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Diastereoselective discrimination of lysine–alanine–alanine peptides by zwitterionic cinchona alkaloid-based chiral selectors using electrospray ionization mass spectrometry

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ABSTRACT

Electrospray ionization-mass spectrometry (ESI-MS) was used to investigate stereoselective interactions between seven zwitterionic alkylsulfonate-modified cinchona alkaloid chiral selectors and biologically relevant lysine–alanine–alanine tripeptide and alanine–alanine dipeptide selectands in modified methanolic solutions. Ion intensities from full scan mass spectra were used to assess degrees of association, the ratios of which were used to calculate selectivities for different selector–selectand pairs. The results support prior work on similar systems using HPLC, in that binding is mediated in these systems primarily through the quinuclidine amine on the selector and the C-terminal carboxylate of the peptide. N^{α}- and N^{α}, N^{ε}-acetylated forms of the tripeptide were used to study the relative contribution to binding imparted by the presence of multiple basic amines on the tripeptide with the selectors; this was not previously investigated by HPLC. The ability of the sulfonate group on the selector to reach and preferentially interact with the N^{ε}-amine on the side chain of lysine was revealed. Overall, in acidic methanol conditions (0.5% acetic acid), degrees of association ranged from 1.5% to 17%, and selectivities ranged from non-selective to a 5.5:1 preference for binding one peptide stereoisomer over another with a given chiral selector. In sodium acetate (100 μM)-modified methanol solutions, significant changes in degrees of association (ranging from 4% to 25%) and selectivities (ranging from non-selective to 4.2:1 preference) were observed. These mass spectrometry experiments help to clarify the chiral recognition mechanism for these selectors and suggest that retention and selectivity could be further modulated in HPLC experiments through the utilization of alkali salt-containing mobile phases.

1. Introduction

Interest in chirality-based research has intensified over the last few decades, due primarily to scientific and economic concerns surrounding the effective action of pharmaceutical and agrochemical compounds. A significant interest exists in developing single-enantiomer products to increase effectiveness and decrease toxicity of chiral compounds in natural systems [1,2]. To achieve this, chiral selectors have been developed to facilitate separation and purification of enantiomers from mixtures of chiral compounds. Chiral selectors can be classified as natural, semisynthetic, and synthetic compounds, which are able to differentially bind one enantiomer form over another. Thus, the formation of diastereomeric complexes between chiral selector and chiral selectand facilitates enantioselective separation, in a variety of formats, but more varied and more versatile chiral selectors are ever needed to keep pace with the development of new chiral compounds in industrial settings [3]. In this work, we describe mass spectrometric-based mechanistic studies surrounding the use of novel semisynthetic zwitterionic cinchona alkaloid-based chiral selectors. These selectors have been shown to separate a wide range of compounds, including biologically relevant chiral amino acids and peptides [4–6]. Soft ionization mass spectrometry methods can help shed light on the details of interactions between selector and selectand pairs, in a manner complementary to liquid phase separation experiments.

The precise mechanisms associated with enantioselective (or diastereoselective) recognition between selector and selectand can often be difficult to ascertain. As the selectand enantiomers approach the chiral selector, physical forces induce an ideal fit of one enantiomer. The ideal fit, or effective binding of one enantiomer over the other, is achieved if there is a steric fit, electrostatic fit, and/or dynamic fit/induced fit [7]. Steric effects

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include size and shape of the selectand, but the chiral selector is usually premade to present a binding pocket for interaction. Electrostatic fit deals with the orientation of functional groups and the complementary groups present on both molecules. These forces include ionic, hydrogen bonding, dipole–dipole, and π–π interactions. Dynamic fit or induced fit refers to maximizing binding interactions by conformational changes during the process of complexation.

Zwitterionic chiral selectors, more precisely those based on a cinchona alkaloid scaffold, have recently been used as chiral stationary phases (CSPs) in HPLC [4]. These selectors consist of fused cation- and anion-exchange moieties in a low molecular mass compound. When bound to a solid support, the resulting CSPs are able to separate the enantiomers and diasteromers of a wide range of ionizable chiral analytes, including chiral acids, chiral amines, chiral amino acids, and peptides [4–6,8]. These studies have shown that the distinct arrangement of the chiral centers and carbamate-linked alkylsulfonates about the C8 and C9 position of cinchona alkaloids are essential for effective enantioselective zwitterion recognition.

Mass spectrometry (MS) has made significant contributions to advance the study of enantioselective recognition [9–11]. A variety of techniques has been developed to enable enantiomeric excess determinations [12–14]. However, these methods generally require calibration with selectands of known enantiomeric excess and are inferior to traditional liquid phase separation methods commonly used for such purposes [15,16]. Yet, a distinct advantage of mass spectrometry, particularly in combination with soft ionization sources, such as electrospray ionization (ESI), is that information on both the solvated and solvent-free recognition mechanisms can be discerned. Full scan mass spectra can often provide a snap-shot of the equilibrium picture in solution, assuming that the ESI process does not alter the relative concentrations of interactants and complex through the droplet desolvation process [17–20]. Once isolated in the gas phase, tandem mass spectrometry can be used to interrogate the complex in the absence of solvation [21,22]. This combination can be useful for a more detailed characterization of recognition mechanisms; however, the full scan solution-phase targeting methods provide information more relevant to assessing the performance of a chiral selector in solution. We have previously used these approaches to study a number of chiral selector systems, including those based on cinchona alkaloids and metallotartrates [23–25]. However, up to now ESI-MS has not been used to help elaborate the mechanisms associated with the recognition of stereoisomeric selectands by zwitterionic cinchona alkaloid selectors.

The goal of this study was to evaluate the solution phase-based mechanism of diastereoselective binding of a wide selection of cinchona-alkaloid-based zwitterionic chiral selectors to biologically relevant lysine–alanine–alanine tripeptides. L-Lysine–D-alanine–D-alanine is well known to be a component of lipid II, which is involved in peptidoglycan formation in bacterial organisms [26–29]. Multiple stereochemical configurations of the peptide, as well as N^{α}- and N^{α},N^{ε}-acetyl-protected lysine forms of the peptide were evaluated. Additionally, all four stereoisomers of alanine–alanine dipeptides were studied to understand (a) the contributions of peptide length on the degree of complex formation, (b) the influence of the N-terminal lysine residue and its enhanced potential for ion pairing with the sulfonate site of the selectors on stereoselective recognition, and (c) the contribution of individual stereocenters of the peptide chain as well as the combinations thereof. Overall, the results show that the binding between the selector and the selectand is primarily supported through the quinuclidine tertiary amine and the C-terminus of the peptide; however, this is altered to an extent with different chiral selector forms. Also, the degree of complex formation and selectivities are altered as the peptide length is shortened and its capacity for ion pairing is altered, and this is consistent with HPLC experiments [6].

2. Experimental

2.1. Materials

Seven zwitterionic chiral selectors, synthesized as previously described [4,8,30], were used in this study. They are termed **CS1**–**CS7** and their structures are shown in Fig. 1. **CS1** and **CS2** are based on the combination of taurine with quinine and quinidine, respectively. Quinine and quinidine are diastereomers, based on inversion of stereochemistry on two out of their five stereocenters, namely C-8 and C-9. These chiral centers predominantly control the favorable stereorecognition characteristics of cinchona-based selectors [31]. **CS1** and **CS2** are characterized by a comparatively flexible ethyl sulfonate linked to the cinchona alkaloid scaffold through a carbamoyl moiety. The remaining selectors are all quinine (8*S*, 9*R*)-based and their sulfonate groups are sterically more demanding. In **CS3** and **CS4**, the pendant sulfonates are linked and conformationally fixed in a *trans*-1,2-cyclohexyl ring. These selectors have opposite configurations about the anionic functional unit. **CS5**, **CS6**, and **CS7** all incorporate the extended alkyl sulfonate pendant to quinine, similar to **CS1**. However, various functional groups are incorporated in a stereospecific manner, β to the sulfonate group, primarily to investigate additional steric effects on stereoselectivity.

Four tripeptides (L-lysine–D/L-alanine–D/L-alanine; denoted as KAA, KAa, KaA, and Kaa, where an upper-case letter indicates the α-amino acid in the L-configuration and a lower-case letter indicates the D-configuration) and four dipeptides (D/L-alanine–D/L-alanine; denoted as AA, Aa, aA, and aa) were synthesized in-house using standard solution phase peptide chemistry and used as chiral selectands in this study. Appropriate synthesis and purification of these compounds was confirmed by NMR and MS measurements. The analyzed peptides were spectroscopically and chromatographically pure. No diastereomers were detected during the preparation of these materials and no degradation of enantiopurity could be detected by the selected peptide synthesis protocol, based on comparison of dipeptide optical activity with literature values. Details of the synthesis, including NMR data, are given in the supplementary information. As a means to differentiate and evaluate the importance of the lysine N^{α}-terminal and N^{ε}-terminal amines, N^{α}-Ac-L-Lys-D-Ala-D-Ala (AcKaa; acetylated N^{α}-terminal amine) and N^{α}-Ac-L-Lys(N^{ε}-Ac)-D-Ala-D-Ala (Ac_2Kaa; acetylated N^{α}-terminal and N^{ε}-terminal amines on Lys) were purchased from Bachem (Torrance, CA), and evaluated concurrently with the unprotected tripeptide selectands.

All solutions were prepared using LC–MS grade methanol (Burdick & Jackson, Muskeegon, MI). Selectors and selectands were first dissolved in methanol to create 1 mM stock solutions. From these, sample solutions for analysis were prepared. Each solution contained 10 μM of a selector, 50 μM of a selectand, and either 100 μM sodium acetate (NaOAc; Sigma–Aldrich, St. Louis, MO) or 0.5% (84 mM) acetic acid (HOAc; J.T. Baker, Phillipsburg, NJ). Sodium acetate was chosen as one solution modifier to normalize background sodium concentration and stabilize sodiated forms of complex ions observed in ESI-MS [32]. Acetic acid was chosen as an alternative to help understand the effect of attenuating ionization of the C-terminal carboxylate on the selectands in solution. It is also a common modifier used in HPLC experiments where these chiral selectors are used as CSPs, and the presence of acid in solution helps the formation of directed proton-supported carboxylate–ammonium electrostatic interactions in these systems [4,6,8].

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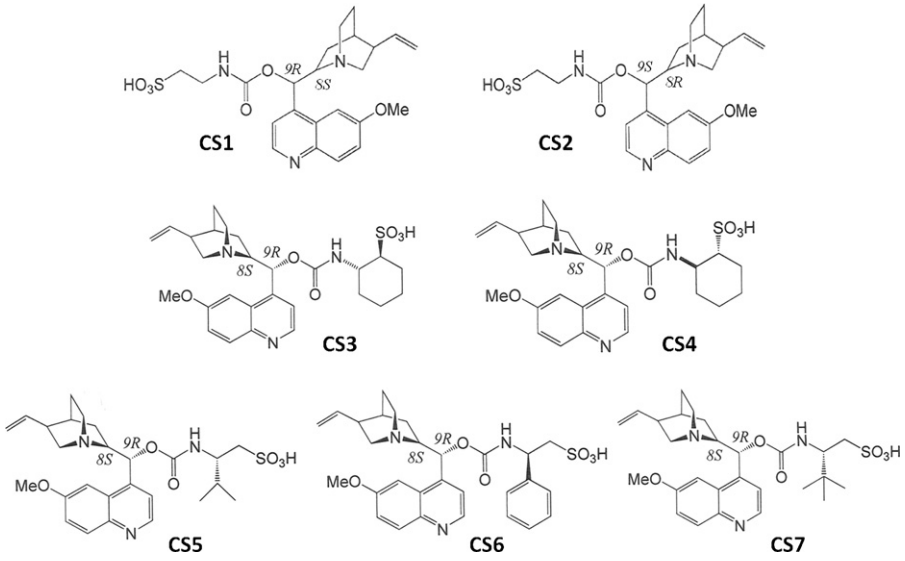

Fig. 1. Structures of zwitterionic chiral selectors investigated in this work.

2.2. Methods

The study was carried out on a Thermo LCQ Deca XP mass spectrometer equipped with a conventional ESI source. Ionization conditions were optimized for a representative selector–selectand complex ion (**CS1** + Kaa in NaOAc) in the positive ionization mode and the conditions were maintained for all subsequent analyses. ESI spray voltage was set to 3.5 kV; transfer line temperature was 200 °C; nebulizer gas flow rate was 20 arbitrary units; and tube lens offset voltage was 20.0 V. Each sample was measured in triplicate by direct infusion at a flow rate of 5 μL/min. Each replicate mass spectrum acquired was composed of an average of approximately 60 scans, where each scan was an average of 3 microscans. After each selector–selectand pair was analyzed, the lines were extensively washed (100% methanol) to ensure no residual analytes remained. The process was repeated for every selector–selectand pair studied. XCalibur (ver. 1.5) was used for data acquisition and the data for all ions of interest were transferred to Microsoft Excel through the use of an in-house-developed data extractor program.

The degree of complex formation (A), as given in Eq. (1), for each selector–selectand combination was calculated and compared to determine relative diastereoselectivity.

$$A = \frac{\sum I_{\text{complex}}}{\sum I_{\text{total}}} \quad (1)$$

The degree of complex formation was assessed as the sum of intensities of all observed ionic complex forms (I_{complex}) divided by the sum of the intensities of all ion forms (I_{total}), according to common convention [9–11]. Ion forms commonly observed in the mass spectra were (CS denotes chiral selector and S denotes chiral selectand): $[CS+H]^+$; $[CS+Na]^+$; $[2CS+Na]^+$; $[2CS-H+2Na]^+$; $[S+H]^+$; $[S+Na]^+$; $[S-H+2Na]^+$; $[S-2H+3Na]^+$; $[2S+H]^+$; $[CS+S+H]^+$; and $[CS+S+Na]^+$. Thus, the ratio of values of A for any two selector–selectand systems can be used as measurable enantioselectivity or diastereoselectivity (α), where any value that does not equal unity, taking into account experimental error, can be referred to as exhibiting enantioselective or diastereoselective discrimination.

3. Results and discussion

The recent years in pharmaceutical industry have seen a move toward significant production of biological compounds as therapeutics [33–35]. Biologically inspired or derived systems are naturally composed of chiral building blocks, and are ideally suited for selective interaction with other biological systems. Eukaryotic systems are largely composed of L-amino acid-containing peptides and proteins. However, an increasing body of literature points to the importance of polymorphisms or post-translational racemizations, as means for D-amino acids to become incorporated into these systems. While the effect of these configurational changes has not been fully assessed, some connections have been made with deleterious conditions, such as Alzheimer's disease. In addition, D-amino acid-containing systems are often less susceptible to normal enzymatic degradation [36–39]. In prokaryotes, the inclusion of D-amino acids is common. D-Amino acid-containing depsipeptide units in bacteria comprise important building blocks of cell walls, and subsequently, they are also important targets for treatment of bacterial infections by therapeutics, such as glycopeptides antibiotics [26–29]. Therefore, systematic investigations of compounds designed to recognize amino acid polymorphisms, in both natural and synthetically manipulated systems, are important to support the further development of medicinal and analytical chemistry in these areas.

A systematic investigation of interactions between seven zwitterionic cinchona-alkaloid-based chiral selectors (Fig. 1) and a series of free and protected lysine–alanine–alanine tripeptides and free alanine dipeptides, of variable stereochemical configuration was performed. It is well know that at least three points of interaction are required for enantioselective recognition [40]. As shown in Fig. 2, these systems are capable of joining by multiple attractive and repulsive interactions, including those created by Coulombic, hydrogen bonding, Van der Waals, and steric forces. Through systematic variation of their structural motifs, a clearer picture of the capacity of these selectors to distinguish between the forms of biologically relevant peptides was accomplished.

3.1. Taurine–quinine/quinidine chiral selectors (CS1 and CS2)

Fig. 3 displays representative mass spectra recorded for the various chiral selector–selectand systems investigated in this study (those for **CS2** with Kaa are specifically depicted). Spectra recorded in the presence of HOAc (Fig. 3A) clearly show the protonated complex ion form with approximately a 9% degree of association (calculated according to Eq. (1)), in this case. Because cinchona

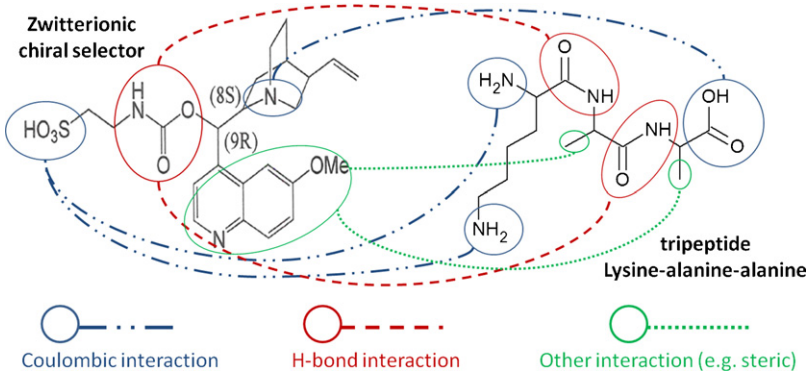

Fig. 2. General depiction of interaction forces between a zwitterionic chiral selector and a Lys–Ala–Ala tripeptide.

alkaloid chiral selectors have been previously investigated using mass spectrometry in the presence of alkali metal salts as cationization reagents, experiments were also performed in the presence of a normalized NaOAc background. In this case (Fig. 3B), multiple protonated and sodiated complex ion forms were observed and the degree of association (calculated including the sum of all ionic complex forms observed) was elevated to 22%. As expected, both the free selector and free selectand were observed, both in monomeric and dimeric ion forms. Given the zwitterionic nature of each, some degree of self-complexation in solution and/or as a result of the electrospray process was expected. While an evaluation of potential contributions from nonspecific binding were not specifically pursued, the presence of 1:1 (and no higher order) binding between selector and selectand provides evidence that only specific complexes were produced using the optimized experimental conditions.

The relative degrees of association for each selector with each selectand can be used to deduce selectivity. Fig. 4 shows the degrees of association recorded for **CS1** (Fig. 4A) and **CS2** (Fig. 4B), in both HOAc and NaOAc media, for each of the tripeptides. Also shown are the effects of blocking the N^{α}- and/or N^{ε}-termini of lysine (Fig. 4C and D) on the degree of association, and the results obtained for dipeptide diastereomers and enantiomers (Fig. 4E and F). Detailed calculations of average enantioselectivities, diastereoselectivities, and their associated precision for all systems are given in Supplemental Information (Fig. S1).

While selectivities were modest, both **CS1** and **CS2** exhibited a preference for binding Kaa over other tripeptides in the NaOAc solution. Selectivity was greater than 2.5 when comparing the change in stereochemical configuration of the C-terminus from D-Ala to L-Ala. In fact, all selectors under all conditions tested exhibited the least degree of association with the KaA tripeptide. In HOAc, **CS2** maintained essentially the same relative binding selectivity as in NaOAc (Kaa > KAA > KAa > KaA). However, **CS1** could not discriminate between any of the stereoisomers, except for KaA (Kaa ≈ KAA ≈ KAa > KaA).

Experiments performed with the blocked tripeptide indicated that binding is predominantly forged through the interaction of the quinuclidine tertiary amine on the chiral selector and the C-terminus of the tripeptide. Prior studies also support the importance of this interaction site [6]; however, the contribution of the sulfonate should not be understated. For both **CS1** and **CS2**, analyzed in NaOAc, progressive protection of the α- and ε-terminal amines on Kaa reduced binding to an appreciable extent. Acetylating the amines on Kaa removes the potential for Coulombic interaction with the sulfonate binding increment on the selector; yet, even with both amines protected, **CS1** and **CS2** still exhibited an 11% and 19%, respectively, degree of association with Ac_2Kaa.

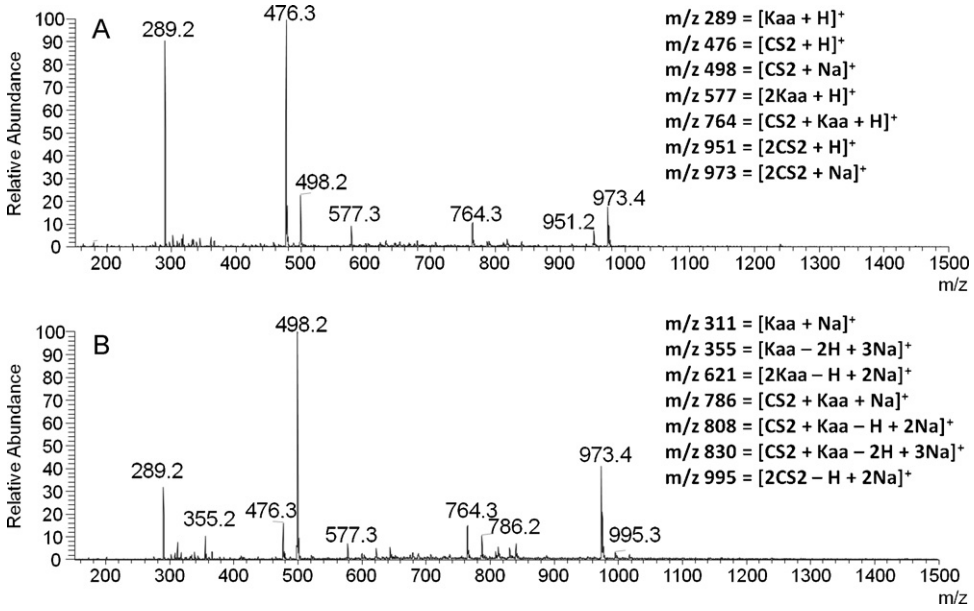

Fig. 3. Representative mass spectra observed for **CS2** and Kaa in (A) methanol + 0.5% HOAc and (B) methanol + 100 μM NaOAc.

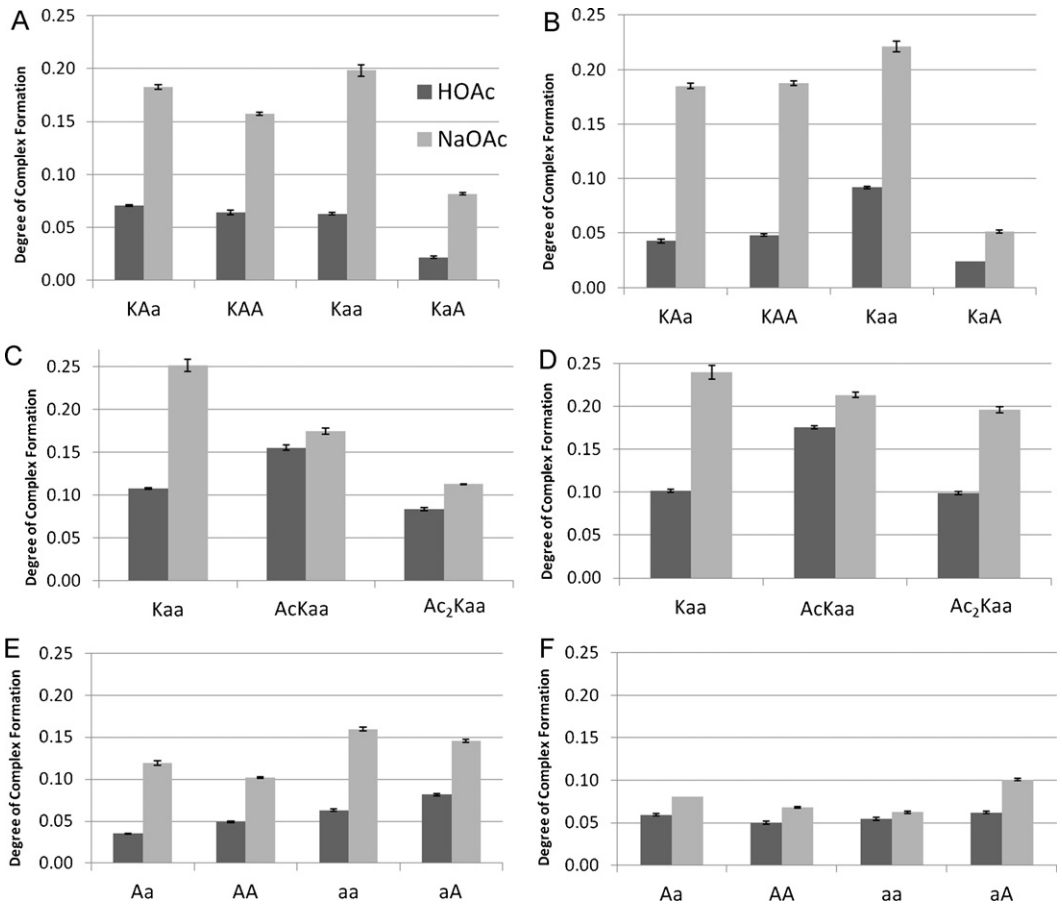

Fig. 4. Degrees of association for **CS1** (A, C, and E) and **CS2** (B, D, and F) with unprotected tripeptides (A and B), protected tripeptides (C and D) and dipeptides (E and F) in methanol plus either 0.5% HOAc or 100 μM NaOAc. The legend given in (A) is valid for all figure panels.

Apparently, electrostatic attraction of the C terminus and the quinuclidine moiety and polar interactions of the peptide backbone with the carbamoyl moiety can partially compensate for the loss of Coulombic interaction connected with the masking of the N-terminal charge(s). It is important to remember that the difference in the cinchona alkaloid scaffold (quinine in **CS1** vs. quinidine in **CS2**) has previously been cited to be responsible for discriminating the stereoisomers of chiral acids [31]. Here, a reversal in binding preference for peptides possessing opposing stereochemistries was not observed. This could be due to the fact that the C-terminal alanines have small side chains, which provide limited steric influence for discrimination, or that the lysine–sulfonate interaction is so strong, it compromises the ability of the cinchona alkaloid motif to exert its expected stereodiscrimination. Additional experiments would be necessary to fully elucidate this picture.

In HOAc, the situation was different. Both **CS1** and **CS2** showed the highest degree of binding with AcKaa. This is another trend, which was consistent throughout all selectors tested. With the N^{α}-terminus of Kaa blocked, the sulfonate on the selector can exclusively interact with the ε-terminal amine, and for **CS1** and **CS2**, this caused the highest degree of binding for any of the peptides studied in these experiments. For these selectors, the flexible alkyl sulfonate appears to have the ability to arrange itself preferentially to interact with the flexible side chain of Lys in this system.

When the lysine was removed, and only Ala–Ala dipeptides were considered, in many cases, binding was diminished, but in some, enhancements relative to the tripeptides were recorded. For **CS1** in NaOAc, significant enantioselectivity was recorded ($\alpha = 1.57 \pm 0.02$ for aa over AA and $\alpha = 1.21 \pm 0.03$ for aA over Aa). Degree of binding was seen to be similar to that for the tripeptides, and the presence of the N-terminal D-Ala provided the strongest interaction for the dipeptides. This preference was maintained in the HOAc solution, but the magnitude of enantioselectivity was altered ($\alpha = 1.28 \pm 0.03$ for aa over AA and $\alpha = 2.31 \pm 0.05$ for aA over Aa). Overall, the magnitude of enantioselectivity and diastereoselectivty for **CS1** was greater than reported for related HPLC experiments [6]. However, this is not uncommon as the mechanism associated with selectivities measured by association/dissociation equilibria in ESI-MS experiments is different from the dynamic theoretical plate-based HPLC separation mechanism [24]. **CS2** interacted with the dipeptides to a much lesser degree than **CS1**, and only minimal selectivities were recorded. Relative to experiments with tripeptides, in HOAc, **CS2** showed similar magnitudes of association with the dipeptides, but the affinity was significantly reduced in the NaOAc solution.

In general, for most systems investigated, tripeptides showed a higher degree of association with the selectors than did dipeptides in NaOAc solution. This could be attributed to additional attachment sites for sodium cations on the lysine residue, as well their effect on mediating favorable interactions between selector and selectand. Our group has previously shown the advantages, with respect to enhanced binding and sensitivity, that alkali metal salts can impart on cinchona alkaloid-based interaction systems [41]. Recent computational evidence also suggested that the cations can mediate salt–bridge interactions between basic amine and carboxylate interaction sites in these complexes [42].

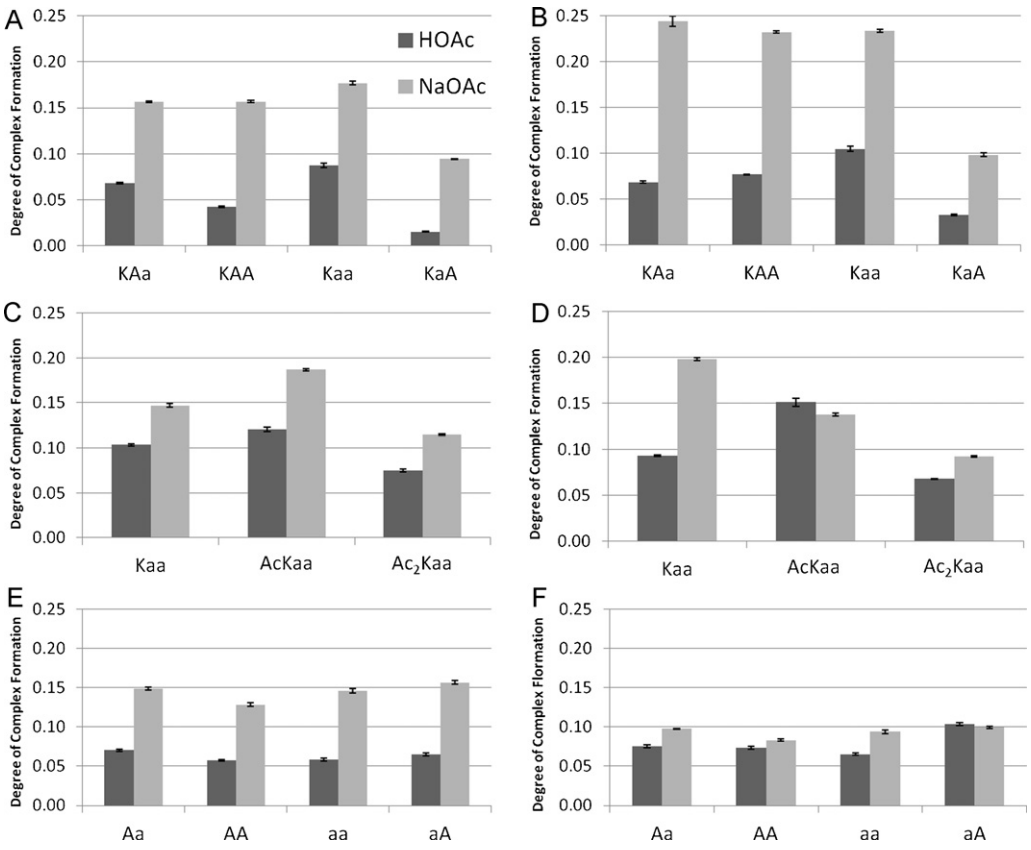

Fig. 5. Degrees of association for **CS3** (A, C, and E) and **CS4** (B, D, and F) with unprotected tripeptides (A and B), protected tripeptides (C and D) and dipeptides (E and F) in methanol plus either 0.5% HOAc or 100 μM NaOAc. The legend given in (A) is valid for all figure panels.

3.2. Cyclohexyl-sulfonate-pendant quinine selectors (CS3 and CS4)

Fig. 5 displays the degrees of association recorded for **CS3** and **CS4** with the free tripeptides (Fig. 5A and B), protected tripeptides (Fig. 5C and D), and dipeptides (Fig. 5E and F). **CS3** and **CS4** are both quinine-based selectors, which vary in the stereochemistry about a pendant, relatively rigid cyclohexyl-sulfonate moiety. This change in stereochemistry imparted some subtle changes in measured selectivities throughout the conditions tested.

Consistent with **CS1** and **CS2**, the highest degrees of association between **CS3** and **CS4** and the tripeptides were observed in the NaOAc solution. However, selectivities recorded among the various stereoisomers were minimal, except against KaA. For **CS3**, the strongest interaction was forged with Kaa (Kaa > KAa ≈ KAA > KaA; $\alpha = 1.88 \pm 0.02$ for Kaa over KaA). Future computational studies could be pursued to better visualize the orientation of the C-terminal L-Ala relative to other residues in the complex with the selectors to better understand the general deleterious effect it has on binding. Since the C-terminal carboxylate forms a key interaction with the rigidly positioned quinuclidine amine, it is conceivable that the change in stereochemistry from D-Ala to L-Ala points this carboxylate away from the amine and induces significant steric repulsion in the binding pocket. Additionally, it is known that hydrogen bonding between the C-terminal peptide backbone amide and the carbamate linker on zwitterionic selectors is important for binding and selective recognition. The change in configuration could perturb the alignment needed for favorable interactions to be formed. For **CS4**, a slight preference in binding was recorded for KAa over KAA and Kaa, but selectivity was minimal. Again, however, a drastic decrease in association was recorded for KaA ($\alpha = 2.49 \pm 0.08$ for KAa over KaA) in the NaOAc solution. In the HOAc solution, the degree of association was reduced to 10% or less for all free tripeptides. Both **CS3** and **CS4** bound most strongly with Kaa, and some selectivity enhancements were recorded, compared to the NaOAc solution data. Again, the presence of a C-terminal L-Ala greatly diminished binding.

Results obtained by evaluating the effect of α-/ε-terminal acetylated tripeptides were very similar to the results obtained for **CS1** and **CS2**, except in two cases. For **CS3**, in both HOAc and NaOAc solutions, the highest degree of association was recorded with AcKaa. In the NaOAc solution, **CS1**, **CS2**, and **CS4**, all showed the highest degree of binding with Kaa, relative to AcKaa and Ac_2Kaa. While blocking the N^{α}-terminal amine is preferential for binding in all cases (this removes competition between the α- and ε-terminal amines, and allows the ε-amine sole access for binding the sulfonate), this effect was stronger for **CS3**. Thus, we can speculate that the stereochemical arrangement of the cyclohexyl-sulfonate group in **CS3** is optimized to interact with the ε-terminal amine on Lys, more so than the other three selectors. A final point to note was that **CS4** showed a slightly higher binding affinity to AcKaa in the HOAc solution than in the NaOAc solution; in virtually all other cases, binding between the tripeptides and the selectors was significantly higher in NaOAc solution.

Dipeptides were largely indistinguishable by **CS3** and **CS4** (minimal selectivities), although some significant differences between the two selectors were noted. In NaOAc, a greater degree of complexation was observed for **CS3** than for **CS4**, or for either in HOAc. The degree of association between **CS3** and the dipeptides in NaOAc was on par with the degree of association for tripeptides in the same conditions ($A \sim 15\%$). The comparison of **CS3** and **CS4** largely mirrored the comparison of **CS1** and **CS2** in these experiments.

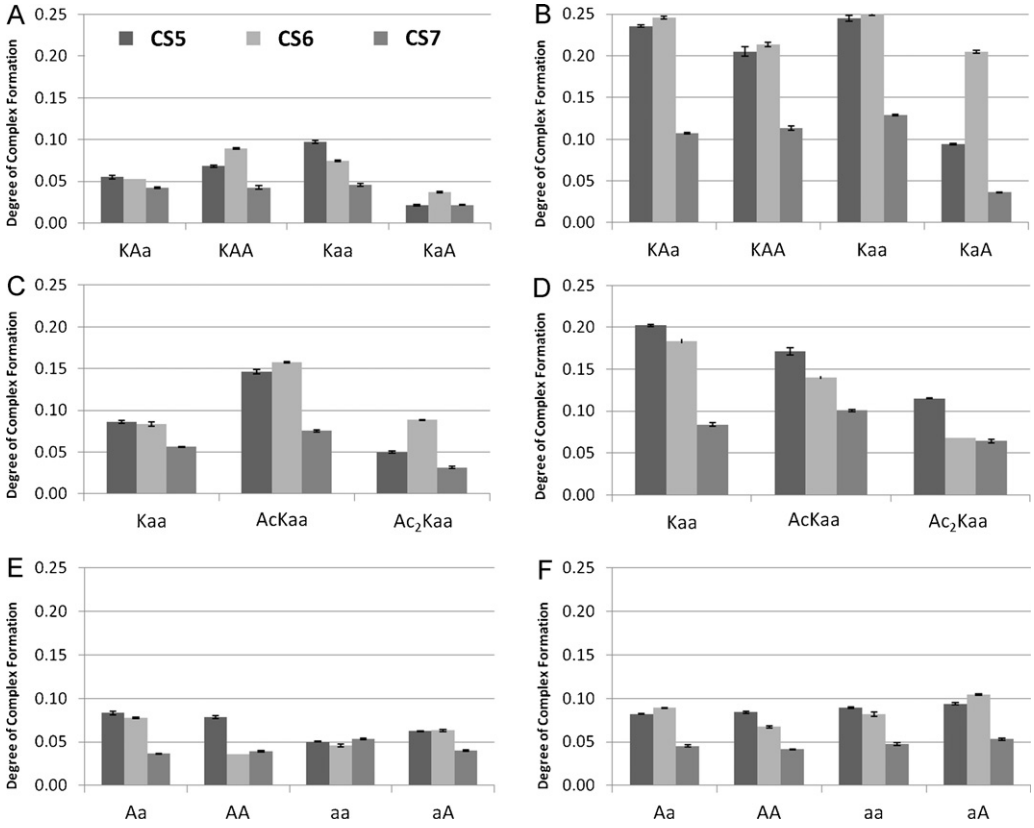

Fig. 6. Degrees of association for **CS5**, **CS6**, and **CS7** with unprotected tripeptides (A and B), protected tripeptides (C and D) and dipeptides (E and F) in methanol plus either 0.5% HOAc (A, C, and E) or 100 μM NaOAc (B, D, and F). The legend given in (A) is valid for all figure panels.

3.3. Sterically hindered alkyl-sulfonate selectors (CS5, CS6, and CS7)

Fig. 6 presents the results obtained for the three alkyl-sulfonates, which contain pendant steric groups: **CS5** (isopropyl); **CS6** (phenyl); **CS7** (*t*-butyl). Fig. 6A, C, and E presents the degrees of association for the selectors in HOAc solution and Fig. 6B, D, and F presents those for NaOAc solutions. The degrees of association for tripeptides were generally higher in NaOAc than they were in HOAc solution, and those for the dipeptides in each solution were comparable. These results were largely consistent with the other data sets.

For the tripeptides, each of the quinine-based **CS5**, **CS6**, and **CS7** selectors followed approximately the same trends for association and selectivity. One notable difference was the reversal of binding preferences between Kaa and KAA for **CS5** and **CS6** in HOAc. For CS5, $\alpha = 1.43 \pm 0.04$ (Kaa over KAA). For **CS6**, $\alpha = 1.20 \pm 0.02$ (KAA over Kaa). As both selectors are comprised of the same alkaloid scaffold (quinine), this switch was attributed to the inverse configurations of their stereocenters β to the sulfonate group (Fig. 1). Though the selectivities are not of high magnitude, this reversal of preference indicated the utility of these pendant steric groups for adjustable selective interaction with this biologically relevant system. While KAA may be encountered in eukaryotic systems, Kaa is found only to a significant extent in nature in prokaryotic bacterial systems. Interestingly, **CS7**, which differs from **CS5** by only the addition of a methyl group, could not distinguish between these two diastereomers ($\alpha = A_{CS7+Kaa}/A_{CS7+KAA} = 1.07 \pm 0.07$). In the NaOAc solution, all selectors showed a slight preference for Kaa over KAA. Again, consistent with all other results, complex formation of the selectors with KaA was the least abundant for all free tripeptides.

Results recorded for the protected tripeptides followed similar trends to the other selectors tested. Taken all together, there were two classes of interactions seen. First, there were those that showed decreased binding with the tripeptides as amines were successively blocked (highest binding with unprotected Kaa): **CS1** and **CS2** in NaOAc; **CS4** in NaOAc; and **CS5** and **CS6** in NaOAc. The presence of sodium seems to largely support the complexation of the selectors with the free tripeptides in these systems, and dominant, though not exclusive, interactions could be attributed to the quinuclidine nitrogen and C-terminal carboxylate. The second class of interactions were those that showed an increased binding with AcKaa over Kaa and Ac_2Kaa: **CS1** and **CS2** in HOAc; **CS3** in HOAc and NaOAc; **CS4** in HOAc; **CS5**, **CS6**, and **CS7** in HOAc; and **CS7** in NaOAc. As mentioned previously, blocking the N^{α}-terminal amine would allow for preferential interaction between the sulfonate and the ε-amine on Lys. Additionally, the presence of an acidic environment (common for all of the selectors in this interaction class; **CS3** and **CS7** also behaved in this fashion in NaOAc), provides some indication that a proton-rich environment can further modulate this interaction. Although these interactions were studied in methanol, it can be assumed that the only ionizable group which could be suppressed under this condition is the C-terminal carboxylate of the peptide. While it is difficult to paint a complete picture of how interaction modes are modulated in different solution conditions, it is clear that some interesting changes in interaction strength and selectivity can be induced by varying the ionic strength, ionic character, and the pH of the environment.

For the free dipeptides in a NaOAc environment, selectivities were minimal and the degrees of association followed similar trends with minor variations. Degrees of association in some cases were diminished relative to the tripeptide systems,

which could be attributable to the increased steric bulk of the functional groups on the alkyl-sulfonate and its ability to limit access to the anionic binding unit by the shorter dipeptides. Even so, in HOAc, binding strengths between selector and dipeptide were comparable to those with the tripeptide. Additionally, some significant selectivities were seen, which varied depending on the selector. **CS5** preferred dipeptides with an N-terminal L-alanine, but the configuration of the C-terminal alanine had little effect. **CS6** preferred Aa over the other dipeptides and the degree of association decreased in the order Aa > aA > aa > AA; here, the relative configuration of both residues mattered. **CS7** preferred to bind aa, but could not easily distinguish between the other three dipeptides. From these experiments, the incorporation of steric functional units on the alkyl-sulfonate chain appears to have considerable ability for tuning interactions, although a logical prediction of these effects would require a larger study of variations, both in terms of selectors and selectands.

4. Conclusions

ESI-MS can be a useful tool for elaborating the mechanistic details underlying enantioselective and diastereoselective interactions. Measurements can be made quickly with minimal amounts of sample and with multiple variations in solution conditions. While it can sometimes be difficult to decouple contributions originating from the electrospray process itself, and while the magnitudes of interaction strengths and selectivities do not always perfectly mirror those given by other techniques, useful information can be gleaned to support further development and application. In this work, a series of chiral selectors, which have been evaluated to an appreciable extent for their use as immobilized CSPs for HPLC to separate free (non-derivatized) peptides, were applied to a biologically relevant peptide system. Systematic variations of selector structures, selectand configurations, and solution conditions afforded insights as to the influence of those parameters on the chiral recognition mechanism and, thus, potential tuning sites for (stereo)selectivity. Worthy to note, the increased association, and in many cases increased selectivity, obtained by introducing NaOAc into these systems, suggests that future enantioselective and diastereoselective separations using HPLC might benefit from the inclusion of alkali metal ions. Work is ongoing in our lab to assess these effects, as well as expand the scope of selectands evaluated by ESI-MS in the presented fashion, to better understand the detailed mechanisms associated with molecular recognition and discrimination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.08.010.

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