DISCOVERY OF POTENTIAL ANTICANCER AND NEUROPROTECTIVE AGENTS FROM NORTH QUEENSLAND PLANTS

Thesis submitted by
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STATEMENT OF THE CONTRIBUTION OF OTHERS

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DECLARATION ON ETHICS

I, the undersigned and author of this work, declare that the research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). Research involving the use of animals followed *The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* and the *Queensland Animal Care and Protection Act 2001*. The proposed research methodology for animal use received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A618_00).

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ABSTRACT

Plants represent a largely untapped resource for drug discovery. There are approximately 25,000 plant species in Australia alone, and 9,000 of these are found in Queensland. This study aimed to discover drug leads from North Queensland plants by screening extracts for pharmacological activity. Two pharmacological targets were selected. The first screen aimed to find novel cytotoxic compounds with potential as anticancer agents. The second screen aimed to find compounds with therapeutic potential in schizophrenia or neurological disorders that involve neuroinflammation and neurodegeneration, such as Huntington's Disease and AIDS-related dementia. Various imbalances in the relative levels of kynurenine pathway metabolites, particularly kynurenic acid, quinolinic acid and 3-hydroxykynurenine, have been implicated in these conditions. In order to discover potential drug leads that might be applied to rectify these imbalances, the extracts were screened for inhibition of three key enzymes of this pathway, namely kynurenine-3-hydroxylase, kynureninase and kynurenine aminotransferase.

Samples (365) from 125 species of plants from North Queensland were collected and extracted. After removal of tannins to prevent interference with the assays, the plant extracts were screened for cytotoxicity and for enzyme inhibition. Cytotoxicity was assessed *in vitro* using the P388D1 mouse lymphoma cell line. The enzyme inhibition assays involved the use of crude enzyme preparations from rat liver or kidney, and products were quantified by HPLC with electrochemical or fluorescence detection. Extracts with the greatest kynurenine-3-hydroxylase inhibition and selected cytotoxic extracts were subjected to bioassay-guided fractionation in order to isolate and identify the active compounds. No extracts possessed sufficient kynureninase or kynurenine aminotransferase inhibition to warrant investigation.

Three cytotoxic quinonemethide triterpenes were isolated from *Maytenus cunninghamii* (Celastraceae). Netzahualcoyoic acid (**25**, IC_{50} value = 0.12 μM, 0.11–0.13 μM 95% CI range) and Δ^{15}-celastrol (**32**, IC_{50} value not determined) are new structures, while celastrol

(**22**, IC_{50} value = 0.37 μM, 0.30–0.45 μM 95% CI range) is a known cytotoxic agent. Δ^{15}-celastrol represents a possible biosynthetic intermediate between celastrol and netzahualcoyoic acid. Further work is needed to assess the cytotoxic profile of netzahualcoyoic acid and its acid-rearranged products in the National Cancer Institute's 60 cell line panel.

The known cytostatic compounds podophyllotoxin (**36**), deoxypodophyllotoxin (**37**) and picropodophyllotoxin (**38**) were isolated from *Callitris intratropica* (Cupressaceae). This is the first reported isolation of podophyllotoxins from this species.

Bioassay-guided fractionation of kynurenine-3-hydroxylase inhibitory extracts led to the isolation of two new triterpenes, 11α,28-dihydroxylupenone (**43**) and 2α,3β-dihydroxyfriedelan-29-oic acid (**53**), from *Maytenus disperma*, as well as two new 24-oxomaytenonic acids (**58** and **60**) and four known triterpenes from *M. cunninghamii*. A biosynthetic pathway was proposed for compounds isolated from *M. cunninghamii* and related compounds. Five other species afforded a total of five additional known triterpenoids.

Triterpenes were identified as a new class of potent and selective competitive inhibitors of kynurenine-3-hydroxylase. The most active was uncaric acid (**78**, K_{ic} = 0.023 ± 0.002 μM), isolated from *Dolichandrone heterophyllum* (Bignoniaceae), and it is one of the most potent kynurenine-3-hydroxylase inhibitors that has been reported. The next most active inhibitors of this study were the 24-oxofriedelan-29-oic acids (**60**, K_{ic} = 0.061 ± 0.005 μM; **62**, K_{ic} = 0.077 ± 0.011 μM; **58**, K_{ic} = 0.12 ± 0.02 μM) and celastrol (**22**, K_{ic} = 0.14 ± 0.03 μM). Important structure-activity relationships relating to triterpene skeleton, functional groups, and ring conformations were observed. It is proposed that the triterpene inhibitors would make ideal drugs for the treatment of many neuroinflammatory and neurodegenerative disorders, and that uncaric acid, 2α-hydroxy-24-oxomaytenonic acid (**60**) and celastrol should be investigated *in vivo*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

[I]	inhibitor concentration
[S]	substrate concentration
1D	one dimensional
2D	two dimensional
3HA	3-hydroxyanthranilic acid
3HAO	3-hydroxyanthranilic acid oxidase
3HK	3-hydroxykynurenine
AA	anthranilic acid
AD	Alzheimer's Disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
aq	aqueous
br d	broad doublet
br s	broad singlet
BRI	Herbarium of Queensland, Brisbane
BSA	bovine serum albumin
C_5D_5N	deuterated pyridine
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane
CH_3COOH	acetic acid
$CHCl_3$	chloroform
CI	confidence interval
CNS	central nervous system
CSF	cerebrospinal fluid
CSIRO	Commonwealth Scientific and Industrial Research Organisation
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
DEPT	Distortionless Enhancement by Polarisation Transfer
dm	doublet of multiplets
DMSO	dimethylsulfoxide
EAAs	excitatory amino acids
EDTA	ethylenediaminetetraacetic acid
ESI-MS	Electrospray Ionisation Mass Spectrometry
EtOAc	ethyl acetate
EtOH	ethanol

FCS	foetal calf serum
*g*COSY	gradient Correlated Spectroscopy
*g*HMBC	gradient Heteronuclear Multiple-Bond Coherence
*g*HMQC	gradient Heteronuclear Multiple-Quantum Coherence
*g*HSQC	gradient Heteronuclear Single-Quantum Coherence
HD	Huntington's Disease
HPLC	High-Performance Liquid Chromatography
IC_{50}	with respect to cytotoxicity: defined as the concentration of compound that inhibits cell-replication by 50% relative to the control
IC_{50}	with respect to enzyme inhibition: defined as the concentration of inhibitor that reduces the formation of product by 50% relative to the control
i.d.	internal diameter
IDO	indoleamine-2,3-dioxygenase
IR	Infrared
J	coupling constant
JCT	Herbarium of James Cook University
JCU	James Cook University
KAT	kynurenine aminotransferase
K_{ic}	the specific (competitive) inhibition constant
K_{iu}	the catalytic (uncompetitive) inhibition constant
K_m	the concentration of substrate at which the rate of product formation is equal to half the maximum, or limiting, rate of product formation(V)
K_m^{app}	the apparent K_m in the presence of an inhibitor
KYN	L-kynurenine
KYNA	kynurenic acid
m	multiplet
MeCN	acetonitrile
MeOH	methanol
min	minutes
mNBA	(*m*-nitrobenzoyl)alanine (**13**)
mp	melting point
NAD	β-nicotinamide adenosine dinucleotide
NADPH	β-nicotinamide adenine dinucleotide phosphoric acid
NAL	nicotinylalanine (**11**)
NCI	National Cancer Institute, Washington DC
NMDA	*N*-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
nOe	nuclear Overhauser effect

NOESY	Nuclear Overhauser Effect Spectroscopy
OD	optical density
oMBA	(*o*-methoxybenzoyl)alanine (**12**)
PCP	phencyclidine (**1**)
PD	Parkinson's Disease
PDA	photodiode-array
PLP	pyridoxal-5'-phosphate
q	quartet
QUIN	quinolinic acid
s	singlet
SAR	structure-activity relationship
sp.	species (singular)
spp.	species (plural)
SRB	sulforhodamine B
syn.	synonym
t	triplet
TCA	trichloroacetic acid
td	triplet of doublets
TDO	tryptophan-2,3-dioygenase
Tris	tris(hydroxymethyl)aminomethane
UV	Ultraviolet
v br	very broad
V	the maximum, or limiting, rate of product formation
v	the measured rate of product formation
V^{app}	the apparent maximum rate of product formation in the presence of an inhibitor
var.	variety
Vis	Visible light

CHAPTER 1.

INTRODUCTION

1.1 MEDICINAL PLANTS AND DRUG DISCOVERY

1.1.1 History of Medicinal Plant Use

Plants and medicine have been intricately linked throughout human history. The oldest evidence exists from a 60,000 year-old Neanderthal burial in Shanidar Cave, Iraq, where pollen from several plant taxa were found distributed in a manner that suggested these plants were placed there for ritual purposes[1,2]. The taxa that were identified, such as *Achillea* sp. (yarrow) and *Ephedra altissima*, are all medicinal plants known both locally and throughout Europe, suggesting that these plants may have been used medicinally by the people of the time[2,3].

The use of medicinal plants by animals implies modern humans and ancestors have also employed them throughout the ages. In fact, many of the medicinal plants used by chimpanzees are also used by people in traditional medicine. One such plant, *Vernonia amygdalina*, is used in traditional human medicine across Africa for a range of complaints including malarial fever, stomach upset, amoebic dysentery and other disorders caused by intestinal parasites[4]. Chimpanzees displaying symptoms of parasitic infection have been observed to chew the bitter pith of *V. amygdalina* and fully recover within 24 hours[5,6]. This plant contains sesquiterpene lactones and steroid glucosides which are active against *Schistosoma*, *Plasmodium* and *Leishmania*, suggesting that the chimpanzees may chew the pith to rid themselves of internal parasites[7]. Chacma baboons have been observed eating small amounts of plants known to be poisonous and hallucinogenic, including two *Datura* species[8]. It is unclear whether they use this to combat parasites or for more scandalous

reasons. European starlings incorporate fresh material of plants containing high levels of biocidal volatile compounds into their nest, possibly to fumigate the nest to reduce the parasite load on their chicks[9]. In eastern Africa, one particular tree of the Boraginaceae is used by the local people to induce labour[10]. This knowledge may not be restricted to humans, as a pregnant elephant has been observed eating unusually large quantities of this tree prior to birth[10]. Indeed, many traditional societies have gained knowledge of medicinal plants by watching animals.

The earliest writings about medicinal plants were engraved onto clay tablets by the Sumerians up to 5,000 years ago[11]. Much of western herbal medicine is based on the ancient scholarship of the Middle East and Greece. One of the oldest texts is the Ebers papyrus written in the 16th century BC that describes many remedies involving herbs such as myrrh, frankincense, senna, willow, opium and laudanum[12]. Significant Greek texts describing hundreds of medicinal plants include those of Hippocrates and Theophrastus in the 4th century BC and Dioscorides in the 1st century AD[13]. It seems that the knowledge of medicinal plants of all great ancient civilisations were influenced by those proceeding them. Egyptian medicine appears to have influenced many civilisations down the years, through Assyrian, Greek, Roman, and Arab to European medicines[12,14].

Ayurvedic, which also influenced Greek medicine[14], and Chinese medicine are other significant ancient systems employing extensive use of medicinal plants. Over 11,000 plant taxa are used medicinally in China[15], while almost 5,000 plants species are used in the Indian subcontinent for Ayurvedic and other traditional medicines[16].

The majority of the world's inhabitants still live traditionally or do not have access to modern western medicine and drugs. An estimated 75–80% of the world's population rely on traditional medicine, predominantly from plants, for their primary healthcare needs[17,18]. Even in western countries, traditional herbal medicine is still a very important source of

healthcare and is in a resurgence. Traditional medicinal plants were the source of the first organic compounds to be used as drugs, and an immense number have been discovered from them since. Plants still represent a largely untapped, almost unlimited resource for drug discovery.

1.1.2 Drug Discovery from Plants

Plants have been a source for the discovery of single-ingredient drugs since the beginning of the 19th century when morphine was first isolated from the opium poppy, *Papaver somniferum*[13]. By the middle of the 19th century, many other important drugs had been isolated from plants, including quinine, berberine, colchicine, hyoscyamine, and salicin[13]. Two hundred years after the first isolation of morphine, plants and other biological organisms still provide a significant resource for drug discovery. Plant-derived drugs make up approximately 25% of prescription drugs worldwide[19] and only a small proportion of the estimated 250,000 plant species have been chemically investigated[20]. Despite other avenues of drug discovery by rational chemical synthesis, combinatorial chemistry, and emerging therapies such as cell delivery of small interfering RNAs, a huge potential for drug discovery from plants still exists. Natural products provide superior structural diversity to combinatorial chemistry and are much more likely to be biologically active[21,18]. In addition, they are often small and have favourable pharmacokinetic properties[21].

Natural products from plants and other biological organisms are normally present with a number of structurally related compounds. Assessment of the pharmacological activity of related compounds can provide valuable information regarding structure-activity relationships (SARs). This allows the selective development of analogues that may demonstrate improved efficacy, selectivity, or pharmacokinetics over the lead compounds. Even if little is known about the SARs, improved derivatives may be discovered by

developing and testing a large number of analogues of a lead compound. In the very least, this will provide missing information about SARs, which may allow more selective development of analogues. Natural product drug derivatives have been developed since the 19th century. Acetylsalicylic acid (aspirin) was released as an alternative to the natural product salicylic acid, which can cause severe gastric distress[22,23]. Heroin is one of the more sinister natural product derivatives that have been developed. Ironically, it was released in 1898 as a less addictive analogue of morphine and was only later realised to be highly addictive[23]. Recently, drugs that were derived from or modelled on natural products accounted for 33% of the 520 new drugs approved in 1983–1994 by the United States Food and Drug Administration and equivalent entities worldwide[24].

1.1.3 Strategies for Drug Discovery from Plants

There are two main approaches for drug discovery from plants. One approach is the chemical investigation of a targeted plant species or herbal extract. The choice of species or extract for investigation can be based on a number of criteria such as its traditional use, chemotaxonomy, toxicity, or ecological observations. Traditional medicinal plants are often investigated in order to isolate and identify the compounds responsible for the activity that is attributed to the plant.

The second approach is the screening of plant extracts for activity in a specific pharmacological assay, such as inhibition of a particular enzyme. There are three common strategies for the selection of plant species to be included in a screen. Plants may be chosen at random, particularly for the large scale screens performed by large companies. Alternatively, in smaller screens, selection may be restricted to plants that have been used traditionally for a specific medicinal use that is pertinent to the pharmacological target. For

example, Bernard and co-workers[25] screened 62 traditional anti-inflammatory plants from South America for inhibition of phospholipase A_2, a target implicated in inflammation.

The third strategy, and the one taken in this study for the selection of plants in pharmacological screens, is a compromise between random selection and selection based on a specific medicinal use. In this strategy, only plants that are likely to be pharmacologically active are included for screening based on certain selection criteria. Thus, restricting screening to plants that are likely to be pharmacologically active should increase the number of active extracts in the screen. The selection criteria may be based on information regarding the medicinal use, chemotaxonomy, toxicity, ecology, or other relevant properties of the plants. Medicinal plants are often chosen for screening purely on the basis that they have been used medicinally, regardless of what conditions or diseases they have been used to treat. Chemotaxonomic information is used to select plants belonging to a genus or family that has produced pharmacologically important compounds. *Callitris intratropica* was one of the plants collected for screening in this study for chemotaxonomic reasons, since the potent cytostatic agents, podophyllotoxins, have been isolated from other *Callitris* species[26,27]. One drawback of this chemosystematic approach is that the bioactive compounds in the selected taxa are more likely to have been previously reported. Toxic plants are selected on the premise that while the active compounds in these plants are toxic at higher doses, they may act as selective drugs at lower doses. The toxicity itself may even be desirable if the pharmacological screen is for cytotoxicity or antimicrobial agents. Following this rationalisation, many plant species were collected in this study because they have been reported as stock poisons. Ecological observations, such as the observation that a particular plant has suffered no insect attack in an area suffering from heavy herbivorous insect infestation, may indicate the presence of insecticidal, cytotoxic or antifungal compounds. Such observations are perhaps more common in benthic marine organisms. Consequently, few plants were collected in this study based on ecological observations.

1.2 ANTICANCER DRUG DISCOVERY

In 2000 there were over 3.5 million reported deaths from cancer worldwide[28]. Cancer is the leading cause of death in Australia and accounted for 29% of deaths in 2001[29]. Most currently used anticancer drugs do not act selectively on cancerous cells and produce severe side effects, such as nausea, vomiting, immunosuppression, hepatotoxicity, nephrotoxicity, neurotoxicity and secondary. Therefore, there has been a strong emphasis on the discovery and development of selective chemotherapeutic agents. Many cancer-specific molecular targets have been investigated in an attempt to find selective anticancer agents. Another approach is that taken by the National Cancer Institute (NCI) in Washington DC, which screens for cytotoxicity against 60 different cancer cell lines to find compounds that are substantially more cytotoxic to some cell lines than others[30]. The mode of action of these selectively cytotoxic compounds would likely be specific for the cell lines affected, so that the compounds would be less likely to considerably affect other cells and therefore have fewer adverse effects *in vivo*. Observed cell line selectivity may also be utilised as an indicator for mode of action, by comparing cell line selectivity with the selectivity of other cytotoxic agents whose mode of action is known.

1.2.1 Screening Plant Extracts for Anticancer Activity

Plants and other biological organisms represent an important source for anticancer drug discovery. As of 1994, 54 of the 87 approved anticancer drugs were natural products, derived from natural products, or modelled on natural products[24].

For decades, the common method for screening both natural products and synthetic compounds for anticancer activity involved *in vivo* tests on tumour-bearing mice, and this

was undertaken on a large scale by the NCI[31]. Some culture methods were also in use for assessing cytotoxicity prior to *in vivo* testing. One of these methods assessed the toxicity of compounds and extracts against brine shrimp, *Artemia salina*[32]; while another method tested compounds and extracts for their ability to inhibit the formation of crown gall tumours, caused by the bacterium *Agrobacterium tumefaciens*, in potato-disc cultures[33]. However, mammalian cell culture methods have become common. These involve testing compounds and extracts for cytotoxicity against mammalian cancer cells, usually human or murine, *in vitro* using microtitre plates and often utilise robotic automation for high-throughput screening. By 1990, the NCI had established a 60 cell line panel to rapidly assess the cytotoxic profile of compounds and extracts against a number of different types of human cancer[30]. *In vitro* pharmacological assays have also been developed that target specific molecular mechanisms of cell replication, cancerous cells, and tumours. These include inhibition assays for DNA polymerases[34-36], DNA topoisomerases[37-39] and growth factor receptor tyrosine kinases[40,41]. Some other pharmacological assays target inhibition of angiogenesis to 'starve' tumour cells[42,43], and inhibition of cell adhesion to prevent metastasis[44,45].

One of the most important anticancer agents that was discovered by screening plant extracts for anticancer activity is taxol, which was isolated from the pacific yew tree, *Taxus brevifolia*, during the NCI's *in vivo* screening program. It is currently in clinical use for a number of cancers such as breast, colon and non-small cell lung cancers[46]. Another important compound discovered in the NCI's *in vivo* screening program is camptothecin, which was isolated from *Camptotheca acuminata*. Two derivatives of camptothecin are in clinical use for the treatment of metastatic colorectal, metastatic ovarian and primary colon cancers, while many other camptothecin derivatives are currently in clinical trials[46].

1.2.2 Selection of the Cytotoxicity Screening Assay

Part of this study involves screening extracts of plants from North Queensland for cytotoxicity in order to isolate and identify new cytotoxic compounds. The P388D1 mouse lymphoma cell line was selected because it is sensitive to cytotoxic agents, easily handled, has a doubling time of only 16 hours, and is a standard cell line used for initial assessment of cytotoxicity. The cytotoxicity assay was based on the tried and tested method employed by the NCI[30], which uses a sulforhodamine B (SRB) colourimetric assay for determination of cell density[47]. This simple protein staining assay is more sensitive than other macromolecular staining methods[47]. It is superior to the MTT and XTT microculture tetrazolium assays that measure the metabolic activity of the cells[30]. Once the 96-well plates are fixed and stained in the SRB assay, they can be stored at 4°C for a long period of time before the optical density (OD) is measured, whereas the OD of the 96-well plates in the microculture tetrazolium assays must be measured within a very narrow window of time. In addition, the SRB assay is less time-consuming than the MTT assay and more sensitive than the XTT assay.

SRB is an anionic protein stain that binds to basic amino acid residues under mildly acidic conditions and can be solubilized by the addition of a weak base in order to measure the OD[47]. In the SRB assay, the OD is outside the range of linearity when measured at the λ_{max} of SRB (564 nm). Therefore, the OD must be measured at a suboptimal wavelength. When measured at a wavelength between approximately 490–530 nm, the OD is linear with cell density and there is still a sufficient signal-to-noise ratio[47]. The wavelength chosen for this study was 492 nm, since the 96-well plate reader available was already fitted with a filter for this wavelength.

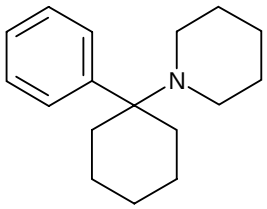
1.3 NEURODEGENERATIVE DISORDERS AND THE KYNURENINE PATHWAY

1.3.1 Excitatory Amino Acids and Excitotoxicity

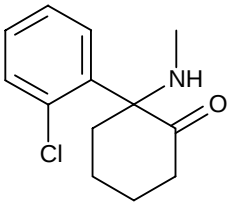
Glutamate and other excitatory amino acids (EAAs) are responsible for fast synaptic transmission between neurons, and are also important for the formation and plasticity of synapses in the developing and mature brain[48]. The excitatory effect of EAAs on postsynaptic neurons is mediated by three ion channel-coupled receptors, which are named according to their selective agonists, *N*-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)[49]. Overexcitation of these receptors leads to excitotoxicity, which is characterised by an excessive influx of Ca^{2+} followed by cell injury and cell death[49]. Excitotoxicity can lead to neuroinflammation, neurodegeneration and neural lesions. NMDA receptor-mediated excitotoxicity, in particular, has been implicated in epilepsy[50], ischaemia[51], and many neurodegenerative disorders such as Huntington's Disease[52] and AIDS-related dementia[53].

1.3.2 Binding-Sites on the NMDA Receptor

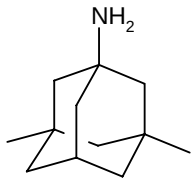
Antagonists of NMDA receptors, such as some of those in Figure 1.1, are in clinical trials or clinical use for the treatment of a number of conditions, including epilepsy, traumatic brain injury, stroke, Parkinson's disease, amyotrophic lateral sclerosis (motor-neuron disease) and chronic pain (see Muir and Lees[54]). NMDA receptors have at least five recognised binding-sites: a site where phencyclidine (PCP) and related drugs bind, a Mg^{2+} binding-site, a polyamine binding-site, a glutamate binding-site and a glycine binding-site (Figure 1.2).



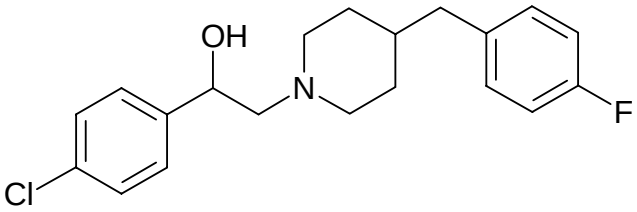
Phencyclidine (PCP) **1**



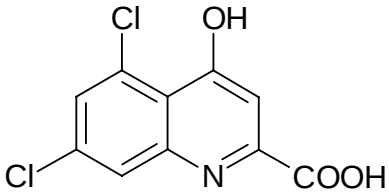
Ketamine **2**



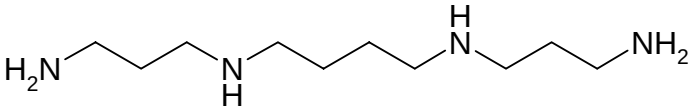
Memantine **3**



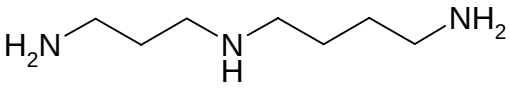
Eliprodil **4**



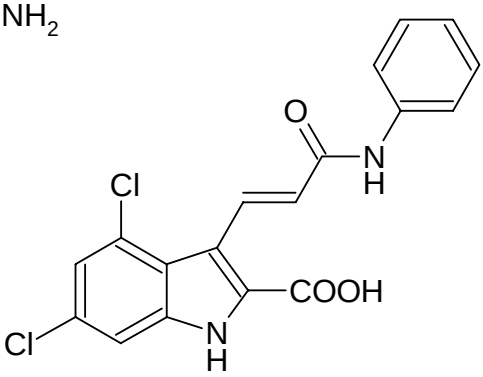
5,7-Dichlorokynurenic acid **7**



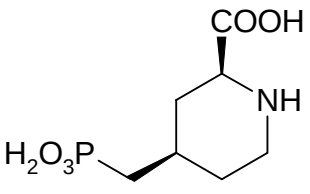
Spermine **5**



Spermidine **6**



Gavestinel **8**



Selfotel **9**

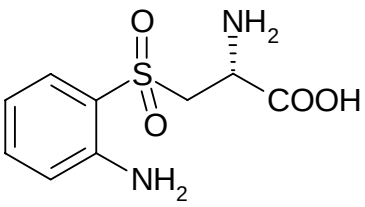


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Figure 1.1. Structures of NMDA receptor antagonists (**1**-**9**) and a kynureninase inhibitor (**10**). **1**–**3** are ion channel blockers; **4** is an antagonist of the polyamine binding-site; **5** and **6** are endogenous antagonists of the polyamine binding-site; **7** and **8** are antagonists of the glycine binding-site; and **9** is an antagonist of the glutamate binding-site.

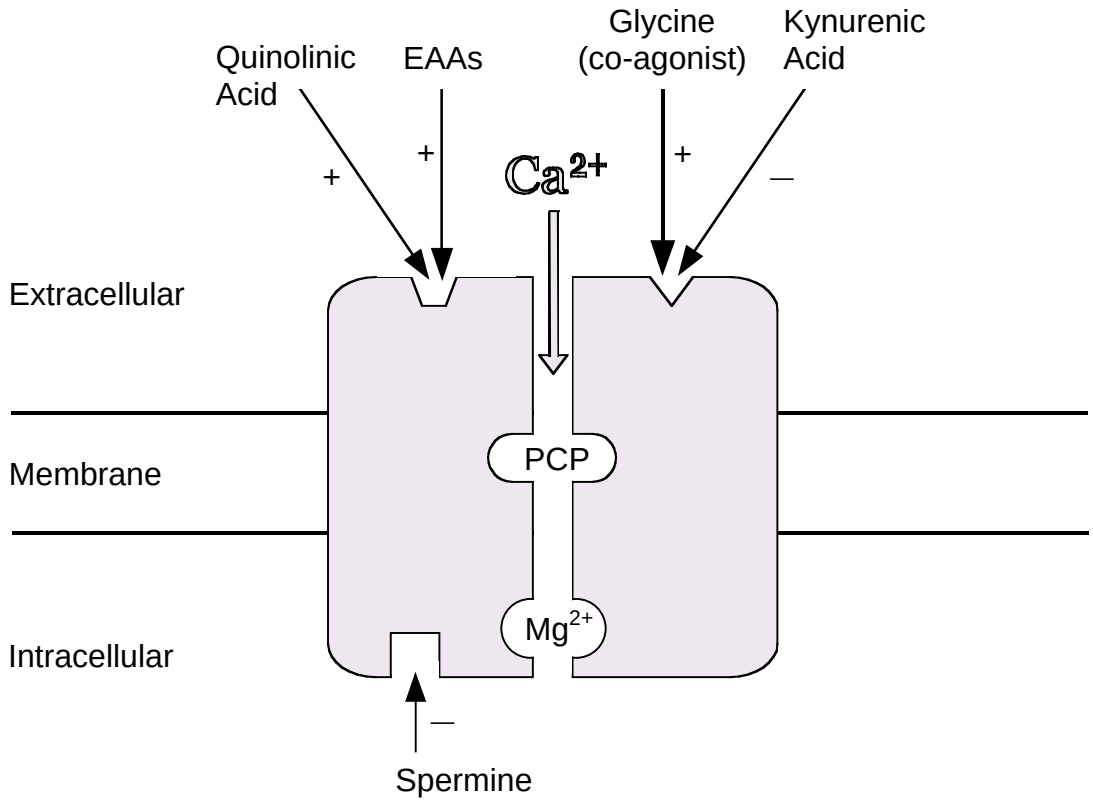


Figure 1.2. Stylised diagram of the ion channel-coupled NMDA receptor showing binding sites. Also shown are examples of agonists (+) and antagonists (-) of the glutamate, glycine and polyamine binding-sites, and the channel blockers Mg^{2+} and PCP (phencyclidine). All substances shown are endogenous with the exception of PCP. Adapted from Muir and Lees[54].

PCP (**1**) and related drugs bind to a site in the ion channel of the NMDA receptor (reviewed by Muir and Lees[54]). PCP was developed as an anaesthetic but is now used mainly for psychiatric research. It produces euphoria and potent analgesia, followed by hallucinations, agitation, paranoia and other schizophrenic symptoms. Some of these actions of PCP have resulted in its use as the recreational drug known as 'angel dust'. Drugs that block the ion channel often have undesirable side effects. Memantine (**3**), for example, is an effective neuroprotectant and is used to treat Huntington's disease and spasticity, but it has long-term side effects that include vertigo, agitation and fatigue.

Mg^{2+} binds at a second binding-site in the ion channel of the NMDA receptor. Physiological relevant concentrations of Mg^{2+} ions are able to bind to this site when the neuron is depolarised and block the ion channel of the NMDA receptor[48]. Administration of Mg^{2+} causes few side effects and it is currently in clinical trials for stroke[55,56].

Spermine (**5**) and spermidine (**6**) are two endogenous polyamines that are produced during amino acid biosynthesis and are antagonists of the polyamine binding-site[54]. Eliprodil (**4**) is a synthetic agent that also binds to the polyamine site and entered clinical trials for stroke and traumatic brain injury, but displayed unacceptable adverse effects[55].

Glycine is a co-agonist of NMDA receptors and is necessary for activation of the NMDA receptor, but is saturating at physiological levels. The glycine binding-site is also the binding-site for the endogenous antagonist, kynurenic acid (KYNA), which is a metabolite of tryptophan. Many KYNA analogues, such as 5,7-dichlorokynurenic acid (**7**), have been developed that are antagonists of the glycine binding-site[53]. Glycine-site antagonists are more advantageous than ion channel blocking drugs since they tend to have a greater therapeutic window between desirable actions and adverse effects[57]. Gavestinel (**8**) is a highly potent antagonist of the glycine site that showed only minor side effects in clinical trials for stroke, but these were discontinued due to lack of efficacy[55]. Similar efficacy

problems of other NMDA receptor antagonists in clinical trials for stroke and traumatic brain injury has led to the conclusion that NMDA receptor antagonists are more likely to be effective for chronic neurodegeneration rather than acute insults[55,58].

Glutamate and other EAAs are the prominent agonists of the glutamate binding-site, but the tryptophan metabolite quinolinic acid (QUIN) is also an agonist of this site. This has important implications in disorders that involve an imbalance in tryptophan metabolism. Selfotel (**9**) is an antagonist of the glutamate binding-site that entered clinical trials for stroke and traumatic brain injury, but these were discontinued due to adverse side effects[55].

1.3.3 The Kynurenine Pathway of Tryptophan Metabolism

One of the important routes of tryptophan metabolism results in the production of serotonin (5-hydroxytryptamine, 5-HT) and melatonin[59]. It is the kynurenine pathway, however, that accounts for the vast majority of tryptophan metabolism[60]. This pathway proceeds via the production of L-kynurenine (KYN), quinolinic acid (QUIN) and nicotinamide and results in the synthesis of the ubiquitous enzyme cofactor, nicotinamide adenosine dinucleotide (NAD) (Figure 1.3)[59]. An important side product of this pathway is the NMDA receptor antagonist and neuroprotectant, kynurenic acid (KYNA).

Tryptophan is metabolised by either indoleamine-2,3-dioxygenase (IDO) or tryptophan-2,3-dioxygenase (TDO) to *N*-formylkynurenine, which is converted to KYN by formamidase. Under physiological conditions, most KYN is metabolised by kynurenine-3-hydroxylase to 3-hydroxykynurenine (3HK)[61,62]. KYN is also metabolised by kynurenine aminotransferase (KAT) to KYNA, by decarboxylases to kynuramine, or by kynureninase to anthranilic acid (AA). AA may undergo hydroxylation to 3-hydroxyanthranilic acid (3HA), but the main source of this product is from the metabolism of 3HK by kynureninase. 3HA is then

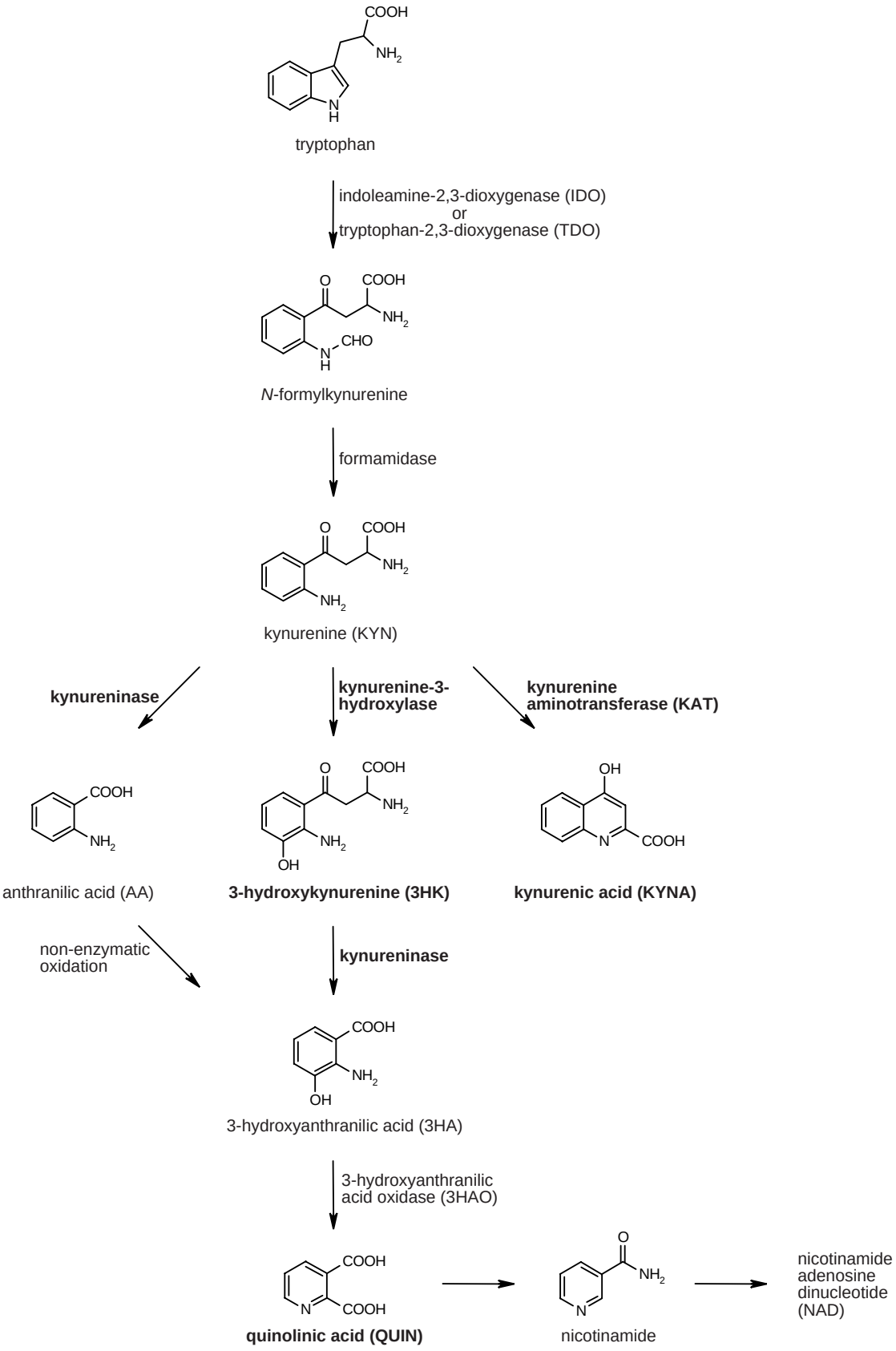


Figure 1.3. Kynurenine pathway of tryptophan metabolism. Only the enzymes responsible for tryptophan metabolism through to 3-hydroxyanthranilic acid (3HA) are given. The names of the metabolites and enzymes that are of particular relevance to this study are in bold text. Adapted from Stone[59].

metabolised by 3-hydroxyanthranilic acid oxidase (3HAO) to QUIN, which is converted to NAD via nicotinamide.

1.3.4 Implications of the Kynurenine Pathway in Neurodegenerative Disorders

An imbalance of the metabolites of the kynurenine pathway has been implicated in a number of disorders. Most of these disorders are associated with an imbalance between QUIN and KYNA. As an agonist of NMDA receptors, QUIN can cause NMDA receptor-mediated excitotoxicity at elevated levels, and may also cause neural toxicity by production of reactive oxygen species[63]. Up-regulation of QUIN also results in elevated levels of the metabolic intermediate 3HK, which can also cause neurotoxicity by production of reactive oxygen species[64].

There is much evidence of altered levels of kynurenine metabolites and altered activities of their metabolic enzymes in neurological disorders. One of these disorders is Huntington's Disease (HD), which is caused by an increased number of polyglutamine repeats in the *huntingtin* gene[65]. QUIN rapidly induces expression of the *huntingtin* gene in rats[66] and produces HD-like symptoms (reviewed by Stone[63]). Although levels of QUIN are not increased in HD patients[67], reduced levels of KYNA have been reported in the cerebrospinal fluid (CSF)[67] and the brain[68]. Consistent with this, HD shows reduced activity of KAT in the brain[68]. Overall, this indicates that the QUIN:KYNA ratio is increased in HD, which likely plays a role in the disease. Elevated levels of 3HK have also been reported[69] and would contribute to the pathology of HD.

There is also evidence for the role of kynurenine metabolites in Alzheimer's Disease (AD) and Parkinson's Disease (PD). As with HD, AD patients have lowered levels of KYNA in the CSF but unaltered levels of QUIN[67]. It has also been demonstrated that the β-amyloid

peptide Aβ1-42 induces QUIN production and IDO expression in macrophages[70], which is typical of the neuroinflammatory process (see below). In PD, which is partly characterised by a loss of dopaminergic neurons in the substantia nigra[71], an elevated level of 3HK has been reported[72]. A link has been demonstrated between nigral dopamine neurons and kynurenine metabolites. Of therapeutic interest to PD, raised KYNA levels caused by administration of a kynurenine-3-hydroxylase inhibitor resulted in activation of dopamine neurons within the substantia nigra[73].

Many neurological disorders involve neuroinflammation, which has a marked effect on kynurenine metabolism in the central nervous system (CNS). Causes of neuroinflammation include ischaemia, trauma, autoimmune diseases and infection[74,60]. These stimuli cause invasion of macrophages into the CNS and activation of microglia[63]. Interferon-γ and other pro-inflammatory cytokines greatly increase the activity of IDO in macrophages and microglia, which are largely responsible for the production of kynurenine metabolites in neuroinflammation[75,60,53]. The flow-on effect of increased IDO activity causes an increase in activity of the kynurenine pathway enzymes, particularly kynurenine-3-hydroxylase and kynureninase[75]. Macrophages and microglia have very low KAT activity, however, and the increase in IDO activity results in a disproportionate increase in the neurotoxic kynurenine metabolites[60]. For example, a 10-fold increase of 3HK and a 50-fold increase of QUIN were observed in the spinal cord of mice with experimental allergic encephalomyelitis, which is a model of multiple sclerosis and is an autoimmune neuroinflammatory disorder[74]. In contrast, only a 1.5-fold increase in KYNA was observed. This disproportionate increase in 3HK and QUIN would have a great effect on the pathology of disorders that involve neuroinflammation.

The effect of infection on kynurenine metabolites in the CNS is most well documented in AIDS-related dementia, which is characterised by increased IDO activity and raised levels of 3HK and QUIN[76-79]. Increased IDO activity and/or neurotoxic kynurenine metabolites have

also been detected in infections associated with cerebral malaria[80], poliovirus infection[81], Lyme Disease[82], and others.

All the disorders discussed in this section thus far have involved an increase in the ratio of QUIN:KYNA, which would result in increased NMDA receptor-mediated excitation and, in combination with raised 3HK levels, neurotoxicity. However, reduced activation of NMDA and other EAA receptors has been implicated in schizophrenia and learning disabilities[60]. Elevated KYNA levels were detected in the brain of schizophrenic patients and may be a causal factor in the disease[83]. Other NMDA antagonists, such as the drugs PCP (**1**) and ketamine (**2**), can produce schizophrenia-like symptoms in normal people and are used as experimental agents for study of the disease[54].

1.3.5 Therapeutic Targets in the Kynurenine Pathway

Many of the enzymes of the kynurenine pathway have been targeted to find modulators of kynurenine metabolite levels. The rate limiting step of the kynurenine pathway is the production of *N*-formylkynurenine from tryptophan[84]. This reaction is catalysed by TDO in the liver and IDO elsewhere in the body[60]. Since pro-inflammatory cytokines cause a great increase in IDO activity, it would seem that this enzyme would make a good target for inhibition. However, inhibition of IDO would cause a substantial shift of tryptophan metabolism to the production of serotonin, which may be useful in disorders that involve serotonin dysfunction but would cause undesirable effects in disorders where serotonin function is normal. While the effect of IDO inhibition on serotonin has been little studied due to a lack of known potent inhibitors, a potent and selective TDO inhibitor has been shown to markedly increase serotonin in the brain[85].

Towards the downstream end of the pathway, 3HAO, which catalyses the formation of QUIN from 3HA, has been a target for inhibition. Halogenated analogues of 3HA have been developed to inhibit 3HAO and thus decrease levels of QUIN without altering KYNA levels[86].

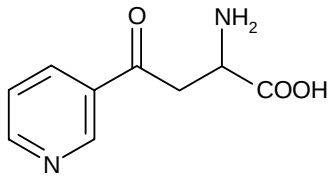
The more commonly investigated targets involve KYN metabolism. Inhibitors of kynureninase, which metabolises KYN to AA and 3HK to 3HA, include *S*-Aryl-L-cysteine *S*,*S*-dioxides (e.g., **10**)[87]. While kynureninase inhibitors increase KYNA levels and decrease QUIN levels, they also increase the levels of 3HK[88], since they inhibit the conversion of 3HK to 3HA.

Most KYN is metabolised by kynurenine-3-hydroxylase to 3HK[61,62] and this enzyme is the most desirable target for modulation of kynurenine metabolite concentrations. From its position in the pathway, inhibition of kynurenine-3-hydroxylase would be predicted to reduce QUIN and 3HK synthesis and drive KYNA production. Studies with kynurenine-3-hydroxylase inhibitors, such as those in Figure 1.4, have confirmed these actions, as discussed.

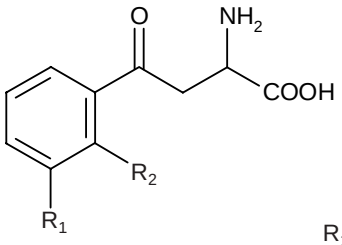
KAT metabolises KYN to KYNA. Since most efforts have been to find modulators that decrease the QUIN:KYNA ratio, few inhibitors of KAT have been reported. However, inhibitors of KAT may be useful in the treatment of schizophrenia, where raised levels of KYNA have been implicated in the disease[83].

1.3.6 Inhibitors of Kynurenine-3-hydroxylase

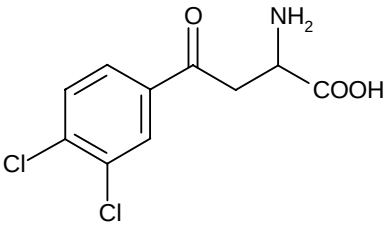
Nicotinylalanine (NAL, **11**) is a KYN analogue that was discovered to be a weak inhibitor of both kynurenine-3-hydroxylase and kynureninase over 40 years ago[89]. A concerted



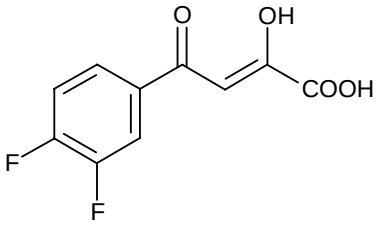
Nicotinylalanine (NAL) **11**



		R_1	R_2
(*o*-methylbenzoyl)alanine (oMBA)	**12**	H	Me
(*m*-nitrobenzoyl)alanine (mNBA)	**13**	NO_2	H



PNU 156561A **14**



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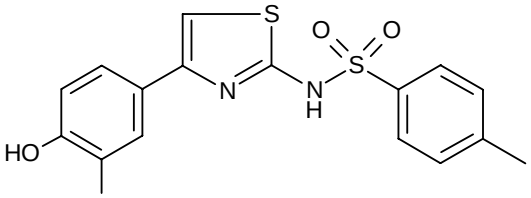

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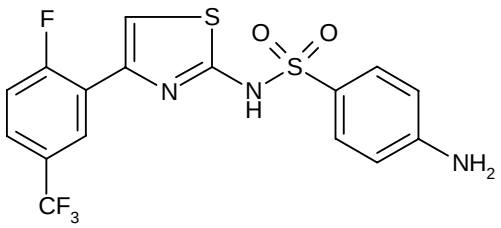

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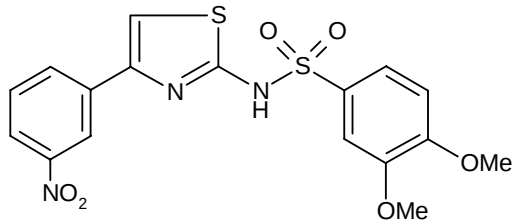

Ro 61-8048 **18**

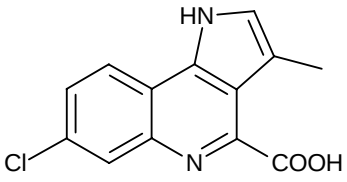

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Figure 1.4. Structures of inhibitors of enzymes in the kynurenine pathway of tryptophan metabolism. **11** is a dual inhibitor of kynurenine-3-hydroxylase and kynureninase; **12** is an inhibitor of kynureninase; and **13**–**19** are inhibitors of kynurenine-3-hydroxylase.

pharmacological interest in kynurenine-3-hydroxylase and kynureninase inhibitors did not appear until many years later. NAL has been shown to reduce seizures in rates by raising KYNA levels in the brain[90,91].

Approximately ten years ago, kynurenine analogues with greatly improved activity and selectivity were developed. A selective kynureninase inhibitor, (*o*-methoxybenzoyl)alanine (oMBA, **12**), and a selective kynurenine-3-hydroxylase inhibitor, (*m*-nitrobenzoyl)alanine (mNBA, **13**), showed 2–3 orders of magnitude greater inhibition of the respective enzymes than NAL[92,88]. As expected, both of these compounds raised KYNA levels and reduced seizures in rats when administered individually, and they displayed an additive effect when co-administered[92,88]. Chiarugi and co-workers[93] showed that mNBA also substantially reduced 3HK levels while oMBA raised 3HK levels in rat brain and blood, which is consistent with their predicted activity and emphasises the preference for kynurenine-3-hydroxylase inhibitors over kynureninase inhibitors.

Further synthesis and inhibitory evaluation of benzoylalanines produced the potent selective kynurenine-3-hydroxylase inhibitor (3,4,-dichlorobenzoyl)alanine (PNU 156561A, **14**). PNU 156561A, formerly known as FCE28833A, was 15-fold more active (IC_{50} = 0.2 μM) than mNBA (IC_{50} = 3.0 μM)[94], and has been demonstrated to raise KYNA levels in rat brain *in vivo*[94,73]. Additional manipulations of the side chain and the aryl group of benzoylalanines resulted in only a slight improvement on activity[95,96]. The IC_{50} of the most active of these side chain variants (**15**) was reported as 0.12 μM[96]. However, this activity cannot be directly compared to PNU 156561A since it was not tested in the same study and different assay conditions, particularly different substrate concentration, can have a great effect on IC_{50} values.

Two other classes of kynurenine-3-hydroxylase inhibitors have been reported. **19** is the most active inhibitor (IC_{50} = 24 μM) of a series of pyrrolo[3,2-*c*]quinolines that were synthesised

by one group[97]. The most successful class of kynurenine-3-hydroxylase inhibitors to date are the *N*-(4-phenylthiazol-2-yl)benzenesulfonamides. The development of these compounds was based on a screening hit (**16**) and mNBA[98]. The two most active compounds, **17** (IC_{50} = 19 nM) and Ro 61-8048 (**18**, IC_{50} = 37 nM), were 6- to 12-fold more active than PNU 156561A (IC_{50} = 237 nM)[98]. Ro 61-8048, as well as mNBA, has been shown to reduce elevated levels of QUIN in immune-stimulated mice, but not effect basal QUIN levels in control mice[99]. Conversely, the inhibitors increased levels of KYNA and KYN in both control and immune-stimulated mice[99]. The *in vivo* neuroprotective effect of both Ro 61-8048 and mNBA has been demonstrated by their ability to greatly reduce neuronal damage resulting from ischaemia[100].

1.3.7 Screening for Inhibitors of Kynurenine-3-hydroxylase

Screening compounds in a chemical library can lead to the discovery of enzyme inhibitors with more structural diversity than can be synthesised based on substrate analogues alone. Until the discovery of the *N*-(4-phenylthiazol-2-yl)benzenesulfonamides, kynurenine-3-hydroxylase inhibitors had been based solely on KYN analogues. The *N*-(4-phenylthiazol-2-yl)benzenesulfonamide screening hit (**16**) had equivalent or better activity than any previously reported compound (IC_{50} = 0.1 μM)[98], and was the lead for rational development of even more active inhibitors.

Aeons of evolution have endowed plants and many other organisms with an array of structurally diverse and biologically active secondary metabolites. As a result, plants and other biological organisms are unrivalled as a resource for drug discovery. For this reason plant extracts were used to establish a library for screening for enzyme inhibitors of the kynurenine pathway in this study.

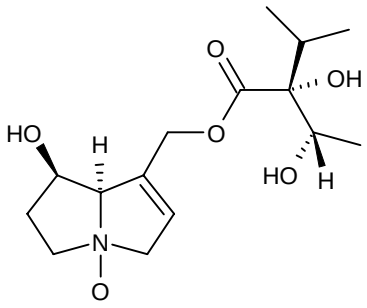
As previously stated (Section 1.3.5), kynurenine-3-hydroxylase represents the most desirable enzyme for inhibition to reduce the elevated QUIN:KYNA ratio and 3HK levels seen in many neurological disorders. Plant extracts were screened for kynurenine-3-hydroxylase inhibition in order to isolate and identify novel inhibitors of this enzyme. *In vitro* 3HK production by kynurenine-3-hydroxylase was measured using HPLC with electrochemical detection[101].

Raised KYNA levels seen in schizophrenia patients suggest that KAT may be a novel target for therapy of this disease[83], yet few KAT inhibitors have been reported. Plant extracts were screened for inhibition of KAT in order to isolate and identify novel KAT inhibitors. The amounts of KYNA and AA were measured simultaneously using HPLC with fluorescence detection[102]. Since AA was able to be quantified simultaneously with KYNA, inhibition of kynureninase by plant extracts were also investigated in order to find novel kynureninase inhibitors.

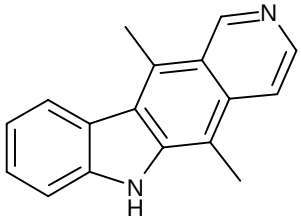
1.4 DRUG DISCOVERY FROM AUSTRALIAN PLANTS

Australia has an estimated 25,000 plant species[103], approximately 85% of which are not found elsewhere[104], and these represent an enormous resource for drug discovery. Two major investigations into the pharmacological activity and chemical composition of Australian plants will be discussed. In the 1940's, the CSIRO initiated a major cross-institutional pharmacological and chemical investigation of plants from Australia and Papua New Guinea that lasted for over three decades. Collins and co-workers[105,106] have provided a detailed summary of the investigation and presentation of the major results. Extracts of thousands of species were tested for the presence of alkaloids, and extracts of nearly 1,500 species were screened for *in vivo* anticancer activity in mice. The chemical constituents of poisonous plants, mainly stock poisons, were also investigated. Subsequent chemical

investigation of extracts and pharmacological evaluation of extracts and isolated compounds resulted in the publication of over two thousand papers from 1940 to 1987. Two of the notable compounds to be isolated as a result were indicine *N*-oxide (**20**) from *Heliotropium* species and ellipticine (**21**) from *Ochrosia* and *Bleekeria* species. Indicine *N*-oxide was investigated in phase II clinical trials for relapsed childhood solid tumours and leukaemia, but caused hepatotoxicity at therapeutic doses[107,108]. On the other hand, derivatives of ellipticine have shown promising activity and acceptable tolerance in clinical trials for metastatic breast cancer[109], solid tumours[110] and in combination therapy for AIDS[111].



indicine *N*-oxide **20**



ellipticine **21**

Approximately ten years ago, a collaboration was established between Griffith University and Astra (now Astra-Zeneca). The collaboration predominantly screens Queensland plants and marine extracts for activity in a range of molecular assays that target different diseases[112]. By mid-1998, over 40% of Queensland's approximately 9,000 species had been collected for their natural product screening library[113]. Since, high-throughput screening began, hundreds of bioactive compounds have been isolated, about a third of which were new[113].

This project aimed to utilise Australia's great biological resources for drug discovery. Specifically, a library of extracts of local North Queensland plants were established and used for pharmacological screening, in order to isolate new pharmacologically active compounds.

1.4.1 Selection of Plants for Pharmacological Screening

Plant species collected for this study were mainly local native species, and some introduced species, from the woodlands, creek banks and vine thickets surrounding the Douglas Campus of James Cook University and the adjacent Mt Stuart Defence Training Area, Townsville, Queensland. Some cultivated plants from the Douglas Campus were also collected. Species were identified and selected for screening if they were suspected of, or known to possess, pharmacological activity. Pharmacological activity was supposed from reports of toxicity[114], alkaloid content[105], or medicinal use[115-117]. All species of Apocynaceae, Lauraceae, Moraceae, Rubiaceae, Rutaceae, Solanaceae and *Crotalaria* (Fabaceae), among others, that were encountered were also selected due to the high proportion of pharmacologically active species known to occur in these taxa. Species were selected in this manner to increase the likelihood of discovering activity against the pharmacological targets tested. Leaves, bark, fruit and other organs were collected from each species based on availability, ease of collection and supposition of pharmacological activity. Samples from a few individuals per plant population were collected and pooled, when possible, to minimise the effect of intrapopulation chemical variation. Due to practical limitations, only one population per species was sampled with the exception of *Maytenus cunninghamii*. Two populations of *M. cunninghamii* were collected and kept separate. One population was from a *Eucalyptus drepanophylla* woodland at the Douglas Campus and the other population was from a *E. drepanophylla / Corymbia* sp. open woodland at Red Falls, approximately 130 km west of Townsville. The Red Falls population was collected due to a report of superior activity of the root-bark extract in a brine-shrimp lethality assay when compared to other populations of *M. cunninghamii* (B. R. Jackes, pers. comm.).

1.4.2 Considerations for Testing Plant Extracts for Pharmacological Activity

Certain ubiquitous plant constituents may interfere with pharmacological assays. Tannins are an extremely diverse group of polyphenolic compounds that are found ubiquitously in plants and are believed to act as antifeedants by binding to proteins[118]. The binding properties of plant phenolics have been reviewed by Loomis and Battaile[119]. Polyphenols form hydrogen bonds with proteins. In addition, many polyphenols are readily oxidised to quinones which are highly reactive compounds that rapidly form covalent bonds with proteins. Due to these properties, polyphenols and quinones are capable of inhibiting enzymes in a non-specific manner. These compounds may also interfere with cell-surface proteins in cell-culture assays. It is necessary to remove both polyphenols and quinones from the crude plant extracts prior to pharmacological screening assays to avoid false positives. Polyamide has been very useful for this purpose[120-122]. Quinones bind irreversibly to the free amino groups at the polyamide chain-ends while phenolic compounds form hydrogen bonds with the amide groups in polyamide[123]. The affinity a phenolic compound has for the polyamide increases with the number of free hydroxyl groups[123]. In general, polyphenols have a greater affinity for polyamide than simpler phenols that have fewer free hydroxyl groups. These simpler phenols, along with non-phenols, may be eluted from the polyamide with water followed by methanol, while polyphenols are retained on the polyamide[120].

1.4.3 Overview and Aims of this Study

The general aim of this study was to utilise North Queensland plants as a resource for drug discovery. This involved collection and extraction of 365 samples from 125 local plant species. Polyphenols and quinones were removed from extracts by polyamide column

chromatography prior to screening to prevent interference of these compounds with the pharmacological assays. Extracts were screened for anticancer activity using a P388D1 cell line in an *in vitro* assay for cytotoxicity. The most potent cytotoxic extracts were sent to the NCI and tested in their 60 cell line panel in order to prioritise the extracts based on selective cytotoxicity and potency. Extracts were also screened in two different assays for inhibition of three key enzymes of the kynurenine pathway: kynurenine-3-hydroxylase, kynureninase, and KAT. The extracts were prioritised according to the potency of enzyme inhibition. In order to isolate and identify the compounds responsible for the activity, the prioritised cytotoxic and enzyme inhibitory extracts were subjected to bioassay-guided fractionation using solvent-solvent partitioning, column chromatography and preparative HPLC. The identities of the isolated active compounds were determined using 1- and 2D-NMR and other spectroscopic techniques. New compounds were fully characterised in this manner. All compounds isolated were quantitatively assessed for cytotoxicity against the P388D1 cell line, and for inhibition of kynurenine-3-hydroxylase, kynureninase, and KAT.

This study had two specific and equal aims. Firstly, it was desired that novel cytotoxic compounds with potential for cancer chemotherapy would be discovered from local North Queensland plants. Secondly, this study aimed to provide agents that could rectify the imbalance of kynurenine metabolites seen in a variety of neurological disorders, by the discovery of novel inhibitors of kynurenine-3-hydroxylase, kynureninase, and KAT from local North Queensland plants.

CHAPTER 2.

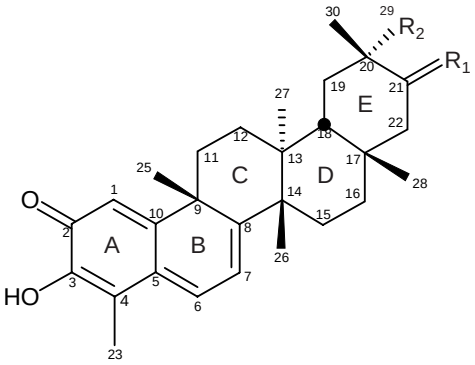
POTENTIAL ANTICANCER AGENTS FROM NORTH QUEENSLAND PLANTS: RESULTS AND DISCUSSION

2.1 QUINONEMETHIDE TRITERPENES FROM *MAYTENUS CUNNINGHAMII*

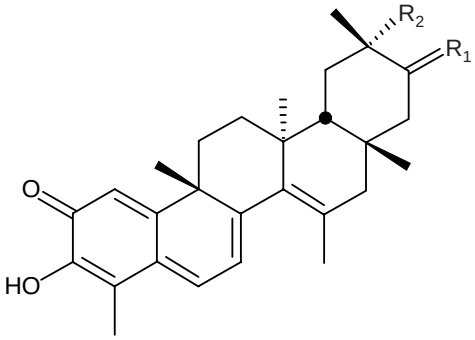
2.1.1 Background

Maytenus is a large genus of approximately 200 tropical and subtropical shrubs and small trees belonging to the family Celastraceae[124]. Various species of *Maytenus* have been used traditionally to treat a range of ailments including skin cancer[125,126], rheumatism[125], fever and colds[126], dysentery[126], and as analgesics and contraceptives[126]. Bioactive compounds that have been isolated from this genus include agarofurans, diterpenes, triterpenes and the highly cytotoxic maytansinoids[127]. The most commonly isolated group of triterpenes from *Maytenus* and other genera in the Celastraceae are the friedelanes, which include the cytotoxic quinonemethide triterpenes.

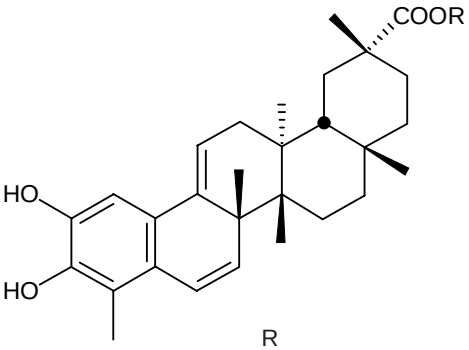
Celastrol (**22**) and pristimerin (**23**) exemplify quinonemethide triterpenes, or celastroloids, which are a group of highly modified 24-nor-friedelanes generally found in the root-bark, and sometimes in the trunk- or stem-bark, of members of the Celastraceae. Isolated occurrences of these compounds have been reported from *Heisteria pallida* (Olacaceae)[128] and *Melia azedarach* (Meliaceae)[129]. Tetracyclic celastroloids with a shionane skeleton (e.g., **34**) have even been isolated from the fungus, *Russula flavida* (Russulaceae)[130]. The repetitive co-occurrence of moderately oxidised friedelanes with celastroloids has led to the proposal of biosynthetic pathways that involve the production of celastroloids from friedelin (**35**) and its derivatives, such as that proposed by Itokawa and co-workers[131] (Scheme 2.1). It has been shown that friedelane derivatives in *Maytenus aquifolium* are produced in the



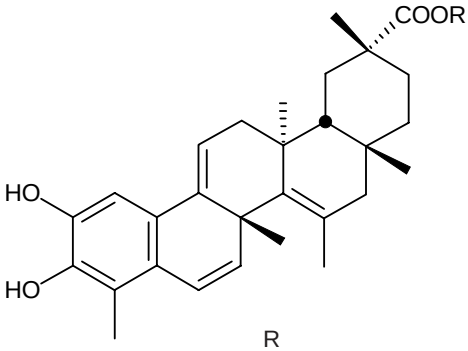
		R_1	R_2
Celastrol	**22**	H_2	COOH
Pristimerin	**23**	H_2	COOMe
Tingenone	**24**	O	H



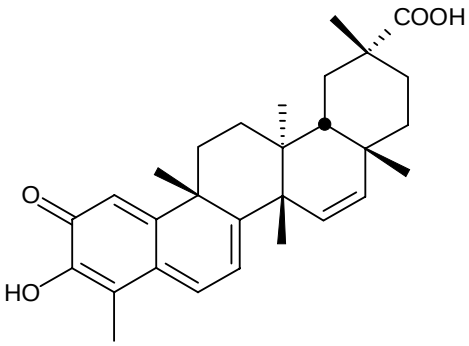
		R_1	R_2
Netzahualcoyoic acid	**25**	H_2	COOH
Netzahualcoyene	**26**	H_2	COOMe
Scutione	**27**	O	H



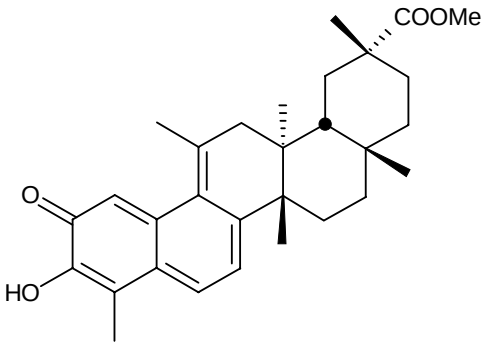
	R
28	H
29	Me



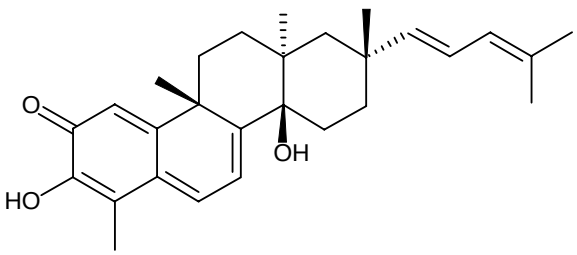
	R
30	H
31	Me



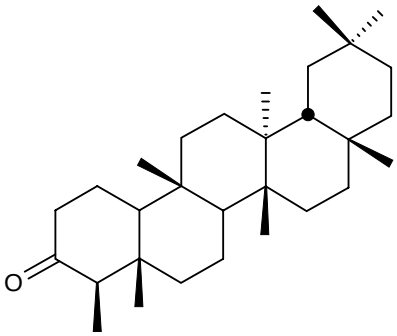
Δ^{15}-Celastrol **32**



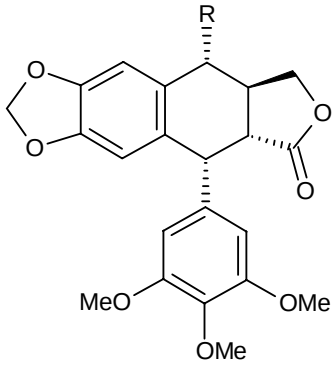
Pristimerinene **33**



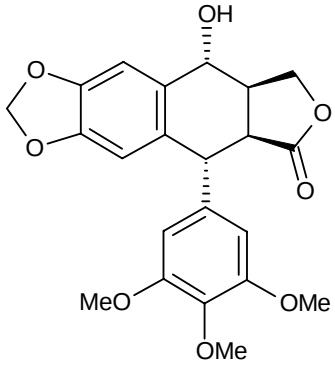
Russulaflavidin **34**



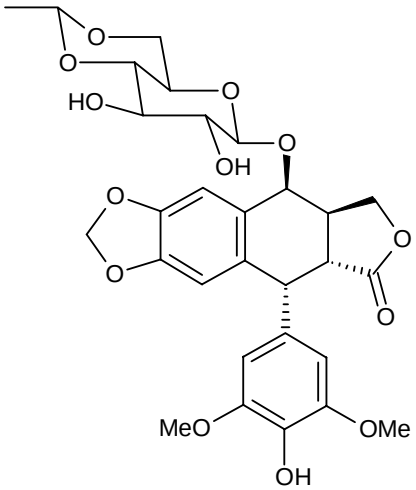
Friedelin **35**



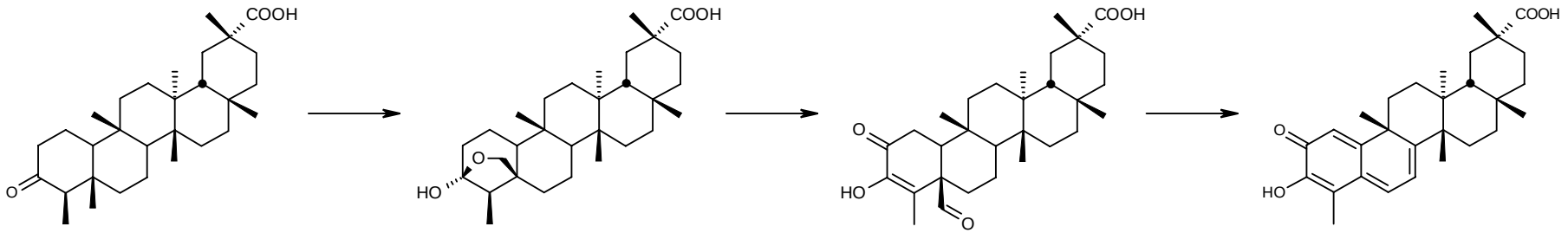
		R
Podophyllotoxin	**36**	OH
Deoxypodophyllotoxin	**37**	H



Picropodophyllotoxin **38**



Etoposide **39**



Scheme 2.1†

† Adapted from Itokawa and co-workers[131]

leaves and translocated to the root bark where they are transformed into celastroloids[132]. The biosynthesis of ring A in celastroloid precursors is discussed further in Section 4.1.2.4.

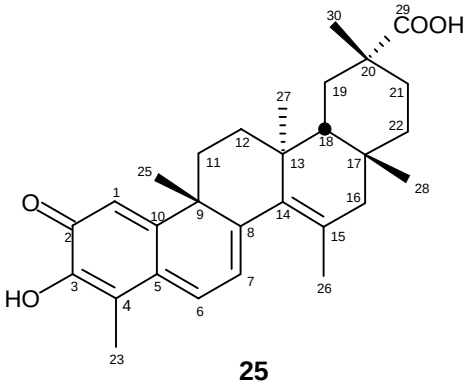
The chromophore of celastroloids may be variously oxygenated or rearranged. For example, the conjugation may be extended into ring D, as in netzahualcoyene (**26**)[133]. Although there are reports of conjugation extension into ring C, as in pristimerinene (**33**)[134], only three structures have been reported and it is likely that they are actually Δ^{14} compounds that have been incorrectly assigned[135]. The chromophores of celastrol and netzahualcoyene are unstable in acidic conditions, where they rearrange to phenolic forms, such as in **28**[136] and **31**[135]. Other variations in the structure of celastroloids commonly involve the level of oxidation of C-21, C-22 and C-29.

Celastroloids possess a large range of biological activities. They have antitumour activity[137,138] and are cytotoxic against a large range of cancer cell lines[139,140], but their non-selective cytotoxicity may prevent their application as drugs. *In vivo* toxicity has been reported for celastroloids[141,138]. Pristimerin, for example, was 100% lethal when administered to mice parenterally at 0.25–0.5 g/kg, but no toxicity was observed when pristimerin was administered orally at 0.5 g/kg each day for 28 days[141], which was most likely due to acidic rearrangement of pristimerin to a phenolic form, such as **29**, in the stomach. However, **29** has been shown to be much less cytotoxic against three cell lines than pristimerin[131]. In addition, Sotanaphun and co-workers[136] have reported that acidic rearrangement of tingenone (**24**) and derivatives caused the loss of nearly all antibacterial activity. Celastroloids possess antibacterial activity by interfering with cellular respiration in gram-positive bacteria, yet are inactive against gram negative bacteria, probably because they are unable to permeate the cell wall[142]. Other biological activities of celastroloids include immunosuppressive[143,144], anti-inflammatory[128,143], antiplasmodial[145], and radical scavenging[146] activity, as well as inhibition of enzymes such as aldose reductase[147].

The yellow-berry bush, *Maytenus cunninghamii*, is a widespread endemic shrub that is found in inland and near-coastal areas of tropical and subtropical Australia[124]. There had been no reported chemical investigation of *M. cunninghamii* prior to this study. Two populations of *M. cunninghamii* were initially collected for screening: one population was collected in woodland at the James Cook University, Townsville, while the other was collected from Red Falls, approximately 130 km west of Townsville. Extracts of root-bark collected from the Red Falls population has previously demonstrated much greater lethality in brine-shrimp assays than other populations (B. R. Jackes, pers. comm.). The root-bark of the University population was not originally collected so no comparisons can be made. Supporting the previous observations, the trunk-bark extract from the Red Falls population showed almost complete cytotoxicity against the P388D1 mouse lymphoma cell line at 5 μg/mL while the trunk-bark extract from the University population showed only approximately 50% cytotoxicity at five times this concentration, demonstrating remarkable population heterogeneity in the cytotoxicity of *M. cunninghamii*. It is not known whether the cytotoxic compounds, most likely celastroloids, were absent from University population or merely present in much lower concentrations. The root-bark of the Red Falls population was used for chemical isolation since it was the most cytotoxic of all *Maytenus* extracts screened. Bioassay-guided fractionation of the root bark afforded celastrol (**22**) and two new celastroloids, netzahualcoyoic acid (**25**) and Δ^{15}-celastrol (**32**). The co-occurrence of these compounds is of biosynthetic importance, as discussed below (Section 2.1.3).

2.1.2 Characterisation of Compounds Isolated from *Maytenus cunninghamii*

2.1.2.1 Netzahualcoyoic acid (**25**)



25

Compound **25** was isolated as red-orange crystals (16.0 mg) and assigned the molecular formula $C_{29}H_{36}O_4$ from ESI-MS. The UV λ_{max} at 448 nm suggested a celastrol-like chromophore with conjugation extended by an extra double bond. The ^{1}H NMR (300 MHz, $CDCl_3$, Table 2.1) spectrum showed four methyl singlets between δ0.80 and δ1.28, two vinylic methyls at δ1.70 and δ 2.27, three olefinic protons with an *ABX* coupling system at δ6.19, δ6.62, and δ7.24, and a broad singlet at δ7.5 corresponding to a hydroxyl proton. The ^{13}C spectrum (75.4 MHz, $CDCl_3$, Table 2.1) showed two carbonyl resonances at δ184.8 and δ177.7, in addition to signals for ten other sp^2 carbons from δ117.8 to δ161.0. The ^{13}C and ^{1}H spectra were almost identical to those of netzahualcoyene (**26**)[148], except **25** lacked a methoxyl group, and the ^{13}C shift at δ184.8 in **25** was δ5.4 more downfield than the corresponding carboxylate shift in **26**. This led to the identification of compound **25** as the 29-carboxylic acid of **26**, named netzahualcoyoic acid.

Multiplicities of ^{13}C signals were determined from a DEPT experiment. One-bond ^{13}C-^{1}H correlations were established from a *g*HSQC experiment and all but two ^{13}C shifts were assigned from *g*HMBC correlations. The assignment of the two remaining carbons, C-19 and C-21, was based on *g*COSY correlations. Selective ^{1}H decoupling at δ1.95 (H_2-11) changed the multiplicity of H-12β (δ2.53) from a triplet of doublets (J = 13.0, 5.5 Hz) to a doublet (J = 13.0 Hz), which confirmed that the protons on C-11 had near identical ^{1}H shifts.

Table 2.1. ^{13}C and ^{1}H NMR shifts (δ), and *g*COSY, NOESY and *g*HMBC correlations for netzahualcoyoic acid (**25**) in $CDCl_3$. Shifts were referenced on the ^{13}C and residual ^{1}H peaks of $CDCl_3$ (δ_C77.0 and δ_H7.24, respectively).

				^{1}H-^{1}H correlations (non-geminal)		^{1}H-^{13}C long-range correlations
C	δ_C		δ_H[a]	*g*COSY	NOESY	*g*HMBC
1	119.8		6.62 (H, d, *J* = 1.1 Hz)	6	25, 11β	C-3, C-5, C-9
2	177.7					
3	146.3		7.5 (OH, br s)			
4	117.8					
5	127.5					
6	135.9		7.24 (H, dd, *J* = 6.6, 1.1 Hz)	1, 7	7, 23	C-5, C-9, C-14
7	121.7		6.19 (H, d, *J* = 6.6 Hz)	6	6, 26	C-4, C-8/10
8	160.3[b]					
9	44.7					
10	161.0[b]					
11	37.5	α, β	1.95 (H_2, m)	12α, 12β	1, 12β, 27	C-8/10, C-9, C-12, C-13, C-25
12	35.6	α	1.30 (H, m)	11α/β	27	
		β	2.53 (H, td, *J* = 13.0, 5.5 Hz)	11α/β	11α/β, 25, 28	C-13, C-27
13	43.3					
14	135.3					
15	128.6					
16	37.7	α	2.65 (H, d, *J* = 16.7 Hz)	26	19α, 21α, 22α	C-14, C-15, C-28
		β	1.26 (H, d, *J* = 16.7 Hz)		26	C-14, C-15, C-18, C-26, C-28
17	33.8					
18	43.8	β	1.41 (H, m)	19α, 19β	19β, 22β, 28, 30	C-17/19
19	33.8	α	1.57 (H, m)	18β	16α, 21α, 27	C-29, C-30
		β	1.70 (H, m)	18β	18β, 27	
20	42.4					
21	28.4	α	1.92 (H, m)	22α, 22β	16α, 19α	C-30
		β	1.46 (H, m)	22β		C-17/19
22	36.0	α	1.30 (H, m)	21α	16α	C-17
		β	1.63 (H, m)	21α, 21β	18β, 28, 30	C-18
23	10.5		2.27 (H_3, s)		6	C-3, C-4, C-5
25	29.5		1.28 (H_3, s)		1, 12β, 26	C-8/10, C-9, C-11
26	22.0		1.72 (H_3, s)	16α	7, 16β, 25	C-14, C-15, C-16
27	24.0		0.80 (H_3, s)		11α, 12α, 19α, 19β	C-12, C-13, C-14, C-18
28	31.5		1.20 (H_3, s)		12β, 18β, 22β	C-16, C-17, C-18, C-22
29	184.8					
30	19.6		1.24 (H_3, s)		18β, 22β	C-19, C-20, C-21, C-29

[a] multiplicities undefined due to overlapping signals are designated 'm'

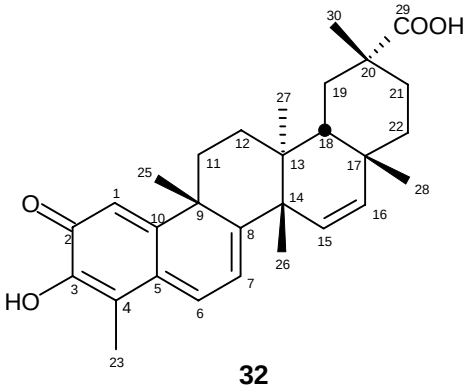
[b] shifts interchangeable

The stereochemistry of the methyl and carboxyl groups attached to C-20 was confirmed by NOESY correlations from δ1.24 (H_3-30) to δ1.41 (H-18β) and δ1.63 (H-22β). The stereochemistries of the remaining protons were assigned from NOESY correlations and coupling constants where definable. NOESY correlations for **25**, particularly H-12β (δ2.53) to H_3-28 (δ1.20), suggest that ring D is in a half-chair conformation where five of the carbons are in the same plane while C-17 sits above the plane (Figure 2.1b). NOESY correlations also indicate that ring E is in a chair conformation that places the C-30 in an axial position and C-29 in an equatorial position. The conformation of rings D and E is in agreement with that evidenced from nOe correlations[149,150] and a crystal structure[151] of other 7,14-dienequinonemethides. Conversely, rings D and E in celastrol (**22**) would most likely exist in a chair-chair conformation (Figure 2.1a), which places C-29 in spatial proximity to C-27 (see Section 4.2.1.2 for further discussion of the conformation of rings D and E in friedelanes).

25 is the first reported 7,14-dienequinonemethide triterpene with a 29-carboxylic acid group. Other 7,14-dienequinonemethides that have been previously isolated from *Maytenus* spp. include scutione (**27**)[152] and the C-29 methyl ester of **25**, netzahualcoyene (**26**)[153].

2.1.2.2 Δ^{15}-Celastrol (**32**)

Compound **32** was isolated as a red-orange solid (1.1 mg) and assigned the molecular formula $C_{29}H_{36}O_4$ from ESI-MS, isomeric with netzahualcoyoic acid (**25**). The UV λ_{max} at 428 nm suggested a celastrol-like chromophore. The ^{1}H NMR (300 MHz, $CDCl_3$, Table 2.2)



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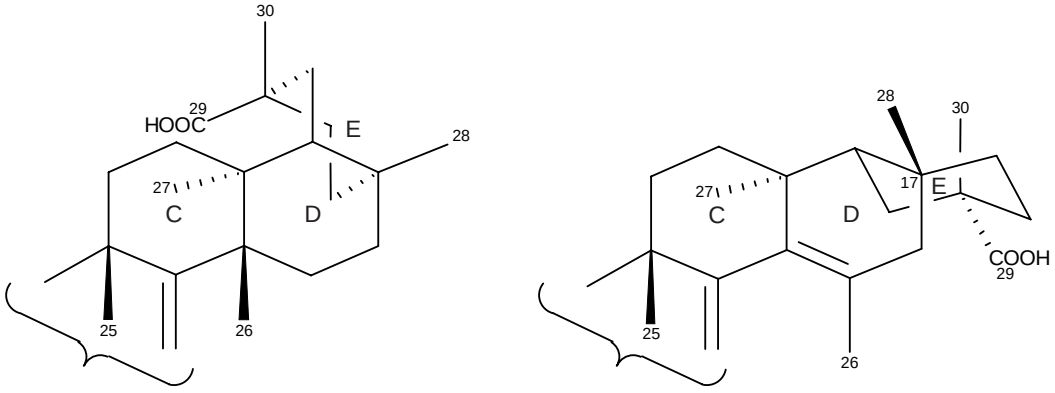


(a) (b)

Figure 2.1. Three-dimensional stick diagrams showing conformation of rings D and E and the position of the carboxylic acid groups in celastrol (**22**, a) and netzahualcoyoic acid (**25**, b), viewed from above the β face.

Table 2.2. Selected ^{1}H NMR shifts (δ) for Δ^{15}-celastrol (**32**) and celastrol (**22**) in $CDCl_3$. Shifts were referenced on the residual ^{1}H peak of $CDCl_3$ (δ_H7.24).

H	Δ^{15}-celastrol (**32**)	celastrol (**22**)[b]
1	6.50 (H, br s)	6.47 (H, d, *J* = 1.1 Hz)
6	7.04 (H, br d, *J* = 7.2 Hz)	7.04 (H, dd, *J* = 7.2, 1.1 Hz)
7	6.54 (H, d, *J* = 7.2 Hz)	6.30 (H, d, *J* = 7.2 Hz)
15	6.09 (H, d, *J* = 10.2 Hz)	
16	5.24 (H, d, *J* = 10.2 Hz)	
19α	2.56 (H, br d, *J* = 15.9 Hz)	2.48 (H, br d, *J* = 15.6 Hz)
23	2.18 (H_3, s)	2.18 (H_3, s)
25	1.38[a] (H_3, s)	1.40 (H_3, s)
26	1.36[a] (H_3, s)	1.22 (H_3, s)
27	0.50 (H_3, s)	0.54 (H_3, s)
28	1.09 (H_3, s)	1.06 (H_3, s)
30	1.26 (H_3, s)	1.26 (H_3, s)

[a] shifts interchangeable

[b] asssignments for celastrol were determined from 2D-NMR experiments

spectrum showed five methyl singlets between δ0.50 and δ1.38, one vinylic methyl at δ 2.18, three olefinic protons with an *ABX* coupling system at δ6.50, δ6.54, and δ7.04, and two olefinic protons with an *AB* coupling system at δ5.24 and δ6.09. The similarity of the ^{1}H spectrum with that of celastrol (**22**; authenticated sample, Table 2.2) suggested that **32** differed from **22** only in the presence of a double bond. The coupling (J = 10.2 Hz) of the olefinic *AB* protons was typical of a *cis* double bond, which restricted its position to either Δ^{11}, Δ^{15} or Δ^{21}. The ^{1}H shift of H-7 in **32** is δ0.24 downfield of H-7 in **22**[139], while H-1 and H-6 are almost unchanged. This difference suggests the H-7 is in the deshielding zone of a double bond between C-15 and C-16. Therefore, compound **32** was tentatively assigned as Δ^{15}-celastrol. This is a novel celastroloid structure and represents the first reported isolation of a friedelane with an unconjugated double bond at this position. ^{13}C and 2D-NMR experiments were not initially attempted due to insufficient material. Re-isolation attempts failed (see Section 3.4.1.3), and partial decomposition of the original sample occurred in the interim.

2.1.2.3 Celastrol (**22**)

Celastrol is commonly isolated from the root bark of *Maytenus* spp. and other members of the Celastraceae. It is cytotoxic against a large range of cancer cell lines[139,140], and shows antibacterial activity[154], anti-inflammatory activity[143] and DNA topoisomerase II inhibition[155]. The anti-inflammatory and antioxidant properties of celastrol have led to the proposal that it might be useful in the treatment of Alzheimer's and other neurodegenerative diseases (see Section 4.3.2)[156]. In this study, celastrol was the major component of the organic fraction of *M. cunninghamii* root bark, but only a portion of this was isolated due to substantial tailing during chromatographic separation.

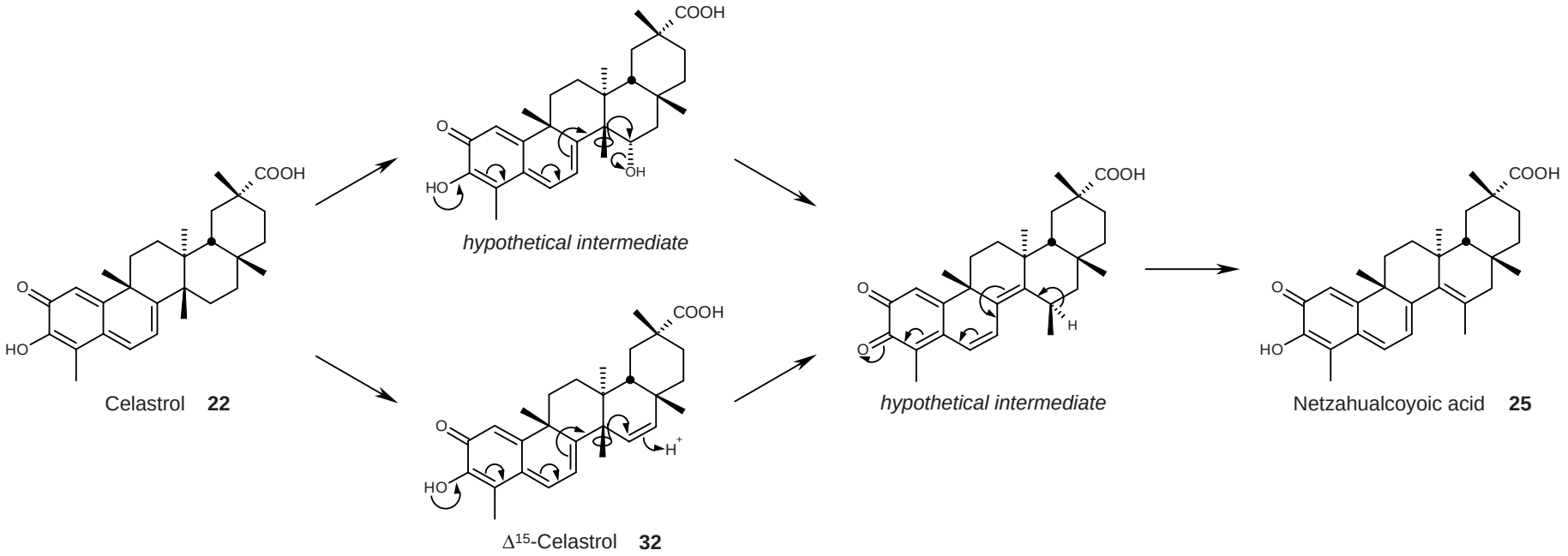
2.1.3 Biosynthetic Aspects

Dehydrogenation and methyl migration of celastrol analogues has been suggested to occur via a 15-hydroxylated intermediate (Scheme 2.2)[149]. This has been supported by the discovery of 15α-hydroxy derivatives in three species of the Celastraceae[157-159], and semi-synthesis of netzahualcoyene (**26**) from 15α-hydroxypristimerin with thionyl chloride in pyridine[158]. It has also been suggested that the biosynthesis may occur via a Δ^{15} intermediate (Scheme 2.2)[149]. The co-occurrence of celastrol (**22**), netzahualcoyoic acid (**25**) and Δ^{15}-celastrol (**32**) in the root bark of *Maytenus cunninghamii* provides evidence for the latter biosynthetic pathway.

Two alternative intermediates in the biosynthesis of Δ^{15}-celastrol from celastrol can be proposed. The first possibility is a 16-hydroxyl derivative, which might be expected to give Δ^{16} and Δ^{17} products with a migrated 28-methyl as well as a Δ^{15} product, as demonstrated by Kikuchi and co-workers[160] in the reactions of 16α- and 16β-hydroxyfriedelane with methanesulfonyl chloride. Alternatively, Δ^{15}-celastrol may be biosynthesised from a 15-hydroxyl intermediate, although only a very small proportion would be expected since methyl migration and double bond formation at Δ^{14} would extend the conjugated system in rings A and B.

2.1.4 Biological Activity and the Relationship with Structure

Netzahualcoyoic acid (**25**) and celastrol (**22**) showed strong *in vitro* cytotoxic activity against the P388D1 mouse lymphoma cell line, with IC_{50} values of 0.12 μM (0.11–0.13 μM 95% confidence interval range) and 0.37 μM (0.30–0.45 μM 95% confidence interval range), respectively. The IC_{50} for Δ^{15}-celastrol (**32**) could not be determined due to partial



Scheme 2.2†

† Adapted from Fernando and co-workers[149]

decomposition prior to testing. The IC_{50} of celastrol against the P388D1 cell line is similar to those reported against 11 other cell lines[140]. As with other celastroloids, the cytotoxicity of netzahualcoyoic acid is also likely to be non-selective, but further testing against other cell lines are required to determine whether this is true.

Comparison of the IC_{50} values of celastrol and netzahualcoyoic acid indicates that the extended conjugation in the latter compound increases cytotoxicity approximately 3-fold. This may partly be due to the different conformation of rings D and E in celastrol and netzahualcoyoic acid that is caused by the extension of the conjugation into ring D. This conformational difference has a great influence on kynurenine-3-hydroxylase inhibition, as discussed in Section 4.2.1.2.

Pristimerin (**23**) and celastrol have almost identical IC_{50} values against 11 cell lines tested by Chang and co-workers[140], which demonstrates that methylation of the C-29 carboxylic acid is not important for cytotoxicity in compounds with a celastrol-like chromophore. It has also been shown that friedelanes with a 29-carboxyl or a 29-methyl carboxylate have the same conformation in rings D and E[161-163]. The similar cytotoxicity of celastrol and pristimerin contrasts greatly with antimicrobial activity, where celastrol has more than 100-fold greater activity than pristimerin against *Staphylococcus aureus*[154], which suggests that celastrol has different modes of action for its cytotoxic and antimicrobial activities.

2.1.5 Further Work

The potential non-selective cytotoxicity of netzahualcoyoic acid might prevent its use as an anticancer agent, but this remains to be tested. Like pristimerin[141], parenteral administration of netzahualcoyoic acid would be likely to produce *in vivo* toxicity whereas oral administration would not. *In vivo* studies in mice are required to confirm this. Oral

administration would be expected to cause the acid-rearrangement of netzahualcoyoic acid to a phenolic form, such as in **30**. Therefore, it is necessary to test these acid-rearranged products for *in vitro* cytotoxicity to assess the anticancer potential of orally administered netzahualcoyoic acid. Following this, it would be desirable to test the acid-rearranged products of netzahualcoyoic acid, as well as netzahualcoyoic acid itself, in the NCI's 60 cell line panel to assess the compounds for selective cytotoxicity.

2.2 PODOPHYLLOTOXINS FROM *CALLITRIS INTRATROPICA*

Callitris (Cupressaceae) is a genus of 19 species that is restricted to Australia and New Caledonia[164]. *Callitris intratropica* (syn. *C. columellaris* var. *intratropica*) is a tropical tree found across northern Australia[164] that has been used by Aborigines as an antiseptic[117]. Sesquiterpenes, common in *Callitris* spp., have been reported from this species[165]. Podophyllotoxins have not been previously reported from *C. intratropica*, but have been isolated from other *Callitris* spp., particularly *C. drummondii*[27] and *C. columellaris*[26].

The podophyllotoxins are a well known group of anticancer lignans primarily found in, but not restricted to, conifers. Podophyllotoxin (**36**) and deoxypodophyllotoxin (**37**) have been shown to prevent cell division by interfering with microtubule assembly, and the trans-lactone in ring D is a requirement for activity[166]. Picropodophyllotoxin (**38**) possesses a cis-lactone and has much less effect on microtubule assembly[166]. Interestingly, etoposide (**39**), a clinically important β-D-glucoside derivative of podophyllotoxin, has a trans-lactone but does not inhibit microtubule assembly[166]. Rather, etoposide interferes with topoisomerase II activity, which results in high levels of DNA strand breaks and leads to apoptosis[167].

In this study, the crude extracts of leaves, stems, bark, and fruit of *C. intratropica* were all completely cytostatic at 5 μg/mL. The leaves and stems of *C. intratropica* were recollected

due to ease of availability, and were extracted together. Bioassay-guided fractionation of this extract led to the isolation of three known podophyllotoxins, namely deoxypodophyllotoxin (**37**, 13.7 mg), podophyllotoxin (**36**, 1.5 mg) and picropodophyllotoxin (**38**, < 0.1 mg). The anticancer activities of these compounds are well known so IC_{50} values were not determined. This is the first reported isolation of picropodophyllotoxin from *Callitris*.

CHAPTER 3.

POTENTIAL ANTICANCER AGENTS FROM NORTH QUEENSLAND PLANTS: EXPERIMENTAL

3.1 GENERAL

3.1.1 High-Performance Liquid Chromatography

Preparative high-performance liquid chromatography (HPLC) was performed using a GBC LC 1150 HPLC pump, a Rheodyne 7725i manual injector with a 2.5 mL sample loop, and various C_{18} columns (described under individual separation procedures). Samples were observed at multiple UV/Vis wavelengths simultaneously with a GBC LC 5100 Photodiode Array Detector (PDA) and WinChrom Chromatography Data System (Version 1.31) software. Flow rates ranged from 1–3 mL/min. Distilled water and LR grade methanol (MeOH) were filtered through a Millipore 0.45 μm HA (water) or FH (methanol) membrane filter, and degassed by sparging with helium for 15 minutes.

3.1.2 Spectroscopy

Melting points (mp) were determined with a Stuart Scientific SMP1 melting point apparatus. Ultraviolet (UV) spectra were gathered in 95% aqueous ethanol (EtOH) on a Varian Cary 5E UV-Vis-NIR spectrophotometer. Infrared (IR) spectra were taken in AR grade $CHCl_3$ using a Nicolet Nexus Fourier Transform IR spectrometer. Nuclear Magnetic Resonance (NMR) spectra were obtained in deuterated chloroform ($CDCl_3$, referenced on δ_H7.24 and δ_C77.0) on a 300 MHz Varian Mercury spectrometer, using standard Varian pulse sequences for ^{1}H, ^{13}C,

*g*COSY, *g*HSQC, *g*HMQC, *g*HMBC and NOESY experiments, unless otherwise stated. Positive-ion electrospray ionisation mass spectrometry (ESI-MS) was performed in HPLC grade MeOH with a Bruker BioApex 47e Fourier Transform Ion Cyclotron Resonance Mass Spectrometer by Mr Rick Willis at the Australian Institute of Marine Science, Townsville.

3.2 PLANT EXTRACTS FOR CYTOTOXICITY SCREENING

3.2.1 Plant Material

Plant species from woodlands, creek banks and vine thickets surrounding the Douglas Campus of James Cook University and the adjacent Mt Stuart Defence Force Training Area were identified and selected for screening if they were suspected of, or known to possess, pharmacological activity (see Section 1.4.1). Some cultivated plants from the Douglas Campus were also collected, based on supposed pharmacological activity. One population of *Maytenus cunninghamii* was collected from Red Falls, approximately 80 km northwest of Charters Towers. A total of 365 organ samples (e.g., leaf, bark) from 125 different angiosperm species were collected for screening (listed in Appendix 2). A few grams of material were collected per sample and stored at 4°C for up to seven days until extraction. Voucher specimens were collected and lodged at BRI (Herbarium of Queensland, Brisbane), with duplicates at JCT (Herbarium of James Cook University, Townsville).

3.2.2 Sample Extraction

Fresh material (1.0 g) from each sample was homogenised in 10 mL of acetone-MeOH (1:1), and extracted at room temperature for three hours. The extract was removed from the

material and replaced with another 10 mL of fresh acetone-MeOH (1:1). This second extract was removed and combined with the initial extract and filtered. Air was blown over the combined extract to remove the solvent, and the extract was stored at -20°C until required.

3.2.3 Extract Treatment

A method to remove polyphenols and quinones prior to testing was adapted from Wall and co-workers[122]. A glass column (i.d. 5.5 mm, length 65 mm) was packed with 0.35 g of polyamide powder (ICN Biomedicals) that had been suspended in hot MeOH, rinsed thoroughly, and soaked in distilled water overnight. Several milligrams of crude extract was resuspended in a minimal quantity of MeOH and applied to the column (gravity-fed). Crude extract material that was insoluble in MeOH was suspended in a minimal quantity of water and applied to the column. The column was eluted with 3 mL of water followed by at least 7 mL of MeOH until the eluent was colourless. The water and MeOH eluents were combined. Air was blown over the combined eluents to remove the MeOH, then the residue was freeze dried using a Dynavac (model FDA/3RH) freeze drying unit to remove the remaining water.

3.3 CYTOTOXICITY ASSAYS

3.3.1 Cells and Cell Maintenance

The P388D1 mouse lymphoma cell line was kindly donated by the National Cancer Institute (NCI), Washington DC. Cells were maintained at approximately 10^5–10^6 cells/mL in antibiotic-free RPMI-1640 (JRH Biosciences) medium containing 5% foetal calf serum

(FCS; JRH Biosciences) at 37°C in a humidified atmosphere containing 5% CO_2 in a Forma Scientific Infrared CO_2 incubator and passaged every 2–3 days. Cell aliquots were resuspended in 1 mL of 10% dimethylsulfoxide (DMSO) in RPMI-1640 with 5% FCS at 1×10^7 cells/mL and stored in liquid nitrogen.

3.3.2 Cytotoxicity Screening

The protocol used for testing the cytotoxicity of plant extracts, fractions, and pure compounds was adapted from Monks and co-workers[30]. The culture medium used for seeding plates and for sample preparation was RPMI-1640 medium, containing 5% FCS and 50 μg/mL of gentamicin sulfate (Gibco BRL) to prevent bacterial contamination.

3.3.2.1 Seeding plates

Aliquots (100 μL) of either a 5×10^4 cells/mL suspension in medium or medium alone (background controls) were pipetted into designated wells (Figure 3.1: *unshaded* and *shaded*, respectively) of a flat-bottomed 96-well plate. The 96-well plate was incubated (37°C, 5% CO_2) for 24 hours to stabilise the cells prior to addition of extract preparations.

3.3.2.2 Extract preparation and application

Each polyphenol-free crude extract was resuspended in the drug vehicle (1:9 MeOH:H_2O) at 2 mg/mL by dissolving the extract in MeOH at 20 mg/mL and dilution to 2 mg/mL by

addition of water. A portion of the resuspended extract was removed and diluted to 100 µg/mL with medium, then further diluted to 20 µg/mL with medium.

Aliquots (100 µL) of the 100 µg/mL and 20 µg/mL concentrations of extract in medium were added to the designated wells (Figure 3.1: *D1a*, *D1b*) of a 96-well plate that had been seeded with cells and incubated for 24 hours, to give a final test concentration of 50 µg/mL and 10 µg/mL, respectively. A total of seven extract preparations at the two concentrations were added to the designated wells (Figure 3.1: *D1a—D7a*, *D1b—D7b*) of the 96-well plate. Aliquots (100 µL) of medium containing 5% drug vehicle, in the absence of extract, were added into the designated wells (Figure 3.1: *Veh*) of the same 96-well plate to serve as a negative control. Aliquots (100 µL) of medium, in the absence of extract and drug vehicle, were added to the designated wells (Figure 3.1: *Med*) of the same 96-well plate to serve as an additional negative control, and for comparison with medium containing 5% drug vehicle to establish that the drug vehicle was not affecting the growth of cells at the concentration employed. The previously described extract preparations and controls were simultaneously aliquoted using an 8-channel multipipette, on a per column basis. Variation between columns pipetted was allowed for by placing the treatments in rows (Figure 3.1). The 96-well plate was incubated (37°C, 5% CO_2) for a further 48 hours (3 cell doubling times) and then fixed.

3.3.2.3 Cell fixation

Each 96-well plate was removed from the incubator and placed on a bench for 5 minutes to allow the cells to settle, as they are non-adherent. In order to fix the cells to the bottom of the wells, 50 µL of cold (4°C) 50% aqueous trichloroacetic acid was gently layered onto the medium in each well. The plates were kept at room temperature for a further 5 minutes, then

	1	2	3	4	5	6	7	8	9	10	11	12
A	*D1a*	*D1a*	*D1a*	*D1a*	*D1a*	*D1a*	*D1b*	*D1b*	*D1b*	*D1b*	*D1b*	*D1b*
B	*D2a*	*D2a*	*D2a*	*D2a*	*D2a*	*D2a*	*D2b*	*D2b*	*D2b*	*D2b*	*D2b*	*D2b*
C	*D3a*	*D3a*	*D3a*	*D3a*	*D3a*	*D3a*	*D3b*	*D3b*	*D3b*	*D3b*	*D3b*	*D3b*
D	*Veh*	*Veh*	*Veh*	*Veh*	*Veh*	*Veh*	*Med*	*Med*	*Med*	*Med*	*Med*	*Med*
E	*D4a*	*D4a*	*D4a*	*D4a*	*D4a*	*D4a*	*D4b*	*D4b*	*D4b*	*D4b*	*D4b*	*D4b*
F	*D5a*	*D5a*	*D5a*	*D5a*	*D5a*	*D5a*	*D5b*	*D5b*	*D5b*	*D5b*	*D5b*	*D5b*
G	*D6a*	*D6a*	*D6a*	*D6a*	*D6a*	*D6a*	*D6b*	*D6b*	*D6b*	*D6b*	*D6b*	*D6b*
H	*D7a*	*D7a*	*D7a*	*D7a*	*D7a*	*D7a*	*D7b*	*D7b*	*D7b*	*D7b*	*D7b*	*D7b*

Figure 3.1. 96-well plate design, showing position of wells A1–H12, for cytotoxicity testing of seven extracts or drugs at two concentrations (*D1a–D7a* and *D1b–D7b*). Negative controls, lacking extracts and drugs, contained media with 5% drug vehicle (*Veh*) and media alone (*Med*). Unshaded areas represent wells seeded with 100 µL of 5×10^4 cells/mL, while shaded areas represent background controls that contained no cells.

gently transferred to a refrigerator and incubated at 4°C for 1 hour. Plates were then removed from the refrigerator and flicked over a sink to remove all liquid contents. The wells were rinsed five times with tap water, flicking to remove all liquid contents after each rinse, then allowed to dry overnight.

3.3.2.4 Cell staining and measurement

A solution of 100 μL of 0.4% (w/v) sulforhodamine B (SRB; ICN Biomedicals) in 1% aqueous acetic acid was pipetted into each well to stain the cells that were fixed to the plate (100 μL final volume). The plates were stained for at least 30 minutes, then flicked over the sink to remove all excess stain. The wells were rinsed five times with 1% aqueous acetic acid, flicking to remove all liquid contents after each rinse, then allowed to dry overnight.

The stain was solubilised by addition of 100 μL of 10 mM Tris buffer (pH 10.5) to each well. The plates were then gently shaken on an orbital shaker (Edwards Bio-Line) for 10 min. The optical density (OD) of the solution in each well was measured at 492 nm using a Labsystems Multiskan EX 96-well plate reader, with Labsystems Genesis Version 3.00 software. The OD data was imported into Microsoft Excel for manipulation.

3.3.2.5 Determination of cytotoxicity

The mean background-corrected OD for each extract at each concentration was expressed as a percentage of the mean background-corrected OD for the drug vehicle control (Equations 3.1–3.3). Since the OD is directly proportional to cell number below 1.8 OD units[47], this

percentage represents the mean number of cells present in each well for each extract at each concentration, relative to the control (Equation 3.3). Example calculations are given below.

$$(OD_{A2} + OD_{A3} + OD_{A4} + OD_{A5} + OD_{A6}) / 5 - OD_{A1} = bcOD^{D1a} \quad (3.1)$$

$$(OD_{D2} + OD_{D3} + OD_{D4} + OD_{D5} + OD_{D6}) / 5 - OD_{D1} = bcOD^{Veh} \quad (3.2)$$

$$N^{D1a} = 100\% \times bcOD^{D1a} / bcOD^{Veh} \quad (3.3)$$

where $bcOD^{D1a}$ is the mean background corrected OD of extract 1 at 50 μg/mL (Figure 3.1: *D1a*), $bcOD^{veh}$ is the mean background-corrected OD of the control containing medium with 5% drug vehicle (Figure 3.1: *Veh*), N^{D1a} is the expression, as a percentage of the control, of the mean number of cells per well after 48 hour incubation in the presence of *D1a*, and OD_{A1} represents the optical density of the well at position A-1 on the 96-well plate (Figure 3.1), and so on for other subscripted *OD*.

All 365 extracts were tested for cytotoxicity and those with the lowest *N* (mean percentage of cells per well relative to the control), i.e., extracts with the greatest cytotoxic or cytostatic activity, were retested in the P388D1 cytotoxicity assay at 25 μg/mL and 5 μg/mL to confirm activity and assist prioritisation. The most active extracts at 5 μg/mL were then sent to the NCI, for testing in the 60 cell line panel established there, to further assist prioritisation. Extracts were chosen for chemical investigation based on the level of cytotoxicity, selective cytotoxicity for specific cell lines, and comparison of activity profiles with known cytotoxic agents using the COMPARE algorithm and database[168], which was performed by the NCI.

3.3.3 IC_{50} Determination of Isolated Compounds.

Selected isolated compounds were assessed for cytotoxicity against the P388D1 cell line as per the previously described procedure (Section 3.3.2), with the following deviations.

3.3.3.1 Extract preparation and application

Each compound was resuspended in the drug vehicle (1:9 DMSO:MeOH) at 2 mg/mL. A portion of the resuspended compound was removed and serially diluted with medium to six concentrations that were double the final test concentrations.

Aliquots (100 µL) of each concentration of compound in medium were added to designated wells of a 96-well plate that had been seeded and incubated for 24 hours. The 96-well plate layout was similar to that used for the screening of plant extracts (Section 3.3.2.2). Subsequent preparation, treatment and cell measurement was identical to that described above. Incubations were performed on three different passages of cells for replication purposes and to ensure that the activity seen did not represent a phenotypic anomaly of a single passage.

3.3.3.2 Analysis of cytotoxicity data

For each compound, the values for the mean percentage of cells per well relative to the control (*N*) for each incubation were plotted against the test concentrations, to give a total of 18 data points. A probit regression for a sigmoidal curve was performed using SPSS 11.01 for Windows to calculate the IC_{50} value and 95% confidence interval. The IC_{50} was defined as the value halfway between the top and bottom plateaux of the sigmoidal curve. For

compounds with data points that included the top plateau, slope and bottom plateau of the sigmoidal curve, all the points were included for the regression. For compounds that lacked data points for one of the plateaux of the curve, only data points on the slope were included for the regression. If the data points of the lowest concentrations did not extend to the top plateau, then the latter was assumed to be at 100% of control. Alternatively, if the data points of the highest concentrations did not extend to the bottom plateau, then the latter was assumed to be at 10% of control, which was the approximate value for the bottom plateau of the celastroloids (**22** and **25**) with complete cytotoxicity.

3.4 ISOLATION OF CYTOTOXIC COMPOUNDS

3.4.1 *Maytenus cunninghamii*

3.4.1.1 Plant material and extraction

Root-bark (130 g) of *Maytenus cunninghamii* (Hook.) Loes. was collected from the original collection site at Red Falls and stored at 4°C until extraction. The material was ground with a hammer at room temperature and then after freezing with liquid nitrogen. The ground material was extracted five times with 400 mL of acetone-MeOH (1:1) in a 1 L conical flask placed in a 10 L ultrasonicating bath (Unisonics) for 30 minutes. The five extractions were combined and filtered. The solvent was removed at 40°C under vacuum on a rotary evaporator (Büchi) to give 13.3 g of crude extract.

3.4.1.2 Crude extract treatment

Polyphenols and quinones were removed using a scaled-up adaptation of the method described earlier (Section 3.2.3). Briefly, a glass column (i.d. 2.2 cm, length 13 cm) was packed with 12 g of polyamide powder that had been suspended in hot MeOH, rinsed thoroughly and soaked in distilled water overnight. A portion (1.0 g) of the crude extract was resuspended in a small volume of MeOH and applied to the column, which was then eluted with 100 mL of water followed by 400 mL of MeOH, under gentle vacuum supplied from beneath using a sidearm conical flask. Solvent was removed from the combined MeOH and water eluents at 50°C under vacuum on a rotary evaporator, and then on a freeze-drier, to give 563 mg of extract.

3.4.1.3 Bioassay-guided fractionation

Following removal of polyphenols and quinones, the extract was subjected to bioassay-guided fractionation. The procedure is summarised in Figure 3.2. For cytotoxicity testing, the fractions were resuspended in the same volumes of solvent, regardless of the weight of individual fractions, in order to effectively compare activity between fractions and the original extract.

The treated extract (563 mg) was resuspended in 40 mL water, aided by ultrasonication, and centrifuged (Piccolo) prior to solvent-partitioning to reduce the considerable problems of emulsion formation and insoluble matter at the interface. The pellet was washed with water and centrifuged again. This second supernatant was added to the first, then partitioned between 200 mL each of dichloromethane (CH_2Cl_2) and water, with backwashing. The pellet from above was added to the CH_2Cl_2 extract, and the solvent was removed at 30°C

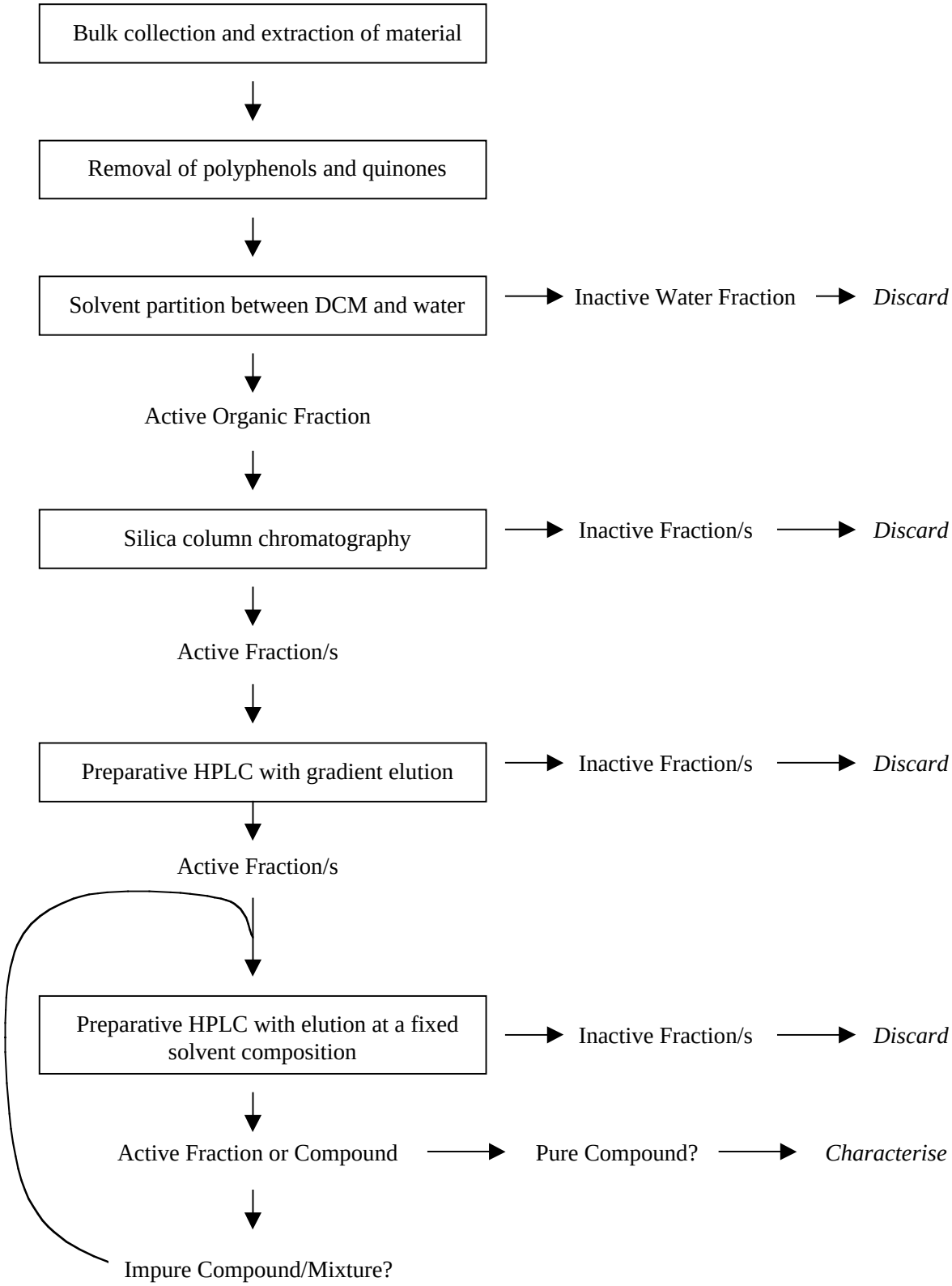


Figure 3.2. Flow-chart of general strategy for bioassay-guided fractionation of selected extracts following bulk collection, extraction and removal of polyphenols and quinones.

under vacuum on a rotary evaporator, leaving 383 mg of organic extract. The water extract was freeze-dried to remove the water, leaving 178 mg.

The cytotoxic fraction (organic extract, 383 mg) was resuspended in a minimal amount of CH_2Cl_2 and applied to a glass column (i.d. 3.0 cm, length 12 cm) that had been dry packed with silica gel (60 H, thin-layer chromatography grade; Merck) and solvated with CH_2Cl_2. The column was eluted under vacuum with 100 mL each of CH_2Cl_2, 40% ethyl acetate (EtOAc) in CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, and MeOH (fractions 1–6). The solvent was removed from each fraction at 30–40°C under vacuum on a rotary evaporator. Fractions 2 (40% EtOAc/CH_2Cl_2, 187 mg) and 3 (60% EtOAc/CH_2Cl_2, 50 mg) were cytotoxic and further separated by HPLC.

A portion (60 mg) of fraction 2 was injected in 1.0 mL MeOH onto a Whatman Partisil ODS-2 column (10 μm, i.d. 9.5 mm, length 500 mm). Stepwise gradient elution from 50%, 70%, 80%, 90%, 92.5% to 100% $MeOH_{(aq)}$ afforded fractions 2.1–2.6. The major component from the cytotoxic fraction 2.3 (80% $MeOH_{(aq)}$, 16.5 mg) was purified by HPLC on a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm) with 80% $MeOH_{(aq)}$ to yield celastrol (**22**, 13.8 mg).

Fraction 3 (50 mg) was injected in 1.0 mL MeOH onto a Whatman Partisil ODS-2 column (10 μm, i.d. 9.5 mm, length 500 mm). Stepwise gradient elution from 50%, 70%, 87%, to 100% $MeOH_{(aq)}$ afforded fractions 3.1–3.4. Fractions 3.2 (70% $MeOH_{(aq)}$, 10.8 mg) and 3.3 (87% $MeOH_{(aq)}$, 16.8 mg) were cytotoxic. Attempts to purify the major component, netzahualcoyoic acid (**25**), from fraction 3.2 proved unsuccessful. Therefore, isolation of this compound was reattempted from the crude extract, guided by thin-layer chromatography and ^1H NMR. The remaining crude extract (12.3 g) was dissolved in CH_2Cl_2 and solvent-partitioned with water. Insoluble material and the water extract were discarded, while 4.5 g of CH_2Cl_2 extract was obtained. This was chromatographed on silica gel (column i.d. 9 cm,

length 13 cm), eluting with 300 mL each of CH_2Cl_2, 15% EtOAc/CH_2Cl_2, 25% EtOAc/CH_2Cl_2, 35% EtOAc/CH_2Cl_2, 45% EtOAc/CH_2Cl_2, 55% EtOAc/CH_2Cl_2, 65% EtOAc/CH_2Cl_2, 100% EtOAc, and 100% MeOH. Due to substantial peak tailing, most fractions contained celastrol as a major component and netzahualcoyoic acid as a minor component. The 45% EtOAc fraction was repeatedly chromatographed on silica (column i.d. 3.0 cm, length 7–10 cm) with 10–15% EtOAc/CH_2Cl_2. Netzahualcoyoic acid was then recrystallised from MeOH and obtained as a pure red-orange solid (16.0 mg).

Fraction 3.3 (16.8 mg) was injected in 0.5 mL MeOH onto a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm). Elution with 75% $MeOH_{(aq)}$ yielded a novel quinonemethide nor-triterpene, Δ^{15}-celastrol (**32**, 1.1 mg, fraction 3.3.3), in addition to 2.5 mg netzahualcoyoic acid (fraction 3.3.2) and 11.7 mg of celastrol (fraction 3.3.4). The 65% EtOAc/CH_2Cl_2 silica column fraction of the remaining 12.3 g of crude extract, described above in the re-isolation of netzahualcoyoic acid, was observed to contain a small amount of Δ^{15}-celastrol. Repeated attempts to isolate Δ^{15}-celastrol from this fraction failed. During this re-isolation attempt, the 1.1 mg sample of Δ^{15}-celastrol isolated earlier had decomposed somewhat either while stored at –20°C or prior to storage. Therefore, structural characterisation relied solely on ^{1}H NMR, ESI-MS and UV/Vis data (HPLC with PDA multi-wavelength UV/Vis detector) on the 1.1 mg of sample that had been obtained prior to partial decomposition of the sample.

Celastrol (22): red-orange solid; mp 211–214°C; UV (95% $EtOH_{(aq)}$) λ_{max} (logε) 426 nm (4.13); IR ($CHCl_3$) ν_{max} 3230 (br), 2944, 2874, 1701, 1582, 1508, 1445, 1378 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$), see Table 2.2; ESI-MS *m/z* 473.2677 (calculated for $[C_{29}H_{38}O_4Na]^+$, 473.2662). Spectroscopic data was consistent with the literature[139].

Netzahualcoyoic acid (25): red-orange crystals; mp, begins decomposing at 215°C; UV (95% $EtOH_{(aq)}$) λ_{max} (log ε) 448 nm (4.02); IR ($CHCl_3$) ν_{max} 3400 (br), 2987, 2930, 1698, 1590, 1508, 1442, 1376, 1286 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 2.1; ^{13}C NMR (75.4 MHz, $CDCl_3$), see Table 2.1; ESI-MS *m/z* 471.2527 (calculated for $[C_{29}H_{36}O_4Na]^+$, 471.2506)

Δ^{15}-Celastrol (32): red-orange solid; UV (75% $MeOH_{(aq)}$) 428 nm; ^1H NMR (300 MHz, $CDCl_3$), see Table 2.2; ESI-MS *m/z* 471.2509 (calculated for $[C_{29}H_{36}O_4Na]^+$, 471.2506).

3.4.2 *Callitris intratropica*

3.4.2.1 Plant material and extraction

Leaves and stems (296 g) of *Callitris intratropica* R.T.Baker & H.G.Sm. were collected from the two individuals cultivated at the Douglas Campus that were originally collected. The fresh material was crushed by hand after freezing in liquid nitrogen, and extracted as described above (Section 3.4.1.1), giving 25.2 g of crude extract.

3.4.2.2 Crude extract treatment

Polyphenols and quinones were removed from a portion (2.0 g) of the crude extract as described above (Section 3.4.1.2), using 25 g polyamide powder in a glass column (i.d. 3.0 cm, length 17 cm), eluting with 300 mL of water followed by 500 mL of MeOH, producing 1.42 g of extract.

3.4.2.3 Bioassay-guided fractionation

The polyphenol/quinone free extract was subjected to bioassay-guided fractionation (see Section 3.4.1.3). The extract (1.43 g) was solvent partitioned between CH_2Cl_2 and water, giving 537 mg of organic extract and 897 mg of water extract.

The cytotoxic fraction (organic extract, 537 mg) was chromatographed with silica gel (column i.d. 3.0 cm, length 12 cm) and eluted with 100 mL each of CH_2Cl_2, 20% EtOAc/CH_2Cl_2, 40% EtOAc/CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, and MeOH (fractions 1–7). Fractions 2 (20% EtOAc/CH_2Cl_2, 149 mg) and 4 (60% EtOAc/CH_2Cl_2, 27 mg) were the most cytotoxic fractions and further separated using HPLC.

Fraction 2 (149 mg) was injected in 1.0 mL MeOH onto a Whatman Partisil ODS-2 column (10 μm, i.d. 9.5 mm, length 500 mm). Elution at 1.8 mL/min with 70% $MeOH_{(aq)}$ for 60 min, followed by continuous gradient elution from 70% to 100% $MeOH_{(aq)}$ over 120 min, afforded 17 fractions (2.1–2.17). The cytotoxic fraction 2.2 (eluted with 70% $MeOH_{(aq)}$; 13.7 mg) appeared to contain only one compound, which had a UV λ_{max} at 287 nm. The compound was identified as deoxypodophyllotoxin by ESI-MS and comparison of ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75.4 MHz, $CDCl_3$) shifts with literature values[166,169].

Fraction 4 (27 mg) was injected in 1.0 mL MeOH onto a Whatman Partisil ODS-2 column (10 μm, i.d. 9.5 mm, length 500 mm). Stepwise gradient elution from 40%, 60%, 80%, to 100% $MeOH_{(aq)}$ afforded fractions 4.1–4.4. The most cytotoxic fraction 4.2 (60% $MeOH_{(aq)}$, 2.0 mg) was injected in 0.5 mL MeOH onto the same column, and eluted with 50% $MeOH_{(aq)}$, affording a trace amount (<0.1 mg) of picropodophyllotoxin and 1.5 mg of podophyllotoxin. These were identified by ESI-MS, UV and comparison of ^{1}H NMR (300 MHz, $CDCl_3$) data with reported values[166].

CHAPTER 4.

POTENTIAL NEUROPROTECTIVE AGENTS FROM NORTH QUEENSLAND PLANTS:

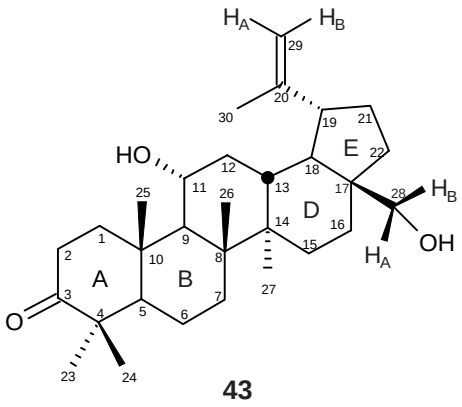
RESULTS AND DISCUSSION

4.1 ISOLATION AND CHARACTERISATION OF KYNURENINE-3-HYDROXYLASE INHIBITORS

4.1.1 Compounds from *Maytenus disperma*

Maytenus is a large genus of approximately 200 tropical and subtropical shrubs and small trees belonging to the Celastraceae[124]. Various species of *Maytenus* have been used traditionally to treat a range of ailments including skin cancer[125,126], rheumatism[125], fever and colds[126], dysentery[126], and as analgesics and contraceptives[126]. Bioactive compounds that have been isolated from this genus include agarofurans, diterpenes, triterpenes and the highly cytotoxic maytansinoids[127]. Friedelane triterpenes are frequently isolated from *Maytenus* and other genera in the Celastraceae. Orangebush, *Maytenus disperma,* is one of several species of *Maytenus* that are endemic to Australia. It is a shrub or tree found in vine thickets of near-coastal and coastal areas of northern New South Wales and Queensland[124]. Three triterpenes have been previously isolated from the root-bark of *M. disperma*, namely 3-oxooleana-11,13(18)-diene[170] and the two quinonemethides dispermoquinone[170] and pristimerin[171]. β-Sitosterol[172], a bis-diterpene[171], a quinonemethide diterpene[172] and three other diterpenes[172] have also been previously isolated from the root-bark. In this study, bioassay-guided fractionation has led to the isolation of two new triterpenes from the bark of *M. disperma*, 11α,28-dihydroxylupenone (**43**) and 2α,3β-dihydroxyfriedelan-29-oic acid (**53**).

4.1.1.1 11α,28-Dihydroxylupenone (**43**)



43

Compound **43** was isolated as a white solid (23 mg) and assigned the molecular formula $C_{30}H_{48}O_3$ from ESI-MS. The ^{1}H NMR (300 MHz, $CDCl_3$, Table 4.1) showed two methyl singlets and one three-methyl singlet from δ0.98-δ1.06, a vinylic methyl (δ1.66), two hydroxymethylene (CH_2OH) protons (δ3.72 and δ3.31), a hydroxymethine (CHOH) proton (δ3.85) and two vinylidene ($H_2C=C<$) protons (δ4.57 and δ4.68). The ^{13}C NMR (75.4 MHz, $CDCl_3$, Table 4.1) indicated a ketone carbonyl (δ219.0) in addition to these functional groups. Multiplicities of the ^{13}C signals were assigned from a DEPT experiment. The shifts of the vinylidene carbons were typical of a lup-20(29)-ene, while the carbonyl shift was typical of a ketone at C-3 in lup-20(29)-enes without further substitution in ring A[173-176]. The vinylic methyl (δ1.66) was consequently assigned to C-30. The hydroxymethylene was assigned to C-28 as this methyl is typically oxidised in lup-20(29)-enes, and substitution at C-23, C-24, C-25 or C-26 would be expected to exert a downfield shift on signals of nearby methyl groups. Thus it appeared that compound **43** was a hydroxylated derivative of 28-hydroxylupenone (**46**).

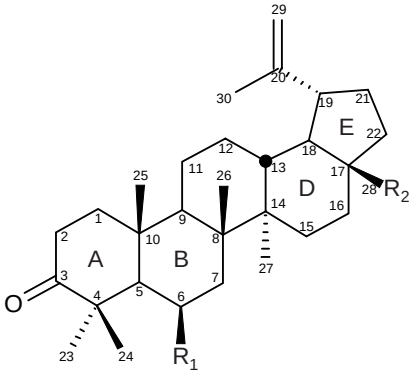
The hydroxymethine proton at δ3.85 had two large couplings (J = 10.9 Hz) and one small coupling (J = 5.4 Hz), which suggested one geminal, one axial-axial, and one axial-equatorial coupling. This narrowed the position of the hydroxyl as equatorial on either C-6, C-11, C-12, or C-21. A *g*HMBC correlation between δ_H3.86 and δ_C38.1, which was established as C-10 due to other *g*HMBC correlations, placed the hydroxyl at either C-6 or C-11. A 6α-hydroxyl would cause a downfield shift of the methyl at C-23, just as the 6β-hydroxyl causes a downfield shift of the methyls at C-24, C-25, and C-26 to

Table 4.1. ^{13}C and ^{1}H NMR shifts (δ), and *g*COSY, NOESY and *g*HMBC correlations for 11α,28-dihydroxylupenone (**43**) in $CDCl_3$. Shifts were referenced on the ^{13}C and residual ^{1}H peaks of $CDCl_3$ (δ_C77.0 and δ_H7.24, respectively).

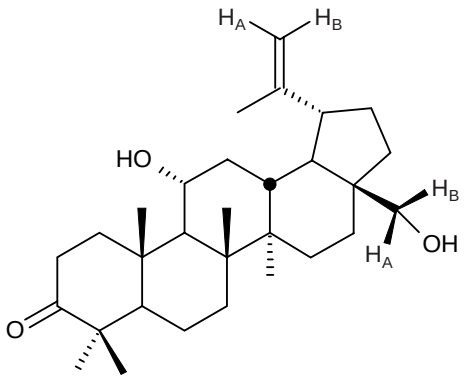
				^{1}H-^{1}H correlations (non-geminal)		^{1}H-^{13}C long-range correlations
C	δ_C		δ_H[a]	*g*COSY	NOESY	*g*HMBC
1	42.0	α	1.58 (H, m)	2β	2α	C-3, C-5/9, C-10, C-25
		β	2.62 (H, ddd, *J* = 13.6, 8.1, 5.7 Hz)	2α, 2β	2α, 2β	C-2, C-3, C-5/9, C-10, C-25
2	34.2	α	2.44 (H, ddd, *J* = 15.3, 8.1, 5.4 Hz)	1α, 1β	1α, 1β	C-1, C-3, C-10
		β	2.36 (H, m)	1α	1β	C-1, C-3, C-10
3	219.0					
4	47.6[b]					
5	54.7[c]	α	1.43[d] (H, m)			C-23, C-24
6	19.6	α, β	1.4 (H_2, m)		23, 25/26	
7	34.1	α, β	1.4 (H_2, m)			
8	42.4					
9	54.6[c]	α	1.45[d] (H, d, *J* = 10.9 Hz)	11β		C-11
10	38.1					
11	70.2	β	3.85 (H, td, *J* = 10.9, 5.4 Hz)	9α, 12α, 12β	12β, 25/26	C-9, C-10, C-12
12	37.2	α	1.15 (H, ddd, *J* = 13.6, 12.5, 10.9 Hz)	11β, 13β	18α	C-11, C-13
		β	1.89 (H, ddd, *J* = 12.5, 5.4, 3.7 Hz)	11β		C-9, C-11
13	36.3	β	1.75 (H, ddd, *J* = 13.6, 11.4, 3.7 Hz)	12α, 18α	15β, 26, 28_A	C-8/14, C-19
14	42.4					
15	26.9	α	1.04 (H, m)		16β	
		β	1.57 (H, m)	16α, 16β	13β, 26, 28_A	C-8/14, C-13, C-17, C-27
16	29.0	α	1.19 (H, m)	15β		C-14, C-28
		β	1.91 (H, m)	15β	15α	
17	47.7[b]					
18	48.0	α	1.63 (H, m)	13β	12α, 27	C-19, C-22, C-28
19	47.4	β	2.35 (H, m)	21β	28_B, 29_B	C-20, C-21, C-29, C-30
20	149.7					
21	29.6	α	1.22 (H, m)		22α	
		β	1.38 (H, m)	19β	22β	C-20
22	33.8	α	1.03 (H, m)		21α	
		β	1.85 (H, m)		21β	C-19, C-21, C-28
23	27.4		1.06 (H_3, s)		5α/6α	C-3, C-4, C-5, C-24
24	20.9		1.02 (H_3, s)		6α/β	C-3, C-4, C-5, C-23
25	16.7[e]		1.02 (H_3, s)		26	C-1, C-5, C-9, C-10
26	16.8[e]		1.02 (H_3, s)		13β, 15β, 25	C-7, C-8/14, C-9
27	14.5		0.98 (H_3, s)		7α/9α, 12α/16α, 18α	C-8/14, C-13, C-15
28	60.3	A	3.72 (H, br d, *J* = 10.9 Hz)		13β, 15β	C-16, C-17, C-22
		B	3.31 (H, d, *J* = 10.9, Hz)		19β	C-16, C-17, C-22
29	110.2	A	4.57 (H, br s)		30	C-19, C-30
		B	4.68 (H, br s)		19β	C-19, C-30
30	19.1		1.66 (H_3, br s)		29_A	C-19, C-20, C-29

[a] unable to define multiplicity due to overlapping signals

[b-e] shifts bearing the same superscipt are interchangeable



		R_1	R_2
Lupenone	**40**	H	Me
	41	OH	Me
	42	H	COOH



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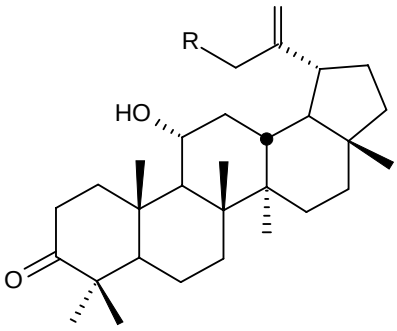
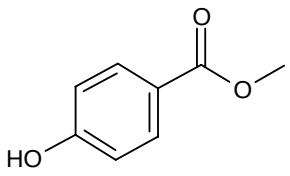
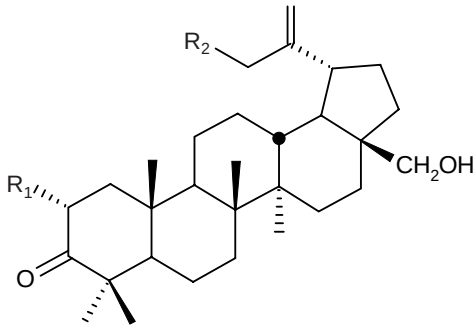


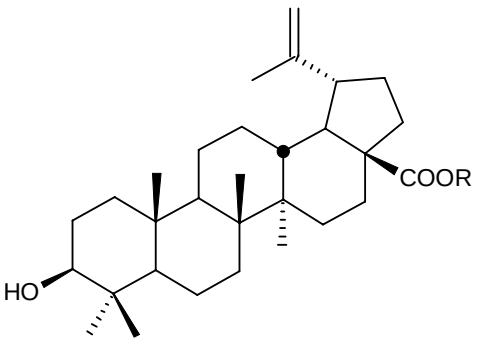
	R
44	H

45

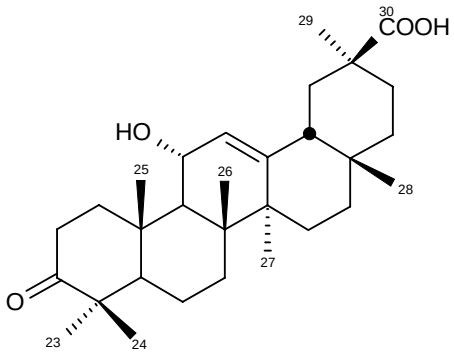




	R_1	R_2
46	H	H
47	OH	H
48	H	OH



		R
Betulinic acid	**49**	H
Methyl betulinate	**50**	Me



51

approximately δ1.4 in 6β-hydroxylupenone (**41**)[177]. The hydroxyl group was assigned to C-11 on this basis. As the hydroxyl was equatorial, compound **43** was assigned the structure 11α,28-dihydroxylupenone (11α,28-dihydroxy-3-oxolup-20(29)-ene).

One-bond ^{13}C-^{1}H connectivities were determined from a *g*HMQC experiment. The majority of ^{13}C shifts were assigned from *g*HMBC correlations. The ^{13}C shifts of C-23 and C-24 were not differentiated at this stage. Twelve other carbon signals remained unassigned due to many overlapping signals. C-9 was assigned from a *g*COSY correlation between δ3.85 (H-11α) and δ1.45. C-13 was assigned from a *g*COSY correlation between δ1.15 (H-12α) and δ1.75. This led to the assignment of C-27, and subsequently C-15, C-26 and C-7, from *g*HMBC correlations. Assignment of C-16 and C-18 relied on *g*COSY correlations from δ1.57 (H-15) to δ1.91, and from δ1.75 (H-13β) to δ1.63, respectively, which led to the assignment of C-22 from *g*HMBC correlations with the hydroxymethylene protons. The remaining three carbons, C-6, C-8 and C-14, were assigned by deduction.

The only NOESY correlations observable for the methyls attached to C-4 were with δ1.42-δ1.43 (H-5α, H-6α, H-6β), so were not informative in determining the stereochemistry at C-4. Assignment of C-23 (δ_H1.06, δ_C27.4) and C-24 (δ_H1.02, δ_C20.9) was therefore based on the ^{13}C shifts of lupenone in a detailed study of ^{13}C NMR spectra of lupenes[173]. This agrees with two recently reported 2D-NMR assignments for dammarane triterpenes with identical A, B and C rings to those of **43**[178,179]. Likewise, due to the lack of discriminating NOESY correlations, the stereochemistries at C-1 and C-2 follow the 2D-NMR assignment of 11α,20α-dihydroxy-3-oxodammarene[178]. Coupling constants and NOESY correlations were used to determine the stereochemistry at C-12 and to confirm the position of the 11α-hydroxyl group. The β protons on C-16 and C-22 were assigned as δ1.91 and δ1.85, respectively, due to predicted through space deshielding by the hydroxymethylene group. The remaining stereochemistries of the five-membered ring were then assigned based on α-α

and β-β NOESY correlations between the C-22 and C-21 protons. The stereochemistry at C-15 was determined by unambiguous NOESY correlations with H-13β, H-26 and H-28_A.

The NOESY correlation between H-29_B (δ4.68) and H-19β indicates that the isopropenyl group favours a conformation that places the vinylidene approximately in plane with the ring and the methyl below the plane of the ring. This is supported by the crystal structure reported for 6β-hydroxylupenone (**41**)[180]. The NOESY correlations of H-28_A (δ3.72) to H-13β and H-15β, and of H-28_B (δ3.31) to H-19β, demonstrate that the hydroxymethylene is sterically hindered, and its preferred conformation is with the hydroxyl facing away from the bulk of the molecule, approximately in line with the bond between C-17 and C-18.

Other triterpenes with an 11α-hydroxyl group that have been reported from *Maytenus* spp. include 11α-hydroxylupenone (**44**)[174,176], its C-30 *p*-hydroxybenzoic acid ester (**45**)[181], and a number of oleananes[182,183], such as 11α-hydroxy-3-oxoolean-12-en-30-oic acid (**51**)[184]. Two other 28-hydroxylupenones have been reported from *Maytenus* spp., namely 2α,28-dihydroxylupenone (**47**)[185] and 28,30-dihydroxylupenone (**48**)[183]. Both lupenone (**40**)[186] and lupenon-28-oic acid (**42**)[187] have also been reported from *Maytenus* spp. Compound **43** (11α,28-dihydroxylupenone) is the first report of a lupenone derivative with hydroxyls at both the 11α and 28 positions.

4.1.1.2 2α,3β-Dihydroxyfriedelan-29-oic acid (**53**)

Compound **53** was isolated as a white solid (2.5 mg) and was assigned the molecular formula $C_{30}H_{48}O_4$ from ESI-MS. The ^{1}H NMR (300 MHz, $CDCl_3$, Table 4.2) spectrum showed six methyl singlets and one methyl doublet (J = 7 Hz) between δ0.83 and δ1.21, and two singlets at δ3.53 and δ3.97, suggesting a dihydroxylated triterpene. A *g*COSY correlation

Table 4.2. Observable ^{1}H NMR shifts (δ) and *g*COSY, NOESY and *g*HMBC correlations for 2α,3β-dihydroxyfriedelan-29-oic acid (**53**) in $CDCl_3$. Shifts were referenced on the residual ^{1}H peak of $CDCl_3$ (δ_H7.24).

		^{1}H-^{1}H correlations (non-geminal)		^{1}H-^{13}C long-range correlations
H	δ_H[a]	*g*COSY	NOESY	*g*HMBC
1α	1.54 (H, m)	2β		
1β	1.78 (H, m)	2β		
2β	3.97 (H, br s)	1α, 1β, 3α		
3α	3.53 (H, br s)	2β, 4α		
4α	1.58 (H, m)	3α, 23		
8α	1.31 (H, m)			
18β	1.52 (H, m)		26	
19α	2.33 (H, br d, *J* = 13.8 Hz)			
19β	1.52 (H, m)			
21α	2.12 (H, dm, *J* = 14.7 Hz)	22β		
21β	1.33 (H, m)	22α, 22β		
22α	1.99 (H, td, *J* = 13.8, 3.6 Hz)	21β		
22β	0.95 (H, m)	21α, 21β		
23	0.91 (H_3, d, *J* = 7 Hz)	4α		C-3, C-4, C-5
24	0.90 (H_3, s)		25	C-4, C-5, C-6, C-10
25	0.84 (H_3, s)		24	C-8, C-9, C-10, C-11
26	0.83 (H_3, s)		18β, 28	C-8, C-13/14, C-15
27	0.93 (H_3, s)			C-12, C-13/14, C-18
28	1.05 (H_3, s)		26	C-16/22, C-17, C-18
30	1.21 (H_3, s)			C-19, C-20, C-29

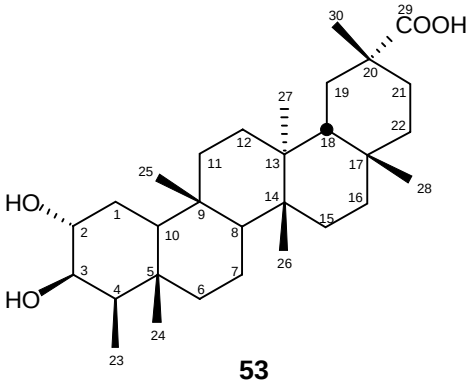
[a] multiplicities undefined due to overlapping signals are designated 'm'

Table 4.3. ^{13}C NMR shifts (δ) for 2α,3β-dihydroxyfriedelan-29-oic acid (**53**) in $CDCl_3$. Shifts were referenced on the $CDCl_3$ peak (δ_C77.0).

C	δ_C	C	δ_C	C	δ_C
1	23.6	11	35.0	21	29.4[a]
2	71.3	12	29.6[a]	22	36.3[c]
3	76.2	13	39.1[b]	23	10.9
4	43.5	14	39.3[b]	24	15.8
5	37.6	15	28.9	25	18.7
6	41.4	16	36.2[c]	26	15.8
7	17.5	17	30.1	27	17.9
8	50.4	18	44.4	28	31.8
9	36.4	19	30.3	29	183.3
10	52.1	20	40.3	30	32.0

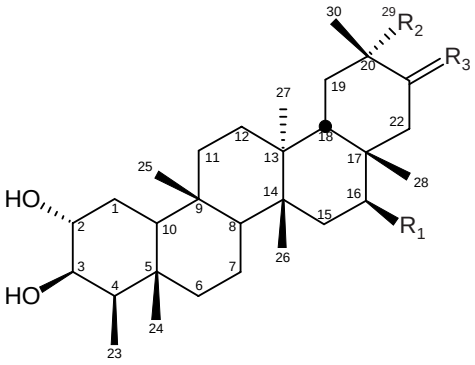
[a-c] shifts bearing the same superscript are interchangeable

between the two oxymethine (CHOR) protons indicated they were on adjacent carbons, while their small couplings in the ^{1}H spectrum suggested the protons were equatorial and the hydroxyls therefore axial. The ^{13}C NMR spectrum (75.4 MHz, $CDCl_3$, Table 4.3) showed a carboxyl carbonyl resonance (δ183.3) and the presence of two oxymethine (CHOR) groups (δ71.3 and δ76.2) was confirmed by a DEPT experiment. A methyl at δ 10.9 was consistent with the C-23 of friedelane triterpenes, which are common in *Maytenus* and the Celastraceae. Comparison of the ^{13}C data with that reported for 2α,3β-dihydroxyfriedelane (**52**)[188] showed good agreement for rings A and B (except C-8) and attached methyls, suggesting that compound **53** was a carboxylic acid derivative of 2α,3β-dihydroxyfriedelane. The remaining ^{13}C shifts, including C-8, were almost identical to those reported for rings C, D and E and attached methyl groups in maytenonic acid (3-oxofriedelan-29-oic acid, **55**)[161] and various ring A derivatives of maytenonic acid[189]. The position of the carboxylic acid in maytenonic acid has been confirmed by X-ray crystallography[161]. A *g*HMBC correlation between δ183.3 (COOH) and δ1.21 (H_3) confirmed that the carboxyl group and a methyl group were attached to C-20. Thus, compound **53** was assigned the structure 2α,3β-dihydroxyfriedelan-29-oic acid.

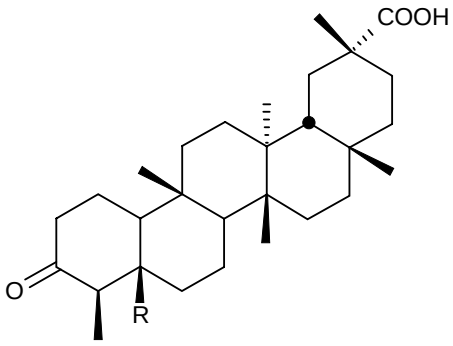


53

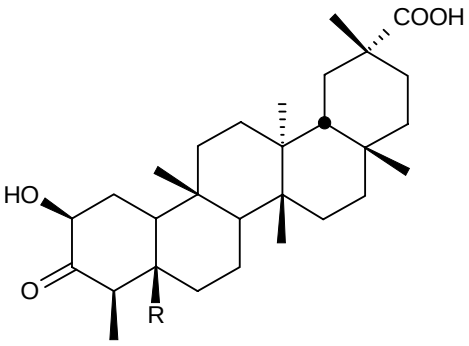
One-bond connectivities (*g*HMQC) between ^{1}H and ^{13}C shifts were observed for all methyls and five methines, but for few methylene protons due to insufficient signal sensitivity. *g*HMBC correlations from the methyl doublet at δ0.91 to δ76.2 and the corresponding *g*HMQC correlation placed the δ3.55 proton at H-3, and δ3.95 at H-2 by inference. *g*COSY correlations confirmed this assignment and allowed the proton shifts on C-1 to be assigned. The stereochemistry of the protons on C-1 were taken from the assignment of 2α,3β-dihydroxyfriedelane (**52**) by Moiteiro and co-workers[188]. The ^{1}H and ^{13}C shifts of



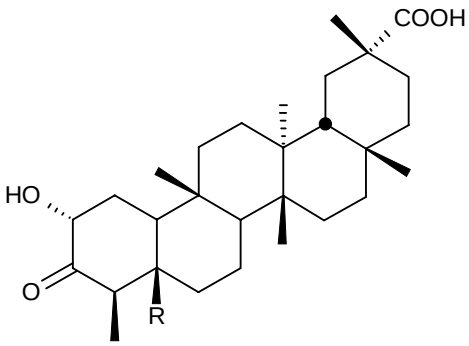
	R_1	R_2	R_3
52	H	Me	H_2
53	H	COOH	H_2
54	Me	COOH	O



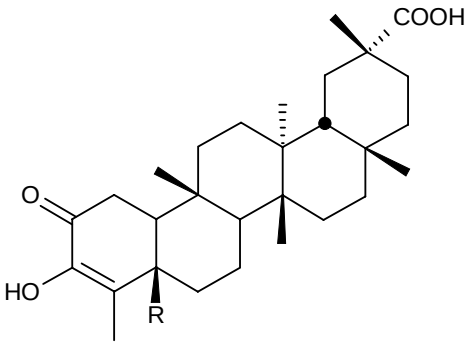
		R
Maytenonic acid	**55**	Me
	56	CHO



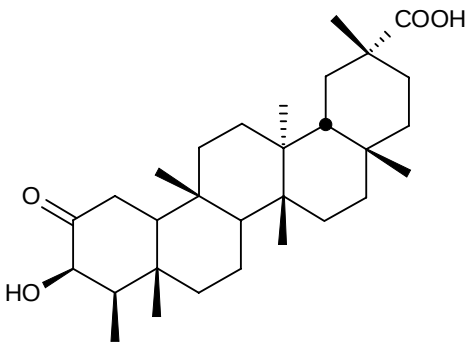
	R
57	Me
58	CHO



	R
59	Me
60	CHO



		R
	61	Me
Cangoronine	**62**	CHO



63

CH_3-23 (δ_H0.91, δ_C10.9), CH_3-24 (δ_H0.90, δ_C15.8) and CH_3-25 (δ_H0.84, δ_C18.7) were assigned from *g*HMQC correlations and *g*HMBC correlations. *g*HMBC correlations of C-18 (δ44.4) to the protons of two methyl groups (δ0.93 and δ1.05) permitted assignment of CH_3-26 (δ_H0.83, δ_C15.8) by deduction. CH_3-28 (δ_H1.05, δ_C31.8) was assigned from a NOESY correlation with H_3-26 (δ0.83), and the final methyl was therefore assigned to C-27 (δ_H0.93, δ_C17.9). Additional *g*HMBC correlations of methyl protons allowed assignment of all ^{13}C shifts except C-1, C-7, C-16, C-19, C-21 and C-22.

Assignment of C-1 and C-7 was based on the shifts reported for these carbons in 2α,3β-dihydroxyfriedelane[188] and maytenonic acid[161]. Of the four remaining unassigned carbons, δ36.3, δ29.4 and δ30.3 had one-bond (*g*HMQC) correlations with ^{1}H shifts at δ1.99, δ2.12 and δ2.33, respectively. The ^{13}C shifts at δ30.3 and δ29.4 exhibited *g*HMBC correlations with the methyl protons of C-30 (δ1.21), which placed them at C-19 and C-21. *g*COSY correlations between the protons on δ29.4 and δ36.3 permitted their assignment to C-21 and C-22 respectively, and therefore assignment of δ30.3 to C-19 and δ36.2 to C-16. The protons at δ1.99, δ2.12 and δ2.33 were considered likely to be the α (below-plane) protons in ring E due to the deshielding effect that a 29-carboxylic acid would impose upon these protons due to spatial proximity. This deshielding effect would only be observed if rings D and E adopted a chair-chair conformation (Figure 4.1b), which would place the carboxyl group in an axial position, rather than the alternative boat-boat conformation (Figure 4.1a), which would place the carboxyl group in an equatorial position. The degree of steric repulsion between the C-27 and C-29 groups is considered responsible for the conformational differences, and 29-carboxyl substitution provides sufficiently low steric repulsion to favour the chair-chair conformation[161-163]. The observed shifts are similar to the α protons in ring E of 2β-hydroxy-24-oxomaytenonic acid (**58**) and 2α-hydroxy-24-oxomaytenonic acid (**60**), reported below (Sections 4.1.2.1 and 4.1.2.2). The corresponding β protons were assigned from the strong geminal *g*COSY correlations.

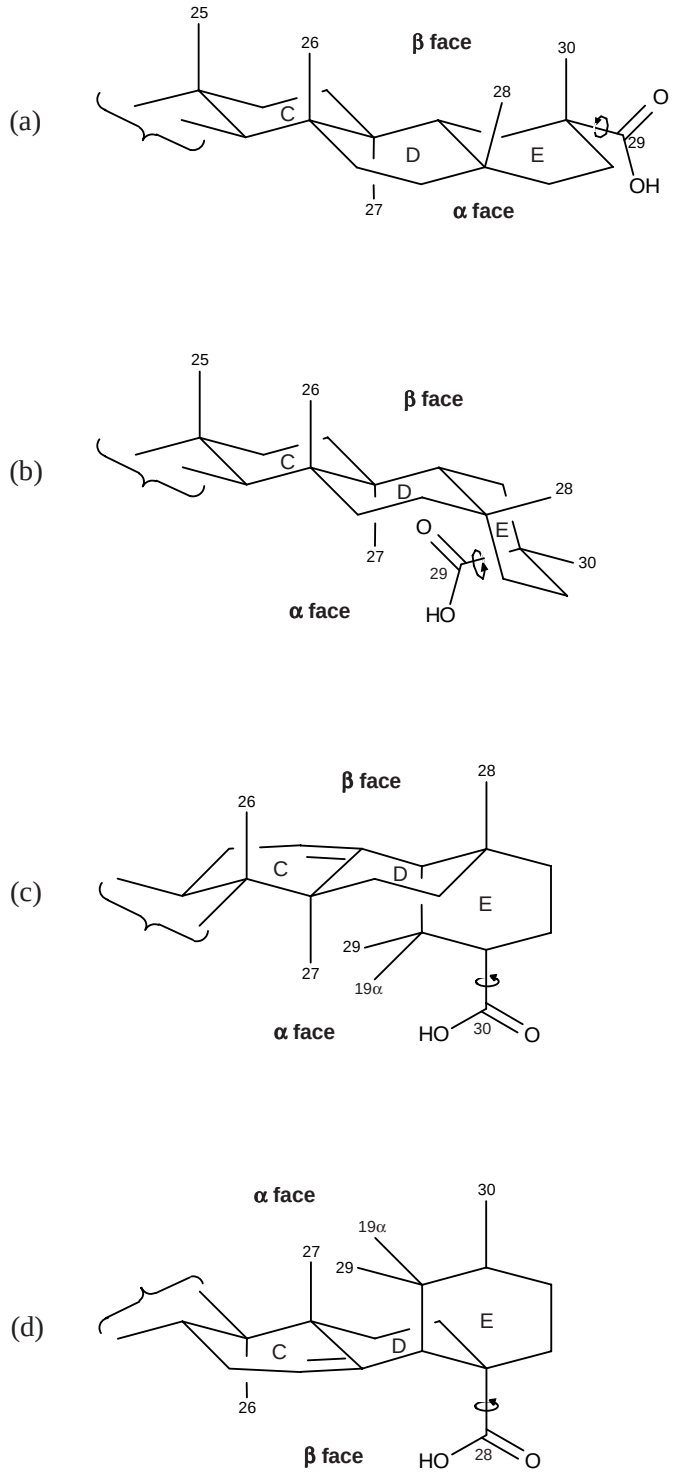


Figure 4.1. Three-dimensional stick diagrams showing the conformations of rings D and E and the position of the carboxyl group in some triterpene acids. Rings D and E in 2α,3β-dihydroxyfriedelan-29-oic acid (**53**) and other 29-carboxyl substituted friedelanes exist primarily in a chair-chair conformation (b) rather than the alternate boat-boat conformation (a). The carboxyl group in 29-carboxyl substituted friedelanes (b) occupies a similar spatial position to that in 30-carboxyl substituted ursanes when viewed from the same face (c) and to that in 28-carboxyl substituted ursanes when viewed from the opposite face (d). A 19α-hydroxyl group, as in uncaric acid (**78**), would provide an additional hydrophilic group on the opposite face to the 28-carboxyl group (d).

A less oxidised form of **53**, 2α,3β-dihydroxyfriedelane (**52**), has been reported in six species across the Celastraceae[190], Fagaceae[191,192], Buxaceae[193] and Passifloraceae[194]. Conversely, compounds **59**[195-201,147], **63**[202,197,189,201], **66**[203-205,199,147] and **54**[206], which are more highly oxidised forms of **53**, have only been reported from the Celastraceae.

4.1.2 Compounds from *Maytenus cunninghamii*

The yellow-berry bush, *Maytenus cunninghamii*, is a widespread endemic shrub that is found in inland and near-coastal areas of tropical and subtropical Australia[124]. There had been no reported chemical investigation of *M. cunninghamii* prior to this study. Two new triterpenes, 2β-hydroxy-24-oxomaytenonic acid (**58**) and 2α-hydroxy-24-oxomaytenonic acid (**60**), and four known triterpenes, 3β-hydroxyurs-12-en-30-oic acid (dulcioic acid, **79**), 2α-hydroxymaytenonic acid (**59**), 24-deoxocangoronine (**61**) and cangoronine (**62**), were isolated from the root-bark of the Red Falls population of *M. cunninghamii*, in addition to the three cytotoxic quinonemethide triterpenes, netzahualcoyoic acid (**25**), Δ^{15}-celastrol (**32**) and celastrol (**22**), described in Section 2.1.2. This is the first report of the occurrence of **61** and **79** in *Maytenus*.

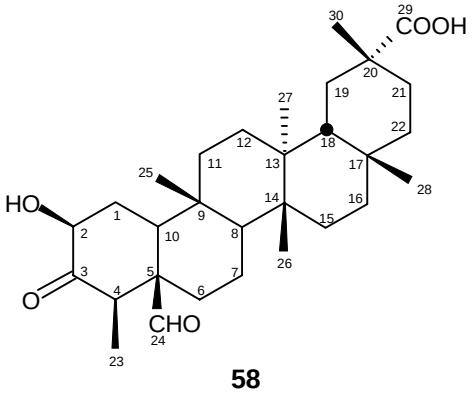
4.1.2.1 2β-Hydroxy-24-oxomaytenonic acid (**58**)

Compound **58** was isolated as a white solid (6.4 mg) and was assigned the molecular formula $C_{30}H_{46}O_5$ from ESI-MS. The ^{1}H NMR (300 MHz, $CDCl_3$, Table 4.4) spectrum showed five methyl singlets and one methyl doublet (J = 6.9 Hz) between δ 0.67 and δ 1.22, a one proton singlet at δ9.64, an oxymethine (CHOR) at δ4.16 and a very broad peak of the corresponding hydroxyl at δ6.2. The peak at δ9.64 remained upon addition of a drop of D_2O, and was

Table 4.4. ^{13}C and ^{1}H NMR shifts (δ), and *g*COSY, NOESY and *g*HMBC correlations for 2β-hydroxy-24-oxomaytenonic acid (**58**) in $CDCl_3$. Shifts were referenced on the ^{13}C and residual ^{1}H peaks of $CDCl_3$ (δ_C77.0 and δ_H7.24, respectively).

				^{1}H-^{1}H correlations (non-geminal)		^{1}H-^{13}C long-range correlations
C	δ_C		δ_H[a]	*g*COSY	NOESY	*g*HMBC
1	30.7	α	2.42 (H, m)	2α	2α, 11β	C-2, C-3, C-5/10
		β	1.82 (H, m)	2α	24, 25	C-2, C-5/10
2	74.4	α	4.18 (H, br d, *J* = 10.5 Hz)	1α, 1β	1α, 4α, 10α	C-1
		β	6.2 (OH, v br s)			
3	210.0					
4	51.6	α	2.23 (H, q, *J* = 6.9 Hz)	23	2α, 6α, 10α, 23	C-3, C-5/10, C-6, C-23, C-24
5	56.7					
6	34.2	α	1.26 (H, m)		4α, 10α	
		β	2.45 (H, br d, *J* = 13.5 Hz)		23, 24	C-5/10
7	18.6	α	1.62 (H, m)			C-5
		β	1.43 (H, m)		24, 25, 26	
8	49.7	α	1.42 (H, m)		10α, 27	C-6
9	37.2					
10	56.7	α	1.80 (H, m)		2α, 4α, 6α, 8α, 11α	C-1, C-2, C-5/10, C-24, C-25
11	33.8	α	1.27 (H, m)		27	
		β	1.42 (H, m)		1α	
12	29.4	α	1.58 (H, m)		27	
		β	1.29 (H, m)		25, 26	
13	39.0					
14	39.2					
15	29.3		1.24 (H, m)			C-26
			1.4 (H, m)			
16	36.0	α	1.37 (H, m)			
		β	1.64 (H, m)		26, 28	
17	30.1					
18	44.1	β	1.53 (H, m)		26, 28	
19	30.2	α	2.32 (H, br d, *J* = 13.2 Hz)		27	C-13, C-17, C-20, C-21, C-29, C-30
		β	1.52 (H, m)		28, 30	C-29
20	40.4					
21	29.5	α	2.15 (H, dm, *J* = 12.0 Hz)	22α	22α, 22β, 30	C-22
		β	1.34 (H, m)	22α, 22β		C-20, C-29
22	36.5	α	1.99 (H, td, *J* = 14.0, 4.0 Hz)	21α, 21β	21α, 27	C-17, C-28
		β	0.97 (H, m)	21β	21α	C-18
23	7.8		0.90 (H_3, d, *J* = 6.9 Hz)	4α	4α, 6β, 24	C-3, C-4, C-5
24	204.6		9.64 (H, s)		1β, 6β, 7β, 23, 25	C-5/10, C-6
25	18.2		0.67 (H_3, s)		1β, 7β, 12β, 24, 26	C-8, C-9, C-10, C-11
26	16.2		0.83 (H_3, s)		7β, 12β, 16β, 18β, 25, 28	C-8, C-13/14, C-15
27	17.8		0.98 (H_3, s)		8α, 11α, 12α, 19α, 22α	C-12, C-13/14, C-18
28	31.8		1.06 (H_3, s)		16β, 18β, 19β, 21β, 26	C-16, C-17, C-18, C-22
29	184.6					
30	31.6		1.22 (H_3, s)		19β, 21α	C-19, C-20, C-21, C-29

[a] multiplicities undefined due to overlapping signals are designated 'm'



58

therefore determined to be an aldehydic proton rather than an –OH stabilised by hydrogen bonding. The ^{13}C (75.4 MHz, $CDCl_3$, Table 4.4) and DEPT spectra confirmed the presence of an aldehyde (δ204.6), and also indicated the presence of a ketone (δ210.0) and a carboxylic acid (δ184.6). The ^{13}C shift of the methyl at δ7.8 suggested that it was the C-23 of a 3-oxofriedelane. Since the typical shift of C-23 in 3-oxofriedelanes is δ6.8[207], the slight downfield shift of the C-23 in **58** suggested that it was functionalised at C-24.

One-bond connectivities between ^{13}C and ^{1}H shifts were determined by a *g*HSQC experiment, which gave much better resolution of methylene correlations than an equivalent *g*HMQC experiment. *g*HMBC correlations indicated that the carboxyl group was attached to C-20. *g*HMBC correlations of CH_3-23 (δ_H0.90, δ_C7.8) to the ketone (δ210.0), a methine carbon (δ51.6) and a quaternary carbon (δ56.7) confirmed the position of the ketone and indicated the C-5 shift was δ56.7. This provided further evidence for the aldehyde at C-24, since the typical C-5 shift of 3-oxofriedelanes is approximately δ42–43[207]. The ^{13}C shift of C-5 was confirmed by *g*HMBC correlations of H-4α (δ2.23) to both the ketone (δ210.0) and the aldehyde (δ204.6). All ^{13}C shifts of compound **58** were assigned from *g*HMBC correlations except for C-1, C-2, C-16, C-21, C-29 and C-30.

The position of the hydroxyl group was narrowed down to either C-1 or C-2 based on *g*HMBC correlations. The *g*COSY correlations of the C-1 and C-2 protons were not discriminating due to the near identical shifts of one of these protons (δ1.82) and H-10α (δ1.80). A NOESY correlation was observed between δ1.82 and δ0.67, which could only be ascribed to a correlation between H-1β and H_3-25. This and another NOESY correlation

between δ2.42 (H-1α) and δ1.42 (H-11β) indicated that the position of the hydroxyl was at C-2. The stereochemistry at C-2 was unambiguously identified as β-hydroxy from the large axial coupling of the oxymethine proton (δ4.16, br d, J = 10.5 Hz) and a NOESY correlation between δ4.16 and δ2.23 (H-4α). The shift of H-4α is almost identical to that reported for 2β-hydroxymaytenonic acid (**57**)[189], and quite different to the lower field shift of H-4α (δ2.79) in 2α-hydroxymaytenonic acid (**59**; authenticated sample, measured in $CDCl_3$) that is caused by a 1,3-diaxial interaction with the hydroxyl group.

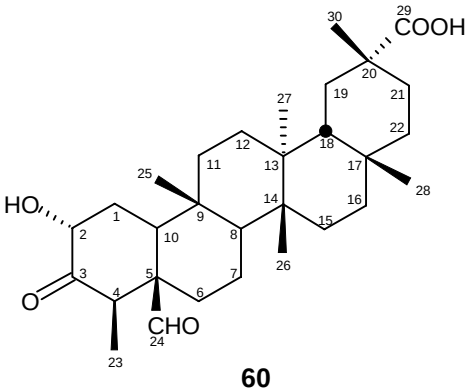
The stereochemistry at C-20 was established from a NOESY correlation between H-19β (δ1.52) and δ1.22, indicating that the methyl was β and therefore the carboxyl group was α. This assignment was confirmed by comparison with the ^{1}H and ^{13}C shifts of 2β-hydroxymaytenonic acid (**57**)[189] and maytenonic acid (**55**), which was substantiated by X-ray crystallography[161]. Thus, the structure of compound **58** was established as 2β-hydroxy-24-oxomaytenonic acid.

*g*COSY correlations of the protons on C-21 were used to assign C-22 and consequently C-16. The multiplicities of some of the overlapping proton shifts were discerned from ^{1}H-^{1}H selective decoupling experiments. The stereochemistries of the protons were determined from NOESY correlations and coupling constants. The coupling constants and downfield shifts of the α protons in ring E are consistent with rings D and E adopting a chair-chair conformation (Figure 4.1b), as described previously in the assignment of 2α,3β-dihydroxyfriedelan-29-oic acid (**53**; Section 4.1.1.2). The NOESY correlations of H-24 (δ9.64) to H-1β, H-6β, H-7β, H_3-23 and H_3-25 suggest that the aldehyde group is not sterically hindered and may freely rotate.

The occurrence of a 2β-hydroxymaytenonic acid derivative is unusual, since 2β-hydroxymaytenonic acid (**57**) itself has only been reported from one species

(*Tripterygium wilfordii*)[189]. Conversely, 2α-hydroxymaytenonic acid (**59**) is much more common (see below, Section 4.1.2.3), as are 2α-hydroxylated triterpenes in general. It is possible that 2β-hydroxy-24-oxomaytenonic acid (**58**) is an artefact arising during isolation from epimerisation of 2α-hydroxy-24-oxomaytenonic acid (**60**), since the hydroxyl is α to a carbonyl group.

4.1.2.2 2α-Hydroxy-24-oxomaytenonic acid (**60**)



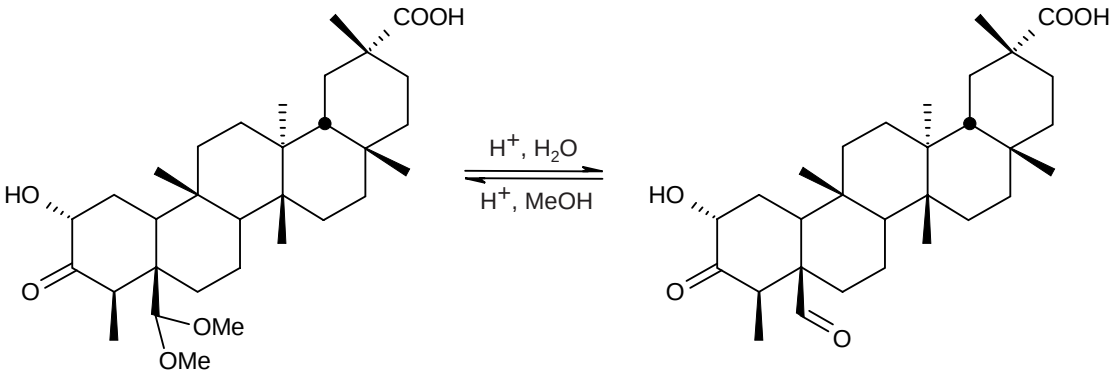
60

Compound **60** was isolated as a white solid (4.7 mg) and was assigned the molecular formula $C_{30}H_{46}O_5$ from ESI-MS. The ^{1}H NMR (300 MHz, $CDCl_3$) spectrum showed five methyl singlets and one methyl doublet (J = 6.9 Hz) between δ 0.67 and δ 1.23, an oxymethine (CHOR) triplet (J = 3 Hz) at δ4.22, a one proton singlet at δ9.68 and a methine proton quartet at δ2.77. The spectrum was almost identical to 2β-hydroxy-24-oxomaytenonic acid (**58**), except that the H-4α quartet was at δ2.77 rather than δ2.23 for **58**, and the coupling of the proton at δ4.22 suggested that the oxymethine was in an equatorial position rather than the axial position seen in compound **58**. The H-4α shift at δ2.77 is almost identical with that of 2α-hydroxymaytenonic acid (**59**; authenticated sample, measured in $CDCl_3$), which is due to a 1,3-diaxial interaction with the hydroxyl group. Therefore, compound **60** was assigned the structure 2α-hydroxy-24-oxomaytenonic acid.

Compound **60** was isolated with a small amount of the corresponding acetal (**64**), which was observed in fractions that contained **60** throughout most of the fractionation procedure and

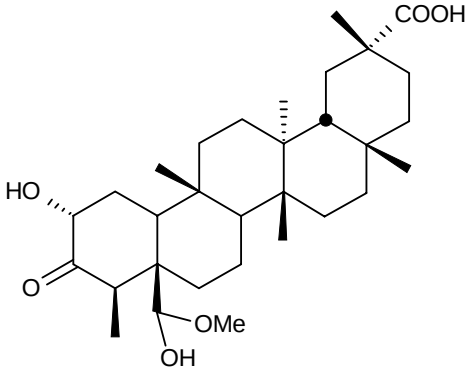
could not be separated. The acetal derivative co-occurred with compound **60** following fractionation of a fraction that clearly did not contain any of the acetal derivative, although it may have contained a hemiacetal derivative (**65**; 1H-singlet at δ4.76, 3H-singlet at δ3.34). Further information in this regard could not be obtained from the crude extract or earlier fractions, as **60** was isolated as only a very minor component. Following this, **64** was considered to be an artefact arising under acidic conditions in the presence of methanol, which was used for recovering the fractions after NMR spectroscopy. The ^{1}H spectrum of **64** showed two methoxyl singlets at δ3.36 and δ3.39 and a one proton singlet at δ4.82. On treatment with 10 μL of 50% aqueous trifluoroacetic acid in approximately 500 μL of $CDCl_3$ and monitored by ^{1}H NMR at 25°C, the height of these peaks reduced while the height of the aldehyde peak of **60** increased and a methanol peak appeared and continued to increase until completion (45 minutes; Scheme 4.1). An alternate proposed structure to the hemiacetal derivative (**65**) is that of 24-methoxyorthosphenic acid (**67**). A similar compound, 3α-deoxy-24-ethoxyorthosphenic acid (**68**), has been isolated from *Tripterygium wilfordii* (Celastraceae)[189], but may have been an artefact from ethanol and acid impurities in the EtOAc used in the isolation procedure.

The full characterisation of compound 60 was performed in C_5D_5N because of slightly improved solubility and complete resolution of two methyls that had overlapping ^{1}H shifts in $CDCl_3$. The ^{1}H and ^{13}C spectra in C_5D_5N (Table 4.5) confirmed that none of the acetal form (**64**) was present. ^{13}C, *g*HSQC, and *g*HMBC experiments confirmed the positions of the ketone (C-3) and aldehyde (C-24) and permitted assignment of all ^{13}C shifts except C-16, C-19, C-21, and C-22. *g*COSY correlations between the protons on the δ30.2 and δ37.1 carbons, along with *g*HMBC correlations, established these as C-21 and C-22 respectively. This allowed C-16 and C-19 to be assigned from *g*HMBC. The C-29 position of the carboxylic acid was confirmed by a NOESY correlation between H_3-30 and H-19β, and comparison with the ^{13}C shifts in C_5D_5N of 2α-hydroxymaytenonic acid (**59**)[198]. The

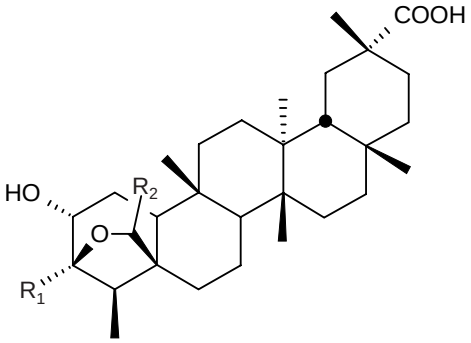


64 **60**

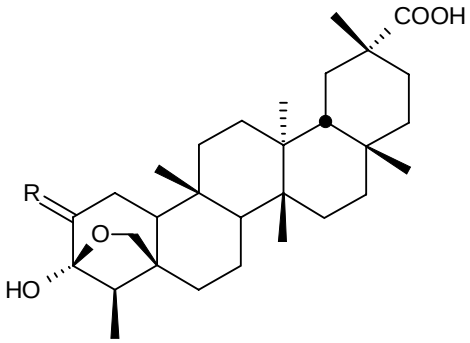
Scheme 4.1



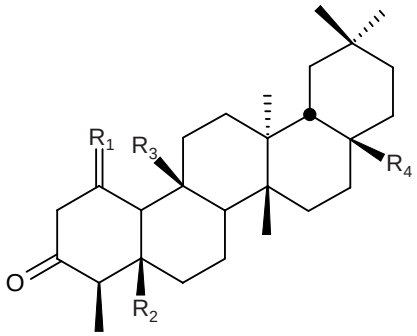
65



		R_1	R_2
Orthosphenic acid	**66**	OH	H
	67	OH	OMe
	68	H	OEt



		R
Salaspermic acid	**69**	H_2
Wilforic acid E	**70**	O



	R_1	R_2	R_3	R_4
71	O	CHO	Me	Me
72	H_2	Me	CHO	Me
73	H_2	Me	Me	CHO

Table 4.5. ^{13}C and ^{1}H NMR shifts (δ), and *g*COSY, NOESY and *g*HMBC correlations for 2α-hydroxy-24-oxomaytenonic acid (**60**) in C_5D_5N. Shifts were referenced on the ^{13}C-4 and residual ^{1}H-4 peaks of C_5D_5N (δ_C135.5 and δ_H7.55, respectively).

				^{1}H-^{1}H correlations (non-geminal)		^{1}H-^{13}C long-range correlations
C	δ_C		δ_H[a,b]	*g*COSY	NOESY	*g*HMBC
1	29.2	α, β	2.17 (H_2, m)	2β, 10α	2β, 11b, 24, 25	
2	73.2	β	4.55 (H, t, *J* = 2.7 Hz)	1α/β	1α/β	C-10
3	210.8					
4	48.7	α	3.21 (H, q, *J* = 6.8 Hz)	23	6α, 10α, 23	C-3, C-5, C-23, C-24
5	57.0					
6	33.7[c]	α	1.18 (H, m)		4α	
		β	2.41 (H, m)		23, 24	
7	18.8	β	1.47 (H, m)		24, 25	
8	49.5	α	1.46 (H, m)		10α, 27	
9	36.8					
10	52.3	α	2.54 (H, m)	1α/β	4α, 8α	C-1
11	33.8[c]	β	1.27 (H, m)		1α	
12	29.5		1.62 (H, m)			
13	39.2					
14	39.2					
15	29.2		1.36 (H, m)			
16	36.4		1.36 (H, m), 1.63 (H, m)			
17	30.2					
18	44.5	β	1.49 (H, m)		26, 28	C-13/14, C-20, C-27, C-28
19	30.6	α	2.61 (H, br d, *J* = 14.1 Hz)		27	C-17/21, C-29
		β	1.63 (H, m)		30	C-20, C-29, C-30
20	40.5					
21	30.2	α	2.46 (H, m)	22α		
		β	1.41 (H, m)	22α, 22β	28	C-30
22	37.1	α	2.23 (H, td, *J* = 14.0, 3.9 Hz)	21α, 21β	27	C-17/21
		β	0.98 (H, m)	21β		
23	7.9		0.92 (H_3, d, *J* = 6.8 Hz)	4α	4α, 6β, 24	C-3, C-4, C-5
24	206.2		9.87 (H, s)		1b, 6β, 7β, 23, 25	C-5
25	17.8		0.66 (H_3, s)		1b, 7β, 24, 26	C-8, C-9, C-10, C-11
26	16.1		0.75 (H_3, s)		18β, 25, 28	C-8, C-13/14, C-15
27	17.6		1.14 (H_3, s)		8α, 19α, 22α	C-12, C-13/14, C-18
28	31.8		1.06 (H_3, s)		18β, 21β, 26	C-16, C-17, C-18, C-22
29	181.2					
30	32.0		1.37 (H_3, s)		19β	C-19, C-20, C-21, C-29

[a] multiplicities undefined due to overlapping signals are designated 'm'

[b] shifts of the four following protons are unknown: H-7α, H-11α, H-12, H-15

[c] shifts interchangeable

designation of the hydroxyl group to C-2 was confirmed by *g*COSY correlations of H_2-1 with H-2β (δ4.55) and H-10α (δ2.54), and NOESY correlations from H_2-1 to H_3-25 (δ0.66) and H-11β (δ1.27). Selective decoupling at δ2.17 (H_2-1) changed the multiplicity of H-2β (δ4.55) from a triplet (J = 2.7 Hz) to a singlet, which confirmed that the protons on C-1 shared the same ^{1}H shift. The stereochemistries of the remaining identifiable protons were determined from NOESY correlations, and were confirmed by coupling constants where definable. The coupling constants and downfield shifts of the α protons in ring E are consistent with rings D and E adopting a chair-chair conformation (Figure 4.1b), as described previously in the assignment of 2α,3β-dihydroxyfriedelan-29-oic acid (**53**; Section 4.1.1.2). As with 2β-hydroxy-24-oxomaytenonic acid (**58**), the NOESY correlations of H-24 (δ9.87) to H-1β, H-6β, H-7β, H_3-23 and H_3-25 suggest that the aldehyde group is not sterically hindered and may freely rotate.

One other reported isolation of a 3,24-dioxofriedelane is 1,3,24-trioxofriedelane (**71**) from *Salacia prenoides*[208]. Other 3-oxofriedelanes with an aldehyde group that have been previously reported from *Maytenus* spp. include 3,25-dioxofriedelane (**72**)[209] and 3,28-dioxofriedelane (**73**)[210]. Zhang and co-workers[211] tentatively assigned the structure of 24-oxomaytenonic acid (**56**) to a compound isolated from *Tripterygium wilfordii*, but the ^{1}H shifts of some methyls (particularly δ1.87 and δ1.49) do not correlate with the proposed structure, indicating it was incorrectly assigned.

4.1.2.3 Other compounds

Dulcioic acid (**79**) was first isolated from *Scoparia dulcis* (Scrophulariaceae)[212], and has since been isolated from *Tripterygium wilfordii* (Celastraceae)[213,143,189], *Anaphalis araneosa* (Asteraceae)[214] and *Lonicera japonica* (Caprifoliaceae)[215]. It is interesting that dulcioic acid

has been reported from four separate families despite the paucity of reported occurrences. The only reported biological activity of dulcioic acid is as an inhibitor of the production of cytokines and thus it may be an immunosuppresant[189].

2α-Hydroxymaytenonic acid (**59**) has been reported from *Maytenus hookeri*[200], *M. canariensis*[196], *Tripterygium wilfordii*[198], *T. hypoglaucum*[197], *T. doianum*[201], *Euonymus mupinensis*[199], *Salacia chinensis*[147], and *Acanthothamnus aphyllus*[195], which all belong to the Celastraceae (including Hippocrateaceae) family. It has not been reported outside this family. No biological activity has been previously reported for **59**.

24-Deoxocangoronine (**61**) and cangoronine (**62**) have also only been isolated from members of the Celastraceae. They have both been found in *Tripterygium wilfordii*[216,189] and *T. hypoglaucum*[197]. **61** has also been isolated from *Euonymus mupinensis*[199] and *Austrolplenckia populnea*[202], while **62** has also been isolated from *T. doianum*[201], *Maytenus ilicifolia*[131] and *Glyptopetalum sclerocarpum*[217]. Neither compound has any previously reported biological activity.

4.1.2.4 Biosynthetic aspects

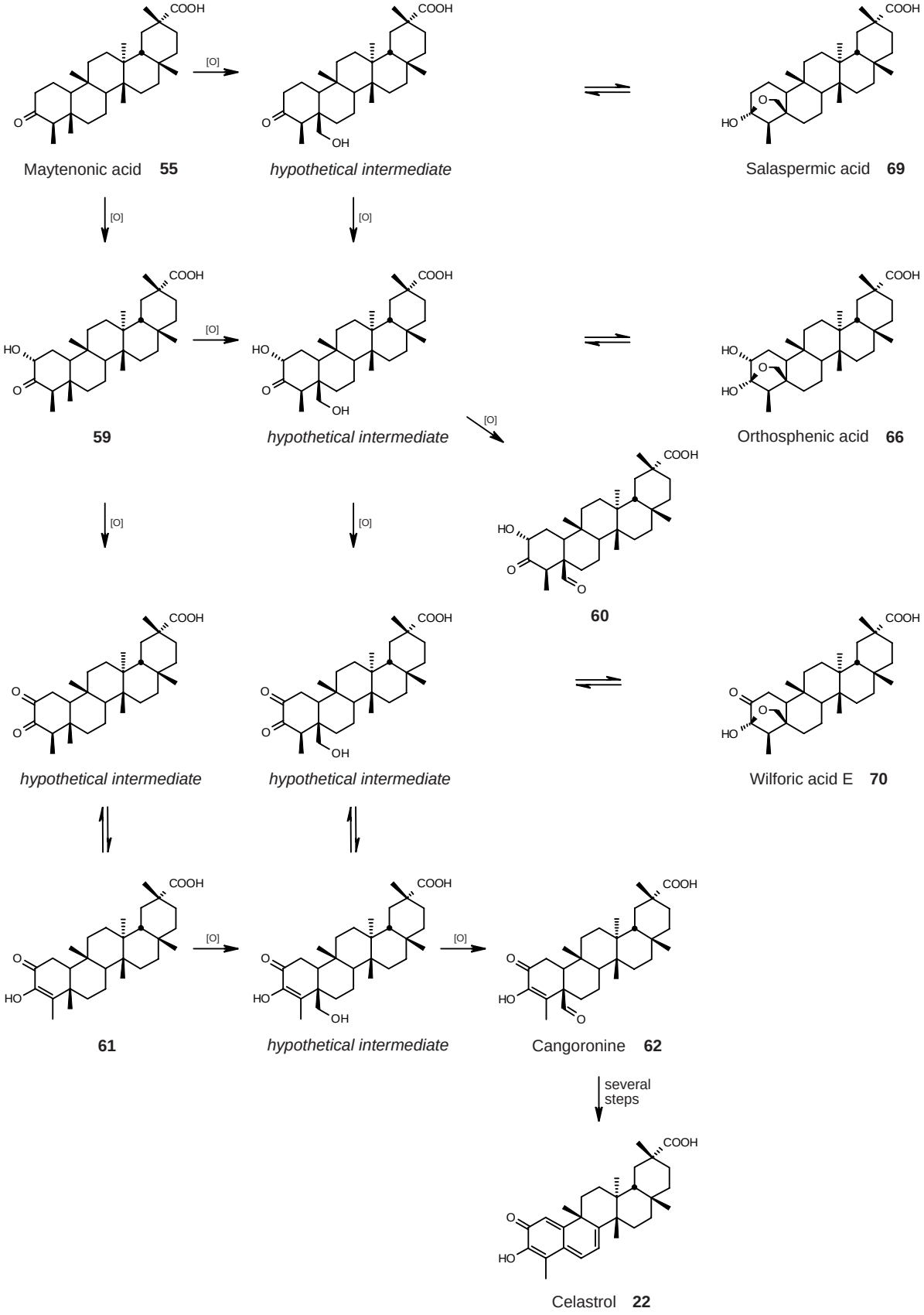
It has been proposed that celastroloids may be biosynthesised from maytenonic acid (**55**) via salaspermic acid (**69**) and orthosphenic acid (**66**)[218,133]. Itokawa and co-workers[131] isolated maytenonic acid, salaspermic acid, pristimerin (**23**) and a new compound, cangoronine (**62**), from *Maytenus ilicifolia*, and proposed that cangoronine may represent an intermediate between salaspermic acid and the celastroloids. Duan and co-workers[189] reported the isolation of wilforic acid E (**70**) from *Tripterygium wilfordii*, along with maytenonic acid, orthosphenic acid, cangoronine and celastrol (**22**). It is proposed here that the hemiacetals (**66**, **69** and **70**) may not be directly involved in the biosynthesis of cangoronine from

maytenonic acid (Scheme 4.2). The pathway may proceed via the intermediates 2α-hydroxymaytenonic acid (**59**) and 24-deoxocangoronine (**61**) that were isolated in this study along with cangoronine and celastrol. The proposed pathway involves progressive oxidation of maytenonic acid or the hypothetical 24-hydroxymaytenonic acid at C-2 to form the α-diketone, which would tautomerise to the diosphenol, and subsequent oxidation at C-24 would lead to cangoronine. Likewise, 2α-hydroxy-24-oxomaytenonic acid (**60**) is likely biosynthesised from maytenonic acid by progressive oxidation at C-2 and C-24 (Scheme 4.2). Epimerisation of 2α-hydroxyl intermediates would allow the biosynthesis of 2β-hydroxy-24-oxomaytenonic acid (**58**).

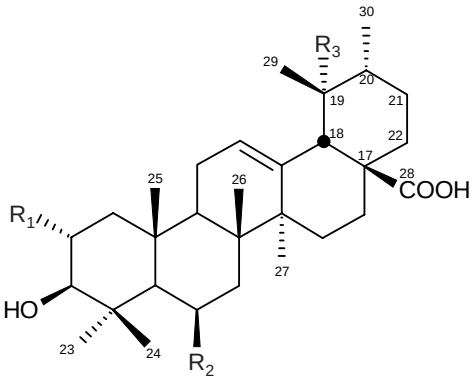
4.1.3 Compounds from *Dolichandrone heterophylla*

Dolichandrone (Bignoniaceae) is a small genus (approximately 9 species) of trees found across the old world tropics from East Africa to New Caledonia[219]. Sterols[220] and flavonoids[221,222] have been isolated from members of the genus. The lemonwood, *Dolichandrone heterophyllum*, is a small endemic tree found in woodlands across northern Australia[223] that has been used by Aborigines as an antiseptic for skin sores and ear infections[117]. This study is the first chemical investigation of *D. heterophyllum*. Bioassay-guided fractionation of the fruits of *D. heterophyllum* led to the isolation of 6β,19α-dihydroxyursolic acid (uncaric acid, **78**). This represents only the second reported occurrence of uncaric acid in the Bignoniaceae[224] and the first reported isolation of a triterpene with an ursane skeleton from the genus *Dolichandrone*.

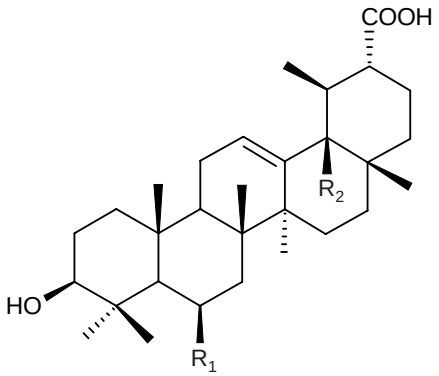
Uncaric acid (**78**) has been previously isolated from *Tabebuia rosea* (Bignoniaceae)[224], *Eriobotrya japonica* (Rosaceae)[225] and *Uncaria* spp. (Rubiaceae)[226-231]. Uncaric acid was first isolated from *U. thwaitesii* (syn. *U. elleptica*) by Herath and co-workers[232] and


COOH
[O]
O
OH
HO
Maytenonic acid 55
hypothetical intermediate
Salaspermic acid 69
59
Orthosphenic acid 66
60
hypothetical intermediate
Wilforic acid E 70
61
Cangoronine 62
several steps
Celastrol 22

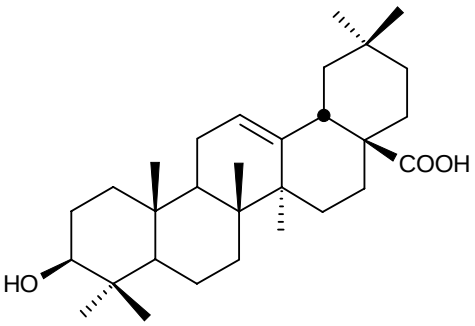
Scheme 4.2



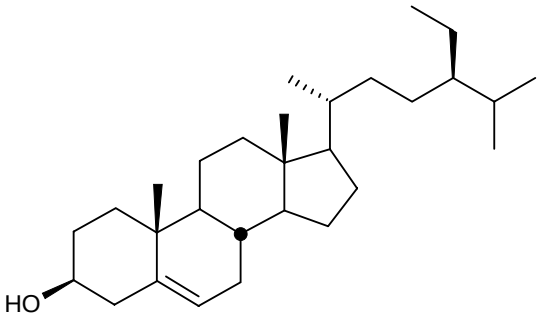
		R_1	R_2	R_3
Ursolic acid	**74**	H	H	H
	75	OH	H	H
	76	H	OH	H
	77	H	H	OH
Uncaric acid	**78**	H	OH	OH



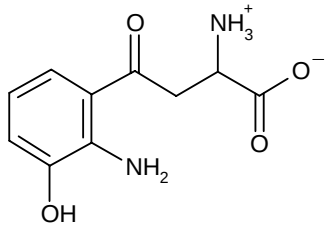
		R_1	R_2
Dulcioic acid	**79**	H	H
	80	OH	OH



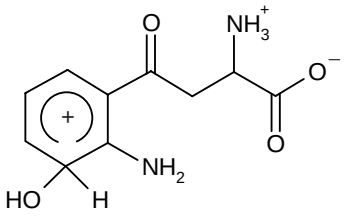
Oleanolic acid **81**



β-Sitosterol **82**



3-Hydroxykynurenine **83**



84

incorrectly identified as 3β,6β,18β-trihydroxyurs-12-en-30-oic acid (**80**). Diyabalanage and co-workers[228] isolated the same triterpene and reassigned the structure, based on 2D-NMR experiments, as 3β,6β,19α-trihydroxyurs-12-en-28-oic acid (**78**), which had since been identified from *E. japonica*[225] and other species of *Uncaria*[226,227]. Uncaric acid has been shown to reduce blood sugar in normal mice and reduce glycosuria in genetically predisposed diabetic mice[233], which is consistent with the traditional use of *E. japonica* for diabetes[234].

4.1.4 Compounds from *Lophostemon grandiflorus*

Lophostemon (Myrtaceae) is an Australasian genus of only four described species[235]. Friedelin[236], sitosterol[237], and three other triterpenoids[237], as well as essential oils[238] and a flavonoid[236], have been isolated from members of this genus. The northern swamp box, *Lophostemon grandiflorus* (syn. *Tristania grandiflora*, *T. suaveolens*) is an endemic tree that occurs in woodlands and watercourses across northern Australia[235]. The only previously chemical investigation of *L. grandiflorus* reported a number of essential oils from the leaves[238]. In this study, bioassay-guided fractionation of the bark of *L. grandiflorus* led to the isolation of betulinic acid (**49**), ursolic acid (**74**) and 2α-hydroxyursolic acid (**75**). This is the first reported occurrence of triterpenes of the lupane and ursane series in a member of the genus *Lophostemon*. Interestingly, β-sitosterol (**82**) was isolated by accident during the fractionation procedure due to its very low solubility in the normally universal solvent, dimethylsulfoxide (DMSO).

Betulinic acid (**49**) is a ubiquitous monohydroxylated triterpene of the lupane series. Its biological activities include inhibition of DNA polymerases α and β[239], antiviral[240,241], anti-inflammatory[242,243] and nematocidal[244] activities. However, the most promising therapeutic

potential of betulinic acid and its derivatives is related to its anticancer effects. Betulinic acid was initially shown to be a selective apoptosis-inducing antimelanoma agent[245]. Reports since suggest, however, that it is cytotoxic against a range of cancer cell lines[246-249], but shows no or only low cytotoxicity against 'normal' cell lines[248,249]. It has been demonstrated to be effective *in vivo* against human melanoma[245] and ovarian[248] xenografts in mice, with no apparent toxicity even at concentrations of 100–500 mg/kg.

Ursolic acid (**74**) is a ubiquitous monohydroxylated triterpene. Like betulinic acid, numerous biological activities have been reported for ursolic acid. These include antiviral[234], anti-inflammatory[250,242,243,251,252], antileishmanial[253], analgesic[250], hepatoprotective[254], antitumour[255,256] and low cytotoxic[257,256] activities, as well as inhibition of HIV-1 protease[258], DNA topoisomerase II[239], and DNA polymerases α and β[239]. Ursolic acid has also been shown to reduce blood sugar in normal mice[259] and in both alloxan-induced[259] and streptozotocin-induced diabetic mice[260].

2α-Hydroxyursolic acid (**75**) is a common dihydroxylated triterpene of the ursane series, normally found in co-occurrence with ursolic acid. Biological activities of 2α-hydroxyursolic acid include antiviral activity[234], low cytotoxicity[257] and protein kinase C inhibition[257]. 2α-Hydroxyursolic acid was also shown to reduce blood sugar in normal mice and reduce glycosuria in genetically predisposed diabetic mice[233]. Both ursolic acid and 2α-hydroxyursolic acid have been isolated from the traditional antidiabetic plants, *Lagerstroemia speciosa*[261] and *Eriobotrya japonica*[262], whose activity can be partially attributed to these triterpenes.

β-sitosterol (**82**) is a ubiquitous phytosterol that is important in human nutrition[263]. It influences serum levels of sterols such as cholesterol[264] and 17β-estradiol[265]. Many

biological activities have been reported for β-sitosterol, including analgesic[266], anthelmininticc[266], antimutagenic[266], angiogenic[267], and antitumour[265] activities.

4.1.5 Compounds from *Lagerstroemia speciosa*

The Pride of India, *Lagerstroemia speciosa* (Lythraceae), occurs naturally across South-East Asia, India and Sri Lanka, and is a popular cultivated tree throughout the tropics[268]. Banaba is a name used in the Philippines for a tea made from the leaves of *L. speciosa* that is traditionally used to treat diabetes[269,270,261]. Its efficacy has been verified by pharmacological studies[271-274,270] and a phase II clinical trial[275]. It has also been used to treat diarrhoea[276,269], urinary complaints[276,269], asthma[276], coughs[276] and wounds[276], and it possesses anti-inflammatory activity[269]. Ellagitannins[277,274], ursolic acid (**74**)[261], 2α-hydroxyursolic acid (**75**)[271,261], β-sitosterol glucoside[261] and triterpenes of the oleanane series[261] have been isolated from the leaves of *L. speciosa*. In this study, bioassay-guided fractionation of *L. speciosa* leaves led to the isolation of 2α-hydroxyursolic acid.

4.1.6 Compounds from *Hyptis suaveolens*

Hyptis suaveolens, is a pan-tropical weed that is believed to have become naturalised in northern Australia either before or shortly after European settlement[278]. It is a member of the Lamiaceae family of aromatic medicinal and culinary herbs. It is traditionally used to treat headache, wounds, fever and nausea[276], and the extract possesses antifungal[279] and antibacterial[280] activity. Essential oils[281,282], diterpenoids[283] and triterpenoids[279,284,285], including ursolic (**74**), betulinic (**49**) and oleanolic (**81**) acids, have been isolated from the

extract. Ursolic acid was isolated in this study from the aerial parts of *H. suaveolens* by bioassay-guided fractionation.

4.1.7 Compounds from *Melaleuca viridiflor*a

The broadleaf paperbark, *Melaleuca viridiflora* (Myrtaceae), is a large shrub to small tree that occurs near watercourses across northern Australian, New Guinea and Melanesia[286,223]. *Melaleuca viridiflora* and other *Melaleuca* species have been used traditionally as antiseptics, decongestants and analgesics[117]. Niaouli oil is the essential oil prepared from the leaves of *M. viridiflora* and/or *M. quinquenervia*. Along with the essential oils of other *Melaleuca* species, it possesses antifungal and antibacterial activity[287,288]. Ursolic (**74**) and 2α-hydroxyursolic (**75**) acids and other triterpenes have been isolated from other species of *Melaleuca*, such as *M. leucadendron*[289,290]. However, the isolation of triterpenes from *M. viridiflora* has not been previously reported. Bioassay-guided fractionation led to the isolation of betulinic acid and ursolic acid from the bark of *M. viridiflora*.

4.1.8 Other compounds investigated

Although not isolated from the plants investigated, oleanolic acid (**81**) and methyl betulinate (**50**) were chosen for a study designed to investigate structure-activity relationships of kynurenine-3-hydroxylase inhibitors. Oleanolic acid is a ubiquitous monohydroxylated triterpene of the oleanane series. Like ursolic acid (**74**) and betulinic acid (**49**), numerous biological activities have been reported for oleanolic acid. These include anti-inflammatory[242,243,252], antitumour[255], antileishmanial[253], and hepatoprotective[254] activity, along with inhibition of HIV-1 protease[258] and DNA polymerases α and β[239]. Oleanolic acid

has also been shown to reduce blood sugar in normal and alloxan-induced diabetic mice[259], but not in streptozotocin-induced diabetic mice[260]. There are a number of reports comparing pharmacological activity of oleanolic acid and ursolic acid, which differ only in the position of a methyl group in ring E. For example, ursolic has been reported to have greater anti-inflammatory[242,252], hypoglycaemic[260,259] and antitumour activities[255] than oleanolic acid. Conversely, oleanolic acid has been reported to be more active against the leishmanial parasite than ursolic acid[253]. However, the alternate positions of the methyl group in ring E do not appear to effect hepatoprotection, as oleanolic and ursolic acids are equally able to protect the liver from injury caused by hepatotoxicants[254].

Methyl betulinate (**50**) is much less frequently isolated than betulinic acid (**49**). Reported biological activities for methyl betulinate include cytotoxicity[291,292,249] and antiplasmodial activity[293]. The cytotoxicity of methyl betulinate compared to betulinic acid is dependent on the cell line tested. Methyl betulinate has been reported as more cytotoxic against KB cells[291] and a mouse melanoma[292], but less cytotoxic against a human melanoma[291], and comparable to betulinic acid against other human melanoma, leukaemia and neuroblastoma cell lines[249]. The two compounds have a different effect on erythrocyte membranes and methyl betulinate is twice as active as betulinic acid at inhibiting the growth of *Plasmodium falciparum*[293]. Conversely, methyl betulinate showed much lower HIV-1 protease inhibition than betulinic acid[258]. Thus, the functional group at C-17 is often important for activity.

4.2 ACTIVITY OF ENZYME INHIBITORS AND STRUCTURE-ACTIVITY RELATIONSHIPS

4.2.1 Inhibition of Kynurenine-3-hydroxylase by Triterpenoids and Relation to Structure

Kinetic parameters for the inhibition of kynurenine-3-hydroxylase by 17 triterpenoids were determined with crude enzyme prepared from rat liver (Table 4.6, Figure 4.2). Most of the compounds tested were potent inhibitors of kynurenine-3-hydroxylase with competitive inhibition constants (K_{ic}) in the nanomolar range. The most active was 6β,19α-hydroxyursolic acid (uncaric acid; **78**), which had a K_{ic} value of 0.023 ± 0.002 μM. The compounds displayed a large range of activities but were all competitive inhibitors of kynurenine-3-hydroxylase, with the exception of methyl betulinate (**50**), which had negligible activity. Three of these competitive inhibitors (**22**, **74** and **81**) also appeared to exhibit a little uncompetitive inhibition (Table 4.6). However, the uncompetitive inhibition constants (K_{iu}) are unreliable due to testing at an inhibitor concentration much less than the calculated K_{iu} values.

Δ^{15}-Celastrol (**32**) was not tested fully due to partial decomposition following spectroscopic analysis. As a part of the bioassay-guided fractionation procedure, however, Δ^{15}-celastrol was tested for kynurenine-3-hydroxylase activity at one concentration of L-kynurenine (KYN) and showed comparable activity to celastrol (**22**).

4.2.1.1 Relationship of triterpenoid-type with activity

The structure-activity relationships of kynurenine-3-hydroxylase inhibition by triterpenes are summarised in Table 4.7 and Figure 4.3. Four types of triterpenes and a sterol were

Table 4.6. Enzyme inhibition of kynurenine-3-hydroxylase, kynurenine aminotransferase (KAT) and kynureninase by triterpenoids. This data is also represented graphically in Figure 4.2. Values are given with their standard errors. K_{ic} is the competitive inhibition constant, K_{iu} is the uncompetitive inhibition constant, and IC_{50} is the concentration of inhibitor that reduces product formation by 50%.

Compound		K_{ic} for Kynurenine-3-hydroxylase (μM)	K_{iu} for Kynurenine-3-hydroxylase (μM)	IC_{50} for KAT (μM)[a]	IC_{50} for Kynureninase (μM)
uncaric acid	**78**	0.023 ± 0.002	-	» 10	» 10
2α-hydroxy-24-oxomaytenonic acid	**60**	0.061 ± 0.005	-	7.8 ± 0.6	» 10
cangoronine	**62**	0.077 ± 0.011	-	> 10	» 10
2β-hydroxy-24-oxomaytenonic acid	**58**	0.12 ± 0.02	-	6.4 ± 1.3	» 10
celastrol	**22**	0.14 ± 0.03	1.4 ± 0.3	> 10	» 10
Δ^{15}-celastrol	**32**	nd[b]	nd[b]	nt[c]	nt[c]
24-deoxocangoronine	**61**	0.18 ± 0.02	-	> 10	» 10
oleanolic acid	**81**	0.21 ± 0.05	1.5 ± 0.4	» 10	» 10
2α,3β-dihydroxyfriedelan-29-oic acid	**53**	0.27 ± 0.03	-	> 10	» 10
2α-hydroxyursolic acid	**75**	0.29 ± 0.03	-	» 10	» 10
2α-hydroxymaytenonic acid	**59**	0.29 ± 0.04	-	> 10	» 10
dulcioic acid	**79**	0.34 ± 0.04	-	9.5 ± 0.7	» 10
ursolic acid	**74**	0.42 ± 0.13	2.2 ± 0.7	» 10	» 10
betulinic acid	**49**	0.72 ± 0.12	-	> 10	» 10
11α,28-dihydroxylupenone	**43**	0.93 ± 0.12	-	> 10	» 10
netzahualcoyoic acid	**25**	1.5 ± 0.2	-	0.50 ± 0.14	» 10
β-sitosterol	**82**	3.4 ± 0.4	-	» 10	» 10
methyl betulinate	**50**	> 10	-	» 10	» 10

[a] > represents 'greater than'; » represents 'much greater than'

[b] not determined, but activity comparable to celastrol (**22**) (see Section 4.2.1)

[c] not tested

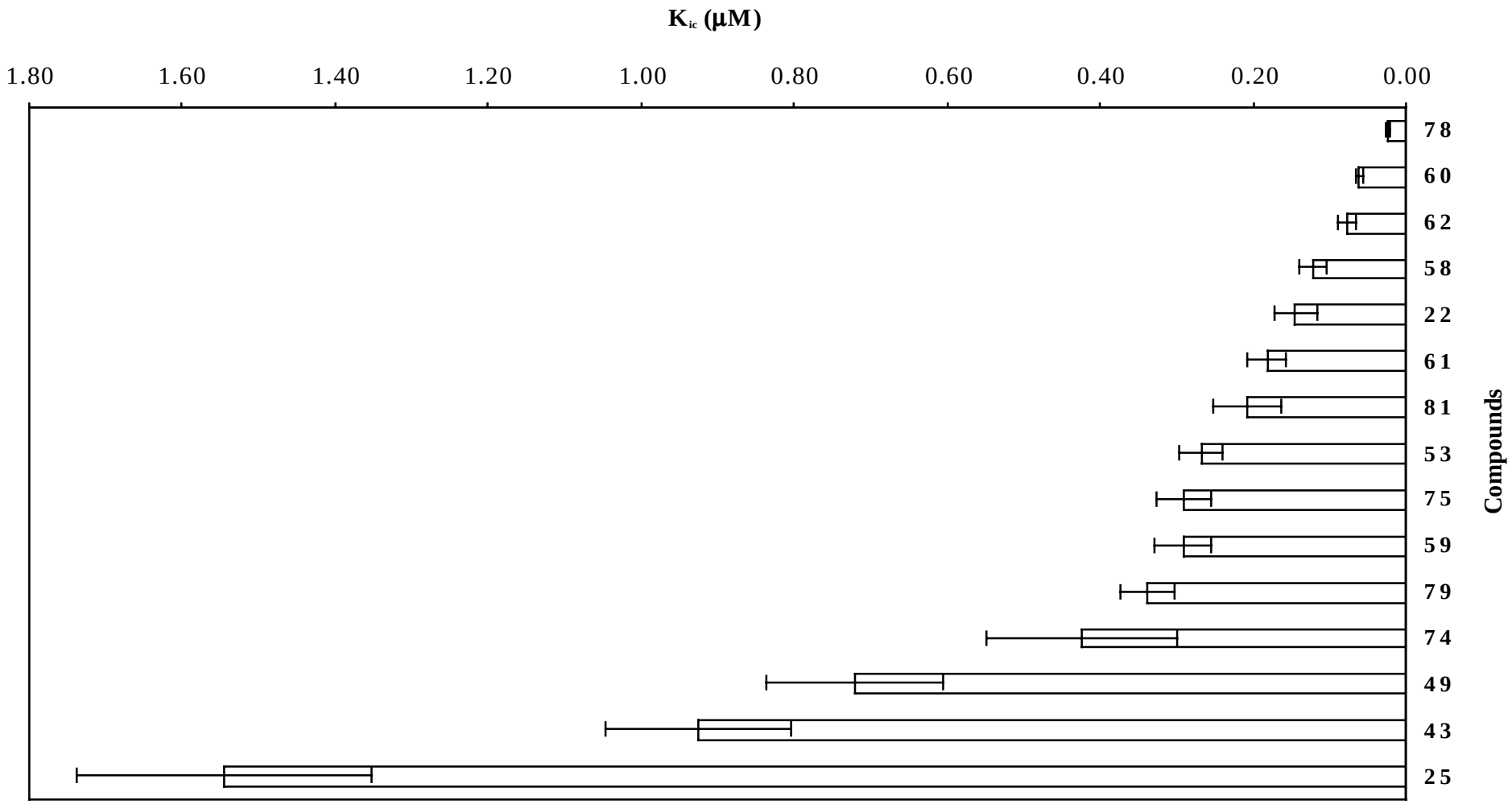


Figure 4.2. Graphical representation of kynurenine-3-hydroxylase inhibition by triterpenoids with K_{ic} values less than 2 μM. Error bars represent the standard errors of the K_{ic} values, which are given in Table 4.6.

Table 4.7. Summary of structure-activity relationships of the triterpene inhibitors of kynurenine-3-hydroxylase. Refer also to Figures 4.3.

	Higher Activity (Lower K_{ic})	Lower Activity (Higher K_{ic})	Approximate Magnitude of Difference
Triterpene Skeleton	Oleanane, Ursane, Friedelane	Lupane	2-3 fold
Ring A Substituents in Friedelanes	24-CHO	24-Me	2-5 fold
Ring E Substituents	COOH, CH_2OH	COOMe	> 10-fold
Conformation of Rings D and E in Friedelanes	Chair–Chair (as in celastrol, **22**)	Half-chair–Chair (as in netzahualcoyoic acid, **25**)	10-fold
Additional substitution	6β,19α-dihydroxy	-	10-15 fold

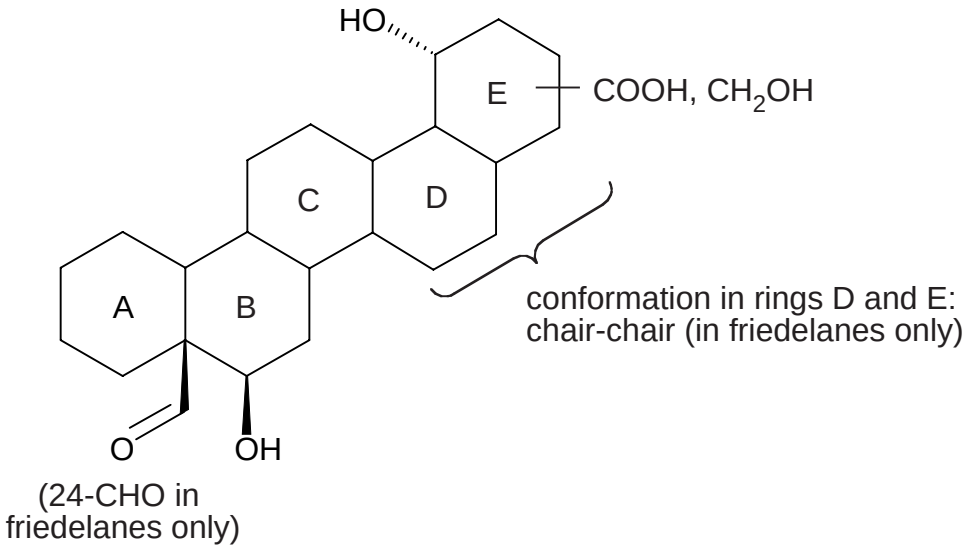


Figure 4.3. Summary of features in ursanes, oleananes and friedelanes that increase inhibition of kynurenine-3-hydroxylase. Refer also to Table 4.7.

represented in this study. The sterol (**82**) was much less active (K_{ic} = 3.4 ± 0.4 μM) than the triterpenes (except for methyl betulinate), although this may be partly due to the lack of a hydrophilic group in the side chain. The two lupenes (**43** and **49**) with a carboxylic acid or a hydroxymethyl at C-28 had K_{ic} values just under 1 μM, and were substantially less active than members of the other three groups of triterpenes. The similarity in activity for both of the 2α,3β-dihydroxy triterpenes (**53**, K_{ic} = 0.27 ± 0.03 μM; **75**, K_{ic} = 0.29 ± 0.03 μM) demonstrates that friedelanes and ursanes are equivalently active. The difference in activity between ursolic acid (**74**, K_{ic} = 0.42 ± 0.13 μM) and oleanolic acid (**81**, K_{ic} = 0.21 ± 0.05 μM) suggests that oleananes may be slightly more active than ursanes. However, the large standard error in the K_{ic} of ursolic acid indicates that the value cannot be relied upon and there may be no actual difference in activity between ursanes and oleananes.

4.2.1.2 Relationship of variations in ring E with activity

Ring E, its substituents and conformation appear important for activity. The triterpenes with a six-membered ring E are more active than the lupenes (**43** and **49**), which have a five-membered ring, and far more active than β-sitosterol (**82**), which has a side-chain rather than a fifth ring. All compounds with a K_{ic} below 1 μM have either a carboxylic acid or a hydroxymethyl attached to ring E. Methylation of the carboxylic acid group produces a remarkable reduction in activity, as evidenced by betulinic acid (**49**, K_{ic} = 0.93 ± 0.12 μM) and its methyl ester (**50**, K_{ic} > 10 μM). Additional oxidation in ring E produces a marked increase in inhibition, demonstrated by 19α-hydroxyl substitution causing a ten-fold decrease in the K_{ic} for uncaric acid (**78**, K_{ic} = 0.023 ± 0.002 μM) when compared to the other ursanes, based on the assumption that the 6β-hydroxyl also present in uncaric acid has little affect on activity.

Within the friedelanes, the conformation of rings D and E are important for activity. When the rings are in the chair-chair conformation the carboxylic acid at C-29 is in an axial position and in proximity to C-27 (Figure 4.1b), whereas in the alternative boat-boat conformation the carboxyl group is in an equatorial position and distal to groups on the other rings (Figure 4.1a). Simple friedelanes exist in solution in an approximately 1:1 mixture of the chair-chair and boat-boat conformations due to steric repulsion between methyls at C-27 and C-29[163]. A carboxylic acid or methyl carboxylate at C-29 would present its flat face to the C-27 methyl and therefore reduce the steric repulsion enough to favour the chair-chair conformation in solution almost completely[161-163]. This is supported by the NMR data for the three new friedelanes described above (Sections 4.1.1.2, 4.1.2.1 and 4.1.2.2). Rings A and B in celastrol (**22**) are planar due to the conjugated system, but the conformation of rings D and E would remain unaffected and, because of the presence a 29-carboxylic acid, would most likely adopt the chair-chair conformation (Figure 2.1a). The extra double bond in ring D of Δ^{15}-celastrol (**32**) would not substantially alter this, and it has comparable activity to celastrol (see above, Section 4.2.1). However, by extending the conjugation of the chromophore of celastrol into ring D, as in netzahualcoyoic acid (**25**), this conformation is no longer favourable. As described previously (Section 2.1.2.1), ring D of netzahualcoyoic acid is in a half-chair conformation, while ring E is in a chair that places the 29-carboxyl group in an equatorial position away from the bulk of the molecule (Figure 2.1b), and quite a distance away from C-27, in contrast to celastrol and the other friedelanes in this study. Netzahualcoyoic acid was ten-fold less active (K_{ic} = 1.5 ± 0.2 μM) than celastrol (K_{ic} = 0.14 ± 0.03 μM), which had comparable activity to the other friedelanes. This infers that when the carboxyl group is distal to the C-27, as seen in netzahualcoyoic acid or in simpler friedelanes that adopt a boat-boat conformation in rings D and E, the activity is markedly reduced. Thus, adoption of a chair-chair conformation in rings D and E in friedelanes is important for activity.

The substitution position of the carboxylic acid does not seem to affect activity, as long as it is attached to ring E. The 30-carboxyl in ursanes such as dulcioic acid (**79**, K_{ic} = 0.34 ± 0.04 μM) sits on the α face of the molecule in a spatially similar position to the 29-carboxyl in friedelanes with a chair-chair conformation in rings D and E (Figure 4.1b,c). Likewise, the 28-carboxyl in ursanes, oleananes and lupanes sits on the β face of the molecule in a similar place to the carboxyl in friedelan-29-oic acids if the molecule is rotated 180° around its long axis (Figure 4.1d). The reduced activity observed in the lup-20(29)-enes (**43** and **49**) may possibly be due to intermolecular steric repulsions between the isopropenyl group, which protrudes from the opposite (α) face to the carboxyl, and a substituent in the enzyme's active site. In uncaric acid (**78**), an ursen-28-oic acid, the hydroxyl group at 19α also sits in a spatially similar position to the carboxyl in the friedelan-29-oic acids, so that it has a hydrophilic substituent in similar positions on both faces of the molecule (Figure 4.1c,d). This may result in a greater affinity for the active site of the enzyme. From this evidence, and from comparison of conformation of rings D and E in friedelanes, it can be argued that the spatial position of the hydrophilic group is important for activity, rather than its point of attachment.

4.2.1.3 Relationship of variations in ring A with activity

With respect to ring A, a hydrophilic group is probably all that is required for reasonable activity. An aldehyde at C-24 in friedelanes is the most influential substituent in ring A observed in this study. Two pairs of compounds (**59** and **60**, **61** and **62**) differing in this manner indicate that an aldehyde at C-24 increase the activity two- to five-fold compared to a methyl at this position. It is possible that this relationship may be restricted to friedelanes.

The presence of a ketone in ring A in addition to, or instead of, a hydroxyl group does not seem to affect activity. Likewise, the presence or absence of a hydrophilic group at C-2 does not appear to affect activity. However, comparison of the activities of 2α- and 2β-hydroxy-24-oxomaytenonic acid (**60**, K_{ic} = 0.061 ± 0.005 μM; **58**, K_{ic} = 0.12 ± 0.02 μM) suggests that a 2β-hydroxyl may reduce the activity slightly in 24-oxofriedelanes.

The similar activities of celastrol (**22**, K_{ic} = 0.14 ± 0.03 μM) and 24-deoxocangoronine (**61**, K_{ic} = 0.18 ± 0.02 μM) indicate that extending the conjugation of the diosphenol in **61**, which results in a planar conformation in rings A and B, has no effect on activity. Thus, the conformation of rings A and B does not appear to be important for activity.

4.2.1.4 Comparison of inhibitor structures with 3-hydroxykynurenine

Although no computer modelling was performed, some mechanistic inferences can be tentatively proposed regarding the inhibition of kynurenine-3-hydroxylase by the friedelanes, ursanes and oleananes in this study. These inferences are based on the assumption that a carboxylic acid in ring E in the right spatial position and a hydrophilic group in ring A are required for activity. In the compounds with K_{ic} values less than 1 μM, the distance between these two functional groups is approximately the same as that between the 3-hydroxyl and the carboxyl group of 3-hydroxykynurenine (3HK, **83**), the product of kynurenine-3-hydroxylase. The 3-hydroxyl of 3HK is unique among the enzyme products of kynurenine metabolism. This suggests that these triterpenes are highly selective competitive inhibitors of kynurenine-3-hydroxylase because they are structural analogues of 3HK and the transitional intermediate (**84**) proposed by Pellicciari and co-workers[92]. This would explain the loss of activity in netzahualcoyoic acid, where the 29-carboxyl group is in a position much further away from ring A when compared to the more active inhibitors such as

celastrol (**22**). In compounds **58**, **60** and **62**, the distance between the ring E carboxylic acid and the 24-aldehyde may provide more favourable interactions in the active site than a C-2 or C-3 hydrophilic group. Alternatively, the 24-aldehyde may increase activity due to an additional interaction with another functional group of the active site, such as that which associates with the molecular oxygen species or the 2-amino group of 3HK. While the 19α-hydroxyl is possibly responsible for the increased activity of uncaric acid (**78**), the 6β-hydroxyl may also be involved by interacting with a functional group in the active site that normal associates with the 2-amino or 7-carbonyl of 3HK. Testing 6β-hydroxyursolic acid (**76**) and 19α-hydroxyursolic acid (**77**) would resolve the relative contributions of the 6β- and 19α-hydroxyl groups to the increased inhibition displayed by uncaric acid.

4.2.2 Inhibition of Kynurenine Aminotransferase by Triterpenoids and Relation to Structure

The IC_{50} values for inhibition of kynurenine aminotransferase (KAT) by 17 triterpenoids were determined with crude enzyme prepared from rat kidney (Table 4.6). All but one compound displayed no or only weak inhibition of KAT, with IC_{50} values greater than 5 μM. In particular, the ursolic acids (**74**, **75** and **78**) and oleanolic acid (**81**) displayed less than 10% inhibition at 10 μM. The inhibition of KAT by netzahualcoyoic acid (**25**, IC_{50} = 0.50 ± 0.14 μM) was unique amongst the compounds tested. In comparison, celastrol (**22**) displayed only 10% inhibition when tested at 10 μM. This great difference in activity is likely due to the different conformation in rings D and E explained above (Section 4.2.1.2), which affects the spatial position of the carboxylic acid. By inference, friedelan-29-oic acids that adopt a boat-boat or similar conformation may also be inhibitors of KAT. Thus, the conformation of rings D and E in friedelan-29-oic acids is not only important for the activity

of KAT and kynurenine-3-hydroxylase inhibitors, it is also important for the selective inhibition displayed by compounds **22**, **53** and **58**–**62**.

4.2.3 Inhibition Studies of Kynureninase

No compounds tested in this study inhibited kynureninase when tested at 10 μM (Table 4.6). This and the previous discussion of KAT inhibitors demonstrates that, with the exception of netzahualcoyoic acid, the triterpenes in this study are highly selective inhibitors of kynurenine-3-hydroxylase.

4.3 THERAPEUTIC POTENTIAL OF THE KYNURENINE-3-HYDROXYLASE INHIBITORS

4.3.1 Potential of Some Traditional Herbal Medicines Containing Kynurenine-3-hydroxylase Inhibitors

Many plants are known that contain reasonable amounts of the inhibitors discovered in this study. Many have been used traditionally for medicinal purposes and have demonstrated very low toxicity. These may have potential in the treatment of neurodegenerative disorders due to their kynurenine-3-hydroxylase inhibitor content. One such plant, *Scoparia dulcis*, has been used traditionally to treat spasms and diabetes[126]. The occurrence of dulcioic acid (**79**)[212] and betulinic acid (**49**)[294] in *S. dulcis* may help to explain its use as an antispasmodic.

The leaves of *Lagerstroemia speciosa* and *Eriobotrya japonica* have been used medicinally as teas for diabetes and other conditions[269,234]. In this study, the leaves of *L. speciosa* yielded a reasonable amount of 2α-hydroxyursolic acid (**75**), approximately 0.02% (fresh weight).

The leaves of *E. japonica* have reportedly afforded 0.03% (dry weight) of ursolic acid (**74**)[295], as well as 0.0005% (dry weight) uncaric acid (**78**) and other triterpenes[262,225]. These plants may be of therapeutic value for neurodegenerative diseases due to their content of kynurenine-3-hydroxylase inhibitors and their safe traditional use.

The most active inhibitor of kynurenine-3-hydroxylase, uncaric acid (**78**), has been isolated from a number of *Uncaria* species[226-228,230,231], including *U. rhynchophylla*[229]. *Uncaria rhynchophylla* is a traditional Chinese medicine used in the treatment of convulsions and epilepsy[296]. The extract is hypotensive[297] and protects against glutamate[298] and kainic acid induced[296] convulsions and NMDA-induced excitotoxicity and cytotoxicity[299,300]. The neuroprotective activity has been attributed to indole alkaloids[298,301]. The discovery of potent kynurenine-3-hydroxylase inhibition by uncaric acid and other triterpenes provides an additional pharmacological mechanism by which the plant is neuroprotective. The reported activities suggests that *U. rhynchophylla* has potential in the treatment of neurodegenerative disorders, such as Huntington's and Parkinson's diseases, severe epilepsy and AIDS-related dementia.

The crude extract of *Maytenus cunninghamii* root-bark may be suitable since it contains five of the six most inhibitory compounds. It also contains large amounts of celastrol (**22**), but oral administration would result in acid-rearrangement of celastrol, greatly reducing its toxicity, as discussed in Section 2.1.1. However, the chemical variation observed between populations could cause a problem with variation of inhibitor concentrations in prepared crude extracts.

4.3.2 Potential of Kynurenine-3-hydroxylase Inhibitors as Drugs

Triterpenes commonly have low toxicity, few adverse side effects, are orally available, and cross the blood-brain barrier. Most of the triterpenes reported here are highly selective competitive inhibitors of kynurenine-3-hydroxylase with moderate to high activity in the nanomolar range. Eleven of the inhibitors were tested against the P388D1 cell line to assess cytotoxicity and make inferences regarding their toxicity. The very low *in vivo* toxicity of compounds **49**[245,248], **74**[256], **75**[302], **81**[303] and **82**[304] has been demonstrated elsewhere so these compounds were not tested for cytotoxicity in this study. Most compounds tested against the P388D1 cell line showed no or only low cytotoxicity against this sensitive cell line (Table 4.8), which suggests that they should be safe at doses that would be employed. The notable exceptions were the quinonemethide triterpenes (**22** and **25**) that had IC_{50} values below 0.4 μM. It is interesting to note that the presence of an aldehyde at C-24 caused a five-fold increase in the IC_{50} of **62** when compared to **61**.

The most promising compound is uncaric acid (**78**) due to its strong inhibition of kynurenine-3-hydroxylase (K_{ic} = 0.023 ± 0.002 μM) and lack of cytotoxicity. It is one of the most potent kynurenine-3-hydroxylase inhibitors to be reported, second only to Ro 61-8048 (**18**, reported K_{ic} = 0.0048 ± 0.0021 μM) and similar *N*-(4-phenylthiazol-2-yl)benzenesulfonamides [98]. *Dolichandrone heterophyllum* may provide a source for the extraction and isolation of uncaric acid, because it was isolated in this study at 0.01% (fresh weight) from the fruit, and may be present in other organs, since the bark and leaves also possessed strong inhibition in the initial screen for inhibitors of kynurenine-3-hydroxylase.

Other promising compounds are cangoronine (**62**, K_{ic} = 0.077 ± 0.011 μM) and two of the newly discovered compounds, 2α-hydroxy-24-oxomaytenonic acid (**60**, K_{ic} = 0.061 ± 0.005 μM) and 2β-hydroxy-24-oxomaytenonic acid (**58**, K_{ic} = 0.12 ± 0.02 μM). These

Table 4.8. Cytotoxicity (IC_{50}) of compounds tested against the P388D1 cell line *in vitro*. IC_{50} values are reported with the 95% confidence interval (CI) range. All compounds with IC_{50} values » 20 μM showed no cytotoxicity at the highest concentration tested (10 μg/mL).

	IC_{50} (μM)[a]	95% CI range (μM)
25	0.12	0.11 – 0.13
22	0.37	0.30 – 0.45
62	3.2	3.0 – 3.3
43	11	10 – 12
61	15	13 – 18
58	19	17 – 21
53	» 20	
59	» 20	
60	» 20	
78	» 20	
79	» 20	

[a] » represents 'much greater than'

friedelanes share a 24-aldehyde moiety and possess activities 3- to 6-fold less than uncaric acid, but are more active than the simpler friedelanes, ursanes and oleanolic acid (**81**). Their synthesis from simpler friedelanes is feasible, whereas the 19α-hydroxyl of uncaric acid may be difficult to selectively introduce, so reliance on natural sources of uncaric acid or similar compounds in the future would be likely. Comparison of the cytotoxicity of **62** (IC_{50} = 3.2 μM), **58** (IC_{50} = 19 μM) and **60** (IC_{50} » 20μM) and their K_{ic} values suggests that **60** has the greatest therapeutic potential of the three compounds.

Celastrol (**22**) possesses strong *in vitro* kynurenine-3-hydroxylase inhibition (K_{ic} = 0.14 ± 0.03 μM) and strong *in vitro* cytotoxicity (IC_{50} = 0.37 μM). This cytotoxicity may not necessarily preclude the therapeutic use of celastrol for neurodegenerative disorders. The *in vivo* toxicity of celastrol is probably analogous to its 29-methyl carboxylate (pristimerin, **23**). Bhatnagar and Divekar[141] found that pristimerin was 100% lethal when administered parenterally to mice at 0.25–0.5 g/kg, but was non-toxic when administered orally at 0.5 g/kg daily for 28 days. The acidic conditions of the stomach would cause the rearrangement of celastrol and pristimerin to the non-toxic phenolic forms, such as in **28** and **29**[136]. Since the kynurenine-3-hydroxylase inhibition of celastrol does not rely on the chromophore, the phenolic form should still have inhibitory activity. However, the K_{ic} values of the acid-rearranged products would need to be determined. Celastrol also possess other pharmacological activities that may be desirable for the therapy of neurodegenerative disorders that involve inflammation and production of radical oxygen and nitrogen species[305,306]. For example, celastrol has *in vivo* anti-inflammatory activity when administered orally at 3 mg/kg[307], and celastroloids in general possess radical scavenging activity[146]. It is these and similar properties that have led to its suggested use as a treatment for Alzheimer's Disease[156].

The lesser activity (K_{ic} values = approximately 0.2–0.4 μM) of the simpler friedelanes, ursanes and oleanolic acid may prove adequate for treatment. They have the advantage of being readily available and potential side effects may be more easily predicted since much is already known about their pharmacological activities.

Netzahualcoyoic acid (**25**) would not be useful as a kynurenine-3-hydroxylase inhibitor because it is a relatively poor inhibitor of kynurenine-3-hydroxylase and it inhibits KAT. However, there is some evidence that KAT inhibitors may be useful in schizophrenia[83]. Nevertheless, the *in vivo* toxicity of netzahualcoyoic acid and its *in vivo* effect on KYNA levels would need to be investigated. It would also be advantageous to test other 7,14-dienequinonemethides to determine the relative effect of different substituents in ring E on the selectivity towards KAT or kynurenine-3-hydroxylase inhibition.

It is interesting that many of the kynurenine-3-hydroxylase inhibitors in this study have also been reported to have hypoglycaemic activity. Various kynurenine metabolites are known to influence serum levels of free insulin[53]. Also, diabetes mellitus is common in many neurodegenerative disorders[71] and is well documented in transgenic mouse models of Huntington's disease[308,309]. As mentioned above, hypoglycaemic activity has been reported for compounds **74**, **75**, **78** and **81** (see Sections 4.1.3, 4.1.4 and 4.1.8). No friedelan-29-oic acids have been reported to possess hypoglycaemic activity, although this may be a property that is yet to be discovered. The hypoglycaemic activity of compounds **74**, **75**, **78** and **81** may be correlated to their kynurenine-3-hydroxylase inhibition, and therefore of lesser concern when considering possible side effects. However, if the mechanism of hypoglycaemic activity is predominantly independent of kynurenine-3-hydroxylase inhibition, a hypoglycaemic side effect may result from the use of these compounds in the treatment of neurodegenerative disorders. Since neurodegenerative disorders are commonly associated with diabetes, some hypoglycaemic activity may even be desirable. Therefore, it would be important to monitor blood glucose levels of individuals undergoing treatment with

kynurenine-3-hydroxylase inhibitors, to ensure there is no undesirable hypoglycaemic effect. If friedelan-29-oic acids were found to contain no or little hypoglycaemic activity, then they may be preferred over **78** and other ursanes and oleananes.

4.3.3 Further Work

A more potent kynurenine-3-hydroxylase inhibitor than uncaric acid (**78**) may be its 24- or 23-aldehyde. The latter has been reported to occur in *Uncaria tomentosa*[227], and it may be desirable to test this. However, the increase in activity due to a 24-aldehyde may be restricted to friedelanes due to the different point of attachment in ring A. As previously mentioned, it would also be desirable to test 6β-hydroxyursolic acid and 19α-hydroxyursolic acid to determine the contribution of the 19α- and 6β-hydroxyl groups to the activity of uncaric acid. Increased oxidation of ring E in the 24-oxofriedelanes (**58**, **60** and **62**) may improve activity, and it may be desirable to oxidise C-28 or one of the ring E methylene groups and assess the activity of these derivatives.

The next stage for uncaric acid (**78**), 2α-hydroxy-24-oxomaytenonic acid (**60**) and celastrol (**22**) is to assess their activity *in vivo*. This would involve sampling KYNA and 3HK levels using a microdialysis probe implanted in the hippocampus of normal rats before and after oral administration of the test compound. Drug efficacy would be demonstrated by an increase in KYNA and a decrease in 3HK levels following administration. Monitoring blood glucose levels would also be desirable to assess the potential for adverse effects. Extracts of plants such as *Lagerstroemia speciosa*, *Eriobotrya japonica* and *Uncaria rhynchophylla* could be similarly tested. After demonstrating that the drugs effectively alter kynurenine metabolite levels *in vivo* in the rat hippocampus, it would be desirable to assess the each

drug's effect in animal models of diseases where a QUIN:KYNA imbalance is implicated, such as in one of the available transgenic mouse models of Huntington's disease.

CHAPTER 5.

POTENTIAL NEUROPROTECTIVE AGENTS FROM NORTH QUEENSLAND PLANTS:

EXPERIMENTAL

5.1 GENERAL

Preparative high-performance liquid chromatography (HPLC) and spectroscopy were performed as outlined in Section 3.1. Specific rotations ($[\alpha]_D$) were performed in AR grade chloroform ($CHCl_3$) using an Optical Activity POLAAR 2001 polarimeter. Oleanolic acid was purchased from Sigma-Aldrich. An authentic sample of methyl betulinate was kindly supplied by Professor W. Taylor of the University of Sydney.

5.2 PLANT EXTRACTS FOR SCREENING FOR ENZYME INHIBITORS

The same treated plant extracts that were used in screening for cytotoxicity were also used in screening for inhibitors of three enzymes of the kynurenine pathway of tryptophan metabolism (see Section 3.2).

5.3 KYNURENINE-3-HYDROXYLASE INHIBITION ASSAYS

5.3.1 Screening Plant Extracts for Inhibition of Kynurenine-3-hydroxylase

The method used to screen extracts for inhibition of kynurenine-3-hydroxylase involves measuring the amount of 3-hydroxykynurenine (3HK) formed by crude enzyme in the presence of plant extract, and comparing this to the amount of 3HK produced in the absence of extract. The amount of 3HK was measured using HPLC with electrochemical detection.

5.3.1.1 Crude enzyme preparation

The preparation of crude enzyme and the incubation procedure were adapted from Chiarugi and co-workers[88]. Mouse liver was homogenised with approximately 8 volumes of cold (4°C) 0.32 M sucrose in a glass homogeniser, then ultrasonicated for 30 seconds. Unhomogenised material was removed and 1.5 mL aliquots of the enzyme preparation were centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the pellet resuspended in approximately 1.5 mL of cold 0.32 M sucrose, and centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the washing procedure repeated twice more. The pellet was finally resuspended in approximately 1.5 mL of cold buffer (20 mM K_2HPO_4, 140 mM KCl, pH adjusted to 7.0 with a saturated KH_2PO_4 solution), and stored at -80°C until required.

5.3.1.2 Incubation procedure

Each extract was tested at 10 μg/mL in a 100 μL incubation mixture containing 100 μM L-kynurenine (KYN; as L-kynurenine sulfate, Sigma-Aldrich), 600 μM NADPH (β-nicotinamide adenine dinucleotide phosphoric acid, tetrasodium salt, Sigma-Aldrich), and 1% crude enzyme preparation as follows. Each polyphenol/quinone free plant extract was resuspended in MeOH, placed in an ultrasonicating bath for 30 seconds, and diluted to a concentration of 100 μg/mL. An aliquot (10 μL) containing 1 μg of extract was pipetted into a 1.5 mL microfuge tube and allowed to dry overnight. Aliquots (10 μL) of MeOH were pipetted into clean 1.5 mL microfuge tubes and allowed to dry overnight, to act as controls.

The samples in the microfuge tubes were assayed in batches of 21 with three controls per batch. Each sample was resuspended in 55 μL of incubation buffer (100 mM K_2HPO_4, 4 mM KCl, pH adjusted to 7.5 with a saturated KH_2PO_4 solution) and ultrasonicated for 30 seconds. Following this, 20 μL of a 500 μM KYN solution, 15 μL of a freshly prepared 4 mM NADPH solution, and 10 μL of 1/10 diluted enzyme preparation were added to each tube, giving a final volume of 100 μL of incubation mixture. The samples were incubated in a 37°C water bath for 60 minutes, terminated by the addition of 100 μL of 0.1 M trichloroacetic acid (TCA), and centrifuged at 10,000 rpm for 10 minutes at 4°C. The amount of 3HK present in the supernatant was determined by HPLC with electrochemical detection.

5.3.1.3 HPLC quantification of 3HK in incubated samples

3HK was quantified by HPLC with electrochemical detection, based on the method of Pearson and Reynolds[101]. HPLC was performed using a GBC LC1150 HPLC pump, an ICI

AS2000 HPLC automatic injector, a Spherisorb ODS-2 column (5 μm, i.d. 3.0 mm, length 100 mm), an Eicom CB-100 electrochemical detector with a glassy carbon electrode (+0.60 V redox potential), and a BBC Goerz Metrawatt SE 120 chart recorder. The mobile phase consisted of 100 mM NaH_2PO_4, 2.5 mM octylsulfonic acid, 50 μM ethylenediaminetetraacetic acid (EDTA), and 6% MeOH (HPLC grade) prepared in deionised (18 ohm) water with the pH adjusted to 3.6 with glacial acetic acid. The mobile phase was degassed by sparging with helium for 15 minutes.

A 0.1 mg/mL solution of 3HK standard (Sigma-Aldrich; in 0.1 M TCA) was used to make small adjustments to the MeOH concentration of the mobile phase so that the 3HK peak appeared approximately 5 minutes after injection at a flow rate of 0.5 mL/min when appropriately modulated. Variable volume injections from 10 μL to 50 μL of 3HK (0.1 mg/mL) demonstrated the linearity of peak heights with the volume injected. At least four consecutive 20 μL injections of 0.1 mg/mL of 3HK were performed at least three times per day to confirm reproducibility of injections, and as a standard to identify the 3HK peak and determine the amount of 3HK produced during the incubations. Supernatant from the incubated samples was injected (20 μL) and the peak height of each sample was expressed as a percentage of control.

All 365 extracts were tested for inhibition of kynurenine-3-hydroxylase. Extracts that showed substantial inhibition, indicated by a low amount of 3HK formation relative to the controls, were assayed twice more for replication purposes. The most inhibitory extracts were chosen for chemical investigation in order to isolate and identify the kynurenine-3-hydroxylase inhibitor/s present.

5.3.2 Bioassay-guided Fractionation of Kynurenine-3-hydroxylase Inhibitors

The method to test inhibition of kynurenine-3-hydroxylase during the bioassay-guided fractionation procedure varied somewhat from the method used to screen crude plant extracts.

5.3.2.1 Crude enzyme preparation

A portion of Sprague-Dawley rat liver was homogenised in approximately 8 volumes of cold 0.32 M sucrose with a motorised homogeniser (Biospec Products Tissue Tearor, Model 985370). The remaining procedure for crude enzyme preparation was identical with that described above (Section 5.3.1.1).

5.3.2.2 Total protein determination of crude enzyme preparation

The total protein content of the enzyme preparation was determined in quadruplicate relative to bovine serum albumin (BSA) using a modification of the method of Lowry and co-workers[310].

5.3.2.3 Incubation procedure

Fractions were tested in a 100 µL incubation mixture that contained 50 µM L-kynurenine (KYN; as L-kynurenine sulfate), 600 µM NADPH, and 0.5% crude enzyme preparation. Fractions were resuspended in the same volume of MeOH, regardless of the weight of

individual fractions. The resuspended fractions were ultrasonicated for 30 seconds and diluted with MeOH to ten-times the desired test concentration. The test concentration was chosen based on the amount of sample in the parent fraction in order to effectively compare activity between sibling fractions and the parent fraction. Aliquots (10 µL) of these suspensions were pipetted into 1.5 mL microfuge tubes and allowed to dry overnight. Aliquots (10 µL) of MeOH were pipetted into clean 1.5 mL microfuge tubes and allowed to dry overnight, to act as controls.

Samples were assayed in batches of up to 21 with three controls per batch. Each sample was resuspended in 50 µL of 100 µM KYN in buffer solution (100 mM K_2HPO_4, 4 mM KCl, pH adjusted to 7.5 with a saturated KH_2PO_4 solution) and ultrasonicated for 30 seconds. Following this, 50 µL of a buffered solution containing 1.2 mM NADPH and 1% crude enzyme preparation was added to this, giving a final volume of 100 µL of incubation mixture. The samples were incubated in a 37°C water bath for 60 minutes, terminated with the addition of 100 µL of 0.1 M trichloroacetic acid (TCA), and centrifuged at 10,000 rpm for 10 minutes at 4°C. The amount of 3HK present in the supernatant was determined by HPLC with electrochemical detection.

5.3.2.4 HPLC quantification of 3HK in incubated samples

HPLC was performed using a GBC LC1150 HPLC pump, an ICI AS2000 HPLC automatic injector, a Phenomenex Luna C18(2) column (5 µm, i.d. 2.0 mm, length 150 mm), a GBC LC1260 electrochemical detector with a glassy carbon electrode (+0.50 V redox potential), and a JJ Instruments CR452 chart recorder. The mobile phase was prepared as previously described (Section 5.3.1.3) and filtered (Millipore 0.45 µm HA filter) prior to addition of MeOH (8% final concentration) and pH adjustment to 3.6 with glacial acetic acid. The

MeOH concentration of the mobile phase was adjusted as previously described with 1 mg/mL of a 3HK standard rather than 0.1 mg/mL, so that the 3HK peak appeared approximately 6 minutes after injection at a flow rate of 0.3 mL/min. Fractions were tested in triplicate.

5.3.3 Determination of Inhibition Constants of Isolated Compounds

The method employed for determining inhibition of kynurenine-3-hydroxylase by isolated compounds was similar to that used for bioassay-guided fractionation (Section 5.3.2), except that different incubation conditions were used and the amount of 3HK formation was expressed per amount of protein per unit time, rather than as a percentage of control.

5.3.3.1 Optimisation of incubation conditions

The incubation time, and NADPH and KYN concentrations were optimised for incubation with 1% crude enzyme preparation before testing pure compounds for inhibition. The calculation of Michaelis-Menten-derived enzyme kinetic parameters assumes that the rate measured is equivalent to the initial rate, or the rate of product formation at time zero[311]. Therefore, duplicate experiments with triplicate time observations from 0 to 60 minutes were performed with 300 μM each of KYN and NADPH to determine the maximum incubation time that best estimated initial velocity. An incubation time of 15 minutes was chosen as the longest time that still sufficiently estimated the initial velocity while staying within the reasonable detection limits of 3HK. A range of concentrations of NADPH from 5 μM to 600 μM were tested with 50 μM KYN and a 15 minute incubation time to determine the concentrations that approach saturating levels. The concentration of 600 μM NADPH was

chosen on this basis. A range of concentrations of KYN from 5 μM to 450 μM were tested with 600 μM NADPH and an incubation time of 15 minutes to estimate the K_m, the concentration of substrate at which the rate of product formation is equal to half the maximum, or limiting, rate of production formation (V). This estimated K_m was used to determine the range of KYN concentrations used to test the inhibitors.

5.3.3.2 Testing of isolated compounds to determine inhibition constants

Each inhibitor was prepared for preliminary testing at up to three different concentrations following the previously described procedure (Section 5.3.1.2). Each concentration of inhibitor was tested in three assay batches of 15 μM, 60 μM, and 150 μM concentrations of KYN, respectively. Each assay batch contained up to 21 samples, three controls with no inhibitor, and four background controls lacking the crude enzyme preparation. To create 3HK standards, two of these blank controls were spiked with 100 μL of 3HK standard in 0.1 M TCA after incubation rather than 100 μL of 0.1 M TCA normally added to terminate the reaction. These standards contained a final concentration of either 0.5 μg/mL or 0.25 μg/mL 3HK, dependent on the KYN concentration tested.

The peak heights of blank controls were subtracted from those of the samples, controls and standards before further calculations were performed. The peak heights of the samples and controls were then converted to values of *v*, the rate of 3HK formation expressed as nmol/min·mg(protein), by comparison with the 3HK standards and subsequent calculations.

The approximate inhibition constants of each inhibitor were calculated from the line of best fit of the Hanes plot of [S]/*v* versus [S], where [S] is the concentration of KYN[311]. These approximate inhibition constants were used to determine the concentrations of the inhibitors

used for more detailed testing. All inhibitors exhibited predominantly competitive inhibition and a small number also displayed some uncompetitive inhibition.

Each inhibitor was tested at an inhibitor concentration equivalent to its estimated K_{ic}. The K_{ic} is the specific (competitive) inhibition constant, which corresponds to the concentration of inhibitor where:

$$\frac{V^{app}}{K_m^{app}} = \frac{1}{2} \times \frac{V}{K_m}$$

where V^{app} and K_m^{app} are the apparent V and apparent K_m , respectively, in the presence of an inhibitor. Inhibitors that displayed some uncompetitive inhibition were also tested at a concentration equivalent to their estimated K_{iu} values. The K_{iu} is the catalytic (uncompetitive) inhibition constant, which corresponds to the concentration of inhibitor where:

$$V^{app} = \frac{1}{2} \times V$$

The inhibitors were tested at 5–6 concentrations of KYN (7.5 µM, 15 µM, 30 µM, 60 µM, 120 µM and 240 µM) with 3–6 replicates per KYN concentration per inhibitor concentration. The amount of substrate consumed during the incubation ranged from less than 1% at 240 µM to 11% at 7.5 µM KYN. The assay batches contained controls, blanks and blanks spiked with 3HK standard as described above for the preliminary inhibitor testing, and the rates (*v*) were similarly calculated from peak heights.

5.3.3.3 Analysis of kynurenine-3-hydroxylase inhibition data

The data from the kynurenine-3-hydroxylase inhibition studies were analysed using the MS-DOS enzyme kinetics data analysis program LEONORA Version 1.0[312]. The K_m and V, in absence of inhibitor were calculated by fitting the control data to the Michaelis-Menten equation defined as:

$$v = \frac{V \times [S]}{K_m + [S]}$$

using a dynamic biweighted robust regression with default parameters, where weights are calculated by the program from the data to minimise the sum of squares. Standard errors were automatically calculated by this method. The V and K_m of kynurenine-3-hydroxylase under these assay conditions was 3.89 ± 0.05 nmol/min·mg(protein) and 9.5 ± 0.4 μM, respectively.

The competitive and uncompetitive inhibition constants (K_{ic} and K_{iu}, respectively) of each inhibitor were calculated by fitting the inhibition data and the control data to the mixed inhibition model, with the equation:

$$v = \frac{V \times [S]}{K_m \times (1 + [I]/K_{ic}) + [S] \times (1 + [I]/K_{iu})}$$

where [I] is inhibitor concentration, using dynamic biweighted robust regression with default parameters, where weights are calculated by the program from the data to minimise the sum of squares. Standard errors were automatically calculated by this method. Only data for one concentration per inhibitor was used with the exception of netzahualcoyoic acid. Meaningful data was measured for two concentrations of netzahualcoyoic acid and analysed simultaneously, together with the control data, using the mixed inhibition model. The uncompetitive component of the inhibition was very small for most inhibitors, and gave

meaningless values of K_{iu}. Therefore, the calculated K_{iu} was only presented in the results for three inhibitors that had somewhat meaningful K_{iu} values and errors.

5.4 KYNURENINE AMINOTRANSFERASE AND KYNURENINASE INHIBITION ASSAYS

5.4.1 Screening Plant Extracts for Inhibition of Kynurenine Aminotransferase and Kynureninase

The method used to screen extracts for inhibition of kynurenine aminotransferase (KAT) and kynureninase simultaneously was adapted from Feng[102]. The assay employs HPLC with fluorescence detection to measure the amount of enzyme products, kynurenic acid (KYNA) and anthranilic acid (AA), produced in an incubation of a crude enzyme preparation in the presence of plant extract, and compare the result with the amount of KYNA and AA produced in the absence of extract.

5.4.1.1 Crude enzyme preparation

Mouse liver was homogenised with approximately 8 volumes of cold buffer (20 mM K_2HPO_4, 140 mM KCl, pH adjusted to 7.0 with a saturated KH_2PO_4 solution). Unhomogenised material was removed and 1.5 mL aliquots of the enzyme preparation were centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was removed and stored at -80°C until required.

5.4.1.2 Total protein determination of crude enzyme preparation

The total protein content of the enzyme preparation was determined in quadruplicate relative to BSA using a modification of the method of Lowry and co-workers[310].

5.4.1.3 Incubation procedure

Each extract was tested at 10 μg/mL in a 100 μL incubation mixture containing 50 μM L-kynurenine (KYN; as L-kynurenine sulfate, Sigma-Aldrich), 5 μM sodium pyruvate (Sigma-Aldrich), 5 μM pyridoxal-5'-phosphate (PLP; Sigma-Aldrich) and 20% crude enzyme preparation in tris(hydroxymethyl)aminomethane (Tris; Sigma-Aldrich) acetate buffer as follows. Samples and controls were prepared as for screening for inhibitors of kynurenine-3-hydroxylase (Section 5.3.1.2).

The samples in the microfuge tubes were assayed in batches of 21 with three controls per batch. Each sample was resuspended in 70 μL of incubation buffer (100 mM Tris, pH adjusted to 8.0 with glacial acetic acid) and ultrasonicated for 30 seconds. Following this, 10 μL of substrate solution (containing 500 μM KYN, 50 μM pyruvate, and 50 μM PLP) and 20 μL of crude enzyme preparation was added to each tube, giving a final volume of 100 μL of incubation mixture. The samples were incubated in a 37°C water bath for 30 minutes, terminated by the addition of 100 μL of 0.1 M trichloroacetic acid (TCA; Sigma-Aldrich), and centrifuged at 10,000 rpm for 10 minutes at 4°C. The amount of KYNA and AA present in the supernatant was determined by HPLC with fluorescence detection.

5.4.1.4 HPLC quantification of KYNA and AA in incubated samples

HPLC was performed using a GBC LC1150 HPLC pump, a GBC LC1610 automatic injector, a Spherisorb ODS-2 column (5 μm, i.d. 2.0 mm, length 150 mm), a post-column Braun-Melsungen syringe infusion pump set to deliver 1 M zinc acetate at 0.1 mL/min for fluorescence enhancement of KYNA[313], a GBC IC 1255 spectrofluorometer (excitation 340 nm, emission 416 nm), and a BBC Goerz Metrawatt SE 120 chart recorder. The mobile phase consisted of 50 mM sodium acetate and 4% MeCN (HPLC grade) prepared in deionised (18 ohm) water with the pH adjusted to 6.2 with glacial acetic acid. The mobile phase was degassed by sparging with helium for 15 minutes.

KYNA and AA standards (Sigma-Aldrich; 0.1 mg/mL) were used to make small adjustments to the MeCN concentration of the mobile phase, so that the second peak (KYNA) appeared approximately 6 minutes after injection at a flow rate of 0.3 mL/min with clear separation from the first peak (AA). Variable volume injections from 10 μL to 50 μL of a combined standard mixture (0.05 mg/mL KYNA and 0.05 μg/mL AA) demonstrated the linearity of peak heights with the volume injected. At least four consecutive 20 μL injections of the combined standard were performed at least three times per day to confirm reproducibility of injections, and as a standard to identify the product peaks and determine the amount of each product produced during the incubations. Supernatant from the incubated samples was injected (20 μL) and the peak height of each sample was expressed as a percentage of control.

All 365 extracts were tested for inhibition of KAT and kynureninase. None of the extracts tested exhibited strong inhibition of either enzyme. The focus among the three enzymes screened was to discover inhibitors of kynurenine-3-hydroxylase. Therefore, no attempt was made to isolate inhibitors of KAT or kynureninase.

5.4.2 Testing of Kynurenine-3-hydroxylase Inhibitors for Inhibition of Kynurenine Aminotransferase and Kynureninase

Kynurenine-3-hydroxylase inhibitors were tested for inhibition of KAT and kynureninase in order to assess the selectivity of these inhibitors for kynurenine-3-hydroxylase. The method differed somewhat to that used for screening of inhibitors of KAT and kynureninase.

5.4.2.1 Crude enzyme preparation

Sprague-Dawley rat kidney was homogenised in approximately 4 volumes of cold buffer (20 mM K_2HPO_4, 140 mM KCl, pH adjusted to 7.0 with a saturated KH_2PO_4 solution) with a motorised homogeniser (Biospec Products Tissue Tearor, Model 985370). Unhomogenised material was removed and 1.5 mL aliquots of the enzyme preparation were centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was removed and centrifuged again at 10,000 rpm for 20 minutes at 4°C. The supernatant was removed and stored at -80°C until required.

5.4.2.2 Total protein determination of crude enzyme preparation

The method of total protein determination was identical to that described for kynurenine-3-hydroxylase (Section 5.3.2.2).

5.4.2.3 Incubation procedure

Each kynurenine-3-hydroxylase inhibitor was tested at 10 μM for inhibition of KAT and kynureninase in a 100 μL incubation mixture that contained 100 μM L-kynurenine (KYN; as L-kynurenine sulfate), 5 μM sodium pyruvate, 5 μM pyridoxal-5'-phosphate (PLP) and 10% crude enzyme preparation as follows. Samples and controls were prepared as for screening for inhibitors of kynurenine-3-hydroxylase (Section 5.3.1.2).

Each sample was resuspended in 50 μL of buffered (100 mM Tris acetate, adjusted to pH 8.0 with concentrated NaOH solution) substrate solution (200 μM KYN, 10 μM pyruvate and 10 μM PLP) and ultrasonicated for 30 seconds. Following this, 50 μL of 20% crude enzyme preparation in Tris-acetate buffer (pH 8.0) was added to each tube, giving a final volume of 100 μL of incubation mixture. The samples were incubated in a 37°C water bath for 30 minutes, terminated by the addition of 100 μL of 0.1 M trichloroacetic acid (TCA), and centrifuged at 10,000 rpm for 10 minutes at 4°C.

Each assay batch contained up to 21 samples, three controls with no inhibitor, and four background controls lacking the crude enzyme preparation. To create the KYNA and AA standards, two of these blank controls were spiked with 100 μL of a combined KYNA and AA standard in 0.1 M TCA after incubation rather than 100 μL of 0.1 M TCA normally added to terminate the reaction. These standards contained a final concentration of 0.2 μg/mL KYNA and 0.2 μg/mL AA.

5.4.2.4 HPLC quantification of KYNA and AA in incubated samples

HPLC was performed using a GBC LC1150 HPLC pump, an ICI AS2000 HPLC automatic injector, a Phenomenex Luna C18(2) column (5 μm, i.d. 2.0 mm, length 150 mm), a JASCO FP-210 spectrofluorometer (excitation 340 nm, emission 416 nm), and a JJ Instruments CR452 chart recorder. The zinc acetate was supplied isocratically in the mobile phase rather than using a post-column syringe infusion pump. The mobile phase consisted of 50 mM sodium acetate and 250 mM zinc acetate in distilled water, and was filtered (Millipore 0.45 μm HA filter) prior to addition of MeCN (HPLC grade, 6% final concentration) and adjustment of pH to 6.2 with glacial acetic acid. The mobile phase was degassed by sparging with helium for 15 minutes. A combined standard solution of 0.5 mg/mL KYNA and 0.5 μg/mL AA was used to adjust the MeCN concentration of the mobile phase, so that the second peak (KYNA) appeared approximately 6 minutes after injection at a flow rate of 0.3 mL/min. Compounds that showed less than 50% inhibition of either enzyme at 10 μM were tested in duplicate at this concentration only. Compounds that showed between 50% and 70% inhibition at 10 μM were tested in duplicate at both 10 μM and 5 μM. Netzahualcoyoic acid (**25**), the only compound to show greater than 70% inhibition, was tested once at 10 μM, and in duplicate at 6.02 μM, 1.2 μM and 0.3 μM.

The peak heights of blank controls were subtracted from those of the samples, controls and standards before further calculations were performed. The peak heights of the samples and controls were then converted to values of *v*, the rate of product formation expressed as pmol/min·mg(protein), by comparison with the KYNA and AA standards and subsequent calculations. The *v* for the controls was calculated as 20.4 ± 0.6 pmol/min·mg(protein) (mean ± standard error of 11 observations) for KAT and 42.0 ± 0.8 pmol/min·mg(protein) (mean ± standard error of 11 observations) for kynureninase. When expressed as the amount of product formation per minute (independent of the amount of protein), the *v* of KAT (3.37

± 0.11 pmol/min) and kynureninase (6.93 ± 0.14 pmol/min) under these incubation conditions were in the same order of magnitude as the V_{max} of kynurenine-3-hydroxylase (12.3 ± 0.2 pmol/min) in the inhibition assay described in Section 5.3.3. This indicates that, for each compound tested, large differences in inhibition displayed against each of the three enzymes can be subjectively compared, albeit with care.

5.4.2.5 Analysis of kynurenine aminotransferase and kynureninase inhibition data

The IC_{50}, the concentration of inhibitor that reduces the formation of product by 50% with respect to the control, of each compound under the described experimental conditions was determined as follows. Compounds that showed inhibition of less than 10% at 10 μM were considered to have an IC_{50} of much greater than 10 μM. Compounds that showed between 10% and 50% at 10 μM were considered to have an IC_{50} of somewhat greater than 10 μM. The IC_{50} of compounds that were tested at 5 μM and 10 μM were calculated from the equation given by a least squares linear regression of the relationship of percent inhibition with inhibitor concentration using SPSS 11.01 for Windows. The inhibition of KAT by netzahualcoyoic acid (**25**) displayed a hyperbolic relationship over the concentration range tested. Therefore, a linear regression by least squares was performed on percentage inhibition versus the reciprocal of concentration, and the IC_{50} calculated from the equation of this regression. The error of each IC_{50} value was calculated from the errors of the regression analysis using standard calculations for propagation of errors.

5.5 ISOLATION OF KYNURENINE-3-HYDROXYLASE INHIBITORS

5.5.1 *Maytenus disperma*

5.5.1.1 Plant material and extraction

Bark (175 g) of *Maytenus disperma* (F.Muell.) Loes. was collected from four shrubs in woodland in the Mount Stuart Defence Force Training Area, and was refrigerated until extraction. The fresh material was crushed with a hammer, then extracted by soaking overnight with enough acetone-MeOH (1:1) to cover it. The material was re-extracted several times, and the extractions were combined and filtered. The solvent was removed at 40°C on a rotary evaporator to give 18.5 g of crude extract.

5.5.1.2 Crude extract treatment

The crude extract (18.5 g) was solvent partitioned between hexane and 90% $MeOH_{(aq)}$ to remove lipids, giving 12.4 g of 90% $MeOH_{(aq)}$ extract. Insoluble material was included with the 90% $MeOH_{(aq)}$ extract, while the hexane extract was discarded. Polyphenols and quinones were removed from a portion (1.0 g) of the 90% $MeOH_{(aq)}$ extract as described earlier (Section 3.4.1.2), using 15 g polyamide powder in a glass column (i.d. 2.2 cm, length 16 cm), which was eluted with 100 mL of water followed by 450 mL of MeOH, to produce 430 mg of extract.

5.5.1.3 Bioassay-guided fractionation

The polyphenol/quinone free extract was subjected to bioassay-guided fractionation (see Section 3.4.1.3). The extract (430 mg) was solvent partitioned between CH_2Cl_2 and water using 120 mL volumes of each, to afford 222 mg of organic extract and 189 mg of water extract.

The active fraction (organic extract, 222 mg) was chromatographed with silica gel (column i.d. 3.0 cm, length 10 cm) and eluted with 100 mL each of CH_2Cl_2, 40% EtOAc/CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, 10% MeOH/EtOAc and MeOH (fractions 1–7). Fraction 2 (40% EtOAc/CH_2Cl_2, 126 mg) and Fraction 4 (80% EtOAc/CH_2Cl_2, 19 mg) showed the highest K3Hase inhibition and were further separated using HPLC.

Fraction 2 (126 mg) was injected in 1.0 mL MeOH onto a Whatman Partisil ODS-2 column (10 μm, i.d. 9.5 mm, length 500 mm). Stepwise gradient elution from 30%, 50%, 80%, 90%, 95% to 100% $MeOH_{(aq)}$ afforded fractions 2.1–2.6. Recrystallisation of fraction 2.3 (80% $MeOH_{(aq)}$) from aqueous methanol afforded a new lupane-type triterpene, 11α,28-dihydroxylupenone (**43**, 23 mg).

Fraction 4 (19 mg) was injected in 0.5 mL MeOH onto a Phenomenex Luna C18(2) column (10 μm, i.d. 4.6 mm, length 250 mm). Stepwise gradient elution from 20%, 40%, 60% to 100% $MeOH_{(aq)}$ afforded fractions 4.1–4.4. The major component of the active fraction 4.4 (100% $MeOH_{(aq)}$, 7.3 mg) was purified by silica column chromatography (column i.d. 1.0 cm, length 7 cm), and eluted with 1:9:10 MeOH:EtOAc:CH_2Cl_2 to afford 2.5 mg of a new friedelane-type triterpene, 2α,3β-dihydroxyfriedelan-29-oic acid (**53**).

11α,28-Dihydroxylupenone (43): white solid; mp, begins decomposing at 285°C; $[\alpha]^{28.4}_D$ +33.6° (*c* 0.0119, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3450 (br), 2950, 2871, 1699, 1461, 1386 cm^{-1}; ^1H

NMR (300 MHz, $CDCl_3$), see Table 4.1; ^{13}C NMR (75.4 MHz, $CDCl_3$), see Table 4.1; ESI-MS *m/z* 479.3495 (calculated for $[C_{30}H_{48}O_3Na]^+$, 479.3496).

2α,3β-Dihydroxyfriedelan-29-oic acid (53): white solid; IR ($CHCl_3$) ν_{max} 3380 (br), 2940, 2872, 1701, 1461 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 4.2; ^{13}C NMR (75.4 MHz, $CDCl_3$), see Table 4.3; ESI-MS *m/z* 497.3603 (calculated for $[C_{30}H_{48}O_4Na]^+$, 497.3601).

5.5.2 *Maytenus cunninghamii*

5.5.2.1 Plant material, extraction and isolation

Root bark of *Maytenus cunninghamii* (Hook.) Loes. was collected, extracted, treated, solvent partitioned and column chromatographed as described above (Section 3.4.1). The 40% EtOAc fraction obtained from silica column chromatography (fraction 2, 187 mg) possessed the highest kynurenine-3-hydroxylase inhibition. The isolation of celastrol (**22**), the major inhibitory component of fraction 2, was described in Section 3.4.1.3. The 100% EtOAc fraction obtained from the same separation (fraction 5, 73 mg) also possessed high K3Hase inhibition and was further separated using HPLC.

Fraction 5 (73 mg) was injected in 1.0 mL MeOH onto a Whatman Partisil ODS-2 column (10 μm, i.d. 9.5 mm, length 500 mm). Stepwise gradient elution from 20%, 40%, 60%, 80%, 90%, 95% to 100% $MeOH_{(aq)}$ afforded fractions 5.1–5.7. Fraction 5.6 (95% $MeOH_{(aq)}$, 25 mg) showed the highest kynurenine-3-hydroxylase inhibition. It contained mainly celastrol (**22**) and a small amount of netzahualcoyoic acid (**25**), so was not separated further. Fraction 5.7 (100% $MeOH_{(aq)}$, 21 mg) was the second most inhibitory fraction. Recrystallisation of fraction 5.7 from methanol afforded the minor component,

3β-hydroxyurs-12-en-30-oic acid (dulcioic acid, **79**, 2.9 mg), identified by comparison of ^{1}H NMR (300 MHz, C_5D_5N) with reported values[143] and confirmed by analysis of the ^{13}C NMR (75.4 MHz, C_5D_5N) spectrum. The concentrated mother liquor was injected in 0.5 mL MeOH onto a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm) and eluted with 95% $MeOH_{(aq)}$, to afford 2α-hydroxymaytenonic acid (**59**, 12.3 mg), identified by comparison of ^{13}C NMR (75.4 MHz, C_5D_5N) and ^{1}H NMR (300 MHz, C_5D_5N) with literature values[314].

During re-isolation attempts of Δ^{15}-celastrol from the 65% EtOAc/CH_2Cl_2 silica column fraction of the 12.3 g of crude extract (Section 3.4.1.3), other compounds were observed by ^{1}H NMR that were considered worthy of isolation. Repeated silica column chromatography of this 65% EtOAC/CH_2Cl_2 using various percentages of EtOAc in CH_2Cl_2 afforded three fractions that contained compounds of interest. The least polar of these fractions was subjected to column chromatography (column i.d. 2.2 cm, length 11 cm) with 30% EtOAc/Hexane, yielding 24-deoxocangoronine (**61**, 3.4 mg) and cangoronine (**62**, 4.2 mg). **61** was identified by comparison of ^{13}C NMR (75.4 MHz, C_5D_5N) and ^{1}H NMR (300 MHz, $CDCl_3$) with reported values[315]. **62** was identified by comparison of ^{13}C NMR (75.4 MHz, C_5D_5N) and ^{1}H NMR (300 MHz, C_5D_5N) with literature values[131]. The next least polar fraction was subjected to column chromatography (column i.d. 2.2 cm, length 11 cm) with gradient elution from 95:5:0.1 CH_2Cl_2:EtOAc:CH_3COOH to 75:25:0.1 CH_2Cl_2:EtOAc:CH_3COOH, to afford 6.4 mg of a new friedelane-type triterpene, 2β-hydroxy-24-oxomaytenonic acid (**58**). The most polar fraction was column chromatographed (column i.d. 2.2 cm, length 11 cm) with gradient elution from 85:15:0.1 CH_2Cl_2:EtOAc:CH_3COOH to 70:30:0.1 CH_2Cl_2:EtOAc:CH_3COOH, to afford 4.7 mg of the 2-epimer of **58**, 2α-hydroxy-24-oxomaytenonic acid (**60**). **60** was isolated with a small amount of the dimethyl acetal (**64**), probably formed by post column reaction of the aldehyde function with MeOH, which was used to recover samples from NMR tubes, under

acidic conditions. A volume (10 μL) of 50% aqueous trifluoroacetic acid was added to the sample containing **60** and **64** dissolved in approximately 0.5 mL of $CDCl_3$, and monitored by ^1H NMR at room temperature until the acetal was consumed and converted to the aldehyde (45 minutes).

2β-Hydroxy-24-oxomaytenonic acid (58): white solid; IR ($CHCl_3$) ν_{max} 3350 (br), 2945, 2871, 1719, 1455, 1388, 1303 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 4.4; ^{13}C NMR (75.4 MHz, $CDCl_3$), see Table 4.4; ESI-MS *m/z* 509.3244 (calculated for $[C_{30}H_{46}O_5Na]^+$, 509.3237).

2α-Hydroxy-24-oxomaytenonic acid (60): white solid; IR ($CHCl_3$) ν_{max} 3400 (br), 2933, 2871, 1717, 1458, 1388, 1303 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 4.5; ^{13}C NMR (75.4 MHz, $CDCl_3$), see Table 4.5; ESI-MS *m/z* 509.3237 (calculated for $[C_{30}H_{46}O_5Na]^+$, 509.3237).

5.5.3 *Dolichandrone heterophylla*

5.5.3.1 Plant material and extraction

Juvenile to mature fruits and seeds (172 g) of *Dolichandrone heterophylla* (R.Br.) F.Muell. were collected from four shrubs in woodland on the Douglas Campus and were refrigerated until extraction. The material was ground with a hammer at room temperature then by hand after freezing with liquid nitrogen. The material was extracted three times with 300 mL of acetone-MeOH (1:1) in a 1 L conical flask placed in an ultrasonicating bath for 30 minutes. The three extractions were combined and filtered. The solvent was removed at 40°C on a rotary evaporator to give 6.0 g of crude extract.

5.5.3.2 Crude extract treatment

Polyphenols and quinones were removed from a portion (1.0 g) of the crude extract as described earlier (Section 3.4.1.2), using 12 g polyamide powder in a glass column (i.d. 2.2 cm, length 14 cm), and eluted with 100 mL of water followed by 400 mL of MeOH, to produce 893 mg of extract.

5.5.3.3 Bioassay-guided fractionation

The polyphenol/quinone free extract was subjected to bioassay-guided fractionation (see Section 3.4.1.3). The extract (893 mg) was solvent partitioned between CH_2Cl_2 and water using 100 mL volumes of each, to yield 83 mg of CH_2Cl_2 extract, 765 mg of water extract, and 15 mg of insoluble material, which was tested separately to the CH_2Cl_2 and water fractions.

The active fraction (CH_2Cl_2 extract, 83 mg) was chromatographed on silica gel (column i.d. 2.2 cm, length 10 cm) and eluted with 100 mL each of CH_2Cl_2, 20% EtOAc/CH_2Cl_2, 40% EtOAc/CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, and MeOH (fractions 1–7). Two fractions displayed high K3Hase inhibition. The fraction that had been tested at the lowest actual concentration, fraction 3 (40% EtOAc/CH_2Cl_2, 10 mg), was further separated using HPLC.

Fraction 3 (10 mg) was injected in 0.5 mL MeOH onto a Phenomenex Luna C18(2) column (10 μm, i.d. 4.6 mm, length 250 mm). Stepwise gradient elution from 40%, 60%, 80% to 100% $MeOH_{(aq)}$ afforded fractions 3.1–3.4. The major component from the most active fraction 3.3 (80% $MeOH_{(aq)}$, 4.5 mg) was purified by injecting in 0.1 mL acetonitrile (MeCN) onto a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm) and

eluting with 60% $MeCN_{(aq)}$. It was identified as 6β,19α-dihydroxyursolic acid (uncaric acid, **78**, 3.2 mg) from ESI-MS and comparison of ^{13}C NMR (75.4 MHz, $CDCl_3$-C_5D_5N (9:1)) and ^{1}H NMR (300 MHz, $CDCl_3$-C_5D_5N (9:1)) with reported values[228].

5.5.4 *Lophostemon grandiflorus*

5.5.4.1 Plant material and extraction

Bark (204 g) of *Lophostemon grandiflorus* (Benth.) Peter G. Wilson & J.T. Waterh. was collected from one tree in a planted rainforest gully on the Douglas Campus and was refrigerated until extraction. The material was frozen in liquid nitrogen and crushed with a hammer, then extracted twice with 600 mL of acetone-MeOH (1:1) in a 2 L conical flask placed in an ultrasonicating bath for 30 minutes. The two extractions were combined and filtered. The solvent was removed at 40°C on rotary evaporator to give 8.7 g of crude extract.

5.5.4.2 Crude extract treatment

Polyphenols and quinones were removed from a portion (1.0 g) of the crude extract as described earlier (Section 3.4.1.2), using 15 g polyamide powder in a glass column (i.d. 3.0 cm, length 10 cm), eluted with 125 mL of water followed by 500 mL of MeOH, to afford 717 mg of extract.

5.5.4.3 Bioassay-guided fractionation

The polyphenol/quinone free extract was subjected to bioassay-guided fractionation (see Section 3.4.1.3). The extract (717 mg) was solvent partitioned between CH_2Cl_2 and water using 200 mL volumes of each, to yield 146 mg of organic extract and 530 mg of water extract.

The active fraction (organic extract, 146 mg) was chromatographed with silica gel (column i.d. 3.0 cm, length 10 cm) and eluted with 100 mL each of CH_2Cl_2, 20% EtOAc/CH_2Cl_2, 40% EtOAc/CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, and MeOH (fractions 1–7). Fractions 2 (20% EtOAc/CH_2Cl_2, 38 mg), 3 (40% EtOAc/CH_2Cl_2, 39 mg) and 4 (60% EtOAc/CH_2Cl_2, 15 mg) showed the highest K3Hase inhibition and were further separated.

Recrystallisation of fraction 2 from approximately 5% dimethylsulfoxide (DMSO) in MeOH afforded pure β-sitosterol (**82**, 2.5 mg), identified by comparison of ^{1}H NMR (300 MHz, $CDCl_3$) data with literature values[316], and an impure mixture of β-sitosterol and betulinic acid in the mother liquor. The solvent was removed from the mother liquor and the resulting solid was recrystallised from aqueous MeOH to remove impurities. The solid was then recrystallised from DMSO, to give **82** as a solid and pure betulinic acid (**49**, 4.2 mg) in the mother liquor. **49** was identified by comparison of ^{13}C NMR (75.4 MHz, $CDCl_3$) and ^{1}H NMR (300 MHz, $CDCl_3$) with the literature[317].

Fraction 3 (39 mg) was injected in 0.5 mL MeOH onto a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm). Stepwise gradient elution from 60%, 80%, 90% to 100% $MeOH_{(aq)}$ afforded fractions 3.1–3.4. A portion (10 mg) of the most active fraction 3.2 (80% $MeOH_{(aq)}$, 26 mg) was injected in 0.5 mL MeOH onto the same column. Elution with

95% $MeOH_{(aq)}$ afforded pure betulinic (**49**, 4.0 mg) and ursolic acids (**74**, 4.6 mg). **74** was identified by comparison of ^{1}H NMR (300 MHz, C_5D_5N) data with reported values[176].

Fraction 4 (15 mg) was injected in 0.5 mL MeOH onto a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm). Stepwise gradient elution from 60%, 80%, 90% to 100% $MeOH_{(aq)}$ afforded fractions 4.1–4.4. The most active fraction 4.2 (80% $MeOH_{(aq)}$, 9.4 mg) was injected in 0.5 mL MeOH onto the same column. Elution with 75% $MeOH_{(aq)}$ afforded pure 2α-hydroxyursolic acid (**75**, 3.0 mg), identified from ESI-MS and comparison of ^{13}C NMR (75.4 MHz, C_5D_5N) and ^{1}H NMR (300 MHz, C_5D_5N) data with the literature[234].

5.5.5 *Lagerstroemia speciosa*

5.5.5.1 Plant material and extraction

Leaves and stems (207 g) of *Lagerstroemia speciosa* (L.) Pers. were collected from two trees cultivated on Douglas Campus and were refrigerated until extraction. The material was ground with a hammer at room temperature and after freezing with liquid nitrogen, then extracted three times with 600 mL of acetone-MeOH (1:1) in a 2 L conical flask placed in an ultrasonicating bath for 30 minutes. The three extracts were combined and filtered. The solvent was removed at 40°C on a rotary evaporator to give 7.5 g of crude extract.

5.5.5.2 Crude extract treatment

Polyphenols and quinones were removed from a portion (1.0 g) of the crude extract as described earlier (Section 3.4.1.2), using 18 g polyamide powder in a glass column (i.d.

3.0 cm, length 12 cm), eluted with 150 mL of water followed by 350 mL of MeOH, to produce 553 mg of extract.

5.5.5.3 Bioassay-guided fractionation

The polyphenol/quinone free extract was subjected to bioassay-guided fractionation (see Section 3.4.1.3). The extract (553 mg) was solvent partitioned between CH_2Cl_2 and water using 300 mL volumes of each, to yield 207 mg of organic extract and 306 mg of water extract.

The active fraction (organic extract, 207 mg) was chromatographed with silica gel (column i.d. 3.0 cm, length 10 cm) and eluted with 100 mL each of CH_2Cl_2, 20% EtOAc/CH_2Cl_2, 40% EtOAc/CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, 10% MeOH/EtOAc and MeOH (fractions 1–8). Two fractions displayed high K3Hase inhibition. The fraction that had been tested at the lowest actual concentration, fraction 6 (EtOAc, 11 mg), was further separated using HPLC.

Fraction 6 (11 mg) was injected in 0.5 mL MeOH onto a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm). Stepwise gradient elution from 40%, 60%, 80% to 100% $MeOH_{(aq)}$ afforded fractions 6.1–6.4. The most active fraction 6.3 (80% $MeOH_{(aq)}$, 5.6 mg) contained 2α-hydroxyursolic acid (**75**), identified from ESI-MS and comparison of ^{1}H NMR (300 MHz, C_5D_5N) data with the literature[234].

5.5.6 *Hyptis suaveolens*

5.5.6.1 Plant material and extraction

Leaves and stems (990 g) of *Hyptis suaveolens* (L.) Poit. were collected from 22 individuals from a creek bank on the Douglas Campus and refrigerated until extraction. The material was crushed by hand after freezing with liquid nitrogen, then extracted as for *Maytenus disperma* (Section 5.5.1.1), to yield 67.2 g of crude extract.

5.5.6.2 Crude extract treatment

The crude extract (67.2 g) was solvent partitioned between hexane and 90% $MeOH_{(aq)}$ to remove lipids, to afford an unweighed amount of 90% $MeOH_{(aq)}$ extract. Insoluble material was included with the 90% $MeOH_{(aq)}$ extract, while the hexane extract was discarded. Polyphenols and quinones were removed from a portion (1.0 g) of the 90% $MeOH_{(aq)}$ extract as described earlier (Section 3.4.1.2), using 12 g polyamide powder in a glass column (i.d. 2.2 cm, length 14 cm), eluted with 100 mL of water followed by 300 mL of MeOH, to produce 770 mg of extract.

5.5.6.3 Bioassay-guided fractionation

The polyphenol/quinone free extract was subjected to bioassay-guided fractionation (see Section 3.4.1.3). The extract (770 mg) was solvent partitioned between CH_2Cl_2 and water using 100 mL volumes of each, to afford 173 mg of CH_2Cl_2 extract, 567 mg of water extract,

and 14 mg of insoluble material, which was tested separately to the CH_2Cl_2 and water fractions.

The active fraction (CH_2Cl_2 extract, 173 mg) was chromatographed on silica gel (column i.d. 3.0 cm, length 10 cm) and eluted with 100 mL each of CH_2Cl_2, 20% EtOAc/CH_2Cl_2, 40% EtOAc/CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, and MeOH (fractions 1–7). Three fractions displayed some K3Hase inhibition. The fraction that had been tested at the lowest actual concentration, fraction 4 (60% EtOAc/CH_2Cl_2, 4.6 mg) was further separated using HPLC.

Fraction 4 (4.6 mg) was injected in 0.5 mL MeOH onto a Hewlett-Packard RP-18 column (7 μm, i.d. 9.5 mm, length 250 mm). Stepwise gradient elution from 60%, 80%, 90% to 100% $MeOH_{(aq)}$ afforded fractions 4.1–4.4. The most active fraction 4.3 (90% $MeOH_{(aq)}$, 1.3 mg) contained pure ursolic acid (**74**), identified from ESI-MS and comparison of ^1H NMR (300 MHz, C_5D_5N) data with reported values[176].

5.5.7 *Melaleuca viridiflora*

5.5.7.1 Plant material and extraction

Bark (580 g) of *Melaleuca viridiflora* Sol. ex Gaertn. was collected from three shrubs in woodland on the Douglas Campus and was refrigerated until extraction. The material was crushed by hand, then extracted as for *Maytenus disperma* (Section 5.5.1.1), to yield 115.5 g of crude extract.

5.5.7.2 Crude extract treatment

The crude extract (115.5 g) was solvent partitioned between hexane and 90% $MeOH_{(aq)}$ to remove lipids, and afforded 57.1 g of 90% $MeOH_{(aq)}$ extract. Insoluble material was included with the 90% $MeOH_{(aq)}$ extract, while the hexane extract was discarded. Polyphenols and quinones were removed from a portion (1.0 g) of the 90% $MeOH_{(aq)}$ extract as described earlier (Section 3.4.1.2), using 9 g polyamide powder in a glass column (i.d. 2.2 cm, length 10 cm), and eluted with 70 mL of water followed by 280 mL of MeOH, to produce 738 mg of extract.

5.5.7.3 Bioassay-guided fractionation

The polyphenol/quinone free extract was subjected to bioassay-guided fractionation (see Section 3.4.1.3). The extract (738 mg) was solvent partitioned between CH_2Cl_2 and water using 200 mL volumes of each, to yield 607 mg of organic extract and 124 mg of water extract.

The active fraction (organic extract, 607 mg) was chromatographed with silica gel (column i.d. 3.0 cm, length 12 cm) and eluted with 100 mL each of CH_2Cl_2, 40% EtOAc/CH_2Cl_2, 60% EtOAc/CH_2Cl_2, 80% EtOAc/CH_2Cl_2, EtOAc, and MeOH (fractions 1–6). Four fractions showed similar amounts K3Hase inhibition. The fraction that had been tested at the lowest actual concentration, fraction 3 (60% EtOAc/CH_2Cl_2, 44 mg), was further separated using HPLC.

Fraction 3 (44 mg) was injected in 1.0 mL MeOH onto a Whatman Partisil ODS-2 column (10 μm, i.d. 9.5 mm, length 500 mm). Stepwise gradient elution from 40%, 60%, 80%, to 100% $MeOH_{(aq)}$ afforded fractions 3.1–3.4. Two fractions showed similar amounts of

inhibition. The fraction that had been tested at the lowest actual concentration, fraction 3.4 (100% $MeOH_{(aq)}$, 13 mg), was injected in 0.2 mL MeOH onto the same column. Stepwise gradient elution from 75%, 80%, 87.5%, 95% to 100% $MeOH_{(aq)}$ afforded 8 fractions. The fraction eluted with 80% $MeOH_{(aq)}$ (fraction 3.4.2, 4.2 mg) contained impure betulinic acid (**49**), identified by comparison of ^1H NMR (300 MHz, $CDCl_3$) with the literature[317]. The first fraction that eluted with 87.5% $MeOH_{(aq)}$ (fraction 3.4.3, 0.5 mg) contained impure ursolic acid (**74**), identified by comparison of ^1H NMR (300 MHz, C_5D_5N) data with reported values[176]. Purification of betulinic and ursolic acids from these fractions was not attempted.

CHAPTER 6.

CONCLUSIONS

6.1 DISCOVERY OF POTENTIAL ANTICANCER AGENTS FROM NORTH QUEENSLAND PLANTS

This study successfully discovered potential anticancer agents by screening extracts of North Queensland plants for cytotoxicity using the P388D1 cell line *in vitro*. Bioassay-guided fractionation of selected cytotoxic extracts led to the isolation of two new and one known quinonemethide triterpenes from *Maytenus cunninghamii* (Celastraceae) and three known podophyllotoxins from *Callitris intratropica* (Cupressaceae). The new compounds were named netzahualcoyoic acid (**25**) and Δ^{15}-celastrol (**32**), while the third quinonemethide was identified as the known cytotoxic agent celastrol (**22**). The structure of netzahualcoyoic acid was fully characterised by 1D and 2D-NMR and other spectroscopic techniques. The structure of Δ^{15}-celastrol was determined solely from ^{1}H NMR, UV/Vis and ESI-MS evidence, due to partial decomposition before a full analysis using 2D-NMR was attempted. Δ^{15}-Celastrol, which has a double-bond in an unusual position, may represent a biosynthetic intermediate between celastrol and netzahualcoyoic acid.

Netzahualcoyoic acid (IC_{50} value = 0.12 μM, 0.11–0.13 μM 95% CI range) was approximately three-fold more cytotoxic than celastrol (IC_{50} value = 0.37 μM, 0.30–0.45 μM 95% CI range). This may be partly due to the difference in the conformations of rings D and E between the two compounds. Rings D and E in celastrol are likely to be in a chair-chair conformation that places the C-29 carboxyl group proximal to the C-27 methyl group (Figure 2.1a). Conversely, the double bond at C-14 in netzahualcoyoic acid causes ring D to

be in a half-chair and ring E to be in a chair conformation, which places the C-29 carboxyl group distal to the C-27 methyl group (Figure 2.1b).

Further work needs to assess the cytotoxic profile of netzahualcoyoic acid and its acid-rearranged products, such as **30**, in the NCI's 60 cell line panel. The acid-rearranged products are probably much less toxic than netzahualcoyoic acid *in vivo*, but they may also be much less cytotoxic. If these compounds display selective cytotoxicity, then *in vivo* toxicity studies followed by *in vivo* antitumour studies may be warranted.

6.2 DISCOVERY OF POTENTIAL NEUROPROTECTIVE AGENTS FROM NORTH QUEENSLAND PLANTS

This study successfully identified triterpenes as a new class of potent and selective competitive inhibitors of kynurenine-3-hydroxylase from a screen of extracts of North Queensland plants. Kynurenine-3-hydroxylase inhibitors divert the metabolism of KYN to the neuroprotective agent KYNA and reduce the production of the neurotoxic metabolites, QUIN and 3HK. Thus, kynurenine-3-hydroxylase inhibitors have therapeutic potential in neurological disorders where an increased QUIN:KYNA ratio and raised levels of 3HK are implicated. Particularly strong evidence exists for this pathophysiology in Huntington's Disease and AIDS-related dementia (see Section 1.3.4). Evidence for this pathophysiology also exists in a number of other disorders that involve neuroinflammation, neurodegeneration or CNS infection, such as Alzheimer's Disease, Parkinson's Disease, cerebral malaria, poliovirus infection and Lyme Disease (see Section 1.3.4).

Kynurenine-3-hydroxylase inhibition was assessed for plant extracts and pure compounds in crude enzyme preparations using HPLC with electrochemical detection. In addition to the three quinonemethides mentioned above, four new and nine known triterpenoids were

isolated, predominantly by bioassay-guided fractionation, from seven plant extracts that possessed strong kynurenine-3-hydroxylase inhibition. The new compounds were identified as 11α,28-dihydroxylupene (**43**) and 2α-3β-friedelan-29-oic acid (**53**) from *Maytenus disperma* (Celastraceae), and 2β- and 2α-hydroxy-24-oxomaytenonic acids (**58** and **60**, respectively) from *M. cunninghamii*. These compounds were fully characterised by 1D- and 2D-NMR and other spectroscopic methods. The ring conformations in each new compound was determined from a combination of NOESY correlations, coupling constants and rationalisation of deshielding effects. The known compounds isolated were: 2α-hydroxymaytenonic acid (**59**), 3-hydroxy-2-oxofriedel-3-en-29-oic acid (**61**), cangoronine (**62**) and dulcioic acid (**79**) from *M. cunninghamii*; uncaric acid (**78**) from *Dolichandrone heterophylla* (Bignoniaceae); betulinic acid (**49**), ursolic acid (**74**), 2α-hydroxyursolic acid (**75**) and β-sitosterol (**82**) from *Lophostemon grandiflorus* (Myrtaceae); 2α-hydroxyursolic acid from *Lagerstroemia speciosa* (Lythraceae); ursolic acid from *Hyptis suaveolens* (Lamiaceae); and ursolic acid and betulinic acid from *Melaleuca viridiflora* (Myrtaceae). A detailed biosynthetic pathway was proposed for compounds isolated from *M. cunninghamii* and related compounds based on progressive oxidation from maytenonic acid (Scheme 4.2).

The inhibition constants of the isolated compounds, plus oleanolic acid (**81**) and methyl betulinate (**50**), were determined for kynurenine-3-hydroxylase (Table, 4.6, Figure 4.2). The most active compound was uncaric acid (**78**, K_{ic} = 0.023 ± 0.002 μM), which is one of the most potent kynurenine-3-hydroxylase inhibitors that have been reported. The next most active inhibitors of this study were the 24-oxofriedelan-29-oic acids (**60**, K_{ic} = 0.061 ± 0.005 μM; **62**, K_{ic} = 0.077 ± 0.011 μM; **58**, K_{ic} = 0.12 ± 0.02 μM) and celastrol (**22**, K_{ic} = 0.14 ± 0.03 μM). Important structure-activity relationships (SARs) relating to triterpene-type, functional groups, and ring conformations were observed (Table 4.7, Figure 4.3). The friedelanes, ursanes and oleanolic acid were more active than the lupenes, probably due to a

steric clash of the isopropenyl group in the lupenes with substituents in the enzyme's active site. Activity was dependent on a carboxylic acid or a hydroxymethyl attached to ring E. In friedelanes, rings D and E needed to be in a chair-chair conformation to place the 29-carboxylic group in the correct spatial position for activity. It is not clear whether the hydroxyl group at 19α or 6β in uncaric acid is responsible for its superior activity. In ring A, a hydrophilic group is likely to be a requirement for activity. Furthermore, an aldehyde attached to C-5 in friedelanes, rather than a methyl, increased activity two- to five-fold. The SARs can be related to the structure of 3HK (**83**) and the transitional intermediate (**84**). For the triterpenes with K_{ic} values less than 1 μM, the distance between the carboxyl or hydroxymethyl group in ring E and the hydrophilic group in ring A is approximately the same as the distance between the carboxyl group and the 3-hydroxyl group of 3HK and **84**.

The selectivity of the kynurenine-3-hydroxylase inhibitors was demonstrated by their complete lack of activity against kynureninase at 10 μM and their very low to absent activity (IC_{50} > 5 μM) against kynurenine aminotransferase (KAT). The one exception to this was netzahualcoyoic acid (IC_{50} = 0.50 ± 0.14 μM for KAT). The high KAT inhibition and relatively low kynurenine-3-hydroxylase inhibition of netzahualcoyoic acid can be related to the different spatial position of the carboxyl group, caused by a different conformation in rings D and E when compared to the other compounds.

With the exception of the quinonemethides, all the triterpenoids have either previously been shown to have low *in vivo* toxicity, or were shown in this study to have low or no *in vitro* cytotoxicity against the sensitive P388D1 cell line (Table 4.8). Acid-rearrangement of celastrol, as would occur in the stomach after oral administration, would greatly reduce its toxicity and may not greatly affect the kynurenine-3-hydroxylase inhibitory activity. Its antioxidant and anti-inflammatory activities would provide additional modes of action in treating neurological disorders that involve inflammation and neurodegeneration.

Triterpenes commonly have good drug-like properties. They commonly have low toxicity, few adverse side effects, are orally available, and cross the blood-brain barrier. This study has shown that many triterpenes, particularly pentacyclic triterpenes with a carboxylic acid substituent in ring E, are potent inhibitors of kynurenine-3-hydroxylase and therefore have great potential in the treatment of neurodegenerative disorders such as Huntington's Disease and AIDS-related dementia. Uncaric acid (**78**), 2α-hydroxymaytenonic acid (**60**) and celastrol (**22**) show the greatest potential. It is recommended that they be investigated *in vivo* firstly in normal mice and then in animal models of disease where an imbalance in kynurenine metabolites has been implicated, such as in one of the available transgenic mouse models of Huntington's disease.

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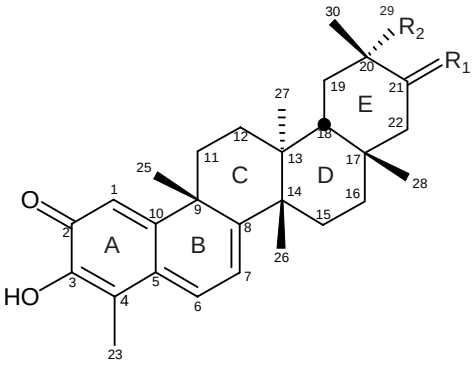
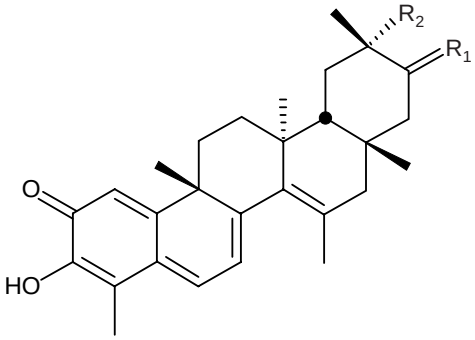
APPENDIX 1.

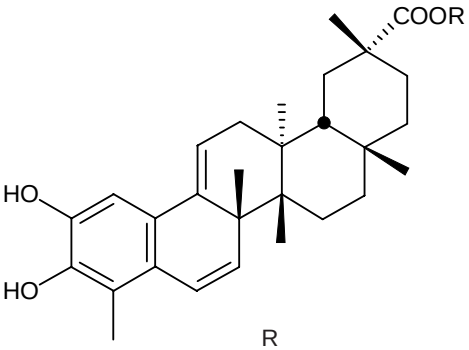
STRUCTURES DISCUSSED IN THE RESULTS AND DISCUSSION CHAPTERS



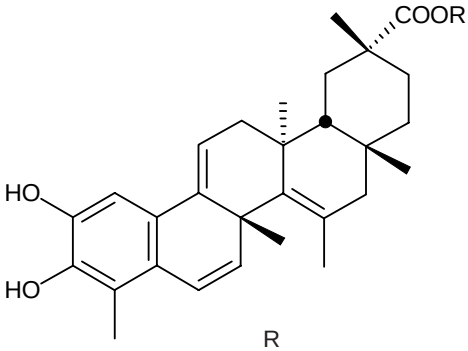
		R_1	R_2
Celastrol	**22**	H_2	COOH
Pristimerin	**23**	H_2	COOMe
Tingenone	**24**	O	H



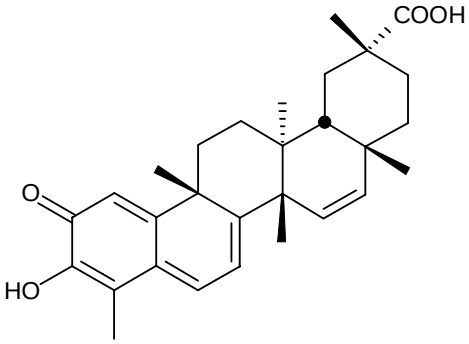
		R_1	R_2
Netzahualcoyoic acid	**25**	H_2	COOH
Netzahualcoyene	**26**	H_2	COOMe
Scutione	**27**	O	H



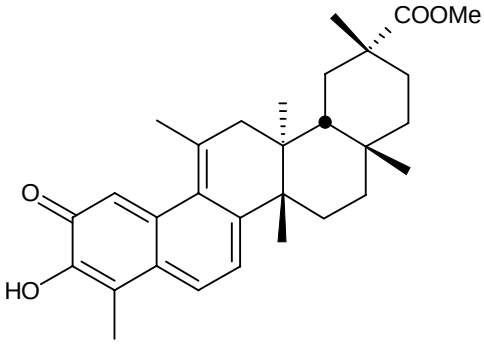
	R
28	H
29	Me



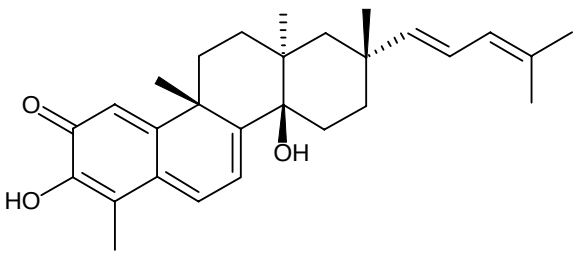
	R
30	H
31	Me



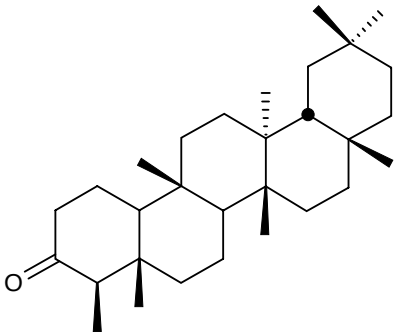
Δ^{15}-Celastrol **32**



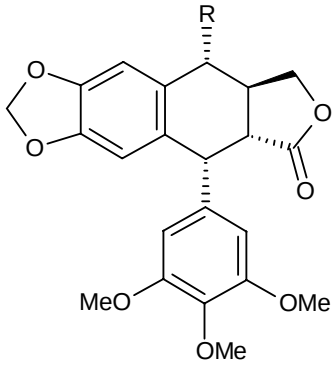
Pristimerinene **33**



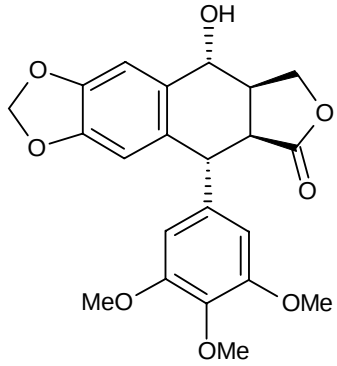
Russulaflavidin **34**



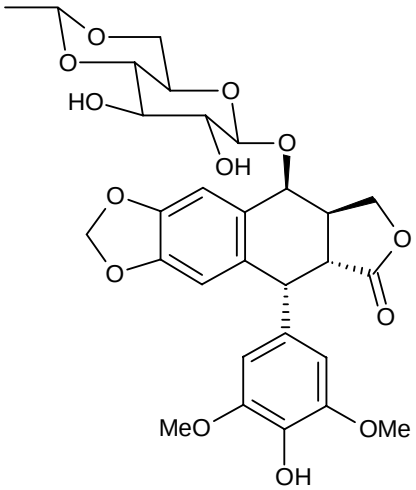
Friedelin **35**



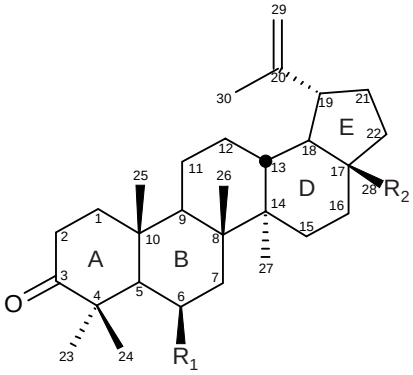
		R
Podophyllotoxin	**36**	OH
Deoxypodophyllotoxin	**37**	H



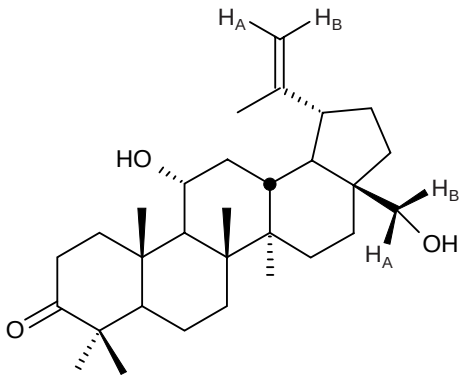
Picropodophyllotoxin **38**



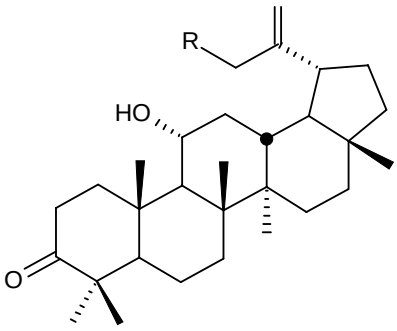
Etoposide **39**



		R_1	R_2
Lupenone	**40**	H	Me
	41	OH	Me
	42	H	COOH

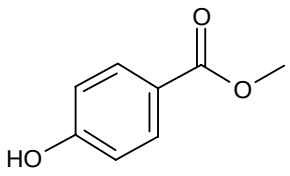


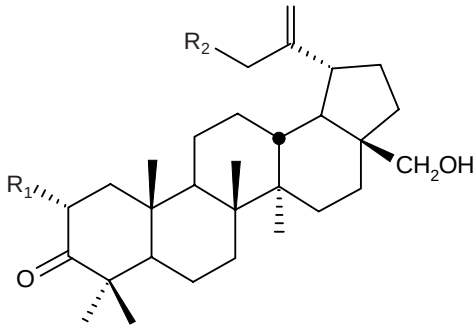
43



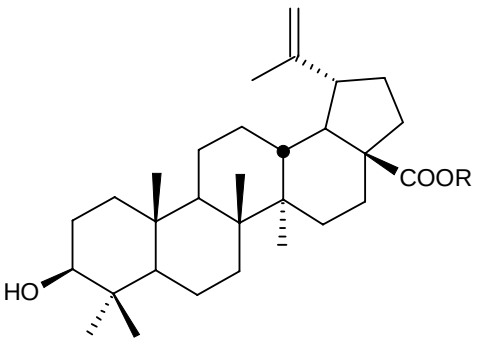
	R
44	H

45

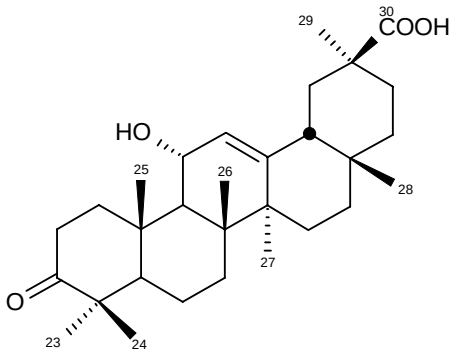




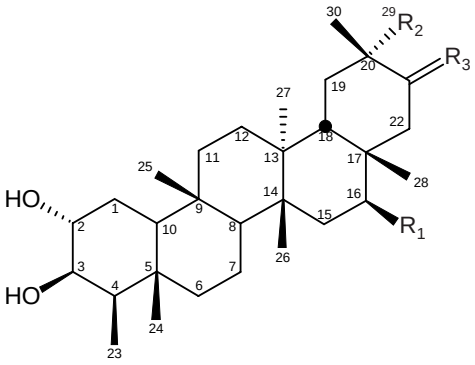
	R_1	R_2
46	H	H
47	OH	H
48	H	OH



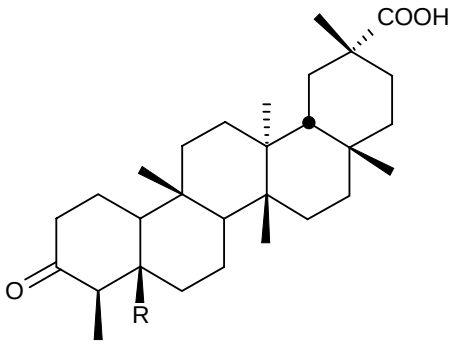
		R
Betulinic acid	**49**	H
Methyl betulinate	**50**	Me



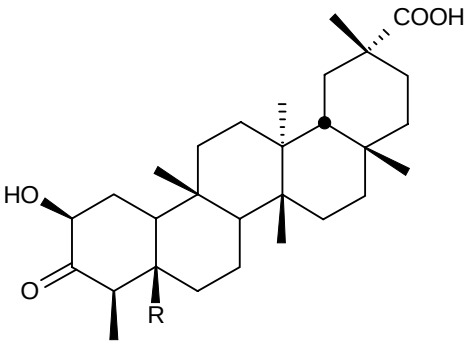
51



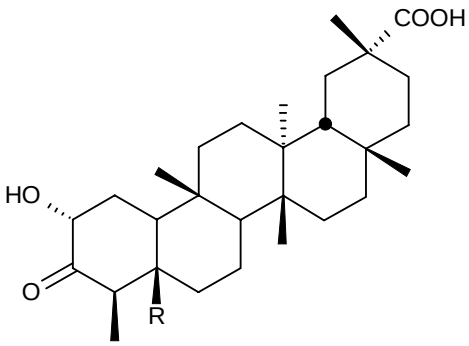
	R_1	R_2	R_3
52	H	Me	H_2
53	H	COOH	H_2
54	Me	COOH	O



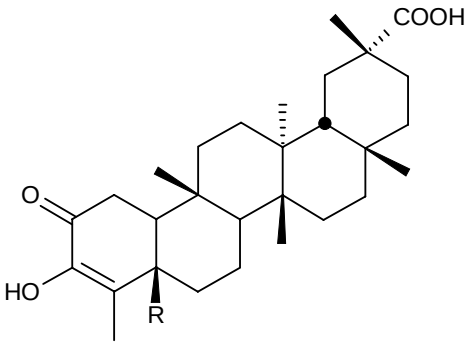
		R
Maytenonic acid	**55**	Me
	56	CHO



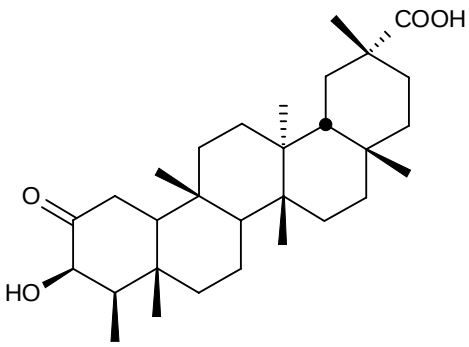
	R
57	Me
58	CHO



	R
59	Me
60	CHO



		R
	61	Me
Cangoronine	**62**	CHO



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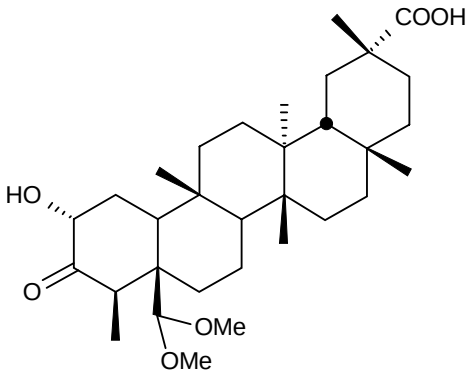

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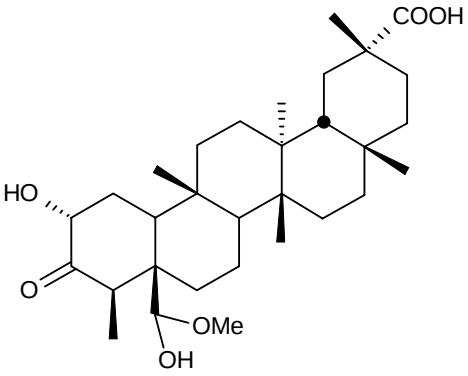

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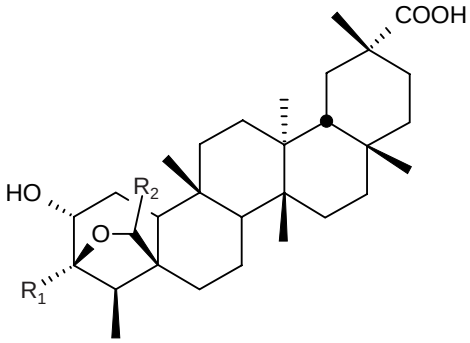
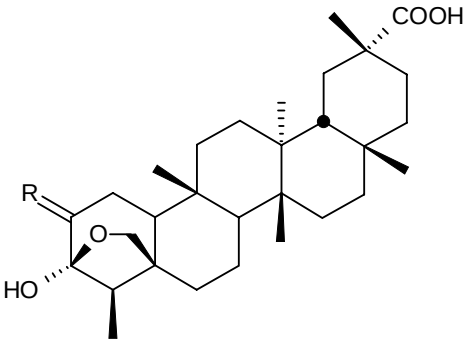
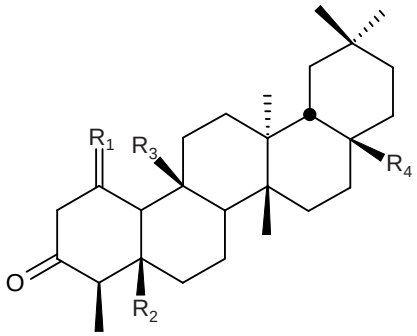


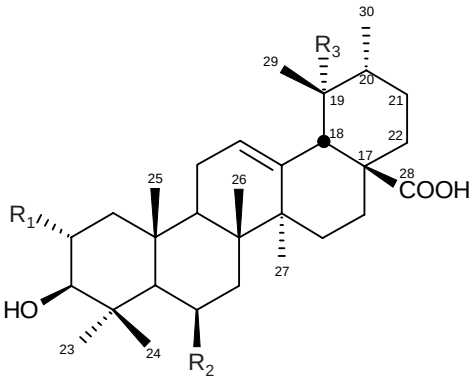
		R_1	R_2
Orthosphenic acid	**66**	OH	H
	67	OH	OMe
	68	H	OEt



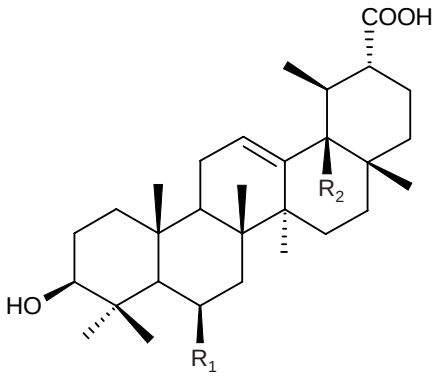
		R
Salaspermic acid	**69**	H_2
Wilforic acid E	**70**	O



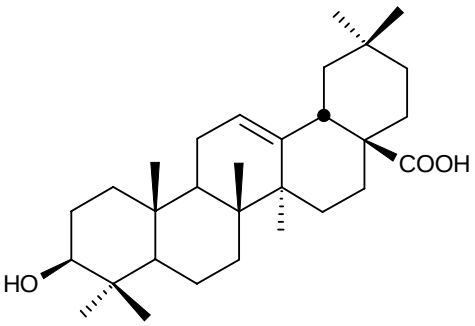
	R_1	R_2	R_3	R_4
71	O	CHO	Me	Me
72	H_2	Me	CHO	Me
73	H_2	Me	Me	CHO



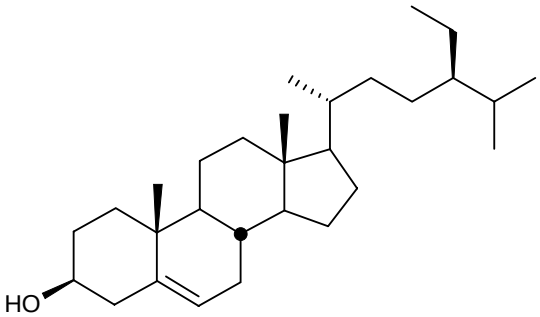
		R_1	R_2	R_3
Ursolic acid	**74**	H	H	H
	75	OH	H	H
	76	H	OH	H
	77	H	H	OH
Uncaric acid	**78**	H	OH	OH



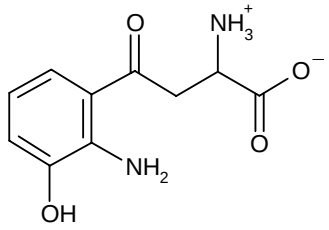
		R_1	R_2
Dulcioic acid	**79**	H	H
	80	OH	OH



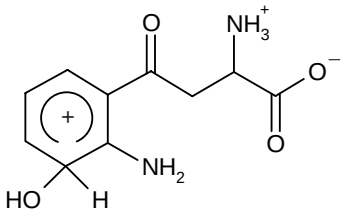
Oleanolic acid **81**



β-Sitosterol **82**



3-Hydroxykynurenine **83**



84

APPENDIX 2. LIST OF PLANT SPECIES AND ORGANS COLLECTED

Family	Species	Separate Organs Extracted
Acanthaceae	*Thunbergia laurifolia* Lindl.	leaves, fruit and inflorescence
Aizoaceae	*Trianthema portulacastrum* L.	aerial parts
Anarcardiaceae	*Mangifera indica* L.	leaves and stem, bark
	Pleiogynium timorense (DC.) Leenh.	leaves, fruit, bark
	Schinus terebinthifolius Raddi	leaves and stem, bark, inflorescence
Apocynaceae	*Alstonia constricta* F. Muell.	bark
	Carissa ovata R.Br.	leaves and stem, bark
	Cascabela thevetia (L.) Lippold	leaves, stem, bark, fruit
	Plumeria rubra L.	leaves, flowers, bark
Asclepiadaceae	*Sarcostemma viminale* subsp. *brunonianum* (Wight & Arn.) P.I.Forst.	stem
Asteraceae	*Ageratum conyzoides* L.	aerial parts
	Bidens bipinnata L.	whole plant
	Emilia sonchifolia (L.) DC.	aerial parts
	Pterocaulon serrulatum Guillaumin	leaves and stem
	Vernonia cinerea (L.) Less.	leaves, inflorescence, root
	Wedelia triloba (L.) Hitchc.	whole plant
	Zinnia elegans Jacq.	leaves and stem, inflorescence and fruit
Araucariaceae	*Araucaria cunninghamii* A.Cunn. var. *cunninghamii*	leaves and stem, branchlets, bark
Bignoniaceae	*Dolichandrone heterophylla* (R.Br.) F.Muell.	leaves, stem, bark, fruit
	Spathodea campanulata P.Beauv.	leaves, flowers, bark, fruit, juvenile fruit
Bixaceae	*Cochlospermum gillivraei* Benth.	leaves, juvenile leaves, fruit, stem, bark, flowers, buds
Boraginaceae	*Trichodesma zeylanicum* R.Br.	leaves, stem, inflorescence and flowers
Bromeliaceae	*Ananas comosus* (L.) Merr.	leaves
Caesalpiniaceae	*Bauhinia hookeri* F. Muell	leaves, stem, bark, flowers, fruit
	Bauhinia variegata L.	leaves, flowers, stem, bark, fruit, seeds

Caesalpiniaceae (cont.)	*Cassia fistula* L.	leaves, stem, bark, unripe fruit, ripe fruit
	Chamaecrista mimosoides (L.) Greene	aerial parts
	Senna alata (L.) Roxb.	leaves, juvenile leaves, stem, bark, fruit, flowers, root
	Tamarindus indica L	leaves, stem, bark, fruit, seeds
Capparaceae	*Cleome viscosa* L.	leaves, stem, fruit
Celastraceae	*Cassine melanocarpa* (F.Muell.) Kuntze	leaves, stem, bark, fruit
	Maytenus cunninghamii (Hook.) Loes.	leaves, stem, bark, root bark, fruit
	Maytenus disperma (F.Muell.) Loes.	leaves, fruit, bark
Clusiaceae	*Calophyllum inophyllum* L.	fruit, leaves, bark, stem
	Garcinia dulcis (Roxb.) Kurz.	leaves, fruit, bark
Combretaceae	*Terminalia catappa* L.	leaves, seeds, bark, fruit
Commelinaceae	*Commelina ensifolia* R.Br.	aerial parts
Convolvulaceae	*Argyreia nervosa* (Burman f.) Bojer	leaves, stem, bark, fruit
	Ipomoea eriocarpa R.Br.	aerial parts
	Ipomoea nil (L.) Roth.	leaves and stem, fruit
	Ipomoea plebeia R.Br.	whole plant
	Ipomoea triloba L.	aerial parts
Cupressaceae	*Callitris intropica* R.T. Baker & H.G. Sm.	leaves, bark, fruit, stem
Ebenaceae	*Diospyros geminata* (R.Br.) F.Muell	leaves, stem, bark, fruit
Euphorbiaceae	*Chamaesyce hirta* (L.) Millsp.	whole plant
	Drypetes deplanchei (Brongn. & Griseb.) Merr	leaves, fruit, bark
	Euphorbia cyathophora Murr.	whole plant
	Euphorbia heterophylla L.	whole plant
	Euphorbia lactea Haw.	leaves and stem
	Flueggea virosa subsp. melanthesoides (F.Muell.) Webster	leaves, fruit, bark
	Macaranga tanarius (L.) Mull.Arg.	stem, bark, leaves
	Petalostigma pubescens Domin.	leaves, bark, stem, fruit, seeds
	Phyllanthus hebecarpus Benth.	aerial parts
	Phyllanthus virgatus G.Forster	whole plant
Fabaceae	*Abrus precatorius* L.	leaves and stem, fruit, seeds
	Castanospermum australe A.Cunn. & C.Fraser ex Hook.	leaves, stem, bark, fruit, seeds
	Clitoria ternatea L	leaves and stem, fruit, root
	Crotalaria calycina Schrank	whole plant

Fabaceae (cont.)	*Crotalaria laburnifolia* L.	leaves, stem, bark, fruit, flowers
	Crotalaria linifolia L.f.	aerial parts
	Crotalaria medicaginea Lam.	whole plant
	Crotalaria pallida Aiton	leaves, stem, flowers and fruit
	Crotalaria verrucosa L.	leaves and stem, fruit
	Erythrina vespertilio Benth.	leaves and stem, bark
	Indigofera colutea (Burm.f.) Merr.	aerial parts
	Indigofera linifolia (L.f.) Retz.	aerial parts
	Pongamia pinnata (L.) Pierre	leaves, stem, bark, fruit
Haemodoraceae	*Haemodorum coccineum* R.Br.	leaves, rhizome, inflorescence and flowers
Lamiaceae	*Hyptis suaveolens* (L.) Poit.	leaves, stem, seeds
Lauraceae	*Cassytha filiformis* L.	aerial parts
Lecythidaceae	*Planchonia careya* (F.Muell.) R.Knuth	leaves, stem, bark, fruit
Leeaceae	*Leea indica* Merr.	leaves, fruit, bark
Liliaceae	*Dianella caerulea* var. *vannata* R.J.F.Hend.	leaves, stem, inflorescence, fruit
Loganiaceae	*Strychnos psilosperma* F.Muell.	leaves, stem, bark, fruit
Lythraceae	*Lagerstroemia speciosa* (L.) Pers.	leaves and stem, fruit, bark
Malvaceae	*Abelmoschus moschatus* subsp. *tuberosus* Borss.Waalk.	aerial parts
	Sida cordifolia L.	leaves and stem, juvenile leaves, inflorescence
	Sida spinosa L.	whole plant
Menispermaceae	*Stephania japonica* var. *timoriensis* (DC.) Forman	aerial parts, root
Mimosaceae	*Acacia holosericea* G.Don	leaves, stem, bark, fruit
	Leucaena leucocephala (Lam.) de Wit	leaves, stem, bark, fruit, flowers, seeds
Moraceae	*Ficus benjamina* L.	leaves and stem, fruit, bark
	Ficus opposita Miq.	leaves, stem, bark, fruit
Myrtaceae	*Corymbia dallachiana* (Benth.) K.D.Hill & L.A.S.Johnson	kino, bark
	Corymbia erythrophloia (Blakely) K.D.Hill & L.A.S.Johnson	leaves and stem, bark and kino
	Eucalyptus robusta Sm.	leaves and stem, bark and kino, inner bark, outer bark

Myrtaceae (cont.)	*Lophostemon grandiflorus* (Benth.) Peter G. Wilson & J.T. Waterh.	bark
	Melaleuca viridiflora Gaertn.	leaves, stem, bark, fruit
Nyctaginaceae	*Boerhavia dominii* Meikle & Hewson	aerial parts, root
Oleaceae	*Jasminum didymum* subsp. *racemosum* (F.Muell.) P.S.Green	leaves and stem, bark
Onagraceae	*Ludwigia octovalvis* (Jacq.) P.H.Raven	leaves, juvenile fruit
Pandanaceae	*Pandanus spiralis* R.Br.	leaves, bark, leaf bases on stem, fruit
Passifloraceae	*Passiflora foetida* L.	leaves, stem, fruit, juvenile fruit
Poaceae	*Arundo donax* var. *versicolor* (Mill.) Stokes	leaves and stem, inflorescence, rhizome and root
	Heteropogon contortus (L.) Roem. & Schult.	leaves, root, inflorescence
	Mnesithea rottboellioides (R.Br.) de Koning & Sosef	leaves, culm, root
	Panicum maximum var. *coloratum* C.T.White	leaves, stem
	Themeda arguens (L.) Hack.	whole plant
Portulacaceae	*Portulaca pilosa* L.	aerial parts
Rhamnaceae	*Alphitonia excelsa* (Fenzl) Benth.	leaves, stem, bark, inflorescence
	Ziziphus mauritiana Lam.	leaves, stem, bark, fruit
Rubiaceae	*Hedyotis corymbosa* (L.) Lam.	whole plant
	Morinda citrifolia L.	leaves, inflorescence, stem, fruit
	Mussaenda philippica A.Rich.	leaves, flowers, bark
Rutaceae	*Geijera salicifolia* Schott	leaves, stem, bark
	Melicope elleryana (F. Muell.) T. Hartley	bark
Santalaceae	*Exocarpos latifolius* R.Br.	leaves, stem, bark, fruit
Sapindaceae	*Dodonaea lanceolata* F.Muell.	leaves and stem, root
	Harpullia pendula F.Muell.	leaves and stem, flowers, bark
Sapotaceae	*Mimusops elengi* L.	leaves, stem, bark, fruit
Scrophulariaceae	*Scoparia dulcis* L.	aerial parts
Smilaceae	*Tinospora smilacina* Benth.	whole plant

Solanaceae	*Physalis minima* L.	leaves and stem, fruit
	Solanum torvum Sw.	leaves, flowers and fruit
Taccaceae	*Tacca leontopetaloides* (L.) Kuntze	leaves, flowers, root
Tiliaceae	*Grewia latifolia* Benth.	leaves, flowers, stem
	Grewia retusifolia Kurz	leaves, fruit, root
Typhaceae	*Typha domingensis* Pers.	leaves and stem, flowers, rhizome and root
Ulmaceae	*Trema tomentosa* var. *viridis* (Planch.) Hewson	aerial parts
Urticaceae	*Pipturus argenteus* (G.Forst.) Wedd.	leaves, stem, bark, inflorescence
Verbenaceae	*Clerodendrum floribundum* R.Br.	leaves, stem, bark, inflorescence and fruit
	Lantana camara L.	leaves, stem, juvenile leaves, fruit
	Stachytarpheta jamaicensis (L.) J.Vahl	leaves, stem, fruit, inflorescence, root
Violaceae	*Hybanthus enneaspermus* (L.) F.Muell. subsp. *enneaspermus*	whole plant
Vitaceae	*Cayratia trifolia* (L.) Domin	leaves, stem, fruit, juvenile fruit