Illuminating the dark space of bioactive peptides with mass spectrometry

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Natural product peptides embody a suite of inherent bioactivities and serve as a template to inspire new chemistries and molecular scaffolds in drug discovery and agrotechnology. Mapping the vast and diverse bioactive peptidome, however, is largely obfuscated by unpredictable molecular transformations in both non-ribosomal sequences and highly post-translationally modified ribosomal protein products. Mass spectrometry is a powerful analytical technique with modern instrumentation achieving unprecedented resolving power, rapid and sensitive gas-phase separations, and versatile multistage fragmentation techniques. As such, mass spectrometry can be (1) leveraged to characterize traditionally difficult-to-sequence natural product peptide modifications *via* enhanced gas-phase technologies and (2) coupled with complementary 'Omics' approaches to predict peptide structure through transcripts, motifs, biosynthetic pathways, and the biomolecular machinery involved in peptide biogenesis. Herein, the challenges of and recent innovations in mass spectrometry towards the discovery and characterization of natural product bioactive peptides are profiled.

1. Inherent challenges in the "dark space" of bioactive peptide discovery

Beyond the basic gene-encoded peptidome lies "dark" chemical space where classes of structurally complex and functionally diverse bioactive peptides remain largely hidden to conventional analytical methodologies. Ribosomally synthesized and post-translationally modified peptides (RiPPs) incorporate nonstandard structures *via* non-canonical amino acids, macrocyclization, and common, complex, or unpredicted post-translational modifications. In RiPP biosynthesis, a ribosomal precursor peptide consisting of discrete recognition and core regions serves as a substrate for extensive post-translational modification by tailoring enzymes genetically co-localized with the precursor peptide in a biosynthetic gene cluster (BGC). Subsequent cleavage by a BGC-encoded protease releases the recognition sequence from the precursor peptide, leaving the post-translationally modified core as the mature active form. Distinction among enzymatic recognition and modification sites on core sequences maintains target selectivity while tolerating core region mutations, permitting the rapid evolution of RiPP structure and function.[1] As such, sequence flexibility in core regions and differential proteolytic processing of RiPPs yields numerous peptidic variants from a single precursor sequence, each with the potential for dramatically different bioactivities.[2,3]

Non-ribosomal peptides (NRPs) are complex natural products manufactured entirely independent of the ribosome. Megadalton systems of non-ribosomal peptide synthetases (NRPSs) construct the NRP peptide backbone from a vast pool of precursor modules and facilitate "tailoring," *e.g.*, methylation, oxidation, reduction, formylation, or epimerization. Separate *trans*-acting BGC-encoded enzymes may add additional modifications on the growing peptide chain or on the natural product peptide after it is released from the NRPS.[4] Significant chemical diversity of NRPs emerges from the incorporation of primary metabolite-derived non-proteinogenic, D-, β-, N-methyl or homo amino acids, often hydroxylated, methylated, or halogenated (Fig. 1).[5] Promiscuity for structurally-analogous amino acids among NRPS domains, enabling nimble adaptation to changing targets and environmental pressures, produces heterogeneous populations of NRP analogs.[5] Additionally, hybrid systems of NRPSs and polyketide synthetases (PKSs), functioning in multienzyme complexes to condense small carboxylic acids into polyketide oligomers, generate increasingly complex NRP-PKS natural products (*e.g.*, lipopeptides) with the ability to access new chemistries and modes of action.

Although mature sequences ultimately present similar post-translational modifications, the challenges associated with the unique RiPP and NRP biosynthetic origins limit bioinformatics in predicting novel structures. While gene-encoded RiPP core sequences are readily accessible through genome-mining, RiPP BGCs are conserved only within RiPP families and thus homology rule-based tools often fail to detect novel RiPPs.[5,6] Additionally, the identification of short RiPP precursor-encoding genes through genomic approaches can yield significant false positives given the number of putative short open reading frames within a genome; setting a minimum length threshold may reduce these false positives, but risks excluding

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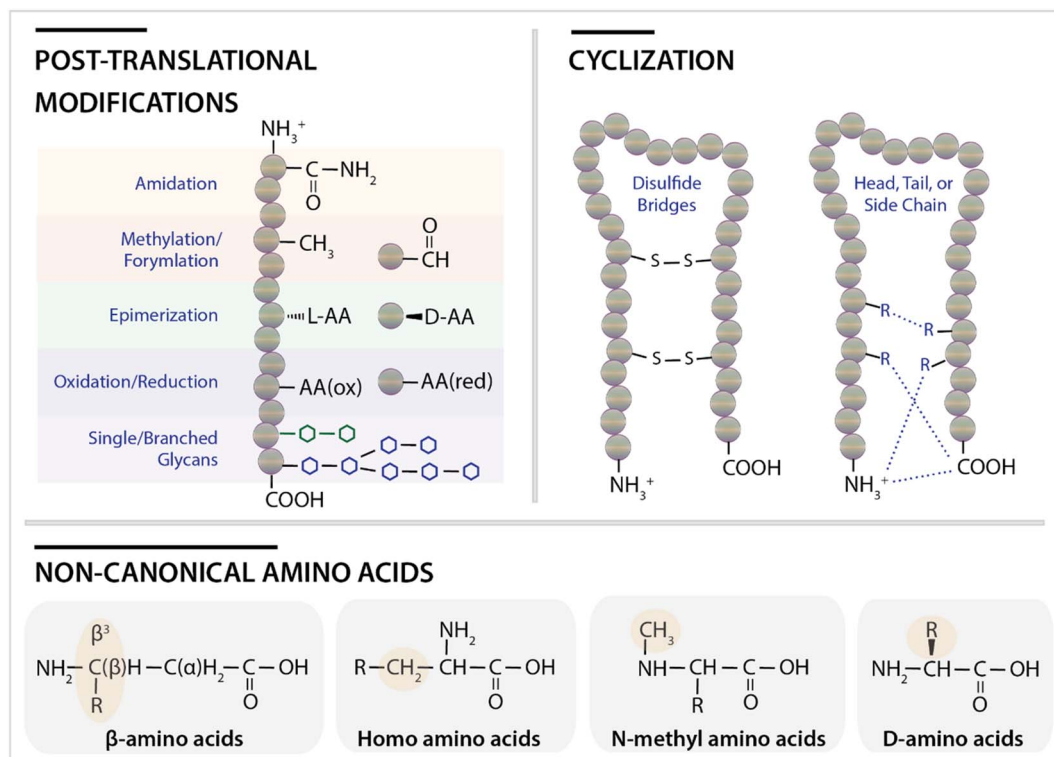This journal is © The Royal Society of Chemistry 2025

Fig. 1 Natural product bioactive peptides challenge traditional characterization methods with extensive post-translational modifications (top, left), head, tail, and/or sidechain cyclization (top, right), and the incorporation of non-canonical amino acids (bottom).

legitimate RiPP sequences.[6] Efforts to characterize and annotate NRPS BGCs have resulted in increasingly intelligent bioinformatic tools for the prediction of mature NRP structures, however, promiscuity in NRPS enzymes hinders the use of genome mining for complete NRP structure prediction. While RiPPs and NRPs hold the capacity for unique and highly-specialized chemistries attractive to medicinal and agricultural biotechnology, the discovery and characterization of new active molecular species is limited by their complexity, indeterminate variability, and unpredictability beyond a finite genetic script.

Alternatively, mass spectrometry (MS) is a dynamic platform with the speed and sensitivity required to analyze highly variable and previously uncharacterized natural product extracts[7] for the detection of mature bioactive peptides with or without genomic or transcriptomic information. Traditional MS characterization methods are challenged by combinatorial additions of post-translational modifications, generating heterogenous molecular populations and increasing source material complexity while decreasing the abundance of any given peptidoform.[8] As such, legacy instruments with

conventional fragmentation modes, *e.g.*, collision-induced dissociation, and basic data processing are generally limited to the characterization of less complex bioactive peptides, merely scratching the surface of the full repertoire of elusive natural product NRPs and RiPPs. Modern technologies, however, sport increasingly high resolving power, innovative multistage fragmentation methods, and can be coupled with refined bioinformatic strategies for powerful peptidomic analyses; the discovery of novel bioactive peptides relies on these advances in MS proteomics to detect and characterize novel molecular species independent of genetic predictions or to complement bioinformatic 'Omics' approaches.

2. Gas—phase technology to elucidate difficult-to-characterize NRPs and RiPPs

The modularity and versatility of multistage mass spectrometry (MS^n) with increasingly accessible high resolving power instruments allows access to data-rich sequence information

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and enables more facile *de novo* peptide sequencing, driving the characterization of natural product peptides in challenging matrices (Fig. 2). Considering the speed, sensitivity, and selectivity of different mass spectrometers, MS^n schema can be tailored to answer specific experimental questions, from untargeted, discovery-based screening to targeted, highly quantitative analyses. Recent peptidomic work profiling toad venoms, used in traditional Chinese medicine practices, demonstrated a hybrid untargeted/targeted LC-MS/MS approach wherein toad venom peptides were identified on a Quadrupole Time-of-Flight (Q-TOF) mass spectrometer and characterized with *de novo* sequencing software.[9] Subsequent quantification of select peptide markers on a Quadrupole-Linear ion-trap mass spectrometer (Q-Trap) *via* Multiple Reaction Monitoring (MRM) and a partial Least Squares Discriminant Analysis (PLS-DA) enabled the characterization of

numerous, diverse venoms. In contrast, a study focused on deeper proteome coverage of the previously uncharacterized Montpellier snake venom employed shotgun proteomics, a 'bottom-up' approach where protein identities in complex samples are inferred from the analysis of enzymatically-digested source material and its corresponding MS^2 spectra. High resolution analysis of the snake venom with a hybrid quadrupole-Orbitrap instrument and subsequent automated *de novo* sequencing revealed 42 protein families with numerous cysteine-rich and post-translationally modified sequences.[10]

Developments in MS^n enable the sequence elucidation of peptides containing structural isomers and populations of complex post-translationally modified peptides inaccessible to traditional mass spectrometric methods. In the absence of genetic information, the discrimination of leucine/isoleucine residues has historically challenged MS-based sequencing of
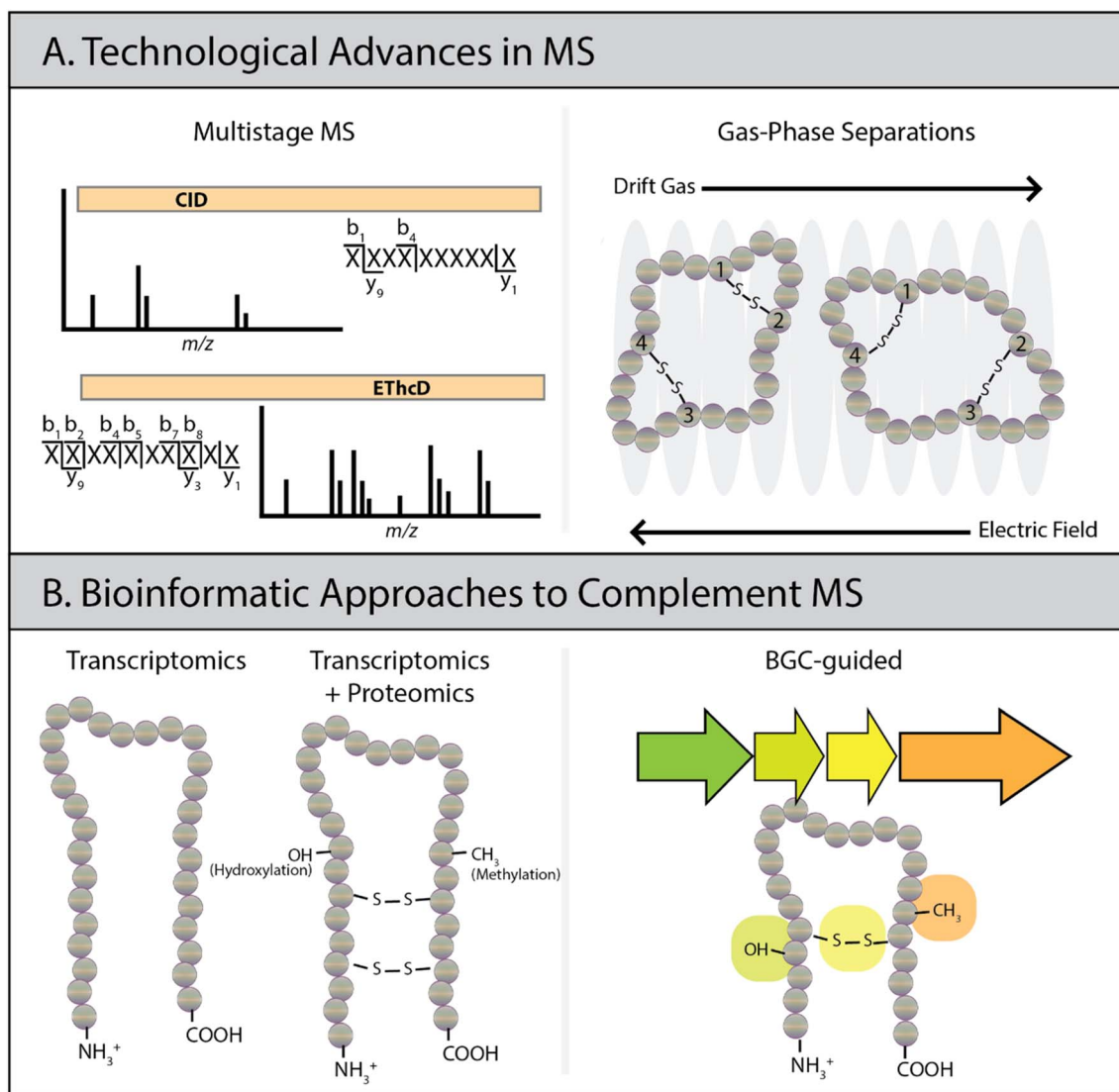

Fig. 2 (A) Technological advances in mass spectrometry, such as innovations in multistage MS (left) and gas–phase separations (right), enhance sequencing capabilities of novel bioactive peptide species and enable advanced separations based on structural conformations. (B) Bioinformatic 'Omics' methods, including transcriptomics (left) and biosynthetic gene cluster-guided approaches (right) complement proteomics for comprehensive RiPP and NRP discovery and characterization.

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peptide primary structure; inaccurate assignment of leucine/isoleucine can have detrimental effects on protein activity and specificity.[11] However, a hybrid multistage mass spectrometry (MS[3]) approach combining HCD (high-energy collisional dissociation) and ETD (electron transfer dissociation) was demonstrated to unambiguously distinguish leucine and isoleucine residues in proteins and peptides up to 3 kDa, and can be applied to increase the accuracy of *de novo* sequencing.[11] The enhanced peptide backbone fragmentation characteristic of less commonly available hybrid HCD/ETD, or EThcD, has also been leveraged for the *de novo* sequencing of difficult-to-sequence natural product peptides. In a botanical extract, a novel bioactive peptide, existing in multiple isobaric peptidoforms, challenged conventional characterization by CID fragmentation alone.[8] Analysis *via* EThcD revealed the incorporation of hydroxyproline variably at three different positions along the peptide backbone, enabling comprehensive sequence characterization. Glycopeptides present diverse, heterogeneous populations of covalent N- or O-linked complex carbohydrates and oligosaccharides. Traditionally, glycoproteomics has relied on *in vitro* enzymatic cleavage of glycans and subsequent mass spectral analyses of glycan chains and associated peptides separately. Although glycan composition can be ascertained, this strategy cannot localize glycan attachment sites and complicates analysis. To reduce data complexity and laborious sample preparation in large-scale *N*-glycopeptidomics, an EThcD fragmentation approach for high-throughput analysis of intact gylcopeptides was implemented, where both HCD and ETD fragmentation information is collected in a single spectrum.[12]

Less commonly implemented gas-phase separations, such as ion mobility, coupled to MS can reveal novel molecular species in complex matrices through multidimensional separations complementary to LC-based separations, *e.g.*, high-performance liquid chromatography (Fig. 2). Found in natural product NRPS and RiPPs,[13] D-amino acids are typically characterized by nuclear magnetic resonance (NMR), requiring milligram quantities of highly purified material. Although standard methods are unable to discriminate enantiomers solely based on mass-to-charge measurements, increased access to commercially available ion mobility instruments has permitted the development of methods for enantioselection; recent work has demonstrated the use of a modified commercial miniature ion trap to break the chiral symmetry of sugars, amino acids, and small molecule drugs[14] with promising future applications to peptides. Additionally, the ability of ion mobility to resolve analytes by collisional cross-section allows for the differentiation of disulfide-rich peptide conformers[15] common in natural products, the characterization of which is essential when evaluating the impact of specific disulfide linkages on peptide bioactivity. In a recent study, the highly complex and dynamic peptidome generated by a germinating seed and its microenvironment was profiled on a commercially available hybrid TIMS (Trapped Ion Mobility Spectrometry)-TOF instrument. A comprehensive analysis of germinating *Phaseolus vulgaris* seed, the common bean, examined peptide variability among eight bean genotypes, identifying >3000 peptides and laying the groundwork for future investigation of bioactive seed-exuded peptides.[16]

3. 'Omics' complement mass spectrometry for NRP and RiPP identification and characterization

Venoms are proteinaceous mixtures rich in highly-targeted, potent peptide toxins[17] with proven applications in the biomedical[18] and agrotechnology fields.[19,20] Transcriptomics have been used to predict the presence and differential expression of antimicrobial peptides in animals without prior peptidomic analysis;[21] despite significant advances in bioinformatics, extensive post-translational modifications common to venom peptides, specifically multiple disulfide bonds, hydroxylation, methylation, amidation, and cyclization (Fig. 1) challenge full venom peptidome characterization solely by genetic/transcriptomic prediction. As such, high-resolution MS has been used in tandem with transcriptomics to identify bioactive peptides in a number of systems. For example, novel pilosulin-like peptides were discovered in ant venom that were undetected with transcriptomic analysis alone.[22] High-resolution MS coupled with transcriptome-assisted *de novo* sequencing deciphered a suite of histone-derived bioactive peptides in Komodo dragon plasma.[23] In cone snail venom, a similar approach revealed >3000 novel conotoxin precursors, 16 previously unknown toxin gene superfamilies, and six cysteine-knot motifs never before found in cone snails.[17] A novel linear, highly stable cationic AMP, LC-AMP-I1, was discovered in Chinese Wolf Spider venom through complementary transcriptomic and proteomic analyses: a cDNA library of the venom gland of *L. coelestis* was compared to a MALDI-TOF top-down analysis of fractionated venom.[24]

The identification of biosynthetic pathways and machinery can guide the discovery of previously unknown sequences, structures, and post-translational modifications. In RiPPs, NRPs, and hybrid NRP-PKSs, cellular machinery aids or fully orchestrates the synthesis of structurally- and functionally-diverse bioactive peptides *via* backbone or side-chain cyclization, single, heterogenous, or branched post-translational modifications, and the incorporation of non-proteinogenic amino acids and chimeric glycan side chains. Minimally, MS can be used to deduce unpredictable mature peptide products from BGCs with unknown functions.[25] When available, genetic information can be paired with MS for the identification of BGCs to guide bioactive peptide discovery, where numerous bioinformatic platforms are available to facilitate large data analysis. Millions of mass specta in the Global Natural Products Social (GNPS) were searched with Metaminer, a spectral networking tool that integrates natural product MS and metagenomic datasets for RiPP discovery and tolerates unknown modifications, against eight genomic datasets; MetaMiner identified 38 known and unknown RiPPs from diverse sources.[26] Interpretation of bacterial genomic data with AntiSMASH, a BGC homology-based tool for the identification of NRPs/PKs and novel BGCs, yielded six RiPP, NRP, and PKS BGCs,

enabling the prediction and subsequent mass spectrometric characterization of mature bioactive peptides.[27] Non-ribosomal peptide discovery from only four GNPS MS datasets and their associated genomes with NRPminer, a modification-tolerant tool that mines non-canonical NRPS assembly lines, identified four novel non-ribosomal peptide families and 180 NRPs.[28] HypoRiPPAtlas, a machine-learning, genomics-derived 'atlas' of predicted natural product sequences compared *in silico* with MS data for RiPP discovery and prediction, searched 46 GNPS MS datasets and identified numerous bioactive RiPPs and a novel post-translational modification.[29] Additionally, MS can provide mechanistic and structural insights into BGCs; transcriptomics targeting BURP-domain peptide-motifs revealed a novel bicyclic peptide cyclase, and the mechanism of catalytic activity was monitored with bottom-up proteomics.[30,31]

4. Conclusion

Natural product RiPPs and NRPs are constructed from ribosomal or enzymatically-synthesized backbones studded with combinatorial additions of post-translational modifications that often elude traditional characterization methods. Advanced MS instrumentation, sophisticated bioinformatic platforms, and BGC-guided discovery aid the identification and characterization of complex non-proteinogenic and highly modified peptides. This technology, however, is not without limitations. It is worth noting that despite the wealth of information generated by these recent innovations in MS and adjacent technologies, detected RiPPs and NRPs do not necessarily exhibit potent or specific bioactivities against desired biological targets. As substantial resources are required for full characterization of natural product peptide primary sequence and post-translational modifications, bioactivity measurements can be coupled with MS-based RiPP and NRP identification to guide discovery efforts towards peptides with targeted bioactivities. Bioassay-guided fractionation is a common strategy for reducing natural product extracts to subsets of components likely responsible for extract bioactivity and simplifying subsequent MS analysis. However, this method is often slow, expensive, and can result in peptide degradation over numerous rounds of fractionation, and thus loss of bioactivity[32] – in addition to the re-identification of known bioactive peptides. To streamline the natural product peptide discovery process and prioritize the discovery of novel peptides with targeted bioactivities, methods have been developed wherein activity-driven screening identifies bioactive species by statistically correlating high resolution MS with molecular networking[33] or bioactivity measurements.[34] This latter pipeline is highly adaptable with the ability to incorporate a variety of natural product source materials and screen against numerous pathogens in any format of bioactivity assay.[35,36]

Beyond bioactive peptide discovery from isolated natural product extracts *in vitro* as discussed herein, further characterization of bioactive peptides *via* the complex relationships among peptide structure and function, localization within source material, and cellular/protein targets can be probed through additional, sophisticated MS strategies. Hydrogen/

Deuterium eXchange Mass Spectrometry (HDX-MS) monitors hydrogen/deuterium exchange kinetics to deduce protein structure and conformational dynamics, *e.g.*, *cis–trans* isomerization of the protein backbone. Antimicrobial peptides often exert bactericidal effects through the disruption of microbial membranes; HDX-MS can be used to examine the interactions and structural changes of proteins upon membrane recruitment[37] and may be leveraged in future studies to examine the mode of action of membrane-acting peptides. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has been implemented to spatially localize endogenous peptides in botanical tissues,[38] with the potential to further our understanding of the functionality of bioactive peptides within its source organism. Cross-linking mass spectrometry (XL-MS) is a maturing technique where interacting proteins are covalently linked prior to MS analysis, and has promising future applications in the elucidation of interactions among bioactive peptides, intracellular protein interactions, and mechanism of action.[39] As a standalone technique or in tandem with powerful 'Omics' strategies, mass spectrometry is a rapidly-evolving, dynamic platform with diverse applications across the discovery, primary sequence identification, and structural characterization of natural product bioactive peptides.

5. Conflicts of interest

There are no conflicts to declare.

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