SEARCHING FOR ANTICANCER AGENTS
AND ANTIMALARIAL AGENTS FROM
MADAGASCAR

Ende Pan

A thesis submitted to the faculty of the Virginia Polytechnic
Institute and State University in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy In Chemistry

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ABSTRACT

In our continuing search for biologically active natural products from Madagascar as part of an International Cooperative Biodiversity Group (ICBG) program, a total of four antiproliferative extracts were studied, leading to the isolation of twelve novel compounds with antiproliferative activity against the A2780 human ovarian cancer line, and one extract with antimalarial activities was studied, which led to the isolation of five new natural products with antimalarial activities against the Dd2 and HB3 malarial parasites.

The plants and their metabolites are discussed in the following order: one new xanthone and two known guttiferones from *Symphonia tanalensis* Jum. & H. Perrier (Clusiaceae); four new diphenyl propanes and one new cyclohepta-dibenzofuran skeleton from *Bussea sakalava* (Fabaceae); four new cardiac glycosides from *Leptadenia madagascariensis* Decne. (Apocynaceae); two new and four known alkaloids from *Ambavia gerrardii* (Baill.) Le Thomas (Annonaceae); five new sesquiterpene lactones from *Polycline proteiformis* Humbert (Asteraceae).

The structures of all compounds were determined by analysis of their mass spectrometric, 1D and 2D NMR, UV and IR spectroscopic and optical rotation data. Other than structure elucidation, this dissertation also involve bioactivity evaluation of all the isolates, synthesis of two interesting alkaloids, as well as a proposal for the possible biosynthetic pathway of the new cyclohepta-dibenzofuran skeleton.
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I. Introduction: natural product drug discovery

1.1 Introduction

The term “Natural product” is used commonly in reference to a chemical substance produced by a living organism. Natural products often have pharmacological or biological activities that are useful for pharmaceutical drug discovery and drug design. The use of natural resources as medicines for human health can be traced back thousands of years, and the use of plants for treating different diseases has been recorded in the medical books of ancient China, India, and North Africa. Many of the aqueous, ethanolic, distilled, condensed, or dried plant extracts used had some beneficial medicinal properties for patients. Today, 80% of the world’s population still relies on traditional medicines.

Beginning in the 19th century, man started to isolate the active components from medicinal plants. The first landmark was made by the French scientists Caventou and Pelletier, who isolated quinine (1.1) from Cinchona bark. This discovery sparked a vast interest in searching for active compounds from plants. Before the 1930s, a series of natural products, such as morphine (1.2), codeine (1.3), digoxin (1.4) and atropine (1.5) were isolated from higher plants, and these compounds continue in clinical use today.
Another milestone was made when the penicillins (1.6) were isolated from a species of *Penicillium* and used for treating serious infections during World War II. In recent years, the isolation of paclitaxel (1.7) in 1967 from the bark of the Western Pacific Yew *Taxus brevifolia* and its use for treating cancers, and the isolation of artemisinin (1.8) from the Chinese herb *Artemisia annua* in 1972 and its use for treating malaria has increased the attention paid to the field of natural product drug discovery.

Natural products provide one of the most important sources for novel drug discovery. Many natural product and natural product derived compounds are being evaluated in clinical trials or in registration, and according to a recent report, 28% of new approved drugs were natural product or products derived (Figure 1.1) from natural products among
the overall 1184 new chemical entities (NCE) covering all diseases/countries/sources in the years 01/1981-06/2006. 

![Figure 1.3 All new chemical entities, 01/1981-06/2006, by source (N) 1184.](image)

**B**: Biological; usually a large (>45 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host.

**N**: Natural product.

**ND**: Derived from a natural product and is usually a semisynthetic modification.

**S**: Totally synthetic drug, often found by random screening/modification of an existing agent.

**S**: Made by total synthesis, but the pharmacophore is/was from a natural product.

**V**: Vaccine.

**Subcategory**: **NM**: Natural product mimic

Of 974 small molecules from the above 1184 NCEs, 34% were natural products or natural product derived. In the area of cancer, from the late 1940s to 06/2006 for 155 approved anticancer small molecule drugs, 73% are other than synthetic products, with 47% actually being either natural products or their derivatives. The antiinfective area (antibacterial, antifungal, antiparasitic, and antiviral) has been largely dependent on the structures of natural product, and 86 of total 180 small molecule drugs approved from 01/1981 to 06/2006 were natural products or derived therefrom. The influence of natural product structures are also quite significant in other areas. Searching for lead natural products as sources of novel structures is still one of the most important approaches for drug discovery.

**1.3 Natural product medicines from different sources**
Today, natural products are obtained not only from terrestrial plants but also from marine organisms, microorganisms and animals. Below are a few examples of natural product medicines from different categories.

1.3.1 Medicines derived from plants

With a long history of plants in traditional medicine, many compounds from plants have entered clinical use. In addition to quinine, paclitaxel and artemisinin, the anticancer drug camptothecin (1.9) was isolated from the bark and stems of the Chinese tree *Camptotheca acuminata*. Galantamine (1.10) from the plant, *Galanthus nivalis*, is used to treat Alzheimer’s disease, while dronabinol (D9-THC) (1.11) and cannabidiol (1.12), which are used as adjunctive treatments for the symptomatic relief of neuropathic pain, were obtained from the *Cannabis* plant.

![Chemical structures of 1.9-1.12](image)

*Figure 1.4 Chemical structures of 1.9-1.12*

1.3.2 Medicines derived from microorganisms
Microorganisms became an important source for natural product drug discovery beginning with the success of the penicillins in treating infections. Fumagillin (1.13) was isolated from *Aspergillus fumigatus* and approved for use in the treatment of intestinal microsporidiosis in France in 2005. Depsipeptide (FR-901228, FK-228) (1.14) is an inhibitor of HDAC (histone deacetylase). It is isolated from *Chromobacterium violaceum* and now in phase II clinical trials for treating cancers. Marketed in Japan since 2002, amrubicin (1.15), a derivative of doxorubicin isolated from *Streptomyces peucetius* var *caesius*, is used in the treatment of lung cancer by inhibiting topoisomerase II.

![Figure 1.5 Chemical structures of 1.13-1.15](image)

1.3.3 Medicines derived from marine organisms

Water covers more than 70% of the earth, but it was only in the 1950s that scientists started to search for medicinal natural products from the sea. The approval of spongouridine (1.16) and spongothymidine (1.17) from the Caribbean sponge *Cryptotheca crypta* as anticancer drugs, catalyzed increased work on the discovery of marine natural products. Ziconotide (1.18), derived from the toxin of the cone snail species *Conus magus*, is used as a treatment for patients suffering from chronic pain.
Ziconotide acts by blocking the N-type calcium channel to inhibit the release of pronociceptive neurochemicals.  

![Chemical structure of Ziconotide](image)

**Figure 1.6** Chemical structures of 1.16-1.18

Trabectedin (1.19) is a tetrahydroisoquinoline alkaloid isolated from the ascidian *Ecteinascidia turbinata*. It was approved by EMEA (European Medicines Agency) in 2007 for the treatment of advanced soft tissue sarcoma. Trabectedin is also in clinical trials for treating ovarian, breast and prostate cancers. Isolated from the bryozoan *Bugula neritina*, bryostatin 1 (1.20) is a protein kinase C inhibitor. It currently in phase II clinical trials for cancer treatment.

![Chemical structures of Trabectedin and Bryostatin 1](image)

**Figure 1.7** Chemical structures of 1.19 and 1.20

1.3.4 Medicines derived from terrestrial animals
Some compounds obtained from various terrestrial vertebrates and invertebrates could also be potential medicines due to their biochemical properties. Exenatide (1.21), a 39 amino acid peptide isolated from the saliva of the Gila monster *Heloderma suspectum*, acts as an adjunctive therapy to treat type 2 diabetes by improving glycemic control.21,22 The skin of the frog *Epipedobates tricolor* in Ecuador yielded epibatidine (1.22), which has been isolated and used as a lead compound for developing drugs for pain relief.23,24

![Chemical structures of 1.21 and 1.22](image)

**Figure 1.8** Chemical structures of 1.21 and 1.22

### 1.4 Anticancer agents and antimalarial agents from natural products

#### 1.4.1 Anticancer agents

Cancer is a class of diseases that causes millions of human deaths each year. It particularly refers to the rapid growth of abnormal cells, cell invasion to adjacent tissues, and sometimes spreading of abnormal cells to other locations. The World Trade Organization (WHO) estimates 7.9 million deaths were due to cancer in 2007 around 13% of all deaths.25

The Search for anticancer drugs from natural products began in the late 1940s. Other than paclitaxel (1.7), there are several anticancer drugs available derived from natural products. Topotecan (1.23) and irinotecan (1.24) are semisynthetic, water soluble analogs of camptothecin (1.9). Topotecan was approved by the FDA in 1996 and is used as second-line therapy for advanced ovarian cancer. Irinotecan was approved in 2000 by
FDA for treating advanced colorectal cancer. By stabilizing the DNA-topoisomerase I covalent binary complex, topotecan and irinotecan inhibit DNA synthesis and cause cell death during the cell cycle.\textsuperscript{20}

**Figure 1.9** Chemical structures of 1.23 and 1.24

Isolated from *Streptomyces peucetius* derived strains, doxorubicin (1.25) was registered in the early 1970s and has been one of the most widely used drugs in cancer chemotheraphy. Its analog epirubicin (1.26), with better performance to doxorubicin in clinical trial, was approved by the FDA in 1999. Doxorubicin and epirubicin are used to treat different cancers by interacting with DNA and inhibiting topoisomerase II.\textsuperscript{20,26}

**Figure 1.10** Chemical structures of 1.25 and 1.26
Vinca alkaloids represent one of the most important classes of anticancer agents. In distinction to paclitaxel, which acts as a promoter of tubulin polymerization, vinca alkaloids inhibit the assembly of tubulin into microtubules, and prevent the cells from undergoing division. Vinblastine (1.27) and vincristine (1.28) were isolated from the leaves of *Cantharanthus roseus*, a plant endemic to Madagascar, in the 1950s. Since the 1960s, the vinca alkaloids vinblastine and vincristine have been widely used for treating different types of cancer.\textsuperscript{20,26}

![Chemical structures of 1.27 and 1.28](image)

**Figure 1.11** Chemical structures of 1.27 and 1.28

\subsection*{1.4.2 Antimalarial agents}

Malaria is a mosquito-borne infectious disease caused by an eukaryotic protist of the genus *Plasmodium*. The disease is found in tropical regions throughout sub-Saharan Africa, Southeast Asia, the Pacific Islands, India, and Central and South America. About half of the world's population lives in malaria-endangered areas. In 2008, malaria kills nearly one million people out of 250 million malaria cases. A child dies of malaria every 30 seconds.\textsuperscript{27} Medicines for treating malaria are needed urgently.
Powdered bark from the cinchona tree containing the plasmodicidal quinoline alkaloids quinine (1.1) and quinidine (1.29) was the first antimalarial medicine and used to treat malarial for centuries.\textsuperscript{6}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{malaria Structures.png}
\caption{Chemical structures of 1.29-1.31}
\end{figure}

Chloroquine (1.30) was synthesized in 1934 and became the foundation of malaria therapy after World War II. However, a drug resistant strain began to emerge in the 1960s. The new antimalarial agent called artemisinin (1.8) was isolated in 1972 from Chinese herb \textit{Artemisia annua}, which is a plant used in traditional Chinese medicine for malaria treatment.\textsuperscript{6} Artemisinin is currently one of the most efficient antimalarial medicines in the market. Arterolane (OZ-277) (1.31), a synthetic trioxolane modelled on the artemisinin pharmacophore, is in phase II clinical trial.\textsuperscript{8}

However, the growing threat of drug resistant strains due to gene mutation demands a more extensive searching for new antimalarial agents from natural products.

\subsection*{1.5 The ICBG (International Cooperative Biodiversity Groups) program}

The tropical rain forests of the world provide a huge source for natural products, holding the potential for drug discovery. However, in the past several decades, those
forests are disappearing at a rapid rate, especially in developing countries, because of continuous logging and conversion of forests to agricultural uses.

In an attempt to address the interdependent issues of drug discovery, biodiversity conservation and economic development, the International Cooperative Biodiversity Groups (ICBG) Program was initiated in 1993. It is currently jointly funded by the U. S. National Institutes of Health (NIH), the National Science Foundation (NSF), the U. S. Department of Agriculture (USDA), the U. S. Department of Energy (DOE) and the National Oceanic and Atmospheric Administration (NOAA).

The Kingston research group at Virginia Tech has been funded by the ICBG program since 1993, and has been collaborating with the Missouri Botanical Garden, Conservation International, the three Madagascar centers Center National d’Application des Recherches Pharmaceutiques (CNARP), Centre National de Recherche Oceanographique, (CNRO), and Centre National de Recherche pour l’Environnement (CNRE) to study tropical plants, marine organisms and microorganisms in Madagascar. The companies Eisai Inc. and Dow AgroSciences are also partners in this cooperative program.

As the fourth biggest island in the world, Madagascar has been isolated from Africa for over 150 million years. For this reason, 75% of the 200,000 plant and animal species found in Madagascar are endemic to the island. The Madagascar ICBG program is searching for new anticancer agents and antimalarial agents by isolation of bioactive natural products from different resources in Madagascar.

Plants, marine organisms and microorganisms are collected and screened for antimalarial activity in Madagascar, and screened for antiproliferative activity at Virginia Tech. Potential active extracts are selective for fractionation and purification under the
guidance of bioassay. In the past twelve years, many new active antiproliferative compounds have been isolated. Due to the great biodiversity of the flora and fauna of Madagascar, the isolated compounds covered various types of natural products from alkaloids, flavonoids to terpenoids and many others with all kinds of skeletons. The structural variety of the isolated compounds may increase the hit rate in the bioassay screening and be beneficial for drug discovery.

Searching for novel bioactive agents from Madagascar is the project which this dissertation will describe. The research was focused on the isolation and structure elucidation of bioactive natural products applying bioassay guided fractionation and modern analytical techniques including LC-MS, NMR, UV, IR, optical rotation and CD (circular dichroism). The A2780 human ovarian cancer cell line was the most frequently used bioassay in addition to the occasional use of other cancer cell lines. HB3 and Dd2 malaria parasites were the most used bioassays for antimalarial extracts. This dissertation also involves the synthesis of bioactive natural products, specifically two new antiproliferative alkaloids from a Malagasy plant. The research work is discussed in detail in the following chapters.

References


II. An antiproliferative xanthone and two guttiferones of *Symphonia tanalensis* from the Madagascar rainforest

This chapter is a slightly expanded version of a published article. Attributions from co-authors of the article are described as follows in the order of the names listed. The author of this dissertation (Mr. Ende Pan) conducted isolation and structure elucidation of the titled compounds, and drafted the manuscript. Dr. Shugeng Cao was a mentor for this work, and in particular, he provided invaluable advice and hints for structure elucidation of those compounds, and he also proofread the manuscript before submission. Ms. Peggy Brodie performed the A2780 bioassay on the isolated fractions and compounds. Dr. James S. Miller, Dr. Fidisoa Ratovoson and Dr. Chris Birkinshaw from Missouri Botanical Garden did the plant collections and identification. Dr. Roland Rakotondrajaona, Dr. Rabodo Andriantsiferana and Dr. Vincent E. Rasamison from Madagascar carried out the initial plant extraction. Dr. David G. I. Kingston was a mentor for this work and the corresponding author for the published article. He provided critical suggestions for this work and crucial revisions to the manuscript.

2.1 Introduction

In our continuing search for biologically active natural products from tropical rainforests as part of an International Cooperative Biodiversity Group (ICBG) program, we obtained an ethanol extract of a plant identified as *Symphonia tanalensis* Jum. & H. Perrier (Clusiaceae) from Madagascar. The extract showed moderate antiproliferative activity against the A2780 human ovarian cancer cell line with an IC\textsubscript{50} value of 19 $\mu$g/mL.
On the basis of the activity and the absence of previous phytochemical study on this species, *S. tanalensis* was selected for further investigation under the guidance of our bioassay.

### 2.1.1 Previous investigations of Symphonia

Previous studies on the genus *Symphonia* reported guttiferone analogues\(^4\,^6\) and xanthone derivatives,\(^5\,^7\,^9\,^10\,^11\) some of which showed anti-HIV,\(^4\) antioxidant\(^5\) and antiplasmodial\(^5\,^7\,^10\,^11\) activities. The cytotoxicities of xanthones\(^9\,^12\) and guttiferones\(^13\,^14\) have also been studied. Bioassay-guided fractionation by applying C\(_{18}\) open column and HPLC on the dichloromethane fraction yielded a new xanthone 2.1 and two guttiferones 2.2 and 2.3. Herein we report the structural elucidation of the new xanthone and the antiproliferative properties (against the A2780 human ovarian cancer cell line) of the isolated compounds.

![Figure 2.1 Compounds isolated from Symphonia.](image-url)
2.2 Results and Discussion

2.2.1 Structure elucidation of compound 2.1

Compound 2.1 was obtained as a yellow solid. Its positive ESI-MS revealed a pseudomolecular ion peak at $m/z = 425.1590$ [M+H]$^+$ corresponding to a molecular formula of C$_{24}$H$_{25}$O$_7$, (calcd for C$_{24}$H$_{25}$O$_7$ 425.1600). The $^1$H NMR spectrum showed signals for one aromatic proton singlet at $\delta_H 7.21$ (s), one methoxyl group at $\delta_H 3.95$ (s), an olefinic proton at $\delta_H 5.22$ (t, $J = 7.5$ Hz), two methylene protons at $\delta_H 3.35$ (d, $J = 7.5$ Hz) and a pair of downfield methyl singlets at $\delta_H 1.68$ (s) and 1.82 (s) which corresponded to a 3-methyl-2-butenyl group. In addition a pair of cis-coupled olefinic doublets ($\delta_H 7.04$, d, $J = 10.0$ Hz; 5.69, d, $J = 10.0$ Hz) and a pair of methyl singlets at $\delta_H 1.48$ (s) suggested the presence of a 3-oxygenated-3-methyl-butenyl group.

The $^{13}$C NMR spectrum of 2.1 exhibited 24 carbon signals (Table 2.1), including one resonances of a carbonyl at $\delta_C 181.7$, one methoxyl group at $\delta_C 56.6$, one 3-methyl-2-butenyl group ($\delta_C 22.0$, 123.5, 131.9, 26.0, 18.1), and one 3-oxygenated-3-methyl-butenyl group ($\delta_C 116.6$, 127.8, 79.1, 28.5, 28.5), as well as twelve aromatic carbons assignable to two isolated aromatic rings, seven of which were oxygenated due to their corresponding down field carbon chemical shifts ($\delta_C 160.8$, 151.4, 159.0, 134.4, 143.8, 143.3, 147.3). The above data suggested that compound 2.1 was a xanthone similar to those previously isolated from the same genus Symphonia.$^5$-$^{11}$ In order to satisfy the degree of unsaturation number (13) implied by the molecular formula C$_{24}$H$_{25}$O$_7$, the 3-oxygenated-3-methyl-butenyl group must be cyclized with a hydroxyl group of the xanthone moiety to form a gem-dimethyl dihydropyran. To assign the attachment of the functionalities, 2D NMR experiments were carried out.
Table 2.1 $^1$H and $^{13}$C NMR Data (δ) in CD$_3$OD for Compounds 2.1

<table>
<thead>
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<th>position</th>
<th>δ$_H$</th>
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<tr>
<td>2</td>
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<td>8</td>
<td>7.21 (s)</td>
<td>96.7</td>
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<tr>
<td>OCH$_3$</td>
<td>3.95 (s)</td>
<td>56.6</td>
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</table>

$^a$δ (ppm) 500 MHz for $^1$H and 125 MHz for $^{13}$C; multiplicities, J values (Hz) in parentheses.

HMOC (Heteronuclear Multiple Bond Coherence) allowed the assignment of all the
signals observed in the 1D NMR spectra. Careful interpretation of the $^1$H–$^1$H COSY, HMBC, ROESY and NOESY allowed us to confirm the xanthone nature of 2.1.

![Chemical structure of compound 2.1.](image)

Figure 2.2 Chemical structure of compound 2.1.

$^1$H–$^1$H COSY correlations between the signal of H-1" ($\delta_H 7.04$) and H-2" ($\delta_H 5.69$) as well as the HMBC correlations between H-1" and the two oxygenated aromatic carbons: C-3 $\delta_C 151.4$, C-4a $\delta_C 159.0$ led us to conclude that the 3,3-dimethylpyran ring was attached at C-3 and C-4. The allocation of the proton on the pentasubstituted aromatic ring was substantiated by the observation of $^3J$ correlations between the singlet aromatic proton at H-8 ($\delta_H 7.21$) and the carbonyl carbon C-9, the two overlapped aromatic carbon signals at C-5a ($\delta_C 143.8$) and at C-6 ($\delta_C 143.3$), and $^2J$ correlations between H-8 ($\delta_H 7.21$) and the aromatic carbon at C-8a ($\delta_C 112.3$) and C-7 ($\delta_C 147.3$). The location of a methoxyl group on C-7 was deduced from the cross peak in the ROESY spectrum between the signals at $\delta_H 3.95$ and at H-8. This was confirmed by the observation of HMBC correlation between the signal of the methoxyl group and that of C-7.
The hydrogen bonded proton observed at $\delta_{H}$ 13.30 in CDCl$_3$ indicated a hydroxyl group must be attached on C-1. $^1$H-$^1$H COSY correlations between the signal at $\delta_{H}$ 3.35 (H$_2$-1') and that at $\delta_{H}$ 5.22 (H-2') as well as a HMBC long-range coupling between the signal at H-2' and those of two methyl groups ($\delta_{H}$ 1.68 and $\delta_{H}$ 1.82) confirm the presence of a 3-methyl-2-butenyl group. The latter was assigned to be attached at the C-2 position due to the HMBC correlations observed from H-1' to C-1 and to C-2. Furthermore, a clear NOESY correlation was observed between the hydrogen-bonded proton and H$_2$-1' in the spectrum of 2.1 measured in CDCl$_3$. On the basis of the molecular formula of 2.1, the remaining two hydroxyl groups must be located at C-5 and C-6. From the above data, the structure of 2.1 was determined to be 2-(3,3-dimethylallyl)-1,5,6-trihydroxy-7-methoxy-2",2"-dimethylpyrano(6",5":3,4) xanthone.

2.2.2 Identification of the known guttiferone I and A

The structures of the two known guttiferone analogues (guttiferone I (2.2) and guttiferone A (2.3)) isolated in the present study were deduced by comparison with data reported in the literature.$^4,15$
2.2.3 Antiproliferative activities of isolated compounds

Compound 2.1 showed a strong antiproliferative activity against the A2780 human ovarian cancer cell lines with an IC$_{50}$ value of 3.8 $\mu$M. Lenta and co-workers isolated a very similar compound [2-(3,3-dimethylallyl)-1,5-dihydroxy-6,7-dimethoxy-2",2"-dimethylpyrano (6",5":3,4)xanthone] (2.4) which showed good anti-plasmodial activity (1.3 $\mu$M).$^5, 7$ Although the cytotoxic activities of xanthones (against KB, MCF-7, HT-29, Hela, et al) $^{16-18}$ have been reported in the literature, only few reports have investigated the properties of xanthones against the A2780 human ovarian cancer cell lines.$^{19}$ Compound 2.1 showed better antiproliferative activity than two xanthones (2.5 and 2.6) previously reported in the literature.$^{19}$

Figure 2.4 Chemical structures of compounds 2.2 and 2.3.

Figure 2.5 Chemical structures of compound 2.4-2.6.
Compounds 2.2 and 2.3 showed IC5₀s of 8.3 μM and 7.8 μM antiproliferative activity against the A2780 cell line.

2.3 Experimental Section

2.3.1. General experimental procedure

UV spectra were measured on a Shimadzu UV-1201 spectrophotometer. NMR spectra were recorded in CD₃OD on either Varian INOVA 400 or JEOL Eclipse 500 spectrometers. The chemical shifts are given in δ (ppm) and coupling constants (J) are reported in Hz. Mass spectra were obtained on an Agilent 6220 TOF Mass Spectrometer. HPLC was performed on a Shimadzu LC-10AT instrument with a semi-preparative C₁₈ Varian Dynamax column (5 μm, 250 mm, 10 mm) and a preparative C₁₈ Varian Dynamax column (8 μm, 250 mm, 21.4 mm). Optical rotation was measured on a JASCO P-2000 polarimeter.

2.3.2. Antiproliferative bioassay

Antiproliferative activities were obtained at Virginia Polytechnic Institute and State University against the drug-sensitive A2780 human ovarian cancer cell line. Human ovarian cancer cells (A2780) grown to 95% confluency were harvested and re-suspended in growth medium (RPMI1640 supplemented with 10% fetal bovine serum and 2 μM L-glutamine). Cells were counted using a hemacytometer and a solution containing 2.5x10⁵ cells per ml was prepared in growth media. Eleven columns of a 96 well microtitre plate were seeded with 199 μL of cell suspension per well, and the remaining column contained media only (one hundred percent inhibition control).
The plate was incubated for 3 hours at 37°C/5% CO₂ to allow the cells to adhere to the wells. Following this incubation, potential cytotoxic agents, prepared in DMSO, were added to the wells in an appropriate series of concentrations, 1 μL per well. One column of wells was left with no inhibitor (zero percent inhibition control), and 4 dilutions of taxol (IC₅₀ 0.015 μg/mL) were included as a positive control. (Scheme 2.1) The plate was incubated for 2 days at 37°C/5% CO₂, then the media gently shaken from the wells and replaced with reaction media (supplemented growth medium containing 1% Alamar blue), and incubated for another 3 hours. The level of Alamar blue converted to a fluorescent compound by living cells was then analyzed using a Cytofluor Series 4000 plate reader (Perseptive Biosystems) with an excitation wavelength of 530 nm, an emission wavelength of 590 nm, and gain of 45. The percent inhibition of cell growth
was calculated using the zero percent and one hundred percent controls present on the plate, and an \( IC_{50} \) value (concentration of cytotoxic agent which produces 50% inhibition) was calculated using a linear extrapolation of the data which lie either side of the 50% inhibition level. Samples were analyzed in triplicate on at least two separate occasions to produce a reliable \( IC_{50} \) value. The A2780 cell line is a drug-sensitive ovarian cancer cell line.\(^{20}\)

2.3.3. Plant material

A sample of the leaves, fruit and inflorescence of Symphonia pauciflora Baker (Clusiaceae) was collected in October 2000 as ROL 136. The collection was made by Rolland Rakotodrajaona et al. from a plant growing in dense humid forest near the Parc National de Zahamena at coordinates 17°33′15″S 48°53′23″E at an elevation of 730 m. Voucher specimens have been deposited at the Parc Botanique and Zoologique de Tsimbazaza (TAN) and at the Centre National d'Application des Recherches Pharmaceutiques (CNARP) in Antananarivo, Madagascar; the Missouri Botanical Garden in St. Louis, Missouri (MO); and the Muséum National d'Histoire Naturelle in Paris, France (P).

2.3.4. Extraction and Isolation

Dried leaves, inflorescence and fruits of *Symphonia tanalensis* (370 g) were ground in a hammer mill, then extracted with ethanol by percolation for 24 hours at room temperature to give the crude extract MG 0724 (35.5 g), of which 7.8 g was shipped to Virginia Polytechnic Institute and State University (VPISU) for further bioassay guided
isolation. The fractionation tree is shown in Scheme 2.2. The extract MG 0724 (IC$_{50}$: 19 μg/mL, 2.3 g) was suspended in aqueous MeOH (MeOH-H$_2$O, 9:1, 100 mL) and extracted with hexane (3 x 100 mL portions). The aqueous layer was then diluted to 60% MeOH (v/v) with H$_2$O and extracted with CH$_2$Cl$_2$ (3 x 150 mL portions). The hexane extract was evaporated in vacuo to leave 242 mg with an IC$_{50}$ value of 11 μg/mL. 1.04 g of residue from CH$_2$Cl$_2$ extract also showed IC$_{50}$: 11 μg/mL activity. The aqueous MeOH extract (998 mg) was inactive. The CH$_2$Cl$_2$ extract was first fractionated by a C$_{18}$ open
column, and four fractions were collected. Fractions A, B, C, and D (61, 748, 134, and 156 mg) had IC₅₀ values of 20, 10, 16, and >25 μg/mL, respectively. Fraction B was separated by C₁₈ preparative HPLC (90% MeOH-H₂O), and 11 sub-fractions were obtained. Using Diol semi-preparative HPLC (5% EtOH in DCM), compound 2.1 (1.4 mg, tᵣ 19.8 min) was purified from sub-fraction B-5. Compounds 2.2 (0.9 mg, tᵣ 25.1 min) and 2.3 (1.2 mg, tᵣ 27.2 min) were isolated from sub-fraction B-9 by Diol semi-preparative HPLC (5% EtOH in DCM).

2.3.5 2-(3,3-Dimethylallyl)-1,5,6-trihydroxy-7-methoxy-2'-2''-dimethylpyrano (6'',5'':3,4) xanthone (2.1)

Yellow solid; UV (MeOH) λₘₐₓ nm (log ε) 206 (4.37), 219 (4.33), 271 (4.31), 337 (3.70), 376 (3.82) nm; ¹H NMR (500 MHz, CD₃OD) and ¹³C NMR (125 MHz, CD₃OD), see table 2.1; ESI-MS m/z 425.1590 [M+H]⁺ (caled for C₂₄H₂₅O₇:425.1600)

2.3.6 Guttiferone I (2.2)

Yellow solid; [α]D -64 (c 0.2 CHCl₃) UV (MeOH) λₘₐₓ nm (log ε) 235 (4.05), 279 (4.15) nm; ¹H NMR (500 MHz, CD₃OD) 7.20 (d, J = 1.9 Hz, H-12), 6.96 (dd, J = 8.3, 1.9 Hz, H-16), 6.67 (d, J = 8.3 Hz, H-15), 5.16 (m, H-30), 5.06 (m, H-25), 4.90 (m, H-18), 2.72 (dd, J = 9.1, 13.5 Hz, H-17a), 2.55 (m, H-17b), 2.51 (m, H-29), 2.11 (m, H-24), 2.04 (m, H-7), 2.00 (m, H-32), 2.00 (m, H-33), 1.98 (m, H-6), 1.69 (s, H-38), 1.68 (s, H-21), 1.64 (s, H-20), 1.64 (s, H-27), 1.57 (s, H-36), 1.53 (s, H-37), 1.48 (s, H-28), 1.23 (s, H-22), 1.01 (s, H-23) and ¹³C NMR (125 MHz, CD₃OD) (209.8 (C-9), 196.0 (C-10), 195.3 (C-3), 195.0 (C-1), 152.6 (C-14), 146.2 (C-13), 139.2 (C-31), 135.7 (C-19), 133.8 (C-26),
132.3 (C-35), 129.5 (C-11), 125.5 (C-25), 125.3 (C-16), 125.3 (C-34), 120.8 (C-18),
120.5 (C-30), 117.9 (C-2), 117.3 (C-12), 115.1 (C-15), 68.0 (C-4), 61.7 (C-8), 47.8 (C-5),
41.0 (C-6), 41.0 (C-32), 40.7 (C-7), 31.8 (C-29), 30.1 (C-24), 27.7 (C-33), 27.3 (C-23),
27.1 (C-17), 26.4 (C-27), 26.0 (C-20), 26.0 (C-36), 23.3 (C-22), 18.3 (C-21), 18.2 (C-28),
17.8 (C-37), 16.8 (C-38); ESI-MS m/z 603.3694 [M+H]+ (caled for C₃₈H₅₁O₆, 603.3686).

2.3.7 Gutiferone A (2.3)

Yellow solid; [α]D +28 (c 0.1 CHCl₃) UV (MeOH) λmax nm (log ε) 235 (4.05), 280
(4.14) nm; ¹H NMR (500 MHz, CD3OD) 7.21 (d, J = 2.0 Hz, H-12), 7.01 (dd, J = 8.4, 2.0
Hz, H-16), 6.69 (d, J = 8.3 Hz, H-15), 5.21 (m, H-30), 5.08 (m, H-35), 2.65 (m, H-17a),
2.61 (m, H-17b), 2.47 (m, H-29a), 2.44 (m, H-29b), 2.09 (m, H-7b), 2.09 (m, H-24a),
2.06 (m, H-24b), 1.97 (m, H-7a), 1.87 (m, H-34), 1.83 (m, H-6), 1.71 (s, H-32), 1.68 (s,
H-21), 1.67 (s, H-33), 1.67 (s, H-37), 1.66 (s, H-27), 1.64 (s, H-27), 1.60 (s, H-20), 1.52
(s, H-28), 1.39 (m, H-23a), 1.24 (s, H-22), 1.20 (m, H-23b) and ¹³C NMR (125 MHz,
CD3OD) 209.7 (C-9), 195.6 (C-10), 195.6 (C-1), 195.5 (C-3), 152.6 (C-14), 146.2 (C-13),
135.7 (C-19), 135.7 (C-31), 133.8 (C-26), 132.9 (C-36), 129.3 (C-11), 125.5 (C-25),
125.1 (C-35), 125.0 (C-16), 120.8 (C-18), 120.8 (C-30), 117.8 (C-2), 117.4 (C-12), 115.2
(C-15), 68.8 (C-4), 62.1 (C-8), 51.9 (C-5), 41.1 (C-6), 40.1 (C-7), 36.9 (C-23), 32.0 (C-
29), 29.8 (C-24), 26.6 (C-17), 26.3 (C-27), 26.3 (C-32), 26.0 (C-20), 25.9 (C-37), 23.6
(C-34), 19.6 (C-22), 18.3 (C-33), 18.2 (C-21), 18.1 (C-28), 17.7 (C-38); ESI-MS m/z
603.3691 [M+H]+ (caled for C₃₈H₅₁O₆, 603.3686).
References


20. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of
III. Four diphenylpropanes and a cycloheptadibenzofuran from

*Bussea sakalava* from the Madagascar Dry Forest

This chapter is a slightly expanded version of a published article. Attributions from co-authors of the article are described as follows in the order of the names listed. The author of this dissertation (Mr. Ende Pan) conducted isolation and structure elucidation of the titled compounds, and drafted the manuscript. Dr. Liva Harinantanaina was a mentor for this work, and in particular, he provided invaluable advice and hints for structure elucidation of those compounds, and he also proofread the manuscript before submission. Ms. Peggy Brodie performed the A2780 bioassay on the isolated fractions and compounds. Dr. James S. Miller, Dr. Richard Randrianaivo, Dr. Fidisoa Ratovoson and Dr. Chris Birkinshaw from Missouri Botanical Garden did the plant collections and identification. Dr. Rabodo Andriantsiferana and Dr. Vincent E. Rasamison from Madagascar carried out the initial plant extraction. Dr. David G. I. Kingston was a mentor for this work and the corresponding author for the published article. He provided critical suggestions for this work and crucial revisions to the manuscript.

3.1 Introduction

In our continuing search for biologically active natural products from tropical rainforests as part of an International Cooperative Biodiversity Group (ICBG) program, we obtained an ethanol extract from the roots of a plant identified as *Bussea sakalava* Du Puy & R. Rabev. (Fabaceae) from Madagascar. This extract showed moderate antiproliferative activity against the A2780 human ovarian cancer cell line with an IC\textsubscript{50}
value of 10 μg/mL. The extract was selected for examination on the basis of this activity and the absence of previous phytochemical studies of the species.

3.1.1 Previous investigations of Bussea

Previous studies on the genus *Bussea* indicated the presence of azetidine-2-carboxylic acid and 3-hydroxyproline in seeds of different *Bussea* species,\(^3\) and the cytotoxicity and high trypanocidal activity of a methanol extract of stem bark of *Bussea occidentalis* has been reported.\(^5\)

![Figure 3.1 Compounds isolated from Bussea](image)

\begin{align}
\text{3-Hydroxyproline} & \quad \text{Azetidine-2-carboxylic acid}
\end{align}

3.2 Results and Discussion

Fractionation of a dichloromethane fraction of an ethanol extract of *B. sakalava* by C\(_{18}\) open column and high performance liquid chromatography (HPLC) yielded four new diphenylpropanes named bussealins A-D (3.1-3.4) and a cycloheptadibenzofuran derivative named bussealin E (3.5). Herein we report the structural elucidation of these new compounds and their antiproliferative properties against the A2780 human ovarian cancer cell line.

3.2.1 Structure elucidation of bussealin A (3.1)
Bussealin A (3.1) was obtained as an off-white solid. Its positive ESI-MS revealed a pseudomolecular ion peak at \( m/z \) 321.1338 [M + H]\(^+\) corresponding to molecular formula C\(_{17}\)H\(_{21}\)O\(_6\). The IR spectrum showed absorptions of OH (3367 cm\(^{-1}\)) and aromatic groups. The \(^1\)H NMR spectrum (Table 3.1) exhibited a singlet at \( \delta_H \) 6.18 (s, 2H) corresponding to a pair of aromatic protons of an A\(_2\) system, two aromatic doublets [\( \delta_H \) 6.50 (d, \( J = 8.4 \) Hz) and 6.38 (d, \( J = 8.4 \) Hz)] of an AB system, two OCH\(_3\) groups [\( \delta_H \) 3.75 (s) and 3.78 (s)], and a multiplet and two triplet methylene groups at \( \delta_H \) 1.79 (m, 2H), 2.52 (t, \( J = 7.7 \) Hz, 2H) and 2.41 (t, \( J = 7.7 \) Hz, 2H) respectively. The \(^{13}\)C NMR spectrum of 3.1 exhibited signals for 17 carbons, including three methylene carbons (\( \delta_C \) 36.5, 33.0, and 30.6), two OCH\(_3\) groups (\( \delta_C \) 56.5 and 60.8), and twelve aromatic carbons assignable to two isolated aromatic rings. Six of the aromatic carbons were oxygenated, as shown by their deshielded carbon chemical shifts (Table 3.1) and were consistent with the molecular formula. The above data suggested that 3.1 had a diphenyl propane skeleton. The complete \(^1\)H and \(^{13}\)C NMR assignments and the connectivities were determined from analysis of a combination of COSY, HMQC, and HMBC data. Three mutually coupled methylene groups were revealed by the cross peaks observed in the COSY spectrum. In the HMBC spectrum, H-1 (\( \delta_H \) 2.41) showed correlations with C-2 (\( \delta_C \) 33.0), C-3 (\( \delta_C \) 30.6), C-1' (\( \delta_C \) 140.2), and with C-2' and C-6', both of which had the same chemical shifts (\( \delta_C \) 108.7). The A\(_2\) substitution pattern of the A ring of 3.1 was established by HMBC correlations from the signal at \( \delta_H \) 6.18 (H-2' and H-6') to C-1 (\( \delta_C \) 36.5), C-1' (\( \delta_C \) 140.2), C-3' (\( \delta_C \) 151.3), C-4' (\( \delta_C \) 134.7) and C-6' and C-2' (\( \delta_C \) 108.7), as well as the correlation from one OCH\(_3\) group at \( \delta_H \) 3.75 to C-4' (\( \delta_C \) 134.7). The proton substitutions on the B ring were assigned based on the \(^3\)J HMBC correlations between H-3 (\( \delta_H \) 2.52) and C-6"
(δ_C 120.5), and between H-5" (δ_H 6.38) and C-1" (δ_C 123.4). Moreover, the H-5" proton showed HMBC correlations to C-6" (δ_C 120.5), C-4" (δ_C 147.8) and C-3" (δ_C 134.9). The location of the remaining OCH3 group was at C-4", as deduced from the HMBC correlation between the signal at δ_H 3.78 and that of C-4". On the basis of the molecular formula of 3.1, the remaining four OH groups were located at C-2" (δ_C 144.7), C-3" (δ_C 134.9), C-3' (δ_C 151.3), and C-5' (δ_C 151.3). Bussealin A is thus assigned the structure 3',5',2",3"-tetrahydroxy-4',4"-dimethoxy-1,3-diphenylpropane (3.1).

![Figure 3.2 Chemical structure of bussealin A-D (3.1-3.4)](image)

### 3.2.2 Structure elucidation of bussealin B (3.2)

Bussealin B (3.2) was obtained as an off-white solid. Its positive ESI-MS revealed a pseudomolecular ion peak at m/z 335.1512 [M + H]^+ corresponding to molecular formula C_{18}H_{23}O_{6}. The 1^H NMR spectrum (Table 1) showed two singlets of an AX system at δ_H 6.58 (s) and 6.60 (s), two aromatic doublets of an AB system at δ_H 6.51 (d, J = 8.4 Hz)
and 6.39 (d, $J = 8.4$ Hz), three OCH$_3$ groups [$\delta_H$ 3.76 (s), 3.80 (s) and 3.83 (s)], and one multiplet and two triplet methylene groups at $\delta_H$ 1.76 and 2.54 (t, $J = 7.8$ Hz) and 2.50 (t, $J = 7.8$ Hz). Inspection of the $^1$H and $^{13}$C NMR spectra of 3.2 revealed close similarities with those of 3.1, except for the presence of an additional OCH$_3$ signal and the chemical shifts of the AX system of ring A. The fact that the chemical shifts of the carbons of ring B of compounds 3.1 and 3.2 were superimposable (Table 3.1) indicated the presence of a 2",3"-dihydroxy-4"-methoxyphenyl group in 3.2. Interpretation of HMBC and NOESY experiments allowed us to determine the location of the OCH$_3$ groups to be at 2', 4', and 4". The two singlet aromatic protons on ring A were assigned according to the observation of $^3J$ HMBC correlations from H-6' ($\delta_H$ 6.60) to C-1 ($\delta_C$ 30.4) and from H-3' ($\delta_H$ 6.58) to C-1' ($\delta_C$ 124.7). Moreover, the proton signal of H-1 ($\delta_H$ 2.50) showed HMBC correlations with C-1' ($\delta_C$ 124.7), C-6' ($\delta_C$ 117.9) and the methoxylated carbon at C-2' ($\delta_C$ 152.2). This indicated that the third OCH$_3$ group must be at C-4' or C-5'. NOESY correlations from H-3' ($\delta_H$ 6.58) to 2'-OMe ($\delta_H$ 3.76) and to 4'-OMe ($\delta_H$ 3.83) established the location of the methoxy group at C-4' and the hydroxy group at C-5'. The structure of bussealin B was thus assigned as 5',2",3"-trihydroxy-2',4',4"-trimethoxy-1,3-diphenylpropane.

3.2.3 Structure elucidation of bussealin C (3.3)

Bussealin C (3.3) was obtained as an off-white solid. Its positive ESI-MS revealed a pseudomolecular ion peak at $m/z$ 305.1384 [M + H]$^+$ corresponding to molecular formula C$_{17}$H$_{21}$O$_5$. Its $^1$H NMR and $^{13}$C NMR spectra (Table 3.1) indicated that 3.3 is also a diphenylpropane with a 2",3"-dihydroxy-4"-methoxyphenyl group substituted at C-3. The
1,3,4-trisubstituted A ring was determined by the proton coupling constants and HMBC correlations from H-2' (δ_H 6.64) and H-6' (δ_H 6.60) to C-1 (δ_C 36.1), and COSY correlations between H-5' (δ_H 6.79) and H-6' (δ_H 6.60). Furthermore, the HMBC spectrum showed a 3J correlation from H-6' to the methoxylated carbon at C-4' (δ_C 147.0), which was confirmed by NOESY correlations between H-5' (δ_H 6.79) and 4'-OMe (δ_H 3.80). The above data coupled with the molecular formula led to assignment of the structure of bussealin C as 3',2'',3''-trihydroxy-4',4''-dimethoxy-1,3-diphenylpropane.

3.2.4 Structure elucidation of bussealin D (3.4)

Bussealin D (3.4) was obtained as an off-white solid. The positive ESI-MS exhibited a pseudomolecular ion peak at m/z 349.1648 [M + H]^+ corresponding to the molecular formula C_{19}H_{25}O_{6}. The ^1H NMR and ^13C NMR spectra (Table 3.1) indicated that 3.4 had the same tetrasubstituted B ring with an OCH₃ group at C-4'' as in compounds 3.1-3.3. In its ^1H NMR spectrum, the coupling patterns and the locations of the aromatic proton resonances of ring A were very similar to those of 3.2.

The presence of three OCH₃ groups and the substitution pattern of ring A of compound 3.4 were deduced by interpretation of the 1D and 2D NMR data. The HMBC spectrum of 3.4 showed correlations from H-1 (δ_H 6.79) to C-1' (δ_C 124.3), C-6' (δ_C 116.3) and to the methoxylated carbon at C-2' (δ_C 153.3). Furthermore, a clear 3J long-range correlation from the singlet proton H-3' (δ_H 6.61) to C-1' (δ_C 124.3) was also observed. Thus, the two remaining OCH₃ groups were determined to be at C-4' (δ_C 149.1) and C-5' (δ_C 144.1). The structure of bussealin D was thus determined to be 2''-dihydroxy-2',4',5',4''-tetramethoxy-1,3-diphenylpropane.
Table 3.1 $^1$H and $^{13}$C NMR data for Bussealin A-D (3.1-3.4) in CD$_3$OD $^a$

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<th>$^1$H ($J$, Hz)</th>
<th>$^{13}$C</th>
<th>$^1$H ($J$, Hz)</th>
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$^a$ $\delta$ (ppm) 500 MHz for $^1$H and 125 MHz for $^{13}$C; multiplicities; $J$ values (Hz) in parentheses.

3.2.5 Structure elucidation of bussealin E (3.5)
The positive ESI-MS of bussealin E (3.5) displayed a pseudomolecular ion peak at \( m/z \) 331.1181 \([M + H]^+\) corresponding to the molecular formula \( C_{18}H_{19}O_6 \). The \(^1\)H NMR spectrum in CDCl\(_3\) showed signals for a singlet aromatic proton at \( \delta_H \) 6.70, two OH groups (\( \delta_H \) 5.75 and 5.69), three OCH\(_3\) groups at \( \delta_H \) 4.24, 4.24, and 4.01, and three methylene groups as multiplets at \( \delta_H \) 3.13, 3.12 and 2.17. The \(^{13}\)C NMR spectrum of 3.5 exhibited 18 signals, assigned to three methylene (\( \delta_C \) 35.5, 28.7, 24.3), three OCH\(_3\) (\( \delta_C \) 60.8, 60.8 and 61.7), and twelve aromatic carbons of two isolated aromatic rings.

![Chemical structure of bussealin E (3.5) and 3.6](image)

**Figure 3.3** Chemical structure of bussealin E (3.5) and 3.6

Seven of the aromatic carbons were oxygenated, based on their deshielded chemical shifts (Table 3.2). The ten degrees of unsaturation implied by the molecular formula \( C_{18}H_{18}O_6 \) required two additional rings. Interpretation of \(^1\)H-\(^1\)H COSY, HMQC, HMBC and NOESY spectra allowed assignment of the locations of the functionalities present in 3.5. In the COSY spectrum, the three methylene groups were mutually coupled. The
assignment of a singlet aromatic proton was substantiated by the observation of HMBC correlations from H-1 ($\delta_H 6.70$) to C-10 ($\delta_C 35.5$), C-3b ($\delta_C 118.2$), and two oxygenated aromatic carbons at C-2 ($\delta_C 146.5$) and C-3 ($\delta_C 129.7$). HMBC correlations from the signal at $\delta_H 5.69$ to C-1 ($\delta_C 110.1$), C-2 ($\delta_C 146.5$) and the methoxylated carbon at C-3 ($\delta_C 129.7$) were observed, substantiating the location of a hydroxy group at C-2. The other hydroxy group was assigned to position 7 based on the observation of HMBC correlations from the signal at $\delta_H 5.75$ to the carbon signals at C-6 ($\delta_C 136.5$), C-7 ($\delta_C 142.3$) and C-7a ($\delta_C 115.0$). In addition, the signal at $\delta_H 5.75$ showed NOESY correlations to H-8 ($\delta_H 3.13$) and 6-OMe ($\delta_H 4.01$). These observations required that the remaining OCH$_3$ group be placed at C-5. Furthermore, the HMBC correlations observed from H-10 ($\delta_H 3.12$) to C-1 ($\delta_C 110.1$), C-10a ($\delta_C 131.7$), C-3b ($\delta_C 118.2$), C-8 ($\delta_C 28.7$) and C-9 ($\delta_C 24.3$) confirmed the location of the cycloheptadiene ring. The above data confirmed the cycloheptadibenzo[klm]furan skeleton of 3.5. Assignments of the $^{13}$C NMR signals of C-3a, C-4a and C-4b were made by comparing the measured data with those calculated by ACD/ChemSketch version 11.01. The calculated shifts were in excellent agreement with the observed values, and were all within the standard deviation of the software (5 ppm), except for C-7a. Therefore, the structure of 3.5 was assigned as 9,10-dihydro-2,7-dihydroxy-3,5,6-trimethoxy-8H-cyclohepta[klm]dibenzofuran.

It is noteworthy that bussealin E is the first cycloheptadibenzo[klm]furan isolated from natural sources, and the cycloheptadibenzo[klm]furan skeleton is rare among synthetic compounds; the only simple synthetic compound with this ring system is 9,10-dihydro-1-methyl-8H-cyclohepta[klm]dibenzofuran (3.6) and its 8-keto derivative.6
Table 3.2 $^1$H and $^{13}$C NMR Data for Bussealin E (3.5)$^a$

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*a.* In CDCl$_3$; $\delta$ (ppm) 600 MHz for $^1$H and 150 MHz for $^{13}$C; multiplicities; $J$ values (Hz) in parentheses.

*b.* Calculated using ACD/ChemSketch version 11.01.

*c.* In CD$_3$OD; (ppm) 600 MHz for $^1$H; multiplicities; $J$ values (Hz) in parentheses.

3.2.6 Possible biosynthesis of bussealin E (3.5)

The presence of diphenylpropanes in *B. sakalava* suggests that bussealin E is biosynthesized by oxidative coupling of an appropriate precursor diphenylpropane. This
could be followed by nucleophilic attack from a phenolate anion on a carbonyl group followed by dehydration to afford the new cycloheptadibenzofuran skeleton (3.5) as indicated in Scheme 3.1.

Scheme 3.1. Possible biosynthesis of cycloheptadibenzofuran 3.5 in B. sakalava

3.2.6 Antiproliferative activities of bussealins A-E (3.1-3.5)

The bioactivity of diphenylpropanes has been widely studied. The diphenylpropane broussonin A inhibited respiratory syncytial-virus (RSV) more effectively than the standard antiviral drug ribavirin,\(^7\) and its anti-aromatase activity has also been evaluated.\(^8\) Broussonin B moderately inhibited a chymotrypsin-like activity of the proteasome.\(^9\) The
anti-inflammatory,\textsuperscript{10-11} antifungal,\textsuperscript{12} antivascular,\textsuperscript{13} adipogenic,\textsuperscript{14} and anti-hCNT3 (human concentrative nucleoside transporter 3)\textsuperscript{15} activities of diphenylpropane analogues have also been reported. Since there have been no previous studies on the properties of diphenylpropanes on human ovarian cancer cells, we investigated the antiproliferative activity of diphenylpropanes \textbf{3.1-3.4} against the A2780 human ovarian cancer cell line. Bussealins A-D (\textbf{3.1-3.4}) showed only weak antiproliferative activities, with IC\textsubscript{50} values of 36, 24, 36, and 40 \(\mu\text{M}\), respectively. Bussealin E (\textbf{3.5}), with a new chemical skeleton, was also tested against the A2780 cell line, but it also only exhibited weak activity with an IC\textsubscript{50} value of 45 \(\mu\text{M}\). The new skeleton of bussealin E thus does not appear to confer any novel antiproliferative activity beyond that which is normal for diphenylpropanes.

\textbf{3.3 Experimental Section}

\textit{3.3.1 General experimental procedures}

UV and IR spectra were measured on a Shimadzu UV-1201 spectrophotometer and a MIDAC M-series FTIR spectrophotometer, respectively. NMR spectra were recorded in CD\textsubscript{3}OD or CDCl\textsubscript{3} on either JEOL Eclipse 500 or Bruker Avance 600 spectrometers. The chemical shifts are given in \(\delta\) (ppm) and coupling constants \((J)\) are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS. HPLC was performed on a Shimadzu LC-10AT instrument with a semi-preparative C\textsubscript{18} Varian Dynamax column (5 \(\mu\text{m}\), 250 x 10 mm).

\textit{3.3.2 Antiproliferative bioassays}
The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported, except that the samples were added in 1µL 100% DMSO per well instead of 20 µL of 1:1 DMSO:H₂O. The A2780 cell line is a drug-sensitive human ovarian cancer cell line.

3.3.3 Plant Material
A sample of root of *Bussea sakalava* Du Puy & R. Rabev. (Fabaceae) was collected on January 25, 2007, near Ambolobozobe, Madagascar at coordinates 12°31'26"S 49°31'29"E, at an elevation of 20 m. Its assigned collection number is Rakotonandrasana et al. 1079. The genus *Bussea* Harms is a small genus including 7 species (5 from Tropical Africa and 2 from Madagascar). *B. sakalava* is endemic to deciduous forest from western to northern Madagascar. The hard wood of this species is used in construction and as firewood. Voucher specimens have been deposited at the Parc Botanique and Zoologique de Tsimbazaza (TAN) and at the Centre National d'Application des Recherches Pharmaceutiques (CNARP) in Antananarivo, Madagascar; the Missouri Botanical Garden in St. Louis, Missouri (MO); and the Muséum National d'Histoire Naturelle in Paris, France (P).

3.3.4 Extraction and Isolation
Dried roots of *B. sakalava* (275 g) were ground in a hammer mill, then extracted with ethanol by percolation for 24 hours at room temperature to give the crude extract MG 4273 (14.4 g), of which 3.0 g was shipped to Virginia Polytechnic Institute and State University (VPISU) for bioassay-guided isolation. The fractionation tree is shown in
Scheme 3.2. Sample MG 4273 (IC$_{50}$ 9.6 $\mu$g/mL, 2.1 g) was suspended in aqueous MeOH (MeOH-H$_2$O, 9:1, 100 mL) and extracted with hexane (3 x 100 mL portions). The aqueous layer was then diluted to 60% MeOH (v/v) with H$_2$O and extracted with CH$_2$Cl$_2$ (3 x 150 mL portions). The hexane extract was evaporated in vacuo to leave 227 mg with an IC$_{50}$ value of 19 $\mu$g/mL. 102.9 mg of residue from the CH$_2$Cl$_2$ extract had an IC$_{50}$ of

10 $\mu$g/mL. The aqueous MeOH extract (1.7 g) was inactive. The CH$_2$Cl$_2$ extract was selected for fractionation using an SPE cartridge over C$_{18}$, and two fractions were collected. Fractions I and II (70.2 mg and 26.8 mg) had IC$_{50}$ values of 8.6 and 15 $\mu$g/mL,

Scheme 3.2 Separation of ethanol extract of *Bussea sakalava*
respectively. Fraction I was separated by C\textsubscript{18} HPLC (65% MeOH-H\textsubscript{2}O), and compounds 3.1 (3.3 mg t\textsubscript{R} 12.5 min), 3.2 (1.7 mg t\textsubscript{R} 18.6 min), 3.3 (2.0 mg t\textsubscript{R} 22.0 min), 3.4 (1.1 mg t\textsubscript{R} 29.5 min) and 3.5 (1.1 mg t\textsubscript{R} 26.5 min) were isolated. HPLC chromatogram of Fraction I is shown in Scheme 3.3.

\begin{center}
\textbf{Scheme 3.3} HPLC Chromatogram of fraction II
\end{center}

\textit{3',5',2'',3''-tetrahydroxy-4',4''-dimethoxy-1,3-diphenyl-propane (3.1)}

Off-white solid; UV (MeOH) \( \lambda_{\text{max}} \) nm (log \( \varepsilon \)) 218 (4.40), 267 (3.69), 294 (3.52); IR \( \nu_{\text{max}} \) cm\textsuperscript{-1}: 3367, 1648, 1450, 1115, 1024. \(^1\)H NMR (500 MHz, CD\textsubscript{3}OD) and \(^{13}\)C NMR (125 MHz, CD\textsubscript{3}OD), see Table 3.1; ESI-MS \( m/z \) 321.1338 [M + H]\(^+\) (calcd for C\textsubscript{17}H\textsubscript{21}O\textsubscript{6}, 321.1336).

\textit{5',2'',3''-trihydroxy-2'',4',4''-trimethoxy-1,3-diphenyl-propane (3.2)}

Off-white solid; UV (MeOH) \( \lambda_{\text{max}} \) nm (log \( \varepsilon \)) 214 (4.25), 229 (sh) (4.10), 290 (3.59) nm; IR \( \nu_{\text{max}} \) cm\textsuperscript{-1}: 3332, 1599, 1444, 1095, 1032; \(^1\)H NMR (500 MHz, CD\textsubscript{3}OD) and \(^{13}\)C NMR (125 MHz, CD\textsubscript{3}OD), see Table 3.1; ESI-MS \( m/z \) 335.1512 [M + H]\(^+\) (calcd for C\textsubscript{18}H\textsubscript{23}O\textsubscript{6}, 335.1495).
3',2'',3''-Trihydroxy-4',4''-dimethoxy-1,3-diphenyl-propane (3.3)

Off-white solid; UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 208 (4.15), 267 (3.54), 289 (3.47) nm; IR $\nu_{\text{max}}$ cm$^{-1}$: 3338, 1656, 1450, 1115, 1024; $^1$H NMR (500 MHz, CD$_3$OD) and $^{13}$C NMR (125 MHz, CD$_3$OD), see Table 3.1; ESI-MS m/z 305.1384 [M + H]$^+$ (calcd for C$_{17}$H$_{21}$O$_5$, 305.1389).

3',2'',3''-Trihydroxy-4',4''-dimethoxy-1,3-diphenyl-propane (3.4)

Off-white solid; UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 210 (4.21), 229 (sh) (4.01), 289 (3.48) nm; IR $\nu_{\text{max}}$ cm$^{-1}$: 3350, 1602, 1450, 1115, 1026; $^1$H NMR (500 MHz, CD$_3$OD) and $^{13}$C NMR (125 MHz, CD$_3$OD), see Table 3.1; ESI-MS m/z 349.1648 [M + H]$^+$ (calcd for C$_{19}$H$_{25}$O$_6$, 349.1651).

9,10-Dihydro-2,7-dihydroxy-3,5,6-trimethoxy-8H-cyclohepta[klm]dibenzo[ffuran (3.5)

Off-white solid; UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 218 (4.25), 270 (3.78), 294 (3.73), 316 (3.47)) nm; IR $\nu_{\text{max}}$ cm$^{-1}$: 3332, 1567, 1449, 1115, 1024; $^1$H NMR (600 MHz, CD$_3$OD and CDCl$_3$) and $^{13}$C NMR (150 MHz, CD$_3$OD), see Table 3.2; ESI-MS m/z 331.1181 [M + H]$^+$ (calcd for C$_{18}$H$_{19}$O$_6$, 331.1182).

References


9. Tsukamoto, S.; Wakana, T.; Koimaru, K.; Yoshida, T.; Sato, M.; Ohta, T., 7-Hydroxy-3-(4-hydroxybenzyl)chroman and broussonin B: Neurotrophic compounds,


IV. Cardenolides of *Leptadenia madagascariensis* from the Madagascar dry forest

This chapter is a slightly expanded version of a published article.² Attributions from co-authors of the article are described as follows in the order of the names listed. The author of this dissertation (Mr. Ende Pan) conducted isolation and structure elucidation of
the titled compounds, and drafted the manuscript. Dr. Liva Harinantanaina was a mentor for this work, and in particular, he provided invaluable advice and hints for structure elucidation of those compounds, and he also proofread the manuscript before submission. Ms. Peggy Brodie performed the A2780 bioassay on the isolated fractions and compounds. Dr. Martin Callmender and Dr. Stephan Rakotonandrasana from Missouri Botanical Garden did the plant collections and identification. Dr. Etienne Rakotobe, and Dr. Vincent E. Rasamison from Madagascar carried out the initial plant extraction. Dr. Karen TenDyke, Dr. Yongchun Shen, and Dr. Edward M. Suh from Eisai Inc. performed the H460 bioassay on the isolated compounds. Dr. David G. I. Kingston was a mentor for this work and the corresponding author for the published article. He provided critical suggestions for this work and crucial revisions to the manuscript.

4.1 Introduction

In our continuing search for biologically active natural products from tropical rainforests as part of an International Cooperative Biodiversity Group (ICBG) program,3-4 we obtained an EtOH extract from the roots of the plant *Leptadenia madagascariensis* Decne. (Apocynaceae) from Madagascar. The extract exhibited good antiproliferative activity against the A2780 human ovarian cancer cell line, with an IC₅₀ value of 10 μg/mL. On the basis of this activity and the absence of any previous phytochemical studies on this species, the extract was selected for fractionation to isolate its active components by bioassay-guided fractionation.

4.1.1 Previous Investigations of *Leptadenia*
There are about twenty species in the genus *Leptadenia*, some of which are used in traditional medicine in Africa and India.\textsuperscript{5-7} Previous phytochemical investigations reported the presence of flavonoids \textsuperscript{Error! Bookmark not defined.}, terpenoids\textsuperscript{6, 8, 10-11} and polyoxypregnane esters\textsuperscript{5, 12}

![Chemical structures](image)

**Figure 4.1** Compounds from the genus *Leptadenia*

pregnane glycosides\textsuperscript{13-14}, cardiac glycosides\textsuperscript{15} and alkaloids\textsuperscript{16} in these species. The medicinal use of plants containing cardiac glycosides was recorded as early as 1500 years ago. As an important class of natural products, cardiac glycosides are widely used for treating cardiac failure,\textsuperscript{17} and their cardiac activities and cytotoxicities are well known.\textsuperscript{Error! Bookmark not defined.} What is less well known is the fact that they
are also beginning to find use in cancer chemotherapy, and the first generation of anticancer cardiac glycosides is in clinical trials.20-21

4.2 Results and Discussion

The EtOH extract of the stems and leaves of L. madagascariensis was subjected to liquid-liquid partitioning to give active dichloromethane and MeOH fractions with IC$_{50}$ values in the A2780 assay of 0.28 and 2.4 µg/mL, respectively. Fractionation by C$_{18}$ open column and High Performance Liquid Chromatography (HPLC) on the MeOH fraction yielded two new cardenolides named madagascarensilide A (4.1) and madagascarensilide B (4.2) (Figure 4.2). Similar purification of the CH$_2$Cl$_2$ fraction yielded the two additional new compounds madagascarensilides C and D (4.3 and 4.4, Figure 3). Herein we report the structural elucidation and the antiproliferative properties of the four isolates.

4.2.1 Structure elucidation of madagascarensilide A (4.1)

Madagascarensilide A (4.1) was obtained as a white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at m/z 849.4255 [M+Na]$^+$, corresponding to a molecular formula of C$_{42}$H$_{66}$O$_{16}$ for 4.1. Its $^1$H NMR spectrum in CD$_3$OD showed signals at $\delta$H 5.04 dd ($J = 18.6, 1.8$ Hz), 4.92 dd ($J = 18.6, 1.8$ Hz), and 5.90 s, characteristic of an $\alpha, \beta$-unsaturated $\gamma$ lactone (Table 4.1). In addition, three anomeric proton signals were observed at $\delta$H 4.91 dd ($J = 9.5, 1.8$ Hz), 4.57 d ($J = 7.7$ Hz) and 4.37 d ($J = 7.7$ Hz). The $^{13}$C NMR spectrum contained 42 signals, which included signals for one methoxyl, four methyls, 12 methylenes (including one oxymethylene), 20 methines (including 15
oxymethines and one olefinic carbon), and five quaternary carbons (including one oxyquaternary carbon, one olefinic carbon and one carbonyl carbon), as indicated by an HMQC spectrum (Table 4.1). The above data suggested that \textbf{4.1} is a cardiac glycoside with three sugar moieties.

\textbf{Figure 4.2} Chemical structures of madagascarsilides A (\textbf{4.1}) and B (\textbf{4.2}).

In the aglycone of \textbf{4.1}, two spin systems CH₂-CH₂-CH-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂ (H₂-1 through H₂-2, H-3, H₂-4, H-5, H₂-6, H₂-7, H-8, H-9 and H₂-11 to H₂-12) in rings A, B and C, and CH₂-CH₂-CH (H₂-15 through H₂-16 to H-17) in ring D (Figure 4.2) were identified in the COSY and TOCSY spectra. The connectivities of rings A, B, C and D were assigned based on the interpretation of the HMBC spectrum. Long-range correlations from H₃-19 to C-1, C-5, C-9 and C-10, and from H₂-1 to C-9 indicated the connectivity of rings A and B. The relationship between rings C and D was established by the observation of correlations from H₃-18 to C-12, C-13, the oxygenated quaternary carbon at C-14 and C-17, as well as those observed from H₂-12 to C-17, and H₂-15 to C-8. Moreover, the \(\alpha\), \(\beta\)-unsaturated \(\gamma\) lactone was deduced to be connected to C-17 by the HMBC correlation from H-17 to C-20, C21 and C22. The Rotating frame Overhauser
Effect SpectroscopY (ROESY) correlation between H$_3$-19 and H-5 indicated that rings A and B are *cis* fused, while the *trans* orientation of H-8 and H-9 was deduced from the presence of correlations between H$_3$-18 and H$_3$-19 to H-8 (Figure 4.3). The correlations of H$_3$-18 to C-14-OH, H-21, and H-22 in the ROESY spectrum in deuterated pyridine indicated *cis* fused C and D rings and the β-orientation of the γ-lactone at C-17. These data, together with a comparison of the $^{13}$C NMR data of 4.1 with those of the aglycone of coroloside and similar digitoxigenin glycosides, established the aglycone of 4.1 as digitoxigenin.$^{22}$

The presence of three sugar units in 4.1 was indicated by the presence of three anomeric proton signals at $\delta_H$ 4.91, 4.57 and 4.37. Their spin systems were determined by COSY and TOCSY correlations: H-1'-H$_2$-2'-H-3'-H-4'-H-5'-H$_3$-6', H-1''-H-2''-H-3''-H-4''-H-5''-H$_3$-6'', and H-1'''-H-2'''-H-3'''-H-4'''-H-5'''-H$_2$-6''. In addition, HMBC correlations from H-1' to C-3, H-1'' to C-4', and H-1''' to C-4'' built up the connectivity of the sugar units from C-1' to C-3, C-1'' to C-4' and C-1''' to C-4''. The relative conformations of the sugar moieties were determined by the coupling constants of the sugar protons and by analysis of the ROESY data. In the $^1$H NMR spectrum of 4.1 in deuterated pyridine, the coupling constants observed for H-1' (d, $J = 9.5$, 1.8) and for H-4' (dd, $J = 9.7$, 2.7) as well as the clear ROESY correlation between H-1' and H-5' indicated that H-1', H-4' and H-5' are all axial, and so H-3' must be equatorial based on its 2.7 Hz coupling constant with H-4'.

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The HMBC correlation between the methoxy protons at 3.69 ppm and C-3'' ($\delta_C$ 85.6) placed the methoxy group at C-3''. In the same manner, the coupling constants observed for H-1" (d, $J = 7.7$ Hz), H-3" (dd, $J = 9.7, 2.9$ Hz) and the ROESY correlation of H-5" with H-1" indicated that H-1", H-2", H-3", H-5" are axial, while H-4" is equatorial. The coupling constants observed for the third sugar unit for H-1"" (d, $J = 7.8$ Hz) and H-2"" (t, $J = 7.8$ Hz), with ROESY correlations from H-1"" to H-3"" and H-5"", as well as from H-2"" to H-4"", led to the conclusion that all the protons in this sugar unit must be axial,
indicating it to be glucopyranose. Therefore, the structure of 4.1 was determined to be digitoxygenin 3-O-β-glucopyranosyl-(1->4)-O-β-digitalopyranosyl-(1->4)-O-β-digitoxopyranoside. The absolute stereochemistry of the glucose unit was assigned as D since L-glucose has never been observed in cardenolides. The digitoxose and digitalose units were also assigned as D-sugars based on their occurrence in other cardenolides in the D-form.

4.2.2 Structure elucidation of madagascarensilide B (4.2)

Madagascarensilide B (4.2) was obtained as a white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \( m/z \) 687.3728, corresponding to a molecular formula of \( C_{36}H_{56}O_{11} \) for 4.2. The \(^1\)H NMR data of 2 in CD\(_3\)OD were very similar to those of 4.1, and thus its structure was indicated to be a cardiac glycoside with two sugar units. The \(^1\)H and \(^13\)C NMR data arising from the aglycone and the sugar moiety attached at C-3 of 4.2 were essentially superposable with those of 4.1. In addition, the spin system H-1''-H-2''-H-3''-H-4''-H-5''-H3-6'' was identified by analysis of the COSY data (Table 4.1). The HMBC correlation observed between the methoxy protons and C-3'' indicated that the second sugar moiety shares the same planar structure as the second sugar of 4.1. The coupling constants observed at H-1'' (d, \( J = 7.7 \) Hz), H-2'' (dd, \( J = 9.7, 7.7 \) Hz), H-3'' (dd, \( J = 9.7, 3.1 \) Hz) and H-5'' (brq, \( J = 6.4 \) Hz), together with the ROESY correlation between H-1'' and H-5'' indicated that H-1'', H-2'', H-3'' and H-5'' are axial while H-4'' is equatorial. Thus the structure of 4.2 was assigned as digitoxigenin 3-O-β-digitalopyranosyl-(1->4)-O-β-digitoxopyranoside.

4.2.3 Structure elucidation of madagascarensilide C (4.3)
Madagascarensilide C (4.3) was obtained as a white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \( m/z \) 845.4315 \([\text{M}+\text{Na}]^+\) corresponding to a molecular formula of \( \text{C}_{43}\text{H}_{66}\text{O}_{15} \) for 4.3. The \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra in deuterated pyridine indicated compound 4.3 to be a cardenolide with three sugar units, with signals for three anomic protons at \( \delta_H \) 5.39, 5.17, and 4.69 ppm and one aldehyde at \( \delta_H \) 10.42 (Table 4.1). In the aglycone of 4.3, three spin systems: \( \text{CH}_2\text{-CH}_2\text{-CH-CH}_2 \) (H-2' through H-2, H-3 to H-4) for ring A; \( \text{CH}_2\text{-CH}_2\text{-CH-CH}_2\text{-CH}_2 \) (H-6 through H-7, H-8, H-9, H-11 to H-12) in rings B and C; and \( \text{CH}_2\text{-CH}_2\text{-CH} \) (H-15 through H-16 to H-17) in ring D were identified by COSY and TOCSY spectra. The connectivities of rings A, B, C and D were assigned based on an analysis of HMBC data. The long-range correlations arising from H-19 at \( \delta_H \) 10.42 to C-1 and H-9 at \( \delta_H \) 1.78 to C-19 at \( \delta_C \) 208.9, as well as the correlations from \( \text{H}_\alpha\)-4 and \( \text{H}_\beta\)-6 to C-10, and from the hydroxyl group signal at C-5 to C-4 and C-6, indicate the connectivity of rings A and B. Meanwhile, the relationship between rings C and D was established by the observation of correlations from H-18 to C-12, C-13, C-14 and C-17, and from the hydroxy group at C-14 to C-8, C-13, C-14 and C-15. The \( \alpha,\beta\)-unsaturated \( \gamma\)-lactone was determined to be at C-17 by the HMBC correlation from H-17 to C-20, C21 and C22. Moreover the assigned \(^{13}\text{C}\) NMR chemical shifts of the aglycone of 4.3 in CD\(_3\)OD (Table 4.1) are very similar to those of strophanthidin.\(^{24}\) From the above data, the planar structure of the aglycone of 3 was deduced to be strophanthidin.

The spin systems H-1'-H2'-H-3'-H-4'-H-5'-H3-6', H-1"-H2"-H-3"-H-4"-H-5"-H3-6", and H-1'''-H2'''-H-3'''-H-4'''-H-5'''-H3-6''' of the sugar units were assigned by COSY and TOCSY correlations. Long-range correlations from H-1' to C-3, H-1" to C-4', and H-1'''
to C-4" established the connectivity of the sugar units as depicted in Figure 4.4. The methoxy groups at C-3" and C-3'" were substantiated by observation of HMBC correlations in deuterated pyridine between the methoxy signals (δ_H 3.51 and 3.40, each singlet) and the two carbon signals at δ_C 78.1 and 79.3 (C-3" and C-3'", respectively). The relative conformations of the sugar moieties were determined by analysis of the ROESY data of 4.3 and the coupling constants of the sugar protons. The values of the coupling constants of H-1' (J = 9.6, 1.9 Hz) and H-4' (J = 9.7, 3.0 Hz) and the clear ROESY correlation between H-1' and H-5' indicated that H-1', H-4' and H-5' are axial, while H-3' is equatorial. The coupling constants of H-1" (dd, J = 9.7, 1.8 Hz) and H-4" (dd, J = 9.7, 2.7 Hz) and the ROESY correlation between H-5" and H-1" suggested that H-1", H-4", and H-5" are axial, while H-3" is equatorial. Similarly, the coupling constants of H-1''' (dd, J = 9.7, 2.1 Hz), H-3''' (ddd, J = 12.1, 4.7, 2.9 Hz) and H-5''' (qd, J = 6.4, 1.3 Hz), and the ROESY correlation between H-1''' and H-5''' indicate that H-1''', H-3''' and H-5''' are axial and H-4''' is equatorial. Therefore, the structure of 4.3 was determined as strophanthidin 3-O-β-diginopyranosyl-(1->4)-O-β-cymaropyranosyl-(1->4)-O-β-digitoxopyranoside.
Table 4.1 $^1$H and $^{13}$C NMR chemical shifts of madagascarensilides A (4.1), B (4.2), C (4.3) and D (4.4)\textsuperscript{a}

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<td>5.44 dd (9.5, 1.8)</td>
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<td>4'</td>
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<td>1.67 d (6.2)</td>
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\(^a\) \(\delta\) (ppm) 500 MHz for \(^1\)H and 125 MHz for \(^13\)C; multiplicities; \(J\) values (Hz) in parentheses.

\(^b\) In CD3OD

\(^c\) In deuterated pyridine
4.2.4 Structure elucidation of madagascarensilide A (4.4)

Madagascarensilide D (4.4) was obtained as a white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \( m/z \) 1007.4844 [M+Na]\(^+\) corresponding to a molecular formula of \( \text{C}_{49}\text{H}_{76}\text{O}_{20} \) for 4.4. The \(^1\)H and \(^{13}\)C NMR spectroscopic data of 4.4 were very similar to those of 4.3, suggesting that 4.4 is a cardenolide derivative with four sugar units (Table 4.1). Inspection of the carbon chemical shifts of 4.4 revealed a close similarity to those of 3 except for the downfield shifts of the signals arising from C-3'' (+1.4) and C-4'' (+6.9) and the upfield shift of the signal of C-5'' (-0.2). These data indicated that the additional sugar unit in 4.4 is linked to the third sugar, and the position of attachment of this fourth sugar unit was confirmed to be at C-4'' due to observation of a clear HMBC correlation between H-1''' and C-4''. The \(^{13}\)C NMR data of the fourth sugar unit (\( \delta_C \) 104.6, 76.0, 78.2, 71.8, 78.0, 63.0) were very similar to the those of the terminal \( \beta \)-glucopyranosyl unit of the tetrasaccharide adoligose B (\( \beta \)-Glc-\( \beta \)-Dgn-\( \beta \)-Cym-\( \beta \)-Cym),\(^{25}\) indicating the sugar moieties of 4.4 to be \( \beta \)-Glc-\( \beta \)-Dgn-\( \beta \)-Cym-\( \beta \)-Dgx. Compound 4.4 is thus strophanthidin 3-\( O-\beta \)-glucopyranosyl-(1->4)-\( O-\beta \)-diginopyranosyl-(1->4)-\( O-\beta \)-cymaropyranosyl-(1->4)-\( O-\beta \)-digitoxopyranoside.
4.2.5 *Antiproliferative activities of madagascarensilide A-D (4.1-4.4)*

Madagascarensilide A (4.1), B (4.2), C (4.3) and D (4.4) were tested for antiproliferative activity against the A2780 human ovarian cancer cell line. Compounds 4.1 and 4.3 were the most potent, having an IC$_{50}$ value of 0.18 and 0.17 µM, while compounds 4.2 and 4.4 were slightly less potent, with IC$_{50}$ values of 0.21 and 0.29 µM, respectively. It appears that the aldehyde group at position 10 between rings A and B on the aglycone does not significantly affect the activity of cardenolides against A2780 cells.
Cardenolides 4.1-4.4 were also evaluated in the H460 human lung cancer cell line. Madagascarensilide A (4.1) showed strong activity with a IC₅₀ value of 0.16 μM. Madagascarensilide B (4.2), C (4.3) and D (4.4) were also active with IC₅₀ values of 0.68, 0.37, and 0.48 μM, respectively.

4.3 Experimental Section

4.3.1 General experimental procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. UV and IR spectra were measured on a Shimadzu UV-1201 spectrophotometer and a MIDAC M-series FTIR spectrophotometer, respectively. NMR spectra were obtained in CD₃OD or deuterated pyridine on either JEOL Eclipse 500 or Bruker Avance 600 spectrometers. The chemical shifts are given in δ (ppm) and coupling constants (J) are reported in Hz. Mass spectra were obtained on an Agilent 6220 TOF Mass Spectrometer. HPLC was performed on a Shimadzu LC-10AT instrument with a semi-preparative C₁₈ Varian Dynamax column (5 μm, 250 x 10 mm).

4.3.2 Antiproliferative bioassays

The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported, except that the samples were added in 1 μL 100% DMSO per well instead of 20 μL of 1:1 DMSO:H₂O. The A2780 cell line is a drug-sensitive human ovarian cancer cell line. Assays against the NCI-H460 lung cancer cell line were carried out at Eisai, Inc., as previously described for similar cell lines.
4.3.3 Plant material

A sample of the roots of *Leptadenia madagascariensis* Decne. (Apocynaceae) was collected in 2007 2 km west of the village of Ambolobozobe, Madagascar, in degraded dry forest. Collection coordinates were 12° 31' 26" S, 49° 31' 29" E, and elevation 20 m. Voucher specimens have been deposited at the Parc Botanique and Zoologique de Tsimbazaza (TAN) and at the Centre National d'Application des Recherches Pharmaceutiques (CNARP) in Antananarivo, Madagascar; the Missouri Botanical Garden in St. Louis, Missouri (MO); and the Muséum National d'Histoire Naturelle in Paris, France (P), voucher number SR1092.

4.3.4 Extraction and Isolation

Dried roots of *Leptadenia madagascariensis* (275 g) were ground in a hammer mill, then extracted with EtOH by percolation for 24 hours at room temperature to give the crude extract MG 4294 (12.7 g), of which 3.34 g was shipped to Virginia Polytechnic Institute and State University (VPISU) for bioassay guided isolation. The fractionation tree is shown in Scheme 4.1. The extract MG 4294 (IC$_{50}$ 3.6 µg/mL, 2.0 g) was suspended in aqueous MeOH (MeOH-H$_2$O, 9:1, 100 mL) and extracted with hexane (3 x 100 mL portions). The aqueous layer was then diluted to 60% MeOH (v/v) with H$_2$O and extracted with CH$_2$Cl$_2$ (3 x 150 mL portions). The hexane extract was evaporated *in vacuo* to leave 352 mg with an IC$_{50}$ value of 15 µg/mL. The residue from the CH$_2$Cl$_2$ extract (223 mg) had an IC$_{50}$ of 0.28 µg/mL and the aqueous MeOH extract (1.22 g) had an IC$_{50}$ of 2.4 µg/mL. Fractionation of the aqueous MeOH extract by C$_{18}$ open column
gave the four fractions I - IV (987.6, 70.4, 11.9 and 5.8 mg), with IC\textsubscript{50} values of 10, 0.14, 16 and 14 μg/mL, respectively.

Scheme 4.1 Separation of ethanol extract of *Leptadenia madagascariensis*

The most active fraction (fr-II) was separated further by C\textsubscript{18} HPLC (solvent system: MeOH-H\textsubscript{2}O 70:30), and compounds 4.1 (2.8 mg, t\textsubscript{R} 15.5 min) and 4.2 (0.7 mg, t\textsubscript{R} 21.4 min) were isolated. Fractionation of the CH\textsubscript{2}Cl\textsubscript{2} extract on a C\textsubscript{18} open column gave the
five fractions A - E (11.5, 67.3, 47.8, 44.6, and 8.8 mg) with IC\textsubscript{50} values: >20, 0.11, 0.12, 0.97, and 6.9 µg/mL, respectively. Fraction B was selected for further purification by C\textsubscript{18}-HPLC (solvent system: gradient from MeOH: H\textsubscript{2}O 60:40 to 70:30 for 40 min) to afford compounds \textnumero4.3 (1.5 mg, t\_R 38.7 min) and \textnumero4.4 (5.0 mg, t\_R 28.0 min).

*Digitoxigenin 3-O-β-glucopyranosyl-(1->4)-O-β-digitalopyranosyl(1->4)-O-β-digitoxopyranoside (4.1, madagascarensilide A)*

Compound \textnumero4.1 was a white solid; [α]_D\textsuperscript{23} +11 (c 0.28, MeOH); UV (MeOH) λ\textsubscript{max} nm (log ε) 215 (4.1); IR ν\textsubscript{max} cm\textsuperscript{-1}: 3396, 2934, 1739, 1449, 1372, 1073 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (500 MHz, CD\textsubscript{3}OD, \textit{d}-pyridine) and \textsuperscript{13}C NMR (125 MHz, CD\textsubscript{3}OD, \textit{d}-pyridine), see Table 4.1; HRESI-MS m/z 849.4255 [M+Na]+ (calcd for C\textsubscript{42}H\textsubscript{66}NaO\textsubscript{16} 849.4249).

*Digitoxigenin 3-O-β-digitalopyranosyl-(1->4)-O-β-digitoxopyranoside (4.2, madagascarensilide B)*

Compound \textnumero4.2 was a white solid; [α]_D\textsuperscript{23} +16 (c 0.07, MeOH); UV (MeOH) λ\textsubscript{max} nm (log ε) 215 (3.6); IR ν\textsubscript{max} cm\textsuperscript{-1}: 3224, 2940, 1739, 1595, 1355, 1078 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (500 MHz, CD\textsubscript{3}OD) and \textsuperscript{13}C NMR (125 MHz, CD\textsubscript{3}OD, \textit{d}-pyridine), see Table 4.1; HRESI-MS m/z 687.3728 [M+Na]+ (calcd for C\textsubscript{36}H\textsubscript{56}NaO\textsubscript{11} 687.3720).

*Strophanthidin 3-O-β-diginopyranosyl-(1->4)-O-β-cymaropyranosyl(1->4)-O-β-digitoxopyranoside (4.3, madagascarensilide C)*

Compound \textnumero4.3 was a white solid; [α]_D\textsuperscript{23} +21 (c 0.15, MeOH); UV (MeOH) λ\textsubscript{max} nm (log ε) 215 (3.9); IR ν\textsubscript{max} cm\textsuperscript{-1}: 3459, 2939, 1740, 1374, 1072 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (500 MHz,
d-pyridine) and $^{13}$C NMR (125 MHz, d-pyridine), see Table 4.1; HRESI-MS $m/z$ 845.4315 [M+Na]$^+$ (calcd for C$_{43}$H$_{66}$NaO$_{15}$ 845.4299).

_Strophanthidin_ 3-O-$\beta$-glucopyranosyl-(1$\to$4)-O-$\beta$-diginopyranosyl-(1$\to$4)-O-$\beta$-cymaropyranosyl-(1$\to$4)-O-$\beta$-digitoxopyranoside (4.4, madagascarensilide D)

Compound 4.4 was a white solid; $[\alpha]_D^{23} +16$ (c 0.51, MeOH); UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 216 (4.1); IR $\nu_{\text{max}}$ cm$^{-1}$: 3445, 2937, 1736, 1372, 1074 cm$^{-1}$. $^1$H NMR (500 MHz, CD$_3$OD) and $^{13}$C NMR (125 MHz, CD$_3$OD), see Table 4.1; HRESI-MS $m/z$ 1007.4844 [M+Na]$^+$ (calcd for C$_{49}$H$_{76}$NaO$_{20}$ 1007.4828).

References


V. Isolation and Synthesis of Antiproliferative Eupolauridine Alkaloids of *Ambavia gerrardii* from the Madagascar Dry Forest

This chapter is a slightly expanded version of a prepared manuscript.\(^1\) Attributions from co-authors of the article are described as follows in the order of the names listed. The author of this dissertation (Mr. Ende Pan) conducted isolation and structure elucidation of the titled compounds, and drafted the manuscript. Dr. Shugeng Cao was a mentor for this work, and in particular, he provided invaluable advice and hints for structure elucidation of those compounds, and he also proofread the manuscript before submission. Ms. Peggy Brodie performed the A2780 bioassay on the isolated fractions and compounds. Dr. Martin Callmander and Dr. Stephan Rakotonandrasana from Missouri Botanical Garden did the plant collections and identification. Dr. Etienne Rakotobe, and Dr. Vincent E. Rasamison from Madagascar carried out the initial plant extraction. Dr. Karen TenDyke, Dr. Yongchun Shen, and Dr. Edward M. Suh from Eisai Inc. performed the H460 bioassay on the isolated compounds. Dr. David G. I. Kingston was a mentor for this work and the corresponding author for the published article. He provided critical suggestions for this work and crucial revisions to the manuscript.

5.1 Introduction

In our continuing search for biologically active natural products from tropical rainforests as part of an International Cooperative Biodiversity Group (ICBG) program, we obtained an ethanol extract from the root of a plant identified as *Ambavia gerrardii* (Baill.) Le Thomas (Annonaceae) from Madagascar. The extract exhibited good antiproliferative activity against the A2780 human ovarian cancer cell line with an IC\(_{50}\)
value of 10 μg/mL. On the basis of the activity and the absence of previous phytochemical study on this species, *A. gerrardii* was selected for fractionation to isolate its active components under the guidance of our bioassay.

### 5.1.1 Previous investigations of Annonaceae

The genus *Ambavia* (Annonaceae) consists of only two species, *A. capuronii* and *A. gerrardii*, both of which are endemic to Madagascar. Previous phytochemical studies of plant species in Annonaceae family have revealed the presence of cytotoxic acetogenins, miliusanes, styrylpyrones, polyacetylenes, diterpenoids, flavanones, and alkaloids.

![Compounds isolated from the family Annonaceae](image)

**Figure 5.1.** Compounds isolated from the family Annonaceae

### 5.2 Results and Discussion
An EtOH extract of the roots of *A. gerrardii* was subjected to liquid-liquid partitioning between hexanes, CH$_2$Cl$_2$ and MeOH to give fractions with IC$_{50}$ values of 14 µg/mL, 2.6 µg/mL and >100 µg/mL, respectively, in the A2780 assay. Fractionation of the active CH$_2$Cl$_2$ fraction by C$_{18}$ open column and high performance liquid chromatography (HPLC) yielded the two new eupolauridine derivatives **5.1** and **5.2**, as well as eupolauridine N-oxide (**5.3**), 8-methoxysampangin (**5.4**), eupolauridine (**5.5**) and sampangin (**5.6**). The structures of **5.1** and **5.2** were proposed on the basis of their UV, IR, HRESIMS and $^1$H NMR spectra data, but the limited samples available combined with the absence of $^{13}$C NMR data and HMBC correlations made it necessary to confirm the structures by synthesis. The synthesis also provided material for the evaluation of the biological activities of **5.1** and **5.2**. Herein we report the isolation and synthesis of **5.1** and **5.2** as well as the bioassay activities of all isolates.

![Chemical structures of compounds 5.1-5.6](image)

**Figure 5.2.** Chemical structures of compounds 5.1-5.6

Eupolauridine N-oxide (**5.3**),$^{11}$ 8-methoxysampangin (**5.4**),$^{12}$ eupolauridine (**5.5**)$^{11}$ and sampangin (**5.6**)$^{13-15}$ were all obtained as yellow solids. 8-Methoxysampangin was
isolated from a natural source for the first time. Eupolauridurine has been previously isolated from the annonaceous plant *Cananga odorata* (Ylang ylang) collected in Madagascar,\textsuperscript{16} and from the eupomatiaceous plant *Eupomatia laurina*.\textsuperscript{17}

### 5.2.1 Structure elucidation of compound 5.1

Compound 5.1 was obtained as a yellow solid. Its UV absorptions in MeOH, with $\lambda_{\text{max}}$ (log $\varepsilon$) 220 (4.15), 240 (3.98), 287 (3.82), 351 (3.28), and 369 (3.35) nm, indicated the presence of an extended aromatic chromophore, and were similar to that of eupolauridurine 5.5. The IR spectroscopic data of compound 5.1, which showed absorptions at 1599, 1580, 1398, 1378, 1202, 848 and 808 cm\textsuperscript{-1}, confirmed the existence of the aromatic and C-O functions. The positive ion HRESIMS of 5.1 revealed a pseudomolecular ion peak at $m/z = 221.0702$ [M+H]\textsuperscript{+} corresponding to a molecular formula of C\textsubscript{14}H\textsubscript{10}N\textsubscript{2}O\textsubscript{2}, (caled for C\textsubscript{14}H\textsubscript{9}N\textsubscript{2}O\textsubscript{2}: 221.0715). $^1$H NMR signals 5.1 in CD\textsubscript{3}OD exhibited AB, A'B' and A"B"X" multiplets at low field: $\delta_H$ 8.64 d (1H, $J = 6.1$ Hz), 8.55 (1H, d, $J = 6.1$ Hz), 7.78 (1H, d, $J = 8.2$ Hz), 7.61 (1H, d, $J = 6.1$ Hz), 7.51 (1H, d, $J = 6.1$ Hz), 7.42 (1H, d, $J = 2.3$ Hz), 6.86 (1H, dd, $J = 8.2$, 2.3 Hz)]. The above data together with the fact that 5.1 and 5.5 (C\textsubscript{14}H\textsubscript{10}N\textsubscript{2}) have the same unsaturation number (12) suggested that compound 5.1 was a hydroxylated eupolauridurine. The position of the hydroxyl group was assigned at C-8 from the coupling patterns of the protons of the benzene ring of 5.1 [$\delta_H$ 7.78 (1H, d, $J = 8.2$ Hz), 6.86 (1H, dd, $J = 8.2$, 2.3 Hz) and 7.42 (1H, d, $J = 2.3$ Hz)] and comparison of the $^1$H-NMR spectroscopic data of 5.1 with those of 5.5 [$\delta_H$ 8.71 (2H, d, $J = 6.0$ Hz), 8.01 (2H, m), 7.67 (2H, d, $J = 6.0$ Hz), 7.55 (2H, m)]. Based on the above data, we proposed the structure of 8-hydroxyeupolauridurine for compound 5.1.
5.2.2 Structure elucidation of compound 5.2

Compound 5.2 was also obtained as a yellow solid. It showed strong UV absorptions (MeOH) at $\lambda_{\text{max}}$ (log $\varepsilon$) 225 (4.03), 254 (3.99), 292 (3.87), and 384 (3.36) nm. Its IR spectrum displayed aromatic absorptions at 1611, 1593, 1569, 1451, 1423, 846, 828 cm$^{-1}$, a C-O stretch absorption at 1022 cm$^{-1}$, and N-O stretch absorptions at 1487 and 1380 cm$^{-1}$. The characteristic alkyl C-H stretch absorptions were also observed at 2919 and 2851 cm$^{-1}$. The positive ion HRESIMS of 5.2 displayed a pseudomolecular ion peak at $m/z = 251.0811$ [M+H]$^+$, corresponding to a molecular formula of $C_{15}H_{11}N_2O_2$, (calcd for $C_{15}H_{11}N_2O_2$: 251.0821). Its proton spectrum [AB, A'B' and A"B"X" aromatic coupling pattern and one OCH$_3$ group: $\delta_H$ 8.58 (1H, d, $J = 6.0$ Hz), 8.23 (1H, d, $J = 7.1$ Hz), 7.95 (1H, d, $J = 8.2$ Hz), 7.92 (1H, d, $J = 2.4$ Hz), 7.79 (1H, d, $J = 7.1$ Hz), 7.57 (1H, d, $J = 6.0$ Hz), 7.09 (1H, dd, $J = 8.2$, 2.4 Hz)] was very similar to that of 5.1 except for the presence of a signal for an additional methyl group at $\delta$ 3.96 (3H, s). The above spectroscopic data thus suggested compound 5.2 to be either 8-methoxyeupolauridine 1-oxide or 9-methoxyeupolauridine 1-oxide, but the available data did not permit a distinction between these two structures. It was thus necessary to synthesize 5.1 and 5.2 to confirm the structure of 5.1 and to provide additional material for bioassay, and to determine the position of the N-oxide on 5.2.

5.2.3 Synthesis of compounds 5.1 and 5.2

The synthesis of 8-hydroxyeupolauridine initially followed Wong’s method for synthesizing eupolauridine, using 5-methoxy-1,3-indanedione as the starting material.
instead of the 1,3-indanedione of the published method. The key step to afford 7-methoxyonychin (5.9) involved the thermal rearrangement of an oxime O-crotyl ether, and this gave a very low yield of product (<10%) in our hands. We thus changed the synthetic strategy by modifying Bracher’s synthesis of eupolauridine (Scheme 1). Ethyl 3-(4-methoxyphenyl)-3-oxopropionate was deprotonated by NaH, which then underwent Michael addition with crotonaldehyde to afford the intermediate α-(1-methyl-3-oxopropyl)-β-oxo-4-methoxyl-benzenepropanoic acetate, which reacted with hydroxylamine hydrochloride to give ethyl 2-(4-methoxy)phenyl-4-methyl-3-pyridinecarboxylate 5.7.

![Scheme 5.1 Synthesis of 5.1, 5.2, and 5.11.](image)

Although polyphosphoric acid was reported to catalyze the formation of onychin from ethyl 2-phenyl-4-methyl-3-pyridinecarboxylate in good yield, a very low yield of product was obtained when 5.7 was used as a substrate in the study. Compound 5.7 was
thus hydrolyzed to the corresponding nicotinic acid 5.8, which was then converted to its acyl chloride by treatment with thionyl chloride. A Friedel-Crafts reaction of the acyl chloride in chlorobenzene under reflux gave 7-methoxy onychin 5.9. The acidic methylene group of 5.9 reacted with dimethylformamide diethyl acetal to afford an enamine intermediate, which yielded 8-methoxy eupolauridine 5.10 through a ring closing reaction of the intermediate in the presence of ammonium acetate at 140 ºC. Cleavage of the methyl ether of 5.10 in 48% hydrobromic acid gave 8-hydroxyeupolauridine in 1.7 % overall yield from ethyl 3-(4-methoxyphenyl)-3-oxopropionate. Comparison of the 1H NMR (Table 5.1) and HRESIMS data of 8-hydroxyeupolauridine with the data of compound 5.1 confirmed the proposed structure of the isolated natural product.

Mono-oxidation of 8-methoxyeupolauridine 5.10 by one equivalent of *meta*-chloroperoxybenzoic acid yielded two methoxyeupolauridine N-oxides in a 3.7:1 ratio in 2.7 % overall yield; the 1H NMR data for the major product matched the corresponding data of the natural product 5.2, and differed from that of the isomeric product 5.11. Comparison of the 1H NMR data of 5.2 and 5.11 (Table 5.1) indicated that the proton signals of H-10 for 5.2 [δH 7.95 (d, J = 8.2 Hz)] were less deshielded than those of 5.11 [δH 8.21 (d, J = 8.4 Hz)]. On the other hand the signals for H-7 of 5.2 [δH 7.92 (d, J = 2.4 Hz)] were more deshielded than those of 5.11 [δH 7.61 (d, J = 2.3 Hz)].
These differences enabled the structures of 5.2 and 5.11 to be assigned. The larger deshielding of H-10 of 5.11 and the larger deshielding of H-7 of 5.2 are consistent with the major resonance structures of each compound (Figure 5.2). HMBC correlations of 5.2 from both downfield shifted H-5 (δH 8.23) and H-7 (δH 7.92) to C-6 (δC 144.0) confirmed it to be the 9-methoxypseudolauridine 1-oxide.
Table 5.1 ¹H and ¹³C NMR spectra of compound 5.1, 5.2, 5.10 and 5.11

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*In CD₃OD, δ (ppm); multiplicities; J values (Hz) in parentheses.

5.2.4 Antiproliferative activities of compounds 5.1-5.6, 5.10 and 5.11

Previous research has shown eupolauridine to have antifungal activity, and sampangin derivatives to have cytotoxic, antimalarial, and antifungal activities. 8-hydroxyeupolauridine 5.1, 9-methoxyeupolauridine 1-oxide 5.2, 8-methoxyeupolauridine 5.10, and 8-methoxyeupolauridine 1-oxide 5.11, as well as eupolauridine N-oxide 5.3, 8-methoxysampangin 5.4, eupolauridine 5.5, and sampangin 5.6 were tested against the
A2780 human ovarian cancer cell line. Among these eight compounds, sampangin (5.6) was the most active against the A2780 cell line with an IC₅₀ value of 0.60 µM, but sampangin derivative 5.4 showed much weaker activity, with IC₅₀ 10.3 µM. Among the eupolauridine analogues, compound 5.3 was the most active with an IC₅₀ value of 3.5 µM.

When tested against the H460 human lung cancer cell line, compounds 5.4 and 5.6 both exhibited strong activity with IC₅₀ values of 0.57 µM and 0.58 µM, respectively. Interestingly, only 5.3 among the eupolauridine analogues showed antiproliferative activity against this cell line, with an IC₅₀ value of 1.77 µM.

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### 5.3 Experimental Section

#### 5.3.1 General experimental procedures

UV and IR spectra were measured on a Shimadzu UV-1201 spectrophotometer and a MIDAC M-series FTIR spectrophotometer, respectively. Melting points were obtained on a B-540 Büchi melting-point apparatus. NMR spectra were recorded in CDCl₃, CD₃OD or D₂O on either JEOL Eclipse 500 or Bruker 600 spectrometers. The chemical shifts are given in δ (ppm) and coupling constants (J) are reported in Hz. Mass spectra were obtained on an Agilent 6220 TOF Mass Spectrometer. HPLC was performed on a
Shimadzu LC-10AT instrument with a semi-preparative C\textsubscript{18} or phenyl Varian Dynamax column (5 \( \mu \)m, 250 x 10 mm).

5.3.2 Plant material

Roots of *Ambavia gerradii* (Baill.) Le Thomas (Annonaceae) were collected on July 15, 2005, in the Ambohibe dry forest near the village of Betsimiranja, Diana, Antsiranana, Madagascar. The collection coordinates were 13° 02’ 42” S, 049° 09’ 11” E, and the elevation was 50 m. The plant sampled was a tree of about 16 m height with a diameter at breast height of 35 cm, and with green fruit; it occurs commonly in this area. The tree was identified by R. Randrianaivo, and its identity was confirmed by G.E. Schatz (Missouri Botanical Garden). Voucher specimens with voucher number RIR 1196 have been deposited in herbaria at the Parc Botanique and Zoologique de Tsimbazaza (TAN), at the Centre National d'Application des Recherches Pharmaceutiques in Antananarivo, Madagascar (CNARP), at the Missouri Botanical Garden in St. Louis, Missouri (MO), and at the Muséum National d'Histoire Naturelle in Paris, France (P).

5.3.3 Extraction and Isolation

Dried roots of *Ambavia gerrardii* (250 g) were ground in a hammer mill, then extracted with EtOH by percolation for 24 hours at room temperature to give the crude extract MG 3311 (6.4 g), of which 3.0 g was available at Virginia Polytechnic Institute and State University (VPISU) for evaluation. The fractionation tree is shown in Scheme 5.2. The extract MG 3311 (IC\textsubscript{50} 3.6 \( \mu \)g/mL, 1.3 g) was suspended in aqueous MeOH
(MeOH-H₂O, 9:1, 100 mL) and extracted with hexanes (3 x 100 mL portions). The aqueous layer was then diluted to 60% MeOH (v/v) with H₂O and extracted with CH₂Cl₂ (3 x 150 mL portions). The hexanes extract was evaporated in vacuo to leave 138 mg with an IC₅₀ value of 14 μg/mL. The residue from the CH₂Cl₂ extract (354 mg) had an IC₅₀ value of 2.6 μg/mL. The aqueous MeOH extract (715 mg) was inactive. The CH₂Cl₂ extract was selected for fractionation, and six fractions of 124, 44, 81, 26, and 38 mg were collected from a C₁₈ open column eluted with MeOH/H₂O (gradient from 60% to 100%). The first four fractions had IC₅₀ values of 3.7, 1.9, 3, and 16 μg/mL, respectively, and the last fraction was inactive. Fractions I and II were selected for further work. Separation of fraction I by C₁₈ preparative HPLC (60% MeOH-H₂O) yielded eight subfractions (IC₅₀ 19 μg/mL, inactive, inactive, 3.2 μg/mL, inactive, 2 μg/mL, 4.9 μg/mL and inactive), and the most active subfractions I-4 (IC₅₀ 3.2 μg/mL), and I-6 (IC₅₀ 2 μg/mL) were selected for further separation by phenyl HPLC (60% MeOH-H₂O). Compounds 5.1 (0.4 mg, tᵣ 28.1 min) and 5.3 (2.0 mg tᵣ 31.4 min) were isolated from subfraction I-4, and compounds 5.2 (0.5 mg, tᵣ 29.8 min), and 5.4 (1.0 mg, tᵣ 36.0 min) were isolated from subfraction I-6. Fraction II was applied to a C₁₈ open column. The most active subfraction (IC₅₀ 0.14 μg/mL) was subjected to phenyl HPLC (70% MeOH-H₂O) to give compounds 5.5 (1.9 mg, tᵣ 25.3 min) and 5.6 (0.6 mg, tᵣ 31.4 min).
Scheme 5.2 Separation of ethanol extract of *Ambavia gerradii*

8-Hydroxy eupolauridine (5.1)

Yellow solid; UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 220 (4.15), 240 (3.98), 287 (3.82), 351 (3.28), 369 (3.35); IR $\nu_{\text{max}}$ cm$^{-1}$: 1638, 1599, 1580, 1398, 1378, 1289, 1243, 1202, 1091, 1060, 1016, 994, 842 and 808 cm$^{-1}$. $^1$H NMR (500 MHz, CD$_3$OD), see Table 5.1; HRESI-MS $m/z$ 221.0713 [M+H]$^+$ (caled for C$_{14}$H$_{10}$N$_2$O, 221.0715).

9-Methoxy eupolauridine 1-oxide (5.2)

Yellow solid; UV (CD$_3$OD) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 225 (4.03), 254 (3.99), 292 (3.87), 384 (3.36); IR $\nu_{\text{max}}$ cm$^{-1}$: 2919, 2851, 1611, 1593, 1487, 1451, 1423, 1380, 1256, 1233, 1022,
973, 846 and 828 cm\(^{-1}\). \(^1\)H NMR (500 MHz, CD\(_3\)OD) and \(^{13}\)C NMR (125 MHz, CD\(_3\)OD), see Table 5.1; HRESI-MS \(m/z\) 251.0820 [M+H]\(^+\) (calcd for C\(_{15}\)H\(_{11}\)N\(_2\)O\(_2\), 251.0821).

**Eupolauridine N-oxide (5.3)**

Yellow solid; LC-MS \(m/z\) 221.1 [M+H]\(^+\) (calcd for C\(_{14}\)H\(_9\)N\(_2\)O, 221.1). \(^1\)H NMR (500 MHz, CD\(_3\)Cl), see appendix.

**8-Methoxysampangin (5.4)**

Yellow solid; LC-MS \(m/z\) 263.1 [M+H]\(^+\) (calcd for C\(_{16}\)H\(_{11}\)N\(_2\)O\(_2\), 263.1). \(^1\)H NMR (500 MHz, CD\(_3\)Cl), see appendix.

**Eupolauridine (5.5)**

Yellow solid; LC-MS \(m/z\) 205.1 [M+H]\(^+\) (calcd for C\(_{14}\)H\(_9\)N\(_2\), 205.1). \(^1\)H NMR (500 MHz, CD\(_3\)Cl), see appendix.

**Sampangin (5.6)**

Yellow solid; LC-MS \(m/z\) 233.0 [M+H]\(^+\) (calcd for C\(_{15}\)H\(_9\)N\(_2\)O, 233.1). \(^1\)H NMR (500 MHz, CD\(_3\)Cl), see appendix.

5.3.4 Synthesis of 8-hydroxy eupolauridine and 9-methoxyl eupolauridine 1-oxide

**Ethyl 2-(4-methoxy)phenyl-4-methyl-3-pyridinecarboxylate (5.7)**

To a solution of 3.84 g (17 mmol) of ethyl 3-(4-methoxyphenyl)-3-oxopropionate in 14 mL dioxane in a 100 mL flask was added 48 mg (2 mmol) NaH, and then 1.68 g (24
mmol) crotonaldehyde dropwise in 6 mL dioxane. After the reaction mixture was stirred for another 30 min at room temperature, 4.9 g of H₂NÖH·HCl (70 mmol) and 20 mL glacial AcOH were added. The reaction mixture was stirred at 100-110 °C for 90 minutes and then poured onto 100 g of ice, made basic with K₂CO₃ and extracted with ether (3 x 100 mL). The combined organic phase was extracted with 2N HCl (3 x 100 mL). The combined acidic aqueous phase was neutralized with K₂CO₃ and extracted with ether (3 x 100 mL), and the ether was dried over K₂CO₃ and evaporated under reduced pressure. The oily crude product was purified by flash silica column chromatography (hexane:EtOAc, 4:1) to afford ethyl 2-(4-methoxy)phenyl-4-methyl-3-pyridinecarboxylate 5.7 (1.47 g, 32%). Yellow oil. ¹H NMR (500 MHz, CDCl₃) δ_H 8.53 (1H, d, J = 5.1 Hz), 7.54 (2H, d, J = 8.8 Hz), 7.08 (1H, d, J = 5.1 Hz), 6.94 (2H, d, J = 8.8 Hz), 4.17 (2H, q, J = 7.2 Hz), 3.82 (3H, s), 2.39 (3H, s), 1.08 (3H, t, J = 7.2 Hz). ¹³C NMR (125 MHz, CDCl₃) δ_C 168.8, 160.0, 156.0, 149.4, 145.4, 132.4, 129.6, 128.9, 123.1, 113.7, 61.3, 55.2, 19.3, 13.7. HRESI-MS m/z 272.1268 [M+H]+ (calcd for C₁₆H₁₈NO₃, 272.1287).

2-(4-Methoxyphenyl)-4-methyl-3-pyridinecarboxylic acid (5.8)

Compound 5.7 (670 mg, 2.5 mmol) was refluxed overnight in aqueous NaOH (40%, 10 mL). The solution was extracted with CHCl₃ to remove any unreacted starting material. The pH value of the mixture was then adjusted to about 6, the solvent was removed under reduced pressure, and the residue was extracted with hot MeOH (3 x 10 mL). The MeOH extracts were combined and concentrated to a residue which was purified on a silica column eluted with MeOH:CH₂Cl₂ (3:1) to give 2-(4-
methoxy)phenyl-4-methyl-3-pyridinecarboxylic acid 5.8 (403 mg, 66%). Off-white solid, Mp 198-200ºC. $^1$H NMR (500 MHz, D$_2$O) $\delta$H 8.43 (1H, d, $J$ = 6.2 Hz), 7.78 (1H, d, $J$ = 6.2 Hz), 7.62 (2H, d, $J$ = 9.0 Hz), 7.13 (2H, d, $J$ = 9.0 Hz), 3.89 (3H, s), 2.59 (3H, s). $^{13}$C NMR (125 MHz, D$_2$O) $\delta$C 171.6, 161.8, 155.4, 147.6, 139.1, 130.3, 126.7, 123.6, 115.0, 55.7, 49.0, 20.2. HRESI-MS $m/z$ 244.0958 [M+H]$^+$ (calcd for C$_{14}$H$_{14}$NO$_3$, 244.0974).

7-Methoxyonychin (5.9)
A solution of 5.8 (93 mg, 0.38 mmol) in 2 mL SOCl$_2$ was refluxed 24 h under N$_2$. After the solvent was removed under vacuum, 51 mg (0.38 mmol) AlCl$_3$ and 2 mL chlorobenzene were added to the flask and the mixture refluxed overnight under N$_2$. The reaction was cooled and quenched by pouring into 10 mL saturated NaHCO$_3$ solution. The resulting mixture was extracted with CH$_2$Cl$_2$ (3 x 10 mL), dried over K$_2$CO$_3$, and the CH$_2$Cl$_2$ phase was evaporated. The residue was purified by silica gel PTLC (hexanes:EtOAc, 6:4) to afford 7-methoxyonychin (5.9) (21 mg, 25%). Yellow solid, Mp 134-136ºC. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$H 8.30 (1H, d, $J$ = 5.2 Hz), 7.79 (1H, d, $J$ = 8.2 Hz), 7.18 (1H, d, $J$ = 2.3 Hz), 7.04 (1H, dd, $J$ = 8.2, 2.3 Hz), 6.90 (1H, d, $J$ = 5.2 Hz), 3.87 (3H, s), 2.59 (3H, s). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$C 192.4, 164.8, 162.5, 151.3, 148.2, 136.9, 134.5, 126.1, 125.0, 122.7, 120.4, 109.0, 55.8, 17.3. HRESI-MS $m/z$ 226.0865 [M+H]$^+$ (calcd for C$_{14}$H$_{12}$NO$_2$, 226.0868).

8-Methoxyeupolauridine (5.10)
A solution of 33 mg (0.15 mmol) of 5.9, plus N,N-dimethylformamide diethyl acetal in 1 mL DMF was stirred 2 h at 120 ºC under N$_2$. NH$_4$OAc (600 mg) was added to the
flask and the reaction mixture was stirred for another 30 min at 140 °C, quenched with 5 mL water, and the solvent was extracted with EtOAc (3 x 5 mL). The organic phase was combined, dried over K₂CO₃ and concentrated to a residue which was separated on a silica TLC plate (hexanes:EtOAc, 6:4) to give 8-methoxy eupolauridine 5.10 (25.4 mg, 74%). Yellow solid, Mp 138-140°C. ¹H NMR (500 MHz, CDCl₃) δH 8.63 (1H, d, J = 5.7 Hz), 8.57 (1H, d, J = 5.7 Hz), 7.81 (1H, d, J = 8.2 Hz), 7.49 (1H, d, J = 1.4 Hz), 7.35 (1H, d, J = 5.7 Hz), 7.28 (1H, d, J = 5.7 Hz), 6.89 (1H, dd, J = 8.2, 1.4 Hz), 3.89 (3H, s). ¹³C NMR (125 MHz, CDCl₃) δC 162.8, 162.5, 162.2, 149.7, 149.6, 142.0, 134.9, 131.9, 123.7, 121.3, 117.8, 116.3, 115.5, 109.0, 55.7. HRESI-MS m/z 235.0875 [M+H]⁺ (calcd for C₁₅H₁₁N₂O, 235.0871).

8-Hydroxyeupolauridine (5.1)

A solution of 5.10 (6 mg, 0.026 mmol) in 1 mL of 48% HBr was refluxed for 24 h. The mixture was then cooled and evaporated in vacuo to give a residue which was purified by PTLC on silica gel (hexanes:EtOAc, 6:4). 8-Hydroxyeupolauridine 5.1 (2.5 mg, 43 %) was collected as a yellow solid. Mp 278-280°C. Its ¹H NMR and HRESI-MS spectrum were identical to those of the isolated material. ¹³C NMR, see Table 5.1.

Preparation of 8-methoxyeupolauridine 1-oxide (5.11) and 9-methoxyeupolauridine 1-oxide (5.2)

A solution of 8-methoxyeupolauridine (5.10, 10 mg, 0.045 mmol) and meta-chloroperoxybenzoic acid, (7.4 mg, 0.043 mmol) in 1 mL CH₂Cl₂ was stirred at room temperature for 24 h. After the solvent was removed, the residue was separated by C₁₈
HPLC (60% MeOH:H₂O) to afford yellow solid 9-methoxyeupolauridine 1-oxide **5.2** (5.9 mg, 55%) (Mp 198-200°C) and 8-methoxyeupolauridine 1-oxide **5.11** (1.6 mg, 15%). The NMR and mass spectroscopic data for **5.2** were identical to those of the natural product.

**8-Methoxyeupolauridine 1-oxide (5.11)**

Yellow solid, Mp 218-220°C. ¹H NMR (600 MHz, CD₃OD): δ_H 8.65 (1H, d, J = 5.8 Hz), 8.21 (1H, d, J = 8.5 Hz), 8.16 (1H, d, J = 7.1 Hz), 7.72 (1H, d, J = 7.1 Hz), 7.65 (1H, d, J = 5.8 Hz), 7.61 (1H, d, J = 2.3 Hz), 7.07 (1H, dd, J = 8.4, 2.4 Hz), 3.96 (3H, s). ¹³C NMR (150 MHz, CD₃OD) of **5.11**: δ_C 164.6, 159.3, 149.8, 145.6, 144.5, 141.1, 130.7, 128.4, 126.3, 125.3, 123.1, 119.3, 116.5, 111.2, 56.5. HRESI-MS m/z 251.0807 [M+H]+ (calcd for C₁₅H₁₁N₂O₂, 251.0821).

**5.3.5 Antiproliferative Bioassays**

The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported, except that the samples were added in 1 µL 100% DMSO per well instead of 20 µL of 1:1 DMSO:H₂O. The A2780 cell line is a drug-sensitive human ovarian cancer cell line. Assays against the NCI-H460 lung cancer cell line were carried out at Eisai, Inc., as previously described.

**References**


23. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of
VI. Five new antimalarial pseudoguaianolides of *Polycline proteiformis* from the Madagascar dry forest

6.1 Introduction

In our continuing search for biologically active natural products from tropical rainforests, we obtained an ethanol extract from the aerial part of a plant identified as *Polycline proteiformis* Humbert (Asteraceae) from Toliara, Madagascar. The extract exhibited good antimalarial activities against the chloroquine-sensitive HB3 strain and the chloroquine-resistant Dd2 strain of the malaria with IC$_{50}$ value of 1.9 $\mu$g/mL (HB3) and 1.6 $\mu$g/mL (Dd2). On the basis of the activities and the absence of previous phytochemical study on this species, *P. proteiformis* was selected for fractionation to isolate its active components under the guidance of our bioassay.

6.1.1 Previous investigations of Asteraceae

There are only four species in the genus *Polycline* (Asteraceae): *P. gracilis*, *P. proteiformis*, *P. psyllioides*, and *P. stuhlmannii*. Previous phytochemical studies of plants belonging to the family Asteraceae have revealed the presence of antimalarial sesquiterpene lactones$^{1-5}$ and flavonoids.$^{6-7}$ Among all the compounds isolated from natural resources (from alkaloids, terpenes, flavonoids, limonoids, chalcones, peptides, xanthones, quinones and coumarins),$^8$ the sesquiterpenoid artemisinin from a traditional Chinese medicinal plant, *Artemesia annua* (Asteraceae)$^1$ is still one of the best antimalarial agent available. Its derivatives have been widely used in artemisinin-based combination therapies (ACTs).$^9-10$ The structures of artemisin and a representative set of compounds isolated from Asteraceae are shown in Figure 6.1.
6.2 Results and Discussion

An EtOH extract of aerial parts of *P. proteiformis* was subjected to liquid-liquid partitioning to give hexanes, dichloromethane (CH$_2$Cl$_2$) and methanol fractions with IC$_{50}$ values of 4 µg/mL, 1.5 µg/mL, and >10 µg/mL, respectively against the HB3 malaria parasite, and 2 µg/mL, 1.0 µg/mL and 8 µg/mL respectively against the Dd2 malaria parasite. Fractionation by C$_{18}$ open column and High Performance Liquid Chromatography (HPLC) of the active CH$_2$Cl$_2$ fraction yielded five new sesquiterpene lactones (6.1-6.5), as well as a flavonoid 6.6. Herein we report the structural elucidation, the antimalarial properties and the cytotoxicities of the isolates.
6.2.1 Structure elucidation of polyclinolide A (6.1)

Polyclinolide A (6.1) was obtained as an off-white needle crystal. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \( m/z \) 437.2176 \([\text{M+H}]^+\), corresponding to the molecular formula of \( \text{C}_{23}\text{H}_{32}\text{O}_8 \). The IR spectrum showed strong absorption in the range 1740-1710 cm\(^{-1}\) which was consistent with the presence of ester, and lactone groups. Its \(^1\text{H}\) NMR spectrum in CDCl\(_3\) showed four singlet methyl groups at \( \delta_{\text{H}} \) 2.10, 2.09, 2.08 and 0.98, three doublet methyl groups at \( \delta_{\text{H}} \) 1.16, 1.16 and 1.05, one exocyclic methylene (\( \delta_{\text{H}} \) 6.27 and 5.65, each doublet, \( J = 3.2 \) Hz, H-13a and H-13b), two sets of methylenes at \( \delta_{\text{H}} \) 2.64 (m, H-3a) and \( \delta_{\text{H}} \) 1.32 (dd, \( J = 16.3 \), 2.7 Hz, H-3b), and at \( \delta_{\text{H}} \) 2.33 (dt, \( J = 12.8 \), 3.1 Hz, H-9a) and \( \delta_{\text{H}} \) 1.55 (m, H-9b), eight methine resonances [\( \delta_{\text{H}} \) 1.88, m (H-10); \( \delta_{\text{H}} \) 2.67, m (H-1); \( \delta_{\text{H}} \) 2.52, septet, \( J = 7.0 \) Hz (H-2'); \( \delta_{\text{H}} \) 3.48, m (H-7); \( \delta_{\text{H}} \) 4.11, ddd, \( J = 12.2 \), 9.0, 3.5 Hz (H-8); \( \delta_{\text{H}} \) 4.95, d, \( J = 4.9 \) Hz (H-4); \( \delta_{\text{H}} \) 5.03, ddd, \( J = 8.7 \), 8.7,
2.7 Hz, (H-2) and δ_H 5.06, d, J = 11 Hz (H-6)], four of which were oxygenated (Table 6.1). The \(^{13}\)C NMR spectrum displayed signals arising from two acetoxy groups (δ_C 169.8 and 20.0) and (δ_C 170.2 and 21.4), one isobutyroxy group (δ_C 176.4, 33.9, 18.8 and 18.7) together with 15 signals [two quaternary carbons at δ_C 51.5 (C-5), 136.2 (C-11), a lactone carbonyl at δ_C 169.2 (C-12), one quaternary and one secondary methyls at δ_C 22.4 (C-15) and 20.0 (C-14), two methylenes at δ_C 37.9 (C-3) and 43.1 (C-9), four oxygen-bearing methines at δ_C 80.9 (C-8), 79.6 (C-4), 78.1 (C-6) and 75.5 (C-2), three methines at δ_C 51.3 (C-1), 46.3 (C-7) and 27.2 (C-10), an exocyclic methylene at δ_C 124.1 (C-13), as indicated by the HMQC spectrum (Table 6.1)] assignable to a sesquiterpene lactone. Inspection of the \(^1\)H NMR spectral data revealed that polyclinolide A is very similar to pseudoguaianolides 6-angeloyloxypuchellin\(^{11}\) and 2-deacetyl-2-isobutyryl-chamissonolide\(^{12}\).

The complete \(^1\)H and \(^{13}\)C NMR assignments and connectivities were established from a combination of HMQC, COSY and HMBC data analyses. The COSY spectrum showed correlations that indicated the presence of spin systems: H-4, H-3, H-2, H-1, H-10, H-9, H-8, H-7, H-6, and H-14, and H-2', H-3' and H-4' of the isobutyroxy moiety. In HMBC spectrum, the correlations from H_3-15 to C-4, C-5, C-1, and C-6 as well as the correlations from H-4 to C-1 and to C-5 corroborated the presence of a five member ring fused at C-1 and C-5, with a seven member ring. The HMBC correlations from H_2-9 to C-1, C-8, C-7 and C-14, and from H-6 to C-5, C-7 and C-8, and from H_2-13 to C-7 and the lactone carbonyl at C-12 suggested the presence of a γ-lactone ring, fused at C-7, C-8. These data indicated that 6.1 was a pseudoguaianolide analogue.\(^{11-12}\) The HMBC correlations from two methyls (H_3-3' and H_3-4'), one septet methine (H-2'), and H-2 to C-
1', H-4 and H₃-2'' to C-1'', H-6 and H₃-2''' to C-1''' suggested the presence of an isobutyrate group at C-2, an acetate group at C-4, and another acetate group at C-6. To determine the relative stereochemistry of 6.1, NOESY (Nuclear Overhauser Effect Spectroscopy) experiment was carried out. In the NOESY spectrum, the correlations from H-1 to H-7, H-9b, and H₃-14, from H₃-15 to H-2, H-4, H-6, H-8 and H-10, from H-8 to H-6, H-9a and H-10 suggested the α orientations of H-1, H-7 and H₃-14, and the β-orientations of H-2, H-4, H-6, H-8, H-10 and H₃-15.

![Figure 6.3](image)  
*Figure 6.3.* a) Key COSY (bold) and HMBC (arrows) correlations of 6.1  
b) Key NOESY correlations for 6.1.

In order to determine the absolute stereochemistry, compound 6.1 was crystallized in methanol to afford a good quality of a single crystal and its structure was confirmed by single crystal X-ray diffraction. An anisotropic displacement ellipsoid drawing is shown in Figure 6.4. The structure of 6.1 was thus established to be (1S,2S,4R,5S,6S,7R,8S,10R)-2-isobutyryloxy-4-acetoxy-6-acetoxyguai-11(13)-en-8,12-olide (6.1, polyclinolide A)
6.2.2 Structure elucidation of polyclinolide B (6.2)

Polyclinolide B (6.2) was obtained as an off-white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \( m/z \) 417.1889 \([\text{M+Na}]^+\), corresponding to the molecular formula of \( \text{C}_{21}\text{H}_{30}\text{O}_7 \). The IR spectrum showed hydroxyl function (3474 cm\(^{-1}\)), and strong absorption in the range 1740-1710 cm\(^{-1}\) which was consistent with the presence of ester, and lactone groups. Its \(^1\text{H}\) NMR spectrum in CDCl\(_3\) showed signals for two singlet methyl groups at \( \delta_H \) 2.10 and 0.98, three doublet methyl groups at \( \delta_H \) 1.14, 1.14 and 1.02, three multiplet methylenes (\( \delta_H \) 2.66, 1.53; 2.42, 1.41; 5.53, 6.40), two of which were olefinic protons, and eight methines (\( \delta_H \) 1.98, 2.24, 2.50, 3.04, 4.41, 4.64, 4.90 and 5.02.), four of which were oxygenated (Table 6.1). Inspection of the \(^1\text{H}\) and \(^{13}\text{C}\) NMR data of 6.2 in CDCl\(_3\) showed a close similarity with the data of the previously isolated 2,4-diacetooxy-6-hydroxyguai-11(13)-en-8,12-olide 6.7,\(^13\) except for the presence of an isobutyrate group and an acetate group instead of two acetate groups at C-2 and C-4, respectively. The HMBC correlations from H-2 to C-1', one septet methine H-2', two methyls H\(_3\)-3' and H\(_3\)-4' to C-1', and from H-4 to C-1'' suggested the isobutyrate group.
was located at C-2 and the acetate group at C-4. In the NOESY spectrum, the correlations from H-1 to H-6, H-7, H-9b, and H₃-14, from H-6 to H-7 and H-1, and from H₃-15 to H-2, H-4, H-8 and H-10, suggested the α orientations of H-1, H-6, H-7 and H₃-14, and the β-orientations of H-2, H-4, H-8, H-10 and H₃-15. The absolute configuration of 6.2 was deduced by the comparison of its CD spectral data with those of 6.1. The characteristic UV absorption of α,β-unsaturated lactone chromophore was shown at around 230 nm.¹⁴ The negative Cotton effect \([\theta] = -1.56 \times 10^3\) of 6.2 at 230 nm, which was very similar to that of 6.1 \(([\theta]_{230\ \text{nm}} = -3.57 \times 10^3)\), assigned the S configuration of C-7 according to the back octant rule.¹⁵ Therefore, polyclinolide B (6.2) was determined to be \((1S,2S,4R,5S,6R,7S,8S,10R)-2\text{-isobutyryloxy-4-acetoxy-6-hydroxyguai-11(13)-en-8,12-olide (6.2, polyclinolide B)}\)

\[ \text{Figure 6.5 Chemical structures of 6.4 and 6.7.} \]

**6.2.3 Structure elucidation of polyclinolide C (6.3)**

Polyclinolide C (6.3) was obtained as an off-white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \(m/z\ 483.2003\ [M+Na]^+\), corresponding to the molecular formula of C₂₅H₃₂O₈ for 6.3. The IR spectrum showed hydroxyl function (3444 cm⁻¹), and strong absorption in the range 1740-1710 cm⁻¹ which was consistent with the presence of ester, ketone and lactone groups. Its \(^1\text{H NMR spectrum in CDCl₃ showed} \]
signals for three singlet methyl groups (δ_H 1.27, 1.26 and 1.07), one doublet methyl group (δ_H 1.41 d, J = 7.2 Hz), two olefinic methyl groups (δ_H 1.78 m; 1.94 dq, J = 7.3, 1.5 Hz), one singlet methylene (δ_H 2.49), and three methines (δ_H 3.55 m, 3.19 m and 2.43 m). Three oxygenated methines were also observed at δ_H 5.48 s, 5.34 (d, J = 3.7 Hz), δ_H 4.66 s. In the downfield region of the spectrum, there were five olefinic methines at δ_H 7.60 dd (J = 6.0, 1.6 Hz), 6.80 s, 6.12 s, 6.12 m, 6.10 m. The 13C NMR spectrum displayed a set of signals ascribable to an (Z)-2-methyl-2-butenoyl (angeloyl) group (δ_C 166.1, 127.0, 139.7, 15.7, 20.4)13 and a 3-hydroxy-3-methylbutanoyl group (δ_C 171.6, 46.4, 69.2, 29.3, 29.2)16 together with 15 signals of a sesquiterpene lactone [two quaternary carbons at δ_C 55.3 and 130.8, a lactone carbonyl at δ_C 162.4 and a conjugated ketone at δ_C 207.7 and one quaternary and one secondary methyl (δ_C 18.8 and 19.2, respectively), two olefinic methines (δ_C 161.0 and 130.3, three oxygen-bearing methines (δ_C 88.5, 76.0 and 64.7), three methines (δ_C 50.5, 45.5 and 50.5), and an exocyclic methylene (δ_C 134.4), as indicated by the HMQC spectrum (Table 6.1)]. The complete 1H and 13C NMR assignments and connectivities were established from a combination of COSY, HMQC, and HMBC data. The COSY spectrum showed correlations that indicated the presence of the two spin systems: H-3, H-2, H-1, H-10, H-9, H-8, H-7, H-6, and H-14, and H-3' and H-4' of the angeloyl moiety. In the HMBC spectrum, the correlations from H3-15 to C-4, C-5, C-1, and C-6 as well as the correlations from H3 to C-4 and C-5 corroborated the presence of an α, β-unsaturated five member ketone ring fused with a seven member ring. The HMBC correlations from H-9 to C-12 at δ_C 162.4 and H-7 to C-11 at δ_C 130.8, C-12 at δ_C 162.4 and C-13 at δ_C 134.4 suggested the presence of a δ-lactone ring, fused at C-7, C-8 and C-9. These data indicated that 6.3 was a pseudoguaianolide analogue.17
Inspection of the $^{13}\text{C}$ NMR data of 6.3 indicated a close similarity to those of pseudoguaianolides previously isolated from *Hymenoxys ivesiana*.$^{17}$ Spectrally, 6.3 differed from compound 6.8 only in the nature of the ester substituent at C-8. The locations of the (Z)-2-methyl-2-butenoate (angelate) group at C-6 and the 3-hydroxy-3-methyl butanoate group at C-8 were substantiated by the observation of HMBC cross peaks from H-6 to C-1', H-3' to C-1', H-4' to C-3' and C-2', and H-5' to C-1', C-2' and C-3' and from H-8 to C-1", H2-2" to C-1", C-3", C-4" and C-5", H3-4" to C-2", C-3", and C-5", and H3-5" to C-2", C-3", and C-4".

![Figure 6.6 a) Key COSY (bold) and HMBC (arrows) correlations for 6.3. b) Key NOESY correlations for 6.3. c) Chemical structure of compound 6.8.](image)

To determine the relative stereochemistry of 6.3, a NOESY experiment was carried out. The NOESY correlations from H3-15 to H-10 and H-6, and H3-14 to H-1, H-9 and H-8, as well as the cross peaks arising from H-7 to H-8 suggested $\alpha$ orientations of H-1, H-7, H-8, H-9 and H3-14 and $\beta$ orientations of H-6, H-10 and H3-15. Therefore, polyclinolide C (6.3) was determined to be 1$\alpha$,7$\alpha$,10$\beta$H-4-oxo-6$\alpha$-angeloyloxy-8$\beta$-(3-hydroxy-3-methyl-butanoyloxy)pseudoguaia-2(3),11(13)-dien-9$\beta$,12-olide, as depicted in Figure 6.6.
6.2.4 Structure elucidation of polyclinolide D (6.4)

Polyclinolide D (6.4) was obtained as an off-white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \( m/z \) 497.2143 \([M+Na]^+\), corresponding to the molecular formula of \( C_{26}H_{34}O_8 \). The IR spectrum showed hydroxyl function (3445 cm\(^{-1}\)), and strong absorption in the range 1740-1710 cm\(^{-1}\) which was consistent with the presence of ester, ketone and lactone groups. The \(^1\)H NMR data of 6.4 in CDCl\(_3\) showed a close similarity of those of 6.3, except for the presence of a set of signals at \( \delta_H \) 2.51 (1H, d, \( J = 15.5 \) Hz), 2.42 (1H, d, \( J = 15.5 \) Hz), 1.52 (2H, qd, \( J = 7.5, 2.6 \) Hz), 0.90 (3H, t, \( J = 7.5 \) Hz) and 1.19 (3H, s) instead of those for the 3-hydroxy-3-methylbutanoate group, which indicated the presence of a different side chain at C-8 in 6.4. The \(^{13}\)C NMR spectrum displayed signals ascribable to a 3-hydroxy-3-methylpentanoate group\(^{18}\) (\( \delta_C \) 171.7, 44.5, 71.4, 34.7, 8.2 and 26.2). The HMBC correlations from H-8 to C-1", H-2" to C-1" and C-3"; H-4" to C-2", C-3", C-5" and C-6", H-5" to C-3", and H-6" to C-2" and C-3" as well as the COSY cross peak from H-5" and H-6" confirmed the presence of a 3-hydroxy-3-methylpentanoate group and its location to be at C-8. The NOESY spectrum of 6.4 showed similar correlations as observed for 6.3. The correlations from H\(_3\)-15 to H-10 and H-6, and H\(_3\)-14 to H-1, H-9 and H-8, as well as the correlation from H-7 to H-8 were observed. Thus the structure of 6.4 was concluded to be \( 1\alpha,7\alpha,10\beta \text{-H}-4\text{-oxo-6\alpha-angeloyloxy-8\beta-(3-hydroxy-3-methyl-pentanoyloxy)pseudoguaia-2(3),11(13)-dien-9\beta,12-olide}. \)
6.2.5 Structure elucidation of polyclinolide E (6.5)

Polyclinolide E (6.5) was obtained as an off-white solid. Its positive ion HRESIMS revealed a pseudomolecular ion peak at \( m/z \) 489.2496 \([M+H]^+\), corresponding to the molecular formula of \( \text{C}_{27}\text{H}_{36}\text{O}_8 \). The IR spectrum showed hydroxyl function (3441 cm\(^{-1}\)), and strong absorption in the range 1740-1710 cm\(^{-1}\) which was consistent with the presence of ester, ketone and lactone groups. The similarity of the \(^1\)H and \(^{13}\)C NMR spectroscopic data of 6.5 as those of 6.4 (Table 6.1) suggested that 6.5 was a close related pseudoguaianolide analogue. The presence of an (E)-3-methyl-2-pentenoate group at C-6 was indicated by the observation of a set of signals at \( \delta_H \) 5.53 (1H, s), 2.16 (2H, \( q J = 7.3 \) Hz), 2.15 (3H, s) and 1.05 (3H, \( t J = 7.3 \) Hz) in the \(^1\)H NMR spectrum, and the corresponding \(^{13}\)C NMR data (\( \delta_C \) 165.5, 113.3, 164.4, 33.9, 11.8 and 19.0) supported this conclusion.\(^{19}\) The HMBC correlations from H-6 to C-1', H-2" to C-1", C-3", H-4" to C-2', C-3', C-5' and C-6', H-5' to C-3' and C-4';and H-6' to C-2', C-3' and C-4' as well as the COSY cross peak from H-4' and H-5' confirmed an (E)-3-methyl-2-pentenoate group at C-6. Moreover, the NOESY correlation observed in 6.5 were similar to those observed in 6.3 and 6.4. Clear correlations from H\(_3\)-15 to H-10 and H-6, from H\(_3\)-14 to H-1, H-9 and
H-8, from H-7 to H-8 of 5 as well as from H-2’ to H-4’ were observed. Thus the structure of 6.5 was concluded to be $1\alpha,7\alpha,10\beta$H-4-oxo-$6\alpha$-(E)-3-methyl-2-pentenoylxy-$8\beta$-(3-hydroxy-3-methyl-pentanoyloxy)pseudoguaia-2(3),11(13)-dien-$9\beta,12$-olide.

6.2.6 Structure elucidation of compound (6.6)

Compound 6.6 was determined to be the previously isolated flavonoid centaureidin by comparison of its MS and $^{13}$C NMR data with literature data.20

6.2.7 Bioactivities of polyclinilide A-E (6.1-6.5) and centaureidin (6.6)

Polyclinolide C (6.3) and D (6.4) were tested for their antimalarial activities against HB3 and Dd2 malaria parasites. Polyclinolide C (6.3), showed strong activities against both malaria parasites with IC$_{50}$ values of 0.7 µM in each case. Polyclinolide D (6.4) had similar activities (IC$_{50}$ 0.8 µM) against both parasites.

The cytotoxicities of compounds 6.1-6.6 were also evaluated against the A2780 human ovarian cancer cell line. Polyclinolide A-E had IC$_{50}$ values of 2.2, 6.0, 0.9, 0.8, and 4.0 µM respectively against A2780 cell line. Centaureidin 6.6 was also cytotoxic with an IC$_{50}$ value of 3.9 µM.

Although polyclinolide C and D showed strong antimalarial activities against both HB3 and Dd2 strains, they will not be good lead compounds for antimalarial drug development due to their cytotoxicities.
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<td>2.16 q (7.3)</td>
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Table 6.1 $^1$H and $^{13}$C NMR chemical shifts of polyclinolides A (6.1), B (6.2), C (6.3), D (6.4) and E (6.5) a
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* In CDCl₃, δ (ppm) 500 MHz for ¹H and 125 MHz for ¹³C; multiplicities; J values (Hz) in parentheses.
6.3 Experimental Section

6.3.1 General experimental procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. UV and IR spectra were measured on a Shimadzu UV-1201 spectrophotometer and a MIDAC M-series FTIR spectrophotometer, respectively. CD analysis was performed on a JASCO J-810 spectropolarimeter with a 1.0 cm cell in methanol. NMR spectra were recorded in CDCl$_3$ on either JEOL Eclipse 500 or Bruker 600 spectrometers. The chemical shifts are given in $\delta$ (ppm) and coupling constants ($J$) are reported in Hz. Mass spectra were obtained on an Agilent 6220 TOF Mass Spectrometer. HPLC was performed on a Shimadzu LC-10AT instrument with a semi-preparative C$_{18}$ Varian Dynamax column (5 $\mu$m, 250 x 10 mm).

6.3.2 Extraction and Isolation

Dried aerial part of Polycline proteiformis were ground in a hammer mill, then extracted with ethanol by percolation for 24 hours at room temperature to give the crude extract N110635 (5 g), of which 3 g was shipped to Virginia Polytechnic Institute and State University (VPISU) for further bioassay guided isolation. The fractionation tree is shown in Scheme 6.1. The extract N110635 [IC$_{50}$: 1.9 $\mu$g/mL (HB3), 1.6 $\mu$g/mL (Dd2), 2 g] was suspended in aqueous MeOH (MeOH-H$_2$O, 9:1, 100 mL) and extracted with hexanes (3 x 100 mL portions). The aqueous layer was then diluted to 60% MeOH (v/v) with H$_2$O and extracted with CH$_2$Cl$_2$ (3 x 150 mL portions). The hexanes extract was evaporated in vacuo to leave 186.5 mg with an IC$_{50}$ value of 4.0 (HB3), 2.0 (Dd2) $\mu$g/mL.
470.1 mg of residue from the CH$_2$Cl$_2$ extract had IC$_{50}$ of 1.5 (HB3), <1.0 (Dd2) μg/mL. The aqueous MeOH extract (1.392 g) was less active with IC$_{50}$ of >10.0 (HB3), 8.0 (Dd2). Therefore, the CH$_2$Cl$_2$ extract was selected for fractionation by C$_{18}$ open column, and four fractions were collected. Fractions I, II, III, and IV (155.7, 149.7, 54.2 and 19.3 mg) had IC$_{50}$ values of > 5.0 (HB3), > 5.0 (Dd2); 0.45 (HB3), 0.42 (Dd2); 5.0 (HB3), 3.5 (Dd2); > 5.0 (HB3), > 5.0 (Dd2) μg/mL, respectively. Fraction II was selected for further separation by C$_{18}$ preparative HPLC (60% CH$_3$CN-H$_2$O). Compound 6.1 (2.2 mg, $t_R$ 22.1
min), 6.2 (2.2 mg, \(t_R\) 14.6 min), 6.3 (2.3 mg, \(t_R\) 13.2 min), 6.4 (2.9 mg, \(t_R\) 16.5 min), 6.5 (1.1 mg, \(t_R\) 20.2 min) and 6.6 (2.3 mg, \(t_R\) 10.0 min) were isolated.

6.3.3 Bioassays

Antiplasmodial assays with the chloroquine-sensitive strain \(P. falciparum\) HB3 and chloroquine-resistant strain \(P. falciparum\) Dd2 were performed by Professor Roepe and his group at Georgetown University. Both assays used the previously reported SYBR green 1 method.21 The A2780 ovarian cancer cell line assay was performed by Ms. Peggy Brodie at Virginia Polytechnic Institute and State University as previously reported,22 except that the samples were added in 1 \(\mu\)L 100% DMSO per well instead of 20 \(\mu\)L of 1:1 DMSO:H2O. The A2780 cell line is a drug-sensitive ovarian cancer cell line.23

6.3.4 Plant material

A sample of the aerial part of \(Polycline proteiformis\) Humbert (Asteraceae) was collected in April 24th, 1998 near Toliara, Madagascar. This aromatic herbaceous plant can grow up to 60 cm height, with white flowers. Voucher specimens have been deposited at Smithsonian Institution, Washington D.C..

\((1S,2S,4R,5S,6S,7R,8S,10R)-2\text{-isobutyryloxy-4-acetoxy-6-acetoxyguai-11(13)-en-8,12-olide} (6.1, polyclinolide A)\)

Compound 6.1 was an off-white needle crystal; \([\alpha]_D^{23}\) -53 (c 0.1, CHCl3); CD \([\theta]_D^{23}\) -3570 (MeOH); UV (MeOH) \(\lambda_{\text{max}}\) nm (log \(\varepsilon\)) 211 (4.0); IR \(\nu_{\text{max}}\) cm\(^{-1}\): 3463, 2965, 1733, 1464, 1375, 1258, 1156, 1052, 1018 cm\(^{-1}\). \(^1\)H NMR (500 MHz, CDCl3) and \(^{13}\)C NMR
(125 MHz, CDCl₃), see Table 6.1; HRESI-MS m/z 437.2176 [M+H]⁺, (calcd for C₂₃H₃₅O₈ (437.2175).

X-ray Crystallography of 6.1

A colorless needle (0.03 x 0.03 x 0.33 mm³) of 6.1 was centered on the goniometer of an Oxford Diffraction SuperNova diffractometer operating with CuKα radiation. The data collection routine, unit cell refinement, and data processing were carried out with the program CrysAlisPro.²⁴ The Laue symmetry and systematic absences were consistent with the monoclinic space groups C₂, Cm, and C₂/m. As the molecule was known to be enantiomerically pure, the chiral space group, C₂, was chosen. The structure was solved using SHELXS-97²⁵ and refined using SHELXL-97²⁶ via OLEX2.²⁶ The final refinement model involved anisotropic displacement parameters for non-hydrogen atoms and a riding model for all hydrogen atoms. The absolute configuration was established from anomalous dispersion effects [Flack x = 0.02(15);²⁷ Hooft P2(true) = 1.000, P3(true) = 1.000, P3(rac-twin) = 0.3x10⁻⁵, P3(false) = 0.7x10⁻²⁵, y = 0.06(9)].²⁸,²⁹ Crystal data were obtained by Dr. Carla Slebodnick: C₂₃H₃₂O₈, Mr = 436.49, monocyclic, a = 32.7331(12) Å, b = 7.1315(3) Å, c = 9.7799(3) Å, α = 90.00, β = 92.961, γ = 90.00, V = 2279.94(13) Å³, 13543 reflections, 287 parameters. The atomic coordinates and equivalent isotropic displacement parameters, as well as a full list of bond distances and angles, and the structure factor table are deposited as supplementary material at the Cambridge Crystallographic Data Centre (Deposition No. CCDC 802814).
(1S,2S,4R,5S,6R,7S,8S,10R)-2-isobutyryloxy-4-acetoxy-6-hydroxyguai-11(13)-en-8,12-olide (6.2, polyclinolide B)

Compound 6.2 was an off-white solid; $[\alpha]_{D}^{23} -4$ (c 0.2, CHCl$_3$); CD $[\theta]_{230} -1560$ (MeOH); UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 211 (3.9); IR $\nu_{\text{max}}$ cm$^{-1}$: 3474, 2972, 1729, 1464, 1376, 1250, 1160, 1044, 1017 cm$^{-1}$. $^1$H NMR (500 MHz, CDCl$_3$) and $^{13}$C NMR (125 MHz, CDCl$_3$), see Table 6.1; HRESI-MS m/z 417.1889 [M+Na]$^+$, calcd for C$_{21}$H$_{30}$NaO$_7$ (417.1889).

1a,7a,10βH-4-oxo-6α-angeloyloxy-8β-(3-hydroxy-3-methyl-butanoyloxy)pseudoguaia-2(3),11(13)-dien-9β,12-olide (6.3, polyclinolide C)

Compound 6.3 was an off-white solid; $[\alpha]_{D}^{23} -69$ (c 0.1, CHCl$_3$); UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 220 (4.2); IR $\nu_{\text{max}}$ cm$^{-1}$: 3444, 2924, 1721, 1458, 1382, 1229, 1154, 1035, 999.8 cm$^{-1}$. $^1$H NMR (500 MHz, CDCl$_3$) and $^{13}$C NMR (125 MHz, CDCl$_3$), see Table 6.1; HRESI-MS m/z 483.2003 [M+Na]$^+$, calcd for C$_{25}$H$_{32}$NaO$_8$ (483.1995).

1a,7a,10βH-4-oxo-6α-angeloyloxy-8β-(3-hydroxy-3-methyl-pentanoyloxy)pseudoguaia-2(3),11(13)-dien-9β,12-olide (6.4, polyclinolide D)

Compound 6.4 was an off-white solid; $[\alpha]_{D}^{23} -66$ (c 0.2, CHCl$_3$); UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$) 220 (4.3); IR $\nu_{\text{max}}$ cm$^{-1}$: 3445, 2927, 1721, 1458, 1382, 1216, 1154, 1035, 999.5 cm$^{-1}$. $^1$H NMR (500 MHz, CDCl$_3$) and $^{13}$C NMR (125 MHz, CDCl$_3$), see Table 6.1; HRESI-MS m/z 497.2143 [M+Na]$^+$, calcd for C$_{26}$H$_{34}$NaO$_8$ (497.2151).
1α,7α,10β-H-4-oxo-6α-(E)-3-methyl-2-pentenoyloxy-8β-(3-hydroxy-3-methyl-pentanoyloxy)pseudoguaia-2(3),11(13)-dien-9β,12-olide (6.5, polyclinolide E)

Compound 6.5 was an off-white solid; [α]D\textsuperscript{23} -33 (c 0.1, CHCl\textsubscript{3}); UV (MeOH) λ\textsubscript{max} nm (log ε) 221 (4.3); IR ν\textsubscript{max} cm\textsuperscript{-1}: 3441, 2923, 1721, 1458, 1380, 1216, 1142, 1034, 1005.5 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) and \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}), see Table 6.1; HRESI-MS m/z 489.2496 [M+H]\textsuperscript{+}, calcd for C\textsubscript{27}H\textsubscript{37}O\textsubscript{8} (489.2488).

References


20. Flamini, G.; Antognoli, E.; Morelli, I., Two flavonoids and other compounds from the aerial parts of Centaurea bracteata from Italy. *Phytochemistry* **2001**, *57*, 559-564.


VII. Miscellaneous Plants Studied

7.1 Introduction

During the search for novel anticancer and antimalarial agents, some extracts yielded only known compounds. These known compounds isolated from various species are reported in this chapter, to provide a complete record of the work that was done and to document the botanical sources of the isolated compounds.

7.2 Anticancer extracts

7.2.1 Chadsia racemosa (Fabaceae)

An ethanol extract of the leaves of *Chadsia racemosa* (Fabaceae) (MG 3288, 1.9 g) yielded 728 mg of an active dichloromethane fraction with an IC$_{50}$ value of 11 μg/mL against the A2780 human ovarian cancer line after liquid/liquid partition. The most active subfraction with an IC$_{50}$ value of 8 μg/mL was separated over a C$_{18}$ open column followed by PTLC to afford the two known flavonoids 7.1 (2.0 mg) and 7.2 (1.2 mg) with IC$_{50}$ values of around 19 μM. Their structures were determined by mass spectrometry to deduce their molecular formulas and by comparison of their $^1$H and $^{13}$C data with literature data.$^1$

![Structure of 7.1](image1.png)  
![Structure of 7.2](image2.png)
7.2.2 *Gastonia duplicata* (Araliaceae)

An ethanol extract of the leaves of *Gastonia duplicate* (Araliaceae) (MG 4016) displayed an IC$_{50}$ value of 4.2 µg/mL against the A2780 human ovarian cancer line. Liquid/liquid partitioning of 110 mg of crude extract followed by separation of 82 mg of the bioactive methanol fraction with an IC$_{50}$ value of 7.2 µg/mL over a reversed phase C$_{18}$ open column, followed by HPLC on a C$_{18}$ column to afford the two known bioactive triterpenoid saponins 7.3 (1.3 mg) and 7.4 (4.2 mg),$^2$ with IC$_{50}$ values less than 1.7 µM. The isolation work was done by Brian T. Murphy and the structure elucidation was done by Ende Pan.

![Chemical structures](image)

7.2.3 *Entada louvelii* (Fabaceae)

Comparison of the antiproliferative activity of the crude extract (MG 3303) with the activities of the fractions from liquid/liquid partitioning of 490 mg crude extract indicated that this procedure did not improve activity. The most active BuOH fraction (293 mg) had an IC$_{50}$ of 17 µg/mL. This fraction was fractionated over a C$_{18}$ open column, followed by HPLC on a cyano column. The activities of the fractions remained around 15 µg/mL. Due to the low bioactivity of the fractions, further separation of the plant was discontinued.

7.2.4 *Entada sp.* (Fabaceae)
The crude bark extract of this unknown species (MG 2125) was active in the A2780 cell line at 14 μg/mL. Liquid/liquid partitioning of 693 mg crude extract yielded 202 mg active n-BuOH fraction with an IC₅₀ of 11 μg/mL. This active fraction was further fractionated over C₁₈ open column. The most active two fractions were active at approximately 6 μg/mL. Proton NMR spectroscopy indicated that there were two or three triterpene saponins in the active fractions. Since these types of compounds are not attractive drug candidates, further separation of the plant was discontinued.

7.2.5 *Leea guineensis* (Vitaceae)

The crude root extract of *Leea guineensis* (MG 1929) was shown to be active against the A2780 cell line at 19 μg/mL. Liquid/liquid partitioning of 74 mg of extract afforded four active fractions. The dichloromethane and the n-butanol fractions were found to be the most active at 15 μg/mL and 17 μg/mL, respectively. Since most of the weight of the sample was in the n-BuOH fraction, further separation work was performed on this fraction. After separation on Sephadex LH20, followed by C₁₈ open column and C₁₈ HPLC, the subfractions showed no interesting activity. Thus, further separation of the plant was discontinued.

7.2.6 *Droceloncia reticulate* (Euphorbiaceae)

Liquid/liquid partitioning of 3.3 g crude leaf extract of *Droceloncia reticulate* (MG 3736) led to three fractions. The dichloromethane fraction was subject to C₁₈ open column and gave four sub-fractions. The most active fraction showed antiproliferative activity against 2780 cell line with an IC₅₀ value about 4μg/mL. This fraction was
subjected to C$_{18}$ HPLC to afford 14 fractions. All the fractions had IC$_{50}$ values higher than 7 µg/mL, so the bioactivities had been lost by the separation. Further separation of this plant was thus discontinued.

7.3 Antimalarial extracts

7.3.1 Phyllanthus muellerianus (Euphorbiaceae)

An ethanol extract of the leaves of *Phyllanthus muellerianus* (Euphorbiaceae) was available from Dr. Patricia Onocha, a Fulbright scholar in the Kingston group from 2007-2008. The bioassay work was carried out by Dr. Paul Roepe, Georgetown University. The extract had IC$_{50}$ values lower than 1 µg/mL against the HB3 strain and 1.5 µg/mL against the Dd2 strain. Liquid/liquid partitioning of 12 g of crude extract followed by separation of the active dichloromethane fraction (1.2 g) over reversed phase C$_{18}$ open column, and C$_{18}$ HPLC has so far afforded the five known compounds 7.5 (0.7 mg), 7.6 (0.8 mg), 7.7 (10. mg), 7.8 (4.3 mg), and 7.9 (1.0 mg). Only 7.9 showed encouraging activities with IC$_{50}$ values about 2 µM against the HB3 and Dd2 malaria parasite strains.
An ethanol extract (10 g) of the roots of *Phyllanthus muellerianus* (Euphorbiaceae) displayed an IC$_{50}$ value 2.2 µg/mL against the Dd2 malaria parasite. The extract was separated by liquid-liquid partition to give an active dichloromethane fraction. This dichloromethane fraction was further separated on a C$_{18}$ open column, and the most active subfraction (0.6 g, IC$_{50}$: < 1 µg/mL) was separated by C$_{18}$ HPLC. Compounds 7.10 (0.6 mg) and 7.11 (0.8 mg) were isolated, but neither of them had any significant activity against the malaria parasite.

The structure determination of 7.10 and 7.11 was done by mass spectrometric analysis of their molecular formula and comparison of their $^1$H and $^{13}$C NMR data and their optical rotation data with literature data.$^{3-6}$

The search for more potent antimalarial compounds from *Phyllanthus muellerianus* is being continued by other group members.

7.3.2 *Microdesmis caseariifolia* (Pandaceae)
An ethanol extract (100 mg) of the leaves of *Microdemis caseariaefoli* (Pandaceae) (N015451) displayed IC$_{50}$ values of 5.4 μg/mL against the HB3 strain and 6.0 μg/mL against the Dd2 strain of *P. falciparum*. Liquid/liquid partitioning of the crude extract followed by separation of 29 mg active dichloromethane fraction [IC$_{50}$: 0.8 μg/mL (HB3) and 0.4 μg/mL (Dd2)] over reversed phase C$_{18}$ open column, then by C$_{18}$ HPLC afforded 1.0 mg of $N^5$-(p-coumaroyl)-$N^1, N^{10}$-diferuloylspermidine 7.12 (keayanidine B) with IC$_{50} > 1$ μg/mL against both parasites. Its structure was confirmed by analysis of its $^1$H NMR data and the comparison of its mass spectrometric data with literature data.$^7$

![Diagram 7.12](image)

### 7.3.3 *Majidea sp.* (Sapindaceae)

Liquid/liquid partitioning of 116 mg crude leaf extract of *Majidea sp.* (MG 2164) led to 3 fractions. The most active methanol aqueous fraction with IC$_{50}$ 11 μg/mL against the FCM29 malaria parasite was subjected to separation on C$_{18}$ and Sephadex LH 20 open columns to give an active fraction (13.8 mg) with IC$_{50}$ value about 4 μg/mL. The
subfractions of this fraction failed to produce any bioactivity lower than 7 μg/mL. Further separation of this plant thus was stopped.

7.3.4 *Terminalia septentrionalis* (Combretaceae)

An ethanol extract (113 mg) of the leaf extract of *Terminalia septentrionalis* (MG 2316) with an IC₅₀ value of 18 μg/mL against the FCM29 malaria parasite was subjected to liquid-liquid partition to lead to three fractions. The most active aqueous methanol fraction (IC₅₀ 14 μg/mL, 53 mg) was separated by C₁₈ open column chromatography and phenyl HPLC. None of the subfractions showed any increase in the bioactivity as compared with the aqueous methanol fraction. Further separation of this plant was discontinued.

7.4 Structure elucidation of compounds isolated in Madagascar

Dr. Vincent Rasamison at CNARP (Centre National des Recherches Pharmaceutique) in Madagascar is working on the isolation of potent antimalarial compounds. As part of the ICBG program, the isolated bioactive compounds were then sent to Virginia Tech for structure determination.

Twigs and leaves of *Vitex cauliflora* (Verbenaceae) were collected in the eastern region of Madagascar in 2005. One triterpenoid called uvaol (7.13) was isolated from the extract. It did not show interesting antimalarial activity.

Compounds 7.14-7.17 were isolated from the roots of *Vepris macrophylla* (Rutaceae), and were determined to be known compounds, with IC₅₀ values 12 μg/mL, 12 μg/mL, 14
μg/mL and not active against the A2780 cell line, respectively. The structure of compound 7.14 was determined by comparing its spectroscopic data with literature values, and the structures of compounds 7.15-7.17 were determined by analysis of their MS and NMR spectra.

References


VIII. General Conclusions

In our continuing search for biologically active natural products from tropical rainforests as part of an International Cooperative Biodiversity Group (ICBG) program, more than fifteen plants were selected for initial isolation. Six of them were further fractionated to yield twelve new and ten known compounds under the guidance of the A2780 human ovarian cancer cell line. Four antimalarial extracts were selected for further separation to give five new and eight known compounds.

8.1 Anticancer extracts

Fractionation of the leaves, fruit and inflorescence of *Symphonia tanenlesis* led to the isolation of one novel xanthone and two known guttiferones. The xanthone showed moderate activity with IC$_{50}$ 3.8 μM, and both guttiferones also showed moderate activity against the A2780 cancer cell line with IC$_{50}$ values of 8.3 μM and 7.8 μM.

Two new compounds were isolated from the roots of *Bussea sakalava*, four of which are diphenyl propanes. The other compound has a cycloheptadibenzofuran skeleton which has not been previously reported. A possible biosynthetic pathway of the cycloheptadibenzofuran was proposed.

Fractionation of the roots of *Leptadenia madagascariensis* led to four new cardenolide glycosides, all of which showed strong antiproliferative activity against A2780 human ovarian cancer cell line (IC$_{50}$: 0.18, 0.21, 0.17 and 0.29 μM) and the H460 lung cancer cell line (IC$_{50}$: 0.16, 0.68, 0.37 and 0.48 μM).
Four known and two new alkaloids were isolated from the roots of *Ambavia gerradii*. The structures of the new alkaloids were confirmed by total synthesis. Among these alkaloids, the known alkaloid sampagine showed the strongest antiproliferative activities against the A2780 cell line (IC$_{50}$: 0.60 μM) and H460 cell line (IC$_{50}$: 0.58 μM).

### 8.2 Antimalarial extracts

Fractionation of aerial parts of *Polyclione proteiformis* guided by assay against HB3 and Dd2 malaria parasites led to the isolation and structure elucidation of five new sesquiterpene lactones called pseudoguaianolides and the flavanoid centaureidin. Two of those new pseudoguaianolide displayed strong antimalarial activities against both parasites with IC$_{50}$ values < 1.0 μM. All of the pseudoguaianolides showed strong antiproliferative activities against the A2780 cell line. However, they are thus not good lead compounds for antimalarial drug development.

Five known compounds were isolated from the leaves of *Phyllanthus muellerianus*, none of which showed strong activities against HB3 and Dd2 malaria parasite, although the crude extract was tested to have strong activities against both malaria strains. Some other fractions were found to have strong antimalarial activities. Time did not permit the isolation of pure compounds from these fractions, and it is recommended that further exploration of this plant be undertaken to isolate potent antimalarial compounds.
APPENDIX
(\textsuperscript{1}H and \textsuperscript{13}C NMR spectra)

\textsuperscript{1}H NMR spectrum of 2.1 in CD\textsubscript{3}OD

\textsuperscript{13}C NMR spectrum of 2.1 in CD\textsubscript{3}OD
$^1$H NMR spectrum of 3.1 in CD$_3$OD

$^{13}$C NMR spectrum of 3.1 in CD$_3$OD
$^1$H NMR spectrum of 3.2 in CD$_3$OD

$^{13}$C NMR spectrum of 3.2 in CD$_3$OD
$^1$H NMR spectrum of 3.3 in CD$_3$OD

$^{13}$C NMR spectrum of 3.3 in CD$_3$OD
$^1$H NMR spectrum of 3.4 in CD$_3$OD

$^{13}$C NMR spectrum of 3.4 in CD$_3$OD
$^1$H NMR spectrum of 3.5 in CDCl$_3$

$^{13}$C NMR spectrum of 3.5 in CDCl$_3$
$^1$H NMR spectrum of 4.1 in d-Pyridine

$^{13}$C NMR spectrum of 4.1 in d-Pyridine
$^1$H NMR spectrum of 4.2 in CD$_3$OD

$^{13}$C NMR spectrum of 4.2 in CD$_3$OD
$^1$H NMR spectrum of 4.3 in d-Pyridine

$^{13}$C NMR spectrum of 4.3 in d-Pyridine
$^1$H NMR spectrum of 4.3 in CD$_3$OD

$^{13}$C NMR spectrum of 4.3 in CD$_3$OD
$^1$H NMR spectrum of 4.4 in CD$_3$OD

$^{13}$C NMR spectrum of 4.4 in CD$_3$OD
$^1$H NMR spectrum of 5.1 in CD$_3$OD

$^{13}$C NMR spectrum of 5.1 in CD$_3$OD
$^1$H NMR spectrum of 5.2 in CD$_3$OD

![1H NMR spectrum of 5.2 in CD$_3$OD](image)

$^{13}$C NMR spectrum of 5.2 in CD$_3$OD

![$^{13}$C NMR spectrum of 5.2 in CD$_3$OD](image)
$^1$H NMR spectrum of 5.11 in CD$_3$OD

$^{13}$C NMR spectrum of 5.11 in CD$_3$OD
$^1$H NMR spectrum of **5.5** in CDCl$_3$

$^1$H NMR spectrum of **5.6** in CDCl$_3$

$^1$H NMR spectrum of **6.1** in CDCl$_3$
$^{13}$C NMR spectrum of 6.1 in CDCl$_3$
$^1$H NMR spectrum of 6.2 in CDCl$_3$

$^{13}$C NMR spectrum of 6.2 in CDCl$_3$

$^1$H NMR spectrum of 6.3 in CDCl$_3$
$^{13}$C NMR spectrum of 6.3 in CDCl$_3$

$^1$H NMR spectrum of 6.4 in CDCl$_3$
$^{13}$C NMR spectrum of 6.4 in CDCl$_3$

$^1$H NMR spectrum of 6.5 in CDCl$_3$
$^{13}$C NMR spectrum of 6.5 in CDCl₃

$^1$H NMR spectrum of 6.6 in CD₃OD
$^{13}$C NMR spectrum of 6.6 in CD$_3$OD