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Naturally occurring circular proteins: distribution, biosynthesis and evolution

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Circular proteins, *i.e.*, proteins with a backbone comprised of a continuous and seamless circle of amino acids, have been discovered over the last 15 years in bacteria, plants, fungi and animals. They function as defence tools in the organisms in which they are expressed and are exceptionally stable. The cyclotides are the largest known family of circular proteins and are expressed by plants of the *Violaceae* (violet), *Rubiaceae* (coffee) and *Cucurbitaceae* (cucurbit) families, where they have a role in plant defence against insect predation. So far there are fewer examples of cyclic peptides in bacteria or animals but we suggest that cyclic peptides are an underdiscovered class of molecules and that many more will be discovered in the near future. There is much interest in understanding the mechanism of cyclization of circular proteins and the role of the cyclic backbone in defining structure and activity. In this review, the families of ribosomally synthesized cyclic proteins reported to date are described and their common features are examined, providing information on their distribution, biosynthesis and evolution. The unusual structure of circular proteins confers them with high stability, and makes them very interesting as scaffolds for drug design, and this has led to the re-engineering of linear proteins to stabilise them and use them for such applications.

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1. Introduction

Amino acids are essentially bifunctional chemical units with an amino functionality and a carboxyl functionality, which link successively to form the backbone chains of peptides and proteins. When represented in simple Lego-format, as shown in Fig. 1, it is clear that there is no reason why it would not be possible for one final step in the protein formation pathway to occur, *i.e.*, the joining of the N-terminus and C-terminus with a peptide bond to form a head-to-tail cyclized protein. This seems to not happen very commonly in nature for ribosomally synthesized proteins. Or does it? We now know that examples of head-to-tail cyclized proteins occur in animals, plants, fungi and bacteria. Although they are less common that their linear counterparts, more than 200 sequences of circular proteins have now been documented[1] and they are being discovered at an ever increasing rate. With recent studies showing that the mechanisms of cyclization are relatively simple, involving protease-catalyzed protein splicing,[2,3,4] it seems probable that many more examples will be discovered in the future.

This review describes the currently known classes of ribosomally synthesized head-to-tail cyclized peptides and proteins (summarized in Table 1), and updates both an earlier general review[5] and several specific reviews on individual classes of circular proteins.[5–14] We explicitly use the descriptor "head-to-tail" cyclized proteins because, strictly speaking, amino acids are not just bifunctional; some are trifunctional *via* reactivity of their side chains. Thus, in addition to potential head-to-tail linkages, side chain-to-backbone linkages are also possible (*e.g.*, from the side chain of an Asp or Glu to the N-terminus, or from a Lys side chain to the C-terminus). Furthermore, side chain-to-side chain interactions can occur as well. Disulfide bonds between cysteine residues most commonly exemplify the latter, but other more exotic possibilities are known and will be briefly mentioned later in this review in relation to the circular bacterial peptide subtilosin A.

We will use the term circular "protein" to refer to head-to-tail cyclized peptides and proteins. In general, the dividing line between peptides and proteins is not well defined, but typically is taken to be around 50 amino acids. Many of the molecules examined in this review are shorter than this but we use the term protein even in these cases because, in general, head-to-tail cyclized peptides have many of the properties of proteins, *i.e.*, they have well defined elements of secondary and tertiary structure and they are synthesized like regular proteins on the ribosome. There is also a large number of cyclic peptides of typically fewer than 12 amino acids that are synthesized by an alternative route, *i.e.*, *via* non-ribosomal peptide synthetases.[15] These cyclic peptides often contain a stunning array of post-translational modifications and non-coded amino acids will not be covered in this review, as our focus is on ribosomally synthesised cyclic proteins.

Naturally occurring circular proteins occur in a diverse range of protein families but most appear to have in common a role in host defence of the organisms in which they are expressed. The presence of a circular backbone in these molecules suggests an advantage of head-to-tail cyclization over conventional linear proteins, probably for favouring resistance to proteases and enhancing stability. In this review, we describe selected examples of ribosomally synthesized circular proteins present in mammals, plants, fungi and bacteria, and discuss their general characteristics and possible evolutionary reasons for their unusual structure.

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2. Circular proteins in animals

Mammals express proteins with antimicrobial activities as part of the innate immune system for combating infection, known as defensins.[16–18] Of the three defensin subfamilies, α, β and θ-defensins, the α- and β-defensins are the best studied, and are disulfide-rich linear peptides of 30–45 amino acids.[19] By contrast,

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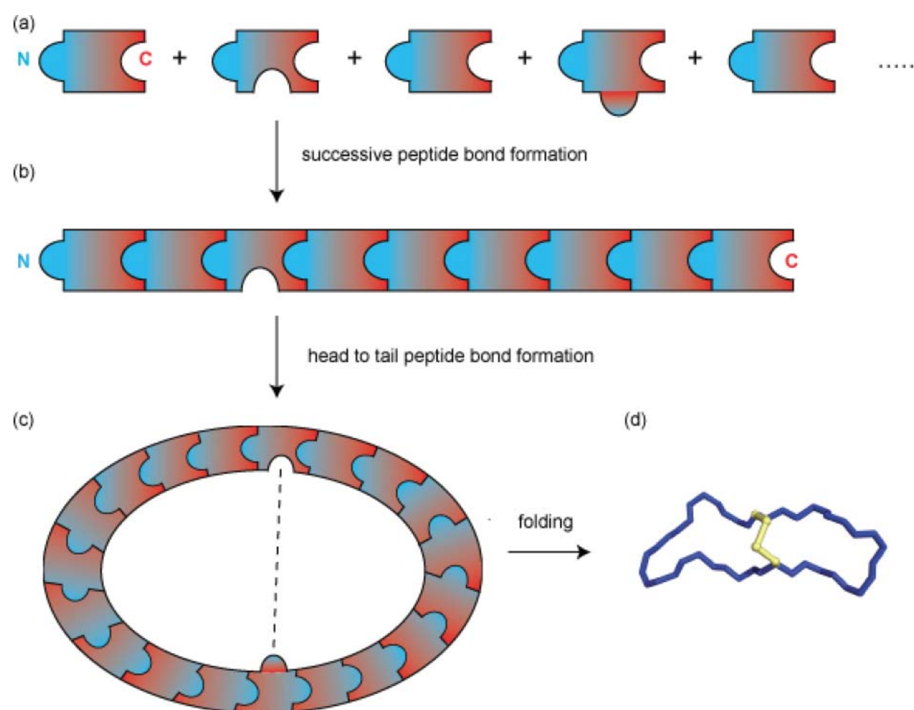

Fig. 1 Schematic representation of the formation of a protein from amino acid building blocks. (a) Shows the bifunctional nature of individual amino acids. (b) Shows a linear peptide chain being assembled from amino acids. (c) Shows that the N- and C-termini can be potentially linked to form a cyclic protein. The dashed line represents a possible side chain-to-side chain linkage, which typically is formed by disulfide bonds between Cys residues. (d) Shows an example of a circular peptide, SFTI-1, having head-to-tail cyclization and a single disulfide bond.

the θ-defensins were discovered more recently, and are both smaller in size and fewer in number. However, they have the important distinguishing feature of a head-to-tail cyclised backbone. The prototypic θ-defensin expressed in animals is Rhesus theta defensin 1 (RTD-1).[20] RTD-1 was originally discovered based on its antimicrobial activities against Gram-negative, Gram-positive bacteria and fungi;[20,21] later studies also showed that it protects against HIV-1 infection.[22]

RTD-1 is 18 amino acids in size and contains six cysteine residues, forming three disulfide bonds in a laddered arrangement (Fig. 2).[23] Not surprisingly, the structure of RTD-1 is rather flexible, as the laddered arrangement of the three disulfide bonds does not produce the globular type structure that is observed in many other examples of cyclic peptides but, despite its flexibility, RTD-1 is very stable and maintains its antibacterial activity under high salt conditions, which is not the case for many antimicrobial peptides.[20] Studies of RTD-1 with membrane mimicking systems suggest that its mechanism of action involves the disruption of membranes.[24]

RTD-1 is biosynthesised from the splicing and ligation of two linear precursor proteins, RTD1a and RTD1b, as shown in Fig. 2.

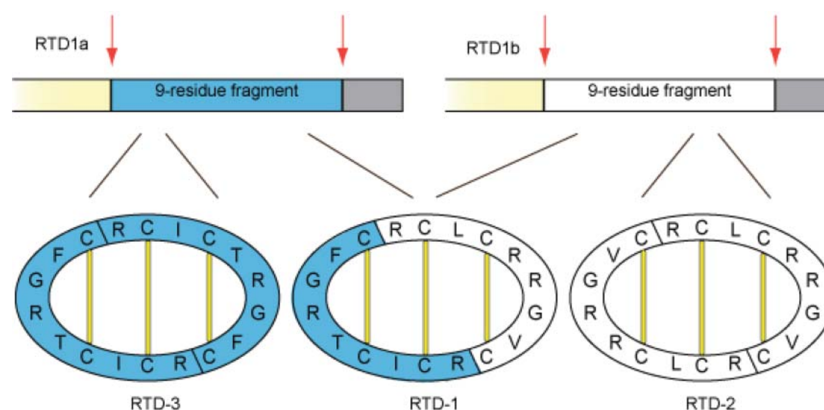

Fig. 2 Schematic representation of the biosynthesis of RTD-1, RTD-2 and RTD-3. Two genes code for two precursor proteins that each provide 9 amino acids towards the 18-residue heterodimer RTD-1. Homodimers of RTD1a and RTD1b form RTD-3 and RTD-2, respectively. The three peptides have six conserved cysteine residues that form three disulfide bonds. These bonds are arranged in a laddered arrangement that has been defined as a "cystine ladder" motif.[42]

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Table 1 Sequences of circular proteins, highlighting "terminal" residues in precursors

SPECIES	FAMILY	PEPTIDE	ORGANISM	A.A.	SEQUENCE	REFERENCE
MAMMALS	θ-DEFENSINS (18 a.a.)	RTD-1	*Macaca mulatta*	18	**R**CLCRRGVC — RCICTRGF**C**	(20)
		Retrocyclin-1	*Homo sapiens*	18	**R**CICGRGIC — RCICGRGI**C**	(22)
FUNGI	AMATOXINS (18 a.a.)	α-Amanitin	*Amanita bisporigera*	8	**I**WGIGCN**P**	(97)
	PHALLOTOXINS (7 a.a.)	Phalloidin	*Amanita bisporigera*	7	**A**WLATC**P**	(98)
		Phalloidin	*Conocybe albipes*	7	**A**WLVDC**P**	(99)
PLANTS	BB-INHIBITORS (14 a.a.)	SFTI-1	*Helianthus annus*	14	**G**RCTKSIPPICFP**D**	(82)
	CYCLOTIDES (28-37 a.a.)	Kalata B1	*Oldenlandia affinis*	29	**G**LPVCGETCVGGTCNTPGCTCSWPVCTR**N**	(45)
		Cycloviolacin O1	*Viola odorata*	30	**G**IPCAESCVYIPCTVTALLGCSCSNRVCY**N**	(43)
		MCoTI-II	*Momordica cochinchinensis*	34	**G**GVCPKILKKCRRDSDCPGACICRGNGYCGSGS**D**	(55)
BACTERIA	BACTERIOCINS (35-70 a.a.)	Subtilosin A	*Bacillus subtilis*	35	**N**KGCATCSIGAACLVDGPIPDFEIAGATGLFGLW**G**	(109)
		Butyrivibriocin AR10	*Butyrivibrio fibrisolvens*	57	**Y**FIADKMGIQLAPAWYQDIVNWVSAGGTLTTGFAIIVGVTVPAWIAEAAAAFGIAS**A**	(121)
		Gassericin A	*Lactobacillus gasseri*	58	**I**YWIADQFGIHLATGTARKLLDAMASGASLGTAFAAILGVTLPAWALAAAGALGATA**A**	(115)
		Acidocin B	*Lactobacillus acidophilus*	58	**I**YWIADQFGIHLATGTARKLLDAVASGASLGTAFAAILGVTLPAWALAAAGALGATA**A**	(120)
		Carnocyclin	*Carnobacterium maltaromaticum*	60	**L**VAYGIAQGTAEKVVSLINAGLTVGSIISILGGVTVGLSGVFTAVKAAIAKQGIKKAIQ**K**	(123)
		Lactocyclicin Q	*Lactococcus* sp strain QU 12	61	**L**IDHLGAPRWAVDTILGAIAVGNLASWVLALVPGPGWAVKAGLATAAAIVKHQGKAAAAA**W**	(126)
		Circularin A	*Clostridium beijerinckIi*	69	**V**AGALGVQTAAATTIVNVILNAGTLVTVLGIIASIASGGAGTLMTIGWATFKATVQKLAKQSMARAIA**Y**	(114)
		Uberolysin	*Streptococcus uberis*	69	**L**AGYTGIASGTAKKVVDAIDKGAAAFVISIISTVISAGALGAVSASADFIILTVKNYISRNLKAQAVI**W**	(122)
		AS-48	*Enterococcus*	70	**M**AKEFGIPAAVAGTVLNVVEAGGWVTTIVSILTAVGSGGLSLLAAAGRESIKAYLKKEIKKKGKRAVIA**W**	(106)
	PILIN (74-77 a.a.)	T pilin	*Agrobacterium tumefaciens*	74	**Q**SAGGGTDPATMVNNICTFILGPFGQSLAVLGIVAIGISWMFGRASLGLVAGVVGGIVIMFGASFLGKTLTGG**G**	(132)
		TrbC	*Escherichia coli*	78	**S**EGTGGSLPYESWLTNLRNSVTGPVAFALSIIGIVVAGGVLIFGGELNAFFRTLIFLVLVMALLVGAQNVMSTFFGR**G**	(132)

Sequences of representative circular proteins from different species. The N- and C-termini are highlighted in bold type. The Cys residues involved in disulfide bridges are highlighted in red. Other residues involved in cross-links are highlighted in blue.

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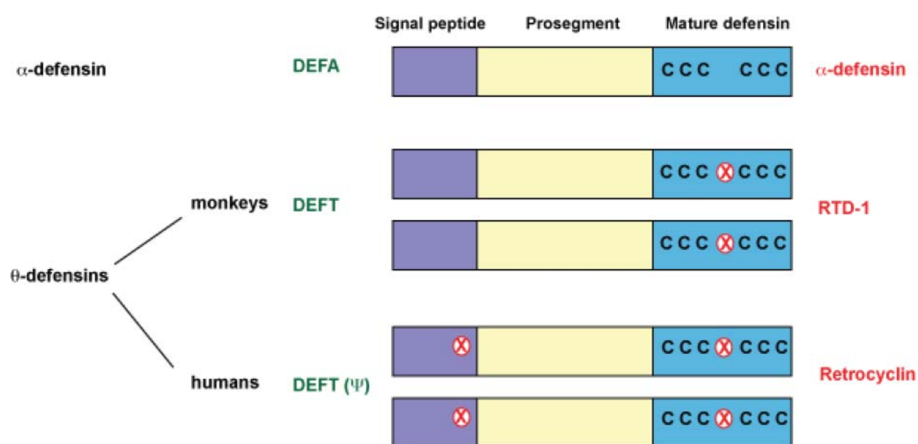

Fig. 3 Schematic representation of genes encoding α-defensins and θ-defensins. α-Defensins are encoded by the gene DEFA and come from one precursor protein. The θ-defensin gene, DEFT, has a stop codon after the third cysteine, and mature RTD-1 results from the excision and ligation of two precursor proteins. The human genome includes a θ-defensin pseudogene, DEFT(ψ), but the corresponding peptide is not expressed due to the premature stop codon in the signal peptide, represented by a red cross. Retrocyclin is derived from the chemical synthesis of a homodimer of nonapeptides of the human θ-defensin pseudogene.

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These precursor proteins are in fact each α-defensin homologues in which the corresponding open reading frame is truncated prematurely by a stop codon after the third of the six cysteines in full-length α-defensins.[20] The post-translational processing steps required for producing mature RTD-1 involve removal of a signal peptide, proteolytic cleavage at sites flanking each of the precursor peptides (RTD1a, RTD1b), and formation of two new peptide bonds and three disulfide bonds.[10] After the initial report on the discovery of RTD-1, two other closely related peptides were found, namely RTD-2 and RTD-3 (Fig. 2).[25] These two peptides derive from homodimers of RTD1b and RTD1a, respectively. RTD-1 is more abundant than RTD-2 and RTD-3 combined.[25]

The θ-defensins are expressed in several species of Old World monkeys and orangutans but not New World primates or humans.[26] Although human θ-defensin pseudogenes are transcribed, the mRNA is not translated effectively due to a mutation that introduces a premature stop codon into the signal peptide, as illustrated in Fig. 3.[22] Nevertheless, the chemical synthesis of a peptide, named retrocyclin, corresponding to the sequence of a θ-defensin human pseudogene showed that this molecule had antimicrobial activity.[11,22] Not surprisingly, the antibacterial properties of retrocyclin resemble those of RTD-1, but more interestingly, retrocyclin was found to be a potent blocker of HIV-1 infection. Numerous analogues have been synthesized, forming what is now known as the retrocyclin family. Various analogues have anti-HIV activity,[11,26–29] anti-HSV-1 activity,[29–31] or anti-influenza A virus activity.[32] Moreover, retrocyclins have been reported to be effective against *Bacillus anthracis*, the causative agent for anthrax, and provide a novel molecular template for designing agents against *B. anthracis* and its toxins.[33]

Retrocyclins act as lectins that bind to gpl20, CD4, and galactosylceramide with high affinity, and their mechanism of action against HIV has been studied extensively.[11,34–36] Retrocyclin binding to glycoproteins at the early stage of the viral infection inhibits the entry of viruses into cells, rather than their replication. Once the virus is in the cell, retrocyclins have little effect as antivirals.[22,29,30,37] Amongst the retrocyclins examined so far, retrocyclin-2 (RC-2) is the most potent θ-defensin against both HIV-1[38] and herpes simplex type 2.[29] It is identical to retrocyclin-1 (RC-1) except for a substitution of a Gly for an Arg in RC-2. Another derivative of retrocyclin, RC-101, that arises from the substitution of an Arg for a Lys, is twice as active as retrocyclin.[39] The enantiomeric counterpart of RC-1, RC-112, shows enhanced antiviral activity.[40] Wang and coworkers[41] recently reported a retrocyclin analogue (RC-111) that has the same sequence but in reverse, which enhances HIV infection, rather than inhibiting it.

Attempts to solve the structure of retrocyclin in aqueous solution initially proved problematic and only after considerable trial and error was it possible to obtain solution conditions under which the structure could be determined. Using a combination of NMR and ultracentrifugation experiments, it was found that retrocyclin self-associates in solution and this, in part, was responsible for poor NMR spectra at typical NMR concentrations in aqueous solution. Because the monomeric form appears to be stabilized by anionic lipids, solutions containing SDS micelles were used in the structure determination process.[42] By extrapolation of the structures determined in SDS and in combination with ultracentrifugation experiments, it was deduced that retrocyclin most probably exists in solution as a trimeric structure, as indicated in Fig. 4. The biological significance of this structure is not known but it has been suggested that because of its small size, self-association might assist in the interaction of retrocyclin with biological membranes.[42]

3. Circular proteins in plants

The cyclotides[43] are by far the largest known family of circular proteins, with current estimates suggesting that around 50 000 members might exist.[44] Fig. 5 shows the structure of the prototypical cyclotide, kalata B1, which comprises 29 amino acids and includes three disulfide bonds arranged in a cystine knot.[45]

Kalata B1 was discovered originally as the active medicinal agent in a tea used by women in the Congo region of Africa to accelerate childbirth.[46] Gran isolated a uterotonic peptidic fraction from the tea and showed that its main constituent, kalata B1, was a

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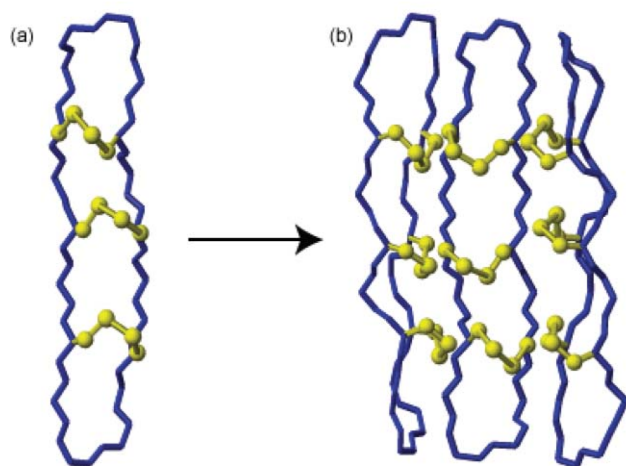

Fig. 4 (a) Structure of the θ-defensin retrocyclin. (b) Shows a schematic representation of a putative trimeric structure proposed for retrocyclin.[42]

peptide of around 30 amino acids, although the peptide could not be fully characterized at the time.[46] We subsequently determined the primary sequence and three-dimensional structure of kalata B1 and showed that it had an unusual topology, *i.e.*, a circular backbone and knotted arrangement of disulfide bonds.[45]

Around the time that the structure of kalata B1 was reported, three other examples of head-to-tail cyclic peptides were also reported, namely violapeptide I,[47] cyclopsychotride A[48] and the circulins.[49] In each case, the peptides were ~30 amino acids in size, with a circular backbone, and had six conserved Cys residues. Based on the conserved features of these peptides, we proposed that they would form part of a larger family, and this indeed turned out to be the case. In 1999 the term cyclotide was introduced to represent what now appears to be a very large family of backbone cyclized peptides produced by plants in the *Rubiaceae* (coffee), *Violaceae* (violet) and *Cucurbitaceae* (cucurbit) families.[43]

It is apparent from Fig. 5 that cyclotides can be regarded as a template built around a central cystine knot, onto which various loops are presented. Since all cyclotides contain exactly six cystine residues and have a circular backbone, there are six loops between the six cystine residues. Two of these loops (loops 1 and 4) comprise part of the embedded ring of the cystine knot and are generally regarded as integral to the core of the molecule. By contrast, loops 2, 3, 5 and 6 vary in both size and residue composition and can be thought of as the exposed bioactive regions of cyclotides. For this

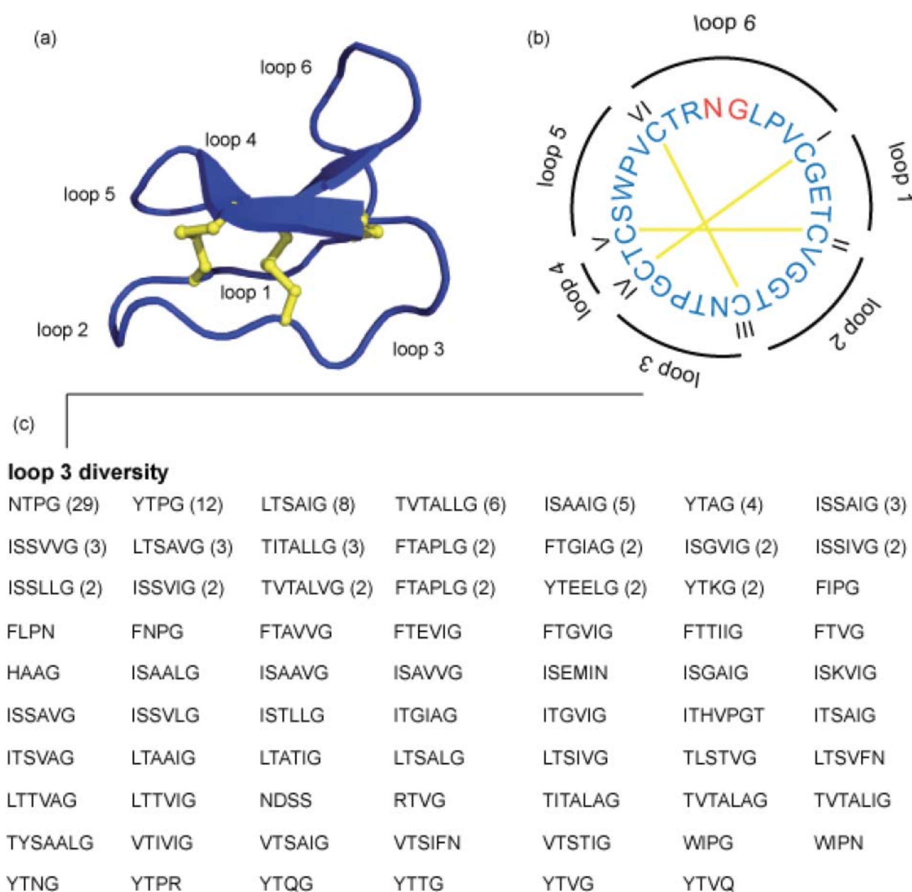

Fig. 5 Structure and sequences of cyclotides. (a) Structure of the prototypic cyclotide kalata B1 (PDB ID: 1NB1).[165] The figure shows the macrocyclic peptide backbone and cystine knot arrangement of three conserved disulfide bonds. In this motif two disulfide bonds and their connecting backbone segments form a ring that is penetrated by the third disulfide bond. Cyclotides have six backbone segments between their cysteine residues that are referred to as loops and numbered 1 to 6 in panel (b). The conserved cysteines are numbered I to VI and the disulfide connectivity is represented by yellow bars. The amino acids involved in the cyclization of the backbone (Gly and Asn) are highlighted in red. (c) Summarizes the diversity of sequences in loop 3 of currently known cyclotides. The sequences are organized according to their frequency of occurrence. The numbers in brackets represent the number of cyclotides where that sequence has been found in loop 3.

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reason cyclotides have been regarded as a natural combinatorial template.[50] One of the interesting properties of cyclotides is that they are exceptionally stable.[51] This is not surprising given the strongly cross-braced structure and the fact that they have no termini, thereby precluding digestion by exoproteases. On the basis of their stability, cyclotides have been proposed as possible scaffolds in drug design applications.[50,52–54]

Cyclotides were originally classified into two subfamilies, depending on the presence or absence of a *cis*-proline residue in loop 5. Möbius cyclotides have a *cis*-proline, which induces a conceptual twist in the backbone (leading to the analogy of a Möbius strip, a circular ribbon containing a single twist), whereas bracelet cyclotides do not.[43] A third subfamily of cyclotides was subsequently introduced based on the discovery of two peptides in the seeds of the tropical vine *Momordica cochinchinensis*, *i.e.*, the trypsin inhibitor cyclotides MCoTI-I and MCoTI-II.[55] The structure of MCoTI-II is similar to other cyclotides, based on two independent structure determinations.[56,57] It comprises a cyclic cystine knot motif that superimposes relatively well with cyclotides of the Möbius and bracelet subfamilies even though the sequences of the trypsin inhibitor cyclotides are quite different from other cyclotides. The trypsin inhibitor cyclotides are also referred to as cyclic knottins.[11]

As well as the uterotonic activity first reported for kalata B1, cyclotides have a wide range of other biological activities, including anti-HIV,[58–62] haemolytic,[63,64] neurotensin antagonism,[48] antimicrobial,[65] antifouling,[66] cytotoxic,[67–69] and trypsin inhibitor activity.[54] Nevertheless, the natural function of cyclotides appears to be as defence agents based on the fact that they potently inhibit the growth and development of insect larvae when incorporated into artificial diets.[14,70–73] They have also been shown to be molluscicidal[74] and nematocidal.[75–77] An individual plant typically expresses many cyclotides[78] with some being produced in very large amounts (up to 2 g per kg wet plant). Their toxic activity against insect and other pests, combined with their high level of expression, is strongly supportive of a natural role in plant defence.

The diverse range of biological activities of cyclotides is initially surprising, but it appears that the different activities can be explained by a common mechanism involving the interaction of cyclotides with membranes. For example, the anti-HIV activity is measured in a cell-based assay that might reflect specific interactions of cyclotides with viral or host cell membranes.[49] Additionally, the proposed insecticidal activity of cyclotides involves disruption of mid-gut membranes in *Helicoverpa* caterpillar species.[72] Support for the membrane hypothesis is provided by the fact that biophysical measurements clearly indicate the interaction of cyclotides with membranes. The first such studies involved biosensor measurements that showed that lipids immobilized on a biosensor chip attracted cyclotides to varying degrees depending on lipid composition, thus indicating that some cyclotides are selective for some membrane types over others.[79] NMR studies have also been used extensively to delineate the mechanism of interaction of cyclotides with membranes. Shenkarev and co-workers used spin labels embedded in DPC micelles to determine that the cyclotide kalata B1 interacts with the micelles only near the surface, rather than embedding deeply.[80] Göransson and co-workers reported the selective interaction of cyclotides with cell membranes and established a link between biological activity and membrane binding.[81]

Another example of a circular protein from plants is the sunflower trypsin inhibitor 1 (SFTI-1). As its name suggests, SFTI-1 is a trypsin inhibitor derived from sunflower seeds[82] and has high sequence homology to a family of serine protease inhibitors known as Bowman-Birk Inhibitors (BBIs).[83,84] SFTI-1 is a 14 amino acid peptide and is the most potent Bowman-Birk trypsin inhibitor known, despite being the smallest. It has also been found to inhibit the enzyme matriptase, which has led to suggestions of applications in anticancer medicine in humans.[85,86]

SFTI-1 was originally reported in 1999, when its structure in complex with trypsin was elucidated.[82] The NMR solution state structure of unbound SFTI-1[87] is virtually superimposable with the crystal structure[82] bound to trypsin (Fig. 6), highlighting that one of the features of circular proteins is that they tend to be relatively rigid molecules (except for RTD-1, which we noted above has a flexible structure).

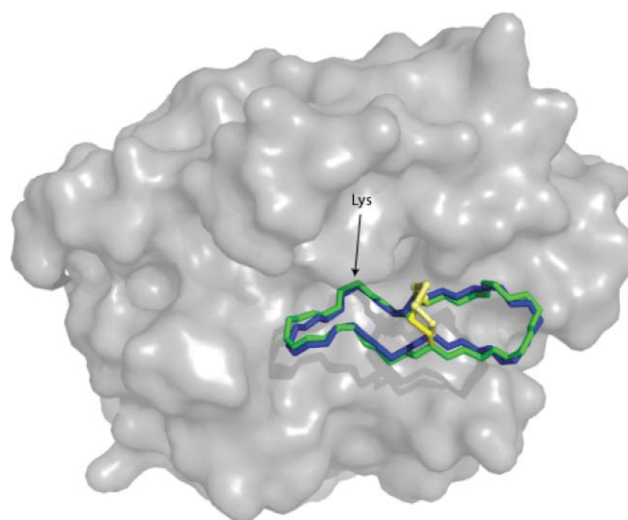

Fig. 6 X-Ray structure of sunflower trypsin inhibitor I (SFTI-1) bound to trypsin (PDB ID: 1SFI).[82] The figure shows a superimposition of the free (blue) and bound structures (green) and highlights their similarity. The residue that confers trypsin inhibitory activity to SFTI-1 (Lys) is indicated with an arrow.

Due to its small size and high stability conferred by the cyclic backbone, SFTI-1 is attractive as a drug scaffold. Its biosynthesis, structure and activity have been reported over recent years:[87–93] SFTI-1 has two loops; the binding loop contains Lys 5, which is responsible for the trypsin inhibitory activity of the peptide; the secondary loop, containing Asp14, is crucial for maintaining the rigid structure of SFTI-1.[94] Asp14 is also thought to be involved in the cyclization process, a point to which we will return later when discussing generic aspects of the biosynthesis of cyclic proteins.[95]

There are a large number of other cyclic peptides in plants that are smaller than SFTI-1 or the cyclotides. These have been reviewed extensively by Tan and Zhou,[96] and since it is probable that these peptides are made by non-ribosomal routes, we will not discuss them further here.

4. Circular peptides from fungi

Fungi have long been known to produce a variety of small cyclic peptides, typically smaller than 12 amino acids in size.

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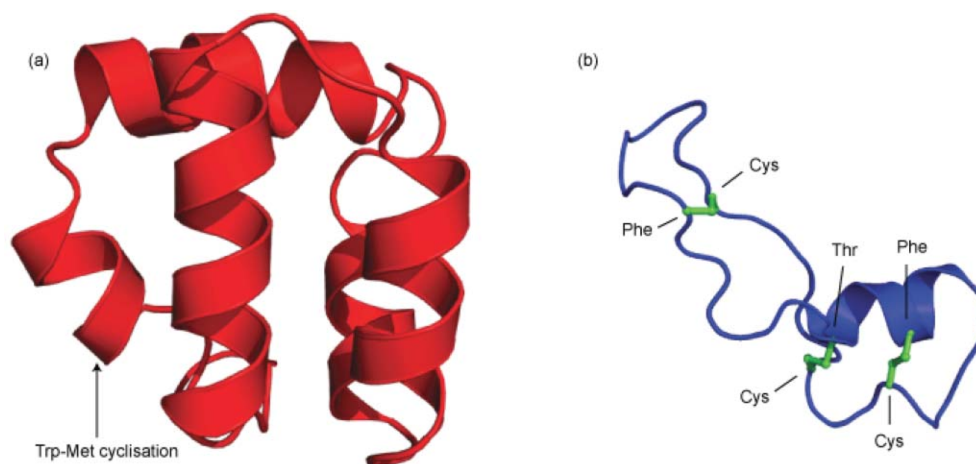

Fig. 7 Structure of bacteriocins. (a) Structure of AS-48 (PDB ID: 1E68).[113] This 70 amino acid peptide comprises five helical sections. Of particular interest is the fact that the termini of the pre-proprotein occur at a site that becomes part of helix 5. Unlike other circular proteins, it is unusual that the termini of the precursor are joined in an element of secondary structure. (b) Structure of subtilosin A (PDB ID: 1PXQ). This 35 amino acid peptide has a head-to-tail cyclized backbone and three cross-links formed between Cys residues and the α-carbons of Thr or Phe, as indicated on the structure. These covalent cross-links are post-translationally formed by a mechanism that is not yet known.

Cyclosporin is the most famous example and is used widely as an immunosuppressive drug.[97] Again though, we will not cover this class of peptides here since in general they are made non-ribosomally. However, there are some exceptions. Until recently it had been thought that all cyclic peptides from fungi were made by non-ribosomal peptide synthetases, until sequences corresponding to toxic cyclic peptides were found in the genome of *Amanita bisporigera*.[98] The ribosomally synthesized cyclic peptides found in fungi are classified into two main groups: the amatoxins and phallocidins, which are octapeptides and heptapeptides, respectively. In addition to their head-to-tail cyclic backbone they have an unusual cross-link formed by the condensation of cysteine and tryptophan residues. This combination of a cyclic backbone stabilised with additional internal cross-links makes them topologically similar to the cyclic peptides in plants and animals discussed above, despite being somewhat smaller and very different in sequence and mode of action.

Despite being similar structurally to one another, the mechanism of action of the amatoxins and phallocidins differs significantly. Amatoxins inhibit RNA polymerase II, whereas phallocidins stabilize F-actin. The predicted protein products of the genes coding for these toxins contain a hypervariable toxin region, potentially capable of encoding a wide variety of peptides of 7–10 amino acids, flanked by conserved sequences. The mechanism of cyclization has been suggested to involve a proline protease in processing a ~35 residue precursor protein.[99]

5. Circular proteins in bacteria

Bacteria produce a great diversity of peptides, and amongst them bacteriocins stand out for their exceptional stability and tendency to include cyclic examples within their family. In general, bacteriocins range from ~35–70 amino acids in size and are ribosomally produced by Gram-positive bacteria. The bacteriocins isolated so far are documented in an online database, named BACTIBASE.[100] Although bacteriocins have been classified in several ways,[101,102] according to Nes *et al.*,[103] they can be divided into three main groups, *i.e.*, classes I, II and III, which are further subdivided. Of interest here is class IId, comprising circular bacteriocins. Table 1 summarizes the main circular bacteriocins known to date. Class II bacteriocins have been recently reviewed by Nissen-Meyer *et al.*[104] and the cyclic bacteriocins have been reviewed by Maqueda *et al*.[105]

Bacteriocins have antimicrobial activities against a broad range of pathogenic microbes, but are mainly directed against Gram-positive bacteria. Some, including enterocin AS-48[106,107] and subtilosin A,[108,109] also display activity against Gram-negative bacteria. The mechanism of action of bacteriocins is the same as most antimicrobial peptides, *i.e.*, by cell membrane disruption.[110] Bacteriocin producers are immune to their own bacteriocins due to the presence of specific immunity proteins, which are typically small highly charged proteins.[111,112] The immunity proteins act together with the secretory ABC system to keep the cytoplasmic bacteriocin concentration levels low to avoid pore formation.[113] Space limitations prevent coverage in detail here, but the specific proteins involved in immunity for various bacteriocins are summarized in a recent review.[105]

Although bacteriocins vary widely in size and primary structure, a common theme among them is a high proportion of hydrophobic residues. Moreover, their secondary structure is composed of mainly α-helices, which gives them a stable compact structure that maintains activity after protease or thermal treatments. In contrast to cyclic peptides made by higher organisms, the bacterial cyclic peptides typically do not contain disulfide cross-links. Fig. 7(a) shows the structure of bacteriocin AS-48,[113] which comprises a five-helix bundle, and is perhaps the best characterised of the bacteriocins. The mechanism of action of AS-48 has been studied in detail and involves interactions with membranes, leading to the formation of pores.[110,113]

Other examples include circularin A, a 69 residue cyclic peptide produced by *Clostridium beijerinckii*[114] that has 60% homology with bacteriocin AS-48. Gassericin A and reutericin 6 are 58 residue cyclic peptides that were originally thought to differ in the number of D-Ala residues in the sequence; one for gassericin A

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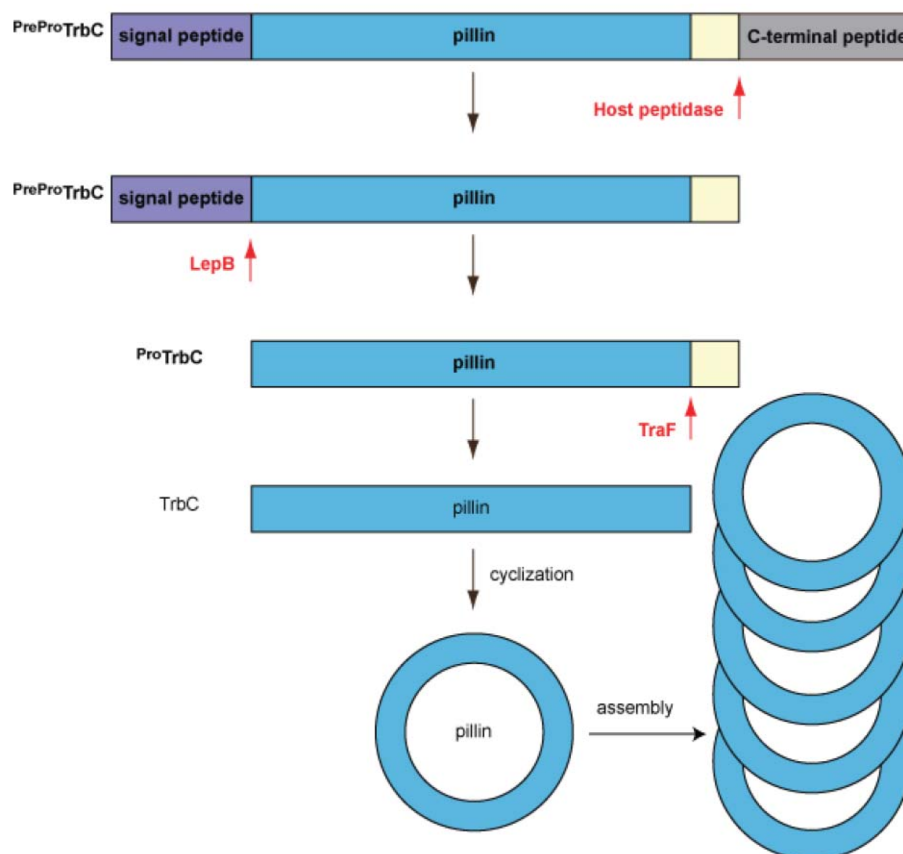

Fig. 8 Schematic representation of the biosynthetic pathway of pilin proteins.[134] For the maturation of the pilin protein, first the C-terminal peptide is cleaved by an unknown host-encoded proteinase. Then a host-encoded proteinase, LepB, removes the N-terminal signal peptide. Finally, TraF cleaves a tetrapeptide and facilitates the formation of a new peptide bond between the N- and C-termini and the production of the circular protein.

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and two for reutericin 6.[115,116] However, a recent paper has shown that they are in fact identical.[117] The gene encoding the immunity protein for gassericin A has been recently identified.[118,119]

Another circular bacteriocin is acidocin B,[120] which differs from gassericin A only by one residue (M24V).[116] The bacteriocin produced by *Butyrivibrium fibrisolvens* AR10, butyvibriocin AR10, also shows high homology with gassericin A.[121] Uberolysin is a 69 residue cyclic peptide produced in *Streptococcus uberis* 42.[122] Recently a new bacteriocin, carnocyclin A, was isolated from fresh pork.[123] It comprises 60 residues and its structure[124] is similar to enterocin AS-48; however, the mechanism of action of carnocyclin A seems to be different to that of AS-48. Rather than forming non-selective pores like AS-48, carnocyclin A forms anion-selective channels.[125]

Another recently reported bacteriocin isolated from cheese,[126] lactocyclicin Q, comprises 61 amino acids and has the highest hydrophobic amino acid content among the cyclic bacteriocins known to date.

Bacillus subtilis produces an atypical bacteriocin, subtilosin A,[109] which is more similar to the cyclic peptides from higher organisms than to other bacteriocins because it contains cross-links between side-chains as well as a circular backbone. In subtilosin A the cross-links are not disulfide bonds like in the cyclic peptides from higher organisms but comprise unusual covalent linkages between Cys residues and the α-carbons of Thr or Phe residues.[127,128] Fig. 7(b) shows the structure of subtilosin A. A mutant of subtilosin A, named subtilosin A1, has been recently isolated and shows haemolytic activity and enhanced antibacterial activity.[129]

Besides bacteriocins, bacteria produce the largest circular proteins known to date, namely the pilin proteins. Pilins are involved in the transfer of genetic material between bacteria.[130,131] Two examples of circular peptides involved in pili assemblage have been reported.[132] *Agrobacterium tumefaciens* produces T-pilin, a 74 residue peptide that arises from a precursor protein, VirB2 propilin,[133] and *Escherichia coli* produces the pilin subunit TrbC, a 78 amino acid circular protein. Its precursor protein, proTrbC, comprises 145 amino acids.[134] In contrast to other circular proteins, the mechanism of cyclization of these two proteins is fully understood and involves enzymatic processing as summarised in Fig. 8.[130,132,135,136] Interestingly, even though other pilin precursor proteins show homology with VirB2 and TrbC,[134,137–139] as far as we are aware, no other circular pilin proteins have been found. The three-dimensional structures of pilin proteins have not yet been reported, probably reflecting the highly hydrophobic nature of these peptides and hence the difficulty in handling them.

Other recent examples of circular proteins come from cyanobacteria, which produce a family of cyclic peptides known as cyanobactins.[140] It was originally believed that cyanobactins were synthesized by non-ribosomal peptide synthetases until a gene cluster from *Prochloron didemmi* was sequenced.[141] The genes that encode the precursor proteins contain hypervariable regions,

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leading to a natural combinatorial library of cyclic peptides.[142] For example, the first cyclic peptides identified in cyanobacteria were patellamides A and C,[141] which result from the cleavage and cyclization of the same precursor protein. Since then, many other cyclic peptides have been isolated from cyanobacteria.[143–145] However, all the cyanobactins reported so far are subject to post-translational modifications that introduce non-peptidic components in their sequence (*e.g.* thiazole and ozazoline rings). These structures are outside the scope of this review and will not be further discussed.

6. "Circular" proteins that never were, or no longer are

One bacterial product that was originally reported to be a cyclic, microcin J-25,[146] is not actually head-to-tail cyclic. It turns out that the original structure determination[146] was erroneous and the peptide is really side chain-to-backbone cyclized. Nevertheless, it is informative to examine the early data for microcin J-25 to highlight the difficulties in characterising cyclic peptides and to point out that there are alternatives to head-to-tail cyclization that also produce highly stable structures. It was initially suggested that the ultra-stable bacterial peptide microcin J-25 had a circular primary sequence of 21 amino acids[146] and its three-dimensional structure was likened to that of kalata B1, with the suggestion that both had a circular backbone folded back upon themselves. However, microcin J-25 does not have disulfide bonds and so it was puzzling to us why such a peptide would be apparently as stable as the cyclotides, and this led us to re-examine the original literature report. Two other groups had similar suspicions and simultaneously reported a revised structure in which the carboxyl side chain of Glu6 is linked *via* an amide bond to the N-terminus to produce a cyclic substructure.[147–149] Remarkably, the remaining peptide chain loops back and its C-terminal tail threads through the N-terminal ring to create a lasso-type structure. Unthreading of the lasso is prevented by bulky residues in the threaded peptide chain on either side of the ring, which explains initial enzyme digestion studies which reported that the peptide stayed in one piece after digestion with thermolysin in the loop region.[146]

The initially incorrect structure determination for microcin J-25 highlights one of the difficulties in working with cyclic peptides, namely that they can be tricky to structurally characterise. It also provides support for the suggestion that there are likely to be many more examples of cyclic peptides existing in nature. Such peptides are not particularly amenable to sequencing, and researchers generally have not been attuned to looking for them, and thus many may have gone undetected.

In contrast to microcin J25, a circular protein that "never was", the retrocyclins mentioned earlier in this article are an example of "once were" cyclic peptides that "no longer are", thanks to an aberrant stop codon in their encoding gene. We mention this to highlight that although cyclic peptides apparently evolved from linear precursors, and presumably persist because their cyclic backbones give them stability advantages over their linear ancestors, evolution is an ongoing process and not even cyclic peptides are immune to fateful mutations that stop their production. Although it is not possible to know whether the evolutionary loss of retrocyclin contributed to the susceptibility of humans to HIV-1 infection, synthetic retrocyclin is a now promising lead for designing agents that can prevent human HIV infection.[22] Recent studies have also shown that it is possible to re-awaken the expression if retrocyclin in human cells *via* treatment with aminogylcoside antibiotics that "read-through" the premature stop codon.[150] This leads us to a discussion of the biosynthetic pathways that facilitated the evolution of cyclic peptides.

7. Biosynthetic pathways leading to cyclization

We focus here on ribosomally synthesized cyclic peptides, *i.e.*, peptides that are translated from a nucleic acid sequence and synthesized on the ribosome. It follows from this biosynthetic pathway that cyclization occurs after assembly of the peptide chain, and the peptide must transition through a linear precursor stage. The genes encoding precursor proteins for the largest family of circular proteins, *i.e.*, the cyclotides, have been well studied and a schematic representation of the generic precursor protein structure is shown in Fig. 9. The precursors have a modular nature and encode one, two or three cyclotides.

Alignment of the nucleic acid sequences for a large number of cyclotide precursors, as well as alignment of the sequences of the mature cyclotides themselves, has helped to identify strongly conserved features in addition to the six cysteine residues that form the signature cystine knot motif. One conserved residue of particular importance is an Asn (or Asp) at the proto-C-terminus of the mature cyclotide domain. This conservation suggests a role for an Asn (or Asp) residue in cyclotide processing and indeed two recent publications have confirmed that the mechanism of cyclization of cyclotides in plants involves asparaginyl endopeptidase enzyme activity.[2,4]

Fig. 9 summarizes our current understanding of the biosynthetic process for cyclotides. It is interesting to note that this mechanism involves the use of an asparaginyl endopeptidase enzyme to ***make*** a peptide bond, which is the reverse of its normal proteolytic function to ***break*** peptide bonds. We propose that cyclotides evolved from ancestral linear proteins that acquired a favourable Asn mutation which subsequently allowed an asparaginyl endopeptidase, presumably already present in plants with another function, to be co-opted to perform the cyclization.

We further propose that this mechanism for cyclization (*i.e.* protease-mediated peptide bond formation) is likely to be common to all classes of ribosomally synthesized cyclic proteins, but the terminal amino acid residues involved in the ligation reaction are vastly different in the various examples of circular proteins, as illustrated in Table 1. For example the bacteriocin AS-48 precursor undergoes a ligation (and corresponding) cyclization event between a Met and a Trp residue.[107] The enzyme involved in this process remains unknown. Remarkably, the cyclization occurs within what becomes an element of secondary structure in the final folded protein, namely helix 5 (Fig. 7a). Since it is commonly believed that a protein sequence encodes the information for driving the formation of secondary and tertiary structure, the finding that a well-defined element of secondary structure is formed from two discontinuous segments (*i.e.*, the N and C termini of the precursor protein) represents a new twist in protein folding. In most other cases of cyclic peptides, the ligation reaction that

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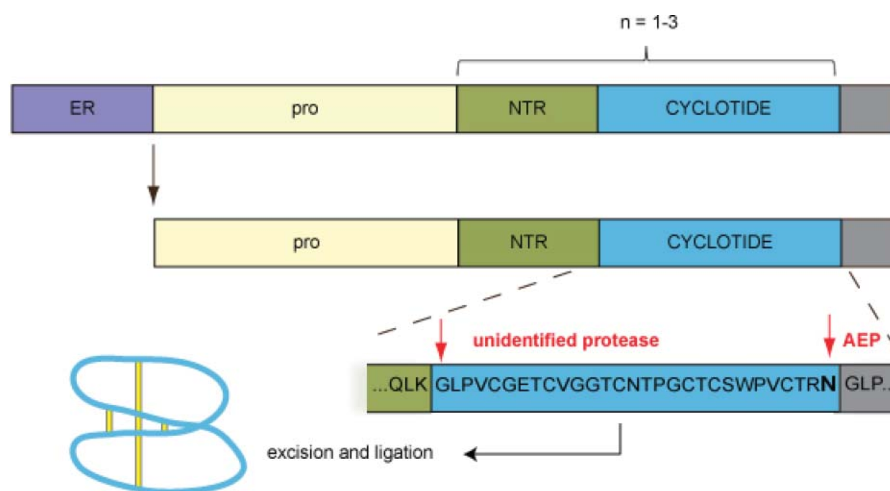

Fig. 9 Schematic representation of our current understanding of the biosynthetic pathway of cyclotides. Genes have been discovered that encode one, two or three copies of mature cyclotide domains, in some cases the same mature cyclotide encoded multiple times in the one gene, and in other cases with multiple copies of different cyclotides encoded in one gene. An asparagyl endopeptidase (AEP) has been found to be responsible for cleavage and cyclization of mature cyclotides.

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joins the ends of the precursor protein occurs in regions without highly defined secondary structure, *i.e.*, in turn or loop regions.

The θ-defensins are particularly remarkable in that they utilise not one, but two genes to build their cyclic backbone. For example, the 18-residue peptide RTD-1 is encoded by two genes, each producing a precursor protein that contributes only nine amino acids to the mature product.[20] The origin of this unusual pathway reflects the evolutionary development of θ-defensins from α-defensin precursors, as noted earlier (Fig. 3), but the mechanism of double head-to-tail ligation and cyclisation remains unknown.

Whatever mechanism drives cyclization in particular cases, it is interesting to consider that since peptide bond formation is energetically unfavourable under physiological conditions of ionic strength and pH, why does cyclisation occur in any case? In early studies, chemists used a variety of approaches to help favour formation of peptide bonds, including enzyme-mediated approaches on esterified peptide substrates,[151] cysteine-activated native chemical ligation approaches [152,153] and activation using selenocysteine-mediated approaches.[154] The discovery of naturally occurring intein-mediated protein splicing[155] demonstrated an alternative biosynthetic way of making peptide bonds, which has since led to novel applications in protein engineering.[156] In brief, in protein splicing, two peptide bonds that flank the protein splicing element (the intein) are cleaved, followed by the ligation of the external protein domains (the exteins) by a new peptide bond. Although some intermediate steps in the process are thermodynamically unfavourable, their coupling to diverse types of self-catalysed irreversible steps drives the overall protein rearrangement to completion.

Although the mechanisms are different, there are certain similarities between protein splicing and peptide cyclization. It seems probable that the cyclization of the circular proteins described in this article preferentially occurs, despite the apparent thermodynamic lability of the extra peptide bond, because the cyclic protein products need to be viewed in the context of the whole organism. The linking peptide bond that cyclizes the proteins requires energy, but since it confers high biological stability it appears to be advantageous enough to justify the energetic cost. Interestingly, despite the fact that cyclic proteins are present in all four kingdoms of life, they are more abundant in plants, bacteria and fungi, where peptides constitute a particularly important mechanism of defence. For this reason, it makes sense that these organisms will invest the extra energy required for the peptide bond involved in cyclization in order to have more biologically stable defence proteins that will protect them from external dangers.

8. Artificially engineered cyclic proteins

An examination of the protein database (PDB) shows that many proteins have their ends close to one another.[12] Therefore it is not surprising that there have been many attempts to produce artificially engineered cyclic proteins. An early attempt involved the cyclization of bovine pancreatic trypsin inhibitor, a 53 amino acid protein.[157] The cyclization was done by a chemical reaction on the native protein, and although a circular product was produced, it did not have any improved properties over the native protein; in fact it was less active and less stable. However, this result appears to be an exception and a range of chemical,[158] recombinant[156,159,160] and chemo-enzymatic[161] approaches have since been used to produce a variety of circular proteins that have improved properties over their linear counterparts. In our laboratory, the focus has been on the cyclization of small disulfide-rich peptides, in particular on the cyclization of conotoxins.[162]

Our prototypical example involved conotoxin MII. This 12 residue conotoxin contains two crossed disulfide bonds and specifically binds to the α3β4 subtype of the nicotinic acetylcholine receptor and has been implicated as a potential treatment for Parkinson's disease as well as other neurological disorders. Analysis of the structure of native MII showed that the two termini potentially could be joined by a linker of five or more amino acids. We synthesized analogues with linkers of five, six or seven amino acids and found that the six and seven amino acid-linked cyclic molecules had equal biological activity to the native conotoxin but

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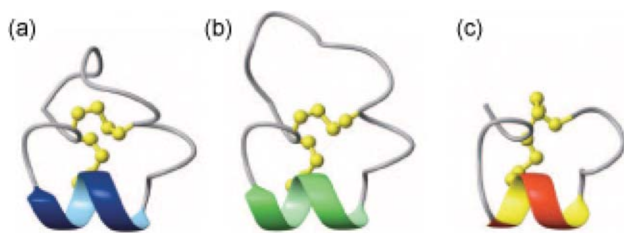

Fig. 10 Structure of α-conotoxin MII and cyclized derivatives containing (a) a six amino acid linker, (b) a seven amino acid linker, and (c) the native linear MII. Figure adapted from Clark *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2005.[162]

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had dramatically enhanced stability. Fig. 10 shows our engineered cyclic analogue of the conotoxin MII.[162]

MII represents a prototypical example for the cyclization of a range of other conotoxins and, in general, we find that improved proteolytic stability is obtained in cyclic analogues. For example, this improved stability is seen in MrIA, a member of the χ-family of conotoxins that targets the noradrenaline transporter and is being developed as a lead molecule for the treatment of neuropathic pain.[163] The χ-conotoxins are similar to α-conotoxins in that they have two disulfide bonds, but the disulfide bond connectivity has a laddered, rather than crossed arrangement. Nevertheless, they have proven to be amenable to cyclization technology, thus demonstrating the generality of this approach. In a recent study, yet another conotoxin, Vc1.1, has been re-engineered with a cyclic backbone, and, as well as being more potent than the native linear conotoxin, is orally active in a rat model of neuropathic pain.[164] The introduction of oral activity into a peptide-based drug lead is a significant breakthrough and highlights the value of cyclization as a peptide engineering tool, a lesson learned from the naturally occurring cyclic peptides seen in nature.

10. Common links

Amongst the various circular proteins from animals, plants, fungi and bacteria there are some common links. The first is literally a peptide link between the N- and C-termini of the mature peptide segment of their linear precursor proteins. Once formed this bond is indistinguishable from all of the other peptide bonds in the protein, so that cyclic peptides have an essentially seamless backbone. The second common link is in their biosynthesis, *i.e.*, in all cases proteolytic enzymes appear to be utilised to perform vital processing steps, including forming the peptide link that culminates in cyclization. The third common link is in their properties: all naturally occurring cyclic peptides have an exceptional degree of stability that is brought about by their seamless, and hence protease-resistant, backbone. Finally, nature has in many cases supplemented the cyclic backbone with additional internal cross-links. Currently known circular proteins include examples with no cross-links, or with one, two or three cross-links. In general, the cross-links are disulfide bonds but in the case of subtilosin A and some fungal toxins other covalent linkages are involved. Furthermore, the cross-links can have various topologies, ranging from a single link in SFTI-1 to two laddered or crossed links in cyclized conotoxins, through to laddered or knotted links in three-disulfide-containing cyclic peptides such as the θ-defensins and the cyclotides, respectively. Overall, it appears that the cross-links have a role in stabilizing the structures rather than in defining the three-dimensional shape. But the supporting role of these cross-links is overshadowed by the main defining characteristic of circular proteins, *i.e.*, a backbone with no break in its chain and hence no chink in its armour that can be pierced by chemical, enzymatic or thermal degradative processes.

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