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Site-Specific Characterization of D-Amino Acid Containing Peptide Epimers by Ion Mobility Spectrometry

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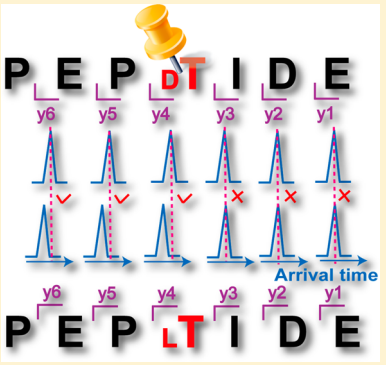
Supporting Information

ABSTRACT: Traditionally, the D-amino acid containing peptide (DAACP) candidate can be discovered by observing the differences of biological activity and chromatographic retention time between the synthetic peptides and naturally occurring peptides. However, it is difficult to determine the exact position of D-amino acid in the DAACP candidates. Herein, we developed a novel site-specific strategy to rapidly and precisely localize D-amino acids in peptides by ion mobility spectrometry (IMS) analysis of mass spectrometry (MS)-generated epimeric fragment ions. Briefly, the D/L-peptide epimers were separated by online reversed-phase liquid chromatography and fragmented by collision-induced dissociation (CID), followed by IMS analysis. The epimeric fragment ions resulting from D/L-peptide epimers exhibit conformational differences, thus showing different mobilities in IMS. The arrival time shift between the epimeric fragment ions was used as criteria to localize the D-amino acid substitution. The utility of this strategy was demonstrated by analysis of peptide epimers with different molecular sizes, [D-Trp]-melanocyte-stimulating hormone, [D-Ala]-deltorphin, [D-Phe]-achatin-I, and their counterparts that contain all-L amino acids. Furthermore, the crustacean hyperglycemia hormones (CHHs, 8.5 kDa) were isolated from the American lobster *Homarus americanus* and identified by integration of MS-based bottom-up and top-down sequencing approaches. The IMS data acquired using our novel site-specific strategy localized the site of isomerization of L- to D-Phe at the third residue of the CHHs from the N-terminus. Collectively, this study demonstrates a new method for discovery of DAACPs using IMS technique with the ability to localize D-amino acid residues.



The isomerization of an L- to D-amino acid is a remarkable post-translational modification of peptides in RNA-based protein synthesis and has been documented in amphibians, invertebrates, and mammals.[1−4] In many cases, the D-amino acid containing peptides (DAACPs) exhibit dramatically higher affinity and selectivity for receptor binding than their all-L counterparts and thus are essential for biological function.[4] Generally, the targeted approaches for discovery of endogenous DAACPs include two steps: screening DAACP candidates in biological samples and then localizing D-amino acid residues.[2,5] Many new DAACPs were found by observing the differences in biological activity or chromatographic retention time between synthetic peptides and naturally occurring peptides.[2−5] In addition, immunoassays based on conformational antibodies have been successfully used to screen DAACPs at the tissue and cellular level.[2,6] For localization of D-amino acids in DAACP candidates, the most popular approach relies on matching chromatographic retention time of the naturally occurring peptide with a panel of synthetic peptides.[2] For example, validation of a deca-DAACP presumably requires testing 10 synthetic peptides, each of which contains a D-amino acid at a varied position, leading to high cost and limited analytical throughput. Other techniques utilize Edman degradation[7] or acid hydrolysis[2,8] to release free amino acids, followed by chromatographic analysis of the free or derivatized amino acids. However, cleavage of amide bond by chemical methods induces a 3−15% level of racemization.[9] Therefore, there is a great demand for development of a simple and low-cost method to localize D-amino acids in a wide range of DAACP candidates.

In the past decade, mass spectrometry (MS) has become a powerful tool for peptidomics studies. However, differentiation of D/L-peptide epimers by MS represents a major analytical challenge because the peptide epimers share identical masses and primary structures.[10] Consequently, L- to D-amino acid isomerization has been largely overlooked in MS-based peptide discovery. Recently, a variety of fragmentation techniques in tandem mass spectrometry (MS/MS), such as collision-induced dissociation (CID),[9,11,12] metastable decomposition,[12] electron capture dissociation,[9,13] and radical-directed dissociation,[14] have been successfully applied in discriminating D/L-peptide epimers through the comparison of fragment ion intensities. Although excellent differentiation and quantitation between D/L-peptide epimers can be accomplished by these strategies, localization of D-amino acid in peptides is still difficult, as measurement of fragment ion intensities cannot provide accurate positional information of D-amino acids. To address this problem, this study introduces a novel ion mobility

Received: October 17, 2013
Accepted: December 10, 2013
Published: December 10, 2013

   dx.doi.org/10.1021/ac4033824 | *Anal. Chem.* 2014, 86, 2972−2981

spectrometry−mass spectrometry (IMS−MS)-based strategy enabling site-specific characterization of DAACP epimers to localize D-amino acids.

The IMS−MS technique has been widely used to probe the gas-phase conformations of biomolecules by measuring their mobility in a buffer gas[15,16] and has shown very broad applicability in the separation and identification of isomeric peptides.[16,17] Previous studies reported that intact D/L-peptide epimers displayed different mobility in IMS due to conformational differences attributed by substitution of an L- to D-amino acid.[18] Presumably, under CID fragmentation the two peptide epimers may produce epimeric fragment ions which contain the same amino acid sequences but differ by substitution of an L- to D-amino acid. This raises a question of whether the epimeric fragment ions derived from D/L-peptide epimers exhibit different mobility in IMS due to conformational differences, and whether or not those differences enable D-amino acid localization. For example, the peptide epimers PEP_DTIDE and PEP_LTIDE may respectively produce the epimeric y_6 ions, EP_DTIDE^+ and EP_LTIDE^+, which show different mobility in IMS analysis due to substitution of D/L-Thr. In contrast, the y_3 ions IDE^+ derived from the two peptide epimers contain the same all-L amino acid residues and thus exhibit the same mobility. Therefore, sequential mobility analysis of epimeric fragment ions may be able to accurately identify D-amino acids in DAACP candidates. On the basis of this concept, we developed a novel liquid chromatography (LC)−MS/MS−IMS strategy which allows site-specific characterization of peptide epimers. The practical utility was demonstrated by analysis of peptide standards, [D-Trp]-melanocyte-stimulating hormone (MSH), [D-Ala]-deltorphin (DTP), [D-Phe]-achatin-I, and their all-L forms. The strategy was then applied to determine the isomerization of an L- to D-Phe in crustacean hyperglycemia hormones (CHHs) isolated from the sinus gland of American lobster, *Homarus americanus*.

EXPERIMENTAL SECTION

Chemicals. Methanol, glacial acetic acid, borane pyridine, and formaldehyde were obtained from Sigma-Aldrich (St. Louis, MO). Optima grade formic acid, acetonitrile (ACN), water, and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Peptide standards, [D-Trp]-MSH and its all-L form were purchased from American Peptide Company; and [D-Ala]-deltorphin and [D-Phe]-achatin-I as well as their all-L forms were synthesized in Biotechnology Center, University of Wisconsin−Madison.

Animals, Tissue Dissection, and Extraction. American lobster *H. americanus* were purchased from Maine Lobster Direct Web site (http://www.mainelobsterdirect.com). All animals were kept in a circulating artificial seawater tank at 10−15 °C. Tissue dissection and extraction was performed according to our previous reports.[19,20] Briefly, animals were anesthetized in ice, and the sinus glands were dissected and collected in chilled acidified methanol and stored in −80 °C freezer prior to further sample processing. The tissues were homogenized and extracted with 100 μL of acidified methanol (methanol/H_2O/acetic acid, 90:9:1, v/v/v) for three times.

LC−MS/MS Coupled to Ion Mobility Spectrometry. The LC−MS/MS−IMS experiments were performed on a Waters nanoAcquity ultraperformance LC system coupled to a Synapt G2 high-definition mass spectrometer. Chromatographic separations were performed on a Waters BEH 300 Å C18 reversed-phase capillary column (150 mm × 75 μm, 1.7 μm). The mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). The peptide sample was injected and loaded onto the Waters Symmetry C18 trap column (180 μm × 20 mm, 5 μm) using 97% mobile phase A and 3% mobile phase B at a flow rate of 5 μL/min for 3 min. The gradient started from 3% to 10% B during the first 5 min, increased to 45% B in the next 65 min, then was kept at 90% B for 20 min. A fixed MS/MS survey was employed to select the peptide molecular ions in a traveling-wave (T-Wave) trap cell for CID fragmentation with adjusted collision energy of 22−30 eV. The resulting fragment ions were online submitted to T-Wave drift tube and time-of-flight analyzer to measure the arrival time. Instrument acquisition parameters used were as follows: an inlet capillary voltage of 2.8 kV, a sampling cone setting of 35 V, and a source temperature of 70 °C. The argon gas pressures in the traveling wave ion guide trap and the traveling wave ion guide transfer cell were 2.44×10^{-2} and 2.61×10^{-2} mbar, respectively. The wave height, the wave velocity, and the nitrogen pressure in the traveling wave IM drift cell were 32.0 V, 800 m/s, and 2.96 mbar, respectively.

Data processing was conducted using Waters MassLynx 4.1 and DriftScope 2.1. The LC−MS/MS−IMS .raw data was opened in DriftScope, and the Selection Tool is used to respectively select the two peptide epimer LC peaks and export the two corresponding Masslynx .raw data by retaining arrival time functions. The arrival time distributions of interested ions were exported from Masslynx.

Calibration of Collision Cross Sections on Ion Mobility Spectrometry. The T-Wave N_2 drift tube of Synapt G2 was calibrated for collision cross section (CCS) measurements using a slightly modified version of the polyalanine method outlined by Bush et al.[21] and the calculation method by Ruotolo et al.[22] A 100 μg mL^{-1} solution (49:49:2, water/acetonitrile/acetic acid) of polyalanine was directly infused and acquired with the same instrument and method settings used to acquire the DAACP analyte. A peak list containing m/z-corrected mobility and arrival times was then exported from DriftScope to a csv for CCS calculation. Further details of the CCS calculation and a representative calibration curve can be found in the Supporting Information. It should be noted that this method allows measurement of CCS_{He} in spite of using nitrogen as drift gas in our experiment.[21] Thus, the CCSs shown in this paper correspond to the helium drift gas.

Tryptic Digestion and Bottom-Up Sequencing of CHHs on Synapt G2 HDMS. For trypsin digestion of CHHs, 1 μL of tissue extract was reduced and alkylated by incubation in 2.5 mM dithiothreitol (DTT) for 1 h at 37 °C followed by incubation in 7 mM iodoacetamide (IAA) in the dark at room temperature for 1 h, and then digested at 37 °C overnight after addition of 50 mM ammonium bicarbonate buffer with 0.5 μg of trypsin (Promega, Madison, WI). The tryptic digest was injected into a Waters nanoAcquity UPLC system coupled to a Synapt G2 HDMS. Chromatographic conditions are the same as described above. A data-dependent acquisition was employed for the MS survey scan and the selection of three precursor ions and subsequent MS/MS of the selected parent ions. The MS scan range was from m/z 400−2000, and the MS/MS scan was from m/z 50−2000.

Top-Down MS/MS Fragmentation of CHHs on Q Exactive. A 1 μL of crude tissue extract was reduced by incubation in 2.5 mM DTT for 1 h at 37 °C and desalted by C18 ZipTip and resuspended in 10 μL of water containing 0.2% formic acid. Online top-down MS was carried out on

dx.doi.org/10.1021/ac4033824 | Anal. Chem. 2014, 86, 2972−2981

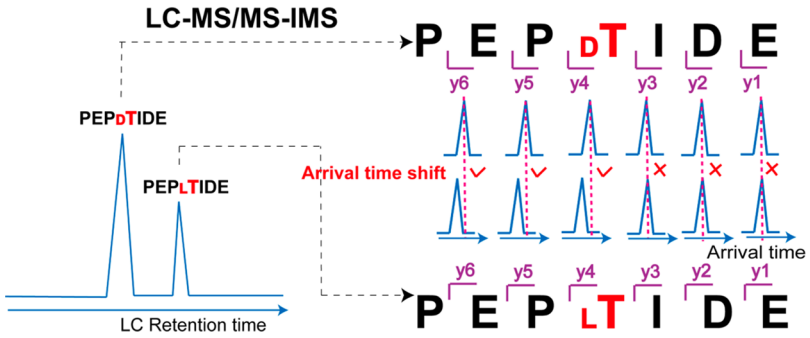


Figure 1. Workflow of the proposed strategy for localization of D-amino acids in peptides. The analysis can be performed in a single LC−MS/MS−IMS run. The two peptide epimers are separated by RPLC and respectively fragmented by CID. Their fragment ions are then submitted to IMS for arrival time measurement. By comparing the arrival time distributions between the two sets of fragment ions, the position of D-amino acid can be determined: √, arrival time shift; ×, no shift. For illustration purpose, only y ions are listed in this workflow. Note that other fragment ions can also be used as indicators for localization of D-amino acids.

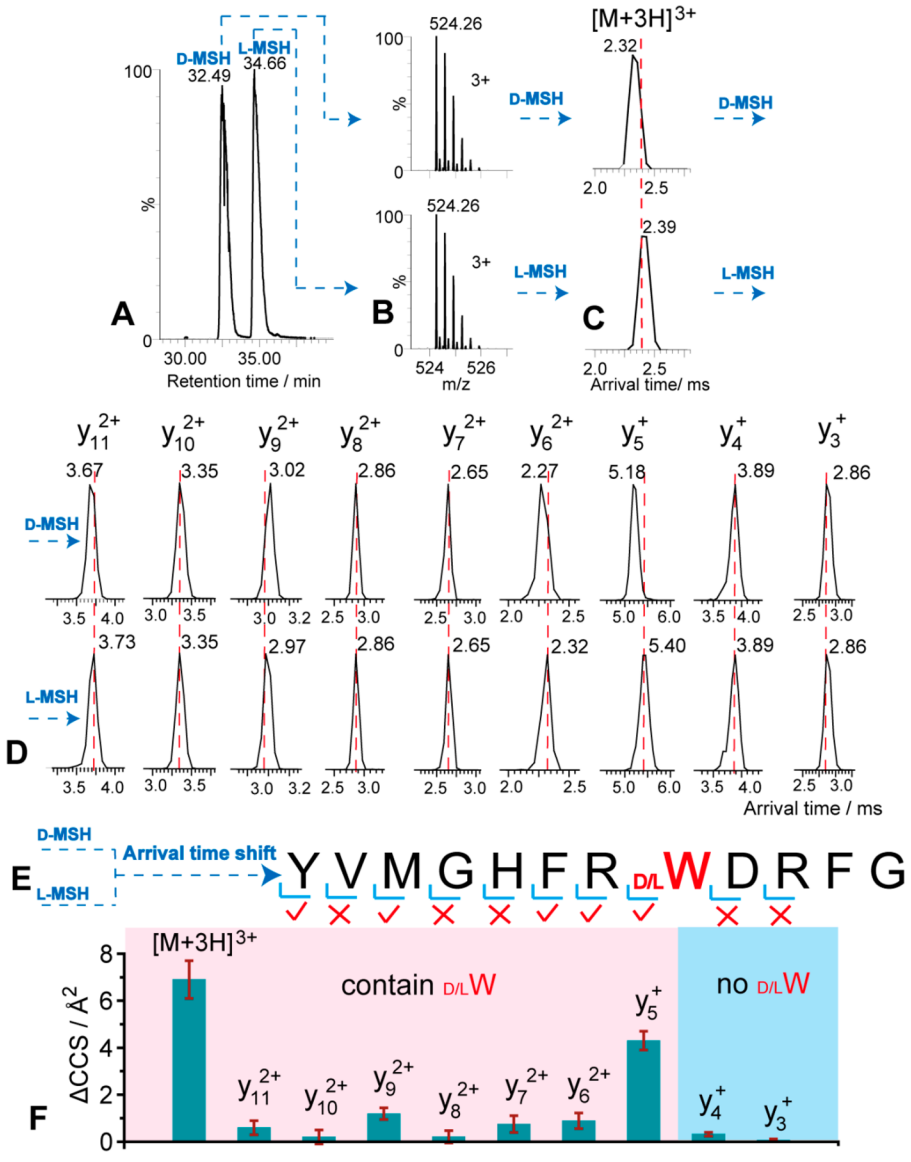


Figure 2. Site-specific characterization of D/L-MSH peptide epimers. (A) Extracted ion chromatogram of LC−MS analysis of D/L-MSH peptides. (B) Molecular ions and (C) the corresponding IMS distributions of D/L-MSH peptides. (D) IMS distributions of fragment ions of D/L-MSH peptides. (E) Localization of D-amino acid residue position by comparison of arrival time shift: √, arrival time shift; ×, no shift. (F) CCS differences (ΔCCS, absolute values) of peptide precusor and fragment ions. Error bars stand for standard deviations.

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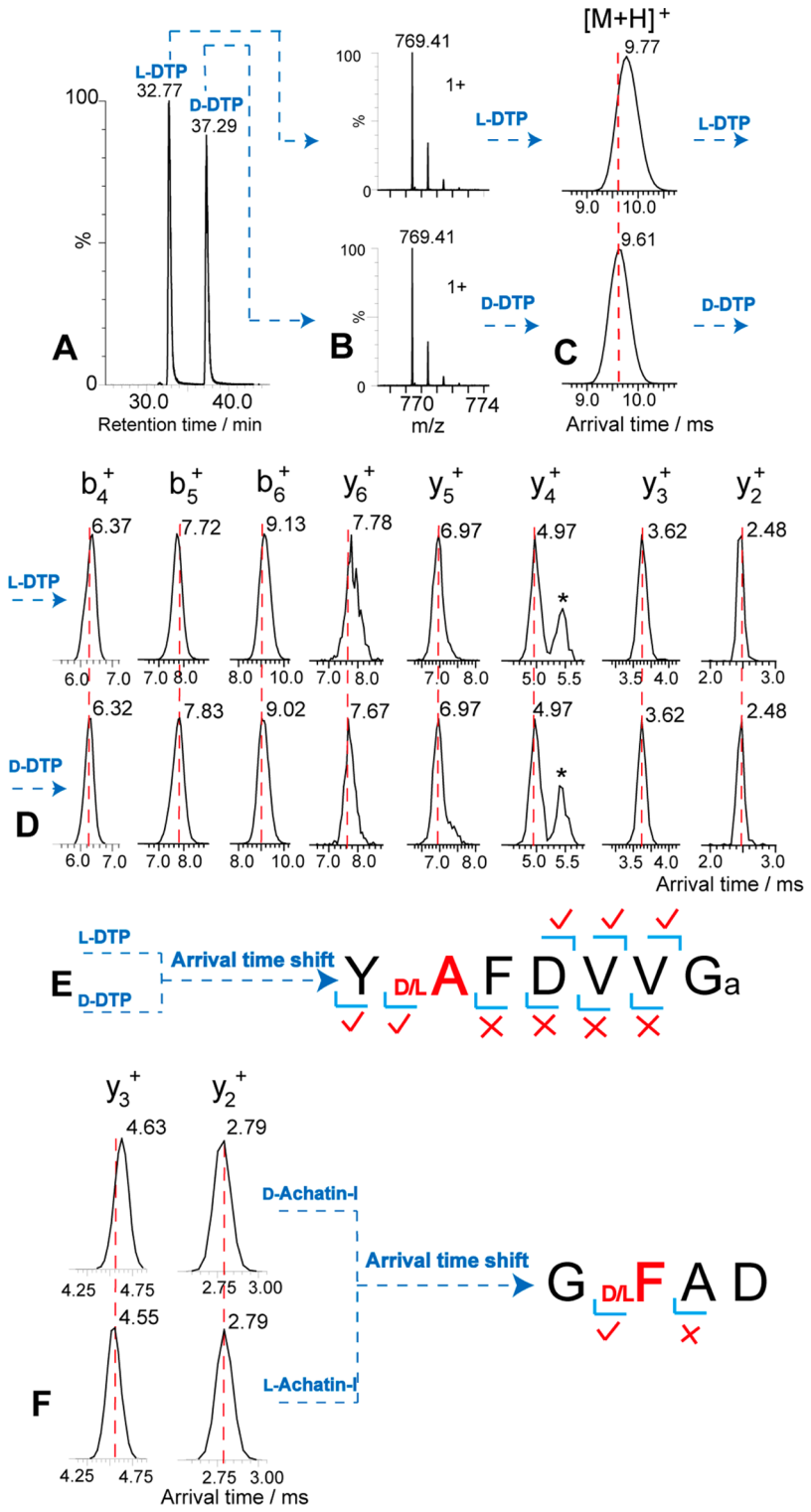

Figure 3. Site-specific characterization of D/L-DTP and D/L-achatin-I peptide epimers. (A) Extracted ion chromatogram of LC−MS analysis of D/L-DTP peptides. (B) Molecular ions and (C) the corresponding IMS distributions of D/L-DTP peptides. (D) IMS distributions of fragment ions of D/L-DTP peptides. (E) Localization of D-amino acid residue position by comparison of arrival time shift. (F) IMS analysis of D/L-achatin-I and localization of D-amino acid position: √, arrival time shift; ×, no shift; ∗, interference ions.

Waters nanoAcquity ultraperformance LC system coupled to a Q Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The peptide sample was injected and loaded onto the Waters Symmetry C18 trap column (180 μm × 20 mm, 5 μm) using 97% mobile phase A and 3% mobile phase B at a flow rate of 5 μL/min for 3 min. A Waters BEH 300 Å C18 reversed-phase capillary column (150 mm × 75 μm, 1.7 μm) was used for separation. The gradient started from 3% to 10% B during the first 5 min, increased to 55% B in the next 65 min, then was kept at 90% B for 20 min. Typical mass spectrometric conditions were as follows: spray voltage, 2.8 kV; no sheath and auxiliary gas flow; heated

dx.doi.org/10.1021/ac4033824 | Anal. Chem. 2014, 86, 2972−2981

capillary temperature, 275 °C; normalized HCD collision energy 30%. The Q Exactive instrument was operated in targeted MS/MS mode with an inclusion list containing the targeted mass of the CHHs. The settings are as follows: resolution 70 000; automatic gain control 2×10^5; maximum ion injection time, 100 ms; isolation window, 6 m/z; fixed first mass, 100 m/z. All MS/MS spectra were processed with Xtract CI-3.0 Software (Thermo Scientific Inc., Bremen, Germany) using an S/N threshold of 1.5 and fit factor of 40% and validated manually. The resulting mass lists were further assigned using the in-house developed "Ion Assignment" software with of 10 ppm of mass error tolerance. The assigned ions were manually validated to ensure the quality of assignments.

RESULTS AND DISCUSSION

Workflow and Rationale of the Proposed Site-Specific Strategy for Localization of D-Amino Acids in Peptide Epimers. Figure 1 illustrates the workflow for the proposed strategy. The analysis can be completed in a single LC−MS/MS−IMS run. The peptide epimers are separated by reversed-phase LC (RPLC) and online-submitted to CID fragmentation. The resulting peptide fragment ions are then subjected to IMS for measurement of arrival time. The epimeric ions of y_6, y_5, and y_4 derived from the two peptide epimers respectively contain the L- or D-Thr, which possibly leads to conformational differences between each epimeric y ion pair, resulting in arrival time shift during IMS analysis. In contrast, the two peptide epimers produce the same y_3, y_2, and y_1 ions containing all-L amino acids, due to the absence of the D- or L-Thr from the peptide chain by CID fragmentation. Thus, these y ion pairs display identical arrival times. By determining at which residue the arrival time shift starts to occur, the D-amino acid can be confidently localized at the threonine. Sometimes, an LC−MS−IMS run without fragmentation is needed to measure the arrival times of peptide molecular ions when the D-amino acid is at the N-terminus. To validate the proposed strategy, we analyzed three pairs of peptide epimers with various molecular sizes: [D-Trp]-MSH (MW 1569.73 Da), [D-Ala]-DTP (768.38 Da), [D-Phe]-achatin-I (407.18 Da), and their counterparts with all-L amino acids.

Site-Specific Characterization of MSH Peptide Epimers for Localization of D-Amino Acids. The MSHs are produced by cells in the intermediate lobe of the pituitary gland, which stimulates the production and release of melanin, and have effects on appetite and sexual arousal.[23] It was reported that the D-Trp-substituted isoform was the most selective analogue for the melanocortin receptors.[23] In our study, the peptide epimers D/L-MSH (YVMGHFR$_D$WDRFG and YVMGHFRWDRFG, 1:1, concentration ratio) were analyzed by LC−MS−IMS on a Waters Synapt G2 HDMS mass spectrometer coupled to a nanoACQUITY UPLC system. Figure 2A shows the extracted ion chromatogram of the two peptides with RPLC baseline separation. The $[M + 3H]^{3+}$ ions of the two epimers exhibit different arrival times at 2.32 and 2.39 ms, respectively (Figure 2, parts B and C). Subsequently, the peptide mixture was analyzed by LC−MS/MS−IMS, where the two peptide epimers were fragmented by CID and then the resulting fragment ions were submitted to IMS for arrival time measurement. In their CID spectra (Supporting Information Figure S-1), a set of y ions, y_3−y_{11} are observed, and the corresponding arrival time distributions are illustrated in Figure 3D. The D/L-epimeric ions of y_5, y_6, y_9, and y_{11} exhibit arrival time shifts, whereas the paired y ions of y_3 and y_4 derived from the two peptide epimers have the identical arrival time distributions. More importantly, the arrival time shift between the epimeric y ions starts from y_5, which is the first y ion that contains tryptophan by counting from y_3 to y_{11}. The results indicate that the D-amino acid is localized at the tryptophan (YVMGHFR$_D$WDRFG) as annotated in Figure 2E. Although the epimeric ions of y_{10}, y_8, and y_7 ions contain D/L-Trp, they do not show arrival time shifts (Figure 2D). These exceptions may be attributed to the possibilities that the conformational differences are too small to be resolvable due to limited resolving power of our IMS instrument, or due to other factors that might have reduced the conformational differences of D/L-Trp.

Previous studies reported that D/L-peptide epimers exhibit conformational differences, resulting in distinct mobilities in IMS.[18] The orientation of the epimeric amino acid residues could produce unique intramolecular interactions that lead to a more extended or compact overall shape. In this study, we hypothesize that the same phenomenon occurs with the CID-produced epimeric fragments. If the fragments are epimers, the different amino acid orientation could still produce unique intramolecular interactions and unique conformations. However, if the fragments are identical, then the intramolecular interactions and resulting conformations may also be identical. To support this assumption, we measured the collision cross sections (CCSs) of the precursor and fragment ions arising from D/L-MSH.[21,22] The results are listed in Supporting Information Table S1, and their CCS differences (ΔCCSs) are illustrated in Figure 2F. The standard deviations only represent the reproducibility of the measurement, while the absolute CCS error can be as large as 3.5%. The epimeric $[M + 3H]^{3+}$ ions of D-MSH and L-MSH show a 6.9 Å^2 of ΔCCS, which reveals their conformational differences attributed to the substitution of D/L-Trp. The epimeric fragment ions of y_5−y_{11} containing D/L-Trp show an averaged ΔCCS of 1.2 Å^2, while the y_3 and y_4 ions without D/L-Trp have an averaged ΔCCS of 0.2 Å^2. More importantly, the epimeric y_5 ions with D/L-Trp at the N-terminus exhibit a remarkable ΔCCS of 4.3 Å^2, which clearly indicates that the D-amino acid is localized at the tryptophan residue. In contrast, the ΔCCSs of epimeric y_{10}, y_8, and y_7 ions are 0.2, 0.2, and 0.7, respectively. These small differences indicate a possible reason why their arrival time shifts are not resolved in Figure 2D. The differences may be small compared to the absolute error, but were very significant given the excellent reproducibility of these measurements. More in-depth investigation will be needed to fully understand the conformational differences between D/L-peptide epimers. This work is primarily focused on developing an alternative analytical methodology for discrimination of peptide epimers. On the basis of the concept described above, two smaller peptides are further investigated to validate the proposed site-specific strategy.

Site-Specific Characterization of Deltorphin and Achatin-I Peptide Epimers for Localization of D-Amino Acids. Deltophins are a family of naturally occurring peptides found in skin extracts of frogs. They have high affinity and selectivity for δ opioid binding sites.[24] Here, we choose [D-Ala]-DTP (Y$_D$AFDVVG−NH$_2$) and its all-L form (YAFDVVG−NH$_2$) to further validate our proposed strategy. The two peptide epimers are baseline-separated by LC and eluted at 32.77 and 37.29 min (Figure 3, parts A and B), respectively. The arrival time measurement indicates a shift of 0.16 ms

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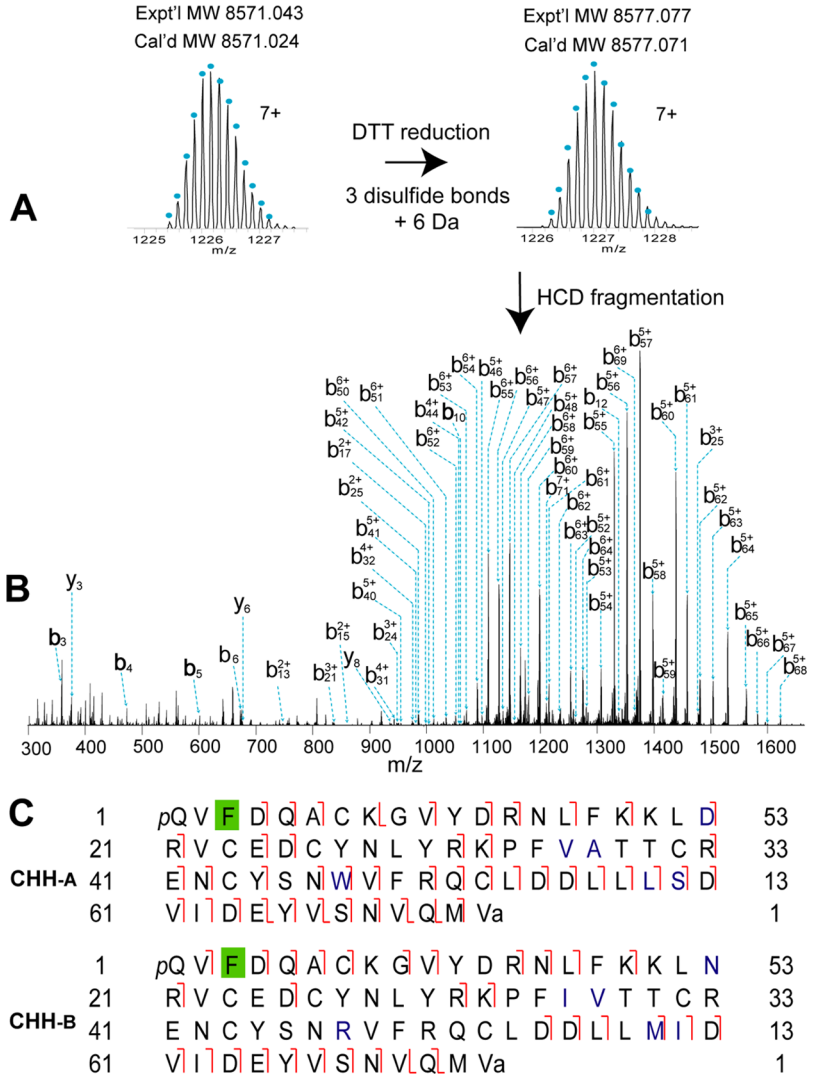

Figure 4. Identification of CHH_{-A} and CHH_{-B} peptides (isolated from the sinus glands of American lobsters) by top-down MS/MS. (A) Isotopic distributions of intact and DTT-reduced CHH_{-A}. (B) HCD MS/MS spectrum of DTT-reduced CHH_{-A}. (C) Top-down fragmentation maps of CHH_{-A} and CHH_{-B}:⌉, b ions; ⌊, y ions. The different residues between the two CHH peptides are highlighted in blue. The D-Phe residue is localized in the third position from the N-terminus, highlighted in green shading.

between the two epimeric $[M + H]^+$ ions (Figure 3C). In the subsequent LC−MS/MS−IMS experiment, the epimeric ions of y_4, b_4, b_5, and b_6 exhibit different mobility, leading to arrival time shift as shown in Figure 3D. In contrast, the paired y ions of y_2−y_5 derived from the two peptide epimers display nearly identical arrival times. Figure 3E summarizes the results of arrival time shifts of these fragment ions, clearly indicating that the D-amino acid is localized at the second position from the N-terminus, an alanine residue ($Y_DAFDVVG$−NH_2).

Achatin-I (G_DFAD) is a neuroexcitatory peptide in *Achatina fulica* ganglia.[25] On our nanoRPLC system, the achatin-I and its all-L form (GFAD) were not baseline-separated due to a short retention (data not shown). Alternative separation techniques, such as chiral chromatography[26] or capillary electrophoresis,[27] could be incorporated into our strategy. To examine the proof of principle, we assume that the two peptide epimers had been off-line separated, and the individual peptide epimer was directly infused into mass spectrometer for MS−IMS and MS/MS−IMS analysis. The $[M + H]^+$ ions of the two peptide epimers show the same arrival time at 5.20 ms. The immeasurable arrival time shift may be attributed to the limited resolution of our IMS instrument. Interestingly, the epimeric y_3 ions derived from the peptide epimers exhibit a 0.08 ms of arrival time shift as shown in Figure 3F. In contrast, the y_2 ions from the two peptide epimers have identical arrival time at 2.79 ms. These results provide precise positional information for localization of D-amino acid at the phenylalanine of G_DFAD (Figure 3F).

Site-Specific Characterization of D-Amino Acids in CHHs Isolated from the American Lobster. To apply the site-specific strategy to identification of DAACPs in real biological samples, we sequenced the CHHs in the American lobster *H. americanus* and determined the D-amino acid position in these large signaling peptides. CHHs are a family of neurohormones released from crustacean sinus glands, which regulates the glycemia through the classical mechanisms of glycogen mobilization.[28] Soyez and co-workers[5,29] reported that the sinus gland of *H. americanus* secrets two peptide hormones, CHH_{-A} and CHH_{-B}, and both of CHH_{-A} and CHH_{-B} occur as two isoforms with D/L-Phe at the third residue from the N-terminus. The biological activities of the D/L-isoforms differ in the kinetics of their hyperglycemic effect.[5]

dx.doi.org/10.1021/ac4033824 | *Anal. Chem.* 2014, 86, 2972−2981

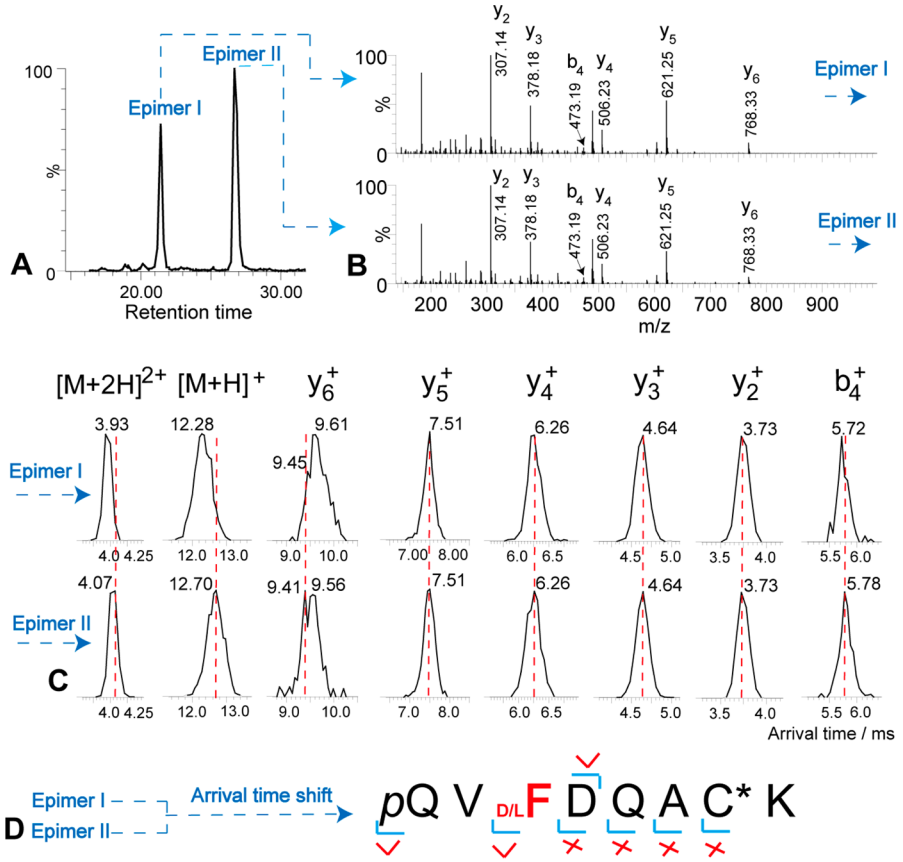

Figure 5. Localization of D-amino acid in tryptic peptides, *p*QV$_{D/L}$FDQAC*K. (A) Extracted ion chromatogram and (B) MS/MS of the tryptic peptide epimers I and II. (C) IMS distributions of fragment ions from tryptic peptide epimers I and II. (D) Localization of D-amino acid residue by comparison of arrival time shift: √, arrival time shift; ×, no shift. It should be noted that the elution order of the two D/L-peptide epimers cannot be determined by our current method, so we use epimer I and II for annotation.

The first step is to identify and sequence the CHHs from the American lobster. The identification is challenging, as CHHs contain more than 70 residues and multiple post-translational modifications (PTMs), including three disulfide bonds and N-terminal *p*Glu modification.[19,28] In this work, we employed an online top-down approach. The intact peptide and dithiothreitol (DTT)-reduced peptide were analyzed on a high-resolution Q Exactive Orbitrap mass spectrometer by targeted MS/MS to acquire high-quality top-down MS and MS/MS spectra. Figure 4A shows the isotopic distributions of the CHH$_{-A}$ before and after reduction of disulfide bonds with an error less than 2 ppm. The mass increase of 6 Da resulting from DTT reduction suggests that the peptides contain three disulfide bonds, which is an important criterion to discriminate CHHs from other peptide families.[28] Figure 4B is the top-down high-energy collisional dissociation (HCD) MS/MS spectrum of the reduced CHH$_{-A}$. Compared with HCD fragmentation of the intact CHH$_{-A}$ (data not shown), the efficiency of peptide fragmentation is dramatically improved due to elimination of disulfide linkage, where 46% of sequence coverage and 62% of amide bond cleavage are achieved. The fragmentation maps of CHH$_{-A}$ and CHH$_{-B}$ are shown in Figure 4C. These results confirm the identities of the two CHHs in American lobster. However, baseline separation of D- and L-CHHs by nanoRPLC is difficult (Supporting Information Figure S-2) because of the negligible differences of the hydrophobicity and sequence-dependent effects of the D/L-isoforms attributed to the third D/L-Phe. Therefore, we adopted a bottom-up approach in the following experiment.

The second step is to find the target tryptic peptide epimers that contain D/L-amino acids. After treatment with DTT and IAA, the crude extract was digested by trypsin and analyzed by RPLC−MS/MS in a data-dependent mode and processed using software PEAKS[30] against a database containing the sequences of CHH$_{-A}$ and CHH$_{-B}$. Supporting Information Table S2 lists all the tryptic peptides and their corresponding LC retention times. The tryptic peptide CHH[1−8] is eluted at 21.42 and 26.69 min, and CHH[1−17] at 33.21 and 35.70 min. The elution pattern of splitting peaks suggests the possibility that the two peptides are D/L-epimer candidates. In contrast, the rest of tryptic peptides are eluted at single time points without splitting. Figure 5A shows the representative extracted ion chromatogram of tryptic peptide *p*QVFDQAC*K (*p*Q, pyro-Gln; *, carbamindomethyl), where the D/L-peptide epimer candidates are baseline-separated. It should be noted that CHH$_{-A}$ and CHH$_{-B}$ share the same N-terminal sequence from ^{1}pyro-Gln to ^{19}Leu, and the residue D/L-Phe is at the third position from the N-terminus.[5,29] To obtain the precise D-amino acid information of each peptide, the two CHHs should be purified and separately analyzed. The goal of our study is to examine the practical utility of the site-specific strategy in a real biological sample. Herein, we used a simplified experimental procedure by directly investigating the entire tryptic digest of the tissue extracts.

dx.doi.org/10.1021/ac4033824 | *Anal. Chem.* 2014, 86, 2972−2981

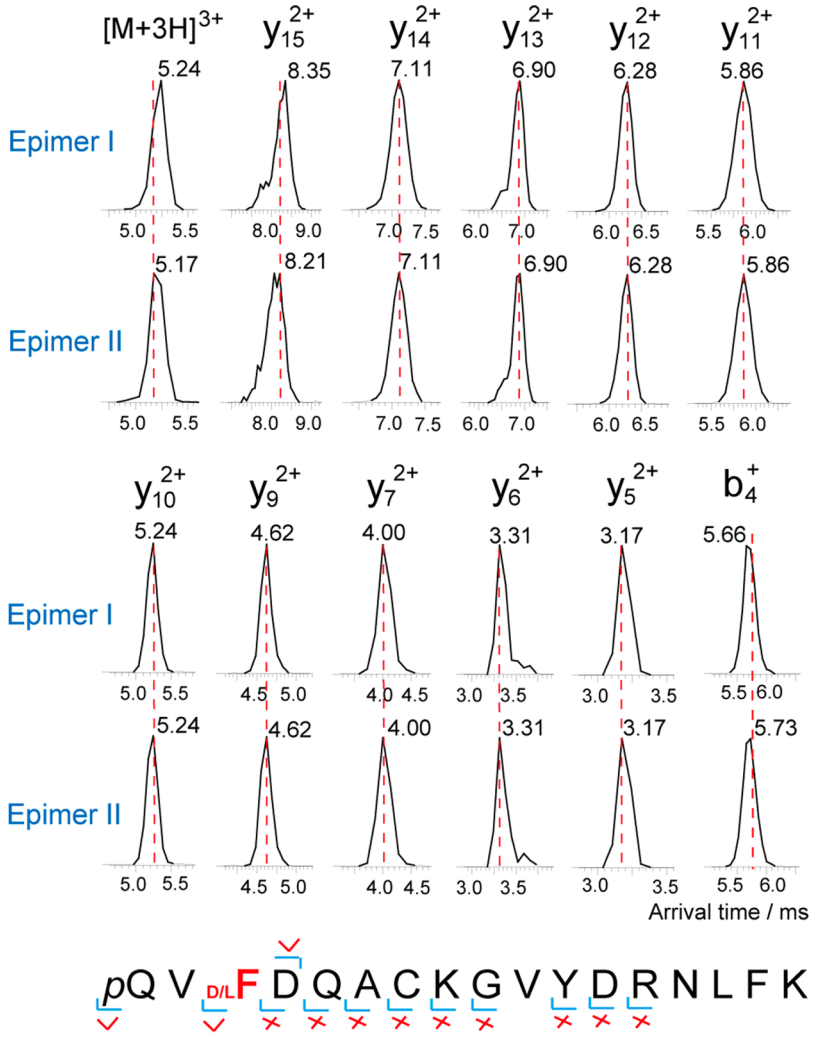

Figure 6. IMS distributions of fragment ions from tryptic peptide epimers, $pQV_{D/L}FDQAC^*KGVYDRNFLK$. Tryptic peptide epimers I and II were eluted from RPLC at 33.21 and 35.70 min, respectively: √, arrival time shift; ×, no shift.

The third step is to localize the D-amino acid in the target tryptic peptides, *p*QVFDQAC*K and *p*QVFDQAC*KGVYDRNLFK. The entire tryptic digest of the tissue extracts was analyzed by LC−MS−IMS and LC−MS/MS−IMS. Parts B and C of Figure 5 show the MS/MS spectra of peptide epimers, $pQV_{D/L}FDQAC^*K$, and the corresponding arrival time distributions of the fragment ions. As summarized in Figure 5D, the paired y ions of y_2−y_5 derived from the two peptide epimers have the same arrival time, whereas those of b_4, y_6, $[M + H]^+$, and $[M + 2H]^{2+}$ display arrival time shifts. Similarly, LC−MS/MS−IMS analysis of peptide epimers *p*QVFDQAC*KGVYDRNLFK (Figure 6) indicates that the paired y ions of y_5−y_{14} show identical arrival time distributions, whereas those of y_{15}, b_4, and $[M + H]^+$ ions exhibit arrival time shifts. These results suggest that the D-amino acid is localized at the phenylalanine of the third residue of the CHHs from the N-terminus.

Practical Utility of the Site-Specific Strategy. In this work, we demonstrated the broad application and practical utility of the site-specific strategy for the characterization of DAACPs and their all-L counterparts. By employing LC−MS/MS−IMS, the position of D-amino acid in the peptide epimers can be rapidly and precisely determined. Specifically, the LC−MS/MS experiment serves as a means to screen for D/L-peptide epimer candidates in biological samples, so the initial candidates of D/L-peptide epimers must display differential LC retention times. An LC−MS−IMS experiment can be carried out to measure the arrival time of the peptide molecular ions to obtain complementary IMS evidence to support the initial identification of peptide epimers. Lastly, the site-specific strategy based on LC−MS/MS−IMS experiment is utilized to localize the D-amino acid.

The coexistence of DAACP and its all-L form counterpart is widely found in biological systems due to incomplete enzymatic isomerization of an L- to D-amino acid. In contrast, some DAACPs are uniquely found without their all-L counterparts.[2,4] Characterization of these DAACPs does not require the online separation prior to MS/MS−IMS analysis. The purified DAACP candidate and the synthetic all-L counterpart can be infused into mass spectrometer for IMS analysis separately, and the D-amino acid can be localized by the site-specific strategy outlined in this study. In addition, our site-specific strategy can be incorporated into the nontargeted strategies for DAACP discovery. For example, Ewing et al.[31] employed microsomal alanyl aminopeptidase to selectively degrade peptides lacking a D-amino acid in the second position from the N-terminus, so the DAACPs can be identified from a complex mixture. Our site-specific strategy can serve as a downstream tool for

dx.doi.org/10.1021/ac4033824 | *Anal. Chem.* 2014, 86, 2972−2981

validation of these putative DAACP candidates. However, we cannot exclude the possibility that some peptide epimers and their CID-produced fragments show identical mobility (maybe slightly different conformations) in spite of containing D/L-amino acids, or their mobility differences are too small to be resolvable by current IMS instrumentation, such as the case for the epimeric y_7, y_8, and y_{10} ions of D/L-MSH (Figure 2D).

In this study, we measured the arrival time distributions of y, b, and molecular ions of peptide epimers, indicative of the D-amino acid position. It is expected that other peptide fragment ions, such as a, c, z, neutral loss ions, etc., could be used as indicators for site-specific characterization because of being sensitive to chirality. Also, the charge state of epimeric fragment ions could be a factor which influences their conformational difference, leading to various arrival time shifts. In this study, we chose the most abundant fragment ions as indicators for comparison of arrival times. One of our ongoing studies is to investigate the effect of charge states on the conformational difference of peptide epimers. In addition, the peptide epimers are eluted at different time points by LC solvents containing different percentage of organic phase. For example, the D-MSH and L-MSH were eluted in 24% and 25% of acetonitrile, respectively. To rule out the possibility of that the arrival time shift was caused by various different organic phase percentages in solvents, we analyzed the L-MSH in 24% and 30% of acetonitrile by direct infusion into MS/MS−IMS and found that the arrival time distributions of peptide precursor ions and fragment ions were identical between two solvent conditions (Supporting Information Figure S-3). This result suggests that the measurement conditions between the two peptide epimers in one LC−MS/MS−IMS run have a negligible effect on the changes of their arrival time distributions in the gas phase in the ion mobility drift cell.

CONCLUSIONS

In this study, we report on a novel strategy for site-specific characterization of peptide epimers, which allows rapid and precise localization of D-amino acids in DAACP candidates. The analysis can be finished in a single LC−MS/MS−IMS run, followed by data processing in a simple and straightforward manner. The efficiency and utility of the strategy have been demonstrated by analysis of a set of peptides with various molecular sizes, MSH, DTP, and achatin-I, and their counterparts possessing all-L amino acids. In the CHHs isolated from the American lobster, our data indicates that the peptides contain an isomerization site of L- to D-Phe at the third residue from the N-terminus. This study represents a new route to obtain positional information about amino acid isomerizations in peptides by elucidating the IMS data of peptide fragment ions. By coupling with efficient screening approaches, the developed strategy is potentially applicable to large-scale discovery and characterization of DAACPs, and the proposed concept is transferable to the characterization of other post-translational isomerizations in large biological molecules.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Professor Jonathan V. Sweedler, Dr. Lu Bai, and Itamar Livnat (UIUC) for helpful discussions and insightful suggestions on this project. This work is supported in part by the National Science Foundation Grant CHE-0957784 and the National Institutes of Health Grants 1R01DK071801 and 1R56DK071801. We acknowledge the NIH shared instrument program for funding the instrument purchase (S10 RR029531). C.B.L. acknowledges an NIH-supported Chemistry Biology Interface Training Program Predoctoral Fellowship (Grant No. T32-GM008505) and an NSF Graduate Research Fellowship (DGE-1256259). L.L. acknowledges an H. I. Romnes Faculty Research Fellowship. We thank the UW School of Pharmacy Analytical Instrumentation Center for access to Q Exactive Orbitrap mass spectrometers.

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