1-1-2012

Antimalarials from Plant Pathogenic Fungi

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ANTIMALARIALS FROM PLANT PATHOGENIC FUNGI

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Mississippi

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December 2012

ABSTRACT

The purpose of this study was to discover antimalarial compounds from necrotropic plant pathogenic fungi**.** Various phytotoxins released by necrotropic plant pathogenic fungi may inhibit the plant-like metabolic pathways in the apicoplast, a chloroplast-like organelle, essential for the survival of apicomplexan parasites, such as *Plasmodium* which cause malaria in humans. This dissertation describes the isolation, characterization, and biological evaluation of secondary metabolites of eight fungal strains. In addition to the antiplasmodial assay, all the extracts were subjected to phytotoxic, antimicrobial, and cytotoxic bioassays. Isolation of metabolites was performed by chromatographic methods and identification was carried out by spectroscopic and spectrometric methods. Absolute configurations of the metabolites were determined by comparing specific rotation values, ECD spectra, or X ray crystallographic analysis.

The dissertation consists of seven chapters. Chapter 1 describes the overview of the current malaria situation in the world and the rationale for using necrotropic plant pathogenic fungi to discover antimalarial leads. Protocols used for isolation and fermentation of endophytic fungi from infected plant parts, as well as those used for biological activities of fungal metabolites are described in Chapter 2. Chapters 3-6 describe the isolation and identification of

metabolites from the plant pathogenic fungi obtained from the American Type Culture Collection (ATCC) (*Septoria pistaciarum*, *S. musiva*, *Phomopsis viticola*, and *Postia balsamea*).

Chapter 7 consists of four sections which describe the isolation and identification of active metabolites from four endophytic fungi (*Xylaria* sp., *Alternaria* sp., *Botryosphaeria dothidea*, and a new fungus, UM #10 M) isolated from the infected leaves and seeds of *Torreya taxifolia.*

From these fungi, 40 compounds including 17 new natural products were isolated and identified. Thirteen compounds displayed antiplasmodial activity, 12 showed phytotoxicity, whereas all 13 exhibited weak to moderate cytotoxicity towards mammalian cells. This indicates that cytotoxicity rather than inhibition of plant-like metabolic pathways in the apicoplast was probably responsible for the observed antiplasmodial activity.

The antiplasmodial compounds with high selectivity indices, septoriamycin A and 19,20-epoxycytochalasin C, were evaluated for antimalarial activity in a mouse model. Septoriamycin A showed no activity or toxicity, whereas 19,20-epoxycytochalasin C, exhibited toxicity and weak suppressive activity.

DEDICATION

To my Parents and all the Teachers

who taught me the richness of education in my life

ACKNOWLEDGMENTS

I wish to express my deepest appreciation and gratitude to my advisors, Dr. D. Ferreira, Chair and Professor of Pharmacognosy, and Dr. N. P. D. Nanayakkara, Research Professor in the Research Institute of Pharmaceutical Sciences, National Center for Natural Products Research (NCNPR), School of Pharmacy, University of Mississippi, for their constant supervision, invaluable guidance, unfailing encouragement, and financial support throughout the course of my study. This research would not have been possible without their advice, constructive criticism, and extensive discussions.

I would like to extend my special thanks to all of my internal advisory committee members, Dr. M. Slattery, Department of Pharmacognosy, Dr. S. Khan, NCNPR and my external advisor, Dr. J. Hoeksema, Department of Biology, for their valuable suggestions and critical discussions during my graduate studies. Special thanks are also due to all the faculty within the Departments of Pharmacognosy, Medicinal Chemistry, and Biology who taught me multidisciplinary courses that helped me gain insight into the research environment.

I would like to extend my appreciation and gratitude to Dr. Edward Croom, Croomia, 1509, Smallwood Dr., Oxford, MS 38655, for suggesting that *Torreya taxifolia*

Arnott should be a rich source of biologically active compounds from endophytic plant pathogens and furnishing the infected plant material used to isolate the endophytic fungi. My sincere thanks are also extended to Dr. Luiz H. Rosa, Laboratório de Microbiologia, Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, MG 35400-000, Brazil, for providing me the endophytic fungi isolated from infected seeds from *T. taxifolia*, and Dr. N. Techen, NCNPR, for her kind assistance in identifying the endophytic fungi isolated from *T. taxifolia*. I also want to express my sincere thanks to Dr. D. E. Wedge and Ms. Linda Robertson, United States Department of Agriculture (USDA), for providing me with training on fungi culture maintenance and microbiology techniques.

Special thanks are also extended to Dr. M. Jacob, Dr. S. Khan, Dr. B. Tekwani, and Dr. S. Duke for providing data on the antimicrobial, antimalarial, antileishmanial, and phytotoxic activities of all the compounds included in this thesis. My thanks are also due to Dr. B. Avula, Dr. M. Wang, and Mr. F. T. Wiggers for recording the HRMS, GCMS, and ^{1}H NMR spectra (600 MHz), and to Dr. F. Fronczek, Department of Chemistry, Louisiana State University, for proving X-ray crystallographic data, which allowed for the successful assignment of the absolute configuration of septoriamycin A.

I would like to extend my special thanks to Dr. K. Meepagala, USDA and Dr. W. Herath, NCNPR for their kind help and generous support. My thanks also go to all the lab

members in Dr. Nanayakkara's lab, Dr. T. B. Herath, Dr. H. R. W. Dharmaratna, and Dr. V. Reddy for their kind assistance and cooperation. Special thanks also go to Ms. Casey Stauber, for her administrative help and constant support. It's my pleasure to acknowledge all the fellow graduate students, my past and present friends, and all individuals who helped me knowingly and/or unknowingly in various ways to achieve success in my graduate studies.

The NCNPR, University of Mississippi, is greatly acknowledged for providing me with a graduate student scholarship award. In addition, the funding sources, i.e. The National Institutes of Health (R21 A1061431-01 and R01 AI 27094) and, in part, by the USDA, ARS, Specific Cooperative Agreement No. 58-6408-2-009 and US DoD CDMRP Investigator Initiated Grant Award W81XWH-09-2-0093, are acknowledged.

Last but not least, I would like to offer my gratitude and thanks to my parents and family for their lovely, never-ending support given to me in all the way to fulfill my dreams. I would like to extend my special thanks to my husband Upul, and to my son Isuru for being with me and giving me love, patience, endless encouragement, and a cheerful understanding atmosphere throughout my graduate career at Ole Miss.

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ANTIMALARIALS FROM PLANT PATHOGENIC FUNGI

1.1. Background and Significance

Malaria is an infectious disease caused by apicomplexan parasites of the genus *Plasmodium* that are transmitted to people through bites of infected *Anopheles* mosquitoes. This disease is endemic throughout the tropics, especially in sub-Saharan Africa and the developing world, threatening about 40% of the world's population.[1] More than 200 million cases of malaria and around 650,000 deaths, the majority of them African children, have been reported in 2010.[1]

Human malaria is caused by *Plasmodium falciparum, P. vivax, P. ovale,* and *P. malariae. P. falciparum* and *P. vivax* are the most prevalent, and *P. falciparum* is responsible for the majority of mortality. *P. vivax, P. ovale,* and *P. malariae* cause a mild form of the disease, and *P. knowlesi,* a zoonosis, also rarely infects humans. Owing to the unavailability of effective vaccines, pharmacotherapy is still the only mode of therapy for malaria.[2] Several drugs, synthetic and natural product-derived, are currently available for the treatment of malaria;[2, 3] however, control of this disease is limited by drug resistance in the parasite and insecticide resistance in

the mosquito vector.[3] Resistance to all available drugs except artemisinin has been reported from many malaria endemic areas. Recent studies indicate the emergence of strains in Cambodia also resistant to artemisinin derivatives.[4] No new class of antimalarials has been approved since 1996,[3] therefore, there is an urgent need to develop drugs with new modes of action to counter this trend.

1.2. The Apicoplast as an Antimalarial Drug Target

The apicoplast is an organelle essential for the survival and viability of the apicomplexan parasites such as *Plasmodium*, *Toxoplasma*, *Theileria*, *Babasia*, and *Eimeria* (Figure 1.1). This organelle is similar to plastids found in the cells of photosynthetic organisms like algae and plants.[5] Plastids are present in plants in several forms such as chloroplasts, leucoplasts, amyloplasts, and chromoplasts with the same genetic compliments.[6] It is believed that apicomplexan parasites acquired the apicoplast by engulfment of an ancestral algae and retention of its plastid.[5] Apicoplasts contain their own genome that encodes for prokaryotic protein synthesis (70 S ribosomes, tRNAs, RNA polymerase, and DNA gyrase) and a small number of proteins.[7] Many biosynthetic pathways in apicoplasts, including heme and ion sulfur clusters, type II fatty acid synthase, and the non-mevalonate pathway for isoprenoids, are absent in the vertebrate hosts of parasites, but are similar to those present in the plastids of plants (Table 1.1).[5, 6, 8] Apicoplast function is essential for both erythrocytic and hepatic development of the plasmodia parasites in mammalian hosts;[7] thus, metabolic pathways found in apicoplasts are good targets for antimalarial drug discovery.[5] Research over the last decade has demonstrated a

number of validated targets in plant-like metabolic pathways in the apicoplast of malaria parasites.[5, 7, 9, 10]

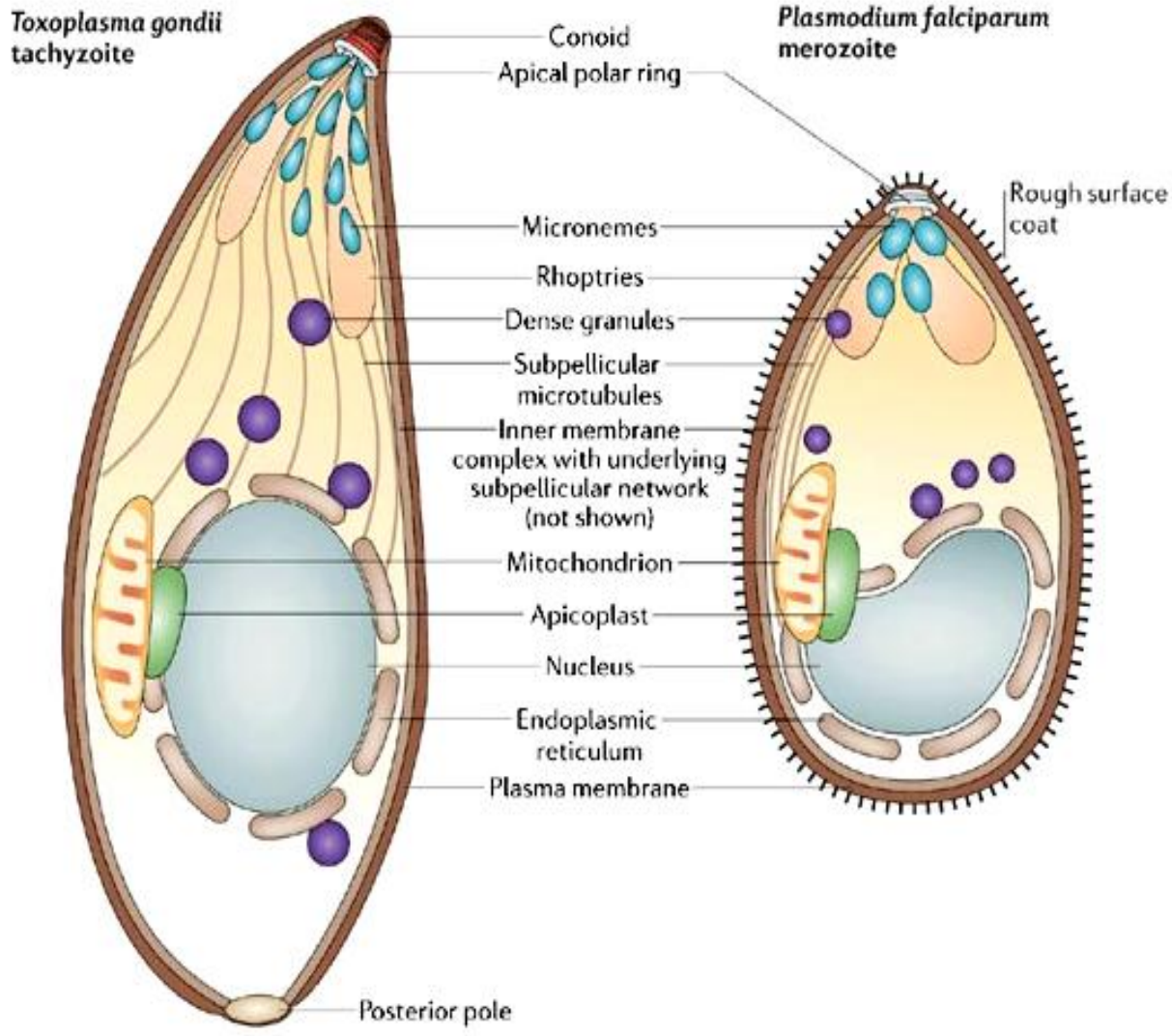

Figure 1.1. General features of apicomplexan cells[11]

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Table 1.1. Drugs and possible apicoplast targets[5, 10]

Drug/herbicide	**Metabolic activity**	**Putative target**
Ciprofloxacin	DNA replication	Plastid DNA topoisomerase II
Clindamycin	Protein translation	Plastid 23S rRNA
Erythromycin		
Azithromycin		
Spiramycin		
Thiostrepton		
Micrococcin		
Chloramphenicol		
Doxycycline		Plastid 16S rRNA
Tetracycline		
Amythiamicin		Elongation factor Tu (thermo unstable)
Rifampicin	RNA transcription	Plastid RNA polymerase β-subunit
Fosmidomycin	Isoprenoid biosynthesis	DOXP reductoisomerase
Thiolactomycin	Fatty acid biosynthesis	*B*-ketoacyl-ACP synthase (Fab H)
Clodinafop	Fatty acid biosynthesis	Acetyl-CoA carboxylase (ACC)
Triclosan	Fatty acid biosynthesis	Enoyl-ACP reductase (Fab I)

Antibiotics such as azithromycin, doxycycline, clindamycin, tetracyclines, rifampicin, and fluoroquinolones which are used for prophylaxis and treatment of malaria in combination with other antimalarial drugs,[7] have been reported to target apicoplast prokaryotic derived protein translation pathways.[5,7,12] Isoprenoid biosynthesis of the apicoplast involves the non-mevalonate pathway which is similar to that in bacteria, and is known as the 1-deoxy-D-xylulose-5-phosphate (DOXP) or 2-C-methyl-D-erythritol-4-phosphate (MEP)pathway.[6] Unlike in humans, these isoprenoids are prosthetic groups for many enzymes involved in apicoplast translation, synthesis of mitochondrial ubiquinones, protein prenylation, and the formation of dolichols which are also involved in membrane proteins of the parasite.[6] Fosmidomycin, an inhibitor of DOXP reductoisomerase (IspC) of isoprenoid biosynthesis in the apicoplast has been reported to be effective in killing in the intra-erythrocytic phase of the malaria parasite.[6] Tonhosolo et al.[13] also reported the presence of carotenoid biosynthesis including the phytoene desaturase enzyme in the apicoplast of the intraerythrocytic stages of *P. falciparum*. Inhibition of parasite growth by the herbicide norflurazon, a phytoene desaturase inhibitor, and chemical rescue by lycopene clearly indicated that this pathway is also a potential target for antimalarial drug therapy.[13] Recent findings of chemical rescue of blood stage *P. falciparum* lacking an apicoplast demonstrated that parasites also exclusively depend on the apicoplast for isoprenoid precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP).[14] In contrast to humans and other eukaryotes without plastids, the apicoplast of the malaria parasite uses bacterial type fatty acid II synthase for the synthesis of lipids required for its function and to form parasitoporous vacuoles (PV) for the invasion of host erythrocytes.[5] Apicoplast fatty acid II synthase biosynthesis involves several distinct enzymes namely, acetyl-CoA carboxylase (ACC),

acyl carrier protein transacylase (FabD), *β*-ketoacyl-ACP synthase (FabH), *β*-ketoacyl-ACP synthase (FabB/F), *β*-ketoacyl-ACP reductase (FabG), *β*-hydroxyacyl-ACP dehydratase (FabZ/A) and *trans*-2-enoyl-ACP reductase (FabI).[15] All these enzymes are potential targets for malaria drug discovery.[15, 16] FabH inhibitors like cerulenin, platensimycin,[17] thiolactomycin, and their analogues,[18] and FabI inhibitors triclosan,[18] and (*E*)-oroidin,[19] many of these of natural origin, have been reported to have herbicidal and antimalarial activities.[5, 9] Several attempts have also been made in designing and in silico screening of analogues of triclosan,[16, 20] and thiolactomycin,[18] as well as flavonoid derivatives[21] for the antimalarial FAS-II activity.

The apicoplast of the malaria parasite also possesses a family of class II histone deacetylase (HDAC) proteins which are also present in other photosynthetic eukaryotes and primitive algae.[22] The enzyme CpHDAC3 (Rpd3 super family classs II) catalyzes deacetylation of histone tails to promote epigenetic regulation and some cellular processes of the parasite.[22-24] Analogues of several known anticancer HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), SB 939, hydroxamic acid (TSA), and apicidin have shown activity against the malaria parasite, some at low nanomolar concentrations.[23, 25-30]

It has been demonstrated that all these apicoplast specific pathways are important for its proper functioning. Plasmodium parasites exclusively depend on the apicoplast for isoprenoid precursors, and it is involved in establishing the parasitophorus vacuole in host cells and invasion of parasites in new host cells.[6, 9, 14] Thus, plant-like metabolic pathways in the apicoplast are potential targets for malaria drug discovery.

1.3. Plant Pathogenic Fungi

Fungi produce a variety of biologically active secondary metabolites with applications in agriculture and medicine.[31] Endophytic fungi reside inter- and-/or intracellularly in plants and their association can vary from latent pathogenesis to mutualistic symbiosis.[31,32] Many endophytic fungi are inert phytopathogens which cause disease symptoms when the host plant is aged and/or stressed under various environmental conditions.[33] Phytopathogenic fungi are ubiquitous in the plant kingdom and their diversity depends on the ecosystem and host species. Plant pathogenic fungi are grouped into three categories based on their mode of nutrition.[34, 35] Biotrophs derive their nutrients from the host plant without killing it, whereas necrotrophs kill host plant cells to derive their nutrition.[34] Hemi-biotrophs have an initial biotrophic phase and then gradually become necrotrophic.[35] Some biotrophs have symbiotic as well as parasitic associations.[35] Parasitic biotrophic fungi such as rusts, smuts, and mildew have a restricted host range.[35] In general, necrotrophs have a broader host range than biotrophs.[36]

The invasion of host cells by necrotrophic fungi with appressoria and haustoria is initiated by active penetration of the surface by germinating fungal spores in favorable environmental conditions.[37] The fungi release degradative enzymes such as pectinases, cellulases, proteases, and toxins to kill host cells.[37] Wood-rotting pathogenic fungi preferentially attack living trees and rapidly depolymerize the cell wall components of internal, vascular, and cambium tissues.[34] Host cells activate plant defense mechanisms by releasing phytoalexins, antifungal pathogenesis related proteins (PR's), and oxidative enzymes.[38] The spread of the

pathogen is controlled by an effective host defense mechanism;[39] however, most of the plant pathogenic fungi (necrotrophs) can overcome the host defense mechanisms and derive nutrition by killing the neighboring cells. According to Schulz and Boyle's hypothesis[39] (Figure 1.2), as long as fungal virulence and host defense are balanced, colonization remains asymptomatic.[39] When the balance is disturbed by environmental factors or by senescence in favor of the fungus, disease develops.[39] Pathogenesis is associated with the amount of phytotoxin produced by the pathogen.

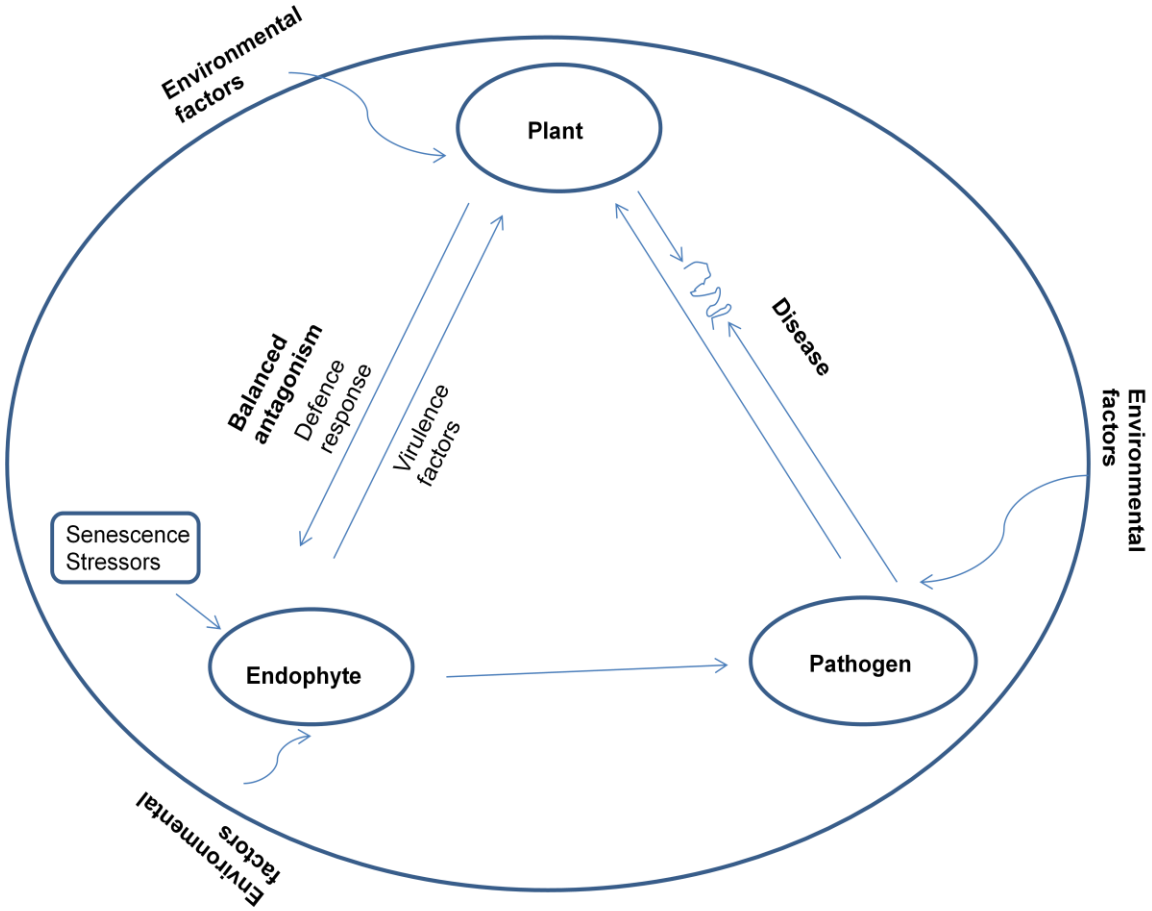


Figure 1.2. Host-fungi interaction[39]

Degradative enzymes and/or toxins released into host cells by necrotrophic fungi cause necrotic lesions, chlorosis, wilting, and finally death of plant parts.[40] Necrotrophic disease is recognized by patches of dead black tissues and identified by different names such as anthracnose, blight, canker, scab, leaf spot, fruit rot, heart rot, butt rot, root rot, and shot hole.[34] Unlike biotrophs, necrotrophs can survive in the absence of their host cells and thus can be cultivated in an artificial media to isolate the biologically active lead molecules.

Some natural and synthetic phytotoxic compounds have been shown to target the plant-like metabolic pathways in the plastid of plants, indicating their potential as inhibitors of metabolic pathways in the apicoplast of malaria parasites.[41]

1.4. Fungi as a Source of Antimalarials

Natural products have been used as antimalarial drugs or pharmacophores in designing potent analogues. Quinine, artemisinin, doxycycline, clindamycin, and azithromycin are some of the currently used malaria drugs that have been isolated from plants or microorganism.[42] Fungal metabolites have several advantages over plant secondary metabolites: large scale preparation of metabolites can be carried out without disturbing biodiversity; optimization can be done using genetic engineering approaches; and results can be obtained in a short time, in many cases in less than one month, reducing the cost and time of drug development processes. Endophytic fungi, including necrotrophic plant pathogenic fungi, have yielded a number of metabolites with a variety of biological activities including antimalarial activity;[31] however, antimalarial fungal

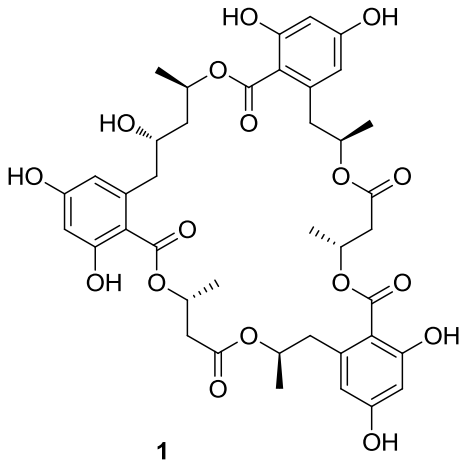
metabolites may also act on targets other than those in the apicoplast such as cytosolic targets (dihydrofolate reductase inhibitors), food vacuole targets (haemozoin inhibitors), and membrane targets (biosynthesis and transport of phospholipids).[5, 43]

Recently, several antimalarial compounds have been isolated and identified from fungi and some of these compounds can be considered potential therapeutic agents. A Scifinder Scholar® survey on antimalarials reported from endophytic, insect pathogenic, and plant pathogenic fungi showed a number of active compounds. These compounds are chemically diverse and showed varying degrees of antiplasmodial and cytotoxic activities (Table 1.2 & Figure 1.3). Of these compounds, radicicol (**30**) was moderately active in vivo at 40 mg/kg and heptelidic acid (**31**) showed hemolysis at this dose.

Table 1.2. Antimalarials reported from fungi

Fungi	Compounds	Activity/μM	Cytotoxicity	Ref.
Menisporopsis theobromae	Menisporopsin A (**1**)	5.0	Cytotoxic	[44]
Menisporopsis theobromae	Dithiodiketopiperazine (**2**)	2.95	Weak	[45]
Cordyceps brunnearubra	Cordyformamide (**3**)	18	Weak	[46]
Paecilomyces cinnamomeus	Paecilodepsipeptides (**4**)	4.9	Cytotoxic	[47]
Hirsutella nivea	Hirsutatins B (**5**)	8.2	Not toxic	[48]
Phomopsis sp.	Phomoxanthones A (**6**) Phomoxanthones A (**7**)	0.14 0.44	Cytotoxic Cytotoxic	[49]
Favolaschia tonkinensis	9-Methoxystrobilurin B (**8**) 9-Methoxystrobilurin G (**9**)	0.85 0.06	Weak to moderate	[50]
Cordyceps militaris	Cordycepin (**11**)	17.8	Not reported	[51]

Geotrichum sp.	Dihydroisocoumarin (**12**)	8.4	Not reported	[52]
Hirsutella kobayasii	Hirsutellide A (**13**)	4.2	Non toxic to vero	[53]
Kionochaeta pughii	Pughiinin A (**14**) Picnidione (**15**)	6.7 0.5	Not toxic Cytotoxic	[54,55]
Microsphaeropsis sp.	Preussomerins (**16**)	2.3	Cytotoxic	[56]
Xylaria sp.	(-)-Depudecin (**17**) (+)-Phaseolinone (**18**) (+)-Phomenone (**19**) 19,20-Epoxycytochalasin Q (**20**)	4.7 1.7 1.2 1.1	Cytotoxic Cytotoxic Cytotoxic Cytotoxic	[57]
Sclerotinia homoeocarpa	Tetranorditerpenoids (**21**)	0.1	Cytotoxic	[58]
Rhizina sp.	Chromanone derivatives (**22**)	14.7	Moderate	[59]
Berkleasmium nigroapicale	Berkleasmin (**23**)	5.4	Cytotoxic	[60]
Torrubiella luteorostrata	Pyrone diterpene (**24**)	8.1	Cytotoxic	[61]
Penicillium sp.	Brefeldin A (**25**)	3.9	Cytotoxic	[62]
Myrothecium verrucaria	Macrocyclic trichothecenes Roridin E acetate (**26**)	0.0001	Cytotoxic	[63]
Cordiceps nipponica	Cordypyridones A (**27**) Cordypyridones B (**28)**	0.23 0.13	Weak Weak	[64]
Streptomyces sp. *Humicola* sp. Soil fungus- FO-4443	Nanomycin A (**29**) Radicicol (**30**) Heptelidic acid (**31**)	0.03 0.02 0.04	Weak Weak Weak	[65, 66] [67]



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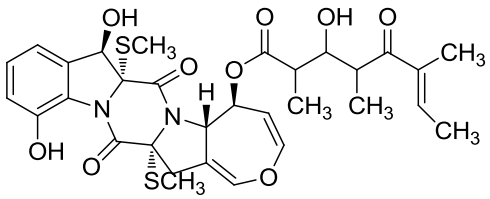

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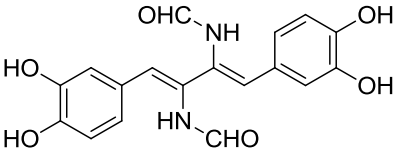

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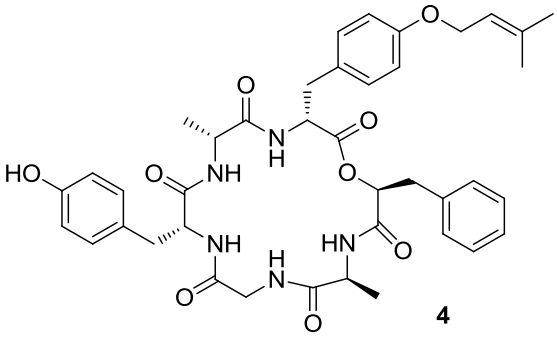

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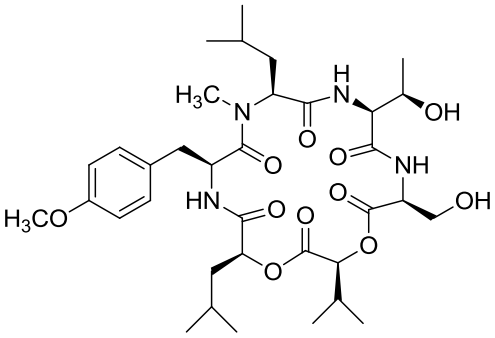

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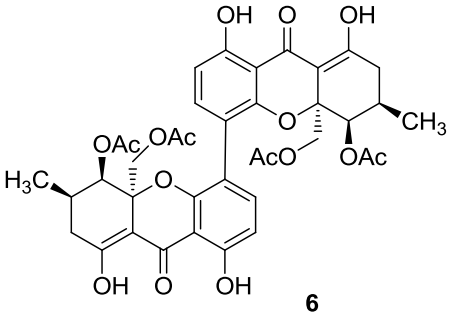

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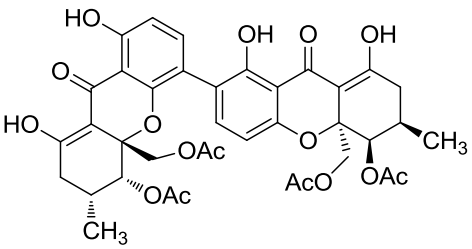

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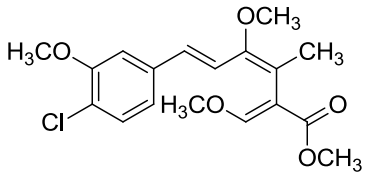

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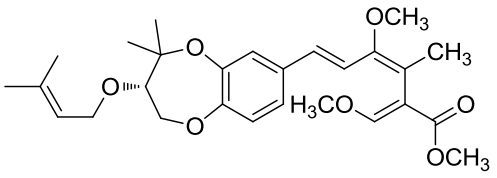

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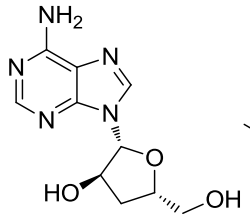

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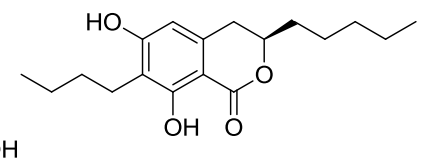

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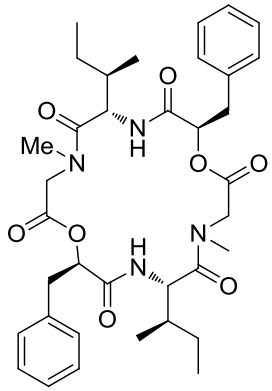

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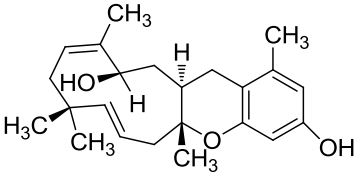

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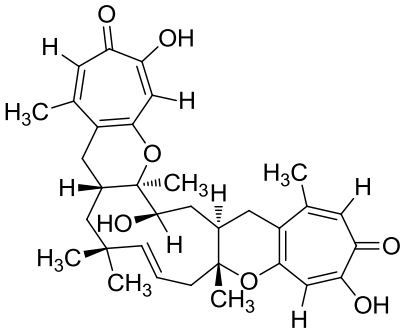

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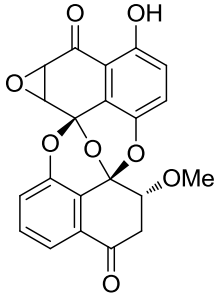

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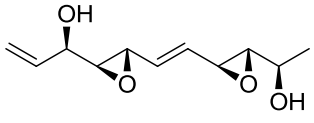

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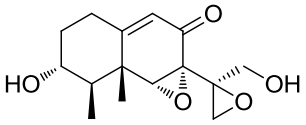

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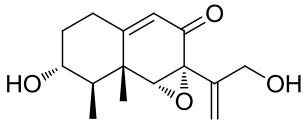

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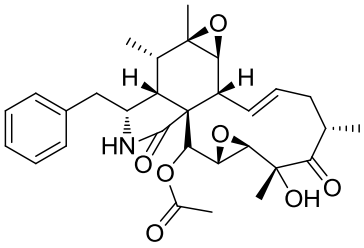

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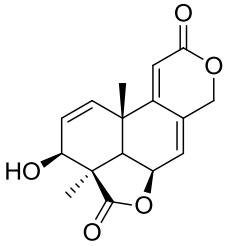

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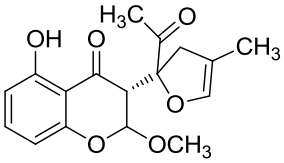

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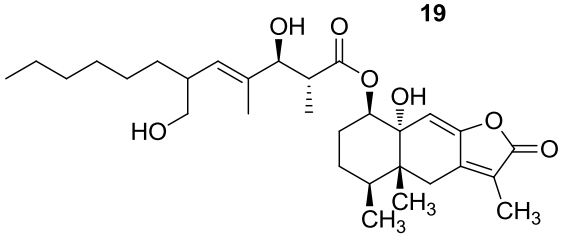

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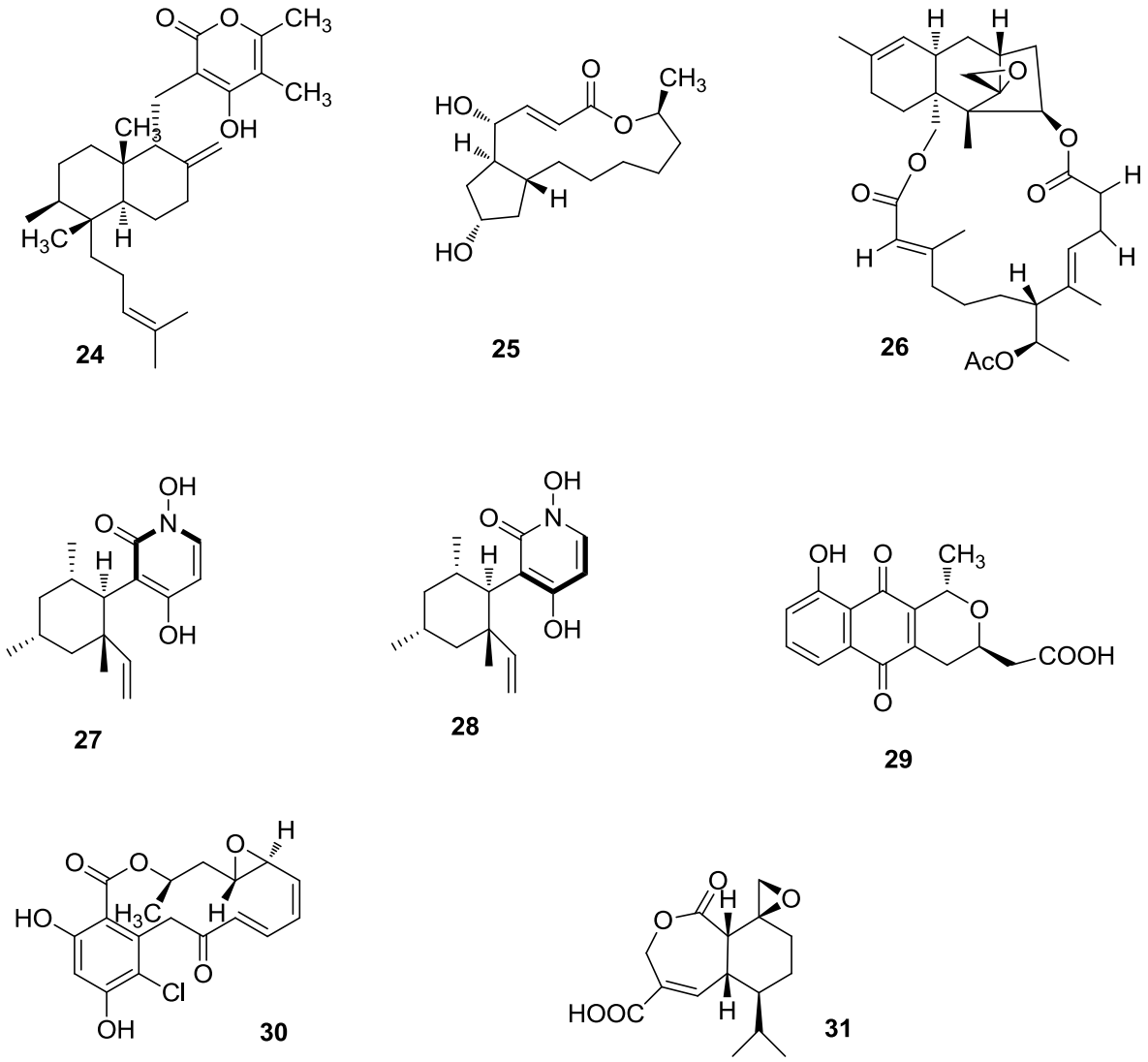


Figure 1.3: Antimalarial compounds reported from fungi

1.5. Aims and Objectives of the Thesis

Malaria, an infectious disease caused by *Plasmodium* parasites, is a major cause of morbidity and mortality in tropical parts of the world. Control of this disease is hampered by drug resistance. No new class of antimalarial drug has been approved since 1996.

The overall aim of this project is to search for antimalarial compounds from necrotrophic plant pathogenic fungi based on the rationale that certain phytotoxins biosynthesized by this class of fungi may inhibit plant-like metabolic pathways in the apicoplast, an essential organelle, of the apicomplexan parasites including *Plasmodium*.

Main objectives were:

1.5.1. Procurement of necrotrophic plant pathogenic fungi

1.5.2. Culture and extraction of fungi

1.5.3. Evaluation of fungal extracts for herbicidal and antiplasmodial activities

1.5.4. Bioassay guided fractionation of active extracts and isolation of compounds responsible for biological activity.

1.5.5. Identification of actives compounds

1.5.6. Evaluation of promising active compounds in animal models

CHAPTER 2: MATERIALS AND METHODS

The overall approach of this research project as summarized in Figure 2.1 was to search for antimalarial actives from necrotrophic plant pathogenic fungi based on the rationale that some phytotoxins released by these fungi may inhibit plant-like metabolic pathways in the apicoplast, an essential organelle, of the apicomplexan parasites.

2.1. Sources of Necrotrophic Plant Pathogenic Fungi

ATCC (American Type Culture Collection): The ATCC database was searched for fungi which caused leaf spot, fruit rot, heart rot, butt rot, and root rot diseases in plants and the available organisms were purchased.

Plant pathology labs: Identified plant pathogenic fungi were obtained from the Biological Control of Pests Research Unit, USDA-ARS, Stoneville, MS 38677, USA and the Center for Bottomland Hardwoods Research, USDA-Forest Service, Southern Hardwoods Laboratory, Stoneville, MS 38677, USA.

Fresh necrotropic plant-pathogenic fungal isolates from infected plant: Leaves which showed cell death (leaf spots) due to fungal infections were collected from cultivated plants and fungi were isolated from infected leaf areas.

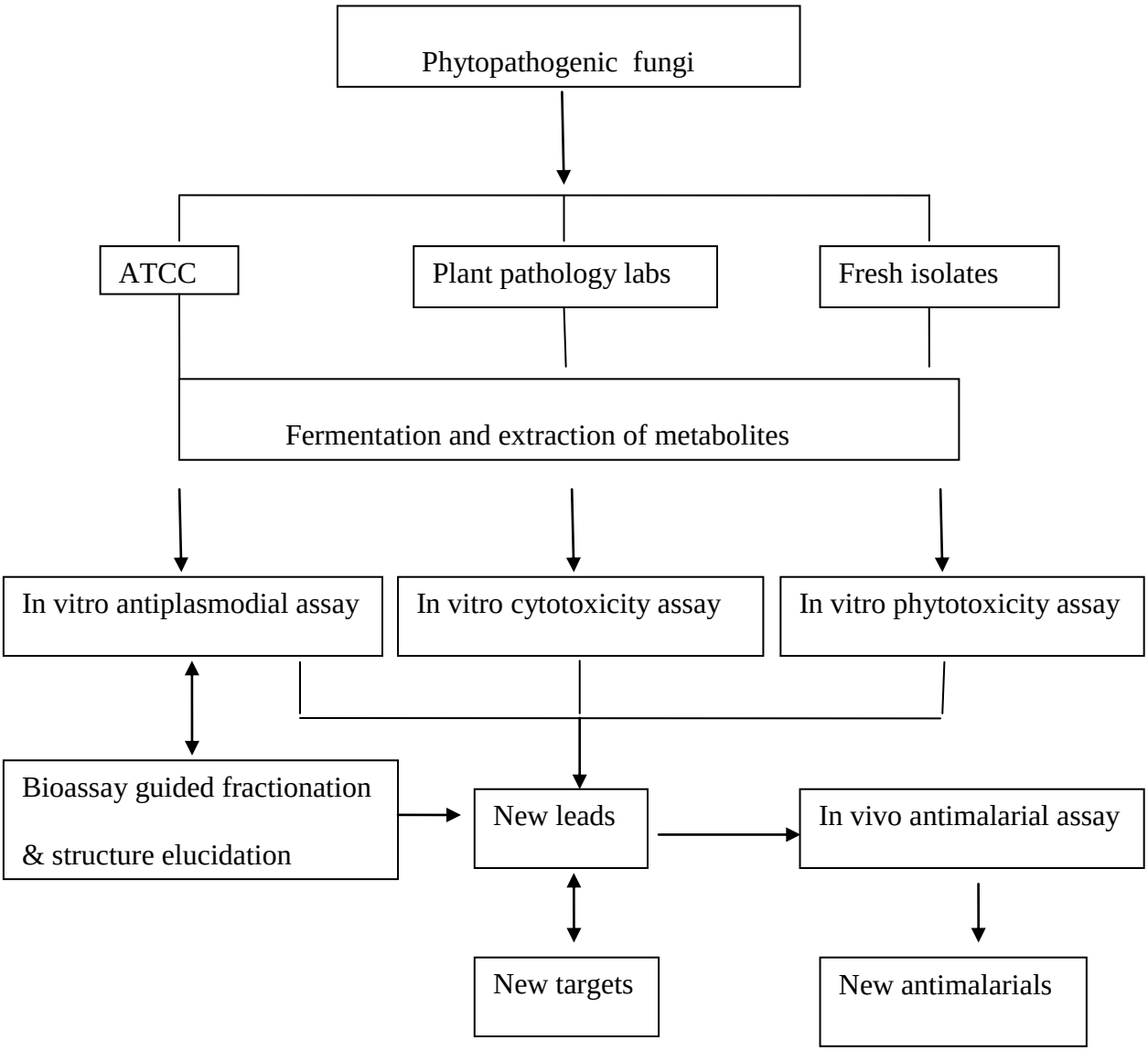


Figure 2.1. The overall design of the research approach

2.2. Isolation of Fungi from Infected Plant Materials

Leaves of the infected plant, *Torreya taxifolia* Arnott, were obtained from Dr. Edward Croom. The plant species identity was determined by Dr. Croom following the taxonomic treatment in the Flora of North America (http://www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=220013607).

2.2.1. From Infected leaves

The infected parts of leaves were surface-sterilized with 5% Clorox for 5 minutes and rinsed with sterile water (×3). Transverse sections from the dried leaf were cut aseptically into small portions and immersed into Potato Dextrose plates (PDA). Plates were incubated for two weeks and sub-cultured to PDA plates to isolate pure fungal strain.

2.2.2. From Infected seeds

Infected seeds of *T. taxifolia* Arnott were furnished by Dr. Edward Croom, Croomia, 1509, Smallwood Dr., Oxford, MS, and isolation of fungi from those was performed by Dr. Luis Rosa, NCNPR, University of Mississippi.

2.3. Identification of Fungi

DNA sequence analysis of fungi was carried out[68] by Dr. N. Techen, NCNPR, University of Mississippi

Preliminary identification of isolated fungi were mainly based on morphological features of colonies and DNA sequence analysis of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA).

2.4. In Vitro Culture and Extraction of Plant Pathogenic Fungi

Pure fungal isolates were grown in PDA plates at 27 ºC for 5-7 days. For preliminary assays, fungi were cultured in 2 conical flasks (1 L) containing 500 mL of potato dextrose broth and incubated at 27 ºC for 2 weeks, 3 weeks, and 4 weeks respectively, on an orbital shaker. Culture medium was separated by filtration and mycelium and broth were extracted with EtOAc (× 3). Fungi which showed antiplasmodial activity were cultured in large scale (20 L, 40 L) media to obtain sufficient amount of extracts. If a fungal extract showed no activity in the antimalarial bioassay, then the fungus was cultured in three additional media selected from the following:

1. PDB
2. Modified Czapex Dox Broth
3. Solid media (Rice)

4. V – 8 (100% vegetable juice)

2.5 Evaluation of In vitro Antimalarial and Phytotoxic Activities of the Extracts

Mycelium and broth extracts were subjected to in vitro antiplasmodial (10.0 mg each) and phytotoxic (1.0 mg of each) assays. Metabolites which inhibit plant-like metabolic pathways in the apicoplast should exhibit both antimalarial and phytotoxic activities. However, cytotoxic compounds also may show positive results in both of these assays. In order to identify these compounds, extracts were also simultaneously evaluated for cytotoxicity and selectivity indices (IC_{50} cytotoxicity / IC_{50} antiplasmodial activity). Active extracts with good selectivity indices (>10) were selected for bioassay-guided fractionation.

2.6. Bioassay Guided Fractionation of Antimalarial Extracts, Isolation, and Structural Elucidation of Bioactive Metabolites.

Active extracts were fractionated using chromatographic methods. A suitable stationary phase was selected on the basis of the chemical profile of the extract. For preliminary fractionation, gel filtration, reversed-phase, or normal phase column chromatography was used. Further separation of active fractions was carried out by the same methods and also by preparative TLC and HPLC. Structure elucidation of active compounds was carried out by spectroscopic/spectrometric methods (UV/Visible, IR, ^{1}H, ^{13}C, NMR, COSY, NOESY/ROESY, HMQC, HMBC, and HRMS). Absolute configuration was determined by comparing specific rotation values, ECD spectra, or X ray crystallography.

Out of the broth and mycelia extracts of 60 plant pathogenic and endophytic fungi screened in different culture media, the extracts as shown in Table 2.1 showed good antiplasmodial and herbicidal activities. Among them the following fungal strains were selected for further isolation and characterization of bioactive metabolites

1. *Septoria pistaciarum* (Chapter 3 & 4)
2. *Septoria. musiva* (Chapter 5)
3. *Phomopsis viticola* (Chapter 5)
4. *Postia balsamea* (Chapter 6)
5. *Xylaria* sp. (Chapter 7 section 1)
6. *Alternaria* sp. (Chapter 7 section 2)
7. *UM #10 M* (new isolate) (Chapter 7 section 3)
8. *Botryosphaeria dothidea* (Chapter 7 section 4)

These extracts were also screened for antimicrobial and antileishmanial activities. Among those, some of plant pathogenic fungal extracts (*Sclerotinia homeocarpa*, *Gleosporium minus*, *Elsinoe corni*, *Resinium bicolor*) and an unidentified endophytic fungus (*# 1 A*) isolated from cultivated *Torreya taxifolia* Arnott showed good antimicrobial activities.

2.7. Preparation of Culture Media

2.7.1. Potato Dextrose Broth (PDB)

24.0 g of PDB powder (Difco^{TM})

1.0 L of distilled water

PDB powder and a magnet was added to an Erlenmeyer flask (1L) containing 1 L of distilled water and heated at boiling point on a stirrer for about 15 minutes. The solution was divided into two Erlenmeyer flasks (500 mL). Flasks were covered with Aluminum foil and then autoclaved at 121 ^{o}C for 15 minutes.

2.7.2. Modified Czapex Dox Broth

Czapex Dox 35 g

Yeast 1.5 g

Malt extract 1.5 g

All ingredients were dissolved in 1 L of distilled water and autoclaved at 121 ^{0}C for 15 minutes.

2.7.3. Solid Media (Rice)

Rice (long grain) 100 g

Distilled water 60 mL

Rice was mixed with distilled water in an Erlenmeyer flask and was allowed to stand for 2 hours. Then it was autoclaved at 121 ^{o}C for 45 minutes. After 24 hours, flasks were re-autoclaved under the same conditions, and allowed to cool to room temperature. Fungi inoculated into PDB media were used to inoculate these solid media.

2.7.4. V-8 (100% vegetable juice)

V-8 100 mL

PDB 12 g

$CaCO_3$ 1.5 g

All ingredients were dissolved in 1 L of distilled water and autoclaved at 121 ºC for 15 minutes.

2.7.5. Potato Dextrose Agar (PDA) plates

39.0 g of PDA powder (Difco^{TM})

1.0 L of double deionized (DDI) water

PDA powder with a small magnet was added to an Erlenmeyer flask (2L) containing 1 L of distilled water and heated at boiling point on a stirrer for about 15 minutes. The solution was divided into two Erlenmeyer flasks (500 mL). The flasks were covered with aluminium foil and then autoclaved at 121 ^{o}C for 15 minutes. This media were used to pour into sterilized petridishes and allowed to solidify over 5 minutes.

Table 2.1. Antiplasmodial and herbicidal activity of plant pathogenic fungi extracts

Fungi	Medium	Antiplasmodial activity IC_{50} (ng/mL)				Cytotoxicity (Vero) IC_{50} (ng/mL)	Phytotoxicity	
		P. falciparum D6	SI	*P. falciparum* W2	SI		Lettuce	Bentgrass
Septoria pistaciarum (Broth + mycelium)	Czapex Dox	1200	3.1	1400	2.6	3700	5	5
S. musiva (Broth + Mycelium)	Czapex Dox	720	4.2	570	5.3	3000	5	5
Postia balsamea (Broth)	PDB	1800	25	1400	32.1	45000	5	5
Phomopsis viticola (Broth)	PDB	11000	>4.3	10000	>4.8	NC	NT	NT
Phomopsis viticola (Broth)	V8	1200	>39.7	1200	>39.7	NC	NT	NT
Phomopsis viticola (Broth)	Czapex Dox	570	83.5	400	119	47600	5	5
Sclerotinia homoeocarpa (Broth)	PDB	700	28.6	1300	15.4	20000	5	5
Amylostereum ferreum (Broth)	PDB	3000	>15.9	2300	>20.7	NC	NT	NT
Amylostereum laevigatum (Broth)	PDB	2400	>19.8	1700	>28	NC	NT	NT

Botryosphaeria dothidea (Broth)	PDB	860	37.2	1300	24.6	32000	5	5
Xylaria sp (Broth)	PDB	62	3.3	87	2.3	200	5	5
UM #10 M (Broth)	PDB	40	170	44	154.5	6800	5	5
Alternaria sp. (Broth + Mycelium)	PDB	6800	1.8	5800	2.1	12000	5	5

Antiplasmodial assay: NC = not cytotoxic , NT = not tested , S. I. (selectivity index) = IC_{50} for cytotoxicity /IC_{50} for antiplasmodial activity, IC_{50}: concentration causing 50% growth inhibition, Phytotoxicity assay: Concentration (mM) = 1 mg/mL, Ranking based on scale of 0 to 5, 0 = no effect, 5 = no growth

2.8. Preparation of Stock Solution (10 % RPMI Buffer Solution) for Long Term Storage of Fungi

2.8.1. RPMI (Roswell Park Memorial Institute) Buffer Preparation

Powdered medium of RPMI 1640 without sodium bicarbonate (10.4 g) was mixed with 34.5 g of MOPS (3-(N-Morpholino)propanesulfonic acid) buffer in a beaker (1L) and dissolved in 750 mL of DDI water. The pH was adjusted to 7.0 with 0.1M NaOH and DDI water was added and stirred to bring the total volume to 1.0 L. RPMI BUFFER was filtered using a 0.45 micron filter unit to sterilize and stored at 4 ºC.

2.8.2. 10% Glycerol/RPMI Buffer Stock Solution

90 mL of RPMI buffer (Stock buffer stored at 4 ^{o}C) and 10 mL of glycerol were vortexed for 2 minutes. It was filtered using Nalgene® (NYL) filter unit, (0.45 microns, 250 mL), and stored at 4 ^{0}C.

2.8.3. Inoculum Stock Solution

50 µL of inoculum was added to 1 mL of 10% RPMI buffer/glycerol stock solution in a 5 mL round bottom tube, vortexed and stored at 4 ^{o}C.

2.8.4. Select Isolate for Storage

Fungal spores were scraped from the PDA plates (4-5) with sterilized DDI water. Spore suspension was filtered through a mirror cloth and collected into a polypropylene conical tube, mixed with a vortex and stored at 4 ^{o}C. After 24 hours, supernatant was removed. 5 mL of 10% glycerol/ RPMI buffer stock was added on to spores and vortexed for 2 minutes. 0.5 mL conidial suspension was added into each cryogenic vial and stored at -80 ^{o}C.

2.9. Evaluation of Pure Compounds for In Vitro Biological Activities

Pure compounds (2.0 mg each) were submitted to the National Center for Natural Products Research (NCNPR) for screening of antimalarial, cytotoxic, antimicrobial (antifungal and antibacterial), and antileishmanial activities.

2.9.1. In Vitro Antiplasmodial Screening

This assay was performed by Dr. S. Khan, NCNPR. University of Mississippi

The antiplasmodial activity of extracts, fractions, and pure compounds was performed against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *P. falciparum* using the in vitro assay as reported.[69] The 96-well microplate assay was based on evaluation of the effect of compounds/extracts on growth of asynchronous cultures of *P. falciparum*, determined by the assay of parasite lactate dehydrogenase (pLDH) activity. The appropriate dilutions of the compounds /extracts were prepared in DMSO or RPMI-1640 medium and it was added to the

cultures of *P. falciparum* (2% hematocrit, 2% parasitemia) set up in clear flat bottomed 96 well plates. The plates were placed into the humidified chamber and flushed with a gas mixture of 90% N_2, 5% CO_2, and 5% O_2. The cultures were incubated at 37 ^oC for 72 hours. The growth of the parasite in each well was determined by pLDH assay using Malsatat® reagent. The medium and RBC controls were also set-up in each plate. In addition to the *P. falciparum* strains, samples were tested against mammalian kidney cell line (Vero) as an indicator of general cytotoxicity. The selectivity indices (SI) – ratio of Vero IC_{50} to D6 or W2 IC_{50} – were calculated. Artemisinin and chloroquine were used as the positive controls while DMSO was the negative control. IC_{50} values were computed from the dose response curves using Microsoft Excel software.

2.9.2. In Vitro Phytotoxicity Activity

This assay was performed by Dr. S. Duke, USDA-ARS, University of Mississippi.

Herbicidal or phytotoxic activity of extracts and test compounds was performed via the published procedure[70] using bentgrass (*Agrostis stolonifera*) and lettuce (*Lactuca sativa* cv. L., Iceberg), in 24-well plates. Test compounds (1 mg each) were dissolved in 100 µL of acetone and a 20 µL aliquot of each solution was pipetted onto the filter paper and dried for 30 min under airflow in a sterile biohazard hood. Water (200 µL) was added after placing the dried and sample impregnated filter paper in the well. The solvent controls were treated identically, using the solvent described above. Phytotoxicity was ranked visually. The ranking of phytotoxic activity was based on a scale of 0 to 5 with 0 showing no effect and 5 no growth.

2.9.3. In Vitro Antimicrobial Activity

This assay was performed by Dr. M. Jacob, NCNPR, University of Mississippi.

All organisms were obtained from the American Type Culture Collection (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113 and *Aspergillus fumigatus* ATCC 90906 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI methods[71, 72] as described.[73] *M. intracellulare* and *A. fumigatus* growth were monitored using Alamar Blue™ (Bio-Source International, Camarillo, CA). Samples dissolved in DMSO were serially diluted using 0.9% DMSO and transferred in duplicate to 96 well flat bottom microplates. Microbial inocula were prepared by diluting saline suspensions of colonies with assay broth to afford recommended colony forming units/mL. Growth (saline only), solvent and blank (media only) controls were included on each test plate. The drug controls ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi were included in each assay. All organisms were read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em (*M. intracellulare*, *A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG Lab Technologies, Germany) prior to and after incubation. The MIC (minimum inhibitory concentration) was defined as the lowest test concentration that allowed no detectable growth.

2.9.4. In Vitro Antileishmanial Assay

This assay was performed by Dr. B. L. Tekwani, NCNPR, University of Mississippi.

The in vitro antileishmanial activity of the isolated compounds was carried out on a culture of *Leishmania donovani* promastigotes and axenic amastigotes by the Alamar Blue assay as described.[74] In a 96 well microplate the samples with appropriate dilution were added to the leishmania promastigotes/axenic amastigote culture (2×10^6 cell/mL). The compounds were tested at six concentrations ranging from 40 to 0.0128 µg/mL. The plates were incubated at 26 °C for 72 hours (37 °C for amastigote) and growth of leishmania promastigotes/ amastigotes was determined. IC_{50} and IC_{90} values were computed from the dose response curves. Pentamidine and amphotericin B were used as standard antileishmanial agents.

2.9.5. In Vitro Macrophage Amastigote Assay

This assay was performed by Dr. B. L. Tekwani, NCNPR, University of Mississippi.

A recently developed[75] promastigote rescue assay by Jain et al. was used. The THPI 1 cells (Human acute monocytic leukemia cell line) were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were prepared prior to each assay and suspended in RPMI1640 medium with 10 % FBS at the cell density of 2.5 $X10^5$ cells/mL. Phorbol 12-myristate 13-acetate (PMA) was added to the cell suspension to achieve final concentration of 25 ng/mL and cells were seeded onto a clear flat-bottom 96 well plate with 200 µL (5×10^4 cells) into each well. The plate was incubated in a 5% CO_2 incubator at 37 °C for at

least 12 hours for differentiation of the THP1 cells to adherent macrophages. After overnight incubation the medium from each well was discarded and adherent cells were gently washed at least twice with serum free RPMI1640 medium. The *Leishmania donovani* promastigotes culture was harvested at the stationary phase (metacyclic infective stage) and suspended into RPMI1640 medium with 2% FBS at the density of 2.5×10^6 cells/mL. The 200 µL of promastigotes (5×10^5) culture was added to each well. The plate was further incubated in a 5% CO_2 incubator at 37 °C for at least 24 hours to allow infection of macrophages with the *Leishmania* parasites. After 24 hours the non-adherent macrophages and unattached *Leishmania* promastigotes were washed off with serum free RPMI1640 medium. The infected macrophages were further incubated at 37 °C and 5% CO_2 in 200 µL RMPI1640 medium and 2% FBS with different concentrations of standard antileishmanial drugs (pentamidine & amphotericin B) or the test compounds for 48 hours. The control wells with medium, uninfected THP1 cells, infected cells without drugs or test compounds were set up simultaneously. The cultures were washed off with serum free RPMI1640 and treated for 30 seconds with 20 µL of 0.05% sodium dodecyl sulfate in RPMI1640 medium for the release of amastigotes from the infected macrophages. To each well 180 µL of RPMI1640 medium with 10% FBS was added and the plate was further incubated at 26 °C for 48 hours to allow transformation of released amastigotes to promastigotes. To each well 20 μL of Alamar blue was added, the plates were incubated at 26 °C for 24 hours and read on a BMG Fluostar microplate reader (BMG Lab Technologies) at excitation wavelength of 544 nm and emission wavelength of 590 nm. Each compound was tested in duplicates at six concentrations, IC_{50} and IC_{90} values were computed from the dose response curves.

2.9.6. Assay for In Vitro Cytotoxicity

This assay was performed by Dr. S. Khan, NCNPR, University of Mississippi.

In vitro cytotoxicity was determined against a panel of mammalian cells that included kidney fibroblast (Vero), kidney epithelial (LLC-PK_{11}), malignant melanoma (SK-MEL), oral epidermal carcinoma (KB), breast ductal carcinoma (BT-549), and ovary carcinoma (SK-OV-3) cells.[76] The assay was performed in 96-well tissue culture-treated plates. Cells were seeded to the wells of 96-well plate at a density of 25,000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined by using Neutral Red dye and IC_{50} values were obtained from dose response curves. Doxorubicin was used as a positive control.

2.9.7. Evaluation of In Vivo Antimalarial Assay

This assay was performed by Dr. B. L. Tekwani, NCNPR, University of Mississippi.

Housing of the Animals- The colony bred mice weighing 15 -20 g were used for maintaining the malaria parasite and also for screening of the test compounds for antimalarial activity. The mice weighing 10-15 g were procured and kept under quarantine for one week. The animals were maintained on standard diet and water *ad libitum* and housed in solid bottom cages. Each cage had 6 mice and control and untreated mice were housed in separate cages in the same room. All the animal procedures were conducted under sterile conditions in the laminar flow hood.

Infection – A group of three to five mice was inoculated intraperitoneally or intravenously with the *Plasmodium berghei* infected mouse blood. The parasitemia was monitored from day 5 onwards by preparation of blood smears from a tail nick. These mice were used as donor mice for infecting a larger batch of mice for in vivo testing. The mice with parasitemia >30% were anesthetized by i.p. injection of ketamine/xylazine (8 mg and 1.2 mg/Kg). Blood was collected from the heart in sterile citrated saline. The blood counts were adjusted to 4×10^7 infected erythroctes /500 µL by dilution with sterile normal saline. Each mouse was inoculated intraparitoneally with 4×10^7 *P. berghei* infected erythrocytes in 500 µL of sterile saline.

Treatment – The mice inoculated with *P. berghei* infected blood were divided into different groups with 6 animals in each group, housed in separate cages, were subjected to the treatment with the test compounds. For multiple dose studies three groups of mice were administered with the test compounds at different dose levels (ranging between 1-100 mg/Kg) by oral route once daily for three days. The doses may be decided on the basis of prior information available with the compounds of similar class. In case of novel compounds in vitro cytotoxity data was used as the guide for dose selection. One group of postive control was treated with chloroquine (30 mg/Kg) while a group of 6 mice was treated with the vehicle.

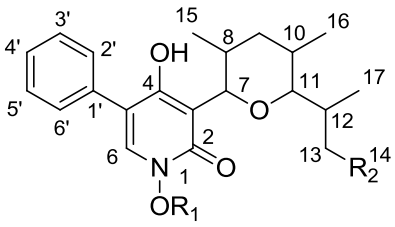
The mice were closely monitored for at least one hour after receiving the treatment and also at least once daily (for recording body weight, movement, and behaviour) till their survival or up to 28 days post infection (the total duration of the experiment). Parasitemia was monitored on day 5,7,10, 14, 21, and 28 post infection by preparing the blood smears from tail nick. The level of parasitemia and mean survival time were compared in treated and vehicle control groups.

Parasitemia was monitored by preparing the thin blood smears from the tail nick and staining with Geimsa stain.

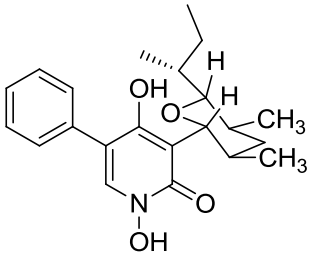
CHAPTER 3: BIOACTIVE 1, 4-DIHYDROXY-5-PHENYL-2-PYRIDINONE ALKALOIDS FROM *SEPTORIA PISTACIARUM*

Results presented in this chapter have been published in the following journal.

Kumarihamy, M.; Fronczek, F. R.; Ferreira, D.; Jacob, M.; Khan S. I.; Nanayakkara, N. P. D. Bioactive 1,4-dihydroxy-5-phenyl-2-pyridinone alkaloids from *Septoria pistaciarum* *J. Nat. Prod.* **2010**, 73, 1250–1253



1 R_1 = H, R_2 = CH_3
2 R_1 = CH_3 , R_2 = CH_3
3 R_1 = CH_3, R_2 = CH_2OH
4 R_1 = CH_3, R_2 = CH_2OAc



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3.1. Summary

Four new 1,4-dihydroxy-5-phenyl-2-pyridinone alkaloids (**1**-**4**) were isolated from an EtOAc extract of a culture medium of *Septoria pistaciarum*. The structures of these compounds were determined by spectroscopic methods, and the absolute configuration of the major compound (**1**) by X-ray crystallographic analysis. Compound **1** exhibited moderate in vitro antiplasmodial (antimalarial) activity against chloroquine-sensitive (D6) and -resistant (W2) strains of *Plasmodium falciparum* and cytotoxic activity to Vero cells. Compound **2** was moderately active against both methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus*.

3.2. Introduction

Plant-like metabolic pathways found in the apicoplast,[10] a chloroplast-like organelle of *Plasmodium* species, have been identified as suitable targets in malaria drug discovery. A number of herbicides and phytotoxins with known molecular targets in the plastid have been evaluated for antimalarial activity.[77,78,41] Various phytotoxins released by plant pathogenic fungi are known[79,80,81] to inhibit metabolic pathways in the apicoplast.[82] As part of our ongoing search for potential sources of new antimalarial compounds, we have screened a number of plant pathogenic fungi for antiplasmodial activity.

Septoria pistaciarum (Ascomycetes) is the causative agent of Septoria leaf and fruit spot disease in pistachio (*Pistachia vera*) in the US[83] and Mediterranean countries.[84]

An EtOAc extract of this fungus showed potent herbicidal activity and moderate antiplasmodial activity against *Plasmodium falciparum* albeit with low selectivity index. No previous chemical work has been reported for this species. Structurally diversed chemical compounds namely, septorine,[85] N-methoxyseptorine,[85] N-methoxyseptorinol (substituted pyrazines),[86] dihydroisocoumarins,[87] mycophenolic acid,[87] and mycosporins[88] have been reported from the culture medium of the phytopathogenic fungus *Septoria nodorum* Berk., a parasite of wheat. Cordipyridones isolated from the insect pathogenic fungus *Cordiceps nipponica* have shown potent antimalarial activity with moderate selectivity.[64]

Bioassay-guided fractionation of the EtOAc extract led to the isolation of a new 1,4-dihydroxy-5-phenyl-2-pyridinone analogue as the active compound. Three additional new but inactive analogues of the same class were also isolated and identified. Several compounds of this class such as tenellin, bassianin, funiculosin, sambutoxin, oxysporidinones, cordipyridones, farinosones, and TMC-69 have been isolated from a number of fungal species and have shown antibacterial,[89,90] antitumor,[90] antifungal,[89,91] and neurotrophic[92] activities.

3.3. Results and Discussion

Compound **1** was isolated as the major active constituent of the EtOAc extract of *S. pistaciarum.* The molecular formula of **1** was determined as $C_{22}H_{29}NO_4$ by HRESIMS. The ^{1}H NMR (Figures 3.1 & 3.2) and COSY spectrum (Figure 3.3) displayed six hydrogens in the aromatic region consisting of a one-proton singlet and an A_2B_2C system due to a monosubstituted phenyl group. The ^{13}C NMR spectra (Figures 3.4 & 3.5) confirmed the presence

of 22 carbon atoms, which comprised five quaternary, 11 methine, two methylene, and four methyl carbons. NMR signals in the aromatic region showed close resemblance to those of the 1,4-dihydroxy-5-phenyl-2-pyridinone moiety of the antitumor agent, TMC-69-6H, and its analogues.[90] IR absorptions (3109, 1642 and 1556 cm^{-1}) and UV maxima (207.1, 241.0, and 296.0 nm) of **1** were consistent with those reported for a compound with a 1,4-dihydroxy-2-pyridinone moiety.[64] In its HMBC spectrum (Figure 3.6), H-2′ and H-6′ (δ_H 7.49) showed cross peaks with C-3′ and C-5′ (δ_C 128.6), C-4′ (δ_C 127.9), C-1′ (δ_C 133.4), and C-5 (δ_C 114.2), and H-6 (δ_H 7.69) with C-2 (δ_C 158.0), C-4 (δ_C 161.4), C-5 (δ_C 114.2), and C-1′ (δ_C 133.4) supporting the partial structure of the substituted pyridinone ring moiety. In the aliphatic region, the ^{1}H NMR spectrum showed two doublets representing two oxymethine hydrogens, and four methyl resonances, comprising three doublets and a triplet. The ^{1}H NMR data additionally indicated the presence of a tetrahydropyrano ring attached to C-3 of the pyridinone ring similar to TMC-69-6H.[90] The H-7 oxymethine doublet (δ_H 4.72) showed cross peaks in the HMBC spectrum with C-4 (δ_C 161.4), C-2 (δ_C 158.0) C-3 (δ_C 110.4), C-11 (δ_C 89.9), C-9 (δ_C 42.2), C-8 (δ_C 36.8), and C-15 (δ_C 17.5). The H-11 oxymethine doublet at δ_H 3.07 similarly displayed HMBC correlations with C-7 (δ_C 81.4), C-9 (δ_C 42.2), C-12 (δ_C 35.6), C-13 (δ_C 22.1), C-16 (δ_C 17.5), and C-17 (δ_C 17.2). The methyl triplet at δ_H 0.98 showed HMBC cross peaks with C-12 (δ_C 35.5) and C-13 (δ_C 22.0) confirming its ω location in the chain. These correlations strongly suggested the gross structure **1** depicted for this compound. The HMBC correlations of other proton resonances and COSY correlations further supported this structure (Figure 3.7).

The relative configuration of compound **1** was determined by the ^{1}H NMR coupling constants and ROESY (Figure 3.9) data. A $^3J_{7,8}$ value of 10.2 Hz suggested that H-7 and H-8

were *trans*-diaxially orientated. A $^{3}J_{10,11}$ value of 10.2 Hz similarly indicated the *trans*-diaxial arrangement of H-10 and H-11. The lack of three-bond coupling of H-11 and H-12 may reflect a C-11 - C-12 conformation having H-11 and H-12 orthogonally oriented in order to minimize Van der Waals interaction between the C-10 and C-11 alkyl groups. In the ROESY spectrum H-7 displayed interactions with H-11 and CH_3-15 indicating the cofacial orientation of the three groups. Similarly, H-11 showed interactions with H-7 and CH_3-16 (Figures 3.7 & 3.8).

Table 3.1. NMR Data for Compounds **1-4** in CDCl3- Methanol-*d*4

Position	**1**		**2**		**3**		**4**	
	δ_C [b]	δ_H [a] (*J* in Hz)	δ_C [b]	δ_H [a] (*J* in Hz)	δ_C [b]	δ_H [a] (*J* in Hz)	δ_C [b]	δ_H [a] (*J* in Hz)
2	158.0		158.0		158.0		158.0	
3	110.4		111.8		111.7		111.7	
4	161.4		161.8		161.8		161.7	
5	114.2		114.3		114.4		114.3	
6	131.8	7.69, s	132.9	7.43, s	133.0	7.42, s	133.4	7.44, s
7	81.4	4.72, d (10.2)	81.3	4.73, d (10.2)	81.4	4.72, d (10.2)	81.5	4.75, d (10.2)
8	36.8	1.88, m	36.9	1.87, m	36.8	1.88, m	36.8	1.88, m
9	42.2	1.82, dt (13.2, 3.6) 1.12, q (12.6)	42.2	1.83, dt (13.2, 3.6) 1.14, q (12.6)	42.1	1.82, dt (13.2, 3.6) 1.13, q (12.6)	42.0	1.85 1.13, q (12.6)
10	32.6	1.76, m	32.6	1.75, m	32.6	1.76, m	32.7	1.71, m
11	89.9	3.07, d (10.2)	89.9	3.07, d (10.2)	89.8	3.08, d (9.6)	89.6	3.10, d (10.2)
12	35.6	1.66, m	35.6	1.65, m	30.1	1.96, m	30.4	1.61, m
13	22.1	1.02, m 1.56, m	22.1	1.00, m 1.54, m	32.3	1.23, m 1.74, m	28.4	1.21, m 1.61, m
14	12.8	0.90, t (7.2)	12.8	0.88, t (7.2)	61.0	3.56, brq (9.0) 3.71, m	63.0	4.02, m 4.13, m
15	17.5	0.73, d (6.6)	17.8	0.88, d (6.6)	17.7	0.86, d (6.6)	17.8	0.88, d (6.6)
16	17.5	0.84, d (6.6)	17.5	0.83, d (6.6)	17.6	0.84, d (6.6)	17.3	0.85, d (6.6)
17	17.2	0.98, d (6.6)	17.1	0.96, d (6.6)	17.4	0.97, d (7.2)	17.6	1.00, d (6.6)
1′	133.4		133.4		133.3		133.0	

2′,6′	129.3	7.49, d (7.8)	129.2	7.45, d (7.2)	129.2	7.42, d (7.8)	129.3	7.45, d (7.2)
3′,5′	128.6	7.43, t (7.8)	128.6	7.40, t (7.8)	128.5	7.38, t (7.8)	128.6	7.40, t (7.2)
4′	127.9	7.35, t (7.2)	127.8	7.33, t (7.8)	127.8	7.13, t (7.8)	128.0	7.34, t (7.2)
OH		9.72, s		9.69, s		9.66, s		9.49, s
OCH_3			65.0	4.01, s	65.0	4.03, s	65.1	4.05
$COCH_3$							21.1	2.01, s
*C*OCH3							171.8	

[a]^{1}H NMR spectra recorded at 600 MHz. [b]^{13}C NMR spectra recorded at 100 MHz.

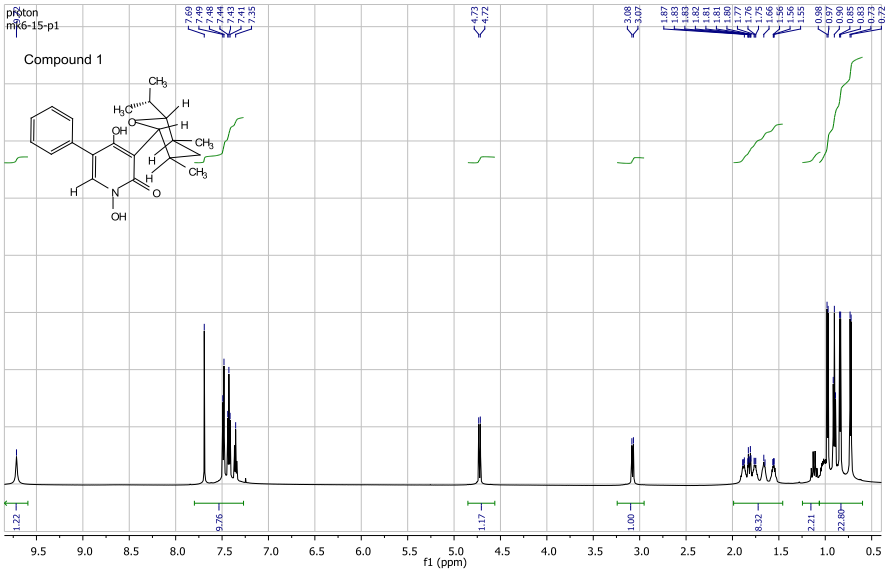


Figure 3.1. ^{1}H NMR spectrum of **1**

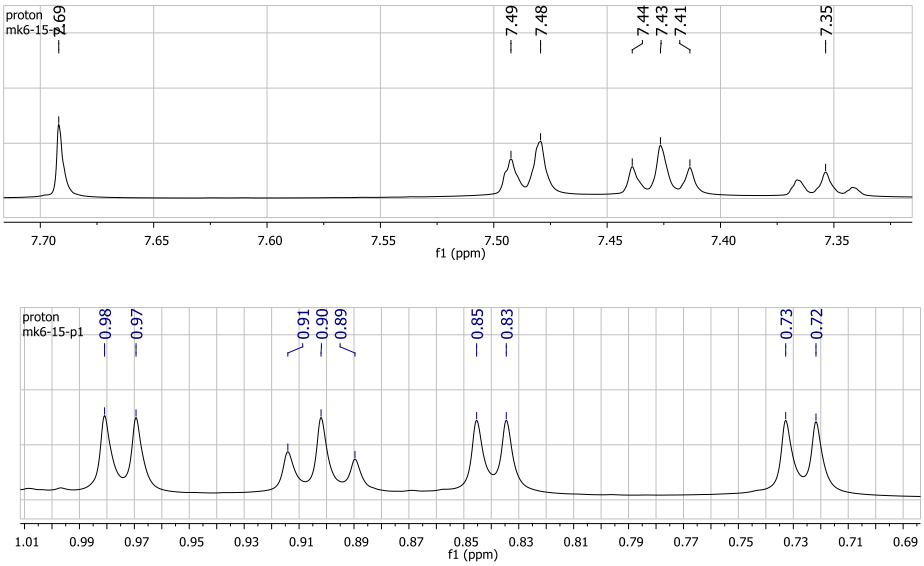


Figure 3.2. Expansion of ^{1}H NMR spectra of **1**

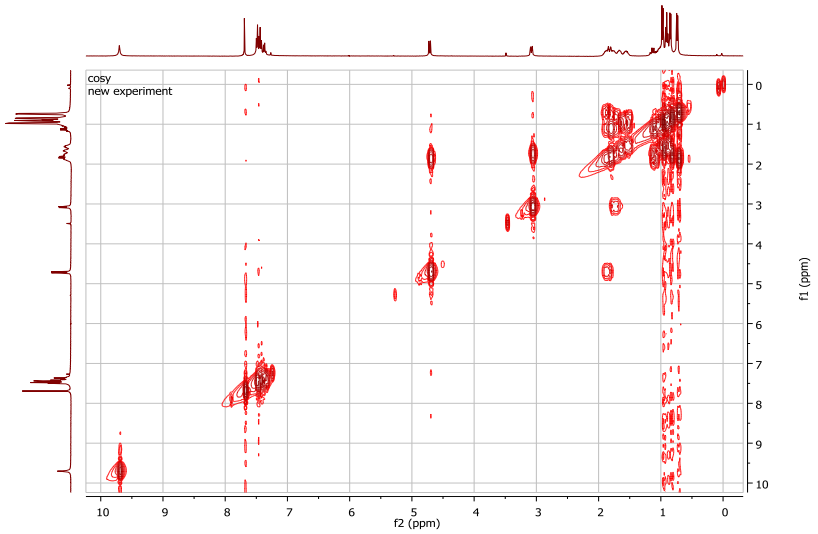


Figure 3.3. COSY spectrum of **1**

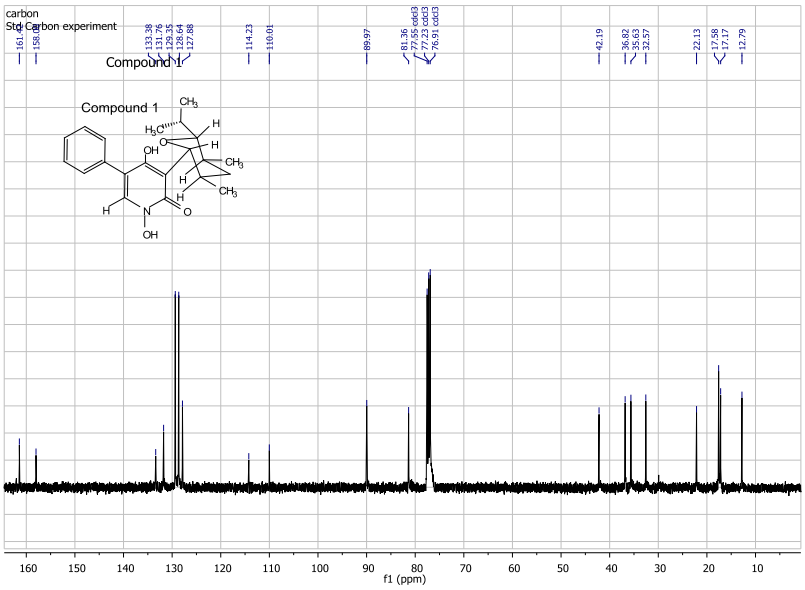


Figure 3.4. ^{13}C NMR spectrum of **1**

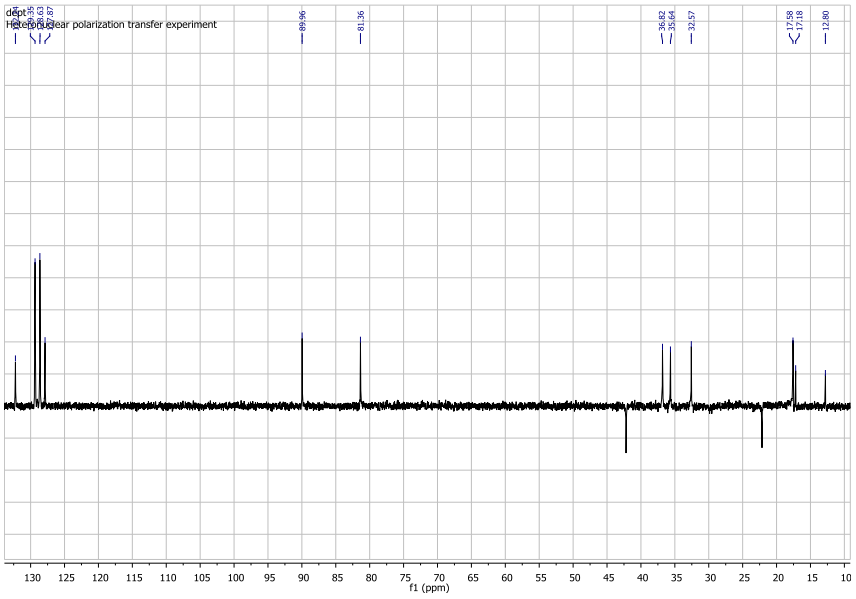


Figure 3.5. DEPT spectrum of **1**

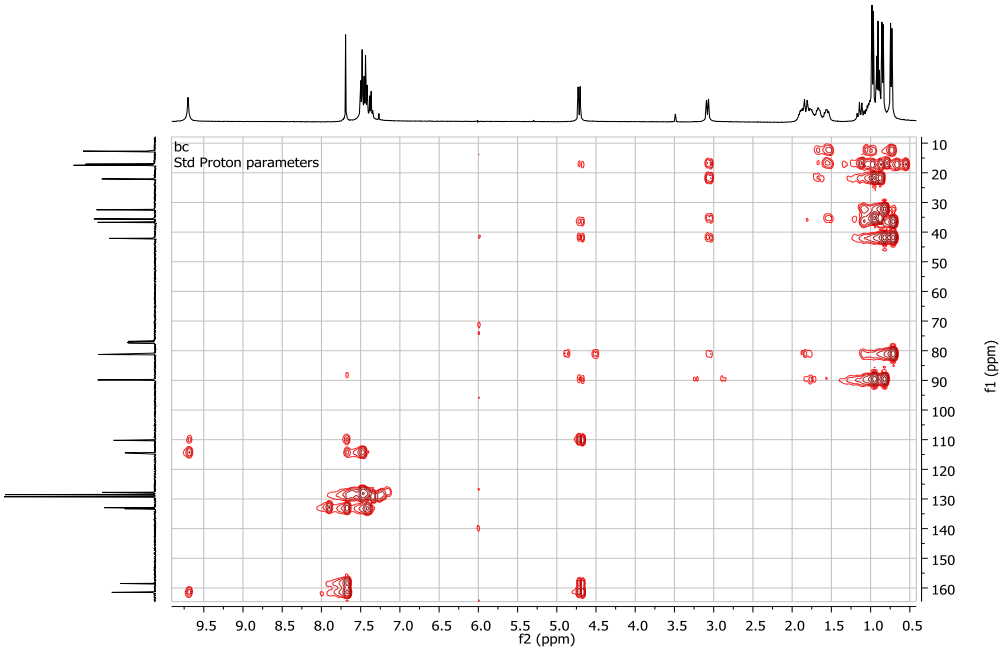
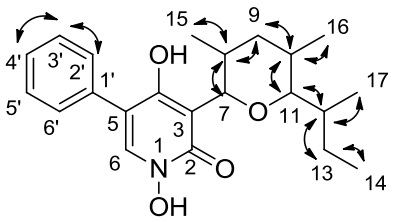
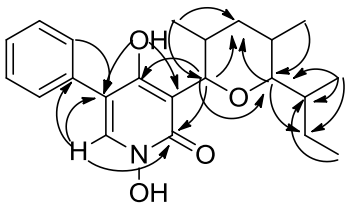


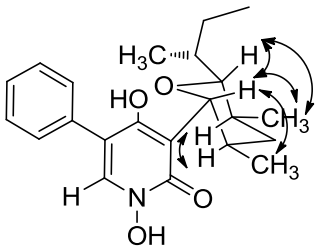
Figure 3.6. HMBC spectrum of **1**



Key COSY ( ) correlations



Key HMBC ( ) correlations



Key ROESY ( ) correlations

Figure 3.7. COSY, HMBC, and ROESY correlations of **1**

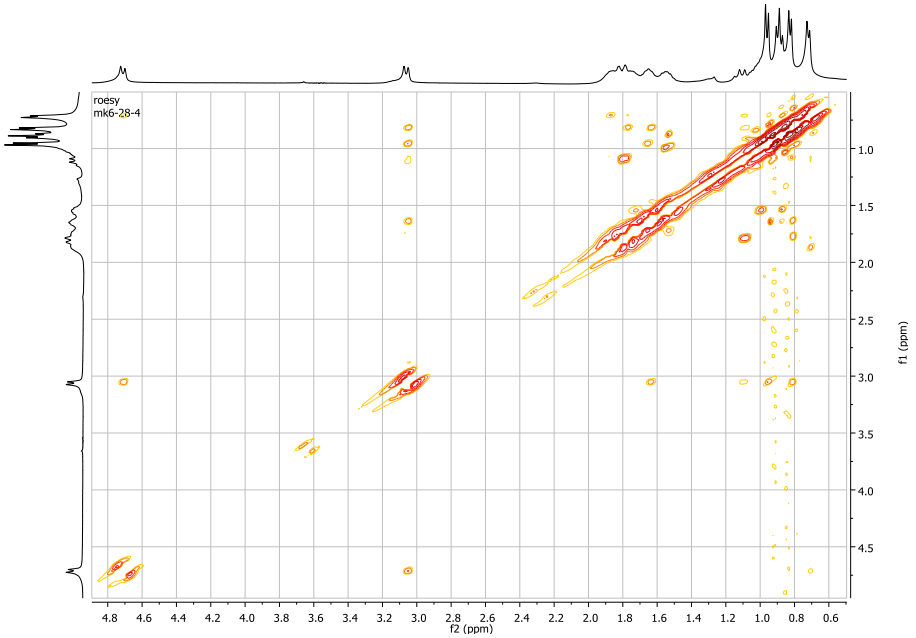


Figure 3.8. ROESY spectrum of **1**

This compound afforded crystals that were suitable for crystallographic analysis. On the basis of Cu Kα X-ray diffraction data, the absolute configuration of compound **1** was assigned as 7*R*, 8*R*, 10*S*, 11*R*, and 12*R*. The solid-state structure contains four independent molecules, one of which is illustrated in Figure 3.9. All four have an intramolecular OH...O hydrogen bond to the tetrahydropyran oxygen, with O...O distances in the range 2.5771(19) to 2.5930 (19) Å. The four molecules differ only slightly in conformation, except for that about the bond joining the heterocyclic and phenyl rings, for which C-C-C-C torsion angles vary from -50.5(3)º to +44.(3)º.

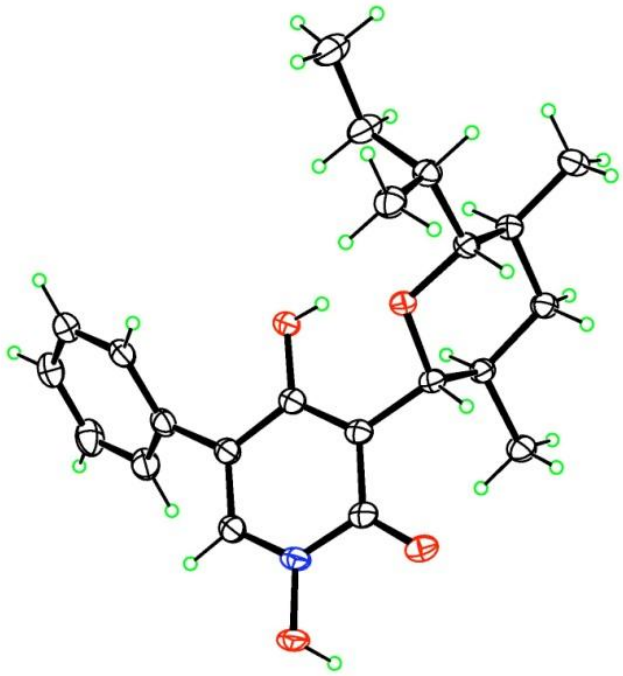


Figure 3.9. Single-crystal X-ray structure of **1,** showing the absolute configuration of one of the four independent molecules

Compound **2** had a molecular formula of $C_{23}H_{31}NO_4$ as determined by HRESIMS. The ^{1}H (Figure 3.10) and ^{13}C NMR (Figure 3.11) spectra were similar to those of compound **1** except for the presence of an *O*-methyl resonance in the spectra of the former. This indicated that compound **2** was an *O*-methyl analogue of compound **1**. The COSY (Figure 3.12), HMQC (Figure 3.13) and HMBC spectra (Figure 3.14) of compound **2** showed correlations identical to those observed for compound **1**. Additionally, the *O*-methyl resonance showed no cross peaks in the HMBC spectrum whereas the OH proton (δ_H 9.69) correlated with C-3 (δ_C 118.8), C-4 (δ_C 161.8) and C-5 (δ_C 114.2) suggesting that the *O*-methyl group was located on the nitrogen. The up-field shift of H-6 of compound **2** relative to that of compound **1** is consistent with the

structural modification. Identical coupling constants and ROESY correlations indicated that compounds **1** and **2** have the same relative configuration.

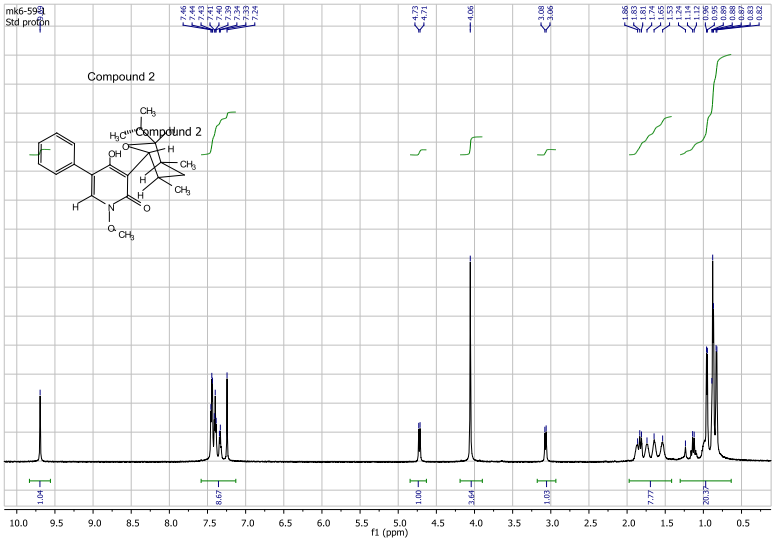


Figure 3.10. ^{1}H NMR spectrum of **2**

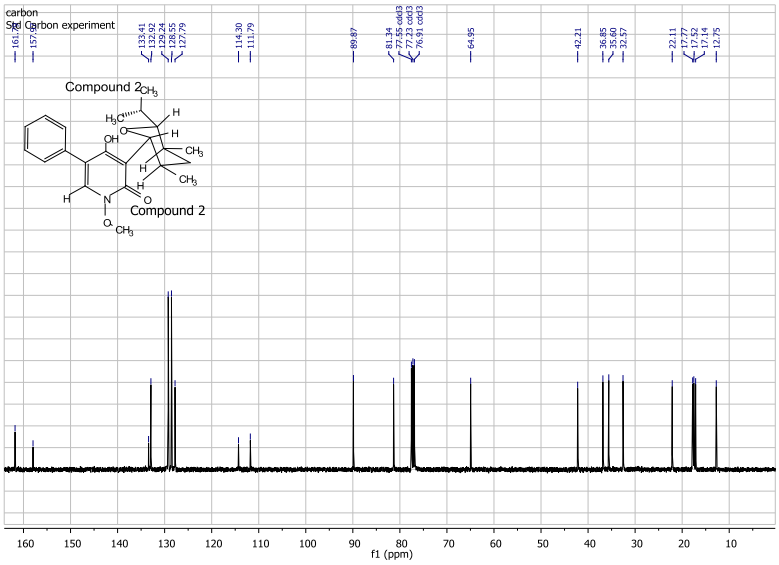


Figure 3.11. ^{13}C NMR spectrum of **2**

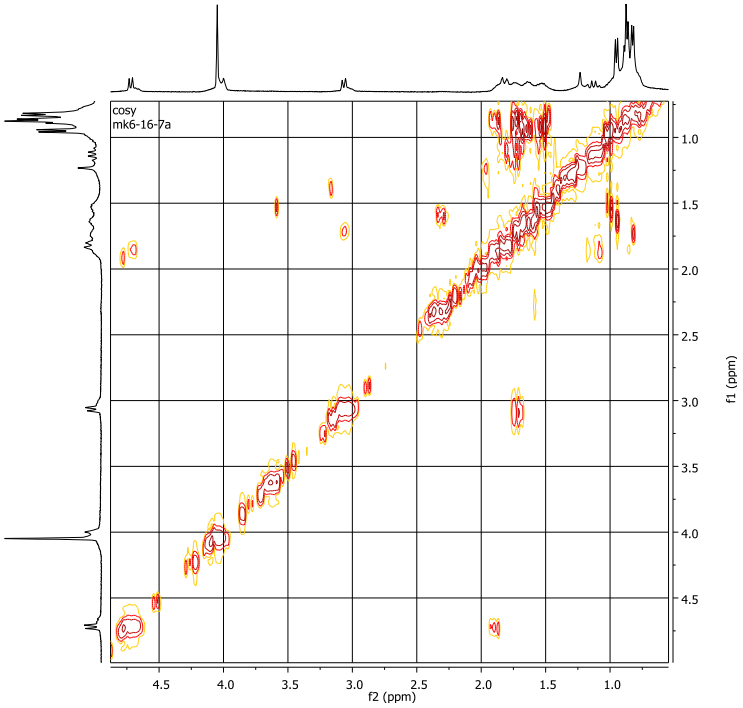


Figure 3.12. COSY spectrum of **2**

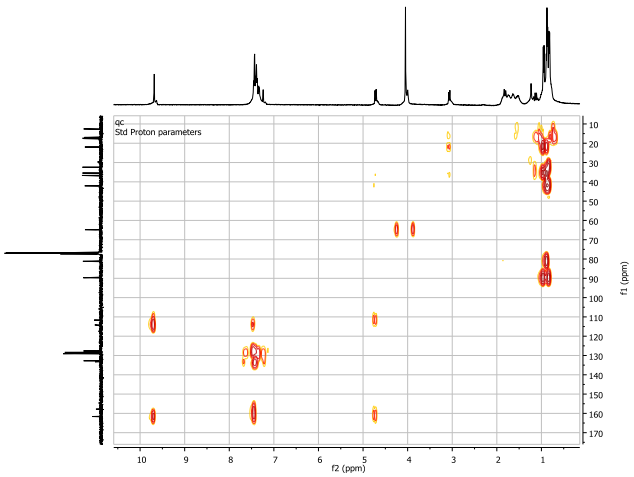


Figure 3.13. HMQC spectrum of **2**

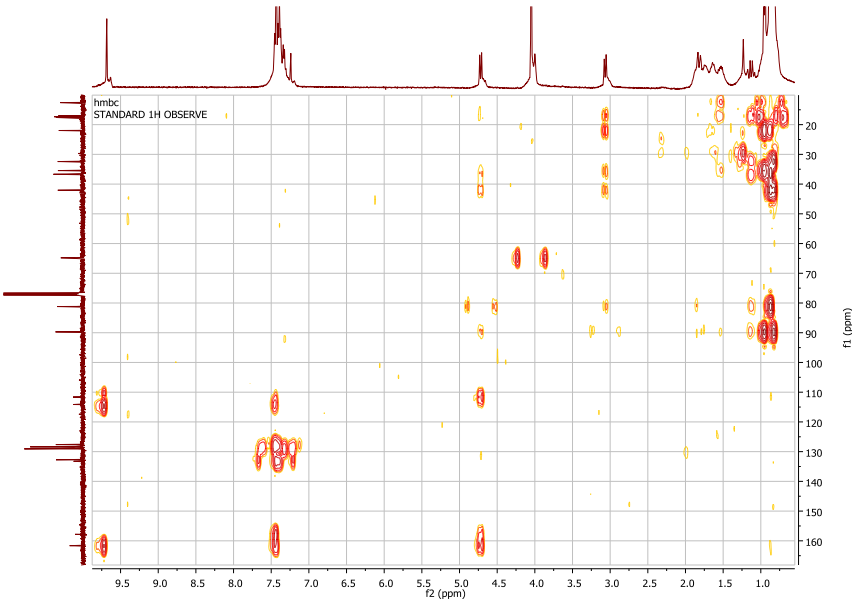


Figure 3.14. HMBC spectrum of **3**

The molecular formula of compound **3** was established as $C_{23}H_{31}NO_5$ by HRESIMS. Its ^{1}H-NMR spectrum (Figure 3.15) was similar to compound **2**, the major differences being the replacement of the methyl triplet by two diastereotopic oxymethylene hydrogens in the spectrum of **3**. This suggested that compound **3** was the C-14 oxygenated analogue of **2**. Comparison of the ^{13}C NMR data (Figure 3.16) of these compounds also confirmed the replacement of a methyl by an oxymethylene group in **3**. The COSY spectrum of compound **3** (Figure 3.17) displayed cross peaks between the C-14 oxymethylene (δ_H 3.58, 3.73) and C-13 methylene protons (δ_H 1.23, 1.75). The COSY correlations for the rest of the hydrogen resonances were identical to those of compound **2**. In the HMBC spectrum (Figure 3.18) the oxymethylene protons showed cross peaks with C-13 (δ_C 32.3) and C-12 (δ_C 30.1). The remaining HMBC correlations were identical to those observed for compound **2**. These observations suggested that compound **3** was the C-14 hydroxy analogue of **2**. ROESY correlations and coupling constants of H-7, H-9, and

H-11 of compound **3** were identical to those observed for compound **1** indicating that these two compounds had the same relative configuration.

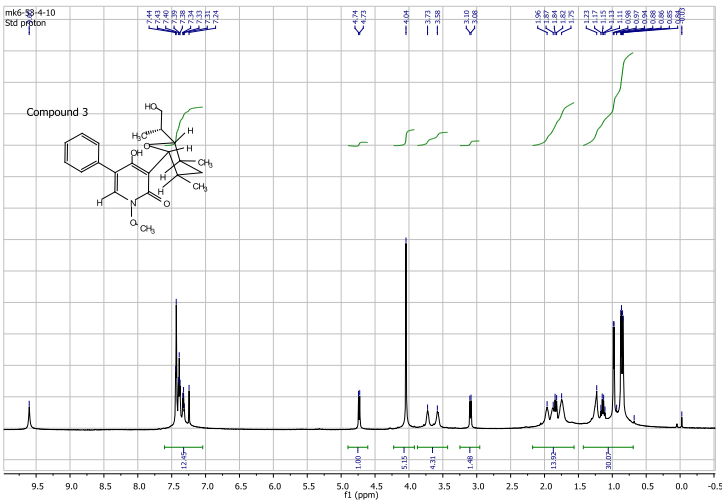


Figure 3.15. ^{1}H NMR spectrum of **3**

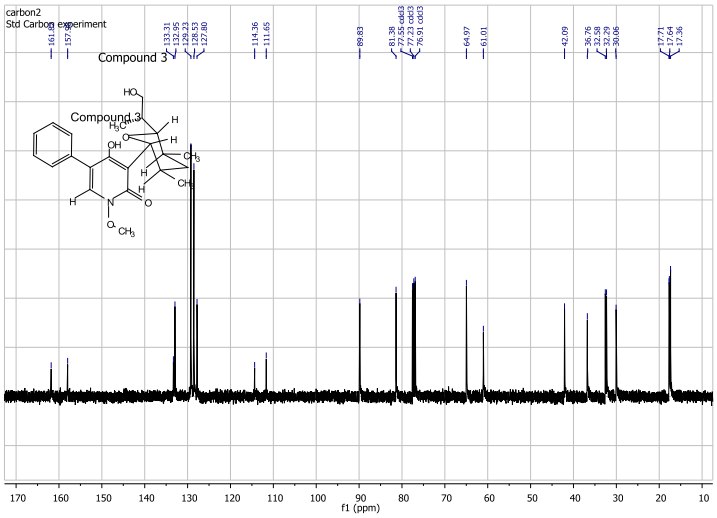


Figure 3.16. ^{13}C NMR spectrum of **3**

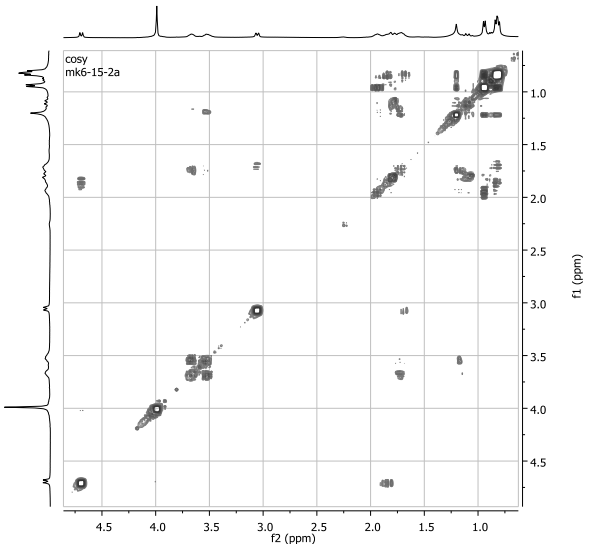


Figure 3.17. COSY spectrum of **3**

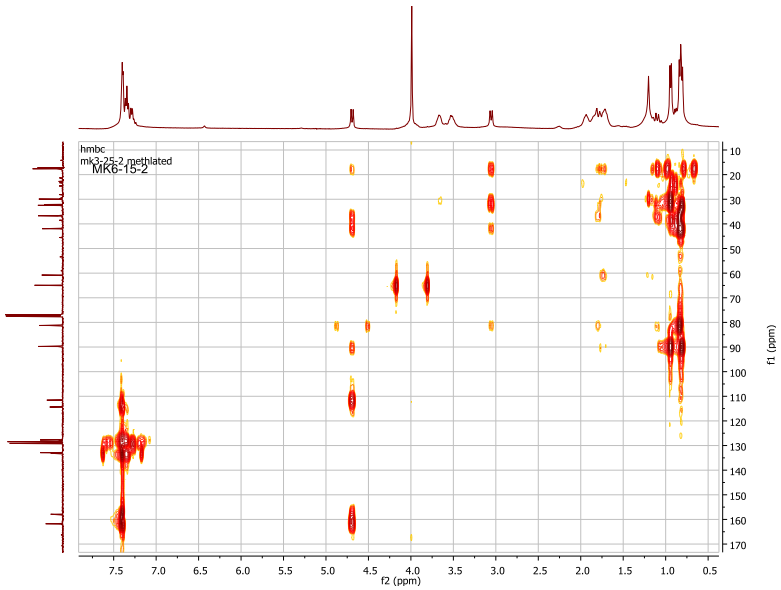


Figure 3.18. HMBC spectrum of **3**

The molecular formula of compound **4** was determined by HRESIMS as $C_{25}H_{33}NO_6$. The ^1H (Figure 3.19) and ^{13}C NMR (Figure 3.20) spectra, COSY (Figure 3.21), and HMBC (Figure 3.22) correlations of this compound were similar to those observed for compound **3**. Major differences involved the presence of an additional methyl singlet (δ_H 2.01) and two carbon signals [δ_C 21.2 (CH_3), 171.8 (CO)] in its ^1H and ^{13}C NMR spectra, respectively. The down-field shift of proton signals due to the C-14 oxymethylene group and HMBC correlations between these protons and the acetyl carbonyl carbon indicated that compound **4** was the 14-*O*-acetyl analogue of compound **3**.

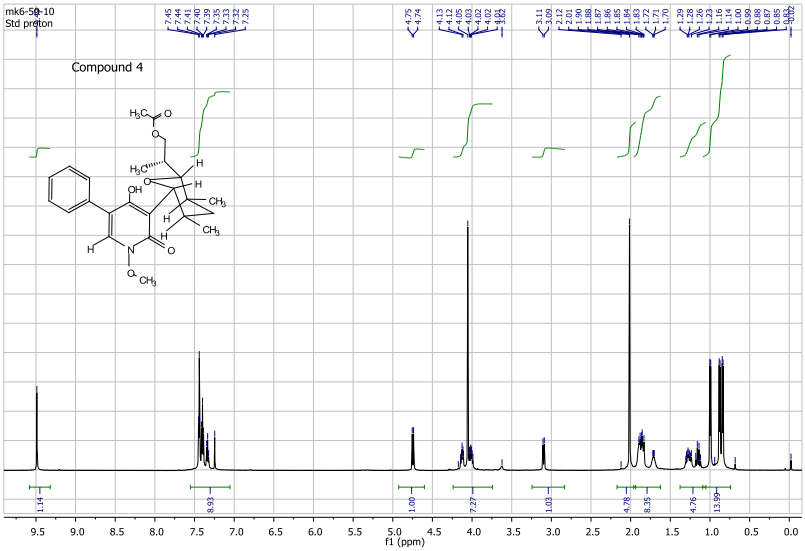


Figure 3.19. ^1H NMR spectrum of **4**

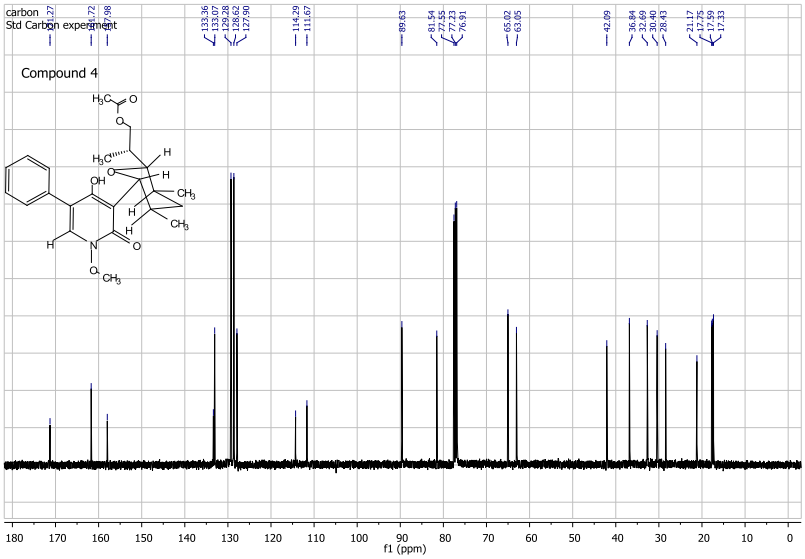


Figure 3.20. ^{13}C NMR spectrum of **4**

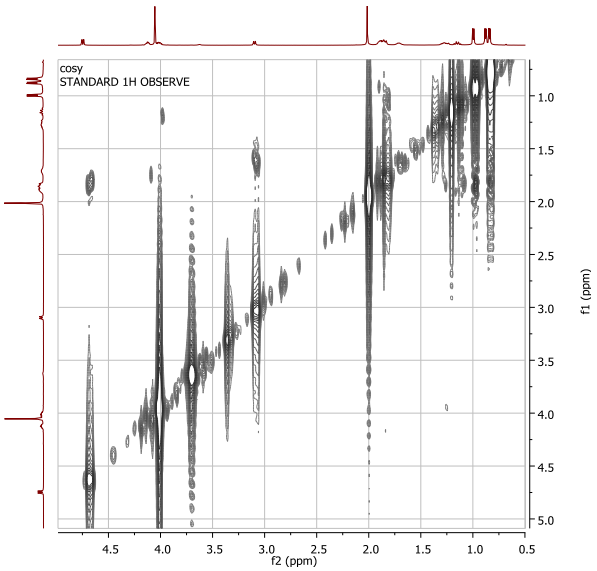


Figure 3.21. COSY spectrum of **4**

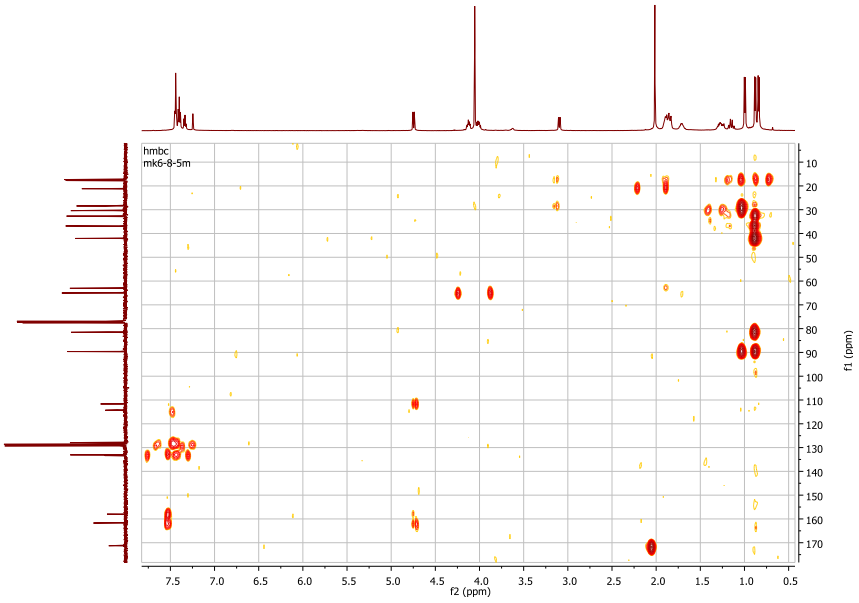


Figure 3.22. HMBC spectrum of **4**

Since the pyridinone analogues **1**-**4** presumably share a common biosynthetic origin involving an aromatic amino acid and an activated polyketide,[93] analogues **2**, **3**, and **4** also possess 7*R*, 8*R*, 10*S*, 11*R*, 12*R* absolute configuration. Such an assumption is supported by the fact that all four compounds are strongly dextrorotatory.

The in vitro antiplasmodial activities of compounds **1**-**4** are summarized in Table 3.2. Compound **1** demonstrated strong antiplasmodial activity whereas compounds **2**-**4** were inactive. The selectivity index of antiplasmodial activity was calculated based on their cytotoxicity to mammalian kidney fibroblasts (Vero cells) determined in parallel experiments. Compound **1** was moderately cytotoxic to Vero cells.

Compound **2** exhibited moderate activity against both methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* (Table 3.3). The cytotoxic potential of these compounds was further evaluated against a panel of solid tumor cell lines (SK-MEL, KB, BT-

549, SK-OV-3) and kidney epithelial cells (LLC-PK_{11}) (Table 3.4). Compound **1** showed moderate cytotoxicity against all the cell lines while compound **2** was inactive. The presence of an *N*-hydroxy group appears to be essential for the activity of these compounds. It is interesting to note that a free *N*-hydroxy group was also a prerequisite for the activity of cordypyridones, a group of 1,4-dihydroxy-2-pyridinone alkaloids, with moderate antiplasmodial activity previously *reported from fungus Cordyceps nipponica*.[64]

3.4. Bioactivity Results

Table 3.2. Antiplasmodial activity of **1-4**

Compound	Chloroquine-sensitive (D6)-clone		Chloroquine-resistant (W2)-clone		Cytotoxicity to Vero cells
	IC_{50} (nM)	S.I.	IC_{50} (nM)	S. I.	IC_{50} (nM)
1	0.07	5.0	0.07	5.4	0.38
2	NA		NA		NC
3	NA		NA		NC
4	NA		NA		NC
chloroquine[a]	0.03		0.31		NC
artemisinin[a]	0.02		0.01		NC

[a] Positive controls

NC: not cytotoxic

NA: not active

S. I. (selectivity index) = IC_{50} for cytotoxicity/IC_{50} for antiplasmodial activity

Table 3.3. Antifungal and antibacterial activity of **1-2.**

Compound	*Candida albicans*		*Aspergillus fumigatus*		*Staphylococcus aureus*		MRSA	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
1	32.5	NT	23.5	NT	NA	NA	NA	NA
2	NA	NA	NA	NA	7.24	12.98	7.76	12.98
amphotericin B	0.45	2.70	1.16	12.6	NT	NT	NT	NT
ciprofloxacin	NT	NT	NT	NT	0.27	0.75	0.15	0.75

IC_{50} and MIC (minimum inhibitory concentration, the lowest test concentration that allows no growth) values are in μM.

NA: not active.

NT: not tested.

Table 3.4. Cytotoxic activity [IC_{50} (μM)] of compounds **1-2**

Compound	**SK-MEL**	**KB**	**BT-549**	**SK-OV-3**	**LLC-PK_{11}**	**Vero**
1	4.04	13.46	4.45	7.0	4.4	3.77
2	NA	NA	NA	NA	NA	NA
doxorubicin[a]	11.03	2.48	1.47	2.2	1.1	>0.9

NA: not active.

[a] Positive control

3.5. Experimental

3.5.1. General

Melting points were measured with a Uni-melt, Thomas Hoover capillary melting point apparatus. Optical rotations were measured using a Rudolph Research Analytical Autopol IV automatic polarimeter model 589-546. UV and IR spectra were recorded on a Varian-50 Bio UV visible spectrophotometer and a Bruker-Tensor-27 infrared spectrophotometer, respectively. NMR spectra were recorded on a Varian-Mercury-plus-400 or Varian Unity-Inova-600 spectrometer using $CDCl_3$/methanol-d_4 as the solvent unless otherwise stated. MS analyses were performed on an Agilent Series 1100 SL equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA). Column chromatography was carried out on Merck silica gel 60 (230-400

mesh). Preparative TLC was carried out using silica gel GF plates (20 X 20 cm, thickness 0.25 mm).

3.5.2. Fermentation, Extraction, and Isolation

Septoria pistaciarum Caracc. (ATCC 22201) was obtained from the American Type Culture Collection, Manassas, VA and grown in potato dextrose agar plates at 27 ºC for 12 days. An Erlenmeyer flask containing 100 mL of potato dextrose broth was inoculated with small agar pieces (0.25 in × 0.25 in) and were incubated at 27 ºC for 7 days on a shaker (RPM = 100). This seed culture was used to inoculate 20 × 1L Erlenmeyer flasks each containing 500 mL of M-2 medium, (modified Czapex Dox medium : Czapex Dox 35.0 g, yeast extract 1.5 g, malt extract 1.5 g in 1L of distilled H_2O) and were incubated at 27 ºC for 28 days on a shaker (RPM = 100). The culture broth and mycelia were extracted with EtOAc (×3) and the organic layer was evaporated under reduced pressure to give an oily residue (5.2 g).

The oily extract (5.0 g) was chromatographed over Sephadex LH -20 and eluted with $CHCl_3$/MeOH (20:80) to give 12 fractions. Fractions 3, 4, 5, 6, and 7 showed antiplasmodial activity (IC_{50} < 529 ng/mL). Fractions 3, 4, and 5 were combined and chromatographed over a reversed phase C_{18} column eluting with a gradient 10% to 100% MeOH / H_2O to yield five fractions. Subfraction 4 was further purified by crystallization from MeOH and CH_2Cl_2 to give compound **1** as white crystals (53.0 mg)**.** Combined fractions 6 and 7 were chromatographed over a silica gel column using CH_2Cl_2/hexanes as the eluent to give 10 subfractions. Subfraction 7 was further purified by Sephadex LH-20 (MeOH) followed by preparative TLC (2% MeOH in CH_2Cl_2) to give compound **2** (20.0 mg) and compound **3** (12.0 mg). Subfraction 4 was

chromatographed over reversed phase C_{18} eluting with CH_3CN / H_2O (40:60) to give compound **4** (3.0 mg**).**

Compound 1**:** white crystals (CH_2Cl_2/MeOH); mp 220 ^{0}C; $[\alpha]^{26}_D$ +201 (*c*, 0.4, CH_3OH); UV (MeOH) λ_{max} (log ε) 207.1 (3.78), 241.0 (3.75), 296.0 (3.03) nm; IR ($CHCl_3$) ν_{max} 3109, 2963, 2927, 1642, 1556, 1454, 1225 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS $[M - H]^-$ *m/z* 370.2012 (calcd for $(C_{22}H_{29}NO_4 - H)^-$, 370.2018).

Compound 2**:** amorphous; $[\alpha]^{26}_D$ +15 (*c*, 0.375, MeOH); UV (MeOH) λ_{max} (log ε) 207.1 (2.15), 241.0 (1.96), 297.0 (0.88) nm; IR ($CHCl_3$) ν_{max} 3318, 2917, 1686, 1047 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS $[M + H]^+$ *m/z* 386.2423 (calcd for $(C_{23}H_{31}NO_4 + H)^+$, 386.2331).

Compound 3**:** amorphous; $[\alpha]^{26}_D$ +190 (*c*, 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 205.0 (3.61), 241.0 (3.46), 290.0 (2.72) nm; IR ($CHCl_3$) ν_{max} 3212, 2929, 2341, 1647, 1557, 1456, 1057 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS $[M + H]^+$ *m/z* 402.236 (calcd for $(C_{23}H_{31}NO_5+H)^+$, 402.228) .

Compound 4**:** amorphous: $[\alpha]^{26}_D$ +47 (*c*, 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 205.0 (2.56), 241.0 (2.69) nm; IR ($CHCl_3$) ν_{max} 3331, 2925, 2853, 1736, 1647, 1227, 1053 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS $[M + Na]^+$ *m/z* 466.2307 (calcd for $(C25H33NO6 + Na)^+$, 466.2205).

3.5.3. Crystallographic Data for Compound 1

X-ray data were collected at low-temperature (90 K) on a Bruker Kappa Apex-II diffractometer equipped with graphite-monochromated CuKα (λ = 1.54178 Å) source, yielding 14,467 independent data to θ_{max} = 69.0º. Crystals were monoclinic, space group $P2_1$, with a = 11.1427(9), b = 13.0004(10), c = 28.073(2) Å, β = 90.430(5)º, Z = 8. Refinement of the model containing four independent molecules resulted in R = 0.038 for 1015 parameters. Resonant scattering, principally from the O atoms, resulted in a Flack[94] parameter X = 0.01(9) and a Hooft[95] parameter Y = 0.09(5) for 6739 Bijvoet pairs. This corresponds to a probability of 1.000 that the illustrated configuration is correct.

3.5.4. Biological Assay

3.5.4.1. Antimicrobial Assay

All organisms were obtained from the American Type Culture Collection (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113 and *Aspergillus fumigatus* ATCC 90906 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI methods[72,71] as described by Samoylenko et al.[73] The drug

controls ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi were included in each assay.

3.5.4.2. Antiplasmodial Assay

In vitro antiplasmodial activity was determined against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *Plasmodium falciparum* as described earlier.[69]

3.5.4.3. Cytotoxicity Assay

In vitro cytotoxicity was determined against a panel of mammalian cells that included kidney fibroblast (Vero), kidney epithelial (LLC-PK_{11}), malignant melanoma (SK-MEL), oral epidermal carcinoma (KB), breast ductal carcinoma (BT-549), and ovary carcinoma (SK-OV-3) cells.[76] The assay was performed in 96-well tissue culture-treated plates. Cells were seeded to the wells of 96-well plate at a density of 25,000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined by using Neutral Red dye and IC_{50} values were obtained from dose response curves. Doxorubicin was used as a positive control.

CHAPTER 4: ANTIPROTOZOAL AND ANTIMICROBIAL COMPOUNDS FROM THE PLANT PATHOGEN *SEPTORIA PISTACIARUM*

Some of the results presented in this chapter have been published in the following journal.

Kumarihamy, M.; Khan, S. I.; Jacob, M.; Tekwani, B. L.; Duke, S. O.; Ferreira, D.; Nanayakkara, N. P. D. Antiprotozoal and antimicrobial compounds from the plant pathogen *Septoria pistaciarum*, *J. Nat. Prod.* **2012**, *75*, 883-889.

4.1. Summary

Four new 1,4-dihydroxy-5-phenyl-2-pyridinone alkaloids, 17-hydroxy-*N*-(*O*-methyl)septoriamycin A (**1**), 17-acetoxy-*N*-(*O*-methyl)septoriamycin A (**2**), 13-(*S*)-hydroxy-*N*-(*O*-methyl)septoriamycin A (**3**) and 13-(*R*)-hydroxy-*N*-(*O*-methyl)septoriamycin A (**4**), together with the known compounds (+)-cercosporin (**5**), (+)-14-*O*-acetylcercosporin (**6**), and (+)-di-*O*-acetylcercosporin (**7**), lumichrome, and brassicasterol were isolated from an ethyl acetate extract of a culture medium of *Septoria pistaciarum*. Methylation of septoriamycin A (**8)** with

diazomethane yielded three di-*O*-methyl analogues, two of which existed as mixtures of rotamers. We previously reported in vitro antimalarial activity of septoriamycin A. This compound also exhibited significant activity against *Leishmania donovani* promastigotes and amastigotes. Compounds **5-7** showed in vitro activity against *L. donovani* promastigotes, chloroquine-sensitive (D6) and -resistant (W2) strains of *Plasmodium falciparum*, methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus*, *Mycobacterium intracellulare*, *Candida glabrata*, and moderate cytotoxicity against a panel of cell lines. Compounds **5**, **6**, and **7** also exhibited moderate phytotoxic activity against both a dicot (lettuce, *Lactuca sativa*) and a monocot (bentgrass, *Agrostis stolonifera*).

4.2. Introduction

Phytotoxins from plant pathogenic fungi may act as inhibitors of plant-like metabolic pathways in the apicoplast, a chloroplast-like organelle, essential for the survival of plasmodium species.[41] Previously we reported[96] the isolation and identification of the 2-pyridinone alkaloid, septoriamycin A[97] **(8)** and three of its derivatives from the causative agent of septoria leaf spot disease in pistachio, *Septoria pistaciarum* (Ascomycetes), as part of our program to identify antimalarial compounds from plant pathogenic fungi. Although we detected several minor alkaloids of the same class in the original extract, their structural elucidation was not possible due to insufficient quantities. In order to isolate the minor compounds, we recultured the same fungus under the identical conditions at a larger scale. In the ethyl acetate extract of this fermentation culture broth, a series of pigments with 2-pyridinone alkaloidal architecture were

detected. The extract showed herbicidal, antimicrobial, antiplasmodial, and antileishmanial activities albeit with low selectivity indices. From this extract, four more new minor septoriamycin A analogues, 17-hydroxy-*N*-(*O*-methyl)septoriamycin A (**1**), 17-acetoxy-*N*-(*O*-methyl)septoriamycin A (**2**), 13-(*S*)-hydroxy-*N*-(*O*-methyl)septoriamycin A (**3**), and 13-(*R*)-hydroxy-*N*-(*O*-methyl)septoriamycin A (**4**), in addition to the parent and its previously reported analogues were identified. Septoriamycin A (**8)**, the major 2-pyridinone alkaloid in this extract showed significant antileishmanial activity in addition to its reported antiplasmodial activity. The pigments were identified as three known perylenequinones, (+)-cercosporin (**5**),[98] (+)-14-*O*-acetylcercosporin (**6**),[98] and (+)-di-*O*-acetylcercosporin (**7**).[99] These pigments have previously been identified as phytotoxins produced by a number of phytopathogenic *Cercospora* species and have been linked to their pathogenicity.[100] Their biosynthesis appeared to be controlled by numerous environmental and physiological factors and the presence of even small amounts of certain compounds in the medium was found to have a strong stimulatory or inhibitory effect on their production.[100, 101] Their ability to generate reactive oxygen species in the presence of light has been attributed to their phytotoxic activity.[100] Cercosporin and its esters have also been reported to have antibacterial and antifungal[102] activities as well as growth inhibitory effects on lettuce[98] and tomato seeds.[102] In this study, the perylenequinones showed antileishmanial, antiplasmodial, and cytotoxic activities in addition to antibacterial and antifungal activities. Two more known compounds, lumichrome (**11**),[103] and brassicasterol (**12**)[104] were also isolated and identified.

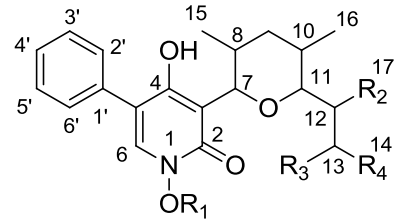
4.3. Results and Discussion

Fractionation of an EtOAc extract of a culture medium of *S. pistaciarum* by Sephadex LH-20 gel column chromatography followed by purification using silica gel and RP C_{18} chromatography afforded four minor 2-pyridinone alkaloids **1-4** in addition to the known septoriamycin A (**8**) and its three derivatives,[96] three known perylenequinones (+)-cercosporin (**5**), (+)-14-*O*-acetylcercosporin (**6**), (+)-di-*O*-acetyl-cercosporin (**7**), lumichrome (**11**), and brassicasterol (**12**).

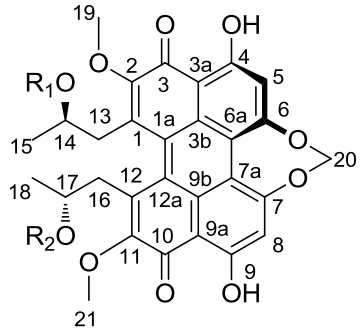
The molecular formula of compound **1** was determined as $C_{23}H_{31}NO_5$ by HRESIMS. UV maxima (205.0, 240.9, and 297.0 nm) and IR absorptions (3400, 3207, 1647, and 1555 cm^{-1}) of **1** were consistent with those of a 2-pyridinone moiety.[96] The aromatic regions of the ^1H NMR (Figure 4.2) and COSY (Figure 4.3) spectra were similar to those observed for 5-phenyl-2-pyridinones previously isolated from this species, and consisted of a one-proton singlet and an A_2B_2C spin system due to a monosubstituted phenyl group.[96] The aliphatic region exhibited two oxymethine and two methyl doublets indicating the presence of a 2,4-dimethyltetrahydropyran moiety and an *N*-methoxy group (Figure 4.3).

HMBC (Figure 4.4) correlations of H-2′ and H-6′ (δ_H 7.43) with C-3′ and C-5′ (δ_C 128.6), C-4′ (δ_C 127.8), C-1′ (δ_C 133.3), and C-5 (δ_C 114.5), and H-6 (δ_H 7.44) with C-2 (δ_C 158.0), C-4 (δ_C 161.9), C-5 (δ_C 114.5), and C-1′ (δ_C 133.3) supported the partial structure of the substituted pyridinone ring moiety. HMBC correlations of the H-7 oxymethine doublet (δ_H 4.73) with C-4 (δ_C 161.9), C-2 (δ_C 158.0), C-3 (δ_C 111.6), C-11 (δ_C 86.3), C-9 (δ_C 36.4), C-8 (δ_C 39.8), and C-15 (δ_C 17.8) supported the partial structure of the tetrahydropyran moiety. The

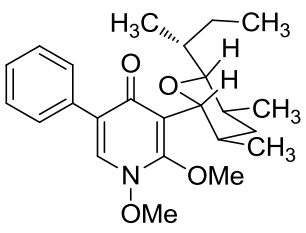
major difference in the rest of the ^{1}H NMR resonances of compound **1** and the reported[96] *N*-(*O*-methyl)septoriamycin A (**9**) is the replacement of the methyl doublet in the chain attached to C-11 of the tetrahydropyran ring by two diastereotopic oxymethylene hydrogens. This indicated that compound **1** was the C-17 oxygenated analogue of **9**. The COSY spectrum of compound **1** displayed cross peaks between the C-17 oxymethylene (δ_H 3.60, 3.49) and C-12 methine proton (δ_H1.87). In the HMBC spectrum the oxymethylene protons showed cross peaks with C-11 (δ_C 86.3), C-12 (δ_C 36.5), and C-13 (δ_C 22.3), and the H-11 oxymethine doublet at δ_H 3.36 with C-7 (δ_C 81.2), C-9 (δ_C 36.4), C-12 (δ_C 36.5), C-13 (δ_C 22.3), C-16 (δ_C 12.6), and C-17 (δ_C 63.6) further confirming that compound **1** was 17-hydroxy-*N*-(*O*-methyl)septoriamycin A. (Figure 4.1)



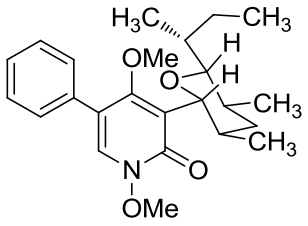
	R_1	R_2	R_3	R_4
1	CH_3	CH_2OH	H	CH_3
2	CH_3	CH_2OAc	H	CH_3
3	CH_3	CH_3	OH (S)	CH_3
4	CH_3	CH_3	OH (R)	CH_3
8	H	CH_3	H	CH_3
9	CH_3	CH_3	H	CH_3



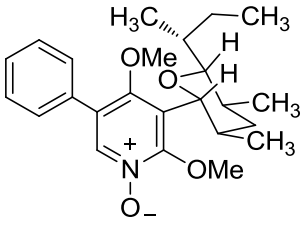
	R_1	R_2
5	H	H
6	OAc	H
7	OAc	OAc



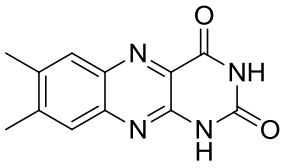
10a



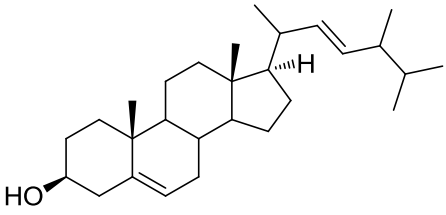
10b



10c



Lumichrome (**11**)



brassicasterol (**12**)

Figure 4.1. Structure of compounds **1 - 12**

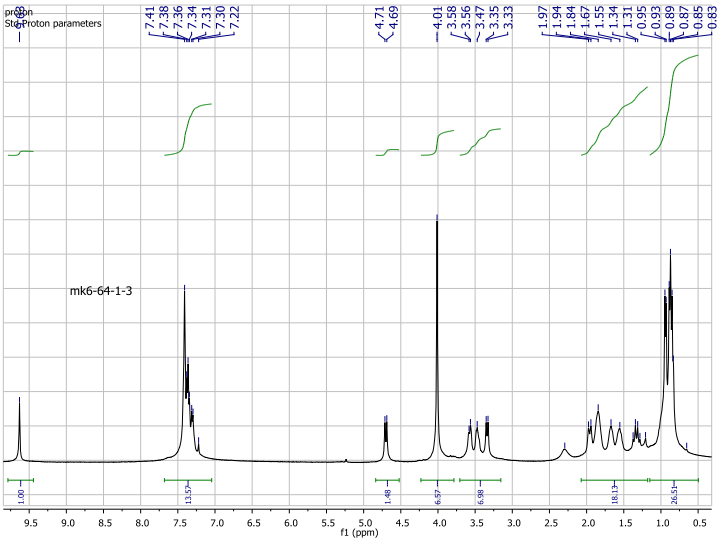


Figure 4.2. ^{1}H NMR spectrum of **1**

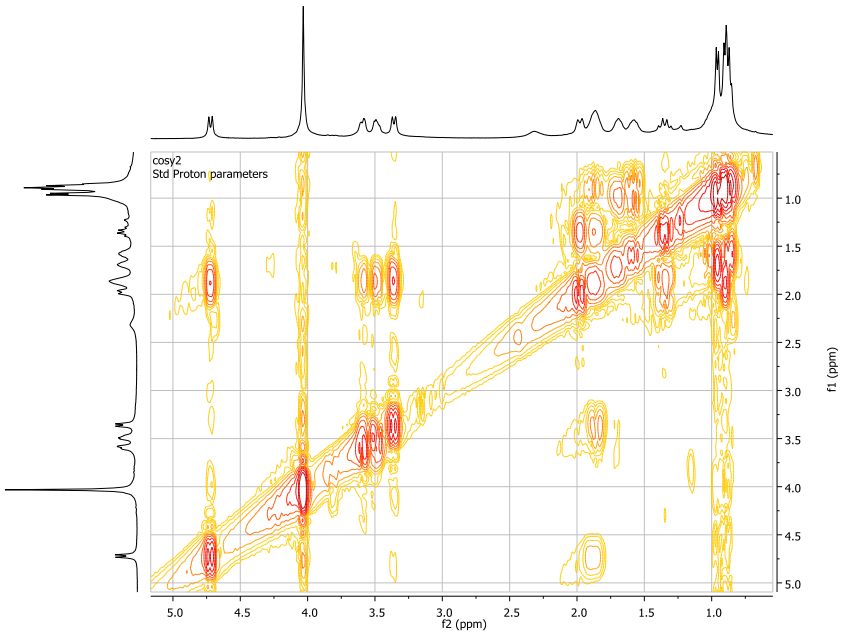


Figure 4.3. COSY spectrum of **1**

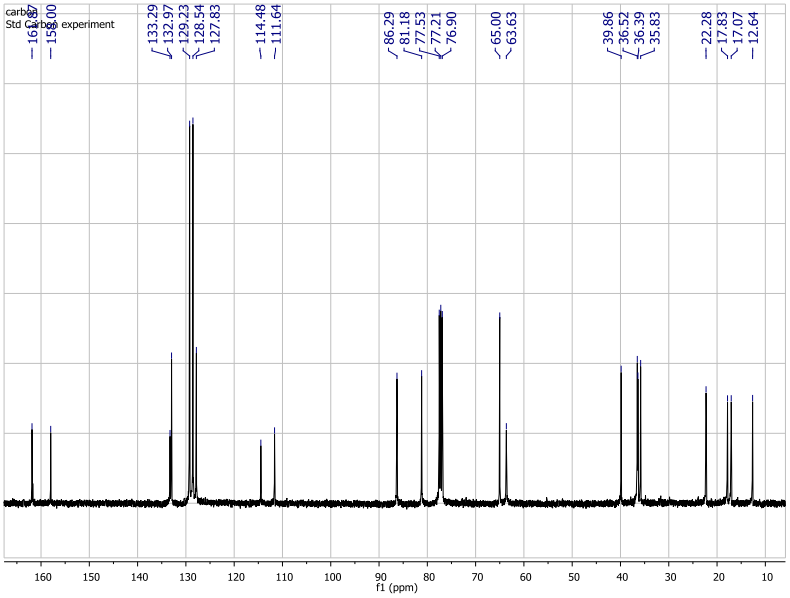


Figure 4.4. ^{13}C NMR spectrum of **1**

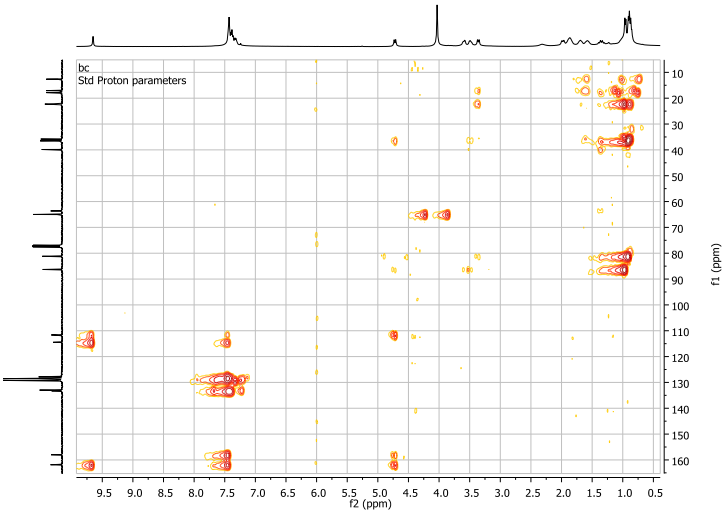


Figure 4.5. HMBC spectrum of **1**

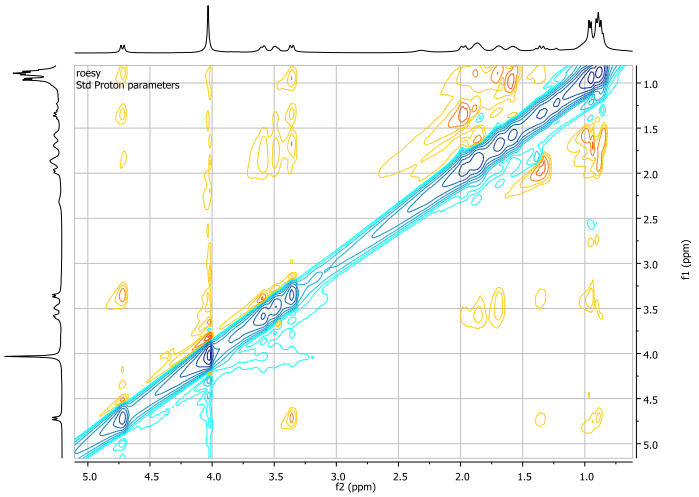


Figure 4.6. ROESY spectrum of **1**

The HRESIMS data established the molecular formula of compound **2** as $C_{25}H_{33}NO_6$. The ^{1}H (Figure 4.7) and ^{13}C NMR spectra (Figure 4.8) were similar to those of **1** except for the presence of additional resonances [(δ_H 2.06) δ_C 21.1 (CH_3), δ_C 171.2 (CO)] originating from an *O*-acetyl group. HMBC (Figure 4.9) correlations between the C-12 oxymethylene group and the acetyl carbonyl carbon indicated that compound **2** was 17-*O*-acetyl-*N*-(*O*-methyl)septoriamycin A. The remaining HMBC and COSY (Figure 4.10) correlations were identical to those of compound **1.** Both $^{3}J_{7,8}$ and $^{3}J_{10,11}$ values were 10.2 Hz indicating that these protons are *trans* – diaxially oriented. ROESY correlations of compound **1** (Figures 4.6 & 4.11) and **2** were identical to those observed for septoriamycin A **(8)** suggesting that these two compounds had the same relative configurations. Since we have previously assigned the absolute configuration of septoriamycin A on the basis of X-ray diffraction data,[96] and all these compounds presumably

share a common biosynthetic origin, compounds **1** and **2** also have 7*R*, 8*R*, 10*S*, 11*R*, and 12*R* absolute configuration. It is further supported by their dextrorotatory specific rotations.

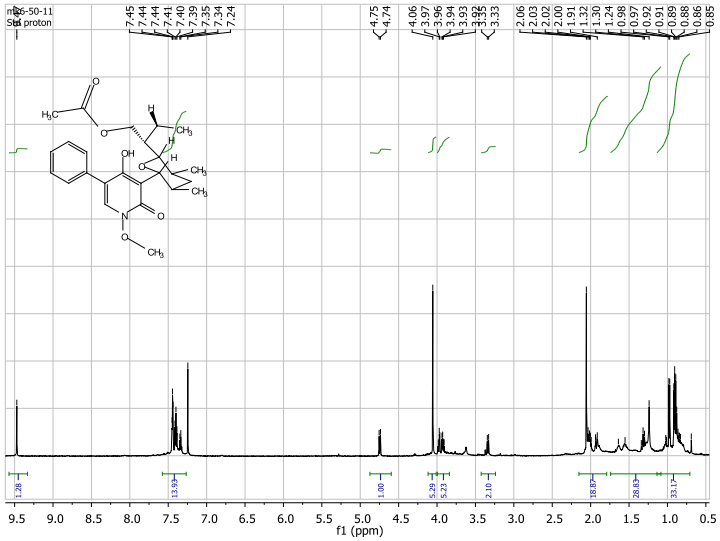


Figure 4.7. ^{1}H NMR spectrum of **2**

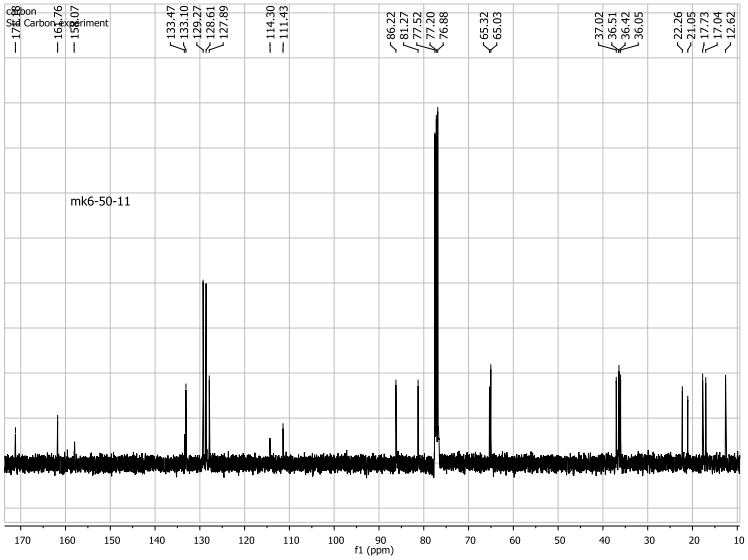


Figure 4.8. ^{13}C NMR spectrum of **2**

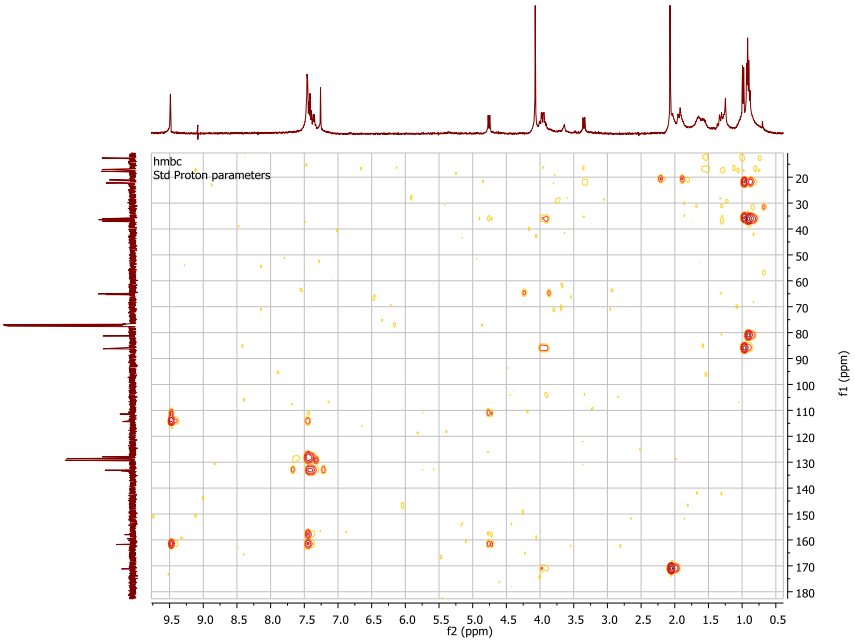


Figure 4.9. HMBC spectrum of **2**

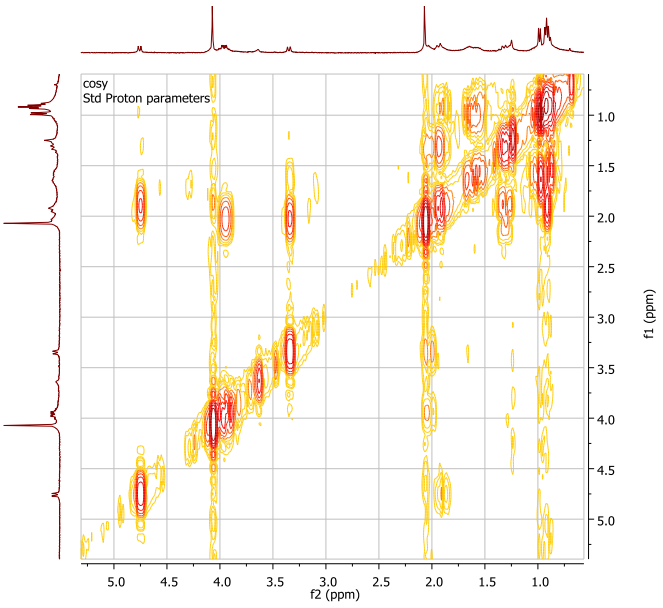
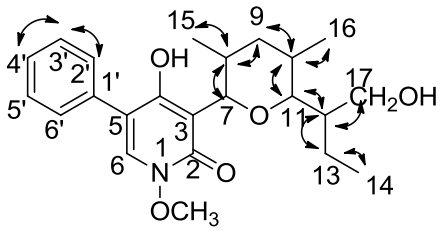
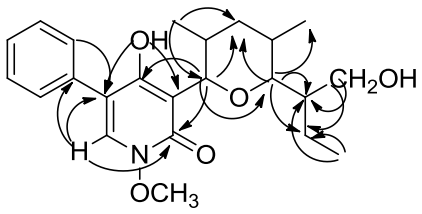


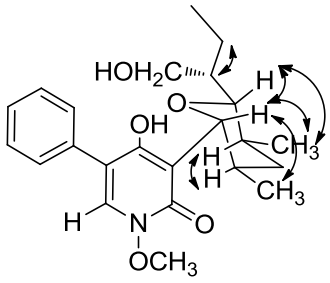
Figure 4.10. COSY spectrum of **2**



Key COSY ( ) correlations



Key HMBC ( ) correlations



Key ROESY ( ) correlations

Figure 4.11. COSY, HMBC, and ROESY correlations of **1.**

The HRESIMS data of **3** established its molecular formula as $C_{23}H_{31}NO_5$. Comparison of ^{1}H NMR (Figure 4.12), ^{13}C NMR (Figure 4.13), COSY (Figure 4.14), and HMBC (Figure 4.15) data of **3** with those of **8** showed that the major difference was the replacement of a methyl triplet (δ_H 0.89) of the latter by a methyl doublet (δ_H 1.16) and an oxymethine doublet (δ_H 3.78) in the former. These changes could be attributable to the substitution of one of the C-13 diastereotopic methylene hydrogens in **8** with a hydroxy group indicating that compound **3** is the 13-hydroxy analogue of *N*-(*O*-methyl)septoriamycin A (**9**)**.** The methyl doublet at δ_H 0.93 (17-CH_3) showed HMBC cross peaks with C-11 (δ_C 88.9), C-12 (δ_C 41.4), and C-13 (δ_C 68.9) and the oxymethine multiplet at δ_H 3.78 with C-11 (δ_C 88.9), C-12 (δ_C 41.4), and C-17 (δ_C 15.9) confirming the C-13 location of the secondary hydroxy group and, thus, compound **3** was 13-hydroxy-*N*-(*O*-methyl)septoriamycin A.

ROESY (Figure 4.16) data and ^{1}H NMR coupling constants of compound **3** showed close correlations to those reported[96] for septoriamycin A (**8**)**.** Our attempts to determine the absolute configuration at C-13 by Mosher analysis were unsuccessful. Treatment of compound **3** with the *R*- and *S*-Mosher's acid chlorides afforded a mixture not worth resolving. Methylation prior to acylation also yielded a mixture of products. The absolute configuration of compound **3** was proposed by a $^3J_{12,13}$ - based comparison of compounds **3** and **4** (Figure 4.17).

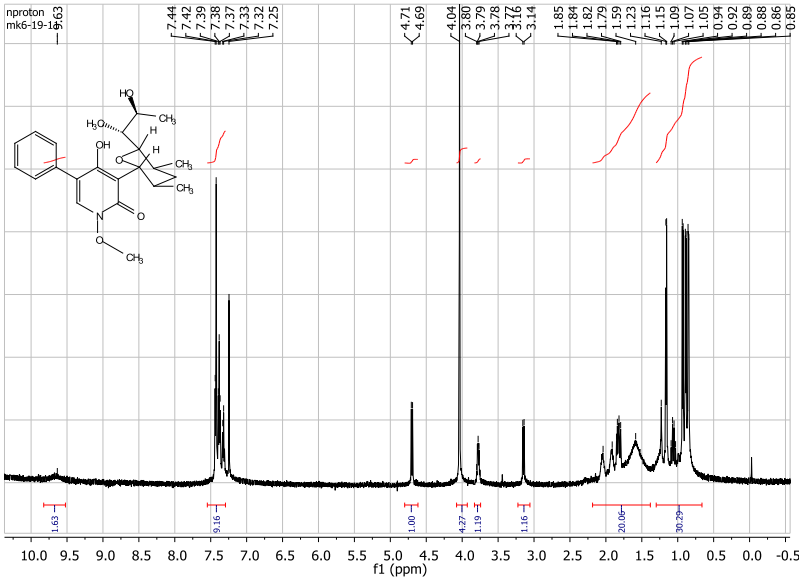


Figure 4.12. ^{1}H NMR spectrum of **3**

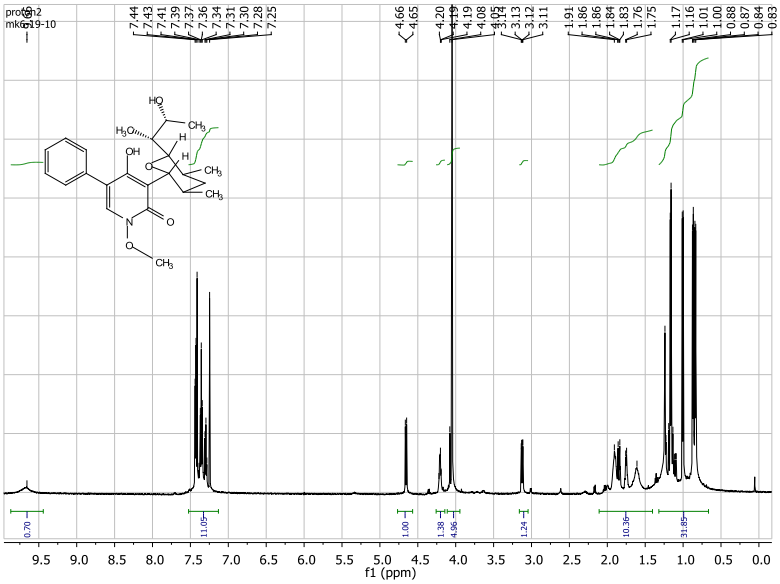


Figure 4.13. ^{13}C NMR spectrum of **3**

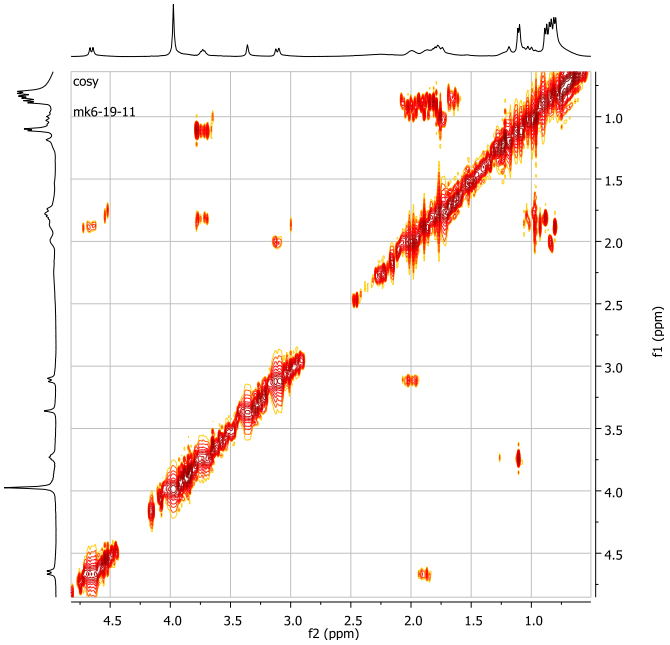


Figure 4.14. COSY spectrum of **3**

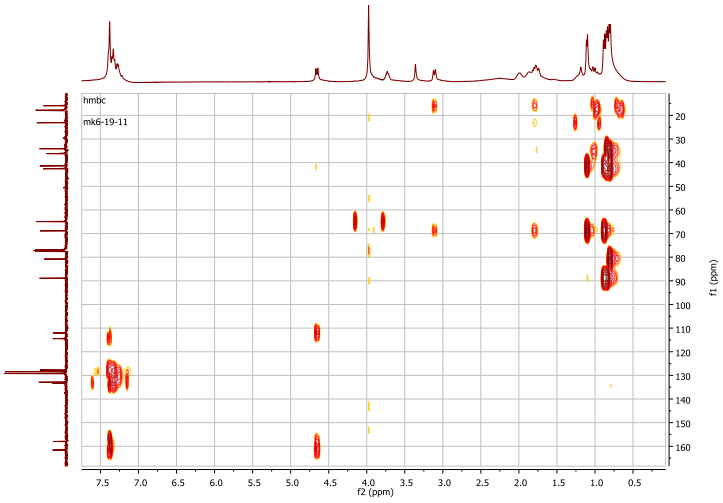


Figure 4.15. HMBC spectrum of **3**

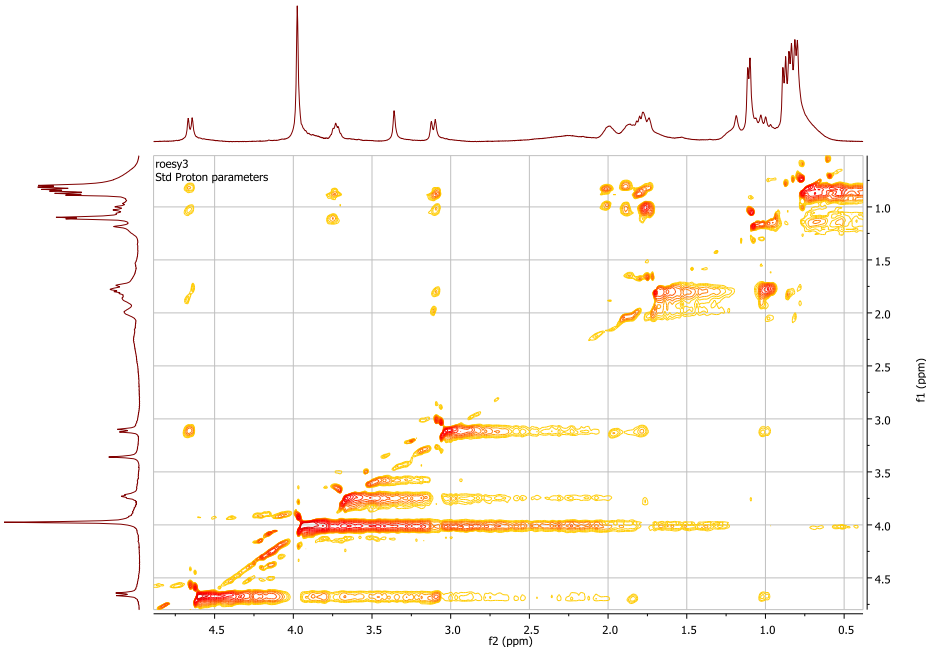


Figure 4.16. ROESY spectrum of **3**

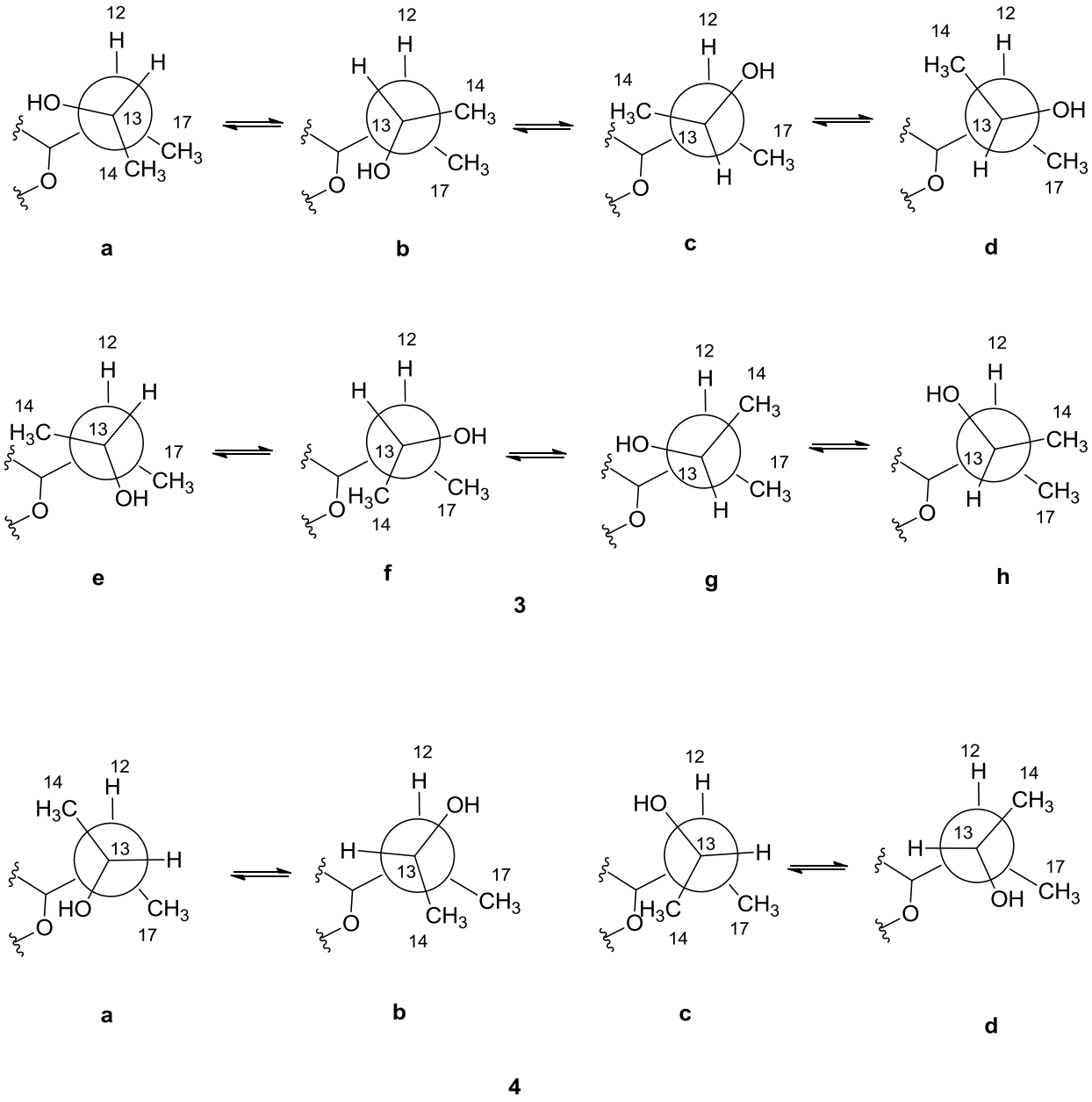


Figure 4.17. Rotamer representation of **3** and **4**

Compound **4** had the same molecular formula, $C_{23}H_{31}NO_5$, as that of **3** based on HRESIMS data. The ^{1}H NMR (Figure 4.18) and ^{13}C NMR (Figure 4.19) spectra of these compounds were similar except for the down-field shift of the oxymethine proton (δ_H 4.20) and the up-field shift of a methyl doublet (δ_H 1.00). The COSY (Figure 4.20) and HMBC (Figure 4.21) spectra of **4** showed the same correlations as those observed for **3** suggesting that both compounds have the same gross structure. Identical coupling constants and ROESY (Figure 4.22) correlations for the protons in the tetrahydropyran moiety indicated that they had the same relative configuration except at C-13. Since compounds **3**, **4,** and **8** presumably share a common biosynthetic origin, compounds **3** and **4** also have 7*R*, 8*R*, 10*S*, 11*R*, 12*R* absolute configuration. As described earlier for compound **3**, our attempts to determine the absolute configuration of the C-13 stereogenic center by Mosher analysis were unsuccessful. Thus, we used a *J*-based approach relying on ^{1}H NMR coupling constants combined with ROESY correlations, to assign the C-13 absolute configuration of compounds **3** and **4**. An observed $^3J_{12,\ 13}$ value of 7.2 Hz of **3,** suggested a dihedral angle of ca 30^o or 150^o between H-12 and H-13. Eight rotamers are possible for **3** with these dihedral angles for the *S* (**3a** – **3d**) and *R* (**3e** -**3h**) epimers (Figure 4.17). Observed ROESY correlations between H-12 and H-13, H-13 and CH_3-17, and CH_3-14 – CH_3-17 ruled out all conformers except **3a** as the probable most abundant rotamer for compound **3** in solution indicating a 13*S* absolute configuration. Similarly, an observed $^3J_{12,\ 13}$ of 1.0 Hz for **4** supported the fact that the dihedral angle between H-12 and H-13 was 90^0 as shown in Figure 4.17 for the 13*R* (**4a** - **4b**) and *S* (**4c** – **4d**) epimers. In the ROESY spectrum, H-12 showed correlation with H-13 and CH_3-14 and absence of interaction between CH_3-14 and CH_3 -17 indicating **4a** as the dominant rotamer and, hence, 13*R* as absolute configuration.

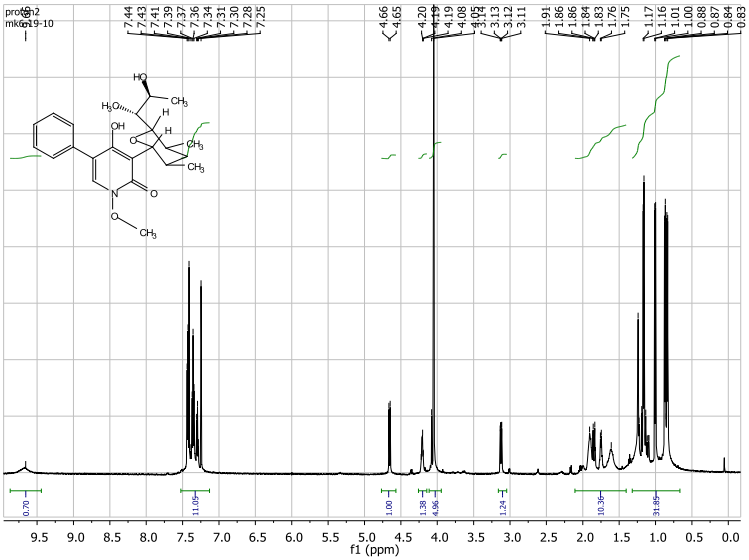


Figure 4.18. ^{1}H NMR spectrum of **4**

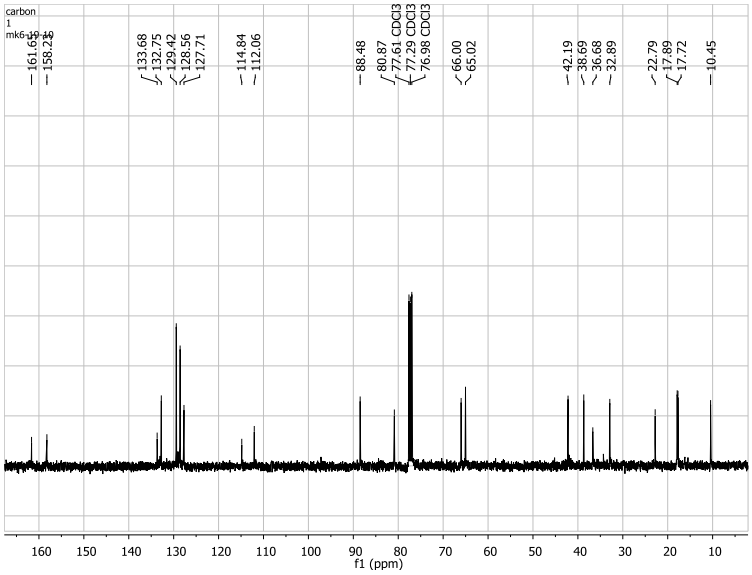


Figure 4.19. ^{13}C NMR spectrum of **4**

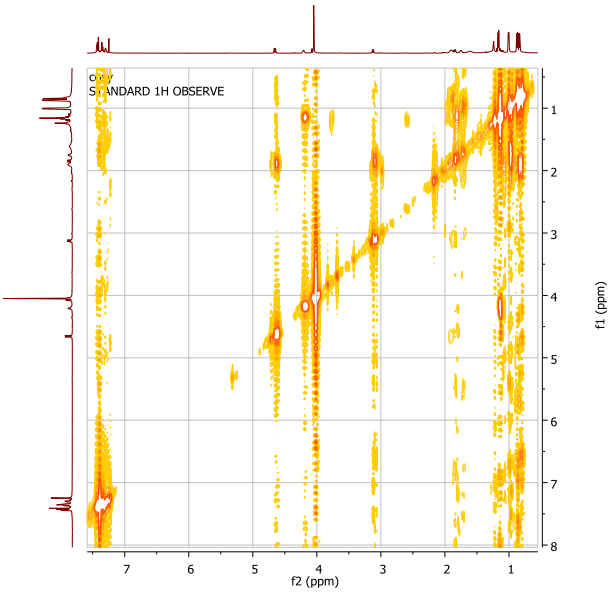


Figure 4.20. COSY spectrum of **4**

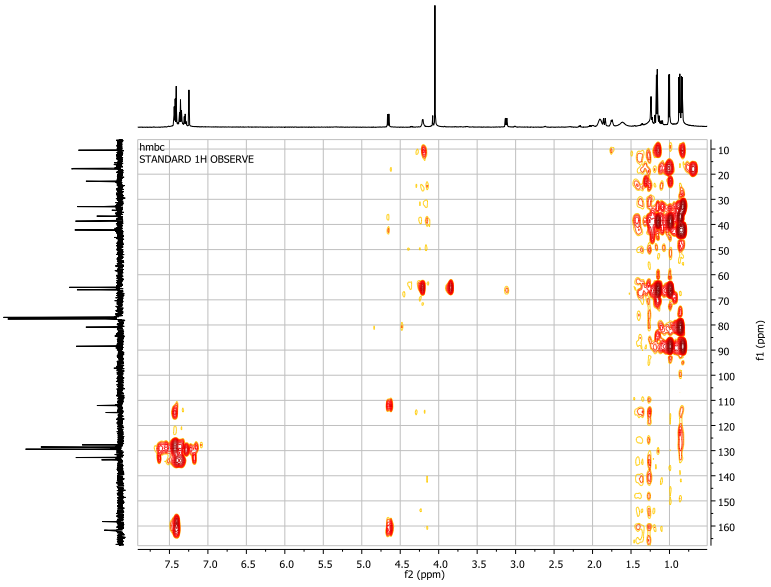


Figure 4.21. HMBC spectrum of **4**

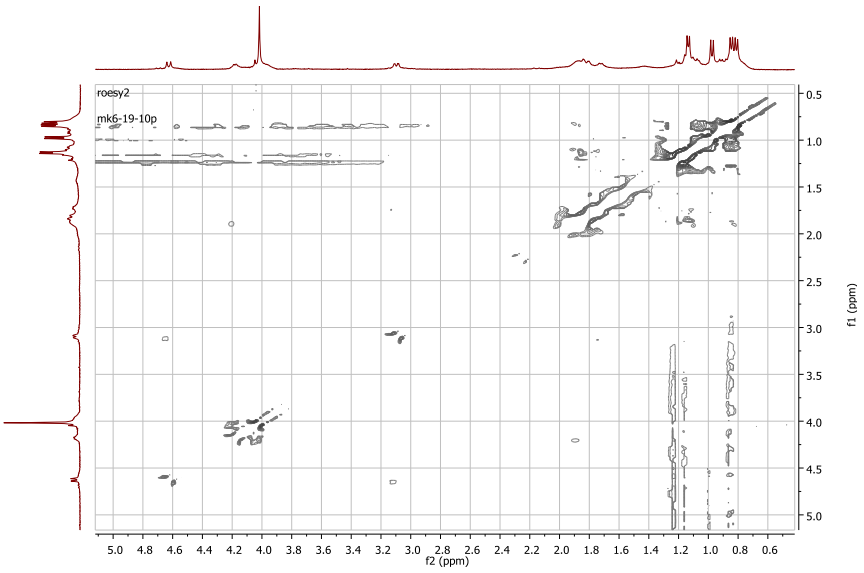


Figure 4.22. ROESY spectrum of **4**

Methylation of compounds **3** and **4** with diazomethane afforded several products. Treatment of septoriamycin A (**8**) with diazomethane as a model gave three products, whereas methylation with MeI and Cs_2CO_3 afforded a single compound which was identified as analogue **9**. The products of diazomethane methylation of septoriamycin A were separated by chromatography. All these products had the same molecular formula, $C_{24}H_{33}NO_4$, by HRESIMS, suggesting that they were di-*O*-methyl derivatives. The ^{1}H NMR (Figure 4.23) spectrum of the least polar compound (**10a**) was similar to that of **9** except for the presence of an additional *O*-methyl resonance in the former. The ^{13}C NMR (Figure 4.24) spectrum of this compound showed changes in resonances in the pyridone ring. A down-field shift of C-4 (δ_C 177) indicated that the carbonyl group now resided at this carbon suggesting **10a** was *N*,2-di-*O*-methylseptoriamycin A.

A hypsochromic shift of the UV absorption (λ_{max} 278.9 nm) when compared to **9** (λ_{max} 295 nm)[105] and COSY and HMBC correlations further supported this structure**.**

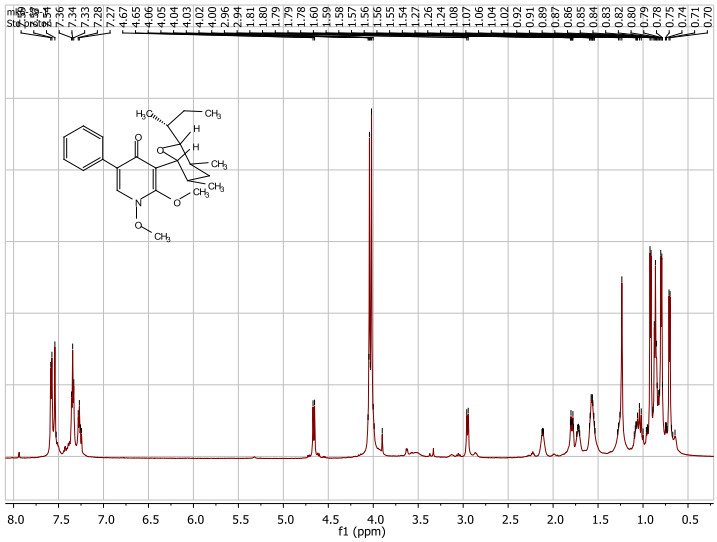


Figure 4.23. ^{1}H NMR spectrum of **10a**

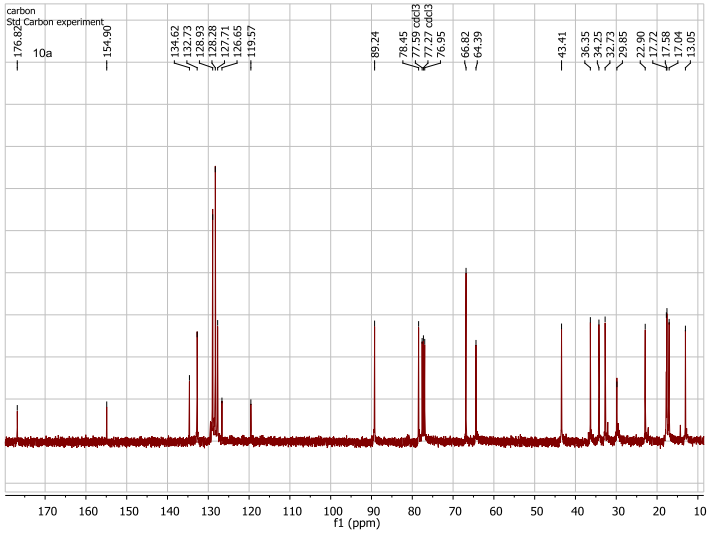


Figure 4.24. ^{13}C NMR spectrum of **10a**

Table 4.1. ^{1}H, and ^{13}C, NMR data for compounds **1-4** in $CDCl_3$/Methanol-d_4.

	1		**2**		**3**		**4**	
position	$\delta_H{}^a$ (J in Hz)	$\delta_C{}^b$	$\delta_H{}^a$ (J in Hz)	$\delta_C{}^b$	$\delta_H{}^a$ (J in Hz)	$\delta_C{}^b$	$\delta_H{}^a$ (J in Hz)	$\delta_C{}^b$
2		158.0		158.0		158.0		158.2
3		111.6		111.4		112.0		112.0
4		161.9		161.8		161.6		161.7
5		114.5		114.3		114.5		114.8
6	7.43, s	133.0	7.44, s	133.1	7.42, s	132.9	7.43, s	132.8
7	4.73, d (10.2)	81.2	4.74, d (10.2)	81.3	4.70, d (10.2)	80.8	4.66, d (10.2)	80.9
8	1.87, m	39.8	1.91, m	36.4	1.91, m	36.2	1.91, m	36.7
9	1.34, q (12.6)	36.4	1.30, q (12.6)	36.5	1.06, q (12.6)	42.6	1.16, q (12.0)	42.2
	1.98, brd (13.2)		2.00, (overlap)		1.80, dt (13.8, 3.6)		1.84, dt (13.2, 3.6)	
10	1.69, m	35.8	1.64, m	36.0	2.03, m	34.1	1.89, m	32.9
11	3.36, d (10.2)	86.3	3.33, d (10.2)	86.2	3.14, d (10.2)	88.9	3.12, dd (10.2, 2.8)	88.5
12	1.87, m	36.5	2.00, m	37.0	2.05, m	41.4	1.75, m	38.7
13	1.02, m, 1.58, m	22.3	1.02, m, 1.55, m	22.3	3.78, m (7.2, 7.2)	68.9	4.20, m (6.6, 1.0)	66.0
14	0.88, t (7.2)	12.6	0.89, t (7.2)	12.6	1.16, d (6.6)	23.1	1.16, d (6.3)	22.8
15	0.91, d (6.6)	17.8	0.91, d (7.2)	17.7	0.85, d (6.6)	17.9	0.87, d (6.6)	17.9

16	0.95, d (6.6)	17.1	0.97, d (7.2)	17.0	0.83, d (6.6)	17.7	0.84, d (6.6)	17.7
17	3.49, dd (10.6, 3.6) 3.60, dd (10.6, 3.6)	63.6	3.91, dd (11.2, 5.4) 3.98, dd (11.6, 4.5)	65.3	0.93, d (7.2)	15.9	1.01, d (7.2)	10.5
1′		133.3		133.4		133.4		133.7
2′, 6′	7.44, d (7.3)	129.2	7.44, d (7.3)	129.3	7.43, d (7.3)	129.2	7.43, d (7.3)	129.4
3′, 5′	7.39, t (7.2)	128.6	7.40, t (7.3)	128.6	7.38, t (7.8)	128.5	7.36, d (7.2)	128.6
4′	7.32, t (7.2)	127.8	7.34, t (7.3)	127.9	7.32, t (7.2)	127.7	7.30, t (7.2)	127.8
OH	9.63, s		9.47, s		9.63, s		9.65, s	
OCH_3	4.04, s	65.0	4.06, s	65.0	4.04, s	65.0	4.05, s	65.0
$COCH_3$			2.06, s	21.1				
$COCH_3$				171.2				

[a]^{1}H NMR spectra recorded at 600 MHz, [b]^{13}C NMR spectra recorded at 100 MHz

Their ^{1}H and ^{13}C NMR spectra were similar to those of compound **9** but had an additional methoxy resonance. HMBC correlations showed that the additional methoxy group correlated to C-4 indicating that **10b** was *N*,4-di-(*O*-methyl)septoriamycin A. At 100 ^{o}C in pyridine, duplicated ^{1}H NMR (Figure 4.27) resonances coalesced but were restored to the original ratio when the temperature returned to ambient conditions (Figure 4.28). Atropisomers of 3-cyclohexyl- or 3-cycloheptyl-*N*,4-dihydroxy-2-pyridone alkaloids have previously been isolated from several fungal species.[64, 105-108] Even though a number of 3-pyrano-*N*,4-dihydroxy-2-pyridone alkaloids similar to septoriamycin A analogues have been identified[109] from fungal sources, none have been reported to exist as rotamers. The introduction of a 4-methoxy group appears to cause restricted rotation about the C-3-C-7 bond giving rise to rotamers. The most polar compound (**10c**) showed ionic characteristics. ^{1}H (Figure 4.29) and ^{13}C NMR (Figure 4.30) data indicated that the compound also existed as a 3:1 mixture of two diastereomeric rotamers. HMBC correlations showed that the methoxy resonance correlated to C-2 and C-4 suggesting that **10c** is the 2,4-di-*O*-methylpyridinone-*N*-oxide analogue of septoriamycin A. The presence of the 4-methoxy group appears to cause restricted rotation about the C-3-C-7 bond. At 100 ^{o}C in pyridine duplicated ^{1}H NMR (Figure 4.31) resonances coalesced. Even though the original ratio of rotamers reappeared at ambient temperature some decomposition of **10c** was also observed (Figure 4.32).

The known perylenequinones, (+)-cercosporin (**5**),[98] (+)-14-*O*-acetylcercosporin (**6**),[98, 99] and (+)-di-*O*-acetylcercosporin (**7**),[99] lumichrome (**11**),[103] and brassicasterol (**12**),[104] were identified by comparing their spectroscopic data with literature data. The absolute configurations of **5-7** were determined by comparing experimental and reported electronic circular dichroism data.[110, 111]

The helicity of compounds **5-7** was confirmed as M (*aS*) and the absolute configuration of both C-14 and C-17 as *R*.[112]

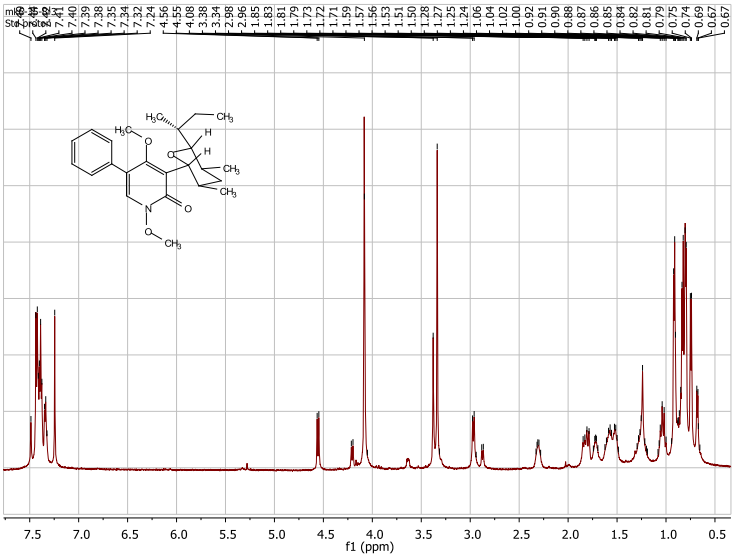


Figure 4.25. ^{1}H NMR spectrum of **10b**

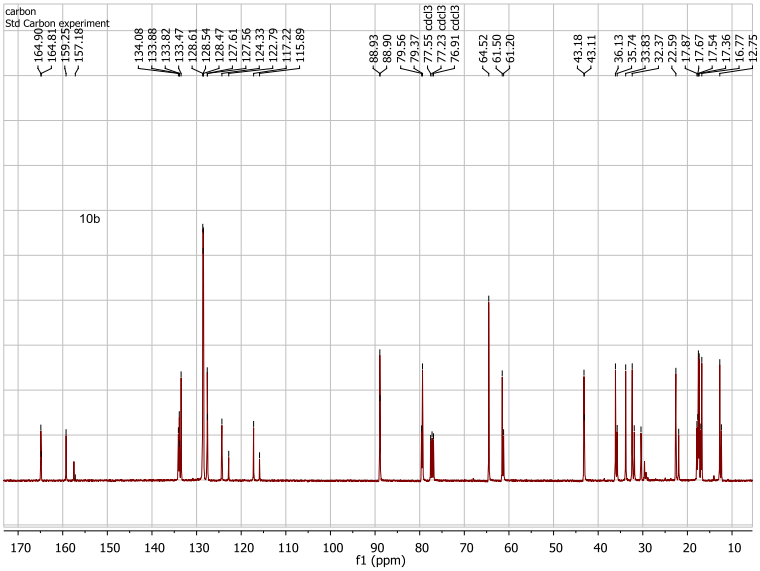


Figure 4.26. ^{13}C NMR spectrum of **10b**

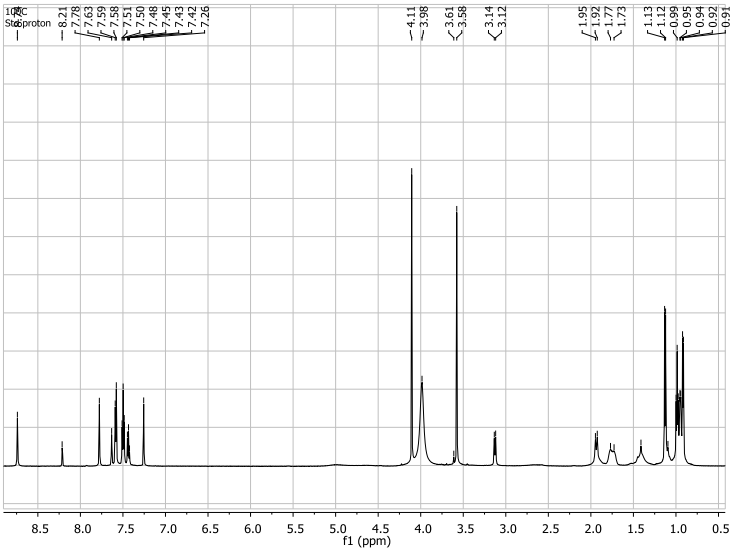


Figure 4.27. ^{1}H NMR spectrum of **10b** (at 100 ^{o}C in pyridine)

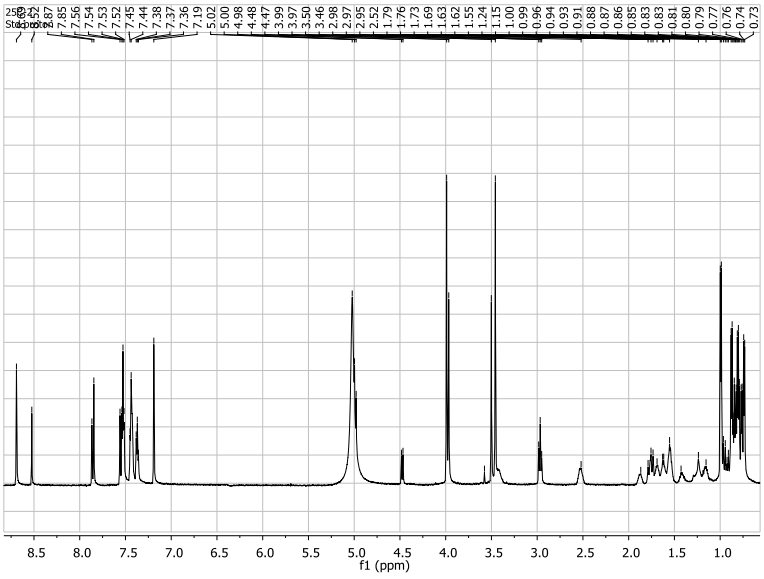


Figure 4.28. ^{1}H NMR spectrum of **10b** (at 25 ^{o}C in pyridine)

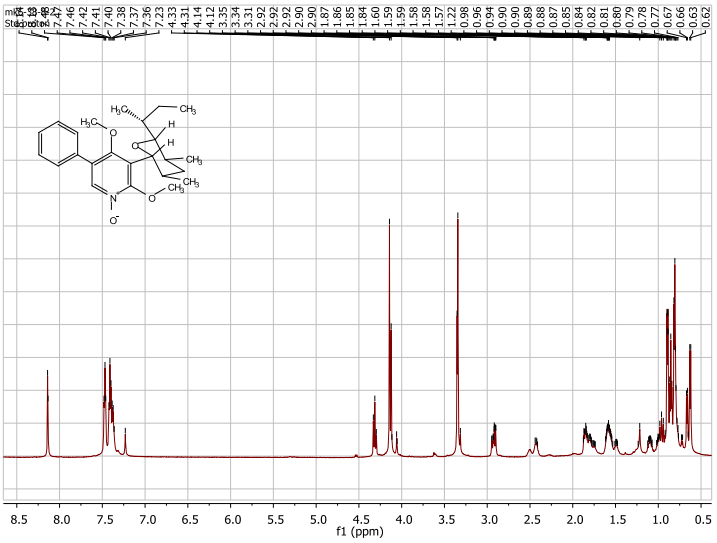


Figure 4.29. ^{1}H NMR spectrum of **10c**

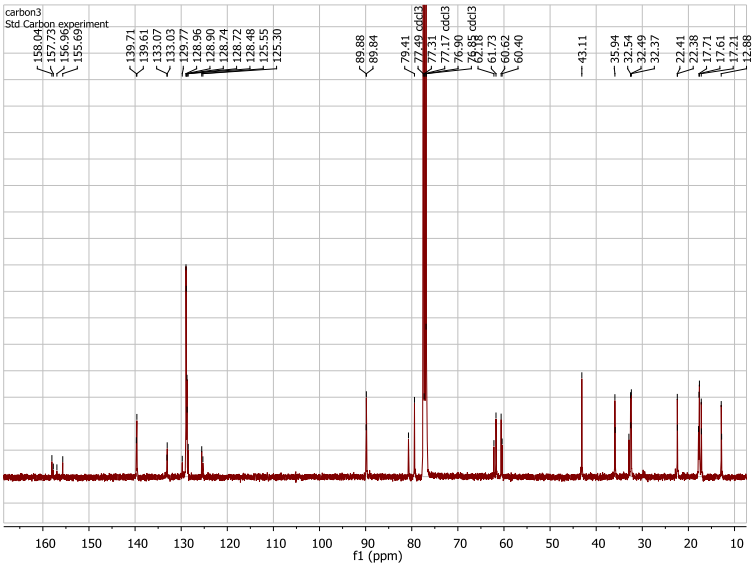


Figure 4.30. ^{13}C NMR spectrum of **10c**

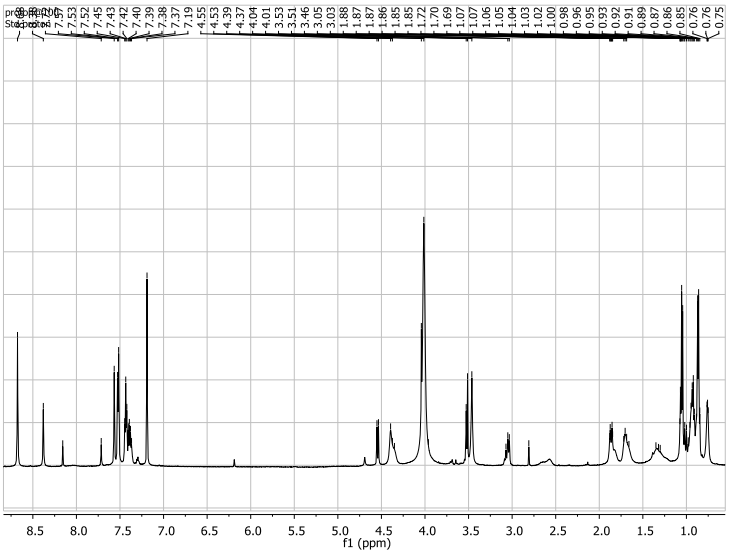


Figure 4.31. ^{1}H NMR spectrum of **10c** (at 100 ^{o}C in pyridine**)**

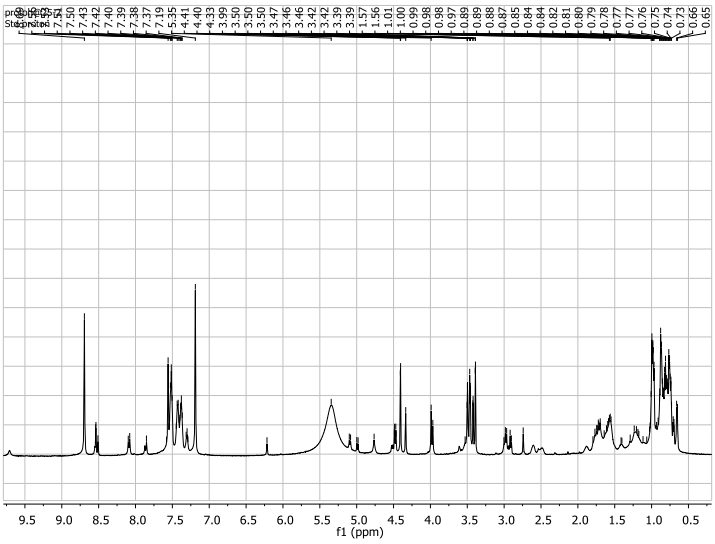


Figure 4.32. ^{1}H NMR spectrum of **10c** (at 25 ^{o}C in pyridine**)**

Table 4.2. ^{1}H and ^{13}C NMR data (methylated compounds **10a**, **10b**, and **10c**) in $CDCl_3$/Methanol-d_4.

position	**10a**		**10b**				**10c**			
	$\delta_H{}^a$ (J in Hz)	$\delta_C{}^b$	$\delta_H{}^a$ (J in Hz)		$\delta_C{}^b$		$\delta_H{}^a$ (J in Hz)		$\delta_C{}^b$	
2		155.0			159.2	157.2			158.0	156.0
3		119.5			124.3	122.8			125.6	125.3
4		177.0			164.9	164.8			155.7	157.0
5		134.6			117.2	115.9			128.5	128.5
6	7.51, s	132.7	7.44, s	7.49, s	133.4	134.0	8.15, s	8.14, s	139.6	139.7
7	4.63, d (10.2)	78.4	4.56, d (10.2)	4.21, d (10.2)	79.4	79.5	4.33, d (10.4)	4.33, d (10.4)	79.4	80.7
8	2.0, m	36.3	2.3, m	2.3, m	33.8	33.8	2.44, m	2.52, m	32.6	32.9
9	1.78, m 1.02, m	43.4	1.79, m 1.04, m	1.84, m 1.06, m	43.2	43.1	1.86, m 0.96, m	1.86, m 0.96, m	43.1	43.1
10	1.69, m	33.7	1.72, m	1.72, m	32.3	31.9	1.88, m	1.88, m	32.4	32.5
11	2.92, d (12.0)	89.2	2.97, d (12.0)	2.87, d (12.0)	89.0	88.9	2.92, d (10.0)	2.94, d (10.0)	89.8	90.0
12	1.54, m	34.2	1.57, m	1.57, m	36.1	36.7	1.60, m	1.60, m	36.0	36.0
13	1.54, m 0.82, m	22.8	1.53, m 0.85, m	1.53, m 0.85, m	22.6	21.9	1.5, m 1.1, m	1.5, m 1.1, m	22.5	22.5

14	0.84, t (7.2)	13.0	0.84, t (6.6)	0.84, t (6.6)	12.7	12.4	0.87, t (6.6)	0.87, t (6.6)	12.9	12.8
15	0.68, d (6.6)	17.6	0.80, d (6.6)	0.80, d (6.6)	17.5	17.8	0.81, d (6.6)	0.81, d (6.6)	17.6	17.6
16	0.77, d (6.6)	17.7	0.75, d (6.6)	0.68, d (6.6)	17.3	17.6	0.64, d (6.4)	0.67, d (6.6)	17.7	17.8
17	0.89, d (6.6)	17.0	0.91, d (6.6)	0.91, d (6.6)	16.8	17.0	0.90, d (6.6)	0.90, d (6.6)	17.2	17.2
1′		126.6			133.8	133.8			133.0	133.0
2′, 6′	7.56, d (7.2)	128.9	7.43, d (7.2)	7.43, d (7.2)	128.6	128.5	7.48, d (6.8)	7.48, d (7.5)	129.0	128.9
3′, 5′	7.33, t (7.2)	128.3	7.39, t (7.2)	7.39, t (7.2)	128.4	128.5	7.42, t (6.8)	7.42, d (7.5)	128.9	128.7
4′	7.25, t (7.2)	127.8	7.34, t (7.2)	7.34, t (7.2)	127.6	127.5	7.39, t (7.3)	7.39, t (7.3)	129.8	129.8
OCH_3	4.01, s	66.8	4.08, s	4.08, s	64.5	64.5	3.36, s	3.37, s	61.7	62.1
OCH_3	4.0, s	64.4	4.01, s	3.34, s	61.5	61.2	4.16, s	4.14, s	60.6	60.4

[a]^{1}H NMR spectra recorded at 600 MHz, [b]^{13}C NMR spectra recorded at 100 MHz

Compounds **1-4** showed no antimicrobial, antifungal, antiprotozoal, phytotoxic, or cytotoxic activity in vitro. Compounds **5-7** showed moderate in vitro antiplasmodial activity (Table 4.3) but were cytotoxic to Vero cells. Their low selectivity indices (ratio of cytotoxicity vs. antiplasmodium activity) preclude them as antimalarial drug leads. Even though the plant pathogen *S. pistaciarum* is host-specific to pistachio, compounds **5-7** showed nonspecific moderate phytotoxic activity towards both bentgrass (*A. stolonifera*) and lettuce (*L. sativa* cv. L., Iceberg) in the presence of light (Table 4.4). General phytotoxicity of phytotoxins from host-specific pathogens is very common. The biosynthesis of cercosporin (**5**) appeared to be controlled by numerous environmental and physiological factors and their production has been linked to the pathogenicity of fungi.[100, 101] The possible mechanism of phytotoxic activity of this type of compounds has previously been attributed to their ability to generate reactive oxygen species in the presence of light.[100] This suggested that the selective inhibition of the plant-like metabolic pathways in the apicoplast of the malaria parasite[41] is not responsible for the observed antimalarial activity of compounds **5-7**. Compounds **5-7** also showed significant antileishmanial activity with IC_{50} values of 1.14 μM, 1.7 μM, and 3.1 μM, respectively (Table 4.5). Compound **5** was active against both methicillin-sensitive and methicillin-resistant *Staph. aureus* with IC_{50} values of 2.2 and 2.3 μM, respectively, and MIC values in the range of 4.7 – 9.4 μM (Table 4.6). This compound also showed weak activity against *M. intracellulare*. Compounds **6** and **7** exhibited weak activity against *C. glabrata*, *M. intracellulare*, and methicillin-sensitive and methicillin-resistant *Staph. aureus*.

Septoriamycin A (**8**), which demonstrated good antiplasmodial and antifungal activities,[96] exhibited significant antileishmanial activity against *Leishmania donovani* promastigotes with an IC_{50} of 0.11 μM and an IC_{90} of 0.29 μM (Table 4.5) and was more potent than the positive

controls pentamidine and amphotericin B. It also showed good activity against *Leishmania donovani* amastigotes with no toxicity to macrophages (Table 4.5).

The cytotoxic potential of compounds **5-7** was further evaluated against a panel of human solid tumor cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and pig kidney epithelial cells ($LLC\text{-}PK_{11}$) (Table 4.7). Moderate cytotoxicity was observed against all the cell lines. The di-*O*-methyl derivatives of septoriamycin A, **10a**, **10b** and **10c** were also evaluated and compound **10c** exhibited weak antibacterial and antiplasmodial activities whereas **10a** and **10b** were inactive in these assays.

Structural features suggest that septoriamycin A may act as a hydroxamic acid analogue. Hydroxamic acid derivatives are also known[25, 113] to target histone deacetylase of the parasites which cause malaria, schistosomiasis, trypanosomiasis, toxoplasmosis, and leishmaniasis. The apicoplast of the malaria parasite also possesses a family of class II histone deacetylase (HDAC) proteins which are also present in other photosynthetic eukaryotes and primitive algae.[22] This unusual enzyme (CpHDAC3) catalyzes the deacetylation of histone tails to promote epigenetic regulation and some cellular processes of the parasite.[22, 23] A number of hydroxamic acid analogues which inhibit histone deacetylase have been synthesized and their in vitro antiplasmodial and in vivo antimalarial activities have been evaluated.[114, 115] Some of these compounds have shown selective potent in vitro activity but low in vivo activity in a mouse and a monkey model due to metabolic instability.[115] Human and mouse liver microsomes rapidly converted the linear hydroxamic acids test compounds to carboxylic acids rendering them inactive.[115] Septoriamycin A (**8**), a cyclic hydroxamic acid was evaluated in a *P. berghei*-infected mouse model. At a dose of 100 mg/kg, the compound showed no toxicity and no

antimalarial activity (Table 4.8). This compound was also submitted for in vivo antileishmanial activity in a mouse model. The results have not yet been received.

4.4. Bioactivity Results

Table 4.3. Antiplasmodial activity of **5-7** and **10c**

Compound	Chloroquine-sensitive (D6)-clone		Chloroquine-resistant (W2)-clone		Cytotoxicity to Vero cells
	IC_{50} nM	S.I.	IC_{50} nM	S. I.	IC_{50} nM
5	1.08	4.8	1.62	3.2	5.24
6	2.78	1.9	3.12	1.7	5.21
7	2.75	1.6	1.94	2.3	4.53
10c	6.76	>1.1	6.51	>1.2	NC
chloroquine[a]	0.03		0.31		NC
artemisinin[a]	0.02		0.01		NC

[a] Positive controls

NC not cytotoxic

S. I. (selectivity index) = IC_{50} for cytotoxicity /IC_{50} for antiplasmodial activity

IC_{50}: concentration causing 50% growth inhibition

IC_{90}: concentration causing 90% growth inhibition

Table 4.4. Phytotoxic activity of **5-7**

Compound	Concentration (mM)	Lettuce	Bentgrass
5	1.87	3	2
6	1.73	2	4
7	1.62	3	4

Ranking based on scale of 0 to 5

0 = no effect

5 = no growth

Table 4.5. Antileishmanial activity of **5-8** in μM

	L.donovani promastigotes		Macrophage amastigotes		Cytotoxicity (Transformed THP1 cells)		
Compound	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	Selectivity
5	1.14	2.81	NT	NT	NT	NT	NT
6	1.7	8.5	NT	NT	NT	NT	NT
7	3.1	9.7	NT	NT	NT	NT	NT
8	0.11	0.29	1.18	1.48	>26.9	>26.9	22.79
pentamidine[a]	2.9	5.58	8.72	20.18	>58.75	>58.75	6.73
amphotericin B[a]	0.18	0.38	0.15	1.88	3.62	8.72	24.13

[a] Positive controls

IC_{50} concentration causing 50% growth inhibition

IC_{90} concentration causing 90% growth inhibition

Selectivity = IC_{50} for cytotoxicity /IC_{50} for macrophage amastigotes activity

NT = Not tested

Table 4.6: Antifungal and antibacterial activity of **5 -7** and **10c**

Compound	*Candida glabrata*		*Staphylococcus aureus*		MRSA		*Mycobacterium intracellulare*	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC50	MIC
5	NA	NA	2.2	4.7	2.3	9.4	3.5	NA
6	4.1	NA	6.9	17.3	5.9	34.7	7.1	NA
7	7.9	NA	20.0	NA	26.4	NA	8.7	NA
10c	NA	NA	23.1	NT	26.8	NT	NA	NA
amphotericin B[a]	0.45	2.70	NT	NT	NT	NT	NT	NT
ciprofloxacin[a]	NT	NT	0.27	1.17	0.27	1.11	0.72	1.5

[a] Positive controls

IC_{50} and MIC (concentration causing 50% growth inhibition and the lowest test concentration that allows no growth) values are in μM

NA: not active

NT: not tested

Table 4.7. Cytotoxic activity [IC_{50} (μM)] of **5-7**

Compound	SK-MEL	KB	BT-549	SK-OV-3	LLC-PK_{11}
5	3.6	7.1	10.8	3.7	3.9
6	3.8	8.5	8.6	4.0	10.1
7	4.9	8.7	8.7	4.8	9.4
doxorubicin[a]	1.6	2.6	2.6	1.5	1.6

SK-MEL = human malignant melanoma

KB = human epidermal carcinoma

BT-549 = human breast carcinoma (ductal)

SK-OV-3 = human ovary carcinoma

LLC-PK_{11} = pig kidney epithelial

Table 4.8. In vivo antimalarial activity of **8**

Treatment (PO)	Dose (mg/kg × # days post infection)	% Parasitemia suppression[1]		Survival[2]	Day of Death	MST[3]	Cure[4]
		Day 5	Day 7				
vehicle	× 3	-	-	0/5	17/19/17/19/17	17.8	0/5
chloroquine[a]	100 × 3	100	100	5/5	28/28/28/28/28	28	0/5
8	100 × 3	37.18	40.37	0/5	17/17/17/17/12	16	0/5

[1]% Suppression in parasitemia is calculated by considering the mean parasitemia in the vehical control as 100%.

Parasitemia suppression < 80% is considered as non-significant

[2]Number of animals that survived day 28/total animals in group (the day of the death-post infection)

[3]% MST – mean survival time (days)

[4]Number of mice without parasitemia (cured) till day 28 post-infection

4.5. Experimental

4.5.1. General

Optical rotations were obtained using a Rudolph Research Analytical Autopol IV automatic polarimeter model 589-546. Melting points were measured with a Uni-melt, Thomas Hoover capillary melting point apparatus. UV and IR spectra were determined on a Varian-50 Bio UV visible spectrophotometer and a Bruker-Tensor-27 infrared spectrophotometer, respectively. NMR spectra were recorded on a Varian-Mercury-plus-400 or Varian Unity-Inova-600 spectrometer using $CDCl_3$ and methanol-d_4 unless otherwise stated. MS data were obtained from an Agilent Series 1100 SL equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA). Column chromatography and preparative TLC were carried out using Merck silica gel 60 (230-400 mesh) and silica gel GF plates (20 x 20 cm, thickness 0.25 mm), respectively. HPLC analysis was conducted on an Hewlett-Packard Agilent 1100 with diode array detector.

4.5.2. Fermentation, Extraction, and Isolation

The plant pathogenic fungus, *Septoria pistaciarum* Caracc. (ATCC 22201) was obtained from the American Type Culture Collection, Manassas, VA and it was grown as previously reported.[2]

The oily extract (3.3 g) was chromatographed over Sephadex LH -20 and eluted with 80% MeOH in $CHCl_3$ to give 16 fractions. Fractions 2 -11 which showed antiplasmodial activity and were combined and chromatographed over a reversed phase C_{18} column eluting with a gradient of 10 to 100% MeOH-H_2O to yield eight fractions. Subfractions 2, 3, and 4 were combined and separated on Sephadex LH-20 (MeOH) to give compounds **1** (5.0 mg) and **8** (35 mg). Subfraction 5 was chromatographed over a silica gel column using CH_2Cl_2-hexanes as the eluent to give compound **2** (8.0 mg)**.** Combined fractions 6, 7, and 8 were chromatographed over a silica gel column using CH_2Cl_2-hexanes as the eluent to give 10 subfractions. Subfraction 2, 3, and 5 were combined and further separated using a C_{18} reversed phase HPLC column eluting with MeCN:H_2O, (1:1), flow rate 3.5 mL/min, to give compounds **3** (3.5 mg) and **4** (8.0 mg). Subfractions 6, 7, and 8 were combined and purified on Sephadex LH-20 (MeOH) to give compounds **5** (14.0 mg), **6** (12.0 mg), and lumichrome (3.0 mg). Subfractions 9 and 10 were combined and chromatographed over a silica gel column using CH_2Cl_2-hexanes as the eluent to give compound **7** (10.0 mg) and brassicasterol (8.0 mg)**.**

Compound 1**:** amorphous powder: $[\alpha]^{26}_{D}$ +112 (*c* 0.05, MeOH); UV (MeOH); λ_{max} (log ε) 205.0 (3.56), 240.9 (3.51), 297.0 (2.79) nm; IR ($CHCl_3$) ν_{max} 3400, 3207, 2967, 2929, 1647,

1555, 1454, 1049 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 1); HRESIMS $[M - H]^+$ *m/z* 400.2118 (calcd for $[C_{23}H_{31}NO_5 - H]^+$, 400.2124) .

***Compound 2*:** amorphous powder: $[\alpha]^{26}_D$ +101 (*c* 0.05, MeOH); UV (MeOH); UV (MeOH) λ_{max} (log ε) 204.0 (3.44), 240.0 (3.25), 295.9 (2.56) nm; IR ($CHCl_3$) ν_{max} 3331, 2967, 2925, 1740, 1651, 1230, 1045 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 1); HRESIMS $[M + H]^+$ *m/z* 444.2370 (calcd for $[C_{25}H_{33}NO_6 + H]^+$, 444.2386).

***Compound 3*:** amorphous powder: $[\alpha]^{26}_D$ -38 (*c* 0.43, CH_3OH); UV (MeOH); λ_{max} (log ε) 208.2 (3.94), 241.9 (3.92), 297.0 (3.19) nm; IR ($CHCl_3$) ν_{max} 3405, 3201, 2965, 2929, 1644, 1551, 1455, 1219, 755 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 1); HRESIMS $[M + H + Na]^+$ *m/z* 425.2172 (calcd for $[C_{23}H_{31}NO_5 + H + Na]^+$, 425.2178).

***Compound 4*:** amorphous powder: $[\alpha]^{26}_D$ +130 (*c* 0.2, MeOH); UV (MeOH); λ_{max} (log ε) 207.1 (3.01), 241.0 (2.95), 295.1 (2.10) nm; IR ($CHCl_3$) ν_{max} 3422, 3159, 2926, 1641, 1541, 1456 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 1); HRESIMS $[M + H + Na]^+$ *m/z* 425.2174 (calcd for $[C_{23}H_{31}NO_5 + H + Na]^+$, 425.2178).

Methylation of Septoriamycin A (8) with MeI:

A mixture of MeI (4 mL), Cs_2CO_3 (10.0 mg), and compound **8** (10.0 mg) in acetone was stirred at room temperature for 5 h. The reaction mixture was filtered, and the solvent was

evaporated. The product was dissolved in CH_2Cl_2 and passed through a plug of florisil to give *N*-(*O*-methyl)septoriamycin **A** (**9**)**.**

Methylation of Septoriamycin A (8) with Diazomethane:

A solution of **8** (60 mg) in MeOH was treated with excess diazomethane in Et_2O at 0 ^oC for 2 h. The solvent was evaporated and the mixture was separated by PTLC (40% EtOAc in hexanes) to yield compounds **10a** (8 mg), **10b**, and **10c**. Compounds **10b** and **10c** were further purified by HPLC using a reversed phase Luna C_{18} column (1 × 25 cm) with MeOH: H_2O (92:8) as the mobile phase at a flow rate of 4 mL/min, to give compounds **10b** (5 mg) and **10c** (6.0 mg).

Compound 10a**:** amorphous powder: $[\alpha]^{26}_D$ +14 (*c* 0.5, MeOH); UV (MeOH); λ_{max} (log ε) 205.0 (3.75), 232.0 (3.68), 278.9 (3.41) nm; IR ($CHCl_3$) ν_{max} 2959, 2929, 2873, 1627, 1547, 1469, 1046 cm^{-1}; ^1H and ^{13}C NMR data (see Table 2); HRESIMS $[M + Na]^+$ *m/z* 422.2307 (calcd for $[C_{24}H_{33}NO_4 + Na]^+$, 422.2307).

Compound 10b**:** amorphous powder: UV (MeOH) λ_{max} (log ε) 204.0 (3.39), 235.0 (3.18), 308.0 (2.65) nm; IR ($CHCl_3$) ν_{max} 2958, 2929, 1740, 1656, 1524, 1457 cm^{-1}; ^1H and ^{13}C NMR data (see Table 2); HRESIMS $[M +H]^+$ *m/z* 400.2472 (calcd for $[C_{24}H_{33}NO_4 + H]^+$, 400.2487).

***Compound 10c*:** amorphous powder: UV (MeOH) λ_{max} (log ε) 207.0 (2.58), 240.9 (2.47), 307.1 (1.70) nm; IR ($CHCl_3$) ν_{max} 2900, 2850, 1575, 1450 cm^{-1}; ^1H and ^{13}C NMR data (see Table 2); HRESIMS $[M + Na]^+$ *m/z* 422.2279 (calcd for $[C_{24}H_{33}NO_4 + Na]^+$, 422.2307).

***Compound 5*:** Red crystals (MeOH): mp 239 ^0C (lit.[110] 240-241.5 ^0C); UV (MeOH) λ_{max} (log ε) 210.0 (3.71), 221.0 (3.72), 267.0 (3.55) , 470.0 (3.42) , 563.0 (2.98) nm; IR ($CHCl_3$) ν_{max} 3396, 2924, 1616, 1267 cm^{-1} ^1H and ^{13}C NMR and CD data were consistent with those reported.[98, 110, 112]

***Compound 6*:** Red crystals (MeOH): mp 135 ^0C (lit.[99] 134 ^0C); UV (MeOH) λ_{max} (log ε) 222.0 (3.9), 269.0(3.74) , 471.0 (3.62) , 563.0 (3.2) nm; IR ($CHCl_3$) ν_{max} 3338, 1735, 1616, 1266 cm^{-1} ^1H and ^{13}C NMR and CD data data were consistent with those reported.[98, 99]

***Compound 7*:** Red amorphous powder: UV (MeOH) λ_{max} (log ε) 222.0 (3.9), 270.0 (3.84) , 470.1 (4.0) , 563.3 (3.26) nm; IR ($CHCl_3$) ν_{max} 2921, 1736, 1617, 1211 cm^{-1} ^1H and ^{13}C NMR and CD data were consistent with those reported.[99]

4.5.3. Biological Assay

4.5.3.1. Antiplasmodial Assay

The antiplasmodial activity was determined against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *Plasmodium falciparum* in an in vitro assay as described earlier.[69] Artemisinin and chloroquine were included as the drug controls and IC_{50} values were computed from the dose response curves using Microsoft Excel software.

4.5.3.2. Phytotoxicity Assay

The bioassay for phytotoxicity was carried out according to the procedure described by Dayan et al.[70] using bentgrass (*Agrostis stolonifera*) and lettuce (*Lactuca sativa* cv. L., Iceberg), in 24-well plates.

4.5.3.3. Antileishmanial Assay

The in vitro antileishmanial activity of the compounds was carried out on a culture of *Leishmania donovani* promastigotes.[74] Pentamidine and amphotericin B were used as standard antileishmanial agents. The IC_{50} values for each compound were computed from the growth inhibition curve using Microsoft Excel software.

4.5.3.4. Macrophage Amastigote Assay

A recently developed promastigote rescue assay by Jain et al. was used.[75]

4.5.3.5. Antimicrobial Assay

All organisms were obtained from the American Type Culture Collection (Manassas, VA) and included the fungi *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906, and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRSA), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI methods[71,72] as described[73] by Samoylenko et al. The drug controls ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi were included in each assay.

4.5.3.6. Cytotoxicity Assay for Mammalian Cells

In vitro cytotoxicity was determined against a panel of mammalian cells that included kidney fibroblast (Vero), kidney epithelial (LLC-PK_{11}), malignant melanoma (SK-MEL), oral epidermal carcinoma (KB), breast ductal carcinoma (BT-549), and ovary carcinoma (SK-OV-3) cells as described earlier.[76] The number of viable cells was determined by using Neutral Red dye and IC_{50} values were obtained from dose response curves. Doxorubicin was used as a positive control.

4.5.3.7. In Vivo Antimalarial Assay

The in vivo antimalarial activity was determined in mice infected with *P. berghei* (NK-65 strain), originally obtained from the Walter Read Army Institute of Research, Silver Spring, MD. Male mice (Swiss Webster strain) weighing 18-20 g were intraperitoneally inoculated with 2 x 10^7 parasitized red blood cells obtained from a highly infected donor mouse. Mice were divided into different groups with at least six mice in each group. Compounds stocks were prepared in DMSO. The mice infected with *P. berghei* were orally administered 100 μL of the compound. The animals were closely observed for at least 2 h after every dose for any apparent signs of toxicity. Blood smears were prepared on different days starting from 5 days post infection (through 28 days) by clipping the tail end, stained with Giemsa, and the slides were observed under microscope for determination of parasitemia. Mice without parasitemia through day 28 postinfection were considered cured.

CHAPTER 5

PART-1: ANTIMALARIAL COMPOUNDS FROM SEPTORIA MUSIVA

5.1.1. Summary

An EtOAc extract of the culture medium of *Septoria musiva* showed antiplasmodial, antimicrobial, antileismanial, and phytotoxic activities. Bioassay-guided fractionation of this extract led to the isolation of septoriamycin A (**1**) and (+)-cercosporin (**2**) as the compounds responsible for antiplasmodial, antimicrobial, and antileishmanial activities. (+)-Cercosporin showed phytotoxic activity whereas both compounds exhibited moderate cytotoxic activity against mammalian cell lines.

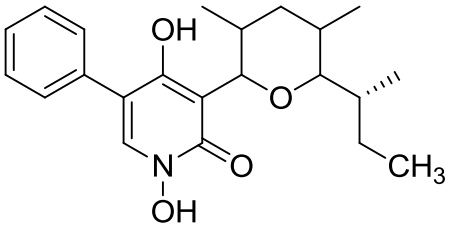
5.1.2. Introduction

Septoria musiva is the causal agent of stem canker and leaf spot disease leading to defoliation in Populus sp (poplar tree). Chemical or biological investigation of *S. musiva* has not

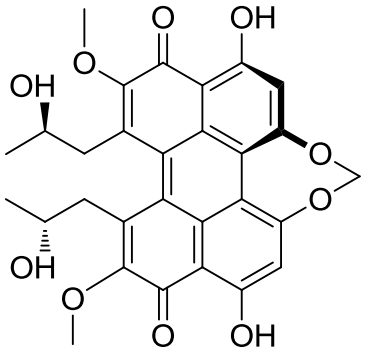
been reported. An EtOAc extract of the culture medium *S. musiva* obtained from the American Type Culture Collection (ATCC 52800) showed antiplasmodial, phytotoxic, antimicrobial, and antileishmanial activities. This extract also exhibited moderate cytotoxicity towards Vero cells. Bioassay-guided fractionation of the EtOAc extract led to the isolation of septoriamycin A (**1**) and (+)-cercosporin (**2**) as the active constituents.

5.1.3. Results and Discussion

S. musiva was obtained from the American Type Culture Collection (ATCC 52800) and grown in potato dextrose agar (PDA) plates. Although the fermentation was carried out in several culture media such as PDB, Czapex Dox, V-8 (100% vegetable juice), and solid rice, only Czapex Dox medium afforded an extract with the antiplasmodial activity [720 ng/mL for chloroquine-sensitive (D6) and 570 ng/mL for chloroquine-resistant (W2) strains]. Chromatographic separation of this extract yielded septoriamycin A (**1**) and (+)-cercosporin (**2**) as the active compounds (Figure 5.1). These compounds showed identical physical, spectroscopic data, and biological activity profiles with those of the same compounds isolated from *S. pistaciarum*.[96, 117]



septoriamycin A (**1**)



(+)-cercosporin (**2**)

Figure 5.1.1. Structure of compounds **1** and **2**

5.1.4. Experimental

5.1.4.1. General

Optical rotations were measured using a Rudolph Research Analytical Autopol IV automatic polarimeter model 589-546. NMR spectra were recorded on a Varian-Mercury-plus-400 using $CDCl_3$/methanol-d_4 as the solvent. MS analyses were performed on an Agilent Series 1100 SL equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA). Column chromatography was carried out on Merck silica gel 60 (230-400 mesh) and reversed phase C_{18}. Preparative TLC was carried out using silica gel GF plates (20 X 20 cm, thickness 0.25 mm).

5.1.4.2. Fermentation, Extraction, and Isolation

The fungus, *S. musiva* (ATCC 52800) was obtained from the American Type Culture Collection (Manassaas, VA, USA) and its fermentation was carried out as previously described.[96]

The EtOAc extract (9.0 g) was chromatographed over a silica gel column using hexanes-CH_2Cl_2-MeOH gradients as mobile phases to give 20 fractions. Fractions 12 and 13 were combined and chromatographed over a reversed phase C_{18} column eluting with a gradient of 10 to 100% MeCN-H_2O to yield nine fractions. Subfraction 4 was recrystallized with MeOH to obtain septoriamycin A (**1,** 100 mg) as the major active constituents. Fractions 18-20 from the original extract were combined and purified on Sephadex LH-20 (MeOH) to give (+)-cercosporin (**2,** 10 mg).

Compound 1 **:** white crystals (MeOH); mp 220 ºC; $[\alpha]^{26}_{D}$ +201 (*c*, 0.4, CH_3OH) mp and $[\alpha]^{26}_{D}$ were consistent with reported[96]; ^{1}H and ^{13}C NMR data[96] (see Chapter 3); HRESIMS $[M - H]^-$ *m/z* 370.2012 (calcd for $(C_{22}H_{29}NO_4 - H)^-$, 370.2018).

Compound 2 **:** Red crystals (MeOH); mp 239 ºC (lit.[110] 240-241.5 ºC); ^{1}H and ^{13}C NMR (see Chapter 4) were consistent with reported.[98, 112] HRESIMS $[M + H]^+$ *m/z* 535.1651 (lit.[112] $[C_{24}H_{33}NO_4 + H]^+$ 535.1604).

5.1.4.3. Biological Assays

Antiplasmodial,[69] phytotoxic,[70] antimicrobial[71],[72] and cytotoxic[76] assays were carried out as described.

PART 11: MALFORMIN A_1 AN ANTIMALARIAL COMPOUND FROM *PHOMOPSIS VITICOLA* (ASCOMYCOTA)

5.2.1. Summary

Malformin A_1 was isolated as the active compound from an extract of *Phomopsis viticola* with antiplasmodial and phytotoxic activities. Even though this compound was not cytotoxic to Vero cells, it showed potent toxicity to a panel of mammalian cancer cell lines precluding it as a potential antimalarial drug lead.

5.2.2. Introduction

P. viticola (Ascomycetes), the agent responsible for Phomopsis cane and leaf spot disease in grapevines, causes serious yield loss. A variety of biologically active secondary metabolites have been reported[119-124] from this genus.

An EtOAc extract of *P.viticola* (ATCC 48153) in Czapex Dox medium showed potent herbicidal and antiplasmodial activities with good selectivity. Bioassay-guided fractionation of this extract led to the isolation of malformin A_1 (**3**) as the active compound (Figure 6.2.1).

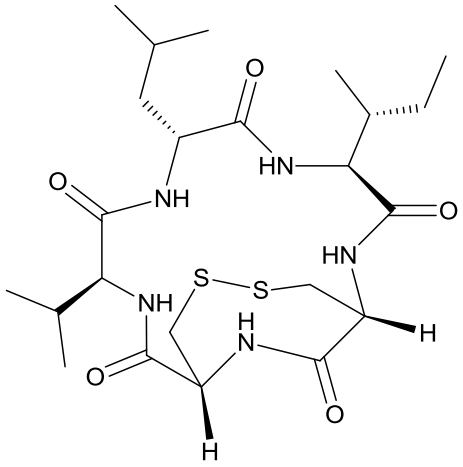


Figure 5.2.1. Structure of malformin A_1 (**3**)

5.2.3. Results and Discussion

P.viticola, (ATCC 48153) was grown in PDB, V8, solid rice, and Czapex Dox culture media. Of those, an EtOAc extract of Czapex Dox culture broth showed potent herbicidal and antiplasmodial activity with good selectivity index (Table 2.1). Bioassay-guided fractionation of this extract by repeated column chromatography furnished malformin A_1 (**3)** as the active compound. The structure of this compound was confirmed by spectroscopic methods.[125] Malformin A_1, a cyclic pentapeptide (cyclo-D-Cys-D-Cys-L-Val-D-Leu-L-Ile) with a disulfide bond between two cysteine moieties, has been isolated from plant pathogenic *Aspergillus* species.[125, 126] The phytotoxic activity of this compound has extensively been studied.[125, 127] Antiplasmodial and antitrypanosomal activities, and structure activity relationships of several synthetic malformin analogues have been reported.[128] Based on these results, the presence of a disulfide bond is required for the biological activity of these compounds.[128]

Malformin A_1 (**3**) showed potent antimalarial activity with IC_{50} values in the range of 0.05-0.07 nM for W2 and D6 strains (Table 5.2.1) and strong phytotoxic (Table 5.2.3) activity. Although this compound exhibited no toxicity to Vero cells at the highest concentrations tested (4760 ng/mL), previous studies have shown it to be toxic to a panel of cancer cell lines.[129] Evaluation of activity of malformin A_1 (**3**) against a panel of human cancer cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and mammalian kidney epithelial cells (LLC-PK_{11}) showed significant cytotoxicity to all the tested cell lines with IC_{50} values in the range of 2-4 μM except for KB cells (9.5μM). Malformin A_1 has shown high intraperitonial toxicity in mice (LD_{50} 3.1 mg/kg); however, it had no oral toxicity at doses ≤ 50 mg/kg.[126] This lack of oral toxicity may be due to poor oral bioavailability of peptides.[130]

5.2.4. Bioactivity Results

Table 5.2.1. Antimalarial activity of **3**

Compound	Chloroquine-sensitive (D6)-clone		Chloroquine-resistant (W2)-clone		Cytotoxicity to Vero cells
	IC_{50} nM	S.I.	IC_{50} nM	S. I.	IC_{50} nM
malformin A_1(**3**)	0.07	>128.2	0.05	>178.0	NC
chloroquine[a]	0.07		0.88		NC
artimisinin[a]	0.06		0.04		NC

[a] Positive controls

NC not cytotoxic

S. I. (selectivity index) = IC_{50} for cytotoxicity /IC_{50} for antiplasmodial activity

IC_{50}: concentration causing 50% growth inhibition

Table 5.2.2. Cytotoxic activity [IC_{50} (μM)] of **3**

Compound	SK-MEL	KB	BT-549	SK-OV-3	LLC-PK_{11}
malformin A_1	2.83	9.45	3.12	4.91	3.11
doxorubicin[a]	1.65	1.65	1.51	3.49	1.13

[a] Positive control.

IC_{50} = concentration causing 50% growth inhibition

SK-MEL = human malignant melanoma

KB = human epidermal carcinoma

BT-549 = human breast carcinoma (ductal)

SK-OV-3 = human ovary carcinoma

LLC-PK_{11} = pig kidney epithelial

Table 5.2.3. Phytotoxic activity of **3**

Compound	Concentration (mM)	Lettuce	Bentgrass
Malformin A_1	1.89	5	5
	0.18	3	4
	0.018	1	1

Ranking based on scale of 0 to 5

0 = no effect, 5 = no growth

5.2.5. Experimental

5.2.5.1. General

Melting points were measured with a Uni-melt, Thomas Hoover capillary melting point apparatus. Optical rotations were measured using a Rudolph Research Analytical Autopol IV automatic polarimeter model 589-546. NMR spectra were recorded on a Varian-Mercury-plus-400 or Varian Unity-Inova-600 spectrometer using pyridine-d_4 as the solvent unless otherwise stated. MS analyses were performed on an Agilent Series 1100 SL equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA). Column chromatography was carried out on Merck silica gel 60 (230-400 mesh) and reversed-phase C_{18} silica gel. Preparative TLC was carried out using silica gel GF plates (20 X 20 cm, thickness 0.25 mm).

5.2.5.2. Fermentation, Extraction, and Isolation

P. viticola (ATCC 48153) was obtained from the American Type Culture Collection (Manassaas, VA, USA) and grown in PDA plates at 29 ^{o}C for 14 days. A flask (1L) with100 mL of PDB was inoculated with agar pieces (0.25 in × 0.25 in) and incubated at 29 ºC for 8 days on a shaker (RPM = 100). Erlenmeyer flasks (38 × 1L) each containing 500 mL of M-2 medium, (modified Czapex Dox medium : Czapex Dox 35.0 g, yeast extract 1.5 g, malt extract 1.5 g in 1L of distilled H_2O) were inoculated with this seed culture and incubated at 29 ºC for 28 days on a shaker (RPM = 100). The broth was separated by filtration and extracted with EtOAc (×3). The organic layer was evaporated under reduced pressure to give an oily residue (3.0 g).

The oily extract (2.9 g) was chromatographed over Sephadex LH -20 and eluted with 100% MeOH to give 12 fractions. Fractions 4 and 5 which showed antiplasmodial activity (≤ 528 ng/ml) were combined and chromatographed over a reversed-phase C_{18} column eluting with a gradient of 10 to 100% MeCN-H_2O to yield malformin A_1 (7.0 mg).

Compound 3 **:** white amorphous powder (MeOH); mp 303 ^{0}C (lit.[110] > 300 ^{o}C); $[\alpha]^{26}_{D}$ -28 (*c*, 0.5, CH_3OH) ($[\alpha]^{26}_{D}$ -39 lit.[128]); ^{1}H and ^{13}C NMR data were consistent with reported[128] values; HRESIMS $[M + H]^{+}$ *m/z* 530.2418 (lit.[128] $[C_{24}H_{33}NO_4 + H]^{+}$ 530.2470)

5.2.5.3. Biological Assays

Antiplasmodial,[69] phytotoxic,[70] and cytotoxic[76] assays were carried out using the procedures as previously described.

CHAPTER 6: PHYTOTOXIC COMPOUNDS FROM THE WOOD ROTTING FUNGUS, *POSTIA BALSAMEA* (BASIDIOMYCETES)

6.1. Summary

Postia balsamea is one of the causal agents of root rot and butt rot in balsam fir (*Abies balsamea*, Pinaceae). Preliminary screening of the EtOAc extract of a fermentation broth of *P. balsamea* showed antiplasmodial and phytotoxic activities. Bioassay-guided fractionation of the EtOAc extract led to the isolation of four polyacetylenes, (2*E*,8*E*)-matricaria ester (**1**), (2*E*,8*E*)-matricarianol (**2**), (2*E*,8*E*)-deca-2-8-diene-4,6-diyne-1,10-diol (**3**), and (3*S*,8*E*)-methyl 3-hydroxydeca-8-en-4,6-diynoate (**4**); one phenolic acid, (*S*)-3-(3,4-dihydroxyphenyl)lactic acid (**5**); three halogenated phenolic acid analogues, (*S*)-3-(3,5-dichloro-4-hydroxyphenyl)lactic acid (**6**), (*S*)-Methyl 3-(3,5-dichloro-4-methoxyphenyl)lactate (**7**), and (*S*)-methyl 3-(3,5-dichloro-4-methoxyphenyl)lactate (**8**); and four triterpenes, eboricoic acid (**9**), acetyleboricoic acid (**10**), sulfurenic acid (**11**), dehydrosulfurenic acid (**12**), and a mixture of minor compounds which showed good antiplasmodial activity. The structures of the pure compounds were determined by

spectroscopic and spectrometric methods. Of these compounds, compounds **4** and **8** are new, and compounds **6** and **7** have been synthesized, but this is the first report of their isolation from a natural source. The conjugated polyacetylenes and halogenated phenolic compounds showed strong phytotoxic activity against monocots (*Agrostis stolonifera* cv. Pencross) and dicots (*Lactuca sativa*).

6.2. Introduction

Postia Fr (Aphyllophorales, Basidiomycota), a widespread genus of brown rot fungi in North America, Europe, and China, has variable morphological characteristics and a wide range of tree hosts including both hardwoods and conifers. [131-133] Species of this genus are also known by several synonyms because of updates in nomenclature, among them *Oligoporous, Postia, Rhodonia, Spongipellis, Tyromyces, Polyporous, Polysticus,* and *Poria*. [131-133]

P. balsamea, an agent responsible for root rot and butt rot in balsam fir (*Abies balsamea,* family Pinaceae), causes considerable economic damage. Balsam fir which grows in the northern United States and Canada is extensively used for pulp and lumber and is also one of the most popular Christmas trees. *P. balsamea* can enter roots and the basal area of trees through wounds caused by insects or mechanical means and attack functional vascular and cambium tissues most likely by releasing phytotoxins first and then depolymerizing enzymes to hydrolyze cellulose and hemicelluloses.[40, 135] Infected trees become susceptible to secondary attacks by insects and wind damage.[135] Thus far, no work has been reported on the chemical constituents of genus under the

named "*Postia*", although there is a possibility that *Postia* has been identified under another synonym.

Preliminary screening of the EtOAc extract of the fermentation broth of *P. balsamea* showed potent antiplasmodial and phytotoxic activities. Bioassay-guided fractionation of this extract afforded a fraction with potent activity and good selectivity. Although the EtOAc extracts of the subsequent large-scale fermentation broths showed potent phytotoxic activity against monocots (*Agrostis stolonifera* cv. Pencross) and dicots (*Lactuca sativa*), they were devoid of antiplasmodial activity. Thin layer chromatographic analysis of the active fraction showed two major and four minor spots. By comparison with the inactive fractions all but one could be eliminated as the possible active constituent (Figure 6.1). Compounds in *P. balsamea* broth belong to three different chemical classes: C-10 polyacetylenes, phenyllactic acid analogues, and lanostane triterpenes. Spectroscopic analysis indicated that constituents only present in the active fraction by TLC analysis did not to belong to any of these classes.

6.3. Results and Discussion

Preliminary small-scale culture of *P. balsamea* (ATTC 64899) in PD broth produced an extract with high antiplasmodial activity but no cytotoxicity. Although the EtOAc extracts of the large scale fermentation broth showed TLC profiles similar to the original extract, it was devoid of antiplasmodial activity. Re-culture of the fungus with the same and different culture media [Czapex dox, V-8 (100% vegetable juice), and rice] and a new sample of the fungus from the ATCC, cultured under original conditions, failed to produce an extract with antiplasmodial

activity. Attempts were also made to produce active metabolites by inoculating the culture medium with the bark and leaf parts of *Abies balsamea* as elicitors. Fractionation of the original active extract yielded a fraction (15 mg) with potent selective activity. TLC analysis revealed a spot in the active fraction which was only present in that fraction (Figure 6.1).

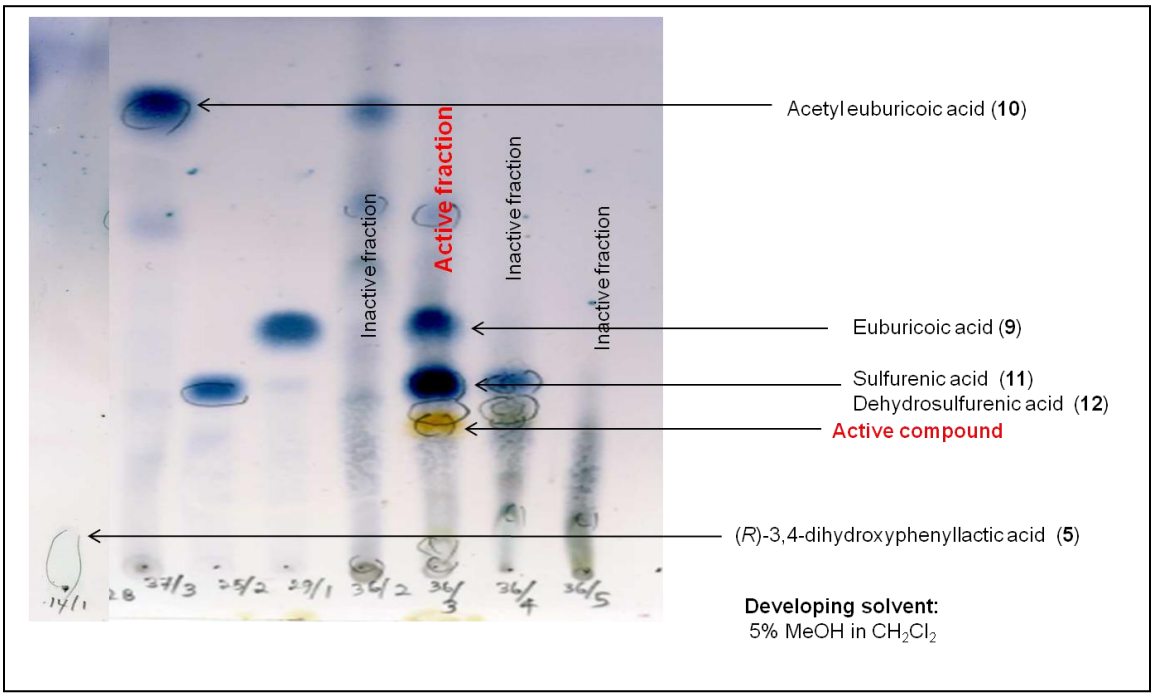


Figure 6.1. TLC comparison of antimalarial active fraction with inactive fractions and compounds

Since the compound or compounds corresponding to this spot could be derivatives of the other compounds present in the extracts, all constituents in the extracts were isolated and identified to facilitate the identification of the active compounds.

EtOAc extracts of broth and mycelia of *P. balsamea* were fractionated by repeated silica gel column chromatography, and the compounds were purified by PTLC to afford polyacetylenes (**1**-**4**), four phenolic compounds (**5**–**8**), and four triterpene acids (**9**-**12**) as shown in Figure 6.2. Compounds **4** and **8** are new, and compounds **6** and **7** have previously been synthesized.[136] The known compounds, (2*E*,8*E*)-matricaria ester (**1**),[137] (2*E*,8*E*)-matricarianol (**2**),[137] (2*E*,8*E*)-deca-2-8-diene-4,6-diyne-1,10-diol (**3**),[137, 138] (*S*)-3-(3,4-dihydroxyphenyl)lactic acid (**5**),[139] (*S*)-3-(3,5-dichloro-4-hydroxyphenyl)lactic acid (**6**),[136] (*S*)-methyl 3-(3,5-dichloro-4-methoxyphenyl)lactic acid (**7**),[136] eboricoic acid (**9**), [140] acetyl eboricoic acid (**10**),[140] sulfurenic acid (**11**),[141] and dehydrosulfurenic acid (**12**)[141] were identified by comparing observed and published spectroscopic data.

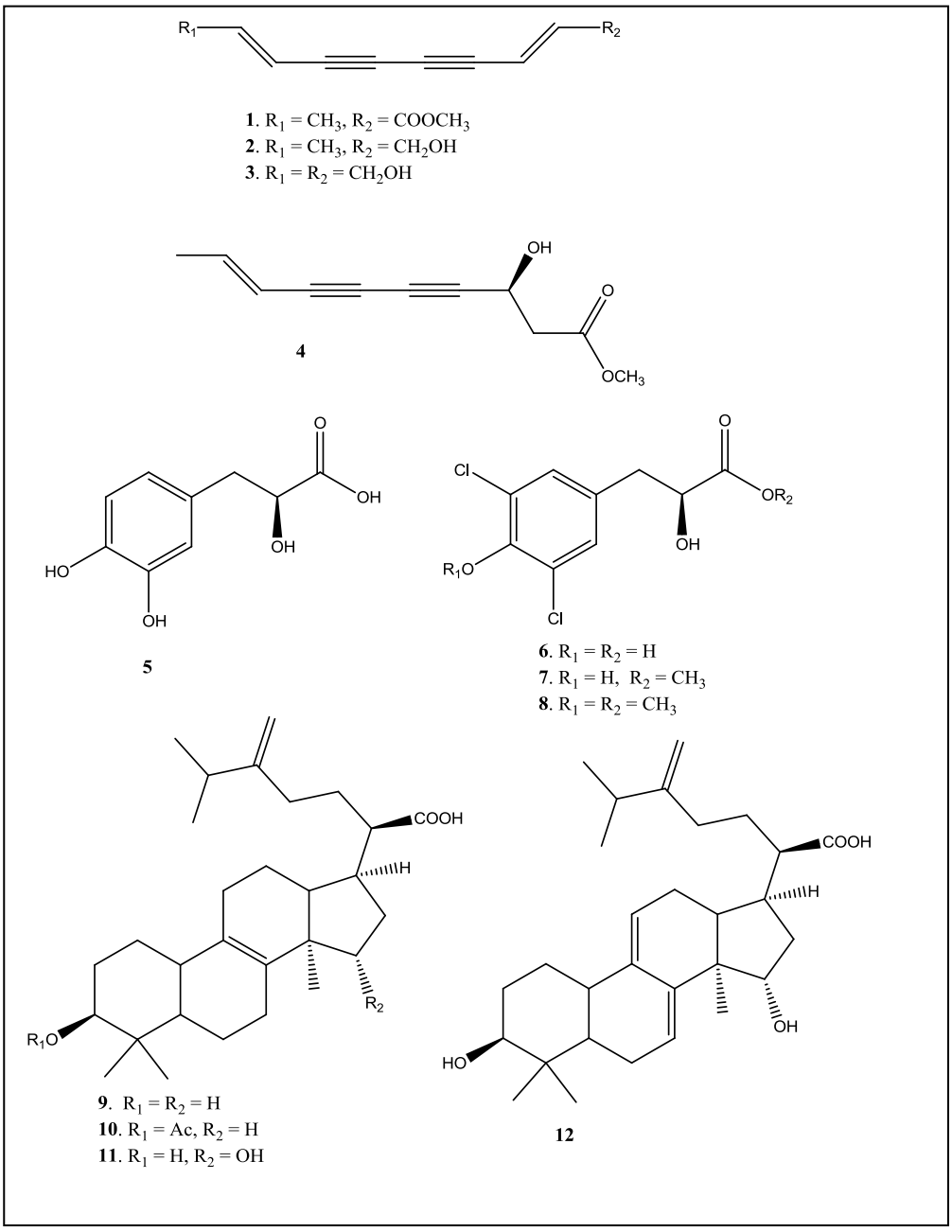


Figure 6.2. Structure of compounds isolated from *P. balsamea*

Compound **4** was obtained as an amorphous powder. The IR spectrum of **4** showed absorption at 3435 cm^{-1} due to a hydroxy group and at 1728 cm^{-1} due to a carbonyl group. The UV spectrum showed characteristic absorptions at 206, 228, 253, 268, and 284 nm, indicating the presence of an ene-diyne chromophore.[142] The ^{1}H NMR spectrum (Figure 6.3) showed spin systems for a methyl-substituted *trans*-olefinic bond [δ_H (1H) 6.34 dq J = 16.0, 7.2 Hz, δ_H 5.52 (1H) brd J = 16.0 Hz, and δ_H 1.82 (CH_3) dd 7.2, 1.6 Hz], similar to that observed for compound **1,**[137] a hydroxymethine adjacent to a methylene [δ_H (1H) 4.85 t J = 6.0 Hz, δ_H (2H) 2.76 d, J = 6.0 Hz], and a methoxy group (δ_H 3.76). The ^{13}C NMR spectrum (Figure 6.4) of **4** showed 11 resonances indicating four acetylenic carbons (δ_C 80.1, 78.4, 71.5, and 70.2), two olefinic carbons (δ_C 109.6, 144.7), one oxymethine carbon (δ_C 59.4), one methoxy carbon (δ_C 52.3), one ester carbonyl carbon (δ_C 171.6), and one methylene carbon (δ_C 41.6). In the HMBC spectrum (Figure 6.5), H-9 showed correlations with the methyl group, C-10, and C-7 (δ_C 78.4). The -CH_3 protons (δ_H 1.80) showed correlations with C-9 (δ_C 144.7), C-8 (δ_C 109.6), C-7 (δ_C 78.4), and C-6 (δ_C 71.5). The oxymethine proton at C-3 showed long range correlations to the carbonyl group (δ_C 171.6), C-4 (δ_C 80.1), C-5 (δ_C 70.2), and C-2 (δ_C 41.6); whereas, the methylene doublet at δ_H 2.76 correlated with the carbonyl (δ_C 171.6), C-3 (δ_C 59.4), and C-4 (δ_C 80.1). The methoxy group (δ_H 3.76) had an HMBC correlation with the carbonyl group. These correlations, as indicated in Figure 6.6, supported the proposed structure as methyl 3-hydroxydeca-8-en-4,6-diynoate (**4**). The absolute configuration at C-3 was determined based on specific rotation. The reported specific rotations for (3*R*)- and (3*S*)-3-hydroxypent-4-ynoic acid methyl esters are +24.5[143] and -19.2,[144] respectively. The specific rotation of compound **4** was -14.5; thus, the absolute configuration of C-3 was assigned as *S*.

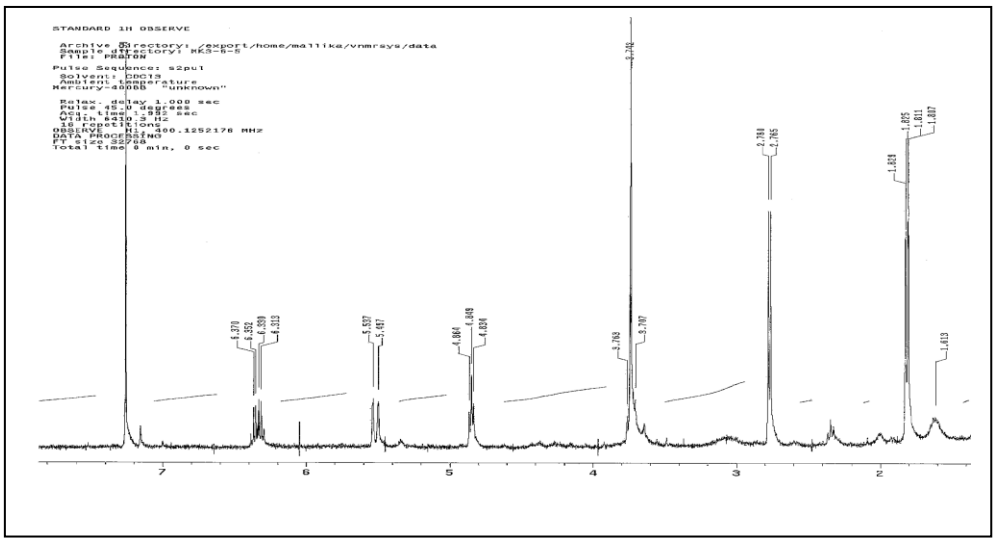


Figure 6.3. ^{1}H NMR spectrum of **4**

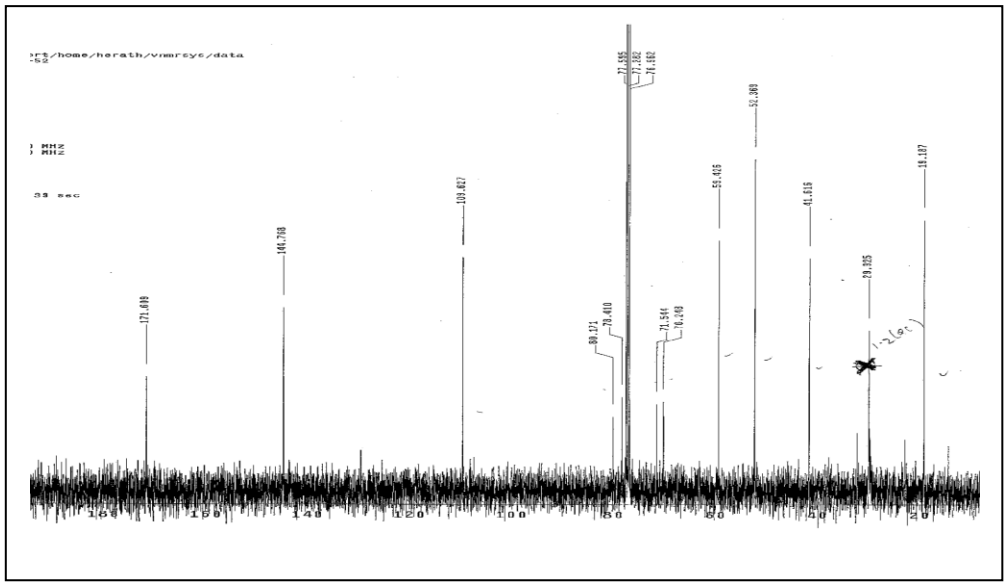


Figure 6.4. ^{13}C NMR spectrum of **4**

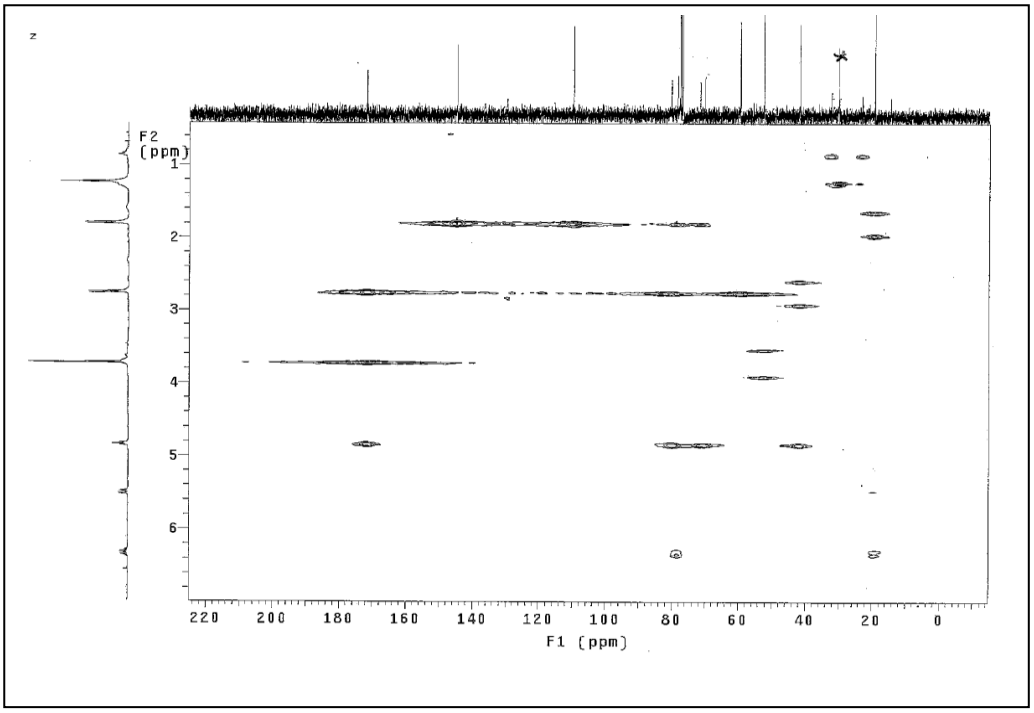


Figure 6.5. HMBC spectrum of **4**

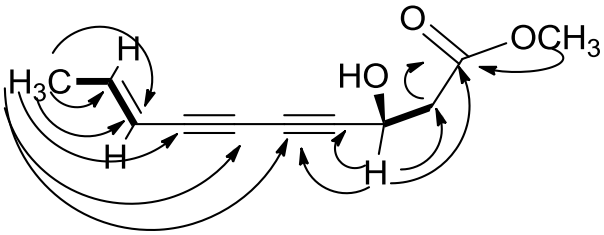


Figure 6.6. COSY (—) and HMBC (◠) correlations of **4**

The NMR spectra of **8** were superimposable with those of **7** except for the presence of an additional resonance for a methoxy group in the spectra of the former (Figure 6.7). Methylation

of compound **7** with trimethylsilyldiazomethane yielded a product which had the same physical and spectroscopic data (Figures 6.7 and 6.8) as those for **8,** confirming that the latter was (*S*)-methyl 3-(3,5-dichloro-4-methoxyphenyl)lactate.

The active fraction from the original extract was separated by column chromatography to afford a subfraction which appeared to be a single compound by TLC. The ^{1}H NMR spectrum indicated that it was a mixture of closely related compounds. This subfraction was further separated by HPLC to yield four compounds in minute amounts. The ^{1}H NMR data of these compounds indicated that they did not belong to any class of compound isolated from these extracts. Owing to lack of material, their structure elucidation was not feasible.

Compounds **1**, **2**, and **3** showed significant phytotoxic activity against both dicots (lettuce, *Lactuca sativa*) and monocots (bentgrass, *Agrostis stolonifera*) (Table 6.1). A number of conjugated polyyne compounds have been isolated from higher plants,[145-148] and basidiomycetes,[137, 149] and exhibited a variety of biological activities.[142, 145, 150, 151] Geometric isomers of compound **1** have been isolated from *Chrysoma pauciflosculosa* and their phytotoxicity as well as allelopathic effects on some Florida sandhill scrub species have been reported.[148]

Compounds **5**, **6**, and **8** exhibited good phytotoxic activity against both dicots (lettuce, *Lactuca sativa*) and monocots (bentgrass) (Table 6.1). Compounds **1**-**8** showed no activity in antimicrobial and antiprotozoal assays.

Compound **5**, the *S* enantiomer, is a rare natural product. Its *R* enantiomer, salvianic acid A, has been isolated from *Salvia miltiorrhiza* root, which is widely used in traditional medicine for the treatment of various cardiovascular diseases [152] and is also a biosynthetic precursor to a number of natural products including rosmarinic acid.[139]

Compounds **6** and **7** have not been isolated from natural sources. They have been found as precursors to compounds isolated from a cyanobacterium[136] and have been synthesized.

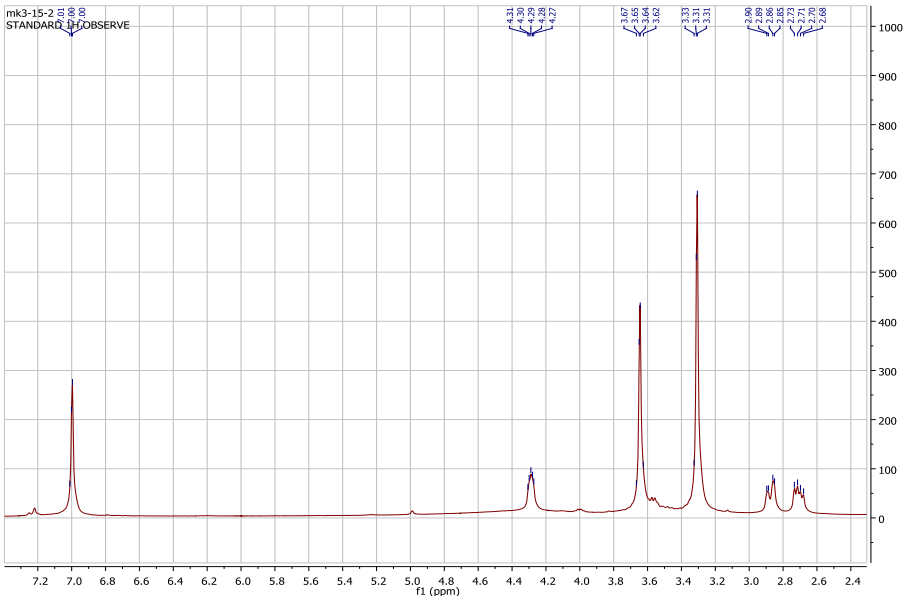


Figure 6.7. ^{1}H NMR spectrum of **8**

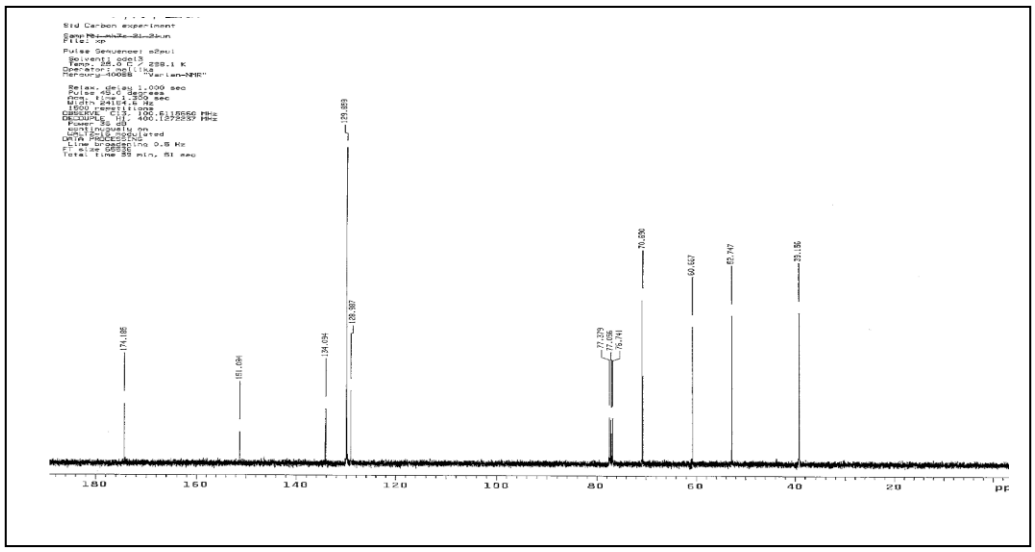


Figure 6.8. ^{13}C NMR spectrum of **8**

A number of chlorinated phenolic compounds have been isolated from basidiomycetes.[142, 153, 154] Some of these compounds have shown antimicrobial[155] and seed germination inhibitory[156] activities. They also have been reported[157, 158] to be involved in lignin degradation[25,26] and inhibition of the enzyme chitin synthase.[29]

Lanostane triterpenes have been isolated from several fungal species of the Polyporaceae, and their antioxidant and cytotoxic activities have been reported.[140, 159-161] Compounds **9-12** showed no activity in antimicrobial, antiprotozoal, and phytotoxic assays.

The variation in metabolite production in culture media and from one culture flask to another within the same media has been reported[45, 119] from several fungi. The failure to produce the antiplasmodial metabolites in large-scale culture broths may be due to the loss of virulence factors and/or specific virulence genes involved in the synthesis of specific toxins, upon

continuous subculturing of the fungus. It may also be due to mutation of genes responsible for biosynthesis of the specific toxins.

6.4. Experimental

6.4.1. General

Optical rotations were obtained using a Rudolph Research Analytical Autopol IV automatic polarimeter model 589-546. Melting points were measured with a Uni-melt, Thomas Hoover capillary melting point apparatus. UV and IR spectra were determined on a Varian-50 Bio UV visible spectrophotometer and a Bruker-Tensor-27 infrared spectrophotometer, respectively. NMR spectra were recorded on a Varian-Mercury-plus-400 or Varian Unity-Inova-600 spectrometer using $CDCl_3/CD_3OD$ as the solvent unless otherwise stated. MS analyses were obtained from an Agilent Series 1100 SL equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA) and GCMS (Hewlett-Packard 6890 series). Column chromatography and preparative TLC were carried out using Merck silica gel 60 (230-400 mesh) and silica gel GF plates (20 × 20 cm, thickness 0.25 mm), respectively. TLC spots were detected under UV irradiation and by heating on a hot plate after spraying anisaldehyde reagent. HPLC analysis was conducted on a Hewlett-Packard Agilent 1100 with diode array detector. Potato dextrose agar (PDA), potato dextrose broth (PDB), and Czapex dox were purchased from Difco™. V-8 and rice were purchased from Walmart in Oxford, MS. *Postia balsamea* (ATCC 64899) was obtained from the American Type Culture Collection (ATCC).

6.4.2. Fermentation and Extraction

P. balsamea obtained from the ATCC was grown on PDA plates at 27 ºC for 12 days. Erlenmeyer flasks (2 × 1 L) containing 600 mL of PDB were inoculated with three small agar pieces (5 × 5 mm) containing *P. balsamea* and were incubated at 27 ºC for 28 days on a shaker (RPM = 100). The culture broth and mycelia were filtered. The broth was extracted with EtOAc (× 3), and the organic layer was evaporated under reduced pressure to give an oily residue (189.0 mg). Mycelia were extracted with MeOH (× 3) and evaporated to give a brown oily residue (200 mg). These extracts showed good phytotoxic and antimalarial activities.

Scale up of fermentation was carried out (× 3) by repeating the above procedure with identical conditions using Erlenmeyer flasks (40 × 1L) to obtain EtOAc broth extracts (3.8, 3.5, and 3.7 g) and mycelia extracts (4.1, 4.8, and 3.4 g). These extracts exhibited good phytotoxic activities but were inactive in the antimalarial assay.

Fermentation was carried out in several other culture media [V8 (100% vegetable juice), Czapex dox, and Rice] and under various conditions such as different volume of broth (100 , 250 , and 500 mL), different Erlenmeyer flask sizes (250, 500 mL, and 1 L), and different fermentation time periods (2, 3, 4, and 5 weeks). Fermentations were also carried out in the presence of small amounts of the bark and leaf parts of a fir tree; however, the above experiments, as well as a fresh *P. balsamea* sample from the ATCC under the original culture conditions, failed to produce extracts with antimalarial activity.

6.4.3. Attempted Isolation of Antimalarial Compounds

The original oily extract (189 mg) which showed good antiplasmodial activity (IC_{50} < 1 µg/mL against *P. falciparum*) and good phytotoxic activity was chromatographed over a gravity column using hexanes, CH_2Cl_2, and MeOH as the eluents to give seven subfractions. Fraction 3 (18.0 mg) showed potent antiplasmodial activity (IC_{50} < 120 ng/mL) with high selectivity. TLC analysis of this fraction showed the presence of two major and four minor spots (Figure 6.1). The active fraction was separated using a reversed-phase Luna C_{18} column (1 × 25 cm) and eluted with MeOH-H_2O (65:35) as the mobile phase at a flow rate of 2.5 mL/min, with wave length set at 254 nm. The major compounds corresponding to the major spots were also present in substantial amounts in the large-scale extracts as well as in mycelia extracts. They were isolated from mycelium extract and identified as eboricoic acid (**9**) and sulfurenic acid (**11**) (vide infra). These compounds were inactive in the antiplasmodial assay. HPLC analysis revealed that the minor spot consisted of at least four related compounds, and their identification was not possible due to insufficient quantities.

Polyacetylenes

The EtOAc extract of the large scale fermentation broth (3.8 g) which showed potent phytotoxic activity against monocots (*Agrostis stolonifera* cv. Pencross) and dicots (*Lactuca sativa*) was fractionated by silica gel column chromatography using hexanes, CH_2Cl_2, and MeOH as eluents to give eighteen fractions. Fractions 1, 2, and 3 showed potent phytotoxic activity against both monocots and dicots. The compounds present in these fractions showed a

tendency to undergo decomposition. Fraction 1 (50.0 mg) was chromatographed over a gravity column using hexanes as the eluting solvent, and further separation was carried out by PTLC using 15% EtOAc in hexanes to yield (2*E*,8*E*)-matricaria ester (**1**)[137] (8.0 mg). Fractions 2 and 3 were combined and subjected to silica gel gravity column chromatography using hexanes and CH_2Cl_2 as the eluents to yield 9 fractions. Subfraction 8 (130 mg) was applied on Sephadex LH-20 and eluted with 100% MeOH to give (2*E*,8*E*)-matricarianol (**2**)[137] (5.0 mg). Subfraction 9 was applied on Sephadex LH-20 and eluted with 100% MeOH followed by PTLC using 30% EtOAc in hexanes as developing solvent to yield (2*E*,8*E*)-deca-2-8-diene-4,6-diyne1,10-diol (**3**)[137, 138] (1.0 mg) and (3*S*,8*E*)-methyl 3-hydroxydeca-8-en-4,6-diynoate (**4)** (2.0 mg).

Phenolic Compounds

Fractions 4 and 5 were combined and chromatographed over a silica gel gravity column followed by PTLC using 35% EtOAc in hexanes as the developing solvent to give three simple phenolic compounds, **5** (*S*)-3-(3,4-dihydroxyphenyl)lactic acid (2.0 mg), **6** (*S*)-3-(3,5-dichloro-4-hydroxyphenyl)lactic acid (7.0 mg), and **7** (*S*)-methyl 3-(3,5-dichloro-4-methoxyphenyl)lactate (10.0 mg). Compound **8** (*S*)-methyl 3-(3,5-dichloro-4-methoxyphenyl)lactate (10.0 mg) was isolated as white crystals from fraction 12 (CH_2Cl_2/MeOH).

Triterpenes

Fractions 13 and 14 were combined and chromatographed over a silica gel column using hexanes and EtOAc as eluents to give eboricoic acid (**9,** 1.0 mg) and acetyl eboricoic acid (**10,** 2.0 mg) as white amorphous powders. The mycelia extract (12.0 g) was chromatographed over

a silica gel column using hexanes, CH_2Cl_2, and MeOH as the eluents to give nine fractions. Further purification of these fractions using repeated column chromatography furnished triterpenes **9** (5.0 mg) and **10** (10.0 mg) and a mixture of the two triterpenes **11** and **12.** NMR and HRESIMS data of the mixture confirmed them to be sulfurenic acid (**11**) and dehydrosulfurenic acid (**12**).

*Compound **4*** **:** amorphous, $[\alpha]^{26}_{D}$ – 14.5 (*c* 0.1, $CHCl_3$); IR ν_{max} 3435, 1728 cm^{-1}; UV ($CHCl_3$): λ_{max} (log ε) 206 (3.20), 228 (3.33), 253 (3.22), 268 (3.20), and 284 (3.14) nm; ESIMS $[M - H]^+$: m/z = 174; ^{1}H NMR: δ1.82 (3H, dd, J = 7.2, 1.6 Hz, H-10), 2.77 (2H, d, 6.0 Hz, H-2)), 3.74 (3H, s, OCH_3), 4.85 (1H, t, J = 6.0 Hz, H-3), 5.52 (1H, d, J = 15.2 Hz, H-8), 6.34 (1H, dq, J = 16.0, 7.2Hz, H-9), ^{13}C NMR: δ19.2 (C-10), 41.6 (C-2), 52.4 (-OCH_3), 59.4 (C-3), 70.2 (C-5), 71.5 (C-6), 78.4 (C-7), 80.1 (C-4), 109.6 (C-8), 144.8 (C-9), 171.6 (C-1)

*Compound **8*** **:** White solid, mp 80^o C, $[\alpha]^{26}_{D}$ -11 (*c* 0.1, $CHCl_3$); IR ν_{max} 3468, 1736 cm^{-1}; UV (MeOH) λ_{max} (log ε): 225 (3.91), 275 (3.19) nm; EIMS $[M + H]^+$ *m/z* 279. HRESIMS $[2M + H + Na]^+$ *m/z* 581.0403 (calcd for ($C_{22}H_{25}Cl_4O_8Na$) 580.0201); ^{1}H NMR δ 2.7 (1H, dd, J = 14.0, 7.2 Hz, H-3), 2.87 (1H, brd, J = 14.0 Hz, H-3), 3.31 (3H, s, OCH_3), 3.64 (3H, s, $COOCH_3$), 4.29 (1H, brs, H-2), 7.00 (2H, s, H-2′ and H-6′); ^{13}C NMR: δ 39.2 (C-3), 52.7 (OCH_3), 60.7 ($COO\underline{C}H_3$), 70.7 (C-2), 129.0 (C-1′), 129.9 (C-2′, 6′), 134.1 (C-3′ and C-5′), 151 (C-4′).

*Methylation of Compound **7***:

Compound **7** (2 mg) in methanol (0.5 ml) was treated with trimethylsilyldiazomethane (50 μL) at room temperature for 10 minutes. Solvent was evaporated and the residue was dissolved in CH_2Cl_2, and filtered through a plug of celite® to yield compound **8** (2.1 mg).

6.4.4. Biological Assay

6.4.4.1. Antiplasmodial Activity

The antiplasmodial activity of extracts, fractions and pure compounds was performed against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *P. falciparum* using the in vitro assay as reported.[69]

6.4.4.2. Phytotoxic Activity

The herbicidal activity of extracts and test compounds was performed using the procedure described by Dayan et al.[111] using bentgrass (*Agrostis stolonifera*) and lettuce (*Lactuca sativa* cv. L., Iceberg), in 24-well plates. The results are collated in Table 6.1.

Table 6.1. Phytotoxic activity of *P. balsamea* compounds

Compound	Concentration (mM)	Lettuce	Bentgrass
1	5.7	5	4
	0.6	1	0
2	6,8	5	5
	0.7	4	3
3	5.2	5	5
	0.52	1	0
4	-	NT	NT
5	3.6	5	4
	0.36	0	0
6	5.1	4	4
	0.5	0	0
7	-	NT	NT
8	4.0	5	5
	0.4	0	0
9	2.1	NA	NA
10	1.9	NA	NA
11	2.1	NA	NA
12	2.1	NA	NA

Ranking based on scale of 0 to 5

0 = no effect, 5 = no growth

NT = Not tested

NA = Not active

CHAPTER 7: ANTIMALARIALS FROM ENDOPHYTIC FUNGI ISOLATED FROM *TORREYA TAXIFOLIA* ARNOTT

Torreya taxifolia Arn., an evergreen tree of the family Taxaceae, known as Florida torreya, stinking-cedar, or gopherwood, can grow to heights up to 20 meters.[162] The native population decline during the last decades has been attributed to both abiotic and biotic causes including fungal diseases.[162] *T. taxifolia* is considered the rarest conifer in North America and is one of the most endangered trees in the world.[162] The symptoms of disease include needle spot, needle death, needle cankers, defoliation, and stem lesions.[163] This tree is in the same plant family as *Taxus brevifolia* (Pacific yew) that produces taxol and taxane derivatives.[164] Although several studies have been carried out to determine the causative agent responsible for the rapid decline of the plant, the identity of a single highly virulent pathogen remains an area of active research, with a *Fusarium* being the most recently reported causative agent.[162, 163] Pathogenicity studies carried out in the past few years, concluded that several pathogens may be responsible for leaf spots, but not for the canker disease. These pathogens included[163, 165, 166] *Macrophoma* sp., *Rhizoctonia solani*, *Sphaeropsis* sp., *Sclerotium rolfsii, Fusarium lateritium,* and *Pestalotiopsis microspora.*

From *T. taxifolia* nine endophytic fungi were isolated from diseased leaves of cultivated plants (Figures 7.1 and 7.2), and six endophytic fungi were isolated from diseased seeds.

EtOAc extracts of fermentation cultures of these fungi were screened for phytoxic activity against both a dicot (lettuce, *Lactuca sativa* L.) and a monocot (bentgrass, *Agrostis stolonifera* L.) and antiplasmodial activity against chloroquine-sensitive (D6) and -resistant (W2) strains of *P. falciparum*. Of the endophytes screened, *Xylaria* sp. (Section 7.1), *Alternaria* sp. (Section 7.2), a new isolate, UM #10 M (Section 7.3), and *Botryosphaeria dothidea* (Section 7.4) showed good antiplasmodial and phytotoxic activities (Table 7.1).

Figure 7.1. Symptoms of diseased needles of cultivated *T. taxifolia*

Figure7.2. Symptoms of diseased needles of cultivated *T. taxifolia*

Table 7.1: Antiplasmodial and herbicidal activity of endophytic fungi extracts

Fungi	Medium	Antiplasmodial activity IC_{50} (ng/mL)				Cytotoxicity (vero) IC_{50} (ng/mL)	Phytotoxicity	
		P. falciparum D6 clone	SI	*P. falciparum* W2 clone	SI		Lettuce	Bentgrass
Botryosphaeria dothidea (Broth)	PDB	860	37.2	1300	24.6	32000	5	5
Xylaria sp (Broth)	PDB	62	3.3	87	2.3	200	5	5
UM #10 M (Broth)	PDB	40	170	44	154.5	6800	5	5
Alternaria sp. (Broth + Mycelium)	PDB	6800	1.8	5800	2.1	12000	5	5

Ranking based on scale of 0 to 5

0 = no effect, 5 = no growth

SECTION 7.1: ANTIMALARIALS FROM *XYLARIA* SP.

7.1.1. Summary

Bioassay-guided fractionation of the potato dextrose culture broth extract of *Xylaria* sp. isolated from a diseased leaf of *Torreya. taxifolia* afforded a known compound, heptelidic acid (**1**), as the active constituent. This compound showed good antiplasmodial activity and phytotoxicity, but was also cytotoxic to vero cells and a panel of solid tumor cell lines.

Figure 7.1.1. PDA plate of *Xylaria* sp.

7.1.2. Introduction

Xylaria sp. is also known as Stag's Horn and Candle-snuff. Its morphology (Figure 7.1.1) shows it contains antler-like ascocarps (fruit bodies) which are black at the base and, white and branched towards the top where the fruit bodies produce white conidia (asexual spores). This is a cosmopolitan fungal genus belonging to the family Xylariaceae and several members of this family have been reported[168-177] to produce a number of chemically diverse, biologically active, and taxonomically significant constituents, including several antimalarial compounds.[57, 178, 179]

An EtOAc extract of the fermentation culture broth of *Xylaria* sp. showed potent herbicidal and antiplasmodial activities. Bioassay-guided fractionation of this extract afforded heptelidic acid (**1**), as the active compound.

7.1.3. Results and Discussion

This fungus was identified to be a *Xylaria sp.* by its characteristic morphological features and the DNA sequencing profile of the fungal internal transcribed spacer (ITS) region.[68] The active EtOAc extract was fractionated by repeated silica gel column chromatography, and compounds were further purified with Sephadex LH-20 to afford heptelidic acid (**1**) (Figure 7.1.2) as the antiplasmodial and phytotoxic constituent. Its identity was confirmed by comparison of the physical and spectroscopic data with those reported.[180] This sesquiterpene lactone has been isolated[180] from *Trichoderma viride*, *Chaetomium globsum*, and *Gliocladium virens*, by Itoh et al., and was demonstrated to possess antiplasmodial activity.[67]

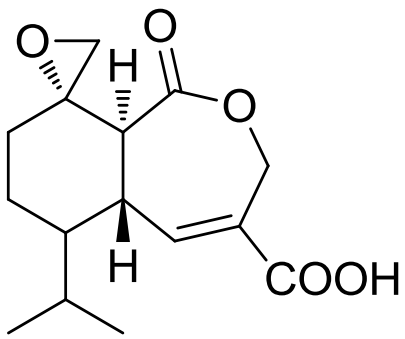


Figure 7.1.2. Heptelidic acid (**1**)

Heptelidic acid (**1**) exhibited moderate antiplasmodial activity (Table 7.1.1) but also showed cytotoxicity against Vero cells (Table 7.1.1), kidney epithelial cells, and a panel of solid tumor cell lines (Table 7.1.3). This compound showed moderate phytotoxic activity against a dicot (Table 7.1.2). The observed general cytotoxicity precludes heptelidic acid as a viable antimalarial drug lead.

7.1.4. Biological Activities

Table 7.1.1. Antiplasmodial activity of **1**

Compound	Chloroquine-sensitive (D6)-clone		Chloroquine-resistant (W2)-clone		Cytotoxicity to Vero cells
	IC_{50} nM	S. I.	IC_{50} nM	S. I.	IC_{50} nM
heptelidic acid (**1)**	0.30	2.0	0.27	2.3	0.61
chloroquine[a]	0.03	496.6	0.31	48.1	NC
artemisinin[a]	0.02	845	0.01	1690	NC

[a] Positive controls

NC, not cytotoxic

S. I. (selectivity index) = IC_{50} for cytotoxicity/IC_{50} for antiplasmodial activity

Table 7.1.2. Phytotoxic activity of **1**

Compound	Lettuce	Bentgrass
heptelidic acid (**1)**	4	1

Ranking based on scale of o to 5

0 = no effect

5 = no growth

Solvent used, 10% acetone

Concentration used, 1mg/mL

Table 7.1.3. Cytotoxic activity [IC_{50} (µM)] of **1**

Compound	SK-MEL	KB	BT-549	SK-OV-3	LLC-PK_{11}
heptelidic acid (**1)**	2.67	3.21	3.4	11.8	2.32
doxorubicin[a]	1.29	2.12	1.83	1.47	1.28

[a] Positive control.

IC_{50} = concentration casusing 50% growth inhibition

SK-MEL = human malignant melanoma

KB = human epidermal carcinoma

BT-549 = human breast carcinoma (ductal)

SK-OV-3 = human ovary carcinoma

LLC-PK_{11} = pig kidney epithelial

7.1.5. Experimental

7.1.5.1. General

Melting points were measured on a Unimelt, Thomas-Hoover capillary melting point apparatus. NMR spectra were recorded on a Bruker 400 MHz spectrometer using $CDCl_3$/methanol-d_4 as the solvent unless otherwise stated. MS analyses were performed on an Agilent Series 1100 SL equipped with an ESI source (Agilent Technologies, Palo Alto, CA). Column chromatography was carried out on Merck silica gel 60 (230-400 mesh) and Sephadex LH-20. Preparative TLC was carried out using silica gel GF plates (20 × 20 cm, thickness 0.25 mm). HPLC analysis was conducted on a Hewlett-Packard Agilent 1100 with diode array detector.

7.1.5.2. Isolation of *Xylaria* sp. from *T. taxifolia*

Diseased leaves were collected from a cultivated *T. taxifolia* plant. Leaves were surface sterilized with 5% Chlorox for 5 minutes and rinsed with sterile water three times. Transverse

sections from the dried leaf were cut aseptically into small portions and immersed in the potato dextrose plates. The plates were incubated for two weeks, and fungal colonies were subcultured to PDA plates to isolate pure fungal *Xylaria* sp.

7.1.5.3. Fermentation, Extraction, and Purification

Xylaria sp. was cultured in two conical flasks (1 L) containing 500 mL of Potato Dextrose Broth and incubated at 27 ºC for 18 days on an orbital shaker at 100 rpm. The media was separated by filtration. The culture broth was extracted with EtOAc (× 3), and the organic layer was evaporated to give an oily residue (410 mg).

The oily extract (400 mg) was chromatographed over a silica gel gravity column using hexanes, CH_2Cl_2, and MeOH gradients as mobile phase to give 12 fractions. Fractions 5-9, which showed antiplasmodial activity, were combined, chromatographed over Sephadex LH-20, and washed with 100% MeOH to give heptelidic acid (**1**, 12.0 mg).

Compound 1 **:** Heptelidic acid; white powder; ^{1}H and ^{13}C NMR were consistent with those reported.[180] $[\alpha]^{26}_{D}$ +2.5 (*c* 0.28, $CHCl_3$) lit[180] +7.7; HRESIMS $[M + H]^+$ *m/z* 281.1422 (calcd for $[C_{24}H_{33}NO_4 + H]^+$ *m/z* 281.1389)

7.1.5.4. Biological Assay

7.1.5.4.1. Antiplasmodial Assay

In vitro antiplasmodial activity against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *Plasmodium falciparum* was determined as described earlier.[69]

7.1.5.4.2. Phytotoxic Assay

The bioassay for phytotoxicity was carried out according to the procedure described by Dayan *et al*.[70] using bentgrass (*Agrostis stolonifera*) and lettuce (*Lactuca sativa* L.cv. "Iceberg"), in 24-well plates.

7.1.5.4.3. Cytotoxicity Assay

In vitro cytotoxicity was determined against a panel of mammalian cells as described earlier.[76]

SECTION 7.2: ANTIMALARIALS FROM *ALTERNARIA* SP.

7.2.1. Summary

An *Alternaria* strain isolated from a diseased leaf of *T. taxifolia* exhibited antiplasmodial and phytotoxic activities. Bioassay-guided fractionation of this extract afforded orsellinic acid (**2**), penicillic acid (**3**), and a diastereomeric mixture of two new compounds, 6-hydroxy-5,6-dihydropenicillic acid (**4**). Penicillic and orsellinic acids showed strong phytotoxic activity and the former showed weak antiplasmodial activity.

7.2.2. Introduction

Figure 7.2.1. PDA plate of *Alternaria* sp.

The genus *Alternaria* is widely distributed and a number of its species are known as plant pathogens.[181] Although several diverse secondary metabolites have been reported from terrestrial,[181] marine,[182] and endophytic *Alternaria* sp., no antimalarial compounds have yet been reported. The EtOAc extract of the broth and mycelia of this fungus had similar chemical profiles and showed weak antiplasmodial activity against chloroquine-sensitive (D6) and -resistant (W2) strains of *P. falciparum* and potent phytotoxic activity against both a dicot (lettuce, *Lactuca sativa*) and a monocot (bentgrass, *Agrostis stolonifera*). Bioactivity-guided fractionation of the combined extracts afforded orsellinic acid (**2**), penicillic acid (**3**), and a diastereomeric mixture of two new compounds, 6-hydroxy-5,6-dihydropenicillic acid (**4**)

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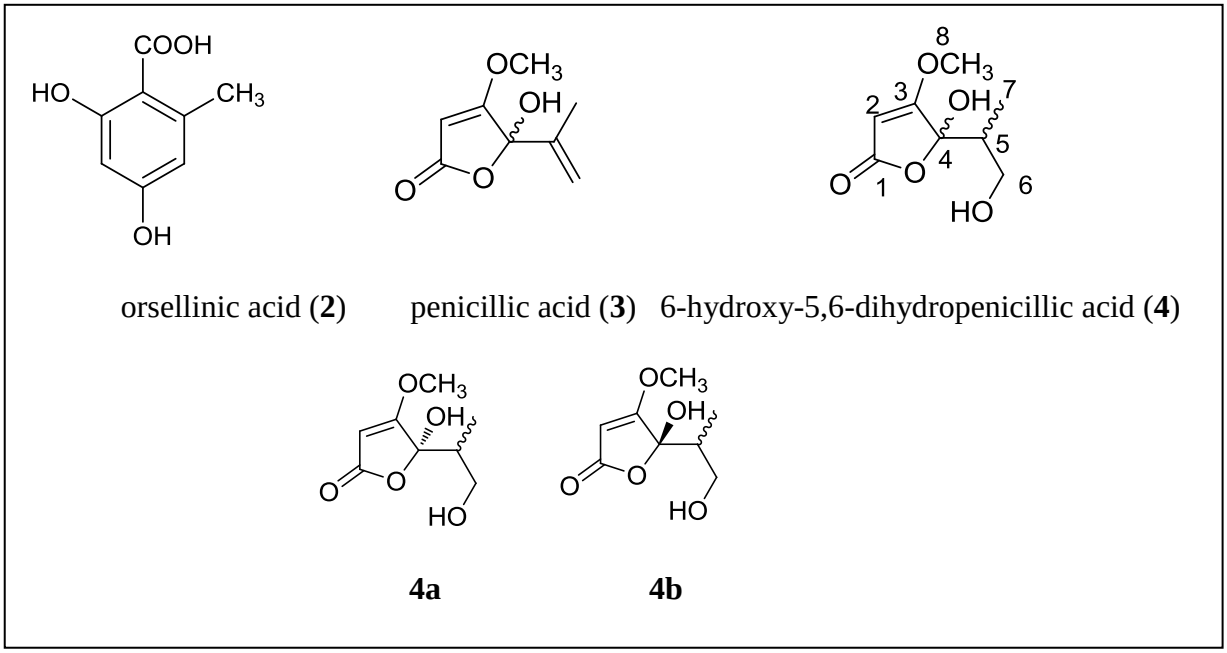


Figure 7.2.2. Compounds isolated from *Alternaria* sp.

7.2.3. Results and Discussion

An *Alternaria* strain was isolated from a diseased leaf of *T. taxifolia*. Identification of this fungus was based on the DNA sequencing profile of the fungal internal transcribed spacer (ITS) region.[68] The active extract was fractionated over silica gel and the active fractions were separated on Sephadex LH-20 to yield orsellinic acid (**2**), penicillic acids (**3**), and compound **4**. The identities of orsellinic (**2**)[183] and penicillic acids (**3**)[184] were confirmed by comparing their physical and spectroscopic data with those reported.

Compound **4** was obtained as a white semisolid, and its molecular formula was assigned as $C_8H_{12}O_5$ by HRESIMS. The ^1H (Figure 7.2.3) and ^{13}C NMR (Figure 7.2.4) data of **4** clearly showed the presence of two related compounds in the ratio of approximately 1:2 (Table 7.2.1). Comparison of spectroscopic data of **4** with those of penicillic acid (**3**) indicated that they were closely related and that the vinyl group of the later has been replaced by a -$CHCH_2OH$ group creating an stereogenic center. As in the case of penicillic acid, this compound also forms epimeric lactols (Figure 7.2.5); however, due to the presence of the additional stereogenic center, compound **4** comprises two diastereomers. COSY (Figure 7.2.6), and HMBC correlations (Figure 7.2.7), were used to assign the resonances of both diastereomers, **4a** and **4b** (Figure 7.2.8). In the COSY spectrum, both the oxygenated methylene (H-6) and the methyl doublet (H-7) had crosspeaks with the upfield methine multiplet (H-5). In the HMBC spectrum, oxygenated methylene (H-6) showed correlations to C-4, C-5, and C-7 supporting the structure proposed for this compound. Additional correlations shown in Figure 7.2.8 further support this structure. A molecular mechanics energy minimization method showed **4a**, as depicted in Figure 7.2.9, or its

enantiomer to be the more stable conformer. The absolute configuration at C-5 remains to be determined.

Orsellinic acid has previously been isolated from lichens[183] and several fungi species. [185] This compound showed good phytotoxicity (Table 7.2.2). Anti-oxidant,[183] antibacterial,[186] and growth inhibitory[187] activities of orsellinic acid have been reported. Penicillic acid has been reported[188-190] from several fungi and is known to be cytotoxic to mammalian cells. Penicillic acid showed weak in vitro antiplasmodial activity against chloroquine-sensitive (D6) and -resistant (W2) strains of *Plasmodium falciparum* (Table 7.2.3) and good phytotoxic activity against both a monocot and a dicot (Table 7.2.2). Phytotoxic activity of penicillic acid against radical growth of *Amaranthus hypochondriacus* has been reported.[191] Orsellinic acid is the intermediate for the biosynthesis of penicillic acid derivatives.[184]

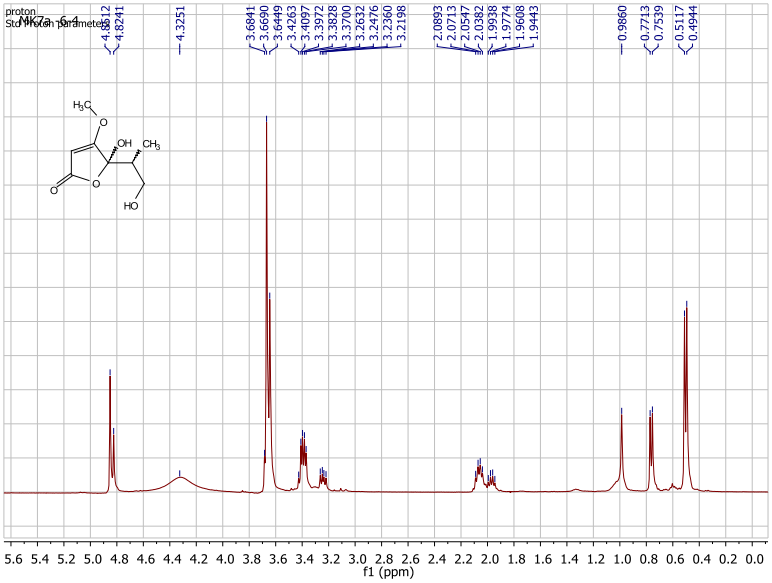


Figure 7.2.3. ^{1}H NMR spectrum of **4**

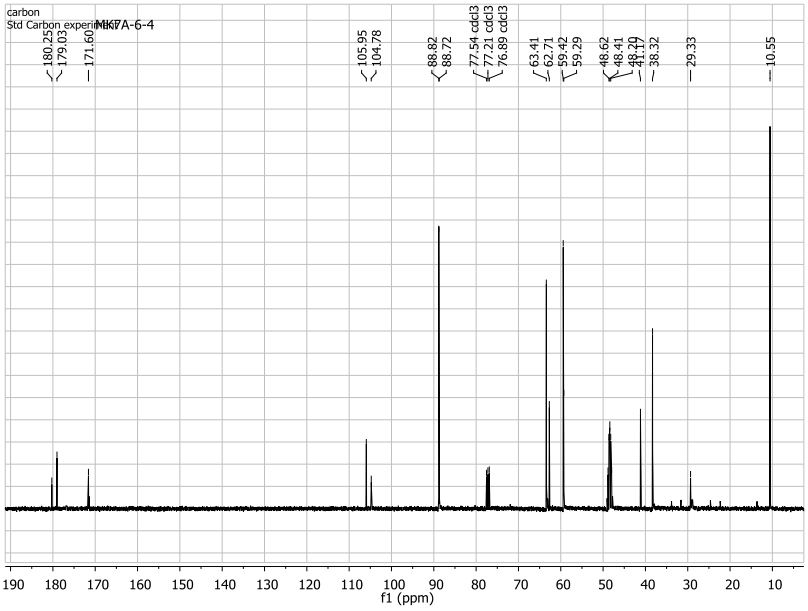
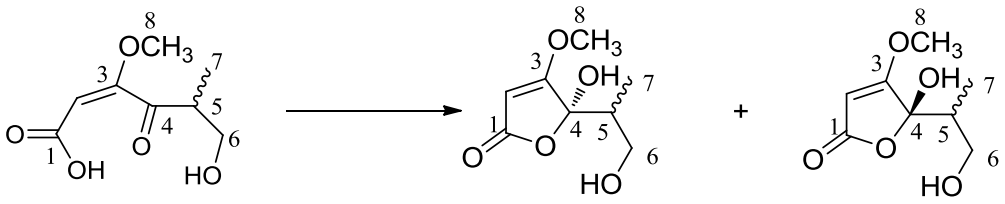


Figure 7.2.4. ^{13}C NMR spectrum of **4**



4a **4b**

6-Hydroxy-5,6-dihydropenicillic acid

Figure 7.2.5. Formation of C-4 diastereomers (**4a** >**4b**, 2:1)

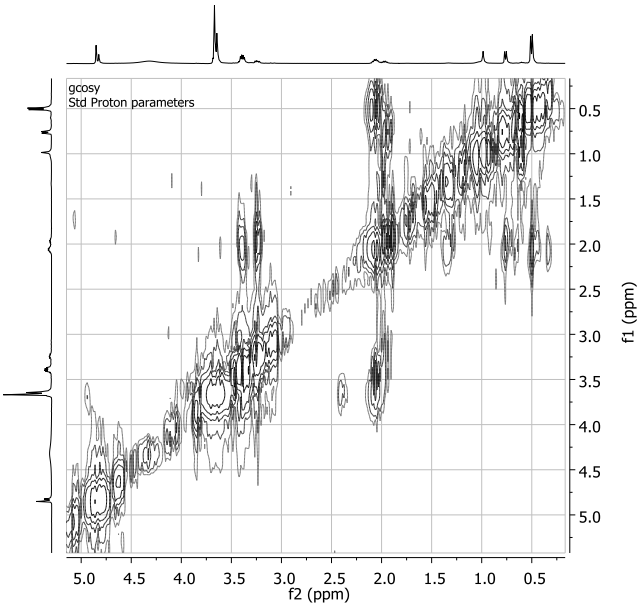


Figure 7.2.6. COSY spectrum of **4**

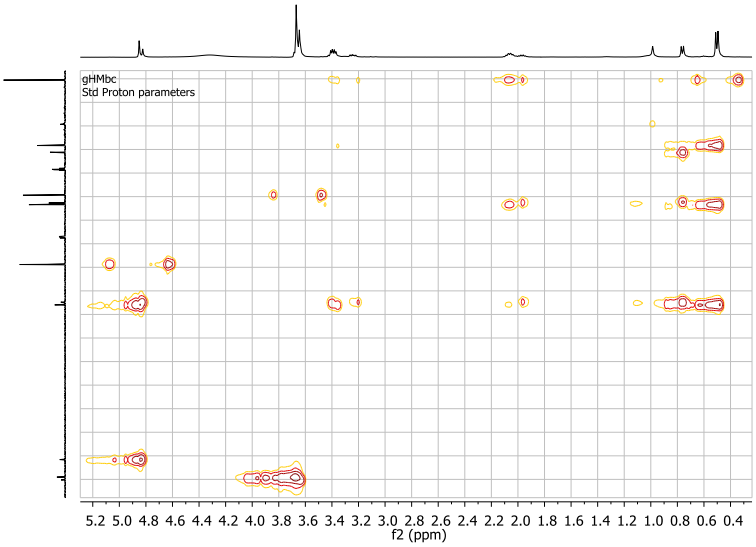


Figure 7.2.7. HMBC spectrum of **4**

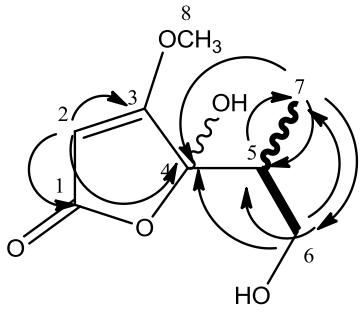
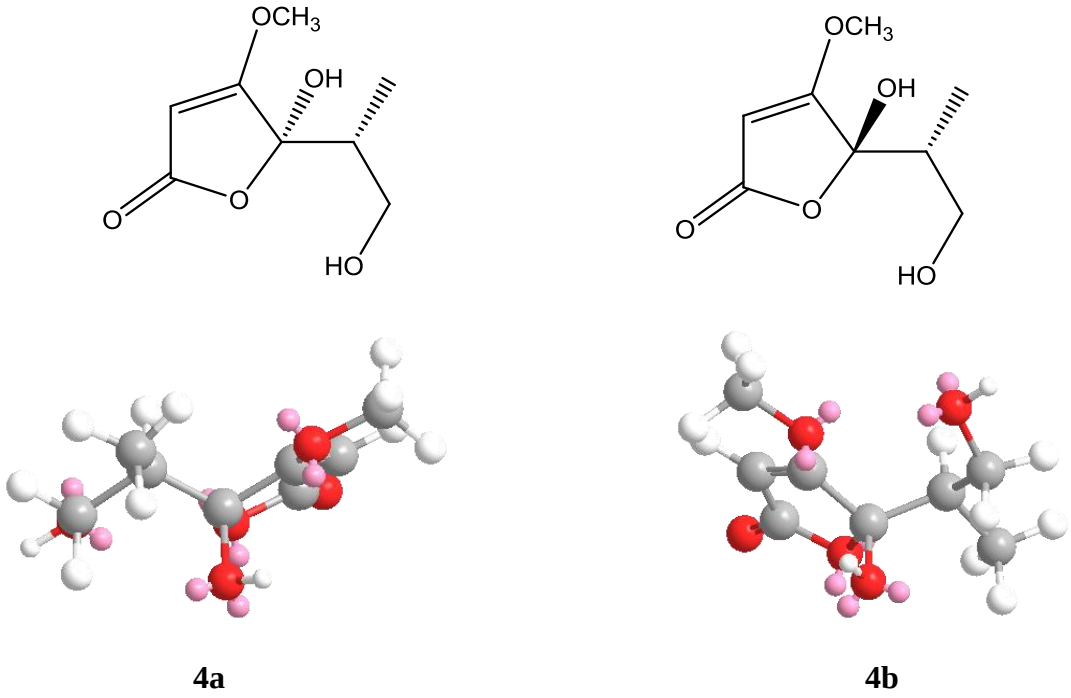


Figure 7.2.8. COSY (▬) and HMBC (↷) correlations of **4**



4a **4b**

Figure 7.2.9. Energy minimized structures (**4a** > **4b**; 2:1)

Table 7.2.1. ^{1}H and ^{13}C NMR of **3**, **4a** and**, 4b** in $CDCl_3$- Methanol-d_4

Carbon	penicillic acid (**3**)		**4a**		**4b**	
	δ_C [b]	δ_H [a] (*J* in Hz)	δ_C [b]	δ_H [a] (*J* in Hz)	δ_C [b]	δ_H [a] (*J* in Hz)
1	172.0		171.6		171.6	
2	89.1	5.48, s	88.8	4.85, s	88.7	4.82, s
3	179.4		179.0		180.2	
4	116.5		105.9		104.8	
5	139.3		38.3	2.05, m	41.1	1.91, m
6	116.5	5.12, s, 5.22, s	63.4	3.63, m	62.7	3.48, m
7	17.1	1.77, s	10.6	0.5, d (8.0)	10.6	0.50, d (8.0)
8	59.9	3.91, s	59.4	3.67, s	59.3	3.64, s

[a1]H NMR recorded at 400 MHz, [b13]C NMR spectra recorded at 100 MHz

7.2.4. Biological Activities

Table 7.2.2. Phytotoxic activity of **2-4**

Compound	Lettuce	Bentgrass
orsellinic acid (**2**)	5	5
penicillic acid (**3**)	4	5
6-hydroxy-5,6-dihydropenicillic acid (**4**)	1	3

Ranking based on scale of o to 5

0 = no effect 5 = no growth

Solvent used, 10% acetone

Concentration used, 1mg/mL

Table 7.2.3. Antiplasmodial activity of **2-4**

Compound	Chloroquine-sensitive (D6)-clone		Chloroquine-resistant (W2)-clone		Cytotoxicity to Vero cells
	IC_{50} nM	S. I.	IC_{50} nM	S. I.	IC_{50} nM
orsellinic acid (**2**)	NA		NA		NC
penicillic acid (**3**)	17.1	>1.6	15.9	>1.7	NC
6-hydroxy-5,6-dihydropenicillic acid (**4a & 4b**)	NA		NA		NC
chloroquine[a]	0.03	496.6	0.31	48.1	NC
artemisinin[a]	0.02	845	0.01	1690	NC

[a] Positive controls

NC, not cytotoxic

NA, not active

S. I. (selectivity index) = IC_{50} for cytotoxicity/IC_{50} for antiplasmodial activity

7.2.5. Experimental

7.2.5.1. General

Carried out as previously described in the section 7.1.4.1

7.2.5.2. Isolation of *Alternaria* sp.

Alternaria sp. was isolated as described in the Section 7.1.5.2

7.2.5.3. Fermentation, Extraction, and Purification

Alternaria sp. was cultured in four conical flasks (2 L) containing 500 mL of PDB and incubated at 27 ^{o}C for 21 days on an orbital shaker at 100 rpm. The mycelium was separated by filtration, and then the broth was extracted with EtOAc (× 3) separately. The organic layer of the broth was evaporated to give a brown residue (1.85 g).

The brown extract (1.8 g) was chromatographed over a silicagel column using hexanes, CH_2Cl_2, and MeOH gradients as mobile phase to yield nine fractions. Further separation of the active fractions (5-8) by Sephadex LH-20 gel filtration (100% MeOH) afforded orsellinic acid (**2**, 30.0.0 mg), penicillic acid (**3**, 15 mg), and a diastereomeric mixture of two new compounds, 6-hydroxy-5,6-dihydropenicillic acid (**4a** and **4b,** 6.0 mg).

Compound 2 **:** Orsellinic acid; ^{1}H ,^{13}C NMR, and HRESIMS were consistent with reported data.[183]

***Compound 3* :** Penicillic acid; colorless crystalline solid (MeOH); mp 80 ^{o}C (lit[190] 82-85 ^{o}C) ^{1}H[190] and ^{13}C NMR[184] were consistent with literature values.

***Compound 4* :** 6-Hydroxy-5,6-dihydropenicillic acid; amorphous powder; ^{1}H and ^{13}C NMR (see Table 7.2.1) HRESIMS $[M + H]^{+}$ *m/z* 535.1651 (calcd for $[C_{24}H_{33}NO_4 + H]^{+}$ 535.1604). The most stable conformer was deduced using molecular mechanics (MM2) energy minimization program (ChemBio3D Ultra 12.0).

7.2.5.4. In Vitro Biological Assay

7.2.5.4.1. Antiplasmodial Assay

In vitro antiplasmodial activity was determined against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *Plasmodium falciparum* as described earlier.[69]

7.2.5.4.2. Phytotoxic Assay

The bioassay for phytotoxicity was carried out as described by Dayan *et al*.[70] using bentgrass (*Agrostis stolonifera*) and lettuce (*Lactuca sativa* cv. L., Iceberg), in 24-well plates.

7.2.5.4.3. Cytotoxicity Assay

In vitro cytotoxicity was determined against a panel of mammalian cells as described[76] earlier.

SECTION 7.3: ANTIMALARIALS FROM A NEW ISOLATE, UM #10 M

7.3.1. Summary

Bioassay-guided fractionation of an EtOAc extract of the broth of the new fungus, UM #10 M, isolated from a diseased *T. taxifloia* leaf, afforded two known cytochalasins, 19,20-epoxycytochalasin C (**5**), 19,20-epoxycytochalasin D (**6**), and a new analogue, 18-deoxy-19,20-epoxycytochalasin C (**7**). All three compounds showed potent in vitro antiplasmodial activity and phytotoxicity with no cytotoxicity to Vero cells. These compounds were also not cytotoxic against a panel of solid tumor cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and kidney epithelial cells ($LLC\text{-}PK_{11}$). Evaluation of in vivo antimalarial activity of 19,20-epoxycytochalasin C (**5**) in a mouse model showed that this compound was toxic to animals at subtherapeutic doses.

7.3.2. Introduction

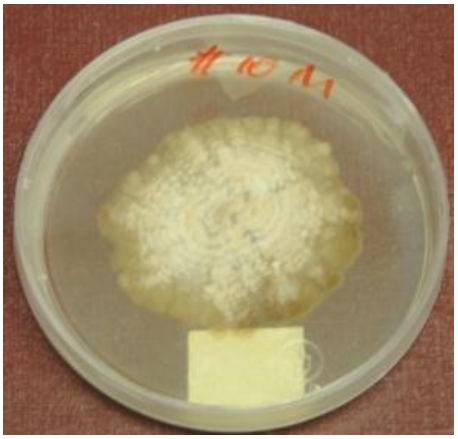


Figure 7.3.1. PDA plate of the new fungus, UM #10 M

Even though this fungus was conclusively identified as a new species, 18S rDNA analysis and morphology indicated that it most probably belongs to the family Xylariaceae. Some members of this family have been identified[192] as endophytes and plant pathogens. A number of cytochalasins have been reported[192] from Xylariaceae and several other plant pathogenic[193, 194] and endophytic[192, 195, 196] ascomycete and basidiomycete genera, namely, *Penicillium*, *Aspergillus*, *Zygosporium*, *Phoma*, *Metarhizum*, *Rosellinia*, *Ascochyta*, *Phomopsis*, *Chaetomium*, *Hypoxylon*, and *Daldinia*. The chemistry and biology of cytochalasins have extensively been studied by several research groups.[192] Generally, the cytochalasin skeleton includes a macrocyclic ring, fused with a phenylsubstituted perhydroisoindolone group. Cytochalasins are known to be involved in fungal virulence, food spoilage, and symbiotic fungus-host relationships.[192] Cytochalasin B[197, 198] and D[199] have been identified as microfilament-targeting molecules capable of blocking several cellular processes involving

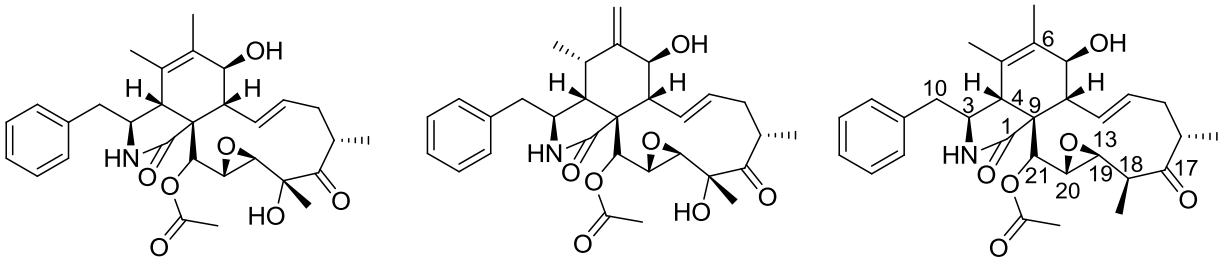
cytokinesis, intracellular motility, and cytosis,[192, 200] resulting in general cytotoxicity. Antiplasmodial[57] and antitoxoplasma[199] activities of cytochalasins have also been reported.

The EtOAc extract of this fungal broth showed potent antiplasmodial activity and low cytotoxicity. Bioassay-guided fractionation yielded two known cytochalasins, 19,20-epoxycytochalasin C (**5**),[201] and 19,20-epoxycytochalasin D (**6**),[202] and a new analogue, 18-deoxy-19,20-epoxycytochalasin C (**7**) as active constituents.

7.3.3 Results and Discussion

Analysis of the 18S rDNA of this fungus indicated[68] that it is related to both Xylariaceae sp., and Sordariomycetes sp., with 93 % sequence homology. Morphologically, (Figure 7.3.1) this fungus showed close similarities to *Muscodor* sp. of the family Xylariaceae originally isolated from *Cinnamomum zeylanicum* by Strobel and coworkers.[203] Since it does not show 100% homologies to all the published sequences, this fungus was designated as a new isolate, UM # 10 M.

An EtOAc extract of the culture broth of UM # 10 M, was fractionated over silica gel, followed by separation over Sephadex LH-20, and PTLC to afford compounds **5**-**7**. Compounds **5**[201] (19,20-epoxycytochalasin C) and **6**[202] (19,20-epoxycytochalasin D) (Figure 7.3.2) have been isolated from *Xylaria obovata*[201] and *X. hypoxylon*,[202] respectively, and were identified by comparing their spectroscopic and physical data with reported data.



5 **6** **7**

Figure 7.3.2. Structure of cytochalasins (**5**-**7**)

Compound **7** was obtained as a white amorphous powder, and HRESIMS determined its molecular formula to be $C_{30}H_{37}NO_6$, one oxygen less than that of **5.** Comparison of the ^{1}H (Figure 7.3.4) and ^{13}C NMR (Figure 7.3.5) resonances of **7** with those of **5** showed similarities except for the changes observed due to the absence of the hydroxy group at C-18 (Table 7.3.1). The ^{1}H NMR spectrum of **7** showed a -CH_3 doublet (δ_H 1.32) for 23-CH_3 instead of a -CH_3 singlet (δ_H 1.54) as in **5** indicating the absence of the C-18 hydroxy group. HMBC correlation (Figures 7.3.6 and 7.3.7) of H-18 (δ_H 2.18) with C-20 (δ_C 57.6), C-19 (δ_C 58.6), and C-17 (δ_C 216.9), in combination with COSY (Figure 7.3.8) cross peaks between the C-18 methine doublet (δ_H 2.18) and the C-19 oxymethine multiplet (δ_H 3.01) supported the gross structure of **7** to be the 18-deoxy analogue of **5,** thus named as 18-deoxy-19,20-epoxycytochalasin C (**7**)**.** Relative configuration at C-18 was assigned from ROESY correlations of **7** (Figure 7.3.9. and 7.3.10). In the ROESY spectrum H-18 displayed interactions with H-19 indicating the cofacial orientation of these protons. Since the analogues **5**-**7** presumably share a common biosynthetic origin, compound **7** also may possess 3*S*, 4*R*, 5*Z*, 7*S*, 8*R*, 9*R*, 13*E*, 16*S*, 18*S*, 19*R*, 20*S*, 21*S* absolute configuration.

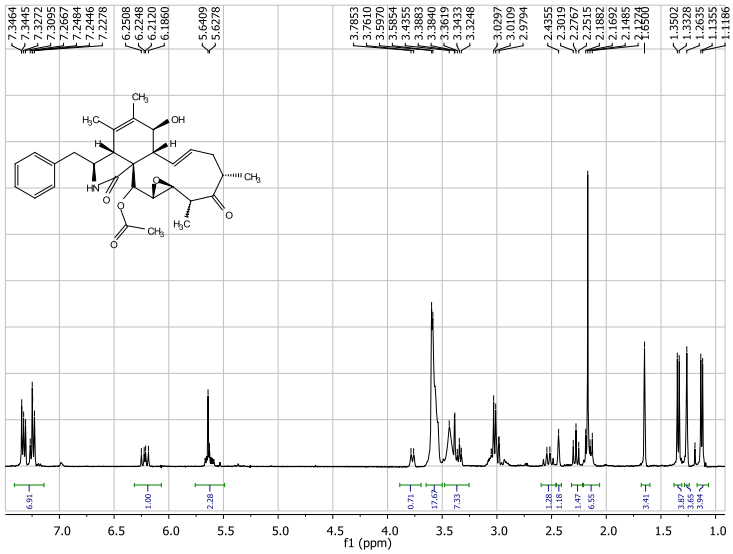


Figure 7.3.3. ^{1}H NMR spectrum of **7**

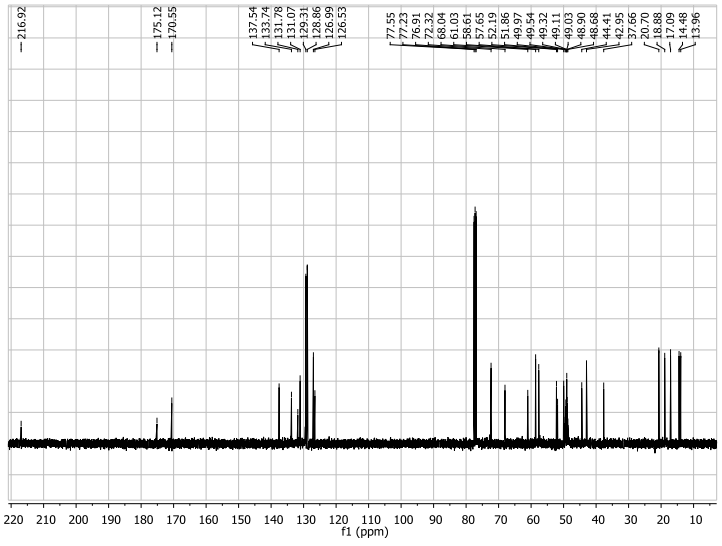


Figure 7.3.4. ^{13}C NMR spectrum of **7**

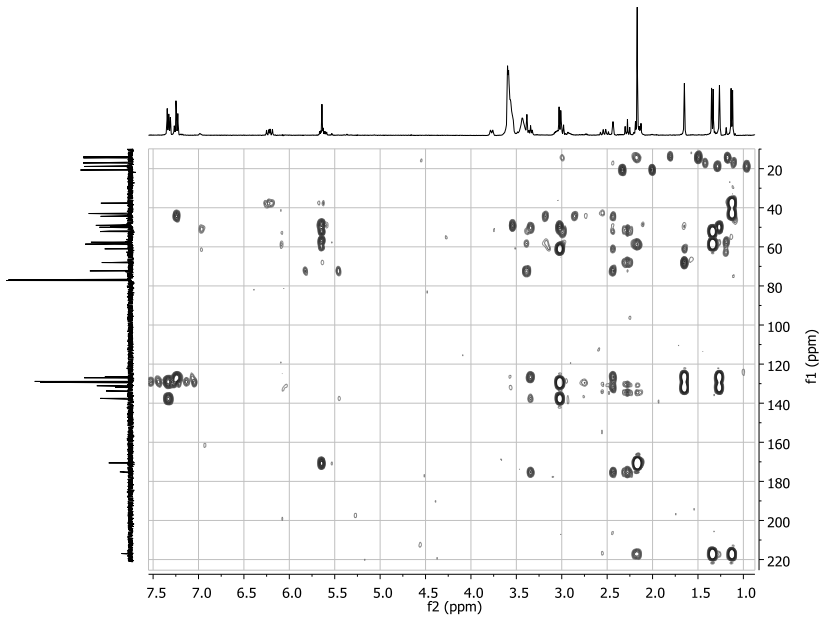


Figure 7.3.5. HMBC spectrum of **7**

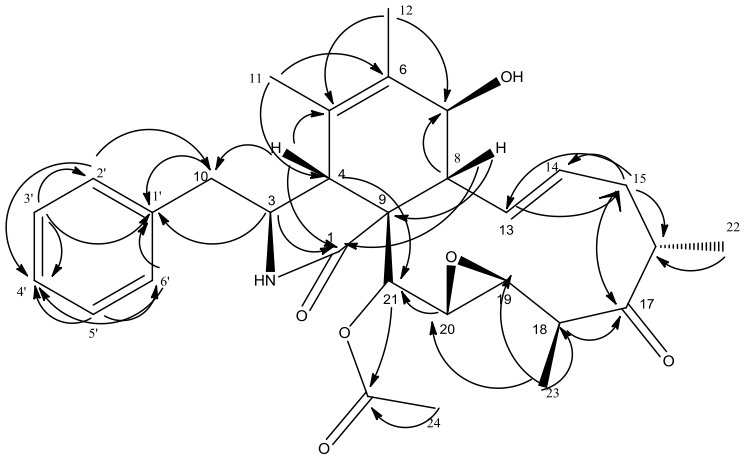


Figure 7.3.6. HMBC (⟶) correlations of **7**

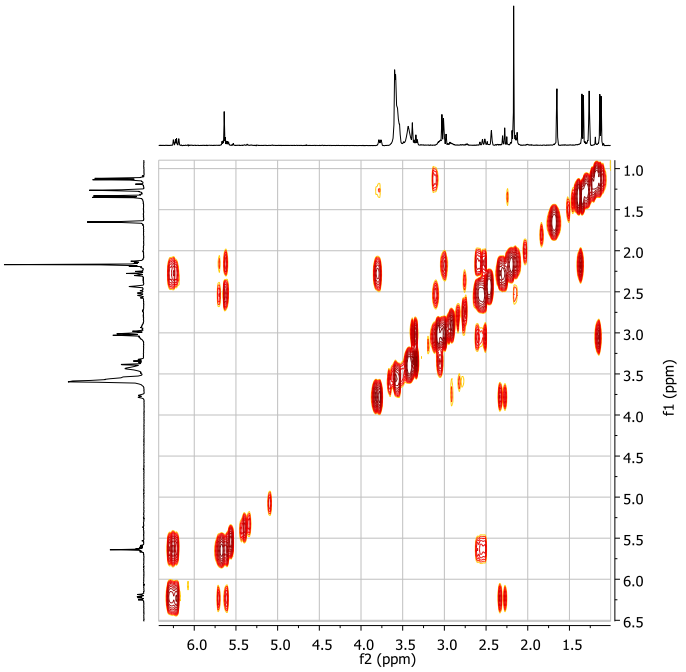


Figure 7.3.7. COSY spectrum of **7**

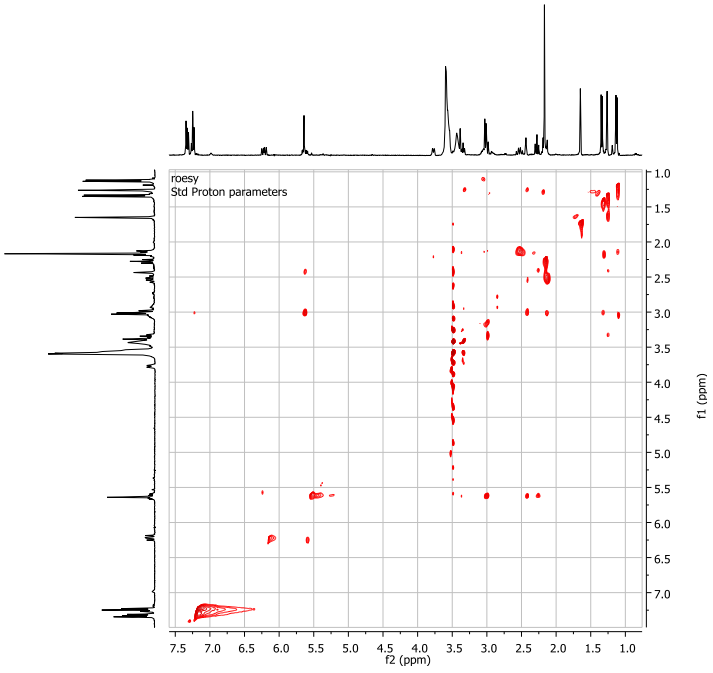


Figure 7.3.8. ROESY spectrum of **7**

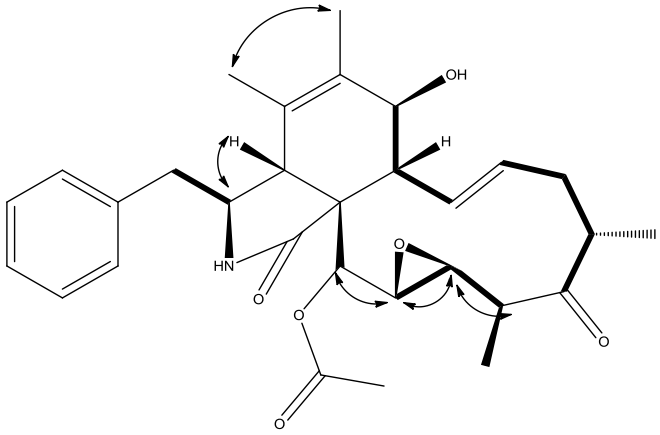


Figure 7.3.9. COSY (—) and ROESY (↷) correlations of **7**

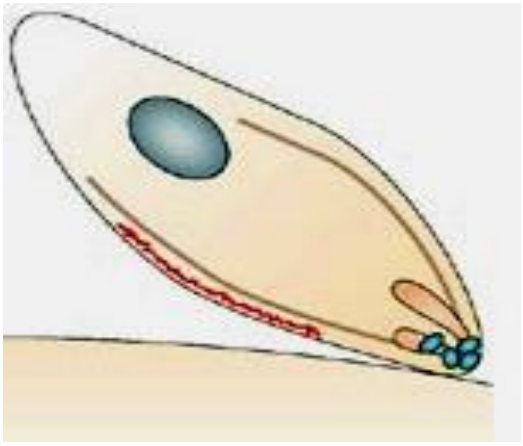


Figure. 7.3.10. Gliding motility in apicomplexan parasites

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Table 7.3.1. ^{1}H and ^{13}C NMR data of **5-7** in $CDCl_3$-Methanol-d_4

Carbon	**5**		**6**		**7**	
	δ_C [b]	δ_H [a] (J in Hz)	δ_C [b]	δ_H [a] (J in Hz)	δ_C [b]	δ_H [a] (J in Hz)
1	175.0		175.0		175.1	
3	61.0	3.35, m	54.1	3.28, s	61.1	3.32, m
4	49.9	2.50, s	49.9	2.25, m	49.9	2.43, bs
5	126.4		32.3	2.53, m	128.5	
6	131.8		148.0		131.7	
7	68.1	3.76, d (12)	70.2	3.78, d, (12.0)	68.1	3.77, d
8	48.7	2.25, t (12)	46.2	2.59, m	49.0	2.27, dd, (10, 10)
9	52.0		53.0		51.8	
10	44.1	3.03, d (10)	44.1	2.84, d, (12.0)	44.4	3.04, bd, (7.5)
11	16.7	1.19, s	13.7	13.1, d, (8.0)	14.1	1.26, s
12	13.8	1.62, s	113.7	5.24, bs, 5.02, bs	13.9	1.65, s
13	131.4	5.5, m	131.8	5.76, m	131.0	6.24, dd, (15.5, 10.4)
14	132.9	5.99, dd (12, 8)	132.9	5.82, m	133.7	5.61, m
15	37.5	2.63, q (12), 2.14, m	37.5	2.65, m	37.6	2.53, q (10.8), 2.14 (m)
16	41.7	3.27, s	41.7	3.24, m	42.9	2.98, d (1.92)
17	215.3		215.3		216.9	
18	76.3		76.2		52.1	2.18, m (overlap)
19	53.2	3.38, s	59.8	3.21, s	58.6	3.01, m (overlap)
20	59.9	3.25, m	53.0	3.46, m	57.6	3.38, d (1.97)
21	72.0	5.75, s	72.0	5.43, s	72.3	5.69, s
22	18.9	1.21, d (12)	18.9	1.19, d, (6.6)	18.8	1.12, d (6.7)
23	21.5	1.54, s	21.5	1.53, s	17.1	1.32, d (6.9)
24	170.4		170.4		170.5	

25	20.5	2.18, s	20.5	2.17, s	20.7	2.15, s
1'	137.3		137.3		136.9	
2',6'	129.2	7.28, m	129.2	7.29, m	129.4	7.26, m
3',5'	128.6	7.33, m	128.6	7.34, m	128.7	7.33, m
4'	126.8	7.25, m	126.9	7.25, m	126.9	7.25, m

[a]^{1}H NMR spectra recorded at 400 MHz, [b]^{13}C NMR spectra recorded at 100 MHz

Compounds **5-7** showed potent selective in vitro antiplasmodial activity against chloroquine-sensitive (D6) and -resistant (W2) strains of *P. falciparum* (Table 7.3.2) and phytotoxic activity against both a monocot (bentgrass, *Agrostis stolonifera*) and a dicot (lettuce, *Lactuca sativa*) (Table 7.3.3). These compounds showed no cytotoxicity against a panel of solid tumor cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and kidney epithelial cells ($LLC\text{-}PK_{11}$) (Table 7.3.4).

Antiplasmodial[57] and antitoxoplasma[199] activities of cytochalasins have been reported. These compounds have been shown[11] to inhibit the actin-based gliding motility (Figure 7.3.11) and impair host cell invasion of apicomplexan parasites of *Toxoplasma gondii*[199] and *P. falciparum*.[204]

Evaluation of antimalarial activity of compound **5** in a mouse model showed that it was toxic to mice at subtherapeutic doses (Table 7.3.5), thus precluding it as an antimalarial lead.

7.4. Biological Activities

Table 7.3.2. Antiplasmodial activity of **5-7**

Compound	Chloroquine-sensitive (D6)-clone		Chloroquine-resistant (W2)-clone		Cytotoxicity to Vero cells
	IC_{50} nM	S. I.	IC_{50} nM	S. I.	IC_{50} nM
19,20-epoxycytochalasin C (**5**)	0.07	>129.8	0.05	>181.8	NC
19,20-epoxycytochalasin D (**6**)	0.04	226.7	0.04	226.7	NC
18-dehydroxy-19,20-epoxycytochalasin C (**7**)	0.56	>7	0.19	>49.6	NC
chloroquine[a]	0.03	496.6	0.31	48.1	NC
artemisinin[a]	0.02	845	0.01	1690	NC

[a] Positive controls

NC, not cytotoxic

NA, not active

S. I. (selectivity index) = IC_{50} for cytotoxicity/IC_{50} for antiplasmodial activity

Table 7.3.3. Phytotoxic activity of **5-7**

Compound	Lettuce	Bentgrass
19,20-epoxycytochalasin C (**5**)	3	2
19,20-epoxycytochalasin D (**6**)	3	3
18-dehydroxy-19,20-epoxycytochalasin C (**7**)	3	4

Ranking based on scale of o to 5

0 = no effect

5 = no growth

Solvent used, 10% acetone

Concentration used, 1mg/mL

Table 7.3.4. Cytotoxic activity [IC50 (μM)] of **5-7**

Compound	SK-MEL	KB	BT-549	SK-OV-3	LLC-PK_{11}
19,20-epoxycytochalasin C (**5**)	8.02	NA	NA	NA	NC
19,20-epoxycytochalasin D (**6**)	>19.1	NA	7.84	NA	8.4
18-dehydroxy-19,20-epoxycytochalasin C (**7**)	12.41	19.7	6.89	NA	>19.7
doxorubicin[a]	1.29	2.12	1.83	1.47	1.28

NA: not active at the highest test concentration of 10 μg/mL.

[a] Positive control.

IC_{50} = concentration casusing 50% growth inhibition

SK-MEL = human malignant melanoma

KB = human epidermal carcinoma

BT-549 = human breast carcinoma (ductal)

SK-OV-3 = human ovary carcinoma

$LLC\text{-}PK_{11}$ = pig kidney epithelial

Table 7.3.5. In vivo antimalarial activity of **5**

Treatment (PO)	Dose (mg/kg × # days post infection)	% Parasitemia suppression[1]		Survival[2]	Day of Death	MST[3]	Cure[4]
		Day 5	Day 7				
Vehicle	× 3	-	-	0/5	13/5/13/14/14	11.8	0/5
chloroquine[a]	100 × 3	100	100	5/5	28/28/28/28/28	28	2/5
5	100 × 3	33.87	71.36	0/5	17/3/5/1/0	5.2	0/2

[1]% suppression in parasitemia is calculated by considering the mean parasitemia in the vehical control as 100%.

Parasitemia suppression < 80% is considered as non-significant

[2]Number of animals that survived day 28/total animals in group (the day of the death-post infection)

[3]% MST – mean survival time (days)

[4]Number of mice without parasitemia (cured) till day 28 post-infection

7.5. Experimental

7.5.1. General

Carried out as previously described in Section 7.1.4.1.

7.5.2. Isolation of the Fungus from *T. taxifolia*

UM # 10 M was isolated as previously described in Section 7.1.5.2.

7.5.3. Fermentation, Extraction, and Purification

Fungus UM # 10 M was cultured in four conical flasks (2 L) containing 500 mL of PDB and incubated at 27 ^{o}C for 30 days on an orbital shaker at 100 rpm. Mycelia and broth were

separated by filtration and extracted with EtOAc (×3). The organic layer from the broth was evaporated to give a brown/black residue (625 mg).

This extract (600 mg) was chromatogtaphed on Sephadex LH-20 and eluted with 80% MeOH in $CHCl_3$ to give 10 fractions. Fractions 2, 3, and 4 showed antimalarial activity. Fraction 2 was subjected to silica gel gravity column chromatography using hexanes, CH_2Cl_2, and MeOH gradient as the mobile phase to yield five fractions. Subfraction 3 which showed antimalarial activity was further separated by preparative thin layer chromatography (PTLC) using 0.4 % MeOH in $CHCl_3$ (× 3) as the developing solvent to obtain 19,20-epoxycytochalasin C (**5,** 85.0 mg). Fractions 3 and 4 were also subjected to PTLC using 0.4 % MeOH in $CHCl_3$ (× 3) as the developing solvent to afford 19,20-epoxycytochalasin D (**6,** 4 mg), and the new analogue, 18-deoxy-19,20-epoxycytochalasin C (**7,** 2 mg)**.**

***Compound 5* :** 19,20-Epoxycytochalasin C; white amorphous powder; ^1H, ^{13}C NMR data (Table 7.3.1) were consistent with literature values.[201] $[\alpha]^{26}_D$ -13 (*c* 0.5, $CHCl_3$) lit[201] -6.8; HRESIMS $[M + H]^+$ *m/z* 524.264 (lit[201] $[M+ H]^+$ 524.2)

***Compound 6* :** 19,20-Epoxycytochalasin D; white amorphous powder; ^1H, and ^{13}C NMR, data (Table 7.3.1) were consistent with literature values.[202] $[\alpha]^{26}_D$ -113 (*c* 0.085, $CHCl_3$) lit[202] -228; HRESIMS $[M + H]^+$ *m/z* 524.283 (lit[202] $[M+ H]^+$ 524.2)

***Compound 7* :** 18-Deoxy-19,20-epoxycytochalasin C; white amorphous powder; ^1H and ^{13}C NMR (see Table 7.3.1.) $[\alpha]^{26}_D$ -2.4 (*c* 0.09, $CHCl_3$); HRESIMS $[M + H]^+$ *m/z* 508.2703 (calcd for $[C_{24}H_{33}NO_4 + H]^+$ 508.2620)

7.5.4. Biological Assay

7.5.4.1. Antiplasmodial Assay

In vitro antiplasmodial activity was determined as described earlier.[69]

7.5.4.2. Phytotoxic Assay

The bioassay for phytotoxicity was carried out as described[70] by Dayan and coworkers.

7.5.4.3. Cytotoxicity Assay

In vitro cytotoxicity was determined against a panel of mammalian cells as described[76] earlier.

7.5.4.4. In Vivo Antimalarial Assay

In vivo antimalarial activity was determined as described in Chapter 4 (4.5.7).

SECTION 7.4: ANTIMALARIALS FROM *BOTRYOSPHAERIA DOTHIDEA*

7.4.1. Summary

The culture broth extract of *Botryosphaeria dothidea* showed antimalarial and phytotoxic activities. Bioactivity-guided fractionation of this extract afforded a mixture of two known phytotoxins, sapinopyridiones (**8a)** and flavipucine (**8b**), one inactive α-alkyl-α,β-unsturated-γ-lactam (**9**), and three antiplasmodial α-alkyl-γ-lactam derivatives (**10**-**12**).

7.4.2. Introduction

Botryosphaeria dothidea was isolated from seeds collected from cultivated *Torreya taxifolia* trees that included plants with disease symptoms. This fungus is a member of the family Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) which includes species of telemorph and anamorph stages of *Diplodia*, *Botryosphaeria*, *Fusicoccum*, *Dothiorella*, *Lasiodiplodia*, and *Sphaeropsis*.[205] *Botryosphaeria* is the teleomorph (sexual) stage of the anamorph (asexual) genus *Sphaeropsis.* Chemical investigation of *Sphaeropsis sapinea* has been reported[206] by Evidente et al.. *S. sapinea* is known[206] to be identical to *B. dothidea*. *S. sapinea*, also known as *Diplodia pinea*, is the causative agent for shoot blight and canker diseases of pines and other conifers.[208]

Several phytotoxins, sapinopyridione,[206] sphaeropsidins,[209-213] sapinofuranones,[214] sphaeropsidones,[215] and dimedone methyl ethers[215] have been reported from *S. sapinea* isolated from conifers. Most of the fungi of this family are known[205] as endophytes and/or latent pathogens and cause diseases with the onset of environmental stress factors due to climate changes. This is the first report of the isolation of a fungus producing compounds capable of severe plant pathogenicity from the seeds of *T. taxifolia.*

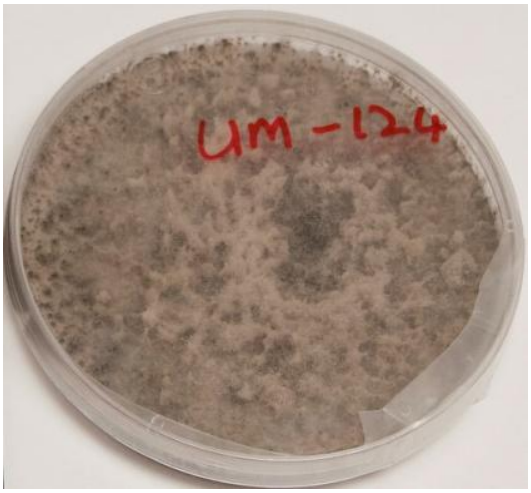


Figure 7.4.1. PDA plate of *B. dothidea*

Bioassay-guided fractionation of an EtOAc extract of the potato dextrose broth of *B. dothidea* resulted in the isolation of a mixture of the known closely related phytotoxins, sapinopyridiones (**8a**)[206, 216] and flavipucine (**8b**),[216, 217] and four new α-alkyl -γ-lactam derivatives (**9**-**12**). Three of these (**10**-**12**) showed potent, selective antiplasmodial activity.

7.4.3. Results and Discussion

Analysis[68] of 18S rDNA of the fungus gave 100 % sequence identities to *B. dothidea.*

An EtOAc extract of *B. dothidea* culture broth was fractionated by silica gel column chromatography followed by Sephadex LH-20 gel filtration (MeOH) and repeated reversed-phase HPLC (MeOH-H_2O) to afford a mixture of two known phytotoxins,[216] sapinopyridione (**8a)** and flavipucine (**8b**), and four related new compounds (**9-12**). Structure elucidation of sapinopyridione (**8a)** and flavipucine (**8b**) was carried out by comparing the spectroscopic data with reported data.[206, 217]

Compound **9** was isolated as a brown amorphous solid, and its molecular formula was determined as $C_{22}H_{29}NO_5$ by HRESIMS. The ^{1}H (Figure 7.4.2) and ^{13}C NMR (Figure 7.4.3) spectra of **9** (Table 7.4.1) indicated the presence of 22 carbons resonances that consisted of eight quaternary, six methine, two methylene, and six methyl carbons. Analysis of ^{1}H and ^{13}C NMR data of **9** indicated that it was an α-alkyl-α,β-unsaturated-γ-lactam derivative, related to epolactaene[218] (Figure 7.4.4). Comparison of the ^{1}H and ^{13}C NMR data of these compounds showed that they had the same side chain but differed in the 2-pyrrolidone ring. These differences were attributable to the replacement of the epoxide and the hydroxy groups in the 2-pyrrolidone ring of epolactaene by a double bond and methoxy group, respectively, in **9**. In the HMBC spectrum of **9** (Figure 7.4.5), observed cross-peaks of H-1 (δ_H 1.72) with C-2 (δ_C 139.9) and C-3 (δ_C 130.3); H-2 (δ_H 6.96) with C-3 (δ_C 130.3) and C-19 (δ_C 167.8); H-4 (δ_H 5.98) with C-21 (δ_C 14.3), C-19 (δ_C 167.8), and C-2 (δ_C 139.9); H-21 with C-6 (δ_C 135.6) and C-4 (δ_C 112.1); H-8 (δ_H 2.35) with C-6 (δ_C 135.6), C-7 (δ_C 128.2), C-9 (δ_C 30.1), and C-10 (δ_C 150.0); and H-22

(δ_H 1.82) with C-10 (δ_C 150.0) and C-6 (δ_C 192.1) confirmed the structure of the side chain. Further, HMBC correlations of the -OCH_3 singlet at δ_H 3.18 with C-15 (δ_C 139.9), the 18-CH_3 singlet at δ_H 1.61 with C-15 (δ_C 89.23) and C-14 (δ_C 149.5), and the H-14 olefinic proton singlet at δ_H 6.83 with C-17 (δ_C 168.3) confirmed the structure of the 2-pyrrolidone moiety and its link to the side chain. Similarly, a methyl triplet (δ_H 1.89) at C-22 showed correlations with C-12 (δ_C 191.4) and C-10 (δ_C 148.6), and other COSY (Figure 7.4.6) and HMBC (Figure 7.4.7) correlations supported this structure (Figure 7.4.8). The configuration of the olefinic bond between C-6 and C-7 was determined to be *E* (*trans*) by the coupling constant (J = 15.6 Hz). Due to the lack of NOESY or ROESY data, the configuration of the remaining double bonds could not been determined. However, ROESY data indicated that olifinic bonds between C-2 and C-3, C-4 and C-5, and C-10 and C-11 in compound **10** were in *E* configuration (vide infra). Since compounds **9** and **10** presumably share a common biosynthetic origin these bonds in compound **9** also have the same configurations.

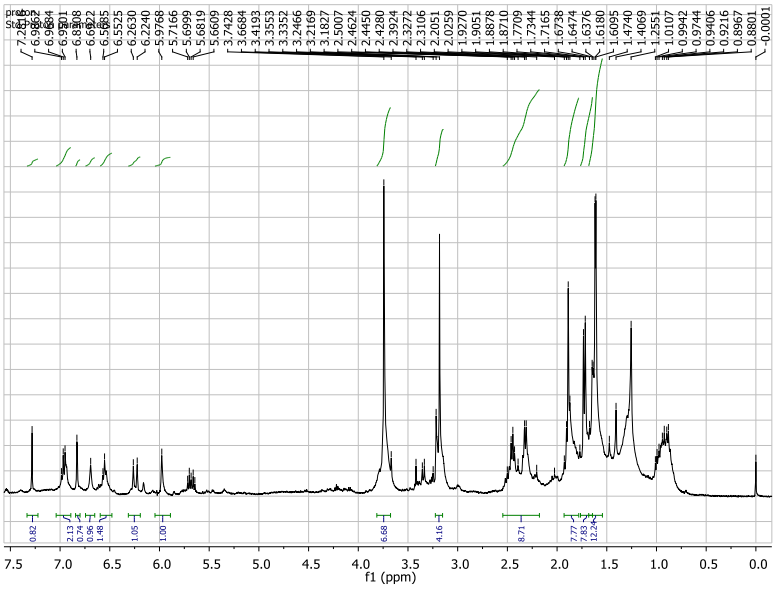


Figure 7.4.2. ^{1}H NMR spectrum of **9**

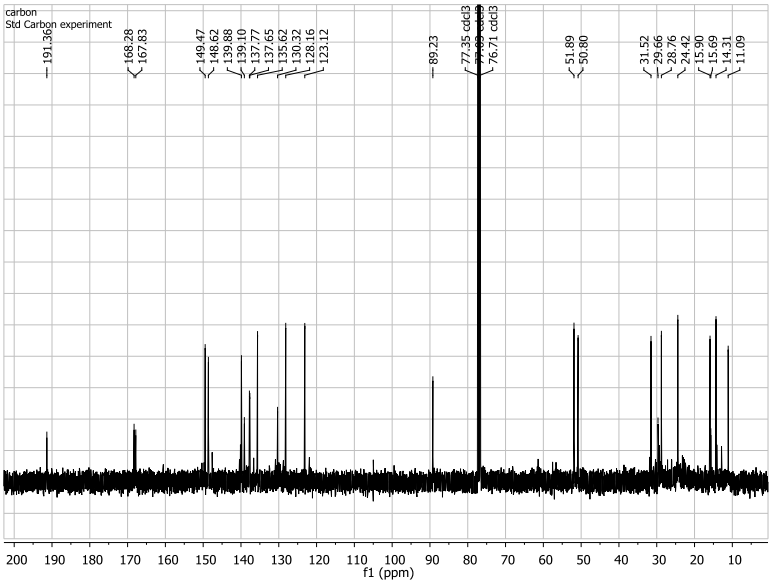


Figure 7.4.3. ^{13}C NMR spectrum of **9**

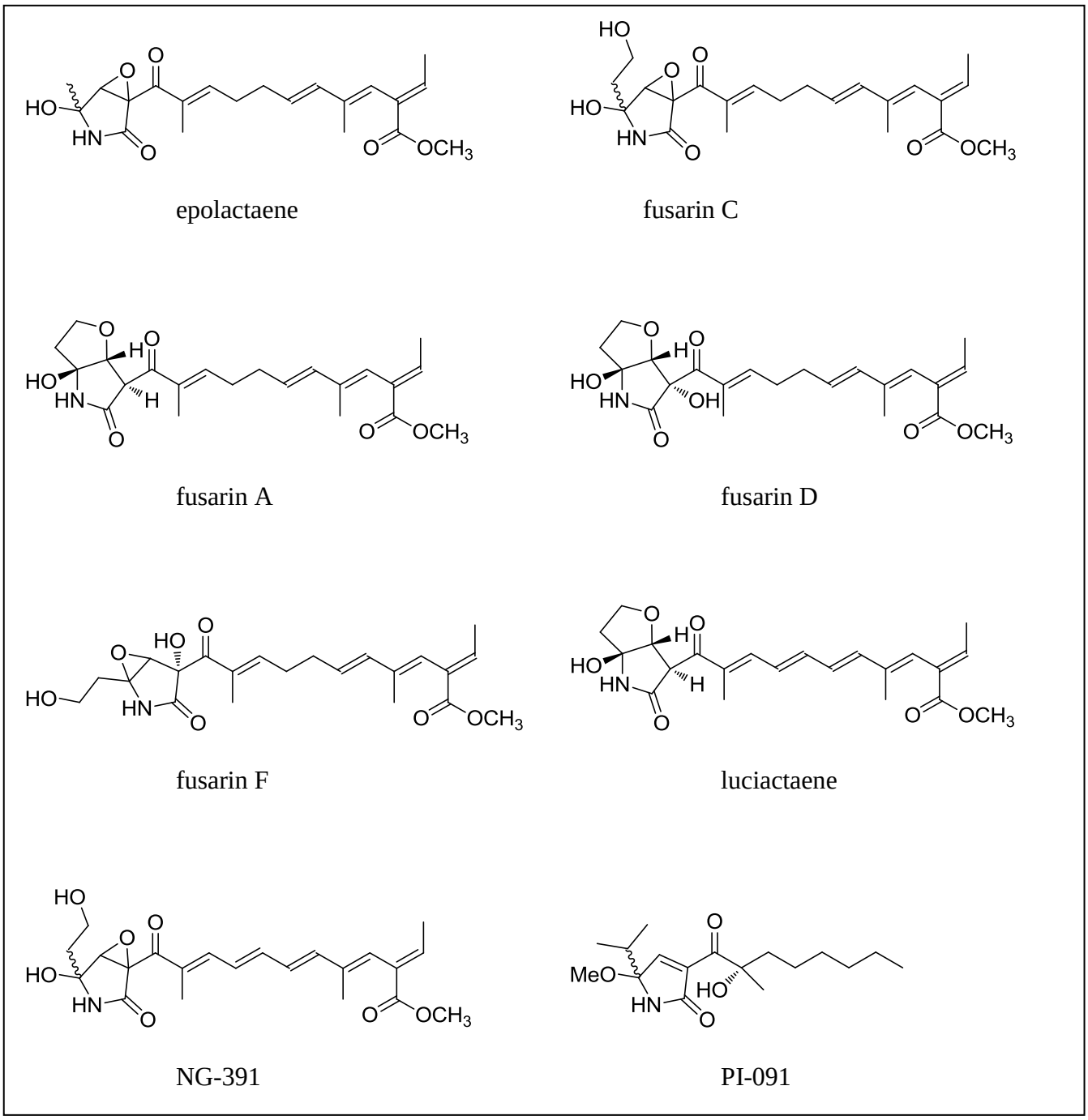


Figure 7.4.4. Structurally related compounds

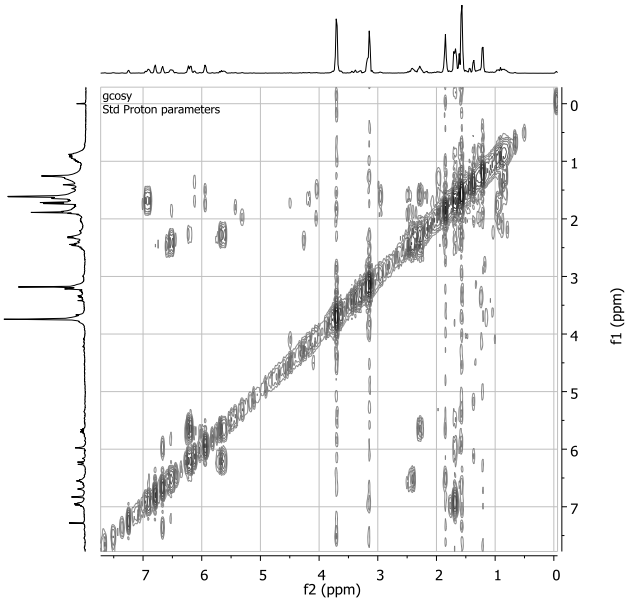


Figure 7.4.5. COSY spectrum of **9**

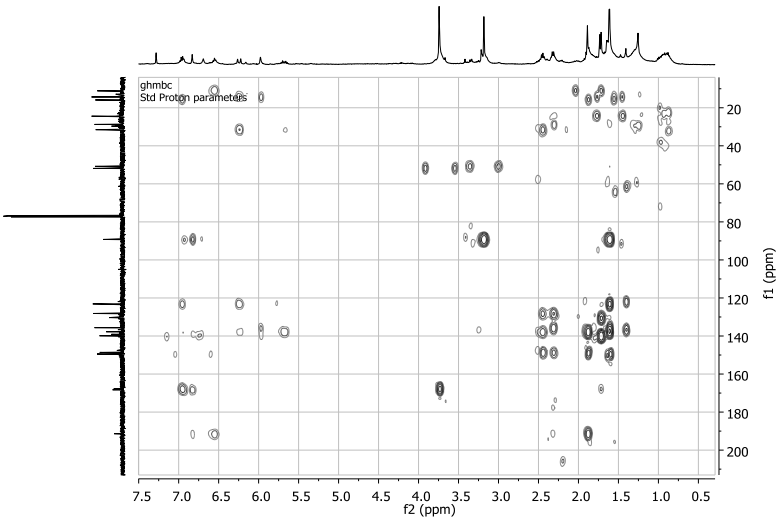


Figure 7.4.6. HMBC spectrum of **9**

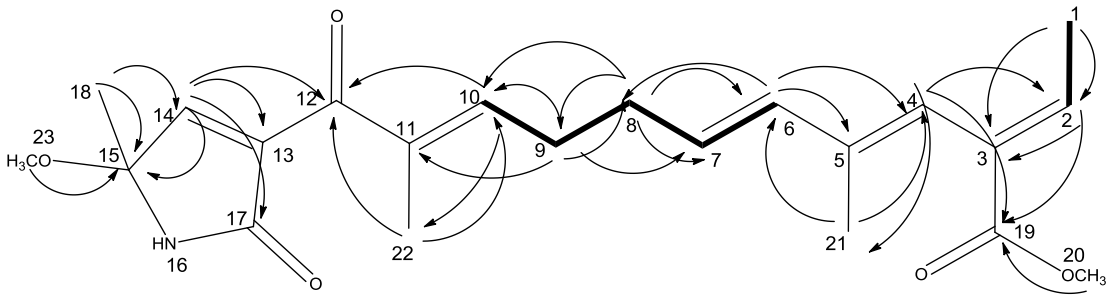
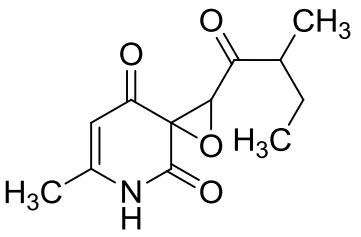


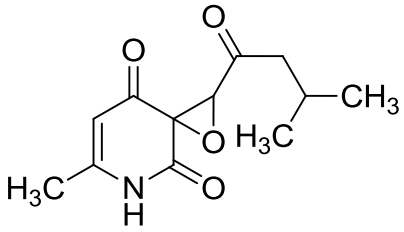
Figure 7.4.7. HMBC ( ) and COSY ( ) correlation of **9**

The antimalarial fraction of the extract was found to be a mixture of three minor diastereomers (**10**-**12**) which was separated by preparative HPLC. Owing to lack of sufficient material and the apparent instability of compounds, only tentative structures could be proposed for these compounds.

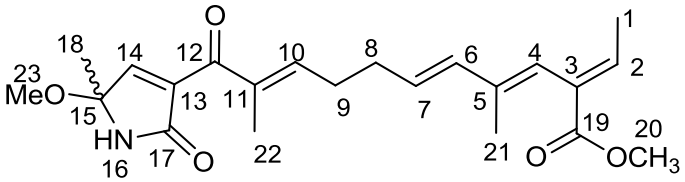
Compound **10** gave the molecular formula, $C_{24}H_{33}NO_7$, by HRESIMS. Its NMR spectra showed resonances due to the same side chain as **9,** and COSY and HMBC data provided confirmatory evidence (Figures 7.4.9–7.4.22). Comparison of the remaining resonances with those of **9** indicated the absence of the olefinic bond and the methoxy group in the 2-pyrrolidone ring and the presence of three oxygenated carbons due to a glycerol moiety.



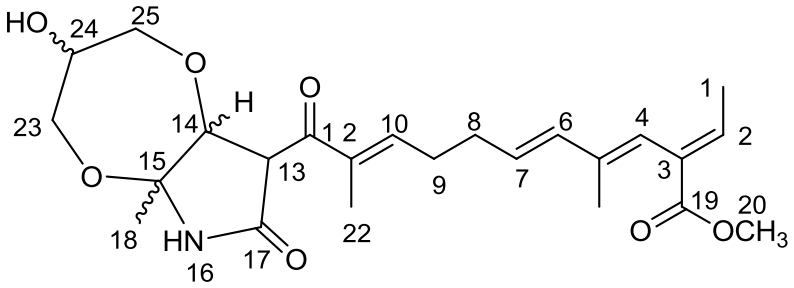
sapinopyridione (**8a**)



flavipucine (**8b**)



9



10-**12**

Figure 7.4.8. Structure of compounds isolated from *B.dothidea*

Table 7.4.1. ^{1}H and ^{13}C NMR data of compounds **9-12** in $CDCl_3$-Methanol-d_4

Position	**Epolactaene**		**9**		**10**		**11**		**12**	
	δ_C [b]	δ_H [a] (J in Hz)	δ_C [b]	δ_H [a] (J in Hz)	δ_C [b]	δ_H [a] (J in Hz)	δ_C [b]	δ_H [a] (J in Hz)	δ_C [b]	δ_H [a] (J in Hz)
1	16.0	1.72, dd, 1.2, 7.3	15.9	1.72, d, 7.1	15.8	1.55, d, 6.9	15.7	1.62, d, 7.1	15.8	1.56, d, 9.5
2	140.9	6.93, dq, 1.0, 7.3	139.9	6.93, q, 7.1	140.1	6.70, q, 10.0	140.0	6.85, q, 7.1	140.1	6.80, m
3	131.9		130.3		130.5		130.3		130.4	
4	123.7	5.94, br s	123.1	5.97, br s	122.6	5.76, s	122.6	5.83,s	122.6	5.77,s
5	139.6		137.6		138.0		138.1		137.9	
6	136.6	6.27, d, 15.6	135.6	6.23, d, 15.6	135.0	6.1, d, 15.6	134.9	6.15, d, 15.4	134.9	6.15, d, 15.7
7	129.7	5.77, dt, 15.6, 7.8	128.2	5.68, dt, 15.2, 6.6	128.8	5.60, dt, 15.4, 6.9	128.7	5.65, dt, 15.6, 7.2	128.8	5.56, m
8	32.6	2.35, m	31.5	2.30, m	31.6	2.20, m	31.5	2.26, m	31.5	2.20, m
9	30.1	2.47, m	28.4	2.40, m	29.4	2.30, m	29.4	2.35, m	29.4	2.30, m
10	150.0	7.01, dt, 1.0, 7.3	148.6	6.56, t, 6.4	146.4	6.59, t, 4.6	146.8	6.67, t, 6.1	147.3	6.61,m
11	137.2		137.8		138.8		137.9		137.9	
12	192.1		191.4		196.2		195.9		195.2	

13	63.9		139.1		50.0	3.21, s	49.28	3.28,s	49.15	3.23,s
14	66.1	3.98, s	149.5	6.83, s	76.5	4.29, s	77.0	4.7, s	77.3	4.07, s
15	84.8		89.2		83.9		83.1		85.2	
17	172.2		168.3		168.3		168.3		168.3	
18	22.2	1.51, s	24.4	1.61, s	20.3	1.46, s	20.2	1.52, s	20.2	1.47, s
19	169.6		167.8		168.3		168.3		168.3	
20	52.4	3.71, s	51.9	3.74, s	51.9	3.55, s	51.8	3.62, s	51.9	3.57, s
21	14.6	1.61, d, 1.3	14.3	1.62, s, 3.4	14.3	1.45, s	14.2	1.51, s	14.3	1.45, s
22	11.1	1.82, s	11.1	1.89, s	11.5	1.67, s	11.6	1.62, s	11.4	1.63, s
23					60.6	3.48, m	61.5	3.64, m	61.6	3.46, m (overlap)
24					70.4	3.65, m	68.3	3.73, m (overlap)	68.3	3.70, m (overlap)
25					61.9	3.40, d, 3.08	61.8	3.54, m	61.8	3.80 (overlap)

[a1]H NMR spectra recorded at 600 MHz. [b13]C NMR spectra recorded at 100 MHz.

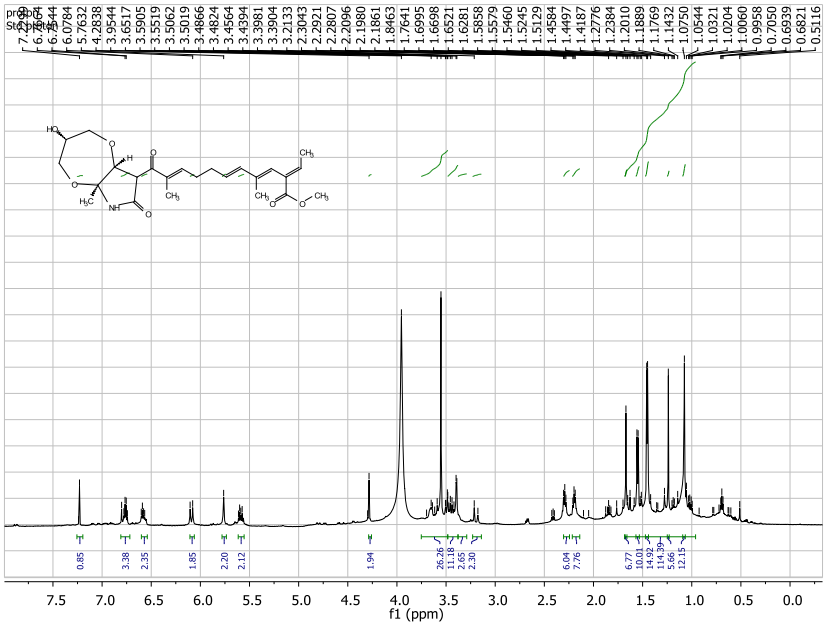


Figure 7.4.9. ^{1}H NMR spectrum of **10**

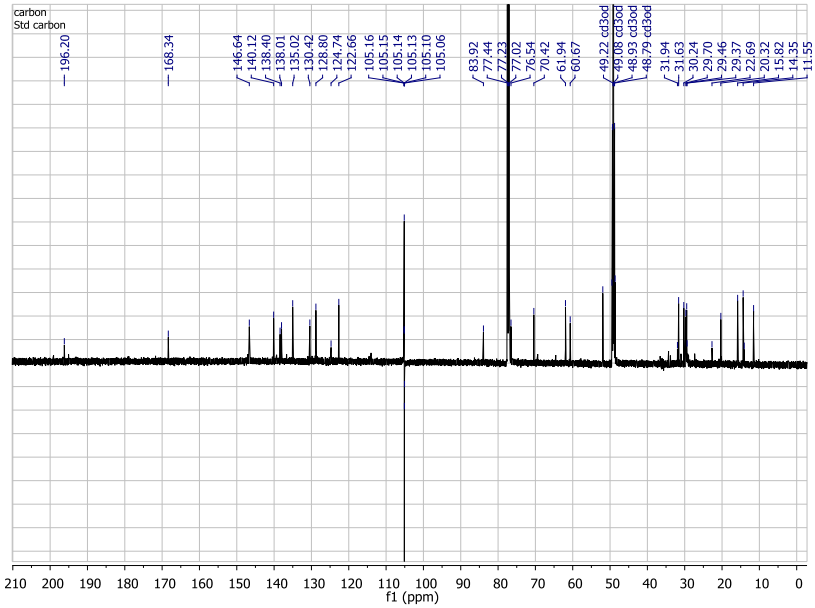


Figure 7.4.10. ^{13}C NMR spectrum of **10**

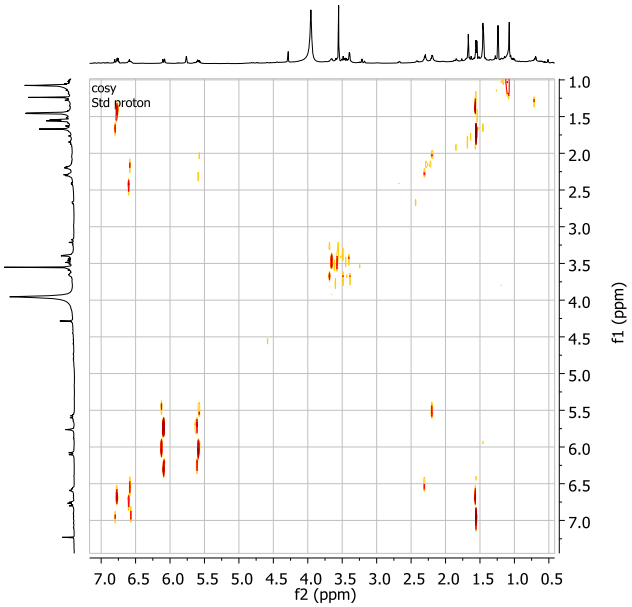


Figure 7.4.11. COSY spectrum of **10**

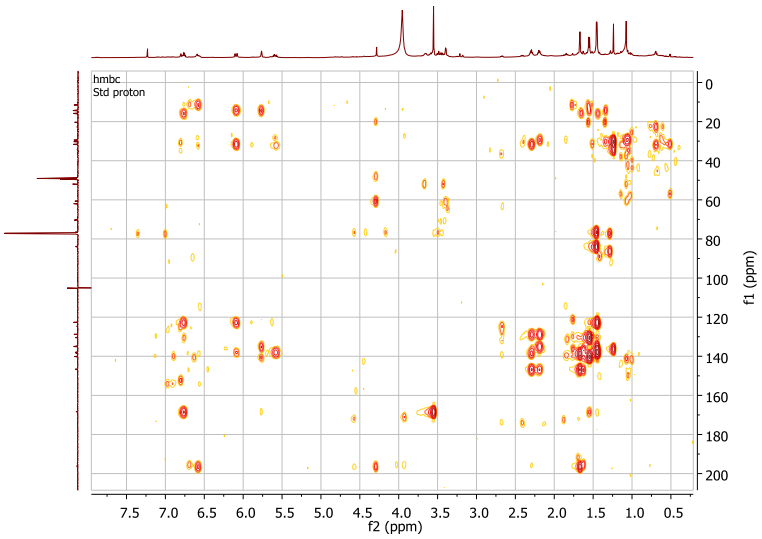


Figure 7.4.12. HMBC spectrum of **10**

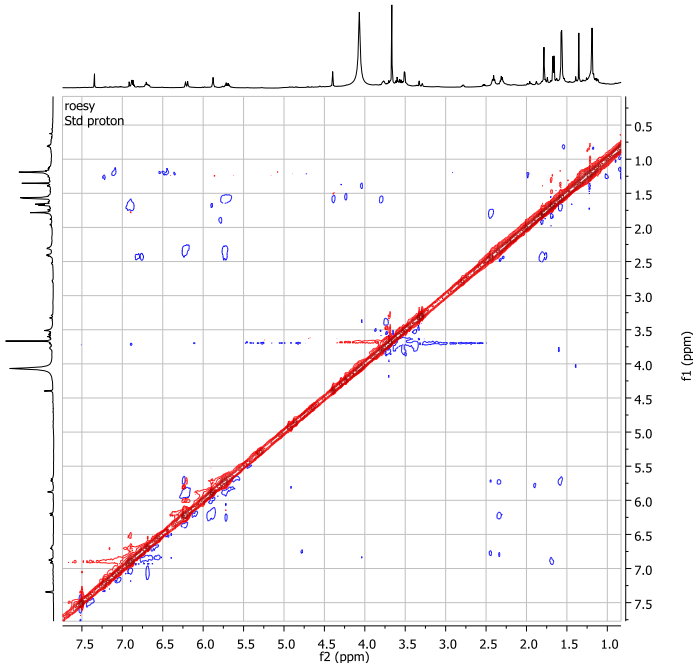


Figure 7.4.13. ROESY spectrum of **10**

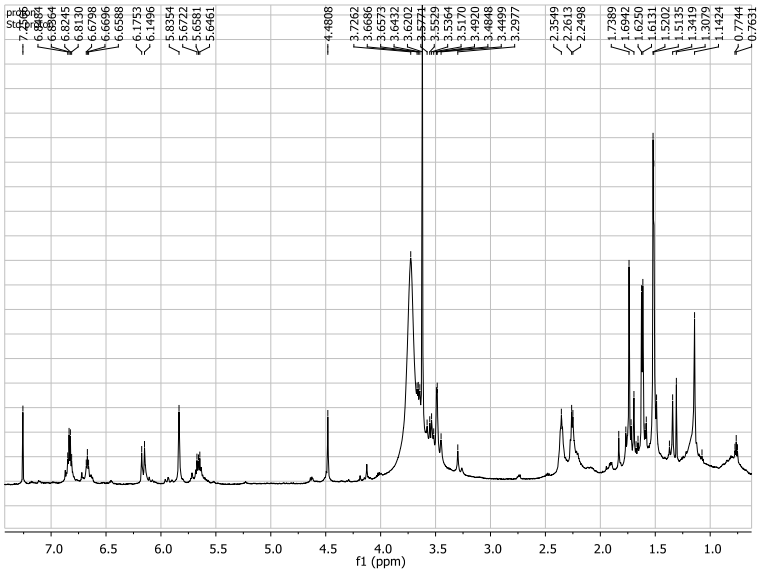


Figure 7.4.14. ^{1}H NMR spectrum of **11**

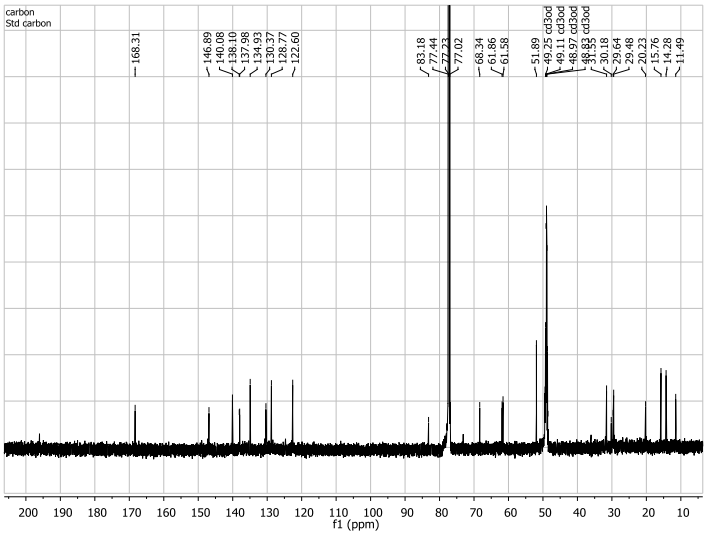


Figure 7.4.15. ^{13}C NMR spectrum of **11**

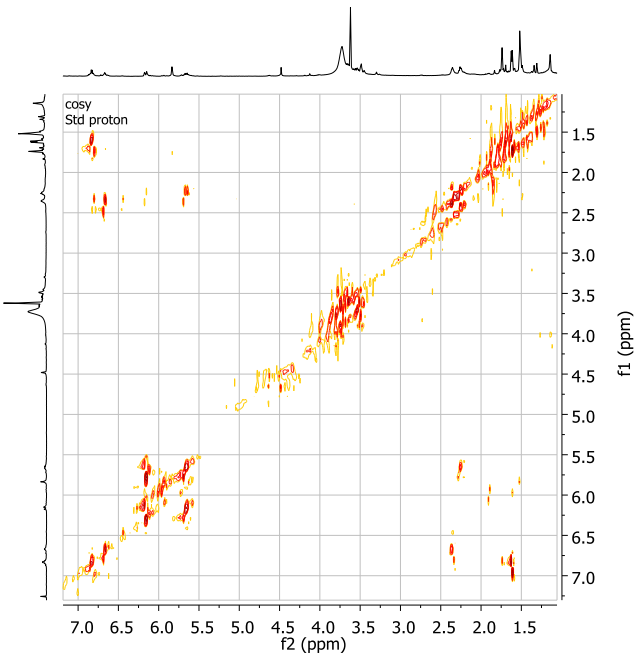


Figure 7.4.16. COSY spectrum of **11**

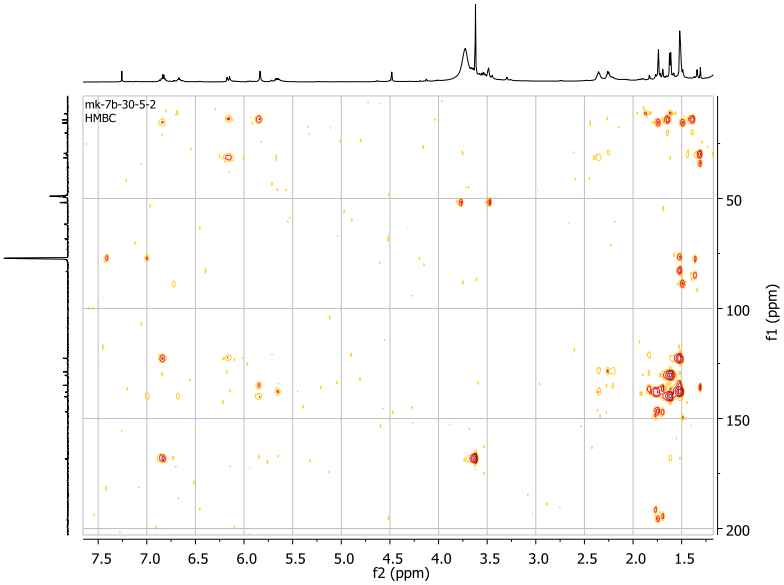


Figure 7.4.17. HMBC spectrum of **11**

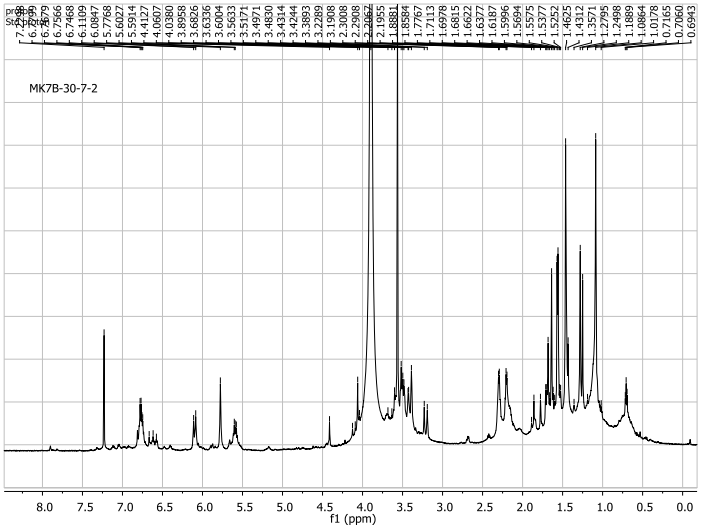


Figure 7.4.18. ^{1}H NMR spectrum of **12**

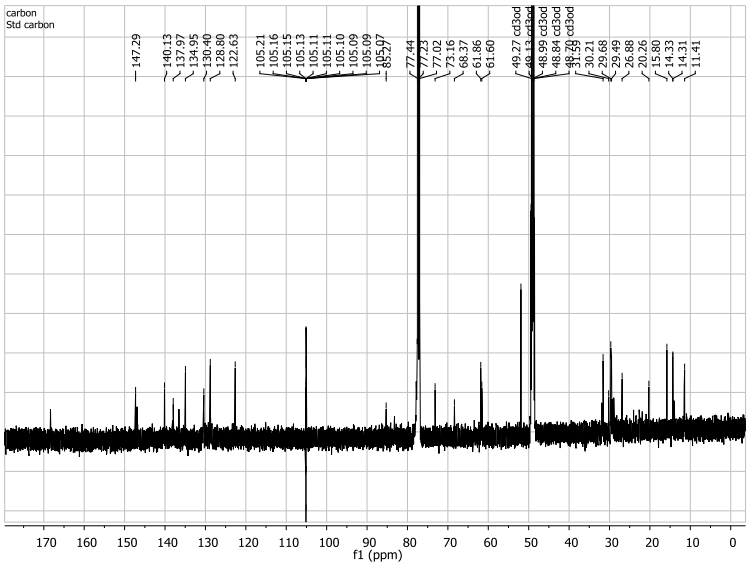


Figure 7.4.19. ^{13}C NMR spectrum of **12**

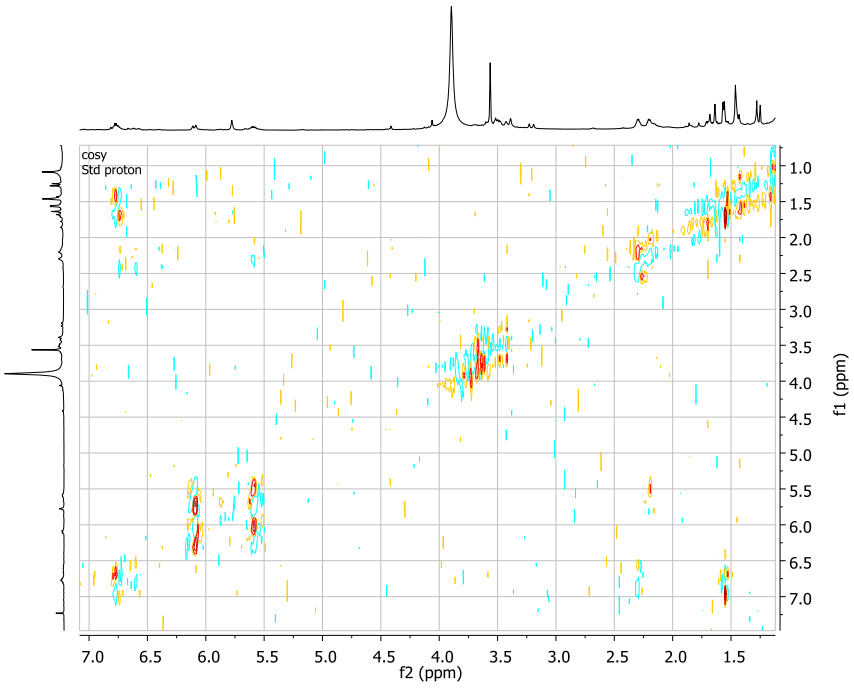


Figure 7.4.20. COSY spectrum of **12**

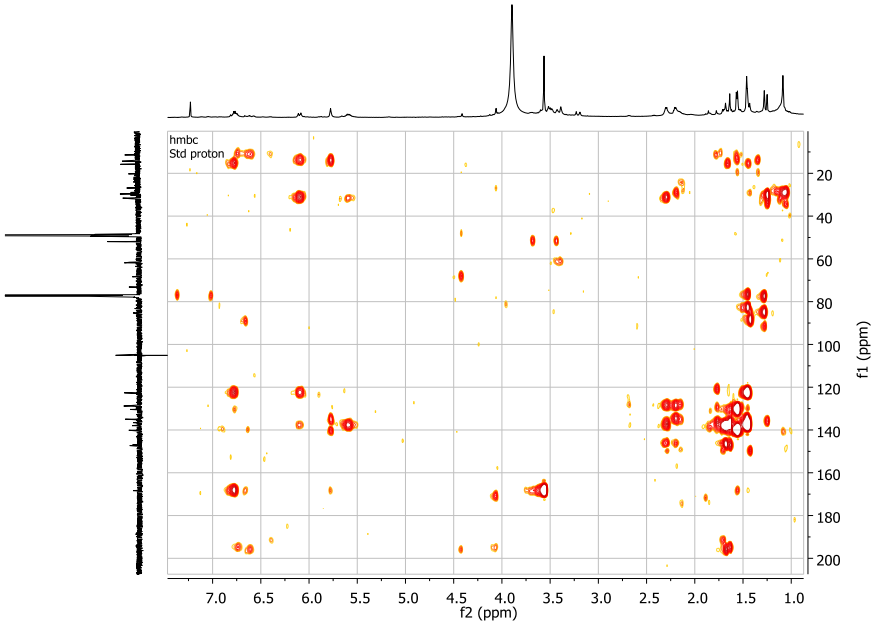


Figure 7.4.21. HMBC spectrum of **12**

The COSY (Figure 7.4.11) and HMBC (Figure 7.4.12) spectra of **10** showed, respectively, two oxymethylenes (δ_H 3.40 and 3.48) coupled with a methine multiplet (δ_H 3.65) and a methylene doublet (δ_H 3.40) correlating with C-23 (δ_C 60.6) and C-24 (δ_C 70.4), confirming the presence of a glycerol moiety. In the HMBC spectrum, H-14 of the 2-pyrrolidone ring had a cross peak with one of the oxygenated methylenes of glycerol, indicating one of its linkages. Based on the molecular formula $C_{24}H_{33}NO_7$, this compound has an additional index of deficiency. The polarity of **10-12** was comparable to that of **9** indicating that additional hydroxy groups were functionalized. Even though no other cross peaks between the glycerol moiety and the 2-pyrrolidone ring were visible in the HMBC spectrum due to high noise levels, it was probable that the second oxygenated methylene group of the former was linked to C-15 of the latter. The tentative structure of **10** and its COSY and HMBC correlations are shown in Figure

7.4.22. Large coupling (J = 15.6 Hz) between H-6 and H-7 showed that the olefin bond between C-6 and C-7 was in *E* (*trans*) configuration. ROESY correlations, as shown in the Figure 7.4.23 indicated the *E* configuration for the other double bonds in the side chain and *cis* orientation for two cyclic rings.

HRESIMS data confirmed that compounds **11** and **12** had the same molecular formula, $C_{24}H_{33}NO_7$ as that of **10**. NMR analysis showed that these compounds had identical side chains and the same gross structure. The addition of the glycerol moiety to the 2-pyrrolidone ring creates four stereogenic centers. Thus, these compounds **10**-**12** are likely diastereomeric as far as C-13, C-14, C-15, and C-24 are concerned? However, complete structure elucidation of these compounds was not possible due to lack of sufficient material.

Mass spectra, calculated for $C_{21}H_{26}NO_4$, of compounds **10**-**12** had base peaks at m/z = 355.1784. This fragment could form from the parent due to the loss of glycerol as shown in the Figure 7.4.24, further supporting the proposed structures. Compound **9** may be the biosynthetic precursor of these compounds.

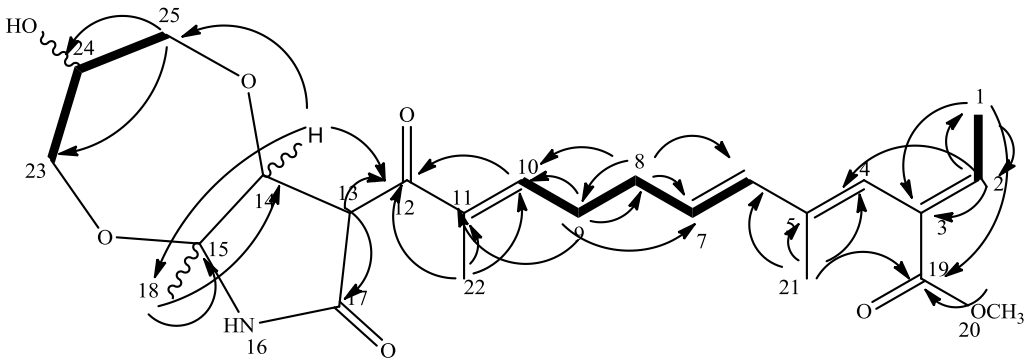


Figure 7.4.22. HMBC ( ) and COSY ( ) correlations of **10**

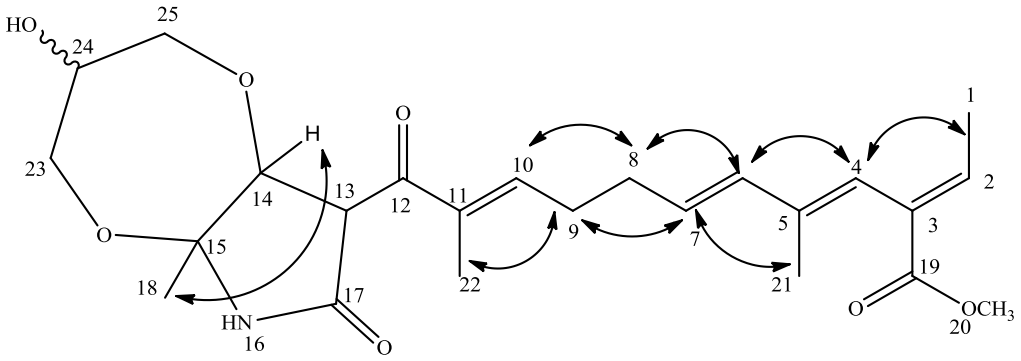


Figure 7.4.23. ROESY (↙↷) correlations of **10**

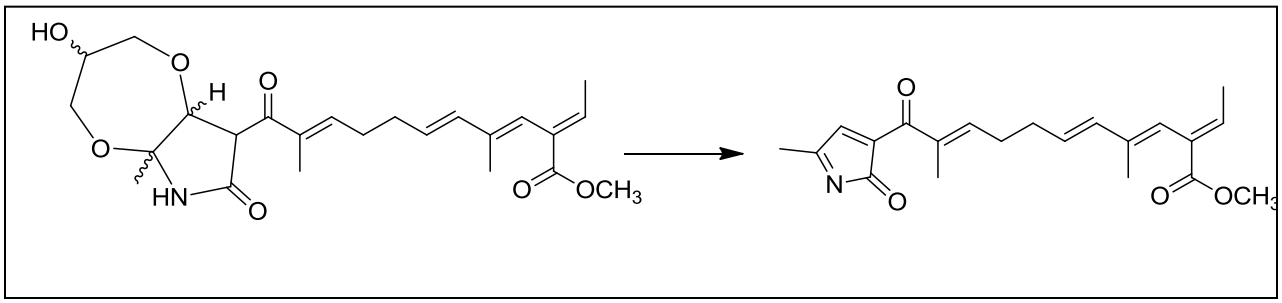
Several compounds (Figure 7.4.4) structurally related to compounds **9**-**12** have been isolated and their biological activities have been studied. Epolactaene,[218] isolated from *Penicillum* sp. has been shown to promote neurite outgrowth and arrest the cell cycle at the G_0/G_1 phase in a human neuroblastoma cell line. In addition, Osada and coworkers reported[219] the ability of epolactaene to bind human Heat shock protein (Hsp60) and inhibit the Hsp60 chaperone activity that is involved in apoptosis, immunoregulatory function, and cell spreading; however, epolataene is known[220] to be labile as it contains a conjugated triene moiety and an epoxy group which is activated by two oxo groups. Several fusarins have been isolated from *Fusarium moniliforme*,[221, 222] and *Nectria coccinea*.[217] Of these compounds, fusarin C has been identified as a mutagen, and one of the compounds responsible for the toxicity associated with *F. moniliforme* has been found in contaminated food.[223, 224] Luciactaene, a cell cycle inhibitor in p53-transfected cancer cells, and NG-391 have been reported from different *Fusarium* sp.[225] A number of analogues of the platelet aggregation inhibitor, PI-091, isolated from *Paecilomyces*

sp.,[226] have been synthesized and their biological activities have been reported.[219] Their biological activity is most likely due to the presence of reactive functional groups such as α, β-unsaturated carbonyl, epoxide, and hemiaminal carbonyl, which could react with biological nucleophiles such as the sulfhydryl functionality of cysteine.[219]

Sapinopyridione (Figure 7.4.8) has been reported from *S. sapinea,* the anamorph (asexual) genus of *B. dothidea*.[206] (+)- and (-)-flavipucine (Figure 7.4.8) has been reported from plant pathogenic fungi *Macrophoma* sp.[216] and *Phoma* sp.[217] respectively.

The fraction containing compounds **10**-**12** showed strong antiplasmodial activity (<1.17 nM) with no cytotoxicity to Vero cells (Table 7.4.2). The lack of material prevented further biological studies on these compounds. However, due to instability and the toxicity reported for this class of compounds, they would not serve as potential antimalarial agents.

Compound **9**, sapinopyridione, and flavipucine were inactive in antiplasmodial assays. The mixture of sapinopyridione and flavipucine showed weak to moderate cytotoxicity (Table 7.4.4). The mixture of sapinopyridione and flavipucine was highly phytotoxic to both monocots and dicots (Table 7.4.3). Phytotoxic activity of sapinopyridione and flavipucine has been studied, and the oxirane ring and the oxo group in the aliphatic side chain have been determined to be prerequisites for this acivity.[80]



Chemical formula $C_{24}H_{34}NO_7$ $[M+H]^+$

Exact mass *m*/*z* = 448.2335

Chemical formula $C_{21}H_{26}NO_4$ $[M+H]^+$

Exact mass *m*/*z* = 356.1862

Figure 7.4.24. Mass fragmentation of compounds **10**-**12** and their common base peak

7.4.4. Biological Activities

Table 7.4.2. Antiplasmodial activity of **8**-**12**

Compound	Chloroquine-sensitive (D6)-clone		Chloroquine-resistant (W2)-clone		Cytotoxicity to Vero cells
	IC_{50} nM	S. I.	IC_{50} nM	S. I.	IC_{50} nM
Mixture of two sapinopyridione (**8a**) and flavipucine (**8b**)	NA		NA		NC
9	NA		NA		NC

10-12	<1.17	>9	<1.17	>9	NC
chloroquine[a]	0.03	496.6	0.31	48.1	NC
artemisinin[a]	0.02	845	0.01	1690	NC

[a] Positive controls

IC_{50} = concentration casusing 50% growth inhibition

NC, not cytotoxic

NA, not active

S. I. (selectivity index) = IC_{50} for cytotoxicity/IC_{50} for antiplasmodial activity

Table 7.4.3. Phytotoxic activity of **8-11**

Compound	Lettuce	Bentgrass
Mixture of two sapinopyridione (**8a**) and flavipucine (**8b**)	5	5
9	NT	NT
10-11	NT	NT

Ranking based on scale of o to 5

0 = no effect

5 = no growth

Solvent used, 10% acetone

Concentration used, 1 mg/mL

Table 7.4.4. Cytotoxic activity [IC_{50} (μM)] of **8-12**

Compound	SK-MEL	KB	BT-549	SK-OV-3	LLC-PK_{11}
Mixture of two sapinopyridione (**8a**) and flavipucine (**8b**)	5.05	21.9	20.3	6.7	7.6
9	NT	NT	NT	NT	NT
10-12	NT	NT	NT	NT	NT
doxorubicin[a]	1.29	2.12	1.83	1.47	1.28

NA: not active at the highest test concentration of 10 μg/mL.

[a] Positive control.

IC_{50} = concentration casusing 50% growth inhibition

SK-MEL = human malignant melanoma

KB = human epidermal carcinoma

BT-549 = human breast carcinoma (ductal)

SK-OV-3 = human ovary carcinoma

LLC-PK_{11} = pig kidney epithelial

7.4.5. Experimental

7.4.5.1. General

Carried out as previously described in the section 7.1.4.1.

7.4.5.2. Isolation of the Fungus from *T. taxifolia*

The fungus was isolated from the infected seeds collected from a cultivated *T. taxifolia* plants. Infected seeds of *T. taxifolia* Arnott were furnished by Dr. Edward Croom, Croomia, 1509, Smallwood Dr., Oxford, MS, and isolation of fungi from those was performed by Dr. Luis Rosa, NCNPR, University of Mississippi.

7.4.5.3. Fermentation, Extraction, and Purification

Botryosphaeria dothidea was cultured in 80 conical flasks (1 L) containing 500 mL of Potato Dextrose Broth and incubated at 27 ^{o}C for 14 days on an orbital shaker at 100 rpm. The mycelium was separated by filtration, and the broth was extracted with EtOAc (× 3). The EtOAc extract was evaporated to give a black residue (3.15 g).

The EtOAc extract (3 g) was chromatographed over silica gel and eluted with a gradient of hexanes, CH_2Cl_2, and MeOH to yield 15 fractions. Fractions which showed antimalarial activity were combined (550 mg) and chromatographed over Sephadex LH-20 and eluted with MeOH to give 12 fractions. A white precipitate observed in subfraction 11 was separated and washed with Et_2O to yield a mixture (1:3) of sapinopyridione (**8a)** and flavipucine (**8b**). Subfractions 6-10, which showed antimalarial activity, were combined (160 mg) and further separated using a C_{18} reversed-phase semi-preparative HPLC column eluting with MeOH-H_2O

(1:4), at a flow rate of 3.0 mL/min, to give eight fractions. Sub-fraction 4, which showed no antimalarial activity, was further purified by Sephadex LH-20 gel filtration with MeOH (100%) to give a new compound (**9,** < 1.5 mg).

Subfractions 5-7, which showed potent antimalarial activity, were further purified by using C_{18} reversed-phase analytical HPLC chromatography and eluted with MeOH-H_2O (1:4) at a flow rate of 1.5 mL/min, to give three minor compounds **10** (1.0 mg), **11** (1.0 mg), and **12** (<1.0mg).

A mixture (1:3) of sapinopyridione (**8a**) and flavipucin (**8b**); white amorphous powder; ^{1}H and ^{13}C NMR, and HRESIMS were compared with the literature data.[206, 217]

Compound 9: epolactaene; HRESIMS $[M + H]^+$ *m/z* 388.2184 (calcd for $[C_{24}H_{33}NO_4 + H]^+$ 388.2124), ^{1}H and ^{13}C NMR data: see Table 7.4.1

Compound 10: HRESIMS $[M + H]^+$ *m/z* 448.2268 (calcd for $[C_{24}H_{33}NO_4 + H]^+$ 448.2335), ^{1}H and ^{13}C NMR data: see Table 7.4.1

Compound 11: HRESIMS $[M + H]^+$ *m/z* 448.2268 (calcd for $[C_{24}H_{33}NO_4 + H]^+$ 448.2335), 1H and 13C NMR data: please see Table 7.4.1

Compound 12: HRESIMS $[M + H]^+$ *m/z* 448.2247 (calcd for $[C_{24}H_{33}NO_4 + H]^+$ 448.2335), ^{1}H and ^{13}C NMR data: see Table 7.4.1

7.4.5.4. Biological Assay

Antiplasmodial,[69] phytotoxic,[70] and cytotoxicity[76] assays were carried out as previously described.

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APPENDIX: STATEMENT OF CONTRIBUTION

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My overall contribution to this dissertation consists of isolation of endophytic fungi from infected leaves collected from cultivated *Torreya taxifolia* Arnott trees; maintenance of fungal cultures; preparation of fungal extracts in different culture media; scale-up culture of active fungi and extractions for bio-assay guided fractionation; isolation, and characterization of metabolites.

The final output of this work is a result of several contributors. I would like to extend my gratitude to the following scientists for their contribution included in this dissertation.

Dr. Edward Croom, provided the infected plant material *T. taxifolia* and its taxonomic identification, Dr. N. Techen, performed DNA sequence analysis to identify the endophytic fungi isolated from *T. taxifolia*, and Dr. Luiz H. Rosa isolated the endophytic fungus, *Botryosphaeria dothidea* from infected seeds from *T. taxifolia*

Dr. M. Jacob, Dr. S. Khan, Dr. B. Tekwani, and Dr. S. Duke monitored antimicrobial, antimalarial, antileishmanial, and phytotoxic bioassays, respectively, to evaluate the biological activities of all the extracts and compounds. Dr. F. Fronczek, performed the X-ray crystallographic data of septoriamycin A, isolated from *Septoria pistaciarum*.

This investigation was carried out under the scientific guidance of Dr. N. P. D. Nanayakkara and Dr. D. Ferreira.

VITA

Mallika Kumarihamy received her Bachelor of Science degree in Chemistry and Master of Philosophy in Natural Products Chemistry from the University of Peradeniya, Sri Lanka. She worked as a Research Assistant at the Institute of Fundamental Studies, Department of Chemistry, University of Peradeniya, and Department of Agriculture, Peradeniya, Sri Lanka. She joined the Department of Pharmacognosy and National Center for Natural Products Research, University of Mississippi as a Graduate Research Assistant in August 2006 to pursue a Ph. D. in Pharmaceutical Sciences. At the University of Mississippi, she gained extensive experience in drug discovery and development. Mallika is a member of American Society of Pharmacognosy and American Chemical Society.