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Enzymatic Synthesis Assisted Discovery of Proline-Rich Macrocyclic Peptides in Marine Sponges

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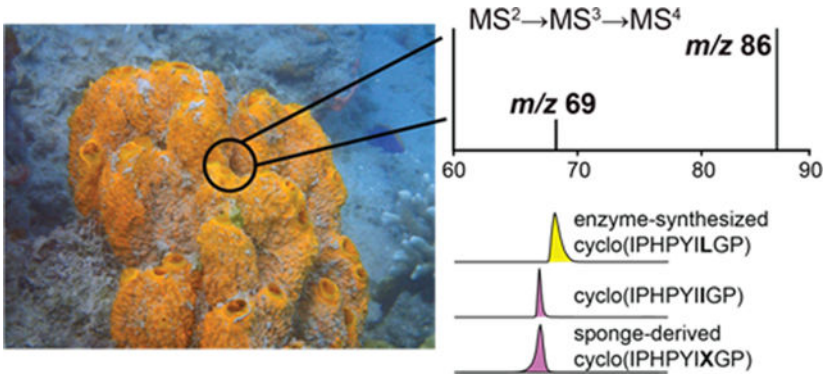
Abstract

Proline-rich macrocyclic peptides (PRMPs) are natural products present in geographically and phylogenetically dispersed marine sponges. The large diversity and low abundance of PRMPs in sponge metabolomes precludes isolation and structure elucidation of each individual PRMP congener. Here, using standards developed via biomimetic enzymatic synthesis of PRMPs, a mass spectrometry-based workflow to sequence PRMPs was developed and validated to reveal that the diversity of PRMPs in marine sponges is much greater than that has been realized by natural product isolation-based strategies. Findings are placed in the context of diversity-oriented transamidative macrocyclization of peptide substrates in sponge holobionts.

Graphical Abstract



By adopting an integrated approach of chemoenzymatic synthesis of standards and HCD-MS^n fragmentation, we have sequenced proline-rich cyclopeptide natural products from marine sponges. Our MS^n fragmentation workflow successfully differentiated between leucine and isoleucine residues in the cyclopeptides and can be used to sequence low abundance cyclic peptides from sub-gram amounts of sponge biomass.

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Keywords

chemoenzymatic synthesis; cyclopeptides; fragmentation; marine sponge

Macrocyclization of ribosomal and non-ribosomal peptides is ubiquitous in natural product chemistry. Macrocyclization imparts pharmacologically desirable properties of stability and rigidity which reduces the entropic cost associated with target binding.[1] From neutrophil defensins and snake venom toxins to cyclic peptide natural products produced by bacteria, across all scales of life, macrocyclic peptides demonstrate potent bioactivities and serve various different biological roles.[2] Benthic marine invertebrates- sponges, are prolific sources of proline rich macrocyclic peptides (PRMPs).[3] PRMPs are detected in marine sponges without restriction of geographical location and are widespread among several sponge phyla. Cell growth inhibitory cytotoxic activities of sponge derived PRMPs has sustained interest in their development as drug candidates.[3] Unlike other natural product chemical classes, the abundance of which dominates the sponge metabolomes, PRMPs in sponges are present in trace amounts. For instance, in *Stylissa* spp. sponges the metabolomes of which are enriched in pyrrole imidazole alkaloids (PIAs),[4] PRMPs were isolated in 0.01% yields (PRMP weight/sponge dry weight).[5] Low abundance of PRMPs interspersed with much higher abundance other natural products in sponge extracts encumbers their structure elucidation using natural product isolation-based strategies. Here, we devise a mass spectrometry-based workflow to sequence sponge-derived PRMPs and validate the spectrometric sequencing using standards furnished by biomimetic enzymatic peptide macrocyclization.

We previously described the detection of PIAs in *Stylissa* and *Axinella* spp. sponges using LC/MS-based untargeted metabolomics.[6] Screening these LC/MS data using the DEREPLICATOR tool[7] hinted at the presence of peptidic natural products in *Stylissa* and *Axinella* spp. extracts consistent with prior reports describing the isolation of PRMPs from these sponge genera.[3] The low abundance of PRMPs and sub-gram amounts of dry sponge biomass which were available to us precluded a natural product isolation-based effort. Thus, we decided to pursue PRMP structure elucidation from mass spectrometry fragmentation data.

In a proline containing peptide, fragmentation preferentially occurs at the N-terminus of the Pro residue.[8] Sequencing PRMPs detected in sponge extracts thus began with the annotation of the ^{N}Pro-Xaa^{C} dipeptide oxonium MS^2 fragment ions per the inventory shown in Table S1. Proceeding from the ^{N}Pro-Xaa^{C} dipeptide, to recover the PRMP sequence, we annotated mass shifts corresponding to proteinogenic amino acids till we reached the parent ion in the MS^2 spectra. As shown in Fig. 1A for a PRMP detected in *Axinella* sp., PRMPs containing more than one proline residue yielded multiple ^{N}Pro-Xaa^{C} dipeptide MS^2 fragments. Progressing from each of these different dipeptide fragments, ^{N}Pro-Phe^{C} (MS^2 *m/z* 245) and ^{N}Pro-Glu^{C} (MS^2 *m/z* 227), the same cyclic sequence, cyclo(FFPE**X**WP), was recovered. Here, **X** denotes Leu/Ile residues that cannot be differentiated based on MS^2 fragmentation alone. Identities of the amino acid constituents in cyclo(FFPE**X**WP), Phe,

Pro, Glu, Leu/Ile, and Trp, were supported by the detection of the individual amino acyl immonium ions in the MS^2 spectra (Fig. S1, Table S2).

To resolve Leu and Ile residues, we developed standards for the two possible PRMPs–cyclo(FFPE**L**WP) and cyclo(FFPE**I**WP). For this, synthetic linear peptides FFPE**L**WPFQA and FFPE**I**WPFQA were obtained. The C-terminal FQA tripeptide was proteolyzed and transamidatively macrocyclized products were furnished by the plant peptidase PCY1.[9] Upon fragmentation of the cyclo(FFPE**L**WP) and cyclo(FFPE**I**WP) products, the MS^2 immonium ion for Leu/Ile, *m/z* 86, was isolated and fragmented. Consistent with prior reports,[10] fragmentation of the Ile-derived MS^2 *m/z* 86 immonium ion generated a characteristic MS^3 *m/z* 69 product ion in high abundance, while the MS^3 *m/z* 69 product ion was detected in lesser abundance when the MS^2 *m/z* 86 precursor ion was derived from Leu. By modulating the MS^3 fragmentation energy (Fig. S2), we generated an unambiguous binary result in which the MS^3 *m/z* 69 product ion was observed only for Ile (Fig. 1B), but not observed for Leu (Fig. 1C). Adopting the thus optimized methodology for the cyclo(FFPE**X**WP) PRMP detected in the sponge extract, we annotated **X** as Leu (Fig. 1D). Validation of the mass spectrometry-based result was obtained by spiking the sponge extract with enzymatically synthesized cyclo(FFPE**L**WP) and cyclo(FFPE**I**WP) standards; the cyclo(FFPE**L**WP) standard coeluted with the sponge-derived PRMP (Fig. 1E) which we term as axinellin D. PRMP congeners detected in this study are highlighted in Table S3.

Employing the now validated mass spectrometry workflow which could differentiate between Leu and Ile residues, we additionally sequenced the PRMPs stylissatin E cyclo(FVPELWP) and stylissatin F cyclo(FPWVPLTP) from *Stylissa* sp. specimens used in this study (Fig. 1F, S3–6, Table S3). We also detected the presence of the PRMPs cyclo(FGPELWP), cyclo(PAVMLRP), and cyclo(FPLTVPWP) in *Axinella* sp. extracts (Fig. S7–12). Primary sequences of these PRMPs correspond to the previously described hymenamide C, hymenamide F, and axinellin B. The presence of axinellin B serves as an internal control as it has been reported from an *Axinella* sponge previously.[11] However, hymenamides C and F were isolated from *Hymeniacidon* sp. sponges (Table S3).[12] *Hymeniacidon* sp. sponges belong to a different phylogenetic order (Suberitidia) as compared to *Axinella* sp. (order Axinellida) within the Demospongiae class of marine sponges.

Abovementioned workflow is applicable when a single Leu/Ile is present in the peptide sequence. When more than one Leu/Ile residues are present, multiple side chains will contribute to MS^2 *m/z* 86 ion which will complicate the $MS^2(86) \rightarrow MS^3$-based Leu/Ile distinction. This scenario presents itself for the cyclo(FYS$\mathbf{X^1}$A$\mathbf{X^2}$P) PRMP that we detected in *Axinella* sp.; $\mathbf{X^n}$ denote Leu/Ile residues. As before, the PRMP was sequenced starting from the ^{N}Pro-Phe^{C} MS^2 *m/z* 245 fragment ion (Fig. 2A, S13). Next, the ion corresponding to the fragment PFYS$\mathbf{X^1}$A, MS^2 *m/z* 679 (Fig. 2A) was isolated and the MS^3 *m/z* 86 Leu/Ile immonium ion was generated. Here, only a single residue, $\mathbf{X^1}$, contributes to the production of the MS^3 *m/z* 86 product ion. Further fragmentation of the MS^3 *m/z* 86 ion did not generate the characteristic MS^4 *m/z* 69 ion (Fig. 2B), denoting that $\mathbf{X^1}$ was Leu.

Next, the cyclo(FYS**L**A**L**P), cyclo(FYS**I**A**L**P), cyclo(FYS**L**A**I**P), and cyclo(FYS**I**A**I**P) standards were generated by PCY1-mediated macrocyclization of the corresponding -FQA appended linear peptides. Fragmenting cyclo(FYS**I**A**L**P) and cyclo(FYS**I**A**I**P) using the $MS^2(679)\rightarrow MS^3(86)\rightarrow MS^4$ procedure yielded the characteristic MS^4 *m/z* 69 ion while fragmentation of cyclo(FYS**L**A**L**P) and cyclo(FYS**L**A**I**P) did not (Fig. S14). To query the identity of $\mathbf{X^2}$ in the PRMP cyclo(FYS$\mathbf{X^1}$A$\mathbf{X^2}$P), the MS^2 *m/z* 185 ion was isolated. This MS^2 ion can be generated by the $\mathbf{X^1}$A, or the A$\mathbf{X^2}$ dipeptides. The $MS^2(185)\rightarrow MS^3(86)\rightarrow MS^4$ fragmentation scheme demonstrated the production of the characteristic MS^4 *m/z* 69 ion (Fig. 2C). As $\mathbf{X^1}$ was Leu (and hence the $\mathbf{X^1}$A fragment could not contribute to the production of the MS^4 *m/z* 69 product ion), these data denote that $\mathbf{X^2}$ was Ile. Analogous $MS^2(185)\rightarrow MS^3(86)\rightarrow MS^4$ fragmentation for the cyclo(FYS**L**A**I**P) standard generated the MS^4 *m/z* 69 ion while the cyclo(FYS**L**A**L**P) standard did not (Fig. S15). Validation of the mass spectrometry-based PRMP sequencing scheme was obtained by spiking the sponge extract with the PCY1 generated standards; the cyclo(FYS**L**A**I**P) standard coeluted with the sponge PRMP which is termed axinellin E (Fig. 2D, Table S3).

Using the workflow developed above, we additionally sequenced the multiple Leu/Ile containing PRMPs axinellin F cyclo(FFPELLP) from *Axinella* sp., and stylissatin G cyclo(WLPLTPLP) from *Stylissa* sp. (Fig. 2E, Fig. S16–19, Table S3). In addition to these PRMPs, we detected the presence of the previously described PRMPs hymenamide H[13]- cyclo(WVPLTPLP) and phakellistatin 18[14]- cyclo(YPIFPIP) from *Stylissa* sp. (Fig. 2E, Fig. S20–23). Hymenamide H was described from *Hymeniacidon* sp. (order Suberitidia) while phakellistatin 18 was described from *Phakellia fusca* (order Axinellida), sponges belonging to orders different from *Stylissa* sp. (order Scopalinida). Together with the detection of hymenamide C in *Axinella* sp., it is evident that PRMP congeners are shared among phylogenetically different sponges.

Unlike axinellin F, cyclo(FFPELLP), where Ile residues occur next to Leu/Ile residues in a PRMP sequence, mass spectrometry alone may be insufficient to provide unambiguous results. This scenario presented itself during the sequencing of the PRMP cyclo($\mathbf{X^1}$PHPY$\mathbf{X^2X^3}$GP) that we detected in *Axinella* sp. As before, the PRMP was sequenced starting from the ^{N}Pro-His^{C} (*m/z* 235), ^{N}Pro-Tyr^{C} (*m/z* 261), and ^{N}Pro-$\mathbf{X^1}^{C}$ (*m/z* 211) dipeptide MS^2 ions (Fig. 3A). Using PCY1 and appropriately designed linear peptide substrates, we generated standards for all eight possible Leu/Ile combinations for cyclo($\mathbf{X^1}$PHPY$\mathbf{X^2X^3}$GP). Residue $\mathbf{X^1}$ was annotated as Ile by isolating the MS^2 *m/z* 211 ion corresponding to the P$\mathbf{X^1}$ dipeptide and following the $MS^2(211)\rightarrow MS^3(86)\rightarrow MS^4$ fragmentation scheme (Fig. 3B). Annotation of $\mathbf{X^1}$ as Ile was supported by analogous fragmentation of the cyclo(**L**PHPY$\mathbf{X^2X^3}$GP) and cyclo(**I**PHPY$\mathbf{X^2X^3}$GP) standards (Fig. S24–25). Similarly, identity of $\mathbf{X^2}$ as Ile was decided by isolating the MS^2 *m/z* 608 ion corresponding to the PHPY$\mathbf{X^2}$ pentapeptide and following the $MS^2(608)\rightarrow MS^3(86)\rightarrow MS^4$ fragmentation scheme (Fig. 3C) and comparing the MS^4 spectra to cyclo(**I**PHPY**L**$\mathbf{X^3}$GP) and cyclo(**I**PHPY**I**$\mathbf{X^3}$GP) standards (Fig. S26). Because $\mathbf{X^2}$ was Ile (which generates the MS^4 *m/z* 69 diagnostic ion) and no MS^2 fragment could be detected which contained only $\mathbf{X^3}$ without $\mathbf{X^1}$ or $\mathbf{X^2}$, identity of $\mathbf{X^3}$ could not be determined by mass spectral fragmentation. To annotate $\mathbf{X^3}$, retention times for the cyclo(**I**PHPY**IL**GP) (X^1,X^2=Ile;

X^3=Leu) and cyclo(**I**PHPY**II**GP) (X^1,X^2=Ile; X^3=Ile) standards were compared to reveal the sponge PRMP as cyclo(**I**PHPY**II**GP) which we term axinellin G (Fig. 3D, Table S3). Among the inventory of PRMPs, cyclo-nonapeptides such as axinellin G are relatively rare (Table S3). Overall, we demonstrate the utility of enzymatic synthesis of cyclic peptide standards for discovery and sequencing of sponge PRMPs. At present, we cannot determine the *cis*/*trans* conformation of the Pro residues.

Amino acid residues reported to date for sponge PRMPs are proteinogenic amino acids in the L- configuration (to the best of our knowledge the only exception is Trp derived L-kynurenine in phakefustatins A–C[15]). It is thus conceivable that sponge-derived PRMPs are ribosomally synthesized and posttranslationally modified peptides[16] and that the PCY1 catalyzed proteolysis of a C-terminal recognition sequence (RS in Fig. 4A; PCY1 RS is FQA[9]) and intramolecular transamidative macrocyclization mimics their physiological biosynthetic route. Peptide macrocyclization by prolyl oligopeptidases and cyanobactin macrocyclases is well established.[16] A recurring feature in biosynthesis of macrocyclic peptides is the requirement of a proline residue or a Ser/Thr/Cys-derived azol(in)e heterocycle immediately upstream of the RS (shaded green in Fig. 4A).[9, 17] Organizing the sponge PRMP octa- and heptapeptide sequences containing a single Pro residue such that the Pro is at the C-terminus leads to the observation that the transamidating N-terminal residues in PRMP linear peptides are hydrophobic amino acids with Trp, Phe, and Tyr predominating (Fig. 4B). Further organizing PRMP sequences containing multiple Pro residues in this sequence alignment then identifies position 2 to be a site for hypervariability.

It is as yet unknown whether the sponge host or a bacterial symbiont produces PRMPs in sponge holobionts. To date, natural products that have been shown to be produced by bacterial symbionts within marine sponges are all derived from high microbial abundance (HMA) sponges.[18] As microbial diversity correlates with microbial abundance for sponge microbiomes that are not dominated by cyanobacteria,[19] PRMP harboring sponges of the genera- *Axinella*, *Stylissa*, *Phakellia*, and *Hymeniacidon* spp. are all LMA sponges (Fig. 4C).

The diversity of natural products in marine sponges is much greater than that has been realized.[21] Here, with the sequencing of 12 macrolactam cyclopeptide sequences of which seven were novel PRMP sequences including a rare cyclononapeptide (Table S3), we demonstrate that inventorying natural product diversity using mass spectrometry and supplementing structural information using enzymatic synthesis can rapidly expand the natural product chemical space without recourse to sacrificing large amounts of sponge biomass which is otherwise necessary for the isolation of low abundance molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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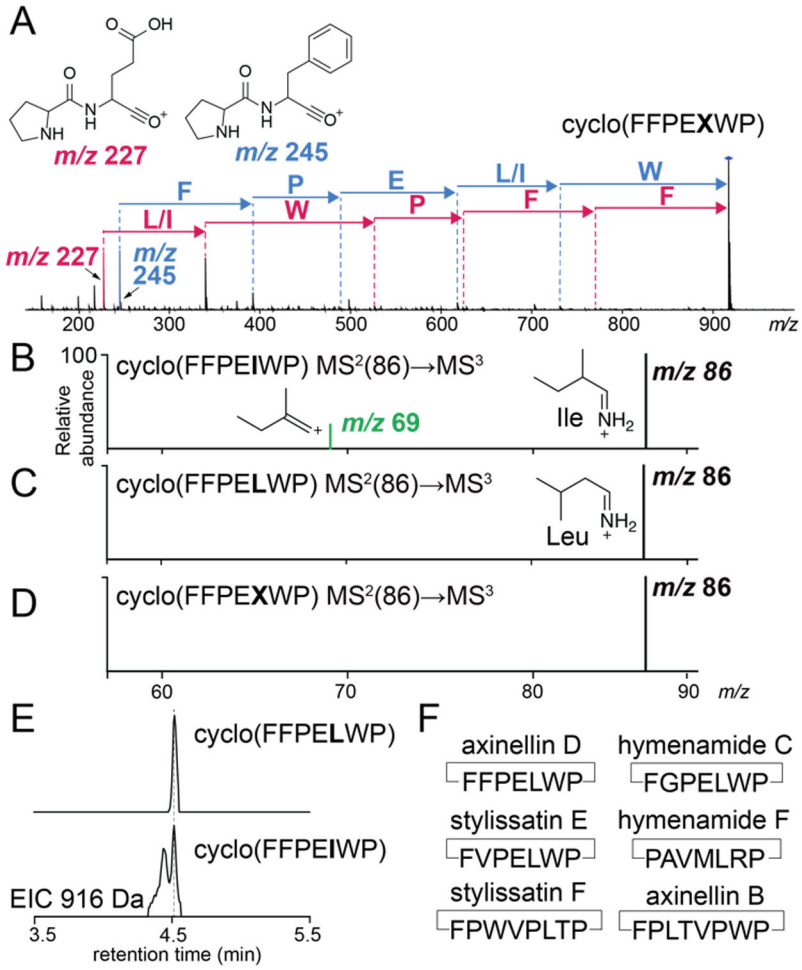

Figure 1.

(**A**) MS^2 spectra for cyclo(FFPE**X**WP). $MS^2(86)\rightarrow MS^3$ spectra for the (**B**) Ile-derived MS^2 *m/z* 86 precursor ion (cyclo(FFPE**I**WP) standard), (**C**) Leu-derived MS^2 *m/z* 86 precursor ion (cyclo(FFPE**L**WP) standard), and the (**D**) Leu/Ile-derived MS^2 *m/z* 86 precursor ion (cyclo(FFPE**X**WP) PRMP). (**E**) Extracted ion chromatograms (EICs) showing co-injection of cyclo(FFPELWP) (top) and cyclo(FFPEIWP) (bottom) standards with the sponge extract. (**F**) Inventory of single Leu/Ile containing PRMPs sequenced in this study.

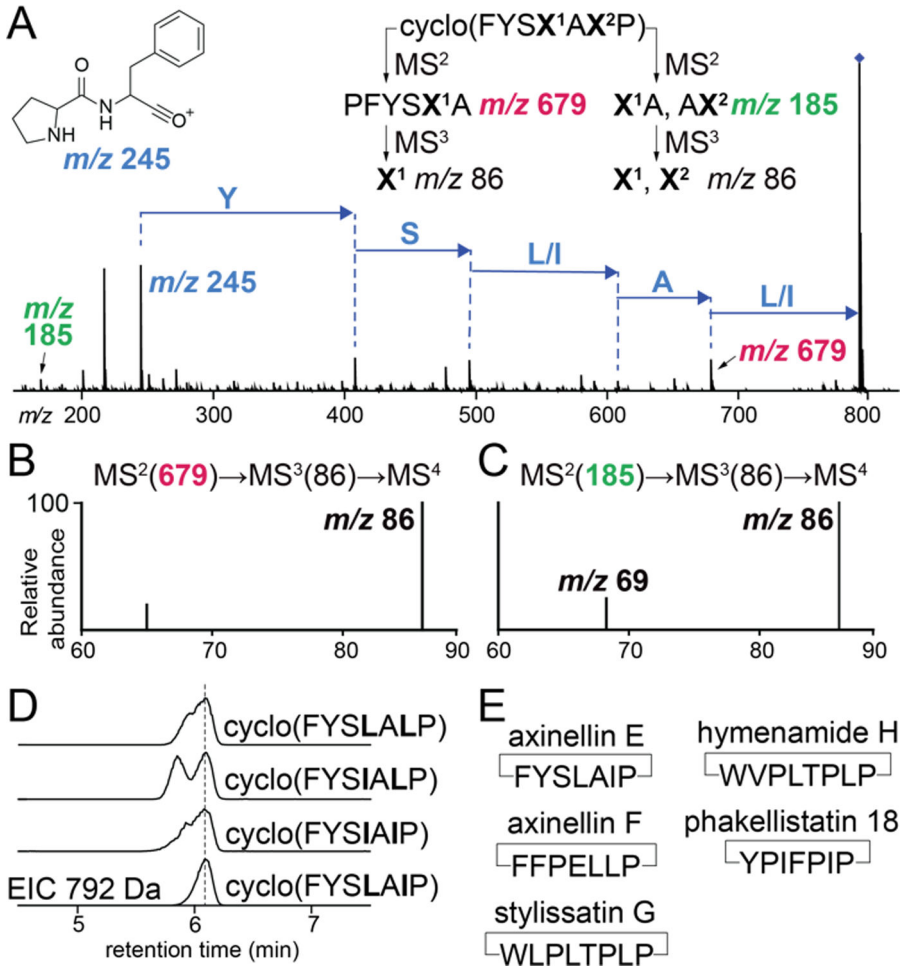

Figure 2.

(**A**) MS^2 spectra for the PRMP cyclo(FYS**X**^{1}A**X**^{2}P) detected in *Axinella* sp. where **X** corresponds to Leu/Ile. MS^n fragmentation strategies to query **X**^{1} and **X**^{2} identities are illustrated. (**B**) Confirmation of **X**^{1} as Leu as MS^4 *m/z* 69 product ion is not detected. (**C**) Confirmation of **X**^{2} as Ile as MS^4 *m/z* 69 product ion is detected. (**D**) EICs showing co-injection of enzymatically synthesized PRMP standards with the sponge extract. (**E**) Inventory of multiple Leu/Ile containing PRMPs sequenced in this study.

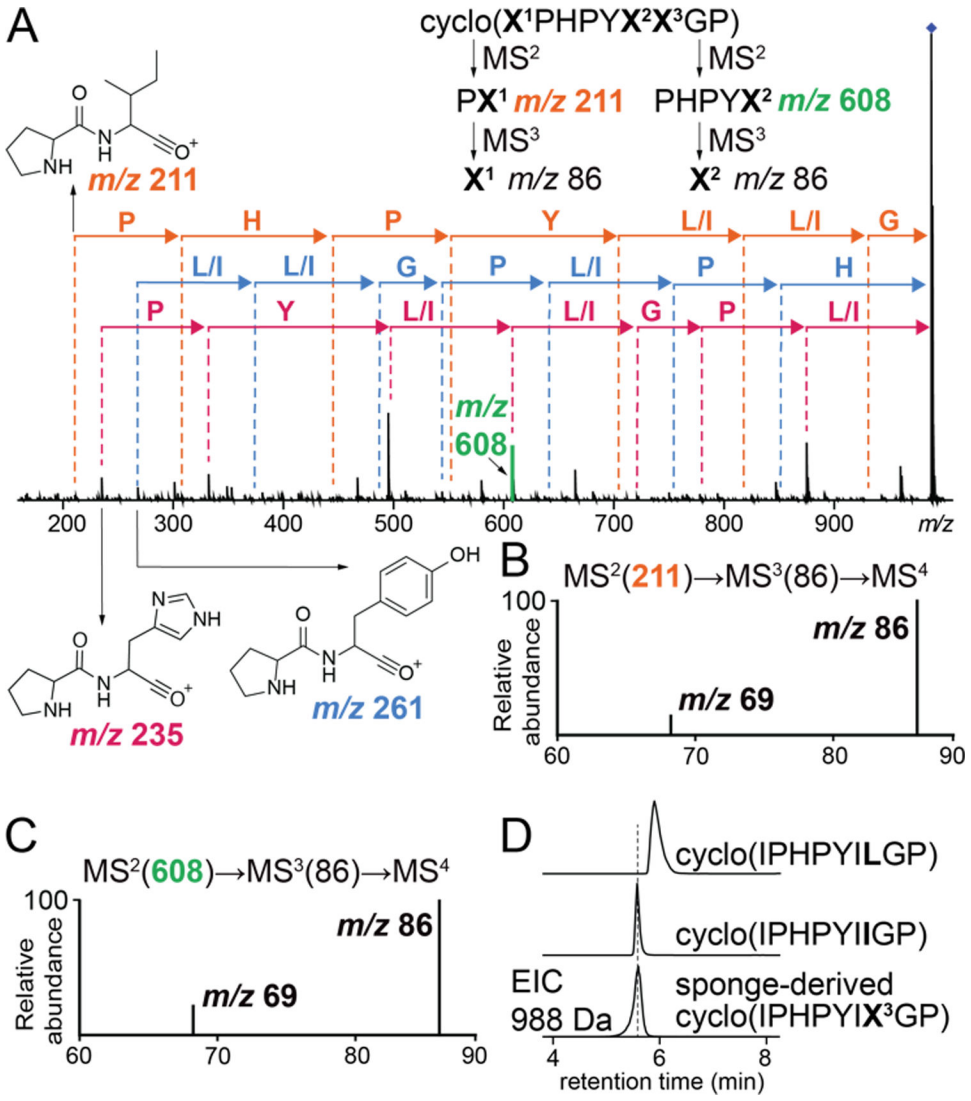

Figure 3.

(**A**) Sequencing of the cyclo(**X**^{1}PHPY**X**^{2}**X**^{3}GP) based on MS^{2} spectra. Note that three Pro residues in this octapeptide generate three different ^{N}Pro-Xaa^{C} MS^{2} dipeptide product ions. Fragmentation strategies to query identities of **X**^{1} and **X**^{2} residues are illustrated. Annotation of (**B**) **X**^{1} and (**C**) **X**^{2} residues as Ile by the detection of the MS^{4} *m/z* 69 product ion. (**D**) Annotation of **X**^{3} as Ile by retention time comparison of the sponge detected PRMP against enzymatically synthesized cyclo(IPHPYILGP) and cyclo(IPHPYIIGP) standards.

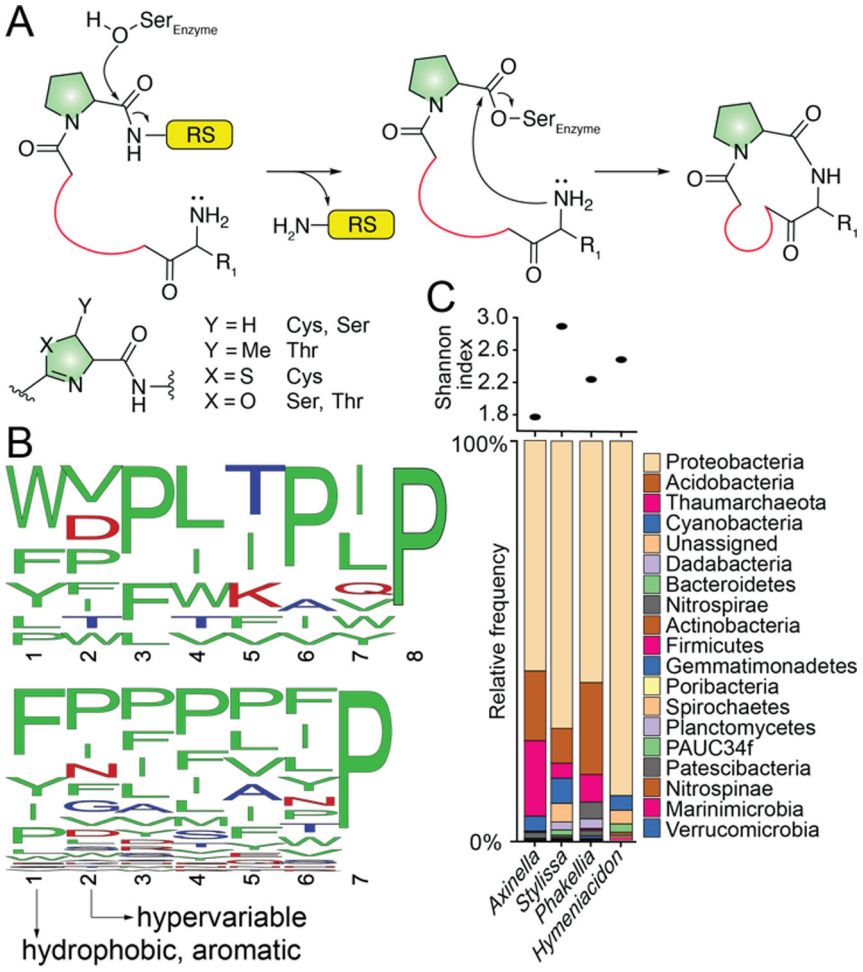

Figure 4.

(**A**) Intramolecular transamidative peptide cyclization catalyzed by serine proteases such as PCY1. (**B**) WebLogo representation of PRMP octa- (top) and heptapeptides (bottom) with a C-terminal Pro. (**C**) Representative phylum-level microbiome architectures for *Axinella*,[6] *Stylissa*,[6] *Phakellia*,[20] and *Hymeniacidon*[20] spp. sponges. Microbiome diversity is denoted by Shannon indices. Shannon indices for HMA sponges are typically greater than 4.