Distribution and Chemical Diversity of Cyclotides from Violaceae

Impact of Structure on Cytotoxic Activity and Membrane Interactions

ROBERT BURMAN
Dissertation presented at Uppsala University to be publicly examined in B21, BMC, Husargatan 3, Uppsala, Friday, November 5, 2010 at 09:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in English.

Abstract

During the last decade there has been increased interest in the cyclotide protein family, which consist of a circular chain of approximately 30 amino acids, including six cysteines that form three disulfide bonds, arranged in a cyclic cystine knot motif. This thesis gives new insights in cyclotide distribution and occurrence in the plant family Violaceae, structure-activity relationships for cytotoxic effects, membrane disruption and adsorption on lipid membranes, and evaluates toxicity and anti-tumor activity in vivo.

A large-scale analysis was done on over 200 samples covering 17 of the 23 genera in Violaceae, and cyclotides were positively identified in almost 150 of approximately 900 known species. Conclusions are that the Violaceae is an extremely rich source of cyclotides, and that they are ubiquitous among all species in that plant family.

After investigating the cyclotides' cytotoxicity it was evident that the effects were immediate and occurred at low micromolar concentrations. To understand the relationships between structure and activity, approximately 30 cyclotides and cyclotide derivatives were assayed for cytotoxicity. Results showed that the overall charge is of minor influence on activity and revealed a strong correlation between an intact hydrophobic molecular surface and cytotoxic effect.

The cytotoxic activity is mainly due to interactions between peptides and target membranes, illustrated by prototypic cyclotides' ability to induce liposome leakage and adsorb to lipid membranes. Cyclotides were strongly lytic against zwitterionic liposomes, less when cholesterol was included, while for anionic liposomes, activity depend on the net charge of cyclotide. A similar pattern was observed for the adsorption of the cyclotides to anionic bilayers, in which strong lytic activity was coupled with high adsorption.

To further evaluate cyclotides cytotoxic effects, in vivo studies were conducted, both for acute toxicity and anti-tumor efficacy in mice. Two different methods were used: hollow fiber method and traditional xenografts, but no significant anti-tumor effects were detected. The results indicate that anti-tumor effects are minor or absent at tolerable doses and that cyclotides have a very abrupt in vivo toxicity profile, with lethality after single injection at 2.0 mg/kg.

Keywords: Cyclotide, Violaceae, Viola, Cytotoxic, Mechanism, In vivo, Structure-activity, Membrane, Mass spectrometry

Robert Burman, Department of Medicinal Chemistry, Division of Pharmacognosy, Box 574, Uppsala University, SE-75123 Uppsala, Sweden.

© Robert Burman 2010

ISSN 1651-6192
urn:nbn:se:uu:diva-131104 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-131104)
The Violet

Down in a green and shady bed,
A modest violet grew;
Its stalk was bent, it hung its head
As if to hide from view.

And yet it was a lovely flower,
Its colour bright and fair;
It might have graced a rosy bower,
Instead of hiding there.

Yet thus it was content to bloom,
In modest tints arrayed;
And there diffused a sweet perfume,
Within the silent shade.

Then let me to the valley go
This pretty flower to see;
That I may also learn to grow
In sweet humility.

Jane Taylor (1783-1824)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with permission from the respective publishers.
Contents

1. Introduction ...................................................................................................................... 11
   1.1 Pharmacognosy ............................................................................................................. 11
   1.2 History of cyclotide discovery .................................................................................... 12
   1.3 Structure and sequence ............................................................................................. 12
   1.5 Distribution and occurrence ....................................................................................... 15
   1.6 Biosynthesis ............................................................................................................... 15
   1.7 Biological activity ........................................................................................................ 16

2. Aims .................................................................................................................................. 18

3. Cyclotides of the Violaceae ............................................................................................. 19
   3.1 Extraction and isolation .............................................................................................. 20
   3.2 Sequence determination .............................................................................................. 21
   3.3 Novel cyclotides .......................................................................................................... 22
   3.4 Distribution of cyclotides in Violaceae ....................................................................... 24
   3.5 Stability of cyclotides for almost 200 years ............................................................... 28

4. Structure-activity relationships ......................................................................................... 29
   4.1 The fluorometric microculture assay .......................................................................... 29
   4.2 Cytotoxic activity of native cyclotides ........................................................................ 30
   4.3 Effects of charged and hydrophobic residues ................................................................ 33
   4.4 Additive effects of cyclotide mixtures ....................................................................... 35

5. Mechanistic studies on cyclotides .................................................................................... 37
   5.1 Kinetics of the cytotoxic effect .................................................................................. 37
   5.2 Membrane disruptive effects ..................................................................................... 39
   5.3 Membrane adsorption ............................................................................................... 41
   5.4 Mode of action ............................................................................................................ 44

6. Toxicity and antitumor activity in vivo ........................................................................... 45
   6.1 Toxicity in mice ............................................................................................................ 45
   6.2 Hollow fiber assay ....................................................................................................... 45
   6.3 Xenograft studies ........................................................................................................ 48

7. Discussion and future perspectives .................................................................................. 50
   7.1 Isolation and structure elucidation .............................................................................. 50
   7.2 Cyclotide evolution and occurrence .......................................................................... 52
   7.3 Toxicity and anti-tumor activity .................................................................................. 53
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK</td>
<td>cyclic cystine knot</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CF</td>
<td>carboxyfluorescein</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DOPA</td>
<td>1,2-dioleoyl-$sn$-glycero-3-phosphate</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-$sn$-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FDA</td>
<td>fluorescein diacetate</td>
</tr>
<tr>
<td>FMCA</td>
<td>fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>inhibitory concentration 50%</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>MGG</td>
<td>May-Grünwald-Giemsa</td>
</tr>
<tr>
<td>MOA</td>
<td>mode of action</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTD</td>
<td>maximum tolerated dose</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-di-methylthiazol-2-yl)-2,3-diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTR</td>
<td>N-terminal repeat</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data base</td>
</tr>
<tr>
<td>RP</td>
<td>reversed phase</td>
</tr>
<tr>
<td>SI</td>
<td>survival index</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
</tbody>
</table>
Three and one letter codes, polarity (or charge) for the amino acids mentioned in the thesis.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>3 letter code</th>
<th>1 letter code</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
<td>positively charged</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
<td>negatively charged</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
<td>negatively charged</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>H</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
<td>positively charged</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>S</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
<td>hydrophobic</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Pharmacognosy

Pharmacognosy is an interdisciplinary subject focusing on medicines from natural sources. The American Society of Pharmacognosy defines it as "the study of the physical, chemical, biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources". Pharmacognosy is derived from the two Greek words pharmakon, meaning drug, and gnos, meaning knowledge. The term was coined by Johann Adam Schmidt (1759-1809) and is mentioned in his textbook “Lehrbuch der Materia Medica” published posthumously in 1811. In the 19th century, the use of pharmacognosy spread throughout the German-speaking areas of Europe, while other countries often kept the older term Materia Medica.

At Uppsala University, Sweden, in the 18th century, Professor Carolus Linnaeus (Carl von Linné) was responsible for teaching Materia Medica. He is best known for introducing a new, binomial system for naming and classifying plants; a key contribution to science. After his death, the responsibility for teaching Materia Medica was passed to succeeding professors until 1851 when the last Linnean professor, Göran Wahlenberg, died. The chair was then divided into many parts, raising uncertainty about which professor should be regarded as the current incumbent of the Linnean chair. Some of the responsibilities were assigned to a new chair in pharmacognosy, pharmacy, physiology and pathological chemistry (Chemiae Medicae et Physiologiae Professor). The term Materia Medica was consequently discarded and replaced by the newer term pharmacognosy. The subject was taught to students for another 100 years before it almost disappeared as a subject at Uppsala University.

Active interest in the subject was although found in other places in Sweden, e.g. Royal Pharmaceutical Institute in Stockholm, which in 1968 moved and became part of Uppsala University and transformed into the Faculty of Pharmacy. Once again, Uppsala had a professor in pharmacognosy, Finn Sandberg, who together with his successors Gunnar Samuelsson and Lars Bohlin have continued, developed and modernized the subject.
1.2 History of cyclotide discovery

In 1965, the professor in pharmcognosy at Uppsala University, Finn Sandberg reported the traditional use of plants after an expedition in the Central African Republic. He saw that the natives used decoctions of the plant *Oldenlandia affinis*\(^1\) to facilitate childbirth (Sandberg, 1965). Some years later in the 1970s, a Red Cross doctor, Lorens Gran, observed that tribes in Congo (Zaire) used the plant and brought samples home to Norway for identification and investigation. He discovered that polypeptides in the plant had remarkably strong uterotonic activity. The main active component was almost completely sequenced and called kalata B1, after the native name of the plant `Kalata-Kalata’ (Gran, 1973b; Sletten, 1973).

The structure of kalata B1 was not fully elucidated until 1995, when its circular backbone and knotted arrangement of disulfide bonds was clarified by nuclear magnetic resonance (NMR) analysis (Saether, 1995). At around this time three different groups independently published studies of macrocyclic peptides with six cysteine residues from the plants *Viola arvensis*, *Chassalia parvifolia* and *Psychotria longipes* (Schöpke, 1993; Gustafson, 1994; Witherup, 1994) The group of described macrocyclic peptides with a cystine knot grew over the following years and a collective term for them was suggested, cyclotides after *cyclo-peptides* (Craik, 1999).

In 1995, the Division of Pharmacognosy initiated a discovery project aimed at plant polypeptides (in the 10-50 amino acid residue size range) with drug development potential or use as pharmacological tools. A fractionation protocol, for the isolation of a highly purified polypeptide fraction from plant biomass was developed (Claeson, 1998). Using this protocol cyclotides was found (Göransson, 1999; Göransson, 2004b) and hence the project re-connected to Finn Sandbergs’ expedition in Africa three decades earlier. Since then focus have been on understanding the cyclotides’ biological functions and to explore their possible use in biotechnological, pharmaceutical and agricultural applications.

1.3 Structure and sequence

Cyclotides are an exceptional family of gene-encoded plant proteins, in that they are cyclic, *i.e.* their N and C termini are joined by a peptide bond, forming a continuous circular backbone. The circular cyclotide chain consists of approximately 30 amino acids, including six Cys residues that form three disulfide bonds arranged in a cyclic cystine knot (CCK) motif (Figure 1)

---

\(^1\) Auctor names are not included in the Latin plant names throughout this thesis. They can be found in the original papers.
The unique cyclotide structure forces hydrophobic residues to be exposed on the surface of the protein making them amphipathic proteins.

The remarkable CCK motif makes cyclotides extremely resistant to enzymatic, chemical and thermal degradation (Colgrave, 2004) and ideal for developing cyclotide based peptides with diverse medical and agricultural applications (Camarero, 2007; Craik, 2007; Kolmar, 2009), especially following recent advances in methods to synthesize and fold cyclotides with correct conformations (Cemazar, 2008; Leta Aboye, 2008; Aboye, 2010).

**Figure 1.** Schematic structure of a Möbius and bracelet cyclotide, together with typical cyclotide sequences from both subfamilies. The structures are based on the protein database (PDB) files 1NB1 and 2KNM. The abbreviated notation cyO2 and cyO19 stand for the cycloviolacins O2 and O19, respectively. Note the unique features of the CCK motif: a cyclic backbone with sequence loops (1-6) and three stabilizing disulfide bonds. These disulfides are arranged in a cystine knot, that is two of the disulfides form a ring structure together with the backbone connecting the four cysteines (I-IV; II-V), while the third disulfide is threaded through the ring (III-VI).

To date, nearly 200 cyclotides have been described, and they are divided into two main subfamilies, the Möbius and the bracelet subfamilies. They are characterized by the presence or absence, respectively, of a $cis$-Pro peptide linkage (Craik, 1999). The subfamilies also differ in size and amino acid contents, the bracelets being the more structurally diverse of the two; to date according to Cybase (the database of cyclic proteins) (Mulvenna, 2006b; Wang, 2008b), 2/3 of the known cyclotides belong to the bracelet subfamily, and the rest to the Möbius subfamily.

Some residues are found in all/most cyclotides, the strictly conserved Cys residues with the intermediate residues defined as loops (Figure 1), a Glu residue in loop 1, and a Gly-Asn/Asp sequence in loop 6 (residues involved in the post-translational ring closure (Jennings, 2001)). The rest of the residues are changeable, and although there are relatively few amino acids in a cyclotide sequence, variations are immense.
The connectivities of the six Cys residues have been debated and can theoretically form 15 possible variants. The real conformation has been deduced from NMR spectra (Saether, 1995; Rosengren, 2003; Nair, 2006), and verified by chemical proofs achieved by partial reduction of the disulfides with stepwise alkylation (Göransson, 2003b) and recently by X-Ray crystallography (Wang, 2009b). The consensus conclusion from these studies is that they connect as follows: CysI-CysIV, CysII-CysV and CysIII-CysVI forming the cystine knot.

A few atypical cyclotides have also been isolated; some lacking the conserved Asn/Asp in loop 6 and instead having a Lys that prevents cyclization, making them “linear cyclotides” (Ireland, 2006b; Gerlach, 2010) and a few others that have features from both subfamilies, making them hybrid cyclotides. However, the latter lack the cis-Pro bond in loop 5 and hence should be regarded as bracelets. In the curbit (Cucurbitaceae) plant family many trypsin-inhibiting proteins with a cystine knot have been isolated; two of which are circular and thus fulfill the criteria for inclusion in the cyclotide family (Hernandez, 2000). However, they have more sequence similarity with their linear counterparts than to cyclotides, another circulation point, and are hence regarded as cyclic knottins (Chiche, 2004). A sequence similarity plot is shown in Figure 2, illustrating the clustering of subfamilies of cyclotides.

![Image](Figure 2. Sequence similarity plot of proteins with the CCK motif. The main two subfamilies of cyclotides are divided into two separate groups with the Möbius/bracelet hybrid group of cyclotides between them. The cyclic trypsin inhibitors fall into a separate branch.)
1.5 Distribution and occurrence

Since the discovery of the first cyclotide-containing plant, *Oldenlandia affinis*, cyclotides have been isolated from approximately 40 plant species. Patterns in occurrence are beginning to be unraveled and most described cyclotides have been isolated from the violet (Violaceae) and coffee (Rubiaceae) families. Although Rubiaceae is a large family of plants, with 600 genera and over 13,000 species, cyclotides have only been found in a minority of species in this family, with the distribution focused to a few tribes (Gruber, 2008). The Violaceae family includes 22 genera and approximately 930 species, predominantly tropical, growing as perennial herbs, shrubs, and trees or treelets (Hekking, 1984; 1988). Cyclotides have been found in many species within the Violaceae (Göransson, 1999; Hallock, 2000; Broussalis, 2001; Göransson, 2003a; Chen, 2005; Wang, 2008a; Zhang, 2009), and the family can be regarded as a very rich source of cyclotides.

Those two plant families are phylogenetically distant and thus this scattered occurrence of cyclotides is remarkable, and the reason is yet unknown. However, their distribution in the plant kingdom is probably wider than the current knowledge suggests, and screenings are already in progress in attempts to find cyclotides in other plant families. Outside the Violaceae and Rubiaceae, cyclotide-like gene sequences have been identified in cereal crops of Poaceae, such as wheat, maize and rice (Basse, 2005; Mulvenna, 2006a), but no expressed cyclotides have been detected in these plants. A recent screening has also found that members of a family closely related to the Rubiaceae, Apocyanaceae, contain small proteins with six Cys residues, which is a good indication of cyclotide content warranting further investigation (Gruber, 2008).

1.6 Biosynthesis

Cyclotides comprise one of few classes of natural macrocyclic gene products discovered to date (Trabi, 2002; Daly, 2009). Analysis of cyclotide precursor sequences obtained from cDNA have shown that the genes encoding them consist of an endoplasmic reticulum (ER) signal domain, a pro-region and one to three mature cyclotide domains, each proceeded by an N-terminal repeat (NTR) sequence (Jennings, 2001; Simonsen, 2005; Herrmann, 2008; Zhang, 2009). Figure 3 shows the cleavage points of a schematic cyclotide after a Lys/Gly/Asn residue in the NTR sequence and the Asn or Asp in the cyclotide domain. Details of the processing of the precursors, including the order of the events, are not fully understood, but involve oxidative folding, excision of the mature cyclotide sequence and head-to-tail cyclization.

An asparaginyl-endoproteinase has been suggested to be involved in cleavage of the C-terminal tail and simultaneous cyclization of the cyclotide.
domain, at least for the prototypic cyclotide kalata B1 (Saska, 2007; Gillon, 2008). Additionally, a protein-disulfide isomerase seems to play a major role in the oxidative folding of cyclotides through re-shuffling (isomerization) of disulfide bonds (Gruber, 2006; Gruber, 2007).

**Figure 3.** Biosynthesis and structure of cyclotides. Cyclotides are synthesized as precursor proteins, with a conserved endoplasmic reticulum (ER) signal region, a pro-region, an N-terminal repeat (NTR) signal, the mature cyclotide sequence and a short C-terminal tail. The NTR and cyclotide region can be repeated up to three times in different precursors, encoding different or identical cyclotides. The arrow below the ER-signal indicates a highly conserved region that has been used (e.g. Paper I) as a target for a degenerative primer encoding for the sequence AAFALPA.

### 1.7 Biological activity

In the early 1990:s, a series of independent reports were published describing cyclotides discovered in bioassay-guided isolations; including the hemolytic violapeptide I (Schöpke, 1993), the neurotensin-binding inhibitor cyclopsychotride A (Witherup, 1994), and the circulins A-B with anti-HIV properties (discovered in efforts supported by the National Cancer Institute (NCI) of America) (Gustafson, 1994). In subsequent assays cyclotides have shown activities in the low micromolar range against a wide range of pests and other organisms; insecticidal effects against *Helicoverpa punctigera* and *H. armigera* larvae (Jennings, 2001), golden apple snails and Nile tilapia fish (Plan, 2008), nematode parasites of sheep (Colgrave, 2008), human hookworms (Colgrave, 2009), and the inhibition of barnacle larvae from settling (Göransson, 2004a). A set of cyclotides, from both subfamilies, has also shown activity against human pathogens including *Escherichia coli*, *Klebsiella oxytoca*, *Staphylococcus aureus* and *Candida kefyr* (Tam, 1999). In contrast, the bracelet cycloviolacin O2 reportedly has only low activity against Gram-positive bacteria, *e.g.* *Staphylococcus*, but potent effects against Gram-negative bacteria and none of the Möbius cyclotides tested have strong effects against any tested bacteria (Pränting, 2010). All these pesticide and anti-pathogen effects support the hypothesis that cyclotides are components of the plant defense systems.
In addition, cytotoxic effects of cyclotides have been reported, initially by members from our laboratory, who found that three cyclotides (varv A, F and cycloviolacin O2) have potent activity against a panel of ten human tumor cell lines. Furthermore, their activity profiles were weakly correlated to those of anticancer drugs in clinical use today (Lindholm, 2002), suggesting that they have a different mode of action (MOA). Since then additional cyclotides have been tested for cytotoxicity (Svangård, 2004; Herrmann, 2008; Gerlach, 2010). Recent studies have shown that cyclotides interact with cell membranes (Huang, 2009), an interaction that is thought to be the main cause of their cytotoxic properties (Svangård, 2007), and may also explain some of their other biological effects.
2. Aims

The work presented in this thesis was part of a research project concerning plant proteins focusing on cyclotides at the Division of Pharmacognosy, Department of Medicinal Chemistry, Uppsala University. The long-term aims of the project are to understand the biological functions of this unique protein family and to explore their possible use in biotechnological, pharmaceutical and agricultural applications.

The specific objectives of the work this thesis is based upon were:

• To further refine analytical and preparative methods and characterize novel cyclotide proteins and precursors in an attempt to understand cyclotide biosynthesis and structural diversity more clearly.

• To map, at large-scale, cyclotide occurrence and distribution in the Violaceae.

• To understand structure-activity relationships of cyclotides, particularly regarding charged and hydrophobic residues.

• To obtain more insights into mechanisms of cyclotides’ activities by investigating their cytotoxicity, membrane-disrupting ability and membrane interactions.

• To determine cyclotides’ properties in tumor cell models in vitro and conduct preliminary studies in vivo to evaluate their toxicity and anti-tumor activity.
3. Cyclotides of the Violaceae

This chapter explains the procedures used for the extraction, isolation and sequence determination of cyclotides in Paper I-VI, summarizes the novel cyclotides found and discuss the distribution and occurrence of cyclotides in Violaceae. The last part illustrates the cyclotides extreme stability through a study of 190-year old plant material.

Despite extensive screenings for cyclotides, they have mainly been found in two phylogenetically distant families: the Violaceae and the Rubiaceae. In Paper I-II the focus was on the Violaceae, which consists of 23 genera and approximately 900 species worldwide. Although the Violaceae has been identified as one of the major sources of cyclotides and cyclotide diversity, only a limited number of species and genera have been examined prior to these studies. Further, the majority of studies have focused on plants of the Viola and Hybanthus genera (e.g. Broussalis, 2001; Simonsen, 2005; Ireland, 2006b; Herrmann, 2008) and there have been few investigations of their occurrence in other genera of the Violaceae (Hallock, 2000; Trabi, 2009).

![Schematic diagram](image)

**Figure 4.** Schematic overview of the procedures to detect novel cyclotides. To first evaluate the cyclotide content of a plant, an aqueous extract is made and analyzed using by liquid chromatography-mass spectrometry (LC-MS). After isolation of pure cyclotides by HPLC, the cysteines are reduced and alkylated then enzymatically digested, yielding in linear products that can be sequenced by MS-MS. The sequence determination can be elusive but in combination with amino acid analysis full sequence coverage are usually obtained. To obtain a complete 3-D structure NMR spectroscopy or X-Ray crystallography can be used.
In a contribution to map cyclotide biosynthesis, structural diversity and distribution in Violaceae more comprehensively, the focus was first on two species of the genus *Gloeospermum* (Paper I) followed by a large-scale screening of over 200 samples (15% of the species) in the Violaceae (Paper II). In the attempt to discover novel cyclotides a procedure has been generated over the years that was followed in these studies (Figure 4).

3.1 Extraction and isolation

The early methods for extracting and fractionating cyclotides from plant biomass were time- and resource-consuming, and established to isolate polypeptides in general (Claeson, 1998). Over time, knowledge has grown regarding the cyclotides and new methods have been incrementally developed, so current procedures are much simpler and adapted (Svangård, 2003; Göransson, 2004b; Svangård, 2004; Herrmann, 2008). For example, the extraction procedure has changed from a pre-extraction with dichloromethane, followed by extraction with 50% aqueous ethanol to extraction with 60% aqueous methanol, with subsequent liquid-liquid extraction against dichloromethane. From the beginning the protocol also included polyamide filtration and size-exclusion chromatography, which was shown to be unessential, and thus removed.

In the present work experimental procedures include: a preliminary overview of the cyclotide contents before proceeding to large-scale extraction (Paper I, III) or for screening purposes (Paper II), a fast small-scale extraction using only milligram quantities of plant material was performed. Following reversed-phase solid phase extractions (RP-SPE) the extract was injected into a C18 column connected to a liquid chromatography-mass spectrometry (LC-MS) system, with a linear gradient from 10 to 60% aqueous acetonitrile in 0.05% formic acid. The cyclotides in the samples were detected by their late retention times and molecular masses between 2.8 and 3.8 kDa.

In Papers I and III-VI, samples were extracted with 60% methanol, and then defatted by partitioning against dichloromethane. The aqueous phase was after subsequent RP-SPE, injected into a C18 column connected to a high performance liquid chromatography (HPLC) system, with a linear gradient from 10 to 60% aqueous acetonitrile in 0.05% trifluoroacetic acid. Pure cyclotides were obtained by repetitive RP-HPLC. The procedure used in Paper II is described in Chapter 3.4.
3.2 Sequence determination

Cyclotide sequence determination can be elusive and full sequence coverage usually requires the combination of different methods. Today, the main method to analyze sequences is by MS-MS, which is a rapid method, and gives detailed characterization of polypeptides. Cyclotide sequences can be obtained through other means than traditional peptide isolation. An approach to sequence cDNA clones of cyclotide precursors and thereby characterize cyclotide content has been successful in Paper I.

3.2.1 MS-MS sequencing

In Papers I-VI, MS-MS determined the sequences of each isolated cyclotide. In order to sequence cyclotides by MS-MS, their disulfide bonds have to be broken and they must be linear. Hence, the disulfides were reduced with dithiothreitol. The free thiols were subsequently S-carbamidomethylated by iodoacetamide. That reaction is also an effective way to confirm the presence of six cysteines (i.e. three disulfide bonds) in a cyclotide, since it increases their mass by 348 Da (6 x 58 Da).

The cyclotides were then enzymatically cleaved targeting the conserved Glu residue in loop 1 using endoproteinase GluC, which usually resulted in a single product, thus also establishing the presence of a cyclic backbone, with mass increase of 18 Da. To obtain shorter, more readily interpretable peptides other enzymes such as trypsin or chymotrypsin were used. There is an inherent problem in distinguishing between isobaric residues (with same mass), i.e. Leu and Ile, using MS-MS. Use of chymotrypsin can sometimes solve the problem (e.g. for vodo O in Paper III) since it cleaves sequences after Leu and not Ile, however if Leu is followed by a Pro cleavage is impossible and hence the results are difficult to interpret.

Reduced, S-carbamidomethylated and enzymatically digested samples were dissolved in 50% methanol, 1% formic acid and analyzed by nanospray MS-MS using a Protana NanoES source mounted on a Thermo Finnigan LCQ ion trap MS. Alternatively, the samples were injected by a syringe through a PicoTip® emitter connected to a Waters Q-Tof Micro™.

3.2.2 Amino acid analysis

To confirm the novel sequences obtained from the MS-MS analysis of globa C, glopa A-C (Paper I) and vodo O (Paper III), they were sent for amino acid composition analysis at the Amino Acid Analyses Centre, Department of Biochemistry and Organic Chemistry, Uppsala University. The proteins were hydrolyzed for 24 h at 100°C with 6 N HCl containing 2 mg/mL phenol, and the hydrolyzates were analyzed using an LKB model 4151 Alpha Plus amino acid analyzer with ninhydrin detection.
3.2.3 Cyclotide precursor screening

In **Paper I**, novel cyclotide sequences were identified through both protein isolation and screening for cDNA clones encoding cyclotide precursors. Such screening not only serves as a complementary method for identifying novel cyclotide sequences, but also provides information on precursor sequences and helpful clues regarding cyclotide biosynthesis.

RNA was extracted from the two investigated species, *Gloeospermum pauciflorum* and *Gloeospermum blakeanum*, using an RNA Aqueous Qiagen® kit and cDNA was prepared from the RNA using an Omniscript RT Kit, following the manufacturer’s recommended protocols. Clones were amplified using oligo(dT) with degenerative forward primer based on the protein sequence AAFALPA in the ER signal region in the cyclotide precursor region (**Figure 4**, on page 19). The resulting PCR products (approximately 600 bp) were purified by gel electrophoresis and cloned into a vector using the TOPO Cloning Kit then transformed into *E. coli* cells. Plasmid DNA was then extracted, purified and sent for sequencing.

3.3 Novel cyclotides

In attempts to map the structural diversity and distribution of cyclotides in the Violaceae, an extensive analysis of a broad collection of samples was conducted from various members of the family. **Paper I** focuses on two species of the genus *Gloeospermum* that yielded in six protein sequences (three from each species), and ten mRNA products encoding putative cyclotides. Intriguingly, only two of the transcript sequences matched any of the isolated protein sequences, and only one sequence had been previously described (vibi E). Hence, 12 of the sequences were novel; five of which were found in *G. pauciflorum* (designated glopa A-E), and seven in *G. blakeanum* (designated globa A-G).

The results showed that it is still possible to find undescribed cyclotides in intensively investigated plants. Notably, we found a late-eluting peak in the RP-HPLC analysis of extracts of *Viola odorata*, one of the most intensively investigated plant in terms of cyclotide contents (Craik, 1999; Svangård, 2003; Dutton, 2004; Ireland, 2006a; Ireland, 2006b; Colgrave, 2010). The cyclotide responsible for this peak was isolated, sequenced and found to be novel and given the name vodo O (**Paper III**).

In **Paper II**, large-scale chemical screening of species covering 15% of the species in Violaceae was conducted. Although only the small amounts of plant material were available (10-100 mg) it was possible to isolate and sequence 12 novel cyclotides. The total number of known cyclotides is now becoming so large (around 200) that a new naming system is required. The
problem is likely to increase exponentially as the number of new sequences increases in the near future.

In total, Paper I-III describes 25 novel cyclotide sequences, as listed in Table 1. The naming of the cyclotides follows a proposed system in which a trivial name for a cyclotide is constructed as an indicative and pronounceable acronym of the Latin binomial of the plant from which it was first isolated, followed by a letter indicating its order of discovery (Broussalis, 2001).

Table 1. Alignment of the amino acid sequences of the 25 novel cyclotides from Papers I-III.

<table>
<thead>
<tr>
<th>Cyclotide</th>
<th>Sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globa A¹</td>
<td>G--IP--CGESCVFIP--CITAA--IGCSCKT--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa B¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa C²</td>
<td>A--P--CGESCVFIP--CITAA--IGCSCKT--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa D¹</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa E¹</td>
<td>GSAFG--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa F¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa G¹</td>
<td>GGVIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa A²</td>
<td>GGSLP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa B²</td>
<td>GGSLP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa C²</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa D¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa E¹</td>
<td>GGSVP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa F¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa G¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa A²</td>
<td>GGSVP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa B²</td>
<td>GGSVP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa C²</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa D¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa E¹</td>
<td>GGSVP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa F¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Globa G¹</td>
<td>G--VIP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Hobo A</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Hyden A³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Memæ A³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Memæ B³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Orto A³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Rigæ A³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Rili A³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Rili B³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Vide A³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Vini A³</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
<tr>
<td>Vodo O</td>
<td>G--IP--CGESCVFIP--CISAV--LGCSCKS--KVCYRN</td>
<td>I</td>
</tr>
</tbody>
</table>

¹Sequences predicted from cDNA precursor clones (Globa A-B was also found as proteins).
²The number of L and I was determined by amino acid analysis. Placements of the isobaric L/I are determined assuming homology to other cyclotides.
³Amino acid analysis was not performed and MS sequencing does not discriminate between L and I so both residues are here represented as L.
3.4 Distribution of cyclotides in Violaceae

Most of the Violaceae genera contain a small number of species or are monotypic and restricted to the New World or Old World tropics. The three largest genera (*Viola*, *Hybanthus* and *Rinorea*) collectively include more than 90% of the species. The largest of these three genera is *Viola*, the “true violets” that are characteristically herbs with bilaterally symmetrical, spurred flowers. The other genera have radially symmetrical flowers and are lianas, shrubs, or either large or small trees.

As shown in Figure 5, Hekking subdivides the Violaceae into three subfamilies, two monotypic ones, containing the genera *Fusispermum* and *Leonia* respectively, and the third, the Violoideae, containing the majority of the genera (Hekking, 1984; 1988). Within the latter subfamily, Hekking recognizes the essentially actinomorphic-flowered tribe Rinoreeae, and the zygomorphic-flowered Violeae, which includes the largest genus, *Viola*.

![Figure 5](image)

**Figure 5.** Classification and distribution of species in Violaceae. Systematics of Violaceae, according to Hekking (Hekking, 1984; 1988), with minor revisions by Munzinger (Munzinger, 2003). The number of recognized species positively identified to contain cyclotides in Paper II is tabulated next to the total number of species. The genera containing isolated and sequenced cyclotides are indicated by bullet points (•).
In **Paper II**, an examination was performed on the distribution of cyclotides in the Violaceae in depth. A large-scale chemical analysis was conducted on 143 species representing 17 genera from all parts of the world. The sampling was made in attempts to cover all parts in the phylogenetic tree of the Violaceae.

Plant material was kindly provided by three of the major herbaria in Sweden, namely those located in Uppsala, Gothenburg and Stockholm, each of which possesses outstanding plant collections. As only a limited amount of plant material could be sampled from each herbarium sheet (5-100 mg), a new approach to extract and identify cyclotides had to be developed, which needed to be simple, fast and sensitive.

The plant material was first extracted with 60% aqueous acetonitrile, diluted and then the cyclotides were captured on a C18-SPE column. The eluate was freeze-dried, re-dissolved to a concentration proportional to the original amount of plant material (10 μL/mg) and analyzed using LC-MS. Following this procedure it was possible to obtain robust LC-MS results using an amount of extract corresponding to only 1 mg of plant material. However, for practical reasons, and to allow repeat analyses, at least 10 mg of plant material was extracted, corresponding to less than 1 cm² of leaf material. **Figure 6** shows base peak chromatograms from six typical samples.

**Figure 6.** Base peak chromatograms (m/z = 800-1900) obtained from analysis of six representative species in the Violaceae. The cyclotide region from 25-40 min is shown, and major components are labeled with molecular weights (Da). Note that the LC-MS traces of *Viola kiangsiensis* and *Viola sepincola* contain a higher number of cyclotide peaks, and also express varv A (mass 2877 eluting at 35 min), which is found in 2/3 of all *Viola* species.
Cyclotide peaks having a unique molecular weight between 2.8 and 3.8 kDa and expected retention time (±1 min) were classified as individual cyclotides. Using these criteria, 730 cyclotides were detected with masses ranging from 2806 to 3716 Da with an average of 3070 Da and a median of 3048 Da. On average five or six unique sequences were found per species and by extrapolation, there are potentially at least 5,000 different cyclotides in the Violaceae alone, which is consistent with earlier estimates (Simonsen, 2005). Nevertheless, this is still likely to be an underestimate of the total number of cyclotides, since it was probable that detection only was of the most abundant cyclotides. Based on the experience of species investigated in detail such as *Viola odorata* (for references, see *Table 2*), the real number is likely to be up to five times higher, approximately 25,000.

When the distribution of different cyclotides was compared among species, it was apparent that many cyclotides occurred in more than one species, following a pattern reflecting the division of genera. In particular, the cyclotide varv A was found in 70% of the analyzed species of the genus *Viola*, varv E and kalata B1 were present in about 50%, and six other cyclotides in 10-30% of the *Viola* species. The majority of the cyclotides were only found in either one or a few (<10%) species.

Cyclotides were positively identified in 17 of the 23 genera, and in 15% of all the species in the Violaceae. The samples represented a wide spread of species across the plant family. All isolated and sequenced cyclotides in Violaceae are listed in *Table 2*. In total, cyclotides have now been fully sequenced from eight genera in the family Violaceae. These eight genera are phylogenetically well spread across the family (*Figure 5*, on page 24). The origin of sequenced peptides covers two of the subfamilies, and several of the subtribes in the Violoideae. Analysis of these results corroborates that the Violaceae is an extremely rich source of cyclotides, and that cyclotides are ubiquitous throughout the family.

**References cited in Table 2 (on next page).**

1. (Simonsen, 2005) 12. (Craik, 1999)
2. (Broussalis, 2001) 13. (Chen, 2005)
5. (Yeshak, In manuscript) 16. (Dutton, 2004)
6. (Claeson, 1998) 17. (Ireland, 2006a)
7. (Göransson, 1999) 18. (Ireland, 2006b)
10. (Zhang, 2009) 21. (Wang, 2008a)
11. (Göransson, 2003a)
Table 2. List of all species that contain isolated cyclotides and/or predicted cyclotide sequences from cDNA in Violaceae. The references are shown on the previous page. Cyclotides in paranthesis are first found in another species.

<table>
<thead>
<tr>
<th>Species</th>
<th>No.</th>
<th>Proteins</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gloeospermum blakeanum</em></td>
<td>7</td>
<td>globa A-G</td>
<td>I</td>
</tr>
<tr>
<td><em>Gloeospermum pauciflorum</em></td>
<td>7</td>
<td>glopa A-G</td>
<td>I, II</td>
</tr>
<tr>
<td><em>Hybanthus calycinus</em></td>
<td>1</td>
<td>hyca A</td>
<td>I</td>
</tr>
<tr>
<td><em>Hybanthus debilissimus</em></td>
<td>1</td>
<td>hyde A</td>
<td>I</td>
</tr>
<tr>
<td><em>Hybanthus denticulatus</em></td>
<td>1</td>
<td>hyden A</td>
<td>II</td>
</tr>
<tr>
<td><em>Hybanthus enneaspernum</em></td>
<td>2</td>
<td>hyen A-B</td>
<td>I</td>
</tr>
<tr>
<td><em>Hybanthus epacroides</em></td>
<td>2</td>
<td>hyep A-B</td>
<td>I</td>
</tr>
<tr>
<td><em>Hybanthus floribundus</em></td>
<td>16</td>
<td>hyfl A-P</td>
<td>I</td>
</tr>
<tr>
<td><em>Hybanthus monopetalus</em></td>
<td>2</td>
<td>hymo A</td>
<td>I</td>
</tr>
<tr>
<td><em>Hybanthus parviflorus</em></td>
<td>1</td>
<td>hypa A</td>
<td>2</td>
</tr>
<tr>
<td><em>Hybanthus stellarioides</em></td>
<td>1</td>
<td>hyst A</td>
<td>I</td>
</tr>
<tr>
<td><em>Hybanthus vernonii</em></td>
<td>1</td>
<td>hyve A</td>
<td>I</td>
</tr>
<tr>
<td><em>Hymenanthera oborata</em></td>
<td>1</td>
<td>hobo A</td>
<td>II</td>
</tr>
<tr>
<td><em>Leonia cymosa</em></td>
<td>4</td>
<td>cycloviol A-D</td>
<td>3</td>
</tr>
<tr>
<td><em>Melicytus ramiflorus</em></td>
<td>17</td>
<td>mral1-5, 14a, 14b, 17a, 18a, 18b, 22-26, 30a</td>
<td>4</td>
</tr>
<tr>
<td><em>Melicytus macrophyllus</em></td>
<td>2</td>
<td>mema A-B</td>
<td>II</td>
</tr>
<tr>
<td><em>Orthion oblaceolatum</em></td>
<td>1</td>
<td>orto A</td>
<td>II</td>
</tr>
<tr>
<td><em>Rinorea gracilipes</em></td>
<td>1</td>
<td>rigra A</td>
<td>II</td>
</tr>
<tr>
<td><em>Rinorea lindeniana</em></td>
<td>2</td>
<td>rili A-B</td>
<td>II</td>
</tr>
<tr>
<td><em>Viola abyssinica</em></td>
<td>5</td>
<td>vaby A-E</td>
<td>5</td>
</tr>
<tr>
<td><em>Viola arvensis</em></td>
<td>8</td>
<td>varv A-H, (tricyclon A, viola peptide 6-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Varv E = cycloviolacin O12</td>
<td></td>
</tr>
<tr>
<td><em>Viola biflora</em></td>
<td>11</td>
<td>vibi A-K, (cycloviolacin O2, O9, varv 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4) A, vitri A)</td>
<td></td>
</tr>
<tr>
<td><em>Viola baoshanensis</em></td>
<td>17</td>
<td>viba 1-17 (varv A, E, kalata B1, cyc-10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>loviolacin Y5)</td>
<td></td>
</tr>
<tr>
<td><em>Viola cotyledon</em></td>
<td>2</td>
<td>vico A-B</td>
<td>11</td>
</tr>
<tr>
<td><em>Viola decumbens</em></td>
<td>1</td>
<td>vide A</td>
<td>II</td>
</tr>
<tr>
<td><em>Viola hederacea</em></td>
<td>7</td>
<td>cycloviolacin H1-4, vhl-1, vhl-2, vhr1 12-14</td>
<td></td>
</tr>
<tr>
<td><em>Viola nivalis</em></td>
<td>1</td>
<td>vini A</td>
<td>II</td>
</tr>
<tr>
<td><em>Viola odorata</em></td>
<td>39</td>
<td>cycloviolacin O1-O11, O13-36,         12, 15-19,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>violacin A, vodo M-O, (cycloviolacin 12, 15-19,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H1, kalata B1, B4 varv A, E1, H)     III</td>
<td></td>
</tr>
<tr>
<td><em>Viola tricolor</em></td>
<td>3</td>
<td>vitri A, tricyclon A-B, (varv A, E1)  7, 20</td>
<td></td>
</tr>
<tr>
<td><em>Viola yedoensis</em></td>
<td>5</td>
<td>cycloviolacin Y1-5, (kalata B1, varv 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3) A, E1)</td>
<td></td>
</tr>
</tbody>
</table>

Total 169

1 Varv E = cycloviolacin O12
2 Viola peptide I are most probable the same as varv A.
3.5 Stability of cyclotides for almost 200 years

Although the exceptional chemical, thermal and biological stability of cyclotides had been demonstrated in earlier studies (Colgrave, 2004), the approach of sampling extensively from dry plant material held in various herbaria gave a unique opportunity to assess cyclotide stability over time (Paper II). Using sweet violet, *Viola odorata*, which has a high level of cyclotide expression (Craik, 1999; Svangård, 2003; Ireland, 2006a) and is represented in a number of different herbarium collections, it was able to sample specimens collected between 1820 and the present day.

The LC-MS chromatograms of the samples are shown in Figure 7. The three dominant cyclotides (cycloviolacin O2, O19 and varv A) had the same peak intensity in all sweet violet LC-MS chromatograms obtained from analyses of both old and recent materials, indicating their concentration to be similar in all samples. The small differences seen are comparable to the seasonal variations observed over the year (Trabi, 2004).

Proteins are usually considered to be rather fragile biomolecules that are easily degraded by chemical and biotic factors. Nevertheless, they may be detected by immunological methods and/or mass spectrometry, even in material from ancient dinosaur fossils (Wick, 2001; Asara, 2007). Moreover, the resilient proteins commonly studied, such as collagen and osteocalcin, are usually associated with “resistant” tissues such as bone. In the case of cyclotides however, the analysis revealed that their full structures are retained intact in preserved leaves for almost 200 years.

![Figure 7. Stability of cyclotides over 190 years. Base peak chromatograms (m/z = 800-1900) obtained from five specimens of *Viola odorata* collected in the years 1820, 1849, 1886, 1948, and 2004. The LC-MS traces are shown in the range of 25-40 min, and the major components are labeled.](image-url)
4. Structure-activity relationships

Following the increasing number of known cyclotides and their activities, the main objective of Paper III was to obtain new insights into structure-activity relationships. The correlations between charged residues and overall hydrophobicity to cytotoxic activity were evaluated. The investigation included analysis of the 22 native cyclotides and to obtain more insights into the importance of certain residues in varv A (Möbius) and cycloviolacin O2 (bracelet). Charged and hydrophobic residues were targeted by chemical modifications and the effects of the changes on the cyclotides’ cytotoxicity were assessed. These studies provided more detailed understanding of residues that are important for high activity. As cyclotides are expressed as cocktails in planta a study to evaluate the effects of cyclotide mixtures was initiated. Thus, in a systematic way mixtures of cyclotides from both sub-families were prepared, and their cytotoxicity was tested to evaluate any possible sub- or superadditive effects.

4.1 The fluorometric microculture assay

The cytotoxicity of the cyclotides was determined using the fluorometric microculture cytotoxicity assay (FMCA) (Larsson, 1989; Lindhagen, 2008). It was used in Paper III-VI, and is a cell viability assay used for measurement of the cytotoxic and/or cytostatic effect in vitro. The assay is based on hydrolysis of the probe, fluorescein diacetate (FDA) by esterases in cells with intact plasma membranes.

The cyclotides were dissolved in 10% ethanol (equal to a final concentration of ethanol of 1% in the assay) and tested at series of concentrations obtained by two-fold dilutions. Microtiter plates were prepared with test solution in triplicates for each concentration. In addition, six solvent-control wells (containing 10% ethanol, corresponding to a final concentration of 1% in the assay), six blank wells (containing medium), and six negative-control wells (containing phosphate buffered saline solution (PBS)) were prepared on each microtiter plate. All experiments were performed three times.

In the assays, the tumor cells were suspended in cell-growth medium, dispensed on the prepared microtiter plates (20,000 cells/180 μL per well) and incubated at 37°C and 5% CO₂. After 72 h incubation, the cells were washed with PBS, and FDA was added to each well. The plates were incubated at
37°C and 5% CO₂ for 40 min, and the generated fluorescence was measured using a scanning fluorometer (excitation/emission wavelengths, 485/538 nm). The fluorescence was directly proportional to the number of living cells, hence cell survival was quantified as a survival index (SI), defined as the fluorescence of the test wells relative to the average fluorescence for control wells (after subtracting with blank values). The IC₅₀-values, which correspond to an SI of 50%, were calculated using non-linear regression.

4.2 Cytotoxic activity of native cyclotides

All the cyclotides tested against the human lymphoma cell line U937-GTB in the FMCA showed potency in the low micromolar range (Table 3, on page 32). The effects are promising compared to drugs used in the clinic that has been tested on this cell line. For example, the IC₅₀ values of doxorubicin, cisplatin and paclitaxel are 0.09, 0.49 and 0.003 μg/mL, respectively (Gullbo, 2004).

The bracelet cyclotides are generally more cytotoxic than Möbius cyclotides (Lindholm, 2002; Svangård, 2004; Herrmann, 2008). Within the bracelet subfamily, it can be noted that cyclotides with several positive residues in loops 5 and 6 have higher activity than those lacking such residues. As the proposed mechanism of action is through membrane interactions and the increased activity may be because they have higher affinity for negatively charged cell membranes, or the polar head groups of the phospholipids in the membrane (Papers IV and VI).

This tendency is not as clear in the Möbius cyclotides, although vaby D, kalata B2 and B13 that are the most potent once, and have additional charged residue(s) in loops 5 and 6. However, these cyclotides also differ from other Möbius cyclotides in other respects, e.g. loop 2 is more hydrophobic as a Phe residue replaces Val/Thr/Ala. Kalata B7 and vibi D also have additional charged residues, but in loops that disturb the overall amphipathicity (Figure 8), and thus activity. Hence, charged residues per se do not promote activity their effect depends on their localization and in relation to the rest of the sequence.

Comparisons of differences in the surface hydrofobicity between Möbius and bracelet cyclotides have shown that both subfamilies are amphipathic, but by different parts of the protein (Wang, 2009a). The hybrid cyclotides kalata B8 and psyle A provide good examples of the importance of the amphipathic structure. They are defined as hybrids due to their similarity to Möbius cyclotides, except for the residues in loop 5, which normally contains hydrophobic residues. In hybrids, loop 5 is similar to a typical bracelet loop, with charged and polar amino acids, disrupting high amphipathicity and thus reducing activity.
Figure 8. Surface representations of the Möbius cyclotides varv A and vibi D and the bracelet cyclotide cycloviolacin O2 (cyO2). The hydrophobic residues (Ala, Leu, Ile, Pro, Trp, Phe, and Val) are in green, cationic residues (Arg and Lys) in blue, anionic residues (Asp and Glu) in red.

In addition to the hybrids, one other atypical cyclotide has been tested for cytotoxic activity: namely the naturally occurring linear cyclotide, psyle C. Instead of an Asn/Asp at the end of the immature cyclotide domain of the precursor sequence it has a Lys that prevents ring closure. However, despite this opening in loop 6 it shows strong cytotoxic activity, with an IC$_{50}$ value of 3.5 µM. This is in contrast to the acyclic permutants of kalata B1, which have been found to have no hemolytic activity, while native kalata B1 possesses at least mild potency (Daly, 2000).
Table 3. Sequences, cytotoxic activities and sequences of all bracelet, hybrid, linear and Möbius cyclotides tested in the FMCA using the human lymphoma cancer cell line U937-GTB. Charges residues are in italics.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>IC_{50}</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bracelet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyO2^1</td>
<td>G-IP-CGESCVWIPC-ISSAIGSCC-KSKVCYRN</td>
<td>0.27-1.8</td>
<td>III, 1-2.</td>
</tr>
<tr>
<td>cyO19^1</td>
<td>G-IP-CGESCVWIPC-ISSVVGSCC-KSKVCYKD</td>
<td>0.52</td>
<td>III</td>
</tr>
<tr>
<td>vitri A</td>
<td>G-IP-CGESCVWIPC-ITSAIGSCC-KSKVCYRN</td>
<td>0.6</td>
<td>3.</td>
</tr>
<tr>
<td>psyle E</td>
<td>G-IP-CGESCVFIIPC-ISSVLCSC-KNKVCYRD</td>
<td>0.76</td>
<td>4.</td>
</tr>
<tr>
<td>vibi G</td>
<td>GTFP-CGESCVFIIPC-LTSAIGSCC-KSKVCYKN</td>
<td>0.96</td>
<td>5.</td>
</tr>
<tr>
<td>vibi H</td>
<td>GLLP-CAESCVYIPC-LTTVIGSCC-KSKVCYKN</td>
<td>1.6</td>
<td>5.</td>
</tr>
<tr>
<td>vibi E</td>
<td>G-IP-CAESCVWIPC-TVTALIGGC-SNKVCY-N</td>
<td>3.2</td>
<td>5.</td>
</tr>
<tr>
<td>vodo O</td>
<td>G-IP-CAESCVFIIPC-TTALLGSCC-SNKVCY-N</td>
<td>3.2</td>
<td>III</td>
</tr>
<tr>
<td>cyO2-kyn^1,2</td>
<td>G-IP-CGESCVWIPC-ISSAIGSCC-KSKVCYRN</td>
<td>5.2</td>
<td>III</td>
</tr>
<tr>
<td><strong>Hybrid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kalata B8</td>
<td>GSVLNCGETCLLGC----YTTGCTCNKYRVCYKD</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>psyle A</td>
<td>G-IA-CGESCVFLGC----FIPGCSVKS-KCYFN</td>
<td>26</td>
<td>4.</td>
</tr>
<tr>
<td><strong>Linear</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>psyle C</td>
<td>---KLCGETCFKFKC----YTPGSCC-SYPFC-K-</td>
<td>3.5</td>
<td>4.</td>
</tr>
<tr>
<td><strong>Möbius</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kalata B2</td>
<td>G-LPVCGETCFGTC---NTPGCSC-TWPICTRD</td>
<td>2.6</td>
<td>III</td>
</tr>
<tr>
<td>vaby D</td>
<td>G-LPVCGETCFGTC---NTPGCSC-CDWPVCYRN</td>
<td>2.8</td>
<td>6.</td>
</tr>
<tr>
<td>kalata B13</td>
<td>G-LPVCGETCFGTC---NTPGCSC-TWPICTRD</td>
<td>3.8</td>
<td>III</td>
</tr>
<tr>
<td>varv E</td>
<td>G-LPVCGETCFGTC---NTPGCSC-SWPVCYRN</td>
<td>4.0</td>
<td>3.</td>
</tr>
<tr>
<td>varv A</td>
<td>G-LPVCGETVGTC---NTPGCSC-SWPVCYRN</td>
<td>6.4-10</td>
<td>III, 1.</td>
</tr>
<tr>
<td>varv F</td>
<td>G-VPIGGETCFLGTG----YTACGC-SWPVCYRN</td>
<td>7.1</td>
<td>1.</td>
</tr>
<tr>
<td>kalata B1</td>
<td>G-LPVCGETCAGGC---NTPGCSC-SWPVCYRN</td>
<td>7.1</td>
<td>III</td>
</tr>
<tr>
<td>vaby A</td>
<td>G-LPVCGETCAGGC---NTPGCSC-SWPVCYRN</td>
<td>7.6</td>
<td>6.</td>
</tr>
<tr>
<td>kalata B7</td>
<td>G-LPVCGETCFLGTG----YTACGC-SWPVCYRN</td>
<td>29</td>
<td>III</td>
</tr>
<tr>
<td>vibi D</td>
<td>G-LPVCGETCAGGC---NTPGCSC-SWPVCYRN</td>
<td>&gt;30</td>
<td>5.</td>
</tr>
</tbody>
</table>

^1 cyO2, O19 = cycloviolacin O2, O19
^2 kyn = kynurenine (The tryptophan in loop 2 are naturally modified into a kynurenine)

References cited in Table 3.
1. (Lindholm, 2002)  4. (Gerlach, 2010)
2. (Herrmann, 2006)  5. (Herrmann, 2008)
3. (Svangård, 2004)  6. (Yeshak, In manuscript)
4.3 Effects of charged and hydrophobic residues

The orientation of the Möbius protein varv A and the bracelet cycloviolacin O2 in membranes are shown in Figure 9. Varv A interacts with the lipid bilayer via the hydrophobic parts of loops 2, 5 and 6, cycloviolacin O2, loops 2 and 3 are buried in the membrane.

To evaluate the charged and hydrophobic residues influence on cytotoxicity, chemical modification was conducted on surface-exposed positive charged residues and the conserved negatively charged Glu (Herrmann, 2006, Paper III). The importance of the hydrophobic properties might also be connected to the low potency of cyclotides, thus the Trp that are deeply penetrated in the membrane was hydroxylized in attempts to disrupt the hydrophobic patch.

![Figure 9](image)

**Figure 9.** Orientation of varv A and cycloviolacin O2 (cyO2) in membranes. The lines represent the upper part of the lipid bilayer, i.e. the parts of the protein buried in the membrane are located beneath the line. The hydrophobic residues (Ala, Leu, Ile, Pro, Trp, Phe, and Val) are in green, cationic residues (Arg and Lys) in blue, anionic residues (Asp and Glu) in red, and other residues in white.

The impact of charged amino acids in the bracelet cycloviolacin O2 showed that the conserved Glu (found in loop 1) plays a key role in its cytotoxicity. As shown in Table 4, a simple methylation caused a 48-fold decrease in potency. Virtually no change in activity was observed when the Arg was modified, but after chemically modifying the two Lys residues, the potency was reduced 3-fold. The derivative with modifications
at both arginine and lysine residues showed a 7-fold loss of potency. To further evaluate the charged and hydrophobic residues influence on cytotoxicity, chemical modification of a cyclotide from each subfamily (varv A and cycloviolacin O2) were conducted (Paper III), targeting all charged residues but also the hydrophobic Trp residue that is buried deep into the membrane (Figure 9).

After masking the Arg residue in the Möbius varv A, no change in potency was observed. Esterification of the Glu residue produced a 3-fold loss in potency and both modifications together gave a 5-fold loss (Table 4). The main reason for the low potency following methylation of the Glu in cycloviolacin O2 can be attributed to breakage of the hydrogen bonds linking Glu to the hydrophobic α-helix in loop 3 (ISSAIG). The loss of the hydrogen bonds significantly affects the conformation of the α-helix, which becomes more flexible as shown by NMR studies (Göransson, 2009). The Glu residue also forms hydrogen bonds to loop 3 in the Möbius cyclotides, but this loop is significantly less hydrophobic and shorter in this subfamily (NTPG in varv A) thus loss of the bonds does not affect their conformation or activity as strongly (Rosengren, 2003).

Table 4. Cytotoxic activity and relative potency of native and chemical modified varv A and cycloviolacin O2.

<table>
<thead>
<tr>
<th>Cyclotide derivative</th>
<th>IC₅₀ (μM)</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>varv A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>native</td>
<td>10 ±2</td>
<td>1</td>
</tr>
<tr>
<td>Arg (CHD)</td>
<td>9.1 ±2</td>
<td>1</td>
</tr>
<tr>
<td>Glu (Me)</td>
<td>34 ±4</td>
<td>1/3</td>
</tr>
<tr>
<td>Arg (CHD) + Glu (Me)</td>
<td>46 ±7</td>
<td>1/5</td>
</tr>
<tr>
<td>Trp (OH)₂</td>
<td>&gt;100</td>
<td>&lt;1/10</td>
</tr>
<tr>
<td>Cycloviolacin O2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>native¹</td>
<td>0.75 ±0.1 / 1.8 ±0.2</td>
<td>1</td>
</tr>
<tr>
<td>Arg (CHD)</td>
<td>0.95 ±0.1</td>
<td>1</td>
</tr>
<tr>
<td>Lys (Ac)₂</td>
<td>2.3 ±0.3</td>
<td>1/3</td>
</tr>
<tr>
<td>Trp (OH)</td>
<td>4.5 ±0.4</td>
<td>1/3</td>
</tr>
<tr>
<td>Trp (OH)</td>
<td>5.1 ±0.5</td>
<td>1/3</td>
</tr>
<tr>
<td>Arg (CHD) + Lys (Ac)₂</td>
<td>5.1 ±0.4</td>
<td>1/7</td>
</tr>
<tr>
<td>Glu (Me)</td>
<td>36 ±4</td>
<td>1/48</td>
</tr>
<tr>
<td>Trp (OH)₂</td>
<td>55 ±12</td>
<td>1/31</td>
</tr>
<tr>
<td>Trp (OH)₂</td>
<td>&gt;100</td>
<td>&lt;1/50</td>
</tr>
</tbody>
</table>

¹ In the study of charged residues in cycloviolacin O2 (Herrmann, 2006) and Paper III, its IC₅₀ was found to be 0.75 and 1.8 μM, respectively. The relative potency is based on the activity in the respective study.
In *Viola odorata*, a natural modification of the cycloviolacin O2 was found; the Trp residue was processed to kynurenine, which are more hydrophilic. The activity of this cyclotide decreased to the same level as that of the singly oxidized species (Table 3, Table 4). The double oxidation of Trp in cycloviolacin O2 reduced the activity even more. This is the first report on a natural degradation of cyclotides in the plant that reduces their cytotoxic activity.

This was also seen for varv A, demonstrating the importance of the hydrophobic properties in loop 5 for the cytotoxic activity of Möbius cyclotides. There are two other examples showing the importance of this residue; both kalata B1 with photo-oxidized Trp and synthesized analogs lacking the Trp show no hemolytic activity (Clark, 2006; Plan, 2007). The hybrid kalata B8, which also have a disturbed amphipathicity, *i.e.* has the sequence of a bracelet cyclotide, but a hydrophilic loop 3 shows low cytotoxic (Table 3) and hemolytic activity (Daly, 2006). The conclusion is that in order to correlate cyclotides’ structures with their potency and to understand their mechanisms of action, it is necessary to consider their structure as a whole rather than focusing exclusively on single residues.

### 4.4 Additive effects of cyclotide mixtures

In plants, cyclotides are expressed as cocktails and act as defense molecules (Jennings, 2001). It is therefore plausible that such cyclotide mixtures might have synergistic effects that would make the combination more effective against external threats such as pathogens and pests than would be expected on the basis of the peptides’ individual activities. To identify such potential combination effects, cyclotides from both subfamilies were mixed in different ratios and the cytotoxicity of these binary cocktails was analyzed. Three cyclotides were chosen for the studies: kalata B1, which is the most intensively studied cyclotide and regarded as a prototype for the Möbius subfamily; kalata B2 which was included for comparison within the subfamily; and a member of the bracelet subfamily, cycloviolacin O2.

A common method for comparing the effects of a combination of bioactive compounds to those of the individual constituents (isoboles) of the combination is to construct an isobologram. The doses of the individual cyclotides required to generate 50% effect were plotted as points on the axes of a Cartesian plot. The straight line connecting the two individual IC$_{50}$ values is the locus of points that will produce this effect in an additive combination. This line of additivity allows comparison with the actual dose pair that produces this effect level experimentally. It should be noted dose combinations that deviate from the line may be either sub-additive (antagonistic) or super-additive (synergistic) (Tallarida, 2001).
Figure 10. Isobolograms of the 50% cytotoxic effect for several combinations of cyclotides. For each of the combinations of cyclotide, mixtures of seven concentration mixtures were prepared and tested for cytotoxicity. The combinations were kalata B1 and B2 (A), cycloviolacin O2 (cyO2) and kalata B1 (B) and cyO2 and kalata B2 (C).

As can be seen in Figure 10, the activity of the cyclotide mixtures were all close to line of additivity, implying that mixtures of cyclotides (including mixtures of cyclotides from both subfamilies) have combined effects that are very similar to the sum of the individual effects. A mixture of all three cyclotides also had additive effects: equal quantities of the stock 60 x IC_{50} solutions of each cyclotide were mixed in a 1:1:1 ratio. A dilution series of this mixture was prepared and tested; its IC_{50} was determined to be exactly 1/60 of the maximum tolerable concentration, which unambiguously demonstrates that more complicated cyclotide mixtures also show additive effects.

The results support the hypothesis that each individual in the large cocktail of cyclotides makes a small contribution to their total effect. Different cyclotides also show effects against different targets, as illustrated in studies using a panel of different assays/targets with a defined set of cyclotides (Tam, 1999; Ireland, 2008). Conclusions are that plants produce a cocktail of cyclotides with individually high activity against certain targets, less against others, but collectively excellent potency against multiple targets. As components of host defense in the plant, this would definitely enhance survival prospects.
5. Mechanistic studies on cyclotides

It has been shown that cyclotides represent a novel class of cytotoxic agents that display strong activity in a dose dependent manner (Lindholm, 2002). The cytotoxic activity was maintained throughout a cell line panel consisting of ten human tumor cell lines, including solid tumor cells. In addition, the activity profile for cyclotides tested in the panel, which is designed to represent defined types of drug resistance, differs significantly from those of antitumor drugs in clinical use suggesting a new mode of action (MOA).

Sparked by these results studies were initiated to understand the MOA for the cytotoxic effect (Paper IV). The study included characterization of the kinetics of the cytotoxic effect, microscopy studies and also descriptions of the morphology of cyclotide treated cells. Further studies were performed (Paper VI) to examine the cyclotides ability to disrupt/adsorb to lipid membranes and influence of electrostatic interactions and secondary structure for activity was investigated by fluorescent spectroscopy, ellipsometry and circular dichroism (CD).

5.1 Kinetics of the cytotoxic effect

A step in characterizing the MOA for the cyclotides cytotoxic effect includes a study of the kinetics. In normal cases are the cells in the FMCA (See Chapter 4.1 for details) incubated with the substance for 72 h. The long time period make sure that cell death with longer on-set periods are included, such as apoptosis that require activation of several intracellular pathways and active gene expression. Apoptosis is considered to be an active cell death and are characterized by nuclear condensation, fragmentation and formation of apoptotic bodies (Kerr, 1972).

A more passive cell death is through necrosis which cause lethal changes through swelling of the cytoplasm and eventually disruption of the cell membrane (Majno, 1995). This pathway is more rapid and can in some cases occur within minutes. In Paper IV investigations was performed on the kinetics of cyclotide cytotoxic activity. First the activity was evaluated down to 4 h incubation in the FMCA. Shorter time points are not possible by using FMCA, instead cell death was assessed by cell morphology.
The kinetics of the cytotoxic effect of cycloviolacin O2 were monitored using the human lymphoma cell line U937-GTB followed by FMCA after 4, 8, 24 and 72 h incubation. The concentration-response curves at the different time points overlapped and the IC$_{50}$ values were calculated to coincide at approximately 0.7 μM (Figure 11).

**Figure 11.** Kinetics in the FMCA. The human lymphoma cell line was exposed to cycloviolacin O2 for 4, 8, 24, and 72 h, and cell viability was assessed using the FMCA. Each point represents the mean while the error bars indicate ± SEM.

Time point of cytotoxic effect down to 5 min of exposure was assessed by preparation of microscope slides after centrifugation of cell suspension (i.e. cytocentrifugation). The harvested cancer cells retain original size and shape but are flattened, giving excellent nuclear presentation. The cytospin slides were stained with May-Grünwald-Giemsa (MGG) and showed signs of disintegrated cell membranes within 5 min, and after 1 h more than half of the cells were necrotic (Figure 12).

**Figure 12.** Summary after manual count of cells in the cytospin slides from 5 min to 24 h. For comparison, control cells, cells exposed to 25 μM etoposide, and cells exposed to 1% triton X-100 are shown. The bars represent mean values + SEM.
5.2 Membrane disruptive effects

**Paper V** assessed the membrane adsorption and disruption of prototypic cyclotides and correlated the findings to the cytotoxic properties of the cyclotides. The cyclotides used were the Möbius kalata B1 and B2, and the bracelet cycloviolacin O2. In order to obtain additional mechanistic information on membrane binding and disruption by these peptides, we also modified selected charged amino acids in cycloviolacin O2 that have been shown to be important for cytotoxic activity (Herrmann, 2006). The membrane interactions of these cyclotides were studied by a method combination of fluorescence spectroscopy, ellipsometry, and circular dichroism, in order to investigate the dependence of membrane affinity and lytic properties on membrane composition, electrostatics, and peptide secondary structure, and to correlate results obtained in model lipid membranes of cytotoxicity.

5.2.1 Comparison to cytotoxic effect

For comparison to lytic effects on liposomes cyclotides’ cytotoxicity was evaluated in the FMCA using three different cancer cell lines, U937-GTB which is considered to be sensitive to a wide range of cytotoxic agents and two resistant cancer cell lines, HT29 and Ht116. As shown in **Table 5**, all cyclotides showed concentration-dependant cytotoxic effects with significant different potencies, but all in the low micromolar range. Cycloviolacin O2 showed the highest potency followed by kalata B2 and B1, and their effects were strongest against the sensitive lymphoma cells, although strong activities were also observed against the more resistant Ht116 and HT29.

**Table 5.** Cytotoxic activity of kalata B1, kalata B2, and cycloviolacin O2 against U937-GTB lymphoma, HT29 colon adenocarcinoma, and Ht116 colorectal cancer cells.

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>kalata B1 IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>kalata B2 IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>cycloviolacin O2 IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937-GTB</td>
<td>5.0 ±0.7</td>
<td>1.7 ±0.5</td>
<td>0.7 ±1.5</td>
</tr>
<tr>
<td>HT29</td>
<td>14.6 ±1.2</td>
<td>5.0 ±0.3</td>
<td>5.3 ±1.0</td>
</tr>
<tr>
<td>Ht116</td>
<td>8.2 ±0.4</td>
<td>2.9 ±0.2</td>
<td>1.9 ±0.1</td>
</tr>
</tbody>
</table>

5.2.2 Lytic effects of native cyclotides

Membrane permeability was measured by monitoring 5(6)-carboxyfluorescein (CF) efflux from the liposomes, *i.e.* loss of self-quenching and consequently an increased fluorescence signal. The results are expressed as percent of total disruption, generated after adding of
Triton X-100 and subtracting the baseline value. Table 6 records the leakage induction observed following the addition of cyclotides to liposomes with different phospholipid compositions. In the tests we used the zwitterionic DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) or DOPC/cholesterol, as well as anionic DOPC/DOPA (1,2-dioleoyl-sn-glycero-3-phosphate) and E. coli liposomes.

In analogy to the cytotoxicity effects, cycloviolacin O2 was the most potent of the native cyclotides against all liposomes, followed by kalata B2 and B1. Furthermore, we note that the condensing effect of cholesterol on lipid chain packing (Needham, 1990), precludes membrane lysis by the native cyclotides. Cholesterol reduced susceptibility to all cyclotides investigated, to the degree that no activity was seen for kalata B1 even at the highest concentration tested.

Cycloviolacin O2 has more positive charged residues than kalata B1 and B2, thus its activity could be more dependent on electrostatic interactions. This hypothesis was corroborated by cycloviolacin O2 having higher activity against the anionic liposomes than the zwitterionic liposomes, while kalata B1 and B2 showed the same or slightly less activity on the anionic liposomes.

Table 6. Membrane-disruption activity as percent leakage relative to total disruption caused by the positive control Triton X-100 in tests with native cyclotides. (-) denotes that the corresponding experiment was not conducted.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>kalata B1 (μM)</th>
<th>kalata B2 (μM)</th>
<th>cycloviolacin O2 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 10 5 1</td>
<td>20 10 5 1</td>
<td>20 10 5 1 0.1</td>
</tr>
<tr>
<td>DOPC</td>
<td>23 14 6 2</td>
<td>68 44 14 4</td>
<td>97 88 45 18 3</td>
</tr>
<tr>
<td>DOPC/Cholesterol</td>
<td>0 0 - -</td>
<td>16 7 4 -</td>
<td>80 70 49 18 4</td>
</tr>
<tr>
<td>DOPC/DOPA</td>
<td>26 11 6 1</td>
<td>28 12 7 1</td>
<td>100 100 83 33 7</td>
</tr>
</tbody>
</table>

5.2.3 Lytic effects of chemically modified cycloviolacin O2

In an earlier study we showed the importance of the Glu and Lys residues for the cytotoxic activity of cycloviolacin O2 (Herrmann, 2006), which was modified in exactly the same ways here as in the former study and the activity of the native and modified variants was tested on anionic DOPC/DOPA and E. coli liposomes. As shown in Table 7, the leakage induced by the Lys modified variant was lower than that induced by native cycloviolacin O2. The Glu modified variant (cyO2-Glu) had stronger effect on anionic liposomes, with almost identical effects on the E. coli liposomes. In the latter case, however, the native variant was more potent.

The results of both changing from zwitterionic to anionic liposomes and removing the two charges on the Lys residues (cyO2-Lys) clearly
indicated that the effects of cycloviolacin O2 are influenced by electrostatic interactions. The modification of the Glu residue was included for another reason; esterification of the conserved Glu residue breaks several internal ion bonds to a helix in loop 3, which becomes disorganized causing a sharp drop in both cytotoxic (Herrmann, 2006; Göransson, 2009) as antibacterial (Pränting, 2010) activities. Nevertheless, the destabilization of the secondary structure caused by eliminating this ion bond renders the protein to more membrane active.

Table 7. Membrane-disruption activity listed as percent leakage relative to total disruption caused by the positive control Triton X-100 in tests with native and chemically modified cycloviolacin O2 (cyO2). (-) denotes that the corresponding experiment was not conducted.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>cycloviolacin O2 (μM)</th>
<th>cyO2 - Glu (μM)</th>
<th>cyO2 - Lys (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 10 5 1 0.1</td>
<td>20 10 5 1 0.1</td>
<td>20 10 5 1 0.1</td>
</tr>
<tr>
<td>DOPC/DOPA</td>
<td>100 100 83 33 7</td>
<td>- 100 100 98 17</td>
<td>7 61 41 15 4</td>
</tr>
<tr>
<td>E. coli lipids</td>
<td>- - - 100 83</td>
<td>- - 100 96 19</td>
<td>- - - - -</td>
</tr>
</tbody>
</table>

5.3 Membrane adsorption

Adsorption to the membrane is essential for membrane-lytic activity, but the degree of lytic potency and the level of adsorption are not always proportional (Strömstedt, 2007; Wessman, 2008; Strömstedt, 2009). Peptide adsorption to supported lipid bilayers was studied by in situ null ellipsometry. After lipid bilayer formation and adjustments for buffer and temperature, peptides were added cumulatively from 1/16, 1/4, 1, 4 to 16 μM.

Cyclotide adsorption was monitored on the DOPC/DOPA mixture, since this phospholipid composition displayed considerable susceptibility to cyclotide-induced membrane lysis. For the presently investigated cyclotides, the adsorption followed the same pattern as the lytic activity. Thus, cycloviolacin O2 displays the strongest lytic potency, and also has the highest affinity to the membranes, while kalata B1 and B2 showed low adsorption as well as relatively weak lytic properties (Figure 13).
Figure 13. Adsorption isotherms of kalata B1, B2 and cycloviolacin O2 to supported DOPC:DOPA bilayers. Low adsorption was observed for kalata B1 and B2 (KB1, KB2), while cycloviolacin O2 (cyO2) displayed more extensive adsorption.

As shown in Figure 14, cyO2-Lys shows reduced adsorption compared to its native sequence partly as a result of decreasing the electrostatic attraction to the anionic membrane the neutralizing its two Lys. The cyO2-Glu, on the other hand, represents an increase in net positive charge by neutralizing its anionic Glu residue. However, the increase in adsorption to the anionic DOPC/DOPA bilayer by neutralizing the Glu residue is so great that electrostatics cannot be the only factor involved. Instead it is likely that the structural role of Glu comes into play by the relaxation of loop 3, which constitute a major part of the hydrophobic patch on the molecular surface it most probably directly associated with the membrane interface upon adsorption.

Figure 14. Adsorption isotherms of cycloviolacin O2 and modified variants to supported DOPC:DOPA bilayers. Cycloviolacin O2 (cyO2) and its chemically modified variants (Lys and Glu) displayed extensive adsorption, particularly the cyO2-Glu variant.
As lipid environments stabilize peptide internal hydrogen bonds, and thus helix formation, the net gain in helices following membrane adsorption will be a driving force for adsorption itself (Wieprecht, 1999; Wieprecht, 2002). Hence, relaxation of an idealized helical loop, prior to adsorption, as in the case of cyO2-Glu, may contribute to high adsorption levels. Due to the complexity of the CD spectra for these highly knotted cyclotides, the exact degree of helix induction by adsorption could not be quantified by certainty. Nevertheless, CD spectra showed that the degree of ordering in the cyclotides’ structure increased upon membrane binding, to a large extent due to helix formation (Figure 15). This effect was most pronounced for cyO2-Glu compared to its analogues, in accordance with our proposed mechanism.

**Figure 15.** CD spectra of native and chemically modified cycloviolacin O2 (cyO2) in the presence and absence of liposomes.

Although it remains to be shown more conclusively, it is likely that the antimicrobial effect of cycloviolacin O2 is due to bacterial membrane rupture. Results of cycloviolacin O2-induced leakage of liposomes prepared from *E. coli* lipid was compared to that for the DOPC/DOPA model membranes (There are a very similar charge density for *E. coli* and DOPC/DOPA liposomes). Strikingly, with *E. coli* liposomes the order of potency for cycloviolacin O2 and cyO2-Glu is similar to the order of their antibacterial activity, but opposite to that observed for DOPC/DOPA.

Quantitatively, the cyO2-Glu shows essentially identical inducing leakage properties towards these two anionic liposomes, while cycloviolacin O2 is about 50 times as potent against the latter. This suggests that membrane lysis by cycloviolacin O2 is sensitive toward variations either in the specific compositions of the alkyl groups or phosphatidyl groups in a manner that is not directly related to the overall membrane charge or liquid order phase, perhaps due to lipid demixing or dependence on a specific membrane thickness.
5.4 Mode of action

Since the discovery of the first cyclotide many reports have been published on their biological activities. The set of activity is mostly related to pesticide effects, against e.g. insects, nematodes, barnacles, bacteria and viruses. Some other effects are uterus-contractive, neurotensin-binding, hemolytic and cytotoxic. The mechanism is suggested to be associated with membrane interactions. It has been shown by NMR spectroscopy that cyclotides can bind to dodecylphosphocholine micelles by electrostatic and hydrophobic interactions (Shenkarev, 2006; Shenkarev, 2008) and several cyclotides have been shown to have affinity to membranes by surface plasmon resonance analysis (Kamimori, 2005). Paper IV and VI showed that a rapid membrane disruption plays a crucial role for the cytotoxic effect of cyclotides.

Studies utilizing analytical ultracentrifugation have shown that the cyclotide, kalata B2 forms oligomers in solution (Nourse, 2004), which could potentially have a role in the formation of membrane-spanning pores. Electrophysiological experiments have recently showed that conductive pores were induced in liposome patches on incubation with kalata B1. The conductance calculated from the current-voltage relationship indicated that the diameter of the pores formed in the bilayer patches is 41–47 Å (Huang, 2009).

However, the exact mechanism of the cyclotides lytic effects is not fully understood and in my opinion, conclusive evidence that cyclotides form well-defined pores remains to be shown. What is known is that the amphipathicity of the cyclotides allows partition into the membrane lipid bilayer and eventually disrupts the membrane. Membranes have shown to be of importance in some of the other effects as well. For example, cyclotides forms holes that lead to cell swelling and lysis in the midgut of lepidopteran larvae (Barbeta, 2008) and hemolysis has also been associated with membrane activity (Ireland, 2006a). Membrane interactions provide a plausible explanation for many of the diverse set of activities of this unique protein family. Nevertheless there might be other targets for the cyclotides that yet are to be discovered.
6. Toxicity and antitumor activity \textit{in vivo}

The results of cyclotides’ potent cytotoxic effects on human cancer cells prompted additional studies \textit{in vivo} (\textbf{Paper VI}). First, a study was initiated to evaluate cyclotides’ effects in a tumor microenvironment using the hollow fiber assay \textit{in vitro}. The results was promising and continued studies was conducted to examine \textit{in vivo} tolerability and anti-tumor activity, by using two different \textit{in vivo} methods: the hollow fiber method with single dosing and traditional xenografts with repeated dosing over two weeks. Before these tests \textit{in vivo}, a maximal tolerated dose (MTD) was determined.

6.1 Toxicity in mice

Eight animals (male NMRI) each received a single intravenous injection of different doses of cycloviolacin O2 (0.5-2.0 mg/kg). The mice were checked daily for survival and general appearance. After one week the animals were sacrificed, weighed and autopsied. Major organs were visually inspected for pathological changes. MTD in mice was determined to be 1.5 mg/kg. At the next dose level (2.0 mg/kg) one of two animals died shortly after injection. Animals receiving high doses showed a non-significant tendency for reduced weight gain, but no unusual observations were made at autopsy for any of the animals. The dose 1.0 mg/kg was considered appropriate for administration in the hollow fiber \textit{in vivo} and xenograft experiments.

6.2 Hollow fiber assay

The hollow fiber assay has the ability to provide quantitative indices of drug efficacy in heterogeneous tumors with minimal expenditures of time and materials (Hollingshead, 1995). It is currently being used as the initial \textit{in vivo} experience for agents found to have reproducible cytotoxic activity \textit{in vitro}. Small hollow fibers (1 mm in diameter, 2 cm long, molecular weight exclusion of 500,000 Da), made of polyvinylidene fluoride and containing cells from human tumors, are inserted underneath the skin and in the body cavity of the mouse. Mice are treated with experimental
agents and the fibers are collected from the mice following treatment and subjected to the stable endpoint MTT assay.

Three human tumor cell lines were used for the hollow fiber assay: the lymphoma U937-GTB, leukaemia CCRF-CEM, and small cell lung cancer NCI-H69 lines. They all displayed dose-dependent in vitro sensitivity in the FMCA (See Chapter 4.1 for details). Hollow fibers of polyvinylidene fluoride were filled with cell suspension and incubated in Petri dishes with supplemented medium for 72 h prior to exposure to the test substance in the in vitro study and 48 h in the in vivo study.

Cycloviolacin O2 was tested at five concentrations obtained by tenfold dilutions using 15 µM as the maximum concentration in the in vitro assay. The hollow fibers encapsulating the cell lines were exposed to cycloviolacin O2 and the living cell density was determined for duplicate fibers and evaluated by staining with 3-(4,5-di-methylthiazol-2-yl)-2,3-diphenyltetrazolium bromide (MTT), which is converted by metabolically active cells to insoluble blue formazan crystals.

The crystals were extracted with dimethyl sulfoxide and the optical density (OD) was analyzed at 570 nm in a plate reader. Blank values for dimethyl sulfoxide were subtracted from each reading. The OD (as measured) is proportional to the number of living cells; cell survival was quantified as a survival index (SI), defined (in percentage) as the absorbance of control wells, after subtracting average values for blank wells. IC_{50} values were calculated using non-linear regression in GraphPad Prism (GraphPad Software, Inc., San Diego).

The workflow of the in vivo study is outlined in Figure 16. Fibers of different color were filled with tumor cell lines, incubated in vitro and subcutaneously implanted into mice. The day after implantation they received one dose of cycloviolacin O2 into their tail veins. Five days after administration were the fibers removed and MTT-stained.

![Figure 16](image)

Figure 16. Study design for the hollow fiber assay in vivo. Hollow fibers were filled with cancer cells, incubated in vitro and subcutaneously implanted into mice. The day after implantation they received one dose of cycloviolacin O2 into their tail veins. Five days after administration were the fibers removed and MTT-stained.
showed no signs of discomfort and gained weight like the controls (Table 8).

**Table 8.** Dose-dependent toxicological effects and observations after administration of cycloviolacin O2 in mice; average values are given ± SD.

<table>
<thead>
<tr>
<th>Number of animals (n)</th>
<th>Dose (mg/kg)</th>
<th>Start weight (g)</th>
<th>End weight (g)</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hollow fiber (8)</td>
<td>Single 1.0</td>
<td>32.5 ±1.7</td>
<td>34.0 ±1.6</td>
<td>Normal</td>
</tr>
<tr>
<td>Hollow fiber, control (8)</td>
<td>0</td>
<td>33.1 ±1.5</td>
<td>34.7 ±1.4</td>
<td>Normal</td>
</tr>
<tr>
<td>Xenograft (4)</td>
<td>Repeated 1.0</td>
<td>22.8 ±1.9</td>
<td>26.8 ±0.7</td>
<td>Discolored tail after 2-3 days, microscopic evaluation showed an inflammatory reaction at injection site. Normal hematology.</td>
</tr>
<tr>
<td>Xenograft (5)</td>
<td>Repeated 0.5</td>
<td>21.2 ±1.7</td>
<td>25.7 ±1.3</td>
<td>Normal (incl. hematology)</td>
</tr>
<tr>
<td>Xenograft, control (8)</td>
<td>0</td>
<td>21.6 ±1.8</td>
<td>26.0 ±1.5</td>
<td>Normal (incl. hematology)</td>
</tr>
</tbody>
</table>

1 Trend for suppression of RBC, Hct, HGB, and PLT as described in Table 9.

After removal, the fibers were incubated with MTT as described above, to evaluate the living cell density within them. To estimate the time-zero cell mass, the viable cell mass was determined from the mean of two fibers on the implantation day. For each fiber, the cell density on retrieval day was expressed as net growth (%) defined as:

\[
\text{Net growth} = \left( \frac{OD_{\text{retrieval day}} - OD_{\text{implantation day}}}{OD_{\text{implantation day}}} \right) \times 100
\]

Hence, a net growth of -100% represents total cell kill, while a value greater than 0% represent growth in the fiber compared with implantation day. Values greater than 0%, but less than control growth, represent a partial effect of the drug (i.e. growth inhibitory effect).

Cycloviolacin O2 showed concentration-dependent cytotoxicity towards all tumor cell lines in the hollow fiber assay in vitro, with IC_{50} values in the low micromolar range. Hence, the cyclotide is able to penetrate the polyvinylidene fiber and the three-dimensional architecture of cells inside, and express activity in the proliferative heterogeneity of the tumor microenvironment. However, despite being robust, the subcutaneous implantation of fibers also provides a highly resistant model that show only modest responses to several standard cytotoxic drugs (Alley,
1988; Hollingshead, 1995). This is probably related to the design of the model, with tumors located in a poorly vascularized environment in which nutrient and drug exchange is dependent on diffusion (Phillips, 1998).

One day after fiber implantation, mice received a single intravenous dose of 1.0 mg/kg cycloviolacin O2. Disappointingly, despite dosing close to MTD no significant effects of the cyclotide were detected after the trial was terminated five days later. There are several possible explanations for its apparent effects in vitro, but lack of in vivo. A fundamental reason for the failure of a compound to induce response in vivo is that the concentration of the compound at the target (which may be influenced by a number of factors, including the route of administration, the administration vehicle, metabolism and excretion) may be insufficient. In solid tumors there may be additional factors, such as poor vasculature and poor tissue penetration (Double, 2004). Therefore, an additional xenograft study was needed, using the tumors established over sufficient time to allow in-growth of capillaries and (hence) greater penetrations of nutrients and drugs. Furthermore, repeated dosing would increase the probability of a positive outcome with respect to anti-tumor effects, given cycloviolacin O2’s high MTD.

6.3 Xenograft studies

Twenty-eight female nude mice (NMRI nu/nu) were engrafted with colorectal adenocarcinoma HT29 cells subcutaneously in the flank. After a 0.2 mL tumor was established, animals were randomly assigned to receive cycloviolacin O2 (1.0 mg/kg) or a control treatment every weekday (i.e. not Saturday and Sunday) for two weeks. After randomization of four animals to the treatment arm it was evident that this dose resulted in discoloration of the tail. Hence the dose was reduced to 50% (0.5 mg/kg) for the remaining animals, and no local effects were observed at this dose (n=5). Treatment continued for two weeks and the tumor volume was measured every other day (Figure 17).

Previously, no signs of acute toxicity were observed at 1.5 mg/kg of cycloviolacin O2. In the xenograft study, mice receiving repeated doses of 1.0 mg/kg suffered from a local reaction at the injection site, probably because repeated puncture of the mid tail vein and the high local concentration of cycloviolacin O2 during injection (and perhaps small venous rafts) resulted in tissue damage. Repeated injections of a less concentrated solution (0.5 mg/kg) did not cause these problems, but had no detected effects on tumor growth in this model.
Figure 17. (A) Tumor growth volume (mm$^3$) in the xenograft study after treatment with cycloviolacin O2. Each point represents the mean ± SEM. (B) Tumor weight at day 14 in the xenograft study after treatment with cycloviolacin O2. Each bar represents the mean ± SEM.

A slight dose-dependent, but not statistically significant, effect on hematology was seen after repeated dosing. Animals receiving repeated injections of cycloviolacin O2 showed no signs of discomfort, gained weight like the controls (Table 8, on page 47) and presented with normal hematology at the end of the study, but with a non-significant trend for suppression of red blood cells, hemoglobin, hematocrite, and thrombocytes after treatment with cycloviolacin O2 (Table 9). Hemolytic effects (on red blood cells) of different cyclotides have been previously described (Simonsen, 2004), but this is the first indication of selectivity between different hematologic lineages.

Table 9. Hematology of animals receiving repeated injections (control, 0.5 or 1.0 mg/kg) of cycloviolacin O2. Average values are given ± SD.

<table>
<thead>
<tr>
<th>Hematology</th>
<th>Control</th>
<th>0.5 mg/kg</th>
<th>1.0 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell (RBC)</td>
<td>8.2 ±0.6</td>
<td>7.6 ±0.7</td>
<td>7.2 ±0.4</td>
</tr>
<tr>
<td>Hemoglobin (Hb)</td>
<td>137 ±9</td>
<td>131 ±9</td>
<td>127 ±4</td>
</tr>
<tr>
<td>Hematocrite (Hct)</td>
<td>0.40 ±0.03</td>
<td>0.39 ±0.03</td>
<td>0.37 ±0.02</td>
</tr>
<tr>
<td>Trombocyte (Trc)</td>
<td>967 ±173</td>
<td>917 ±111</td>
<td>856 ±234</td>
</tr>
</tbody>
</table>
7. Discussion and future perspectives

7.1 Isolation and structure elucidation

Since the beginning of cyclotide research, methods for their isolation and structure elucidation have developed dramatically. The early methods were very time and resource consuming. Over time, knowledge has grown regarding the cyclotides and new methods for investigating them have been developed and are now in use. Methods like MS have simplified their sequencing, but is still time consuming. To date, approximately 200 cyclotide sequences have been described, 2/3 of which belong to the bracelet subfamily, the rest to the Möbius subfamily.

In Paper II the number of cyclotides in the Violaceae was estimated to be in the range 5,000 - 25,000, while in the Rubiaceae the number has been previously estimated to be between 10,000 - 50,000 (Gruber, 2008). Adding these values gives a total between 15,000 and 75,000, which clearly illustrates the plasticity of the cyclotide scaffold and presumably important role of cyclotides in plant defense. It also makes the cyclotides one of the largest known groups of plant proteins, and considering that they may also be present in other plant families, the total number of naturally occurring cyclotides are probably even higher.

Figure 18 shows the loops between each of the cysteines of the two subfamilies. By combining these loops with each other, theoretically the immense total of over 600 million different possible cyclotides can be formed. We can also use our knowledge concerning sequence data to synthesize new non-native cyclotide sequences for structure-activity studies, or to design artificial cyclotides, with active sequences from other sources (Craik, 2007).

In order to map the occurrence and distribution of this vast number of cyclotides advances in analytical techniques and instrumental sensitivity are required. Present methods for extracting, isolating and elucidating the structure by MS-MS involve too many steps and are too time and resource consuming for high-throughput analysis. If convenient methods could be developed to solve problems associated with these features they would be highly beneficial not only for cyclotide research but for all work on disulfide-rich peptides, e.g. scorpion and spider toxins, defensins, thionins and conotoxins.
Figure 18. Intercysteine loops of Möbius and bracelet cyclotides illustrating the plasticity of the cyclotide scaffold. The peptide backbone is depicted in black, disulfides are in grey, and the β-sheet is indicated by arrows. The numbers of different loops are displayed in parentheses. The hybrid cyclotides are included within the bracelet subfamily, linear cyclotides are excluded.
7.2 Cyclotide evolution and occurrence

Presently, cyclotides have only been found in two phylogenetically distant plant families, the Violaceae and Rubiaceae. All species of Violaceae examined to date contain cyclotides (Paper II), and it would be of interest to look for them in closely related plant families. In the family Rubiaceae cyclotides have only been found in a minority of species, mostly members of a few tribes (Gruber, 2008). In the related family Apocynaceae small peptides with a cyclized backbone and six cysteines have been found, but not sequenced. Further, it has been predicted that cyclotides will be found in a much wider group of plant families than is currently known (Craik, 2010). The lack of discovery so far may due to the cyclic peptides being intrinsically more difficult to sequence than conventional linear peptides and/or to cyclotides being present in lower abundance in some plant families with the paucity of peptide screens in them and/or natural product discovery have been focused on molecules with molecular weight under 1000.

A cyclotide is defined as a peptide of approximately 30 amino acids with a cyclized backbone and six cysteine residues involved in three disulfide bonds, i.e. containing the CCK motif (Craik, 1999). Suggestions have been made to expand the cyclotide family to include two circular trypsin inhibitors with the CCK motif from the Cucurbitaceae. Considering the strict definition, they should be included, but these two proteins have more sequence similarity with other, linear variants of trypsin inhibitors, hence they do not have the same phylogenetic history as the other cyclotides.

The same problem, but in reverse is posed by the two linear variants of the cyclotides, violacin A (Ireland, 2006b) and psyle C (Gerlach, 2010), which by the definition are not cyclotides, their sequences lack a key Asn (or Asp) residue involved in processing. As do the recently reported cyclotide-like nucleic acid sequences that are present in monocotyledonous plants such as rice, wheat and corn, which are part of the Poaceae plant family (Basse, 2005; Mulvenna, 2006a). However, none of these nucleic acid sequences have been detected at the peptide level, and it remains to be discovered whether these mRNAs are translated into proteins.

This raises questions about whether cyclotides should be redefined, i.e. whether we should continue to use the current, strictly structural definition of cyclotides or develop a new one, including peptides presumed to have the same phylogenetical origin, excluding small proteins like the trypsin inhibitors of the Cucurbitaceae. I favor the latter, covering only cyclotides with common ancestry, and in my opinion the current knowledge support the fact that cyclotides have evolved gradually from linear precursors, hence linear variants should be included as cyclotides. I am confident that we will find expressed cyclotides or traces of the ancient
genes in increasing number of species from a wide range of plant families and that in the future we will be able to explain the diverse occurrence the cyclotides now seem to have.

7.3 Toxicity and anti-tumor activity

This thesis focuses on the potent cytotoxic effects of cyclotides. **Paper III** presents new insights into their structure–activity relationships, and we know that the cyclotides do not share a mechanism of action with any clinically used anticancer agent (Lindholm, 2002). **Paper IV** and **V** extended our knowledge of their action by investigating the kinetics of their cytotoxicity, proposing a mode of action, considering their membrane affinities and disrupting abilities, and correlating the findings to their cytotoxic properties.

In **Paper VI**, we further extended the cyclotide investigations by examining their effects on animals. First, the MTD was determined for cycloviolacin O2 (2.0 mg/kg) to establish a tolerable dose in mice for the efficacy tests. The observations are congruent with results for the first discovered cyclotide, kalata B1 (Gran, 1973a), which has been studied *in vivo* in rats and rabbits (Gran, 1973b). In that study animals received intravenous doses of kalata B1, which was found to have a marked effect on their circulation. Electrocardiography showed that it caused ventricular tachycardia and the lethal dose, which resulted in ventricular fibrillation, was estimated to be 1.0 mg/kg for rats and 1.2 mg/kg for rabbits.

The anti-tumorous effects of cycloviolacin O2 *in vivo* were evaluated using two different mouse models. First, hollow fiber assay *in vitro* showed that the cyclotide can penetrate several cell layers and retain its activity in proliferate heterogeneity of tumor microenvironment, but despite dosing close to MTD no significant effects in hollow fiber assays *in vivo* were detected. Subsequently we turned to a xenograft model using repeated administration; cycloviolacin O2 did not show significant activity in that assay either.

Thus, the studies this thesis is based upon highlight a series of requirements to facilitate continued exploration of the cytotoxic activity of cyclotides, and their possible utility as anticancer agents. These include needs: to find ways to increase the cyclotide concentrations in the proximity of tumors, *e.g.* by clarifying cyclotide distribution and clearance routes; to increase their selectivity; to change the route of administration; and/or to increase the therapeutic window, primarily by minimizing their acute toxicity.

Although the results from this *in vivo* study are discouraging for the direct application of cycloviolacin O2 as an anticancer agent, the study has providing insights into the process of extending cyclotides applica-
tions. Indeed, to decrease or abolish cyclotides’ effects is one key to their future as a grafting scaffold, i.e. bearer of introduced biological effect.

7.4 Potential applications

A unique feature of cyclotides is their extreme thermal, chemical, and enzymatic stability (Colgrave, 2004). Proteins are otherwise usually considered to be rather fragile biomolecules that are easily degraded by chemical and biotic factors. In Paper II we show that their full structures are retained intact in preserved leaves for almost 200 years, and I am confident that they could be found intact in even older material. Clearly, cyclotides have an extreme structural stability that lacks parallels in other protein families. Their natural role is thought to be as plant defense agents, most notably against insect pests, but they also have potential applications in drug design and agriculture.

There has been some interest in commercializing cyclotides and a number of patents concerned with cyclotides have been awarded, including (inter alia) the following:

- WO2006041917 (Herrmann et al) discloses that cyclotides are active against homopteran insects.
- WO0127147 (Craik et al) discloses a novel cyclic molecular framework comprising so called cyclotides, i.e. cyclic peptides. These compounds are claimed to be usable for treatment or prophylaxis of disease conditions in animals, mammals and plants.
- WO0068265 (Ouelette et al) discloses pharmaceutical compounds based on cyclic peptides.
- WO2004055044 (Bohlin et al) discloses cyclotides as an on-growth inhibiting agent, for the inhibition and/or prevention of on-growth of biological organisms on objects or living beings.
- WO2006076189 (Lu et al) discloses compositions and methods for regulating expression of heterologous nucleotide sequences in a plant. Compositions include a novel nucleotide sequence for a root-preferred promoter for the gene encoding Cyclo1. The invention relates to the construction of a gene encoding all or a portion of plant cyclotides.

Whether cyclotide patents will be further developed and commercially used remains to be seen. The patents mainly concern aspects of cyclotide activity or inventions regarding cyclotide genes. As I see it, cyclotides can be exploited in many ways both in agricultural and pharmaceutical applications (Figure 19). Some developments toward such applications have already been made, e.g. a cyclotide gene has been transferred to
crop plants in an attempt to improve the crops’ natural defenses against pests (Gillon, 2008). The successful start i.e. production of transgenic plants, is promising, but the studies so far have relied on the insertion of only a single gene. Co-expression with auxiliary proteins such as folding and circularization enzymes from cyclotide-bearing plants has the potential to increase the yields.

Figure 19. Overview of potential pharmaceutical (to the right) and agricultural (to the left) applications of cyclotides. In agricultural applications, cyclotide gene sequences could be expressed in crop plants to enhance resistance to pests, while in pharmaceutical applications biologically active peptide epitopes could be grafted into the CCK framework of natural cyclotides. A link between pharmaceutical and agricultural applications is indicated with an arrow; pharmaceutically modified cyclotides might, in the future, be produced in plants or plant cell cultures, via transformation with genes encoding modified cyclotides.

Another potential application is to directly use cyclotides’ pesticide effects directly to inhibit the growth of bacteria, algae, and fungi. Cyclotides have already been shown to have potent, nontoxic and reversible effect against fouling barnacles (Göransson, 2004a). Further investigations are required to optimize the cyclotides’ potency against additional organisms growing on surfaces or degrading building material. A suitable formulation (e.g. a paint) to deliver the cyclotides also has to be developed.

Pharmaceutical applications include the incorporation of small peptide epitopes with desired biological functions into the CCK motif, essentially using the cyclotide framework as a delivery vehicle for stabilizing peptides. For this, synthetic methods for producing and modifying cyclotides are required. This concept has been proven (Gunasekera, 2008; Thongyoo, 2009), but further preclinical and clinical tests are needed. All peptides are susceptible to degradation, and to enhance their therapeutic potential different approaches for improving stability have been de-
veloped. With the inspiration of the cyclotides’ stability cyclization of the peptide backbone via the joining of the N- and C-termini with peptide linkers are favored. An example is a recent study of a natural α-conotoxin, Vc1.1 with exciting potential for the treatment of neuropathic pain (by inhibiting the HVA Ca\(^{2+}\) channel currents via activation of GABA\(_B\) receptors) (Clark, 2010). By engineering a cyclic Vc1.1 analogue they were able to increase the stability in gastric fluid, simulated intestinal fluid, and human serum. Funds are at the moment raised so enough preliminary experiments can be done to file an Investigational New Drug Application.

After a lead compound has been identified, the development of large-scale processes is complex and expensive. However, a possible solid phase peptide synthesis approach for generating high-value, pharmaceutically grafted cyclotides is to generate plant cultures that express them. Suspension cultures and hydroponic plants of *Oldenlandia affinis* have already been established that produce milligrams of cyclotide per gram dry weight (Seydel, 2007; Dörnenburg, 2008).

We also have traditional use of a few *Viola* species using extracts containing cyclotides, *e.g.* the use of *Viola yedoensis* as a tea to treat toxic heat, swelling, carbuncles, sores, boils, snake bites, bronchitis, hepatitis, acute nephritis, appendicitis, and enteritis (Qin, 1995) and use of *Viola tricolor* in folk medicine externally and internally as an adjuvant in treating various skin conditions such as eczema, impetigo, acne and pruritus also internally as an auxiliary agent to promote metabolism (Bisset, 1994) and the European Medicines Agency is in the process of making a monograph over *Viola tricolor* with the indication symptomatic treatment of mild seborrhoeic skin condition.

7.5 Concluding remarks

In this thesis, work has been done in refining the analytical and preparative methods of cyclotides. Totally, 25 novel cyclotide proteins and precursor sequences have been characterized. From the large-scale mapping of cyclotides conclusions were that cyclotides are expressed in plants as a cocktail of up to 25 different cyclotides per species. The cyclotides in the cocktail have individually high activity against certain targets, less against others, but collectively excellent potency against multiple targets.

Most cyclotides found in a species are also unique to that species, although it was apparent that many cyclotides occurred in more than one species. In particular, the cyclotide varv A, E and kalata B1 were abundant in the *Viola* species. The total number of different cyclotides in the Violaceae has been estimated to be in the range 5,000-25,000. The large-scale mapping of cyclotide covered approximately 1/6 of all species in
the Violaceae, such that that we can now definitely conclude that cyclotides are ubiquitous in this plant family.

To understand structure-activity relationships of cyclotides, particularly regarding charged and hydrophobic residues approximately 30 native and chemically modified cyclotides have been tested for cytotoxicity. The results show that an intact hydrophobic patch is important for maintaining cytotoxic activity. Charged amino acids in the right positions are also favorable for additional activity, probably through electrostatic interactions with the target membrane.

The thesis also includes work the show that cytotoxic properties are connected to membrane-disrupting effects, which also can explain many of the cyclotides other biological activities. The effects generally appear quick, *e.g.* the cell death occur within minutes. The adsorption to the membrane and lytic effects are affected by the phospholipid composition and may explain the different activity profiles in different assays.

*In vivo* tolerability and anti-tumor activity of cyclotides were examined, using two different methods: the hollow fiber method with single dosing and traditional xenografts with repeated dosing over two weeks. Before these tests a maximal tolerated dose (MTD) was also determined. The injections of cyclotide did not give effects on tumor growth in these models.

For the future, I am eager to know if the cyclotides may be found in larger parts of the plant kingdom and how it may be such a sprinkled distribution of cyclotides in nature. The evolution of cyclotides is intriguing and studies in this field may give the answers. Another question is if we can exploit the cyclotides biological activities and unique stability to develop them for therapeutic or agricultural use. Research takes time and money, and advances are made step by step. The final conclusions concerning the implications of cyclotide lies in the future ahead.

### 7.6 Personal reflections

I started my research carrier in the fun and enlightening research summer school (SOFOSKO) on CYP enzymes and fatty acids supervised by Professor *Ernst Oliw* and Doctor *Katarina Stark*, in which I worked on techniques as PCR, western blotting, HPLC and MS. This led to my first publication and I am grateful to both of them for that great introduction to the scientific community. During work for my master thesis I changed department and started my current studies on cyclotides at the Division of Pharmacognosy.

Before the start of my doctoral studies I was involved in a project to collect species from the Panamanian forests. My contribution was to extract and screen species from the Rubiaceae and Violaceae for cyclotide
contents. When I came back home to Sweden to continue my project I was hooked on the fascinating cyclotides. It was then I realized that I wanted to continue working on cyclotides as a post-graduate student.

The aim of my doctoral studies was, briefly, to study the cyclotide occurrence in Violaceae and to investigate cyclotides’ activity and mechanism. It has been an intriguing journey that has concerned many parts of the subject pharmacognosy, from meeting native Indians in the rainforest to using modern techniques in a well-equipped lab. I have also been fortunate enough to be awarded scholarships to participate in congresses in various parts of the world, most recently in Australia where I met the discoverer of the cyclotides, Lorents Gran (Figure 20).

When I first started I was the youngest member of the Division of Pharmacognosy, a novice who probably asked a lot of questions. Now I am one of the most experienced and people ask me for advice. The last five years has been five years well spent on a most intriguing journey from a young fledgling to a full-fledged scientist!

Figure 20. A photograph of Lorents Gran and myself, taken during the 1st International Conference on Circular Proteins in Australia, October 2009.
Människan har sedan urminnes tider använt växter som läkemedel men genom utvecklingen i samhället har vi i modern sjukvård lämnat användandet av örtteér, växtextrakt och tinkturer. Vi har numera övergått till att inrika oss på att finna enskilda substanser i dessa växter, som vi använder istället för själva växten. Utvecklingen har inte gått så långt att vi klarar oss utan växterna eller andra levande organismer. Naturen är fortfarande den största källan till att finna nya läkemedel och dessutom odlas de ofta för att tillverka de läkemedel som redan finns ute på marknaden.

Den här avhandlingen handlar om en grupp substanser som kallas cyklotider. Upptäckten av cyklotiderna går tillbaka till 60- och 70-talen då professor Finn Sandberg och en norsk läkare vid namn Lorents Gran obeboende av varandra observerade att afrikanska kvinnor använde en ört för att påskynda barnafödandet vid förlossning. Lorents Gran tog med sig växten (Oldenlandia affinis) tillbaka till Norge och isolerade en substans som kunde orsaka livmodersammandragningar. Det var upptäckten av den första cyklotiden. Idag nästan 40 år senare har forskningen på cyklotiderna tagit fart och det har nu skrivits mer än 200 artiklar i ämnet.

Cyklotider är en grupp av proteiner som har speciella egenskaper, vilket skiljer dem ifrån andra proteiner. Vanliga proteiner består av en linjär kedja med aminosyror och har alltså två ändar. Cyklotiderna är som namnet antyder cykliska och saknar alltså ändar på sin kedja av aminosyror och dessutom är de väldigt kompakt. Dessa egenskaper gör dem ytterst stabila mot värme, kemiska och enzymatiska påfrestningar, vilket är mycket ovanligt för proteiner som annars är väldigt lätt nedbrutna. Som bevis för cyklotidernas extrema stabilitet visas i denna avhandling att de är intakta i torkat växtmaterial i nästan 200 år!

Från några violarter har vi isolerat och lyckats strukturutreda samman-
tagat 25 nya cyklotider, bl.a. luktviol som växer i Sverige men även andra arter som växer på vitt skilda platser över hela världen. Det kan ses som få jämfört med de tusental som finns totalt men isolering och strukturut-
redning är en tids- och kostnadskrävande procedur och hittills har bara totalt runt 200 strukturutretts.

Några av de isolerade cyklotiderna testades sedan för celldödande egenskaper på odlade cancerceller. Efter att ha testat tillräckligt många cyklotider kunde deras struktur jämföras mot den celldödande aktiviteten och på så sätt få har vi fått en ökad förståelse för vad i strukturen hos cyklotiden som är viktig för dess effekt. Cyklotiderna har också visas sig ha en mängd andra intressanta aktiviteter t.ex. så är de antibakteriella, antivirala, och hämmar insekt- och larvtillväxt. Orsaken till att växten producerar dessa substanser tror vi är till försvar mot yttre angrepp.

Efter att ha fått en god förståelse över vad som är viktigt i strukturen hos cyklotider för den celldödande effekten ville jag förstå exakt hur de dödar cancerceller, dvs utreda cyklotidernas mekanism. Detta har stude-
rats bl.a. genom att undersöka cancerceller som blivit utsatta för en dos av cyklotider i mikroskåp. Resultaten visade att cyklotiderna dödade can-
cercellerna mycket snabbt, inom 15 minuter genom att förstöra cellens yttre lager, det så kallade cellmembranet. Mer detaljer hur de binder in och förstör cellmembranet har sedan undersömts i efterföljande studier.

Cellers membran består bland annat av fosfolipider som i sin tur är uppbyggda av ett vattenlösligt huvud med två svansar av fettsyror. Från ren fosfolipider kan man tillverka runda liposomer som modell för en cells membran men som har andra kemiska egenskaper. Om man dessut-
om kapslar in en fluorescerande molekyl inuti liposomerna kan man måta förstörelsen av liposomer efter tillsats av substans, i detta fall cyklotider. Analysen från denna studie gav oss en ökad förståelse på vilket sätt cyk-
lotiderna kan förstöra cellmembran och därigenom döda cancerceller.

Som ett sista projekt har vi studerat cyklotidernas effekt på möss. Mössen klarar av att få doser av cyklotid upp till 1,5 mg/kg utan att de påverkas nämnvärt. En något lägre dos på 1,0 mg/kg gavs till möss som hade mänskliga tumörer och sedan mättes tillväxten hos tumören. Resultat-
ten jämfördes med möss som enbart fick koksaltlösning och tyvärr sågs ingen skillnad i effekt mellan de två olika behandlingarna. Detta tror vi beror på att cyklotiderna har svårt att nå tumören i mössen och vi på nå-
got sätt måste lösa det innan vi kan gå vidare med dessa studier.

Denna avhandling spänner från upptäckt av nya cyklotider, deras can-
cercellödande effekt till att utreda deras mekanik och verkan i biolo-
giska system. Forskningen på cyklotiderna fortsätter och i framtiden vet vi förhoppningsvis mer och kanske kan vi utnyttja deras unika stabila och potenta effekter.
9. Acknowledgement

The work presented in this thesis was carried out mainly at the Division of Pharmacognosy, Department of Medicinal Chemistry, Faculty of Pharmacy, Uppsala University. Travel grants and stipends from Apotekare C.D. Carlssons stiftelse and IF:s stiftelse enabled me to attend scientific congresses and go to field trips.

I wish to express my sincere gratitude to all those who contributed to the realization of this work. I would like to thank all of them, particular the following:

My outstanding supervisor, Ulf Göransson, for your commitment and interest in my scientific work, your critical optimism and for all your help in the lab as well as in the writing of papers. You have been my mentor and have become a really good friend.

My assistant supervisor Lars Bohlin for your unlimited enthusiasm for the subject, for giving me excellent chances to network and for your warm heart. I also wish to thank Lars and Janne Bruhn for introducing me to pharmacognosy about ten years ago!

All former and present members of the cyclotide group: Per Claeson for making Ulf such a good scientist, Erika Svedlund for arousing interest in cyclotides in me, Anders Herrmann for teaching me HPLC and MS, Teshome Leta Aboye and Mariamawit Yeshak for introducing me to your indigenous food and culture, Sunithi Gunasekera for sharing office space with me without complaining about any of my habits, SungKyu Park for continuing with work on cyclotides and Adam Strömstedt for being my membrane mentor.

All other “pharmacognosist”: Anders Backlund for all your botanical knowledge you so kindly shared with me, Hesham El-Seedi for your unlimited devotion to science, Jenny Felth for your amicableness and as my first choice travel companion to conferences. I also wish to show my gratitude to: Ulrika Melin, Petra Lindholm, Sonny Larsson (also for all the memorable lunches at Pharmen), Martin Sjögren, Erik Hedner (also for all the laps in Stabby Forest), Catarina Ekenäs and Josefin

61
Rosén for showing the way to go! Stefan Svahn, Cecilia Alsmark, Christina Wedén and Elisabet Vikeved for expanding the subject both scientifically and by the coffee table.

The supporting staff, Kerstin Ståhlberg, Maj Blad, Gunilla Eriksson and Anna-Maria Andersson. Without you all work stops at the department!

The undergraduate students over the years that has been involved in the project: Kristina Rizzardi, Gustav Karlsson, Marta Bajona Roig, Linn Kristin Born, Rasha Tamin, Anette Jansson, Neeti Vaghela, Stefano Melillo, Linnan Hao, Rosetti Tran and Hanna Justad.

All the people at the Division of Analytical Pharmaceutical Chemistry; Curt, Douglas, Per, Ylva, Olle, Henrik, Matilda, Annica, Marika, Anita, Jacob, Victoria, Alex and Axel, for all nice discussions around the coffee table.

Co-authors and collaborators at the Division of Clinical Pharmacology for all the help over the years. I would especially like to thank Rolf Larson for providing excellent facilities and Joachim Gullbo for all your knowledge and help in the cancer field. I also wish to show my gratitude to Henrik Lövborg, Sadia Hassan, Malin Wickström, Linda Richardsson, Christina Leek and Lena Lenhammar.

Co-authors and collaborators at Medical Biochemistry and Microbiology, Dan Andersson, Maria Pränting and Camilla Lööv for showing cyclotides to be antimicrobial.

Co-authors and collaborators at Pharmaceutical Physical Chemistry, Martin Malmström, Lovisa Ringstad and Lotta Wahlberg for all your help and knowledge shared during our studies on liposomes and membrane adsorption.

Co-author and collaborator at Department of Ecology and Evolutionary Biology, Tulane University, USA, Samantha Gerlach, for all the nice moment during your stay in Uppsala.

To my colleagues in Australia, David Craik for increasing interest in cyclotides in the scientific community, Christian Gruber for reintroducing me to the biochemical side of things, Richard Clark for letting me sleep in your basement (feel free to sleep in mine) and Johan Rosengren for all the NMR teaching you have given me.
To Mahabir P. Gupta and all others at his lab at the University of Panama for introducing me to your nice country. I also want to thank Therese Landolsi for taking the opportunity to go with me to Panama, without you I would not have gone and I think back on this time as one of the best.

My close friends, Clara Hallén for the chit-chat by the HPLC as master students, Johan Alsiö for being like me in so many ways, Einar Larsson and Cecilia Tegelberg for always being there when you were most needed, Per Stenqvist for all the good times, and Jonas Engfors for all the magical moments.

To Hanno Lindroth, Daniel and Isa Petrini for welcoming me into your family.

My mother Birgit and late father Lage, my brother Joachim, his wife Sara and their wonderful children, Filippa, Hanna and Lovisa. You have always been there and supported me during the ups and downs in life. I never have to feel lonely knowing you all are there!

To all other friends and relatives, not mentioned here. You mean a lot to me and I am glad to be so fortuned as to have you all in my life!

Last but far from least, to my love for life Jessica and our expanding family! 💖


Qin P, Chen QP, Wang BR, Shi LW. In Species Systematization and Quality Evaluation of Commonly Used Chinese Traditional Drugs. Lou ZQ, Qin P, editors. Beijing: Beijing Medical University and Beijing Union Medical University; 1995;


Wang CK, Colgrave ML, Ireland DC, Kaas Q, Craik DJ. Despite a conserved cystine knot motif, different cyclotides have different membrane binding modes. Biophys J. 2009a, 97(5):1471-1481.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 130

Editor: The Dean of the Faculty of Pharmacy

A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy”.)