CHIRAL RECOGNITION AND BINDING CONSTANT DETERMINATION IN MONOVALENT AND BIVALENT SYSTEMS USING ELECTROSPRAY IONIZATION MASS SPECTROMETRY

by

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ABSTRACT

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Electrospray ionization mass spectrometry (ESI-MS) is a soft ionization method which is capable of transferring weakly bound host-guest noncovalent complexes from the solution phase to the gas phase. This capability makes ESI-MS an ideal tool for the quantification of binding affinities of different host-guest noncovalent complexes.

There is a growing interest in using ESI-MS for chiral recognition studies. In this work ESI-MS was employed to investigate the binding affinities for cinchona alkaloid chiral selectors binding N-blocked leucine enantiomers, as well as for macrocyclic antibiotics binding a variety of derivatized and underivatized amino acids. The effect of multivalency on binding affinity was also evaluated. This ESI-MS method is very promising for the binding studies of different host-guest systems. Merits of this method are speed, sensitivity and low sample consumption.

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CHAPTER 1

INTRODUCTION

Electrospray Ionization (ESI) and Matrix Assisted Laser Desorption/Ionization (MALDI) are mass spectrometry techniques which emerged around the same time and were awarded the Nobel prize in 2002. Since John B. Fenn discovered ESI in 1988, it has become an important tool for qualitative and quantitative analysis in the chemical and biological sciences [1,2]. Electrospray ionization has proven to be a decisive technique for structural elucidation of biological macromolecules, understanding reaction mechanisms, and investigating binding affinities for non-covalent complexes. The predominant merits of this technique are its speed, sensitivity, and specificity of analysis. Growing interest in chiral recognition using electrospray ionization mass spectrometry (ESI-MS) is clear, according to Figure 1.1, as number of publications have increased in last decade.

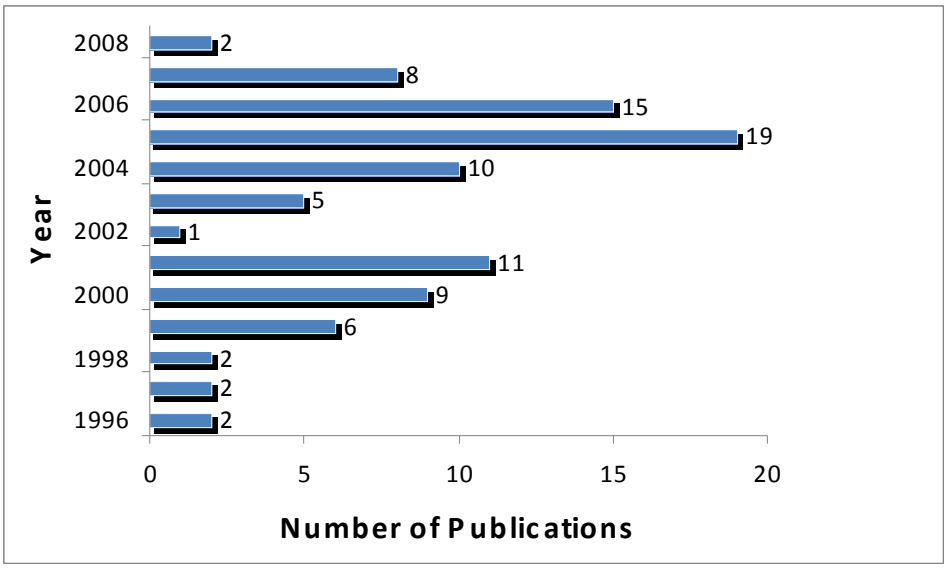


Figure 1.1 Number of publications for "chiral recognition using ESI-MS" according to Scifinder Scholar.

1.1Electrospray Ionization Mass Spectrometry as a Tool

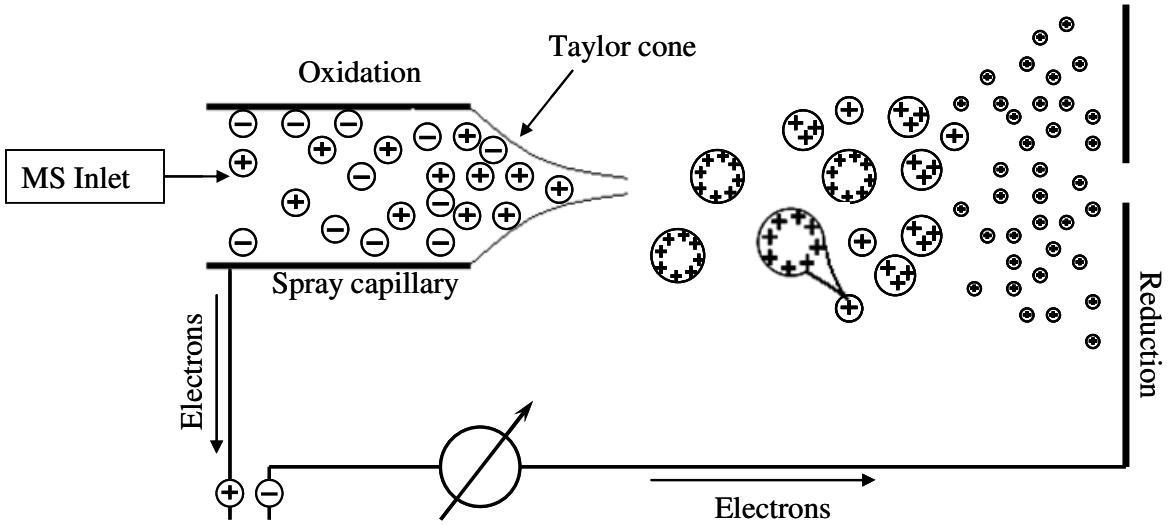


Figure 1.2 Electrospray ionization process

Figure 1.2 depicts the ESI process, which can mechanistically be described by three different steps: (1) nebulization of a sample solution into electrically charged droplets; (2) release of ions from droplets; and (3) transportation of ions from the atmospheric pressure region to the high vacuum mass analyzer. A typical analysis might be a dilute solution of 10^{-6} to 10^{-4} M of an analyte with a flow rate of 10 to 50 microliter per minute sprayed from the capillary upon which a high potential of 3-5 kV is applied. Electrostatic forces affect the spray and cause charge separation at the liquid surface, eventually leading to the deformation of the emerging liquid surface, known as the Taylor cone. This deformation results in many small droplets which are desolvated and decompose as they traverse the spray chamber on their way to the mass spectrometer inlet. The diameter of the droplet is affected by different parameters such as applied voltage, flow rate of the solution, and solvent composition [3]. The electrostatic repulsion increases as these highly charged droplets experience solvent evaporation and droplet fission occurs at the Raleigh limit which is considered the point at which electrostatic repulsion overcomes the surface tension holding the droplet together [4]. The ions released from these droplets are sampled by the mass analyzer and separated based on their mass to charge ratio.

1.2 Chiral recognition using ESI-MS

Chiral recognition plays an important role in biological systems, including antibody-antigen, enzyme-substrate, and drug-target recognition [5-7]. It is well-known that living systems can exhibit different responses to different enantiomers of a chiral molecule. For example, one of the enantiomers of the drug can be very effective for the prevention or cure of the disease where the other enantiomer can be highly toxic. In chiral recognition, a particular chiral host molecule **(H)** can engage in enantioselective intermolecular interactions with a particular chiral guest molecule **(G)** to form a diastereomeric noncovalent complex. Noncovalent interactions are commonly driven by electrostatic forces which can be further strengthened by additional dipole-dipole, hydrogen bonding, and hydrophobic interactions [8].

The electric fields present in a mass spectrometer may be considered achiral and thus incapable of discriminating between two enantiomers. In fact, mass spectra for *D* and *L* enantiomers are identical. In mass spectrometry, as well as in most solution phase methods, a chiral selector is introduced to form a diastereomeric complex with enantiomers in order to achieve discrimination. Different solution phase spectroscopic methods like nuclear magnetic resonance spectroscopy (NMR) [9,10], calorimetry [11,12], potentiometry [13,14], ultraviolet-visible spectroscopy (UV-VIS) [15, 16] have been employed to investigate and quantify these host-guest noncovalent interactions. Since Fales and Wright [17] observed chiral discrimination in their studies of tartarate dimer ion formation, the interest for chiral recognition using mass spectrometry has consistently grown. In early days, chemical ionization (CI) [18-28] as well as fast atom bombardment (FAB) [29-39] were applied for chiral recognition studies. Henion *et al*. introduced ESI for the first time as a tool for the investigation of protein-ligand noncovalent complexes [40]. Later on, ESI [41-46] become a primary mode of investigation for chiral recognition as it is considered to be the soft ionization method and capable of transferring the

weakly bound noncovalent complexes intact from solution phase to the gas phase. It is possible to tune the ionization condition to preserve the transfer of noncovalent complexes or species of interest to the analyzer by varying applied voltage and temperatures in the ionization source. ESI has been employed widely for qualitative and quantitative analysis. Adequate literature is available where ESI also has been used for quantitation of enantiomeric excess [47-52]. Cooks *et al*. used the Kinetic Method, based on ESI-MS/MS analysis of metal-mediated diasteromeric complexes, to determine enantiomeric excess of α-amino acids with good accuracy [43].

To better understand the process involving transfer of charged species from solution phase to gas phase during ESI, an equilibrium partitioning model (EPM) has been developed by Enke for simple charged analytes [53] and later expanded by Brodbelt and coworkers for application to host-guest complexes [54, 55]. The model describes the droplet in two different regions: An electro-neutral droplet core and a charged droplet surface. Charged analyte species in the droplet compete for the limited number of charged sites at the droplet surface and partition depending on the nature of the species. Polar species tend to stay at the core of the droplet whereas nonpolar species tend to migrate to the surface of the droplet. This leads to ion response which is highly dependent on analyte properties. Solution pH, solvent polarity, and analyte solvation energy play particularly important roles in the observed response factor. However, merit for this method stays high and several reviews published describing chiral recognition of noncovalent host-guest complex using ESI-MS [56-58].

In the work which comprises this thesis, ESI-MS has been successfully employed for the chiral recognition study and quantification of binding affinity of different systems was performed using a static titration method [59]. In the static titration method, a series of discrete samples are prepared and analyzed where the host (**H**) and guest (**G**) are added together. The concentration of one of the components (generally **H**) is kept constant whereas the

concentration of the other component (*i.e.* **G**) is varied by at least one order of magnitude. Ideally, the complex peak intensity increases with an increase in guest concentration, keeping host concentration constant. The assumption here is that the host, guest and complex peak intensities in the mass spectra are representative of equilibrium concentrations of host, guest and complex in the solution as shown in Equation 5. If this assumption holds and if all species in equilibrium can be directly observed, then the dissociation constant for the complex can be determined directly using Equation 6. However, in a more practical sense, titration models are derived which incorporate the relative ion abundances and response factors of particular species, as well as initial concentrations of host and guest, to determine binding constants through curve fitting procedures.

$$H + G \underset{K_d}{\overset{K_a}{\rightleftharpoons}} HG \qquad (1)$$

$$K_d = \frac{[H][G]}{[HG]} \qquad (2)$$

The dynamic titration method for dissociation constant determination has been developed by Schug and coworkers [60]. In this method, the host solution (10-20 µM) flows at a constant flow rate (10-60 µL/min) and a small plug (e.g. 2 µL) of guest (100 µM-1000 µM) is injected into the flow of the host. As the plug passes through a substantial length of tubing, band-broadening occurs and results in an approximately Gaussian profile. This Gaussian profile of guest forming complex has been utilized to calculate the dissociation constant using 1:1 binding model prepared in-house.

In the current study, macrocyclic antibiotic chiral selectors like vancomycin **(V)**, teicoplanin **(T)**, teicoplain aglycon **(A)** and ristocetin A **(R)** are investigated for their ability to

discriminate derivatized and underivatized amino acid enantiomers. These macrocyclic antibiotics are known to be very effective chiral selectors in high performance liquid chromatography (HPLC) [61-65] and capillary electrophoresis (CE) [66-70]. The main goal of this work is to investigate the solution phase binding affinity of these chiral selectors for different amino acids using ESI-MS with the static and dynamic titration methods. The effect of different solvent and different buffer conditions on the binding was investigated and an effort was made to study the trend of the binding of noncovalent complexes by monitoring gas phase ion abundances. The simplest and easiest way to investigate binding affinity of particular system is to perform host-guest screening experiment. Host-guest screening provides speed to quickly screen relative binding affinity in solution and indicates probable trends for the particular system. However, reproducibility can be an issue and results should only be taken as semi-quantitative. As we know the susceptibility of the technique to different solvent conditions, pH condition, and buffer conditions, the host-guest screening method gives a broad view of the system's behavior in different mediums. Host-guest screening was applied to the macrocyclic antibiotic-amino acid system to observe the behavior of these systems in different solvent conditions like 100% H_2O, 50/50 H_2O/methanol and 100% methanol. Different buffer conditions NH_4OAc 100uM **(1)** and NH_4OAc 100uM with 0.5% HOAc **(2)** were also evaluated. For enantiomerically pure compounds, the degree of association $\mathbf{A_{(R)}}$ was calculated by taking ratio of the complex ion peak intensity ($i_{[(R)\text{-}H+(R)\text{-}G]}$) and sum of ion intensities of host and complex ($i_{[(R)\text{-}H]}+i_{[(R)\text{-}H+(R)\text{-}G]}$) as shown in Equation 3. The selectivity **α** obtained as shown in Equation 4 by taking the ratio of the value obtained for each enantiomer as shown in equation 3.

$$A(R) = \frac{i[(R)-H+(R)-G]}{(i[(R)-H]+i[(R)-H+(R)-G])} \quad (3)$$

$$\alpha = \frac{A(R)}{A(S)} = \frac{i[(R)-H+(R)-G]/(i[(R)-H]+i[(R)-H+(R)-G])}{i[(R)-H+(S)-G]/(i[(R)-H]+i[(R)-H+(S)-G])} \quad (4)$$

This screening technique might not give us an accurate quantification of binding affinities. Therefore, more rigorous host-guest titration techniques like static titration and dynamic titration are required to get relatively accurate value of binding affinity. Linearization methods like Scatchard plot, Benesi-Hildebrand plot and the method of Scott are available for extracting binding constants [59]. Several examples of using the scatchard plot method for determining the binding constant in combination with ESI-MS have been reported [71-73]. However, nonlinear curve fitting methods are more preferable for getting binding constant as they are more versatile, customizable, and easily evaluated using modern computers. In this study, static titration method and dynamic titration methods are employed for obtaining dissociation constants of different systems. Good correlation was found by means of enantiomeric preference and selectivity in many cases. Discrepancies were observed for some of the systems due to the mechanistic differences between HPLC, CE and ESI-MS. Overall, chiral recognition was successful for macrocyclic antibiotic-amino acid system using basic screening method, static titration method and dynamic titration method.

In a second model system, the effect of multivalency has been investigated on the chiral recognition where the binding affinity of the monovalent system consisting of *tert*-butylcarbamoylquinine (tBuCQN) and dipropoxybenzoyl-leucine (DPB-Leucine) was compared with the binding affinity of the bivalent system containing bis-*tert*-butylcarbamoylquinine (bis-tBuCQN) and C_6-bis-alkoxy-leucine. Solution phase measurements were made for monovalent, bivalent and multiple monovalent systems to measure the binding affinity using static titration method. A gas phase affinity determination method based on collision induced dissociation (CID) was also applied to measure the difference in binding affinity between the monovalent and bivalent system. In CID measurements, a singly protonated noncovalent complex is isolated in the ion trap and subjected to multiple collisions with He gas to induce dissociation.

As the collision energy is increased, the parent ion peak intensity for complex decreases, releasing protonated host and neutral guest in a unimolecular decay process.

Overall, the static titration method is successfully employed to determine binding affinity using ESI-MS. This technique provides good speed and sensitivity for this type of study. The main advantage of this method is low consumption of the sample (micrograms) compared to conventional methods like NMR, UV, calorimetry, and potentiometry, all of which require more amount of the sample compound (milligrams). For investigating the selectivity of novel chiral selector (which may be available in only limited quantity), this technique is very promising. Overall, the use of mass spectrometry for chiral recognition is still a maturing field and possibilities are there that it can blossom to effectively provide useful mechanistic information in an efficient manner.

CHAPTER 2

INVESTIGATION OF MONOVALENT AND BIVALENT ENANTIOSELECTIVE MOLECULAR RECOGNITION BY ELECTROSPRAY IONIZATION – MASS SPECTROMETRY AND TANDEM MASS SPECTROMETRY

2.1 Abstract

In this work is described the investigation of bivalent versus monovalent enantioselective molecular recognition in the context of enantioselective separations. Electrospray ionization – mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) are used for evaluating enantioselective systems through the measurement of i) relative solution phase binding constants via titration and ii) relative gas phase binding via collision threshold dissociation. In HPLC, a cinchonane-type chiral stationary phase (CSP) based on tert.-butylcarbamoylquinine provides vastly increased retention and enantioselectivity for separation of bivalent versus monovalent alkoxy-benzoyl-N-blocked leucine enantiomers. The bivalent enantiomers are able to span and simultaneously interact with multiple interaction sites on the CSP surface, leading to enhanced separation. ESI-MS titration measurements also show an increased avidity for binding between bivalent selector and bivalent selectand, compared to the monovalent system. However, enhanced enantioselectivities measured in HPLC for the bivalent system cannot be reproduced by MS due to inherent mechanistic differences. Assumed discrepancies in relative response factors also give rise to systematic errors which are discussed. The results of MS/MS gas phase experiments show that enantioselectivity is essentially lost is the absence of solvation, but that dissociation thresholds can provide a measure of relative dissociation energy in the bivalent interaction system relative to the monovalent counterpart. Such measurements may prove useful and efficient in better understanding multivalent effects in line with current theoretical

considerations of effective concentrations and ion trap effects. This is the first application of mass spectrometric methods for assessing increased avidity of binding in multivalent enantioselective molecular recognition.

2.2 Introduction

Multivalent molecular recognition refers to a process where noncovalent association is induced by a receptor which employs multiple copies of an identical functional unit to bind to a ligand with similarly arranged copies of a complementary functional unit [74-75]. Multivalent interactions profit from an enhancement in favorable enthalpic contributions to binding due to multiple contact sites on individual molecules, while unfavorable entropic costs associated with the binding process are similar to that for the monovalent system. In other words, the entropic penalty for binding (disruption of solvent molecules which solvate the analytes) is paid for by the first contact of complementary receptor-ligand functional units at one site, bringing other interaction sites into close proximity, and resulting in increased affinity. Huskens *et al*. have provided a detailed treatment of the thermodynamics of multivalent interactions at solution-surface interfaces [76]. This model requires the explicit consideration of "effective concentrations," which are defined by the characteristics of the surface-bound receptors (display, orientation, flexibility, arrangements, smoothness of surface, relative spacing of selectors from each other, etc.) and the properties of the multivalent analytes (nature of linkers, flexibility, rotational freedom, number of interaction site motifs incorporated). In simpler terms, the phenomena and associated nomenclature for multivalent interactions are shown in Figure 2.1.

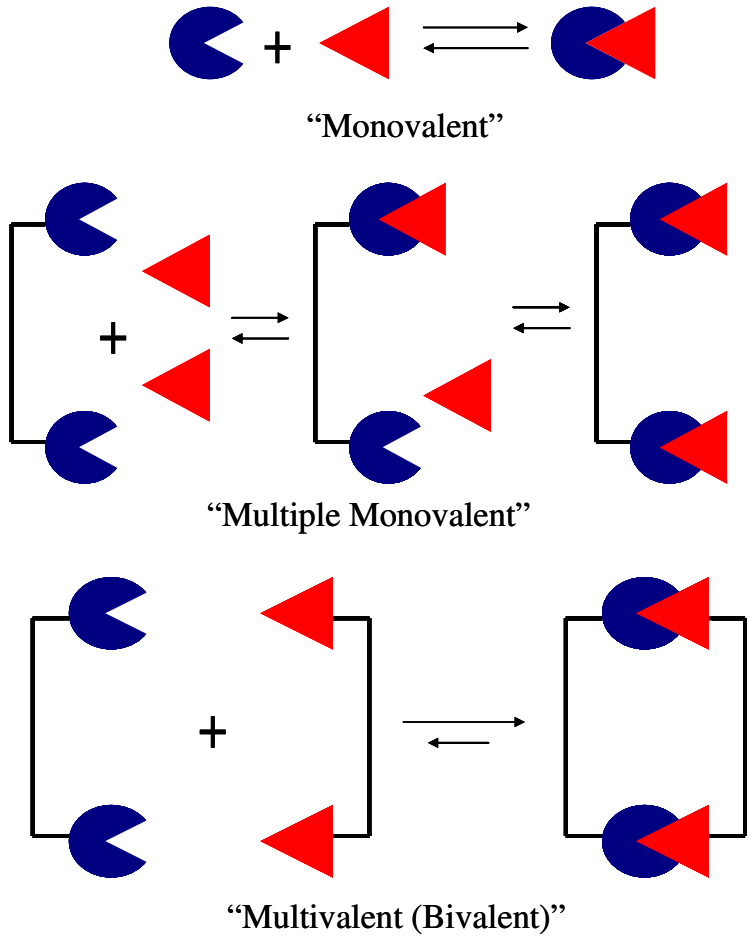


Figure 2.1 Schematic and nomenclature for monovalent and bivalent interaction systems.

The scientific literature is rich with examples where multivalent synthetic receptors and ligands are employed to increase avidity. Whitesides and coworkers reported the synthesis of model bivalent and trivalent vancomycin – bacterial cell wall tripeptide systems and their subsequent binding affinity determinations [77-79]. Whereas the monovalent vancomycin – Ac-*L*-Lys-*D*-Ala-*D*-Ala interaction is characterized by a dissociation constant (K_d) of approximately 10^{-6} M [80, 81], the bivalent and trivalent arrangements (both the receptor and the ligand were each synthesized to properly orient multiple functional units with appropriate spacers) returned enhanced binding affinities, giving K_d = 10^{-9} and 10^{-17} M, respectively. Breslow and Zhang reported cholesterol recognition using a synthesized β-cyclodextrin (CD) dimer, where the

bivalent receptor (K_d = 1.8 x 10^{-7} M) showed a 300-fold enhancement in binding, relative to monovalent β-CD (K_d = 5.9 x 10^{-5} M) [82]. Stoddart and coworkers have spent considerable effort studying the binding of multivalent ammonium cation arrangements with multivalent crown ether-based receptors, showing substantial increases in affinity relative to monovalent counterparts [83-85]. Related work has included collaborative efforts to incorporate multivalency into the design of nanomachines, such as molecular elevators [86]. Reinhoudt, Huskens, and coworkers have also made substantial contributions to the use and understanding of multivalency in nanofabrication processes [75,76,87].

Researchers have invoked the model of multivalency and polyvalency to better understand and influence life processes in the biochemical arena [74,88]. Novel bivalent intercalating binders based on [n]-polynorbornane have been developed to improve DNA recognition by Waring, Hollfelder and coworkers [89]. In a similar effort, Luger, Edayathumangalam, and coworkers designed hairpin polyamide dimers to target a nucleosomal supergroove on chromatin [90]. Hol, Fan, and coworkers synthesized a series of bivalent ligands to study their ability to inhibit the activity of cholera toxin, returning significant potency gains [91]. Others have used a phage display optimization approach to reveal bivalent ligands based on proline-rich peptide segments to recognize and inhibit SH3 domains involved in diverse signaling pathways [92]. Also, homo- and heterobivalent inhibitors based on aldehyde peptide head groups have been reported for enhanced inhibition of proteosome, a multicatalytic protease [93]. Taken together, these works represent only a fraction of the studies demonstrating the effects of multivalent molecular recognition architectures in biochemical systems.

In the field of separation science, one of the most challenging tasks is the separation of enantiomers [94]. New “chiral selectors” (receptors capable of enantioselective discrimination) are constantly in demand to meet the challenges of separating and purifying new chiral entities

produced by pharmaceutical and agrochemical industries. Chiral selectors achieve enantioselectivity by displaying a chiral scaffold which can differentially bind one enantiomer over another. In most cases, chiral selectors are highly specialized in the types of enantiomers they can effectively discriminate. Minute differences in binding affinity must often be optimized in order to achieve resolution of the desired target enantiomers. It would thus, seem reasonable to invoke the multivalency concept as a means to amplify enantioselectivity provided by monovalent systems where resolution is less than acceptable.

Pirkle and coworkers demonstrated this advantage when they studied the inter-functional distance between enantioselective recognition motifs on a chiral stationary phase (CSP) using synthesized bivalent analyte stereoisomers [95, 96]. Bis-amide linked 3,5-dinitrobenzoyl-leucine (DNB-Leu) enantiomers, incorporating a homologous series of spacer units, were chromatographed on N-(2-naphthyl)alanine-derived CSPs. Selectivities (α) were compared to separation, on the same CSP, of monovalent DNB-Leu enantiomers. For the optimum case, the n-hexyl bis-amide of DNB-Leu was separated with α = 121, a substantial increase over that obtained for monovalent DNB-Leu (α = 10.5). The bivalent ligand displayed appropriate spacing for simultaneous enantioselective interaction with two complementary bound stationary phase units to achieve an approximate doubling of ΔΔG for enantiodiscrimination. More recently, Ling *et al* investigated the beneficial effects of multivalency when they described the preparation of dendritic CSPs based on L-proline indananilide chiral selectors on polymeric beads [97]. Enantioselectivities up to α = 31 were recorded for a series of N-dinitrobenzoylated amino acids. In general, the enhancements provided by these multivalent enantioselective systems are impressive, providing an additional experimental and theoretical basis for studying the resultant increase in avidity. However, the general applicability of such approaches is limited, due to the time-consuming nature associated with preparing bivalent ligands from their monovalent

counterparts prior to separation. For purification purposes, the bivalent ligands would need to be decoupled following separation.

Despite apparent limitations in practical implementation, the concept of enantioselective multivalent recognition fits well within the efforts of ongoing research in our group to develop and evaluate mass spectrometric methods for measuring binding affinities in small molecule molecular recognition systems [58-60,98]. The investigation of molecular recognition and noncovalent binding systems by both solution-phase- and gas-phase-based mass spectrometric methods is a growing area of research over the last decade [58, 98-105]. The cited review articles indicate a wealth of applications in the areas of protein (enzyme)-ligand and oligonucleotide (RNA, DNA)-ligand interaction systems. With respect to small molecule [106-113] and chiral recognition systems [59, 60, 98, 114-117], far fewer quantitative solution phase affinity studies have been reported utilizing ESI-MS. The vast majority of work relating the application of mass spectrometry to studying enantioselective interactions has centered on gas phase tandem mass spectrometric (e.g. by ion/molecule reactions and the kinetic method) or desorption mass spectrometric experiments (e.g. by fast atom bombardment) [31,118-126]. Although many of these approaches are applicable for analysis of enantiomeric excess, given suitable calibration, the nature of the experiments provides less information about mechanisms associated with enantioselective interactions in the solution phase. On a related note, previous examples of the study of multivalent binding by mass spectrometry include work by Klassen and coworkers [127], on the binding between Shiga-like toxin with globotriaoside, and by Meijer and coworkers [128], on the collisional dissociation of multiple monovalent dendrimeric binding interactions in the gas phase.

In this work, is described the application of electrospray ionization – mass spectrometry (ESI-MS)-based titration measurements and collision activated dissociation threshold tandem

mass spectrometry measurements to study multivalent enantioselective molecular recognition. Our model system is composed of monovalent (**1**) and bivalent (**2**) forms of the cinchonane-type chiral selector, *tert*-butylcarbamoylquinine (tBuCQN; (8*S*, 9*R*)), binding the enantiomers of mono- (**3**) and bis-N-alkoxy-benzoylated leucine (**4**). Solution-phase-targeting mass spectrometric-based titration experiments using 1:1 and 1:2 binding models are employed to compare monovalent, multiple monovalent, and bivalent interaction strengths. Gas phase collision threshold measurements are performed to study binding in the gas phase for the bivalent compared to the monovalent systems in the absence of solvation. Enantioselectivities obtained by ESI-MS measurements are compared with HPLC separations of **3** and **4** on a cinchonane-type CSP (based on tBuCQN **1**).

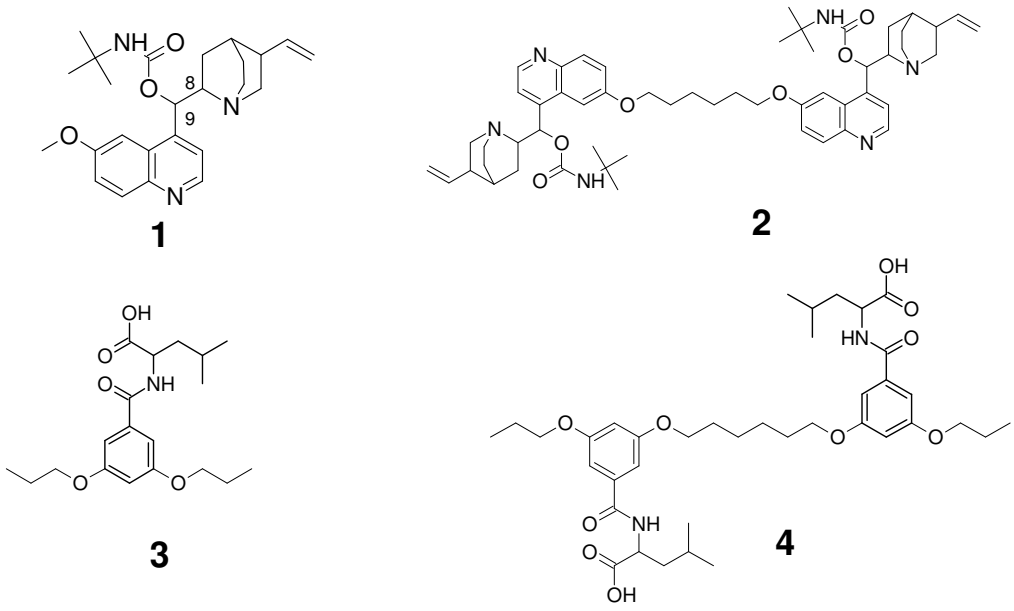


Figure 2.2 Structures of *tert*-butylcarbamoylquinine **1**, C_6-bis-tert-butylcarbamoylquinine **2**, dipropeoxybenzoylleucine **3** and C_6-bis-3,5-dinitrobenzoylleucine **4**

A clear distinction should be noted concerning the experimental approaches employed in this investigation. In HPLC separations, multiple association and dissociation events give rise to a series of step-by-step solid-liquid equilibria (multiple theoretical plates) characterized by the guest binding to a surface presenting multiple host receptor sites. A selectivity value is calculated

as a ratio of HPLC retention factors. In the MS experiment, selectivity is represented as a ratio of dissociation constant values taken from the measurement of a single equilibrium in solution (one theoretical plate). However, the underlying equilibria in both are cases are essentially the same with the exception of HPLC retention effects arising from the bonding chemistry (tether) used to create the CSP. The goal of this work is to evaluate ESI-MS and MS/MS techniques [58, 99, 101] for probing the increased avidity (relative interaction energies) and enantioselectivity (configurational preference) offered by bivalent compared to monovalent recognition systems in the solution phase and compared to HPLC separation processes. MS may not be an ideal choice for probing surface-based interactions, but it is the only tool that allows direct observation of all relevant species in the multivalent equilibria.

Prior results in this research area, published by our group, have shown that the absolute affinity of enantioselective binding interactions measured by ESI-MS titrations can be shifted to higher affinity compared to those values obtained by more traditional solution phase techniques, leading inevitably to poor accuracy, despite excellent precision, for absolute binding constant determinations in these small molecule systems [58-60]. However, the relative binding affinities (ratio of binding constants), indicative of the sought enantioselective performance of new cinchona alkaloid-based recognition systems, match very well those relative values obtained by complementary solution phase methods (e.g. by microcalorimetry) and HPLC separations on a CSP. The data and results presented here provide complementary information for the investigation of multivalent interactions and are presented with a critical discussion of the merits and limitations of the mass spectrometric experiments. This is the first time measurements of relative binding affinities for multivalent enantioselective recognition systems, based on ESI-MS titration and collision threshold dissociation methods and in the context of evaluating the performance of potential chiral separation media, have been reported.

2.3 Experimental

Chemicals and Synthesis

LC-MS grade water (H_2O) and HPLC grade methanol (MeOH) from J.T. Baker (Phillipsburg, NJ), composed the bulk of the sample solutions prepared for MS analysis. Sodium acetate (NaOAc) (EMD chemicals, Gibbstown, NJ) and ammonium acetate (NH_4OAc) (J.T. Baker) were used as solution phase modifiers. All samples were prepared in 50:50 (v/v) MeOH/H_2O with 100 micromolar (μM) NH_4OAc and 10 μM NaOAc. This composition was chosen to mimic solution phase conditions employed for enantioselective ion exchange separation of N-block amino acid enantiomers on cinchonane-type CSPs by HPLC [129, 130]. It is also consistent with previously published work related to ESI-MS binding affinity studies using cinchona alkaloid host systems [59, 60].

The enantiomerically-pure chiral selector (host) and chiral selectand (guest) monovalent and bivalent systems investigated in this work were synthesized at the Institute for Analytical Chemistry and Food Chemistry at the University of Vienna. Monovalent selector **1** was prepared following a procedure described previously [131]. Bivalent selector **2** was synthesized from quinine in 6 steps in 31% overall yield. Monovalent and bivalent analytes **3** and **4** were prepared from 3,5-dihydroxy benzoic acid in 6 and 9 steps in 51% and 16% overall yields, respectively. A detailed description of the synthetic procedures will be provided in a following paper specifically focusing in the chromatographic aspects of multivalent chiral recognition.

Bivalent Selector: 1,6-Bis-(O6´-[9O-tert.-butylcarbamoyl-cupreine])-hexane; **2**

Colorless solid. ^{1}H NMR ($CDCl_3$) δ: 8.80 (d, 2H), 7.47 (s, 2H), 7.35 (m, 4H) 6.43 (d, 2H), 5.85 (m, 2H), 4.99 (m, 4H), 4.72 (s, 2H); 4.14 (m, 4H); 3.31 (m, 2H), 3.04 (m, 4H); 2.62 (m, 4H), 1.95-1.42 (overlapped m´s , 18H) and 1.28 (s, 18H). ^{13}C NMR ($CDCl_3$) δ: 187.8, 147.8, 145.2, 144.6, 143.0, 132.0, 127.9, 122.5, 119.0, 114.8, 102.7, 72.9, 68.6, 59.5, 57.0, 51.0, 42.8, 40.3, 29.6, 29.3, 28.2,

28.3, 26.5 and 24.5 ppm. ATR-IR (solid): 3239, 2934, 1725, 1621, 1591, 1510 and 1459 cm^{-1}. MS: $[M+H]^+$ at 901.6 *m/z*; $[M+2H]^{2+}$ at 451.3 *m/z*. Optical rotation: $[\alpha]_{546}$ = -18.1 ° (c=1.0, MeOH, 25°C).

*Monovalent Analytes: (R)- and (S)--2-(3,5-Dipropoxy-benzylamino)-4-methyl-pentanoic acid; (R)-***3** *and (S)-***3**

Colorless solid. ^1H NMR ($CDCl_3$) δ: 10.33 (s, broad, 1H), 6.89 (s, 2H), 6.59 (s, 1H), 6.51 (d, 1H), 4.81 (m, 1H), 3.93 (t, 4H), 1.79 (m, 6H), 1.69 (m, 1H), 1.03 (t, 6H) and 0.97 (m, 6H). ^{13}C NMR ($CDCl_3$) δ: 177.4, 168.1, 160.8, 136.0, 105.9, 105.2, 70.3, 51.76, 41.7, 25.4, 23.3, 22.9, 22.3 and 10.9 ppm. ATR-IR (solid): 3928, 2965, 2879, 1707, 1634, 1592, 1533 and 1436 cm^{-1}. MS: $[M-H]^-$ at 350.2 *m/z* for both enantiomers. Optical rotation: (*S*)-3, $[\alpha]_{546}$ = - 12.2° (c = 1.0, MeOH, 25°C); (*R*)-3, $[\alpha]_{546}$ = + 12.1° (c = 1.0, MeOH, 25°C).

*Bivalent Analytes: (2R, 2´R)- and (2S, 2´S)-2-(3-{6-[3-((2R´)-1-Carboxy-3-methyl-butylcarbamoyl)-5-propoxy-pehnoxy]hexyloxy}-5-propoxy-benzylamino)-4-methyl-pentanoic acid; (2R,2R´)-***4** *and (2S,2S´)-***4**

Colorless solid. ^1H NMR ($CDCl_3$) δ: 10.06 (s, broad, 2H), 6.89 (s, 2H), 6.87 (s, 2H) 6.64 (s, 1H), 6.62 (s, 1H), 4.81 (m, 2H), 3.97 (t, 4H); 3.90 (t, 4H); 1.78 (m, 10H), 1.67 (m, 2H); 1.52 (m, 4H), 1.03 (t, 6H) and 0.97 (m, 12H). ^{13}C NMR ($CDCl_3$) δ: 177.5, 168.4, 160.8, 160.71, 136.1, 106.08, 70.25, 68.50, 51.76, 14.57, 29.40, 26.15, 25.39, 23.27, 22.89, 22.30 and 10.87 ppm. ATR-IR (solid): 3306, 2935, 2864, 1717, 1640, 1593, 1526 and 1454 cm^{-1}. MS: $[M-H]^-$ at 699.4 *m/z*; $[M-2H]^{2-}$ at 349.2 *m/z*; $[M-2H+Na]^-$ at 721.4 *m/z* for both enantiomers. Optical rotation: (2*S*,2*S*´)-**4** - enantiomer: $[\alpha]_{546}$ = -11.2° (c=1.0, MeOH, 25°C); (2*R*,2*R*´)-**4**: $[\alpha]_{546}$ = + 11.3° (c = 1.0, MeOH, 25°C).

Instrumental Analysis

All mass spectrometric-based titration and collision threshold dissociation measurements were performed using a Surveyor HPLC system (pump and autosampler) hyphenated to a LCQ Deca XP quadrupole ion trap mass spectrometer with an in-built syringe pump manufactured by Thermo-Fisher Scientific, Inc. (West Palm Beach, FL). Source parameters were optimized for the observation of relevant "host-guest" (receptor-ligand, selector-selectand, etc.) complexes for monovalent, multiple monovalent, and bivalent interaction systems in the positive ionization mode using a conventional electrospray ionization source. In all cases, a spray capillary voltage of 5 kV was applied using a coaxial sheath gas (N_2) flow rate of 20 arbitrary units. The transfer capillary temperature was set to 200 °C and the tube lens offset voltage was set to 20 V. Variation of the latter parameter was assessed over a wide range to check its influence on the observed ionic complexes (data not shown). It was found that the intensity of the complex ions do not vary substantially over a reasonable range of tube lens offset voltages, indicating that temporal variations in this instrumental setting should not appreciably affect the transfer of the species of interest to the mass analyzer. For the monovalent system (binding between **1** and **3**), the pump flow rate was set to 15 μL/min, providing identical analysis conditions to those employed in previous experiments. In the case of the multiple monovalent (binding between **2** and **3**) and the bivalent (binding between **2** and **4**) interaction systems, a flow rate of 50 μL/min was employed due to the better signal quality observed under this setting.

For titration experiments, a discrete set of 1 mL sample solutions in 50/50 MeOH/H_2O were prepared containing the host molecule (**1** or **2**) at a constant concentration (10 μM for monovalent **1** and 5 μM for multiple monovalent and bivalent **2** systems) and the guest molecule (**3** or **4**) in a range of concentration spanning at least two orders of magnitude (0.1 to 100 μM, typically). The samples were introduced by an autosampler using the above-specified pump flow rate for each system, so that a plateau signal lasting two minutes for each sample injection was

obtained. Each data point for the titration was measured in triplicate to obtain mass spectra which were represented by an average of 50 scans, where each scan was a composite of 3 microscans. A suitable flushing step was incorporated in between each triplicate sample measurement to reduce potential carry-over effects. Each complete titration was repeated three times with fresh solutions.

For collision threshold experiments [132-134], single sample solutions containing 10 μM each of host and guest, along with 100 μM NH_4OAc and 10 μM NaOAc in 50/50 H_2O/MeOH, were introduced into the electrospray source on the LCQ Deca XP via an integrated syringe pump at 15 μL/min. The observed 1:1 host-guest complex was isolated in the ion trap and subjected to collision activated dissociation (CAD). The collision activation voltage was varied from 0% to 75% (where 100% represents 5 V applied potential according to manufacturer specifications), and the loss of precursor ion signal was monitored relative to the appearance of other components observed in the MS/MS spectra. A constant q-value of 0.250 and an activation time of 30 ms were employed for all measurements. The software setting for collision energy normalization was turned off. At least 30 scans were averaged to obtain each data point and each measurement was performed in triplicate.

The chromatographic measurements were carried out using a Merck Hitachi LaChrom HPLC system, consisting of an L-7159 pumping system, and L-7250 programmable autosampler, an L-7455 diode array detector and a D-7000 data interface. Data acquisition and manipulation was achieved using the Merck Hitachi HPLC system manager software (Ver. 4.0), installed on a personal computer. Column temperature was maintained at 25°C (298 K) by immersion into an electronically-controlled thermostatted water bath (Haake C4, Germany). All measurements were carried out on a commercial Chiralpak AX-QN CSP (150 x 4.6 mm I.D., Chiral Technologies Europe, Illkirch, France) comprising selector **1** covalently immobilized onto the surface of 5 μm

spherical silica particles. A mixture of MeOH/HOAc/NH_4OAc 98:2:0.5 (v/v/w) was employed as mobile phase, with all components being of HPLC grade quality (Merck, Darmstadt, Germany). A flow rate of 3.0 mL/min was used. Peaks were detected at 254 nm. Samples were prepared in mobile phase, and the injected sample volumes (total amounts) were 10µL (10 µg) for monomeric analyte **2** and 20 µL (40 µg) for dimeric analyte **3**. The enantiomer elution order of the analytes **3** and **4** were confirmed by injection of enantiomerically enriched samples. Thiourea was used as a marker for the hold up time (t_{hu}) of the chromatographic system. The column void time (t_0) used for the calculation of the chromatographic parameters of **3** and **4** was corrected for extra-column contributions (t_{exc}) of the chromatographic system ($t_0 = t_{hu} - t_{exc}$). The reported values for the retention factors k_i ($k_i = (t_i-t_0)/t_0$) and enantioselectivity ($\alpha_{ij} = k_i/k_j$ with $k_i/k_j > 1$) are the mean values from three independent chromatographic measurements.

Titration Models

To determine apparent dissociation constants based on the mass spectrometric data, a theoretical binding equilibrium model based on standard arguments (including an appropriate minimization procedure for fitting to the experimental data) was used to account for 1:1 binding in the monovalent and bivalent, and 1:2 binding in the multiple monovalent, interaction systems. For the latter case, interaction of bivalent host (H) with monovalent guest (G) consists of two interconnected equilibria characterized by two dissociation constants (K_{d1}, K_{d2}):

$$HG \leftrightarrow H + G \;\; K_{d1} = \frac{[H][G]}{[HG]} \quad \text{Eq. 5}$$

$$HG_2 \leftrightarrow HG + G \;\; K_{d2} = \frac{[HG][G]}{[HG_2]} \quad \text{Eq. 6}$$

where H is host, G is guest, HG and HG_2 are the 1:1 complex and 1:2 complex, respectively, and square brackets denote equilibrium concentrations.

In order to obtain the dissociation constants from mass spectrometric titration data, it is useful to introduce host distribution coefficients α_0, α_1 and α_2, incorporating suitable mass balance equations to relate equilibrium concentrations to initial concentrations used to set up the experiment. In this paper, we assume that mass spectral intensities of free host (i_H) and the 1:1 (i_{HG}) and 1:2 complexes (i_{HG2}) truly reflect their solution concentrations and can be therefore used to determine the value of the distribution coefficients directly from mass spectra (rightmost terms in Eqs 7a-7c). The goodness of fit of the gas phase ion abundance data to the solution phase binding model provides an indication of the correlation between the two.

$$\alpha_0 = \frac{[H]}{c_{0,H}} = \frac{[H]}{[H]+[HG]+[HG_2]} = \frac{i_H}{i_H + i_{HG} + i_{HG_2}} \quad \text{Eq. 7a}$$

$$\alpha_1 = \frac{[HG]}{c_{0,H}} = \frac{[HG]}{[H]+[HG]+[HG_2]} = \frac{i_{HG}}{i_H + i_{HG} + i_{HG_2}} \quad \text{Eq. 7b}$$

$$\alpha_2 = \frac{[\mathrm{HG_2}]}{\mathrm{c_{0,H}}} = \frac{[\mathrm{HG_2}]}{[\mathrm{H}]+[\mathrm{HG}]+[\mathrm{HG_2}]} = \frac{i_{HG_2}}{i_H + i_{HG} + i_{HG_2}} \quad \text{Eq. 7c}$$

With the knowledge of total concentrations of host ($c_{0,H}$) and guest ($c_{0,G}$) and the distribution coefficients read from spectra, it is possible to determine the equilibrium concentrations of H, HG, HG_2 (Eq. 7a-c) and, based on the mass balance equation of G (Eq. 8), combined with Eq. 7a-c, the guest equilibrium concentration (Eq. 9):

$$\mathrm{c_{0,G}} = [\mathrm{G}]+[\mathrm{HG}]+2[\mathrm{HG_2}] \quad \text{Eq. 8}$$

$$[\mathrm{G}] = \mathrm{c_{0,G}} - \mathrm{c_{0,H}}(\alpha_1 + 2\alpha_2) \quad \text{Eq. 9}$$

Therefore it is at least theoretically possible to calculate the K_d values for any one-point measurement by substituting for equilibrium concentration in Eq. 1 and Eq. 2 from Eq. 3a-c and Eq. 5:

$$K_{\mathrm{d1}} = \frac{\alpha_0}{\alpha_1\left[\mathrm{c_{0,G}} - \mathrm{c_{0,H}}(\alpha_1 + 2\alpha_2)\right]} \quad \text{Eq. 10a}$$

$$K_{d2} = \frac{\alpha_1}{\alpha_2\left[c_{0,G} - c_{0,H}(\alpha_1 + 2\alpha_2)\right]}$$ Eq. 10b

The result of titration experiments is a series of (α_0, α_1, α_2) sets corresponding to one data point for every combination of $c_{0,H}$ and $c_{0,G}$. Since it is preferable to obtain K_d values from the complete titration data set, rather then calculating a value for each titration point (and then taking their average, for example), we instead generate sets of distribution coefficients for different combinations of K_{d1} and K_{d2} and picked those dissociation constants that yielded sets as close to the experimental ones as possible. The dissociation degrees can be calculated by replacing the α_0 in Eq. 7a with $1-\alpha_1-\alpha_2$ (since $\alpha_0 + \alpha_1 + \alpha_2 = 1$) and then solving Eqs. 10a and 10b as a set of two equations with two unknowns (α_1, α_2). Expressing α_2 from Eq. 10b and further substitution of α_2 in Eq. 10a yields a cubic expression (Eq. 11) that can be solved for α_1 as it is the only unknown:

$$\left[(K_{d2} - 4K_{d1})c_{0,H}{}^2\right]\alpha_1{}^3 + \left(c_{0,H}K_{d2}{}^2 - 2c_{0,H}{}^2K_{d2} - 4c_{0,H}K_{d1}K_{d2}\right)\alpha_1{}^2 + \left(2c_{0,H}c_{0,G}K_{d2} - c_{0,G}K_{d2}{}^2 - c_{0,G}{}^2K_{d2} - c_{0,H}K_{d2}{}^2 - K_{d2}{}^2K_{d1}\right)\alpha_1 + K_{d2}{}^2c_{0,G} = 0$$ Eq. 11

To judge the degree of agreement between generated and experimental series of distribution coefficient sets, the sum of differences between generated ($\alpha_{0,gen}$, $\alpha_{1,gen}$, $\alpha_{2,gen}$) and experimental ($\alpha_{0,exp}$, $\alpha_{1,exp}$, $\alpha_{2,exp}$) values across all N experimental points (Δ) is used. The sought pair of K_{d1}, K_{d2} is determined by the minimal value of Δ associated with it.

$$\Delta = \sum_{i=1}^{N}\left(\left|\alpha_{0,gen} - \alpha_{0,exp}\right| + \left|\alpha_{1,gen} - \alpha_{1,exp}\right| + \left|\alpha_{2,gen} - \alpha_{2,exp}\right|\right)$$ Eq. 12

The K_d extraction from titration data for 1:1 binding is a simpler analogy of the procedure employed for 1:2 binding. Only one equilibrium is present in the system (Eq. 9) and therefore only two host distribution coefficients are introduced. As before, their values are obtained directly from the mass spectra:

$$\alpha_0 = \frac{[H]}{c_{0,H}} = \frac{[H]}{[H]+[HG]} = \frac{i_H}{i_H + i_{HG}}$$ Eq. 13a

$$\alpha_1 = \frac{[HG]}{c_{0,H}} = \frac{[HG]}{[H]+[HG]} = \frac{i_{HG}}{i_H + i_{HG}}$$ Eq. 13b

The analogous treatments for the mass balance of G (Eq. 8) and the guest equilibrium concentration (Eq. 9) are reduced for 1:1 binding and incorporation of these expressions into Eq. 5 provides an expression for K_d in terms of host distribution coefficients and initial concentrations of host and guest:

$$K_d = \frac{\alpha_0}{\alpha_1\left(c_{0,G} - c_{0,H}\alpha_1\right)}$$ Eq. 14

The α_0 in Eq. 14 can be replaced with $1-\alpha_1$ (since $\alpha_0 + \alpha_1 = 1$) and the equation can be then solved for α_1:

$$\alpha_1 = \frac{c_{0,H} + c_{0,G} + K_d - \sqrt{(c_{0,H} + c_{0,G} + K_d)^2 - 4c_{0,H}c_{0,G}}}{2c_{0,H}}$$ Eq. 15

Again, a series of (α_0, α_1) sets were generated for different K_d values. Using the criterion given in Eq. 16, a dissociation constant giving the distribution coefficients fitting best into the experimental data is chosen.

$$\Delta = \sum_{i=1}^{N}\left(\left|\alpha_{0,gen} - \alpha_{0,exp}\right| + \left|\alpha_{1,gen} - \alpha_{1,exp}\right|\right)$$ Eq. 16

The experimental data for both 1:1 and 1:2 binding were processed with a computer program written in-house according to the equations and procedures described above using the Microsoft C# 2005 Express Edition.

2.4 Results & Discussion

Cinchonane-type derivatives, such as **1**, have found widespread use as selective separation agents ("chiral selectors") for discriminating the enantiomers of chiral acids, specifically, N-blocked amino acids [94,121,131]. Virtually every mode of liquid phase enantioselective separation has been investigated (except supercritical chromatography) and the

interaction mechanism has been well characterized [130, 135, 136]. The protonated tertiary amine on the quinuclidine group of the host selector induces coulombic attraction with the deprotonated carboxylate group on the guest enantiomer. Simultaneously, multiple contact sites through hydrogen bonding, π-π, and van der Waals interactions are induced to provide a high degree of stereoselectivity in binding to the guest enantiomers. The competition by achiral anions (acetate) in the mobile phase provides a crucial component to the enantioselective ion-exchange chromatographic separation mechanism. A strict 1:1 interaction between host and guest (e.g. **1** and **3**) has been shown previously by NMR and isothermal titration calorimetry experiments (using slightly different guest enantiomers of 3,5-dinitrobenzoyl-leucine (DNB-Leu)) [135].

This interaction system has been exploited with an aim of developing high throughput screening methods based on ESI-MS titration measurements in previous work [59, 60]. Thus, the study of multivalency effects in the cinchonane-type chiral selector systems were pursued in order to investigate: 1) Whether tethering guest enantiomers to create bivalent forms would show increased retention and enantioselectivity in "chiral HPLC" (indicating the use of stationary phases to which is bound the chiral selector **1** for the purpose of differential retention of guest enantiomers), in a similar manner to that reported by Pirkle and coworkers [95, 96]; and 2) if mass spectrometric methods can offer complementary information in terms of interaction affinity, selectivity, and energetics of enantioselective multivalent chiral recognition systems.

Initial experiments by chiral HPLC were performed by comparing the retention and selectivity for enantiomers of monovalent **3** and bivalent **4** N-blocked chiral acids. An initial separation of the enantiomers of **3** on a tBuCQN (**1**)-based CSP in polar organic mode returned capacity factors of $k'_R = 1.19$ and $k'_S = 7.66$ ($\alpha_{HPLC} = k'_S / k'_R = 6.4$, $\Delta\Delta G = 4.61$ kJ/mol). A representative chromatogram is shown in Figure 2A. When the enantiomers of the bivalent guest **4** were separated under identical conditions on the same CSP, values of $k'_{R,R} = 7.72$ and $k'_{S,S} =$

312.20 ($\alpha_{HPLC} = k'_{S,S} / k'_{R,R} = 40.7$, $\Delta\Delta G = 9.18$ kJ/mol), were measured. A representative chromatogram is shown in Figure 2.3B.

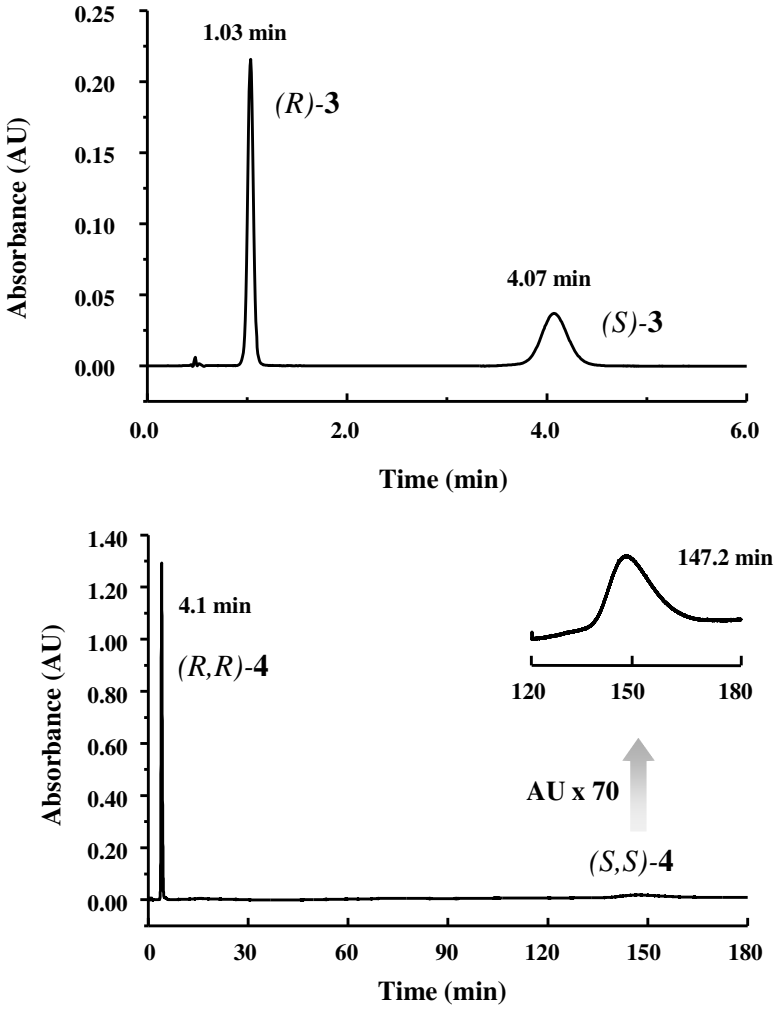


Figure 2.3 HPLC separation of enantiomers of monovalent **3** (A.) and bivalent **4** (B.) on a cinchonane-type CSP based from chiral selector **1**.

These results indicate that the bivalent receptor with a C_6 tether unit possesses the ability to interact with multiple selector sites on the CSP surface, simultaneously, thus increasing interaction affinity by a factor of two, and enantioselectivity more than 5-fold. The data successfully demonstrate the concept first illustrated by Pirkle and coworkers, a technique which was used originally to study the distance between selector sites on CSPs [95, 96].

To test the multivalency effect in mass spectrometric and tandem mass spectrometric experiments, the host molecules **1** and **2**, and the guest molecules **3** and **4** (all enantiomer forms) were synthesized and purified, using C_6 tether units in **2** and **4** to allow appropriate and consistent spacing for concerted interactions in solution. Initial screening experiments, performed by simply mixing host and guest in suitable concentrations, and represented by the spectra in Figure 3, showed responses of expected ion forms for the monovalent (**1** + **3**, Figure 2.4A), bivalent (**2** + **4**, Figure 2.4B), and multiple monovalent (**2** + **3**, Figure 2.4C) interaction systems. For the monovalent interaction case, the predominant ions observed were identified as protonated host and protonated host-guest complex. For the bivalent system, the mass spectrum is dominated by protonated and doubly-protonated host ion responses, as well as protonated and doubly protonated host-guest complex ions. For the multiple monovalent interaction system, similar and consistent ion forms were also observed. A response for the 1:2 (H:G) ionic complex can be detected, albeit in lower abundance compared to the 1:1 ionic complex, as seen in Figure 2.4B. Higher guest concentrations (up to 200 μM) were used to titrate the 1:2 interaction equilibria.

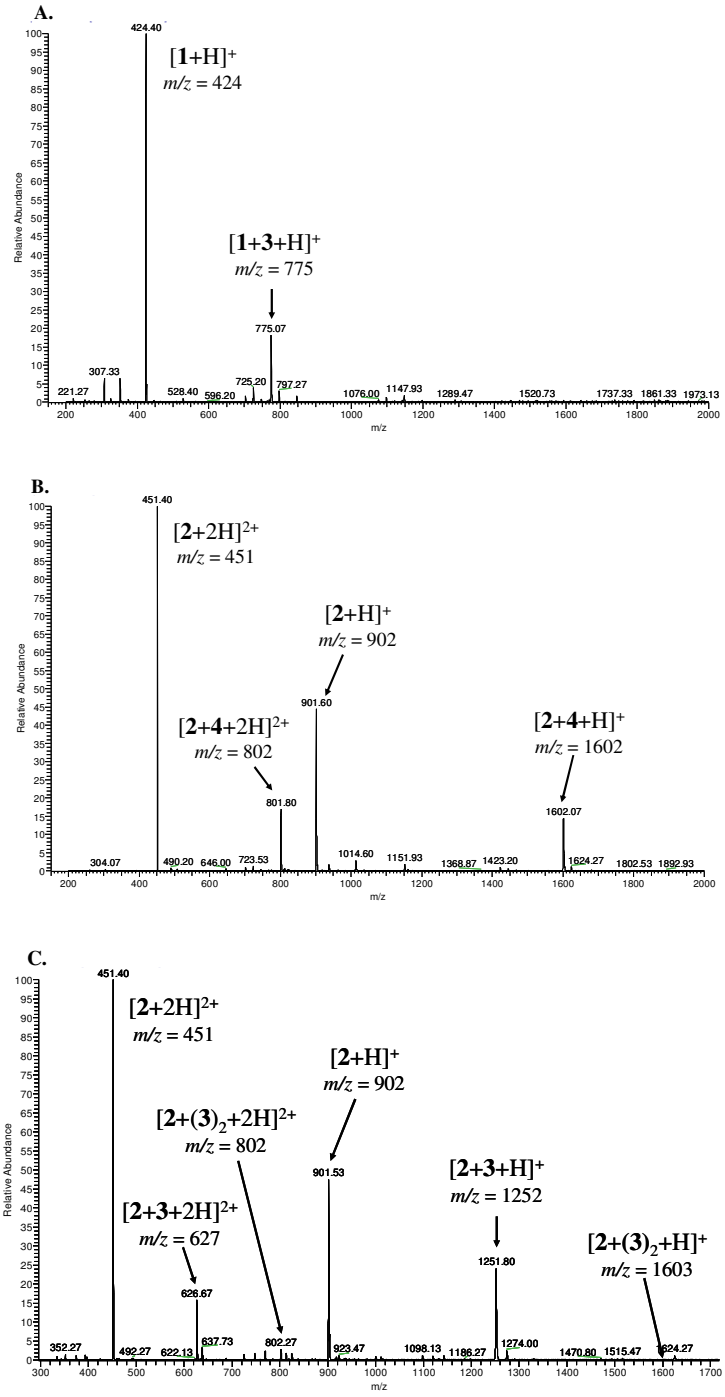


Figure 2.4 Representative mass spectra obtained through screening experiments (10 μM each of host and guest) for monovalent (A.), bivalent (B.), and multiple monovalent (C.) interaction systems. Assigned major ion signals of interest are labeled.

In order to test the quantitative nature of the interaction (through the measurement of concentration independent K_d values) and the correlation between gas phase ion abundances and solution phase equilibrium concentrations (by fitting the mass spectral data to a solution phase-based interaction model), a series of MS-based titration experiments were performed whereby a series of mixtures comprising different host and guest concentrations were successively flow injected through the ESI source. Figure 2.5 shows the fit of the mass spectral data ($I = i_H/i_{HG}$; based on the summed ion intensities of all signals related to the host (i_H) divided by the summed ion intensities of signals related to the host-guest complex (i_{HG})), plotted against initial guest concentration ($c_{0,G}$), to the derived model for 1:1 interaction stoichiometry, for the monovalent and bivalent interaction systems.

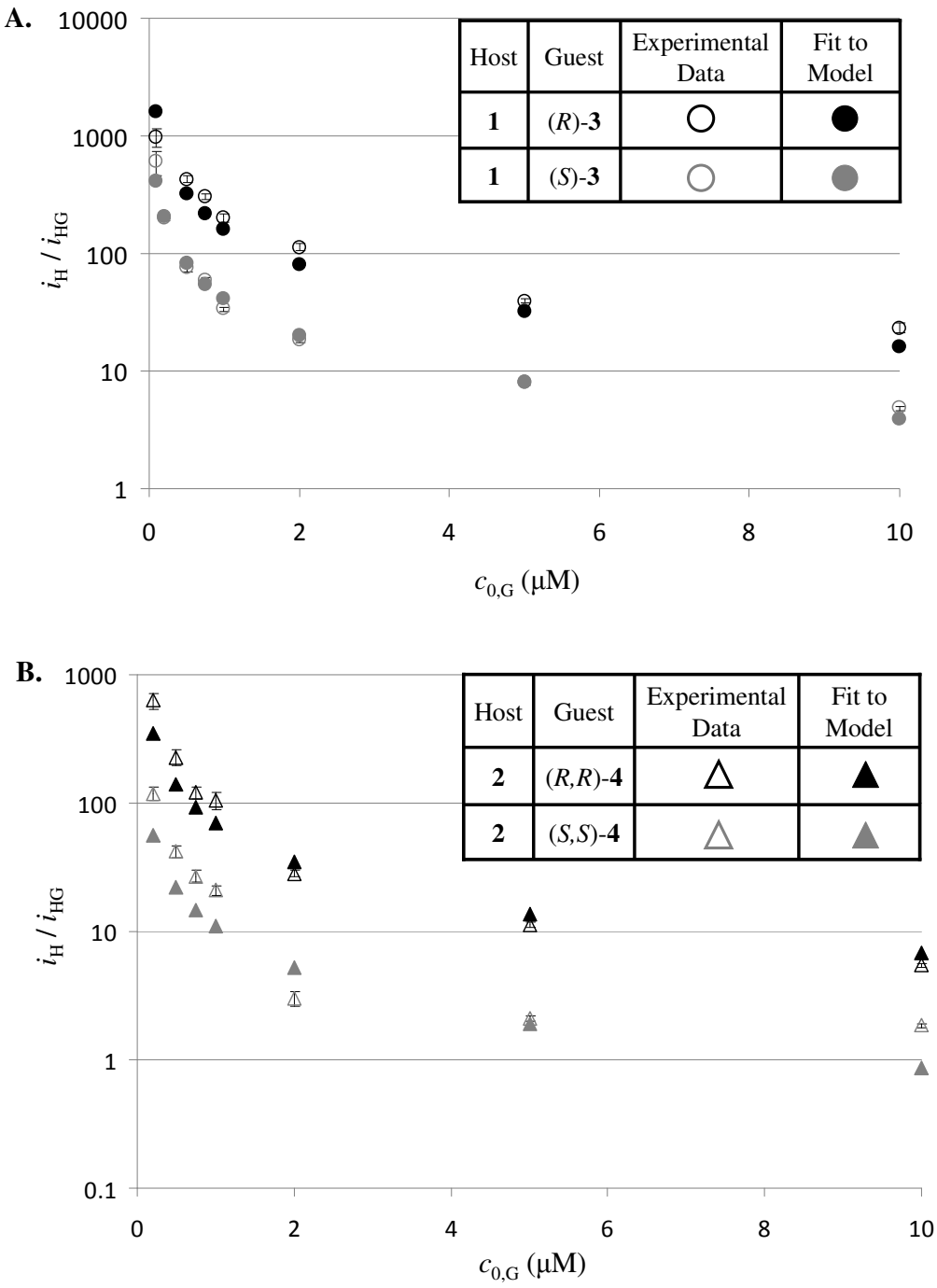


Figure 2.5 Mass spectrometric-based titration experimental data for monovalent (A.) and bivalent (B.) interaction systems. Error bars on experimental data represent standard error (N = 9) for each point.

The fit of the data to the theoretical solution phase model indicates that the gas phase ion abundances correlate reasonably well with solution phase concentrations. Points at low concentration which give rise to a large i_H/i_{HG} (between 100 and 1000) may be subject to increased uncertainty due the relative ion abundances incorporated, and thus, the use of low concentration points were restricted to those which could be recorded with <50% relative standard error (RSE). With $i_H/i_{HG} < 500$, points were recorded with good precision (< 15% RSE). Similarly, points at high concentration begin to deviate as the limit of linearity of the ESI process is approached. Error bars for standard error in each data point in Figure 2.5 are presented for N = 9 (3 replicates of 3 pseudo-replicates; see Experimental section). Overall, data are presented and evaluated over the widest range of concentrations possible to judge the position of the equilibria in the most comprehensive manner.

The use of ESI-MS titrations as a tool to study solution phase binding affinity in a quantitative manner is still a maturing art. Although specificity, sensitivity, and speed of analysis are strong advantages, it is prudent to discuss some of the limitations of the method. Previously, it was explained that association degree can be calculated directly from gas phase ion abundances when we assume they directly reflect the solution phase concentration in solution (Eqs. 3a-c, 9a-b). This is a safe assumption when working in the linear response regime of the ESI source (typically, < 50 μM). In the linear response range, the gas phase ion abundance of each species is correlated with its equilibrium solution phase concentration by a correlation coefficient, or response factor (i.e. $i_X = f_X[X]$, where f_X denotes the response factor of any species X at equilibrium concentration[X]). In principle, it is impossible to deduce the equilibrium concentration of a resultant complex without first knowing the binding constant. Without knowing the equilibrium concentration, it is not possible to deduce an accurate response factor for the complex. This problem is similarly inherent to other spectroscopic titration techniques, as well [137]. In ESI-MS titration experiments, it is commonly assumed that the response factor of the

complex is approximately equivalent to the response factor of the free host (the guest response is not followed in this treatment). This assumption is most valid for situations where a) the free host and host-guest complex(es) are similar in size and where b) the free host and the host-guest complex display identical charge states in the mass spectrum. When these assumptions are fulfilled, the physicochemical character of free host and host-guest complex ions would be similar and be expected to give rise to similar ion response.

In this study, we deal with small molecule monovalent and bivalent diastereomeric complexes. It is clear that systematic error in determined binding constants for these systems may be present due to the virtual doubling of size of the host-guest complex compared to the free host. In contrast, however, the results should be comparable on a relative basis. The charge states for the free host and the host-guest complex are preserved. Also, because the binding of enantiomers to a particular host are considered, the relative ionization efficiency of the diastereomeric complexes incorporating the different enantiomers should be similar (i.e. this is a best case scenario for studying small molecule binding by ESI-MS). In comparing the monovalent and bivalent interaction systems, it is reasonable to assume that the relative response factors of the free host and the host-guest complexes are preserved due to the consistent structural make-up, giving rise to similar systematic error in each case. Because all responses for complexes are normalized to that of the free host in the applied model, the relative binding constants for the monovalent versus the bivalent systems should be comparable, and the results support this estimate.

Table 2.1 Experimental results for mass spectrometric-based titration experiments investigating monovalent (**1** + **3**), bivalent (**2** + **4**), and multiple monovalent (**2** + **3**) interaction systems.

Host	Guest	K_{d1} ± SE (μM) [a]	K_{d2} ± SE (μM) [a]	α_{MS} (= $K_{d,R}/K_{d,S}$) [b]
1	(*R*)-**3**	170 ± 9	--	6.1 ±0.4
	(*S*)-**3**	28 ± 1	--	
2	(*R,R*)-**4**	63 ± 2	--	10 ±3
	(*S,S*)-**4**	6 ± 2	--	
2	(*R*)-**3**	230 ± 50	220 ± 30	2.1± 0.6, 1.9±0.7 [c]
	(*S*)-**3**	110 ± 20	110 ± 30	

a. Average (N = 3) ± standard error.
b. Uncertainty given based on propagation of error from $K_{d,R}/K_{d,S}$.
c. α_{MS} value based on K_{d1}, K_{d2} from multiple monovalent interaction system.

Table 2.1 shows the results of the titration experiments. A 2 – 3 fold enhancement in binding affinity for the bivalent interaction system, compared to that for the monovalent case, is measured, in good agreement with that observed by HPLC. The RSE of the dissociation constants, determined from the average of three replicate measurements are acceptable, ranging from ~5 – 25%, across the different interaction systems investigated. The uncertainties in the K_d values are propagated to provide the uncertainties in the resultant MS-based selectivity values. The enantioselectivity value for the monovalent case ($\alpha_{MS} = K_{d,R} / K_{d,S} = 6.1$) is similar in magnitude (*ab initio*) to that obtained by HPLC ($\alpha_{HPLC} = 6.4$); and the configurational preference is maintained (i.e. the enantiomer in the (*S*)-configuration binds more strongly to chiral selector **1**). The enantioselectivity in the bivalent case is much lower for the MS-based measurement (α_{MS} = 10), compared to HPLC ($\alpha_{HPLC} = 40.7$); however, this may be explained by the mechanistic differences in the separation processes, as explained previously. More importantly, the MS analysis provides a consistent result for the increased avidity of the enantioselective bivalent interaction system, and the configurational preferences are maintained.

Evaluating the multiple monovalent interaction system (**2** + **3**), key findings include: a) The binding affinity of the first (K_{d1}) and second (K_{d2}) association events are diminished relative to the monovalent (**1** + **3**) interaction systems; b) the corresponding selectivity (α_{MS} = 2.1) for the first binding event is also decreased in comparison to the monovalent system (α_{MS} = 6.1), but the expected configurational preference is maintained; c) the binding affinity of the second association event (K_{d2}) is similar in magnitude to the first association event (K_{d1}) for the multiple monovalent system, indicating the approximate equivalence and independence of the two association events under these solution conditions; and d) the selectivity of the second binding event (α_{MS} = 1.9) is very close to that for the first binding event (α_{MS} = 2.1) in the multiple monovalent interaction system. The latter points, c) and d) above, are consistent with expected results for a well behaved multiple monovalent interaction system. The discrepancies described in the former points, a) and b), are most likely due to systematic errors which can be ascribed to the relative response factor variation. In other words, the measurements for the multiple monovalent system are of interest to show the equivalence of binding sites, but the magnitude of the dissociation constants are probably not comparable to that for the monovalent and bivalent systems. The relative responses of free host and host-guest complex in the multiple monovalent systems are less likely to conform to that for the monovalent and bivalent interaction systems. The relative responses of free host and host-guest complex in the multiple monovalent systems are less likely to conform to that for the monovalent and bivalent interaction systems. In other words, it is assumed that f_H/f_{HG} (monovalent) ≈ f_H/f_{HG} (bivalent) ≠ f_H/f_{HG} (multiple monovalent), because for the monovalent and bivalent systems, a correspondingly similar increment of guest is added to the host in each case to form the complex. In contrast, for the multiple monovalent case, especially where bivalent host binds to monovalent guest ($K_{d,1}$), the structure, and thus the physicochemical characteristics, of the resulting complex are not changed in a regular increment compared to the monovalent and bivalent systems. This speculative reasoning highlights the problem of comparing dissociation constants for interaction systems that may exhibit significant

relative response differences when analyzed by ESI-MS titration experiments. Relative K_d values (selectivity) are of use to assess enantioselective performance (especially for monovalent systems), but absolute K_d values still need to be rigorously compared with values taken by complementary solution phase methods.

Table 2.2 Experimental determination of collision threshold dissociation values (V_{50}, n = 3) for monovalent and bivalent interaction systems.

Host	Guest	Isolated complex	V50 (V)	Corrected V50 (V) [a]
1	(*R*)-**3**	[**1**+(*R*)-**3**+H]+	0.994 ± 0.003	0.994
1	(*S*)-**3**	[**1**+(*S*)-**3**+H]+	0.982 ± 0.002	0.982
2	(*R,R*)-**4**	[**2**+(*R,R*)-**4**+H]+	1.916 ±0.003	0.919
2	(*S,S*)-**4**	[**2**+(*S,S*)-**4**+H]+	1.931 ± 0.007	0.929

[a.] Average V_{50} value corrected based on RRKM arguments. Values for bivalent system are normalized by a factor (0.48) derived from the relative degrees of freedom in the monovalent (351) system to that for the bivalent (729) system.

By virtue of transferring the noncovalent diastereomeric complexes into the gas phase via ESI, and the use of an ion trap mass analyzer, relative binding affinities can also be investigated through gas-phase collision threshold measurements to obtain useful information in the absence of salvation [139, 140]. Figure 2.6 shows this experimental determination for the monovalent (**1** + **3**) and the bivalent (**2** + **4**) interaction systems. Table 2.2 gives the activation voltage necessary in each case to dissociate 50% of the parent ion complex (V_{50}), a measure of their relative stability. For both the monovalent and bivalent systems, unimolecular decomposition of the protonated 1:1 host-guest complex giving the free protonated host is consistently observed (neutral loss of guest is assumed) through the range of activation voltages investigated.

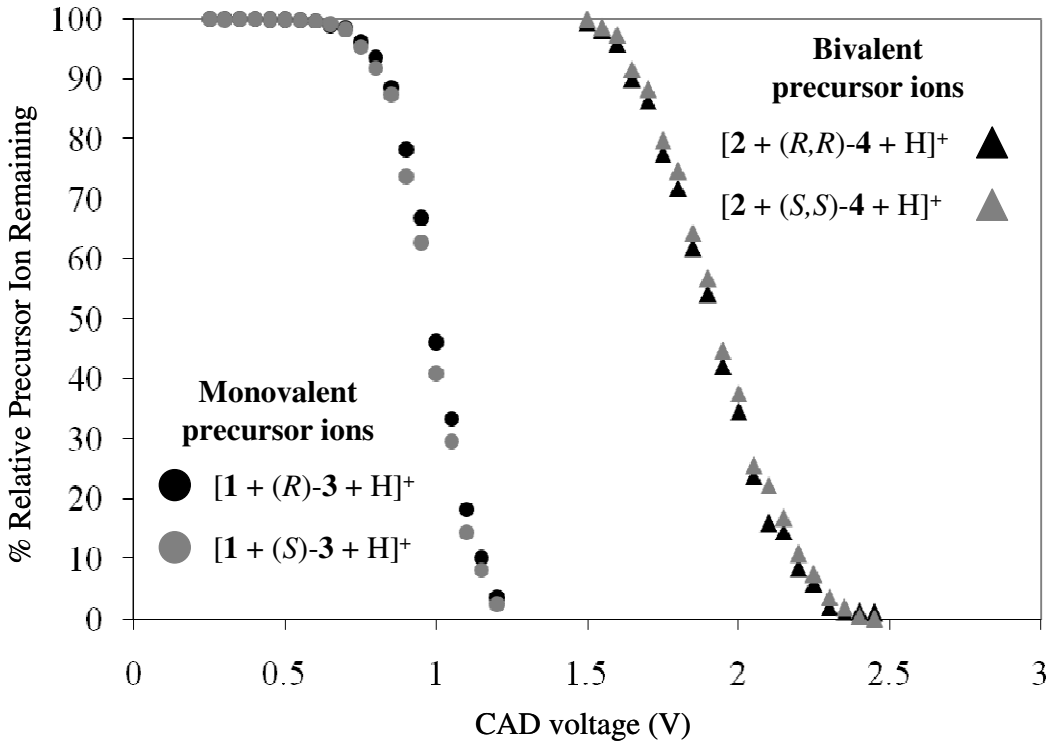


Figure 2.6 Experimental data for determination of collision threshold dissociation values for monovalent and bivalent singly-charged ionic complexes.

First, it is notable that enantioselectivity is significantly (if not completely) diminished in the gas phase relative to the solution phase for both the monovalent and bivalent interaction systems. Previous work has emphasized the delicate balance of noncovalent forces which give rise to enantioselectivity in these systems in the solution phase. An ion exchange mechanism best describes the role of competing (achiral) anions in the solution phase which give rise to enantioselective interactions for these chiral selector systems [132, 138, 139]. Complexes stripped from solvent molecules tend to overexpress electrostatic binding increments, due to the reduced dielectric of the gas phase medium, and thus distort the solvent-based enantioselective effects to reveal a different picture. Lacking a suitable ion exchange competitor for binding, both the monovalent and bivalent chiral selectors lack appreciable enantioselective capacity for differentiating the binding of complementary analyte enantiomers in the gas phase.

Even more interesting is to compare the measured V_{50} values between the monovalent and bivalent interaction systems. It may be reasonable to conclude that the increased (approximately doubled) activation voltage necessary to dissociate the bivalent system translates to a concomitant increase in interaction affinity in the gas phase relative to the monovalent system. However, RRKM effects should be considered. The V_{50} values measured by collisional activation in an ion trap are subjected to entropic contributions based on the larger number of degrees of freedom in the bivalent system composed from a larger number of atoms. The unimolecular decay of the bivalent system will be slower. Using simple RRKM arguments, the last column in Table 2 gives a corrected average V_{50} value for the bivalent system, taking into account the entropic contributions to dissociation by normalizing the measured V_{50} value by a factor of 0.48 (the ratio of degrees of freedom in the monovalent system (3n-6 = 351; n = 119 atoms) to that for the bivalent system (3n-6 = 729; n = 245 atoms)). This normalization puts the corrected V_{50} values for the bivalent system in similar magnitude to but slightly lower than the monovalent system.

If we assume that RRKM effects are significant, then this is an interesting finding in terms of investigating multivalent interaction systems by tandem mass spectrometry. In the work described previously by Huskens *et al*. [77], an intricate mathematical model is described to compare multivalent interactions indirectly based on dissociation constants (similar to the titration measurements discussed above). In contrast, the measurement of relative collisional activation voltages in tandem mass spectrometry may allow the direct measurement of relative interaction affinity at the level of dissociation energies. Although obtaining *ab initio* absolute energy values from collisional dissociation experiments in an ion trap is substantially (if not impossibly) complicated by the multi-collision environment present, the fact that similar values for dissociation energies (based on corrected relative V_{50} values) are obtained in these systems is not surprising. The systems investigated in this work are designed so that the bivalent system has twice the

mass (and chemical bond repertoire) of the monovalent system. By applying the normalization procedure to the bivalent system, it is shown that the measurements provide the average dissociation energy of the underlying monovalent system directly and efficiently.

Whether RRKM are significant in this analysis remains to be comprehensively elucidated. While it is true that the bivalent complex will decay more slowly than the similarly-activated monovalent complex based on its greater number of degrees of freedom, this difference will affect the observed threshold only if the observation period is short compared to the decay time, or if ion cooling rates are of similar magnitude to decay rates. In a quadrupole ion trap, the observation period is relatively long. Additionally, under activation conditions, collisions should generally be activating rather than deactivating, and radiative cooling rates should be slow relative to the time scale of the experiment in an ion trap. Thus, it is still reasonable to suggest that the observed difference in dissociation threshold measured for the monovalent versus the bivalent system is mainly due to real energetic differences. Further experiments are underway to more comprehensively investigate the role of RRKM effects in the collisional dissociation of these systems.

2.5 Conclusion

Quantitative characterization of interaction strengths for multivalent (bivalent) enantioselective interaction systems using ESI-MS solution phase- and MS/MS gas phase-based methods have been demonstrated for the first time. The results indicate the viability of these methods in terms of studying multivalent recognition and that they can add new insight into the solvent-mediated enantioselective performance of cinchonane-based chiral selectors. It is concluded that MS is an attractive tool in this regard, allowing the ability to monitor each component in the equilibria of interest. However, the inherent mechanistic differences which give rise to the enantioselectivity

values in MS and HPLC measurements are amplified in light of the multivalent interaction systems. Whereas, in previous studies, and here for the monovalent interaction systems, a suitable empirical correlation between the two techniques has been observed, MS-based solution phase titration experiments cannot adequately account for the vastly enhanced enantioselectivities obtained for bivalent systems by HPLC. In this regard, consideration of effective concentrations for multivalent surface-based enantioselective equilibria should be the focus of further experimentation aimed to reveal a more complete thermodynamical picture.

Evaluation of solution phase binding affinity by ESI-MS titrations is a maturing art, providing accurate results in a system dependent manner. In this work, relative binding affinities are assumed comparable by virtue of a) the similarity of the diastereomeric complexes being investigated, b) the careful design of comparable monovalent versus bivalent interaction systems, and c) the procedure by which complex ion responses are normalized to that of the respective free host ion. Still, the inability to determine response factors for the complexes induces systematic error that makes it more difficult to assess the accuracy of the absolute dissociation constant values. The study of multiple monovalent interactions in this light is especially problematic. Even so, increased avidity for binding due to the relative binding constants is apparent and configurational preferences are maintained.

The gas phase dissociation experiments provide a different picture than that which is present in the solution phase. Enantioselectivity is lost in the gas phase due to strengthened electrostatic forces and the lack of suitable competitors for supporting an ion exchange-type mechanism. However, the comparison of collisional dissociation thresholds via ion trap tandem MS demonstrates a potentially efficient means to directly study relative interaction affinities of multivalent systems. Further work is still needed in this area to assess the general applicability and limits of precision for such measurements, but these preliminary studies are encouraging.

CHAPTER 3

MACROCYCLIC GLYCOPEPTIDE ANTIBIOTICS AS A CHIRAL SELECTOR FOR ENANTIODISCRIMINATION USING ELECTROSPRAY IONIZATION-MASS SPECTROMETRY

3.1 Abstract

In high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), macrocyclic antibiotics have been found to be effective “chiral selectors” for enantioselective discrimination of amino acids, peptides and neutral molecules. In order to maximize speed and sensitivity related to new target enantiomers which can be differentiated, the initiative has been taken to apply electrospray ionization – mass spectrometry (ESI-MS) as a complementary tool to screen enantioselective performance of chiral selectors. Binding constants have been measured by ESI-MS-based titration experiments for the macrocyclic antibiotics vancomycin, teicoplanin and teicoplanin aglycon interacting with the enantiomers of various amino acids and related derivatives. Results of these experiments include: Binding constant determination for each interaction pair; enantioselectivity calculated as the ratio of the binding constants for a pair of enantiomers binding a particular selector; and the configurational preference of the interaction (e.g., which enantiomer binds more tightly). These results have been compared with published data taken by HPLC and CE. Good agreement in enantioselectivity and configurational preference has been found for many of the systems. Discrepancies, however, can occur due to the inherent differences in separation mechanisms and experimental conditions when comparing HPLC, CE and ESI-MS measurements. The ESI-MS binding constant screening approach for determining enantioselective performance of chiral selectors is promising, but requires further

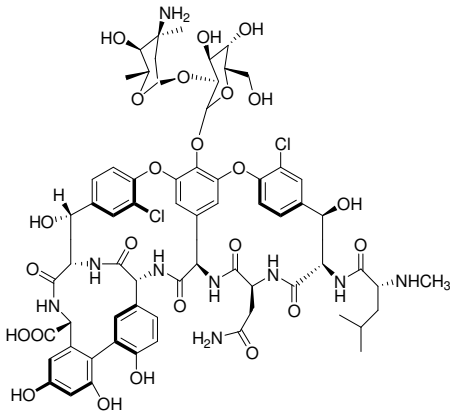
development. The versatility of the method with respect to the range of solution conditions which can be studied, limited sample requirements, and speed of analysis are major advantages to be capitalized upon. Thus, this work represents an application for the purposes of developing this technology, but also to study its use in gaining new insight into the enantioselective capabilities of macrocyclic antibiotics against analytes of biological and pharmaceutical significance.

3.2 Introduction

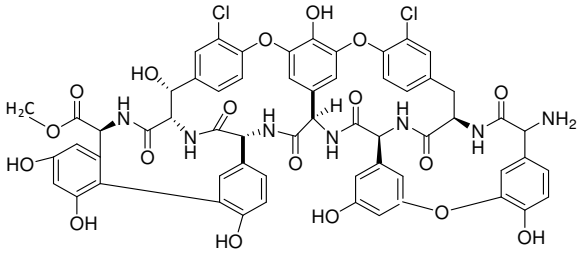
A significant number of macrocyclic antibiotics with different structural types are available. Among all these macrocyclic antibiotics, glycopeptide antibiotics represent a discrete class of drugs which is effective against aerobic and anaerobic gram positive bacteria [138]. Vancomycin (**V**), teicoplanin (**T**), teicoplanin aglycon (**A**) and ristocetin A (**R**) are the representatives of macrocyclic glycopeptide antibiotics. Vancomycin and teicoplanin are produced as fermentation products of *streptomyces orientalis* and *actinoplanes teichomyceticus*, respectively. Vancomycin was discovered by scientists at Eli Lilly Company from a *Streptomyces orientalis* in the 1950s [67]. As we know evolution is a continuous process, bacteria can evolve at a fast pace, leading to strains of drug-resistant bacteria . For example, vancomycin is considered to be the last drug of resort against methicillin-resistant *Staphylococcus aureus*.

Besides their biological significance, it turned out that these chiral macromolecules are also chemically very important for enantiomeric separation of many different compounds [62-66]. These macrocyclic glycopeptide antibiotics consist of an aglycon basket shape portion of fused macrocyclic rings and glycopeptide moieties attached to aglycon basket. Their molecular weights vary from 1200 to 2200 amu. Although they have analogous structures, they do differ in number of stereogenic centers, number of macrocycles, number of sugar moieties and number of aromatic rings, which makes significant difference in their ability for chiral recognition [138].

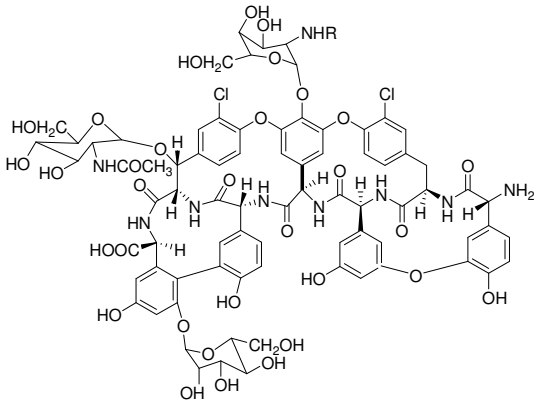
These macromolecules are soluble in water, partly soluble in polar aprotic solvents and insoluble in nonpolar solvents.



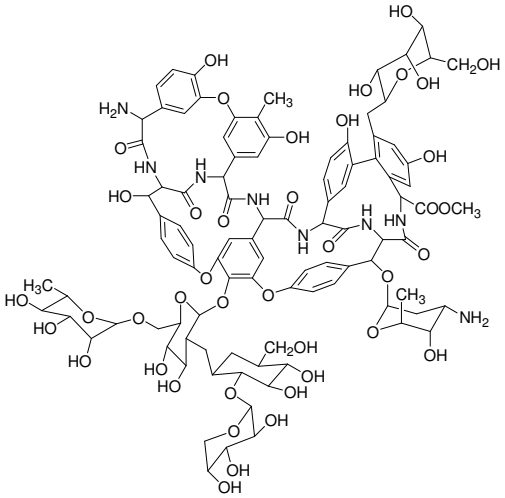
Vancomycin **(V)**
1449 g/mol



Teicoplanin Aglycon **(A)**
1197 g/mol



Teicoplanin **(T)**
1877 g/mol



Ristocetin A **(R)**
2166 g/mol

Figure 3.1 Structures of macrocyclic antibiotics

Macrocyclic glycopeptide antibiotics have analogous ionizable groups which control their charge and which have been shown to play a role in chiral recognition. In 1994, for the first time, Armstrong *et. al*. introduced these macrocyclic antibiotics as a new chiral selector for the separation of optically active enantiomers using HPLC [62,63,139-141] capillary electrophoresis

[64-68] and TLC [142]. These chiral macrocyclic glycopeptide antibiotics have more than one stereogenic centers which make them highly stereoselective and efficient chiral selectors. Macrocyclic glycopeptide antibiotics are very effective chiral selector because they are amphoteric, containing both hydrophobic and hydrophilic moieties, which makes them soluble in water as well as aqueous buffers and partially soluble in organic solvents [61]. Moreover they are reasonably stable in aqueous condition. Macrocyclic glycopeptide antibiotics have been proven very effective chiral stationary phases for both normal-phase as well as reverse-phase HPLC [62]. In addition, they are very stable and form chiral stationary phases that exhibit high loading capacity [62].

An effort has been made by Lim *et al.*, to investigate the binding affinity of chiral host molecules, vancomycin and ristocetin, with a tri-peptide guest molecule using ESI-MS [70]. In another study, Jorgensen *et al.* obtained the binding constant for vancomycin and diacetyl-*L*-lysyl-*D*-alanyl-*D*-alanine where no complex peak was observed for diacetyl-*L*-lysyl-*L*-alanyl-*L*-alanine [70]. This is the good illustration of the stereospecific binding of these macrocyclic antibiotics in the solution phase. Noncovalent interaction of vancomycin and the tripeptide L-lysyl-D-Alanyl-D-Alanine has been studied thoroughly by Perkins et. al.[81] and dissociation constant of the system was calculated (K_d ~10^{-6} M). This value of the dissociation constant is in good agreement with the value of the dissociation constant (K_d ~3 x 10^{-6} M) of vancomycin and Ac-*L*-lysyl-*D*-Alanyl-*D*-Alanine measured in our lab using solution phase 1:1 binding model (unpublished data). Whitesides and coworkers also studied the bivalent and trivalent system of vancomycin and peptide using a variety of solution phase methods. It has been shown that binding affinity increases as we go from monovalent to multivalent system (dissociation constant for trivalent system K_d ~4 x 10^{-17} M)[79]. In 1991, Williams *et al.* applied fast atom bombardment (FAB) mass spectrometric methods to study chiral recognition of N-acetyl-*D*-Ala-*D*-Ala and N-acetyl-*L*-Ala-*L*-Ala by looking at their degree of association with

vancomycin and ristocetin A chiral selectors. However, ESI is more amenable for providing a better picture of the binding in the solution phase compare to FAB. The glycopeptide antibiotics can interact through multiple interactions like hydrogen bonding, hydrophobic, dipole–dipole, π–π interactions and steric interactions [143,144]. More importantly, they possess an electrostatic or charge-to-charge interaction [71] which make them more suitable for our noncovalent binding studies. However, chiral recognition of small molecules, like blocked and unblocked amino acids by these macrocyclic antibiotics has not been previously examined using ESI-MS.

We have successfully employed these macrocyclic glycopeptide antibiotics as a chiral selector in the chiral recognition study using ESI-MS. Basic screening of these chiral selectors with different enantiomerically pure amino acids was performed in different solvent compositions like 100% H_2O, 50:50 H_2O:MeOH and 100% MeOH. Moreover, different buffer conditions, such as NH_4OAc (100μM) and NH_4OAc (100μM) with 0.5% HOAc were also investigated. Screening provides us with the necessary information like conformational preferences and change in selectivity at different pH conditions. Static titration method [59] is employed to obtain relatively accurate binding constant values. Solution phase 1:1 binding model has been employed successfully to study the stereospecific binding of the chiral host molecules vancomycin, teicoplanin ,teicoplanin aglycon and ristocetin A with chiral guest molecules including underivatized and derivatized amino acids. The dissociation constant is calculated for all systems using a solution phase 1:1 binding model and selectivity is obtained by taking the ratio of the dissociation constants for *D* and *L* amino acid binding with each macrocyclic glycopeptide antibiotic. Here it has been assumed that the host, guest and complex peak intensities in the mass specta are the representatives of the equilibrium concentration of host, guest and complex. Speculation are made about the correlation of mass spectral peak intensities with the equilibrium concentrations of the species as there is a significant effect of pH, solvation energies and solvent polarity which ultimately results in unequal response for the existing species. However, there are

several studies showing strong correlation of noncovalent complex in gas phase and solution phase especially stereospecificity of the noncovalent interactions [101].

3.3 Experimental

Materials

LC-MS grade water (H2O) and HPLC grade methanol (MeOH) were obtained from J.T. Baker (Phillipsburg, NJ). Ammonium acetate (NH4OAc) was purchased from J.T. Baker. Acetic acid was purchased from EMD Sciences, Gibbstown, NJ, USA. The enantiomerically-pure chiral selectors (host), vancomycin (V), teicoplanin (T) and teicoplanin aglycon (A) were generously provided by Advanced Separation Technologies, Inc. (Whippany, NJ, USA). Ristocetin A was purchased from MP Biomedicals (Solon, OH, USA). Blocked and unblocked amino acids Z-*D*-Leucine, Z-*L*-Valine, Z-*D*-Phenylalanine, *D*-Leucine and *L*-Leucine were purchased from Fluka, Deisenhofen, Germany. Z-*L*-Leucine and z-*D*-Phenylglycine were purchased from Fluka, Buchs, Switzerland. Z-*D*-Valine was purchased from Advanced Chemtech, (Louisville, KY, USA). The Z-blocking group (also commonly referred to as Cbz) represents a carbonylbenzoxy unit attached to the N-terminus amino group of the amino acid. *D* & *L* 3,4-dihydroxyphenylalanine (DOPA) was purchased from Sigma (St. Louis, MO).

Instrumental Set up

All measurements were performed using the LCQ Deca XP ion trap mass spectrometer from Thermo Fisher Scientific (San Jose, CA, USA) equipped with a conventional electrospray ion source. Capillary temperature was set to 200 ^{o}C and sheath gas flow was set to 20 arbitrary units. Applied spray voltage was 5 kV. Instrument method was tuned for each and every system in order to get the maximum response. Other parameters like offset voltage, tube lens voltage

were different for different tune methods. Data was collected and processed using Xcaliber software from Thermo.

Basic screening method

For screening experiment, samples were prepared in three different solvent conditions: 100% water (H_2O); 50:50 (v/v) MeOH/H_2O; and 100% MeOH. Two different buffer conditions: 100 micromolar (µM) NH_4OAc; and 100 µM NH_4OAc with 0.5% acetic acid (HOAc) were investigated. Degree of association (A_R) was obtained for each system by taking ratio of complex peak intensity (i_{HG}) and total peak intensities of host and complex ($i_H + i_{HG}$) as per Equation 3 of Chapter 1. Each measurement was performed three times and a ratio of average values of degree of association for the interaction between a macrocyclic antibiotic and each enantiomer were used to obtained selectivity ($\alpha = A_R/A_S$) as per Equation 4 of chapter 1.

Static titration method

Static titration method was applied to screen different systems and to obtain relatively accurate values for binding affinity [59]. In this experiment, a series of samples were prepared where the concentration of chiral host molecule were kept constant and chiral guest molecule concentration was varied at least one fold of magnitude as shown in Figure 3.2.

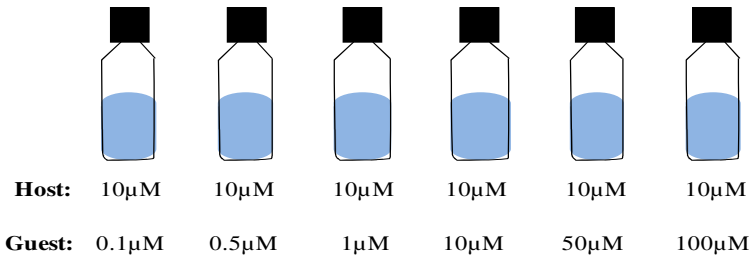


Figure 3.2 Sample preparation for static titration

Each additive formulation was investigated in separate experiments. The samples were introduced using an autosampler (25 µL injection) and solvent flow rate was set to 10 µL/min. Mass spectra were acquired for two minutes during elution of the sample mixture, and 50 scans

were averaged, where each scan was a composite of 3 microscans. Each sample solution was measured three times sequentially to obtain reproducible mass spectra. Total host peak intensity and total complex peak intensity were extracted by summing up all host peak intensities and all complex peak intensities. All the measurements using static titration method were done in 50/50 H_2O/MeOH. Solution phase 1:1 binding model was used for extracting binding constant for different systems where the ratios of host peak intensity (i_H) and complex peak intensity (i_{HG}) were plotted against initial guest concentration. The model for solution phase static titration is described in detail in Chapter 2 where it has been used to extract binding constant for monovalent and bivalent *tert*-butylcarbamoylquinine and N-alkoxybenzoylleucine systems.

3.4 Results & Discussion

Basic Screening results

Basic screening is an easy and quick way to obtain relative enantioselectivity and conformational preference for binding of a guest molecule to the chiral host molecule. In Table 3.1 presents basic screening performed for macrocyclic antibiotic chiral selectors with unblocked (Leucine) and blocked (*Z*-Leucine) amino acids under a range of solvent conditions. The complex peak for **V** and **A** chiral selectors was observed for both blocked and unblocked amino acid in all solvent systems. Contrarily, a complex peak for **T** was only observed in some solvent and buffer conditions and no complex peak was observed for **R** in all different solvent and buffer conditions.

Other interesting information about conformational preference can be extracted from Table 3.1. Conformational preference for the enantiomerically pure guest molecule (indicated in parentheses) which has higher degree of association with chiral host molecule. For the unblocked amino acid leucine, conformational preference for *D* was observed in all different solvent and buffer conditions except for **V** and **A** in MeOH when buffer condition 1 was employed. The effect of different buffer conditions is also observed in macrocyclic host molecule and blocked amino

acid systems. A conformational preference toward the *D* enantiomer was observed for macrocyclic antibiotic chiral selectors and leucine system in all different buffer and solvent conditions. In MeOH solvent condition, conformational preference of V, T and A reverts from *D* to *L* as buffer condition was changed from 1 to 2.

Table 3.1 Basic screening of vancomycin, teicoplanin and teicoplanin aglycon with leucine and Z-leucine enantiomers. Buffers 1 and 2 denote NH_4OAc 100μM and NH_4OAc 100μM with 0.5% HOAc, respectively.

Guest	**Solvent**	**Buffer**	**Selectivity (α)**		
			Vancomycin	**T-Aglycon**	**Teicoplanin**
Leucine	H_2O	1	1.3±0.60(*D*)	1.12±0.12(*D*)	-
		2	1.6±0.13(*D*)	1.09±0.02(*D*)	1.04±0.11(*D*)
	H_2O/MeOH	1	2.5±0.29(*D*)	1.09±0.11(*D*)	1.24±0.04(*D)*
		2	1.1±0.01(*D*)	1.03±0.02(*D*)	1.14±0.04(*D*)
	MeOH	1	1.3±0.18(*L*)	1.06±0.06(*L*)	1.06±0.06(*D*)
		2	1.0±0.08(*D*)	1.09±0.05(*D*)	1.10±0.07(*D*)
Z-Leucine	H_2O	1	1.1±0.04(*L*)	1.12±0.10(*D*)	-
		2	1.0±0.04(*L*)	1.10±0.16(*L*)	-
	H_2O/MeOH	1	1.1±0.05(*D*)	1.05±0.03(*D*)	1.22±0.09(*L*)
		2	1.0±0.01(*D*)	1.08±0.05(*D*)	1.23±0.28(*L*)
	MeOH	1	1.0±0.07(*D*)	1.04±0.01(*D*)	1.33±0.29(*D*)
		2	1.0±0.02(*L*)	1.20±0.01(*L*)	1.11±0.06(*L*)

Static titration results

Static titration method was used to obtain more reliable binding constant value of different host-guest systems. Dynamic range for the titration was determined by screening the host-guest

system at different guest concentrations. For example, Figure 3.3 describes the mass spectra of **A** and *D*-DOPA system where concentration of *D*-DOPA changes from 10 micromolar to 100 micromolar. The concentration of **A** is 10 µM and NH4OAc is 100 µM with 0.5% HOAc added as acid modifier. The increase in the complex peak (1394 m/z) intensity was observed as guest concentration was increased. Here peak at 1198 m/z represents the singly charged host peak.

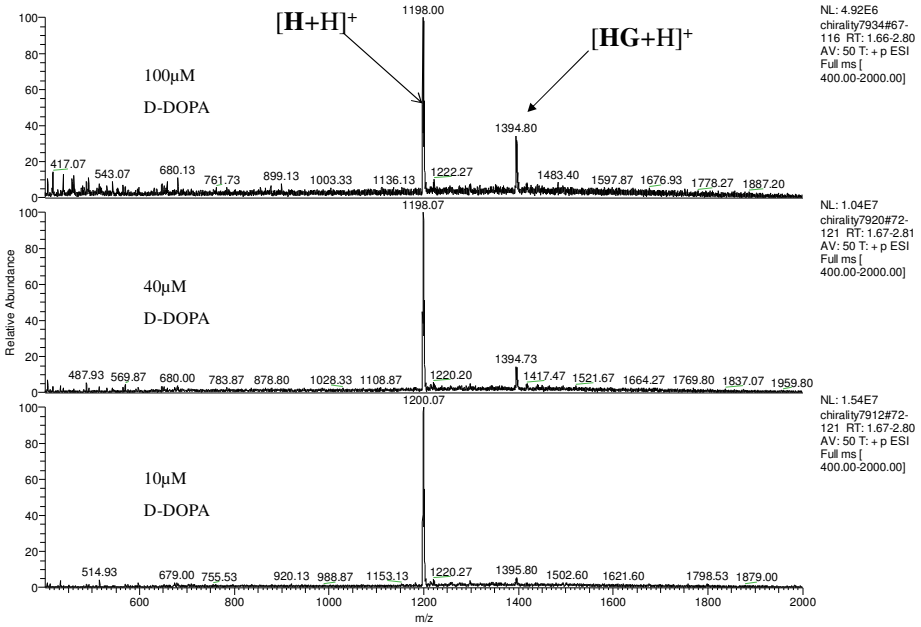
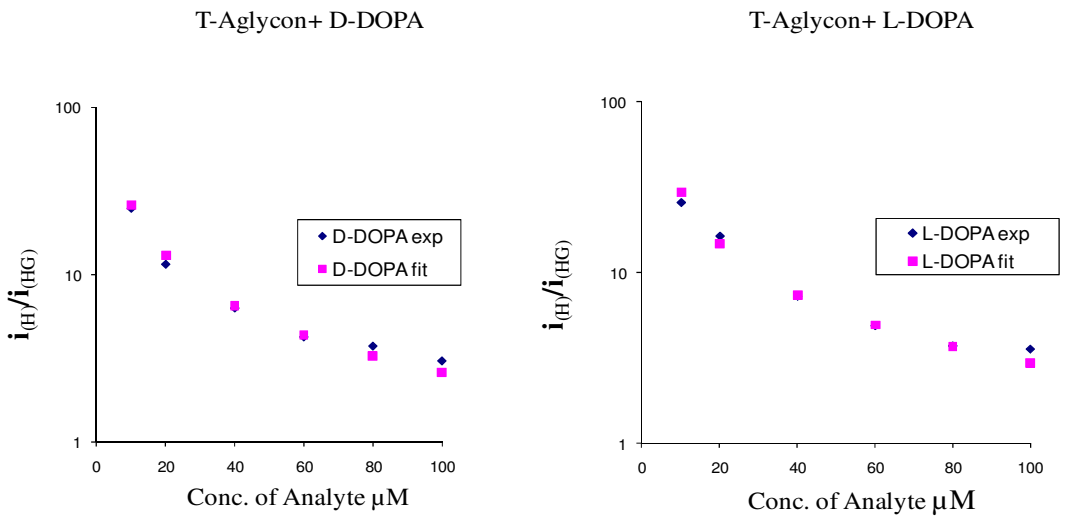


Figure 3.3 Representative mass spectra obtained at different concentrations of *D*-DOPA and teicoplanin aglycon (10µM) where 100µM NH4OAc with 0.5% HOAc was used as a solvent modifier.

Static titration was performed for the teicoplain aglycon and *D*-DOPA system where concentration of *D*-DOPA was varied from 1µM to 100µM. Similar titration experiments were performed for teicoplain aglycon and *L*-DOPA. A solution phase 1:1 binding model was applied to acquire binding constants for these systems, where the ratio of host peak intensity and complex peak intensity was plotted against initial guest concentration. The typical non linear curve fitting for teicoplain aglycon and *D*-DOPA as well as teicoplanin aglycon and *L*-DOPA systems are shown in Figure 3. Selectivity is obtained by taking ratio of binding constants.



$$\alpha = \frac{K_d \text{ of L-DOPA}}{K_d \text{ of D-DOPA}} = \frac{290}{250} = 1.1$$

Figure 3.4 Fit to the solution phase 1:1 binding model where ratio i_H/i_{HG} plotted against initial concentration of guest

Table 3.2 below lists systems to which static titration method was employed and binding constants were measured. All measurements were performed in 50:50/H_2O:MeOH solvent condition with the indicated modifier. Macrocyclic antibiotic host molecules **V**, **T** and **A** were titrated with blocked amino acid guest molecules. These results are in good agreement with both the screening study with respect to conformational preferences and selectivities. All static titration measurements show a conformational preference for the *D* enantiomer, consistent with the expected conformational preference based on the anti-bacterial mode of action of these macrocyclic antibiotics.

Table 3.2 Expimental values of binding constant for different host-guest systems determined using the static titration method.

Host	Guest	Buffer	K_d (μM)	α	LC (CE) Results
V	*D*-DNB Leucine	NH_4OAc 100μM	14	1.2	1.1(LC) 1.1(CE)
	L-DNB Leucine	NH_4OAc 100μM	17		
V	*D*-DNB Leucine	TEA 0.5%	16	1.8	1.1(LC) 1.1(CE)
	L-DNB Leucine	TEA 0.5%	30		
T	*D*-DNB Leucine	NH_4OAc 100μM	64	1.3	5(LC)
	L-DNB Leucine	NH_4OAc 100μM	85		
A	*D*-DOPA	0.5% HOAc	250	1.1	8(LC)
	L-DOPA	0.5% HOAc	290		
V	*D*-DOPA	NH_4OAc 100μM +0.5% HOAc	450	1.0	1.2(LC) 1.2(CE)
	L-DOPA	NH_4OAc 100μM +0.5% HOAc	460		
V	*D-Z*-Leucine	NH_4OAc 100μM +0.5% HOAc	19	1.3	N/A
	L-Z-Leucine	NH_4OAc 100μM +0.5% HOAc	24		
V	*D-Z*-Phenylalanine	NH_4OAc 100μM +0.5% HOAc	3	1.3	N/A
	L-Z-Phenylalanine	NH_4OAc 100μM +0.5% HOAc	4		
V	*D-Z*-Phenylglycine	NH_4OAc 100μM +0.5% HOAc	12	1.1	N/A
	L-Z-Phenylglycine	NH_4OAc 100μM +0.5% HOAc	13		
V	*D*-Leucine	NH_4OAc 100μM	267	1.7	1.5(CE)
	L-Leucine	NH_4OAc 100μM	461		
A	*D-Z*-Leucine	NH_4OAc 100μM +0.5% HOAc	31	1.8	N/A
	L-Z-Leucine	NH_4OAc 100μM +0.5% HOAc	56		

Vancomycin was further titrated with leucine and *Z*-leucine and the binding constant values are indicative of the fact that vancomycin has higher binding affinity towards blocked amino acids compare to unblocked amino acids. Selectivity values were compared to high performance liquid chromatography (HPLC)[61-65] and capillary electrophorasis (CE) data [66-70]. Good agreement was found in many cases, but some discrepancies were observed in a few systems, such as **A** binding DOPA and **T** binding DNB-leucine. One of the main reasons for these discrepancies is mechanistic difference between HPLC, CE and ESI-MS methods. These discrepancies are also due to the phase change as noncovalent complex goes from solution phase to the gas phase. The earlier study conducted by Dearden et. al. shows that valinomycin has higher affinity for the alkali metal cations in the gas phase compared to the solution phase [145]. The study shows the possible difference in the binding affinity as noncovalent complex goes from gas phase and the solution phase. Overall, static titration method was successfully employed and chiral recognition of blocked and unblocked amino acids is done successfully where three macrocyclic antibiotics **V**, **T** and **A** are used as host molecules.

Effect of different additives

The effect of different additives was investigated for different chiral recognition systems. Below Figure 3.5 describes the effect of different additives on the vancomycin and *Z-D*-leucine system where both host and guest present in equimolar concentrations. Doubly protonated noncovalent complex peak (858 m/z) intensity and host peak (725, 1450 and 1470 m/z) intensity are different in different additive containing solutions. It has been observed with these macrocyclic chiral host and amino acid systems that acetic acid enhances the response of the noncovalent complex significantly which is illustrated in the following Figure 3.5.

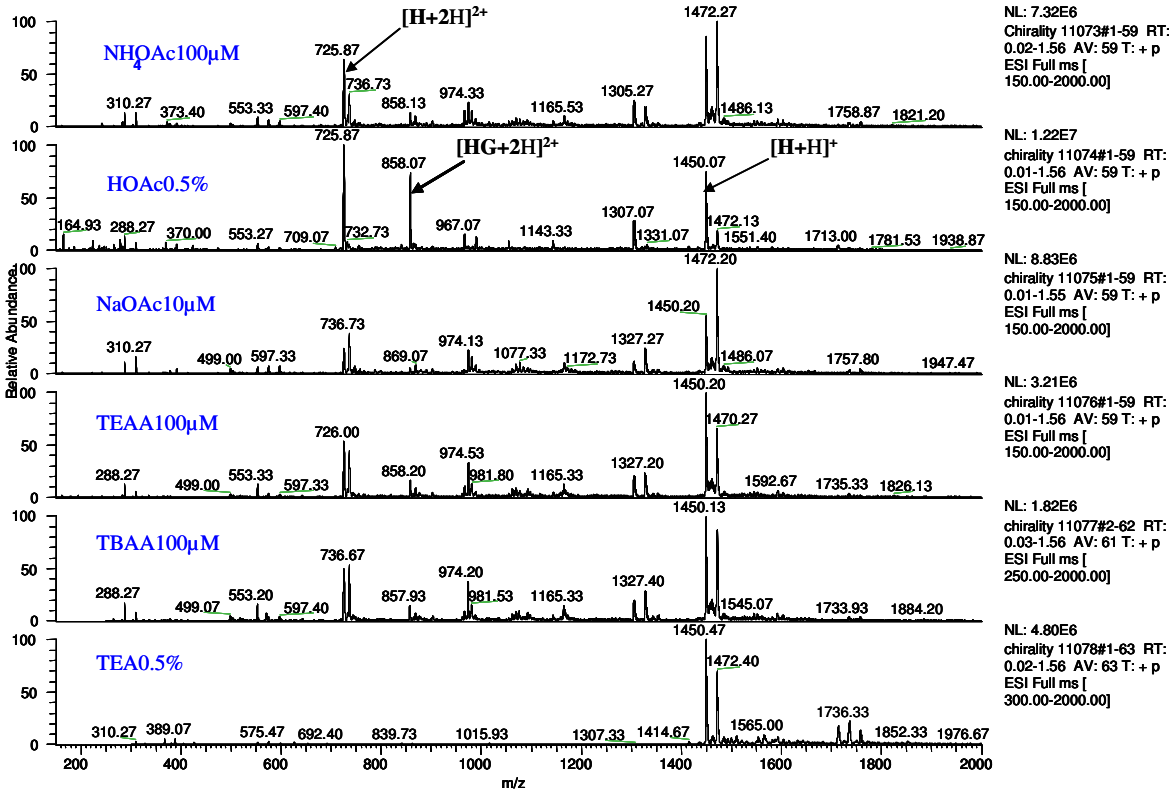


Figure 3.5 Effect of different additives investigated on vancomycin 10µM and *Z-D*-leucine 10µM system.

Effect of different buffer concentration

Figure 3.6 describes the effect of different buffer concentration on vancomycin and Z-*L*-leucine system. Three different NH_4OAc concentrations of 1000 µM, 500 µM and 100 µM were evaluated. All these measurements were performed in 50/50 H_2O/MeOH solvent system with acetic acid as an additive. Signal to noise ratio decreases as buffer concentration increases from 100 µM NH_4OAc to 1000 µM NH_4OAc. Moreover, complex peak intensity decreases by three- to five-fold as NH_4OAc concentration increases by one fold of magnitude.

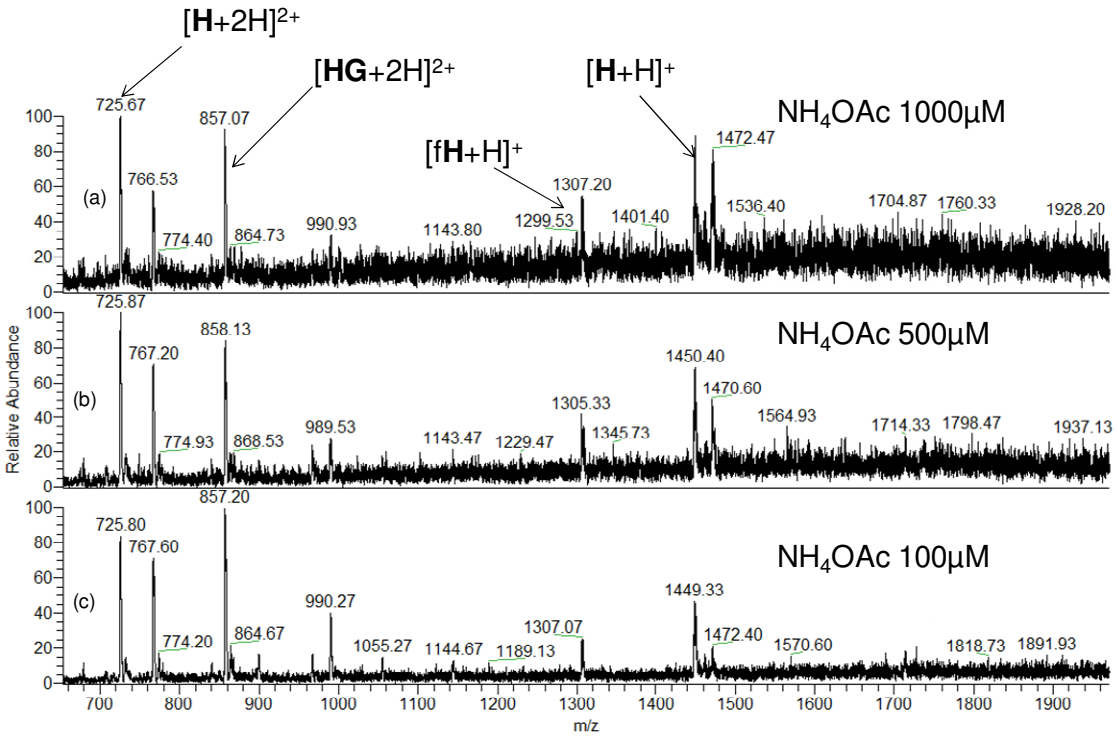


Figure 3.6 Mass spectra of Vancomycin 10µM, Z-*L*-leucine 10µM, HOAc 0.5% with different NH_4OAc concentrations (a) 1000 µM, (b) 500 µM and (c) 100 µM. H = Host, fH = vancomycin aglycon and HG = complex.

To evaluate the effect of different buffer concentration on binding constant determination static titration method was employed where chiral host molecule vancomycin was titrated against Z-leucine, Z-phenylalanine and Z-phenylglycine at two different NH_4OAc concentrations, 100 µM and 1000 µM.

Table 3.3 Effect of different buffer concentration on determination of binding constant for Vancomycin 10µM and blocked amino acids (N = 3).

Host	NH_4OAc	Guest	K_d (µM)	α
Vancomycin	100µM	z-D-Leucine	19 ± 4	**1.3**
		z-L-Leucine	24 ± 7	
		z-D-Phenylalanine	3 ± 0.2	**1.3**
		z-L-Phenylalanine	4 ± 0.7	
		z-D-Phenylglycine	12 ± 1.7	**1.1**
		z-L-Phenylglycine	13 ± 3	
	1000µM	z-D-Leucine	46 ± 0.7	**1.3**
		z-L-Leucine	59 ± 0.7	
		z-D-Phenylalanine	21 ± 5	**2.3**
		z-L-Phenylalanine	49 ± 9	
		z-D-Phenylglycine	15 ± 0.7	**1.5**
		z-L-Phenylglycine	22 ± 0.1	

In Table 3.3. the comparison of binding constant in two different buffer conditions has been done for vancomycin and blocked amino acid systems. All measurements were performed in 50/50 H_2O/MeOH solvent condition. All above measurements were performed three times and average value for the dissociation constant with standard deviation is reported in Table 3.3. The value of dissociation constant increases for all three systems as the concentration of NH_4OAc increases from 100µM to 1000µM. Results in Table 3.3 are evident of an increase in the selectivity of the same system when buffer concentration is increased from 100µM to 1000µM. Interestingly, for vancomycin and blocked amino acid systems no selectivity observed when NH4OAc 100µM is used. Significant selectivity observed for vancomycin and blocked amino acid systems at higher NH4OAc concentration (i.e.1000µM).

3.5 Conclusion

Vancomycin, teicoplanin and teicoplanin aglycon were evaluated as a chiral selector for enantiodiscrimination using ESI-MS. Basic screening and static titration methods were use to investigate the binding affinity of host-guest systems consisting of macrocyclic antibiotic and amino acid systems. Vancomycin and teicoplanin aglycon proved to be good chiral selectors for molecular recognition using ESI-MS. Teicoplanin shows moderate or no binding towards blocked and unblocked amino acids in different solvent and buffer conditions where ristocetin A does not demonstrate any binding towards blocked and unblocked amino acids. Effect of different buffer composition and different buffer concentration was also evaluated to investigate the effect on binding affinity. Chiral recognition for these macrocyclic antibiotics was evaluated successfully. The main advantages speed and sensitivity of these ESI-MS methods were exploited.

CHAPTER 4

CONCLUSION

Electrospray ionization mass spectrometry is widely used for the noncovalent binding study as it is a soft ionization method and capable of transferring weakly bound noncovalent complexes from the solution phase to the gas phase. In these studies, ESI-MS was employed successfully for the investigation of chiral recognition in different host-guest systems. Quantification of binding affinity was performed using different solution phase ESI-MS techniques like basic screening and static titration methods. Here, it is assumed that the mass spectrum peak intensities represent the solution phase equilibrium concentration of appropriate species. Speculations are made about the validity of binding affinities measured using this method as drastic change occur in bulk solution as analyte species transferred from the solution phase to the gas phase.

Solution phase titration method was applied to monovalent, bivalent and multiple monovalent systems of tert-butylcarbamoylquinine and N-alkoxybenzoyl leucine in order to investigate the binding affinity and multivalency on chiral recognition. Significant increase in the binding affinity of bivalent system observed compared to monovalent system using solution phase static titration method. Gas phase collision induced dissociation(CID) measurements are also done for monovalent and bivalent system. V_{50} (energy required to break the parent complex ion peak to 50 percent) was measured for monovalent and bivalent systems. RRKM analysis was applied to normalize the collision energy, but further work is needed to understand

entropic contributions to the measured dissociation threshold obtained by ion trap mass spectrometry.

The solution phase titration method was also employed for investigating the binding affinity of macrocyclic antibiotic chiral selector and amino acid systems. Vancomycin, teicoplanin and teicoplanin aglycon were successfully demonstrated as effective chiral selectors whereas no binding observed for ristocetin A and amino acid systems. Effect of different buffer conditions as well as different solvent conditions were evaluated. Good agreement found for many systems in terms of conformational preference.

An important quantitative information was obtained about noncovalent binding of different host-guest systems using ESI-MS solution phase titration method. The main advantage of ESI-MS over other physicochemical methods is its speed, sensitivity and low sample consumption. Moreover, it also provides us with stochiometric information about noncovalent complex. ESI-MS provide us with an efficient analysis of noncovalent binding affinity with certain limitations. Continuous efforts are made to enhance the reliability of ESI-MS methods by justifying a few complex issues addressing response factor of different species. Overall, ESI-MS is a novel tool to study binding affinity of different host-guest systems in solution phase as well as in the gas phase.

Macrocyclic antibiotics turned out to be effective chiral selectors for the chiral recognition study using ESI-MS. Recently, dynamic titration method developed by Schug et. al to investigate the binding affinity of noncovalent complex using ESI-MS [60]. Application of this dynamic titration method can be studied further for different host-guest systems containing macrocyclic antibiotics as a host molecule. Results of dynamic titration method can be compared with the current results obtained by static titration method.

APPENDIX A

CHAPTER 1 CITATION

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APPENDIX B

CHAPTER 2 CITATION

Manuscript in preparation

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BIOGRAPHICAL INFORMATION

Manish Joshi was born in India where he spent 25 years of his life. He earned his Bachelor of Science degree in chemistry with minor in physics and biology from South Gujarat University at India. He continued his education in chemistry by earning Master of Science degree in organic chemistry from the same university. He worked as a chemist for a year in the process development lab of Cadila Pharmaceuticals Ltd., one of the top bulk drugs producing company in India. In August 2005, he joined the University of Texas at Arlington where he pursued his research under guidance of Dr. Kevin Schug and Dr. Daniel Armstrong. Focus of his research was chiral recognition and binding constant determination using electrospray ionization mass spectrometry. He worked as a graduate teaching assistant at University of Texas at Arlington for two and half years. He gained crucial industrial research experience by doing internship at Pfizer. He is also a member of the American Society for Mass Spectrometry since 2006. Master of Science degree in analytical chemistry was awarded to him in August, 2008.