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Recent progress on cyclotides: 2021–2024

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ABSTRACT: Cyclotides are ultra-stable macrocyclic peptides. This article is part of a special issue celebrating the 50th Anniversary of *ScienceAsia*. It reviews progress in the cyclotide field of research in the period 2021 to 2024. More than 100 papers on cyclotides were published in this period along with multiple patents, book chapters and conference presentations. Here we discuss the findings from 84 original research papers on the discovery of native cyclotides and their biological activities, as well as describing a range of experimental and computational techniques that have been reported over the last four years to advance cyclotide research. The aim is to provide a useful resource for researchers to be updated on the discovery of native cyclotides and to explore the chemistry, biology and applications of engineered cyclotides over recent years.

KEYWORDS: cyclic peptide, drug design, insecticide, kalata B1

INTRODUCTION

Cyclotides are macrocyclic peptides from plants that were first classified as a family and named in 1999 [1]. Some early members of the family were characterized in the period 1970–1998, including the prototypic member, kalata B1, whose structure is shown in Fig. 1, which summarizes the main features of cyclotides- i.e., they are plant-derived peptides of around 30 amino acids that have a head-to-tail cyclic backbone and a knotted arrangement of three disulfide bonds. The combination of these two structural features makes cyclotides exceptionally stable and, most notably, they are highly resistant to proteases. Currently, the sequences of more than 700 members of the cyclotide family have been determined and are documented in the on-line database CyBase [2] (www.Cybase.org.au). The six backbone loops between successive Cys residues are hypervariable and are responsible for the wide range of activities of cyclotides.

Cyclotides are of interest because of their native function as host defense peptides, which lends them to applications in agriculture, but also for their applications as ultra-stable scaffolds in peptide-based drug design. They are classified into three main subfamilies known as bracelet, Möbius and trypsin inhibitor cyclotides, with prototypic sequences and structures for each subfamily shown in Fig. 1. A fourth subfamily, referred to as 'hybrid' recognizes that some cyclotides contain sequence elements that are hybrid between two or more of the other subfamilies. So far cyclotides have been found in more than 100 species from five major plant families, including the Violaceae, Rubiaceae, Cucurbitaceae, Solanaceae and Fabaceae.

Cyclotides have been extensively reviewed since their original discovery and readers are referred to recent reviews for background [3–5]. The current article is a mini review that focusses solely on developments in the cyclotide field in the period 2021 to 2024. Considering the inclusion of this article in a special issue celebrating the 50th anniversary of the journal *ScienceAsia*, we have highlighted selected studies from Asia reported in this period.

LITERATURE SURVEY AND SCOPE OF REVIEW

The content of this mini review is based on a literature search conducted on November 20th, 2024, that surveyed entries in the "All Database" section of Web of Science using the keyword "cyclotide" to search in the topic field and the date range 2021–2024. The search returned 150 entries, which included 25 reviews, seven patents and a small number of book chapters, abstracts and other articles. The dataset analyzed in this article comprises the original research papers identified in that survey along with a few key earlier articles. We categorized the research papers into the topics of '**Discovery and characterization**' (19 papers) [6–24], '**Technologies for cyclotide analysis**', which included biophysical methods (9 papers) [25–33], computational and genomic approaches (11 papers) [34–44], 'synthetic and biosynthetic approaches' (11 papers) [45–55] and '**Applications**', including in agriculture [56–59], pharmaceuticals [60–81] and biotechnology [82–89]. While there is clearly overlap of these topics in many individual papers, we assigned the papers to one of these categories based on the study design and major message of each paper.

[†]2015 FAOBMB Award for Research Excellence; 2023 Medal, Australian Academy of Science; Editorial Board of ScienceAsia 2023-present.

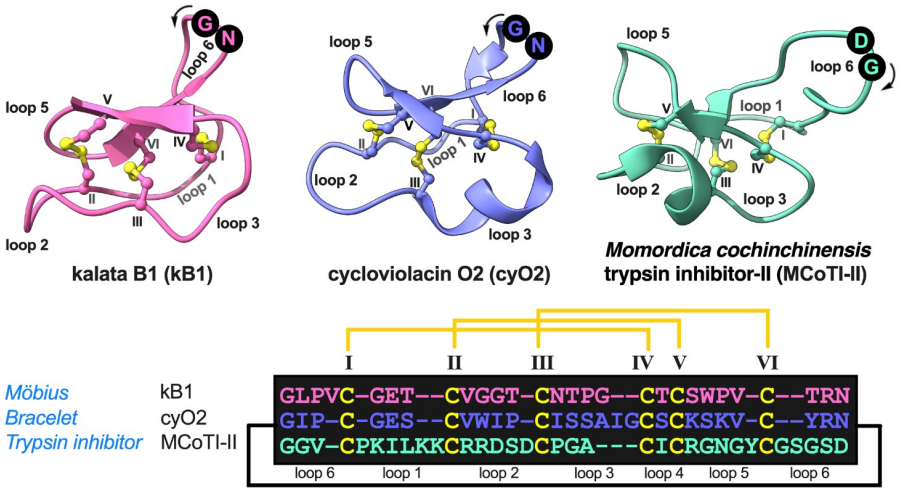

Fig. 1 Representative three-dimensional structures and sequences of cyclotides from each of the three subfamilies, Möbius: kalata B1 (pink, PDB: 1NB1), bracelet: cycloviolacin O2 (purple, PDB: 2KNM), and trypsin inhibitor: MCoTI-II (green, PDB: 1IB9). Top: Ribbon representation of the backbone of the cyclotide molecules. Disulfide bonds are illustrated in yellow. The black curve arrow indicates the beginning of the sequence from the glycine residue. Bottom: The numbering scheme for the cysteine residues is indicated by I–VI. The backbone loops between the six conserved cysteine residues are labelled (loop 1–6) and shown in yellow lines. The cyclic backbone is indicated by the black line.

DISCOVERY AND CHARACTERISATION

There were 19 papers in the review period whose primary focus was the discovery of new cyclotides or cyclotide-bearing extracts. These studies reported new cyclotides mainly from plants in the Rubiaceae [6–8] and Violaceae [9–20] families, both of which had earlier been known as cyclotide-bearing families. Reports of cyclotides were also made from species in the Solanaceae [21] and Fabaceae families [22] based on screening at the nucleic acid level.

Table 1 lists the sequences, plant origins, and activities of the recently discovered novel cyclotides, along their categorization into cyclotide subfamilies and an indication of the method used to determine their sequence. (See on-line version for full list, print version contains a shorter summary Table). The table also includes examples where a known cyclotide was reported during the review period in a plant different to that in which it was originally discovered [12, 13], or a where known cyclotide was found during the review period to have a previously unreported activity [15]. The table contains a total of 166 cyclotides and 39 acyclotides (acyclic versions of cyclotides described in more detail below). It highlights that there are ongoing discoveries of new cyclotide sequences and the diversity of sequences in the six backbone loops of cyclotides is very high.

Of the 19 primary discovery papers, many of the novel cyclotides were found plants belonging to the Rubiaceae or Violaceae families (Table 1). Among them, five novel cyclotides were isolated from *Palicourea sessilis*, a Brazilian plant from the Rubiaceae family, and demonstrated immune-suppressive activity on human lymphocytes [6]. Another notable Rubiaceae species, *Carapichea ipecacuanha*, was the source of caripe 11 [7], which was reported to have activity against the cholecystokinin-2 receptor. This plant is well known in medical circles as the source of the emetic preparation 'syrup of ipecac' and was widely used in Western clinical practice until the late 20th century. In another example from the Rubiaceae family, Gunasekera and colleagues reported a systematic survey of *Geophila repens* from Sri Lanka [8]. That study provided a detailed outline of methods used for cyclotide discovery, resulting in the identification of eight novel cyclotides, named gere 1–8, believed to function as plant defense molecules.

A majority of the newly reported cyclotides were derived from various species within the Violaceae family. This is not surprising as the Violaceae is the only family so far thought to be ubiquitous in its expression of cyclotides. Until now, approximately 40 of the 900 species in this family have been examined for cyclotides, and all those investigated have contained cyclotides. In the new papers from 2021–2024, the Violaceae species examined included *Viola betonicifolia*, *V. communis*, *V. japonica*, *V. odorata*, *V. philippica*, and *V. dalatensis* Gadnep [9–17]. One point worthy of caution is that some discovery papers inadvertently report discovery of a novel cyclotide and give it a name, but inspection of the sequence later reveals the sequence to be the same as an already known cyclotide. Several examples of this unfortunate occurrence are in-

Table 1 Sequences of novel cyclotides reported in the period 2021–2024 (includes some known cyclotides in new plants or with new activities).

Name	Sequence	Plant species	Function/target	Sub-family	Sequencing method	Ref.
pase A	GLPVCGETCVGGTCNTPGCVCSWPVCTRN	*Palicourea sessilis*	Immunosuppressant	Möbius	MS/MS	[6]
pase B	GLPVCGETCVGGTCNTPGCVCSWPICTRN	*Palicourea sessilis*	Immunosuppressants	Möbius	MS/MS	[6]
pase C	GLPTCGETCVGGTCNTPGCVCSWPICTRN	*Palicourea sessilis*	Immunosuppressants	Möbius	MS/MS	[6]
pase D	GLPVCGETCVGGTCNTPGCVCAWPICTRN	*Palicourea sessilis*	Immunosuppressants	Möbius	MS/MS	[6]
pase E	GLPVCGETCVTGSCYTPGCSCSWPVCKRN	*Palicourea sessilis*	Immunosuppressants	Möbius	MS/MS	[6]
caripe 11	GVIPCGESCVFIPCISTVIGCSCKKKVCYRN	*Carapichea ipecacuanha*	Binds cholecystokinin-2 receptor (CCK2R)	Bracelet	MS	[7]
gere 1	GIPCGESCVWIPCISSAIGCSCKNKVCYKN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	RNAseq, MS/MS	[8]
gere 2	GIACGESCAYFGCWIPGCSCKDKVCYIN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	MS/MS	[8]
gere 3	GVPCGESCVFIPCITTVIGCSCKDKVCTYN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	MS/MS	[8]
gere 4	GVPCGESCVFIPCFTTVVGCSCKDKVCYNN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	MS/MS	[8]
gere 5	GVACGESCAYFGCWIPGCSCKDKVCYFN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	RNAseq	[8]
gere 6	GVPCGESCVFIPCLTSVVGCSCKDKVCYNN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	RNAseq	[8]
gere 7	GLPTCGETCFTGKCYTPGCTCGAYFVCLKN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	RNAseq	[8]
gere 8	GMPCGESCVWIPCLSAIAGCSCKNKVCYNN	*Geophila repens*	Antimicrobial & cytotoxic	Bracelet	RNAseq	[8]
vibe 1	GIPCGESCVWIPCITSAIGCSCSSKVCYRN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq	[9]
vibe 2	GIPCGESCVWIPCLTSAIGCSCSSKVCYKN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq	[9]
vibe 3	GTFPCGESCVFIPCISSVVGCSCKSKVCYKN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq, MS	[9]
vibe 4	GFPCGESCVYIPCLTAAIGCSCKNKVCYKN	*Viola betonicifolia*	Not reported	Acyclotide	RNAseq	[9]
vibe 5/Viba 24*	GKIPCGESCVWIPCITTVVGCSCSNKVCYKN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq	[9]
vibe 6	GSVPCGESCVWIPCISSVVGCSCSNKVCYMN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq, MS	[9]
vibe 7	GLKAAVPCGESCVWIPCVTSVVGCSCSNKVCYN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq	[9]
vibe 8	GLGRVPCGESCVYIPCFTSIAGCSCSDKVCWHN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq	[9]
vibe 9/Viba 18*	GIHCAETCLWGTCRTAYIGCSCENKICYKN	*Viola betonicifolia*	Not reported	Bracelet	RNAseq, MS	[9]
vibe 10	GIPCGESCAYAPCFTSLISCTCSRKGCYH	*Viola betonicifolia*	Not reported	Acyclotide	RNAseq	[9]
vibe 11	GAFCFETCVFLPCFSSGIGCYCAWHYCVQD	*Viola betonicifolia*	Not reported	Acyclotide	RNAseq, MS	[9]
vibe 12	GIPCGESCAYAPCFTSLISCTCSRKGCYH	*Viola betonicifolia*	Not reported	Bracelet	RNAseq, MS	[9]
vibe 13	GLPVCGETCVGGSCYTPGCTCSWPVCTRN	*Viola betonicifolia*	Not reported	Möbius	RNAseq	[9]
vibe 14	GVPICGESCFKGACYTPGCTCNWPVCERN	*Viola betonicifolia*	Not reported	Möbius	RNAseq, MS	[9]
vibe 15	GSYISCGETCVKLKCYTPGCKCTWPACKKN	*Viola betonicifolia*	Not reported	Möbius	RNAseq, MS	[9]
vibe 16	ASSTCGKTCFGGICNTPGCSCSSWPMCMKN	*Viola betonicifolia*	Not reported	Möbius	RNAseq	[9]
vibe 17	GIVYCGETCGGTRCYTPGCSCRYPYCSKN	*Viola betonicifolia*	Not reported	Möbius	RNAseq, MS	[9]
vibe 18	GSIFNCGETCVFGTCYTPGCSCVYGACSKD	*Viola betonicifolia*	Not reported	Hybrid	RNAseq, MS	[9]
vibe 19	GDRAVCGETCFTGICYTPICVCGKWDLCRMN	*Viola betonicifolia*	Not reported	Hybrid	RNAseq, MS	[9]
vibe 20	GDYYACRESCHKTKCHTPGCICGWPGLCAKN	*Viola betonicifolia*	Not reported	Möbius	RNAseq	[9]
vibe 21	GLNCGESCWGFHCDRHDCTCGLTWPYCSKN	*Viola betonicifolia*	Not reported	Möbius	RNAseq	[9]
vibe 22	GSFSRCSGSCRNTPCPPGCFCTTRFTCI	*Viola betonicifolia*	Not reported	Acyclotide	RNAseq	[9]
vibe 23	GTIFDCGETCALGTCYTPHCSCGKFFLCYGTD	*Viola betonicifolia*	Not reported	Hybrid	RNAseq	[9]
vibe 24	GTIFDCGETCFLGKCYTPGCSCGEYKVCYGTN	*Viola betonicifolia*	Not reported	Hybrid	RNAseq	[9]
vibe 25	GYNCGETCWGFHCDRDDCSCGLTWPYCSKN	*Viola betonicifolia*	Not reported	Möbius	RNAseq	[9]
Vodo L1	GLPICGETCTLGTCYTVGCTCSWPICTRN	*Viola odorata*	Not reported	Möbius	MS/MS	[10]
Vodo L2	ALPVCGETCVGGTCNTPGCSCSWPVCTRN	*Viola odorata*	Not reported	Möbius	MS/MS	[10]
Vodo L3	SKDNCGESCFAGKCYTPGCTCEYPICMNN	*Viola odorata*	Not reported	Möbius	MS/MS	[10]
Vodo L4/Vodo P3/Cter5*	GGEFLKCGESCVQGECYTPGCSCDYPICKNN	*Viola odorata*	Not reported	Möbius	MS/MS	[10, 27]
Vodo L5	GTIPCGESCVFIPCLTSAIGCSCKKVCYKN	*Viola odorata*	Not reported	Bracelet	MS/MS	[10]
Vodo L6	GIPCGESCVWIPCITGTIGCSCKSKVCYTN	*Viola odorata*	Not reported	Bracelet	MS/MS	[10]
Vodo L7	GIPCGESCVFIPCISSIVGCSCKSKVCYKN	*Viola odorata*	Not reported	Bracelet	MS/MS	[10]
Vodo L8	GGEFLKCGESCVQGECYTPGCSCDWPICKKN	*Viola odorata*	Not reported	Möbius	MS/MS	[10]
Vodo L9	GSIPCGESCVFIPCLTSAIGCSCKSKVCYRN	*Viola odorata*	Not reported	Bracelet	MS/MS	[10]
Vodo L11	GSIPCGESCVFIPCISSIVGCSCKSKVCYR	*Viola odorata*	Not reported	Acyclotide	MS/MS	[10]
Vodo L12	GVPCGESCVWVPCTVTALMGCSCVREVCRIS	*Viola odorata*	Not reported	Acyclotide	MS/MS	[10]
Vodo L13/Vodo P4	GAIYCGESCVLIPCISSVIGCRCENKVCVHR	*Viola odorata*	Not reported	Bracelet	MS/MS	[10, 27]
Vodo L14/cT38*	KIPCGESCVWIPCFTSAFGCYCQSKVCYHS	*Viola odorata*	Not reported	Acyclotide	MS/MS	[10]
Vodo L18	GSVIKCGESCLLGKCYTPGCTCSRPICKGK	*Viola odorata*	Not reported	Acyclotide	MS/MS	[10]
Vodo L19	GHPDGAVPCFESCVFVPCISSVVGCRCENNVCVK	*Viola odorata*	Not reported	Acyclotide	MS/MS	[10]
Vodo P1	GYELCGETCALSGGCNTGGCQCAWPVCIKN	*Viola odorata*	Not reported	Möbius	MS/MS	[27]
Vodo P2	GIPCGESCVFIPCLTSAIGCSCKSKVCYKN	*Viola odorata*	Not reported	Bracelet	MS/MS	[27]
Vcom1	AAVPCGESCVWIPCFSSVIGCSCKNKVCYN	*Viola communis*	Insecticidal	Bracelet	RNAseq, MS/MS	[11]
Vcom2	GAPVCGETCFGGTCNTPGCTCDPWPVCSKN	*Viola communis*	Insecticidal	Möbius	RNAseq, MS/MS	[11]
Vcom3	GLPVCGETCVTGSCYTPGCSCSWPVCTRN	*Viola communis*	Insecticidal	Möbius	RNAseq	[11]
Vcom4	GIPCGESCVWIPCFSSAIGCSCKNKVCYRN	*Viola communis*	Insecticidal	Bracelet	RNAseq	[11]
Vcom5	GVPCGESCVIIPCVTGIVGCSCRSNVCYLN	*Viola communis*	Insecticidal	Bracelet	RNAseq	[11]
Vcom6	GVIPCGESCVFIPCITSVVGCSCKSKVCYKN	*Viola communis*	Insecticidal	Bracelet	RNAseq	[11]
Vcom7	GLPCGESCVFIPCLTSAIGCSCKSKVCYKN	*Viola communis*	Insecticidal	Bracelet	RNAseq	[11]
Vcom8	GTLPCGESCVWIPCISSVVGCSCKSKVCYRN	*Viola communis*	Insecticidal	Bracelet	RNAseq	[11]
Vcom9	GLPVCGETCVGGTCNTPGCSCTWPVCSRN	*Viola communis*	Insecticidal	Möbius	RNAseq	[11]
Mra30*	GIPCGESCVFIPCLTSAIGCSCKSKVCYRN	*V. dalatensis* Gadnep	Antimicrobial	Bracelet	MS/MS	[12]
Cycloviolacin O17*	GIPCGESCVWIPCISAAIGCSCKNKVCYRN	*V. dalatensis* Gadnep	Antimicrobial	Bracelet	MS/MS	[12]
Cycloviolacin O2*	GIPCGESCVWIPCISSAIGCSCKSKVCYRN	*V. dalatensis* Gadnep	Antimicrobial	Bracelet	MS/MS	[13]
Cycloviolacin O4*	GIPCGESCVWIPCISSAIGCSCKNKVCYRN	*V. dalatensis* Gadnep	Antimicrobial	Bracelet	MS/MS	[13]
viba 7/cycloviolacin B12*	GVIPCGESCVFIPCISSVIGCSCKSKVCYRN	*V. dalatensis* Gadnep	Antimicrobial	Bracelet	MS/MS	[13]
chassatide C6*	GVIPCGESCVFIPCISSVIGCSCKNKVCYRN	*V. dalatensis* Gadnep	Antimicrobial	Bracelet	MS/MS	[13]

Table 1 Continue ...

Name	Sequence	Plant species	Function/target	Sub-family	Sequencing method	Ref.
vija 1	GTIFDCGETCFLGKCYTPHCLCGKYKFCYGQD	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 2	GNYIPCAESCVYIPCTVTAYVFGCSCKDKVCWN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 3	GRAVCGETCFTGICYTPICVCGKWDLCRMN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 4	GLNCGETCWGFTCDRPDCSCGLTYPFCAKN	*Viola japonica*	Antimicrobial	Möbius	RNAseq, MS	[14]
vija 5	GIPCAESCVFLPCVTVVLGCSCKDKVCYN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 6	GTIFDCGESCFLGKCYTPHCSCGEYFFCYGTD	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 7	GPIDCRETCVWTPCKSVLMNCRCRQGICFRN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 8	GEFCGETCVAFPCFSTAHGCGCYQMGCVKN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 9	GLPVCGETCTLGTCYTAGCSCSWPVCTRN	*Viola japonica*	Antimicrobial	Möbius	RNAseq, MS	[14]
vija 10	GSIPCGESCVFIPCISALLGCSCSSKVCYKN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq, MS	[14]
vija 11	GIHCAETCFWGTCRTAYIGCSCENKICYKN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq, MS	[14]
vija 12	GSYISCGETCVKLKCYTPGCTCTWPACKKN	*Viola japonica*	Antimicrobial	Möbius	RNAseq, MS	[14]
vija 13	GSVPCGESCVWIPCISSLAGCSCSNKVCYLN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 14	GKSCGESCVFIPCITSAIGCYCSSNVCSRN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 15	GLPTCGETCFTGVCYTPGCQCDWPMCTKN	*Viola japonica*	Antimicrobial	Möbius	RNAseq	[14]
vija 16	GSFPCGESCVWIPCLTGPLGCSCKNKVCYYN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 17	GIPCAESCVFIPCTVTALLGCSCSSKVCYN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 18	GMPVCGETCVTGSCYTPGCSCSWPVCTQN	*Viola japonica*	Antimicrobial	Möbius	RNAseq	[14]
vija 19	GWPCVETCIFANWCATSVIGCSCHRGECENN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 20	GIPCAESCVWIPCTVTALLGCSCSSKVCYN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 21	GVPCGESCVFIPCLTGVIGCSCSSNVCYLN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 22	GLPTCGETCFTGVCYTPGCQCDWPICTRN	*Viola japonica*	Antimicrobial	Möbius	RNAseq, MS	[14]
vija 23	GSIFNCGETCILGTCYTPGCSCVYGACSKN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 24	GEPCGETCTENFCATKFFGCFCSNGVCIN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 25	GVVPCGESCVFIPCLTTVIGCSCKSNVCYKN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 26	GLPTCGETCFTGVCYTPGCQCDWPICTKN	*Viola japonica*	Antimicrobial	Möbius	RNAseq	[14]
vija 27	GMPVCGETCVGGTCNTPGCSCSWPVCTRN	*Viola japonica*	Antimicrobial	Möbius	RNAseq	[14]
vija 28	GVPVCGETCVTGSCYTPGCSCSWPVCTQN	*Viola japonica*	Antimicrobial	Möbius	RNAseq, MS	[14]
vija 29	GSTPCGESCVWIPCISSVVGCSCSNKVCYMN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 30	GVPICGESCIQGTCYTPGCTCNWPVCERN	*Viola japonica*	Antimicrobial	Möbius	RNAseq	[14]
vija 31	GSVTGCGETCFKFKCFTPGCKCAKYPLCSKN	*Viola japonica*	Antimicrobial	Möbius	RNAseq, MS	[14]
vija 32	GAPICGETCFQGACYTPGCTCDWPVCKRN	*Viola japonica*	Antimicrobial	Möbius	RNAseq	[14]
vija 33	GTIFDCGETCLLGTCYTPGCSCGDYKLCYGTN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 34	GSIFNCGESCIFGTCYTPECSCVYGACSKN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 35	GIHCAETCLWGTCRTAIIGCSCENRICYKN	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vija 36	GTIFDCGETCAWGKCYTPHCSCGKYFFCYGTD	*Viola japonica*	Antimicrobial	Bracelet	RNAseq	[14]
vaby A*#	GLPVCGETCAGGTCNTPGCSCSWPICTRN	*Viola odorata*	Immunosuppressant	Möbius	MS	[15]
Viba30*#	GPPVCGETCVGGTCNTPGCSCSWPVCTRN	*Viola odorata*	Immunosuppressant	Möbius	MS	[15]
kalata B12*#	GSLCGDTCFVLGCNDSSCSCNYPICVKD	*Viola odorata*	Immunosuppressant	Möbius	MS	[15]
Hypa A*#	GIPCAESCVYIPCTITALLGCSCKNKVCYN	*Viola odorata*	Immunosuppressant	Bracelet	MS	[15]
cT30*#	GDPLKCGESCFAGKCYTPGCTCSRPICKKN	*Viola odorata*	Immunosuppressant	Möbius	MS	[15]
cT31*#	GDPLKCGESCFAGKCYTPGCTCDRPICKKN	*Viola odorata*	Immunosuppressant	Möbius	MS	[15]
HB8*#	GIPCGESCAFLPCLTSLLGCTCQNKVCYRD	*Viola odorata*	Immunosuppressant	Bracelet	MS	[15]
viphi I	GRLCGERCVIEATRAWCRTVGCTCSLQTLECFRN	*Viola philippica*	Metal-binder/nematocidal	Bracelet	DNA sequencing	[16]
vian 1	GLPVCGETCVGGTCNTPGCTCSWPVCSRN	*Viola anagae*	Antifungal	Möbius	RNAseq, MS	[17]
vian 2	GFPXCGETCXNRLCSXGCSCTWPTCTRN	*Viola anagae*	Antifungal	Möbius	RNAseq, MS/MS	[17]
vian 3	GSIFNCGETCIMGTCYTPGCSCVYGACSKN	*Viola anagae*	Antifungal	Bracelet	RNAseq	[17]
vian 4	ALPVCGESCFQGACYTPGCVCSWPVCVQN	*Viola anagae*	Antifungal	Möbius	RNAseq	[17]
vian 5	GIPCGESCVYIPCISAVIGCSCSSKVCYRN	*Viola anagae*	Antifungal	Bracelet	RNAseq, MS	[17]
vian 6	GAFPCGESCVYIGCITSIAGCSCSDNVCYKN	*Viola anagae*	Antifungal	Bracelet	RNAseq, MS	[17]
vian 7	LPLCGGETCTFGTCDTPGCTCGYWPFCTKD	*Viola anagae*	Antifungal	Möbius	RNAseq	[17]
vian 8	GSIGPCGESCFKGKCYTPGCTCDWPICKKN	*Viola anagae*	Antifungal	Möbius	RNAseq	[17]
vian 9	SDTGYCNESCGTNECTTLGCICRKKVCVID	*Viola anagae*	Antifungal	Bracelet	RNAseq	[17]
vian 10	SLPCGESCVYIPCISGLLGCSCKNKVCYYN	*Viola anagae*	Antifungal	Bracelet	RNAseq	[17]
vian 11	GLNCGETCWGFHCDSPGCSCGLTWPYCSKN	*Viola anagae*	Antifungal	Möbius	RNAseq, MS	[17]
vian 12	IFDCGESCIMGKCYTPGCSCGSWKMCYGTN	*Viola anagae*	Antifungal	Bracelet	RNAseq	[17]
vian 13	GRAVCGETCFTGICYTPVCVCGKWDLCRMN	*Viola anagae*	Antifungal	Bracelet	RNAseq, MS	[17]
vian 14	GFTEPCGETCRILACGIPGCQCYARICYWN	*Viola anagae*	Antifungal	Bracelet	RNAseq	[17]
vian 15	GKFCSETCMSFPCFTKAIGCSCESKRCYKN	*Viola anagae*	Antifungal	Bracelet	RNAseq, MS	[17]
vian 16	LDLCFESCIYLGCISSAFGCYCYNLLCVKD	*Viola anagae*	Antifungal	Bracelet	RNAseq, MS	[17]
vian 17	GIPCAETCLWRPCHTAIIGCSCEYNFCHKN	*Viola anagae*	Antifungal	Bracelet	RNAseq	[17]
vian 18	GIHCAETCLWRPCHTAIIGCSCEHNICYKN	*Viola anagae*	Antifungal	Bracelet	RNAseq, MS	[17]
vian 20	RDLCFETCITFGCISASFGCYCYNLLCVKD	*Viola anagae*	Antifungal	Bracelet	RNAseq, MS	[17]
anpy A	GSVPCGESCVWIPCISGILGCSCSNKVCYYN	*Anchietea pyrifolia*	Cytotoxic activity	Bracelet	MS	[18]
anpy B	SIPCGESCVWIPCTVTALAGCSCKNKVCYKN	*Anchietea pyrifolia*	Cytotoxic activity	Bracelet	MS	[18]
anpy C	GLPCGESCVFLPCTVTAIIGCSCKSKVCYKN	*Anchietea pyrifolia*	Cytotoxic activity	Bracelet	MS	[18]
ribe 1	AIPCGESCVYLPCFTKVFKCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 4	AIPCGESCVYIPCLTSVIKCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 5	AIPCGESCVYIPCISKILGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 6	QQYCGESCYVLPCFTQGCHCVSGQCVRDN	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 7	IPCGESCVWIPCLTSVFKCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 8	IPCGESCVYIPCISQVIGCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 9	AIPCGESCVWIPCISKVIGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 10	AIPCGESCVYIPCISQVIGCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 11	IPCGESCVFIPCISQVVGCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 12	AIPCGESCVFIPCISQVVGCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]

Table 1 Continue ...

Name	Sequence	Plant species	Function/target	Sub-family	Sequencing method	Ref.
ribe 13	AIPCGESCVWIPCISAAIGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 14	VIPCGESCVYIPCISTVIGCSCQNKVCYRN	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 15	AIPCGESCVWIPCISSVVGCKCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 16	AIPCGESCVWIPCISAAIGCSCRSKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 17	IPCGESCVYIPCLTAAIGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 18	IPCGESCVYIPCISKVIGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 19	ZLPCGESCVWIPCISSVIGCSCRNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 20	FQCGESCGMITTYCFTSVIGCSCKGRVCVR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 21	AIPCGESCVYIPCISAVIGCKCQNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 22	AIPCGESCVYIPCISSVLGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 23	AIPCGESCVFIPCISSVIGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 24	AIPCGESCVYIPCLTAAIGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 25	ZLPCGESCVWIPCISSVIGCSCRNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 26	AIPCGESCVYIPCISKVIGCSCKNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 27	ETPCGESCVWIPCLTGVIGCSCRNKVCYM	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 28	VPCGESCVFLGCFIPGCSCKNKVCYF	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 29	ZLPCGESCVWIPCISSVIGCSCRNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 30	AIPCGETCVILGCFIPGCKCKNKVCYF	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 31	AIPCGESCVYIPCISVVIGCSCRNKVCYR	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS/MS	[19, 20]
ribe 32	AVPCGESCVFLGCFIPGCSCKNKVCYF	*Rinorea bengalensis*	Cytotoxic and insecticidal	Acyclotide	RNAseq, MS	[19, 20]
ribe 33	GIPCGESCVYIPCTVTALLGCSCQDKVCYKN	*Rinorea bengalensis*	Cytotoxic and insecticidal	Bracelet	RNAseq, MS/MS	[19, 20]
Cter_38	GIPCGEFCVYIPCTITALLGCSCKSKVCYKN	*Clitoria ternatea*	Not reported	Bracelet	WGS, MS/MS	[22]
Cter_39	GIPCGESCVWIPCITGAIGCSCKNRVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_40	GVISCGESCVFIPCISTVIGCSCKNKVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_41	GIPCGEYCVWIPCITGAIGCSCKNRVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_42	GVIPCGESCVFIPCISTIIGCSCKNKVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_43	GIPCGESCVFIPCISGFLGCSCKNKVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_44	GIPCGESCVFIPCISGVFGCSCKNKVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_45	GIPCGESCVIFPCFIPGCSCSNKVCYLN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_46	GIPCGESCVFIPCFTAAIGCSCRSRVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_47	GIPCGESCVFIPCFTAAIGCSCKSRVCYRN	*Clitoria ternatea*	Not reported	Bracelet	WGS, MS/MS	[22]
Cter_48	SPTCGETCFGGTCYTPNCVCDLWPICTKN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_49	GLPICGETCFGGTCNTPNCVCDPWPICTRN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_50	GLPICGETCFGGTCNTPNCVCDPWPICTKN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_51	GIPCGESCVWIPCTVTALLGCSCSNKVCYKN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_52	GDPFACGETCVLQKCYTPGCSClVAICTQN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_53	GLSICGETCFKTKCYTKGCSCSYPICKKN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_54	GSAFCGETCVLGTCYTPDCSCTAIVCIKN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_55	SAFCGETCVLGTCYTPSCSCKAVVCLNN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_56	GNPFACGETCVFQKCYTPGCSCSVAVCTQN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_57	STVPCRESCVFIPCITGIIAPCSCKNKVCYLN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_58	GDPFPCGELCFQGTCYTPGCVCDPWPICTKN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_59	GTVPCGESCVFIPCITGIVAPCSCKNKVCYLN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_60	GSVPCGESCVFIPCITRIAGCSCKNKVCYLN	*Clitoria ternatea*	Not reported	Bracelet	WGS, MS/MS	[22]
Cter_61	SSVPCGESCVFIPCITGIIGCSCKNKVCYLN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_62	GDPLKCGETCFGGTCYTPNCTCDPWPICTNN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_63	GIPCGEPCVFIPCIITALLGCSCKDKVCYKN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_64	GSIRCGERCLLGRCHRPGCTCIRRMCRRN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_65	GVSWICDQTCLMQGKCYRSGCTCDRPICKKN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_66	NSAFCGETCVLGTCYTPDCSCKAVVCIKN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_67	SIPCGESCVYLPCLTTIVGCSCKNNVCYTN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_68	KACGESCLYLPCVTPMFGCYCHGYRGICYHN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_69	GLPICEETCFTGKCYTSSCTCSYLICKKN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_70	GLPICGETCFTGKCYTPGCTCSYPVCEKN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
Cter_71	DLPCSSTCVHIPCIASVCYCKDKLCYKN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_72	KIPCGESCVWIPCLTGYFGCYCQSKVCYHN	*Clitoria ternatea*	Not reported	Bracelet	WGS	[22]
Cter_73	HILICGETCFTGTCYTPDCTCDWPVCMKN	*Clitoria ternatea*	Not reported	Möbius	WGS	[22]
kalata B23	GIPCGESCVYIPCLSTVIGCSCSNKVCYRN	*Oldenlandia affinis*	Not reported	Bracelet	RNAseq, MS	[50]

Footnote: The cited studies also list many previously known cyclotide sequences that are not shown in this table. Z: pyroglutamic acid. X: Ile/Leu. *: a known cyclotide reported during the review period in a plant different to that in which it was originally discovered. #: a known cyclotide reported during the review period to have a previously unreported activity. Bracelet and Möbius: cyclotides classified based on the presence or absence of a *cis*-Pro in loop 5. Hybrid: hybrid cyclotides having loops similar to both Möbius and bracelet groups (notation as assigned in the cited publication). RNAseq: transcriptome sequencing. DNA: sequence encoding cyclotide precursor was amplified from genomic DNA. WGS: whole genome sequencing. MS: sequences were characterized with information only on their molecular weights. MS/MS: sequences identified through mass spectrometry sequencing with information on b- and y- ion fragmentation.

dicated in Table 1, including Vodo L4, a 'new' cyclotide being reported from *V. odorata* [10], but in fact having the same sequence as Vodo P3 reported in a different paper by the same authors [27] and also matching the sequence of Cter 5, reported earlier from the plant *Clitoria ternatea*. A similar redundancy in sequence

is also apparent for Vodo L13 and Vodo L14. Such occurrences highlight the need for authors to carefully check known sequences before claiming a new name for a sequence.

Interestingly, 129 cyclotide-like masses were discovered in Violaceae species on the Canary Islands that spanned diverse habitats ranging from subtropical forests to high-altitude volcanic peaks [17]. The greatest diversity of peptides was recorded in *V. anagae*, a species inhabiting subtropical forests, which yielded novel putative cyclotides vian 1–20 with potent antifungal activity. Amongst their range of findings, the authors suggested that cyclotides could serve as valuable chemosystematic markers.

Although the *Viola* genus was the most studied genus within the Violaceae over the reviewed period, novel cyclotides were also reported from two other Violaceae species, i.e., *Anchietea pyrifolia* [18] and *Rinorea bengalensis* [19, 20]. Notable among these studies was the report of an acyclotide from *R. bengalensis* [20]. Acyclotides are typically very similar in sequence and structure to cyclotides apart from the fact that they lack a cyclic backbone. Although many of the early studies on cyclotides found only cyclic versions of such peptides in the studied species, there have been increasing numbers of reports of acyclotides in recent years, as reviewed recently [5]. In some cases, these acyclic molecules have similar biological activities to the cyclic form, but in others, they are less potent or less stable. In the case of acyclotide ribe 31 from *R. bengalensis*, the structure was determined (Fig. 2) and shown to be similar to that of typical cyclotides despite its lack of a head-to-tail cyclic backbone. This peptide exhibited insecticidal activity against *Drosophila*, demonstrating that a cyclic backbone is not essential for insecticidal activity [20].

An analysis of the sequences of the novel cyclotides and acyclotides in Table 1 shows that there is considerable diversity in the amino acid content and sequences of the backbone loops between successive Cys residues. Fig. 3 shows the range of amino acid residues observed at each of the positions within the cyclotide loops. In this 'diversity wheel' representation, the amino acids seen at a given position in any of the new sequences are shown, with their relative frequency decreasing outward along the radial spokes. This representation highlights that ongoing discoveries in this field continue to demonstrate that cyclotides can be considered as a natural combinatorial template, i.e. a diverse range of sequences is displayed on an ultrastable cystine knot core.

Many of the recent studies reporting the discovery of new cyclotides focused on screening plants based on their phylogenetic proximity to known cyclotide-bearing species. Peptide extracts or isolated and purified peptides were then typically screened for a target

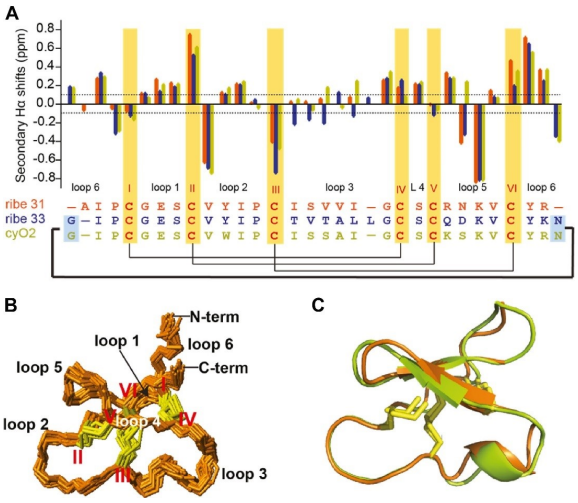

Fig. 2 Secondary Hα NMR chemical shift comparison between cyO2 and ribe 31 and 33 and the three-dimensional NMR structure of ribe 31. (A), The comparison of secondary Hα chemical shifts of ribe 31 (orange), ribe 33 (blue) and cyO2 (lime green). The chemical shifts of cyO2 were obtained from the Biological Magnetic Resonance Data Bank (BMRB, ID: 16073). All cysteines are highlighted in red text with yellow boxes. The cyclic backbones of ribe 33 and cyO2 are indicated with a thick black line, and disulfide bond connectivities ($Cys^{I–IV}$, $Cys^{II–V}$ and $Cys^{III–VI}$) are shown as thin black lines. (B), Superposition of 20 conformers representing the 3D NMR structure of acyclotide ribe 31. (C), The superimposition of 3D NMR structure of cyclotide cyO2 (lime green) and acyclotide ribe 31 (orange). Figure and caption reproduced with permission from The Journal of Biological Chemistry [20].

activity. These included immunosuppressive, gastrointestinal, antifungal, nematocidal, metal-binding, insecticidal, antimicrobial, and cytotoxic activities, as are highlighted in Table 1 for the tested novel cyclotides. In these cases, the cyclotides were typically identified by MS/MS sequencing (as noted in Table 1).

Another approach to cyclotide discovery is to use bioinformatics methods to discover cyclotide-like sequences from nucleic acid sequencing data, including transcriptomic and genomic data. Both the peptide-based and nucleic acid-based approaches have been successful, but the latter promises to accelerate cyclotide discovery as more plant transcriptomes and genomes become available. Such an approach was recently used to identify additional putative cyclotide sequences from plants in the Solanaceae [21] and Fabaceae [22] families.

An example of the power of the nucleic acid-based approach is illustrated by our recent determination of the genome of the cyclotide-bearing plant *Clitoria ternatea* [22] colloquially known as butterfly pea. That genome was mined for the presence of known and new cyclotides and to explore the mechanism behind the

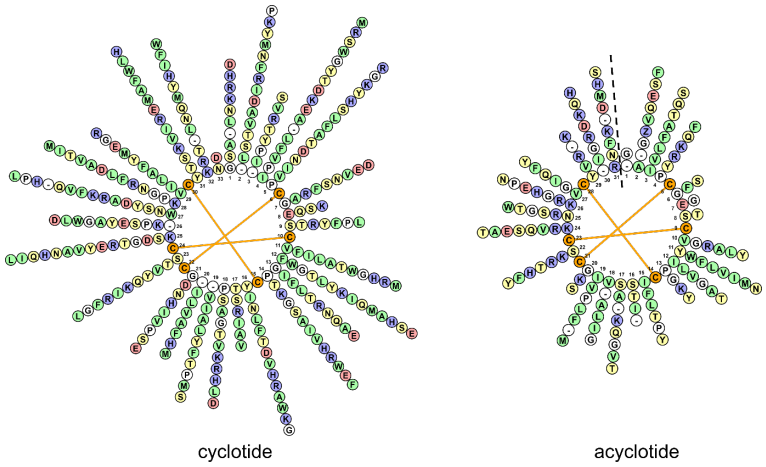

Fig. 3 Sequence diversity wheels of novel cyclotides and acyclotides reported in the period 2021 to 2024. The image was created by aligning peptide sequence using MUSCLE alignment in Genious Prime® 2024.0.5 (www.geneious.com), and Sequence Diversity Wheel tool (https://cybase.org.au/index.php?page=circles) to generate the diversity wheels. The alignment of acyclotides was manually curated to remove alignment errors. Positions with a percentage of gaps above 90% were removed. In the acyclotide diversity wheel, the dashed line indicates the absence of a bond forming between the two amino acids.

evolution of cyclotides in this leguminous plant from the Fabaceae family. By comparing the genome of *C. ternatea* to grain legumes, which are not natural cyclotide-producer plants, we identified a significant expansion of the albumin-1 gene family in *C. ternatea*, which appears to have allowed for the diversification of loci encoding cyclotides. Iterative rounds of gene duplication and diversification led to the creation of genomic islands enriched with cyclotides in this plant. Additionally, we identified an ancestral asparaginyl endopeptidase (AEP) that underwent neofunctionalization and multiple duplications to generate ligase-type AEPs crucial for cyclotide maturation and in particular to the cyclization of their backbone [22]. We return later in this article to the biotechnological applications of such ligase-type enzymes.

Another recent report [23] also noted the value of genome mining to discover cyclotides and to attribute them to subfamilies at a genome-scale. In that study cyclotide subfamily specific (CSS) markers were used as an effective approach to screen for cyclotide subfamilies in a range of plants without prior sequencing knowledge. Surprisingly, the study reported bracelet family cyclotides to be the least abundant, despite the fact that at the peptide level, the distribution of cyclotides between the bracelet and Möbius subfamilies remains roughly at 2:1 amongst the newly discovered cyclotides (e.g., 108 bracelet vs. 54 Möbius sequences in Table 1).

To conclude this section on cyclotide discovery, we note that the presence of cyclotides, in itself, has now been used as a taxonomic marker for the African plant *Oldenlandia affinis* (Rubiaceae), the first plant species in which kalata B1 was reported [24]. This plant is notable for its widespread use in traditional medicines in Africa, including as a uterotonic agent. It seems fitting that the presence of cyclotides can now be used as an aid to plant classification and highlights the importance of this unique class of molecules to plant biology and taxonomy.

TECHNOLOGIES FOR CYCLOTIDE ANALYSIS

A number of technologies that have been developed or improved over the last decade have contributed to the increasing pace of cyclotide discovery, not just from the genomics approaches mentioned above but also from developments in mass spectrometry, mass spectrometry imaging (MSI), microfluidics, and a range of protocols for assessing function, stability and structure. We now discuss selected examples of these technologies, grouping them under the broad headings of biophysical, computational, and synthetic and biosynthetic technologies.

Biophysical technologies

Mass spectrometry (MS) has been featured strongly amongst the technologies for cyclotide characterization, and tandem MS has been a key, but now routine, methodology used for cyclotide sequencing. In the last few years, there has been a trend towards more detailed MS analysis to definitively elucidate cyclotide sequences. For example, Kalmankar et al [25] reported on the use of MS to discover cyclotides from *Clitoria ternatea* (Fabaceae), noting the value of using Xaa-Pro bond fragmentation patterns to position Pro residues in the sequence. More recently, Yu et al [26] used MS to map the presence of cyclotides in *V. philippica*. They identified 65 known and 18 potential novel cyclotides,

which constitutes the largest dataset of cyclotides in *V. philippica*. In a number of cases the exact sequence was not determined; for example, it was not possible to definitively confirm Leu vs Ile from the MS data, as they are isobaric. In fact, this is a common limitation of MS/MS sequencing but is one that is readily solved if nucleic acid sequencing data are available. For example, a peptide with the name Viphi I was reported in papers independently in 2024 by Yu et al [26] and Zhang et al [16], with the later using the lower case spelling, viphi I. Yu et al reported Viphi I only as a putative cyclotide, different from the sequence reported by Zhang et al [16], which was supported by bioinformatics analysis of cyclotide precursor sequences. Therefore, here we opted to include only Zhang et al's sequence in Table 1 [16].

Another example of the value of a combined LC-MS/MS and nucleic acid precursor driven approach was similarly used to map cyclotides in *V. odorata*, whereby the authors identified four putative novel cyclotides, and three acyclotides [27]. They also detected 47 precursor sequences encoding for 15 putative cyclotides. *V. odorata*, also known as the 'sweet violet' is now a very extensively studied cyclotide-producing plant and an excellent model system for cyclotide research given the vast amount of peptide and nucleic acid data available for it.

Direct mass spectrometry imaging (MALDI-MSI) of plant tissue is a powerful new approach recently applied to cyclotide discovery and characterization by Niyomploy and Sangvanich [28]. They noted that they could directly characterize a plant sample with 10 to 20 times less material than needed for traditional analysis, as no extraction and purification processes are necessary. Gilding et al [22] demonstrated how the expression of different cyclotides can be monitored in different tissues and at different developmental stages by using MALDI-MSI to visualize the distribution of cyclotides in a wide range of tissues in *C. ternatea* plants. Although this technology is not widely available, but it will be interesting to follow its development.

Reflecting the current interest in the emerging approach of microfluidics in peptide characterization, two 2023 papers on the use of microfluidics in cyclotide discovery have been well cited. Ebrahimi et al [29] reported that the use of microfluidic-based separation for the purification, isolation and separation of cyclotides was a quick, economical, and straightforward approach compared to standard HPLC approaches. Didarian et al [30] also reported the efficiency of using microfluidics for separating Vigno 1–5 cyclotides from extracts of *Viola ignobilis*. It is likely that we will see increasing use of this technology in cyclotide discovery and bioassay in the near future.

Stability is one of the hallmarks of cyclotides. In an article aimed at standardizing measurements of stability, we reported a detailed protocol for assessing cyclotide stability [31]. In another study related to stability we used scanning mutagenesis on kalata B1 to determine residues important for the stability and insecticidal activity of this molecule [32]. The work showed that a single mutation to avoid deamidation could enhance stability, which has implications for the use of cyclotide-bearing products in crop protection applications. That study provides an example of the value of systematic mutagenesis of cyclotide frameworks. An earlier study on the bracelet cyclotide hyen D [33] had shown that certain residues are crucial for folding. Bracelet cyclotides are notoriously difficult to fold, and this work facilitates biotechnology applications for this subgroup of cyclotides.

Cyclotides are known to interact with membranes, and their membrane-binding activity is critical for their insecticidal activity. A wide range of biophysics approaches have been used in such membrane binding studies. In a new approach not previously used for cyclotides Gupta et al [34] employed surface-sensitive thermodynamic methods to understand the interaction of kalata B1 with various phospholipids, including 1,2-dipalmitoyl-sn-glycero-3- phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DPPG), and 1,2-dis-tearoyl-sn-glycero-3-ethylphosphocholine chloride salt (DSEPC), focusing on their electrostatic properties. They reported that kalata B1 has a thermodynamically more favorable interaction with the anionic lipid, DPPG. Although it has been known that the presence of phosphatidylethanolamine (PE) phospholipid is essential for the activity of cyclotides towards membranes, their study did not include PE lipids. It would be interesting to see how the inclusion of PE lipids in this approach would influence the findings.

Computational technologies

Computational approaches have been used to shed light on multiple aspects of cyclotides, including their oxidative folding to form the cystine knot motif. In two recent papers, Venkatesan and Roy [35, 36] employed molecular dynamics (MD) simulations to examine the role of the cystine knot in cyclotide folding. They explored its role in the structural reorganization of cyclotides and developed some insights into why the disulfide bonds appear in a particular combination.

In another computational study that may help to underpin future drug design applications of cyclotides as stable peptide scaffolds, Ilieva et al [37] studied the effects of grafting an octapeptide sequence into each of the loops of MCoTI-II on its structural stability. They found that the dynamics of the grafted structures varied depending on the grafting location and that grafting into loop 3 led to the most stable and, hence, the most suitable structure for drug design applications.

One of the very useful features of computational

technologies is that they can generate cyclotide sequences not found in nature. We recently used ancestral sequence reconstruction to generate novel ancestral sequences for Möbius and bracelet cyclotides [38]. That work provided new insights into the diversity of available cyclotide sequences and thus could also underpin future drug design and grafting studies. Specifically, the study showed that the reconstructed sequences were highly temperature stable, bound to PE membranes at micromolar concentrations, and inhibited the growth of insect cells. One of the 'ancestral' cyclotides had a higher propensity for correct folding than comparable native cyclotides, consistent with the general principle that ancestral proteins evolved in a higher temperature environment and thus may be more stable than present-day proteins.

In silico analysis has also been used to provide insights into antibacterial [39] and antifungal [40] activities of cyclotides. In both studies, the authors found that potent antibacterial and antifungal cyclotides contain more hydrophobic amino acids than non-potent cyclotides. Their *in silico* analysis showed that potent cyclotides possess low transfer-free energy and deeper membrane penetration, suggesting enhanced binding stability and membrane disruption capability.

Computational studies over recent years using MD simulations have been pivotal in defining how cyclotides interact with membranes [41, 42]. Such interactions are central to their role in host defense. In an extensive study using all-atom MD simulation, Roseli et al [41] showed that the kalata B1 can specifically and selectively bind to PE membranes in the presence of only 10% POPE lipid in the membrane model. That work highlighted the key role of particular amino acid residues in allowing kalata B1 to recognize and bind to PE-phospholipids selectively. Lei et al [42] conducted an MD simulation of the bracelet cyclotide, cycloviolacin O2, with three different membrane models composed of 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), 1-palmitoyl-2-oleo-yl-sn-glycero-3-phosphoglycerol (POPG)-doped POPE (POPE/POPG = 3:1), and 1-palmitoyl-2-oleoylphosphatidyl-choline (POPC) lipid bilayers. They found that cycloviolacin O2 forms a stable binding complex with membranes composed of POPE lipids. Both studies suggest that the binding of cyclotides to pure POPC lipids is not favorable due to bulky methyl groups of the POPC lipids, which leads to steric hindrance when interacting with the cyclotides.

In another computational approach, Zielesny and coworkers employed dissipative particle dynamics on the microsecond timescale to quantify kinetic rate constants for cyclotide-induced bilayer membrane disruption by lipid extraction [43, 44]. In that study, the authors introduced a 'sandwich' interaction model whereby cyclotides are placed in a water layer between two bilayer membranes, and they found that their model successfully predicts experimental trends on the bioactivities of cyclotides towards membranes.

Synthetic and biosynthetic technologies

Solid phase peptide chemistry, including automated Fmoc-based SPPS approaches, continues to be a mainstay of cyclotide studies and we used it in several mutagenesis studies over recent years to understand structure-activity relationships and factors affecting the folding and stability of cyclotides. For example, based on our finding that bracelet cyclotides ribe 33 and Cter27, which differ only in a single residue at position 24, are unusually efficient at folding for bracelet cyclotides [19], we examined the impacts of single amino acid substitutions at position K24 on the structure of Cter 27 [45]. This position is at the beginning of loop 5 of the cyclotide framework, a β-turn loop that has been implicated as important in templating cyclotide folding. The Cter 27 variants were successfully folded without structural changes, albeit with slightly lower yields than for the parent peptide. That work helped to confirm loop 5 as important in cyclotide folding. To further understand cyclotide folding and improve folding efficiency, the effect of β-turn nucleation on oxidative folding was investigated by Tian et al [46]. Insertion of $_{D}$-Pro-Gly into loop 5 of Möbius, bracelet, or trypsin inhibitor cyclotides had a beneficial effect on cyclotide folding.

In what we think will be a significant advance in the drug design potential of cyclotides, we recently developed a modular "plug and play" synthesis approach to address impaired oxidative folding often occurring in grafted cyclotides [47]. This convenient and robust approach allows bioactive epitopes to be easily inserted into a folded cyclotide scaffold.

Although many cyclotides are amenable to chemical synthesis, there are significant concerns about production costs and the environmental damage caused by the extensive use of chemicals in large-scale production of peptides in general. In this regard, *Pichia pastoris* and *Escherichia coli* are appealing recombinant expression systems for producing cyclotides and their variants due to their rapid growth, high yields, and well-established methods for therapeutic products. A protocol for yeast-based bioproduction of the cyclotide MCoTI-II was published in Nature Protocols in 2021 [48]. The method includes four main steps: (1) Designing vectors for expression of MCoTI-II precursor and the ligase-type AEP enzyme [C247A]OaAEP1b separately, (2) recombinant production of a linear form of MCoTI-II in *P. pastoris*, (3) recombinant production of [C247A]OaAEP1b in *E. coli*, and (4) *in vitro* cyclization of *P. pastoris*-derived acyclic MCoTI-II into the mature cyclic form using the recombinant [C247A]OaAEP1b. It was estimated that the cost of recombinant production of MCoTI-II using the yeast system is approximately 16 times lower than that of

SPPS. Additionally, the use of hazardous and toxic solvents is reduced by about 10 times. In an alternative approach to sustainable production, Yayci et al [49] established *E. coli* based-recombinant production of kalata B1 using conditional split inteins. This method also requires *in vitro* cyclization by mixing two fragments of a split intein.

Cyclotides are cyclic peptides derived from plants; therefore, utilizing plant cell suspension and tissue cultures of cyclotide-producing plants offers an alternative method for producing endogenous cyclotides. During the period 2021–2024, two publications focusing on cell suspension culture for the production of cyclotides in *O. affinis*, *C. ternatea*, *H. enneaspermus* [50] and *V. uliginosa* [51] were reported. Doffek et al [50] reported that cell suspension cultures of *O. affinis* and *H. enneaspermus* showed a less diverse cyclotide profile than the plant organs, which offers reduced complexity in subsequent purifying cyclotides from cultures. Importantly, the yield of two bracelet cyclotides, cyO2 and cyO13, was significantly increased in the *H. enneaspermus* cell suspension culture. Additionally, 3 mM sodium chloride increased the accumulation of cyO2 and kalata B23 (a new cyclotide) about 3-fold compared to the untreated culture. In *V. uliginosa* cell suspension culture, jasmonic acid significantly improved the accumulation of the three bracelet cyclotides cyO3, cyO13, and viul M. Another study by Slazak et al [51] examined the role of plant stress hormones in optimizing cyclotide production. Together, the publications highlight the advantages of plant cell suspension culture for the production of natural cyclotides. This approach is particularly beneficial for producing bracelet cyclotides, which are challenging to synthesize chemically, as noted earlier [45].

Since cyclotides are gene-encoded plant peptides, recombinant production using plant-based systems has been developed for a wide range of native and engineered cyclotides. Plant-based recombinant expression systems provide post-translation modification, serum-free production, freedom from zoonotic contaminants, and large-scale production with low-cost inputs of water, light, and fertilizer. In non-cyclotide host plant-based recombinant systems, the production of cyclotides or engineered cyclic peptides requires co-expression of a ligase-type AEP and a suitable peptide precursor gene [90, 91].

Nicotiana benthamiana continues to be a prominent choice for use as a plant factory, facilitating the transient expression of cyclotides and cyclic peptides [53, 54]. We recently demonstrated that the yield of [T20K]kalata B1 (T20K), which is a single amino acid variant of kalata B1 and is in clinical trials as a peptide-based drug candidate for the treatment of multiple sclerosis, reached about 0.3 mg/g dry mass in whole infiltrated *N. benthamiana* plant and close to 1.0 mg/g dry mass in individual infiltrated spots [54]. Achieving this yield required optimizing the peptide precursor arrangement, transgene regulatory regions, and selection of ligase-type AEPs. We earlier reported [53] that biosynthesis pathways of cyclotides can be exploited to produce other linear or cyclic bioactive peptides. Notably, genome-edited *N. benthamiana* with knocking out several endogenous AEPs, allows the accumulation of potential therapeutical peptides for treating prostate cancer, neuropathic pain, and Netherton syndrome, as well as the production of a potent insecticidal peptide derived from garden pea (*Pisum sativum*). Plant-stable transformation is another approach for producing recombinant cyclotides. Transgenic rice expressing kalata B1 showed potential in protecting rice from golden apple snails (*Pomacea canaliculata*) and reducing the use of chemically synthesized molluscicides [55].

In conclusion, we note that the range of technology developments described above, ranging from biophysical methods to computational methods to (bio)synthetic methods has underpinned a range of applications for cyclotides that would not have been possible without these technology developments. We now describe selected examples of this applications.

APPLICATIONS

Cyclotides lend themselves to a wide range of agricultural, pharmaceutical and biotechnology applications. Table 2 provides details of patents published during the 2021 to 2024 survey period, most directed at pharmaceutical applications, including for multiple sclerosis, cancer cardiovascular, and antimicrobial applications, but there was one for insecticidal applications in agriculture. With the current interest in peptide-based solutions in both the pharmaceutical and agricultural industries, it is likely that there will be a significant number of as yet unpublished patents. Table 2 is presented here to provide an indication of published patent activity in the 2021–2024 period.

Agriculture applications

Cyclotides have shown a wide range of applications in agriculture. Grover et al [4] provided an extensive review of their potential uses, highlighting those based on their insecticidal, antibacterial, antifungal, molluscicidal, and nematocidal properties. Butterfly pea (*Clitoria ternatea*) is the plant source for a marketed insecticide called Sero-X (https://innovate-ag.com.au/sero-x/), which is made up of a natural suite of more than 70 cyclotides. As noted earlier, the recently reported genome of this plant [22] has been useful in confirming the presence of both known and previously undetected cyclotides (Table 1).

Eteme et al [56] recently reviewed the anti-helminthic activities of cyclotides and other cyclic peptides. Several original research studies published between 2021 and 2024 reported on the applications of

Table 2 Recent published patents on applications of cyclotides.

Patent no.	Application title	Inventors	Filing date	Patent jurisdiction
US20240269230	Cyclotides in combination with kappa opioid receptor ligands for MS therapy	Christian Gruber, Edin Muratspahic	19.03.2021	US
EP4121085	Cyclotides in combination with kappa opioid receptor ligands for MS therapy	Christian Gruber, Edin Muratspahic	09.06.2021	EP
EP4416282A1	Proteinaceous molecules and uses thereof	David Craik, Simon de Veer, Yen-Hua Huang, Joakim Swedberg, Hiroaki Suga, Wenyu Liu, Toby Passioura	14.10.2021	AU, US, EP, WO
US20240049725A1	Compositions comprising cyclotides and other insecticidal peptides and uses thereof	Aurelien Bigot, Fides Benfatti, David Craik, Yen-hua Huang, Quentin Kaas, Mark Schiebler, Conan Wang	21.12.2021	WO, US, MX, EP, CL
WO2023215032A2	Potent anti-cancer cyclotides	Julio A. Camarero Palao, Dipankar Chaudhuri	03.03.2023	WO
WO2023225488A1	Blood-brain barrier translocating peptides and related molecules and methods of use thereof	Duncan McGregor, William Eldridge, Vaughn Smider, Charles Melancon	15.05.2023	WO
US20240092846A1	Engineered cyclotides with potent broad antimicrobial activity	Julio A. Camarero Palao, Paul Beringer, Mansour Dughbaj, Rajasekaran Ganesan	20.06.2023	US

Source: World Intellectual Property Organization & Google patent search; 'cyclotide' used as a keyword; US: United States; EP: Europe; WO: Worldwide; MX: Mexico; CL: Chile.

cyclotides in agriculture. For instance, Slazak et al [57] demonstrated the potential role of cyclotides in *V. uliginosa* and *V. odorata* in combating the two-spotted spider mite (*Tetranychus urticae*). Bajpai et al [58] reported that cyclotides had nematocidal activity when tested in crude extracts, cyclotide-enriched fractions, or individually as purified peptides, against *Caenorhabditis elegans*. The mechanism of action involves either damage to the pharynx and intestines of young larvae after ingestion or the formation of membrane blebs upon exposure. Other examples of the bioactivities of cyclotides against pests and pathogens relevant to agriculture are indicated in Table 1.

Three papers in the review period highlighted other potential benefits of cyclotides in the environment that are relevant to agriculture. As noted earlier, Saad et al [55] demonstrated that transgenic rice expressing kalata B1 cyclotide could be an environmentally friendly approach to protect rice against golden apple snail (*Pomacea canaliculata*). Sychta et al [59] investigated the involvement of cyclotides in the heavy metal tolerance of several *Viola* species. That work highlighted a potential application of cyclotides in protecting plants against heavy metals and those plants having a role in remediating contaminated soils. In a similar vein, Zhang et al [16] focussed on *V. philippica*, a plant known to be a metallophyte, and hypothesized a Cd-binding activity for the new cyclotide viphi I, which would be consistent with a role for cyclotide-bearing plants in soil remediation from mine sites for example.

With an increasing focus on sustainable solutions for pest control in agriculture and a growing awareness of the need to protect the environment, it is anticipated that more applications of cyclotides in agriculture will be explored in the future. The diversity of new cyclotide sequences noted above and reported in just the last four years augers well for the discovery of new cyclotides with commercial potential in agriculture.

Pharmaceutical applications

Cyclotides have demonstrated significant potential across a range of pharmaceutical applications, showcasing their versatility as therapeutic agents. One active area in recent years has been on the targeting of G-protein coupled receptors (GPCRs). For example, Gruber and colleagues [60] noted how plant extracts rich in cyclotides, cyclotide-like peptides, or knottin peptides derived from species such as *Carapichea ipecacuanha*, *Psychotria poeppigiana*, *Momordica charantia*, *Beta vulgaris* and *Sambucus nigra*, as well as synthetically designed cyclotides such as T20K, are ligands for the κ-opioid receptor (KOR). This receptor is a critical target for developing analgesics and multiple sclerosis therapies, highlighting the potential of these plant-derived peptides as novel KOR modulators [60].

Another type of GPCR explored using cyclotides is the cannabinoid type 2 receptor (CB_2R). A recent study investigated the plant-derived cyclotide vodo-C1 from *Viola odorata* as a potent CB_2R agonist, demonstrating its potential as a cannabinoid receptor modulator [61]. By designing vodo-C1-based bicyclic peptides (vBCL1 to vBCL4), the authors explored ways to improve potency and reduce size. They found that the peptides

functioned as negative allosteric modulators or neutral antagonists. That work highlighted cyclotides as a promising template for developing synthetic peptide-based drugs targeting CB_2R, with applications for chronic inflammation and fibrosis treatments.

Cyclotides have featured prominently in anti-cancer applications in recent years. For example, CyO2, CyO13, kalata B1, and varv peptide A derived from *V. odorata*, were reported to have dose-dependent cytotoxicity against glioblastoma and neuroblastoma cells [62]. Notably, co-treatment with temozolomide significantly enhanced cell death, positioning these peptides as promising adjuvants in glioblastoma chemotherapy [62]. Expanding on the anticancer potential of cyclotides another study examined their role in natural killer (NK) cell-mediated cytotoxicity of tumor cells [63]. The authors found that cyclotide-containing extracts from *Carapichea ipecacuanha* enhance NK cell killing of tumor cells. They further found that one isolated cyclotide, caripe 8, boosts NK cell degranulation and directly targets tumor cells, suggesting its dual therapeutic potential in cancer treatment. These findings highlight the promising role of cyclotides in enhancing immunotherapy approaches in cancer treatment [63]. They also highlight the importance of cyclotides as synergizing agents when acting in concert with other bioactive molecules.

Other cyclotide-containing plant extracts have also featured in studies of immunomodulation therapies. For example, Retzl et al [64] showed that plant extracts and purified kalata S from *Viola tricolor* were able to modulate immune cell responses at the cytokine level. The authors suggested that the work provided further understanding of the role of cyclotide-containing extracts from this plant for future applications in immune disorders, such as inflammatory bowel disease [64].

Cyclotides have also been explored for applications against infectious diseases during the review period. For example, cyclotides from *V. tricolor* have been reported to have activity against HIV-1, reinforcing their potential as natural scaffolds for antiviral therapeutics [65]. In an example relevant to parasite-mediated infectious disease, cyclotide extracts from the Himalayan violet *Viola canescens* were reported to have anti-plasmodial activity and thus relevance to malaria treatment [66]. In another example of cyclotide extract screening, Tran et al. investigated cyclotide-rich fractions from *Clitoria ternatea* mature pods and demonstrated them to have antimicrobial activity [67]. As noted earlier, this plant is particularly rich in cyclotides and has been extensively studied for both pharmaceutical and agricultural applications.

In a quite different application of this cyclotide-bearing plant, Kalmankar et al investigated a wide range of tissues of *C. ternatea*, including pods, stems, leaves, flowers, and roots, and demonstrated neuroprotective effects against β-amyloid-induced neurotoxicity in Alzheimer's disease models, supporting their potential development as pharmacophore scaffolds for neurodegenerative therapies [68]. A subsequent study by the same group explored the mode of action between cyclotides and β-amyloid fibrils using molecular dynamic simulations [69]. These applications to β-amyloid-induced neurotoxicity have yet to be independently explored by other groups.

Beyond making use of the intrinsic pharmacological activities of natural cyclotides, the MCoTI-I and MCoTI-II cyclotide frameworks from the trypsin inhibitor subfamily of cyclotides have emerged as robust platforms for grafting bioactive sequences. Loop 6 of MCoTI-I and MCoTI-II has been frequently selected for incorporating desired bioactive sequences due to its high flexibility and tolerance to substitution. For example, Camarero and coworkers recently reported the successful insertion of bioactive peptides such as porcine protegrin (PG-1) into MCoTI-I, and demonstrated antimicrobial activity against multiple ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) and efficacy in *in vivo* models of acute *P. aeruginosa* peritonitis [70]. Similarly, the grafting of a potent 9-mer antimicrobial peptide, optP7, into MCoTI-II showed enhanced proteolytic stability, highlighting its potential for future antimicrobial drug development [71].

In our group we have focussed largely on MCoTI-II as a preferred scaffold for drug design applications. For instance, a short linear Nrf2 motif was grafted into MCoTI-II, creating MCNr-2c, which targets Keap1 and shows potential as a treatment for oxidative stress-related diseases [72]. We also demonstrated that the MCoTI-II framework could enhance the stability and activity of the anti-angiogenic P15 peptide, resulting in MCoP15 as a cyclic and stable therapeutic candidate for cancer therapy [73].

In collaboration with the Suga group [74], we leveraged mRNA display to develop cMCoFx1, a potent and selective inhibitor of coagulation factor XIIa (FXIIa) based on the MCoTI-II scaffold. The binding mode of this inhibitor, three times more effective and selective against FXIIa than other serine proteases, was delineated in a cocrystal structure with FXIIa, showcasing its potential for coagulation therapy [74]. Further expanding on this therapeutic application, and using a complementary rational design approach rather than display-based screening, we recently showed that modifications within loop 6 of MCoTI-II, such as generating the compound [Q3R, L8G]M5, significantly improved FXIIa-driven coagulation inhibition in human plasma [75].

Other groups have also recognized the value of MCoTI-II as a scaffold for the design of selective protease inhibitors. For example, Mishra and colleagues

altered MCoTI-II's specificity from trypsin to cysteine protease inhibition by grafting IHL residues into loop 1, demonstrating its adaptability for diverse protease targeting [76].

Highlighting cyclotides under clinical development, T20K, a single mutant of kalata B1, is being explored for multiple sclerosis and, at the time of writing, was in phase 1 clinical trials. It has been subject to many SAR studies and one recent study revealed that the acyclic analog of T20K is inactive in modulating the immune system, underscoring the necessity of the cystine knot and cyclic backbone motifs for bioactivity in this case [77]. Interestingly, T20K has shown promising results in treating anaplastic large cell lymphoma (ALCL), as evidenced by reduced tumor weight and enhanced apoptosis in mouse models [78].

Chemical modifications to cyclotides can expand their therapeutic applications by addressing pharmacokinetic limitations. For example, lipidation of the CXCR4 antagonist cyclotide MCo-CVX-5c significantly enhances its half-life by reducing renal clearance [79]. Interestingly, the palmitoylated version retained potent CXCR4 antagonistic activity, whereas other lipid modifications compromised biological efficacy. This finding highlights the importance of strategic lipidation in optimizing pharmacokinetics while maintaining therapeutic activity [79].

Innovative delivery strategies have been used to improve the intracellular targeting capabilities of cyclotides. For example, a cyclotide-based peptide targeting p53:MDM2/X interactions was optimized for intracellular delivery by conjugation to the cyclic cell-penetrating peptide cR10, achieving $>$ 35-fold improved inhibitory activity, enhanced cellular uptake, and effective induction of apoptosis in cancer cells without membrane disruption [80]. This demonstrates the potential of cyclotide scaffolds for delivering large polar peptides to modulate intracellular protein-protein interactions in cancer therapy.

The blood-brain barrier presents significant challenges for delivering peptide-based drugs to the central nervous system. Melander and co-workers [81] explored the use of the cyclotide kalata B1 and sunflower trypsin inhibitor-1 (SFTI-1) as scaffolds for drug delivery, identifying SFTI-1 as a promising candidate for delivering drugs to extracellular targets in the central nervous system. That work underscored the potential of cyclic peptide-based scaffolds in addressing critical drug delivery challenges.

Biotechnology applications

Cyclotides and their associated biosynthetic enzymes have been used in a range of biomedical and biotechnological applications in the last few years. We first discuss recent studies in which the cyclotides themselves have been used in biomedical applications. Here, we note that in addition to their direct therapeutic applications described above, cyclotides are being explored for their potential in drug delivery systems, such as antimicrobial nanofibers. One recent study [82] developed antimicrobial nanofibers by electrospinning polyvinyl alcohol with cyclotide-rich fractions. After extraction and separation by C18 flash chromatography and RP-HPLC, the molecular weights of the cyclotides were determined by quadrupole time-of-flight liquid chromatography-mass spectrometry. The resulting nanofibers, containing 100% cyclotide-rich fractions, displayed regular fiber textures and were confirmed to contain peptides by RP-HPLC. One of the cyclotide-rich fractions demonstrated antimicrobial activity against *Bacillus cereus*, highlighting the potential of cyclotide-containing nanofibers for pharmaceutical applications.

In two other studies, the potential of cyclotide-based materials for biological applications was explored in terms of stability and biocompatibility. Dayani et al [83] used optical microscopy, polarized optical microscopy, scanning electron microscopy, transmission electron microscopy, and fluorescence microscopy methods to study the self-association of cyclotides extracted from *V. odorata* into nanostructures. It was felt that such structures might find application in drug delivery and tissue engineering. Indeed, in another study the authors demonstrated that the cyclotide nanotubes were stable, biocompatible, and capable of self-assembly, with successful encapsulation of coumarin [84]. The stability of the nanotubes was confirmed by differential scanning calorimetric and field emission scanning electron microscopy. *In vivo* and cytotoxicity assays showed that the nanotubes were non-toxic, suggesting their potential as novel carriers for biological agents.

One of the most active and exciting areas of cyclotide research that has broadened the field has been in the use of their biosynthetic enzymes as biotechnology tools for splicing, cyclizing, and tagging proteins. Underpinning these studies has been the development of efficient methods for discovering, characterizing, and efficiently producing these enzymes. The discovery of AEPs has been reviewed extensively already, so here we focus on recent approaches to identify other processing auxiliaries involved in cyclotide biosynthesis. Specifically, we recently investigated the biosynthesis of the cyclotide kalata B1 in both cyclotide-producing *Petunia* $\times$ *hybrida* and non-cyclotide-producing *Nicotiana benthamiana* using proximity labelling [85]. Several proteins involved in kalata B1 maturation were identified, including endoplasmic reticulum (ER) resident chaperones, protein disulfide isomerases, a papain-like cysteine protease, and an asparaginyl endopeptidase. Overexpressing the papain-like cysteine protease improved the yield of cyclic kalata B1, while overexpressing protein disulfide isomerases did not, despite their confirmed interaction.

These findings enhance our understanding of the auxiliary factors involved in cyclotide production in plants.

With regard to the production of key cyclotide processing enzymes, a protocol for the expression of a modified *O. affinis* AEP (OaAEP1b) in *E. coli* and its use in cyclizing a range of peptides was reported earlier [48]. For scale-up purposes, yeast-based expression has some advantages, and a recent study used *Pichia pastoris* for this purpose [86]. Expression was optimized by integrating multiple copies of the OaAEP1b gene, achieving high protein yields. The recombinant enzyme exhibited strong cyclizing activity, confirmed through a range of assays, including the cleavage of substrate peptides and the cyclization of small ubiquitin-like modifier (SUMO) proteins. The study confirmed the potential of recombinant OaAEP1b as a powerful tool for peptide engineering and cyclotide production, offering a scalable method for biotechnological applications in drug design [86].

Significant progress has been made in understanding the basis for hydrolysis vs ligase activity in AEPs. For example, a recent study reported crystal structures of VyPAL2, a highly active ligase-type AEP from *Viola yedoensis*, in its activated state with and without a bound substrate [87]. The structures revealed how the enzyme recognizes its substrates, and specifically how the Asx residue inserts into the S1 pocket and the importance of a hydrophobic residue at the P2' position. Additionally, the role of the 'Gatekeeper' residue in the S2 pocket was identified, influencing the enzyme's activity toward ligation or hydrolysis. These insights provide a foundation for designing AEPs with tailored specificities for bioengineering applications.

Another study explored the use of N^{γ}-hydroxyasparagine, Asn(OH), as an unnatural P1 substrate for peptidyl asparaginyl ligases to create cyclic peptides [88]. By incorporating Asn(OH), the researchers synthesized potent matrix metalloproteinase 2 (MMP2) inhibitors, with the hydroxamic acid moiety serving as the key pharmacophore. The study also demonstrated that Asn(OH) can be oxidized to Asp, enabling the synthesis of bioactive cyclic peptides like MCoTI-II, kalata B2, sunflower trypsin inhibitor-1 (SFTI), and Arg-Gly-Asp (RGD) peptides, which are otherwise challenging to cyclize using traditional peptidyl asparaginyl ligase (PAL) substrates. This approach expands the utility of AEPs in therapeutic peptide synthesis.

Finally, in an illustration of the versatility of peptide cyclizing enzymes, a recent study investigated the use of the subtilisin-like macrocyclase PatGmac, produced by the marine cyanobacterium *Prochloron didemni*, for cyclizing larger peptides beyond its natural substrates, the patellamides [89]. PatGmac recognizes a specific C-terminal sequence (-AYDG) to cyclize peptides. The enzyme successfully cyclized the 14-amino acid SFTI-1 but was less efficient in cyclizing the 29-residue cyclotide kalata B1. Plant derived AEPs and their engineered analogs remain as the most efficient auxiliaries for cyclization of cyclotides.

CONCLUDING REMARKS

We hope this article has provided a valuable update on cyclotide research over the last four years. The main conclusions are that the field is highly active in terms of discovery, mechanistic understanding and applications. In terms of discovery, the diversity of cyclotide sequences continues to expand, although interestingly, the previously seen size range of 28–37 amino acids for cyclotides has not been breached in any of the new sequences. Perhaps most noteworthy as far as discovery efforts are concerned has been the increasing prevalence of acyclotide sequences being reported. Significant advances have been made in our understanding of cyclotide biosynthesis and evolution. Overall, the papers reviewed here collectively emphasize the expanding applications of cyclotides and their associated biosynthetic auxiliaries, from protease inhibition to immunomodulation and oncology, highlighting their transformative potential in pharmaceutical research.

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