Dereplication, Sequencing and Identification of Peptidic Natural Products: from Genome Mining to Peptidogenomics to Spectral Networks

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Dereplication, Sequencing and Identification of Peptidic Natural Products: from Genome Mining to Peptidogenomics to Spectral Networks

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Abstract

While recent breakthroughs in discovery of peptide antibiotics and other Peptidic Natural Products (PNPs) raised the challenge of developing new algorithms for their analysis, the computational technologies for high-throughput PNP discovery are still lacking. We discuss the computational bottlenecks in analyzing PNPs and review recent advances in genome mining, peptidogenomics, and spectral networks that are now enabling the discovery of new PNPs via mass spectrometry. We further describe the connections between these advances and the new generation of software tools for PNP dereplication, *de novo* sequencing, and identification.

Contents

1 Introduction

The golden age of antibiotics, that started in the 1940s, was followed by a decline in the pace of antibiotics discovery in the 1990s[1]. However, antibiotics and other natural products (including immunosuppressive, antiproliferative, herbicidal, insecticidal, fungicidal and antiparasitic drugs) are again in the center of attention as exemplified by the recent discovery of teixobactin[2,3]. Recent launch of the Global Natural Products Social (GNPS) Molecular Networking project[4] (the first large community project in natural product discovery) brought together over a hundred laboratories that have already generated over a billion mass spectra of natural products. While these spectra represent a gold mine for future antibiotics discovery (over 70 million of them are publicly available), their interpretation remains a challenging computational problem.

While first computational methods for analyzing mass spectra of small molecules were developed in the 1960s[5–8], three decades before their proteomics counterparts[9,10], computational mass spectrometry of small molecules is often viewed as a more complex (and less mature!) field as compared to computational proteomics[11,12]. See[12–16] for recent reviews of computational approaches to analyzing small molecules. Depending on their building blocks, natural products are classified into a variety of chemical classes that include Peptidic Natural Products (PNPs), the focus of this review. Starting from penicillin, PNPs have an unparalleled track record in pharmacology: many antibiotics, antiviral and antitumor agents, immunosuppressors, and toxins are PNPs.

While recent breakthroughs in PNP discovery[2,17,18] raised the challenge of developing new algorithms for dereplication, *de novo* sequencing and identification of PNPs, the computational technologies for high-throughput PNP discovery are still lacking. The traditional process of PNP discovery is to elucidate structure of the compound by chemical assays (such as Nuclear Magnetic Resonance) and association of the chemical compound to its biosynthetic gene cluster by genome manipulations. This process is time-intense, laborious, and requires large amounts of highly purified material. Moreover, rather than discovering novel PNPs, it often rediscovers known PNPs resulting in wasted efforts.

Recently, mass spectrometry (MS) has become a cheap, fast, and reliable complementary approach for the traditional PNP discovery techniques[19,20]. However, compared to traditional applications of MS in proteomics, application of MS for PNP discovery faces additional computational challenges due to higher complexity of the compounds and unusual fragmentation patterns. Some of these challenges are now addressed through *genome mining*, *peptidogenomics*, and *spectral networks*:

- **Genome mining.** Sequencing many bacterial and fungal genomes in the last decade opened an era of genome mining for PNP discovery. Genome mining refers to using information about the biosynthetic genes (responsible for synthesizing a PNP) to infer information about the PNP itself. Discovery of coelichelin in *Streptomyces coelicolor* was one of the first successes of genome mining[21,22] that was followed by characterization of many PNPs from sequenced genomes.

- **Peptidogenomics.** Given a mass spectrum and a peptide database, peptide identification refers to finding a peptide in the database (or its variant) that generated the given spectrum. While peptide database in traditional proteomics consists of *known* peptides, peptide databases in peptidogenomics are often dominated by *putative* peptides derived via genome mining. Since many PNPs are not directly encoded in genomes, genome mining often fails to generate the database of putative PNPs that contains the exact amino acid sequence of a PNP corresponding to a given spectrum. Instead, it produces a database containing an error-prone template that makes matching spectra against such a template difficult. Therefore,

popular proteomics tools such as Sequest[9] and Mascot[10] fail to identify PNPs. Also, identification of spectra derived from PNPs is more difficult than traditional peptide identification in proteomics because many PNPs are non-linear peptides with extensive modifications that generate complex spectra (the standard proteomics tools fail to identify non-linear peptides).

- **Spectral networks.** Bandeira et al.,[23] introduced the concept of spectral networks (also known as *molecular networks*[18]) that reveal spectra of related compounds (without knowing what these compounds are) using *spectral alignment* algorithms[24,25]. Nodes in the spectral networks correspond to spectra while edges connect *spectral pairs*, i.e., pairs of spectra that are generated from related peptides (e.g., peptides differing by a single mutation or a modification). Spectral networks enable discovery of novel variants of known PNPs as well as novel PNP families. Thus, since most PNPs form families of related peptides[18]), spectral networks are ideally suited for analyzing PNPs.

PNPs are produced by two types of biosynthetic machineries: Non-Ribosomal Peptide Synthetase (NRP synthetase) [26,27] and Ribosomally synthesized and Posttranslationally modified Peptide synthetase (RiPP synthetase)[28,29]. NRP and RiPP synthetases produce Non-Ribosomal Peptides (NRPs) and Ribosomally synthesized and Posttranslationally modified Peptides (RiPPs), respectively. NRPs are widely distributed and biomedically important natural products that are not directly inscribed in genomes but instead are encoded by NRP synthetases using non-ribosomal code[30]. In addition to standard amino acids, NRPs often include non-proteinogenic amino acids such as ornithine. Known NRPs include hundreds of non-proteinogenic building blocks and some NRPs like kutznerides[31] are build entirely from non-proteinogenic amino acids. Since the non-ribosomal code remains poorly understood, accurate prediction of PNPs from NRP synthetases remains challenging.

While RiPPs are encoded in the genome, the genes encoding RiPPs are often short making it difficult to annotate them (short genes often evade gene prediction methods[32,33]). Moreover, RiPPs often have many unusual Post Translational Modifications (PTMs) making it difficult to identify them via MS. Heavily modified peptides with more than two *blind modifications* often evade identification algorithms such as InsPecT[25] and MODa[34] designed for discovery of unexpected PTMs.

Analysis of over 1000 bacterial genomes from the Joint Genome Institute (JGI) database revealed that 71% of them harbor at least one RiPP protein family (Pfam) domain and 69% harbor at least one NRP synthetase Pfam domain[17]. Recent analysis of 830 *Actinobacteria* genomes revealed that *Actinobacteria* encodes thousands of potential drug leads[35]. These and other studies[18] suggest that we only saw a tip of the iceberg with respect to PNP discovery and raise the challenge of developing new methods for PNP discovery.

Understanding how PNP biosynthetic machineries work is a prerequisite to genome mining and peptidogenomics that involve two steps; predicting the candidate gene clusters responsible for the synthesis of a PNP and connecting them to their chemical products by MS. However, connecting biosynthetic gene clusters to their products is a non-trivial task since the rules defining how a gene cluster specifies its products remain poorly understood. For example, the existing tools for predicting NRPs from NRP synthetases remain error-prone. The transition from a gene cluster to its product becomes particularly difficult in the case of modifications involved in maturation of PNPs. For example the gene cluster for coelichelin (NRP synthetase) was elucidated in 2000[21], but coelichelin itself (NRP) was sequenced only in 2005[22].

Below we review recent advances in genome mining, peptidogenomics, and spectral networks (**section 2**) and further describe PNP dereplication (**section 3**), PNP sequencing (**section 4**), and PNP identification (**section 5**). We remark that, in difference from dereplication (that reveal

known PNPs or their variants), PNP sequencing and identification may reveal previously unknown PNPs. **Figure 1** illustrates various approaches to PNP discovery.

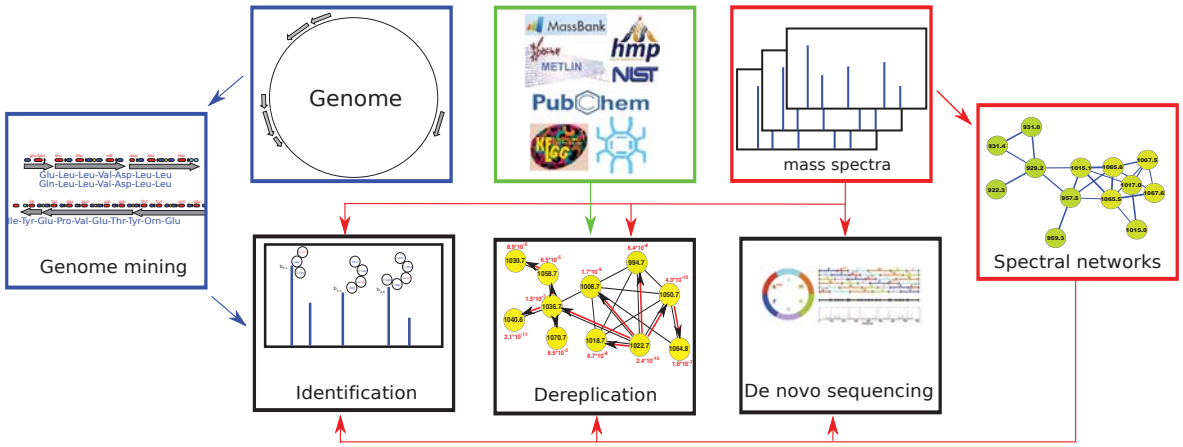


Figure 1: Three computational approaches to PNP discovery.

2 Genome mining, peptidogenomics, and spectral networks

2.1 Genome mining for PNPs

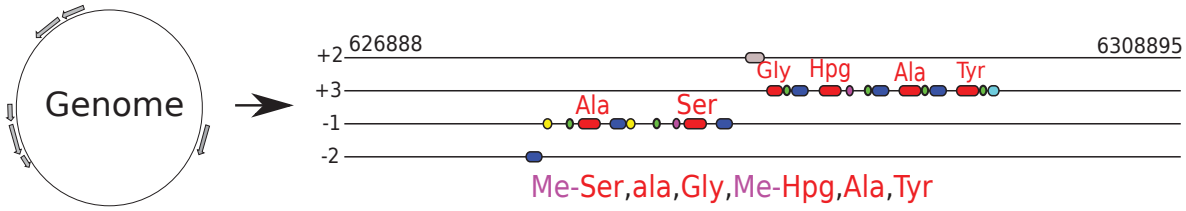
Genome mining tools for identification of NRP synthetase gene clusters and prediction of NRPs they produce include ClustScan[36], NP.searcher[37], NRPSpredictor[38], NRPSpredictor2[39], and antiSMASH[40–42]. For polyketide synthetase gene cluster predictors see [43–47]. **Figure 2** illustrates how NRP genome mining tools work. Medema et al.[48] recently developed Pep2Path genome mining tool that works for both NRPs (NRP2Path) and RiPPs (RiPP2Path) by matching *peptide sequence tags* in the spectra against the biosynthetic gene clusters that have the highest likelihood of generating PNPs containing these tags.

NRP synthetases are formed by an array of distinct modular sections, each of which is responsible for incorporation or modification of a single amino acid into the final NRP. Minimum of three domains are required for each NRP synthetase module, adenylation domain (A-domain), peptidyl carrier domain (PCP-domain) and condensation domain (C-domain). The A-domain is responsible for picking the specific amino acids that are to be incorporated into the NRP. Hundreds of different A-domain specificities have been classified, each one recruiting a specific amino acid. This allows us to determine the sequence of the putative NRP by looking at the order of A-domains along the assembly line and assigning a specific amino acid to each A-domain using the non-ribosomal code. However, since the non-ribosomal code is still poorly understood, the tools for defining specificities of A-domains remain error-prone. These tools often use *profile Hidden Markov Models (HMMs)* to align conservative amino acids within each A-domain (red amino acids in Figure 2(b)) against previously analyzed A-domains. The constructed alignment reveals variable amino acids within A-domains (purple amino acids in Figure 2(b)) that define the non-ribosomal code. The genome mining tools further use various machine learning technique to derive the amino acid in the NRP defined by the non-ribosomal code.

RiPPs are classified into more than 20 classes (such as lanthibiotics, thiopeptides, cyanobactins,

lasso peptides, and many others) based on structural and biosynthetic commonality[49]. Various software tools for RiPP genome mining have been reviewed in[50]. BAGEL, a genome mining tool for bacteriocins, revealed 150 putative lanthipeptide gene clusters[51,52]. ThioFinder, a genome mining tool for thiopeptides, predicted 53 novel thiopeptide producing gene clusters[53]. Recent genome mining studies predicted 79 lasso peptides[54] and 27 cyanobactin-producing *Anabaena* strains[55]. Development of RiPP genome mining tools is tied to construction of databases of known RiPPs, such as Bactibase, a database of 177 bacteriocins[56] or a bacteriocin database[51,52] consisting of 483 bacteriocins (236 class I, 160 class II and 93 class III as of August 2015). Other examples include Thiobase, a database of 39 thiopeptides[53], and MIBiG, a natural product structure and biosynthetic gene cluster repository with over 169 RiPPs from different classes[42]. Availability of these databases for diverse RiPP classes speeds up development of novel machine learning techniques aimed at genome mining for RiPPs[50].

AntiSMASH is one of the most popular genome mining tools for analyzing both NRPs and RiPPs as well as polyketides. AntiSmash pipeline includes the following steps : (i) genes are extracted or predicted from the genome using Glimmer3[32], (ii) biosynthetic gene clusters are identified using profile HMMs, (iii) biosynthetic gene clusters are annotated (iv) the core chemical structure of natural products are predicted based on the annotated gene clusters. Optionally, comparative analysis of the biosynthetic gene clusters can be done using ClusterBlast[40].



(a)

```
YAFDLGYTCMFPVLLGGGELHIVQKETYTAPDEIAHYIKEHGITYIKLTPSLFHTIVNTASFAFDANFESLRLIVLGGEKIIPIDVIAFRKMYGHTE-FINHYGPTEATIGA
-AFDVSAGDFARALLTGGQLIVCPNEVKMDPASLYAIIKKYDITIFEATPALVIPLMEYI-YEQKLDISQLQILIVGSDSCSMEDFKTLVSRFGSTIRIVNSYGVTEACIDS
IAFDASSWEIYAPLLNGGTVVCIDYYTTIDIKALEAVFKQHHIRGAMLPPALLKQCLVSA----PTMISSLEILFAAGDRLSSQDAILARRAVGSGV-Y-NAYGPTENTVLS
```

```
LTKVGHIG  Asp
VGEIGSID  Orn
AWMFAAVL  Val
```

(b)

Figure 2: (a) Predicting NRPs based on NRP synthetase analysis using tools such as NRPSpredictor2[39] and antiSMASH[40]. The following domains are shown: A-domains (red), PCP-domains (green), C-domains (blue), methylation domains (yellow), and thioester domains (purple). Note that different modules of the same NRP synthetase can appear in different frames. (b) Extracting signature sequences (non-ribosomal code) from A-domains (only a short segments of the A-domains are shown). Various A-domains have conserved residues (shown in red) that enable their accurate multiple alignment using profile HMMs. The non-ribosomal code postulates that certain amino acids in the resulting multiple alignment (shown in purple) define a single amino acid in the NRP loaded by this domain. The three A-domains shown here define 8 amino acid signatures LTKVGHIG, VGEIGSID, WMFAAVL corresponding to amino acids Asp, Orn, and Val, respectively. The 8 amino acid signature shown here represent a simplified representation of the non-ribosomal code, e.g., NRPSpredictor2 uses longer signatures to predict amino acids for each A-domain.

2.2 Peptidogenomics of PNPs

The key difficulties in peptidogenomics are that (i) many PNPs are non-linear peptides, (ii) many PNPs (all NRPs) are not directly encoded in the genomes, (iii) even when a PNP is encoded in a genome (all RiPPs), they often have many modifications making it difficult to identify them using standard MS/MS searches, and (iv) many PNPs are encoded in the alphabet of 100s building blocks rather than in the alphabet of 20 proteinogenic amino acids. Also, many PNPs fragment poorly due to multiple complex modifications and multicyclic structure. For example, spectra of RiPPs often feature very few peaks making it nearly impossible to identify them using conventional MS/MS database search tools.

Kersten et al., 2011[17] discovered many novel PNPs using a manual peptidogenomic approach for connecting PNPs to their biosynthetic genes and matching them against mass spectra. However, the manual peptidogenomics approach to PNP discovery, while useful[57], is somewhat limited in analyzing large spectral datasets (such as LC-MS/MS datasets from bacterial extracts) and complex patterns of modifications. Moreover, this approach relies on identifying long peptide sequence tags (4-5 amino acids) to reduce the search space[48]. Such long tags are often not available for multicyclic peptides such as lanthipeptides or for NRPs with non-standard amino acids. Also, since the manual approach does not provide estimates of statistical significance (a pre-requisite for analyzing large spectral datasets) an automated peptidogenomics software tool is needed.

Peptidogenomics is based on comparison of experimental spectra with the *theoretical spectrum* of a PNP. Various *bond disconnection* algorithms[58–64] generate a list of bonds between atoms in a compound (excluding hydrogens) and assign them the *breakage score* based on the likelihood of each bond being disconnected. The theoretical spectrum is constructed from masses and breakage scores of all substructures resulting from bond disconnections. Tools such as MetFrag[59] attempt to explain the peaks in the experimental spectrum using the likely substructures formed by disconnecting some bonds. The alternative machine learning approaches use large collections of MS/MS spectra for learning the rules governing MS/MS fragmentation process[65–67]. Alternative approaches to bond disconnetction algorithm have also been suggested[65–68].

Theoretical spectra of PNPs are formed by disconnecting only amide bonds (rather than all bonds)[69,70] (see **Figure 3**). Since the number of fragmented substructures grow quadratically with the PNP length (under the assumption that at most two amide bonds are disconnected), theoretical spectra of PNPs have large number of masses making it difficult to analyze them since only a fraction of these masses have counterparts in the experimental spectra. In spite of this complication, some studies used general metabolite dereplication tools to successfully dereplicate PNPs[35].

A *Peptide-Spectrum Match* (PSM) is a pair of a peptide and a spectrum with the same precursor mass (up to an error δ). In the context of PNP discovery, a *PSM score* is often defined as the number of peaks shared between a theoretical spectrum and an experimental spectrum. Given a spectrum, a peptide that forms a PSM with the highest score against this spectrum (among all peptides in a peptide database) is reported as a potential annotation of the spectrum.

It is well known in the context of traditional proteomics that PSM scores often poorly correlate with statistical significance of PSMs such as *p-values*[71]. This observation is greatly amplified for non-linear peptides since scoring PSMs formed by non-linear peptides is currently more primitive than scoring PSMs formed by linear peptides due to the lack of a large learning sample of PSMs formed by non-linear peptides.

To address this challenge, Ibrahim et al., 2013[70] proposed additional statistical measures to distinguish between correct and erroneous PSMs formed by PNPs in their iSNAP approach. Mohimani *et al.*,[72] developed the MS-DPR algorithm for computing p-values of PSMs formed by arbitrary

PNPs. MS-DPR addresses the problem of deciding whether a given spectrum was generated by a linear, cyclic, or branch cyclic peptide since it enables evaluation of statistical significance of peptides with diverse structures[72] (see **Figure 4**).

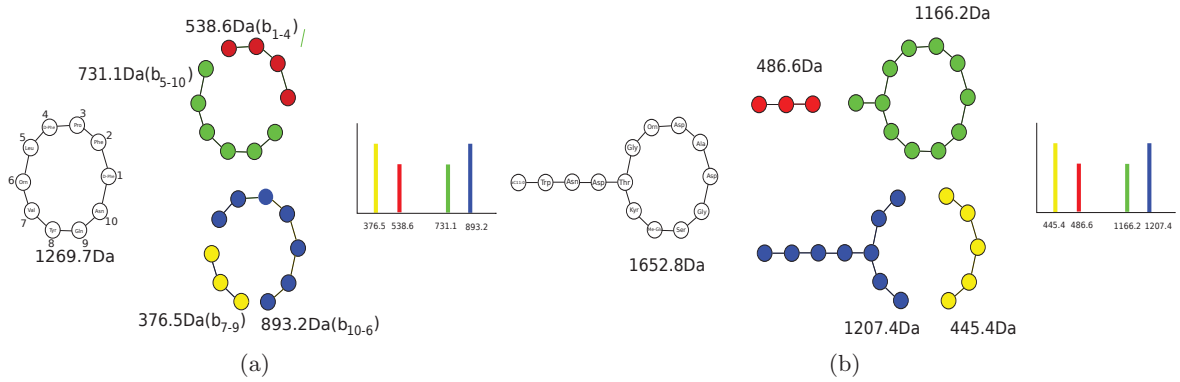


Figure 3: (a) Generating the theoretical spectrum of a cyclic (tyrocidine) and (b) branch cyclic (daptomycin) peptides. Only four out of $9 \cdot 10 = 90$ ($9 \cdot 10 + 4 \cdot 2 = 98$) theoretical peaks in tyrocidine (daptomycin) are shown. For a cyclic PNP of length n, the theoretical spectrum contains $n(n-1)$ masses. For a branch cyclic PNP with a cycle of length n and a branch of length m, the theoretical spectrum contains $n(n-1)+2m$ masses.

2.3 Spectral networks of PNPs

Spectral networks allow one to enlarge the set of identified PNPs (and sometimes get rid of incorrectly identified PNPs) by analyzing multiple spectra to simultaneously dereplicate, sequence, or identify *related* unknown peptides. The advantage of this approach (as compared to analyzing individual spectra) is that finding peptides that simultaneously explain all spectra in a spectral network may result in more accurate spectral interpretations. Thus, an *individual* PSM deemed statistically insignificant may become reliable in the context of multiple related PSMs revealed by a spectral network (and vice versa). Since most PNPs form families of related peptides, spectral networks can be used to reveal relationships between different spectra without knowing the amino acid sequences corresponding to these spectra.

Given a set of peptides $P_1, \dots P_m$, their *peptide network* is a graph with nodes $P_1, \dots P_m$, and edges connecting two peptides if they differ by a single amino acid modification. **Figure 5 (a)** shows the peptide network for nine variants of tyrocidine, a family of NRPs from *Bacillus brevis*[73]. For example, peptide 1 (tyrocidine B1) in this network (red node) is connected to four peptides differing from tyrocidine B1 by a single modification: tyrocidine A1 (peptide 2), tyrocidine B (peptide 5), tyrocidine C1 (peptide 8), and a previously unreported peptide with mass 1338.7 (peptide 9). However, it is not connected to peptides 3, 4, 6 and 7 since they differ from peptide 1 by multiple modifications. Six of these nine tyrocidines (1, 2, 3, 5, 7, 8) are contained in the database of putative NRPs generated by NRPSpredictor2 (without modifications) and three more differ from these variants by one or two modifications/mutations.

In reality, we are not given peptides $P_1, \dots P_m$ but only their spectra $S_1, \dots S_m$. Nevertheless, one can approximate the peptide network by constructing the spectral network on nodes $S_1, \dots S_m$ where spectra S_i and S_j are connected by an edge if they can be aligned against each other using *spectral alignment*[23,25,75]. **Figure 5** shows the peptide and spectral networks of nine tyrocidines and illustrates that the spectral network captures all edges of the peptide network. While the

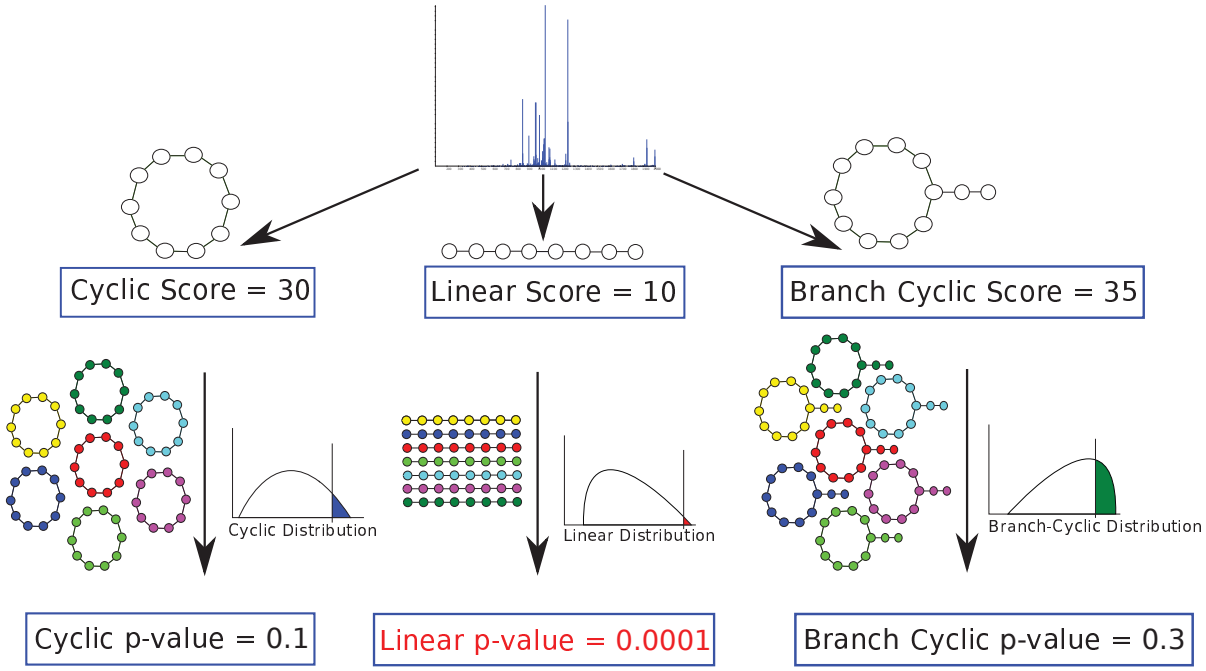
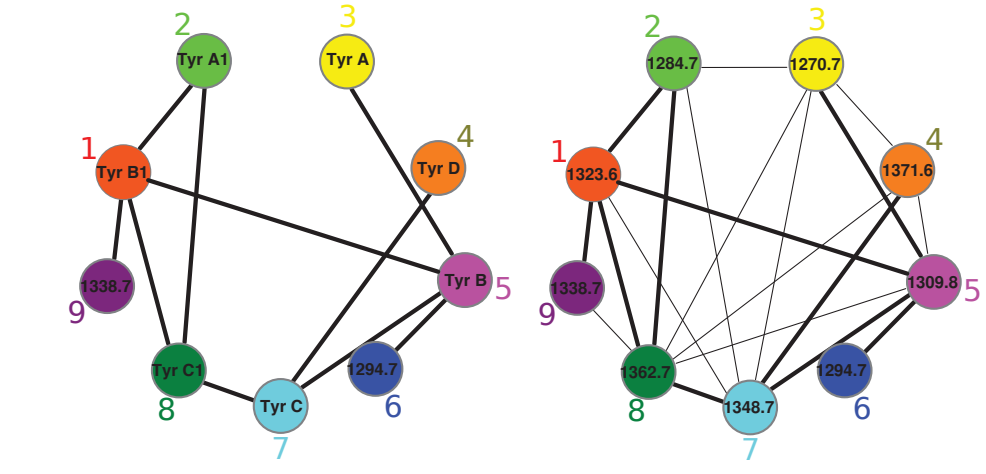


Figure 4: Deciding whether a peptide that produced a spectrum is linear, cyclic or branch cyclic. Given a spectrum, MS-DPR[72] considers various structure assumptions for a peptide that generated the spectrum (e.g. linear, or cyclic, or branch cyclic), and derives a p-value of PSMs resulting from each such assumption. For each structure, MS-DPR explores many putative amino acid sequences (shown by different colors) to estimate the p-value. If one of the structures results in a small p-value (e.g. linear structure with p-value of 0.0001 shown in red), that structure is accepted as the most likely structure for a given spectrum. Note that even though the linear peptide in this example has the lowest score, it is the most statistically significant among the three structures. The figure is reproduced from[72] by permission from ACS publications.

peptide and spectral networks in **Figure 5** are not identical, their shared edges usually allow one to interpret the peptides corresponding to the nodes of the spectral network using the *spectral network dereplication algorithm*[76]. The algorithm starts from a node with a known annotation in the spectral network, and propagates annotations from known to unknown peptides through the edges of the network.

3 PNP dereplication

PNP researchers face the challenge of maximizing the discovery of new compounds while minimizing the re-evaluation of already known PNPs. The process of using the information about the chemical structure of a previously characterized compound to identify this compound in an experimental sample (without having to repeat the entire isolation and structure-determination process) is called *dereplication*. In many cases, a PNP in the new sample is absent in the database of known PNPs, but its variant is present in this database with a modification. Identification of a PNP from its variants is called *variable dereplication*.



1 Phe-Pro-Trp-Phe	-Asn	-Gln-Tyr	-Val	-Orn(+14.7)	-Leu (p-value = 4.7e-16)
2 Phe-Pro-Trp-Phe(-38.3)	-Asn	-Gln-Tyr	-Val	-Orn(+14.1)	-Leu (p-value = 2.6e-12)
3 Phe-Pro-Trp-Phe	-Asn(-38.3)	-Gln-Tyr	-Val	-Orn	-Leu (p-value = 8.4e-09)
4 Phe-Pro-Trp-Phe(+39.8)	-Asn	-Gln-Tyr(+23.0)	-Val	-Orn	-Leu (p-value = 1.2e-10)
5 Phe-Pro-Trp-Phe	-Asn	-Gln-Tyr	-Val	-Orn	-Leu (p-value = 1.2e-10)
6 Phe-Pro-Trp-Phe	-Asn(-15.2)	-Gln-Tyr	-Val	-Orn(+32.8)	-Leu (p-value = 6.9e-10)
7 Phe-Pro-Trp-Phe(+39.8)	-Asn	-Gln-Tyr	-Val	-Orn	-Leu (p-value = 2.7e-12)
8 Phe-Pro-Trp-Phe(+39.0)	-Asn	-Gln-Tyr	-Val	-Orn(+14.7)	-Leu (p-value = 5.9e-15)
9 Phe-Pro-Trp-Phe	-Asn	-Gln-Tyr	-Val(+14.8)	-Orn(+14.7)	-Leu (p-vlaue = 1.6e-08)

(c)

Figure 5: The peptide network (a) and the spectral network (b) of tyrocidines[74]. The numbers within nodes represent precursor masses. Edges in the peptide network connect two peptides if they differ by a single amino acid modification. Shared edges between peptide and spectral networks are shown by thick lines. For example, peptide 1 (tyrocidine B1) in this network (red node) is connected to four peptides differing from tyrocidine B1 by a single modification: tyrocidine A1 (peptide 2), tyrocidine B (peptide 5), tyrocidine C1 (peptide 8), and a previously unreported peptide with mass 1338.7 (peptide 9). However, it is not connected to peptides 3, 4, 6 and 7 since they differ from peptide 1 by multiple modifications. In part (c), annotation of each node in the spectral network is shown. The spectral network revealed two novel tyrocidine variants at masses 1294.7 and 1338.7. The figure is reproduced from [74] by permission from ACS publications.

3.1 Dereplication via chemical databases

Development of chemical structure databases such as PubChem[77] (≈60 million compounds, as of August 2015), ChemSpider[78] (≈34 million compounds, as of August 2015), mzCloud[63] (≈3 thousand compounds), KEGG[79] (≈16 thousand compounds), MetaCyc[80] (≈10 thousand compounds), Norine[81] (≈1000 compounds), MIBiG[82] (≈1200 compounds with biosynthetic gene cluster), and AntiMarin, the result of a merger between AntiBase and MarinLit databases[83] (≈60 thousand compounds) paved the way for development of bioinformatics tools for natural product dereplication. However, the number of PNPs in these databases remains limited, e.g., AntiMarin contains only 3462 compounds with more than five amide bonds.

Ng *et al.*,[69] proposed the first method for dereplication of cyclic PNPs. Ibrahim *et al.*,[70] proposed an alternative dereplication approach, iSNAP, that is not limited to cyclic NRPs but extends to branch cyclic and linear peptides.

iSNAP analyzes each spectrum using the following steps: (i) identify all amide bonds for each NRP in the chemical database, (ii) generate theoretical spectrum for each NRP by cleaving at most

two amide bonds at a time, (iii) generate PSMs formed by the experimental spectrum and all NRPs in the database whose mass matches the precursor mass of the spectrum, (iv) score resulting PSMs, estimate their statistical significance, and report statistically significant PSMs.

3.2 Dereplication via spectral libraries

Since some natural products feature atypical fragmentation patterns[84], their experimental spectra have low scores against their theoretical spectra. In such cases, instead of dereplication via search in chemical databases, researchers search *spectral libraries* of natural products by comparing the experimental spectrum of interest against previously identified spectra. Development of large metabolite spectral databases such as mzCloud[63] (≈200 thousands spectra), NIST[85] (≈120 thousand spectra), METLIN[86] (≈55 thousand spectra), MassBank[87] (≈36 thousand spectra), HMDB[88] (≈1000 human metabolite spectra), and GNPS spectral library[4] (≈1600 natural product spectra) enabled MS/MS library searches for metabolites[84,89–95].

While dereplication via the spectral library search is more accurate than dereplication via search in a chemical database, the spectral libraries still contain only a fraction of PNPs present in chemical databases, e.g., as of August 2015, only 81 out of 1607 annotated spectra in GNPS Molecular Networking dataset[4] represented PNPs. Therefore, applications of spectral libraries to PNP dereplication remain limited. For example, Milman and Zhurkovich[96] described dereplication of toxic NRPs based on a small spectral library consisting of only 263 spectra.

3.3 Dereplication via spectral networks

The spectral network approach to PNP dereplication analyzes *connected components* of a spectral network. In contrast to the traditional spectral library approaches that compare spectra with the same precursor mass, spectral networks reveals relationships between spectra with different precursor masses thus enabling analysis of PNP variants. As long as there is at least one annotated spectrum in a connected component of a spectral network, its annotation can be *propagated* to all spectra in this connected components[23]. Ng *et al.*,[69] and Mohimani *et al.*,[74,97] described variable PNP dereplication algorithms using spectral networks and identified many variants of previously known PNPs.

Watrous *et al.*,[18], constructed spectral networks of various bacterial extracts and dereplicated many PNPs using manual analysis of connected components in these networks. Various studies reported success in utilizing spectral networks for discovery of natural products[4,97–108].

For example, Mohimani *et al.*,[97] discovered a lanthipeptide informatipeptin as a doubly charged ion with mz 1065.5 using RiPPquest algorithm. This PNP belonged to a connected component of the spectral network and was connected with three doubly charged ions with mz of 929.2, 957.5, and 1015.1, Comparing the mass shifts between these ions and informatipeptin provided a hint that these peptides are N-terminal derivatives of informatipeptin. While the three resulting PSMs had borderline statistical significance and RiPPquest did not report them as significant discoveries, the fact that they clustered with informatipeptin in the spectral network provided evidence that they are indeed N-terminal derivatives of informatipeptin (**Figure 6**).

4 PNP sequencing

While availability of genome sequences enables PNP discovery via genome mining, many PNPs are produced by difficult-to-cultivate organisms whose genomes are still unknown. If a genome is

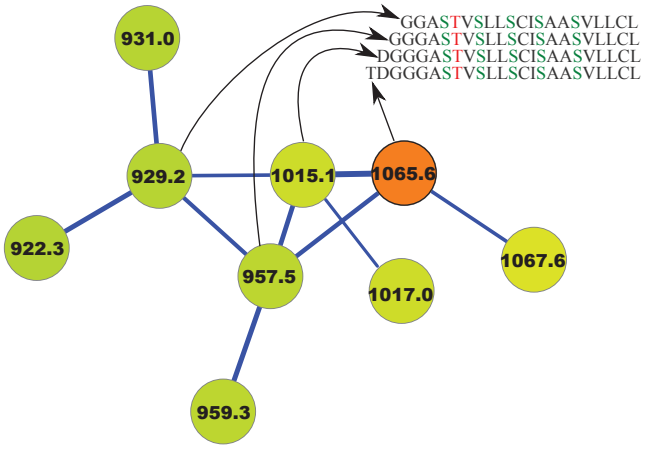


Figure 6: Spectral network analysis leads to variable dereplication of RiPP informatipeptin (shown in orange) into 3 variant PNP[97]. Ser → Dha and Thr → Dhb conversions in this lanthipeptide are shown in green and red, respectively. The figure is reproduced from[97] by permission from ACS publications.

unavailable and if the dereplication of a PNP fails, *de novo* sequencing[69,76,109,110] remains the last resort.

Allmer *et. al.*[111] recently reviewed various approaches to *de novo* sequencing of linear peptides. However, while dozens of tools for *de novo* sequencing of linear peptides have been proposed[112–115], techniques for *de novo* sequencing of non-linear peptides are still at the early stage of development. Ng *et al.*,[69], proposed the first algorithm for sequencing of cyclic peptides that however works only for very well-fragmented spectra. Novak et al.,[116] recently developed CycloBranch that takes advantage of high resolution mass spectrometry to improve the accuracy of *de novo* sequencing of cyclic, branched, and branch cyclic peptides.

Mohimani *et al.*,[76] developed *multiplex de novo peptide sequencing* algorithm for the case when spectra of multiple related peptides are available. Multiplex peptide sequencing starts from constructing the spectral network and identifying clusters of related compounds (connected components in the spectral network). It further attempts to sequence *all* compounds in each connected component (see **Figure 7**). In difference from PNP dereplication via spectral networks (when at least one spectrum in the connected component represents a known compound), *de novo* PNP sequencing works even when all nodes in the connected component represent unknown compounds. The advantage of spectral networks for PNP sequencing is that finding PNPs that *simultaneously* explain all spectra in a connected component of a spectral network results in a more accurate approach than sequencing each individual spectrum. When tandem mass spectrometry (MS^2) fails to sequence a PNP, one can attempt multistage (MS^n) mass spectrometry and apply *multistage de novo peptide sequencing* approach[109,117] (see **Figure 8**).

5 PNP identification

For both RiPPs and NRPs, the PNP identification consists of the genome mining step for detecting the biosynthetic gene clusters and their putative PNPs, and the peptidogenomics step for identifying a spectrum that matches one of the putative PNPs and finding modifications in this putative PNP. Below we describe these steps for RiPP identification and NRP identification.

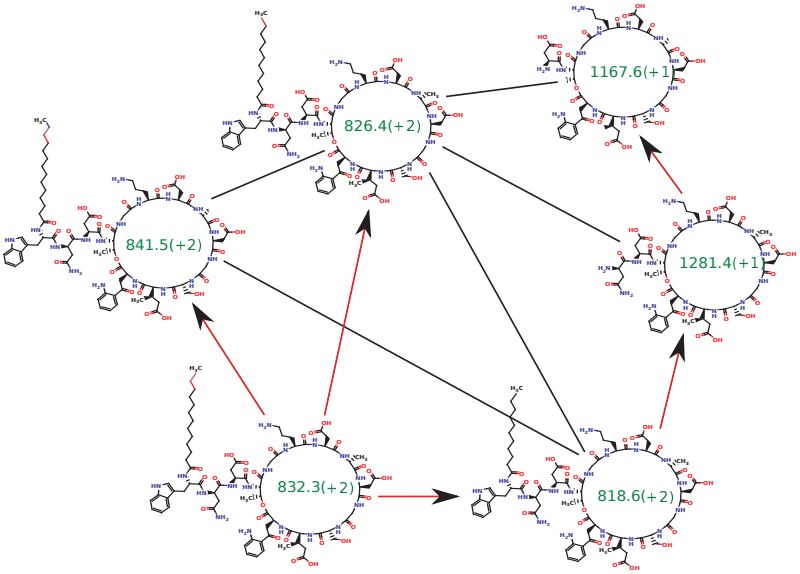


Figure 7: The *spectral network dereplication algorithm* from[76] attempts to *de novo* sequence all spectra in a spectral network in a coordinated fashion. It starts from a putative interpretation of one of the spectra (bottom left node) and propagates this interpretation to other nodes using red edges. The propagation typically fails if the initial putative interpretation is incorrect and succeeds if it is correct. Thus, the propagation process allows one to reject the incorrect initial interpretations. The spectral network dereplication algorithm generates many putative interpretations of the spectrum and propagate them through the spectral network in an attempt to decide which one is correct.

5.1 RiPP identification

A RiPP biosynthetic gene cluster usually includes a gene encoding a single *core peptide* and several genes encoding modification enzymes that are responsible for conversion of the *core peptide* to *mature peptide*. The standard MS/MS database search tools are limited with respect to identification of complex RiPPs with more than two modifications. This limitation makes them inadequate for analyzing such RiPPs as lanthipeptides that often have more than five modifications. Moreover, even if these tools were able to efficiently search for peptides with more than two modifications, the resulting PSMs often would not be reported as statistically significant since many RiPPs are poorly fragmented (due to presence of multicyclic modifications). Since search for multiple variable modifications is statistically equivalent to the search in a huge virtual database of all modified peptides, it often results in a high false discovery rate (FDR) even for microbial organisms with small proteomes[118].

Even when the core RiPP sequence is known and the types PTMs in a RiPP can be predicted, multiple possible PTM sites typically result in thousands of structures that are difficult to analyze. Due to this complications, computational approaches to RiPP identification did not keep pace with rapid progress in RiPP discovery in recent years. Cycloquest[119], a tool for RiPP identification, is limited to cyclic peptides with very few modifications. Also, since Cycloquest does not take advantage of genome mining, it is unable to identify poorly fragmented peptides (e.g., lanthipeptides).

Genome mining is crucial for the success of RiPP identification efforts. The statistical significance (E-values) of the found PSMs deteriorates with the increase in the size of the protein

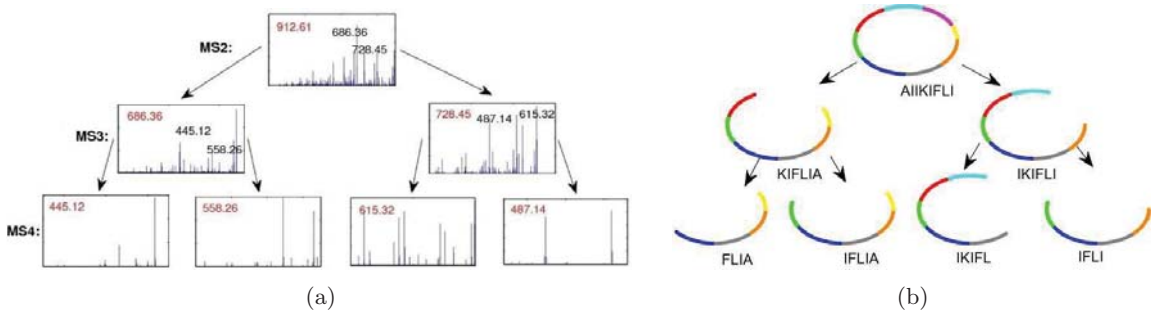


Figure 8: Illustration of an algorithm for peptide sequencing by multistage mass spectrometry. For each candidate peptide, multistage peptide sequencing algorithm scores how well each subpeptide is explained by MS^n data[109]. The figure is reproduced from[109] by permission from Wiley publications.

database. Thus, one way to make PSMs formed by poorly fragmented spectra statistically significant is to reduce the *effective* size of the protein database. Fortunately, most RiPPs appear in small windows of ≈20,000 nucleotides around biosynthetic gene clusters, and these clusters can be identified by searching for conserved biosynthetic enzymes. Thus, limiting the search space to this small region of the genome has the potential to reduce the E-values of found PSMs by orders of magnitude thus separating them from false PSMs.

RiPPquest[97] is a RiPP database search tool that addresses these complications and uses a more involved pipeline than peptide identification tools in traditional proteomics (compare **Fig. 9(a)** with **Fig. 9(b)**). While RiPPquest is currently limited to lanthipeptide analysis, it can be extended to other RiPP classes as soon as (i) it implements a genome mining rational for a specific RiPPs class, and (ii) it implements a biosynthetic rationale for transforming core into mature peptide for a specific RiPP class.

Zhang et al.[120] recently developed the Hypothetical Structure Enumeration and Evaluation (HSEE) algorithm for RiPP identification and applied it for identification of the lanthipeptide prochlorosin. HSEE is based on matching spectra against a collection of hypothetical structures predicted based on the biosynthetic gene cluster. HSEE generates a theoretical spectrum for each hypothetical structure and scores structures based on the shared peak count between the theoretical and experimental spectrum. The structure with the highest score is reported as a putative interpretation of an experimental spectrum.

We illustrate the PNP identification pipeline using RiPPquest[97] that includes the following steps: (i) identifying RiPP synthetases in the genome, (ii) extracting candidate open reading frames (ORFs) in a window around the gene cluster, (iii) adding proper modifications, (iv) matching spectra against the database of putative RiPPs and computing p-values of resulting PSMs, and (v) refining and enlarging the set of identified RiPPs using spectral networks (**Fig. 9(b)**). Below is a brief description of RiPPquest pipeline:

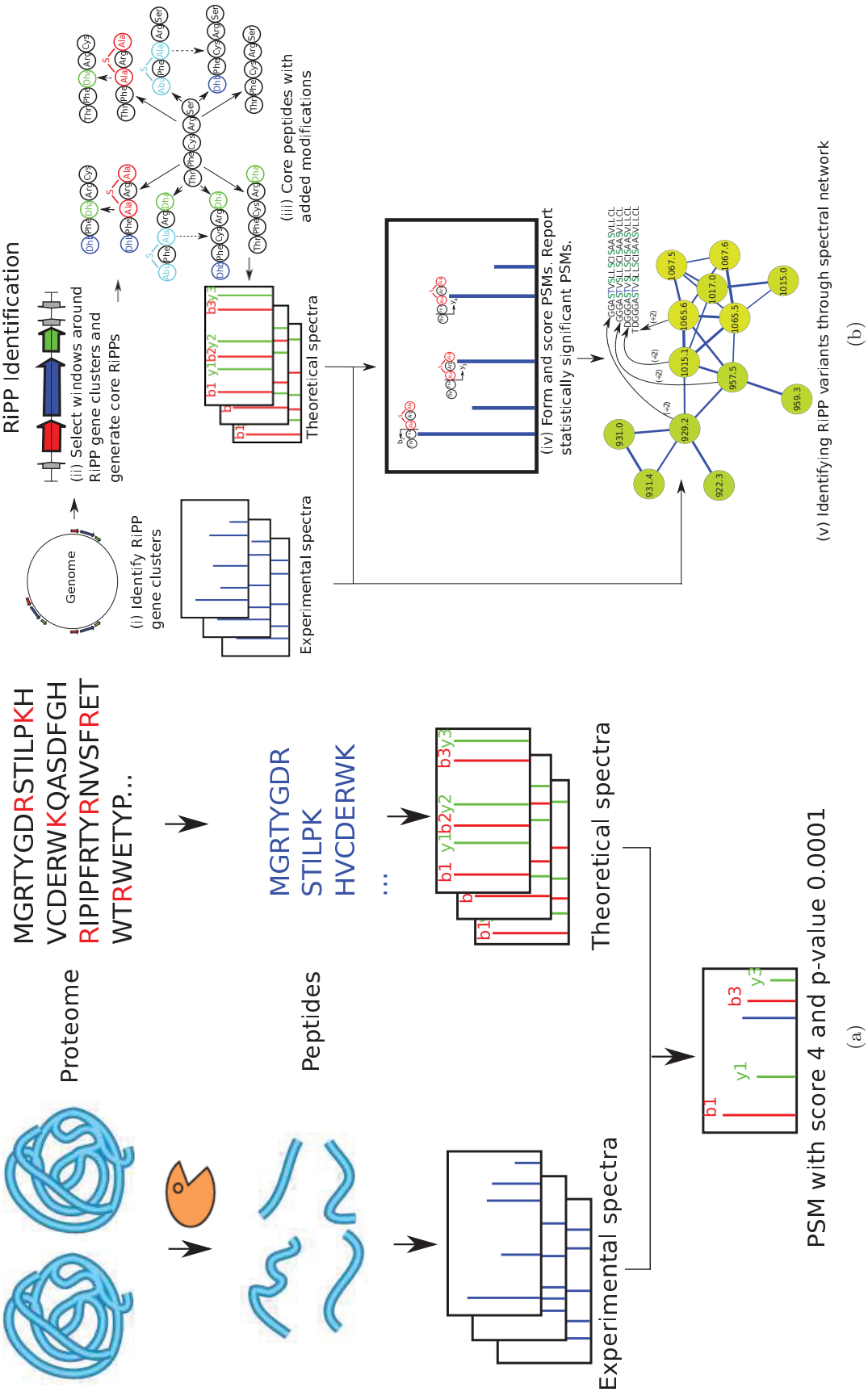


Figure 9: (a) Standard proteomic database search tools (e.g., Sequest[9]) are based on digesting the proteins by an enzyme, and collecting tandem spectra of the resulting peptides. Each spectrum is matched against theoretical spectra of all peptides in a protein database and PSMs with highest scores/lowest p-values are reported. (b) RiPPquest[97] includes the following steps : (i) identifying RiPP synthetases in the genome, (ii) extracting candidate ORFs in a window around the gene cluster, (iii) adding proper modifications, (iv) matching spectra against the database of putative RiPPs and and computing p-values of resulting PSMs, and (v) refining and enlarging the set of identified NRPs using spectral networks. The figure is reproduced from [97] by permission from ACS publications.

RiPPquest uses genome mining tools such as BAGEL[51,52], ThioFinder[53]), and antiSMASH[40–42,121] for identification of RiPP gene clusters. Limiting the search to small windows centered at RiPP gene clusters decreases the search space by two orders of magnitude as compared to the entire *Streptomyces* genome. Candidate core peptides are extracted from short ORFs found in these windows, and transformed to mature peptides according to the biosynthetic enzymes in the gene cluster. In the case of lanthipeptides, the most essential modifications are dehydration of serine and threonine, and formation of the lanthionine and methyl-lanthionine bridges[49].

Most classes of RiPPs form families of related peptides, making spectral networks helpful in RiPP analysis[18]. In particular, spectral networks revealed related lanthipeptides with stepwise N-terminal leader processing and different dehydration numbers[97] (see **Fig. 6**).

5.2 NRP identification

While genome mining techniques accurately identify NRP synthetases in the genome, accurate determination of specificities of A-domains remains difficult, especially for non-proteinogenic amino acids that are common in NRPs. While most NRPs go through modifications such as backbone macrocylization and addition of fatty acid chains, existing genome mining tools fail to predict most of these modifications. That is why NRP identification algorithms have to implement a *blind* MS/MS search that allows for multiple unexpected modifications and mutations. Blind searches refer to the case when the set of possible modifications is not restricted (different from typical searches for PTMs in traditional proteomics). This is a difficult computational problem even in the case of linear peptides[25,34], let alone non-linear peptides.

NRPquest[74] uses a genome sequence and a spectral dataset as an input and includes the following steps (i) identifying NRP synthetases in the genome, (ii) using non-ribosomal code to construct the database of putative NRPs generated by each NRP synthetase, (iii) matching spectra against the database of putative NRPs and computing p-values of resulting PSMs, and (iv) refining and enlarging the set of identified NRPs using spectral networks (**Fig. 10**).

NRPquest uses NRPSpredictor2[39] and antiSMASH[40–42] to identify NRP synthetases in the genome, and to predict the set of all possible amino acids generated by each A-domain. NRPquest further searches the genome for methylation domain, epimerization domain, and side chain bond formation domain, and accounts for the corresponding modification in the database of putative NRPs. Each spectrum is matched against each putative peptide in the database of putative NRPs using a brute force algorithm that allows for up to two blind modifications. These blind modifications account for possible inaccurate prediction of specificities of A-domains that are particularly common for non-proteinogenic amino acids and modifications. Similar to RiPPquest, NRPquest scores the PSMs using the scoring function from[114] and computes p-values using MS-DPR algorithm[72].

NRPquest constructs a spectral network[23] to refine and enlarge the set of identified PSMs. After constructing the spectral network, its connected components are extracted and the spectral network dereplication algorithm from Mohimani et al., 2011[76] is used for identification of all peptides represented by spectra forming this connected component. Moreover, the peptide propagation through the spectral network[23,69] allows one to identify peptides with many modifications that NRPquest missed during blind searches of individual spectra (since blind searches with more than two modifications are prohibitively time-consuming).

6 Discussion

Despite the important biomedical applications of PNPs, most pharmaceutical companies are now focusing on synthetic compounds and do not utilize the biosynthetic capacity of bacteria and fungi. However, the rise of high-throughput DNA sequencing has revealed a wealth of new PNP biosynthetic gene clusters in various genomes that exceeds the previous expectations by orders of magnitude. These new discoveries suggest that there may be a reversal of focus in pharmaceutical industry that could lead to revival in biomedical applications of natural products. Arguably, one of the key bottlenecks for accomplishing such a transformation is the shortage of computational tools for PNP discovery. Here we have reviewed recently developed approaches for PNP discovery and computational technologies (genome mining, peptidogenomics, and spectral networks) that enabled these methods. While these approaches have made a rather modest progress towards PNP discovery, further developments of the algorithms for PNP discovery may enable a systematic and high-throughput exploration of PNPs.

We described three approaches to PNP discovery with their own merits and limitations: PNP dereplication, PNP sequencing and PNP identification. For example, while PNP dereplication requires a chemical structure database to be available and can only identify known compounds and their variants, PNP identification requires the genome sequence to be available and can identify new compounds. The fragmentation quality of spectra required for success of these approaches is vastly different, as they perform searches in vastly different computational spaces.

While the search space for PNP dereplication is usually small, the search space for PNP sequencing is very large since it includes all peptides with a given mass. PNP identification, for both NRPs and RiPPs, has a search space that typically includes under a million putative peptides, standing in between PNP dereplication and PNP sequencing with respect to the search space. Thus, while PNP sequencing can succeed only with extremely high quality spectra, PNP identification can succeed with a medium quality spectra, and PNP dereplication can succeed even with poorly-fragmented spectra. Since PNP sequencing using a single tandem mass spectrum rarely succeeds, researchers have tried to utilize information from multiple spectra / multistage MS to overcome this limitation[23,76,109,117,122].

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References

[1] J. Li and J. Vederas, *Science*, 2009, **325**, 161–165.

[2] L. Ling, T. Schneider, A. Peoples, A. Spoering, I. Engels, B. Conlon, A. Mueller, T. Schberle, D. Hughes, S. Epstein, M. Jones, L. Lazarides, V. Steadman, D. Cohen, C. Felix, K. Fetterman, W. Millett, A. Nitti, A. Zullo, C. Chen and K. Lewis, *Nature*, 2015, **517**, 455–459.

[3] A. Harvey, R. Edrada-Ebel and R. Quinn, *Nat. Rev. Drug Discov.*, 2015, **14**, 111–129.

[4] M. Wang *et al.*, *Natrue Biotechnol.*, 2015.

[5] J. Lederberg, *Proc. Natl. Acad. Sci.*, 1965, **53**, 134–139.

[6] J. Lederberg, *ACM Conf. on the History of Medical Informatics*, 1987, 5–9.

[7] I. Mun and F. Mclafferty, *ACS Symposium Series*, 1981, **9**, 117–124.

[8] D. Smith, N. Gray, J. Nourse and C. Crandell, *Anal. Chim. Acta.*, 1981, **133**, 471–497.

[9] J. Eng, A. McCormack and J. Yates, *J. Am. Soc. Mass Spectrom.*, 1994, **5**, 976–989.

[10] D. Perkins, D. Pappin, D. Creasy and J. Cottrell, *Electrophoresis*, 1999, **20**, 3551–3567.

[11] J. Gasteiger, W. Hanebeck and K. Schulz, *J. Chem. Inf. Comput. Sci.*, 1992, **32**, 264–271.

[12] K. Scheubert, F. Hufsky and S. Bocker, *J. Cheminform.*, 2013, **5**, 12.

[13] S. Nuemann and S. Bocker, *Anal. Bioanal. Chem.*, 2010, **398**, 2779–2788.

[14] T. Kind and O. Fiehn, *Bioanal. Rev.*, 2010, **2**, 23–60.

[15] J. F. Xiao, B. Zhou and H. Ressom, *Trends Analyt. Chem.*, 2012, **32**, 1–14.

[16] A. Vaniya and O. Fiehn, *Trends Analyt Chem.*, 2015, **69**, 52–61.

[17] R. Kersten, Y. Yang, P. Cimermancic, S. Nam, W. Fenical, M. Fischbach, B. Moore and P. Dorrestein, *Nat. Chem. Biol.*, 2011, **7**, 794–802.

[18] J. Watrous, P. Roach, T. Alexandrov, B. Heath, J. Yang, R. Kersten, M. van der Voort, K. Pogliano, H. Gross, J. Raaijmakers, B. Moore, J. Laskin, N. Bandeira and P. Dorrestein, *Proc. Nat. Acad. Sci.*, 2012, **109**, E1743–1752.

[19] A. Bouslimani, L. Sanchez, N. Garg and P. C. Dorrestein, *Nat. Prod. Rep.*, 2014, **31**, 718–729.

[20] T. Ito and M. Miyako, *Nat. Prod. Rep.*, 2014, **67**, 353–360.

[21] G. Challis and J. Ravel, *FEMS microbiology letter*, 2000, **187**, 111–114.

[22] S. Lautru, R. Deeth, L. Bailey and G. Challis, *Nat. Chem. Biol.*, 2005, **1**, 265–269.

[23] N. Bandeira, D. Tsur, A. Frank and P. Pevzner, *Proc. Natl. Acad. Sci.*, 2007, **104**, 6140–5.

[24] P. Pevzner, Z. Mulyukov, V. Dancik and C. Tang, *Genome Res.*, 2001, **11**, 290–299.

[25] D. Tsur, S. Tanner, E. Zandi, V. Bafna and P. Pevzner, *Nat. Biotechnol.*, 2005, **23**, 1562–1567.

[26] M. Marahiel, T. Stachelhaus and H. Mootz, *Nat. Prod. Rep.*, 1997, **7**, 2651–2674.

[27] D. Schwarzer, R. Finking and M. Marahiel, *Nat. Prod. Rep.*, 2003, **20**, 275–287.

[28] T. Oman and W. van der Donk, *Nat. Prod. Rep.*, 2010, **6**, 9–18.

[29] J. McIntosh, M. Donia and E. Schmidt, *Nat. Prod. Rep.*, 2009, **26**, 537–59.

[30] T. Stachelhaus, H. Mootz and M. Marahiel, *Chem. Biol.*, 1999, **6**, 493–505.

[31] A. Broberg, A. Menkis and R. Vasiliauskas, *J. Nat. Prod.*, 2006, **69**, 97–102.

[32] A. Delcher, D. Harmon, S. Kasif, O. White and S. Salzberg, *Nucleic Acids Res.*, 1999, **27**, 673–679.

[33] K. Severinov, E. Semenova, A. Kazakov, T. Kazakov and M. Gelfand, *Mol Microbiol.*, 2007, **65**, 1380–94.

[34] S. Na, N. Bandeira and E. Paek, *Mol. Cell. Proteomics*, 2012, **11**, M111.010199.

[35] J. Doroghazi, J. Albright, A. Goering, K. Ju, R. Haines, K. Tchalukov, D. Labeda, N. Kelleher and W. Metcalf, *Nat Chem Biol.*, 2014, **10**, 6963–8.

[36] A. Starcevic, J. Zucko, , J. Simunkovic, P. Long, J. Cullum and D. Hranueli, *Nucleic Acids Res.*, 2008, **36**, 6882–6892.

[37] M. Li, P. Ung, J. Zajkowski, S. Garneau-Tsodikova and D. Sherman, *Nucleic Acids Res.*, 2009, **10**, 185–194.

[38] C. Rausch, T. Weber, O. Kohlbacher, W. Wohlleben and D. Huson, *Nucleic Acids Res.*, 2005, **33**, 5799–808.

[39] M. Rottig, M. Medema, K. Blin, T. Weber, C. Rausch and O. Kohlbacher, *Nucleic Acids Res.*, 2011, **39**, W332–7.

[40] M. Medema, K. Blin, P. Cimermancic, V. Jager, P. Zakrzewski, M. Fischbach, T. Weber, E. Takan and R. Breitling, *Nucleic Acids Res.*, 2011, **39**, W339–W346.

[41] K. Blin, M. Medema, D. Kazempour, M. Fischbach, R. Breitling, E. Takano and T. Weber, *Nucleic Acids Res.*, 2013, **41**, W204–12.

[42] T. Weber, K. Blin, S. Duddela, D. Krug, H. Kim, R. Bruccoleri, S. Lee, M. Fischbach, R. Muller, W. Wohlleben, R. Breitling, E. Takano and M. Medema, *Nucleic Acids Res.*, 2015, **43**, W237–43.

[43] S. Anand, M. Prasad, G. Yadav, N. Kumar, J. Shehara, M. Ansari and D. Mohanty, *Nucleic Acids Res*, 2010, **38**, W487.

[44] N. Khaldi, F. Seifuddin, G. Turner, D. Haft, W. Nierman, K. Wolfe and N. Fedorova, *Fungal Genet Biol.*, 2010, **47**, 736.

[45] M. Umemura, H. Koike, N. Nagano, T. Ishii, J. Kawano, N. Yamane, I. Kozone, K. Horimoto, K. Shin-ya, K. Asai, J. Yu, J. Bennett and M. Machida, *PLOS one*, 2013, **8**, e84028.

[46] T. Weber, C. Rausch, P. Lopez, I. Hoof, V. Gaykova and D. H. Huson, *Journal of Biotechnology*, 2009, **140**, 13.

[47] H. Tae, E. Kong and K. Park, *BMC Bioinformatics*, 2007, **8**, 327.

[48] M. Medema, Y. Paalvast, D. Nguyen, A. Melnik, P. Dorrestein, E. Takano and R. Breitling, *PLoS Comput Biol.*, 2014, **10**, e1003282.

[49] P. Arnison *et al.*, *Nat. Prod. Rep.*, 2013, **30**, 108–160.

[50] J. Velsquez and W. van der Donk, *Curr. Opin. Chem. Biol.*, 2011, **15**, 11–21.

[51] de Jong A., van Heel A.J., J. Kok and O. Kuipers, *Nucleic Acids Res.*, 2010, **38**, W647651.

[52] A. van Heel, A. de Jong, M. Montalban-Lopez, J. Kok and O. Kuipers, *Nucleic Acids Res.*, 2013, **41**, W448–53.

[53] J. Li, X. Qu, X. He, L. Duan, G. Wu, D. Bi, Z. Deng, W. Liu and H. Ou, *PLoS One.*, 2012, **7**, e45878.

[54] M. Maksimova, I. Pelczerb and J. Link, *Proc. Natl. Acad. Sci.*, 2012, **109**, 15223–15228.

[55] N. Leikoski, D. Fewer, J. Jokela, M. Wahlsten, L. Rouhiainen and K. Sivonen, *Appl Environ Microbiol*, 2010, **76**, 701–709.

[56] R. Hammami, A. Zouhir, J. Ben Hamida and I. Fliss, *BMC Microbiol.*, 2007, **7**, 89–94.

[57] Y. Xu, R. Kersten, S. Nam, L. Lu, A. Al-Suwailem, H. Zheng, W. Fenical, P. Dorrestein, B. Moore and P. Qian, *J. Am. Chem. Soc.*, 2012, **134**, 8625–8632.

[58] A. Hill and R. Mortishire-Smith, *Rapid Commun. Mass Spectrom.*, 2005, **19**, 3111–3118.

[59] S. Wolf, S. Schmidt, M. Mller-Hannemann and S. Neumann, *BMC Bioinformatics*, 2010, **11**, 148.

[60] M. Krauss, H. Singer and J. Hollender, *Anal Bioanal Chem.*, 2010, **397**, 943–951.

[61] L. Ridder, J. J. J. van der Hooft, S. Verhoeven, R. C. H. de Vos, R. J. Bino and J. Vervoort, *Anal Chem.*, 2013, **85**, 6033–6040.

[62] Y. Wang, G. Kora, B. Bowen and C. Pan, *Anal Chem.*, 2014, **86**, 9496–503.

[63] R. Mistrik, J. Lutisan, Y. Huang, M. Suchy, J. Wang and M. Raab, *9th International Conference of the Metabolomics Society, Glasgow, Scotland*, 2013.

[64] A. Klitgaard, A. Iversen, M. Andersen, T. Larsen, J. Frisvad and K. Nielsen, *Anal Bioanal Chem.*, 2014, **406**, 1933–43.

[65] H. Shen, N. Zamboni, M. Heinonen and J. Rousu, *Metabolites*, 2013, **3**, 484–505.

[66] F. Allen, A. Pon, M. Wilson, R. Greiner and D. Wishart, *Nucleic Acids. Res.*, 2014, **42**, W94–9.

[67] F. Allen, R. Greiner and D. Wishart, *Metabolomics*, 2015, **11**, 98–110.

[68] M. Gerlich and S. Neumann, *J. Mass. Spectrom.*, 2013, **48**, 291–8.

[69] J. Ng, N. Bandeira, W. Liu, M. Ghassemian, T. Simmons, W. Gerwick, R. Linington, P. Dorrestein and P. Pevzner, *Nat. Methods*, 2009, **6**, 596–599.

[70] A. Ibrahim, L. Yang, C. Johnston, X. Liu, B. Ma and N. Magarveya, *Proc. Natl. Acad. Sci.*, 2012, **109**, 19196–19201.

[71] S. Kim, N. Gupta and P. Pevzner, *J. Prot. Res.*, 2008, **7**, 3354–3363.

[72] H. Mohimani, S. Kim and P. Pevzner, *J. Prot. Res.*, 2013, **12**, 1560–1568.

[73] X. Tang, P. Thibault and R. Boyd, *Int. J. Mass Spectrom. Ion Processes*, 1992, **122**, 153–179.

[74] H. Mohimani, W. Liu, R. Kersten, B. Moore, P. Dorrestein and P. Pevzner, *J. Nat. Prod.*, 2014, **77**, 1902–1909.

[75] P. Pevzner, V. Dancik and C. Tang, *J. Comput. Biol.*, 2000, **7**, 777–787.

[76] H. Mohimani, W. Liu, Y. Liang, S. Gaudenico, W. Fenical, P. Dorrestein and P. Pevzner, *J. Comput. Biol.*, 2011, **18**, 1371–1381.

[77] E. Bolton, Y. Wang, P. Thiessen and S. Bryant, *Annu. Rep. Comput. Chem.*, 2008, **4**, 217–241.

[78] H. Pence and A. Williams, *J. Chem. Educ.*, 2010, **87**, 1123–1124.

[79] M. Kanehisa, S. Goto, Y. Sato, M. Furumichi and M. Tanabe, *Nucleic Acids Res.*, 2012, **40**, 109–114.

[80] R. Caspi, T. Altman, R. Billington, K. Dreher, H. Foerster, C. A. Fulcher, T. A. Holland, I. M. Keseler, A. Kothari, A. Kubo, M. Krummenacker, M. Latendresse, L. A. Mueller, Q. Ong, S. Paley, P. Subhraveti, D. S. Weaver, D. Weerasinghe, P. Zhang and P. D. Karp, *Nucleic Acids Res.*, 2014, **42**, D459–D471.

[81] S. Caboche, M. Pupin, V. Leclre, A. Fontaine, P. Jacques and G. Kucherov, *Nucleic Acids Res.*, 2008, **36**, D326–D331.

[82] M. H. Medema *et al.*, *Nature Chemical Biology*, 2015, **11**, 625–631.

[83] J. Blunt, M. Munro and H. Laatsch, *University of Canterbury; Christchurch, New Zealand: University of Gottingen; Gottingen, Germany*, 2007.

[84] H. Lam, E. Deutsch, J. Eddes, J. Eng, N. King, S. Stein and R. Aebersold, *Proteomics*, 2007, **7**, 655–667.

[85] S. Heller, *Todays Chemist at Work*, 1999, **8**, 45–46.

[86] D. Wishart, C. Knox, A. Guo, R. Eisner, N. Young, B. Gautam, D. Hau, N. Psychogios, E. Dong, S. Bouatra, R. Mandal, I. Sinelnikov, J. Xia, L. Jia, J. Cruz, E. Lim, C. Sobsey, S. Shrivastava, P. Huang, P. Liu, L. Fang, J. Peng, R. Fradette, D. Cheng, D. Tzur, M. Clements, A. Lewis, A. De Souza, A. Zuniga, M. Dawe, Y. Xiong, D. Clive, R. Greiner, A. Nazyrova, R. Shaykhutdinov, L. Li, H. Vogel and I. Forsythe, *Nucleic Acids Res.*, 2009, **37**, D603–10.

[87] H. Horai, M. Arita, S. Kanaya, Y. Nihei, T. Ikeda, K. Suwa, Y. Ojima, K. Tanaka, S. Tanaka, K. Aoshima, Y. Oda, Y. Kakazu, M. Kusano, T. Tohge, F. Matsuda, Y. Sawada, M. Hirai, H. Nakanishi, K. Ikeda, N. Akimoto, T. Maoka, H. Takahashi, T. Ara, N. Sakurai, H. Suzuki, D. Shibata, S. Neumann, T. Iida, K. Tanaka, K. Funatsu, F. Matsuura, T. Soga, R. Taguchi, K. Saito and T. Nishioka, *J Mass Spectrom.*, 2010, **45**, 703–14.

[88] C. Smith, G. O'Maille, E. Want, C. Qin, S. Trauger, T. Brandon, D. Custodio, R. Abagyan and G. Siuzdak, *Ther Drug Monit.*, 2005, **6**, 747–51.

[89] P. Ausloos *et al.*, *J. Am. Soc. Mass Spectrom.*, 1999, **10**, 287–299.

[90] S. Stein and D. Scott, *J. Am. Soc. Mass Spectrom.*, 1994, **5**, 859–866.

[91] L. Domokos, D. Hennberg and B. Weimann, *Anal. Chim. Acta*, 1984, **165**, 61–74.

[92] J. Halket, D. Waterman, A. Przyborowska, R. Patel, P. Fraser and P. Bramley, *J. Exp. Bot.*, 2005, **56**, 219–243.

[93] R. Craig, J. Cortens, D. Fenyo and R. Beavis, *J. Prot. Res.*, 2006, **5**, 1843–1849.

[94] H. Lam and R. Aebersold, *Methods Mol Biol*, 2010, **604**, 95–103.

[95] H. Lam, E. Deutsch and R. Aebersold, *J. Prot. Res.*, 2010, **9**, 605–610.

[96] B. Milman and I. Zhurkovich, *Anal. Chem. Res.*, 2014, **1**, 8–15.

[97] H. Mohimani, R. Kersten, W. Liu, M. Wang, S. Purvine, S. Wu, H. Brewer, L. Pasa-Tolic, B. Moore, P. Pevzner and P. Dorrestein, *ACS Chem. Biol.*, 2014, **9**, 1545–1551.

[98] D. Nguyen, C. Wu, W. Moree, A. Lamsa, M. Medema, X. Zhao, R. Gavilan, M. Aparicio, L. Atencio, C. Jackson, J. Ballesteros, J. Sanchez, J. Watrous, V. Phelan, C. van de Wiel, R. Kersten, S. Mehnaz, R. De Mot, E. Shank, P. Charusanti, H. Nagarajan, B. Duggan, B. Moore, N. Bandeira, K. Palsson, B.. nd Pogliano, M. Gutierrez and P. Dorrestein, *Proc. Natl. Acad. Sci.*, 2013, **110**, E2611–20.

[99] W. Liu, A. Lamsa, W. Wong, P. Boudreau, R. Kersten, Y. Peng, W. Moree, B. Duggan, B. Moore, W. Gerwick, R. Linington, K. Pogliano and P. Dorrestein, *J. Antibiot.*, 2014, **67**, 99–104.

[100] J. Yang, L. Sanchez, C. Rath, X. Liu, P. Boudreau, N. Bruns, E. Glukhov, A. Wodtke, R. de Felicio, A. Fenner, W. Wong, R. Linington, L. Zhang, H. Debonsi, W. Gerwick and P. Dorrestein, *J. Nat. Prod.*, 2013, **76**, 1686–99.

[101] W. J. Moree, V. V. Phelana, C. H. Wu, N. Bandeira, D. S. Cornett, B. M. Duggan and P. C. Dorrestein, *Proc. Natl. Acad. Sci.*, 2012, **109**, 13811–13816.

[102] K. Duncan, M. Crsemann, A. Lechner, A. Sarkar, J. Li, N. Ziemert, M. Wang, N. Bandeira, B. Moore, P. Dorrestein and P. Jensen, *Chem Biol.*, 2015, **22**, 460–471.

[103] M. Traxler, J. Watrous, T. Alexandrov, P. Dorrestein and R. Kolter, *MBio.*, 2013, **4**, e00459–13.

[104] J. Winnikoff, E. Glukhov, J. Watrous, P. Dorrestein and W. Gerwick, *J. Antibiot.*, 2014, **67**, 105–12.

[105] M. Wilson, T. Mori, C. Rckert, A. Uria, M. Helf, K. Takada, C. Gernert, U. Steffens, N. Heycke, S. Schmitt, C. Rinke, E. Helfrich, A. Brachmann, C. Gurgui, T. Wakimoto, M. Kracht, M. Crsemann, U. Hentschel, I. Abe, S. Matsunaga, J. Kalinowski, H. Takeyama and J. Piel, *Nature.*, 2014, **506**, 58–62.

[106] A. Bouslimani, , C. Rath, M. Wang, Y. Guoc, A. Gonzalez, D. Berg-Lyon, G. Gail Ackermann, G. Christensen, T. Nakatsujig, L. Zhang, A. W. Borkowskig, M. J. Meehan, K. Dorrestein, R. Gallog, N. Bandeira, R. Knight, T. Alexandrov and P. Dorrestein, *Proc. Nat. Acad. Sci.*, 2015, **112**, E2120–2129.

[107] A. Edlund, Y. Yang, S. Yooseph, A. Hall, D. Nguyen, P. Dorrestein, K. Nelson, X. He, R. Lux, W. Shi and J. McLean, *ISME J.*, 2015.

[108] M. I. Vizcaino and J. M. Crawford, *Nature Chem.*, 2015, **7**, 411–417.

[109] H. Mohimani, Y. Liang, W. Liu, P. Hsieh, P. Dorrestein and P. Pevzner, *J. Proteomics*, 2011, **11**, 3642–3650.

[110] D. Kavan, M. Kuzma, K. Lemr, K. Schug and V. Havlicek, *J Am Soc Mass Spectrom.*, 2013, **24**, 1177–84.

[111] J. Allmer, *Expert Revi Proteomic*, 2011, **8**, 645–657.

[112] V. Dancik, T. Addona, K. Clauser, J. Vath and P. Pevzner, *J. Comput. Biol.*, 1999, **6**, 327–42.

[113] B. Ma, K. Zhang, C. Hendrie, C. Liang, M. Li, A. Doherty-Kirby and G. Lajoie, *Rapid Commun Mass Spectrom.*, 2003, **17**, 2337–42.

[114] A. Frank and P. Pevzner, *Anal. Chem.*, 2005, **77**, 964–983.

[115] J. A. Taylor and R. S. Johnson, *Anal. Chem*, 2000, **73**, 2594–2604.

[116] J. Novak, K. Lemr, K. A. Schug and V. Havlicek, *J. ASMS*, 2015, **1780-6**, 26.

[117] N. Bandeira, J. Olsen, M. Mann and P. Pevzner, *Bioinformatics*, 2008, **24**, 416–423.

[118] A. Guthals, C. Boucher and N. Bandeira, *J Comput Biol.*, 2014, **22**, 353–66.

[119] H. Mohimani, W. Liu, J. Mylne, A. Poth, M. Colgrave, D. Tran, M. Selsted, P. Dorrestein and P. Pevzner, *J. Prot. Res.*, 2011, **10**, 4505–4512.

[120] Q. Zhang, M. Ortega, Y. Shi, H. Wang, J. Melby, W. Tang, D. Mitchell and W. van der Donk, *Proc. Natl. Acad. Sci.*, 2014, **111**, 12031–12036.

[121] K. Blin, D. Kazempour, W. Wohlleben and T. Weber, *PLoS One*, 2014, **9**, 489420.

[122] N. Bandeira, V. Pham, P. Pevzner, D. Arnott and J. Lill, *Nat. Biotechnol.*, 2008, **26**, 1336–8.

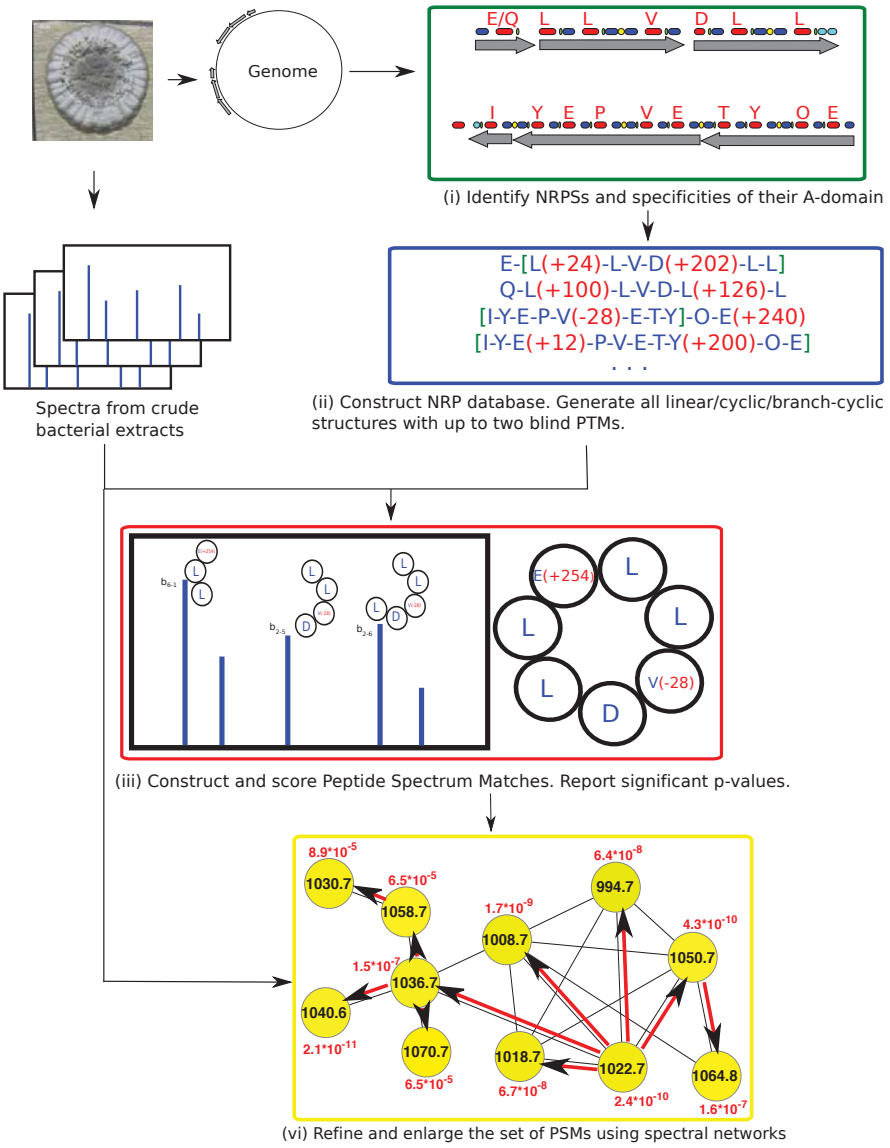

Figure 10: NRPquest[74] pipeline includes the following steps : (i) identifying NRP synthetases in the genome, (ii) using non-ribosomal code to construct the database of putative NRPs generated by each NRP synthetase, (iii) matching spectra against the database of putative NRPs and and computing p-values of resulting PSMs, and (iv) refining and enlarging the set of identified NRPs using spectral networks. The figure is reproduced from [74] by permission from ACS publications.