

The synthesis of δ -amides of eflornithine to improve oral bioavailability

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"Nothing in the world can take the place of persistence. Talent will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education alone will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent. The slogan "press on" has solved and always will solve the problems of the human race."

John Calvin Coolidge

Abstract

The oral route of drug administration has for years remained the mainstay drug delivery route because of its ease of use and good patient compliance. Orally administered drugs need to pass through a number of barriers before entering the systemic blood circulation. The biological membranes of the gastro-intestinal tract are lipophilic in nature and contain various proteins responsible for active or facilitated transport of polar and large molecules. Apart from active and facilitated transport, passive diffusion is one of the major absorption processes for most drugs. Water soluble drugs have a greater difficulty in crossing these membranes due to their hydrophilic nature compared to their lipophilic counterparts. For hydrophilic molecules, in order to passively cross the lipophilic membranes, they need to be rendered lipophilic. One way to address this problem is through linkage of the hydrophilic drugs to lipophilic moieties.

Human African trypanosomiasis (HAT), or sleeping sickness, is a vector-borne parasitic disease caused by protozoa of the species *Trypanosoma brucei*. HAT is responsible for 40 000 to 50 000 deaths each year. The disease covers 15% of Africa's population with 0.5 to 0.8% of that population contracting the disease each year. There are currently only 4 drugs (suramin, pentamidine, melarsoprol and eflornithine) approved for the treatment of HAT. The latest of these drugs, eflornithine, was approved 20 years ago.

Eflornithine (DFMO) is a selective irreversible inhibitor of ornithine decarboxylase (ODC), an enzyme responsible for polyamine synthesis in humans and trypanosoma. Eflornithine is the second line treatment for late stage *T. b. gambiense* infections or melarsoprol relapse patients. The drug is very hydrophilic and is primarily administered intravenously which contributes to it being expensive and labour intensive. Eflornithine can however be given orally but is not favoured due to a low oral bioavailability of 54%. Consequently the drug needs high doses to achieve the minimum effective concentration of 50 μ M in the brain. This is explained by the hydrophilic nature of the drug limiting its oral absorption as well as transport over the blood-brain barrier.

The object of this study was to synthesise lipophilic amides of DFMO, determine their physicochemical properties, evaluate their intrinsic activity and assess their oral absorption in an attempt to improve the bioavailability of this drug.

Seven δ -amides were synthesised by means of acylation whereby lipophilic moieties were attached to the δ -amino group of eflornithine through amide bond formation. The structures of the products were confirmed by nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS).

The aqueous solubility of DFMO (control) and all its derivatives were determined experimentally in phosphate buffer (pH 7.4) at 37 °C. All the derivatives except 2-amino-2-(difluoromethyl)-5-acetamidopentanoic acid demonstrated a decrease in water solubility ranging from 28 to 19 mg/ml compared to that of DFMO (34.96 mg/ml), which corresponds to an increase in log D in the range of 4.6 to 9.47 mg/ml. 2-amino-2-(difluoromethyl)-5-(2-phenylacetamido)pentanoic acid (S_w = 11.13 mg/ml, log D = -0.07) was the most lipophilic and was therefore expected to be the most absorbed. The biggest increases in lipophilicity were observed with aryl-containing derivatives.

The *in vivo* oral absorption tests conducted at the University of Göteborg, Sweden, were done on Sprague-Dawley rats after oral administration of the compounds. Blood samples were drawn and analysed with HPLC. Results for the compounds tested showed no metabolism into eflornithine, possible due to the stable amide bond. The *in vivo* results do not represent the concentration of the synthesised compound but that of eflornithine in the blood stream. Thus no conclusive evidence was attained to confirm oral absorption.

T. b. brucei was used to determine the intrinsic activity of the synthesised compounds *in vitro* and was expressed as IC_{50} values. 2-amino-2-(difluoromethyl)-5-propanamidopentanoic acid and 2-amino-2-(difluoromethyl)-5-[(4-methoxyphenyl)formamido]pentanoic acid showed a moderate increase in activity of 32.05 and 35.45 μ M respectively, compared to that of eflornithine (36.22 μ M).

No correlation was found between physicochemical properties, oral absorption and intrinsic activity. The study does, however, prove that derivatisation can influence the lipophilicity. Only 2-amino-2-(difluoromethyl)-5-propanamidopentanoic acid and 2-amino-2-(difluoromethyl)-5-[(4-methoxyphenyl)formamido]pentanoic acid showed an increased lipophilicity and intrinsic activity.

Opsomming

Na al die jare word die orale roete weens die gemaklike toediening van geneesmiddels en goeie pasiëntmeewerkendheid nog steeds as die eenvoudigste roete van toediening gesien. Orale geneesmiddels moet eers deur verskeie hindernisse beweeg voordat dit die bloedsirkulasie bereik. Die biologiese membrane van die spysverteringstelsel is lipofilies van aard en bevat verskeie proteïene verantwoordelik vir die aktiewe en gefasiliteerde transport van polêre and groot molekules. Behalwe aktiewe en gefasiliteerde transport bly passiewe diffusie die mees algemene meganisme van absorpsie. Die absorpsie van wateroplosbare geneesmiddels word grootendeels beperk deur die polêre aard van die molekules. Vir 'n molekule om deur middel van passiewe diffusie geabsorbeer te word, moet dit dus meer lipofiel wees. Een manier om hierdie probleem aan te spreek, is deur lipofiele derivatisering.

Menslike Afrika tripanosomiase (MAT) of slaapsiekte is 'n vektor-gedraagde parasitiese siekte wat veroorsaak word deur protosoë wat deel van die spesie *Trypanosoma brucei* is. MAT is verantwoordelik vir 40 000 tot 50 000 sterftes per jaar. Die siekte affekteer 15% van Afrika se bevolking waarvan 0.5 tot 0.8% jaarliks geïnfekteer word. Daar is tans slegs vier geneesmiddels wat vir die behandeling van MAT goedgekeur is. Die jongste middel van hierdie vier, eflornitien, is 20 jaar gelede goedgekeur.

Eflornitien (DFMO) is 'n selektiewe onomkeerbare antagonist van ornitiendekarboksilase (ODK), 'n ensiem verantwoordelik vir poli-amiensintese in die mens en tripanosome. Eflornitien word gebruik as tweedeliniebehandeling vir laat fase *T. b. gambiense*-infeksies of in pasiënte wat nie op melarsoprol reageer nie. Die geneesmiddel word hoofsaaklik intraveneus toegedien wat arbeidsintensief is en tot die hoë koste van die behandeling bydra. Eflornitien kan oraal toegedien word, maar dit het 'n lae orale biobeskikbaarheid van slegs 54%. Gevolglik moet die geneesmiddel teen hoë dosisse gegee word om die minimum effektiewe konsentrasie van 50 μM in die brein te bereik. Hierdie lae biobeskikbaarheid word deur die hidrofiliese karakter van die geneesmiddel verklaar wat die orale absorpsie sowel as transport oor die bloed-breinskans benadeel.

Die doel van hierdie studie was om lipofiele amiede van DFMO te sintetiseer, die fisies-chemiese eienskappe daarvan te bepaal, die intrinsieke aktiwiteit te evalueer en die orale

absorpsie te assesser in 'n poging om die biobeskikbaarheid van die geneesmiddel te verbeter.

Sewe δ -amiede is deur asilering gesintetiseer waartydens lipofiele kettings deur die vorming van 'n amiedbinding aan die δ -aminogroep van eflornitien gekoppel is. Die strukture van die produkte is deur kernmagnetiseresonansiespektrometrie (KMR) en massaspektrometrie (MS) bevestig.

Die wateroplosbaarheid in fosfaatbuffer (pH 7.4) by 37 °C van DFMO (kontrole) en al die derivate is eksperimenteel bepaal. Al die derivate, behalwe 2-amino-2-(difluoormetiel)-5-asetamidopentanoësuur, het 'n laer wateroplosbaarheid (28 tot 19 mg/ml) as DFMO (34.96 mg/ml) wat met 'n verhoging in log D in die gebied van 4.6 tot 9.47 mg/ml korreleer.

Dit is verwag dat die mees lipofiele derivaat, 2-amino-2-(difluoormetiel)-5-(2-fenielasetamido)pentanoësuur ($S_w = 11.13$ mg/ml, log D = -0.07) die grootste absorpsie sal vertoon. Die grootste toename in lipofiliteit is by arielbevattende derivate waargeneem.

Die *in vivo* orale absorpsie toetse van die derivate is geëvalueer by die Universiteit van Göteborg, Swede, deur gebruik te maak van Sprague-Dawley rotte. Bloedmonsters is geneem en geanaliseer deur HDVC. Die resultate van die monsters het geen metabolisme na eflornitien getoon nie weens moontlike stabiele amiedbinding. Die *in vivo* resultate verteenwoordig nie die konsentrasie van die gesintetiseerde derivaat nie, maar die konsentrasie van eflornitien in die bloedstroom. Geen duidelike getuigenis om orale absorpsie te staaf is gevind nie.

T. b. brucei is gebruik om die *in vitro* intrinsieke aktiwiteit, uitgedruk as IK_{50} waardes, van die gesintetiseerde middels te bepaal. Twee middels, 2-amino-2-(difluoormetiel)-5-propanamidopentanoësuur en 2-amino-2-(difluorometiel)-5-[(4-metoksifeniel)formamido]pentanoësuur, het elk met IK_{50} waardes van 32.05 en 35.45 μ M respektiewelik 'n effense hoër intrinsieke aktiwiteit as eflornitien (36.22 μ M) getoon.

Geen korrelasie tussen die fisies-chemiese eienskappe, orale absorpsie en intrinsieke aktiwiteit is gevind nie. Die studie toon wel dat derivatisering 'n invloed op lipofiliteit het. Slegs 2-amino-2-(difluoormetiel)-5-propanamidopentanoësuur en 2-amino-2-(difluoormetiel)-5-[(4-metoksiefeniel)formamido]pentanoësuur het 'n hoër lipofiliteit en intrinsieke aktiwiteit as eflornitien getoon.

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List of Abbreviations

Å - Angstrom

°C – Degrees celcius

μA - Microampere

amu – Atomic mass units

APCI – Atmospheric pressure chemical ionisation

ATP – Adenosine triphosphate

Bat – Broad-scope amino acid transporter

BBB – Blood-brain barrier

Cat – Cationic amino acid transporter

CATT – Card agglutination test for *T. b. gambiense*

CNS – Central nervous system

CSF – Cerebrospinal fluid

d – Doublet

DALY – Disability adjusted life years

DCM - Dichloromethane

DFMO – Difluoromethylornithine

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DRC – Democratic Republic of Congo

DSC – Differential scanning calorimetry

ELISA – Enzyme-linked immunosorbent assay

F_{abs} – Fraction absorbed

G. – Glossina

HAT – Human African trypanosomiasis

HIA – Human intestinal absorption

HPLC – High pressure liquid chromatography

IFA – Immunofluorescent assays

IC₅₀ – 50 % Inhibitory concentration

IgM – Immunoglobulin M

INF- γ – Interferon gamma

IU/ml – International units per millilitre

LDL – Low density lipoproteins

Log D – Logarithmic partition coefficient at specific pH

m – Multiplet

Mel T – Melarsen oxide trypanothione

MHz – Megahertz

Mp – Melting Point

MS – Mass spectroscopy

M_w – Molecular weight

N – Normality

NMR – Nuclear magnetic resonance

NO – Nitric oxide

ODC – Ornithine decarboxylase

PCR – Polymerase chain reaction

Pgp – P-glycoproteins

PLP – Pyridoxyl 5' phosphate

ppm – Parts per million

PBS – Phosphate buffer solution

q – Quartet

rpm – Revolutions per minute

s – Singlet

SAMDC – S-adenosylmethionine decarboxylase

SEM – Standard error of mean

spp. – Subspecies

t – Triplet

T. b. – Trypanosoma brucei

TLC – Thin layer chromatography

TLTF – T-lymphocyte triggering factor

TMS – Tetramethylsilane

TNF- α – Tumour necrosis factor alpha

t_R – Peak retention time

USFDA – United States Food and Drug Administration

V – Volts

VSG – Variant surface glycoproteins

v/v – Volume per volume

WHO – World Health Organization

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Chapter 1

Introduction and Aims of the Study

1.1 Introduction

Human African trypanosomiasis (HAT), or sleeping sickness, is a tropical disease which is transmitted through the bites of tsetse flies infected with the trypanosoma parasite. The disease was controlled in the 1960s, but due to political conflict in most of the infected countries and lack of human and/or financial resources invested in combating the disease, the disease re-emerged in the 1980s (WHO, 1998). The extent of the re-emergence is such that the prevalence of the disease is now the same as it was in the 1920s (Barrett, 1999; Delespaulx and De Koning, 2007). As a result 15% of Africa's population is at risk with 0.5 to 0.8% of that population contracting the disease each year (Barrett *et al.*, 2007; Seed, 2000; WHO, 1998). Countries hit hardest by this epidemic are Sudan, Uganda, Democratic Republic of Congo (DRC) and Angola. In the 1990s, 2% of the DRC population had 70% prevalence in certain communities (Barrett, 1999). The WHO estimates the annual deaths due to HAT to be between 40 000 and 50 000 each year (WHO, 2006). Although 50% of all newly diagnosed cases are fatal, the number of deaths is fortunately on the decline (Barrett, 1999; Bogitsh *et al.*, 2005). In the late 1990s, the cost of treating HAT with eflornithine was an estimated US\$ 750 per patient (Barrett, 1999). In the current financial and health crisis situation, HAT is still regarded as a major problem and remains a serious threat.

The disease is caused by two species of extracellular protozoa: *Trypanosoma brucei rhodesiense* and *T. b. gambiense*. *T. b. rhodesiense*, which is prevalent in eastern and southern African countries, causes the acute form of the disease while *T. b. gambiense*, primarily found in West and central Africa, causes the chronic form (Simarro *et al.*, 2008; WHO, 1998). HAT has two stages, namely an early or haematolymphatic stage and a late or meningoencephalitic stage. The early stage is usually undiagnosed and non symptomatic but characterised by swollen lymph nodes behind the neck (Winterbottom's sign). As the parasite crosses the blood-brain barrier and migrates to the brain, the disease progresses to the late stage which is distinguished by wide spread neurological symptoms such as muscle weakness and sleep disturbances (Bogitsh *et al.*, 2005; WHO, 1998). Eventually coma and death ensue (Katz *et al.*, 1989). The disease will only progress into the second stage, which is usually fatal, if the first stage is left untreated (Terada and Inui, 2004).

Only four drugs that have been approved in the past century are available for the treatment of HAT. Suramin (1922) and pentamidine (1937) are used for early stage treatment while melarsoprol (1949) and eflornithine (1990) remain the drugs of choice for late stage infections (Bouteille *et al.*, 2003). Nifurtimox (mid 1970s) is considered a third alternative against late stage infection, especially in melarsoprol refractory patients. Studies have shown a low efficacy rate of mono therapy against *T. b. rhodesiense* infections (Bouteille *et al.*, 2003; De Koning, 2001; Delespaux and De Koning, 2007; Phillips and Stanley, 2001). Melarsoprol is a highly toxic arsenical drug and causes fatal encephalopathy in 5-10% of patients treated (Balasegaram *et al.*, 2009). There is also a growing resistance against melarsoprol with a relapse rate of 30% in certain endemic areas (Balasegaram *et al.*, 2009; De Koning, 2001; Matovu *et al.*, 2001).

Eflornithine (DFMO) was initially synthesized as an antitumor agent, but was later found to have activity against early and late stage trypanosome infections (Bacchi and Yarett, 2002; Bouteille *et al.*, 2003). Eflornithine is a selective irreversible inhibitor of ornithine decarboxylase (ODC) (Bouteille *et al.*, 2003; Denise and Barrett, 2001; McCann and Pegg, 1992). The drug is primarily used in severe second stage infections or melarsoprol refractory patients. Conversely, eflornithine is only effective against *T. b. gambiense* infections (Burri and Brun, 2003; Phillips and Stanley, 2001). Eflornithine is a highly hydrophilic drug with a log D value of -0.82 (pH 7.4). Due to this hydrophilicity, the drug's transport over the blood-brain barrier is very low. Consequently a dosing regimen of 400 mg/kg of body weight divided into four intravenous doses administered each day for 14 days are needed to obtain the minimum effective concentration of 50 μ M in the brain (Burri and Brun, 2003; Phillips and Stanley, 2001; WHO, 1998). Pharmacokinetic studies found that orally administered eflornithine had a racemic plasma concentration of approximately 50% of that observed for intravenous administration (Na-Bangchang *et al.*, 2004). The costs of treating patients with eflornithine are very high and the mode of administration creates difficulties in rural conditions (Balasegaram *et al.*, 2009). The physicochemical (log D) and pharmacokinetic properties (bioavailability, intrinsic activity) of DMFO makes derivatisation with lipophilic moieties a prospect to be investigated to improve its bioavailability.

The log D value is a good indication of the ability of a molecule to cross biological membranes. A log D value of lower than 5 is required for good oral absorption or permeation (Lipinski, 2000). Another important property is the aqueous solubility of a drug. Because log D is an indication of lipophilicity, aqueous solubility was used as a qualitative

measure to prove the increase in lipophilicity. Derivatisation, by substituting lipophilic moieties to the δ -amino group of eflornithine, may increase the log D consequently increasing both oral absorption and bioavailability theoretically. Intrinsic activity refers to the ability of a molecule to elicit a pharmacological response. Derivatisation also creates new potential lead compounds. These compounds may have an increased efficacy, better stability or reduced adverse effects.

1.2 Aims of the study

The primary objective of this study was to synthesise a series of new derivatives of the anti-trypanosomal drug eflornithine, and to evaluate their oral absorption and intrinsic activity.

In order to achieve this objective, the following aims were set:

- Screen proposed compounds to be synthesised and tested.
- Synthesise a series of δ -amides of eflornithine and confirm their structures.
- Experimentally determine the physicochemical properties such as the partition coefficient and aqueous solubility of eflornithine and its synthesised derivatives and compare them with calculated values from commonly used prediction software.
- Experimentally determine the oral absorption for eflornithine and its synthesised derivatives *in vivo*.
- Experimentally determine the intrinsic activity of eflornithine and its synthesised derivatives *in vitro* on eflornithine sensitive *T. b. brucei* strains.
- Examine the effect of derivatisation on oral absorption and intrinsic activity.

Chapter 2 gives a literature overview on HAT and gastrointestinal absorption. Chapter 3 contains an article for submission and includes a short introduction, research methodology, results and discussion. Chapter 4 provides an overall summary of the study as well as future prospects envisaged during the study.

Chapter 2

Literature Overview

2.1 Introduction

Of all the diseases plaguing the world, Africa always seems to be hit the hardest. Malaria, tuberculosis and HIV have been prevalent in Africa for many years. Research done on treatment, vaccines and quarantine methods has led to a better understanding and control of these infectious diseases. But somehow in the shadow and aftermath of AIDS and its devastating effects on a country's economical, social and nutritional well-being, the world has forgotten about one disease in particular that is becoming a growing problem in Africa, namely human African trypanosomiasis. Human African trypanosomiasis (HAT) is fatal if left untreated. Although four drugs are currently available to treat this fatal disease, their chemotherapy remains unsatisfactory. The substandard treatment of HAT and limited drug availability, stresses the importance of new research and treatment strategies. Improving pharmacokinetic and pharmacodynamics properties of current drugs can be a possible solution.

The study of pharmacokinetics involves the kinetics of pharmaceutical drugs in terms of absorption, distribution, metabolism and elimination in the body. Pharmacokinetics utilizes experimental and theoretical methods to analyse, interpret and model the behaviour of drugs in the body. It is usually studied in conjunction with pharmacodynamics, which refers to the relationship between the drug concentration at the site of action and the pharmacologic response it elicits. This link between pharmacokinetics and pharmacodynamics is crucial in drug development, dose optimization and drug characterization (Shargel and Yu, 1999).

2.2 Gastrointestinal absorption

Oral administration is the most common drug delivery route. Due to its good compliance it is usually preferred by patients. For a drug to be absorbed into the general blood circulation, it must pass through or between more than one layer of cells. The amount of active drug that enters the systemic circulation after administration is referred to as bioavailability (Silverman, 2004). The permeability of a drug is closely related to its molecular structure and to the physical and/or biochemical properties of the cell membrane. Most drugs enter the cell by

transcellular absorption where the drug moves across the cell. Some polar drugs are not able to move across the cell, but instead pass through gaps between cells, a process called paracellular absorption. The fluid mosaic model explains transcellular diffusion of polar molecules. The cell membrane contains globular proteins embedded in a lipid bilayer matrix (Figure 2.1). These proteins provide the means for selective transport of polar molecules through the lipid bilayer (Shargel and Yu, 1999).

In principal small compounds can either be absorbed paracellularly or transcellularly. Transcellular absorption can further be divided into passive and carrier mediated processes (Breves *et al.*, 2007; Narawane and Vincent, 1994).

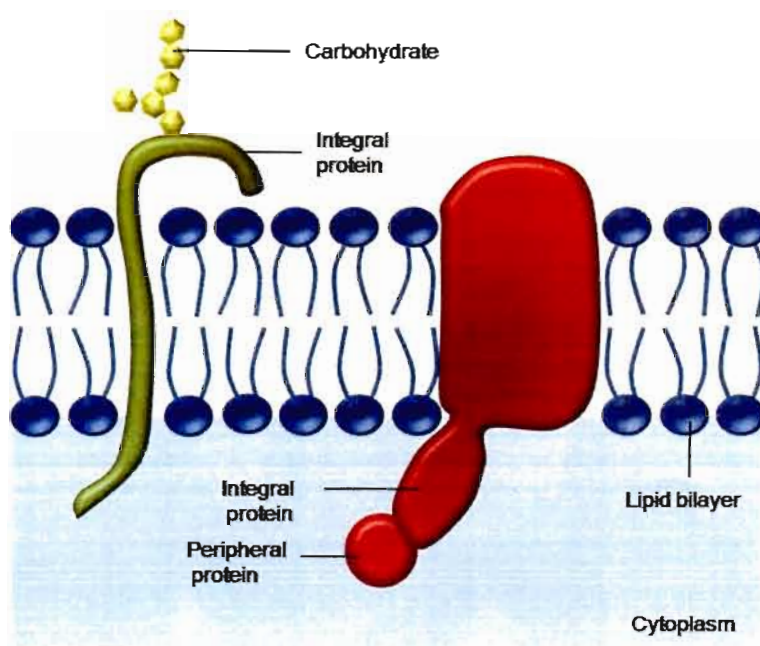


Figure 2.1 A diagram of the human cell membrane (adapted form (Shargel and Yu, 1999).

The advantage of carrier-mediated transport processes over passive diffusion and facilitated diffusion is the ability to transport molecules against a concentration gradient (Breves *et al.*, 2007). The transcellular route will briefly be discussed.

2.2.1 Passive diffusion

Passive diffusion is the spontaneous movement of molecules from a region of high concentration to a lower concentration region. The process is passive because no energy is expended (Shargel and Yu, 1999). This route of transport is preferred by small and lipophilic molecules and is the most common way of absorption for orally administered drugs (Lennernäs, 2007; Narawane and Vincent, 1994).

2.2.2 Carrier-mediated transport

Numerous specialised carrier-mediated transport such as active transport and facilitated diffusion are present in the intestine for the absorption of ions and nutrients that are too polar or too big to cross passively or paracellularly.

2.2.2.1 Active transport

As mentioned earlier, active transport is the transport of molecules against a concentration gradient, utilising energy derived from adenosine triphosphate (ATP) hydrolysis. Studies suggest that active transport plays a pivotal role in the absorption of large hydrophilic compounds (MW > 250 – 300) (Fagerholm *et al.*, 1997; Lennernäs, 1998; Lennernäs, 2007).

2.2.2.2 Facilitated diffusion

Facilitated diffusion differs from active transport in that it does not utilise energy. Transport of molecules is facilitated by specific transport proteins from a high to a low concentration (Narawane and Vincent, 1994; Shargel and Yu, 1999).

Oral drug absorption is determined by the interaction of physicochemical properties of the drug (such as molecular size, hydrogen bonding, conformation and lipophilicity) and physiological characteristics of the gastrointestinal tract.

2.3 Lipinski's rule of five

Lipinski and his colleagues discovered a correlation between four parameters with the solubility and permeability of a compound in the gastrointestinal tract (Lipinski, 2000). These parameters are molecular weight, log P, the number of H-bond donors and the

number of H-bond acceptors. From the data collected, the “rule of 5” was created. The “rule” consists of five statements that if met could lead to poor absorption or permeation.

Lipinski’s rule of five states the following for poor absorption or permeation:

- A molecular weight more than 500 g/mol.
- The log P exceeding 5.
- More than ten hydrogen bond acceptors (expressed as the sum of nitrogen and oxygen atoms).
- More than five hydrogen bond donors (expressed as the sum of nitrogen or oxygen atoms with one or more hydrogen atoms).
- Compounds that are substrates for biological transporters are exceptions to the rule.

The log P value on its own is an important parameter for potential derivatisation. It was found that hydrophilic compounds exhibited P values smaller than 1 (negative log P), and conversely lipophilic compounds a bigger P value than 1 (positive log P). Thus, an increase in log P value causes an increase in lipophilicity that in turn increases cellular absorption (Silverman, 2004).

2.4 Human African Trypanosomiasis

Human African trypanosomiasis (HAT), more commonly known as sleeping sickness generally occurs in remote rural areas of sub-Saharan Africa where health systems are weak or non-existent. The disease spreads in poor settings and displacement of populations, war and poverty are important factors leading to increased transmission. According to the World Health Organization’s (WHO) global burden update in 2004, the effects of parasitic diseases, measured in disability adjusted life years (DALY) loss due to trypanosomiasis, is estimated to be 1.7 million, consequently HAT ranks third in the world of infectious diseases next to malaria and schistosomiasis (Blum *et al.*, 2006; WHO, 2004). Together with inadequate surveillance and control human African trypanosomiasis is still regarded as a major problem and remains a serious threat (Barrett, 1999).

2.4.1 History

From the first day a human set foot upon the domain of the tsetse fly, African sleeping sickness has plagued human inhabitants (Bogitsh *et al.*, 2005). Human African trypanosomiasis is an age old disease that has been present on earth for millions of years. It is estimated that all Salivarian trypanosomes, to which African trypanosomes belong, diverged from other trypanosomes 300 million years ago (Haag *et al.*, 1998). Evidence of this old disease's existence was recorded by the ancient Egyptians, and comes from the Veterinary Papyrus of the Kahun Papyri, dating from the 2nd millennium BC. It described a cattle disease resembling nagana (disease caused by *Trypanosoma brucei brucei*) (Steverding, 2008).

It is only in the middle ages that one of the initial historical accounts for trypanosomiasis is described. During his journey into Africa in the 1200s, the geographer, Abu Abdallah Yaqut reportedly found a village where the inhabitants and even dogs were asleep. Hundred years after Yaqut's death the first case of African sleeping sickness were documented by the Arabian historian, Ibn Khaldun. He reported that the Emperor of Mali died of an illness that resembled African trypanosomiasis (Steverding, 2008).

But it is in the early 1700s, with an increased awareness of trypanosomiasis, that the most written reports about African sleeping sickness are found. The English naval surgeon John Atkins published the first medical report in 1734, describing only the neurological symptoms of late stage African sleeping sickness. Thomas Winterbottom, an English physician, discovered swollen lymph nodes at the back of the neck, the characteristic sign of early stage sleeping sickness, and published it in a report in 1803 (Cox, 2004). This discovery was so profound that he named the symptom after himself, the Winterbottom sign. At this stage no one really had an idea about the progress of the sickness until David Livingstone (Figure 2.2), the Scottish missionary, reported in 1852 that African sleeping sickness is transmitted by the bite of the tsetse fly. Forty years later, pathologist and microbiologist, Sir David Bruce (Figure 2.3) made one of the greatest discoveries about the disease, by proving that trypanosomes were the causative agent of cattle trypanosomiasis (cattle nagana).

In 1902, physician and pathologist Aldo Castellani found trypanosomes in the cerebrospinal fluid (CSF) and suggested that they cause sleeping sickness. Bruce, who at that time, believed that the disease was mechanically transmitted by tsetse flies, changed his opinion

after Friedrich Karl Kleine, another surgeon, discovered in 1909 the cyclical transmission of trypanosome in tsetse flies (Steverding, 2008). During the 20th century, three major epidemics terrorized Africa: the first lasting from 1896 to 1906 and the other two occurring in 1920 and 1970 respectively (WHO, 2006). This sparked major research in the fields of drug discovery, implementation of vector control as well as unravelling the mystery of African trypanosomiasis.



Figure 2.2 Dr David Livingstone, the Scottish missionary who hypothesised the transmission of HAT through the bite of the tsetse fly



Figure 2.3 Sir David Bruce, microbiologist who identified *T. b. brucei* as the causative agent in nagana disease (Steverding, 2008).

2.4.2 Epidemiology

HAT is a vector-borne parasitic disease affecting humans and animals and is fatal if left untreated (Simarro *et al.*, 2008). It is caused by protozoa of the species *Trypanosoma brucei* and transmitted by the tsetse fly (*Glossina* genus) who acquired the infection from humans or animals harbouring the pathogen (Simarro *et al.*, 2008; WHO, 1998). The disease is endemic in regions of sub-Saharan Africa (Figure 2.4), between latitudes 14 °N and 29 °S, corresponding to the distribution of their vectors (Schmidt *et al.*, 1996; Simarro *et*

al., 2008; WHO, 1998). Thus the ecology and behaviour of tsetse flies have a significant epidemiological effect on disease transmission (Ukoli, 1984). Yet, interestingly enough, there are areas where tsetse flies are found, but sleeping sickness not (WHO, 2006). This is because *Glossina* have highly focal distributions due to specific habitat requirements (Fèvre *et al.*, 2006)

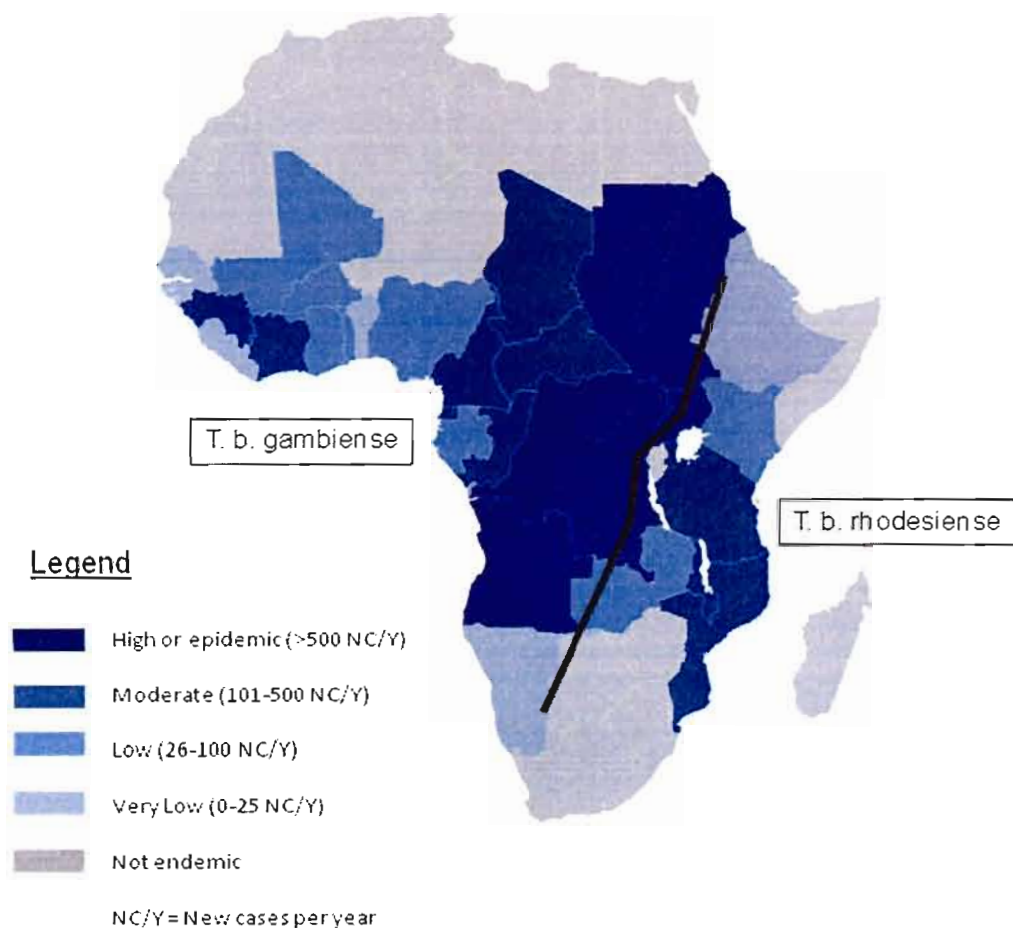


Figure 2.4 The distribution human African trypanosomiasis (adapted from Pépin *et al.*, 2001; WHO, 2000).

HAT has two forms, morphologically indistinguishable, but that varies in infectivity (Schmidt *et al.*, 1996). *Trypanosoma brucei gambiense* (*T. b. gambiense*), which is found in west and central Africa, causes the chronic form of the disease and is responsible for more than

90% of reported cases. *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) is found in eastern and southern Africa. It represents less than 10% of cases, and causes an acute infection (Simarro *et al.*, 2008; WHO, 2006). The disease covers 60 million of the 400 million people inhabiting 36 African countries and has around 200 active foci (Barrett, 1999; Pépin and Méda, 2001; WHO, 1998). Only 5 to 10% of the endemic population is under surveillance, with 25 000 new cases diagnosed but an estimated 300 000 to 500 000 are infected annually (Barrett, 1999; Seed, 2000; WHO, 1998).

The disease was almost eradicated in the 1960s but due to lack of proper control and funding the disease has yet again become a big threat (Barrett, 1999; WHO, 1998). The toll on human victims is astonishing: 50% of all newly diagnosed cases are fatal and the remaining 50% result in permanent brain damage (Bogitsh *et al.*, 2005). War-torn countries such as Sudan, Uganda, Democratic Republic of Congo (DRC) and Angola are hit hardest by these epidemics (Barrett, 1999; Legros *et al.*, 2002). For example, 2% of the DRC population has 70% prevalence in some communities. Fortunately, the number of cases and annual deaths is on the decrease (Barrett, 1999).

2.4.3 Life Cycle of *Trypanosoma brucei* spp.

African trypanosomes live extracellular, both in the mammalian and insect host. The life cycle of *T. b. gambiense* and *T. b. rhodesiense* are identical thus the explanation given, according to Figure 2.5, applies to both. Several *Glossina* species act as vectors for the disease. The insect vectors of *T. b. rhodesiense* are *G. morsitans*, *G. pallipides* and *G. swynnertone*, whereas those of *T. b. gambiense* are *G. palpalis* and *G. tachinoides*. Both sexes of the fly can transmit the disease (Katz *et al.*, 1989; Schmidt *et al.*, 1996). The life cycle is as follows:

1. Before its blood meal, the fly injects saliva, containing parasites, into the mammalian dermis. This dilates the blood vessels, prevents coagulation of blood and simultaneously secretes the metacyclic trypomastigote, the infective form of the protozoa. One fly may inoculate its host with several thousand protozoa in a single bite. The minimum infective dose is around 400 organisms for most hosts. At this stage the metacyclic trypomastigote is morphologically blunt, with no free flagellum. The parasite needs to be in this morphological state within the vector to be infective to its host.

2. Once in the bloodstream the metacyclic trypomastigote transforms into long slender trypomastigotes, the blood form of the trypanosome. This sparks a series of divisions by binary fission at the bite site, which leads to the formation of the primary chancre
3. The trypomastigotes multiplies rapidly in the blood by more divisions, reaching populations of more than 1500 trypomastigotes/mm³, eventually entering the cerebrospinal fluid, lymph and interstitial spaces. In the bloodstream, trypomastigotes exhibit three forms:
 - a long slender form, 29 µm long with a free flagellum,
 - an intermediate form, 23 µm long, with a shorter free flagellum,
 - and a short stumpy form, 18 µm long, with no flagellum.
4. In order to complete the parasite's life cycle and consequently transmit the disease, the tsetse fly must ingest the short stumpy trypomastigote. In this form the parasite is adapted to live within the insect vector. This stage of the cycle is also relevant for diagnostic purposes.
5. The tsetse fly now becomes infected after a blood meal from an infected host.
6. The stumpy trypomastigotes elongate, lose their antigenic surface coats and transform into procyclic trypomastigotes in the midgut of the fly. Here they multiply by longitudinal binary fission for ten days. Two to ten days later, the slender organisms migrate and are found in the foregut.
7. Then they migrate further into the salivary gland (Figure 2.6) and transforms into epimastigotes, the dominant form in the oesophagus and buccal cavity of the fly.
8. In the salivary gland division continues, whilst attached to the epithelium by their flagella. When division is completed, epimastigotes transform into metacyclic trypomastigotes and detach into the lumen of the gland.

The entire cycle lasts 25-50 days, depending on fly and trypanosome species and temperature. Each fly remains infected for 2-3 months. From the above mentioned cycle it becomes evident that the trypomastigotes and epimastigotes play crucial roles in the life cycle (Bogitsh *et al.*, 2005; Katz *et al.*, 1989; Schmidt *et al.*, 1996; Ukoli, 1984).

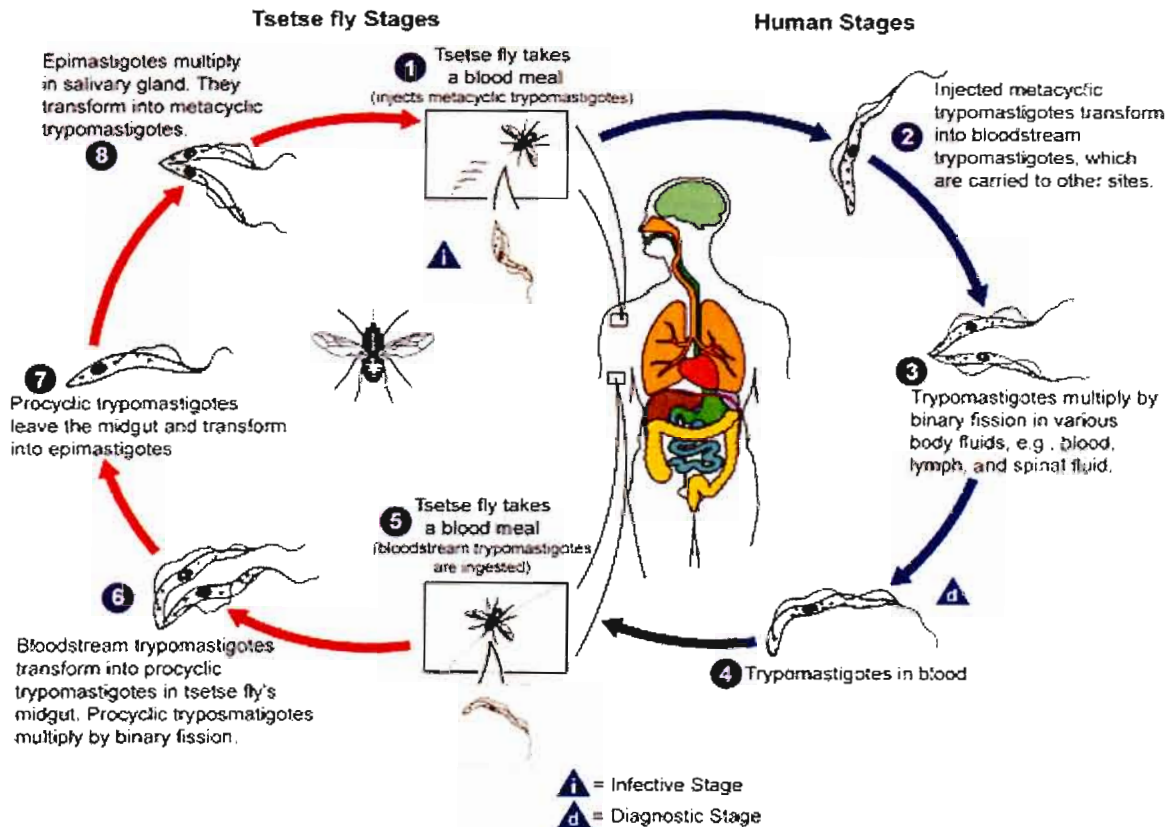


Figure 2.5 Life cycles of the *Trypanosoma brucei* parasites (CDC, 2009).

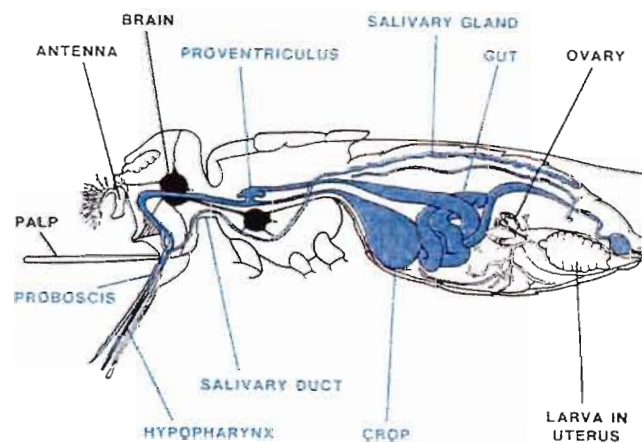


Figure 2.6 Diagram of the internal organs of the female tsetse fly (taken from ILRAD, 1984).

2.4.4 Symptoms and Clinical Features

Both trypanosomes cause similar signs and symptoms that characterize sleeping sickness, only differing in the pathogenic timeline. Infections with *T. b. rhodesiense* present an acute form of onset, showing signs and symptoms within days following infection. Due to its short incubation period of two to three weeks, the onset is usually severe involving neurological systems within three to four weeks. *T. b. gambiense*, on the other hand, has an incubation period of several weeks to months, presenting a chronic form with months and sometimes years passing by before symptoms are observed (Katz *et al.*, 1989; WHO, 1998).

These signs and symptoms are classified in two stages according to the clinical progression of the disease:

- a haematolymphatic or early stage, and
- meningoencephalitic or late stage.

Clinical signs are generally unspecific, showing differences in frequency between individuals and disease foci (WHO, 1998). In Table 2.1 the different major symptoms in both stages are listed, but only the most relevant symptoms will be addressed in this section. It is important to note that symptoms from both stages can be noticed. This is because the migration of the parasite from the blood to the CNS is not immediate, but rather a progressive process.

2.4.4.1 Haematolymphatic stage

The chancre is the primary lesion visible at the site of inoculation and develops between a few days to 2 weeks after the bite. It resembles a small, erythematous and swollen wound and disappears eventually within 2 to 3 weeks (Berkow and Beers, 1999). As the parasites multiply and migrate into the bloodstream and lymph nodes, fever and headaches become more frequent. Characteristic enlargement of lymph glands (Winterbottom's sign), especially behind the neck and supraclavicular areas, become visible. The lymph nodes are firm, painless and vary in size (Bogitsh *et al.*, 2005; Katz *et al.*, 1989; WHO, 1998).

2.4.4.2 Meningoencephalitic stage

The late stage manifests predominantly as neurological disorders. Initially headache followed by sleep disturbances and depression are some of the debilitating symptoms (Katz *et al.*, 1989). With regards to sleep, the circadian rhythm of sleep and wakefulness

disappears. During a study conducted on 2541 stage two patients, Blum and his colleagues found that patients in their population complained more frequently about nocturnal insomnia (56.8%) than daytime sleep (41%) (Blum *et al.*, 2006). A mental deterioration progress further and is associated with tremors, seizures and palsies (Katz *et al.*, 1989). Eventually coma and death ensue. Deaths are rarely caused by the parasite directly, but rather indirectly due to malnutrition, heart failure or other parasitic infections (Schmidt *et al.*, 1996).

Table 2.1 Major signs and symptoms in sleeping sickness (adapted from WHO, 1998).

Haematolymphatic stage	Meningoencephalitic stage
• Chancre • Lymphadenopathy • Fever • Headache • Pruritus • Skin rash • Splenomegaly • Musculoskeletal pains • Anaemia • Oedema • Ascites • Cardiovascular disorders • Endocrinological disorders • Renal impairment • Intercurrent lung infections	• Sleep disturbances • Alteration of mental state • Abnormal reflexes • Tone disorders • Abnormal movements • Sensory disorders • Coordination disorders • Other neurological disorders ○ Convulsions ○ Neurovegetative disorders ○ Hemiplegia ○ Deterioration of consciousness ○ Coma

2.4.5 Pathology and Pathogenesis

As mentioned before, trypanosomes divide and spread quickly with severe consequences, especially in the acute form of the disease caused by *T. b. rhodesiense*. The pathology of trypanosome infections involve degenerative, necrotic and inflammatory changes within the

tissue and organs the parasite invades (Ukoli, 1984). Infections are widespread throughout the body, where the lymphoid system, brain, heart and lungs are mostly affected. The late stage symptoms typically found include cerebral and meningeal oedema, as well as myocarditis causing an enlargement of the heart. This is mainly due to inflammation caused by infiltration of lymphocytes, plasma cells and Mott cells, which are plasma cells filled with immunoglobulins. Mott cells are indicative and characteristic of trypanosome infections. There is however no correlation between the inflammatory changes in the brain and the distinct neuropsychiatric symptoms of the disease (WHO, 1998).

2.4.6 Immunology

As with the pathology, the immune response in human hosts infected with trypanosomes is very complex and presents a number of challenging immunological problems (Donelson *et al.*, 1998; Schmidt *et al.*, 1996). Trypanosomes have evolved an amazing mechanism for evading the host's defences. The parasites accomplish this by means of antigenic variation, which is the changing of variant surface glycoproteins (VSGs) surrounding the plasma membrane of trypanosomes. The VSGs form a thick coat protecting the parasite against macromolecules and lytic elements found in the host's serum (Bisser *et al.*, 2006). VSGs have two functions. Firstly, it elicits a cascading immune response when trypanosomes are lysed and destroyed leading to prolonged parasite survival in the blood. Secondly, the ability of the VSGs to undergo variation allows some of the parasites to survive (Bisser *et al.*, 2006; Donelson *et al.*, 1998). Engstler *et al.* reported that trypanosomes have the ability to change the entire VSG coat within 12 minutes (Engstler *et al.*, 2004) which is possibly essential for the parasite to evade immune responses (Pays, 2005).

Two overwhelming immune responses characterise an African trypanosome infection: a non specific polyclonal activation of B cells and a generalised suppression of humoral and cellular immune functions (Pépin and Donelson, 2006). VSG molecules activate polyclonal B cells resulting in a massive production of immunoglobulin M (IgM), the first type of immunoglobulin generated by the appearance of foreign antigens (Bisser *et al.*, 2006; Donelson *et al.*, 1998).

Trypanosomes inhibit many secondary immune responses in a subtle way through specific substances. Macrophages can be activated directly through a substance called trypanin. Trypanin, formerly called T-lymphocyte triggering factor (TLTF) is a microtubule-binding

protein found in the flagellum (Hill *et al.*, 2000; Hutchings *et al.*, 2002). It binds to T-lymphocytes (CD8 cells) triggering and enhancing the proliferation of interferon gamma (INF- γ) by macrophages (Figure 2.7). INF- γ in turn acts as a growth stimulus promoting trypanosome proliferation (Bakhiet *et al.*, 1996; Gobert *et al.*, 2000). On the other hand, activated macrophages produce other substances such as interleukin and prostaglandins that stimulate tumour necrosis factor- α (TNF- α) and nitric oxide (NO), both these substances having trypanocidal activity. Amazingly, trypanosomes can evade the NO trypanocidal effects. NO is produced from L-arginine by NO synthase (Vincendeau *et al.*, 2003). The parasite does this by using the substrate in their polyamine metabolism, thus depleting substrate levels or using the host's own metabolism. According to Gobert *et al.* the majority of L-arginine was metabolized by arginase (Gobert *et al.*, 2000) to produce ornithine, a molecule used for trypanosome growth (Vincendeau *et al.*, 2003). Increased levels of certain cytokines are seen during trypanosomiasis. Th2 cytokines induce arginase lowering L-arginine concentrations leading to reduced NO levels (Gobert *et al.*, 2000).

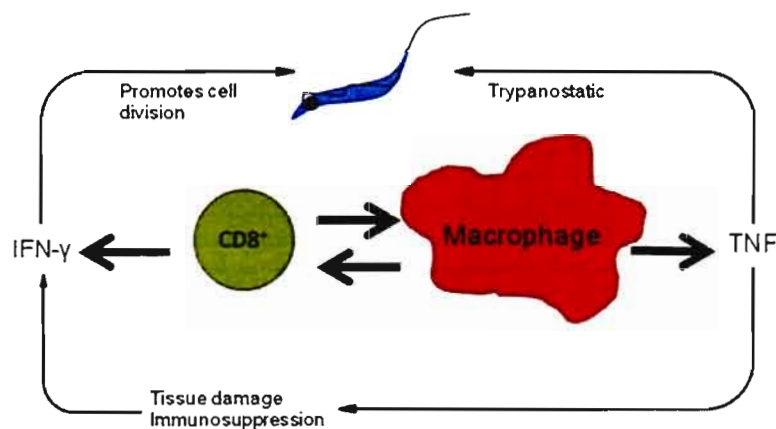


Figure 2.7 The effects of macrophage-derived factors during HAT infections (adapted from WHO, 1998).

It is evident that trypanosomes use elaborated yet successful methods for evading the host's defences. This and the fact that this disease is an age old one, might suggest that HAT will still be a problem in the years to come. The research done so far on the immunology of trypanosomes opens up new doors for drug development, vector control and diagnosis.

2.4.7 Diagnosis

Trypanosomes are extracellular parasites, found in the blood, CSF and lymph. Diagnosis utilizes the screening of these elements. Laboratory screening is used to accurately diagnose the disease because no single diagnostic sign or symptom exists (WHO, 1998).

Direct detection of parasites by microscopy is the 'gold standard' diagnostic technique for trypanosomes (Fèvre *et al.*, 2006). Due to the high parasitemia in the blood during the early stages of HAT, thick stained blood smears have been found to be very useful in diagnosing *T. b. rhodesiense* infections (Ukoli, 1984). Microscopy however is only accurate at or above concentrations of 10^4 parasites/ml, a parasite concentration well above those found in *T. b. gambiense* infections (Fèvre *et al.*, 2006). Diagnosing *T. b. gambiense*, stained exudates from lymph nodes or CSF is usually effective (Ukoli, 1984). In late stage HAT, trypanosomes may only be found in centrifuged CSF. An elevated leukocyte count of more than 5 cells/ μ l or increased protein concentrations above 37 mg/100 ml are indicative of central nervous system (CNS) invasion (WHO, 1998).

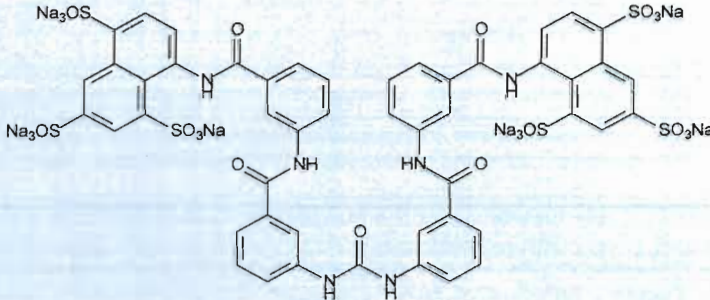
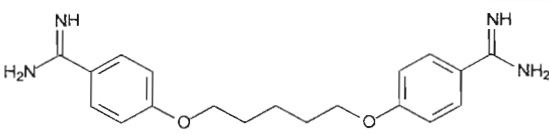
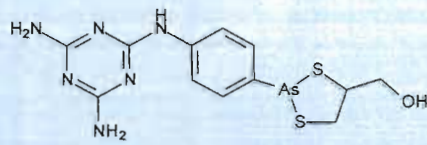
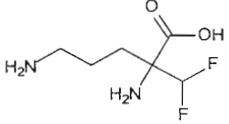
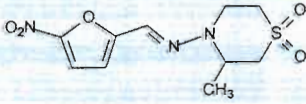
Antibody tests like immunofluorescent assays (IFA), indirect haemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) are also very effective (Berkow and Beers, 1999). High IgM levels caused by the disease, especially during CNS involvement, are also diagnostically relevant (Berkow and Beers, 1999; Ukoli, 1984). Another diagnostic method that utilizes the multiplication of specific deoxyribonucleic acid (DNA) fragments is the polymerase chain reaction (PCR). PCR has been used to determine *T. b. gambiense* concentrations in the blood and CSF. The method is also recognised as the most sensitive diagnostic method with a detection limit of 25 trypanosomes/ml of human blood (Fèvre *et al.*, 2006).

Although laboratory screening is preferred for diagnosis, trypanosome concentrations in body fluids are often below the limit of detection as with *T. b. gambiense* infections. It also limits field testing that requires quick and easy tests without the immediate use of laboratory equipment. Diagnosis utilizing the detection of antibodies or circulating antibodies are effective for field testing. The serological screening test CATT (card agglutination test for *T. b. gambiense*), is very sensitive and results can be obtained within 5 minutes (WHO, 1998).

2.4.8 Treatment

There are currently four drugs used for the treatment of HAT. Three out of the four drugs were developed over 50 years ago (Fairlamb, 2003). The treatment varies according to the stage and severity of the disease. Table 2.2 shows the different drugs used in each stage.

Table 2.2 Structures of the drugs used in early and late stage HAT (adapted from Fairlamb, 2003).

Haematolympathatic stage drugs	 Suramin
	 Pentamidine
Meningoencephalitic stage drugs	 Melarsoprol
	 Eflornithine
	 Nifurtimox^a

^a Nifurtimox is also effective against early stage infections but is used primarily in combination with eflornithine or melarsoprol against late stage infections.

Early stage trypanosomiasis is treated with suramin or pentamidine, but these drugs are ineffective in late stage diseases, presumably because the blood-brain barrier (BBB) prevents them from reaching trypanocidal levels in the CSF (WHO, 1998). Late stage trypanosomiasis is treated with melarsoprol, a toxic arsenic derivative that has severe side effects such as reactive encephalopathy which is fatal in 2-12% of cases (Bouteille *et al.*, 2003). Eflornithine can also be used to treat late stage trypanosomiasis, but has been used with little success because of difficult dosage regimes and high cost. Together with this predisposition eflornithine is not effective against *T. b. rhodesiense*. Fairlamb adds that all of the current therapies used against HAT are unsatisfactory due to unacceptable toxicity, poor efficacy, undesirable route of administration and drug resistance (Fairlamb, 2003).

Eflornithine is the only drug to be approved against HAT in the last half century and is currently the first choice for non-responding patients to melarsoprol treatment (Delespaulx and De Koning, 2007). Nifurtimox is currently registered for the treatment of Chagas disease but has shown activity against early and late stage HAT (Fairlamb, 2003). Success as monotherapy has been limited but its use in drug combinations has been of great interest and results seem promising (Barrett *et al.*, 2007; Priotto *et al.*, 2007).

2.4.8.1 Suramin

Suramin is a colourless polysulphonated, symmetrical naphtylamine derivative (Denise and Barrett, 2001; Fairlamb, 2003; Pepin and Milord, 1994; Phillips and Stanley, 2001), and its discovery is based on the trypanocidal activity of the dyes trypan red, trypan blue and afridol violet (Phillips and Stanley, 2001).

Suramin was introduced in 1920, making it one of the oldest drugs still used today and remains the drug of choice for treatment of early stage *T. b. rhodesiense* infections (Fairlamb, 2003; Phillips and Stanley, 2001). Suramin is highly water soluble due to its ionic nature and is therefore administered intravenously. This has the advantage of avoiding local inflammation and necrosis caused by subcutaneous and intramuscular injections (Pepin and Milord, 1994). It is 99.7% bound to serum proteins and has a high potential of binding to low density lipoproteins (LDL). It has been speculated that the uptake of the drug occurs *via* endocytosis bound to LDL, which would explain the slow accumulation of the drug in the trypanosomes (Vansterkenburg *et al.*, 1993). The protein binding, hydrophilicity and slow onset of action, explains the low CNS penetration (~ 1% of serum levels) and long

terminal half life (90 days). Even though suramin has been used for over 85 years, no significant resistance has emerged (Fairlamb, 2003; Phillips and Stanley, 2001).

Not much is known about the mechanism of action, but it is suspected that several enzymes, especially those of the glycolytic pathway are inhibited (Figure 2.8). As a result many metabolic processes such as DNA and protein synthesis are blocked (Delespaux and De Koning, 2007; Frayha *et al.*, 1997; Pepin and Milord, 1994). Treatment of trypanosomiasis with suramin should not be started until 24 hours after a diagnostic lumbar puncture. This is to exclude CNS involvement (Phillips and Stanley, 2001). A normal single dose for an adult is 20 mg/kg given intravenously (no more than 1 g per injection), but it is advisable to start with a test dose of 200 mg to detect sensitivity. After that the normal dose is given in a series of five injections on days 1, 3, 7, 14 and 21 (Bouteille *et al.*, 2003; Phillips and Stanley, 2001; WHO, 1998). Suramin causes a variety of side effects of which nausea, fatigue and malaise are most common. In severe cases where several doses are required, renal toxicity and neurological complications are mainly encountered (Bouteille *et al.*, 2003; Pepin and Milord, 1994; Phillips and Stanley, 2001).

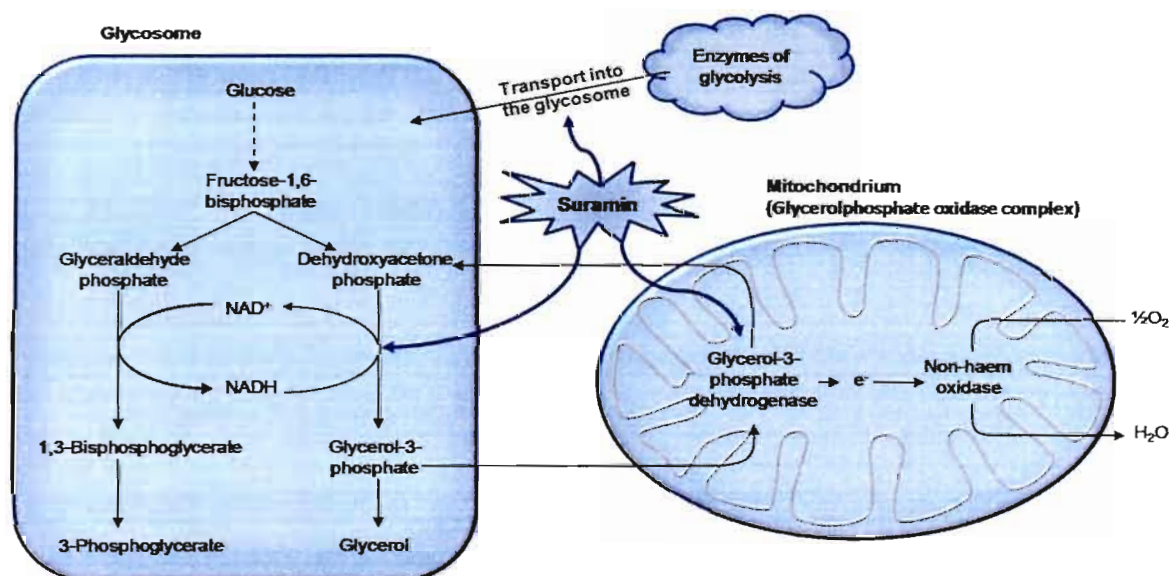


Figure 2.8 Proposed mechanism of action of suramin (adapted from Fraya *et al.*, 1997).

2.4.8.2 Pentamidine

Pentamidine is an aromatic, positively charged diamine at a physiological pH (Bray *et al.*, 2003; Denise and Barrett, 2001; Pepin and Milord, 1994; Phillips and Stanley, 2001). The discovery in 1937 was made after the observation that a hypoglycaemic inducing drug, synthalin, had anti-trypanosomal activity (Bray *et al.*, 2003; Denise and Barrett, 2001; Phillips and Stanley, 2001). Fifty years later, pentamidine is still used as an alternative to suramin in treating early stages of the infection (Frayha *et al.*, 1997). Pentamidine has low oral bioavailability due to its high protonated characteristic, and is thus administered parentally. This drug is less reliable in treating *T. b. rhodesiense* infections, because it does not cross the BBB (Fairlamb, 2003). As a result, it is primarily used in early stage *T. b. gambiense* infections (Denise and Barrett, 2001; Phillips and Stanley, 2001).

Transport of pentamidine into the trypanosome utilizes at least three active transporters, of which the P2-transporter is most significant (Carter *et al.*, 1995) and concentrates the drug in the cytoplasm to very high levels (Denise and Barrett, 2001). Loss of these transporters due to inhibition, especially the P2-transporter creates resistance to diamidines (Bray *et al.*, 2003; Denise and Barrett, 2001). Once inside the trypanosome, the drug interacts electrostatically with cellular polyanions, such as circular DNA molecules found in all kinetoplastid flagellates (Denise and Barrett, 2001; Frayha *et al.*, 1997). Pentamidine also reversibly inhibits the enzyme S-adenosylmethionine decarboxylase (SAMDC) (Figure 2.9), preventing the methylation of putrescine into spermidine (Bouteille *et al.*, 2003; Frayha *et al.*, 1997; Phillips and Stanley, 2001).

In terms of pharmacokinetics, early trials have suggested that the drug exhibits prolonged action and slow rate of excretion (Pepin and Milord, 1994). The elimination half-life is very long lasting from weeks to months and is 70% bound to plasma proteins (Bouteille *et al.*, 2003; Phillips and Stanley, 2001). Pentamidine therapy consists of a series of seven to ten intramuscular injections with a recommended dosage of 4 mg/kg/day (Bouteille *et al.*, 2003; Pepin and Milord, 1994; Phillips and Stanley, 2001; WHO, 1998). The drug is generally well tolerated, although 50% of individuals receiving the recommended dose show some adverse reactions such as renal, cardiac or pancreatic toxicity, rashes and haematological disturbances (Bouteille *et al.*, 2003; Pepin and Milord, 1994; Phillips and Stanley, 2001). It is therefore advisable to monitor patients receiving treatment.

Like with diamidines, melarsoprol actively accumulates in the trypanosome cell by an amino-purine transporter (Carter *et al.*, 1995; Denise and Barrett, 2001). This explains the induced resistance due to decreased transport into the cell (De Koning, 2001). Due to the molecule's high reactivity and potential to bind covalently to thiol groups, inhibition of various energy metabolism pathways occurs (Frayha *et al.*, 1997). One of these pathways, the trypanothione formation, has been described in great detail in recent years. Melarsoprol inhibits trypanothione reductase (Frayha *et al.*, 1997), an enzyme used in the formation of trypanothione (Figure 2.9), thereby slowing down the formation of an essential metabolic building block. Apart from inhibiting the enzyme, it also binds reversibly to trypanothione forming melarsen oxide-trypanothione (Mel T), a potent inhibitor of trypanothione reductase. Both these processes are suspected to result in trypanosome cell death (Fairlamb, 2003; Phillips and Stanley, 2001).

According to studies, 30 – 60% of melarsoprol is excreted, mostly (70 – 80%) in the faeces, and the rest in urine (Pepin and Milord, 1994; Phillips and Stanley, 2001). It has an elimination half-life of 35 hours, yet does not accumulate. CSF concentrations are low, between a 0.5 and 5%, yet these concentrations seem to be therapeutic (Bouteille *et al.*, 2003; Pepin and Milord, 1994). There is no standard treatment protocol even though it has been derived empirically more than 40 years ago (Bouteille *et al.*, 2003). A commonly used protocol consists of 3.6 mg/kg daily intravenous injections for three to four days, repeated three times in weekly intervals (Bouteille *et al.*, 2003; Phillips and Stanley, 2001; WHO, 1998). A study conducted by Burri *et al.* concluded that a treatment protocol of 2.2 mg/kg/day for ten days could reduce the total quantity of melarsoprol used by 30% and reduce the length of hospitalization by 16 days, with comparable efficacy to the commonly used protocol (Burri *et al.*, 2000).

Neurotoxicity is commonly found with melarsoprol treatment (Fairlamb, 2003; Pepin and Milord, 1994). Other neurotoxic symptoms such as drug induced encephalopathy, seizures, headache and peripheral neuropathy have been documented. Reactive encephalopathy is a serious and usually fatal adverse reaction. Five to 10% of patients receiving melarsoprol treatment develop reactive encephalopathy, of which 50 – 70% of these patients die due to this adverse reaction (Fairlamb, 2003; Pepin and Milord, 1994; Phillips and Stanley, 2001; Schmid *et al.*, 2005). Reactive encephalopathy is more common in *rhodesiense* than in *gambiense* trypanosomiasis and occurs six to 10 days after treatment which suggest that it is rather an immunological effect than a cumulative toxic effect (Checkley *et al.*, 2007; Pepin

and Milord, 1994; Phillips and Stanley, 2001). Common side effects include hypertension, myocardial damage, albuminuria, vomiting, abdominal pain and skin rash (Fairlamb, 2003; Phillips and Stanley, 2001).

2.4.8.4 Nifurtimox

Since its appearance in the 1960s nifurtimox has been used to treat American trypanosomiasis or Chagas disease (Bouteille *et al.*, 2003; Rodgers, 2009; Rodgers, 2009). However, nifurtimox has been reported to be curative against both early and late stage HAT caused by *T. b. gambiense*. Its efficacy against *T. b. rhodesiense* is not well known (Bouteille *et al.*, 2003; Delespaux and De Koning, 2007; Phillips and Stanley, 2001). Although nifurtimox is not registered for the use in HAT, it has been used to treat patients with melarsoprol refractory *T. b. gambiense* infections (Bouteille *et al.*, 2003; Rodgers, 2009). As monotherapy, nifurtimox has been disappointing with reports showing a nearly 50% relapse rate after treatment (Coura and De Castro, 2002; Pepin, 2007). Combination therapy with eflornithine has been proven efficacious and addresses some disadvantages of eflornithine as monotherapy such as drug resistance, cost and logistics (Priotto *et al.*, 2007).

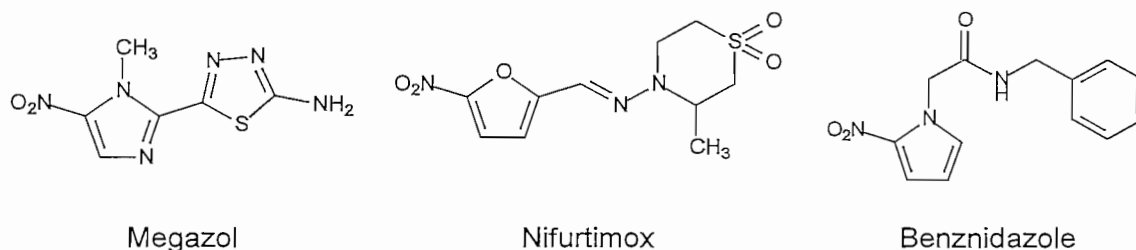


Figure 2.10 Structure of some nitroheterocyclic trypanocides.

Nifurtimox is a 5-nitrofuran belonging to the nitroheterocyclic trypanocides alongside megazol and benznidazole (Figure 2.10). The drug contains a nitro group that is crucial for activity. Nifurtimox functions as a prodrug and therefore needs to be activated to induce a pharmacological response (Wilkinson *et al.*, 2008). The mechanism of action relies on the generation of free radicals by nitrofuran reduction (Frayha *et al.*, 1997; Townson *et al.*, 1994). This reduction which activates the drug is accomplished by a NADH-dependent, bacterial like, type 1 nitroreductase (NTR) located in the trypanosomal mitochondria (Wilkinson *et al.*, 2008). Cyclical reduction and oxidation of the nitro group produces

superoxides, hydrogen peroxide and free radicals (Fairlamb, 2003). The oxidative stress induced by these free radicals result in cellular damage as they react with components such as DNA, membrane lipids and proteins (Fairlamb, 2003; Pepin and Milord, 1994; Phillips and Stanley, 2001).

Monotherapy regimens involve eight to 20 mg/kg doses per day in four divided doses for 90 to 120 days. Nifurtimox is given orally and is well absorbed after administration with peak plasma levels observed after one to three hours. Despite this, assays have demonstrated low plasma concentrations of the drug (~4 µM) after a single dose of 15 mg/kg (Barrett *et al.*, 2007; Phillips and Stanley, 2001). Drug clearance occurs rapidly with an elimination half-life of about three hours (Phillips and Stanley, 2001). Nifurtimox also crosses the BBB readily reaching levels half of that in plasma (Barrett *et al.*, 2007). According to Enanga *et al.* trypanosomes are not very susceptible to nifurtimox exhibiting an IC₅₀ value of 5 µM compared to 10 nM of melarsen oxide. This would explain the limited success of nifurtimox monotherapy (Barrett *et al.*, 2007; Enanga *et al.*, 2003).

Although drug-related side effects are common, children tolerate nifurtimox better than adults. Side effects range from hypersensitivity reactions to dose- and age dependent complications. Gastrointestinal disorders such as anorexia, vomiting, nausea and neurological symptoms such as headache, sleep disorders, confusion and agitation are very common after prolonged treatment (Phillips and Stanley, 2001; Priotto *et al.*, 2007). Toxic effects to the central and peripheral nervous system have also been reported (Castro *et al.*, 2006). However toxicity can be reduced with a treatment schedule of 14 or 21 days of three daily doses of 5 mg/kg (Bouteille *et al.*, 2003).

2.4.8.5 Eflornithine

Due to the specificity and focus of the project, eflornithine will be described in more detail.

Discovery and clinical use

Eflornithine (DFMO, α-difluoromethylornithine) was developed in the early 1980s, making it the newest molecule registered in over half a century (Burri and Brun, 2003). DFMO was synthesised as an antitumour agent, but was considered too toxic (Bouteille *et al.*, 2003; Na-Bangchang *et al.*, 2004). It was later found to have activity against both early and late stage trypanosome infections in mice (Bacchi *et al.*, 1987; Bacchi and Yarlett, 2002). DFMO was

approved and registered in 1990 by the United States Food and Drug Administration (USFDA), as Ornidyl, for the treatment of HAT (Bacchi and Yarlett, 2002; Na-Bangchang *et al.*, 2004). Currently it is primarily used in patients with severe CNS complications, or who have relapsed after arsenical treatment (Burri and Brun, 2003; Phillips and Stanley, 2001). Even though the drug is curative for late stage *gambiense* infections, it is ineffective against *rhodesiense* infections (Phillips and Stanley, 2001). The drug has other disadvantages such as high cost, complex dosage regimes and production shortages, making it far from ideal (Fairlamb, 2003; Phillips and Stanley, 2001).

Mechanism of action

DFMO, an ornithine analogue, is a specific and irreversible suicide inhibitor of ornithine decarboxylase (Bouteille *et al.*, 2003; Denise and Barrett, 2001; McCann and Pegg, 1992; WHO, 1998), the enzyme that catalyses the first rate limiting step in polyamine biosynthesis (Figure 2.9) (Delespaux and De Koning, 2007; Fairlamb, 2003; Phillips and Stanley, 2001). This step involves the decarboxylation of ornithine into putrescine (Bouteille *et al.*, 2003; Frayha *et al.*, 1997). Consequently putrescine is completely depleted, spermidine and trypanothione reduced by 25 to 50% and methionine metabolism is altered (Phillips and Stanley, 2001).

Putrescine, spermidine and trypanothione are some of the polyamines necessary for cell division and differentiation of trypanosomes. Putrescine is condensated with the aminopropyl moiety of decarboxy-S-adenosylmethionine, a reaction that is mediated by spermidine synthase. In trypanosomes spermidine is conjugated with glutathione to form the antioxidant trypanothione that is specific to the kinetoplastids (Reguera *et al.*, 2005). Trypanothione is a reducing agent with many regulatory and protective functions in the trypanosomatids, such as protection against oxidative damage caused by free radicals and peroxides (Heby *et al.*, 2003; Reguera *et al.*, 2005). The inhibition of these polyamines would lead to the cessation of cell growth or death (Bouteille *et al.*, 2003; Phillips and Stanley, 2001). DFMO is essentially cytostatic and requires a functional host immune system to kill the static cells (Denise and Barrett, 2001; Matovu *et al.*, 2001a). Thus the general effects of ODC inhibition are:

- loss in putrescine and decrease of spermidine levels,
- decrease in trypanothione levels, and
- disturbance in S-adenosylmethionine metabolism (Fairlamb, 2003).

DFMO has similar affinity for the mammalian and trypanosomal ODC (Coffino, 2001), yet specificity to the parasite arises because trypanosomal ODC has a longer turn-over rate than that of mammalian ODC (Denise and Barrett, 2001; Ghoda *et al.*, 1990; Heby *et al.*, 2003). Mammalian ODC is rapidly inactivated and degraded giving it a half-life of 10 to 20 minutes. *T. b. gambiense* has an ODC turn-over rate of 18 to 19 hours and that of *T. b. rhodesiense*, 4.2 to 4.4 hours. This could explain why *T. b. rhodesiense* has an innate resistance against DFMO (Iten *et al.*, 1997). Mammalian cells have the capability to synthesise polyamines *de novo*, as well as transporters capable of transporting polyamines into their cells, preventing eflornithine from blocking of the polyamine influx. Trypanosomes on the other hand do not have such an extensive transport mechanism, and cannot import sufficient quantities of polyamines when treated with eflornithine (Heby *et al.*, 2003; Reguera *et al.*, 2005).

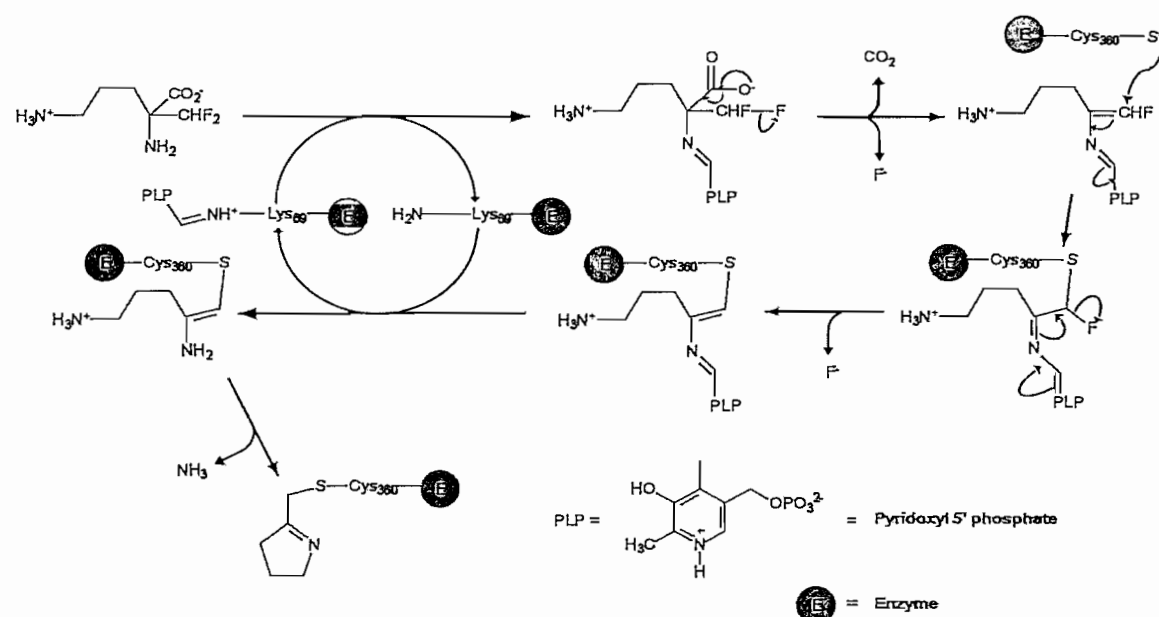


Figure 2.11 Schematic representation of the inactivation of ornithine decarboxylase by eflornithine (adapted from McCann and Pegg, 1992).

The proposed binding of DFMO to the ODC enzyme is depicted in the scheme in Figure 2.11. At the active site, DFMO forms a Schiff base to pyridoxal 5' phosphate (PLP), which is bound to Lys-69. Decarboxylation followed by elimination of a fluoride anion generates a conjugated imine that is strongly electrophilic. The imine alkylates the nucleophilic thiol group of Cys-360. A second conjugated imine is formed after the other fluoride anion is removed, which undergoes a transaldimination reaction with the amino group of Lys-69, to

yield an enamine. At this stage the enamine may cyclise with the consequent loss of ammonia to give a cyclic imine (McCann and Pegg, 1992; Poulin *et al.*, 1992). The scheme also depicts two important factors needed for binding. Both the α -carboxyl and α -amine groups of DFMO are essential for binding to ODC.

Treatment

The treatment of *T. b. gambiense* infections with DFMO is well documented (Khonde *et al.*, 1997; Pépin *et al.*, 2002; Priotto *et al.*, 2008). The current preferred adult dosage regimen is 400 mg/kg/day intravenously for 14 days as a two hour infusion (Phillips and Stanley, 2001; WHO, 1998). A study conducted by Khonde *et al.* proved that a seven day course with eflornithine was effective to treat relapse cases (Khonde *et al.*, 1997). Studies have shown that 10% of patients relapse 12 months after treatment (Balasegaram *et al.*, 2009; Priotto *et al.*, 2008). Children younger than 12 years require a higher dose of 600 mg/kg/day, probably due to an increased clearance rate or low CSF bioavailability (Bouteille *et al.*, 2003; Chappuis *et al.*, 2005; Phillips and Stanley, 2001). The increase in dose had no noteworthy difference compared to the standard dosage regimen (Priotto *et al.*, 2008).

Combination therapy with DFMO has been used with great effect in recent years (Bacchi *et al.*, 1987; Pepin and Milord, 1994; Simarro and Asumu, 1996). One study conducted in 1987 found that synergism between 9-deazinosine, a C-C nucleoside analogue, and DFMO was effective in treating *T. b. brucei* infections *in vivo*. Further studies however had to be conducted to prove this effectiveness against *gambiense* and *rhodesiense* subspecies (Bacchi *et al.*, 1987). Another case study involves the treatment of a 26 year old man, having relapsed from *T. b. gambiense* disease after being treated with melarsoprol and eflornithine individually. After receiving melarsoprol and eflornithine combined, the man was declared cured after being followed up for 2 years (Simarro and Asumu, 1996). Nifurtimox, an orally active agent against Chagas disease, has been used in combination with DFMO to treat relapse cases. Even though nifurtimox has some severe toxic side effects, the combination offers improved safety over melarsoprol, which causes reactive encephalopathy. Data suggest that the effectiveness of the combined treatment is similar to that of standard eflornithine treatment, but has some advantages such as decreased toxicity, cost reduction and simpler, shorter dosage regimes (Priotto *et al.*, 2007).

Adverse reactions

The most frequent side effect found in patients is bone marrow suppression (Pepin and Milord, 1994). Anaemia (48%) and leucopenia (27%) are also commonly observed in patients receiving intravenous treatment (Phillips and Stanley, 2001). Diarrhoea (39%) is also very common, especially after oral administration. Anaemia is reversible with the completion of treatment. Convulsions occur in 7% of treated patients, especially in arseno-resistant cases (Pepin and Milord, 1994; Phillips and Stanley, 2001). Other side effect, such as seizures, gastrointestinal disorders, fever, thrombocytopenia and headache, occur in less than 10% of treated patients (Gibbon, 2005; Na-Bangchang *et al.*, 2004; Phillips and Stanley, 2001; Priotto *et al.*, 2008). Ototoxicity resulting in reversible hearing loss can occur after prolonged treatment but is very rare (Na-Bangchang *et al.*, 2004; Phillips and Stanley, 2001).

Pharmacokinetics and pharmacodynamics

DFMO is a chiral drug and is administered as a racemic mixture of two enantiomers, D/L-DFMO (Malm and Bergqvist, 2007). Both L- and D-DFMO are potent inhibitors of ODC, but the L-enantiomer is more active compared to the D-enantiomer (Qu *et al.*, 2003). This is probably due to the 10 – 20 times higher affinity the L-form has for ODC than the D-form (Jansson *et al.*, 2008a). DFMO is either given orally or intravenously. Several studies have been conducted in recent years on the oral bioavailability of DFMO (Carbone *et al.*, 2000; Haegele *et al.*, 1981; Jansson *et al.*, 2008b; Na-Bangchang *et al.*, 2004). Jansson *et al.* reported that the L-isomer accounted for 35% of the oral bioavailability in the rats and the D-isomer for the remaining 65% (Jansson *et al.*, 2008b). For the racemic oral dose, the bioavailability was 54% with peak plasma levels achieved after 4 hours (Phillips and Stanley, 2001).

Table 2.3 Pharmacokinetic and physicochemical properties of eflornithine.

Molecular weight	182.17 g/mol	Half-life	4-20 hours
Log P	-0.82	Clearance	1.5 ml/min/kg
Melting point	149 °C	Excretion	Renally (80%)
Oral bioavailability	54-58%	Volume of distribution	0.3 l/kg
Metabolism	Not metabolized		

DFMO does not bind to plasma protein. The CSF:plasma ratio after a 14 day intravenous course was reported to be 0.91 (Milord *et al.*, 1993; Phillips and Stanley, 2001). This low CSF concentration of DFMO can be attributed to the active or passive efflux of DFMO from the CSF (Na-Bangchang, 2003). Although facilitated and active transport have been suggested (Hawkins *et al.*, 2006; O'Kane *et al.*, 2006; O'Kane *et al.*, 2006), recent studies have shown that transport of DFMO in and out of the BBB occurs by passive diffusion (Sanderson *et al.*, 2008). Eflornithine has a short elimination half-life of over three hours, and is mainly (80%) renally excreted, unchanged (Burri and Brun, 2003; Phillips and Stanley, 2001). Table 2.3 lists the pharmacokinetic and physicochemical properties of eflornithine.

Gastrointestinal absorption

Eflornithine is a cationic amino acid analogue of ornithine and has some structural similarities with lysine (Figure 2.12) (Frayha *et al.*, 1997; Sanderson *et al.*, 2008). Cationic amino acid transport occurs *via* two families of proteins found in various tissues. Cat (cationic amino acid transporter), referred to as system y^+ , and Bat (broad-scope amino acid transporter), which contains carriers $b^{0,+}$, $B^{0,+}$ and y^+L (O'Kane *et al.*, 2006). These systems can either be Na^+ -dependent or Na^+ -independent (Breves *et al.*, 2007).

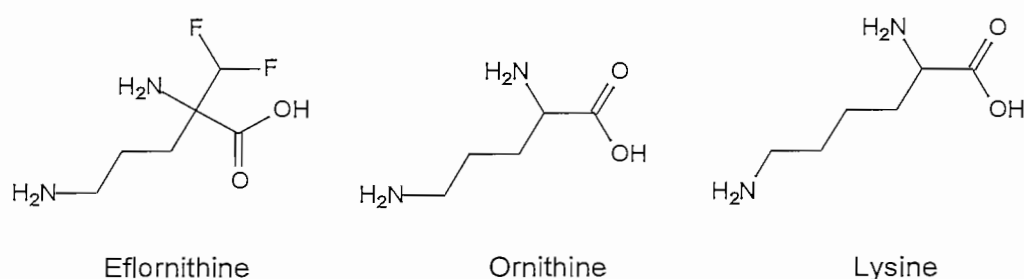


Figure 2.12 The similarities between eflornithine, ornithine and lysine.

Chillarón *et al.* proposed a model depicting the various amino acid transporters and their functional interconnections (Chillarón *et al.*, 1996). In Figure 2.13, neutral amino acids are taken up against their gradient *via* a Na^+ /Amino acid symporter, called B^0 , which is energised by the influx of Na^+ . This transmembrane electrochemical Na^+ gradient is maintained by the Na^+/K^+ -ATPase located in the basolateral enterocyte membrane (Breves *et al.*, 2007; Chillarón *et al.*, 1996). A number of neutral amino acids compete with each other for this transporter (Esposito, 1984). The gradient of neutral amino acid efflux into the extracellular

compartment, drives the uptake of cationic amino acids *via* the $b^{0,+}$ antiporter, located in the brush border membrane, against their concentration gradient. Cationic and neutral amino acids leave the enterocyte in the basolateral membrane *via* systems y^+L and L respectively (Breves *et al.*, 2007).

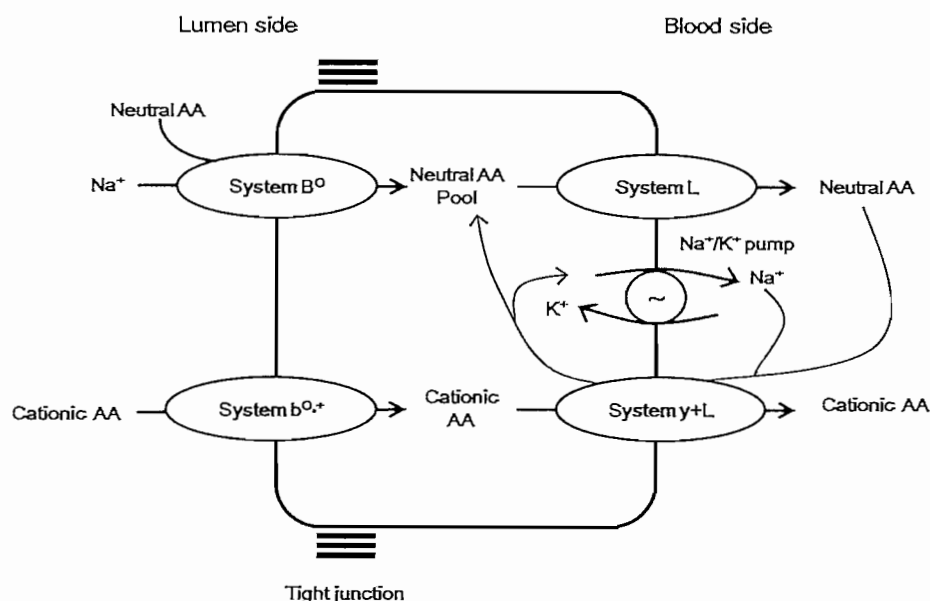


Figure 2.13 A model for the absorption of cationic and neutral amino acids (AA) via various carriers coupled to the in- and efflux of Na⁺ (adapted from Breves, 2007).

Due to the structural similarity of DFMO to the natural amino acids lysine and ornithine, it could be expected that DFMO would be actively transported as explained above, yet in terms of molecular weight it is small enough to be absorbed paracellularly (Jansson *et al.*, 2008b; Seiler and Dezeure, 1990). This could be explained by the various factors affecting drug absorption such as pH of the absorption site, gastric motility, physicochemical properties of the molecule, perfusion of the gastrointestinal tract and enzymatic degradation. It must be noted that amino acid transport has been studied in great detail and that the absorption of DFMO is based on the structural similarity to ornithine and lysine. The precise intestinal absorption route of DFMO requires further investigation.

2.5 Drug resistance

Drug resistance in HAT has been known for years, but the mechanism of resistance in HAT has only recently become clear (Mäser *et al.*, 2003). Trypanosomal resistance can be described at four levels, namely the host, vector, drug and the parasite (Matovu *et al.*, 2001b). Parasite derived resistance and its effects on drug transport and drug binding has been studied in more detail to get a better understanding on the mechanisms of drug resistance (De Koning, 2001; Delespaux and De Koning, 2007; Iten *et al.*, 1997; Mäser *et al.*, 2003).

Alterations in ODC characteristics account for resistance against DFMO, especially in *T. b. rhodesiense* (Iten *et al.*, 1997). As described in section 3.8.5.2, DFMO inhibits ODC, preventing polyamine synthesis. The selectivity of DFMO to the parasite ODC enzyme is due their slower turn-over rates (Ghoda *et al.*, 1990; Heby *et al.*, 2003). Although *T. b. rhodesiense* has a slower turn-over rate than mammalian ODC, the parasite produces enough ODC to counter the inhibitory effects of the drug (Iten *et al.*, 1997; Matovu *et al.*, 2001b).

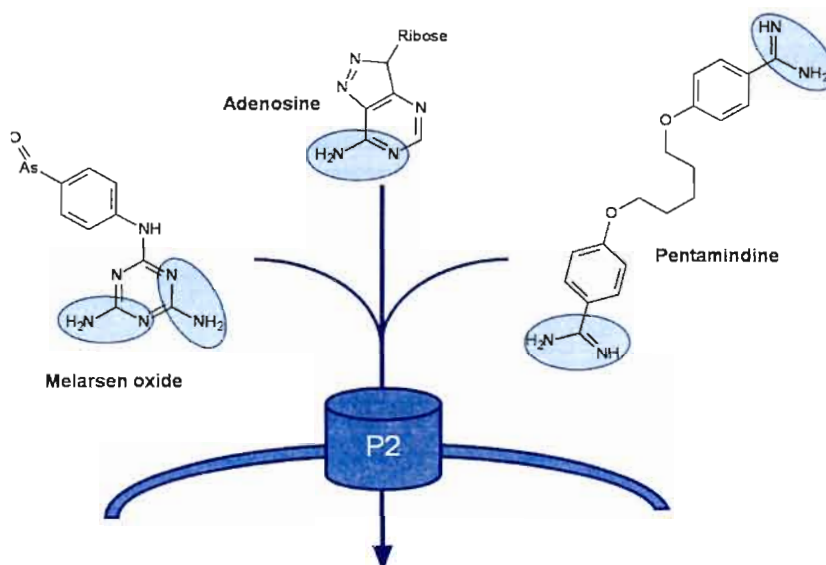


Figure 2.14 Structural similarities between adenosine, melanophenyl arsenicals (melarsen oxide) and diamidines (pentamidine) (adapted from Matovu, 2001b).

Most of the drugs used today still target polyamine synthesis. Putrescine, spermidine and trypanothione are synthesised by the cell and are needed for cell division and differentiation (Bouteille *et al.*, 2003; Phillips and Stanley, 2001). On the other hand purines such as adenosine is also needed for cell division but cannot be synthesised. Instead it is scavenged from the host and transported into the cell *via* transporters, P1 and P2 (Matovu *et al.*, 2001b). P1 is specific for adenosine and inosine (Mäser *et al.*, 1999), whereas the P2-transporter has been implicated in the uptake of melaminophenyl arsenical drugs (melarsoprol) and diamidines (pentamidine) together with adenosine and inosine (Carter *et al.*, 1995). The transport of these trypanocides may lie in their structural similarities (Figure 2.14), as they all contain amidine moieties (Matovu *et al.*, 2001b). This however has not yet been proven. Resistance arises through down regulation of the genes coding the P2-transport proteins, thus decreasing drug transport into the cell (De Koning, 2001).

Other transporters also play a role in drug influx or efflux. One such class is the ATP-binding cassette (ABC) transporters, which are ubiquitous membrane proteins that utilize ATP (adenosine triphosphate) to transport substrate across cell membranes (Borst and Oude Elferink, 2002; Delespaux and De Koning, 2007). These include P-glycoproteins (Pgps), used in drug efflux (Ambudkar *et al.*, 1999; Upcroft, 1994), and the multidrug-resistance associated proteins (MRPs) that export lipophilic anionic conjugates of drugs out of the cell (König *et al.*, 1999). Recent studies have found ABC transporters in many protozoa including *T. brucei* (Klokouzas *et al.*, 2003; Maser and Kaminsky, 1998), although their role in drug resistance is still unfounded (Matovu *et al.*, 2001b).

2.6 Conclusion

Human African trypanosomiasis still ranks third in the world of parasitic diseases next to malaria and filariasis. Within the endemic population up to 500 000 people are infected annually. The parasite requires both a vector and host to complete its life cycle. The disease can be divided into two distinct stages, a haematolymphatic and a meningoencephalitic stage. It is during the meningoencephalitic stage where the most profound and well-known symptoms such as depression, sensory disorders and sleep disruptions are observed. Suramin and pentamidine are both polar macromolecules that cannot cross the BBB, thus are ineffective for late stage infections. Melarsoprol, a toxic arsenical drug, and eflornithine are used for late stage infections. Eflornithine however, requires large doses to achieve the necessary effective concentrations in the CNS.

Resistance against most of the drugs have been documented and increases the need for new drugs development.

Chapter 3

Article for Submission

Chapter 3 contains the manuscript of an article to be submitted to Pharmaceutical Research. The article contains the background, aims, all the experimental methods and results of this study, including the physicochemical properties and biological evaluation results of efloornithine and its derivatives. The article is prepared according to the author guidelines that can be found on the website of this journal (<http://www.springer.com/biomed/pharmaceutical+science/journal/11095?detailsPage=contentItemPage&CIPageCounter=145121>), except that for easy reading, some formatting guidelines have been ignored.

Synthesis, metabolism and intrinsic activity of δ -amides of eflornithine

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Abstract

Purpose

The purpose of this study was to synthesise a series of δ -amides of the anti-trypanosomal drug eflornithine (DMFO - α -difluoromethylornithine) in an attempt to improve its low bioavailability, to assess the metabolism, if any, of the derivatives into the parent drug, and to determine their intrinsic activity against trypanosomiasis.

Methods

δ -amides were synthesised by derivatisation of eflornithine on its δ -amino group by amidation with acyl chlorides. Oral absorption was determined in Sprague-Dawley rats with blood analysed by HPLC at pH 7.4. Log D and aqueous solubility values of each compound was determined in phosphate buffer at pH 7.4. *In vitro* evaluation of intrinsic activity was done on eflornithine sensitive *Trypanosoma brucei brucei* strains.

Results

The derivatives were successfully synthesised and their structures confirmed by NMR and mass spectrometry. No metabolism of the derivatives into eflornithine was observed. Six of the seven derivatives had an increase in lipophilicity. One of the four compounds evaluated showed a moderate increase in intrinsic activity compared to DMFO.

Conclusions

Although an increase in lipophilicity was achieved, no conclusive results were obtained to verify improved oral bioavailability. Two of the compounds tested showed moderate increase in intrinsic activity.

Keywords

Eflornithine; Derivatives; Oral bioavailability; Human African trypanosomiasis (HAT); Log D

1 Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a vector-borne parasitic disease affecting humans and animals and is uniformly fatal if left untreated (1). HAT is considered to rank third in the world of parasitic diseases next to malaria and filariasis (2). The disease affects 60 million people living in 36 endemic African countries, primarily in sub-Saharan Africa (3-5). Only 5-10% of the endemic population is under surveillance, with 25 000 new cases diagnosed each year. An estimated 300 000 to 500 000 people are infected annually due to this disease (3, 6, 7).

The human disease is caused by two species of extracellular kinetoplastid protozoa that are morphologically indistinguishable, but vary in infectivity (8). *T. b. rhodesiense* causes the acute form of the disease, while *T. b. gambiense*, representing over 90% of all reported

cases, causes the chronic form (1). The signs and symptoms caused by HAT are classified in two stages namely an early (haematolymphatic) stage and a late (meningoencephalitic) stage. Early stage symptoms such as fever, headache, lymphadenopathy and anaemia appear as the parasite multiplies in the blood. Late stage symptoms occur as the parasite migrates over the blood-brain barrier and enters the brain. This stage is characterised by neurological disorders such as sensory disorders, convulsions and sleep disturbances.

Melarsoprol, an organo-arsenical drug, and eflornithine (DFMO) are the only two drugs registered for the treatment of late stage HAT. Melarsoprol is highly toxic and causes fatal encephalopathy in five to ten percent of patients treated, with approximately 40% of these patients dying as a result of these adverse reactions (9). There is also a growing resistance against melarsoprol with a relapse rate of 30% in certain endemic areas (9-12).

Eflornithine is an ornithine analogue, and relies on the mechanism of specifically and irreversibly inhibiting ornithine decarboxylase (ODC) (7, 13-15). The drug is only effective against *T. b. gambiense* infections (16), and is currently primarily used in patients with severe CNS complications such as convulsions and hemiplegia, or who have relapsed after melarsoprol treatment. Eflornithine is highly hydrophilic and displays an estimated oral bioavailability of 54% (17). Subsequently, high doses of eflornithine are needed to reach the minimum effective concentration of 50 μ M in the brain, where the parasite resides during the late stage. Because the drug is primarily given intravenously, the drug is very expensive and the mode of administration requires medical protocol and procedures, which would explain why so many patients are left untreated (9). Should orally administered eflornithine be available, it would have some advantages such as easier administration and better patient compliance.

The objective of this study was to synthesise lipophilic amides of DFMO, determine their physicochemical properties, evaluate their intrinsic activity and assess their oral absorption in an attempt to improve the bioavailability of this drug

2 Materials and methods

2.1 Materials

The following chemicals were purchased from Sigma Aldrich, South Africa Ltd: thiophenecarbonyl chloride, p-anisoyl chloride 99%, acetyl chloride 98%, and nicotinoyl chloride hydrochloride 97%. Phenylacetyl chloride, propionyl chloride and benzoyl chloride were purchased from Fluka, South Africa. Eflornithine hydrochloride was generously donated by the World Health Organisation (WHO). All the chemicals and reagents were of analytical grade.

2.2 General procedures

The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance[™] III 600 spectrometer (at a frequency of 600.17 and 150.913 MHz, respectively) in dimethyl sulfoxide (DMSO). Chemical shifts are reported in parts per million δ (ppm) using tetramethylsilane (TMS) as internal standard. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublet), dt (doublet of a triplet), dq (double of quartet), t (triplet), q (quartet) and m (multiplet).

Mass spectra (MS) were recorded in positive mode on a Thermo Electron LXQ[™] (Thermo Fisher Scientific Inc, Waltham, MA) ion trap mass spectrometer with APCI source set at 300 °C and Xcalibur 2.2 data acquisition and analysis software by using direct infusion with a Harvard syringe pump at a flow rate of 10 $\mu\text{l}/\text{min}$. A full scan from 100-1200 amu was obtained in 1 sec, with a capillary voltage of 7 V while the corona discharge was 10 μA .

Melting points were determined by differential scanning calorimetry (DSC) on a Shimadzu DSC-60A instrument (Shimadzu Corp., Kyoto, Japan). Thin-layer chromatography (TLC) was performed using silica gel plates (60F254 Merck). Preparative column chromatography was done on silica gel (230-240 mesh, G60 Merck) or silica gel 60 (70-230 mesh ASTM, Fluka).

2.3 High performance liquid chromatography (HPLC) analysis

The HPLC system (Agilent Technologies, Palo Alto, CA) consisted of an Agilent 1200 series auto sampler, quaternary gradient pump and a variable wavelength detector (VWD). A Synergetic Hydro-RP 4 μm (250 x 4.60 mm) column (Phenomex, Torrance, CA) and the Agilent Chemstation v08.03A for LC systems software package for data analysis was used. The compounds were quantified using a gradient method (A = 0.2% triethylamine in water, pH 7.0, B = acetonitrile) at a flow rate of 1 ml/min with 10 μl standard sample injections. The gradient consisted of 5% of solvent B for 8 min, then increased linearly to 70% of B in 8 min, and held for 10 min. Stop time was 16 min. The wavelength used for HPLC detection for DFMO and all the derivatives was at 205 nm. The peak retention times (t_R) were 3.67 min for (**3a**), 4.96 min for (**3b**), 6.04 min for (**3c**), 5.43 min for (**3d**), 6.13 min for (**3e**), 6.38 min for (**3f**), 5.70 min for (**3g**) and 2.33 min for (**1**).

2.4 General procedure for the acylation of the δ -amine of eflornithine hydrochloride

The synthesis of δ -amides (**3a** – **3g**) required the coupling of an acyl chloride to the δ -amino group of eflornithine and is outlined in Figure 1. This was achieved by adapting the

method reported by Zhang *et al.* (18). Eflornithine hydrochloride (1) (3 g, 13.5 mmol) was dissolved in 15 ml of NaOH solution (5N) and stirred in an ice bath for 10 min. The corresponding acyl chloride (2) (3 equiv. relative to eflornithine) was added drop wise under careful control of the pH. When the pH dropped below 11, NaOH (5N) was added. The reaction mixture was stirred in the ice bath for 12 h and then at room temperature for another 36 h. Thereafter the reaction mixture was acidified with HCl (5N) to pH ~ 1. The precipitate that formed was filtered off, and washed with diethyl ether (3 x 50 ml) and dried under vacuum. The target compound was isolated after silica gel chromatography eluted with a mixture of MeOH and DCM in different ratios depending on the compound being isolated.

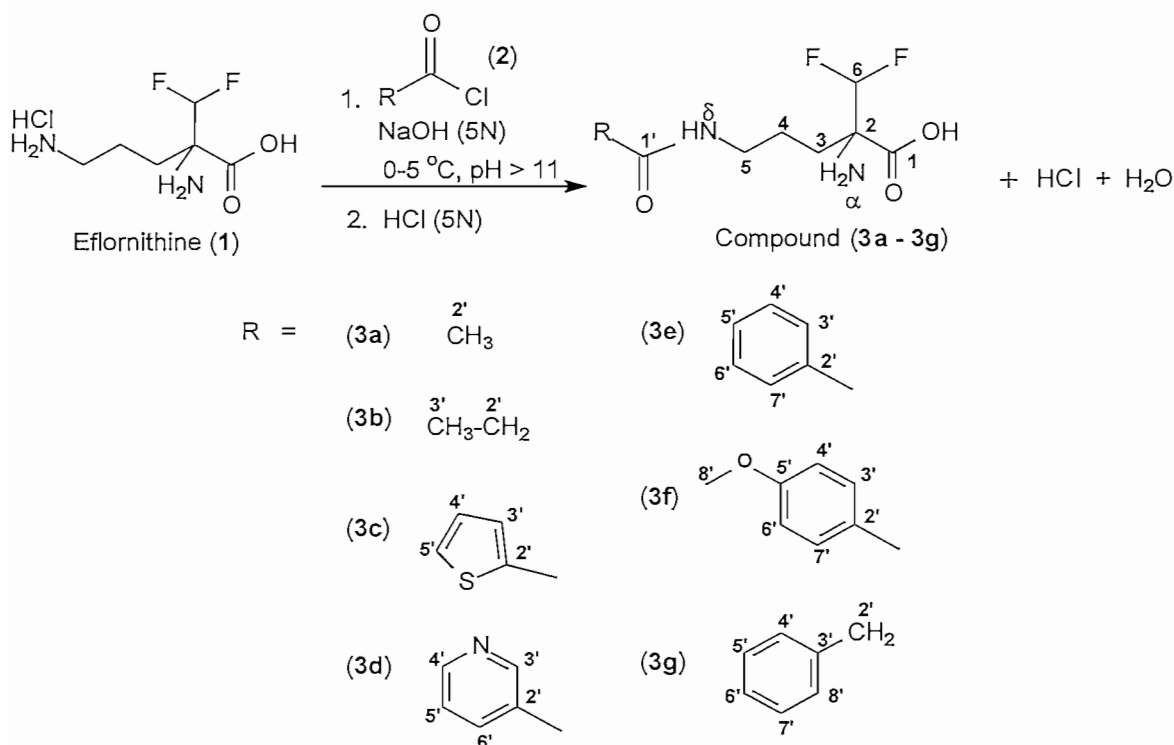


Figure 1. Scheme of the synthesis of δ -amides of eflornithine.

2.4.1 2-amino-2-(difluoromethyl)-5-acetamidopentanoic acid (3a)

Compound (3a) was purified by column chromatography, eluting with DCM:MeOH (2:1 v/v) to give a white powder weighing 1.94 g (64%) yield. Mp 181.6 °C, C₈H₁₄F₂N₂O₃, M_w 224.21. ¹H NMR δ (ppm) (DMSO): 7.94 (t, J = 5.4 Hz, 1H, H- δ), 6.19 (t, J = 54.6 Hz, 1H, H-6), 2.96 (dd, J = 12.3, 6.2 Hz, 2H, H-5), 1.76 (s, 3H, H-2'), 1.70 (dd, J = 14.7, 10.8 Hz, 1H, H-3a), 1.51 (m, overlapping, 2H, H-3b, H-4a), 1.30 (dq, J = 14.3, 7.2 Hz, 1H, H-4a). ¹³C NMR δ (ppm): 169.11 (C-1), 166.89 (C-1'), 117.35 (C-6), 63.67 (C-2), 38.51 (C-5), 29.63 (C-3),

23.43 (C-4), 22.63 (C-2'). m/z (APCI,%): $[M+H]^+$ 224.71 (100), 246.74 (15), 278.92 (10), 300.1 (4), 448.5 (62), 470.71 (33), 492.74 (30), 493.82 (6), 546.9 (6), 716.57 (11), 738.7 (17), 760.39 (9).

2.4.2 2-amino-2-(difluoromethyl)-5-propanamidopentanoic acid (3b)

Compound (3b) was purified by column chromatography, eluting with DCM:MeOH (2:1 v/v) to give a white powder weighing 1.86 g (58% yield). Mp 181.7 °C, $C_9H_{16}F_2N_2O_3$, M_w 238.23. ^1H NMR δ (ppm) (DMSO): 7.91 (t, J = 5.4 Hz, 1H, H- δ), 6.22 (t, J = 54.5 Hz, 1H, H-6), 2.96 (dd, J = 12.3, 6.3 Hz, 2H, H-5), 2.03 (m, 2H, H-2'), 1.71 (t, J = 11.5 Hz, 1H, H-4a), 1.54 (t, J = 11.3 Hz, 2H, H-3), 1.31 (m, 1H, H-4b), 0.95 (t, J = 7.6 Hz, 3H, H-3'). ^{13}C NMR δ (ppm): 172.83 (C-1), 166.86 (C-1'), 117.16 (C-6), 63.61 (C-2), 38.40 (C-5), 29.39 (C-3), 28.51 (C-2'), 23.40 (C-4), 10.04 (C-3'). m/z (APCI,%): $[M+H]^+$ 238.68 (100), 239.8 (10), 476.62 (8), 498.76 (10), 520.83 (8), 752.94 (2).

2.4.3 2-amino-2-(difluoromethyl)-5-(thiophen-2-ylformamido)pentanoic acid (3c)

Compound (3c) afforded a yield of 2.7 g (61%) as a yellow powder after being purified by column chromatography eluting with MeOH. Mp 154.6 °C, $C_{11}H_{14}F_2N_2O_3S$, M_w 292.30. ^1H NMR δ (ppm) (DMSO): 7.91 (t, J = 5.4 Hz, 1H, H- δ), 6.22 (t, J = 54.5 Hz, 1H, H-6), 2.96 (dd, J = 12.3, 6.3 Hz, 2H, H-5), 2.03 (m, 2H, H-2'), 1.71 (t, J = 11.5 Hz, 1H, H-4a), 1.54 (t, J = 11.3 Hz, 2H, H-3), 1.31 (m, 1H, H-4b), 0.95 (t, J = 7.6 Hz, 3H, H-3'). ^{13}C NMR δ (ppm): 172.83 (C-1), 166.86 (C-1'), 117.16 (C-6), 63.61 (C-2), 38.40 (C-5), 29.39 (C-3), 28.51 (C-2'), 23.40 (C-4), 10.04 (C-3'). m/z (APCI,%): $[M+H]^+$ 292.69 (100), 293.74 (12), 414.93 (6), 584.7 (8).

2.4.4 2-amino-2-(difluoromethyl)-5-(pyridin-3-ylformamido)pentanoic acid (3d)

A yield of 1.5 g (41%) of a yellow powder was obtained after column chromatography eluting with MeOH. Mp 106.7 °C, M_w 287.27, $C_{12}H_{15}F_2N_3O_3$, M_w 287.27. ^1H NMR δ (ppm) (DMSO): 8.99 (s, 1H, H- δ), 8.83 (s, 1H, H-3'), 8.67 (d, J = 3.5 Hz, 1H, H-5'), 8.19 (d, J = 7.9 Hz, 1H, H-4'), 7.47 (dd, J = 7.5, 5.0 Hz, 1H, H-6'), 6.04 (t, J = 56.4 Hz, 1H, H-6), 3.22 (s, 2H, H-5), 1.76 – 1.62 (m, overlapping, 2H, H-3a, H-4a), 1.49 – 1.35 (m, overlapping, 2H, H-3b, H-4b). ^{13}C NMR δ (ppm): 170.34 (C-1), 164.57 (C-1'), 151.68 (C-3'), 148.42 (C-4'), 134.93 (C-6'), 123.43 (C-5'), 118.65 (C-6), 62.94 (C-2), 40.04 (C-5), 31.27 (C-3), 23.56 (C-4). m/z (APCI,%): $[M+H]^+$ 287.73 (100), 308.81 (28), 392.84 (24), 404.92 (37), 414.96 (19), 469.82 (37), 504.86 (52), 505.99 (17), 574.83 (36), 596.75 (22), 618.85 (19), 641 (15), 701.69 (15), 723.87 (28), 725.09 (14).

2.4.5 2-amino-2-(difluoromethyl)-5-(phenylformamido)pentanoic acid (3e)

Purification by column chromatography, eluting with DCM:MeOH (2:1 v/v) to give a off-white powder weighing 2.62 g (68%) yield. Mp 182.6 °C, $C_{13}H_{16}F_2N_2O_3$, M_w 286.28. ^1H NMR δ (ppm) (DMSO): 8.57 (s, 1H, H- δ), 7.84 (d, J = 7.4 Hz, 2H, H-3', H-7'), 7.49 (t, J = 7.3 Hz, 1H, H-5'), 7.43 (t, J = 7.3 Hz, 2H, H-4', H-6'), 6.15 (t, J = 55.3 Hz, 1H, H-6), 3.21 (d, J = 5.5 Hz, 2H, H-5), 1.75 (t, J = 10.6 Hz, 1H, H-3a), 1.68 (d, J = 8.2 Hz, 1H, H-3b), 1.49 (dd, J = 24.6, 12.5 Hz, 2H, H-4). ^{13}C NMR δ (ppm): 166.05 (C-1), 162.36 (C-1'), 134.63 (C-2'), 131.01 (C-5'), 128.22 (C-4', C-6'), 127.19 (C-3', C-7'), 117.84 (C-6), 63.33 (C-2), 35.81 (C-5), 30.28 (C-3), 23.51 (C-4). m/z (APCI,%): $[M+H]^+$ 286.74 (100), 308.73 (10), 402.95 (10), 422.90 (5), 572.54 (12), 616.80 (9).

2.4.6 2-amino-2-(difluoromethyl)-5-[(4-methoxyphenyl)formamido]pentanoic acid (3f)

Compound (3f) was purified by column chromatography, eluting with DCM:MeOH (1:1 v/v) to give a yellow powder weighing 1.71 g (40%) yield. Mp 138.4 °C, $C_{14}H_{18}F_2N_2O_4$, M_w 316.30. ^1H NMR δ (ppm) (DMSO): 8.54 (s, 1H, H- δ), 7.83 (d, J = 8.0 Hz, 2H, H-3', H-7'), 6.96 (d, J = 8.0 Hz, 2H, H-4', H-6'), 6.54 (t, J = 52.8 Hz, 1H, H-6), 3.78 (s, 3H, H-8'), 3.23 (dd, J = 14.5, 6.3 Hz, 2H, H-5), 1.97 (t, J = 11.9 Hz, 1H, H-3a), 1.86 (t, J = 12.0 Hz, 1H, H-3b), 1.73 (d, J = 4.1 Hz, 1H, H-4a), 1.44 (d, J = 4.7 Hz, 1H, H-4b). ^{13}C NMR δ (ppm): 167.19 (C-1), 165.86 (C-1'), 161.58 (C-5), 129.11 (C-2'), 126.65 (C-3', C-7'), 114.62 (C-6), 113.52 (C-4', C-6'), 63.71 (C-2), 55.44 (C-7), 38.60 (C-5), 28.60 (C-3), 23.06 (C-4). m/z (APCI,%): $[M+H]^+$ 316.81 (100), 317.79 (16), 338.78 (8), 462.99 (15), 464.96 (8), 522.93 (7), 632.56 (16), 654.84 (8), 725.36 (5).

2.4.7 2-amino-2-(difluoromethyl)-5-(2-phenylacetamido)pentanoic acid (3g)

A yield of 2.26 g (56%) of a yellow powder was obtained after column chromatography eluting with MeOH:DCM (2:1 v/v). Mp 172.6 °C, $C_{14}H_{18}F_2N_3O_3$, M_w 300.31. ^1H NMR δ (ppm) (DMSO): 8.13 (s, 1H, H- δ), 7.30 – 7.17 (m, 5H, H-4'–H-8'), 6.09 (t, J = 55.4 Hz, 1H, H-6), 3.36 (s, 2H, H-2'), 2.97 (d, J = 5.8 Hz, 2H, H-5), 1.68 (t, J = 11.1 Hz, 1H, H-3a), 1.54 (d, J = 2.8 Hz, 1H, H-4a), 1.38 (t, J = 12.1 Hz, 1H, H-3b), 1.31 (d, J = 6.4 Hz, 1H, H-4b). ^{13}C NMR δ (ppm): 169.91 (C-1), 162.36 (C-1'), 136.61 (C-3'), 128.99 (C-4', C-8'), 128.16 (C-5', C-7'), 126.25 (C-6'), 118.03 (C-6), 63.27 (C-2), 42.42 (C-2'), 35.81 (C-5), 30.48 (C-3), 23.48 (C-4). m/z (APCI,%): $[M+H]^+$ 300.80 (100), 301.81 (18), 334.87 (7), 418.87 (7), 430.398 (19), 450.87 (10), 600.72 (21), 622.85 (10), 644.86 (16), 645.92 (5), 718.81 (5).

2.5 In silico predictions

2.5.1 Docking studies

Docking studies were done in silico with Discovery Studio 1.7[®] (19). The crystallographic structure of ornithine decarboxylase (ODC) (1F3T.pdb) was retrieved from the RCSB protein data bank (20), and the co-crystallized ligand, putrescine, was removed. To optimise hydrogen bonding patterns and prevent any unfavourable steric clashes, the Charm force field was applied to the enzyme (chain A) and the binding site subsequently identified (site 1). The active site on ODC can be found between the cofactor (where the co-crystallized ligand was removed), cysteine 360 from the one domain and lysine 69 from the neighbouring domain. The ligands to be docked were first prepared with DS Viewer using the 'Prepare Ligands' module of Discovery Studios 1.7[®]. Hydrogens were added and the structure was configured to the minimum energy state. Docking was performed in the 'Dock Ligands' module (Ligandfit) utilizing the 'Smart Minimizer' algorithm. In order to evaluate the accuracy of the docking procedure eflornithine was also docked within the active site using the RMS fit protocol.

2.5.2 Pharmacokinetic parameters

Pharmacokinetic parameters such as fraction absorbed (F_{abs}) were calculated using the computer program Simcyp, Ltd (21). Another parameter used to describe oral absorption is by means of human intestinal absorption (HIA) calculated as a percentage. HIA values were calculated using PreADMET, a web-based application for predicting ADME data (22). Human intestinal absorption data are derived from the sum of the bioavailability and absorption data evaluated from the ratio of excretion or cumulative excretion in urine, bile and faeces (23).

2.6 Aqueous solubility

The aqueous solubility values of compounds **3a** - **3g** were obtained by preparing saturated solutions in phosphate buffer at pH 7.4. The slurries were stirred with magnetic bars in a water bath at 37 °C for 24 h. An excess of solute was present at all times to provide saturated solutions. After 24 h, the solutions were filtered and analyzed directly by HPLC to determine the concentration of solute dissolved in the solvent. The measurements were performed in triplicate.

2.7 Experimental log D

Equal volumes of *n*-octanol and phosphate buffer solution (PBS) of pH 7.4 were saturated with each other under vigorous stirring for at least 24 h. Accurately weighed 3 mg of

eflornithine hydrochloride (0.3 mg/ml) as well as of each derivative was dissolved in 10 ml of pre-saturated PBS, stoppered and agitated for 10 min in 15 ml graduated tubes (0.5 ml division). Subsequently 3 ml of pre-saturated *n*-octanol was transferred to the tubes containing the before-mentioned solutions and agitated for 45 min and then centrifuged at 4000 rpm for 30 min. The *n*-octanol and aqueous phases were allowed to separate at room temperature for 5 min and thereafter the volume ratio (*n*-octanol:buffer 1:1 v/v) was established. In all cases, the ratio was found to be 1. Then 1.5 ml of aqueous phase was extracted, HPLC analyzed and the concentrations determined. Consequently, the concentrations in *n*-octanol phases were deduced. The log D values [(log (octanol:buffer partition coefficient))] were calculated as logarithmic ratios of the concentrations in the *n*-octanol phase to the concentrations in the buffer. The experiment for each compound was performed in triplicate and the log D values are expressed as means.

2.8 *In vivo* oral absorption studies and intrinsic activity

The following section details the materials and methods used in the *in vivo* biological studies (by M. Ashton and C. Johansson at the University of Göteborg, Sweden) and the intrinsic activity determination (by S.K. Vodnala at the Tumour and Cell Biology Karolinska Institute, Stockholm, Sweden) that was done.

2.8.1 Animals

Male Sprague-Dawley rats (Charles River, Germany), weighing between 260 and 350 g, were after their arrival at the certified animal facility (Experimental Biomedicine at Gothenburg University, Sweden) were left to acclimatise for at least 5 days. The rats were kept in 12 h light-dark cycles, at 25 - 27 °C and at 60 - 65% humidity. There were four rats per cage up until surgery, where after they were kept separately. Food (Harlan, USA) and tap water were available before and after surgery *ad libitum* and was removed eight hours prior to drug administration. All experiments were done during the light phase of the cycle. The study was approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden (253/2008) (24).

2.8.2 Chemicals used for *in vivo* experiments

Isoflurane (Forene; Abbot Scandinavia AB, Solna, Sweden) and heparin (Leo Pharma AB, Malmö, Sweden) were obtained from Apoteket AB (Sweden). Eflornithine hydrochloride was obtained from WHO/TDR (Geneva, Switzerland). N-acetyl-L-cysteine and o-phthalaldehyde were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane, di-sodium tetraborate, hydrochloric acid and methanol were

obtained from Fischer Scientific (Waltham, MA, USA). Deionised water was prepared by a Milli-Q® deionised water system (Millipore, Bedford, USA). All chemicals were of analytical grade, and all solvents were of high performance liquid chromatography (HPLC) grade.

2.8.3 Animal surgery procedure

The animals were anesthetized by inhalation of isoflurane (2.9 - 3.7%) in air. The left jugular vein was catheterized using a MRE040 1.02-mm outer-diameter, 0.64 mm inner-diameter tube (AgnThos, Lidingö, Sweden) prefilled with 100 IU/ml of a heparin in saline solution. The catheter was tunnelled subcutaneously to emerge at the back of the neck. To prevent clotting, catheters were kept patent with heparinised saline solution (20 IU/ml) between sampling. All animals were allowed to recover for at least 16 h after surgery before the tests were continued (24).

2.8.4 Oral drug formulation

The drug solutions for oral administration were prepared by dissolving the amount of derivative needed in a saline solution (24).

2.8.5 Instrumentation and chromatographic conditions

The HPLC system consisted of a 48-well plate Prospekt 2 auto injector (SparkHolland, Emmen, Holland), two interconnected Shimadzu LC10AD pumps and a Shimadzu SPD 10-A UV-VIS detector (Shimadzu, Kyoto, Japan), set at 340 nm. Data acquisition was performed using Chromatographic Station for Windows® version 1.2.3 (Data-apex, Prague, Czech Republic).

For improved separation, derivatised eflornithine enantiomers were eluted on two Chromolith Performance columns (RP-18e 100 mm × 4.6 mm I.D.) that were serially connected and protected by a Chromolith® Guard RP-18e column (10 mm × 4.6 mm I.D.) (VWR International, Darmstadt, Germany).

Firstly, an isocratic flow was used for the separation of the derivatised diastereomers followed by a gradient flow to elute endogenous compounds. The mobile phase that was used for the isocratic flow (phase A) consisted of 82% tris (hydroxymethyl) aminomethane dissolved in deionised water (0.1 M; pH 6.8) and 18% methanol. Mobile phase B consisted of 70% methanol and 30% deionised water. The flow rate was set at 2 ml/min and the following gradient program was used: $t = 0 - 16$ min, A 100%; $t = 16 - 17$ min, linear decrease of A from 100 to 0%; $t = 17 - 20$ min, linear increase of A from 0 to 100%. The typical retention times for L- and D- eflornithine were 12.7 and 14.8 min, respectively.

2.8.6 Preparation of calibration standard samples and the samples used for quality control

Stock solutions of D/L-eflornithine hydrochloride (100 mM) were prepared in deionised water. Working solutions of D/L-eflornithine ranging from 50 to 50 000 μ M were prepared by serial dilution of the stock solution in deionised water. These solutions were used to spike blank plasma samples yielding calibration standards at eight different concentrations (not including blank plasma) ranging from 1.5 to 1250 μ M for each enantiomer. The calibration curve was prepared in batches of 10 ml, divided into aliquots (75 μ l) and stored at -37 °C until analysis. Quality control samples, used for determination of accuracy, precision, and recovery were prepared at three concentrations (3, 400, and 1000 μ M of each enantiomer) and divided into 75 μ L aliquots and stored at -37 °C. All spiked calibrators and QC solutions contained between 2 and 4% of working solutions (v/v).

2.8.7 Sample preparation

Plasma samples (75 μ l) were precipitated with cold methanol (300 μ l, -37 °C). The samples were agitated on a mixer for approximately 15 sec, were kept at 4 °C for 30 to 60 min and then mixed for another 15 sec. After centrifugation for 10 min at 12000 rpm, the samples were kept at -37 °C for 10 min to freeze the pellet. The supernatants were decanted into new tubes and evaporated to dryness at room temperature under a gentle stream of nitrogen for 2 – 3 h. The dried samples were re-dissolved in 75 μ l of deionised water, transferred to injection vials and then placed in the auto injector for analysis.

2.8.8 Pre-column derivatisation

The auto injector was programmed to add the derivatisation reagent (100 μ l) to prepared samples, generating diastereomers. The derivatisation reagent was prepared before each analytical run, by mixing *o*-phthalaldehyde (44 mg), *N*-acetyl-L-cysteine (54 mg), methanol (2 ml) and 8 ml borate buffer (0.09 M; pH 8.9). The mixture of the derivatisation reagent and sample was then programmed to stand in the auto injector for a duration of 4.85 min, at 20 °C prior to injection.

2.8.9 Experimental design

A total of 20 rats were administered compounds **3a** - **3g** orally by gavage (5 ml/kg) at an equimolar dose of eflornithine of 100 mg/kg of body weight. Blood samples were drawn from the jugular vein and flushed with heparinised saline solution (20 IU/ml) after each sampling occasion. Sample volumes were replaced with an equal volume of saline solution. Eight blood samples were taken up to eight hours after drug administration (250 μ l per sample).

Plasma was separated by centrifugation for 8 min at 12 000 rpm within 30 min after blood collection and kept at -22 °C until analysis.

2.8.10 *In vitro* drug susceptibility assay

In vitro susceptibility assay was done with some modifications using the method as described by Vodnala and co-workers (25). Bloodstream forms of *T.b. brucei* (AnTat1.1E), freshly isolated from infected C57BL/6 mice (3 days post infection with 16107 parasites/mouse) were separated by DEAE-cellulose chromatography under sterile conditions. *T. b. brucei* parasites were incubated in D-MEM containing 10% heat-inactivated calf serum, 28 mM HEPES, 0.14% glucose, 1.5% NaHCO₃, 2 mM L-glutamate, 0.14 mg/ml gentamycin, 0.3 mM dithiothreitol, 1.4 mM sodium pyruvate, 0.7 mM L-cysteine, 28 µM adenosine, and 14 µM guanosine at 37 °C. To measure drug sensitivity, 8000 parasites were cultured in 96 flat-bottomed well culture plates with serial drug dilutions covering a range from 20 to 0.16 µM, for 72 h at 37 °C. Cultures (100 µl) were incubated for 2 h with 10 µl of WST-1 reagent (Roche, Mannheim, Germany). Viability was measured by the conversion of WST-1 reagent to formazan, recorded by a multiwell scanning spectrophotometer at an excitation wavelength of 450 nm. Fluorescence development was measured and expressed as percentage of the control. Data were analysed using Graphpad Prism 5 software which calculated IC₅₀ values. DFMO served as control.

3 Results

3.1 Chemistry

All compounds were successfully synthesised with yields varying between 40 and 68%. The δ-amino group reacted with the acyl chlorides in an alkaline mixture forming amides which were converted to the free acid form after addition of HCl. Structures were confirmed as δ-amides by NMR and mass spectroscopy

3.2 *In silico* predictions

Results from computer modelling together with the pharmacokinetic parameters were used in this study to screen the best seven molecules to be synthesised from 27 envisaged candidates (see Annexure B).

3.2.1 Docking studies

All proposed compounds were docked in silico with no errors at physiological pH 7.4. All of the compounds screened exhibited lower docking scores than DFMO (control) scoring

116. Compounds **3g** and **3c** showed the best docking scores of 28.91 and 28.52 respectively. Please note that a higher docking score indicates a better ligand fit to the enzyme. All of the compounds were docked as the salt free form to simulate ideal binding to the active site. The results are given in Table I.

3.2.2 Pharmacokinetic parameters

Structures of compounds **3a** - **3g** were constructed and evaluated with pharmacokinetic prediction software such as Simcyp and Pre-ADMET. Table II shows the values of the different pharmacokinetic parameters. All compounds displayed slightly lower F_{abs} values than eflornithine, but higher HIA values.

3.3 Aqueous solubility and lipophilicity

All the compounds were crystalline and highly water-soluble. There is some correlation between the predicted and experimental log D values. An increase in lipophilicity was obtained with all compounds with the exception of **3a** that showed a decrease. Compound **3g** showed the highest increase in lipophilicity with an aqueous solubility (S_w) of 11.13 mg/ml compared to that of DFMO at 34.96 mg/ml. Compounds **3b** to **3f** also showed considerable decrease in aqueous solubility with S_w values ranging between 28 and 19 mg/ml. The physicochemical properties (log D and aqueous solubility) are summarised in Table I.

3.4 *In vivo* oral absorption studies

Eflornithine was used as a reference to determine whether compounds **3a** - **3g** had increased intestinal absorption in the rat after oral administration. After oral administration of eflornithine, samples were taken at precise selective time intervals and analysed. Figure 2 represents the chromatogram of administered eflornithine. Each line represents a sample taken at particular time interval. The first peak ($t_R = 10.5$ min) represents the L-isomer and the second peak ($t_R = 12$ min) the D-isomer. Results from analysed samples containing compounds **3a**, **3c**, **3d** and **3f** are shown in Figure 3. The HPLC results show that no metabolism to eflornithine occurred.

3.5 Intrinsic activity

The intrinsic activity of all the compounds (**3a** - **3g**) with DFMO as control were evaluated *in vitro* and are expressed as IC_{50} values. All the compounds tested showed activity but only compounds **3b** and **3f** showed a moderate increase in activity with an IC_{50} value of 32.05 μ M and 35.45 μ M respectively, in comparison with DFMO (36.22 μ M). Results of intrinsic activity assays are shown in Table II.

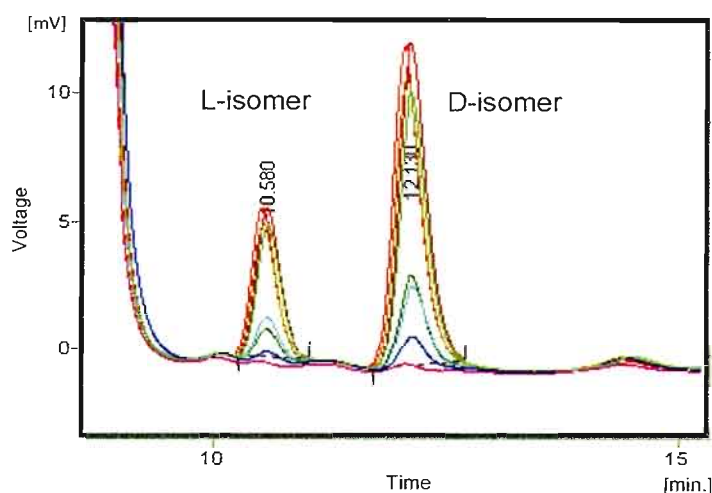


Figure 2. HPLC chromatograms of eflornithine from samples taken on various time intervals showing the D- and L-isomers of DFMO.

Table I. Docking scores, predicted and experimental log D, aqueous solubility (S_w) and IC_{50} values of DFMO (control) and synthesised compounds (**3a – 3g**).

Compound	Docking score	Log D ^a	Log D ^b	Log D ^c	S_w (mg/ml)	IC_{50} ($\times 10^{-6}$ μ M)
DFMO	115.67	-0.82	-0.77	-0.96 ± 0.88	34.96 ± 0.37	36.22
3a	23.23	-1.08	-1.03	-1.21 ± 0.53	36.38 ± 0.12	> 100
3b	22.57	-0.33	-0.08	-0.78 ± 1.07	28.74 ± 0.36	32.05
3c	28.91	0.7	0.87	-0.19 ± 0.77	25.47 ± 0.65	43.18
3d	27.31	-0.35	-0.23	-0.66 ± 0.98	27.26 ± 0.27	38.74
3e	26.74	0.81	0.91	-0.13 ± 0.99	19.67 ± 0.67	> 100
3f	25.8	0.99	0.9	-0.28 ± 0.95	24.13 ± 0.34	35.45
3g	28.52	1.2	0.95	-0.07 ± 1.08	11.13 ± 0.32	> 100

Partition coefficient, log D (*n*-octanol-PBS, pH 7.4).

^a Calculated with Simcyp Ltd. ^b Calculated using pre-ADMET v1.0 online software. ^c Experimental data represent the mean and standard error of mean (SEM) of 3 measurements.

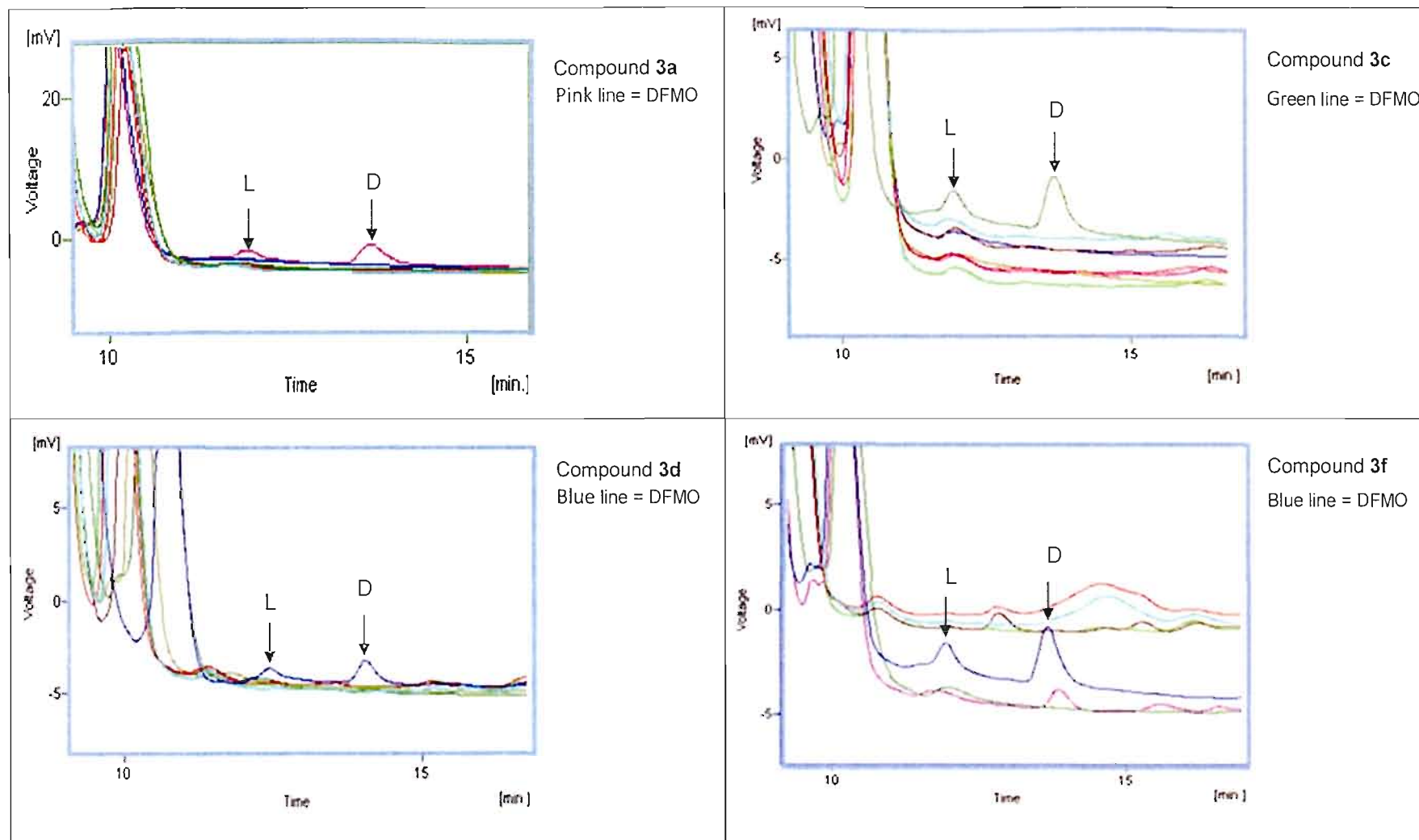


Figure 3. Chromatograms of compounds 3a, 3c, 3d and 3f after oral administration. The peaks indicated are that of the two isomers of DFMO after oral administration of DFMO.

Table II. Pharmacokinetic parameters of DFMO (control) and synthesised compounds (**3a - 3g**).

Compound	M _w (g/mol)	Mp (°C)	F _{abs} ^a	HIA (%) ^b	H Donors ^a	H Acceptors ^a
DFMO	182.17	149.5	0.44	55.2	3	5
3a	223.2	181.6	0.412	56.3	3	5
3b	237.23	181.7	0.412	59.6	3	5
3c	292.3	154.6	0.412	70.1	3	5
3d	287.27	106.7	0.321	71.6	3	6
3e	286.28	182.6	0.412	83.9	3	5
3f	316.3	138.4	0.345	80.4	3	6
3g	300.31	172.6	0.398	85.8	3	5

Molecular weight (M_w), melting point (Mp), fraction absorbed (F_{abs}), human intestinal absorption (HIA).

^a Calculated with Simcyp Ltd. ^b Calculated using pre-ADMET v1.0 online software.

4 Discussion

4.1 Chemistry

Eflornithine is an amino acid analogue of ornithine. Due to the pK_a of the two amino groups ($\delta\text{-NH}_2 = 10.1$; $\alpha\text{-NH}_2 = 6.84$) and of the carboxylic group ($\alpha\text{-COOH} = 1.22$), the molecule is able to be protonated at virtually any pH value. This makes intestinal absorption difficult. Two major factors have to be considered when creating drugs for oral administration, namely solubility and permeability (26). An ideal drug should be sufficiently water soluble to dissolve in the gastric environment yet be lipophilic enough to pass through biological membranes. Coupling lipophilic substituents to the δ -amino group of eflornithine should increase its lipophilicity, but still maintain the hydrophilic potential from the free α -carboxylic and α -amino groups. The molecule is thus amphiphilic. N-acylated amines are not commonly used due to their metabolic stability (27). However, due to this resilience to enzymatic degradation the extent of oral absorption for each of the derivatives can be determined to a greater degree of accuracy.

Eflornithine is highly soluble in alkaline aqueous medium. The δ -amino group of eflornithine reacted with the acyl chloride and was converted to amides. The free acid form was obtained after fast acidification with hydrochloric acid (33%) to pH 1.

Eflornithine exhibited ^1H NMR signals at 2.75 ppm (H-5), 1.5 ppm (H-4) and 1.75 ppm (H-3) respectively. Substitution on the δ -amino group as opposed to the α -amino group was confirmed by the downfield shift of the signal of H-5 to an area around 3 to 3.5 ppm. Coupling to the α -amino group would be observed by chemical shifts of H-3 and H-4 downfield, which was not the case for compounds **3a** – **3g**. Chollet *et al.* found that the α -amino group was very unreactive, possibly due to steric hindrance or electron withdrawal by the difluoromethyl group (28). This was also confirmed in this study by the chemical shifts observed in the ^1H NMR spectra.

The presence of eflornithine was confirmed by the resonance of H-6 observed in the ^1H NMR spectra by the triplet in a region at 6 – 6.5 ppm downfield with coupling constants of all the compounds varying between 56.4 and 52.8 Hz. The protons of H-3 and H-4 were also clearly visible in the 1.75 and 1.5 ppm regions respectively. Due to the close proximity of protons H-3 and H-4 to the chiral centre of the molecule, the resonance of H-3 and H-4 should be confirmed by two distinct peaks in ^1H NMR. And indeed, they are discernable in the spectra of compounds **3c**, **3f** and **3g** while in other cases they overlapped each other.

The MS data for the compounds confirmed the presence of molecular ions (m/z) at $[\text{M}+\text{H}]^+$ 224.71 (**3a**), 238.68 (**3b**), 292.69 (**3c**), 287.73 (**3d**), 286.74 (**3e**), 316.81 (**3f**) and 300.80 (**3g**), corresponding to the molecular formulae $\text{C}_8\text{H}_{14}\text{F}_2\text{N}_2\text{O}_3$ (**3a**), $\text{C}_9\text{H}_{16}\text{F}_2\text{N}_2\text{O}_3$ (**3b**),

$C_{11}H_{14}F_2N_2O_3S$ (**3c**), $C_{12}H_{15}F_2N_3O_3$ (**3d**), $C_{13}H_{16}F_2N_2O_3$ (**3e**), $C_{14}H_{18}F_2N_2O_4$ (**3f**) and $C_{14}H_{18}F_2N_2O_3$ (**3g**). All of the compounds show results of dimerisation which explains, in most cases, the occurrence of the second highest peaks being twice that of the highest peak. Due to fragmentation caused by electron impact ionisation, only nominal mass could be determined by means of APCI.

4.2 In silico predictions

4.2.1 Computer modelling

The docking scores obtained in this study are the average of all the docking score parameters (LigScore 1, LigScore 2, PLP 1, PLP 2, Jain, PMF, Ludi Energy Estimate1, Ludi Energy Estimate 2 and Ludi Energy Estimate 3) (29). It should be emphasized that computer modelling does not take into account factors such as pre-enzymatic degradation, allosteric variation, pH of the target area or intrinsic activity. It is however a good representation of binding affinities and is useful in identifying lead compounds.

The docking score for eflornithine confirmed excellent binding to ODC which correlates well with the mechanism of action of eflornithine. Although the docking scores for compounds **3a** – **3g** are below that of the control, it should be noted that only the α -carboxyl and α -amino groups are involved in binding to the enzyme. Thus the derivatives should theoretically be able to bind to the ODC enzyme. However, the substituents on the δ -amino group may play a role in entering the enzyme cavity or by stabilizing the molecule inside the cavity. Thus the substituents may influence the ligand fit to a certain extent and consequently affect the docking score. Computer modelling together with the pharmacokinetic parameter results were used in this study to obtain the best seven molecules to be synthesised from 27 possible candidates.

4.2.2 Pharmacokinetic parameters

HIA values were surprisingly high considering the polar nature of the compounds. According to Table III compounds **3a** – **3g** are classified as moderately to well absorbed, with compound **3g** having the highest HIA value of 85.8. This was interesting as **3g** exhibited the third lowest F_{abs} value of 0.38. However it should be noted that calculation of HIA are based on molecular properties and are effective for oral absorption estimation but not oral bioavailability (30). F_{abs} is the fraction of the dose transported (absorbed) across the cell membrane into the cellular space of the enterocyte. This fraction might be affected by two factors namely the first pass extraction of the drug in the intestinal wall and the first pass extraction of the drug in the liver (31).

Table III. Classification of compounds according to HIA values (adapted from (29)).

Classification	HIA (Human Intestinal Absorption)
Poorly absorbed compounds	0 ~ 20 %
Moderately absorbed compounds	20 ~ 70 %
Well absorbed compounds	70 ~ 100 %

All compounds demonstrated slightly lower F_{abs} values than that of eflornithine, with **3d** being the lowest. Studies have shown that peptide transporters, responsible for the transport of di- or tripeptides over the intestinal wall, demonstrate different affinities for different ionic species of a given electrically charged peptide substrate (32). Thus the polar groups at the α -terminal of the derivatives and the non-polar aliphatic chains at the δ -terminal of the eflornithine backbone may have debilitated the transport across the intestinal wall with a subsequent decrease of F_{abs} . This might explain the slight variation in F_{abs} values. Thus the pharmacokinetic parameters provide an insight to the behaviour of these derivatives in terms of oral absorption.

4.3 Aqueous solubility and lipophilicity

Lipophilicity is an important property for orally administered drugs as the membranes of the gastro intestinal tract are lipophilic in nature and generally favour lipophilic drugs. Yet, drugs need to be hydrophilic enough to go in solution. Log D expressed as a ratio of octanol solubility to aqueous solubility at pH 7.4 is found in almost every physicochemical analysis to be related to absorption (34, 35). One of the most reliable methods in medicinal chemistry to improve in vitro activity is to incorporate properly positioned lipophilic groups.

Lipinski expresses lipophilicity as one of the four parameters used to determine poor permeability and absorption. In the rule of 5 a log D value higher than 5 contributes to poor absorption (35). This is possible due to the molecule's inability to solubilise in the hydrophilic environment. There is a good correlation between the experimental and theoretical log D values, yet the experimental values remain more reliable even though they are lower. Increased polarity contributes to increased hydrophilicity and thus a lower log D value.

All the compounds (**3b – 3g**) with the exception of **3a** showed a greater solubility in octanol than DFMO. Thus the increase in lipid solubility exhibited by compounds (**3b – 3g**) occurs because the lipophilic moiety masks the hydrogen bond acceptor, in this case the δ -NH₂ of eflornithine. Eflornithine has an experimental log D value of -0.96 which correlates

well with the theoretical value of -0.82 (Calculated with Simcyp, Ltd). The S_w values for (**3a** – **3g**), as expected, decreased with an increase in log D, thus validating the structure-lipophilicity relationships within the series.

The molecular weights of DFMO and the derivatives (**3a** – **3g**), ranging from 182.2 to 316.3 g/mol, are below 500 g/mol. The number of H-donors and acceptors for each compound are below 5 and 10, respectively. Considering the results, each of the compounds adheres to Lipinski's "rule of five". In this regard compounds **3a** – **3g** should display increased oral absorption or permeation.

4.4 *In vivo* oral absorption studies

The biological absorption studies did not detect any of the derivatives in the blood samples because the HPLC method only detects eflornithine that is released from the derivatives after enzymatic cleavage of the amide bond. After this cleavage DFMO is then able to bind to the derivatisation reagent at the δ -amine position of eflornithine and is then detected. Conversely, if the amide bond does not break, no eflornithine is detected. This explains why no eflornithine was detected for compounds **3a**, **3c**, **3d** and **3f**. Thus the HPLC results examined in this study do not represent the concentration of the derivative in the blood but that of eflornithine. Further investigations are needed however to validate these results.

4.5 Intrinsic activity

Intrinsic activity refers to the resulting pharmacological response induced by a compound that binds to a specific receptor. Activity is expressed in this study as IC_{50} , which represents the concentration needed to inhibit the enzyme ODC by 50%.

Intrinsic activity was determined after the observation that no metabolism into eflornithine had occurred during oral absorption. The mechanism of action of eflornithine involves two major groups namely the α -amino group, acting as the pharmacophore, and the α -carboxyl group, which serves as an auxophore. Thus the δ -amino group of eflornithine is not involved in binding to ODC. All of the compounds have intrinsic activity due to the fact that the major binding groups needed for binding to ODC are not blocked by derivatisation. However, the binding of the molecule to the receptor may vary due to steric hindrances caused by the lipophilic chain attached to the δ -amine of compounds **3a** – **3g**. This explains the variation in IC_{50} values of the compounds tested.

Only compound **3b** and **3f** showed a moderate increase in activity, which is remarkable considering that **3b** has the second lowest log D value (excluding DFMO). Although this

dataset was small one can assume that lipophilicity in this study does influence, to some extent, the activity of the drug.

5 Conclusion

The results show that δ -substituted derivatives of eflornithine were successfully synthesised and structures confirmed by NMR and MS. Biological results show no metabolism to eflornithine in the blood due to the stable amide bond at the δ -amine position. Although no correlation and comparison between intestinal absorption and log D could be established, an increase in lipophilicity was achieved for all the derivatives with the exception of compound **3a**. Although not significant, all except compounds **3b** and **3f** showed an increase in activity.

In closing, the theoretical and experimental data illustrates that an increase in oral absorption and activity can be achieved by derivatisation of eflornithine. However, it seems feasible that further evidence and a bigger datasets are needed to substantiate these findings.

6 Abbreviations

amu – Atomic mass unit	Mp – Melting point
APCI – Atmospheric pressure chemical ionisation	MS – Mass spectrometry
CNS – Central nervous system	M _w – Molecular weight
DFMO – Difluoromethylornithine	NMR – Nuclear magnetic resonance
DMSO – Dimethyl sulfoxide	ODC – Ornithine decarboxylase
F _{abs} – Fraction absorbed	PBS – Phosphate buffer solution
HAT – Human African trypanosomiasis	SEM – Standard error of mean
HIA – Human intestinal absorption	S _w – Aqueous solubility
HPLC – High performance liquid chromatography	T. b. – Trypanosoma brucei
IC ₅₀ – 50% Inhibitory concentration	TLC – Thin layer chromatography
Log D – Logarithmic partition coefficient at a specific pH	TMS – Tetramethylsilane
	t _R – Peak retention time
	WHO – World Health Organisation

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Chapter 4

Summary and Future Prospects

This section gives a summary and the final conclusions of the study, including results from Chapter 3 (article) as well as Annexure A and B (not in article). It therefore strives to unify the outcomes of the whole study and draws overall conclusions from all data, points of relevance and specific scientific advances made by the study and finally points out remaining scientific questions and proposes appropriate prospective studies.

The oral route of drug administration is the most common drug delivery route due to its many advantages such as better patient compliance and ease of use. Yet, it has disadvantages as well such as first pass metabolism and unwanted side-effects. One of the most important factors in oral formulations is the amount of drug entering the bloodstream. Orally administered drugs need to cross a number of barriers before entering the systemic circulation. Water-soluble drugs have a greater difficulty in crossing biological membranes due to their polar nature (Gao *et al.*, 2008). One of the ways to address this problem is to synthesise lipophilic derivatives that have more suitable physicochemical properties for oral administration.

Human African trypanosomiasis (HAT) is caused by two species of extracellular protozoa namely *T. b. gambiense* and *T. b. rhodesiense*. *T. b. rhodesiense* causes the acute form of the disease while *T. b. gambiense* causes the chronic form. The disease is transmitted to humans by the bite of infected tsetse fly during its blood meal. HAT is primarily found in sub-Saharan Africa and corresponds to the migration of its vectors. Only four drugs have been approved for the treatment of HAT in the last 50 years namely suramin, pentamidine, melarsoprol and eflornithine. The latter two are used against late stage infections. Eflornithine is commonly used for the treatment of late stage *T. b. gambiense* infections or patients relapsing after melarsoprol treatment. Eflornithine is a selective irreversible inhibitor of ornithine decarboxylase (ODC), an enzyme responsible for polyamine synthesis in humans and trypanosoma. Eflornithine has an oral bioavailability of 50% and is highly hydrophilic (log P of -0.82). Subsequently high doses of eflornithine are needed to reach the minimum effective concentration of 50 µM in the brain, where the parasite resides during the late stage. The drug is also very expensive and requires medical personnel for its administration and monitoring of the infection in order to prevent relapse.

This study was primarily founded on the idea of increasing the oral bioavailability of eflornithine by means of linkage to lipophilic moieties at its δ -amino group. Much attention was given to the choice of suitable moieties in order to obtain the best ligand fit to the ODC enzyme by adhering to Lipinski's rule of 5.

The primary objective of this study was to synthesise a series of new derivatives of the anti-trypansomal drug eflornithine featuring increased lipophilicity in an attempt to improve its oral bioavailability, and to assess their intrinsic activity.

The following aims were set:

- Screen proposed compounds to be synthesised and tested.
- Synthesise a series of δ -amides of eflornithine and confirm their structures.
- Experimentally determine the physicochemical properties like the partition coefficient and aqueous solubility for eflornithine and its synthesised derivatives and compare the experimental data with calculated values from commonly used prediction software.
- Experimentally determine the oral absorption for eflornithine and its synthesised derivatives *in vivo*.
- Experimentally determine the intrinsic activity of eflornithine and its synthesised derivatives *in vitro* against DFMO sensitive *T. b. brucei* strains.
- Examine the effect of derivatisation on oral absorption and intrinsic activity.

Screening of proposed compounds was done by means of docking studies and *in silico* determination of pharmacokinetic parameters such as HIA and F_{abs} . All the derivatives displayed lower docking scores than eflornithine. Of the 27 compounds screened, compounds **3g** and **3c** showed the best docking scores of 28.91 and 28.52, respectively. All compounds displayed slightly lower F_{abs} values than eflornithine, but higher HIA values. However, results from docking studies and pharmacokinetic parameters were solely employed to find seven compounds with the best ligand fit and pharmacokinetic properties from 27 possible candidates. Only seven compounds were chosen to achieve a practical and accurate data set to reach the aims of this study.

The eflornithine derivatives were successfully synthesised and their structures were confirmed by ^1H and ^{13}C NMR and MS spectroscopy.

The experimental partition coefficient (log D) of eflornithine (-0.96) was lower than that of its derivatives ranging from -0.07 to -0.78 with the exception of **3a** (-1.21). Compound **3g** with a log D of -0.07, was the most lipophilic of all compounds evaluated. Compounds **3c** (-0.19), **3e** (-0.13) and **3f** (-0.28), all containing the aryl ring obtained the greatest increase in lipophilicity. The S_w values for compounds (**3b** – **3g**) ranged between 19 and 28 mg/ml compared to that of DFMO at 34.96 mg/ml. Log D values correlated well with the aqueous solubility (S_w) values, with compounds (**3b** – **3g**) showing a decrease in solubility.

In vivo oral absorption studies of compounds **3a**, **3c**, **3d** and **3f** show no metabolism into eflornithine. Thus no free eflornithine was found in the blood after their oral administration. The findings can possibly be explained by the stable amide bond between the lipophilic moiety and eflornithine. Consequently no conclusive evidence was attained to confirm increase oral absorption of DFMO

Intrinsic activity of compounds tested revealed compounds **3b**, featuring a propionyl moiety and **3f** a methoxybenzoyl moiety to have increased activity with an IC_{50} of 32.05 μ M and 35.45 μ M respectively, compared to that of eflornithine (36.22 μ M).

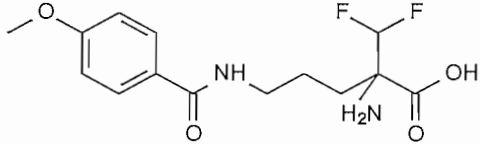
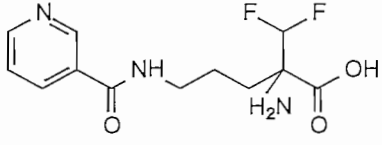
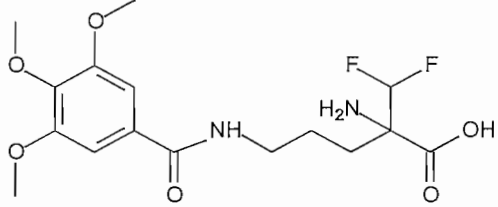
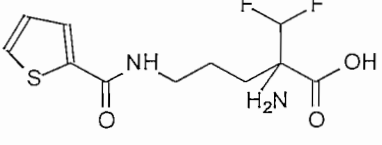
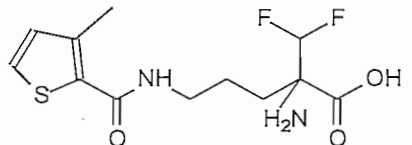
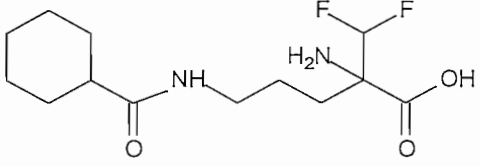
Although the results are not significant, this study demonstrated that derivatisation had an effect on oral absorption and intrinsic activity. Overall compound **3b** and **3f** showed promising results and proves that derivatisation can improve both oral absorption and intrinsic activity.

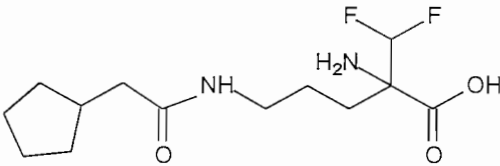
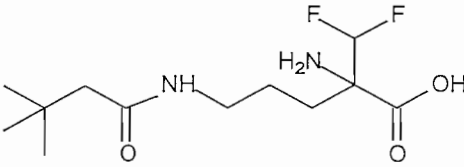
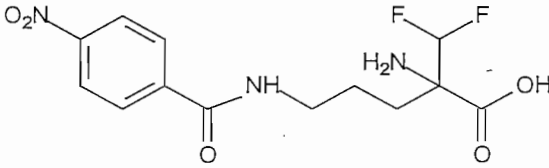
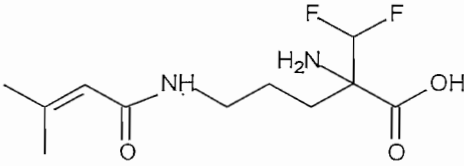
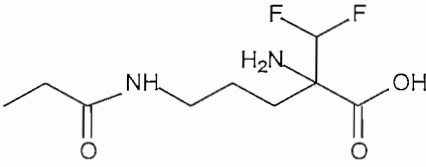
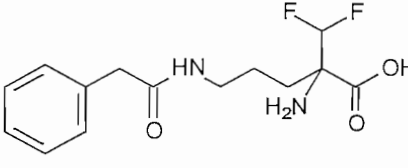
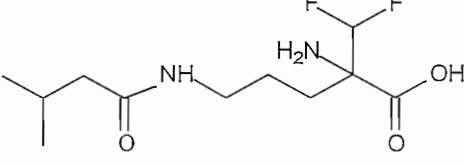
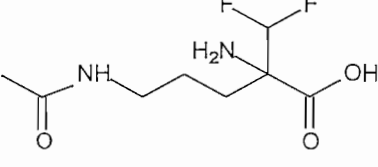
Future possibilities were envisaged during the progress of this study. These are the following:

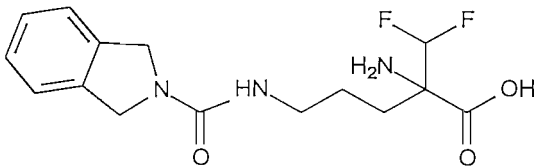
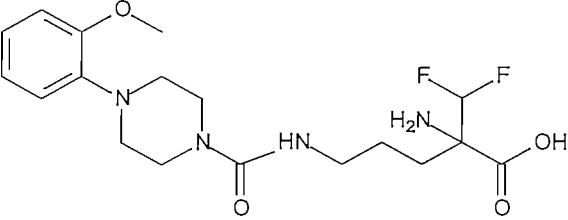
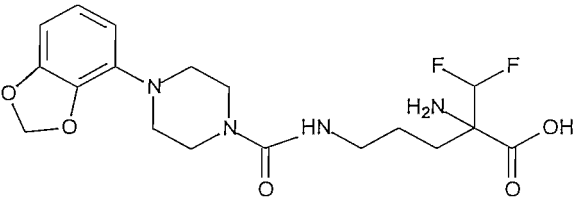
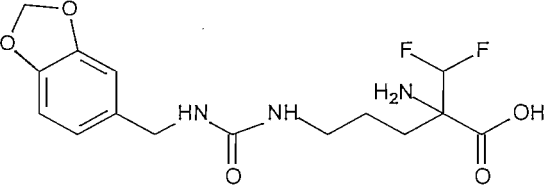
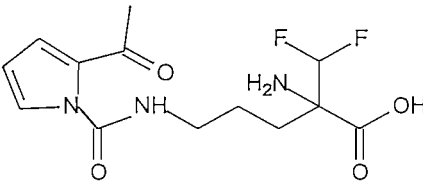
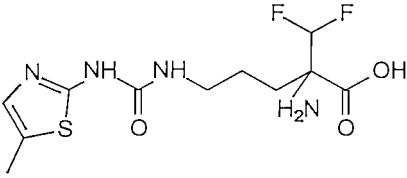
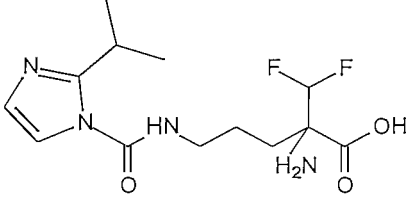
- Improved HPLC method for the *in vivo* determination of oral absorption for eflornithine and its derivatives.
- One important factor that should be considered is the determination of transport over the blood-brain barrier for each of the derivatives. Thus a better efficacy profile for each of the derivatives *in vivo* can be obtained.
- Toxicity assays of the derivatives synthesised.

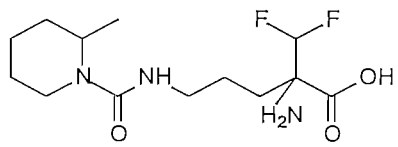
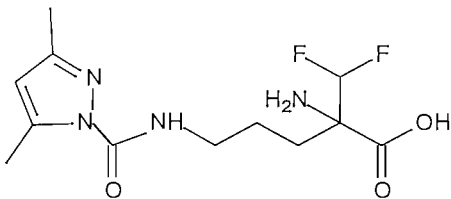
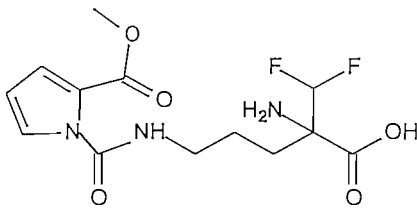
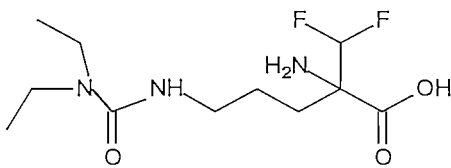
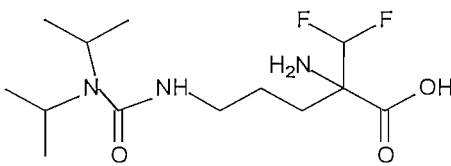
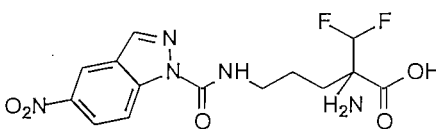
Annexure A

Proposed Compounds

Name ^a	Structure	M _w	Docking score
4-Methoxybenzoyl-DFMO		344.4	25.8
Nicotinoyl-DFMO		287.3	27.3
3,4,5-Trimethoxybenzoyl-DFMO		376.4	20.5
2-Thiophenecarbonyl-DFMO		292.3	28.9
3-Methyl-2-thiophenecarbonyl-DFMO		306.3	22.8
Cyclohexanecarbonyl-DFMO		292.3	20.5

Cyclopentanepropionyl-DFMO		334.4	20.4
3,3-Dimethylbutyryl-DFMO		280.3	15.8
4-Nitrobenzoyl-DFMO		331.3	21.4
3,3-Dimethyacryloyl-DFMO		264.3	22.4
Propionyl-DFMO		237.2	24.6
Phenylacetyl-DFMO		300.3	28.5
Isovaleryl-DFMO		266.3	19.3
Acetyl-DFMO		223.2	23.2

Indoline-DFMO		327.3	20.1
1-(2-Methoxyphenyl) piperazine-DFMO		400.4	18.6
1-Piperonylpiperazine-DFMO		414.4	20.9
Piperonylamine-DFMO		314.3	14.6
Acetylpyrole-DFMO		317.3	16.7
2-Amino-5-methylthiazole-DFMO		322.3	20.4
2-Isopropylimidazole-DFMO		318.3	21.4

2-Methylpiperidine-DFMO		307.3	21.3
3,5-Dimethylpyrazole-DFMO		304.3	19.8
Pyrrole-2-methylcarboxylate-DFMO		333.3	18.1
Diethylcarbonyl-DFMO		281.3	21.7
N,N-Disopropylcarbonyl-DFMO		309.4	15.7
6-Nitroindazole-DFMO		371.3	20.7

Molecular weight (M_w), eflornithine (DFMO).

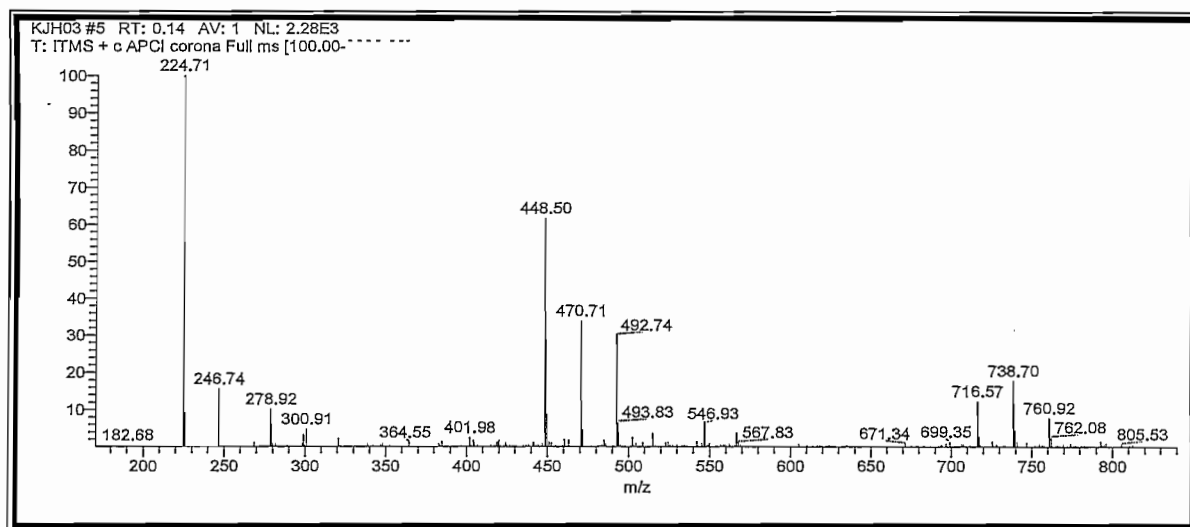
^a Nomenclature not according to IUPAC standards due to space limitations as well as for ease of reading.

Annexure B

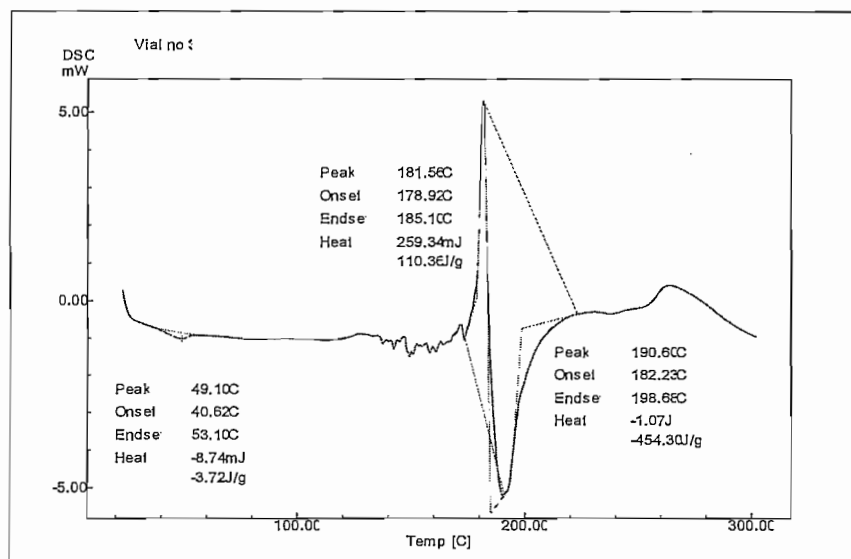
Physical Data

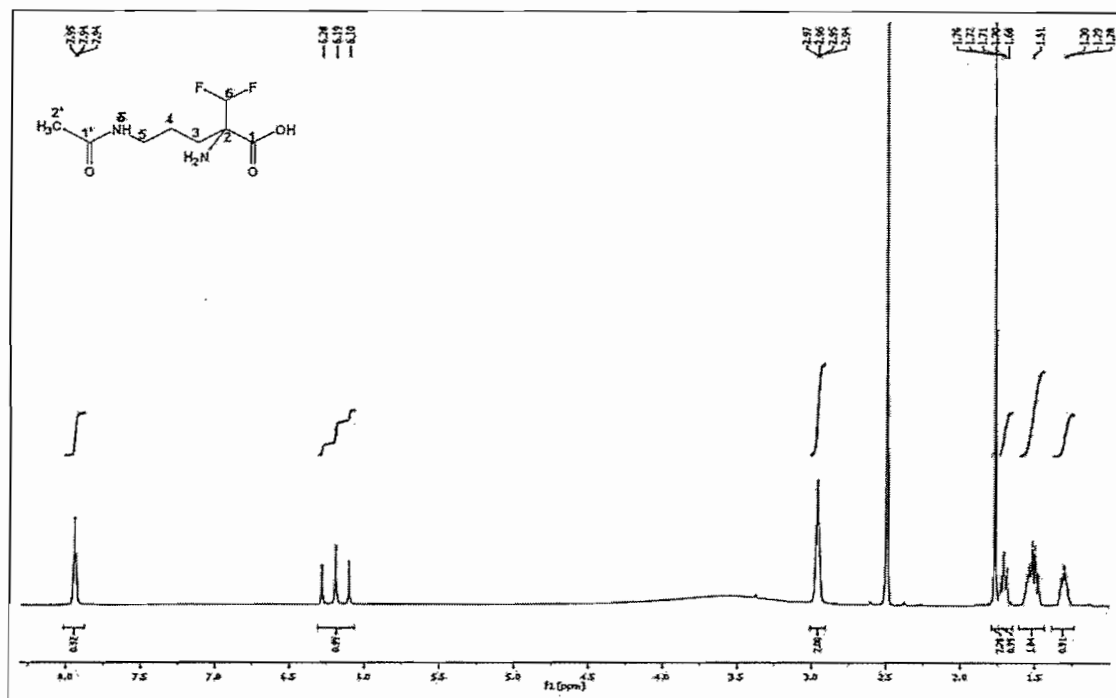
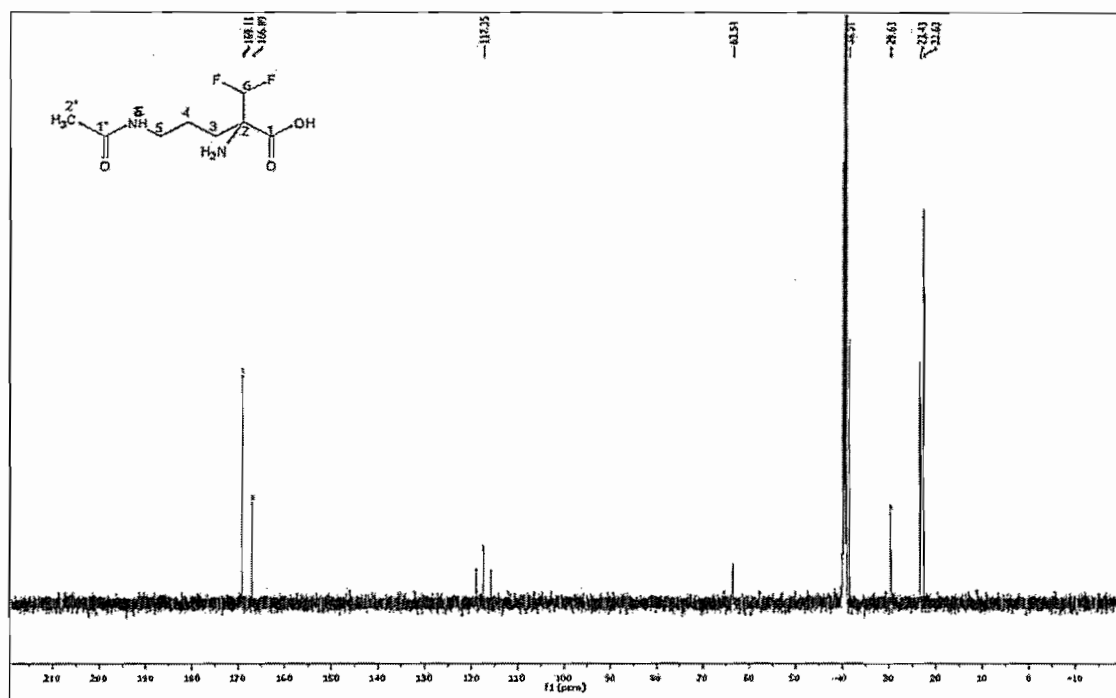
2-amino-2-(difluoromethyl)-5-acetamidopentanoic acid (3a)

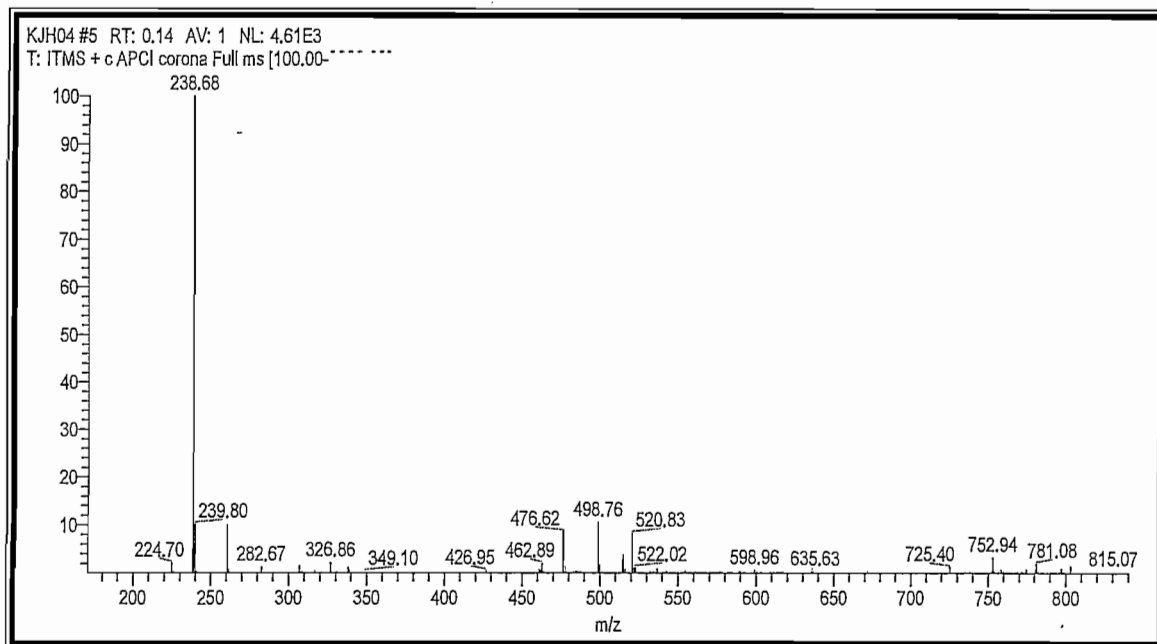
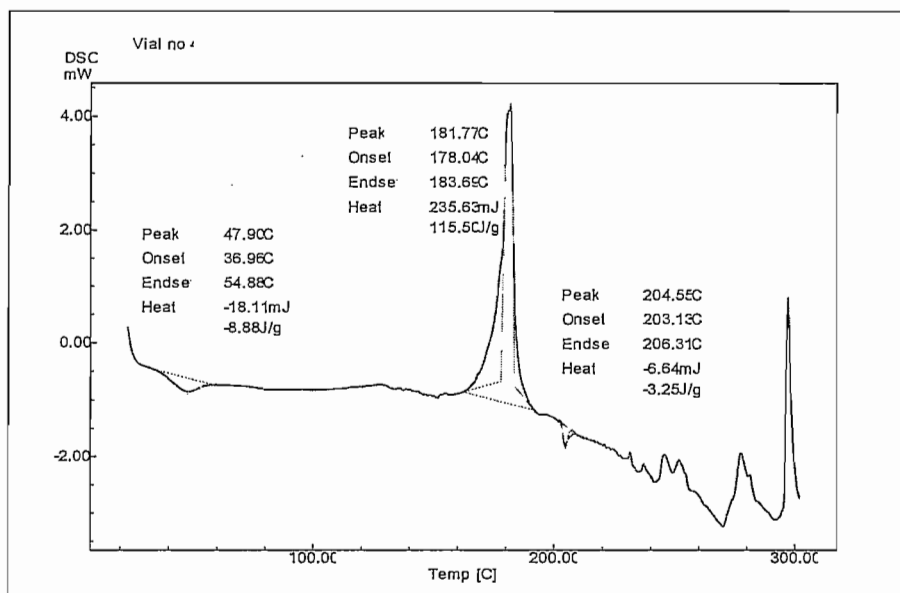
MS

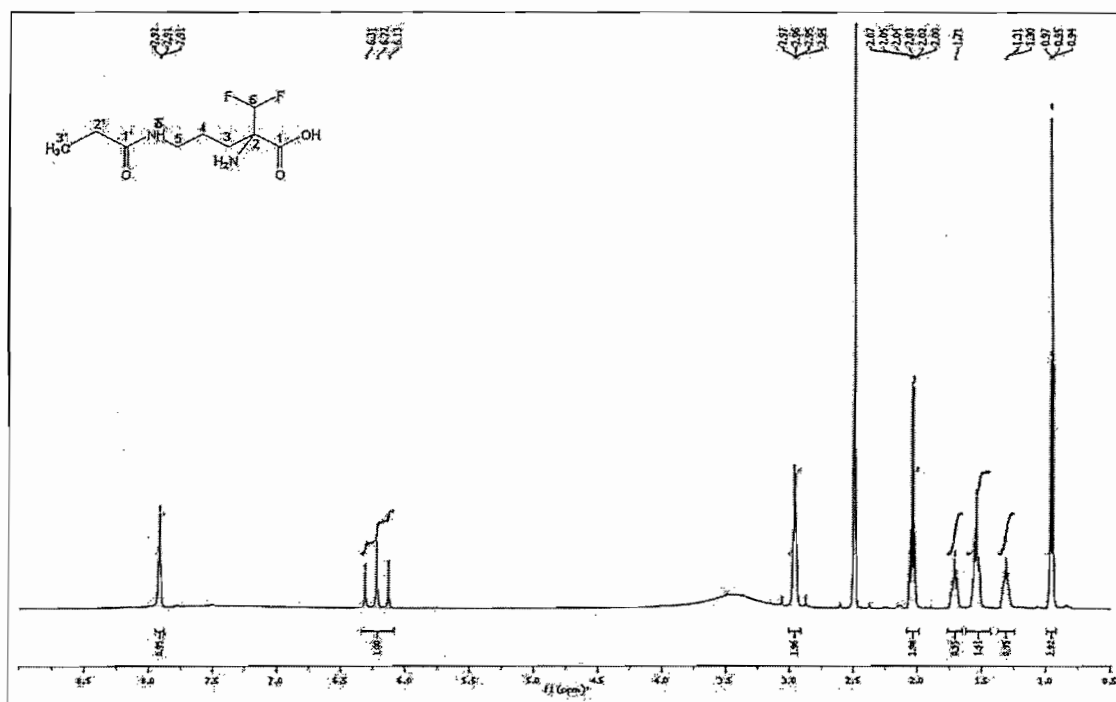
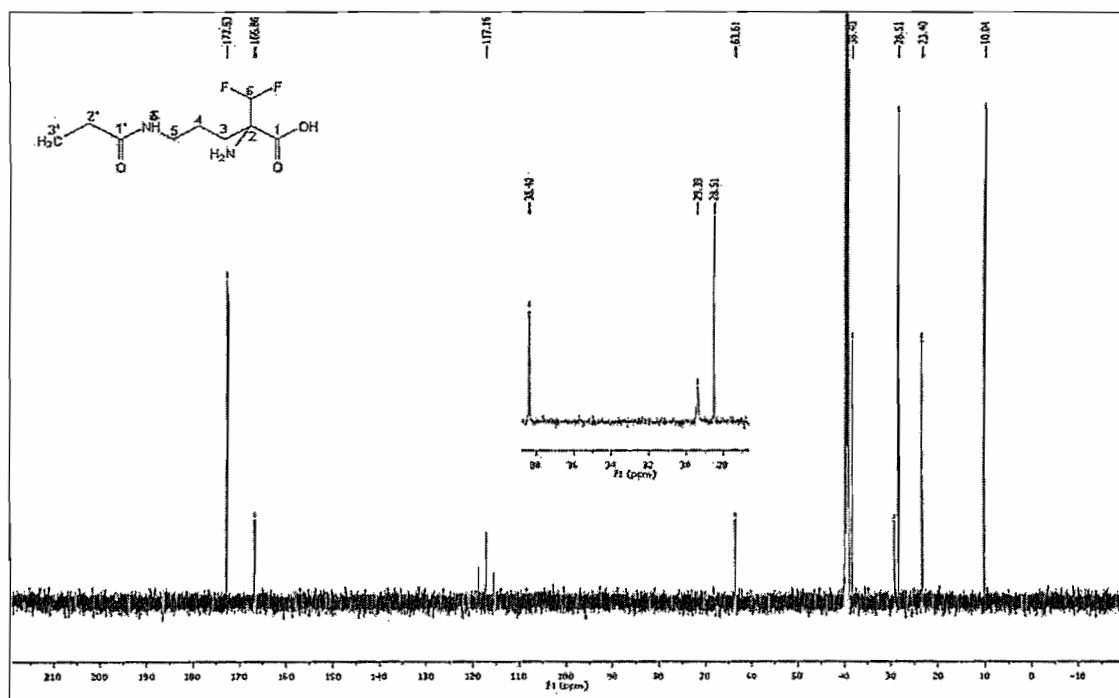


DSC



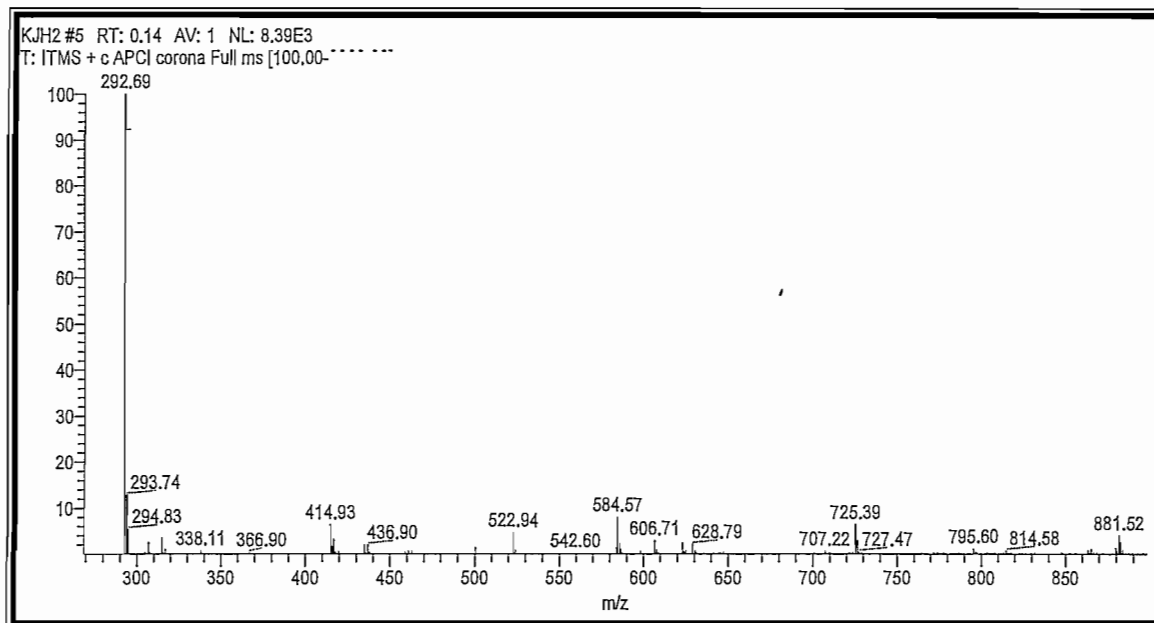
NMR (^1H)NMR (^{13}C)

2-amino-2-(difluoromethyl)-5-propanamidopentanoic acid (3b)**MS****DSC**

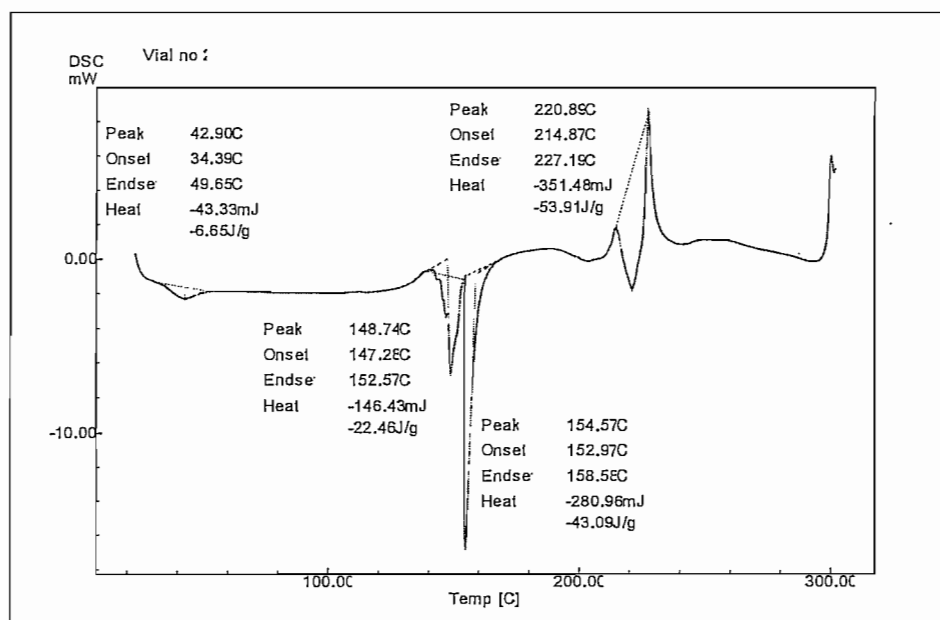
NMR (^1H)NMR (^{13}C)

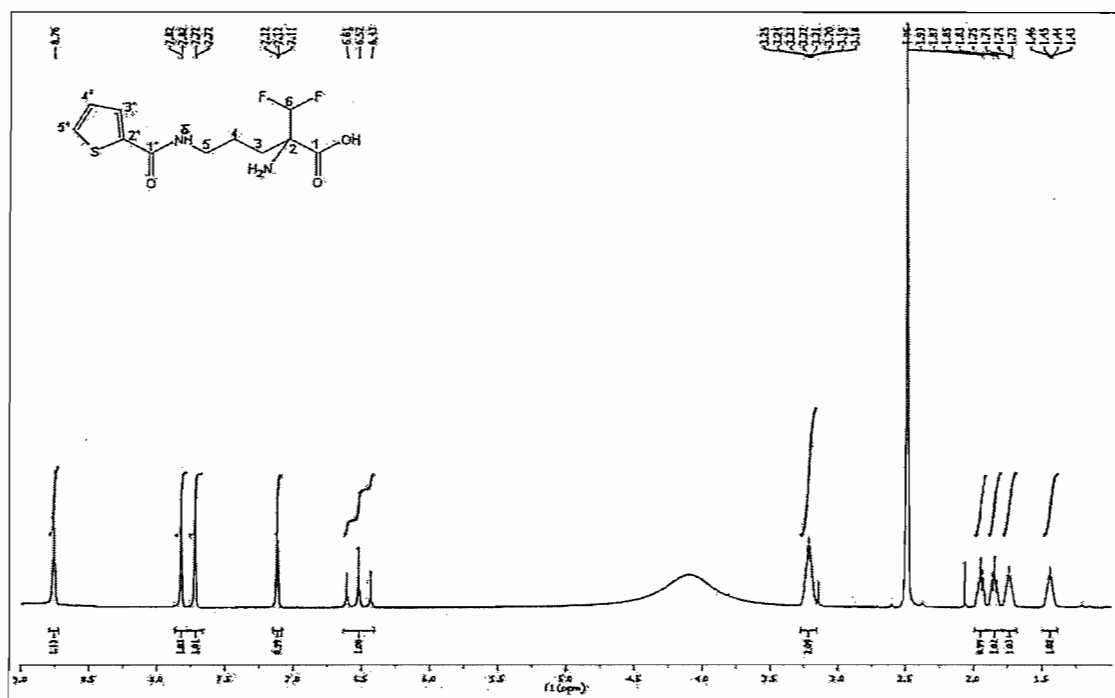
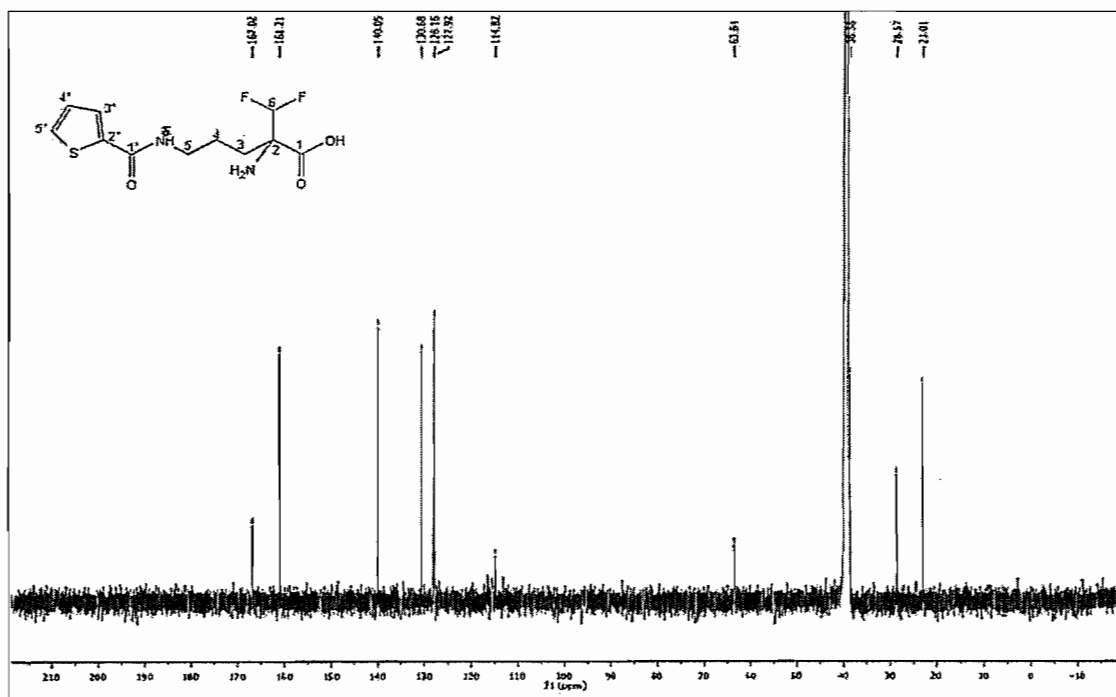
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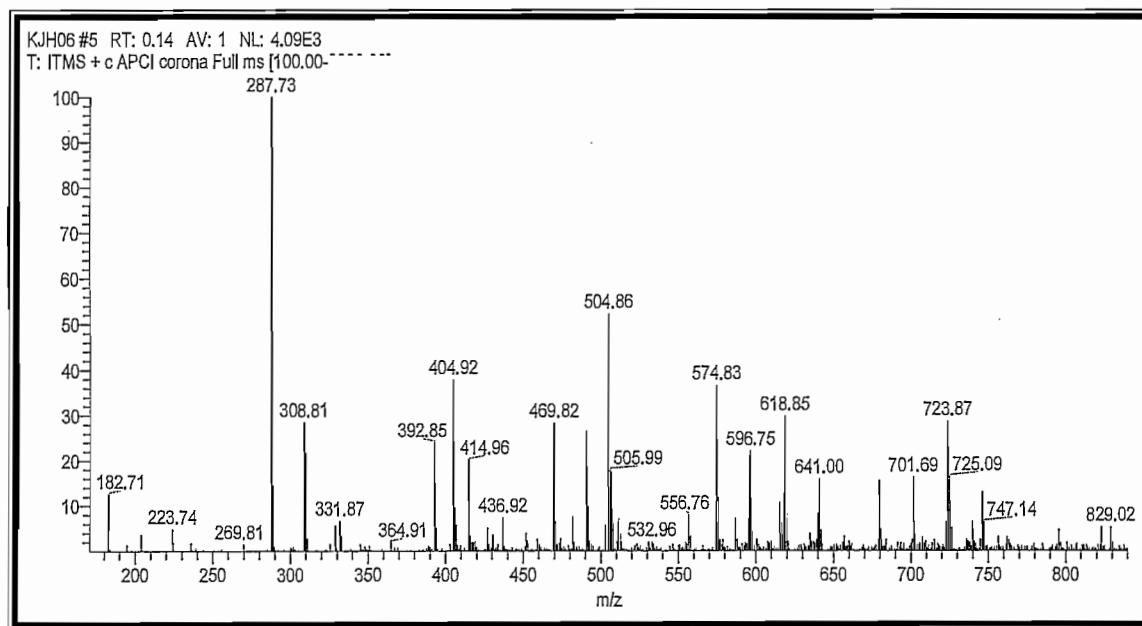
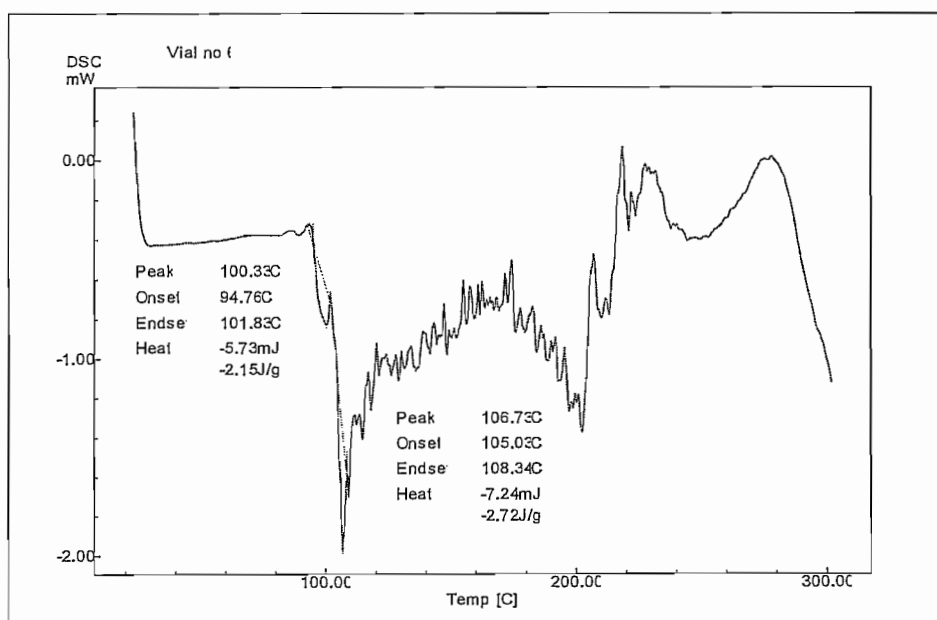
MS

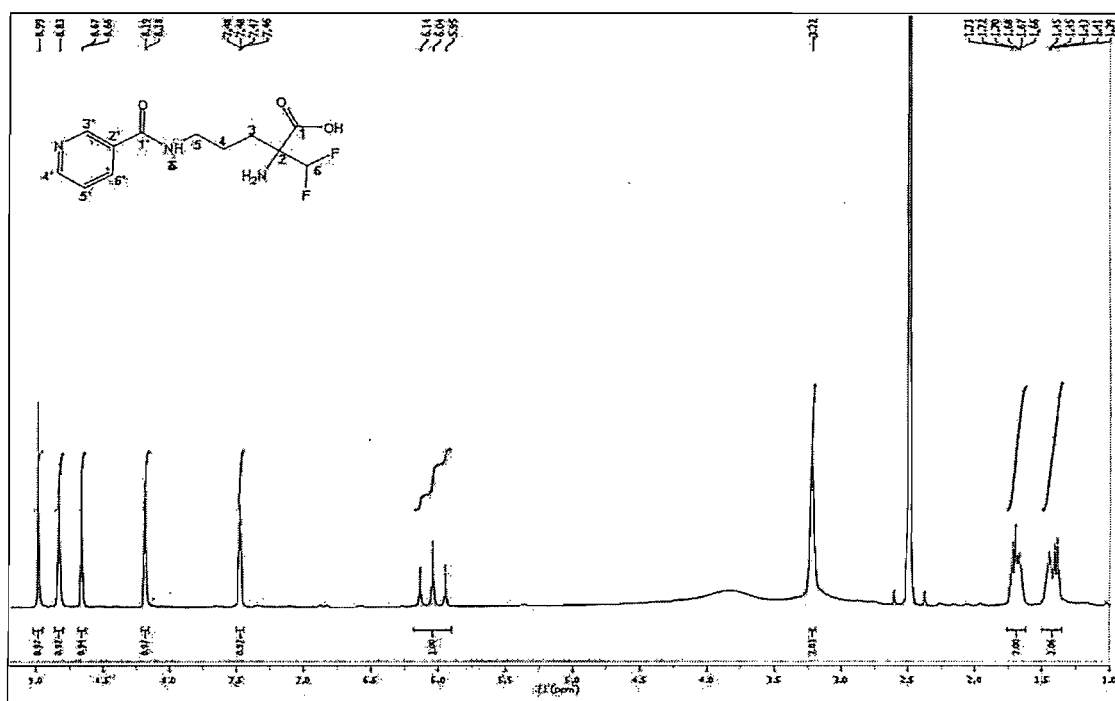
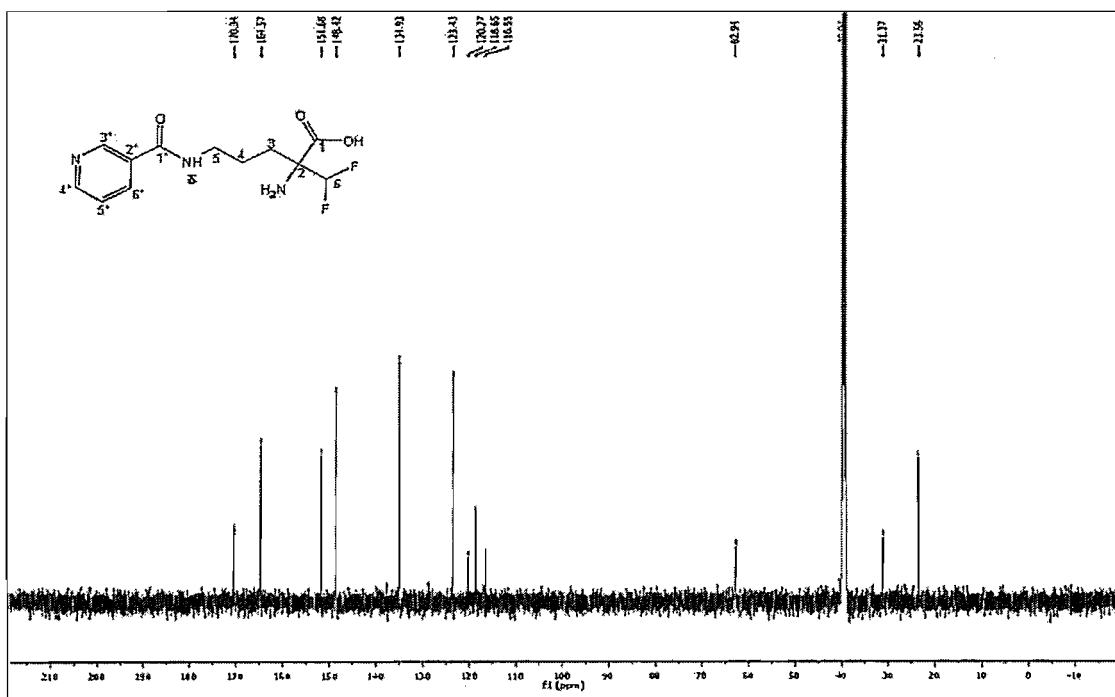


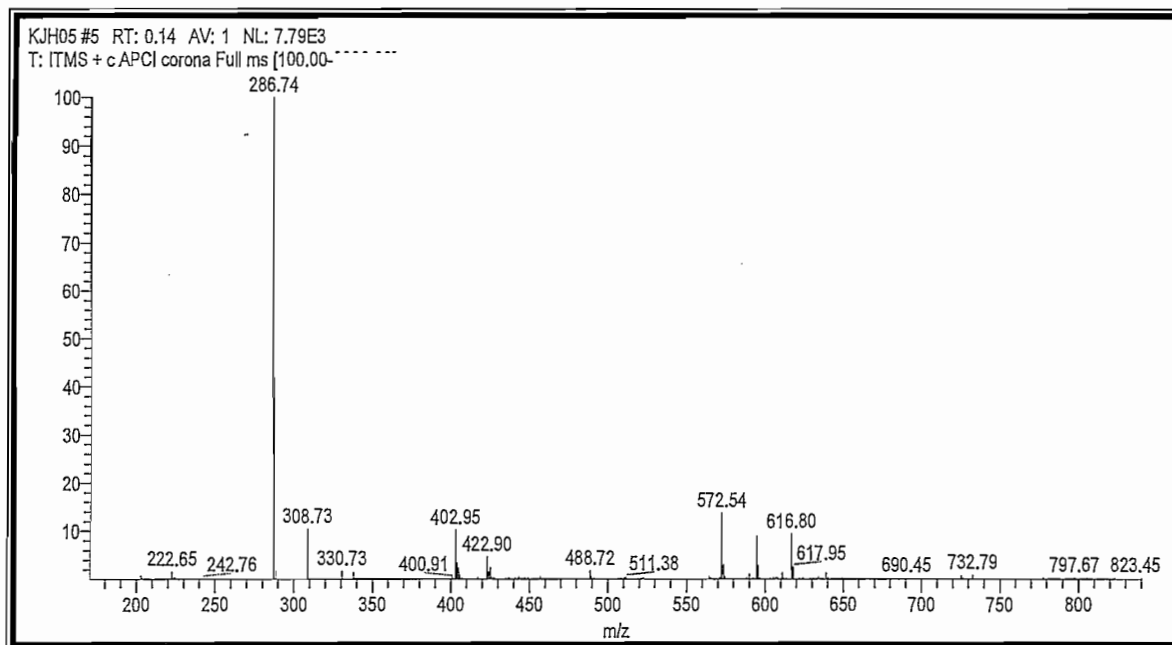
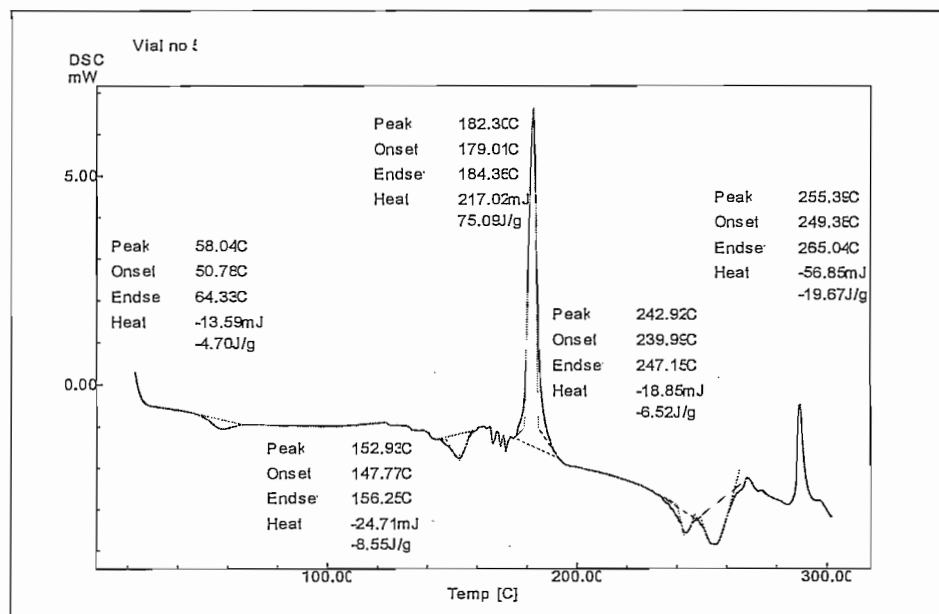
DSC

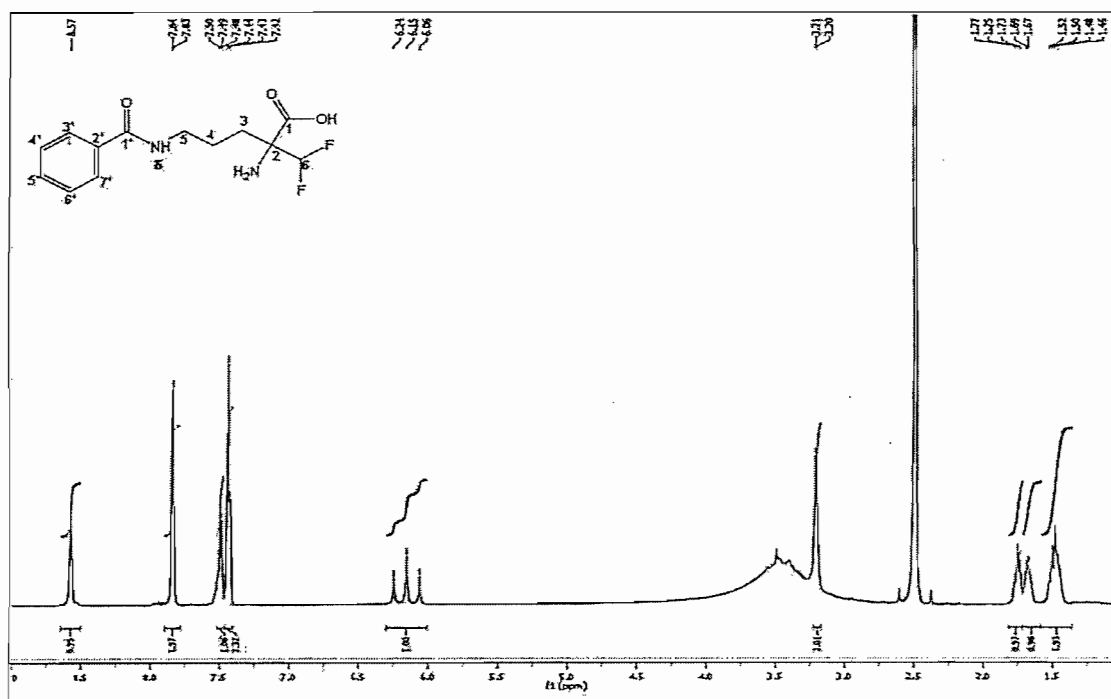
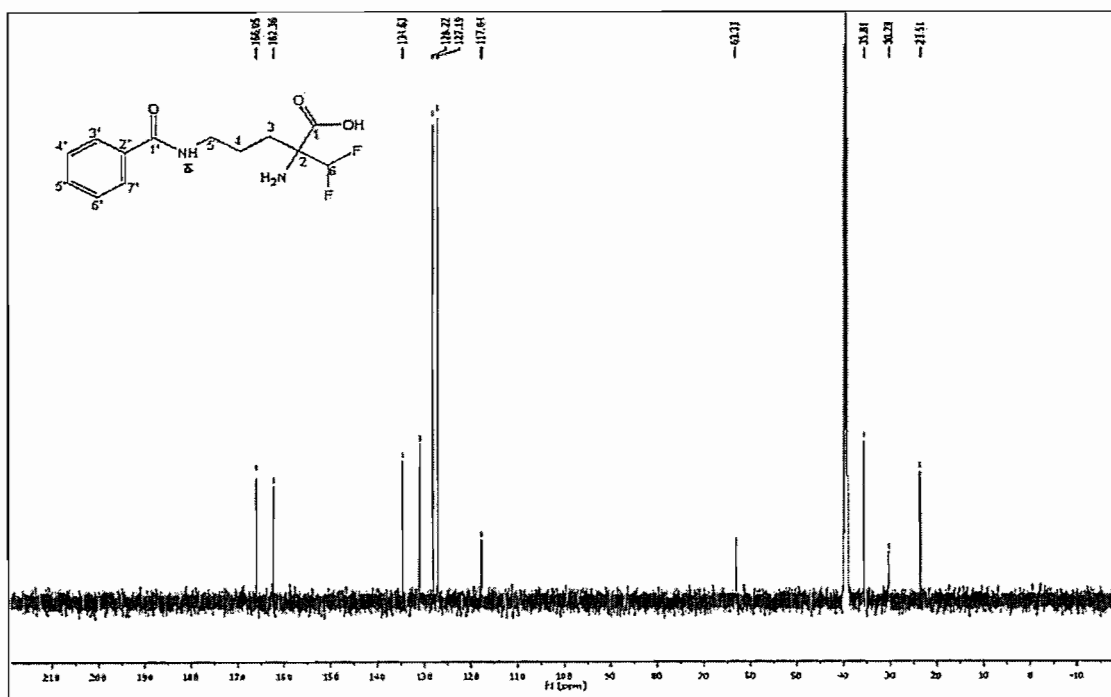


NMR (^1H)NMR (^{13}C)

2-amino-2-(difluoromethyl)-5-(pyridin-3-ylformamido)pentanoic acid (3d)**MS****DSC**

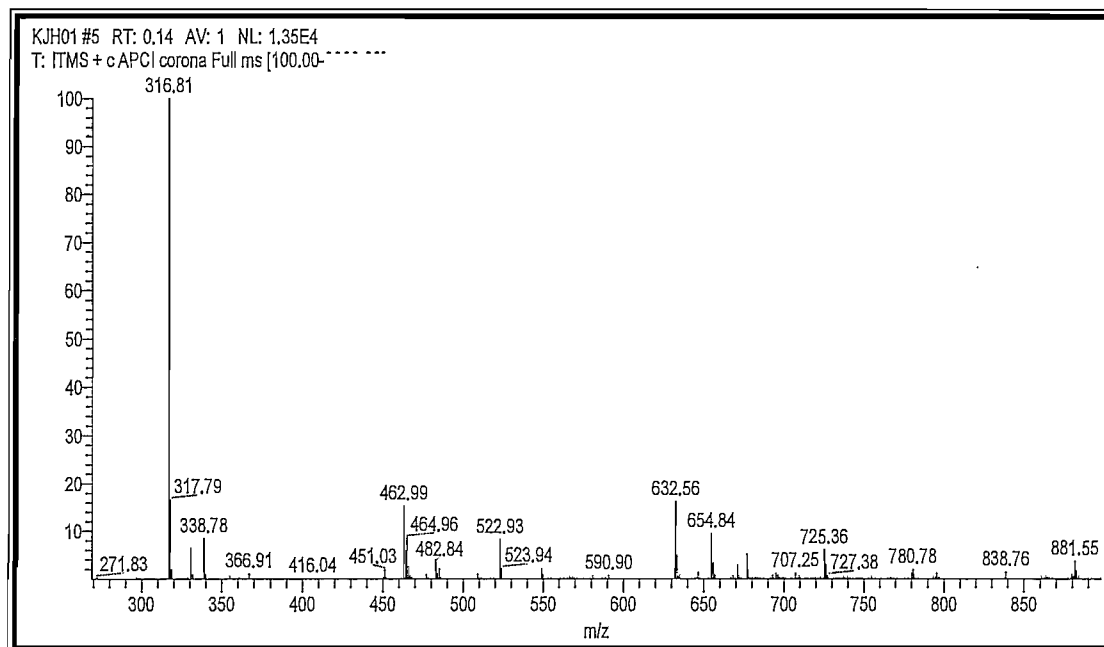
NMR (^1H)NMR (^{13}C)

2-amino-2-(difluoromethyl)-5-(phenylformamido)pentanoic acid (3e)**MS****DSC**

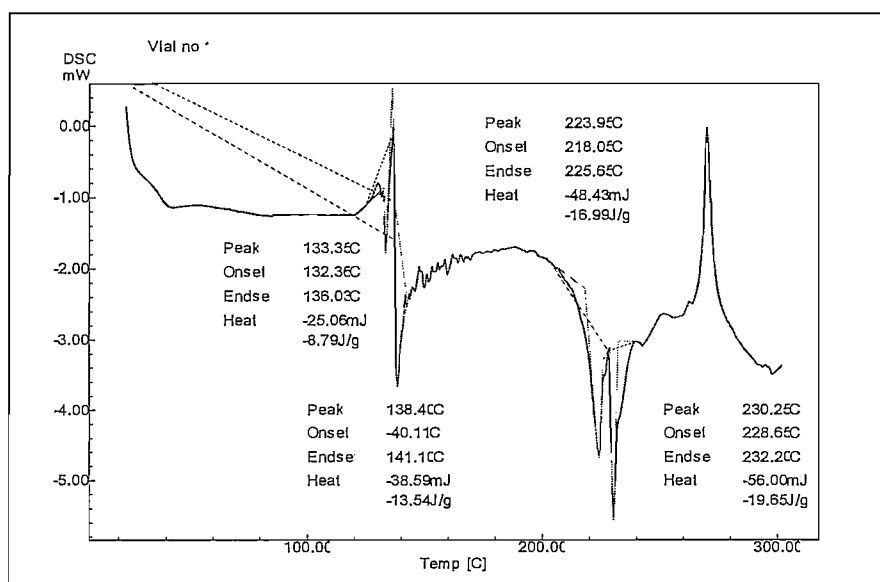
NMR (^1H)NMR (^{13}C)

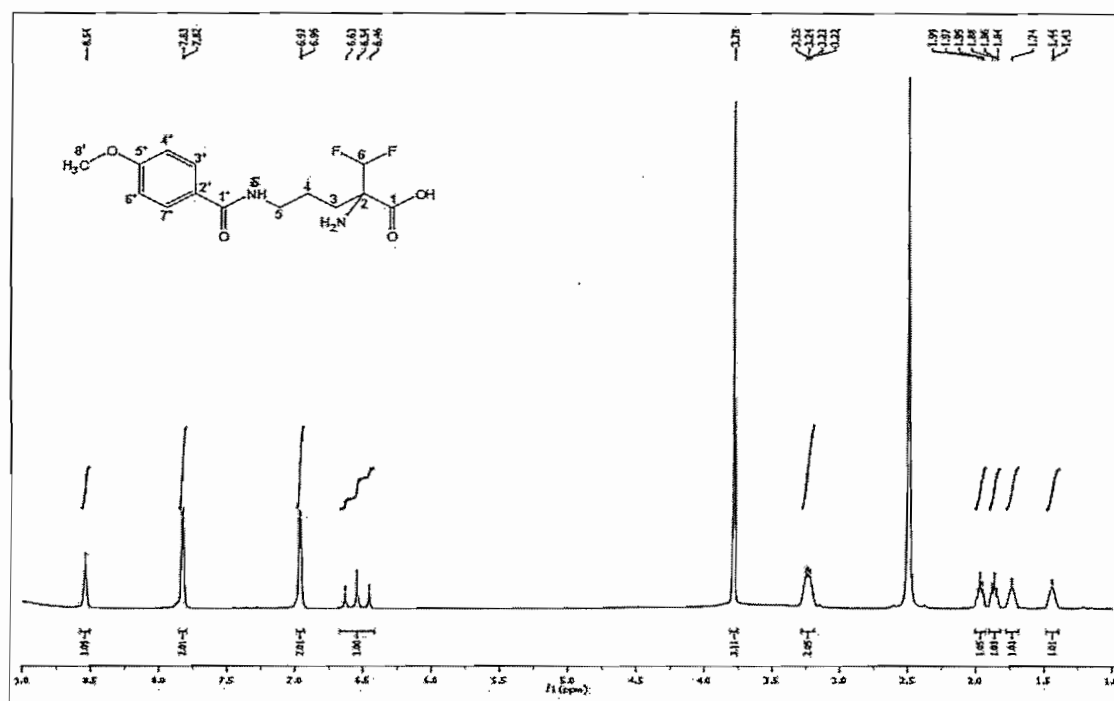
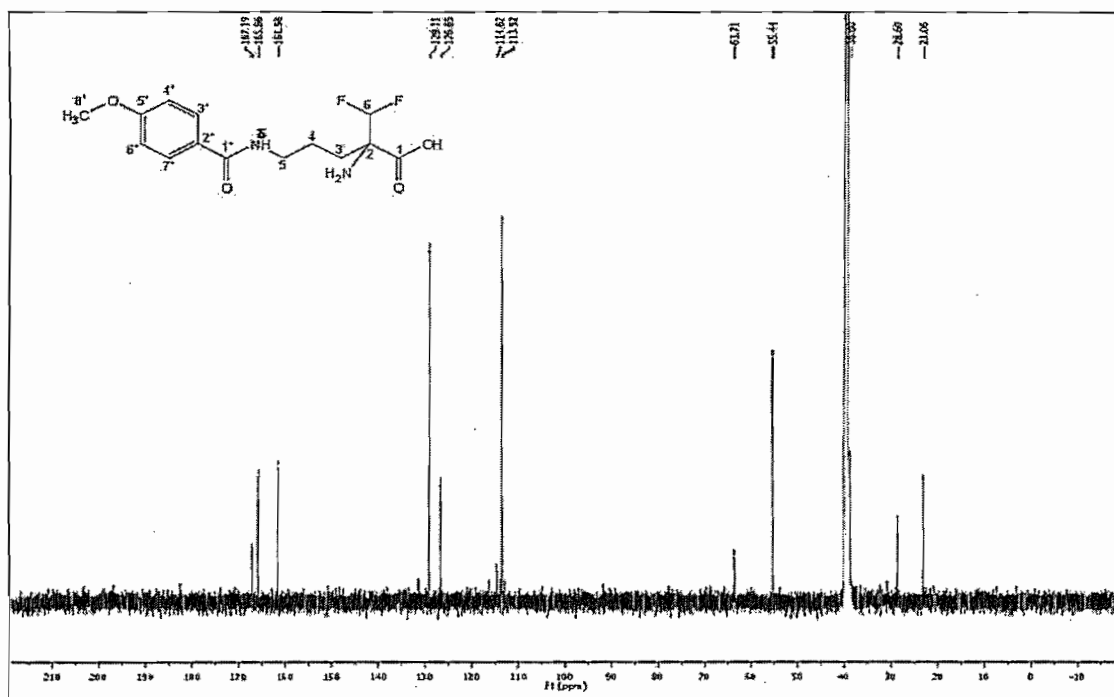
2-amino-2-(difluoromethyl)-5-[(4-methoxyphenyl)formamido]pentanoic acid (3f)

MS



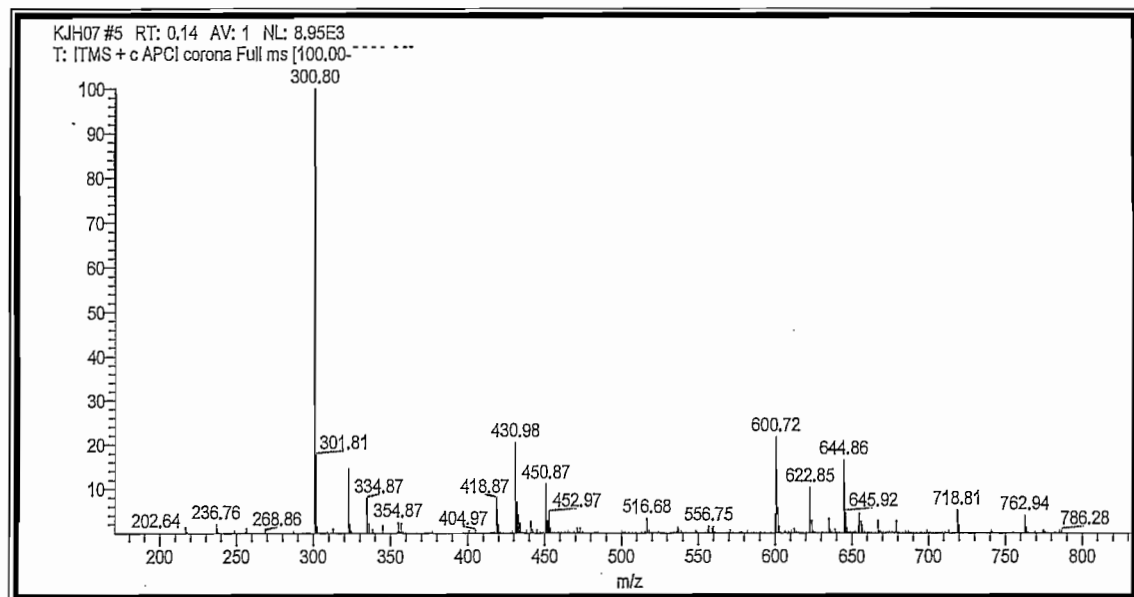
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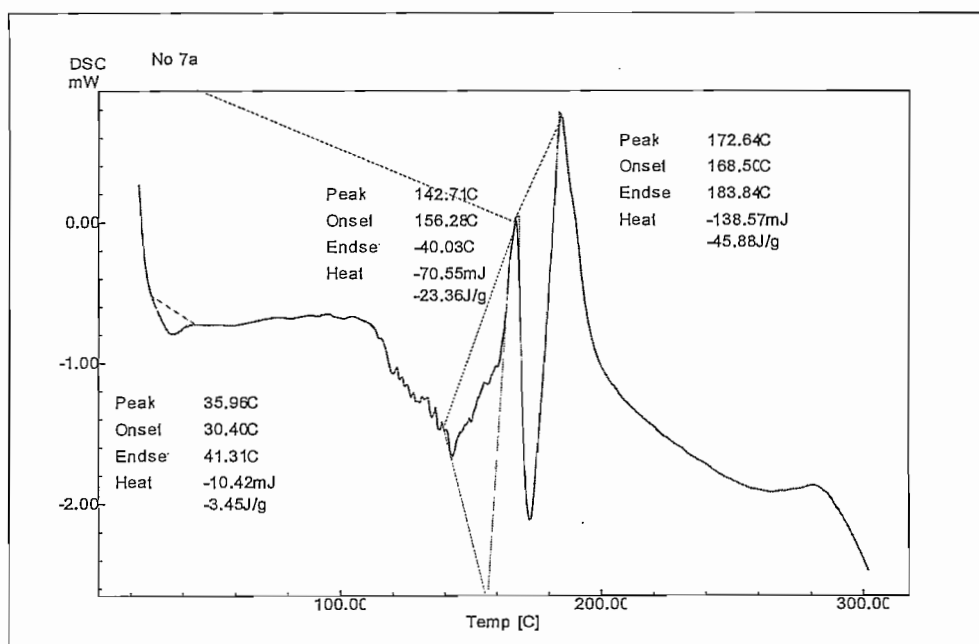
NMR (^1H)NMR (^{13}C)

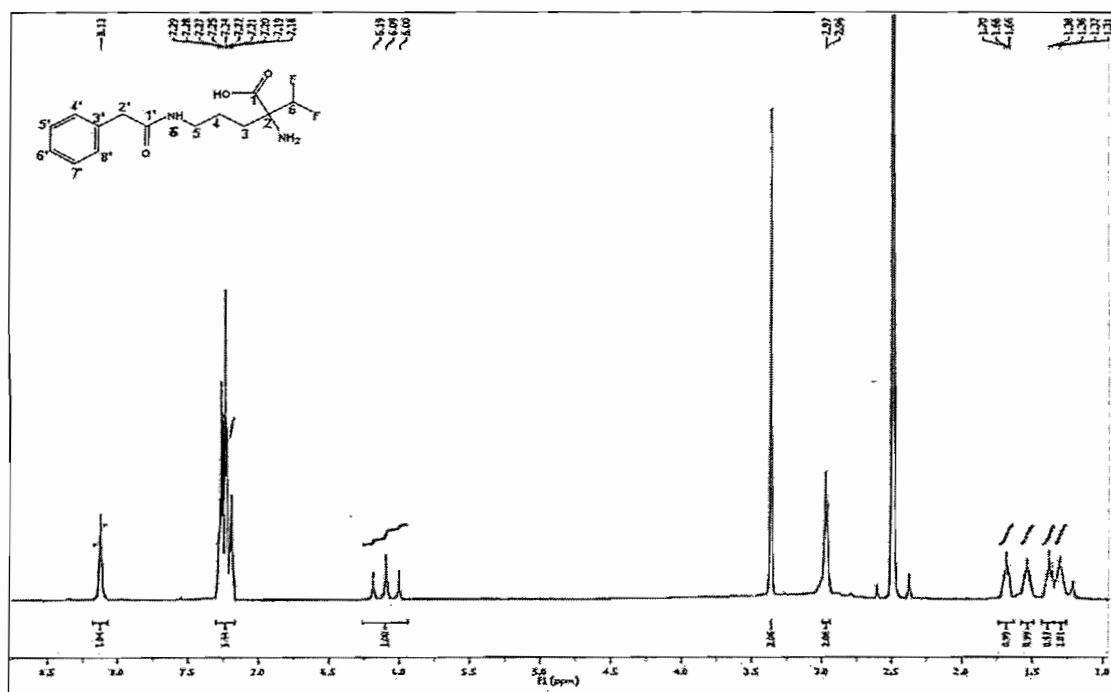
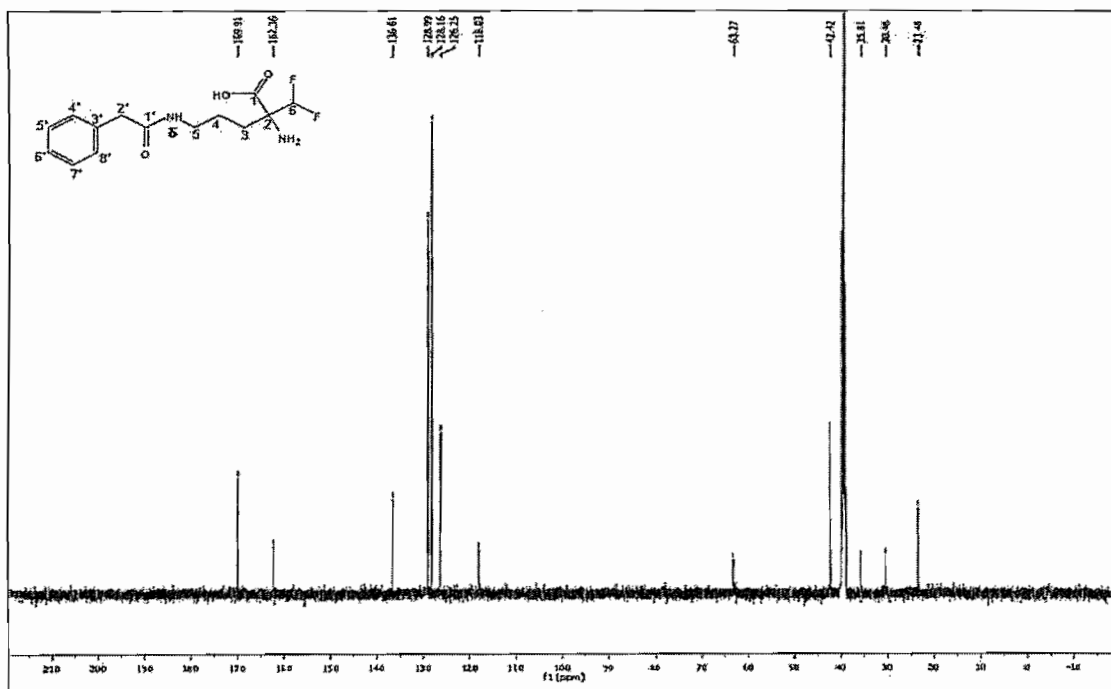
2-amino-2-(difluoromethyl)-5-(2-phenylacetamido)pentanoic acid (3g)

MS



DSC



NMR (^1H)NMR (^{13}C)

Annexure C

Conference Participation

THE 5TH INTERNATIONAL CONFERENCE ON PHARMACEUTICAL AND PHARMACOLOGICAL SCIENCES

Powerpoint Presentation

Synthesis of δ -amides of eflornithine to improve oral bioavailability

KJ Helena, JC Breytenbach, DD N'Da

Pharmaceutical Chemistry, School of Pharmacy,
North-West University, Potchefstroom 2531

Introduction

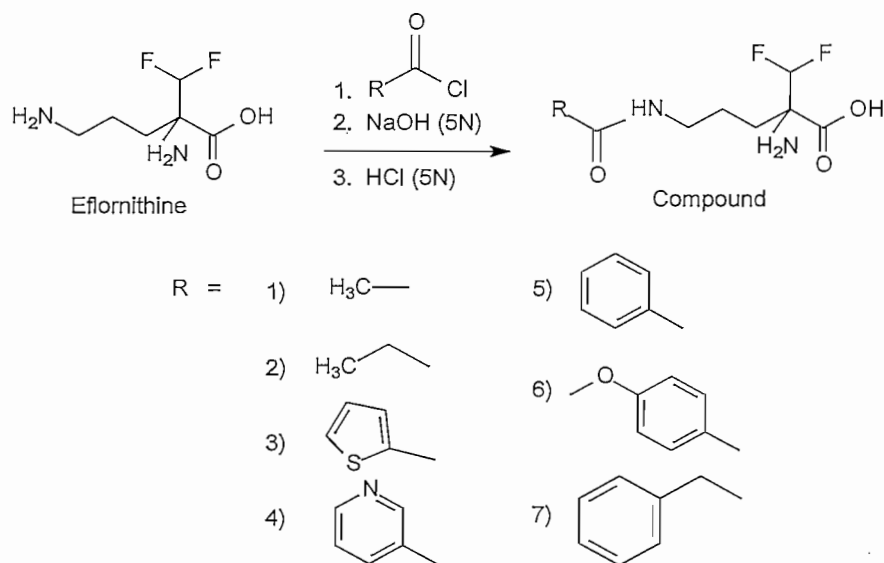
Human African trypanosomiasis (HAT) is an endemic disease of sub-Saharan Africa caused by two species of parasitic protozoa, *Trypanosoma brucei gambiense* and *T.b. rhodesiense*, which are transmitted to humans by the bite of various species of *Glossina* (tsetse fly). Eflornithine, given intravenously, is the drug of choice for late stage trypanosomiasis but has been used with little result due to difficult dosage regimes and high cost. This can be attributed to low cerebrospinal fluid (CSF) concentration. Increasing the lipophilicity of eflornithine would enhance its oral bioavailability and thus administer the drug orally with greater efficiency. This could have several advantages such as easier dosage regimes and fewer side effects.

Aim of study

The aim of this study is to synthesise lipophilic derivatives of eflornithine, determine their physicochemical properties namely log D and evaluate their oral bioavailability.

Method

Molecular modelling was used to determine ODC cavity binding of several derivatives, given in scheme 1. To decrease the polarity of eflornithine, heterocyclic rings or hydrocarbon chains were attached to the δ amino groups (**1-8**) by means of acylation. The target compounds were obtained after purification by silica gel column chromatography.



Scheme 1 – Reaction pathways in the synthesis of eflornithine derivatives

Bioassays, conducted by an outside institution (Goteborg University, Sweden) will be done on Sprague-Dawley rats. Blood samples will be analysed after oral administration by HPLC. Log P determination of derivatives 1-7 will be done in phosphate buffer at pH 7.4.

Results

δ -Substituted derivatives of eflornithine have been synthesized and their structures confirmed by nuclear magnetic resonance (NMR). Biological results show no conclusive evidence of oral absorption. Furthermore, there is no indication of metabolism into eflornithine for any of the derivatives (1-7).

Young Scientist Competition

Joined runner-up in the category Adcock Ingram APSSA Young scientist presentation entitled **Synthesis of δ -amides of eflornithine to improve oral bioavailability**

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