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Plant Cyclopeptides and Possible Biosynthetic Mechanisms

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Abstract: Plant cyclopeptides are a large group of small molecular weight natural products, typically with 4 - 10 amino acids, isolated from the leaves, stem barks, roots, and seeds of a wide variety of plant species throughout the world. The peptides are present in many Chinese medicinal plants, and their potentials have not been well exploited because of the lack of knowledge in their biosynthetic origin and mechanism. The cyclopeptides often have complex chemical structures distinct from common polypeptides (proteins) that are non-cyclic and gene-coded. This review discussed the potential origin of the cyclopeptides and their possible biosynthetic mechanisms.

Key words: Plant Cyclopeptide; Biosynthesis; Endophyte; Nonribosomal peptide

Plant cyclopeptides represent a new, largely untapped source for human medicines. Many of the natural products have fascinating structures and potent biological activities, such as sedative sanjoinine-A (Han *et al.*, 1989), immunosuppressive cycloleonurinin (Morita *et al.*, 1997), antitumor RA-VII (Itokawa *et al.*, 1983; Hitotsuyanagi *et al.*, 2004) and uterotonic kalata B1 (Saether *et al.*, 1995). These are a large group of natural products and classified into eight types based on their structures and distributions in plants (Tan and Zhou, 2006). However, none of the cyclopeptides, except the Type-VIII (cyclotides) that are gene-coded products, have been studied for their biosynthetic mechanisms. An understanding of the biosynthetic mechanism for the bioactive compounds is an essential step toward utilization of the natural products as human medicines. This is because the biosynthesis is a feasible approach for utilization of these products.

Many of the cyclopeptides have complex chemical structures that preclude the chemical total synthesis approach from commercial application . Their bioavailability is generally low that makes it unsustainable for large scale extraction of the products directly from plants . The knowledge of the biosynthetic mechanism will help design new and rational approaches to increase and improve the yield of the compounds in the native hosts or to produce the compounds in user-friendly heterologous hosts through genetic engineering . The biosynthetic approach may also lead to a lower toxicity of some of the compounds, such as the potent antitumor RA-VII, which was in phase II clinical trial but dropped off due to the toxicity .

Another need of studying the biosynthetic mechanism for plant cyclopeptides comes from the fact that many of the cyclopeptides are isolated from traditional Chinese herb medicines . There are more than 5, 000 plants and plant products in Chinese herb pharmacopoeia, and many of the plants have been used to treat various diseases for thousands of years without knowing the exact natures of the active factors (Bensky, 1993) . The understanding of their biosynthesis would provide new molecular insights into the understanding of the pharmacological and medical theories of traditional Chinese medicines . This in turn will help develop safer and more defined use of the medicines to treat human diseases .

1   Small Molecular Weight Peptides

Small molecular weight peptides can be classified into two groups according to their biosynthetic mechanisms, the ribosomal peptides (RPs) and nonribosomal peptides (NRPs) . RAs are synthesized by translation of mRNA on the ribosome . These peptides are derived from gene-encoded precursors (pre-peptides) that undergo posttranslational modifications, including proteolysis, dehydration, and cyclization . For example, the antibiotic microcin MccB17 produced by various *E. coli* strains is derived from the 69-aa MccB17 precursor McbA (encoded by *mcbA*) (Li *et al.*, 1996; Milne *et al*., 1999) . Three genes, *mcbB*, *mcbC*, and *mcbD*, encode the three components of the MccB17 synthetase

required for posttranslational modifications of McbA to produce the 43-residue final product, MccB17 . The modifications consist of the removal of the 26-residue leader peptide and the formation of eight heterocycles, four oxazole rings and four thiazole rings . The oxazoles and thiazoles are formed via cyclization-dehydration-oxidation of serine residues and cysteine residues, respectively, with adjacent residues . Extensive posttranslational modifications are also found in the biosynthesis of lantibiotics, such as lacticin 481 that is matured from a 51-aa prepeptide (LctA) through removal of the 24-aa leader peptide, dehydration of serines and threonines, and intramolecular cyclic thioether formation (Xie *et al.*, 2004; Xie and van der Donk, 2004) . Amazingly, this process is catalyzed by one single enzyme, LctM . Because of the gene-encoded nature, RPs are composed only from the 21 proteinogenic amino acids .

NRPs are assembled by specific enzymes, independent of the ribosome . For example, glutathione, an important antioxidant in cells, is a linear tripeptide synthesized by two ATP-dependent enzymes, -glutamylcysteine synthetase (glutamate cysteine ligase) and glutathione synthetase . Glutamate cysteine ligase is a heterodimeric enzyme consisting of a catalytic and modulatory subunit . The most actively studied NRPs are a vast array of small peptides isolated from microbes . They are assembled on enzyme complexes called nonribosomal peptide synthetases (NRPS), which are modular enzymes composed from a series of functional units (domains) (Marahiel *et al.*, 1997) . These peptides are highly diverse in their chemical structures and biological activities . More than 300 precursors are known to serve as building blocks for these NRPs . These include many nonprotenogenic amino acids and modified proteinogenic amino acids . The modifications include hydroxylation, acylation, *N*-methylation, glycosylation, halogenation, and epimerzation (D-amino acids) . The huge precursor pool contributes to the vast structural diversity of these peptides, including lipopeptides, depsipeptides, and peptidolactones that can be linear, cyclic, or cyclic branched . Interestingly, many plant cyclopeptides also share these structural

features, which are hallmarks for the nonribosomally synthesized peptides . The peptides possess an extremely broad range of biological activities and pharmacological properties, including antibiotics (such as penicillin, vancomycin and daptomycin), anticancer agents (such as epothilone and bleomycin), immunosuppressants (such as cyclosporine A), siderophores (such as entorobactin and myxochelin A), and toxins (such as microcystins) .

2   Plant Cyclopeptides

Plant cyclopeptides are a large group of small molecular weight natural products, typically containing 4 - 10 amino acid residues . They have been isolated from the leaves, stem barks, roots, and seeds of a wide variety of plant species throughout the world . Tan and Zhou reviewed 455 plant cyclopeptides that were isolated from 120 species, spreading in 65 different genera of 26 plant families (Tan and Zhou, 2006) . The peptides exhibit numerous biological activities, including anticancer, antibacterial, antifungal, anti-HIV, antimalarial, and sedative effects . These plant cyclopeptides are grouped into eight types (I to VIII) based on their chemical structures and distributions (Fig. 1) (Tan and Zhou, 2006) . Type VIII (cyclotides) is a distinct group with a chain (usually 30 residues) much longer than the rest of cyclopeptides . This group is also the best understood plant cycloptides in terms of their biosynthetic mechanism . Similar to microcins and lantibiotics from bacteria, cyclotides are gene-products synthesized via the ribosomal mechanism (Jennings *et al.*, 2001) . For example, the prototypic kalata B1 is a 29-residue cyclopeptide isolated from *Oldenlandia affinis* . It is derived from a 124-residue precursor protein that is encoded by the Oak1 gene . The precursor protein consists of a 20-residue endoplasmic reticulum signal sequence, a 65-residue non-conserved pro-region, a highly conserved 3-residue region known as the N-terminal repeat, the 29-residue cyclotide domain and a 7-residue hydrophobic C-terminal tail . The final mature kalata B1 is formed through a series of posttranslational proteolysis and disulfide bond formation, which forms the three characteristic interlocked disulfide bonds, the

" cyclic cystine knot" . The six cysteine residues that form the knots are absolutely conserved throughout the cyclotides .

So far, the Type VIII cyclopeptides (cyclotides) are the only group whose biosynthetic mechanism has been studied . None of the other seven types have been studied for their biosynthetic mechanism (note that the term" cyclopeptides" refers to Type I-VII and is the target of the rest discussion in the article) . Structurally, these cyclopeptides are more similar to typical nonribosomal peptides found in microorganisms . For example, at least 34 amino acids are found in these cyclopeptide alkaloids (Type I), many of them are nonproteinogenic amino acids or modified proteinogenic amino acids, such as -hydroxyl, N-methyl, N-aldehyde, N, N-dimethyl, N-aldehyde- N-methyl, N-oxo- N, N-dimethyl (N O) amino acids . An especially notable and frequently occurring type of amino acids is the so-called" ring bond amino acids", which are linked to the rings of other amino acids or moieties by bonds other than an amide bond, such as the ether, carbon-carbon, or carbon-nitrogen bond (Fig. 1) . In addition, the cyclopeptides are generally smaller than 10 amino acid residues . These structural features make them distinct from cyclotides and more closely related to NRPs .

3   Cyclopeptides Isolated from Microbial Endophytes of Plants

Before discussing the biosynthetic mechanism for plant cyclopeptides, it is important to briefly mention a group of cyclopetides that are isolated from microorganisms living within plants, the so-called endophytes . These compounds could be regarded as a link between the biosynthetically well-known, microbe-originated NRPs and the plant cyclopeptides, for which the biosynthetic mechanism and origin remain unclear . Although the plant cyclopeptides are isolated from plant materials, there are three possible biosynthetic origins, the plants, potential endophytic microbes, and both . Several dozens of plants' genomes have been sequenced or are being sequenced . Within the two genomes completed (*Arabidopsis thaliana* and *Oryza sativa* Japonica group), there is no sign of presence of NRPS-type genes
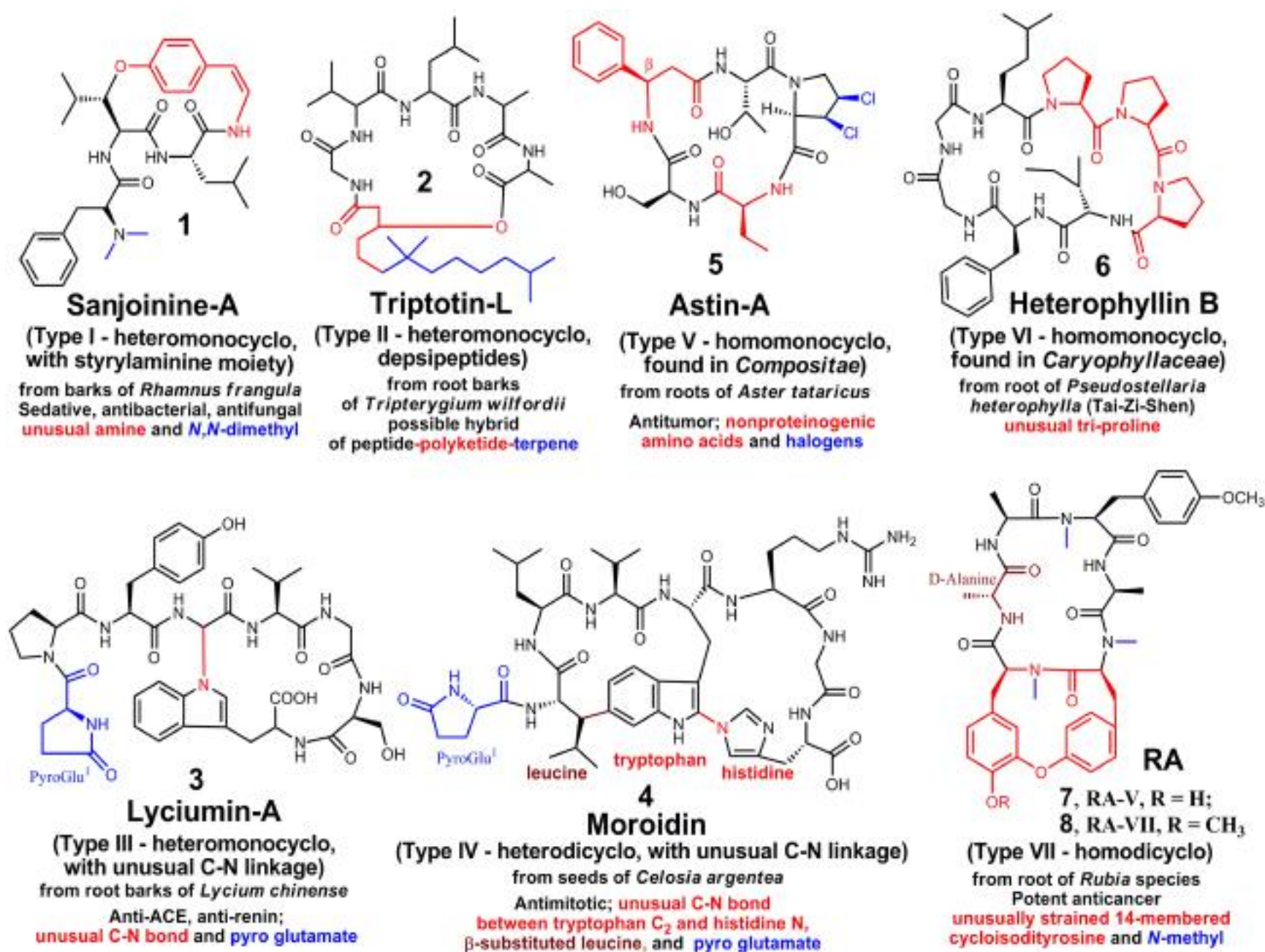
Sanjoinine-A
(Type I - heteromonocyclo,
with styrylaminine moiety)
from barks of *Rhamnus frangula*
Sedative, antibacterial, antifungal
unusual amine and *N,N*-dimethyl

Triptotin-L
(Type II - heteromonocyclo,
depsipeptides)
from root barks
of *Tripterygium wilfordii*
possible hybrid
of peptide-polyketide-terpene

Astin-A
(Type V - homomonocyclo,
found in *Compositae*)
from roots of *Aster tataricus*
Antitumor; nonproteinogenic
amino acids and halogens

Heterophyllin B
(Type VI - homomonocyclo,
found in *Caryophyllaceae*)
from root of *Pseudostellaria
heterophylla* (Tai-Zi-Shen)
unusual tri-proline

Lyciumin-A
(Type III - heteromonocyclo,
with unusual C-N linkage)
from root barks of *Lycium chinense*
Anti-ACE, anti-renin;
unusual C-N bond and pyro glutamate

Moroidin
(Type IV - heterodicyclo, with unusual C-N linkage)
from seeds of *Celosia argentea*
Antimitotic; unusual C-N bond
between tryptophan C₂ and histidine N,
β-substituted leucine, and pyro glutamate

RA
7, RA-V, R = H;
8, RA-VII, R = CH₃
(Type VII - homodicyclo)
from root of *Rubia* species
Potent anticancer
unusually strained 14-membered
cycloisodityrosine and *N*-methyl

Fig . 1   Chemical structures of plant cyclopeptides . One representative compound from each of the seven groups (Type I to VII) is shown .
The NRPs-like , unusual structural features are highlighted by colors , and the activities and occurrences of the peptides are also indicated

(Blastp search by Du , unpublished observation) . While it is not known if these two plants produce cyclopeptides , it is clear that these plants do not contain such giant , multi-domain enzyme complexes as NRPS found in microbes . Although it is possible that different groups of cyclopeptides may have different biosynthetic mechanisms and origins , the structural features suggest that at least some of the plant cyclopeptides might be originated from endophytic microbes using the NRPS mechanism .

Numerous natural products have been isolated from endophytes (Strobel , 2002 ; Ge and Tan , 2009 ; Zhang *et al.*, 2006) , including cyclopeptides of plant endophytes (Fig.2) . Cryptocandin A , a potent antimycotic , was isolated from *Cryptosporiopsis quercina* , which is an endophytic fungus of the medicinal plant *Tripterygium wilfordii* (Strobel *et al.*, 1999) . This compound contains several unusual hydroxylated amino acids and a novel amino acid , 3-hydroxy-4-hydroxymethyl proline . Pseudomycin A is one of the antifungal lipodepsipeptides produced by *Pseudomonas syringae* MSU 16H , a plant-associated pseudomonas (Ballio *et al.*, 1994) . The compound contains several unusual amino acids , including chlorothreonine , hydroxyaspartic acid , and diaminobutryic acid . Epichlicin , another potent antifungal cyclopeptide , was isolated from *Epichloe typhina* , an endophytic fungus of the timothy plant , *Phleum pretense* (Seto *et al.*, 2007) . The peptide contains an unusual -amino acid , 3-amino tetradecanoic acid . Inturin A2 , A3 and A6 , with a structure and activities similar to epichlicin , were isolated from *Acinetobacter baumannii* , an unusual endophytic bacterium of the medicinal plant *Cinnamomum camphora* (Liu *et al.*, 2007) . Besides , a number of structurally not-well-resolved cyclopeptides have also been isolated from plant endophytes . For example , munum-

9 Cryptocandin A

10 Pseudomycin A

11 Epichlicin
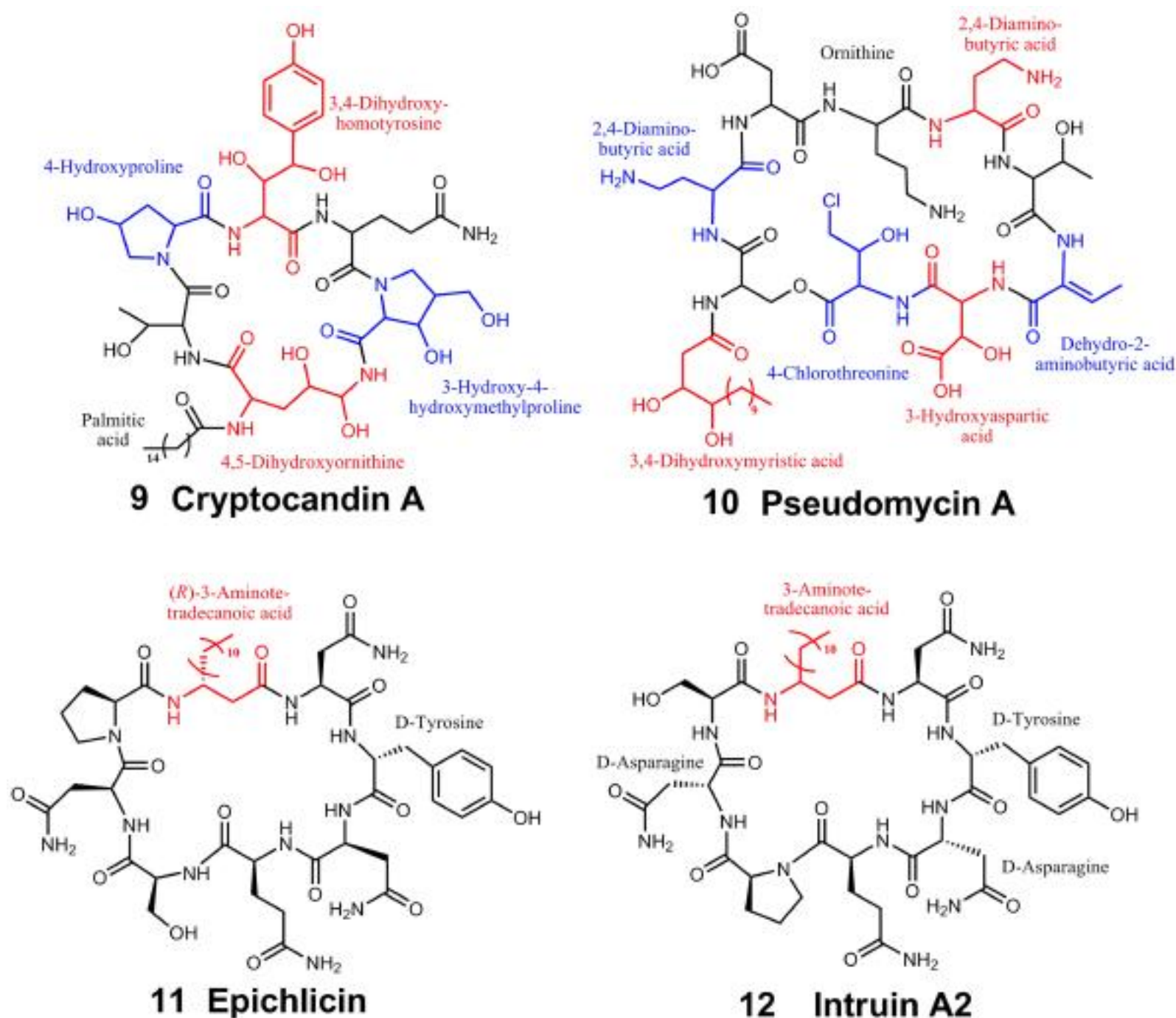
12 Intruin A2

Fig . 2   Chemical structures of cyclopetides isolated from endophytic microorganisms of plants . The unusual structural features are highlighted by colors . All the compounds exhibit antifungal activities . Cryptocandin A (9) was isolated from *Cryptosporiopsis quercina*, which is an endophytic fungus of *Tripterygium wilfordii*, a medicinal plant native to Eurasia (Strobel *et al.*, 1999) . Pseudomycin A (10) was isolated from *Pseudomonas syringae* MSU 16H that is associated with plants (Ballio *et al.*, 1994) . Epichlicin (11) was isolated from *Epichloe typhina*, which is an endophytic fungus of plant *Phleum pretense* (Seto *et al.*, 2007) . Intruin A2 (12) was isolated from *Acinetobacter baumannii* LCH001, which is an endophytic bacterium of plant *Cinnamomum camphora* (Liu *et al.*, 2007) .

bicins, broad spectrum peptide antibiotics, were isolated from *Streptomyces* sp . strain NRRL 30562, which is a novel endophyte of snakevine, *Kennedia nigriscans*, a bush medicine in Australia (Castillo *et al.*, 2002) . Coronamycins, another complex of peptides with antifungal activities, were isolated from a verticillate *Streptomyces* sp ., which is an endophyte from an epiphytic vine, *Monstera* sp (Ezra *et al.*, 2004) . These prior examples suggest that endophytes are a likely source for many of the cyclopeptides isolated from plants .

In marine natural products, it has been recognized that some compounds originally isolated from marine invertebrates are actually synthesized by microbes associated with the invertebrates (Schmidt, 2005, 2008) . Some plant terpenoids and polyketides that were originally isolated from the plants turned out to be of microbial origins . For example, maytansinoids, the 19-membered macrocyclic lactams, were initially isolated from the tropic plant *Mallotus nudiflorus* . Recent evidence suggests that the core structure of maytansinoids, which is related to ansamycin antibiotics of microbial origin, is synthesized by endophytic microbes (Yu *et al.*, 2002) . Recently, it was shown that a novel fungal endophyte isolated from the inner bark of the medicinal plant *Camptotheca acuminata* was able to produce camptothecin and analogs through fermentation (Kusari

et al., 2009). Camptothecin is a potent antineoplastic agent, originally isolated from the plant. Interestingly, the yield of the compounds sharply decreased over seven successive subculture generations of the endophyte, suggesting that the production of these metabolites probably requires unknown factors of the host plant origin. Very little is known about the interactions between endophytes and their hosts. The understanding the interactions is clearly important in order to use endophytes as alternate sources of plant secondary metabolite production.

4   Heterophyllin B and RA-VII—Two Prototypical Plant Cyclopeptides

For the purpose of illustrating the possible biosynthetic mechanism, we choose to focus on two prototypical plant cyclopeptides, heterophyllin B (6) and RA-VII (8), in the following sections. Heterophyllins (Type VI) are isolated from the Chinese medicinal plant, Tai-Zi-Shen (*Pseudostellaria heterophylla*) (Tan *et al.*, 1993; Morita *et al.*, 1994), which is used to treat palpitation, sweating, fatigue, cough, and loss of appetite (Fig.1). At least 12 cyclopeptides, most with inhibitory activities against tyrosinase or melanogenesis, have been isolated from this plant. Heterophyllin B (HB) is the best known one and has served as a model cyclopeptide in many studies (Tan and Zhou, 2006). Besides, HB is the only plant cyclopeptide for which an in vitro enzymatic assay and a tissue culture with the HB biosynthetic activity have been established (Jia *et al.*, 2006). Structurally, HB contains three sequential proline residues, which is an unusual feature in NRPs. An understanding of HB biosynthetic mechanism will set the foundation to exploit this group of cyclopeptides in Tai-Zi-Shen as well as other type cyclopeptides.

The RAs are a group of bicyclic hexapeptides (Type VII) isolated from several *Rubia* species in 1980s. Among them, *R. yunnanensis* is a traditional Chinese medicinal plant (Xiao-Hong-Shen) (Fig.1), which is used to treat anemia, injury, rheumatism, gastritis, lipoma, and menoxenia. At least 18 cyclopeptides (RA-I through RA-XVIII) have been identified. Most RAs have the same amino acid sequence and differ only in the numbers of modification groups (methyl and hydroxyl). The bioavailability of RAs is extremely low, about 0.001 to 0.00001% of dry plants. The most recent member of this family is RA-XVIII, which was isolated from the dried roots of *R. cordifolia* (Lee *et al.*, 2008). From 55 kg dried roots, 4.8 mg RA-XVIII was obtained through a series of extraction and separation. The RAs are probably the most interesting plant cyclopeptides in terms of their biological activities and structural features. They all exhibit very potent anticancer activities (IC$_{50}$ at nM level) and have distinctive structural features. The most interesting structural feature is the unusually strained 14-membered cycloisodityrosine that was proposed to be the pharmacophore for its activities (Boger *et al.*, 1991; Boger and Yohannes, 1993; Boger and Zhou, 1995). The anticancer activities were observed with P-388 leukemia, ascites tumors, L1210, B-16 melanoma and solid tumors, colon 38, Lewis lung carcinoma, and Ehrlich carcinoma (Itokawa, 1984). The effective dose ranges (five days of *i.p.* administration) were 0.01 - 4.0 mg kg. Among them, RA-V (deoxybouvardin, 7) was especially potent on MM2 mammary carcinoma in mice (two of six mice given 5 mg kg and one of seven mice given 10 mg kg recovered). RA-VII (8) was in phase II clinical trials in Japan in the 1980s (Itokawa, 1984; Inoue, 1986). It exhibited almost the same chemosensitivity compared to that of five standard anticancer drugs (adriamycin, mitomycin C, cisplatin, vinblastine and 5-FU). However, RA-VII also caused nausea and vomiting, fever, stomachache, mild hypotension and slight abnormality of electric-cardiogram. The mode of action is believed through interacting with eukaryotic 80S ribosomes to inhibit protein synthesis (SirDeshpande and Toogood, 1995). Recently, it was shown to cause conformational changes of F-actin and stabilization of actin filaments to induced G2 arrest (Fujiwara *et al.*, 2004).

5   Biosynthetic Genes and Possible Nonribosomal Biosynthetic Mechanism for Plant Cyclopeptides

None of the plant cyclopeptides have been studied

for their biosynthetic genes and enzymes . However, biosynthetic genes for peptides polyketides have been cloned from endophytes of insects and marine animals (Schmidt, 2008; Piel, 2006) . Pederin was the first natural product from an endophyte whose biosynthetic gene cluster has been sequenced (Piel, 2002) . It has to be emphasized that the cyclopeptides exemplified in Fig.2 were isolated from plant endophytes that are " culturable" . Their identification followed the common route of identifying" interesting plants", isolating and identifying microbes from the plants, culturing the microbes and isolating bioactive natural products . Thus, this approach can only identify those products made by culturable endophytes and would miss the" unculturable" endophytes . Furthermore, even within the culturable endophytes, the approach could also miss those products that are not synthesized under the culture conditions . Thus, the uncertainty always exists between what an endophyte produces in cultures and what it may produce in nature . One way to circumvent the disadvantages is to use the metagenomic approach (Wang *et al.*, 2008), which has been successfully used to exploit biosynthetic genes from marine sponges, insects, and human microbiota (microbiome) . This approach has recently been exploited in plant endophytes by enriching the microbiota from plants (Wang *et al.*, 2008) .

Below, we will use RA-VII as an example to propose a possible NRPS-catalyzed biosynthetic pathway (Fig.3) . This proposal is based on the general paradigm established for microbial cyclopeptides and the structural features of RA-VII . The biosynthesis follows the" clockwise" cycle of the cyclopeptide . L-Tyrosine-1 is proposed to be the starter . This is mainly for the convenience of discussion, because the biosynthesis could start from other amino acids and is only be sure by experimental determination . The putative RA-VII synthetase could consist of the starter module (activating, thiolating and *N*-methyl transferring to L-Tyr-1) and five elongation modules (incorporating D-Ala-2, L-Ala-3, L-Tyr-4, L-Ala-5, and L-Tyr-6) . Among them, module-2 would contain an epimerase domain to convert L-Ala to D-Ala, and module-1, 4 and 6 would

contain a methyltransferase domain to add a methyl group to the amide nitrogen . The physical distribution of the modules could not be predicated, as they could be on multiple proteins or on a single protein . The biosynthesis would be terminated by the nucleophilic attack of L-Tyr-1 nitrogen on the carbonyl of L-Tyr-6 to produce a cyclohexapeptide precursor . This could be catalyzed by a thioesterase domain located at the end of the synthetases . The cyclized precursor would be further processed to become a mature cyclopeptide . This includes the O-coupling of the two phenyl rings of Tyr-1 and Tyr-6 . This is probably the most interesting step in the biosynthesis of RA-VII, as it leads to the formation of the unusually strained 14-membered cycloisodityrosine unit, which is important for its anticancer activity . This type of phenolic O-coupling is known from lignins and could be readily rationalized through oxidative radical coupling mechanism . The reaction could be catalyzed by a peroxidase or a cytochrome P450-type enzyme . In addition, the cyclized precursor would require two O-methylations at the phenolic oxygen of Tyr-1 and Tyr-4 . This type of methylation is usually catalyzed by a separate methyltransferase, rather than the MT domain of the NRPS .

6   Final Remarks

Finding alternative drug sources is obviously an important goal in light of the constant occurrence of multi-drug resistant pathogens and tumor cells . This is especially significant for multi-drug resistant pathogens and cancers . Unlike other types of plant natural products, cyclopeptides represent an untapped source for new drugs and drug leads that could be used in the battle against the multi-drug resistant pathogens and cancers . To realize this goal, it is crucial to have an understanding of the biosynthetic origin and the biosynthetic mechanism . This knowledge is essential for developing rational approaches toward the utilization of the natural resources through metabolic engineering . In the review, we intend to propose an endophytic origin and a nonribosomal peptide biosynthetic mechanism . However, the real answer to the questions will ultimately have to come from the experiments .
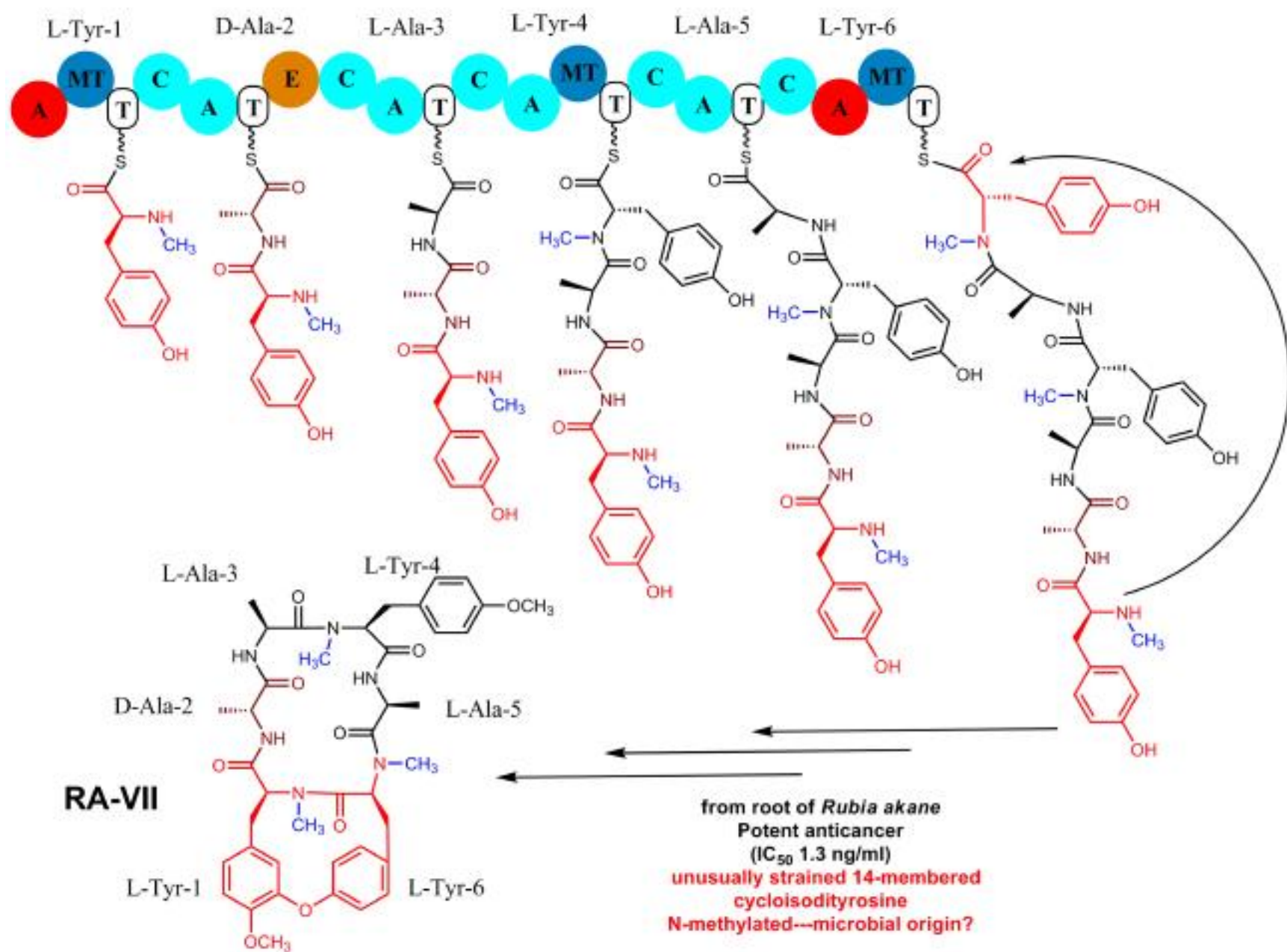
Fig . 3 A proposed biosynthetic pathway for RA-VII . Abbreviations: A, adenylation domain; C, condensation domain;
E, epimerase domain; MT, methyltransferase domain; T, thiolation domain (peptidyl carrier protein) .

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