Natural Products from Australian Celastraceae Plants and Their Leucine Transport Inhibition in Prostate Cancer Cells

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Abstract

Natural products are the main source of approved medicines with more than half of the drugs on the market today being either natural products or natural product derivatives. Moreover, a significant number of these drugs are of plant origins. However, it has been estimated that of the >422,000 plant species currently described, only 15% of this plant diversity have been phytochemically explored. Therefore, there still exists a huge potential in the plant kingdom for the discovery of new chemistry, some of which may result in the identification of lead compounds or drugs for the pharmaceutical industry. Several notable examples of plant-derived natural product drugs are the anticancer compounds vinblastine, vincristine, and paclitaxel, the antimalarial agents artemisinin and quinine, and the Alzheimer drugs galantamine and huperzine A. In regards to plant-derived lead compounds, two pertinent examples include camptothecin, which lead to the development of the anticancer agents, topotecan and irinotecan, and papaverine, which was the lead model for the antihypertension verapamil.

The Celastraceae plant family is found worldwide, especially in tropical and subtropical regions; currently there are 88 genera and 1300 species belonging to this flowering plants family. Various molecules possessing numerous biological activities have been reported from Celastraceae. One noteworthy example of an interesting natural product and cancer lead compound from Celastraceae is the alkaloid maytansine, which was further developed into the antibody drug conjugate treatment, trastuzumab emtansine, and is currently used for breast cancer treatment. However, the characteristic secondary metabolites of Celastraceae are the dihydro-β-agarofuran sesquiterpenoids, which have been reported in the literature as both chemotaxonomic markers and privileged structures.

The L-type amino acid transporters (LATs) are responsible for the uptake of various amino acids (including leucine) into cells. Leucine is a regulator amino acid of the mTORC1 signalling pathway and one of the essential amino acids that is transported by LATs. Though LATs are expressed in both normal and cancer cells, the overexpression of LATs has been reported in various cancer cells, including those associated with prostate cancer. Thus, the inhibition of leucine uptake may be a novel drug target for cancer treatment. To date, very few natural product LATs inhibitors have been identified. Venulosides, which are all monoterpenoid glycosides, were the first and only
natural products (reported by Quinn et al.) that had been reported to inhibit LATs prior to these PhD studies. Their recent discovery highlights the potential of natural products in the discovery of new LAT inhibitors. This knowledge coupled with an interest in the chemistry of the hitherto under-investigated Australian plant family, Celastraceae, motivated us to identify new small molecules from this particular biota and evaluate all compounds isolated or semi-synthesised for their ability to inhibit leucine uptake on the human prostate cancer cell line, LNCaP. The plants that were investigated during these PhD studies included *Maytenus bilocularis*, *Denhamia pittosporoides*, and *Celastrus subspicata* and *Denhamia celastroides*.

In Chapter 2, phytochemical studies of the leaves of the Australian rainforest plant *Maytenus bilocularis* led to the identification of three new dihydro-β-agarofurans, bilocularins A–C, and six known compounds, namely celastrine A, 1α,6β,8α-triacetoxy-9α-benzoyloxydihydro-β-agarofuran, 1α,6β-diacetoxy-9α-benzoyloxy-8α-hydroxydihydro-β-agarofuran, Ejap-10, 1α,6β-diacetoxy-9β-benzoyloxydihydro-β-agarofuran, and Ejap-2. Bilocularin A was used to generate four semisynthetic ester analogues. The absolute configuration of bilocularins A and B was established by X-ray crystallography study; for bilocularin C, the absolute was determined by comparison of ECD spectra. All compounds were found to be inactive in a cytotoxicity assay against the human prostate cancer cell line LNCaP. However, several compounds were found to exhibit similar potency to verapamil in reversing multidrug resistance in the human leukemia cells (CEM/VCR R). Moreover, 1α,6β,8α-triacetoxy-9α-benzoyloxydihydro-β-agarofuran was shown to inhibit leucine uptake in LNCaP cells with IC₅₀ value of 15.5 µM, which was more potent than the leucine analogue 2-aminobicyclo[2.2.1]-heptane-2-carboxylic acid (BCH).

Motivated by these findings with dihydro-β-agarofurans from the leaves of *M. bilocularis*, we extended our studies (see Chapter 3) to the roots of this plant, which had never been phytochemically explored. Six new dihydro-β-agarofuran sesquiterpenoids (bilocularins D–I), together with three known compounds, namely 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran, pristimerin, and celastrol were successfully isolated. Moreover, the absolute configuration of bilocularin D was established by single-crystal X-ray diffraction analysis. Bilocularins D and G, 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran, and celastrol inhibited leucine uptake in the LNCaP cells with IC₅₀ values ranging from 2.5–27.9 µM. This study identified bilocularins D–F as the first dihydro-β-agarofurans possessing a hydroxyacetate group.
Chapter 4 details the identification of two previously undescribed dihydro-β-agarofurans, denhaminols I and J together with four related and known metabolites, 1α,2α,6β,15-tetraacetoxy-9α-benzoyloxy-8-oxodihydro-β-agarofuran, wilforsinine F, 1α,2α,6β,8α,15-pentaacetoxy-9α-benzoyloxydihydro-β-agarofuran, and 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran. These metabolites were purified from the CH\textsubscript{2}Cl\textsubscript{2} extract of the leaves of \textit{D. pittosporoides}. The structure of denhaminol I was further confirmed by X-ray crystallography analysis, which also established its absolute configuration. Denhaminol I and wilforsinine F were shown to exhibit leucine transport inhibitory activity in LNCaP cells with IC\textsubscript{50} of 51.5 \( \mu \)M and 95.5 \( \mu \)M, respectively.

Chapter 5, reports on the chemical investigation of CH\textsubscript{2}Cl\textsubscript{2} extract of the leaves of the Australian endemic vine, \textit{Celastrus subspicata}, which afforded seven previously unknown dihydro-β-agarofurans, celastrofurans A–G and two known compounds, (1S,4R,5S,7R,9S,10S)-9-benzoyloxy-1-furoyloxydihydro-β-agarofuran and (1R,2R,4R,5S,7R,9S,10R)-2-acetoxy-9-benzoyloxy-1-furoyloxydihydro-β-agarofuran. X-ray diffraction and ECD studies were undertaken to define the absolute configurations of celastrofurans A–D. All the isolated compounds from this vine were found to inhibit leucine transport in the human prostate cancer cell line LNCaP with IC\textsubscript{50} values ranging from 7.0 to 98.9 \( \mu \)M, which were more potent than the L-type amino acid transporter (LAT) family inhibitor, BCH.

Finally, Chapter 6 describes the development of an analytical method using UHPLC-MS that was applied to 16 crude CH\textsubscript{2}Cl\textsubscript{2} extracts from Australian Celastraceae plants. A subset of the available Celastraceae plants from Griffith University’s NatureBank resource was accessed during these studies that included three barks, one fruit, one leaf, seven roots, two twigs and two mixed samples all of which were collected from the State of Queensland. The data generated were analysed and dereplication performed using scientific databases such as the Dictionary of Natural Products and SciFinder in order to identify new natural products from Celastraceae plants. These investigations led to the large-scale extraction and isolation work on the prioritised \textit{Denhamia celastroides} fruits sample, which resulted in the purification of the new natural products, denhaminol O–R. and a known analogue denhaminol G.

In summary, this thesis describes the isolation and characterisation of 18 new natural products and 12 known metabolites from three Australian Celastraceae plants. The chemical structures of all compounds were determined by detailed interpretation of 1D/2D NMR and MS data and X-ray crystallography studies. Full spectroscopic and spectrometric characterisation of all new compounds was performed using NMR, UV,
IR, ECD and specific rotation. The compounds were screened for their leucine uptake inhibition in LNCaP cells and several compounds were identified as leucine uptake inhibitors, which were more potent than the LAT family inhibitor and currently used positive control, BCH. This thesis describes for the first time the inhibition of leucine uptake in prostate cancer cells by dihydro-β-agarofurans; the most potent inhibitor of LAT (bilocularin G, IC_{50} = 2.5 \mu M) to date was also reported during these studies. Finally, the new UHPLC-MS methodology developed during these studies was used to rapidly analyse 16 Celastraceae plants, and subsequently prioritise three samples for future metabolomics investigations. The fruits of *D. celastroides* were chosen for chemical investigation due to most encouraging data. An additional four new compounds (denhaminols O–R) were successfully isolated from the CH$_2$Cl$_2$ of the fruits of *D. celastroides*. This study exemplified the advantage of UHPLC-MS in combination with scientific databases data analysis in natural products dereplication.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Mario Wibowo

14 August 2018
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>1D</td>
<td>one-dimensional</td>
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<td>Ac</td>
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<td>ACT</td>
<td>artemisinin combination therapies</td>
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<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
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<td>DMSO</td>
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<td>deuterated DMSO</td>
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<td>HMBC</td>
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<tr>
<td>HPLC</td>
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<tr>
<td>HRESIMS</td>
<td>high-resolution electrospray ionisation mass spectrometry or</td>
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spectrum
Hz  Hertz
IC$_{50}$ half maximal inhibitory concentration
IR infrared spectroscopy
$J$ coupling constant
LC-MS liquid chromatography/mass spectrometry
LRESIMS low-resolution electrospray ionisation mass spectrometry or spectrum
LRMS low-resolution mass spectrometry or spectrum
M molarity
m metre(s)
m multiplet
$m/z$ mass to charge ratio
MeOH methanol
mg milligram(s)
MHz mega Hertz
min minute(s)
$mL$ millilitre(s)
MS mass spectrometry
Mw molecular weight (g/mol)
N normal
$N_2$ nitrogen
NMR nuclear magnetic resonance
NPs natural products
Nic nicotinoyl
ORTEP Oak Ridge thermal ellipsoid plot
Pv pivaloyl
ppm parts per million
q quartet
ROESY rotating frame overhauser effect spectroscopy
rpm revolutions per minute
rt room temperature
SAR structure-activity relationship
s singlet
SPE solid phase extraction
sh shoulder
\( t \)  

triplet

\( t_R \)  

retention time

TFA  

trifluoroacetic acid or trifluoroacetate

THF  

tetrahydrofuran

Tig  

tigloyl

TLC  

thin-layer chromatography

TOF  

time of flight

UHPLC  

ultra-high-pressure liquid chromatography

UV  

ultraviolet

wt  

weight

WHO  

World Health Organisation

\( \delta \)  

chemical shift

\( ^\circ C \)  

degrees Celsius

\( \mu m \)  

micrometre(s)

\( \mu g \)  

microgram(s)

\( \mu L \)  

microliter(s)
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Finally and most importantly, I thank God, the omnipotent power, for allowing me to experience the beauty of science and life through this PhD.
Acknowledgement of Papers Included in This Thesis

Section 9.1 of the Griffith University Code for the Responsible Conduct of Research (“Criteria for Authorship”), in accordance with Section 5 of the Australian Code for the Responsible Conduct of Research, states:

To be named as an author, a researcher must have made a substantial scholarly contribution to the creative or scholarly work that constitutes the research output, and be able to take public responsibility for at least that part of the work they contributed. Attribution of authorship depends to some extent on the discipline and publisher policies, but in all cases, authorship must be based on substantial contributions in a combination of one or more of:

- conception and design of the research project
- analysis and interpretation of research data
- drafting or making significant parts of the creative or scholarly work or critically revising it so as to contribute significantly to the final output.

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Researchers are expected to:

- offer authorship to all people, including research trainees, who meet the criteria for authorship listed above, but only those people.
- accept or decline offers of authorship promptly in writing.
- include in the list of authors only those who have accepted authorship
- appoint one author to be the executive author to record authorship and manage correspondence about the work with the publisher and other interested parties.
- acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge. Obtain written consent to name individuals.

Included in this thesis are papers/manuscripts in Chapters 2–6 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details/status for these papers including all authors, are:
Chapter 2:
Mario Wibowo, Claire Levrier, Martin C. Sadowski, Colleen C. Nelson, Qian Wang, Jeff Holst, Peter C. Healy, Andreas Hofmann, and Rohan A. Davis
Bioactive Dihydro-β-agarofuran Sesquiterpenoids from the Australian Rainforest Plant Maytenus bilocularis
Journal of Natural Products 2016, 79, 1445–1453
Supporting Information placed in Appendix 1

Chapter 3:
Mario Wibowo, Qian Wang, Jeff Holst, Jonathan M. White, Andreas Hofmann, and Rohan A. Davis
Dihydro-β-Agarofurans from the Roots of the Australian Endemic Rainforest Tree Maytenus Bilocularis Act as Leucine Transport Inhibitors
Phytochemistry 2018, 148, 71–77
Supporting Information placed in Appendix 2

Chapter 4:
Mario Wibowo, Qian Wang, Jeff Holst, Jonathan M. White, Andreas Hofmann, and Rohan A. Davis
Dihydro-β-agarofurans from the Australian Endemic Rainforest Plant Denhamia pittosporoides Inhibit Leucine Transport in Prostate Cancer Cells
Supporting Information placed in Appendix 3

Chapter 5:
Mario Wibowo, Qian Wang, Jeff Holst, Jonathan M. White, Andreas Hofmann, and Rohan A. Davis
Celastrofurans A–G: Dihydro-β-agarofurans from the Australian Rainforest Vine Celastrus subspicata and Their Inhibitory Effect on Leucine Transport in Prostate Cancer Cells
Journal of Natural Products 2017, 80, 1918–1925
Supporting Information placed in Appendix 4
Chapter 6:
Mario Wibowo, Paul I. Forster, Gordon P. Guymer, Andreas Hofmann, and Rohan A. Davis

Using UHPLC-MS Profiling for the Discovery of New Dihydro-β-agarofurans from Australian Celastraceae Plant Extracts

*Journal of Chromatography B: Manuscript in Preparation*

Supporting Information placed in Appendix 5

Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in each paper.

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Mario Wibowo

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Supervisor: Associate Professor Rohan Davis

14 August 2018
Chapter 1. Introduction

1.1 Natural products and drug discovery

Natural products are small molecules isolated from biological sources, such as plants, microbes, or animals. Natural products have been playing a critical role in providing vital medicines for human health for thousands of years. It has been estimated that only 27% of medicines approved by the US Food and Drug Administration (FDA) between 1981 and 2014 were purely synthetic, while >50% were either natural products or derivatives of natural products.\(^1, 2\)

The distinct property of natural products is their immense structural and chemical diversity that cannot be matched by any synthetic compound library.\(^3\) In fact, ~40% of natural products scaffold cannot be found in today’s medicinal chemistry synthetic libraries.\(^4\) Structural diversity, nevertheless, is not the only reason why natural products are interesting for drug discovery and development. Another important reason is that they often display selective and specific biological effects based on their mode of action.\(^3\) The biological activity of natural products arises from the hypothesis that generally all natural compounds have some receptor-binding activity,\(^3\) due to biosynthetic processes, which involve various interactions with modulating enzymes. Many natural products exhibit greater binding potential with different biological targets compared with synthetic compounds.\(^4, 5\) Moreover, they continue to inspire and provide insights into chemical biology as they have also been used as chemical probes to explore biological systems. Chemical probes are important pharmacological tools for assembling biochemical pathways and are exploited to unravel complex biological functions, which ultimately identify novel biological targets, that may be exploited in drug discovery and development.\(^6\)

Over the last century, natural products have continued to supply leads or drugs that have entered clinical trials.\(^7\) Examples of approved drugs (Figure 1.1) include galantamine (1.1), huperzine A (1.2), and daptomycin (1.3). Galantamine (1.1) is an alkaloid isolated from the herbaceous plant, *Galanthus woronowii*, which is used in the treatment of Alzheimer’s disease.\(^8\) Another alkaloid used for Alzheimer’s treatment is huperzine A (1.2), a naturally occurring compound found in the clubmoss *Huperzia serrata*.\(^9\) Alzheimer’s disease is characterised by low levels acetylcholine in the brain. Both galantamine (1.1) and huperzine A (1.2) are known to inhibit acetylcholinesterase,
the enzyme responsible for the breakdown of acetylcholine and thus these drugs maintain the level of this important neurotransmitter.8, 9

Daptomycin (1.3) is an antibiotic used for the treatment of skin infections by Gram-positive pathogens, such as Staphylococcus aureus, Streptococcus pyogenes, Clostridium difficile, and, Propionibacterium acnes.10 Daptomycin (1.3) is a cyclic lipopeptide that was first identified in the fermentation of Streptomyces roseosporus that was obtained from a soil sample from Mount Ararat (Turkey).11, 12 The production of 1.3 critically depends on the addition of decanoic acid during the fermentation process.11 Daptomycin was approved by FDA in 2003 for the treatment of complicated skin and skin structure infections caused by specific Gram-positive bacteria.12

![Chemical structures of the FDA approved Alzheimer’s drugs galantamine (1.1), huperzine A (1.2), and the antibiotic daptomycin (1.3).](image)

**Figure 1.1:** Chemical structures of the FDA approved Alzheimer’s drugs galantamine (1.1), huperzine A (1.2), and the antibiotic daptomycin (1.3).

Some examples of lead compounds from Nature (Figure 1.2) are khellin (1.4), galegine (1.6), and papaverine (1.8). Khellin (1.4), isolated from the Egyptian flowering plant, Ammi visnaga, was used as a bronchodilator until it was proven to produce severe side effects, such as vomiting and nausea. The discovery of khellin (1.4) led to the synthesis of a more potent bronchodilator with fewer side-effects in 1996 named, sodium cromoglycate (1.5), which is utilised to treat broncoconstriction in allergic
asthma patients. Galegine (1.6) was isolated from the plant *Galega officinalis*, which was used as a herbal medicine in medieval Europe.\textsuperscript{13} Compound 1.6 was the basis of the development of metformin (1.7) as an important antidiabetic drug, which entered the US market in 1995;\textsuperscript{13} while papaverine (1.8) from the opium poppy, *Papaver somniferum* was the lead model for verapamil (1.9), an L-type calcium channel antagonist with antihypertensive effect, which has been used successfully since FDA approval back in 1982.\textsuperscript{14, 15}

\textbf{Figure 1.2}: Chemical structures of the lead natural products khellin (1.4), galegine (1.5), papaverine (1.6), and the commercial drugs sodium cromoglycate (1.7), metformin (1.8), and verapamil (1.9).

\subsection*{1.1.1 Plant-derived natural products}

As noted above, >50\% of the approved drugs between 1981–2001 are of natural product origin\textsuperscript{1, 2} and a substantial number of these therapeutics were discovered from plants.\textsuperscript{16} Among the various sources of natural products, plants, in particular, have been the major source of natural products research. The earliest evidence of herbal drugs is the use of plant-derived extracts (mainly in the form of oils) in Mesopotamia (2600 BC). Examples include oils from *Cupressus sempervirens* (cypress) and *Glycyrriza glabra* (licorice), which are still used today to treat human ailments like coughs and colds.\textsuperscript{17} Since these times, plants have continued to be an excellent source of bioactive
compounds and have been continuously used by human as medicinal herbs over the centuries. It was estimated by the World Health Organisation (WHO) in 1985 that approximately 80% of the world’s population relied on medicinal herbs for their primary health therapy. Although plants have been extensively investigated, it has been estimated that only 6% of existing terrestrial plant species have been pharmacologically studied and only 15% have been phytochemically explored. Thus, there exists vast and unexplored plant diversity, which may contain bioactive constituents that could serve as new leads or drugs for the pharmaceutical industry.

A classical example of a plant-derived drug is morphine (1.10), an analgesic and sleep-inducing agent that was first isolated from the opium poppy (Papaver somniferum) by a German scientist, Friedrich Sertürner, at the beginning of 19th century. Morphine (1.10) is one of the first examples of a purified natural product drug. This was the beginning of modern drug discovery and saw a major focus on plants with the evaluation of other medicinal herbs and isolation of many bioactives, such as codeine (1.11), quinine (1.12), nicotine (1.13), cocaine (1.14), and atropine (1.15). Codeine (1.11) and morphine (1.13) are still used today for treating both acute and chronic pain, while cocaine (1.14) and atropine (1.15) are used for anaesthetic purposes.

![Chemical structures of some of the first purified natural products that were used as drugs](image)

**Figure 1.3:** Chemical structures of some of the first purified natural products that were used as drugs, which include morphine (1.10), codeine (1.11), quinine (1.12), nicotine (1.13), cocaine (1.14), and atropine (1.15).

The discovery of artemisinin (1.16) in 1971 is another success story of how plant-derived natural products have inspired and contributed to modern day drug discovery.
Artemisinin is an antimalarial sesquiterpene lactone featuring a rare endoperoxide bridge isolated from the Chinese plant, *Artemisia annua*, also known as sweet wormwood. Artemisinin and its semi-synthetic analogues, such as dihydroartemisinin (1.17) and artemether (1.18) are now used in many countries for malaria treatment, typically in combination with other antimalarial drugs that have different mechanisms of action.\(^2^2\) Artemisinin combination therapies (ACT) are currently the WHO gold standard in drug treatment for infections by the malaria parasite, *Plasmodium falciparum*.\(^2^3\) The endoperoxide moiety is the important functionality for the antimalarial effect,\(^2^4\) as it can be cleaved by heme iron, which results in the generation of free radical species that can attack the parasite’s proteins.\(^2^4\) In addition, artemisinin (1.16) has been reported to directly target malarial mitochondria via specific mitochondria activation.\(^2^4, 2^5\)

![Figure 1.4: Chemical structures of the antimalarial compounds artemisinin (1.16), dihydroartemisinin (1.17), and artemether (1.18).](image)

Plant secondary metabolites have also had a long and successful history in the treatment of cancer. Among the plant-derived anticancer drugs, some well-known examples are the vinca alkaloids (Figure 1.5), vinblastine (1.19) and vincristine (1.20), from the Madagascar pink periwinkle *Catharanthus roseus* (previously known *Vinca rosea*).\(^2^6, 2^7\) Compounds 1.19 and 1.20 were discovered in 1950’s. These alkaloids work by interacting with tubulin and microtubule function disruption, causing metaphase arrest.\(^2^6\) The vinca alkaloids where first used as disinfectant and to treat high blood pressure, but even today they are some of the most important drugs used in chemotherapy and are used against various type of cancers, such as breast cancer, acute leukemia, neuroblastoma, rhabdomyosarcoma, and testicular cancer.\(^3, 2^6\)
One of the most exciting plant-derived anticancer drugs identified in the past 40 years is paclitaxel (1.22), which was discovered from the bark of the Pacific Yew tree, *Taxus brevifolia* in 1962.\textsuperscript{28} This compound was found to have an interesting mode of action, which includes promoting the assembly of tubulin into microtubules.\textsuperscript{29} Paclitaxel was first approved as a drug for ovarian cancer in 1992. Since then, the demand for paclitaxel has increased significantly, and this important drug is successfully used in the treatment of a variety of cancers like non-small cell lung cancer, pancreatic cancer, and breast cancer.\textsuperscript{28} The isolation of paclitaxel from *T. brevifolia*, however, was not ideal because of the low concentration of paclitaxel in the slow growing yew bark (0.004–0.01% dry weight).\textsuperscript{30, 31} Therefore, the main method used nowadays to produce paclitaxel is a semisynthesis from 10-deacetylbaclatin III (1.21), which can be isolated in higher yield (1 g/kg of dry leaves) from the related European yew tree, *Taxus baccata*.\textsuperscript{32} Furthermore, other semisynthetic derivatives of taxol (1.21) such as cabazitaxel (1.23) and docetaxel (1.24), are used to treat prostate, breast, stomach, and head and neck cancers.\textsuperscript{33-36} The 6-step semisynthetic route from 10-deacetylbaclatin III (1.21) to produce cabazitaxel (1.23) with reagents/conditions and yield specified is shown below in Scheme 1.1.\textsuperscript{35}
Figure 1.6: Chemical structure of the non-cytotoxic taxoid, 10-deacetylbaccatin III (1.21), the precursor of the anticancer drugs paclitaxel (1.22), cabazitaxel (1.23), and docetaxel (1.24).

Scheme 1.1: Semisynthesis of cabazitaxel (1.23) from 10-deacetylbaccatin III (1.21). Reagents and conditions: (a) 1. Et₃SiCl, pyridine, rt, 51%; 2. NaH, MeI, DMF, 76%; (b) Et₃N·3HF, CH₂Cl₂, rt, 77%; (c) NaH, MeI, DMF, 0 °C, 74%; (d) oxazolidinecarboxylic acid, DCC, DMAP, EtOAc, 76%; (e) 0.1 M HCl, EtOH, 0 °C, 32%.35

1.2 Plants of the family Celastraceae

The Celastraceae is a family of trees and shrubs consisting of 88 genera and 1300 species that are distributed worldwide, but mostly in tropical and subtropical regions of the world.37 They have simple, alternate, subopposite or opposite leaves. The flowers are bisexual or sometimes unisexual.38 The largest genera of this family are *Maytenus*, *Euonymus*, *Cassine*, and *Celastrus*.39 Among the 88 genera of Celastraceae, 14 genera
are found in Australia (Table 1.1), some of which are endemic to Australia (Figure 1.7 and Figure 1.8.). Several species of the Celastraceae are claimed to be useful in medicine and folk agriculture. For instance, the extract of the thunder-god vine *Tripterygium wilfordii* has been used in the treatment of leukemia by the Chinese. Similarly, in several areas in the Amazonian river basin, *Maytenus* species, including *M. chuchuhuasha*, *M. krukonovii*, *M. colasii*, and *M. laevis*, have been used to treat both skin cancers and sores.

![Figure 1.7: The Celastraceae plant distribution in Australia. This figure is made available under the Open Database License: http://opendatacommons.org/licenses/odbl/1.0/. Any rights in individual contents of the database are licensed under the Database Contents License: http://opendatacommons.org/licenses/dbcl/1.0/](#)
Figure 1.8: Several Australian Celastraceae endemic plants, (a) *Denhamia celastroides*, (b) *Hedraianthera porphyropetala*, (c) *Denhamia pittosporoides*, and (d) *Maytenus bilocularis*. Reproduced with permission of Rohan Davis.

Table 1.1. Celastraceae genera found in Australia and reports of chemistry.

<table>
<thead>
<tr>
<th>Genera</th>
<th>No. of species in the world</th>
<th>No. of species in Australia</th>
<th>No. of reported chemical studies&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Celastrus</em></td>
<td>30</td>
<td>2</td>
<td>271</td>
</tr>
<tr>
<td><em>Denhamia&lt;sup&gt;a&lt;/sup&gt;</em></td>
<td>10</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td><em>Elaeodendron</em></td>
<td>80</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td><em>Euonymus</em></td>
<td>180</td>
<td>2</td>
<td>292</td>
</tr>
<tr>
<td><em>Gymnosporia</em></td>
<td>100</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td><em>Hedraianthera&lt;sup&gt;a&lt;/sup&gt;</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Hexaspora&lt;sup&gt;a&lt;/sup&gt;</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Hippocrates</em></td>
<td>100</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td><em>Hysophila&lt;sup&gt;a&lt;/sup&gt;</em></td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>Lophopetalum</em></td>
<td>20</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td><em>Maytenus</em></td>
<td>200</td>
<td>9</td>
<td>333</td>
</tr>
<tr>
<td><em>Pleurostylia</em></td>
<td>6</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td><em>Salacia</em></td>
<td>150</td>
<td>3</td>
<td>174</td>
</tr>
<tr>
<td><em>Siphonodon</em></td>
<td>7</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Endemic to Australia.

<sup>b</sup>http://scifinder.cas.org
1.2.1 Chemical diversity of Celastraceae and their biological activity

The chemistry of Celastraceae plants is of interest due to many reasons, such as the interesting chemical diversity and the different types of biological activities associated with the secondary metabolites. Many bioactive compounds and numerous chemical structures have been reported from Celastraceae. This family has proven to be a plentiful source of terpenoids, including triterpenes, diterpenes, sesquiterpenes, and monoterpenes. A total of 118 diterpenoids were reported between 2000 and 2016 as reviewed by Bazzochi et al. Some examples of diterpenoids isolated from Celastraceae are shown in Figure 1.9.

![Diterpenoids isolated from Celastraceae](image)

Figure 1.9: Diterpenoids isolated from Celastraceae, ent-kauranol (1.25) from *Tripterygium doianum*, tripterlide A (1.26) from *Tripterygium wilfordii*, and cuzcol (1.27) from *Maytenus cuzcoina*.

Moreover, Celastraceae plants are a rich source of natural Diels-Alder terpenoid adducts. A recent review covering literature between 1961 and 2017 also by Bazzochi et al. highlights 84 natural products isolated from Celastraceae plants which were hypothesised to occur via biosynthesis involving Diels-Alder reactions. Several examples are drawn in Figure 1.10.
Figure 1.10: Diels-Alder adducts isolated from Celastraceae, blepharodin (1.28) from *Maytenus magellanica*, cangorosin A (1.29) from *Maytenus ilicifolia*, and xuxuarine Ka (1.30) from *Maytenus chuchuscas*.

Figure 1.11: Proposed biosynthesis of the 1,4-dioxane ring in blepharodin (1.28). Hetero Diels-Alder reaction between the quinone form of blepharodol (1.28b) and sinapyl acetate (1.28a) could yield blepharodin (1.28).
In addition to diverse terpenoids, several bioactive maytansinoids (e.g. maytansine, 1.31), alkylamines (e.g. cathinone, 1.34), and flavonoids (e.g. quercetin, 1.35) have also been isolated. The interest in Celastraceae increased significantly following the discovery of the highly cytotoxic maytansine (1.31) from the African plant, *Maytenus ovatus*. The semi-synthetic analogue of maytansine, trastuzumab emtansine (Kadcyla®) was granted FDA approval as a drug for the treatment of patients with HER-2 positive metastatic breast cancer in 2013. Trastuzumab emtansine (1.33) is an antibody drug conjugate consisting of the anti-HER-2 anti-body, trastuzumab linked to the microtubule inhibitor agent, DM1 (1.32). DM1 is an analogue of the natural product maytansine, which by itself has limited clinical benefit and high toxicity. However, the safety was improved and the clinical activity was demonstrated by the conjugation of DM1 to trastuzumab. Interestingly, many studies have shown that endophytic microbes are the “hidden producers” of maytansinoids. Consequently, the biosynthesis of maytansinoids was proposed based on a type I modular polyketide synthase (PKS) from an aromatic starter unit.

![Chemical structures of maytansine, DM1, trastuzumab emtansine, cathinone, and quercetin.](image)

**Figure 1.12:** Chemical structures of the lead compounds maytansine (1.31), DM1 (1.32) and the anticancer drug trastuzumab emtansine (1.33) and examples of alkylamine (cathinone, 1.34) and flavonoid (quercetin, 1.35) isolated from Celastraceae.

Terpenoids are the most bioactive substances of Celastraceae. Monoterpenes like β-thujone (1.36) are found in the freshly picked leaves of *Catha edulis*. The diterpene
triptolide (1.37) from the thunder-god vine *T. wilfordii* was found to be the active constituent of this plant with anti-leukemic activity. Tingenone (1.38), a pentacyclic triterpene is commonly found in Celastraceae, and displays cytotoxic and antibacterial activities.

Figure 1.13: Chemical structures of terpenoids isolated from Celastraceae including, thujone (1.38), triptolide (1.39), and tingenone (1.40).

The characteristic secondary metabolites of this plant family are a group of highly oxygenated tricyclic sesquiterpene polyesters, which are based on the C\textsubscript{15} dihydro-\(\beta\)-agarofuran skeleton (Figure 1.14). These unique polyoxygenated sesquiterpenes are also known as a chemotaxonomic marker of the family. The dihydro-\(\beta\)-agarofurans are interesting because of the extent of their biological activities and the structural features they possess, especially the tetrahydrofuran ring, as this structural characteristic is not found in other sesquiterpenes. This structural class is regarded as a privileged structure, which is defined as a single molecular skeleton capable of providing ligands for diverse receptors. These sesquiterpene polyesters are found in a variety of oxygenated forms, bearing two to nine ester groups. Recently, Celastraceae dihydro-\(\beta\)-agarofurans have been reported to display cytotoxic, anti-HIV, multidrug resistance reversal, and neuroprotective activities. They have attracted much interest as shown by a large number of new dihydro-\(\beta\)-agarofurans reported since the 1990s (462 new compounds were reported between 1990 and 2006). Some pertinent examples of dihydro-\(\beta\)-agarofurans are given below.

Figure 1.14: The dihydro-\(\beta\)-agarofuran skeleton.
Bazzocchi et al. isolated four new macrolide sesquiterpene pyridine alkaloids (1.41–1.44) from the leaves of *M. chiapensis* collected in El Salvador. Compounds 1.41 and 1.42 were evaluated for their cytotoxicity against insect Sf9 and mammalian CHO cells. Compounds 1.41 and 1.42 had moderate to low cytotoxicity (EC50 value of 0.039 µg/mL and 0.066 µg/mL, respectively) towards insect Sf9 cells, while neither natural products were active against mammalian CHO cells.

![Chemical structures of dihydro-β-agarofurans](image)

**Figure 1.15**: Chemical structures of dihydro-β-agarofurans isolated from *Maytenus chiapensis* (1.41–1.44) and from *Celastrus subspicata* (1.45 and 1.46).60,64

In 2009, two new dihydro-β-agarofurans (1.45 and 1.46) were successfully isolated from the seeds of the Australian rainforest vine, *Celastrus subspicata*.60 The compounds showed minimal inhibition on a pro-inflammatory assay at 100 µM. However, compounds 1.45 and 1.46 were cytotoxic against HeLa cancer cells at a concentration of 100 µM with 73% and 85% inhibition, respectively.60

Lee et al. evaluated the anti-HIV activity of 20 macrocyclic alkaloid dihydro-β-agarofurans (1.47–1.66) isolated from several Chinese plants of the genus *Tripterygium*.61 Among these secondary metabolites, twelve compounds (1.52–1.55, 1.57–1.59, and 1.62–1.66) exhibited anti-HIV activity with EC50 values ranging from 2.54 to <0.1 µg/mL, while the remaining alkaloids were inactive. Compounds 1.52, 1.53, and 1.58 were the most active with EC50 values of <0.1 µg/mL.61
Figure 1.16: Chemical structures of macrocyclic alkaloidal dihydro-β-agarofurans (1.47–1.66) from Chinese Tripterygium plants.

A series of new dihydro-β-agarofurans named denhaminols was recently isolated from the leaves of the Australian endemic plant, *Denhamia celastroides*. Denhaminols A–H (1.67–1.74) were evaluated for their cytotoxicity against the human prostate cancer cell line LNCaP. Denhaminols A (1.67) and G (1.73) were also tested for their effects on the lipid content of LNCaP cells. All compounds were found to have low cytotoxicity against LNCaP cells with denhaminol G (1.73) showing the best activity with an IC\textsubscript{50} value of 32 µM. Furthermore, compounds 1.67 and 1.73 affected lipid homestasis and decreased the lipid content of LNCaP cells. Compounds affecting lipogenesis in cancer cells have been identified as potential anticancer agents, since...
alteration of lipogenesis is found in almost all cancers and is associated with cancer cell survival.\textsuperscript{65,66}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{denhaminols}
\caption{Chemical structures of denhaminols A–H (1.67–1.74) which were isolated from the leaves of the endemic Australian plant, \textit{Denhamia celastroides}.\textsuperscript{65}}
\end{figure}

Recently, a group of Chinese scientists tested the neuroprotective effect of 62 natural dihydro-β-agarofurans from medicinal plants of the genus \textit{Celastrus} on Aβ\textsubscript{25-35}-induced neurotoxicity in SH-SY5Y cells.\textsuperscript{63} Among the evaluated compounds, two dihydro-β-agarofurans (1.75 and 1.76) showed neuroprotective effects by significantly improving the cell viability from 64.6\% to more than 74.0\% at 1 \textmu M.\textsuperscript{63}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{neuroprotective_dihydro_β_agarofurans}
\caption{Chemical structures of neuroprotective dihydro-β-agarofurans 1.75 and 1.76 isolated from Chinese \textit{Celastrus} sp.\textsuperscript{63}}
\end{figure}

Three new dihydro-β-agarofurans 1.77–1.79 were isolated from the roots of the South Korean plant \textit{Celastrus orbiculatus}. These compounds showed moderate inhibition in
NO (nitric oxide) production with IC\textsubscript{50} values of 50.4, 51.2, and 43.6 \textmu M, respectively.\textsuperscript{67}

![Chemical structures of antiinflammatory constituents dihydro-\(\beta\)-agarofurans 1.77–1.79 from the roots of \textit{Celastrus orbiculatus}.\textsuperscript{67}]

Figure 1.19: Chemical structures of antiinflammatory constituents dihydro-\(\beta\)-agarofurans 1.77–1.79 from the roots of \textit{Celastrus orbiculatus}.\textsuperscript{67}

Driven by the potent biological activities of dihydro-\(\beta\)-agarofurans, several research groups from around the world have started to design and synthesise screening libraries based on these natural products in order to optimise their potency. One salient example involves the identification of dihydro-\(\beta\)-agarofurans from Celastraceae plants, which are potent inhibitors of P-glycoprotein (P-gp) in multidrug resistance (MDR) cells. To discover more effective MDR reversal agents, Bazzocchi, \textit{et al.} generated an 81-membered library based on the lead compound 1.80, which was isolated from the leaves of \textit{Celastrus vulvanicola}.\textsuperscript{68} The compound analogues were semi-synthesised via esterification, isomerisation, nucleophilic substitution, hydrogenation, and various other reactions. All analogues were tested for their ability to inhibit P-gp mediated daunomycin efflux in MDR cells. Five derivatives (1.81–1.85) were shown to exhibit better potency than the parent compound 1.80 [reversal index (RI*) = 5.6] with RI of 10.0, 15.8, 11.8, 11.8, and 10.8, respectively. The semisynthetic routes of compounds 1.81–1.85 from the natural dihydro-\(\beta\)-agarofuran 1.80 are shown in Schemes 1.2–1.4.

\textsuperscript{*The reversal index (RI) was defined as ratio between IC\textsubscript{50} values of MDR cells without and with daunomycin addition.
Scheme 1.2: Synthesis of aminoester analogue 1.81. Reagents and conditions: (a) Ac₂O, Et₃N or pyridine, DMAP, CH₂Cl₂, rt, 81.8%; (b) bromoacetyl bromide, Et₃N, DMAP, ZnO, CH₂Cl₂, ultrasound, 0 °C, 45%; (c) isobutylamine, CH₂Cl₂, Et₃N, rt, 11.4%. ⁶⁸

Scheme 1.3: Synthesis of analogue 1.82. Reagents and conditions: (a) succinic anhydride, DMAP, 2,6-lutidide, 100-110 °C, 62.3%; (b) EtOH, Et₃N, DMAP, DCC, N,N’-dicyclohexylurea, CH₂Cl₂, Ar, 0 °C, 7.1%. ⁶⁸

Scheme 1.4: Synthesis of acyl analogues 1.83–1.85. Reagents and conditions: 1.83 (a) nicotinic acid, CH₂Cl₂, DCC, rt, 15.3%; or (a) dimethylcarbamoyl chloride (for 1.84) or Ac₂O (for 1.85), Et₃N or pyridine, DMAP, CH₂Cl₂, rt, 52.7% (for 1.84) or 25.6% (for 1.85). ⁶⁸
1.3 L-type amino acid transporter inhibitors in cancer therapy

Lately, there has been an increased interest in the research of drug transporters, which has revealed their important role in regulating processes such as drug absorption, distribution, and excretion.\(^69\),\(^70\) The involvement of transporters in these processes is critical to drug discovery and development since they provide the opportunity to deliver the drug to the target organ and to avoid distribution of the drug to other organs, thereby reducing potential side effects.\(^70\) Of the known transporters in humans, the L-type amino acid transporters (LAT) have attracted significant interest due to their role in amino acids uptake, which is vital for protein synthesis.\(^71\) By blocking LATs with small molecule drugs, cellular protein production is shut down thus leading to cell death, which is critical for most cancer therapies.

The L-type amino acid transporters (LAT) transport amino acids into the cell in a Na\(^+\) dependent manner. One of the major LAT substrates is leucine, which is a regulator amino acid of the mTORC1 (mammalian target of rapamycin complex 1) growth pathway.\(^72\),\(^73\) The LATs are the main transporters that control uptake of leucine into cells, therefore regulating mTORC1 signalling and protein synthesis. Even though LATs are found in normal and cancer cell membranes, the overexpression of LATs have been found in numerous cancer cells.\(^74\) The LAT family consists of four members, namely LAT1, LAT2, LAT3, and LAT4, among which LAT1 and LAT3 are selectively expressed in cancer cells. Cancer cells, like normal cells, require a sufficient supply of oxygen and nutrients to grow. Some of the key nutrients are glucose and amino acids. Therefore, compounds that constrain LATs by inhibiting leucine uptake offer scientists a novel drug target for cancer therapy.\(^72\)
Figure 1.20: mTORC1 pathway showing activation via amino acid (leucine) uptake affecting cell growth and proliferation. Image modified and adapted from Dodd, et al.\textsuperscript{73} and Kim, et al.\textsuperscript{75}. Reproduced and modified with permission of Shelly Gordon.\textsuperscript{76}

Since the substrates of LATs are amino acids, such as leucine, the development of LAT family inhibitors mainly focused on compounds that have similar chemical structures with LAT substrates, and thus could compete for amino acid binding. This approach has resulted in the identification of leucine analogue LAT inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH, 1.72). However, BCH is still not suitable for clinical development since it targets all members of the LAT family. Consequently, recent development of new LAT inhibitors has focused on specifically targeting LAT1 and/or LAT3. Several important examples of LAT1 or LAT3 small molecule inhibitors are detailed below.

KYT-0353 (1.75) is a tyrosine analogue, which has been demonstrated to selectively inhibit LAT1 \textit{in vitro} and \textit{in vivo}. Compound 1.75 can inhibit the HT29 (human colon adenocarcinoma) cell growth in both cultured cells (IC\textsubscript{50} = 13.9 µM) and a nude mouse-transplanted tumor cell system.\textsuperscript{74}

Giacomini et al. investigated the anti-proliferative effects of two compounds, namely 3-iodo-L-tyrosine (1.76) and acivicin (1.77), in the LAT1-expressing glioblastoma multiforme cell line T98G (both control cells and cells with LAT1 expression knocked down).\textsuperscript{77} The anticancer acivicin (1.77) was found to be more potent in inhibiting the control T98G cells (75% growth reduction) than the LAT1-knocked down T98G cells
(51% growth reduction). In a similar manner, 3-iodo-L-tyrosine (1.76) reduced the
growth of the control T98G cells by 27%, but had no effect on the knocked down cells.
The results implied that compounds 1.76 and 1.77 inhibited LAT1 activity, thereby
reduced cancer cells proliferation.

Recently, high-throughput screening using a prefractionated natural product library
was undertaken with the aim of finding new LAT inhibitors.78 Two novel monoterpane
glycosides, venuloside A (ESK246, 1.78) and venuloside B (ESK242, 1.79) were
purified from a Queensland collection of the plant *Pittosporum venulosum*79. Venulosides A (1.78) and B (1.79) were shown to inhibit leucine uptake with IC$_{50}$
values of 8.1 µM and 29.6 µM, respectively. Furthermore, compound 1.79 was found to
inhibit LAT1 and LAT3, while 1.78 specifically inhibited LAT1. Further studies using
$^1$H NMR fingerprint on the chemistry of *Pittosporum venulosum* undertaken by the
same research group, led to the isolation of two minor monoterpane glycosides,
venulosides C (1.80) and D (1.81).79 Compounds 1.80 and 1.81 were found to inhibit
leucine transport in LNCaP prostate cancer cells with IC$_{50}$ values of 11.5 and 39.7 µM.
Compounds 1.78–1.81 are the first reported natural products that inhibit leucine
transport in cancer cells. The discovery of venulosides exemplifies the potential of
natural products in the discovery of new LAT inhibitors.

![Chemical structures of the LAT inhibitors](image)

**Figure 1.14**: Chemical structures of the LAT inhibitors, BCH (1.74), KYT-0353 (1.75), 3-iodo-L-
tyrosine (1.76), acivicin (1.77), and venulosides A–D (1.78–1.81).
1.4 PhD research goals

As reviewed above, natural products have been a rich source of medicinal agents for millennia, with numerous useful drugs developed from plant sources. Furthermore, plants from the family Celastraceae are a source of diverse chemical compounds with various bioactivities. Australian plants belonging to this plant family have rarely been studied for their chemistry or biology. Therefore, the aims of this PhD project were to:

1. Isolate new secondary metabolites from Australian Celastraceae plants.
2. Elucidate the chemical structures of all isolated compounds using spectroscopic techniques.
3. Synthesise analogues of new compounds (where possible) in order to assist structure-activity relationship (SAR) studies.
4. Evaluate the leucine transport inhibitory activity of all secondary metabolites and their analogues and elucidate SAR.

1.5 Significance

Natural products have played an important role in drug discovery and the potential of natural sources, such as plants, has been under-investigated, especially in terms of Australian biota. Isolation and biological evaluation of new compounds from unique Australian plants has the potential to identify future medicinal agents or chemical probes that will help unravel biological pathways. This study will provide a set of new natural compounds, which may contain potential drugs, lead molecules, or chemical probes.

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76. Gordon, S. Characterisation of natural products from the Australian endemic rainforest plant *Denhamia celastroides* that inhibit leucine transport in cancer cells. Griffith University, 2017.


Chapter 2. Bioactive Dihydro-β-agarofuran Sesquiterpenoids from the Australian Rainforest Plant *Maytenus bilocularis*

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

**Mario Wibowo, Claire Levrier, Martin C. Sadowski, Colleen C. Nelson, Qian Wang, Jeff Holst, Peter C. Healy, Andreas Hofmann, and Rohan A. Davis**


Supporting Information can be found in Appendix 1.

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**My contribution to the paper involved:**

I conducted all the plant extractions, compound isolation experiments, chemical reactions, and recorded and analysed the spectroscopic and spectrometric data for all compounds. The biology and X-ray diffraction experiments were performed by our collaborators. I was the major contributor to the preparation of the manuscript.

14 August 2018

Mario Wibowo

14 August 2018

Corresponding author of paper: Rohan A. Davis

14 August 2018

Supervisor: Rohan A. Davis
Bioactive Dihydro-β-agarofuran Sesquiterpenoids from the Australian Rainforest Plant Maytenus bilocularis

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Supporting Information

ABSTRACT: Chemical investigations of the CH₂Cl₂ extract obtained from the leaves of the Australian rainforest tree Maytenus bilocularis afforded three new dihydro-β-agarofurans, bilocularins A–C (1–3), and six known congeners, namely, celastrine A (4), 1α,6β,8α-triacetoxy-9β-benzoyloxydihydro-β-agarofuran (5), 1α,6β,8α-diacyctoxy-9β-benzoyloxydihydro-β-agarofuran (6), Ejp-10 (11), 1α,6β,8α-triacetoxy-9β-benzoyloxydihydro-β-agarofuran (12), and Ejp-2 (13). The major compound 1 was used in semisynthetic studies to afford four ester derivatives (7–10). The chemical structures of 1–3 were elucidated following analysis of 1D/2D NMR and MS data. The absolute configurations of bilocularins A (1) and B (2) were determined by single-crystal X-ray diffraction analysis. All compounds were evaluated for cytotoxic activity against the human prostate cancer cell line LNCaP; none of the compounds were active. However, several compounds showed similar potency to the drug efflux pump inhibitor verapamil in reversing the drug resistance of the human leukemia CEM/VCR R cell line. In addition, similar to verapamil, compound 5 was found to inhibit leucine uptake in LNCaP cells (IC₅₀ = 15.5 μM), which was more potent than the leucine analogue 2-aminobicyclo[2.2.1]-heptane-2-carboxylic acid. This is the first report of secondary metabolites from Maytenus bilocularis.

The Celastraceae family is widely found in tropical and subtropical regions of the world. The potential of Celastraceae plants as producers of secondary metabolites that possess a broad range of bioactivity has attracted much interest, as shown by the large number of natural products isolated from this family over the last 30 years. Terpenoids, including monoterpenoids, sesquiterpenoids, diterpenoids, and triterpenoids, are the most common bioactive constituents found in this family. However, the typical metabolites of the Celastraceae that have attracted the most interest are a class of polyoxygenated tricylic sesquiterpenoids known as dihydro-β-agarofurans. They are known to exhibit a broad range of biological effects, such as cytotoxic, anti-HIV, multidrug resistance reversal, neuroprotective, and chemopreventive activities.

The genus Maytenus, which consists of approximately 225 plant species, is one of the largest genera in the Celastraceae family. Maytenus species have widely been used in folk medicine as remedies for the treatment of several diseases, such as gastric ulcers, arthritis, and inflammatory disease. The interest in Maytenus increased significantly following the discovery of the highly cytotoxic compound maytansine from an African plant, Maytenus ovatus. Maytenus bilocularis, locally known as “orangebark”, is an endemic Australian tree growing up to 10 m tall in the rainforests of New South Wales and Queensland. Although many recent studies have reported the discovery of new bioactive constituents possessing various activities from Maytenus, M. bilocularis has not been phytochemically investigated. Owing to an ongoing interest in the chemistry of Australian Celastraceae species, we selected M. bilocularis for chemical investigation. The isolation and structure elucidation of three new and six known dihydro-β-agarofuran sesquiterpenoids from a CH₂Cl₂ extract of the leaves of M. bilocularis are reported. All compounds were tested for their cytotoxicity toward the

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LNCAP human prostate cancer cell line. Furthermore, the drug resistance reversal ability of a subset of compounds in the vinca alkaloid-resistant leukemia CEM/VCR R cell line and their effects on neutral amino acid uptake are also reported.

RESULTS AND DISCUSSION

The CH₃Cl₂ extract of the leaves of M. bilocularis was subjected to silica gel flash column chromatography and further purified by RP-HPLC to yield three new dihydro-β-agarofurans, bilocularins A–C (1–3), and six known congeners: celestine A (4), 1α,6β,8α-triacetox-9α-benzoyloxydiydro-β-agarofuran (5), 1α,6β-diacetox-9α-benzoyloxy-8α-hydroxydihydro-β-agarofuran (6), Epap-10 (11), 1α,6β-diacetox-9β-benzoyloxydihydro-β-agarofuran (12), and Epap-2 (13) (Figure 1).

![Figure 1. Chemical structures of natural products (1–6 and 11–13) and semisynthetic analogues (7–10).](image1)

Bilocularin A (1) was obtained as stable colorless crystals and was assigned the molecular formula C₇₇H₇₆O₃₉ following analysis of the (+)-HRESIMS sodium adduct ion at m/z 559.2197 [M + Na]⁺ (calcd for C₇₇H₇₆O₃₉Na, 559.2201). The 1H NMR spectrum of 1 revealed the signals corresponding to six methyl groups at δH 1.00, 1.42, 1.46, 1.50, 2.10, and 2.29, four oxygenated methine protons (δH 4.42, 5.40, 5.55, and 6.56), a pair of coupled oxymethylene doublets (δH 4.60, 4.92, J = 12.9 Hz), and one benzoyl group (5H, δH 7.46, 7.57, 8.06). The 13C NMR and edited HSQC spectra suggested the presence of 28 carbon atoms, consisting of six methyls, three methylenes, 11 methines, and eight nonprotonated carbons. The 13C NMR resonances at δC 163.1, 169.8, 170.1, and 170.5 were indicative of four ester groups in 1. Two oxygenated tertiary carbon signals were observed at δC 81.2 and 90.7, which were characteristic of the ethereal carbons of a dihydro-β-agarofuran. Fragments H-1/H₂-2/H₃-3/H₄-4/H₅-14 and H-6/H-7/H-8/H-9 were readily established from the 1H–1H COSY data of 1 (Figure 2). The remaining structure of 1 was deduced following analysis of HMBC data. HMBC correlations from both H-1 and H-4 to C-10, as well as from H-4 and H-14 to C-5, constructed a partial structure of cyclohexane ring A substituted by a methyl group at C-4 (Figure 2). The HMBC spectrum of 1 also showed correlations from H-6 to C-5 and C-10, from H-7 to C-5, and from H-9 to C-1 and C-10, revealing the presence of another six-membered ring B, which was fused to ring A (Figure 2). HMBC correlations from H-6 and H-7 to C-5 and C-11, along with the 13C NMR resonances of C-5 (δC 80.7) and C-11 (δC 81.2), suggested that C-5 and C-11 were oxygenated tertiary carbons linked through an ether bond. A gem-dimethyl group was located on the oxygenated tertiary carbon C-11 based on HMBC correlations from H-12 and H-13 to C-7 and C-11; these correlations also confirmed that the gem-dimethyl group and C-7 were connected through the nonprotonated carbon C-11. Further HMBC correlations from H-13 to C-2, C-3, C-7, and C-10 located CH₂-15 at C-10. The presence of three acetox groups was indicated by HMBC correlations from the methyl protons at δH 1.46, 2.10, and 2.29 to carbonyl resonances at δC 170.1, 169.8, and 170.5, respectively. These considerations indicated that 1 was a dihydro-β-agarofuran sesquiterpenoid containing one benzoxa and three acetox groups. The benzoxa moiety was attached at C-9 based on HMBC correlations from both aromatic protons at δH 8.06 and 5.55 (H-9) to an ester carbonyl group at δC 165.1. Similarly, the three acetox groups were located at C-1, C-6, and C-15 on the basis of HMBC correlations from H-1, H-6, and H-15 to carbonyl carbons at δC 170.1, 169.8, and 170.5, respectively. Finally, a hydroxy group was positioned at C-8 by considering the deshielded 1H and 13C NMR resonances of CH-8 (δH 4.42 and δC 69.0). On the basis of these data, the 2D structure of 1 was established.

The relative configuration of 1 was assigned following analysis of 1H–1H coupling constants and the ROESY data (Figure 3). Large coupling constants between H-1 and H-2 (J1,2 = 12.0, 4.6 Hz) revealed the β-orientation of H-1. ROESY correlations between H-1 and H-9 and between H-1 and H-3β

![Figure 2. COSY and selected HMBC correlations of bilocularin A (1).](image2)

![Figure 3. Key ROESY correlations of bilocularin A (1).](image3)
Figure 4. ORTEP drawings of bilocularin A (1) (left) and bilocularin B (2) (right).

showed that these protons were cofacial. The coupling constant of 4.2 Hz between H-7 and H-8 suggested that H-7 and H-8 were α- and β-oriented, respectively. A small coupling constant between H-6 and H-7 (J_6,7 = 1.1 Hz) indicated a dihedral angle close to 90° between these protons and thus supported an α-orientation of H-6. Notably, in all dihydro-β-agarofurans possessing an ester group at C-6, H-6 is α-oriented. Finally, the α-orientation of CH_3-14 and CH_2-15 was determined on the basis of ROESY correlations between H-6 and H_3-14, H-6 and H_2-15, and H-2α and H-15.

A single-crystal X-ray diffraction experiment using Cu Kα radiation was performed on compound 1 (Figure 4), which unambiguously established the structure and absolute configuration of bilocularin A as (1S,4R,5S,6R,7R,8S,9S,10S)-1,6,15-triacetoxy-9-benzoyloxy-8-hydroxydihydro-β-agarofuran.

Compound 2, obtained as colorless crystals, had the molecular formula C_{22}H_{18}O_{10} assigned following analysis of the (+)-HRESIMS and NMR data. The 1H NMR spectrum of 2 was similar to that of 1, except for the absence of a methane proton signal in 2, which corresponded to H-8 in 1. Analysis of the NMR and MS data of 2 and comparison with 1 revealed that both compounds shared the same dihydro-β-agarofuran scaffold. However, the secondary hydroxy group in 1 was oxidized to a carbonyl group (C-8) in 2. This was determined by HMBC correlations from H-6 (δ_6 6.42), H-7 (δ_7 3.05), and H-9 (δ_9 5.87) to a carbonyl carbon at δ_5 198.6. The ROESY spectrum of 2 exhibited correlations between H-1 and H-9, H-6 and H_3-14, and H-6 and H_2-15, implying the relative configuration of 2 to be the same as compound 1. The structure of 2 (bilocularin B) was assigned as (1S,4R,5S,6R,7R,9S,10S)-1,6,15-triacetoxy-9-benzoyloxy-8-hydroxydihydro-β-agarofuran. It is worth mentioning that oxidation of bilocularin A (1) using Dess-Martin periodinane afforded bilocularin B (2) with 93% yield.

The minor metabolite, compound 3, was isolated as a colorless gum and was assigned the molecular formula C_{19}H_{16}O_{10} by HRESIMS. These data indicated that 3 was a constitutional isomer of 1. While the H and 13C NMR spectra of 3 showed a high degree of similarity with 1, several key differences were readily identified. For instance, the H-6 resonance was shielded from δ_6 5.65 in 1 to δ_6 5.25 in 3, while the H-8 signal was deshielded from δ_8 4.42 in 1 to δ_8 5.63 in 3. A COSY correlation between H-6 and an exchangeable OH-6 proton (δ_6 1.69), as well as HMBC correlations from both H-8 and the methyl protons at δ_3 0.20 to an ester carbonyl carbon at δ_5 169.8, indicated that in compound 3 H-6 was attached to a carbon bearing a hydroxyl group, while H-8 was part of an acetoxyemethine group. The relative configuration of 3 was deemed to be identical to that of 1 following ROESY data analysis. On the basis of biosynthetic considerations and comparison of chirophtal and NMR data for this series, the absolute configuration of bilocularin C (3) was assigned as (1S,4S,5S,6R,7S,8S,9S,10S)-1,8,15-triacetoxy-9-benzoyloxy-8-hydroxydihydro-β-agarofuran.

The known compounds were characterized as celestrine A (4), 1α,6β,8α-triacetoxy-9β-benzoyloxydihydro-β-agarofuran (5), 1α,6β-diaceetoxy-9α-benzoyloxy-8α-hydroxydihydro-β-agarofuran (6), Ejap-10 (11), 1α,6β-diaceetoxy-9β-benzoyloxydihydro-β-agarofuran (12), and Ejap-2 (13) by comparison of their spectroscopic data with the literature. In this report, we have also included [α]_D^20 values for compounds 4 and 12 along with 13C NMR and [α]_D^20 data for compounds 11 and 13, which were not reported in the original papers. In
addition, the electronic circular dichroism (ECD) data of compounds 1–13 are also reported. The 1H NMR data of 11 were consistent with the reported values,58 however a discrepancy in the [α]D sign was observed. Our experimental data showed a specific rotation of −47 (c 0.1, EtOH), while the literature reported an [α]D value of +25 (c 0.1, EtOH).29 This observation suggested either that the [α]D of 11 was misassigned or that it was the enantiomer of 11. Attempts to obtain authentic material for reanalysis were not successful.

A number of ester analogues were semisynthesized from the major compound 1 in order to facilitate preliminary structure–activity relationship studies for this type of compound. Compound 1 was reacted with pivaloyl chloride, methoxyacetyl chloride, benzoyl chloride, and p-bromobenzoyl chloride in dry pyridine to generate 7, 8, 9, and 10, respectively. Their structures were confirmed by 1D/2D NMR and HIRESIMS data. Benzoylation of 1 yielded the benzoylated derivative 9 (celaspenc A).53 The ECD spectrum of 9 showed a negative Cotton effect at 238 nm (Δε = −60.0) and a positive Cotton effect at 223 nm (Δε = +13.3) due to exciton coupling of the two benzene chromophores at C-8 and C-9α, which were in accordance with the reported data.59 Accordingly, the structure and absolute configuration of 9 were assigned as (1S,4R,5S,6R,7R,8R,9S,10S)-1,6,15-triaceetoxy-8,9-dibenzyloxy-dihydro-β-agarofuran. The absolute configuration of 9 established by the ECD studies was identical to that of 1 assigned through X-ray crystallographic analysis.

Owing to our continuing interest in the discovery and development of new prostate cancer active agents from nature,50-53 compounds 1–13 were initially evaluated for their cytotoxicity toward the LNCaP human prostate cancer cell line (lymph node metastasis, androgen-sensitive) using a cell viability assay that is based on metabolic activity (alamarBlue). None of the compounds showed >30% cell viability inhibition toward LNCaP cells at 10 μM after 72 h (see Table S31 Supporting Information, for full biological results). Compounds 5, 7, and 9 exhibited the higher activity with 14.6%, 25.6%, and 21.2% inhibition at 10 μM, respectively. In order to further investigate the biological effects of dihydro-β-agarofurans toward cancer cells, the major compounds, 1 and 2, and the most active compounds, 5, 7, and 9, were subjected to further biological testing.

One of the main challenges in cancer treatment is the development of the multidrug resistance (MDR) phenotype due, in part, to the overexpression of multidrug transporters, which act as efflux pumps to reduce the intracellular concentration of chemotherapeutic agents.54,55 P-Glycoprotein (P-gp, ABCB1), MDR protein 1 (MRP1/ABCC1), and breast cancer resistance protein (BCRP/ABCG2) are the best characterized multidrug efflux pumps. Overexpression of P-gp has been detected in many cancer types in humans and therefore is an attractive target for cancer treatment therapy.56 Recently, dihydro-β-agarofurans have been reported as promising MDR reversal agents by binding specifically to P-gp.57-59 Owing to these findings, the drug resistance reversal ability of a subset of dihydro-β-agarofurans (compounds 1, 2, 5, 7, and 9) was studied.

The IC50 value of the vinca alkaloid microtubule inhibitor vinblastine was measured in the absence or presence of compounds 1, 2, 5, 7, and 9 (all at 5 μM) in the vinca alkaloid-resistant cell line CEM/VCR R, which is a subline of the human T-cell acute lymphoblastic leukemia cell line CEM.60,61 The MDR cell line CEM/VCR R was demonstrated to overexpress P-gp mRNA and protein, exhibiting multidrug resistance to vincristine, vinblastine, doxorubicin, daunorubicin, actinomycin D, teniposide, and tretacycline.62,63 The tretacycline resistance was completely reversed with the P-gp inhibitors cyclosporine A and verapamil.62 The latter is a calcium channel blocker that is routinely used as an inhibitor of multidrug resistance,63 as it has been shown to bind to the efflux transporter P-gp.64 Our data demonstrate that CEM/VCR R cells were 477.2-fold less sensitive to vinblastine than the parental CEM cells (IC50 1336.2 nM vs 2.8 nM, Table 3). Compounds 1, 2, 5, 7, and 9 showed 1H NMR (500 MHz) Spectroscopic Data of Bilucinosins A–C (1–3) in CDCl3.

<table>
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<th>position</th>
<th>δH mult. (J in Hz)</th>
<th>δH mult. (J in Hz)</th>
<th>δH mult. (J in Hz)</th>
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<td>2.21, m</td>
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<td>6.42, d (1.0)</td>
<td>5.25, d (4.4)</td>
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<td>5.03, d (12.5)</td>
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</table>

OH-6: 1.69, d (4.4)

OH-8: 1.69, d (4.4)

*NMR data corresponding to ester groups are provided in the Experimental Section. Overlapping signals. Not observed.

did not show any cytotoxicity toward CEM or CEM/VCR R cells on their own (data not shown) and slightly sensitized the CEM cells to vinblastine (Table 3). However, when CEM/
Table 3. Drug Resistance Reversal Ability of Compounds 1, 2, 5, 7, and 9 (all at 5 μM) in a Drug-Resistant Cell Line

<table>
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<th>Treatment</th>
<th>CEM</th>
<th>CEM/VCR R</th>
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<tbody>
<tr>
<td>vinblastine</td>
<td>IC50 (nM)</td>
<td>RI</td>
</tr>
<tr>
<td>+compound 1</td>
<td>1.6 ± 1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>+compound 2</td>
<td>1.5 ± 0.8</td>
<td>1.9</td>
</tr>
<tr>
<td>+compound 5</td>
<td>1.4 ± 0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>+compound 7</td>
<td>1.9 ± 1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>+compound 9</td>
<td>1.3 ± 0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>verapamil</td>
<td>1.0 ± 0.0</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*IC50 values were determined using a viability assay (alamarBlue) in two independent experiments performed in triplicates (mean ± SD). Verapamil (5 μM) was used as positive control. RI: reversal index. The RI was calculated as the ratio of the IC50 (vinblastine) to the IC50 (vinblastine + tested compound at 5 μM).

VCR R cells were treated with compounds 1, 2, 5, 7, and 9 in combination with vinblastine, they strongly sensitized cells, as indicated by the reduced IC50 values relative to that of vinblastine treatment alone (Table 3). Notably, at equimolar concentrations (5 μM), compounds 7 and 9 showed similar potency based on their reversal indices (RI) to the P-gp inhibitor verapamil, while the remaining compounds scored a lower RI (Table 3). Thus, compounds 1, 2, 5, 7, and 9 are active in inhibiting the MDR phenotype of CEM/VCR R cells, suggesting that they inhibit P-gp. Comparison of the MDR reversal activity for 1, 2, 7, and 9 showed that compounds with an ester group at C-8 (7 and 9) were more potent than those with a hydroxy (1) or carbonyl (2) group at this position. These data indicated that ester substitution at C-8 improved MDR reversal activity for this series of dihydro-β-agarofurans.

Since verapamil has been shown to reduce amino acid transport by blocking calcium flux,14 the leucine uptake in LNCaP cells was next examined. Verapamil significantly reduced leucine uptake to 82.7% of control at 10 μM; however, it was not as effective as 2-amino-bicyclo[2.2.1]heptane-2-carboxylic acid (BCH, 40.2% of control), an α-type amino acid transporter (LAT) family inhibitor (Figure 5A). In LNCaP cells, the essential amino acid leucine is delivered predominantly by LAT3 and regulates protein synthesis through mTORC1 signaling, thereby controlling cancer cell growth.44–47 To determine the effects of our compounds on leucine uptake, a dose–response analysis for each compound was performed. Compound 5 inhibited leucine uptake with an IC50 value of 15.5 μM (Figure 5B). Compounds 1 (IC50 = 124.5 μM) and 2 (IC50 = 151.2 μM) also inhibited leucine uptake at higher concentrations, while 7 and 9 did not show any effects on leucine transport (Figure 5B). These results showed that when C-15 is not substituted with an oxygen functionality (i.e., 5 vs 1/2), leucine transport inhibition is more significant. Furthermore, ester substitution at C-8 (7/9 vs 1/2) resulted in loss of activity. Apart from the LAT family inhibitor BCH, several other natural compounds (monoterpene glycoside isolated from Pittosporum venulorum) have been shown to inhibit LAT3-mediated leucine transport.48,49 While the dihydro-β-agarofurans structure class has had several biological activities reported to date,1,2 this is the first report of dihydro-β-agarofurans inhibiting leucine uptake.

In conclusion, three new and six known dihydro-β-agarofurans were obtained from M. bidulcarius. Four semi-synthetic ester analogues were prepared from the major dihydro-β-agarofuran. Several compounds exhibited similar potency to the reference drug verapamil in reversing the drug resistance of the CEM/VCR R cells. In addition, one compound was found to inhibit leucine uptake in LNCaP cells and was more potent than the leucine analogue BCH. These preliminary findings warrant future mechanistic studies on how these compounds inhibit P-gp and LAT activity.

**EXPERIMENTAL SECTION**

General Experimental Procedures. Melting points were measured using a Cole-Parmer melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO P-1020 polarimeter. UV spectra were obtained using a JASCO V-650 UV/vis spectrophotometer. CD spectra were recorded on a JASCO J-715 spectropolarimeter. IR data were acquired using an attached Universal Attenuated Total Reflectance (UATR) Two module on a PerkinElmer spectrophotometer. NMR spectra were recorded on a Bruker AVANCE III HD 500 MHz NMR spectrometer at 25 °C. The 1H and 13C NMR chemical shifts were referenced to the solvent peaks for CDCl3 at δH 7.26 and δC 77.16, respectively. HRESIMS data were recorded on a Waters ZQ ESI mass spectrometer. HRESIMS data were acquired on a 12 T SolarX XR FT-ICR-MS. X-ray diffraction data were collected on an Oxford-Diffraction Gemini S Ultra CCD diffractometer utilizing Crysalis software.50 Alltech Davisl 30–40 μm 60 Å silica gel packed into an open glass column (25 × 90 mm) was used for flash column chromatography. Thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F254 precoated aluminum plates and was observed using UV light. Activon phenyl- bonded silica was used for preadsorption work before HPLC separations, and the resulting material was packed into an Alltech stainless steel guard cartridge (10 × 30 mm). Phenomenex polypropylene solid-phase extraction (SPE) cartridges were used for purifications. A Waters 600 pump fitted with a Waters 996 photodiode array detector and Gilson 717-plus autosampler was used for semipreparative HPLC separations. A Phenomenex Luna 5 μm phenyl-hexyl column (250 × 10 mm) was used for semipreparative HPLC separations. A Fritsch Universal Cutting Mill Pulverisette 19 was used to grind the air-dried plant material. An Edwards Instrument Company BioHive orbital shaker was used for plant extraction. All solvents used for chromatography, optical rotation, ECD, UV, and MS were Lab-Scale HPLC grade. H2O was Millipore Milli-Q PF filtered. All compounds were analyzed for purity by 1H NMR spectroscopy and shown to be >95% pure, unless otherwise stated. All chemical reagents were purchased from Sigma-Aldrich.
Plant Material. Leaves of *M. bicalcarata* (F. Muell.) Loes (Celastraceae) were collected from The Gap, Queensland, Australia, in May, 2015. A voucher specimen (RAD076) has been deposited at the Ekiti Institute, Griffith University, Brisbane, Australia.

Extraction and Isolation. The air-dried and ground leaves of *M. bicalcarata* (10 g) were extracted with CH$_2$Cl$_2$ (2 × 250 mL) at room temperature under constant shaking. The organic solvent was filtered and evaporated under reduced pressure to give 720 mg of CH$_2$Cl$_2$ extract, which was subsequently chromatographed on a silica gel flash column (25 × 90 mm) using a stepwise gradient solvent system of n-hexane/CH$_2$Cl$_2$ (100% n-hexane to 100% CH$_2$Cl$_2$), affording 100 fractions (100 × 10 mL). Each fraction was analyzed by TLC (n-hexane/CH$_2$Cl$_2$), and those displaying the same TLC profile were combined to afford 16 fractions (F1–F16); all these fractions were analyzed by H NMR spectroscopy. Fractions F3, F9, and F11 were pure and identified as 1α,6β-diacetoxy-9β-benzoyloxy-

Bilobalide C (3): stable, colorless gum; $[\alpha]_D^{22}$ +65 (c 0.04, MeOH); ECD $\lambda_{	ext{max}}$(MeOH) 212 ($\Delta$–1.2), 244 ($\Delta$–1.2) nm; UV (MeOH) $\lambda_{	ext{max}}$(log $\varepsilon$) 231 (4.28), 273 (3.18) nm; IR (KBr) $\nu_{	ext{max}}$ 3484, 2945, 1733, 1370, 1232, 1078, 1105, 1038, 715 cm$^{-1}$; H NMR (CDCl$_3$, 500 MHz) 6.801 (2H, m, O–H), 7.561 (1H, m, O–H), 7.744 (2H, m, O–H), 6.092 (2H, br s, O–H), 1.479 (3H, s, O–H), 0.498 (3H, s, O–A), and other signals, see Table 3; (+)-LREIMS m/z 531 (100) [M+H$^+$]; (+)-HREIMS m/z 535.2040 [M+Na$^+$] (calcd for C$_{35}$H$_{40}$O$_{14}$Na, 555.2044). Infrared-active signals.

Bilobalide C (3): stable, colorless gum; $[\alpha]_D^{22}$ +65 (c 0.04, MeOH); ECD $\lambda_{	ext{max}}$(MeOH) 212 ($\Delta$–1.2), 244 ($\Delta$–1.2) nm; UV (MeOH) $\lambda_{	ext{max}}$(log $\varepsilon$) 231 (4.28), 273 (3.18) nm; IR (KBr) $\nu_{	ext{max}}$ 3484, 2945, 1733, 1370, 1232, 1078, 1105, 1038, 715 cm$^{-1}$; H NMR (CDCl$_3$, 500 MHz) 6.801 (2H, m, O–H), 7.561 (1H, m, O–H), 7.744 (2H, m, O–H), 6.092 (2H, br s, O–H), 1.479 (3H, s, O–H), 0.498 (3H, s, O–A), and other signals, see Table 3; (+)-LREIMS m/z 531 (100) [M+H$^+$]; (+)-HREIMS m/z 535.2040 [M+Na$^+$] (calcd for C$_{35}$H$_{40}$O$_{14}$Na, 555.2044).

Celastrine A (4): white, amorphous powder; $[\alpha]_D^{22}$ +15 (c 0.1, MeOH); ECD $\lambda_{	ext{max}}$(MeOH) 228 (±0.7), 245 (±0.5) nm; IR (KBr) $\nu_{	ext{max}}$ (solvent not reported) 226 (Δ–3.8) nm; (+)-LREIMS m/z 597 (100) [M+Na$^+$]; 1α,6β-Diacetoxy-9α-benzoyloxoy-

Bilobalide C (3): stable, colorless gum; $[\alpha]_D^{22}$ +65 (c 0.04, MeOH); ECD $\lambda_{	ext{max}}$(MeOH) 212 ($\Delta$–1.2), 244 ($\Delta$–1.2) nm; UV (MeOH) $\lambda_{	ext{max}}$(log $\varepsilon$) 231 (4.28), 273 (3.18) nm; IR (KBr) $\nu_{	ext{max}}$ 3484, 2945, 1733, 1370, 1232, 1078, 1105, 1038, 715 cm$^{-1}$; H NMR (CDCl$_3$, 500 MHz) 6.801 (2H, m, O–H), 7.561 (1H, m, O–H), 7.744 (2H, m, O–H), 6.092 (2H, br s, O–H), 1.479 (3H, s, O–H), 0.498 (3H, s, O–A), and other signals, see Table 3; (+)-LREIMS m/z 531 (100) [M+H$^+$]; (+)-HREIMS m/z 535.2193 [M+Na$^+$] (calcd for C$_{35}$H$_{40}$O$_{14}$Na, 555.2301).

Celastrine A (4): white, amorphous powder; $[\alpha]_D^{22}$ +15 (c 0.1, MeOH); ECD $\lambda_{	ext{max}}$(MeOH) 228 (±0.7), 245 (±0.5) nm; IR (KBr) $\nu_{	ext{max}}$ (solvent not reported) 226 (Δ–3.8) nm; (+)-LREIMS m/z 597 (100) [M+Na$^+$]; 1α,6β-Diacetoxy-9α-benzoyloxoy-

Bilobalide C (3): stable, colorless gum; $[\alpha]_D^{22}$ +65 (c 0.04, MeOH); ECD $\lambda_{	ext{max}}$(MeOH) 212 ($\Delta$–1.2), 244 ($\Delta$–1.2) nm; UV (MeOH) $\lambda_{	ext{max}}$(log $\varepsilon$) 231 (4.28), 273 (3.18) nm; IR (KBr) $\nu_{	ext{max}}$ 3484, 2945, 1733, 1370, 1232, 1078, 1105, 1038, 715 cm$^{-1}$; H NMR (CDCl$_3$, 500 MHz) 6.801 (2H, m, O–H), 7.561 (1H, m, O–H), 7.744 (2H, m, O–H), 6.092 (2H, br s, O–H), 1.479 (3H, s, O–H), 0.498 (3H, s, O–A), and other signals, see Table 3; (+)-LREIMS m/z 531 (100) [M+H$^+$]; (+)-HREIMS m/z 535.2193 [M+Na$^+$] (calcd for C$_{35}$H$_{40}$O$_{14}$Na, 555.2301).
Pivaloylation of Bisacryl A (1). Compound 1 (117. mg, 0.0220 mmol) was dissolved in dry pyridine (2 drops) before pivaloyl chloride (200 μL, 1.6 mmol) was added. The solution was stirred for 16 h at room temperature. The reaction mixture was dried and purified by silica SPE cartridge (10 × 40 mm) using a 5% stepwise gradient from n-hexane to 20% EtOAc–n-hexane (10 mL elutions). The fraction that eluted with 20% EtOAc–80% n-hexane contained the pivaloylated product 7 (5.3 mg, 25%).

8-Pivaloyloxybacilin A (7): white, amorphous powder; [α]D20 −51 (c 0.1, MeOH); ECD λmax (MeOH) 240 (Δε +0.5), 273 (Δε −0.2) nm; UV (MeOH) λmax (log ε) 232 (4.06), 274 (2.93) nm; IR (UATR) 3097, 1736, 1369, 1271, 1229, 1149, 1096, 714 cm−1; 1H NMR (CDCl3, 500 MHz) δ 7.58 (3H, d, J = 11.1 Hz, H-7), 5.68 (1H, d, J = 5.7 Hz, H-7), 5.60 (1H, d, J = 5.7, 3.9 Hz, H-8), 5.40 (1H, d, J = 11.9, 4.8 Hz, H-1), 4.96 (1H, d, J = 13.3 Hz, H-15a), 4.71 (1H, d, J = 13.3 Hz, H-15b), 2.42 (1H, br, d, J = 3.9 Hz, H-5), 2.32 (1H, m, H-4), 2.11 (1H, m, H-3b), 2.10 (3H, s, OAc-5), 1.79 (1H, m, H-2b), 1.36 (3H, d, J = 6.7 Hz, H-4a), 0.93 (3H, t, J = 6.7 Hz, H-3a), 0.92 (3H, d, J = 6.7 Hz, H-4a), 0.89 (3H, d, J = 6.7 Hz, H-3a), 0.77 (3H, d, J = 6.7 Hz, H-3a), 0.50 (3H, s, C(3)-C(6)), 0.39 (4H, C(5)-O(1)), 0.37 (1H, C(14)-C(11)), 0.14 (3H, OAc-1), 0.18 (1H, Me, H-1a), 0.14 (1H, Me, H-1a), 0.10 (1H, d, J = 7.6 Hz, H-14); 13C NMR (CDCl3, 125 MHz) δ 172.6 (C-O, OAc), 171.0 (C-O, OAc), 170.1 (C-O, OAc), 169.8 (C-O, OAc), 164.9 (C-O, OAc), 133.5 (CH, OAc), 129.8 (2CH, OAc), 129.4 (2CH, OAc), 90.9 (C-O, C(3)), 81.5 (C-O, C(1)), 79.6 (CH, C(3)), 73.9 (CH, C(9)), 70.3 (CH, C(8)), 61.5 (CH, C(15)), 50.3 (CH, C(7)), 50.8 (C, C(10)), 39.0 (C, O(OH)), 33.7 (CH, C(4)), 30.5 (CH, C(13)), 27.2 (2CH, OAc), 26.8 (CH, C(2)), 24.5 (CH, C(12)), 23.4 (CH, C(2)), 21.7 (CH, OAc-15), 21.4 (CH, OAc-6), 20.9 (CH, OAc-1), 15.2 (CH, C(14)); (+)-HR-ESIMS m/z 639.2773 ([M + Na]+) (calcd for C37H53NO12Na, 639.2776).

Methodacetylation of Bacilin A (1). Compound 1 (121 mg, 0.0227 mmol) was dissolved in dry pyridine (2 drops) before 200 μL (2.2 mmol) of methacryloyl chloride was added. The solution was stirred overnight at room temperature. The reaction mixture was dried and purified using the same protocol described for the purification of compound 4 to give compound 5 (8.3 mg, 45%), tR 36–37 min.

8-Methodoxybacilin A (8): white, amorphous powder; [α]D20 −20.0 (c 0.3, MeOH); ECD λmax (MeOH) 230 (Δε +0.5), 250 (Δε −0.4) nm; UV (MeOH) λmax (log ε) 232 (4.02), 273 (2.92) nm; IR (UATR) 3070, 1700, 1457, 1371, 1232, 1127, 1171 cm−1; 1H NMR (CDCl3, 500 MHz) δ 8.02 (2H, t, J = 7.7 Hz, H-8), 0.007 (1H, m, OAc-9), 2.21 (3H, s, OAc-5), 0.16 (1H, Me, H-1a), 0.13 (1H, Me, H-1a), 0.11 (1H, Me, H-1a), 0.08 (1H, Me, H-1a), 0.07 (1H, Me, H-1a), 0.06 (1H, Me, H-1a), 0.05 (1H, Me, H-1a), 0.04 (1H, Me, H-1a), 0.03 (1H, Me, H-1a), 0.02 (1H, Me, H-1a), 0.01 (1H, Me, H-1a), 0.00 (1H, Me, H-1a), 0.00 (1H, Me, H-1a); 13C NMR (CDCl3, 125 MHz) δ 172.6 (C-O, OAc), 171.0 (C-O, OAc), 170.1 (C-O, OAc), 169.8 (C-O, OAc), 164.9 (C-O, OAc), 133.5 (CH, OAc), 129.8 (2CH, OAc), 129.4 (2CH, OAc), 128.6 (2CH, OAc), 90.9 (C-O, C(3)), 81.5 (C-O, C(1)), 79.6 (CH, C(3)), 73.9 (CH, C(9)), 70.3 (CH, C(8)), 61.5 (CH, C(15)), 50.3 (CH, C(7)), 50.8 (C, C(10)), 39.0 (C, O(OH)), 33.7 (CH, C(4)), 30.5 (CH, C(13)), 27.2 (2CH, OAc), 26.8 (CH, C(2)), 24.5 (CH, C(12)), 23.4 (CH, C(2)), 21.7 (CH, OAc-15), 21.4 (CH, OAc-6), 20.9 (CH, OAc-1), 15.2 (CH, C(14)); (+)-HR-ESIMS m/z 639.2773 ([M + Na]+) (calcd for C37H53NO12Na, 639.2776).

X-ray Crystallography Analysis. Colorless crystals of bacilin A (1) and bacilin B (2) were obtained by crystallization from EtOH. Unique data sets for 1 and 2 were measured at 200 K on an Oxford-2020 Gemini 5 Ultra CCD diffractometer with Cu Kα radiation using the CryoFlex software. The crystal structures were obtained by the direct methods program SIR2004 and refined by full matrix least-squares refinement on F2 using the WinGX software package incorporating SHELXL-2013. Anisotropic thermal parameters were refined for non-hydrogen atoms; (x, y, z, Uiso) were included and constrained at estimated values. Conventional residuals at convergence are quoted; statistical weights were employed. The absolute configurations of 1 and 2 were determined by anomalous dispersion effects (1, 1955 Bijvoet pairs, Flack parameter 0.02(6); 2, 1825 Bijvoet pairs, Flack parameter 0.05(6)); ORTEP-32 and PLATON32 software were utilized to prepare material for publication. Full cif deposition resides with the Cambridge Crystallographic Data Centre (CCDC Nos. 1453302 and 1453303). Copies can be obtained free of charge on application at the following address: http://www.ccdc.cam.ac.uk/cgi-bin/芡ree.cif?crageg.
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REFERENCES

Chapter 3. Dihydro-β-Agarofurans from the Roots of the Australian Endemic Rainforest Tree *Maytenus Bilocularis* Act as Leucine Transport Inhibitors

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

**Mario Wibowo, Qian Wang, Jeff Holst, Jonathan M. White, Andreas Hofmann, and Rohan A. Davis**


Supporting Information can be found in Appendix 2.

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**My contribution to the paper involved:**

I conducted all the plant extractions, compound isolation experiments, and recorded and analysed the spectroscopic and spectrometric data for all compounds. The biology and X-ray diffraction experiments were performed by our collaborators. I was the major contributor to the preparation of the manuscript.

14 August 2018

Mario Wibowo

Corresponding author of paper: Rohan A. Davis

Supervisor: Rohan A. Davis
Dihydro-β-agarofurans from the roots of the Australian endemic rainforest tree *Maytenus bilocularis* act as leucine transport inhibitors

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ABSTRACT

Phytochemical studies of the roots of the Australian plant, *Maytenus bilocularis*, resulted in the identification of six previously undescribed dihydro-β-agarofuran sesquiterpenoids, bilocularins D–I, along with three known natural products, namely 1α,6α,15-triaceotony-9β-benzooyloxydihydro-β-agarofuran, pristimerin, and celastrol. The structures of all compounds were characterized via analysis of 1D/2D NMR and MS data. The absolute configuration of bilocularin D was determined by X-ray crystallography analysis. Bilocularins D and G, 1α,6α,15-triaceotony-9β-benzooyloxydihydro-β-agarofuran, and celastrol inhibited leucine transport in the human prostate cancer cell line LNCaP with IC₅₀ values ranging from 2.5–279 μM, which were more potent than the L-type amino acid transporter (LAT) family inhibitor 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH). Bilocularins D–F are the first examples of dihydro-β-agarofurans bearing a hydroxyacetate group.

1. Introduction

L-Type amino acid transporters (LATs) facilitate the transport of neutral amino acids, such as leucine, isoleucine, and valine through cell membranes (Grkovic et al., 2015). LAT family members are crucially important for cell survival since they mediate the uptake of essential amino acids, which are necessary for energy and protein production. The overexpression of LATs, such as LAT1 (SLC7A5) and LAT3 (SLC43A1), has been observed in various human cancers, including prostate cancer (Wang et al., 2011; Wang et al., 2013b). Furthermore, the inhibition of LAT1 and LAT3 has been reported to restrict leucine uptake, thereby regulating the activity of mammalian target of rapamycin complex 1 (mTORC1) (Wang et al., 2011; Wang et al., 2013b). Leucine is an essential amino acid needed for mTORC1 activation (Kimball et al., 1999). Since protein translation can only commence at a sufficient intra-cellular level of leucine, the inhibition of leucine transport into the cell presents an opportunity to inhibit the growth of cancer cells (Wang et al., 2011), and the LAT family transporters may therefore prove to be a novel drug target for future cancer therapies (Wang and Holst, 2015).

Recently, we have reported the chemical investigation of the leaves of the Australian rainforest plant, *Maytenus bilocularis* (F. Muell.) Loes (Celastraceae), that resulted in the discovery of the previously undescribed natural products, bilocularins A–C (1–3) (Wibowo et al., 2016a). Bilocularins A (1) and B (2) were the first reported dihydro-β-agarofurans, which inhibited leucine uptake in prostate cancer cells (Wibowo et al., 2016a). Moreover, several other dihydro-β-agarofuran natural products isolated from two other Australian Celastraceae plants, *Denhamia pittosporoides* and *Celastrus subspicata*, were subsequently shown by our group to possess leucine uptake inhibitory activity in the human prostate cancer cell line, LNCaP (Wibowo et al., 2016b, 2017). Driven by our...
previous discovery of dihydro-β-agarofurans that inhibited leucine uptake from the leaves of *Maytenus bilocularis*, which so far had not been phytochemically characterized. This paper reports the structures of six previously undescribed compounds, namely bilocularins D–I (4–9) together with three known natural products, 1α,2α,6β,15-tetraacetoxy-9β-benzoxyldihydro-β-agarofuran (10) (Tu, 1990), pristimerin (11) (Alvarenga et al., 1999), and celastrol (12) (Ngassapa et al., 1994). Furthermore, a subset of the discovered compounds was characterized with respect to inhibition of leucine uptake in the human prostate cancer cell line, LNCaP.

2. Results and discussion

The CH$_2$Cl$_2$ extract obtained from the roots of *Maytenus bilocularis* was separated by various chromatographic techniques to afford six previously undescribed dihydro-β-agarofuran sesquiterpenoids, bilocularins D–I (4–9), and three known natural products (Fig. 1).

Bilocularin D (4) was isolated as stable white crystals. The molecular formula C$_{37}$H$_{42}$O$_{13}$ was determined by (+)-HRESIMS data (m/z 717.2515 [M + Na]$^+$, calc. for 717.2518). The $^1$H NMR spectrum of 4 exhibited signals for five methyl singlets (δ$_H$ 1.56, 1.57 (6H), 2.11, and 2.14), four sets of methylene protons (δ$_H$ 2.03/2.22, 2.22/2.62, 3.89/3.92, and 4.88/4.96), 17 methine protons (δ$_H$ 2.03, 5.30, 5.76, 5.84, 6.17, 6.31, 7.38 (3H), 7.53 (4H), 7.62, 7.67, and 8.18 (2H)), and two hydroxy groups (δ$_H$ 1.56, 1.57 (6H), 2.18 and 2.96); the latter two signals were confirmed following HSQC experiment. The $^{13}$C NMR and HSQC spectra revealed 37 carbon resonances including five methyls, four methylenes, 17 methines, and 11 non-protonated carbons. The presence of five ester groups in 4 was suggested by $^{13}$C NMR signals at δ$_C$ 165.6, 166.8, 170.0, 170.2, and 172.4. These carbon resonance at δ$_C$ 170.2 and 170.2, respectively. The presence of the hydroxyacetate group was confirmed by HMBC correlations from the exchangeable proton (δ$_H$ 2.18) and the oxymethylene (δ$_H$ 3.89/3.92) to the carbonyl carbon at δ$_C$ 172.4. Furthermore, an HMBC correlation from H-1 (δ$_H$ 5.76) to the carbon signal at δ$_C$ 172.4 positioned the hydroxyacetate at C-1. Finally, a hydroxy group was attached at C-4 by considering the deshielded $^{13}$C NMR resonance of C-4 (δ$_C$ 70.0) and the HMBC correlation from the hydroxy proton signal at δ$_H$ 2.96 to C-4.

The relative configuration of 4 was assigned following analysis of $^1$H-$^1$H coupling constants and the ROESY spectrum (Fig. 2). The β-orientation of H-1 and H-2 was assigned based on the small coupling constant between the two protons (J$_{1,2}$ = 3.5 Hz) (Gao et al., 2016; Núñez et al., 2016). The ROESY spectrum of 4 exhibited correlations between H-1 and H-3β, indicating that these two atoms were co-facial. Furthermore, ROESY cross-peaks between H-6 and H$_2$-15, as well as between H$_2$-14 and H-6 determined the α-orientation of H-6, CH$_3$-14, and CH$_2$-15. The structure of 4 was confirmed by X-ray crystallography studies (Fig. 3), which also defined its absolute configuration as (1R,2S,4S,5S,6R,7R,9S,10R)-2,6-diacetoxy-15-benzoxyloxy-9-cinnamoyloxy-1-hydroxyacetoxy-4-hydroxydihydro-β-agarofuran.

Compound 5, which was purified as a white amorphous powder, had a molecular formula of C$_{39}$H$_{44}$O$_{13}$, which was assigned
following analysis of the (+)-HRESIMS data. The 1H and 13C NMR
data of 5 were similar to those of 4, indicating that both compounds
possessed the same dihydro-β-agarofuran core structure. Detailed
analysis of NMR data of 5 and comparison with 4 showed that the
benzoate group in 4 was replaced by a trans-cinnamoyl moiety in 5.
The 1H resonances at δH 7.87 (1H, J = 16.1 Hz), 7.69 (1H, d, J = 16.0 Hz), 7.63 (2H, m), 7.54 (2H, m), 7.39 (3H, m), 6.56 (1H, d, J = 16.1 Hz), and 6.32 (1H, d, J = 16.0 Hz) were evident of
the presence of the two trans-cinnamoyl moieties in 5 (García et al.,
2013). The location of the trans-cinnamoyl group at C-15 in 5 was
further supported by HMBC correlations from H2-15 (δH 4.69/4.91)
and an olefinic proton at δH 7.87 to a carbonyl resonance at δC 166.6.
The ROESY spectrum exhibited correlations between H2-14 and H-15,
between H2-15 and H-8, as well as H2-15 and H-6, implying the
relative configuration of 5 to be the same as compound 4. Finally,
on the basis of biosynthetic considerations (Núñez et al.,
2016) the absolute configuration of 5 (bilocularin E) was estab-
lished as (1R,25,45,55,6R,7R,9S,10R)-2,6-diacetoxy-9,15-
dicinnamoyloxy-1-hydroxyacetoxymethoxy-4-hydroxydihydro-β-
agarofuran.

Bilocularin F (6), a white amorphous powder, was assigned the
molecular formula C33H30O12 following analysis of the MS data. The
NMR (Tables 1 and 2) and MS data suggested that 6 was structurally
similar to 4, except that the benzoate in 4 was replaced by a hy-
droxy moiety in 6. The H2-15 protons were shifted upfield from δH 4.88/4.96 in 4 to δH 4.18/4.33 in 6, which supported the attachment
of the hydroxy at C-15 in 6. The ROESY data showed that the relative
configuration of 6 was the same as that of 4. By considering
biosynthetic pathways and comparison of chiroptical data of 6 and
4, the absolute configuration of 6 was assigned as (1R,25,45,55,6R,7R,9S,10R)-2,6-diacetoxy-9-cinnamoyloxy-1-
hydroxyacetoxymethoxy-4,15-dihydroxydihydro-β-agarofuran. To the best
of our knowledge, bilocularins F–F (4–6) are the first reported
dihydro-β-agarofurans bearing a hydroxycetate group.

Compound 7 was assigned the molecular formula C32H30O12 on the
basis of the (+)-HRESIMS data. The NMR (Tables 1 and 2) and
MS data revealed that 7 was a dihydro-β-agarofuran sesquiterpe-
noid decorated with five ester groups. Comparison of the NMR data
of 7 and 4 identified a high degree of similarity between these
compounds. The hydroxycetate and the benzoate functionalities
signals in 4 were not present in the NMR spectra of 7, however two additional acetate group signals were observed. HMBC correlations (Fig. 4) from H-1 (δH 5.59) to a carbonyl at δC 169.7 and from H2-15 (δH 4.34/4.96) to a carbon signal at δC 170.7 readily placed the two acetates at C-1 and C-15 in 7. The same
relative configuration previously assigned to 4 was also determined
for 7 following ROESY data (Fig. 4) analysis. Comparison of ECD
spectra of compounds 4 and 7 implied that both compounds had
identical absolute configurations. Therefore, the structure and ab-
solute configuration of 7 was elucidated as (1R,25,45,55,6R,7R,9S,10R)-12,6,15-tetraacetoxy-9-cinnamoyloxy-
4-hydroxydihydro-β-agarofuran and was given the trivial name
bilocularin G (7).

Table 1
1H NMR (800 MHz) spectroscopic data of bilocularins D–I (4–9) in CDCl3.a

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<th>Position δH, mult. (J in Hz)</th>
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<th>6</th>
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a NMR data of the ester groups are provided in the Experimental Section.
b Interchangeable signals.
c Overlapping signals.
\na Not observed.
The molecular formula of bilocularin H (8) was determined as C32H37NO9 by (+)-HRESIMS and NMR data analysis. The 1D NMR spectra of 8 showed it to have a similar chemical structure to that of 7. Detailed analysis of HMQC spectrum of 8 revealed that the acetate groups at C-2 and C-15 in 7 were replaced by hydroxy moieties in 8. The chemical shift of H-2 was shifted upfield from δH 9.49 in 7 to δH 4.29 in 8, which supported the presence of an α-OH group on C-2. Similarly, H2-15 protons were also shielded from δH 4.96/4.34 in 7 to δH 4.33/4.27 in 8 which indicated that the latter natural product contained an α-C2H2OH moiety. The 2D NMR data further confirmed the location of the remaining esters in compound 8. The relative configuration of compound 8 was identical to that of 7 following ROESY data analysis. The ECD spectrum of 8 was similar to that of 7, which suggested that both compounds had the same absolute configuration. Consequently, the structure of bilocularin H (8) was assigned as (1R,2S,4S,5S,6R,7R,9S,10R)-1,6-diacytioxy-9-cinnamoyloxy-2,4,15-trihydroxydihydro-β-agarofuran. Bilocularin I (9) was obtained as a colorless gum and was assigned the molecular formula C32H37NO9 based on (+)-HRESIMS data. Analysis of NMR and MS data showed that 9 was an alkaloid possessing a dihydro-β-agarofuran core structure. The structure of 9 was found to be similar with that of 8. However, the acetate group at C-6 in 8 was replaced by a nicotinate in 9. The presence of the nicotinate moiety was confirmed by the characteristic 1H resonances at δH 9.37 (1H, d, J = 1.9, 0.7 Hz), 8.80 (1H, dd, J = 4.8, 1.9 Hz), 8.51 (1H, ddd, J = 7.9, 1.9, 1.9 Hz), and 7.42 (1H, ddd, J = 7.9, 4.8, 0.7 Hz) (Wang et al., 2013a; Ning et al., 2015). HMBC correlations from both H-6 (δH 5.75) and the proton at δH 8.80 to a carbonyl resonance at δC 165.0 further confirmed the position of the nicotinate ester at C-6. In addition, comparison of the NMR data also revealed that the –C2H2OH moiety in 8 was replaced by a methyl group in 9. This was confirmed by HMBC correlations (Fig. 5) from a methyl at δH 1.60 to C-1, C-5, C-9, and C-10. The relative configuration of compound 9 was ascertained to be identical with that of 8 following ROESY studies (Fig. 5). The same absolute configuration previously assigned for 8 was also defined for 9 taking into account biosynthetic reasoning. Accordingly, the structure of bilocularin G (9) was determined as (1R,2S,4S,5S,6R,7R,9S,10R)-1-acetoxy-9-cinnamoyloxy-2,4-dihydroxy-6-nicotinoyloxydihydro-β-agarofuran. The structures of the known compounds, 1a,2α,6β,15-tetraacetoxo-9β-benzyloxydihydro-β-agarofuran (10) (Tu, 1990), pristinomycin (11) (Alvarenga et al., 1999; Chang et al., 2003), and celastrol (12) (Ngassapa et al., 1994; Chang et al., 2003) were characterized by comparison of their spectroscopic data with the literature. Compound 10 was initially isolated from the Celastraceae plant Euonymus burgeanus (Tu, 1990), while pristinomycin (11) and celastrol (12) have been identified in a variety of plant species from the Celastraceae family (Camello et al., 2015).

Owing to our previous discovery of natural products that inhibited leucine uptake (Wibowo et al., 2016a; Wibowo et al., 2016b, 2017), several metabolites (compounds 4–7 and 10–12) were evaluated for their effect on leucine uptake in the androgen-responsive prostate cancer cell line LNCaP. Bilocularins A (1) and B (2) had previously been tested in this assay, and exhibited IC50 values of 124.5 and 151.2 μM, respectively (Wibowo et al., 2016a). Compounds 8 and 9 could not be purified in sufficient quantities to allow for biological evaluation. Bilocularins D (4) and G (7) inhibited leucine uptake in LNCaP cells, with IC50 values of 3.1 and 2.5 μM, respectively, however they only inhibited leucine uptake by around 50%. By contrast, compounds 10 (IC50 = 27.9 μM; Fig. 6B) and 12 (IC50 = 5.7 μM, Fig. 6C) showed higher IC50 concentrations, but more completely inhibited leucine uptake, perhaps reflecting inhibition of multiple LAT family members. Compounds 5, 6, and 11 showed little or no effect on leucine transport (Fig. 6A and C). Based on these bio-assay data, some preliminary structure-activity relationships were delineated. Compounds with a benzoate (4) or an acetate (7) at C-15 had better activity compared with the molecules with a trans-cinnamate (5) or a hydroxy (6) group at the same position. This suggested that a benzoate or an acetate moiety at C-15 for this series of sesquiterpenoids might play an important role in the inhibitory effect of leucine uptake. Sesquiterpenoids 4, 7, and 10 exhibited better activity than the currently utilized LAIs family inhibitor 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH, IC50 = 4060 μM) (Wang et al., 2014). Previously, several natural products obtained from Pittosporum venulosum (Wang et al., 2014; Grkovic et al., 2015), Denhamia pittosporoides (Wibowo et al., 2016b), and Celastrus subspicata (Wibowo et al., 2017) have also been reported to inhibit leucine uptake in prostate cancer cells. In an extension of investigations of Australian Celastraceae plants for bioactive constituents we have here identified compounds from the roots of M. bilocularis that constitute the most potent leucine uptake inhibitors among small molecule effectors thus far. This research supports the continued investigations of Australian Celastraceae plants for bioactive constituents.

3. Experimental

3.1. General experimental procedures

Melting points were measured using a Cole-Parmer melting point apparatus and are uncorrected. Specific rotations were acquired on a JASCO P-1020 polarimeter. UV spectra were recorded using a JASCO V-650 UV/vis spectrophotometer. ECD spectra were obtained on a JASCO J-715 spectropolarimeter and processed using the software SDAR (Weeratunga et al., 2012). IR data were acquired using an attached Universal Attenuated Total Reflectance (UATR) two module on a PerkinElmer spectrophotometer. NMR spectra were recorded on a Bruker AVANCE HDX 800 MHz NMR spectrometer equipped with a TCI cryoprobe at 25 °C. The 1H and 13C NMR chemical shifts were referenced to the solvent peaks for CDCl3 at δH 7.26 and δC 77.16, respectively. LRESIMS data were recorded on a Waters ZQ ESI mass spectrometer. HRESIMS data were acquired on a 12 T Solarix XR FT-ICR-MS. Alltech Davisil 90–130 μm 60 Å Si gel was used for column chromatography. Thin-layer chromatography (TLC) and preparative TLC was carried out on Merck Si gel 60 F254 pre-coated aluminum plates and was observed using UV light. Alltech C18-bonded Si (35–75 μm, 150 Å) was used for preadsorption work, and the resulting material was packed into an Alltech stainless steel guard cartridge (10 x 30 mm) prior to HPLC separations. A Waters 600 pump fitted with a Waters 996 photodiode array detector and Gilson 717-plus autosampler was used for semi-preparative HPLC separations. Phenomenex Luna 5 μm 90–110Å C18 (250 x 10 mm), and Thermo Hypersil-Keystone Betalis 5 μm 100 Å C8 (150 x 21.2 mm) columns were used for semi-preparative HPLC separations. A Fritsch Universal Cutting Mill Pulverisette 19 was used to grind the air-dried plant material and...
an Edwards Instrument Company Bio-line orbital shaker was used for plant extraction. All solvents used for chromatography, specific rotation, ECD, UV and MS were Honeywell Burdick & Jackson (BDJ) HPLC grade. H₂O was Millipore Milli-Q PF filtered. All compounds were analyzed for purity by ¹H NMR spectroscopy and shown to be >95%, unless otherwise stated. NMR spectra were processed using MestReNova version 11.0.

3.2. Plant material

The roots of Maytenus bilocularis (F. Muell.) Loes (Celastraceae) were collected in Brisbane, Australia on the 22 October 2016 and identified by D. Sommerville from Save Our Watersways Now. A voucher specimen (RAD076-R) has been deposited at the Griffith Institute for Drug Discovery, Griffith University, Brisbane, Australia.

3.3. Extraction and isolation

The air-dried and ground roots of M. bilocularis (150 g) were extracted with CH₂Cl₂ (2 x 1 L for 16 h each) to afford 3.17 g of a crude extract (red gum). The extract was chromatographed over a Si-gel column (4 x 20 cm) eluted with a stepwise CH₂Cl₂/MeOH gradient solvent system (100% CH₂Cl₂ to 10% MeOH/CH₂Cl₂) to give 98 fractions (~20 mL each). Similar fractions were pooled following (+)-LRESIMS analysis to afford 15 fractions (A1-A15). Fraction A10 (364.4 mg) was further separated using a Si-gel column chromatography (2.5 x 11 cm) using a stepwise n-hexane/MeOH gradient solvent system (5% EtOAc/n-hexane to 50% EtOAc/n-hexane) to afford 58 subfractions. The subfractions were combined following TLC analysis to yield fractions B1–B6. Fraction B2 contained pure pristimerin (11, 95.3 mg, 0.0635% dry wt) Fraction B4 (93.8 mg) was preadsorbed to C₁₈ bonded Si-gel (~1 g), packed into a guard cartridge and attached to a semi-preparative C₁₈ HPLC column (250 x 10 mm). A linear gradient from 40% MeOH/H₂O to 100% MeOH at a owrate of 9 mL/min was run over 60 min to obtain 1a,2a,6,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran (10, 27.6 mg, tᵣ 23–25 min, 0.0184% dry wt).

Fraction A11 (1.43 g) was preadsorbed to C₁₈ bonded Si-gel (~1 g), which was divided into 5 portions, each portion was packed into a guard cartridge and further purified using a semi-preparative C₁₈ HPLC column (150 x 21.2 mm) eluted with a gradient solvent system of 60% MeOH/H₂O to 100% MeOH over 60 min at a flowrate of 9 mL/min. Bilocularin D (4, 54.1 mg, 0.0361% dry wt) was obtained by crystallization of fractions eluting at 24–26 min, while fractions eluting at 42–43 min contained celastrol (12, 152.2 mg, 0.0105% dry wt). The fraction eluting at 21 min (16.1 mg) was chromatographed on preparative TLC (1% MeOH/CH₂Cl₂) to obtain 11.5 mg of bilocularin G (7, 0.0077% dry wt). The fractions eluting at 27–35 min (111.1 mg) were combined then preadsorbed to C₁₈ bonded Si-gel (~1 g), packed into a guard cartridge and attached to a semi-preparative C₁₈ HPLC column (250 x 10 mm). A linear gradient solvent system of 70% MeOH/H₂O to 90% MeOH/H₂O at a flowrate of 4 mL/min was performed over 60 min to afford semi-pure bilocularin E (22.5 mg, tᵣ 26–28 min), which was further purified by preparative TLC (35% EtOAc/n-hexane) to yield pure bilocularin F (5, 4.8 mg, 0.0032% dry wt).

Fraction A13 (51.1 mg) was preadsorbed to C₁₈ bonded Si-gel (~1 g), packed into a guard cartridge and attached to a semi-preparative C₁₈ HPLC column (250 x 10 mm). A linear gradient from 50% MeOH/H₂O to 95% MeOH/H₂O at a flowrate of 4 mL/min was employed over 60 min to afford bilocularin F (6, 1.5 mg, tᵣ 27–28 min, 0.0010% dry wt) and bilocularin I (9, 1.0 mg, tᵣ 38 min, 0.0007% dry wt). Fraction A17 (26.1 mg) was preadsorbed to C₁₈ bonded Si-gel (~1 g), packed into a guard cartridge and attached to a semi-preparative C₁₈ HPLC column (250 x 10 mm). A linear gradient from 45% MeOH/H₂O to 100% MeOH at a owrate of 4 mL/min over 60 min was employed to afford bilocularin H (8, 0.2 mg, tᵣ 31–32 min, 0.0001% dry wt).

3.3.1. Bilocularin D (4)

Stable white crystals (MeOH); mp 231–234 °C; [α]D₂⁰ +38 (c 0.185, MeOH); ECD (λmax (MeOH) 219 (31.5), 266 (+34.8) nm; UV (MeOH) λmax (log ε) 224 (5.21), 281 (5.26) nm; IR (UATR) max 3535, 2970, 1722, 1246, 1152, 712 cm⁻¹; ¹H NMR (CDCl₃, 800 MHz) 8.18 (2H, m, Obz-15), 7.67 (1H, d, J = 16.0 Hz, OtCin-9), 7.62 (1H, m, Obz-15), 7.53 (2H, m, Obz-15), 7.53 (2H, m, OtCin-9), 7.38 (3H, m, OtCin-9), 6.31 (1H, d, J = 16.0 Hz, OtCin-9), 3.92 (1H, dd, J = 17.3, 5.3 Hz, OHAcO-1), 3.89 (1H, dd, J = 17.3, 3.9 Hz, OHAcO-1), 2.18 (1H, br m, h, OHAcO-1), 2.14 (3H, s, OAc-2), 2.11 (3H, s, OAc-6), for other signals, see Table 1; ¹³C NMR (CDCl₃, 200 MHz) δC 172.4 (C, OHAcO-1), 170.2 (C, OAc-6), 170.0 (C, OAc-6), 166.8 (C, Obz-15), 165.6 (C, Obz-15), 146.7 (CH, OtCin-9), 134.1 (C, OtCin-9), 133.7 (CH, Obz-15), 130.8 (CH, OtCin-9), 129.9 (2CH, Obz-15), 129.6 (C, Obz-15), 129.1 (2CH, OtCin-9), 128.9 (2CH, Obz-15), 128.4 (2CH, OtCin-9), 117.1 (CH, OtCin-9), 60.8 (CH₃, OHAcO-1), 21.6 (CH₃, OAc-6), 21.3 (CH₃, OAc-2) for other signals, see Table 2; (+)-LRESIMS m/z 717 (100) [M+Na]+; (+)-HRESIMS m/z 717.2518 [M+Na]+ (calcld for C₉H₁₄O₁₃Na, 717.2518). Overlapping signals.

3.3.2. Bilocularin E (5)

White amorphous powder; [α]D₂⁰ +57 (c 0.240, MeOH); ECD (λmax (MeOH) 218 (30.6), 261 (23.3), 293 (+67.8) nm; UV (MeOH) λmax (log ε) 217 (2.58), 281 (5.46) nm; IR (UATR) max 3477, 2980, 1709, 1634, 1237, 1150, 768 cm⁻¹; ¹H NMR (CDCl₃, 800 MHz) 7.87 (1H, d, J = 16.1 Hz, OtCin-15), 7.69 (1H, d, J = 16.0 Hz, OtCin-15), 7.54 (2H, m, OtCin-9), 7.42 (3H, m, OtCin-9), 7.39 (3H, m, OtCin-9), 6.56 (1H, d, J = 16.1 Hz, OtCin-15), 6.32 (1H, d, J = 16.0 Hz, OtCin-9), 3.92 (1H, d, J = 17.1 Hz, OHAcO-1), 3.90 (1H, d, J = 17.1 Hz, OHAcO-1), 2.13 (3H, s, OAc-2), 2.12 (3H, s, OAc-6) for other signals, see Table 1; ¹³C NMR (CDCl₃, 200 MHz) δC 172.4 (C, OHAcO-1), 170.2

Fig. 6. [³H]L-Leucine dose response in the presence of 4, 5, 6 (A), 7, 10 (B), 11 and 12 (C) in LNCaP cells (n ≥ 3, mean ± S.E.M). The IC₅₀ values of 4, 7, 10, and 12 were 3.1, 2.5, 27.9, and 5.7 µM, respectively.
3.3.3. Bilocularin F (6)

White amorphous powder; \[^{1}^H\]NMR (CDCl\(_3\), 800 MHz) \(7.86 \text{ (1H, d, } J = 16.0 \text{ Hz, Cin-9)}\); \(7.42 \text{ (1H, d, } J = 15.9 \text{ Hz, Cin-9)}\); 7.39 (3H, m, Cin-9), 7.39 (3H, m, Cin-9), 7.14 (2H, d, \(J = 7.9 \text{ Hz, Cin-9)}\); 7.04 (1H, d, \(J = 16.0 \text{ Hz, Cin-9)}\), 7.69 (1H, d, \(J = 16.0 \text{ Hz, Cin-9)}\), 7.39 (3H, m, Cin-9), 7.39 (3H, m, Cin-9), 6.35 (1H, d, \(J = 16.0 \text{ Hz, Cin-9)}\), 2.25 (3H, s, OAc-1), 2.110 (3H, s, OAc-2), 2.06 (3H, s, OAc-2), for other signals, see Table 1; \[^{13}C\]NMR (CDCl\(_3\), 200 MHz) \(170.7 \text{ (C, OAc-6)}\), 170.4 (C, Cin-9), 169.9 (C, OAc-6); 162.2 (C, Cin-9), 146.5 (CH, Cin-9), 134.2 (C, Cin-9), 130.8 (CH, Cin-9), 129.1 (2CH, Cin-9), 128.5 (2CH, Cin-9), 117.5 (CH, Cin-9), 60.9 (CH\(_2\), OAc-1), 21.8 (CH\(_3\), OAc-6), 21.4 (CH\(_3\), OAc-6), for other signals, see Table 2; (+)-HRESIMS m/z 613 (100) [M+Na\(^+\)]; (+)-HRESIMS m/z 613.2252 [M+Na\(^+\)] (calcd for \(C_{35}H_{44}O_{13}Na\), 555.2202)

3.4. X-ray crystallography analysis

Intensity data for bilicarol D (4) were collected with an Oxford Diffraction SuperNova CCD diffractometer using Cu-K\(_{a}\) radiation, the temperature during data collection was maintained at 130.0(1) K using an Oxford Cryosystems cooling device. The structure was solved by direct methods and difference Fourier Synthesis (Sheldrick, 2008). Hydrogen atoms bound to carbon atoms were placed at their idealized positions using appropriate HFIX instructions in SHELXL, and included in subsequent refinement cycles. The hydrogen atom attached to oxygen was located from difference Fourier maps and refined freely with isotropic displacement parameters. Thermal ellipsoid plots were generated using the program ORTEP-3 (Farrugia, 1997) integrated within the WINGX suite of programs (Farrugia, 1999). Crystallographic data for bilicarol D (4), CCDC No. 1575458 have been deposited to the Cambridge Crystallographic Data Centre. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

3.4.1. Crystal data for bilicarol D (4)

\(C_{24}H_{32}O_{13}\). \(\text{CH}_3\text{OH}, M = 726.75, T = 130.0(2) K, \mu = 1.5418 \text{ Å}, \text{Orthorhombic, space group P}_{2}1_{2}1_{2}\), \(a = 9.2192(2), b = 13.5542(2), c = 29.2363(1) \text{ Å}, V = 3652.87(10) \text{ Å}^3\), \(Z = 4, D_c = 1.321 \text{ Mg m}^{-3}\), \(\mu = 0.843 \text{ mm}^{-1}\), \(F(000) = 1544\), crystal size 0.60 \times 0.37 \times 0.03 mm, \(\theta_{\text{max}} = 77.34^\circ\), 63245 reflections measured, 7697 independent reflections (\(R_{\text{int}} = 0.067\)) final \(R = 0.0320 \{2>2\sigma(I)\}, 7262 \text{ data points}\) and \(wR(P) = 0.0822\) (all data), Absolute structure parameter 0.07(6), GOOF = 1.028.

3.5. Leucine transport inhibition assay

The LNCaP human prostate cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The LNCaP cell identity was confirmed by STR profiling in 2014 (Cellbank, Australia). Cells were cultured in RPMI 1640 medium (Life Technologies, Australia) containing 10% (v/v) fetal bovine serum (FBS), penicillin-streptomycin solution (Sigma-Aldrich, Australia) and 1 mM sodium pyruvate (Life Technologies, Australia). Cells were maintained at 37 °C in a fully humidified atmosphere containing 5% CO\(_2\). The \([^{1}H]\)-leucine uptake assay was performed as detailed previously (Wang et al., 2011). Briefly, cells were cultured in 6-well plates in RPMI medium. After collecting and counting, cells (3 \times 10\(^{4}\)/well) were incubated with 0.3 μCi \([^{1}H]\)-leucine (200 nM; PerkinElmer) in leucine-free RPMI media (Invitrogen) with 10% (v/v) dialyzed FBS in the presence of different concentrations of compounds for 15 min at 37 °C. The re-suspended cells were collected, transferred to filter paper using a scintillation mat harvester (PerkinElmer), dried, exposed to scintillation fluid and counts measured using a MicroBeta2 scintillation counter (PerkinElmer). GraphPad Prism 6 was used to determine the IC\(_{50}\) of each compound. Each data point was determined in triplicate, and repeated in three independent experiments. BCH was used as the positive control.
positive control \( IC_{50} = 4060 \pm 1.1 \mu M \) (Wang et al., 2014).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.phytochem.2018.01.009. These data include MOL files and InChIKeys of the most important compounds described in this article.

References


Chapter 4. Dihydro-β-agarofurans from the Australian Endemic Rainforest Plant Denhamia pittosporoides Inhibit Leucine Transport in Prostate Cancer Cells

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Mario Wibowo, Qian Wang, Jeff Holst, Jonathan M. White, Andreas Hofmann, and Rohan A. Davis


Supporting Information can be found in Appendix 3.

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Direct link to publication:
https://doi.org/10.1002/ajoc.201600462

My contribution to the paper involved:
I conducted all the plant extractions, compound isolation experiments, and recorded and analysed the spectroscopic and spectrometric data for all compounds. The biological and X-ray diffraction experiments were performed by our collaborators. I was the major contributor to the preparation of the manuscript.

14 August 2018

Mario Wibowo

14 August 2018

Corresponding author of paper: Rohan A. Davis

14 August 2018

Supervisor: Rohan A. Davis
Abstract: Two previously unknown dihydro-β-agarofuran sesquiterpenoids, denhaminol I (1) and denhaminol J (2), together with four related and known metabolites, 1α,2α,6β,15-tetraacetoxy-9α-benzoyloxy-8-oxodihydro-β-agarofuran (3), wilforosine F (4), 1α,2α,6β,8α,15-pentaacetoxy-9α-benzoyloxydihydro-β-agarofuran (5), and 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran (6), were isolated from the leaves of an Australian rainforest plant, Denhamia pittosporoides. The structures of compounds 1 and 2 were determined by analysis of their 1D/2D NMR and MS data. The absolute configuration of compound 1 was established by single-crystal X-ray diffraction analysis. Compounds 1 and 4 were shown to inhibit leucine transport in the human prostate cancer cell line LNCaP, with IC₅₀ values of 51.5 and 95.5 μM, respectively. Both compounds 1 and 4 were more potent than the L-type amino acid transporter (LAT) family inhibitor 2-aminobicyclo[2.2.1]-heptane-2-carboxylic acid (BCH). This is the first report of dihydro-β-agarofurans from D. pittosporoides.

Dihydro-β-agarofurans are structurally diverse tricyclic sesquiterpenoid polyester natural products that possess the 5,11-epoxy-5β,10α-eudesman-4(14)-ene scaffold. Dihydro-β-agarofurans have been extensively investigated owing to their range of bioactivities and unique structural features, in particular the tetrahydropyran ring, which is not observed in other sesquiterpenoids. The Celastraceae plant family, which is primarily found in tropical and subtropical regions of the world, is regarded as a major source of dihydro-β-agarofurans.[5] Dihydro-β-agarofurans have attracted much attention because they are considered to be privileged structures that exhibit numerous biological activities, owing to their unique molecular skeleton, which is capable of providing ligands for various biological receptors.[1a, 2] Recently, Celastraceae-derived dihydro-β-agarofurans have been reported to display neuroprotective,[3] anti-HIV,[4] anti-Mycobacterium,[5] cancer chemopreventive,[6a, 6b] multidrug-resistance phenotype reversal,[7] and leucine-uptake-inhibitory effects.[6b]

Although Denhamia (Celastraceae) is an endemic Australian rainforest genus that consists of 10 species,[8] there remain a paucity of investigations into their chemistry, with only two phytochemical studies reported to date.[9] Denhamia pittosporoides, also known as “orange boxwood”, grows as a shrub or tree up to 7m tall in eastern Queensland and northeastern New South Wales.[8, 10] Previous phytochemical investigations that were undertaken on the bark of this species led to the isolation of a triterpenoid quinone methide, pristimerin.[9b] In addition, a recent chemical study on the leaves of D. celastroides yielded eight new dihydro-β-agarofurans, denhaminols A–H.[9b]

As part of our ongoing interest in dihydro-β-agarofurans from the Australian Celastraceae plant family,[7b, 9a, 11] we conducted a chemical investigation on the leaves of D. pittosporoides. Herein, we report the isolation and characterization of two new dihydro-β-agarofurans from the leaves of this plant.

The CH₂Cl₂ extract of air-dried and ground leaves of D. pittosporoides was subjected to extensive purification by column chromatography to afford two new dihydro-β-agarofurans, denhaminol I (1) and denhaminol J (2), as well as four known compounds, 1α,2α,6β,15-tetraacetoxy-9α-benzoyloxy-8-oxodihydro-β-agarofuran (3), wilforosine F (4), 1α,2α,6β,8α,15-pentaacetoxy-9α-benzoyloxydihydro-β-agarofuran (5), and 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran (6; Figure 1).
Denhaminol I (1), which was obtained as stable colorless block crystals, was assigned the molecular formula C_{26}H_{32}O_{15}, based on HRMS (ESI+) analysis. The 1H NMR spectrum (Table 1) of compound 1 exhibited signals that were attributed to six methyl protons (δ_H = 1.33, 1.52, 1.55, 1.60, 2.03, and 2.13 ppm), two sets of methylene protons (δ_H = 4.78/5.05 ppm and δ_C = 4.78/5.05 ppm), six methine protons (δ_H = 4.4, 4.15, 5.65, 5.67, 6.45, and 6.47 ppm), and one benzoate group (5 H; δ_C = 165.3, 165.4, and 170.6 ppm). The 13C NMR spectrum (Table 2) and edited HSQC spectrum of compound 1 revealed signals for 28 carbon atoms, which were attributed to six methyl, two methylene, 11 methine, and nine unprotonated carbon atoms. A ketone 13C NMR signal was observed at δ_C = 198.2 ppm, whilst signals at δ_C = 165.3, 169.2, 169.7, and 170.6 ppm indicated the presence of four ester groups in compound 1. These data suggested that compound 1 belonged to the dihydro-β-agarofuran class of compounds.\[^{7b,11}\] The COSY spectrum of compound 1 established the partial structures of the H-1/H-2/H-3/H-4/H-13 and H-6/H-7 atoms (Figure 2). The ketone moiety was located at the C8 position, based on HMBC correlations between the H-6 (δ_H = 6.45 ppm), H-7 (δ_H = 3.09 ppm), and H-9 atoms (δ_H = 5.87 ppm) and the ketone carbon atom (δ_C = 198.2 ppm). Similarly, the positions of the ester groups in compound 1 were determined by using HMBC analysis (Figure 2). A HMBC correlation between the H-9 atom (δ_H = 5.87 ppm) and the ester carbonyl carbon atom at δ_C = 165.3 ppm enabled us to locate the benzoate group at the C9 position, whereas the attachment of three acetoxymieties was assigned based on HMBC correlations between the H-1 (δ_H = 5.65 ppm), H-6 (δ_H = 6.45 ppm), and H-7/15 atoms (δ_H = 4.78/5.05 ppm) and the carbonyl carbon atoms at δ_C = 169.7, 169.2, and 170.6 ppm, respectively. Finally, a hydroxy group was attached to the C2 atom by considering

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**Table 1.** 1H NMR (800 MHz) spectroscopic data for denhaminol I (1) and denhaminol J (2) in CDCl₃.

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[a] Interchangeable signals.

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**Figure 1.** Natural products (1-6) that have been isolated from Denhamia pitoxiporoides.

**Figure 2.** COSY and key HMBC/ROESY correlations for compound 1.
the molecular formula of compound 1 and the downfield resonances of the CH-2 atom (δ_H = 4.15 ppm; δ_C = 69.4 ppm).

The relative configuration of compound 1 was established on the basis of ROESY data (Figure 2) and 1H−1H coupling constants. The β-orientation of the H-1 and H-2 atoms was indicated by the coupling constant between the two atoms (J(H1,H2) = 3.0 Hz), whilst the ROESY spectrum of compound 1 showed correlations between the H-1 and H-8 atoms, thus indicating that these two protons were coplanar. In addition, ROESY correlations between the H-6 and H-2 atoms, indicated that the H-6, CH_3-14, and CH_2-15 atoms all adopted α-orientations. The absolute configuration of compound 1 was defined by single-crystal X-ray diffraction analysis by using a Cu Kα source and unequivocally confirmed the structure of compound 1 as (1R,2S,4R,5S,6R,7R,9S,10S)-1,2,6,15-tetraacetoxy-9-benzoyloxy-8-oxodihydro-β-agarofuran. An ORTEP of denhaminol I (1) is shown in Figure 3.

Denhaminol J (2) was isolated as a white amorphous powder. The HRMS (ESI+) molecular ion peak at m/z = 569.1992 (m/z calcld for C_{50}H_{40}O_{14}Na: 569.1993 [M+Na]^+) indicated that compound 2 was an isomer of compound 1. The 1H and 13C NMR spectra of compound 2 were almost identical to those of compound 1, thus indicating that compound 2 had the same dihydro-β-agarofuran scaffold and the same ester functionalities as compound 1. However, the 1H NMR resonance of the H-6 proton was shifted upfield from δ_H = 6.45 ppm in compound 1 to δ_H = 5.28 ppm in compound 2, which supported the assignment of a hydroxy group on the C6 atom, rather than an acetoxy group. HMBC correlations between the H-2 (δ_H = 5.39 ppm) and methyl atoms (singlet, δ_H = 2.12 ppm) and an ester carbonyl carbon atom at δ_C = 169.9 ppm indicated the presence of an acetoxy group on the C2 atom (Figure 4). The 2D NMR data further confirmed the position of the remaining ester groups in compound 2. The relative configuration of compound 2 was shown to be identical to that of compound 1 following ROESY analysis. On the basis of biogenetic considerations and comparison of the chiroptical and spectroscopic data of compounds 1 and 2, the absolute configuration of compound 2 was established as (1R,2S,4R,5S,6R,7R,9S,10S)-1,2,6,15-tetraacetoxy-9-benzoyloxy-8-oxodihydro-β-agarofuran.

The chemical structures of the known dihydro-β-agarofurans, 1α,2α,6β,15-tetraacetoxy-9α-benzoyloxy-8-oxodihydro-β-agarofuran (3),[10] wilforsinine F (4),[11] 1α,2α,6β,8α,15-pentaacetoxy-9α-benzoyloxydihydro-β-agarofuran (5),[12] and 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran (6),[13] were determined by comparison of the NMR, MS, and specific rotation data with literature values. Herein, we also include the electronic circular dichroism (ECD) data of known compounds 3–6, which were not reported in the original publications. Comparison of the ECD spectra (Figure 5) and specific rotations of com-

![Figure 3. ORTEP of a single molecule of denhaminol I (1). Hydrogen atoms are omitted for clarity; thermal ellipsoids are set at 30% probability.](image)

![Figure 4. Selected HMBC correlations for compound 2.](image)

![Figure 5. ECD spectra of compounds 1–3 in MeOH.](image)
port (Figure 6). Both compounds 1 and 4 showed leucine-transport inhibition that was more potent than the L-type amino acid transporter (LAT) family inhibitor 2-aminobicyclo[2.2.1]-heptane-2-carboxylic acid (BCH), which had an IC_{50} value of 4060 μM in LNCaP cells.\(^{[16]}\) In LNCaP cells, leucine transport is mainly mediated by LAT3 and is required for the growth and progression of prostate cancer.\(^{[17]}\) Several studies have isolated and purified new leucine-transport inhibitors from various plants, such as Pittosporum venulosum and Maytenus biolocularis.\(^{[7b, 16, 18]}\) Our study has identified new dihydro-β-agarofurans from D. pittosporoides, which can be used as leucine-transport inhibitors in the treatment of prostate cancer.

All of the dihydro-β-agarofurans (1–6) that are reported in this study have been added to the Davis Open-Access Compound Library, which is housed at Comounds Australia, Griffith University, and is a resource that can be accessed for drug discovery and chemical biology research.\(^{[19]}\)

In summary, this report describes the first isolation and structure elucidation of two new dihydro-β-agarofurans, denhaminol I (1) and denhaminol J (2), together with four known compounds (3–6), from the leaves of D. pittosporoides. Compounds 1 and 4 were found to inhibit leucine transport in the human prostate cancer cell line LNCaP, with IC_{50} values of 51.5 and 95.5 μM, respectively.

### Experimental Section

#### Plant Materials

Leaves of Denhamia pittosporoides (F. Muell.; Celastraceae) were collected from Bunya, Queensland, Australia on 8 June, 2015. A voucher specimen (RAD078) has been deposited at the Eskitis Institute of Griffith University, Brisbane, Australia.

#### Extraction and Isolation

The leaves of D. pittosporoides (10 g) were air-dried, ground, and exhaustively extracted with CH\(_2\)Cl\(_2\) (2 x 250 mL). The crude extract (350 mg) that was obtained after evaporation under reduced pressure was subjected to flash column chromatography on silica gel (column dimensions: 25 x 50 mm; n-hexane/EtOAc, stepwise from 0:1 to 0:1) to afford 97 separate fractions (ca. 10 mL each). Similar fractions were combined following TLC analysis (n-hexane/EtOAc) to give 15 fractions (F1–F15).

Fraction F8 (7.6 mg) was preadsorbed onto C\(_18\)–bonded silica (ca. 1 g), which was packed into a guard cartridge and attached to a semipreparative C\(_18\) HPLC column. A linear gradient from 60% MeOH (0.1% trifluoroacetic acid, TFA)/40% water (0.1% TFA) to 85% MeOH (0.1% TFA)/15% water (0.1% TFA) at a flow rate of 4 mL/min over 60 min was used to afford 1α,2α,6β,15-tetraacetoxy-9β-benzoxyldihydro-β-agarofuran (6; 1.4 mg, t\(_R\) = 40–41 min, 0.014% dry weight).

Fraction F10 (27.9 mg) was preadsorbed onto phenyl-bonded silica (ca. 1 g), which was packed into a guard cartridge and attached to a semipreparative phenyl-hexyl HPLC column. Initially, isocratic conditions of 50% MeOH (0.1% TFA)/50% water (0.1% TFA) were employed for 5 min, followed by a linear gradient to 100% MeOH (0.1% TFA) over 45 min, and finally isocratic conditions of 100% MeOH (0.1% TFA) for 10 min, all at a flow rate of 4 mL/min. In total, 60 fractions (60 x 1 min) were collected from the start of the HPLC run. The fractions that eluted at 38–39 min contained 1α,2α,6β,15-tetraacetoxy-9β-benzoxyloxy-8-oxodihydro-β-agarofuran (3; 12.4 mg, 0.122% dry weight).

Fraction F11 (21.1 mg) was further purified according to the same HPLC procedure as described above for Fraction F10. Following this separation process, the fraction that eluted at 33 min was subjected to chromatography on a phenyl-hexyl HPLC column with a linear gradient from 60% MeOH (0.1% TFA)/40% water (0.1% TFA) to 90% MeOH (0.1% TFA)/10% water (0.1% TFA) at a flow rate of 4 mL/min over 60 min to give pure denhaminol I (1; 3.5 mg, t\(_R\) = 28–30 min, 0.035% dry weight). The fraction that eluted at 39 min was purified on a semipreparative C\(_18\) HPLC column with a linear gradient from 60% MeOH (0.1% TFA)/40% water (0.1% TFA) to 85% MeOH (0.1% TFA)/15% water (0.1% TFA) at a flow rate of 4 mL/min over 60 min to afford pure denhaminol I (1; 3.5 mg, t\(_R\) = 30 min, 0.016% dry weight).

Fraction F12 (45.9 mg) was separated according to the same HPLC procedure as described above for Fraction F10. The fractions that eluted at 31–33 min (35 mg) from this purification step were pooled and subjected to chromatography on a semipreparative biphenyl HPLC column with a linear gradient from 60% MeOH (0.1% TFA)/40% water (0.1% TFA) to 90% MeOH (0.1% TFA)/10% water (0.1% TFA) at a flow rate of 4 mL/min over 60 min to afford pure denhaminol J (2; 3.7 mg, t\(_R\) = 22 min, 0.011% dry weight), semipure denhaminol J (3.7 mg, t\(_R\) = 35 min), and semipure denhaminol I (22.0 mg, t\(_R\) = 36–40 min).

The fraction eluting at 35 min (3.7 mg) was further purified on a biphenyl HPLC column under isocratic conditions of 65% MeOH (0.1% TFA)/35% water (0.1% TFA) to give denhaminol J (2; 1.1 mg, t\(_R\) = 23–27 min, 0.011% dry weight), whilst the fractions eluting between 36–40 min (22.0 mg) were combined and purified on a semipreparative C\(_18\) HPLC column with a linear gradient from 60% MeOH (0.1% TFA)/40% water (0.1% TFA) to 90% MeOH (0.1% TFA)/15% water (0.1% TFA) at a flow rate of 4 mL/min over 60 min to afford pure denhaminol J (2; 8.2 mg, t\(_R\) = 22 min, 0.082% dry weight).

Denhaminol I (1): stable colourless block crystals (EtOH/CH\(_2\)Cl\(_2\), 9:1); m.p. 246–248°C; [α]_D=+16.7 (c = 0.18, MeOH); \(^{1}H\) NMR (CDCl\(_3\), 800 MHz): see Table 1; \(^{13}C\) NMR (CDCl\(_3\), 200 MHz): see Table 2; UV/
Vis (MeOH): $\lambda_{max}$(log e) = 231 (4.72), 275 nm (3.62); IR (UATR): $\nu_{max}$ = 3535, 1733, 1722, 1366, 1227, 1108, 1034, 709 cm$^{-1}$; ECD (MeOH): $\lambda_{max}(\Delta\lambda)$ = 231 (0.18), 248 (0.82), 290 nm (4.57 mol$^{-1}$ cm$^{-1}$); MS (ESI+): m/z (%): 569 (100) [M+Na]$^+$; HRMS (ESI+): m/z calcd for C$_{32}$H$_{38}$O$_{34}$Na: 569.1938 [M+Na]$^+$; found: 569.1895.

\textbf{Denhamiol J (2):} white amorphous powder; [\alpha]$_D^{20}$ = +4.8 (c = 0.05, MeOH); $\delta$H NMR (CDCl$_3$, 500 MHz): see Table 1; $\delta$C NMR (CDCl$_3$, 125 MHz); see Table 2; UV/Vis (MeOH): $\lambda_{max}(log e)$ = 231 (4.05), 274 nm (2.90); IR (UATR): $\nu_{max}$ = 3517, 1743, 1719, 1368, 1224, 1115, 1044, 714 cm$^{-1}$; ECD (MeOH): $\Delta\lambda_{max}$ (1.68), 289 nm ($\pm$0.18 1 mol$^{-1}$ cm$^{-1}$); MS (ESI+): m/z (%): 569 (100) [M+Na]$^+$; HRMS (ESI+): m/z calcd for C$_{32}$H$_{38}$O$_{34}$Na: 569.1934 [M+Na]$^+$; found: 569.1929.

$(1R,2S,4R,5S,6R,7R,9S,10S)$-1,6,15-Triacetoxy-9-benzoyloxy-2-hydroxy-8-oxodihydro-$\beta$-agarofuran (3): colorless gum; $[\alpha]_{D}^{20}$ = +3.2 (c = 0.6, MeOH); lit. $[\alpha]_{D}^{20}$ = 3.4.2 (c = 0.01, MeOH);$^{[1]}$ ECD (MeOH): $\Delta\lambda_{max}$ (247 (0.29), 292 nm (4.485); MS(ESI+): m/z: 611 [M+Na]$^+$.

\textbf{Wilforssinine F (4):} white amorphous powder; $[\alpha]_{D}^{20}$ = +16.7 (c = 0.2, MeOH); lit. $[\alpha]_{D}^{20}$ = 5.1; MeOH);$^{[2]}$ ECD (MeOH): $\Delta\lambda_{max}$ (231 (4.16), 256 nm (0.32); MS(ESI+): m/z: 563 [M+Na]$^+$.

\textbf{X-ray Diffraction Analysis}

Intensity data for compound 1 were collected on an Oxford Diffraction SuperNova CCD diffractometer by using Cu Kα radiation; the temperature during data collection was maintained at 130.0(1) K by using an Oxford Cryosystems cooling device. The structure was solved by using direct methods and difference Fourier Synthesis.$^{[29]}$ Hydrogen atoms that were bound to carbon atom were placed at their idealized positions by using appropriate HFIX instructions in SHELXL, and included in subsequent refinement cycles. The hydrogroup atom that was bound to the oxygen atom was located by using difference Fourier maps and refined freely with isotropic displacement parameters. Thermal ellipsoid plots were generated by using the ORTEP-3 program$^{[30]}$ and integrated within the WINGX suite of programs.$^{[31]}$

CCDC 1494636 contains the supplementary crystallographic data for this paper. These data are provided free of charge by The Cambridge Crystallographic Data Centre.

Crystal data for 

\textbf{Leucine Transport Inhibition Assay}

Human prostate cancer cell line LNCaP was purchased from American Type Culture Collection (Manassas, VA). We confirmed the identity of the LNCaP cells by STR profiling in 2014 (CellBank, Australia).

\textbf{Acknowledgements}

The authors acknowledge the National Health and Medical Research Council (NHMRC) for financial support (Grant No. APP1024314 to R.A.D.) and thank the Australian Research Council (ARC) for support towards purchasing NMR and MS equipment (Grant Nos LE0668477 and LE0237908) and for financial support (Grant No. LP120200339 to R.A.D.). This work was supported by grants from Movember through the Prostate Cancer Foundation of Australia (Grant No. Y10813 to Q.W.) and the Australian Movember Revolutionary Team Award Targeting Advanced Prostate Cancer (Grant No. MRTA1 to J.H. and Q.W.); Cancer Council NSW (Grant No. APP1080503) to J.H.; and The University of Sydney through a Sydney Medical School PhD ECR Supervisor Grant to Q.W. and a HMR+ Implementation Grant to J.H. The authors thank R. Whyte, M. Novak, and R. Harisson from Save Our Waterways (SOWN) for performing the plant collection and identification. W. Loa-Kum-Chung is acknowledged for performing the HRMS measurements. M.W. thanks Griffith University for the provision of PhD scholarships (GUPRS and GUIPRS).


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Chapter 5. Celastrofurans A–G: Dihydro-β-agarofurans from the Australian Rainforest Vine Celastrus subspicata and Their Inhibitory Effect on Leucine Transport in Prostate Cancer Cells

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Mario Wibowo, Qian Wang, Jeff Holst, Jonathan M. White, Andreas Hofmann, and Rohan A. Davis

Celastrofurans A–G: Dihydro-β-agarofurans from the Australian Rainforest Vine Celastrus subspicata and Their Inhibitory Effect on Leucine Transport in Prostate Cancer Cells. *Journal of Natural Products* 2017, 80, 1918–1925.

Supporting Information can be found in Appendix 4.

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My contribution to the paper involved:
I conducted all the plant extractions, compound isolation experiments, and recorded and analysed the spectroscopic and spectrometric data for all compounds. The biological and X-ray diffraction experiments were performed by our collaborators. I was the major contributor to the preparation of the manuscript.

14 August 2018

Mario Wibowo

Corresponding author of paper: Rohan A. Davis

14 August 2018

Supervisor: Rohan A. Davis
Celastrofurans A–G: Dihydro-β-agarofurans from the Australian Rainforest Vine *Celastrus subspicata* and Their Inhibitory Effect on Leucine Transport in Prostate Cancer Cells

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**Supporting Information**

**ABSTRACT**: Seven new dihydro-β-agarofurans, celastrofurans A–G (1–7), along with two known secondary metabolites, 9β-benzozyloxy-1α-furoxoyldihydro-β-agarofuran (8) and (1R,2R,4R,5S,7R,9S,10R)-2-acetoxy-9-benzozyloxy-1-furoxoyldihydro-β-agarofuran (9), were obtained from the leaves of the Australian rainforest vine, *Celastrus subspicata*. The structures of the new compounds were determined by detailed spectroscopic (1D/2D NMR) and MS data analysis. The absolute configurations of compounds 1–4 were defined by ECD and single-crystal X-ray diffraction studies. All compounds were found to exhibit inhibitory activity on leucine transport in the human prostate cancer cell line LNCaP with IC₅₀ values ranging from 7.0 to 98.9 μM. Dihydro-β-agarofurans 1–9 showed better potency than the L-type amino acid transporter (LAT) family inhibitor, 2-aminobicyclo[2.2.1]-heptane-2-carboxylic acid (BCH).

**RESULTS AND DISCUSSION**

The CH₂Cl₂ extract of air-dried and ground leaves of *C. subspicata* was subjected to extensive chromatography to yield leaves of *Celastrus subspicata* Hook. (Celastraceae), which had never been analyzed before. *Celastrus subspicata* is a vine that is found in the rainforests of Queensland and New South Wales. Historically, the genus *Celastrus* has been broadly used in Chinese indigenous medicine. While plants of the genus *Celastrus* have been reported to produce sesquiterpenoids, diterpenoids, triterpenoids, and flavonoids, the chemistry of *C. subspicata* has been superficially explored, with only one phytochemical study reported to date. A previous study on the seeds of this plant reported the isolation of two new dihydro-β-agarofurans (10 and 11). Herein, we report the isolation and structure elucidation of seven new (1–7) and two known (8 and 9) dihydro-β-agarofurans as well as their leucine transport inhibitory effects on the human prostate cancer cell line, LNCaP.
seven new dihydro-β-agarofuran, celastrofurans A–G (1–7) and two known analogues; 9β-benzyloxy-1α-furoloxydihydro-β-agarofuran (8) and (1R,2R,4R,5S,7R,9S,10R)-2-acetoxy-9-benzoyloxy-1-furoloxydihydro-β-agarofuran (9) (Figure 1).

Celastrofuran A (1) was isolated as a white amorphous powder with a molecular formula of C_{29}H_{44}O_{8} as suggested by the (+)-HRESIMS data (m/z 485.2529 [M + Na]^+), calcd 485.2298. The 1H NMR spectrum (Table 1) of 1 showed signals of four methyl protons (δH 1.12, 1.23, 1.38, and 1.39), four sets of methylene protons (δH 1.48/2.32, 1.72/1.99, 2.09/2.21, and 2.04/2.09), four methane protons (δH 1.91, 2.01, 5.04 and 5.70), and two benzoate groups (10H, δH 7.24, 7.31, 7.41, 7.46, 7.58, and 7.84). The 13C NMR (Table 2) and edited HSQC spectra revealed the presence of 29 carbons corresponding to four methyl, four methylene, 14 methine, and seven nonprotonated carbons. The 13C NMR resonances at δC 165.75 and 165.81 suggested the presence of two ester functionalities in 1, which was further supported by the IR data that showed an absorption at 1711 cm⁻¹. These data indicated that compound 1 belonged to the dihydro-β-agarofuran class of compounds.10,11 The COSY spectrum (Figure 2) of 1 established the fragments of H-1/H-2/H-3/H-4/H-14 and H-6/H-7/H-8/H-9. The core structure of dihydro-β-agarofuran in 1 was determined by HMBC correlations (Figure 3).
Table 2. $^{13}$C NMR (200 MHz) Spectroscopic Data of Celastrofurans A–G (1–7) in CDCl$_3$.$^a$

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$^a$NMR data of the ester groups are provided in the Experimental Section.

Figure 2. COSY and selected HMBC/ROESY correlations for celastrofuran A (1).

The two benzoate groups were located on C-1 and C-9 based on HMBC correlations from H-1 (δ$_H$ 5.70) and H-9 (δ$_H$ 5.04) to the ester carbonyl carbons at δ$_C$ 165.75 and 165.81, respectively.

The relative configuration of 1 was determined based on the analysis of ROESY data (Figure 2) and $^1$H–$^1$H coupling constants. The β-orientation of H-1 was suggested by the large coupling constants between H-1 and H-2 (J$_{1,2}$ = 12.0 Hz), while the ROESY correlations between H$_2$-14 and H$_2$-15 as well as between H-9 and H$_2$-15 indicated that CH$_3$-14, CH$_2$-15, and H-9 were all α-oriented. The absolute configuration of 1 was resolved by the dibenzoate chirality method, which has been used routinely to determine the absolute configuration of dihydro-β-agarofuranins bearing two benzoate chromophores. The ECD spectrum of 1 (Figure 4) exhibited a Davydov-type splitting with a positive first Cotton effect at 240 nm (Δε = +4.08) and a negative second Cotton effect at 223 nm (Δε = -2.87) due to the coupling of the two benzoates at C-1α and C-9β. Thus, the absolute configuration of 1 was assigned as 1S,4R,5S,7R,9S,10S-1,9-dibenzoyloxydihydro-β-agarofuran.

Celastrofuran B (2) was purified as stable colorless crystals and was assigned the molecular formula C$_{11}$H$_{14}$O$_4$ following analysis of the (+)-HRESIMS data. The $^1$H and $^{13}$C NMR spectra of 2 were similar to those of 1, except for the presence of an acetate group (δ$_H$ 1.79 and δ$_C$ 21.2, 170.9) and an additional oxygenated methine (δ$_H$ 5.33 and δ$_C$ 69.5) in 2. Detailed analysis of the HMBC spectrum revealed that the acetate group was located at C-2 in 2, since correlations from both H-2 (δ$_H$ 3.33) and the methyl protons at δ$_H$ 1.79 to an ester carbonyl carbon at δ$_C$ 170.9 were observed. The relative configuration of 2 was assigned following analysis of $^1$H–$^1$H coupling constants and the ROESY spectrum. A large coupling constant (J$_{1,2}$ = 10.6 Hz) between H-1 and H-2 indicated the β-orientation of H-1 and the α-orientation of H-2. The ROESY correlations between H$_2$-14 and H$_2$-15, and between H-9 and H$_2$-15, indicated that CH$_3$-14, CH$_2$-15, and H-9 were cofacial. The structure of 2 was confirmed by a single-crystal X-ray diffraction study (Figure 3), which also established its absolute configuration as 1R,2R,4R,5S,7R,9S,10R-2-acetoxy-1,9-dibenzoyloxydihydro-β-agarofuran.

The molecular formula of compound 3 was determined as C$_{11}$H$_{14}$O$_4$ based on the (+)-HRESIMS data. The $^1$H and $^{13}$C NMR spectra of 3 were very similar to those of 2. Comparison of the NMR and MS data between 2 and 3 revealed that both compounds had the same ester substitution, but the MS data indicated that the latter natural product contained a –CHO– moiety. The hydroxy group was positioned at C-8 based on the deshielded resonances of CH-8 (δ$_H$ 4.12 and δ$_C$ 72.8), and was further supported following detailed HMBC analysis (see Supporting Information). The α-orientation of OH-8 was assigned by the ROESY correlation between H-8 and H$_2$-12. The ECD spectrum of 3 (Figure 4) exhibited a positive Cotton effect at 241 nm (Δε = +10.58) and a negative Cotton effect at 221 nm (Δε = -4.37) due to the coupling of the two benzoate chromophores at C-1α and C-9β. Therefore, the structure of 3
Celenofuran E (5), a stable colorless gum, was found to possess the molecular formula C_{20}H_{20}O_{10} following interpretation of its (+)-HRESIMS and NMR data. The H and ^{13}C NMR data of 5 closely resembled those of 4. However, NMR data comparison showed that the C-8 ketone functionality in 4 was reduced to a secondary hydroxy in 5. The presence of an additional oxymethine signal (CH-8, δ_{H} 4.45 and δ_{C} 68.2) and HMBC correlations (Figure 6) from H-6, H-7, and H-9 to C-8 (δ_{C} 68.2) further supported the presence of the secondary hydroxy group at C-8 in 5. Analysis of the ROESY spectrum (Figure 6) and H-H coupling constants allowed determination of the relative configuration of 5. A large coupling constant (J_{H1,2} = 10.3 Hz) between H-1 and H-2 indicated that both protons were axially oriented. The β-orientation of H-8 and H-9 was suggested by a small coupling (J_{H8,9} = 4.6 Hz) between H-8 and H-9. This was further confirmed by ROESY correlations from both H-8 and H-9 to H_{21}-12. A ROESY correlation between H_{1}-14 and H_{2}-15 revealed that CH_{14}-14 and CH_{21}-15 were α-oriented. Consequently, the structure of 5 was established as 2β-acetoxy-9α-benzoyloxy-1α-furoxyloxy-8α-hydroxydihydro-β-agarofuran.

The molecular formula of compound 6 was assigned as C_{21}H_{20}O_{10} by (+)-HRESIMS and ^{13}C NMR data analysis. The NMR spectra of 6 showed it to have similar features to those of 5, with the only difference being the replacement of the benzoate group at C-9 in 5 by a furoate group in 6. This was supported by HMBC correlations (Figure 5) from H-1 (δ_{H} 5.86) to the carbonyl carbon of the furoate group at δ_{C} 161.3. In addition, the secondary hydroxy group in 3 was oxidized to a carbonyl (C-8) in 4. HMBC correlations from H-6, H-7, and H-9 to a carbonyl carbon at δ_{C} 205.6 further confirmed the presence of a ketone functionality at C-8 in 4. The relative configuration of 4 was ascertained to be the same as that of 3 following ROESY data (Figure 5).
7 following analysis of $^1$H–$^1$H coupling constants and the ROESY spectrum. Therefore, the structure of compound 7 was elucidated as 2$\alpha$,9$\alpha$-diacetoxy-9$\alpha$-benzoyloxy-1$\alpha$-furoxyloxy-dihydro-$\beta$-agarofuran.

The known compounds 9$\beta$-benzoyloxy-1$\alpha$-furoxyloxydihydro-$\beta$-agarofuran (8) and (1R,2R,4R,5S,7R,9S,10R)-2-acetoxy-9$\beta$-benzoyloxy-1$\alpha$-furoxyloxydihydro-$\beta$-agarofuran (9) were identified by comparison of their spectroscopic data with those reported in the literature.\textsuperscript{20,21} Compound 8 was previously isolated from the root bark of Oyster lanceolata (Santalaceae), while compound 9 was obtained originally from the fruits of Maytenus jelskii (Celastraceae). It is worth mentioning that compound 9 has been reported to block P-glycoprotein in human MDRI cells.\textsuperscript{22} Herein, the ECD data are included for the known compounds 8 and 9, which were not reported in the original papers. Comparison of ECD spectra (Figure 4) and the specific rotations of compounds 1 and 8 showed that both compounds had identical absolute configurations. Thus, the structure and absolute configuration of compound 8 were defined as (1S,4R,5S,7R,9S,10S)-9-benzoyloxy-1$\alpha$-furoxyloxydihydro-$\beta$-agarofuran. Previous studies on the seeds of C. subspicata have led to the isolation of 10 and 11,\textsuperscript{13} but neither of these compounds was identified during our studies on the leaves of this vine.

Due to our previous discovery of dihydro-$\beta$-agarofurans that inhibited leucine transport,\textsuperscript{10,11} compounds 1–9 was evaluated for their effects on leucine transport in the LNCaP human prostate cancer cell line. All compounds inhibited leucine uptake in LNCaP cells, with IC<sub>50</sub> values ranging from 7.0 to 98.9 μM (Table 3 and Figure 7), which was lower than the currently utilized LAT family inhibitor BCH (IC<sub>50</sub> = 4060 μM).\textsuperscript{22}

Comparison of the leucine uptake inhibition of compounds 1–3, 6, and 9 showed that compounds without a hydroxy moiety at C-8 (1, 2, 8, and 9) were more active than the natural product 3, which has C-8 hydroxylation. This indicated that the presence of OH-8 decreased the leucine uptake inhibition. Furthermore, substituting the C-8 methylene unit in 9 with a ketone functionality in 4 resulted in a 2.6-fold decrease in the activity. Compounds 5–7 also exhibited leucine uptake inhibition, with 7 being the most active in this series with an IC<sub>50</sub> value of 15.2 μM. These data suggested that for this particular agarofuran series acetylation rather than hydroxylation at C-8 conferred better activity. For example, replacing the hydroxy group (OH-8) of 5 with an acetoxy group in 7 resulted in a 6.5-fold increase in leucine uptake activity. Moreover, the minimal difference between the activity of 5 and 6 indicated that the type of ester substitution for this series had no profound effect on bioactivity. In conclusion, these studies identified celastrol B (2) as the most active compound that inhibited leucine uptake, with an IC<sub>50</sub> value of 7.0 μM (Figure 7). This activity is comparable or better than the recently reported natural products from the plants Pittosporum venulosum, Maytenus bicornularis, and Denhamia pittsperoides.\textsuperscript{19,11,22,23} These findings warrant future investigation on how these compounds inhibit leucine uptake in cancer cells.

### Table 3. Leucine Uptake Inhibition of Compounds 1–9 in the LNCaP Cell Line

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</tr>
<tr>
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### EXPERIMENTAL SECTION

#### General Experimental Procedures.

Melting points were measured using a Cole-Parmer melting point apparatus and are uncorrected. Specific rotations were determined on a JASCO P-1020 polarimeter. UV spectra were recorded using a JASCO V-650 UV/vis spectrophotometer. ECD spectra were obtained on a JASCO J-715 spectropolarimeter and processed using the software SDAR.\textsuperscript{15} IR data were acquired using an attached Universal Attenuated Total Reflectance (ATR) two module on a PerkinElmer spectrophotometer. NMR spectra were recorded on a Bruker AVANCE HDX 800 MHz NMR spectrometer equipped with a TCI cryoprobe at 25 °C. The 1H and 13C NMR chemical shifts were referenced to the solvent peaks for CDCl<sub>3</sub> at δ<sub>H</sub> 7.26 and δ<sub>C</sub> 77.16, respectively. LRESIMS data were recorded on a Waters ZQ ESI mass spectrometer. HRESIMS data were acquired on a 12 T SOLAniX XR FT-ICR-MS. Alltech Davison 30–40 μm 60 Å Si gel packed into an open glass column (25 × 130 mm) was used for flash column chromatography. Thin-layer chromatography (TLC) and preparative TLC were carried out on Merck Si gel 60 F<sub>254</sub> precoated aluminum plates and were observed using UV light. Activon phenyl-bonded or Altech C<sub>18</sub>-bonded Si (35–75 μm, 150 Å) was used for preadsorption work, and the resulting material was packed into an Alltech stainless steel guard cartridge (10 × 30 mm) prior to HPLC separations. A Waters 600 pump fitted with a Waters 996 photodiode array detector and Gilson 717-plus autosampler was used for semipreparative HPLC separations. Phenomenex Luna S 5 μm 90–110 Å phenyl-bonded (250 × 10 mm), Phenomenex Luna S 5 μm 90–110 Å C<sub>18</sub> (250 × 10 mm), and Betasil S 5 μm 100 Å C<sub>18</sub> (150 × 21.2 mm) columns were used for semipreparative HPLC separations. A Fritsch Universal Cutting Mill Pulverisette 19 was used to grind the air-dried plant material. An
Edwards Instrument Company Bioline orbital shaker was used for plant extraction. All solvents used for chromatography, specific rotation, ECD, UV, and MS were Honeywell Bardick & Jackson (B&J) HPLC grade. H₂O was Millipore Mili-Q, PF filtered. All compounds were analyzed for purity by ¹H NMR spectroscopy and shown to be >95%, unless otherwise stated. NMR spectra were processed using MestReNova version 11.0.

**Plant Material.** Leaves of *Celastrus subspicata* were purchased from Borungbar Rainforest Nursery (Borrungbar, New South Wales, Australia) in March 2016. A voucher specimen (RAD079) has been deposited at the Griffith Institute for Drug Discovery, Griffith University, Brisbane, Australia.

**Extraction and Isolation.** The air-dried and ground leaves of *C. subspicata* (20 g) were extracted with CHCl₃ (2 × 250 mL) at room temperature. The organic solvent was removed under reduced pressure to afford 1.9 g of CHCl₃ extract. The extract was chromatographed on a Si gel flash column using a stepwise gradient solvent system of n-hexane/EtOAc (100% n-hexane to 100% EtOAc) to give 89 fractions (20 mL each). Similar fractions were combined following (+)-LR/RESIMS analysis to give 22 fractions (F1-F22). Fraction F3 (82.1 mg) was preadsorbed to phenyl-bonded Si gel (+1 g), packed into a guard cartridge, and attached to a semi-preparative phenyl-hexyl HPLC column. A linear gradient solvent system of 70% MeOH/H₂O to 95% MeOH/H₂O at a flow rate of 4 mL/min over 60 min was then employed to afford celastrol A (1, 3.6 mg, tₚ 40–41 min, 0.018% dry wt). Fraction F4 (64.9 mg) was preadsorbed to phenyl-bonded Si gel (+1 g), packed into a guard cartridge, and attached to a semi-preparative phenyl-hexyl HPLC column. A linear gradient solvent system of 60% MeOH/H₂O to 95% MeOH/H₂O at a flow rate of 4 mL/min over 60 min was then employed to furnish compound 8 (10.8 mg, tₚ 43–44 min, 0.054% dry wt). Fraction F7 (63.7 mg) was preadsorbed to phenyl-bonded Si gel (+1 g), packed into a guard cartridge, and attached to a semi-preparative phenyl-hexyl HPLC column. A linear gradient solvent system of 55% MeOH/H₂O to 100% MeOH/H₂O at a flow rate of 4 mL/min over 60 min was then employed to afford celastrol B (2, 19.9 mg, tₚ 45–46 min, 0.0995% dry wt). Fraction F9 (63.7 mg) was preadsorbed to C₁₈-bonded Si gel (+1 g), packed into a guard cartridge, and attached to a semi-preparative C₁₈ HPLC column (250 × 10 mm). A linear gradient solvent system of 60% MeOH/H₂O to 95% MeOH/H₂O at a flow rate of 4 mL/min over 60 min was then employed to afford compound 9 (6.1 mg, tₚ 33 min, 0.0305% dry wt). Fraction F12 (60.0 mg) was further purified using the same protocol detailed for the separation of fraction F7 to afford celastrol G (7, 5.9 mg, tₚ 39 min, 0.0295% dry wt). Fraction F13 (188.6 mg) was preadsorbed to C₁₈-bonded Si gel (+1 g), packed into a guard cartridge, and attached to a semi-preparative C₁₈ HPLC column (150 × 2.12 mm). A linear gradient solvent system of 50% MeOH/H₂O to 95% MeOH/H₂O at a flow rate of 9 mL/min over 60 min was then employed to yield celastrol C (3, 2.6 mg, tₚ 33 min, 0.013% dry wt) and celastrol F (6, 91.6 mg, tₚ 28–29 min, 0.0458% dry wt). Fraction F14 (62.7 mg) was preadsorbed to phenyl-bonded Si gel (+1 g), packed into a guard cartridge, and attached to a semi-preparative phenyl-hexyl HPLC column. A linear gradient solvent system of 45% MeOH/H₂O to 90% MeOH/H₂O at a flow rate of 4 mL/min over 60 min was then run. Sixty fractions were collected from the start of the HPLC run. Fractions eluting at 47–48 min were pooled and further purified using a semi-preparative C₁₈ HPLC column (250 × 10 mm). A linear gradient solvent system of 50% MeOH/H₂O to 95% MeOH/H₂O at a flow rate of 4 mL/min over 60 min was run to afford celastrol D (4, 5.9 mg, tₚ 34–35 min, 0.0295% dry wt). Fraction F16 (61.3 mg) was preadsorbed to C₁₈-bonded Si gel (+1 g), packed into a guard cartridge, and attached to a semi-preparative C₁₈ HPLC column. A linear gradient solvent system of 50% MeOH/H₂O to 88% MeOH/H₂O at a flow rate of 4 mL/min over 60 min was then employed. Sixty fractions were collected from the start of the HPLC run. The fraction eluting at 32 min (13.9 mg) was further purified using preparative TLC (1% MeOH/CH₂Cl₂) to give celastrol F (6, 7.7 mg, 0.0385% dry wt).
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Notes
The authors declare no competing financial interest.

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REFERENCES
Chapter 6. Using UHPLC-MS Profiling for the Discovery of New Dihydro-β-agarofurans from Australian Celastraceae Plant Extracts

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Mario Wibowo, Paul I. Forster, Gordon P. Guymer, Andreas Hofmann, and Rohan A. Davis

UPLC-MS Profiling of Australian Celastraceae Plants for New Dihydro-β-Agarofurans
Journal of Chromatography B: Manuscript in Preparation, to be submitted before 9 November 2018.
Supporting Information can be found in Appendix 5.

My contribution to the paper involved:

I conducted all the plant extractions, compound isolation experiments, and recorded and analysed the spectroscopic and spectrometric data for all compounds. I was the major contributor to the preparation of the manuscript.

Mario Wibowo  
14 August 2018

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14 August 2018
Using UHPLC-MS profiling for the discovery of new dihydro-β-agarofurans from Australian Celastraceae plant extracts

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ABSTRACT
An analytical method using UHPLC-MS was developed and applied to 16 crude CH$_2$Cl$_2$ extracts from Australian Celastraceae plants; the endemic plant materials were accessed from Griffith University’s NatureBank resource and included bark, fruit, leaf, seven roots, twigs and mixed samples all of which were collected from Queensland, Australia. The generated UHPLC-MS data were analysed and dereplicated using the scientific databases Dictionary of Natural Products and SciFinder Scholar in order to potentially identify new dihydro-β-agarofurans from Celastraceae plants. These investigations led to the large-scale extraction and isolation work on a prioritised fruit sample that belonged to the rainforest plant, Denhamia celastroides. Chemical investigations resulted in the purification of four new natural products, denhaminols O–R (1–4), along with the related and known compound, denhaminol G (5). The structures of all the new compounds were determined via detailed analysis of NMR and MS data.
1. Introduction

Dihydro-β-agarofurans are a class of structurally unique polyoxygenated tricyclic sesquiterpenoids, which incorporate a trans-decalin and a tetrahydrofuran, and are commonly found in the Celastraceae plant family.\(^1\) This class of natural products has gained much attention due to various promising bioactivities, such as multidrug resistance reversal,\(^2\) antitumor-promotion,\(^3\) acetylcholinesterase inhibition,\(^4\) antifungal,\(^5\) α-glucosidase inhibition,\(^6\) antiplasmodial,\(^7\) and leucine uptake inhibition.\(^8\) The biological effects of dihydro-β-agarofurans are related not only to several stereocentres, but also to acyl groups attached to the tricyclic core scaffold. One of our group’s current research interests is the isolation of new natural products (i.e dihydro-β-agarofurans) from Australian Celastraceae native plants and their biological evaluations.\(^8\)-\(^11\)

Within the workflow of natural product research, the time-consuming re-isolation of previously identified compounds presents a major obstacle and can significantly delay discovery efforts.\(^12\) However, rapid and detailed dereplication methodologies can solve this problem. In the context of our continuing interest in the identification of new natural products from Celastraceae plants,\(^8\)-\(^11\) we sought to establish a new UHPLC-MS dereplication method that can guide prioritisation of biota samples and expedite the discovery of new chemical entities.

UHPLC-MS is becoming an important tool in natural product dereplication as it allows for fast fingerprinting and profiling analysis.\(^13\)-\(^15\) Moreover, the use of MS in conjunction with the UHPLC system also provides key structural information, such as molecular weight and diagnostic fragments.\(^13\),\(^16\),\(^17\) Therefore, in the current work, we utilised UHPLC–MS and scientific database (Dictionary of Natural Products\(^18\) and SciFinder Scholar\(^19\)) analysis to rapidly undertake dereplication and prioritise Celastraceae plant samples for detailed chemical investigation work, with the ultimate goal of identifying new dihydro-β-agarofurans. A number of Celastraceae plants from NatureBank\(^\dagger\),\(^20\) at Griffith University were accessed for this study. The plant biota included three bark, one fruit, one leaf, seven roots, two twigs and two mixed samples collected from Queensland, Australia. The CH\(_2\)Cl\(_2\) extract of all 16 plant samples were subjected to UHPLC-MS. A total of three samples were prioritised for potential large-scale extraction and isolation studies; specifically, the selected samples included the fruits of Denhamia celastroides since UHPLC-MS data in conjunction with database

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\(^\dagger\) NatureBank is a unique biodiversity and biodiscovery resource based on natural product extracts and fractions derived from Australian plants, fungi, and marine invertebrates, which also archives more than 30,000 biota samples.
analyses suggested the presence of new dihydro-β-agarofurans. Large-scale extraction and MS guided-isolation of the fruits of *D. celastroides* led to the discovery of four previously undescribed dihydro-β-agarofurans (denhaminols O–R, 1–4) and a known congener, denhaminol G (5). The structures of all new compounds were assigned by 1D/2D NMR and MS data analysis.

This report describes a simple and rapid method for the generation of Celastraceae plant extracts and subsequent UHPLC-MS analysis, dereplication and prioritisation that has successfully led to the identification of new chemical entities.

2. **Materials and Method**

2.1 General experimental

Values of specific rotation were determined with a JASCO P-1020 polarimeter and UV spectra were recorded using a JASCO V-650 UV/vis spectrophotometer. ECD spectra were obtained on a JASCO J-715 spectropolarimeter and processed using the software SDAR v3.2.\(^{21}\) IR data were acquired using an attached Universal Attenuated Total Reflectance (UATR) Two module on a PerkinElmer spectrophotometer. NMR spectra were acquired from a Bruker AVANCE HDX 800 MHz NMR spectrometer equipped with a TCI cryoprobe at 25 °C. The \(^1\)H and \(^13\)C chemical shifts were referenced to the residual solvent signal of CDCl\(_3\) at \(\delta_H 7.26\) and \(\delta_C 77.16\) ppm, respectively. A Fritsch Universal Cutting Mill Pulverisette 19 was used to grind the air-dried plant material and an Edwards Instrument Company Bio-line orbital shaker was used for plant extraction. Phenomenex Strata solid phase extraction (SPE) cartridges (3 cc, polypropylene, single fritted, catalogue# AH0-7806) were used for the small-scale plant extractions. The UHPLC-MS was performed on Ultimate 3000 RS UHPLC coupled to a Thermo Fisher Scientific MSQ Plus single quadruple ESI mass spectrometer using an analytical Waters ACQUITY UPLC CSH C\(_{18}\) column (2.1 × 50 mm, 1.7 \(\mu\)m, 130 Å). Thermo Fisher Scientific Dionex Ultimate 3000 UHPLC was used for semi-preparative HPLC separations. Phenomenex Luna C\(_{18}\) (250 × 10 mm, 5 \(\mu\)m, 90–110 Å) column was used for semi-preparative HPLC separations. Alltech C\(_{18}\)-bonded Si (35–75 \(\mu\)m, 150 Å) was used for pre-adsorption work, and the resulting material was packed into an Alltech stainless steel guard cartridge (10 × 30 mm) prior to semi-preparative HPLC separations. Merck Si gel (0.040–0.063 mm, 230–400 mesh)
was used for Si gel column chromatography. All solvents (CH$_2$Cl$_2$ and CH$_3$CN) used for chromatography, specific rotation, ECD, UV and MS were RCI Labscan HPLC grade. H$_2$O was Sartorius arium pro VF filtered. All compounds were analysed for purity by $^1$H NMR spectroscopy and shown to be $>$95%, unless otherwise stated. NMR spectra were processed using MestReNova version 11.0.

2.2 Plant materials

The 16 Celastraceae plant samples were obtained from the NatureBank biota library housed at the Griffith Institute for Drug Discovery, Griffith University, Australia. All samples were collected in Queensland and taxonomically identified by the Queensland Herbarium. Voucher specimens have been deposited at the Queensland Herbarium, Australia. The fruits of *Denhamia celastroides* (F. Muell.) Jessup used for large scale extraction were collected on the 26 November 1997 in Mt Windsor Tableland rainforest, Queensland. A voucher specimen (NB016128) has been deposited at the Griffith Institute for Drug Discovery, Griffith University, Brisbane, Australia. All plant specimens were air-dried, ground, and stored at room temperature prior to extraction.

2.3 Preparations of plant extracts for UHPLC-MS analyses

Each of the air-dried and ground Celastraceae plant material (300 mg) was packed into an SPE cartridge and extracted under gravity with 8 mL of CH$_2$Cl$_2$. The CH$_2$Cl$_2$ extracts were dried, weighed, and resuspended in CH$_3$CN in order to generate a stock solution, which had a concentration of 1 mg/mL (minimum stock solution volume = 0.5 mL).

2.4 UHPLC-MS conditions

All CH$_2$Cl$_2$ extracts were subjected to UHPLC-MS analysis (5 $\mu$L injection volume). UHPLC-MS experiments were performed with an Ultimate 3000 RS UHPLC coupled to a Thermo Fisher MSQ Plus single quadrupole ESI mass spectrometer using an analytical Waters ACQUITY UPLC CSH C$_{18}$ column (2.1 $\times$ 50 mm, 1.7 $\mu$m, 130Å). Employing a flow rate of 0.3 mL/min, a gradient of 10% CH$_3$CN (0.1% formic acid) in H$_2$O (0.1% formic acid) to 100%
CH₃CN (0.1% formic acid) was applied over 15 min, followed by isocratic elution of CH₃CN (0.1% formic acid) for 5 min.

2.5 Large-scale extraction and isolation of the fruits of *D. celastroides*

The air-dried and ground fruits of *D. celastroides* (10 g) were extracted with CH₂Cl₂ (2 × 500 mL for 16 h each) to afford 245.8 mg of a crude extract. The extract was subjected to chromatography using a Si-gel column (3 × 8 cm) and a step-wise gradient system of *n*-hexane/EtOAc (100% *n*-hexane to 100% EtOAc, 10% increment, 100 mL each) to afford 11 fractions (fractions 1–11). All 11 fractions were analysed by UPLC-MS and fractions 7–9 were chosen for further purification based on UPLC-MS data analysis. Fraction 7 (22.4 mg) was pre-adsorbed to C₁₈ bonded Si-gel (~1 g), packed into a guard cartridge and attached to a semi-preparative C₁₈ HPLC column. A linear gradient from 45% CH₃CN/H₂O to 90% CH₃CN/H₂O at a flowrate of 4 mL/min was run over 60 min to obtain denhaminol O (1, 10.4 mg, *t*R_{R} 18–19 min, 0.104% dry wt) and denhaminol Q (2, 1.5 mg, *t*R_{R} 21 min, 0.015% dry wt). Fraction 8 (38.4 mg) was pre-adsorbed to C₁₈ bonded Si-gel (~1 g), packed into a guard cartridge and attached to a semi-preparative C₁₈ HPLC column. A linear gradient from 45% CH₃CN/H₂O to 80% CH₃CN/H₂O at a flowrate of 4 mL/min was run over 60 min to yield denhaminol G (5, 13.9 mg, *t*R_{R} 24 min, 0.139% dry wt). Fraction 9 (29.4 mg) was pre-adsorbed to C₁₈ bonded Si-gel (~1 g), packed into a guard cartridge and attached to a semi-preparative C₁₈ HPLC column. A linear gradient from 40% CH₃CN/H₂O to 70% CH₃CN/H₂O at a flowrate of 4 mL/min was run over 60 min to yield denhaminol P (3, 10.2 mg, *t*R_{R} 27–28 min, 0.102% dry wt) and denhaminol R (4, 2.2 mg, *t*R_{R} 29–30 min, 0.022% dry wt).

2.5.1 Denhaminol O (1)

Colourless gum; [α]_{D}^{24} −40.2 (c 0.520, MeOH); ECD λ_{ext} (MeOH) 214 (−4.63), 231 (0.19), 267 (−3.55) nm; UV (MeOH) λ_{max} (log ε) 281 (4.36) nm; IR (UATR) ν_{max} 3409, 2979, 1747, 1705, 1634, 1386, 1256, 1197, 1161, 1077, 973, 768 cm⁻¹; ¹H NMR (CDCl₃, 800 MHz) see Table 2; ¹³C NMR (CDCl₃, 200 MHz) see Table 3; (+)-LRESIMS *m/z* 615 [M+H]+; (+)-HRESIMS *m/z* 637.2595 [M+Na]+ (calcd for C₃₃H₄₂O₁₁Na, 637.2619).
2.5.2 Denhaminol P (2)

Colourless gum; \([\alpha]_D^{24} -36.0 (c 0.075, \text{MeOH}); \text{ECD } \lambda_{\text{ext}} (\text{MeOH}) 217 (-4.79), 268 (-3.81) \text{ nm}; \text{UV (MeOH) } \lambda_{\text{max}} (\log c) 282 (4.38) \text{ nm}; \text{IR (UATR) } v_{\text{max}} 2952, 1739, 1705, 1373, 1197, 1162, 1076, 975, 769 \text{ cm}^{-1}; \text{H NMR (CDCl}_3, 800 \text{ MHz}) \text{ see Table 2; } ^{13}\text{C NMR (CDCl}_3, 200 \text{ MHz}) \text{ see Table 3; } (+)-\text{LRESIMS } m/z 657 [M+H]^+; (+)-\text{HRESIMS } m/z 679.2693 [M+Na]^+ \text{ (calcld for C}_{35}\text{H}_{44}\text{O}_{12}\text{Na, 679.2725}).

2.5.3 Denhaminol Q (3)

Colourless gum; \([\alpha]_D^{24} -33.3 (c 0.510, \text{MeOH}); \text{ECD } \lambda_{\text{ext}} (\text{MeOH}) 216 (-4.60), 253 (-2.01), 301 (0.29) \text{ nm}; \text{UV (MeOH) } \lambda_{\text{max}} (\log c) 630 (4.441) \text{ nm}; \text{IR (UATR) } v_{\text{max}} 3419, 2971, 1747, 1710, 1391, 1278, 1120, 1163, 1078, 974, 713 \text{ cm}^{-1}; \text{H NMR (CDCl}_3, 800 \text{ MHz}) \text{ see Table 2; } ^{13}\text{C NMR (CDCl}_3, 200 \text{ MHz}) \text{ see Table 3; } (+)-\text{LRESIMS } m/z 631 [M+H]^+; 653 [M+Na]^+; (+)-\text{HRESIMS } m/z 653.2531 [M+Na]^+ \text{ (calcld for C}_{33}\text{H}_{42}\text{O}_{12}\text{Na, 653.2568}).

2.5.4 Denhaminol R (4)

Colourless gum; \([\alpha]_D^{24} -18.2 (c 0.110, \text{MeOH}); \text{ECD } \lambda_{\text{ext}} (\text{MeOH}) 216 (-5.37), 235 (0.92), 279 (-1.31) \text{ nm}; \text{UV (MeOH) } \lambda_{\text{max}} (\log c) 224 (4.42), 281 (4.39) \text{ nm}; \text{IR (UATR) } v_{\text{max}} 3414, 2981, 1746, 1709, 1635, 1277, 1199, 1163, 1077, 973, 712 \text{ cm}^{-1}; \text{H NMR (CDCl}_3, 800 \text{ MHz}) \text{ see Table 2; } ^{13}\text{C NMR (CDCl}_3, 200 \text{ MHz}) \text{ see Table 3; } (+)-\text{LRESIMS } m/z 653 [M+H]^+; 675.2400 [M+Na]^+ \text{ (calcld for C}_{35}\text{H}_{40}\text{O}_{12}\text{Na, 675.2412}).

3. Result and Discussion

The \text{CH}_2\text{Cl}_2 extracts of samples from 16 Australian Celastraceae plants were prepared using a small amount of the air-dried and ground samples. All extracts were subjected to UHPLC-MS profiling (Figure 1 and Figure S1, Supplementary data). The data molecular weights of the major UV-active compounds in each of the extracts were determined from either the negative or positive total ion chromatogram (TIC). These data were then analysed using SciFinder Scholar and the Dictionary of Natural Products (DNP). Examination of respective UHPLC chromatograms and scientific databases provided a preliminary overview of the constituents of the extracts. The number of hits (from the database search) of the molecular weights generated from MS data was used as a filter. Of the 16 plant extracts subjected to UHPLC-MS, three plant samples...
(Denhamia celastroides fruits, Hysophila halleyana F.Muell. bark, and Perrottetia arborescens (F.Muell.) Loes. roots) were prioritised since distinct molecular ions were detected (less than five hits reported in the databases, Table S2, Supporting Information). Since the UHPLC traces of the fruits of D. celastroides showed the best separation among all analysed plant extracts, this sample was given the highest priority.

The scientific databases search (Table 1 and Table S2, Supporting Information) indicated the possibility of new dihydro-β-agarofurans present in the fruits of Denhamia celastroides CH₂Cl₂ extract since it contained several distinct molecular ions in the (+)-ESI mode (m/z 615 [M+H]⁺; 631 [M+H]⁺; and 657 [M+H]⁺), which were only found in a few dihydro-β-agarofurans (less than five hits) reported in SciFinder Scholar and DNP search (keywords: “Celastraceae” and “agarofuran”). To confirm the presence of the new compounds and to unambiguously identify the structures, we conducted a large-
scale extraction of the plant material and MS-directed isolation to purify the targeted compounds with the distinct molecular weights before NMR experiments were conducted.

![Diagram](image)

**Figure 2.** UHPLC chromatogram (254 nm) of the CH$_2$Cl$_2$ extract of *D. celastroides* fruits; retention times for major UV peaks are indicated.

<table>
<thead>
<tr>
<th>Retention time ($t_R$, min)</th>
<th>[M+H]$^+$ m/z</th>
<th>Molecular weight</th>
<th>No. of SciFinder Scholar hits$^a$</th>
<th>No. of DNP hits$^a$</th>
<th>Compounds</th>
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<tr>
<td>9.873</td>
<td>615</td>
<td>614</td>
<td>1</td>
<td>0</td>
<td>Denhaminol O (1)</td>
</tr>
<tr>
<td>10.097</td>
<td>657</td>
<td>656</td>
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<td>0</td>
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<tr>
<td>10.410</td>
<td>673</td>
<td>672</td>
<td>10</td>
<td>0</td>
<td>Denhaminol G (5)</td>
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<tr>
<td>11.037</td>
<td>631</td>
<td>630</td>
<td>4</td>
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<td>Denhaminol Q (3)</td>
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<tr>
<td>11.513</td>
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<td>652</td>
<td>17</td>
<td>0</td>
<td>Denhaminol R (4)</td>
</tr>
</tbody>
</table>

$^a$Accessed on the 15 June 2018

The fruits of *D. celastroides* (10 g) were sequentially extracted with CH$_2$Cl$_2$. Subsequent purifications using Silica gel column chromatography and RP-HPLC afforded five dihydro-$\beta$-agarofurans (**Figure 3**). The targeted compounds with molecular ions $m/z$ [M+H]$^+$ of 615, 631, 657 were confirmed to be new natural products, which were given the trivial names denhaminols O–Q (1–3), respectively. During the isolation of the three targeted compounds, another new dihydro-$\beta$-agarofuran (denhaminol R, 4) and a known congener, denhaminol G (5), were also obtained. The complete structure elucidation of the new compounds is detailed below.
Denhaminol O (1) was isolated as a colourless gum with a molecular formula of C_{33}H_{42}O_{11} based on HRESIMS data (m/z 637.2595). The $^1$H NMR spectrum (Table 2) exhibited signals of six methyl protons ($\delta_H$ 1.33, 1.59, 1.63, 1.78, 1.79, and 1.88), five methylene protons ($\delta_H$ 1.53/1.95, 1.69/1.86, 2.03/2.26, 4.31/4.45, and 4.52/4.69), and 12 methine protons ($\delta_H$ 2.37, 4.44, 4.88, 5.39, 6.40, 6.81, 7.36 (3H), 7.56 (2H), and 7.65). The $^{13}$C (Table 3) and HSQC spectra of 1 suggested a total of 33 carbons, including six methyls, five methylenes, 12 methines, 10 non-protonated carbons. The $^{13}$C resonances at $\delta_C$ 165.9, 167.1, 167.6, and 170.2 suggested the presence of four ester groups in 1. These data indicated that compound 1 was a dihydro-$\beta$-agarofuran bearing four ester groups,\textsuperscript{8-11} which was confirmed by COSY and HMBC experiments (Figure 4).
Table 2. \(^1\)H (800 MHz) NMR data for denhaminols O–R (1–4) in CDCl\(_3\).

<table>
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<th>Position</th>
<th>(\delta_H), multiplicity (J in Hz)</th>
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<tr>
<td>1</td>
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<td>2α</td>
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<tr>
<td>2β</td>
<td>1.95, m</td>
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<td>7</td>
<td>2.37, br dd (3.5, 3.0)</td>
</tr>
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<tr>
<td>34</td>
<td>-</td>
</tr>
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<td>35</td>
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</table>

The positions of the ester groups were determined following HMBC data analysis. HMBC crosspeaks from two olefinic protons \((\delta_H 6.40 \text{ and } 7.65, \text{ d, } J = 16.0)\) and H-9 \((\delta_H 4.88)\) to an ester carbonyl carbon at \(\delta_H 165.9\) located a trans-cinnamate group at C-9. The \(^1\)H NMR resonances at \(\delta_H 1.78\) (3H, m), 1.79 (3H, m), and 6.81 (1H, m) are the characteristic of tigloyl moiety.\(^{22}\) The tigloyl group was located at C-12 based on HMBC correlations from a pair of diastereotopic methylene protons \((\delta_H 4.52 \text{ and } 4.69)\), a methyl at \(\delta_H 1.79\), and an olefinic proton at \(\delta_H 6.81\) to a carbonyl resonance at \(\delta_C 167.6\). The HMBC spectrum of 1 also exhibited correlations from a set of methylene protons at \(\delta_H 4.31\) and 4.45 to two carbonyl carbon resonating at \(\delta_C 167.1\)
and 170.2. These data along with further HMBC correlation from H-1 ($\delta_H$ 4.88) to the carbonyl carbon at 167.1 suggested the location of an acetoxyacetate functional group at C-1. Finally, two hydroxy moieties were positioned at C-4 and C-6 by considering the molecular formula of 1 and the deshielded NMR resonances of C-4 ($\delta_C$ 73.3) and CH-6 ($\delta_H$ 4.44 and $\delta_C$ 79.4). The relative configuration of denhaminol O (1) was established by ROESY (Figure 4) and $^1$H-$^1$H coupling constant data analysis. The large coupling constant of H-1 ($J_{1,2} = 12.1$ Hz) indicated the $\beta$-orientation of H-1. Similarly, the $\alpha$-orientation of H-9 was assigned based on the coupling constant ($J_{8,9} = 7.4$ Hz). ROESY crosspeaks between H$_3$-14 and H-6, between H-6 and H-9, as well as between H-9 and H$_3$-15 suggested that these protons were cofacial. It is worth mentioning that ROESY correlations were also observed between H$_2$-12 and H-8$\beta$, as well as between H$_3$-13 and H-7. Consequently, the structure of 1 was established as 1$\alpha$-acetoxyacetate-8$\beta$-cinnamoyloxy-4$\beta$,6$\beta$-dihydroxy-12-tigloyloxydihydro-$\beta$-agarofuran.

![Figure 4. Diagnostic 2D NMR correlations for denhaminol O (1).](image)

Denhaminol P (2, colourless gum) had a molecular formula of C$_{35}$H$_{44}$O$_{12}$ as concluded from (+)-HRESIMS data. Comparison of $^1$H and $^{13}$C NMR spectra of 2 and 1 showed a high degree of similarity between the two compounds. However, the $^1$H resonance of H-6 was shifted downfield from $\delta_H$ 4.44 in 1 to $\delta_H$ 5.48 in 3, which suggested the attachment of an ester group at C-6 in 3. HMBC correlations from a methyl singlet signal at $\delta_H$ 2.15 and H-6 ($\delta_H$ 5.48) to a carbonyl carbon at $\delta_C$ 170.6 identified the presence of an acetate group at C-6 in 2. The 2D NMR data (Figure S28, Supporting Information) further confirmed the attachment of the remaining ester moieties in 2. Comparison of ROESY data of 1 and 2 revealed the same relative configurations for these compounds. Thus, the structure of 2 was elucidated as 6$\beta$-acetoxy-1$\alpha$-acetoxyacetate-8$\beta$-cinnamoyloxy-4$\beta$-hydroxy-12-tigloyloxydihydro-$\beta$-agarofuran.
Table 3. $^{13}$C (200 MHz) NMR data for denhaminols O–R (1–4) in CDCl$_3$.

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<thead>
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| Interchangeable signals |

Compound 3 was isolated as a colourless gum, had a molecular formula of C$_{33}$H$_{42}$O$_{12}$ as suggested by HRESIMS. The NMR data of 3 were similar to those of 1, except for the presence of an additional hydroxy in 3. The hydroxy group was positioned at C-8 based on the deshielded resonances of CH-8 ($\delta$H 4.30 and $\delta$C 70.3). HMBC correlations (Figure S29, Supporting Information) from H-8 to C-6, C-9, and C-11 further confirmed the location of OH-8. The $\beta$-orientation of the hydroxy moiety at C-8 was assigned by ROESY correlations (Figure S29, Supporting Information) between H-8 and H$_3$-15. Therefore, the structure of denhaminol Q (3) was determined.
as α-acetoxyacetate-8β-cinnamoyloxy-4β,6β,8β-trihydroxy-12-tigloyloxydihydro-β-agarofuran.

During the isolation of the major compounds 1–3, denhaminol R (4) was obtained. The molecular formula of compound 4 was C_{33}H_{40}O_{12} as indicated by (+)-HRESIMS data. Analysis of NMR and MS data showed that the structure of 4 was similar to that of 3. However, the tigloyl group at C-12 in 3 was replaced by a benzoate in 4. The presence of the benzoate group was confirmed by the characteristic {^1}H NMR resonances at δ_{H} 7.45 (2H, m), 7.57 (1H, m), and 8.03 (2H, m). The position of the benzoate at C-12 was assigned by HMBC correlations (Figure S30, Supporting Information) from H₂-12 (δ_{H} 4.88 and 5.02) and the proton at δ_{H} 8.03 to an ester carbonyl carbon at δ_{C} 166.4. The relative configurations of 4 were ascertained to be the same with those of 3 by ROESY experiment. Accordingly, the structure compound 4 was assigned as α-acetoxyacetate-12-benzoyleoxy-8β-cinnamoyloxy-4β,6β,8β-trihydroxydihydro-β-agarofuran.

Previously, we reported the isolation of dihydro-β-agarofuran sesquiterpenoids from two Australian plants belonging to the Denhamia genus, D. celastroides and D. pittosporoides. The chemical investigation of the leaves of D. celastroides afforded eight dihydro-β-agarofurans (denhaminol A–H), while two new dihydro-β-agarofurans (denhaminols I and J) were obtained from the leaves of D. pittosporoides.11

4. Conclusion

In this study, we demonstrated how UHPLC-MS data and database mining can be used to extract molecular features related to characteristic secondary metabolites of a plant family. This approach was successfully applied to 16 Australian Celastraceae plants extracts and a UHPLC-MS protocol and dereplication process was developed for the rapid identification of new dihydro-β-agarofurans from Celastraceae plants. Using the developed methodology, the extract obtained from the fruits of D. celastroides was prioritised and four previously undescribed dihydro-β-agarofurans (denhaminol O–R, 1–4) along with the known compound denhaminol G (5) were successfully isolated and characterised. Other Celastraceae samples that were prioritised for large-scale extraction and isolation studies will be pursued in the future. The pure compounds reported in this paper will be added to the Davis Open Access Compound library which is housed at Compounds Australia, Griffith University^23-27 and will be tested in various in vitro assays in the future. It is worth mentioning and well known that the chemical profile of a plant can vary due to different geographical locations and collection seasons, however,
even though this research utilised only Queensland Celastraceae plants, the UHPLC-MS method developed here should be applicable to the chemical profiling of any plant sample. Furthermore, this UHPLC-MS methodology should be adaptable to the chemical investigation of any biota material, including not only plants but also microbes, marine invertebrates and fungi.

Acknowledgement

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References


Chapter 7. General Conclusions and Future Directions

Plants are a major source and focus for natural products research around the world and have been playing an important role in modern drug discovery research for over two centuries. Plants from the Celastraceae family are a source of numerous chemical entities with various biological effects. Australian plants belonging to the Celastraceae family have been superficially explored for their chemistry and pharmacological properties. This PhD thesis describes the chemical investigation of four Australian Celastraceae plants along with the leucine uptake inhibitory evaluation in LNCaP human prostate cancer cells of the isolated natural products from these endemic botanical flora. The plants that were studied during this PhD program included *Maytenus bilocularis*, *Denhamia pittosporoides*, and *Celastrus subspicata* and *Denhamia celastroides*.

The importance of natural products in drug discovery and chemical biology research is reviewed in Chapter 1. Moreover, the first chapter of this thesis also outlines the role of leucine transport in cancer cells, and why this type of amino acid transporter is now considered by many as a novel target for cancer therapeutic research.

Chapter 2 describes the isolation and structure elucidation of three new (bilocularins A–C) and six known dihydro-β-agarofuran sesquiterpenoids from the crude CH$_2$Cl$_2$ extract of leaves of the Australian rainforest plant *M. bilocularis*. The known compounds were identified as celastrine A, 1α,6β,8α-triacetoxy-9α-benzoyloxydihydro-β-agarofuran, 1α,6β-diacetoxy-9α-benzoyloxy-8α-hydroxydihydro-β-agarofuran, Ejap-10, 1α,6β-diacetoxy-9β-benzoyloxydihydro-β-agarofuran, and Ejap-2. Bilocularin A was utilised in the generation of four ester analogues. All natural products and semi-synthetic analogues were evaluated for their cytotoxicity against the human prostate cancer cell line LNCaP; none of them were cytotoxic. Several of the compounds were found to exhibit MDR-reversal effect in the vinca alkaloid resistant cell line CEM/VCR_R. Furthermore, the known compound 1α,6β,8α-triacetoxy-9α-benzoyloxydihydro-β-agarofuran was shown to inhibit leucine uptake in LNCaP cells with an IC$_{50}$ value of 15.5 μM.

Chapter 3 of this thesis reports on the chemical investigation of the roots of *M. bilocularis*, which resulted in the discovery of six new dihydro-β-agarofuran sesquiterpenoids (bilocularins D–I), along with three known congeners, namely 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran, pristimerin, and celastrol. Bilocularins D and G, 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-
agarofuran, and celastrol exhibited leucine uptake inhibition in the LNCaP cells with IC$_{50}$ values ranging from 2.5–27.9 μM. Furthermore, this study identified bilocularins D–F as the first dihydro-β-agarofurans bearing a hydroxyacetate group.

The extraction and isolation of the leaves of the Australian endemic plant *D. pittosporoides* were described in Chapter 4. The isolation studies afforded two previously undescribed (denhaminols I and J) and four known (1α,2α,6β,15-tetraacetoxy-9α-benzoxyloxy-8-oxodihydro-β-agarofuran, wilforsinine F, 1α,2α,6β,8α,15-pentaacetoxy-9α-benzoxyloxydihydro-β-agarofuran, and 1α,2α,6β,15-tetraacetoxy-9β-benzoxyloxydihydro-β-agarofuran) compounds. Several compounds were tested for their leucine uptake inhibition in LNCaP cells; denhaminol I and wilforsinine F were shown to inhibit leucine uptake with IC$_{50}$ of 51.5 and 95.5 μM, respectively.$^5$

The fifth chapter of this thesis focuses on the isolation and structure determination of seven previously unknown dihydro-β-agarofurans from the leaves of *C. subspicata*, which were given the trivial names celastrofurans A–G. In addition, two known analogues, (1S,4R,5S,7R,9S,10S)-9-benzoxyloxy-1-furoxyloxydihydro-β-agarofuran and (1R,2R,4R,5S,7R,9S,10R)-2-acetoxy-9-benzoxyloxy-1-furoxyloxydihydro-β-agarofuran were also obtained. Biological evaluation of the isolated compounds identified that all compounds displayed leucine uptake inhibitory activity in LNCaP cell with IC$_{50}$ values ranging from 7.0 to 98.9 μM.$^4$

Finally, Chapter 6 reports an analytical UHPLC-MS method, which was utilised together with scientific database analysis to prioritise and rapidly dereplicate 16 CH$_2$Cl$_2$ Australian Celastraceae plant extracts. The main aim of this study was to identify new dihydro-β-agarofurans using the developed method. A subset of extracts (three in total) were prioritised for potential large-scale extraction and isolation studies, one of which included the fruits of *D. celastroides* since UHPLC-MS data and database analyses indicated the presence of new dihydro-β-agarofurans. The large-scale extraction and isolation studies of the fruit sample afforded four previously undescribed dihydro-β-agarofurans, which were given the trivial name denhaminols O–R and a known analogue, denhaminol G. This study has successfully demonstrated the use of UHPLC-MS methodology in conjunction with database analysis for the rapid identification of new chemical entities in plant extracts.

The structures of all compounds presented in this thesis were established by detailed analysis of NMR, MS, UV, IR, and specific rotations data. The absolute configurations of several compounds were defined by X-ray diffraction studies and/or ECD data
analysis. The studies reported in this PhD thesis further validate the importance of natural products as drugs or drug leads. All compounds both natural products and semi-synthetic compounds will be deposited into the Davis Open-Access Compound Library, which is curated by Compounds Australia (https://www.compoundsaustralia.com). Compounds Australia is part of the Griffith Institute for Drug Discovery (GRIDD) that securely stores and curates sample libraries submitted by Australian based chemists. This highly specialised facility enables health and medical researchers worldwide to outsource compound and natural product management and logistics. By adding all compounds from this thesis to the Davis library, they will become accessible for further biological evaluations by both national and international collaborators in the drug discovery or chemical biology research areas.

In conclusion, the studies presented in this thesis further exemplified the prominence and importance of natural products to drug discovery. However, some challenges were encountered during the course of this research. The supply of Celastraceae plants was one of the main limitations in this PhD project with limited quantity of several plant samples resulting in only small amounts of some isolated compounds, which prohibited detailed evaluation of their biological activity.

Furthermore, 19 Australian Celastraceae plant samples were collected and analysed at the beginning of this PhD project, however after detailed chemical studies, only four samples were shown to give interesting chemical profiles and worthy of investigating. The remaining 14 Celastraceae plant samples were devoid of identifiable or interesting chemistry, hence we conclude that not all plants belonging to this family are worthy of chemical investigation. Therefore the simple UHPLC-MS method described in Chapter 6, which can be employed in future research as more Celastraceae plants become available through new collecting trips, was developed in order to assist sample prioritisation and to accelerate the process of new natural product discovery.

We have identified for the first time, dihydro-β-agarofuran sesquiterpenoids that inhibit leucine transport in cancer cells. This interesting bioactivity warrants further investigation as to how this chemotype specifically inhibits leucine uptake. In the future, the isolated compounds could be chemically modified and/or more dihydro-β-agarofurans could be isolated in order to elucidate additional structure-activity relationships. More detailed mechanism of action studies on these plant metabolites is warranted.
References


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S5 HMBC Spectrum of Bilocularin A (1) in CDCl$_3$
S6 ROESY Spectrum of Bilocularin A (1) in CDCl₃
$^1$H NMR (500 MHz) Spectrum of Bilocularin B (2) in CDCl$_3$
S8 $^{13}$C NMR (125 MHz) Spectrum of Bilocularin B (2) in CDCl$_3$
S9 HSQC Spectrum of Bilocularin B (2) in CDCl₃
S10 COSY Spectrum of Bilocularin B (2) in CDCl₃
S11 HMBC Spectrum of Bilocularin B (2) in CDCl$_3$
ROESY Spectrum of Bilocularin B (2) in CDCl$_3$
S13 $^1$H NMR (500 MHz) Spectrum of Bilocularin C (3) in CDCl$_3$
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S16 COSY Spectrum of Bilocularin C (3) in CDCl₃
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S29 COSY and Selected HMBC Correlations of Compounds 2 and 3

S30 Key ROESY Correlations of Compounds 2 and 3
### Inhibitory Effect of Compounds 1–13 against the LNCaP Cell Line$^a$

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$^a$Cell viability was measured using the alamarBlue reagent after 72 h of treatment (n=4, mean ± SD). Vinblastine was used as positive control (IC$_{50}$ = 3.2 ± 0.1 nM)
Appendix 2: Supporting Information for Chapter 3

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<td>S19</td>
<td>$^1$H NMR (800 MHz) spectrum of bilocularin G (7) in CDCl$_3$</td>
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<td>$^{13}$C NMR (200 MHz) spectrum of bilocularin G (7) in CDCl$_3$</td>
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<td>S21</td>
<td>HSQC spectrum of bilocularin G (7) in CDCl$_3$</td>
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<td>COSY spectrum of bilocularin G (7) in CDCl$_3$</td>
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<td>ROESY spectrum of bilocularin G (7) in CDCl$_3$</td>
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<td>$^1$H NMR (800 MHz) spectrum of bilocularin H (8) in CDCl$_3$</td>
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<td>$^{13}$C NMR (200 MHz) spectrum of bilocularin H (8) in CDCl$_3$</td>
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<td>HSQC spectrum of bilocularin H (8) in CDCl$_3$</td>
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<td>COSY spectrum of bilocularin H (8) in CDCl$_3$</td>
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<td>S29</td>
<td>HMBC spectrum of bilocularin H (8) in CDCl$_3$</td>
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<td>ROESY spectrum of bilocularin H (8) in CDCl$_3$</td>
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<td>$^1$H NMR (800 MHz) spectrum of bilocularin I (9) in CDCl$_3$</td>
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<td>$^{13}$C NMR (200 MHz) spectrum of bilocularin I (9) in CDCl$_3$</td>
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<td>COSY spectrum of bilocularin I (9) in CDCl$_3$</td>
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<td>S36</td>
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S3 HSQC spectrum of bilocularin D (4) in CDCl₃
S4 COSY spectrum of bilocularin D (4) in CDCl₃
S5 HMBC spectrum of bilocularin D (4) in CDCl$_3$
S6 ROESY spectrum of bilocularin D (4) in CDCl₃
S\textsuperscript{7} $^1$H NMR (800 MHz) spectrum of bilocularin E (5) in CDCl\textsubscript{3}
$^{13}$C NMR (200 MHz) spectrum of bilocularin E (5) in CDCl$_3$
S9 HSQC spectrum of bilocularin E (5) in CDCl₃
S10 COSY spectrum of bilocularin E (5) in CDCl₃
S11 HMBC spectrum of bilocularin E (5) in CDCl₃
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S13 $^1$H NMR (800 MHz) spectrum of bilocularin F (6) in CDCl$_3$
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$^{13}$C NMR (200 MHz) spectrum of bilocularin G (7) in CDCl$_3$
S21 HSQC spectrum of bilocularin G (7) in CDCl₃
S22 COSY spectrum of bilocularin G (7) in CDCl$_3$
S23 HMBC spectrum of bilocularin G (7) in CDCl₃
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S25 $^1$H NMR (800 MHz) spectrum of bilocularin H (8) in CDCl$_3$
$^{13}$C NMR (200 MHz) spectrum of bilocularin H (8) in CDCl$_3$
S27 HSQC spectrum of bilocularin H (8) in CDCl₃
S28 COSY spectrum of bilocularin H (8) in CDCl₃
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S14 ECD Spectra of Compounds 4–6

![Graph showing ECD Spectra of Compounds 4, 5, and 6](image-url)
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S5 HMBC Spectrum of Celastrofuran A (1) in CDCl$_3$
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HSQC Spectrum of Celastrofuran C (3) in CDCl₃
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S17 HMBC Spectrum of Celastrofuran C (3) in CDCl₃
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S28 COSY Spectrum of Celastrofuran E (5) in CDCl₃
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S39 HSQC Spectrum of Celastrofuran G (7) in CDCl$_3$
S40 COSY Spectrum of Celastrofuran G (7) in CDCl$_3$
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- **Denhamia celastroides** (F.Muell.) Jessup roots
- **Perrottetia arborescens** (F.Muell.) Loes. twigs
- **Denhamia celastroides** (F.Muell.) Jessup bark
- **Denhamia fasciculiflora** (Jessup) M.P.Simmons roots
- **Perrottetia arborescens** (F.Muell.) Loes. roots
- **Elaeodendron melanocarpum** F.Muell. roots
- **Perrottetia arborescens** (F.Muell.) Loes. leaves
- **Elaeodendron melanocarpum** F.Muell. bark
S1 Cont’d UHPLC chromatograms of 16 CH₂Cl₂ extracts of Australian Celastraceae plants

- *Hippocratica barbata* F.Muell. mixed
- *Hypsophila halleyana* F.Muell. bark
- *Denhamia celastroides* (F.Muell.) Jessup fruits
- *Denhamia cunninghamii* (Hook.) M.P.Simmons bark
- *Denhamia pittosporoides* F.Muell. subsp. pittosporoides roots
- *Denhamia cunninghamii* (Hook.) M.P.Simmons roots
- *Denhamia cunninghamii* (Hook.) M.P.Simmons roots
- *Hypsophila dielsiana* Loes. mixed
S2 UHPLC-MS data of the major UV peaks of 16 CH₂Cl₂ extracts of Australian Celastraceae and scientific databases analysis.

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<th>[M–H]⁻ ( m/z )</th>
<th>Molecular weight</th>
<th>No. of SciFinder Scholar hits⁹</th>
<th>No. of DNP hits⁹</th>
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*Accessed on the 15 June 2018*
S3 $^1$H NMR (800 MHz) spectrum of denhaminol O (I) in CDCl$_3$. 
$^{13}$C (200 MHz) NMR spectrum of denhaminol O (I) in CDCl$_3$
S5 COSY spectrum of denhaminol O (1) in CDCl$_3$
S6 HSQC spectrum of denhaminol O (1) in CDCl$_3$
S7 HMBC spectrum of denhaminol O (1) in CDCl₃
S8 ROESY spectrum of denhaminol O (1) in CDCl₃
S9 $^1$H NMR (800 MHz) spectrum of denhaminol P (2) in CDCl$_3$
S10 $^{13}$C NMR (200 MHz) spectrum of denhaminol P (2) in CDCl$_3$
S11 COSY spectrum of denhaminol P (2) in CDCl₃
S12 HSQC spectrum of denhaminol P (2) in CDCl$_3$
S13 HMBC spectrum of denhaminol P (2) in CDCl₃
S14 ROESY spectrum of denhaminol P (2) in CDCl₃
S15 $^1$H NMR (800 MHz) spectrum of denhaminol Q (3) in CDCl$_3$
$^{13}$C NMR (200 MHz) spectrum of denhaminol Q (3) in CDCl$_3$
S17 COSY spectrum of denhaminol Q (3) in CDCl$_3$
S18 HSQC spectrum of denhaminol Q (3) in CDCl$_3$.
S19 HMBC spectrum of denhaminol Q (3) in CDCl₃
S20 ROESY spectrum of denhaminol Q (3) in CDCl$_3$
S21 $^1$H NMR (800 MHz) spectrum of denhaminol R (4) in CDCl$_3$
$^{13}$C NMR (200 MHz) spectrum of denhaminol R (4) in CDCl$_3$
S23 COSY spectrum of denhaminol R (4) in CDCl$_3$. 

![COSY spectrum of denhaminol R (4) in CDCl$_3$.](image-url)
S24 HSQC spectrum of denhaminol R (4) in CDCl₃
S25 HMBC spectrum of denhaminol R (4) in CDCl₃
S26 ROESY spectrum of denhaminol R (4) in CDCl₃
ECD spectra of denhaminols O–R (1–4) and denhaminol G (5) in MeOH

Diagnostic 2D NMR correlations for denhaminol P (2)
S29 Diagnostic 2D NMR correlations for denhaminol Q (3)

S30 Diagnostic 2D NMR correlations for denhaminol R (4)
Appendix 6: DVD containing NMR, MS, and X-ray crystallography Data Related to this Thesis

NMR, MS, and X-ray crystallography data associated with Chapters 2–6