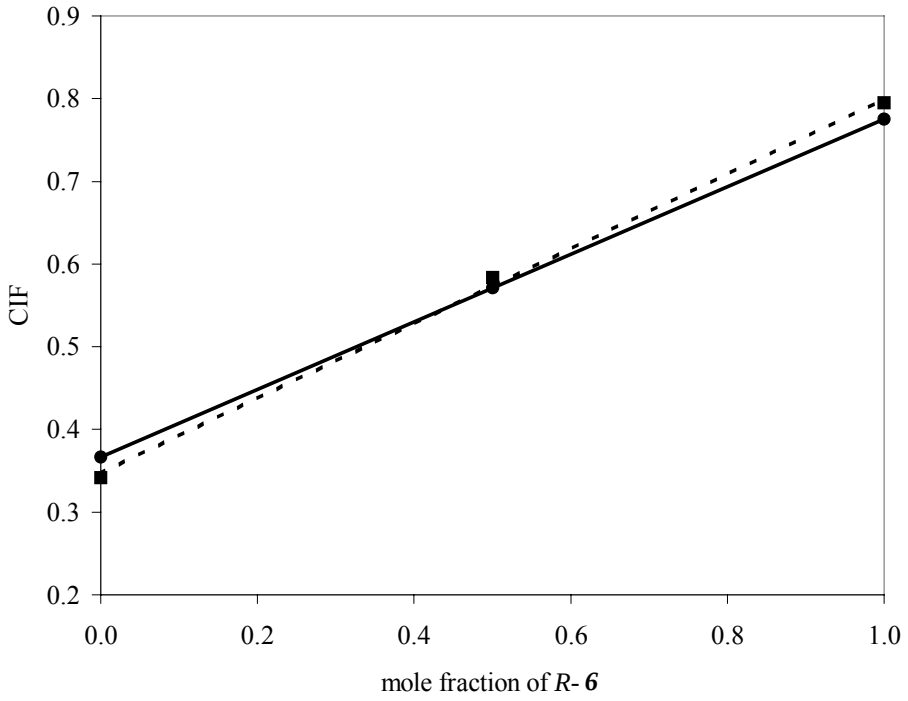


Figure 5 - 9. Calibration line constructed using *pseudo*-enantiomeric selectors (*S*)-**24** / (*R*)-**4** (250 μM each) and analyte **6** at two different concentrations. (The dotted line is 12.5 μM, and the continuous line is 125 μM; the concentration of HCl is 5.0 mM).

Table 5-3 presents the enantiomeric composition assays of samples of **6** at five different compositions. Each sample of **6** was analyzed at four different concentrations spanning an order of magnitude (20 μM to 200 μM). Each sample was also analyzed by chiral HPLC using CSP **23** to allow comparisons between enantiomeric composition determinations by these two methods. As can be seen from the data, enantiomeric composition values that are accurate enough for screening applications are obtained. The majority of the data using either calibration line are within 0.05 of the mole fraction. In fact, the standard deviation for the difference between the HPLC and MS enantiomer determinations is 0.045 and 0.030 for the calibration lines constructed at a concentration of analyte **6** of 125 μM and 12.5 μM, respectively (See Figure 5-9).

Table 5 - 3. Determination of the enantiomeric composition of five different samples of analyte **6** by mass spectrometry at four different concentrations using two calibration lines constructed at different analyte concentrations.

	Mole fraction (*R*)-**6**		
[**6**], μM	HPLC[a]	MS[b,d]	MS[c,d]
200	0.974	0.96	0.91
100	0.974	1.01	0.95
50	0.974	1.03	0.97
20	0.974	1.02	0.97
200	0.749	0.74	0.71
100	0.749	0.78	0.75
50	0.749	0.77	0.74
20	0.749	0.81	0.78
200	0.547	0.56	0.55
100	0.547	0.59	0.58
50	0.547	0.59	0.57
20	0.547	0.57	0.56
200	0.259	0.29	0.31
100	0.259	0.26	0.27
50	0.259	0.23	0.26
20	0.259	0.23	0.25
200	0.039	0.04	0.08
100	0.039	-0.02	0.02
50	0.039	-0.08	-0.03
20	0.039	-0.01	0.03

[a] CSP **23**; MeOH / Water (82 : 18), 1.2 mL min^{-1}
[b] calibration line: [**6**] = 125 μM,
[c] calibration line: [**6**] = 12.5 μM
[d] [**24**]=[**4**] = 250 μM; [HCl] = 5.0 mM; CH_3CN / H_2O (1:1); desolvation temp 200 °C, cone voltage 8 V, syringe pump 8 μL / min

5.3.2 Using anilide derivatives of trans-4-hydroxyproline, (2S, 4R)-25 and (2R, 4S)-26 as chiral selectors

Design and preparation of chiral selectors

Initially, the anilide derivatives of *trans*-4-hydroxyproline (**25** and **26**) were used as new chiral selectors, given that the hydroxyl group is likely an ionization site in the molecule. The preparations of hydroxyproline-based chiral selectors are presented in Figure 5-10. The *trans*-4-hydroxy-(*L*)-proline was used for the preparations of both *trans*-*N*-pivaloyl-4-hydroxy-(*L*)-proline-(3,5-dimethyl)anilide, (*2S*, *4R*)-**25**, and *trans*-*N*-pivaloyl-4-hydroxy-(*D*)-proline-(4-methyl)anilide, (*2R*, *4S*)-**26**. Generally, hydroxyprolines were first converted to *N*-pivaloyl hydroxyprolines, and then coupled with aniline to form anilide derivatives. To synthesize (*2R*, *4S*)-**26**, the chirogenic center at C-2 of *trans*-4-hydroxy-(*L*)-proline was first inversed by refluxing in the mixture of acetic anhydride and acetic acid, and then converted to *cis*-4-hydroxy-(*D*)-proline, (*2R, 4R*)-**26**; while the chiral center at C-4 was epimerized following the steps: (1) converting hydroxyl group of *cis*-*N*-pivaloyl-4-hydroxy-(*D*)-proline-(3,5-dimethyl)anilide, (*2R, 4R*)-**26** to α-chloroacetyl ester, and (2) replacing it with acetate group to give *trans*-*N*-pivaloyl-4-acetyloxy-(*D*)-proline-(3,5-dimethyl)anilide, and (3) hydrolyzing the acetate group in EtONa / EtOH to afford *trans*-*N*-pivaloyl-4-hydroxyl-(*D*)-proline-(3,5-dimethyl)anilide (*2R, 4S*)-**26**. The substitution of methyl groups on the aromatic ring accounts for the mass difference of the *pseudo*-enantiomeric chiral selectors, (*2S*, *4R*)-**25** / (*2R, 4S*)-**26**. Again, it is assumed that this type of substitution will typically afford only a minor perturbation of the extent of observed chiral recognition.

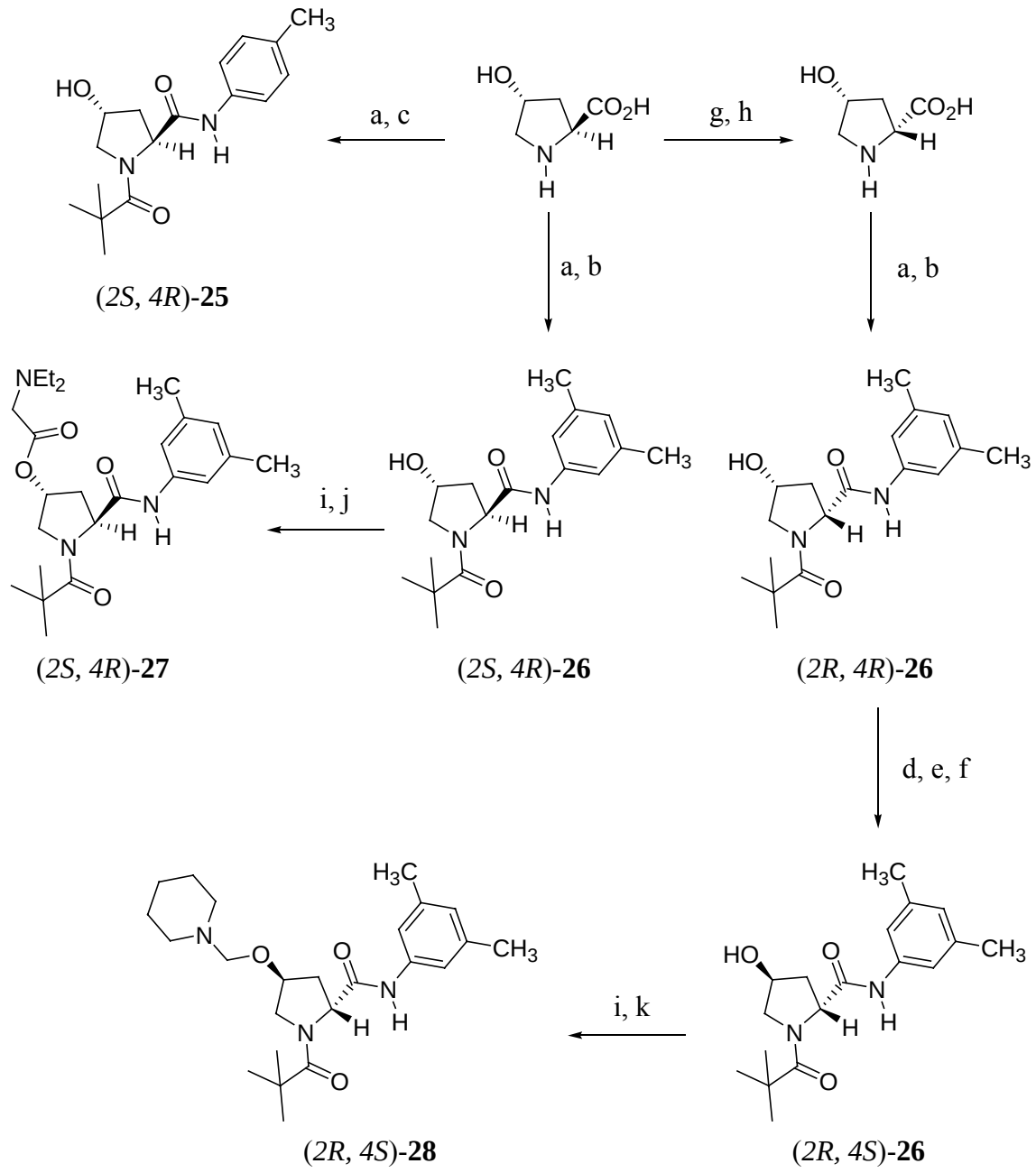


Figure 5 - 10. Preparation of chiral selectors.

[Reagents and conditions: (a) $(CH_3)_3CCOCl$, NaOH (aq), 0^0C; (b) 3,5-dimethylaniline, HBTU, DIPEA, 0^0C; (c) *para*-toluidine, EEDQ, 0^0C; (d) MsCl, 0^0C; (e) $Bu_4N^+ {}^-OAc$; (f) EtONa / EtOH, rt; (g) Ac_2O, HOAc, reflux; (h) HCl (aq), Δ; (i) α-chloroacetylchloride; (j) diethylamine; (k) piperidine.]

Chiral recognition with lithium chloride as the additive

Solutions were prepared in the same way as before. Lithium chloride was first used as additives. Figure 5-11 presents the mass spectrum of a solution containing chiral selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** (1.0 mM), (*rac*)-**6** and lithium chloride in methanol / water (1 : 1). The lithiated selectors and analyte ions were observed at *m/z* 311 [**25**+Li^+], 325 [**26**+Li^+] and 387 [**6**+Li^+], respectively. The lithiated dimeric ions were observed at *m/z* 615 [$\mathbf{25}_2$+Li^+], 629 [**25**+**26**+Li^+], 643 [$\mathbf{26}_2$+Li^+], and 767 [$\mathbf{6}_2$+Li^+]. The selector-analyte complexes were observed at *m/z* 691 [**25**+**6**+$Li]^+$, 703 [**26**+**6**+$Li]^+$.

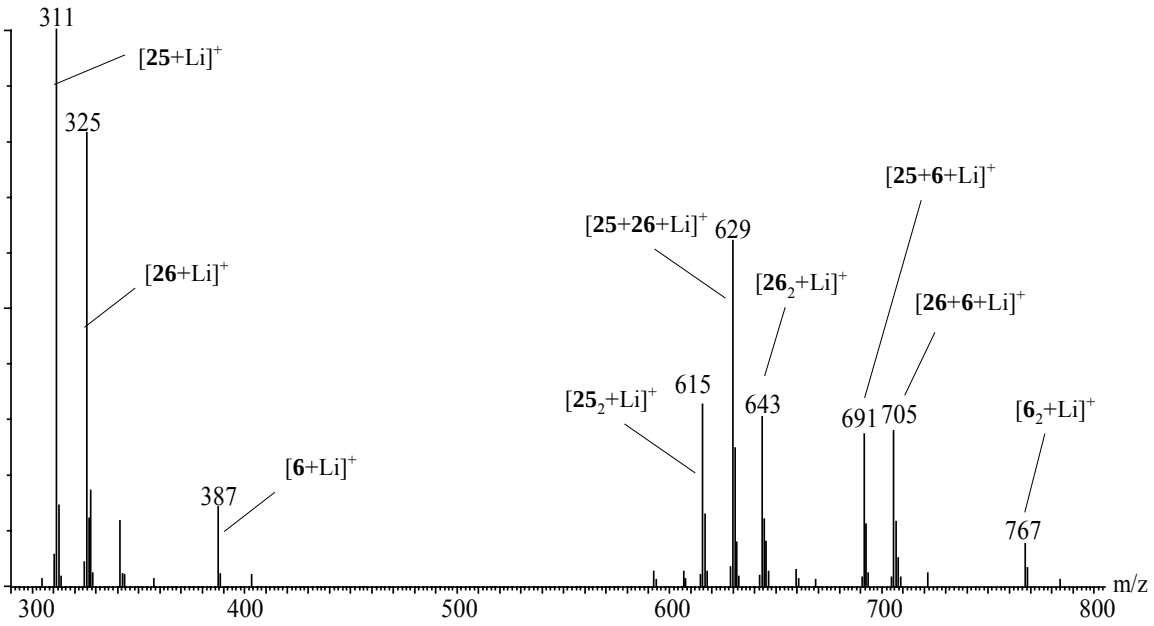


Figure 5 - 11. Electrospray ionization mass spectrum of a solution containing *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** (1.0 mM), racemic analyte **6** (0.5 mM), and hydrogen chloride (5.0 mM) in methanol / water (1 : 1).

Figure 5-12 presents the portions of the mass spectrum showing the selector-analyte complexes that are observed at three different enantiomeric composition of analyte **6**. It can clearly be seen that the relative intensity of the selector-analyte complexes changes regularly with the enantiomeric composition of analyte **6**. (*R*)-**6** has a greater affinity with the (*R*)-enantiomer of the chiral selector, and that (*S*)-**6** preferentially binds to the (*S*)-enantiomer of the chiral selector. The sense of chiral recognition agrees with what is observed using proline-based selectors (*S*)-**24** and (*R*)-**4**.

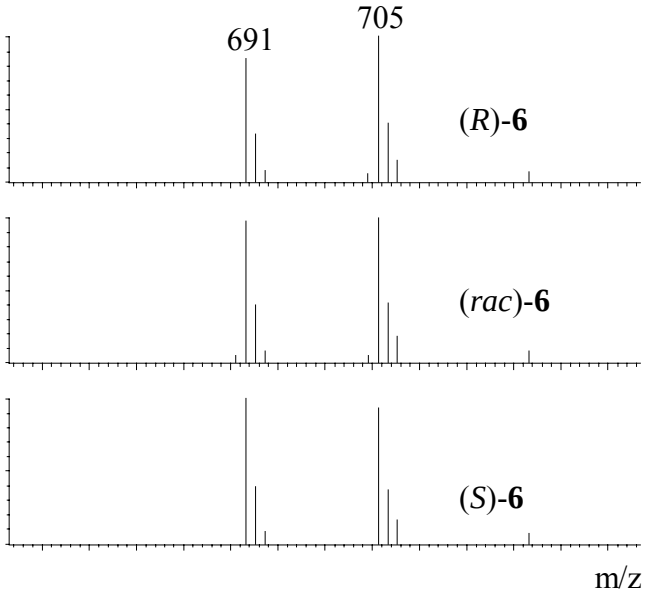


Figure 5 - 12. Electrospray ionization mass spectra of solutions containing *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** (1.0 mM), racemic analyte **6** (0.5 mM), and lithium chloride (5.0 mM) in methanol / water (1 : 1). Enantiomeric composition of analyte is noted in the figure.

Chiral recognition with hydrogen chloride as the additive

Subsequently, ESI-MS experiments were performed for solutions with added hydrogen chloride as additives. Figure 5-13 presents the mass spectrum of a solution of

the chiral selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** (1.0 mM) with racemic-**6** and hydrogen chloride in acetonitrile / water (1 : 1). The protonated selectors and analyte ions were observed at *m/z* 305 [**26**+H^+], 319 [**26**+H^+] and 381 [**6**+H^+], respectively. The homo-dimeric ions were observed at *m/z* 609 [$\mathbf{25}_2$+H^+], 637 [$\mathbf{26}_2$+H^+], and 761 [$\mathbf{6}_2$+H^+], respectively, while the hetero-dimeric complexes were observed at *m/z* 623 [**25**+**26**+H^+], 685 [**25**+**6**+H^+], 699 [**26**+**6**+H^+].

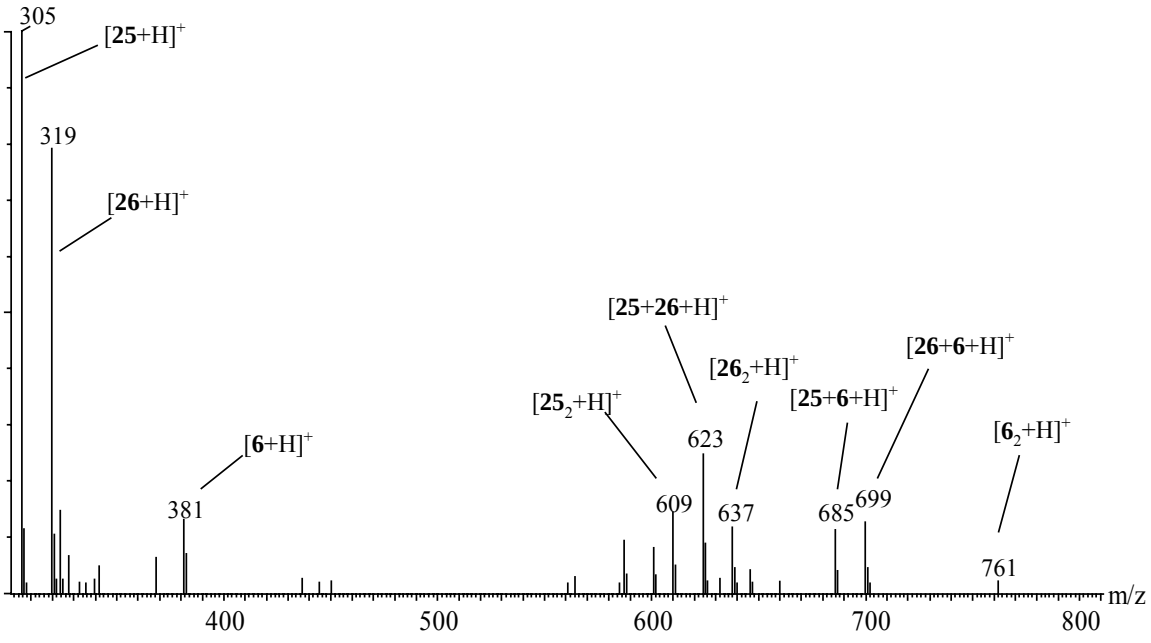


Figure 5 - 13. Electrospray ionization mass spectrum of a solution containing *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** (1.0 mM), racemic analyte **6** (0.5 mM), and hydrogen chloride (5.0 mM) in acetonitrile / water (1 : 1).

Figure 5-14 presents three partial mass spectra showing the selector-analyte complexes for solutions containing analyte (*R*)-**6**, (*rac*)-**6**, and (*S*)-**6**, respectively. Similarly, the regular changes of peak intensities are observed with the change of

enantiomeric composition of the analyte, and the sense of chiral recognition agrees with what is observed using proline-based selectors (*S*)-**24** and (*R*)-**4**.

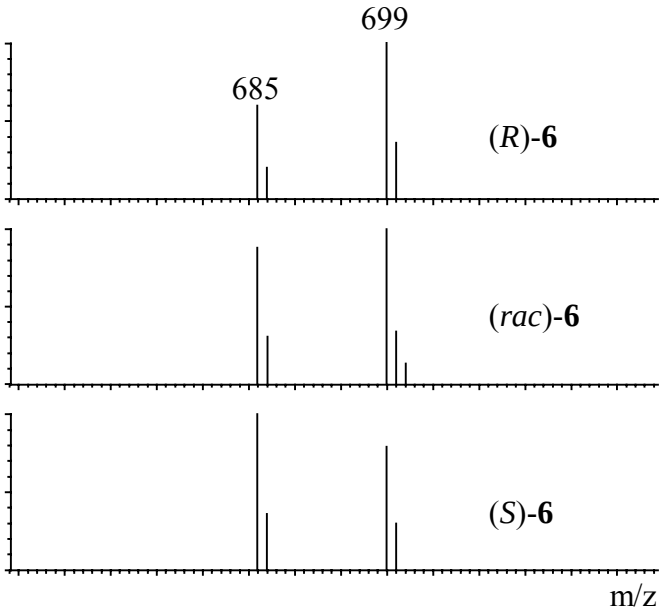


Figure 5 - 14. Electrospray ionization mass spectra of solutions containing chiral selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** (1.0 mM), (*rac*)-**6** (0.5 mM), and HCl (10.0 mM) in acetonitrile / water (1 : 1). Enantiomeric composition of analyte is noted in the figure.

Comparison of enantioselectivities between the use of lithium chloride and the use of hydrogen chloride as additives

Table 5-4 presents the observed mass spectrometric enantioselectivities, α_{MS}, using selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** for analytes **6**, **29**, **30**, **32**, **36**, and **37** with either added lithium chloride or added hydrogen chloride. The concentrations were 1.0 mM for the selectors, 0.5 mM for the analyte and 5 mM for the additive in every case. The desolvation temperature was set as 150 ^{0}C, and cone voltage was 8.0 volts for solutions containing hydrogen chloride; the desolvation temperature was 350 ^{0}C, and the cone voltage was 15 volts for lithium chloride. These are the optimized conditions where the

highest α_{MS} values were observed (the optimization procedure is similar to what is demonstrated for proline-based selectors, but detailed data are not shown here). It can be seen that the enantioselectivities in every case are largely diminished when compared with those using proline-base selectors (*S*)-**24** and (*R*)-**4** (*cf.* Table 5-2).

Table 5 - 4. Observed enantioselectivities (α_{MS}) using *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**25** and (*2R, 4S*)-**26** [a] with added hydrogen chloride or lithium chloride for ionization.

Entry	Analyte	Additive [b]	α_{MS}
1	**6** [c]	HCl	1.46
2	**29** [c]	HCl	1.12
3	**30** [c]	HCl	N/A [e]
4	**6** [d]	LiCl	1.25
5	**32** [d]	LiCl	1.02
6	**36** [d]	LiCl	1.00
7	**37** [d]	LiCl	1.21

[a] [**25**] = [**26**] = 1.0 mM; [analyte] = 0.5 mM; MeOH / H_2O = 1 : 1.
[b] [additive]= 5 mM.
[c] desolvation temp 150 ^{0}C, cone voltage 8 V.
[d] desolvation temp 350 ^{0}C, cone voltage 15 V.
[e] no selector-analyte complexes observed.

It is reasoned that the binding ability of hydroxyl oxygen is likely equal or less competitive than other groups in the selectors. The possible interaction between the hydroxyl group and the chiral recognition sites of the analyte might even deteriorate the extent of enantioselectivities.

5.3.3 Enantiomer analysis using tertiary amine appended derivatives of trans-4-hydroxyproline-(3,5-dimethyl)anilide, (2S, 4R)-27 and (2R, 4S)-28 as chiral selectors

Chiral recognition

To overcome the obstacle encountered with chiral selectors **25** / **26**, a tertiary amine group was appended onto the chiral selectors. This amine group is designed to attach through the hydroxyl group on C-4 of **26**. It is aimed to remove the ionization site from the requisite chiral recognition sites. An additional advantage is that the possible interference of hydroxyl group to the chiral recognition sites is eliminated (the hydroxyl group is converted to ester). As is shown in Figure 5-10, the tertiary amine group on (*2S*, *4R*)-**27** was from diethylamine, while that on its (*pseudo*)-antipode, (*2R*, *4S*)-**28** was from piperidine. Since this difference is occurring on the remote site from the requisite chiral recognition sites, they can act as a "true" pair of enantiomeric chiral selectors.

Solutions for ESI-MS were prepared in a similar way to before (the concentrations were noted in captions for the mass spectrum). Figure 5-15 shows the ESI-mass spectrum of a solution containing chiral selectors, (*2S*, *4R*)-**27** and (*2R*, *4S*)-**28**, and racemic-**6**, with added hydrogen chloride in acetonitrile / water. The protonated selectors and analyte were observed at *m/z* 381 $[\mathbf{6}+H]^+$, 432 $[\mathbf{27}+H]^+$, and 444 $[\mathbf{28}+H]^+$ for compound **6**, **27**, and **28**, respectively. The protonated selector dimmers were observed at *m/z* 864 $[\mathbf{27}_2+H]^+$, 876 $[\mathbf{27}+\mathbf{28}+H]^+$, and 888 $[\mathbf{28}_2+H]^+$, while the protonated selector-analyte complexes were observed at *m/z* 812 $[\mathbf{6}+\mathbf{27}+H]^+$, and 824 $[\mathbf{6}+\mathbf{28}+H]^+$.

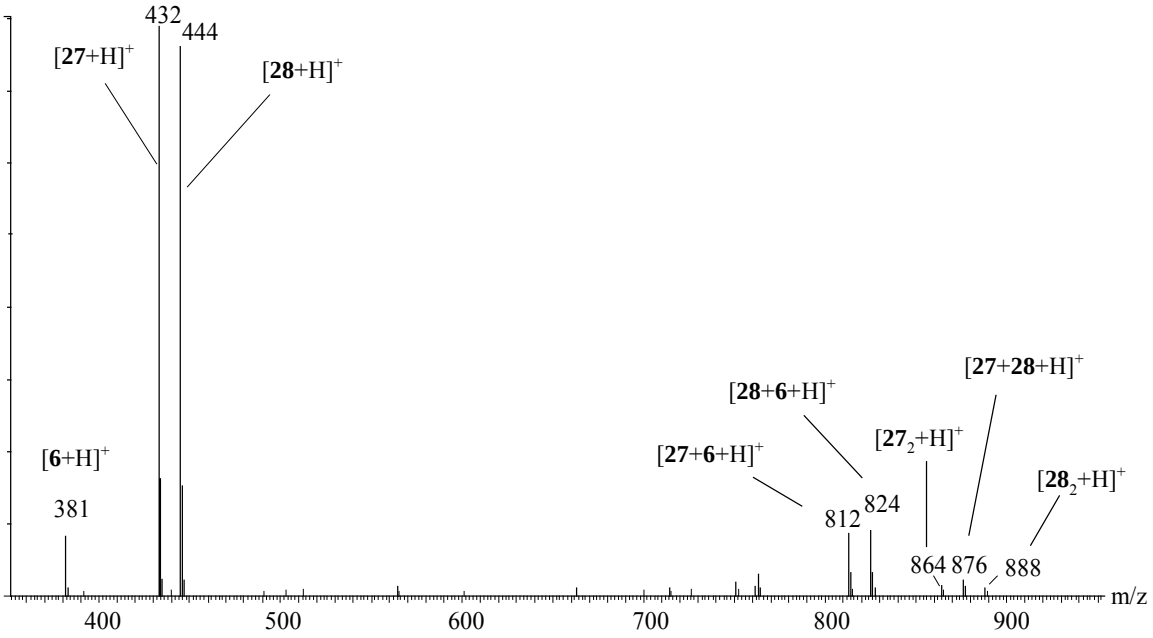


Figure 5 - 15. Electrospray ionization mass spectrum of a solution containing *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**27** and (*2R, 4S*)-**28** (250 μM), racemic-**6** (125 μM), and hydrogen chloride (5 mM) in acetonitrile / water (1 : 1).

Figure 5-16 presents the partial mass spectra, only showing the selector-analyte complexes that were observed at three different enantiomeric compositions of analyte **6**. The relative intensity of the selector-analyte complexes changes regularly with the enantiomeric composition of analyte **6**, and again, the sense of chiral recognition is consistent to what is observed using **24** / **4** as chiral selectors.

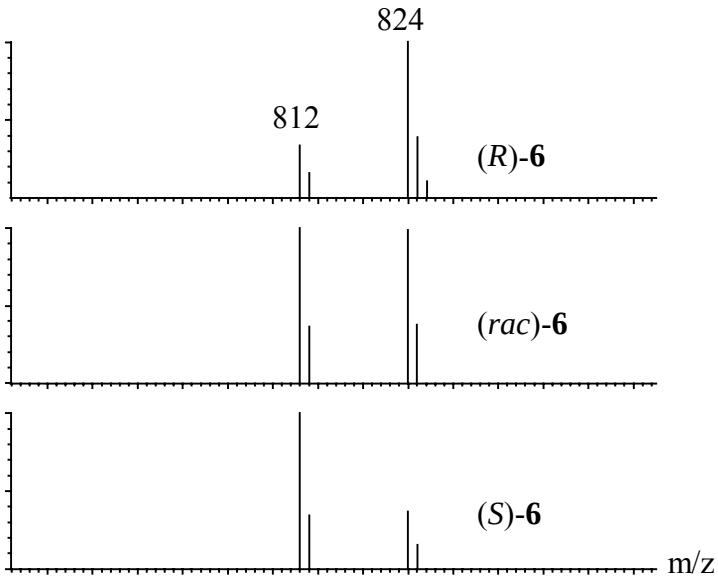


Figure 5 - 16. Electrospray ionization mass spectra of solutions containing *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**27** and (*2R, 4S*)-**28** (250 μM), analyte **6** (125 μM), and ammonium chloride (500 μM) in acetonitrile / water (1 : 1). Enantiomeric composition of analyte is noted in the figure.

A plot of CIF *vs* the mole fraction of (*R*)-**6** affords a straight line with a slope of 0.4291, and a correlation coefficient of 0.9997. The enantioselectivity, α_{MS}, is calculated to be 2.50, which is a significant improvement compared to the original chiral selectors, (*2S, 4R*)-**25** and (*2R, 4S*)-**26** (Figure 5-17).

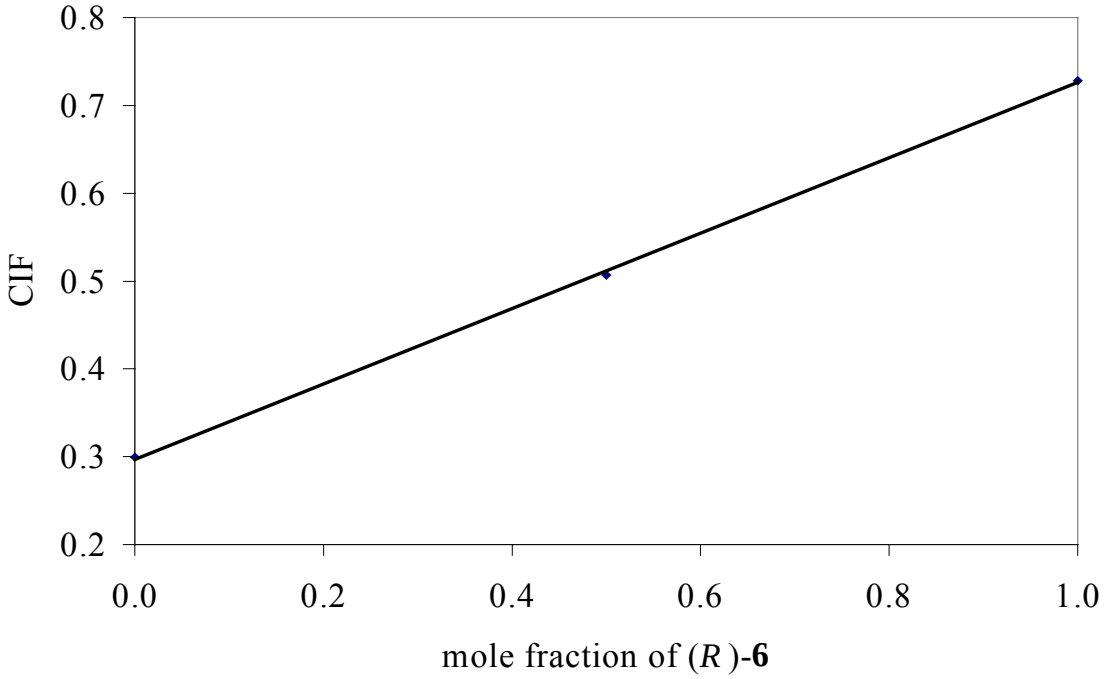


Figure 5 - 17. Plot of CIF *vs* mole fraction of (*R*)-**6**. *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**27** and (*2R, 4S*)-**28** (250 μM), analyte **6** (125 μM), and ammonium chloride (500 μM) in acetonitrile / water (1 : 1). Slope = 0.429, intercept = 0.297, correlation coefficient = 1.000.

Effects of additives on the enantioselectivities and the abundances of selector-analyte complexes

Observation of enantioselectivities of chiral selectors **27** / **28** was made with different acid additives, including ammonium chloride, acetic acid, formic acid, and hydrogen chloride. The ESI-MS conditions were: capillary voltage 3.5 kV, cone voltage 8 V, desolvation temperature 225 ^{0}C, desolvation gas flow 408 L/hr, cone gas flow 60 L/hr, source temperature 80 ^{0}C. The additives afford the following order of enantioselectivities: ammonium chloride ~ acetic acid > formic acid > hydrogen chloride for the α_{MS} values.

Figure 5-18 presents enantioselectivity data using *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**27** and (*2R, 4S*)-**28**, analyte **6**, as a function of the concentration of ammonium chloride. The concentrations of the selectors and the analyte were 250 μM and 125 μM, respectively throughout. The concentration of ammonium chloride was varying as: 0 mM, 0.5 mM, 1 mM, 2 mM, and 5 mM. The mass spectrometry conditions were kept constant throughout: capillary voltage, 3.5 kV, cone voltage, 8 V; desolvation temperature, 150 ^{0}C; source temperature, 80 ^{0}C. In the lack of any additives (0 mM of ammonium chloride), the total ion count in the mass spectrum was rather low so that it was not useful in measuring the actual CIF. Once a small amount of ammonium chloride (0.5 mM) was added, the total ion count was increased significantly. The total ion count reached the highest value at about 2 mM of ammonium chloride and kept relatively constant beyond this point. The highest enantioselectivity, however, was observed at 1 mM of ammonium chloride (2 eq. to each chiral selector). Similar results have been reported by Schug *et al*[132], that the concentration of added ions has effect on α_{MS} value.

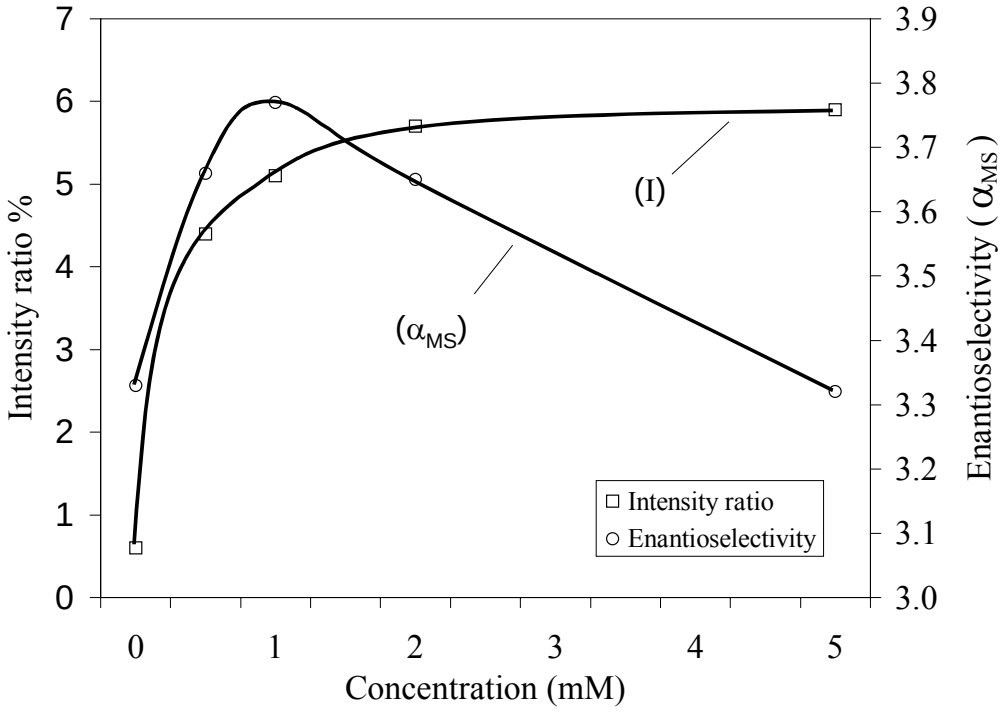


Figure 5 - 18. Observed mass spectrometric enantioselectivities (α_{MS}) (circles) and percentage ratio of the sum of the selector-analyte ion counts and total ion counts (squares) as a function of cone voltage as a function of the concentration of added ammonium chloride. [**27**] = [**28**] = 250 μM; [**6**] = 125 μM; desolvation temp. 150 ^{0}C; acetonitrile / water = 1 : 1; cone voltage 8 V, capillary voltage 3.5 kV, syringe pump 5 μL / min.

Optimization of capillary voltage and desolvation temperature

A varying voltage was applied to the capillary when the sample was being introduced into the ESI. It was observed that the enantioselectivity was relatively invariant with the change of the capillary voltage (data not shown). In addition, enantioselectivities as a function of desolvation temperature were evaluated. Clear relation was not observed, although the highest enantioselectivity was observed at 150 ^{0}C (data not shown).

Analyte survey

The mass spectrometry enantioselectivities, using *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-27 and (*2R, 4S*)-28 for a number of analytes, are presented in Table 5-5. Using this pair of *pseudo*-enantiomeric chiral selectors makes possible the direct analysis of DNB-amino acids, such as 10, 11, 40, and 41. Compared to the chromatographic chiral recognition, this will not only speed up the analysis, but also eliminate inaccuracy resulted from the peak tailing on chiral HPLC.

It can be seen from the table that, in every case where the selector-analyte complexes were observed in the ESI-mass spectrum, the α_{MS} values were greater than those observed using proline-based chiral selectors, (*S*)-**24** and (*R*)-**4**. Additionally, the sense of chiral recognition is consistent with what was observed before.

Determination of the enantiomeric composition with constructed standard line

The validity of this pair of *pseudo*-enantiomeric chiral selectors was tested in accurately determining enantiomeric composition independent of analyte concentration. To accomplish this, the enantiomeric composition of five different samples was determined at four different concentrations, using the calibration line that was constructed at the concentration different from each of these. The calibration line was constructed by fitting a plot of the CIF value *vs* mole fraction of (*R*)-**6** to a straight line. Three enantiomeric compositions of **6** were used to construct the plot: (*R*)-**6**, *rac*-**6**, and (*S*)-**6**.The concentrations were 250 μM for the chiral selectors, 125 μM for the analyte and 1.0 mM for ammonium chloride using acetonitrile and water as the solvents (1 : 1).

Table 5 - 5. Comparison of the chromatographic separation factors (α_{HPLC}) for the enantiomers of analytes on **CSP 23**[a], and observed mass spectrometric enantioselectivities (α_{MS}) using *pseudo*-enantiomeric chiral selectors (*2S, 4R*)-**27** and (*2R, 4S*)-**28,** with ammonium chloride for ionization.

analyte	k_1[b]	α_{HPLC}[c]	α_{MS}[d]
6	2.79	3.53	3.40
29	2.31	4.80	1.66
11	1.77	1.94	2.34
31	2.14	2.77	2.97
32	1.95	2.71	1.15
10	1.37	1.44	1.99
34	3.18	2.86	2.20
35	2.79	3.23	1.27
40	1.95	1.66	1.68
37	1.72	1.93	2.85
38	1.47	3.25	1.56
41	0.99	1.35	1.55

[a] eluent: acetonitrile / water (60 : 40), 1.0 mL / min for butyl amide and diethyl amide analytes; acetonitrile / water (60 : 40) with 0.1% acetic acid, 1.5 mL / min for acids analytes.
[b] retention factor for the first eluted enantiomer
[c] ratio of the retention factors for the analyte enantiomers
[d] [**27**] = [**28**] = 250 μM; [analyte] = 125 μM; [NH_4Cl] = 1.0 mM; acetonitrile / water = 1 : 1; desolvation temp. 150 ^{0}C, cone voltage 8 V, capillary voltage 3.5 kV, syringe pump 5 μL / min, desolvation gas flow 408 L / hr, cone gas flow 60 L / hr.

Table 5 - 6. Determination of the enantiomeric composition of five different samples of analyte **6** by mass spectrometry at four different concentrations using a calibration line constructed at different analyte concentrations.

	enantiomeric composition (*R*)-**6**	
[**6**], μM	HPLC[a]	MS[b,c]
200	0.974	0.910
100	0.974	0.992
50	0.974	1.026
20	0.974	1.016
200	0.749	0.686
100	0.749	0.748
50	0.749	0.758
20	0.749	0.773
200	0.547	0.524
100	0.547	0.523
50	0.547	0.546
20	0.547	0.556
200	0.259	0.278
100	0.259	0.252
50	0.259	0.252
20	0.259	0.268
200	0.039	0.102
100	0.039	0.027
50	0.039	0.026
20	0.039	0.017

[a] CSP **23**: MeOH / Water (82 : 18), 1.2 mL min^{-1}

[b] calibration line: [**6**] = 125 μM

[c] [**27**]=[**28**] = 250 μM; [NH_4Cl] = 1.0 mM; CH_3CN / H_2O (1:1); desolvation temp 150 °C, cone voltage 8 V, syringe pump 5 μL / min.

Table 5-6 presents the enantiomeric composition assays of samples of **6** at five different compositions. Each sample of **6** was analyzed at four different concentrations spanning one order of magnitude (20 μM to 200 μM). Each sample was also analyzed by chiral HPLC using **CSP 23** to allow comparisons between enantiomeric composition determinations by these two methods. As can be seen from the data, enantiomeric composition values that are accurate enough for screening applications are obtained. The majority of the data are within 0.07 of the mole fraction obtained by this method.

5.4 SUMMARY

Observations of chiral recognition in the electrospray ionization mass spectra were demonstrated, using proline-based selectors and *trans*-4-hydroxy-proline-based chiral selectors, respectively. A number of *N*-(3,5-dinitrobenzoyl)amino acid analytes were successfully assayed with these chiral selectors. The observed precision for enantiomeric composition determinations is more than adequate for most high-throughput analyses where one is often willing to trade some precision for analysis time.

In a number of instances optimization of the chiral selectivity in the mass spectrometric experiments afforded enantioselectivities comparable to what is observed by chiral HPLC. It was found that the desolvation temperature has an important effect on the enantioselectivity. The added additive is another factor that has effects on the extent of the selectivity. With the proline-based chiral selectors, the greatest enantioselectivity was observed in the presence of hydrogen chloride as additives. With the tertiary amine appended hydroxyproline-based selectors the highest enantioselectivity was observed with ammonium chloride at an appropriate concentration.

Given the correlation between the enantioselectivities observed chromatographically and by mass spectrometry, one would expect the scope of analytes that one can assay by this method should be comparable to scope of analytes that can be enantio-resolved on the corresponding chiral stationary phase. It is also expected that this method will have utility as a screening method for the discovery of new chiral selectors.[31, 133, 134]

CHAPTER VI

DISCOVERY OF NOVEL CHIRAL SELECTORS BY SCREENING A LIBRARY OF TERTIARY AMINE APPENDED DERIVATIVES OF *N*-(3,5-DINITROBENZOYL) DI-PEPTIDES PREPARED THROUGH COMBINATORIAL SYNTHESIS

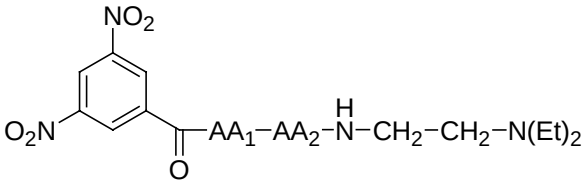
6.1 INTRODUCTION

In order to explore the enantioselectivities of dipeptides, derivatives of *N*-(3,5-dinitrobenzoyl) di-peptides were synthesized through combinatorial peptide synthesis. Although there are a number of methods[135, 136] reported for the discovery of chiral selectors, none of them applies mass spectrometry as the screening tool. Mass spectrometry is well-suited to the fast screening of combinatorial libraries because of its intrinsic rapidity.[137-139]

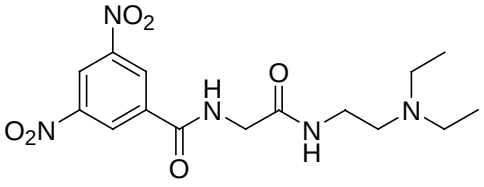
In this study, the enantioselectivities of dipeptides were evaluated using the ESI-mass spectrometry. Since the extent of chiral recognition by mass spectrometry is comparable to chromatographic chiral recognition in many cases, chiral selectors showing great enantioselectivities within mass spectrometry will likely be effective chromatographically. This will avoid the burden in preparing CSPs[133] for validation.

Combinatorial library synthesis can afford a large number of structurally diverse compounds with limited synthetic steps.[134, 140] Generally, it can be divided into two

categories:[141] (1) solution-phase synthesis and (2) solid-phase synthesis. The reaction in solution phase is rapid, but extraction / column chromatography may be needed. On the other hand, solid phase synthesis only requires washing of excess reagent. However, solid phase synthesis demands a functional group attached to a solid support that likely limit the diversity of the library. It also requires an additional cleavage step upon completion of synthesis.



AA_1, AA_2: (*L*)-amino acid



42

Figure 6 - 1. General structures of a library of chiral selectors and the structure of the internal standard, **42**.

Peptide syntheses are benefited from the appearance of different types of coupling reagents and synthetic strategies[142] for both solution phase[143] and solid phase[144]. Herein, are presented the syntheses of small libraries of DNB-dipeptides and the evaluation of enantioselectivities using ESI-mass spectrometry for the discovery of chiral selectors.

Figure 6-1 presents a general structure of dipeptides selectors. A DNB group is attached to the nitrogen end of the first amino acid (AA_1), and a diethyl amino group is

attached to the second amino acid (AA_2) through a two-carbon alkyl chain. Compound **42** is a tertiary amine appended derivative of DNB-glycine, which is used as an internal standard. It maintains a similar affinity to analyte as DNB-peptides do, but has no differentiation due to its non-chirality.

6.2 EXPERIMENTAL

Synthesis of N-(3,5-dinitrobenzoyl)glycine-(N,N-diethylethane-1,2-diamino)amide

The synthesis was accomplished in the same way as that of *N*-(3,5-dinitrobenzoyl) amino acids presented in Chapter 2.

N-(3,5-dinitrobenzoyl)glycine: ^{1}H NMR, acetone-d_6, δ 9.11 (s, 3H), 8.89 (br.s, 1H), 4.23 (s, 2H). ^{13}C NMR, acetone-d_6, δ 171.8, 164.9, 150.5, 139.0, 129.2, 122.8, 43.1.

42: ESI-MS: *m/z* 368 $[M+H]^+$.

General procedure for solution phase synthesis of dipeptide chiral selectors

To an oven dried r.b flask were added 2 mmol (*S*)-DNB-leucine, **11** and 2 mmol *N*-hydroxysuccinimide (HOSU), followed by the addition of 5 mL dry THF under N_2. The reaction mixture was cooled to 0 ^{0}C and added 2 mmol DIC was added via syringe dropwise. The white solid precipitated after stirring for several minutes. The reaction mixture was then allowed to stir at 0 ^{0}C for 2 hours and then stood in the refrigerator for half an hour. The precipitate was filtered through cotton. The filtrate was dried *in vacuo* to afford a white floppy solid, **59**.

10 mmol Na_2CO_3 was dissolved in 15 mL water in a 25 mL Erlenmeyer flask. A mixture of five amino acids: glycine, (*S*)-alanine, (*S*)-proline, (*S*)-valine, and (*S*)-leucine (0.4 mmol each) was added to the above aq. solution, followed by the addition of the intermediate, **59** in 10 mL acetonitrile. The mixture was allowed to stir at r.t for 40 min before adding 2 M HCl to acidify the solution to pH~1. The mixture was extracted with ethyl acetate (100 mL × 2), washed with saturated sodium chloride solution (20 mL × 2), dried over sodium sulfate, and evaporated *in vacuo* to afford 0.64 g of brown solid, **60** (mixture of 5 DNB-dipeptides). It was directly used for the next step without purification.

The mixture **60** was estimated to have a total of ~2 mmol DNB-dipeptides. It was dissolved in 5 mL acetonitrile and added to excess *N*,*N*-diethyl-1,2-diamine in 15 mL acetonitrile. The solution was stirred at r.t for 1 hour, then evaporated *in vacuo*, re-dissolved in methylene chloride, washed with water, dried over Na_2SO_4, and evaporated *in vacuo* to provide a brown solid. ESI-MS: *m/z* 481 [**43**+H]$^+$, 495, [**44**+H]$^+$, 521 [**45**+H]$^+$, 523 [**46**+H]$^+$, and 537 [**47**+H]$^+$.

Preparation of ketone resin

5 g of polystyrene-2% divinylbenzene copolymer resin was suspended in 30 mL nitrobenzene at 0 ^{0}C. To the stirring suspension were added 10 mmol nitrobenzoyl chloride, followed by 5 mmol aluminum chloride in several portions. The suspension was allowed to stir at 0 ^{0}C for 2 hours then at r.t overnight. The formed ketone resin was collected by vacuum filtration and successively washed with 40 mL dioxane, 40 mL dioxane / 3 M HCl (3 : 1), 30 mL dioxane, 30 mL methanol, 30 mL dichloromethane, and 30 mL methanol. The resin was dried *in vacuo* overnight.

48: IR: KBr, (cm^{-1}): 1665, 1600, 1525, 1492, 1451, 1347, 1309. The IR data agrees with the literture.[145]

Preparation of oxime resin

Ketone resin, **48** was suspended in 50 mL ethanol. To the stirring suspension was added 6 mL pyridine, followed by addition of 8.6 mmol hydroxylamine hydrogen chloride. The suspension was refluxed for 21 hours, cooled to r.t, collected by vacuum filtration, and washed successively with ethanol, 50% ethanol, ethanol, acetone, and finally dichloromethane (30 mL each). The resin was dried *in vacuo* overnight. It is showed in the IR spectrum that no carbonyl absorbance is observed at 1665 cm^{-1}, while there is a new peak at 3501 cm^{-1} for hydroxyl group. This resin, **49**, was directly used as solid support for peptide synthesis.

General procedure for solid phase parallel synthesis of dipeptide chiral selectors

The procedure is based on the reported methods.[134, 146] Oxime resin, **49** was added to the reaction vessel equipped with a stir bar and a septum. To a 10 mL test tube was added the *N*-Boc-amino acid (0.54 mmol), HBTU (0.54 mmol), DIPEA (0.72 mmol) and 3 mL anhydrous DMF. All solids were dissolved before being transferred to the suspension of oxime resin in 2 mL DMF. The resultant suspension was stirred slowly for 23 hours at r.t. The resin was washed with DMF (2 mL × 6), and then the reaction procedure was repeated for a second time. The resin was washed with DMF (2 mL × 6), and dichloromethane (2 mL × 6). The boc group was removed by treating the resin with 2 mL of 25% TFA in dichloromethane for 2 hours, and then washed with dichloromethane (2

mL × 5), 10% DIPEA in dichloromethane (2 mL × 5), and DMF (3 mL × 2). A mixture of 0.54 mmol (*S*)-DNB-leucine, **11**, 0.54 mmol HBTU, and 0.72 mmol DIPEA in 3 mL DMF was added to the above resin and the suspension was stirred for 23 hours. The resin was washed with DMF (2 mL × 6), and the same procedure was repeated for another time. The resin was then washed in the same way as before. The dipeptide was cleaved by 100 μL *N*,*N*-diethylethane-1,2-diamine in 3 mL dichloromethane for 15 hours. The collected filtrate was washed with water (2 mL × 3), dried over $MgSO_4$, and evaporated *in vacuo* to afford a brown solid. The crude product was used without further purification.

Preparation of solutions

A. For products prepared in solution phase. Stock solution of five dipeptides, **43-47** was prepared by dissolving a total of 13.0 mg (~2.6 mM, the average M.W of dipeptides was taken as ~500 Da) of a solid mixture in 5 mL methanol (~5 mM). Stock solution of internal standard, **42** was also prepared in methanol (2.5 mM). Mixing of the above two solutions at one to one ratio afforded the stock solution of chiral selectors (containing both the mixture of **43-47** and the internal standard, **42**). Stock solution of acetic acid (20 mM) was prepared in water. Stock solutions of analyte, (*R*)-**17** and (*S*)-**17** (5 mM) were made in methanol, respectively. Metered amount of the above solutions were combined to afford the final solutions for ESI-MS. The concentrations were 0.25 mM for all chiral selectors together, **43-47**, 0.1 mM for **42**, 0.25 mM for analyte **17**, and 2.0 mM for acetic acid in methanol / water (1 : 1).

B. For products prepared in solid phase. Each dipeptide was dissolved in methanol to afford a solution at a concentration of 2.0 mM. The solution of internal standard, **42**, was

also prepared at 2.0 mM in methanol. They were mixed at equal amount to provide the stock solution of chiral selector (1.0 mM for both the chiral selector and the internal standard). The stock solution of analyte was made in methanol with a concentration of 0.5 mM. The stock solution of ammonium chloride was prepared in water with a concentration of 0.4 mM. Metered amount of stock solutions of the chiral selector, the analyte, and ammonium chloride were combined to provide the final solutions for ESI-MS. The concentrations were 0.1 mM for the chiral selector, 0.1 mM for the internal standard, 0.05 mM for the analyte, and 0.2 mM for ammonium chloride in methanol / water (1 : 1).

Mass spectrometry

Solutions were infused with a syringe pump into the ESI source. Spectrometer conditions will be given in the corresponding section. For each experiment, data were collected for approximately 1 min, each full scan requiring 1 s, with all the scans averaged to afford the final spectrum.

6.3 Results And Discussion

6.3.1 Solution phase combinatorial peptide synthesis of DNB-dipeptides

Figure 6-2 presents the synthesis for a small library of chiral selectors **43-47** in solution phase. (*S*)-DNB-leucine, **11**, which was from the previous study, was converted to the succinimide derivative by reacting with HOSU using DIC as coupling reagent. This intermediate was allowed to react with a mixture of amino acids in a basic solution to

afford a library of DNB-leucine-amino acids. Finally, the mixture of DNB-leucine-amino acids were allowed to couple with *N*,*N*-diethylethane-1,2-diamine to afford a library of tertiary amine appended derivatives of DNB-leucine-amino acids, **43**-**47**.

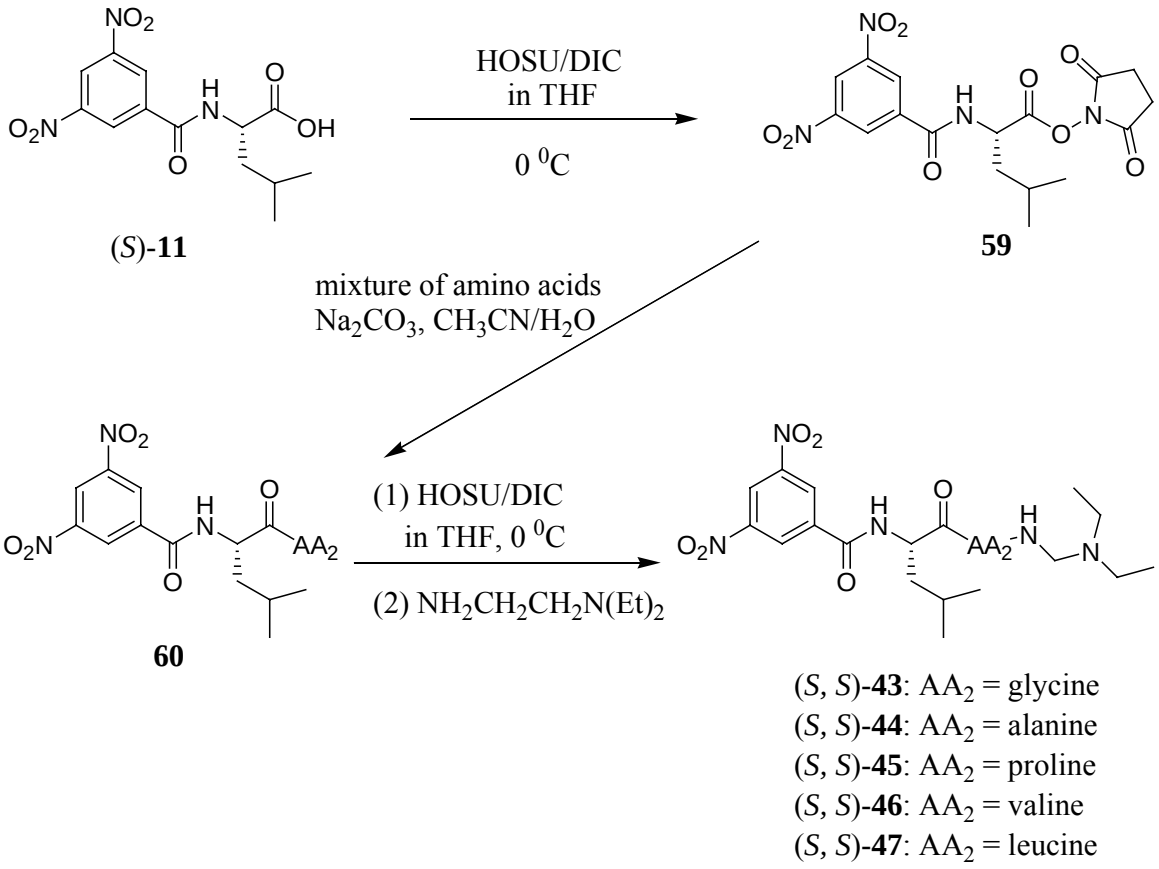


Figure 6 - 2. Solution phase combinatorial synthesis of a library of chiral selectors using (*S*)-DNB-leucine as the starting material.

Screening of the library

Solutions containing the mixture of five dipeptides, internal standard, and analyte were infused with a syringe pump into the ESI source at a rate of 20 μL / min (Figure 6-3). Spectrometer conditions were as follows: capillary, 3.0 kV; cone voltage, 12 V;

extractor, 1.0 V; RF lens, 0.5 V; source temperature, 80 ^{0}C; desolvation temperature, 325 ^{0}C; cone gas flow, 70 L / h; desolvation gas flow, 757 L / h.

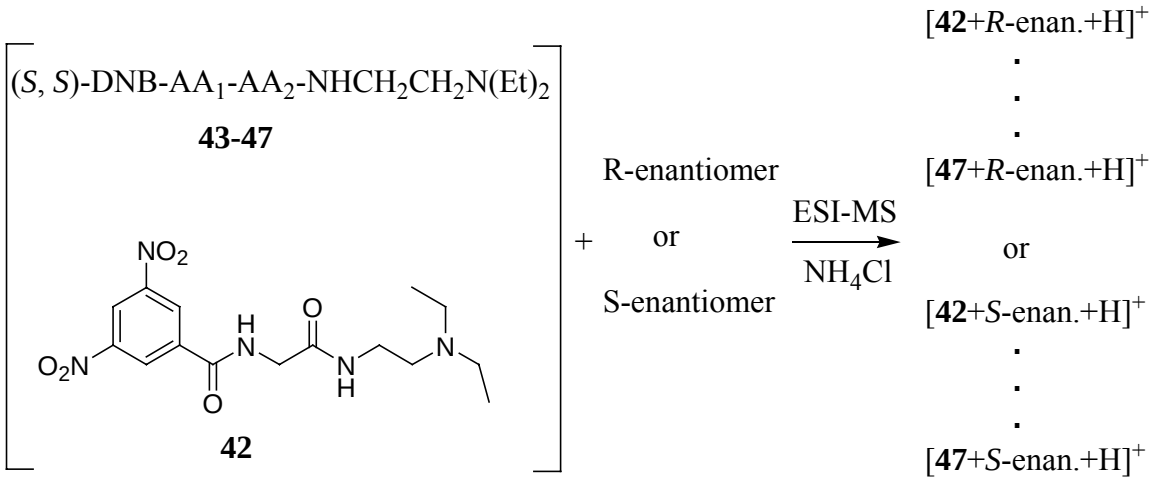


Figure 6 - 3. Screening of the library containing selectors **43**-**47** with **42** as internal standard and added ammonium chloride as additives using electrospray ionization mass spectrometry.

Figure 6-4 presents two mass spectra for solutions containing chiral selectors **43**-**47**, internal standard **42**, analyte **17** (the top spectrum is for (*S*)-**17**, and the bottom one for (*R*)-**17**), with added acetic acid for protonation. The protonated selectors were observed at *m/z* 424 [**13**+H]$^+$,* 481 [**43**+H]$^+$, 495, [**44**+H]$^+$, 521 [**45**+H]$^+$, 523 [**46**+H]$^+$, and 537 [**47**+H]$^+$, respectively. The protonated internal standard was observed at *m/z* 368 [**42**+H]$^+$, while the protonated analyte was not clearly observed at *m/z* 319, though its dimmer was observed at *m/z* 659 [**17**$_2$+H]$^+$. The protonated selector-analyte complexes were observed at *m/z* 742 [**13**+**17**+H]$^+$, 799 [**43**+**17**+H]$^+$, 813 [**44**+**17**+H]$^+$, 839

* During the synthesis in solution phase, the starting material did not 100% convert to the dipeptide, and the leftover was converted to **13** in the final step; the enantioselectivity of **13** has been discussed in Chapter 4.

[**45**+**17**+H]$^+$, 841 [**46**+**17**+H]$^+$, and 855 [**47**+**17**+H]$^+$, respectively. The protonated internal standard-analyte complex was observed at *m/z* 686 [**42**+**17**+H]$^+$.

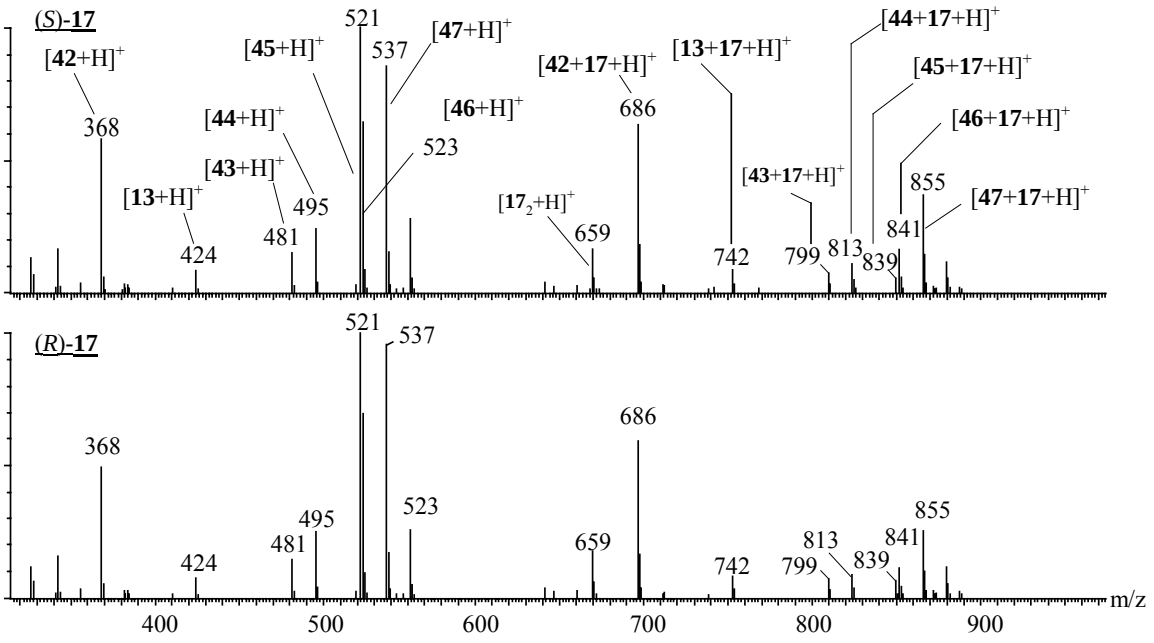


Figure 6 - 4. Mass spectra of solutions containing chiral selectors **43**, **44**, **45**, **46**, and **47**, analyte **17** and acetic acid in 1:1 methanol / water. Internal standard, **42**. The sense of analyte **17** is noted in each spectrum.

The peak intensity, *I*, of each selector-analyte complex was normalized to the intensity of internal standard-analyte complex (the normalized intensity was designated as $I^{'}$). The enantioselectivity was expressed as the ratio of $I^{'}_R$ to $I^{'}_S$ ($I^{'}_R$ is for (*R*)-enantiomer of analyte and $I^{'}_S$ is for (*S*)-enantiomer). The calculated $I^{'}_R / I^{'}_S$ values for selectors **13**, **43-47** were 1.05, 0.97, 1.50, 0.85, 1.33, and 3.26, respectively (entry 4 in Table 6-1).

Table 6 - 1. Observed enantioselectivity, (I_R'/I_S')[a], of solutions containing mixtures of chiral selectors **43-47**, internal standard **42**, and one of analytes **4**, **15-19**, **21**, and **22** in each case with added ammonium chloride as additives.[e]

Entry	Analyte[b]	**13**	**43**	**44**	**45**	**46**	**47**
1	**4**	1.16	1.10	1.71	1.19	2.06	2.12
2	**15**	1.14	1.07	1.55	0.91	1.46	1.60
3	**16**	1.06	1.08	1.49	0.94	1.31	1.46
4	**17**	1.05	0.97	1.50	0.85	1.33	3.62
5	**18**	1.06	1.00	1.32	0.84	1.33	1.46
6	**19**	1.18	1.14	1.47	1.00	1.50	1.79
7	**21**[c]	1.00	0.96	0.92	0.89	0.93	0.91
8	**22**[d]	1.05	1.11	1.32	0.97	1.23	1.37

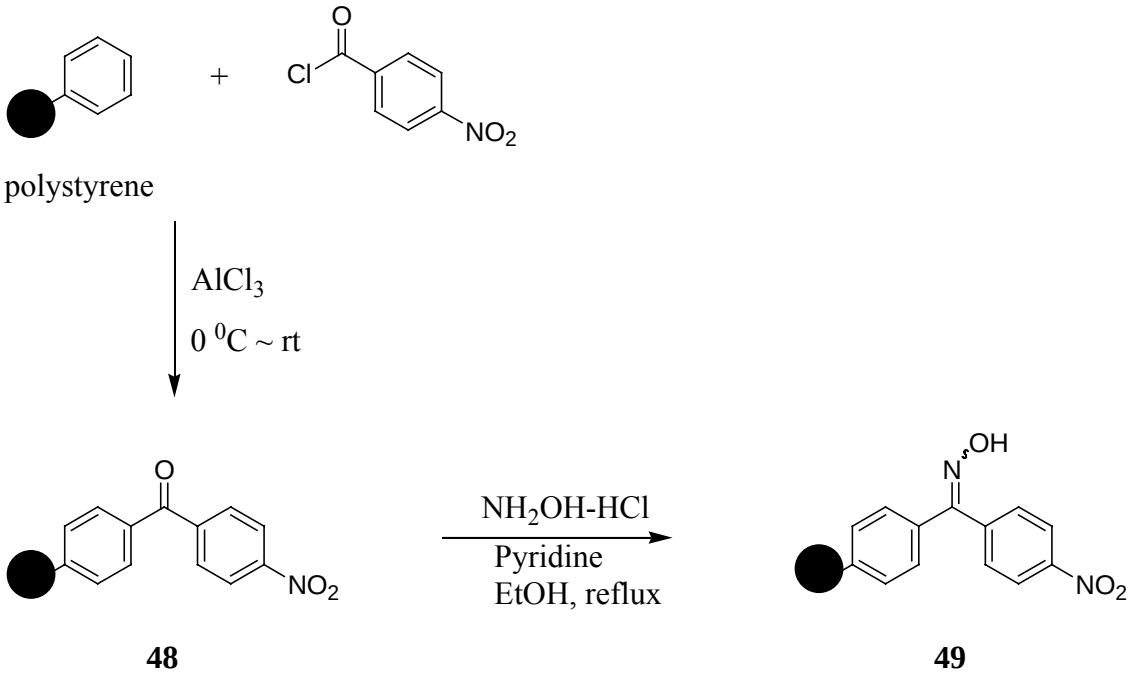
[a] $I_R' = I_R / I_{\text{(internal standard)}}$, $I_S' = I_S / I_{\text{(internal standard)}}$.
[b] See Figure 4-1 for analyte structures.
[c] (*R, R*)-enantiomer is 100%, while "(*S, S*)-enantiomer" is 72%.
[d] (*S*)-enantiomer is 100%, while "(*R*)-enantiomer" is 74%.
[e] The total concentration of all selectors is approximately 0.5 mM; [**42**] = 0.5 mM; [analyte] = 1 mM; [NH_4Cl] = 2.0 mM; solvents: 1:1 methanol / water; desolvation temperature 325 ^{0}C, cone voltage 12 V.

Table 6-1 also presents I_R'/I_S' values for analytes **4**, **15**, **16**, and **18-22** with the dipeptide selectors. Any value that is not equal to "one" indicates the existence of chiral recognition. The highest selectivity is from the selector **47** toward analyte **17**, I_R'/I_S' = 3.62. This selector also shows superior selectivities for the rest analytes compared to other selectors in the library (see the last column in the table).

6.3.2 Solid phase parallel peptide synthesis of chiral selectors

In order to validate the enantioselectivity data obtained from the mixture of chiral selectors, the single DNB-dipeptide was used as chiral selector, which was prepared through solid phase peptide synthesis. Oxime resin was used as the solid support in the synthesis. Figure 6-5 presents the preparation of oxime resin. The commercially available

polystyrene resin was modified by the reaction with *para*-nitrobenzoyl chloride to afford ketone resin, **48**. It was then converted to the oxime resin, **49** upon treatment with hydroxylamine hydrogen chloride. The IR spectrum of **49** lacks the absorbance of the carbonyl group at 1661 cm^{-1} that is present in **48**, indicating the existence of hydroxyl group of oxime.[147]



48 **49**

Figure 6 - 5. Synthesis of oxime resin.

Figure 6-6 presents the synthetic route of parallel syntheis of tertiary amine appended derivatives of DNB-dipeptides, **43**-**47**, and **50**-**58**. The Boc-protected amino acid was attached to the oxime resin through the hydroxyl group. Then the boc group was removed to release the amino group that was used to couple with a second amino acid (in this case, it is (*S*)-DNB-leucine). The DNB-leu-AA_2 dipeptide was cleaved by *N*,*N*-diethylethane-

1,2-diamine to afford the final product with a tertiary amino group attached on the carbon end.

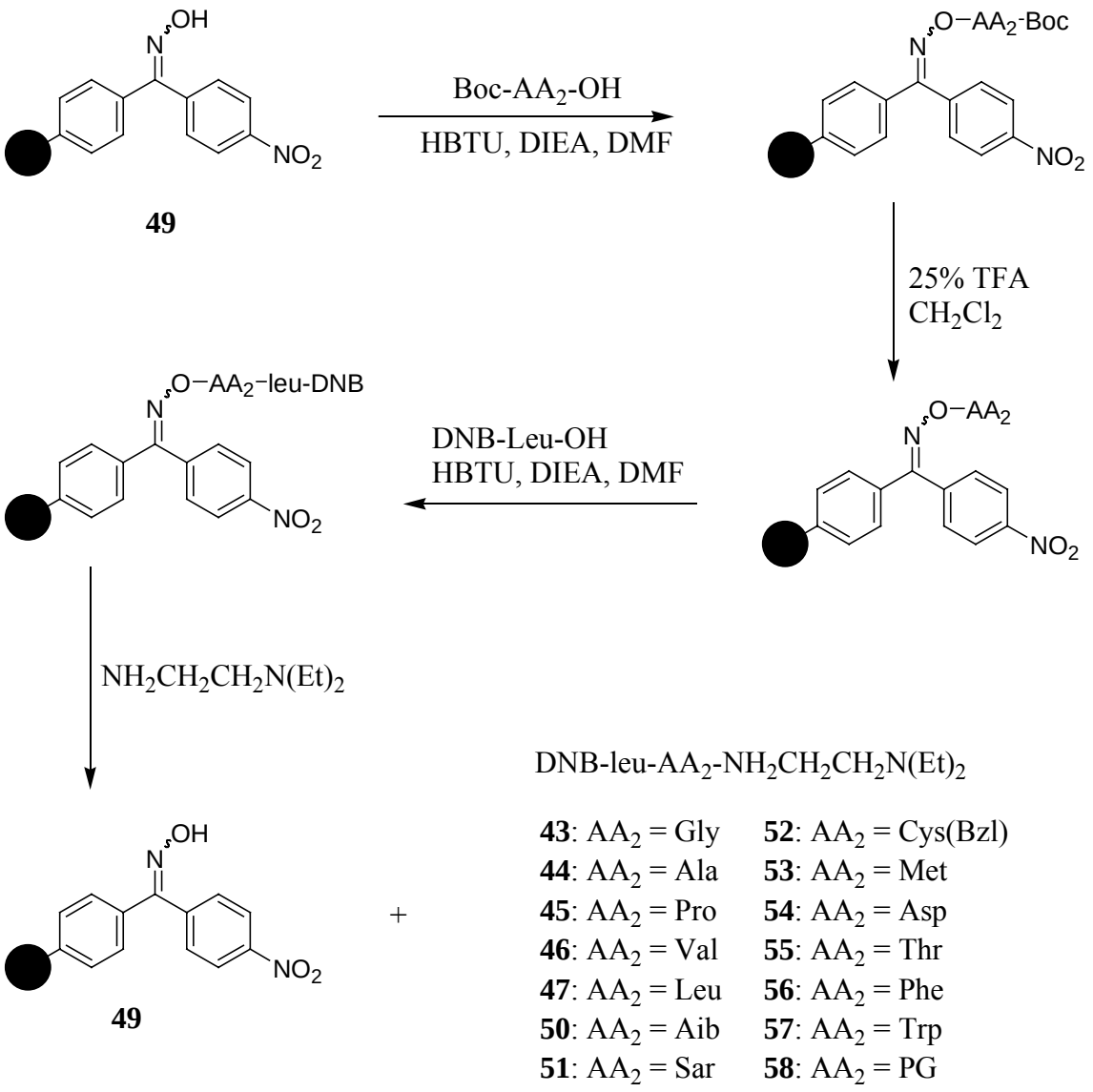


Figure 6 - 6. Solid phase parallel synthesis of a library of chiral selectors.

Screening of the library

Figure 6-7 presents two mass spectra for solutions containing chiral selector **55**, internal standard **42**, analyte **17** (the top spectrum is for (*R*)-**17** and the bottom one (*S*)-**17**), with added ammonium chloride. The protonated analyte, internal standard, and selector were observed at *m/z* 319 [**17**+H]$^+$, 368 [**42**+H]$^+$, and 615, [**55**+H]$^+$, respectively. The protonated internal standard-analyte and selector-analyte complexes were observed at *m/z* 686 [**42**+**17**+H]$^+$, and 933 [**55**+**17**+H]$^+$, respectively. The calculated $I_R^{'}/I_S^{'}$ value is 1.36.

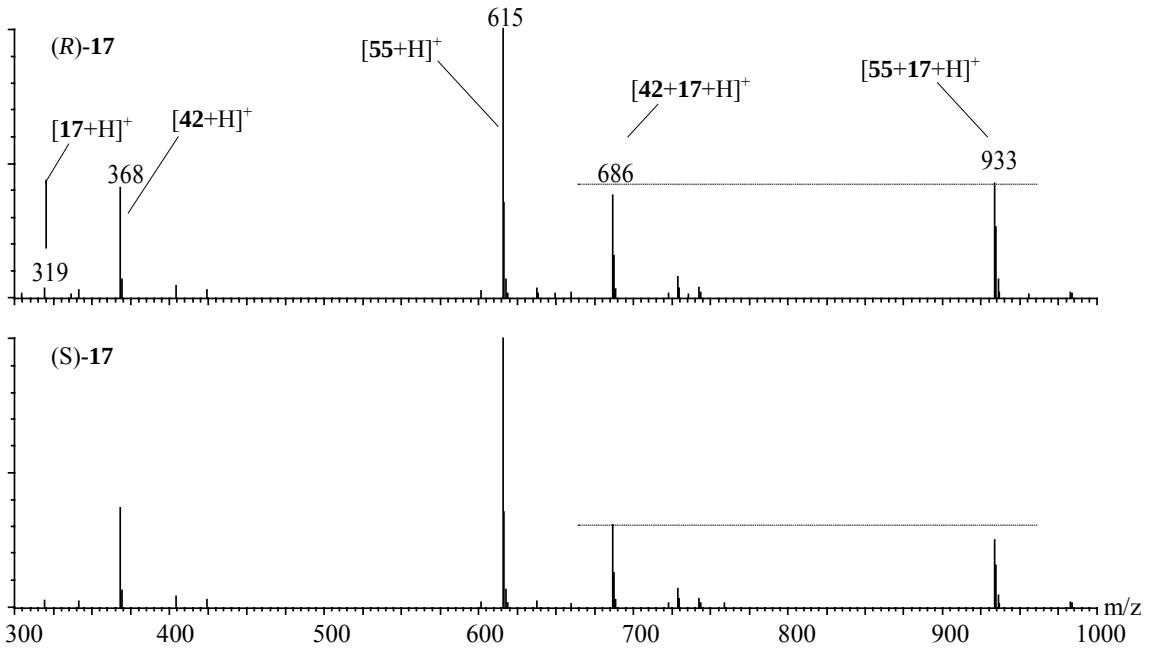


Figure 6 - 7. Mass spectra of solutions containing chiral selectors **55**, and analyte **17** with added ammonium chloride in 1 : 1 methanol / water. Internal standard, **42**. The sense of analyte **17** is noted in each spectrum.

In the same way the $I_R^{'}/I_S^{'}$ value of chiral selector (*S*)-**13** was measured to be 0.71. This indicates that it has greater affinity to the (*S*)-analyte. The sense of chiral recognition

is consistent with what is observed in previous chapters where the *pseudo*-enantiomeric chiral selectors were used. Compared to the result obtained using the mixture of chiral selectors as shown for the entry 4 in Table 6-1, where almost no chiral recognition of **13** was observed ($I_R^{'}/I_S^{'}$, 1.05), the data measured using the single chiral selector is reliable.

The remaining selectors in the library also were tested in the same way. The enantioselectivity data are presented in Table 6-2. It was observed that the extent of enantioselectivity of every DNB-dipeptide selector is more or less diminished compared to the chiral selector **13**. Among all the dipeptide chiral selectors, the largest enantioselectivity was observed for the selector **55**, which is 1.36. It can be seen that the sense of the chiral recognition is reversed to what is observed with the single amino acid chiral selector **13**.

Table 6 - 2. Observed enantioselectivity, $(I_R^{'}/I_S^{'})$[a], of solutions containing one of pure chiral selector **43**-**47**, **50**-**57**, internal standard **42**, and analyte **17** in each case with added ammonium chloride as additives. [b]

selector	$I_R^{'}/I_S^{'}$	selector	$I_R^{'}/I_S^{'}$
13	0.71	**52**	0.80
43	0.99	**53**	0.98
44	1.00	**54**	0.95
45	1.28	**55**	1.36
46	1.03	**56**	1.15
47	0.96	**57**	1.18
50	0.98	**58**	1.15
51	1.04		

[a] $I_R^{'} = I_R / I_{\text{(internal standard)}}$, $I_S^{'} = I_S / I_{\text{(internal standard)}}$.
[b] [selector] = [**42**] = 100 μM; [analyte] = 50 μM; [NH_4Cl] = 200 μM; solvents: 1:1 methanol / water; desolvation temperature 200 ^{0}C, cone voltage 8 V.

6.4 SUMMARY

The libraries of tertiary amine appended dipeptide chiral selectors were prepared through both solution phase and solid phase methods. The measured enantioselectivities for the mixture of DNB-dipeptides are suspicious of being off the real data, possibly due to the complication of the compositions in the solution. For the selectors prepared through solid phase parallel synthesis, the compound was made and screened individually, and the measured enantioselectivities are reliable since I_R'/I_S' value of **13** is consistent with what is observed using the strategy of *pseudo*-enantiomeric chiral selectors. In both cases, the tertiary amine appended DNB-glycine **42** was used as an internal standard to eliminate the fluctuation between measurements.

Future work of this project will be centered on the synthesis of a much larger library of DNB-dipeptides in order to increase the probability in hitting a successful selector. Libraries of DNB-tripeptides should be explored as a new source of selector candidates. Others may replace the DNB group in order to develop a novel type of selectors different from the classical Pirkle-type ones. More analytes, especially those with pharmaceutical applications may be tested with the synthesized selectors in the library. Finally, the successful selectors will be anchored onto the silica gel to make a CSP for chromatographic analysis or separation of enantiomers.

CHAPTER VII

CONCLUSIONS

Chiral recognition in the electrospray ionization mass spectra, using soluble analogues of Pirkle type CSPs, has been demonstrated for a number of analytes. Electrospray ionization of a solution of the analyte and a one-to-one mixture of mass-labeled *pseudo*-enantiomeric chiral selectors affords selector-analyte complexes in the mass spectrum where the complex intensity fraction for either of the selector-analyte complexes varies linearly with the enantiomeric composition of the analyte. This relationship provides a measure of the extent of enantioselectivity, and allows quantitative determination of the enantiomeric composition. For example, when using one to one mixture of Butyl amide of (*S*)-DNB-leucine, **6** and pentyl amide of (*R*)-DNB-leucine, **7** as *pseudo*-enantiomeric chiral selectors, the enantioselectivity, α_{MS} value, was obtained as 1.34 for analyte **4** in the presence of added lithium chloride; The α_{MS} value was 1.12 with amide derivatives of DNB-phenylglycine, (*R*)-**8** / (*S*)-**9** as chiral selectors. The presence of added ions likely interferes with the requisite selector-analyte hydrogen bonds needed for effective chiral recognition, though the added ions (*e.g.* Li^+) are necessary for the ease of ionization. When the conjugate bases of various DNB-amino acids were used as chiral selectors, effectively removing this additional complication, the enantioselectivities were obviously improved, but with poor precision in the

determination of enantiomeric composition. Tertiary amine appended derivatives of amino acids were also prepared. The amine was appended to provide a site for ready ionization (through protonation). It was observed that the extent of enantioselectivities was comparable to that by chiral HPLC, and the precision in the determination of enantiomeric composition was also improved.

The sense of chiral recognition observed with mass spectrometry agrees with what is observed chromatographically on the corresponding CSP. For all the cases investigated, the homochiral complexes are more stable than the heterochiral complexes. The relative intensities for the two *pseudo*-diastereomeric selector-analyte complexes change regularly with the enantiomeric composition of analyte.

Given the correlation between the enantioselectivities observed chromatographically and by mass spectrometry, it is expected that the scope of analytes by this mass spectrometry method is similar to that of chiral HPLC on the corresponding chiral stationary phase. This is ascertained by performing analyte survey with chiral selectors, including (*R*)-**8** / (*S*)-**9**, (*S*)-**24** / (*R*)-**4**, and (*2S, 4R*)-**27** / (*2R, 4S*)-**28**. In most cases the α_{MS} values are comparable with α_{HPLC} values. It is reasonable for the mass spectrometry method to be used as guidance in the discovery of novel CSPs.

The effect of the additives on the extent of enantioselectivity was investigated. The additives afford the following order: hydrogen chloride> lithium chloride ~ sodium chloride > potassium chloride for the α_{MS} values with chiral selectors, (*S*)-**24** / (*R*)-**4**. With respect to the tertiary amine appended hydroxyproline derivatives, (*2S, 4R*)-**27** / (*2R, 4S*)-**28** as chiral selectors, different acid additives were evaluated, and the order for the α_{MS} values were observed as: ammonium chloride > acetic acid > formic acid >

hydrogen chloride. The concentration of ammonium chloride also has effects on the enantioselectivity. The highest α_{MS} value is observed with two equivalences of ammonium chloride to the selector.

The α_{MS} value can be influenced by the relative concentrations of selector to analyte. The relative higher concentration of the analyte will saturate the selectors, therefore dimininshing the enantioselectivity. As long as the concentrations of the selectors are greater than the analyte, the α_{MS} value will be relatively invariant. Also, once the selector / analyte ratio is kept constant, their absolute concentrations do not have significant influence on the enantioselectivity.

Solvent effects on the enantioselectivity were evaluated with tertiary amine appended DNB-Leucine chiral selectors, (*R*)-**8** / (*S*)-**9**. It was demonstrated that water is an indispensable solvent for efficient ionization. Other solvents can be added without greatly influencing the enantioselectivity, providing that all the solvents are miscible.

Libraries of tertiary amine appended derivatives of DNB-dipeptides were prepared through both the solid phase synthesis and the solution phase synthesis. The libraries were screened using ESI-mass spectrometry in the presence of tertiary amine appended derivative of DNB-glycine, **42**, as internal standard. The normalized intensities of the selector-analyte complexes were obtained. The ratio of the two normalized intensities between the (*R*)-enantiomer and the (*S*)-enantiomer of analyte gave the enantioselectivity. The enantioselectivity data from the use of the mixture of the dipeptides are off from the truth, due to the complication of the composition in the solution; while those from the use of the single dipeptide are reliable.

A mass spectrometry method for the chiral recognition and the determination of enantiomeric composition has been developed. This method is promising in high throughput analysis, for each sample requires less than one minute for analysis. The observed precision using this method is more than adequate for most high-throughput analyses where one is often willing to trade some precision for analysis time. Additionally, it is preliminarily demonstrated that this method will be a useful tool for the discovery of new chiral selectors, particularly by combinatorial methods, which can then in turn be used for the preparation of new chiral stationary phases.

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