DEVELOPMENT, CHARACTERIZATION AND APPLICATION OF CHIRAL STATIONARY PHASES

By

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Abstract

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Cyclofructans are cyclic oligosaccharides consisting of a crown ether core with pendent fructofuranose units. These unique macrocycles were reported recently to be powerful chiral selectors. Native cyclofructans show little enantioselectivity. However, when they are derivatized and bonded to silica, they make excellent chiral selectors for HPLC. In this study, several new derivatives of cyclofructan 6 were prepared by introducing aromatic selectors with electron-withdrawing chloro and nitro group(s) and electron-donating methyl group. Their enantioselectivities were evaluated in the normal phase mode in comparison to the commercially available cyclofructan columns (LARIHC CF6-P, LARIHC CF6-RN and LARIHC CF7-DMP). In several cases, the new columns showed improved enantioselectivity compared to the existing commercially available stationary phases. Furthermore, an evaluation of

the number and position of chloro and methyl groups on the phenyl derivatizing agent is discussed in terms of their ability to alter enantioselectivity. For the other project, high-performance liquid chromatographic and gas chromatographic methods were developed for the separation of enantiomers of twelve novel aziridines. The separations were performed on cyclodextrin, cyclofructan, amylose, cellulose and macrocyclic-glycopeptide based chiral stationary phases. The amylose based chiral stationary showed good selectivity toward the aziridines while having low capacity factors. It was also shown that the high-performance liquid chromatography provided improved separations compared to gas chromatography. Effective separations for ten aziridines were developed.

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Chapter 1

Introduction

Enantiomeric Separations

Chirality is a fundamental trait of nature and is one of the most fundamental aspects of life. For instance, living organisms are composed of chiral biomolecules such amino acids, sugars, proteins and nucleic acids. These biomolecules exist dominantly in a single enantiomeric form (e.g. D-sugars and L-amino acids). Because of their chirality, living organisms show different biological responses to enantiomers of chiral drugs, pesticides, or waste compounds.[1]

Enantiomeric separations have drawn significant attention in analytical chemistry over the past few decades. In the modern pharmaceutical industry, chirality is subject of interest because many drug molecules have asymmetric centers and they can exist as enantiomers.[2]

Stereospecific chiral catalysts are very important in asymmetric synthesis. They are also vital in studies for determining reaction mechanisms and reaction pathways. Chirality has importance in agrochemical, food, flavor, and fragrance industries as well. The chiral character of a given molecule is a major concern of pharmaceutical drug manufacturers because enantiomers often have different biological

activities, pharmacokinetics or toxicities. The human body is amazingly chiral selective and may interact and metabolize each enantiomer of a racemic drug in a different way. One of the isomers may produce the desired therapeutic effect, while the other may be inactive or, in worst cases, produce unwanted effects.[3]

A major tragedy was caused by the use of racemic thalidomide in early 1960s (see Figure 1). At that time, the racemic drug of n-phthalyl-glutamic acid imide was marketed as the sedative thalidomide. Unfortunately, it wasn't discovered until after numerous births of malformed infants that the S-(-) enantiomer had teratogenic effects and lead to serious fetal deformation.[4] After this incident, it became very critical to address stereochemistry in drug development.

In 1992, the US Food and Drug Administration (FDA) issued a guideline concerning the development of new stereogenic drugs.[5] It stated that each enantiomer of the drug must be studied separately for its pharmacological and metabolic pathways. The FDA policy resulted in a great demand for developing improved methods for enantiomeric separations. The ability to separate and/or analyze enantiomers has become a prerequisite for almost all areas of research that involves chirality.

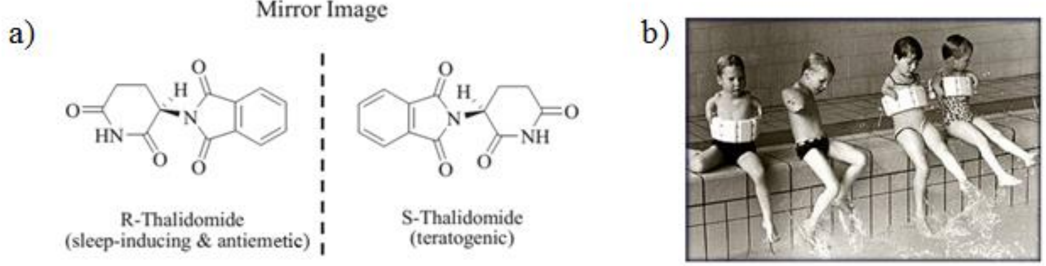

Figure 1. a) structures of thalidomide enantiomers; b) example of thalidomide tragedy.
(http://www.chm.bris.ac.uk/motm/thalidomide/first.html)

The analysis of enantiomers can be performed in both non-chromatographic and chromatographic ways. Among the non-chromatographic methods, various techniques such as polarimetry, nuclear magnetic resonance, enzyme techniques etc. have been used for the analysis of enantiomers. The major disadvantages of these techniques are that they need very pure samples, and there is no separation involved. Chromatography, on the other hand, is a highly sensitive, selective and rapid method which presents both analysis as well as separation of enantiomers simultaneously. The most commonly used chromatographic methods that have been being used for chiral separations are gas chromatography (GC), liquid chromatography (LC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE). High

performance liquid chromatography (HPLC) with chiral stationary phases is the most successful technique in the field of chiral separation because of its good reproducibility, wide selectivity and capability for achieving both analytical and preparative scale separations.

Chiral separations using HPLC

The development and maturity of chiral HPLC methods have played an important role in driving the pharmaceutical industry towards producing enantiomerically pure drugs.[6] Developing an enantioselective chromatographic method was not always easy. The most challenging aspect for chiral method development is to choose the right chiral stationary phase (CSP). Currently, there are over a hundred CSPs available commercially [7]. However, there are only a few types of stationary phases that dominate the field of enantiomeric separation. Those includes polysaccharide based stationary phases,[8] macrocyclic antibiotic based stationary phases,[7] cyclodextrin based stationary phases[7] and cyclofructan based stationary phases.[9] The large number of CSPs can be as listed in Table 1. However, even with these CSPs, it can be a challenging task to choose the right CSP and chromatographic conditions for certain specific compound.

Therefore, researchers continue developing new HPLC chiral stationary phases in order to (a) find broader applicability than existing stationary phases, (b) superior separations for specific groups of compounds, or (c) fill important unfulfilled separation niches.[9b] Apart from the CSP, it is also important that analyte and mobile phase should be taken into consideration when developing a separation method. Thus, an understanding of the possible chiral recognition mechanisms on a given CSP is key for successful enantiomeric separation of a particular class of compounds. In addition to considering some practical factors including the solubility of analyte in the mobile phase, analysis time, column cost, column robustness and column capacity should be considered.

Chiral separations using Gas Chromatography (GC)

Chiral separations using GC can be performed indirectly or directly. The indirect approach includes derivatization of chiral compound resulting in diastereomers which are subsequently separated on an achiral stationary phase. On the other hand, no derivatization is necessary for the direct approach unless the analytes are very polar. The direct approach utilizes chiral stationary phases as a selector. The CSP interacts enantioselectively with chiral molecules and this is the approach now commonly used in industry.[10]

Table 1 Different classes of chiral stationary phases

Class	Examples	Retention mechanisms	Primary interaction
Polymeric	Polysaccharides	Insertion in helical structures	H-bond or dipolar or steric
	Proteins	Multiple bonding sites	Variable
	Synthetic polymers	Diastereomeric selector/analyte complex	H-bond
Macrocyclic	Crown ether	Inclusion complexation	Ion (primary amino group) dipole
	Glycopeptides	Multiple-bonding sites	Variable
	Cyclodextrin	Inclusion complexation	H-bond
	Cyclofructans	Multiple bonding sites	Variable
ligand exchange	Hydroxyproline/ Penicillamine	Diastereomeric selector/ metal ion/analyte	Coulomb or ion-dipole (lone electron pair coordination) coulomb
π- π association	π- complex selector	Transient 3-point	π- π interaction
Miscella-neous and hybrid	Cross-linked tartaric acid deriavtives		

Pertinent references for each class and type of chiral selector can be found in references.[7, 11]

Gil-Av et al. was the first one to successfully introduce a chiral stationary phase for GC in 1960s.[12] They were able to separate derivatized chiral amino acids using a packed column for semi preparative

separation with GC. Early on, only a few selected chiral compounds could be separated by GC. Nowadays, an appropriate an cyclodextrin-based CSP is available for most volatile racemates. Some 24,000 GC enantiomeric separations, in over 2400 publications, have been reported.[10]

There are three principal CSPs that have been thoroughly investigated using GC:[10, 13]

- CSPs based on non-racemic chiral amino acid derivatives
- CSPs based on non-racemic chiral metal coordination compounds and
- CSPs based on cyclodextrin derivatives

Among them, the mostly utilized chiral stationary phases for gas chromatography are based on cyclodextrin derivatives, which are commercially known as CHIRALDEX GC columns.

Cyclodextrins are macrocyclic oligosaccharides composed of 6 or more D (+)-glulcose units bonded through α-glycosidic linkages. According to the number of glucose units they are classified into three types: α-cyclodextrin (six units), β-cyclodextrin (seven units) and γ-cyclodextrin (eight units). Native cyclodextrin, because of its high crystallinity and insolubility in most organic solvents, is difficult to formulate into GC stationary phases. However, when they are derivatized with some specific

groups (e.g. trifluoroacetyl, pentyl etc.), they act as excellent selectors towards a wide variety of racemic analytes.

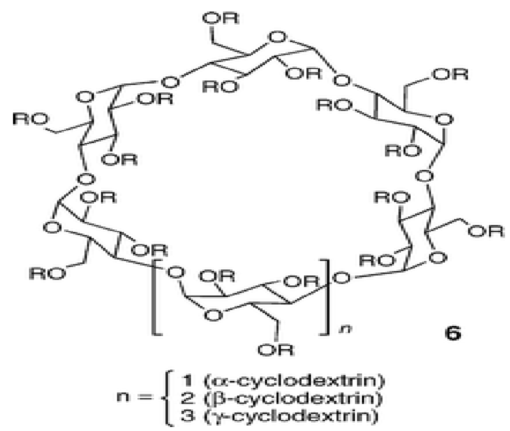

Figure 2. Cyclodextrin (R= H or derivatives).

Chapter 2

New Aromatic Derivatives of Cyclofructan 6 as Chiral Stationary Phases for HPLC

Introduction

Enantiomeric separations has been in the spotlight over the past few decades because of its importance in the field of chemistry, biochemistry, pharmaceuticals, etc. Initially, separating enantiomers was a very challenging problem. Extensive research, however, has turned it into a routine laboratory task. The development of a large number of HPLC chiral stationary phases (CSPs) is one of the driving force for the success of this technique.[9b] Developing new CSPs is still important because no commercially available CSP can separate every newly synthesized compound as well as a large number of existing chiral compounds.

Cyclofructans (CF) are cyclic oligosaccharides consisting of a crown ether core with pendant fructofuranose units (see Figure 3). It is composed of six to eight D-fructofuranose units, which are known as CF6, CF7 and CF8. These unique macrocycles were introduced recently by the Armstrong research group as powerful chiral selectors. It has been reported earlier that native cyclofructans show little enantioselectivity, however, when they are derivatized and bonded to silica, they make

excellent chiral selectors for HPLC.[9] The three derivatives (now commercially available) that have been most successful are as follows.

LARIHC CF6-P (isopropyl derivative of cyclofructans 6) is an alkyl derivatized CF6, which demonstrates pronounced enantioselectivity toward all types of primary amines, such as amino alcohols, amino esters, and amino amides. Unlike all current crown ether chiral stationary phases, this new CSP works more effectively with organic solvents and supercritical fluids. It also has excellent capabilities for preparative-scale separations. A recent study showed that this stationary phase alone can separate 93% of tested racemic primary amines.[9a]

LARIHC CF6-RN (R-napthylethyl functionalized CF6) is aromatic CSP that shows excellent chiral selectivity toward various types of analytes, including chiral acids, secondary and tertiary amines, alcohols, and many neutral compounds. This stationary phase can be operated in all three mobile phase modes, but the normal phase approach was found to be the preferred mode of operation. This is an excellent CF based CSP choice for enantiomers that are not primary amines.[9b, c]

LARIHC CF7-DMP (3,5-dimethylphenyl carbamates of CF7) is aromatic stationary phase that also separates a wide variety of chiral compounds. It can be used with all common HPLC mobile phase, but like other aromatic derivatized columns, it works most effectively in the normal

phase mode. The LARIHC CF7-DMP phase is the only commercialized cyclofructan 7 based column. Most importantly, the LARIHC CF7-DMP CSP demonstrates complementary enantioselectivity when compared to the LARIHC CF6-RN phase.[9b, c]

With all these columns, cyclofructans have been proven to be a unique and effective chiral selector. However, developing new cyclofructan based HPLC columns is still necessary, because a) a new column may separate some compounds that weren't separated before; b) there are some compounds for which commercial columns do not have baseline separation; c) as organic chemists are synthesizing new chiral entities, it is always necessary to develop new columns with different enantioselectivity for particular applications.

Extensive studies on different chiral stationary phases revealed that there are definite correlations between their chiral selectivity and their electronic and structural properties. It is reported that chiral recognition ability depends largely on the type and position of the substituents introduced on to the phenyl moiety of the derivatives.[8a, b]

Chankvetadze et al.[8a] reported eight different chiral stationary phases based on cellulose (polysaccharide) by introducing electron-donating methyl and electron-withdrawing chloro on the phenyl moiety of the derivatized glucose units of cellulose. These new CSPs were

evaluated for their chiral recognition abilities using HPLC and it was demonstrated that choromethylphenylcarbamate derivative of cellulose showed superior enantioselectivity over dichloro and dimethylphenyl derivatives of cellulose for the separation of several racemic compounds. They showed that the enantioselectivity was dependent on the positions of the CSP substituents. They also reported that the CSPs with meta- and para- disubstituted derivatives showed higher chiral recognition capabilities than the CSPs with ortho-, meta- or para- mono-substituted derivatives. They also found that any aromatic derivatized CSPs that had ortho- substituted groups on the phenyl ring exhibited poor chiral recognition ability.

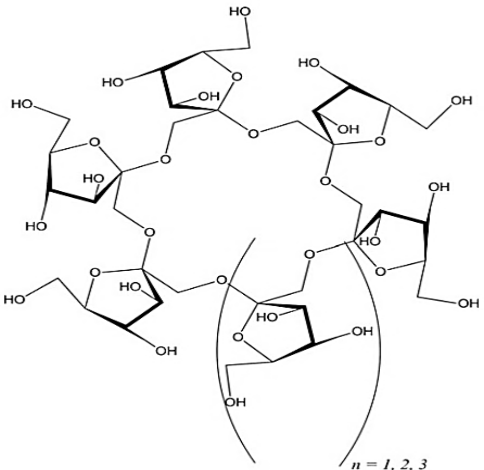

Figure 3. Molecular structure of cyclofructans, n=1, CF6; n=2, CF7; n=3, CF8.

Following the trends of the work of Chankvetadze et al.,[8a] the work reported here aimed at evaluating new classes of cyclofructan based CSPs by introducing similar types of phenyl derivatives to the CF6. The new derivatives of cyclofructan 6 were prepared by derivatization with aromatic groups substituted by electron-withdrawing (chloro and nitro) and electron-donating (methyl) groups at different positions on the phenyl ring. Their enantioselectivities were assessed in the normal phase mode, and their performanc was compared with the commercially available cyclofructan based stationary phases (LARIHC CF6-P, LARIHC CF6-RN and LARIHC CF7-DMP). Furthermore, a comparison of the number and position of chloro and methyl groups on the phenyl moiety of the derivatizing agent was also evaluated in terms of their ability to alter enantioselectivity.

Experimental

Materials

Cyclofructans are usually produced via fermentation of inulin by an extracellular enzyme from strains of various microorganisms e.g. for example *Bacillas circulans* OKUMZ 31B and *B. circulans* MCI-2554.[14] For this project, cyclofructans were obtained from AZYP, LLC (Arlington, TX, USA). The solvents used for mobile phases and packing purposes are

heptane, hexane, ethanol, 2-propanol (IPA) acetonitrile. All were of HPLC grade (≥99.5 pure) and obtained from Sigma-Aldrich (Milwaukee, WI, USA). Daiso silica gel (5 μm, 100 Å pore size and 450 m^2/g surface area) was used as supporting material for stationary phases in the packing columns. Toluene, anhydrous pyridine and 3-(triethoxysilyl) propyl isocyanate were also obtained from Sigma Aldrich. A total of ten newly developed and three commercial columns with dimensions of 25 cm x 0.46 cm (i.d.) were screened by HPLC. The stationary phases of the new and commercial columns are listed in Table 2. The three commercial columns LARIHC CF6-P, LARIHC CF6-RN and LARIHC CF7-DMP were generously donated by AZYP, LLC (Arlington, TX, USA). Most of the isocyanate derivatization reagents were also obtained from Sigma-Aldrich. They include 4-methylphenyl isocyanate, 4-chlorophenyl isocyanate, 3-chlorophenyl isocyanate, 3,5-dichlorophenyl isocyanate, 3,4-dichlorophenyl isocyanate, 3-chloro-4-methyl phenyl isocyanate and 4-chloro-2-nitrophenyl isocyanate. Others, 4-chloro-3-methylphenyl isocyanate, 4-chloro-3-nitrophenyl isocyanate and 4-chloro-2-methylphenyl isocyanate were obtained from SynQuest Labs, Inc. (Alachua, Fl, USA), TCI America (Portland, OR, USA) and Alfa Aesar (Ward Hill, MA, USA) respectively. Water was purified using Mili-Q water purification system (Millipore Billerica, MA).

Table 2 Stationary phases of the new and commercial columns

Column type	Column name	Stationary phase
New	4C3MP-CF6	4-chloro-3-methylphenylcarbamate-CF6
	4C2MP-CF6	4-chloro-2-methylphenylcarbamate-CF6
	3C4MP-CF6	3-chloro-4-methylphenylcarbamate-CF6
	35DCP-CF6	3,5-dichlorophenylcarbamate-CF6
	34DCP-CF6	3,4-dichlorophenylcarbamate-CF6
	4CP-CF6	4-chlorophenylcarbamate-CF6
	3CP-CF6	3-chlorophenylcarbamate-CF6
	4MP-CF6	4-methylphenylcarbamate-CF6
	4C3NP-CF6	4-chloro-3-nitrophenylcarbamate-CF6
	4C2NP-CF6	4-chloro-2-nitrophenylcarbamate-CF6
Commercial	LARIHC CF6-P	isopropylcarbamate-CF6
	LARIHC CF6-RN	R-naphthylethylcarbamate-CF6
	LARIHC CF7-DMP	3,5-dimethylphenylcarbamate-CF7

HPLC method

The HPLC column packing system includes and air driven fluid pump (HASKEL, DSTV-122), an air compressor, a pressure regulator, a low pressure gauge, two high pressure gauges (40 and 70 MPa, respectively), a slurry chamber (300 ml) and check valves. The stainless-steel columns (25 cm x 0.46 cm i.d.) were slurry packed with the synthesized stationary phases. The packing system and other materials were generously provided by AZYP, LLC (Arlington, TX, USA).

The HPLC chromatographic system used was an Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA), consisting of a diode

array detector, an autosampler, a binary pump and a temperature-controlled column chamber. Data acquisition and analysis were performed using Chemstation software Rev. B.03.02[341]. For all HPLC experiments, the injection volume and flow rate were 5 μL and 2 mL min^{-1} respectively. The column was at room temperature (~23.5°) for all tests. Each sample was run at least twice to confirm the enantiomeric separation. The mobile phases were degassed by ultrasonication under vacuum for 5 min before use. The analytes were dissolved in ethanol with a concentration of 2-3 mg mL^{-1}. The chromatograms were obtained in the normal phase mode, where heptane or hexane was used as the main mobile phase constituent and ethanol was used as the modifier. For the calculation of retention factors (k_1, k_2), the dead time, t_0, was determined by injecting 1,3,5-tri-tert-butylbenzene in the normal phase mode.

Synthesis of cyclofructan-based CSPs

Synthesis of cyclofructan derivatives

Cyclofructan 6 derivatives of chloro and/or methyl and/or nitrophenyl carbamate were prepared as described previously. [9b, 15] The derivatization was performed by reacting dried CF6 with molar equivalents of isocyanate reagents in anhydrous pyridine at 95°C.

Figure 4. Synthesis of CF6 derivatives.

Specifically, 2.06 mmol (2 g) of CF6 (dried overnight in vacuum oven) was dissolved in 60 ml of anhydrous pyridine at 45°C under argon atmosphere. Then, 12.36 mmol (1:6 isocyanate:CF6) of the isocyanate derivatization reagent was dissolved in 10 ml of anhydrous pyridine and added slowly into the reaction vessel with constant stirring. The temperature of the reaction vessel was raised to 95°C and the mixture was allowed to react for an additional five hours. After the reaction, the solution was rotary evaporated under vacuum followed by drying in vacuum oven over night. Structures of the derivatized CF6 is given in Figure 5. Mass spectrometry (ESI-MS) results showed that in most cases an average of five degrees of substitution was achieved. There were two exceptions 3,4-dichlorophenyl carbamate CF6 (34DCP-CF6) and 4-chloro-2-methylphenyl carbamate CF6 (4C2MP-CF6), for which the average degrees of substitution were four and six respectively (Table 3).

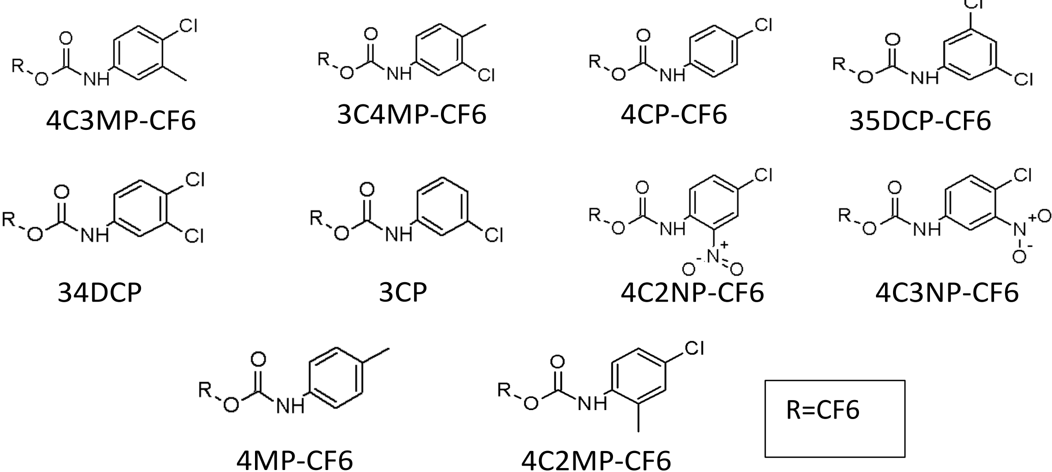

Figure 5. Structures of derivatized CF6.

Preparation of stationary phases

Several ways have been reported to bond derivatized cyclofructans to silica support in order to prepare the column packing materials.[9b, 15] In this study, cyclofructan derivatives were bonded to silica gel by 3-(triethoxysilyl) propyl isocyanate as a linker to bind them. First, 2 g of CF6 derivatives were dissolved in anhydrous pyridine at 45°C followed by the addition of the linker with a proportion to 1.5 to 1 (linker to CF6). The temperature was then increased to 95°C, and the mixture was reacted for approximately five hours.

Table 3 Properties of newly prepared CSPs

CSP	Avg. degrees of substitution	%C loading	%N loading	% Mass Loading (from C data)	Mass loading (μM/m2 SP)
4C3MP-CF6	5	15.85	*0.92*	31.7	0.54
4C2MP-CF6	6	14.74	1.08	29.1	0.44
3C4MP-CF6	5	14.85	1.34	29.7	0.49
4C3NP-CF6	5	13.24	1.87	30.5	0.47
4C2NP-CF6	5	14.23	1.91	32.8	0.52
35DCP-CF6	5	12.84	0.71	27.1	0.41
34DCP-CF6	4	12.16	0.94	27.1	0.46
3CP-CF6	5	15.28	1.28	31.4	0.55
4CP-CF6	5	14.37	1.39	29.5	0.50
4MP-CF6	5	13.07	0.81	23.8	0.40

Meanwhile, 4 g of silica gel (dried in heating oven) was dissolved in 330 ml of toluene. The mixture was heated well above the boiling point of water-toluene azeotropic mixture (b.p. of water-toluene azeotrope is 84.1°C) and a Dean-Stark trap was used to remove the azeotropic mixture to make sure that the whole system was anhydrous. After the reaction of CF6 derivative and isocyanate linker, the product was added to silica gel-toluene mixture, and the reaction was allowed to reflux overnight (approx. 12 hours). Then, the system was cooled to room temperature, and filtered using vacuum filtration over a fine fritted glass funnel. The residue was washed thoroughly with IPA, methanol, acetone and dichloromethane.

The resultant product was the stationary phase and after drying in vacuum overnight, it was ready to be packed into a stainless steel column. The structure of the stationary phase is shown in Figure 7. Elemental analysis was performed to confirm the successful bonding of derivatized CF6 to silica gel. Percent and μmol/m^2 SP carbon loading data was calculated from elemental analysis results. The results are reported in Table 3.

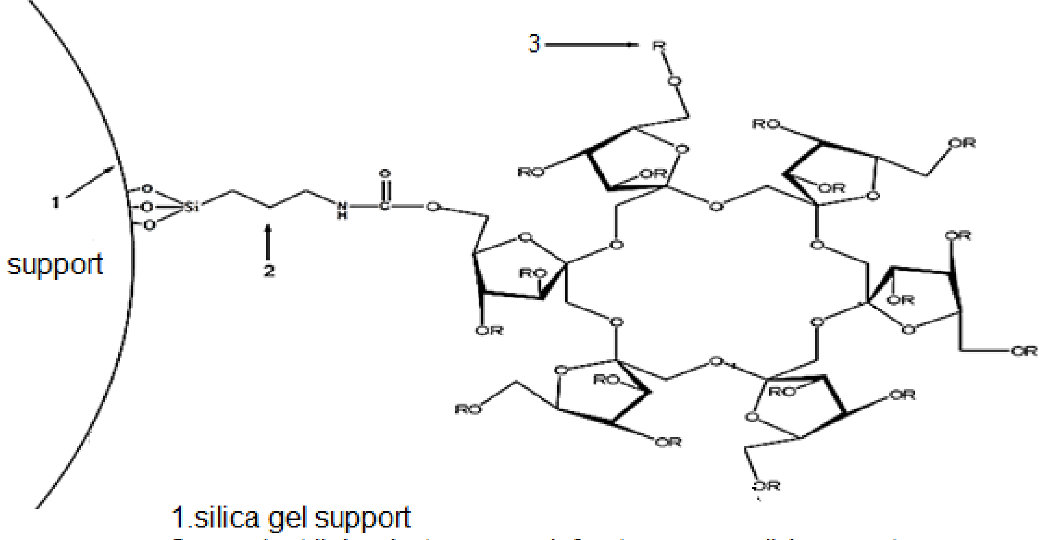

Figure 6. Structures of New Chiral Stationary Phases.

Results and Discussion

In this study, a total of ten new aromatic derivatives of CF6 were bonded to silica gel and evaluated as unique chiral stationary phases (CSPs) for HPLC. The enantiomeric separation results of seven test chiral compounds (Figure 8) were used to probe the new aromatic derivatized cyclofructan 6 based CSPs as well as the commercially available stationary phases (see Table 4).

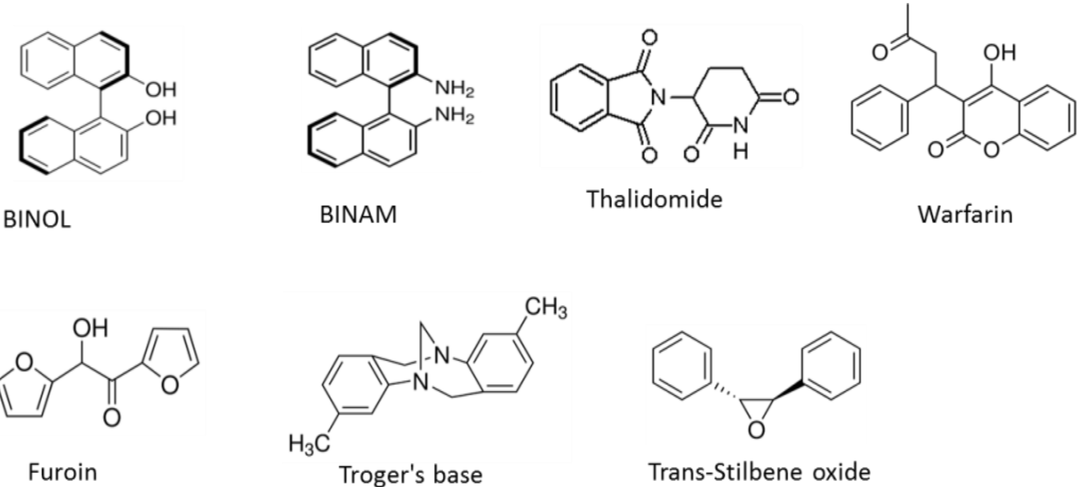

Figure 7. Name and structures of the probe chiral analytes.

The overall performance of the new HPLC columns was gauged by comparing them to the three commercially available cyclofructan columns. The new HPLC columns exhibited improved chiral selectivity in several cases. For example, the new CSPs have better separation (i.e higher

selectivity (α) values) for BINOL, Tröger's base, warfarin, trans-stilbene oxide and thalidomide, while the commercial columns provided a better separation BINAM, and a similar separation for Furoin (see Table 4).

An overview of the total number of compounds separated in each tested CSPs is shown in Figure 8, which shows that eight out of the ten new CSPs separated all probe analytes, while the three commercial columns separated three, five and six compounds individually. Moreover, the new columns provided a maximum three baseline separations while the commercial columns provided two baseline separations.

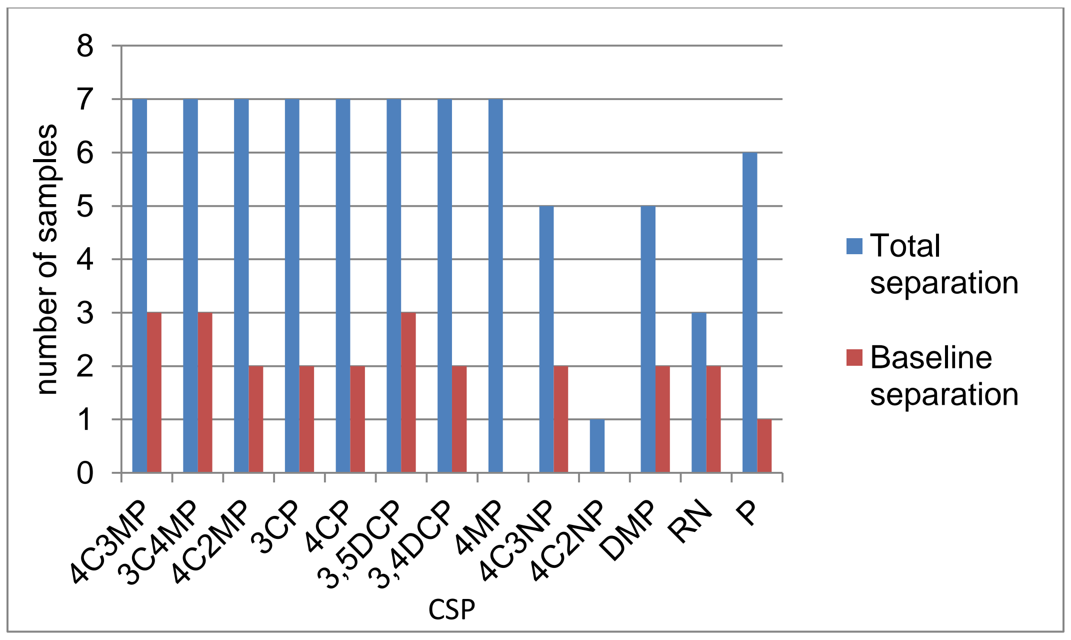

Figure 8. Number of separation of each CSPs tested.

In this study, two of the CSPs provided the best performance. One of them (4C3MP-CF6) showed the broadest selectivity with high enantioselectivity values and the other one (4C2MP-CF6) exhibited unique enantiomeric selectivity for compounds with axial chirality among the tested compounds.

Examples of enantiomeric separations obtained with the new CSPs compared to the existing commercial CSPs are shown in Figure 9. The comparison was carried out using two parameters, selectivity (α) and resolution (R_s). It is obvious that the new CSPs outperformed the commercial columns in these examples. In fact, for five out of seven compounds, the new HPLC columns showed better selectivity and/or resolution than the commercial columns. Although the commercial LARIHC CF7-DMP column proved most useful in the separation of BINAM, it is notable that four of the new CSPs (4C2MP-CF6, 4C3MP-CF6, 3C4MP-CF6 and 35DCP-CF6), also had good selectivity with baseline separation of BINAM (Table 4). Moreover, Sun et al. showed that CF7-DMP often exhibited better enantioselectivity than CF6-DMP.[9c] This project was limited to the study of CF6 because it was readily available. Therefore, further studies of the effect of derivatives, reported herein, on CF7 may provide further improvements in enantioselectivity.

Table 4 Enantiomeric separation of racemic compounds using new CSPs and commercial CSPs*

Compound	4C3MP-CF6			3C4MP-CF6			4C3NP-CF6			4C2NP-CF6		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
BINOL	6.51	1.36	3.8	5.84	1.21	2.1	7.99	1.26	2.5	8.22	1.00	0
Tröger's base	2.47	1.48	4.2	2.30	1.44	3.1	2.93	1.24	1.5	2.85	1.00	0
Warfarin	4.55	1.09	1.4	3.86	1.08	1.1	8.17	1.06	1.0	4.14	1.00	0
Trans-Stilbene oxide	1.41	1.08	1.1	1.57	1.07	0.8	1.84	1.00	0	1.92	1.00	0
Thalidomide	4.06	1.07	0.9	8.84	1.05	0.8	13.6	1.00	0	10.8	1.00	0
Furoin	10.6	1.02	0.6	10.5	1.03	0.8	10.9	1.02	0.6	11.4	1.00	0
BINAM	8.31	1.21	2.9	7.74	1.25	2.5	10.3	1.12	1.2	11.1	1.09	1.3

Compound	4CP-CF6			3CP-CF6			35DCP-CF6			34DCP-CF6		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
BINOL	5.14	1.24	1.8	5.07	1.18	1.6	4.89	1.26	2.1	4.17	1.28	2.0
Tröger's base	1.93	1.23	1.6	1.77	1.28	1.8	2.08	1.38	2.2	2.14	1.33	2.0
Warfarin	3.80	1.14	1.4	3.38	1.12	1.3	4.32	1.04	0.8	4.35	1.07	1.1
Trans-Stilbene oxide	1.14	1.07	1.0	1.21	1.07	0.9	1.23	1.05	0.8	1.03	1.05	0.8
Thalidomide	6.51	1.06	1.0	5.75	1.04	0.8	5.56	1.02	0.5	7.91	1.04	0.5
Furoin	9.28	1.02	0.7	8.87	1.02	0.7	8.99	1.03	0.8	9.16	1.03	0.7
BINAM	6.40	1.13	1.4	6.34	1.13	1.4	6.10	1.17	1.6	5.88	1.19	1.4

.

Compound	4MP-CF6			4C2MP-CF6			LARIHC CF7-DMP**		
	k_1	A	R_s	k_1	α	R_s	k_1	α	R_s
BINOL	4.81	1.08	1.2	7.21	1.75	6.9	4.17	1.08	1.1
Tröger's base	1.68	1.15	1.3	3.05	1.20	1.4	1.37	1.39	2.3
Warfarin	2.30	1.06	1.0	5.04	1.06	1.0	2.18	1.00	0
Trans-Stilbene oxide	0.80	1.06	0.8	1.73	1.07	1.0	0.73	1.07	0.8
Thalidomide	4.81	1.05	0.9	10.7	1.11	1.1	2.83	1.05	0.8
Furoin	7.51	1.02	0.6	13.2	1.02	0.7	6.08	1.00	0
BINAM	6.28	1.13	1.4	11.5	1.33	2.5	5.18	1.50	4.2

Table 4 (continued)

Compound	LARIHC CF6-RN**			LARIHC-CF6-P**		
	k_1	α	R_s	k_1	α	R_s
BINOL	6.24	1.04	0.9	5.84	1.04	0.9
Tröger's base	1.43	1.40	2.0	1.28	1.09	1.0
Warfarin	2.70	1.00	0	1.92	1.03	0.6
Trans-Stilbene oxide	0.89	1.00	0	0.75	1.07	0.9
Thalidomide	4.95	1.00	0	4.85	1.00	0
Furoin	7.91	1.00	0	8.44	1.03	0.7
BINAM	7.89	1.14	1.6	6.60	1.13	1.5

*Abbreviations of CSPs: CF-cylcofructan; C-chloro, M-methyl, and N-nitro group; k_1-retention factor; α-selectivity; R_s- resolution;
** commercial columns

Screening protocol
Mobile phase:99.5/0.5 heptane/ethanol for trans-Stilbene oxide; 70/30 heptane/ethanol for thalidomide; 95/5 heptane/ethanol for all other compounds;
Flow rate: 2 ml/min, UV at 254mn, 220 nm and 210nm

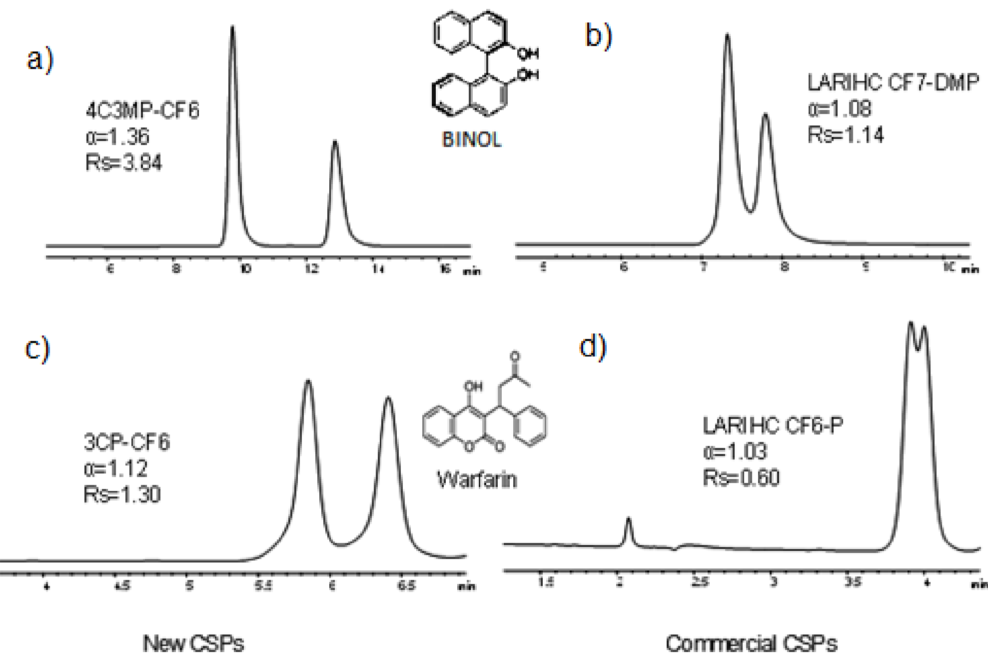

Figure 9. Separation of BINOL(a and b) and warfarin (c and d) using new CSPs compared to commercial CSPs; mobile phase: 95/5 heptane/ethanol
Flow rate: 2 ml/min UV at 254 nm.

Effects of the CSP substituents on enantioselectivity

The new CSPs, are derivatized with phenylcarbamate groups which have different substituents e.g. electron withdrawing (chloro and nitro) groups and electron donating (methyl) group in the meta-, para- and ortho-positions of the phenyl ring.

By comparing the CSPs composed of 4-chlorophenyl carbamate (4CP-CF6) and 4-methylphenyl carbamate of CF6 (4MP-CF6), it was

observed that having electron withdrawing group at the para position provides increased selectivity compared to having an electron donating group at the same position. However, when both are introduced together in the meta- and para- position (i.e. 4C3MP-CF6, 3C4MP-CF6), their enantiomeric selectivity increases significantly in most cases. These two CSPs showed improved separations for five out of seven test analytes. This was more than any of the other new CSPs tested (Table 4). It was also observed that chloro-methylphenyl substituted CSPs showed better enantioselectivity than dichlorophenyl substituted group (3C4MP vs 34DCP). Another observation was that monochloro (4CP and 3CP) substituted CSPs and dichloro substituted CSPs (35DCP and 34DCP) showed complementary selectivity. For example, 4CP-CF6 and 3CP-CF6 showed better selectivity in the separation of warfarin and trans-stilbene oxide, while 3,5DCP-CF6 and 3,4DCP-CF6 showed better selectivity of BINOL and Tröger's base. Examples of the effect of the CSP substituent are shown in Figure 10.

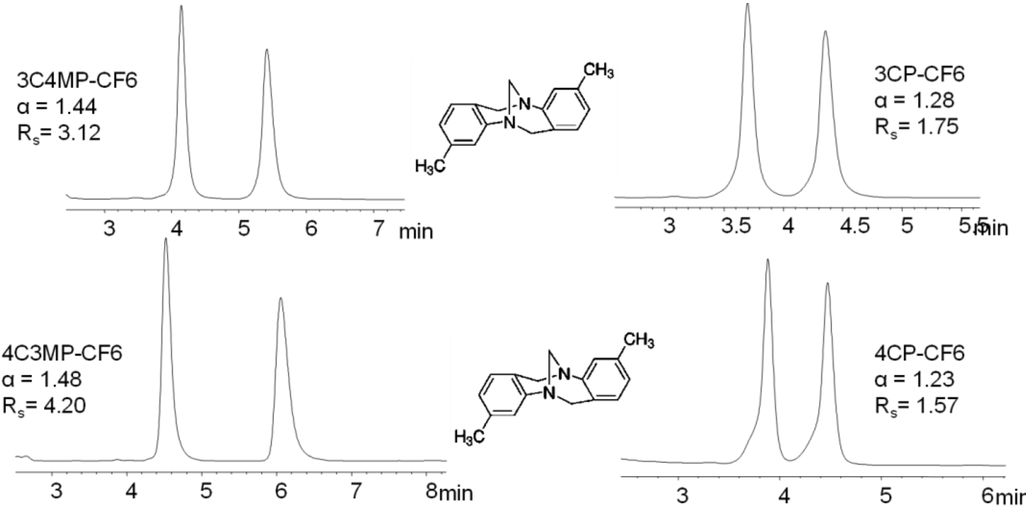

Figure 10. Effect of substitution group on separation of Tröger's base. Flow: 2 ml/min; mobile phase: 95/5 heptane/ethanol; UV at 254 nm.

Lastly, by comparing 4C3MP-CF6, 3,4DCP-CF6 and 4C3NP-CF6 it was interesting to see that ,for disubstituted CSPs where the chloro group is fixed at para position, enantiomeric recognition decreases as the second substituent at meta position changes from a methyl group (electron donating) to a chloro group (electron withdrawing) to nitro group (strongly electron withdrawing). Table 4 shows that for almost all the tested racemic compounds, 4C3MP-CF6 showed higher enantioselectivity than 34DCP-CF6 which showed better selectivity than 4C3NP-CF6. An example of this phenomenon is given in the Figure 11.

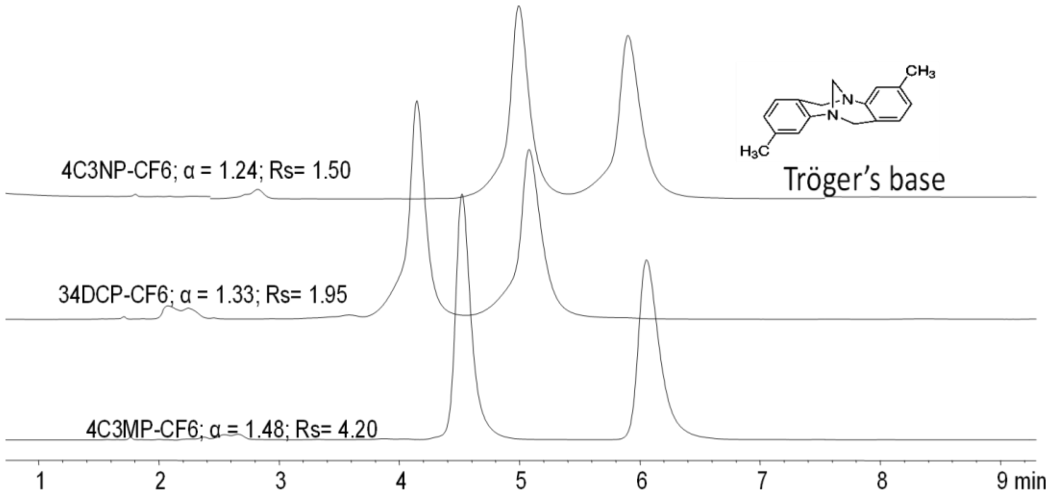

Figure 11 Separation of troger's base comparing the effect of CSP substituent.

Like polysaccharide based CSPs, it was found that when the derivatized CF6 has a substituent at th ortho position, it drastically loses its selectivity [12]. For example, comparing 4C3NP-CF6 and 4C2NP-CF6, it is clear that 4C3NP-CF6 separated five compounds, while 4C2NP separated only one (see Table 4).

To further probe this ortho substitution effect, a 4-chloro-2-methylphenyl carbamate CF6 CSP (which has both electron withdrawing and electron donating groups and one of them is at ortho- position) was studied. Surprisingly and also unexpectedly, this stationary phase worked quite well for a specific class of chiral compounds. Table 4 shows that this unique CSP showed the best selectivity for BINOL and BINAM, though, it did show the typical ortho- substitution effect by exhibiting poor selectivity

for some other compounds. BINOL and BINAM have axial chirality (i.e. they are atropisomers). The results indicate that the 4C2MP-CF6 CSP excels in the separation of axially chiral compounds. The reason for this result is unclear, but future separations of such chiral compounds may warrant the use of this unique CSP. Figure 12 shows the separation of atropisomers using 4C2MP-CF6 CSP compared to the 4C3MP-CF6, a CSP which was the best overall.

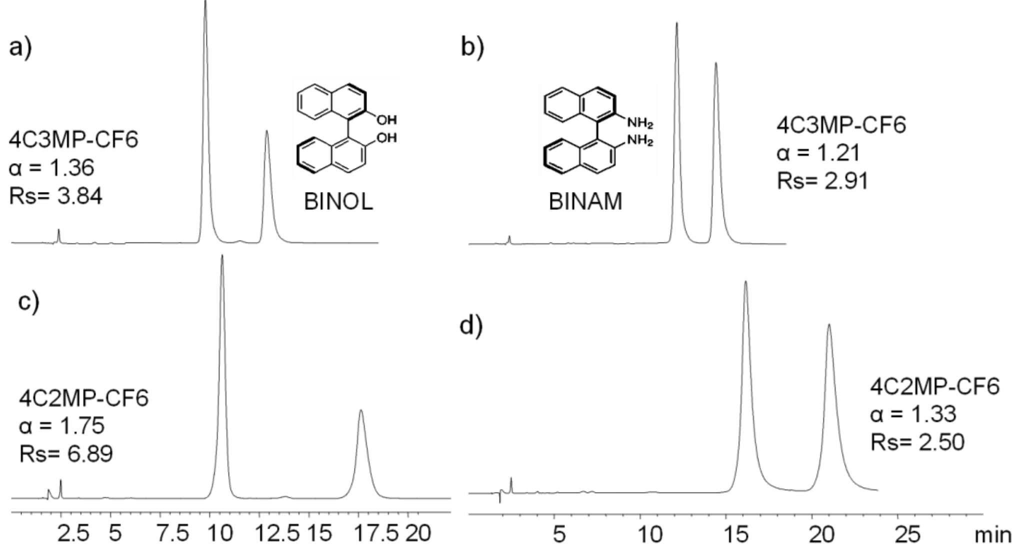

Figure 12. Separation of BINOL and BINAM using 4C2MP-CF6 compared to 4C3MP-CF6; mobile phase: 95/5 heptane /ethanol; Flow rate: 2 ml/min; UV at 254 nm.

Conclusions

The chiral recognition abilities of ten new aromatic derivatives of cyclofructan 6 were evaluated for HPLC, and they were compared with three commercially available stationary phases. Though, it has been reported that the commercial columns are good chiral selectors toward a variety of compounds including acidic, basic and many neutral molecules. In this study, it was established that in several cases the newly developed CSPs showed improved selectivity. It was also observed that the type and position of the substituents on the phenyl group of derivatizing reagents affected selectivity and enantiomeric separations. Among the new CSPs, the 4-chloro-3-methylphenyl isocyanate derivative of CF6 generally showed highest resolving powers for most of the tested.

The derivatized CSP with ortho substituents generally showed poor enantioselectivity. However, the one unique chiral selector 4-chloro-2-methylphenyl carbamate CF6 showed better enantioselectivity for atropisomers than any other CSP tested.

Chapter 3

Enantiomeric Separations of Aziridines using GC and HPLC

Introduction

Aziridines are an analog of the 3-membered epoxide ring, where the oxygen is replaced with a nitrogen.[16] These molecules have a bond angle of 59.7° which is significantly less than thermodynamically stable hydrocarbon bond angle of 109.47°.[16a] The decreased angle leads to ring strain which contributes to the high reactivity of these heterocyclic compounds.[16a, b] In addition, due to decrease of the C-N-C bond angle, the C-N bonds increase their p-character resulting the lone pair of electrons on the nitrogen and the N-H bond to exhibit increased s-character. Consequently, aziridines experience less basicity (pKa of 7.98 for its conjugate acid) than its complementary acyclic aliphatic amine.[16a] Aziridines are subject to the Thorpe-Ingold effect, where there is an increase in the pyramidalization angle because of the decrease in the C-N-C bond angle.[16a] This effect results in an increase in basicity when such heterocyclic ring systems increase in size from three- to six-membered cyclic amines.[16a]

Natural and synthetic aziridines have been intensely studied due to their biological properties and reactivity.[16b] As alkylating agents, aziridines

exhibit cytotoxicity. It has been subjected to intensive investigation for their synthesis and pharmacological activity.[16a] Consequently, aziridines were reported to have antitumor and anticancer activities against some cancer cell lines as well as solid growth tumors.[16a] In addition, they can also be used as antibacterial and antimicrobial agents. Among the more well-known examples are the Mitosanes, natural products, which were found in *Streptomyces verticillatus*.[16c] Mitosanes have both antitumor and antibacterial properties.[16c] The three-membered aziridine ring has been identified as essential for DNA interactions, which lead to antitumor activities.[16c] In other work, lipophilic aziridines have been used as probes and markers for protease, an enzyme which degrades proteins.[17]

Aziridines have many applications as starting materials in the synthesis of catalysts and chiral auxiliaries.[16b, 16d, 18] They are commonly used reagents in the formation of α and β-amino acids and various irreversible protease inhibiters.[17] Because of the alkylation capability, aziridines undergo attack by nucleophiles such as the nitrogenous bases in DNA.[16a] In addition, their basicity, hydrogen bonding capability, rigid structure and high reactivity make them ideal as interaction agents with proteins.[16a] Furthermore, the ability to control ring-opening reactions leads them to undergo regioselective and stereoselective transformations. This property makes aziridines as desirable chiral auxiliaries.[16b, 16d]

Previously the synthesis of aziridines met with difficulties due to ring opening, degradation and side reactions.[16b, 19] The two main methods for the synthesis of aziridines required either strong oxidants or strong electron withdrawing N-protecting groups.[16b, 19] When olefins or imines are used as starting materials, the 3-membered rings produced were often unstable and/or allylic C-H amination products were formed.[16b, 19] Until recently, there were no protocols for the direct synthesis of N-H and N-alkyl aziridines. However, in 2014, a simple method utilizing a rhodium catalyst to directly convert olefins to aziridines was developed.[16b] This approach was shown to be chemoselective and stereospecific. In addition, the new protocol was applied to a wide range of olefins in order to create a variety of aziridines.[16b] Thus far, there have been limited reports on the enantiomeric separations of chiral aziridines. In one case 5 compounds were separated utilizing capillary electrophoresis with aqueous and nonaqueous run buffers.[17] The aziridines had limited solubility in the aqueous phase and, therefore, nonaqueous system was preferred.[17] In this work, we reported the separations of a wide variety of newly available aziridine racemates using both enantioselective HPLC and GC. The two approaches were compared and contrasted. To our knowledge, there have been no reported enantiomeric separations of these or any related compounds by any of these methods.

Experimental

Method

An Agilent 1200 HPLC system consisting of a diode array detector (DAD), an autosampler, a binary pump and a temperature-controlled column chamber (Agilent Technologies, Palo Alto, CA, USA) was used. Data acquisition and analysis were obtained from the Chemstation software version Rev. B.03.02[341]. Separations were performed at room temperature (23.5 °C), if not specified otherwise. The injection volume and flow rate was 5 μL and 1 mL min^{-1} respectively. The wavelength of the UV detector was set at 210, 220, 254, and 280 nm. All GC enantiomeric separations were performed on an Agilent 6850 GC series system equipped with a flame ionization detector (FID) and an autosampler (Agilent Technologies, Palo Alto CA). Data acquisition and analysis were done with Chemstation plus software (Rev. B.01.02). All analysis were performed with a helium carrier gas and at a flow rate of 1 mL/min and a split ratio of 5:1. The oven temperature was held isothermally. The injection port and detector were set to 250 °C.

Materials

The HPLC separations were performed on 25 cm x 0.46 cm (i.d.) columns. The LARIHC-CF6-P (isopropyl carbamate-cyclofrucatn) was

obtained from AZYP, LLC (Arlington, TX).[9a, b] The two Chirobiotic columns, Chirobiotic TAG (teicoplanin aglycone) and Chirobiotic V2 (vancomycin), were obtained from Supelco, LLC (Bellefonte, PA).[20] The polysaccharide based CSPs, Chiralpak IB (cellulose 3,5-dimthylphenylcarbamate), Chiralpak IC (cellulose 3,5-dichlorophenylcarbamate), Chiralpak ID (amylose 3-chlorophenylcarbamate), Chiralpak IE (amylose-3,5-dichlorophenylcarbamate) and Chiralpak IF (amylose-3-4-methylphenylcarbamate) were obtained from Chiral Technologies (West Chester, PA, USA). The GC separations were performed on a 30m x 0.25mm i.d., 0.12 μm film thickness capillary columns. A total of six cyclodextrin based commercial GC columns were used for this study. Those were CHIRALDEX G-TA (2,6-di-O-pentyl-3-trifluoroacetyl-γ-cyclodextrin), CHIRALDEX B-PM (2,3,6-tri-O-methyl-β-cyclodextrin), CHIRALDEX B-DM (2,3-di-O-methyl-6-t-butyl silyl-β-cyclodextrin), CHIRALDEX B-PH ((S)-2-hydroxy propyl methyl ether-β-cyclodextrin), CHIRALDEX B-TA (2,6-di-O-pentyl-3-trifluoroacetyl-β-cyclodextrin) and CHIRALDEX B-DA (2,6-di-O-pentyl-3-propionyl- β-cyclodextrin), obtained from Supelco, LLC (Bellefonte, PA).[21] All the HPLC grade solvents, heptane, hexane, methanol, ethanol, and acetonitrile as well as the diethylamine additive were obtained from Sigma-Aldrich (Milwaukee, WI).

The 12 aziridines, listed in Table 5, were synthesized are reported in the reference 16b.

Table 5 Structures, abbreviated names and the number associated with the compound are listed

Compound number	Chemical Structure
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	

Results and Discussion

All racemic aziridine samples were analyzed on 6 GC chiral columns (see the materials section) and six of these compounds were found to be resolved for the enantiomers. Optimal separations are given in Table 6. The CHIRALDEX B-PM column had the broadest selectivity among the tested GC columns in separating the tested aziridines as it was able to separate maximum four compounds. A comparative study for the performance of different GC CSPs in separating the probe aziridines is shown in Figure 13. An example chromatogram for the separation of compound 2 is shown in Figure 14. Although some analytes were separated with good selectivity, the resolutions were compromised by band broadening and large retention factors.

The analysis performed by GC, did not always provide ideal retention times. With the Chiraldex B-PM CSP, runtimes of hours were often required in order to obtain adequate resolution. Only one of the enantiomeric separations produced by the GC had a retention time under 1 hour, as seen in Figure 14. Given the novelty of most of these compounds, we were also sample limited for many determinations.

Table 6 Optimized separations of 5 aziridines utilizing GC. The retention factors (k), selectivities (α), and resolutions (Rs) are also given

Compound	GC column	Temperature	k	α	R_s
1	G-TA	100	45.9	1.04	0.5
2	B-PH	100	9.8	1.04	1.0
5	B-PM	120	109.0	1.04	1.80
6	B-PM	120	109.0	1.04	1.8
8	B-PM	120	90.1	2.37	2.2
11	G-TA	100	40.0	1.10	2.0

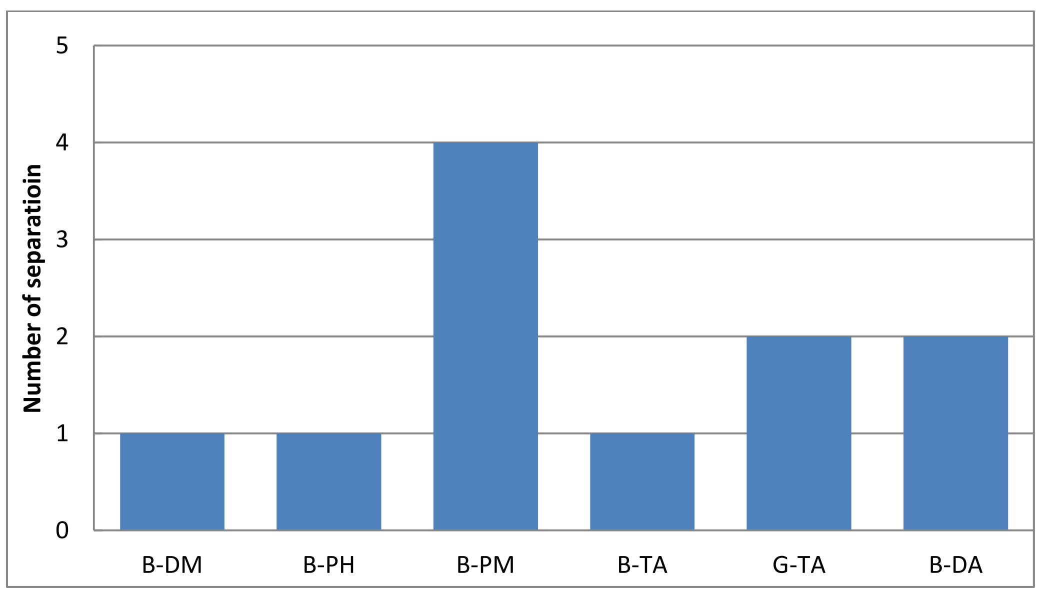

Figure 13. Total separations accomplished by GC chiral stationary phase. See the experimental for an explanation of the abbreviated name of each CSP.

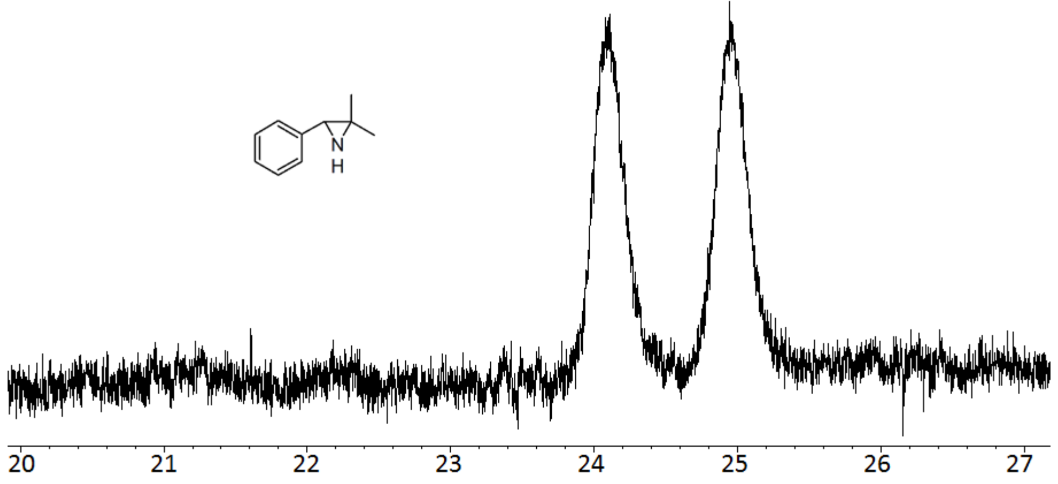

Figure 14. Optimized separation of compound 2 on the Chirldex B-PH, with a split ratio of 5:1 split ratio at 100 °C.

Interesting results were obtained for compound 6 on the Chiraldex B-PM column. Unlike the other used chiral stationary phases (CSP) where only the enantiomers could be separated, on the CHIRALDEX B-PM column, the major enantiomeric pair, compound 6, was partially separated and the minor enantiomeric pair, compound 5, was baseline separated. These two compounds, compounds 5 and 6, are cis/trans-isomers (Table 5). In this case, compound 5 apears as a small impurity formed during the synthesis of compound 6 and therefore referred to as the minor component pair. The major pair of enantiomers, as seen in Figure 15A, elutes after 5 hours at 100 °C and the minor pair of enantiomers elutes after the major enantiomeric pair. The temperature

was therefore increased to 120 °C in order for the minor pair of enantiomers to elute in less time, as seen in Figure 15B, while maintaining its baseline separation.

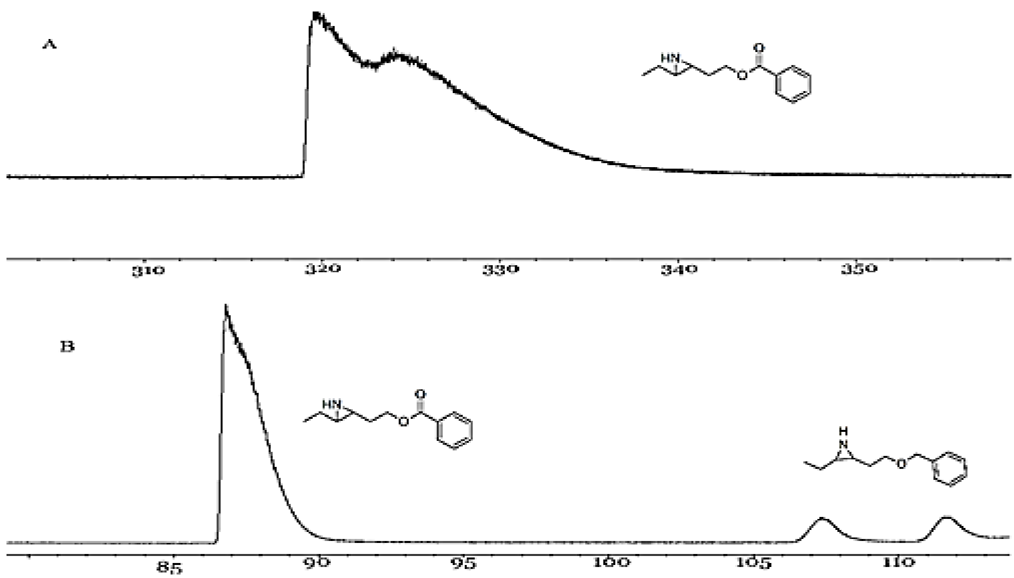

Figure 15. GC chromatograms showing the separation of the major and minor enantiomeric peaks of compound 6 on the B-PM chiral stationary phase. Chromatogram A the major enantiomers separated at 100 °C. Chromatogram B is the minor enantiomers separated at 120 °C.

Using chiral HPLC, a total of 9 compounds were separated, and the optimal separations are shown in Table 8. Chiralpak ID and Chiralpak IF proved to be the best LC chiral stationary phases for enantiomeric separation of the tested aziridines. These two stationary phases separated the largest number of compounds (Figure 16). When comparing these two

stationary phases, the Chiralpak ID separated six aziridines with three baseline separation and the Chiralpak IF separated five compounds with four baseline separation. The Chiralpak ID column was able to separate two compounds which the Chiralpak IF was not, *i.e.,* the compounds 6 and 12 (Table 5). However, the Chiralpak IF was the only column able to separate compound 7. While the Chirobiotic V2, Chiralpak IB and Chiralpak IC columns were able to separate a few of the aziridines, the selectivities and resolutions were less than Chiralpak ID and IF. The cyclofructan based column LARIHC CF6-P produced only one separation, compound 5, and surprisingly it was the best separation among all the CSPs tested. Representative optimized HPLC chromatograms are shown in Figure 17 for three of the aziridine racemates.

Table 7 Optimized separations and conditions of 9 aziridine compounds. The separation factors (k), selectivities (α), and resolutions (Rs) are also given

Compound	HPLC column[a]	k	α	Rs	Mobile phase
2	Chiralpak IB	0.7	1.29	2.4	95/5 Hexane/Ethanol
3	Chiralpak ID	1.4	1.14	1.5	95/5 Heptane/Ethanol
4	Chirobiotic V2	1.0	1.12	0.7	100/0.1 AcN/DEA
5	LARIHC CF6-P	3.3	1.14	1.5	90/10 Heptane/Ethanol
6	Chiralpak ID	9.8	1.07	1.5	98/2/0.1 Heptane/Ethanol/DEA
7	Chiralpak IF	3.0	1.13	1.5	95/5 Hexane/Ethanol
8	Chiralpak IC	4.8	1.20	1.5	100 Acetonitrile
11	Chiralpak ID	2.8	1.47	3.3	95/5 Heptane/Ethanol
12	Chiralpak ID	4.0	1.20	1.2	95/5 Heptane/Ethanol

[a] All columns were 25 X 0.46 (i.d.) and contained particles 5 μm diameter (see Experimental).

The HPLC analysis times were significantly reduced compared to GC and were under 40 minutes and mostly less than 20 minutes. The HPLC separations had relatively narrow and symmetrical peaks. Most of optimized separations were performed without using any additives. However, for compound 4 and 6, it was found out that using basic additive (dielthylamine) provided better separation. (see Table 8). This is important for these particular compounds in terms of their improved chromatographic resolution, because it has been shown that the presence of acids causes the aziridines to decompose.

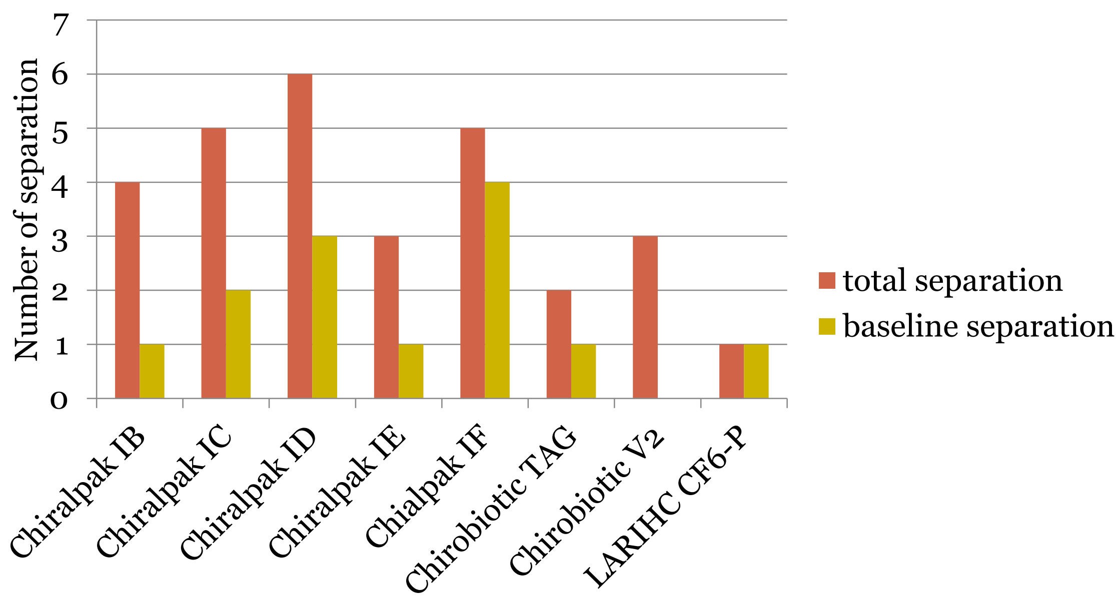

Figure 16. The graph illustrated the total number of separations performed by each HPLC chiral stationary phase.

When the aziridines were evaluated using reverse phase HPLC with mobile phase composed of water and methanol, degradation was observed. As the analysis temperature was increased, the degradation was also found to be increased. An increase in the temperature from 10°C to 40°C showed that considerable on-column degradation was occurring as shown in Figure 18. As the analysis temperature is increased, the analyte peak at 6.5 minutes loses intensity and broadens while the number of early eluting degradation peaks increases.

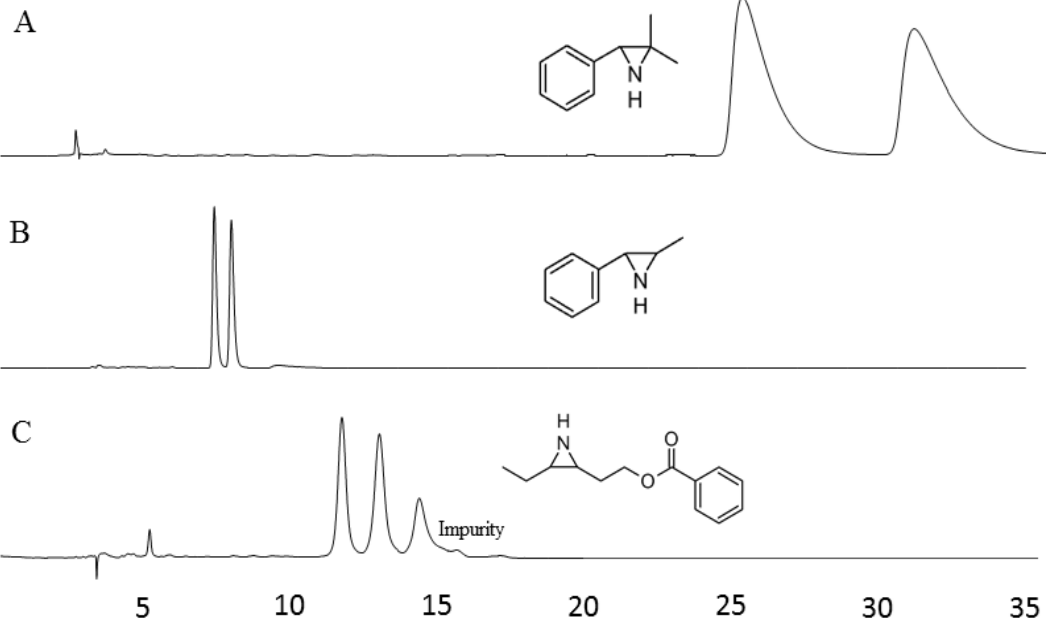

Figure 17. Example chromatograms for three different separations on 3 different chiral stationary phases. A) separation of compound 2 using Chirobiotic-TAG column, a macrocyclic-glycopeptide based chiral stationary phases; mobile phase: 90/10 heptane/ethanol. B) separation of compound 3 using a amylose based chiral stationary phases, Chiralpak ID column; mobile phase: 95/5 heptane/ethanol. C) separation of compound 5 on the Larihc CF6-P column, a cyclofructan based chiral stationary phase; mobile phase: 90/10 heptane/ethanol.The third peak was determined to be a nonchrial impurity using a circular dichroism detector.

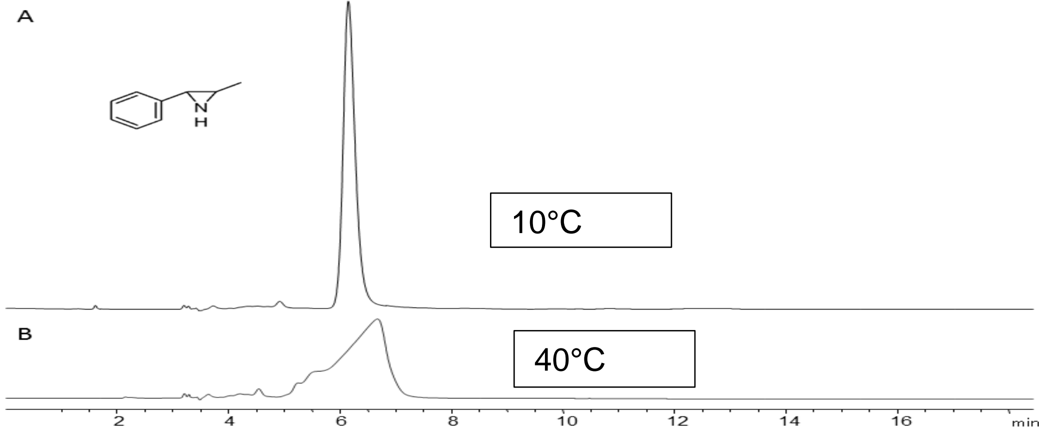

Figure 18. Chromatogram A is for compound 3 analyzed on the Cyclobound RSP column using a 50/50 water/methanol mobile phase at 10 °C. Chromatogram B is for compound 3 analyzed on the Cyclobound RSP column using a 50/50 water/methanol mobile phase at 40 °C. Clearly decomposition is occurring on the chromatographic time scale at the higher temperature and under these solvent conditions.

Conclusions

GC and LC were capable of resolving 10 out of 12 newly available aziridines. It was observed that the best column for GC was the Chiraldex B-PM whereas Chiralpak ID and IF were the best columns for separations using HPLC. HPLC was shown to separate enantiomers for 9 out of the 12 aziridines and the GC was able to separate 6 racemates. HPLC also benefited from lower retention factors and higher resolutions than GC.

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