MASS SPECTROMETRIC DIFFERENTIATION OF LINEAR PEPTIDES COMPOSED OF L–AMINO ACIDS FROM ISOMERS CONTAINING ONE D–AMINO ACID RESIDUE

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Abstract. MS/MS of electrosprayed ions is shown to have the capacity to discriminate between peptides that differ by configuration about their α-carbons. It is not necessary for the peptides to possess tertiary structures that are affected by stereochemistry, since five epimers of the pentapeptide, H_2N-Gly-Leu-Ser-Phe-Ala-OH (GLSFA) all display different collisionally activated dissociation (CAD) patterns of their protonated parent ions. The figure of merit, ***r***, is a ratio of ratios of fragment ion abundances between stereoisomers, where ***r***=1 corresponds to no stereochemical effect. Values of ***r*** as high as 3.8 are seen for diastereomer pairs. Stereochemical effects are also seen for the diprotonated dodecapeptide H_2N-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Gln-Lys-OH (LVFFAEDVGSNK), a tryptic fragment from the amyloid β-protein. Triply-charged complexes of the protonated dodecapeptide with cobalt (II) ions undergo CAD at lower collision energies than do doubly-protonated LVFFAEDVGSNK ions. Statistically significant ($p<0.01$) differences between the all-**L** dodecapeptide and the ones containing a **D**-serine or a **D**-aspartic acid are observed.

Living cells construct proteins from α–amino acids. Of these amino acids, only glycine is achiral. So far as is known, protein biosynthesis uses only the **L**-configurations of the chiral amino acids. Nevertheless, the presence of a single **D**-amino acid residue is well documented in a variety of naturally occurring peptides.[1-3] Epimerized carbons can arise via two processes: spontaneous racemization and post-translational modification. On one hand, the rate of spontaneous racemization for most peptidic amino acid residues (except aspartic acid, Asp[4]) is so slow that it finds application as an archaeological dating technique.[5] On the other hand, specific enzymes that catalyze epimerization of serine residues (Ser) have been characterized.[6]

Here we report the use of electrospray ionization-MS/MS to distinguish peptides that contain only **L**–amino acids from those containing one **D**–amino acid residue. The ability of mass spectrometry to differentiate acyclic stereoisomers has been known for 4 decades.[7] The most efficient discrimination is achieved by comparing ratios of competing fragmentation pathways, but only lately have the magnitudes of measured differences been found to be large enough for analytical purposes.[7-13]

The figure of merit is $\boldsymbol{r}_{A,B}$, the intensity ratio for two decompositions to give fragments A and B from one stereoisomer divided by the intensity ratio of those same fragments from another stereoisomer: [7]

$$\boldsymbol{r}_{A,B} = \frac{(I_A/I_B)_{\text{isomer 1}}}{(I_A/I_B)_{\text{isomer 2}}} \qquad (1)$$

Experimental $\boldsymbol{r}$ values depend not only on the choice of A and B, but also upon the conditions of ionization and fragmentation. The precision of mass spectrometric ion intensity measurements is sufficient that values $\boldsymbol{r} \geq 1.2$ or $\boldsymbol{r} \leq 0.8$ permit quantitation of the relative abundances of two stereoisomers in a mixture. This has been demonstrated for diastereomers that differ only by the configuration of deuterium relative to that of a functional group[8,9,11] and in the ESI-MS/MS of the amino acid threonine and its stereoisomer *allo*-threonine.[12]

Heretofore, mass spectrometrists have explored stereoisomeric peptides to only a limited extent. In 1985 Tabet, Kagan, *et al.* showed that the *S,S* and *R,S* diastereomers of the acetylated dipeptide ester Ac-Phe-Phe-OCH_3 can be differentiated using MS/MS.[14] More recently, Zubarev and coworkers used collisionally activated dissociation (CAD) and electron capture

dissociation neutralization to correlate fragmentation patterns of an eicosapeptide with changes in its tertiary structure resulting from **D**–amino acid substitutions.[15]

This paper describes a straightforward MS/MS approach for differentiating diastereomeric peptides of neurochemical relevance. The examples chosen include terminal pentapeptides of ω–agatoxins IVB and C from the venom of the spider *Agelenopsis aperta* (which act as specific calcium channel blockers[16]) and the tryptic dodecapeptide Aβ17-29 from the amyloid β-protein, the principal component of senile plaques in Alzheimer's disease.[17] ω–Agatoxin IVC is composed entirely of **L**-amino acid residues, while ω–agatoxin IVB, the more active naturally occurring stereoisomer, has the same sequence, but with a **D**-serine residue. Cleavage of the toxin with the endopeptidase Glu-C yields the terminal pentapeptide, which includes the **D**-serine. The present work compares the CAD fragmentation patterns of all of the variations of the terminal pentapeptide that contain a single **D**-amino acid. These peptides are too small to have tertiary structure (as the concept is generally understood), yet the differences among stereoisomers are significant.

The amyloid β-protein comprises a family of peptides, which contain on the order of 40 amino acid residues, resulting from cleavage of a larger, integral membrane protein. Amino acid analysis indicates that Asp and Ser in plaques containing the amyloid β-protein display an unusually high proportion of epimerization. A specific Ser has been pinpointed immunochemically.[17] After synthesizing the tryptic fragment that contains this Ser, we find that metal complexation permits MS/MS to distinguish the peptide containing a **D**–serine or a **D**–aspartic acid residue from the peptide composed of only **L**–amino acids.

EXPERIMENTAL

Protonated peptides were introduced into a commercial quadrupole time-of-flight mass spectrometer (Q-Tof Ultima-Global, Micromass, UK) by electrospray ionization through a 0.3 mm aperture and introduced into the quadrupole via a differentially pumped Z-spray source. The capillary voltage was set to 3.3V in positive ion mode. For unmetallated peptides, source block and desolvation gas (nitrogen) temperatures were set at 80 ^{o}C and 120^{o}C, with cone voltage set at 70V. For metallated peptides, the cone voltage was reduced to 40V and the desolvation gas temperature set at 100^{o}C. Collisional activation was performed using argon gas (*ca* 4 psi) in a hexapole (rf only) collision cell. Identities of ions from the source and of fragment ions from CAD were confirmed by exact mass measurements.

The pentapeptide GLSFA and its stereoisomers were synthesized and purified by HPLC on a C18 column (Agilent, Waldbronn, Germany). The peptides were run 6μl/min in reverse-phase mode with a gradient increased from 2 to 65% mobile-phase B (mobile-phase B was 0.1% trifluoroacetic acid in acetonitrile and mobile-phase A was 0.1% TFA in water) for 65 minutes. The gradient was held steady for 10 minutes then increased to 90% mobile-phase B for 10 minutes. Eluting peaks were recorded on chromatograms using a diode-array detector, collected manually in 0.5mL siliconized Eppendorf tubes, diluted to *ca* 10mM, and injected into the ESI source at a flow rate of 6μL/min. Mass-selected, singly protonated peptide ions from the first stage of mass selection were collided with argon gas at 4-12eV collision energy (laboratory frame). At the quadrupole mass filter, the LM and HM resolutions were set at 10 (arbitrary units) with an ion energy of 0.9V. Statistically significant variation among a group of diastereomeric peptides was assessed by performing an analysis of variance (ANOVA) on a given set of CAD peak intensities (relative to the intensity of a fragment chosen as reference). For cleavages where the ANOVA corresponded to $p<0.01$, *t*-tests were used to isolate statistically significant differences ($p<0.01$) between mean intensities of a given fragment ion for different stereoisomers.

NH_2-Gly-Leu-Ser-Phe-Ala-OH (GLSFA) and its epimers were synthesized using Schlenk type glassware. In a typical procedure, Fmoc-Ala-Wang resin (100 – 200 mesh) (0.25g, 0.168mmol) was suspended in 15 mL 20% piperidine solution (20:80 v/v piperidine:DMF). The solution was stirred under nitrogen for 15 minutes, the solid filtered, Fmoc-Phe-OH (0.138g, 0.356mmol) added to it, followed by 2.8mL 5% DCC solution (5:95 v/v 1,3-dicyclohexylcarbo-diimide: DMF), and the suspension allowed to stir for 45 min at room temperature under nitrogen. Successive amino acid residues were added in the same manner, after which the beads were filtered, washed with 2 x 7mL methanol, and dried under vacuum for 16 hours. Pentapeptide was cleaved from the resin by stirring the solid beads with 7 mL neat trifluoroacetic acid under nitrogen for 3 hours. The solid was filtered, the filtrate added to 12mL water, and lyophilized until approximately one-tenth of the solvent remained. Pure pentapeptide was isolated from this solution by HPLC using the conditions described above.

An authentic sample of synthetic all-**L** fragment 17-28 from the amyloid β-protein, ***H_2N-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-OH*** (LVFFAEDVGSNK), was purchased from Sigma, analyzed, and used without further purification. The dodecapeptide stereoisomers

containing **D**-serine or **D**-aspartic acid residues were synthesized starting with Fmoc-Lys(Boc)-Wang resin (100–200 mesh) on an automated peptide synthesizer (with addition of 1-hydroxyybenzo-triazole) and purified via HPLC (using aforementioned conditions). Samples of the peptides in 1:1 (v/v) acetonitrile:water with 0.1% formic acid were mixed (1:1) with 1mM divalent metal salt ($MnCl_2$ or $CoCl_2$) in water. Poor yields of metallated ions were observed when divalent nitrate salts were used. Samples were injected directly into the electrospray source in 10μL quantities contained in vaults with a separate continuous 6μL/min flow of solvent. Collision energies for multiply charged ions were varied in order to optimize fragment yields: doubly protonated peptide ions at 24eV (laboratory frame) , while triply charged ions $[peptide + Metal + H]^{3+}$ dissociated well at 12eV (laboratory frame).

All sample peptides, metallated and unmetallated, were run in repetition of at least three times over multiple days in order to afford proper error analysis. Ab initio geometries of the protonated pentapeptides and their dehydrated cyclic analogues were optimized at the RHF level using the 6-31G* basis set using the GAUSSIAN program suite.

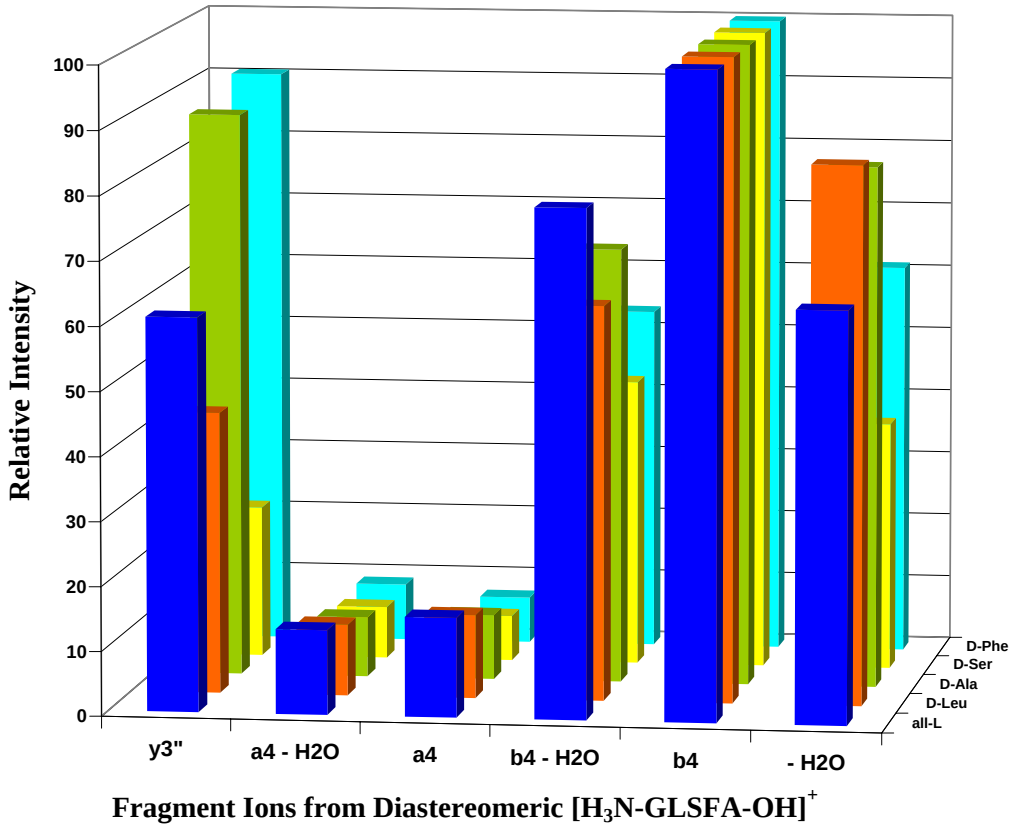
Results

The protonated pentapeptides corresponding to the C-terminals of ω-agatoxins IVB and IVC (and their stereoisomers), GLSFA, yield fragment ions under ESI-MS/MS conditions that are characteristic of typical peptide bond cleavages, which undergo further water losses. The most prominent are summarized by the bar graph in Figure 1. Although each diastereomeric pentapeptide gives the same decomposition fragments, intensities vary significantly from one another. CAD fragment ion abundances are normalized to that of the most intense fragment ion (b_4).

Our initial hypothesis supposed that water loss should parallel previous results on protonated threonine diastereomers, where backside displacement from the sidechain occurs. Consistent with that supposition, the **D**-serine-containing pentapeptide shows significant differences for water loss, relative to the all-**L** pentapeptide. However, all of the diastereomeric pentapeptides exhibit observable differences, although a **D**-serine residue exerts the most pronounced effects.

The notion that displacement of water from a protonated Ser sidechain by the N-terminus (to form a 9-membered ring) would provide the most favorable pathway was explored computationally. Water loss from the C-terminus would, by contrast, form a 15-membered ring.

Figure 1. Bar graphs showing the 6 most abundant fragments from CAD (10eV, laboratory frame) of protonated GSFLA and its diastereomers, with intensities normalized to the most abundant fragment.



Contrary to original expectation, ab initio calculations predict that the latter water loss is 0.7 eV less endothermic than the former. Water loss might also take place from backbone amide groups.[18] In any event, the computational results suggest that the Ser sidechain does not provide the most favored oxygen for water loss.

As noted above, all the diastereomeric pentapeptides display significant stereochemical effects. Energy-minimized protonated parent pentapeptide ions adopt quasicyclic structures. This implies the ease of transferring the "mobile proton" from the N-terminus to oxygen in preparation for displacement of water.

The *y*-cleavage that produces a protonated Ser-Phe-Ala fragment displays the most dramatic stereochemical consequences. By convention[19] this ion is designated as y_3'', where the double-prime superscript indicates that it carries a single positive charge and has two more hydrogens than would the uncharged radical produced by homolysis of the C-N bond of a neutral

peptide. As Figure 1 summarizes, it is possible to differentiate all of the diastereomers based on the characteristic intensities of their MH^+-H_2O and y_3'' fragments relative to the intensity of the b_4 fragment ion. Table 1 summarizes ratios of relative ion intensities.

As noted in the introduction, the figure of merit for diastereomeric differentiation is the ratio of ratios, ***r***, defined as shown in eqn 1. Values of ***r*** for the fragment ions relative to the b_4 ion are compared for each diastereomeric pentapeptide in Table 1. With respect to eqn 1, fragmentation A stands for the pathway listed at the top of Table 1, and fragmentation B corresponds to the b_4 cleavage, where isomer 1 represents the all **L**-peptide (represented by dark blue bars) and isomer 2 the peptide containing the indicated **D**-amino acid residue. Though all ***r*** values show promise for quantitation of a mixture, the **D**-serine containing pentapeptide exhibits the most robust differences. If one is interested in distinguishing between two **D**-amino acid containing peptides, ***r*** for that pair is equal to the quotient of the corresponding ***r*** values in Table 1. For instance, ***r*** has the value 2.59/0.673 = 3.8 for the y_3''-cleavage of the peptide containing a **D**-serine (GLdSFA, represented by yellow bars) relative to the one containing a **D**-phenylalanine (GLSdFA, (represented by turquoise bars).

The other neurochemically significant peptide studied, the amyloid β-protein fragment 17-28, contains detectable levels of epimerized Ser or Asp.[17] Again the objective of this study was to measure differences in diastereomeric peptides containing a singly substituted **D**-amino acid. The tryptic fragment, LVFFAEDVGSNK, required MS/MS analysis of multiply charged peptide ions. Diprotonated peptides containing single **D**-amino acids show significant ($p<0.01$) variations with stereochemistry when fragment ion ratios are compared. All the possible y''-cleavage ions are observed with intensities $\geq$5% that of the parent $[M+2H]^{++}$ ion (except for y_1''), as well as the *b*-cleavage fragments corresponding to the most abundant y'' ions (b_1, b_2, and b_3). Also, y_{10}''', the doubly-charged analogue of the most intense *y*-cleavage peak, occurs with abundance greater than that of any of the other *b* or *y* cleavages. This sort of pattern is typical of doubly protonated tryptic peptides. Figure 2 summarizes the abundances of the diprotonated parent ion, $[M + 2H]^{++}$, and the eleven most intense CAD peaks relative to the most abundant CAD fragment, y_{10}'' (error bars represent measurement standard deviations).

Unlike the monoprotonated pentapeptides, stereochemical effects in the diprotonated dodecapeptides do not permit an easy distinction among all diastereomeric peptides containing

single **D**–amino acid residues. A **D**-serine does lead to small changes in the intensities of y_3'' and y_4'' fragments, cleavages near Ser (although only the latter is statistically significant). By contrast, the cleavages that produce the protonated cyclic peptide Phe-Phe-Ala-Glu and the y_7'' fragments show greater sensitivity to the stereochemistry of the Asp. Also, the parent $[M + 2H]^{++}$ ion dissociates to a much larger extent when it contains a **D**-aspartic acid residue.

While the stereochemical effects on CAD of diprotonated dodecapeptide ions are reproducible, they do not provide much scope for distinguishing between the all-**L** dodecapeptide and one containing a **D**-serine. In part, the high collision energy (24eV lab frame) might account for this. Consequently, we have explored complexation with divalent metals.

Transition metals, manganese (II) and cobalt (II), were chosen on the basis of their monoisotopic character and their expected propensity for chelation by carboxyl groups, as has previously been exploited in negative ion mass spectrometry.[20] We first examined the doubly charged, peptide-complexed metal ions. These species required collision energies ≥30eV in

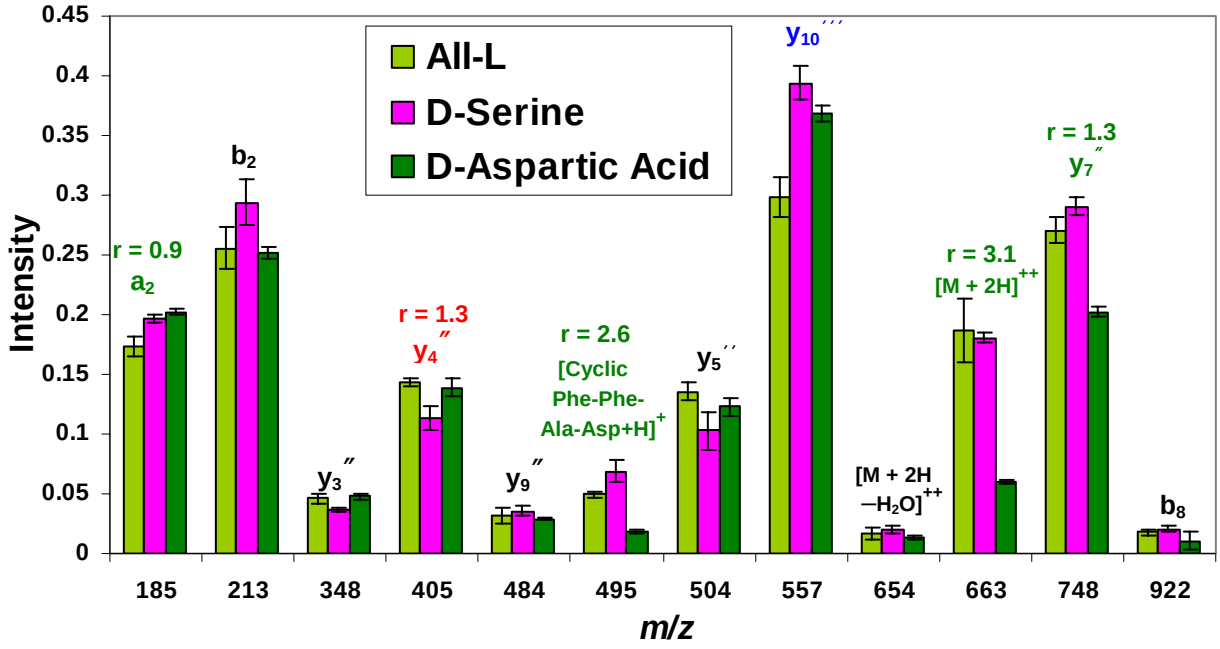


Figure 2: CAD (24eV, laboratory frame) fragment ion intensities from diastereomeric dodecapeptide $[M + 2H]^{++}$ parent ions, relative to the most abundant fragment, y_{10}''(*m/z* 1114). Colored labels (*a*, *y*, *etc*) indicate ion ratios that show significant differences ($p<0.01$) for the diastereomeric peptide versus the all-**L** peptide: green ions represent significance for **D**-aspartic acid; red for **D**-serine; and blue means that both are statistically significantly different from the all-**L** peptide. Error bars represent standard deviations.

order for fragment ions to be observed. Not surprisingly, the CAD patterns at this high energy did not reveal significant differences between diastereomeric peptides.

Because the doubly charged parent ions gave little prospect for diastereomeric differentiation of Ser epimers, we then looked at the MS/MS of triply charged, metal-complexed protonated dodecapeptide ions. Figure 3 summarizes the abundances of the twelve most abundant CAD fragments relative to the intensity of the metallated y_{10} CAD peak (by analogy to the most abundant fragment in the CAD of the diprotonated dodecapeptide; error bars represent measurement standard deviations). The collision energy was optimized at 12eV (lab frame). At this fragmentation energy Co(II)-complexed protonated dodecapeptides display significant stereochemical effects from epimerized Ser. The **D**-serine-containing dodecapeptide exhibits an increase in its y_3 cleavage (to form y_3'' singly-charged fragments) and the appropriate counterpart, b_9 metal-complexed, doubly charged ions. These ions complement one another in retaining the

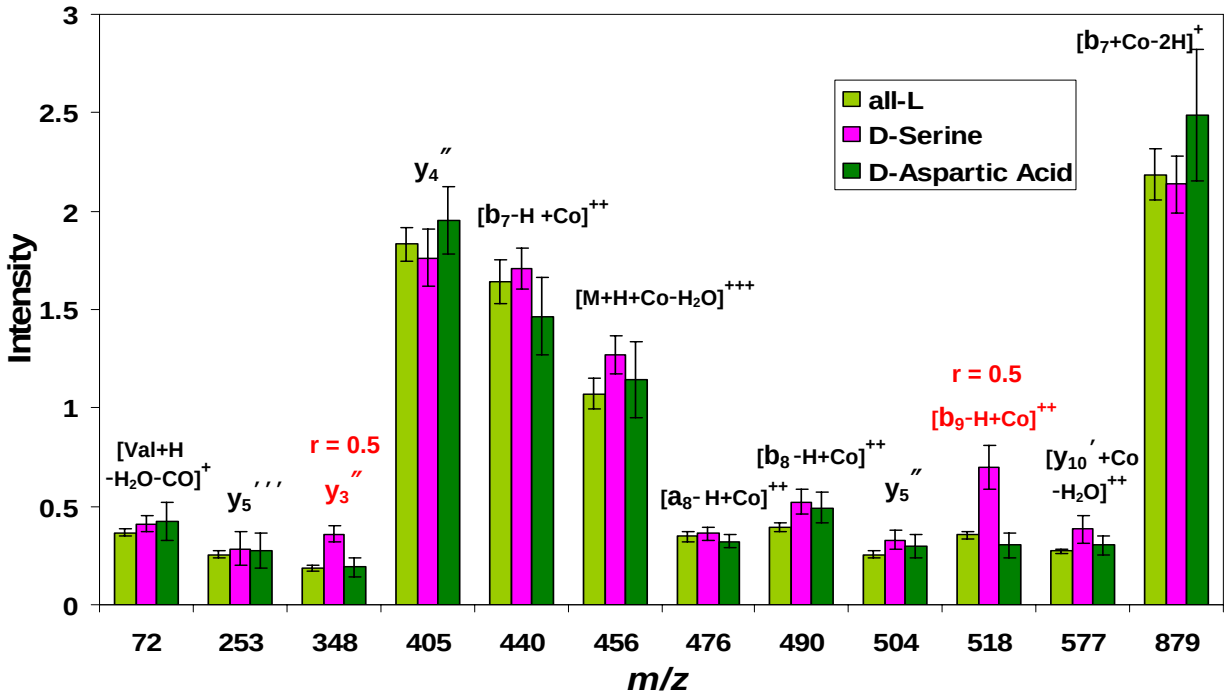


Figure 3: CAD (12eV, laboratory frame) fragment ion intensities for diastereomeric, cobalt-complexed protonated dodecapeptide parent ions, $[M + Co + H]^{+++}$, relative to $[y_{10}' + Co]^{++}$ (*m/z* 586). Ions labeled in red indicate fragments whose relative abundances show significant ($p<0.01$) differences between the peptide containing a **D**-serine residue and the all-**L** peptide. Error bars represent standard deviations.

original mass and charge balance of the parent ion. The cleavage site lies next to the Ser peptide bond. By contrast, Mn(II) complexed, protonated parent ions show a low extent of fragmentation and did not allow diasteromeric differentiation within statistical limitations, as Figure 4 summarizes. Clearly, increasing the charge of the parent ion so as to reduce collision energy does not guarantee that MS/MS will be able to discriminate between stereoisomers.

The catalogue of fragments from metallated parent ions in Figures 3 and 4 requires some explanation. Conventional nomenclature for peptide mass spectrometry denotes a singly charged fragment ion resulting from cleavage of a bond to the peptide carbonyl (*a*, *b*, or *y*″). Following this convention, a doubly charged, unmetallated *y*-ion is designated as *y*‴ and a neutral peptide as *y* ′.[19] A doubly-charged, metallated *y*-cleavage fragment corresponds to a neutral peptide with a divalent metal attached. For instance, the ion that serves as the intensity reference in Figures 3 and 4 can be designated as $[y_{10}' + \text{Metal}]^{++}$. Similarly, the aforementioned b_9 metal-complexed, doubly charged ion is designated as $[b_9 - \text{H} + \text{Co}]^{++}$ (*i.e.* a *b* ion minus a proton plus a divalent

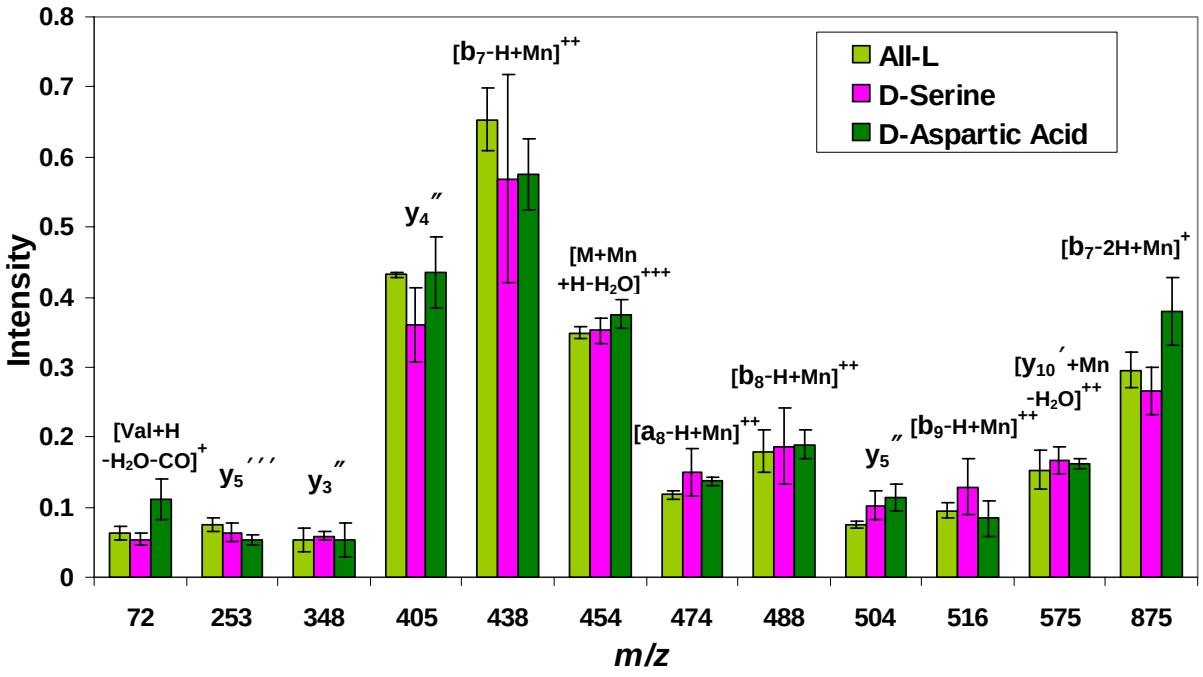


Figure 4: CAD (12eV, laboratory frame) fragment ion intensities from diastereomeric, manganese-complexed protonated dodecapeptide parent ions, $[\text{M} + \text{Mn} + \text{H}]^{+++}$, relative to $[y_{10}' + \text{Mn}]^{++}$ (*m/z* 584). Analogues of the ions summarized in Figure 3 do not exhibit significant stereochemical effects. Error bars represent standard deviations.

metal). A singly-charged, metallated *b*-type ion corresponds to the *b* fragment minus two protons plus the divalent metal (*e.g.* $[b_7 - 2H + Co]^+$).

In metal-complexed tryptic peptides, the accessible fragmentation pathways become limited, relative to the CAD of diprotonated peptides. Only four specific cleavages of the triply charged dodecapeptide occur to any major extent: the aforementioned y_3/b_9 couple; a y_{10}-complexed metal ion (the intensity reference) with its b_2 partner; a y_4'' fragment with its b_8 metal-complexed partner (along with further fragmentation of the latter ion to give a_8 and both singly and doubly charged b_7-metal complexed ions); and singly and doubly charged y_5 ions with the complementary b_7–metal complexed ions. With regard to competition among fragmentation pathways, the **D**-serine containing peptide is more easily distinguished from the all-**L** peptide than is the peptide that contains a **D**–aspartic acid residue.

DISCUSSION

The two sets of peptides studied here suggest several inferences. For diastereomeric differentiation via collisionally activated dissociation (CAD), the parent ion must give substantial decomposition at a suitably low energy. For the pentapeptides, the protonated, singly-charged ion undergoes a usable degree of fragmentation at 10 eV (lab frame). Doubly-charged dodecapeptides show more stereochemical sensitivity when the charge comes from double protonation (CAD at 24eV) than when it comes from complexation by a divalent metal ion (CAD at 30eV). Dissociation of doubly protonated parent ions exhibits stereochemical sensitivity, as Figure 2 summarizes, but the effect is much more pronounced for epimerized Asp than for epimerized Ser.

Triply-charged ions from metal complexation of a protonated dodecapeptide permit the effect of Ser epimerization to be measured at 12 eV collision energy. While the Co(II)-complexed, protonated dodecapeptides exhibit significant differences between diastereomers, the Mn(II)-complexed ones give much less fragmentation and do not display stereochemical effects. It is also worth noting that the choice of metal salt plays a role in the observed abundance of metal-complexed ions from the electrospray source: chlorides give satisfactory ion intensities, but nitrates do not.

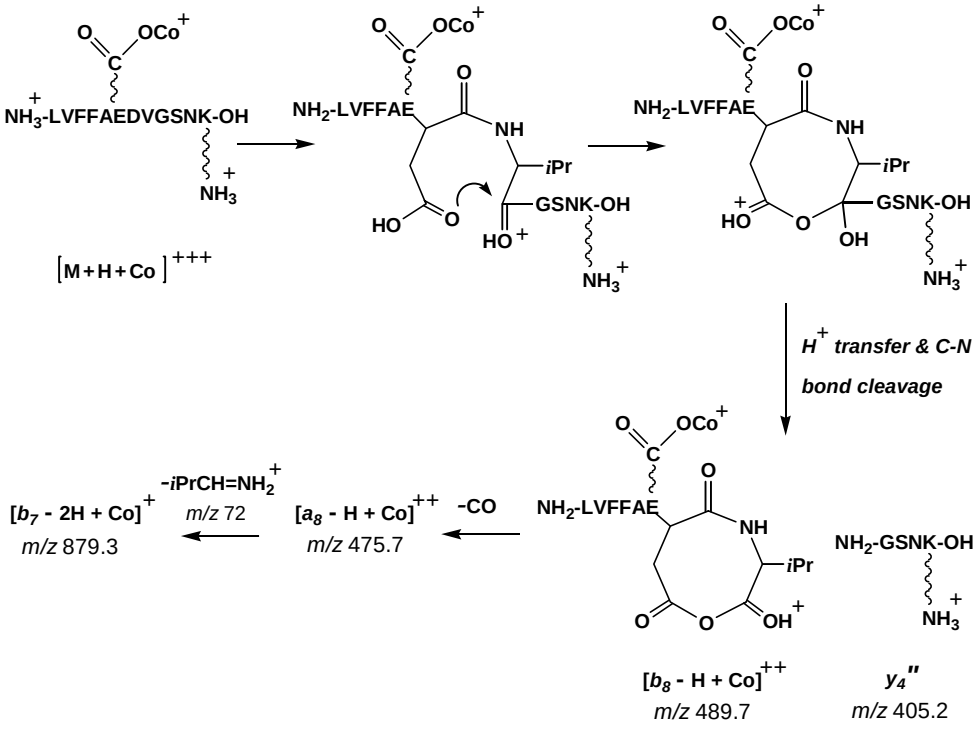
The survey of 5 stereoisomers of the terminal pentapeptide from ω-agatoxin IVB/C indicates that epimerization of Ser leads to the greatest observed difference, relative to the all **L**-

peptide. The largest effects are seen for cleavages of peptide bonds flanking the Ser residue (y_3 and b_4), as well as for water loss from the protonated parent ion. Epimerization of Ser in the tryptic dodecapeptide from the amyloid β-protein has a more pronounced effect in dissociating triply charged ions than that of Asp. Here, too, stereochemistry exerts its greatest effect on a peptide bond to Ser (the complementary pair y_3 and $[b_9\text{-H} + \text{Co}]^{++}$).

A strategy for measuring Ser *vs* Asp epimerization can thus be developed for the dodecapeptide. CAD of the doubly protonated parent ion shows the stereochemical effect of Asp, while CAD of the triply charged parent ion shows the effect of Ser. MS/MS of these two ions (which are both present when the peptide is electrosprayed with $CoCl_2$) gives ***r*** values that should be far enough from unity to permit quantitation of diastereomers in a mixture.

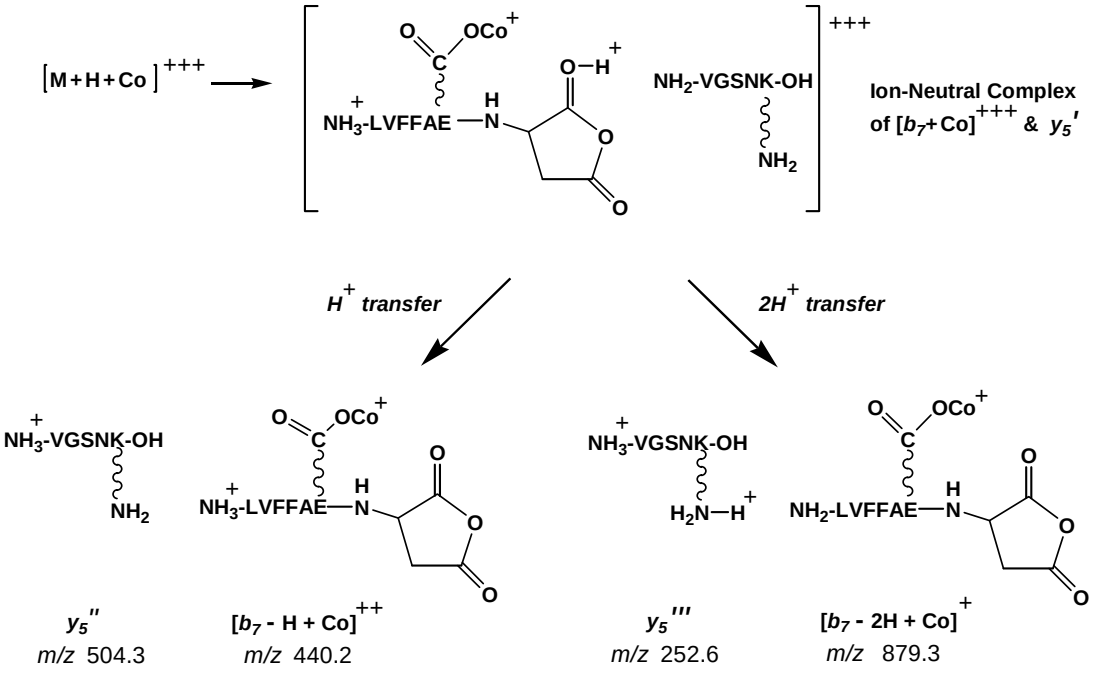
The principal pathways by which metallated protonated dodecapeptide ions dissociate can be described by analogy to a well-precedented mechanism,[21,22] in which an Asp sidechain carboxylic group acts as a nucleophile towards a protonated peptide carbonyl. Scheme 1 illustrates the consequences of this cyclization in the decomposition of a triply-charged, metallated protonated parent ion to form the y_4''/metal-complexed b_8 or a_8 couple. The $[a_8 - \text{H} + \text{Co}]^{++}$ ion can subsequently expel the $i\text{PrCH}{=}\text{NH}_2^+$ immonium ion (the observed $[\text{Val+H} - \text{H}_2\text{O} - \text{CO}]^+$ fragment) to produce the $[b_7 - \text{H} + \text{Co}]^+$ ion.

Scheme 1

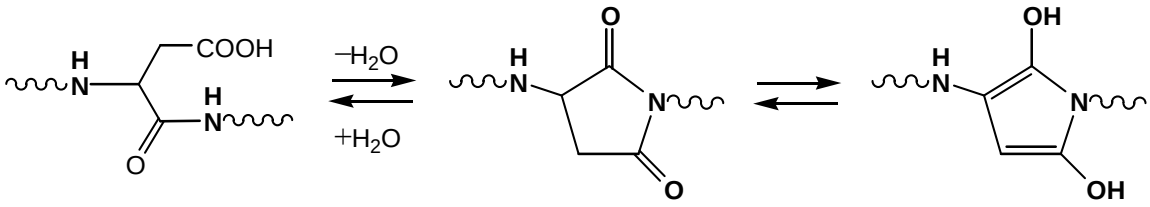


Scheme 2 depicts an alternative, direct route to y_5/b_7 cleavage. This mechanism accounts for the production of doubly-charged y_5''' fragments in addition to the singly-charged y_5''. Cleavage of the cyclized parent ion (not illustrated) forms an ion-neutral complex containing a triply-charged, metallated $[b_7 + \mathrm{Co}]^{+++}$ ion and neutral y_5'. Proton transfer within the complex yields the *y*-fragments drawn in Scheme 2. Two protons have to transfer simultaneously in order to form the y_5''' fragment, since a complex between two charged partners cannot persist. Regardless of how fragment ions are formed, all cleavages retain the metal ion in the fragment that contains the glutamate residue.

Scheme 2

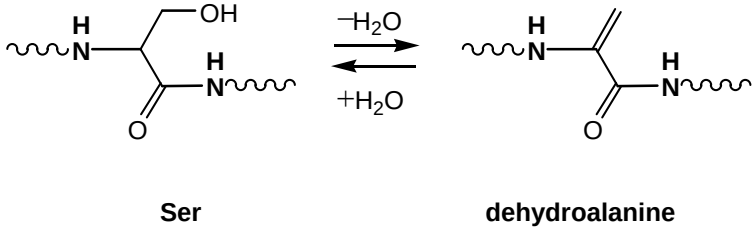


From an analytical standpoint, the mass spectrometric sensitivity to amino acid stereochemistry may offer a useful tool. While Asp is known to racemize spontaneously,[2c,4] epimerization of Ser within peptides occurs very slowly. The relative ease with which the Asp side chain cyclizes to a succinimide via reversible water loss, as drawn in the first step of eqn 2, accounts for its lability. Tautomerization of the succinimide to an aromatic dihydroxypyrrole can explain the rapid loss of stereochemistry at the α-carbon.



(2)

One pathway for epimerization of a Ser involves loss of water to form dehydroalanine, followed by rehydration, as eqn 3 illustrates. Available evidence suggests that the first-order rate constant for uncatalyzed, thermal expulsion of water from the Ser sidechain of the hexapeptide RRASVA has a value on the order of 0.5 hr^{-1} at 200° C.[23] If the Arrhenius preexponential factor for that reaction is assumed to be $\geq 10^{11}$ sec^{-1}, then the half-life for Ser at 37° C should be >10,000 years. The formation of **D**-serine residues in the amyloid β-protein therefore signals some kind of catalysis. The ability to measure the relative rates of epimerization of specific Asp and Ser residues may lead to clues as to the nature of this catalysis.



(3)

Enzymic production of dehydroalanine within peptides is well-documented. For example, the first step in post-translational modification of peptides by microorganisms to produce the dimeric amino acid lanthionine occurs via Ser dehydration, a reaction for which an entire family of enzymes has been identified.[24] In the absence of an enzyme that catalyzes further reaction of dehydroalanine, one might well imagine that rehydration would occur, possibly without catalysis. While this appears not to be the mechanism for the conversion of ω-agatoxin IVC to ω-agatoxin IVB,[25] dehydration/rehydration might operate as a biocatalytic pathway in other contexts. Given that Ser epimerization could prove to be a marker for aberrant enzymic activity *in vivo*, further exploration of analytical methods for differentiating diastereomeric peptides is warranted.

Acknowledgment.

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Supporting Information.

Gaussian optimized geometries (Cartesian coordinates) and electronic energies for protonated H_2N-Gly-Leu-Ser-Phe-Ala-OH and two isomeric fragment ions from water loss (10 pp). This material available free of charge via the Internet at http://pubs.acs.org.

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Table 1. ***r*** values ($\pm$ 95% confidence intervals) for diastereomeric pentapeptides relative to the all-**L** pentapeptide, where CAD peak intensities are calculated relative to that of the b_4 fragment ion, *m/z* 405 (values tabulated only for fragmentations for which $p<0.01$).

GLSFA peptide	**Fragment ion (*m/z*) from MH^+**				
	y_3''(324)	a_4-H_2O (359)	a_4 (377)	b_4-H_2O (387)	MH^+-H_2O (476)
D-Leu	1.39 $\pm$ 0.02	1.19 $\pm$ 0.10	1.19 $\pm$ 0.08	1.28 $\pm$ 0.09	0.76 $\pm$ 0.02
D-Ser	2.59 $\pm$ 0.06	1.62 $\pm$ 0.10	2.20 $\pm$ 0.12	1.76 $\pm$ 0.05	1.65 $\pm$ 0.02
D-Phe	0.67 $\pm$ 0.01	1.49 $\pm$ 0.08	2.17 $\pm$ 0.06	1.47 $\pm$ 0.03	–
D-Ala	0.69 $\pm$ 0.07	1.41 $\pm$ 0.10	1.54 $\pm$ 0.08	1.16 $\pm$ 0.05	0.78 $\pm$ 0.04