Mistletoes and Thionins

as Selection Models in Natural Products Drug Discovery

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Abstract

The process of drug discovery from natural products starts with the selection of study object. In this project recent knowledge and methods are incorporated to investigate the process of such selection for pharmacognostic investigations. As the model and object of study mistletoes and their content of the small cytotoxic peptides thionins are chosen.

The thionins are compared in silico to other proposed plant innate defense peptides. Utilizing analysis of amino acid sequences and secondary structures, the thionins are shown to be one of eight distinct groups of cystein-rich plant polypeptides analysed. Common features of thionins are exploited in an investigation of isolation methods, where a simple acidic extraction is equally efficient to isolate thionins as the laborious methods hitherto used.

An effort to study the relationships of the order Santalales was done. To infer phylogenetic relationships from DNA sequences, we increased the taxon sampling for utilized genes and regions such as rbcL, atpB and ribosomal 18S and 26S rDNA sequences within the Santalales. Analysing these together with published sequences for other tricolpate taxa a position for Santalales as sister to caryophyllids and basal to asterids is implied. This indication is supported by chemical characters such as the presence of cyclopeptide alkaloids of a kind only known from Gentianales.

To validate the chemosystematic implications from thionin distribution extracts of mistletoes collected in Panama, Taiwan and Madagascar, and the relative Osyris alba (Santalaceae) collected in Spain, were screened with the established fluorescence microculture cytotoxicity assay using the thionin-sensitive human lymphoma cell-line U937GTB. Bioassay guided isolation concludes that the cytotoxic compounds in Loranthaceae may however constitute another group of peptides.

In conclusion this work shows that the incorporation of informatic techniques may aid prediction and decision making when planning pharmacognostic research.

Keywords: drug discovery, mistletoe, phylogeny, cytotoxic peptides, Santalales

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urn:nbn:se:uu:diva-7705 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7705)
...his task had never been to undo what he had done,
but to finish what he had begun.

A Wizard of Earthsea
*Ursula K. Le Guin*
List of Papers

This thesis is based on the following papers, referred to in the text by their roman numerals:

I  Larsson, S. & Backlund, A.  
Delineating small plant polypeptides – with a proposal on their classification and circumscription.  
(submitted to *Proteins: Structure, Function and Genetics*)

II Larsson, S., Tamimi, H., Jonsson, M., Gupta, M. & Backlund, A.  
Comparison of extraction methods for alkaline thionins from mistletoes  
(submitted to *Peptides*)

III El-Seedi, H. R., Larsson, S. & Backlund, A.  
Chemosystematic value of cyclopeptide alkaloids from *Heisteria nitida* (Olacaceae)  

IV Larsson, S., Chase, M. W. & Backlund, A.  
An asterid connection for the order Santalales  
(*manuscript*)

Screening for small cytotoxic peptides in Loranthaceae and Santalaceae  
(submitted to *Journal of Biomolecular Screening*)
## Abbreviations and conventions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rDNA</td>
<td>nuclear gene for the 18S subunit of ribosomal nucleic acid component</td>
</tr>
<tr>
<td>26S rDNA</td>
<td>nuclear gene for the 26S subunit of ribosomal nucleic acid component</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid/s</td>
</tr>
<tr>
<td>APG</td>
<td>Angiosperm Phylogeny Group</td>
</tr>
<tr>
<td>APG2</td>
<td>APG-classification latest update</td>
</tr>
<tr>
<td>atpB</td>
<td>chloroplast gene for the β-subunit of adenosine triphosphate synthase</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>HPLC with ultraviolet absorption detection</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>matK</td>
<td>chloroplast gene for the lysine transport ribonucleotide acid maturase</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>rbcL</td>
<td>chloroplast gene for the large subunit of ribulose-bisphosphate carboxylase/oxidase</td>
</tr>
<tr>
<td>SI</td>
<td>survival index</td>
</tr>
</tbody>
</table>

Appendix A. Scientific nomenclature of botanical entities discussed in this thesis, with comments.

Appendix B. List of thionins, including synonyms and references, discussed in this thesis.
1. Introduction

1.1 Pharmacognosy

The word pharmacognosy is derived from two Greek words *fármako* (φάρμακο), meaning drug, and *gnósi* (γνώση), meaning knowledge. In explaining the role of the pharmacognosist, the definition of the subject by Samuelsson (Samuelsson 2004) can be used:

The subject of pharmacognosy deals with natural products used as drugs or for the production and discovery of drugs.

In retrospective this echoes one of the original definitions for the pharmacist, from the emergence of the profession in the 11th century (as cited in (Tschanz 2003)):

[The pharmacist is defined] as the professional who is specialized in the collection of all drugs, choosing the very best of each simple or compound, and in the preparation of good remedies from them, following the most accurate methods and techniques as recommended by experts in the healing arts.

from as-Saydanah fit-Tibb by Abu ar-Rayhan al-Biruni (*973–†1048)

And since all drugs in the 11th century were derived from Nature, every pharmacist at this time was also a pharmacognosist. However, today the knowledge of drugs has become the working field for a number of specialists. The pharmacologist works with the mechanism and action of drugs, the pharmaceutics expert formulates the drugs for best possible administration to patients, the pharmacokineticists studies how often and how big the doses should be taken and given, the medicinal chemist synthesizes new compounds to use as drugs, and so forth. The subject of pharmacognosy still depends on a multidisciplinary approach. In dealing with natural products as drugs the identification of the crude material constituting the drug is a necessary step and requires knowledge in botany, zoology or geology, depending on the drug. For the production of the drug insight in all forms of chemistry is a prerequisite and in drug discovery, the most sought after subdiscipline, you may have to turn to a spectrum of tools ranging from chemo- and bioinformatics, physical chemistry and molecular biology over to such “soft” sciences as history or anthropology.
In an effort to contain this multidisciplinary feature of pharmacognosy in an easily explained model, Bruhn and Bohlin have proposed a three-point/three-edge model (Bruhn and Bohlin 1997). This model, as redrawn in figure 1, shows the relationship between the three entities the organism, the biological activity, and the chemical structure.

![Figure 1. This explanatory model describes the connections between biology, pharmacology and chemistry in the multidisciplinary science of modern pharmacognosy (Bruhn and Bohlin 1997).](image)

Along the interconnecting edges this model contains elements necessary for drug discovery using natural products. As an example, the classic ethnopharmacological approach relies on the observation of the effect (biological activity) of a remedy (organism) in a certain disease (bioassay) when prepared (separation) and administered in a particular way (depending on the chemical characteristics). It is these recurring connections between the three points of the triangle that characterize the science of pharmacognosy.

### 1.2 Ethnopharmacology

Drug discovery based on ethnopharmacology is probably one of the oldest strategies for finding new drugs, effectively used since the origins of human history. The fundamental principle is that if the use of a specific organism for treatment of a specific illness has survived through the changing history of a human culture, the organism is bound to actually have an effect on this disease.

No person willingly will take an inactive drug when he or she is sick. No human will patronize an ineffective medicine man when he or she is ill. In consequence of this natural selection, most of the plant drugs used in primitive areas have some real benefit and relatively little toxicity.

Ethnopharmacology, as defined in the inaugural editorial for the Journal of Ethnopharmacology, truly encompasses the multidisciplinary approach (Rivier and Bruhn 1979), and at the beginning of the 21st century it still seem to be one of the main reasons for pharmacognostical studies. This since no less than 64% of published articles in Planta Medica during July 2003–June 2004, referred to studies instigated for ethnobotanical reasons (Dr Anders Backlund, personal communication). As a measure of the success of ethnopharmacology Fabricant and Farnsworth presents 122 naturally occurring compounds used as drugs, and out of these 88 are specified as having pharmacological effects in accordance to the use of their corresponding traditional botanical drug (Fabricant and Farnsworth 2001). The remaining 34 will be discussed in chapter 1.4 later, in another perspective relating to screening. Example substances derived from ethnopharmacological efforts and approved as drugs in Sweden include digoxin and digitoxin from foxgloves (Digitalis species), ephedrine from Ephedra species, and codeine and morphine from the opium poppy (Papaver somniferum). However successful, there are several challenges to modern ethnopharmacology, e.g. how to acknowledge the intellectual property rights (Soejarto, Fong et al. 2005), to take the holistic approach of many ethnomedical systems into account for evaluating and measuring ethnopharmacological effects (Verpoorte, Choi et al. 2005), and how to return scientific findings to the ethnopharmacological practitioner (Jäger 2005).

The mistletoes and their relatives within the order Santalales, have a strong ethnopharmacological track record. Sandalwood oil from Santalum album is used in traditional Indian medicine as e.g. a genito-urethral remedy, a soothing component for treating headaches and as a diaphoretic and diuretic (Roberts 1931; Khare 2004). When this highly valued medicinal oil through trade became available in Arabic medicine during early medieval times, it resulted in the new profession of the pharmacist being known as sandalani – [he] who trades in sandalwood oil (Tschanz 2003). The spectacular parasitic habit of mistletoes, and the fact that they often are evergreen, seems to have enticed cultures throughout their distribution range. In northern Europe Viscum album, besides its place as a charm against all evil in druidic tradition and as the modus operandi in the killing of the Viking god Balder, have been used for epilepsy, cancer and as a cardiotonic (Anderson and Phillipson 1982; Wagner, Feil et al. 1984; Franz 1985). The Maori tradition says that the mistletoes grows in the crowns of other trees because when the creator Tane saw the last plant in his planting basket he decided that he could not leave it on the ground (Riley 1994). The New Zealand mistletoes are reported to be used as cardiotonics and in the form of poultices for treating itches (Brooker, Cambie et al. 1981). In Africa many mistletoes are known as “all-healers” and bone-setting drugs (Dalziel 1937), in Southeast Asia they are used against malaria, as tonics and against cancer (Lohézic-Le Dévéhat, Bakhtiar et al. 2002). In South America the uses as
cardiotonics and cancer remedies imported with the European settlers (Fernández, Wagner et al. 1998; Varela, Fernández et al. 2004), are in agreement with the traditional use against cancer (Rios, Salinas et al. 2001; Rios and Aguilar-Guadarrama 2004). In addition to these, the uses of mistletoes include that as an antiseptic against erysipelas (Frei, Heinrich et al. 1998), for headache, and as a diuretic or contraceptive (Filipov 1994).

1.3 Systematics and chemosystematics

Systematic and classificatory efforts to understand the world around us have been boldly made throughout human history, and in a more comprehensive way since the classic era of Dioscorides. The science of systematics deals with conceptions of relatedness. Up until the establishment of the Darwinian ideas of evolution and its underlying mechanisms, this relatedness did not necessarily have anything to do with kinship in an evolutionary sense, but could be based on completely different perspectives. The plant system devised by Linnaeus (Linnaeus 1753) is based on the simple estimate of number and arrangement of male and female parts of the flower. These are criteria easy to follow, and for compiling a catalogue over God’s creation extremely efficient and user friendly. This system was not, however, free from criticism, and arguments that classification should take all possible characters into account was put forward already by contemporary workers, e.g. the French botanist Adanson (Adanson 1763).

These thoughts were the foundation for the successive emergence of first a natural classification, and as the Darwinian theories gained weight also the primordia of a modern phylogenetic classification, e.g. as described and implemented by Haeckel, the result depicted in figure 2 (Haeckel 1866), and later Hennig (Hennig 1966). Within a phylogenetic classification taxa are arranged in an evolutionary sequence. This starts with plesiomorphic groups featuring primitive traits and end with derived, apomorphic, groups united by shared advanced features, synapomorphies. The evolutionary processes are taken into account as each group should consist of all descendants, and only the descendants, of their common ancestor. If this is true for a group, it is said to be monophyletic. Which characters that should be used, and in what way they should be arranged and considered to infer phylogenies, was to begin with largely a question of the practising systematists opinion. To the original morphological characters technical advances during the 19th and 20th century have added e.g. anatomical and palynological characters from microscopy.
Figure 2. Conspectus of main evolutionary groups among life on Earth, as presented by Haeckel (Haeckel 1866).
Numerous reviews and textbooks have been written on the development of methods for phylogenetic analysis. Different schemes of analysis with large differences in their philosophical underpinnings were devised with *e.g.* cluster analysis and neighbour joining (Kimura 1983; Nei 1987; Saitou and Nei 1987) emerging among molecular biologists, maximum likelihood (Felsenstein 1973) from geneticists, and parsimony analysis (Hennig 1950; Hennig 1966; Kluge and Farris 1969) and most recently Bayesian inference (Huelsenbeck, Ronquist et al. 2001) from systematic biologists. Regardless of the ingenious algorithms and computational shortcuts, the probably most important aspect of these methodological advances lies in the greatly improved philosophical basis and discussion on the phylogenetic hypothesis *per se*. The strength in these hypotheses can be seen during the last decades of the 20th century when the widespread angiosperm classifications of Cronquist (Cronquist 1981), Dahlgren (Dahlgren 1980), and Takhtajan (Takhtajan 1997), were challenged by techniques to isolate and describe stretches of DNA combined with rigid phylogenetic framework of analysis and evaluation.

Starting at the end of the 19th century, a systematic effort to connect observations of chemical compounds with knowledge on biological systematics fostered what is known as chemosystematics. Initially the purpose was to better comprehend patterns of observations, but gradually this evolved into a tool for elucidating evolutionary pathways. A huge potential break-through in the understanding of chemosystematics came with the emergence of modern methods for phylogenetic analysis. The basis for this is that characters or traits, also in the shape of biosynthesis machineries, are inherited along the evolutionary lineages. If these traits provide an increase in organism fitness, the traits will be retained. On the other hand, as the overwhelming proportion of novel mutations is non-beneficial or even lethal, the *de novo* appearance of such traits is very rare. The loss of a single enzymatic step within such biosynthetic machinery may however eradicate the presence of an *e.g.* medicinal compound. This can be seen in the case of *Papaver somniferum*, the only source of the analgesics codeine and morphine, where a single induced gene mutation makes the plants accumulate biosynthetic precursors but no active compounds (Millgate, Pogson et al. 2004). However, in most cases where the presence of such secondary metabolites has a measurable importance with regard to fitness, there are also complex and strong mechanisms striving to retain and conserve such important machinery. Hence, a specific and functioning biosynthetic pathway is a very strong phylogenetic argument for shared ancestry.

Extending this line of thought implies that a phylogenetic classification by itself provides a predictive value and framework for evaluating how traits are passed down through the lines of ancestry. And in reverse this also makes it possible to use such a classification for predictions *e.g.* of the presence of pharmacologically active substances.
1.4 Bioassay guided screening

Screening programs are based on the availability of a biological assay able to represent the underlying reason for disease and a rational for testing compounds or extracts in that assay. This is empirical drug discovery, and a field that has been very much affected by technical advancements and ethical considerations.

The most basal form of pharmacological bioassay is the Hippocratic screening, where the tested drug is injected intraperitoneal on rats and a number of reactions are observed during a specified time (Malone and Robichaud 1962; Malone 1983). As this is done in series with a number of animals for each of the tested drug concentrations, with careful observation of reactions after specific intervals of time, often extending over days of work, it is a cumbersome and expensive method. It has been followed by a range of more specific assays, in order of lower complexity: isolated organs exposed to drugs such as the guinea pig ileum assay measuring contraction or inhibition of contraction by smooth muscle; isolated or cultivated cells e.g. measuring survival; subcellular systems such as enzyme activity of microsomes or protein synthesis of ribosomes; isolated enzymes or receptors measuring binding affecting a biological activity (Vlietinck 1999). In efficacy terms they can also be labelled as low-throughput and high-throughput assays, since the laborious Hippocratic screening can handle only a few samples at a time, while specific mechanism-based assays such as enzyme inhibition often can be done automatically with many samples at the same time. Using assays in broad screenings makes it possible to discover biological activities that are not in accordance with ethnopharmacological plant use or have new mechanisms of action. Fabricant and Farnsworth identified 34 drugs having such a history, among them the well known cancer drugs vincristine and vinblastine from *Vinca rosea*, a plant originally used to treat diabetes (Fabricant and Farnsworth 2001).

In a recent review by Sams-Dodd (Sams-Dodd 2006) screening approaches in drug discovery are presented. The two major methods are the physiology-based and the mechanism-based screenings. The physiology-based method is the oldest, utilizing isolated tissues or whole animals to study drug effects (*i.e.* alleviation of symptoms), and the mechanism-based method is more recent and relies upon specific targets such as enzymes or receptors (*i.e.* a specific mode of action). The third approach mentioned, the function-based method, aims to normalize a function instead of just simply alleviate symptoms, but as “normal function” is the result of a multitude of processes the set-up and evaluation of such a screening is in need of technical developments.

In practical pharmacognostic research, the biological assay is one of the most important tools, and in many cases the only way to detect, confirm and follow the active substance through an isolation process.
1.5 The origin of drugs

So where do drugs come from? As stated above all drugs were derived from natural products from the beginning. According to the World Health Organization traditional medicine, such as herbal drugs, accounts for 80% of the primary care in Africa. They also state that 25% of our modern pharmaceuticals are derived from plants with traditional use (WHO 2003). In a survey of new registration drugs for the period 1981-2002 Newman et al. (Newman, Cragg et al. 2003) argues that this figure actually is 57%, maybe even as high as 67% if compounds modelled on a natural compound or a natural compound receptor site is included. In figure 3, showing NCE registration by year, it can be seen that the recent decline in registration of new drugs is due to the fact that fewer totally synthetic compounds reach the market.

Figure 3. Diagram showing the number of new chemical entities approved as drugs by the FDA from 1981–2002 (Newman, Cragg et al. 2003). When split into compounds with natural origin, including those modeled on natural substances and receptor sites (green), and synthetic compounds (red), it is evident the number of drugs derived natural products have outnumbered the synthetic compounds for almost twenty years.

Contemplating these impressive figures, it appears clear that Nature will also in the future be a major source not only of resources, but also biological and chemical inspiration. A well know, and often quoted, example in pharmaceutical sciences is that of Lipinski’s’ rule of five (Lipinski, Lombardo et al. 1997). This set of rules summarized the physical-chemical properties that could be expected of a successful drug-like chemical compound. However, scrutinizing this set of rules, it is completely clear that Lipinski based them
on characteristics of a comparably limited set of compounds that would be absorbed after oral administration. Applying similar methods, but with a somewhat larger scope, it can be convincingly demonstrated that the known biologically active compounds largely invalidates these rules. One way to express this in a graphical way is to apply chemometric methods and plot compound characteristics in a corresponding chemical space. Several studies (e.g. (Larsson, Gottfries et al. 2005; Lloyd, Golfis et al. 2006; Larsson, Gottfries et al. 2007)) have shown that there are vast stretches of chemical space still to explore, that are not void of possibly interesting, biologically active, and natural chemical compounds.

1.6 Old and replaced?

But in the end new discoveries in pharmacology and basic knowledge of disease can lead to a re-evaluation of already known compounds.

In the Iliad by Homer (7-8th century BC.) the shipmates of Odysseus are saved from Circe’s poisonous spell by the drug moly, which has been proposed to be the snowdrop Galanthus nivalis of the family Amaryllidaceae (Plaitakis and Duvoisin 1983). Later reports from the 1950’s state that snowdrops were used against poliomyelitis in the Ural region, and that the compound galanthamine antagonize the effects of non-depolarising neuromuscular blocking agents such as curare (Heinrich and Teoh 2004). The latter effect is the result of the alkaloids inhibition of acetylcholine esterase and allosteric agonism on nicotinergic acetylcholine receptors (Läkemedelsindustriföreningen 2006). Galanthamine was approved in Sweden for use against Alzheimer’s disease in March 2000 (Swedish Medical Products Agency, website www.lakemedelsverket.se), thus bringing to market a drug that may trace it’s history over almost 3000 years.
2. Aims of the thesis

The work presented in this thesis is part of the research program on selection at the Division of Pharmacognosy, Department of Medicinal Chemistry, Uppsala University, aimed at exploring means of rational selection, and eventually prediction, when choosing objects of study for pharmacognostic endeavours.

The specific objectives of the present thesis are:

- to use a combined chemical and bioinformatic approach in circumscribing and testing the integrity of groupings of thionins and other small plant derived peptides,
- to compare and refine the extraction protocols for small plant peptides with the specific purpose to increase the yield of thionins, thus enabling the necessary rapid isolation and analysis needed for thorough screening,
- to probe the usefulness of phytochemical data in conjuncture with proposed phylogenies to understand patterns of distribution of natural products/chemical substances,
- to explore the thionins evolutionary history by use of DNA sequencing in conjuncture with phylogenetic analysis in an attempt to place Santalales within the tree of life,
- to validate the phylogenetically based prediction on presence of cytotoxic peptides in Loranthaceae and not previously investigated Santalaceae.
3. The interest for small peptides

Small proteins and peptides play an important role in our daily life. Examples from human physiology are: insulin (51 aa, ~5.8 kDa) and glucagon (29 aa, ~3.5 kDa) i.e. responsible for regulating glucose metabolism in the body; dynorphin (17 aa, ~1.6 kDa) as one of several neuropeptides functioning as endogenous ligands to opiate receptors (endorphins); and angiotensin II (8 aa, ~1.0 kDa) involved in upholding normal blood pressure (Guyton 1992).

That peptides could play systemic roles also in plants was proved in 1991, when Pearce and co-workers showed that it is an 18 aa peptide, systemin, which is responsible for the induction of proteinases after wounding of tomato leaves, as discovered twenty years earlier (Green and Ryan 1972; Pearce, Strydom et al. 1991). Today several other systemically active peptides, as well as their receptors, have been described from plants (Lindsey, Casson et al. 2002; Matsubayashi and Sakagami 2006), and similarities of plants and animals have resulted in the proposition to use plants as disease models for various aspects of human illness (Guttman 2004; van Baarlen, van Belkum et al. 2007).

The possible gains from this, both ethical and scientifically, are obvious.

3.1 Small defence peptides in plants

During the last few decades, interest in small peptides as part of our defense against pathogenic bacteria, fungi and even multicellular parasites have intensified the research into this probably ancestral system. Defense peptides have been described from many organisms and are present also in those lacking a cell-mediated immunosystem, such as bivalves and arthropods. Structural investigation have confirmed their similar structures (Pardi, Zhang et al. 1992; Landon, Sodano et al. 1997; Yang, Mitta et al. 2000).

Some groups of defense peptides are also described from unicellular organisms such as bacteria. Plectasin, a peptide derived from the filamentous fungi Pseudoplectania nigrella (Sarcosomataceae) confirms the presence of the structural-type of the defensins among not only plants, insects, arthropods and mammals, but also in the fungi (Mygind, Fischer et al. 2005). From an evolutionary standpoint this appears highly appealing (e.g. earlier discussed by Hoffmann, Kafatos et al. 1999)).

Historically, comparison of peptides was done by amino acid composition, size, electrophoretic mobility, and in vitro biological activity (e.g.
(Daley and Theriot 1987)). In hindsight, and with the aid of technical breakthrough in amino acid sequencing and peptide structure elucidation, it can be concluded that this in some cases led to a great nomenclatural confusion. For the thionin family, research started in the early 1940’s when the extraction of wheat flour showed that a factor lethal to yeast consisted of a peptide mixture. Once purified the peptides were named thionins after their high sulfur content (Balls, Hale et al. 1942). In a somewhat later and unrelated investigation into the hypotensive mistletoe component visco toxin, it was shown that this entity also consisted of a high-content cystein peptide mixture (Samuelsson 1958). In 1965 a very hydrophobic peptide, crambin, was isolated from Crambe with an equally high content of cystein (VanEtten, Nielsen et al. 1965). This has been followed by a virtual flood of thionins, both isolated chemically and discovered through means of molecular biology (exemplified in table 1). Reviews of thionins include, for the chemically isolated ones (Garciá-Olemdo, Rodríguez-Palenzuela et al. 1989; Bohlmann and Apel 1991; Bohlmann 1994; Florack and Stiekema 1994; Garciá-Olemdo, Molina et al. 1998), and also incorporating some of those implicated from molecular biology (Stec 2006).

Many of these reviews also treat other groups of putative defence peptides from plants, such as e.g. plant defensins, lipid transfer proteins, hevein-like and knottin-like peptides. When compiling information about thionins, the first of these exemplified groups causes numerous false hits in text-based searches. This is due to their unfortunate name, γ-thionins, coined for peptides isolated from Triticum and Hordeum with assumed homology to thionins due to structural and functional similarities, such as number and composition of amino acids, and inhibition of protein synthesis in cell-free in vitro assays (Colilla, Rocher et al. 1990; Mendez, Moreno et al. 1990). When the NMR structure of these peptides were solved it was evident that they had virtually no structural resemblance to thionins, but instead contained a motif found in arthropod toxins and defensins (Bruix, Jiménez et al. 1993). This lead to a proposal to name these peptides as plant defensins (Broekaert, Terras et al. 1995), but the implementation of this name have thus far had only limited success, as shown by recent reviews of the group (Thomma, Cammue et al. 2002; Pelegrini and Franco 2005). Examples of other ambiguous names for plant defensins include the likewise unfortunate pseudothionin (Moreno, Segura et al. 1994), and ω-thionin (Mendez, Rocher et al. 1996). The most manifest example of this nomenclatural confusion between thionins and defensins, is the report of a defensin from Vigna unguiculata, simply named cowpea-thionin by the authors (Melo, Rigden et al. 2002).
Table 1. Origin and sequence of selected thionins, with names used in this thesis (complete list, synonyms and references can be found in Appendix B).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Thionin</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>arathionin A</td>
<td>KICCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>arathionin B</td>
<td>KICCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>arathionin C</td>
<td>KICCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>arathionin D</td>
<td>KICCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td>Avena sativa</td>
<td>avenothionin A</td>
<td>KSCCMTLRCYHLCRCRGAPKL--CATVCRCKISSGLS-CPKDFPK-</td>
</tr>
<tr>
<td></td>
<td>avenothionin B</td>
<td>KSCCMTLRCYHLCRCRGAPKL--CATVCRCKISSGLS-CPKDFPK-</td>
</tr>
<tr>
<td></td>
<td>avenothionin C</td>
<td>KSCCMTLRCYHLCRCRGAPKL--CATVCRCKISSGLS-CPKDFPK-</td>
</tr>
<tr>
<td></td>
<td>avenothionin D</td>
<td>KSCCMTLRCYHLCRCRGAPKL--CATVCRCKISSGLS-CPKDFPK-</td>
</tr>
<tr>
<td></td>
<td>avenothionin E</td>
<td>KSCCMTLRCYHLCRCRGAPKL--CATVCRCKISSGLS-CPKDFPK-</td>
</tr>
<tr>
<td>Brassica juncea</td>
<td>brassijuthionin</td>
<td>KCCCPSTQAMYLYTCNSRLTP-1CISHTGIEK-1CISHTGIEK-1CISHTGIEK-CPGPPY-</td>
</tr>
<tr>
<td>Brassica rapa</td>
<td>brassirathionin</td>
<td>KCCCPSTQAMYLYTCNSRLTP-1CISHTGIEK-1CISHTGIEK-CPGPPY-</td>
</tr>
<tr>
<td>Crambe hispanica subsp. abyssinica</td>
<td>crambethionin A</td>
<td>TTCCPSIVARANSVLQCLPSTPEAL-CAYTTCI1IPGAT-CPGIAN-</td>
</tr>
<tr>
<td></td>
<td>crambethionin B</td>
<td>TTCCPSIVARANSVLQCLPSTPEAL-CAYTTCI1IPGAT-CPGIAN-</td>
</tr>
<tr>
<td></td>
<td>crambethionin C</td>
<td>TTCCPSIVARANSVLQCLPSTPEAL-CAYTTCI1IPGAT-CPGIAN-</td>
</tr>
<tr>
<td></td>
<td>crambethionin D</td>
<td>TTCCPSIVARANSVLQCLPSTPEAL-CAYTTCI1IPGAT-CPGIAN-</td>
</tr>
<tr>
<td></td>
<td>crambethionin E</td>
<td>TTCCPSIVARANSVLQCLPSTPEAL-CAYTTCI1IPGAT-CPGIAN-</td>
</tr>
<tr>
<td>Dendrophthora clavata</td>
<td>denclathionin B</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td>Helleborus purpurascens</td>
<td>hellethionin A</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>hellethionin B</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>hellethionin C</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>hellethionin D</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>hellethionin E</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td>hordothionin A</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
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<td>hordothionin B</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
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<tr>
<td></td>
<td>hordothionin C</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>hordothionin D</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>hordothionin E</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>osthionin A</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>osthionin B</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
<tr>
<td></td>
<td>osthionin C</td>
<td>KCCCPSTQAKHTGTVVCRKLRFSPK--CMQVSGCQNSDT---CPRGWVN-</td>
</tr>
</tbody>
</table>
Oryza sativa  

| osthionin D | KSCCPYTARNIYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| osthionin E | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| osthionin F | KSCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| osthionin G1 | KSCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| osthionin G2 | KSCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Phoradendron liga  

| ligathionin A | KSCCPYTARNIYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| ligathionin B | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Phoradendron serotinum  

| phorathionin A | KSCCPYTARNIYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| phorathionin B | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| phorathionin C | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| phorathionin D | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| phorathionin E | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| phorathionin F | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Phyllostachys pubescens  

| bamboothionin A | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| bamboothionin B | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Polygonum sibiricum  

| polygonumthionin | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Pyrularia pubera  

| pyrulariathionin | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Salvia miltiorrhiza  

| salviathionin | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Secale cereale  

| secalethionin | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Triticum aestivum  

| purothionin A1a | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| purothionin A1b | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| purothionin B | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Tulipa gesneriana  

| tulipathionin A | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| tulipathionin B | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| tulipathionin C | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| tulipathionin D | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| tulipathionin E | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |

Viscum album  

| viscothionin A1 | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin A2a | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin A2b | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin A3a | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin A3b | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin B | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin C1 | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin D | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin E | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
| viscothionin P1 | KSCCPSTARNYHBCFALGFTQRE-CASLSGCKKVVD-GK-CWAYH |
In an effort to test the integrity of thionins as a plant peptide group, an analysis of +1100 amino acid sequences derived from BLASTp (Altschul, Gish et al. 1990) searches was performed. The searches were seeded by selected amino acid sequences of published proposed antimicrobial peptides, as presented in table 2, with an emphasis on small peptides rich in cysteins.

Table 2. The peptides used in BLASTp searches to compile the matrix used for peptide group comparisons.

<table>
<thead>
<tr>
<th>Group in reviews</th>
<th>Peptide</th>
<th>Origin</th>
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</thead>
<tbody>
<tr>
<td>thionin</td>
<td>purothionin B</td>
<td>Triticum aestivum</td>
</tr>
<tr>
<td>γ-thionin type I</td>
<td>pseudothionin</td>
<td>Solanum tuberosum</td>
</tr>
<tr>
<td>γ-thionin type II</td>
<td>defensin 2</td>
<td>Spinacia oleracea</td>
</tr>
<tr>
<td>defensin type I</td>
<td>antifungal peptide 1</td>
<td>Heuchera sanguinea</td>
</tr>
<tr>
<td>defensin type II</td>
<td>antimicrobial peptide 1</td>
<td>Aesculus hippocastanum</td>
</tr>
<tr>
<td>lipid transfer protein type 1</td>
<td>barley LTP 1</td>
<td>Hordeum vulgare</td>
</tr>
<tr>
<td>lipid transfer protein type 2</td>
<td>onion LTP 1</td>
<td>Allium cepa</td>
</tr>
<tr>
<td>cyclotide, Moebius type</td>
<td>kalata B2-precursor</td>
<td>Oldenlandia affinis</td>
</tr>
<tr>
<td>cyclotide, bracelet type</td>
<td>vitri A</td>
<td>Viola tricolor</td>
</tr>
<tr>
<td>cyclotide, anomalous</td>
<td>palicourein</td>
<td>Palicourea condensata</td>
</tr>
<tr>
<td>hevein-like peptide I</td>
<td>hevein</td>
<td>Hevea brasiliensis</td>
</tr>
<tr>
<td>hevein-like peptide II</td>
<td>antimicrobial peptide 1</td>
<td>Mirabilis jalapa</td>
</tr>
<tr>
<td>hevein-like peptide III</td>
<td>antimicrobial peptide 1</td>
<td>Amaranthus caudatus</td>
</tr>
<tr>
<td>protease inhibitor, Cucurbitaceae type</td>
<td>trypsin inhibitor 1</td>
<td>Trichosanthes kiriowii</td>
</tr>
<tr>
<td>protease inhibitor, Bowman-Birk type</td>
<td>double-headed</td>
<td>Dolichos biflorus</td>
</tr>
<tr>
<td>protease inhibitor, Kunitz type</td>
<td>Bowman-Birk inhibitor</td>
<td>Glycine max</td>
</tr>
<tr>
<td>protease inhibitor, soybean trypsin inhibitor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results were checked to eliminate identical hits in different searches, and trimmed to contain the longest form for each peptide (i.e. every mature peptide with identical sequence in a retrieved precursor peptide was deleted), this to avoid entries of only partially sequenced peptides. For the defensin- and “γ-thionin”-groups, lipid transfer proteins type 1 and 2, the cyclotide types and the hevein-like peptides I–III, a large number of identical hits were found in the different searches. These groups were thus subsequently pruned not to include redundant sequences. The resulting sequence data was, for each group, subjected to alignment with ClustalX 1.83 software (Higgins, Thompson et al. 1996), and retrospectively adjusted by hand taking into account also structural features not interpretable by ClustalX. Among the retrieved sequences were several groups of peptides circumscribed and published elsewhere represented, including e.g. lectins and chitinases, miraculin-like peptides and sporamin-type storage proteins.
The corresponding structural files deposited in the Protein Data Bank (Berman, Westbrook et al. 2000), were collected, and in conjuncture with these an amino acid sequence matrix for the mature peptides (excluding signal peptides, and in some cases C-terminal peptides) was constructed. The alignment between the different peptide groups maximized the comparison between simplified structural motifs (α-helices and β-sheets, see figure 4) and position of cysteines.

![Figure 4. The simplified secondary structures for the eight groups of peptides in I.](image)

The matrix was subjected to clustering analysis by neighbour joining (Saitou and Nei 1987), and the resulting tree diagram is shown in figure 5. This tree diagram is not intended to show any phylogenetic relationship between the groups of peptides, as there are no assumptions of homology apart from what is discussed above. The methodology is here used to test means of how to implement structural information in delimitation of groups with similar functionality. The results indicate that the thionins are a distinct group excluding the γ-thionins, however, the proposed division of thionins into subgroups from amino acid sequences is not supported. In the resulting tree diagram there are eight groups of well supported ‘peptide families’ as deduced from the structural alignment: defensins (including γ-thionins and insect α-amylase inhibitors), chitin binding peptides (including hevein-like peptides
and lectins), thionins, cyclotides, Bowman-Birk type protease inhibitors, Cucurbitaceae type protease inhibitors, Kunitz type protease inhibitors (including miraculin-like and sporamin-type peptides), and lipid transfer proteins.

Figure 5. Cluster analysis tree diagram of plant polypeptide amino acid sequences for the eight well-defined families in I: defensins, chitin-binding peptides (CBP), thionins, cyclotides, Bowman-Birk type protease inhibitors (BBI), Cucurbitaceae protease inhibitors (CPI), Kunitz type protease inhibitors (KPI), and lipid transfer proteins (LTP).

3.2 Methods for small peptide isolation

Since peptides are made up of amino acids their characteristics will be dependent upon the amino acid composition, sequence, and the resulting structure. This is readily seen when comparing isolation methods for thionins. The two first described groups of thionins are purothionins from wheat flour (Balls and Hale 1940; Balls, Hale et al. 1942), and viscothionins from the European mistletoe (Enders, Feuchtinger et al. 1940; Winterfeld and Bijl 1949). The methods used in these respective isolations differ significantly. While the wheat flour was extracted with the lipophilic solvent mixture petroleum ether, the viscothionin mixture was instead isolated with hydrophilic glacial acetic acid. The lipophilic extraction method used by Balls and Hale for the purothionins (Balls and Hale 1940), probably extracts the thionins in
the form of a peptide-lipid complex. This conclusion is supported since the purified purothionins themselves are not soluble in petroleum ether or any other lipophilic solvent. The hydrophilic extraction protocol devised by Enders and co-workers, on the other hand, makes perfect sense with respect to the thionin primary sequence. Both the purothionins and the viscothionins contain significant proportions of the alkaline amino acids arginine and/or lysine, giving them a positive net charge, and consequently good solubility in aqueous solvents and high isoelectric points. These facts were broadly employed during the three decades of extensive investigations of mistletoe thionin isolation by Samuelsson and co-workers (Samuelsson 1958; Samuelsson 1959; Samuelsson 1961; Samuelsson and Ekbлад 1967; Mellstrand and Samuelsson 1973; Samuelsson, Pettersson et al. 1977; Thunberg and Samuelsson 1982; Thunberg 1983).

In the late 1990’s a research program aiming towards broad investigations of small plant peptides was initiated at the Division of Pharmacognosy at Uppsala university, and as a result of this a method for their isolation was developed (Claeson, Göransson et al. 1998). The aim of this protocol was a final fraction enriched in small to medium sized peptides by removing low- and high-molecular compounds such as polyphenols, polysaccharides, enzymes and other large proteins. This protocol was successful in the isolation of the first varv cyclotide peptides from Viola arvensis (Göransson, Luijendijk et al. 1999), and have been further used and modified for isolation of cyclotides from other violets and related genera in Violaceae (Göransson, Luijendijk et al. 1999; Broussalis, Göransson et al. 2001; Svangård, Göransson et al. 2003; Göransson, Svangård et al. 2004; Svangård, Göransson et al. 2004). The non-exclusivity of any such wide aiming isolation protocol is however demonstrated by the identification of cardiac glycosides as the active cytotoxic compounds in the fraction thus obtained from Digitalis purpurea (Johansson, Lindholm et al. 2001; Lindholm, Gullbo et al. 2002).

While the technical advances for peptide isolation during the last decades are readily shown by the isolation of four additional thionins from Phoradendron serotinum in extracts previously shown to contain phoratoxin A and B (Samuelsson and Ekbлад 1967; Mellstrand and Samuelsson 1973; Thunberg 1983; Johansson, Gullbo et al. 2003), and while the non-exclusivity mentioned above can be traced and evaluated, one major obstacle to large scale screening for plant peptides still remained – the time and labour requirements for each isolation. The work from plant collection to reasonably purified, detectable and testable peptide in 1998 ranged from one to several weeks per sample. So how should one go about to investigate on a broader scale the presence of thionins in plants?

This question is addressed in II by comparing different protocols for extracting a thionin-enriched fraction from Viscum album. Four protocols were modified from previously published methods: one from the plant peptide protocol developed at the division (Claeson, Göransson et al. 1998); one
developed for cyclotides, exploiting their hydrophobic character (Broussalis, Göransson et al. 2001); and two specifically used for the isolation of viscothionins (Tonevitsky, Agapov et al. 2001; Tabiasco, Pont et al. 2002). In this comparison all these methods ended with the identical combination of an ion exchange chromatography step, followed by a desalting size exclusion chromatography prior to the final high-pressure liquid chromatography. In addition to these four methods, a further simplification was investigated where the ion exchange step (and thus also the desalting) was omitted. In figure 6, the different steps included in each method are outlined for easy comparison.

Figure 6. Schematic comparison of the extraction protocols investigated in II. Methods I-IV are modified from previously published protocols: I (Tonevitsky, Agapov et al. 2001), II (Claeson, Göransson et al. 1998), III (Broussalis, Göransson et al. 2001), and IV (Tabiasco, Pont et al. 2002). Method V is the modified protocol here proposed to be used in screening for cytotoxic thionins.
The resulting chromatograms after extraction of equal amounts of plant material, see figure 7, show that the simple acetic acid extract (method I) yields the highest amounts of thionins, and that the complicated protocol incorporating three subsequent partitionings (method IV) gives the lowest yield of thionins. In addition, the investigation shows that even though thionins and cyclotides share a structural feature of pronounced amphipathicity, the butanolic extraction that appears to work very well for cyclotides (method III), is not suitable for thionin isolation. The modification of the protocol developed for the screening of peptides within plant material (method II), seem to be almost as efficacious as the simple acid extraction but involves several more steps.

![Figure 7](image_url)

*Figure 7. Qualitative comparison between the four extraction procedures from RP-HPLC. The chromatograms are from the extraction methods as described in figure 6. As shown the same peaks can be found in all four methods.*

As the ion exchange chromatography is a laborious step, or in need of specific technical apparatus, a protocol omitting this step was eventually devised (method V). Since the extraction method V is intended to simplify isolation of thionins as well as to supply fractions testable for biological activity, a polyamid filtration step was included between the acidic extrac-
tion and the high-pressure liquid chromatography step. This process utilizes the feature that polyamid gels have a strong affinity for polyphenols (also called tannins) at acidic pH, thus removing substances often known to evoke false positive responses in biological assays (Loomis and Battaile 1966; Cardellina II, Munro et al. 1993; Wall, Wani et al. 1996). Omitting the ion exchange step saves considerable time but on the other hand results in an extract rich also in compounds other than thionins. For the actual isolation of thionins this seem to be of minor importance, as the thionin peaks in the *Viscum album*-extract are still separable from the rest of the compounds, but naturally it has to be taken into consideration that results from bioassays using such an extract may be influenced by these impurities.

To verify that the isolation protocol was applicable to thionin screening, it was used for material from *Phoradendron quadrangulare*, a species which had not been previously investigated for thionins. The results, as shown in figure 8, clearly displays the presence of thionins as compared to the corresponding extract of *Viscum album*.

![Figure 8](image_url)

**Figure 8.** Superimposed chromatograms for extracts of *Viscum album* and *Phoradendron quadrangulare* made with method V from II, showing the empirically determined thionin-elution time window in grey.

### 3.3 Structure dependent activity

As to emphasize the variation in Nature a thionin without the otherwise characteristic high content of arginine or lysine was in 1965 isolated from *Crambe hispanica ssp. abyssinica* through an acetone/water extraction followed by removal of the acetone and thus precipitating the raw peptides (VanEtten, Nielsen et al. 1965).
Crambin is thus far the only hydrophobic thionin isolated, and this feature made it ideal for early 3D structure elucidation (Teeter and Hendrickson 1979; Llinás, De Marco et al. 1980; Hendrickson and Teeter 1981). By modelling the major purothionin and viscothionin amino acid sequences on the crystal structure of crambin, it could be unambiguously shown that these three peptides share the same conformation and should be treated within the same peptide group (Whitlow and Teeter 1985). Several 3D structures also for other thionins are now described, e.g. the X-ray crystallography structure of purothionin A1a (Teeter, Ma et al. 1990), the nuclear magnetic resonance spectroscopy structures of viscothionin A3a (Romagnoli, Ugolini et al. 2000), and hellethionin D (Milbradt, Kerek et al. 2003), confirming the common structure. A more comprehensive summary can be found in table 3.

The structural features of thionins can be described, in a generalized form utilising the secondary structures, as a $\beta$-$\alpha$-$\alpha$-$\beta$ backbone stabilized with interconnecting disulfides through presence of six or eight cysteines. The 3D structure resembles the greek letter $\gamma$ (or an inverted latin L), where the short arm represents the small antiparallel $\beta$-sheet and the long arm is composed by the two antiparallel $\alpha$-helices, as can be seen in figure 9.

Figure 9. 3D structure of four thionins from *Viscum album* viscothionin A2b (1JMN), A3a (1ED0), B (1JMP) and C1 (1ORL) demonstrating structural homogeneity. In the representation above $\beta$-sheets are marked with yellow (with the exception for viscothionin B, as in the corresponding PDB file $\beta$-sheet borders are not defined) and $\alpha$-helices by red.
<table>
<thead>
<tr>
<th>Taxon</th>
<th>Peptide</th>
<th>Technique</th>
<th>PDB entry</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Triticum aestivum</em></td>
<td>purothionin A1a</td>
<td>X-ray diffraction</td>
<td>2PLH</td>
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<td>1WUW</td>
</tr>
<tr>
<td><em>Viscum album</em></td>
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<td>viscothionin B</td>
<td>NMR spectroscopy</td>
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<td>1ORL</td>
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<td><em>Helleborus purpurascens</em></td>
<td>hellethionin D</td>
<td>NMR spectroscopy</td>
<td>1NBL</td>
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<td>X-ray diffraction</td>
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<td>NMR spectroscopy</td>
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</table>
4. Prediction as a tool for systematic screening

4.1 Angiosperm phylogenetics

During the last decades there have been an enormous activity in inferring phylogenies of the angiosperms in order to deduce the evolution of flowering plants. In this work molecular techniques have been imperative and thus inferred molecular phylogenies have made it possible to re-evaluate the characters used before the emergence of polymerase chain reaction and genetic sequencing. The APG classification for the angiosperms in figure 10 (Angiosperm Phylogeny Group 1998; Angiosperm Phylogeny Group 2003), is compiled from several workers broad phylogenetic analyses, and tries to maximize the information content in each group without violating the criteria of monophyly (see e.g. (Backlund and Bremer 1998) for a discussion on the concept). As can be seen from figure 10, there are still unresolved relationships between several groups, which in most cases are subject for further studies. One of the groups that at present remains without a firm systematic placement is Santalales. This may be explained by factors such as their reduced flowers and common parasitic life modes making homology assumptions difficult.

As the substance class of alkaloids in many cases are comparably easy to isolate, often have a somewhat restricted distribution between species, and often a spectacular pharmacological activity, they early become popular chemosystematic characters. This even though the simple presence of alkaloids as tested through colorimetric reactions or precipitation with chemical reagents such as the Dragendorff reagent, have no bearing on phylogeny as the class is biosynthetically heterogenous (Hegnauer 1988). That there are three different routes for the biosynthesis of piperidine alkaloids is perhaps the strongest argument for the necessity of minding biosynthetic pathways when interpreting alkaloid characters. It also illuminates the restricted value of broad compilations making general statements on the presence or absence of alkaloids (e.g. (Willaman and Li 1970)).
Figure 10. The orders of the phylogenetic classification of the angiosperms suggested by the APG (Angiosperm Phylogeny Group 2003), with the here used informal superordinal names following proposals of Bremer and co-workers, and Judd and Olmstead (Bremer, Bremer et al. 2002; Judd and Olmstead 2004).

For Santalales there are yet another obstacle in interpreting the occurrence of alkaloids, as many species through their parasitic connection can accumulate secondary metabolites synthesized in their host. This is for example known for pyrrolizidine and quinolizidine alkaloids detected in *Osyris alba* (Santalaceae) when growing near alkaloid producing hosts (Woldemichael and Wink 2002). In III the isolation of the cyclopeptide alkaloid anorldianine from the non-parasitic santalaceous plant *Heisteria nitida* (Olacaceae) prompted us to investigate the potential chemosystematic information contained in their structures.
The probable precursors for the cyclopeptide alkaloids are the corresponding unmodified tetra- or pentapeptides, as shown from studies on callus cultures of the Rhamnaceae-species of *Ceanothus americana* (Baig, Banthorpe et al. 1993), as outlined in figure 11.

![Figure 11](image-url)  
*Figure 11. The proposed biosynthetic pathway of cyclopeptide alkaloids, showing two possible paths to a saturated bridge in the tyrosine-derived substructure.*

No congruent classification of cyclopeptide alkaloid structures seem to exist. In recent reviews they are divided based on the identity of the \(\beta\)-hydroxy amino acid necessary for ring closure, sometimes in combination with the number of amino acids (Gournelis, Laskaris et al. 1997; Pomilio, Battista et al. 2006; Tan and Zhou 2006). When applying this criterion four groups can be discerned, increasing to seven if they are split according to size: 1) \(\beta\)-hydroxyleucine with four or five amino acids, 2) \(\beta\)-hydroxyphenylalanine with four amino acids, 3) \(\beta\)-hydroxyproline with four or five amino acids, and 4) with any other \(\beta\)-hydroxy acid and four or five amino acids. Within the last group the review by Tan and Zhou only place ceanothine D from *Ceanothus americanus* (Rhamnaceae) with \(\beta\)-hydroxyisoleucine and four amino acids, and hymenocardine from *Hymenocardia acida* (Phyllanthaceae) with \(\beta\)-hydroxyvaline and five amino acids (Tan and Zhou 2006). The chemosystematic information contained in this classification is very low. This as only the third group has a restricted distribution, and thus potential phylogenetic information, in Rosales.

In **III** we instead propose groups based on the presence or absence of proline in the macrocycle and the degree of saturation of the carbon chain of the C-terminal tyrosine. This yields five groups for the hitherto isolated cyclopeptide alkaloids (positions according to a hypotetical tetrapeptide precursor): 1) absence of proline, 2) both absence of proline and a saturated side chain of the C-terminal tyrosine, 3) presence of proline in the third posi-
tion, 4) presence of proline in the second and third position, and 5) presence of proline in the second position. An overview of the structural comparisons of the groups can be seen in figure 12.

![Figure 12](image1.png)

*Figure 12.* The proposed structural classification from III, based on presence and position of proline, and saturation of carbon chain of the tyrosine residue.

With these criteria the chemosystematic information increases as the four groups 2-5 may infer relationships. Group 1 have been detected in all cyclopeptide alkaloid containing orders of angiosperms except Fabales. The saturated cyclopeptide alkaloids lacking proline are only reported from fabids, and alkaloids retaining the carboxylic function of tyrosine have been isolated from Fabaceae (Sugawara, Ishimoto et al. 1996). Further investigations into the biosynthesis of this group are however needed to fully evaluate any phylogenetic implications since the saturation of this bond can be due to biosynthetic reactions both preceding or succeeding the double bond. As stated above the alkaloids with proline in the second position are only found in Rosales. The group to which anordianine belongs, with proline only in position three, is exclusive for Olacaceae and Rubiaceae (Boulvin, Ottinger et al. 1969; Dongo, Ayafor et al. 1989; El-Seedi, Gohil et al. 1999), thus suggesting an asterid affinity for Santalales (see figure 13).
Figure 13. The presence of cyclopeptide alkaloids of groups 1-5 plotted on the ordinal classification of APG2 (Angiosperm Phylogeny Group 2003). Black marks denotes the presence of, and white marks designated no reports, of such cyclopeptide alkaloids. The phylogenetic implications of group 2 in the fabids, group 4 and 5 in Rosales, and group 3 joining the asterids and Santalales are marked by dotted boxes.

From chemosystematic view there are also other implications for the affinities of Santalales. One feature significant for the order is the presence of acetylenic fatty acids in the seed oils. Such compounds are rare among the angiosperms (Badami and Patil 1981). Even if the first report, published already in 1892, relate to the rosid taxon *Picramnia tariri* DC. (Picramniaceae) and the isolation of 6-octadecynoic acid (Arnaud 1892), the foremost sources of acetylenic fatty acids, and corresponding amides and alcohols, besides Santalales are the campanulid families of Asteraceae, Apiaceae and Araliaceae (Sørensen 1963; Bu'Lock 1966).

In manuscript IV a molecular biology approach is exploited in an attempt to obtain additional support for a firm placement of Santalales.
The order Santalales comprise circa 2000 species in 150-160 genera (Brummit 1992; Mabberley 1997). In the phylogenetic analysis of 18S rDNA and *rbcL* sequences by Nickrent and Malécot (Nickrent and Malécot 2001), it was shown that the familial relationships within the order are in need of realignment. The family Olacaceae is shown to be a paraphyletic assembly from which the genus *Schoepfia* should be excluded, as is further studied by Malécot (Malécot 2002). The mistletoe families Eremolepidaceae and Viscaceae should on the other hand be included in an expanded Santalaceae, since the former family makes Santalaceae polyphyletic and the latter renders it paraphyletic. The resulting composition of Santalales here used, is presented in table 4. Further work on the intraordinal phylogeny of Santalales are under way (cf. Daniel Nickrent and *The Parasitic Plant Connection* at http://www.parasiticplants.siu.edu), but is outside the scope of this thesis.

Table 4. Families of Santalales with approximate number of genera and species (Brummit 1992; Mabberley 1997), and notes on circumscription and distribution.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genera</th>
<th>Species</th>
<th>Circumscription</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loranthaceae</td>
<td>68-77</td>
<td>900</td>
<td></td>
<td>tropics</td>
</tr>
<tr>
<td>Misodendraceae</td>
<td>1</td>
<td>8</td>
<td></td>
<td>Chile</td>
</tr>
<tr>
<td>Olacaceae</td>
<td>26</td>
<td>180</td>
<td><em>sensu lato</em>, including <em>Erythrophalum</em> and <em>Octoknema</em></td>
<td>tropics</td>
</tr>
<tr>
<td>Opiliaceae</td>
<td>10</td>
<td>32</td>
<td></td>
<td>tropics</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>44</td>
<td>936</td>
<td>including Eremolepidaceae and Viscaceae</td>
<td>worldwide</td>
</tr>
<tr>
<td>Schoepfiaceae</td>
<td>1</td>
<td>23</td>
<td>excluded from Olacaceae</td>
<td>N and S America, SE Asia</td>
</tr>
</tbody>
</table>

Since the publication of the influential classification by Bentham and Hooker in *Genera Plantarum* (Bentham and Hooker 1873), Santalales have been associated with Rosidae. In several of the more recent systems Santalales have been placed in the vicinity of groups containing rosid taxa with inconspicuous and/or reduced flowers such as *Celastrus*, *Rhamnus* and *Vitis* (Dahlgren 1980; Cronquist 1981; Takhtajan 1997). The first broad molecular analysis of angiosperm phylogeny, using nucleotide sequences of *rbcL*, revealed that the traditional subdivision of flowering plants was largely artificial (Chase, Soltis et al. 1993). Santalales were not associated with the traditional Rosidae, instead the order was retrieved (together with *e.g.* Gunneraceae and Paeoniaceae) in the vicinity of Asteridae. Several broad studies of angiosperm phylogenies have since then been published utilizing other nucleotide sequences or combinations of nucleotide sequences, but none have been able to fully resolve the relationship of Santalales to the rest of the tricolpates. In figure 14 the tricolpate subset of angiosperm relationships are shown for the APG2 consensus tree and three recent broad studies, two of
which have a resolved position for Santalales (Soltis, Soltis et al. 2000; Hilu, Borsch et al. 2003; Soltis, Senters et al. 2003).

As can be seen from the figure statistical support, in the form of jackknife percentages, are low for the placement of Santalales. This can be due to several factors e.g. limited amounts of nucleotide sequence data or too scarce sampling of santalaleous taxa. Limitations in sequence data can be avoided by adding sequences, i.e. other genes. The scarce sampling is usually more laborious to expand, since collecting additional taxa may prove to be a cumbersome task. In the three published analyses discussed above, the santalaleous sampling have indeed been quite poor, including only few taxa representing no more than three families from the order. Whether one should add more sequences or more taxa to increase resolution and support is still
open to debate (e.g. (Bremer, Jansen et al. 1999; Mitchell, Mitter et al. 2000; Pollock, Zwickl et al. 2002; Hillis, Pollock et al. 2003)), and has been expanded in a recent thesis (Erixon 2006).

In IV an effort to combine both of these two strategies lead us to compile sequences for 18S and 26S rDNA, \textit{atpB}, \textit{matK}, and \textit{rbcL} for phylogenetic analysis. Published sequences were compiled from GenBank, and added to by collection of \textit{Bakerella} species and \textit{Socratina kerauderiana} in Madagascar, \textit{Osyris alba} in Spain, and \textit{Viscum album} and \textit{Thesium alpinum} in Sweden. Further sampling was also done from herbarium specimen at the Botany Section of the Museum of Evolution (UPS). Thus 26 taxa from Santalales, see table 5, were subjected to DNA isolation, sequence amplification by the polymerase chain reaction followed by automated sequencing.

**Table 5. Inclusive list of plant material used for sequencing in IV.**

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Voucher (UPS)</th>
<th>Provenience</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loranthaceae</td>
<td>\textit{Bakerella clavata}</td>
<td>Harder et al. 1563</td>
<td>Antsiranana, Madagascar</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Bakerella grisea}</td>
<td>Larsson et al. L96</td>
<td>Antananarivo, Madagascar</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Bakerella sp.}</td>
<td>Larsson et al. L08</td>
<td>Fianarantsoa, Madagascar</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Englerina inaequilatera}</td>
<td>Mwasumbi 16171</td>
<td>Mbeya, Tanzania</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Erianthemum lindense}</td>
<td>Bidgood et al. 2067</td>
<td>Masasi, Tanzania</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Hyphear tanakae}</td>
<td>Togasi 1540</td>
<td>Honshu, Japan</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Oncella curviramea}</td>
<td>Iversen et al. 86799</td>
<td>Tanga, Tanzania</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Onococalyx doberae}</td>
<td>Thulin et al. 8283</td>
<td>Hadramaut, Yemen</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Plicosepalus curviflorus}</td>
<td>Thulin et al. 8480</td>
<td>Abyan, Yemen</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Socratina kerauderiana}</td>
<td>Larsson et al. L90</td>
<td>Toliary, Madagascar</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Taxillus incanus}</td>
<td>Samuelsson et al. 13</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>Loranthaceae</td>
<td>\textit{Taxillus sclerophyllus}</td>
<td>Samuelsson et al. 11</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>Olacaceae</td>
<td>\textit{Dulacia spruceana}</td>
<td>Asplund 14372</td>
<td>Loreto, Peru</td>
</tr>
<tr>
<td>Olacaceae</td>
<td>\textit{Olax pentandra}</td>
<td>Vollesen 4056</td>
<td>Tanzania</td>
</tr>
<tr>
<td>Opiliaceae</td>
<td>\textit{Cansjera rheedei}</td>
<td>Ryding 886</td>
<td>Rayong, Thailand</td>
</tr>
<tr>
<td>Opiliaceae</td>
<td>\textit{Opilia campestris}</td>
<td>Thulin 6373</td>
<td>Shabeellaha Dhexe, Somalia</td>
</tr>
<tr>
<td>Opiliaceae</td>
<td>\textit{Opilia celtidifolia}</td>
<td>Nawa et al. 33</td>
<td>Zambia</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>\textit{Arjona patagonica}</td>
<td>Moore et al. 343</td>
<td>Tierra del Fuego, Argentina</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>\textit{Comandra richardsiana}</td>
<td>Sjörs 28</td>
<td>Ontario, Canada</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>\textit{Comandra umbellata}</td>
<td>Stevens 1548</td>
<td>Michigan, USA</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>\textit{Osyridicarpus linearifolius}</td>
<td>Borhidi et al. 84054</td>
<td>Tanga, Tanzania</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>\textit{Osyris alba}</td>
<td>Backlund</td>
<td>Spain</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>\textit{Thesium alpinum}</td>
<td>Backlund</td>
<td>Sweden</td>
</tr>
<tr>
<td>Santalaceae</td>
<td>\textit{Viscum album}</td>
<td>Larsson et al.</td>
<td>Sweden</td>
</tr>
<tr>
<td>Schoepfiaceae</td>
<td>\textit{Schoepfia jasminodora}</td>
<td>TNS 1369</td>
<td>Kyushu, Japan</td>
</tr>
<tr>
<td>Schoepfiaceae</td>
<td>\textit{Schoepfia schreberi}</td>
<td>Eggers 3890</td>
<td>Acklin Island, The Bahamas</td>
</tr>
</tbody>
</table>
Phylogenetic analysis was performed using PAUP*4.0b10 (Swofford 2002) for the combined five genes/regions as well as for each single gene/region. The matrices were constructed by primary alignment using ClustalX 1.83 software (Higgins, Thompson et al. 1996), with subsequent adjustments by hand. The combined data set comprise 207 taxa, having complete or almost complete sequences for a minimum of three out of the five selected genes/regions, and 9604 characters of which 3640 were found to be parsimony informative. The most parsimonious solution unambiguously, albeit not with strong support, place Santalales basal to a larger clade consisting of the sister groups Caryophyllales (including Dilleniacae) and asterids shown in figure 15.

This tree is in complete congruence with the chemical data discussed above, and disproves the notion of a rosid affinity for Santalales. Celastraceae, Vitaceae and Rhamnaceae (as represented by Ceanothus) are well separated from Santalales. However, two families included in Santalales by Cronquist (Cronquist 1981), the digeneric Medusandraceae and the monotypic Dipentodontaceae, do have rosid affinities. The two genera of Medusandraceae are not closely related to each other as the genus Soyauxia is placed in Saxifragales (Davis and Chase 2004), and Medusandra is reported to belong in Malpighiales (Soltis, Soltis et al. 2005). The placement of Dipentodon sinicus is shown to be as sister to the rosid family Tapisciaceae, itself unplaced to order within the APG classification (Peng, Chen et al. 2003).
Figure 15. The single most parsimonious tree from the successively re-weighted combined matrix with 207 taxa and 9604 characters. Nodes with bold markings have bootstrap support values over 80%.
4.2 Cytotoxicity due to evolutionary dependent structure

Since mistletoe thionins recently showed interesting cytotoxicity profiles (Johansson, Gullbo et al. 2003), and the mistletoe plants have a long tradition of use for a number of ailments, including cancer (e.g. (Anderson and Phillipson 1982; Wagner, Feil et al. 1984; Franz 1985; Fernández, Wagner et al. 1998; Rios, Salinas et al. 2001; Ishizu, Winarno et al. 2002; Lohézic-Le Dévéhat, Bakhtiar et al. 2002; Lohézic-Le Dévéhat, Tomasi et al. 2002; Ohashi, Winarno et al. 2003; Rios and Aguilar-Guadarrama 2004; Varela, Fernández et al. 2004)), these were chosen as a suitable model for in-depth studies and probing aspects of evolutionary based selection. One apparent advantage of using taxa with active polypeptides is that the functional filtering via a complex biosynthetic machinery becomes obsolete. As the thionins are gene encoded, ribosomal peptides, any evolutionary pressure will act directly on, and be mirrored by, the encoding genes.

In the earlier screening for toxic peptides from mistletoes by Samuelsson and co-workers, 80 taxa from Loranthaceae and Santalaceae were subjected to acidic extraction, precipitation by acetone and/or ion exchange chromatography (Samuelsson 1966; Samuelsson 1969; Samuelsson, Borsub et al. 1981) (all taxa are included in appendix A). The biological effect was evaluated by whole-animal toxicity and the result indicated that toxic alkaline peptides could be found in 13 taxa from Santalaceae. In addition, two Loranthaceae taxa were indicative as possibly containing toxic neutral or acidic peptides, this since their respective acetone precipitations were toxic but this activity was lost after ion exchange chromatography. On this basis it was suggested that the presence of toxic thionins appeared to be restricted to the viscod taxa of Santalaceae sensu lATO. The taxa positive for cytotoxicity are presented in table 6.

Further investigation have isolated, and thus proven the presence of, thionins from Dendrophthora clavata (Samuelsson and Pettersson 1977; Samuelsson, Pettersson et al. 1977), Phoradendron liga (Thunberg and Samuelsson 1982; Li, Gullbo et al. 2002), and Phoradendron serotinum (Samuelsson and Ekblad 1967; Mellstrand and Samuelsson 1973; Mellstrand and Samuelsson 1974; Thunberg 1983; Johansson, Gullbo et al. 2003).

To validate the previous results and confirm presence of cytotoxic peptides, the isolation method from II was used to screen 14 taxa from Loranthaceae and Santalaceae in V, see table 7. The presence of thionins in the two mistletoes Viscum album and Dendrophthora clavata (Santalaceae) has been firmly established, and these taxa were used as positive controls. Cytotoxicity of the samples was evaluated as per centage survival for the human lymphoma cell-line U937-GTB using the fluorometric culture cytotoxicity assay. Methods are detailed in the work Larsson and Nygren (Larsson and Nygren 1989), and the application for screening has previously been tested.
Table 6. Compilation of the santalaleous species tested positive for toxic peptides in the early screening of mistletoes by Samuelsson and co-workers (Samuelsson 1966; Samuelsson 1969; Samuelsson, Borsub et al. 1981).

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Santalaceae</td>
<td><em>Dendrophthora clavata</em></td>
</tr>
<tr>
<td></td>
<td><em>Dendrophthora subtrinervis</em></td>
</tr>
<tr>
<td></td>
<td><em>Korthalsella japonica</em></td>
</tr>
<tr>
<td></td>
<td><em>Notothixos subaureus</em></td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron forestierae</em></td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron liga</em></td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron piperoides</em></td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron rhipsalinum</em></td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron serotinum</em></td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron subfalcatum</em></td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron villosum</em></td>
</tr>
<tr>
<td></td>
<td><em>Viscum heyneanum</em></td>
</tr>
<tr>
<td></td>
<td><em>Viscum verrucosum</em></td>
</tr>
<tr>
<td>Loranthaceae</td>
<td><em>Scurrula parasitica</em></td>
</tr>
<tr>
<td></td>
<td><em>Scurrula philippinensis</em></td>
</tr>
</tbody>
</table>

on plant extracts by e.g Lindholm and co-workers (Lindholm, Gullbo et al. 2002). Two of the tested taxa, *Oryctanthus alveolatus* and *Psittacanthus pusillus*, showed a survival index above 80% at the highest tested concentration and were thus regarded as devoid of toxicity. One taxon, from a previously not investigated genus, *Bakerella grisea*, showed low survival indices comparable to those of *Viscum album* and *Dendrophthora clavata*.

All extracts that were tested positive for cytotoxicity, showing a survival index <80% at the highest test concentration, were submitted to high-pressure liquid chromatography and mass spectrometry to investigate presence of peptides. Since even the more elaborate peptide isolation protocol devised by Claeson and co-workers (Claeson, Göransson et al. 1998), yielded cardiac glycosides as the cytotoxic principle (Johansson, Lindholm et al. 2001), it could be argued that this screening does not prove the existence of cytotoxic peptides in the tested species. One reason for this precaution is that the test solution investigated for biological activity is indeed a very crude extract. To alleviate this concern, a re-investigation of the active extract fractions from the two species of *Phthurusa* was performed with mass spectrometry. From this it could be demonstrated that the results most probably are due to peptides. An isolated and purified fraction containing compounds with the mass 3,1 kDa from *Phthurusa pyrifolia* showed a survival index of less than 1 when tested at a concentration corresponding to the crude extracts original 2 mg/ml. This indicates an approximative 190-fold increase of cytotoxicity with reference to concentration from the additional purification. For a purified and active fraction with compounds weighing 4,0
kDa from *Phthirusa retroflexa* the change is not as astounding but a survival index of 31% as compared to 57%, is almost a 2-fold increase of cytotoxicity.

In the HPLC-UV analyses only *Phoradendron quadrangulare* showed typical thionin peaks as designated in II, and by comparison to the extracts of *Dendrophthora clavata* and *Viscum album*. All other extracts lack these late eluting peaks, including that from *Phoradendron acinacifolium*, from the same genus. In the latter extract the only fraction that showed activity in the cytotoxicity testing elutes at an equivalent position in the chromatogram to the cytotoxic 3,1 kDa compound found in *Phthirusa pyrifolia*. Also in the very active extract of *Bakerella grisea* the same region of the chromatogram contains peaks from peptides, here with the mass 3,2 kDa. In the latter case it still remains to be confirmed that the cytotoxicity is due to these peptides and not another compound in the fraction.

Table 7. The sources of the crude extracts screened for cytotoxicity on the human lymphoma cell-line U937-GTB, with the survival index for the tested concentrations.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>20 µg/ml</th>
<th>200 µg/ml</th>
<th>2000 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loranthaceae</td>
<td>Bakerella grisea</td>
<td>≥100</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Orictanthus alveolatus</td>
<td>≥100</td>
<td>≥100</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td><em>Phthirusa pyrifolia</em></td>
<td>≥100</td>
<td>98</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td><em>Phthirusa retroflexa</em></td>
<td>91</td>
<td>92</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td><em>Psittacanthus pusillus</em></td>
<td>≥100</td>
<td>≥100</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td><em>Taxillus liquidambaricola</em></td>
<td>87</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td><em>Taxillus lonicerifolius</em></td>
<td>98</td>
<td>52</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><em>Taxillus ritozanensis</em></td>
<td>≥100</td>
<td>≥100</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td><em>Taxillus tsaii</em></td>
<td>≥100</td>
<td>≥100</td>
<td>78</td>
</tr>
<tr>
<td>Santalaceae</td>
<td><em>Dendrophthora clavata</em></td>
<td>≥100</td>
<td>6</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td><em>Osyris alba</em></td>
<td>≥100</td>
<td>≥100</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron acinacifolium</em></td>
<td>≥100</td>
<td>72</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Phoradendron quadrangulare</em></td>
<td>≥100</td>
<td>97</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td><em>Viscum album</em></td>
<td>77</td>
<td>2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

In *Phthirusa retroflexa* and *Taxillus liquidambaricola* compounds with expected thionin masses in the range of 4,6-4,8 kDa could be detected, but no cytotoxicity could be attributed to these fractions. Further investigations are needed to confirm the presence of thionins, but we have for the first time in V shown the presence of small and cytotoxic peptides within Loranthaceae. Previous investigations of cytotoxic peptides in this family have described compounds with masses of 14 to 90 kDa (Chen, Zeng et al. 1992; Fernández, Wagner et al. 1998).
5. Discussion

5.1 Conclusion

The conclusions from the different aspects of this thesis are that:

- the thionins are a group of circa 5 kDa peptides thus far containing cationic and neutral peptides, and even though they share biological activity and surface features with the defensins the two groups are distinct from each other,

- thionins can be readily extracted by acetic acid, and following this by polyamide filtration before high pressure liquid chromatography purification enables a rapid screening for thionin content in plants, and yielding a suitable fraction for bioassay testing,

- phytochemical data may be used to provide support in questions of unresolved phylogenetic placement, but care must be taken to incorporate information about biosynthesis as in the case of the cyclopeptide alkaloids of *Heisteria nitida*,

- the order Santalales is shown to have asterid affinities, and is not closely related to the plant groups in whose vicinity it has been classified in recent systems,

- there are small cytotoxic peptides present also in the mistletoes of the family Loranthaceae, but these peptides have masses differing from thionins.

As this project has spanned over all aspects of pharmacognosy, another conclusion is that the model for pharmacognosy should be expanded. The technical advances during the last ten years have opened up new aspects of natural science and produced immense amounts of data — and new kind of data. To incorporate this into the model of Bruhn and Bohlin (Bruhn and Bohlin 1997) would show that pharmacognosy is still at the forefront of drug discovery and explicitly deals with aspects today known as metabolomics and systems biology. The new model proposed is presented in figure 16, and will be expanded in a forthcoming paper (Larsson, Backlund & Bohlin, in prep.).
5.2 Future perspectives

The mistletoes and the thionins are by no means exhausted as study objects, and there are several scientific challenges remaining to be dealt with.

The largest mistletoe family, Loranthaceae, is in great need of revision. To fully evaluate the ethnopharmacology of these plants their taxonomy, systematics and phylogenetic relationships must be made clear. For example the circumscription of the mistletoe genera *Scurrula*, *Taxillus* and *Bakerella* have direct bearing on the results in this thesis.

For the thionins the question of their role within the plants is still elusive. Are both viscothionins and crambins important parts of the plant innate immunity despite their very different physicochemical appearances? And how do they exert the observable activities? Which features of the thionins are responsible for the differential effects seen on different cancer cells?

We have not yet been successful in our efforts to develop a PCR-based screen to identify thionin genes, and thus the potential for their presence, before attempting their isolation. Such a screening would also make more directed isolation efforts possible, concentrating the work on isolating the most diverse thionins possible, and thus adding data to structure-activity relational studies.
6. Populärvetenskaplig sammanfattning

Det här är en avhandling i ämnet farmakognosi, ett namn som kommer från grekiskans *fármako* (φάρμακο) som betyder läkemedel, och *gnósi* (γνώση) som betyder kunskap. Eftersom läkemedelskunskap idag omfattar många olika specialismområden brukar ämnet avgränsas med förklaringen att farmakognosi är vetenskapen om läkemedel och läkemedelsutveckling från naturprodukter. Då denna definition fortfarande är mycket vid, används också en modell där farmakognosi förklaras som studier av relationerna mellan, 1) en organism, 2) organismens kemiska innehåll, och 3) dessa substansers biologiska effekter. För att genomföra sådana studier används ofta etnofarmakologisk information – hur används organismer i olika kulturer som inte använder modern västerländsk medicin; kemosystematik – det faktum att närbesläktade organismer ofta har likartade kemiska föreningar; och olika former av biologiska tester (så kallade bioassays) – modeller för sjukdomar eller deras symtom såsom djurförsök eller isolerade enzym i provrör.

I det här arbetet används organismgruppen sandelträdsväxterna (ordningen Santalales), som i Sverige representeras av den vanliga misteln (*Viscum album*) och spindelörten (*Thesium alpinum*). Mistlarna, och flera av deras släktingar, har en lång användning som mediciner i flera kulturer. I Europa har de ansetts skydda mot allt ont och bota epilepsi och hjärtsvårt; i Afrika anses många mistlar besitta förmågan att läka brutna ben; i Asien är de stärkande medel och malariamediciner; och i Sydamerika används de mot rosfeber (en hudinfektion av streptokocker) och som påstått preventivmedel. Dessutom används mistlar i alla dessa områden i extrakt som sägs bota tumörsjukdomar som till exempel cancer.

Sedan mitten på 1900-talet har det varit känt att vissa mistlar innehåller en grupp små proteiner som är mycket giftiga då de förstör cellmembran och därmed dödar celler. Denna grupp proteiner kallas för tioniner, och samma grupp finns också beskriven från flera andra grupper av växter, framför allt från våra vanliga sädesslag. Det har visat sig att olika tioniner från en amerikansk mistel förstör olika typer av cancerceller och friska celler med varierande framgång, och därför har det här arbetet försökt undersöka hur man skulle kunna leta efter tioniner som bara förstör cancerceller. Detta har gjorts genom att använda olika former av datoranalys, kemiska och genetiska metoder samt biologisk testning på odlade cancerceller.

Först undersöktes hur tioninerna förhåller sig till andra små proteiner som finns i växter. Eftersom proteiner bildas genom att genetisk information i


När det finns en tioninisoleringsmetod är nästa steg att välja organismer för att försöka extrahera fram dessa. Sandelträdsväxterna, och framför allt julmistlarna och deras närmaste släktingar, har visat sig vara rika på tioniner. För att försöka hitta nya aminosyrasekvenser hos tioniner skulle man vilja genomföra isoleringar från växter som är besläktade med Santalales men själva tillhör någon annan växtordning. Tekniska genombrott för genetiska analyser har gjort det möjligt att, på liknande sätt som proteinerna ovan, jämföra gener eller andra DNA-bitar för att slå fast släktet mellan stora grupper av växter. Vissa växtgrupper har dock varit svåra att hitta några ”närmaste” släktningar till, och dit hör ordningen Santalales. I dessa fall kan man ibland få ledtrådar från kemiska substanser som finns i växterna. Sådana ledtrådar diskuteras i det tredje delarbetet där en grupp ämnen kallade cyclopeptidalkaloider undersöks. När man tar hänsyn till hur dessa alka-loider byggs upp i växterna ser det ut som om sandelträdsväxterna skulle vara närmare släkt med den stora växtgrupp som exempelvis innehåller tomat och potatis, maskrosor, dill, kummin och murgröna (gruppen kallas a-

I det sista arbetet testas ett antal mistlar för toxiska effekter på odlade cancerceller, och resultaten härfrån visar att även om dessa mistlar dödar cancercellerna innehåller de andra toxiska peptider än tioniner.

Som sammantagen slutsats slås fast att den använda modellen för farmakognosi baserad på relationer mellan en organism, dess kemiska substanser, och dessa kemiska substansers biologiska aktivitet, behöver utvidgas. Genom att inkorporera en fjärde aspekt – datorbehandling av olika typer av information – erhålls en modell som är uppdaterad och anpassad för alla former av data som erhålls från de nya metoderna som utvecklats de senaste decennierna med avseende på genetisk, kemisk och biologisk information.
7. Acknowledgement

This research was carried out mainly at the Division of Pharmacognosy, Department of Medicinal Chemistry, Faculty of Pharmacy, Uppsala University. Travel grants and stipends from the Swedish Academy of Pharmaceutical Sciences, the Royal Swedish Academy of Sciences, and Uppsala University have made it possible for me to attend scientific congresses and go on field trips, and are here by acknowledged. Among all the people who have been around during my work with this thesis I would like to acknowledge:

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All the other “pharmacognosists”: professor emeritus Finn Sandberg and his wife Ilse – hope you still think I am a real pharmacognost; professor emeritus Gunnar Samuelsson – thanks for starting everything half a century ago; docent Per Claeson – for introducing me to pharmacognostical research; docent Jan G. Bruhn, professor Wenche Rolfsen and Dr Mervi Vasâange and Dr Håkan Tunö; Hesham El-Seedi – for all your enthusiasm and constant smile; all the past and present research students – Dr Ulf Göransson, Dr Senia Johansson, Lic Åke Stenholt, Dr Therese Ringbom, Charlotte af Klercker, Lic Wimahl Pathmasiri, Dr Ulrika Huss – thanks for all your support, Dr Erika Svangârd – for setting the standards, Dr Petra Lindholm – for knowing how it is, Dr Martin Sjögren and his companion Dr Mia Dahlström, Sofia Ortlepp – thanks for sharing the stage, Erik Hedner, Anders Herrmann, Catarina Ekenäs, Jenny Pettersson, Josefina Larsson and Robert Burman – thanks for not commenting on my disorganised part of our room; and the ones responsible for all practical things Kerstin Stählborg, Maj Blad and Siv Berggren. Thank you all!
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The field trip to Madagascar would not have been what it became without Dr Sylvain Razafimandimbison, and the rest of the expedition: professor Arne Anderberg, Dr Torsten Eriksson, Dr Jenny Smedmark, Dr Johannes Lundberg, Dr Frida Eggens, Anja Rautenberg, Cajsa Lisa Anderson, Dr Barbro Axellius, Julia Borg, Ulrika Manns, Markus Englund, Kent Kainulainen, Magnus Lundberg and Anbar Khodabanah.

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And of course thanks to those who are always there, and tries to be interested in what I do – my family. Thank you. Thank you all!
8. References


Daley, L. S. and L. J. Theriot (1987). "Electrophoretic analysis, redox activity, and other characteristics of proteins similar to purothionins from tomato (Lycopersicum esculenta), mango (Mangifera indica), papaya (Carica papaya), and walnut (Juglans regia)." Journal of Agricultural and Food Chemistry 35.


Appendix A

Taxonomic list of species names in this thesis, with notes on nomenclatural discrepancies.

**Brassicaceae**

Taxonomy following results shown in molecular studies by Warwick and Gugel (Prina 2000; Warwick and Gugel 2003).

*Brassica hispanica* L. subsp. *abyssinica* (Hochst. ex R.E.Fries) A.Prina

*Brassica abyssinica* Hochst ex R.E.Fries (VanEtten, Nielsen et al. 1965)

**Rubiaceae**

*Psydrax arnoldiana* (De Wild. & T.Durand) Bridson

*Canthium arnoldianum* (De Wild. & T.Durand) Hepper

III (El-Seedi, Larsson et al. 2005)

*Canthium arnoldianum* De Wild. & Th.Dur. [sic!]

(Dongo, Ayafor et al. 1989)

basionym: *Plectronia arnoldiana* De Wild. & T.Durand

*Psydrax splendens* (K.Schum.) Bridson

*Canthium euryoides* Bull.

(Boulvin, Ottinger et al. 1969)

basionym: *Plectronia splendens* K.Schumann

synonym: *Canthium nitens* Hiern

*Psydrax odorata* (G.Forst.) A.C.Sm. & S.P.Darwin

*Plectronia odorata* Benth. & Hook.f.

*Canthium odoratum* Seem.

(Gournelis, Skalsounis et al. 1989)

basionym: *Coffea odorata* G.Forst

**Loranthaceae**

Loranthaceae is in need of revision, as is evident from the two published molecular phylogenies published where some genera are represented by more than one species (Han, Hao et al. 2004; Wilson and Calvin 2006). Names in this list are ”as published” or, if marked by •, with consideration taken to the following publications: *Tristerix* (Kuijt 1988), African Loranthaceae (Polhill and Wiens 1998), *Amyema* (Barlow 1992), *Amylotheca* (Barlow 1993), *Benthamina* (Barlow 1966). *Bakerella* and *Socratina* (Balle 1964; Balle 1966). *Taxillus* (Chiu 1996) but note the discussion regarding circumscription of *Taxillus* and *Scurrula* in Yang et al. (Yang, Lu et al. 1997), *Elytranthe* and *Scurrula* (Qiu, Chiu et al. 2003), *Oryctanthus*, *Phthirusa* and *Psittacanthus* (Kuijt 1978). Herbaria for located vouchers are given.

The name used in each publication cited in this thesis is included as synonym.
Aetanthus columbianus A.C. Smith  
(Samuelsson 1969)

•Agelanthus natalitius (Meisn.) Polh. & Wiens subsp. zeyheri (Harv.) Polh. & Wiens  
Tapinanthus zeyheri (Harv.) Dans.  
Loranthus zeyheri Harv.  
(Samuelsson 1969)

•Amyema bifurcata (Benth.) Van Tiegh.  
(Samuelsson 1966)

•Amyema congener (Schult.) Van Tiegh.  
(Samuelsson 1966)

•Amyema conspicua (Bail.) Dans.  
(Samuelsson 1966)

•Amyema pendula (Sieber ex Spreng.) Van Tiegh.  
(Samuelsson, Borsub et al. 1981)

•Amyema whitei (Blakely) Dans.  
Loranthus whiteii Blakely  
(Samuelsson 1966)

•Amylotheca dictyophleba (F.Muell.) Van Tiegh.  
Loranthus dictyophlebus F.Muell.  
(Samuelsson 1966)

•Bakerella clavata (Desr.) S. Balle  
 IV UPS

•Bakerella grisea (Sc. Elliot) S. Balle  
 IV, V UPS, S, TAN, MO

•Benthamina alyxifolia (F.Muell. ex Benth.) Tieghem  
Amyema alyxifolia (F.Muell. ex Benth.) Dans.  
(Samuelsson 1966)

Dendrophthoe falcata (L.f.) Ethingsh.  
(Samuelsson, Borsub et al. 1981) UPS

Dendrophthoe ligulata (Thw.) Van Tiegh.  
(Samuelsson, Borsub et al. 1981) UPS

Dendrophthoe neelgherrensis (W. & A.) Van Tiegh.  
Loranthus neelgherrensis W. & A.  
(Samuelsson 1966)

Dendrophthoe suborbicularis (Thw.) Danser  
(Samuelsson, Borsub et al. 1981) UPS

•Elytranthe parasitica (L.) Danser  
Macrosolen parasiticus (L.) Danser  
(Samuelsson, Borsub et al. 1981) UPS

•Englerina inaequilatera (Engl.) Gilli  
 IV UPS
• Erianthemum dregei (Eckl. & Zeyh.) Van Tiegh.
  Loranthus dregei E. & Z.
  (Samuelsson 1969)

• Erianthemum lindense (Sprague) Danser
  IV UPS

• Erianthemum ngamicum (Sprague) Dans.
  Loranthus ngamicum Sprague
  (Samuelsson 1969)

Hyphaear tanakae (Franchet & Savatier) Hosokawa
  Loranthus tanakae Franchet & Savatier
  IV UPS  (This genus is not in current use.)

Loranthus europaeus Jacq.
  Hyphaear europaeum (Jacq.) Dans.
  (Samuelsson 1969)

• Oncella curviramea (Engl.) Danser
  IV UPS

• Oncocalyx doberae (Schweinf.) Wiens & Polh.
  IV UPS

• Oryctanthus alveolatus Kuijt
  V UPS, PMA

Oryctanthus grahamii (Benth.) Engl.
  (Samuelsson, Borsub et al. 1981)

• Pedistylis galpinii (Schinz ex Sprague) Wiens
  Emelianthe galpinii (Sprague) Dans.
  Loranthus galpinii Schinz
  (Samuelsson 1969)

Phrygilanthus tetrandrus Eich.
  (Samuelsson, Borsub et al. 1981) UPS

Phrygilanthus verticillatis (Ruiz & Pav.) Eich.
  (Samuelsson, Borsub et al. 1981) UPS

Phthirusa iodocarpa Diels
  (Samuelsson, Borsub et al. 1981) S

Phthirusa paniculata (H.B.K.) Macbr.
  (Samuelsson 1969)

• Phthirusa pyrifolia (H.B.K.) Eich.
  V UPS, PMA
  (Samuelsson, Borsub et al. 1981) S

• Phthirusa retroflexa (Ruiz & Pav.) Kuijt
  V UPS, PMA

• Plicosepalus curviflorus (Benth. ex Oliv.) Tieghem
  IV UPS
• *Plicosepalus kalachariensis* (Schinz) Dans.
  *Loranthus kalachariensis* Schinz
  (Samuelsson 1969)

• *Plicosepalus sagittifolius* (Sprague) Dans.
  *Loranthus sagittifolius* Sprague
  (Samuelsson 1969)

• *Plicosepalus undulatus* (E.May) Van Tiegh.
  *Loranthus undulatus* E.May
  (Samuelsson 1969)

• *Psittacanthus calyculatus* (DC.) Don
  (Samuelsson, Borsub et al. 1981)

  (Samuelsson, Borsub et al. 1981) s

• *Psittacanthus nodosus* (Desr.) G.Don
  *Aetanthus nodosus* (Desr.) Engl.
  (Samuelsson, Borsub et al. 1981) s

• *Psittacanthus pusillus* Kuijt
  V UPS, PMA

*Scurrula cordifolia* (Wall.) G.Don
  (Samuelsson, Borsub et al. 1981) UPS

• *Scurrula parasitica* L.
  (Samuelsson, Borsub et al. 1981) UPS

  *Loranthus parasiticus* Merr.
  (Samuelsson 1966)

• *Scurrula atropurpurea* (Blume) Danser
  *Scurrula philippinensis* (Cham. & Schlecht.) G.Don
  *Loranthus philippinensis* Cham. & Schlecht.
  (Samuelsson 1966)

• *Socratina kerauderiana* S. Balle
  IV UPS

*Struthanthus alni* Bartlett
  (Samuelsson, Borsub et al. 1981)

*Struthanthus cf. flexicaulis* Mart.
  (Samuelsson, Borsub et al. 1981) s

*Struthanthus mexicanus* Calderón
  (Samuelsson, Borsub et al. 1981)

• *Struthanthus orbicularis* (H.B.K.) Blume
  (Samuelsson, Borsub et al. 1981) s

*Struthanthus polyrhizus* Mart.
  (Samuelsson, Borsub et al. 1981) UPS

*Struthanthus cf. polystachys* (R. & P.) Blume
  (Samuelsson, Borsub et al. 1981) s
Olacaceae

Dulacia spruceana Kuntze

Heisteria nitida Engl.

Olax pentandra Sleumer

Opiliaceae

In the last revision of the genus *Opilia* the species *O. celtidifolia* was synonymized with *O. amentacea* as the eastern extreme form of this variable species distributed from Africa to Malesia (Hiepko 1982). The name is here retained awaiting confirmation from genetic studies.
Cansjera rheedei J.F.Gmel.
   IV UPS

Opilia campestris Engl.
   IV UPS

Opilia celtidifolia Endl. ex Walp.
   IV UPS

Santalaceae – 'Santaloideae'

Arjona patagonica Homb. & Jacq. ex Decne
   IV UPS

Comandra richardsiana Fernald
   IV UPS

Comandra umbellata Nutt.
   IV UPS

Osyridicarpus linearfolius Engl.
   IV UPS

Osyris alba L.
   IV, V UPS

Thesium alpinum L.
   IV UPS

Santalaceae – Viscoideae

Names in this list are "as published" or, if marked by •, with consideration taken to the following publications: Arceuthobium (Nickrent, Garcia et al. 2004), Phoradendron and Dendrophthora (Kuijt 1978; Ashworth 2000; Ashworth 2000), and Viscum (Danser 1941).

• Arceuthobium abietis-religiosae Heil.
   (Samuelsson, Borsub et al. 1981)

• Arceuthobium americanum Nutt. ex Engl. ex.
   (Samuelsson 1969)

• Arceuthobium campylocarpum Engl. ex.
   (Samuelsson 1969)

• Arceuthobium divaricatum (Engelm.) Gill
   Arceuthobium I. divaricatum (Engelm.) Gill
   (Samuelsson 1969)

• Arceuthobium globosum Hawksw. & Wiens
   (Samuelsson, Borsub et al. 1981)

• Arceuthobium oxycedri Bieb.
   (Samuelsson 1966)

• Arceuthobium vaginatum (Willd.) Presl
   Arceuthobium vaginatum subsp. cryptopodum (Engelm.) Hawks. & Wiens
   (Samuelsson 1969)
Dendrophthora chrysostachya (Presl) Urban  
(Samuelsson, Borsub et al. 1981) s

•Dendrophthora clavata (Benth.) Urb.  
(Samuelsson 1969; Samuelsson and Pettersson 1977; Samuelsson, Pettersson et al. 1977)

Dendrophthora dodsonii Kuijt  
(Samuelsson, Borsub et al. 1981) s

Dendrophthora subtrinervis Urban  
(Samuelsson, Borsub et al. 1981)

Korthalsella japonica (Thunb.) Engler  
(Samuelsson, Borsub et al. 1981) UPS

Notothixos subaureus Oliv.  
(Samuelsson 1966)

•Phoradendron acinacifolium Eichler  
UPS, PMA

•Phoradendron bolleanum (Seem.) Eichl. subsp. densum (Torrey) Wiens  
(Samuelsson 1969)

•Phoradendron brachystachyum (DC.) Nutt.  
(Samuelsson, Borsub et al. 1981)

•Phoradendron capitellatum Torr. ex Trel.  
(Samuelsson 1969)

•Phoradendron forestierae Rob. & Greenm.  
(Samuelsson, Borsub et al. 1981)

•Phoradendron juniperinum  
(Samuelsson 1969)

Phoradendron liga (Gill.) Eichl.  
(Samuelsson, Borsub et al. 1981; Thunberg and Samuelsson 1982)

Phoradendron obliquum (Presl) Eichl.  
(Samuelsson 1969)

•Phoradendron piperoides (H.B.K.) Nutt.  
(Samuelsson, Borsub et al. 1981)

•Phoradendron quadrangulare (H.B.K.) Krug & Urban  
UPS, PMA

•Phoradendron rhipsalinum Rzedowski  
(Samuelsson, Borsub et al. 1981)

•Phoradendron serotinum (Raf.) M.C. Johnst.  
Phoradendron flavescens (Pursh) Nutt.  
(Samuelsson 1966)

Phoradendron tomentosum (DC.) Engelm. subsp. macrophyllum (Cockerell) Wiens  
(Samuelsson 1969; Mellstrand and Samuelsson 1973; Mellstrand and Samuelsson 1974;  
Thunberg 1983; Johansson, Guillio et al. 2003)
Phoradendron subfalcatum Abbiatti  
(Samuelsson, Borsub et al. 1981)

•Phoradendron villosum Nutt.  
(Samuelsson 1966)

•Phoradendron villosum Nutt. subsp. coryae (Trel.) Wiens  
(Samuelsson 1969)

Viscum album L.  
IV, V

Viscum album L. subsp. austriacum (Wiesb.) Vollmann  
(Samuelsson and Jayawardene 1974)

Viscum articulatum Burm.  
(Samuelsson 1966; Samuelsson 1969)

Viscum capitellatum Smith  
(Samuelsson, Borsub et al. 1981) UPS

Viscum combreticola Eng.  
(Samuelsson 1969)

Viscum engleri Van Tieg.  
(Samuelsson, Borsub et al. 1981) UPS

•Viscum heyneanum DC.  
(Samuelsson, Borsub et al. 1981) UPS

•Viscum orientale Wild.  
(Samuelsson 1966; Samuelsson 1969)

Viscum rotundifolium L.f.  
(Samuelsson 1969)

Viscum verrucosum Harv.  
(Samuelsson 1969)

Santalaceae – 'Eremolepideae'

Eremolepis cf. glaziovii (Tieg.) Engl.  
(Samuelsson, Borsub et al. 1981) S

Schoepfiaceae

Schoepfia jasminodora Siebold & Zucc.  
IV UPS

Schoepfia schreberi J.F.Gmel.  
IV UPS

APPENDIX REFERENCES


Appendix B

List of thionins discussed in this thesis, including synonyms, accessions for NCBI databases references and selected references. Only the mature thionin is taken into account, and thus different accessions may differ in signal- and/or C-terminal-peptides. The amino acid sequences are gapped for reasons of comparison.

tulipathionin A
KSCC RNTV ARNC YNVC RIPG TPRF V-CA ATCD CKLI TGTK -CPFG YEK-
Tu-AMP 1  peptide

tulipathionin B
KSCC RNTT ARNC YNVC RIPG’TPRF V-CA ATCD CKII TGTK -CPFG YEK-
Tu-AMP 2  peptide

tulipathionin C
KSCC RTTA ARNC YNVC RLGG TPQT L-CA RTCD CIHI TTGN -CPRS HPK-
thionin class 1, Th1 1  CAA57350

tulipathionin D
KSCC RNTT ARNC YNVC RLGG TPFR V-CA ATCD CKII SSF -CPFG YEK-
thionin class 1, Th1 2  CAA57351
thionin class 1, Th1 3  CAA57352

tulipathionin E
KSCC PSTA ARNC YNVC RFGP TPRF V-CA ATCD CKII TGTK -CPFD YPK-
thionin class 1, Th1 4  CAA57353

tulipathionin F
KCF RTTA ARNC YNVC RLGG TPQT L-CA RTCD CIHI TTGN -CPRS HPK-
thionin class 4, Th4 1  CAA57354
Tulipa gesneriana (Fujimura, Ideguchi et al. 2004)

bamboothionin A
KSCC RSTQ ARNI YNAP RFAG GSRP L-CA LGSG CKIV DDKK --TPP ND--
Pp-AMP 1  peptide

bamboothionin B
KSCC RSTT ARNI YNGC RVPG TARP V-CA KKSQ CKIQ EAKK -CPEP YD--
Pp-AMP 1  peptide
Phyllostachys pubescens (Fujimura, Ideguchi et al. 2005)

avenothionin A
KSCC RNTL GRNC YNLC RSRG APKL --CA TVCR CKIS SGLS -CFKD FPK-
avenothionin α  peptide, 0807220a

avenothionin B
KSCC RNTL GRNC YNLC RAGG APKL --CS TVCR CKLT SGLS -CFKD FPK-
avenothionin β  peptide, 0807220a

avenothionin C
KSCC KDIN ARNC YNVC RIPG TPRF V-CA TCTR CKII SGNK -CFKD YPK-
leaf thionin Asthi1  BAB93112

avenothionin D
KSCC KDTT ARNC YNVC RIPG TPRF V-CA TCTR CKII SGNK -CFKD YPK-
leaf thionin Asthi2  BAB93113

avenothionin E
NTCC KDGI ARNC YNVC RTPF I-CA NMCN CIIT RRNE -CFND YPK-
leaf thionin Asthi3  BAB93114
avenothionin F

KSCC KSTT AINC YNVC RLAG APRF V-CA GPCG CKLL DVTT -CFSD WPK-
thionin Asthi4

Ba93115

avenothionin G

KSCC PSTS ARNC YNVC RLTG TSRF R-CA SLGC CKIV DG-T -CFDG YSK-
thionin Asthi5

Ba93116

*Avena sativa* (Bekes and Lasztity 1981)

hordothionin A

KSCC RSTL GRNC YNLC RVRG AQKL --CA GVCR CKLT SSGK -CPTG FPK-
α-hordothionin

AA32966, CA29330, p01545

purothionin

0603243A

hordothionin B

KSCC RSTL GRNC YNLC RVRG AQKL --CA GVCR CKLT SSGK -CPTG FPK-
β-hordothionin

CA78352, p21742

hordothionin

1206255A

hordothionin C

KSCC KSTL ARNC YNTC RFAG GSRP V-CA GACR CKII SGPK -CFSD YPK-
leaf thionin Bth6

AA91047, p09618

thionin

AA21531

leaf specific thionin

1408170A

hordothionin D

KSCC KNTT GRNC YNLC RVRG AQKL --CA GVCR CKLT SSGK -CPTG FPK-
leaf thionin

AA91048, Q42838

thionin

AA32976, AA32977, AA32978

leaf thionin

p09617

hordothionin E

KSCC KSTL ARNC YNTC HFAG HFSP V-CA GACR CKII SGPK -CFSD YPK-
unnamed product

CA29082

leaf thionin DB4

p08772

hordothionin F

KSCC KNTT GRNC YNLC RFAG GSRN V-CA TACG CKII SGPT -CFRD YPK-
probative thionin

CAD48489

probative leaf thionin

Q8h065

hordothionin G

KSCC KSTL ARNC YNTC HFAG HFSP V-CA GACR CKII SGPK -CFSD YPK-
leaf specific thionin

1404366A

Hordeum vulgare (Bohlmann, Clausen et al. 1988; Rodríguez-Palenzuela, Pintor-Toro et al. 1988; Apel, Bohlmann et al. 1990; Holtorf, Apel et al. 1995)

hordothionin H

KSCC KNTT GRNC YNAC RLFG TPRF V-CA KLGLS CKII SGPT -CFRD YPK-
leaf thionin

AA82352, AA82153

Hordeum jubatum, Hordeum marinum (Bunge, Wolters et al. 1992)

osthionin A

KSCC PSTS ARNV YNSC RFAG GSRS V-CA KLGS CKIV D-GN -CKPP YVHH

Os06g0512700

NP_001057732, BAF19646

Os06g0514800

NP_001057734, BAF19648

OSINBA0085C03

BA62131

OSINBA0022006.16

BA62233

OSINBA0022006.28

BA62239

OSINBA0022006.36

BA62232

OSINBA0022006.46

BA62237

OSINBA0061G23.50

BA62479

Osth1

BA03111

osthionin B

KSCC PSTS ARNV YNSC RFAG GSRS V-CA KLGS CKIV D-GN -CFDP YVHH

Os06g0514400

NP_001057733, BAF19647

P0597AS07.39

BAD62258
osthionin C
KSCC PTTT ARNI YNAC RFAG GTRE R-CS KLSG CKIV D-GK -CKPP YIHH
Os06g0517700  NP_001057737, BAF19651
OSJNBa0020P04.23  BAd61980

osthionin D
KSCC PTTT ARNI YNAC RFAL GTRE R-CS KLSG CKIV D-GK -CKPP YIHH
Os06g0517700  NP_001057737, BAF19651
OSJNBa0020P04.11  BAd61980
OSJNBa0085C03.54  BAd62154

osthionin E
KSCC PTTT ARNI YNSC RFAG GTRE R-CS KLSG CKIV D-GK -CKPP YIHH
Os06g0517700  NP_001057737, BAF19651
OSJNBb0071G09.18  BAd62228

osthionin F
KSCC PTTT VRNV YNSC RFAG GSRE A-CA KLST CKHF D-GS -CQPP Y---
Os06g0517700  NP_001057737, BAF19651
OSJNBa0085C03.20  BAd62140

osthionin G1
KSCC PTTT ARNI YNSC RFAG GSRE A-CA KLST CKHF D-GS -CQPP Y---
Os06g0517700  NP_001057737, BAF19651
OSJNBa0085C03.23  BAd62143 (double)

osthionin G2
GGCC PSST ARNI YTSC RFVG GSYP S-CA RLSG CKID F-GR -CQPP Y---
OSJNBa0085C03.23  BAd62143 (double)

Oryza sativa
purothionin A1a
KSCC RSTL GRNC YNLC RVRG AQKL --CA GVCR CKLT SSGK -CPTG FPK-
a1-purothionin, purothionin AII  p01544, 0310202A
a-purothionin  CAA65313
a2-purothionin  CAA50004

purothionin A1b
KSCC RSTL GRNC YNLC RARG AQKL --CA GVCR CKIS SGLS -CPKG FPK-
PA-II  peptide
PURB1 – a1-purothionin

purothionin A2
KSCC RSTL GRNC YNLC RSRG AQKL --CA TVCR CKLT SGLS -CPKG FPK-
a2-purothionin  p32032
a-purothionin  CAA65315
a1-purothionin  BAA12336, CAA50003

purothionin B
KSCC RSTL GRNC YNLC RARG AQKL --CA NVCR CKLT SGLS -CPKG FPK-
-β-purothionin  AAB71137, CAA65312
purothionin AI  p01543
purothionin β  763623a
PA-I  peptide
PURAI – β-purothionin

Triticum aestivum (Mak and Jones 1976; Ohtani, Okada et al. 1977; Van Campenhout, Sági et al. 1998)

secalethionin
KSCC RSTL GRNC YNLC RTRG AQKL --CA NFCT CKLI SSTS -CFKE FPK-
-purothionin  CAA65316
PURB1 – ryethionin

Secale cereale (Van Campenhout, Sági et al. 1998)
hellethionin A
KSCC RNTL GRNC YNGC RFTG GSQP T-CG RLCD CIEV TTTT -CPSS HPS-
hellethionin A  peptide

hellethionin B1
KSCC RNTL GRNC YNAC RFTG GSQP T-CG RLCD CIEV TTTT -CPSS HPS-
hellethionin B1  patent application
hellethionin B2
KSCC RNTL ARNC YNAC RFTG GSPQ T-CG RLCD CIHV TTPT -CFSS HPS-
- hellethionin B2, patent application

hellethionin B3
KSCC RNTL GRNC YNAC RFTG T-CA TLCD CIHV TTPT -CFSS HPR-
- hellethionin B3, patent application

hellethionin B4
KSCC RNTL ARNC YNAC RFTG TSQP Y-CA RLCD CIHV TTPT -CFSS HPR-
- hellethionin B4, patent application

hellethionin B5
KSCC RNTL ARNC YNAC RFTG GSQP T-CA TLCD CIHV TTPT -CFSS HPS-
- hellethionin B5, patent application

hellethionin B6
KSCC RNTL GRNC YNVC RFGG GSQA Y-CA RFCD CIHV TTST -CPSS HPS-
- hellethionin B6, patent application

hellethionin C
KSCC RNTL GRNC YNAC RLTG TSQA T-CA TLCD CIHV TATT -CPPP YPS-
- hellethionin C, patent application

hellethionin D
KSCC RNTL ARNC YNAC RFTG GSQP T-CG ILCD CIHV TTTT -CPSS HPS-
- hellethionin D, patent application, E00057

hellethionin E1
KSCC RNTL ARNC YNAC RLTG LFSQ EQCA RLCD CITV TTPT PCPRT HPS-
- hellethionin E, peptide

hellethionin E2
KSCC RNTL GRNC YNAC RLTG TSQA EQCA RLCD CITV TTPT PCPRT HPS-
- hellethionin E2, patent application

*Helleborus purpurascens* (Milbradt, Kerek et al. 2003; Kerek 2006)

**arathionin A**

KICC PSNQ ARNG YSVC RIRF SKGR --CM QVSG CQNS DT-- -CFRG WYN-

thionin 2.1, *Arabidopsis thaliana*

NP 565038, Q42596

thionin

AAC41678, AAG51790, AAM63655, 2204399A

putative thionin

AAL87264

**arathionin B**

KICC PTGD DRSV YFVC MLSV SSQF Y-CL LKSK CKNT SQTI -CFPG YTN-

AthTH1, *Arabidopsis thaliana*

PCR product

NP 198567, AAM62681, Q42597

thionin

AAC41679, BA611632, 2204399b

**arathionin D**

NICC PSIQ ARTF YNAC LFAV GSPS S-CI RNSS CLDI SEST -CFRG YTN-

thionin 2.2, *Arabidopsis thaliana*

PCR product

NP 176784

putative thionin

AAG51299

At1g66100/F15E12.20

AAC55733, AAL06815

putative thionin 2.4

Q98106

**arathionin C**

KTCO PSQS TRKE FEDC ISEG NLQI L-CS AESG CRDT YVGY -CFPG FPY-

toxin receptor binding

NP 179105

putative thionin

AAG03358

putative thionin 2.3

Q8V2K8

**arathionin E**

KTCO PSQS TRKG FEDC ISEG NLQI L-CS AESG CRDT YVGY -CFPG FPY-

putative thionin

AAL36398, BAA95310

*Arabidopsis thaliana* (Epple, Apel et al. 1995)

**brassijuthionin**

KSCC PSTA ARMA YILC TNSW PLTP L-CI SHTG CIE- SETT -CFPG YPI-
thionin    ABM30200

*Brassica juncea*

brassirathionin

KICC PFTI DRNI YNAC RLZG ASMT N-CA NSLG CKIV SGTT -CPFG YTH-
CFT mRNA

thionin    AAA21800

thionin    q9jia8.8

(Jung, Choi et al. 2001)

*Brassica rapa* subsp. *pekinensis*

crambethionin A1

TTCC PSIV ARSN FNVC RLPG TPEA L-CA TYTG CIII FGAT -CPGD YAN-
crambin

peptide, R01542

crambethionin A2

TTCC PSIV ARSN FNVC RLPG TPEA I-CA TYTG CIII FGAT -CPGD YPN-
crambin

peptide

crambethionin B

TTCC PSIV ARSN FNVC RLPG TSEA I-CA TYTG CIII FGAT -CPGD YPN-
crambin

peptide

crambethionin C

KSCC PTPI ARKT YVVC RLZG STIA S-CA KYSG CITI SGTQ -CFNG YPH-
crambin Thi2Ca2

AAA33004

crambethionin D

KSCC PTMA ARIQ YNAC RALG TPRP V-CA ALSG CKIL DVTK -CPFD YR--
crambin Thi2Ca3

AAA33005

crambethionin E

NICC PNTT ARSN FNVC RLPG TASEP I-CA TDG CIII FGAT -CPGD YPN-
crambin Thi2Ca4

s52548

crambethionin F

NICC PNTT ARSN FNVC RLPG TASEP I-CA TDG CIII FGAT -CPGD YPN-
crambin Thi2Ca5

AAA33006

crambethionin G

TTCC PSIV ARSN FNVC RLPG TASEP I-CA TDG CIII FGAT -CPGD YPN-
crambin Thi2Ca6

s52550

crambethionin H

KSCY PTKS ARNT FDVC RLZG TSMG L-CA AISL CKIL SVTK -CPFL LF--
crambin Thi2Ca9

AAA33008

crambethionin I

KSCC PFTI ARNT YNIC RLPG TPRP V-CA TLSG CIQ SDST -CKKP YP--
crambin Thi2Ca10

AAA33009

crambethionin J

KCCS PFTI ARNT YNIC RLPG TPRP V-CA AISL CKIL SVTK -CPFL LF--
crambin Thi2Ca11

AAA33010

crambethionin K

KSCY PTKS ARNT FDVC RLZG TSMG L-CA AISL CKIL SVTK -CPFL LF--
crambin Thi2Ca12

AAA33011

*Crambe hispanica* subsp. *abyssinica* (Teeter and Hendrickson 1979; Teeter, Mazer et al. 1981)

croponumthionin

KSCC QTTT ARNT YNSC RLAG GSRE R-CA SLSG CKHV TQNT -CSPG WEK-
putative thionin

AAA15789

*Polygonum sibiricum*

croponumthionin

KSCC QTTT ARNT YNSC RLAG GSRE R-CA SLSG CKHV TQNT -CSPG WEK-
pyrulariathionin

P07504
<table>
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<tr>
<th>Name</th>
<th>Sequence</th>
<th>Accession</th>
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</thead>
<tbody>
<tr>
<td>Pyrularia pubera</td>
<td>Dendclathionin B: KSCC PTTA ARNQ YNIC RLFG TPRF V-CA ALSG CKII SGTG -CFPG YRH-</td>
<td>P01541</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Ligathionin A: KSCC PTTT ARNI YNTC RLIG TSRP T-CA SLSG CKII SGST -CBSG WHN-</td>
<td>P01540</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Ligathionin B: KSCC PTTT ARNI YNTC RLIG ASRS V-CA SLSG CKII SGST -CDSG WHN-</td>
<td>P59358</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phoradendron ligata</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ligathionin A: KSCC PTTT ARNI YNTC RLIG ASRS V-CA SLSG CKII SGST -CDSG WHN-</td>
<td>P01539</td>
</tr>
<tr>
<td></td>
<td>Ligathionin B: KSCC PTTT ARNI YNTC RLIG APRP T-CA KLSG CKII SGST -CD-- ----</td>
<td>P01538</td>
</tr>
<tr>
<td></td>
<td>Phorathionin A: KSCC PTTT ARNI YNTC RFGG GSRE V-CA KLSS CKII SGT K -CD-- ----</td>
<td>P32880</td>
</tr>
<tr>
<td></td>
<td>Phorathionin B: KSCC PTTT ARNI YNTC RFGG GSRE V-CA KLSS CKII SGT K -CD-- ----</td>
<td>P01539</td>
</tr>
<tr>
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<td>Phorathionin C: KSCC PTTT ARNI YNTC RFGG GSRE V-CA KLSS CKII SGT K -CD-- ----</td>
<td>P59358</td>
</tr>
<tr>
<td></td>
<td>Phorathionin D: KSCC PTTT ARNI YNTC RFGG GSRE V-CA KLSS CKII SGT K -CD-- ----</td>
<td>P59358</td>
</tr>
<tr>
<td></td>
<td>Phorathionin E: KSCC PTTT ARNI YNTC RFGG GSRE V-CA KLSS CKII SGT K -CD-- ----</td>
<td>P59358</td>
</tr>
<tr>
<td></td>
<td>Phorathionin F: KSCC PTTT ARNI YNTC RFGG GSRE V-CA KLSS CKII SGT K -CD-- ----</td>
<td>P59358</td>
</tr>
<tr>
<td></td>
<td>Phorathionin A2a: KSCC PNTT GRNI YNTC RFGG GSRE V-CA SLSG CKII SAST -CPSD YPK- viscothionin A2</td>
<td>P32880</td>
</tr>
<tr>
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<td>Phorathionin A2b: KSCC PNTT GRNI YNTC RFGG GSRE V-CA SLSG CKII SAST -CPSD YPK- viscothionin A2</td>
<td>P32880</td>
</tr>
<tr>
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<td>Phorathionin A3a: KSCC PNTT GRNI YNAC RLFQ APRF T-CA KLSS CKII SGST -CFPS YPK- viscothionin A3</td>
<td>P01538, VIVAA3 1e00 10KH</td>
</tr>
<tr>
<td></td>
<td>Phorathionin A3b: KSCC PNTT GRNI YNAC RFAG APRF T-CA KLSS CKII SGST -CFPS YPK- viscothionin A3 Thi2Val1.2</td>
<td>AAB29759</td>
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<tr>
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<td>Phorathionin B: KSCC PNTT GRNI YNAC RFAG GSRE R-CA SLSG CKII SAST -CFPS YPK- viscothionin B</td>
<td>P01538, VIVAA3 1e00 10KH</td>
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<tr>
<td></td>
<td>Phorathionin C1: KSCC PNTT GRNI YNAC RFAG GSRE R-CA SLSG CKII SAST -CFPS YPK- viscothionin C1</td>
<td>P83554, 10RL</td>
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viscothionin D
KSCC RTNT GRNC YHAC RVFP YPRF V^CA SLCD CKII SGSK ^CPAD YPR^-
thionin Thi1Va1

viscothionin E
KICC RAPA GKKC YNLC TALL SSHT --CA NTCT CKDV SGET --CPAD YF--
thionin Thi1Va1

viscothionin P1
KSCC PSTT GRBI YBTC RFGG GSRZ V^CA RISG CKII SAST ^CPBS YPK^-
viscotoxin 1-PS
p01537
Viscum album (Samuelsson, Seger et al. 1968; Samuelsson and Pettersson
1971; Olson and Samuelsson 1972; Samuelsson and Jayawardene 1974;
Schrader and Apel 1991; Schrader-Fischer and Apel 1993; Romagnoli,
Ugolini et al. 2000; Romagnoli, Fogolari et al. 2003)

salviathionin
KSCC BRNT ARNI YNTC RLRL PASS --CA DLSG CKVI DGGT ^CPTG WTN^-
thionin
Salvia miltiorrhiza

APPENDIX REFERENCES

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class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the

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468.

inducible via a signal transduction pathway different from that for pathogenesis-

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