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Genome Mining Reveals High Topological Diversity of ω-Ester-Containing Peptides and Divergent Evolution of ATP-Grasp Macrocyclases

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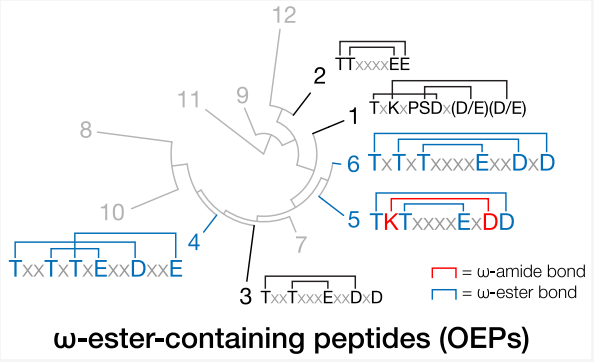
ABSTRACT: ω-Ester-containing peptides (OEPs) are a family of ribosomally synthesized and post-translationally modified peptides (RiPPs) containing intramolecular ω-ester or ω-amide bonds. Although their distinct side-to-side connections may create considerable topological diversity of multicyclic peptides, it is largely unknown how diverse ring patterns have been developed in nature. Here, using genome mining of biosynthetic enzymes of OEPs, we identified genes encoding nine new groups of putative OEPs with novel core consensus sequences, disclosing a total of ∼1500 candidate OEPs in 12 groups. Connectivity analysis revealed that OEPs from three different groups contain novel tricyclic structures, one of which has a distinct biosynthetic pathway where a single ATP-grasp enzyme produces both ω-ester and ω-amide linkages. Analysis of the enzyme cross-reactivity showed that, while enzymes are promiscuous to nonconserved regions of the core peptide, they have high specificity to the cognate core consensus sequence, suggesting that the enzyme−core pair has coevolved to create a unique ring topology within the same group and has sufficiently diversified across different groups. Collectively, our results demonstrate that the diverse ring topologies, in addition to diverse sequences, have been developed in nature with multiple ω-ester or ω-amide linkages in the OEP family of RiPPs.



INTRODUCTION

Natural products have served as the main source of therapeutic leads by virtue of chemical and functional diversity.[1] With the explosion of available genome sequences, bioinformatic approaches are now widely used to explore novel natural products.[2] Analysis of genes encoding homologous proteins of known biosynthetic enzymes and their neighboring genes enables the identification of the biosynthetic gene clusters (BGCs) for new natural products and the prediction of their structures. This approach has been successfully applied to ribosomally synthesized and post-translationally modified peptides (RiPPs), a rapidly growing class of natural products.[3−5] RiPPs are initially synthesized as precursor peptides by the ribosome and subsequently undergo enzymatic modifications, such as macrocyclization and backbone modification, which lead to biological activity and metabolic stability.[6,7] The precursor peptide is mainly composed of leader and core peptides, which are sites for enzyme recognition and modification, respectively. Each subfamily of RiPPs displays a distinct chemical modification on the precursor peptide, illustrating the high chemical diversity of RiPPs.

Several genome-mining methods have been developed to explore novel RiPPs *in silico*[8−12] and demonstrate that putative BGCs for RiPPs are widely distributed in nature. More focused analysis on each RiPP subfamily using bioinformatic tools and biochemical characterization not only expands the chemical space of RiPPs but also enhances our understanding of biosynthetic pathways in a reliable manner.[13−22] In particular, the characterization of a phylogenetically distinct but unattended subgroup often uncovers a novel modification reaction and linkage,[21−24] revealing that much remains to be discovered.

Microviridins are small tricyclic RiPPs that contain intramolecular ω-ester and ω-amide bonds between Thr/Ser/Lys and Glu/Asp in the conserved TxKYPSDx(E/D)(D/E) core motif (Figure 1A).[25,26] Unlike the lasso peptides in which the end-to-side connection generates a macrolactam ring, microviridins have only side-to-side macrocyclic linkages, which enable the introduction of multiple rings into a short peptide. Recently, we reported that the microviridin-like modifications are also found in two groups of RiPPs with novel bicyclic

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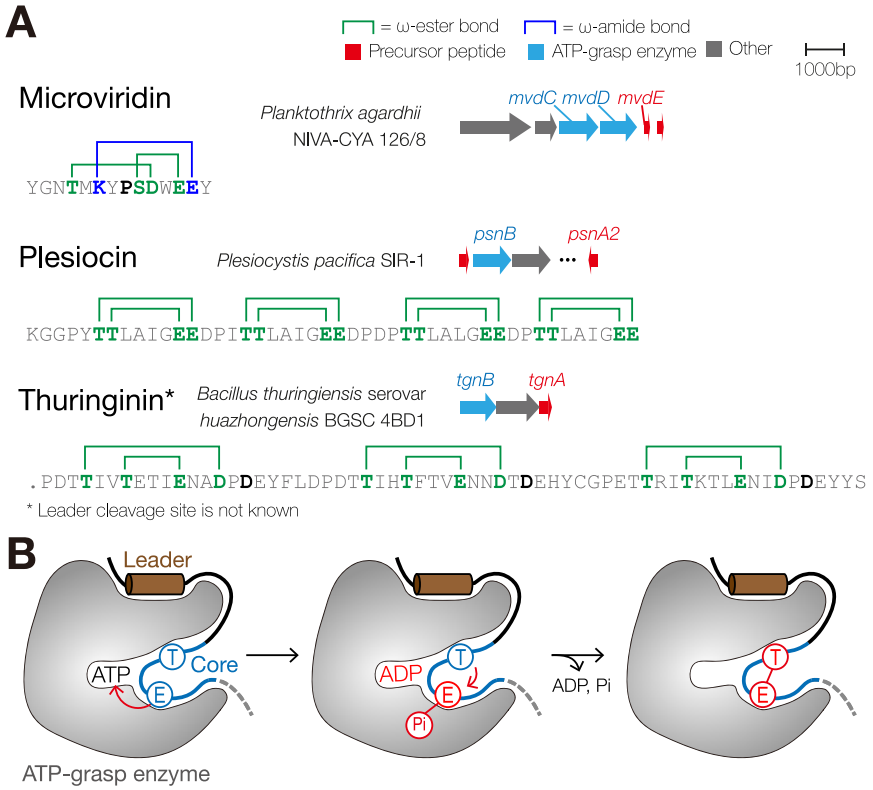

Figure 1. Overview of OEPs. (A) Structures and biosynthetic gene clusters (BGCs) of three representative OEPs with distinct ring topologies. Genes encoding the precursor peptide and the ATP-grasp enzyme are colored red and light blue, respectively. The green and blue lines indicate *ω*-ester and *ω*-amide linkages, respectively. (B) Mechanism of macrocyclization in OEP biosynthesis. When a precursor peptide and an ATP bind to an ATP-grasp enzyme (left), the carboxyl side chain of Glu (or Asp) is phosphorylated (middle). Then, the hydroxyl group of Thr (or that of Ser or the *ε*-amine group of Lys) attacks the mixed carboxylate−phosphate anhydride to generate the *ω*-ester (or *ω*-amide) bond (right).

structures, plesiocins[27] and thuringinins,[28] which contain distinct TTxxxxEE and TxxTxxxExxDxD core motifs, respectively (Figure 1A). Therefore, we proposed that microviridins, plesiocins, and thuringinins compose an expanded RiPP family, termed *ω*-ester-containing peptides (OEPs). ATP-grasp enzymes bind to the leader region of the precursor peptide and mediate the cross-linking reaction of the core region by phosphorylating the carboxyl side chain of Asp or Glu with ATP (Figure 1B).[26,29] By an extensive bioinformatic analysis of several amide-bond-forming enzymes, Aravind and colleagues previously suggested that a subset of ATP-grasp enzymes is likely to be involved in the modification of ribosomally synthesized peptides.[30] However, this study reported only a small number of BGCs and does not encompass the large amount of genomic data generated in the last 10 years. More recent bioinformatic analysis focused only on microviridins,[20] leaving an open question concerning the natural scope of OEPs. In particular, although the multiple side-to-side connections have a potency to generate topologically diverse macrocyclic peptides that vary in the number and size of rings, it is largely unknown what kind of distinct OEPs are present in nature, besides the three OEP groups mentioned above.

Here, through the genome-mining approach, we identified genes for nine new groups of putative OEPs containing novel core consensus sequences, which significantly expand the OEP family to now include a total of ∼1500 members in 12 groups. Connectivity analysis revealed that members of three new groups, designated as groups 4, 5, and 6, indeed present novel tricyclic architectures. In contrast to microviridins, group 5 OEPs show a unique biosynthetic pathway where a single ATP-grasp enzyme creates two *ω*-ester and one *ω*-amide bond. We also analyzed the cross-reactivity of the ATP-grasp enzymes and found that an ATP-grasp enzyme in each group generally has high specificity to peptides with a cognate core consensus sequence. These results suggest that the ring topology of OEPs is determined not only by the core sequence but also by the ATP-grasp enzyme.

RESULTS AND DISCUSSION

Bioinformatic Analysis Significantly Expands the OEP Family. To broaden the scope of OEPs, we first identified new gene clusters that have a high chance of synthesizing OEPs. The minimal components in the biosynthetic gene cluster of OEPs are genes encoding a precursor peptide that contains a distinct consensus sequence for *ω*-ester or *ω*-amide formation and an ATP-grasp enzyme for macrocyclization. New members of a RiPP family have often been identified by searching for proteins homologous to enzymes that mediate the class-defining modification.[13−22] Therefore, with the four known ATP-grasp enzymes (MvdC/MvdD, PsnB, and TgnB for biosynthesis of micoriviridin, plesiocin, and thuringinin, respectively) as individual queries, we used Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) to find homologous enzymes.[31] In searches using each enzyme as a query, the threshold of 10^{-35} was used to retrieve both the other three query enzymes and putative ATP-grasp enzymes that are more likely to be involved in OEP biosynthesis. This procedure yielded a total of 5276 nonredundant homologous proteins (Figure S1).

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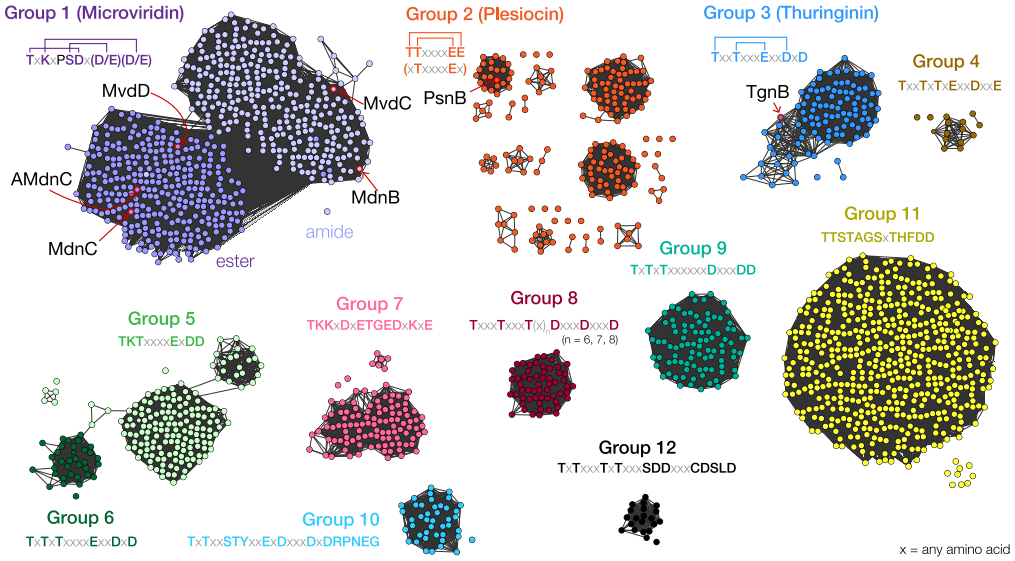

Figure 2. A sequence similarity network (SSN) of 2005 ATP-grasp enzymes in 12 OEP groups (alignment score = 70). Nodes are colored by the core consensus sequence. Each group has a distinct core consensus sequence (x means any amino acid). Two distinct ATP-grasp enzymes of microviridin are colored purple (ester) and light purple (amide), respectively. Some representative nodes that produce known OEPs are highlighted by red circles and arrows.

Biosynthetic enzymes in RiPPs have often strongly coevolved with precursor peptides.[16−22,32] To investigate the evolutionary relationship between the ATP-grasp enzymes and the precursor peptides in OEPs, we constructed a phylogenetic tree of 5276 homologous enzymes using the maximum-likelihood method (Figure S1) and searched for the precursor genes that contain the core consensus sequence of microviridin, plesiocin, or thuringinin within 3 kbp from the ATP-grasp genes. The precursor genes of each OEP group mostly cluster in distinct branches of the phylogenetic tree where the known ATP-grasp enzyme in the same group is found (Figure S1). This suggests that the ATP-grasp enzymes of three OEP groups have coevolved with the core peptides and that the examination of other branches of the phylogenetic tree may help find the precursor peptides with a novel core consensus sequence.

To test the idea, we extracted sequences of all proteins with less than 200 residues that are encoded within 3 kbp of the genes encoding 5276 homologous proteins and inspected if those within a specific clade contain any highly conserved sequence patterns mainly constituted of Thr/Ser/Lys and Glu/Asp by sequence alignment. We could identify nine new putative groups of OEPs with novel core sequences, with which the maximum of 2−5 ω-esters or ω-amides are expected in a single core repeat (Figures S1 and S2). For convenience, we designate group numbers for groups containing 10 or more members as follows: First, microviridins, plesiocins, and thuringinins are referred to as group 1, 2, and 3 OEPs. Second, three new groups in which consensus sequences are often presented three or four times in each precursor peptide are assigned to groups 4, 5, and 6. Finally, the remaining six new groups are numbered groups 7 to 12. Members in the 12 groups are associated with 2005 ATP-grasp enzymes and 1504 nonredundant precursor peptides in 1728 unique BGCs (Figure S2). Several BGCs can contain the same precursor peptide sequence but have different ATP-grasp enzymes, resulting in the discrepancy between numbers of BGCs and precursor peptides. For example, group 11 presents 534 BGCs, but contains only 293 nonredundant precursor peptides. Although we could not find any noticeable consensus sequence in peptides that are associated with the remaining ∼3200 homologous enzymes, it is highly probable that those presumably containing only one macrocycle, a relatively large protein domain, or variable core sequences, easily escape our inspection but still have the same modifications to compose novel OEP groups. Alternatively, some ungrouped ATP-grasp enzymes may be involved in the primary metabolic pathways.[30]

The 12 Groups Suggest Divergent Evolution of OEPs. To obtain the overview of the 12 groups, we constructed a sequence similarity network (SSN) of 2005 ATP-grasp enzymes in which nodes were colored based on the core consensus sequence of the precursor peptides (Figure 2). The SSN clearly illustrates coevolution of the ATP-grasp enzymes and the precursor peptides in the 12 groups, with minor variations in some groups. The group 1 OEPs, microviridins, compose the second largest group of OEPs with 438 nonredundant precursors in 335 BGCs, most of which were previously reported.[20] The two ATP-grasp enzymes for ω-ester and ω-amide formation in this group are present in two separate clusters. The group 2 OEPs, plesiocins, compose the most fragmented or diversified group with 203 precursors in 204 BGCs. The detailed analysis of this group based on the conserved sequences of the leader or core peptides allows us to divide it into six subgroups, 2a−2f (Figure S3). Of note, groups 2b, 2d, and 2f do not have the conserved leader sequence, and groups 2e and 2f present only one Thr/Glu pair that forms the inner ring of plesiocin.

The group 3 OEPs, thuringinins, and the group 4 members share two conserved Thr, one Glu, and one Asp, while the latter has one additional Thr and Glu. ATP-grasp enzymes of these two groups also cluster in two adjacent clades in the phylogenetic tree (Figures S2 and S4), indicating that the group 3 and 4 OEPs may have a relatively close relationship. We also distinguish a three-membered subgroup, designated as group 3b, whose consensus sequence is similar to those in groups 3 and 4 with some variations (Figure S4). Precursor

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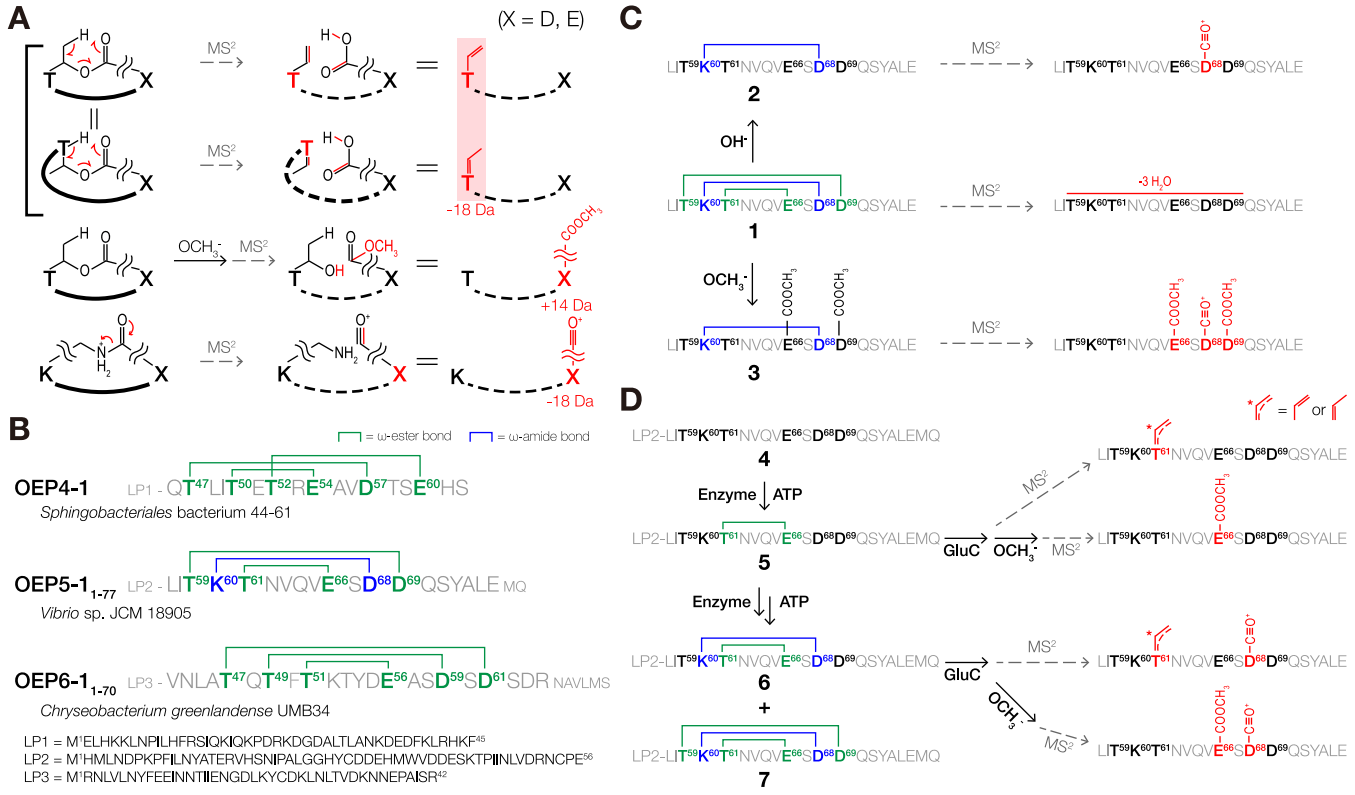

Figure 3. Connectivity analysis of the group 4−6 OEPs. (A) Determination of the positions of the ester-forming threonine (top, in bracket), the ester-forming acidic residue (middle), and the amide-forming acidic residue (bottom) by tandem mass analysis. The modified peptides undergo chemical reaction or rearrangement in mass analyzer (red arrow) and present the fragmentation patterns for the peptides shown at right. The indicated changes in mass value are observed for the residues colored red in the MS^2 spectra. (B) Novel tricyclic structures of three OEPs from group 4−6. The green and blue lines indicate ω-ester and ω-amide linkages, respectively. The bacterial strain that has the BGC is shown under each OEP. Sequences of the leader peptides are given at the bottom. (C) Initial connectivity analysis of **OEP5−1**$_{1-77}$. Hydrolysis and methanolysis of GluC-digested **OEP5−1**$_{1-77}$ (**1**) yielded **2** and **3**, respectively. MS^2 analysis of **1**, **2**, and **3** suggests that D68 generates the ω-amide bond, and E66/D69 generate the two ω-ester bonds. (D) Determination of the connectivity and cross-linking order of **OEP5−1**$_{1-77}$ by the *in vitro* reaction. The precursor peptide (40 μM) and ATP-grasp ligase (2 μM) were mixed and incubated at 25 °C for 0.1 min, 0.5 h, 2 h, and 8 h. Reaction mixtures were quenched, and the leader peptide was digested by endoproteinase GluC at 37 °C for 16 h. Partially or fully modified core fragments were purified by HPLC, and connectivities were analyzed by MS^2 either directly or after methanolysis as shown at the right. The fragmentation patterns from MS^2 spectra and the HPLC chromatogram (Figure S13A) collectively suggest the connectivity of the product and the reaction order of cross-linking reaction as shown (7).

peptides in groups 5 and 6 also share two conserved Thr, one Glu, and one Asp, while they contain one additional Lys and Asp and one additional Thr and Asp, respectively. The phylogenetic tree of the ATP-grasp enzymes indicates that groups 5 and 6 are closely related to one another (Figures S2 and S5). Interestingly, we could separate group 5 into four subgroups, 5a−5d, by the conserved sequences of the leader or core peptides (Figure S5). Members of one subgroup, 5c, has multiple repeats in which either one additional Lys, one Thr, and two Asp are present in a single repeat or the consensus sequences for groups 5 and 6 show up alternatively in multiple repeats of a precursor. We also found a five-membered subgroup, 5d, which presents a variation in the core consensus sequence (Figure S5). Members of group 8 inherently have variable numbers (6−8) of intervening residues between the three conserved Thr and the three conserved Asp, which do not correlate with any specific sub-branches of the phylogenetic tree (Figure S6).

Many groups have a conserved leader motif spanning 6−10 residues mostly composed of aromatic and hydrophobic residues (Figures S3, S5, and S7). This sequence is often the only conserved region in the leader peptides of group 1−3 OEPs, whereas the other nine groups generally show additional conserved sequences including a highly conserved Trp (Figures S5 and S7), suggesting a more complex leader−enzyme interaction or additional roles of the leader region. BGCs of the 12 groups are found in various phyla of bacteria including actinobacteria, proteobacteria, and bacteroidetes, and a small number of BGCs from group 2 and 7 are even found in archaea (Table S1). Nevertheless, the BGCs of each OEP group are mainly distributed in only 1−3 phyla. Similar to the ATP-grasp enzymes for the ω-ester formation in the group 1 OEPs,[20] the ATP-grasp enzymes from each phylum in several groups are mainly found in the same cluster (Figures S3, S4, S5, and S8), suggesting that they have evolved independently. The comparison of GC content of the genes for ATP-grasp enzymes with the whole genomes suggests that horizontal gene transfer of BGCs occurred recently in the majority of the groups except for groups 1, 2, 11, and 12 (Figure S9). These observations collectively demonstrate the distinct evolutionary pathway of each OEP group.

Members of Three New OEP Groups Present Novel Tricyclic Structures. To confirm that members of the new groups indeed contain the OEP-like modifications, we first

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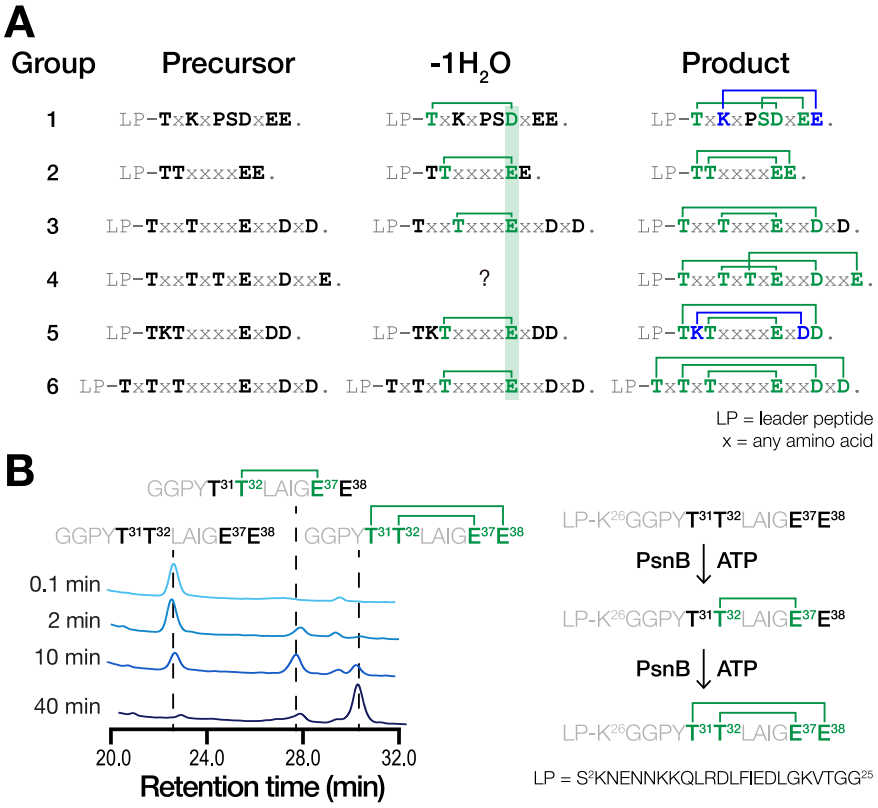

Figure 4. Characterization of the cross-linking order of the group 1−6 OEPs. (A) The first conserved acidic residues of the core peptides (green shadow) generally form the first rings. (B) Determination of the cross-linking order of the plesiocin core peptide (group 2). The reaction with PsnA2$_{2-38}$ (40 μM) and PsnB (0.4 μM) at 37 °C was quenched at designated time points. Then, the leader peptide was digested by trypsin and core fragments were analyzed by HPLC (left). The appearance and disappearance of the partially modified core peptide in the HPLC chromatograms (left) and the connectivity analysis of the observed core fragments (Figure S16) suggest the order of the cross-linking reaction (right).

tested whether the three precursor peptides that belong to group 4, 5, or 6 are modified by their cognate ATP-grasp enzymes (Table S2). We reasoned that members of these three groups have a higher probability to become OEPs because they often present multiple repeats of the same consensus sequence in single precursor peptides, which are also observed in group 1−3 OEPs.[27,28,33] To facilitate the analysis, we used either a single-repeat precursor peptide (group 4) or truncated precursor peptides (groups 5 and 6) that contain only the first repeat of the core peptide. Heterologous coexpression of the precursor peptides and the cognate ATP-grasp enzymes in *Escherichia coli* yielded modified peptides whose molecular weights indicate the loss of three water molecules in the major products, suggesting the formation of three macrocycles in these three peptides (Figure S10).

Next, we determined their macrocyclic connectivity using a previously reported method, which combines tandem mass (MS^2) analysis with ester hydrolysis or methanolysis.[27,28] In this method, the macrocycle-forming residue shows a mass change in the MS^2 spectrum of the modified peptide compared to that of the unmodified peptide. Specifically, the position of the ester-forming threonine and acidic residue can be identified by the loss of 18 Da in the MS^2 spectrum of the modified peptide and by the gain of 14 Da in the MS^2 spectrum of the methanolyzed modified peptide, respectively (Figure 3A, top and middle). Moreover, we found here that the ω-amide linkage is often broken in the MS^2 analysis, and thus the position of the amide-forming acidic residue can be determined by the loss of 18 Da in the MS^2 spectrum (Figure 3A, bottom).

The overall analysis suggests that the three modified peptides from group 4, 5, or 6 present novel tricyclic structures (Figure 3B). The group 4 peptide (**OEP4-1**) contains two ω-esters (Thr47-Asp57 and Thr50-Glu54) that are also shown in the group 3 OEPs, thuringinins, and one additional ω-ester (Thr52-Glu60) that connects the middle of the hairpin to the C-terminal region (Figure S11). This ω-ester bond, besides one ω-ester in microviridins, becomes the second cross-ring linkage shown in OEPs. The group 5 peptide (**OEP5-1**$_{1-77}$) and the group 6 peptide (**OEP6-1**$_{1-70}$) show two different hairpin-like or "ring-within-a-ring" structures (Figures 3C,D and S12−S15; see below for the connectivity determination of **OEP5-1**$_{1-77}$). They both contain two ω-esters that are formed between the two conserved pairs of Thr and Glu/Asp (Thr59-Asp69 and Thr61-Glu66 for **OEP5-1**$_{1-77}$; Thr49-Asp59 and Thr51-Glu56 for **OEP6-1**$_{1-70}$). However, **OEP5-1**$_{1-77}$ has an additional ω-amide (Lys60-Asp68) that makes the middle ring, whereas **OEP6-1**$_{1-70}$ has the third ω-ester (Thr47-Asp61) that constructs the outer ring. Taken together, we demonstrate that the modified peptides from groups 4, 5, and 6 are indeed members of the OEP family of RiPPs and present novel tricyclic structures.

A Single ATP-Grasp Enzyme Makes Both ω-Ester and ω-Amide Linkages in the Biosynthesis of the Group 5 OEP. Of the six groups of OEPs that are biochemically characterized, **OEP5-1**$_{1-77}$ presents a unique biosynthetic

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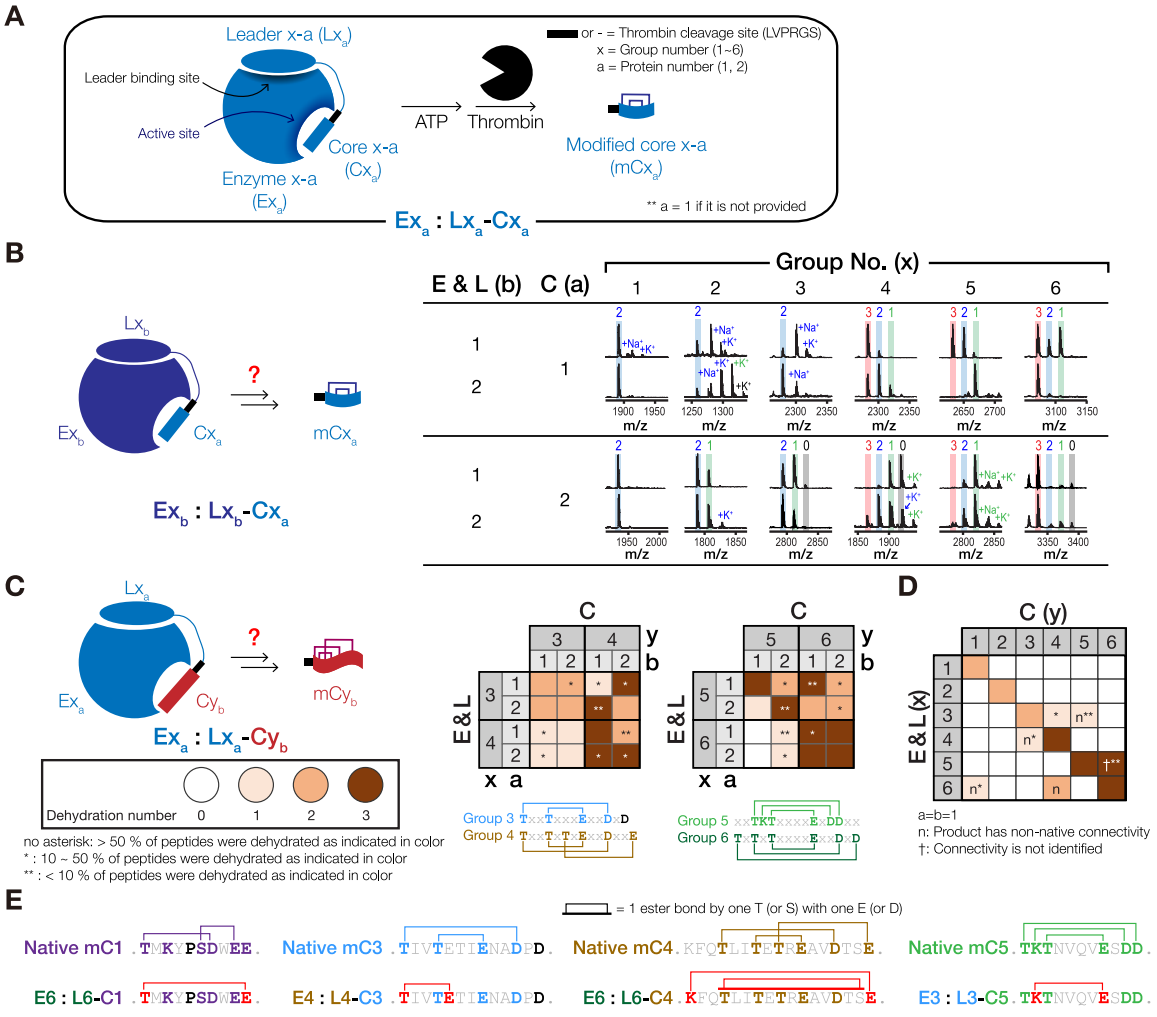

Figure 5. ATP-grasp enzymes are highly specific to the cognate core consensus sequences. (A) Scheme of the modular biosynthesis of OEPs. When the designed precursor peptide, containing a thrombin cleavage site between the leader and the core peptide (Lx$_a$-Cx$_a$), binds to the ATP-grasp enzyme (Ex$_a$), the core peptide (Cx$_a$) is modified by consuming an ATP molecule(s). Digestion of the modified precursor peptide with thrombin yields the modified core peptide (mCx$_a$). "x", "a", and dash between Lx$_a$ and Cx$_a$ (−) indicate the group number (1−6), the protein number (1 or 2, Table S2), and the thrombin cleavage site, respectively. (B) Scheme of the reaction for testing the cross-reactivity within the same group (left) and the resulting mass spectra using two selected OEPs for each group (right). Designed precursor peptides were coexpressed with appropriate ATP-grasp enzymes, purified, and co-incubated with ATP *in vitro* to complete the cross-linking reactions. Peaks highlighted in red, blue, green, and gray indicate the loss of three, two, one, and zero water molecules. Observed and calculated mass values are in Table S3. (C) Scheme of the reaction for testing the cross-reactivity between two different groups (left) and the extents of dehydration in reactions combining group 3 and 4, or group 5 and 6 (right). Different colors were used to illustrate the products with the largest dehydration numbers in the reactions. Various numbers of asterisks (*) were used to show the yields, estimated by the intensities of peaks in MALDI-TOF-MS spectra, of the products. The connectivities of the core peptides of groups 3−6 are illustrated under the tables. MALDI-TOF-MS spectra used to depict the tables are in B ($x = y$) or in Figure S23 ($x \neq y$). (D) Dehydration numbers in reactions using the 36 different combinations of an enzyme−leader pair and a core peptide in group 1−6 OEPs. Same experimental procedure, colors, and number of asterisks (*) as panel C are used to monitor and illustrate the result of cross-linking reactions. "*n*" in the table denotes that the observed products have distinct connectivity when they are modified by cognate ATP-grasp enzymes. "†" indicates that the connectivity of modified product is not identified due to the low yield from coexpression. MALDI-TOF-MS spectra used to depict the table are in panel B ($x = y$) or Figure S24 ($x \neq y$). (E) Non-native connectivities of four products with partial dehydration. The connectivity of the ester bond in E6 : L6-C4 is not clearly determined. MALDI-TOF-MS/MS spectra used to determine the connectivities are in Figures S25 and S26.

pathway in which a single ATP-grasp enzyme mediates the formation of both ω-ester and ω-amide linkages. Microviridins (group 1) also have an ω-amide linkage, but it is formed by a separate ATP-grasp enzyme. Linkages and connectivity of the GluC-treated **OEP5-1**$_{1-77}$ (**1**) were analyzed as follows. First, the direct MS^2 analysis of **1** indicates that the three rings are formed within the sequence containing the highly conserved residues (Figures 3C, middle, and S12). Second, hydrolysis and methanolysis only destroyed two out of three cross-links, suggesting the presence of an ω-amide and two ω-ester linkages. MS^2 analysis of their products (**2** and **3**) demonstrates that Asp68 constitutes an ω-amide, while the other two conserved acidic residues (Glu66 and Asp69) form ω-ester bonds (Figures 3C, top and bottom, and S12). Because Lys60 is the only residue that can form an amide linkage with Asp68, the ω-amide should be formed between these two residues. Third, the *in vitro* reaction of the ATP-grasp enzyme and the truncated precursor peptide (**4**) provided a single-ring peptide (**5**) as an intermediate, whose MS^2 analysis with or without methanolysis after GluC treatment reveals the ester

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linkage between Thr61 and Glu66 (Figures 3D and S13). The longer reaction yielded a two-ring intermediate (**6**) whose analysis is consistent with the presence of the inner macrolactone ring and the middle macrolactam ring. The leader-cleaved **6** was also obtained by partial hydrolysis of **1** (Figure S14). This *in vitro* result also demonstrates that a single ATP-grasp enzyme is capable of creating both ω-ester and ω-amide linkages. Finally, the only possible third linkage is an ω-ester bond between Thr59 and Asp69.

Common Features of OEPs. To understand the properties of OEPs better, we seek to find some features that are commonly or frequently observed in their structures or biosynthesis. First, only the highly conserved residues participate in the ring formation. Although we do not exclude the possibility of some noncanonical macrocycles in particular OEPs, there has been no such linkage in all biochemically characterized OEPs. This observation supports the idea that other members of each OEP group are likely to be modified in the same fashion and that the ATP-grasp enzymes in OEP biosynthesis in general have, if not perfect, high tolerance in the unmodified positions of precursors, which was previously demonstrated in microviridins[34,35] and many other RiPPs.[36−39]

Second, of the two residues that form the ω-ester or ω-amide bonds in the known OEPs, the nucleophilic residue (Thr/Ser/Lys) always precedes the acidic residue (Glu/Asp) in the precursor sequence. Although the group 7 peptides, which have not been biochemically characterized, may show an exception with the consensus sequence of TKKxDxET-GEDxKxE, other uncharacterized groups also present the similar sequence patterns in general. By contrast, both arrangements of the two ring-forming residues are observed in lanthipeptides.[40,41] It remains to be studied why the core sequence of OEPs shows the preferential arrangement of ring-forming residues, but one possibility is that the binding of both the leader peptide and the acidic residue to the enzyme at the initial step of biosynthesis preorganizes the spatial position of the nucleophilic residue and thereby facilitates the nucleophilic addition.

Third, of the conserved acidic residues in the core peptides, the first one from the N-terminus usually creates the first macrocycle when modified by the cognate ATP-grasp enzymes (Figure 4A). The order of ring formation was previously reported for the group 1 and 3 OEPs[28,34] and here characterized either by *in vitro* reactions (groups 2 and 5; Figures 3D, 4B, S13, and S16) or by the ordered coexpression of the ATP-grasp enzyme and the precursor peptide (group 6; Figures S17 and S18). Although we could not reliably determine the reaction order of **OEP4-1** and do not exclude the possibility of exceptions with uncharacterized OEPs, the ATP-grasp enzymes may often be evolved to preferentially recognize the first conserved acidic residue for the first macrocyclization reaction. The directionality of modification was identified in other classes of RiPPs as either N to C,[42−47] C to N,[40,48−52] or random.[53]

Taken together, we could describe the common features observed in six groups of OEPs. These features may not be strict rules or requirements in the OEP biosynthesis but become general guidelines to find other members in the same family or to expand the scope of the OEP family.

ATP-Grasp Enzymes Have High Specificity to the Core Peptides with Cognate Consensus Sequences. One of the major aspects of RiPPs that allow expansion of chemical diversity is the high substrate tolerance of the modifying enzymes.[54] As we suggested above and the previous report has shown for microviridins, ATP-grasp enzymes are likely to have high tolerance for nonconserved positions in the core peptide.[34,35] Previously, enzymes involved in microviridin biosynthesis could not modify mutant substrates in which the position of the ring-forming threonine is shifted by a single residue, suggesting that these enzymes are highly specific to the cognate core consensus sequence.[34] The enzyme for plesiocin biosynthesis is also highly specific given that the modification of similarly mutated substrates yields only monocyclic products with low yield.[55] However, given the diverse core consensus sequences of the 12 OEP groups, it is unknown if an ATP-grasp enzyme in one group can modify precursor peptides in different groups.

We address the question of substrate specificity by the systematic variation of modules in the macrocyclization reaction of six OEP groups that are biochemically characterized (groups 1−6). The canonical macrocyclization reaction in OEP biosynthesis requires three modules—an ATP-grasp enzyme, the leader peptide, and the core peptide—of which the leader and core peptides are present in a single precursor peptide and interact with the leader-binding site and the active site of the enzyme, respectively (Figure 5A). By using cognate or chimeric minimal precursor peptides that contain a leader peptide, a thrombin cleavage site, and a single core repeat, we tested the cross-reactivity of enzymes in three different contexts as follows: First, we tested if the leader peptides are compatible with enzymes from different groups by combining cognate pairs of the enzyme and the core repeat with different leader peptides in the coexpression of an enzyme and a chimeric precursor (Figure S19). Of the total 36 combinations, only those in which three modules belong to the same group (diagonal positions) showed full dehydration in the major products, whereas those with noncanonical leader peptides showed either partial reactions or no expression of the precursor peptide or the enzyme (Figure S19). Because some enzymes or precursors are stably expressed only when they are coexpressed, these results collectively indicate that six enzymes are highly specific to the cognate leader peptides. By contrast, heterocyclases of cyanobactin are known to efficiently modify the substrates containing noncognate leader peptides.[56]

Second, to confirm that if the ATP-grasp enzymes are promiscuous within the same OEP group, we selected two OEPs from each group (protein number 1 and 2; Figure 5A) and combined the cognate pairs of the enzyme and the leader with different core peptides in the same OEP group (Figure 5B, left). Of the six OEP groups, four groups (groups 1, 2, 3, and 6) showed high cross-reactivity, among which the ring connectivities of six OEPs in group 1, 2, or 3 were further confirmed to be canonical (Figures 5B, right, and S20−S22). In groups 4 and 5, the cognate combinations of the second members presented partial reactions, and the level of dehydration was largely maintained in noncognate combinations. These results indicate that enzymes often have a high level of promiscuity to the core peptides within the same group of OEPs. Biosynthetic enzymes of lanthipeptides have also been shown to modify the core peptides with similar sequences, as shown with class I lanthipeptides, nisin and subtilisin,[57,58] and class II lanthipeptides, lacticin 481 and nukacin ISK-1.[59]

Third, to check whether the ATP-grasp enzymes can modify the core peptides with different consensus sequences, we combined the cognate pairs of the enzyme and the leader with

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the core peptides from different OEP groups (Figure 5C, left). We started with two pairs of groups that show relatively close relationships, groups 3 and 4 and groups 5 and 6. When we tested the combinations with two OEPs in each group, we found that the core peptides are often dehydrated more by enzymes from the same group than by those from the different group, indicating that the intergroup reactivity is generally lower than the intragroup reactivity (Figure 5C, right, and Figure S23). Next, we chose one OEP in each group and tested all the possible 36 combinations. Eighty percent and 17% of noncanonical combinations (24 and 5 out of 30) revealed no reaction and partial dehydration, respectively, indicating very low cross-reactivity of enzymes (Figures 5D and S24). Although the low expression level deterred us from analyzing the connectivity of the product in the highly cross-reactive combination (combination of the group 5 enzyme with the group 6 core peptide), we could determine the connectivity of five products with partial dehydration. We found that, surprisingly, four products contain non-native connections (Figures 5E, S25, and S26), whereas only one product with the combination of the group 3 enzyme and the group 4 core peptide presents the native connectivity (Figure S27). Interestingly, the group 6 enzyme and the group 3 enzyme, both of which do not natively make any ω-amide linkage, appear to create non-native ω-amide bonds in the group 4 core and the group 5 core peptides, respectively (Figures 5E and S26). This result suggests that ATP-grasp enzymes in OEP biosynthesis are inherently able to introduce both ω-ester and ω-amide linkages.

Taken together, while the ATP-grasp enzymes have relatively high plasticity for nonconserved positions, they show high specificity to the cognate core consensus sequence, suggesting the divergent evolution of enzymes across the different groups. Previously, it has been shown that some modification enzymes in other RiPP families have high cross-reactivity to noncognate substrates containing very different sequences. For example, the epimerization pattern of a core peptide generated by several noncognate radical S-adenosyl methionine peptide epimerases (RSPEs) was very similar to that made by the cognate enzyme−core pair.[52] The biosynthetic enzymes of nisin and prochlorosin, NisBC and ProcM, could produce the correct rings of epilancin 15X and lacticin 481, respectively, whose ring topologies are completely different from those of nisin and prochlorosin.[32] Also, several studies showed that biosynthetic enzymes of nisin can generate bioactive class II lanthipeptides.[60,61] These observations collectively suggest that the modification patterns of these RiPPs are largely determined by the core sequences rather than by the modification enzymes.[62,63] By contrast, our results suggest that the ring topology of OEPs is dictated by both the core sequence and the ATP-grasp enzyme.

CONCLUSION

The explosion of genomic data has changed the paradigm of the discovery of natural products from the traditional activity-based methods to the bioinformatic approach. We herein significantly expand the OEP family of RiPPs by genome mining and classified OEPs into 12 groups based on the conserved core sequences. Although this listing may not be comprehensive, our results support the idea that substantial topological diversity of multicyclic peptides containing ω-ester and ω-amide linkages has been developed in nature, compared to what was previously known. The diverse ring topologies, not just the diversity in sequences, are found in only a few RiPP families that have side-to-side macrocyclic linkages for major modification, such as OEPs, lantipeptides, sactipeptides, and ranthipeptides.[22,41] We believe that more rigorous bioinformatics analyses will uncover additional OEP groups with distinct structures and properties, thereby providing insights on biosynthetic mechanisms and other tailoring modifications. We also determined the ring topologies of three new groups and showed that they have novel tricyclic structures in which the macrocyclic patterns are unique. Through biochemical characterization, we demonstrated that several ATP-grasp enzymes produce both ω-ester and ω-amide bonds, naturally or unnaturally, suggesting a common mechanism of the bond formation.

Finally, we found that the ATP-grasp enzymes present two different levels of substrate specificity; the enzymes are relatively plastic to the nonconserved positions of the core peptide, but are highly unyielding to the conserved core sequence in the macrocyclization reaction. This result suggests that although the nonconserved region of the core sequence has been diversified with low restriction, the conserved core sequence has been diversified in line with enzymes. Also, we found that the leader peptides are conserved within each group, and the ATP-grasp enzymes are specific to the cognate leader peptides for the efficient modification of the core peptides. These findings have implications for engineering new OEPs: New OEPs may be easily made within the same group, as shown in the previous engineering of microviridin variants.[35] To generate diversity at a higher level, an orthogonal set of enzymes from different groups can be used to make separate libraries with distinct ring topologies. In addition, because the ATP-grasp enzymes sometimes produce noncanonical rings on noncanonical substrates, matching enzymes with random peptides may also generate, albeit with lower efficiency, macrocyclic peptides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b12076.

Materials and methods, supplementary figures, and supplementary tables (PDF)

Data set 1: Biosynthetic enzymes and precursor peptides of OEPs (XLSX)

Data set 2: Sequence similarity network (SSN) of ATP-grasp enzymes (ZIP)

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Notes

The authors declare no competing financial interest.

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