Fragment correlation mass spectrometry enables direct characterization of disulfide cleavage pathways of therapeutic peptides

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ABSTRACT: Therapeutic peptides that are connected by disulfide bonds are often difficult to analyze by traditional tandem mass spectrometry without chemical modification. Using fragment correlation mass spectrometry, we measured 56 pairs of fragment ions from an equimolar (10 µM) mixture of three cyclic peptides, with sequence coverages for octreotide, desmopressin, and the structural analog of desmopressin to be 86%, 100%, and 75%, respectively. In all detected fragment ion pairs, only 20% of the fragments are terminal ions, with most of the measured MS^2 signals only made available by fragment correlation mass spectrometry. From the peak volumes in the covariance map, we calculated branching ratios of each disulfide fragmentation pathway, providing direct measures of disulfide fragmentation probabilities without altering analytes' chemical structures.

Disulfide bonds exist not only in post-translational modifications of proteins[1,2] but also in various therapeutic cyclic peptides.[3,4] Identification of disulfides is analytically important because they directly affect biological function.[5] To tackle the analytical challenges of mapping disulfides, they are typically reduced to thiols which then could be subject to further chemical labeling for fluorescent,[6] mass spectrometric,[7] or other analytical methods.[8,9] Therapeutic cyclic peptides connected by disulfides[10] are especially difficult to analyze using label-free tandem mass spectrometric methods. This is because backbone cleavages are preferential in fragmentation processes such as collision-induced dissociation, while disulfides are generally resistant to gas-phase fragmentation and often remain intact.[11] Alternatively, electrochemical reduction has been used for such analysis.[12,13] Various mass spectrometric approaches have emerged,[14] such as electron transfer dissociation or other means,[15–17] which typically require much more expensive instrumentation or complex setups. Without introducing complexity in chemical derivatization or instrumental modification, a method based on commercial ion trap collision-induced fragmentation conditions is desirable. We developed such a method based on fragment correlation mass spectrometry, enabling a simple experimental workflow to directly measure disulfides in a mixture.[18]

In the current experiment, we first made an equimolar solution of three cyclic peptides which are structurally constructed via disulfides: octreotide, desmopressin, and desmopressin analog. Mpa is the commonly used abbreviation for 3-mercaptopropionic acid in the structure of desmopressin, which is sometimes referred to as deamino-cysteine. β-Mercapto-β,β-cyclopentamethylenepropionyl was abbreviated as Mcp in the structure of desmopressin analog as shown in **Figure 1**. We injected 10 µL of the equimolar solution at 10 µM, without prior separation or sample preparation, into an unmodified linear ion trap using nanoelectrospray ionization. We continuously collected tandem mass spectrometry (MS^2) scans under the same condition, in positive mode using a collision-induced dissociation (CID) energy with normalized collision energy of 11, with a 40-Th isolation width centered at m/z 525. A detailed description of the methods and materials is presented in the Supporting Information (SI). Note that our method is particularly advantageous for mixture analysis especially when analytes coelute in traditional separation techniques, as explained in the SI.

We used these recorded MS^2 spectra scans to calculate contingent covariance between ion intensities at each bin of m/z location, to reveal intrinsic correlation between fragments generated from the same fragmentation pathway.[18] When pairs of fragment ions produce positive contingent covariance peaks, this is a result of coupled fluctuations in their signal intensity. The correlated pairs of fragment ions were then ranked using correlation scores, defined in the SI. Each data point in **Fig. 1** gives the two specific m/z values of fragment 1 and 2 that are correlated and assigned to each structure by our automated analysis procedure. From this dataset, sequence coverage maps of precursor peptides in the mixture were generated, providing 86%, 100%, and 75% of sequence coverage for octreotide, desmopressin, and desmopressin analog, respectively. Among 56 correlated fragment pairs depicted in **Fig. 1**, five times more paired fragment peaks were measured after including non-terminal fragment ions (generated from disulfide cleavages as well as internal ions with intact disulfides) compared to using terminal ions alone. Fragment correlation mass spectrometry offers a critical advantage in this aspect by dramatically reducing the false positive fragment assignment rate for internal ions.[19] In particular, when using fragment correlation mass spectrometry to analyze octreotide (shown in purple), whose disulfide bond is formed between the second and seventh amino acids, the sequence coverage was greatly improved, with all detected fragments arising from non-terminal ions (shown in purple circles and diamonds).

In addition to the m/z values of correlated fragment ions, the volume of a correlation island is also directly proportional to precursor concentration and branching ratio of the relevant fragmentation pathway.[20] We use this information to calculate the relative probability of specific disulfide cleavage pathways, as branching ratios. **Figure 2** shows an example of a region of a contingent covariance map before further jackknife resampling, highlighting a few disulfide fragmentation pathways generated from octreotide. Peaks are shown in dark blue in the map by setting a cutoff to be 0.1% of the height of the tallest peak on the entire covariance map from the mixture analysis. In this region, three peaks related to disulfide cleavage pathways from octreotide were identified, providing rich information about fragmentation patterns, including S-S cleavages (such as pathway 1) and C-S cleavages (such as pathway 2), as described in the scheme of **Fig. 2**.
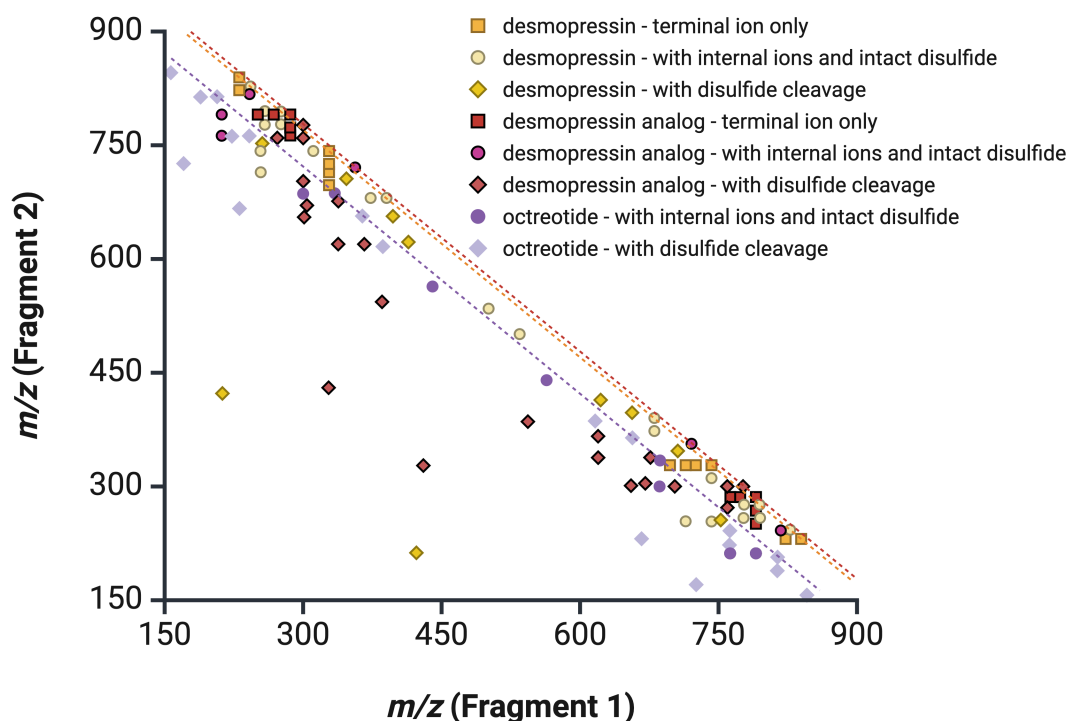
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Figure 1 (Top) A correlation plot identifying paired fragments from the same fragmentation pathways using fragment correlation mass spectrometry after (1) calculating contingent covariance between signal intensities of two pieces of fragments in tandem mass spectrometry; (2) ranking correlation peaks following the jackknife resampling analysis as described in the SI; (3) matching m/z values of the paired fragments generated from the same precursor in the mixture. Data points on the correlation plot were categorized based on types of fragmentation pathways (terminal ions only in squares, internal ions with intact disulfides in circles, and disulfide cleavages in diamonds) using matched fragment structures, with each color representing one precursor ion. (Bottom) Sequence coverage maps generated for each cyclic peptide in the mixture using all data points in the correlation plot for three types of fragmentations (in blue, green and grey).

To calculate the branching ratio for each disulfide fragmentation pathway, we identify the volume of a correlation peak that is generated from a disulfide bond cleavage, normalized to the sum of the volumes of all such detected disulfide cleavage correlation peaks that are fragmented from the same structure. For example, in **Fig. 1** all peaks matched to octreotide disulfide fragmentation are plotted in purple diamonds, with branching ratios calculated in Table S1 in the SI. As shown in **Fig. 2**, branching ratios of pathway 1, pathway 2, and another pathway which is similar to pathway 1 but with an additional water molecule lost from its C terminus, were calculated to be 6%, 19%, and 6%, respectively. In this manner, we provide a direct measure of disulfide cleavage probability without changing analytes' chemical nature by chemical derivatization.

In summary, using fragment correlation mass spectrometry, we directly measured disulfide bond breakage by collision-induced fragmentation in a linear ion trap without the need of chemical modification or instrument modification. Without prior separation of the mixture, we identified independent fragmentation pathways and their branching ratios.
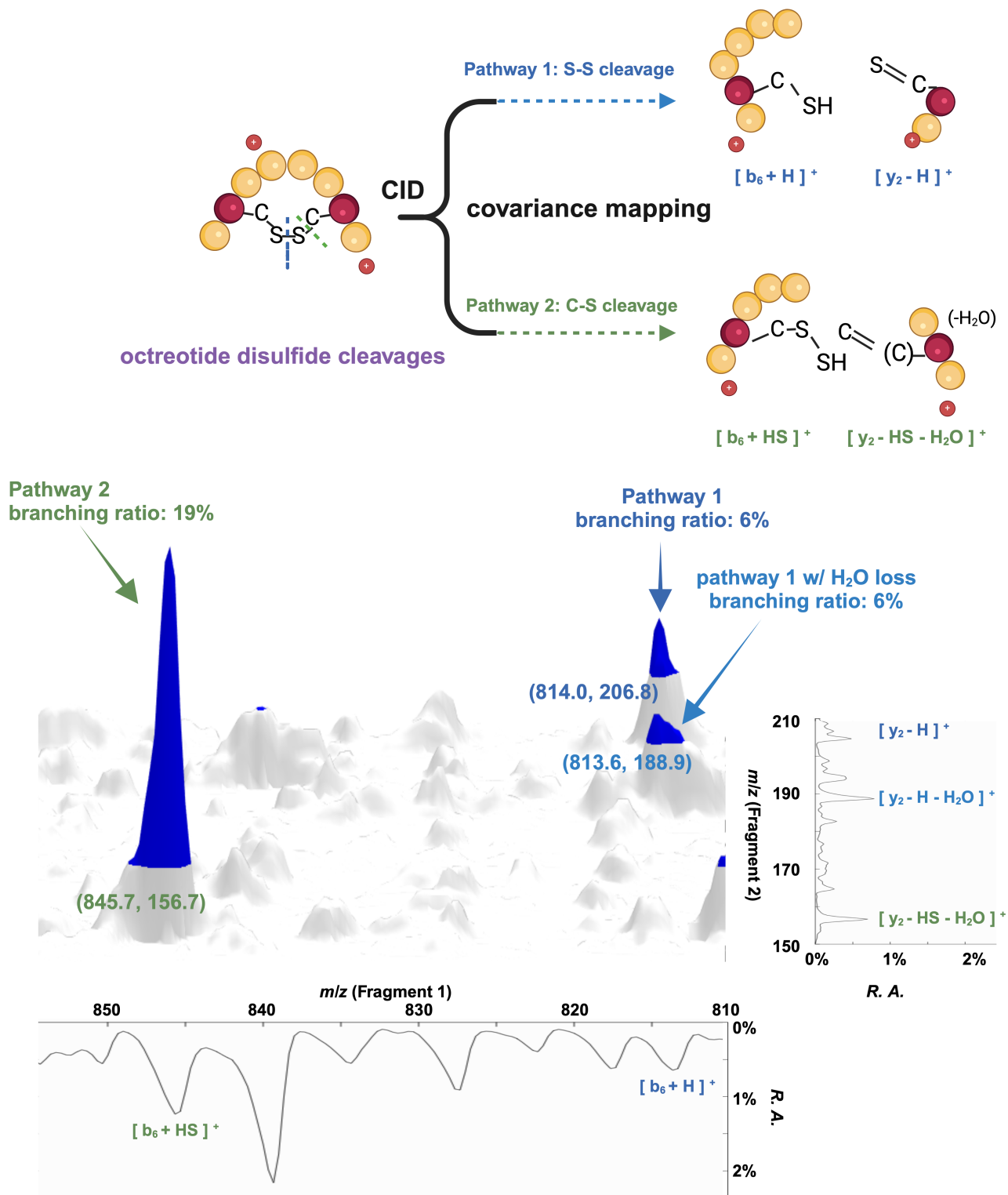
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Figure 2 (Top) A fragmentation pathway scheme using the cyclic peptide octreotide and its two possible disulfide cleavage pathways as examples: S-S cleavage with thiols generated in the b fragment in pathway 1 (blue) and C-S cleavage near C terminus in pathway 2 (green); (Bottom) A contingent covariance map before jackknife resampling to show three independent disulfide cleavage pathways of octreotide with branching ratios in this region of the map. The cutoff for identifying peaks (shown in dark blue with m/z labels of both fragments in brackets) in the map was selected using 0.1% of relative abundance ($R.\ A.$) of the highest peak in the entire map (with $R.\ A.$ below 0.1% shown in light grey). Branching ratios were calculated using each peak volume from each disulfide cleavage pathway divided by the sum of all measured disulfide cleavage peak volumes of octreotide. Black traces show that traditional averaged tandem mass spectra failed to identify specific independent pathways, because for example signals of the fragment $[b_6+H]^+$ could come from both pathway 1 and pathway 1 with (w/) H_2O loss while in the two-dimensional covariance map, they show up at different locations as independent fragmentation pathways.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed description of materials and methods, detailed discussions, and branching ratios of octreotide disulfide fragments in Table S1. (PDF)

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Author Contributions

Y.L. designed and performed experiments, analyzed data, generated figures, and wrote the first draft. T.D. designed the algorithm and wrote the code for contingent covariance analysis. Y.L. and T.D. developed the logic and codes for matching fragmentation pathways of cyclic peptides with disulfides. G.C. and T.D. conceptualized the study and developed the contingent covariance method. R.N.Z. revised the manuscript.

Notes

Taran Driver and Guy Cavet are co-inventors on US Patent Application # 18/479,114.

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The direct characterization of disulfide bond cleavages is challenging using collision-induced dissociation in tandem mass spectrometry without chemical modifications. This complicates mass spectrometric analysis in the presence of disulfide bonds, even for small therapeutic peptides for which the disulfide bond is critical to function. We employed fragment correlation mass spectrometry to directly characterize disulfide cleavage pathways to improve sequence coverages of therapeutic cyclic peptides in a mixture of three such peptides. Moreover, we measured the branching ratio of each disulfide cleavage pathway in the collision-induced dissociation process.