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Novel Cyclotides and Uncyclotides with Highly Shortened Precursors from *Chassalia chartacea* and Effects of Methionine Oxidation on Bioactivities

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Running Head: Novel chassatides with highly shortened precursors

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**Keywords:** cyclotides; uncyclotides; chassatide

Background: Cyclotides are biologically active, plant-derived macrocyclic peptides. Only three naturally occurring linear variants have been discovered.

Results: We discovered 14 novel cyclotides and four uncyclotides from *Chassalia chartacea*. Their precursors are the shortest of all known cyclotides.

Conclusion: Uncyclotides have comparable activities to cyclotides. Oxidation of Met disrupts the hydrophobic patch and causes loss of activity.

Significance: Our study broadens knowledge of cyclotides and uncyclotides.

SUMMARY

Cyclotides are a new class of plant biologics that display a diverse range of bioactivities with therapeutic potentials. They possess an unusual end-to-end cyclic backbone combined with a cystine-knot arrangement making them exceptionally stable to heat, chemical and enzymatic degradation. Currently, over 200 cyclotides have been discovered but only three naturally occurring linear variants (also known as uncyclotides) have been isolated. In this study, we report the discovery of 18 novel peptides, chassatide C1 to C18, composed of 14 new cyclotides and four uncyclotides from *Chassalia chartacea* (Rubiaceae family). Thus far, this is the largest number of uncyclotides being reported in a single species. Activity testings showed that the uncyclotides not only retain the effectiveness but also are the most potent chassatides in the assays for antimicrobial, cytotoxic, and hemolytic activities. Genetic characterization of novel chassatides revealed that they have the shortest precursors of all known cyclotides hitherto isolated, and which represents a new class of cyclotide precursors. This is the first report of cyclotide genes in a second genus, the *Chassalia*, besides the *Hedyotis* (*Oldenlandia*) of the Rubiaceae family. In addition, we also report the characterization of two Met-oxidized derivatives of chassatide C2 and C11. The oxidation of Met residue causes loss of bioactivities, strengthening the importance of the hydrophobic patch for membrane interaction.

Cyclotides are ultra-stable macrocyclic peptides from plants that display a diverse range of biological activities such as anti-HIV (1), antimicrobial (2, 3), insecticidal (4), cytotoxicity (5), and uterotonic activity (6). They have been isolated from four plant families: the Rubiaceae, Violaceae, Cucurbitaceae, and Fabaceae (7-12). Cyclotides, composed of 28-37 amino acids with a cystine-knot structure embedded in a circular peptide backbone, display a great stability against thermal, chemical, and enzymatic degradation (13, 14). The wide range of cyclotide bioactivities and their unusual stability have stimulated a growing interest for their applications in agricultural and pharmaceutical industries (15).

Topologically, cyclotides are divided into two major subfamilies, the Möbius and bracelet. The main difference between them is the presence of a cis-Pro bond in loop 5 of the Möbius cyclotides, which is not found in the bracelets (16). Thus far, about 200 cyclotide sequences have been reported and approximately two-thirds belong to the bracelet subfamily (17). A number of hybrid sequences between two subfamilies were also identified although their occurrence appears to be rare, with only about a dozen sequences characterized.
The biosynthesis of cyclotides is a complex process. They are ribosomally synthesized as linear precursors which are posttranslationally modified to join the N- and C- termini to produce mature cyclic peptides (18). Their encoding genes were first cloned from the African plant *Oldenlandia affinis* of the Rubiaceae family (4). Subsequently, cyclotide-encoding cDNAs have also been characterized in several species of the Violaceae and Fabaceae families (7, 19, 20). The predicted precursors of cyclotides have low sequence homology among the families, presumably due to their distant evolutionary relationship. The gene architecture, however, is strikingly similar between the Rubiaceae and Violaceae (21) but is significantly different from the Fabaceae family (7, 20). In the Rubiaceae and Violaceae families, cyclotide genes such as *hbc1* and *voc1* contain an endoplasmic reticulum (ER) signal sequence, an N-terminal pro-domain (NTPP), an N-terminal repeat region (NTR), a cyclotide domain, and a C-terminal propeptide (CTPP) (21, 22). In the Fabaceae family, the cyclotide precursors of cliotides contain no NTPP and NTR domains, and their ER signal sequence is followed directly by the cyclotide domain, a short linker region and an albumin-1 chain a domain (7, 20).

Recently, a few linear variants of cyclotides (also known as uncyclotides) have been identified, including violacin A from *Viola odorata* (23), psyle C from *Psychotria leptothyrsa* (24), and hedyotide B2 from *Hedyotis biflora* (22). Genetic characterization showed that both violacin A and hedyotide B2 have similar biosynthetic processing (22, 23). They are produced as linear precursors that are unable to cyclize. A nonsense mutation found at their C-terminal ends inhibits the translation of the highly conserved C-terminal Asn/Asp residue which is essential for backbone cyclization. These two uncyclotides also display a marked reduction in biological activities (22, 23). Violacin A has low hemolytic activity, and hedyotide B2 is inactive against all tested bacteria as compared to other cyclotides. The structure-activity study has also shown that the cyclic structure appears to be important for the biological functions, and linearization of kalata B1 causes loss of activity indicated by a complete lack of hemolytic properties of various acyclic permutants of kalata B1 (25). Interestingly, the uncyclotide psyle C maintains a moderate cytotoxicity, prompting the question of the importance of the cyclic backbone for bioactivity (24).

In this study, we report the isolation and characterization of novel cyclotides and uncyclotides from *Chassalia chartacea* (synonym *Chassalia curviflora*), a local species commonly found in the Singapore forest. *Chassalia chartacea* is a medium-sized tree, around 1-2 m tall, and produces inflorescences with either white or red pedicels (Figure S1). It is used in the Malay traditional medicine for treatment of malaria, coughs, wounds, and ulcers (26). Within the Rubiaceae, the *Chassalia* is amongst the earliest genera discovered to produce cyclotides (27). Few subsequent studies on cyclotides, however, characterized species from this genus. Thus far, cyclotides have been isolated from only two *Chassalia* species, *C. parviflolia* and *C. discolor*. The discovery of cyclotides in *C. parviflolia* was guided by an anti-HIV bioassay (27), which led to the characterization of six novel cyclotides, circulin A-F. They exhibited anti-HIV activity that ranged from 50 to 275 nM depending on the viral strains and cell lines used in the assay (27, 28). The discovery of cyclotides in the second *Chassalia* species, *C. discolor*, was through a random screening program that aimed to understand the distribution and diversity of cyclotides in the Rubiaceae family (29). One novel cyclotide was isolated from this species. Its biological activity, however, was not investigated further.

Exhaustive screening of cyclotide content in *C. chartacea* in this study has led to the discovery of 18 novel sequences, chassatide C1 to C18 (chaC1-C18), comprising 14 new cyclotides and four uncyclotides. Biological testing showed that the uncyclotides have comparable activities to cyclotides. Genetic characterization of novel chassatides revealed that their precursors are highly shortened, likely due to the absence of the NTR domain. In addition, we also report the isolation of two Met-oxidized derivatives of chassatide C2 and C11. The oxidation of methionine to methionine sulfoxide (MetO) causes a complete loss of biological activities. Overall, our study provides new insights into the structural diversity, biological activity, and biosynthetic pathway of this unique family of proteins.

**EXPERIMENTAL PROCEDURES**
**Isolation and Purification of Novel Chassatides**

The whole *C. chartacea* plant (40 g) was extracted with 400 mL of 10% ethanol. After removal of plant debris, the extract was fractionated by preparative HPLC using a C18 Vydac column (250 x 21 mm) on a Shimadzu system at a flow rate of 8 ml/min. A linear gradient of 1%/min of 0–80% buffer B (100% acetonitrile, 0.05% trifluoroacetic acid) was applied. The fractions obtained were repurified by semi-preparative HPLC using a C18 Vydac column (250 x 10 mm) at a flow rate of 3 ml/min with the same gradient. Isolation of individual cyclotides was obtained by analytical HPLC.

**Sequence Determination**

10 µg of each peptide was dissolved in 50 µL of NH4HCO3 buffer (100 mM, pH 7.8) containing 10 mM dithiothreitol (DTT), and incubated for 1 hr at 37 °C. The S-reduced peptides were digested with endoproteinase Glu-C (EndoGlu-C), trypsin or chymotrypsin, and sequenced by MALDI-MS/MS as described previously (7). Assignments of isobaric residues Ile/Leu and Lys/Gln were based on cDNA sequences, amino acid analysis, enzymatic digestion patterns, and homology to known cyclotides. Amino acid analysis was performed for five cyclotides without gene sequences including chassatide C1, C3, C5, C6, and C10 (supplemental Tables S1-S5). Because of sample limitation, the Ile/Leu assignments of chassatide C9 and C12 were based solely on chymotryptic digestions and sequence homology. MS/MS spectra of cyclotides that do not have the gene sequences (including chassatide C1, C3, C5, C6, C9, C10, and C12) have been provided in the supplemental materials (Figure S1-S9).

**Cloning of chassatide genes**

RNA was prepared from fresh leaves and converted to single-stranded cDNA. Partial encoding genes of cyclotides were amplified by 3′ RACE PCR (Invitrogen, catalog number 18373-019) using degenerate forward primers targeting loop 1 and loop 2 sequences: 5′-CGATCGATTGYGGIGARAGTTG-3′ encoding CGESC sequence, 5′-GGGGATCCTGYGGIGARAC-ITG-3′ encoding CGETC sequence, and 5′-TGCGTTGATHCCNTGCA-3′ encoding CVWIPCI sequence. The remaining encoding genes were obtained by 5′ RACE PCR (Invitrogen, catalog number 18374-058) using reverse primers based on the cDNA sequences obtained from 3′ RACE PCR. To identify intron locations, genomic DNA from fresh leaves was extracted. PCRs on DNA templates were then conducted with specific primers designed against 5′- and 3′-untranslated regions of each chassatide.

**Antibacterial Assay**

Three bacterial strains from the American Type Culture Collection (ATCC) were used including *Staphylococcus aureus* ATCC 12600, *Staphylococcus epidermidis* ATCC 14990, and *Escherichia coli* ATCC 25922. All the strains were cultured in trypticase soy broth (TSB). The antimicrobial activities of novel chassatides were examined using the radial diffusion assay as described previously (22). D3R was used as the positive control (30).

**Cytotoxicity Assay**

Cytotoxicity assay of novel chassatides against HeLa cells was performed as described previously (7). Kalata B1 was used as the positive control. The cytotoxicities of chassatides were indicated by IC50 values (concentration that gives a survival index of 50%). Their dose-response curves were provided in the supplemental materials (Figure S10).

**Hemolytic Assay**

Blood type A was taken from a healthy volunteer. Hemolytic assay was performed as described previously (7). Kalata B1 and melittin were used as positive controls. The hemolytic activities of chassatides were indicated by HD50 values (concentration that causes 50% lysis of red blood cells). Their dose-response curves were provided in the supplemental materials (Figure S10).

**Homology Modeling**

Computer models of chassatide C2 and C11 were built using the structure prediction module Knoter1D3D available from the protein analysis tool kit PAT at [http://pat.cbs.cnrs.fr](http://pat.cbs.cnrs.fr) (31, 32). The structures were analyzed and represented by PyMOL. For chassatide C2A and C11A, their sequences contain an unusual amino acid MetO which is not supported by the module Knoter1D3D. Therefore, their models were represented by the respective models of chassatide C2 and C11 but with Met residues colored in white to indicate the polar side chain of the MetO as opposed to the hydrophobic side chain of the Met residue (colored in green).

**RESULTS**

**Isolation and Discovery of Novel Chassatides from Chassalia chartacea**.

The whole *C. chartacea* plant (40 g) was extracted with 10% ethanol. After three rounds of purifications by RP-HPLC, 15 peptides were isolated and sequenced by MS/MS, comprising circulin A, nine novel cyclotides, three novel uncyclotides...
and two Met-oxidized derivatives (Table 1). Each novel peptide was termed “chassatide C” followed by a number according to their order of discovery. The novel cyclotides consist of five bracelet, two Möbius and two hybrid cyclotides. All three novel uncyclotides (chassatide C7, C8, and C11) belong to the bracelet subfamily, all lacking the Asn/Asp residue at their C-termini which is crucial for backbone cyclization (22, 23).

During the characterization of novel cyclotides and uncyclotides, we isolated two Met-oxidized derivatives of chassatide C2 and C11, named as chassatide C2A and C11A, respectively. The oxidation of Met to MetO causes a mass increase of 16 Da, corresponding to the addition of an oxygen molecule. Although the MetO residue has the same molecular mass as Phe, it could be easily distinguished from Phe by a facile loss of methanesulfenic acid CH₃SOH (64 Da) during the MS/MS fragmentation (33). Figure 1 shows the MS/MS spectra of the EndoGlu-C-treated fragments of chassatide C2A and C11A to illustrate the identification of the oxidized Met. Another diagnostic feature was that the oxidized peptides eluted earlier in the RP-HPLC as compared to the native peptides (Figure S2).

Expression profiles of cyclotides in Chassalia chartacea. To study the tissue-specific distribution of chassatides in C. chartacea, five different plant parts including leaves, stems, roots, pedicels, and fruits were extracted separately. As shown in Figure 2, leaves and roots displayed strikingly different expression patterns. Leaves expressed predominantly two cyclotides, chassatide C1 and C4, both of which belong to the Möbius subfamily. In contrast, roots produced mainly bracelet cyclotides and uncyclotides, with virtually no Möbius cyclotides detectable under our experimental conditions. Interestingly, fruits, pedicels, and stems shared similar MS profiles, and their chassatide contents appeared to be a combined expression of both roots and leaves. These results suggested that these tissues are heavily protected against insults, indicative of their importance for plant survival.

Next, we compared the cyclotide profiles of the white and red pedicel varieties of C. chartacea. Interestingly, both showed identical cyclotide expression patterns in leaves, stems, pedicels, fruits, and roots (data not shown), suggesting the production of cyclotides in C. chartacea is independent of the pedicel-color variations.

Cloning of the Chassatide Encoding Genes from Chassalia chartacea. Encoding cDNAs of chassatides were cloned by 3′ RACE PCR using degenerated primers targeting loop 1 and loop 2 sequences (CGETC, CGESC, and CVWIPCI). Full-length genes were subsequently obtained by 5′ RACE PCR. This resulted in identifying two partial clone encoding chassatide C7 and C11, and nine full-length clones encoding chassatide C2, C4, C8, and six new sequences, chassatide C13-C18. Chassatide C17 is a putative uncyclotide due to its absence of the C-terminal Asn/Asp residue at the processing site attributed to a nonsense mutation.

To compare the genetic structures of chassatides at both DNA and mRNA levels, four chassatide precursor genes including chassatide C13, C15, C16 and C18 were cloned from the leaf DNA using cDNA-derived sequences as primers. The DNA clones revealed a single intron located in the signal peptide region of the chassatide genes (Figure S11). The intron location is similar to other known cyclotide genes of the Rubiaceae family such as kalata B1 (29) and hedyotide B1 (22). This is also the first time that cyclotide and uncyclotide genes were characterized from another genus, the Chassalia, besides the Hedyotis (Oldenlandia) of the Rubiaceae family.

Translated precursors of chassatide genes are shown in Figure 3. Their primary sequences were aligned with the precursors of other rubiaceous cyclotides including hedyotide B1 and B2 (H. biflora), kalata B1 and B7 (O. affinis), and hcf-1 (H. centranthoides). Chassatide precursors share a high sequence homology among each other but are significantly different from other rubiaceous cyclotides. Interestingly, they have the shortest precursors of all known cyclotides hitherto isolated, and are significantly shorter than the others with only 75-78 residues as compared to kalata B1 (124 residues) or hedyotide B1 (107 residues). Their NTPP, NTR, and CTPP domains are all reduced in size. The NTR domain, in particular, is oversimplified and nearly absent in most chassatide genes. It is interesting to note that their CTPP domains are also the shortest, with many comprised of only two residues “EL” as compared to at least three residues in other cyclotide precursors.

For chassatide C7, C8, and C11, a premature stop codon is found precisely at the
C-terminal Asn/Asp position in their precursors, which provides a biosynthetic explanation for their linear structures. The absence of the conserved Asn/Asp also confirms the crucial role of the C-terminal Asn/Asp residue at the processing point for backbone cyclization.

**Antimicrobial Activities of the Novel Chassatides.** Antimicrobial activities of chassatides C1/C4 (tested as a mixture due to coelution with a molar ratio of chassatide C1:C4 approximately 2:1), C2, C2A, C7, C8, C10, C11, and C11A were tested against three bacteria strains: *E. coli*, *S. aureus*, and *S. epidermis*. D$_5$R, an antimicrobial peptide synthesized in our laboratory, was used as the positive control (30). Chassatide C1/C4, C2, C2A, C10, and C11A were not effective against all tested strains. Unexpectedly, all three uncyclotides displayed moderate activities against *E. coli* with MIC values of 6.4, 6.6, and 8.5 µM for chassatide C7, C8, and C11, respectively (Table 2). None of the tested chassatides were active against *S. aureus* and *S. epidermis* under our experimental conditions.

**Cytotoxicities of the Novel Chassatides.** Cytotoxicities of novel chassatides against HeLa cells were assessed using the MTT assay. Kalata B1 was used as the positive control. Two Met-oxidized derivatives, chassatide C2A and C11A, did not show any cytotoxicity up to 15 µM. Other tested chassatides showed very sharp dose-response curves with IC$_{50}$ values ranging from 1.0 to 9.8 µM (Table 2). Interestingly, three uncyclotides, chassatide C7, C8, and C11, were the most potent among the tested samples with IC$_{50}$ values of 1.2, 1.0, and 1.2 µM, respectively. Chassatide C1/C4 was the least cytotoxic with IC$_{50}$ value of 9.8 µM.

**Hemolytic Activities of the Novel Chassatides.** The hemolytic effects of chassatides were evaluated on human type A erythrocytes. Melittin and kalata B1 were used as positive controls. Their HD$_{50}$ ranged from 11.6 to 51.9 µM (Table 2), and were significantly less potent than melittin (2.1 µM). Similar to the cytotoxic effect, the three uncyclotides, chassatide C7, C8, and C11, were the most active peptides with HD$_{50}$ of 11.6, 25.5, and 13.3 µM, respectively. Chassatide C1/C4 was the least hemolytic with HD$_{50}$ value of 51.9 µM. Hemolytic activities of chassatide C2, C2A, C10, and C11A were not determined due to insufficient samples obtained and their low toxicities to red blood cells with HD$_{50}$ >25 µM.

**DISCUSSION**

The work presented here contributes to our understanding about the diversity and distribution of cyclotides in the *Chassalia* genus. From *C. chartacea*, we have successfully characterized 18 novel sequences using both proteomic and genomic approaches. Consistent with previous reports on other cyclotides’ tissue distributions, chassatides display a tissue-specific expression, suggesting that they may have differential physiological functions in different plant parts. In addition, we also studied the medicinal values of novel chassatides by examining their antimicrobial, cytotoxic and hemolytic activities, which provides a more comprehensive knowledge about the membrane-active property of cyclotides and uncyclotides.

**Distribution of Cyclotides in the Chassalia Genus.** *Chassalia chartacea* is the third species of its genus found to produce cyclotides. Little is known about the distribution of cyclotides in this genus. Prior to this work, only three *Chassalia* species were screened including two cyclotide-positive plants, *C. parvifolia* and *C. discolor*, and one non-cyclotide containing plant, *C. Comm. ex Poir. sp* (27, 29). Although only four *Chassalia* species have been tested, it appears that the occurrence of cyclotides in this genus is also sporadic similar to the *Hedyotis* and other rubiaceous genus. This suggests a complicated evolution, and distribution of cyclotides in the Rubiaceae family.

Thus far, only seven cyclotides have been purified and identified from the *Chassalia* species, six of which are from *C. parvifolia* and one from *C. discolor*. In-depth analysis of the cyclotide content from *C. chartacea* in our work has led to the discovery of 18 new cyclotides and uncyclotides, which triples the number of cyclotides isolated in this genus. Of the novel sequences discovered, four are uncyclotides from the bracelet subfamily. This finding is significant as there are very few examples of acyclic versions of cyclotides reported in the literature, and currently only one example of bracelet uncyclotide, hedyotide B2 from *H. biflora*, was discovered (22). Bracelet uncyclotides may thus be more common than previously thought. It is also noteworthy that the uncyclotides are among the most abundant peptides expressed in both *H. biflora* and *C. chartacea*, suggesting their biologically relevant roles in plant defense similar to cyclotides. However, more work are clearly needed to
unlock the cyclotide and uncyclotide goldmine in the _Chassalia_ which comprises more than 70 species (29), _Distinctive Features of the Novel Chassatide Precursors_. With no prior cyclotide genes being isolated from other genus besides the _Hedyotis_ of the Rubiaceae family, it is of interest to characterize the cDNAs encoding for novel chassatides. Eleven unique clones were obtained, six of which encoded for novel peptides. Sequence comparison with known cyclotides from _O. affinis, H. biflora_, and _H. centranthoides_ revealed several unexpected surprises and distinctive features about the chassatide precursors.

Firstly, they have the shortest precursors of all known cyclotide clones which are 35 to 39% shorter than kalata B1 precursor. It is uncertain why _C. chartacea_ expressed such short cyclotide precursors. One possible explanation is that _C. chartacea_ is an understory tree where sunlight is a limiting factor as opposed to the African species _O. affinis_ which is grown in full sunlight. When less light is available, less energy is available for the plants, and most understory plants evolve to be efficient energy users (34). The reduced size of the cyclotide precursors may provide an economic advantage for plants as less energy will be required for producing cyclotides. This may be part of the shade-tolerant mechanisms developed for plant adaptation to low-light conditions. However, this hypothesis is just a pure speculation which will need a more proper control in future study.

Secondly, the NTR domain is absent in most, if not all, chassatide genes characterized, accounting for the reduced size of the chassatide precursors. The absence of the NTR domain suggests that it is not essential for cyclotide biosynthesis, but may have other physiological functions in plants. _In vitro_ experiments have also shown that the NTR domain has no direct role in the folding of kalata B1 (data not shown), and thus explaining its abridgement in the chassatide precursors.

Thirdly, the CTPPs of chassatide C2, C13 to C16 consist of only two residues “EL”, the shortest CTPPs documented for cyclotide genes which usually contain three to eleven residues. It has been previously proposed that the C-terminal tripeptide motif is essential for the production of cyclic peptides (18). Our finding suggests that only two residues on the CTPPs are sufficient for the biosynthesis of cyclotides.

Fourthly, both chassatide C14 and C16 precursors contain a tetrapeptide motif RNEL and KNEL at their C-termini, respectively. These peptide motifs resemble the classical ER retention signal, C-terminal KDEL sequence. Mutagenesis studies have demonstrated that variants of the KDEL such as RDEL and KNEL can direct protein retention in the ER lumen (35). It is thus of interest to determine in future studies if the chassatide C14 and C16 precursors are retained in the ER or processed for extracellular transport.

Fifthly, there is a clear difference of residues at the N-terminal processing sites between the cyclotide and uncyclotide domains of the chassatide precursors. In both instances, they bear more resemblance to those of the Violaceae family than those of the _Hedyotis_ genus (Rubiaceae). The N-terminal cleavage occurs after the dipeptide motif Val/Leu-Gly for uncyclotides, and X-Asn for cyclotides. Although the Val/Leu-Gly and X-Asn motifs are commonly seen in the cyclotide genes of the Violaceae family (36), this is the first time that they are reported for the Rubiaceae family.

Finally, the biosynthesis of three uncyclotides, chassatide C7, C8, and C11, is likely similar to hedyotide B2 and violacin A, in which their linear structures are genetically predetermined. A premature stop codon is found at the C-terminal Asn/Asp position which inhibits its translation. Our finding reinforces the essential role of the C-terminal Asn/Asp residue in the backbone cyclization during the biosynthesis of cyclotides, and its deficiency will lead to uncyclotide formation. _Biological Activities Study of the Novel Chassatides_. Cyclotides have been proposed to exert their biological activities by permeabilization of the lipid membrane of the target cells, ultimately leading to pore formation and cell death (37-39). In this work, the membrane-active properties of novel chassatides were indirectly assessed by using antimicrobial, hemolysis and cytotoxicity assays, which evaluate their effects on three different membrane types of bacteria, erythrocytes, and HeLa cells.

Structure, net charge, and hydrophobicity (Table S6) show varying degrees of correlation to the activities of chassatides. The end result of membrane permeabilization usually determines by the combined effects of electrostatic and hydrophobic interactions between peptides and target membranes (40-42). Net charge or
electrostatic interaction appears to be the dominant factor determining the antimicrobial activity. Except for chassatide C11, all chassatides with neutral or negative net charges are inactive against all the tested bacteria. Chassatides with +1 net charge display bactericidal activity on E. coli. Interestingly, none of the chassatides are active against two tested Gram-positive strains. It is possible that the thick peptidoglycan layer of Gram-positive bacteria protects them by preventing the interaction between chassatides and the bacterial membranes.

For cytotoxicity, there is a strong influence of hydrophobicity on the chassatide activities. Those with hydrophobicity ratio more than 50% (chassatide C2, C7, C8, and C11) are the most active. Consistently, chassatide C10 (37%) and chassatide C1/12 (41%) with a hydrophobicity ratio less than 50% have weaker cytotoxicity. Net charge in this instance has a less important role. Chassatide C2, for example, displayed a strong cytotoxicity with an IC₅₀ value of 2.44 μM despite its neutral charge. A similar trend appears to be true for hemolytic activity. Those with strong cytotoxicity also display potent hemolytic effects.

**Cyclic Backbone is Not an Essential Requirement for Biological Activities.** The discovery of novel uncyclotides from the bracelet subfamily, perhaps, is one of the most important findings of this work. Characterization of their biological activities provides new understanding about functional correlations between linear and cyclic forms of cyclotides. Previous studies on synthetic linear variants of kalata B1 and the uncyclotide violacin A reported that linearizing the circular backbone structure resulted in a total loss of or reduced hemolytic activity (23, 25). Surprisingly, three uncyclotides isolated in C. chartacea not only maintained the membranolytic activities, but also were the most active of all tested chassatides. They exhibit stronger antimicrobial, cytotoxic, and hemolytic effects than several cyclotides such as kalata B1, chassatide C1/4, C2, and C10 under the same experimental conditions. This suggested that the cyclic backbone may not be an essential requirement for membranolytic activity. This finding is significant as not all plants may possess an efficient cyclization machinery. Transgenic expression of kalata B1 encoding gene (Oak1) in non-cyclotide-containing plants (Arabidopsis thaliana, Nicotiana tabacum, and Nicotiana benthamiana) only produced a marginal level of circular form as compared to the linear form of kalata B1 (18). Therefore, it may be more practical to use uncyclotides instead of cyclotides for transgenic engineering for crop protection. Our work opens up a new avenue of research for the agricultural applications of uncyclotides.

Recently, a moderate cytotoxic activity has also been reported for the uncyclotide psyle C with an IC₅₀ value of 3.5 μM against human lymphoma cell line U937-GTB (24). Our works on chassatide C7, C8, and C11 have further strengthened that uncyclotides can possess a comparable membranolytic activity as their cyclic counterparts. The circular backbone though may provide an additional advantage in terms of stability, it is not an indispensible functional requirement. In the case of violacin A, it seems that its hydrophilic nature is the main reason behind the low hemolytic value. For kalata B1, opening its cyclic backbone may cause a loss of the structural epitope important for activity. While the physiological roles of the cyclic backbone are still uncertain, it is clear that plants do display a wide variety of both linear and cyclic variants, and they are likely to have functional significance in plants.

**Methionine Oxidation Causes Loss of Chassatide Activities.** The oxidation of Met to MetO causes a complete loss of bioactivities of chassatide C2A and C11A. The sulfur atom of Met residue can be oxidized to sulfoxide by the addition of an oxygen atom. This phenomenon has been observed in a number of native proteins and is especially relevant in aging tissues (43). Oxidation of Met causes a significant change in its biophysical properties and often leads to an alteration of protein functions. Met has a long and non-polar side chain, which becomes polar upon oxidation. The hydrophobicity index of MetO has been estimated to be similar to that of Asn (44). Therefore, the oxidation of Met can be considered as a substitution of a hydrophobic for a hydrophilic amino acid, and is expected to have pronounced structural and functional consequences.

In both chassatide C2 and C11, the Met residue is located in loop 3 and its oxidation likely causes disruption of the hydrophobic patches (Figure 4). It has been proposed that bracelet cyclotides rely on patches of hydrophobic residues stretching over loop 2 and loop 3 for membrane interactions (38). These hydrophobic patches are interrupted in the Met-
oxidized variants, chassatide C2A and C11A, explaining their losses of cytotoxic, hemolytic and antimicrobial properties. It should be noted that chassatide C2A and C11A possess cyclic and linear structures, respectively. The hydrophobic patches are thus important for both cyclotides and uncyclotides for exerting their biological activities. A similar phenomenon has been reported for kalata B1, varv A, and cycloviolacin O2 (40, 45). In these studies, the oxidation occurred on the Trp residue which also led to the abrogation of the hemolytic activity (40, 45). The oxidation of the methionine and tryptophan residues may thus be the natural degradation pathways that result in the deactivation of the intrinsic biological activities of cyclotides and uncyclotides in plants.

In summary, our data provide the first description of cyclotides and uncyclotides in Chassalia chartacea. The study has resulted in the discovery of 18 novel sequences, and significantly expanded the number of naturally occurring uncyclotides from three to seven. Their precursors are highly shortened due to the absence of the NTR domain. Our results also indicate that the CTPPs consisting of only two residues are sufficient for the biosynthesis of cyclotides. Interestingly, bioassay testings demonstrated that uncyclotides have comparable activities to cyclotides, suggesting that the cyclic backbone may not be an essential requirement for activity. Overall, the discovery and characterization of novel cyclotides and their precursors continue to produce unexpected surprises of their diversity and biosynthesis as proteinaceous natural products. This trend will likely continue as more cyclotides are identified from various plant families together with an increased interest of cyclotide applications in the agricultural and pharmaceutical industries.

REFERENCES

FOOTNOTES

This work was supported by A*STAR Biomedical Research Council Grant 09/1/22/19/612 and Academic Research Fund Grant ARC21/08 from the Ministry of Education in Singapore. The nucleotide sequences for chassatide C2, C4, C7, C8, C13, C14, C15, C16, C17, C18, C13 (DNA), C15 (DNA), C16 (DNA), C18 (DNA) reported in this paper have been deposited in the GenBank database under GenBank accession numbers JQ309962, JQ309963, JQ309964, JQ309965, JQ309966, JQ309967, JQ309968, JQ309969, JQ309970, JQ309971, JQ309972, JQ309973, JQ309974, JQ309975, respectively.

FIGURE LEGENDS

Figure 1. MS/MS identification of the Met-oxidized products. A) MS/MS spectrum of EndoGlu-C-treated fragment of chassatide C2A. B) MS/MS spectrum of EndoGlu-C-treated fragment of chassatide C11A. Peaks with a neutral loss of methanesulfenic acid (64 Da) from the side chain of methionine sulfoxide are indicated by the arrows.

Figure 2. Tissue-specific distribution of chassatides in *Chassalia chartacea*. Plant specimens were divided into five different parts: leaves, stems, roots, pedicels and fruits. They were extracted and profiled separately by mass spectrometry.

Figure 3. (A) Schematic comparison of the genetic arrangements of kalata B1, hedyotide B2, chassatide C2, and chassatide C8. (B) Multiple sequence alignment of chassatide precursors with kalata B1, kalata B7, hcf-1, hedyotide B1 and hedyotide B2 precursors. The mature cyclotide domains are boxed. The putative ER domain is colored in black, NTPP domain in yellow, NTR domain in purple, cyclotide domain in light blue and the CTPP domain in green color. Asterisks indicate stop codon. The inverted triangle indicates the intron location.

Figure 4. Disruption of the hydrophobic patch in the Met-oxidized chassatides. (A) Computer model of chassatide C2, and (B) representative model of chassatide C2A. (C) Computer model of chassatide C11, and (D) representative model of chassatide C11A. Hydrophobic residues are colored green, polar residues are white, negatively charged residues are red, positively charged residues are blue, Gly are cyan, and Cys are yellow. The arrows indicate the position of the Met residues.
### Table 1. Novel cyclotides and uncyclotides in *Chassalia chartacea*.

<table>
<thead>
<tr>
<th>Chassatide</th>
<th>Amino acid sequence</th>
<th>MW (Da) (^a)</th>
<th>Subfamily</th>
<th>Approach (^d)</th>
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<td>chaC1</td>
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<td>2990</td>
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<td>P</td>
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<td>P, cDNA</td>
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(a) Molecular weights are shown in monoisotopic masses. (b) Chassatide C7, C8, C11 and C17 are uncyclotides. (c) Predicted sequences from cDNA. (d) Peptide sequences were determined by proteomic approach (P), cDNA or DNA gene cloning. Cys residues are highlighted in yellow. Oxidized Met (MetO) residues are in gray.
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<th>Gram Positive</th>
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<th>HD$_{50}$ (µM)</th>
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</table>

Abbreviations are as follows: ND (Not determined), MT (melittin), MIC (Minimal inhibitory concentration), IC$_{50}$ (concentration that gives a survival index of 50%), HD$_{50}$ (concentration that causes 50% lysis of red blood cells).
Figure 1
Figure 2
Figure 3
Figure 4