UPPSALA
UNIVERSITET

*Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 7*

# Cytotoxic Compounds of Plant Origin – Biological and Chemical Diversity

PETRA LINDHOLM

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2005

ISSN 1651-6192
ISBN 91-554-6197-2
urn:nbn:se:uu:diva-5728

Dissertation presented at Uppsala University to be publicly examined in Room C8:301, BMC, Uppsala, Friday, April 22, 2005 at 09:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in Swedish.

**Abstract**
Lindholm, P. 2005. Cytotoxic Compounds of Plant Origin – Biological and Chemical Diversity. (Cytotoxiska föreningar från växter – biologisk och kemisk diversitet). Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 7. 71 pp. Uppsala. ISBN 91-554-6197-2.

The development of resistance by tumour cells to chemotherapeutic agents is a major problem in cancer treatments. One way to counter this is to find compounds with cytotoxic mechanisms other than those of drugs in clinical use today. The biological and chemical diversity encountered in Nature provide opportunities to discover completely new chemical classes of compounds. Some of these may represent previously unknown anticancer agents, and in some cases, novel, potentially relevant cytotoxic mechanisms.

The selection of plants for the cytotoxic investigation in this project was designed to cover large parts of the angiosperm system, providing a broad representation of species. Extracts of the plants were subjected to a polypeptide fractionation protocol, followed by bioassay-guided isolation, yielding series of fractions with increasing purity and cytotoxicity. The cytotoxicity assay included tumour cells from patients and a cell-line panel including ten different cell lines representing several types of resistant and non-resistant tumours. This screening strategy allowed fractions and compounds acting with novel mechanisms to be detected at an early stage.

The compounds isolated represent substantial chemical diversity and originate from diverse parts of the phylogenetic spectrum examined. They include the highly potent cytotoxic alkaloid, thiobinupharidine, the structure of which was determined by NMR techniques. Furthermore, two types of compound were shown to have previously unreported cytoxic activity: cyclotides (small macrocyclic polypeptides, in this case from violets) and polypeptides, possibly of thionine type, of loranthaceaeous mistletoes (collected in Panama). The well known cardiac glycosides from the foxglove, Digitalis, were identified as being responsible for the anti-tumour activity of this species.

In conclusion, the results obtained in this project show that selection based on phylogenetic information, together with a robust and reliable method to detect cytotoxicity, can be a useful approach for exploring the plant kingdom for cytotoxic substances.

*Keywords:* pharmacognosy, cytotoxicity, antitumour, Nuphar alkaloid, cyclotide, thionin, cardiac glycoside

*Petra Lindholm, Department of Medicinal Chemistry, Division of Pharmacognosy, Box 574, Uppsala University, SE-75123 Uppsala, Sweden*



ISSN 1651-6192
ISBN 91-554-6197-2
urn:nbn:se:uu:diva-5728 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-5728)

*"I alla svar ryms nya frågor."*
Okänd

*Till Liten*

# List of Papers

This thesis is based on the following papers, which will be referred to in the text by the corresponding Roman numerals.

**I** **Lindholm P**.*, Gullbo J.*, Claeson P., Göransson U., Johansson S., Backlund A., Larsson R., Bohlin L. (2002) Selective cytotoxicity evaluation in anticancer drug screening of fractionated plant extracts. *Journal of Biomolecular Screening*, 7(4):333-340.

**II** **Lindholm P**., El-Seedi H., Göransson U., Gullbo J., Larsson R., Backlund, A. Bioassay-guided isolation of a cytotoxic quinolizidine alkaloid from *Nuphar luteum* (Submitted to *Chemistry & Biodiversity*).

**III** **Lindholm P**.*, Göransson U.*, Johansson S., Claeson P., Gullbo J., Larsson R., Bohlin L., Backlund A. (2002) Cyclotides: a novel type of cytotoxic agents. *Molecular Cancer Therapeutics*, 1(6):365-369.

**IV** **Lindholm P**., Larsson S., Gupta, M., Larsson R., Backlund A. Cytotoxicity of small peptides of mistletoes in the family Loranthaceae, Santalales (Manuscript).

**V** Johansson S., **Lindholm P**., Gullbo J., Larsson R., Bohlin L., Claeson P. (2001) Cytotoxicity of digitoxin and related cardiac glycosides in human tumor cells. *Anticancer Drugs*, 12(5):475-483.



*These authors contributed equally to this work

# Contents

# Abbreviations

| | |
|---|---|
| 1D | one dimensional |
| 2D | two dimensional |
| AGC | accelerating gradient chromatography |
| AUC | area under curve |
| $CH_2Cl_2$ | dichloromethane |
| CLL | chronic lymphocytic leukaemia |
| DIAS | Dynatech immuno assay system |
| DMSO | dimethyl sulphoxide |
| DTP | Developmental Therapeutics Program |
| EtOH | ethanol |
| FDA | fluorescein diacetate |
| FMCA | fluorometric microculture cytotoxicity assay |
| GSH | glutathione |
| HAc | acetic acid |
| HPLC | high performance liquid chromatography |
| $IC_{50}$ | test concentration giving 50% survival |
| IEC | ion exchange chromatography |
| IR | infrared |
| MDR | multidrug resistance |
| MeOH | methanol |
| MRP | multidrug resistance associated protein |
| MS | mass spectrometry |
| NCI | National Cancer Institute |
| NMR | nuclear magnetic resonance |
| NSCLC | non-small cell lung cancer |
| OVCA | ovarian carcinoma |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| P-gp | P-glycoprotein |
| RP | reversed phase |
| SCLC | small cell lung cancer |
| SI | survival index |
| SPE | solid phase extraction |
| TFA | trifluoroacetic acid |
| TLC | thin layer chromatography |
| UV | ultraviolet |

# Introduction

## Cytotoxicity

When a compound is described as being toxic we mean it is “poisonous”. However, everything is toxic in excessive doses, as expressed by the maxim *Dosis sola facit venenum* (Dosage alone makes the poison), attributed to Paracelcus (1493-1541). Analogously, cytotoxicity means toxicity to the cells. But in the search for cytotoxic compounds with potential as anticancer drugs, not all compounds that exhibit cytotoxicity are of interest. The doses and concentrations required are highly relevant, as is the need for the compounds to meet key clinical criteria. The demonstrated cytotoxicity should preferably be of a level, and mediated through a mechanism, that allows healthy cells to survive, but not tumour cells. The primary goal for cytotoxic drugs, or cytostatic agents, is to prevent the growth of cancer cells. This is usually achieved by targeting mechanisms that directly affect DNA replication or transcription, or by disturbing functions of the cell that are important in mitosis. Since cytotoxic compounds affect all cells, there are inevitably side effects, but the healthy cells can usually cope with them or repair the resulting damage more easily than tumour cells. However, tumorous cells often develop resistance to cytotoxic compounds. Thus, selectivity and resistance are major issues in attempts to identify such compounds, both of which are discussed below.

## Cancer and chemotherapy

Cancer is a term describing conditions characterized by uncontrolled cellular proliferation and differentiation (Ponder 2001). These are very common diseases (41400 new cases were reported in Sweden in 2003) and their incidence is increasing at an average annual rate of 1.2%. Furthermore, the prevalence, the number of persons resident in Sweden with a diagnosed cancer, is increasing by as much as 3% per year. The main factors responsible for this trend are the increasing average age of the population (which accounts for about half of this increasing incidence) and improvements in diagnostic methods. There has also been an increase in the age-adjusted incidence, and improvements in treatment strategies, which have resulted in prolonged survival of patients with chronic cancer disease,

demonstrating the growing needs for additional means of therapy, and both palliative and curative treatments. The strategies available today, however sophisticated, are only able to treat (with curative intention) 60% of the cases – while 40% of these diagnosed with cancer will eventually die of their disease (Verweij and Jonge 2000; Talbäck *et al.* 2003; Socialstyrelsen 2004).

The development of cancer, carcinogenesis, is a multistage process. The initiating agent, the carcinogen, is any substance or agent that increases the incidence of tumours, *e.g.* a virus, an exogenous chemical compound, or ionizing radiation. These carcinogens may be excreted (chemicals), deactivated (chemicals or radiation), or inhibited (viruses) after entering the body. There is a high risk that any carcinogen that is not neutralised by one of these mechanisms will activate a normal cell and eventually induce a genetic mutation. The first step, known as the initiating step, is when a carcinogen interacts with DNA, thereby modifying a gene, causing strands to break or, more often, reacting with a nucleotide and thus forming an adduct. If the genome happens to be replicated before the damage is enzymatically repaired, a DNA polymerase may replicate the injured sequence and permanently fix an inheritable error in the genome. In most cases this will not affect the cell, but if the modification occurs in a coding sequence, or some other essential element, *e.g.* a transcription promoter region, the cell may grow or develop abnormally.

The initiated cell is further stimulated to proliferate by promoters and may eventually develop into a benign tumour, or preneoplastic lesion. This happens through the promotion or selective clonal expansion step, characterised by the development of defects in terminal differentiation and growth control. The benign tumour may then further develop, via the conversion or progression step, into a malign tumour, undergoing more changes that increase the probability that it will become neoplastic. Each of these steps can be activated by onco genes (*e.g*. *ras* and *myc*) or deactivated by suppressor genes (*e.g*. *Rb* and *p53*).

A malign tumour, which is less regulated than a benign tumour, may convert over time into a primary tumour, thus becoming a "clinical cancer". The primary tumour cells can subsequently pass through a progression of steps leading to a heterogenic tumour, which can begin to form metastases. These are cancer cells that invade surrounding tissues, transported via the blood or lymphatic system, and attach to target organs, then invade, and grow, eventually forming a metastatic colony within the target organ. The stepwise process of carcinogenesis requires multiple genetic changes, and often mutations.

Chemotherapy has been used in cancer treatment for more than 50 years, sometimes in combination with or parallel to surgery and radiotherapy, and has proven to be one of the most efficient strategies for treating cancer. Chemotherapy is most often directed towards proliferating cells, and chemotherapeutic agents may be administered by injection directly into the

bloodstream or absorbed into the blood after oral dosing. In contrast to surgery and radiotherapy, chemotherapy is a form of systemic therapy as the dose is distributed throughout the body. This means that undesired side effects may occur, but also that chemotherapy is the only conventional method that has the potential to treat every malignant cell of a metastatic cancer. This is a great advantage due to the technical difficulties involved in locating metastatic cells.

The first modern chemotherapeutic agents were products of programs designed to prepare for chemical warfare during the First World War. Studies of the toxic effects of exposure to mustard gas led to the observation that alkylating agents can cause marrow and lymphoid hypoplasia. This led in 1943 to the first documented treatment of a patient with lymphoma, but the results were not published until 1946 (Gilman 1963).

There are 76 registered chemotherapeutic substances in clinical use for cancer treatment in Sweden today (S.-E. Hillver, MPA, personal communication; FASS 2005). According to a recent SBU (the Swedish Council on Technology Assessment in Health Care) report (Glimelius *et al.* 2001) these chemotherapeutic drugs can be classified into seven main groups based on their mechanisms of action: (1) alkylators and alkylator-like agents; (2) antimetabolites; (3) DNA interacting agents; (4) membrane perturbers; (5) topoisomerase (topo) inhibitors; (6) microtubuli interacting agents; and (7) amino acid depletors. Examples of drugs from these groups are shown in Table 1.

Table 1. *Classification of chemotherapeutic agents.*

| **Mechanism of action** | **Drug example(s)** |
|---|---|
| Alkylation | melphalan |
| Antimetabolism | methotrexate |
| DNA interaction | bleomycin |
| Membrane perturbation | miltefosin |
| Topoisomerase (topo) inhibition | doxorubicin, podophyllotoxin |
| Microtubuli interaction | vincristine, paclitaxel |
| Amino acid depletion | asparaginase |

## Mechanisms of action and cellular resistance

The resistance of tumour cells to chemotherapeutic agents is a major problem in the clinical treatment of cancer. Tumours occasionally develop mechanisms of cellular resistance to the cytotoxic activity of certain chemotherapeutic agents, a syndrome known as multidrug resistance, MDR (Ford 1995). Tumours can develop this resistance to several drugs after a single drug has been administrated due to changes in the genome and/or the stimulated expression of specific key proteins (such as absorbing proteins, detoxifying enzymes, or target proteins) a phenomenon known as acquired

resistance. A tumour that initially responded to chemotherapeutics, will then eventually progress in spite of continued treatment (Hellman and Vokes 1996; Lehnert 1996). The development of MDR has been clinically observed as a response to several chemotherapeutic agents such as the *Vinca*-alkaloids, anthracyclines, antibiotics, and epipodophyllotoxins (Beck *et al.* 1987; Danks *et al.* 1987).

Tumour cell resistance mechanisms are largely related to the primary targets of drug action described earlier, such as interference with transcription, DNA synthesis and mitosis, blockage or equilibrium disturbance of polymerisation and depolymerisation of microtubules, DNA strand breaks, and binding to plasma membranes. Figure 1 summarizes the key enzymes or proteins that are targets of, or act on, different anticancer agents. Their inhibition or altered expression has been traditionally regarded as being related to cellular and molecular drug mechanisms. However, novel concepts, related to cell cycle or cell death proteins, also need to be considered in attempts to describe and understand a drug's anticancer mechanism (Hellman and Vokes 1996; Beck and Dalton 1997).

Several processes are presumed to be involved in the development of resistance, such as alterations in drug accumulation; changes in sub-cellular drug distribution, leading to altered drug concentrations at the target sites; differences in DNA repair capabilities; and changes in cellular metabolic systems that facilitate detoxification of the agent (Bellamy *et al.* 1991). MDR in tumour cell lines has been linked in some cases to over-expression of P-glycoproteins (P-gp) or MDR-associated proteins (MRP). Both P-gp and MRP are members of the ATP-binding (adenosine triphosphate-binding) cassette superfamily, also known as the ABC-superfamily, of membrane transport proteins. Increased expression of these membrane transport proteins decreases drug accumulation in the cell, alters the sub-cellular drug distribution, or both (Gollapudi *et al.* 1997). One way to overcome resistance problems resulting from the over-expression of P-gp or MRP is to develop chemosensitizers for MDR cells, called MDR-modulators. Inhibition of P-gp activity (Ford 1995), or inhibition of substances with selective or enhanced activity in MRP over-expressed cells are other possible strategies. One such substance, probenicid, reverses MDR in certain cell lines, albeit not in P-gp over-expressed cell lines (Gollapudi *et al.* 1997).

Clinical resistance may be caused by numerous additional factors, which differ between tumour cells as well as between patients. Apart from resistance at the cellular level, clinical resistance can also be caused by the degradation of anti-tumour drugs, resulting in the doses applied being insufficient to kill the targeted cells (Larsson and Nygren 1993).

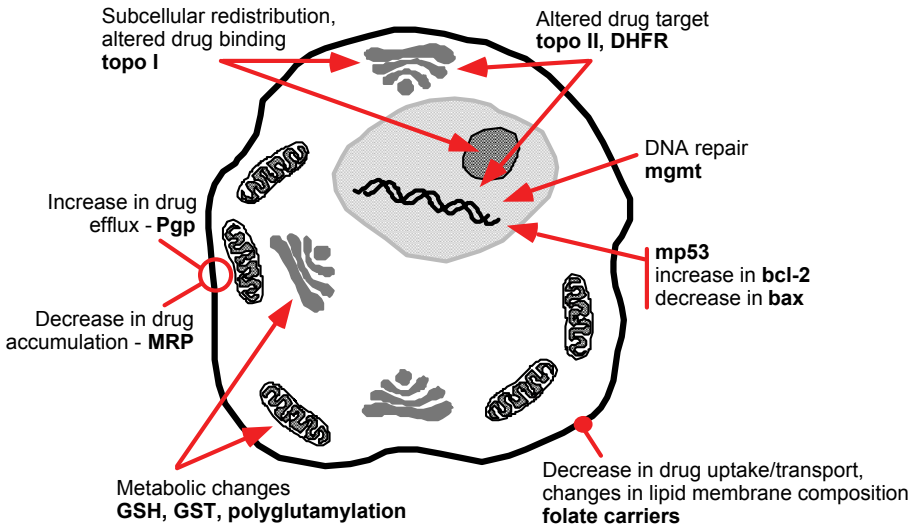



*Figure 1.* Cellular mechanisms of drug resistance. Targets or actions of different anticancer agents. Redrawn after Beck and Dalton (1997).

Solid tumours such as non-small cell lung cancer (NSCLC), renal carcinoma, and gastrointestinal carcinoma are among several other types of tumour that have an initial drug resistance (Glimelius *et al.* 2001).

Others types of tumour, such as small cell lung cancer (SCLC), ovarian cancer, and leukaemia, may respond initially to applied drugs, but then develop clinical resistance later. In such cases resistance often develops not only to the drugs initially used, but also to drugs that the patient has never previously been exposed to. This phenomenon, known as cross-resistance, is analogous to patterns observed in many cases of allergy (Mirski *et al.* 1987; Bellamy *et al.* 1991; Botling *et al.* 1994), which appears to be due to different allergens having sufficient functional resemblance to induce similar responses.

The actual mechanisms of action are still unclear for many drugs used in cancer treatment. These drugs can, nonetheless, be roughly classified by testing them on a cell-line panel representing several types of resistant or non-resistant tumours. The activity profile, *i.e.* the distribution of activity efficiency across cell lines of each compound or drug can be compared with that of substances with known anticancer mechanisms. In this way it is possible to suggest a classification of each compound according to its mode of action (Boyd and Paull 1995; Dhar *et al.* 1996). A profile with little or no correlation to any of the previously studied substances that have well-defined mechanisms is tentatively thought to represent a new mechanism (Dhar *et al.* 1996; 1998).

## Anticancer drugs and natural products

From the above discussion on MDR and mechanisms of action of anti-cancer drugs, it is evident that a wide array of selective and potent compounds is needed to match the growing problems associated with cancer.

Natural products play an important role in chemotherapy (Pezzuto 1997; Cragg 1998; Lee 1999; 2004; Schwartsmann 2000). They offer a valuable source of compounds with a wide variety of biological activities and chemical structures, and provide important prototypes for the development of novel drugs (Cassady *et al.* 1981; Cragg 1998; Verpoorte 1998; Vuorela *et al.* 2004). This is also true for anticancer agents, most of which have been derived from natural sources; directly as pure, native compounds, or as semisynthetic analogues, or as models for synthetic compounds. Some anticancer drugs of natural origin in clinical use in Sweden (FASS 2005) are listed in Table 2. Chemical structures of selected drugs representing several different mechanisms and compound classes, are given in Figure 2.

Anticancer drugs of natural origin, and their semisynthetic analogues, exert their effects (on the cancer cell) with distinct, definable mechanisms. For example, the topoisomerase inhibitors, of which podophyllotoxin, topotecan, and etoposide are examples, interfere with transcription, DNA synthesis, and mitosis by blocking the enzymes DNA topoisomerase I and II. In contrast, the *Vinca*-alkaloids and taxanes, *e.g.* vincristine and paclitaxel, block the polymerisation and depolymerisation, respectively, of microtubuli, thereby interfering with key steps in cell division, such as organisation of the mitotic spindle and thus the mitotic arrangement of the chromosomes.

Camptothecin promotes DNA-strand breaks, thus disturbing DNA replication, whereas anthracyclines, including doxorubicin and daunorubicin, likely have multiple sites of action that include intercalation into DNA, inhibition of DNA topoisomerase II, production of free radicals, and binding to plasma membranes (*e.g.* Beck and Dalton 1997; Glimelius *et al.* 2001).

The biodiversity and chemodiversity encountered in nature also provide opportunities to discover completely new chemical classes of compounds (Cassady *et al.* 1981), some of which may represent previously unknown anticancer agents, and consequently, novel and potentially relevant mechanisms (Schwartsmann 2000).

Table 2. *Examples of anticancer substances of natural origin, and selected drugs in which the compounds are used; their origin, therapeutic use, first scientific report, developmental status, and a brief description of their mechanism of action, when known (cited references and Farnsworth 1988).*

| Substance & drug name | Origin | Therapeutic use | Publication | Mechanism of action |
|---|---|---|---|---|
| **Camptothecin** | Alkaloid first found in *Camptotheca acuminata* | Used clinically in China against gastrointestinal tumours | 1966 (Wall *et al.* 1966) | Enhances binding of topoisomerase I to DNA, thus promoting DNA strand breaks |
| **Daunorubicin** *Cerubidin®* *DaunoXome®* | *Streptomyces peuceticus* and *S. coeruleorubidus* | Antineoplatsic agent against AIDS-related Kaposis disease | 1964 (Arcamone *et al.* 1964) | Inhibition of replication and transcription |
| **Docetaxel** *Taxotere®* | From the needles of *Taxus baccata* | Antineoplastic agent for treatment of ovarian, breast and bronchial carcinomas | 1989 (Mangatal *et al.* 1989) Launched 1995 (South Africa) | Binding to tubulin subunits and stabilization of microtubuli |
| **Doxorubicin** *Adriamycin®* *Caelyx®* | *Streptomyces peuceticus,* mutant strain | Antineoplastic agent in therapies against skin carcinoma, Hodgkin's disease and testicular cancer | 1969 (Arcamone *et al.* 1969) | Unclear. Inhibition of DNA, RNA, and protein synthesis |
| **Etoposide** *Eposin®* *Etopofos®* *Exitop®* *Vepesid®* | Semisynthetic podophyllo-toxin derivative from *Podophyllum* sp. | Antineoplastic agent against SCLC, leukaemia, non-Hodgkin lymphoma, Hodgkin's disease and testicular cancer | 1971 (Keller-Juslén *et al.* 1971) | DNA topoisomerase II inhibitor |
| **Irinotecan** *Campto®* | Semisynthetic, synthesised from camptothecin | Antineoplastic agent against colorectal carcinoma | 1987 (Kunimoto *et al.* 1987) Launched 1994 (Japan) | DNA topoisomerase I inhibitor |
| **Krisantaspas** *Erwinase®* | L-asparaginase from *Erwinia chrysanthemi* | ALL | 1963 (Tower *et al.* 1963) | Inhibition of malign, asparagine dependent cells |
| **Paclitaxel** *Taxol®* | The stem bark of *Taxus brevifolia* and *T. cuspidata* (Taxaceae) Possibly also in the fungus *Taxomyces andaeanae* | Antineoplastic agent against breast carcino-mas and metastasing NSCLC | 1971 (Wani *et al.* 1971) Launched 1993 (US, Canada, Sweden) | Binding to tubulin subunits and stabilization of microtubuli |
| **Peplomycin** *Bleomycin®* | Semisynthetic from *Streptomyces verticullus* | Antineoplastic agent used in combination therapies against melanoma, Hodgkin's disease and testicular cancer | 1977 (Tanaka 1977) | Unclear. Inhibition of DNA synthesis and cell division |
| **Teniposide** *Vumon®* | Closely related to Etoposide | Antineoplastic agent, used in combination therapies | 1970 (Stähelin 1970) | DNA topoisomerase II inhibitor |
| **Topotecan** *Hycamtin®* | Analogue of camptothecin with improved aqueous solubility | Antineoplastic agent against ovarian carci-noma | 1989 (Kingsbury *et al.* 1991) Launched 1996 (US) | DNA topoisomerase I inhibitor |
| **Vinblastine** *Velbe®* | Alkaloid from *Vinca rosea* (Apocynaceae) | Antineoplastic agent used in treatment of Hodgkin's disease and choriocarcinoma | 1959 (Neuss *et al.* 1959; Neuss *et al.* 1962; Neuss *et al.* 1964) | Antimitotic. Inhibition of cell division |
| **Vincristine** *Oncovin®* *Vincristine®* | Alkaloid from *Vinca rosea* (Apocynaceae) | Antineoplastic agent used in therapies for leukaemia, SCLC and malign lymphoma | 1958 (Farnsworth 1988) | Antimitotic |
| **Vinorelbine** *Navelbine®* | Semisynthetic, synthesised from vinblastine | Antineoplastic agent used in treatment of NSCLC and breast cancer | 1979 (Mangeney *et al.* 1979) Launched 1989 | Antimitotic. Inhibition of tubulin polymerisation |

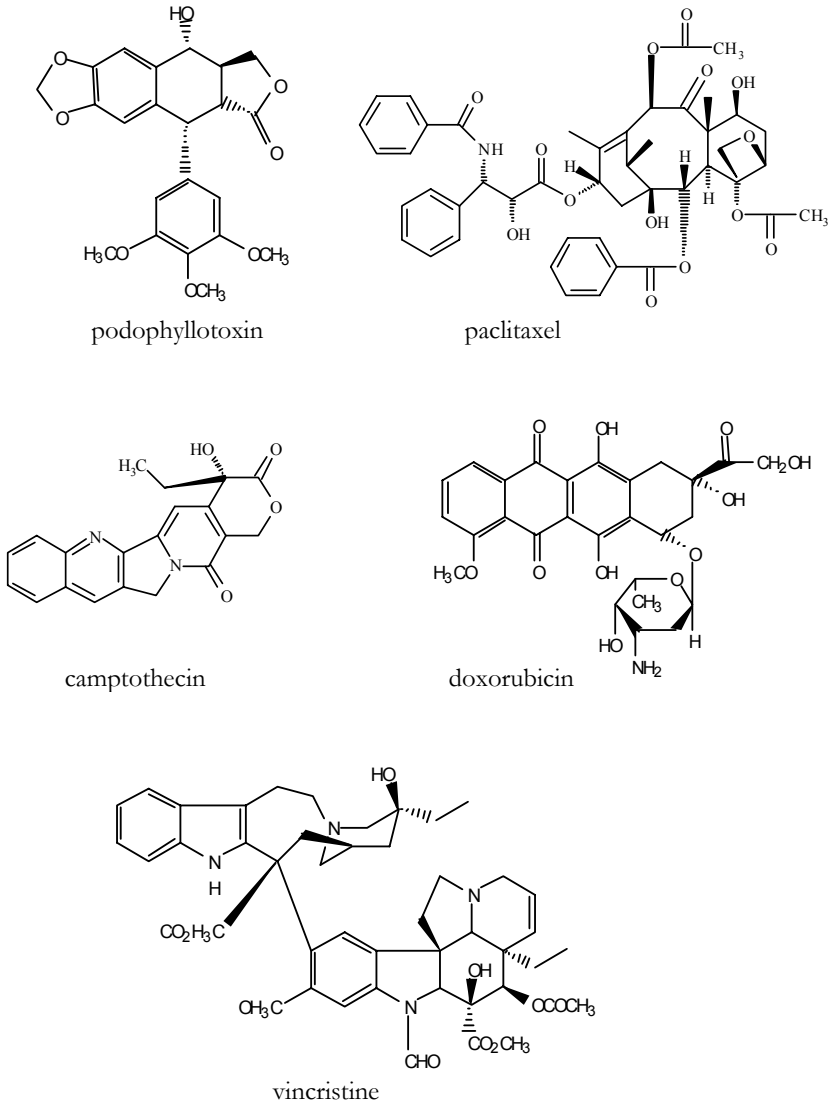



*Figure 2.* Chemical structures of some of the compounds listed in Table 1. Podophyllotoxin, the precursor for etoposide, is itself also a DNA topoisomerase II inhibitor; paclitaxel is a tubulin interferent; camptothecin is an inducer of DNA strand breaks; doxorubicin is an inhibitor of the synthesis of DNA, RNA and protein; and vincristine is an antimitotic compound.

## Anticancer drug discovery and development

The United States National Cancer Institute, the NCI, was established in 1937 to "provide for, foster and aid in coordinating research related to cancer," (Cragg 1998). In 1955 the institute initiated a large-scale screening program for national drug development in the United States. Initially, transplantable L1210 and P388 mouse leukaemias were used as tumour models for screening (DeVita 1997). Several drugs with activity against leukaemia and lymphomas were soon identified and developed.

Solid tumour models were introduced in 1975, but the primary screening model used was still the murine leukaemia P388. Only compounds with observed activity in the P388 model were passed on for testing with the solid tumour panel. However, increasing numbers of compounds with anti-lymphoma and leukaemia activity were found to have little or no activity against solid tumours, or to be analogues of drugs already in use.

Since 1985, a cell-line panel screening strategy has been used instead in the NCI's Developmental Therapeutic Program (Boyd and Paull 1995). The panel utilized for this purpose, consisting of more than 60 cell lines, is designed to represent the most important forms of human cancers, with the aim of identifying novel chemotherapeutic agents. To represent the major classes of solid tumours in an appropriate fashion, the NCI further developed the cell-line panel for use in mechanism-based screening (Chabner 1990). The cell lines are organized into sub-panels representing seven major types of cancers: leukaemia, melanoma, and cancers of the lung, colon, kidney, ovary, and central nervous system (Monks *et al.* 1991). The experimental design and management of the acquired data also allows *in vitro* responses to be classified in relation to different types of known mechanisms. The new screening panel has a central role in the NCI's anticancer drug development program.

In screening for cytotoxic activity, the most commonly determined parameter is the $IC_{50}$ value. For a cytotoxic substance this describes the concentration at which the substance reduces the survival of cells by 50% via its cytotoxic activity, as estimated from dose-response curves. For each substance tested against all ten cell lines in the cytotoxicity assays in the work underlying this thesis, the overall mean $\log_{10}$ $IC_{50}$ was determined, defined as the mean of the log10 values for all ten cell lines in the panel. Subsequently, the mean $\log_{10}$ $IC_{50}$ was subtracted from the $\log_{10}$ value of each cell line to yield a variable defined as delta.

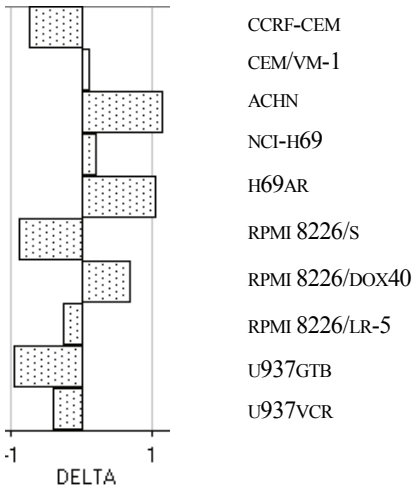



*Figure 3.* Activity profile for doxorubicin.

A mean graph, *i.e.* an activity profile, consisting of the averaged delta values for each drug across the cell-line panel could then be constructed to visualize differences in the cytotoxicity patterns of the drugs (Paull *et al.* 1989). Thus, positive and negative values indicate cell lines that are more sensitive and less sensitive, respectively, than average for a particular drug. Figure 3 shows an example of an activity profile for doxorubicin.

Using the COMPARE algorithm (Paull *et al.* 1989), different compounds can be tested and rank-ordered for their similarity to a mean-graph profile, based on their delta values. Tested compounds may be found to have lower toxicity, or different pharmaceutical properties or anti-tumour spectra. To calculate comparable activity profiles, $\log_{10}$ $IC_{50}$ values for each tested cell line is compared to the cell lines' average $\log_{10}$ $IC_{50}$ values for all tested substances, and the difference between them is expressed as the delta value.

COMPARE software, in addition to calculating an activity profile serving as a fingerprint for agents tested in the cell-line panel, analyses each such profile, comparing it with other profiles obtained from known drugs (Paull *et al.* 1989; Sikic 1991; Weinstein *et al.* 1992; Boyd and Paull 1995).

Different cytotoxic mechanisms result in different activity profiles, *i.e.* different characteristic patterns of responses amongst the cell lines; in reverse, different activity profiles can reflect differences in cytotoxic mechanisms.

Selected activity profiles are discussed in this thesis and are presented in Figures 7, 10, and 12 under Results.

Dhar *et al.* (1996) have shown that a cell-line panel with a selection of only 10 different cell lines, representing a smaller number of defined types

of cytotoxic drug resistance, could be used in an initial evaluation and preliminary mechanistic classification of anticancer agents. Using this cell-line panel, the responses to 37 drugs (some with known and others with unknown mechanisms) were measured. The drugs tested included eight topoisomerase II inhibitors, eight anti-metabolites, eight alkylating agents, eight tubulin-active agents, as well as five compounds with other known or unknown mechanisms, including one topoisomerase I inhibitor. Attempts to classify these drugs in four categories, each representing a different mechanism (topoisomerase II inhibitors, alkylators, tubulin-active agents, and anti-metabolites) gave highly reproducible results (Dhar *et al.* 1996), validating the strategy of using a limited number of cell lines for preliminary prediction of drugs' anticancer mechanisms. The method is well suited for providing important initial information, not only on the mechanistic action of novel anticancer drugs, but also on their susceptibility to defined mechanisms of resistance at the molecular level.

In another study by Dhar *et al*. (1998), primary cultures of cells from patients with chronic lymphocytic leukaemia (CLL) and ovarian carcinoma were compared with renal carcinoma (ACHN) and lymphocytic tumour (CCRF-CEM) cell lines in terms of their responses to a set of test compounds. The results, presented as areas under the tumour-cell survival-concentration curve (AUC), showed that some compounds were cytotoxic to both the cell lines and primary cultures, while others were non-cytotoxic to either the cell lines or primary cultures. Thus, primary cultures of human tumour cells may be useful models for anticancer drug screening, at least in moderate-scale programs, and may have advantages as indicators of cytotoxity *in vivo* compared with cell lines.

These screening approaches form the strategy followed in the studies this thesis is based upon: testing a cell-line panel with a limited number of human tumour cells, complemented with the use of primary cultures of cells from patients, and comparing the activity profiles of the tested compounds (Papers **I**, **III** and **IV**).

## Polypeptides and natural products

Polypeptides have long been known to have important biological functions (Lam *et al.* 1998; Pinson *et al.* 2000; Pearce *et al.* 2001). Several current drugs have been developed based on knowledge of these polypeptides. The majority of such polypeptides, such as hormones and toxins, have thus far been isolated from humans and other animals, while relatively few have come from plants. However, many studies indicate that plants comprise an important and largely unexplored source of novel polypeptides, and even polypeptide classes, as illustrated in Table 3. In many cases the function(s) of these polypeptides in the plants is still unknown. Yet, some

known biological activities, such as use of polypeptides as signal substances, *e.g.* the systemins, and their occasional expression in large quantities (up to 0.1‰$_w$), suggest that many of these polypeptides may be involved in plant defences. Some plant peptides with cytotoxic activity have been isolated, such as the thionins isolated from several species of orders such as the Poales and Santalales (Li *et al.* 2002; Johansson *et al.* 2003); the astins, cyclic pentapeptides isolated from *Aster tataricus* (Asteraceae) (Morita *et al.* 1996b); yunnanins, cyclic polypeptides isolated from *Stellaria yunnanensis* (Caryophyllaceae) (Morita *et al.* 1996a); and the glycoprotein ARS2 from *Chlorella vulgaris* (Oocystaceae) (Noda *et al.* 1996).

Table 3. *Some known polypeptides of plant origin.*

| Compound | Size* | Origin | Biological activity | Described | Reference example |
|---|---|---|---|---|---|
| Astin | 5 | *Aster tataricus* | Cytotoxic | 1995 | (Morita *et al.* 1996b) |
| Yunnanins | 6-7 | *Stellaria yunnanensis* | Cytotoxic | 1994 | (Morita *et al.* 1996a) |
| Cycloleonurinin | 12 | *Leonurus artemisia* | *Unknown* | 1991 | (Kinoshita *et al.* 1991) |
| Peptide PII | 14 | *Panax ginseng* | Insulinomimetic | 1980 | (Ando *et al.* 1980) |
| ARS2 | 15 | *Chlorella vulgaris* | Cytotoxic | 1995 | (Noda *et al.* 1996) |
| Systemin | 18 | *Lycopersicon esculentum* | Plant hormone | 1991 | (Pearce *et al.* 1991) |
| Violapeptid-I | 28 | *Viola tricolor* | Haemolytic | 1993 | (Schöpke *et al.* 1993) |
| Kalata B1 | 29 | *Oldenlandia affinis* | Uterus contractive | 1995 | (Saether *et al.* 1995) |
| CMTI-I | 29 | *Cucurbita maxima* | Trypsin inhibitor | 1987 | (Polanowski *et al.* 1987) |
| Varv peptide A-H | 29 | *Viola* spp. | Cytotoxic | 1998 | (Claeson *et al.* 1998; Göransson *et al.* 1999) |
| Circulins A & B | 30-31 | *Chassalia parvifolia* | HIV-inhibitor | 1994 | (Gustafson *et al.* 1994) |
| Cyclopsychotride A | 31 | *Psychotria longipes* | Neurotensin antagonist | 1994 | (Witherup *et al.* 1994) |
| Hypogin | 40 | *Arachis hypogaea* | Antifungal | 2001 | (Ye and Ng 2001) |
| Palicourein | 37 | *Palicourea condensata* | HIV-inhibitor | 2001 | (Bokesch *et al.* 2001) |
| Dodecandrin | +32 | *Phytolacca dodecandra* | Ribosome inactivating | 1984 | (Ready *et al.* 1984) |
| Thionins | 45-48 | *e.g.* Poaceae, Viscaceae | Antifungal, cytotoxic etc. | 1948 | (Winterfel and Bijl 1948; Samuelsson 1973; Bohlmann and Apel 1991) |

*number of amino acid residues

# Phylogeny and evolutionary space

The vast chemical diversity displayed in nature is the result of eons of evolution and continuous changes in biosynthesis driven by adaptive selection. In contrast to many outdated theories, recent phylogenetic studies of molecular sequence data indicate that animals, fungi, and higher plants diverged from a common ancestor only a few hundred million years ago (see, for instance, Figure 4; from Sogin 1991).

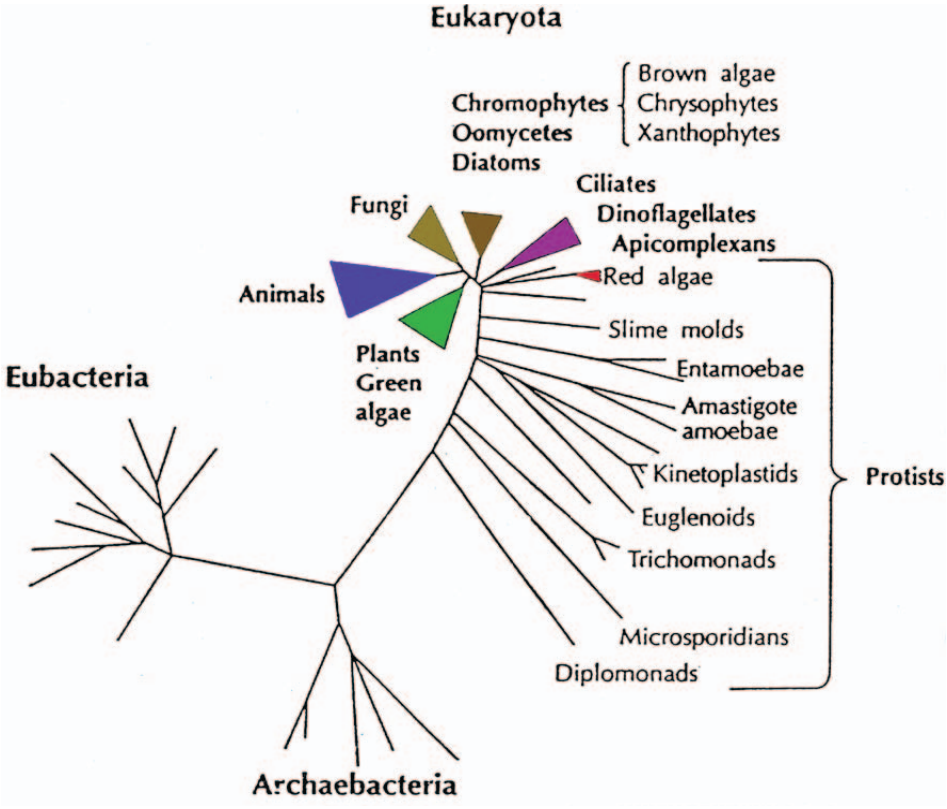



*Figure 4.* Phylogenetic tree of the living organisms on earth, indicating the three major domains: eubacteria, archebacteria, and eukaryotes, from Sogin 1991.

This long-lasting and shared history is presumably one of the reasons that so many common enzymatic pathways are now found among these morphologically diverse groups of organisms. Consequently, it seems reasonable to suppose that many of the substances for which biosynthetic pathways and mechanisms have been developed in plants during the course of evolution, may also have biological activities in animals – including man.

That such couplings occur are obvious, and easily demonstrated *e.g.* by plant derived substances affecting the ubiquitous GABA receptors in animals. Similarity is even so profound that plant models have been suggested for studies of human pathogenesis (Guttman 2004). However, the massive amounts of information available require analytical tools to help abstract patterns that we can interpret. Both nucleotide sequence data, with thousands of aligned nucleotides for phylogenetic analysis, and the multi-dimensional space of a principal component analysis of *e.g.* chemometric or

microarray data, contain such complexity that they need to be simplified before conclusions can be drawn and hypotheses formulated.

Analogously to the way chemometrics has been used to explore structural and functional relationships amongst chemical compounds, the concept of phylogenetics has been used in systematic and evolutionary biology to navigate evolutionary space. Initially building on Darwinian foundations, explicit diagrams indicating relationships and sequences of development were introduced by Haeckel (1866). Through the work by Hennig (*e.g.* 1966) and others the modern concept of phylogenetics subsequently developed, which has contributed greatly in the last 20 years to our understanding of evolutionary processes.

Phylogenetic hypotheses, suggestions of evolutionary relationships, are often expressed in the form of a tree-diagram, and are normally conceived by analyses of sets of data.

Such diagrams for phylogenetic reconstruction were generally based on morphological information initially, but nowadays the most important types of data are nucleotide sequences. Whatever sort of information is used, there are several fundamentally different ways to perform the analyses and approach the same ultimate goal – a well supported, evolutionary hypothesis. Once obtained, this hypothesis can be used for several different purposes.

Formulating new evolutionary hypotheses are beyond the scope of this study, but the comprehensive work by the Angiosperm Phylogeny Group (APG 1998; 2003) has been used as a phylogenetic framework for both selecting study objects and interpreting the obtained results.

Phylogenetics is now an integral component of systematic analyses, including taxonomic studies. Taxonomy is the science of naming and classifying diverse organisms, in this case plants. Phylogeny describes the connections between, and evolutionary history of, sets of organisms, in a biological group.

# Aims of the study

The main aims of the studies underlying this thesis were to find and identify naturally occurring compounds that exhibit cytotoxic activity towards tumour cells. This was done as follows. The first step was *a priori* selection, based on literature surveys, of plants for the cytotoxic investigation, aiming to cover a wide taxonomic range of species. Extracts of these plants were subjected to a protocol designed to identify and retain fractions with polypeptides. Compounds in these plants with anticancer potential, *i.e.* pronounced cytotoxic activity, were then identified and purified by screening all obtained fractions using two successive cytotoxicity assays that can compare different types of anti-tumour activity. Further selectivity assessments and verification procedures were then applied to the selected fractions, using tumour cells from patients in order to detect compounds acting with new mechanisms. This involved evaluating the identified compounds' cytotoxicity using cell lines representing four different resistance mechanisms, and comparing the resulting activity profiles with activity profile databases compiled in-house, and analysis (by several different approaches) of structure-activity relationships of pure, isolated, and structurally related compounds.

The rationale for the overall project design was based on two general assumptions, as presented on the following page.

Cancer is a growing problem and this is further complicated by the MDR syndrome, which many tumours develop sooner or later during chemo-therapy. One way to partly overcome the MDR problem is to challenge tumours with additional compounds with novel structures acting through new mechanisms. Theoretically, such compounds can be synthesised by organic chemists, but the number of *possible* compounds is estimated to exceed $10^{60}$ purely in the limited, so-called 'drug-like space' (Bohacek *et al.* 1996). In Nature, on the other hand, compounds with diverse biological activities have been developed, tested and selected over hundreds of millions of years. These compounds have not been produced by chance; instead compounds with activities that do not have adaptive benefit (or less benefit than other alternatives) have been eliminated by intense evolutionary pressures. The understanding that evolution and biosynthesis are intimately connected was clearly expressed at an early stage by Helen DeSilver Abbott Michael in the late 19th century:

> "The evolution of chemical constituents follows parallel lines with the evolutionary course of plant forms, the one being intimately connected with the other..."
>
> (Abbott in Tarbell and Tarbell 1982)

Hence, to obtain naturally occurring chemical substances of wide chemical diversity, they should be sought from diverse biological systems. The biological diversity exploited here was defined in a phylogenetic framework.

Second, to select suitable objects for study with the aim of providing new tools to combat MDR, early opportunities to obtain hints of novel or uncommon modes of action are important. However, detailed mechanistic investigations are laborious, and the most important aspect was to identify compounds acting via different mechanisms from those of drugs already in use. This can be done by comparing activity profiles from a cell-line panel representing a set of different drug-tolerant types. Thus, such comparisons were included early in the selection process to facilitate the detection of substances with new modes of action as quickly as possible.

Considering the chemical complexity of Nature, and the time frame for the project, two restrictions on the scope of the investigation were imposed: the biological diversity was, limited to angiosperms (after study **I**), and the chemical diversity was limited to polypeptides that could be covered in a single fractionation protocol, albeit a protocol that was modified and developed during the course of the study to improve its efficiency and performance.

# Materials and Methods

For more detailed descriptions of the materials and procedures used in the specific studies, please see the *Material and methods* sections in the respective papers and manuscripts.

## Selection

The initial set of plants examined in Paper **I** were selected to represent wide biological diversity, the extent of which can be seen in Figure 5. In addition, known and thoroughly studied medicinal plants were selected when possible. From Figure 5, it can be seen that 25 of the 45 orders of flowering plants currently recognized by the Angiosperm Phylogeny Group (APG, 1999; 2003), and two additional families, yet without ordinal classification, were represented.[1]

The coverage thus obtained can be considered in proportion to the numbers of cytotoxic compounds of plant origin that have been reported during the last 20 years. In Figure 5, the numbers of such compounds found in these studies, and those previously reported (compiled from reviews and extensive database searches) are listed beside the APG phylogeny. From this comparison, it is clear that the screening in Paper **I** made a substantial contribution to the compounds covered by cytotoxic studies to date.

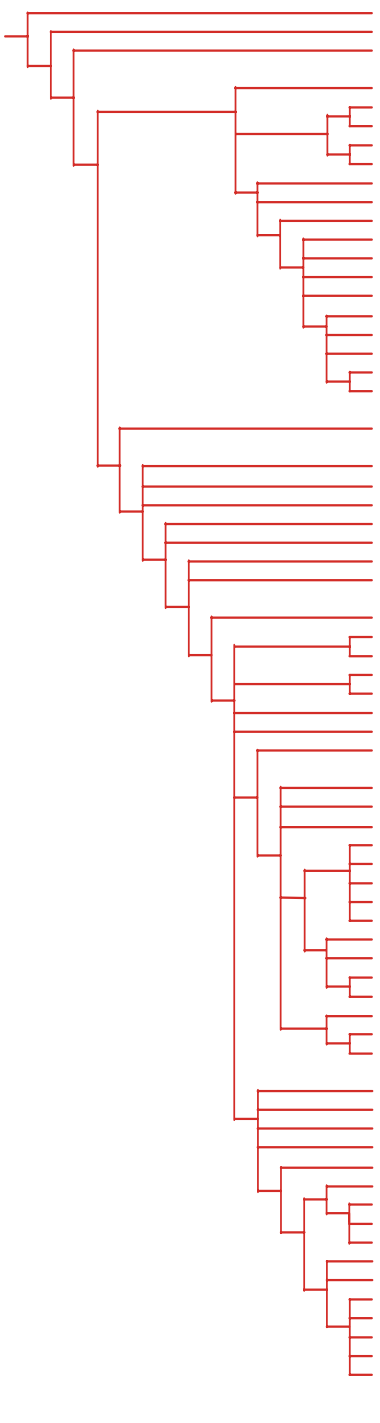
Figure 5 also presents a compilation of the numbers of extracts tested thus far in the extensive screening performed by the NCI and its Developmental Therapeutics Program (DTP) (Backlund *et al*., in prep.). These screenings have provided information from more than 70 000 plant samples to date.

---

*Figure 5*. (Next page) APG2 classification of orders and families of flowering plants (APG, 2003), with appended information on the sampling in Papers **I-V** of this thesis, published data on explicit cytotoxicity testing of compounds from extensive searches of journals and electronic databases including **PubMed**, and sampling in the **NCI** cytotoxicity screening effort. Highly conservative estimates of number of families, **fam** (for orders), genera, **gen**, and species, **sp.**, are given for the systematic entities here considered, data mainly from Mabberley (1998).

---

[1] **Nomenclatural note on the mistletoe *Viscum heyneanum* analysed in study I.** This species is usually considered to be conspecific with *Viscum orientale*, but Danser (1941) examined the type material for both species and re-established *Viscum heyneanum*.



| | Pap. I-V | PubMed | NCI | fam. | gen. | sp. |
|---|---|---|---|---|---|---|
| Amborellaceae | 0 | 0 | 0 | – | 1 : | 1 |
| Nymphaeaceae | 1 | 0 | 12 | – | 6 : | 60 |
| **Austrobaileyales** | 1 | 0 | 33 | 4 | 6 : | 95 |
| Chloranthaceae | 0 | 0 | 66 | – | 4 : | 56 |
| **Canellales** | 0 | 0 | 82 | 2 | 10 : | 76 |
| **Piperales** | 0 | 1 | 462 | 5 | 29 : | 2375 |
| **Laurales** | 0 | 12 | 2168 | 7 | 90 : | 2879 |
| **Magnoliales** | 1 | 53 | 3362 | 6 | 157 : | 2695 |
| **Acorales** | 0 | 0 | 0 | 1 | 1 : | 2 |
| Petrosaviaceae | 0 | 0 | 0 | – | 1 : | 2 |
| **Alismatales** | 0 | 2 | 249 | 14 | 156 : | 3361 |
| **Asparagales** | 2 | 6 | 287 | 25 | 935 : | 20718 |
| **Dioscoreales** | 0 | 2 | 84 | 3 | 30 : | 797 |
| **Liliales** | 1 | 1 | 143 | 10 | 276 : | 1787 |
| **Pandanales** | 0 | 0 | 138 | 5 | 30 : | 1191 |
| Dasypogonaceae | 0 | 0 | 0 | – | 1 : | 2 |
| **Arecales** | 0 | 0 | 776 | 1 | 198 : | 2650 |
| **Poales** | 2 | 0 | 391 | 18 | 890 : | 17058 |
| **Commelinales** | 0 | 0 | 100 | 5 | 70 : | 743 |
| **Zingiberales** | 0 | 3 | 672 | 8 | 92 : | 2031 |
| **Ceratophyllales** | 0 | 0 | 0 | – | 1 : | 2 |
| Balanophoraceae | 0 | 0 | 4 | – | 18 : | 44 |
| Medusandraceae | 0 | 0 | 0 | – | 1 : | 2 |
| **Ranunculales** | 4 | 18 | 290 | 10 | 203 : | 3525 |
| Sabiaceae | 0 | 0 | 112 | – | 3 : | 48 |
| **Proteales** | 0 | 1 | 239 | 3 | 77 : | 1359 |
| Buxaceae | 0 | 0 | 14 | – | 5 : | 60 |
| Trochodendraceae | 0 | 0 | 0 | – | 1 : | 1 |
| **Gunnerales** | 0 | 0 | 32 | 2 | 2 : | 42 |
| Aextoxicaceae | 0 | 0 | 0 | – | 1 : | 1 |
| Berberidopsidaceae | 0 | 0 | 0 | – | 1 : | 2 |
| Dilleniaceae | 0 | 0 | 296 | – | 12 : | 300 |
| **Caryophyllales** | 4 | 3 | 645 | 29 | 596 : | 9763 |
| **Santalales** | 8 | 1 | 819 | 6 | 153 : | 2126 |
| Vitaceae | 0 | 1 | 330 | – | 13 : | 80 |
| **Saxifragales** | 2 | 0 | 241 | 14 | 131 : | 2479 |
| **Crossosomatales** | 0 | 0 | 93 | 3 | 9 : | 41 |
| **Geraniales** | 1 | 0 | 18 | 6 | 19 : | 753 |
| **Myrtales** | 1 | 3 | 3951 | 14 | 438 : | 10736 |
| **Celastrales** | 0 | 0 | 681 | 4 | 97 : | 1317 |
| Huaceae | 0 | 0 | 22 | – | 2 : | 3 |
| Zygophyllaceae | 0 | 0 | 34 | – | 27 : | 250 |
| **Malpighiales** | 9 | 20 | 10933 | 37 | 680 : | 15326 |
| **Oxalidales** | 0 | 0 | 949 | 6 | 64 : | 1863 |
| **Fabales** | 3 | 29 | 6353 | 4 | 680 : | 17359 |
| **Rosales** | 5 | 12 | 3612 | 9 | 283 : | 6415 |
| **Cucurbitales** | 1 | 0 | 263 | 7 | 133 : | 1703 |
| **Fagales** | 3 | 3 | 651 | 8 | 29 : | 1415 |
| **Brassicales** | 6 | 7 | 372 | 16 | 422 : | 3251 |
| **Malvales** | 0 | 18 | 3352 | 11 | 208 : | 2422 |
| **Sapindales** | 3 | 44 | 7002 | 11 | 474 : | 5183 |
| **Cornales** | 0 | 2 | 174 | 7 | 50 : | 554 |
| Boraginaceae | 1 | 2 | 529 | – | 154 : | 2500 |
| Icacinaceae | 0 | 0 | 424 | – | 60 : | 320 |
| Bruniaceae | 0 | 0 | 0 | – | 11 : | 69 |
| **Ericales** | 2 | 6 | 4298 | 26 | 383 : | 10002 |
| **Garryales** | 0 | 0 | 7 | 3 | 3 : | 17 |
| **Gentianales** | 17 | 28 | 7714 | 5 | 1285 : | 17053 |
| **Lamiales** | 15 | 15 | 3231 | 21 | 1215 : | 20537 |
| **Solanales** | 2 | 0 | 877 | 5 | 151 : | 4275 |
| **Aquifoliales** | 0 | 0 | 171 | 5 | 7 : | 441 |
| Sphenostemonaceae | 0 | 0 | 24 | – | 1 : | 7 |
| **Asterales** | 5 | 20 | 1630 | 13 | 1442 : | 23710 |
| **Apiales** | 1 | 8 | 1033 | 10 | 491 : | 4178 |
| Escalloniaceae | 0 | 0 | 122 | – | 1 : | 39 |
| Columelliaceae | 0 | 0 | 2 | 2 | 2 : | 6 |
| **Dipsacales** | 3 | 0 | 126 | 7 | 48 : | 1027 |
| SUM | 104 | 337 | 70703 | 440 | 13070 : | 231185 |

## Plant material

Primary collections of plant material were obtained from the area around Uppsala and the Uppsala Botanical Garden. Voucher specimens are kept at the Division of Pharmacognosy to be deposited at the Uppsala University herbarium (UPS). Plant material for the mistletoe study (Paper **IV**) was collected in Panama.

A comprehensive list of all plant material used in the studies that this thesis is based upon is given in appendix A.

## Polypeptide isolation

As described in Paper **I** the primary plant-extract fractions were produced according to a previously developed fractionation protocol for isolating polypeptides from plant material (Claeson *et al.* 1998). The dried and powdered plants were extracted with dichloromethane ($CH_2Cl_2$) and ethanol (EtOH). The extract from the EtOH extraction was filtered through polyamide to remove tannins. Compounds of molecular weights greater than 700 Da were isolated by size exclusion chromatography using a fast protein liquid chromatograph (FPLC; Pharmacia Biotech, Uppsala, Sweden). Finally, salt and polysaccharides from the fractions were removed using a solid phase extraction (SPE) column with reversed phase (RP) material.

The primary extracts thus obtained, the "*p* fractions" were further fractionated to varying extents, as described in Papers **II**-**V**.

In the investigations described in Paper **II** the original fractionation protocol was modified to retrieve fractions with high cytotoxic activity. Figure 6 shows an overview of both fractionation protocols in parallel tracks. In the modified protocol the dried, ground plant material was pre-extracted with $CH_2Cl_2$ and then extracted with 25% AcN and 0.1% TFA instead of 50% EtOH. Both protocols involved the use of polyamide filtration to remove tannins, after which a strong cation exchanger was used in the modified protocol to extract cationic analytes, followed by fractionation on a semi-preparative High Performance Liquid Chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with an SPD-M10Avp photodiode array detector and a 250 x 10 (i.d.) mm column from Hichrom Ltd, UK ($C_{18}$, 5 μm).

The fractionation protocol used in study **IV** had further modifications, since a comparison of extraction methods for basic thionins identified a more efficient extraction procedure for these compounds (Larsson 2004). In this protocol the plant material was extracted with 0.2 M acetic acid followed by freeze-drying. Filtration through polyamide was done to remove tannins, and thereafter HPLC was performed using an RP column.

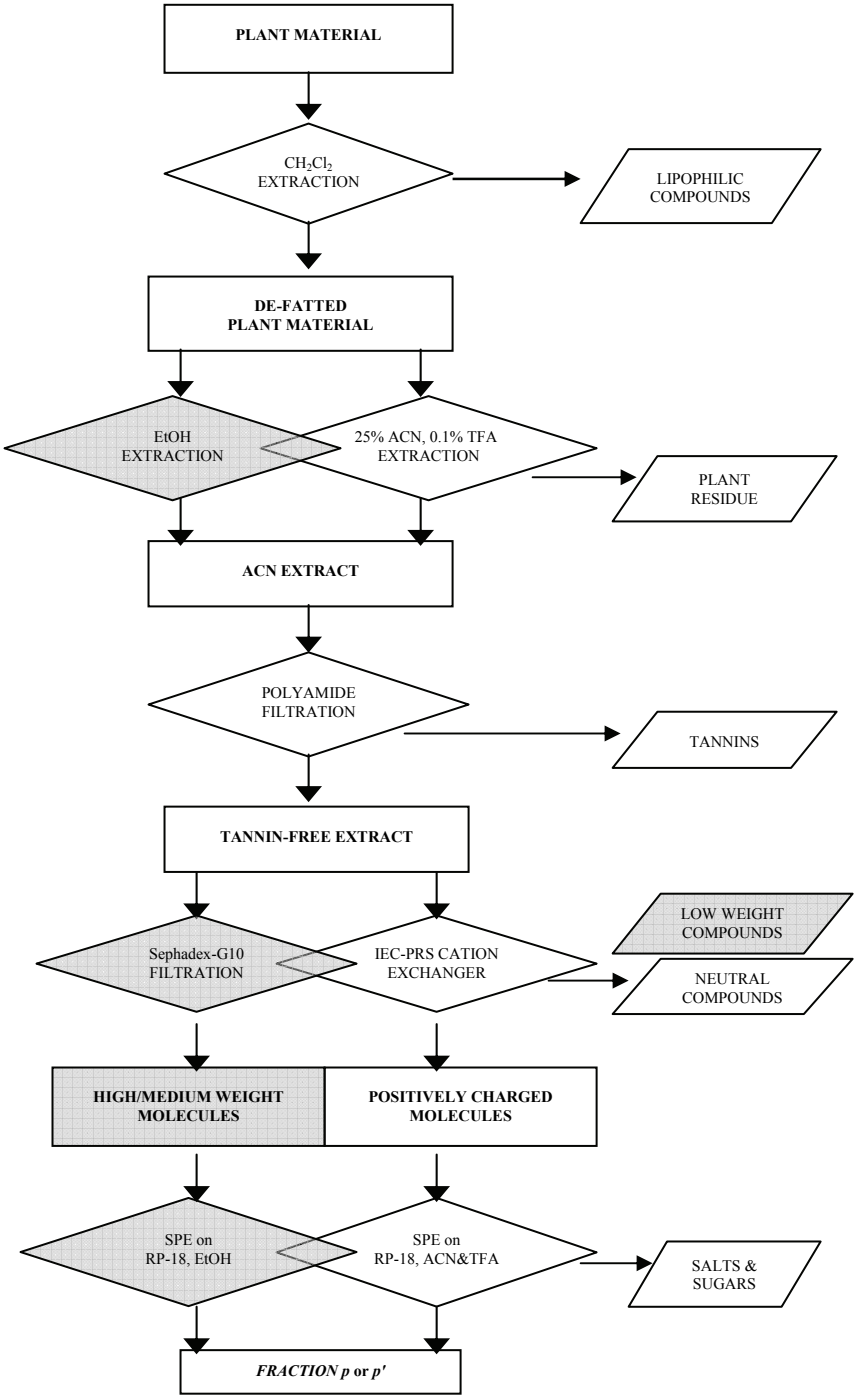



*Figure 6.* Initial fractionation and modified protocols for isolating polypeptide *fraction p* and *fraction p'*, respectively, from plants, redrawn from Claeson *et al.* 1998 giving *fraction p'*. The original protocol was subsequently modified in studies **II** and **IV**.

# Cytotoxicity screening

## Design

The original fractionation protocol (Figure 6) was applied to a final set of more than 100 plant extracts. In collaboration with Medivir AB, 98 of these fractionated plant extracts were initially screened for anti-viral and cytotoxic activity including their ability to inhibit the growth of MT4 and HEL cells, *i.e.* human T-cells and embryonic lung cells, respectively. A second species of one genus, *Digitalis*, was added as an internal control, resulting in 100 fractionated plant extracts after the first selection. Of these 29 were selected for further study, including some extracts apparently lacking cytotoxicity for use as controls.

The screening procedure is described in detail in Paper **I**. Using DIAS (the Dynatech immuno assay system), fractionated plant extracts were tested against tumour cells from patients – including peripheral blood mononuclear cells (PBMC), ovarian cancer cells (OVCA), chronic lymphatic leukaemia cells (CLL) – and against the renal adenocarcinoma cell line ACHN and the T-cell leukaemia cell line CCRF-CEM.

Characteristics of the cell lines are detailed below. This study was approved by the local ethics committee at the Uppsala University hospital, approval number Dnr 21/93 – 930125.

After pre-screening fractionated plant extracts showing cytotoxicity, indicated as AUC<10,000, towards more than one type of cell, were selected. This corresponds to less than 50% survival. The selection of fractions was biased in favour of fractions that were selectively active against solid tumours, because drugs with high activity against such tumours are rare (Larsson *et al.* 1994; Nygren *et al.* 1994). Furthermore, clinical data have shown that haematological tumours are generally more easily treated than solid tumours (Csoka *et al.* 1994). Ratios of AUC values obtained for the different tumour cells (*e.g.* $AUC_{CLL}/AUC_{OVCA}$, $AUC_{PBMC}/AUC_{CLL}$, and $AUC_{CEM}/AUC_{ACHN}$) that deviate significantly from one indicate selectivity, and the magnitude of the deviation indicates the degree of selectivity.

| Criteria for selection: | |
|---|---|
| AUC<10,000 | |
| Solid tumour selectivity | AUC ratio>1: $AUC_{CLL}/AUC_{OVCA}$ |
| Tumour selectivity | AUC ratio>1: $AUC_{PBMC}/AUC_{CLL}$ |
| Tumour cell line selectivity | AUC ratio>1: $AUC_{CEM}/AUC_{ACHN}$ |

### Preparation of microtiter plates

For the screening, experimental 96-well microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 µl per well of plant extract solution (in 10% EtOH) at 10x final concentration, dispensed by a programmable pipetting robot (Propette, Perkin Elmer, Norwalk, CT), after which the plates were transferred to, and stored in, a freezer at -70°C for a maximum of 10 weeks. The final concentrations used in the first-line screening with DIAS were 2.00, 20.0, and 200 µg per ml, each solution and concentration being tested in duplicate. In the second-line screening using the Fluorometric Microculture Cytotoxicity Assay (FMCA), the final concentrations were 0.630, 2.00, 6.32, 20.0, 63.3, and 200 µg/ml, each solution and concentration being tested in triplicate. Test concentrations were selected to provide evenly distributed and comparable dose-response curves.

In both the DIAS and FMCA assays, the plates were prepared using six wells with phosphate buffered saline (PBS) as controls, and three wells with 10% EtOH (as solvent controls), and triton 1% as positive controls.

## Cytotoxicity assays

### Reagents and medium

Alamar Blue (Alamar, Sacramento, CA), a fluorometric (and spectrophotometric) redox indicator, was obtained as a stock solution and stored in the dark at 4°C. Prior to addition to the assay the indicator was diluted ten-fold with PBS in accordance with the manufacturer's instructions. Fluorescein diacetate (FDA, Sigma) was dissolved in dimethyl-sulfoxide (DMSO) to a concentration of 10 mg/ml, and kept in the dark as a frozen stock solution. The cell-growth medium RPMI-1640 (Sigma) was supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma), 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin.

### Primary cultures of human tumour cells

Cell samples were obtained by routine sampling of peripheral blood or bone marrow (CLL), or by a surgical procedure (OVCA). Leukaemic cells were isolated from the bone marrow or peripheral blood by density gradient centrifugation in 1.077 g/ml Ficoll-Paque (Amersham Pharmacia-Biotech, Uppsala, Sweden), following the procedure described by Larsson *et al.* (1992). Tissues from OVCA samples were minced into small pieces, and tumour cells were isolated by collagenase dispersion followed by Percoll Paque (Amersham Pharmacia-Biotech) density gradient centrifugation (Csoka *et al.* 1994). The purified tumour cells were resuspended in fetal bovine serum (FBS) containing 10% DMSO (Sigma), and stored in liquid

nitrogen after freezing for 24 h at -70$^0$C. Cryopreservation by this method has been shown to have no effects on sensitivity (Larsson *et al.* 1992). Frozen cell suspensions to be used in the assay were rapidly thawed and then washed twice in room-tempered complete RPMI medium before testing.

## Human tumour cell lines

The selection and handling of cells in the cell-line panel followed previously described methods (Dhar *et al.* 1996).

The cell-line panel consisted of four sensitive parental cell lines (CCRF-CEM, NCI-H69, RPMI 8226/S and U937/GTB), five drug-resistant sublines (CEM/VM1, H69AR, 8226/DOX40, 8226/LR-5 and U937/VCR), and one cell line with primary resistance (ACHN).

CCRF-CEM is a T-cell leukaemia cell line and its subline CEM/VM1, selected for teniposide resistance, has a topoisomerase II-associated resistance mechanism (atypical MDR phenotype). NCI-H69 is a small cell lung cancer derived cell line and its subline H69AR, selected for doxorubicin (DOX) resistance, has an MRP-associated resistance mechanism. RPMI 8226/S is a myeloma cell line and its resistant subline RPMI 8226/DOX40, selected for DOX resistance, has a P-glycoprotein 170-associated resistance mechanism and RPMI 8226/LR-5, selected for melphalan (Mel) resistance, has a glutathione (GSH)-associated resistance mechanism. U937/GTB originates from histiocytic lymphoma and the resistant subline U937/VCR, selected for vincristine (VCR) resistance, shows a tubulin-associated MDR mechanism. ACHN is a renal adenocarcinoma with primary MDR (Dhar *et al.* 1996). A summary of the cell lines and their properties is presented in Table 4.

Table 4. *Human tumour cell-line panel selected by Dhar* et al. *(1996), representing a defined set of drug resistance types.*

| **Cell lines parental : resistant** | | **Origin** | **Selecting agent** | **Resistance type** |
|---|---|---|---|---|
| RPMI 8226/S : | RPMI 8226/DOX 40 | myeloma | doxorubicin | P-gp associated |
| RPMI 8226/S : | RPMI 8226/LR-5 | myeloma | melphalan | GSH associated |
| CCRF-CEM : | CEM/VM-1 | T-cell leukaemia | teniposide | Topo II associated |
| NCI-H69 : | H69AR | SCLC | doxorubicin | MRP associated |
| U937-GTB : | U937-VCR | histiocytic lymphoma | vincristine | tubulin associated |
| | ACHN | renal adenocarcinoma | vincristine | primary MDR |

SCLC = small cell lung cancer, P-gp = P-glycoprotein (classical MDR), GSH = glutathione, Topo II = topoisomerase II (atypical MDR), MRP = MDR associated protein, MDR = multidrug resistance. ACHN exhibits primary resistance and hence has no parental cell line.

## DIAS

DIAS (Dynatech Laboratories, UK) is a fully automated screening system (Dhar *et al.* 1997) based on measurements of fluorescence generated from the reduction of Alamar Blue by cells with retained metabolic activity. In each 96-well plate six wells containing cells (but without test compound) served as controls and six wells containing only culture medium as blanks. After loading, plates were incubated for 72 hrs at 37°C in a humidified atmosphere containing 5% $CO_2$. At the end of the incubation period, the plates were transferred manually to the DIAS equipment for washing, Alamar Blue dispensation, incubation (6 hrs), and reading. Absorbance of Alamar blue was measured at 570 nm, using 630 nm as a reference wavelength. The microtiter plates were then transferred by hand to a scanning fluorometer (Fluoroscan II, Labsystems Oy, Helsinki, Finland) for fluorescence measurements at excitation and emission wavelengths of 544 nm and 590 nm, respectively. DIAS results were quantified by calculating the cell survival index (SI), defined as the fluorescence or absorbance in experimental wells, as a percentage of the corresponding measurement in control wells, after subtraction of control (blank) values. The data are presented in Paper **I** as AUC values, calculated by the trapezoidal method.

## FMCA

The FMCA approach is based on measurements of fluorescence generated from the hydrolysis of FDA to fluorescein by cells with intact plasma membranes. The procedure followed protocols described by Larsson *et al.* (1992).

The substances were dissolved in EtOH/DMSO (less than 1% after further dilution with PBS and cell growth medium) and dispensed in microtiter plates. The cells were subsequently dispensed on the plates and then incubated at 37°C in a 5% $CO_2$ atmosphere for 72 hours without changing medium. After the incubation period the cells were washed with phosphate-buffered saline (PBS), and FDA dissolved in a physiological buffer was added to each well. The plates were incubated for 40 minutes and the generated fluorescence was measured in the Fluoroscan (485/528 nm).

The reproducibility and clinical correlations of results obtained by the FMCA method have been described previously (Larsson and Nygren 1990; Larsson *et al.* 1990; 1992).

## Quality control and quantification of results

The criteria defining a successful analysis included a fluorescence signal in the control wells of more than five or ten times the mean blank value (for primary human tumour cells and cell lines, respectively), and a mean coefficient of variation (CV) in the control wells of less than 30% (Dhar *et al.* 1996).

Cytotoxic activities of fractionated plant extracts are presented as $IC_{50}$ values in Table 5. The $IC_{50}$ value was defined as the concentration giving a SI of 50%, where SI is the fluorescence in experimental wells, as a percentage of that in control wells, after subtraction of blank values.

## Calculations of cytotoxic activity

Two viability probes with fundamentally different mechanisms were used for the cytotoxicity measurements. In the first line screening, using the fully automated DIAS (Dhar *et al.* 1998), Alamar Blue was used as a fluorometric redox indicator of metabolic activity. Another option – measuring optical density (the reduced form of Alamar Blue is red) – was rejected because it is less sensitive. For the second line screening, the FMCA cytotoxicity assay was used with the reagent FDA, after washing with PBS, providing a measure of the proportion of cells that retained esterase activity, and thus the proportion with intact plasma membranes, in each well (Dhar *et al.* 1996). These two methods have been demonstrated to produce reproducible and comparable results (J. Gullbo, unpublished data).

The cytotoxicity criteria for defining the fractionated plant extracts as active or non-active was based on AUC calculations. Extracts were selected that satisfied two requirements: (a) being sufficiently active (*i.e.* yielding <50% of the survival rates obtained with non-toxic controls), or, operationally, having AUC values <10,000 in more than one cell line, and (b) yielding $AUC_{CLL}/AUC_{OVCA}$, $AUC_{CEM}/AUC_{ACHN}$, or $AUC_{PBMC}/AUC_{CLL}$ ratios greater than one, indicating selectivity for solid tumours, tumour cell lines or malignant tissues, respectively.

## Data analysis

The fluorescence obtained in the assays described above is proportional to the numbers of living cells in the respective wells and cell survival is presented as SI, which is defined as the fluorescence of test wells in percentage of control levels, after subtraction of blank values. The $IC_{50}$ values (the concentration giving a SI of 50% from the dose-response curves) and the areas under the dose-response curve (AUC) were calculated.

# Isolation of cytotoxic compounds

## Bioassay-guided isolation of *Nuphar luteum* (Paper **II**)

Leaves from *Nuphar luteum* Sibth. & Sm. (Nymphaeaceae), were first included in parts of the screening study described in Paper **I**. The fractionated plant extract (isolated by the fractionation protocol described earlier) from *Nuphar luteum* was tested for cytotoxicity, but the obtained results indicated an unexpected potency, prompting additional tests.

The active component was separated and isolated by the original fractionation protocol described earlier, in parallel with a modified fractionation protocol involving the use of other solvents and ion exchange chromatography instead of size exclusion chromatography (see Figure 6).

The column was eluted with a linear gradient from 10% AcN with 0.1% TFA to 60% AcN with 0.1% TFA over 60 minutes. Fractions were collected and dried under a stream of $N_2$. The dried fractions were dissolved in 10% of EtOH for cytotoxicity testing with the cell line U937GTB using FMCA. The choice of U937GTB was based on initial data from the screening procedure (DIAS and FMCA), and its suitability, as a sensitive, well-characterised, single cell suspension with good *in vitro* proliferation parameters (making it easy to work with) that was included in my cytotoxicity studies.

For *Nuphar luteum* the modified protocol resulted in a primary *fraction p'* with even higher cytotoxicity than the *p* fraction obtained with the initial protocol. This primary fraction was further separated by HPLC, giving a new set of fractions that were subjected to further bioassay-guided rounds of HPLC, resulting in successively increasing purification and finally isolation of the active compound(s).

The lipophilic $CH_2Cl_2$ extract from the first step in the fractionation protocol was further separated, in the modified protocol, by accelerating gradient chromatography (AGC) using a solid phase of silica and solvent systems consisting of petroleum ether, $CH_2Cl_2$, and methanol (MeOH), as follows. The $CH_2Cl_2$ extract was dried and re-dissolved in the smallest possible amount of $CH_2Cl_2$, ground in silica and transferred to a column that was successively eluted with 50 ml-portions of: 100% petroleum ether; 12.5:87.5 petroleum ether:$CH_2Cl_2$; 3:97 petroleum ether:$CH_2Cl_2$; 1:99 petroleum ether:$CH_2Cl_2$; 100% $CH_2Cl_2$; then $CH_2Cl_2$:MeOH in the proportions 99:1; 98:2, 97:3, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50, 20:80, 10:90, and finally 100% MeOH.The AGC method is described in more detail in El-Seedi *et al.* (2003).

The fractions from the AGC were collected after inspection of silica-thin layer chromatography (TLC) where components with similar mobilities from different fractions were pooled. The pooled $CH_2Cl_2$ fractions were tested, as were the *p′* fractions, for cytotoxic activity using the cell line U937GTB in the FMCA cytotoxicity test.

The structure of the active component was elucidated by infrared (IR) analyses, mass spectrometry (MS), one dimensional (1D) and extensive two dimensional (2D)-NMR analyses.

## Isolation of cyclotides (Paper **III**)

Polypeptides from *Viola arvensis* Murr. (Violaceae)[2] were extracted for the initial screening according to the original fractionation protocol illustrated above in Figure 6. The macrocyclic polypeptides varv A and varv F of the cyclotide family were isolated using reversed-phase chromatography after adsorption chromatography on Sephadex LH-20 (Göransson *et al.* 1999). Cycloviolacin O2, described by Craik and co-workers (1999), was isolated from a butanol-soluble fraction of *Viola odorata* L. with a combination of high-performance cation exchange and reversed-phase chromatography, and identified using nanospray MS. Purified cyclotides were dissolved in 10% EtOH for the cytotoxicity assay.

[2] **Nomenclatural note on *Viola* *arvensis***. The species *Viola arvensis* Murr. has been formally included in *Viola tricolor* L., which hence is the proper scientific name (IPNI with IK 2005). Throughout this thesis, however, the invalid name *Viola arvensis* will be retained for consistency with the cited studies.

## Isolation of cytotoxic compounds of mistletoes (Paper **IV**)

A series of Panamanian mistletoes was extracted following a highly simplified protocol. In previous studies (Larsson 2004) it has been demonstrated that several isolation steps could be omitted in the extraction of thionins to increase efficiency while retaining quality. In this study, seven mistletoes, four from the family Loranthaceae (*Oryctanthus alveolatus* Kuijt, *Phthirusa retroflexa* Kuijt, *Loranthus retroflexus* Ruíz & Pav., and *Psittacanthus pusillus* Kuijt) and three from the family Viscaceae (*Phoradendron acinacifolium* Mart. Ex Eichl., *Phoradendron guascanum* Trel., and *Phoradendron rubrum* Nutt.) were investigated.[3]

Following the simplified procedure, the plant material was dried, pulverized, and extracted with 0.2 M acetic acid (HAc) in the proportions 1:25 (w:v), followed by freeze-drying. A 0.5 g portion of the dried extract was reconstituted in 10 ml of 0.2 M HAc. The extract was filtered through a column packed with polyamide to remove tannins. The solution was concentrated to 2 mg/ml and fractionated on a HPLC system with a C18-column (4.0 x 125 mm, 5 µm, Bischoff Chromatography, Leonberg, Germany) and eluted with a linear gradient from 15% AcN with 0.1% TFA to 50% AcN with 0.1% TFA over 40 minutes.

[3] **Nomenclatural note on the species and genera of mistletoes studied in paper IV.**
There are several unresolved nomenclatural issues related to the validity of numerous mistletoe names. Of the taxa included in the studies this thesis is based upon, such issues relate specifically to the following names used in Paper **IV** and the thesis summary.
*Phoradendron guascanum* Trel. – This species has been reduced under *Dendrophthora obliqua* (Presl) Wiens by Kuijt and Kellogg (1996), based on the observation of unilocular anthers. However, this change has not yet been accepted, *e.g.* in Index Kewensis, where the species *Phoradendron guascanum* is retained. We have followed the original classification and retained this taxon in the genus *Phoradendron*.
*Phoradendron quadrangulare* Krug & Urb. – This species has been reduced in Index Kewensis under *Phoradendron rubrum*, but without defining author for the latter species. The situation becomes complex as there are several entries for *Phoradendron rubrum* under different authors, one of which has subsequently been included in the genus *Viscum* as *Viscum rubrum*, a suggestion that has not gained wider acceptance. Other entries have also been reduced to different species of *Phoradendron*, but without the possibility to establish a link between *Phoradendron quadrangulare* and either of the *Phoradendron rubrum* we have retained the former name.
*Phthirusa pyrifolia* Eichl. and *Phthirusa retroflexa* (Ruíz & Pav.) Kuijt – both of these species have been re-assigned to the genus *Loranthus* according to Index Kewensis. However, this suggestion has not gained wider acceptance, and as the circumscription of the genus *Loranthus* has been under debate since the late nineteenth century, we have followed the widely accepted view that the genus *Loranthus* consists of only one species, *Loranthus europaeus* Jacq. (see, for instance, Mabberley, 1998). The generic placement in *Phthirusa* has thus been retained. It has also been suggested that *Phthirusa retroflexa*, should be under *Phthirusa stelis*, together with a number of other species (see the series of papers by Kuijt, 1978, 1980; and Kuijt and Kellogg, 1996). However, these suggestions have not been endorsed by Index Kewensis, and hence their status is unclear. Consequently we also retain the name *Phthirusa retroflexa* here.

Fractions showing UV spectra typical of small peptides were collected and their mass/charge ratios were determined using an electrospray ion trap mass spectrometer. The fractions thus obtained were tested for cytotoxic activity with FMCA using the chemo-sensitive lymphoma cell line U937GTB.

## Isolation of digitoxin (Paper **V**)

*Digitalis purpurea* Ehrl. (Plantaginaceae)[4] was fractionated for the initial screening according to the original fractionation protocol described above. The plant extract thus obtained, *fraction p*, was dissolved in a mobile phase of aqueous 25% AcN and 0.1% TFA, and further fractionated on a semi-preparative HPLC system (Shimadzu, Kyoto, Japan) equipped with an SPD-M10Avp photodiode array detector and a 250 x 10 (i.d.) mm Dynamax column ($C_{18}$, 5 µm, pore size 300 Å). The column was eluted with a linear gradient from 25% AcN with 0.1% TFA to 75% AcN with 0.1% TFA over 30 minutes. Fractions were collected and tested for cytotoxicity in the cell-line panel. The most potent fraction was further purified by HPLC to yield a homogenous component, which was unambiguously identified as the cardiac glycoside digitoxin by spectroscopic methods. The $^{1}$H nuclear magnetic resonance (NMR) spectra (recorded at 600 MHz) and electrospray mass spectra were in agreement with those of an authentic sample of digitoxin (Sigma).

[4] **On the familial classification of** *Digitalis***.** *Digitalis* was previously placed in the family Scrophulariaceae, however, recent phylogenetic studies imply that the proper placement should be in the Plantaginaceae (*e.g.* Olmstead and Reeves 1995; Reeves and Olmstead 1998; Olmstead *et al.* 2001).

# Results

## Results of the initial screening (Paper **I**)

### Initial selection

Plants for this study were selected *pro primo* to reflect biodiversity in a phylogenetic sense, and *pro secundo* to include, as far as possible, well studied medicinal plants. This resulted in a set of 98 plant extracts, which were extracted and fractionated according to the methods described above. The *p* fractions from these extracts were initially screened and the extracts that indicated cytotoxic properties were selected along with some non-active extracts as controls. Together with the *fraction p* from *Digitalis purpurea*, which was added afterwards as a control, this resulted in 30 extracts, which passed on to the second screening. Data and details are presented in Paper **I**.

### Pre-screening: Cytotoxic activity in primary human tumour cells

Using two primary human tumour samples, and tumour cell line systems for second-line screening, the 30 fractionated plant extracts (29 from the first-line screening and one from *Digitalis purpurea*) at three concentrations were screened for cytotoxic activities, followed by selection based on the cytotoxicity criterion (*i.e.* AUC<10,000 in more than one cell line) and *in vitro* selectivity determined as at least one of the three AUC-ratios (discussed above) being greater than one. Ten of these 30 extracts were selected for further characterisation in the human tumour cell-line panel.

### Cytotoxic activity characterisation – human tumour cell lines

Using the FMCA, dose-response curves of each of the 10 finally selected extracts were analysed at six concentrations using all 10 cell lines in the panel. Overall cytotoxic activity varied between extracts; and for each extract, activity varied between cell lines (Table 5).

Table 5. *Cytotoxicity screening $IC_{50}$ values (μM) of human tumour cell lines, detected using FMCA. Species indicated by asterisks (*) were identified as prime targets. An $IC_{50}$ value of 200.0 indicates no effect on the cell line even at the highest test concentration.*

| Species | | Family | RPMI 8226/S | RPMI 8226/ DOX40 | RPMI 8226/ LR-5 | CCRF-CEM | CEM/ VM-1 | NCI-H69 | H69AR | U937-GTB | U937-VCR | ACHN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Asperula tinctoria* | | Rubiaceae | 200.0 | 89.0 | 200.0 | 200.0 | 200.0 | 200.0 | 187.0 | 200.0 | 200.0 | 200.0 |
| *Colchicum autumnale* | * | Colchicaceae | 15.8 | 39.8 | 13.0 | 15.4 | 16.4 | 103.0 | 82.2 | 13.6 | 14.9 | 200.0 |
| *Digitalis lanata* | * | Plantaginaceae | 4.0 | 5.4 | 5.2 | 3.3 | 4.3 | 1.1 | 1.9 | 6.0 | 12.0 | 1.6 |
| *Digitalis purpurea* | * | Plantaginaceae | 1.1 | 1.5 | 1.1 | 0.1 | 1.4 | 1.4 | 0.6 | 1.0 | 1.2 | 1.4 |
| *Gentiana punctata* | | Gentinaceae | 200.0 | 188.0 | 200.0 | 200.0 | 200.0 | 200.0 | 137.3 | 200.0 | 200.0 | 200.0 |
| *Helleborus cyclophyllus* | * | Ranunculaceae | 44.2 | 63.2 | 43.8 | 35.7 | 19.9 | 38.4 | 29.8 | 37.1 | 48.5 | 17.9 |
| *Menyanthes trifoliata* | * | Menyanthaceae | 41.2 | 96.9 | 43.8 | 46.7 | 42.4 | 122.0 | 132.3 | 36.2 | 33.4 | 69.4 |
| *Pteridium aqulinum* | | Pteridaceae | 117.7 | 103.0 | 47.0 | 138.6 | 123.0 | 99.0 | 26.2 | 151.0 | 160.2 | 167.0 |
| *Silene rubrum* | | Caryophyllaceae | 100.0 | 51.0 | 46.8 | 70.0 | 111.8 | 200.0 | 200.0 | 31.2 | 41.5 | 154.5 |
| *Viola arvensis* | * | Violaceae | 14.9 | 16.3 | 13.0 | 41.3 | 37.5 | 43.9 | 42.2 | 38.8 | 45.0 | 22.4 |
| *Viola patrinii* | * | Violaceae | 13.7 | 13.6 | 10.1 | 17.6 | 46.6 | 18.8 | 53.2 | 18.4 | 18.8 | 19.2 |

The $IC_{50}$ values obtained from four or more of the cell lines were below 50 μg/ml for the fractionated extracts of seven plants: *Colchicum autumnale* L. (Colchicaceae), *Digitalis lanata* Ehrh., *Digitalis purpurea* L. (Plantaginaceae), *Helleborus cyclophyllus* Boiss. (Ranunculaceae), *Menyanthes trifoliata* L. (Menyanthaceae), *Viola arvensis* Murr., and *Viola patrinii* Ging. (Violaceae).

Activity profiles were then compared with entries in the database. Five of the extracts showed very low correlations with the standard drugs, while the *Menyanthes trifoliata* extract showed moderate but unspecific correlation with several drugs, and the *Colchicum autumnale* extract showed high (0.94) correlation with vincristine, representing the tubulin-interacting agents. From these seven extracts, four were selected for further investigations: the two *Viola* extracts (as described in Paper **III**) and the two *Digitalis* extracts (Paper **V**).

# Cytotoxic compounds in *Nuphar luteum* (Paper **II**)

## Identification of cytotoxic compounds in *Nuphar luteum*

The cytotoxicity of the *Nuphar luteum fraction p'* obtained using the modified fractionation protocol, was found to have an $IC_{50}$ value of less than 0.05 μg/ml, the lowest concentration tested, compared to an $IC_{50}$ value of 148.8 μg/ml for *fraction p*, obtained by the original protocol (unpublished data). The active substance in *fraction p'* from *Nuphar luteum* was isolated by bioassay-guided fractionation.

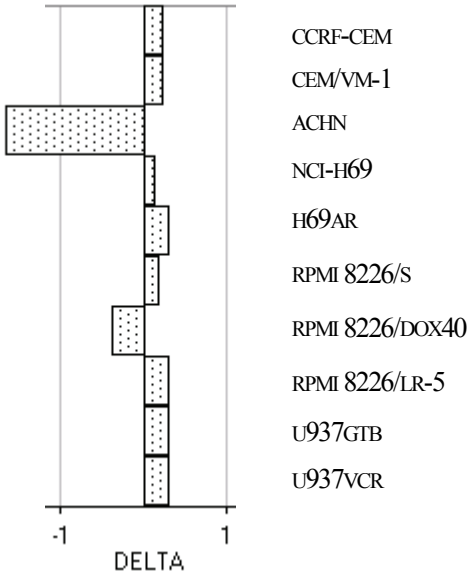



*Figure 7.* Activity profile, mean graph, of *fraction p'* from *Nuphar luteum*.

Comparison of the activities of *fraction p'* with those of other substances in the database yielded the activity profile shown in Figure 7. This activity profile shows insignificant correlations to the profiles of all of the drugs and compounds included in the available databases, implying that one or more components in it has/have a novel mechanism of action.

The procedure applied in the bioassay-guided fractionation is described schematically in Figure 8. After isolation, NMR structural elucidation and high performance mass spectrometric analysis, the cytotoxic active substance was identified as the *Nuphar* alkaloid immonium thiobinupharidine (Figure 9). No $IC_{50}$ value for the pure compound in the lymphoma cell line U937GTB could be determined, due to the miniscule amounts required, but conservative estimates indicate that it is well below 1 nM.

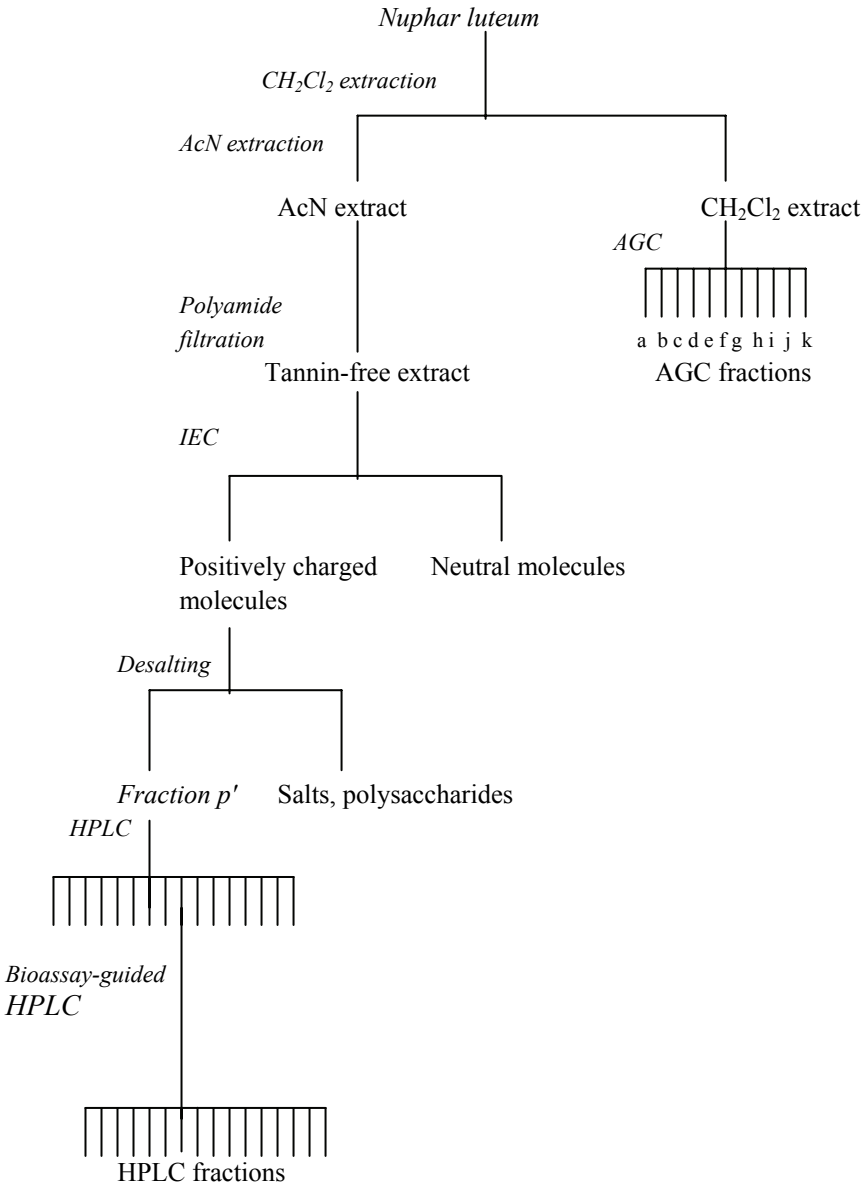



*Figure 8*. Procedure followed during the bioassay-guided isolation of cytotoxic compounds from *Nuphar luteum*.

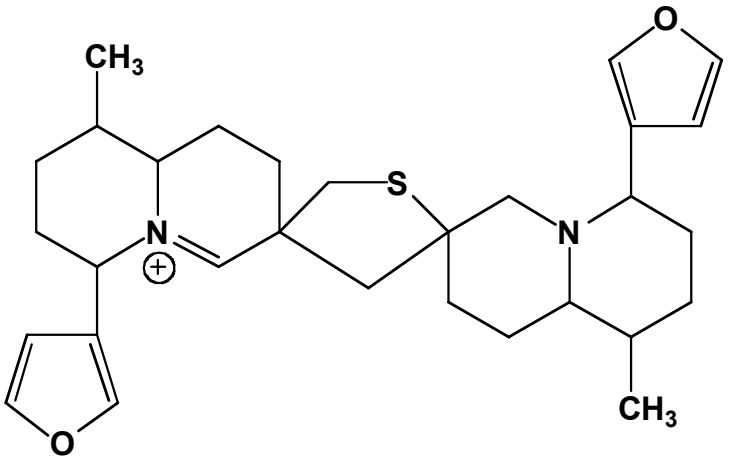



*Figure 9.* Structure of immonium thiobinupharadine.

In the fractions obtained from *Nuphar luteum*, the $CH_2Cl_2$ extract from the first step of de-fatting also exhibited pronounced cytotoxic activity. For further testing, fractionation by AGC was employed. Table 6 shows the cytotoxicity in $IC_{50}$ values for these AGC fractions.

Table 6. *Cytotoxicity of AGC fractions tested against cell line U937GTB.*

| Fractions from AGC | Designation | $IC_{50}$ µg/ml |
|---|---|---|
| 1-21 | a | - |
| 22 | b | 294 |
| 23-24 | c | 509 |
| 25-30 | d | 259 |
| 31-35 | e | 115 |
| 36-41 | f | 270 |
| 42-51 | g | 119 |
| 52-59 | h | 19 |
| 60-70 | i | 99 |
| 71-88 | j | 30 |
| 89-115 | k | 101 |

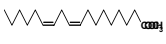
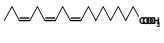
According to the NMR spectra and MS data, the most active fractions (designated h) contained fatty acids. The $^1H$ NMR spectra show an intense peak at δ 1.29 ppm, indicating a long intermediary methylene chain, while the multiplicity signal pattern at δ 0.93 ppm compatible with terminal methyl group; a combination which is typical of fatty compound. The presence of polyunsaturated fatty chains, indicated by cross peaks at δ 2.80 ppm, together with the peak at δ 5.25 ppm, confirm the partial

structure [CH=CH-$CH_2$-(CH=CH-CH)$_n$] with n≥1, while peaks at δ 2.38 and 1.69 ppm are assigned for $\underline{CH_2}COOH$ and $\underline{CH_2}$-$CH_2COOH$ groups, respectively. These data are consistent with the $^{13}C$ NMR spectrum, which showed the COOH group at δ 180.5 ppm, and double bonds at δ 130.3 and 130.0 ppm, with the methyl group and methylene chain from δ 14.4 to 34.3 ppm.

The mixture of fatty acids was methylated using methanol in the presence of *p*-toluene sulphonic acid as a catalyst. After sample preparation the mixture was subjected to gas chromatography-MS analysis. The major compounds were found to be palmitic acid (69%), myristic acid and steric acid from their from the retention times, molecular ion peaks, fragmentation patterns and comparison with authentic samples (Table 7).

Based on IR analyses, high resolution-MS, 1D- and extensive 2D-NMR analyses in addition to comparison with literature data of closely related structures (LaLonde *et al.* 1973; LaLonde *et al.* 1975; LaLonde and Wong 1975), it was finally concluded that the novel structure, reported for the first time as a naturally occurring compound, was immonium thiobinupharidine in the axial form.

Table 7. Fragmentation and elucidation data for the cytotoxic fraction h from the $CH_2Cl_2$ extract of Nuphar luteum.

| Name | Structure | Molecular formula (Molecular weight) | Fragmentation |
|---|---|---|---|
| Palmetic acid methyl ester | $CH_3(CH_2)_{14}COOCH_3$ | $C_{17}H_{34}O_2$ (270) | 239, 227, 213, 199, 185, 171, 157, 143, 129 |
| Myrestic acid methyl ester | $CH_3(CH_2)_{12}COOCH_3$ | $C_{15}H_{30}O_2$ (242) | 211, 199, 185, 171, 157, 143, 129, |
| Steric acid methyl ester | $CH_3(CH_2)_{16}COOCH_3$ | $C_{19}H_{38}O_2$ (298) | 267, 255, 241, 227, 213, 199, 185, 171, 157, 143, 129, 115, 101 |
| Linoleic acid methyl ester |  | $C_{19}H_{34}O_2$ (294) | 294, 263, 220, 150, 123 |
| α-Linoleic acid methyl ester |  | $C_{19}H_{32}O_2$ (292) | 261, 236, 223, 207, 191, 173, 163, 149, 135, 121 |

# Cytotoxic compounds in *Viola* (Paper **III**)

## Identification of cytotoxic compounds in *Viola*

The active substance from the cytotoxic-fractionated plant extract of *Viola arvensis* was shown to be the cyclotide varv A, isolated according to the fractionation protocol and HPLC procedures, described by Claeson and co-workers (Claeson *et al.* 1998). In tumour cells from patients (CLL and OVCA) the $IC_{50}$ values for varv A ranged from 1.2 to 1.3 μM (data from Paper **III**). The mean $IC_{50}$ value for the cell lines was 4.4 μM (Table 8). The cytotoxicity activity profile for varv A is shown below in Figure 10.

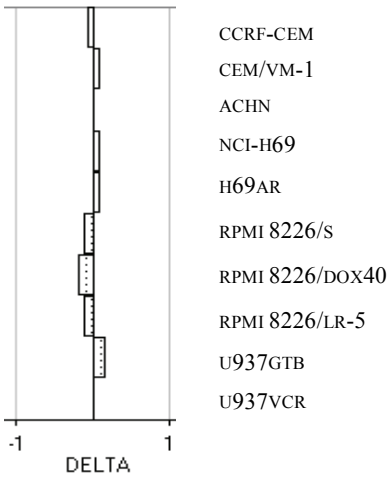



*Figure 10.* Activity profile, mean graph, of varv A from *Viola arvensis*.

## Structure-activity relationship

Because of the pronounced anti-tumour activity shown by the fractionated plant extract of *Viola arvensis*, three purified cyclotides previously isolated from species of *Viola* were tested for cytotoxic activity towards both the cell-line panel and patient samples (Paper **III**). For each of these peptides, the fraction from which it was later isolated also showed cytotoxic activity. The cyclotides varv A, varv F, and cycloviolacin O2, isolated from the two violets *Viola arvensis* and *Viola odorata*, exhibited strong cytotoxic activities at low concentrations. Of these, cycloviolacin O2 proved to be the most potent, with $IC_{50}$ values of 0.10-0.26 μM (Table 8). This is the first time that cytotoxicity of these three cyclotides has been reported. Figure 11 shows the amino-acid sequences and three disulfide bonds of these cyclotides.

Table 8. *Cytotoxic activity as $IC_{50}$ values (in μM) of the cyclotides for a panel of 10 human tumour cell lines*

| Cell line | varv A | varv F | cycloviolacin O2 |
|---|---|---|---|
| RPMI 8226/S | 3.24 | 5.90 | 0.12 |
| RPMI 8226/LR-5 | 3.19 | 6.31 | 0.12 |
| RPMI 8226/DOX40 | 2.73 | 3.14 | 0.12 |
| CCRF-CEM | 3.56 | 7.13 | 0.11 |
| CEM/VM-1 | 4.97 | 7.15 | 0.14 |
| NCI-H69 | 4.88 | 7.49 | 0.12 |
| H69AR | 4.89 | 7.12 | 0.26 |
| U937GTB | 6.36 | 7.07 | 0.26 |
| U937VCR | 4.84 | 7.45 | 0.20 |
| ACHN | 4.19 | 2.63 | 0.22 |

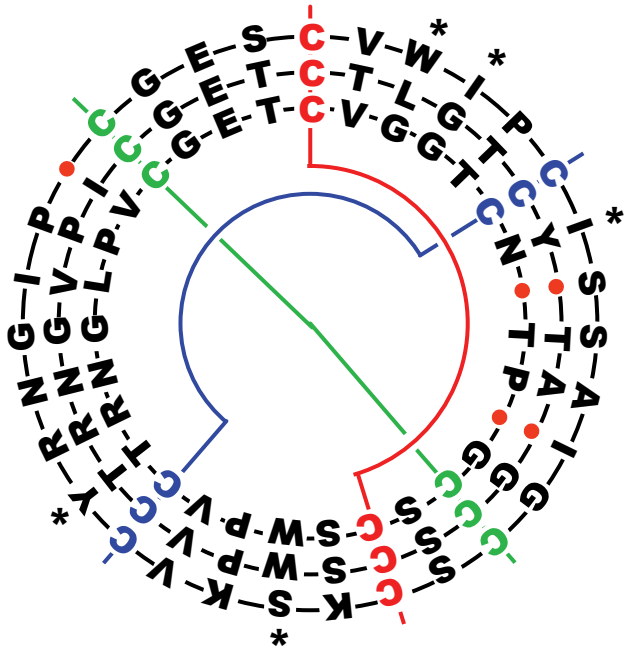


*Figure 11.* Alignment of amino-acid sequences showing the cyclic structure of the cyclotides varv A, varv F and cycloviolacin O2, from inside out. Lines through the centre represent the three disulfide bonds. Asterisks (*) indicate amino acid changes with probability values for random occurrence in fewer than 1 in 1000 substitutions, according to the PAM250 (Dayhoff *et al.*1978) and BLOSUM62 (Gonnet *et al.* 1992) substitution matrices. Figure from Paper **III**.

## Cytotoxic compounds in mistletoes (Paper **IV**)

In the initial screening presented in Paper **I** one mistletoe, *Viscum heyneanum* of the family Viscaceae, was included. Results from tests on HEL and MT4 cells showed surprisingly low activity, considering the previously indicated cytotoxicity of mistletoes (Samuelsson 1966; Samuelsson 1969; Samuelsson *et al.* 1981) and their century-long use in cancer therapy. In study **IV** further investigations on the *in vitro* activity of mistletoes were carried further and expanded into testing extracts from seven Panamanian mistletoe species. Four out of seven mistletoe species tested showed cytotoxicity in the first FMCA screening, namely *Phthirusa pyrifolia* and *Phthirusa retroflexa* from Loranthaceae, and *Phoradendron acinacifolium* and *Phoradendron quadrangulare* from Viscaceae. The $IC_{50}$ values ranged from 1.2 mg/ml to 1.8 mg/ml, corresponding to concentrations of approximately 0.25 mM to 0.38 mM, based on an approximate thionin mass of 4.8 kDa (Table 9).

Table 9. *Cytotoxic activity towards the lymphoma cell line U937GTB in $IC_{50}$ values (μM) of the HAc extracts from different mistletoes.*

| Species | Family | $IC_{50}$ |
|---|---|---|
| *Oryctanthus alveolatus* | Loranthaceae | >2000 |
| *Phthirusa pyrifolia* | Loranthaceae | 1186 |
| *Phthirusa retroflexa* | Loranthaceae | 1644 |
| *Psittacanthus pusillus* | Loranthaceae | >2000 |
| *Phoradendron acinacifolium* | Viscaceae | 1577 |
| *Phoradendron guascanum* | Viscaceae | >2000 |
| *Phoradendron quadrangulare* | Viscaceae | 1785 |

The extract was fractionated by HPLC, and the resulting fractions were scanned by a photodiode array detector for characteristic peptidoid spectra. MALDI-TOF mass spectral analysis was used to check that peptides were present, by seeking characteristic mass/charge ratios of peptide fragments. Both the ultraviolet (UV) spectra and mass analyses indicated that *Phoradendron quadrangulare* contains thionins. However, no typical thionin UV peaks or characteristic fragments were detected in the *Phoradendron acinacifolium* analyses. The loranthaceous mistletoes yielded peaks of lower intensity, indicating that thionins were only present in low concentrations, if at all. Extracts from both *Phthirusa pyrifolia,* and *Phthirusa retroflexa* showed spectra indicating the presence of thionins, but no confirmatory masses were detected. Instead, lower than expected masses were detected, indicating the presence of polypeptide. The spectra and masses obtained are all shown in Paper **IV**.

# Cytotoxic compounds in *Digitalis* (Paper **V**)

## Identification of cytotoxic compounds in *Digitalis*

The active cytotoxic substance from *Digitalis purpurea* indicated in **I**, was isolated by means of a bioassay-guided fractionation. The substance proved to be the clinically well-known cardiac glycoside digitoxin. In tumour cells from patients (OVCA, breast carcinoma and CLL) the $IC_{50}$ range was 55 to 150 nM (data from Paper **V**). Over all the cell lines, the mean $IC_{50}$ value for digitoxin was 37 nM.

## Structure-activity relationships

To further investigate structure-activity relationships, on a small scale, we performed cytotoxicity tests with digitoxin, the saponin digitonin, the aglycone digitoxigenin, and four other cardiac glycosides. All compounds were tested against different human tumour cells, both from cell lines and

primary cultures of human tumour cells from patients (Paper **V**). The chemical structures and activity profiles of these compounds are presented in Figure 12. Of these seven compounds, proscillaridin A proved to be the most potent, with $IC_{50}$ values ranging from 6.4 to 76 nM, then in order of decreasing potency: digitoxin, ouabain, digoxin, lanatoside C, digitoxigenin, and digitonin. The $IC_{50}$ values of cardiac glycosides and digitonin for the human tumour cell-line panel are shown in Table 10. Digitoxin and digoxin were selectively toxic to haematological tumour cells, while proscillaridin A was equally toxic to solid and haematological tumour cells.

Table 10. *$IC_{50}$ values (nM) of cardiac glycosides and digitonin for the tumour cell-line panel.*

| Cell line | Digitoxin | Digitoxigenin | Digoxin | Lanatoside C | Ouabain | Proscillaridin A | Digitonin |
|---|---|---|---|---|---|---|---|
| RPMI 8226/S | 34 | 242 | 84 | 220 | 73 | 13 | 34 |
| RPMI 8226/DOX40 | 59 | 395 | 172 | 339 | 148 | 20 | 1070 |
| RPMI 8226/LR-5 | 25 | 201 | 64 | 150 | 57 | 15 | 1310 |
| CCRF-CEM | 25 | 283 | 49 | 127 | 57 | <6.4* | 2650 |
| CEM/VM-1 | 40 | 441 | 63 | 190 | 74 | 9 | 4430 |
| NCI-H69 | 12 | 635 | 56 | 137 | 70 | 66 | 2600 |
| H69AR | 31 | 200 | 41 | 154 | 45 | 10 | 4730 |
| U937GTB | 32 | 251 | 68 | 142 | 66 | 6.4 | 1880 |
| U937VCR | 36 | 344 | 74 | 133 | 63 | 6.4 | 1100 |
| ACHN | 76 | 658 | 125 | 602 | 126 | 76 | 7000 |

* The estimated $IC_{50}$ value was below the tested concentrations; hence the lowest tested concentration is given.

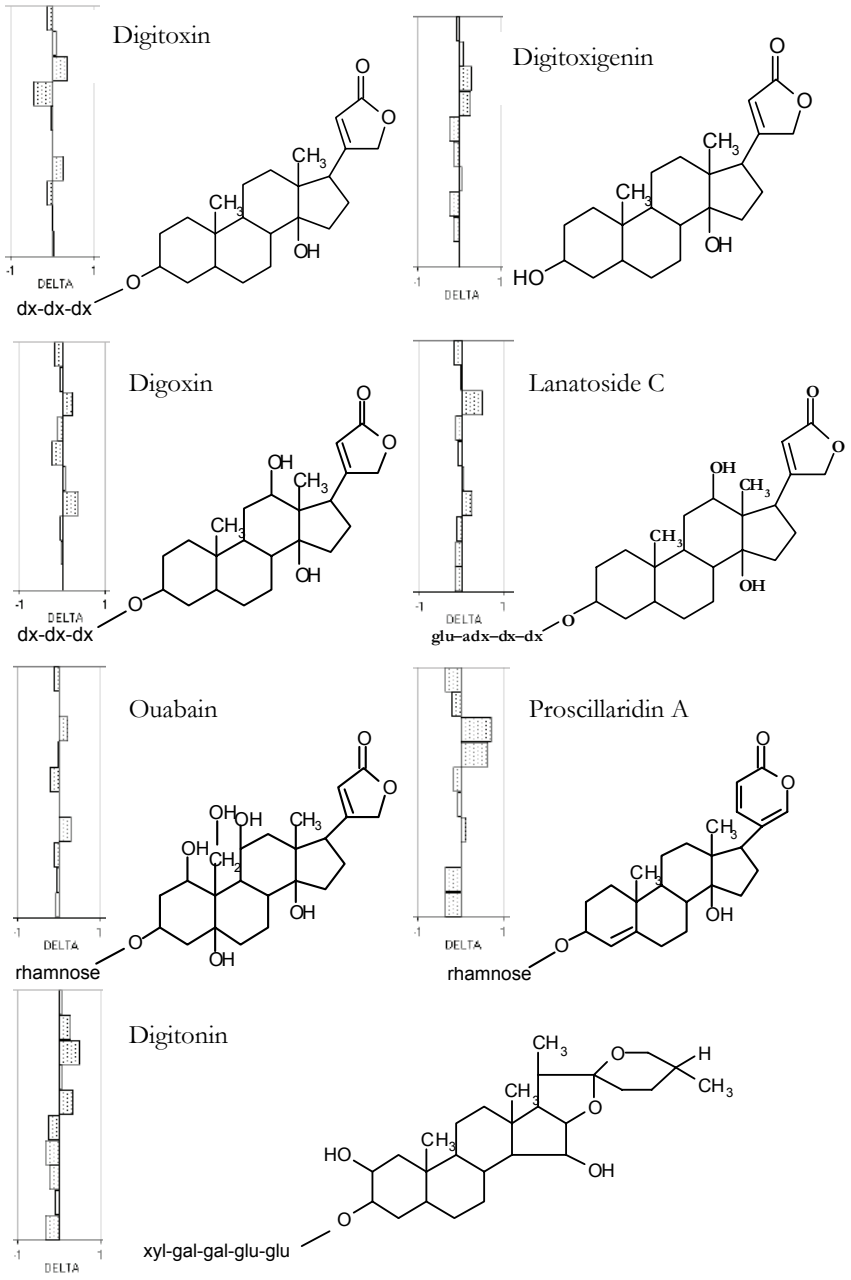



*Figure 12.* Chemical structures and activity profiles of the cardenolides. Abbreviations: dx (digitoxose), glu (glucose), adx (acetyldigitoxose), xyl (xylose), gal (galactose). The 10 cell lines referred to in the activity profiles are, from the top: CCRF-CEM, CEM/VM-1, ACHN, NCI-H69, H69AR, RPMI 8226/S, RPMI 8226/Dox40, RPMI 8226/LR-5, U937GTB and U937VCR.

# Discussion

## Screening and phylogeny (Paper **I**)

The screening efforts described in Paper **I** were designed to identify natural products with the highest potency, greatest chemical diversity, and most promising, distinct anticancer mechanisms possible from a manageable selection of plants. The rationale of the project was based on the assumption that if knowledge of plant phylogeny and relevant systematic information can be used to make a well-founded selection of study objects at the beginning of such a program, the speed at which the structural diversity found in Nature can be explored and exploited should be significantly enhanced.

The results from Paper **I** suggest that compounds with cytotoxic activities are distributed in most parts of the plant kingdom, thus the selected set of plants should include representatives of a wide range of families. Furthermore, natural products from plants have enormous molecular diversity, and numerous strategies have been developed to access and explore this wealth of different structures, and the mechanisms of action related to them. It seems intuitively likely that wide systematic diversity will be reflected in a great diversity of chemical compounds, since each plant species has the capacity, developed over millions of years of evolution, to generate a unique set of chemicals. This capability is retained in the plant genome in the form of genes encoding a large array of biosynthetic enzymes. Phylogeny can thus be used as a tool, both to find new substances, and to help us to decide where to search.

The combination of a well planned and performed phylogenetic selection together with a relevant bioassay method with well-defined criteria contributed to the distinct results in all papers presented in this thesis. The screening in Paper **I** led to a set of plants with cytotoxic substances, from which the cyclotides varv A, varv F, cycloviolacin O2, and digitoxin, a heart drug in clinical use, could be isolated and investigated, together with structurally related substances, as described in Papers **III** and **V**. In Paper **II** unexpected positive results from Paper **I** were further investigated. Unexpected, but negative results from Paper **I** were futher investigated in Paper **IV.**

The strategy of using a robust selection method and selection criterion (cytotoxicity, as determined by appropriate assays) provides a way to address, on a smaller scale, similar questions to those investigated in the NCI's DTP. The NCI attempts to identify interesting compounds by using huge scale screening programs (testing more than 70000 extracts of angiosperms against a 60-cell-line panel looking for anti-tumour substances), while a selection of approximately of 1/4000 this size was screened in the studies underlying this thesis. Nevertheless, the low correlations of the activity profiles of the selected substances with those of standard chemotherapeutic drugs, support the hypothesis that the cytotoxicity displayed by several of these compounds may act by novel, unknown mechanisms.

## *Nuphar luteum* – cytotoxicity of a *Nuphar* alkaloid (Paper **II**)

In summary, Paper **II** demonstrated that an alkaloid isolated from *Nuphar luteum*, representing what is thought to be one of the earlieast lineages of angiosperms to diverge, has cytotoxic activity. This observation, in combination with the activity of paclitaxel in Taxaceae, further corroborates the finding that cytotoxic substances do not only occur in advanced angiosperm families such as Apocynaceae. Consequently, we should use available phylogenetic information to ensure adequate coverage of biological diversity in the whole group of organisms under investigation.

The alkaloid was retrieved by bioassay-guided isolation and shown to have high cytotoxic activity with an apparently unique activity profile. The structure was elucidated by IR, MS, 1D- and extensive 2D-NMR analyses, together with comparison with literature data for closely related structures, and it was identified as immonium thiobinupharidine in the axial form (Figure 9). This substance has not been previously reported, although the bisimmonium ion has been obtained as an intermediate during the synthesis of hydroxyneothiobinupharidine by Wong and LaLonde, (1975).

This is the first report of the bioassay-guided isolation of a cytotoxic *Nuphar* alkaloid. The observed activity is remarkably high both towards the primary resistant renal adenocarcinoma ACHN and the doxorubicin-selected myeloma 8226/DOX40, with $IC_{50}$ values below 1 nM. This is particularly noteworthy considering the generally resistant nature of these cell lines (which also show generally higher resistance than the other lines in the cell-line panel). Thus, the *fraction p'* obtained from *Nuphar luteum* had a unique activity profile (Figure 7), which is presumably similar to that of the pure compound, although this has not been tested due to the miniscule amounts of the substance isolated.

The lipophilic extract from the *Nuphar luteum* contained a mixture of fatty acids and showed cytotoxic activity, but not as potent as the *Nuphar* alkaloid. They were structural elucidated and the major component was determined to be palmetic acid.

## Cyclotides in *Viola* – cytotoxic macrocyclic polypeptides (Paper **III**)

The circulins (see Table 3) were initially found in a random-screening program targeting anti-HIV activity (Gustafson *et al.* 1994; Derua *et al.* 1996; Daly *et al.* 1999). Cyclopsychotride A, which inhibits neurotensin binding to cell membranes (Witherup *et al.* 1994), was isolated from *Psychotria longipes*. Kalata B1, which has uterotonic effects (Gran 1973; Saether *et al.* 1995; Gran *et al.* 2000), was found in *Oldenlandia affinis* DC. Cytotoxicity and haemolytic activity have been shown by both kalata B1, and the previously mentioned circulins A and B, and cyclopsychotride (Tam *et al.* 1999). Cycloviolins A-D, which have anti-HIV activity, have been isolated from *Leonia cymosa* (Hallock *et al.* 2000).

Turning to polypeptides from violets, the haemolytic activity of viola-peptid I led to its discovery (Schöpke *et al.* 1993), and subsequently to the isolation of varv A-H from *Viola arvensis* (Claeson *et al.* 1998; Göransson *et al.* 1999). In Paper **III**, we present evidence for yet another activity of these cyclic polypeptides, namely cytotoxic activity against human tumour cells. More recently, vitri A has been isolated from *Viola tricolor*, and shown to have cytotoxic activity (Svangård *et al.* 2004), as have a series of other *Viola*-cyclotides.

The cyclotides isolated from *Viola* show pronounced anti-tumour activity (Table 8), leading to total cell death at low concentrations in both primary cultures and cell lines. The activity profile in Figure 10 shows that the differences between the sensitive and resistant cell lines are very large. The low variability in $IC_{50}$ values and, thus, activity profiles amongst the different cell lines indicates that the compounds may have an unspecific mechanism of action, such as pore formation (for details of general pore formation theory see Craik *et al.* 2001; Svangård *et al.* 2003). The cyclotides show a dose-dependent cytotoxicity, and the dose-response curves exhibit a sharp profile, similar to dose-response curves for the cytotoxic human neutrophil defensin HP-1, which has been shown to disrupt cell membranes by forming pores (Kagan *et al.* 1990; Bateman *et al.* 1992; Ganz and Lehrer 1999; Heller *et al.* 2000).

More recent studies have confirmed the cytotoxic activity of the cyclotides and the general traits reported in Paper **I** (Göransson *et al.* 2004; Svangård *et al.* 2004). The unique family of cyclotides, macrocyclic proteins

with unusual stability and potency, are highly interesting from a medical perspective. Three disulfide bonds stabilize the unique head-to-tail cyclicized backbone of these substances, and cysteine residues form the cystine knot motif, forming a very stable yet versatile scaffold, as discussed in Paper **III**. Protease digestion of these polypeptides is only possible after reduction of the disulfide bonds to eliminate the stability provided by the cystine knot (Craik *et al.* 1999).

In plants nothing is known about the function of the cyclotides. However, these rather complex molecules do presumably have some biological functions in plants, since evolutionary pressures tend to prevent the production of substances that do not increase reproductive fitness. In addition to these evolutionary considerations, observed biological activities and affinities for plant polypeptides to animal receptors suggest that the cyclotides may have important functions in plant defences.

Craik *et al.* (2001), and Epand and Vogel (1999) suggest that the cytotoxic, haemolytic, and antimicrobial effects of cyclotides are due to their similarities to other antimicrobial peptides. For instance, they have structural similarity to the defensins, a family of polypeptides hypothesized to disrupt cell membranes by forming membrane pores (Kagan *et al.* 1990; Ganz and Lehrer 1999; Yang *et al.* 2000). Because of their positive charge, the cyclotides may interact with negatively charged groups of membranes, thus causing disruptions that affect membrane permeability (Hancock 2001). This could be the mechanism responsible for the cytotoxic effects of the cyclotides.

## Mistletoes (Paper **IV**)

The mistletoes contain peptides with similar activities to the cyclotides, and they have been used in traditional medicine since throughout recorded history, initially for their antimicrobial properties, in wound healing, and recently more often in cancer treatment. Extracts from mistletoes have shown cytotoxic activities in a series of studies (Samuelsson 1966; Samuelsson 1969; Samuelsson *et al.* 1981; Li *et al.* 2002; Johansson *et al.* 2003).

The material screened in Paper **I** included *Viscum heyneanum* (see nomenclatural notes on page 36), a mistletoe from the family Viscaceae, sometimes included in the Santalaceae *sensu latissimo*. Although results of previous reports have indicated the presence of compounds with cytotoxic activities in mistletoes, no significant cytotoxic activity was found in our initial screening; only 5% inhibition of MT4 cells being observed at the highest tested concentration.

Using the *in vitro* FMCA method for cytotoxicity testing, and a fractionation protocol designed for isolating thionins, this study was

performed to further test the previously shown cytotoxicity of mistletoes towards the tested cell lines. The samples studied were selected to compare species from the Viscaceae and Loranthaceae that have not previously been investigated. In this study, fractionated extracts were tested in much the same way as in the NCI's DTP screening program. The results of this more detailed study indicate that cytotoxic compounds are present in the mistletoes, contrary to the indications obtained in the initial screening (Paper **I**). This apparent discrepancy will be further investigated in future studies.

Paper **IV** presents the first report of small cytotoxic polypeptides, as well as possible thionins, in the family Loranthaceae. The masses of compounds detected in the cytotoxic fractions from *Phthirusa pyrifolia* and *Phthirusa retroflexa* as determined with MALDI-TOF indicate the presence of small peptides of 3-5 kDa. Previously published reports of cytotoxic peptides in the Loranthaceae have detected larger, medium-sized, peptides with molecular weights of 14-19 kDa (Mary *et al.* 1994; Fernandez *et al.* 1998).

## Digitoxin in *Digitalis* (Paper **V**)

This study confirmed a new activity for an already well known molecule. The observed cytotoxicity of digitoxin prompted further investigation of the structure-activity relationships of other structurally related compounds. The results revealed pronounced differences in cytotoxicity between the cardiac glycosides, in terms of potency, selectivity, and cytotoxicity mechanisms from those of commonly used anticancer drugs. The cytotoxicity of *Digitalis* has been known for a long time, but epidemiological studies have shown that digitoxin has anti-tumour effects even in the low concentrations that can be used therapeutically (Stenkvist 1999; Haux *et al.* 2001).

Activity profile analysis, using COMPARE software, showed that the profiles of the cardiac glycosides and digitonin were very weakly correlated (with Pearson's correlation coefficients of just 0.30-0.70; Pearson 1900) to those of substances with known cytotoxicity mechanisms (Paper **V**). These findings suggest that the mechanism involved in the cardiac glycosides' activity, has not been previously characterised, and thus indicate further possible therapeutic applications for the cardiac glycosides that are in clinical use today. Other studies support the hypothesis that the cardiac glycosides have anti-tumour activity and the mechanism involved has been suggested to be based on oestrogen-like effects, or the inhibition of proliferation and induction of apoptosis in various cell lines (by digitoxin) (Cove and Barker 1979; Falconer *et al.* 1983), or effects on intracellular $Ca^{2+}$ levels (Yeh *et al.* 2001).

# Summary, Conclusions, and Future Prospects

In the studies presented here an attempt was made to select a set of plants based on systematic information and the connections between evolution, biological diversity and chemical diversity. In this work, naturally occurring compounds with cytotoxic activity towards tumour cells were sought, detected and evaluated. Using fractionation by a primary protocol, then bioassay-guided isolation (cytotoxicity assays using human tumour cell lines and primary cultures of human tumour cells), several compounds with pronounced cytotoxicity were isolated. These compounds, representing substantial chemical diversity and originating from diverse parts of the phylogenic spectrum examined, showed promising cytotoxic activity. Furthermore, their activity appeared to be based on novel mechanisms (according to activity profile comparisions). They included a cytotoxically potent *Nuphar* alkaloid, cytototoxic cyclotides, cytotoxic polypeptides from Loranthaceae, and cardiac glycosides with anti-tumour activity. The approach appears to be valid and useful, since there is an urgent need for new cytostatic substances, preferably with novel mechanisms of action that can act on tumour cells that are resistant to currently used drugs.

As mentioned in the aims presented on page 23, the selection of plants for the cytotoxic investigation was designed to cover large parts of the angiosperm system, providing broad representation of plants across a wide range of species. Extracts were subjected to polypeptide fractionation protocols. Screening was performed with tumour cells from patients and cell lines, selecting for anti-tumour activity and selectivity to detect compounds acting with novel cytotoxic mechanisms. This resulted in the detection and identification of following cytotoxic compounds: a *Nuphar* alkaloid, three cyclotides, polypeptides from Loranthaceae and Viscaceae, and cardiac glycosides from *Digitalis*.

In conclusion, the results show that selection based on phylogenetic information, together with a robust and reliable method to detect cytotoxicity, can be a helpful approach for exploring the plant kingdom for cytotoxic substances. These findings show that the effiency of the screening procedures applied in searches for active compounds can be improved by using a systematic biodiversity-based search with two levels of explicit selection (first of objects for study, then at the different testing steps). Nature can then be more effectively explored for new substances with promising pharmacological properties.

## Future prospects

The results presented in this thesis confirm the value of exploring the vast biological and chemical diversity available for cytotoxic compounds of plant origin, and for new potential leads in the development of novel drugs for cancer treatment

To conclude this thesis, I propose that future studies should be undertaken to further investigate:

- representative species from some of the orders and major families that were not covered in this thesis. Particular attention could then be paid to the systematic entities that have not yet been investigated by the NCI;
- the cytotoxic compounds in *Nuphar luteum*, and related species, testing cytotoxicity in the cell-line panel and examining their mechanisms of action and selectivity;
- mechanisms of action and selectivity of the cyclotides (work that is already in progress at the Division of Pharmacognosy, Uppsala University);
- mechanisms of action and selectivity of the polypeptides in the mistletoes, and the sources encoding polypeptide (DNA sequences) to assess the thionin and polypeptide contents of these plants (also work in progress at the Division);
- the fractionation and isolation methods, considering the unexpected occurrence of cardiac glycosides and alkaloids in a fraction designed to retrieve polypeptides;
- the clinical potential of the compounds described here, by testing their activity on other types of tumour and normal blood cells that have not been affected by tumours as well as, eventually, *in vivo* studies. The *in vitro* studies are admittedly far from the clinical response, but they can be used for identifying cytotoxic activity and preliminary mechanistic classifications. The data thus obtained need to be confirmed, for instance by testing the compounds on primary cultures of human tumour cells, followed by appropriate animal studies;
- connections between the mechanisms of action and the chemical structures in the chemical space spanned by the identified substances (also work in progress at the Division).

# Swedish Summary

## Cytotoxiska föreningar från växter – biologisk och kemisk diversitet

Tumörer utvecklar ofta resistens mot de verkningmekanismer dagens läkemedel mot cancer, cytostatika, använder sig av. Det är därför viktigt att hitta nya cytotoxiska föreningar som hämmar tumörtillväxt för att kunna utveckla ny cytostatika med andra verkningmekanismer.

Naturen uppvisar en otrolig kemisk mångfald och kan erbjuda nya föreningar med potential som läkemedel eller farmakologiska verktyg.

Genom att göra ett systematiskt urval kan man lättare leta efter dessa cytotoxiska föreningar. Växter är en enhetlig och tydligt avgränsad grupp där man har studerat deras evolutionära utveckling. Med ett tydligt schema från början kan man få en bättre utgångspunkt och använda växternas systematik för att hitta ny kemi. I det här arbetet har ett urval gjorts för att täcka in stora delar av de blommande växterna, angiospermerna, genom att systematiskt undersöka olika växtgrupper.

Över 100 olika växter har fraktionerats enligt ett fraktioneringsprotokoll framtaget för att isolera polypeptider. Av dessa fraktionerade växtextrakt valdes 29 ut baserat på cyototoxiska data. Dessa, plus en kontroll, fick genomgå en screening i två steg. Första steget var en cytotoxisk testning med en försöksmodell som kallas DIAS (Dynatech Immuno Assay System) och som utförs på blodceller från friska blodgivare – PBMC, blod- eller benmärgsceller från patienter med kronisk lymfatisk leukemi – CLL, och bortopererat material från ovarialcancerpatienter – OVCA) samt på två cellinjer (solid njurtumörcellinje – ACHN och T-cellsleukemicellinje – CCRF-CEM). Genom att välja växtextrakt som uppfyllde vissa kriterier avseende tillräcklig cytotoxicitet återstod 10 för vidare testning. Dessa fick genomgå en liknande cytotoxicitetstestning kallad FMCA (Fluorometric Microculture Cytotoxicity Assay) som baseras på en cellinjepanel bestående av tio olika cellinjer representerande flera olika verkningsmekanismer. Sju av dessa tio växtextrakt visade även i den andra försöksmodellen intressanta cytotoxiska resultat. De var extrakt från: *Colchicum autumnale* (tidlösa), *Digitalis lanata* (grekisk fingerborgsblomma), *Digitalis purpurea* (fingerborgsblomma), *Helleborus cyclophyllus* (en julros utan svenskt namn), *Menyantes trifoliata* (vattenklöver), *Viola arvensis* (åkerviol) och *Viola patrinii* (saknar svenskt namn).

Genom att titta närmare på några av dessa kunde de cytotoxiskt aktiva substanserna isoleras och identifieras till digitoxin från *Digitalis lanata* och *Digitalis purpurea* samt polypeptiden, en cyklotid varv A från *Viola arvensis*. Dessa substanser studerades tillsammans med strukturellt närstående substanser och uppvisade mycket goda effekter av tidigare delvis okänt slag.

Den gula näckrosen, *Nuphar luteum*, uppvisade i den första undersökningen så oväntade resultat att den studerades separat. Cytotoxicitetsstyrd isolering av de aktiva föreningarna i *Nuphar luteum* resulterade i identifieringen av flera potentiellt intressanta föreningar, bland annat den mycket cytotoxiska alkaloiden immonium-thiobinupharidine.

Sju extrakt från mistlar från familjerna Viscaceae och Loranthaceae, insamlade i Panama, testades för cytotoxicitet med FMCA. Fyra extrakt visade viss aktivitet på en känslig lymfomcellinje (U937GTB) och renades ytterligare med HPLC. Resultatet indikerar att aktiviteten beror på små peptider, tioniner. Tioniner har tidigare visat cytotoxicitet från flera mistlar från familjen Viscaceae, men det här är första rapporten av polypeptider med tioninliknande egenskaper i familjen Loranthaceae.

Som slutsats kan sägas att genom utnyttjande av kunskap om den biologiska diversiteten tillsammans med robusta och pålitliga testmetoder, har inom projektet en serie potenta cytotoxiska substanser med sinsemellan olika typer av biologisk aktivitet/mekanism påträffats i fyra vitt skilda grupper av växter.

# Acknowledgements

The studies presented in this thesis were carried out at the Division of Pharmacognosy, Department of Medicinal Chemistry, Faculty of Pharmacy, Uppsala University, Sweden, during the years 1998-2005. Many persons have contributed either directly or indirectly to the studies, and I would like to express my sincere gratitude to all of them for their kindness and help. I am especially grateful to the following persons who helped me accomplish the work and prepare the thesis:

My main supervisor from 2001, Dr. Anders Backlund; you encouraged me with your never-ending enthusiasm and support. Above all, I enjoyed our frank dialogue and appreciate your friendship, trust in my work, and confidence in my abilities. Thanks for all your help!

My former supervisor, docent Per Claeson, for all your comments, advice, and help. Prof. Lars Bohlin for introducing me to this interesting research field, valuable discussions, and for providing good laboratory conditions.

Dr. Jan G. Bruhn, Prof. Wenche Rolfsen, the late docent Premila Perera Ivarsson, Prof. Finn Sandberg, Prof. Gunnar Samuelsson, Dr. Mervi Vasänge, Dr. Håkan Tunón, and docent Anders Dahlberg, for your support, advice and inspiring scientific discussions.

Prof. Rolf Larsson from the Division of Clinical Pharmacology, Department of Medical Sciences, Uppsala Academic Hospital, for providing kindly and helpful comments and advice.

My co-authors and colleagues for great companionship and support: Dr. Senia Johansson (good spirit), Dr. Ulf Göransson (for *excellent* work), Dr. Joachim Gullbo (your friendly sharing of knowledge is highly appreciated), and Lic. Sonny Larsson (your wide knowledge and tremendous memory are amazing).

Special thanks to Dr. Hesham El-Seedi, the guru of separation and structural elucidation, who taught me so much.

My other colleagues for their encouragement and support: Lic. Erika Svangård, Dr. Ulrika Huss, Dr. Therese Ringbom, Lic. Martin Sjögren, Lic. Wimal Pathmasiri, Dr. Shisheng Li, Mrs. Charlotte af Klercker, Mr. Erik Hedner, Mr. Anders Herrmann, Ms. Josefin Larsson, Ms. Jenny Pettersson, Ms. Sofia Lindgren, and Lic. Åke Stenholm.

My students over the years: Karna Fågelberg, Fredrik Johansson, and Anna Ekström.

The fellows at the Division of Analytical Pharmaceutical Chemistry, especially Mr. Henrik Lodén and Lic. Ingela Sundström for your sympathy.

Research technician Rolf Andersson, for his vast knowledge of NMR and willingness to share it.

All persons at the Division of Clinical Pharmacology, Uppsala Academic Hospital, for your assistance, kindness and helpful comments, especially Dr. Sumeer Dhar, Mrs. Christina Leek, and Mrs. Lena Lenhammar.

Mrs. Kerstin Ståhlberg and Mrs. Maj Blad for administrative support.

Dr. John Blackwell for a great editing service.

On a personal note, I would like to thank my parents, my sister, the "Lindholm family", all my friends outside the "academic world" and, most importantly, the love of my life, Johnny, for your concern, and nutritious food, for helping me value the important things in life, and for giving me a wonderful family, and Charlie; you made this work a challenge with all your "assistance" – love you!

# References


Ando, T., Muraoka, T., Yamasaki, N. and Okuda, H. (1980). Preparation of anti-lipolytic substance from *Panax ginseng*. Planta Medica **38**: 18-23.

APG (1998). An ordinal classification for the families of flowering plants. Annals of the Missouri Botanical Garden **85**: 531-553.

APG (2003). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. Botanical Journal of the Linnean Society **141**: 399-436.

Arcamone, F., Franceschi, G., Orezzi, P., Cassinelli, G., Barbieri, W. and Mondelli, R. (1964). Daunomycin. I. The structure of daunomycione. Journal of the American Chemical Society **86**: 5334-5335.

Arcamone, F., Franseschi, G., Penco, S. and Selva, A. (1969). Adriamycin (14-hydroxydaunomycin), a novel antitumor antibiotic. Tetrahedron Letters **13**: 1007-1010.

Backlund, A. Newman, P. and Bohlin, L. Systematic coverage of NCI cytotoxicity screening efforts. Manuscript in preparation for Journal of Natural Products.

Bateman, A., Singh, A., Jothy, S., Fraser, R., Esch, F. and Solomon, S. (1992). The levels and biologic action of the human neutrophil granule peptide HP-1 in lung tumors. Peptides **13**: 133-139.

Beck, W.T., Cirtain, M.C., Danks, M.K., Felsted, R.L., Safa, A.R., Wolverton, J.S., Suttle, D.P. and Trent, J.M. (1987). Pharmacological, molecular, and cytogenic analysis of "atypical" multidrug-resistant human leukemic cells. Cancer Research **47**: 5455-5460.

Beck, W.T. and Dalton, W.S. (1997). Mechanisms of Drug Resistance. *CANCER: Principles & practice of oncology*. DeVita Jr., V.T. *et al.* Philadelphia, Lippincott-Raven Publishers**:** 498-513.

Bellamy, W.T., Dalton, W.S., Gleason, M.C., Grogan, T.M. and Trent, J.M. (1991). Development and characterization of a melphalan-resistant human multiple myeloma cell line. Cancer Research **51**: 995-1002.

Bohacek, R.S., McMartin, C. and Guida, W.C. (1996). The art and practice of structure-based drug design: a molecular modeling perspective. Medicinal Research Reviews **16**: 3-50.

Bohlmann, H. and Apel, K. (1991). Thionins. Annual Review in Plant Physiology and Plant Molecular Biology **42**: 227-240.

Bokesch, H.R., Pannell, L.K., Cochran, P.K., Sowder II, R.C., McKee, T.C. and Boyd, M.R. (2001). A novel anti-HIV macrocyclic peptide from *Palicourea condensata*. Journal of Natural Products **64**: 249-250.

Botling, J., Liminga, G., Larsson, R., Nygren, P. and Nilsson, K. (1994). Development of vincristine resistance and increased sensitivity to cyclosporin A and verapamil in the human U-937 lymphoma cell line without overexpression of the 170-KDa P-glycoprotein. International Journal of Cancer **58**: 269-274.

Boyd, M.R. and Paull, K.D. (1995). Some practical considerations and applications of the National Cancer Institute *in vitro* anticancer drug discovery screen. Drug Development Research **34**: 91-109.

Cassady, J.M., Chang, C.-J. and McLaughlin, J.L. (1981). Recent advances in the isolation of structural elucidation of antineoplastic agents of higher plants. *Natural Products as Medicinal Agents*. Beal, J. L. *et al.* Stuttgart, Hippokrates**:** 93-124.

Chabner, B.A. (1990). In defense of cell-line screening. Journal of the National Cancer Institute **82**: 1083-1085.

Claeson, P., Göransson, U., Johansson, S., Luijendijk, T. and Bohlin, L. (1998). Fractionation protocol for the isolation of polypeptides from plant biomass. Journal of Natural Products **61**: 77-81.

Cove, D.H. and Barker, G.A. (1979). Digoxin and hormone receptors. Lancet **2**: 204.

Cragg, G.M. (1998). Paclitaxel (Taxol®): A success story with valuable lessons for natural product drug discovery and development. Medicinal Research Reviews **18**: 315-331.

Craik, D.J., Daly, N.L., Bond, T. and Waine, C. (1999). Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. Journal of Molecular Biology **294**: 1327-1336.

Craik, D.J., Daly, N.L. and Waine, C. (2001). The cystine knot motif in toxins and implications for drug design. Toxicon **39**: 43-60.

Csoka, K., Larsson, R., Tholander, B., Gerdin, E., de la Torre, M. and Nygren, P. (1994). Cytotoxic drug sensitivity testing of tumor cells from patients with ovarian carcinoma using the fluorometric microculture cytotoxicity assay (FMCA). Gynecologic Oncology **54**: 163-170.

Daly, N.L., Koltay, A., Gustafson, K.R., Boyd, M.R., Casat-Finet, J.R. and Craik, D.J. (1999). Solution structure by NMR of circulin A: a macrocyclic knotted peptide having anti-HIV activity. Journal of Molecular Biology **285**: 333-345.

Danks, M.K., Yalowich, J.C. and Beck, W.T. (1987). Atypical multiple drug resistance in a human leukemic cell line selected for resistance to teniposide (VM-26). Cancer Research **47**: 1297-1301.

Danser, B.H. (1941). The British-Indian species of *Viscum* revised and compared with those of South-Eastern Asia, Malaysia, and Australia. Blumea **4**:261-319.

Dayhoff, M.O., Schwartz, R.M. and Orcutt, B.C. (1978). A model of evolutionary change in proteins. *Atlas of protein sequence and structure*. Dayhoff, M. O. Washington DC, National Biomedical Research Foundation **5:** 345-352.

Derua, R., Gustafson, K.R. and Pannell, L.K. (1996). Analysis of the disulfide linkage pattern in circulin A and B, HIV-inhibitory macrocyclic peptides. Biochemical and Biophysical Research Communications **228**: 632-638.

DeVita, V.T. (1997). Principles of cancer management: chemotherapy. *Cancer: Principles & practice of oncology*. Philadelphia, Lippincott-Raven Publishers**:** 333-347.

Dhar, S., Gullbo, J., Nilsson, K., Nygren, P. and Larsson, R. (1998). A nonclonogenic cytotoxicity assay using primary cultures of patient tumor cells for anticancer drug screening. Journal of Biomolecular Screening **3**: 207-216.

Dhar, S., Nygren, P., Csoka, K., Botling, J., Nilsson, K. and Larsson, R. (1996). Anti-cancer drug characterisation using a human cell line panel representing defined types of drug resistance. British Journal of Cancer **74**: 888-896.

Dhar, S., Nygren, P., Csoka, K. and Larsson, R. (1997). Feasibility of a nonclonogenic cytotoxicity assay of primary human tumor cells for new anticancer drug screening (Meeting abstract). Proceedings of the Annual Meeting of the American Association for Cancer Research.

El-Seedi, H.R., Ringbom, T., Torssell, K and Bohlin, L. (2003). Constituents of *Hypericum larcifolium* and their cyclooxygenase (COX) enzyme activities. Chemical and Pharmaceutical Bulletin (Tokyo) **51**: 1439-1440.

Epand, R.M. and Vogel, H.J. (1999). Diversity of antimicrobial peptides and their mechanisms of action. Biochimica et Biophysica Acta **1462**: 11-28.

Falconer, I.R., Beresford, A.M., Jones, A. and Vacek, A.T. (1983). Effect of digoxin on DNA synthesis and cell viability in human breast tumour tissue in organ culture. Chemotherapy **29**: 368-372.

Farnsworth, N.R. (1988). *Screening plants for new medicines. Chapter 9*. Washington, D.C, National Academy Press.

FASS 2005. Published on the Internet http://www.fass.se [accessed 1 March 2005].

Fernandez, T., Wagner, M.L., Varela, B.G., Ricco, R.A., Hajos, S.E., Gurni, A.A. and Alvarez, E. (1998). Study of an Argentine mistletoe, the hemiparasite *Ligaria cuneifolia* (R. et P.) Tiegh. (Loranthaceae). Journal of Ethnopharmacology **62**: 25-34.

Ford, J.M. (1995). Modulators of multidrug resistance. Hematology/Oncology Clinics of North America **9**: 337-361.

Ganz, T. and Lehrer, R.I. (1999). Antibiotic peptides from higher eukaryotes: biology and applications. Molecular Medicine Today **5**: 292-297.

Gilman, A. (1963). The initial clinical trial of nitrogen mustard. American Journal of Surgery **105**: 574-578.

Glimelius, B., Bergh, J., Brandt, L., Brorsson, B., Gunnars, B., Hafström, L., Haglund, U., Högberg, T., Janunger, K.G., Jönsson, P.-E., Karlsson, G., Kimby, E., Lamnevik, G., Nilsson, S., Norrlander, M., Nygren, P., Permert, J., Ragnhammar, P., Sörenson, S., Blomqvist, P. *et al.* (2001). *Cytostatikabehandling vid cancer*. Stockholm, SBU, The Swedish Council on Technology Assessment in Health Care.

Gollapudi, S., Kim, C.H., Tran, B.-N., Sangha, S. and Gupta, S. (1997). Probenecid reverses multidrug resistance in multidrug resistance-associated protein-overexpressing HL60/AR and H69/AR cells but not in P-glycoprotein-overexpressing HL60/Tax and P388/ADR cells. Cancer Chemotherapy and Pharmacology **40**: 150-158.

Gonnet, G.H., Cohen, M.A. and Benner, S.A. (1992). Exhaustive matching of the entire protein sequence database. Science **256**: 1443-1445.

Gran, L. (1973). On the effect of a polypeptide isolated from "kalata-kalata" (*Oldenlandia affinis DC)* on the oestrogen dominated uterus. Acta Pharmacologicae et Toxicologicae Scandinavicae **33**: 400-408.

Gran, L., Sandberg, F. and Sletten, K. (2000). *Oldenlandia affinis* (R&S) DC. A plant containing uteroactive peptides used in African traditional medicine. Journal of Ethnopharmacology **70**: 197-203.

Gustafson, K.R., Sowder II, R.C., Henderson, L.E., Parsons, I.C., Kashman, Y., Cardellina II, J.H., McMahon, J.B., Buckheit Jr, R.W., Pannell, L.K. and Boyd, M.J. (1994). Circulins A and B: novel HIV-inhibitory macrocyclic peptides from the tropical tree *Chassalia parvifolia*. Journal of the American Chemical Society **116**: 9337-9338.

Guttman, D.S. (2004). Plants as models for the study of human pathogenesis. Biotechnology Advances **22**: 363-382.

Göransson, U., Luijendijk, T., Johansson, S., Bohlin, L. and Claeson, P. (1999). Seven novel macrocyclic polypeptides from *Viola arvensis*. Journal of Natural Products **62**: 283-286.

Göransson, U., Svangård, E., Claeson, P. and Bohlin, L. (2004). Novel strategies for isolation and characterization of cyclotides: the discovery of bioactive macrocyclic plant polypeptides in the Violaceae. Current Protein and Peptide Science **5**: 317-329.

Haeckel, E. (1866). *Generelle morphologie der organismen*. Berlin, Georg Reiner.

Hallock, Y.F., Sowder II, R.C., Pannell, L.K., Hughes, C.B., Johnson, D.G., Gulakowski, R., Cardellina II, J.H. and Boyd, M.R. (2000). Cycloviolins A-D, anti-HIV macrocyclic peptides from *Leonia cymosa*. Journal of Organic Chemistry **65**: 124-128.

Hancock, R.E. (2001). Cationic peptides: effectors in innate immunity and novel antimicrobials. The Lancet Infectious Diseases **1**: 156-164.

Haux, J., Klepp, O., Spigset, O. and Tretli, S. (2001). Digitoxin medication and cancer; case control and internal dose-response studies. BMC Cancer **1**: 11.

Heller, W.T., Waring, A.J., Lehrer, R.I., Harroun, T.A., Weiss, T.M., Yang, L. and Huang, H.W. (2000). Membrane thinning effect of the beta-sheet antimicrobial protegrin. Biochemistry **39**: 139-145.

Hellman, S. and Vokes, E.E. (1996). Advancing current treatments for cancer. Scientific American **275**: 118-123.

Hennig, W. (1966). *Phylogenetic systematics*. Urbana, Illinois, University of Illinois Press.

IPNI The International Plant Names Index (2005). Published on the Internet http://www.ipni.org [accessed 1 March 2005].

Johansson, S., Gullbo, J., Lindholm, P., Ek, B., Thunberg, E., Samuelsson, G., Larsson, R., Bohlin, L. and Claeson, P. (2003). Small, novel proteins from the mistletoe *Phoradendron tomentosum* exhibit highly selective cytotoxicity to human breast cancer cells. Cellular and Molecular Life Sciences **60**: 165-175.

Kagan, B.L., Selsted, M.E., Ganz, T. and Lehrer, R.I. (1990). Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. Proceedings of the National Academy of Sciences, USA **87**: 210-214.

Keller-Juslén, C., Kuhn, M. and von Wartburg, A. (1971). Synthesis and antimitotic activity of glycosidic lignan derivates related to podophyllotoxin. Journal of Medicinal Chemistry **14**: 936-940.

Kingsbury, W.D., Boehm, J.C., Jakas, D.R., Holden, K.G., Hecht, S.M., Gallagher, G., Caranfa, M.J., McCabe, F.L., Faucette, L.F., Johnson, R.K. and Hertzberg, R.P. (1991). Synthesis of water-soluble (aminoalkyl)camptothecin analogues: inhibition of topoisomerase I and antitumor activity. Journal of Medicinal Chemistry **34**: 98-107.

Kinoshita, K., Tanaka, J., Kuroda, K., Koyama, K., Natori, S. and Kinoshita, T. (1991). Cycloleonurinin, a cyclic peptide from *Leonuri fructus*. Chemical and Pharmaceutical Bulletin **39**: 712-715.

Kuijt, J. (1978). Commentary on the mistletoes of Panama. Annals of the Missouri Botanical Garden **65**: 736-763.

Kuijt, J. (1980). Miscellaneous mistletoe notes 1-9. Brittonia **32**: 518-529.

Kuijt, J. and Kellogg, E.A. (1996). Miscellaneous mistletoe notes 20-36. Novon **6**: 33-53.

Kunimoto, T., Nitta, K., Tanaka, T., Uehara, N., Baba, H., Takeuchi, M., Yokokura, T., Sawada, S., Miyasaka, T. and Mutai, M. (1987). Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, a novel water-soluble derivate of camptothecin, against murine tumors. Cancer Research **47**: 5944-5947.

LaLonde, R.T., Donvito, T.N. and Tsai, A.I.-M. (1975). $^{13}C$ chemical shifts of quinolizidines. 2. 13C spectra of some *Nuphar* alkaloids. Canadian Journal of Chemistry **53**: 1714-1725.

LaLonde, R.T. and Wong, C. (1975). Thiobinupharidines epomeric at C-1 and C-1'. Canadian Journal of Chemistry **53**: 3545-3550.

LaLonde, R.T., Wong, C.F. and Das, K.C. (1973). Thiospiran, C30, *Nuphar* alkaloids. Structure and evidence for intramolecular sulfur-immonium ion interactions. Journal of the American Chemical Society **95**: 6342-6349.

Lam, H.-M., Chiu, J., Hsieh, M.-H., Meisel, L., Oliveira, I.C., Shin, M. and Coruzzi, G. (1998). Glutamate-receptor genes in plants. Nature **396**: 125-126.

Larsson, R., Fridborg, H., Liliemark, J., Csoka, K., Kristensen, J., de la Torre, M. and Nygren, P. (1994). *In vitro* activity of 2-chlorodeoxyadenosine (CdA) in primary cultures of human haematological and solid tumours. European Journal of Cancer **30A**: 1022-1026.

Larsson, R., Kristensen, J., Sandberg, C. and Nygren, P. (1992). Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). International Journal of Cancer **50**: 177-185.

Larsson, R. and Nygren, P. (1990). Pharmacological modification of multi-drug resistance (MDR) *in vitro* detected by a novel fluorometric microculture cytotoxicity assay. Reversal of resistance and selective cytotoxic actions of cyclosporin A and verapamil on MDR leukemia T-cells. International Journal of Cancer **46**: 67-72.

Larsson, R. and Nygren, P. (1993). Laboratory prediction of clinical chemotherapeutic drug resistance: a working model exemplified by acute leukaemia. European Journal of Cancer **29A**: 1208-1212.

Larsson, R., Nygren, P., Ekberg, M. and Slater, L. (1990). Chemotherapeutic drug sensitivity testing of human leukemia cells *in vitro* using a semiautomated fluorometric assay. Leukemia **4**: 567-571.

Larsson, S. (2004). Phytochemistry and phylogeny – deeds of decisive data mining. *Licentiate thesis at the* Faculty of Pharmacy, Department of Medicinal Chemistry. Uppsala, Uppsala University**:** 48.

Lee, K.-H. (1999). Novel antitumor agents from higher plants. Medicinal Research Reviews **19**: 569-596.

Lehnert, M. (1996). Clinical multidrug resistance in cancer: a multifactorial problem. European Journal of Cancer **32A**: 912-920.

Li, S.-S., Gullbo, J., Lindholm, P., Larsson, R., Thunberg, E., Samuelsson, G., Bohlin, L. and Claeson, P. (2002). Ligatoxin B, a new cytotoxic protein with a novel helix-turn-helix DNA-binding domain from the mistletoe *Phoradendron liga*. Biochemical Journal **366**: 405-413.

Mabberley, D.J. (1998). *The Plant-book*. 2nd ed., Cambridge, Cambridge University Press, GB.

Mangatal, L., Adeline, M.-T., Guénard, D., Guéritte-Voegelein, F. and Potier, P. (1989). Application of the vicinal oxyamination reaction with asymmetric induction to the hemisynthesis of taxol and analogues. Tetrahedron **45**: 4177-4190.

Mangeney, P., Andriamialisoa, R.Z., Lallemand, J.-Y., Langlois, N., Langlois, Y. and Potier, P. (1979). 5'-nor anhydrovinblastine: Prototype of a new class of vinblastine derivates. Tetrahedron **35**: 2175-2179.

Mary, K.T., Girija, K. and Ramadasan, K. (1994). Partial purification of tumour reducing principle from Helicanthis elasticus (Fam. Loranthaceae). Cancer Letter **81**: 53-57.

Mirski, S.E.L., Gerlach, J.H. and Cole, S.P.C. (1987). Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. Cancer Research **47**: 2594-2598.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J. and Boyd, M. (1991). Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. Journal of the National Cancer Institute **83**: 757-766.

Morita, H., Kayashita, T., Shimomura, M., Takeya, K. and Itokawa, H. (1996a). Cyclic peptides from higher plants. 24. Yunnanin C, a novel cyclic heptapeptide from *Stellaria yunnanensis*. Journal of Natural Products **59**: 280-282.

Morita, H., Nagashima, S., Uchiumi, Y., Kuroki, O., Takeya, K. and Itokawa, H. (1996b). Cyclic peptides from higher plants. XXVIII. Antitumor activity and hepatic microsomal biotransformation of cyclic pentapeptides, astins, from *Aster tataricus*. Chemical and Pharmaceutical Bulletin **44**: 1026-1032.

Neuss, N., Gorman, M., Boaz, H.E. and Cone, N.J. (1962). Vinca alkaloids, XI. Structures of leurocristine (LCR) and vincaleukoblastine (VLB). Journal of the American Chemical Society **84**: 1509-1510.

Neuss, N., Gorman, M., Hargrove, W., Cone, N.J., Biemann, K., Büchi, G. and Manning, R.E. (1964). Vinca alkaloids. XXI. The structures of the oncolytic alkaloids vinblastine (VBL) and vincristine (VCR). Journal of the American Chemical Society **86**: 1440-1442.

Neuss, N., Gorman, M., Svoboda, G.H., Maciak, G. and Beer, C.T. (1959). Vinca alkaloids. III. Characterization of leurosine and vincaleukoblastine, new alkaloids from *Vinca rosea Linn*. Journal of the American Chemical Society **81**: 4754-4755.

Noda, K., Ohno, N., Tanaka, K., Kamiya, N., Okuda, M., Yadomae, T., Nomoto, K. and Shoyama, Y. (1996). A water-soluble antitumor glycoprotein from *Chlorella vulgaris*. Planta Medica **62**: 423-426.

Nygren, P., Fridborg, H., Csoka, K., Sundström, C., de la Torre, M., Kristensen, J., Bergh, J., Hagberg, H., Glimelius, B., Rastad, J., Tholander, B. and Larsson, R. (1994). Detection of tumor-specific cytotoxic drug activity *in vitro* using the fluorometric microculture cytotoxicity assay and primary cultures of tumor cells from patients. International Journal of Cancer **56**: 715-720.

Olmstead, R.G., DePamphilis, C.W., Wolfe, A.D., Young, N.D., Elisons, W.J. and Reeves, P.A. (2001). Disintegration of the Scrophulariaceae. American Journal of Botany **88**: 348-361.

Olmstead, R.G. and Reeves, P.A. (1995). Evidence for the polyphyly of the Scrophulariaceae based on chloroplast *rbc*L and *ndh*F sequences. Annals of the Missouri Botanical Garden **82**: 176-193.

Paull, K.D., Shoemaker, R.H., Hodes, L., Monks, A., Scudiero, D.A., Rubinstein, L., Plowman, J. and Boyd, M.R. (1989). Display and analysis of patterns of differential activity of drugs against human tumor cell lines: Development of mean graph and COMPARE algorithm. Journal of the National Cancer Institute **81**: 1088-1092.

Pearce, G., Moura, D.S., Stratmann, J. and Ryan, C.A. (2001). Production of multiple plant hormones from a single polyprotein precursor. Nature **411**: 817-820.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991). A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science **253**: 895-898.

Pearson, K. (1900). On the criterion that a given system of deviations from the probable in the case of a correlated system of variables in such case that it can be reasonable supposed to have arisen from random sampling. Philosophical Magazine **50**: 157-175.

Pezzuto, J.M. (1997). Plant-derived anticancer agents. Biochemical Pharmacology **53**: 121-133.

Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J. and Skarnes, W.C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature **407**: 535-538.

Polanowski, A., Cieslar, E., Otlewski, J., Nienartowicz, B., Wilimowska-Pelc, A. and Wilusz, T. (1987). Protein inhibitors of trypsin from the seeds of Cucurbitaceae plants. Acta Biochimica Polonica **34**: 395-406.

Ponder, B.A.J. (2001). Cancer genetics. Nature **411**: 336-341.

Ready, M.P., Adams, R.P. and Robertus, J.D. (1984). Dodecandrin, a new ribosome-inhibiting protein from *Phytolacca dodecandra*. Biochimica et Biophysica Acta **791**: 314-319.

Reeves, P.A. and Olmstead, R.G. (1998). Evolution of novel morphological and reproductive traits in a clade containing *Antirrhinum majus* (Scrophulariaceae). American Journal of Botany **85**: 1047-1056.

Saether, O., Craik, D.J., Campbell, I.D., Sletten, K., Juul, J. and Norman, D.G. (1995). Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide Kalata B1. Biochemistry **34**: 4147-4158.

Samuelsson, G. (1966). Screening of plants of the family Loranthaceae for toxic proteins. Acta Pharmaceutica Suecica **3**: 353-362.

Samuelsson, G. (1969). Screening of plants of the family Loranthaceae for toxic protiens. II. Acta Pharmaceutica Suecica **6**: 441-446.

Samuelsson, G. (1973). Mistletoe toxins. Systematic Zoology **22**: 555-569.

Samuelsson, G., Borsub, L., Jayawardene, A.L., Falk, L., Ziemilis, S. and Nilsson, Ö. (1981). Screening of plants of the families Loranthaceae and Viscaceaee for toxic proteins. Part III. Acta Pharmaceutica Suecica **18**: 179-184.

Schwartsmann, G. (2000). Marine organisms and other novel natural sources of new cancer drugs. Annals of Oncology **11**: 235-243.

Schöpke, T., Agha, M.I.H., Kraft, R., Otto, A. and Hiller, K. (1993). Hämolytisch aktive Komponenten aus *Viola tricolor* L. und *Viola arvensis* Murray. Scientia Pharmaceutica **61**: 145-153.

Sikic, B.I. (1991). Anticancer drug discovery. Journal of the National Cancer Institute **83**: 738-740.

Socialstyrelsen (2004). Published on the Internet http://www.sos.se/sos/statisti.htm [accessed 1 March 2005] Statistics - health and diseases 2004:10. Cancer incidence in Sweden 2003.

Sogin, M.L. (1991). Early evolution and the origin of eukaryotes. Current Opinion in Genetics and Development **1**: 457-463.

Stenkvist, B. (1999). Is digitalis a therapy for breast carcinoma? Oncol Rep **6**: 493-496.

Stähelin, H. (1970). 4'-Demethyl-epipodophyllotoxin thenylidene glucoside (VM 26), a podophyllum compound with a new mechanism of action. European Journal of Cancer **6**: 303-311.

Svangård, E., Göransson, U., D, D.S., Verma, C., Backlund, A., Bohlin, L. and Claeson, P. (2003). Primary and 3-D modelled structures of two cyclotides from Viola odorata. Phytochemistry **64**: 135-142.

Svangård, E., Göransson, U., Hocaoglu, Z., Gullbo, J., Larsson, R., Claeson, P. and Bohlin, L. (2004). Cytotoxic cyclotides from *Viola tricolor*. Journal of Natural Products **67**: 144-147.

Svangård, E., Göransson, U., Smith, D., Verma, C., Backlund, A., Bohlin, L. and Claeson, P. (2003). Primary and 3-D modelled structures of two cyclotides from Viola odorata. Phytochemistry **64**:135-142.

Talbäck, M., Stenbeck, M., Rosen, M., Barlow, L. and Glimelius, B. (2003). Cancer survival in Sweden 1960-1998--developments across four decades. Acta Oncologica **42**: 637-659.

Tam, J.P., Lu, Y.-A., Yang, J.-L. and Chiu, K.-W. (1999). An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. Proceedings of the National Academy of Sciences, USA **96**: 8913-8918.

Tanaka, W. (1977). Development of new bleomycins with potential clinical utility. The Japanese Journal of Antibiotics **30**: S41-S48.

Tarbell, A.T. and Tarbell, D.S. (1982). Helen Abbott Michael: Pioneer in Plant Chemistry. Journal of Chemical Education **59**: 548-549.

Tower, D.B., Peters, E.L. and Curtis, W.C. (1963). Guinea pig serum L-asparaginase. The Journal of Biological Chemistry **238**: 983-993.

Wall, M.E., Wani, M.C., Cook, C.E. and Palmer, K.H. (1966). Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. Journal of the American Chemical Society **88**: 3888-3890.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P. and McPhail, A.T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. Journal of the American Chemical Society **93**: 2325-2327.

Weinstein, J.N., Kohn, K.W., Grever, M.R., Viswanadhan, V.N., Rubinstein, L.V., Monks, A.P., Scudiero, D.A., Welch, L., Koutsoukos, A.D., Chiausa, A.J. and Paull, K.D. (1992). Neural computing in cancer drug development: Predicting mechanisms of action. Science **258**: 447-451.

Verpoorte, R. (1998). Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. Drug Discovery Today **3**: 232-238.

Verweij, J. and de Jonge, M.J.A. (2000). Achievements and future of chemotherapy. European Journal of Cancer **36**: 1479-1487.

Winterfel, K. and Bijl, L.H. (1948). Viscotoxin ein neuer Inhaltstoff der Mistel (*Viscum album* L.). Liebigs Annalen der Chemie **561**: 107-115.

Witherup, K.M., Bogusky, M.J., Anderson, P.S., Ramjit, H., Ransom, R.W., Wood, T. and Sardana, M. (1994). Cyclopsychotride A, a biologically active, 31 residue cyclic peptide isolated from *Psychotria longipes*. Journal of Natural Products **57**: 1619-1625.

Wong, C.F. and LaLonde, R.T. (1975). 6- and 6'-hydroxyneothiobinupharidine. Monohemiaminals from *Nuphar luteum* and their possible role in thiasparine nuphar alkaloid biogenesis. Experientia **31**: 15-16.

Vuorela, P., Leinonen, M., Saikku, P., Tammela, P., Rauha, J.-P., Wennberg, T. and Vuorela, H. (2004). Natural products in the process of finding new drug candidates. Current Medicinal Chemistry **11**: 1375-1389.

Yang, L., Weiss, T.M., Lehrer, R.I. and Huang, H.W. (2000). Crystallization of antimicrobioal pores in membranes: magainin and protegrin. Biophysical Journal **79**: 2002-2009.

Ye, X.Y. and Ng, T.B. (2001). Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen. Journal of Peptide Research **57**: 330-336.

Yeh, J.Y., Huang, W.J., Kan, S.F. and Wang, P.S. (2001). Inhibitory effects of digitalis on the proliferation of androgen dependent and independent prostate cancer cells. The Journal of Urology **166**: 1937-1942.

# Appendix A

List of all species included in Papers **I–V** arranged alphabetically, familial and ordinal assignments for angiosperms according to APG2 (2003).

| ***Species*** | **Family** | **Order** |
|---|---|---|
| *Acalypha diversifolia* | Euphorbiaceae | Malpighiales |
| *Achillea millefolium* | Asteraceae | Asterales |
| *Actaea alba* | Ranunculaceae | Ranunculales |
| *Allium sativum* | Alliaceae | Asparagales |
| *Alluaudia humbertii* | Didiereaceae | Caryophyllales |
| *Alnus glutinosa* | Betulaceae | Fagales |
| *Aloe lateritia* | Alliaceae | Asparagales |
| *Andrographis paniculata* | Acanthaceae | Lamiales |
| *Anemone canadensis* | Ranunculaceae | Ranunculales |
| *Anthriscus silvestris* | Apiaceae | Apiales |
| *Aphelandra flava* | Acanthaceae | Lamiales |
| *Arctostaphylus uva-ursi* | Ericaceae | Ericales |
| *Arnica mollis* | Asteraceae | Asterales |
| *Asclepias tuberosa* | Apocynaceae | Gentianales |
| *Asperula tinctoria* | Rubiaceae | Gentianales |
| *Betula verrucosa* | Betulaceae | Fagales |
| *Boswellia frereana* | Burseraceae | Sapindales |
| *Brassica rapa* | Brassicaceae | Brassicales |
| *Bunias orientalis* | Brassicaceae | Brassicales |
| *Caesalpinia benthamianum* | Fabaceae | Fabales |
| *Carica papaya* | Caricaceae | Brassicales |
| *Chassalia kolly* | Rubiaceae | Gentianales |
| *Cinchona pubescens* | Rubiaceae | Gentianales |
| *Coffea arabica* | Rubiaceae | Gentianales |
| *Colchicum autumnale* | Colchicaceae | Liliales |
| *Commiphora guidotii* | Burseraceae | Sapindales |
| *Commiphora myrrha* | Burseraceae | Sapindales |
| *Convolvulus arvensis* | Convolvulaceae | Solanales |
| *Corylus avellana* | Betulaceae | Fagales |
| *Crambe maritima* | Brassicaceae | Brassicales |
| *Cytisis scoparius* | Fabaceae | Fabales |
| *Digitalis lanata* | Plantaginaceae | Lamiales |
| *Digitalis purpurea* | Plantaginaceae | Lamiales |
| *Elymus repens* | Poaceae | Poales |
| *Erica carnea* | Ericaceae | Ericales |
| *Euphorbia hirta* | Euphorbiaceae | Malpighiales |
| *Fumaria officinalis* | Papaveraceae | Ranunculales |
| *Galium album* | Rubiaceae | Gentianales |

| | | |
|---|---|---|
| *Galium aparine* | Rubiaceae | Gentianales |
| *Galium boreale* | Rubiaceae | Gentianales |
| *Galium odoratum* | Rubiaceae | Gentianales |
| *Galium saxatile* | Rubiaceae | Gentianales |
| *Galium verum* | Rubiaceae | Gentianales |
| *Gentiana punctata* | Gentianaceae | Gentianales |
| *Geranium silvaticum* | Geraniaceae | Geraniales |
| *Ginkgo biloba* | Ginkgoaceae | GINKGOPHYTA |
| *Helleborus cyclophyllus* | Ranunculaceae | Ranunculales |
| *Hylocereus* sp. | Cactaceae | Caryophyllales |
| *Ipomoea quamoclit* | Convolvulaceae | Solanales |
| *Isatis tinctoria* | Brassicaceae | Brassicales |
| *Justitia adhatoda* | Acanthaceae | Lamiales |
| *Justitia betonica* | Acanthaceae | Lamiales |
| *Lamium album* | Lamiaceae | Lamiales |
| *Laportea aestuans* | Urticaceae | Rosales |
| *Leonurus cardiaca* | Lamiaceae | Lamiales |
| *Linum austriacum* | Linaceae | Malpighiales |
| *Lonicera chrysanta* (2 extracts) | Caprifoliaceae | Dipsacales |
| *Lycopus virginica* | Lamiaceae | Lamiales |
| *Magnolia kobus* | Magnoliaceae | Magnoliales |
| *Matricaria inodora* | Asteraceae | Asterales |
| *Menyanthes trifoliata* | Menyanthaceae | Asterales |
| *Mitriostigma axillare* | Rubiaceae | Gentianales |
| *Momordica charantia* | Cucurbitaceae | Cucurbitales |
| *Nuphar luteum* | Nymphaeaceae | *Angiosperm root* |
| *Nymphoides peltata* | Menyanthaceae | Asterales |
| *Oenothera* sp. | Onagraceae | Myrtales |
| *Oldenlandia corymbosa* | Rubiaceae | Gentianales |
| *Oryctanthus alveolatus* | Loranthaceae | Santalales |
| *Paeonia potanini* | Paeoniaceae | Saxifragales |
| *Phoradendron acinacifolium* | Viscaceae | Santalales |
| *Phoradendron guascanum* | Viscaceae | Santalales |
| *Phoradendron quadrangulare* | Viscaceae | Santalales |
| *Phthirusa pyrifolia* | Loranthaceae | Santalales |
| *Phthirusa retroflexa* | Loranthaceae | Santalales |
| *Picea abies* | Pinaceae | PINOPHYTA |
| *Plantago major* | Plantaginaceae | Lamiales |
| *Poa pratensis* | Poaceae | Poales |
| *Polypodium vulgare* | Polypodiaceae | PTERIDOPHYTA |
| *Populus tremula* | Salicaceae | Malpighiales |
| *Pteridium aqulinum* | Pteridaceae | PTERIDOPHYTA |
| *Psittacanthus pusillus* | Loranthaceae | Santalales |
| *Reseda luteola* | Resedaceae | Brassicales |
| *Rhamnus* sp. | Rhamnaceae | Rosales |
| *Rheum* x *hybridum* | Polygonaceae | Caryophyllales |
| *Rubia tinctorum* | Rubiaceae | Gentianales |
| *Rubus idaeus* | Rosaceae | Rosales |
| *Saintpaulia ionantha* | Gesneriaceae | Lamiales |
| *Schisandra chinensis* | Schisandraceae | Austrobaileyales |
| *Sedum rosea* | Crassulaceae | Saxifragales |
| *Silene rubrum* | Caryophyllaceae | Caryophyllales |
| *Sorbus aucuparia* | Rosaceae | Rosales |
| *Stachys byzantina* | Lamiaceae | Lamiales |
| *Strobilanthes* sp. | Acanthaceae | Lamiales |

| | | |
|---|---|---|
| *Symphytum asperum* | Boraginaceae | *Euasterids I* |
| *Syringa vulgaris* | Oleaceae | Lamiales |
| *Tabernaemontana dichotoma* | Apocynaceae | Gentianales |
| *Teucrium lucidum* | Lamiaceae | Lamiales |
| *Trifolium pratense* | Fabaceae | Fabales |
| *Urtica dioica* | Urticaceae | Rosales |
| *Valeriana officinalis* | Valerianaceae | Dipsacales |
| *Vinca minor* | Apocynaceae | Gentianales |
| *Viola arvensis* | Violaceae | Malpighiales |
| *Viola cornuta* | Violaceae | Malpighiales |
| *Viola odorata* | Violaceae | Malpighiales |
| *Viola patrinii* | Violaceae | Malpighiales |
| *Viola tricolor* | Violaceae | Malpighiales |
| *Viscum heyneanum* | Viscaceae | Santalales |

Acta Universitatis Upsaliensis

*Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 7

Editor: The Dean of the Faculty of Pharmacy

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

Distribution: publications.uu.se
urn:nbn:se:uu:diva-5728

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2005