JOC The Journal of Organic Chemistry

PERSPECTIVE

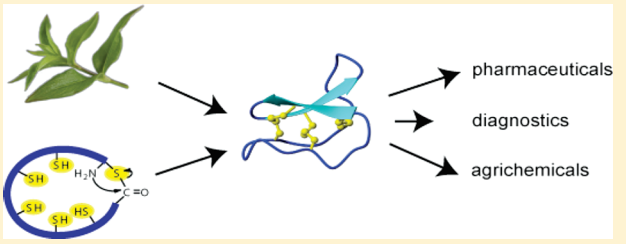
pubs.acs.org/joc

The Chemistry of Cyclotides

David J Craik* and Anne C Conibear

Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, Brisbane QLD 4072, Australia

ABSTRACT: Cyclotides are head-to-tail cyclic peptides that contain a cystine knot motif built from six conserved cysteine residues. They occur in plants of the Rubiaceae, Violaceae, Cucurbitaceae, and Fabaceae families and, aside from their natural role in host defense, have a range of interesting pharmaceutical activities, including anti-HIV activity. The variation seen in sequences of their six backbone loops has resulted in cyclotides being described as a natural combinatorial template. Their exceptional stability and resistance to enzymatic degradation has led to their use as scaffolds for peptide-based drug design. To underpin such applications, methods for the chemical synthesis of cyclotides have been developed and are described here. Cyclization using thioester chemistry has been instrumental in the synthesis of cyclotides for structure−activity studies. This approach involves a native chemical ligation reaction between an N-terminal Cys and a C-terminal thioester in the linear cyclotide precursor. Since cyclotides contain six Cys residues their syntheses can be designed around any of six linear precursors, thus providing flexibility in synthesis. The ease with which cyclotides fold, despite their topologically complex knot motif, as well as the ability to introduce combinatorial variation in the loops, makes cyclotides a promising drug-design scaffold.



INTRODUCTION

Cyclotides[1] are plant-derived peptides that, in addition to having a head-to-tail cyclic backbone, contain three conserved disulfide bonds forming a cystine knot motif. They comprise approximately 30 amino acid residues, and their unique structure engenders them with exceptional stability;[2] cyclotides retain their biological activity after boiling and are remarkably resistant to enzymatic degradation. In a sense, they can be regarded as molecules that have the structure of proteins but the stability of organic chemicals. The prototypical member of the cyclotide family, kalata B1, was originally discovered in the 1970s as an active uterotonic agent in a medicinal tea used in Africa to accelerate childbirth, although it was not structurally characterized at the time.[3,4] Following later independent reports in the 1990s of a few cystine-rich macrocyclic peptide sequences[5,6] and structures,[7] a large number of macrocyclic peptides of similar structure were discovered in plants from the Rubiaceae (coffee) and Violaceae (violet) families, leading to the recognition and naming of the family as cyclotides (*cyclo*pep*tides*) in 1999.[1] It is estimated that around 50000 cyclotides might exist,[8] although so far, the sequences of only ∼160 have been reported.[9,10]

Figure 1 shows the characteristic cyclic cystine knot (CCK) structure of cyclotides and illustrates the diversity of sequences present in the various loops in their structures. The loops are defined as the backbone segments between successive cysteine residues. Loops 1 and 4, combined with their connecting disulfide bonds, form an embedded ring through which a third disulfide bond is threaded to make the cystine knot motif. In contrast with the relatively conserved nature of residues in loops 1 and 4, loops 2, 3, 5, and 6 show a remarkably diverse range of sequences. This diversity, displayed on a conserved core, has led to the description of cyclotides as nature's combinatorial template.[11]

Cyclotides fall into two main subfamilies, Möbius and bracelet, depending on the presence or absence, respectively, of a *cis*-X-Pro peptide bond in loop 5 of the sequence, which introduces a conceptual "twist" in the cyclic peptide backbone.[1] A third subfamily, the trypsin inhibitor cyclotides, contains just two members, MCoTI-I and MCoTI-II, which are also referred to as cyclic knottins.[12]

The majority of cyclotides discovered so far have been found in plants of the Rubiaceae and Violaceae families. However, their distribution within these families differs significantly.[8] The hit rate for the detection of cyclotides in Rubiaceae plants is about 5%, whereas in the Violaceae family, it is 100% for the species examined so far.[8] Individual plants typically contain many cyclotides, which are found in varying quantities in different tissues.[13] The trypsin inhibitor cyclotides MCoTI-I and MCoTI-II are the only macrocyclic peptides reported so far in the Cucurbitaceae plant family.[14,15] We include them under the category of cyclotides as they have a characteristic cyclic cystine knot motif and are derived from plants; however, except for the six conserved cysteine residues, their sequences are very different from other cyclotides.[12] In addition to the Rubiaceae, Violaceae, and Cucurbitaceae plant families, recently we reported the discovery of cyclotides in *Clitoria ternatea*, a plant from the Fabaceae family.[16] This discovery represents an important new development because the Fabaceae is the third largest family of plants on Earth, comprising ∼18000 species, some of which are

Received: March 9, 2011
Published: April 28, 2011

used widely in human nutrition, including for example, peas and beans.

Cyclotides are of interest because of their unique topological features, remarkable stability, widespread geographical distribution in plants, and promising applications in drug design and in agriculture. Our focus here is on their chemistry. Figure 2 gives an overview of the important role that chemistry has had in the discovery and applications of cyclotides; the listed biological activities are separated into "natural" functions, associated with their host defense (agricultural) role, and "unnatural" activities as far as plant function is concerned. The latter activities typically were discovered in random screening programs of plants for human pharmaceutical applications. Such an approach to screening for pharmacological bioactivities is not restricted to cyclotides and is just one example of a rich history of drug discovery from plant sources.[17] This discovery route serves to illustrate the similarities between cyclotides and conventional druglike organic molecules: a theme we would like to emphasize.

There have been several reviews on cyclotides that have covered aspects of their discovery,[12,18−21] characterization,[22] biological activities,[23] and applications.[21,24] These topics will not be addressed in detail here; rather, the focus will center on the organic chemistry of cyclotides and, in particular, on chemical approaches to their synthesis and folding. Consistent with this chemistry focus, we begin with a brief discussion of their chromatographic and spectroscopic properties, including the use of mass spectrometry for sequencing and NMR for structure determination.

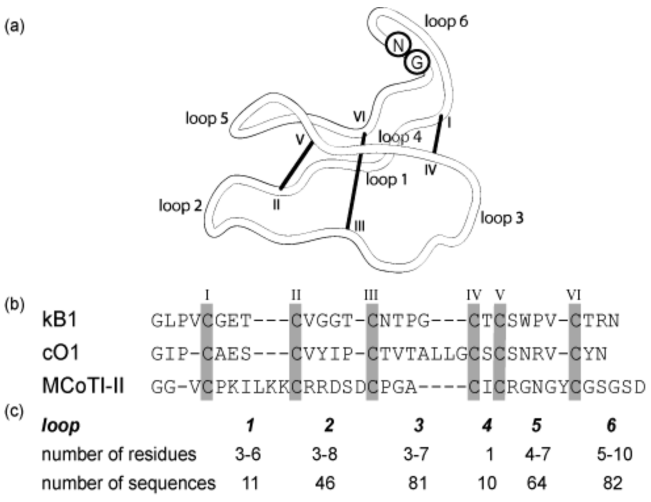


Figure 1. Structure of the cyclic cystine knot framework of the cyclotides. (a) The framework displays six loops, separated by six successive cysteine residues, numbered I−VI according to their order in the linear precursor protein. The ring formed by loops 1 and 4 with their connecting disulfide bonds is penetrated by the third disulfide bond between cysteine residues III and VI to form the cystine knot motif. (b) Example sequences from the Möbius (kalata B1, kB1), bracelet (cycloviolacin O1, cO1), and trypsin inhibitor (MCoTI-II) subfamilies of cyclotides are shown, with the conserved cysteine residues shaded. (c) Statistics from the cyclotides discovered so far are summarized, showing the number of residues and the number of different sequences in each of the loops.

ISOLATION AND SEQUENCING

Although cyclotides were formally recognized as a family only a decade ago,[1] the discovery of the prototypical member occurred indirectly in the early 1970s when Norwegian researchers partially characterized a peptide, kalata B1 (kB1), from the African plant *Oldenlandia affinis*.[25−27] Edman chemistry was used to obtain sequences from enzyme digests of a uterotonic peptide that was deduced to be approximately 30 amino acids in size. Based on an apparently blocked N-terminus and supporting enzyme fragmentation data, they initially hinted at the possibility of the peptide having a macrocyclic structure but later rejected this hypothesis on the basis of inconsistency with carboxypeptidase digestion studies.[25] With the benefit of hindsight, we believe some of these cleavage studies might have been confounded by the presence of contaminating amounts of endopeptidases in the carboxypeptidase digest, leading to spurious fragmentation. It was not until 1995 that the 29-amino acid macrocyclic nature of kalata B1 was unequivocally established using a combination of enzyme digestion (after reduction of disulfide bonds), mass spectrometry and NMR methods.[7] These studies coincided with the discovery of several other macrocyclic peptides of similar size and sequence from other Rubiaceae plants,[5,6] as well as an example from a Violaceae plant.[28]

Since these early studies, a range of approaches to isolate cyclotides from plant tissues has been developed.[1,5,29] Most recently, a three-step systematic approach to the identification of cyclotides from plant extracts was reported[8] and includes the following steps after a methanol-based extraction: (1) identification of the late-eluting fractions on reversed-phase HPLC; (2) confirmation that their mass is consistent with typical cyclotides, i.e., 2700−3800 Da; and (3) observation of an increase in mass of 18 Da on

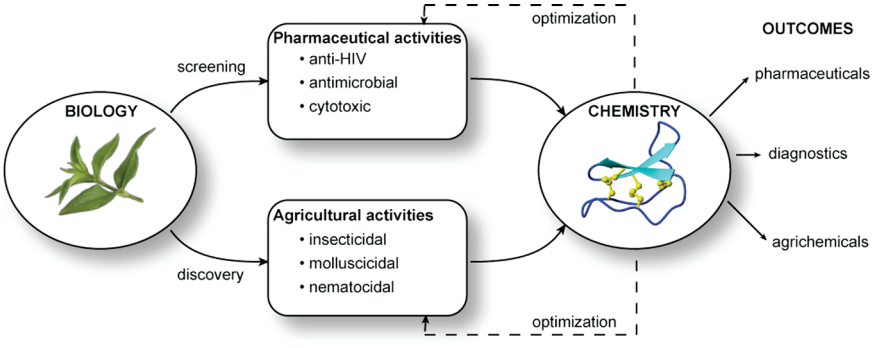


Figure 2. Overview of the partnership between chemistry and biology in the discovery and application of cyclotides. Cyclotide bioactivities were originally discovered while screening for pharmaceutical activities, but their activities were later broadened in scope by targeted studies of their natural functions. Knowledge of cyclotide structures and activities can be used as a starting point for chemical modifications to produce pharmaceuticals, diagnostics, and agrichemicals, which take advantage of the exceptional stability of the cyclotide framework relative to conventional peptides.

dx.doi.org/10.1021/jo200520v |*J. Org. Chem.* 2011, 76, 4805–4817

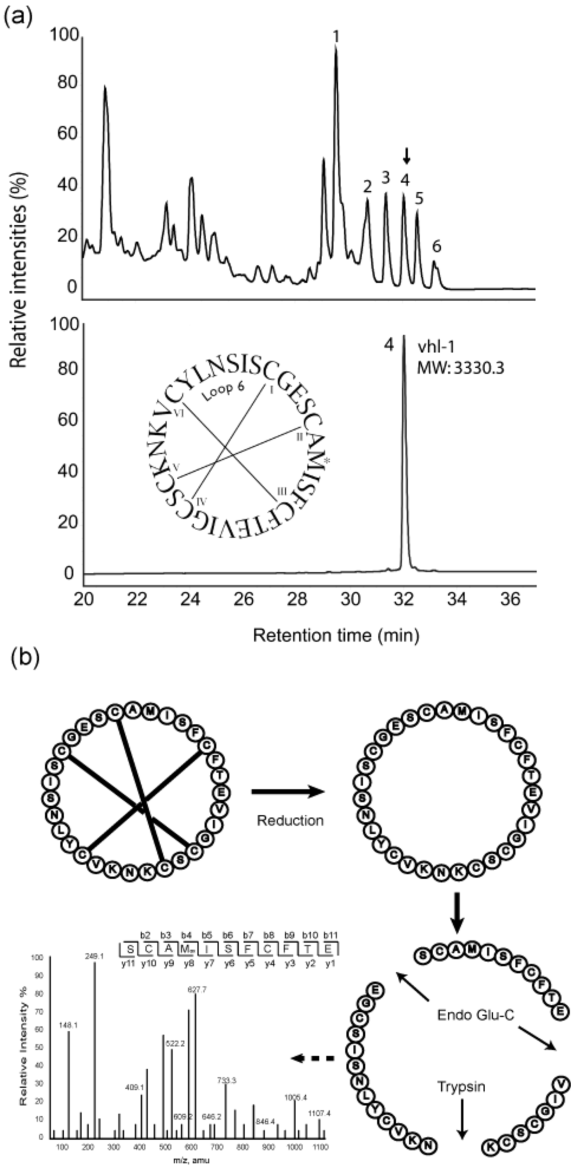


Figure 3. Overview of the isolation and sequencing of cyclotide vhl-1 from *V. hederacea*. (a) Late-eluting fractions on reversed-phase HPLC of a leaf extract were identified (upper trace) and purified (lower trace). (b) Reduction of the disulfide bonds followed by partial enzymatic digestion with trypsin and endo-GluC yielded fragments, which were then sequenced using mass spectrometry and subsequently combined to give the cyclotide sequence.[13]

cleavage with endo-GluC.[16] The characteristic late elution in reversed-phase HPLC[18,30] (Figure 3) arises from the presence of a patch of hydrophobic residues on the surface of cyclotides.[7,31]

Cyclic peptides present a fundamental difficulty for sequencing because they have neither an N- nor a C-terminus, thus blocking conventional sequencing approaches such as Edman degradation as well as more recent MS-based approaches. Furthermore, cyclotides are completely resistant to enzymatic proteolysis in their native oxidized state.[2] Therefore, the standard method for preparing cyclotides for sequencing has been to reduce the disulfide bonds, alkylate the free thiols, and then cleave the cyclic peptide with selected enzymes to liberate termini for sequencing.[32] Endoproteinase GluC (endo-GluC) has been extensively used for this purpose and has the advantage that so far, all cyclotides, with the exception of kalata B12, have a conserved Glu in loop 1 but few other Glu residues in the sequence.[2] Thus, there is generally only one target site for endo-GluC. Trypsin has also been used extensively in sequencing efforts, as has a combination of both enzymes.[13] Edman sequencing has now been essentially replaced with MS-based approaches in which a series of fragment ions can be used to successfully trace the amino acid sequence.[13]

Figure 3 shows an example of the isolation and sequencing of the cyclotide vhl-1 from leaf tissue of the native Australian violet, *Viola hederacea*. A combination of trypsin- and endo-GluC-cleavage produced three peptide fragments that were individually sequenced and then stitched together to deduce the circular peptide.[13] This particular cyclotide is unusual in that it contains multiple Glu residues, but the method works well provided at least one Glu is present.

It is becoming more common to conduct the sequencing of natural peptides based on discovering their encoding nucleic acids, and this has been particularly successful for cyclotides. Using primers designed against conserved sequence elements near the endoplasmic reticulum (ER) signal sequence of cyclotide precursors has proved to be extremely useful in isolating novel cyclotide sequences.[33−40] There is often a close interplay between sequence discovery at the protein and nucleic acid levels, with each approach providing information that can be used to guide discovery at the other chemical (peptide or nucleic acid) level.

A web-based database (www.cybase.org.au) has been developed to curate the sequences of cyclotides and other circular proteins, both at the amino acid and nucleic acid levels.[9,10] The website contains a number of tools to allow analysis of cyclotide sequences and statistics and was used, for example, to generate the loop statistics in Figure 1. Other tools include mass spectroscopy fingerprint searching for sequencing and prediction of digestion fragments, as well as tools for analysis of linear and cyclic permutants and synthetic analogues of cyclotides.[9,10]

THREE-DIMENSIONAL STRUCTURES

The three-dimensional structures elucidated so far for cyclotides have been derived almost exclusively by NMR,[41] with the exception of one cyclotide, varv F, for which both an NMR and X-ray crystal structure exists,[42] and a few cases where the structures have been modeled.[43,44] In all cases, the cystine knot forms the core of the molecule and is surrounded by a β-sheet structure comprising a well-defined β-hairpin and a less ordered third β-strand. The unique cyclic cystine knot structure of the prototypic cyclotide kalata B1 was first reported in 1995.[7] As is typically the case for determining the structure of peptides of this size, a series of distance restraints was derived from NOESY spectra and used in simulated annealing calculations. These distance restraints were supplemented by hydrogen bonding restraints derived from amide exchange experiments in which a sample of kalata B1 was dissolved in D_2O and the rate of disappearance of the amide protons was monitored over time. Kalata B1 contains three proline residues among its 29 residues, and therefore, a total of 26 backbone amides. Unusually for a protein of this small size, 10 of these 26 backbone amide protons were slowly exchanging in D_2O, providing an immediate indication that the structure is well folded and contains extensive internal hydrogen bonding interactions.[7]

dx.doi.org/10.1021/jo200520v |*J. Org. Chem.* 2011, 76, 4805–4817

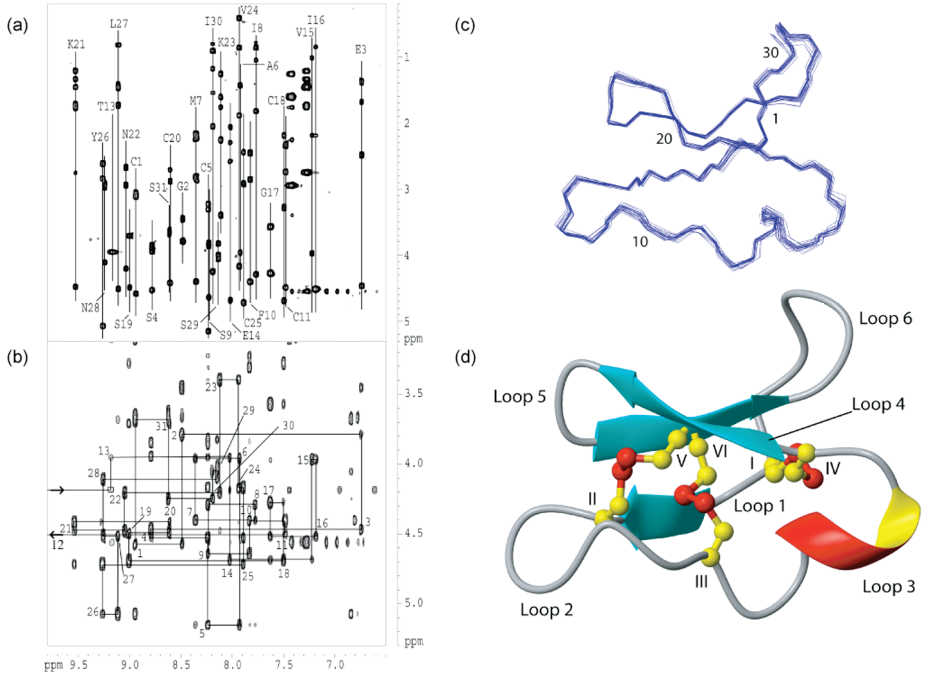


Figure 4. Structure determination of cyclotide vhl-1 by NMR. Cross-peaks in the two-dimensional NMR spectra are well dispersed and allow spin systems and sequential connectivities to be determined from the TOCSY (a) and NOESY (b) spectra, respectively. The sequential walk confirms the cyclic nature of the peptide backbone. The structure was determined using interproton distances, coupling constants and deuterium exchange data. Structure calculations using torsion angle simulated annealing resulted in an ensemble of 20 energy-minimized conformers shown in (c). The first, and every 10th, residue is numbered. The ribbon representation of vhl-1 shown in (d) highlights the key features of a cyclic cystine knot motif and the secondary structural elements.[13]

At the time that the structure of kalata B1 was first determined, it had not proved possible to determine disulfide connectivities using chemical means, and they were instead deduced from the NMR spectra.[7] This is not a trivial task because the assumption that the observation of NOEs between two cysteine residues implies connectivity is not always correct. We used a detailed analysis of energies and geometries of alternative disulfide-bonded forms to deduce the likely knotted topology. The dangers of the simple assumption of cross-Cys NOEs implying disulfide connectivity were highlighted in a study of kalata B1 by another group who incorrectly proposed a nonknotted disulfide connectivity based on this assumption.[45] However, after subsequent studies[46] providing chemical evidence for the knotted disulfide connectivity, and additional NMR studies,[31,47] it is clear that the knotted disulfide form is the correct structure. X-ray structures have also confirmed this connectivity.[42]

Figure 4 shows NMR data used to calculate the three-dimensional structure of vhl-1,[13] including a TOCSY spectrum (Figure 4a) used to assign amino acid spin systems and a NOESY spectrum (Figure 4b) used to generate distance restraints. The full three-dimensional structure was determined using simulated annealing based on distance and angle restraints and, as is typical for NMR structures, is represented, in the first instance, as a family of superimposed structures that match the experimental restraints (Figure 4c). The ensemble is well-defined and highlights the relative rigidity of the cyclotide scaffold. Figure 4d shows a cartoon representation of the global fold, which can be considered to be a conserved CCK onto which various loops are superimposed. The amino acid side chains in these loops dictate the primary interactions of cyclotides with their biological targets, thought to be membranes,[48] rather than protein-based receptors.

CHEMICAL MODIFICATION OF CYCLOTIDES

Cyclotides are amenable to a number of chemical modifications, including reduction and alkylation of their disulfide bonds and/or functionalization of individual amino acid residues. As already noted, the reduction and alkylation of the disulfide bonds is an important routine step for the sequencing of cyclotides, but reduction has also been applied in a number of studies that have looked at the role of the cyclic cystine knot in the structure and activities of cyclotides. In one such study, reduction and alkylation of the disulfide bonds in cycloviolacin O2 (cO2) resulted in complete loss of cytotoxic activity against human lymphoma cells.[49] The proposed explanation was that loss of the stability engendered by the cyclic cystine knot resulted in distortion of the structure and possible burial of bioactive residues within the protein.[49] Similar results have been found for kalata B1.[2]

Other chemical modifications that have been applied to native cyclotides include the derivatization of key functional groups. Göransson and co-workers showed, for example, that the surface-exposed Glu, Lys, and Arg residues in cO2 have a role in its cytotoxicity against human lymphoma cells.[49] Chemical modification of each of these residues, individually and in combination, as shown in Figure 5, allowed the effect of specific charged residues to be studied. Whereas masking either the single Arg residue or the two Lys residues resulted in minimal or no loss of potency, modification of all positively charged residues caused a 7-fold decrease in activity, thus suggesting the importance of at least one cationic residue. Methylation of the single Glu residue resulted in a 48-fold decrease in activity, indicating that this negatively charged residue is crucial, either for the interaction of the cyclotide with its target or for maintenance of the three-dimensional structure.[49]

dx.doi.org/10.1021/jo200520v |J. Org. Chem. 2011, 76, 4805–4817

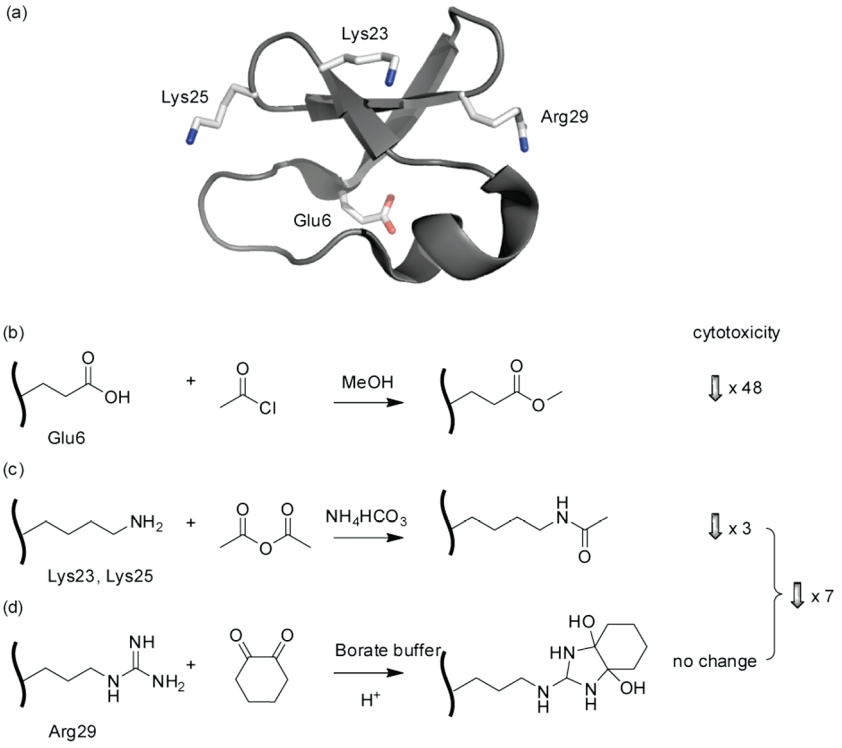


Figure 5. Chemical modification of charged residues in cycloviolacin O2 (cO2).[49] (a) Structure of cO2[50,51] showing the location of side chains that have been the target of chemical modifications to explore structure−activity relationships. (b) Esterification of Glu6 by reaction with acetyl chloride in methanol. (c) Acetylation of Lys23 and Lys25 with acetic anhydride. (d) Reaction of Arg29 with 1,2-cyclohexanedione. The peptide backbone of cO2 is represented by a bold line in (b)−(d). The relative decrease in cytotoxicity as a result of single or multiple modifications is indicated by the down arrows.

Native cyclotides have also been chemically labeled with various tags to study their biological properties or assist with their sequencing. Greenwood et al.,[52] for example, labeled the Lys residues of MCoTI-II with biotin to study the cell-penetrating properties of this cyclotide. Binding of streptavidin to the biotin sites allowed the intracellular location of MCoTI-II to be identified. Göransson and co-workers[32] used aminoethylation of the Cys residues of a cyclotide to develop a loop sequencing method. This approach adds a positivley charged group to the Cys residues, turning them into pseudo-Lys residues that are then amenable to cleavage by trypsin.

In general, all of the chemical reactions that have been applied to cyclotides work well in quantititive yield and benefit from the exceptional stability of cyclotides. The products may be readily purified by HPLC and characterized by MS and NMR. These observations support the proposal that in many ways cyclotides can be considered to be more like stable small molecules than peptides. Notwithstanding this impression, cyclotides are indeed peptides, and this gives them some advantages in their de novo synthesis relative to organic molecules; i.e., they can be made using repetitive and reproducible methods of solid-phase peptide synthesis.

SYNTHESIS OF CYCLOTIDES

Although cyclotides can be purified in high yields from plant material (up to 1 g/kg of plant tissue), synthetic chemical methods can provide access to modified and "grafted" versions of cyclotides. Furthermore, although naturally occurring cyclotides contain a wealth of sequence diversity, the capacity to chemically synthesize cyclotides provides opportunities to explore the structure−function relationships of individual amino acids by making mutants with specific sequence perturbations as well as to develop cyclotides with new biological activities. A range of methods has thus been developed over the past decade for the de novo synthesis of cyclotides. Figure 6 outlines, in general, the steps involved in cyclotide synthesis, the first of which is the assembly of the polypeptide chain.

Chain Assembly by Solid-Phase Peptide Synthesis. Solid-phase peptide synthesis (SPPS), the standard method for peptide synthesis, involves coupling successive N-protected amino acids to a growing peptide chain which is attached, by the C-terminus, to a polymer resin.[56] The heterogeneity of the reaction enables byproducts and excess reagents to be easily washed away, thereby greatly simplifying purification and increasing yields. The process can also be automated, and this allows for rapid synthesis of peptides of up to approximately 50 residues in length.

Regioselectivity of the amide bond formation is achieved by N-protection of the coupling amino acid, most commonly with either the *tert*-butyloxycarbonyl (Boc) or fluorenylmethyloxycarbonyl (Fmoc) protecting group. The Boc protecting group is acid-labile and is removed with trifluoroacetic acid (TFA), whereas the Fmoc protecting group is base-labile and is removed with piperidine.[57] Compared to Boc SPPS, Fmoc SPPS has some advantages for automation and for synthesizing linear peptides because of the use of less corrosive reagents and simpler cleavage from the resin.[58] However, Boc SPPS is currently the method of choice for cyclotides because of its compatibility with the chemistry involved in the cyclization process. In particular, the native chemical ligation reaction used for the cyclization requires a thioester linker at the C-terminus of the peptide. A disadvantage of Fmoc SPPS for the synthesis of cyclotides is that the

dx.doi.org/10.1021/jo200520v |J. Org. Chem. 2011, 76, 4805–4817

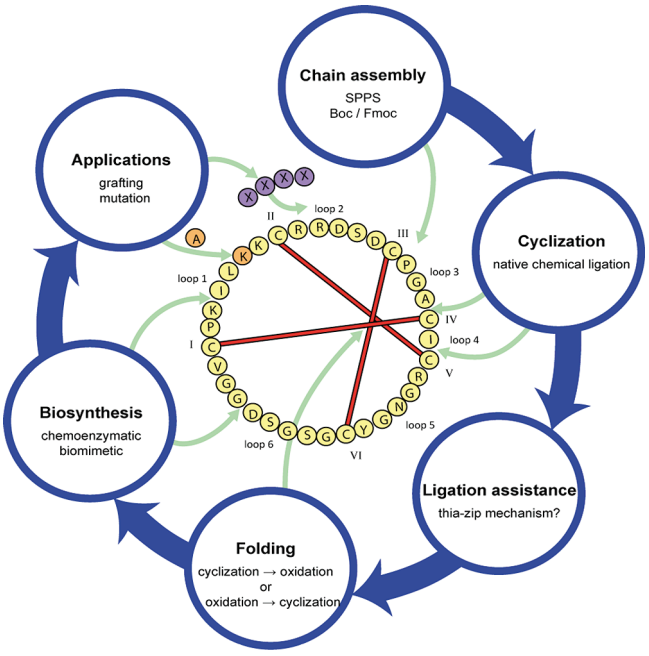


Figure 6. Overview of the chemistry associated with the synthesis, cyclization, and folding of cyclotides. Discrete steps in the process are indicated by blue circles and commence with chain assembly using solid-phase peptide synthesis (SPPS) with either Boc or Fmoc protecting groups. Cyclization is typically achieved using native chemical ligation chemistry,[53] which requires that the sequence be assembled with a cysteine residue at the N-terminus. Cyclotides contain six cysteine residues (numbered with roman numerals), and therefore, there are six possible starting points, two of which are indicated by green arrows on the right of the diagram adjacent to Cys^{IV} and Cys^{V}, respectively. The peptide sequence on the inner circle, indicated by one-letter amino acid codes with disulfide bonds as red bars, is that of MCoTI-II, the prototypical trypsin inhibitor cyclotide. It has been proposed that cyclization is assisted by a "zip" mechanism in which successive intermediate thiols assist in bringing together the N and C termini.[54] As indicated at the bottom of the diagram, folding and oxidation are usually done after cyclization, typically in an ammonium bicarbonate buffer. In principle, oxidation could be done first, followed by cyclization. However, cyclization followed by oxidation is preferable as higher yields are obtained. A number of biomimetic routes to the synthesis of cyclotides have been proposed and the starting points for these are indicated by green arrows. In the chemo-enzymatic approach, a linear peptide is assembled and oxidized and subsequently cyclized using an enzyme.[55] The in vivo biosynthetic starting point in loop 6 is indicated by a green arrow. Finally, the upper left of the diagram indicates possible applications of the chemistry of cyclotides, including the substitution of individual residues (mutagenesis) or grafting (i.e., the replacement of a small group of amino acids with another group of "foreign" amino acids).

thioester is cleaved by piperidine. However, the advantages of a rapid, automated synthetic method based on Fmoc chemistry mean that there is much interest in exploring new methods of cyclotide synthesis.

Attempts are currently being made to adapt either the thioester linker or the Fmoc synthesis protocol so that that Fmoc-based chemistry[58,59] can be used in the synthesis of cyclic peptides. Such an approach was demonstrated recently for the synthesis of the bracelet cyclotide cO2.[60] Standard SPPS building blocks and Fmoc chemistry were used to assemble the peptide chain and cleave it from the resin. The thioester required for ligation of the N and C termini was then formed in solution by reaction of the protected peptide chain with a thiol.

Native Chemical Ligation for Cyclization. The native chemical ligation strategy now typically used for the cyclization of cyclotides was originally developed for the joining of two or more peptide segments to allow synthetic access to large peptides. Because of the ever-decreasing total yields obtained in stepwise chain assembly of large peptides it is preferable to synthesize them by the chemical ligation of two or more peptides to produce a single chain. Early approaches to the problem of ligating multiple chains involved incorporating a nonpeptide bond at the ligation site,[61,62] but in 1994, Kent and co-workers introduced the technique of native chemical ligation (NCL). In this method, two unprotected peptide segments are joined with a peptide bond, thus producing a "native" backbone.[53]

As shown in Figure 7, NCL involves nucleophilic attack on the C-terminal α-thioester by the side-chain thiol of the N-terminal cysteine to form a thioester-linked intermediate. An S,N-acyl transfer rearrangement then produces the native peptide bond backbone and the free cysteine thiol.[53,63] Formation of the thioester-linked intermediate locates the N-terminal amine in the vicinity of the C-terminus for the subsequent formation of the peptide bond, thereby providing "entropy activation".[64,65] The proposed thioester intermediate was isolated as the ligation product of a C-terminal thioester and a peptide bearing an acetylated N-terminal cysteine.[53] When the N-terminal amine was not acetylated, the ligation product was resistant to nucleophilic attack and could form disulfide bonds, indicating that S,N-acyl transfer had taken place.[53] Involvement of an ionized thiolate was suggested by the reduced reaction rates below pH 6. Camarero and Muir first reported the synthesis of a cyclic peptide via this approach,[66] and native chemical ligation has since been widely used for the cyclization of a variety of natural and engineered cyclic peptides. The approach was readily adapted to cyclotide synthesis in the late 1990s.[30,67,68]

Ligation Assistance: The Thia-Zip Mechanism? The involvement of an N-terminal cysteine in native chemical ligation is well established, but the role, if any, played by internal thiols in the sequence is not as well understood. In cyclotides, which have five internal cysteine residues, it has been proposed that these nonterminal cysteine residues also assist in the cyclization process. The first reports of the use of native chemical ligation suggested the possible involvement of internal cysteine residues but not in increasing the rate of ligation.[53] In 1996, Tam and co-workers suggested a "thia-zip" mechanism, in which the reversible formation of thioesters between internal cysteine residues and the C-terminal carboxyl helps to increase the rate of cyclization.[54,72] This thiol−thiolactone exchange with side-chain thiols is thought to be analogous with the aza-zip mechanism, providing entropy activation by gradually increasing the ring size.[54,72] In the thia-zip reaction, as described by Tam et al., internal cysteine residues undergo reversible transthioesterification with the C-terminal thioester, gradually increasing the ring size until a thiolactone is formed between the N-terminal cysteine and the C-terminal carboxyl as shown in Figure 8.[67] An irreversible, spontaneous S,N-acyl transfer then takes place to form the peptide bond, completing the native backbone of the cyclic peptide.

Although the thia-zip reaction appears to be a plausible explanation for the rate enhancement observed in the presence of internal thiols, Tam et al. were unable to establish whether the transthioesterification is sequential along the chain, as depicted in Figure 8, or whether all internal thiols are involved.[54] The

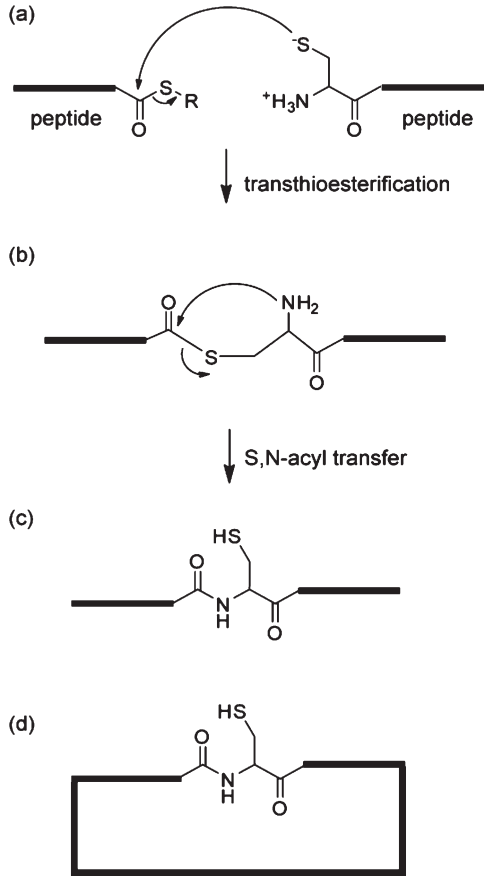


Figure 7. Mechanism of the native chemical ligation strategy for joining two peptide segments with a "native" amide bond. The reaction requires a peptide bearing a C-terminal thioester and a peptide bearing an N-terminal cysteine. (a) Transthioesterification results in the formation of an intermediate thioester (b) in which the two peptides are joined with a non-native backbone. (c) Irreversible S,N-acyl transfer then regenerates the cysteine thiol side-chain and forms the native peptide bond. (d) A single linear peptide with a C-terminal thioester and an N-terminal cysteine can be cyclized by the native chemical ligation strategy. Although Cys is a rare amino acid in most proteins and several studies have investigated alternatives to the need for an N-terminal cysteine,[63,69−71] Cys is remarkably abundant in cyclotides, thus making native chemical ligation an ideal strategy for their cyclization. A convenient cysteine position for ligation can be chosen and the high effective molarity of the reaction results in high yields.[30]

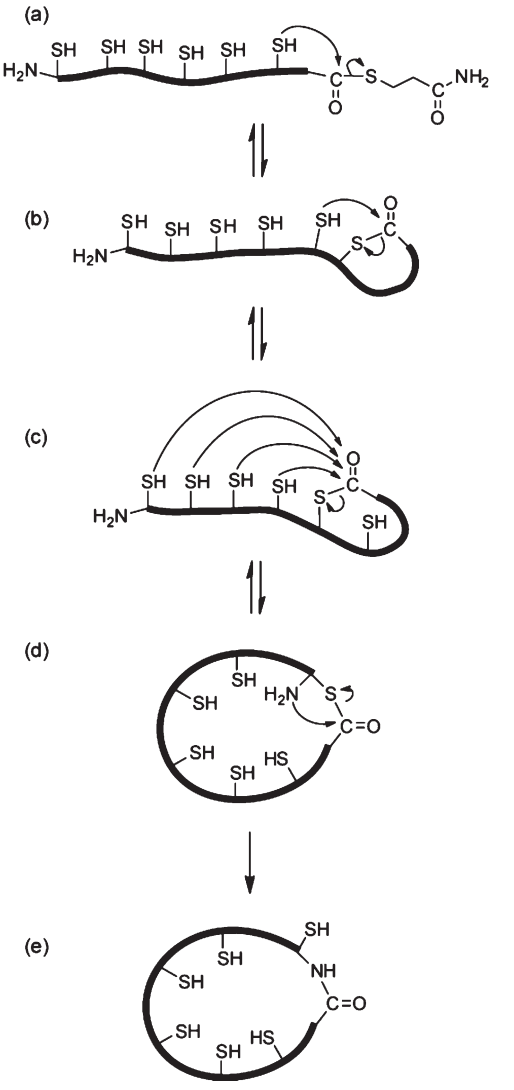


Figure 8. Proposed mechanism of the thia-zip reaction. Nucleophilic attack (a) by a cysteine thiol close to the C-terminal thioester forms an intramolecular thiolactone. Reversible transthioesterification (b) then results in a gradual increase in the ring size of the thiolactam (c). This brings the N and C termini into close proximity for an S,N-acyl transfer to take place (d), forming the cyclic peptide backbone (e).

proposed mechanism, however, is supported by the observation that the formation of the thiolactone is rate-limiting and sequence-dependent. The involvement of internal cysteine residues was also indicated in another study in which glycine or alanine spacers were placed between the cysteine residue and the N-terminus and it was found that the highest ligation rates (compared to a cysteine-lacking control) were obtained when the cysteine was 5−6 residues from the N-terminus.[73] Consideration of the proposed ring−chain tautomeric equilibrium and reversible S-acylation makes the thia-zip mechanism an intuitive explanation for the increased cyclization rate on the grounds of equilibrium principles.[72] When several internal cysteine residues are present, the availability of several isomeric thiolactone conformations with different ring sizes would shift the equilibrium in favor of the product.[55]

Since the early studies, the cyclization of several classes of cysteine-rich cyclic peptides, including cyclotides and α-defensins, has been attributed to the thia-zip mechanism.[54,74] Studies on the different ring sizes formed during S,N-acyl transfer seem to indicate that some macrocyclic ring sizes (such as 5- and 17-membered rings) are favored over others (8-, 9-, 11-, and 12-membered rings) in terms of the reaction rate.[54,72,73] However, there have been no systematic studies on the correlation of the positions of internal thiols with reaction rate. Although the thia-zip reaction may aid in the cyclization of cysteine-rich peptides and, thus, prove to be an additional advantage of these peptides as potential drug scaffolds, it is not essential for cyclization. Direct ligation of peptides lacking internal cysteine residues occurs readily, especially at unhindered ligation sites.[73]

FOLDING OF CYCLOTIDES

The complete synthesis of a functional cyclotide requires not only chain assembly and cyclization but also requires the oxidation of the Cys residues to form into their correct pairs of

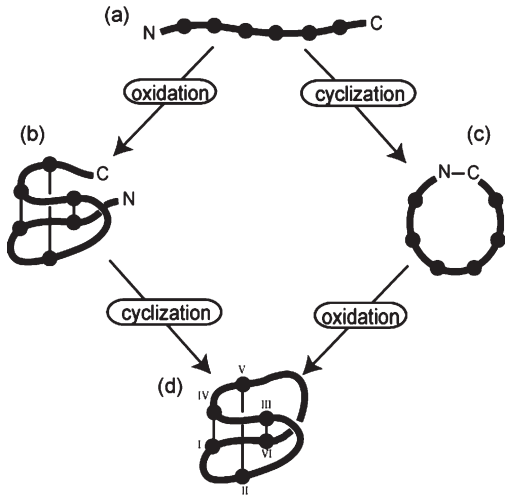


Figure 9. Alternative strategies for the cyclization and oxidation of cyclotides. The linear peptide (a) can be oxidized first to form the cystine knot and bring the N and C termini together (b). Cyclization then forms the native peptide backbone (d). Alternatively, the linear peptide (a) may react first to form the cyclic peptide backbone (c) and then be oxidized to form the disulfide connectivities as shown in (d). Cysteine residues are denoted by dots on the peptide chain.

disulfide bonds. The latter process is referred to as oxidative folding.[75] In principle, this folding can be carried out before or after backbone cyclization, as illustrated in Figure 9. One early synthesis of cyclotides was achieved using the strategy of oxidation before cyclization,[30] as it was predicted that formation of the disulfide bonds would bring the N and C termini into close proximity and accelerate the cyclization reaction as had been suggested for the cyclization of other folded protein domains.[76,77] This strategy would preclude any involvement of the thia-zip mechanism as described above. Although oxidation before cyclization was successful, higher yields of correctly folded protein were obtained by the second strategy, in which cyclization is carried out prior to oxidation.[30] It was suggested that entropic factors might have a role in the improved folding efficiency, as entropic losses on folding are presumably less in the cyclic than the linear peptide.[30]

Some naturally occurring cyclotides, such as kalata B1 and MCoTI, readily form the correct disulfide bond connectivities directly from their unprotected precursors,[30,78] whereas others require a two-step method using two orthogonal pairs of cysteine protecting groups. The latter strategy was employed to prevent the formation of incorrect disulfide isomers in an early synthesis of circulin A.[67] Four of the cysteine residues were protected with a 4-methylbenzyl protecting group, which was removed during the HF cleavage of the peptide from the resin. After formation of the first two disulfide bonds, the remaining two acetamidomethyl-protected cysteine residues were deprotected and oxidized.[67]

The mechanistic details of the folding pathways, i.e., the order in which the various disulfide bonds are formed, have been studied for several cyclotides, including kalata B1[30,79] cO2[60] and MCoTI-II.[75,78] In a few cases, stable two-disulfide intermediates have been identified on the folding pathway. Interestingly, in one case (MCoTI-II), the intermediate is a direct precursor of the final folded product, whereas in the other (kalata B1) it is a kinetic trap that must be shuffled to an alternative form for productive folding.[77] In such studies of cyclotide folding pathways, the ability to make chemical mutants of cyclotides has helped to elucidate the nature of intermediates and pathways. For example, Daly et al. synthesized a di-Ala mutant in which two of the Cys residues were replaced by Ala. This mutant effectively mimicked a two-disulfide intermediate and showed that it had a native-like structure.[79]

Several studies have focused on identifying conditions that favor the folding of cyclotides.[30,60,80] It was noted early on that since cyclotide structures have a small surface-exposed patch of hydrophobic residues, formation of this patch might be favored by doing the folding reactions in a hydrophobic environment. Indeed, this proved to be the case and marked improvements in yields were obtained by conducting the folding reactions in buffers that contained up to 50% isopropanol.[30] Studies have also been reported on the use of a protein disulfide isomerase (PDI) isolated from *O. affinis* to accelerate the folding of kalata B1.[81]

Although Möbius cyclotides appear to fold easily in vitro, the bracelet subfamily has proven more challenging.[60] A recent study of synthetic chimeric cyclotides containing elements of both subfamilies identified two crucial regions thought to be responsible for the difficult folding of bracelet cyclotides in vitro. Simultaneous addition of a Thr in loop 6 and replacement of an Ile in loop 2 with a Gly resulted in a marked improvement in folding yield of the bracelet cyclotide cycloviolacin O1.[82] Both changes make the modified cycloviolacin O1 more like kalata B1, a Möbius cyclotide, but the chimera is still formally a bracelet cyclotide. In another approach to obtaining correctly folded bracelet cyclotides, mis-folded intermediates on the folding pathway of cycloviolacin O2 were identified, and folding conditions were optimized to shuffle them to the native form.[60]

In a more recent folding study, a range of buffer conditions, cosolvents, and redox agents was examined to optimize folding, especially of bracelet cyclotides.[80] It was found that the folding of cyclotides was heavily influenced by the concentration of redox agents, with both the folding rate and yield of native isomers greatly enhanced by high concentrations of oxidized glutathione. As noted previously, the addition of hydrophobic solvents to the buffer also enhanced the folding rates and, in some cases, appeared to alter the folding pathway. In an interesting finding, it was noted that the ability of 2-propanol to stabilize the native form of kalata B1 was also useful in preventing its deamidation. Kalata B1 contains an Asn-Gly sequence that is solvent exposed, consistent with it being the site of backbone cyclization. This makes it susceptible to potential deamidation via formation of a succinimide, followed by ring-opening to form Asp or iso-Asp as illustrated in Figure 10. A detailed HPLC and MS analysis of the folding process found that, for reactions carried out in ammonium bicarbonate buffer lacking isopropanol, additional peaks were found that had a mass one unit higher than the native peptide.[80] MS−MS analysis localized the mass increase to the Asn residue, consistent with conversion of Asn to Asp or iso-Asp. These derivatives constitute the major products under conditions when the folding rates are slow and in the absence of 2-propanol. Thus, besides demonstrating an important stabilizing effect of 2-propanol, the data suggest that deamidation can occur when the peptide is flexible in solution.[80]

BIOSYNTHESIS: FOLLOWING WHAT NATURE DOES?

Cyclotides are biosynthesized via processing from precursor proteins that encode one, two, or three mature cyclotide

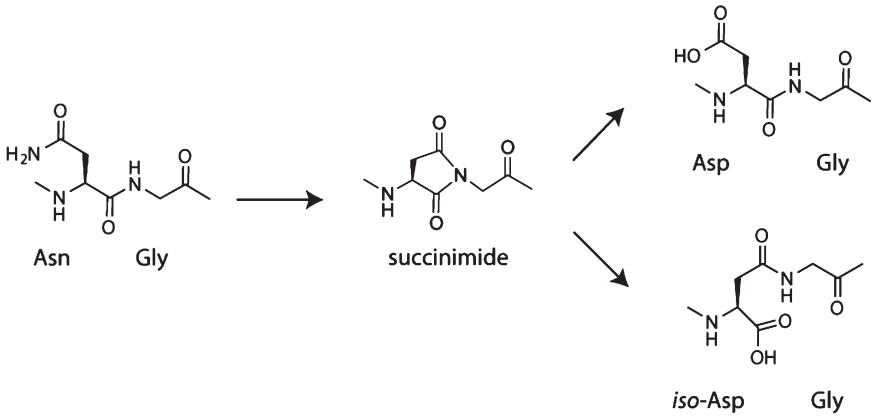


Figure 10. Deamidation of Asn in an Asn-Gly sequence. Peptides, such as kalata B1, containing Asn-Gly sequences are sensitive to deamidation by formation of a succinimide followed by ring-opening to form either Asp or iso-Asp. This reaction occurs spontaneously in solution at neutral pH.

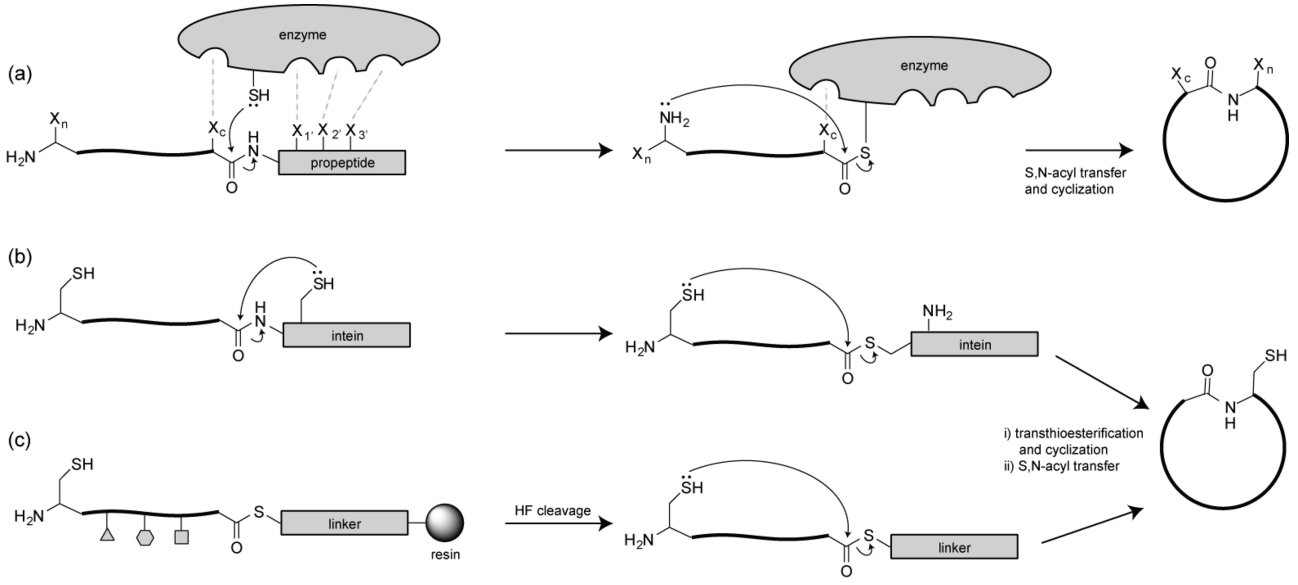


Figure 11. Overview of the common features of enzymatic, intein, and chemical approaches to cyclization. Panel (a) shows the proposed mechanism of cyclotide cyclization.[84] In the first step, an AEP enzyme recognizes the cyclotide precursor substrate, which includes the cyclotide domain (flanked by the N-terminal and C-terminal residues X_n-X_c), and a C-terminal propeptide. X_n is typically Gly, and X_c is universally Asn (or Asp). The latter is important for recognition by AEP. Additional recognition occurs through the first few residues of the C-terminal propeptide domain residues $X_{1'}-X_{3'}$. In many cyclotide sequences, this is the amino acid triplet Gly-Leu-Pro. The acyl-enzyme intermediate that is formed via attack of the active site thiol of the cysteine protease is subsequently resolved by nucleophilic attack from the N-terminal residue of the cyclotide domain, leading to a cyclic peptide containing a native amide bond. Panel (b) summarizes the use of engineered inteins to make cyclotides and other cyclic peptides.[88−91] In this case, a transthioesterification reaction leads to expulsion of the intein unit and cyclization. Panel (c) highlights the parallels of these biological approaches with the NCL approach, which involves a C-terminal thioester.

domains.[33] In some cases, the precursors encode multiple copies of the same cyclotide, and in others, mixtures of different cyclotides are present.[33,34,39] The cyclotide domains are flanked by both N-terminal and C-terminal propeptide sequences. This multiple-cyclotide-per-precursor strategy is consistent with the large number of cyclotides typically produced in a single plant. Although not all of the details of the processing events that excise cyclotides from their precursors are known, recent studies have suggested that certain peptidases, which normally hydrolyze peptide bonds, may also act in "reverse" and catalyze the formation of peptide bonds to form the cyclic peptide backbone.[83−86] In particular, the ubiquitous presence of an Asn (or Asp) residue at the C-terminal processing point of cyclotide precursor proteins has implicated asparaginyl endoproteinase (AEP) in the cyclization of cyclotides.[83,84]

AEP is a cysteine protease that is very common in plants and is involved in a range of proteolytic processing reactions important in plant physiology. It has been proposed that it has fortuitously evolved to also be utilized for cyclotide processing and that cyclization occurs at the same time as cleavage of the C-terminal propeptide from the cyclotide precursor protein.[84,85] Such a transpeptidation would occur by an acyl-transfer mechanism in which the carboxyl group from the cleaved peptide bond is attached to the enzyme to form an acyl-enzyme intermediate as illustrated in Figure 11. Nucleophilic attack of the free amine at the N-terminus of the cyclotide domain on this

dx.doi.org/10.1021/jo200520v |J. Org. Chem. 2011, 76, 4805–4817

acyl-enzyme intermediate then forms a new (cyclizing) peptide bond and releases the peptide from the enzyme. Sortase, another cysteine protease, has a similar acyl-transfer mechanism and has been adapted from its native role of attaching proteins to peptidoglycans in bacteria to be utilized for peptide cyclization.[85,87]

Although native chemical ligation does not involve transpeptidation or the formation of an acyl-enzyme intermediate, it has been termed "biomimetic" on the basis of the acyl transfer from the thioester to the cysteine side chain and finally to the N-terminal amine.[67] Figure 11 highlights some of the parallels between natural enzyme-mediated cyclization and the SPPS chemical ligation approach, as well as another recent approach involving the use of engineered inteins for the production of cyclic peptides in bacterial cells.[88−91] The common theme of all approaches is the attack by a nucleophile (SH or NH_2) residing at the N-terminus of the cyclotide domain on an activated thioester at the C-terminus of the cyclotide domain.

A combination of chemical and native/biomimetic strategies was achieved by a "chemo-enzymatic" synthesis of MCoTI-I.[55] In this approach, the linear precursor was synthesized using Fmoc SPPS and subsequently cyclized using a polymer-supported trypsin.[55] This method was reported to have a higher efficacy than chemical cyclization by NCL, but the specificity of the enzyme means that cyclization can occur only at the Lys recognition site. In MCoTI-II this corresponds to a Lys-Ile bond, as shown in Figure 6. This approach of enzyme-mediated in vitro cyclization followed a previous study in which trypsin was demonstrated to cyclize a linearized version of the cyclic peptide sunflower trypsin inhibitor, SFTI-1.[92] These two studies suggest that protease-mediated cyclization can be a general and efficient process for producing cyclic peptides. In contrast with the in vitro trypsin-mediated reaction for SFTI-I, in the natural biosynthetic mechanism, AEP is thought to be the enzyme involved in cyclization,[93,94] as is the case for cyclotides.

In another biological approach to cyclotide synthesis, Camarero and co-workers achieved the biosynthesis of kalata B1 by expressing linear kalata B1, linked to an "engineered intein" in *E. coli*.[88] Cleavage of the intein by an N,S-acyl transfer resulted in the formation of a C-terminal thioester between the linear kalata B1 precursor and a side-chain thiol on the intein.[88] Cyclization then took place by a native chemical ligation mechanism.[88] It was suggested that the in vivo cleavage of kalata B1 from the intein is promoted by internal cysteine residues and could thus involve a type of "thia-zip" mechanism.[88] This type of approach has been extended to produce libraries of several classes of cyclic peptides, including MCoTI-II and SFTI-I, in cells.[88−91]

APPLICATIONS OF CYCLOTIDES

The natural function of cyclotides is thought to be in plant defense on the basis of their range of pesticidal activities.[11,95,96] For example, kalata B1 causes severe disruption of the gut wall cells of the plant pest *Helicoverpa armigera*, resulting in greatly retarded growth of larvae.[33,96] Other defense-related activities include molluscicidal,[97,98] nematocidal,[99,100] and hemolytic activities.[101] In addition to these defense roles, screening studies have revealed that cyclotides possess a wide range of pharmaceutically relevant biological activities, including uterotonic,[3] anti-HIV,[19] antitumor,[102] and antimicrobial properties.[103] So far, no cyclotide has progressed to clinical development, probably because their potencies in any of these activities are not sufficiently high relative to their toxic activities, which include hemolytic[101] and cardiotoxic[4] effects.

An alternative to using native cyclotides directly as drugs involves modification of an existing cyclotide sequence in order to confer a new bioactivity on the framework. Several recent chemical studies have underpinned such grafting applications. One SPPS study in which all noncysteine residues of kalata B1 were synthetically replaced one at a time by Ala showed that the cyclotide framework was robust to point mutations.[104] The same study found that a cluster of hydrophobic residues was responsible for the insecticidal and hemolytic activity of kalata B1 and that substitution of these residues could be used to remove these toxic activities. In another study, several polar residues were substituted in place of hydrophobic residues in exposed loops of kalata B1 to study the effect of residue substitutions on the folding and hemolytic activity of this cyclotide.[105] This study highlighted the tolerance of the cyclotide framework to substitution and confirmed it was possible to "engineer out" undesired hemolytic activity.

A strategy for cyclotide engineering based on the above findings involves the "grafting" of a bioactive peptide sequence into an existing cyclotide scaffold, thus combining the stability of the cyclotide framework with the bioactivity of the introduced peptide epitope.[11,106] The amenability of cyclotides to chemical synthesis provides an efficient means of generating a library of grafted cyclotides as synthesis can be continued to a common branch point in the SPPS chain assembly, with the resin then divided and the synthesis continued to produce different analogues.[105] The general approach is illustrated in Figure 12.

In a pharmaceutically directed example of this strategy, the cyclotide framework of kalata B1 was shown to be amenable to the grafting of an antiangiogenic peptide epitope.[107] The aim was to produce a molecule that might reduce blood vessel growth in tumors and thus be a useful anticancer agent. When grafted into loop 3, the bioactivity of the epitope was retained.[107] The need for a balance between flexibility and rigidity when selecting a peptide epitope and a site for grafting was highlighted in this study. Whereas rigidity of the grafted loop makes the protein less susceptible to proteolytic cleavage, sufficient flexibility of the epitope must be retained to allow for binding to the desired target.[107] The MCoTI-II framework has also proved attractive for grafting studies, and in a recent example, loop 1 was substituted to produce a micromolar lead with activity against a protease from foot and mouth disease virus.[55] Similar approaches to an exciting range of therapeutic and diagnostic applications have been applied for knottin peptides that are effectively linear versions of cyclotides.[108−110] Interestingly, most acyclic permutants of cyclotides, i.e., linear analogues with the backbone broken in one of the six loops, fold correctly; an early study to explore the role of the cyclic backbone showed that cyclotide backbone loops 2, 3, 5, and 6 were tolerant to chain breakage, and only breaks in loops 1 and 4 resulted in a failure to fold into the native conformation,[111] consistent with their central role in the cystine knot motif.

PERSPECTIVES AND OUTLOOK

Cyclotides have been recognized as a distinct protein family for a little over a decade now, and substantial progress has been made in defining their distribution in the plant kingdom, their biosynthesis, and their biophysical properties. Chemical methods of making cyclotides have been pivotal in understanding their

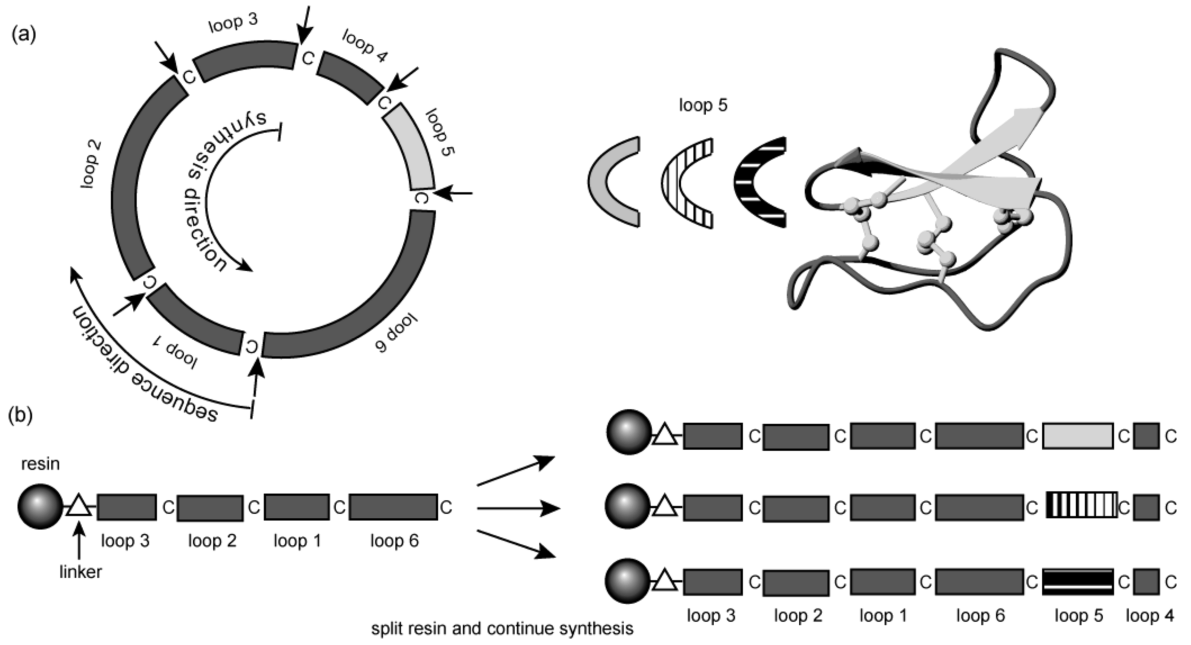


Figure 12. Principles of grafting bioactive peptide sequences into a cyclotide framework. (a) The cyclotide framework can be regarded as a circular arrangement of six loops or "cassettes" that can be substituted with foreign sequences to introduce new biological activities. Practically, the loops are not actually "substituted", but each peptide is synthesized from scratch using solid-phase peptide synthesis (SPPS). Panel (b) schematically illustrates how the synthesis process can be designed for combinatorial variation of individual loops in the sequence. Since chain assembly in SPPS is carried out from the C to N terminus and the synthesis is typically designed to have an N-terminal Cys, any one of six starting points, indicated by arrows in (a), can be used in the synthesis. If one loop is to be combinatorially varied it is desirable to start the synthesis adjacent to this loop so that it is assembled late in the sequence, allowing for the splitting of the solid phase resin and the continuation of a separate synthesis for different sequences in the desired variable loop. In the example shown, loop 5 is the target loop and so synthesis is arranged so that its assembly occurs late in the sequence.

structure—activity relationships and developing their applications as stable peptide templates.

The remarkable stability of cyclotides to chemical, thermal, and enzymatic degradation, coupled with their synthetic accessibility by SPPS, native chemical ligation, and oxidation make them extremely promising potential scaffolds for drug design. More broadly, the lessons learned from cyclotides can be extended to conventional peptides. Many linear peptides have great potential as drug leads, but their susceptibility to proteolysis and poor bioavailability have hindered their application as pharmaceuticals. One strategy for increasing the stability of such peptides is to cyclize the peptide backbone, either directly, or using a peptide linker. As many bioactive proteins have a cystine knot structure, cyclization produces a "pseudo cyclotide", which can have greater stability than the native peptide bearing a cystine knot alone. This strategy has also been used successfully in the cyclization of other disulfide-rich peptides. For example, α-conotoxins have promising efficacy in the treatment of pain, and cyclization has been shown to remarkably improve their biopharmaceutical properties.[112,113]

We believe that organic chemistry will continue to play a significant role in developing new applications of cyclotides, in particular by making use of the sequences and structures that have been determined for natural cyclotides and modifying them using chemical approaches. On the other hand, since cyclotides are produced in high yields in plants, the possibility also exists to use molecular biology approaches to reinsert modified cyclotide genes into plants that can then act as "biofactories" for the biological production of cyclotides for pharmaceutical applications. Similarly, the option to identify a natural cyclotide with insecticidal properties and place the corresponding gene in a plant that does not produce cyclotides opens up the possibility of introducing in-built insecticidal protection into new host plants. We expect that even in these biological applications, chemistry will continue to play a central role by underpinning the exploratory studies that generate new bioactive sequences for pharmaceutical or agricultural applications. Finally, it is worth emphasizing that cyclotides are just one family of head-to-tail cyclic peptides[114] and that it seems likely that more examples of these topologically intriguing molecules will be discovered in coming years.

AUTHOR INFORMATION

Corresponding Author

*E-mail: d.craik@imb.uq.edu.au.

ACKNOWLEDGMENT

Work in our laboratory on cyclotides is supported by grants from the Australian Research Council (DP0984390) and the National Health & Medical Research Council (1009267). D.J.C. is a NHMRC Professorial Fellow (569603). We thank our colleagues listed in the references for their valuable contributions to cyclotide research and many useful discussions. We thank David Wilson for assistance in the preparation of diagrams.

REFERENCES

(1) Craik, D. J.; Daly, N. L.; Bond, T.; Waine, C. *J. Mol. Biol.* **1999**, *294*, 1327.

(2) Colgrave, M. L.; Craik, D. J. *Biochemistry* **2004**, *43*, 5965.
(3) Gran, L. *Medd. Nor. Farm. Selsk.* **1970**, *12*, 173.
(4) Gran, L.; Sandberg, F.; Sletten, K. *J. Ethnopharmacol.* **2000**, *70*, 197.
(5) Gustafson, K. R.; Sowder, R. C. I.; Henderson, L. E.; Parsons, I. C.; Kashman, Y.; Cardellina, J. H. I.; McMahon, J. B.; Buckheit, R. W. J.; Pannell, L. K.; Boyd, M. R. *J. Am. Chem. Soc.* **1994**, *116*, 9337.
(6) Witherup, K. M.; Bogusky, M. J.; Anderson, P. S.; Ramjit, H.; Ransom, R. W.; Wood, T.; Sardana, M. *J. Nat. Prod.* **1994**, *57*, 1619.
(7) Saether, O.; Craik, D. J.; Campbell, I. D.; Sletten, K.; Juul, J.; Norman, D. G. *Biochemistry* **1995**, *34*, 4147.
(8) Gruber, C. W.; Elliott, A. G.; Ireland, D. C.; Delprete, P. G.; Dessein, S.; Goransson, U.; Trabi, M.; Wang, C. K.; Kinghorn, A. B.; Robbrecht, E.; Craik, D. J. *Plant Cell* **2008**, *20*, 2471.
(9) Mulvenna, J. P.; Wang, C.; Craik, D. J. *Nucleic Acids Res.* **2006**, *34*, D192.
(10) Wang, C. K.; Kaas, Q.; Chiche, L.; Craik, D. J. *Nucleic Acids Res.* **2008**, *36*, D206.
(11) Craik, D. J.; Čemažar, M.; Wang, C. K. L.; Daly, N. L. *Biopol. Pept. Sci.* **2006**, *84*, 250.
(12) Chiche, L.; Heitz, A.; Gelly, J. C.; Gracy, J.; Chau, P. T.; Ha, P. T.; Hernandez, J. F.; Le-Nguyen, D. *Curr. Protein Pept. Sci.* **2004**, *5*, 341.
(13) Chen, B.; Colgrave, M. L.; Daly, N. L.; Rosengren, K. J.; Gustafson, K. R.; Craik, D. J. *J. Biol. Chem.* **2005**, *280*, 22395.
(14) Heitz, A.; Hernandez, J. F.; Gagnon, J.; Hong, T. T.; Pham, T. T.; Nguyen, T. M.; Le-Nguyen, D.; Chiche, L. *Biochemistry* **2001**, *40*, 7973.
(15) Hernandez, J. F.; Gagnon, J.; Chiche, L.; Nguyen, T. M.; Andrieu, J. P.; Heitz, A.; Trinh Hong, T.; Pham, T. T.; Le Nguyen, D. *Biochemistry* **2000**, *39*, 5722.
(16) Poth, A. G.; Colgrave, M. L.; Philip, R.; Kerenga, B.; Daly, N. L.; Anderson, M. A.; Craik, D. J. *ACS Chem. Biol.* **2011**, *6*, 345.
(17) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461.
(18) Göransson, U.; Svangard, E.; Claeson, P.; Bohlin, L. *Curr. Protein Pept. Sci.* **2004**, *5*, 317.
(19) Gustafson, K. R.; McKee, T. C.; Bokesch, H. R. *Curr. Protein Pept. Sci.* **2004**, *5*, 331.
(20) Daly, N. L.; Rosengren, K. J.; Craik, D. J. *Adv. Drug. Deliv. Rev.* **2009**, *61*, 918.
(21) Craik, D. J. *Toxicon* **2010**, *56*, 1092.
(22) Ireland, D. C.; Clark, R. J.; Daly, N. L.; Craik, D. J. *J. Nat. Prod.* **2010**, *73*, 1610.
(23) Craik, D. J.; Čemažar, M.; Daly, N. L. *Curr. Opin. Drug Discov. Devel.* **2007**, *10*, 176.
(24) Daly, N. L.; Craik, D. J. *Fut. Med. Chem.* **2009**, *1*, 1613.
(25) Sletten, K.; Gran, L. *Medd. Nor. Farm. Selsk.* **1973**, *7*−*8*, 69.
(26) Gran, L. *Lloydia* **1973**, *36*, 174.
(27) Gran, L. *Lloydia* **1973**, *36*, 207.
(28) Schöpke, T.; Hasan Agha, M. I.; Kraft, R.; Otto, A.; Hiller, K. *Sci. Pharm.* **1993**, *61*, 145.
(29) Claeson, P.; Göransson, U.; Johansson, S.; Luijendijk, T.; Bohlin, L. *J. Nat. Prod.* **1998**, *61*, 77.
(30) Daly, N. L.; Love, S.; Alewood, P. F.; Craik, D. J. *Biochemistry* **1999**, *38*, 10606.
(31) Rosengren, K. J.; Daly, N. L.; Plan, M. R.; Waine, C.; Craik, D. J. *J. Biol. Chem.* **2003**, *278*, 8606.
(32) Göransson, U.; Broussalis, A. M.; Claeson, P. *Anal. Biochem.* **2003**, *318*, 107.
(33) Jennings, C.; West, J.; Waine, C.; Craik, D.; Anderson, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10614.
(34) Dutton, J. L.; Renda, R. F.; Waine, C.; Clark, R. J.; Daly, N. L.; Jennings, C. V.; Anderson, M. A.; Craik, D. J. *J. Biol. Chem.* **2004**, *279*, 46858.
(35) Mulvenna, J. P.; Sando, L.; Craik, D. J. *Structure* **2005**, *13*, 691.
(36) Simonsen, S. M.; Sando, L.; Ireland, D. C.; Colgrave, M. L.; Bharathi, R.; Goransson, U.; Craik, D. J. *Plant Cell* **2005**, *17*, 3176.
(37) Ireland, D. C.; Colgrave, M. L.; Nguyencong, P.; Daly, N. L.; Craik, D. J. *J. Mol. Biol.* **2006**, *357*, 1522.
(38) Herrmann, A.; Burman, R.; Mylne, J.; Karlsson, G.; Gullbo, J.; Craik, D.; Clark, R.; Göransson, U. *Phytochemistry* **2008**, *69*, 939.
(39) Trabi, M.; Mylne, J. S.; Sando, L.; Craik, D. J. *Org. Biomol. Chem.* **2009**, *7*, 2378.
(40) Zhang, J.; Liao, B.; Craik, D. J.; Li, J.-T.; Hu, M.; Shu, W.-S. *Gene* **2009**, *431*, 23.
(41) Craik, D. J.; Daly, N. L. *Mol. Biosyst.* **2007**, *3*, 257.
(42) Wang, C. K.; Hu, S. H.; Martin, J. L.; Sjogren, T.; Hajdu, J.; Bohlin, L.; Claeson, P.; Göransson, U.; Rosengren, K. J.; Tang, J.; Tan, N. H.; Craik, D. J. *J. Biol. Chem.* **2009**, *284*, 10672.
(43) Svangard, E.; Göransson, U.; Smith, D.; Verma, C.; Backlund, A.; Bohlin, L.; Claeson, P. *Phytochemistry* **2003**, *64*, 135.
(44) Sze, S. K.; Wang, W.; Meng, W.; Yuan, R.; Guo, T.; Zhu, Y.; Tam, J. P. *Anal. Chem.* **2009**, *81*, 1079.
(45) Skjeldal, L.; Gran, L.; Sletten, K.; Volkman, B. F. *Arch. Biochem. Biophys.* **2002**, *399*, 142.
(46) Göransson, U.; Craik, D. J. *J. Biol. Chem.* **2003**, *278*, 48188.
(47) Nair, S. S.; Romanuka, J.; Billeter, M.; Skjeldal, L.; Emmett, M. R.; Nilsson, C. L.; Marshall, A. G. *Biochim. Biophys. Acta* **2006**, *1764*, 1568.
(48) Huang, Y. H.; Colgrave, M. L.; Daly, N. L.; Keleshian, A.; Martinac, B.; Craik, D. J. *J. Biol. Chem.* **2009**, *284*, 20699.
(49) Herrmann, A.; Svangard, E.; Claeson, P.; Gullbo, J.; Bohlin, L.; Göransson, U. *Cell. Mol. Life Sci.* **2006**, *63*, 235.
(50) Wang, C. K.; Colgrave, M. L.; Ireland, D. C.; Kaas, Q.; Craik, D. J. *Biophys. J.* **2009**, *97*, 1471.
(51) Göransson, U.; Herrmann, A.; Burman, R.; Haugaard-Jönsson, L. M.; Rosengren, K. J. *ChemBioChem* **2009**, *10*, 2354.
(52) Greenwood, K. P.; Daly, N. L.; Brown, D. L.; Stow, J. L.; Craik, D. J. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 2252.
(53) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.
(54) Tam, J. P.; Lu, Y. A.; Yu, Q. *J. Am. Chem. Soc.* **1999**, *121*, 4316.
(55) Thongyoo, P.; Roqué-Rosell, N.; Leatherbarrow, R. J.; Tate, E. W. *Org. Biomol. Chem.* **2008**, *6*, 1462.
(56) Merrifield, B. *Science* **1986**, *232*, 341.
(57) Atherton, E.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1985**, 165.
(58) Camarero, J. A.; Mitchell, A. R. *Protein Pept. Lett.* **2005**, *12*, 723.
(59) Heidler, P.; Link, A. *Bioorg. Med. Chem.* **2005**, *13*, 585.
(60) Aboye, T. L.; Clark, R. J.; Craik, D. J.; Göransson, U. *ChemBioChem* **2008**, *9*, 103.
(61) Kemp, D. S.; Galakatos, N. G. *J. Org. Chem.* **1986**, *51*, 1821.
(62) Kemp, D. S.; Galakatos, N. G.; Bowen, B.; Tan, K. *J. Org. Chem.* **1986**, *51*, 1829.
(63) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891.
(64) Kemp, D. S.; Carey, R. I. *J. Org. Chem.* **1993**, *58*, 2216.
(65) Liu Chuan, F.; Tam, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 4149.
(66) Camarero, J.; Muir, T. *Chem. Commun.* **1997**, *15*, 1369.
(67) Tam, J. P.; Lu, Y. I. A. N. *Protein Sci.* **1998**, *7*, 1583.
(68) Tam, J. P.; Lu, Y.-A. *Tetrahedron Lett.* **1997**, *38*, 5599.
(69) Hondal, R. J.; Nilsson, B. L.; Raines, R. T. *J. Am. Chem. Soc.* **2001**, *123*, 5140.
(70) Quaderer, R.; Hilvert, D. *Chem. Commun.* **2002**, 2620.
(71) Brik, A.; Ficht, S.; Yang, Y. Y.; Bennett, C. S.; Wong, C. H. *J. Am. Chem. Soc.* **2006**, *128*, 15026.
(72) Zhang, L. S.; Tam, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 2363.
(73) Haase, C.; Seitz, O. *Eur. J. Org. Chem.* **2009**, 2096.
(74) Yu, Q. T.; Lehrer, R. I.; Tam, J. P. *J. Biol. Chem.* **2000**, *275*, 3943.
(75) Čemažar, M.; Daly, N. L.; Haggblad, S.; Lo, K. P.; Yulyaningsih, E.; Craik, D. J. *J. Biol. Chem.* **2006**, *281*, 8224.
(76) Camarero, J. A.; Pavel, J.; Muir, T. W. *Angew. Chem., Int. Ed.* **1998**, *37*, 347.
(77) Camarero, J. A.; Cotton, G. J.; Adeva, A.; Muir, T. W. *J. Pept. Res.* **1998**, *51*, 303.
(78) Čemažar, M.; Gruber, C. W.; Craik, D. J. *Antiox. Red. Sign.* **2008**, *10*, 103.

(79) Daly, N. L.; Clark, R. J.; Craik, D. J. *J. Biol. Chem.* **2003**, *278*, 6314.
(80) Aboye, T. L.; Clark, R. J.; Burman, R.; Roig, M. B.; Craik, D. J.; Göransson, U. *Antiox. Red. Sign.* **2011**, *14*, 77.
(81) Gruber, C. W.; Cemazar, M.; Clark, R. J.; Horibe, T.; Renda, R. F.; Anderson, M. A.; Craik, D. J. *J. Biol. Chem.* **2007**, *282*, 20435.
(82) Gunasekera, S.; Daly, N. L.; Clark, R. J.; Craik, D. J. *Antioxid. Redox Signaling* **2009**, *11*, 971.
(83) Saska, I.; Gillon, A. D.; Hatsugai, N.; Dietzgen, R. G.; Hara-Nishimura, I.; Anderson, M. A.; Craik, D. J. *J. Biol. Chem.* **2007**, *282*, 29721.
(84) Gillon, A. D.; Saska, I.; Jennings, C. V.; Guarino, R. F.; Craik, D. J.; Anderson, M. A. *Plant J.* **2008**, *53*, 505.
(85) Conlan, B. F. G.; A, D.; Craik, D. J.; Anderson, M. A. *Biopolymers* **2010**, *94*, 573.
(86) Cascales, L.; Craik, D. J. *Org. Biomol. Chem.* **2010**, *8*, 5035.
(87) Parthasarathy, R.; Subramanian, S.; Boder, E. T. *Bioconjugate Chem.* **2007**, *18*, 469.
(88) Kimura, R. H.; Tran, A. T.; Camarero, J. A. *Angew. Chem., Int. Ed.* **2006**, *45*, 973.
(89) Austin, J.; Wang, W.; Puttamadappa, S.; Shekhtman, A.; Camarero, J. A. *ChemBioChem* **2009**, *10*, 2663.
(90) Sancheti, H.; Camarero, J. A. *Adv. Drug Deliv. Rev.* **2009**, *61*, 908.
(91) Austin, J.; Kimura, R. H.; Woo, Y. H.; Camarero, J. A. *Amino Acids* **2010**, *38*, 1313.
(92) Marx, U. C.; Korsinczky, M. L.; Schirra, H. J.; Jones, A.; Condie, B.; Otvos, L., Jr.; Craik, D. J. *J. Biol. Chem.* **2003**, *278*, 21782.
(93) Mulvenna, J. P.; Foley, F. M.; Craik, D. J. *J. Biol. Chem.* **2005**, *280*, 32245.
(94) Mylne, J. S. D., N. L.; Colgrave, M. L.; Chanson, A.; Elliot, A. G.; McCallum, E. J.; Jones, A.; Craik, D. J. *Nat. Chem. Biol.* **2011**, *7*, 257.
(95) Gruber, C. W.; Cemazar, M.; Anderson, M. A.; Craik, D. J. *Toxicon* **2007**, *49*, 561.
(96) Barbeta, B. L.; Marshall, A. T.; Gillon, A. D.; Craik, D. J.; Anderson, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 1221.
(97) Plan, M. R.; Saska, I.; Cagauan, A. G.; Craik, D. J. *J. Agric. Food. Chem.* **2008**, *56*, 5237.
(98) Göransson, U.; Sjogren, M.; Svangard, E.; Claeson, P.; Bohlin, L. *J. Nat. Prod.* **2004**, *67*, 1287.
(99) Colgrave, M. L.; Kotze, A. C.; Huang, Y. H.; O'Grady, J.; Simonsen, S. M.; Craik, D. J. *Biochemistry* **2008**, *47*, 5581.
(100) Huang, Y. H.; Colgrave, M. L.; Clark, R. J.; Kotze, A. C.; Craik, D. J. *J. Biol. Chem.* **2010**, *285*, 10797.
(101) Barry, D. G.; Daly, N. L.; Clark, R. J.; Sando, L.; Craik, D. J. *Biochemistry* **2003**, *42*, 6688.
(102) Svangard, E.; Göransson, U.; Hocaoglu, Z.; Gullbo, J.; Larsson, R.; Claeson, P.; Bohlin, L. *J. Nat. Prod.* **2004**, *67*, 144.
(103) Tam, J. P.; Lu, Y. A.; Yang, J. L.; Chiu, K. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8913.
(104) Simonsen, S. M.; Sando, L.; Rosengren, K. J.; Wang, C. K.; Colgrave, M. L.; Daly, N. L.; Craik, D. J. *J. Biol. Chem.* **2008**, *283*, 9805.
(105) Clark, R. J.; Daly, N. L.; Craik, D. J. *Biochem. J.* **2006**, *394*, 85.
(106) Craik, D. J.; Simonsen, S.; Daly, N. L. *Curr. Opin. Drug Discov. Devel.* **2002**, *5*, 251.
(107) Gunasekera, S.; Foley, F. M.; Clark, R. J.; Sando, L.; Fabri, L. J.; Craik, D. J.; Daly, N. L. *J. Med. Chem.* **2008**, *51*, 7697.
(108) Kolmar, H. *Curr. Opin. Pharmacol.* **2009**, *9*, 608.
(109) Jiang, L.; Kimura, R. H.; Miao, Z.; Silverman, A. P.; Ren, G.; Liu, H.; Li, P.; Gambhir, S. S.; Cochran, J. R.; Cheng, Z. *J. Nucl. Med.* **2010**, *51*, 251.
(110) Kimura, R. H.; Cheng, Z.; Gambhir, S. S.; Cochran, J. R. *Cancer Res.* **2009**, *69*, 2435.
(111) Daly, N. L.; Craik, D. J. *J. Biol. Chem.* **2000**, *275*, 19068.
(112) Clark, R. J.; Fischer, H.; Dempster, L.; Daly, N. L.; Rosengren, K. J.; Nevin, S. T.; Meunier, F. A.; Adams, D. J.; Craik, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13767.
(113) Clark, R. J.; Jensen, J.; Nevin, S. T.; Callaghan, B. P.; Adams, D. J.; Craik, D. J. *Angew. Chem., Int. Ed.* **2010**, *49*, 6545.
(114) Craik, D. J. *Science* **2006**, *311*, 1563.