Published in final edited form as:

J Proteome Res. 2013 April 05; 12(4): 1560–1568. doi:10.1021/pr300453t.

A new approach to evaluating statistical significance of spectral identifications

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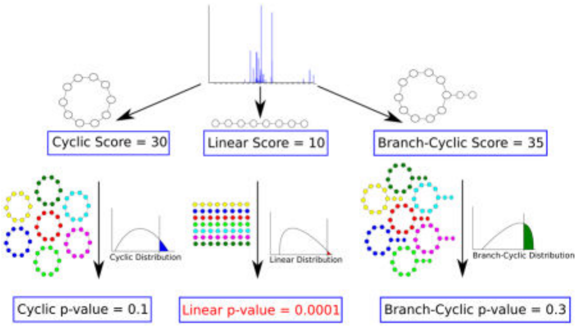
Abstract

While non-linear peptide natural products such as Vancomycin and Daptomycin are among the most effective antibiotics, the computational techniques for sequencing such peptides are still in their infancy. Previous methods for sequencing peptide natural products are based on Nuclear Magnetic Resonance spectroscopy and require large amounts (milligrams) of purified materials. Recently, development of mass spectrometry-based methods has enabled accurate sequencing of non-linear peptide natural products using picograms of material, but the question of evaluating statistical significance of Peptide Spectrum Matches (PSM) for these peptides remains open. Moreover, it is unclear how to decide whether a given spectrum is produced by a linear, cyclic, or branch-cyclic peptide. Surprisingly, all previous mass spectrometry studies overlooked the fact that a very similar problem has been successfully addressed in particle physics in 1951. In this paper, we develop a method for estimating statistical significance of PSMs defined by any peptide (including linear and non-linear). This method enables us to identify whether a peptide is linear, cyclic or branch-cyclic, an important step toward identification of peptide natural products.

Graphical Abstract



Keywords

Mass Spectrometry; Natural Products; Statistical Significance

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Introduction

The dominant technique for sequencing cyclic peptides is nuclear magnetic resonance (NMR) spectroscopy, which requires large amount (milligrams) of highly purified materials that are often nearly impossible to obtain.[1] Tandem mass spectrometry (MS/MS) provides an attractive alternative to NMR because it allows one to sequence a peptide from picograms of non-purified material. Recently, new algorithms have been developed for interpreting mass spectra of cyclic peptides using de novo sequencing[2–4] and database search.[5]

MS/MS coupled with database search is the most popular method for identification of (linear) peptides. A database search engine selects candidate peptides from a database of protein sequences that match the precursor mass from a mass spectrum. Then for each candidate peptide, the software compares a theoretical MS/MS derived from the peptide to the experimental mass spectrum, and reports a peptide with best score.

In the last decade, much effort has been invested in computing statistical significance of Peptide Spectrum Matches (PSMs). Many of these studies stem from the pioneering paper by Fenyo and Beavis[6] that proposed approximating the statistical significance of PSMs by first modeling the distribution of PSM scores (e.g. by Gumbel distribution[6]) and further using this distribution to compute p-values.[7–13] Unfortunately, this approximation approach, while useful in many applications, often fails when one has to estimate extremely small p-values typical for mass spectrometry (e.g. PSM p-values of the order 10^{-10} are often required to achieve 1% FDR[14]). Fortunately, the challenge of estimating the probability of extremely rare events has already been addressed by particle physicists in 1950s,[15] and communication systems engineers in 1980s.[16] However, the mass spectrometry community has overlooked these fundamental studies (directly relevant to mass spectrometry) resulting in inaccurate p-value estimation in some mass spectrometry studies.[17]

In the late 1940s, many top mathematicians worked on the *neutron shielding* problem that was crucial for designing nuclear facilities.[18,19] In this problem, one has to compute the probability that a neutron, doing a random walk, would pass through a slab, an extremely rare event. Two general methods emerged for evaluating extremely rare events by Monte Carlo random sampling (using computers that became available in mid 1940s); *importance sampling* and *multilevel splitting*. Both were developed for nuclear-physics calculations by Fermi, Harris, Kahn, Metropolis, Ulam, von Neumann, and their colleagues, during the production of the first nuclear bomb.[15,18–21] Importance sampling is based on the notion of modifying the underlying probability distribution in such a way that the rare events occur much more frequently. Multilevel splitting uses a selection mechanism to favor the trajectories deemed likely to lead to the rare events of interest. While importance sampling is the most popular rare event simulation method today, the main advantage of the multilevel splitting approach is the fact that it does not need to modify the probabilistic model governing the system. This makes multilevel splitting applicable to any system represented as a black box,[19] and specifically applicable to mass spectrometry studies. Kahn and Harris solved the neutron shielding problem using multilevel splitting in 1951.[15] Later, similar rare event estimation techniques found applications in communication systems,[16] financial mathematics,[22] air traffic management,[23] and chemistry.[24] However, this powerful approach has never been applied to mass spectrometry. This is surprising because there is a clear analogy between statistical significance evaluation in mass spectrometry, and the neutron shielding problem, where a spectrum plays the role of a neutron, a peptide plays the role of a slab, and the rare event “spectrum gets a high score against a peptide” plays the role of an event “neutron passes through a slab”.

Currently, the dominant technique for statistical evaluation of a set of PSMs is to compute the False Discovery Rate (FDR) using the Target Decoy Approach (TDA).[25] TDA is attractive for proteomics studies because it is widely applicable to different instrument platforms and database search algorithms. However, TDA is not applicable to non-linear peptide studies, because in these studies researchers usually work on a few non-linear peptide at a time, whereas TDA is best suited for statistical analysis of large spectral datasets.[25] Even in the case of linear peptides, some popular database search tools are not TDA-compliant.[26]

An alternative technique is to compute a p-value for an *individual* PSM.[17] Given a PSM (*Peptide*, *Spectrum*) of score *t*, the p-value of (*Peptide*, *Spectrum*) is defined as the fraction of random peptides with a score equal to or exceeding *t*.[17] Unlike the FDR that is defined on a set of PSMs, the p-value is defined on a single PSM. Therefore computing the p-value is adequate for non-linear peptide studies, where a single or a few non-linear peptides are considered at a time. Since our results can be applied to both cyclic peptides (e.g. surfactin) and branch-cyclic peptides (e.g. daptomycin), we will use the same term ‘cyclic’ to refer to both cyclic and branch-cyclic peptides.

For cyclic peptide studies, computing p-values offers additional advantages. In studies of peptide natural products, we are given a mixture of spectra of linear and cyclic peptides, from which a small number of spectra of cyclic peptides should be separated and investigated independently. Therefore we need a method that identifies whether a given spectrum represents a linear or a cyclic peptide. This is difficult because different scoring functions are used for linear and cyclic peptides. Since scores from different scoring functions are not usually comparable,[27,28] we need to convert them into p-values (Fig. 1).[26]

In the case of linear peptides, Kim *et al.*, 2008[17] presented a polynomial time algorithm for computing p-values, called MS-GF. However, MS-GF is only applicable to scoring functions that can be represented as a dot-product of vectors, i.e. *additive scoring functions*. Moreover, MS-GF is only applicable to linear peptides, and no one has generalized MS-GF to non-linear peptides yet.

Fenyo and Beavis[6] constructed an empirical score distribution of low-scoring (erroneous) peptide identifications and extrapolated it to evaluate the p-value of high-scoring peptide identifications in the tail of the distribution. Similar approaches are now used in many tools, that provide p-value or E-value of individual PSMs, e.g. OMSSA.[29] However, this approach was demonstrated to be inaccurate.[17] While the pitfalls of such approaches are well recognized in genomics, they remain under-appreciated in proteomics. Waterman and Vingron[30] argued that it is difficult to accurately estimate the extreme tails of a distribution in general, requiring accurate estimation of rare event probability. To do so, one may consider estimating p-values by a Monte-Carlo simulation generating a population of millions of peptides and estimating the probability distribution of scores on this population.[31] This approach becomes time-consuming for estimating extremely low p-values, since it requires calculating scores of billions of randomly generated peptides for accurate estimation of p-values as low as 1 in a billion.

In this paper, we propose MS-DPR (MS-Direct Probability Redistribution), a new method for estimating p-values of PSMs based on rare event probability estimation by multilevel splitting. We show that MS-DPR reports p-values similar to those reported by MS-GF in the case of linear peptides, confirming that it accurately estimates p-values. Furthermore, we show that unlike MS-GF, MS-DPR can compute p-values of PSMs when an arbitrary (non-additive) scoring function is used or when the peptide is non-linear.

Materials and Method

In contrast to importance sampling, which changes the probability laws driving the model, multilevel splitting[15,20] constructs a *Markov chain* and uses a selection mechanism to favor the *trajectories* in the Markov chain deemed likely to lead to rare events. Multilevel splitting is composed of three steps. First, decompose the trajectories to the rare events of interest into shorter sub-trajectories whose probability is not so small. Second, encourage the realizations that take these sub-trajectories (leading to the events of interest) by giving them a chance to reproduce by introducing *reproduction probabilities*. Third, discourage the realizations that go in the wrong direction by killing them with some positive *killing probability*. The sub-trajectories are usually delimited by levels. Starting from a given level, the trajectories that do not reach the next level will not reach the rare event, but those that do will split into multiple copies when they reach the next level. Each copy pursues its evolution independently from then on. This creates an artificial drift toward the rare event by favoring the trajectories that go in the right direction. In the end, an unbiased estimator can be recovered by multiplying the contribution of each trajectory by the appropriate weight.[15]

While multilevel splitting has wide applicability across diverse fields, it is not clear how to select the reproduction and killing probabilities, and the number of offsprings in mass spectrometry applications. Inspired by Kahn and Harris[15] and proposed by Haraszti and Townsend,[32] *Direct Probability Redistribution* (DPR) is a realization of multilevel splitting for estimating the probability of rare states in a Markov chain. Given a Markov chain, DPR implicitly constructs a modified Markov chain where probabilities of states are increased by an arbitrary order of magnitude. For a Markov chain with n states and (unknown) equilibrium probabilities $p_1, \cdots, p_n$, given *oversampling factors* $\mu_1, \cdots, \mu_n$, DPR constructs a Markov chain with (unknown) equilibrium probability $p_1^{'}=\mu_1 p_1/\sum\mu_k p_k, \cdots, p_n^{'}=\mu_n p_n/\sum\mu_k p_k$. For example, take a two-state Markov chain with equilibrium probabilities $p_1 = 0.999$ and $p_2 = 0.001$. If we choose $\mu_1 = 1$ and $\mu_2 = 999$, we end up with equilibrium probability $p_1^{'}=0.5$ and $p_2^{'}=0.5$, illustrated in Fig. 2(A–B). If one decides to estimate probability distribution of Fig. 2(A) by Monte Carlo, thousands of simulations are required (since $p_2 = 0.001$ is small). However, if one tries to estimate probability distribution of Fig. 2(B), only a few simulations are sufficient (since $p_1 = p_2 =$

0.5 is not small). This contrast in the number of simulations is the key idea of DPR. Here we descibe how to apply DPR to the problem of estimating probability distribution of PSM scores.

For simplicity, we define a spectrum as a set of integer masses. A peptide of length k is defined as a string of k positive integers $Peptide = m_1 m_2 \cdots m_k$. The mass of the peptide is defined as the sum of all the integers in the string. A score of a PSM ($Peptide$, $Spectrum$) is denoted by $Score(Peptide, Spectrum)$. Note that the proposed algorithm works for an arbitrary set of amino acid alphabets, not only for the alphabet of 20 standard amino acids. Since nonribosomal peptides often contain non-standard amino acids, in this section we consider peptides in the alphabet of all integers. In the Result section we also consider the case of the standard 20 amino acid alphabet.

Note that while a linear peptide of length k has a unique representation $m_1, \cdots, m_k$, a cyclic peptide of length k can have up to k equivalent represantations. For example, peptide (3,7,1) could also be presented as (7,1,3) and (1,3,7). One can choose an arbitrary representation among these representations, e.g., the representation where the first residue has minimum mass.

Given $Peptide = (m_1, \cdots, m_i, m_{i+1}, \cdots, m_k)$, integer residue index $1 \le i \le k$, and integer mass $-m_i < \delta < m_{i+1}$, we define $Peptide(i,\delta)$ as a peptide $(m_1, \cdots, m_i + \delta, m_{i+1} - \delta, \cdots, m_k)$. These peptides are called *sister peptides*. Note that sister peptides have equal lengths and equal (parent) masses, and all amino acids masses but at most two are the same (see Fig. 3(A)). Note that there are many alternative ways to define the notion of a sister peptide. $RandomTransition(Peptide)$ is a $Peptide(i,\delta)$, where i and δ are integer random variables, i chosen from the uniform distribution on $[1, k]$, and δ chosen from the uniform distribution on $[-m_i, m_{i+1}]$. Supplementary Material discusses the conditions on a $RandomTransition$ that are needed for MS-DPR to work properly. We define $PeptideSpace$ as the set of all peptides with length k and mass m. Consider the following Markov chain defined on $PeptideSpace$:

$$Peptide_{t+1} = RandomTransition(Peptide_t)$$

where $Peptide_0$ is chosen from $PeptideSpace$ with uniform distribution. Then the problem of finding probability distribution of all scores of peptides from $PeptideSpace$ against $Spectrum$ is equivalent to finding equilibrium distribution of the above Markov chain. We use the DPR technique to accurately estimate the total probability of all peptides with high scores (rare events) in this Markov chain. Figure 3(B) illustrates this Markov chain.

Assume the set of all feasible scores (called *score states*) is $ScoreSpace = \{1,\ldots,n\}$, with (unknown) probabilities $p_1, \cdots, p_n$. Assume arbitrary oversampling factors $\mu_1, \cdots, \mu_n$ are given. Then the DPR approach provides a way to modify the transition probabilities such that in the equilibrium distribution of the resulting Markov chain, the probability of states with score i are oversampled by a factor μ_i, i.e. $p_1^{'} = \mu_1 p_1 / \sum \mu_k p_k, \cdots, p_n^{'} = \mu_n p_n / \sum \mu_k p_k$. An example of this procedure is shown in Fig. 2(C–D). Figure 4(A) shows the MS-DPR algorithm, which is a modification of the original DPR algorithm.[32] Glasserman *et. al.*, 1998,[33] show that the optimal choice of $\mu_1, \cdots, \mu_n$ (with respect to reducing the number of trials to achieve the required accuracy for estimation of score distribution) is the one that makes all score states equiprobable, *i.e.* $(\mu_1, \cdots, \mu_n) = (1/p_1, \cdots 1/p_n)$. However, since in practice $p_1, \cdots, p_n$ are unknown beforehand, one needs their rough estimate to efficiently implement DPR. Our idea is to first run the algorithm with $\mu_1 = \cdots = \mu_n = 1$, and obtain a rough estimate of $p_1, \cdots, p_n$. Then we choose $\mu_k = 1/p_k$ in the next iteration. This procedure is summarized in Fig. 4(B).

Results

We used the Standard Protein Mix database consisting of 1.1 million spectra generated from 18 proteins using eight different mass spectrometers.[34] For this study, we considered the charge 2 spectra generated by Thermo Electron LTQ where 1,388 linear peptides of length between 7 and 20 are identified with false discovery rate 2.5% using Sequest[35] and PeptideProphet[36] in the search against the *Haemophilus influenzae* database appended with sequences of the 18 proteins (567,460 residues). For testing MS-DPR on cyclic peptides, we use the dataset from the Cycloquest paper,[5] that includes cyclopeptides SFTI-1 and SFT-L1 from *Helianthus annuus*, as well as a linear and a cyclic peptide, SDP and SKF, from *Bacillus subtilits*.

To apply MS-DPR, we first need to define scoring functions for linear and cyclic peptides. Linear theoretical spectrum of a peptide $Peptide = (m_1, \cdots, m_k)$, $LinearSpectrum(Peptide)$, is a set of $k - 1$ b-ions and $k - 1$ y-ions, where each b-ion is the mass of a prefix of the peptide plus rounded H^+ mass, $m_1 + \cdots + m_{j-1} + 1$, and each y-ion is the mass of a suffix of the peptide plus rounded H^+ and H_2O mass, $m_j + \cdots + m_k + 19$. Similarly to the Cycloquest paper, [5] The cyclic theoretical spectrum of the peptide, $CyclicSpectrum(Peptide)$, is defined as the set of masses of its $k(k - 1)$ substrings of the peptide, $m_i + \cdots + m_{j-1}$ $(m_i + \cdots + m_k + m_1 + \cdots + m_{j-1}$, if $i \geq j)$, illustrated in Fig. 5(A). For branch-cyclic peptide *Peptide*, $BranchCyclicSpectrum(Peptide)$ is defined as the union of $LinearSpectrum(Peptide_l)$ and $CyclicSpectrum(Peptide_c)$, where $Peptide_l$ is the linear part of *Peptide* with cyclic tail assumed as a modification, and $Peptide_c$ is the cyclic part of *Peptide* with the linear tail assumed as a modification, illustrated in Fig. 5(B).

Similarly to the Cycloquest paper,[5] $CyclicScore(Peptide, Spectrum)$ and $BranchCyclicScore(Peptide, Spectrum)$ are defined as the number of shared masses between *Spectrum* with $CyclicSpectrum(Peptide)$ and $BranchCyclicSpectrum(Peptide)$, respectively. For simplicity, score of linear peptide *Peptide* and a spectrum *Spectrum*, $LinearScore(Peptide, Spectrum)$, is defined as the number of shared masses between *Spectrum* and $LinearSpectrum(Peptide)$ (In our experiments we will also use advanced MS-GF scores for linear peptides). We emphasize that while we use the same “shared peak count” principle, the resulting scoring functions are very different in the case of linear, cyclic and branch-cyclic peptides.

In addition to the p-value computed by MS-DPR (denoted by p_{DPR}), we also compute the empirical p-value (denoted by p_E), using a Monte Carlo approach by generating millions (or even billions) of random peptides and estimating probability distribution. Moreover, p_{MS-GF} stand for p-value of MS-GF software tool[17] (with 20 standard amino acid assumption), while p_{GF} stands for exact score probabilities computed by the generating function approach[17] for the case of arbitrary masses of amino acids.

Figure 6 shows the evolution of μ and **p** in three iterations of MS-DPR. $\mathbf{p} = (p_1, \cdots p_n)$ is the original probability distribution, $\mathbf{p}' = (p'_1, \cdots, p'_n)$ is the modified probability distribution, and $\mu = (\mu_1, \cdots \mu_n)$ is the vector of oversampling factors. $\mathbf{p}'$ converges to uniform distribution, and **p** converges to the correct distribution p_{GF}.

To evaluate the accuracy of the MS-DPR approach, we used all 1388 identifications from the ISB database. We compared p_{DPR} and p_{GF} (Fig. 7(A)), under the following assumptions: (i) all integers are considered as possible masses of amino acids (typical assumption for analyzing non-ribosomal peptides in the alphabet of arbitrary amino acid masses[4]), (ii) p-values are computed under the assumption that peptides have fixed known length, and (iii) the shared peak count is used as score. A correlation $R^2 = 0.9998$ between the two p-values shows that our method accurately estimates the probability distribution. Fig. 7(B) shows the comparison with the p-values computed by actual MS-GF software tool for the case of the standard amino acids alphabet[17] (correlation of 0.9990). These small deviations of MS-DPR from the theoretical value are acceptable, as the accuracy of a Monte Carlo algorithm depends on the number of simulations.

To validate MS-DPR for cyclic peptides, we designed the following experiment. For cyclic peptide *Peptide* = (10,20,40), and the spectrum *Spectrum* = *CyclicSpectrum*(*Peptide*) = (10,20,30,40,50,60,70), *CyclicScore*(*Peptide*, *Spectrum*) = 7. In this case we have total of $\binom{70}{2}$ peptides of length three with mass 70, and six of them (rotations and reverse rotations of (10,20,40)), have score 7. Therefore, the exact p-value for score 7 in this case is equal to $6/\binom{70}{2}=0.0025$, while MS-DPR returns 0.0021. Table 1 shows comparison of theoretical and estimated p-value for some cyclic PSM of variable length.

To validate our approach for cyclic peptides and branch-cyclic peptides in practice, we compared p_{DPR} and p_E for Tyrocidine A and Daptomycin A21978C2 spectra. Tyrocidine A is a cyclic peptide with length 10 and mass 1269.7Da, and Daptomycin A21978C2 is a branch-cyclic peptide with length 14 and mass 1652.8Da. Three different scores are used: the *CyclicScore*, *MultiStageCyclicScore* defined in the multistage de novo sequencing paper,[4] and *BranchCyclicScore*. Figure 8 demonstrates that these approaches produce similar results for probabilities higher than 10^{-6}.

To validate efficiency of MS-DPR in identifying whether a spectrum is from a linear, or a cyclic peptide, we compare each spectrum in our dataset against the corresponding proteome. Cycloquest,[5] a database search for identification of linear and cyclic peptides from the mass spectra, is used for searching these peptides, and MS-DPR is used to re-rank top scoring PSMs given by Cycloquest. For *Helianthus annuus*, we used the EST database described in the Cycloquest paper,[5] for *B. subtilis* we used the genome available from Uniprot, and for ISB dataset, we used the 18 protein sequences. By calculating p-values of all PSMs, the method correctly identifies SFTI-1 and SFT-L1 as cyclic peptides with lowest p-values. SDP and SKF are also identified as linear and cyclic peptides with lowest p-values (Table 2). Among 1388 linear peptides from ISB dataset, 1358 (97.8%) are correctly identified as linear peptides, and 99.6% of linear peptide identifications have identical sequences with the ones found by the InsPecT database search tool.[37] Note that the standard ISB dataset does not contain any cyclic peptide, and all 31 cyclic PSMs are non-significant (p-values assigned are larger than 0.01). Lets define $p_{lin}(Spectrum)$ as the p-value of the most statistically significant linear PSM of *Spectrum*, and $p_{cyc}(Spectrum)$ as the p-value of the most statistically significant cyclic PSM. Figure 7(C) shows p_{lin} versus p_{cyc} for SFTI-1, SFT-L1, SKF, SDP and all spectra in ISB dataset. The figure shows that MS-DPR distinguishes cyclic peptides from their linear counterparts.

MS-DPR takes about one second per spectrum in the non-standard amino acid case and about one minute per spectrum in the standard amino acid case with MS-GF score. MS-DPR is specifically designed for computing p-values for cyclic, branch-cyclic and other non-linear peptides, where no alternative tools are available. We do not suggest using MS-DPR for linear peptides in the case of additive scoring function, where fast analytical solution is available.[17] However, some MS/MS database search tools use non-additive scoring function and compute empirical estimates of p-values or E-values. Since these estimates may be inaccurate,[14] MS-DPR may be used for validating or correcting these estimates.

Discussion

Most of the computational techniques developed in mass spectrometry focus on linear rather than non-linear peptides. Hence, computational mass spectrometry has not benefited the field of natural products yet, where the majority of interesting peptides are cyclic or branch-cyclic. One of the important questions in the field of peptide natural products is how to determine the structure (linear/cyclic/branch-cyclic) and amino acid sequence of a peptide from its spectrum. Since scoring functions for linear, cyclic and branch-cyclic peptides are very different, converting these scores to p-values is the first step toward automated MS-based discovery of peptide natural products.

We presented MS-DPR, a method for estimating statistical significance of PSMs in mass spectrometry. In contrast to existing methods for estimating p-values, MS-DPR can work with arbitrary scoring functions and non-linear peptides. Comparison of p-values estimated by MS-DPR with the p-values given by the generating function approach[17] validated MS-DPR in the case of additive scoring function and linear peptides. While there is no method for computing exact p-value of cyclic PSMs for a comprehensive evaluation of MS-DPR in the case of cyclic peptides, incorporating p-values in the recently developed Cycloquest algorithm[5] improved its performance (e.g. identification of cyclic peptide SFTI-1 missed by Cycloquest in previous study).

In the case of non-linear peptides, we used the shared peak count to score PSMs. While advanced scoring algorithms accounting for peak intensities increase the number of identifications of linear peptides at a given FDR, such scoring methods are not currently available for non-linear peptides. This is partially due to the fact that there are not enough annotated non-linear peptide spectra to train scoring algorithms. Recently, the natural product community has started collecting large scale mass spectrometry datasets. Thus, development of more comprehensive scoring algorithms will be possible in the near future.

While we tested MS-DPR only on linear, cyclic, and branch-cyclic peptides, our method is independent of a specific peptide structure and specific score scheme used. By defining a proper scoring function and random mutation for each peptide structure, MS-DPR can convert the score to an accurate p-value.

Cycloquest web-server reporting MS-DPR p-values is available at http://cyclo.ucsd.edu. The source code for MS-DPR is freely available at http://proteomics.ucsd.edu.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Pieter Dorrestein and Wei-Ting Liu for making the spectral dataset for SKF and SDP available, and Michelle Colgrave, Aaron Poth and Joshua Mylne for making the spectral dataset for SFTI-1 and SFT-L1 available. This work was supported by the U.S. National Institutes of Health 1-P41-RR024851-01. We are grateful to Kira Vyatkina for insightful suggestions.

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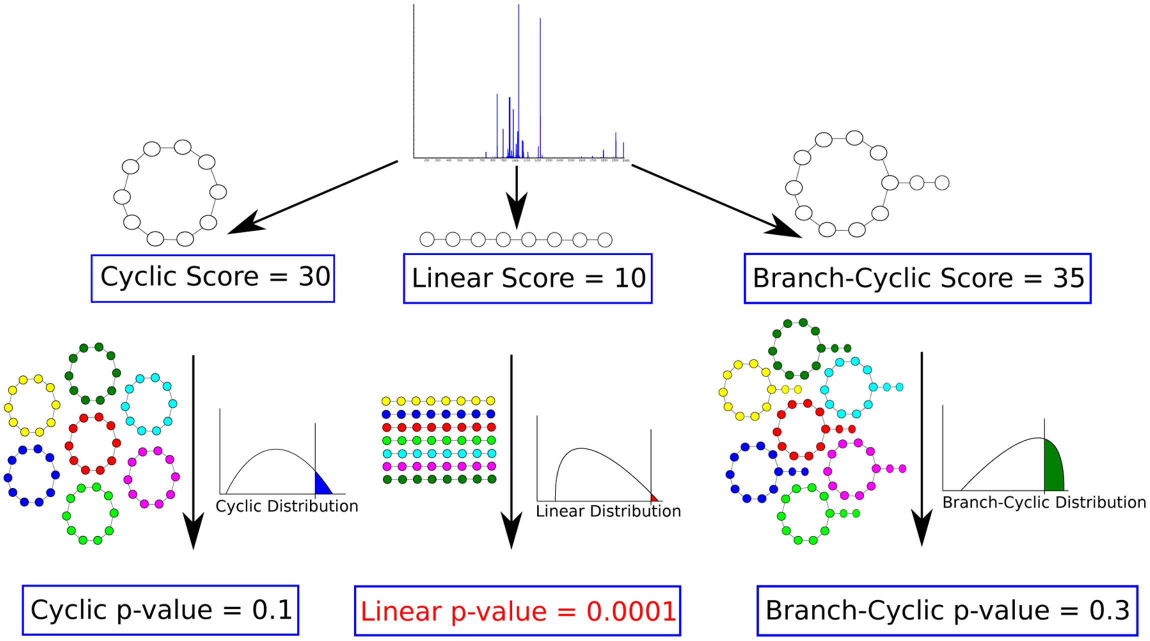


Figure 1.

Deciding whether a peptide that produced a spectrum is linear, cyclic or branch-cyclic. Given a spectrum with unknown structure, we compute its score under different structure assumptions (e.g. linear/cyclic/branch-cyclic), and derive a p-value for each assumption. If one of the structures result in a very small p-value (e.g. linear structure with p-value of 0.0001), that structure is accepted as the most likely structure.

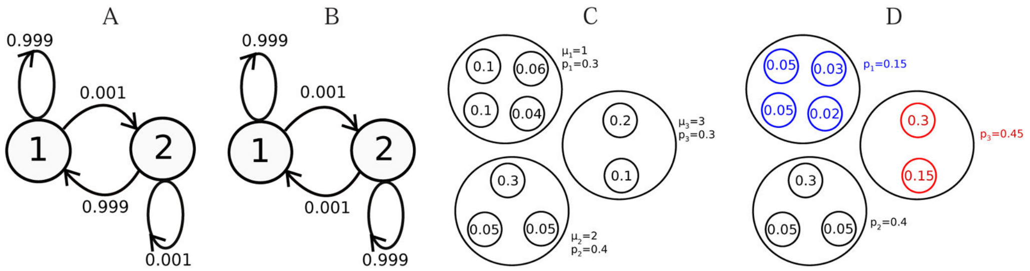


Figure 2.

A) Markov chain before performing DPR, with equilibrium probabilities (0.999,0.001). B) Markov chain after performing DPR, with equilibrium probabilities (0.5,0.5). C) An example of a Markov chain with nine peptides in three score states D) Probability distribution after performing DPR with oversampling factors (μ_1,μ_2,μ_3) = (1, 2,3). The states with decrease in probability are shown in blue, and the states with increase in probability are shown in red.

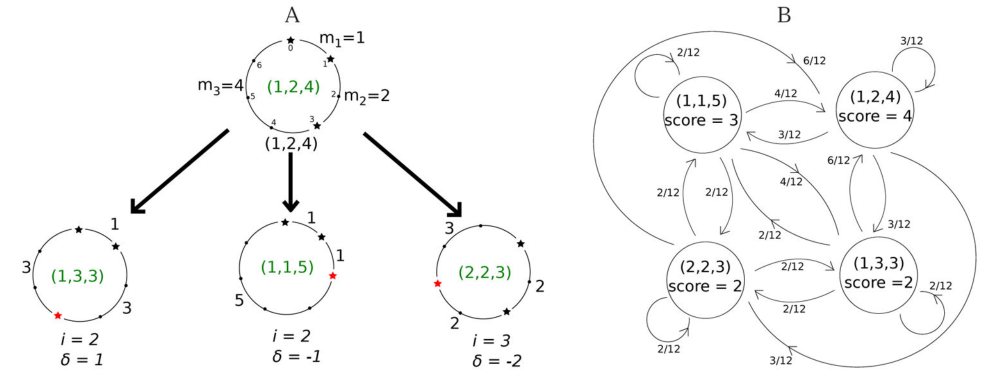
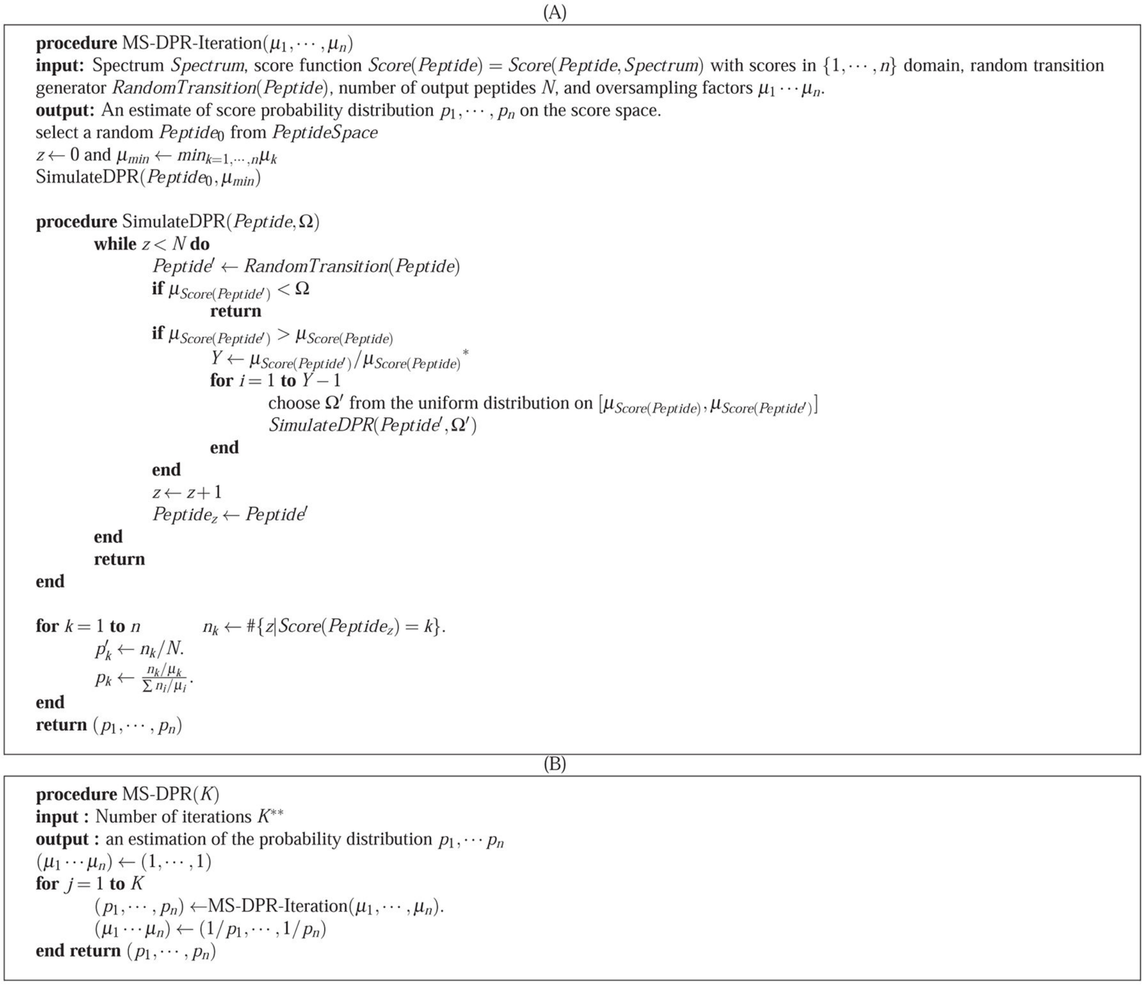


Figure 3.

A) Illustration of all sister peptides (1,3,3), (1,1,5) and (2,2,3) for the cyclic peptide (1,2,4). B) Illustration of the Markov chain for cyclic peptides of length 3 and mass 7. We have total of four different cyclic peptides, (1,1,5), (1,2,4), (1,3,3), and (2,2,3). Each random mutation is determined by selecting *i* (three cases), and *δ* (four cases), giving rise to a total of twelve equiprobable mutations. Transition probabilities between different states of the Markov chain, derived from the uniform mutation probabilities (1/12), are also shown for each edge in the Markov chain.



(A)

```
procedure MS-DPR-Iteration($\mu_1, \cdots, \mu_n$)
input: Spectrum $Spectrum$, score function $Score(Peptide) = Score(Peptide, Spectrum)$ with scores in $\{1, \cdots, n\}$ domain, random transition generator $RandomTransition(Peptide)$, number of output peptides $N$, and oversampling factors $\mu_1 \cdots \mu_n$.
output: An estimate of score probability distribution $p_1, \cdots, p_n$ on the score space.
select a random $Peptide_0$ from $PeptideSpace$
$z \leftarrow 0$ and $\mu_{min} \leftarrow min_{k=1,\cdots,n}\mu_k$
SimulateDPR($Peptide_0, \mu_{min}$)

procedure SimulateDPR($Peptide, \Omega$)
    while $z < N$ do
        $Peptide' \leftarrow RandomTransition(Peptide)$
        if $\mu_{Score(Peptide')} < \Omega$
            return
        if $\mu_{Score(Peptide')} > \mu_{Score(Peptide)}$
            $Y \leftarrow \mu_{Score(Peptide')}/\mu_{Score(Peptide)}{}^*$
            for $i = 1$ to $Y - 1$
                choose $\Omega'$ from the uniform distribution on $[\mu_{Score(Peptide)}, \mu_{Score(Peptide')}]$
                $SimulateDPR(Peptide', \Omega')$
            end
        end
        $z \leftarrow z + 1$
        $Peptide_z \leftarrow Peptide'$
    end
    return
end

for $k = 1$ to $n$          $n_k \leftarrow \#\{z | Score(Peptide_z) = k\}$.
    $p'_k \leftarrow n_k/N$.
    $p_k \leftarrow \frac{n_k/\mu_k}{\sum n_i/\mu_i}$.
end
return $(p_1, \cdots, p_n)$
```

(B)

```
procedure MS-DPR($K$)
input : Number of iterations $K^{**}$
output : an estimation of the probability distribution $p_1, \cdots p_n$
$(\mu_1 \cdots \mu_n) \leftarrow (1, \cdots, 1)$
for $j = 1$ to $K$
    $(p_1, \cdots, p_n) \leftarrow$MS-DPR-Iteration$(\mu_1, \cdots, \mu_n)$.
    $(\mu_1 \cdots \mu_n) \leftarrow (1/p_1, \cdots, 1/p_n)$
end return $(p_1, \cdots, p_n)$
```

Figure 4.

(A) MS-DPR-Iteration($\mu_1, \cdots, \mu_n$) algorithm[32] adapted for estimating statistical significance of PSMs. The algorithm produces peptide process $Peptide_0$, $Peptide_1$, ⋯, $Peptide_N$, and their scores $Score(Peptide_0)$, $Score(Peptide_1)$, ⋯, $Score(Peptide_N)$, with equilibrium probability distribution $p'_1, \cdots, p'_n$ satisfying $p'_k = c\mu_k p_k$ for a constant c. (*) Most of the times $\mu_{Score(Peptide')}/\mu_{Score(Peptide)}$ is not integer. In that case Y would be a random variable, taking $\lceil \mu_{Score(Peptide')}/\mu_{Score(Peptide)} \rceil$ with probability $p = \mu_{Score(Peptide')}/\mu_{Score(Peptide)} - \lfloor \mu_{Score(Peptide')}/\mu_{Score(Peptide)} \rfloor$ and $\lfloor \mu_{Score(Peptide')}/\mu_{Score(Peptide)} \rfloor$ with probability $1 - p$. Note that in case of $\mu_1 = \cdots = \mu_n = 1$, this reduces to simple Monte Carlo estimation of probability distribution from N peptides. (B) MS-DPR(K) algorithm for estimating the probability distribution of scores. (**) While MS-DPR uses the same global variables as MS-DPR-Iteration, these variables are omitted for brevity.

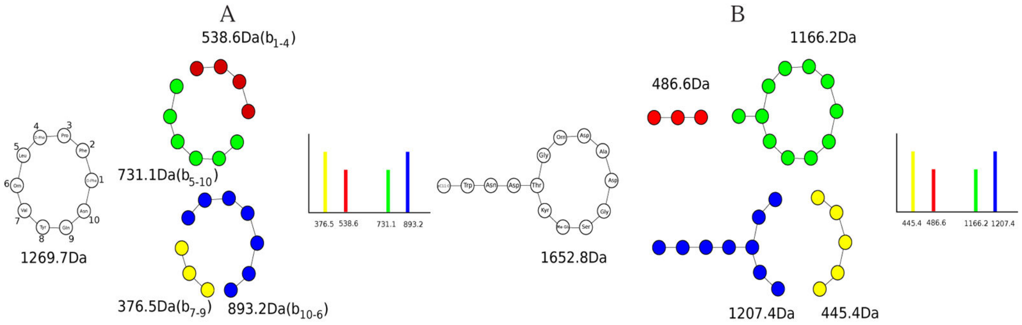


Figure 5.

(A) Illustration of *CyclicSpectrum*(*Tyrocidine*). (B) Illustration of *BranchCyclicSpectrum*(*Daptomycin*).

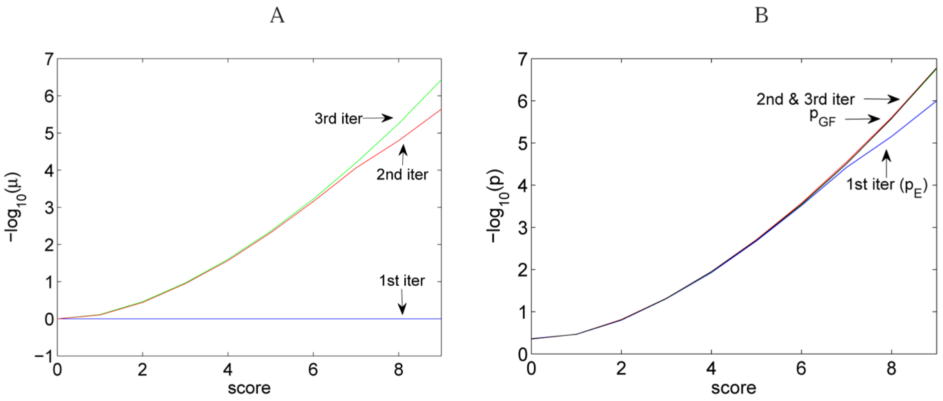


Figure 6.

Evolution of (A) μ_k (B) p_k for three iterations of MS-DPR. The analysis is performed for N = 1,000,000 simulated peptides of length 7, and a spectrum of peptide KYIPGTK from standard ISB database with parent mass 787. Blue, red and green plot stands for first, second, and third iterations respectively. In part (B) p_{GF} is plotted by black. Note that the blue plot in part (B) corresponds to first iteration of MS-DPR, which simply gives the empirical p-value, p_E. From the second iteration on, p_{DPR} is very similar to p_{GF}.

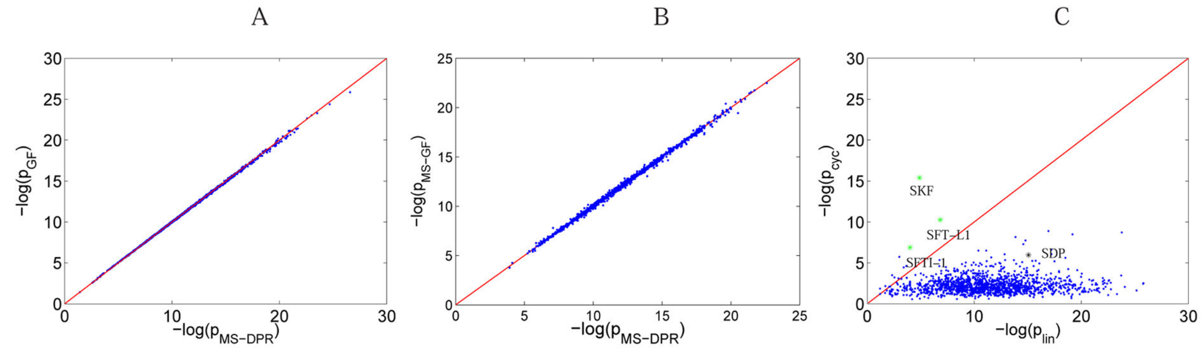


Figure 7.

(A) Comparison of $-log_{10}$ of generating function p-value with MS-DPR p-value for 1388 peptides from ISB database. Red line shows the $x = y$ line. Correlation between the two p-values is 0.9998. Non-standard amino acid model is used, assuming each peptide has a fixed known length, and peak count score. MS-GF approach[17] is modified accordingly, to satisfy these assumptions. (B) Comparison of $-log_{10}$ of the original, publicly available MS-GF p-value with MS-DPR p-value. Correlation between the two p-values is 0.9990. Standard amino acid model is used, with the variable peptide length assumption and MS-GF score. [17] (C) Comparison of $-log_{10}$ of p_{lin}, versus $-log_{10}$ of p_{cyc} for SFTI-1, SFT-L2, SKF, SDP, and spectra from the ISB dataset. Cyclic peptides SFTI-1, SFT-L2 and SKF are shown as green stars, and linear peptide SDP is shown as a black star. Blue dots show spectra from ISB dataset, and red line shows the $x = y$ line.

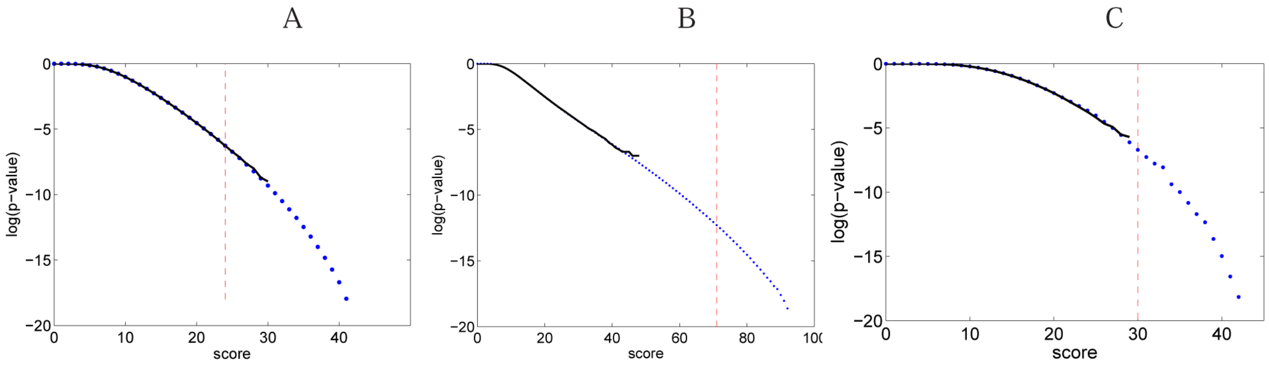


Figure 8.

(A) Estimating the score distribution for PSMs formed by the cyclic peptide Tyrocidine A (single-stage MS). Solid line shows the distribution of scores of 10^9 peptides that are randomly generated. The dots show the MS-DPR p-values. (B) Similar results for the *MultiStage* score defined in the multistage de novo sequencing paper, [4] for 10^7 peptides. Red dashed lines represent the scores of the correct peptide. The figure shows that MS-DPR p-values and empirical p-values are well correlated. Moreover, the p-value of the correct peptide is lower for multi-stage score $(5e-13)$ single-stage score $(5e-07)$, illustrating the advantage of multi-stage mass spectrometry. MS-DPR enables comparisons between arbitrary scoring functions. (C) Similar results for the score distribution for PSMs formed by the branch-cyclic peptide A21978C2 (single-stage MS).

Table 1

Comparison of theoretical p-value of cyclic PSM (*Peptide*,*CyclicSpectrum*(*Peptide*)), with the p-value estimated by MS-DPR with a million simulations.

Peptide	score	theoretical p-value	p_{DPR}
(10, 20, 40)	7	0.0025	0.0021
(10, 20, 40, 80)	13	1.42e-05	1.35e-05
(10, 20, 40, 80, 160)	21	2.59e-08	2.49e-08
(10, 20, 40, 80, 160, 320)	31	1.45e-11	1.09e-11
(10, 20, 40, 80, 160, 320, 640)	43	2.40e-15	6.49e-15
(10, 20, 40, 80, 160, 320, 640, 1280)	57	1.15e-19	2.71e-20

Table 2

Top score reconstructions of three cyclic and one linear peptides from the Cycloquest paper (A) Top score reconstruction of SFT-L1 peptide from a singly charged ion-trap spectrum. Correct reconstructions are shown in bold. PME stands for Parent Mass Error. (B) Top score reconstruction of SFTI-1 peptide from a singly charged spectrum. (C) Top score reconstruction of SKF peptide from a triply charged ion-trap spectrum. (D) Top score reconstruction of linear SDP peptide from a triply charged ion-trap spectrum. Note that the previous version of Cycloquest [5] (that lacked the algorithm for computing p-values) was unable to identify SFTI-1.

Peptide	score	p-value	PME	length	structure
GCIEGSPVCFPD	49	5.2e-11	0.036	12	cyclic
ICTQGNCQLEP	13	1.5e-7	0.069	11	linear
LNICCNVEVAQ	11	9.9e-6	0.105:x:x	11	linear
GRCTKSIPPICFPD	42	1.7e-7	0.024	14	cyclic
ICKQRVACWKNKG	36	8.7e-7	0.083	13	cyclic
KKCQKEVIENVCL	35	2.2e-6	0.082	13	cyclic
PSTHCWHHGMTHC	35	2.2e-6	-0.137	13	cyclic
PPMTTQCNICSFSS	10	0.00017	-0.092	14	linear
CMGCWASKSIAMTRVCALPHPAMRAI	167	1.7e-15	0.007	26	cyclic
GERTKVAGVKEANKENVKAWLKD	120	7.9e-12	-0.055	23	cyclic
ESLLKAVRSLEADVYHLELKDAA	119	1.1e-11	-0.077	23	cyclic
KEDAEKRVKSNLTLEAIAKAENL	119	1.1e-11	-0.045	23	cyclic
LGVLFIWLVAASIIKWRRFTY	16	6.6e-06	0.040	21	linear
CGLYAVCVAAGYLYVVGVNAVALQTAAAVTTAVWKYVAKYSS	39	9.1e-15	0.24	42	linear
CLLHDPKVLILDEPTNGLDPAGIREIRDHLKKLTRERG	180	3.4e-6	0.37	38	cyclic
YLPQLRGPMMIFTKVGRMSLTCYLLHSIIGTVLFLRY	168	9.9e-6	0.34	37	cyclic