PREFACE

Cyclotides are peptides (mini-proteins) that are characterized by their unusual structural features of a head-to-tail cyclized backbone and a knotted arrangement of three cross-linking disulfide bonds. It is thought that their role in plants is as defense agents, a hypothesis based on their observed activity against certain insects, nematodes, and mollusk's, although it is by no means certain that defense is their primary or indeed only function. So far cyclotides have been discovered in five major families of angiosperms, namely the Violaceae, Rubiaceae, Cucurbitaceae, Solanaceae, and Fabaceae and, accordingly are present in some of the most economically important plant families. However, they are sparsely distributed in the plant kingdom, occurring in only a small fraction of species from all of these families, apart from the Violaceae, where they appear to be ubiquitous. One plant may contain from dozens to hundreds of cyclotides.

Cyclotides originally came to notice based on an observation that a plant (*Oldenlandia affinis*, Rubiaceae) containing the prototypical cyclotide, kalata B1, is used in African folk medicine to accelerate child birth. The discovery that kalata B1 has an unusual cyclic and knotted structure was pivotal in explaining the ability of this peptide to survive boiling to make the medicinal tea. It is this unusual structure and associated exceptional stability that sparked interest in cyclotides as potential frameworks in drug design.

Despite more than 25 years of research since the name "cyclotide" was coined in 1999, so far there have been relatively few studies on cyclotides among plant biologists, with most of the cyclotide literature being focused on their applications in drug design. The overall goal of this book is to promote knowledge of cyclotides among plant biologists, with the aim of encouraging the further study of the functions and distribution of this fascinating family of plant proteins. This book comprises 10 chapters arranged to highlight the progression of cyclotide research from discovery to structure to function and applications.

Chapter 1 provides an overview of the history of the discovery and of cyclotides and identifies some of the milestones associated with their structural characterization, synthesis, bioassay, and applications in drug design. In Chapter 2, Ulf Göransson and colleagues from Uppsala University provide an overview of cyclotides in the Violaceae. As noted already, the Violaceae is the only plant family known in which cyclotides occur in every species examined so far. This observation raises interesting questions about the

evolution and biosynthesis of cyclotides. What is special about the Violaceae compared to other plant families with respect to cyclotides?

Chapter 3 provides a complementary discussion of cyclotides in the Rubiaceae, where the incidence of cyclotide-bearing species is less than 5%. In this chapter, Gruber and colleagues provide some practical protocols for the isolation of cyclotides from this important plant family, analyze their sequence diversity, discuss their evolution, and summarize their bioactive properties. Chapter 4 focuses on cyclotides from Chinese plants with a particular focus on plants with a history of uses in Chinese Traditional Medicines. In particular, Tan and colleagues describe cost- and time-effective detection and extraction methods to assist in the isolation and characterization of new cyclotides from Chinese plants.

Chapter 5 focuses on methods for the determination of the amino acid sequences of cyclotides. Sequencing studies are challenging for cyclic peptides, which by definition lack termini, and given the resistance of native cyclotides to proteolysis, this was one of the reasons why early studies in the 1970s did not successfully sequence cyclotides. However, the fact that all cyclotides have a conserved glutamic acid in loop 1 and may be cleaved by endo-GluC after reduction, allows linearization of the backbone, and hence subsequent sequence analysis. In early studies this was done by Edman sequencing but this has largely been supplanted by Mass spectrometry MS/MS studies.

Chapter 6 continues on the theme of structural analysis but focuses on the 3D structures of cyclotides. Most such studies have been carried out using NMR spectroscopy because cyclotides, like other small disulfide-rich peptides are notoriously difficult to crystallize. The few crystal structures that have been done have confirmed the NMR analyses and show the cyclic cystine knot structure.

Chapter 7 describes the natural functions and structure–activity relationships of cyclotides. Such studies include screening for pharmaceutical and agricultural (i.e., pesticidal) applications of native cyclotides as well as the chemical modifications of cyclotides to develop structure–activity relationships. The underpinning chemical syntheses for these applications are described in the following two chapters. In particular, Chapter 8 describes the biosynthesis of cyclotides. Nature biosynthesizes these fascinating molecules as conventional linear precursor proteins that are subsequently cyclized via asparaginyl endopeptidases (AEPs), which are common plant enzymes that typically perform proteolytic functions. The "repurposing" of AEPs as

ligases is an example of the efficient reuse of an existing enzyme to create a new class of molecules.

Chapter 9 focuses on a range of chemical and biological approaches for the nonnatural synthesis of cyclotides. As noted earlier such technologies have helped to underpin a wide range of structure–activity relationship studies of cyclotides and also a wide range of biotechnological applications as described in Chapter 10. These applications include pesticidal (e.g., insecticidal or nematocidal sprays, or via genetic engineering for insect resistance), pharmaceutical (e.g., for controlling angiogenesis in cancer, antiobesity, multiple sclerosis, antipain, and immunosuppressants), and antifouling and biofilm formation.

Overall, I hope that this book will introduce cyclotides to a new audience and extend research in this area. Although currently there are around 300 sequences published, it has been estimated that the number of cyclotides existing in angiosperms is probably in the tens of thousands. Many questions remain to be studied: Why does one plant produce a suite of many similar cyclotides? Why do different tissues contain different suites of cyclotides? Is pesticidial activity their primary function or might there be other physiological roles for cyclotides? How can these molecules be used in biotechnology applications?

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