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Virulence caught green-handed

Laura M. Sanchez and Pieter C. Dorrestein

Skaggs School of Pharmacy & Pharmaceutical Sciences, Departments of Pharmacology, Chemistry and Biochemistry, University of California - San Diego, La Jolla, California, USA

Pieter C. Dorrestein: pdorrestein@ucsd.edu

Abstract

Many of us eat mushrooms, but few of us have probably ever thought about — let alone witnessed — the epic battle of kingdoms that can occur between this delicacy and its bacterial pathogens. Now, imaging mass spectrometry has enabled the identification of a bacterium's potent antifungal weapon of choice.

Fungal infections are on the rise and there are limited treatments that are effective¹. There are only five classes of antifungals, the majority of which have been derived from natural sources². This gap in anti-infectives requires alternative strategies and new tools to discover effective therapeutic leads. One such strategy is through ecological therapeutic lead discovery. Ecological lead discovery establishes the native ecological role of specialized molecules first, and then natural products designed for a suitable biological function are evaluated for therapeutic potential. The rationale being that understanding their native ecological role will aide in identifying specialized metabolites that can be developed into therapeutic leads³.

Uncovering the native role of specialized metabolites and how they interact with the host is still challenging, even with current modern analytical methods. Until recently, there have been very few analytical methods for studying specialized metabolites in their native environments but tools that make this faster and easier are emerging. Imaging mass spectrometry (IMS) is one tool that has been developed to enable researchers to visualize the spatial distributions of molecules within a 2D sample, and therefore help map molecules to their ecological functions^{4–6}.

Writing in *Angewandte Chemie International Edition*, Christian Hertweck and co-workers now describe the use of a combination of advanced analytical methods to investigate the infection process of a mushroom⁷. Imagine mass spectrometry and genome-mining methodologies were employed to uncover the biological role of a single specialized metabolite from the mushroom pathogen *Janthinobacterium agaricidamnosum*. This specialized metabolite causes superficial lesions on mushroom tissue similar to those seen when the mushroom is infected with the pathogen *J. agaricidamnosum*. Furthermore, Hertweck and colleagues correctly hypothesized that any molecule responsible for killing the mushroom could be a potent fungicide against human pathogenic fungi.

To probe the ecological and therapeutic potential of *J. agaricidamnorum*, the bacterium was inoculated on slices of the button mushroom *Agaricus bisporus*. Once rot was induced, the mushroom slice was analysed by matrix-assisted laser desorption/ionization (MALDI)-IMS. The IMS data showed an unknown molecular ion with a mass-to-charge ratio (m/z) of 1,181. Computationally displaying the spatial distribution of the ion as a bright green overlay showed that this ion was only present in the infected regions of the mushroom. Co-localization with the rotting areas suggested the molecule responsible for the peak in the spectra might be a major factor involved in the infection process — and that the molecule could possess antifungal activities. Figure 1 shows a schematic representation of the process.

Although the ion was observed by IMS, the detection of the molecule from liquid cultures was not straightforward. Liquid fermentation studies were conducted to produce enough of the compound for a full structure elucidation. In a deviation from standard laboratory culture conditions, it was found that mushroom cubes needed to be added to the broth before the compound with an m/z of 1,181 could be detected. Subsequent use of a more complex media gave higher yields allowing for large-scale fermentation. High-resolution mass spectrometry revealed the molecular formula to be $C_{56}H_{85}N_{12}O_{16}$, (1,181 equal to the protonated molecular ion $[M+H]^+$), which was consistent with a non-ribosomal peptide based on molecular weight and elemental composition (that is, near equivalent numbers of oxygen and nitrogen atoms). This new metabolite was named jagaricin.

As the potential biosynthetic genes responsible for production of jagaricin had already been identified (the *jag* gene locus) before fermentation and IMS studies, it was possible to mine and identify the putative non-ribosomal peptide synthetase involved. A comparison of natural products produced by similar biosynthetic pathways and MS/MS data suggested that jagaricin was a cyclic lipopeptide, which allowed Hertweck and colleagues to predict the amino acid sequence. From MS/MS, 1D and 2D NMR spectroscopy, and Marfey's analysis — which uses chemical derivatization to determine stereochemistry — the linear structure with absolute configuration was assigned. However, the placement of the final ester bond remained elusive owing to a lack of correlations in the 2D NMR spectrum, but once again Hertweck and co-workers came up with a creative solution for this.

To identify which threonine residue was involved in cyclization, the *jag* genes were examined. The thioesterase domain (responsible for release from biosynthetic machinery via cyclization to form ester bonds in cyclic peptides) was compared with other thioesterase domains where the ring size of the peptide and genes have been previously identified. An examination of the similarities and differences between the genes revealed the *jag* thioesterase is similar to a group of thioesterases that produce cyclooctapeptides. This structure and position of cyclization was then confirmed through peracylation and NMR spectroscopic analysis of peracylated-jagaricin. Knockout mutants were constructed to provide further confirmation that the *jag* genes were responsible for jagaricin production, and no jagaricin could be detected when the knockout mutant was grown on mushroom tissue and examined with IMS.

Jagaricin's native biological role was examined by applying the pure compound to mushroom tissue. These tests showed it creates lesions similar to those seen in mushrooms

infected with a disease called soft rot. Further antimicrobial screening showed that jagaricin possesses potent antifungal effects against the common human fungal pathogens *Candida albicans*, *Aspergillus fumigatus* and *A. terreus*.

Connecting structure to function is notoriously difficult for microbially derived specialized metabolites, but Hertweck and co-workers have successfully demonstrated how IMS can be used to make this connection. Imaging mass spectrometry is a powerful tool that gives researchers rare glimpses of specialized metabolites in their native environments, especially when used in conjunction with efficient identification and characterization strategies such as genome mining. The sensitivity of mass spectrometry enables the detection of specialized metabolites that might not be discovered using standard laboratory culturing methods or by activity-guided isolations. Obtaining enough pure compound to determine the full structure can be a barrier when using IMS to guide discovery of new molecules; however, in this case, the liquid fermentation conditions were amenable to optimization.

Ecological therapeutic lead discovery, as exemplified by jagaricin, will be imperative in uncovering novel anti-infectives in the future. However, finding systems that can be adapted to the laboratory setting is crucial. Access to the host was critical in uncovering jagaricin; however ecological discovery becomes increasingly difficult if the biological organism is too large or cannot be cultured in a laboratory setting. Along these same lines, access to genomic data greatly increases the odds of success using ecological discovery — but is not necessarily required. Genome mining can aid in compound identification, but full structure elucidations are still necessary. As tools such as IMS and genome mining become more efficient in providing insights into the ecological roles of specialized metabolites, we may once again, after decades of neglect, gain the upper hand in the battle against human pathogens.

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Figure 1.

A schematic showing the procedure for identifying jagaricin using imaging mass spectrometry. The button mushroom *A. bisporus* was thinly sliced then inoculated with the bacterium *J. agaricidamnorum*. After infection, the mushroom slice was mounted, matrix was applied and subjected to MALDI-IMS (the laser desorption/ionization process is represented by a lightning bolt). Analysis of the spectra showed a molecular ion with $m/z = 1,181$ $[M+H]^+$ (highlighted in green) that is only present in the infected areas — as demonstrated by the green area on the mushroom.