

**Cloning and expression of deoxyhypusine synthase from
Plasmodium vivax and *Theileria parva* as an approach for
target evaluation in anti-parasitic chemotherapy**

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Dedication:

This thesis is dedicated to my wife Phyllis Njeri Kamau, to the memory that during its process, fate placed on her path the challenge of going through the rest of her life blinded by 'idiopathic intracranial hypertension'.

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Summary

An important issue facing global health today is the need for new, effective and affordable drugs against malaria and a veterinary parasitic disease named Theileriosis, particularly in resource-poor countries. The parasite *P. vivax* which causes benign malaria and *T. parva* causing East Coast Fever (ECF) or *Theileria annulata* causing Tropical Theileriosis (MCF) belong to the group of *Apicomplexa* which have an apicoplast. The apicoplast is a relic of the chloroplast which is necessary for the parasite to invade its host. Genes of the apicoplast have shown to be very important drug targets since they do not exist in their human or animal counterpart. However nuclear encoded genes which show significant differences with respect to the structural domains of their proteins might also be attractive drug targets. Here we report about the cloning and characterization of the deoxyhypusine synthase gene (DHS) from both parasites. DHS catalyzes the first committed step in the biosynthesis of the unique amino acid hypusine in eukaryotic initiation factor 5A (eIF-5A). The enzyme transfers an aminobutyl moiety from the triamine spermidine to a specific lysine residue of the precursor protein. Deoxyhypusinylated eIF-5A is subsequently hydroxylated by deoxyhypusine hydroxylase (DOHH) which completes eIF-5A activation. Surprisingly we identified 4-saturated piperidone monoesters as putative DOHH inhibitors with antiplasmodial activity.

Recent results have shown that DHS is a valuable drug target in *P. falciparum* for the therapy of cerebral malaria. The putative DHS protein from *P. vivax* displays a FASTA score of 74 relative to that from the human parasite *P. falciparum*. The ORF encoding 456 amino acids was expressed under control of IPTG-inducible T7 promoter, and expressed as a protein of approximately 50 kDa (theoretically 52.7 kDa) in *E. coli* BL21 DE3 cells. The N-terminal histidine-tagged protein was purified by Nickel-chelate affinity chromatography under denaturing conditions. DHS has a theoretical pI of 6.0 and its specific enzymatic activity was determined as 1268 U/mg protein. The inhibitor, N-guanyl-1, 7-diaminoheptane (GC7), suppressed specific activity by 36-fold. The *Theileria parva* gene encodes for an ORF of 371 amino acids with a theoretical pI of 5.4 and a calculated molecular weight of 44,8 kDa. *Theileria parva dhs* has a FASTA score

of 49 to its host *Bos taurus*. Expression of the histidine tagged protein in pET28a in *E. coli* BL21 DE3 cells failed.

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III List of abbreviations

aa	Amino acid
ACT	Artemisinin-based combination therapy
AdoMetDC	S-adenosylmethionine decarboxylase
Amp	Ampicillin
APS	Ammoniumperoxidsulfate
AT	Adenine thymidine content
ATP	Adenosintriphosphate
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CMH	Commission on Macroeconomics and Health
CPD-A	Citrate-phosphate-dextrose-adenine
CQ	Chloroquine
CQR	Chloroquine resistance strain
CQS	Chloroquine sensitive strain
CTL	cytotoxic T lymphocyte
DEPC	Diethyl Pyrocarbonate
DFMO	Difluoromethylornithine
DHS	Deoxyhypusinsynthase
DMSO	Dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	Desoxynucleoside-Triphosphate
DOHH	Deoxyhypusinhydroxylase
dpi	days post infection
<i>E. coli</i>	<i>Escherichia coli</i>
ECF	East Coast Fever
EDTA	Ethylenediaminetetraacetic acid
eIF-5A	Eukaryotic initiation factor 5A ()
FCS	fatal calf serum

GC-	guanosine-cytosine content
GC7	N1-guanyl-1,7-Diaminoheptan
GDP	Gross Domestic Product
HSS	Homospermidinsynthase
IPTG	Isopropyl-1-thio- β -galactopyranosid
kDa	Kilodalton
LB-Medium	Luria-Bertani Medium
Min.	Minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
NAD+	Nicotinamide adenine dinucleotide
OD	Optical Density
ODC	Ornithindecaboxylase
<i>P. berghei</i>	<i>Plasmodium berghei</i>
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
<i>P. malariae</i>	<i>Plasmodium malariae</i>
<i>P. ovale</i>	<i>Plasmodium ovale</i>
<i>P. vivax</i>	<i>Plasmodium vivax</i>
PAGE	polyacrylamide-gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
Pf/NF-54	<i>Plasmodium falciparum</i> /NF-54
R –Strain	<i>Plasmodium falciparum</i> /k1
RBCs	red blood cell
ReV	Regulator of Virion
RNA	Ribonucleic acid
rpm	rotation per minute
rRNA	ribosomal RNA
RT	room temperature
SDS	Sodium dodecyl sulphate

SP	Sulphadoxinepyrimethamine
<i>T. annulata</i>	<i>Theileria annulata</i>
<i>T. parva</i>	<i>Theileria parva</i>
TBE	TRIS-Borate-EDTA
TEMED	N, N, N', N',-Tetramethylendiamin
v/v	volume over volume
WHO	World Health Organization
X-Gal	5-Bromo-4-Chlor-3-Indol-®-D-Galactopyranosidion

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1.0 INTRODUCTION

1.0 The Apicomplexa

Apicomplexa are a phylum in the kingdom Protista and single-celled parasites of animals [1, 2]. This large group of protozoa includes such diverse organisms as *Coccidia*, *Piroplasms*, *Plasmodium* and others. Infection by these parasitic protozoa causes incalculable morbidity and mortality to humans and agricultural animals [3]. Apicomplexa of medical importance include *Plasmodium spp* the causative agents of malaria [4]; *Toxoplasma gondii* a source of congenital neurological birth defects; while *Cryptosporidium* and *Cyclospora* (along with *Toxoplasma*) have emerged as opportunistic infections associated with immunosuppressive conditions [5]. *Theileria parva* and *T. annulata* are some of the many apicomplexan parasites of veterinary importance, including *Babesia*, *Eimeria*, *Neospora* and *Sarcocystis*. *Theileria parva* and *T. annulata* are the causes of East Coast fever and Tropical Theileriosis respectively. These are two lymphoproliferative diseases imposing significant constraints on cattle farming in sub-Saharan Africa [6].

1.1 Apicomplexa and their morphological traits

Apicomplexan parasites share a variety of morphological traits that are considered diagnostic for this phylum. These protists have an elongated shape and a conspicuous specialization of the apical region [7]. Many of the distinct characteristics constitute a collection of unique organelles termed the apical complex. These organelles include the rhoptries, the micronemes, the apical polar ring, and the conoid. Rhoptries and micronemes are unique secretory organelles that contain products required for motility, adhesion to host cells, invasion of host cells, and establishment of the parasitophorous

vacuole [7] The apical polar ring is a hallmark organelle of all members of the Apicomplexa [8,9].It serves as one of the three microtubule-organizing centers in these parasites as shown in Figure 1.1. This work focuses on parasites causing malaria and theileriosis with respect to medical and agricultural importance.

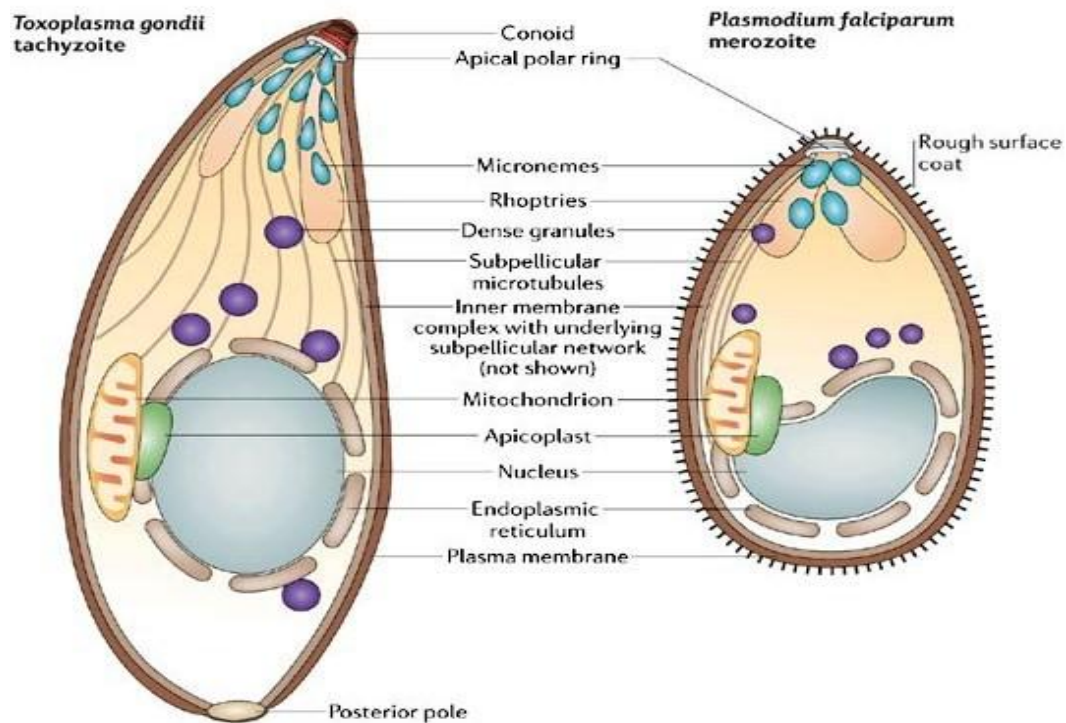


Figure 1.1: Presentation of two apicomplexans *T. gondii* and *P. falciparum*, a comparative morphology between a tachyzoite of *Toxoplasma gondii* and the blood-stage *Plasmodium falciparum* merozoite (not to scale; actual length is ~10 μm for the tachyzoite and 1.5 μm for the merozoite). Figure and legend are adapted from Nature Reviews Microbiology (2006)[116].

1.1.1 Plasmodium parasites

The parasite *Plasmodium vivax* is the most frequent and widely distributed cause of *Malaria tertiana* which persists in the liver and recurs frequently. It is less virulent than *Plasmodium falciparum*, the most severe of the four human parasites and seldom fatal. *P. vivax* is passed on by the female *Anopheles* mosquito, since it is the only gender that bites [14, 15, 22, 23].

Microscopically, the parasitised red blood cell is up to twice as large as a normal cell and fine pink Schüffner's stippling are seen on the cell's surface. The parasite within is often wildly irregular in shape (described as "amoeboid"). Schizonts of *P. vivax* have up to twenty merozoites within them. It is rare to have cells infected with more than one parasite. Merozoites will only attach to immature red blood cells (reticulocytes) which causes a low infection rate of 3% [15]. The incubation period for the infection usually ranges from ten to seventeen days and sometimes up to a year. Persistent liver stages caused relapse of up to five years after elimination of red blood cell stages and clinical cure. The gametocytes of *P. vivax* are commonly found in the peripheral blood at about the end of the first week of parasitemia [22].

Plasmodium falciparum is a protozoan parasite, another of the species of *Plasmodium* which causes malaria in humans. *P. falciparum* causes *Malaria tropica* and is transmitted by *Anopheles* mosquitoes. *P. falciparum* has the highest rates of complications and mortality. In addition it accounts for 80% of all human malarial infections and 90% of the deaths. It is more prevalent in sub-Saharan Africa than in other regions of the world [12, 16, 24].

The life cycle of this organism is complex as shown in figure 1.2 with the parasite alternating between sexual reproduction in an invertebrate vector and asexual reproduction in a vertebrate host. Infection in humans begins with the bite of an infected female *Anopheline* mosquito. Sporozoites are released from the salivary glands of the mosquito and enter the bloodstream during feeding and quickly invade liver cells (hepatocytes). Sporozoites are cleared from the circulation within 30 minutes. During the next 14 days in the case of *P. falciparum*, the liver-stage parasites differentiate and undergo asexual multiplication resulting in tens of thousands of merozoites which burst from the hepatocyte. Individual merozoites invade red blood cells and undergo an additional round of multiplication producing 12- 16 merozoites within a schizont[13]. The length of this erythrocytic stage of the parasite life cycle depends on the parasite species: 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale* and 72 hours for *P. malariae*. The clinical manifestations of malaria which are fever and chills, are associated with the synchronous rupture of the infected erythrocyte. The released merozoites continue to invade additional erythrocytes. Not all of the merozoites divide into schizonts, some differentiate into sexual forms, male and female gametocytes. These gametocytes are taken up by a female *Anopheles* mosquito during a blood meal. Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division, producing 8 flagellated microgametes which fertilize the female macrogamete. The resulting ookinete traverses the mosquito gut wall and encysts in the exterior of the gut wall to form oocyst. Soon the oocyst ruptures and releases hundreds of sporozoites into the mosquito body cavity where they eventually migrate to the mosquito salivary gland [13,14,16].

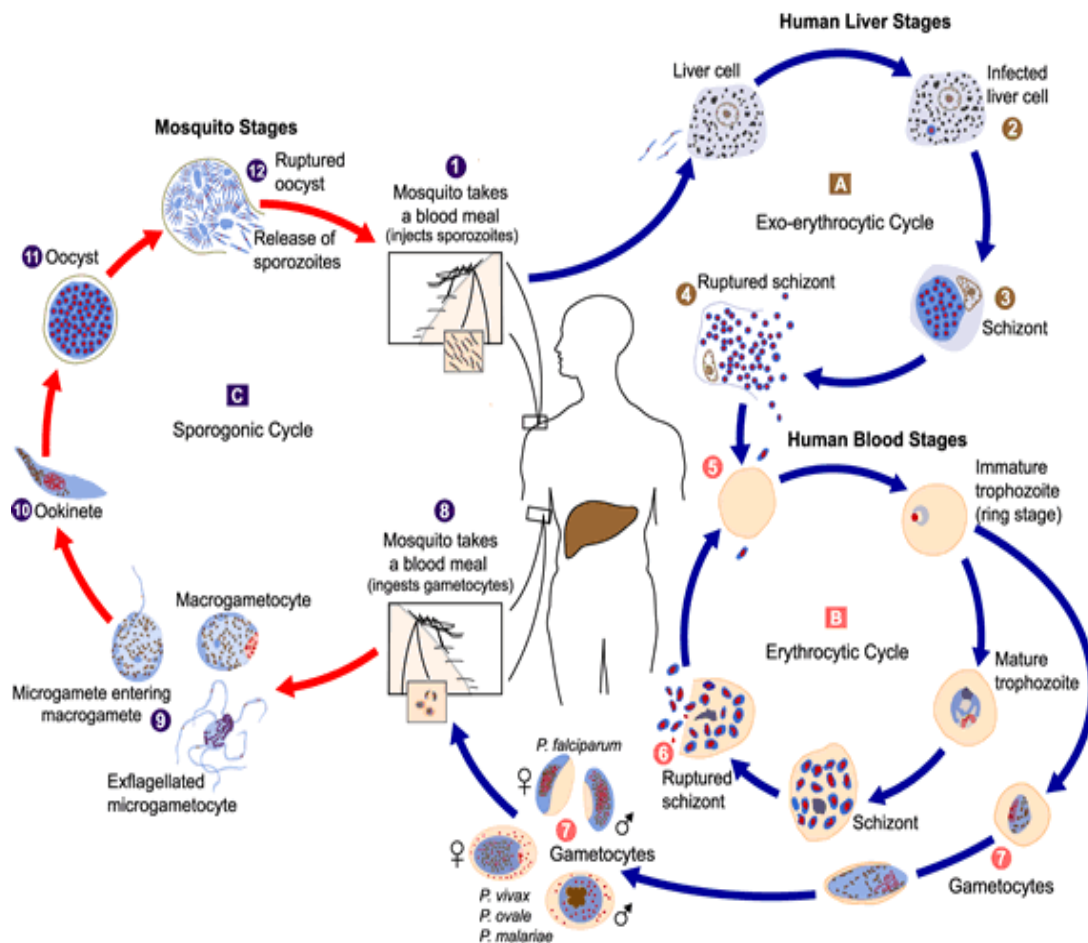


Figure 1.2.: The complex life cycle of the human malaria parasites, *Plasmodium spp* involves a vertebrate host, man, and an invertebrate host, the female *Anopheles* mosquito, adapted from <http://www.cdc.gov/malaria/lifecycle.html>. [117].

1.1.2 Malaria

The name malaria comes from the Italian *male aria*, meaning bad air. It comes from the linkage suggested by Lancisi in 1717 of malaria with the poisonous vapours of swamps [10]. The organism itself was first seen by Laveran in 1880 at a military hospital in Constantine, Algeria, when he discovered a microgametocyte exflagellating [11]. Manson in 1894 hypothesised that mosquitoes could transmit malaria. This hypothesis was experimentally confirmed independently by Giovanni Battista Grassi and Ronald

Ross in 1898. The complex nature of the Plasmodium life cycle was predicted when Grassi 1900 proposed an exo-erythrocytic stage which was later confirmed by Short, Garnham, Covell and Shute in 1948 who found *Plasmodium vivax* in the human liver [12,13]. They concluded what is known today about proliferation of the parasite.

On a global scale, malaria remains a major public health concern. A third of the world's population is exposed to the risk of malaria. The greatest disease burden falls on Africa, where *Plasmodium falciparum* is estimated to cause at least a million deaths of children each year [17]. It has been estimated that malaria lowers the average "per capita" growth of the Gross Domestic Product (GDP) up to 1.3% per year in some of the most affected African countries [18]. Malaria causes various types of costs including costs of the health system and at the household level. The disease negatively affects the current and future potential for development through eroding human resources and the social capital. Currently around US\$ 100 million per year is spent globally for malaria prevention and control [19]. The WHO calculates that this figure will increase to US\$ 4 billion per year as of 2015 for effective malaria prevention and treatment. Malaria has been a scourge throughout history and has killed more people than all wars and other plagues combined. Figure 1.3 shows the world wide distribution of malaria with higher incidence in the tropics. Malaria claims the lives of more children worldwide than any other infectious disease [19,20]. Since 1900 the area of the world exposed to malaria has been halved but in this time two billion more people are presently exposed. Infection rates in children in endemic areas are of the order of 50%. Chronic infection has been shown to reduce school scores by up to 15%. Reduction in the incidence of malaria coincides with increased economic output [20].

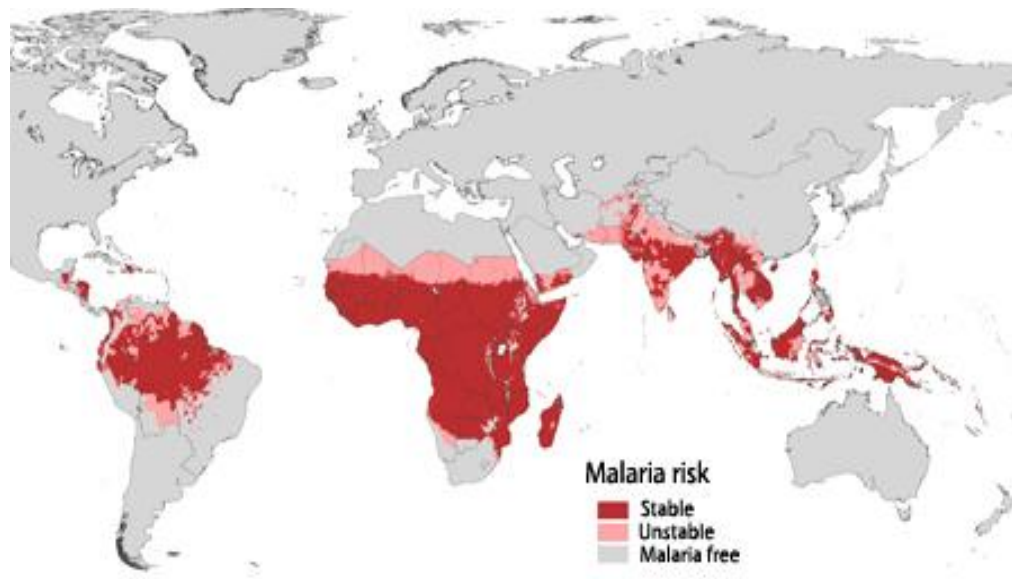


Figure1.3: The world map with defined areas for malaria risk. Stable regions are shown by dark-red shading; Unstable regions (infrequent) are shaded pink. Regions of no risk (are shaded light grey). The borders of the 87 countries defined as *P. falciparum* endemic are shown. Highland areas where risk was excluded due to temperature are also shaded in light grey. Figure taken from Snow (2006)[118].

1.1.3 Drug resistance

Drugs have been employed for centuries for treatment but a vaccine is currently under evaluation in Africa, early results show protection is only for a limited period [94]. A successful vaccine would be a useful tool in the control and final eradication of the disease. For the past half-century, the malaria parasites of humans have been under tremendous selection pressure to evolve mechanisms of resistance to the prevailing anti-malarial drugs. Chloroquine (CQ) and sulphadoxine/pyrimethamine (SP) have become largely ineffective in therapy for the treatment of *Plasmodium falciparum*. The

world health organization (WHO) now recommends artemisinin-based combination therapy (ACT) as first-line treatment for all malaria infections in endemic areas [21].

1.2.1 *Theileria parva*

Theileria parva is a tick-borne parasite that causes a fatal disease in cattle known as East Coast Fever (ECF). This disease, which kills over 1 million cattle each year in sub-Saharan Africa, results in economic losses exceeding \$200 million annually [88]. Unlike other apicomplexans, penetration of host cells by *T. parva* is not orientation-specific. Rhoptries and microspheres discharge after invasion, coincident with dissolution of the surrounding host cell membrane, leaving the parasite free in the host cell cytoplasm. Morbidity and mortality due to ECF are attributed to the ability of the schizont stage to malignantly transform its host cell, the bovine lymphocyte [26,27]. Parasitosis increases exponentially because the schizont divides in synchrony with the host cell and infected cells infiltrate all tissues. Cattle die because this lymphoproliferative disease 4 weeks after infection. The tick infective, red blood cell stage (piroplasm) causes less pathology compared to the schizonts stage in lymphocytes [25, 28].

T. parva is transmitted by the brown-ear tick *Rhipicephalus appendiculatus* [30]. Sporozoites are transferred into the host animal by the bite of an infected tick. Subsequently the parasites invade host lymphocytes (Figure 1.4), where they further develop into intracytoplasmic multinucleated schizonts. The presence of the parasite within the lymphocyte induces the malignant transformation of the host cell. The host cell and the schizont divide synchronously, resulting in the clonal expansion of the infected lymphocytes. Infected animals develop a lymphoma-like disorder that is rapidly fatal. Some parasites form merozoites and are released into the bloodstream by rupture

of the host cell where they invade erythrocytes and develop into intra-erythrocytic forms called piroplasms. Ticks ingest the piroplasms during a blood meal. Following a sexual cycle in the gut, kinetes migrate to the salivary glands of the tick. Sporogony is initiated when the tick attaches to a host animal, resulting in the release of sporozoites into the salivary glands, ready for transmission to the host [25].

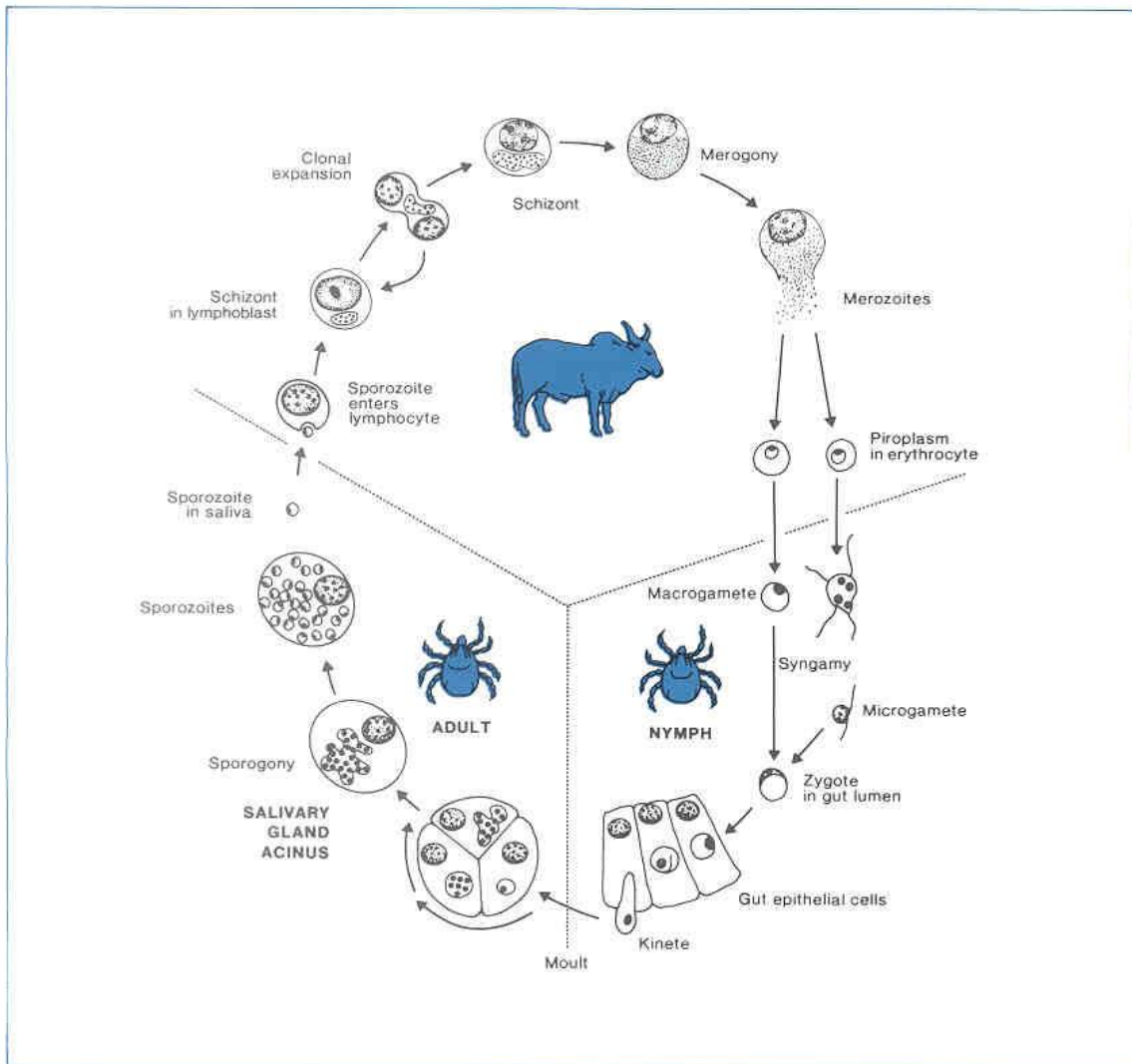


Figure1.4: Life cycle of *Theileria* parasites. Clockwise from upper right: kinete forms of the parasite, which move from the tick gut to the salivary gland; sporozoite forms (Sp), which are able to infect animal hosts, in a tick salivary gland; a sporozoite that in tick feeding has entered an animal host and invaded a

white blood cell (lymphocyte); schizont forms (Sc), which develop in the bovine lymphocyte from the sporozoite forms; a schizont producing merozoite forms, which invade the red blood cells to form a piroplasm. A piroplasm form of the parasite is able to infect ticks. *Piroplasms* will be ingested by a feeding tick, develop into kinete forms in the tick gut, and the life cycle will be repeated *Image and legend from adapted from Norval & Young, The epidemiology of Theileriosis in Africa (Academic Press, 1992[6].*

1.2.2 Theileriosis

Theileriosis results from infection with obligate intracellular protozoa of the genus *Theileria*. The two most important species are *T. parva*, which causes East Coast fever and Corridor disease, and *T. annulata*, which causes Tropical Theileriosis (Mediterranean Theileriosis) [28]. The diseases East coast fever and Tropical Theileriosis are of the great economic impact. *Theileria* parasites are of considerable biological interest, since they are the only eukaryotic pathogens known to transform lymphocytes. Parasite sporozoites invade lymphocytes, escape from the invasion vacuole, interact with the host cell cytoskeleton and alter cellular signaling pathways [27].

A number of other *Theileria* species can infect ruminants. Many of them cause mild or asymptomatic infections. *T. parva* infects cattle, African buffalo, Indian water buffalo, and waterbucks. Symptomatic infections are common only in cattle and Indian water buffalo. African buffalo and waterbucks are reservoirs for this infection [35]. *T. annulata* infects cattle, yak, and buffalo, with milder infections usually seen in buffalo [35].

The distinct clinical symptoms of East Coast Fever are swelling of the draining lymph node followed by generalized lymphadenopathy, fever, anorexia, and a rapid loss of condition. Other symptoms can include lacrimation, nasal discharge, corneal opacity, an

increased respiratory rate, and diarrhea. Death is common in fully susceptible cattle, but rare in Zebu cattle in endemic areas. Terminally, animals often develop pulmonary edema, severe dyspnea, and a frothy nasal discharge [32]. Cattle with East Coast fever may also develop a fatal condition called “turning sickness.” In this form of the disease, infected cells block capillaries in the central nervous system and cause neurologic symptoms. Some animals recover from East Coast fever and become asymptomatic carriers while others may have poor productivity and stunted growth [33,34,35].

Tropical Theileriosis resembles East Coast fever but jaundice and anemia may also occur. Common clinical signs in Tropical Theileriosis include fever, enlarged lymph nodes, pale mucous membranes, a rapid loss of condition, and sometimes hemoglobinuria [32].

1.2.3 Distribution of Theileriosis

East Coast fever is found from southern Sudan to South Africa and eastern Congo to the east African coast as shown in figure 1.5. The tick vectors can be found from sea level to over 8,000 feet, in any area where the annual rainfall exceeds 20 inches [25]. Tropical Theileriosis is seen in North Africa, southern Europe, the southern republics of the former U.S.S.R., the Indian subcontinent, China, and the Middle East [32].

Infection by *Theileria* parasites limits the movement of cattle between countries and can result in production losses and high mortality in susceptible animals.

Distribution of major *Theileria* species of cattle

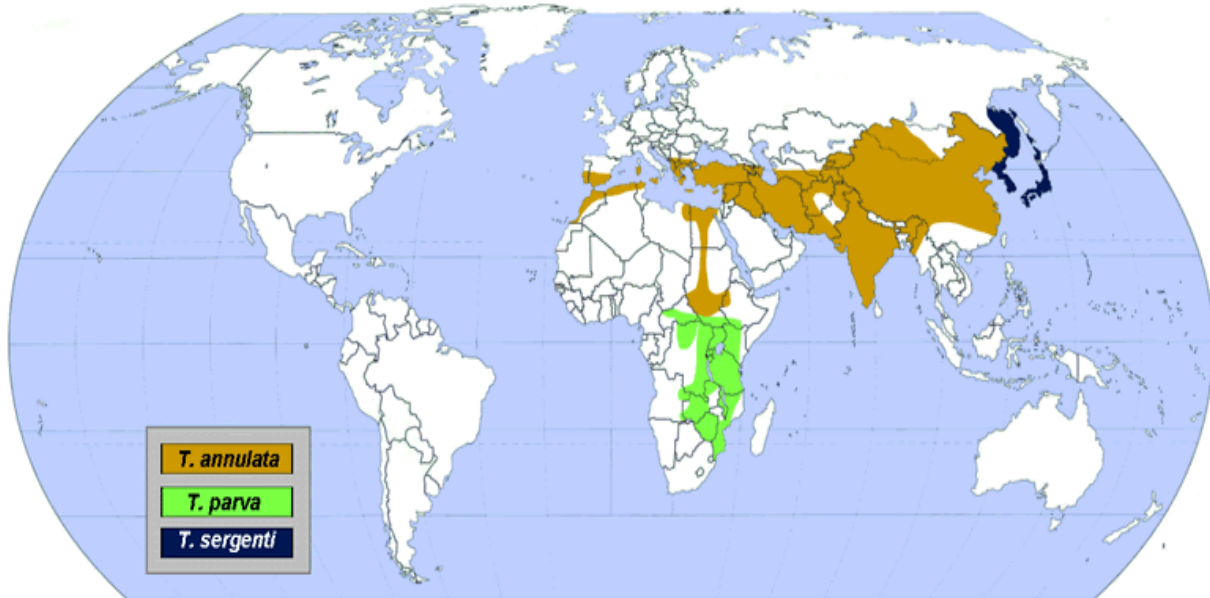


Figure 1.5: The distribution of cattle *Theileria* parasites in the world. Theileriosis disease affects sheep, goats and domestic buffalo, in Africa, the Middle East and Asia. (Image adapted from www.theileria.org/pictures/largemap.gif)[119].

T. parva and *T. annulata* are spread by ticks and are difficult to control where the T vectors (ticks) are readily available [25]. The most important vector for transmitting *T. parva* is *Rhipicephalus appendiculatus*. In southern Africa *R. zembeziensis* is the transmitting tick, while *R. duttoni* responsible for transmission in in Angola. *T. annulata* is transmitted by ticks in the genus *Hyalomma*. Morbidity and mortality vary with the host's susceptibility and the strain of the parasite. The mortality rate from East Coast fever can be up to 100% in naive cattle. The mortality rate for Tropical Theileriosis can also vary from 3% to nearly 90%, depending on the strain of parasite and the susceptibility of the animals [32,35, 25].

Only a few drugs are available for treatment of Theileriosis such as parvaquone and buparvaquone[92,93]. These drugs work in early diagnosed infections but late diagnosis

is mostly fatal. Recovery from one strain of *T. annulata* confers cross-protection against other strains. Cross-protection does not occur with *T. parva* [35]. Cattle that recover from *Theileria* infections usually become carriers [29,31].

1.3 *Plasmodium* and *Theileria* Genomes

The availability of two *Theileria* genomes [43, 44] together with numerous sequences for other apicomplexans such as *Plasmodium* has provided plenty of data for comparative analysis. The *Theileria* genome about 4000 genes in 8.4 Mb. It is reduced in both metabolic complexity and size in comparison to the genomes of other eukaryotes *i.e Plasmodium*. Metabolic deficiencies are noted in the synthesis of purines, polyamines, fatty acids, and porphyrin [45]. The parasites are able to carry out glycolysis and the tricarboxylic acid cycle. The parasite apicoplast and mitochondrial genomes have also been sequenced and moderate levels of synteny are observed between *Theileria* and *Plasmodium* genomes [45, 46].

Like *P. falciparum*, *T. parva* chromosomes contain one extremely A+T-rich region about 3 kbp in length which might be the centromere region. The structure of the subtelomeric regions in *T. parva* is much less complex than that in *P. falciparum*, where arrays of repeats extend up to 30 kbp [44, 45].

The *T. parva* nuclear genome contains about 4035 protein-encoding genes, 20% fewer than *P. falciparum*. The *T. parva* genome exhibits higher gene density and shorter intergenic regions. The functional role of the *T. parva* apicoplast is reduced and there is parasite dependence on the host for many functions [44, 45].

1.4 Hypusination

Hypusination [46] is a posttranslational modification unique in the archaeal and eukaryotic initiation factor 5A (aIF5A and eIF-5A.) eIF-5A precursor is the only cellular protein known to contain a specific lysine residue (Lys50) which is converted into the unique amino acid hypusine, thereby activating eIF-5A. Hypusination occurs shortly after the synthesis of eIF-5A precursor and is the only known specific polyamine-dependent posttranslational modification [46,47,48].

Two enzymes are involved in the formation of hypusine The first step is catalyzed by the enzyme deoxyhypusine synthase [EC 1.14.2249] (DHS), which results in the formation of deoxyhypusine by the transfer of 4-aminobutyl moiety from spermidine to a specific lysine residue in the eIF-5A precursor. The second step involves hydroxylation of the side chain of the deoxyhypusine intermediate by a second enzyme called deoxyhypusine hydroxylase [EC 1.14.99.29]. Hypusination converts eIF-5A to its active form [49, 50, 51].

1.4.1 Biological Function of eIF-5A

Many attempts have been made to elucidate the biological function of eIF-5A but its exact function remains mysterious. Evidence suggests that eIF-5A facilitates protein synthesis by promoting nuclear export of specific mRNA and facilitates the translation of specific subsets of mRNA [52,53,54]. It has also been shown that eIF-5A may be involved in mRNA turnover, acting downstream of decapping. These results have suggested that hypusine-containing eIF-5A facilitates translation of the subset of mRNAs required for cell division and is necessary for cell proliferation. For example, yeast cells in which eIF-5A has been inactivated are incapable of dividing and simply enlarge [55,56].

A series of results suggests that eIF-5A plays a key role in cell growth and differentiation [57]. Isoforms of eIF-5A have been isolated from plant tissue and from numerous organisms. These isoforms might fulfil a role in the translation of a subset of mRNAs required for specific physiological functions like photosynthesis, early development of seedlings and senescence induced programmed cell death [58,59].

Hypusinylated eIF-5A contributes to the life cycle of human immunodeficiency virus by interacting with the retroviral REV protein, thereby participating in the nuclear export of unspliced and incompletely-spliced viral mRNA [59,60].

The X-ray of the crystal structure of the eIF-5A [61,62] reveals that this protein consists of two well-defined domains: the N-terminal domain, which contains the hypusine modification site in an exposed loop, and the C-terminal domain, which is similar to the oligonucleotide-binding domain found in several RNA-binding proteins (see figure 1.6). RNA binding depends on both the presence of the hypusine residue in the eIF-5A protein and conserved core motifs of the target RNA [63].

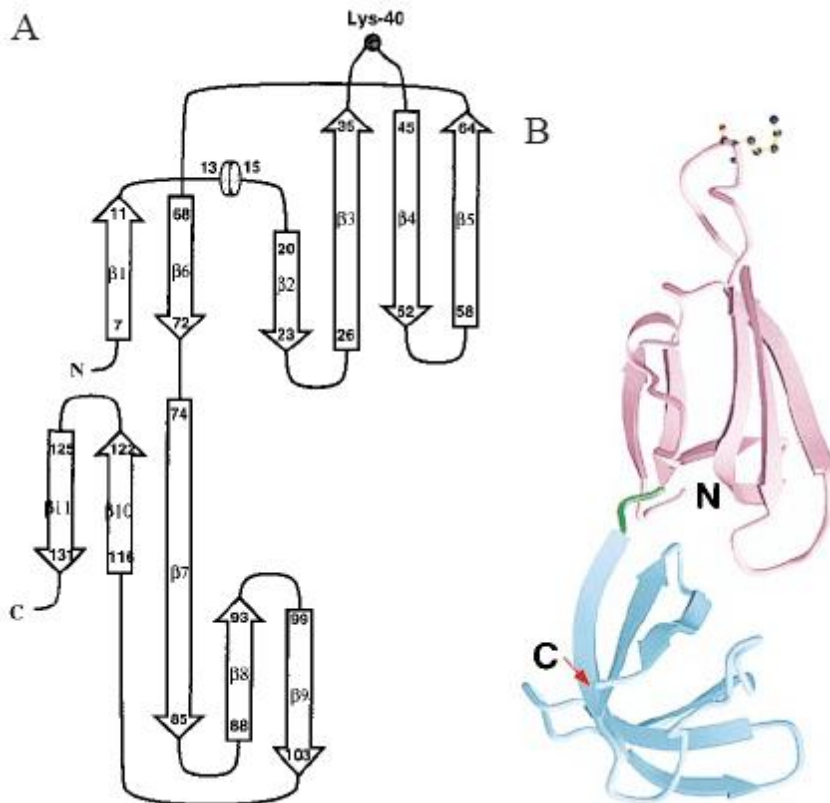


Figure 1.6: Crystal structure of eukaryotic translation initiation factor 5A from *Methanococcus jannaschii* at 1.8 Å resolution (A) Topology diagram of the *M. jannaschii* eIF-5A structure. The arrows represent b-strands and the short cylinder represents a 310 helix. The lysine modification site is represented by a gray circle. (B) Ribbon diagram of *M. jannaschii* eIF-5A structure in C2 crystal form. The arrows represent b-strands. Two domains are colored magenta and blue and connected by a green linker. The side chain of Lys-40 is shown as a ball-and-stick model. Adapted from Kim *et al* (1998) [115].

1.4.2 Deoxyhypusine synthase (DHS)

Deoxyhypusine synthase catalyzes the first step in the posttranslational synthesis of hypusine (N ϵ -(4-amino-2-hydroxybutyl)lysine) in eIF-5A precursor protein. Deoxyhypusine synthase requires NAD⁺ as a cofactor [65] and catalyzes the transfer of the 4-aminobutyl moiety from spermidine to its protein substrate, the eIF-5A precursor. The amino acid sequence of deoxyhypusine synthase is highly conserved among

various eukaryotic species [66]. Both eIF-5A and deoxyhypusine synthase are functionally conserved throughout eukaryotic evolution. Deoxyhypusine synthases from several species share similar physical and catalytic properties. Experimental evidence from gel filtration and ultracentrifugation studies suggests that the native enzyme from *Saccharomyces cerevisiae* exists as a homotetramer [67, 64] with a high affinity between the enzyme and eIF-5A precursor. Deoxyhypusine synthase does not modify free lysine or the lysine residue in a synthetic peptide with the sequence of 16 amino acids surrounding the lysine residue (Lys50) of the relevant precursor. Previous studies involving step wise truncation of the eIF-5A precursor protein from the N- or C terminus, or both, provided evidence that a large portion of the eIF5A precursor molecule (a minimum of 50 amino acids) is required for modification by the enzyme [66]. Recently the crystal structure of human deoxyhypusine synthase containing the competitive inhibitor N1-guanyl-1,7-diaminoheptae (GC7) which binds to the active site of enzyme[89]. was published. The crystal structure identified lys³²⁹ as the residue to which the butylamine moiety of spermidine is transferred. At the active site within a tunnel at the dimer interface a number of charged amino acids can be found (figure 1.7). In particular Asp³¹⁶ and Glu²³² have close contacts with the GC₇ guanidine group.

They presumably are in contact with the one of the to the primary amino acids groups of spermidine. Near the entrance of the tunnel is Asp²⁴³, which forms a salt bridge with the GC7 terminal amino group. A second hydrogen bond is formed to this amino group by the side chain of Asn²⁹² (figure 1.7).

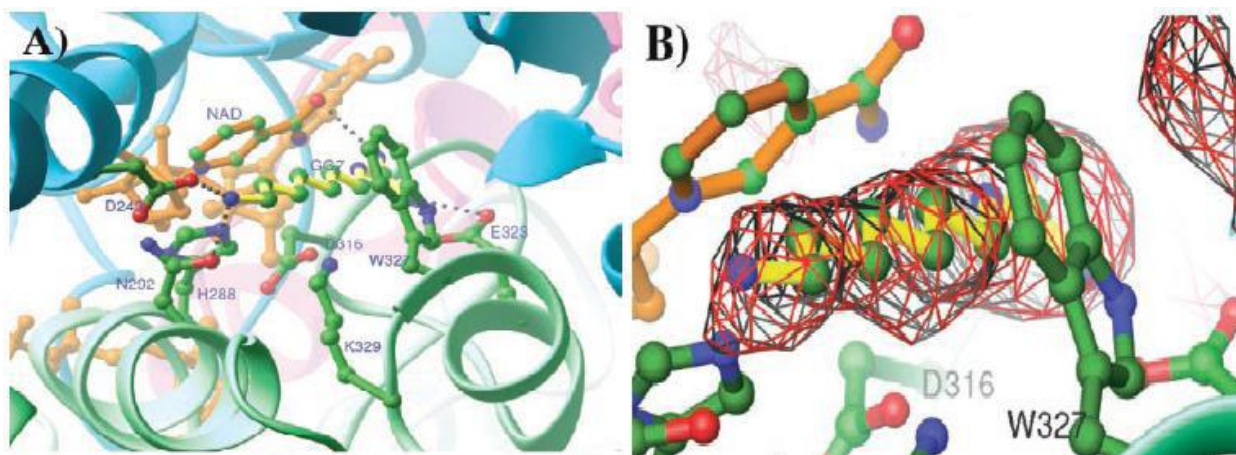


Figure 1.7: The crystal structure of DHS. The inhibitor GC₇ (yellow bonds) is bound to the DHS active site. (A) The charge interactions between GC₇ and DHS are denoted by black spheres. Figure (B) crystal structure of before inclusion of GC₇ and legend from Umland *et al* 2004 [88].

1.4.2.2 Homospermidine synthase (HSS)

Homospermidine synthase catalyzes an analogous reaction to DHS but uses putrescine instead of eIF-5A(lys) as substrate yielding the symmetric polyamine homospermidine as the product. Homospermidine is an essential precursor in the biosynthesis of pyrrolizidine alkaloids, an important class of plant defense compounds against herbivores [59]. Sequence comparisons of the two enzymes indicate an evolutionary origin of homospermidine synthase from deoxyhypusine synthase. Protein-protein binding and kinetic substrate competition studies confirmed that homospermidine synthase, in comparison to deoxyhypusine synthase, lost the ability to bind the eIF-5A(lys) [59]. Instead homospermidine synthase transfers an aminobutyl moiety to putrescine. Both enzymes share comparable specific enzymatic activities and exhibit similar Michaelis kinetics. In conclusion, homospermidine synthase behaves like a deoxyhypusine synthase that lost its major function (aminobutylation of eIF-5A

precursor protein) but retained unaltered first side activity (aminobutylation of putrescine). It is considered as a gene evolved from DHS by gene duplication that obtained a new function in the production of secondary metabolites [71].

1.4.3. Deoxyhypusine hydroxylase (DOHH)

Deoxyhypusine hydroxylase (DOHH) is the second enzyme in the posttranslational modification of eIF-5A. DOHH completes the hypusine pathway by hydroxylation of deoxyhypusine.

The gene encoding deoxyhypusine hydroxylase has been recently cloned and expressed from human, yeast and bovine [70,72,74]: DOHH is a HEAT-repeat protein with a symmetrical super helical structure consisting of 8 (HEAT motifs) organized in a symmetrical diad as shown in the figure 1.8. The metalloenzyme [36 37,38] contains tightly bound iron(Fe^{2+}) at the active sites. Four strictly conserved His-Glu pairs were identified as iron coordination sites as shown in Figure1.8.

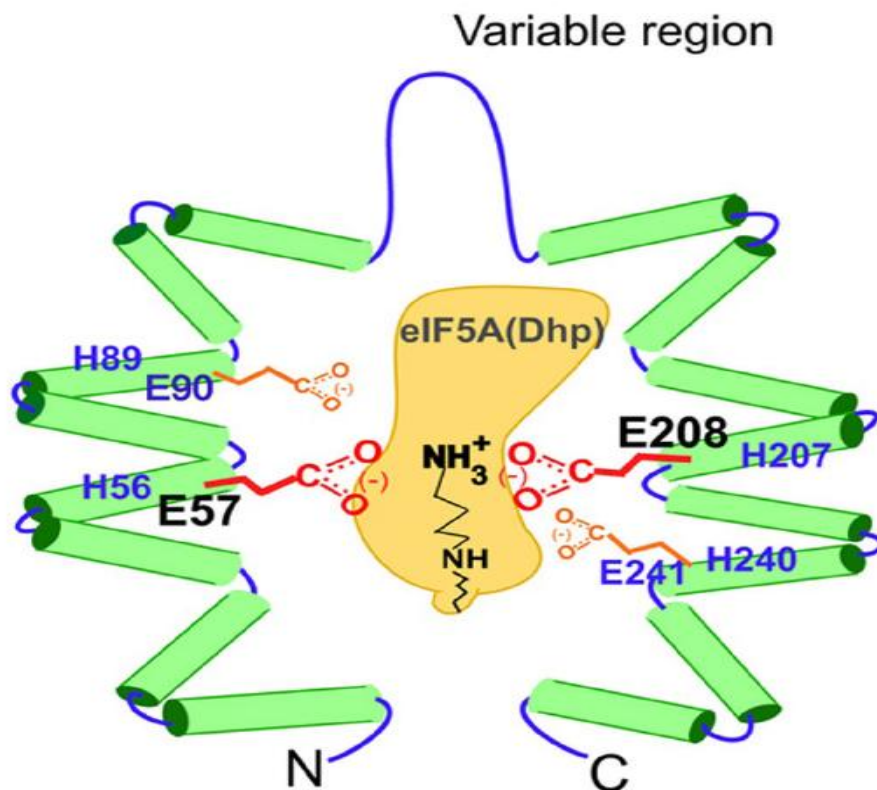


Figure 1.8: The proposed model for the binding of eIF-5A (Dhp) to DOHH. The active site His-Glu residues is important for binding of iron. Histidine residues which interact with deoxyhypusined eIF-5A are numbered. Side chain carboxyl groups of Glu57 and Glu208 are proposed to interact with the amino group(s) of the deoxyhypusine side chain of eIF-5A (Dhp). The γ - carboxyl groups of Glu90 and Glu241 may also contribute to the substrate binding by interaction with the deoxyhypusine residue or other basic residues surrounding it. The six residues, His56, His89, Glu90, His207, His240 and Glu241 implicated in iron binding are in blue. Iron atoms are not included in the diagram, since substrate protein binding does not depend on iron binding. This scheme represents a simplified hypothetical diagram of the DOHH/eIF-5A (Dhp) complex, indicating the key residues involved in the binding without specific indication of orientation of the two proteins. Figure and legend taken from *et al.*2007[67].

In contrast to DHS, the structural and catalytic properties of DOHH are not well understood. This enzyme appears to share some properties with the non-heme Fe(II)- and 2-oxoacid-dependent dioxygenases, such as collagen prolyl 4-hydroxylase and

lysyl hydroxylases. In common with these enzymes, DOHH of mammalian cells or tissues is inhibited by various metal chelators, including 2,2'-dipyridyl, mimosine, deferoxamine, 1,10-phenanthroline, deferiprone, and ciclopiroxolamine [68,109]. DOHH belongs to a family of HEAT-repeat like proteins, which includes human Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A, and the Target of rapamycin. These HEAT-repeat like proteins also occur in importin proteins, -catenin, and clathrin-associated adaptor proteins. In a variety of bacterial and eukaryotic proteins, termed HEAT-repeat-containing proteins, the HEAT motif, an α -helical hairpin (a pair of α - helices) of 50 aa, is tandemly repeated to form super helical structures [36]. Many of these HEAT-repeat like proteins mediate protein–protein interactions and are involved in nucleocytoplasmic transport, vacuolar transport, and cytoskeletal organization [36, 37].

1.4.4 Targeting the polyamine pathway for antiparasitic chemotherapy

It has been shown for many organisms that growth and differentiation processes depend on adequate intracellular concentrations of the polyamines putrescine, spermidine, and spermine [38, 40].

Spermidine is an important precursor for the biosynthesis of hypusine and homospermidine in eukaryotes. Interference with polyamine biosynthesis by inhibition of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) has been discussed as a potential chemotherapy of cancer and parasitic infection [39,41]. Blocking spermidine and spermine synthesis in *P. falciparum*-infected erythrocytes with irreversible inhibitors of AdoMetDC prevents the growth of the parasite *in vitro* [42, 90].

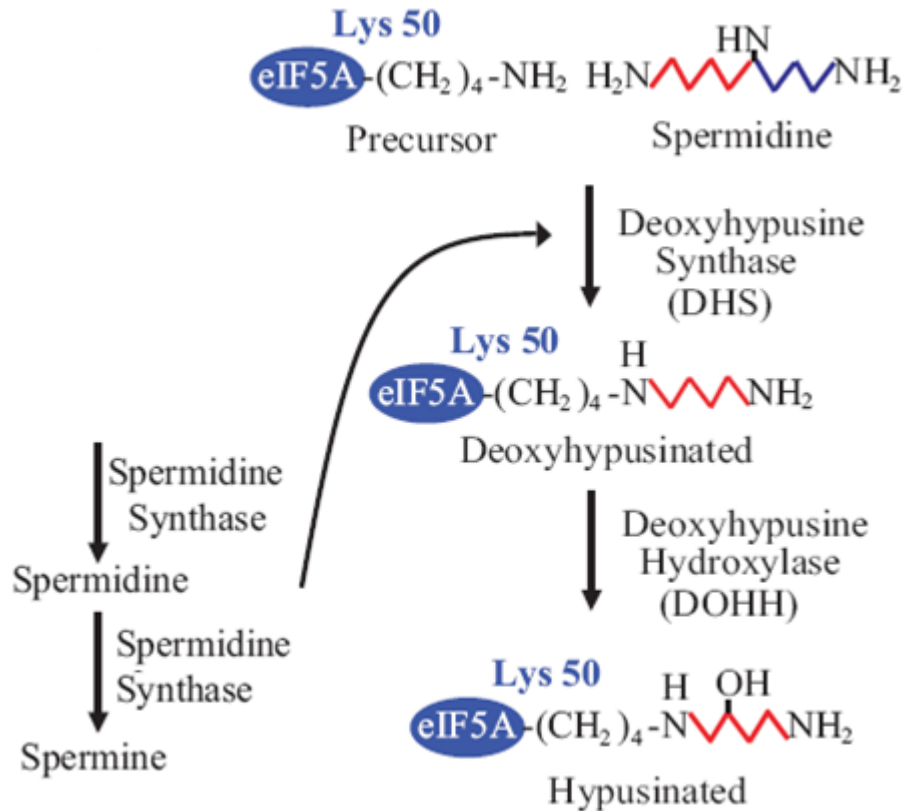


Figure 1.9: Pathway of polyamine biosynthesis in *P. falciparum* and hypusine modification of eIF-5A. Hypusine modification involves two sequential steps. DHS transfers the 4-aminobutyl moiety from spermidine to the ε-amino group of one specific lysine residue (Lys-50 in the human protein) in eIF-5A generating eIF-5A intermediate, which is then hydroxylated by deoxyhypusine hydroxylase to form the mature hypusinated eIF-5A. Figure adapted from Huang 2006[90].

Moreover, the addition of DFMO to human *P. falciparum*-infected red blood cells in continuous culture decreased parasite growth and intracellular putrescine concentrations [41.] DFMO also blocked exoerythrocytic schizogony of *Plasmodium berghei* in mice and in cultured human hepatoma cells and these effects were also reversed by administration of exogenous spermidine [40]. 1,7-Diaminoheptane, an inhibitor of deoxyhypusine synthase, which could decrease the intracellular spermidine content, inhibits the proliferation of malaria parasites of *P. falciparum* NF54 strain in

vitro [90]. These results confirm previous observations that the polyamines, especially spermidine, are crucial for differentiation and proliferation of malarial parasites. In consequence, interference with polyamine biosynthesis may be an attractive for the chemotherapy of malaria [107, 108]. The numerous steps of this pathway are shown by Figure 1.9 and could serve as drug targets for chemotherapy.

1.4.5 The Potential of eIF-5A for Vaccine Development and Antichemotherapy

There is an urgent need for novel drugs and vaccines which are safe, effective and affordable for therapy of malaria and theileriosis.

The eIF-5A modification appears to have potential in vaccine and antichemotherapy development. Two different strategies exist for inhibition of eIF-5A modification: first the inactivation of DHS enzyme and second the inhibition of DOHH enzyme [105]. Recently, it has been shown that inhibition of hypusination suppressed the replication cycle of HIV. Moreover DHS is now a target in cancer, HIV (AIDS) and malaria therapy [40,54,60,77,79].

Homologs of eIF-5A and DHS from different *Plasmodium* species have been cloned but hitherto the cloning of *dhs* from *P. vivax* had not been done.

The eIF-5A protein from *T. parva* has shown some potential as a vaccine candidate. Animals which have acquired immunity develop cytotoxic T lymphocytes that are specifically primed against the eIF-5A protein. EIF-5A is being used as an antigen in a *T. parva* vaccine which is currently under evaluation [78].

Such a vaccine if successful would be important for control of the Theileriosis as it would be an improvement over the current live vaccines that sometimes results in protected animals becoming carriers of the parasite. The success of such a vaccine could be important in the development of a similar one against other apicomplexa diseases.

1.5. Aims of this Study

The increasing drug resistance of *P. falciparum* against conventional drugs enforces new strategies to combat malaria. Only a few drugs are effective against *Theileria* parasites. There is currently no drug available for the late stage of the disease. The novel drugs must meet the requirements of rapid efficacy, minimal toxicity and be of low cost. We have focused in particular on the DHS protein from the apicomplexans for target evaluation.

The Thesis has two objectives:

1. (a) The molecular cloning and expression of the *dhs* gene from *P. vivax* in order to compare it with the ortholog from *P. falciparum* with respect to its pathogenicity and phylogeny.
(b) The molecular cloning and characterization of *T. parva dhs gene*, since the eIF-5A protein from the parasite is used for vaccination.
2. The second objective was to investigate piperidones as potential inhibitors of DOHH for their antiplasmodial activity *in vitro*.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Agarose:	Top Vision™ Le Gq Agarose
Ammoniumchloride:	Roth, Germany
Ampicilin:	Sigma-Aldrich, Germany
Antibiotic-Antimycotic Solution :	Sigma-Aldrich, Germany
1-Brom-3-Chloro-Propan:	Sigma-Aldrich, Germany
Bicarbonate (NaHCO ₃):	Sigma-Aldrich, Germany
Bromphenolblue:	Sigma-Aldrich, Germany
BSA:	Roth, Karlsruhe, Germany
Chloroquine Diphosphatesate:	Sigma-Aldrich, Germany
DEPC-Water:	Ambon, Uk
Dimethylsulfoxide (DMSO):	Sigma-Aldrich, Germany
Ethanol:	Merck, Germany
Ethidiumbromide:	Biomol, Germany
FCS:	PAA, Austria
Ficoll®:	Sigma-Aldrich, Germany
Free RNA&DNA Water:	Millipore
Gentamycin:	Cambrex, Usa
Giemsa Azur-Eosin:	Merck, Germany
Glycerol:	Sigma-Aldrich, Germany
Haematocrit Capillary Tube:	Brand, Wertheim, Germany
Hepes Buffer:	MP Biomedicals, UK
Hydrochloric Acid:	Sigma, Germany
Hydrogen Peroxide 30%:	Sigma-Aldrich, Germany

Hypoxanthine:	Sigma-Aldrich, Germany
Immersion Oil:	Fluka, Germany
Incubator:	Binder, Germany
IPTG:	Sigma-Aldrich, Germany
Isopropanol:	Merck, Germany
LB Agar:	Sigma-Aldrich, Germany
MBI Markers	Fermentas, Germany
Methanol:	Merck, Germany
Methyleneblue-Solution:	Merck, Germany
PBS Buffer Tablets	Merck, Germany
Percoll®:	Sigma-Aldrich, Germany
Potassium Dihydrogen Phosphate:	Merck, Germany
Potassium Hydrogen Sulfate:	Merck, Germany
RPMI-Medium:	Invitrogen, Germany
Sodium Chloride:	Roth, Germany
Sodium Dihydrogen Phosphate:	Merck, Germany
Sodium Hydroxide:	Merck, Germany
Sorbitol:	Sigma-Aldrich, Germany
Tris-Hydrochloride:	Roth, Germany
Trizol®:	Invitrogen, UK
Tween®20:	Sigma-Aldrich, Germany
X-Gal:	Roth, Germany
Xylencyanol:	Merck, Germany

2.2.2 Laboratory supplies

CELLSTAR®, 96-well flat culture plates:

Greiner bio-one, Germany

Disposable pipettes:

Eppendorf, Germany

Eppendorf-Reaction-test tube:

Eppendorf, Germany

Filters (0.2 micron):

Satorious, Germany

Glassware:

Schott AG, Mainz, Germany

Nitrocellulose membrane:

Satorious, Germany

PCR tubes:

Eppendorf, Germany

Pipette tips:

Eppendorf, Germany

Syringes:

B/Braun, Germany

Tissue Culture Dishes 60/15mm:

Greiner Bio-One, Germany

2.2.3 Kits

Ni-NTA Spin Kit:

Qiagen, Germany

Seqlab Plasmid Miniprep Kit:

Sequence Lab, Germany

Qiaex II Gel Extraction Kit:

Qiagen, Germany

Qiaquick Gel Extraction Kit

Qiagen, Germany

Qiaquick PCR Purification Kit:

Qiagen, Germany

Original TA Cloning® Kit:

Invitrogen, Germany

pSTBlue-1 AccepTor™ Vector Kit:

Novagen, Germany

pETBlue-1 AccepTor™ Vector Kit:

Novagen, Germany

pGEM easy Vector

Kit Promega, Germany

2.1.4 Deoxyhypusine Hydroxylase (dohh) Inhibitors

mimosine: Sigma-Aldrich,
ciclopiroxolamine: Spectrum, Chemicals, USA.
DOHH Inhibitors: Professor Dr. Holzgrabe, Institute

2.1.5 Primers

2.1.5.1 *P. vivax dhs*

Forward primer1: 5>ATG ACG AAC CAA GGG GCT TTT3'

Reverse 2 primer2: 5>'TCA CCT GAG CTG CGC TTC ACC3'

Expression forward primer 3: 5>-AAC CCC ATA TGA CGA ACC AAG GGGCTT TT-3'

Expression reverse primer 4: 5>-AAAA GGA TCC TCA CCT GAG CTG CGC TTCp3'

T. parva dhs

Forward primer 5: 5>ATG ACA GAG AAT AAC CTA AAC

Reverse primer 6: 5>TCA TTC GAA TTG ACG ATT AAC,

Expression forward Primer7: 5>AAAAAA CAT ATG ACA GAG AAT AAC CTA3'

Expression reverse Primer 8: 5 >AAAAAGGATCC TCA TTCGAA TTGACG ATT'

2.1.6 Media and Buffers

2.1.6.1 RPMI-1640 media for *P. falciparum* strains in-vitro cultivation:

Two liters of distilled water containing 20.8g RPMI-1640 media, 11.9 g HEPES buffer and 100 mg Hypoxanthine, minimum 99%, were mixed by a magnetic stirrer for 30 min, and the pH was adjusted to 7 with 1M Sodium Hydroxide solution. The prepared media was sterilized by micro filters 0.20 µM GF (Sartorius), and then stored at 4°C.

2.1.6.2 Bicarbonate Buffer (NaHCO₃):

100 ml of distilled water containing 7.5 mg NaHCO₃, were mixed by a magnetic stirrer for 1 hour and then sterilized by micro-filter 0.20 µM. Aliquots of 50ml were stored at 4°C in falcon test tubes.

2.1.6.3 Solution for cryopreservation of Plasmodium:

28% glycerol, 3% sorbitol and 0.65% NaCl

72ml of 4.2% sorbitol were mixed in 0.9% NaCl with 28 ml glycerol and sterilized by filtration and then stored at 4° C.

2.1.6.4 Thawing solution :

A sterilized solution of 3.5% NaCl

2.1.6.5 TBE-buffer 10x: each 1 Litre (pH 8) containing

0.89 M TRIS

0.89 M Boric acid

50 mM EDTA

2.1.6.5 Giemsa Stain:

1 ml Giemsa Azure-Eosin-Methylene blue solution was dissolved in 19 ml buffer solution pH6.8.

2.1.6.6 Luria-Bertani medium:

LB medium: 10 g Peptone, 5 g NaCl, and 5 g yeast extract were dissolved in 1000ml H₂O and sterilized.

2.1.6.7 Luria-Bertani agar plates

To 1000 ml of the above medium (Luria-Bertani medium) 15 g agar was added.

2.1.6.8 SOB

20 g tryptone, 5 g yeast extract, 0.5 g NaCl, and 10 ml 250 mM KCl were dissolved in 1000 ml H₂O.

2.1.6.9 SOC medium

The SOC medium consisted of 20 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 0.58 g/l NaCl, and 0.19 g/l KCl. The pH was adjusted to 7.0 with NaOH, and sterilized by autoclaving. Then 1 ml of 2 M MgCl₂ was added.

stock and 1 ml of 2 M glucose was added to 98 ml of autoclaved medium.

2.1.7 *Plasmodium falciparum* strains used for the in vitro assays

Plasmodium falciparum/NF-54 (Pf/NF-54), Chloroquine sensitive strain (CQS), and *Plasmodium falciparum*/R-stamm), Chloroquine resistance strain (CQR), were maintained as cryo-preserved stocks in infected red blood cells.

2.1.8 Instruments

Centrifuge (Eppendorf 5415 R):	Eppendorf AG, Hamburg, Germany
Centrifuge (Multifuge 4KR): Germany	Heraeus Holding GmbH, Hanau, Germany
Ceramic beads	Percelllys, UK
Cuvettes	Eppendorf, Germany
Electrophoreses system	BioRad, Germany
Freezer (-20°C):	Bosch GmbH:Stuttgart, Germany
Freezer (-80°C): Germny	Heraeus Holding GmbH, Hanau, Germany
Fridge:	Bosch GmbH; Stuttgart, Germany
Haematocrit Centrifuge	Becton-Dickinson, Germany
Heating block	Eppendorf , Germany
Ice machine (Scotsman AF 80):	Gastro Handel GmbH, Wien, Austria
Incubator Hereaeus B5050:	Hereaeus, Germany
Microscope :	Zeiss Axioscope 135, Germany
PH meter:	Mettler Toldo GmbH, Giessen, Germany
Photometer:	Eppendorf, Germany
Pipetboy (pipetus®-akku) Hirschmann:	Laborgeräte, Eberstadt, Germany
Rotorgene PCR machine:	Corbett research, Sydney, Australia

Thermo magnetic stirrer:
Germany

IKA® GmbH & Co.KG, Staufen,

Thermomixer comfort,

ROTH, Germany

Vortex mixer (Minishaker):
Germany

IKA® GmbH & Co.KG, Staufen,

Water bath:

VWR Lab Shop, Batavia, USA

Scales:

Sartorius AG, Goettingen, Germany

2.2 Methods

2.2.1.0 In vitro cultures

2.2.1.1 Preparation of blood and serum for *P. falciparum* in vitro culture

The *in vitro* culture was performed in an Isoflow safety hood to ensure sterile conditions.

Purified erythrocytes (of human blood type A⁺) and human A⁺ serum were kindly provided by the blood bank Medical Care Unit, Bonn University. The serum was inactivated in a water bath at 56°C for 60 min. Serum fractions were pooled into a 500ml glass bottle and aliquots of 50 ml were stored at -20°C in 50ml falcon test tubes.

Blood samples from the same source as above were collected in tubes containing 10 ml heparin. These samples were centrifuged for 5 min at 3000 RPM. The supernatant which contained the plasma was discarded and the cells were washed, 2-3 times, with RPMI-1640 by centrifugation for 5 min at 3000 RPM.

2-3 ml of serum was added to the obtained red blood cells (RBCs) in each sample and stored at 8°C until use.

2.2.1.2 *P. falciparum* in vitro cultures

P. falciparum strains, were retrieved from the liquid nitrogen storage tank and thawed by mixing with the thawing solution at a 1:1 ratio. The thawed parasites were centrifuged at 3000 rpm for 5 min, the supernatant was discarded. The sediment containing the infected cells was mixed with 0.8 ml of RBCs in 5 cm Petri dishes. The cultures were maintained according to a protocol from Traeger and Jensen [95]. The incubation was done in a gaseous phase of 90 % N₂, 5% CO₂ and 5% O₂. Parasites were cultured in human erythrocytes (blood group A⁺) suspended in RPM1640 medium and supplemented with 25 mM HEPES, 20 mM sodium hydrogen carbonate, and 10 % heat

inactivated human A⁺ serum at 10 % (v/v) hematocrit. The cultures were incubated at 37°C in a closed jar under continuous gaseous phase as described above.

2.2.1.3 Inhibition of Growth in cultured *P. falciparum*

Cultures of *P. falciparum* were maintained in small Petri dishes (5 cm) according to a protocol briefly described above. Cultures were adjusted to a parasitemia of 1.5% with red blood cells suspended in RPMI-medium. Aliquots were dispensed into 12-well micro-culture trays and incubated at 37°C in a jar with the favourable gas atmosphere. Growth medium was changed every day for four days. The inhibitors under evaluation were added to the media as indicated for each test.

Inhibitor	Concentrations in µM	DMSO %
DOHH1	10,20,30	0.0%
DOHH 2	1,3,9	0.25%
DOHH 3	10,20,20	0.40%
DOHH 4	1,3,8	0.25%
DOHH 5	9	0.25%
DOHH 6	1,3,9	0.30%
DOHH 7	10,20,30	0.25%

Table 2.1: The table presents the various inhibitors and their concentrations as used in the inhibition study. The amount of DMSO used for dissolving the inhibitors in RPMI 1640 media is given as a percentage.

2.2.1.4 Monitoring growth inhibition by microscopy

A thin blood smear was prepared every 24 hours and parasites were observed microscopically in Giemsa-stained smears. Percentage parasitemia was calculated from the ratio of the number of infected RBCs to the total number of red blood cells counted. Red blood cells were counted in at least 10 independent fields each with approximately

200 cells. This was done using a light microscope employing a 100X–fold oil immersion objective to score visually. Parasitemia levels and stage distribution were estimated as triplicates each with 2000 erythrocytes counted.

2.2 1.5 Calculation of the IC₅₀ value

IC₅₀ values were determined by linear regression analysis of growth curves plotting the percentage of inhibition against the concentration of inhibitor in μM . The IC₅₀ is defined as the concentration which is necessary to obtain 50% inhibition of the parasite [96,97].

2.2.2.0 Molecular Techniques

Standard molecular biology techniques were applied according to Sambrook[98] and Asubel[99] .

2.2.2.1 Polymerase Chain Reaction (PCR)

DNA amplification for screening was carried out in 20 µl reactions. 50 ng of each primer and 0,5 U Taq DNA polymerase (5 units/µl) (Promega), 250 mM of each of the four dNTP's, 2.75 mM MgCl₂, and 2 µL of template were used.

Thermocycling was carried out on a Rotorgene PCR machine. After an initial denaturation step at 96 °C for 3 minutes, the DNA was amplified in three step cycles:

1. denaturation at 95 °C for 30 s
2. annealing at 60°C for 45 s
3. extension at 72°C for 60 s

After 30 cycles, the DNA was given a final extension step at 72°C for 5 minutes.

2.2.2.2 Agarose gel electrophoresis

Conventional agarose gel electrophoresis was used to analyze DNA fragments. The electrophoretic mobility of DNA fragments mainly depends on the fragment size. Agarose gels can resolve DNA from 50 bp to 20 kbp in length. The gels prepared were 1% agarose in TAE and contained 1 µg/ml ethidium bromide for visualization of the DNA bands. The electrophoresis was run in 1x TAE buffer.

2.2.2.3. Purification of PCR Products

A 100 µl PCR volume was set to produce a large amount of the DNA fragment for purification. After separation on an agarose gel, the gel extraction kit from Qiagen was applied according to the manufacturer's instructions for PCR product purification from

agarose gels. The purified PCR product was sent for sequencing to MWG Biotech, Munich.

2.2.3.4 Transformation

Chemocompetent bacteria of *E. coli* strain JM109 were thawed on ice for 5-10 min. For a single transformation 1 aliquot (50 µl) of the competent cells was mixed with 1-2 µl of ligation product in an Eppendorf tube. This equals approximately 1-10 ng of circular plasmid DNA. Immediately after heat-shock (42°C for 30s cells) the tube was placed on ice for 2 min. 950 µl of SOC-medium was added and phenotypic expression continued under shaking at 37°C for 1 h. The transformation (100 µl) was plated on an LB agar plate containing the appropriate antibiotic and selection supplements for the plasmid of interest and subsequent incubation at 37°C overnight.

2.2.3.5 Plasmid Preparation

3 ml LB media supplemented with appropriate selection antibiotics were inoculated with a single colony and grown overnight at 37°C on a shaker. Cells were centrifuged at 3,000 rpm for 10 min. Plasmid DNA was isolated using the SeqLab Kit (Sequence Lab Germany). The DNA pellet was washed two times with 70% ethanol and dissolved in 30 µl 1x TE buffer.

2.2.3.6 Plasmid DNA Preparation for Sequencing and Restriction Digestion.

Preparation of plasmid DNA for sequencing was performed according to the Qiagen Plasmid Midi Kit protocol (see very low-copy plasmid/cosmid purification protocol in Qiagen plasmid purification handbook). About 400 ng of plasmid DNA purified with the Qiagen kit was sequenced by MWG Biotech, Munich.

2.2.4.1 *T. parva* cultures

A *Theileria parva* strain (Muguga stock) which was originally isolated from a cow in Kenya was used in this study [101]. The strains had been maintained in bovine lymphoblasts culture and cryopreserved to a stabilate [100,102,103].

The strain was retrieved from the liquid nitrogen tank and thawed at room temperature. The infected cells i.e. bovine lymphoblasts were cultured in RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, amycostatin (75 units/ml) in 25-ml plastic screw-cap tissue-culture flasks. Medium was changed every 3–4 days. The presence of bright refractile cells which are present in the medium (on examination using a phase-contrast or inverted microscope) indicate an infection. Passage was achieved by decanting the medium, adding 0.025% EDTA for 15 minutes to monolayer cultures, dispersing the cells, then counting and dispensing according to flask size. Approximately 10^6 cells are transferred into a 25 cm² flask. Serum was tested for toxicity through three passages in an established cell line before use. All cultures were incubated at 38.5°C in a humidified atmosphere of 5% CO₂ in air [29 103 102].

2.2.4.2 Purification of schizonts from infected lymphoblasts

The cultured lymphoblasts were harvested by centrifugation at 3000 rpm for 10 min. Sufficient growth was obtained within 10 days. The pellet was suspended in PBS buffer containing 20 mM nocodazole and incubated for 30 min. The preparation was treated further by addition of PBS containing 10 mg aerolysin and incubated for 4 °C for 10 min. Complete lysis was obtained after further 10 minutes at 37 °C. The released schizonts

were centrifuged onto a Ficoll cushion (to avoid pelleting). The interface between the lysate and the Ficoll was collected and contained the schizonts.

2.2.4.3 Preparation of Genomic DNA

Genomic DNA was isolated using a DNA purification kit from Promega (USA). The extraction was done using the procedure as described by the manufacturer. After digestion with Proteinase K (20 µg/ml) samples were applied to the columns for absorption and washing of DNA. Finally, DNA was eluted in 200 µl of buffer available from the kit and evaluated by agarose electrophoresis and spectrophotometry.

2.2.4.4 Preparation of Electro-competent bacteria

JM109 *E. coli* cells were grown over night in 20 ml of LB-medium at 37°C. Further 200 ml of LB-medium was added and the bacteria were grown to an $OD_{600nm} = 0.5-0.6$. Cells were cooled on ice for 20 min. then centrifuged at 4000 x g for 15 min. The pellet was washed two times with 200 ml of ice-cold distilled water. After the final centrifugation the pellet was resuspended in 20 ml 10% glycerol. Cells were aliquoted on dry ice into 50 µl aliquots and stored at -80°C until use.

3.0 RESULTS.

3.1.1. Chloroquine (CQ) inhibition of *in vitro* cultures of *P. falciparum*

Inhibitor experiments were performed with chloroquine to establish a control for the compounds to be tested. The chloroquine susceptible (QS) *P. falciparum* strain NF54 and the chloroquine resistant R strain (QRS) were used to test different concentrations of chloroquine. The IC_{50} values were 0.014 μM for Pf/NF-54 strain and 0.4 μM for the R-strain respectively. The obtained data are comparable to previously published results by Smeijsters and colleagues [113].

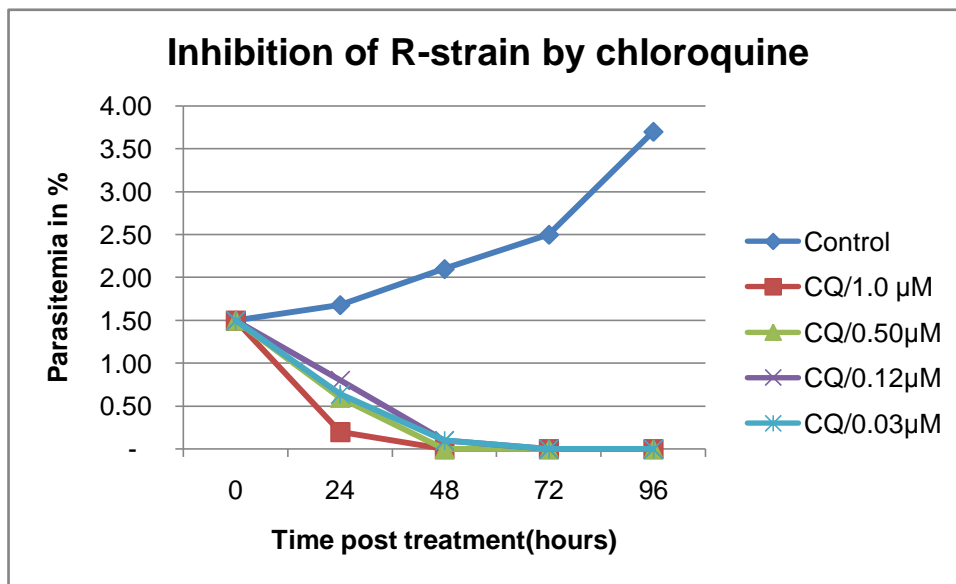


Figure 3.1: Parasitemia of R-strain after chloroquine inhibition.

3.1 2. Control experiment with Dimethyl Sulfoxide (DMSO) on *in vitro* cultures

Some of the inhibitors which were used in this study were dissolved in DMSO and it was therefore important to determine the effects of DMSO on the growth of the parasite. To evaluate these effects, cultures containing various concentrations ranging from 0.25% to 2% DMSO were applied. The results show that DMSO at low concentrations does not have any effect on the growth of *P. falciparum* parasites *in vitro* cultures.

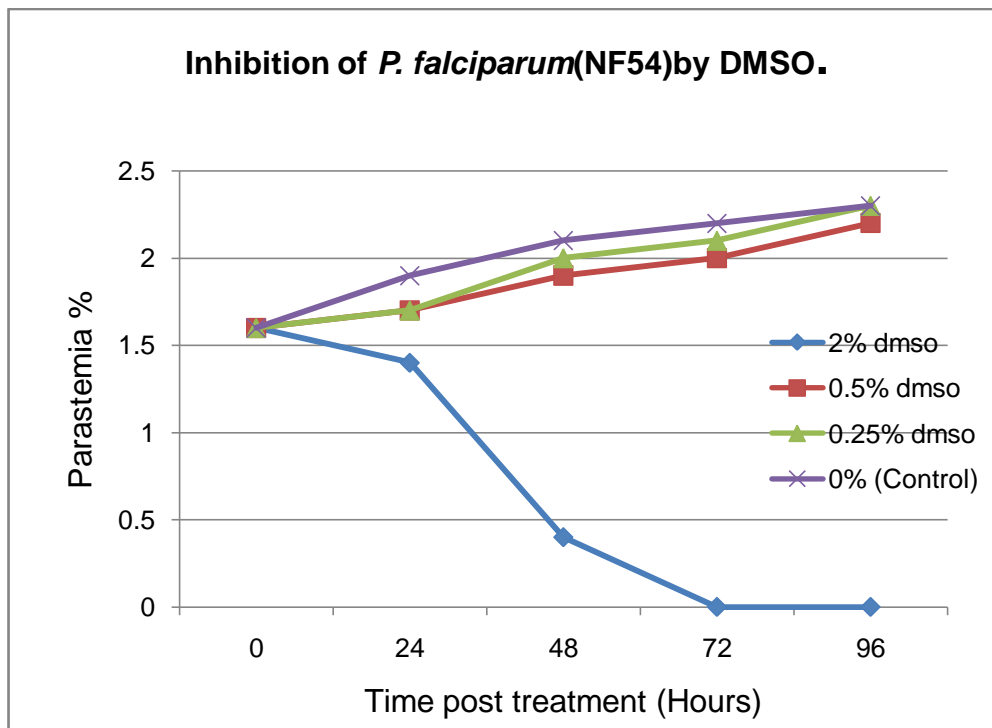


Figure 3 .2: parasitemia of *P. falciparum* PF/NF 54 strain after exposure to DMSO

3.1 3. Mimosine inhibition of *in vitro* cultures of *P. falciparum*

L-Mimosine, β -[N-(3-Hydroxy-4-pyridone)]-aminopropionic acid is a naturally occurring rare amino acid derivative, isolated from *Leucaena leucocephala* seeds. It can reversibly block mammalian cells at late G1 phase and leads to a notable reduction in the steady-state level of mature eIF-5A by means of DOHH inhibition [104]. These results suggested testing the compound for a potential inhibitory effect on *Plasmodium falciparum* *in vitro* cultures. The substance was tested at concentrations of 3.12 μ M, 6.25 μ M and 25 μ M.

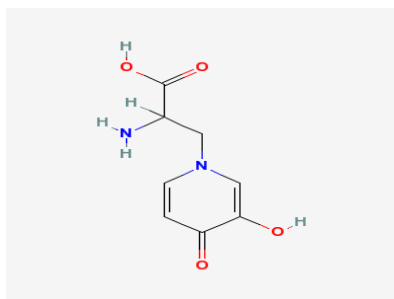


Figure 3.3: The structure of mimosine a rare plant derived amino acid.

L-Mimosine is an iron chelator. Iron chelators have been reported to inhibit cell growth by modulating gene expression and causing G1 phase arrest. This might be a possible mechanism of growth inhibition of mimosine in *P. falciparum*. In practice mimosine was not readily soluble in water and was first dissolved in 10% sodium hydrogen carbonate solution and then added to the culture media.

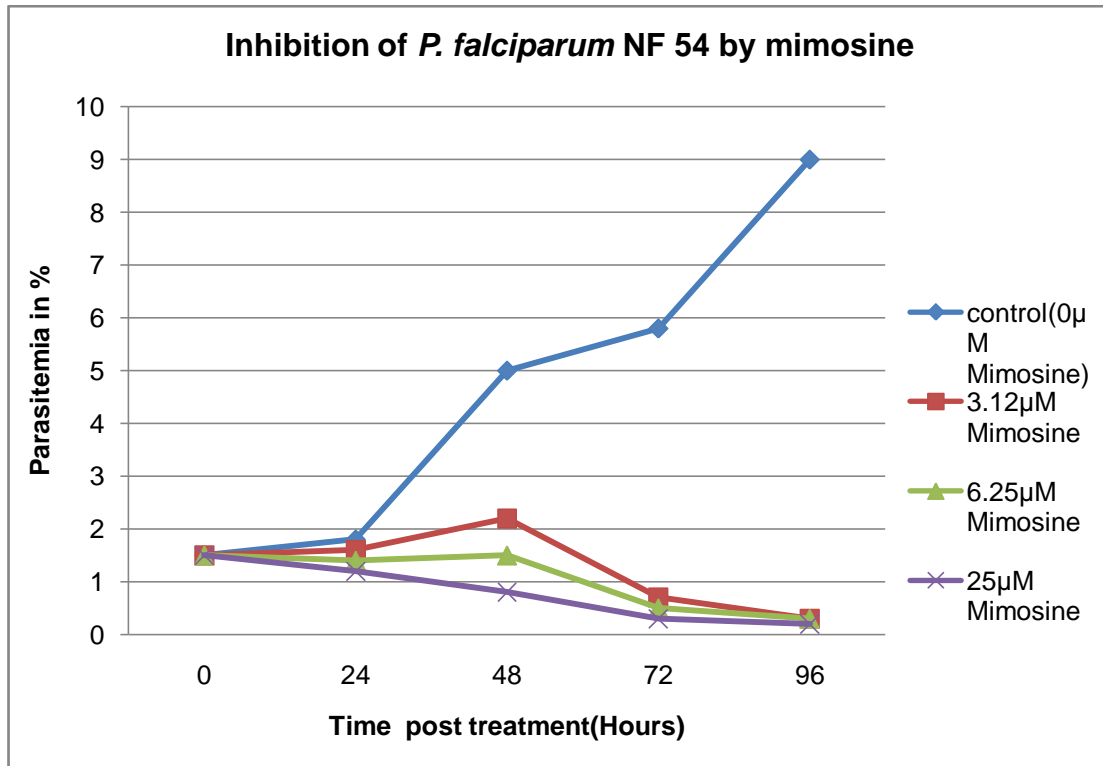


Figure 3.4: Parasitemia after *P. falciparum* NF 54 inhibition by mimosine

L-mimosine concentrations higher than 25 mM resulted in a turbid appearance and affected the Giemsa staining procedure. The obtained IC_{50} value for mimosine was 32 μ M for the chloroquine susceptible (CQS) strain PF/NF-54. The parasitemia after inhibition is shown in figure 3.4. The chloroquine resistant (CQR) strain had an IC_{50} value of 39 μ M. The distribution of trophozoites and schizonts showed more schizonts than trophozoites as shown in figure 3.5.

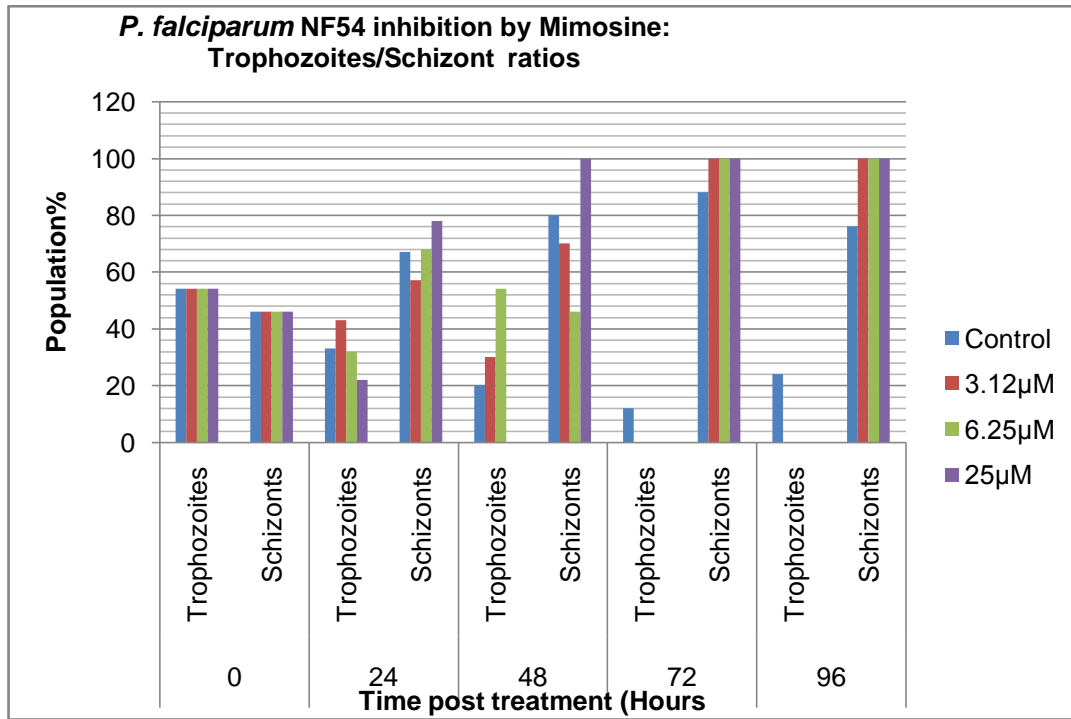


Figure 3.5: Trophozoites and schizont populations in % after inhibition with mimosine.

3.14. Ciclopiroxolamine inhibition of *in vitro* cultures FROM *P. falciprum*

Ciclopiroxolamine is a 6-Cyclohexyl-1-hydroxy-4-methyl-2[1H]-pyridone. It is a broad-spectrum antifungal which has an activity spectrum similar to that of the imidazoles. Previous inhibitor experiments of DOHH performed with extracts from rat testis showed inhibition of the enzyme presumably because of chelate formation with the metalloenzyme which consists of ferrous iron [105]. We tested the compound in concentrations of 3 μM , 6 μM , 9 μM , and 12 μM .

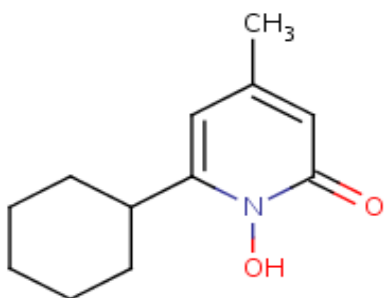


Figure 3.6: The structure of ciclopiroxolamine a hydroxypyridone

The CQR strain, R-Stamm was used in this study. Treatment with ciclopiroxolamine inhibits parasitemia growth in culture as shown in figure 3.7. The number of trophozoites and schizonts present were comparable at 96 hrs is shown in figure 3.8. The IC_{50} value for ciclopiroxolamine was 8.2 μM .

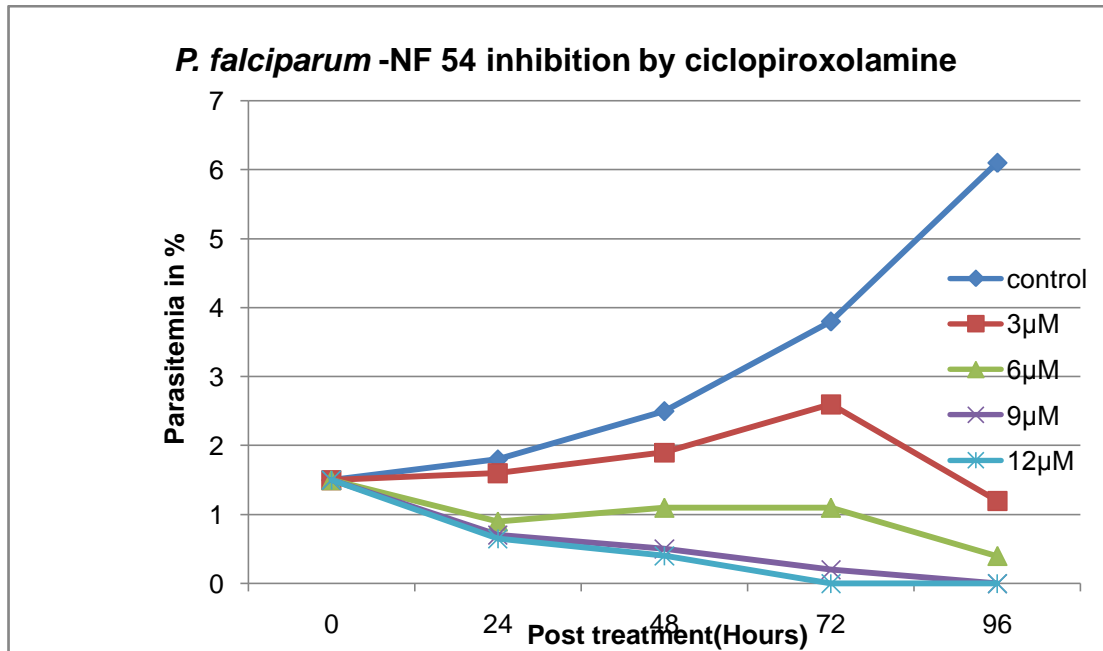


Figure 3.7: parasitemia of R-Strain after inhibition with cyclopiroxolamine

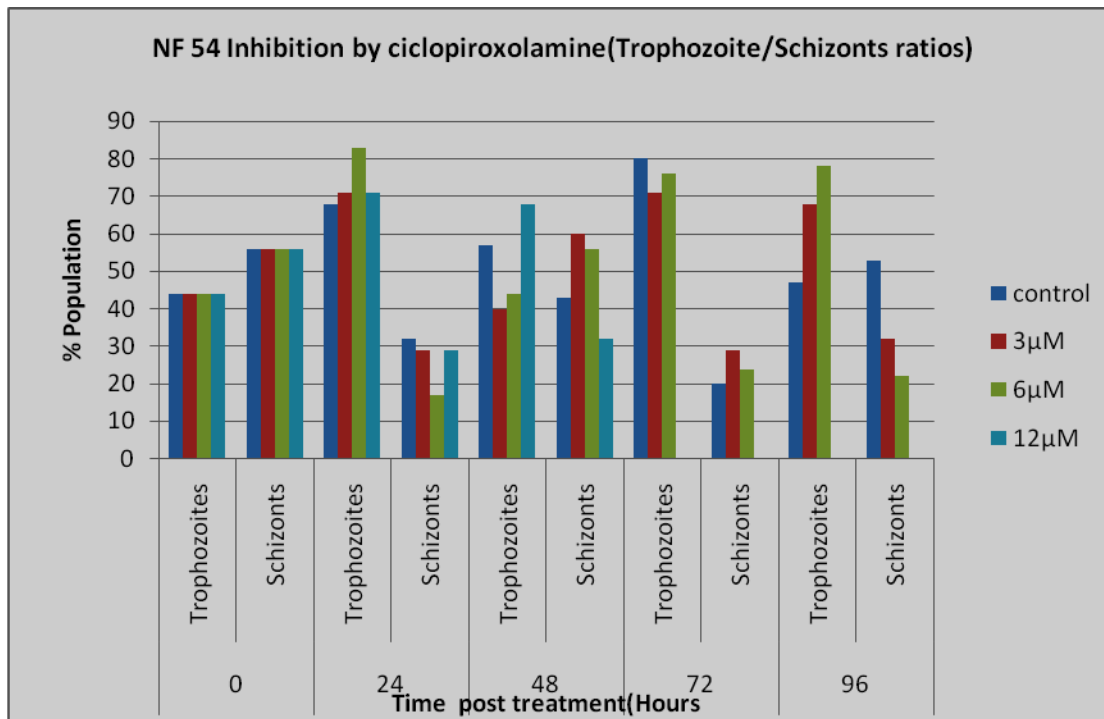


Figure 3.8: R-Strain, trophozoites and schizont populations in % after inhibition with cyclopiroxolamine.

3.1 5. Inhibition by Piperidones

Ciclopiroxolamine and mimosine showed promising results with respect to their ant-malarial activity. The lead structures with the enolizable β -ketoester moiety are able to complex the ferrous iron from a putative DOHH. This prompted us to test differently substituted piperidones from a compound library (Prof. Holzgrabe) in *P. falciparum in vitro* cultures. The seven series prospective DOHH inhibitors were used in various concentrations ranging between 1 and 40 μM being and dissolved in either dimethylsulfoxide (DSMO) or directly in RPMI medium.

Table 3.1 summarizes the *in vitro* inhibition activity of the seven different saturated and non-saturated mono- and diesters tested in CQS *P. falciparum* strains NF54 and CQR R. The saturated 4-piperidone monoesters show the most prominent inhibitory effect (DOHH3, DOHH7) while a 4-piperidone diester (DOHH1) was less efficient in growth inhibition *in vitro*. The average IC_{50} values obtained for the 4-oxo-piperidine monoesters in *P. falciparum* strain NF54 were 1.7 μM for an N-p-chlorobenzyl substitution (DOHH3) and 1.4 μM for an N-allyl substituted derivative (DOHH7), suggesting that the N-substitution is of insignificant value to the inhibition. The 4-oxopiperidine diester DOHH1 shows a higher IC_{50} value of 10.2 μM (DOHH1). The oxidation products, the dihydro- and tetrahydropyridin- monoester (DOHH2, DOHH4, and DOHH5, DOHH6), though structurally similar to the lead compound mimosine, were less efficient in growth inhibition. Most notably, the highest average IC_{50} value was determined to be 18.0 μM for CQS NF54 strain with the DOHH4 inhibitor, a tetrahydropyridine. The dihydropyridine monoesters (DOHH5 and DOHH6) resulted in IC_{50} values of 9.4 and 9.1 μM for the NF54 strain suggesting that dihydropyridine esters have a higher antiplasmodial effect than the tetrahydropyridine derivatives. Therefore, the non-

saturated compounds are superior to the lead compounds mimosine and ciclopiroxolamine in inhibition activity.

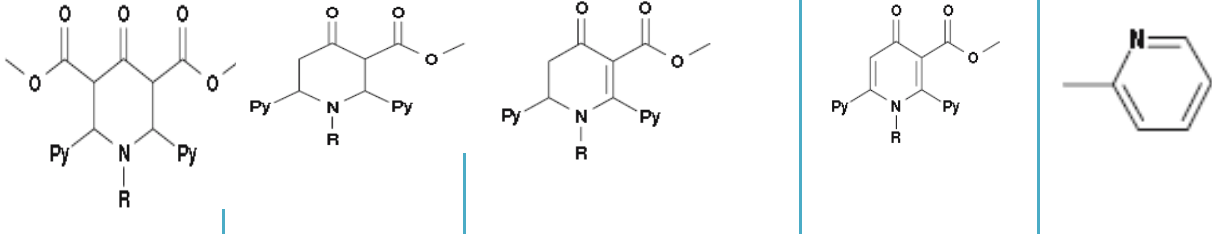
DOHH	Type	R	<i>P. falciparum</i> strain tested	Average IC ₅₀ [μM]
DOHH 1	I	Benzyl	NF54	10.2±5.9
DOHH 2	III	Benzyl	NF54	4.7±2.7
DOHH 3	II	4-Cl-benzyl	NF54	1.7±0.9
DOHH 4	III	4-Cl-benzyl	R	18.0±10.4
DOHH 5	IV	4-Cl-benzyl	R	9.4±5.4
DOHH 6	IV	4-CH ₃ -benzyl	R	9.1±5.2
DOHH 7	II	Allyl	NF54	1.4±0.8
				
Type I	Type II	Type III	Type IV	Py

Table 3.1: Structural formulae of the compounds studied and their determined IC₅₀ values in *P. falciparum* chloroquine susceptible (CQS) and chloroquine resistant (CQR) strains NF54 and R respectively

3.2. Molecular cloning of a putative *dhs* gene from *P. vivax*

The *dhs* amino acid sequence of *P. falciparum* strain NF54 was used to screen a library of expressed sequence tags(EST) from *P. vivax* strain PEST Salvador I. Referring to the 5' and 3' ends of the putative *dhs* gene two primers were constructed to amplify the gene from genomic DNA of *Plasmodium vivax* strain PEST Salvador I.

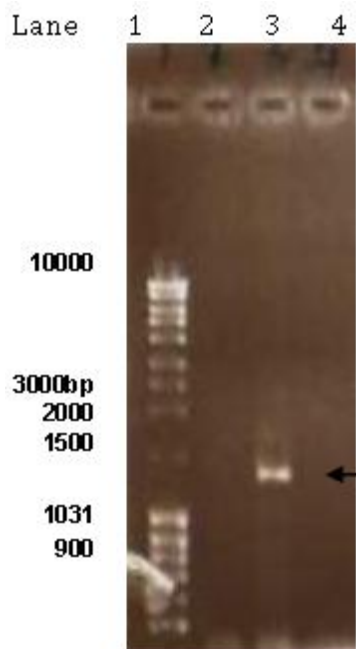


Figure.3.9: A PCR product of 1369 base pairs was obtained from amplification of genomic DNA from *P. vivax* (PEST Salvador I strain) in lane 3. Lane 1 contains the Fermentas ladder mix markers in bp.

The amplification reaction comprised 89 ng genomic DNA of *P. vivax* strain PEST Salvador I, 200 pmol forward primer 1# ATG ACG AAC CAA GGG GCT TTT and reverse primer 2# TCA CCT GAG CTG CGC TTC ACC, 10 mM dNTP, 75 mM MgCl₂, 2 µl 10 fold PCR buffer and 5units *Taq* polymerase (Qiagen) in a total volume of 20 µl.

Amplification was performed using a temperature profile of 94°C for 5', 94°C for 1', 60°C for 1', 72°C for 1' for 30 cycles, and an elongation step at 72°C for 10'. The amplified genomic DNA resulted the PCR product shown on figure 3.9.

3.2.1 Sub cloning of the amplified *P. vivax* gene into pSTBlue Vector

The PCR products were first cloned into pSTBlue-1 using the Acceptor™ kit (Novagen). They were subsequently transformed into *E. coli* Nova Blue™ cells following the manufacturer's protocol. Resulting transformants were selected by growth on LB containing carbenicillin and kanamycin. The transformants were subjected to blue/white screening. Insert positive clones were grown in 1ml LB media and screened by PCR. The positive clone was sequenced. The nucleic acid sequence coding for the *dhs* gene is shown in figure 3.10. The information from the sequencing data shows that the open reading frame has a size of 456 amino acids. The *dhs* gene has an AT content of 52%, and a comparable GC content of 48.


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1 atgacgaacc aaggggcttt taaggagggtt aacaaaatca gaagcgaaag cgacgatgga
61 gagagcagcg atgagaaaag cggaatcgag gacgccaaagt cgtccgtggt tgtcaagtcg
121 aacaaaattc ccgaaaacac agatgtgggtg aaaggaatta atttcgaaga agaagtgaat
181 ttgcaccaat ttgtaaacca gtacaagtac atgggggtttc aggccaccaa cttgggcata
241 ggaattgacg aggtgaacaa aatgattcat ttttaagtatg ccgagggggg agaagggacg
301 caagatggcc atgacaatga ccatgaccag gacagcgatg acgagaggca agcgctgccc
361 aaaaaaaaaa aatgcctaata atggttatcc ttcacctcaa acatgatatac tagcgggtta
421 cgtgaaatat tcgtgtacct cgcaagaag aagttcatag acgtggttgt gaccactgct
481 gggggagtag aggaagatat catcaagtgc ttctccaaaa cgtacttggg cgattttaac
541 ctcaacggga aaaaactaag gaagaagggg tggaacagaa taggcaactt gattgtcccc
601 aatgacaatt actgcaaatt tgaggactgg ctgcagcccc tgctgaataa gatgctgcac
661 gagcagaaca gaaaaaacga agagctcttt ttgaggaagc tggacaagcg caggagggga
721 ggagggcagc ggggggagag ggagccgcc cgcaccgc cacatacgcc acatgcacca
781 tcgccgcctt caccttgca cagctccgat gaagacgaat cggacatggt ctacttgagc
841 ccctccgagt tcatagacaa actgggggag gaaataaacg acgaaagctc cctcatatac
901 tgggtccaca aaaatgacat ccccgatttc tgcccggggc taacagatgg gtccctgggg
961 gataacttat tttttcacia ctatgggaag aaaataaaaa ataatttgat tttagacatt
1021 gtaaaggata ttaagaagat taactccttg gccctgaact gtaagaagtc tgggatcacc
1081 attttggggg ggggtctacc gaaacatcac gtctgtaacg ccaatttgat gagaaacgga
1141 gccgattttg cagtgtatgt gaacacggct aatgagtacg acggcagtga tagtggcgca
1201 aatactacgg aggcctttatc gtggggcaaa attaaggcgg ggcacacgaa caaccatggt
1261 aaagtttttg gcgacgccac gattttggtt cccctgatgg ttctcaacac gttttacttg
1321 cacgatcggg gggggaggca caactcgggt gaagcgcagc tcaggtga

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Figure 3.10: The *DHS* nucleic acid sequence from *P. vivax* has a length of 1369 bp

3.2.2 Cloning of the *P. vivax* *dhs* gene into pETblue-1 Vector

The amplified and purified *dhs* gene was cloned into pETBlue-1 vector (Novagene), which enables expression but no affinity purification. The PCR products were purified using a Qiagen kit. The amplified PCR product was ligated into the pETBlue1 vector according to the manufacturer's protocol. The construct was used to transform Nova Blue cells (Novagen) and subjected to blue/white screening.

PCR positive plasmids from positive (white) clones were isolated and subjected to a restriction digest to ascertain insert size and orientation and subsequently used to transform Tuner (DE3) cells for expression. The expressed product is shown in figure 3.

11.

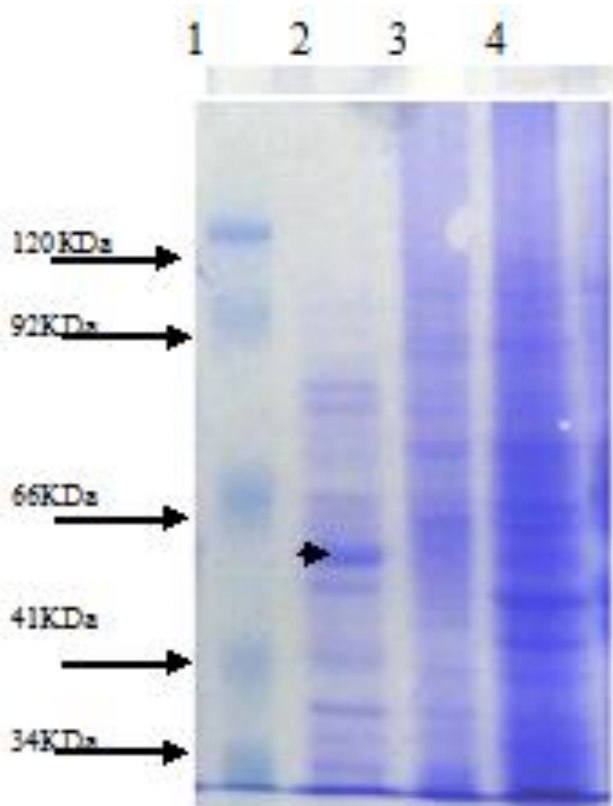


Figure 3.11: Protein expression of the *dhs* gene from *P. vivax* in pETBlue vector. lane 1. 10 kDa ladder (Roth), lane2 induced *dhs* gene; lane 3 uninduced *dhs* gene; lane 4 protein extract of non-transformed Tuner DE cells. The protein exhibited a molecular weight of approximately 50 kDa.

3.2.3 Sub cloning of the *dhs* gene into pET15b expression vector

For expression the *dhs* gene from *P. vivax* was cloned into pET15b vector which enables addition of a six-histidine tag at the N-terminus of the gene. Two primers containing the *NdeI* and *BamHI* restriction sites respectively were used to amplify the *dhs* gene with high fidelity *Pfu* DNA polymerase from genomic DNA of *P. vivax* Salvador PEST strain. The forward primer (primer 3) 5'-AAC CCC ATA TGA CGA ACC AAG

GGGCTT TT-3' contained the NdeI restriction site and the reverse primer (primer 4) 5'-AAAA GGA TCC TCA CCT GAG CTG CGC TTC-3') the Bam HI restriction site respectively. Figure 3.12 shows that the amplified PCR product had the expected size of 1369 bp. Figure 3.13 shows the successful subcloning into pET15B vector by PCR.

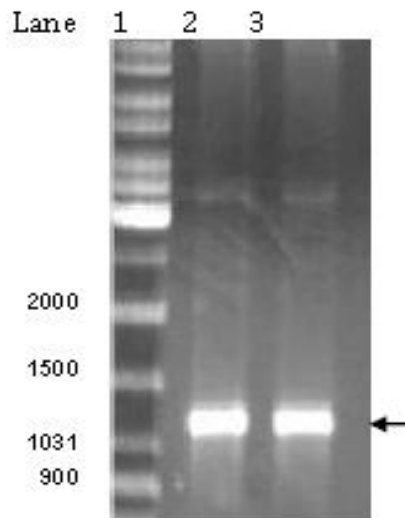


Figure 3.12: PCR (lanes 2 and 3) product made using expression primers and for cloning to pET15b for expression with a 6xhistag. lane 1 has Fermentas ladder mix markers.

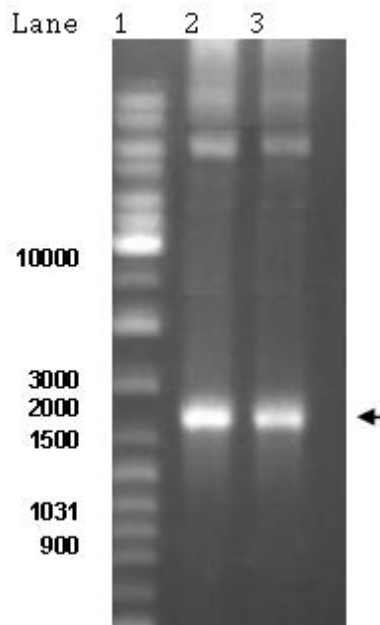


Figure 3.13: PCR amplificate of the 1369bp *dhs* gene obtained with a set of primers for expression after successful cloning

3.2.4 Expression of the *dhs* gene in *E. coli* cells and affinity purification

Protein extracts prepared from *E. coli* cells harboring the recombinant *dhs* pET15b expression plasmid after 4 h of induction with IPTG exhibited a prominent band of approximately 50 kDa on an SDS-PAGE gel (Figure 3.14, lane 3). In contrast, no band was detectable in the uninduced control culture (Figure 3.14, lane 2.) The recombinant protein was expressed with a six-histidine tag at the N-terminus, allowing purification by Nickel-chelate affinity chromatography. Purified protein was analyzed by SDS-PAGE. The protein was detected in both eluate fractions (Figure 3.14), lanes 6 and 7.

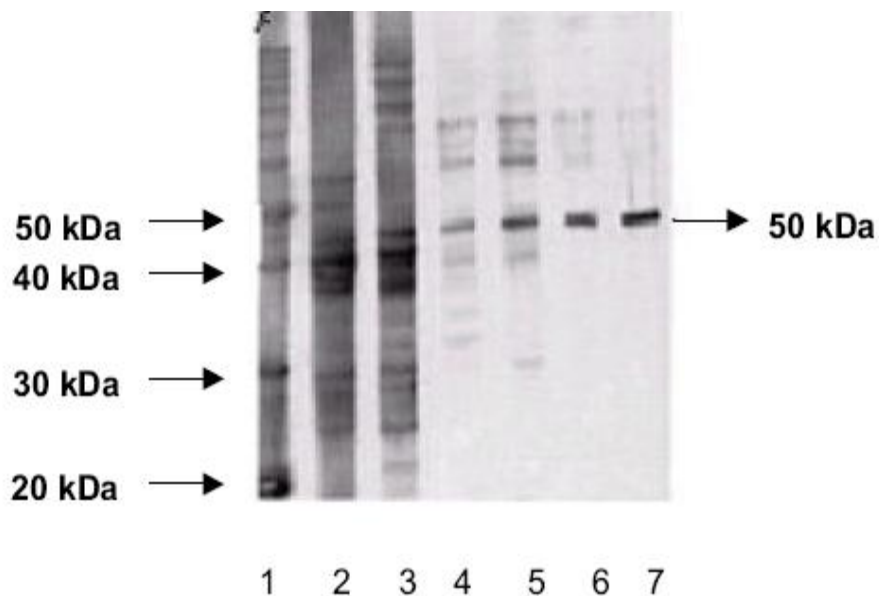


Figure 3.14: Expression and purification of recombinant putative deoxyhypusine synthase from *P. vivax* by Nickel-chelate chromatography on a 12%SDS PAGE protein gel: lane 1) protein marker: 10 kDa ladder; lane 2) non induced bacterial cell lysate; lane 3) induced bacterial cell lysate with 100 mM IPTG after 4 hours of induction; lanes 4 and 5) wash fractions obtained after purification; lanes 5 and 6) eluate fractions of purified putative deoxyhypusine synthase.

3.2.5 Modification eIF-5A Precursor Protein by *P. vivax* deoxyhypusine synthase

The *dhs* activity assay was performed by Marwa Nassar (German University in Cairo).

For the *dhs* activity assay, the purified eIF5A precursor protein and *dhs* enzyme from *P. vivax* were applied. The incorporation of radioactively labeled [¹⁴-C] spermidine into the substrate precursor protein was assayed using a filter paper assay as described earlier. Purified samples were immediately used to determine specific enzymatic DHS activity.

The average specific enzymatic activity from two experiments was 1268 U/mg protein.

A	B	C
eIF5A .lys	water	50 μ M GC7
¹⁴ [C]-spermidine	¹⁴ [C]-spermidine	¹⁴ [C]-spermidine
1268 U	n.d.	35 U
<i>P. vivax</i> DHS	<i>P. vivax</i> DHS	<i>P. vivax</i> DHS
Incubation at 37°C	Incubation at 37°C	Incubation at 37°C

Table 3.2: column A) complete enzymatic assay B) control: DHS enzyme was substituted by water C) inhibition of DHS by the inhibitor GC7 (50 μ M).

suggesting that *dhs* protein from *P. vivax* is able to modify the eIF-5A precursor protein (Table 3.2, column A). In a control experiment, no specific enzymatic activity was detected when water was used instead of *dhs* (Table 3.2, column B). The specific enzymatic activity of *P. vivax dhs* was suppressed 36-fold with the inhibitor N-guanyl-1,7-diaminoheptane(GC7) (Table 3.2, column C).

3.3. Molecular cloning of a putative gene *dhs* from *Theileria Parva*

The *dhs* gene of *T. parva* was first identified *in silico*. The gene was amplified in a PCR amplification step from the genomic DNA isolated from *Theileria parva* with specifically designed primers, a forward primer : 5>ATG ACA GAG AAT AAC CTA AAC and a reverse primer 6 5>TCA TTC GAA TTG ACG ATT AAC.

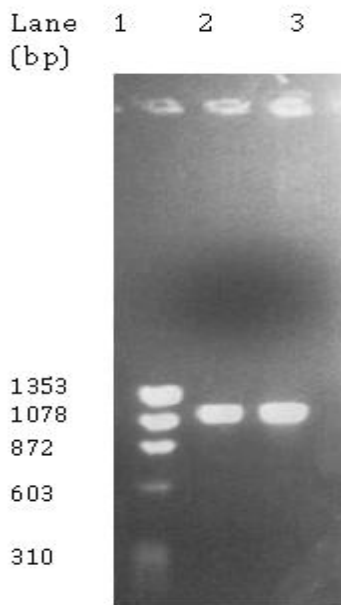


Figure 3.15. Amplified PCR product of *T. parva* (TpM D409) genomic DNA. Lane 1, Phi X 174/Hae III marker, Lanes 2 and 3, putative *dhs* gene amplicate from *T. parva*.

The PCR reaction was composed of: 101 ng genomic DNA of *T. parva* Muguga strain 200 pmol forward primer (primer 5) 5-ATG ACA GAG AAT AAC CTA AAC-3' and reverse primer (primer 6) 5>TCA TTC GAA TTG ACG ATT AAC-3', 10 mM dNTP, 75 mM MgCl₂, 2 µl 10 fold PCR buffer and 5 units *Taq* polymerase (Promega) in a total volume of 20 µl. Amplification was performed using a temperature profile of 94°C for 5', 94°C for 1', 60°C for 1', 72°C for 1' for 30 cycles, and 72°C for 10'. Fig. 3.15 presents

the obtained amplificate with a size of 1113bp. Two clones were obtained which contained the fragment of 1113bp in the plasmid pGEM vector (see Figure.3.17). The nucleotide sequence of the putative *dhs* gene which was isolated from *T. parva* is shown in Figure 3.16. It encodes an ORF of 370 amino acids a theoretical pI of 5.20 / and a MW of 44.9.

```

1 atgacagaga ataacctaaa caattccatt cccaaagttg ctttgggaagc agtcctgcaa
61 accaatgctc aagtgactga gaacatgtta ccagtctccg ggattgagta cgatgacggt
121 ctggccatag attctcttct tgagaagttt agggctcttg gatttcaagc cacgaatttg
181 ggacttgccg ccgagatggt ggaccggatg tattcttggc gactttcaga cgatccgcta
241 aacgaatccg acgaagggac gccttttgcc gatcctgagg tccgccgtaa gaccaagtgt
301 acaatctggg tttccttcac ttcgaatatg atttcttgtg gtcttcgaga agcctttgtg
361 tttatggcca aacacaaatt agttgacggt ttcgtgacta gtggtggagg tgtcgaagag
421 gacctgatca agtgcctggg ccatacttac attggaaaat tcaatcttga tggcgcagac
481 ctcaggaaca agggttggaa cagaaatggg aacctgctcc tccctaataga aaactactgt
541 gcatttgaag actggctcca gcctatthttg gacgagatgc acaccgaaca agtggagaaa
601 gggactatct ggactccttc atcacttatt gaccttctcg ggagcaggat aaacgacgaa
661 acttccttgt actactggtg ccataagaac aagatccctg tattttgccc tggactcaca
721 gatggttctc ttggtgataa tctttatttc cacacctacc gtaagtccag tcttacaacg
781 ctctacctag atatagttaa ggacattagg gccattaatg atttggctgt ccggtgtaag
841 aatcaggcc ttataatcct ggggtggtggt cttccgaaac atcatgtttg taactcgaat
901 ctcatgagaa acggcgctga tttcgccatt tacatctcca ccgccaaga gtatgatggc
961 tcagactctg gtgctaatec cgacgaggct gtttcttggg ggaaaatcaa accgaacaca
1021 gacctgtaa aggttcacgc cgatgettcc atagttttcc cactaattgt cgcgggtggt
1081 ttaaaaaaac acgttaatcg tcaattcgaa tga

```

Figure 3.16: The nucleic acid sequence from *T. parva dhs* with a length of 1113 base pairs.

3.3.1 Expression *Theileria parva* of *dhs* in *E. coli* cells and affinity purification

Expression of the putative *dhs* gene from *T. parva* was performed in the expression vector pET28b. However, there was no expression of the protein after analysis of an SDS- PAGE gel (data not shown). Several attempts were made by changing the *E. coli* host cells BL21(DE3)pLysS to BL21-AI™, One Shot®, BL21 Star™ Cells, and BL21 Gen-X, but expression of the protein was not achieved.

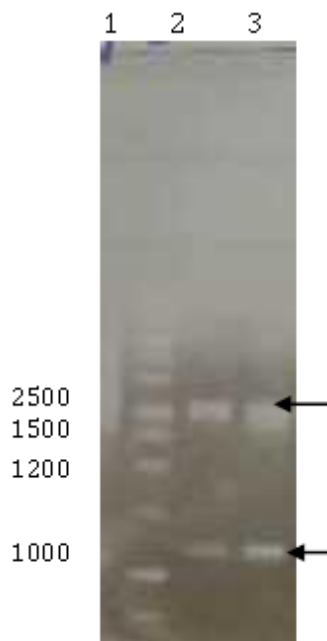


Figure 3.17: Analysis of recombinant clones after restriction digestion of pGEM vector with NdeI and Bam HI (upper arrow) yielding a 1113 bp fragment(Lower arrow, for subcloning into the expression vector pET28b

3.3.2 Western blot analysis of the *Theileria parva* DHS protein with an anti-His-tag antibody

To check whether the *T. parva dhs* protein still contained the His-Tag an anti-Histidin tagged mouse antibody was used as a probe. This presence of his-tag was revealed by further development with a second antibody anti-mouse conjugated to horseradish peroxidase and under DAB substrate.

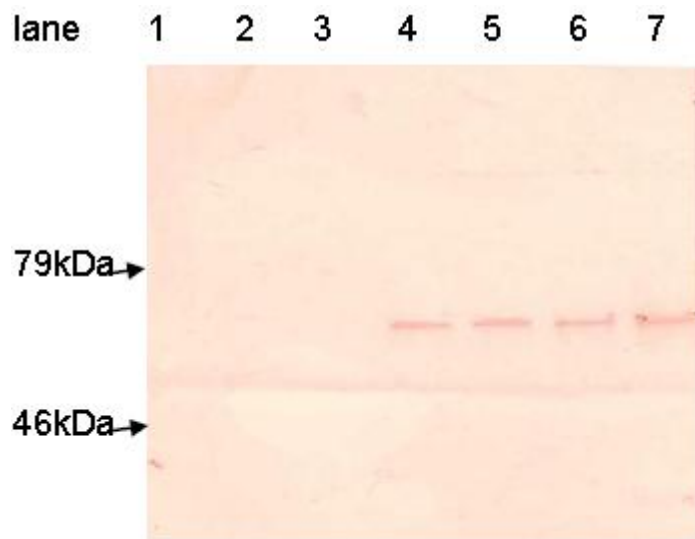


Figure 3.18: Western blot of the expressed *T. parva* DHS probed with an anti-his tag antibody mouse antibody conjugated with horseradish peroxidase (HRP). Lane 1-7, protein extracts from *E. coli* BL21 (DE3) cells with expressed pET28b obtained after different time points of induction , lane3, not induced, lane 4, 1hr after induction, lane 5, 2 hrs, lane 6, 3hrs, lane7, 4 hrs, lane 8 5 hrs, lane 9, 20hrs.

3.3.3 Multiple alignments of DHS Homologs

3.4.1 *P. vivax* DHS gene

The deduced amino acid sequence of the *P. vivax* protein was aligned with different putative *dhs* sequences from other *Plasmodium* species, i. E., *P. falciparum* Dd2 and 3D7 strains (figure 3.19, lanes 1 and 2), rodent malaria parasite *P. yoelii* (figure 3.19 , lane 3), and *Anopheles Gambiae* (figure 3.19, lane 6). The *P. vivax dhs* amino acid sequence displayed FASTA scores of 74 and 73 relative to the *P. falciparum* strains Dd2 and 3D7, respectively. Notably, *P. vivax dhs* exhibits 66% amino acid identity between positions 1–236 and 91% identity between positions 276–443 to the *P. falciparum* Dd2 strain. There are several gaps in the amino acid stretches at positions 103, 240, 257 and 442 in the *P. vivax* DHS protein, which are absent in the *P. falciparum* homolog (numbering refers to the amino acid sequence from *P. falciparum*). The *P. vivax* DHS protein has a FASTA score of 75 to its homolog from the rodent malaria parasite *P. Yoelii*, while amino acid identity to the human DHS protein (Fig. 1, lane5) is only 44%. The lowest FASTA score of 49 was obtained between *P. vivax* and *Anopheles Gambiae* DHS proteins (Figure 1, lane 6)

two *P. falciparum* species. Lane 1: *P. falciparum* strain Dd2 (accession number AF290977); lane 2: *P. falciparum* strain 3D7 (accession number NC_004317); lane 3: *P. yoelii* (XM-724232); lane 4: *P. vivax* (AJ549098) ; lane 5: *Homo sapiens* (U26266), lane 6: *Anopheles gambiae* (XM-316567). The spermidine binding site (243–329 referring to human DHS numbering) is marked by bold amino acids. The NAD binding site from serine 105 to aspartic acid 342 is marked in bold letters. The active center of the DHS protein from glutamine 324 to lysine 329 is bolded black.

3.4.2 *T. Parva* DHS gene

The deduced amino acid sequence of the *T. parva* protein was aligned with different putative dhs sequences from other species, *i. e.* *P. falciparum* Dd2 strain; *P. vivax*; *P. berghei*, a rodent malaria parasite, *T. parva*, *T. annulata*, *Homo sapiens* and *Bos taurus*. The *T. parva* DHS amino acid sequence displayed FASTA scores of 50 to its host *Bos Taurus* and a comparative identity to *T. annulata* (FASTA scores 94). The FASTA scores to *P. falciparum* and *P. vivax* are 52 and 54 respectively. There are several gaps in the sequence in comparison to the *P. falciparum* sequence at amino acid position e.g. between amino acids 96 to 116 (Numbering refers to the amino acid sequence from *T. parva*). The other gaps occur at similar positions as in *P. falciparum* DHS protein. The spermidine binding is highly conserved. 246–342 (*T. parva* DHS numbering). The active site includes a region of six amino acids from Glu 323 to Lys 329. The NAD-binding site (positions 122–349, *T. parva*. DHS numbering) is also highly conserved in relation to the other homologs.

```

P. falciparum      MVDHVSFKEVNKKIRSDDECDADSHNEGDNIEDAKASVFKSSLIPEKTDVVKGGLNFDKKEV 60
P. berghei        -MDGV-FKEVNNKIKNESETEDNDDNNG-GINDAKSSVFKSTKIPEKTDVVKGGINFEKNV 57
P. vivax          MTNQGAFFKEVNNKIRSESDDGESSDEKS-GIEDAKSSVFKSNKIPENTDVKGINFEVEEV 59
T. parva         MTENNLNNSIPKV-----ALEAVLQNTAQVTENMLPVSGIEYDDVL 41
T. annulata      MTENELNESIPKV-----ALEAVLQANAQVTENMLPVSGIEYDGAL 41
H. sapien        -MEGSLEREPAPG-----ALAAVLKHSSTLPPESTQVRGYDFNRGV 40
B. taurus        -MEGPQERVPAP-----ALAAVLKHSALPFETAQVRGYDFNRGV 40
                  :      . . . . . * : * : . : : * * : : :
P. falciparum      DLHEFINNYYKMGFQATNLGISIDEINKMIYYKYKDENIKSEPNENNLLNCNNVSEDLNK 120
P. berghei        NLHEFINQYKYMGFQATNLGIGIDEINKMIHYKFFDN-----KEDITKN 100
P. vivax          NLHQFVNQYKYMGFQATNLGIGIDEVNMKIHFKYAEGGEGTQDG-----HNDNDHQ 110
T. parva         AIDSLLEKFRVFGFQATNLGLAAEMVDRMFSWRLSDD-----PLNESDE 85
T. annulata      AIDSMLEKFRVFGFQATNLGLAAEMVDRMFSWRLSDD-----PLQESDE 85
H. sapien        NYRALLEAFGTTGFQATNFGRAVQQVNAMIEKKLEPL-----SQDE 81
B. taurus        DYRALLEAFSTTFQATNFGRAVQQVNAMIEKKLEPL-----SEDE 81
                  : : : * : : * : : * : : * : :
P. falciparum      DQENHLYHYEKKKKSCIWLFSFTSNMISSGLREIFVYLAKNKFIDVVVTTAGGIEEDIK 180
P. berghei        DS---ITYDNKKK-CMIWLSFTSNMISSGLREIFVYLAKNNYIDVVVTTAGGIEEDLIK 155
P. vivax          DSD---ITQALPKKKK-CLIWLSFTSNMISSGLREIFVYLAKKKFIDVVVTTAGGVEEDIK 169
T. parva         GTPFADPEVRRKTK-CTIWSFSTSNMISGLREAFVFMALHFLVDVFPVTSGGGV EEDLIK 144
T. annulata      GTPYADPEVRRKTK-CTIWSFSTSNMISGLREAFVFMALHRLVDVFPVTSGGGV EEDLIK 144
H. sapien        DQHADLTQSRRLPTSCTIFLGYTSNLISSGIRETIRYLVQHNMVDVLTAGGVEEDLIK 141
B. taurus        DQHADLTQSRRLPTGCTIFLGYTSNLISSGIRETIRYLVQHNMVDVLTAGGVEEDLIK 141
                  . . . . . * : : : * : : * : : * : : * : : * : : * : : * : : * : :
P. falciparum      CFSNTYIGDFNLNGKLRKKGWNRIGNLIVPNDNYCKFEDWLQPIILNKMHLHEQNEKNEQM 240
P. berghei        CFSKTYLGDFFNLNGSKLRKKGWNRIGNLIVPNDNYCMFEDWVQPLLDKILREQNEKNEEL 215
P. vivax          CFSKTYLGDFFNLNGKLRKKGWNRIGNLIVPNDNYCKFEDWLQPLLNKMHLHEQNRKNEEL 229
T. parva         CLGHYYIG FNLGDADLN GWN IGNLLLPENYCAFEDWLQPIILDEMHTEQVE G--- 201
T. annulata      CLGHYYIGFNLGDADLRNKGWNRIGNLLLPENYCAFEDWLQPIILDEMHTEQIEKGG--- 201
H. sapien        CLAPTYLGEFSLRKGELRENGINRIGNLIVPNDNYCKFEDWLMPILDQMVMQNTTEG--- 198
B. taurus        CLAPTYLGEFSLRKGELRENGINRIGNLIVPNDNYCKFEDWLMPILDQMVLEQNTTEG--- 198
                  * . * : * : * * * : * : * : * : * : * : * : * : * : * :
P. falciparum      NKKNHINNYINNYDSDSDQCDMYYLSPSEFINTLGKEINDESSLIYWCYKNDIPIFCPG 360
P. berghei        -----FDEEEDD---MFYLSPELINRLGKEINDESSLIYWCYKNNIPIFCPG 290
P. vivax          -----SPCDSSEDESDFYLSPESEFIDKLGEEINDESSLIYWCHKNDIPIVFCPG 313
T. parva         -----TIWTPSSLIDLLGSRINDESSLIYWCHKNKIPVFCPG 238
T. annulata      -----TIWTPSSFIDLLGSKINDESSLIYWCHKNKIPVFCPG 238
H. sapien        -----VKWTPSKMIARLGKEINNPESVYVWAQKNHPIVFSFA 235
B. taurus        -----VKWTPSKMIARLGKEINNPESVYVWAQKNHPIVLSFA 235
                  : * : * * * : * : * : * : * : * : * :
P. falciparum      LTDGSLGDNLFFHNYGKKIKNNLILDIVKDIKKINSLAMNCEKSGI IILGGGLPKHHVCN 420
P. berghei        LTDGSLGDNLFFHNYGKKMKNNLILDIVKDIKKINSLALCKHSGI IIVLGGGLPKHHVCN 350
P. vivax          LTDGSLGDNLFFHNYGKKIKNNLILDIVKDIKKINSLALNCKKSGI IILGGGLPKHHVCN 373
T. parva         LTDGSLGDNLYFHTYIKSSPTTLYLDIVDIRAINDLAVCKKSGLI IILGGGLPKHHVCN 298
T. annulata      LTDGSLGDNLYFHTYIKSPTTLYLDIVKDIRLINDFAVKCKKSGI IILGGGLPKHHVCN 298
H. sapien        LTDGSLGDMIFHHSYKNP---GLVLDIVEDLRLINTQAIFAKCTGM IILGGGVVHHIAN 292
B. taurus        LTDGSLGDMIFHHSYKNP---GLVLDIVEDLKLINTQAIFAKRTGM IILGGGMVHHIAN 292
                  * * * * : * : * : * : * : * : * : * : * : * : * : * :
P. falciparum      ANLMRNGADFAVYVNTASEYDGSDSGANTTEALSWGKIKYQNTNNHVKVFGDATILFPLM 480
P. berghei        ANLMRNGADFAVYVNTANEYDGSDSGANTTEALSWGKIKSGNNSHVVFGDATILFP-M 409
P. vivax          ANLMRNGADFAVYVNTANEYDGSDSGANTTEALSWGKIKAGHTNNHVKVFGDATILFPLM 433
T. parva         SNLMRNGADFAIYVSTAQYDGSDSGANPDEAVSWG IKP---NTD PVVHADASIVFPLI 356
T. annulata      SNLMRNGADFAIYVSTAQYDGSDSGANPDEAVSWG IKP---HTD PVKVVHADASIVFPLI 356
H. sapien        ANLMRNGADYAVYINTAQEFDGSDSGARPDVAVSWGIRV---DAQPVKVYADASLVFPLL 350
B. taurus        ANLMRNGADYAVYINTAQEFDGSDSGARPDVAVSWGIRM---DAQPVKVYADASLVFPLL 350
                  : * * * * : * : * : * : * : * : * : * : * : * :
P. falciparum      VLNSFYLYDQKRKKDM----- 496
P. berghei        VLNSFYLYNHGEEKKSD--- 427
P. vivax          VLNTFYLHDRGGRHNSGEAQLR 455
T. parva         VAGVLKHHVNRQFE----- 370
T. annulata      VAGVLTKHHVNRQFE----- 370
H. sapien        VAETFAQKMDAFMHERKNEDE--- 369
B. taurus        VAETFAQKVDAFTPEKNEDE--- 369
                  *

```

Figure 3.20: Amino acid alignment of a putative DHS protein from various species. lane1 *P. falciparum* Dd2 strain lane .2 *P. vivax* (PEST Salvador I) lane 3 *P. berghei* rodent malaria parasite lane 4 *T. parva* lane 5 *T annulata* lane 6. *Homo sapiens* lane 7.

Gaps (-) were introduced to obtain maximum alignment. Arterisks label amino acid identities, colons (:) and dots (.) label amino acid similarities. The spermidine binding site (246–349 referring to *T. parva* DHS numbering) is marked by bold letters. The NAD binding site from serine122 to aspartic acid 349 is highlighted.

4.0 DISCUSSION

The life cycles of apicomplexans such as *Plasmodium* spp. are complex. A life cycle consists of a succession of developmental stages in which cell proliferation oscillates between cell cycle arrest (as in e.g. the sporozoites in the salivary glands of the mosquito vector) and intense cell multiplication (as in the erythrocytic stages of the vertebrate human host) [120,121]. The completion of the parasitic life cycle requires rapid changes in its environment such as stimulation and inhibition of cell division.

One of the most important issues facing global health today is the need for new, effective and affordable drugs against malaria, particularly in resource-poor countries [122,123]. Moreover, the currently available antimalarials are limited by factors ranging from parasite resistance to safety, compliance and cost [124]. Innovations in medicinal chemistry are presently lacking. Consistent with this view, there are only a few drugs available for the treatment of Theileriosis, a serious disease of *Bovidae* due to the infection by the protozoa *Theileria parva* causing East Coast Fever (ECF) or *Theileria annulata* causing Tropical Theileriosis or Mediterranean Coast Fever (MCF). Both parasites are transmitted by ticks and cause an enormous economical damage to African farmers. Currently hydroxyanthraquinones i.e. parvaquone and buparvaquone are used but are not effective in the late stage of infection [125]. Cattle can be immunized against *T. parva* by infecting the animals with the sporozoite form of the parasite while at the same time treating the cattle with a long-acting formulation of the antibiotic oxytetracycline. This infection-and-treatment method is the only practical and effective form of immunization against *T. parva* but carries the risk of converting some

animals into disease carriers [126]. With respect to both parasitic diseases, there is still a lack of drugs and therefore a necessity to develop new drugs:

The triamine spermidine has been implicated as essential in the proliferation of malaria parasites [107] and Spermidine is an essential substrate in the biosynthesis of hypusine [N (epsilon)-(4-amino-2-hydroxybutyl) lysine], a novel amino acid present in eukaryotic initiation factor 5A (eIF-5A). Hypusine is formed in a post-translational modification that involves two sequential enzymatic steps catalyzed first by deoxyhypusine synthase [EC 1.114.22499929] (DHS) and in a second step by deoxyhypusine hydroxylase (DOHH) [EC 1.14.9929] [68]. While DHS activity catalyzes the transfer of the aminobutyl moiety to a specific lysine residue in the eIF-5A precursor protein, the DOHH activity completes hypusine biosynthesis through hydroxylation and thereby the eIF-5A is activated.

Enzymes of the hypusine pathway have shown to be valuable drug targets for treatment of different diseases. Inhibition of DHS has shown to be a powerful tool to suppress HIV-replication since eIF-5A is an important co-factor of the Rev protein [127]. Moreover it has recently been shown that the compound CNI-1493 [79] which is in clinical phase II for the treatment of Crohn's disease inhibits the DHS protein of the malaria parasite significantly.

4.1 Inhibitor studies of DOHH

In summary the data from the inhibition experiments indicate that saturated 4-piperidone monoesters show the most prominent inhibitory effect (DOHHI-3, DOHHI-7) while a 4-piperidone diester was less efficient in growth inhibition *in vitro*. The average IC_{50} values which we obtained for the 4-oxo-piperidine mono esters in *P. falciparum* strain NF54 were determined to be 1.7 μ M for an N-p-chlorobenzyl substitution (Table 4.1, type II,

DOHHI-3) and 1.4 μM for an N-allyl substituted derivative (Table 4.1, type II, DOHHI-7), respectively, suggesting that the N-substitution is of minor importance.

DOHH Inhibitors	Type	R	H	P. falciparum	Average $\text{IC}_{50}/[\mu\text{M}]$
1.	I	benzyl	24	NF54	10.2 \pm 5.9
2.	III	benzyl	24	NF54	4.7 \pm 2.7
3.	II	4-Cl-benzyl	48	NF54	1.7 \pm 0.9
4.	III	4-Cl-benzyl	24	R	18 \pm 10.4
5.	IV	4-Cl-benzyl	36	R	9.4 \pm 5.4
6.	IV	4-Cl-benzyl	24	R	9.1 \pm 5.2
7.	II	allyl	24	NF54	1.4 \pm 0.8

Table 4.1. Structural formulae of the compounds studied and their determined IC_{50} values in *P. falciparum* Chloroquine susceptible (CQS) and Chloroquine resistant (CQR) strains NF54 and R respectively.

The 4-oxo- piperidine diester DOHHI-1 shows a higher IC_{50} value of 10.2 M (DOHHI-1) in vitro in comparison to the mono- ester. The oxidation products, the tetrahydropyridinemono ester (DOHHI-2, DOHHI-4) and dihydropyridine monoesters (DOHHI-5, DOHHI-6) although both being structurally rather similar to the lead compound mimosine were less efficient in growth inhibition compared to saturated 4-piperidone monoesters. Most notably the highest average IC_{50} value was determined to be 18.0 μM for CQS NF54 strain with the DOHHI-4 inhibitor, a tetrahydropyridine monoester. Due to the double bonds in DOHHI-2, 4, 5, and 6, the carbonyl function in position 4 cannot form an enol which is favourable to a metal chelation. Thus, the high IC_{50} values of the dihydro- and tetrahydropyridines points to the importance of the metal

ion complexation of the enzyme for an efficient inhibition. Obviously, the two pyridine rings cannot serve as complexation partner for the metal as the -hydroxycarbonyl function does. These inhibition studies show that DOHH can be a targeted in the development of drugs for malaria parasites and the substances studied here can lead to the design of substances with other more useful characteristics.

4.2 Structural features of *P. vivax* and *T. parva* DHS in respect a prospective drug design

Plasmodium vivax is the most widespread human malaria parasite. However, genetic information about its pathogenesis is limited at present, due to the lack of a reproducible *in vitro* cultivation method. Sequencing of the *Plasmodium vivax* genome suggested the presence of a gene homolog of deoxyhypusine synthase (DHS) from *P. falciparum*, the key regulatory enzyme in the first committed step of hypusine biosynthesis. DHS is involved in cell proliferation, and thus a valuable drug target for the human malaria parasite *P. falciparum*. A comparison of the enzymatic properties of the DHS enzymes between the benign and severe *Plasmodium species* should contribute to our understanding of the differences in pathogenicity and phylogeny of both malaria parasites.

The cloning of a 1368 bp putative deoxyhypusine synthase gene (*dhs*) sequence from genomic DNA of *P. vivax* PEST strain Salvador I (Accession number AJ549098) was performed after touchdown PCR. The corresponding protein was expressed and functionally characterized as deoxyhypusine synthase by determination of its specific activity [128] (Nassar et al. 2006).

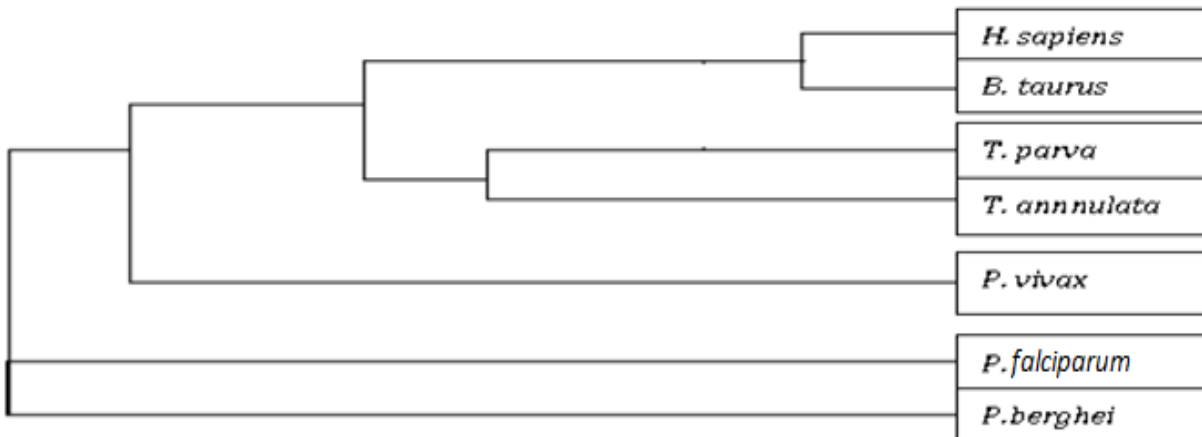


Figure 4.1 A Phylogenetic tree of the DHS protein from *P. falciparum*, *P. vivax*, *P. berghei*, *T. parva*, *T. annulata* and their respective hosts i.e. *H. sapiens* and *B. Taurus* generated using CLUSTAL W[129].

The putative *dhs* gene from *T. parva* sequence is encoded by an ORF of 370 amino acids with a FASTA score of 94 to *T. annulata*, a phylogenetically closely related organism. The *T. parva* FASTA score to *P. vivax* is 54 and 52 to *P. falciparum* strain 3D7. The FASTA score of *T. parva* and *T. annulata* to their bovine host is 50. The phylogenetic relationship of DHS for apicomplexan *P. vivax*, *T. parva*, *T. annulata*, *P. falciparum*, *P. berghei* and that of the corresponding hosts *H. sapiens*, and *B. taurus* is shown in figure 4.1. Phylogram is a branching diagram (tree) is assumed to be an estimate of a phylogeny. Branch lengths are proportional to the amount of inferred evolutionary change. *T. parva* and *T. annulata* are evolutionary close as compared to *P. falciparum* and *P. vivax* while their hosts *H. sapiens* and *B. Taurus* are also evolutionary close.

T. parva gene was not expressed in the pET28 vector although the His-tag was present. It has a theoretical molecular weight of 41.3 KDa and a pI of 5.2. Previous studies have shown that expression of the *dhs* gene from *P. falciparum* was successfully performed after truncation of 22 amino acids from the N terminus [81]. The extreme A+T bias and the insolubility of proteins has been a recurrent problem in expression of *Plasmodium* genes [130]. Perhaps different expression systems such yeast could serve as an alternative [131]. DHS from *Theileria parva* and *Plasmodium vivax* have been shown in this study to be highly conserved as in other species. The *T. parva* DHS retains conserved amino acids at the spermidine- and NAD- binding sites [132].

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