SCREENING OF C-9154 ANTIBIOTIC DERIVATIVES FOR INHIBITORY ACTIVITIES ON TRYPANOSOMAL ALTERNATIVE OXIDASE AND THEIR ANTI-TRYPANOSOMAL POTENTIALS

BY

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AHMADU BELLO UNIVERSITY,

ZARIA NIGERIA

JULY, 2018

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BY

Bashiru IBRAHIM, B.Sc Biochemistry (ABU) 2014

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER DEGREE IN BIOCHEMISTRY

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JULY, 2018

DECLARATION

I, declare that the work in this dissertation entitled **"Screening of C-9154 antibiotic derivatives for inhibitory activities on trypanosomal alternative oxidase and their trypanocidal potentials"** has been carried out by me in the Department of Biochemistry, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree or diploma at this or any other institution.

IBRAHIM BASHIRU _____ _____

P15SCBC8003 Signature Date

CERTIFICATION

This Dissertation entitled "SCREENING OF C-9154 ANTIBIOTIC DERIVATIVES FOR INHIBITORY ACTIVITIES ON TRYPANOSOMAL ALTERNATIVE OXIDASE AND THEIR TRYPANOCIDAL POTENTIALS" by Bashiru IBRAHIM meets the regulations governing the award of the degree of Master of Science in Biochemistry of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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Dean, School of Postgraduate studies	Signature	Date

DEDICATION

I dedicate this Dissertation to Almighty Allah and my Dad Alhaji Ibrahim Barau.

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ABSTRACT

African trypanosomiasis is a neglected tropical disease caused by flagellated parasites of the genus *Trypanosoma* and *Trypanosoma brucei* is a representative specie. It is a disease of humans and livestock. Available drugs have unsatisfactory outcomes such as toxicity and emerging resistance hence the need to search for new remedies. The typanosomal alternative oxidase (TAO), a terminal oxidase of the mitochondrial electron transport chain is an essential enzyme for the parasites' respiration and aerobic metabolism for ATP. This enzyme is absent in the mammalian host and has been considered an attractive molecular target for drug discovery. In this study, a library of thirty four (34) derivatives of the C-9154 antibiotic was screened for their inhibitory activities on TAO and also for their anti-trypanosomal potentials determined by cessation of motility. Eight of the derivatives were found to significantly reduce the activity of the enzyme with compound **32** eliciting the highest inhibitory potency. Investigation into the inhibition kinetics showed that the compound inhibited the enzyme competitively with K_m, $V_{max}$and K_i values of 393.3 µM, 70.9 µmol/min/mg and 1.25 µM, respectively. Molecular docking studies of the compounds was performed and the predicted binding energies is suggestive of tight binding to the enzyme. Compound **32** had the highest binding affinity -9.0 Kcal/mol, corroborating the inhibitory data. The predictive binding mode of the compound in the enzyme active site suggests strong interactions between the compound and Arg118, Thr219, Leu122, and Leu212. Finally, *in vitro* test for trypanocidal activity was performed by checking drop or cessation in motility in the presence of the test compounds on *Trypanosoma brucei brucei*and *Trypanosoma congolense*. Interestingly, while ten of the derivatives reduced the motility in both parasites, only compound **32** was trypanocidal in the presence of 5mM glycerol. In conclusion, derivatives of C-9154 antibiotic was found to be trypanocidal through inhibition of TAO.

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CHAPTER ONE

1.0 INTRODUCTION

African trypanosomes are unicellular extracellular protozoan parasites responsible for sleeping sickness and naganatransmitted by cyclicaltsetse flies.Human African trypanosomiasis (HAT), is a life threatening neglected tropical disease caused by two protozoan parasites, *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*), and is transmitted through the bite of infected tsetse flies belonging to the genus *Glossina*(Malvy and Chappuis, 2011). The two trypanosome subspecies causing HAT are morphologically indistinguishable and genetically similar to the parental*T. b. brucei*, a pathogen of nagana in cattle and other animals (CDC, 2015; Petrelli*et al.,* 2017). Other species of African trypanosomes infect cattle, causing enormous economic losses in the tsetse belt of Africa.Of the animal trypanosomes, *Trypanosoma congolense* is considered the most prevalent and widespread pathogenic trypanosome in tropical Africa. There is wide range of animal hosts, which includes ruminants, pigs, dogs and even wild animals throughout the tsetse belt (Peacock *et al*., 2012).

HAT is endemic in sub-Saharan Africa where it affects primarily rural and low-income populations (Holmes, 2013). Over 20,000 new cases are reported annually,in addition, it is a threat to 65 million human lives (WHO, 2017). Infected patients progress gradually to a coma and severe organ failure which ultimately is fatal (Franco *et al.,* 2014). Even though HAT cases are declining over the last 20 years, the disease is still fatal unless treated. Over 95% of the cases are attributed to the chronic form of infection caused by *T. b. gambiense* and many months to years are required for complete healing(Brun*et al.,* 2001). In contrast, *T. b. rhodesiense* infections are acute and death occurs in few months after infection. Both parasites present two phases of the infection; an early haemolymphatic stage and a late meningoencephaliticstage. Usually, death proceeds the second stage if left untreated.The meningoencephalitic phase is

defined by central nervous system infection after trypanosomal traversal of the blood-brain barrier. Unfortunately, only a few drugs are available and problems such as narrow spectrum, treatment failures owing to resistance, high cost and cases of toxicity have been reported (Brun*et al.,* 2001).

The parasites have a complicated life cycle that involves procyclic forms (PCFs) and bloodstream forms (BSFs). The BSFs(*i.e.*, the form present in the mammalian host) of African trypanosomes rely exclusively on glycolysis for energy (ATP) production as they have no oxidative phosphorylation, no cytochrome-mediated electron transport systems, and no tricarboxylic acid cycle (Verner*et al*., 2016). Interestingly, the BSFs of these parasites possess several structural and metabolic features that are present in the parasite which are absent in the mammalian hosts. Such distinctive features, which provide valid drug targets, include compartmentalization of their glycolysis into an organelle called glycosome, their sole dependence on glycolysis for their energy needs (Haanstra*et al.,* 2008) and the presence of a rudimentary mitochondrion that houses an indispensable cytochrome-independent alternative oxidase (TAO) (Chaudhuri*et al.,* 2006).

TAO(EC 1.10.3.11) is the cytochrome-independent terminal oxidase of the mitochondrial electron transport chain that catalyses the oxidation of ubiquinol. It provides an alternative route for electron passage through the electron transport chain to reduce oxygen. It is anon-proton pumping ubiquinol oxidase that catalyse four-electron reduction of dioxygen to waterbut insensitive to cyanide and requiring a diiron cofactor. TAO is the essential terminal oxidase for reoxidization of NADH produced during glycolysis by these parasites, and in addition to glycerol-3-phosphate dehydrogenase, it is a primary mitochondrial electron transport protein (Kita *et al*., 2003). Because NADH reoxidation is essential for parasite survival and mammalian hosts do not possess an ortholog of this protein, TAO is considered to be a unique target for

development of new antitrypanosomaldrugs (Nihei *et al.,* 2002).There are few compounds (Salicylhydroxamic acid (SHAM) and ascofuranone) that have been shown to inhibit TAO. These compounds all show structural similarity to the ubiquinolsubstrate and are thought to act as competitive inhibitors, by binding to the ubiquinol binding site. Of these known inhibitors of TAO, the antibiotic ascofuranone inhibits the enzyme and cures infection in mice (Minagawa*et al*., 1997; Yabu*et al*., 2003; Ebiloma*et al*., 2018).

Similar to ascofuranone produced by the fungus *Acremoniumsclerotigenum*, the compound C-9154 is an antibiotic produced by *Streptomyces ishigakiensis*and is found to inhibit the growth of various microorganisms including some bacteria and fungi (Hasegawa *et al*., 1975). The analogs and derivatives of C-9154 have been synthesized and were shown to have antibacterial activity against *Staphylococcus aureus*and *Escherichia coli*(Jumina*et al*., 2005; Bello *et al*., 2012). These similarities between Ascofuranone and C-9154 gave the idea for investigating the possible inhibitory activity of this antibiotic and its derivatives on TAO.

1.1 Statement of Research Problem

Owing to the potential fatality and morbidity associated with African trypanosomiasis,the diseases are of great global health and economic importance. The World Health Organization (WHO) estimates that 20,000 new cases of HAT occur each year with a further 65 million people at risk of infection (Keating *et al.*, 2015;WHO, 2017). In addition, the therapeutic agents used to treat this disease are far from ideal since they have numerous severe side effects including, hypoglycemia, hypotension, encephalopathic syndrome, peripheral neuropathy and hepatic toxicity (Munday*et al.*, 2015). In addition to these issues, treatment of HAT is becoming more problematic due to the emergence of resistance to the drugs.Therefore, the need to search for new, safer, affordable and more effective drugs with a different mechanism and broader spectrum of action is of great public health importance (Delespaux and Koning 2007).

1.2 Justification

African trypanosomiasismay be a fatal disease if left untreated. Due to the fact that therapeutic options are limited and threatened by drug resistance, the search for new safe and effective trypanocidal drugs remains an important goal in tropical medicine (Delespaux and Koning 2007).In contrast to mammalian cells, which contain hundreds of mitochondria per cell, trypanosomes possess a single mitochondrion that is involved in vital cellular functions, one of which is energy metabolism. Therefore, the mitochondrial respiration of the parasite is an attractive target for rational drug discovery (Gonzalez *et al*., 2017). Interestingly, TAO is a key enzyme for the respiration of the parasite and is a validated target for chemotherapy. Earlier reports in literature have shown that Salicylhydroxamic acid (SHAM) and ascofuranone in combination with glycerol have trypanocidal effects on *T. brucei*, although the concentrations required for the cure are toxic to the host (Clarkson and Brohn 1976; Grady *et al.,* 1993; Balogun *et al*., 2013; Ott*et al.,* 2016). Hence the need to search for new TAO inhibitors cannot be overemphasized.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this research was to screen for TAO inhibitors from a library of C-9154 antibiotic derivatives andinvestigate their trypanocidal potentials on *T. b. brucei* and *T. congolense*.

1.3.2 Specific objectives

The specific objectives were to:

i Determine the inhibitory activity of the C-9154 derivatives on TAO.

ii Perform inhibition kinetic studies of the inhibitors on TAO.

iii Conduct an *in vitro* trypanocidal activity of the compounds on *T. b. brucei* and *T. congolense.*

iv Investigate the possible binding modes of the inhibitors on TAO using computational modeling.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African Trypanosomiasis

African trypanosomiasis is one of the life threatening diverse groups of neglected tropical diseases. The tsetse fly, *Glossina species* is the main vector for trypanosomes, the parasites that cause trypanosomiasis. The disease is caused by protozoan parasites of the genus *Trypanosoma* that live and multiply extracellularly in the bloodstream and lymph of the mammalian hosts (Rangbarian, 2017).Trypanosomiasis is prominent on the World Health Organization (WHO) list of neglected tropical diseases and a major problem to the poor countries in the world, especially throughout sub-Saharan Africa (WHO, 2010). The disease occurs between latitude 140° N and 290° S covering 10 million Km^2 spanning across 37 countries in Africa (Lozano, 2012). It is estimated that about 65 million people and 50 million cattle are at risk of contracting the disease in Africa alone (Samdi*et al.*, 2010). The socio-economic impact of trypanosomiasis is extremely difficult to estimate however losses have been estimated to amount to over 5 billion US dollars annually in milk, meat, lost in traction power and control program (Shaw, 2009).

The disease is caused by several species of trypanosomes that results in Human African Trypanosomiasis (HAT;also known as sleeping sickness in humans) and Animal African trypanosomiasis (AAT; popularly referred to as nagana) (Kennedy, 2013). The term "sleeping sickness" describes the deregulation of the sleep-wake cycle and the intrasleep cycle which is observed in the late stage of the disease (Bugue*et al*., 2005).

HAT occurs as a highly focal disease (Cecchi*et al*., 2009). The causative agents of HAT are *Trypanosoma brucei gambiense* (Tbg) and *Trypanosoma brucei rhodesiense* (Tbr). They are hemo-parasitic unicellulareukaryotes (Balogun *et al*., 2014). Trypanosomiasis is still of great public health concerndue to lack of a vaccine and satisfactory treatment (CDC, 2015). Two

forms of the disease exist: the slow-progressing form, caused by Tbg, which is endemic in western and central Africa; and, the faster-progressing form, caused by Tbr, found in eastern and southern Africa (Simarro*et al*., 2010). On the other hand, AAT is widespread across most of the 38 countries of sub-Saharan Africa that are considered endemic for tsetse flies and the disease is considered to be a major factor limiting agricultural production (Shaw *et al*., 2014). Both the diseasesand their vectors have been the focus of intense scientific interest since David Bruce first described the link between tsetse flies and HAT (Bruce, 1895). Another human infective trypanosome,*Trypanosoma cruzi*, the etiological agent of *'Chagas'* disease is responsible for the American trypanosomiasis. This condition affects millions of people in central and southern America, causing 15, 000 death annually (Murcia *et al*., 2010).

2.2 Trypanosomes

Trypanosomes are pathogenic unicellular protozoans of the family *Trypanosomatidae*and order *kinetoplastida*that are transmitted cyclically and mechanically by their vector, the tsetse fly (Brun*et al*., 2010). Trypanosomes (20–30 μm× 1.5–3.5 μm) are blood-borne parasites dwelling in various body and tissue fluids. These parasites are motile due to the undulatory motion of their flagellum (Figure 2.1) (Baral, 2010). Trypanosomes share most common morphological features such as the presence of flagellum, kinetoplast, nucleus and an undulating membrane (Brun*et al*., 2010).

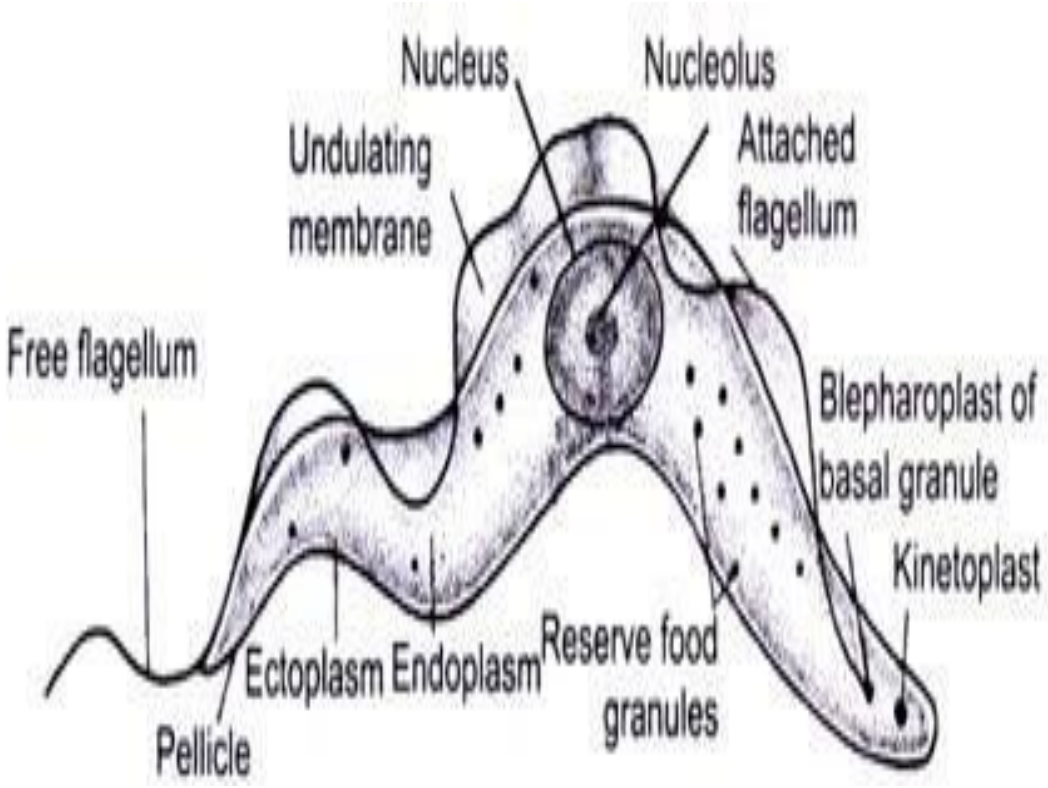


Figure 2.1: Structure of African trypanosome

Source: https://vdsstream.wikispaces.com/file/view/trypanosoma.jpg/576083569/

The trypanosome family (trypanosomatidae) has many genera and species which are transmissible by the vector to a mammalian host (Figure 2.2). The tsetse fly is of the genus *Glossina*and many different species of this fly exists which are capable oftransmitting the trypanosomes (Brun*et al*., 2010). The salivarian trypanosome include the *Trypanozoon, Nanomonas*and *Duttonella*, these classifications are based on the site of their development. *Trypanozoon*includes the *T. brucei* family, which complete their final developmental stage in the insect salivary gland. *Nanomonas*such as *T.congolense*, completes their final maturation in the vector's mouth part (Ngure*et al.,* 2008). *Duttonella*groups includes *T. vivax*and complete their development in the proboscis of the tsetse vector (Dyer *et al*., 2013).The *stercorarian*unlike the salivarian trypanosome, is transmitted by triatomine bug. They undergoes their development maturation in the midgut of the insect vector. *T. cruzi* the etiological agent of '*chagas'* disease falls into this category. The fly is unique in that it is viviparous (gives birth to live developed larva) and mates only once in its life, both of which have important implications in its eradication strategies (WHO 2010).

In HAT, the two different strains of the same species (*T. b. gambiense* and *T. b. rhodesiense*) infect human populations only, but are found in slightly different habitats and reservoirs. Both protozoan species are morphologically indistinguishable, but have drastically different epidemiological features. Both forms of the sleeping sickness strains affect the central nervous system (Baral, 2010).The *T. b. gambiense* strain is an anthroponotic strain causing the chronic form of sleeping sickness that transmits from human-to-human and humans function as the main reservoir. The *T. b. rhodesiense* strain on the other hand is a zoonotic strain and causes the acute form of sleeping sickness that transmits from animal to human (Brun*et al*., 2010). Many animals (in particular the bush pig) canact as reservoirs for this strain (WHO 2010).

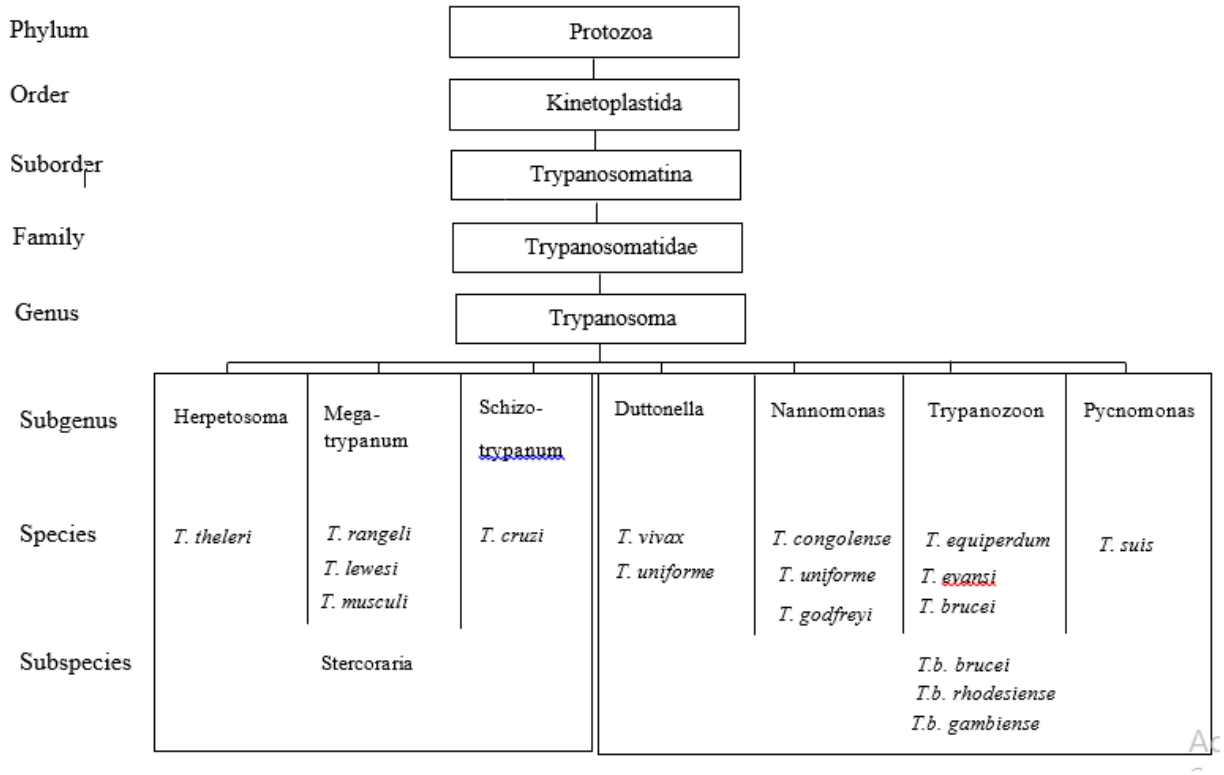


Figure 2.2: Classification of Trypanosomes

Source: https://www.hindawi.com/journals/bmri/2010/389153.fig.002.jpg

2.3 The Organisms (*T. brucei* and *T. congolense*)

T. brucei belongs to the *Trypanosomatidae*, a family consisting of exclusively parasitic organisms found world-wide in vertebrates and insects (Hoare, 1972). The species of*T. brucei* includes three morphologically indistinguishable subspecies. *T. b. brucei*, which causes AAT, is not infective to humans. *T. b. rhodesiense* and *T. b. gambiense* can infect humans as they developed the ability to resisttrypanolytic factor which is made up ofapolipoprotein A (ApoL1), a serum protein that triggers death in other trypanosomes (Vanhollebeke and Pays 2010; Pays *et al*., 2014). *T. brucei* cells contain one central nucleus, one single mitochondrion with its own DNA comprising the kinetoplast situated at the posterior end of the cell, and a flagellum attached to the cell by an undulating membrane. Also, it is a unicellular organism that lives in the blood, lymphatic system and interstitial spaces of organs (Kennedy, 2013). Recently, the parasite is reported to occupy and adapt functionally to the adipose tissue (Trindate*et al*., 2016). It is transmitted through the bite of a tsetse and quickly adapts to the mammalian host to become what is known as a ‘‘slender’’ bloodstream form (BSF). As parasitemia increases, slender forms are capable of sensing population density, and this triggers differentiation to the stumpy form, which is pre-adapted to life in the transmitting tsetse vector and, once there, further differentiates into procyclic form (PCF).

Trypanosoma congolense is one of the most prevalent and widespread pathogenic trypanosome of the livestock trypanosomes in tropical Africa, being found in ruminants, pigs, dogs and other domestic animals throughout the tsetse belt (Bugue*et al.*, 2005, Desquesnes*et al*., 2012). In the mammalian bloodstream, *T. congolense* is a small trypanosome (10μm), shorter in length than *T. brucei* and without a conspicuous undulating membrane (Peacock *et al*., 2012). In the tsetse fly vector, both species develop and multiply in the midgut initially, before onward migration to the mouthparts; infective metacyclics develop in the proboscis for *T. congolense* and in the salivary

glands for *T. brucei*. This difference in vector developmental site led to the subgeneric classification in current use for the African tsetse-transmitted trypanosomes: trypanosomes that develop in the midgut and proboscis are in subgenus Nannomonas, while those that use the midgut and salivary glands are in subgenus Trypanozoon (*T. brucei*) (Hoare, 1972).

2.4 Life cycle of theAfrican trypanosomes

African trypanosomes are transmitted between mammalian hosts by tsetse flies. However, in each host, the parasites undergo many life cycle stages involving forms with discrete morphologies, patterns of gene expression, and proliferation status. In each case, these developmental changes are programmed precisely (Figure 2.3) (Matthews, 1999). Infection in the mammalian host begins when the infective stage, known as the metacyclic stage, is injected while feeding intradermally by the tsetse fly. The organisms rapidly transform into the blood-stage trypomastigotes (long, slender forms), and divide by binary fission in the interstitial spaces at the site of the bite. The buildup of metabolic wastes and cell debris leads to the formation of a "chancre". In the mammalian host, the metacyclic parasites rapidly undergo cell cycle reentry and morphological changes, and exchange the restricted repertoire for antigenic variation that is the characteristics of the metacyclic forms with a more elaborate system of the bloodstream forms (Barry and McCullock, 2001).

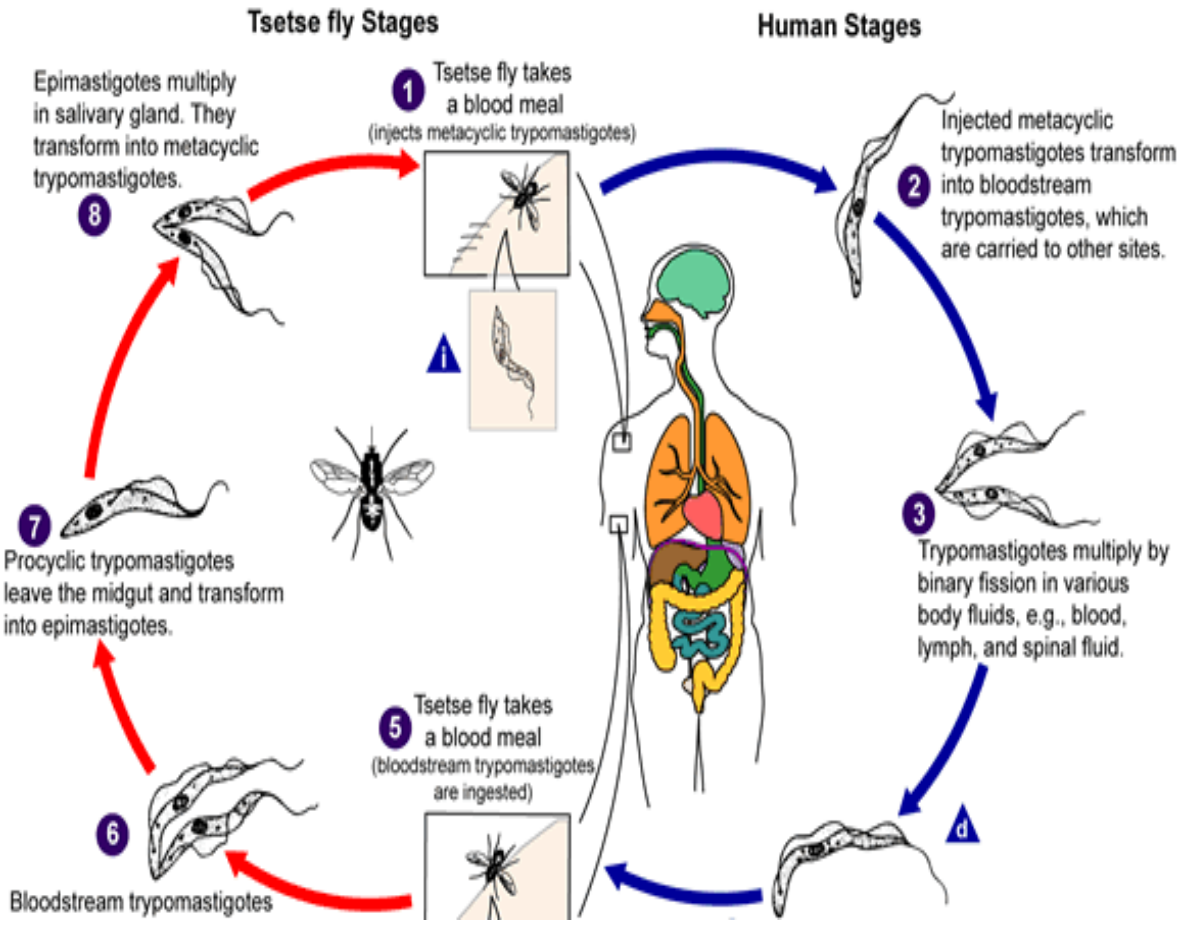


Figure 2.3: Life Cycle of Trypanosomes

Source: http://www.cdc.gov/globalhealth/ntd/diseases/

Once established in the mammalian host, the bloodstream parasite is heterogeneous (Vickerman, 1985; Vickerman, 1965) comprising the proliferative slender forms during the ascending phase of parasitemia and the non-proliferative stumpy forms at the peak of parasitemia (Matthews *et al*., 2004). The transition from the morphological extremes (*i.e.*, the slender versus stumpy forms) involves a progression from proliferation to cell cycle arrest, accompanied by a series of biological andmorphological transformations (Vasella*et al*., 1999; Tyler *et al*., 1997). Once stumpy forms develop during the course of parasitemia, the population is pre-adapted for transition to the procyclicforms, which occupy and proliferate in the midgut of the tsetse. When the fly vector (tsetse) bites an infected individual, it takes the parasites with the blood meal. The parasites undergo metabolic changes in the midgut of the fly. They lose their surface coat, which consists of about 10^7molecules of the Variant Specific Surface Glycoprotein (VSG), and transform into the proliferative procyclic forms. In this form, they express their own surface proteins called the Procyclic Acidic Repetitive Proteins (PARPs, or procyclins). The definingevents of the differentiation from the bloodstream formsto the procyclic forms are the loss of VSG and gain of the procyclins (Balal, 2010).

2.5 Pathogenesis of trypanosomiasis

Trypanosomiasis elicits several biochemical and haematological changes within the host. Trypanosomes rapidly multiply and spread into the lymphatic system and blood stream.Once in the blood stream, the host defense system mounts a series of immune response via macrophage-mediated responses that clear the parasite by opsonization and phagocytosis (Gueganet *al.*, 2013). Nevertheless, trypanosomes are capable of evading subsequent immune response by switching and presenting a distinct variant surface glycoproteincoat (VSG), which is not recognized by existing antibodies (Magez*et al*., 2008). Thus, this event marked the onset of the pathogenesis of trypanosomiasis with several pathologies such as anaemia, organ damage and

immunosuppression. Anaemia is a prominent clinical feature of trypanosomiasis through free radicals or reactive oxygen species and partly the enzyme sialidase, which cleaves sialic acid on the erythrocytes surfaces thus exposing it to specific macrophages attack (Umar *et al.*, 2007) amongst other postulated factors. Also the loss of negative charge on the surface caused the red blood cells to cluster leading to thrombosis (Hendrik *et al.*, 2011).

Progressively, the disease advances into immunosuppression status, cardiomegaly, hepatomegaly and splenomegaly (Ajakaiye*et al.*, 2013). Hepatosplenomegaly is induced due to the generation of free radicals, immune activation, high concentration of inflammatory products such as interferon gamma, tumour necrosis factor alpha and counter inflammatory cytokine, interleukine 10 (Bezie*et al*., 2014). Evidently, in humans' invasion of the central nervous system occurs causing meningenoencephalitis, infiltration of the white matter with inflammatory cells, perivascular and macrophages stimulation (Namangala, 2011). Invasion of the central nervous system triggers immunoglobin response, production of cytokine and chemokine. At this stage if not treated, the infection leads to severe complications, organs failure, coma and eventually death.

2.6 Energy Metabolism of the African trypanosome

In the life cycle of *T. brucei*, the parasite switches between its bloodstream form (BSF) and procyclic form (PCF) during development; this represents a very effective strategy for successful transmission between the mammalian and insect hosts. In addition, *T. brucei* takes advantage of unique energy metabolism pathways conserved in its genome, enabling the parasite to rapidly adapt to the abrupt changes in nutritional environment associated with transmission between hosts.The 'procyclic' form that multiplies in the tsetse depends for ATP generation mainly on the mitochondrial metabolism of amino acids (Michels*et al*., 2000). On the other hand, the BSFs, unlike the PCFs and the host cells, contain a largely repressed mitochondrion that lacks the full

complement of Krebs cycle and oxidative phosphorylation enzymes (Figure 2.4)(Le Febvre and ill, 1986; Nolan and Voorheis, 2000), hence, all their energy needs are derived from the glycolysis (Hannaert*et al*., 2003). The glycolytic pathway of these parasites is compartmentalized within the glycosomeswhich is a peroxisome-like organelle. These organelles sequester the first seven glycolytic enzymes, as well as two enzymes of glycerol metabolism including the NAD-dependent glycerol-3-phosphate dehydrogenase and glycerol kinase (GK) (Opperdoes and Borst, 1977; Guerra-Giraldez*et al*., 2002). In BSFs, a net of two ATP molecules per glucose catabolized are produced from glycolysis, which is tightly coupled to the activity of the mitochondrial localized trypanosome alternative oxidase (TAO) during aerobic respiration. But when TAO is inhibited or during anaerobiosis, the parasite is able to survive from one ATP produced through the reaction catalyzed by GK in the reverse direction (Balogun *et al*., 2014).

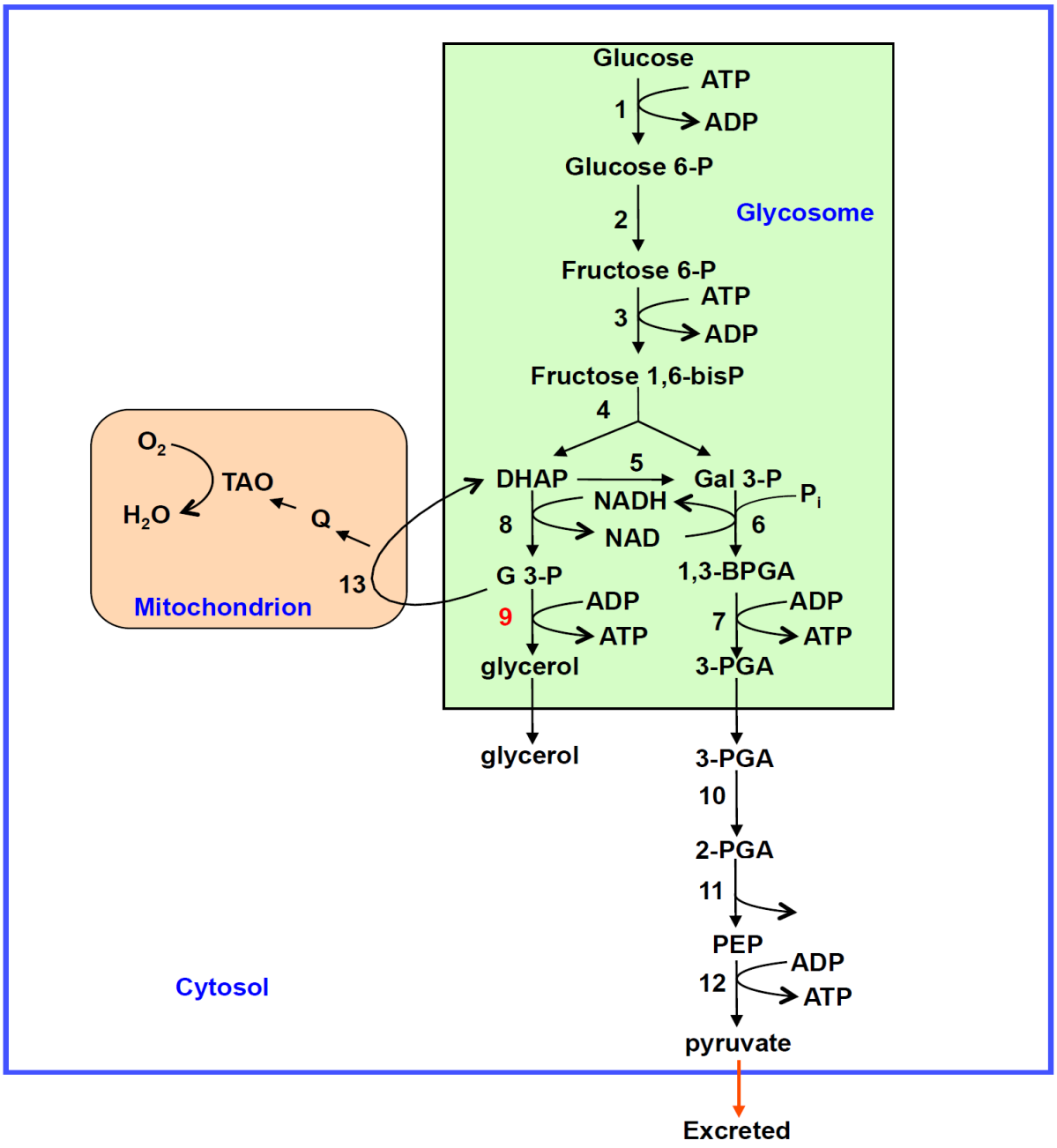


Figure 2.4: *Trypanosoma brucei* energy metabolism (Balogun *et al.*, 2014).

2.7 Trypanosome Alternative Oxidase

The alternative oxidase is a mitochondrial inner-membrane non-proton motiveprotein that accepts electrons directly from ubiquinol and use it to reduce oxygen to water without involving cytochrome-linked electron transport chain (Moore and Albury, 2008; Liu and Guo, 2017).Although the BSFs do not contain any detectable forms of cytochromes, they respire at high rate. The enzyme system responsible for this respiration was discovered in 1960 as the glycerol-3-phosphate oxidase (GPO) system (Grant and Sargent, 1960). This was later found to consist of at least two enzymes: glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate oxidase (Grant and Sargent, 1960; Hill, 1976). Glycerol3-phosphate is the substrate for the glycerolphosphate dehydrogenase component, which transfers electrons to the oxidase component and subsequently to oxygen. Earlier studies revealed that GPO is present in a particulate fraction of the trypanosomes that consists of some type of microbody (Bayne *et al*., 1969). It was shown that the dehydrogenase component of the GPO system is connected to the terminal oxidase through the coenzyme Q9 (Clarkson *et al.,* 1989). Thus, the oxidase componentof GPO is a ubiquinol oxidase and transfers electrons to molecular oxygen (Grant and Sargent, 1960; Clarkson *et al.,* 1989). The electron transfer through this terminal oxidase is not coupled to proton translocation and ATP production in the mitochondria (Clarkson *et al.,* 1989). AOXs that have similar characteristics to those of GPO have been found in many higher plants and fungi (Affourtit*et al*., 2002; Moore *et al*., 2002).Thus, from these similarities, and because ubiquinol not aglycerol3-phosphate is the substrate, the trypanosome GPO was renamed trypanosome alternative oxidase (TAO) by Clarkson's group and the terminology has been accepted by others (Clarkson *et al.,* 1989).

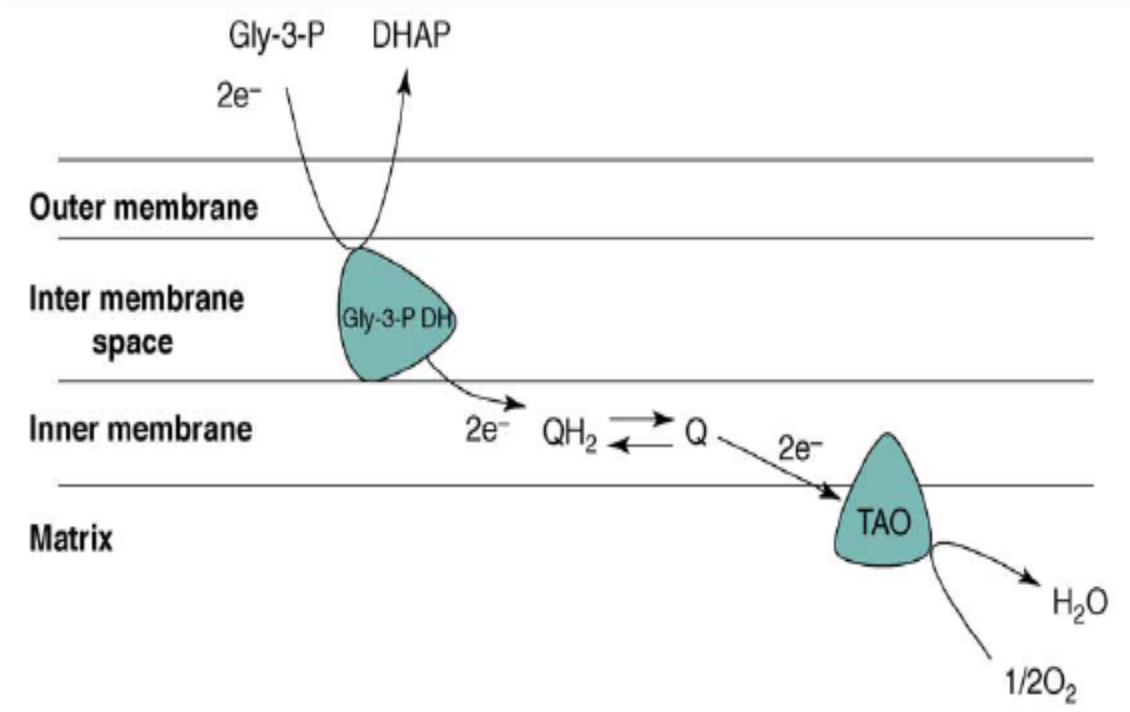


Figure 2.5: The mitochondrial electron transport system in the BSFs of *T. brucei*(Chaudhuri*et al*., 2006).

TAO is the only terminal oxidase of the mitochondrial electron transport chain in the BSFs of *T. brucei* (Figure 2.5). These forms rely on blood glucose as their sole source of energy and suppress most of their mitochondrial activities (Hill, 1976). Glucose enters the cell and is metabolized via glycolysis to pyruvate, which is secreted by the parasite (Figure 2.4). The reducing equivalents generated during glycolysis are reoxidizedthrough a glycerol3-phosphate/dihydroxyacetone phosphate shuttle between glycosomes, the unique glycolytic compartments of the parasite, and the mitochondrion (Figure 2.4) (Balogun *et al*., 2014). Because electron transport through TAO does not produce any ATP, under aerobic conditions the net production of ATP is two molecules per molecule of glucose in the bloodstream form. Under anaerobic conditions or when TAO is inhibited, ATP production is reduced to half with the production of equimolar pyruvate and glycerol (Figure 2.4) (Balogun *et al*., 2014). Under these conditions, the glycosomalNAD^+/NADH balance is maintained by the conversion of glycerol3-phosphate to glycerol by glycerol kinase, which is kinetically unfavorable under normal conditions. Thus, it was thought that the bloodstream form of *T. brucei* could survive using an anaerobic pathway while TAO is inhibited. However, a recent finding suggests that this might not be true. The bloodstream forms of *T. brucei*do not survive under anaerobic conditions for long time periods (Helfert*et al*., 2001). But it was later shown that SHAM alone kills the *in vitro*culture of the BSFswithin 24 h and a reduction in the TAO level by RNAi is detrimental for parasite growth (Helfert*et al*., 2001). They further showed that triosephophateisomerase (TPI), which convertsdihydroxyacetone phosphate to glyceraldehyde- 3-phosphate and vice versa, is essential for the survival of the bloodstream form (Helfert*et al*., 2001). A reduction in the TPI level to less than 15% of the wild type by RNAi reduced the growth of the parasite to half. Together, these results suggest that the BSFs cannot survive under anaerobic conditions by producing equimolar amounts of glycerol and pyruvate, otherwise TPI probably would not be

essential. TAO is therefore considered to be an essential enzyme for the survival of the bloodstream trypanosomes hence it is a potential chemotherapeutic target(Chaudhuri*et al*., 2006).

TAOis a monomeric proteinthat is localised in the mitochondrion (Chaudhuri*et al*., 2005). It consists of α helices with a non-haem di-iron center held by two conserved EXXH (Glutamic acid-X-X-Histidine) motifs at residues 162–165 and 266–269. It has two hydrophobic stretches, similar to other alternative oxidases (Chaudhuri*et al*., 1998). The iron, which is crucial to function, was detected initially by the EPR signal of the mixed valence reaction intermediate and visualized recently *via* protein crystallization (Ajayi*et al*., 2002; Shiba*et al*., 2013). TAO is anmonotopic protein embedded only in the inner leaflet of the mitochondrial lipid bilayer with its ubiquinol binding site opening into the mitochondrial cytoplasm (Shiba*et al*., 2013).

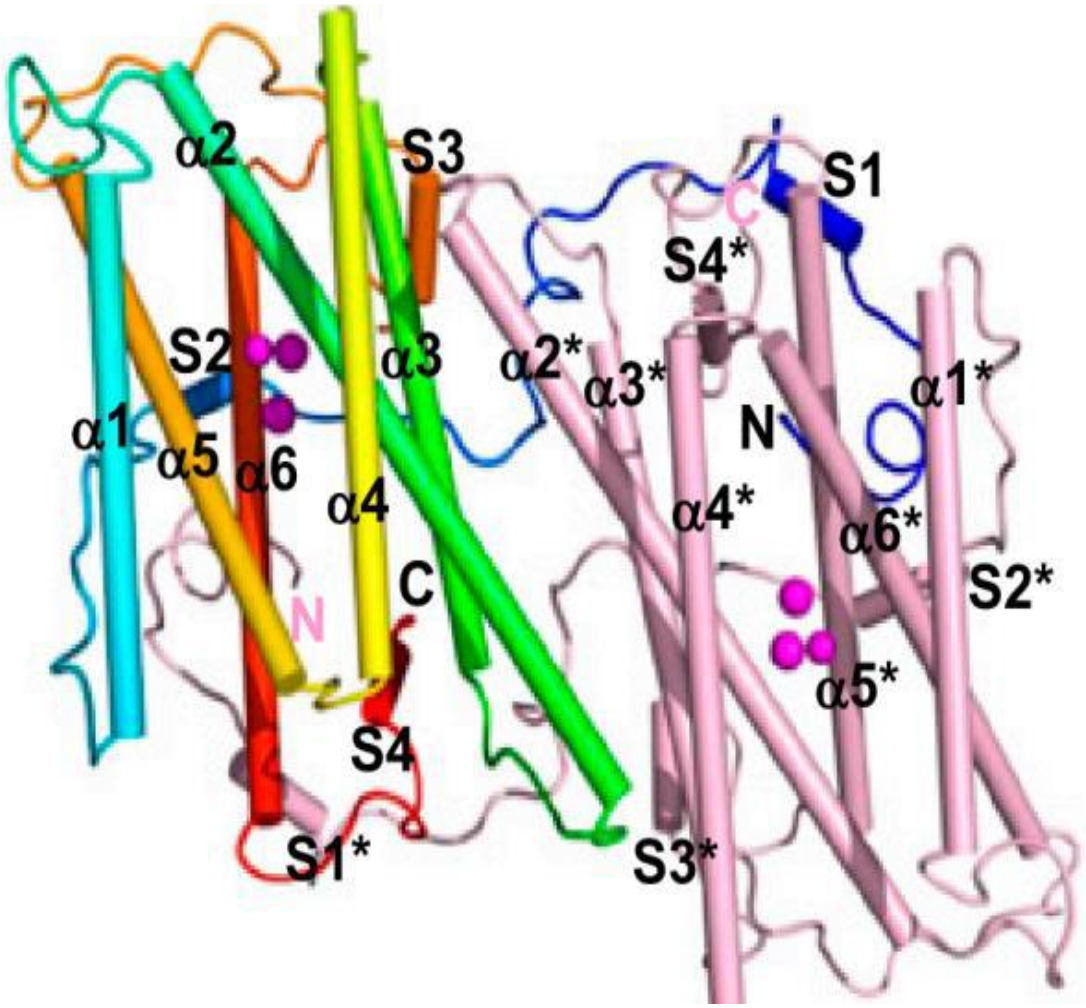


Figure 2.6: Crystallized structure of TAO (Shiba*et al*., 2013).

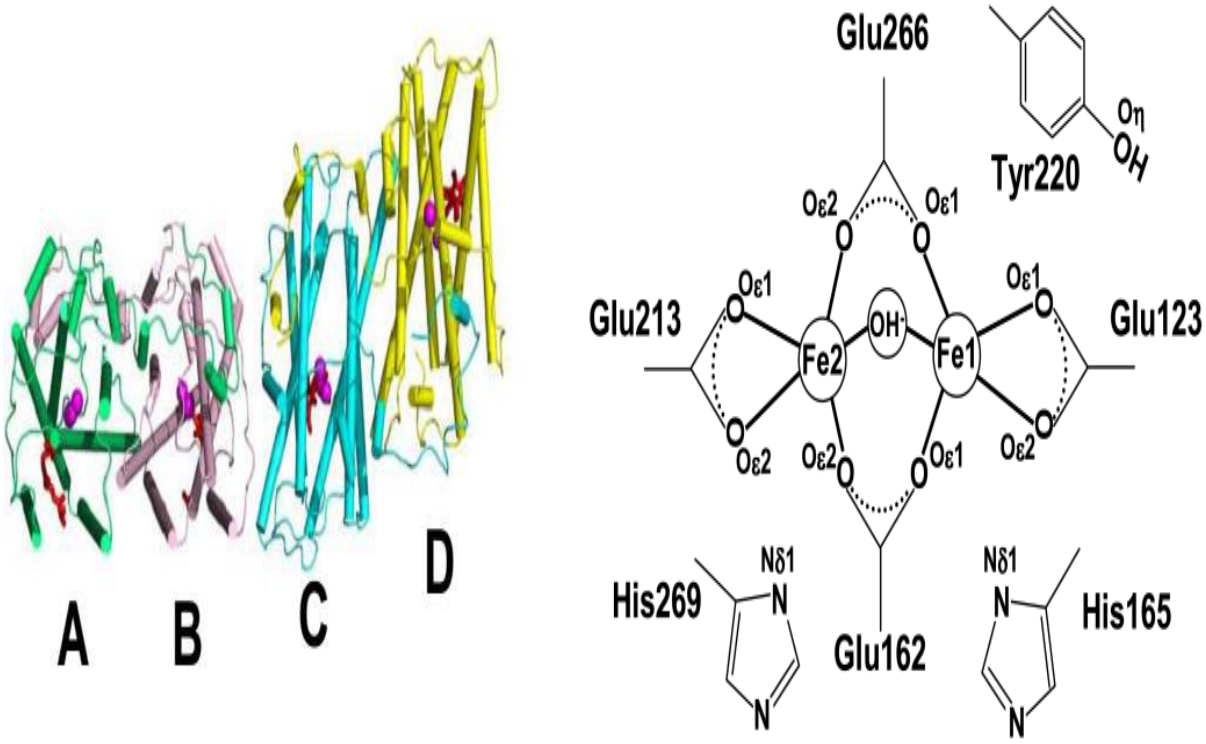


Figure 2.7: - Homodimericcrystalysed form of AOX monomer (left). Five co-ordinate diironcentres at TAO active site (right) (Shiba*et al*., 2013).

The active site, which is located in a hydrophobic environment deep inside the TAO molecule (Figure 2.7), is composed of the diironcentre and four glutamate (E123, E162, E213, and E266) and two histidine residues (H165 and H269), all of which are completely conserved across alterative oxidases (AOXs)(Ajayi*et al*., 2002). These residues bind the diiron center in a five co-ordinate manner possessing a distorted square pyramidal geometry, observed across all four crystallized units.

The diiron molecules bound to (A) chain A, (B) chain B, (C) chain C and (D) chain D are shown as yellow sticks with nitrogen and oxygen atoms colored in blue and red (Figure 2.8). The diiron (Fe-OH--Fe) is drawn as magenta spheres. Contour levels are 1.0 σ (blue) and 3.0 σ (orange)(Shiba*et al*., 2013).

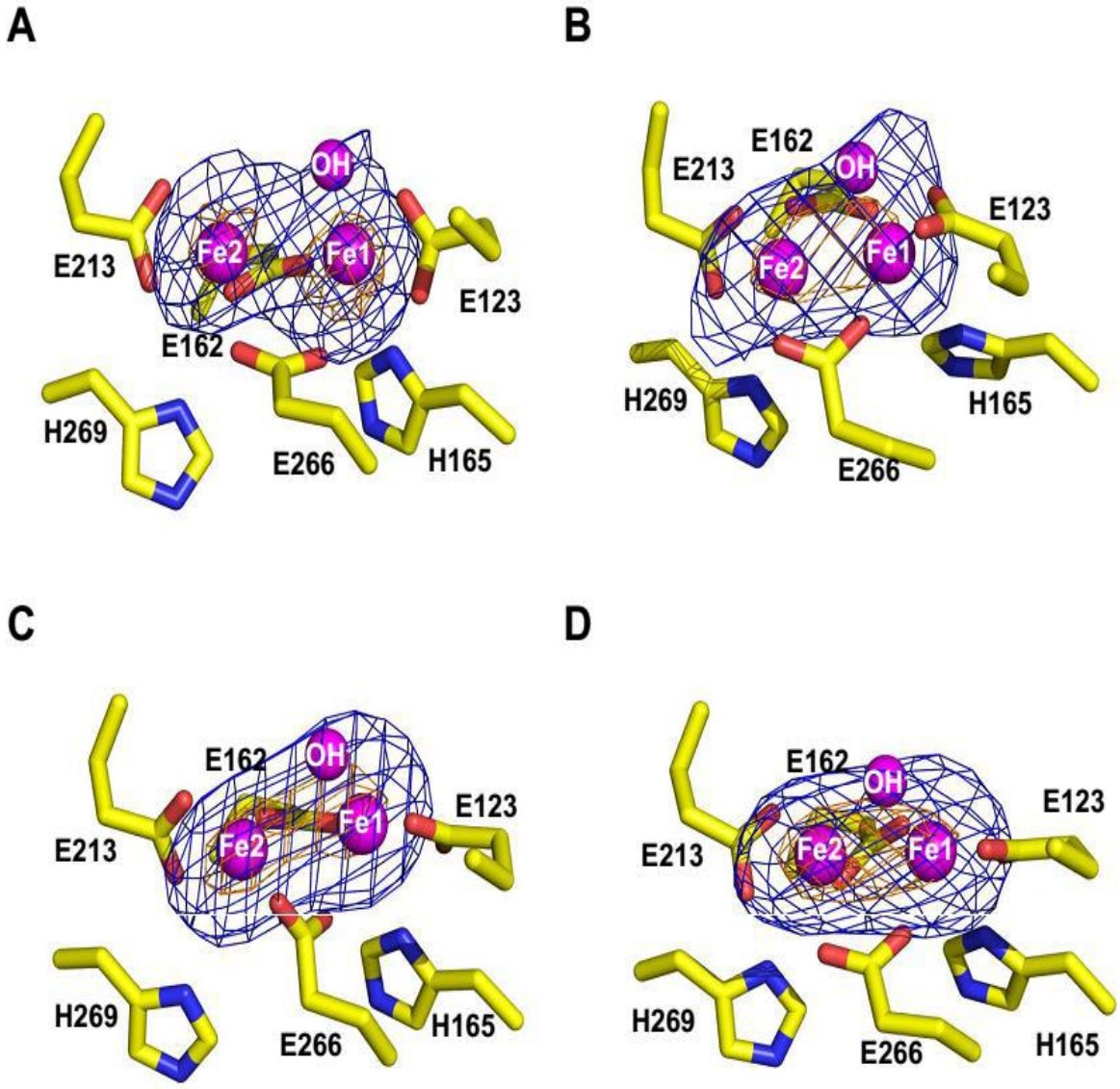


Figure 2.8: The structure of diiron sites (Shiba*et al*., 2013).

2.8 Inhibitors of TAO

Owing to the fact that TAO is unique and essential to the BSFs of trypanosomes and not found in mammals, it represents a potential chemotherapeutic target. However, several previous approaches showed that the inhibition of TAO by SHAM or different benzohydroxamide derivatives does not kill the parasite, but the addition of excess glycerol with SHAM kills the parasite very quickly both *in vitro* and *in vivo* (Clarkson and Brohn, 1976). The reason for the requirement for glycerol along with SHAM is that this combination inhibits both the aerobic and anaerobic pathways for ATP production. Although initially the SHAM plus glycerol combination was demonstrated to kill trypanosomes within several minutes, it was alsoshown that the BSFs could not survive under anaerobic conditions for longer time periods. SHAM at a concentration of 40 mMkills the BSFs of the parasite within 24 h under *in vitro* conditions (Helfert*et al*., 2001).Yabu*et al*.also showed that the repeated administration of ascofuranone, a very potent inhibitor of TAO, could effectively cure infection in mice without the addition of glycerol (Yabu*et al*., 2003; Yabu*et al*., 2006). These new findings alter the previous concept and suggest that the inhibition of TAO alone is trypanocidal. More recent reports have shown the increased potency of these inhibitors by synthesizing the derivatives of both SHAM and ascofuranone (Grady *et al*., 1993; Gonzolez*et al*., 2017; West *et al.*, 2017; Ebiloma*et al*., 2018). All these findings serve to increase the potential of TAO as a target for chemotherapeutic intervention in African trypanosomiasis.

2.9 Chemotherapy of Trypanosomiasis

Tragically, for decades the treatment of trypanosomiasis depends largely on chemotherapy, while the search for an ultimate vaccine is still ongoing, yet looks elusive due to trypanosome antigenic variation, immunosuppression amongst others factors that researchers need to overcome (Ratnaike, 2003). However, current chemotherapy of human African trypanosomiasis mainly

relies on six drugs that have been in use for decades (suramin, pentamidine, melarsoprol, eflornithine, arsobal and mel B) (WHO, 2017), Others such as homidium, isometamidium and diminazeneaceturate are used in African animal trypanosomiasis infections. Each of these drugs is besieged by one or more of these challenges: high cost, high toxicity, adverse side effects, difficult routes of administration and parasites increasing resistance (Maser *et al*., 2003). The Drugs for Neglected Diseases *initiative* (DND*i*) is developing fexinidazole, a new oral drug for human African trypanosomiasis with some promising results. It has entered Phase II/III clinical study in patients with late-stage sleeping sickness (Kaiser *et al*., 2011). It is hoped that fexinidazole would solve most of the problems and limitations of current chemotherapeutic options.

2.10 C-9154 Antibiotic

During studies on screening for antibiotics that showed activity against bacteria resistant to various known antibiotics, a new antibiotic with a broad antibacterial spectrum was isolated from the whole agar culture of a *Streptomyces*strain, NR-7GGI. This *Streptomyces* species was called *Streptomyceskurssanovii*and the isolated antibiotic referred to as fumaramidmycin (Maruyama *et al.,* 1975). Another researcher working independently and slightly earlier than the previous researcher also found that a new species of *Streptomyces*(*Streptomyces ishigakiensis*), produced a novel antibiotic which was named C-9154 (Fig. 2.7) (Hasegawa *et al*., 1975).

Figure 2.9: Structure of C-9154 Antibiotic

The two new antibiotics were found from structural studies to be the same compound (Hasegawa *et al*., 1975; Suhara*et al*., 1975). These new antibioticsare found to inhibit the growths of various microorganism at concentrations between 10–100 μg/mL (Hasegawa *et al*., 1975). It was also shown to be active against certain strains that were resistant to ampicillin, cephalosporin, chloramphenicol, gentamicin, kanamycin, macrolides, neomycin, sulfonamides, streptomycin, and tetracyclinesat concentrations between 3.12–200 μg/mL (Maruyama *et al.,* 1975). Its intraperitoneal LD_{50}value in mice was found to be 75–100 mg/kg while its oral $LD_{50}$was found to be 1.25–2.5 g/kg (Maruyama *et al.,* 1975).Analysis of the structure of C-9154 antibiotic showed that it was made up of two fragments, namely, phenylaceticacid and fumaramide(Hasegawa *et al*., 1975; Suhara*et al*., 1975).

Analogues of the C-9154 antibiotic have been previously synthesized (Suhara*et al*., 1975; Jumina*et al*., 2005). These analogues were shown to have antibacterial activity against *Staphylococcus aureus*and *Escherichia coli* ranging from 15 μg/mL to 4000 μg/mL (Jumina*et al*., 2002). Also derivatives of the C-9154 antibiotic were synthesized and shown to have biological activity in the range of 0.625–10 μg/mL against both Gram positive and negative bacteria and some fungi (Bello *et al*., 2012; Bello *et al.,* 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

All the chemicals and reagents that were used for this research are of analytical grade which were obtained from Sigma (USA) and WAKO (Japan). The enzyme (rTbbTAO) and the substrate (Ubiquinol) was graciously donated by Dr. Balogun Emmanuel Oluwadare of the Department of Biochemistry, Ahmadu Bello University, Zaria. Already synthesized C-9154 antibiotic derivatives were donated by Dr. Bello Isaacof the Department of Chemistry, Ahmadu Bello University, Zaria

3.1.2 History and source of parasites

*T.congolense*was isolated from an infected cow at an abattoir in Kaduna State, Nigeria. Serial passages in albino rats and cryostabilates was used to maintain the parasites.*T. b. brucei* was obtained from stabilates at Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna, Kaduna State, Nigeria andmaintained in the laboratory by continuous passage in rats until required. Passages were considered necessary when parasite levelreaches 16 – 32 parasites per field (usually 3 - 5 days post infection). In passaging, 1×10^6parasites was introduced intraperitoneally into rats in 0.1 - 0.2 ml blood/PBS solution.

3.1.3 Determination of the parasitemia in the infected animals

Parasitemia wasmonitored in blood obtained from the tail, pre-sterilizedwith methylated spirit. The number of parasites wasdetermined microscopically at ×400 magnification using the "Rapid Matching" method of Herbert and Lumsden (1976). Briefly, the method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with phosphate

buffered saline (PBS, pH 7.2). Logarithm values of these counts obtained by matching with the table of Herbert and Lumsden (1976) wasconverted to antilog which provided theabsolute number of trypanosomes per ml of blood.

3.2 Methodology

3.2.1 Isolation of the parasites

Trypanosomes were separated from the blood by the method of Lanham and Godfrey (1970). At peak parasiteamia, experimentally infected rats were sacrificed and the blood collected into an EDTA container by cardiac puncture using a 1 ml syringe. The blood was diluted with 10 ml phosphate buffered saline glucose (pH 8.0) and loaded onto a column packed with DEAE-52 cellulose. The parasites were eluted with the same buffer. Fractions containing parasites were pooled together and centrifuged at 10,000 ×g for 15min. The pellets, which contain only the parasites were re-suspended with phosphate buffer glucose saline and stored at -4°C.

3.2.2 Molecular identification of trypanosome species

3.2.2.1 DNA extraction (DNeasy Blood and Tissue kit, Qiagen)

The DNA extraction was performed as described by the Chechet, (2015). Exactly 100 µlanticoagulated blood was pipetted into 1.5/2ml microcentrifuge tube. Proteinase K (20µl) was added and adjusted to 220µl with PBS followed by the addition of200 µllysis buffer vortexing for thorough mixing before incubation at 56°Cfor 10 minutes. Two hundred microliter ofethanol (96–100%) was added to the solution and mixed thoroughly by vortexing. The mixture was pipetted into the DNeasy Mini spin column placed in a 2ml collection tube and centrifuged at 6000 ×g for 1 minute using a refrigerated centrifuge (Eppendorf). The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed into a new 2ml collection tube and 500µl buffer was added, and centrifuged for 1 minute at 6000 ×g. The eluent

and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2ml collection tube, and 500µl buffer was added, and the tube was centrifuged for 3 minutes at 20,000 ×g to dry the DNeasy membrane. The DNeasy Mini spin column was again placed in a clean 1.5ml microfuge tube, and 200µlElution Buffer (AE) was pipetted directly onto the DNeasy membrane. The tube was incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000×g to elute.

3.2.2.2 Primers

The following primers were used (Chechet, 2015) for the parasite identification.

Name	Primer Sequence 5'-3'
1424	AAG CCA AGT CAT CCA TCG
1423	TAG AGG AAG CAA AAG
1426	TGC AAT TAT TGG TCG CGC
1425	CTT TGC TGC GTT CTT

3.2.2.3 Nested PCR

Two consecutive PCR reactions were carried out: a set of outer primers (1425 and 1426) was used in the first round reaction, followed by inner primers (1423 and 1424) in the second round. One microliter of the PCR product from the first round reaction was added in the second round reaction in a fresh PCR tube. The nested PCR was performed using a 25µl reaction volume containing 100 ng of template DNA, 25µM of each primer, 10mMdNTPs, 25mM 10× Dream Taq polymerase buffer, 5U/µl Dream Taq polymerase to a reaction volume of 25µl. Cycling conditions were as follows: 1 cycle at 95°Cfor 300 seconds followed by 30 cycles at 95°Cfor 60 seconds, 56°Cfor 60 seconds, 72°Cfor 30 seconds, and final extension 72°Cfor 220 seconds. The products were resolved by electrophoresis (45 minutes to 1 hour at 100V) on 1.5% Agarose gels stained with G-Stain.

3.2.3 Preparation of ubiquinol-1 (UQ1H2) from ubiquinone-1 (UQ1)

The quality of Ubiquinol-1 [2,3-Dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinol] is critical to the overall study of TAO kinetics. This is due to the ability of ubiquinol-1 to quickly re-oxidize to either the partially reduced (semiquinone or ubisemiquinone) form, or the fully oxidized (ubiquinone) in the presence of molecular oxygen. Owing to this fact, the following procedure was carefully followed with all precautions duly observed. Three bottles of UQ1 [2,3-Dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4- benzoquinone] corresponding to 10 mg/bottle were emptied into 3 mL of absolute ethanol, then transferred to a clamped separatory funnel containing 15 mL of0.25 M sucrose, 0.1 M sodium phosphate buffer pH 7.5. This was followed by the addition of 2 mL of the first separating solvent, cyclohexane, the mixture was subjected to N_2 (g) bubbling. An equal volume of the second separating solvent, ethanol (2 mL) was added to the mixture. This was followed by the addition of aspatula-full of the reducing agent Dithionite, and the separating funnel with its content subjected to N_2 flushing. The mixture was vigorously mixed until the hexane part became clear, and a phase separation appeared in ~ 2 – 3 minutes. After the lower phase had been let down the funnel, the upper phase (hexane phase) was collected with a Pasteur pipette into a pre-weighed (W1) clean 20 mL Pyrex Eggplant-type flask. Because the ubiquinol formed would still be trapped in the aqueous phase, and to exhaustibly extract the UQ_1H_2, the ethanol phase was returned to the separating funnel after which 2 mL of fresh hexane was added to it, and it was mixed vigorously and left until the phase separation appeared. The hexane phase was collected and added to that previously collected in the pre-weighed (W1) clean 20 mL Pyrex Eggplant-type flask followed by N_2 flushing. This process was repeated three more times to exhaustibly extract the trapped UQ_1H_2. The content of the Pyrex Eggplant-type flask (UQ_1H_2 in hexane) was vacuum dried in a rotary evaporator set at 40°C until the content became syrupy and there was no more hexane left. The flask was re-weighed (W2)

and the weight of UQ1H2 was calculated as W2 – W1. This was followed by the addition of 2 mL acidified ethanol to recover the ubiquinol from the flask. The concentration was determined spectrophotometrically at 290 nm after the spectrophotometric values were fitted to Beer-Lambert's law using an extinction coefficient of 5.0 $mM^{-1}cm^{-1}$. Aliquots were made in 5 mL amber bottles, and were deoxygenated with N_2 flushing before storage at -20 °C.

3.2.4 Enzyme inhibition assay

The derivatives of C-9154 antibiotic were evaluated as inhibitors of TAO activity using the ubiquinol oxidase assay described by Kido *et al.* (2010). In the assay, 1 ml of the reaction mixture contain 987.5 µl of Tris-HCl buffer (pH-7.3), 10 µl of the derivatives, 1µl (15 ng/µl) TAO. The activity was measured by recording the absorbance change of 150 µM ubiquinol (1.5µl) at 278 nm, in the presence or absence of test compound at 25 $^{\circ}C$ for two minutes at 30 seconds interval. The enzyme activity was determined by recording the change of absorbance per time.

3.2.5IC_{50} determination

The IC_{50} of the compound that inhibited the enzyme (inhibitor) was determined by varying the concentration (10, 1. 0.1 0.01 and 0.001) of the inhibitor and the enzyme activity was plotted against the concentration. Graphpad prism software (5.03) was used to determine the IC_{50} usingnon-linear regression plot.

3.2.6 Enzyme kinetic studies

The effect of the inhibitors on the kinetics of the TAO was determined by varying the concentration of the substrate and the inhibitors. Lineweaver Burk plot was used to determine the

Km and $V_{max}$of the uninhibited enzyme and for the diagnosis of the pattern of TAO inhibition by the compound.

3.2.7 *In vitro* test for trypanocidal activity

Assessment of *in vitro* trypanocidal activity was carried out as described by Atawodi*et al.* (2003). Infected blood was collected by cardiac puncture from a donor rat at peak parasitemia (1×10^6 parasite per ml) and transferred into EDTA coated bottle and phosphate buffer glucose saline maintained on ice pack. Aliquots of 10 µl of the compounds (100 µM) was incubated with 90 µl of the infected blood. The compound was replaced with phosphate buffered saline (pH 7.4) and diminazemeaceturate for the negative and positive controls respectively. After 5 minutes of incubation, 2 µl of test mixture was placed on microscope slides and the motility of the parasites was observed under the microscope (Mg×400) at 5 minutes interval for an hour. Same procedure was performed for the isolated parasites and the inhibitors with 5 mM glycerol. Cessation or drop in motility of the parasites in the inhibitor treated blood compared to that of parasite-loaded control blood without inhibitor was taken as a measure of trypanocidal activity.

3.2.8 Computational molecular docking studies

Models of respective derivatives of C-9154 antibiotic were obtained using Chemdraw software. The compound models were converted to their respectivethree dimensional (3D) structures using Sparntan software.The 3Dcrystal structure of *Trypanosoma brucei brucei* Alternative Oxidase (TbbTAO) with ascofuranone (PDB ID: 3VVA) was downloaded from PDB (http://www.rcsb.org/pdb/) andTbbTAO chain A was selected. This 3D compound model structures was docked with TbbTAO chain A which was generated through molecular docking analysis. Implemented docking program, PyRx software withAutodock 4 (http://pyrx.sourceforge.net, Scripps Institute) was used to predict and compare the binding positions of the compounds on the enzyme.The search efficiency was set at maximumto examine the docking conformational space comprehensively. The highest binding affinity (the lowest docking energy) score was chosen to explore the binding mode of docked compounds in the

TbbTAO using PyRx program. Nine conformers were considered for the analysis of the docking calculations for each ligand-macromolecule complex, and the resulting docking clusters was calculated with a 2.0Å root mean squared deviation (RMSD) tolerance on the heavy atoms. The two-dimensional (2D) and 3D molecular interaction models of the docked compounds on the TbbTAO and receptor surface shape modeling (involving hydrogen bonding) was displayed using the DS v. 4.5 and pymolsoftwares (Kim and Ryu, 2016).

3.3 Statistical Analysis

Data recording was done on Microsoft excel. Analyses were carried out using both descriptive and inferential statistics with mean and standard deviation (SD) respectively. Microsoft Excel and Word in Windows 2010 was used for graphs and tables. T-test was used to test for significant differences in means of groups. $P \leq 0.05$ was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Inhibitory Activities of C-9154 Antibiotic Derivatives on TAO

The inhibitory activities of C-9154 antibiotic derivatives (10 µM) on TAO revealed that fourteen of the compounds significantly ($P< 0.05$) inhibited the enzyme although three of the derivatives activated it with compound **32** eliciting the highest inhibitory activity ($P= 0.001$) when compared with the control (Figure 4.1).

4.2 IC_{50} Determination

The result of the inhibitory activity showed that compound **32** has the highest statistical significance ($P< 0.05$) when compared with the control. The IC_{50} of this compound on TAO was determined to be 0.41 µM (Figure 4.2).

4.3 Inhibition Kinetic Studies of the Most Potent Inhibitor

The inhibition kinetics of the most potent inhibitor showed a competitive inhibition with K_m, $V_{max}$and K_i values of 393.3 µM,70.9 µmol/min/mg protein and 1.25 µM, respectively (Figure 4.3).

4.4 *In vitro* Test for Trypanocidal Activity of the Compounds on *T. b. brucei* and *T. congolense*

In vitro test for trypanocidal activity in the presence of the test compounds on *Trypanosoma brucei brucei*and *Trypanosoma congolense*(Figure 4.5).

The parasites were seen motile (× 400 magnifications) in the blood (*T. b. brucei)* (untreated) throughout the period of the experiment (Plate 4.2). When the parasites were incubated with standard drug (DiminazeneAceturate) there was total cessation of motility. (Plate 4.3). Plate 4.4

shows the isolated parasite (*T. congolense)*. Plate 4.5 shows non motile parasite (*T. b. brucei)* incubated with compound **32** and glycerol.

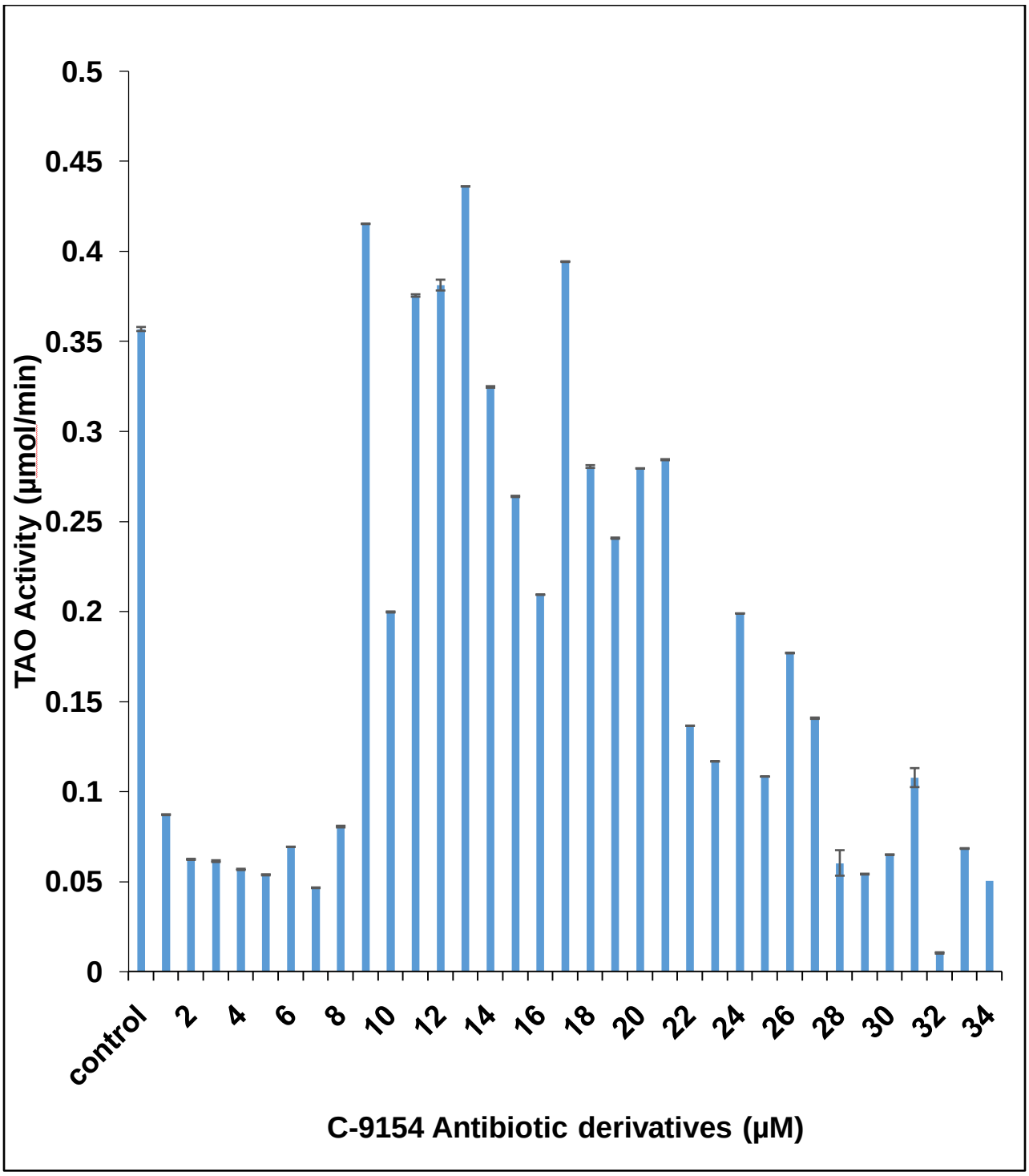


Figure 4.1: Inhibitory activities of C-9154 Antibiotic derivatives on TbbTAO

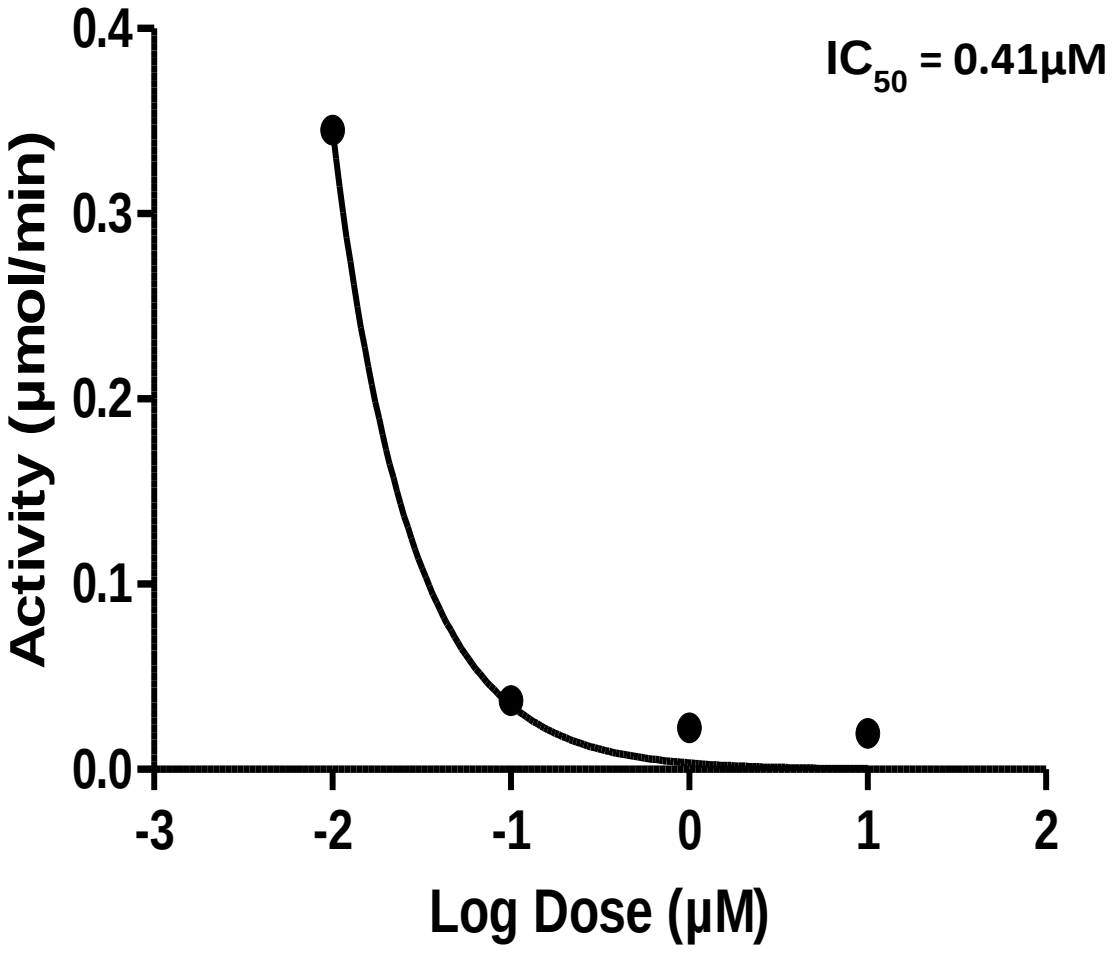


Figure 4.2: Dose response curve showing the IC_{50} of the most potent inhibitor

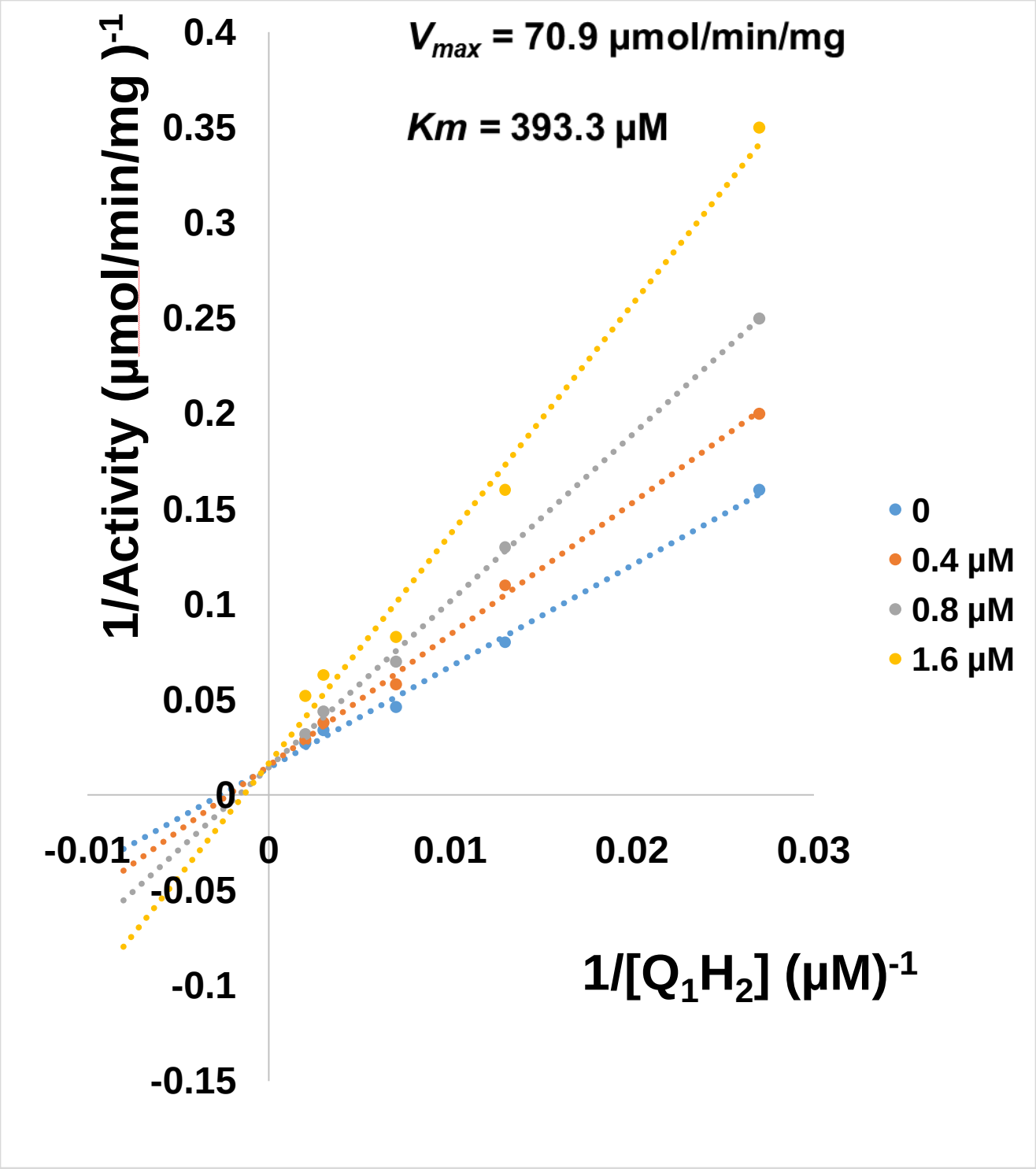


Figure 4.3: Double Reciprocal plot showing the mode of inhibition of compound 32 on TbbTAO

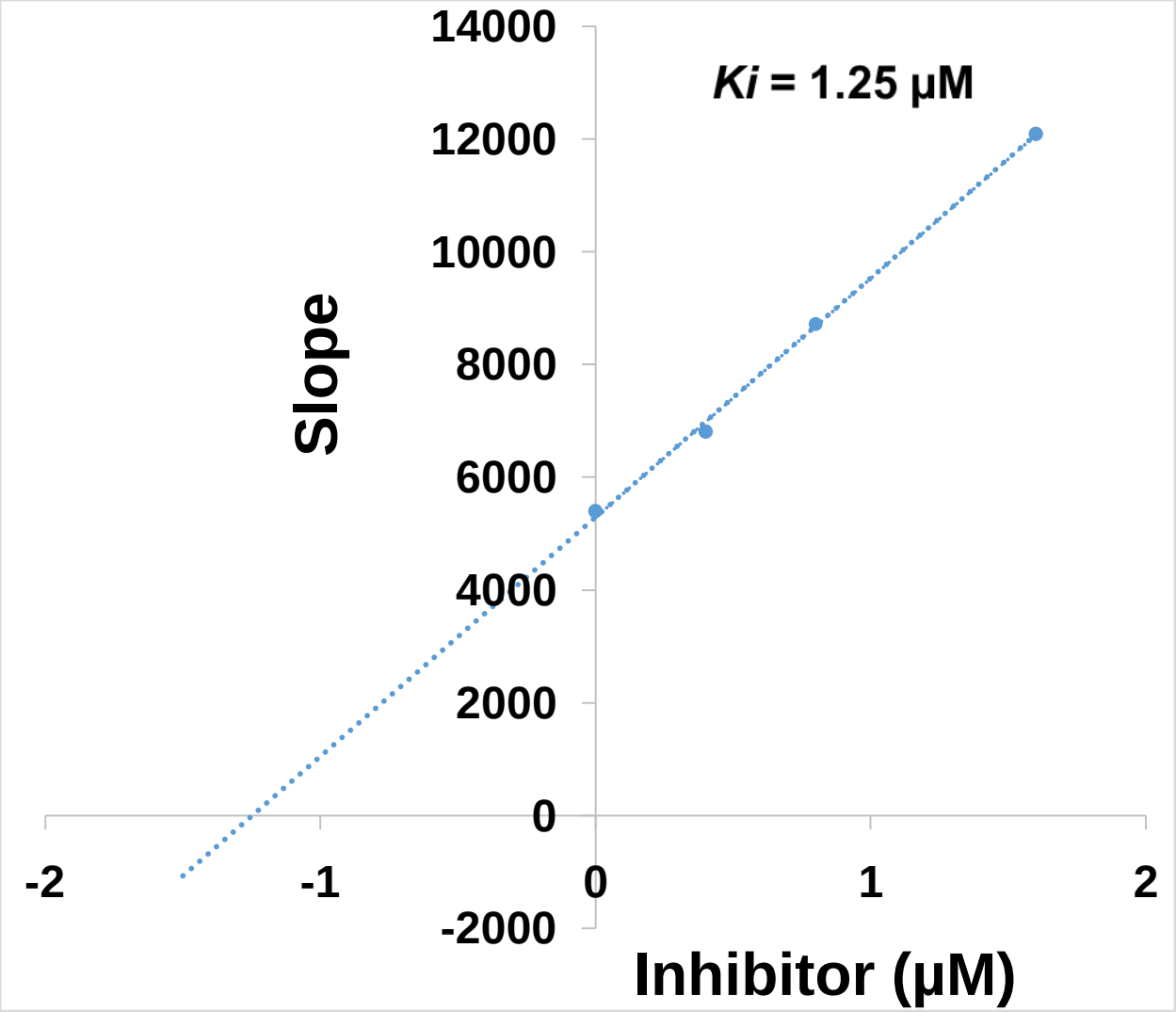


Figure 4.4: Dixon secondary plot showing the K_i of the most potent inhibitor on TbbTAO



Plate 4.1: Agarose gel electrophoregramshowing PCR result; Lane M is the DNA marker, 1 is positive for *T. b. brucei,* 2 is positive for *T. congolense* and 3 is negative

Table 4.1. Expected band size

ITS-1 Band size	Expected species base on ITS
640	*T. congolense savanna*
560	*T. congolense kilifi*
430	*T. brucei*
380	*T. simiae*

Adams *et al*. 2006

Table 4.2: *In vitro* trypanocidal activity of Derivatives of C-9154 antibiotic on trypanosomes

S/No	Compounds	Time (min) after which motility ceased, reduced drastically(+) or reduced slightly(++) with the same concentration of the inhibitor(μM)			
		T.b.brucei	*T.b.brucei* + 5mM glycerol	*T. congolense*	*T. congolense* + 5 mM glycerol
		100 μM	100 μM	100 μM	100 μM
01	1	-	30^{++}	-	-
02	2	-	-	-	-
03	3	-	-	-	-
04	4	-	-	-	-
05	5	-	-	-	-
06	6	50^{++}	-	-	-
07	7	-	-	-	-
08	8	30^{++}	-	-	-
09	9	-	-	-	-
10	10	-	-	-	-
11	11	-	40^{+}	20^{++}	-
12	12	-	-	-	-
13	13	-	-	-	-
14	14	-	-	-	-
15	15	-	-	-	-
16	16	-	-	-	-
17	17	-	-	-	-
18	18	25^{++}	-	-	-
19	19	15^{++}	-	-	-
20	20	-	-	-	-
21	21	-	40^{+}	-	-
22	22	-	-	-	-
23	23	-	-	-	-
24	24	-	-	-	-
25	25	-	-	-	-
26	26	-	-	-	-
27	27	-	-	-	-
28	28	-	-	-	40^{+}
29	29	-	-	-	-
30	30	-	-	-	30^{++}
31	31	-	-	-	-
32	32	-	50^{DEAD}	-	55^{+}
33	33	-	-	-	-
34	34	-	-	-	-

KEY

- = No noticeable effect on motility after 60 minutes

++ = slightly reduced motility

+ = drastically reduced motility

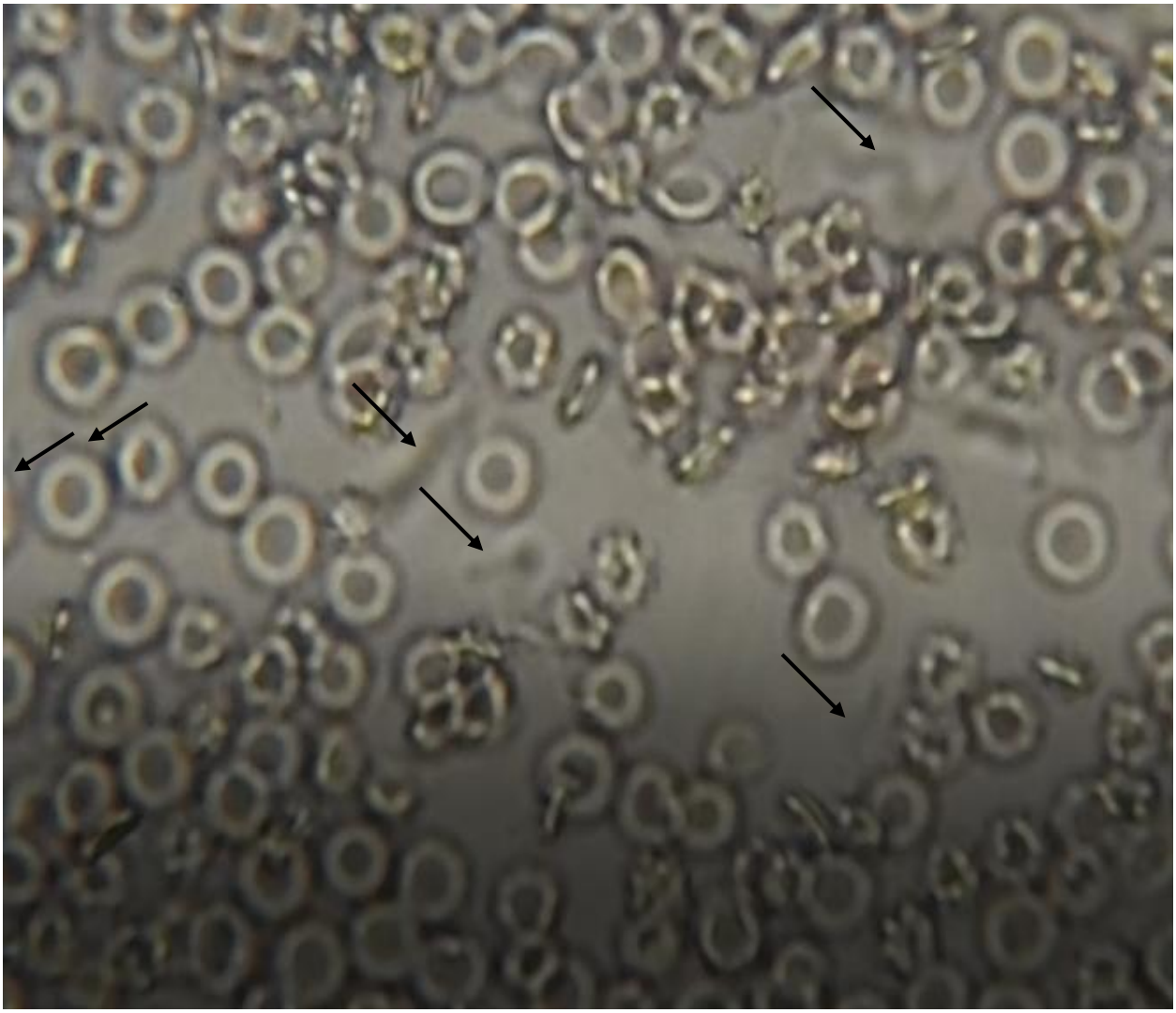


Plate 4.2: Wet mount image of *T. b. brucei* infected blood control(× 400 magnifications).

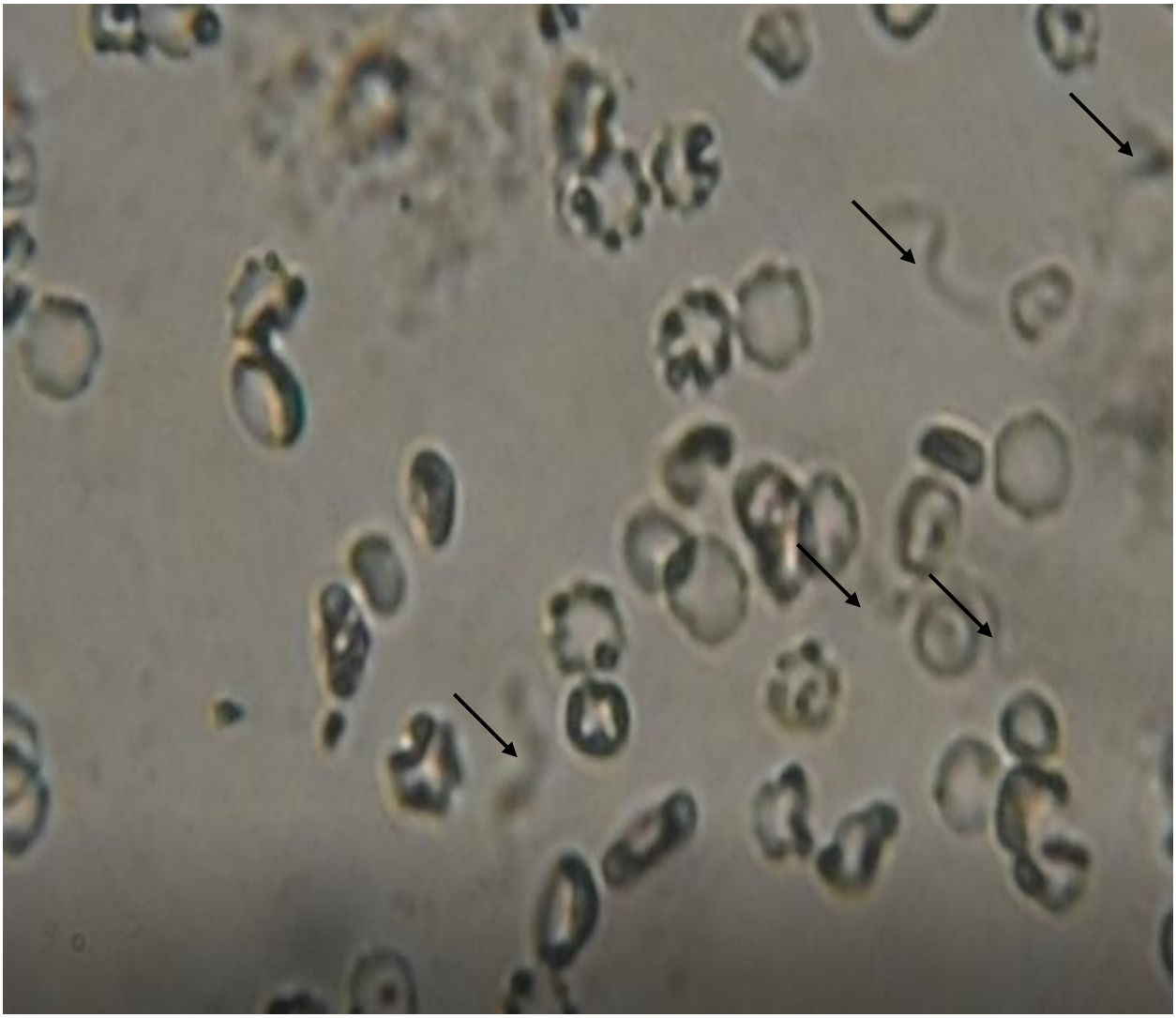


Plate 4.3: Wet mount image of *T. b. brucei* infected blood incubated with DiminazeneAceturate (positive control) (× 400 magnifications).

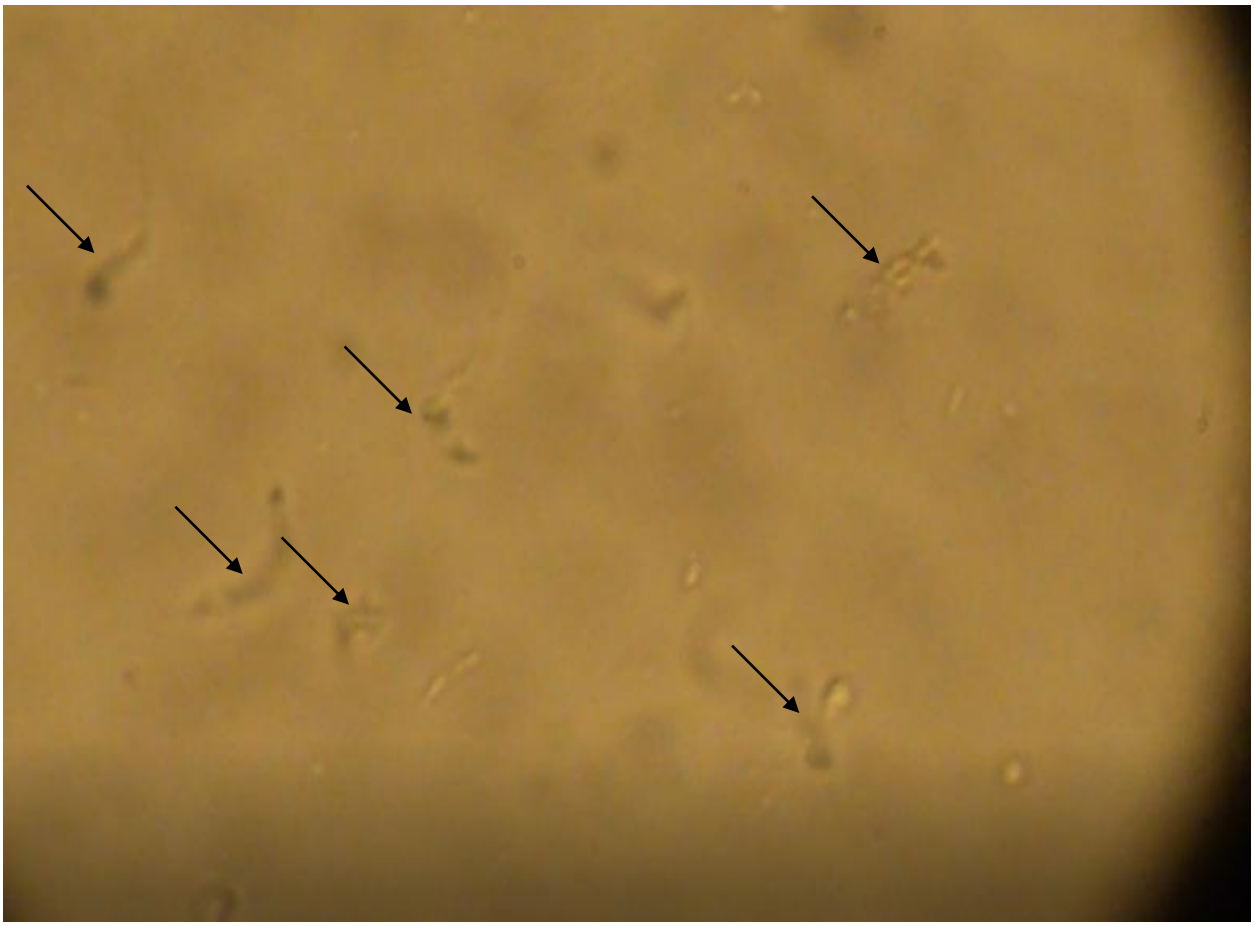


Plate 4.4: Wet mount image of isolated parasite (*T. congolense*) (× 400 magnifications).

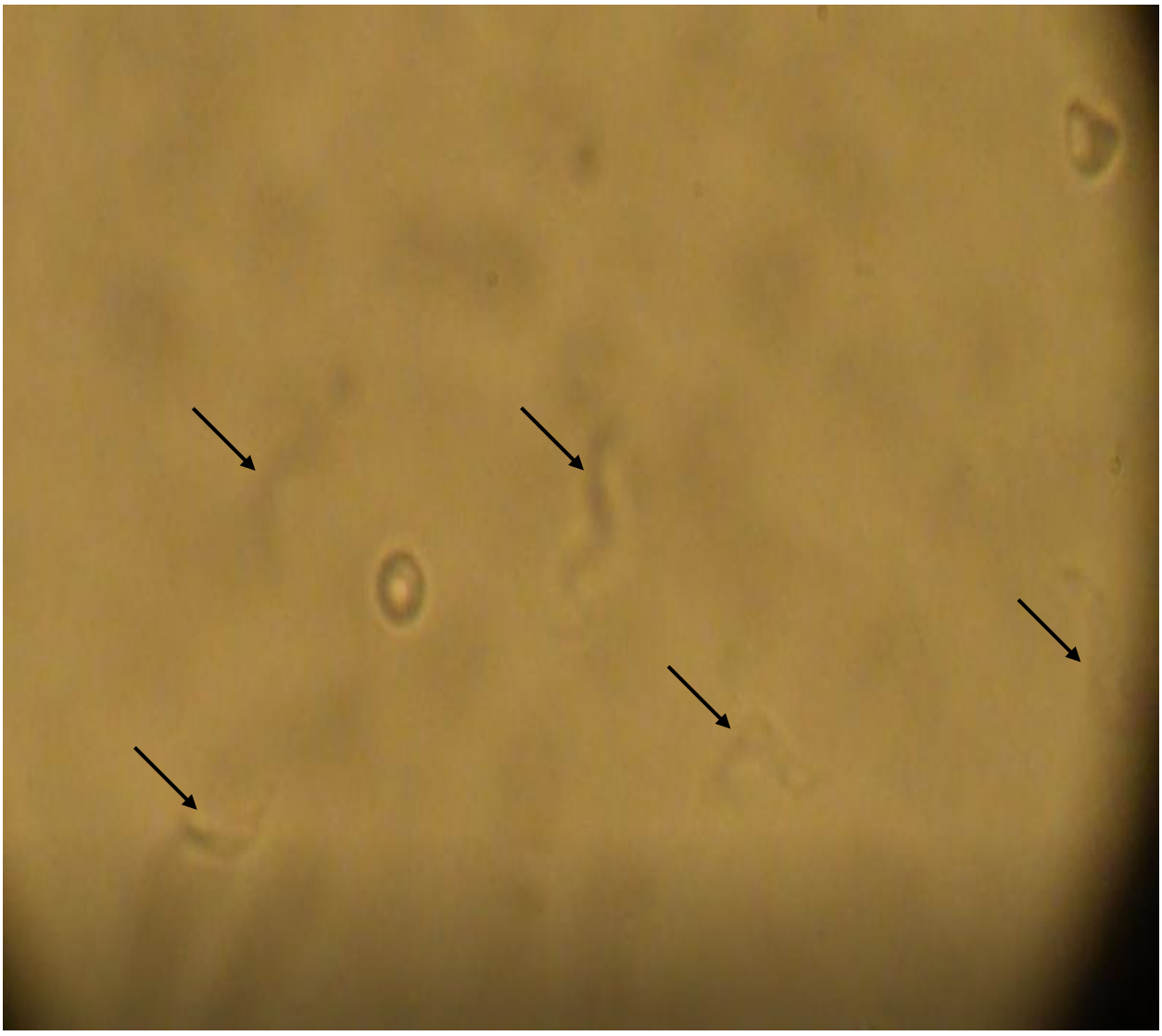


Plate 4.5: Wet mount image of isolated parasite (*T. b. brucei*) incubated with the compound 32 and glycerol (× 400 magnifications).

4.5 Three Dimensional (3D) Structure of *Trypanosoma brucei*TAO

The three dimensional (3D) structure of *Trypanosoma brucei* TAO was retrieved from protein data bank (PDB) with assertion number RCSB PDB -3VV9 (Figure 4.5).

4.6 Molecular Docking Studies of C-9154 Antibiotic Derivatives with *Trypanosoma brucei* TAO

Molecular docking interaction between derivatives of C-9154 antibiotic and *Trypanosoma brucei* TAO revealed the predictive binding energies (kcal/mol) of the ligands on the receptor with compound **32** having the lowest binding energy (-9.0 kcal/mol) (Figure 4.6).

4.7 Molecular Docking Interaction of Compound 32 on *Trypanosoma brucei*TAO

Molecular docking interaction between compound **32** with *T.brucei* TAO revealed the formation of a stable complex in the enzyme catalytic pocket as shown in figures 4.9-4.10. The predictive binding mode of the compound in the enzyme active site suggests strong interactions between the compound and Arg118, Thr219, Leu122, and Leu212 (Figure 4.7).



Figure 4.5: Three Dimensional (3D) structure of *Trypanosoma brucei* Alternative oxidase

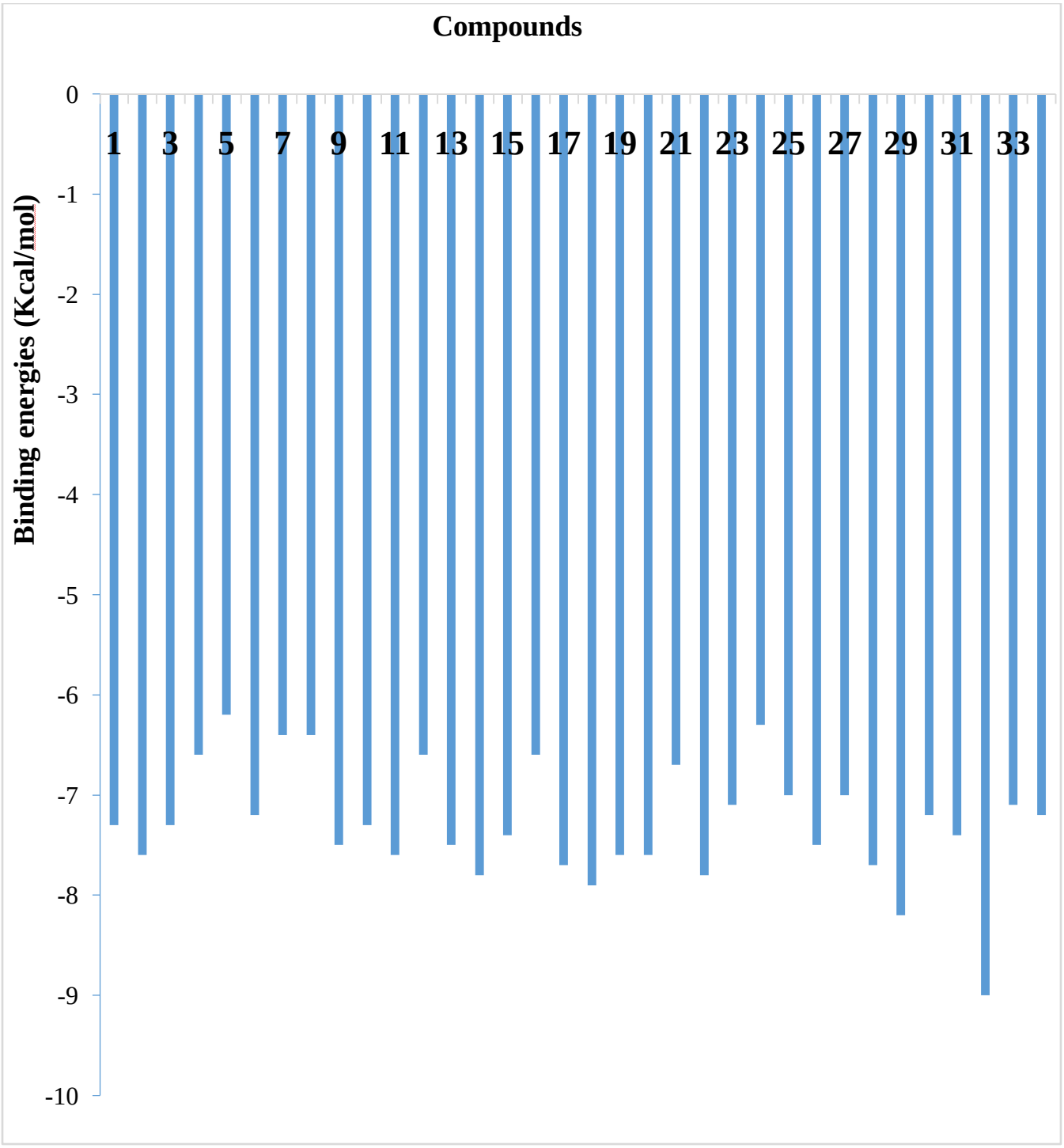


Figure 4.6:Binding affinities of C-9154 Antibiotic Derivatives on TbbTAO

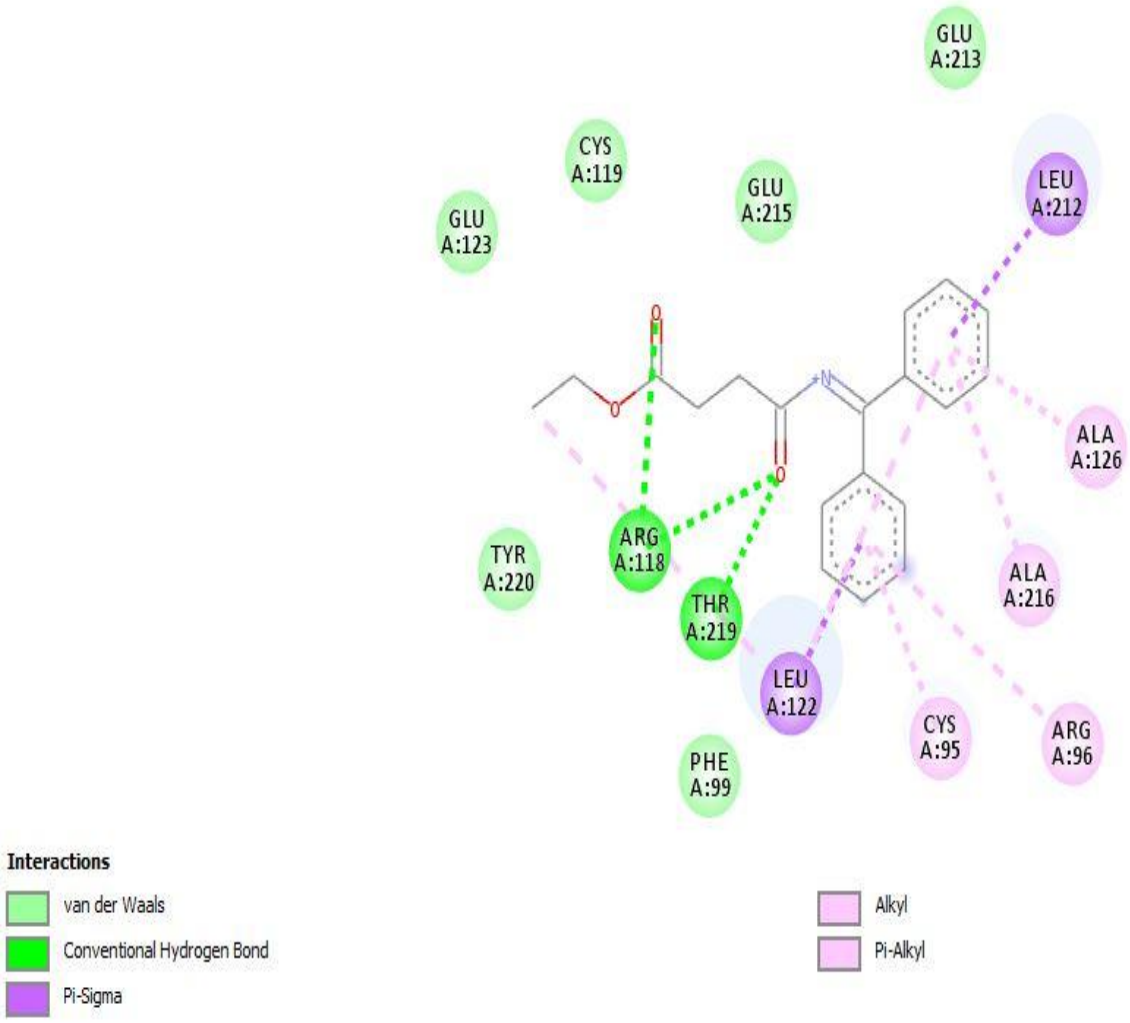


Figure 4.7: 2-D Molecular Docking interaction between the most potent inhibitor and TAO

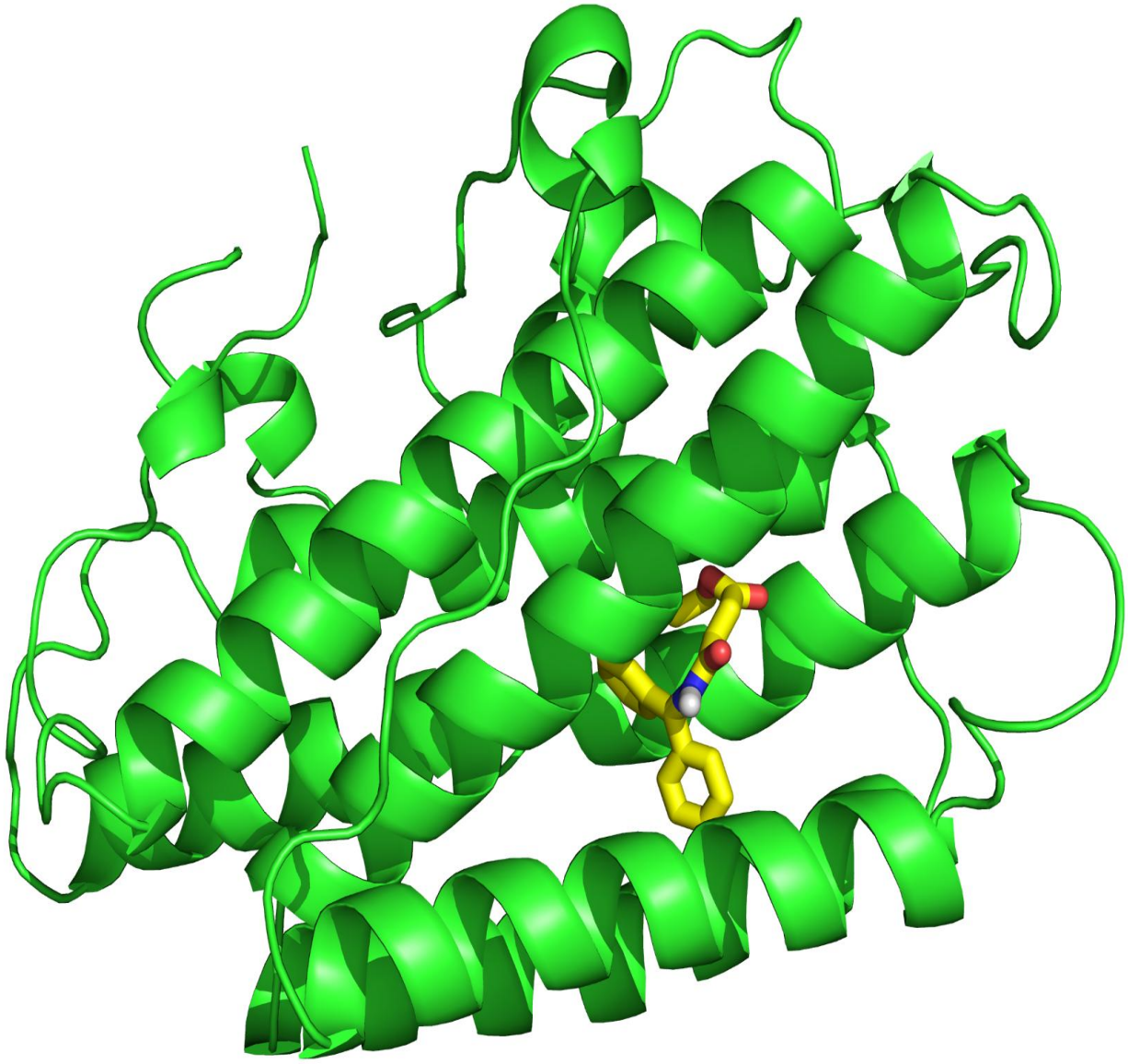


Figure 4.8: 3-D Cartoonmodel interaction between compound 32 and the TAO

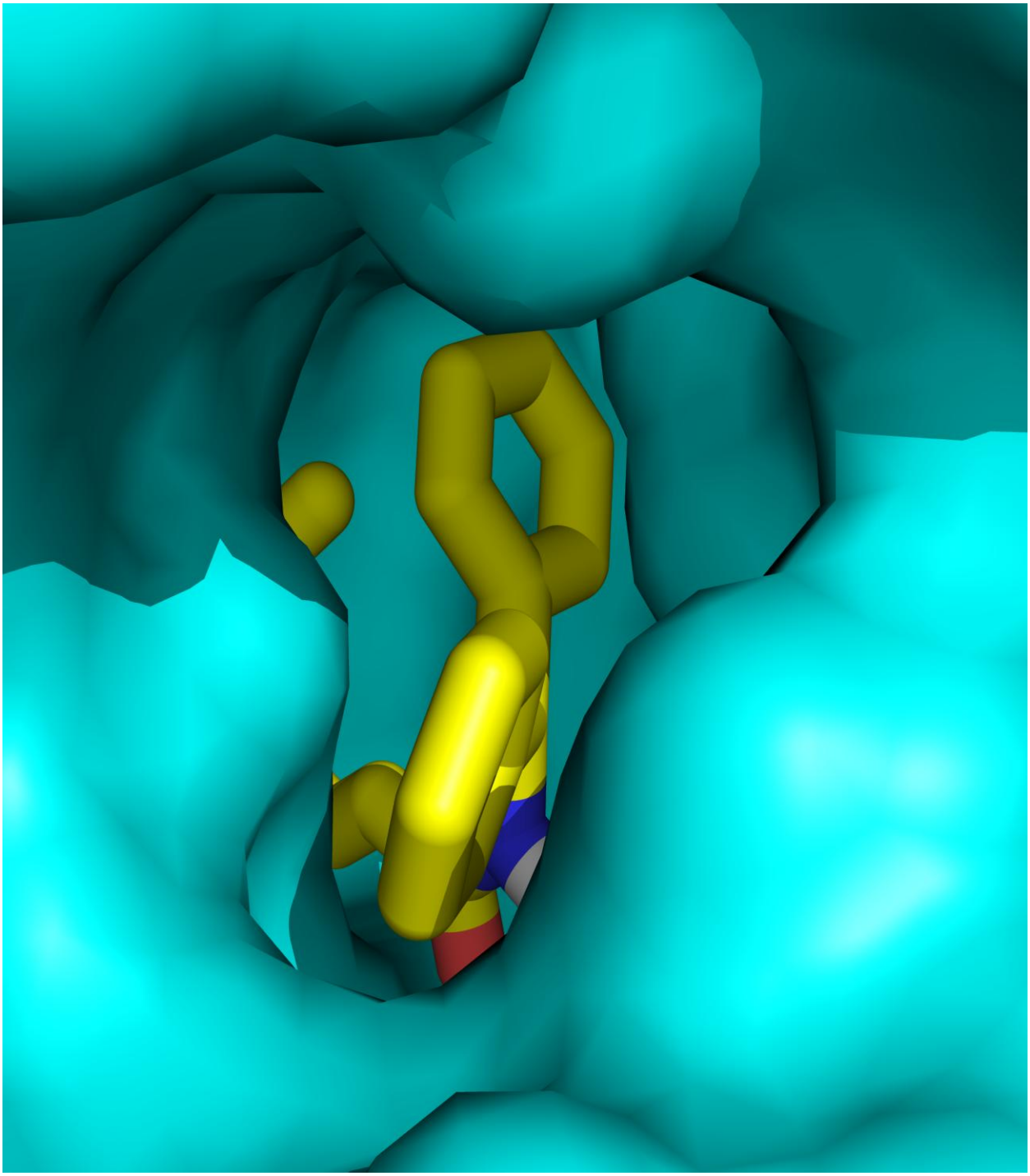


Figure 4.9: 3-D surface model of compound 32 and TAO.

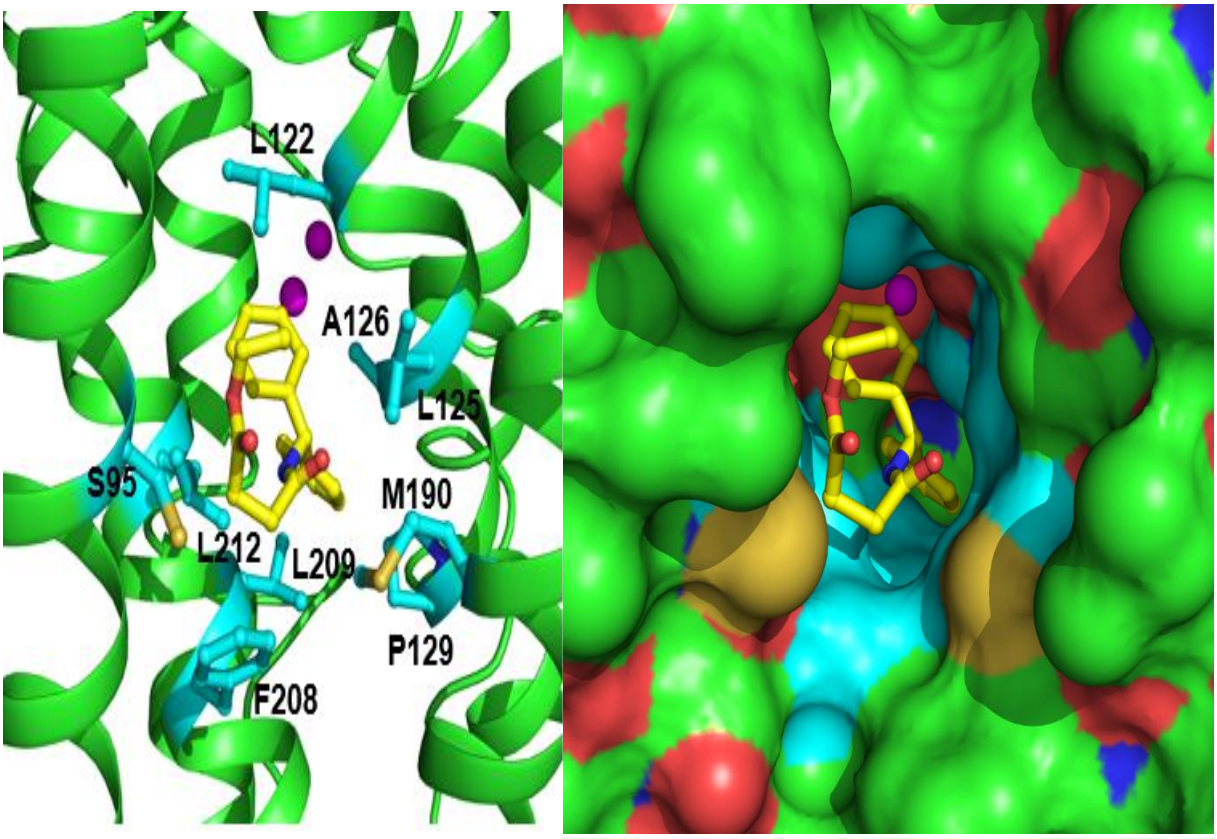


Figure 4.10: Interaction of compound 32 at the TAO active site with the residues around 4 Å

CHAPTER FIVE

5.0 DISCUSSION

The trypanosomal alternative oxidase has been validated as atarget for discovery of drug against African trypanosomiasis. At present, literatures revealed that only ascofuranone, SHAMand their derivatives inhibit this enzyme (Clarkson and Brohn, 1976; Grady *et al.,* 1993; Gonzalez *et al*., 2017; West *et al*., 2017, Ebiloma*et al*., 2018). Although these compounds have been known for their inhibitory activities *in vitro* and *in vivo* over two decades,not much progress has been made towards the development of a potential drug agents from these chemicals. In an attempt to search for new inhibitors againstTAO, thirty four (34) derivatives of C-9154 antibiotic were synthesized with structural modification to improve desirable qualities or remove undesirable properties from the parent compound (C-9154 antibiotic). Such modifications include increasing the chain length and introducing other side chains aimed at increasing their stability in aqueous blood environment and increasing their permeability across lipid bilayer of the membranes.

These compounds were screened for their inhibitory activities on TAO. Although three of the derivatives activated TAO, fourteen of the derivatives inhibited the enzymewith respect to the control (Figure 4.1) with compound **32**significantly ($p<0.05$) having the highest effect. The concentration of the compound that inhibited the oxidation of ubiquinol by oxygen (IC_{50}) of this compound was further determined to be 0.41 µM. In drug discovery, the acceptable cut off for inhibitory compounds to be further developed is 10 µM, implying that compound **32** is a potential drug candidate that may be useful against trypanosomiasis.

Results for the kineticstudiesrevealed a competitive inhibition pattern for compound **32** on TAO,implying that the compound binds only to the free enzyme. This pattern is similar to what was recently reported for the new class of SHAM derivatives designed by Ebiloma*et al.,* (2018) but displaying a different property when compared to the mechanism of ascofuranonne and

derivatives (Shiba*et al*., 2013). Also, it has a K_M of 393.3 µM and a V_{max} of 70.9 µmol/min/mg. The inhibition binding constant (K_i) which is the measure of potency of an inhibitor was estimated as 1.25 µM. Although this value is relatively high when compared to ascofuranone and the new series of SHAM derivatives, the result is still encouraging considering that the hit compound is from a primary screen effort. In order to develop compound **32**further, there is a need to conduct structure-activity relationship studies aimed at arriving at nanomolar potency derivatives.

In this study, it appears reasonable to speculate that inhibition of TAO can only have static action since it is unable to block the energy metabolism of the parasite completely. This agrees with the findings of (Helfert*et al*., 2001) which shows that inhibition of TAO only blocks the aerobic respiration of the parasite, hence the need to co-administer the inhibitor with glycerol.The reason for the requirement for glycerol along with the inhibitor is that the combination inhibits both the aerobic and anaerobic pathways for ATP production *i.e.*the ability of the parasite to use the reverse reaction of glycerol kinase (GK) to be able to produce 1 mole of ATP that is sufficient for the parasite to survive is inhibited when the TAO dependent aerobic respiration is inhibited. In the presence of 5 mM glycerol, it was observed that the motility of the parasites completely ceased.

Molecular docking is one of the most frequently used methods in drug discovery because of its ability to predict, with a substantial degree of accuracy, the conformation of small-molecule ligands within the appropriate target binding site (Meng*et al.,* 2011). Molecular docking has been proved very safe and efficient tool for novel drug discovery for targeting protein. It is a reliable cost effective and time saving technique as it searches for an appropriate ligand that fits both energetically and geometrically into the protein's binding site (Daniel and De Groot, 2010). In the computational molecular docking analysis, the 3-D structure of *T. b. brucei*TAO (TbbTAO)

was downloaded from protein data bank (PDB). The current study looks at the screening of C-9154 antibiotic derivatives on TAO and the possible binding mode of the inhibitor on the enzyme using Schrodinger and Autodocks docking software. Indeed, the results obtained showed that compound **32** has the highest binding affinity (lowest binding energy) compared to the other derivatives (Kim and Ryu, 2016). This result agrees with the earlier objective where the same compound was found to have the highest inhibitory activity on the enzyme. Also, a closer look at the predictive 2-D interaction between the ligand and the receptor shows that the ligand binds at the enzyme active site (Shiba*et al*., 2013) with hydrogen bonding interaction between R118, and T219. There was also a predictive hydrophobic interaction between the two aromatic rings of the ligands and the receptor on L112, L212, C95, R96, A126, and A216. The more negative ΔG_{Bind} value indicates more stability of ligand-protein complex. Since this is the first reported findings of predictive molecular docking interaction between derivatives of C-9154 antibiotic against any receptor, it is challenging to point out clearly or link this findings with previous investigation.

In addition, it was revealed from the 3-D interaction that the compound was able to occupy the catalytic pocket of the enzyme. This is very interesting as it agrees with the results of the inhibition kinetic study that the compound is able to bind the enzyme via competitive inhibition. Though there is lack of structural similarity between this compound and the substrate (ubiquinol), it is possible for the compound to occupy the active site which could lead to change in conformation of the enzyme and prevent the substrate from binding.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

In this study, thirty four derivatives of the antibiotic C-9154 were screened for their inhibitory activities on TAO and also for their trypanocidal potentials. Some of the derivatives were found to significantly reduce the activity of the enzyme with compound **32** having the highest effect. Investigation into the inhibition kinetics showed that the compound inhibited the enzyme competitively with K_m, $V_{max}$and K_i values of 393.3 µM,70.9 µmol/min/mg and 1.25 µM, respectively. Molecular docking studies was further performed to predict the possible binding mode of the most potent inhibitor (Compound **32**) on the crystal structure of the TbbTAO, revealed a strong interaction at the enzyme active site. Finally, *in vitro* test for trypanocidal activity was performed by checking drop or cessation in motility in the presence of the test compounds on *Trypanosoma brucei brucei*and *Trypanosoma congolense*.

6.2 Conclusion

In this study, some derivatives of C-9154 antibiotic were shown to significantly inhibit TAO. The enzyme inhibition kinetics of the most potent inhibitor showed that it inhibited the enzyme competitively. These findings agrees with the results obtained from the predictive molecular docking studies where compound **32** was found to have the best binding affinity. A closer look at the mode of interaction showed that the compound binds to the enzyme catalytic pocket.

Also from the study, some of the derivatives where found to reduce the motility of *T. b. brucei* and *T. congolense* in the presence and absence of glycerol.

6.3 Recommendation

1. *In vivo* studies should be carried out with compound **32** to ascertain its trypanocidal potential.
2. Structural optimization of the compound should be performed and further structure activity relationship.
3. Toxicological analysis should also be performed to determine the safety of the compound.

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APPENDICES

APPENDIX 1.0

Contribution to knowledge

This is the first time enzyme studies was performed on the derivatives of C-9154 antibiotic. The inhibitory activities was established with compound **32** eliciting the highest inhibitory potency.

The inhibition kinetic studies of compound **32** was also established showing the mode of inhibition, K_m, V_{max} and K_i.

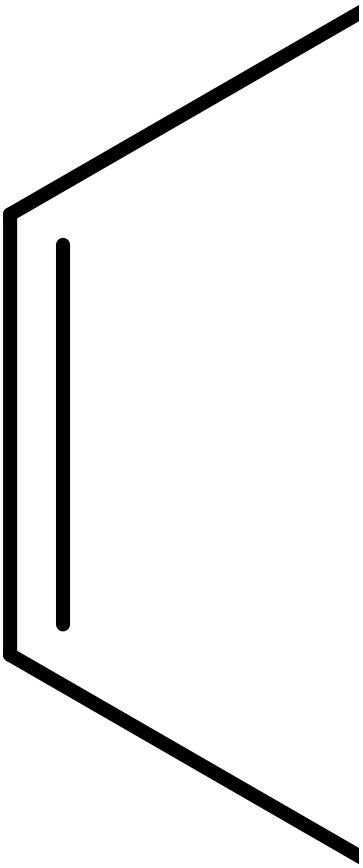
In addition, the predictive mode of interaction of the most potent inhibitor was ascertained occupying the TAO catalytic pocket with strong interaction at the active site

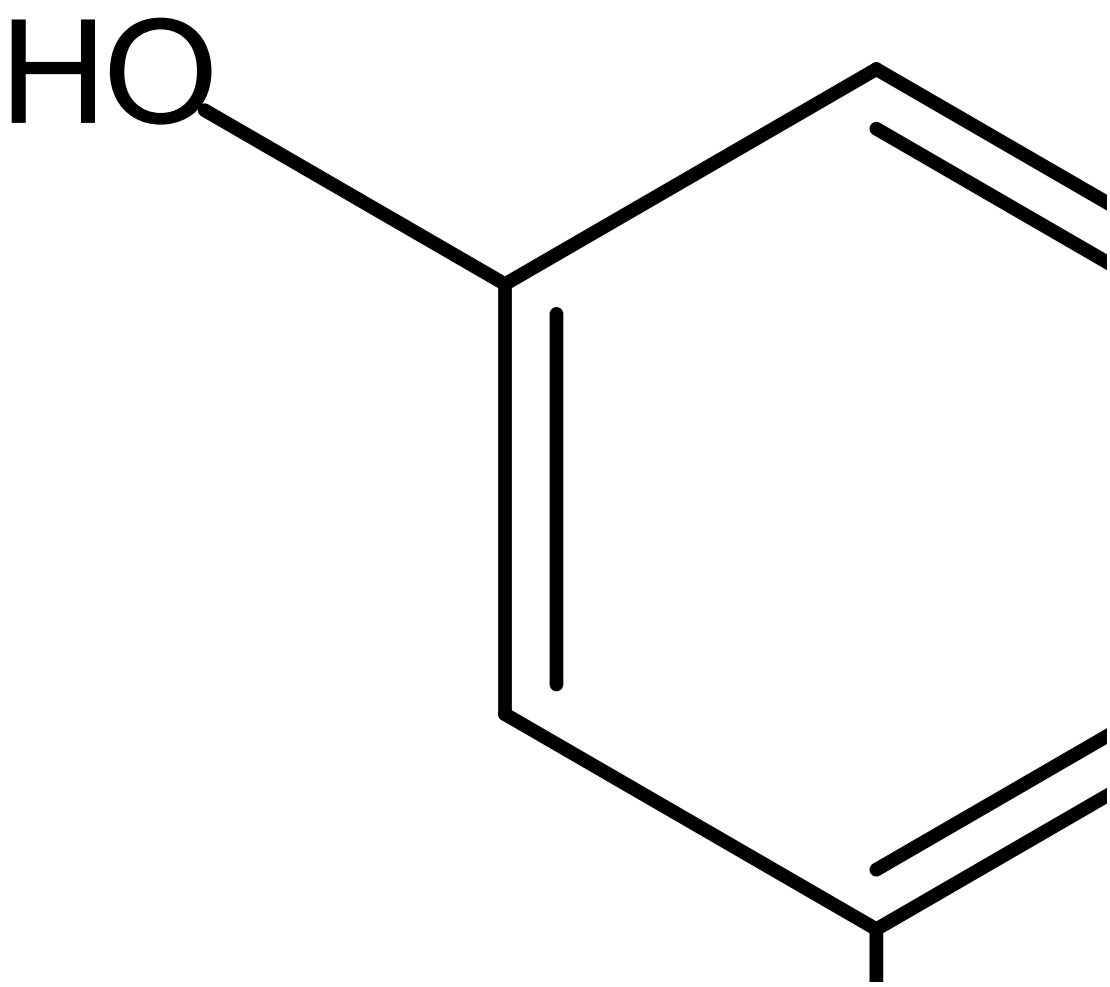
Finally, study also established the trypanocidal potentials of C-9154 antibiotic derivatives.

APPENDIX 2.0

Structures of C-9154 antibiotic derivatives

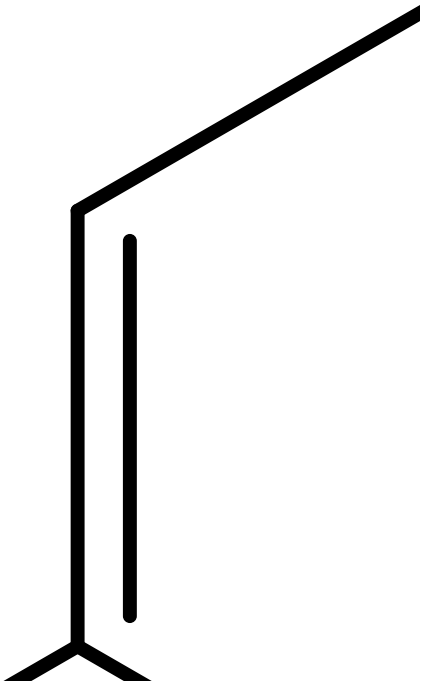
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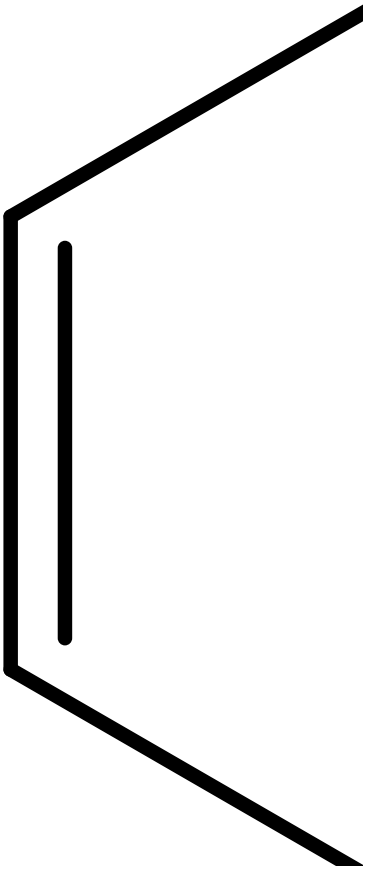

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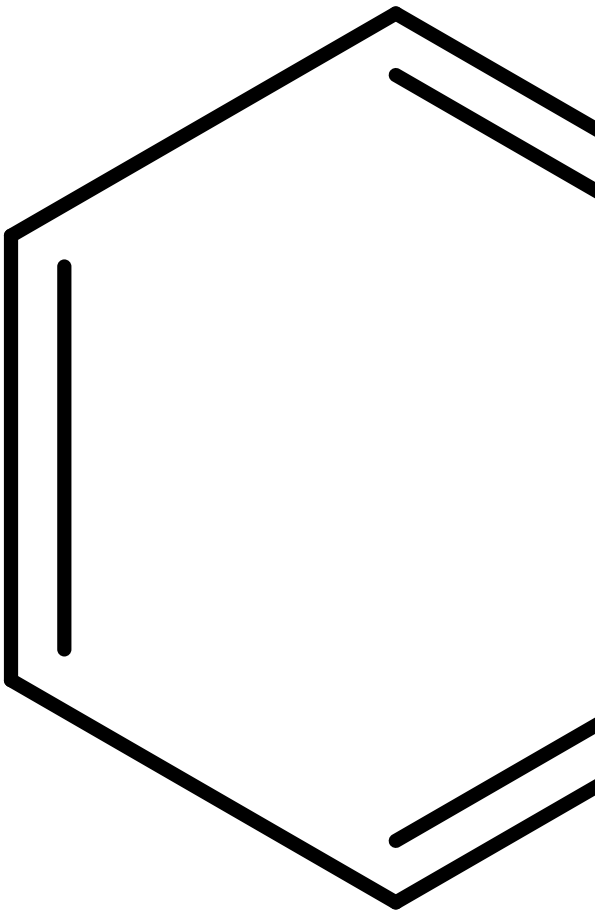


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APPENDIX 3.0

Preparation of Reagents

3.1 Preparation of C-9154 antibiotic derivatives

To prepare 1 mM stock of each of the (34) compounds, the mass (g/dm^3) of each of the compound was determined. 1% (10 µL) DMSO and 990 µL was used to dissolve each of the compounds.

3.2 Preparation of 50 mMTris Buffer

About 121.14 g ofTriswas dissolved in 800 ml distilled water. The pH was adjusted to 7.3 with the appropriate volume of concentrated HCl. The final volume was made up to 1 liter with distilled water.

3.3 Preparation of Phosphate Buffer Saline

0.1 mM phosphate buffer pH 8.0that was used for this work was prepared by dissolvingof di-sodium hydrogen phosphate (Na_2HPO_4) and sodium di-hydrogen phosphate (NaH_2PO_4) and 9 grams of sodium chloride (NaCl) in 1 litre of distilled water. The pH of the solution was adjusted to a desired pH.