Protoberberine-type Alkaloids as Lead Compounds for the Treatment of African Sleeping Sickness, Leishmaniasis, and Malaria

Dissertation

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Abstract

Natural products have played traditionally a pivotal role in the field of drug discovery. The impact of natural products in the biomedical sciences can be shown by a few distinct examples: The discovery of receptor sites (as in the case of nicotinic, muscarinic, and opioid receptors), the isolation of compounds with a unique activity for a disease for which there was no practical treatment (such as quinine in malaria and penicillin for bacterial infections), the identification of a novel mechanism of action in a known disease (such as the tubulin-stabilizing effect of paclitaxel in cancer), and the provision of lead molecules that can be further optimized (such as mefloquine, based on the lead compound quinine). The importance of natural products is also reflected when it is considered that over 30% of all the new small-molecule drugs approved for clinical use in Western countries and Japan during the twenty-five year period from 1981 to 2006 were either natural products or their derivatives, or synthesized molecules based on natural product pharmacophores (Newman et al., 2007).

In the present study, a library of 128 plants available at the College of Pharmacy, The Ohio State University, was screened for antileishmanial and antitrypanosomal activities, following a standardized solvent extraction scheme (Wall et al., 1996). Among the active extracts, the entire plant of *Thalictrum lucidum* L. (Ranunculaceae) was selected as a potential lead and was extracted in a larger scale, with a bioactivity-guided fractionation procedure employed to identify
the active principle or principles. Bioactivity studies led to the identification of berberine (85) as the main active principle of *T. lucidum*, with two other protoberberine-type alkaloids, jatrorrhizine (87) and palmatine (89) isolated.

Following this lead, a small library of semisynthetic berberine derivatives was screened for antileishmanial and antitrypanosomal activities. Among the compounds evaluated in the *in vitro* test systems, samples that were labeled as 8,8-diethyldihydroberberine (HI salt) and 8,8-dimethyldihydroberberine (HI salt) were active against *Leishmania donovani* parasites with IC$_{50}$ values of 12 and 15 ng/mL, respectively. However, both samples proved to be mixtures in NMR spectroscopic and chromatographic studies, possibly due to oxidation during prolonged storage. In an effort to identify the active principle, a semisynthetic study was carried out. Accordingly, the berberine analogue, 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129) was synthesized in sufficient amounts for further biological testing. 5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine chloride showed nanomolar level potency against the *in vitro* models of leishmaniasis, malaria, and trypanosomiasis used as well as activity in an *in vivo* model of visceral leishmaniasis. Therefore, this compound was elucidated as a bioactive oxidized form of the original 8,8-diethyldihydroberberine sample.

Overall, this study describes the discovery of a berberine analogue through a semisynthetic approach with an approximately thousand-fold improvement in activity against leishmaniasis, malaria, and trypanosomiasis as compared to the parent compound. Since the starting material, berberine, is relatively inexpensive, the discovery of 8,8-dialkyldihydroberberine derivatives may lead to a new class of affordable drugs in the field of protozoal diseases.
This dissertation is dedicated to my parents, my children, and my wife. None of this work would be possible without their support, patience, encouragement, and guidance.
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PUBLICATIONS


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LIST OF ABBREVIATIONS

1D, 2D: one- or two-dimensional

Calcd: calculated

CDCl$_3$: deuterated chloroform

CHCl$_3$: chloroform

COSY: correlation spectroscopy

$\delta$ (ppm): chemical shift in parts per million

$\delta_C$: carbon-13 chemical shift

$\delta_H$: proton chemical shift

DEPT: distortionless enhancement by polarization transfer

HMBC: heteronuclear multiple bond correlation spectroscopy

HPLC: high-performance liquid chromatography

HRESI-MS: high resolution electrospray ionization mass spectroscopy

HSQC: heteronuclear single quantum coherence spectroscopy

Hz: hertz

IC$_{50}$: sample concentration that inhibits cell growth by 50% compared to untreated control
IR: infrared spectroscopy

$J$: coupling constant

$\lambda$: wavelength in nanometer

M: molar concentration

MeOH: methanol

min: minute

m.p.: melting point

$m/z$: mass to charge ratio

NMR: nuclear magnetic resonance

NOESY: nuclear Overhauser enhancement spectroscopy

$R_f$: Retention factor

$\nu$ (cm$^{-1}$): infrared absorption frequency in reciprocal centimeter

$t_R$: retention time

TLC: thin-layer chromatography

UV: ultraviolet
“What seems to us more important, more painful, and more unendurable is not really what is more important, more painful, and more unendurable but merely that which is closer to home. Everything distant which for all its important moans and muffled cries, its ruined lives and millions of victims, that does not threaten to come rolling up to our threshold today we consider endurable and of tolerable dimensions.”

CHAPTER 1. THE STATUS AND CHEMOTHERAPY OF NEGLECTED TROPICAL DISEASES AND THEIR IMPACT

A1. Introduction and Terminology

Neglected Tropical Diseases (NTDs) constitute a diverse group of mostly infectious diseases that affect some 2.7 billion of the world’s poorest inhabitants, of whom most live in sub-Saharan Africa, Asia, and the Americas. NTDs are responsible for an estimated number of 534,000 deaths annually and cause suffering that extends from life-long disabilities, to disfigurement, reduced economic productivity, and social stigma (Toreele et al., 2004; Hotez et al., 2006a; Hotez et al., 2007; Anonymous, 2009). In the western world, the understanding of the gravity and the impact of tropical diseases on vast human populations seems to be very much underappreciated and is limited not only by both geographic and emotional distance from the people and countries affected, but also by the complexity of these disease forms, which may not be readily apparent.

Diseases covered under this category are diverse in origin and are caused by various pathogens including bacteria, protozoans, viruses, and worms (Guerrant et al., 2005). Approximately 25% of annual global human deaths are due to infectious diseases, but this figure reaches 45% in developing countries (2008). While the reasons for this substantial difference are wide-ranging in origin, these data demonstrate that a
remarkable number of deaths in developing countries are, in actuality, avoidable. Some authors have termed neglected diseases as “forgotten diseases”, and probably rightfully so. At the completion of the first decade of the 21st century, 500 million people still suffer in rural parts and cities of the developing world, with no matching relief efforts (Hotez, 2009).

Although control of several NTDs is possible through proven strategies, the burden of morbidity they cause has not changed remarkably over the last two decades. Hence, there is an essential need for basic research, drug and vaccine development efforts, along with a favorable prioritization by both governmental and international agencies (Feasey et al., 2010).

The diversity of NTDs spans a variety of biological vectors (e.g., sandflies, mosquitos, and snails), and causative agents (e.g., bacteria, helminthes, protozoans, and viruses), and these occur in multiple geographical regions of the world. A common feature of most of these diseases is their prevalence in poverty-stricken parts of the world (Hotez et al., 2009b). The “barriers of health”, as defined by the biosocial model, indicates that significant morbidity due to tropical diseases persists even far from the tropics. Whether it is primarily the living conditions that accompany poverty that leads to greater susceptibility to these diseases or if it is indeed these diseases that cause the poverty and lack of economic development in these same areas, a successful intervention effort needs to address both of these factors (Hotez et al., 2009b). The World Health Organization (WHO), which has a critical role in both the policy-making and disease
prevention aspects of NTDs, regards the control of such diseases as an imperative condition for the eradication of extreme poverty (Health., 2001).

In particular, there are thirteen neglected diseases of exceptionally high prevalence in Africa. These diseases comprise bacterial (Buruli ulcer, leprosy, and trachoma), protozoan (human African trypanosomiasis and visceral leishmaniasis), and helminth (ascariasis, dracunculiasis, hepatobiliary schistosomiasis, hookworm infections, lymphatic filariasis, onchocerciasis, trichuriasis, urinary schistosomiasis) infections. It is estimated that up to 90% or more of the world’s disease burden from these diseases occurs in Africa, especially in countries south of the Sahara desert (Molyneux et al., 2005).

There is some ambiguity in the literature as to which diseases are classified as “Neglected Tropical Diseases”, and there seems to be no standard definition for this term. Major organizations such as WHO, the Global Network for Neglected Tropical Diseases, the Public Library of Sciences Neglected Tropical Diseases, and the Neglected Tropical Disease Program (USAID) differ in their definitions of “neglect”, and consequently have separate, and changing, diseases under the category “NTD” (Table 1) (Liese et al., 2010). A recent review has documented two approaches these organizations utilize in their listings. While the first approach takes neglect into regard as the defining characteristic, the second approach emphasizes the shared features of these diseases and their impact on development and poverty (Liese et al., 2010). The first systematic approach to define neglected diseases was formulated as recently as 2003, at the International Workshop on Intensified Control of Neglected Diseases in Berlin. This body identified three levels of
<table>
<thead>
<tr>
<th>Organization</th>
<th>Diseases Defined as “NTD”</th>
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<tr>
<td>Global Network for Neglected Tropical Diseases</td>
<td>Ascariasis, Buruli ulcer, dengue, dracunculiasis, human African trypanosomiasis, hookworm, leishmaniasis, leprosy, lymphatic filariasis, onchocerciasis, schistosomiasis, trachoma, and trichuriasis.</td>
</tr>
<tr>
<td>Neglected Tropical Disease Program (USAID)</td>
<td>Lymphatic filariasis, trachoma, onchocerciasis, schistosomiasis, and soil-transmitted helminthiasis.</td>
</tr>
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Table 1. List of diseases grouped under the term “Neglected Tropical Diseases” by different organizations. (Adapted from Liese et al., 2010). (Diseases to be further investigated in this dissertation are bolded).
“neglect” specifically shared by all neglected diseases. These criteria are “neglected locally”, “neglected nationally”, and “neglected internationally” (World Health Organization, 2004). Consequently, it can be inferred that all NTDs are diseases for which the control, eradication, improvement, research, and surveillance, for numerous reasons, fall far behind what is deemed necessary, from either a public health or a human rights perspective (Trouiller et al., 2002; Hunt, 2007). Due to the intrinsic relationship between economic disadvantages and disease, some NTDs are referred to as “diseases of poverty” (Ridley, 2007; Manderson et al., 2009). While all NTDs do indeed exist in tropical regions, extreme poverty appears as a more pertinent factor for defining the foci of these diseases, rather than purely geographical considerations, and thus, today, the title “Neglected Tropical Diseases” can be considered a misnomer (Hotez et al., 2006a; King et al., 2008; Hotez et al., 2009b).

A list of NTDs in the literature commonly incorporates African sleeping sickness, bacterial pneumonia and meningitis, Buruli ulcer, Chagas’ disease, dengue and dengue fever, diarrheal diseases, dracunculiasis, fascioliasis, helminthic diseases, HIV/AIDS, leishmaniasis, leprosy, lymphatic filariasis, malaria, onchocerciasis, rheumatic fever, trachoma, tuberculosis, and typhoid and paratyphoid fevers. For the purposes of this dissertation, HIV/AIDS, malaria, and tuberculosis will not be included in the NTD list, in accordance with recent reviews on this topic (Hotez et al., 2007; Hotez et al., 2009b).

The intricacies of controlling, treating, and potentially eradicating NTDs at a global level has led to challenges that are exceptionally diverse and multi-faceted (Hotez et al., 2007). Limitations in the control of infectious tropical diseases lie not only in the
availability of effective inexpensive drugs, but also in social and cultural factors in affected regions, such as access to healthcare, a brain drain (especially in the case of sub-Saharan Africa), civil conflicts, political instabilities, and social stigma, of which all are usually accompanied by the lack of comprehensive public health policies (Walton et al., 2006).

A2. Description and Current Impact of Human African Trypanosomiasis, Leishmaniasis, and Malaria

While the term “NTD” is relatively new, the diseases embraced therein have been around since antiquity. In contrast to diseases such as avian flu, Ebola, HIV, SARS, and swine flu, which emerged in the last century, resulting in considerable public awareness, most NTDs have been in evidence since the beginning of history (2003). It has been speculated that diseases due to the guinea worm and the hookworm, as well as leprosy, schistosomiasis, trachoma, and other NTDs are described in the Bible, the Talmud, Papyrus Ebers, the Kahun papyrus, along with the writings of Hippocrates (Hotez et al., 2006a). Below are descriptions of human African trypanosomiasis, leishmaniasis, and malaria, and their current status.

Human African trypanosomiasis (HAT), or “African sleeping sickness”, is caused by two morphologically identical subspecies of trypanosomes, Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense. Both parasites are transmitted to humans by tsetse flies. T. brucei demonstrates a complex life-cycle that takes place in the mammalian bloodstream and several species of tsetse flies. Once a tsetse fly bites an
infected person or animal, trypanosomes are ingested with the bloodmeal. At this stage of their life-cycle, bloodstream trypanosomes transform into a procyclic stage. The procyclic trypanosomes eventually migrate to the salivary gland of the fly and transform into the metacyclic form. Following a bite, parasites enter the bloodstream of infected humans and complete their life-cycle.

HAT caused by *T. b. gambiense* manifests clinically as either an early stage in asymptomatic patients with trypanosomes present in the blood or lymph node aspirates or as a late stage with central nervous system involvement including somnolence, other neurologic symptoms, and the presence of trypanosomes in the cerebrospinal fluid. HAT caused by *T. b. rhodesiense* presents as a more acute febrile illness if untreated, and can be fatal in a matter of weeks (Guerrant *et al.*, 2005).

Currently, sixty million people are at risk for HAT, of whom most live in Angola, the Democratic Republic of the Congo, Sudan, and Uganda. Owing to ongoing conflicts, starting with 1975, the prevalence of HAT in these countries has increased to levels not seen since the 1920s. In the early 20th century, outbreaks in the Congo and Uganda alone caused 750,000 deaths. It has been reported that some recent epidemic periods of HAT caused greater levels of mortality in some villages than are evident from HIV/AIDS (Reddy *et al.*, 2007).

It is estimated that 500,000 people are infected currently with HAT and that 50,000 people die annually. Fortunately, the intervention efforts seem to be effective and the incidence of HAT was at an all-time low in 2009, with only 9,878 new cases reported
(Anonymous, 2012). Effective control of HAT can be achieved through rapid treatment of confirmed cases and survey teams that perform active detection in rural areas. However, adding to the complications from these diseases, even the clinical diagnosis of HAT has its shortcomings. While present screening methods lack specificity, parasitological diagnosis lacks sensitivity (Legros et al., 2002). Additionally, the most commonly used drug for the therapy of HAT is extremely toxic and difficult to use. All these factors, along with high drug costs and the emerging incidences of drug resistance designate HAT as a disease well deserving of enhanced research efforts (Hotez et al., 2004).

Leishmaniasis is caused by *Leishmania* sp. parasites that are members of the family Trypanosomatidae, order Kinetoplastida. *Leishmania* species occur in every continent except Australasia and Antarctica. In their life-cycle, parasites exist in their extracellular promastigote form in the gut of their insect vectors, the phlebotomine sand flies. Once in their mammalian host, they transform to intracellular amastigotes within the macrophages in mammals. Traditionally, leishmaniasis has been categorized into three major diseases based on the clinical manifestation evident, namely, cutaneous, mucosal, and visceral. Cutaneous leishmaniasis usually presents at the site of inoculation. At this location, promastigotes absorbed by mononuclear phagocytes subsequently transform to amastigotes. Following the multiplication of parasites, a nodule develops as a result of mononuclear cells recruitment to the area. This nodule turns into an ulcer and is ultimately self-healing. Mucosal leishmaniasis often presents with nasal stuffiness and inflammation, followed by the ulceration of the nasal mucosa and septum. This ulceration
may spread to other tissues, including the lips, cheeks, soft palate, pharynx, and larynx, leading to extensive disfigurement. Visceral leishmaniasis is characterized by fever, weight loss, hepatosplenomegaly, neutropenia, and hyper-γ-globulinemia and may ultimately lead to death.

While cutaneous leishmaniasis is common in Asia (particularly in the Indian subcontinent), Latin America, and the Middle East, visceral leishmaniasis affects mostly the populations of Bangladesh, eastern India, the Sudan, and northeastern Brazil. Visceral leishmaniasis has recently emerged as an opportunistic disease in Spain, southern France, and Italy in patients with drug-induced immunosuppression and AIDS. According to the WHO, 350 million people in 88 countries around the world are estimated to be at risk for leishmaniasis. Approximately 12 million people are thought to be infected with a form of leishmaniasis annually, with about 1 to 2 million estimated new cases occurring each year (Anonymous, 2009; Guerrant et al., 2005).

The control of these three forms of leishmaniasis is complex and requires interventions at multiple levels. For all types of leishmaniasis, both zoonotic and anthroponotic transmission is possible with multiple animal reservoirs present. Hence, the theoretical scenario of treating all patients with leishmaniasis at a given time would still not eradicate leishmaniasis. Visceral leishmaniasis (VL) is the most deadly type of leishmania infection. Out of the 500,000 cases of VL and 1.5 million cases of cutaneous leishmaniasis (CL), 90% of VL cases occur in Bangladesh, Brazil, India, Nepal, and the Sudan, while 90% of CL cases are seen in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. (Anonymous, 2010; Guerin et al., 2002). For VL, major obstructions in
treatment include invasive diagnostics, long durations of treatment (30 days), and emerging drug resistance (up to 40% in India) (Olliaro et al., 2005). Other than biological factors, social and environmental factors such as famine, deforestation, human migration, new irrigation schemes, and HIV/AIDS all contribute to the emergence of VL and CL (Hotez et al., 2004). Diagnosis for leishmaniasis requires biopsy studies, and the drugs currently used for treatment, such as antimonials and amphotericin, are either toxic or expensive. Recent advances, however, include the development of insecticide-treated bednets and use of miltefosine (Hotez et al., 2004).

Malaria is an infectious disease caused by parasites belonging to the genus *Plasmodium*. In humans, four species, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax*, cause malaria. All four parasites share the female *Anopheles* mosquito as a vector. Following a bite, the mosquito inoculates uninucleated sporozoites, which are destined to reach the liver. Here, parasites invade hepatocytes through a specific receptor-ligand interaction and develop into a mature liver-stage schizont with uninucleated merozoites. Once this schizont matures and ruptures, merozoites are released into the bloodstream where they mature into asexual erythrocytic stage schizonts with an average of 16 uninucleated merozoites. Upon maturation of the schizont, the erythrocyte ruptures releasing the merozoites, starting the intra-erythrocytic stage development (Guerrant et al., 2005).

The symptoms seen in malaria are the direct result of the destruction of both infected and uninfected erythrocytes and the periodic release of parasites into the bloodstream, and the host reaction to these events. In particular, *P. falciparum*-infected
erythrocytes specifically hinder the microcirculation of vital organs, and lead to a cascade of events that might cause death if left untreated.

In 2008 alone, there were 247 million cases of malaria globally, leading to one million deaths. Of these, 90% occurred in tropical Africa, taking an especially heavy toll on children. *P. falciparum* is responsible for more deaths in children less than five years of age than any other single infectious disease, including HIV/AIDS.

Through vast scientific advances in especially the areas of epidemiology, genetics, medicinal chemistry, medicine, molecular biology, and pathophysiology, our understanding of virtually every infectious disease has increased exponentially over the last fifty years. Despite all the progress made, however, it would not be wide of the mark to state that these advances, except for under very limited circumstances, have not yet had a significant impact on the overall picture in relation to these “diseases of the poor”. No single tropical disease, even those for which an effective treatment is available, has been brought under effective control or eradicated. Moreover, some of the diseases once thought to be near eradication have made a significant comeback in incidence in the last twenty years. Adding to the problem, co-infection with human immunodeficiency virus (HIV) has made the management of other infectious diseases more difficult. Especially in sub-Saharan Africa, where 12.5 million people are estimated to be infected with HIV, co-infections with tuberculosis, malaria, and leishmaniasis are common due to their geographic overlap (Harms *et al.*, 2002). In the case of leishmaniasis, it has been postulated that HIV and leishmaniasis may increase synergistically the intracellular multiplication of both organisms (Berhe *et al.*, 1999). On a clinical level, leishmaniasis
occurs in unusual organs and tissues in those with HIV, and, in the absence of effective HIV management, relapse is virtually unavoidable (Murray et al., 2005). HIV co-infection also decreases the efficiency for the chemotherapy for leishmaniasis and thus the infected individuals continue to serve as a pool for *Leishmania* parasites (Harms et al., 2002).

**A3. Poverty, Setting Priorities, and Effective Policy-Making**

**A3.1. Right to Health and Obligations**

The right to health is a primary principle included in the Universal Declaration of Human Rights (UDHR), adopted by the United Nations General Assembly in 1947. Article 25 of this document states explicitly “…everyone has the right to a standard of living adequate for the health and well-being of himself and his family, including food, clothing, housing and medical care and necessary social services…” (Anonymous, 1948).

While the inclusion of this article has established that the right to health is inherent, in being solely a declaration, UDHR does not mention distinctly any consequences for those states failing to adhere to the declaration, and hence possesses no obligatory power (Attaran, 1999). As an illustration of a “soft law”, UDHR serves only as a moral compass. UDHR was followed in 1966 by the International Covenant on Civil and Political Rights (ICCPR) and the International Covenant on Economic, Social and Cultural Rights (ICECSR). These documents served to legislate what was already declared in UDHR, and the three documents together are commonly referred to as the International Bill of Human Rights. Article 21 of ICECSR states “…The States Parties to
the present Covenant recognize the right of everyone to the enjoyment of the highest attainable standard of physical and mental health…” In contrast to the generalized statement of Article 25 of UDHR, this article proceeds to define four main areas of development to realize the right to health. These areas include the reduction of stillbirth-rate and infant mortality, improvement of environmental and industrial hygiene, the prevention, treatment, and control of diseases, and the creation of conditions necessary to assure access to health services. These two covenants along with UDHR establish unequivocally the right to health as a universal right. This right comes with legal obligations for the parties responsible for providing health services.

In most western countries, the pharmaceutical industry leads drug development efforts. No different than other industries, pharmaceutical companies aim to be profitable and they achieve this goal through developing successful and marketable drugs (MacDonald et al., 2001; Yamey et al., 2002). Unfortunately, an intrinsic ethical concern presents itself in the very definition of a successful drug. In a world driven by revenues, “success” is almost synonymous with “profitable”. Currently, pharmaceutical companies aim almost exclusively to develop drugs for profitable ailments such as baldness, cancer, heart disease, and pain (Yamey et al., 2002). From a business point of view, this approach has indeed been rewarding for the U.S. pharmaceutical industry, leading to a rate of return on investment more than twice the national industrial average (MacDonald et al., 2001). The drawbacks of this approach are apparent in a survey of new drugs marketed between 1975 and 1999. In this period, out of 1393 new drugs marketed, 16 were for neglected diseases, not remotely matching their respective global disease
burden. Perhaps more representative of the problems caused by a profit-driven approach in the drug discovery field, two thirds of all new drugs could be categorized as “me-too” drugs that have negligible effects on the overall disease burden (Yamey et al., 2002).

Eflornithine presents a striking illustration of the ethical dilemma that may arise when “success” means being “marketable”. Melarsoprol is an arsenic drug that has been a first-line drug for HAT for nearly 50 years. While mostly effective, melarsoprol is extremely toxic and may cause death in up to 10% of the patients administered (Yamey et al., 2002). Due to this high level of toxicity, when an alternative therapeutic, eflornithine, was licensed in 1990, it was welcomed with great expectations by both physicians and other experts in the field. Unfortunately, production was halted in 1999 as a result of a lack of market penetration (Legros et al., 2002). Following persistent demands by Médecins Sans Frontières and other charitable organizations for the manufacturer to reconsider, in 2001, the company concerned agreed to provide as much eflornithine as is needed, together with melarsoprol and the early-stage drug pentamidine, for five years, along with a $25 million donation to WHO (Boseley, 2001). How this interruption of therapy involving eflornithine lasting two years affected the lives of HAT patients is debatable.

As a derivative of the broader right to health, a human right to essential medicines is established in this accord. Under the auspices of the right to health, state agencies are mandated to enforce that all essential medicines are available, accessible (both financially accessible or affordable), acceptable, and of appropriate quality. Some policymakers have advised governments to develop an Essential Medicines List based on the guidance of the
WHO Model List. It is also suggested that governments are under an obligation to
guarantee that no rights are violated by non-state participants, such as drug companies.
Keeping in mind the case of eflornithine, these suggestions if implemented might make a
major difference in the treatment of NTD (Committee on Economic, Social, and Cultural
Rights, 2000).

A3.2. Policy-Making

Decision-makers in governmental and international health agencies face
continuously the challenge of understanding and prioritizing diseases in an effort to
allocate the appropriate funds for where they are needed most (Mathers et al., 2007).
Diseases that are categorized of being high importance naturally receive more funds and
effort required for research and intervention programs. Since this ranking may ultimately
mark the difference between eradication and neglect, the following question begs to be
asked: What is it precisely that makes a disease “high priority”? Perhaps more crucially,
when can a disease be safely deemed “low-priority” and stripped of efforts for
improvement? Unsurprisingly, in the case of NTDs, the attempts to implement successful
programs are hindered by this very preliminary step.

The success of a public health policy is highly dependent on the criteria used
initially to prioritize diseases. The development of effective health policies starts with an
accurate disease-burden assessment based on utilization of data that incorporates the
scale and trends of diseases and their causes. Accurate assessment of the disease burden
requires the data for different diseases to be comparable and suitable for integration.
Some factors that have obstructed historically this process include fragmented and unreliable data, the lack of comprehensive epidemiologic knowledge about certain disease states, and also the framework chosen for prioritization. Additionally, priority-setting processes can be value-laden and political and require robust and legitimate institutions to serve as regulatory agents to assure fair processes (Mathers et al., 2007).

In the public health arena, different methods used to produce the same data may draw significantly distinct results. For instance, an approach that determines the burden of disease by analyzing mortality rate instead of years of potential life lost (YPLL), does not define the causes of premature death. Similarly, YPLL analyses do not emphasize the importance of chronic diseases such as NTDs that cause disabilities but do not change life expectancies significantly. In the 1980s, these methodological factors largely restricted an attempt by WHO to quantify global death patterns of a broad cause of death groups (Mathers et al., 2007).

The Global Burden of Disease Study (GBD) has emerged as a result of a collaboration between the WHO and the World Bank, to serve as a framework that might help define health-sector priorities more reliably (Mathers et al., 2007). GBD, as an analytical framework, aims to produce comparable, comprehensive, and reliable information with high relevance allowing cost-effective intervention packages for different countries at different levels of development. GBD employs a novel time-based metric, the disability-adjusted life year (DALY), with the ultimate goal of permitting standardized measurement across diseases, injuries, and risk factors. DALY calculations take into account not only causes of mortality, but also premature mortality, morbidity,
impairment, and disability, and therefore more precisely reflect the burden of conditions that cause suffering and loss of health but little mortality. The overall results are appreciably different than earlier approaches that analyzed the pattern of causes represented in mortality statistics, and underline the need for key changes in funding policies.

The first global burden of disease survey started in 1990 and classified human afflictions into categories such as “communicable”, “non-communicable” and “trauma”. In this scheme, “Infectious and Parasitic Diseases” are included as a sub-group of communicable diseases. One of the most significant findings of the study was that in the developing world, communicable diseases presented the leading cause of morbidity and mortality. In Africa, communicable diseases accounted for 73% of the total disease burden and 71% of all deaths (Engels et al., 2006).

The DALY approach, while proven to be a game changer in assessing the burden of disease and widely used in both national and international levels, has not been without its critics (King et al., 2008; Liese et al., 2010). Some experts have pointed to shortcomings of DALY in reflecting the burden of NTDs sufficiently and have suggested instead the use of quality-adjusted life year (QALY), a concept which has its own critics. Consequently, it can be inferred that accurately defining the burden of disease for NTDs is fairly complicated and NTDs are possibly under-represented in recent global burden of disease studies (Engels et al., 2006).
The ongoing lack of research effort in the NTDs area is commonly illustrated with the “10:90 gap” concept. This phrase refers expressly to only 10% of available global funding being directed at the diseases that affect 90% of the current world population. Unfortunately, at the end of the first decade of the 21st century, the divide between health conditions in developed and developing countries remains staggering. While 93% of preventable deaths (measured as years of potential life lost) can be attributed to developing countries, in 1986, of the $30 billion global investment in health research, only 5% was designated for diseases of the developing world. Based on these figures, it may be deduced that more than 200 times as much is spent on health research for each year of potential life in industrialized countries versus developing countries (1990; Attaran, 1999). Showing a similar trend, in 1990 the average median age at death for sub-Saharan Africa was 5, compared to a value of 75 for countries with established market economies. This tremendously low figure points to the severe toll high infant mortality takes in Africa (Mundial, 1993).

The distribution of the limited funding for neglected diseases also is not very balanced. This might be due to the perceived significance of NTDs in global public-health terms when only DALYs are taken into account. Remarkably, this does not seem to be the case towards diseases such as Ebola, West Nile virus, Nipah virus, and SARS that affect far fewer people, yet lead to widely disproportionate resources being spent on them (Molyneux, 2004). In 2008, out of the reported $2.96 billion invested for research and development of new neglected disease products, nearly 75% was shared between “the big three”, namely HIV/AIDS, malaria, and tuberculosis, leaving only about $600
million worldwide for all other NTDs combined per year. In this setting, diseases such as HAT and leishmaniasis shared $139 million along with other kinetoplastid infections (Moran et al., 2009).

It has been argued that from a disease burden perspective the NTDs are second only to HIV/AIDS in causing approximately 57 million DALYs annually, suggesting an uneven distribution of funding (Hotez et al., 2006b). Sustainable control and elimination of NTDs (if possible), can only be achieved through coordination and efforts of local health ministries for the integration of various vertical strategies into the public health system. Therefore, improvements in awareness and funding mechanisms for local control programs will benefit directly from the prioritization of NTDs at health ministries of local governments (Hotez et al., 2010).

Fortunately, there have been some recent developments that together provide some optimism (Hotez et al., 2010). Transforming the NTDs into an attractive area of investment for pharmaceutical companies has been the focal point of a few enterprises. Some of the suggested initiatives that might create favorable conditions include the cost-reduction of company research through publically funded grants, public support for clinical trials, tax credits, and the introduction of purchase funds. The Medicines for Malaria Venture, the International AIDS Vaccine Initiative, and the Global Alliance for TB Drugs Development represent the successful implementation of these efforts, while most NTDs still do not represent sufficiently prominent markets (Yamey et al., 2002).
Recently, the U.S. FDA introduced priority vouchers for NTDs and a European counterpart of this process is being considered (Waltz, 2008; Ridley et al., 2010). It is pertinent to draw similarities between the legislation regarding orphan diseases, specifically, the “Orphan Drug Act” of 1983, and the current lack of research and development regarding neglected diseases by pharmaceutical companies. By definition, orphan diseases relate to rare (i.e., fewer than 200,000 patients in the United States) diseases for which there is not much financial incentive for pharmaceutical companies to conduct drug discovery and development work. There has been significant progress when favorable incentives were created with the aforementioned legislation, such as bestowing pharmaceutical companies the unusual privilege of selling the drug without competition for ten years, even if it is a molecule that cannot be patented (“temporary monopolies”), as well as grants, clinical trial tax incentives, and free price setting. These vouchers are applicable towards new drug applications (NDA) and can cut the FDA review process by half, providing a profitable incentive for drug companies.

The WHO Special Program for Research and Training in Tropical Disease Research (TDR) was established in 1975 with the goal of developing new tools to control ten specific diseases through strengthening the research capabilities of target countries. TDR aims to promote basic and applied research and develop cost-effective interventions for NTDs (Remme et al., 2002). For this purpose, TDR categorizes these ten diseases based on three criteria: “Emerging or uncontrolled and lacking effective interventions”, “persisting despite available (but variably effective) strategies”, and “decreasing as a result of effective and applied control strategies” (Remme et al., 2002).
Recently, WHO initiated a Department of Neglected Tropical Diseases, concentrating specifically on NTDs, while an already available program, the Special Programme for Research and Training in Tropical Diseases (WHO-TDR), with the support from other UN agencies, member states, and private philanthropies, established a ten-year strategic plan, called the “Ten Year Vision and Strategy” in 2007 (Hotez et al., 2010). On a governmental level, both the U.K. and the U.S. have boosted substantially their financial commitment for integrated NTD preventive chemotherapy to an annual combined figure of $100 million. In addition, product development partnerships such as those supported by the Bill and Melinda Gates Foundation, Médecins Sans Frontières (MSF), and a few European governments, contribute greatly to efforts made in controlling NTDs (Hotez et al., 2010).

In 2008, owing in large part to the Bill and Melinda Gates Foundation, absolute year-on-year funding for NTDs increased by 3.9% compared to 2007. Interestingly, in the same period, funding from High-Income Countries (HICs), Innovative Developing Countries (IDCs) and multilaterals, suffered decreases in year-on-year funding (Moran et al., 2009). On a national level, in 2009 the U.S. National Institutes of Health defined NTDs as a “research priority.” In the pharmaceutical arena, the U.K.-based charity, the Wellcome Trust, established an agreement with a pharmaceutical company, Merck & Co., in an effort to locate and assign sizeable funds for a joint, not-for-profit research center in India. This facility will focus primarily on the development of affordable “antipoverty vaccines” for NTDs (Hotez et al., 2009a).

A3.3. Poverty
The correlation between extreme poverty and poor health is well-defined, since diseases are known to soar in incidence in poverty-stricken parts of the world. Explaining the causality between the two, on the other hand, proves to be a particularly more complex task. In order to accomplish this undertaking, each factor that plays into the overall health and poverty of a population needs to be identified, quantified, and isolated. Only the consequent analysis and comparison of these data can distinguish the causation relationships from coincidental ones. As expected, this proves to be an especially arduous undertaking when dealing with a group of diseases that often affects rural areas and then goes unnoticed. In the case of NTDs, development of an effective program relies heavily on the incorporation of as many factors surrounding the disease as possible.

Most economic models mention NTDs as a factor in “poverty traps”. “Poverty Trap” is defined as a situation where a self-reinforcing mechanism causes poverty to persist (Azariadis et al., 2005). This model suggests that wealth, through better nutrition and sanitary conditions, offers a level of protection against infectious diseases, but poverty deprives a society of essential human health required for economic productivity, hence leading to a vicious-cycle situation (Bonds et al., 2010). Another apparent vicious cycle emerges between conflicts and NTDs. While poverty is a well-established risk factor for NTDs, there seems to be an association increasingly evident between NTD prevalence and conflict and violation of human rights (Beyrer et al., 2007). In sub-Saharan Africa, political destabilization can be instigated by NTDs via their impact on agricultural efficiency and thus food security, among other factors. The resulting conflict,
in return, contributes greatly to the emergence of NTDs, especially HAT, leishmaniasis, and onchocerciasis (Hotez et al., 2009d).

Specialists in NTDs have persistently advocated the control of NTDs as a means to fight and eliminate poverty. Since most of the NTDs can be combatted with relatively cheap drugs, a serious commitment and political determination may lead to sustained reductions in the burden of NTDs, relieving the most disadvantaged peoples on Earth of the burden of these diseases (Feasey et al., 2010). To exemplify, in the case of HAT, almost a complete interruption of transmission was achieved by the mid-1960s as a result of mobile screening and treatment teams. Nonetheless, when these efforts were discontinued, the disease re-emerged by the 1980s, and by 1997 approximately 450,000 people were infected with HAT (Anonymous, 1998).

In some developing countries, the NTDs are so prevalent that the devastation caused by these diseases is hardly limited to the intense suffering or death of an individual, but rather only starts there. In these areas, NTDs promote extreme poverty due to their negative effects on worker productivity, child development, child mortality, and pregnancy outcome, and ultimately alter the economic viability of people already dealing with poverty. In sub-Saharan Africa, a geographical region struck with multiple diseases including NTDs, 73% of the population lives on less than $2 U.S. per day. Similarly, in South America, 40% of the population lives below the poverty line (Hotez et al., 2008; Hotez et al., 2009b; Hotez et al., 2009c). Consequently, the control and elimination of NTDs have been prioritized as a means to achieve United Nations Millennium Development Goals (MDGs) and also for sustainable poverty reduction (Paul, 2008).
Moreover, NTDs pose a constant challenge to global security and fundamental human rights (Beyrer et al., 2007; Hotez et al., 2007).

Social inequities including poverty have an intrinsic relationship with the prevalence of infectious diseases (Farmer, 1996). Remarkably, poverty by its own accord may dictate the overall public health level in a community, even when all other considerations that are crucial for health, such as the implementation of effective hygiene and public health policies, and access to comprehensive healthcare are otherwise favorable. For example, in a fully developed country such as the United States, underprivileged communities at large have higher disease prevalence and lower life expectations compared to the general population and the outcome of a disease may be considerably worse in those living below poverty level (Hotez, 2007).

The Millennium Development Goals (MDGs) is an international agreement signed by all 192 member nations of the United Nations that addresses extreme poverty. MDGs state eight international development goals to be achieved by the year 2015. Reducing the number of people living in absolute poverty by 50% by 2015 is one of the main goals. The U.N. Millennium Project also incorporates specific health-related targets, but, only HIV/AIDS, malaria, and tuberculosis were identified by name. Consequently, NTDs were relegated to the category of “other diseases”, though they affect as many poor people as the “big three” combined (Molyneux et al., 2005).

The difficulties that surround a global initiative to tackle NTDs are multiple. As stated earlier, there is not a consensus on what diseases should be classified within this
category. Furthermore, elimination and control efforts are hindered by the fragmented nature of individual efforts. Current international initiatives, financing efforts, and substantial drug programs have been described in a recent review (Liese et al., 2010). Different programs have distinctive standards for mapping, data reporting, and monitoring. The efficacy of intervention efforts is highly dependent on the identification of best practices for preventive chemotherapy and transmission control methods. This can be achieved by the development of coordinated processes between global programs and partnerships so as to alleviate the burden on national health systems (Liese et al., 2010).

Another possible way to reach people affected by NTDs may be through combining efforts with other programs, which mainly target the “big three.” This approach may be particularly successful for NTDs that have readily available cures. For instance, a strategy of integrated chemotherapy linked to the “big three” was suggested for the treatment of helminthic diseases. Unfortunately, diseases such as HAT and leishmaniasis do not fit with such provisions. In conclusion, currently the most effective method to tackle NTDs may lie in new and innovative research and development with the aim of discovering affordable, simple, and safe drugs (Torreele et al., 2006). This research effort coupled with government support for the implementation of effective interventions seems crucial for meaningful progress to be made. Drugs for Neglected Diseases Initiative (DNDi) has spearheaded an international appeal urging governments to take part in setting global public health priorities, to fund research for neglected diseases, and also to establish new rules to stimulate essential research (Torreele et al., 2006).
Despite the promising progress made in the last few years in terms of NTDs, it remains highly unlikely that the development of highly active, cheap, more non-toxic drugs by itself will lead to the global control of any disease. Better comprehension of the reality of NTDs in developing countries requires the consideration of the social and political implications of the burden, rather than approaching the problem solely as “diseases that need a cure”. In the same vein, curing these diseases will likely impact more than just the affected individuals leading to significant state-level economic revival in the aforementioned countries (Canning, 2006).

It is estimated that the combined global economy mounts up to a figure of $100 trillion USD. It has been argued that out of this amount, an annual commitment of $2–3 billion to the treatment of NTDs could provide “a modest yet highly cost-effective” mechanism for easing the extreme poverty that approximately one billion inhabitants face (Sachs, 2008). This commitment could fulfill successfully the ethical and human rights responsibility that accompanies the NTDs problem (Beyrer et al., 2007). Furthermore, these intervention programs ultimately invest in human capital, implicating their importance beyond the health sector. Human capital is commonly described as the combined acquired and useful abilities of all the inhabitants or members of the society. Under this consideration, a major reform changing the focus from cost-effectiveness estimates (the cost per unit of health outcome) to the projected rate of return for these efforts would permit the evaluation of intervention efforts in the same vein as other human capital investments, such as skill-building efforts and education (Canning, 2006). This shift in understanding might give NTD efforts a boost that is urgently needed.
Supportive of this outlook change, efforts in the NTD field in the past have consistently offered high-return rates, and improvements in health were accompanied by economic stimulation (Molyneux, 2008).

A4. Current Chemotherapy of Human African Trypanosomiasis, Leishmaniasis, and Malaria

As early as 1936, pathogenic organisms belonging to protozoa were known to be highly differentiated organisms and it was postulated that the more advanced an organism is, the more likely multiple targets suitable for chemotherapeutic intervention would be readily present. The authors showed their optimism for the eradication of these diseases with the following statement: “There is scarcely a protozoal disease of man which cannot be cured nowadays by early treatment with the appropriate synthetic drug” (Horlein, 1936). Nearly 70 years after this declaration, the treatment of these diseases still presents a concrete challenge and is further complicated by resistance. In this section, the current chemotherapy options for human African trypanosomiasis, malaria, and leishmaniasis will be discussed.

A4.1. African Sleeping Sickness (Human African Trypanosomiasis)

West African trypanosomiasis and East African trypanosomiasis represent the two different forms of human African trypanosomiasis and their causative agents and prognosis are distinctly different from each other. While the West African form is
characterized by a lengthy early-stage infection and a late neurologic stage, the East African form develops very rapidly and may result in death within one to three months without medical intervention (Linares et al., 2006). As expected, the treatment of early-stage infection, when the trypanosome parasites are present in the blood circulation and lymph nodes is less challenging than the fatal neurologic-stage when trypanosomes are present in the cerebrospinal fluid.

The lack of drug discovery efforts on African sleeping sickness is apparent from the short list of drugs recommended for the treatment of this disease: Three out of the four drugs approved for the chemotherapy of human African trypanosomiasis, suramin (1), pentamidine (2), and melarsoprol (3), were discovered more than 60 years ago, with eflornithine (4) being an exception. Furthermore, all these current therapies fall short of representing an ideal drug, due to various factors such as drug resistance, poor efficacy, unacceptable toxicity, and an undesirable route of administration (Fairlamb, 2003). The structures of compounds 1-4 are shown in Figure 1.
Chemically, suramin (1) is a polyanionic sulfonated naphthylamine, and was introduced for the treatment of HAT in the early 1920s and is still the drug of choice for the early phase of *T. b. rhodesiense* infections. Suramin needs to be given by the i.v. route and life-threatening immediate side effects, such as collapse and shock, and delayed side effects, such as agranulocytosis and hemolytic anemia, are common. Due to its highly ionic nature, suramin does not penetrate the central nervous system well, and its use is limited to early-stage trypanosomiasis (Fairlamb, 2003). While the mechanism of action for suramin is not thoroughly understood, this drug demonstrates selectivity towards trypanosomal glycolytic enzymes, and inhibits several enzymes including serine oligopeptidase, and dehydrogenases and kinases. The lack of resistance through its 80
years of use has led to the assumption that suramin possibly has several targets and multiple mechanisms of action (Fries et al., 2003).

Pentamidine (2) is an aromatic diamidine and has been the drug of choice for early *T. b. gambiense* infections since its introduction in the 1940s (Fairlamb, 2003). Pentamidine is somewhat less reliable in the treatment of *T. b. rhodesiense* infections and is reserved only as a second-line therapy in cases where suramin cannot be used (Pepin et al., 1994). Pentamidine is injected by the i.m. route due to its poor oral bioavailability, and similar to suramin, does not readily pass the blood-brain barrier, and hence is not used in late-stage trypanosomiasis. The cure rate using pentamidine is estimated to be approximately 90% in early-stage trypanosomiasis, with resistance to this drug having been slow to develop. Owing to these properties, pentamidine was the drug of choice in the mass chemoprophylaxis campaigns in the 1950s and 1960s in West and Equatorial Africa in an attempt to eradicate Gambian sleeping sickness (Fairlamb, 2003).

Major side effects of pentamidine (2) include dysglycemia, hypotension, pancreatitis, and cardiac, hepatic, and renal toxicity. The direct mechanism of action of this compound is not fully understood. Following administration, pentamidine is taken up by at least three transporters and accumulates in the mitochondria of *T. brucei*. Pentamidine is a known selective inhibitor of kinetoplast topoisomerase II and is also a minor groove binder with specificity towards the adenosine-thymidine (AT) sequence (Shapiro et al., 1990; Edwards et al., 1992). Another possible mode of action has been suggested to be through the inhibition of the enzyme *S*-adenosylmethionine decarboxylase, thus disrupting the polyamine biosynthesis in *T. brucei* parasites, although
this effect was not observed in *in vivo* studies (Berger *et al.*, 1993). Like suramin, pentamidine is indicated for only early-stage HAT.

Melarsoprol (3) is an organic arsenic compound developed in the 1940s specifically for the treatment of late-stage HAT caused by both *T. b. gambiense* and *T. b. rhodesiense* (Fairlamb, 2003). Melarsoprol is insoluble in water and its administration is limited to i.v. injections in propylene glycol, a highly irritant solvent. The high efficiency of melarsoprol in HAT is overshadowed by a diverse set of serious side effects. Most notably, the administration of melarsoprol causes serious reactive encephalopathy in up to 10% of patients and half of these reactions may prove fatal (Pepin *et al.*, 1994). Other side effects associated with melarsoprol include abdominal colic, arthralgia, peripheral neuropathy, and thrombophlebitis (a side effect of propylene glycol).

Melarsoprol (3) is a pro-drug and is synthesized by reacting melarsen oxide with 2,3-dimercaptopropanol, also known as British anti-Lewisite, a compound that was originally developed as an antidote for arsenical-based nerve gases. Upon administration, melarsoprol is quickly converted into the active metabolite, melarsen oxide. Melarsen oxide, like other trivalent arsenicals, acts as a promiscuous inhibitor of many enzymes with vicinal thiol groups. Following a selective uptake by the trypanosomal P2-purine transporter into the parasite, melarsoprol interferes with several metabolic and transport mechanisms (Carter *et al.*, 1993). The most widely accepted theory to explain the mechanism of action of melarsoprol points to a combination of trypanothione depletion and a concurrent inhibition of trypanothione reductase, with both events leading to loss of cell integrity and death in trypanosomes (Fairlamb, 2003).
Eflornithine (difluoromethyl ornithine, DFMO, 4) is the newest member of the short list of drugs indicated for the treatment of HAT. Eflornithine was developed in the early 1990s and is indicated for use against late-stage HAT caused by *T. b. gambiense*, but not *T. b. rhodesiense*. Eflornithine acts by inhibiting ornithine decarboxylase (ODC), a key enzyme in the polyamine biosynthetic pathway. While eflornithine inhibits both mammalian and trypanosomal ODCs quite efficiently, the rapid turnover of ODC in humans makes the use of eflornithine as a drug possible (Heby *et al.*, 2003). However, this might not be the only factor explaining how mammalian systems recover (Fairlamb, 2003). Inhibition of ODC causes a loss of putrescine and a decrease in spermidine levels, along with a decrease in trypanothione levels and profound disturbance in *S*-adenosylmethionine metabolism. These effects, in turn, lead to a generalized decrease in DNA, RNA and protein synthesizes, and morphological and biochemical changes.

### A4.2. Leishmaniasis

Among different forms of leishmaniasis, cutaneous leishmaniasis (CL) is the most prevalent worldwide, with the causative agents belonging to different species of the genus *Leishmania*. In the Mediterranean basin, the Middle East, and Africa, CL is caused mainly by *L. major* and *L. tropica* while the responsible organisms in Mexico, Central America, and South America include *L. braziliensis*, *L. mexicana*, and related species. The mucosal form (ML) of the disease is usually caused by *L. braziliensis* in the Americas, with *L. donovani* being the main cause in the Old World, while additional species have been also associated with ML (Guerra *et al.*, 2011). The more lethal form of the disease, visceral leishmaniasis (VL), is caused commonly by *L. donovani* and *L.
*infantum* in the Old World, and by *L. chagasi* in South America. This variance leads to differences in the efficacy of treatment regimens since different species respond differently to a given drug, even though the clinical manifestations may be almost indistinguishable (Croft *et al.*, 2006). While a spontaneous cure is almost the case for all forms of leishmaniasis except for the visceral form, complete healing may take months or years, so, therefore, chemotherapeutic intervention is imperative (Reithinger *et al.*, 2007). The structures of drugs used to treat leishmaniasis are given in Figure 2.

The first successful chemotherapeutic treatment of leishmaniasis became possible when pentavalent antimonials (Sb\(^V\)) were introduced in 1945. Over sixty years later, in many areas of the world, pentavalent antimonials, such as sodium stibogluconate (Pentostam\(^®\), 5) and meglumine antimoniate (Glucantime\(^®\), 6), have persisted as first-line treatments for both cutaneous and visceral leishmaniasis, and are endorsed by WHO for the treatment leishmaniasis in the Mediterranean area (Gradoni *et al.*, 1995). Despite their wide usage, pentavalent antimonials are far from being ideal drugs, due to their adverse effects and high-toxicity profiles (Delgado *et al.*, 1999). Moreover, the molecular mechanisms responsible for their activity are not fully understood.
Figure 2. Drugs currently used for the treatment of leishmaniasis.

The putative enzyme targets for antimonials in *Leishmania* spp. are trypanothione synthetase (TSa) and trypanothione reductase (TR). Both these enzymes play a part in the uniquely thiol-based metabolism that is seen in the members of the family Trypanosomatidae, and are absent in the host. Recently, a study elucidating the mechanism of inhibition of the TR activity by Sb$^{III}$ was published (Baiocco *et al*., 2009).

The side-effects of the parenteral use of antimonial drugs include changes in liver function tests such as increased amylase and lipase levels, and myalgias and arthralgias,
abdominal pain, nausea, and less frequently, thrombocytopenia, leucopenia, ECG changes, and cardiotoxicity (Murray et al., 2005). Besides these biological changes, some practical issues further complicate the use of antimonials, such as the phlebotoxic properties of Pentostam® (5), which lead to difficulties in finding a vein after the few doses in clinical settings.

Resistance to the antimonial drugs is an especially limiting factor for their use. While it is generally expected that 95% of previously untreated patients will respond to antimonial treatment, this is not the case in isolated regions where resistance is the rule rather than the exception. For example, in Bihar Province of India, where visceral leishmaniasis is endemic, 43% of the cases were reported to be unresponsive to antimonials (Croft et al., 2006). This increasingly common phenomenon has been attributed to various factors including genetic changes in individual pathogenic species as a consequence of evolutionary pressure and the varying sensitivity levels of different species of Leishmania to antimonials. Since treatment of patients is not accompanied commonly by efforts to identify the specific pathogen, the outcome may not always be predictable. Other currently used antileishmanial therapeutics include amphotericin B (AmB, 7), paromomycin (8), and miltefosine (9).

Amphotericin B (7) was isolated in 1955 from the filamentous bacterium Streptomyces nodosus, which was discovered in a soil sample from Venezuela (Donovick et al., 1955; Stiller et al., 1955). Amphotericin B, a polyene-type macrolide, was the first commercially significant antifungal drug when it was approved for human use and is still regarded as the “gold standard” for the treatment of life-threatening systemic fungal
infections and leishmaniasis (Gallis et al., 1990; Cohen, 2010). The name of this molecule was coined after its amphoteric properties. Similar to some other drugs of the macrolide class, AmB has amphiphilic properties, which lead to multi-molecular aggregates of AmB in biological membranes. This results in the formation of two distinct ion channels, effectively disrupting the homeostasis of the parasite with lethal consequences (Cohen, 2010).

The high-efficacy of AmB (7), however, is complicated by acute and delayed adverse reactions. The acute side effects following parenteral observation have been observed in up to 79% of patients receiving AmB and range from chills, fever, and headache to systemic problems including hypotension, bronchospasm, and allergic reactions. Severe delayed reactions such as hypokalemia, hypomagnesaemia, and nephrotoxicity are also common (Walker et al., 1998).

Liposomal amphotericin B (AmBisome) was developed in the early 1990s in order to reduce the severity of these side effects. Liposomes, along with other vesicular drugs and particulate structures are removed by macrophages in reticuloendothelial structures. Since Leishmania parasites are intracellular parasites in their infectious stage, the liposomal form of AmB (7) offers the advantage of targeting parasites more efficaciously, thus offering a higher therapeutic window when compared to the parent drug (Smith et al., 1995). Other parenteral pharmaceutical forms of AmB have also been developed, mostly with very limited success. However, the use of both AmB and AmBisome, apart from issues regarding toxicity and expenses, is impractical since the patients are required to visit a clinic for parenteral injections throughout the duration of
their treatment schedule. This is especially an issue in rural areas where patients may need to move away from their home villages for treatment, impacting their economic well-being. Despite these limitations, the use of Amb against VL in India is significant. Research efforts have for some time focused on developing an orally available form of Amb and some successful results have been reported in animal studies (Wasan et al., 2010).

Paromomycin (Humatin®, 8) is another natural product drug indicated for leishmaniasis. Paromomycin is an aminoglycoside class antibiotic that was isolated in 1950s from Streptomyces krestomuceticus. While the spectrum of activity of paromomycin, similar to other aminoglycosides, covers most Gram-negative and many Gram-positive bacteria, it is the only molecule of this class of clinical significance when used against leishmaniasis (Davidson et al., 2009). The antileishmanial properties of paromomycin were discovered in 1961, though its poor oral bioavailability limited its use to intramuscular injections. While the mode of action of paromomycin in bacteria is well established as being through binding to the 30S ribosomal subunit and thus impairing protein synthesis, research indicates that the mode of action in Leishmania parasites might be more complex and involve inhibition of mitochondrial respiration along with its effects on metabolism.

Paromomycin (8) exerts its effects on both the parasitic promastigote and amastigote stages. In clinical settings, the common side effects of the aminoglycoside class of antibiotics, such as cochlear, renal, and vestibular toxicity are negligible at the dosage administered. As such, paromomycin is used against various forms of
leishmaniasis as intramuscular or topical preparations with great success and is one of the most inexpensive treatments available today. Injectable paromomycin was approved for use in India in 2007 and is now included in the *WHO Model List of Essential Medicines* for the treatment of VL (Anonymous, 2011). As is the case in other therapeutics, resistance can develop in response to drug pressure. Accordingly, combination therapies have been suggested to counter such resistance (Davidson *et al.*, 2009).

Miltefosine (9) was developed synthetically in an effort to develop more potent derivatives of a lead anticancer compound, edelfosine (Sindermann *et al.*, 2006). While a topical form of miltefosine initially went into clinical trials and was eventually approved for the treatment of skin lesions from breast cancer, the oral form of the drug caused dose-limiting side effects and was not further developed past phase II studies for this purpose. Due to a previous discovery that demonstrated the leishmanicidal activity of a compound of the same class, 1-\(O\)-octadecyl-glycerol, though at relatively high doses, the possible use of miltefosine for leishmaniasis was investigated further (Berman, 2006). In the initial testing, possibly owing to the poor intake of the drug at the lesion sites, the topical form of miltefosine did not demonstrate consistent efficacy against CL. However, the oral form of miltefosine did indeed possess antileishmanial activity in mice and since the drug had already been in phase II studies at this point and its safety profile was established, a pilot study for the treatment of visceral leishmaniasis was quickly sanctioned in 1998 (Sundar *et al.*, 1998; Berman, 2006). Consequently, oral miltefosine was further developed for its use in leishmaniasis and has been accepted by TDR (a
Special Programme for Research and Training in Tropical Diseases) and the World Health Organization.

As is the case with other alkyl-lysophospholipids, the mode of action of miltefosine (9) has not been fully elucidated. Most research data indicate that miltefosine causes death by promoting apoptosis (Sundar et al., 2007). Most commonly accepted hypotheses suggest that this effect is via inhibition of the synthesis of phosphatidyl choline (PC), without which the synthesis and integrity of cellular membranes are severely disrupted (Wright et al., 2004; Sundar et al., 2007).

Miltefosine (9), along with AmB (7), remains the drug of choice for antimonial-resistant cases of leishmaniasis. Of significance, miltefosine is the first oral drug effective against leishmaniasis. While miltefosine-resistant parasites can be produced under laboratory conditions, there are hardly any reports of resistance against miltefosine in the field and its use is only limited by dose-dependent side effects and teratogenicity (Ouellette et al., 2004).

A4.3. Malaria

As early as in the 1630s, long before the biology of malaria was revealed, “Jesuit’s Bark” was being used to treat the fevers and chills that are one of the hallmarks of malaria. Jesuit’s Bark is originally from Peru and is obtained from several Cinchona spp. (Rubiaceae). The active ingredient of the bark, quinine (10), a quinoline-type
alkaloid, was first isolated by Pelletier and Caventou in 1820, and the first chemotherapeutic for malaria was being used long before the causative agents were discovered in 1870. The total alkaloid content of the bark may be as high as 17% by weight, with quinine, quinidine, cinchonidine, and cinchonine being the most prominent quinoline alkaloids (Guerrant et al., 2005).

The structures of compounds used to treat malaria are shown in Figure 3. Quinine (10) remained one of the few available drugs for malaria till the 1930s, when chloroquine (12) was synthesized and became clinically available. Quinine has served as a lead compound for many of the modern synthetic anti-malarial drugs, including amodiaquine (11), chloroquine (12), mefloquine (13), primaquine (14), and tafenoquine (15).

These compounds are generally referred to as quinoline-like or quinoline-related compounds and constitute one of the four major classes of antimalarials. The digestion of hemoglobin, during the intraerythrocytic asexual reproduction cycle, leads to production of monomeric $\alpha$-hematin, a pro-oxidant compound toxic to the parasite. To avoid $\alpha$-hematin accumulation, *Plasmodium* species utilize a biocrystallization process and convert $\alpha$-hematin into chemically inert and insoluble $\beta$-hematin crystals, called
Figure 3. Drugs currently used for the treatment of malaria, along with sulfachrysoidine (16) and sulfanilamide (17), which were previously used.

Compounds belonging to the quinoline class of antimalarials are lysosomotropic, owing to the pKa of the quinoline nitrogen atom and are “trapped” in the acidic food vacuole of the parasite. Once at this site, quinoline-type drugs lead to heme (ferroprotoporphyrin, FP) build-up by capping hemozoin molecules thus hindering the essential bio-crystallization process. Furthermore, quinoline-type drugs can also
**Figure 3 (continued).** Drugs currently used for the treatment of malaria.

- **cycloguanil (18)**
- **pyrimethamine (19)**
- **trimethoprim (20)**
- **artemisinin (21)**
- **dihydroartemisinin (22)**
- **arteether (23)**
- **artemether (24)**
- **sodium artesunate (25)**
- **lumefantrine (26)**
- **sulfadoxine (27)**
- **atovaquone (28)**
make complexes with FP, which are highly toxic to the parasite. This is widely believed to be the main mode of action for this class of compounds.

The second major class of drugs in use today exhibit their activity by interfering with the folic acid metabolism and are often referred to as antifolate drugs. Folate metabolism is an ubiquitous process used in the cell cycles, including those of bacteria, plants, protozoans, and animals. The cascade of events in folate metabolism provides cofactors that are acceptors for the transfer of one-carbon units and is essential for the survival of rapidly growing cells (Stokstad et al., 1967). Unlike humans, who are not capable of de novo synthesis of folic acid and are dependent on nutrient sources, Plasmodium spp. employ parasite-specific enzymes for the biosynthesis of folic acid. Among these enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) have emerged as specific targets for chemotherapeutic intervention (Yuthavong et al., 2006).

Sulfachrysoidine (16) and sulfanilamide (17) were the first drugs found to exhibit antimalarial activity through interfering with folate synthesis (Hall, 1938). Since this initial discovery, folate precursors, folate metabolites, folate end-products, and folate analogues have been the subject of intense research for their possible use against malaria (Ferone, 1977). The drugs in this class currently used for malaria include cycloguanil (18), pyrimethamine (19), and trimethoprim (20).

A major breakthrough in the treatment of malaria in the last century was the discovery of artemisinin (21), also known as Qinghaosu, from the plant Artemisia annua
(Asteraceae). This finding was a result of a concentrated effort of Chinese scientists to identify compounds with antimalarial activity from Chinese traditional herbs (Miller et al., 2011). Artemisinin and its antimalarial activity was first described in 1979 in an article that was not attributed to any specific author (Anonymous, 1979).

Structurally, artemisinin is a sesquiterpene lactone, with an unusual endoperoxide bridge that is needed for the antimalarial activity of the compound. Many antimalarials structurally related to artemisinin have been isolated or synthesized since its initial discovery, most notably dihydroartemisinin (22), arteether (23), artemether (24), and sodium artesunate (25).

Artemisinin and its derivatives constitute one of the most effective classes of compounds for the treatment of malaria and are effective against multidrug-resistant strains of Plasmodium parasites. From a clinical perspective, the artemisinins demonstrate the broadest time window of activity of any antimalarials by inhibiting parasites in both the asexual blood stages and the sexual gametocytes (O'Neil et al., 2010) and they exhibit activity at nanomolar concentrations (Meshnick et al., 1996).

While the activity of artemisinin was shown originally to be through the bioactivation of 1,2,4 trioxane by iron (II) and the consequent generation of toxic activated oxygen (Meshnick et al., 1991), currently there are two suggested models for the trioxane ring opening, namely, the reductive scission model and open peroxide model. The known targets for the artemisinins include heme, plasmodial ATPase6, several parasitic proteins, and parasite membranes (O'Neil et al., 2010).
Artemisinin derivatives have been determined to be most effective in combinations with other antimalarials that are eliminated more slowly. Common examples of artemisinin combination treatments (ACT) are artemether (24) and lumefantrine (26), artesunate (25) and amodiaquine (11), artesunate (25) and mefloquine (13), and artesunate (25) and sulfadoxine (27)/pyrimethamine (19) (Pearson et al., 2006).

Atovaquone (28), a naphthoquinone, is an antimalarial compound with a unique mode of action and is currently the only drug in its class. Atovaquone selectively targets the mitochondrial respiratory chain through inhibition of the mitochondrial electron transport in the parasite (Looareesuwan et al., 1999). An analogue of ubiquinone, atovaquone possesses broad-spectrum antiprotozoal activity and used commonly in combination with proguanil, under the trade name Malarone® (Looareesuwan et al., 1999; Baggish et al., 2002). This particular combination has been shown to be more effective than either single component alone and also more effective than other available forms of treatment, especially in areas where resistance is an issue. Owing to its novel mechanism of action, and activity against resistant parasite strains, Malarone® remains one of the most effective drugs in the relatively limited arsenal of drugs against malaria (Looareesuwan et al., 1999).
CHAPTER 2. LITERATURE REVIEW ON THE GENUS *THALICTRUM* (RANUNCULACEAE): CHEMISTRY AND BIOLOGICAL ACTIVITY OF PROTOBERBERINE-TYPE ALKALOIDS

B1. Botanical Background

B1.1. Botanical Information on the Ranunculaceae

The plant family Ranunculaceae is known commonly as the “buttercup family” and is related taxonomically to the families Berberidaceae and Papaveraceae. Among the angiosperms, the Ranunculaceae constitutes evolutionarily one of the most primitive families and is divided into four subfamilies, namely, Coptidoideae, Helleboroideae, Ranunculoideae, and Thalictroideae. The genera in this family include *Aconitum*, *Clematis*, *Delphinium*, *Ranunculus*, and *Thalictrum*. The family is known to biosynthesize a diverse set of alkaloids and many plants belonging to Ranunculaceae have been used medicinally. The botanical diagnostic characteristics of plants in the family Ranunculaceae and their distribution are described in the *Flora of North America* as follows (Whittemore *et al.*, 1997):

Herbs, sometimes woody or herbaceous climbers or low shrubs, perennial or annual, often rhizomatous. Stems unarmed. Leaves usually basal and cauline, alternate or sometimes opposite, rarely whorled, simple or
variously compound; stipules present or absent; petioles usually present, often proximally sheathing. Leaf blade undivided or more commonly divided or compound, base cordate, sometimes truncate or cuneate, margins entire, toothed, or incised; venation pinnate or palmate. Inflorescences terminal or axillary, racemes, cymes, umbels, panicles, or spikes, or flowers solitary, flowers pedicellate or sessile. Flowers bisexual, sometimes unisexual, inconspicuous or showy, radially or bilaterally symmetric; sepaloid bracteoles absent; perianth hypogynous; sepals usually imbricate, 3-6(-20), distinct, often petaloid and colored, occasionally spurred; petals 0-26, distinct (connate in Consolida ), plane, cup-shaped, funnel-shaped, or spurred, conspicuous or greatly reduced; nectary usually present, rarely absent; stamens 5-many, distinct; anthers dehiscing longitudinally; staminodes absent (except in Aquilegia and Clematis ); pistils 1-many; styles present or absent, often persistent in fruit as beak. Fruits achenes, follicles, or rarely utricles, capsules, or berries, often aggregated into globose to cylindric heads. Seeds 1-many per ovary, never stalked, not arillate; endosperm abundant; embryo usually small. Genera ca. 60, species 1700 (22 genera, 284 species in the flora): worldwide.

**B1.2. Botanical Information on the Genus *Thalictrum* L.**

*Thalictrum* L. is a large genus of the Ranunculaceae, with about 120-200 species distributed from tropical to temperate regions. The genus is known commonly as “meadow-rue” or “pigamon” and was first described by the father of modern taxonomy, Carl Linnaeus (Linnaeus, 1753). Selected botanical diagnostic characteristics of plants of
the genus *Thalictrum* are described as follows in the *Flora of North America* (Park et al., 1997):

Herbs, perennial, from woody rhizomes, caudices, or tuberous roots. Leaves basal and cauline, proximal leaves petiolate, distal leaves sessile; cauline leaves alternate. Leaf blade 1-4×-ternately or -pinnately compound; leaflets cordate-reniform, obovate, lanceolate, or linear, sometimes 3-lobed or more, margins entire or crenate. Inflorescences terminal, sometimes also axillary, (1-)2-200-flowered panicles, racemes, corymb, umbels, or flowers solitary, to 41 cm; involucres absent or present, involucre bracts 2-3 (these compound, often resembling whorl of 6-9 simple bracts), leaflike, not closely subtending flowers. Flowers all bisexual, bisexual and unisexual on same plant, or all unisexual with sexes on same or different plants, radially symmetric; sepals not persistent in fruit, 4-10, whitish to greenish yellow or purplish, plane, lanceolate to reniform or spatulate, 1-18 mm; petals absent; stamens 7-30; filaments filiform to clavate or distally dilated; staminodes absent between stamens and pistils; pistils 1-16, simple; ovule 1 per pistil; style present or absent. Fruits achenes, usually aggregate, sessile or stipitate, ovoid to obovoid, falcate, or discoid, sides prominently veined or ribbed; beak present or absent, terminal, straight to coiled, 0-4 mm. x = 7. Species 120-200 (22 in the flora): nearly worldwide, mostly temperate.

**B1.3. Botanical Information on *Thalictrum lucidum* L.**

Botanical diagnostic characteristics of *Thalictrum lucidum* L. include the following description from the *Flora of Turkey and the East Aegean Islands* (Davis et al., 1965):
Glabrous herb. Stems erect, strict, up to 100 cm. Leaves ± oblong the two lateral divisions smaller than the middle division. Ultimate segments of the lower leaves narrowly oblong, those of the upper leaves very narrow, linear. Inflorescence dense, the branches erect, the flowers aggregated together in fascicles. Achenes ellipsoid, not compressed, with 8-10 ribs. 

Fl. 6-7. Ditches, marshy places, s.I.-1400 m.

B2. Constituents of the Genus Thalictrum

The genus Thalictrum has been investigated extensively for its phytochemical constituents. Secondary metabolites from the genus are structurally diverse, spanning from cycloartane- and oleanane-type triterpenoid glycosides, to flavonoids, cyanogenic glycosides, fatty acids, phenolic compounds, and phytosterols (Khamidullina et al., 2006). Most notably, the genus is known for its rich content of alkaloids, commonly referred to as “Thalictrum alkaloids”, and a great deal of research has focused on this aspect. Consequently, the structural diversity of alkaloids isolated from this genus will be described following a structural classification used in a previous review (Schiff, 1996).

B2.1. Monomeric Alkaloids

B2.1.1. Aporphines and Related Alkaloids

Compounds based on aporphine (29) constitute a large class of alkaloids, with over 500 examples reported to date. From a biosynthetic perspective, aporphines possess an isoquinoline ring system and are biosynthesized from the precursor molecule, (S)-reticuline (30). Their distribution is especially abundant in the families Berberidaceae, Papaveraceae, and Ranunculaceae, all from the order Ranunculales. Alkaloids of this
class have demonstrated anti-HIV (Kashiwada et al., 2005), antileishmanial (Fournet et al., 2007), antitrypanosomal (Fournet et al., 2007), antimalarial (Kaur et al., 2009), cytotoxic (Stevigny et al., 2005), dopaminergic and serotonergic (Cabedo et al., 2009), and human topoisomerase I and II inhibitory (Hoet et al., 2004) activities. More than fifty compounds of this type have been isolated previously from Thalictrum spp., including (+)-acutifolidine (31) from Thalictrum acutifolium (Guinaudeau et al., 1994), (+)-leucoxylonine N-oxide (32) from Thalictrum simplex (Velcheva et al., 1995), and magnoflorine (33) from Thalictrum podocarpum (Wu et al., 1977b). The structures of selected aporphine alkaloids from the genus Thalictrum are shown in Figure 4.

B2.1.2. Betaine-type Alkaloids

Betaines are named traditionally after N,N,N-trimethylglycine (34), a compound first isolated from Beta vulgaris, the sugar beet, and refer structurally to a specific zwitterion molecule that has a positively charged cationic functional group such as a quaternary ammonium and a negatively charged functional group such as a carboxylate group. Betaine-type compounds assume many biologically significant roles in mammals. Acetylcholine, the major neurotransmitter in the cholinergic system in humans, is synthesized from choline (35) and acetyl-CoA, by the enzyme choline acetyltransferase (ChAT). Betaine is also important as an osmolyte and functions as a methyl donor in transmethylation reactions (Craig, 2004). Choline chloride has been previously isolated from Thalictrum spp., including Thalictrum revolutum (Wu, J. et al., 1980). The structures of selected betaine alkaloids from the genus Thalictrum are shown in Figure 5.
Figure 4. Structures of some aporphine-type alkaloids isolated previously from the genus *Thalictrum* and the aporphine precursor molecule, (S)-reticuline (30).

Figure 5. Structures of some betaine-type alkaloids previously isolated from the genus *Thalictrum*.

**B2.1.3. Diterpenoid alkaloids.**

The main skeleton of the diterpenoid alkaloids is biosynthesized mainly via the mevalonate and methylerthritol phosphate pathways, from geranylgeranyl diphosphate
Diterpenoid alkaloids represent a highly modified subclass of this diverse class of compounds, and hence their biosynthesis differs greatly from those of classical alkaloids, which are biosynthesized almost entirely from amino acids. The origin of the nitrogen atoms in diterpene alkaloids is not always readily apparent, since few have been identified through labeling studies (Cherney et al., 2011). The Thalictrum genus, along with the evolutionarily related Delphinium and Consolida spp., accumulate a large variety of diterpene alkaloids, some of which have been shown to be biologically active. An example of a diterpene alkaloid isolated from Thalictrum is the spiradine A-type (36) alkaloid, (+)-thalicessine (37), from Thalictrum simplex (Velcheva et al., 1993). The structures of these compounds are shown in Figure 6.

**Figure 6.** Structure of a diterpenoid alkaloid isolated previously from the genus *Thalictrum*.

B2.1.4. *Isoquinolines, Isoquinolones, and Related Alkaloids*
Isoquinoline-type (38) alkaloids are biosynthesized from tyrosine and are an especially abundant class of compounds in the plant kingdom. Isoquinoline- and isoquinolone-type (44) alkaloids, along with their derivatives such as tetrahydroisoquinolines (39), benzylisoquinolines (40), and benzyltetrahydroisoquinolines (41), are abundant both as monomers, and also in dimeric form with other classes of alkaloids in Thalictrum species. Two examples of the monomeric form of isoquinoline alkaloids include rugosinone (42) and tembetarine (43) from Thalictrum foliolosum (Chattopadhyay et al., 1983) and are shown in Figure 7. Thalifoline (45) from Thalictrum minus var. adiantifolium (Doskotch et al., 1969), and thalactamine (46) from Thalictrum minus (Dutschewska et al., 1982) are examples of isoquinolone-type alkaloids from Thalictrum (Figure 8).

The incorporation of a phenylethyl group into the isoquinoline (38) skeleton is relatively common and leads to the biosynthesis of benzyltetrahydroisoquinoline-type (41) alkaloids. This class of alkaloids is found especially in five plant families, namely, Berberidaceae, Fumariaceae, Menispermaceae, Papaveraceae, and Ranunculaceae, representing upwards of 2500 compounds (Dewick, 2009). A highly modified class of benzyltetrahydroisoquinoline-type alkaloids are the protoberberines (47), which are biosynthesized through a specific enzyme, the berberine bridge enzyme, catalyzing the conversion of (S)-reticuline (30) to (S)-scoulerine (48) to form a carbon-carbon bridge between the N-methyl group and the phenolic ring through oxidative cyclization (Kutchan et al., 1995).
Two examples of this structural type include 8-oxocoptisine (49), from *Thalictrum glandulosissimum* (Lou *et al.*, 1992) and thalidastine (50) from *Thalictrum cultratum* (Shamma *et al.*, 1965), and are shown in Figure 9.
A further modification of the protoberberine skeleton, namely, cleavage of the heterocyclic ring adjacent to the nitrogen atom, yields the protopine (51) skeleton. Protopine-type alkaloids have been shown to inhibit platelet aggregation (Chia et al.,
Figure 10. Structures of some protopine-type alkaloids isolated previously from the genus *Thalictrum*.

2006), and demonstrate significant hepatoprotective activity *in vivo* (Rathi *et al.*, 2008). Cryptopine (49) (Figure 10) was isolated previously from the roots of *Thalictrum flavum* (Ismailov *et al.*, 1968), while protothalipine (50) (Figure 10) was isolated from *Thalictrum rugosum* (Wu *et al.*, 1976a).

**B2.1.5. Morphinan-type Alkaloids**

Morphinan-type alkaloids (54), unlike protoberberine and phenanthrene-type alkaloids, are biosynthesized from the (R)-isomer of reticuline (30). The transformation from (R)-reticuline to salutaridine (55) is achieved by the one-electron oxidation of phenol groups followed by a radical coupling facilitated by the enzyme salutaridine synthase (Dewick, 2009). Morphinan-type alkaloids do not have a large distribution among the plant kingdom, and, as exemplified by *Papaver* species, their occurrence might be limited to only a few species even within the same genus. The observation of morphinan-type alkaloids from the genus *Thalictrum* is remarkable, since it points to a biosynthetic machinery in the genus that can produce all of the aporphine-,
protoberine-, and morphinan-type skeletons. Two morphinan-type alkaloids isolated from the genus *Thalictrum* are ocobotine (56) (Figure 11) from *Thalictrum fauriei* (Lee et al., 1996) and (+)-pallidine (57) from (Figure 11) *Thalictrum dioicum* (Shamma et al., 1973).

**Figure 11.** Structures of some morphinan-type alkaloids isolated previously from the genus *Thalictrum*.

**B2.1.6. Pavine- and Isopavine-type Alkaloids**

Pavines (58) and isopavines (61) constitute a small subset of alkaloids. From the biosynthetic standpoint, they share a benzylisoquinoline precursor and are functionalized in the B-ring system. Their distribution is limited to the families Berberidaceae, Lauraceae, Papaveraceae, and Ranunculaceae. Even within these families, their
occurrence is not widespread in multiple genera and within the Ranunculaceae pavines have been reported only from the genus *Thalictrum* (Gözler et al., 1983). *Thalictrum* also is the only genus where the occurrence of pavines coincides with presence of isopavines.

Selected pavine alkaloids that were isolated from *Thalictrum* species are (-)-argemonine (59) (Figure 12) from *Thalictrum revolutum* (Wu et al., 1980a), and (-)-eschscholtzidine (60) (Figure 12) from *Thalictrum minus* (Sidjimov, 1997). (-)-Thalisopavine (62) (Figure 12) from *Thalictrum dasycarpum* and (-)-thalidine (63) (Figure 12) from *Thalictrum dioicum* (Shamma et al., 1976) are examples of the isopavine-type alkaloids isolated from *Thalictrum* species.

Figure 12. Structures of some pavine- and isopavine-type alkaloids isolated previously from the genus *Thalictrum*. 

![Figure 12](image-url)
B2.1.7. Phenanthrene-type Alkaloids

Phenanthrene-type compounds (64) represent a small class of alkaloids possessing a phenanthrene ring that are relatively abundant in the genus Thalictrum. Some examples include thalflavidine (65) (Figure 13) from Thalictrum rugosum (Wu, W. N. et al., 1980) and thalicthuberine (66) (Figure 13) from Thalictrum minus (Sidjimov et al., 1984).

B2.2. Dimeric Alkaloids

B2.2.1. Aporphine-Benzylisoquinoline and Dehydroaporphine-Benzylisoquinoline Alkaloids

Adiantifoline (67) (Figure 14) was isolated from the roots and above-ground parts of Thalictrum minus var. adiantifolium (Doskotch et al., 1968). Another alkaloid with the same skeletal type, northalicarpine (68) (Figure 14), was isolated from the roots of Thalictrum revolutum (Wu et al., 1980b). Dehydroaporphine-benzylisoquinoline-type alkaloids are formed through full aromatization of the aporphine ring between the C-6a

![Chemical structures](image)

**Figure 13.** Structures of some phenanthrene-type alkaloids isolated previously from the genus Thalictrum.
and C-7 positions. An example of a compound based on this skeleton is 6a,7-dehydromethoxyadiantifoline (69) (Figure 14) from *Thalictrum foetidum* (Schiff, 1996).

**B2.2.2. Aporphine-Pavine Alkaloids.**

Aporphine-pavine dimers also constitute a small subgroup of dimeric *Thalictrum* alkaloids, probably due to the relatively low abundance of compounds bearing the pavine skeleton among the species in this genus. The first examples of this subtype to be reported were pennsylpavine (70) and pennsylpavoline (71) (Figure 16), both from *Thalictrum polygamum* (Shamma *et al*., 1974).

![Chemical structures](image)

**Figure 14.** Structures of some aporphine-benzylisoquinoline-type alkaloids isolated previously from the genus *Thalictrum*.
Figure 15. Structures of some aporphine-pavine-type alkaloids isolated previously from the genus *Thalictrum*.

### B2.2.3. Bisbenzylisoquinoline and Secobisbenzylisoquinoline Alkaloids.

Bisbenzylisoquinoline alkaloids constitute a large set of compounds and their structural diversity varies widely depending on the changes in the position of the ether bridge, the presence of one or two ether bridges, and the substitution patterns found in the benzylisoquinoline units. Bisbenzylisoquinoline alkaloids occur in multiple genera and their distribution and diversity have been reported previously in extensive reviews on the topic (Schiff, 1987; Schiff, 1997). Some examples for compounds of this skeletal type isolated from *Thalictrum* species include thaligosidine (72) and thaligosinine (73) (Wu et al., 1978) from *Thalictrum rugosum* (Figure 16). Revolutinone (74) (Figure 16) was isolated from *Thalictrum revolutum* (Wu et al., 1980a) and serves as an example of a secobisbenzylisoquinoline alkaloid, where one of the benzylisoquinoline units is cleaved.
Figure 16. Structures of some bisbenzylisoquinoline and secobisbenzylisoquinoline alkaloids isolated previously from the genus *Thalictrum*.

**B3. Constituents of *Thalictrum lucidum* L.**

The only extensive previous phytochemical studies on the alkaloids of *Thalictrum lucidum* have been carried out at the College of Pharmacy, The Ohio State University, and the findings were published in two separate articles (Wu *et al.*, 1976b; Wu *et al.*, 1976c). For these studies, an alkaloid isolation scheme was followed.

Using this approach, oven-dried and powdered roots were percolated with 95% ethanol and the dried residue from this step was suspended with 2% aqueous citric acid solution. This extract was partitioned with ethyl acetate to remove acidic and neutral substances. The aqueous extract was further partitioned with NH₄OH in diethyl ether (pH = 9-10) and CHCl₃ successively to yield diethyl ether, CHCl₃, and aqueous layers. The
diethyl ether layer was partitioned with 5% NaOH to yield diethyl ether and aqueous fractions used for isolation studies.

The diethyl ether partition yielded homoaromoline (75), O-methylthalicberine (76), obaberine (77), 8-oxyberberine (78), thalidasine (79), thaliglinone (80), and thalrugosine (81). The aqueous layer was once more partitioned with NH₄OH in diethyl ether and obamegine (82) and oxyacanthine (83) were isolated by column chromatography from the resulting diethyl ether layer. The alkaloids thaliglinone (80) and thalrugosine (81) were reisolated from the CHCl₃ layer along with the artifactual 8-trichloromethylidihydroberberine (84).

The aqueous layer was treated with a 2% aqueous ammonium reineckate solution and the salt precipitates were mixed with 50% aqueous acetone. After filtration, an IRA-400 ion-exchange resin was employed to provide the quaternary alkaloids as their chloride salts. Further chromatography of this fraction yielded berberine (85), columbamine (86), jatrorrhizine (87), magnoflorine (88), palmatine (89), and thalifendine (90). The isolated alkaloids were tested for their biological activity and O-methylthalicberine (76) showed significant hypotensive properties at 2 mg/kg in a dog model, while homoaromoline (75), obamegine (82), obaberine (77), thaliglinone (80), and thalrugosine (81) had modest activity in the same model (Wu et al., 1976c). In a continuation of this study, two further bis-benzylisoquinoline alkaloids, aromoline (91) and thalicberine (92), were reported (Wu et al., 1976b). The structures of the compounds isolated to date from _T. lucidum_ are shown in Figure 17.
Figure 17. Structures of compounds isolated previously from *Thalictrum lucidum.*
B4. Biosynthesis of the Alkaloidal Protoberberine Skeleton

Alkaloids encompass a large number of natural products. The definition of which compounds should be called “alkaloids” presents a debate among experts in the field. Generally speaking, most alkaloids are small nitrogen-containing molecules with no apparent role in the internal economy of the producing organism. Out of over 27,000 alkaloids known to date, 21,000 have been isolated from terrestrial plants (Dewick, 2009). All “true” alkaloids possess one or more nitrogen atom, usually in a heterocyclic ring (unlike “protoalkaloids”, which have the nitrogen atom on a side chain).
According to at least one definition, “true” alkaloids are biosynthesized from amino acids with further modifications, as opposed to “pseudoalkaloids”, such as those which are synthesized from purine bases or terpene skeletons (Cordell, 1981; Dewick, 2009).

The name “alkaloid” originates from the term “alkali” due to the basic properties offered by the nitrogen atom, although the degree of the basicity of an alkaloid may differ significantly depending on the presence of other functional groups and the location of the nitrogen atom. While this is the case, most alkaloids indeed have basic properties and can be made into salts, and this property was utilized greatly in the early separation and identification of alkaloids long before chromatographic and more advanced laboratory methods became available. It was probably owing to this physical property that the first natural products isolated in the 19th century were indeed alkaloids. Notably, the ethnobotanical uses of many plants may indeed take advantage of relevant physicochemical properties. While most alkaloids contain a nitrogen atom as primary, secondary, or tertiary amines, alkaloids with quaternary nitrogens are also found in nature, as is in the case of berberine (85) (Grycova et al., 2007; Dewick, 2009).

The biosynthesis of true alkaloids involves amino acid precursors, and, while the carboxylic acid is usually lost through a decarboxylation reaction, the skeleton of the amino acid is usually somewhat conserved in the final product. The acetate, methylerthyritol, and shikimate pathways may also be involved in the biosynthesis of alkaloids and hence contribute greatly to their chemical diversification. In this way, the
analysis of the structure of an alkaloid can yield hints about what its amino acid precursor may be. However, this approach is not always conclusive without extensive labeling studies, since some alkaloids acquire only the nitrogen atom from an amino acid through a transamination reaction and the remainder of the skeleton is formed via alternative pathways, such as those mentioned previously.

Since one organism can indeed utilize more than one amino acid source for its complement of alkaloids, the most commonly accepted method to group alkaloids is to utilize a chemical classification system rather than a taxonomic approach. In this manner, alkaloids can be classified based on their respective amino acid precursor, and, in the case of alkaloids that are biosynthesized through an alternate pathway, these particular pathway(s) that contributed to their accumulation can be used for classification purposes.

The biosynthetic pathway leading to protoberberine-type alkaloids shares the same initial steps leading to the biosynthesis of (S)-reticuline (30), which is the key molecule leading to the aporphine-, benzophenanthridine-, and morphinan-type skeletons. The phenyltetrahydroisoquinoline unit in the protoberberine skeleton is provided by two tyrosine (93) units. In the early steps in the biosynthesis, one unit of L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA, 94), while another tyrosine unit goes through pyridoxal 5'-phosphate (PLP)-mediated transamination, forming 4-hydroxyphenylpyruvic acid (95). L-DOPA goes through a decarboxylation via a process mediated by the aromatic amino acid decarboxylase (DOPA decarboxylase) and is converted to dopamine (96). The decarboxylation of the 4-hydroxyphenylpyruvic acid unit leads to the formation of 4-hydroxyphenylacetaldehyde (97).
These two units, 96 and 97, are coupled in a Mannich-like reaction mediated by the enzyme norcoclaurine synthase, forming (S)-norcoclaurine (98), the first compound in the series with a benzyltetrahydroisoquinoline backbone. (S)-Norcoclaurine, in turn, goes through a methylation reaction mediated by norcoclaurine 6-O-methyltransferase, yielding (S)-coclaurine (99). The enzyme (R,S)-coclaurine N-methyltransferase methylates the nitrogen atom, giving arise to (S)-N-methylcoclaurine (100). In a step employing O2 and NADPH, the enzyme (S)-N-methylcoclaurine 3’-hydroxylase facilitates a hydroxylation reaction at the 3’ position, producing (S)-3’-hydroxy-N-methylcoclaurine (101). The methylation of (S)-3’-hydroxy-N-methylcoclaurine at 4’ by the enzyme (R,S)-3’-hydroxy-N-methylcoclaurine 4’-O-methyl transferase leads to the formation of a key compound in the biosynthetic cascade, (S)-reticuline (30).

In a successive step, (S)-reticuline is transformed enzymatically to the first protoberberine-type alkaloid in the series, (S)-scoularine (48), by the berberine bridge enzyme, in a reaction involving the oxidation of the tertiary amine in (S)-reticuline to a transient iminium ion. This step requires O2 as the oxidizing agent and forms H2O2 in the process as a by-product. Consecutively, in a SAM mediated reaction, the phenol group in the D ring of (S)-scoularine is methylated by scoularine 9-O-methyltransferase, forming (S)-tetrahydrocolumbamine (102), followed by the oxidation of the ortho-methoxy phenol ring through a cytochrome, NADPH, and O2-dependent reaction facilitated by (S)-canadine synthase, forming the methylenedioxy-bridge in (S)-canadine (103). The oxidation of (S)-canadine through an iminium cation in a two-step process leads to the formation of the aromatic system in the final product, berberine (85) (Dewick, 2009).
This overall pathway is shown in Scheme 1.

Scheme 1. Biosynthetic steps leading to berberine (85) with L-tyrosine (93) as the starting unit (Adapted from Dewick, 2009).
B5. Biological Activities of Protoberberine-type Alkaloids

Protoberberine- and berberine-type compounds have been of scientific interest for their biological activity and over a thousand research papers have been published on these alkaloids since the early 1970s. Plants containing berberine (85) have been used ethnobotanically for 3000 years or more in both the traditional Chinese and Indian systems of medicine, especially for their antidiarrheal activity (Sack et al., 1982).

The production of certain protoberberine- and berberine-type compounds has been attributed to a possible role in the producing plant for chemical defense against microorganisms and herbivores, through a broad spectrum of biological activities for both enzymes (e.g., acetylcholine esterase, butyrylcholinesterase, and choline acetyl transferase) and various receptors (e.g., α₁ and α₂ adrenergic, nicotinergic, muscarinergic, and serotonergic) (Schmeller et al., 1997).

It has been postulated that the quaternary alkaloid berberine (85) derivatives inhibit the reverse transcription process and may exert antiviral properties through this mechanism (Grycova et al., 2007). Berberine (85) as a pure compound demonstrates broad-spectrum antibacterial, antifungal, antileishmanial, and antiprotozoal activities. Some of the earlier scientific reports indicate its activity against Entamoeba histolytica and Vibrio cholera, the causative agents for amoebiasis and cholera, respectively (Subbaiah et al., 1967; Modak et al., 1970; Munshi et al., 1972). Berberine and related
compounds have also been of interest for their tumor-specific cytotoxicity and apoptosis-inducing activities against many cancer cell lines (Inoue et al., 2005).

The biological activities of quaternary protoberberine alkaloids was recently reviewed and they were categorized by the authors into “interactions with biomacromolecules, cytotoxic activity and apoptosis, antimicrobial activity, antiinflammatory activity, antimalarial activity, and other effects” (Grycova et al., 2007). The next sections of this chapter will employ a similar approach.

B5.1. Interactions of Berberine (85) with Biomacromolecules.

Structurally, berberine (85) is planar, corresponding approximately to the size of a base pair in the DNA double helix, and it has been suggested to be an intercalating agent for both double-helical and denatured DNA, in addition to RNA (Krey et al., 1969). In this respect, modern NMR studies have proven indeed the preferential affinity of berberine for AT-rich sequences in a model with several oligonucleotides (Mazzini et al., 2003) and, in a recent study employing X-ray diffraction, berberine was described as a non-classical intercalator (Ferraroni et al., 2011). Some protoberberines have been shown to be topoisomerase-I and topoisomerase-II poisons and mechanistic studies indicate that the mode of action of a closely related analogue of berberine (85), berberrubine (104, Figure 18), was through stabilizing topoisomerase II-DNA cleavable complexes thus indicating a different mechanism of action from that of etoposide, a known topoisomerase-II inhibitor (Kim et al., 1998). A semi-synthetic analogue of berberine,
coralyne (105, Figure 18), with potent antileukemic activity, on the other hand, was shown to act mainly as a topoisomerase I inhibitor (Gatto et al., 1996).

**B5.2. Cytotoxic and Apoptosis-related Activity of Berberine (85) and Related Compounds.**

Berberine (85) and its analogues have demonstrated *in vitro* cytotoxic and apoptotic effects on a diverse set of cell lines at micromolar concentrations, including HeLa (uterine carcinoma), SVKO3 (ovarian carcinoma), and Hep-2 (laryngeal carcinoma) (Orfila et al., 2000). The cytotoxic mechanism of berberine against MCF-7 breast cancer cells was shown to be through a mitochondria- and caspase-dependent apoptotic pathway (Patil et al., 2010).

The cytotoxic and apoptotic effects of berberine (85) and palmatine (89) against promyelocytic leukemia HL-60 cells were reported previously (Kuo et al., 1995). In this study, treatment of cell lines with berberine at 25 µg/mL resulted in strong cell-cycle arrest, and apoptotic events such as morphological changes and internucleosomal DNA fragmentation were also observed. However, palmatine (89), although being able to form complexes with DNA at the same dose, failed to induce apoptosis, suggesting that
mechanisms other than DNA interactions may play a role in the apoptotic response. Two other mechanisms potentially responsible for apoptotic activity include caspase and mitochondrial activation (Hwang et al., 2006; Burgeiro et al., 2011). An ability of berberine (85) to interfere with the mevalonate pathway was recently ruled out as a mechanism through which berberine infers its cytostatic and cytotoxic effects (Issat et al., 2006).

In a study using hepatocellular carcinoma, berberine induced cell cycle arrest and promoted apoptosis through nonsteroidal anti-inflammatory drug-activated gene (NAG-1) activation (Auyeung et al., 2009). Berberine (85) inhibits the enzyme cyclooxygenase-2 (COX-2), which is largely expressed in colon cancer cells, and thus might act as a chemopreventive agent against colon tumor formation (Fukuda et al., 1999). Berberine (85) has also been studied for its potential chemopreventive activity against chemically induced neoplasia. In an in vivo N-nitrosodiethylamine-induced model in mice, berberine caused significant reduction of the markers of hepatic injury, such as liver weight, γ-
glutamyl transpeptidase activity and glutathione S-transferase, indicating a possible ability to protect against carcinogenesis in this model (Anis et al., 2001).

**B5.3. Antimicrobial Activity of Berberine (85) and Related Compounds.**

Berberine (85) has been described previously as a “potentially excellent antimicrobial” drug, since the membrane potential drives the accumulation of this alkaloid in cells due to its positive charge, and acts mainly at two immutable sites, namely, DNA and the membrane (Amin et al., 1969; Severina et al., 2001), along with other molecular targets (Boberek et al., 2010). While highly water-soluble in the salt form, berberine (85) forms a hydrophobic cation in aqueous solutions and its accumulation in the target cells causes leakage of the cellular contents through the membrane. While the development of drug-resistance against berberine (85) through target modification is highly unlikely, some bacteria are reported to employ multidrug-resistance (MDR) pumps in response to treatment with this compound (Hsieh et al., 1998). Interestingly, some plant species are known to biosynthesize MDR inhibitory compounds along with berberine (85), increasing the resultant antimicrobial effects of berberine significantly (Stermitz et al., 2000a; Stermitz et al., 2000b; Belofsky et al., 2004; Belofsky et al., 2006). Berberine (85) and several other protoberberine alkaloids possess broad-spectrum antifungal activity, and inhibit the human cytomegalovirus (Ficker et al., 2003; Hayashi et al., 2007).

In a study screening for antitubercular activity in a multi-drug resistant strain of *Mycobacterium tuberculosis*, berberine (85) was shown to be moderately active in an *in
vitro model of tuberculosis. In the same study, two structurally similar compounds, canadine (103) and 8-oxotetrahydrothalifendine (106, Figure 19), were found to be inactive (Gentry et al., 1998).

![8-oxotetrahydrothalifendine (106)](image)

**Figure 19.** Structure of 8-oxotetrahydrothalifendine (106).

**B5.4. Antiprotozoal Activity of Berberine (85) and Related Compounds.**

While many reports in the literature point to potent antimicrobial activity of berberine (85) and structurally related compounds, the antiprotozoal activities of this class were not investigated thoroughly until about 20 years ago (Vennerstrom et al., 1988; Vennerstrom et al., 1990).

In a study probing structure-activity relationships in protoberberine alkaloids, a library comprised of natural and semi-synthetic compounds was screened in both *in vitro* and *in vivo* models of malaria. The compounds tested included berberine (85), berberinephenolbetaine (berberine betaine, 107), canadine (103), 8-
cyanodihydroberberine (108), 7,8-dihydroberberine (109), jatrorrhizine (87), N-methylcanadine (110), 8-oxyberberine (78), palmatine (89), and tetrahydroberberine (canadine) N-oxide (111). The structures of some of these compounds are shown in Figure 20. While berberine, 7,8-dihydroberberine, and 8-cyanodihydroberberine demonstrated IC₅₀ values comparable to that of quinine in the in vitro studies, none of the compounds tested showed in vivo activity at the concentrations used in a mouse model. It was also noted that the compounds with more potent in vitro activity had greater in vivo toxicity against the infected animals (Vennerstrom et al., 1988).

Figure 20. Structures of protoberberine analogues tested previously for antileishmanial and antimalarial activities.
In a further study, berberine (85), berberinephenolbetaine (berberine betaine, 107), canadine (104), 8-cyanodihydroberberine (108), 7,8-dihydroberberine (109), N-methylcanadine (110), 8-oxyberberine (78), palmatine (89), and tetrahydroberberine (canadine) N-oxide (111) (Figure 20) were screened for their in vivo activity against *Leishmania donovani* and *L. braziliensis panamensis* in a golden hamster model of leishmaniasis. In the *L. donovani* model, 8-cyanodihydroberberine and canadine were shown to suppress parasite burdens by 50% at 208 and 416 mg/kg, respectively, while showing signs of toxicity at these doses. Berberine and 8-cyanodihydroberberine were the most active compounds against *L. braziliensis panamensis* showing approximately 50% suppression of lesion size at 208 mg/kg (Vennerstrom *et al.*, 1990). More recently, another protoberberine alkaloid, coralyne (105), was shown to be a potent inhibitor of nuclear DNA topoisomerase II in *Leishmania panamensis* promastigotes (Cortazar *et al.*, 2007).

**B5.5. Miscellaneous Biological Activities of Berberine (85) and Related Compounds.**

Berberine (85) and other protoberberine alkaloids have been investigated for their activities using additional bioassay systems. In a study investigating the effects of berberine in the central nervous system (CNS), this compound was shown to modulate the N-methyl-D-aspartate, nitric oxide and serotonin neurotransmitter systems. Another study reported the effects of berberine (85) in pentylenetetrazole-, maximal electroshock (MES)-, and kainic acid (KA)-induced convulsion models in mice and was found to be a potent anticonvulsant agent with potential use in epilepsy (Bhutada *et al.*, 2010). Berberine was found to exhibit acetylcholine esterase (AChE) inhibitory activity at low
micromolar concentrations (Hung et al., 2008). In a mouse model for Alzheimer’s disease, berberine (85) was shown to inhibit the accumulation of amyloid-β peptide derived from amyloid precursor protein, and was purported to be an especially promising agent due to its low cytotoxicity in this assay system (Asai et al., 2007). A phytochemical study to discern the principles responsible for the sedative effects of Corydalis cava demonstrated that those protoberberine-type alkaloids possessing a saturated C ring demonstrated potent γ-aminobutyric acid receptor A (GABA\textsubscript{A}) activity, while compounds with an aromatized C ring showed a complete loss of such activity (Halbsguth et al., 2003).

Other areas of previous study on the protoberberines include their activity on the cardiovascular system. Berberine (85), along with several analogues and a number of bisbenzylisoquinolines, has demonstrated antiarrhythmic, negative chronotropic, positive inotropic, and vasodilatory properties (Patil et al., 1965; Wu et al., 1976c; Wu et al., 1977a; Lau et al., 2001). In addition, berberine (85) was shown recently to decrease plasma cholesterol and triglycerides in hypercholesterolemic patients via increasing the expression of the hepatic low density lipoprotein receptor, a mechanism significantly different than that of most commonly used class of drugs for hypercholesteremia, the statins (Kong et al., 2004). Related mechanistic studies have shown that berberine also inhibits lipid synthesis in human hepatocytes via AMP-activated protein kinase (AMPK) activation (Brusq et al., 2006). Berberine also induces human serum paraoxonase (PON1), an enzyme thought to be protective against atherosclerosis (Cheng et al., 2011) and inhibits the development of cardiac hypertrophy in a rat model (Hong et al., 2003).
Furthermore, a recent study points to a possible role of berberine in the inhibition of cholesterol absorption in the intestines and stimulation of bile acid synthesis (Wang et al., 2010). However, berberine was also shown to have prothrombotic effects on vascular cells, a finding that might hinder the development of this agent as a drug in patients with cardiovascular problems (Holy et al., 2009).

An increasing number of reports point to the potential use of berberine (85) in diabetes treatment (Cui et al., 2009). Berberine (85) increases glucose uptake ability of adipocytes and myocytes in an insulin-independent fashion and lowers hyperglycemia in diabetic mice (Chen et al., 2010). The observed hypoglycemic effects of berberine may be attributed partially to the activation of the transport function of insulin-insensitive glucose transporter (GLUT1) (Cok et al., 2011).

B6. Semi-Synthetic Investigations on Protoberberine Alkaloids

Due to the diverse biological activities exhibited by berberine (85) and its analogues, compounds of this class have been the subject of many semi-synthetic modifications. In one of the first such investigations, several protoberberine derivatives were synthesized in order to evaluate their hypotensive activity (Kametani et al., 1974). In another study, an 8-alkyl derivative, coralyne (105), was shown to have in vivo activity against leukemia in mice (Zee-Cheng et al., 1972). The following section summarizes several types of semi-synthetic modifications performed on the protoberberine alkaloids, which follows the same organization as a previous review on this topic (Grycova et al., 2007).
Scheme 2. Conversion of berberine from salt form to free base form under basic conditions through the addition of a hydroxy group at C-8.

The berberine skeleton exhibits certain chemical attributes different from most alkaloids, since it is characteristically sensitive to nucleophilic attack at C-8. Reactants leading to adducts with substitution at this position include ammonia, chloroform, cyanide, and methoxide (Dostal, 2004). Most significantly, treatment of berberine (85) with a strong base affords a product (112) with a covalently bonded hydroxy group at C-8 (Dostal, 2004; Grycova et al., 2007), reflecting the polarized nature of the iminium bond between N-7 and C-8 (Scheme 2).

In a similar fashion, the nucleophilic addition of OR⁻ groups under alkaline conditions is achievable and 8-ethoxy- and 8-methoxy-7,8-dihydroprotoberberines are formed commonly during the phytochemical isolation process as artifacts, such as 113, in an alkaline medium in the presence of ethanol or methanol (Scheme 3). N-Nucleophiles, such as liquid ammonia, lead to the formation of 8-amino derivatives (Man et al., 2001). Another example resulting in the formation of a C-C bond at C-8 has been observed in
chemical isolation studies with chloroform, forming 114 through the insertion of a 8-trichloromethyl group (Scheme 4). The sensitivity at C-8 of berberine (85) has also been utilized for the addition of C-C bonds through the use of Grignard reagents (Scheme 5). Additions of this type lead to the incorporation of an alkyl group at C-8 and many such compounds have been reported previously (Iwasa et al., 1998).

**Scheme 3.** Nucleophilic addition of a methoxy group at C-8 of a berberine salt (85) under basic conditions.

**Scheme 4.** Addition of a trichloromethyl group at C-8 of a berberine salt (85) in chemical isolation studies using chloroform.
8-Oxyberberine derivatives can be obtained through the treatment of a berberine salt with KOH, although this method yields 8-oxyberberine along with the dihydroberberine derivative (Scheme 6). Alternatively, the use of oxidizing reagents, such as K$_3$Fe(CN)$_6$, have been reported to yield quantitative amounts of 8-oxyberberine (Scheme 7) (Grycova et al., 2007).

The reaction of berberine with hot dilute nitric acid leads to formation of a betaine, berberidic acid (115, Scheme 8). The reaction of 8-oxyberberine (78) with phosphorous oxychloride produces 8-chloroberberine (116, Scheme 9), a compound sensitive to nucleophilic attacks by primary amines, carbanions, and Grignard reagents (Moniot et al., 1979). 8-Chloroberberine (116), in turn, serves as a starting material of a new set of compounds, most notably berberine analogues with dialkyl substitution at C-8 (Scheme 10).
Scheme 6. Oxidation of protoberberine skeleton under basic conditions leading to the formation of 8-oxo and 7,8-dihydro derivatives.

Scheme 7. Formation of 8-oxyprotoberberine derivatives in an exclusive manner with the use of $\text{K}_3\text{Fe(CN)}_6$.

Scheme 8. Formation of berberidic acid (115) upon treatment of berberine (85) with nitric acid.
Scheme 9. Synthesis of 8-chloroberberine chloride (116) from 8-oxyberberine (78).

Scheme 10. Synthesis of 8,8-disubstituted protoberberine alkaloids using 8-chloroberberine (116) as the starting material.

Reduction of the protoberberine skeleton can be achieved selectively, based on the reducing agent (Bhakuni et al., 1986). While the use of LiAlH₄/ether or NaBH₄/pyridine leads to a partial reduction generating a dihydroprotoberberine system, such as 7,8-dihydroberberine (109), the use of H₂/Pt or Zn/HCl produces the tetrahydroprotoberberine system, such as that found in canadine (103).
Scheme 11. Partial or full reduction of the protoberberine-type alkaloids based on the selection of reducing agent.
CHAPTER 3. SCREENING OF SELECTED PLANTS FOR IN VITRO ANTILEISHMANIAL AND ANTITRYPANOSOMAL ACTIVITIES

C1. Statement of Research Problem

As stated in Chapter 1 of this dissertation, there is an urgent need for new and inexpensive drugs in the field of neglected tropical diseases. Historically, natural products from terrestrial and marine organisms have provided numerous bioactive compounds with novel mechanisms of action. In fact, a recent review states that ca. 50% of all small molecules that were filed as “new chemical entities” between the years 2000 and 2010 were natural products (Newman and Cragg, 2012). Markedly, in the field of tropical diseases many of the drugs that are currently used (i.e. artemisinin and its derivatives, amphotericin B, and quinine) are either natural products or natural product analogues (Guerrant et al., 2005).

The biological screening of standardized plant extracts, followed by a bioactivity-guided fractionation scheme has been proven to be a reliable and efficient method for the identification of natural product lead compounds. In this study, this approach was coupled with screening studies in in vitro models of leishmaniasis and trypanosomiasis to identify novel and potent natural products from a library of plant material housed at College of Pharmacy, The Ohio State University.
C2. Background

For the purposes of this dissertation project, a collection was utilized comprised of several native Ohio plants and non-native plants, most notably a collection of *Thalictrum* species, which were mostly grown in a dedicated greenhouse at The Ohio State University. The plants were taxonomically authenticated, and taken from the collections of the late Professor Jack L. Beal and Emeritus Professor Raymond W. Doskotch (College of Pharmacy, The Ohio State University).

This library of dried and ground plants stored at the College of Pharmacy, The Ohio State University, Columbus, Ohio included many species not previously screened for antiprotozoal activity and presented a unique opportunity to employ our group’s well-established bioactivity-guided fractionation experience to identify active lead compounds. The work presented in this part of this dissertation was made possible through a collaboration between the groups of Dr. A. Douglas Kinghorn, which was responsible for the botanical, database, and phytochemical aspects of the project, and Dr. Karl A. Werbovetz, responsible for the biological screening of the plant samples in *in vitro* tests for leishmaniasis and trypanosomiasis.

C3. Preparation of Plant Samples for Bioassay Studies

The samples for the bioassays were obtained by an extraction and partitioning scheme described previously (Wall *et al.*, 1996), aiming for the partial removal of tannins and other plant polyphenols, which are known to interfere with many bioassay systems (Figure 21). This protocol has been previously utilized successfully in other projects and
is suitable for the preparation of crude extracts in a rapid and reliable manner, even with small amounts of plant material (Kinghorn et al., 2009).

Following the above methodology, a 50 g aliquot of each plant sample was macerated with 300 mL of methanol overnight and this process was repeated three times. The methanolic portions were then combined and fractionated first with 250 mL aliquots of hexanes three times to remove waxes and chlorophyll and then, after the addition of adequate amounts of water to avoid emulsions, were subjected to partitioning with 250 mL aliquots of chloroform three times. These chloroform extracts were then washed with 250 mL portions of 1% aqueous NaCl three times to partially remove vegetable tannins, and subsequently combined, dried in a rotary evaporator, and weighed. These dried

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**Figure 21.** Partitioning scheme used for obtaining test samples for bioassay studies (Wall et al., 1996).
samples were then dissolved in dimethyl sulfoxide to produce a standardized 10 mg/mL concentration and consequently were submitted to bioassay studies to determine their effectiveness in *in vitro* models of leishmaniasis and trypanosomiasis (Salem and Werbovetz, 2005, Appendix A).

For the purposes of this study, activity was described as an IC$_{50}$ value of 25 µg/mL in one or both of the systems. Active chloroform-soluble extracts were further tested in Vero cells (Delfin *et al.*, 2006, Appendix A) and only extracts exhibiting five-fold or more selectivity were identified as lead plants and subjected to isolation studies.

**C4. Results of the Initial Bioassay Studies**

Out of the 147 plant chloroform-soluble extracts that were screened, 26 of them showed IC$_{50}$ values of 25 µg/mL or lower in either one or both of the *in vitro* test systems used (Table 2). After a literature search, 11 of these samples were submitted to a Vero cell assay to determine their selectivity towards parasites versus mammalian cells (Appendix 1). Of the samples tested, two samples, *Eupatorium sessilifolium* L. (Asteraceae) and *Thalictrum lucidum* L. (Ranunculaceae), had 10-fold and 40-fold selectivity towards parasites, respectively, and were identified as potential lead plants. For the purposes of this dissertation the *T. lucidum* sample was chosen for further investigation.
<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Plant Family</th>
<th>Leishmania donovani</th>
<th>Trypanosoma b. brucei</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Abies balsamea</em> (L.) Mill.</td>
<td>Pinaceae</td>
<td>29</td>
<td>12.5-25</td>
</tr>
<tr>
<td><em>Acer negundo</em> L.</td>
<td>Aceraceae</td>
<td>21</td>
<td>50-100</td>
</tr>
<tr>
<td><em>Chrysanthemum anethifolium</em> (Willd.) Steud.</td>
<td>Asteraceae</td>
<td>21</td>
<td>50-100</td>
</tr>
<tr>
<td><em>Cornus florida</em> Hook.</td>
<td>Cornaceae</td>
<td>50-100</td>
<td>13</td>
</tr>
<tr>
<td><em>Cotinus coggygrica</em> Scop.</td>
<td>Anacardiaceae</td>
<td>25</td>
<td>50-100</td>
</tr>
<tr>
<td><em>Cryptostegia grandiflora</em> R.Br.</td>
<td>Asclepiadaceae</td>
<td>50-100</td>
<td>&lt; 3.1</td>
</tr>
<tr>
<td><em>Eupatorium maculatum</em> L.</td>
<td>Asteraceae</td>
<td>45</td>
<td>13</td>
</tr>
<tr>
<td><em>Eupatorium sessilifolium</em> L.</td>
<td>Asteraceae</td>
<td>&lt;3.1</td>
<td>50-100</td>
</tr>
<tr>
<td><em>Faramea stenopetala</em> Mart.</td>
<td>Rubiaceae</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td><em>Grindelia squarrosa</em> (Pursh) Dunal</td>
<td>Asteraceae</td>
<td>44</td>
<td>8.2</td>
</tr>
<tr>
<td><em>Ocimum canum</em> Sims</td>
<td>Lamiaceae</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td><em>Piscidia erythrina</em> L.</td>
<td>Leguminosae</td>
<td>20</td>
<td>25-50</td>
</tr>
<tr>
<td><em>Thalictrum dipterocarpum</em> Franch.</td>
<td>Ranunculaceae</td>
<td>50-100</td>
<td>6.4</td>
</tr>
<tr>
<td><em>Thalictrum foetidum</em> L.</td>
<td>Ranunculaceae</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td><em>Thalictrum lucidum</em> L.</td>
<td>Ranunculaceae</td>
<td>5.7</td>
<td>13</td>
</tr>
<tr>
<td><em>Trichilia maynasiana</em> C.DC.</td>
<td>Meliaceae</td>
<td>50-100</td>
<td>12.5-25</td>
</tr>
<tr>
<td><em>Vernonia altissima</em> Walp.</td>
<td>Asteraceae</td>
<td>34</td>
<td>6.5</td>
</tr>
<tr>
<td><em>Zanthoxylum martinicense</em> DC.</td>
<td>Rutaceae</td>
<td>3.1 – 6.3</td>
<td>25-50</td>
</tr>
<tr>
<td>Pentamidine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>&lt;3.1</td>
<td>ND</td>
</tr>
<tr>
<td>Suramin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>ND</td>
<td>0.77 ± 0.31</td>
</tr>
</tbody>
</table>

**Table 2.** IC<sub>50</sub> values (µg/mL) of selected active plant extracts in an initial screening when tested against *in vitro* models of *L. donovani* and *T. b. brucei*. <sup>a</sup>: The IC<sub>50</sub> values for pentamidine and suramin are provided in µM.
CHAPTER 4. BIOACTIVITY-GUIDED FRACTIONATION STUDIES ON THE PLANT LEAD, *THALICTRUM LUCIDUM* L. (RANUNCULACEAE)

D1. General Experimental Procedures

Melting points were measured using a Fisher-Johns melting point apparatus. UV spectra were measured with a Perkin-Elmer Lambda 10 UV/vis spectrometer. CD spectra were measured on a JASCO J-810 spectrometer. IR spectra were run on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. NMR spectroscopic data were recorded at room temperature on a Bruker Avance DPX-300 or DRX-400 NMR spectrometer at The Ohio State University. High-resolution mass spectra were recorded on a LCT-TOF mass spectrometer. Column chromatography was performed with 65-250 or 230-400 mesh silica gel (Sorbent Technologies, Inc., Norcross, GA). Analytical thin-layer chromatography (TLC) was conducted on 250 μm thickness Partisil silica gel 60 F254 glass plates (Whatman, Clifton, NJ) or 200 μm-thick aluminum-backed silica gel plates (Sorbent Technologies, Inc.) with shortwave UV (254 nm) light and Dragendorff reagent (a solution of potassium bismuth (III) iodide) employed for the visualization of alkaloids. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

D2. Plant Material

The seeds for the plant sample used in this project were obtained from the Botanical Garden, Zagreb, Croatia (former Yugoslavia), and the Botanical Garden, University of Munich, Munich, Germany (former West Germany) and were originally labeled as *Thalictrum bulgaricum*. The seeds were planted and grown in the former The
Ohio State University (OSU) Medicinal Plant Garden and later identified as *Thalictrum lucidum* L. (Ranunculaceae) by Bernard Boivin, a botanist then at the Central Experimental Farm, Plant Research Institute, Department of Agriculture, Ottawa, Ontario, Canada. The plants were harvested in 1972 and the ground sample was preserved as a part of a collection housed at the College of Pharmacy, OSU (Wu et al., 1976c).

**D3. Solvent Extraction of *Thalictrum lucidum***

The oven dried, ground, and stored whole plants of *Thalictrum lucidum* (2 kg) were extracted with methanol overnight ($6 \times 4$ L). The macerate was concentrated *in vacuo* (392 g) and partitioned following the same scheme used previously except for the sodium chloride detannification step (Figure 21). In brief, the dried methanol extract was partitioned between 450 mL of a 9:1 methanol-water mixture and a 450 mL portion of hexanes, in 40 g aliquots. The hexanes layers were combined to give a 25.7 g hexanes partition, while the resulting methanol-water partition was dried in a rotary evaporator to yield a thick tar, which was further partitioned between 450 mL of CHCl$_3$ and 450 mL of water to yield chloroform (32 g) and aqueous partitions (330 g).

**D4. Column Chromatography of the Chloroform-soluble Extract of *Thalictrum lucidum***

The chloroform extract of *Thalictrum lucidum* (30 g) was fractionated initially by open column chromatography using silica gel (320 g, Aldrich, Si gel 60, 63-200 mesh) using first 100% dichloromethane, followed, in turn, by gradient mixtures of
dichloromethane/methanol increasing polarities leading to 100% methanol. The gradient employed in this step was as follows: 1 L dichloromethane, 1 L 95% dichloromethane in methanol, 1 L 90% dichloromethane in methanol, 1 L 85% dichloromethane in methanol, 1 L 80% dichloromethane in methanol, 1 L 75% dichloromethane in methanol, 1 L 50% dichloromethane in methanol, 1 L 25% dichloromethane in methanol, 6 L methanol. The eluted fractions were collected in 200 mL portions, with the exception of 100% methanol portion which was collected as one fraction.

Each 200 mL fraction obtained from the column was examined by TLC and visualized under UV light at 254 and 366 nm and the use of Dragendorff reagent. The fractions obtained were combined into a total of thirteen fractions (F01-F13) based on their thin-layer chromatography profiles. Berberine (85, 92 mg) was obtained as a yellow crystalline precipitate from fraction F07 (148 mg). Fraction F08 (418 mg) yielded orange crystals, which were determined to be jatrorrhizine (87, 48 mg).

Fraction F06 (612 mg) tested positive for alkaloids with Dragendorff’s reagent and was further chromatographed using a neutral alumina chromatography (Aldrich, neutral aluminum oxide, 150 mesh, 3.0 × 60 cm), beginning with pure dichloromethane, followed by a gradient of increasing polarity composed of mixtures of dichloromethane and methanol, and washed with 100% methanol. Subsequently, 19 combined fractions (AF1-AF19) were obtained and AF-6 yielded yellow crystals, which were determined to be palmatine (89, 18 mg).

All pure compounds were run through an ion-exchange column (Sigma, IRA 400 resin in Cl⁻ form) to substitute Cl⁻ as the counter-ion. The ion-exchange procedure was
carried out in the following manner. The isolated compounds were dissolved in a solution of 50% acetone in water and were applied to a column packed with 600 g of IRA 400 ion-exchange resin and eluted with 50% acetone in water. The effluents were collected, combined, and dried in a rotary evaporator. The ion-exchange resin was washed and recycled with an aqueous solution of hydrochloric acid between samples (Wu et al., 1976c).

D5. Characterization of Compounds Isolated from *Thalictrum lucidum*

D5.1. Characterization of Berberine Chloride (85)

Berberine chloride (85, 92 mg) exhibited the following physical and spectroscopic data: pale yellow needle crystals; mp 199 °C; UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 230 (4.6), 267 (4.1), 344 (4.3), 352 (4.1), 432 (3.9) nm; $^1$H NMR (MeOD) $\delta$H 9.77 (1H, s, H-8), 8.60 (1H, s, H-13), 8.07 (1H, d, $J = 9.2$ Hz, H-11), 7.97 (1H, d, $J = 8.9$ Hz, H-12), 7.57 (1H, s, H-1), 6.93 (1H, s, H-4), 6.08 (2H, s, -OCH$_2$O-), 4.94 (2H, t, $J = 6.3$ Hz, H-6), 4.18 (3H, s, 9-OCH$_3$), 4.07 (3H, s, 10-OCH$_3$), 3.25 (2H, t, $J = 6.3$ Hz, H-5); $^{13}$C NMR (MeOD) $\delta$C 152.1 (C-10), 152.0 (C-3), 149.8 (C-2), 146.5 (C-8), 145.7 (C-9), 139.5 (C-13a), 135.0 (C-12a), 132.0 (C-4a), 128.0 (C-11), 124.6 (C-12), 123.2 (C-8a), 121.8 (C-13b), 121.4 (C-13), 109.4 (C-4), 106.4 (C-1), 103.6 (-OCH$_2$O-), 62.6 (9-OCH$_3$), 58.0 (10-OCH$_3$), 57.2 (C-6), 28.2 (C-5); HRESIMS $m/z$ 336.1229 [M]$^+$ (calcd. for [C$_{20}$H$_{18}$NO$_4$]$^+$, 336.1236). The purity of berberine (85) was verified by employing thin-layer chromatography (TLC) with three solvent systems (5:1 chloroform-methanol-0.01%
ammonium hydroxide, \( R_f = 0.22 \); 6:2:2:2 ethyl acetate-acetonitrile-methanol-water, \( R_f = 0.72 \); 8:2:2:2 ethyl acetate-acetonitrile-methanol-water, \( R_f = 0.70 \).

**D5.2. Characterization of Jatrorrhizine Chloride (87)**

Jatrorrhizine chloride (87, 18 mg) exhibited the following physical and spectroscopic data: pale yellow needle crystals; mp 204 °C; UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 224 (4.7), 288 (4.2), 342 (4.3) nm; \(^1\)H NMR (MeOD) \( \delta_H \) 9.72 (1H, s, H-8), 8.77 (1H, s, H-13), 8.08 (1H, d, \( J = 9 \) Hz, H-11), 7.99 (1H, d, \( J = 9 \) Hz, H-12), 7.64 (1H, s, H-1), 6.84 (1H, s, H-4), 4.93 (2H, t, \( J = 6.2 \) Hz, H-6), 4.18 (3H, s, 9-OCH\(_3\)), 4.09 (3H, s, 10-OCH\(_3\)), 4.01 (3H, s, 2-OCH\(_3\)), 3.19 (2H, t, \( J = 6.2 \) Hz, H-5); \(^{13}\)C NMR (MeOD) \( \delta_C \) 151.9 (C-3), 151.7 (C-9), 149.6 (C-2), 146.2 (C-8), 140.3 (C-13a), 130.3 (C-4a), 135.4 (C-10), 128.0 (C-12), 124.4 (C-11), 123.2 (C-12a), 120.9 (C-13), 119.3 (C-13b), 115.9 (C-4), 110 (C-1), 101.0 (C-8a), 62.5 (9-OCH\(_3\)), 57.6 (10-OCH\(_3\)), 57.4 (2-OCH\(_3\)), 57 (C-6), 27.7 (C-5); HRESIMS \( m/z \) 338.1388 [M]\(^+\) (calcd. for \([\text{C}_{20}\text{H}_{20}\text{NO}_4]\)^+, 338.1392). The purity of jatrorrhizine (87) was verified by employing thin-layer chromatography (TLC) with three solvent systems (5:1 chloroform-methanol-0.01% ammonium hydroxide, \( R_f = 0.23 \); 6:2:2:2 ethyl acetate-acetonitrile-methanol-water, \( R_f = 0.69 \); 8:2:2:2 ethyl acetate-acetonitrile-methanol-water, \( R_f = 0.67 \)).

**D5.3. Characterization of Palmatine Chloride (89)**

Palmatine chloride (89, 18 mg) exhibited the following physical and spectroscopic data: pale yellow needle crystals; mp 205 °C; UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 227 (4.3), 276 (3.8), 344 (4.0) nm; \(^1\)H NMR (MeOD) \( \delta_H \) 9.74 (1H, s, H-8), 8.78 (1H, s, H-13),
8.08 (1H, d, J = 9.1 Hz, H-12), 8.00 (1H, d, J = 9.1 Hz, H-11), 7.61 (1H, s, H-1), 7.02 (1H, s, H-4), 4.94 (2H, t, J = 6 Hz, H-6), 4.20 (3H, s, 9-OCH₃), 4.08 (3H, s, 10-OCH₃), 3.98 (3H, s, 2-OCH₃), 3.93 (3H, s, 3-OCH₃) 3.28 (2H, t, J = 6 Hz, H-5); ¹³C NMR (MeOD) δ C 153.7 (C-3), 151.8 (C-10), 150.8 (C-2), 146.3 (C-8), 145.6 (C-9), 139.7 (C-13a), 135.2 (C-8a), 130.0 (C-4a), 128.0 (C-12), 124.5 (C-11), 123.2 (C-12a), 121.2 (C-13), 120.4 (C-13b), 112.2 (C-4), 109.9 (C-1), 62.6 (9-OCH₃), 57.6 (10-OCH₃), 57.3 (C-6), 57.1 (2-OCH₃), 56.7 (3-OCH₃), 27.8 (C-5); HRESIMS m/z 352.1538 [M]+ (calcd. for [C₂₁H₂₂NO₄]+, 352.1549). The purity of palmatine (89) was verified by employing thin-layer chromatography (TLC) with three solvent systems (5:1 chloroform-methanol-0.01% ammonium hydroxide, Rf = 0.24; 6:2:2 ethyl acetate-acetonitrile-methanol-water, Rf = 0.64; 8:2:2:2 ethyl acetate-acetonitrile-methanol-water, Rf = 0.65).

D6. Bioactivity-guided Fractionation of Thalictrum lucidum Whole Plants

Extracts and fractions of the aerial parts of T. lucidum were screened in an in vitro model of leishmaniasis following the previously mentioned bioactivity-guided fractionation scheme (Figure 21). In the screening process, only fractions F7 and F8, containing berberine (85) and jatrorrhizine (87) showed IC₅₀ values of less than 10 µg/mL against L. donovani parasites and were regarded as active. Therefore, berberine (85) and jatrorrhizine (87) were identified as the potential antileishmanial principles in the extract of Thalictrum lucidum.
D7. Discussion

D7.1. Identification of Compounds Isolated from *Thalictrum lucidum*

D7.1.1. Identification of Berberine Chloride (85)

The molecular formula of berberine (85) was determined as C\textsubscript{20}H\textsubscript{18}NO\textsubscript{4}, from the positive-ion molecular peak at \( m/z \) 336.1229 (calcd. for [C\textsubscript{20}H\textsubscript{18}NO\textsubscript{4}]\textsuperscript{+}, 336.1236) in the HRESIMS (Figure 22). The presence of two methoxy group signals in \textsuperscript{1}H NMR spectrum (Figure 23) at \( \delta_H \) 4.07 and 4.18 corresponding to six protons, along with a -OCH\textsubscript{2}O- signal at \( \delta_H \) 6.08, and the characteristically downfield signal at \( \delta_H \) 9.77 corresponding to H-8, strongly suggested a berberine-type skeleton (Grycova *et al.*, 2007). Aromatic singlet proton signals corresponding to positions C-1, C-4, and C-13 were observed at \( \delta_H \) 7.57, 6.93, and 8.60, respectively. The two aromatic proton signals at 8.07 and 7.97 were observed as doublets with a mutual J value of 8.9 Hz, providing evidence for vicinal coupling. The aliphatic proton signals at \( \delta_H \) 3.25 and 4.94 were observed as triplets and were assigned to H-5 and H-6, respectively, based on their observed mutual coupling with J values of 6.3 Hz. In the \textsuperscript{1}H-\textsuperscript{1}H COSY experiment (Figure 24) correlations occurred supporting the H-5–H-6 and H-11–H-12 vicinal couplings. Based on the \textsuperscript{13}C NMR and HSQC experiments (Figures 25 and 26, respectively), the protonated carbon atoms at positions C-1, C-4, C-5, C-6, C-8, C-11, C-12, and C-13 were found to resonate at \( \delta_C \) 106.5, 109.4, 28.2, 57.2, 146.5, 128.0, 124.7, and 121.4, respectively.
Figure 22. HRESIMS data for berberine chloride (85).

Figure 23. $^1$H NMR spectrum of berberine chloride (85).
Figure 24. $^1$H-$^1$H COSY NMR spectrum of berberine chloride (85).

Figure 25. $^{13}$C NMR spectrum of berberine chloride (85).
Figure 26. $^1$H-$^{13}$C HSQC NMR spectrum of berberine chloride (85).

Figure 27. $^1$H-$^{13}$C HMBC NMR spectrum of berberine chloride (85).
The C-9 and C-10 signals were assigned based on their HMBC correlations (Figure 27) with 9-OCH$_3$ and 10-OCH$_3$, as $\delta_C$ 145.6 and 151.8, respectively. Bridgehead atoms in the NMR spectra of berberine chloride (85) were assigned from the correlations observed in the HMBC spectrum (Figure 27). The downfield signal at $\delta_C$ 152.0 showed HMBC correlations with H-4, H-5, and the methylenedioxy moiety, and therefore could be assigned to C-3. Similarly, the downfield signal at $\delta_C$ 149.8, showed strong HMBC correlations with H-4, and also with H-5 and the methylenedioxy moiety and was assigned to C-2. The resonance at $\delta_C$ 132.0 showed HMBC correlations with signals corresponding to H-4, H-5, and H-6 and was assigned to C-4a. The signals at $\delta_C$ 139.50 and 121.79 demonstrated HMBC correlations with H-4 and H-5, and H-1, H-8, H-13, respectively, and were assigned unambiguously to C-13a and C-13b. Finally, the resonance observed at $\delta_C$ 135.00 showed HMBC correlations with signals corresponding to H-8, H-11, and H-12 and was attributed to C-12a. Therefore, the structure of this isolate was determined as berberine (85) and this compound exhibited comparable physical and spectroscopic data to literature values (Grycova et al., 2007). The chloride counter ion was inferred from the experimental conditions used during the purification of this compound.

**D7.1.2. Identification of Jatrorrhizine Chloride (87)**

The molecular formula of jatrorrhizine (87) was determined as C$_{20}$H$_{20}$NO$_4$, from the positive molecular ion peak at $m/z$ 338.1388 [M]$^+$ (calcd. for [C$_{20}$H$_{20}$NO$_4$]$^+$, 338.1392) in the HRESIMS (Figure 28) and from the $^{13}$C NMR (Figure 29) and the $^{13}$C DEPT experiment (Figure 30). In the $^1$H NMR experiment in MeOD (Figure 31), in
contrast with berberine, a signal indicating a methylenedioxy group was lacking, but this spectrum showed three methoxy group signals at $\delta_H$ 4.18, 4.09, and 4.01, which were assigned unambiguously as 9-OCH$_3$, 10-OCH$_3$, and 2-OCH$_3$, respectively.

The characteristically downfield signal at $\delta_H$ 9.72 was assigned to H-8. Aromatic proton signals corresponding to positions C-1, C-4, and C-13 were observed as singlets at $\delta_H$ 7.64, 6.84, and 8.77, respectively. Two aromatic proton signals at $\delta_H$ 8.08 and 7.99 were observed as mutual doublets with $J$ values of 9 Hz, consistent with a vicinal coupling, and were attributed to H-11 and H-12, respectively. The aliphatic proton signals at $\delta_H$ 3.19 and 4.93 were observed as triplets and were assigned as H-5 and H-6, respectively, based on their observed coupling with a $J$ value of 6.2 Hz. The $^1$H-$^1$H COSY experiment (Figure 32) further supported the H-5–H-6 and H-11–H-12 vicinal couplings. The protonated carbon atoms at positions C-1, C-4, C-5, C-6, C-8, C-11, C-12, and C-13 were accorded to the resonances observed at $\delta_C$ 110, 115.9, 27.7, 57, 146.2, 124.4, 128, and 120.9, respectively, based on the HSQC spectrum (Figure 33). C-2, C-9 and C-10 were assigned based on their HMBC correlations (Figure 34) with 2-OCH$_3$, 9-OCH$_3$ and 10-OCH$_3$, as $\delta_C$ 149.6, 151.7, and 135.4, in turn. Bridgehead atoms were assigned based on the correlations observed in the HMBC spectrum. Similarly, the downfield signal at $\delta_C$ 149.6, had strong HMBC correlations with H-4, H-5, and 2-OCH$_3$, and was assigned to C-2. The resonance at $\delta_C$ 130.3 showed HMBC correlations with signals corresponding to H-1, H-5, and H-6 and was assigned to C-4a. Signals at 140.3 and 119.3 demonstrated HMBC correlations with H-1, H-4, H-8, and H-13, and H-1, H-4, H-5, and H-13, respectively, and were assigned to C-13a and C-13b. The signal occurring
at $\delta_C$ 123.2 showed HMBC correlations with signals corresponding to H-8, H-11, and H-12, and was assigned as C-12a. A NOESY spectrum (Figure 35) was utilized to further verify the spatial assignments. Therefore, the structure of this isolate was determined as jatrorrhizine (87) and this compound exhibited comparable physical and spectroscopic data to literature values (Grycova et al., 2007). The chloride counter ion was inferred from the experimental conditions used during the purification of this compound.

**Figure 28.** HRESIMS data for jatrorrhizine chloride (87).
Figure 29. $^{13}$C NMR spectrum of jatrorrhizine chloride (87).

Figure 30. $^{13}$C DEPT NMR spectrum of jatrorrhizine chloride (87).
Figure 31. $^1$H NMR spectrum of jatrorrhizine chloride (87).

Figure 32. $^1$H-$^1$H COSY NMR spectrum of jatrorrhizine chloride (87).
Figure 33. $^1$H-$^{13}$C HSQC NMR spectrum of jatrorrhizine chloride (87).

Figure 34. $^1$H-$^{13}$C HMBC NMR spectrum of jatrorrhizine chloride (87).
Figure 35. $^1$H-$^1$H NOESY NMR spectrum of jatrorrhizine chloride (87).

D7.1.3. Identification of Palmatine Chloride (89)

The molecular formula of palmatine was determined as $C_{21}H_{22}NO_4$, from the positive molecular ion peak at 352.1538 [M]$^+$ (calcd. for $[C_{21}H_{22}NO_4]^+$, 352.1549) in the HRESIMS (Figure 36) and from the $^{13}$C NMR spectrum (Figure 37) and the $^{13}$C DEPT experiment (Figure 38). The $^1$H NMR spectrum in MeOD (Figure 39), in a similar manner to jatrorrhizine (87), lacked a signal indicating a methylenedioxy bridge, and additionally showed four methoxy group signals in the $^1$H NMR spectrum at $\delta_H$ 3.98, 3.93, 4.20, and 4.08, which were assigned, in turn, to 2-OCH$_3$, 3-OCH$_3$, 9-OCH$_3$, and 10-OCH$_3$. The characteristic downfield signal at $\delta_H$ 9.74 was attributed to H-8. Aromatic proton signals corresponding to positions C-1, C-4, and C-13 were observed as singlets at $\delta_H$ 7.61, 7.02, and 8.78, respectively. Two mutually coupled aromatic proton signals at
8.00 and 8.08 were observed as doublets with $J$ values of 9.1 Hz, consistent with vicinal coupling, and were assigned as H-11 and H-12, respectively. The aliphatic proton signals at $\delta_{H}$ 3.28 and 4.94 were observed as triplets and assigned to H-5 and H-6, respectively, based on their observed mutual couplings with $J$ values of 6 Hz. The $^1$H-$^1$H COSY experiment (Figure 40) further supported the H-5–H-6 and H-11–H-12 vicinal couplings. The protonated carbon atoms at positions C-1, C-4, C-5, C-6, C-8, C-11, C-12, and C-13 were assigned to $\delta_C$ 109.9, 112.2, 27.8, 57.3, 146.3, 124.4, and 127.8, respectively, aided by a HSQC experiment (Figure 41). The C-2, C-3, C-9 and C-10 signals were assigned based on their HMBC correlations (Figure 42) with 2-OCH$_3$, 3-OCH$_3$, 9-OCH$_3$ and 10-OCH$_3$, as $\delta_C$ 150.8, 153.7, 145.6, and 151.8, respectively. Bridgehead atoms were assigned from the correlations observed in the HMBC spectrum. The downfield signal at $\delta_C$ 153.7 showed HMBC correlations with H-1, H-4, and 3-OCH$_3$, further supporting its assignment as C-3. In turn, C-2 was shown to have correlations with H-1, H-4, and 2-OCH$_3$. Similarly, the resonance at $\delta_C$ 130 showed HMBC correlations with the signal corresponding to H-1 and was assigned as C-4a. The signals at 139.7 and 120.3 demonstrated HMBC correlations with H-1, H-8, H-and H-13, and H-1, H-4, and H-13, respectively, and were assigned to C-13a and C-13b. The resonance at $\delta_C$ 123.2 showed HMBC correlations with signals corresponding to H-8, H-11, and H-13 and was assigned as C-12a. Therefore, the structure of this isolate was determined as palmatine (89) and this compound exhibited comparable physical and spectroscopic data to literature values (Grycova et al., 2007). The chloride counter ion was inferred from the experimental conditions used during the purification of this compound.
Figure 36. HRESIMS data for palmatine chloride (89).

Figure 37. $^{13}$C NMR spectrum of palmatine chloride (89).
Figure 38. $^{13}$C DEPT NMR spectrum of palmatine chloride (89).

Figure 39. $^1$H NMR spectrum of palmatine chloride (89).
Figure 40. $^1$H-$^1$H COSY NMR spectrum of palmatine chloride (89).

Figure 41. $^1$H-$^{13}$C HSQC NMR spectrum of palmatine chloride (89).
D7.2. Bioactivity-guided Fractionation of *Thalictrum lucidum* Whole Plant Parts

A literature search carried out in tandem with this phytochemical analysis pointed to protoberberine-type alkaloids as the likely active principles (Vennerstrom *et al.*, 1990). The justification of this laboratory work was to rule out the presence of other active alkaloids with different skeletons or non-alkaloid active principles of *Thalictrum lucidum* (Wu *et al.*, 1976b; 1976c) (Figure 17). Since the antiprotozoal potential of the natural protoberberine alkaloids are well known (Vennerstrom *et al.*, 1990) and these compounds demonstrate only limited activity in *in vivo* studies, no further isolation or biological screening efforts were focused on this plant sample.

**Figure 42.** $^1$H-$^{13}$C HMBC NMR spectrum of palmatine chloride (89).
CHAPTER 5. BIOASSAY STUDIES ON A LIBRARY OF SEMISYNTHETIC ANALOGUES OF BERBERINE (85) AND SEMISYNTHESIS OF 5,6-DIDEHYDRO-8,8-DIETHYL-13-OXODIHYDROBERBERINE CHLORIDE (129)

E1. Bioassay Studies on a Library of Semisynthetic Analogues of Berberine (85)

E1.1. Background

As mentioned in the previous chapter, from the bioactivity-guided fractionation studies carried out on the plant *Thalictrum lucidum* L. (Ranunculaceae), berberine (85) was identified as the potential principle responsible for the bioactivity observed in the initial *in vitro* antileishmanial screening on this plant. While the demonstrated *in vitro* activities of these compounds have not translated very well to *in vivo* studies thus far (Vennerstrom *et al.*, 1988; Vennerstrom *et al.*, 1990), the antiprotozoal potential of this alkaloid carbon skeletal-type was pointed out previously.

Following up on the activity of berberine (85), a library of semisynthetic protoberberine analogues was screened in *in vitro* models of leishmaniasis and trypanosomiasis (Salem and Werbovetz, 2005, Appendix A). The compounds included in this study were synthesized at Pennsylvania State University, University Park, PA, by the group of Dr. Maurice Shamma (Moniot *et al.*, 1979) and were transferred to the late Dr. Jack L. Beal of College of Pharmacy, The Ohio State University (OSU), Columbus, OH.
Upon Dr. Beal’s retirement, the compounds were passed on, in turn, to Dr. Popat N. Patil, Division of Pharmacology, College of Pharmacy, OSU, and were preserved in his laboratory in a dedicated refrigerator.

The compounds in this library included berberidic acid (117), 8-berberinylidenemalonitrile (118), 8-berberinylideneimine (119), ethyl 8-berberinylideneacetooacetate (120), diethyl 8-berberinylidenemalonate (121), 8-canadinalcetic acid (122), 8-berberinylidene-n-propylamine (123), 8-berberinyl-acetic acid (124), 8,8-diethyldihydroberberine (125), and 8,8-dimethyldihydroberberine (126) (Figure 43). Other available protoberberine-type alkaloids, thalifendine (90), berberubine (104), D-tetrahydropalmatine (127), and L-tetrahydropalmatine (128), were also included in this study. For the purposes of this study, a standardized concentration (100 µg/mL) of the samples was prepared in DMSO and subjected to bioassay studies.

**E1.2. Results of the Bioassay Studies**

In this study, fourteen samples were screened, of which ten gave IC₅₀ values of 10 µg/mL or lower in either one or both of the *in vitro* test systems used (Salem and Werbovetz, 2005, Appendix A, Table 3). Of these samples, 8,8-diethyldihydroberberine (125) and 8,8-dimethyldihydroberberine (126) showed exceptionally high potency, with IC₅₀ values of 0.012 and 0.015 µg/mL against *L. donovani* and 0.001 and 2.2 µg/mL against *T. b. brucei* parasites, respectively, with further research of this particular substitution at C-8 clearly merited. 8,8-Diethyldihydroberberine (125) was given priority
over 8,8-dimethyldihydroberberine (126), since it had equally potent activity in both biological test systems used.

Figure 43. Structures of the semi-synthetic berberine analogues tested in *in vitro* models of leishmaniasis and trypanosomiasis.
<table>
<thead>
<tr>
<th>Sample Label</th>
<th>Activity against L. donovani (IC$_{50}$: µg/mL)</th>
<th>Activity against T.b. brucei (IC$_{50}$: µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine (85)</td>
<td>0.75</td>
<td>N/A</td>
</tr>
<tr>
<td>Thalifendine (90)</td>
<td>0.86</td>
<td>N/A</td>
</tr>
<tr>
<td>Berberrubine (104)</td>
<td>9.3</td>
<td>N/A</td>
</tr>
<tr>
<td>Berberidic acid (117)</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>8-Berberinylidenemalononitrile (118)</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>8-Berberinylideneimine (HCl salt, 119)</td>
<td>7.0</td>
<td>0.17</td>
</tr>
<tr>
<td>Ethyl 8-berberinylideneacetoacetate (120)</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Diethyl 8-berberinylidenemalonate (HCl salt, 121)</td>
<td>8.1</td>
<td>4.7</td>
</tr>
<tr>
<td>8-Canadinylacetic acid (122)</td>
<td>27</td>
<td>&gt;10</td>
</tr>
<tr>
<td>8-Berberinylidene-n-propylamine (123)</td>
<td>0.27</td>
<td>0.74</td>
</tr>
<tr>
<td>8-Berberinyl-acetic acid (124)</td>
<td>9.4</td>
<td>4.3</td>
</tr>
<tr>
<td>8,8-Diethylidihydroberberine (HI salt, 125)</td>
<td>0.012</td>
<td>0.001</td>
</tr>
<tr>
<td>8,8-Dimethylidihydroberberine (HI salt, 126)</td>
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</tr>
<tr>
<td>D-Tetrahydropalmatine (127)</td>
<td>1.8</td>
<td>N/A</td>
</tr>
<tr>
<td>L-Tetrahydropalmatine (128)</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Table 3.** Results of the initial screening of natural and semisynthetic berberine analogues against *in vitro* models of leishmaniasis and trypanosomiasis.
E1.3. Discussion

Since the batch of the semi-synthetic berberine samples tested was dated 1979, thin-layer chromatography, NMR spectroscopy, and high-resolution mass spectrometry were utilized to check on their purity and structural features. These studies proved that the sample tested of 125 (Table 3) was a mixture of four compounds with protoberberine skeletons. Unfortunately, the limited quantity (<2 mg) of the sample available prevented the purification and identification of the active principle. Analysis of the $^1$H and $^{13}$C NMR spectra of the authentic sample (125, provided in Appendix B) suggested the compounds that made up the sample were oxidized, as would be expected in a sample that has been exposed to air for a long period of time. In an effort to identify the active principle, semi-synthetic studies were carried out following a synthetic scheme similar to that described by Shamma group (Moniot et al., 1979; Scheme 12). The individual steps in Scheme 12 are described in Section E2 below.

Scheme 12. Synthetic scheme for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).
E2. Semisynthesis of 5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine Chloride (129)


Melting points were measured using a Fisher-Johns melting point apparatus. UV spectra were measured with a Perkin-Elmer Lambda 10 UV/vis spectrometer. CD spectra were measured on a JASCO J-810 spectrometer. IR spectra were run on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. NMR spectroscopic data were recorded at room temperature on a Bruker Avance DPX-300 or DRX-400 NMR spectrometer at The Ohio State University. Further NMR experiments for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129) were carried out on a Bruker 500 MHz instrument at Carnegie Mellon University, PA, and $^{13}$C-$^{13}$C INADEQUATE experiments were performed on a Bruker 600 MHz instrument with a cryoprobe at Bruker Biospin Corporation in Billerica, MA. High-resolution mass spectra were recorded on a LCT-TOF mass spectrometer. Column chromatography was performed with 65-250 or 230-400 mesh silica gel (Sorbent Technologies, Inc., Norcross, GA). Analytical thin-layer chromatography (TLC) was conducted on 250 μm thickness Partisil silica gel 60 F$_{254}$ glass plates (Whatman, Clifton, NJ) or 200 μm-thick aluminum-backed silica gel plates (Sorbent Technologies, Inc.) with shortwave UV (254 nm) light and Dragendorff reagent (a solution of potassium bismuth (III) iodide) employed for the visualization of alkaloids. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).
E.2.2. Synthesis of 8-Oxyberberine (78) from Berberine Hemisulfate (85).

Berberine hemisulfate (85, 10 g, 26.0 mmol) was dissolved in 200 mL of H2O and added subsequently to a 500 mL solution of sodium hydroxide (40 g, 200 mmol) in a drop-wise fashion over 1 h, in a 1000 mL three-necked round-bottomed flask equipped with a mechanical stirrer, a condenser, and an addition funnel. The mixture was refluxed continuously for a period of 8 h. The resulting yellow precipitate was then collected by paper filtration and washed with 500 mL of 0.1 N warm hydrochloric acid and 500 mL of H2O, consecutively. The filtrate was dried at room temperature for 8 h and then for 2 h under high vacuum to yield a yellow-orange solid (8 g). This solid was chromatographed in an open normal-phase gravity silica gel column (320 g, 400 mm) with a gradient solvent system from 100% CH2Cl2 to 100% MeOH, with 160 20 mL fractions being collected. 8-Oxyberberine (78, 4 g, 11.38 mmol, 44%) was isolated as pale yellow crystals from fractions F111-F118, and was further purified by recrystallization from MeOH.

E.2.3. Characterization of 8-Oxyberberine (78).

8-Oxyberberine (78, 4 g, 11.38 mmol, 44%) exhibited the following physical and spectroscopic data: pale yellow needle crystals; mp 199 °C; UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 218 (4.6), 312 (3.8), 342 (4.2), 368 (4.0) nm; IR (film) \( \nu_{\text{max}} \) 2931, 2838, 1649, 1384, 1278, 1231, 1041 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)) \( \delta_H \) 7.31 (1H, d, \( J = 8.7 \text{ Hz, H-11} \)), 7.26 (1H, d, \( J = 8.7 \text{ Hz, H-12} \)), 7.20 (1H, s, H-1), 6.71 (1H, s, H-13), 6.69 (1H, s, H-4), 6.00 (2H, s, -OCH\(_2\)O-), 4.28 (2H, t, \( J = 5.4 \text{ Hz, H-6} \)), 4.00 (3H, s, 9-OCH\(_3\)), 3.94 (3H, s, 10-OCH\(_3\)),
2.88 (2H, t, J = 5.4 Hz, H-5); $^{13}$C NMR (MeOD) δC 160.2 (C-8), 151.5 (C-10), 149.6 (C-9), 148.5 (C-3), 147.4 (C-2), 135.7 (C-4a), 132.4 (C-8a), 130.1 (C-13a), 123.9 (C-13b), 122.4 (C-12), 119.5 (C-12a), 119.0 (C-11), 108.0 (C-4), 104.8 (C-1), 101.5 (-OCH$_2$O-), 101.4 (C-13), 61.7 (9-OCH$_3$), 57.0 (10-OCH$_3$), 39.5 (C-6), 28.8 (C-5); HRESIMS m/z 374.0978 [M+Na]$^+$ (calcd. for [C$_{20}$H$_{17}$NO$_5$+Na]$^+$, 374.1004).

E.2.4. Synthesis of 8-Chloroberberine (116) from 8-Oxyberberine (78) and its Characterization.

8-Oxyberberine (78, 3 g, 8.54 mmol) was dried under vacuum overnight and added into a 1000 mL three-necked round-bottomed flask equipped with a mechanical stirrer, a condenser, and an addition funnel. POCl$_3$ (2.5 g, 16 mmol) was added drop-wise and refluxed for 3 h at 60 °C. The resulting orange crystalline precipitate (2.1 g, 5.67 mmol, 66%) was collected by filtration, washed with 200 mL dichloromethane (CH$_2$Cl$_2$), dried under high vacuum, and stored under argon.

E.2.5. Characterization of 8-Chloroberberine Chloride (116).

8-Chloroberberine exhibited the following physical and spectroscopic data: pale yellow needle crystals; $^1$H NMR (CF$_3$COOD) δH 8.69 (1H, s, H-13), 8.19 (2H, s, H-11, H-12), 7.62 (1H, s, H-1), 7.02 (1H, s, H-4), 6.19 (2H, s, -OCH$_2$O-), 5.25 (2H, t, J = 6.1 Hz, H-6), 4.21 (3H, s, 9-OCH$_3$), 4.16 (3H, s, 10-OCH$_3$), 3.37 (1H, t, J = 6.1 Hz, H-5); $^{13}$C NMR (CF$_3$COOD) δC 156.1 (C-9), 152.9 (C-3), 150.4 (C-2), 149.8 (C-8), 146.9 (C-10), 141.9 (C-13a), 135.9 (C-8a), 132.4 (C-4a), 127.8 (C-11), 127.3 (C-12), 123.2 (C-13b), 122.7 (C-12a), 122.6 (C-13), 109.6 (C-4), 107.8 (C-1), 104.3 (-OCH$_2$O-), 63.0 (10-
8-Chloroberberine chloride (116, 1 g, 2.5 mmol) was suspended in dry diethyl ether in a fire-dried flask and allowed to react with ethyl magnesium bromide (EtMgBr) in dry diethyl ether (4 mL, 12 mmol) under argon for 2 h, and was monitored with TLC. Following the completion of the reaction, excess of EtMgBr was quenched over ice. Subsequently, 3 mL of concentrated H₂SO₄ was added and the organic layer was partitioned in a separatory funnel. The organic layer was salted out by bubbling HCl gas through the solution, forming a precipitate. Upon drying under a vacuum, 2.8 g of an orange solid were obtained. This sample was pre-fractionated on a normal-phase silica column (140 g, 250 mm) in a gradient fashion from 100% CH₂Cl₂ to 100% MeOH to yield seven fractions (F1-F7). F6 was determined to be alkaloid-rich based on its visualization with the Dragendorff reagent on TLC and was further fractionated using a semi-preparative reversed phase phenol column (YMC pack-ph, 150 mm × 20 mm i.d.), employing MeOH-H₂O (40:60, 8 mL/min) as the mobile phase, to afford a bright yellow compound (407 mg, tᵣ = 11.4 min) that crystallized readily from solution.

In contrast to the final product reported previously by Moniot et al., 1979, the major product in this synthesis was determined to be the new berberine analogue, 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129, 0.9 mmol, 36% yield).
The structure of this compound was confirmed after a series of rigorous computational and spectroscopic studies were conducted.

E.2.7. Characterization of 5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine Chloride (129).

5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine (129) chloride exhibited the following physical and spectroscopic data: yellow needle crystals (MeOH/H$_2$O), mp 159 °C; UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 212 (4.33), 241 (4.18), 287 (4.09) nm; IR (film) $v_{\text{max}}$ 3417, 1659, 1581, 1460, 1290, 1027 cm$^{-1}$; $^1$H NMR (300 MHz, MeOD) $\delta_H$ 9.22 (1H, d, $J = 7.2$ Hz, H-6), 9.09 (1H, s, H-1), 8.61 (1H, d, $J = 7.2$ Hz, H-5), 8.26 (1H, d, $J = 8.9$ Hz, H-12), 7.68 (1H, s, H-4), 7.54 (1H, d, $J = 8.9$ Hz, H-11), 6.45 (2H, s, -OCH$_2$O-), 4.10 (3H, s, 10-OCH$_3$), 4.03 (3H, s, 9-OCH$_3$), 3.22 (2H, dd, $J = 15.4$, 7.6 Hz, H-1"), 2.70 (2H, dd, $J = 15.4$, 7.4 Hz, H-1'), 0.38 (6H, t, $J = 7.4$ Hz, H-2' and H-2''); $^{13}$C NMR (75 MHz, MeOD) $\delta_C$ 178.5 (C-13), 160.9 (C-10), 158.3 (C-2), 156.1 (C-3), 145.0 (C-9), 142.4 (C-13a), 142.1 (C-4a), 133.3 (C-8a), 132.3 (C-6), 129.3 (C-5), 127.0 (C-13b), 126.6 (C-12), 124.7 (C-12a), 115.8 (C-11), 106.6 (C-1), 106.3 (-OCH$_2$O-), 104.4 (C-4), 81.1 (C-8), 61.7 (9-OCH$_3$), 57.0 (10-OCH$_3$), 35.1 (C-1' and C-1'"), 8.2 (C-2' and C-2'"); HRESIMS $m/z$ 406.1650 [M]+ (calcd. for C$_{24}$H$_{24}$NO$_5$, 406.1654).

E.2.8. Biological Activity of 5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine Chloride (129)

The synthesized derivative, 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129), was tested in an intracellular amastigote assay for leishmaniasis (Buckner
et al., 2005; Appendix A), an in vitro model of trypanosomiasis (Salem and Werbovetz, 2005; Appendix A), a malaria assay employing flow cytometry (Liu, et al., 2005; Appendix A), and Vero cells to determine the selectivity of the compound (Delfin et al., 2006, Appendix A) and the results are provided in Table 4. Additionally, the in vivo activities of 129 were determined in a mouse model for visceral leishmaniasis, using a protocol described in Appendix A.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Trypanosoma brucei brucei</th>
<th>Leishmania amazonensis</th>
<th>Plasmodium falciparum</th>
<th>Vero cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B&lt;sup&gt;b&lt;/sup&gt; (7)</td>
<td>N/A</td>
<td>0.12 ± 0.03</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Chloroquine (12)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td>0.0087 ± 0.0021</td>
<td>N/A</td>
</tr>
<tr>
<td>Suramin&lt;sup&gt;b&lt;/sup&gt; (1)</td>
<td>0.25 ± 0.11</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Berberine hemisulfate (85)</td>
<td>1.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17 ± 5</td>
<td>0.80 ± 0.31</td>
<td>N/A</td>
</tr>
<tr>
<td>5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129)</td>
<td>0.0091 ± 0.0032</td>
<td>0.18 ± 0.00</td>
<td>0.036 ± 0.014</td>
<td>91 ± 6</td>
</tr>
</tbody>
</table>

Table 4. Biological activity of of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129) against Trypanosoma brucei brucei, Leishmania amazonensis, Plasmodium falciparum, and Vero cells (IC<sub>50</sub> values in µM).<sup>a</sup> Mean ± standard deviation unless otherwise noted (n ≥ 3); <sup>b</sup> Positive controls; <sup>c</sup> Mean ± range (n = 2).
Table 5. *In vivo* activity of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129) in a mouse model of visceral leishmaniasis. \(^a\) 1 mg/kg/ip; \(^b\) Positive control, 10 mg/kg/ip.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Reduction in liver parasitemia (mean ± standard deviation, (n = 4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129)(^a)</td>
<td>47±4</td>
</tr>
<tr>
<td>Miltefosine (9)(^b)</td>
<td>98±1</td>
</tr>
</tbody>
</table>

E.3. Discussion.

E.3.1. Identification of Synthetic Intermediates and Structure Elucidation of 5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine Chloride (129).

E.3.1.1. Identification of 8-Oxyberberine (78)

The molecular formula of 8-oxyberberine (78) was determined as C\(_{20}\)H\(_{17}\)NO\(_5\), from the sodiated molecular ion peak at \(m/z\) 374.0978 [M+Na]\(^+\) (calcd. for [C\(_{20}\)H\(_{17}\)NO\(_5\)+Na]\(^+\), 374.1004) in the HRESIMS (Figure 44) and the \(^{13}\)C NMR spectra (Figures 45 and 46). In a similar fashion to berberine (85), in the \(^1\)H NMR spectrum (Figure 47), two methoxy group signals appeared at \(\delta_H\) 4.00 and 3.94, corresponding to six protons, along with a -OCH\(_2\)O- signal at \(\delta_H\) 6.00. The \(^{13}\)C NMR spectrum (Figure 45) revealed an amide signal at \(\delta_C\) 160.2, showing oxidation to have occurred at C-8. Aromatic singlet proton signals corresponding to positions C-1, C-4, and C-13 were observed at \(\delta_H\) 7.20, 6.69, and 6.71, respectively. The two aromatic proton signals at \(\delta_H\)
7.31 and 7.26 were observed as doublets with mutual $J$ value of 8.7 Hz, and the vicinal coupling between these two protons was supported by the $^1$H-$^1$H COSY spectrum (Figure 48). The aliphatic proton signals at $\delta_H$ 2.88 and 4.28 were observed as triplets and assigned to H-5 and H-6, respectively, based on their observed mutual coupling with a $J$ value of 5.4 Hz. The $^1$H-$^1$H COSY experiment further showed correlations supporting both the H-5–H-6 and H-11–H-12 vicinal couplings. Based on the HSQC experiment (Figure 49), the protonated carbon signals at $\delta_C$ 104.8, 108.0, 28.8, 39.5, 119.0, 122.4, 101.4 were assigned to C-1, C-4, C-5, C-6, C-11, C-12, and C-13, respectively. The C-9 and C-10 carbon atoms were assigned based on their HMBC correlations (Figure 50) with the 9-OCH$_3$ and 10-OCH$_3$ signals, as $\delta_C$ 149.6 and 151.5, respectively. Bridgehead atoms in the molecule of 8-oxyberberine (78) were attributed based on the correlations observed in the HMBC spectrum. The downfield signal at $\delta_C$ 135.7 showed HMBC correlations with H-1, H-6, and H-13 and was assigned as C-4a. Similarly, the downfield signal at $\delta_C$ 132.4, showed strong HMBC correlations with H-11 and was assigned to C-8a. A signal resonating at $\delta_C$ 119.5 showed HMBC correlations with signals corresponding to H-11 and H-13, and was assigned as C-12a. The signals at $\delta_C$ 130.1 and 123.9 demonstrated HMBC correlations with H-1, H-5, and H-6, and H-1, H-3, and H-5, respectively, and were assigned as C-13a and C-13b. The spatial arrangement of the molecule was confirmed using a $^1$H-$^1$H NOESY experiment (Figure 51). Therefore, the structure of this isolate was determined as 8-oxyberberine (78), and this compound exhibited comparable physical and spectroscopic data to literature values (MacLean et al., 1987).
Figure 44. HRESIMS data for 8-oxyberberine (78).

Figure 45. $^{13}$C NMR spectrum of 8-oxyberberine (78).
Figure 46. $^{13}$C DEPT NMR spectrum of 8-oxyberberine (78).

Figure 47. $^1$H NMR spectrum of 8-oxyberberine (78).
Figure 48. $^1$H-$^1$H COSY NMR spectrum of 8-oxyberberine (78).

Figure 49. $^1$H-$^{13}$C HSQC NMR spectrum of 8-oxyberberine (78).
Figure 50. $^1$H-$^1$C HMBC NMR spectrum of 8-oxyberberine (78).

Figure 51. $^1$H-$^1$H NOESY NMR spectrum of 8-oxyberberine (78).
E.3.1.2. Identification of 8-Chloroberberine Chloride (116)

The molecular formula of 8-chloroberberine (116) was determined as C₂₀H₁₇ClNO₄, from the positive molecular ion peak at m/z 370.0863 [M]⁺ (calcd. for [C₂₀H₁₇ClNO₄]⁺, 370.0846) in the HRESIMS (Figure 52) and from the ¹³C NMR spectra (Figures 53 and 54). In a similar fashion to 8-oxyberberine (78), the ¹H NMR spectrum (Figure 55) exhibited two methoxy group signals at δ_H 4.21 and 4.16, corresponding to six protons, along with a -OCH₂O- signal at δ_H 6.19. Aromatic singlet proton signals corresponding to positions C-1, C-4, and C-13 were observed at δ_H 7.62, 7.02, and 8.69, respectively. The aliphatic proton signals at δ_H 3.37 and 5.25 were observed as triplets and assigned to H-5 and H-6, respectively, based on their observed mutual coupling with a J value of 6.1 Hz. The ¹H-¹H COSY experiment (Figure 56) was used to further show correlations supporting the H-5–H-6 vicinal coupling. Interestingly, the two aromatic proton signals of H-11 and H-12 were not resolved, similar to an observation reported earlier for this compound (Moniot et al., 1979), with a signal occurring as a singlet at δ_H 8.19 that integrated for two protons. The corresponding carbon signals were indeed resolved in both the ¹³C NMR spectra (Figures 53 and 54) and the HSQC (Figure 57) experiments. Based on the HSQC spectrum, the protonated carbon atoms at positions C-1, C-4, C-5, C-6, C-11, C-12, and C-13 were attributed to δ_C 107.8, 109.6, 28.5, 53.9, 127.8, 127.3, and 122.6, respectively. The C-9 and C-10 resonances were found to occur at δ_C 156.1 and 146.9, respectively, based on their HMBC (Figure 58) correlations with 9-OCH₃ and 10-OCH₃. The bridgehead atoms were again assigned based on the correlations observed in the HMBC spectrum. The downfield signal at δ_C 132.4 showed
HMBC correlations with the H-1 signal and was assigned as C-4a. Similarly, the
downfield signal at $\delta_C$ 135.9, showed strong HMBC correlations with H-6, H-11 and H-
12, and was assigned to C-8a. Finally, the signal observed at $\delta_C$ 141.9 showed HMBC
correlations with H-1, H-4, H-6, and H-13 and was assigned to C-13a. Therefore, the
structure of this isolate was determined as 8-chloroberberine (116), and this compound
exhibited comparable physical and spectroscopic data to literature values (Moniot et al.,
1979).

Figure 52. HRESIMS data for 8-chloroberberine chloride (116).
Figure 53. $^{13}$C NMR spectrum of 8-chloroberberine chloride (116).

Figure 54. $^{13}$C DEPT NMR spectrum of 8-chloroberberine chloride (116).
Figure 55. $^1$H NMR spectrum of 8-chloroberberine chloride (116).

Figure 56. $^1$H-$^1$H COSY NMR spectrum of 8-chloroberberine chloride (116).
Figure 57. $^1$H-$^{13}$C HSQC NMR spectrum of 8-chloroberberine chloride (116).

Figure 58. $^1$H-$^{13}$C HMBC NMR spectrum of 8-chloroberberine chloride (116).
E.3.1.3. Structure Elucidation of 5,6-Didehydro-8,8-diethyl-13-oxodihydroberberine Chloride (129)

The molecular formula of the parent compound, 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129) was determined as C_{24}H_{24}NO_{5}, from the positive molecular ion peak at m/z 406.1650 [M]+ (calcd. for C_{24}H_{24}NO_{5}, 406.1654) in the HRESIMS (Figure 59) and from the $^{13}$C NMR spectrum (Figure 60). The UV spectrum showed absorption maxima at 212, 241, and 287 nm. A comparison of the $^1$H NMR spectrum (Figure 61) with that of berberine (85), revealed no significant chemical shift change for the methylenedioxy moiety on ring A, that resonated at $\delta_H$ 6.45. The chemical shifts of the 9- and 10-OCH$_3$ groups on ring D were observed at $\delta_H$ 4.11 and 4.04, respectively. However, a significant change compared to berberine (85) was the loss of aliphatic proton signals, suggesting oxidation between the positions C-5 and C-6. These protons were observed as two aromatic proton signals at $\delta_H$ 8.62 and 9.23, respectively, showing mutual coupling with a $J$ value of 7.2 Hz. The proton signals corresponding to C-5, C-6, C-11, and C-12, along with the protons belonging to the ethyl groups, showed correlations in the COSY experiment (Figure 62). As expected from the synthesis step leading to the generation of this compound, two sets of ethyl group proton signals at $\delta_H$ 3.22 (2H, dd, $J = 15.4$, 7.6 Hz, H-1”), 2.71 (2H, dd, $J = 15.4$, 7.4 Hz, H-1”), and 0.39 (6H, H-2' and H-2") were observed. Protonated carbon atoms were attributed to positions C-1, C-4, C-5, C-6, C-11, and C-12, based on their HSQC correlations, to signals at $\delta_C$ 106.6,
104.4, 129.3, 132.4, 115.9, and 126.6, respectively (Figure 63).

These variations suggested that ring B of 8-chloroberberine (116) was now aromatized with ring C substituted by two ethyl groups as a result of the Grignard reaction performed. In addition to these changes in the \(^1\)H NMR spectrum, a signal at \(\delta_C 178.5\) in the \(^{13}\)C NMR spectrum (Figure 60) pointed to a conjugated carbonyl carbon that was assigned unambiguously to C-13, based on both the HMBC (Figure 64) and \(^{13}\)C-\(^{13}\)C INADEQUATE (Incredible Natural Abundance Double QUantum Transfer Experiment, Appendix B) experiments. The C-9 and C-10 signals were observed at \(\delta_C 145.0\) and 160.9, based on their HMBC correlations with the respective methoxy proton signals at these positions. The downfield signal at \(\delta_C 142.1\) showed HMBC correlations with H-1 and H-6, and was assigned to C-4a. Similarly, the downfield signal at \(\delta_C 133.3\), showed HMBC correlations with H-1', H-1", H-11, and H-12 and was assigned to C-8a. The C-13a signal was observed at \(\delta_C 142.4\) and showed HMBC correlations with H-1 and H-6. The positions of the carbonyl group and the ethyl groups were determined unambiguously through the analysis of correlations observed in all 2D NMR experiments, including the \(^1\)H-\(^1\)H COSY, HSQC, HMBC, and NOESY spectra. HMBC correlations between the protons of the methyl and methylene units of the ethyl groups and C-8 at \(\delta_C 81.1\) proved the substitution of two ethyl groups at the C-8 position. Cross peaks observed in the NOESY spectrum (Figure 65) between the methyl group and methoxy protons at \(\delta_H 4.04\), as well as between the methylene protons and H-6 at \(\delta_H 9.23\) further supported this assignment. Correlations observed between H-12 at \(\delta_H 8.27\) to C-13 at \(\delta_C 178.5\) in the HMBC experiment clearly indicated the carbonyl group to be located
on ring C. Finally, the NMR carbon-carbon correlations network was supported by a $^{13}\text{C}$-$^{13}\text{C}$ 2D INADEQUATE spectrum (Appendix B). Thus, the structure of the synthesized compound was determined as 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129), with the chloride counterion inferred from the experimental conditions used for the preparation of this compound.

**Figure 59.** HRESIMS data for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).
Figure 60. $^{13}$C NMR spectrum of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).

Figure 61. $^1$H NMR spectrum of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).
Figure 62. $^1$H-$^1$H COSY NMR spectrum of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).

Figure 63. $^1$H-$^{13}$C HSQC NMR spectrum of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).
Figure 64. $^1$H-$^{13}$C HMBC NMR spectrum of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).

Figure 65. $^1$H-$^1$H NOESY NMR spectrum of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129).
A main challenge in the structure elucidation of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129) was the lack of a strong carbonyl signal in the IR spectrum as might be expected in a structure of this type. Structural and spectroscopic properties of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride were investigated computationally to obtain better insight regarding the missing carbonyl signal in the IR spectrum (Appendix B). Briefly, using a meta-GGA hybrid M06 functional and the 6-31+G** basis set, the suggested structure for 129 was optimized for density functional theory (DFT). Subsequently, the structures were computed in vacuo and using the polarizable continuum model (PCM) with chloroform and methanol parameters as implemented in Gaussian09 and vibrational frequencies were obtained through the analytic computation of the Hessian matrix at the same level. In this study, the weak intensity observed in the IR spectra for the carbonyl stretching was successfully reproduced in the DFT computed vibrational spectrum and the computed and experimental IR spectra matched each other nearly identically.

Consequently, while the physicochemical factors that led to a significantly weak IR signal for the carbonyl group in 129 are not elucidated, this finding was replicated in a highly accurate computational model. Furthermore, \(^{13}\text{C}-^{13}\text{C}\) INADEQUATE connectivity studies were carried out to ascertain the position of the carbonyl group in the molecule through correlations, and these findings confirmed the suggested structure (Figure 66). Though the oxidation found in 129 was unforeseen, similar oxidations in other isoquinoline-type alkaloids have been reported previously (Li and Yang, 2005) and a mechanism leading to the formation of 129 is proposed in Scheme 13.
Figure 66. Observed correlations for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine chloride (129) in COSY, HMBC, NOESY, and INADEQUATE experiments.

Scheme 13. Scheme explaining the possible mechanism of the oxidation at C-13 and the oxidation of C5–C6 bond, leading to the formation of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129).
E.3.1.4. Discussion of Biological Activity

The IC$_{50}$ values obtained for the new semi-synthetic compound 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129), have been presented in µM values in Table 4. These results show that 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) possesses remarkably high nanomolar level inhibitory potency in *in vitro* models of three protozoal diseases, namely, *Trypanosoma b. brucei* (human African trypanosomiasis), *Leishmania amazonensis* (leishmaniasis), and *Plasmodium falciparum* (malaria), when compared to the parent natural product compound, berberine (85). Notably, in the case of human African trypanosomiasis, compound 129 demonstrated higher potency than that of the positive control drug used, suramin (1). In the case of malaria, compound 129 showed potency comparable to that of chloroquine (12). Selectivity studies using Vero (green monkey epithelial kidney) cells indicated that the selectivity of 129 for protozoal cells in comparison to mammalian cells is >1000-fold.

Following up on the potency observed in the *in vitro* systems, an efficacy study was performed in an *in vivo* model of visceral leishmaniasis in mice (Table 5). In this study, while 129 was shown to be more potent than the clinically used drug, miltefosine (9), some toxicity in mice was observed at higher doses, hindering further dose response studies with this particular berberine derivative.
E.4. Conclusions

This dissertation project, aiming at the discovery of novel lead compounds with antileishmanial and antitrypanosomal activities, started with the biological screening of a library of taxonomically authenticated plant samples and concluded with the discovery of the lead compound, 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129). This synthetic compound is a member of a potentially new class of natural product-based compounds that might be useful in the treatment of African sleeping sickness, different forms of leishmaniasis, and, another major protozoan disease, malaria.

In the plant screening method utilized in this study, crude chloroform-soluble extracts of 147 plants prepared in a standard manner were screened, with 26 exhibiting IC$_{50}$ values of 25 µg/mL or lower in either or both of the in vitro models of leishmaniasis and trypanosomiasis used. These studies led to the determination of Thalictrum lucidum L. (Ranunculaceae) as a lead plant sample, so bioactivity-guided fractionation studies were carried out. An in vitro-active fraction was obtained containing berberine (85) and jatrorrhizine (87). Protoberberine-type alkaloids, such as berberine (85), jatrorrhizine (87), palmatine (89), and thalifendine (90), and their semi-synthetic derivatives, were described previously as having promising activity in vitro against leishmaniasis and malaria (Vennerstrom et al., 1988; Vennerstrom et al., 1990). However, these activities reportedly did not carry over to potent in vivo effects, with the exception of berberine
and 8-cyanoberberine (108), which were found active in an animal model of leishmaniasis (Vennerstrom et al., 1988; Vennerstrom et al., 1990).

From a pharmacognostic perspective, isolation studies on T. lucidum, a member of a genus abundant with protoberberine-type alkaloids, were carried out to rule out the possibility that the observed activity stemmed from a constituent other than a protoberberine alkaloid. The protoberberine alkaloids berberine (85) and jatrorrhizine (87) were isolated and identified during the initial column chromatography, along with palmatine (89) from a similar fraction. After the fractions were combined and tested, the observed bioactivity corresponded strongly with the presence of protoberberine alkaloids. Since the aim of the project was the identification of novel compounds and this particular plant has been studied extensively (Wu et al., 1976b; Wu et al., 1976c), no further phytochemical work was focused on the sample of Thalictrum lucidum.

Since a library of semi-synthetic protoberberine alkaloids was on hand, these compounds were evaluated in in vitro models of leishmaniasis and trypanosomiasis. Of the fourteen compounds tested, ten had IC$_{50}$ values of 10 µg/mL or less in either one or both of the in vitro test systems used (Salem and Werbovetz, 2005; Appendix A, Table 3). Two of these samples, 8,8-diethylidihydroberberine (125) and 8,8-dimethylidihydroberberine (126) demonstrated remarkably high potency, with IC$_{50}$ values of 0.012 and 0.015 µg/mL against L. donovani and 0.001 and 2.2 µg/mL against T. b. brucei parasites, respectively. Of the two, 125 was prioritized since it had equally high potency in both systems, while 126 was considerably less active in the in vitro model of trypanosomiasis.
Unfortunately, a closer inspection of the authentic sample through chromatographic and spectroscopic methods, revealed the active sample, 125, to be a mixture of at least four closely related protoberberine-type alkaloids and it was decided to follow the original scheme described in the literature to determine the active principle in this sample (Moniot et al., 1979).

As a result of this synthetic effort, the lead compound 129 was isolated and structurally elucidated. The major challenge in the structure elucidation of this compound found was the lack of a clear carbonyl signal in the IR spectrum, although a signal corresponding to this carbon was present in the $^{13}$C NMR spectrum and the HRMS data supported the presence of an extra oxygen atom in the compound. To ascertain the structure proposed, a $^{13}$C-$^{13}$C INADEQUATE NMR experiment was employed to probe the carbon connectivity in the molecule, and this spectrum provided positive evidence that the proposed structure of 129 was indeed accurate. Furthermore, computational models (Appendix B) were able to reproduce this unusual feature as observed in the IR spectrum.

Subsequently, the biological activity of 129 was evaluated against several protozoan parasites including in vitro models of leishmaniasis, malaria, and trypanosomiasis, for which the results are included in Table 4. The selectivity of 129 for parasites versus mammalian cells was determined in a Vero cell assay. In all test systems used, 129 showed highly potent activity and these findings were followed up by an in vivo model of leishmaniasis, in which the IC$_{50}$ value of 129 was determined to be 0.18
µM, a value comparable to that of amphotericin B, the positive control. However, further dose escalation studies in vivo were hindered by the toxicity observed in the mice.

In conclusion, 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) is a potent compound, which might be further modified structurally to reduce the toxicity observed in the in vivo model of leishmaniasis. Furthermore, this dissertation work represents a valid example of how a natural product biological screening approach may produce a promising lead compound, for which the activity may be enhanced greatly by semi-sythetic optimization.
References


Cheng, C. C., Hsueh, C. M., Liang, K. W., Ting, C. T., Wen, C. L., and Hsu, S. L. Role of Jnk and C-Jun Signaling Pathway in Regulation of Human Serum Paraoxonase 1


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APPENDIX A

All biological studies were carried out in the laboratory of Dr. Karl A. Werbovetz at the College of Pharmacy, The Ohio State University (OSU), OH, with the exception of the antimalarial assay, which was conducted in the laboratory of Dr. Mark E. Drew, Colleges of Medicine and Pharmacy, OSU. The protocols used in these studies are described in the following paragraphs.

Antitrypanosomal Assay.

The bloodstream-form of *Trypanosoma brucei brucei* (MITat 1.2, variant 221) was cultivated axenically in HMI-9 medium. For this experiment, a 100 μL aliquot of late log-phase parasites was incubated in a 96-well plate for 72 h with a starting concentration of 10⁵ cells/mL at 37 °C, in a humidified 5% CO₂ atmosphere, with or without test compound. In order to evaluate the activity of each compound, a 25 μL portion of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each plate and left to incubate for 2 h. Following the addition of a 100 μL-aliquot of 10% SDS lysis buffer, plates were incubated for another 4 h, and optical densities were measured at 570 nm using a SpectraMax Plus microplate reader. The IC₅₀ values were determined with the aid of SoftMAX Pro software (Amersham Biosciences, Piscataway, NJ) using the dose-response equation \( y = [(a - d)/(1 + (x/c)^b)] + d \), where \( x \) is the drug concentration, \( y \) is the Abs570, \( a \) is the upper asymptote of a four-parameter
curve, b is the slope, c is the IC\textsubscript{50} value, and d is the lower asymptote (Salem and Werbovetz, 2005). The test results for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) are provided in Figure A1.

![Figure A1: IC\textsubscript{50} curve of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) against T. brucei brucei parasites. (C: IC\textsubscript{50} value in ng/mL)](image)

**Figure A1.** IC\textsubscript{50} curve of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) against T. brucei brucei parasites. (C: IC\textsubscript{50} value in ng/mL)

**Antileishmanial Assay Using Axenic Amastigotes.**

The antileishmanial potency of the samples was screened *in vitro* in a model employing *Leishmania donovani* amastigote-like parasites (WHO designation: MHOM/SD/62/1S-CL2\textsubscript{D}), in a 72-hour assay using tetrazolium dye-based CellTiter reagent by Promega, Madison, WI. For this experiment, 10\textsuperscript{6} cells/mL of *Leishmania* amastigotes were seeded in the medium, along with a serial dilution of the test samples, to achieve a final working volume of 60 µL per well. After incubation for 72 h in a humidified 5% CO\textsubscript{2} atmosphere at 37 °C, 12 µL of CellTiter reagent was added to each well. After allowing 3-6 h for color development at 37 °C, the optical densities were measured at 490 nm using a SpectraMax Plus plate reader. The IC\textsubscript{50} values were
determined with the aid of SoftMAX Pro software (Amersham Biosciences, Piscataway, NJ) using the dose-response equation \( y = \frac{(a - d)}{(1 + (x/c)^b)} + d \), where \( x \) is the drug concentration, \( y \) is the Abs490, \( a \) is the upper asymptote of a four-parameter curve, \( b \) is the slope, \( c \) is the IC\(_{50}\) value, and \( d \) is the lower asymptote (Salem and Werbovetz, 2005).

**Intracellular Amastigote Assay (Antileishmanial Assay).**

The antileishmanial efficacy of compounds against intracellular parasites was measured using a colorimetric assay developed by the Buckner laboratory. Starch-elicited peritoneal macrophages were harvested from CD-1 mice, added to 96-well plates, and allowed to adhere overnight. Then, host cells were infected with \( \beta \)-lactamase expressing *Leishmania amazonensis* promastigotes at a parasite-macrophage ratio of 5:1 at 34 °C in a 5% CO\(_2\) atmosphere. On the next day, old medium was replaced with 200 µL of medium containing either the test compound or vehicle. After three days, cells were lysed with a solution containing 0.1% Triton X-100 and 100 µM nitrocefin in PBS. Plates were incubated at 37 °C for 4 h and then read at 490 nm using a SpectraMax Plus microplate reader, and IC\(_{50}\) values were calculated (Buckner *et al.*, 2005). The test results for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) are provided in Figure A2.
Figure A2. IC<sub>50</sub> curve of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) against intracellular *Leishmania amazonensis* parasites. (C: IC<sub>50</sub> value in ng/mL)

Mouse Model of Visceral Leishmaniasis.

*Leishmania donovani* low-pass promastigotes (5 x 10<sup>7</sup>), obtained from spleen homogenates prepared from an infected hamster, were administered to female 6-8-weeks old BALB/c mice by the i.v. route in a BSL-2 facility. Infected mice were randomly sorted into groups of four. On days 7-11 of infection, groups of animals were treated with 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129), miltefosine, or vehicle by either i.p. injection or by the p.o. (oral) route. On day 14 of infection, all animals were euthanized and livers and spleens were removed and weighed. Liver smears were prepared on glass slides, and then were fixed with methanol and stained with Giemsa. The number of amastigotes/1000 host liver macrophage nuclei were counted for each mouse by light microscopy, allowing the activity of the test and control compounds to be determined in comparison to the vehicle control group (Delfin *et al*., 2009).
Malaria Assay Employing Flow Cytometry.

The asexual stage *Plasmodium falciparum* clone 3D7 was cultured in human O+ erythrocytes at 2% hematocrit under 5% CO₂, 5% O₂, and 90% N₂, and grown in rich medium (RPMI 1640 medium supplemented with 27 mM NaHCO₃, 11 mM glucose, 0.37 mM hypoxanthine, 10 µg/mL gentamicin, and 5 g/L Albumax (Invitrogen, Carlesbad, CA). Assays were performed using highly synchronous *P. falciparum* parasites incubated in triplicate with increasing concentrations of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129). After 48 h, a 5 µL-aliquot of parasite culture was sampled by transferring into 100 µL of 1.0 µg/mL acridine orange in PBS, incubating for 10 min, and the percent infected cells were determined using a BD Biosciences Canto II flow cytometer fitted with an high-throughput screening sampler. Growth (relative to DMSO controls) was calculated using FlowJo software (Treestar, Ashland, OR). IC₅₀ determinations were calculated using GraphPad Prism software (GraphPad Software, La Jolla, CA) (Liu, *et al.*, 2005). The test results for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) are provided in Figure A3.

![Graph](image)

**Figure A3.** IC₅₀ curve of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129, on left) against *Plasmodium falciparum* parasites compared with the positive control, chloroquine (12, on right).
**Vero Cell Toxicity Assay.**

Vero cells (green monkey epithelial kidney cells; ATCC, Rockville, MD) were maintained in minimum essential medium alpha modification containing Glutamax-I (Invitrogen, Calsbad, CA), and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 50 units/mL of penicillin, and 50 µg/mL of streptomycin. The cells (2 x 10^4), in a volume of 50 µL, were plated in each of the wells in a 96-well plate, and incubated at 37 °C overnight in a humidified environment with 5% CO₂, except for the negative controls. The test compounds were added to the appropriate wells, and two-fold dilutions were made to permit a range of concentrations to be examined for each compound. The cells in a final volume of 100 µL in each well were incubated at 37 °C for three days in a humidified environment with 5% CO₂. Cell proliferation was determined by a colorimetric assay using the tetrazolium dye-based CellTiter reagent (Promega, Madison, WI). After the addition of 20 µL of dye to each of the wells in the 96-well plate, the absorbance was observed 24 h later at 490 nM on a SpectraMAX Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). IC₅₀ values were determined with using SoftMAX Pro software (Amersham Biosciences, Piscataway, NJ), from the dose-response equation {y = [(a - d)/(1 + (x/c)b)] + d}, where x is the drug concentration, y is the Abs490, a is the upper asymptote of a four-parameter curve, b is the slope, c is the IC₅₀ value, and d is the lower asymptote. Each compound was tested in three separate experiments (Delfin *et al.*, 2006). The test results for 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) are provided in Figure A4.
Figure A4. The IC$_{50}$ curve of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129) against Vero Cells. (C: IC$_{50}$ value in ng/mL)
APPENDIX B

$^1$H and $^{13}$C NMR Spectra of the Authentic Sample, labeled as 8,8-diethylidihydroberberine (HI salt) 125.

NMR spectroscopic data were recorded at room temperature on a Bruker Avance DPX-DRX-400 NMR spectrometer at The Ohio State University.

Figure B1. $^{13}$C NMR spectrum of the sample labeled as 8,8-diethylidihydroberberine (125) depicting the presence of a mixture of four oxidized species with carbonyl groups in downfield region, along with evidence for ethyl substitution in the upfield region.
Figure B2. $^1$H NMR spectrum of the sample labeled as 8,8-diethyldihydroberberine (125) depicting the presence of a mixture of four protoberberine species, along with evidence for ethyl substitution in the upfield region.

INADEQUATE Studies on 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129)

$^{13}$C-$^{13}$C INADEQUATE (Incredible Natural Abundance Double QUAntum Transfer Experiment) studies were carried out by Dr. Clemens Anklin of Bruker BioSpin, Billerica, MA to determine the carbon connectivity of 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129). This experiment provided positive evidence for the assignment of the carbonyl group at C-13 in compound 129 (Figure B3).
Computational Studies on 5,6-didehydro-8,8-diethyl-13-oxodihydroberberine (129)

The computational data presented herein were carried out by Dr. Armando Navarro Vázquez of Universidade de Vigo, Spain and were partly published previously (Bahar et al., 2011). Structural and spectroscopic properties of 129 were investigated computationally regarding the missing carbonyl signal in the IR spectrum. For the study conducted, the proposed structure for 129 was optimized using the meta-GGA hybrid M06 functional and the 6-31+G** basis set. Structures were computed in vacuo using the PCM solvation model with chloroform and methanol parameters as implemented in Gaussian09. Subsequently, vibrational frequencies were then obtained by analytic computation of the Hessian matrix at the same level. The
The computed vibrational IR spectrum (Figure B4) precisely predicted the experimental IR spectrum (Figure B5).

Figure B4. Computed M06/PCM(CHCl₃)/6-31+G** harmonic vibrational spectrum for 129. A scaling factor of 0.950 was applied to all frequencies. Spectrum was simulated using a line width of 10 cm⁻¹.
Figure B5. The IR spectrum of 129 obtained in neutralized chloroform.