

**ANTI-MALARIAL ACTIVITY AND PHYTOCHEMICAL STUDIES OF
CISSAMPELOS MUCRONATA AND *STEPHANIA ABYSSINICA***

By

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DECLARATION

This thesis is my original work and has never been presented for a degree in any other University.

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DEDICATION

To my parents Henry Shem Omole and Rosemary Omole; To my brothers Shem, Dan, Joab and Booker; To my sisters Milly and Jacque; You have been true comrades!

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LIST OF ABBREVIATIONS AND ACRONYMS

Art	Artemisinin
BCAs	Biological control agents
CC	Column chromatography
CDC	Centre for disease control
CDCl ₃	Deuterated chloroform
CD ₃ OD	Deuterated methanol
COSY	Correlation spectroscopy
¹³ C NMR	Carbon 13 nuclear magnetic resonance
CQ	Chloroquine
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-Diethyl- <i>m</i> -toluamide
DEPT	Distortionless enhancement by polarization transfer
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulphoxide
EC	Effective concentration
EIMS	Electron impact mass spectroscopy
EM	Environmental management
EtOAc	Ethyl acetate
¹ H NMR	Proton nuclear magnetic resonance
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HPLC	High performance liquid chromatography
Hz	Hertz
IGRs	Insect growth regulators
IR	Infra red
IRS	Indoor residual spraying
ITNs	Insecticide treated nets
KEMRI	Kenya Medical Research Institute
MEM	Minimum essential medium
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
ppm	Parts per million
PTLC	Preparative thin layer chromatography
RBC	Red blood cells
R _f	Relative fronts
SSA	Sub-Saharan Africa
TLC	Thin layer chromatography
TMS	Trimethyl silane
USA	United States of America
UV	Ultra violet
WHO	World Health Organization

ABSTRACT

Malaria remains the most important public health problem in the tropics. Each year, about 500,000,000 and 2,700,000 malaria cases and related deaths, respectively, are reported globally with Africa accounting for 90%. In Kenya, more than 4 million people are infected annually, the majority being children under five years and pregnant women. The disease is caused by parasites in the genus *Plasmodium* and is transmitted through bites of infected female anopheline mosquito. It is the most serious disease due to its prevalence, virulence and drug resistance. Chemotherapy remains the main strategy in malaria control but has become less effective due to widespread drug resistance, high cost and inadequate armory of drugs for treatment of malaria. Consequently there is need for research and the discovery of new more effective anti-malarial agents with different mode of action. Plants are considered as important sources of lead compounds in drug development owing to their successful use in treatment of various human ailments since antiquity. In search for new anti-malarial principles, bio-evaluation of *Cissampelos mucronata* and *Stephania abyssinica* which are used in treatment of malaria by people living in southern Nyanza was undertaken. The crude extracts were sequentially extracted starting with hexane, DCM, EtOAc and MeOH. Isolation of the compounds was done using TLC, CC and PTLC. Identification of the compounds was done by ¹H NMR, COSY, ¹³C NMR, DEPT, HMQC, HMBC, mass and IR spectroscopy. Preliminary activity and cytotoxicity studies were done on Vero cells 199. Extracts were screened against *Plasmodium falciparum* D₆ strain and the following anti-plasmodial activity noted: *C. mucronata* (hexane: IC₅₀ 8.73±1.81 µg/ml, DCM: IC₅₀ 10.09±1.56 µg/ml, EtOAc: IC₅₀ <3.91 µg/ml and MeOH: IC₅₀ 19.78±2.38 µg/ml) and *S. abyssinica* (DCM: IC₅₀ <3.91 µg/m, EtOAc: IC₅₀ 5.09±0.041 µg/m and MeOH: IC₅₀ 9.61±0.11µg/m). Cytotoxicity bioassay of crude extracts was done using vero cells 199 and *S. abyssinica* hexane extracts, DCM, EtOAc and MeOH (CC₅₀ 82.12 µg/ml, 100 µg/ml, 100 µg/ml and 100 µg/ml, respectively) were not toxic to vero cells 199. Ethyl acetate extract of *C. mucronata* exhibited toxicity to vero cells 199 (CC₅₀ 10.34 µg/ml) although it was the most active extract against *P. falciparum* (D6) while hexane: (CC₅₀ 90.34 µg/ml), DCM (CC₅₀ 100.00 µg/ml) and MeOH (CC₅₀ 66.33 µg/ml) extracts were not toxic. (-)-Stigmasterol (**204**) and (-)-curine (**205**) were isolated from *C. mucronata* while (+)-nonacosan-10-ol (**206**), (-)-5-oxoaknadinine (**207**), (-)-pseudocurine (**208**) and (-)-isocurine (**209**) were reported from *S. abyssinica* for the first time. Anti-plasmodial activity was exhibited by (-)-curine (**205**): IC₅₀ 0.24±0.03 µg/ml, 0.22±0.06 µg/ml), (+)-nonacosan-10-ol (**206**) (IC₅₀ 13.79±1.02, 4.35±2.45 µg/ml), (-)-5-oxoaknadinine (**207**) (IC₅₀ 10.25±1.84, 3.45±2.22 µg/ml), (-)-pseudocurine (**208**): IC₅₀ 0.29±0.00 µg/ml, 0.31±0.01 µg/ml) and (-)-isocurine (**209**) (IC₅₀ 0.75±0.11 µg/ml, 1.65±0.03 µg/ml) against D6 and W2 strains, respectively. It was confirmed that some of the compounds isolated from the two plants had strong anti-malarial activity making them good candidates for further scientific research on anti-plasmodial drug discovery and development. The results thus validate the ethnobotanical medicinal use of the two plants for the treatment of malaria despite potential toxicity exhibited by organic extracts.

CHAPTER 1

INTRODUCTION

1.1 Background

From time immemorial, malaria has been one of the most prevalent diseases affecting people in tropics (WHO, 1986). Majority of the affected are young children under five years and pregnant women due to the undeveloped and low immunity, respectively (Abdullah *et al.*, 2001). It also poses a risk to travelers and immigrants, with imported cases increasing in non-endemic areas (WHO, 2006). Each year, there are about 500,000,000 and 2,700,000 reported malaria cases and deaths, respectively, worldwide (WHO, 2002; Green wood *et al.*, 2005). Malaria in Africa accounts for approximately 90% of the global malaria burden (WHO, 1996). In eastern Africa 25.3 million cases of malaria are reported annually, of which 8.2 million cases are from Kenya out of a national population of 30 million as compared to 8.6, 5.3, 2.0 and 1.2 million cases for Tanzania, Uganda, Burundi and Rwanda, respectively (Jean-Marie, 2002). Although widespread, malaria is now mainly confined to Africa, Asia and Latin America (Figure 1) (<http://www.cdc.gov/malaria/distribution-epi/distribution.htm>).

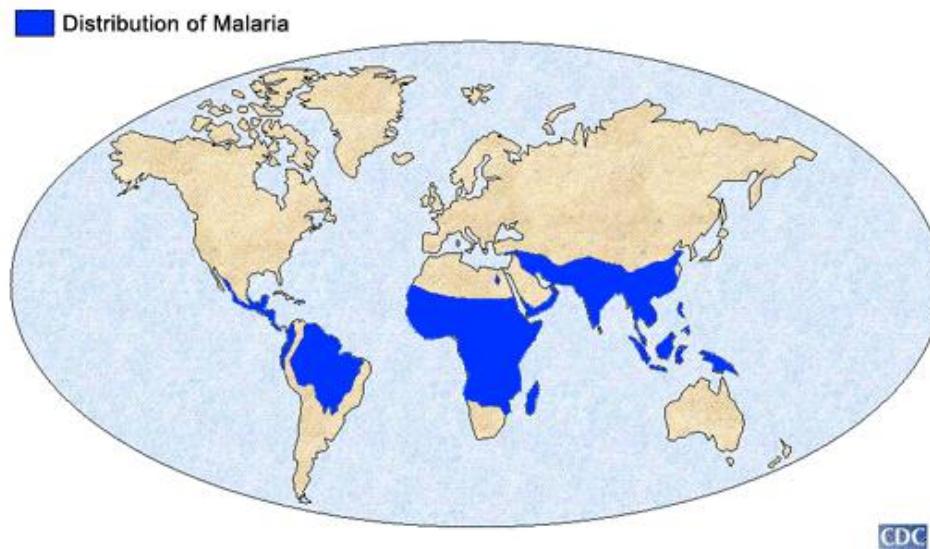


Figure 1: Map of distribution of malaria in the world

1.2 Economic costs of malaria

Malaria still remains a major health problem in tropical countries, with substantial burden on the economy. The high morbidity and mortality in some countries with intense transmission rates has reduced per capita income by up to 1.3%. A 10% reduction in malaria burden has been associated with higher economic growth (Gallup & Sachs, 2001). The cost of treatment and prevention alone is vast and many productive man-hours are lost each day from those suffering from malaria or looking after such patients. Many pregnant women suffer severely resulting in about 800,000 infant mortalities, a substantial number of miscarriages and very low birth weight (VLBW) babies, annually, as well as high risk of death due to the disease (Greenwood *et al.*, 1987; 1992). Moreover, the economic costs involved as a result of deaths from malaria are high, not to mention the pain and suffering associated with the disease. In addition, the spread of drug resistant malaria strains substantially raises the cost of treatment (Greenwood *et al.*, 1987; Molyneux *et al.*, 1999). In the long term malaria negatively affects flow of trade and foreign investments, such as tourism in regions with a high risk of infection (Gallup & Sachs, 2001).

1.3 The malaria parasite and life cycle

Malaria is caused by the parasites in the genus *Plasmodium* which are transmitted to humans through bites by infected female mosquitoes of the genus *Anopheles*. The four species of malaria parasites include *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. They cause *malignant tertian*, *benign tertian*, *quartan* and *ovale* malaria, respectively (Richard, 1985). *Plasmodium falciparum* causes the most severe form of malaria, which accounts for about 90% of all cases and deaths worldwide (WHO, 2002). *Plasmodium vivax* predominates in Asia, South and Central America but it is rarely found in Africa. *Plasmodium malariae* has the same range as *P. falciparum* but it is less virulent while *P. ovale* occurs mainly in tropical West Africa and sporadically in West Pacific region (Rozendaal, 1997). Malaria caused by *P. falciparum* is characterized by periodic chills, fever, and sweating often leading to severe anaemia, enlarged spleen and other complications which results in loss of life, especially among infants (Neil *et al.*, 1992).

The life cycle of malaria parasites occur in two phases: the asexual phase (schizogony) in the vertebrate host; and the sexual phase (sporogony) in the mosquito (Richard, 1985). The sporozoites develop in the salivary glands of the female *Anopheles* mosquito and are injected into the human host when the insect bites for a blood meal. They travel in the blood stream to the liver, invade parenchyma (hepatocytes), differentiate and undergo division (the exo-erythrocytic cycle) forming schizonts (Richard, 1985). Mature tissue schizonts release thousands of merozoites after 5-15 days, depending on the species. The merozoites invade erythrocytes where they appear initially as ring stage followed by the growing trophozoite stage that develops into the dividing asexual schizont stage (Rang *et al.*, 1999). The trophozoite divides (schizogony) and the erythrocyte raptures releasing more merozoites which invade other erythrocytes. The periodic rupture of the infected erythrocytes results into regular paroxysm of fever after the end of each schizogonic cycle (asexual cycle). The duration of asexual cycle depends on the parasite species: 36-48, 72, 48 h for *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*, respectively (Richard, 1985). At some point during the erythrocytic stage, there is development of male and female gametocytes instead of merozoites. When the sexual stage of the parasite is taken up in a blood meal by mosquitoes fertilization occurs and the life cycle is completed. (Figure 2) (<http://www3.niad.win.gov/tropics/malaria/life cycle.htm>).

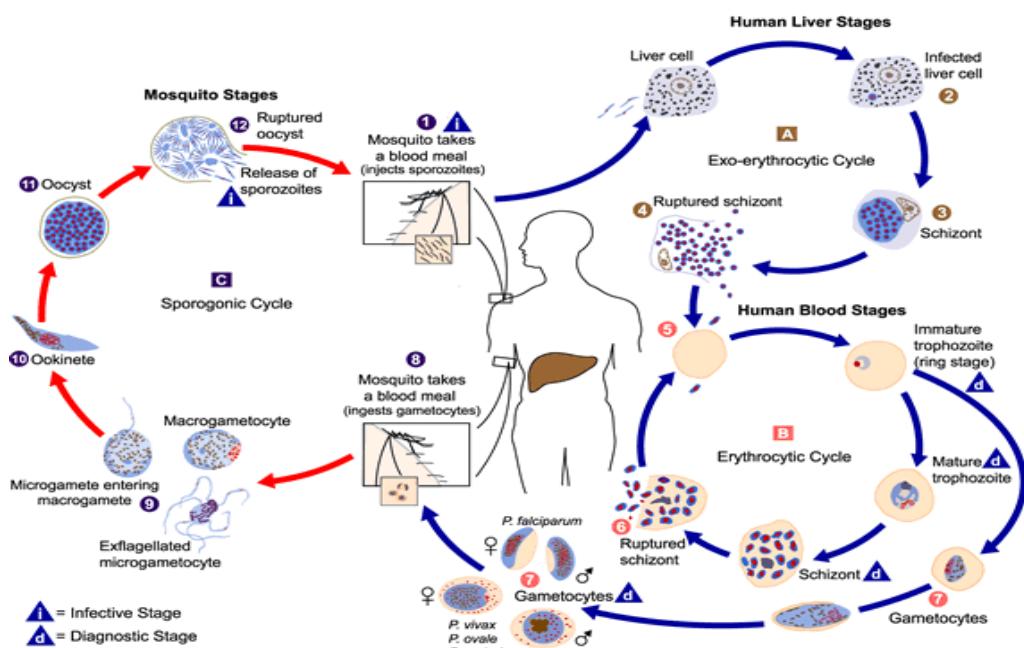


Figure 2: Lifecycle of malaria parasites

1.4 The malaria vector

Malaria is transmitted to humans through bites by infected female anopheline mosquito (Richard, 1985). There are approximately 3,200 species and sub-species of mosquitoes belonging to 42 genera, all within the family Culicidae (Clements, 1992). Only 80 anopheline mosquito species are important malaria vectors (Richard, 1985). The main malaria vector is *Anopheles gambiae* (Plate 1) (<http://www.cdc.gov/malaria/spotlight/index-052704.htm>). Anopheline mosquitoes can be distinguished from the other species by their unique posture while resting. The proboscis and body lies slanted at an angle to the surface (Richard, 1985). Female anopheline mosquitoes suck blood from vertebrates for the protein required for the development of eggs (Richard, 1985).



Plate1: Photograph of *Anopheles gambiae*

1.5 Malaria control

The control strategies for malaria involve vector control, chemoprophylaxis, chemotherapy and vaccine development (WHO, 1998).

1.5.1 Vector control

Vector control targets reduction of anopheline mosquito population at the larval and adult stage. This is based on the application of residual insecticides and preventing human-vector contact. However, chemical control of the vector is becoming increasingly

difficult due to the resistance developed against available commercial insecticides (WHO, 1994).

1.5.1.1 Larval control

The method is used to manage and reduce population of mosquito larva before they emerge into adults. It involves use of larvicides, insect growth regulators (IGR), biological control agents (BCAs) and environmental management (EM) (WHO, 1997).

According to WHO (2004), EM for vector control involves planning, organization and manipulation of environmental factors or their interaction with a view of preventing or minimizing vector development and reducing man vector contact. This include building dams at high altitudes or far away from settlements, draining water in permanent (swamps) and temporary breading habitats (tyres, tins, water storage tanks), intermittent irrigation, desiccation by planting trees and improved housing (Ghebreyesus *et al.*, 1999). However, environmental control has suffered a major setback because some vectors have undergone adaptations that make their larvae survive in temporary water collections in the holes of tree trunks, animal hoof prints, old disused tins, tyres and marshy grounds (Githeko *et al.*, 2000)

BCAs involve the use of predators, parasites and pathogens to control the disease vectors. Factors to consider when choosing a suitable biological agent include: high virulence of the organism to targets species, high specificity/selectivity (harmless to non-target organisms) and ease of production and storage (for long periods of time without loss of virulence).

Entomo-parasitic nematodes such as *Romenomermis culicivovax* have been used to control mosquito larvae (Rawlings, 1998). The nematodes usually enter the insect through the breathing holes, mouth, anus or penetrate thin areas of the insect cuticle (Lacey, 1998). Once inside the body of the insect, they produce toxins that kill the host after a few days (Skovmand *et al.*, 2000).

Fungal bio-control agents like *Entomophaga maimaga*, *Metarizium anisoplae*, *Beauveria bassiana*, *Mediga saviva* and *Cordyceps militaris* have also been used for mosquito larval control (Brown & Knodson, 1994).

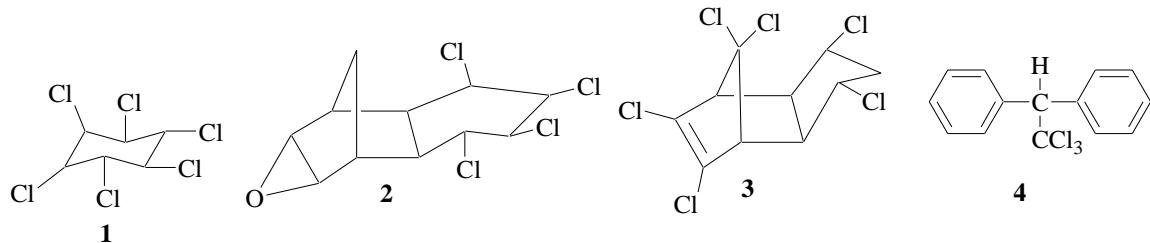
Bacteria like *Bacillus thurengiensis* var *israelensis* (sero-type H14) has been tested as mosquito larvicides and shown to be effective under field and laboratory conditions. Toxins produced by the bacteria show high virulence and selective activity to larval instars (Berry *et al.*, 1987). Viral control agents like baculoviruses have also been developed to control mosquito larva (Brown & Knodson, 1994).

The best known vertebrate BCAs is the mosquito fish, *Gambusia affinis*, a native of USA, which had been introduced in many tropical and sub-Saharan regions to control mosquito larva (Edoh *et al.*, 1997).

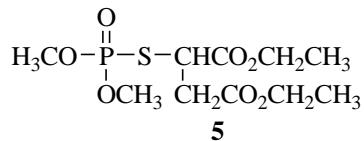
However, biological control of malaria has several limitations. *Bacillus thurengiensis* has been found to lack residual activity. It persists for months in larval habitats and that is not easy to culture (Berry *et al.*, 1987). Bacterial agents have slow mode of action as compared to chemical larvicides. More over, they are sensitive to ultra violet light (Berry *et al.*, 1987). It has also been found that the living system for production of viral control agents against mosquitoes is quite expensive (Weiss *et al.*, 1994). In addition to this, most species of the fungi are difficult to produce and their primary conidia are short lived making timing of inoculative application ineffective (Weiss *et al.*, 1994). The larvivorous fishes can only thrive in specific habitats and cannot be employed in managing some larval habitats especially temporary ones like holes in tree trunks, hoof prints, tyres prints, wells, old tyres and tins (Bay, 1967).

Chemical larvicides have also been used to control mosquito larva. Use of oil sprays on stagnant water bodies to suffocate and kill mosquito larvae is one of the earliest chemical methods used in large-scale operation for mosquito control. Nevertheless their use has been discouraged due to the toxic effect on non-target organisms and development of resistance by mosquito larvae (Wigglesworth, 1976). Synthetic

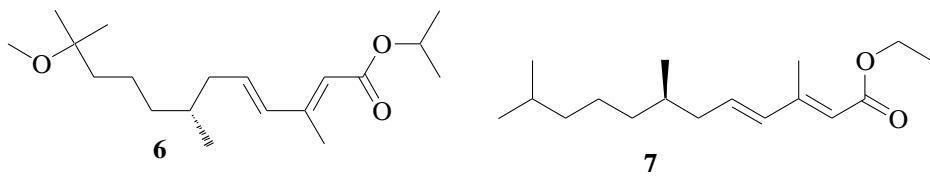
inorganic compounds such as paris green, $(\text{Cu}(\text{H}_2\text{O})_2 \cdot 3\text{Cu}(\text{AsO}_2)_2$ and copper metarsenite, $\text{Cu}(\text{AsO}_2)_2$ have also been used as larvicides (Metcalf & Flint, 1962). Chlorinated synthetic organic compounds like indane (**1**), dieldrin (**2**), chlordane (**3**) and DDT (**4**) have been used as larvicides. However, they are not biodegradable and have caused serious environmental pollution (Metcalf & Flint, 1962).



Organophosphates such as methylparathion (**5**) have been used as larvicides. They are bio-gradable, but not selective in their mode of action (Metcalf & Flint, 1962). Resistance to organophosphates was first reported in certain species of mosquitoes about 50 years ago (Matsumara & Brown, 1961).



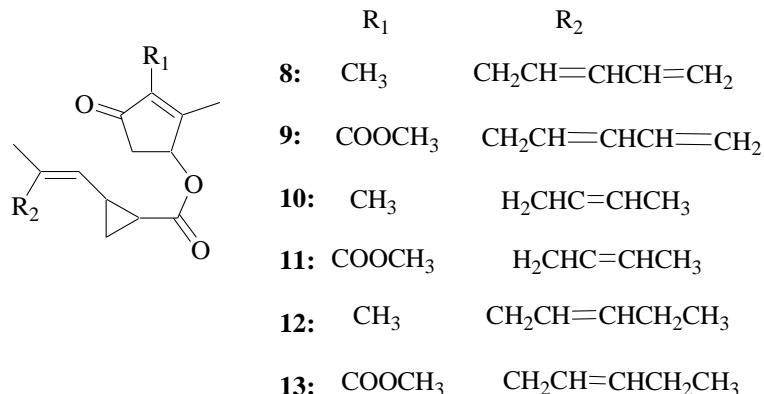
IGRs are materials that interrupt or inhibit the life cycle of an insect. Methoprene (**6**), an ingredient in precorTM, precor 2000TM and Extinguish Fire Ant BaitTM (EFAB), prevents the eggs and the larvae of mosquitoes from developing into adults (Weiss *et al.*, 1994). It has a residual activity of 3.7 months and minimal environmental hazards. Methoprene granules have become an important tool in reducing mosquito larval population in areas where there is standing water or other permanent mosquito breeding habitats (Weiss *et al.*, 1994). Hydrophene (**7**) is used as active ingredient in GentolTM, Gentol Point SourceTM and GentolTM aerosol. It is unstable to light and therefore recommended for indoor use only (Weiss *et al.*, 1994).



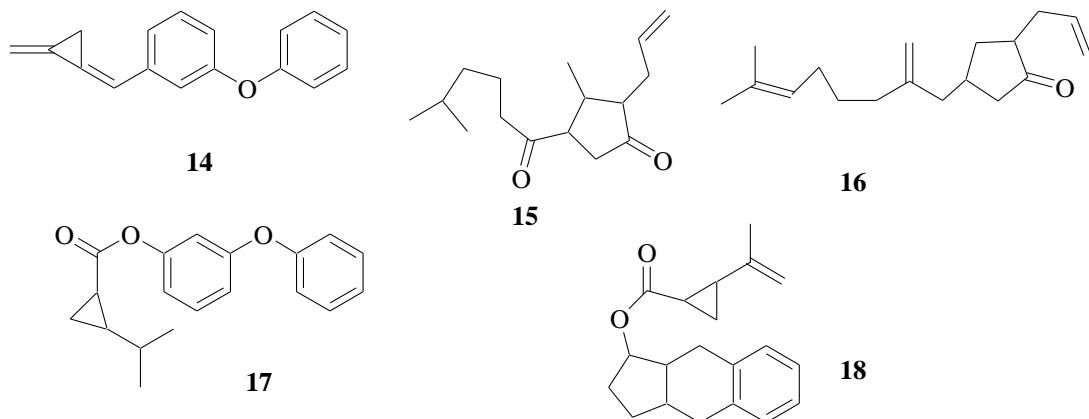
1.5.1.2 Adult control

Killing adult mosquitoes or preventing access to the human host can control the spread of malaria by adult mosquitoes. These include use of IRS with insecticides, insecticide treated bed nets (ITNs), repellents and attractants.

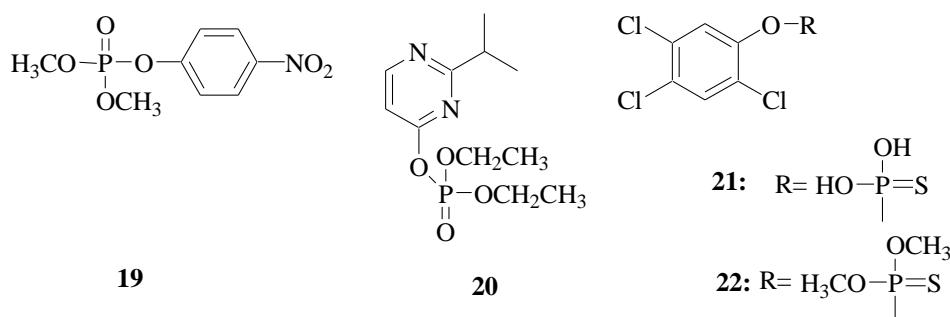
Natural pyrethrins from the flowers of *Chrysanthemum cinerariaefolium* have been used as powerful insecticides because they have low mammalian toxicity. The powder from the flower contain a mixture of six insecticidal esters which include pyrethrin I (**8**) and II (**9**), cinerin I (**10**) and II (**11**) and jasmolin I (**12**) and II (**13**). Use of natural pyrethrins depends on the rapid knock down effect but lack of prolonged residual action has restricted their application (Kumar, 1984).



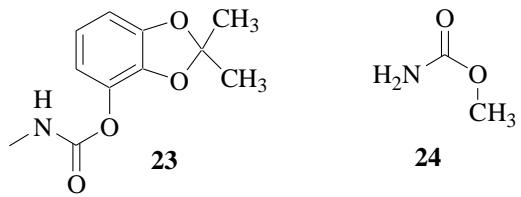
This led to the development and use of synthetic pyrethroids such as permethrin (**14**), alethrin (**15**), bioalethrin (**16**), cymethrin (**17**) and tetramethrin (**18**) among others (Knox *et al.*, 2003).



Due to resistance to synthetic pyrethrins against adult mosquitoes potent insecticides are used. Other synthetic insecticides including organochlorines like DDT (Rozendaal, 1997), organophosphates and carbamates have been used for mosquito control. Use of DDT has been discouraged because it is non-biodegradable and toxic to non-target organisms (Charles *et al.*, 1995). Organophosphates like malathion (**19**) and diazinon (**20**) are highly toxic to mammals including man and other non-target species but are much more stable than organochlorines (Motoyama *et al.*, 1997). They have also been used as adulticides. Other organophosphates that have been used to control mosquitoes include phosphorothioc acid (**21**) and methoxyphosphorothioc acid (**22**).



Carbamates have also been used as insecticides. The most commonly used includes 2,2-dimethyl-1,3-benzodioxol-4-ylcarbamate (**23**) and methyl carbamate (**24**) (Nobert, 2003).



Insecticide treated nets (ITNs) have been shown to reduce the contact between the host and vector leading to significant reduction of malaria transmission. Evidence of the impact of insecticide treated nets (ITNs) on morbidity and mortality from malaria has been provided from studies in Tanzania, Gambia and Guinea Bissau (Abdulla *et al.*, 2001). Since most anopheline bite at or near midnight, it has been found that the use of ITNs has reduced the prevalence and malaria cases in trial areas (WHO, 2002).

Currently, only pyrethroid insecticides have been approved for use on ITNs. These insecticides have very low mammalian toxicity but are highly toxic to insects and have a rapid knock-down effect, even at low doses. Pyrethroids have a high residual effect, they do not rapidly breakdown unless washed or exposed to sunlight. To maintain the efficacy of the ITNs, the nets are treated at interval of 6-12 months or more frequently if the nets are washed. Retreatment is done by simply dipping nets in a mixture of water and insecticide and allowing nets to dry in a shade. Kits for retreating nets are available in most countries.

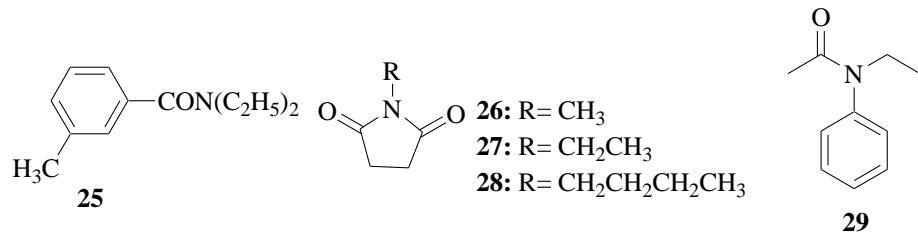
Several companies have developed long lasting ITNs that theoretically retain lethal concentrations of insecticide for the life of the net (3-5 years). Centre for disease control (CDC) is currently testing several of these products in Atlanta, Georgia and Kenya (<http://www.cdc.gov/malaria/distribution-epi/distribution.htm>).

ITNs reduced deaths from life threatening malaria by 44% lowered the hospitalization of children with malaria by 41% and reduced childhood deaths by 33% in Kenya. In Ghana's northern savannah region where malaria transmission is very high, nets reduced childhood deaths by 17%. Based on these studies, scientists estimate that the widespread distribution and use of ITNs could save lives of at least 500,000 children a year in Africa

alone (<http://www.cdc.gov/malaria/distribution-epi/distribution.htm>). Unfortunately, neither the nets nor the insecticides required are widely available or affordable in most sub-Saharan Africa (SSA). The need for frequent retreatments is also one of the most difficult barriers to full effectiveness of ITNs in malaria endemic countries. The additional costs of the insecticide and the lack of understanding of its importance result in very low bed net treatment rates in most African countries (<http://www.cdc.gov/malaria/control-prevention/vector-control.htm>, August 15, 2006). Another setback of the ITNs is that, many people in the malaria endemic rural areas do not have beds (Lines & Addington, 2001).

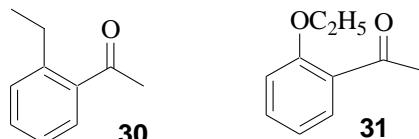
Many synthetic insecticides are highly toxic to man and other non-target organisms in addition to resistance developed by the target organisms (Matsumura *et al.*, 1961). This has contributed to the urgent need for other control strategies such as use of repellents and attractants to control mosquitoes (Kirk & Othmer, 1981).

Repellents are used to deter mosquitoes from accessing vertebrate hosts like humans by making the environment unattractive and offensive (Kirk & Othmer, 1981). These include pyrethrins and pyrethroids. Currently, the most commonly used synthetic repellent is *N,N*-diethyl-*m*-toluamide (DEET) (**25**) though its safety is questionable (Kirk & Othmer, 1981). DEET is available in different concentrations of multiple formulations including solutions, lotions, creams, gels, aerosol pump sprays and impregnated towelettes (Shreck, 1995). Many DEET analogues have been developed to control mosquitoes. These include *N*-methyl succinimide (**26**), *N*-ethylsuccinimide (**27**), *N*-butyl succinimide (**28**) and *N*-ethyl-*N*-phenyl acetamide (**29**) (Gryboski *et al.*, 1994). Another synthetic repellent closely related to DEET is IR3535 (Fradin & Day, 2002).



Permethrin (**14**) causes toxicity to central nerves system leading to death or knockdown of insects (Lindsay *et al.*, 1988). It has low toxicity in mammals, is poorly absorbed by the skin and rapidly inactivated by ester hydrolysis. It maintains its potency for at least two weeks (Lindsay *et al.*, 1988). A combination of permethrin treated clothing and skin application of DEET based repellents creates a formidable barrier against mosquito bites (Kline & Shreck, 1989). In a field trial tried in Alaska, persons wearing permethrin treated uniforms and polymer based 35% DEET product had more than 99.9% protection for over 8 hours (Kline & Shreck, 1989).

Omolo (2005), showed that some mosquito allomones: undecanal, 4-ethylacetophenone (**30**) and 4-ethoxyacetophenone (**31**) constituting the human foot odour have strong repellent activity and may be used in keeping mosquitoes away from human hosts.



Attractants have also been used to control adult mosquitoes. Host-attractant baited traps and targets are promising technologies currently being considered for mosquito control (Mathenge *et al.*, 2002). It is widely used for the surveillance of mosquitoes is the CDC trap. The trap uses carbon dioxide and a light source to attract mosquitoes (Bellamy & Reeves, 1952; Sudai & Chamberlain, 1962; Newhouse *et al.*, 1966; Kline, 1994). However, CDC miniature light traps are unsatisfactorily for field surveys because they consistently produce too small catches for evaluation (Miller *et al.*, 1969).

Mosquitoes use chemical attractants to locate their mates and hosts for blood meals (Takken *et al.*, 2001). Host attractants therefore appear to offer potential application for use in traps and targets in the effort to minimize the threat of human diseases transmitted by mosquitoes. However, attractants of disease vectors and human pests are not adequately studied (WHO, 1996) and therefore not well understood. Despite the array of vector control tools, vaccines offers hope for effective malaria prevention.

1.5.2 Vaccine development

The development of vaccines against malaria is an area where a lot of effort has been directed but without any tangible success partly due to the many different development stages in the life cycles. In order to develop appropriate vaccine, all development stages of the parasites should be taken into account in the design. Some vaccines are directed against sporozoites while others against the asexual blood stage (merozites) and the sexual reproductive stage of the parasite in the mosquito (pre-erythrocytic) (WHO, 1996). Several candidates have been developed using the latest breakthrough in vaccine technology and undergone field-testing in Asia, Africa, and USA (<http://www.epha.org/r/40>).

Another approach in malaria vaccine development involves use of monoclonal antibodies. Several vaccines under development involve multi-component DNA technology (WHO, 1996). These have shown promising results in rodent malaria models (Denise & Stephen, 2001).

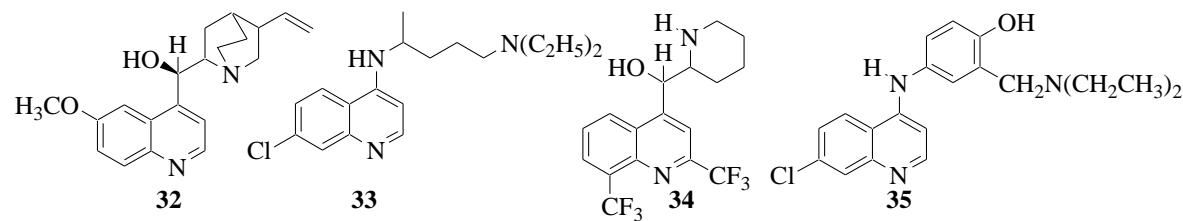
The first multi-component synthetic peptide vaccine (SPf66) based on the merozoite surface protein (MSP) has been widely tested (WHO, 1998). It gave mixed results in field trials in South America, Africa and South East Asia (Peter, 2001). Recently, another multi-component vaccine (RTS, S) was developed. It combines the three pre-erythrocytic proteins (asexual blood stage antigens) and a transmission-blocking candidate. It was initially restricted to human clinical trials in the USA (Doherty *et al.*, 1999). However, an improved candidate vaccine, RTS, S/AS02A, has been tested in southern Mozambique demonstrating efficacy of 30, 45 and 58% against clinical malaria attacks, primary infection with *P. falciparum* and severe disease, respectively, safety and tolerance in children (Alonso *et al.*, 2005). The search for an effective vaccine is still on going since some of the trials have shown little or short-lived efficacies like RTS, S/AS02A. The final assault on malaria remains chemotherapy which is useful for prophylaxis or treatment.

1.5.3 Chemotherapy and chemoprophylaxis

Malaria chemotherapy involves the use of anti-malarial drugs to treat the disease. Chemoprophylaxis is the use of drugs before infection takes place or prior to its manifestations with the aim of preventing either of these occurrences (WHO, 1986). Anti-malarial drugs given at their usual dosages can affect one or more developmental stages of *Plasmodia* species (Kirk & Othmer, 1979). Apart from 8-aminoquinolines and the anti-folates, all anti-malarial drugs are suppressive rather than prophylactic (Peter, 2001). Anti-folates have schizonicidal effect on the erythrocytic and exo-erythrocytic forms of *P. falciparum* in the liver (Rang *et al.*, 1999).

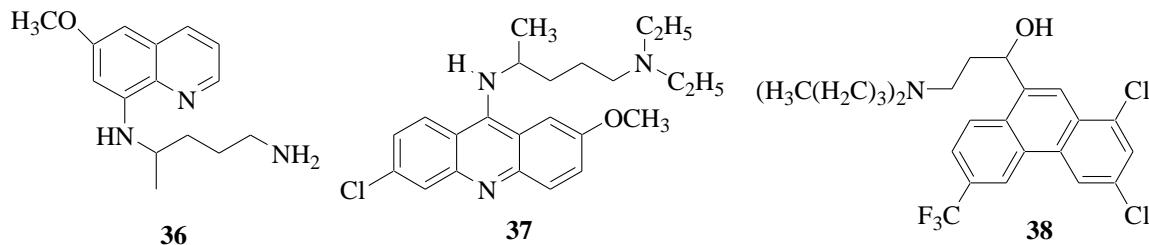
1.5.2.1 Single therapy

The most widely used drugs are quinine (**32**), its derivatives and analogs. It was isolated in 1820 from a Peruvian tree, *Cinchona ledgeriana* and is effective against immature gametocytes of *P. falciparum* malaria but not mature ones (Farnsworth *et al.*, 1985). It is an effective gametocide in *vivax*, *ovale* and *quartan* malaria. However, it is a poor drug for general gametocytocidal prophylaxis (WHO, 1986). Chloroquine (**33**), a 4-aminoquinoline analogue of quinine, was synthesized in 1934 but its usefulness has been compromised by the widespread development of resistance by *P. falciparum* (Foley & Tilley, 1997). Mefloquine (**34**), another analogue of quinine, is effective against chloroquine-resistant parasites but resistance has been reported recently (Wongsrichanalai *et al.*, 2002). Amodiaquine (**35**), an analogue of chloroquine, is effective in the treatment of malaria though at reduced sensitivity by *P. falciparum* has been reported (Rang *et al.*, 1999).

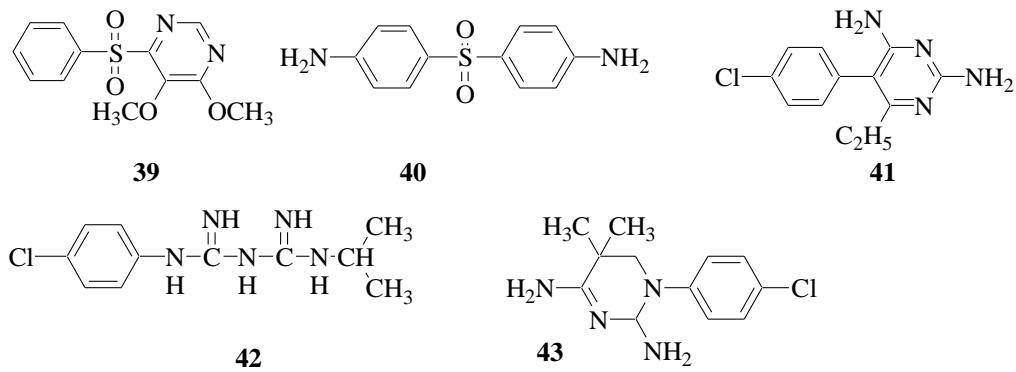


Primaquine (**36**) is highly effective on gametocytes of all species of malaria parasites (WHO, 1986). Mepacrine (**37**) is largely obsolete as an anti-malarial. Although it has

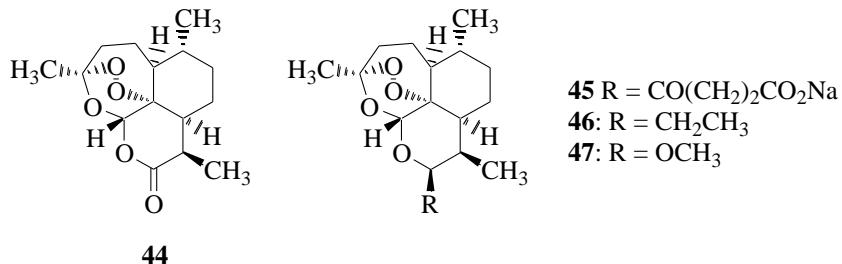
distinct advantages over quinine, these are offset by many side effects. It dyes the skin and conjunctiva yellow and occasionally causes alarming, though transient, mental disturbances (WHO, 1986). Halofantrine (**38**), a 9-phenthrenemethanol, is effective against *P. vivax* and *P. falciparum* strains.



Sulfadoxine (**39**) and dapsone (**40**) are highly effective against the asexual blood forms of *P. falciparum*. However, they are less effective against other *Plasmodium* species. Their action is too slow for use alone. They are more effective in combination with other drugs. Although there has been development of resistance by *P. falciparum* to pyrimethamine (**41**), it is a cheap anti-malarial drug and is active against asexual blood stages of all malaria parasites (Rang *et al.*, 1999). However, it is slow acting and not recommended for treatment of acute malaria attack (WHO, 1986). Proguanil (**42**) is highly effective against primary exo-erythrocytic forms of *P. falciparum*, and has a fleeting inhibitory action on *P. vivax* (WHO, 1986). Proguanil acts after conversion to a triazine metabolite, cycloguanil (**43**) by binding to an enzyme required by malaria parasite, dihydrofolate reductase (DHFR) (WHO, 1986).



Artemisinin (**44**) was originally isolated from the Chinese plant, *Artemisia annua*, and is effective against chloroquine sensitive and resistant strains of *P. falciparum* (Farnsworth *et al.*, 1985). Its derivatives artesunate (**45**), arteether (**46**) and artemether (**47**) are more effective anti-malarial drugs than artemisinin itself (Peters, 1987; Dhingira *et al.*, 2000).



Malaria parasites develop resistance to the single therapy rapidly (Kain, 1995). Due to development of resistance of malaria parasite to the available drugs, the search for effective, safe and affordable drugs for resistant malaria is one of the most pressing and urgent health priorities worldwide.

1.5.2.2 Double therapy

The combination of sulfadoxine with pyrimethamine (FansidarTM) achieved wide acceptance because of its simplicity, reliability and absence of adverse effects (WHO, 1986). FansidarTM acts against the asexual intra-erythrocytic forms of *P. falciparum* in humans. Sulfalene-pyrimethamine (MetakelfinTM) allows double attack in different points of the same metabolic pathway of the parasite. The persistence of the two components in blood and the similarity of the half-life times allow the treatment of malaria with single dose and prophylaxis with a delayed dose assure consistency of synergism (Rang *et al.*, 1999). Atavaquone is used in combination with a second drug, such as proguanil (atavaquone-proguanil) (MalaroneTM) or tetracycline (atavaquone-tetracycline) to delay resistance development (Peter, 2001).

Although double therapy has proved to be more effective than single therapy in treatment of malaria, parasite resistance against some of the combinations has been reported. Resistance to FansidarTM has been reported in N.E. Tanzania (Mutibingwa *et al.*, 2001).

1.5.2.3 Triple therapy

Some of the combinations in triple therapy include chloroquine with sulfadoxine-pyrimethamine and amodiaquine with chloroprogua-nil-dapsone (Wongsrichanalai *et al.*, 2001). They are effective and affordable.

Although triple therapy has proved effective in management of malaria parasites, there is no guarantee that resistance will never be developed against them. Therefore, the search for new anti-malarials with different modes of action has continued to receive considerable attention.

Plants have provided many medicinal compounds including quinine, aspirin, morphine, ephedrine, artemisinin (Balandrin *et al.*, 1993), and earlier studies on the anti-malarial plants from southern Nyanza revealed that *C. mucronata* and *S. abyssinica* are widely used in treatment of malaria (Muregi *et al.*, 2003; 2004). For this reason *Cissampelos mucronata* (A. Rich) and *Stephania abyssinica* (Dillon & A. Rich) were chosen for investigations.

1.6 Statement of research problem

Due to the development of resistance to available anti-malarial drugs by the parasites and their toxicity, there is need for new and more effective but less toxic herbal alternatives. Several plant-based herbal drugs have been used by indigenous people to treat malaria, without knowledge of safety, efficacy and the bioactive principles. *Cissampelos mucronata* and *Stephania abyssinica* used as traditional anti-malarial drugs in southern Nyanza have not been validated for efficacy and safety. Furthermore, their anti-malarial principles safety and efficacy is not known despite widespread use.

1.7 Hypothesis

Cissampelos mucronata and *Stephania abyssinica* used in traditional anti-malarial therapy in southern Nyanza contain natural products with unique and stable chemical structures that are potential drugs against malaria.

1.8 Research objectives

1.8.1 General objective

To evaluate the anti-plasmodial activity and toxicity of the extracts of *Cissampelos mucronata* (A. Rich) and *Stephania abyssinica* (Dillon & A. Rich) and to isolate and identify anti-plasmodial principles therein.

1.8.2 Specific objectives

- (i) To evaluate the *in vitro* anti-plasmodial activity of extracts of *C. mucronata* and *S. abyssinica*;
- (ii) To undertake cytotoxicity assay of extracts of *C. mucronata* and *S. abyssinica* using vero cells;
- (iii) To isolate the anti-plasmodial principles of *C. mucronata* and *S. abyssinica*;
- (iv) To identify the bio-active constituents of *C. mucronata* and *S. abyssinica*;
- (v) To determine the *in vitro* anti-plasmodial activity of compounds isolated from *C. mucronata* and *S. abyssinica*.

1.9 Justification

The discovery of two important anti-malarial drugs: quinine and artemisinin from plants and their use as molecular templates for synthetic analogues has encouraged phytochemical and pharmacological studies on plants used in traditional malaria therapy by indigenous communities. The development of resistance to the available anti-malaria drugs and their toxicity rationalizes the continuous search for new, more effective and less toxic remedies. *Cissampelos mucronata* and *Stephania abyssinica* used for traditional anti-malarial therapy in southern Nyanza have not been investigated either phytochemically or pharmacologically.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant-derived anti-malarials

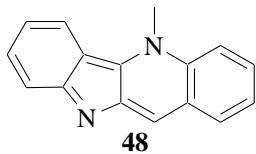
Several plants are known to produce anti-plasmodial agents that are presently in clinical use. Quinine (**32**) which was first isolated from *Cinchona ledgeriana* (Rubiaceae) in 1820 is among the pharmalogically potent plant derived natural products in clinical use (Nkunya, 1992). It has also been used as a template for synthetic amino-quinoline based anti-malarial analogues such as chloroquine (**33**), amodiaquine (**35**), primaquine (**36**) and mefloquine (**34**) (Nkunya, 1992). Artemisinin (**44**) isolated from a Chinese anti-malarial plant *Artemisia annua* (Asteraceace) is currently the most potent and safe plant-derive anti-malarial drug (Nkunya, 1992).

Phytochemical and pharmacological screening has shown that some of the plants used in traditional medicines contain alkaloid, terpenoid, phenolic and flavonoid compounds with promising anti-plasmodial activity (Phillipson *et al.*, 1990).

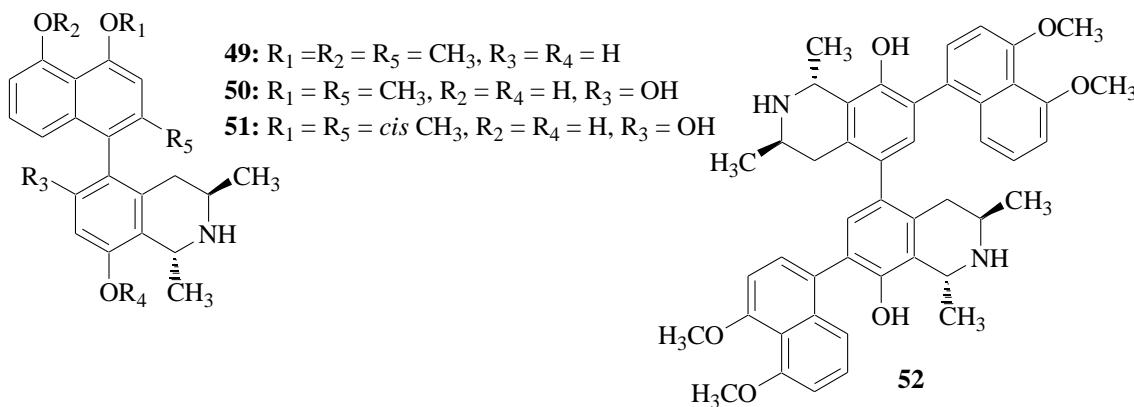
2.1.1 Alkaloids

Quinolines like quinine were the first to be used for treatment of malaria. Quinine (**32**) provided lead structure in the discovery of synthetic derivatives like chloroquine (**33**), mefloquine (**34**) and amodiaquine (**35**) that have higher anti-malarial activity (Kayser *et al.*, 1998).

Indoles alkaloids comprise of compounds with varied biological activity. Crytolepine (**48**) and related indole quinolines isolated form *Cryptolepis sanguinolenta* exhibit anti-plasmodial activity against *P. falciparum* (W2, D6 and K1) though they are mildly active *in vivo* (10.8-19.4% suppression of *P. yoelii* at 100 mg/kg/day) (Kayser *et al.*, 1998).



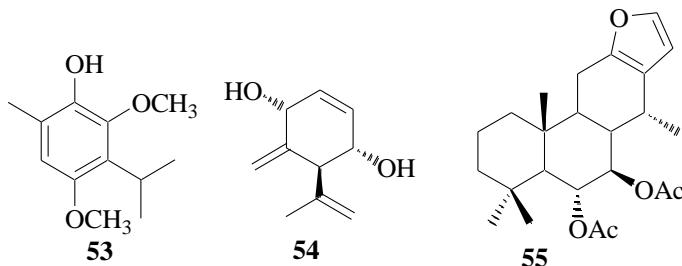
Naphthylisoquinoline alkaloids isolated from tropical lianas form a unique class of promising anti-plasmodial natural products. These compounds consist of naphthalene and isoquinoline moiety joined by a binary axis. Korupensamines A (**49**) and B (**50**) isolated from a liana indigenous to Cameroon (*Ancistrocladus korupensis*) exhibited strong anti-plasmodial activity (IC_{50} 0.31 and 0.17 $\mu\text{g}/\text{ml}$) against K1 and NF54, respectively (Bringmann, 1996). Dioncophylline A (**51**) exhibited mild activity against chloroquine-resistant strains of *P. falciparum* (IC_{50} 13.6 $\mu\text{g}/\text{ml}$) *in vitro* (Bringmann, 1996). Some dimmers of the bioactive principles showed improved bioactivity. Jozimine A (**52**), the dimer of dioncophylline A (**51**) also exhibited strong anti-plasmodial activity (IC_{50} 0.075 $\mu\text{g}/\text{ml}$) (Bringmann, 1996). These compounds constitute novel lead structures for the development of anti-malarial drugs.



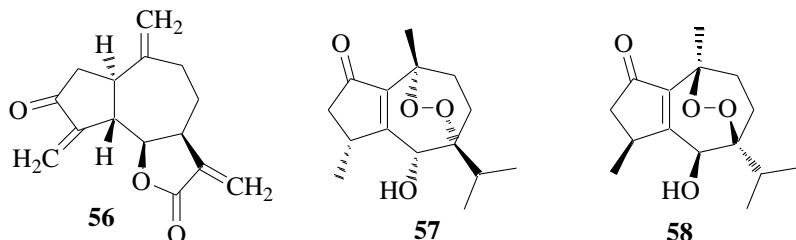
2.1.2 Terpenoids

Monoterpenes like espintanol (**53**) isolated from *Oxandra espinata* (Annonaceae) exhibit mild anti-plasmodial activity (IC_{50} of 25-100 $\mu\text{g}/\text{ml}$) against *P. falciparum* (Kayser *et al.*, 1998). Piquerol A (**54**) isolated from the same plant had an IC_{50} of 100 $\mu\text{g}/\text{ml}$ against *P. falciparum* (Kayser *et al.*, 1998). Japanese researchers isolated cassane-type diterpene $6\alpha, 7\beta$ -diacetoxyvouacapane (**55**) from the seeds of *Bowdichia nitida* (Fabaceae), a

Brazilian Amazon species. It showed promising *in vitro* anti-plasmodial activity against parasite *P. falciparum* 3D7 strain ($IC_{50} = 1 \mu\text{m}$) (Matsuno *et al.*, 2008).

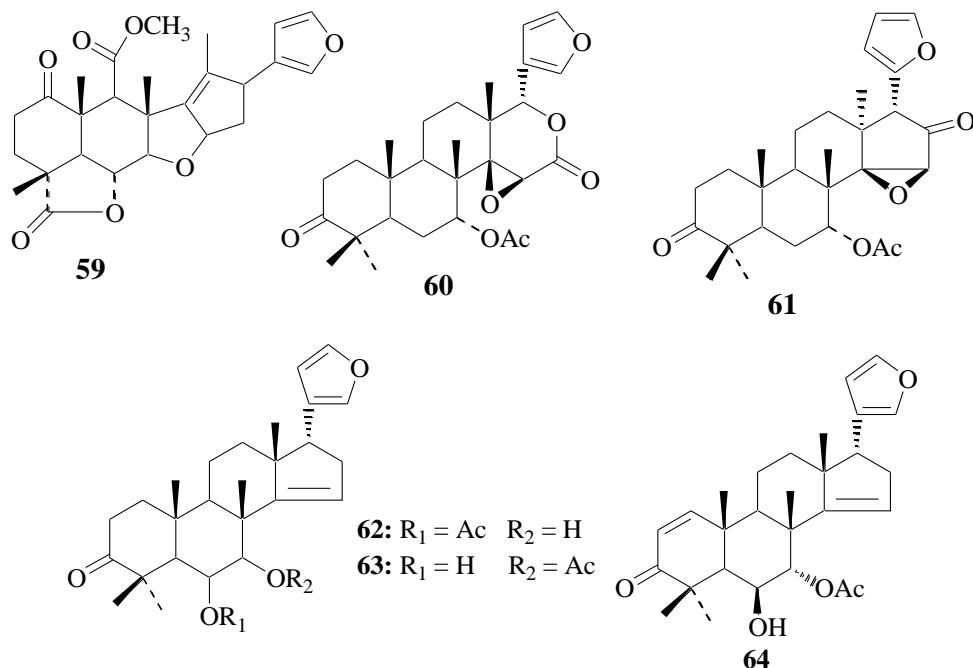


Sesquiterpenes especially artemisinin (**44**) and its derivatives have been identified as new anti-malarial drugs with high clinical efficacy. The sesquiterpenes lactone parthein (**56**) also show moderate anti-plasmodial activity ($EC_{50} 1.29 \mu\text{g/ml}$) against *P. falciparum* *in vitro* (Picman *et al.*, 1979). Nardoperoxide (**57**) and isonardoperoxide (**58**) isolated from the roots of *Nardostachys chinensis* showed a strongest anti-malarial activity ($EC_{50} 1.50 \times 10^{-6}$ and $6.0 \times 10^{-7} \text{ M}$, respectively) against NF 54 strain (Takaya, 1998; Saxena *et al.*, 2003).

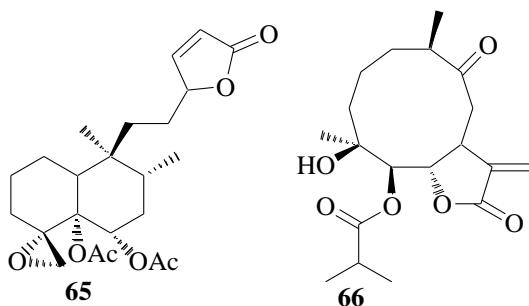


Limonoids from *Azadirachta indica*, the neem tree widely used as an anti-malarial plant in Asia belongs to this family. Nimbolide (**59**) ($EC_{50} 0.95 \mu\text{g/ml}$, *P. falciparum* K1) was the first to be identified as the active anti-plasmodial principle of the neem tree (Rochanakij *et al.*, 1985). Several limonoids from the Meliaceae have since been shown to exhibit anti-plasmodial activity with gedunin (**60**) from *A. indica* being the most active against *P. falciparum* Dd2, the chloroquine resistant strain, *in vitro* ($IC_{50} 0.02 \mu\text{g/ml}$) (Mackinnon *et al.*, 1997). Nimbinin (**61**), meldenin (**62**), isomeldenin (**63**) and nimocinol (**64**) isolated from *Azadirachta indica* showed anti-plasmodial activity (IC_{50}

0.77, 5.23, 50 and 50.1 $\mu\text{g/ml}$, respectively) against K1 strain (Bray *et al.*, 1990; Saxena *et al.*, 2003).

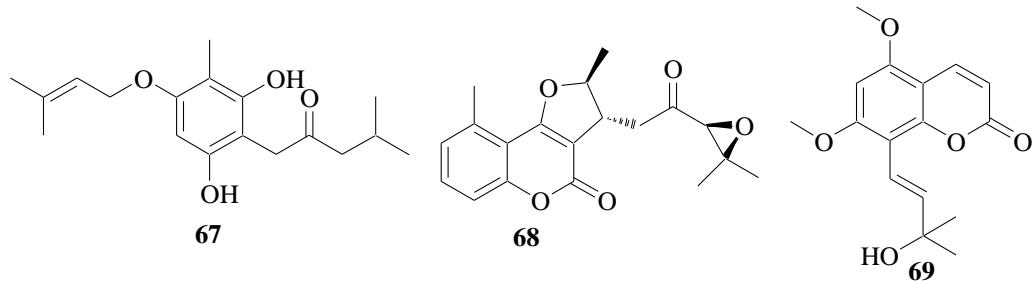


Ajuga remota a commonly occurring medicinal plant in Kenya is widely used as anti-malarial remedy (Kokwaro, 1976; Kuria *et al.*, 2002). Ajugarine (65), isolated from this plant is mildly active (IC_{50} 23 $\mu\text{g/ml}$) against *P. falciparum* FCA20/GHA, the CQ sensitive strain, *in vitro* (Kuria *et al.*, 2002). The chloroform-soluble fraction obtained from methanol extract of *Carpesium rosulatum* (Asteraceae) yielded ineupatorolide A (66) which displayed a very impressive anti-plasmodial activity against chloroquine resistant D10 strain of *P. falciparum* (IC_{50} 0.019 μM) (Moon, 2007). Korean researchers demonstrated that compound (66) show potent *in vivo* anti-malarial activity when tested against *P. berghei* in mice at doses of 2, 5 and 10 mg/kg/day (Chung *et al.*, 2008; 2009).



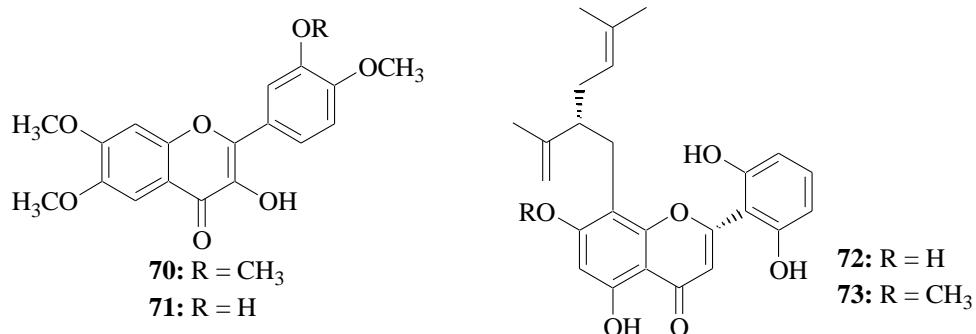
2.1.3 Phenols

Prenylated phloroglucinol derivative (**67**) isolated from *Hypericum calycinum* (*Hypericaceae*), inhibited EC₅₀ 0.88 µg/ml *P. falciparum* *in vitro* (Decosterd *et al.*, 1991). Anti-plasmodial activity of 2'-epicycloisobrachycoumarinone epoxide (**68**) and its stereo isomer isolated from *Vernonia brachylalyx* (Asteraceae) has also been reported. Both stereoisomers show similar *in vitro* activity against both chloroquine-sensitive and chloroquine resistant strains of *P. falciparum* (EC₅₀ 0.11 and 0.15 µg/ml, respectively) (Oketch-Rabah *et al.*, 1997a). Another coumarin derivative, 5,7-dimethoxy-8-(3'-hydroxy-3-methyl-1'-butene)-coumarin (**69**) isolated from *Toddalia asiatica* and was found to be mildly active (EC₅₀ 16.2±1.4 and 8.8±1.6 µg/ml) against chloroquine-sensitive (ENT30) and chloroquine resistant (NF54) strains *P. falciparum*, respectively (Oketch-Rabah *et al.*, 2000).

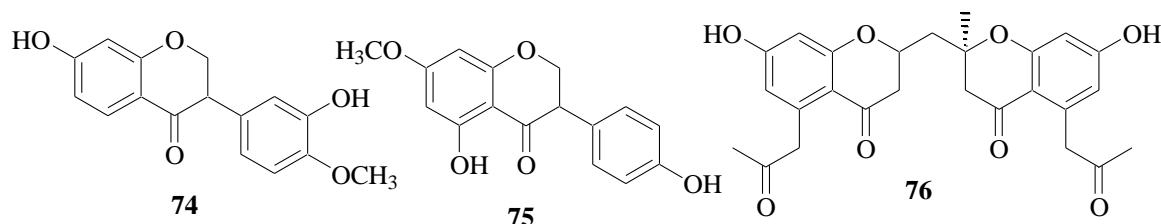


2.1.4 Flavonoids and related compounds

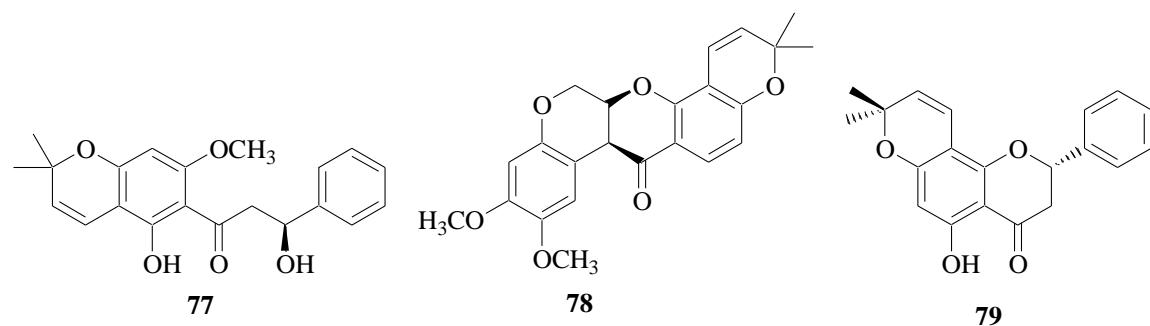
Flavonoids from *Artemisia annua* have been shown to have anti-plasmodial activity. Elford *et al* (1985) demonstrated that methoxylated flavonones, artemisinin (**70**) and casticin (**71**), act synergistically with artemisinin against *P. falciparum* *in vitro*. Exiguaflavone A (**72**) and B (**73**) isolated from *Artemisia indica* exhibited *in vitro* activity against *P. falciparum* (EC₅₀ 4.6 and 7.1 µg/ml, respectively) (Rachanda *et al.*, 1998).



Calycosin (**74**) and genistein (**75**) isolated from *Andira inermis* showed moderate anti-plasmodial activity (IC_{50} 9.8 and 4.1 $\mu\text{g/ml}$, respectively) against Dd2, a chloroquine resistant strain (Kraft *et al.*, 2000; Saxena *et al.*, 2003). A new bischromone, chrobisiamone A (**76**) with a good *in vitro* anti-plasmodial activity (IC_{50} 9.8 μM) against parasite *P. falciparum* 3D7 a chloroquine sensitive strain has been isolated from the leaves of *Cassia siamea*, a Fabaceae species widely used in traditional medicine, particularly treatment of periodic fever and malaria in Indonesia (Oshimi *et al.*, 2008).

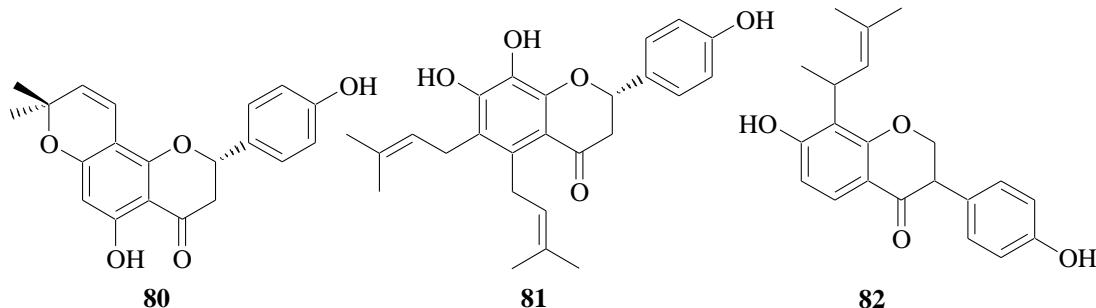


Seeds of *Tephrosia elata* (Fabaceae) have yielded a new β -hydroxydihydrochalcone (**77**) along with flavonoids deguelin (**78**) and obovatin (**79**). Among the flavonoids so far isolated the β -hydroxydihydrochalcone (**77**) exhibited the highest anti-plasmodial activity (IC_{50} 8.2 ± 0.8 and $16.3 \pm 0.9 \mu\text{M}$, respectively) against D6 and W2 strains while compound (**78**) and (**79**) showed IC_{50} values ranging from 12.4 to 27.6 μM against these strains (Muiva *et al.*, 2009).



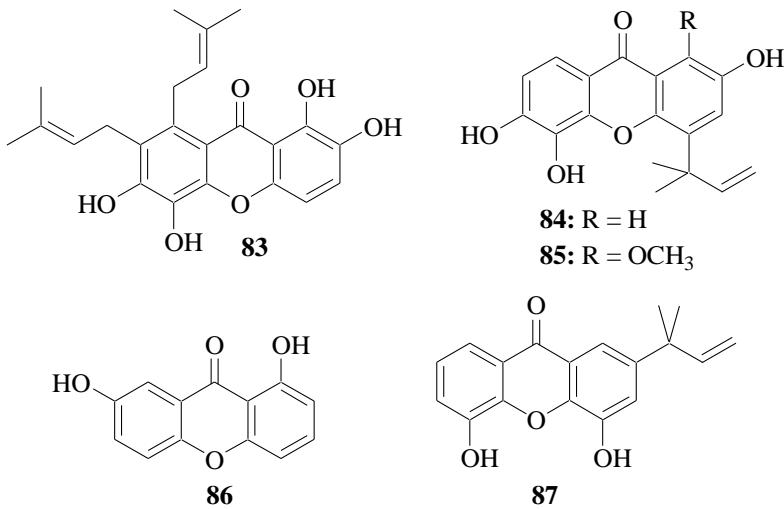
In their studies of screening of medicinal plants from Thailand with anti-malarial activity against the multi-drug resistant *P. falciparum* (K1 strain), Khaomek *et al.*, 2008, isolated three flavonoids citflavanone (**80**), lonchocarpol A (**81**) and 8-prenyldaidzein (**82**) from ethyl acetate extract of the stem bark of *Erythrina fusca* Lour with anti-

plasmodial activity <12.5 µg/ml. Diprenylated flavanone (**82**) (IC_{50} 3.9 µM) displayed the most potent activity among these compounds.



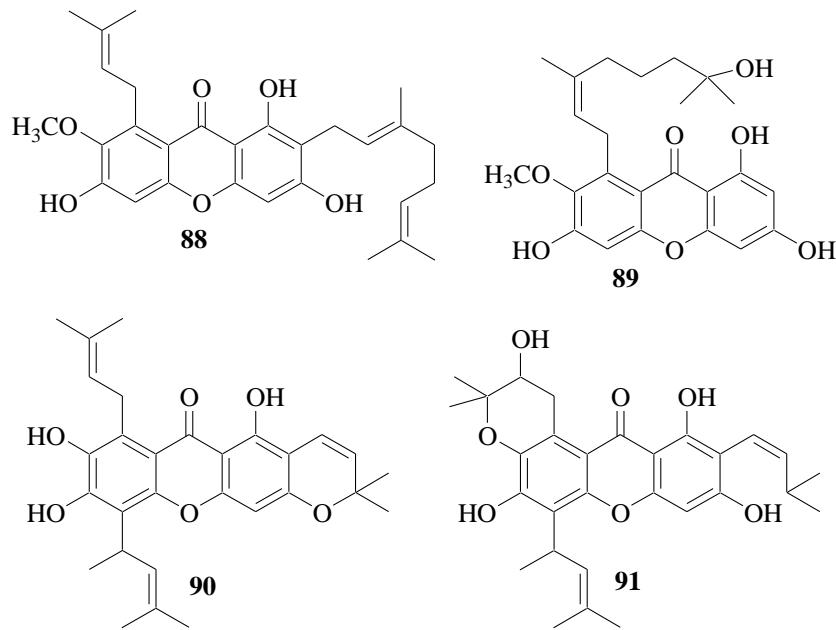
2.1.5 Xanthones

The ethanol extract of the bark of *Garcinia dulcis* yielded five xanthones. Garciniaxanthone (**83**), symphoxanthone (**84**), 1-*O*-methylsymphoxanthone (**85**), 1,7-dihydroxyxanthone (**86**) and 12 β -hydroxy-D-garcigerrin-A (**87**). They exhibited anti-malarial activity (IC_{50} 0.96, 3.75, 3.71, 3.88 and 2.08 µg/ml, respectively) against K1 strain. Garciniaxanthone (**83**) showed the highest activity against *P. falciparum* of IC_{50} 0.96 µg/ml (Likhithwitayawuid *et al.*, 1998).



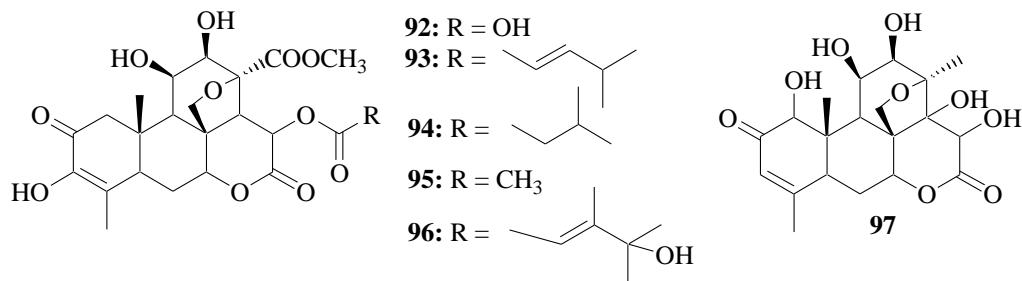
From the stem bark of *Pentadesma butyracea* (Clusiaceae) four new xanthones named butyroxanthones A-D (**88-91**) were isolated and assayed *in vitro* for anti-plasmodial activity against the *P. falciparum* chloroquine resistant FcB1 strain. All the xanthones

isolated exhibited good anti-plasmodial activity with IC₅₀ values ranging from 4.4 to 8.0 μM (Zelefack *et al.*, 2009).

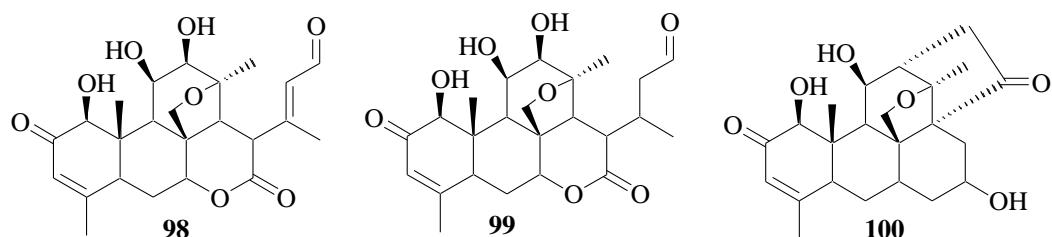


2.1.6 Quassinooids

They are heavily oxygenated lactones with majority having C₂₀ basic skeleton. However, C₁₈, C₁₉ and C₂₅ quassinooids are also known. Plant derived anti-malarial quassinooids have been reported. Brusatol (**92**), bruceitine (**93**) bruceine A (**94**), bruceine B (**95**), bruceine C (**96**) and bruceine D (**97**) have been isolated from *Brucea javanica* with anti-plasmodial activity (ED₅₀ 0.003, 0.0008, 0.11, 0.11, 0.005 and 0.015 $\mu\text{g/ml}$, respectively) against K1 strain (Anderson *et al.*, 1991; Sharma & Agarwal, 1993).



Gutolactone (**98**), simalikactone (**99**) and cedronin (**100**) isolated from *Simaba guinesensis* exhibited anti-plasmodial activity (IC_{50} 4.0, 1.6 and 0.25 $\mu\text{g/ml}$, respectively) against W2 strain (Cabral *et al.*, 1993; Saxena *et al.*, 2003).



However, quissinoids are known to be cytotoxic (Anderson *et al.*, 1991).

Indigenous local communities in southern Nyanza have used several local plant species to treat malaria for centuries. The project undertook the investigation of *Cissampelos mucronata* and *Stephania abyssinica* for *in vitro* anti-plasmodial activity, toxicity, isolation and chemical identification of the bio-active constituents. Both plants belong to the family Menispermaceae.

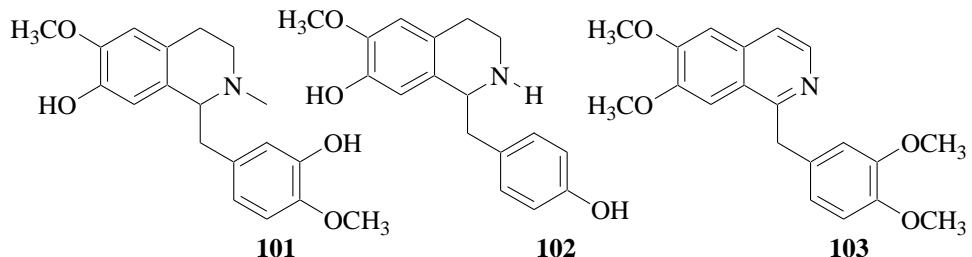
2.2 Menispermaceae

The family Menispermaceae consists of 70 genera and 450 species (Barbosa-Filho *et al.*, 2000). They are usually twining vines and are dioecious (male and female flowers on separate plants). The flowers are trimerous with floral parts in whorls of three. Fruits are one-sided drupes with taxonomical characteristics related to embryo size, shape and endosperm characteristics (Beentje, 1994). The South American dart poison ‘curare’ is derived from some species in this family. Incidentally, there are some species, which have medicinal value (Kokwaro, 1993). Previous phytochemical studies have shown that the family Menispermaceae yielded different types of alkaloids based on the several basic skeleton structures. These include benzylisoquinoline (BIQ), bisbenzylisoquinoline (BBIQ), proaporphine, aporphine, tropoloisoquinoline and azafluoranthrene, morphinan, protoberberine, hasubanane, erythrine, stephaoxocane, phenanthrene, aristolochic acid-derived, isoxxoarporphine, pavine, benzazepine, corhisine, hirsutine,

acutumine, eribidine, isoquinoline and phenethylcinnamide alkaloids (Barbosa-Filho *et al.*, 2000).

2.2.1 Benzylisoquinoline (BIQ) alkaloids

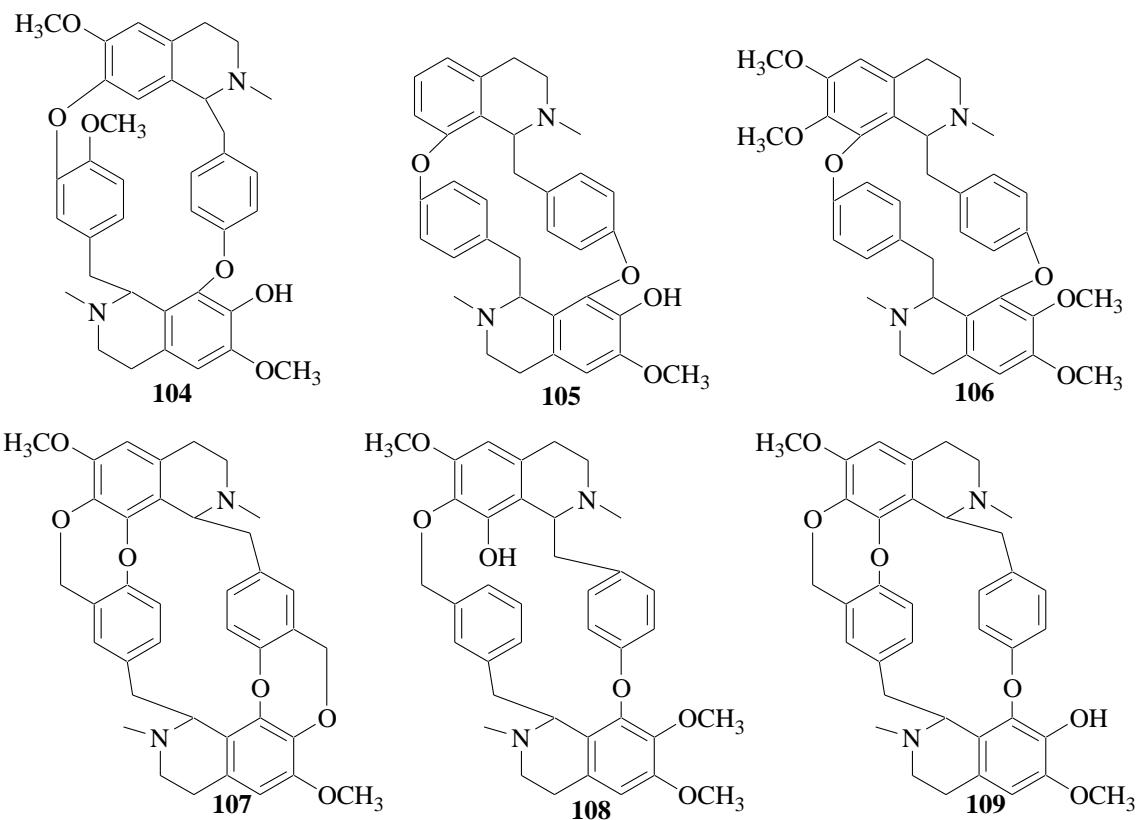
Alkaloids of this subtype represent about 4% of the alkaloids so far isolated from the family Menispermaceae. Benzylisoquinoline alkaloids have been isolated from the genera *Cissampelos* and *Stephania*. Reticuline (**101**) and isococlaurine (**102**) have been reported in *Cissampelos mucronata* and *Cissampelos hirta*. Papaverine (**103**) has been identified in *Stephania gracilenta* (Khosa *et al.*, 1987).



Papaverine is the most important of BIQ group from pharmacological point of view. It inhibits aldol reductase, glucose response in chemosensory cells and phosphodiesterase (Wink, 1993). *In vivo*, it increases coronary artery flow and causes dilation. Papaverine has an LD₅₀ of 27.5 mg/kg *in vivo* in mouse (Wink, 1993).

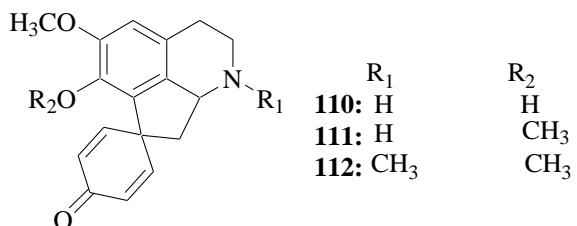
2.2.2 Bisbenzylisoquinoline (BBIQ) alkaloids

This type of alkaloids represents 40% of alkaloids so far isolated from the family (Barbosa-Filho *et al.*, 2000). They have been isolated from the genera *Cissampelos*, *Abuta*, *Albertisia*, *Artizoma*, *Menispemum* and *Stephania*. 12-O-methylcurine (**104**) has been isolated from *Cissampelos hirta*. Isochondrodendrine (**105**) and cyleanine (**106**) have been isolated from *Cissampelos mucronata*. Cissacapine (**107**), cyleaneonine (**108**) and insulanoline (**109**) have been reported from *Artizoma miersiana* (Kashiwaba *et al.*, 1997). Isochondrodendrine isolated from *Epinetum villosum* exhibited strong anti-plasmodial activity (IC₅₀ 0.10 µg/ml) (Borris & Schaeffer, 1992; Phillipson *et al.*, 1993; Otshundi *et al.*, 2005).

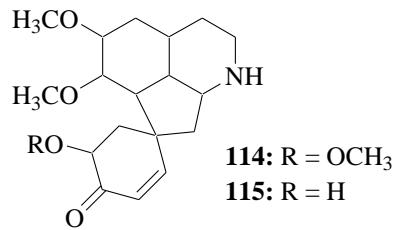
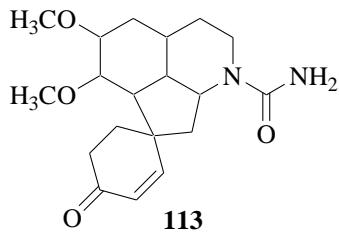


2.2.3 Proaporphine alkaloids

Proaporphine alkaloids represent 2% of the total alkaloids so far isolated from Menispermaceae, predominantly in the genera *Stephania*, *Caryomene*, *Cocculus* and *Legnephora* (Barbosa-Filho *et al.*, 2000). These include crotsparine (**110**), glaziovine (**111**) and pronuciferine (**112**).

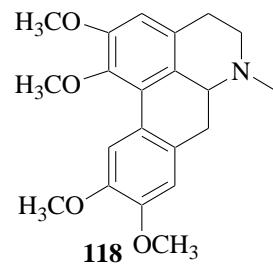
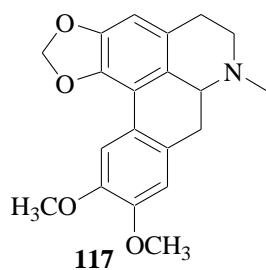
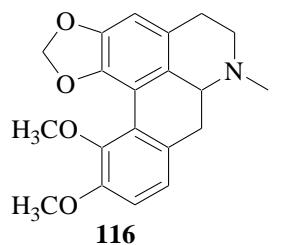


Three proaporphine, (+)-*N*-carboxamodostephavine (**113**), (-)*O*-methylstephevinosine (**114**) and (-) stephevinosine (**115**), alkaloids were isolated from the rhizomes of *Stephania venosa* (Bamrung, 1992).

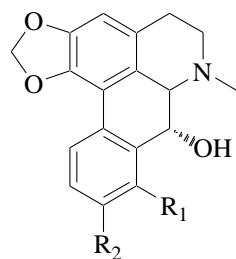


2.2.4 Aporphine alkaloids

It is the second most abundant class (20%) of alkaloids so far isolated from the family Menispermaceae. The genus *Stephania* is the richest in this type of alkaloids followed by *Coccolus* (Barbosa-Filho *et al.*, 2000). Bulbocapnine (**116**), dicentrine (**117**) and lauroschotzine (**118**) have been reported from *Stephania dinklagei*, *Cissampelos pariera* L and *Antizoma miersiana* (Ahmad *et al.*, 1992; Camacho *et al.*, 2000; Barbosa-Filho *et al.*, 2000). Dicentrine (**117**) has also been isolated from *Cissampelos mucronata* (Brown *et al.*, 1962)



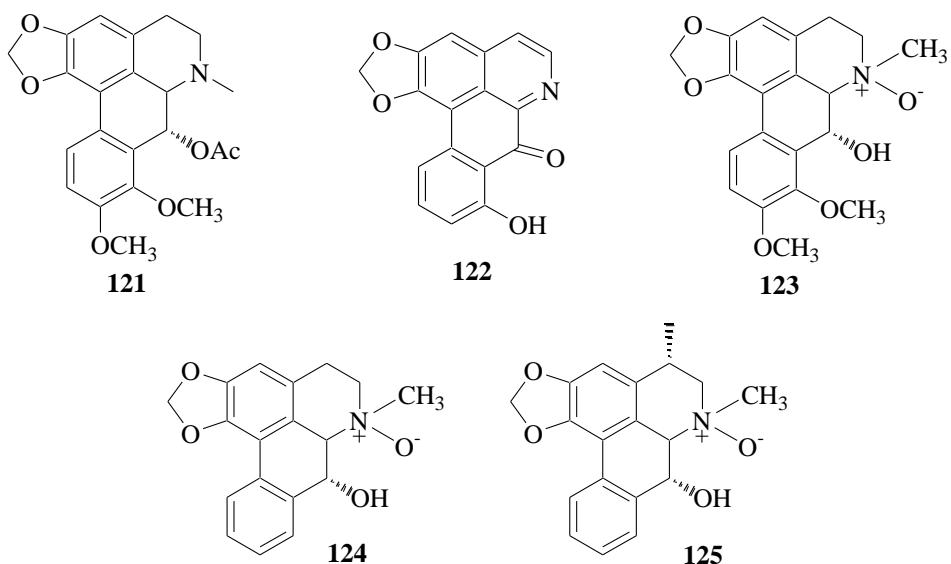
From the dried tuberous root powder of *Stephania venosa* two new 7-hydroxylated aporphine alkaloids have been isolated ayuthianine (**119**) and sukhodianine (**120**) (Bamrung, 1992).



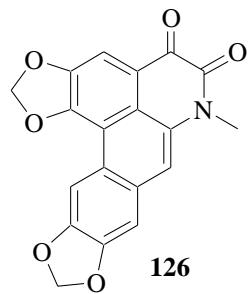
119: R₁ = OCH₃ R₂ = H

120: R₁ = OCH₃ R₂ = OCH₃

From the leaves of *Stephania venosa* (-)-*O*-acetylsukhodianine (**121**) and oxostephanosine (**122**) have been isolated. Sukhodianine- β -*N*-oxide (**123**), ushinsunine- β -*N*-oxide (**124**) and (-)-stephadiolamine- β -*N*-oxide (**125**) have also bee isolated from this plant (Bamrung, 1992).

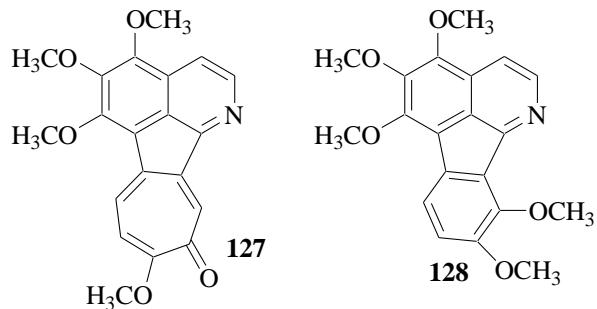


Stephadionine (**126**) a 4,5-dioxoaporphine have been isolated from arial parts of *Stephania tetrandra* (Duan *et al.*, 1992)



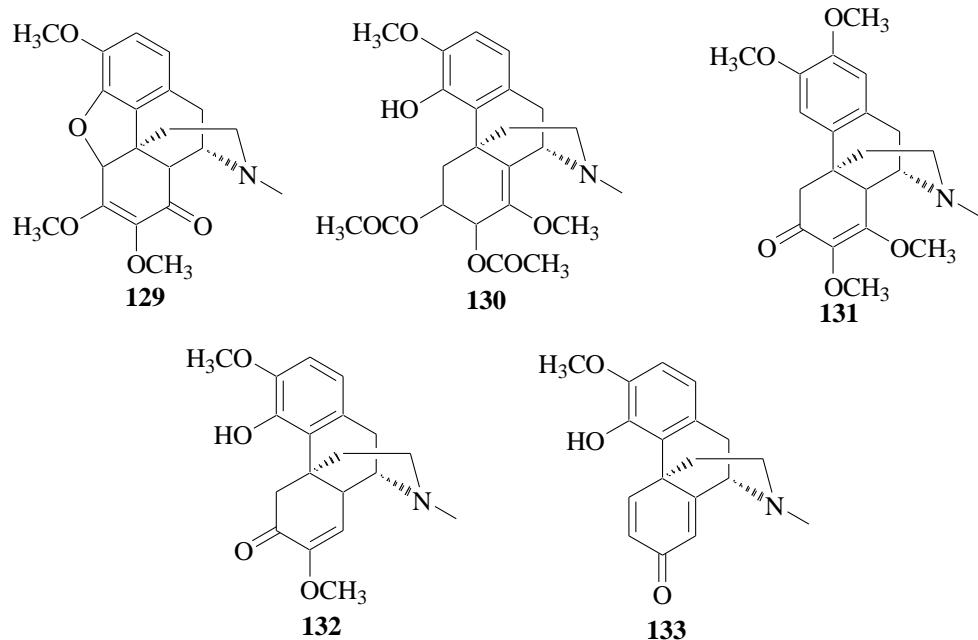
2.2.5 Tropoloisoquinoline and azafluoranthrene alkaloids

This type of alkaloid has been isolated in the genera *Abuta* and *Cissampelos*. Imerubrine (**127**) and imeluteine (**128**) have been reported (Barbosa-Filho *et al.*, 2000). Alkaloids of this structural type have inhibitory effects on *Candida albicans* in low concentration as low as 1.56 μ g/ml (Clarke & Hufford, 1992). Imerubrine exhibits anti-leukaemic activity (IC_{50} 0.33 μ g/ml) (Barbosa-Filho *et al.*, 2000).



2.2.6 Morphinane alkaloids

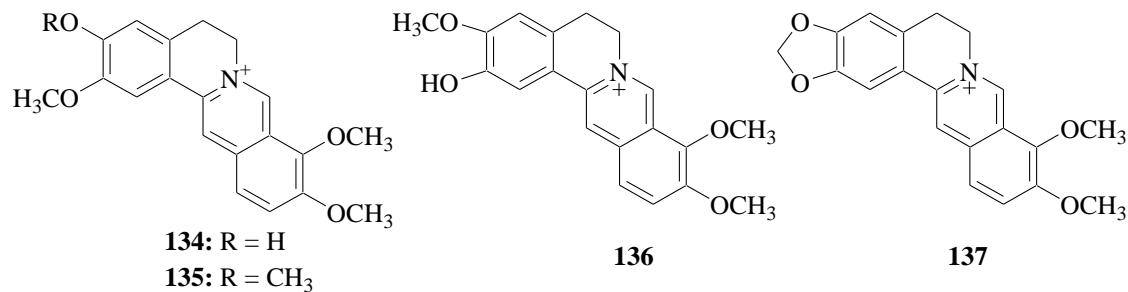
Morphinane type of alkaloids occurred in 4% of the samples tested in this family (Barbosa-Filho *et al.*, 2000). Five morphinane alkaloids cephazamine (**129**), cephakicine (**130**), tannagine (**131**), 14-episinomenine (**132**) and sinoacutine (**133**) were isolated from *Stephania cepharantha hayata* cultivated in Japan (Kashiwaba *et al.*, 1996).



2.2.7 Protoberberine alkaloids

This type of alkaloid has been isolated from *Tinispora* species (Barbosa-Filho *et al.*, 2000). Jatrorrhizine (**134**) and palmatine (**135**) have been reported from *Tinispora tenera* (Barbosa-Filho *et al.*, 2000). Columbamine (**136**) and berberine (**137**) have been isolated

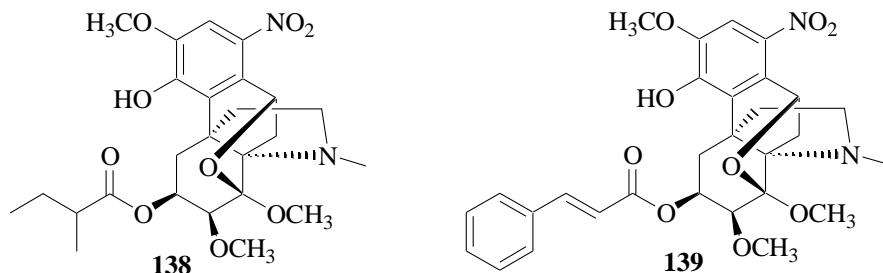
from several *Tinospora* species: *T. fragosa*, *T. crispa*, *T. cordifolia* and *T. capillipes* (Bisset & Nwaiwu, 1983; Samra *et al.*, 2009).



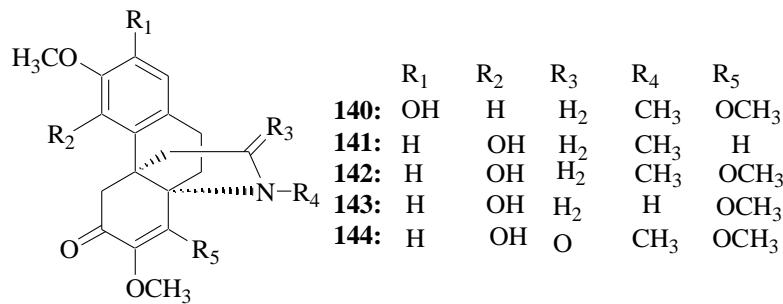
Jatrorrhizine (**134**) and palmatine (**135**) are active against *P. falciparum* (Wink, 1993). Jatrorrhizine (**134**) and palmatine (**135**) exhibited anti-plasmodial activity (IC_{50} 0.24 ± 0.002 , $0.08 \pm 0.01 \mu\text{g/ml}$ respectively) *in vitro* against *P. falciparum* K1 strain (Malebo, 2009). Berberine has anti-microbial, trypanocidal, anti-amebic, anti-fungal, anthelmic, leishmanicidal and tuberculostatic properties (Wink, 1993). This alkaloid inhibits the growth of *Trypanosoma cruzi* (Wink, 1993). It is also widely used in treatment of malaria, amoebiasis and leishmaniasis. The IC_{50} against *P. falciparum* ranges from 0.14 to 0.36 $\mu\text{g/ml}$. However it lacks *in vivo* activity in mice (Borris & Schaeffer, 1992; Philipson *et al.*, 1993). Columbamine iodide and other protoberberine alkaloids are active inhibitors of the HIV-1 reverse transcriptase system (Tan *et al.*, 1991).

2.2.8 Hasubanane alkaloids

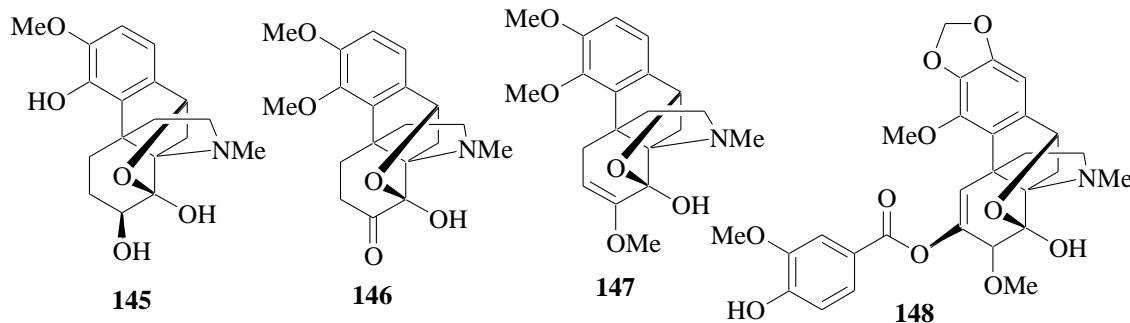
All alkaloids so far isolated from this class are from the genus *Stephania* (Barbosa-Filho *et al.*, 2000). Two nitro-substituted hasubanan-type alkaloid stepholine J (**138**) and K (**139**) were isolated from *Stephania longa* (Zhang *et al.*, 2006).



Five hasubanane alkaloids cephatonine (**140**), cephramine (**141**), aknadinine (**142**), acknadicine (**143**) and acknadilactam (**144**) have been isolated from *Stephania cepharantha hayata* cultivated in Japan (Kashiwaba *et al.*, 1996).

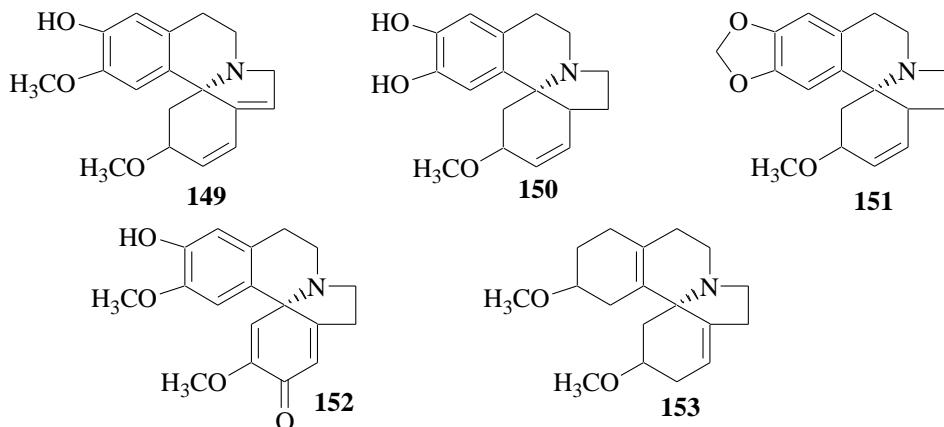


Four hasubanane alkaloids stephaboline (**145**), stephavamine (**146**), methaphamine (**147**) and stephabyssine (**148**) have been isolated from *Stephania abyssinica* (Kupchan *et al.*, 1973; Dagne *et al.*, 1993). Stephavamine (**146**) and stephabyssine (**148**) have also been isolated from *Stephania longa* while methaphamine was also isolated from *Stephania japonica* (Buckingham, 2001).



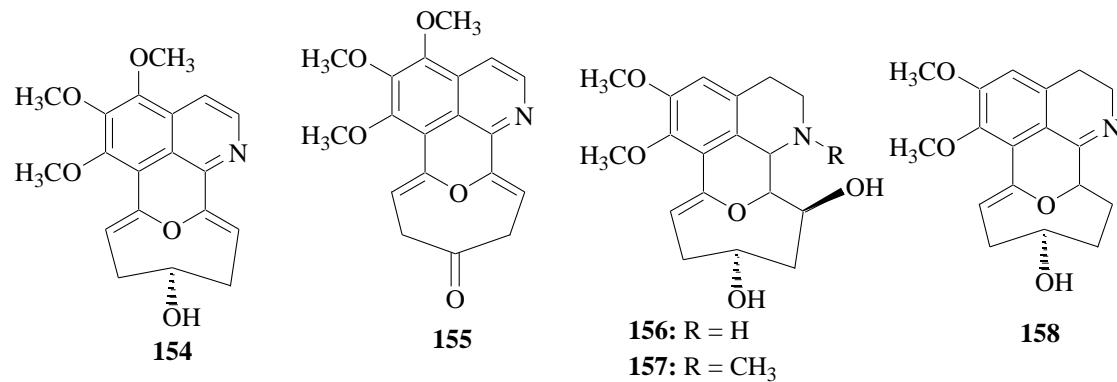
2.2.9 Erythrine alkaloids

This class of alkaloids has been isolated from the genera *Cocculus*, *Hyberbaena* and *Pachygona*. Erysodiene (**149**), erysopine (**150**), erythraline (**151**), erysodienone (**152**) and cocculinidine (**153**) have been reported (Fodor, 1980). Cocculinidine nitrates have been reported to show hypotensive action in dogs (Dyke & Quessy, 1981). Cocculinidine isolated from *Cocculus trilobus* has been used as an insecticides (Dyke & Quessy, 1981; Wink, 1993).



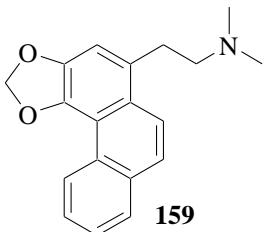
2.2.10 Stephaoxocane alkaloids

It is a relatively new class of alkaloids that has been isolated from this family. So far, it has been isolated from the genera *Cissampelos* and *Stephania* (Barbosa-Filho *et al.*, 2000). Eletefine (**154**) and oxoeletefine (**155**) has been isolated from *Cissampelos glaberrima* (Da-Cuhna *et al.*, 1998). Excentrine (**156**) and *N*-methylexcentrine (**157**) have been isolated from *Stephania excentrica* while stephaoxocanine (**158**) has been reported in *Stephania cepharantha* (Barbosa-Filho *et al.*, 2000).



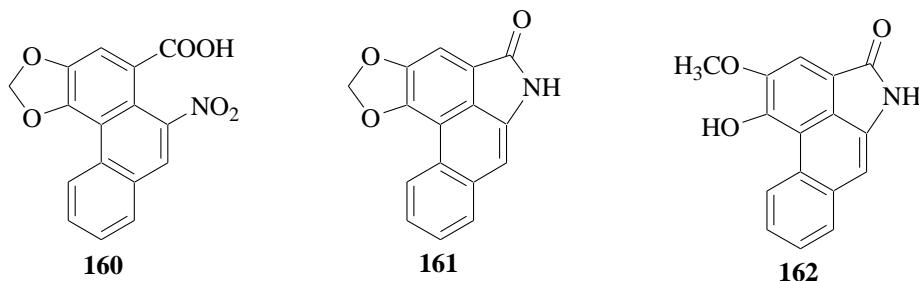
2.2.11 Phenathrene alkaloids

It is a very rare type of alkaloid. Of this type of alkaloid, only stephanthrine (**159**) has been isolated from the family Menispermaceae, being present in *Stephania tetrandra* and *Anisocycla cymosa* (Barbosa-Filho *et al.*, 2000).



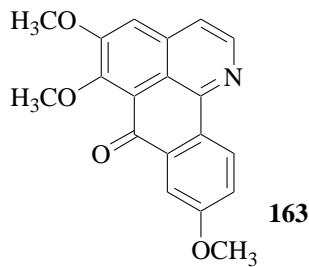
2.2.12 Aristolochic acid-derived alkaloids

In the family Menispermaceae only three alkaloids have been isolated. Aristolochic acid I (**160**) from *Cocculus tribolus* and the aristolactams cepharanone A (**161**) and cepharanone B (**162**) were isolated from *Stephania chepharantha* (Barbosa-Filho *et al.*, 2000).



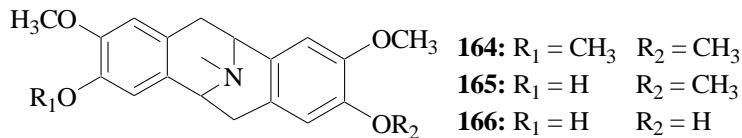
2.2.13 Isooxoaporphine alkaloids

Only one alkaloid has been reported so far from this family. Menisporphine (**163**) has been isolated from *Menispermum dauricum* (Barbosa-Filho *et al.*, 2000).



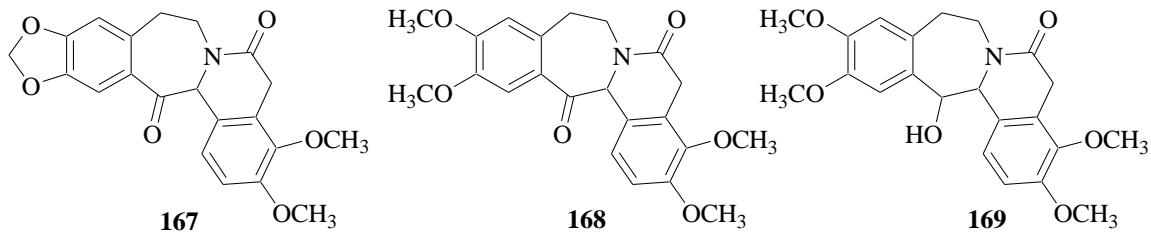
2.2.14 Pavine alkaloids

In the family Menispermaceae only three alkaloids have been isolated so far from the genera *Chasmanthera* and *Cyclea*. Bisnorargemonine (**164**) from *Chasmanthera dependens*, argemonine (**165**) and norargemonine (**166**) have been reported from *Cyclea atjhehensis* (Barbosa-Filho *et al.*, 2000; Gozler, 1987).



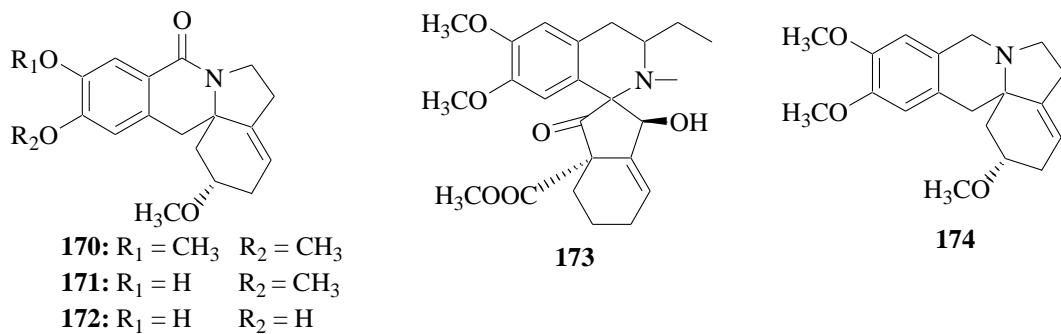
2.2.15 Benzazepine alkaloids

This type of alkaloids has a similar structure skeleton similar to the benzodiazepine drug. Only three compounds from this class have been isolated. Puntarenine (**167**) has been isolated from *Berberis empetrifolia*, while saulatine (**168**) and dihydrosalutine (**169**) were both isolated from *Abuta bullata* (Barbosa-Filho *et al.*, 2000).



2.2.16 Cohirsine alkaloids

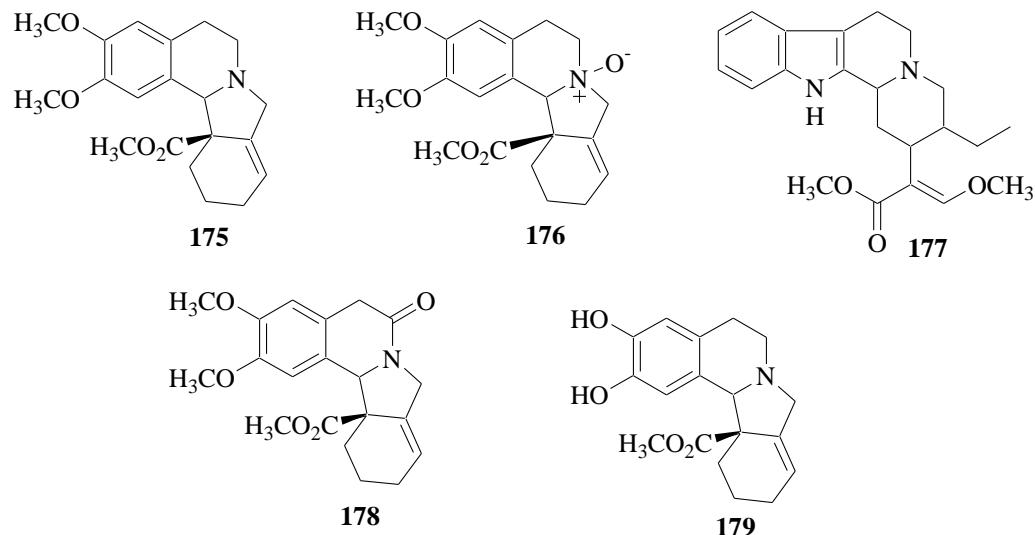
This type of alkaloid has only been isolated five times and all of them from *Cocculus hirsutus*. These alkaloids are corhisine (**170**), corhisinine (**171**), shaheenine (**172**), corhisitine (**173**) and corhistinine (**174**) (Barbosa-Filho *et al.*, 2000; Ahmad & Iqbal, 1992). Corhisine was first isolated in 1987 (Ahmad *et al.*, 1987a).



2.2.16 Hirstine alkaloids

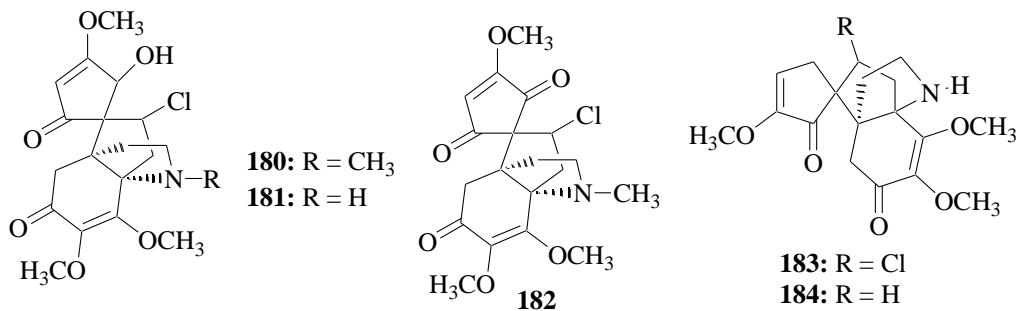
Only five alkaloids of this type have been described in the literature all from *Cocculus hirsutus*. These include jamtine (**175**), jamtine-*N*-oxide (**176**), hirsutine (**177**), jamtinine

(178) and haiderine (**179**) (Rasheed *et al.*, 1991; Barbosa-Filho *et al.*, 2000; Ahmad *et al.*, 1987b).



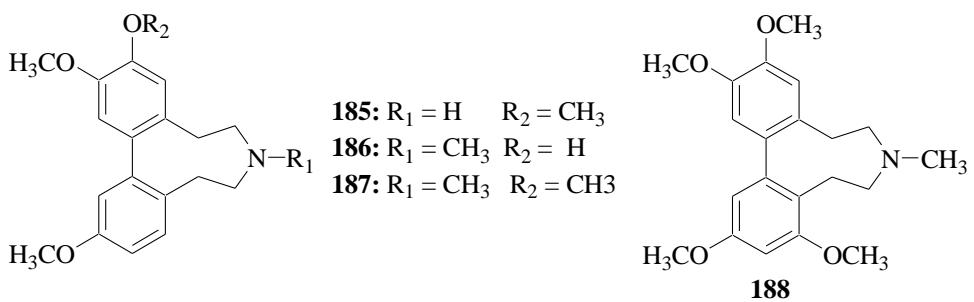
2.2.17 Acutumine alkaloids

The acutumine alkaloids are another class of alkaloids present only in the family Menispermaceae. All five alkaloids isolated were from the genera *Limacia*, *Menispermum* and *Sinomenium* (Barbosa-Filho *et al.*, 2000). They include acutumine (**180**), acutumidine (**181**), acutuminine (**182**), clolimalongine (**183**) and limalalongine (**184**).



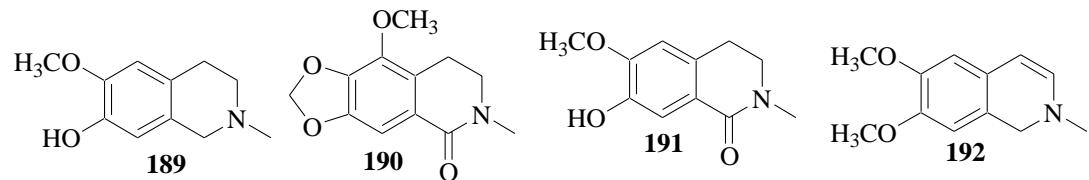
2.2.18 Eribidine alkaloids

They are rare class of secondary metabolites. From *Cocculus* were isolated laurifine (**185**), laurifinine (**186**), laurifonine (**187**) and from *Hyperbaena* and *Stephania*, protostephanine (**188**) has been reported (Barbosa-Filho *et al.*, 2000).



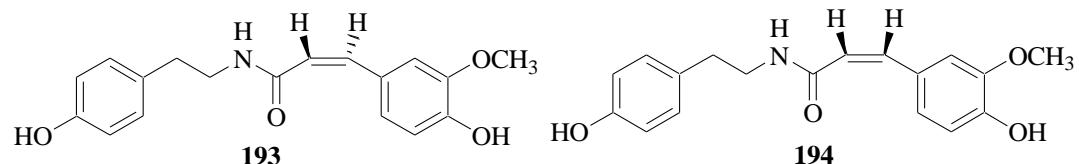
2.2.19 Isoquinoline alkaloids

In Menispermaceae they are very rare. Up to the moment, the only compounds isolated from plants in this family were corypalline (**189**) and thalflavine (**190**) from *menispermum dauricum*, thalifoline (**191**) from *Abuta pahni* and 6,7-dimethoxy-2-methylisoquinoline (**192**) (Barbosa-Filho *et al.*, 2000).



2.2.20 Phenethylcinnamide alkaloids

Only two different compounds have been isolated from this family. *N-trans*-feruloyltyramine (**193**) has been reported from the genera *Penianthus*, *Somomenium* and *Tinospora* and *N-cis*-feruloyltyramine (**194**) from the genus *Tinospora* (Barbosa-Filho *et al.*, 2000).



2.3 Biosynthetic pathway for alkaloids in Menispermaceae

(*S*)-Norcoclaurene, a benzyltetrahydroisoquinoline, was identified as a very important intermediate in the formation of alkaloids found in plants of the Menispermaceae family

(Barbosa-Filho *et al.*, 2000). The biosynthetic pathway for the formation of (*S*)-norcoclaurine is detailed in figure 3. Phosphoenolpyruvic acid (PEP) (**195**) reacts with erythrose-4-phosphate (**196**) to form shikimic acid (**197**) which reacts with phosphoenolpyruvic acid, followed by a series of steps to form phenylpyruvic acid (**198**) which after reductive amination is transformed into phenylalanine (**199**). The amino acid (**199**) undergoes transformation to L-tyroside (**200**) which may then undergo deamination to form phenylacetaldehyde (**201**) or decarboxylation to form phenylethylamine (**202**). These compounds may combine to form (*S*)-norcoclaurine (**203**) (Barbosa-Filho *et al.*, 2000)

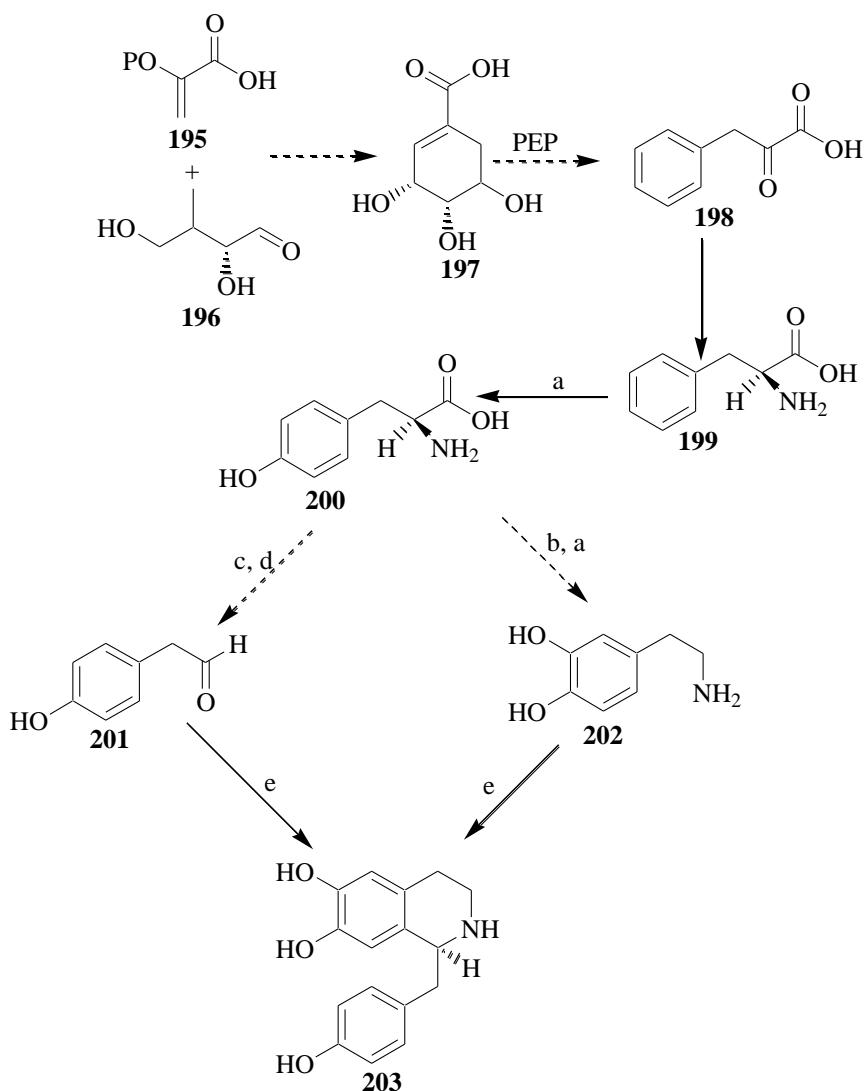


Figure 3: Biosynthetic pathway for *S*-norcoclaurine

a: phenolase, b: L-tyrosine decarboxylase, c: L-tyrosine transaminase, d: *p*-hydroxyphenylpyruvate decarboxylase, e: (*S*)-norcoclaurine synthase.

Most alkaloids in Menispermeceae are formed through this pathway. Other chemical modifications occur to give rise to the many compounds isolated from Menispermaceae. *Cissampelos mucronata* and *Stephania abyssinica* belong to the family Menispermaceae.

2.4 Plants under investigation

The investigation focused on *Cissampelos mucronata* and *Stephania abyssinica* that were identified during a survey of traditional anti-malarial plants from southern Nyanza (Muregi *et al.*, 2003; 2004).

2.4.1 *Stephania abyssinica* (Menispermaceae)

The East African species include *Stephania abyssinica* and *Stephania cepharantha* (Hiern) (Beentje, 1994). *Stephania abyssinica* is a liana wood at the base, 2-3 m high. Leaves are peltate, broadly ovate, round base and obtuse or acute base. Flowers are cream or reddish. Fruits are yellow or pinkish green round and 5-8 mm in diameter. The plant is commonly found in the forest margins, marine, bamboo and hagenia zone (Beentje, 1994). The leaves are crushed and applied to wounds resulting from tortoise bites. The roots are used as an aphrodisiac (Kokwaro, 1993). In recent studies, *S. abyssinica* water extract showed good anti-plasmodial activity (IC_{50} 22.90 $\mu\text{g/ml}$) against K39 a laboratory adopted chloroquine-sensitive local *P. falciparum* isolate (Muregi *et al.*, 2003; 2004).

Phytochemical investigations on *S. abyssinica* ethanol root extract revealed the presence of hasubanan alkaloids: stephaboline (**145**), stephavamine (**146**), methaphamine (**147**) and stephabbyssine (**148**) (Kupchan *et al.*, 1973; Dagne *et al.*, 1993).



Plate 2: Photograph of *S. abyssinica*

2.4.2 *Cissampelos mucronata* (Menispermaceae)

The plant (plate 3) is traditionally known as *Esibasu* (Lunyore), *Higerekya Mpiti* (Lusoga), *Kishiki cha Buga* (Swahili), *Musa* (Padhola) and *Olandra* (Luo). Traditionally the roots are used to relieve swollen testicles in newly born male children (Kokwaro, 1976). The leaves are pounded, added to hot water and the juice is given to the sick child to drink twice a day. Roots are also used for relieving abdominal pains and as an antidote for snake bite. The pounded leaves are mixed with pounded stem-bark of *Vernonia amygdalina*, steeped in cold water and the extract given to the cows after birth to facilitate the expulsion of placenta (Kokwaro, 1976).

In Nigeria, fresh aerial parts are used for amenorrhoea in adults (Elujoba *et al.*, 1995) and dried roots as uterine relaxant (Nwafor *et al.*, 2002). Dried leaves are used as an anti-spasmodic taken as an infusion. In Tanzania, roots are used to treat indigestion whereas in South Africa the dried root infusion is used for schistosomiasis (Sparg *et al.*, 2000). The water extract has spasmolytic activity in Guinea pig (Offiah *et al.*, 1996).



Plate 3: Photograph of *Cissampelos mucronata*

Alkaloids like isochondodendrine (**104**) and dicentrine (**118**) were isolated from this plant (Brown & Kupchan 1962; Lengo *et al.*, 2000).

CHAPTER 3

BIOASSAYS RESULTS AND DISCUSSIONS

3.1 Plant extracts

The plants were extracted sequentially using hexane, DCM, ethyl acetate (EtOAc) and methanol (MeOH). The yield of the plant extracts are summarized in table 1.

Table 1: Yield of solvent extracts of *Cissampelos mucronata* and *Stephania abyssinica*

Plant species	Amount (kg)	Crude extracts	Amount (g)	% yield
<i>C. mucronata</i> (R)	1.57	Hexane	21.8	1.4
		DCM	7.8	0.5
		EtOAc	3.1	0.2
		MeOH	15.1	1.0
<i>S. abyssinica</i> (L)	0.9	Hexane	9.3	1.0
		DCM	8.1	0.9
		EtOAc	14.8	1.6
		MeOH	29.6	3.4

R- roots, L- leaves, DCM – dichloromethane, EtOAc- ethyl acetate, MeOH- methanol

The yields for *S. abyssinica* were as follows: hexane>MeOH>DCM>EtOAc while *C. mucronata* were as follows MeOH>EtOAc>hexane>DCM.

3.2 Bioassay of plant extracts

3.2.1 Anti-plasmodial activity

The hexane, DCM, ethyl acetate and methanol extracts of the two plants were screened for *in vitro* plasmodial activity using D6 strain of *P. falciparum* (CQ-susceptible) according to Trager & Jensen, (1976). The mean concentrations of the extract that inhibit 50% of *P. falciparum* (IC_{50}) are summarized in table 2 for all the extracts.

Table 2: *In vitro* anti-plasmodial activity (IC_{50}) of *Cissampelos mucronata* and *Stephania abyssinica* extracts against *Plasmodium falciparum* D6 strain

Plant species	Crude extracts	$IC_{50}\pm SD$ ($\mu\text{g/ml}$)
<i>C. mucronata</i> (R)	Hexane	8.73 ± 1.81
	DCM	10.09 ± 1.56
	EtOAc	<3.91
	MeOH	19.28 ± 2.38
<i>S. abyssinica</i> (L)	DCM	<3.91
	EtOAc	5.09 ± 0.041
	MeOH	9.61 ± 0.011

R- roots, L- leaves, CH_2Cl_2 – dichloromethane, EtOAc- ethyl acetate, MeOH- methanol, IC_{50} CQ 1.16 ± 0.00 ng/ml against *P. falciparum* (D6).

Hexane and dichloromethane extracts of *C. mucronata* showed moderate anti-plasmodial activity of ($IC_{50} 8.73 \pm 1.81$ and $10.09 \pm 1.56 \mu\text{g/ml}$ respectively) against *P. falciparum* D6 isolate. Ethyl acetate extract showed strong anti-plasmodial activity ($IC_{50} < 3.91 \mu\text{g/ml}$) while methanol extract showed mild activity ($IC_{50} 19.78 \pm 2.38 \mu\text{g/ml}$) against *P. falciparum* D6 strain. The order of the decreasing anti-plasmodial activity of *C. mucronata* against chloroquine-sensitive *P. falciparum* D6 isolate was as follows: ethyl acetate < hexane < dichloromethane < methanol.

Dichloromethane extract of *S. abyssinica* showed strong anti-plasmodial activity ($IC_{50} < 3.91 \mu\text{g/ml}$) against CQ-sensitive *P. falciparum* D6 isolate. Ethyl acetate and methanol extracts exhibited moderate anti-plasmodial activity ($IC_{50} 5.09 \pm 0.041$ and $9.61 \pm 0.11 \mu\text{g/ml}$, respectively) against CQ-sensitive *P. falciparum* D6 strain. The order of decreasing anti-plasmodial activity was as follows: dichloromethane < ethyl acetate < methanol. The anti-plasmodial activity for hexane extract for *S. abyssinica* was not done because earlier study done by Muregi *et al.* (2003) showed that it was inactive ($IC_{50} > 100 \mu\text{g/ml}$) against chloroquine sensitive K39 strain.

3.2.2 Cytotoxicity

Cytotoxic activity of extracts against vero cells was undertaken according to Mosmann (1983). The results are summarized in table 3.

Table 3: *In vitro* cytotoxicity of *Cissampelos mucronata* and *Stephania abyssinica* extracts against vero cells (199)

Plant species	Extract	$CC_{50} (\mu\text{g/ml})$
<i>C. mucronata</i>	Hexane	90.34
	DCM	100.00
	EtOAc	10.34
	MeOH	66.33
<i>S. abyssinica</i>	Hexane	82.12
	DCM	100.00
	EtOAc	100.00
	MeOH	100.00

Chloroquine (CQ) $CC_{50} 77.05$ and podophylotoxin (PPT) $CC_{50} 67.35$, were used as the control standards for the experiments

The definition of cytotoxicity was as follows: $CC_{50} < 10 \mu\text{g/ml}$, high toxicity; $CC_{50} 11-50 \mu\text{g/ml}$, moderate toxicity; $CC_{50} 51-100 \mu\text{g/ml}$, mild toxicity; and $CC_{50} > 100 \mu\text{g/ml}$ not toxic. None of the extract tested was highly toxic though ethyl acetate of *C. mucronata* was moderately toxic. Methanol and hexane extracts of *C. mucronata* were mildly toxic while dichloromethane extract was not toxic. Only hexane extract of *S. abyssinica* was mildly toxic while dichloromethane, ethyl acetate and methanol extracts were not toxic.

The cytotoxicity of the extracts was compared to the standard toxin, podophyllotoxin (PPT) and the standard drug, chloroquine (CQ). The results of the comparison of toxicity of extracts, CQ and podophyllotoxin are presented on table 4.

Table 4: Comparison of cytotoxicity (CC_{50}) of extracts of *Cissampelos mucronata* and *Stephania abyssinica* with podophyllotoxin (PPT) and chloroquine (CQ)

Plant species	Extract	$CC_{50}/CC_{50}\text{PPT}$	$CC_{50}/CC_{50}\text{CQ}$
<i>C. mucronata</i> (R)	Hexane	1.30	1.20
	DCM	1.48	1.30
	EtOAc	0.15	0.13
	MeOH	0.98	0.86
<i>S. abyssinica</i> (L)	Hexane	1.23	1.07
	DCM	1.48	1.30
	EtOAc	1.48	1.30
	MeOH	1.48	1.30

Apart from the ethyl acetate extract of *C. mucronata*, the cytotoxicity of the rest of extracts were comparable to podophyllotoxin and chloroquine.

3.2.3 Selectivity indices

The selectivity index is defined as the ratio of the CC_{50} value for the vero cells (type 199) to the IC_{50} for the *P. falciparum* D6 strain (Chung & Hyung-In, 2009). Selectivity indices (SI) were calculated using the formula:

$$SI = CC_{50}/IC_{50}$$

They are summarized in table 5.

Table 5: Selectivity indices of *Cissampelos mucronata* and *Stephania abyssinica*

Plant species & extract	CC ₅₀ (µg/ml)	IC ₅₀ ±SD (µg/ml)	SI
<i>C. mucronata</i> (R)			
Hexane	90.34	8.73±1.81	10.35
DCM	100.00	10.09±1.56	9.91
EtOAc	10.34	<3.91	>2.61
MeOH	66.33	19.28±2.38	3.35
<i>S. abyssinica</i> (L)			
Hexane	82.12	ND	ND
DCM	100.00	<3.91	>25.58
EtOAc	100.00	5.09±0.041	19.65
MeOH	100.00	9.61±0.011	10.41

R- roots, L- leaves, DCM – dichloromethane, EtOAc- ethyl acetate, MeOH- methanol, ND- not determined

The extracts with moderate anti-plasmodial activity exhibited mild selectivity (SI 2.26-10.35 and 10.41-25.58 for *C. mucronata* and *S. abyssinica*, respectively). DCM extract of *S. abyssinica* (IC₅₀ <3.91 µg/ml, SI >25.58) was the most selective followed by ethyl acetate extract (IC₅₀ 5.09±0.041 µg/ml, SI 19.65). The methanol extract of *S. abyssinica* (IC₅₀ 9.61±0.011 µg/ml, SI 10.41) was mildly selective. *Cissampelos mucronata* ethyl acetate extract (IC₅₀ <3.91, SI >2.61) was the least selective followed by methanol extract (IC₅₀ 19.28±2.38 µg/ml, SI 3.35), dichloromethane extract (IC₅₀ 10.09±1.56 µg/ml, SI 9.91) and hexane extract (IC₅₀ 8.73±1.81 µg/ml, SI 10.35).

3.3 Anti-plasmodial activity of the compounds isolated from *Cissampelos mucronata* and *Stephania abyssinica* extracts

The extracts were fractionated and purified by column chromatography (CC) and TLC. The anti-plasmodial activity for isolated compounds was done using CQ susceptible (D6) and CQ resistant (W2) *P. falciparum* strains according to Trager & Jensen (1976). The results are summarized in table 6.

Table 6: *In vitro* anti- plasmodial activity IC₅₀ for isolated compounds

Compound	<i>P. falciparum</i> (D6) IC ₅₀ ±SD(µg/ml)	<i>P. falciparum</i> (W2) IC ₅₀ ±SD(µg/ml)
Stigmasterol (204)	>5.0	>5.0
(-)Curine (205)	0.24±0.03	0.22±0.06
(+)-Nonaconsan-10-ol (206)	13.79±1.02	4.35±2.45
(-)5-Oxoaknadinine (207)	10.25±1.84	3.45±2.22
OR/SA/D1	0.34±0.03	0.55±0.11
(-)Pseudocurine (208)	0.29±0.00	0.31±0.01
(-)Isocurine (209)	0.75±0.11	1.65±0.03

IC₅₀ CQ 1.16±0.00 ng/ml against *P. falciparum* (D6) and 1.69±0.14 ng/ml *P. falciparum* (W2) and IC₅₀ Art 8.34±0.14 ng/ml against *P. falciparum* (D6) and 56.87±1.27 ng/ml *P. falciparum* (W2).

Apart from stigmasterol (**204**), (+)-nonaconsan-10-ol (**206**) and (-)-5-oxoaknadinine (**207**) which were inactive, (-)-curine (**205**), (-)-pseudocurine (**208**), (-)-isocurine (**209**) and OR/SA/D1 showed strong anti-plasmodial activity against *P. falciparum* (D6 and W2) strains *in vitro*. For D6 strain the IC₅₀ range for active compounds was 0.24±0.03-13.79±1.02 µg/ml while for W2 the IC₅₀ range was 0.22±0.06-4.35±2.45 µg/ml. (-)-Curine (**205**) exhibited the strongest anti-plasmodial activity (0.24±0.03 µg/ml and 0.22±0.06 µg/ml) against *P. falciparum* D6 and W2 strains, respectively.

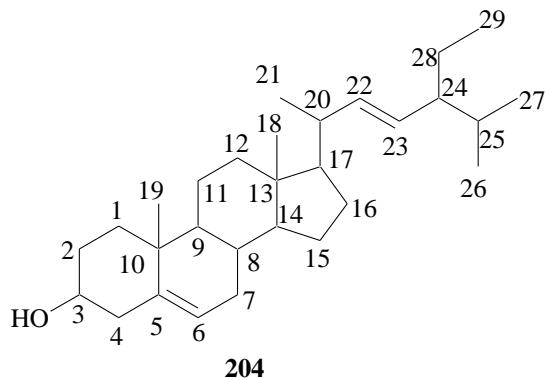
CHAPTER 4

STRUCTURAL ELUCIDATION AND DISCUSSION

4.1 Structure elucidation of compounds isolated from *Cissampelos mucronata*

4.1.1 (-)-Stigmasterol (204) (OR/CM/D1)

This compound was isolated as white crystals (24 mg, $[\alpha]^{25}_D -49^\circ$ (1.5, CHCl_3), R_f 0.5, 15% EtOAc/ hexane) and melting point 165-167 °C (lit. 170 °C, Budavari, 1996). The IR spectrum in appendix 1d showed characteristic bands at 3422 cm^{-1} (OH) and 1653 cm^{-1} (C=C bonds).



The ^1H NMR data in appendix 1a is presented in table 7.

Table 7: ^1H NMR (200 MHz, CDCl_3) data for (-)-stigmasterol (**204**) (OR/CM/D1)

Position	δ_{obs}	δ_{lit}	Multiplicity	Integral
3	3.51	3.52	m	1H
6	5.35	5.36	br d	1H
18	0.69	0.70	s	3H
19	1.00	1.01	s	3H
21	0.92	0.92	d	3H
22	5.14		m	2H
23	5.02		m	
26	0.85	0.84	d	3H
27	0.81	0.81	d	3H
29	0.67		s	3H
Others	0.8-2.28		m	26H

Literature data (δ_{lit}) from Morales *et al.*, 2003

Methyl signals were observed at δ 0.67 (s, 3H, H-29), 0.69 (s, 3H, H-18) and 0.81 (d, 3H, 27), 0.85 (d, 3H, H-26), 0.92 (d, 3H, H-21) and 1.00 (s, 3H, H-19). The signal at δ 3.51 (m, 1H) was assigned to C-3 which suggested the presence of a α -proton typical of

hydroxylated sterols. The signal at δ 5.35 (br, d, 1H) was assigned to an olefinic proton. The multiplet at δ 5.02-5.14 (2H) suggested presence of two olefinic protons. The rest were a complex mass of multiplets spread between δ 0.80-2.27.

The ^{13}C NMR in appendix 1b and DEPT data in appendix 1c are summarized in table 8.

Table 8: ^{13}C NMR (200 MHz, CDCl_3) and DEPT data for (-)-stigmasterol (**204**) (OR/CM/D1)

Position	δ_{obs}	δ_{lit}	DEPT	Position	δ_{obs}	δ_{lit}	DEPT
1	37.5	37.3	CH_2	16	29.1	29.0	CH_2
2	31.9	31.7	CH_2	17	56.2	56.1	CH
3	72.0	71.8	CH	18	12.1	12.1	CH_3
4	42.4	42.4	CH_2	19	19.6	19.4	CH_3
5	140.9	140.8	C	20	40.7	40.5	CH
6	121.9	121.7	CH	21	21.3	21.1	CH_3
7	33.9	33.6	CH_2	22	138.5	138.4	CH
8	32.1	31.9	CH	23	129.5	129.3	CH
9	50.4	50.2	CH	24	51.5	51.3	CH
10	36.7	36.6	C	25	31.9	31.9	CH
11	21.4	21.1	CH_2	26	21.4	21.3	CH_3
12	39.9	39.7	CH_2	27	19.2	19.0	CH_3
13	42.5	42.4	C	28	25.6	25.4	CH_2
14	56.9	56.9	CH	29	12.3	12.3	CH_3
15	24.5	24.4	CH_2				

Literature data (δ_{lit}) from Morales *et al.*, 2003

^{13}C NMR revealed 29 signals of which four olefinic carbons at δ 121.9 (C-6), 129.5 (C-23), 138.5 (C-22) and 140.9 (C-5) suggested the presence of double bonds. Six (06) methyl groups appeared at δ 12.3 (C-29), 12.1 (C-18), 19.2 (C-27), 21.4 (C-26), and 21.3 (C-21) as revealed by DEPT analysis. Similarly, nine (9) methine carbon resonances were observed at δ 72.0, 33.9, 50.4, 56.9, 56.2, 40.7, 51.5 and 31.9. Signals at δ 140.9, 36.7 and 42.4 revealed quaternary carbons. The signal at δ 72.0 suggested oxygenation hence C-3.

The EIMS revealed molecular ion peak at m/z 412 [M^+] corresponding to the formula $\text{C}_{29}\text{H}_{48}\text{O}$. Other prominent peaks were observed at m/z 394 [$\text{M}-\text{H}_2\text{O}$], 327 [$\text{M}-\text{C}_5\text{H}_9\text{O}$] $^+$, 301 [$\text{M}-\text{C}_7\text{H}_{11}\text{O}$] $^+$, 273 [$\text{M}-\text{C}_{10}\text{H}_{19}\text{O}$] $^+$ and 255 [$\text{M}-\text{C}_{10}\text{H}_{22}\text{O}$] $^+$ which are consistent with (-)

β -stigmasterol (**204**) structure. The mass fragmentation pattern in appendix 1f is shown in the figure 4.

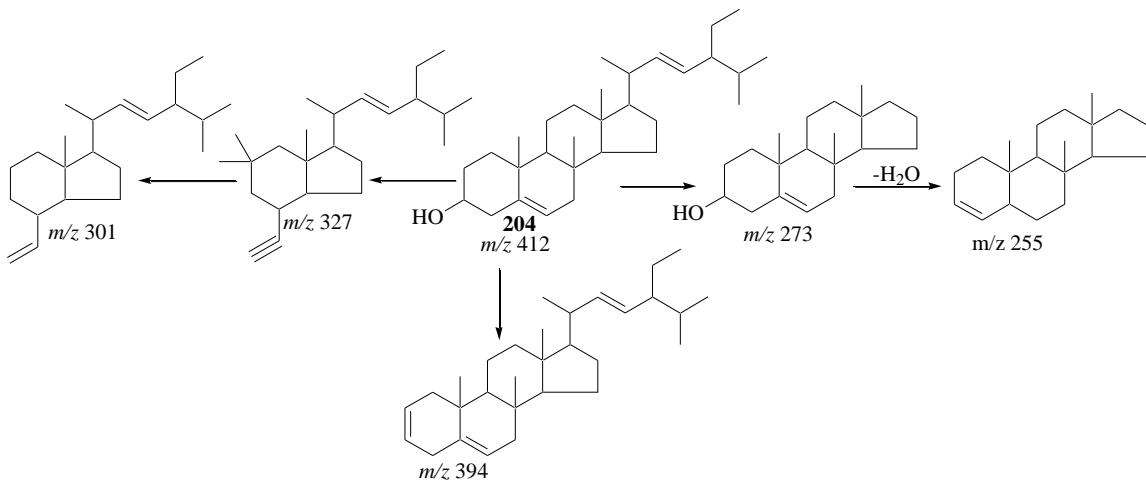
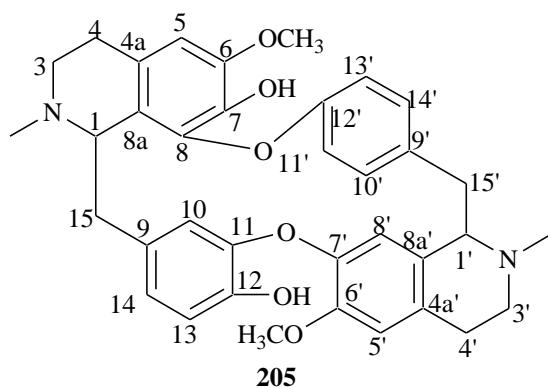


Figure 4: Mass fragmentation pattern for (-)-stigmasterol

All the spectroscopic data confirmed the structure of (-)-stigmasterol (**204**) and were consistent with literature values (Morale *et al.*, 2003; Forgo & Köver, 2004)

4.1.2 (-)-Curine (**205**) (OR/CM/D3)

This compound was isolated as white amorphous solid (14 mg, $[\alpha]^{25}_D -258^\circ$ (3.4, MeOH), R_f 0.72, 1:19 MeOH: DCM mp 215-217 °C (lit. 214-216 °C, Wang *et al.*, 2010). The IR spectrum showed the presence of a hydroxyl group at 3384 cm⁻¹ and aromatic C=Cstr at 1507cm⁻¹.



¹H NMR data is in appendix 2a summarized in table 9.

Table 9: ^1H NMR (400 MHz, CDCl_3) data for (-)-curine (**205**) (OR/CM/D3)

Position	δ_{obs}	δ_{lit}	J (Hz)	Multiplicity	Integral
1	3.55	3.52	8.0, 2.1	dd	1H
3 _{ax}	2.81	2.81	12, 4.0	dd	1H
3 _{eq}	3.32	3.31	12, 4.0	dd	1H
4 _{ax}	2.46	2.42	4.0	d	1H
4 _{eq}	2.97	2.97	4.0	d	1H
5	6.55	6.56		s	1H
10	6.64	6.63	2.4	d	1H
13	6.79	6.78	8.2	d	1H
14	6.93	6.92	8.2, 2.4	dd	1H
15 _{ax}	2.76	2.78		m	1H
15 _{eq}	2.50	2.48		m	1H
1'	3.46	3.47	8.0, 2.5	dd	1H
3' _{ax}	2.79	2.83		m	1H
3' _{eq}	3.28	3.28		m	1H
4' _{ax}	2.71	2.70		m	1H
4' _{eq}	2.91	2.90		m	1H
5'	6.67	6.66		s	1H
8'	5.96	5.92		s	1H
10'	6.44	6.47	8.2	bd	1H
11'	6.67	6.66	8.2	bd	1H
13'	6.67	6.66	8.1	bd	1H
14'	7.08	7.12	8.1	bd	1H
15' _{ax}	2.50	2.51	12.0	d	1H
15' _{eq}	3.13	3.10	12.0	d	1H
2-NMe	2.28	2.26		s	3H
2'-NMe	2.49	2.53		s	3H
6-OMe	3.87	3.86		s	3H
6'-OMe	3.89	3.88		s	3H

Literature values are derived from Lengo *et al.*, 2000 and Banyingela *et al.*, 1997.

The ^1H NMR spectra revealed 10 aromatic protons with signals at δ 6.67 (s, 1H, C-5'), 5.96 (s, 1H, C-8'), 6.44 (s, 1H, C-10'), 6.67 (m, 1H, C-11'), 6.67 (m, 1H, C-13'), 7.07 (d, 1H, C-14'), 6.53 (s, 1H, C-5), 6.64 (s, 1H, C-10), 6.79 (d, 1H, C-13), 6.94 (dd, 1H, C-14). Other peaks at δ 3.87 (s, 3H, 6-OMe), 3.89 (s, 3H, 6'-OMe) showed presence of two methoxy groups while signals at δ 2.28 (s, 3H, 2-NMe) and 2.49 (s, 3H, 2'-NMe) indicated presence of alkyl amine thus the two N-methyl groups.

The ^{13}C NMR data in appendix 2b is summarized in table 10.

Table 10: ^{13}C NMR (400 MHz, CDCl_3) and DEPT data for (-)-curine (**205**) (OR/CM/D3)

Position	δ_{obs}	δ_{lit}	DEPT	Position	δ_{obs}	δ_{lit}	DEPT
1	60.4	60.2	CH	1'	65.3	65.1	CH
3	43.9	43.7	CH_2	3'	45.5	45.2	CH_2
4	21.9	21.8	CH_2	4'	25.1	24.8	CH_2
4a	125.0	124.8	C	4a'	129.4	129.8	C
5	108.1	108.1	CH	5'	112.3	112.4	CH
6	146.7	146.7	C	6'	148.7	148.4	C
7	137.3	137.3	C	7'	143.3	143.5	C
8	138.7	138.4	C	8'	120.3	120.1	CH
8a	124.5	124.5	C	8a'	129.4	129.4	C
9	133.7	133.5	C	9'	132.3	132.1	C
10	120.7	120.9	CH_2	10'	132.1	131.7	C
11	144.3	144.2	CH_2	11'	113.4	113.1	CH
12	146.3	146.2	C	12'	155.4	155.3	C
13	115.4	115.4	CH_2	13'	115.2	115.4	CH
14	126.6	126.6	CH_2	14'	129.6	129.4	CH
15	39.7	39.7	CH_2	15'	39.9	39.8	CH_2
2-NMe	41.7	41.4	CH_3	2'-NMe	42.0	41.7	CH_3
6-OMe	56.0	56.0	CH_3	6'-OMe	56.1	56.0	CH_3

Literature values are derived from Lengo *et al.*, 2000 and Banyingela *et al.*, 1997.

^{13}C NMR revealed 36 signals. DEPT analysis indicated 6 methylenes at δ 43.9 (C-3), 21.9 (C-4), 39.7 (C-15), 45.5 (C-3'), 25.1 (C-4') and 39.9 (C-15'). Fourteen (14) signals at δ 124.5 (C-4a), 146.7 (C-6), 129.4 (C-4a'), 155.4 (C-12), 133.7 (C-9), 125.0 (C-8a), 148.7 (C-6') 144.3 (C-7'), 129.4 (C-8a'), 132.3 (C-9'), 143.2 (C-11), 146.3 (C-12), 138.7 (C-8) and 137.3 (C-7) were quaternary carbons. It also indicated presence of 12 methine groups at δ 60.4 (C-1), 108.1 (C-5), 120.3 (C-8'), 115.4 (C-13), 126.3 (C-14), 65.3 (C-1'), 120.7 (C-10), 132.1 (C-10'), 113.4 (C-11'), 115.2 (C-13') and 129.6 (C-14'). From the NMR analysis and comparison with published data the structure of curine (**205**) OR/CM/D3 was proposed.

The spectral data obtained was in agreement with those reported for curine (Koike *et al.*, 1981; Lengo *et al.*, 2000). The identity of the compound was further confirmed by comparison of the melting point (Wang *et al.*, 2010)..

EIMS for curine revealed the molecular ion peak at m/z 594 [M^+] corresponding to $C_{36}H_{38}N_2O_6$. The mass fragmentation pattern in appendix 2e is shown in the figure 4.

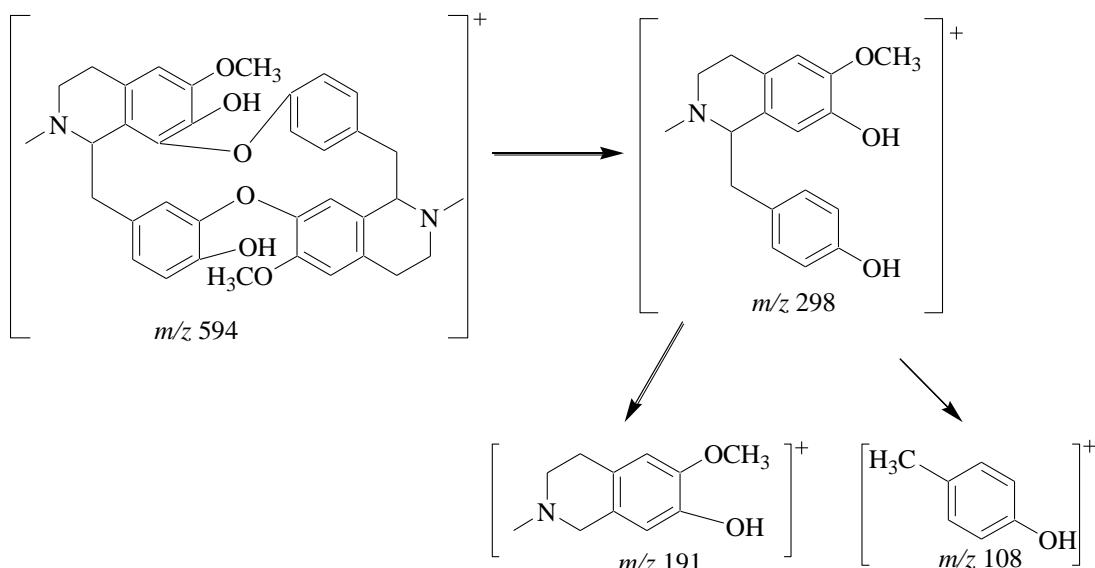


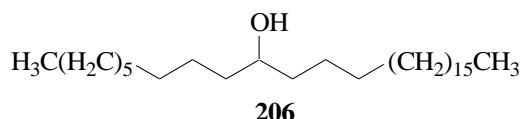
Figure 5: Mass fragmentation pattern for (-)-curine (**205**)

(-)Curine was previously isolated from *Isolona ghesquiereina* showed strong *in vitro* anti-plasmodial activity (IC_{50} 353 ± 17 ng/ml) against the chloroquine resistant *P. falciparum* strain FMC 29 (Lengo *et al.*, 2000).

4.2 Structure elucidation of compounds isolated from *Stephania abyssinica*

4.2.1 (+)-Nonacosan-10-ol (206) (OR/SA/H1)

It was isolated as white crystals (17.3 mg, $[\alpha]^{25}_D +87.5^\circ$ (1.6, $CHCl_3$), R_f 0.50, 1:1 hex: DCM, mp 79-81 °C) (lit. 81-81.5 °C, Guatam *et al.*, 2005; Aarnoud *et al.*, 2007). The structure was established by 1H NMR, ^{13}C NMR, DEPT NMR and MS data.



The 1H NMR data in appendix 3a is summarized in table 11.

Table 11: ^1H NMR (200 MHz, CDCl_3) data for (+)-nonacosan-10-ol (**206**)

Position	δ_{obs}	δ_{lit}	Multiplicity	Integral
1	0.89	0.88	m	3H
9	1.60	1.60	m	2H
10	3.56	3.58	m	2H
11	1.40	1.43	m	1H
29	0.89	0.88	m	3H
Others	1.24	1.26	m	48H

^1H NMR revealed signals at δ 0.89 (m, H-1), δ 1.60 (m, H-2), δ 3.56 (m, H-10), δ 1.40 (m, H-11), δ 0.89 (m, H-29) and δ 1.24 (m). H-10 was assigned to the signal at δ 3.56 due to being attached to an oxygenated centre. The number of protons resonating at δ 1.24 were determined using mass spectroscopy and found to be 48. The ^{13}C NMR data in appendix 2b and DEPT data in appendix 2c are summarized in table 12.

Table 12: ^{13}C and DEPT NMR (200 MHz, CDCl_3) data for (+)-nonacosan-10-ol (**206**)

Position	δ_{obs}	δ_{lit}	DEPT
1	14.1	14.5	CH_3
2	22.7	23.0	CH_2
3	29.3	29.7	CH_2
4	31.9	31.9	CH_2
8	25.7	26.0	CH_2
10	72.1	72.4	CH
11	37.9	37.9	CH_2
Others	29.7	29.9	CH_2

^{13}C NMR in appendix 3b revealed signals at δ 14.1 (C-1), δ 22.7 (C-2), δ 29.3 (C-3), δ 31.9 (C-4), δ 25.7 (C-8), δ 72.1 (C-10), δ 37.9 (C-11) and a number of methines at 29.7. DEPT analysis in appendix 3c indicated 2 methyls, 1 methine and 26 methylenes which were determined using the mass spectroscopy.

The IR spectrum in appendix 3d showed the presence of a hydroxyl group at 3337.6 cm^{-1} . Important clue for the structure was obtained from mass spectrum. EIMS in appendix 3f revealed molecular ion peak at m/z 424 [M^+] corresponding to the formula $\text{C}_{29}\text{H}_{60}\text{O}$ and consistent with nonacosan-10-ol (**206**). The peak at m/z 297 indicated loss of $\text{CH}_3(\text{CH}_2)_{18}\text{CH}(\text{OH})$ group while the one at m/z 157 indicated loss of $\text{CH}_3(\text{CH}_2)_8\text{CH}(\text{OH})$.

group. The mass fragmentation pattern was consistent with the fact that –OH group at 10th position. The structure was confirmed from the mass spectral fragmentation pattern (Figure 6).

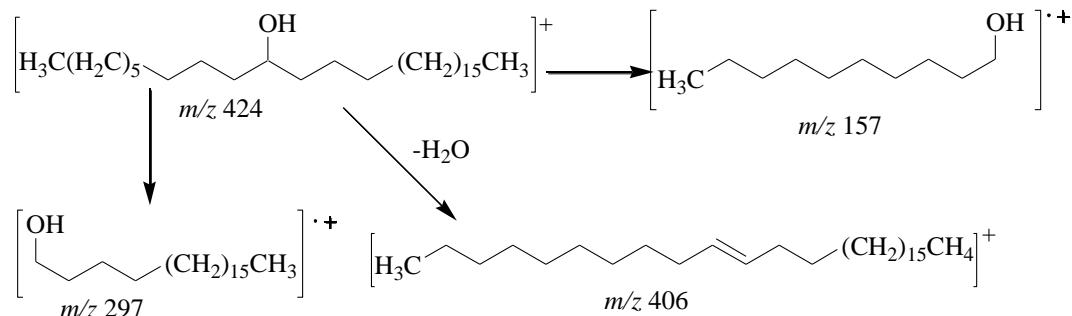
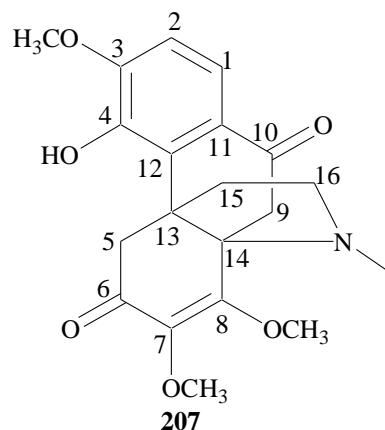


Figure 6: Mass fragmentation patterns for nonacosan-10-ol (206)

(+)-Nonacosan-10-ol (**206**) has been previously isolated from *Cocculus hirsutus*, *Araucacia araucana*, *Agathis robusta*, *Wollemia nobilis* and *Podocarpus neriifolius* (Ahmad *et al.*, 1987c; Guatam *et al.*, 2005; Dragota & Riederer, 2008). Pharmacological and toxicological activity of (+)-nonacosan-10-ol has not been reported.

4.2.2 (-)-5-Oxoaknadinine (207) HOR/SA/D4

This compound was isolated as light yellow crystals (23.8 mg, $[\alpha]^{25}_D -291^\circ$ (1.5, MeOH), R_f 0.6 1: 24 MeOH: DCM). It gave a positive chemical reaction for alkaloids with Dragendoffs reagent. UV (280 nm) spectra indicated the presence of α , β -unsaturated carbonyl moiety. The structure was partially determined on the basis of 1D (1H , ^{13}C and DEPT) and 2D (HMQC, HMBC and COSY) NMR experiments.



The 1H NMR in appendix 4a and COSY data in appendix 4e are summarized in table 13.

Table 13: 1D and 2D ^1H NMR (400 MHz, CD_3OD) data for (-)-5-oxoaknadinine (**207**)
HOR/SA/D4

Position	δ_{obs}	Multiplicity	J_{obs}	Integral	COSY
1	6.51	d	8.3	1H	H-2
2	6.71	d	8.3	1H	H-1
5 _{eq}	3.40	d	16.0	1H	H-5 _{ax}
5 _{ax}	2.70	d	16.0	1H	H-5 _{eq}
9 _{eq}	2.19	d	16.2	1H	H-9 _{ax}
9 _{ax}	2.00	d	16.2	1H	H-9 _{eq}
15 _{eq}	2.50	m		1H	H-15 _{ax} , H-16 _{eq}
15 _{ax}	2.33	m		1H	H-15 _{eq} , H-16 _{ax}
16 _{eq}	2.72	m		1H	H-16 _{ax} , H-15 _{eq}
16 _{ax}	2.58	m		1H	H-16 _{eq} , H-15 _{ax}
3-OCH ₃	3.81	s		3H	
7-OCH ₃	3.55	s		3H	
8-OCH ₃	4.08	s		3H	
N-CH ₃	2.53	s		3H	

The ^1H NMR revealed two aromatic protons δ 6.51 (H-1, d, J 8.3 Hz) and δ 6.71 (H-2, d, J 8.3 Hz). The COSY spectrum displayed correlations between H-1 and H-2 showing that they are neighbours. Coupling constant (J) values confirmed that H-1 and H-2 are *ortho* position to each other. H-5_{eq} exhibited COSY interaction with H-5_{ax} while H-9_{ax} with H-9_{eq}. H-15_{eq} exhibited COSY interaction with H-15_{ax} and H-16_{eq}. H-15_{ax} exhibited COSY interaction to H-15_{eq} and H-16_{ax}. Three methoxy groups resonating at δ 3.81, 3.55 and 4.08 were noted together with N-methyl at δ 2.53. The COSY interactions are shown in the figure 6.

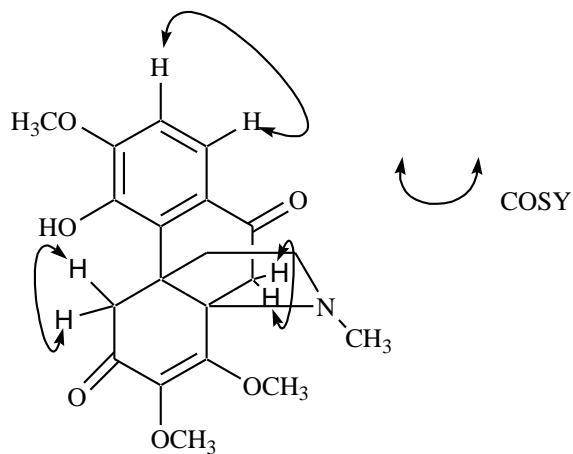


Figure 7: COSY interactions for (-)-5-oxoaknadinine (**207**) HOR/SA/D/4.

¹³C NMR, DEPT, HMBC and HMQC data is summarized in table 14

Table 14: 1D and 2D ¹³C NMR (400 MHz, CD₃OD) data for (-)-5-oxoaknadinine (**207**)
HOR/SA/D4

Position	δ_{obs}	HMQC	HMBC
1	120.0	6.51	C-12, C-3
2	110.6	6.71	C-11, C-4
3	147.3		
4	144.4		
5	44.0	3.40, 2.70	C-13
6	194.0		
7	139.9		
8	166.0		
9	23.7	2.00, 2.19	C-14
10	196.0		
11	129.9		
12	128.0		
13	47.7		
14	69.9		
15	35.2	2.50, 2.33	C-13, C-16
16	52.9	2.72, 2.58	N-Me
3-OMe	56.6	3.81	C-3
7-OMe	61.2	3.55	C-7
8-OMe	61.4	4.08	C-18
N-Me	36.9	2.53	C-14, C-16

The DEPT analysis in appendix 3c revealed four methylene groups at δ 52.9, 44.0, 23.7 and 35.2. Three bond correlations between H-1 and C-12 (δ 128.0) and C-3 (δ 147.3) were observed in HMBC experiments and between H-2 and C-11 (δ 129.9) and C-4 (δ 144.4). This confirmed the assignment of H-1 and H-2. Methoxy groups δ 3.81 (δ 56.6), δ 3.55 (δ 61.2) and δ 4.08 (δ 61.4) showed HMBC C-3, C-7 and C-8, respectively, hence the assignment at these positions. N-Methyl group at δ 2.53 showed three bond correlation to C-16 (δ 52.9) and quaternary centre at C-14 (δ 69.9). ¹H and ¹³C NMR Data from Kashiwaba *et al.*, 1996 was used to partially assign the structure. The HMBC interactions are shown in the figure 8.

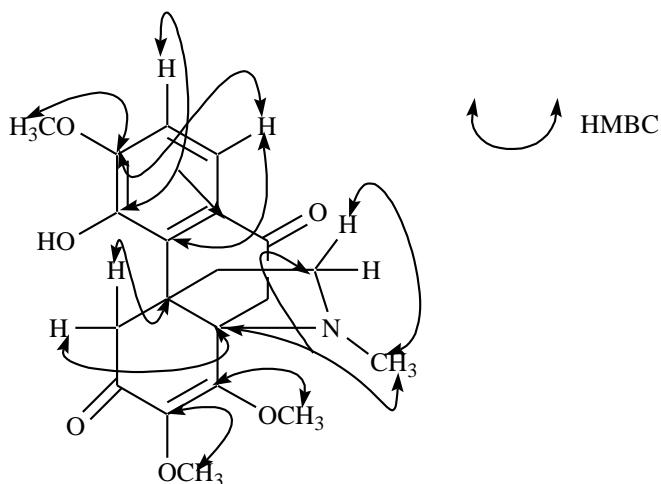


Figure 8: HMBC for (-)-5-oxoaknadinine (**207**) (HOR/SA/D/4)

The NMR data for the compound was compared to that of aknadinine (**142**).

Table 15: Comparison of ^{13}C and ^1H NMR (400 MHz, CD_3OD) data for 5-oxoaknadinine (**207**) (HOR/SA/D4) and aknadinine (**142**)

Position	$\delta_{\text{H}} \text{207}$	$\delta_{\text{H}} \text{142}$	$\delta_{\text{C}} \text{207}$	$\delta_{\text{C}} \text{142}$
1	6.51	6.56	120.0	119.1
2	6.71	6.66	110.6	108.6
3			147.3	147.0
4			144.4	143.8
5	3.40, 2.70	3.50, 2.64	44.0	43.3
6			194.0	194.8
7			139.9	138.1
8			166.0	165.2
9	2.00, 2.19	1.90, 2.19	23.7	23.1
10		2.56, 2.79	196.0	25.2
11			129.9	128.8
12			128.0	128.4
13			47.7	47.1
14			69.9	67.8
15	2.50, 2.33	2.47, 2.11	35.2	34.0
16	2.72, 2.58	2.67, 2.83	52.9	51.3
3-OMe	3.81	3.83	56.6	56.2
7-OMe	3.55	3.65	61.2	60.1
8-OMe	4.08	4.07	61.4	61.2
NMe	2.53	2.53	36.9	36.4

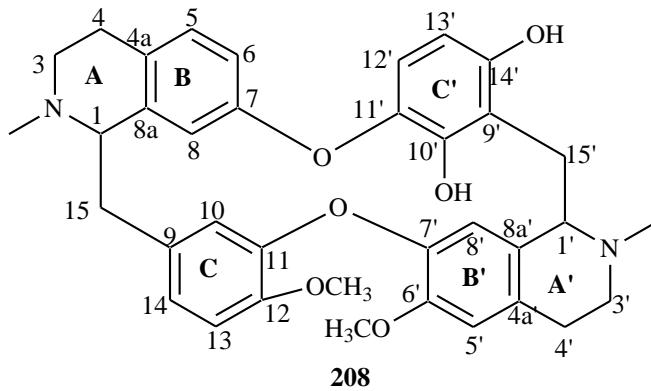
The NMR data for (-)-5-oxoaknadinine (**207**) HOR/SA/D4 and aknadinine (**142**) were comparable apart from C-10 (δ 196) for (-)-5-oxoaknadinine (**207**) where there was a

carbonyl while C-10 (δ 25.2) aknadinine (**142**) was a methylene with H_{ax} (δ 2.56) and H_{eq} (δ 2.79).

The compound was assigned the structure (**207**) and christened (-)-5-oxoaknadinine (**207**). (-)-5-Oxoaknadinine (**207**) is structurally related to aknadinine (**142**) isolated from *Stephania cepharantha hayata* (Kashibawa *et al*, 1996). Biosynthetically it may be derived from aknadinine (**142**) through oxidation of the benzylic position. This compound is being reported for the first time.

4.2.3 (-)-Pseudocurine (**208**) OR/SA/D2

This compound was isolated as a brown amorphous solid (43.6 mg, $[\alpha]^{25}_D -262^\circ$ (2.1, MeOH), R_f 0.60, 1:49 MeOH: DCM, mp 169-171 °C). The IR spectrum showed the presence of hydroxyl group at 3352.1 cm⁻¹, aromatic C-Hstr at 2943.3 cm⁻¹ and C=Cstr 1450.4 cm⁻¹. It gave a positive reaction for alkaloids with Dragendorff's reagents



The ¹H NMR in appendix 5a and COSY NMR data in appendix 5d are summarized in table 15

Table 16: 1D and 2D ^1H NMR (500 MHz, CD_3OD) data for (-)-pseudourine (**208**)
OR/SA/D2

Position	δ_{obs}	Multiplicity	J_{obs} (Hz)	Integral	COSY
1	3.78	d	13.8, 8.9	1H	H-15 _{eq}
3 _{ax}	2.92	m		1H	H-3 _{eq}
3 _{eq}	3.42	ddd	13.0, 5.2, 4.5	1H	H-3 _{ax}
4 _{ax}	2.57	dd	17, 4.6	1H	H-4 _{eq}
4 _{eq}	3.03	m		1H	H-4 _{ax}
5	6.67	d	8.2	1H	H-6
6	6.43	dd	8.2, 1.7	1H	H-5
8	6.67	d	1.7	1H	
10	6.45	d	1.8	1H	
13	6.78	d	8.3	1H	H-14
14	7.05	dd	8.3, 1.8	1H	H-13
15 _{ax}	2.71	d	9.8	1H	H-15 _{eq}
15 _{eq}	2.88	d	9.8	1H	H-15 _{ax}
1'	3.82	d	10.0	1H	H-15 _{ax} '
3 _{ax} '	3.07	m		1H	H-3 _{eq} '
3 _{eq} '	3.50	m		1H	H-3 _{ax} '
4 _{ax} '	2.99	m		1H	H-4 _{eq} '
4 _{eq} '	3.08	m		1H	H-4 _{ax} '
5'	6.90	s		1H	
8'	5.75	s		1H	
12'	7.20	d	7.5	1H	H-13'
13'	6.67	d	7.5	1H	H-12'
15 _{ax} '	2.73	dd	10.0, 2.1	1H	H-15 _{eq} '
15 _{eq} '	3.24	dd	10.0, 2.1	1H	H-15 _{ax} '
12-OMe	3.90	s		3H	
6'-OMe	3.89	s		3H	
2-NMe	2.31	s		3H	
2'-NMe	2.68	s		3H	

The ^1H NMR spectrum in appendix 5a revealed 10 aromatic protons with signals at δ 6.67 (H-5, d, J 8.2 Hz), δ 6.43 (H-6, dd, J 8.4, 1.7 Hz), δ 6.67 (H-8, d, J 1.7 Hz), δ 6.45 (H-10, d, J 1.8 Hz), δ 6.78 (H-13, d, J 8.3 Hz), δ 7.05 (H-14, dd, J 8.3, 1.8 Hz), δ 6.90 (H-5', s), δ 5.75 (H-8', s), δ 7.20 (H-12', d, J 7.5 Hz), δ 6.67 (H-13', d, J 7.5 Hz). Two methoxyl groups at δ 3.90 (12-OMe, s) and δ 3.89 (6'-OMe, s) were also observed. Signals at δ 2.31 (2-NMe, s) and δ 2.68 (2'-NMe, s) indicated presence of two methyl groups.

The ^{13}C , HMQC and HMBC NMR data in appendices 5b, 5c and 5d, respectively are summarized in table 16.

Table 17: 1D and 2D ^{13}C NMR (500 MHz, CD_3OD) data for (-)-pseudourine (**208**) (OR/SA/D2)

Position	δ_{obs}	HMQC	HMBC
1	60.2	3.78	C-15, C-8a, C-4a
3	43.6	3.40, 2.92	C-1, C-4
4	21.4	3.03, 2.57	C-5, C-4a
4a	123.9		
5	108.0	6.67	C-7, C-8a
6	131.7	6.43	C-8, C-4a
7	156.0		
8	114.7	6.67	C-6, C-4a
8a	123.0		
9	132.6		
10	121.1	6.45	C-14, C-12, C-15
11	142.0		
12	146.7		
13	115.1	6.78	C-11, C-9
14	125.3	7.01	C-15, C-10, C-12
15	39.2	2.88, 2.71	C-9, C-1, C-14, C-10
1'	64.7	3.82	C-8a', C-15'
3'	45.0	3.07, 3.50	C-4a'
4'	23.7	3.08, 2.99	C-8a', C-4a', C-3'
4a'	126.1		
5'	112.6	6.90	C-8a', C-7', C-4'
6'	149.3		
7'	144.3		
8'	117.6	5.75	C-6', C-4a', C-1'
8a'	126.6		
9'	130.9		
10'	138.8		
11'	148.0		
12'	129.2	7.20	C-7
13'	113.5	6.67	C-9', C-11'
14'	137.7		
15'	37.9	2.73, 3.24	C-8a', C-1', C-9', C-14'
12-OMe	55.3	3.89	C-12
6'-OMe	55.3	3.90	C-6'
2-NMe	40.1	2.31	C-1, C-3
2'-NMe	40.2	2.68	C-1', C-3'

The aromatic protons: H-5 (δ 6.67) exhibited HMQC to C-5 (δ 108.0), H-6 (δ 6.43) to C-6 (δ 131.7), H-8 (δ 6.67) to C-8 (δ 114.7), H-10 (δ 6.45) to C-10 (δ 121.1), H-13 (δ 6.78)

to C-13 (δ 115.1), H-14 (δ 7.01) to C-14 (δ 125.3), H-5' (δ 6.90) to C-5' (δ 112.6), H-8' (δ 5.75) to C-8' (δ 117.6), H-12' (δ 7.20) to C-12' (δ 129.2) and H-13' (δ 6.67) to C-13' (δ 113.5). By correlating two hydrogens to one carbon, HMQC revealed that the compound had 6 methylene groups: H-3 (δ 3.40, 2.92) to C-3 (δ 43.6), H-4 (δ 3.03, 2.57) to C-4 (δ 21.4), H-15 (δ 2.88, 2.71) to C-15 (δ 39.2), H-3' (δ 3.07, 3.50) to C-3' (δ 45.0), H-4' (δ 3.08, 2.99) to C-4' (δ 23.7) and H-15' (δ 2.73, 3.24) to C-15' (δ 37.9). COSY interactions showed correlation between H-5 (δ 6.67) and H-6 (δ 6.43) thus confirming the *ortho* relationship. It also confirmed that H-14 (δ 7.01) and H-13 (δ 6.78) are *ortho* to one another. The observed COSY interactions in appendix 5d are represented in figure 9.

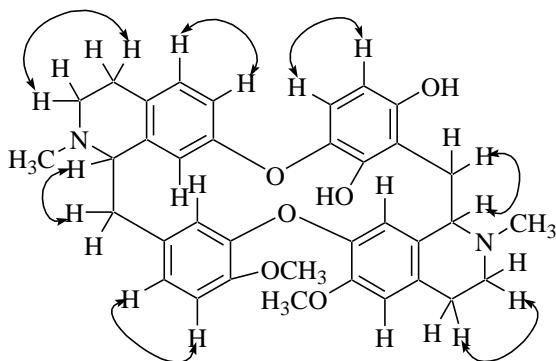


Figure 9: COSY for (-)-pseudocurine (**208**) OR/SA/D2

H-1 (δ 3.78) showed a rare two bond correlation to C-15 (δ 39.2), C-8a (δ 123.0) and three bond correlation to C-4a (δ 123.9). H-5 (δ 6.67) showed HMBCs to C-4 (δ 21.4), C-7 (δ 156.0) and C-8a (δ 123.0), H-6 (δ 6.43) showed three bond correlations to C-8 (δ 114.7) and C-4a (δ 123.9). 2-N-CH₃ (δ 2.31) showed HMBCs to C-1 (δ 60.2) and C-3' (δ 43.7). This led to assignments in ring A and B. H-10 (δ 6.45) showed HMBCs to C-12 (δ 146.7), C-15 (δ 39.2) and C-14 (δ 125.3). H-13 (δ 6.78) exhibited HMBCs to C-9 (δ 132.6) and C-11 (δ 142.0). H-14 (δ 7.01) showed three bond correlations to C-15 (δ 39.2), C-10 (δ 121.1) and C-12 (δ 146.7). 12-OCH₃ (δ 3.89) showed HMBC to C-12 (δ 146.7). This led to assignments in ring C.

H-1' (δ 3.82) showed a rare two bond correlation to C-15' (δ 37.9) and C-8a' (δ 126.6). H-5' (δ 6.90) showed three bond correlations to C-7' (δ 144.3), C-4' (δ 23.7) and C-8a'

(δ 126.6). H-8' (δ 5.75) showed HMBCs to C-1' (δ 64.7), C-4a' (δ 23.7), C-6' (δ 149.3), and C-8a' (δ 126.6). H-13' (δ 6.67) showed three bond correlation to C-11' (δ 148.0) and C-9' (δ 130.9). 2'-NCH₃ (δ 2.68) showed HMBCs to C-1' (δ 64.7) and C-3' (δ 45.0). 6'-OCH₃ (δ 3.90) showed a three bond correlation to C-6' (δ 149.3). This led to assignments of rings A', B' and C'. Some observed HMBC correlations are presented on figure 10.

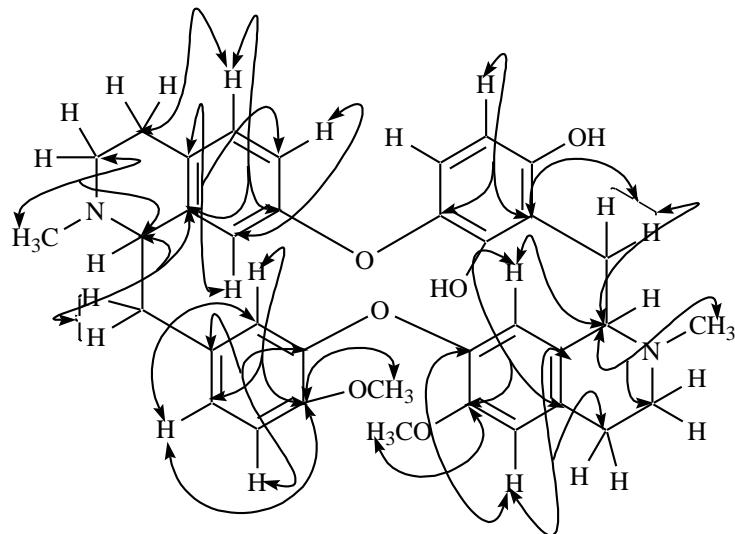


Figure 10: HMBC for (-)-pseudourine (**208**) OR/SA/D2

The EIMS in appendix 5i showed a molecular ion peak at m/z 594 [M^+] corresponding to C₃₆H₃₈N₂O₆. Other prominent MS peaks in appendix 5h appeared at m/z 299, 298, 282, 267, 191, 177 and 107. The observed MS could be rationalized by the fragmentation pattern in figure 11.

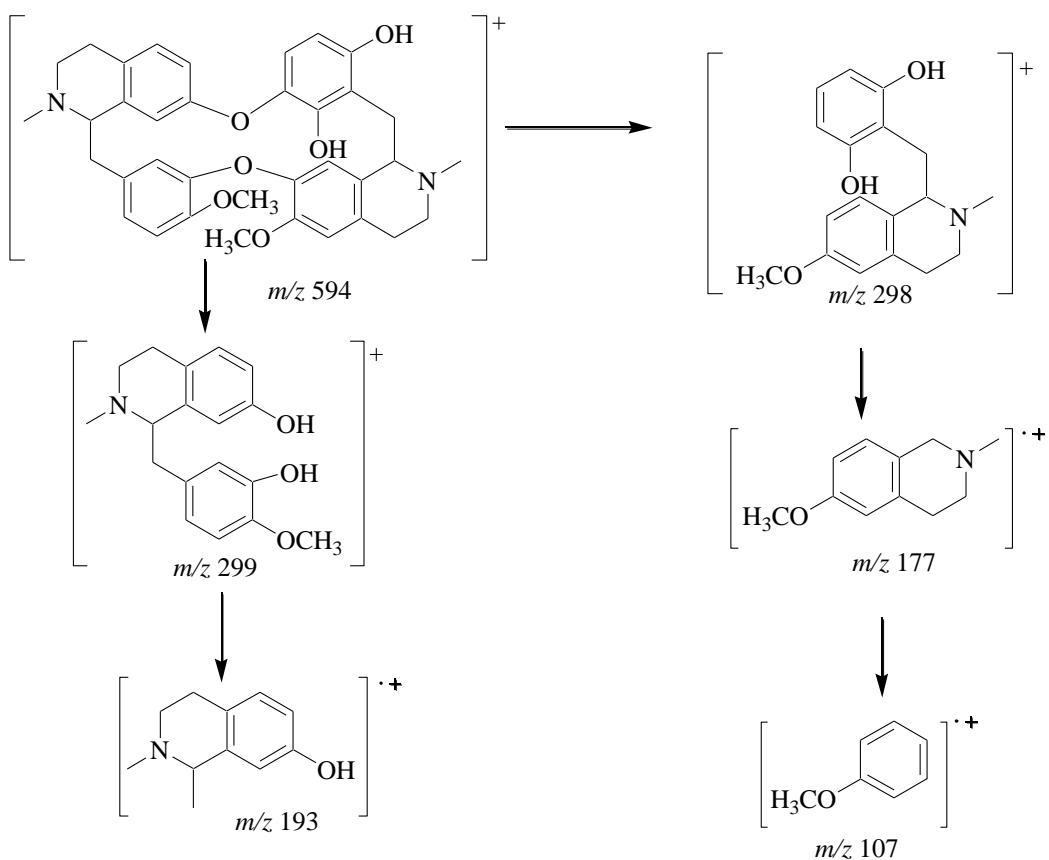
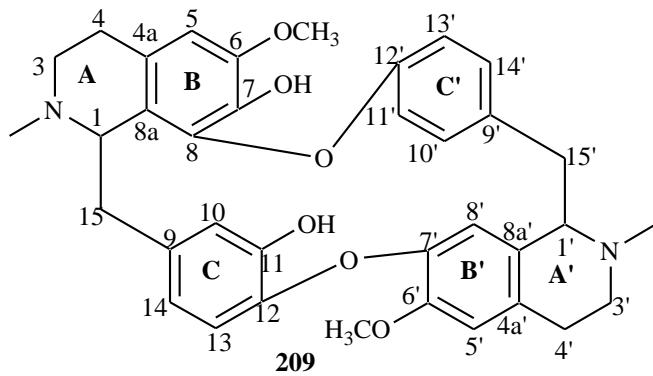


Figure 11: Mass fragmentation pattern for (-)-pseudocurine (**208**) OR/SA/D2

This compound is being reported for the first time.

4.2.4 (-)-Isocurine (**209**) OR/SA/D3

This compound was isolated as a brown amorphous solid (21.8 mg, $[\alpha]^{25}_{D} -158^{\circ}$ (3.5, MeOH), R_f 0.45, 1:49 MeOH: DCM, mp 158-160 °C). The IR spectrum showed the presence of hydroxyl group at 3352.1 cm^{-1} , aromatic C-Hstr at 2943.3 cm^{-1} and C=Cstr 1450.4 cm^{-1} . It gave a positive reaction for alkaloids with Dragendorff's reagent.



The ^1H NMR in appendix 6a and COSY data in 6d for (-)-isocurine are summarized in table 19

Table 18: 1D and 2D ^1H NMR (500 MHz, CD_3OD) for (-)-isocurine (**209**) OR/SA/D3

Position	δ_{obs}	Multiplicity	J_{obs} (Hz)	Integral	COSY
1	4.50	d	9.8	1H	H-15 _{eq}
3 _{ax}	3.13	m		1H	H-4 _{eq}
3 _{eq}	3.70	m		1H	H-3 _{ax}
4 _{ax}	2.91	m		1H	H-3 _{ax}
4 _{eq}	3.14	m		1H	H-4 _{ax}
5	6.77	s		1H	
10	6.48	d	1.7	1H	
13	6.81	d	8.4	1H	H-14
14	6.91	dd	8.4, 1.7	1H	H-13
15 _{ax}	2.88	dd	16.0, 6.0	1H	H-15 _{eq}
15 _{eq}	3.00	dd	16.0, 6.0	1H	H-15 _{ax}
1'	4.15	d	7.5	1H	H-15 _{ax} ', H-15 _{eq}
3' _{ax}	3.28	m		1H	
3' _{eq}	3.77	m		1H	
4' _{ax}	2.95	m		1H	
4' _{eq}	3.14	m		1H	
5'	6.95	s		1H	
8'	5.65	s		1H	
10'	6.43	bd	8.4,	1H	
11'	6.89	dd	8.4, 2.2	1H	H-11'
13'	6.74	dd	8.4, 2.2	1H	H-10', H-13'
14'	7.25	bd	8.4	1H	H-14'
15' _{ax}	2.95	dd	14.7, 6.6	1H	
15' _{eq}	3.32	dd	14.7, 6.6	1H	
12-OMe	3.92	s		3H	
6'-OMe	3.91	s		3H	
N-Me	2.52	s		3H	
N'-Me	2.81	s		3H	

The ^1H NMR spectrum in appendix 6a revealed 10 aromatic protons with signals at δ 6.77 (H-5, s), 6.48 (H-10, d J 1.7), 6.81 (H-13, d J 8.4 Hz), 6.91 (H-14, dd J 8.4, 1.7 Hz), 6.95 (H-5', s), 5.65 (H-8', s), 6.43 (H-10', bd J 8.4 Hz), 6.89 (H-11', dd J 8.4, 2.2 Hz), 6.74 (H-13', dd J 8.4, 2.2 Hz) and 7.25 (H-14', bd J 8.4 Hz). Two methoxy groups at δ 3.92 (12-OMe, s) and 3.91 (6'-OMe, s) were observed while signals at δ 2.52 (2-NMe, s) and δ 2.81 (2'-NMe, s) indicated presence of two N-methyl groups. The COSY for isocurine interactions are summarized in figure 12.

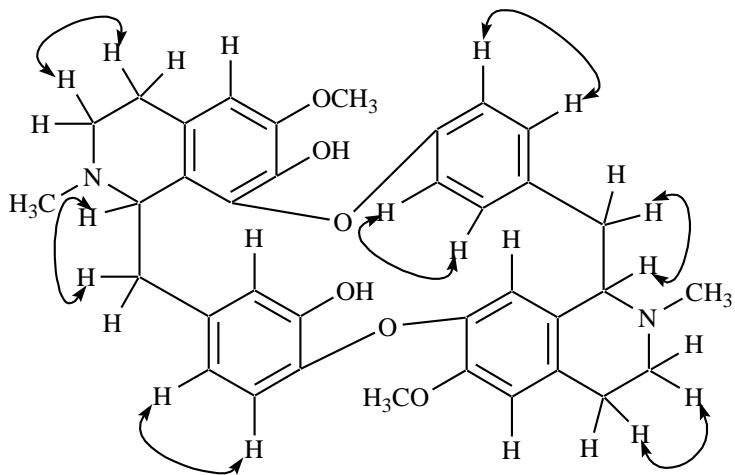


Figure12: COSY for (-)-isocurine (**209**) (OR/SA/D3)

COSY interactions confirmed that H-14' (δ 7.25) and H-13' (δ 6.74) are neighbours. It also revealed that H-10' (δ 6.43) and H-11' (δ 6.89) are *ortho* to each other. H-13 (δ 6.81) and H-14 (δ 6.91) were also confirmed to be neighbours through COSY interactions. Other protons that showed COSY interactions are H-15 and H-1, H-3 and H-4, H-15' and H-1', H-3' and H-4'. Although there was a lot of overlapping of the signals in 2D NMR spectra, COSY, HMQC and HMBC in appendix 6d, 6e, 6f and 6g, respectively led to confirmation of these assignments.

The ^{13}C HMQC and HMBC NMR data is summarized in table 18.

Table 19: 1D and 2D ^{13}C NMR (500 MHz, CD_3OD) data for (-)-isocurine (**209**) (OR/SA/D3)

Position	δ_{obs}	HMQC	HMBC
1	60.0	4.50	C-15, C-8a, C-4a
3	44.5	3.13, 3.70	C-1, C-4
4	22.3	3.14, 2.91	C-5, C-4a
4a	122.1		
5	108.0	6.77	C-4, C-7, C-8a
6	148.7		
7	138.7		
8	137.4		
8a	121.4		
9	132.6		
10	121.4	6.48	C-14, C-12, C-15
11	142.6		
12	147.6		
13	116.7	6.81	C-11, C-9
14	126.3	6.91	C-15, C-10, C-12
15	38.5	3.00, 2.88	C-9, C-1, C-14, C-10
1'	64.2	4.15	C-8a'
3'	45.3	3.77, 3.28	C-4a'
4'	22.9	3.14, 2.95	C-8a', C-4a', C-3'
4a'	125.3		
5'	112.4	6.95	C-8a', C-7', C-4'
6'	149.4		
7'	144.9		
8'	116.1	5.65	C-6', C-4a', C-1'
8a'	123.3		
9'	129.6		
10'	132.5	6.43	C-15', C-12', C-14'
11'	114.7	6.89	C-13', C-9'
12'	156.0		
13'	113.1	6.74	C-9', C-11'
14'	130.0	7.25	C-10', C-12',
15'	37.0	2.95, 3.32	C-8a', C-1', C-9', C-14'
6-OMe	55.3	3.92	C-12
6'-OMe	55.3	3.91	C-6'
2-NMe	40.3	2.52	C-1, C-3
2'-NMe	40.1	2.81	C-1', C-3'

H-5 (δ 6.77) showed HMBCs to C-4 (δ 22.3), C-7 (δ 138.7), C-8a (δ 121.4) and it showed a rare two bond correlation to C-6 (δ 148.7). 2-NCH₃ (δ 2.52) exhibited three bond correlations to C-3 (δ 44.5) and C-1 (δ 60.1). This led to assignments of ring A and

B. In ring C, H-10 (δ 6.48) exhibited HMBCs to C-15 (δ 38.5), C-12 (δ 147.6) and C-14 (δ 126.3). H-14 (δ 6.91) exhibited HMBCs to C-15 (δ 38.5), C-12 (δ 147.6) and C-10 (δ 121.4). H-13 (δ 6.81) revealed HMBCs to C-11 (δ 142.6) and C-9 (δ 129.4). The methoxyl group at δ 3.92 showed HMBCs to C-6 (δ 148.7) confirming its position on C-6 in ring B. These observations led to assignments on ring A, B and C.

H-5' (δ 6.95) exhibited HMBC with C'-4 (δ 22.9), C-7' (δ 144.9) and C-8a' (δ 123.3). H-4' (δ 3.14) showed HMBCs with C-5' (δ 112.4), C-4a' (δ 125.3), C-8a' (δ 123.3) and C-3' (δ 45.3). H-8' (δ 5.65) revealed HMBCs with C-1' (δ 64.2), C-6' (δ 149.4) and C-4a' (δ 125.3). 6'-OCH₃ (δ 3.91) exhibited HMBCs to C-6' (δ 149.4) leading to assignment of the position of the methoxyl group to C-6' in ring B'. H-1' (δ 4.15) showed HMBCs to C-15' (δ 37.2), C-3 (δ 45.3), C-4a (δ 125.3) and C-8a' (δ 123.3). 2'-NCH₃ (δ 2.81) exhibited HMBCs C-1' (δ 64.2) and C-3' (δ 45.3). H-14' (δ 7.25) revealed HMBCs to C-15' (δ 37.2), C-12' (δ 156.0) and C-10' (δ 132.5). H-13' (δ 6.74) showed three bond correlation to C-11' (δ 114.7) and C-9' (δ 129.6) while H-10' (δ 6.43) exhibited HMBCs to C-12' (δ 156.0), C-14' (δ 130.0) and C-15' (δ 37.0). The observations led to assignments of rings A', B' and C'. Some of the observed HMBCs are presented in figure 13.

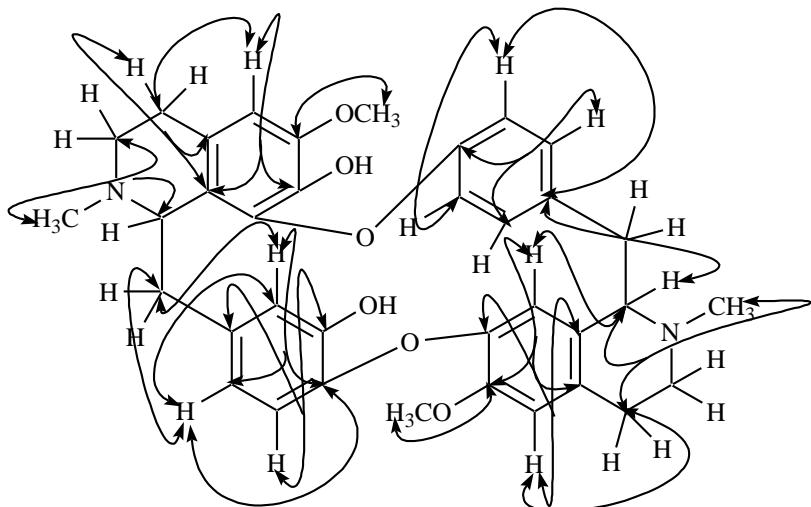


Figure 13: HMBCs for (-)-isocurine (**209**) OR/SA/D3

The EIMS in appendix 6j showed a molecular ion peak at m/z 595 [M^++1] corresponding to $C_{36}H_{38}N_2O_6$. Other prominent peaks in appendix 6i appeared at m/z 299, 282 and 267. The observed MS peaks could be rationalized by the fragmentation pattern in figure 14.

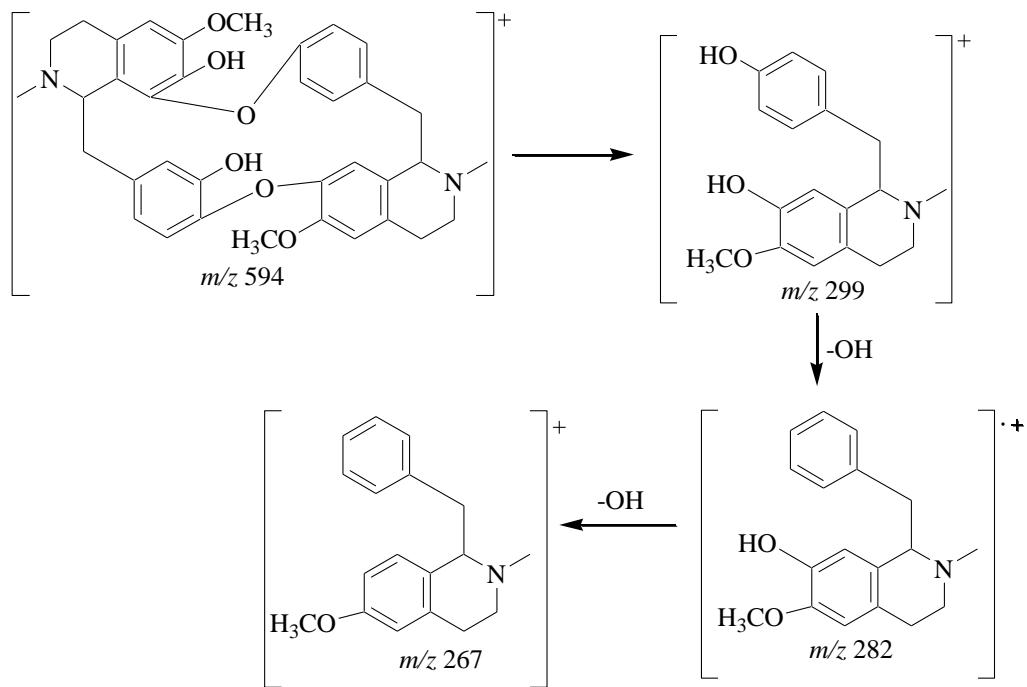


Figure 14: Mass fragmentation pattern for (-)-isocurine (**209**) (OR/SA/D3)

This compound is being reported for the first time.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

It was confirmed that two plants *Cissampelos mucronata* and *Stephania abyssinica* used in traditional anti-malarial therapy in southern Nyanza exhibited strong *in vitro* anti-plasmodial activity. The ethyl acetate extract of *C. mucronata* was the most active but toxic to the vero cells 199 ($IC_{50} < 3.91\mu\text{g}/\text{ml}$, $CC_{50} 10.34 \mu\text{g}/\text{ml}$) while dichloromethane extract of *S. abyssinica* was the most active but non-toxic to the vero cells 199 ($IC_{50} < 3.91\mu\text{g}/\text{ml}$, $CC_{50} 100.00 \mu\text{g}/\text{ml}$, respectively). The ethyl acetate extract of *S. abyssinica* was moderately active and moderately selective ($IC_{50} 5.09 \pm 0.041 \mu\text{g}/\text{ml}$, SI 19.65, respectively). The methanol extract ($IC_{50} 19.28 \pm 2.38 \mu\text{g}/\text{ml}$, SI 3.35), dichloromethane extract ($IC_{50} 10.09 \pm 1.56 \mu\text{g}/\text{ml}$, SI 9.91) and hexane extract ($IC_{50} 8.73 \pm 1.81 \mu\text{g}/\text{ml}$, SI 10.35) of *C. mucronata* had a mild *in vitro* anti-plasmodial activity and moderate selectivity. Methanol extract of *S. abyssinica* had a moderate *in vitro* activity against *P. falciparum* and moderate selectivity ($IC_{50} 5.09 \pm 0.041 \mu\text{g}/\text{ml}$, SI 19.65, respectively).

Phytochemical investigation of *C. mucronata* led to isolation of a triterpene, (-)-stigmasterol (**204**), and an alkaloid, (-)-curine (**205**). The triterpene was inactive while the alkaloid showed strong anti-plasmodial activity against D6 and W2 strains of *P. falciparum* ($IC_{50} 0.24 \pm 0.03$ and $0.22 \pm 0.06 \mu\text{g}/\text{ml}$, respectively).

Five compounds were isolated from *S. abyssinica*: (+)-nonacosan-10-ol (**206**), (-)-5-oxoaknadinine (**207**), OR/SA/D1, (-)-pseudocurine (**208**) and (-)-isocurine (**209**). It was not possible to determine the structure of OR/SA/D1 since it was isolated in small amount with some impurities and could not be separated. However it showed a strong anti-plasmodial activity against *P. falciparum* D6 and W2 strains *in vitro* ($IC_{50} 0.34 \pm 0.03$ and $0.55 \pm 0.11 \mu\text{g}/\text{ml}$, respectively). (-)-Pseudocurine was the most active against *P. falciparum* D6 and W2 strains *in vitro* ($IC_{50} 0.29 \pm 0.00$ and $0.31 \pm 0.01 \mu\text{g}/\text{ml}$, respectively). (-)-Isocurine (**209**) had strong and moderate anti-plasmodial activity ($IC_{50} 0.75 \pm 0.11$ and $1.65 \pm 0.03 \mu\text{g}/\text{ml}$) against D6 and W2 strains, respectively. (+)-Nonacosan-10-ol (**206**) showed a mild anti-plasmodial activity against *P. falciparum* D6 and W2

strains *in vitro* (IC_{50} 13.79±1.02 and 4.35±2.45 µg/ml, respectively) with (-)-5-oxoaknadinine (**207**) also showing a mild anti-plasmodial activity against *P. falciparum* D6 and W2 strains *in vitro* (IC_{50} 10.25±1.84 and 3.45±2.22 µg/ml). (-)-5-oxoaknadinine (**207**), (-)-pseudourine (**208**) and (-)-isourine (**209**) were isolated for the first time with their anti-plasmodial activity being reported for the first time.

The hypothesis of the study stated that *C. mucronata* and *S. abyssinica* used in traditional anti-malarial therapy in southern Nyanza contain natural products with unique chemical structures that are potential drugs against malaria. It was confirmed that some compounds isolated from the two plants had strong anti-malarial activity making them good candidates for further scientific research on anti-plasmodial drug discovery and development.

5.2 Recommendations

It is recommended that the promising extracts with good anti-plasmodial activity and the isolated compounds from the two plants should be subjected to further efficacy and safety evaluations against *P. falciparum* *in vivo* in animal models such as mice.

Toxicity studies should be carried out on the compounds isolated from the two plants that were active against *P. falciparum* to establish their safety levels for use by humans. This will help in calculating the safe dose of the herbal preparations if the amounts of the compounds in the plant extracts can be estimated.

Further isolation of compounds from methanol extracts of *C. mucronata* and *S. abyssinica* should be carried out in order to identify the bioactive compounds in these extracts and their contribution to the activity of the plant extracts.

OR/SA/D1 should be isolated in large amounts to facilitate further purification and structural elucidation since it showed strong anti-plasmodial activity.

CHAPTER 6

EXPERIMENTAL

6.1 Materials, reagents and equipment

6.1.1 Reagents

Organic solvents like acetone, *n*-hexane, ethyl acetate, dichloromethane, chloroform and methanol were purchased from Sciencescope Ltd, Nairobi, Kenya. All solvents used for extraction, fractionation and crystallization were of analar grade. Acetic acid, citric acid, dextrose, Giemsa stain, glycerol and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Company, St. Louis, U.S.A. [³H]-hypoxanthine, methanol, sodium hydrogen carbonate, sodium chloride, Rosewell Park Memorial Institute 1640 (RPMI-1640) powdered medium were purchased from Glico Laboratories California, U.S.A. The culture gas mixture was acquired from British Oxygen Company (BOC), Nairobi, Kenya.

6.1.2 Equipment for the *in vitro* culture

Laminar flow hood, liquid scintillation counter, microscopes, refrigerators (-4 and -80 °C), incubator, gas-tight box, cell harvester, analytical balance (with sensitivity of 0.1 mg), vacuum pump, centrifugal machine adjustable volume Eppendorf micro-pipette, automatic pipette pump, vibro mixer and electrically heated water bath were used at the Kenya Medical Research Institute (KEMRI).

6.1.3 Disposable plastics and glassware

Anti-coagulant free blood-collecting bags and sterile gloves (Trifex®), 15 and 50 ml centrifuge tubes (Brinkmann Instruments Company, Westbury U.S.A), 50 and 150 ml culture flasks (Corning®, U.S.A), microscopes slides and cover slips (Sigma Chemical Company, U.S.A), 0.45 and 0.22 µm filter units (Naglene®, Naglene Company, U.S.A), serological Pasteur pipettes (Fischer Scientific, Pittsburg, U.S.A) were acquired through Diagnostics, U.S.A.

6.1.4 Recycled glassware

Re-usable glassware was soaked in hot water with liquid detergent. They were then washed and rinsed thoroughly with tap water, several times with distilled water, ethanol and acetone. They were then dried at 110 °C in an electric oven for at least 1 h and allowed to cool slowly to room temperature before use.

6.1.5 Sterilizing materials

All culture experiments were carried out in a lamina flow hood, sterilised Pasteur pipettes, lids and disposable pipettes were passed over Bunsen burner flame several times before use. Ethanol (70%) was used to sterilize the hood and other equipment. The used Pasteur pipettes were put in 20% sodium hypochlorite to disinfect them before washing. Disposable apparatus were similarly disinfected before being discarded.

6.2 Spectroscopy

6.2.1 Infra-red (IR) spectroscopy

IR spectra were measured at the department of chemistry Jomo Kenyatta University on a HYPER-IR spectrophotometer using potassium bromide pellets and methanol as a solvent. Resonance frequencies were given in cm^{-1} .

6.2.2 Ultra violet spectroscopy

UV spectra were measured at the department of chemistry Kenyatta University on a CECIL 2041 ultra violet spectrometer. Methanol and chloroform were used as solvents. 200-400 nm the ultra violet and visible region was used as the range.

6.2.3 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were measured on a Bruker drx 500 (^1H 500MHz; ^{13}C NMR 125 MHz) in the United Kingdom and Bruker Avance 400 (^1H 400MHz; ^{13}C NMR 101 MHz) in the University of Kwazulu Natal on South Africa. Deuterated CDCl_3 , DMSO and CD_3OD were used as solvents. Chemical shifts were given in δ (ppm) values with TMS (δ 0) as an internal standard. Resonances were designated as s- singlet, d- doublet and m- multiplet. Coupling constant (J) were reported in Hz.

6.2.4 Mass spectroscopy

Mass spectra were measured on mass spectrometer VG-12-250 (EIMS). The m/z are reported in amu with corresponding intensities in parentheses.

6.3 Melting points

Melting points of recrystallized solids were measured using a Gallen Kamp apparatus and are uncorrected.

6.4 Optical rotation

Optical rotation was done at the department of food science, Jomo Kenyatta on a polarimetr POLAX-2L. Methanol and chloroform were used as solvents.

6.5 Sampling of plant materials

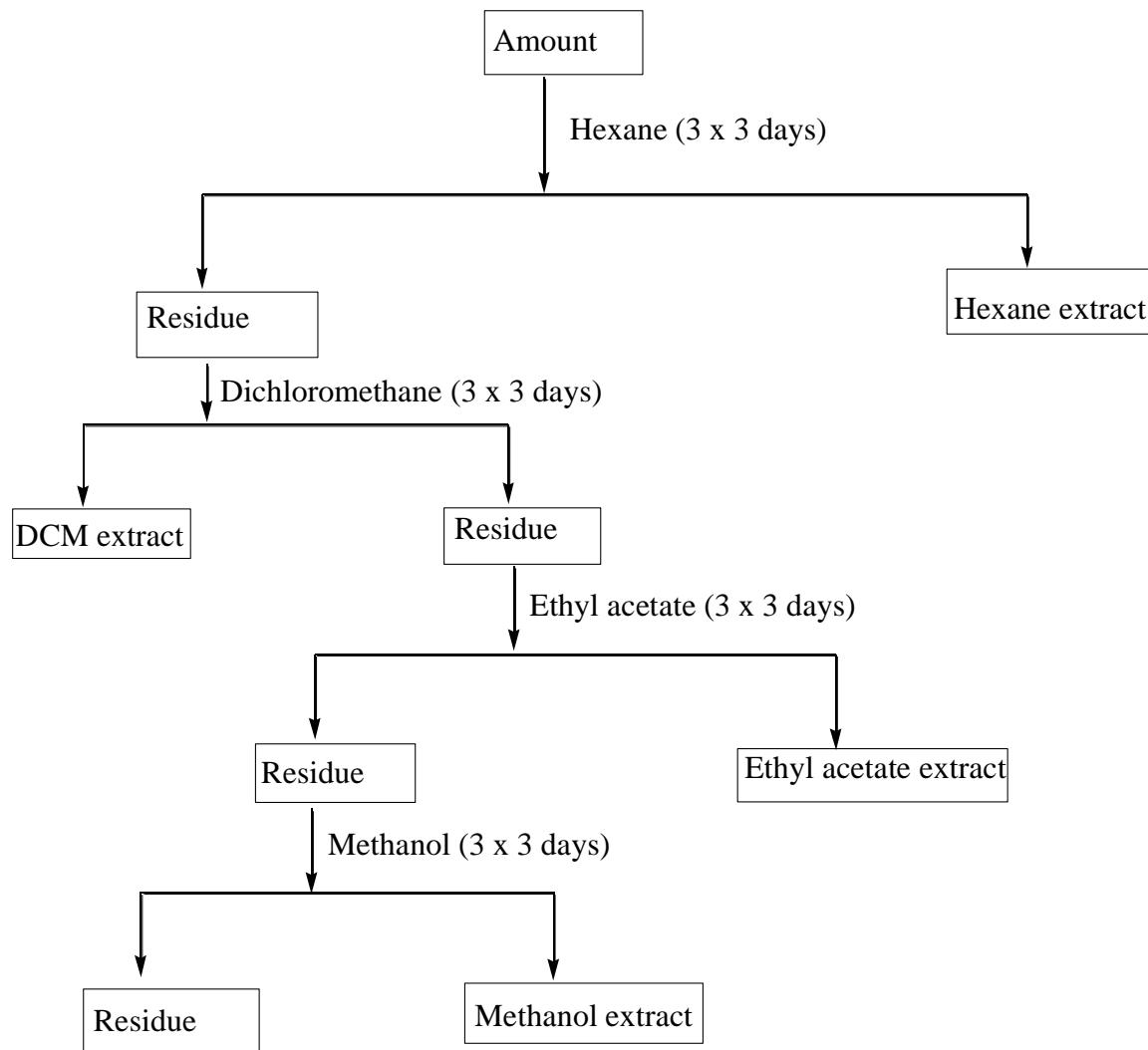
Roots of *Cissampelos mucronata* and leaves of *Stephania abyssinica* were collected from southern part of Nyanza. *Cissampelos mucronata* was obtained from Kabondo village Rachuonyo District while *Stephania abyssinica* from the Kisii highlands. The plants were identified during a survey of traditional plants from southern Nyanza by Muregi *et al.* (2003; 2004). They were identified by Mr. Simon Mathege, a plant taxonomist from Department of Botany, University of Nairobi. The voucher specimens with reference numbers RO/01/2006 and RO/02/2006 for *C. mucronata* and *S. abyssinica*, respectively, were deposited at the University of Nairobi, Herbarium in the Department of Botany.

6.6 General procedures

6.6.1 Extraction

Roots of *C. mucronata* and leaves *S. abyssinica* were dried under shade for 14 days ground in powder using miller (8 Lab Mill Christy and Morris Limited, England) and soaked at room temperature in solvents of increasing polarity in the order of hexane, dichloromethane, ethyl acetate and methanol. Each soaking lasted 3 days and the extract was decanted and filtered through Whatman filter No 1 while the mercerate was steeped again for 24 h. The extraction process was repeated until a clear extract was obtained.

The filtered extracts were combined and solvents removed under reduced pressure. The dried extract samples were weighed and stored in a freezer (-4 °C) for further chemical and biological analysis. Scheme 1 shows the sequential extraction process for the ground material from leaves of *S. abyssinica* and roots of *C. mucronata*.



Scheme 1: Sequential extraction of *Cissampelos mucronata* and *Stephania abyssinica*

6.6.2 *In vitro* cytotoxicity assay

In vitro cytotoxicity assay of the extracts was carried out following a colometric assay using vero type 199 kidney epithelial monkey cells (Mosmann, 1983). Cells were maintained in minimum essential medium (MEM), (GIBCO, Grand Island, New York)

containing 10% fetal bovine serum (FBS). Cell suspension (2×10^5 cells/ml) was seeded in 96 well micro-titer plates row (A-H) and incubated at 37 °C under 5% CO₂ for 12 h to

allow cells to attach after which drugs were added. Row H carried the highest drug concentration and serial dilution was carried out to row B. Row A wells 3, 6, 9 and 12 served as blanks while the remaining eight wells were negative controls (cells without drugs). The cells were incubated for 48 h at 37 °C under 5% CO₂, after which 10 µl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye was added and the mixture incubated for another 4 h. The media was removed from the wells using a micro-pipette and 100 µl of DMSO added. The plates were read on a scanning multi-well spectrometer (Multiscan Exlassystems, U.K) at 562 and 620 nm as reference. Podophylotoxin, a natural product isolated from *Podophyllum* species and its semisynthetic derivative etopoxide, were used as positive standards (Newmann *et al.*, 2000).

Data was analysed as follows:

$$\text{Cell viability (\%)} = [\text{OD}_{\text{sample}562} - \text{OD}_{620}] / [\text{OD}_{\text{control}562} - \text{OD}_{620}] \times 100$$

Where OD = optical density

Data was transferred onto a graphic programme (EXCEL) and expressed as percentage of the untreated controls. The 50% cytotoxicity concentration (CC₅₀) was calculated from linear regression analysis.

6.6.3 *In vitro* anti-plasmodial assay

6.6.3.1 Parasite

In this study *P. falciparum* D6 and W2 strains were used. These strains were obtained from the Centre for Biotechnology Research and Development (CBRD), Malaria Parasite Bank, KEMRI, Nairobi, Kenya.

6.6.3.2 Parasite cultivation

Parasite cultivation was done based on the *in vitro* technique described by Trager and Jensen (1976). It was carried out aseptically in a laminar flow hood.

6.6.4 Culture preparation

6.6.4.1 RPMI 1640/ HEPES medium

This was prepared according to Trager & Jensen (1976). Briefly, it contained 25 ml HEPES (5.94 g/l) and 10.5 g RPMI 1640 powdered medium without *p*-amino benzoic acid (PABA) and lactic acid (LA) dissolved in 960 ml of distilled and autoclaved water. It was filtered and sterilized using a vacuum pump and 0.22 µm filter, and stored at 4 °C before use within 4 weeks.

6.6.4.2 Wash Medium (WM)

It was prepared according to Rowe *et al.* (1968) by mixing 95.8% v/v of the RPMI 1640 and HEPES medium and 4.2% v/v of 5% w/v sodium carbonate.

6.6.4.3 Uninfected erythrocyte

The method of obtaining uninfected erythrocytes was adopted from Trager & Jensen (1976). Briefly, uninfected blood group O rhesus positive from Kenya Medical Research Institute (KEMRI) recruited volunteers was drawn into 15% (v/v) acid-citrate-dextrose (ACD). Enzyme-linked immunosorbent assay (ELISA) method was used to screen blood for human immune virus (HIV) and hepatitis B infections at Centre for Virology Research (CVR), KEMRI. Before blood donation, it was ascertained that the individuals had not contacted malaria or visited a malaria endemic area in the past two months. It was also ascertained that the donor had not taken any anti-malarial or anti-biotic drugs.

The blood was washed free of plasma and white blood cells before use in the culture by centrifugation at 3600 rpm for 10 min at 4 °C. The plasma and buffy coat at the top of the cell pellet was aspirated and discarded. The red cell pellet was washed twice with 2 ml of wash medium (WM) and the resulting suspension centrifuged at 3600 rpm for 10 min at 4 °C. After the last wash, the packed cells were re-suspended in an equal volume

of WM to obtain a haematocrit of 50%. The cells were exposed to a gas mixture of 92% N₂, 5% CO₂ and 3% O₂) and stored at 4 °C and used within two weeks.

6.6.4.4 Preparation of human serum

The method of preparation human serum was adopted from Trager & Jensen (1976). Blood from donors was collected aseptically into blood bags without anti-coagulants and allowed to clot by leaving it at room temperature for 90 min. followed by overnight (18 h) storage at 4 °C. The following day, the serum was carefully dispensed into sterile 50 ml centrifuge tubes and centrifuged at 3000 rpm for 10 min. at 4 °C. The serum was aseptically aliquoted into 10 ml snap-top tubes and heat inactivated in a water bath at 56 °C for 40 min. The tubes were placed in the upright position at -20 °C overnight and then kept at -70 °C until they were used.

6.6.4.5 Complete culture medium with serum (CMS)

The CMS was prepared by mixing 86.22% (v/v) of RPMI 1640 and HEPES, 3.78% (v/v) of 5% NaHCO₃ and 10% (v/v) human serum. The CMS was stored at 4 °C and used within one week of preparation.

6.6.4.6 Thawing of the malaria parasite

The malaria parasites were prepared according to Rowe *et al.* (1968). Briefly, laboratory strains of malaria parasites preserved in liquid nitrogen were removed quickly thawed in a water bath maintained at 37 °C. The ampoules were surface sterilized with 70% ethanol. The stocks were then gently agitated and transferred to a sterile 15 ml centrifuge plastic tube while still cold. The cells were centrifuged in a thermostated centrifugal machine at 1500 rpm for 5 min. at 20 °C. The supernatant (SN) was aspirated and discarded. The packed cells were immediately re-suspended on 0.3 ml of filter sterilized 3.5% (w/v) sodium chloride in distilled autoclaved water (DAW) and immediately re-centrifuged and the supernatant aspirated and discarded again, to prevent osmotic lysis during the removal of the glycerol cells. The parasites were finally re-suspended in 1 ml of CMS, centrifuged and supernatant aspirated and discarded.

6.6.4.7 Setting of the culture

The procedure of Trager & Jensen (1976) was adopted. Briefly, after washing the parasites, the packed cell volume (PCV) of the parasitized erythrocytes was estimated and the volume of the RBC adjusted to 6% (v/v) (6% haematocrit (hct) by the addition of the CMS. The culture flasks were exposed to a gas mixture (92% N₂, 5% CO₂ and 3% O₂) and incubated at 37 °C for 24 h. The medium was changed daily to remove the toxic compounds and smears prepared after every 48 h to determine the percentage parasitaemia (%P), the growth rate and to monitor contamination.

6.6.4.8 Determination of parasitaemia and parasite growth rates

This was done according to Trager & Jensen. (1976). Briefly, thin blood smears were prepared using sterile plugged Pasteur pipettes after carefully aspirating and discarding the spent medium. A small drop of cell suspension was placed on a clean frosted glass microscope slide and a thin film made by touching the drop with the edge of another slide held at angle of 45° to the first. This spread the cells across the width of the slides and smear were air-dried, fixed with absolute methanol and stained with 10% Giemsa stain for 10 min. The slides were rinsed gently under flowing tap water, air-dried and observed in oil immersion under microscope ($\times 100$). Dilution or sub-culturing was usually done when the percentage parasitaemia was high and no contamination found on examination of slides under microscope. The necessary volumes of culture 50% fresh erythrocytes and medium needed for 5 ml, 6% hematocrit culture were calculated from the formulae:

$$\text{Culture volume (CV)} = \text{S/D}$$

$$50\% \text{ erythrocyte volume (EV)} = 6/(50-\text{CV})$$

$$\text{Medium volume} = (\text{CV}+\text{EV})$$

Where D is the reciprocal factor of the desired dilution factor (D=10 for 1:10 dilution).

The appropriate volume of 50% RBC and medium were mixed together in a new 25 cm³ culture flasks using sterile technique, gassed (92% N₂, 5% CO₂ and 3% O₂) and incubated for 20 min. at 37 °C. The desired volume of old culture was then added, gassed (92% N₂, 5% CO₂ and 3% O₂) and incubated.

6.6.4.9 Freezing of parasites (cryopreservation)

This was done according to Rowe *et al.* (1968) to ensure enough supply of laboratory adapted isolates as well as having manageable culture flask. Briefly, thick smear was usually made to ascertain the cultures to be frozen are not contaminated. The culture to be cryopreserved was transferred into 50 µl centrifuge tube and centrifuged at 1500 rpm (400 g) for 5 min. at 20 °C. After aspirating the supernatant, packed cell volume (PCV) was estimated and one equivalent of Rowe's cryosolution added. Aliquots of 0.25 ml were then put into 2 ml cryonals (Nunc®, U.S.A) placed in aluminium canes, which were placed into liquid nitrogen freezer.

6.6.4.10 *In vitro* sensitivity test

The semi-automated micro-dilution technique of Desjardins *et al.* (1979) for assessing *in vitro* anti-plasmodial activity as modified by Le Bras & Deloron (1983) was adopted for the drug sensitivity studies for chloroquine, plant extracts and isolated compounds against *P. falciparum* isolates. The 96 flat-bottom well micro-titre plates (8 rows ×12 columns) were set such that all wells except the controls contain 25 µl of doubling concentrations of drug solutions. Parasitized red blood cells (200 µl) were added so that the total volume per well is 225 µl.

6.6.4.10.1 Preparation of plant extracts and chloroquine for *in vitro* assays

The dry plant extract samples were retrieved from the freezer (4 °C) and dissolved in distilled water so that the final highest concentration in the micro-titre plates was 250 µg/ml. For these experiments, 0.045 g of the plant extract was dissolved to a final volume of 20 ml (stock solution of 2,250 µg/ml). Since the final volume in each well was 225 µl, this stock solution was meant for the first row at a concentration of 250 µg/ml using the formula:

$$C_1 V_1 = C_2 V_2$$

Where C_1 = Initial concentration, V_1 =Initial volume, C_2 = Final concentration and V_2 = final volume

Taking into account that the volume of each drug in each well was 25 µl, the highest concentration (250 µg/ml) was calculated so that

$$2,250 \text{ } \mu\text{g/ml} \times V_1 = 250 \text{ } \mu\text{g/ml} \times 225 \text{ } \mu\text{l}$$

$$V_1 = 250 \times 225 / 2250 = 25 \text{ } \mu\text{l}$$

$V_1 = 25 \text{ } \mu\text{l}$ in the first row of the micro titre plate

For drugs which were not readily soluble in water, especially non-polar extracts of hexane and chloroform, they were dissolved in 50 μl of dimethylsulphoxide (DMSO) (solvent concentration in tests did not exceed 0.02%) and the volume adjusted to 20 ml with distilled water (Elueze *et al.*, 1996).

6.6.4.10.2 Preparation of micro-titre plates

This was done according to Desjardins *et al.* (1979). The 96 well flat bottomed micro-titre plates (Nunc®, U.S.A) with covers were used for drug sensitivity tests. Under sterile conditions in the laminar flow hood (Bellco Glass Inc., U.S.A), the plates were laid along the columns (1-12) and sterilized deionised water (25 μl) was added with a multi-channel pipette from row B to H, exempting row A (two columns were used for each drug and one plate therefore accommodated 6 drugs in duplicate). Two-fold dilution were done by transferring 25 μl of the drug with a multi-channel micro-pipette from row A down to row G and the last 25 μl from G discarded. Row H wells were exempted since they served as controls. Thus, wells in row A had a concentration of 250 $\mu\text{g/ml}$, row B 125 $\mu\text{g/ml}$ as concentrations halved down to row G, which had the lowest concentration at 3.905 $\mu\text{g/ml}$. The plates covered and kept at 4 °C for 48 h.

6.6.4.10.3 Addition of parasites to the pre-dosed plates

This was done according to Desjardins *et al.* (1979). Briefly, the test culture at ring stage, having a percentage parasitemia (%P) $\geq 4\%$ and growth rates (GR) $\geq 3\%$ was used for sensitivity tests. After examining the parasite under microscope, the % P of the test culture to be added to the wells of pre-dosed plates was adjusted to 0.4% and haemactocrit (hct) adjusted to 1.5% with 50% RBC. The mixture (200 μl) was then added into each well except for H₇ to H₁₂. If for instance the %P of the test culture (V_i) was 4% and the number of plates to be set was 1 (n=1), the following calculations were done to the cultures maintained at 5 ml and 6% heamatocrit.

$$C_i V_i = C_f V_f$$

Where C_i and C_f = initial and final concentrations, respectively

V_i and V_f = initial and final volumes, respectively

$$C_i = 4\%$$

$$C_f = 0.4\%$$

The volume of the plate (V_f) was calculated as follows approximating 96 wells to 100 wells:

$$V_f = 1 \text{ plate} \times 100 \text{ wells} \times 200 \mu\text{l} (\text{volumes of culture per well})$$

$$= 2000 \mu\text{l} = 20 \text{ ml}$$

The volume of the test culture (5 ml, 6% hct), which was used (V_i) was calculated as follows;

$$C_i V_i = C_f V_f$$

$$4\% \times V_i = 0.4\% \times 20 \text{ ml}$$

$$V_i = 2 \text{ ml}$$

Since 5 ml has 6% hct or $6/100 \times 5 \text{ ml} = 0.3 \text{ ml}$ (100% RBC)

2 ml culture has 0.12 ml (100% RBC)

To adjust haemotocrit to 1.5% of V_f

$$1.5/100 \times 20 \text{ ml} = 0.3 \text{ ml}$$
 (100% RBC)

But the V_i (2 ml) has 0.12 ml (100% RBC) and $(0.3 - 0.12) = 0.18 \text{ ml}$ (100% RBC) were required. This requires the addition of 50% RBC. Since the remaining 0.18 ml haemocrit is 100% RBC, $0.18 \text{ ml} \times 2 = 0.36 \text{ ml}$ of % RBC is needed.

The final volume of 20 ml, needed is achieved by addition of CMS to 2 ml test culture and 0.36 ml (50% RBC)

$$\text{CMS needed} = 20 \text{ ml} - (2 + 0.36) \text{ ml}$$

$$= 17.64 \text{ ml}$$

This means that to set one plate using a culture whose %P = 4, 17.64 ml CMS, 0.36 ml (50% RBC) and 2ml test culture to achieve 0.4% P and 1.5 haemotocrit is required. The pre-warmed CMS (37 °C) was put into 25 cm³ flask, the appropriate volume of 50% RBC added, flushed with gas 92% N₂, 5% CO₂ and 3% O₂ mixture (BOC, Kenya) and kept at 37 °C incubator for 5 min.

Using sterile technique in a laminar flow hood, the appropriate volume of test culture was added in the flask containing CMS and 50% RBC, and gently swirled in a circular motion to mix. The pre-dosed plates were warmed at 37 °C for about 20 min. retrieved, placed in the laminar flow hood and the test culture put into sterile tissue culture dishes (Lux®, U.S.A). Using 1-200 µl pipette, aliquots of 200 µl were dispensed into wells except for H₇ to H₁₂ (6 wells). To these, unparasitized red blood cells (UPRBC) were added (negative control) so that H₁-H₈ served as parasitized RBC (PRBC) (positive control) since they had no drug and the former served as UPRBC control.

For 1 plate (6 wells):

$$\begin{aligned}\text{Volume} &= 6 \text{ wells} \times 200 \mu\text{l} = 1200 \mu\text{l} \\ &= 1.2 \text{ ml}\end{aligned}$$

$$\begin{aligned}1.5 \text{ hct} &= 1.5/100 \times 1.2 \text{ ml} = 0.018 \text{ ml (100% RBC)} \\ &= 0.036 \text{ ml (50% RBC)}\end{aligned}$$

$$\begin{aligned}\text{CMS} &= (1.2 - 0.036) \text{ ml} \\ &= 1.164 \text{ ml}\end{aligned}$$

0.36 ml of 50% RBC was mixed with 1.164 ml CMS and 200 µl was aliquoted into wells H₇ to H₁₂ using a multi-channel pipette. The same procedure and calculations were done for n number of plates (n = 2, 3, 4....etc)

6.6.4.10.4 Incubation of plates

This was done according to Desjardis *et al.* (1979). Briefly, after replacing the lids of micro-titre plates gently, they were placed into a gas-tight box lid which had a damp tissue to maintain a humid atmosphere in the chamber. The gas box lid was replaced and the air-tight box flushed with gas mixture (92% N₂, 5% CO₂ and 3% O₂) and incubated at 37 °C. After 48 h, [G-³H] hypoxanthine (1 µCi/well) was pulsed in aliquots of 25 µl into each well and the plates incubated for further 48 h.

6.6.4.10.5 Harvesting of cells and scintillation counting

This was done according to Desjardis *et al.* (1979). Briefly, the cells were harvested using a multiple semi-automatic cell harvester (Skarton®, Norway) onto glass fibre filters (Skarton®, Norway) for each row from A to H. The filters were then dried at 37 °C overnight (18 h), introduced into scintillation vials, 1 ml of scintillation fluid (ecolume) added, and the vials loaded into a liquid scintillation β-counter (1211 Minibeta, England). Disintegrations per minute were calculated for each sample represented the incorporation of [G-³H] hypoxanthine into the parasite nucleic acids.

6.6.4.10.6 Inhibitory concentration 50 (IC₅₀)

This was done according to Sixsmith *et al.* (1984). The IC₅₀ refers to the drug concentration inhibiting 50% of the parasite incorporation of [G-³H] hypoxanthine found in the drug free PRBC wells. The UPRBC's counts per minute (CPM) values were taken as background count and corrected CPM values of each well by subtracting UPRBC CPM values from each wells CPM values of each well. To calculate the IC₅₀ values, the mid-point (Y₅₀) was calculated by the formula:

$$Y_{50} = (\text{PRBC CPM values} - \text{UPRBC CPM values})/2$$

IC₅₀ was calculated from the formula:

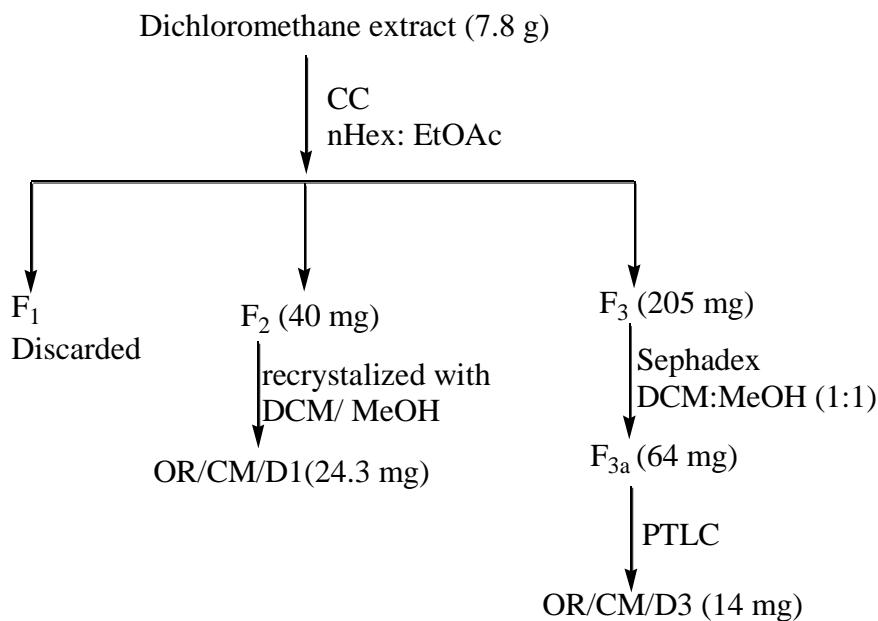
$$\text{IC}_{50} = \text{Antilog}(\log X_1 + [(\log Y_{50} - \log Y_1)(\log X_2 - \log X_1)])/\log Y_2 - \log Y_1$$

Where IC₅₀ = Inhibitory concentration 50, X₁ and X₂ lower and higher concentrations respectively, Y₁ = CPM values which correspond with X₂

6.7 Isolation of compounds from the roots of *Cissampelos mucronata*

The dichloromethane extract (7.8 g) was subjected to fractionation by column chromatography on silica gel with hexane: ethyl acetate gradient (100:0-0:100) giving 45 fractions. These were pooled on the basis of the R_f of the spots and concentrated *in vacuo* to give three fractions F₁, F₂ and F₃. F₂ and F₃ which were subjected to further purification and two compounds OR/CM/D1 and OR/CM/D3 obtained. OR/CM/D1 was obtained through recrystallization of F₂ using dichloromethane and methanol. It gave a positive colour test for steroids with *p*-anisaldehyde. F₃ was re-chromatographed (sephadex LH-20, DCM: MeOH 1:1) to obtain fraction F_{3a} which was subjected to preparative thin layer chromatography (5% MeOH/DCM in NH₃). OR/CM/D3 gave a

positive reaction for alkaloids with Dragendorff's reagent. The process of isolation of compounds from *C. mucronata* extract is summarized in scheme 2.



Scheme 2: Isolation of compounds from the dichloromethane extract of *Cissampelos mucronata*

(-)-*Stigmasterol* (**204**): was isolated as white crystals (24.3 mg); mp 165-167 °C; $[\alpha]^{25}_D$ -49°; UV: λ_{max} 289 (1.01)nm; ν_{max} (KBr) 3422 and 1653 cm⁻¹; EIMS: *m/z* 412 (74), 400 (100), 396 (32), 382 (48), 300 (35), 273 (35), 255 (64), 145 (41), 107 (37), 95 (34), 81(42), 55 (44), 43 (30); ¹H NMR (CDCl₃) (Table 7), ¹³C (CDCl₃) NMR (Table 8).

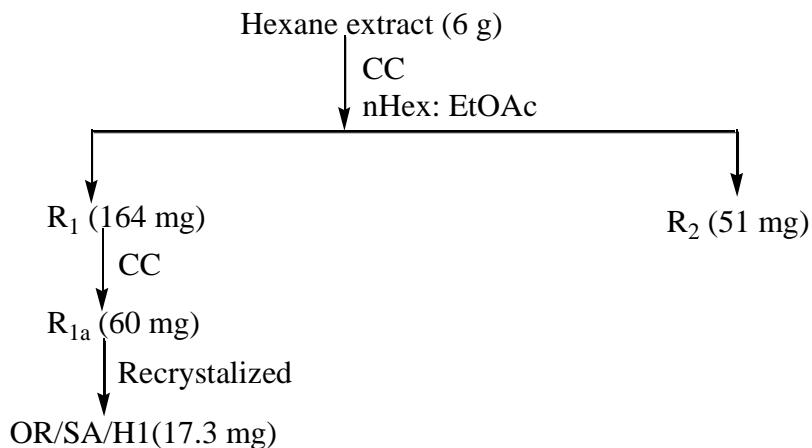
(-)-*Curine* (**205**): was isolated as white amorphous solid (14 mg); mp 215-217 °C; $[\alpha]^{25}_D$ -258° (3.4, CH₃OH); UV: λ_{max} 230 (2.36), 238 (2.51), 246 (2.55) 269 (2.67) and 285 (2.43) nm; IR: ν_{max} KBr 3384, 1507, 2930, 1653 cm⁻¹; EIMS: *m/z* 594 (12), 298 (100), 296 (15), 266 (10) 190 (14); ¹H NMR (CDCl₃) (Table 9); ¹³C NMR (CDCl₃) and DEPT (Table 10).

6.8 Isolation of compounds from the leaves of *Stephania abyssinica*

The hexane extract (12 g) was subjected to column chromatography on silica gel with hexane: ethyl acetate gradient (100:0-0:100) giving 38 fractions. These were combined together on the basis of their R_f values to yield R₁ and R₂. R₂ was not worked on because

it contained many compounds but the amount was very small. R₁ was subjected to repeated column chromatography using DCM: hexane to yield R_{1a} which was then recrystallized using DCM: hexane (1: 19) to obtain (+)-nonacosan-10-ol (**206**) (OR/SA/H1) (17.3g). It gave positive test with *p*-anisaldehyde. The process of isolating OR/SA/H1 is summarized in scheme 3.

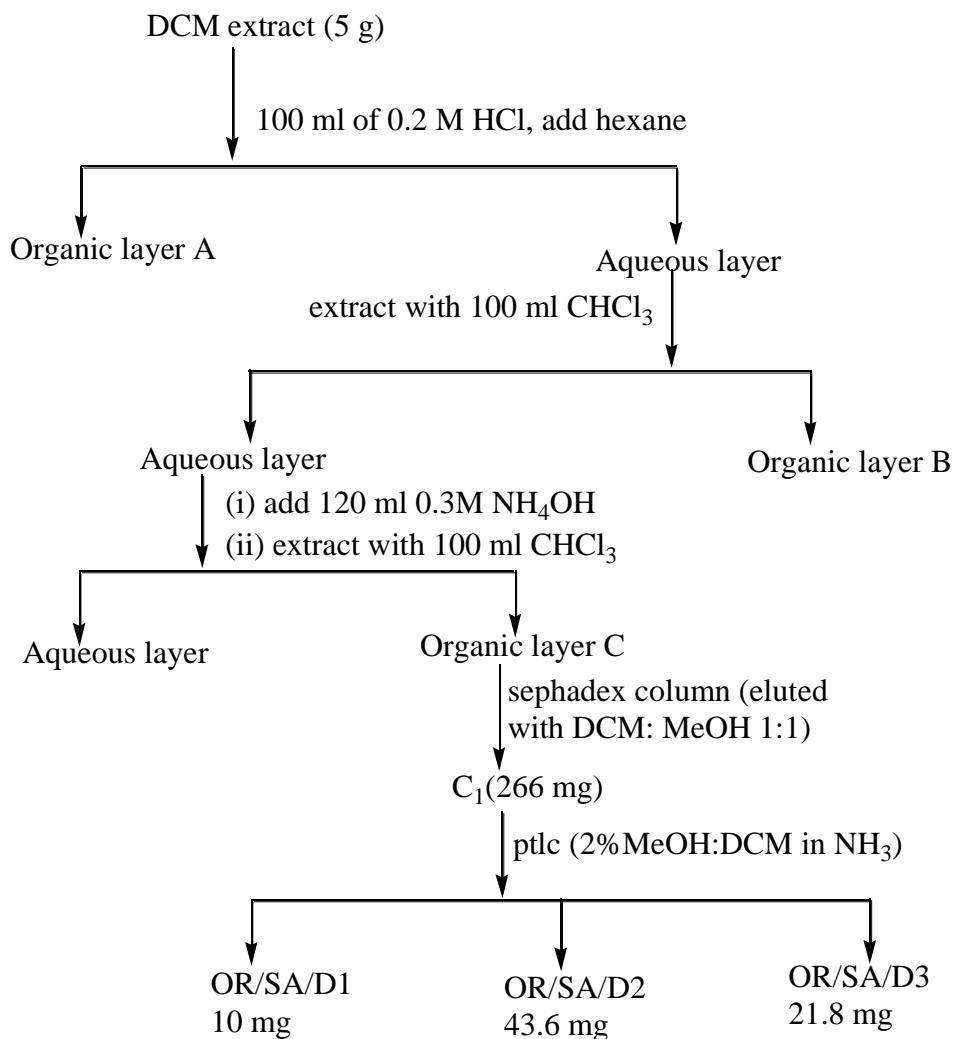
Scheme 3: Isolation of compounds from hexane extract of *Stephania abyssinica*



(+)-*Nonacosan-10-ol* (**206**): was isolated as white powder (17.3 mg): mp 79-81 °C; $[\alpha]^{25}_D +87.5^\circ$ (1.6, CHCl₃); IR: ν_{max} KBr 3337, 2849, 1470 cm⁻¹; UV: λ_{max} 222 (2.05) nm; EIMS: *m/z* 424 (2), 406 (100), 298 (25), 297 (90), 278 (12), 157 (55), 129 (32), 105 (61), 83 (46), 77 (35) 57 (32). ¹H NMR (CDCl₃) (table 11); ¹³C NMR (CDCl₃) and DEPT (Table 12).

100 ml of 0.2 M HCl was added to 5 g dichloromethane extract of *Stephania abyssinica* and heated at 80 °C for 15 min and MeOH: CHCl₃ (3:7) added. It formed two layers which were separated using a separating funnel. The organic layer was concentrated under vacuo to give fraction A. To the aqueous layer 100 ml of CHCl₃ was added and allowed to settle. The organic layer was separated and concentrated to give fraction B. 120 ml of 0.3 M NH₄OH was added to the aqueous layer and then extracted with 100 ml CHCl₃ to obtain organic layer C. Fraction A and B were combined together based on the R_f values of the spots but were not worked on further because the amount was small and it had many compounds (spots). Fraction C was loaded into sephadex LH-20 column

and eluted with DCM: MeOH (1:1). Fraction C₁ 266 mg was obtained and purified by preparative thin layer chromatography to produce OR/SA/D1 (10 mg), pseudocurine (OR/SA/D2) (43.6 mg) and isocurine (OR/SA/D3) (21.8 mg). From the NMR analysis OR/SA/D1 had impurities and could not be purified further due to the small amount of sample. However, anti-plasmodial bioassay was done for OR/SA/D1. A summary of the isolation process for OR/SA/D1- OR/SA/D3 is given in scheme 4.



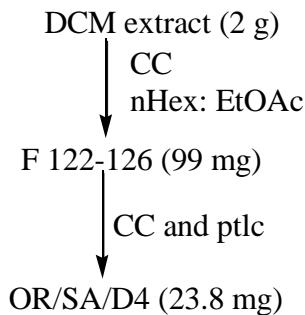
Scheme 4: Isolation of compounds from the dichloromethane extract of *Stephania abyssinica*

(-)-Pseudocurine (208) (OR/SA/D2): was isolated as brown amorphous solid (43.6 mg): mp 168-170 °C; $[\alpha]^{25}_D -262^\circ$ (2.1, CH₃OH); UV: λ_{max} 235 (2.43), 240 (2.48), 271 (2.51), 278 (2.44) and 287 (2.53) nm; IR: ν_{max} (CH₃OH) 3352.1, 2943.2, 2831.3, 1450.4, 1114.8

and 1029.9 cm⁻¹; EIMS: *m/z* 594 (100), 552 (15), 386 (23), 298 (100), 282 (67) 267 (55), 253 (28) 174 (20) 147 (22) 107 (10). ¹H NMR (CD₃OD) and COSY (Table 15); ¹³C NMR, HMQC and HMBC (Table 16).

(-)Isocurine (**209**) (OR/SA/D3): was isolated as brown amorphous solid (21.8 mg): mp 158-160 °C; [α]²⁵_D -158° (3.5, CH₃OH); UV: λ_{max} 216 (2.17), 224 (2.31), 228 (2.50) and 280 (0.90) nm; IR: ν_{max} (CH₃OH) 3352.1, 2943.2, 2831.3, 1450.4, 1114.8 and 1029.9 cm⁻¹; EIMS *m/z* 595 (100), 552 (23), 521 (15), 315 (12), 298 (100), 282 (28) 267 (20), 177 (5); ¹H NMR (CD₃OD) and COSY (Table 17); ¹³C NMR, HMQC and HMBC (Table 18).

Repeated column chromatography on another portion of dichloromethane extract eluted with hexane: EtOAc (100:0-0:100) and methanol: EtOAc (15:85) and gave 126 fractions. Fractions 122-126 (99 mg) were combined based on the R_f values of the spots, re-chromatographed on sephadex LH, (MeOH: CH₂Cl₂ 1:1) and purified by preparative thin layer chromatography to give HOR/SA/D4 which gave a positive reaction for alkaloids with Dragendorff's reagent. A summary of the isolation process for (-)-5-oxoaknadinine (HOR/SA/D4) is given in scheme 5.



Scheme 5: Isolation of (-)-5-oxoaknadinine (OR/SA/D4)

(-)5-Oxoaknadinine (**207**) (OR/SA/D4): was isolated as light yellow crystals (23.8 mg): [α]²⁵_D -291° (1.5, CH₃OH); UV: λ_{max} 231 (2.62), 237 (2.72), 250 (2.84) 272 (2.67) and 280 nm (2.58); : ¹H NMR (CD₃OD) and COSY (Table 13); ¹³C NMR (CD₃OD), HMQC and HMBC (Table 14).

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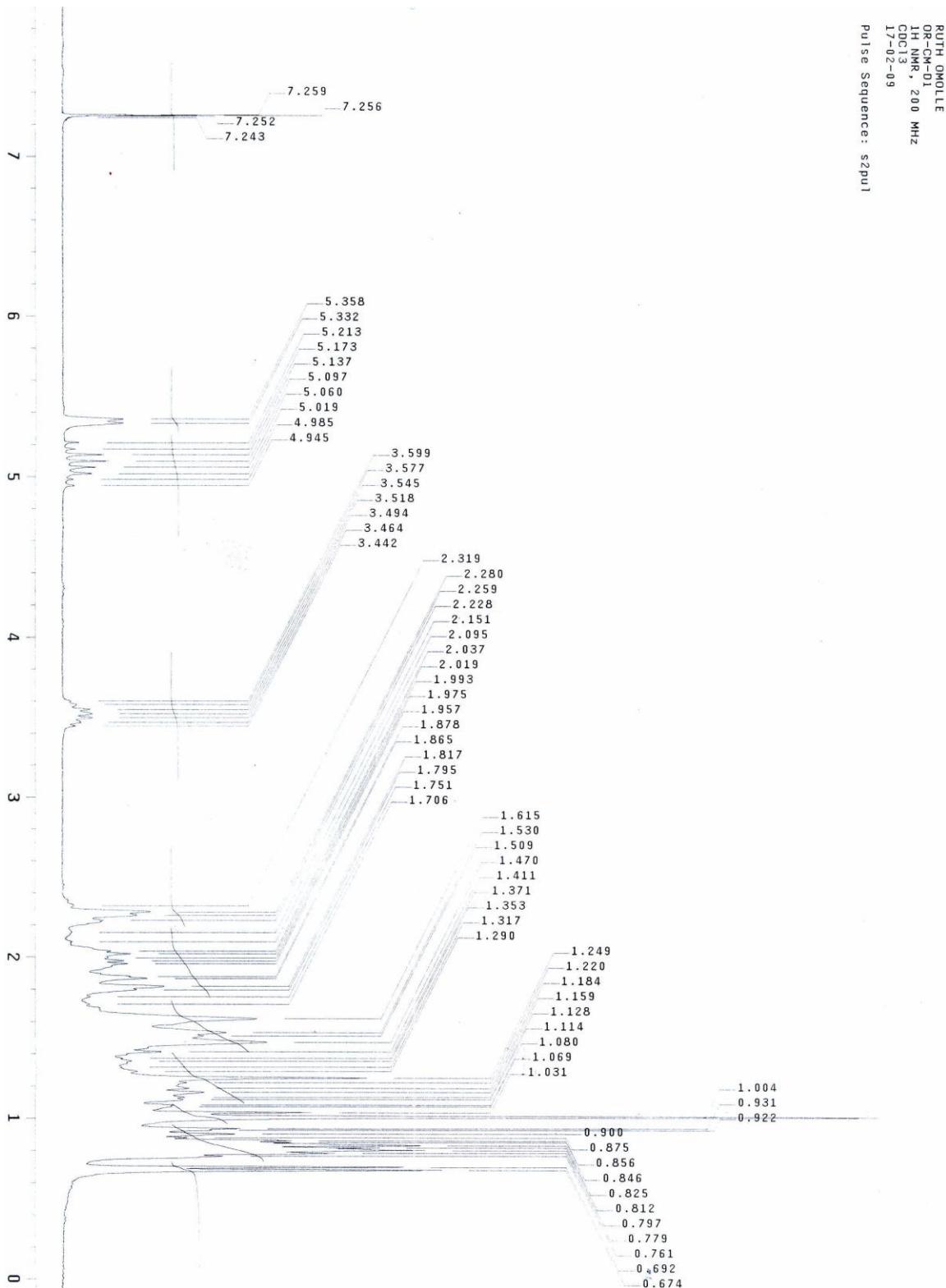
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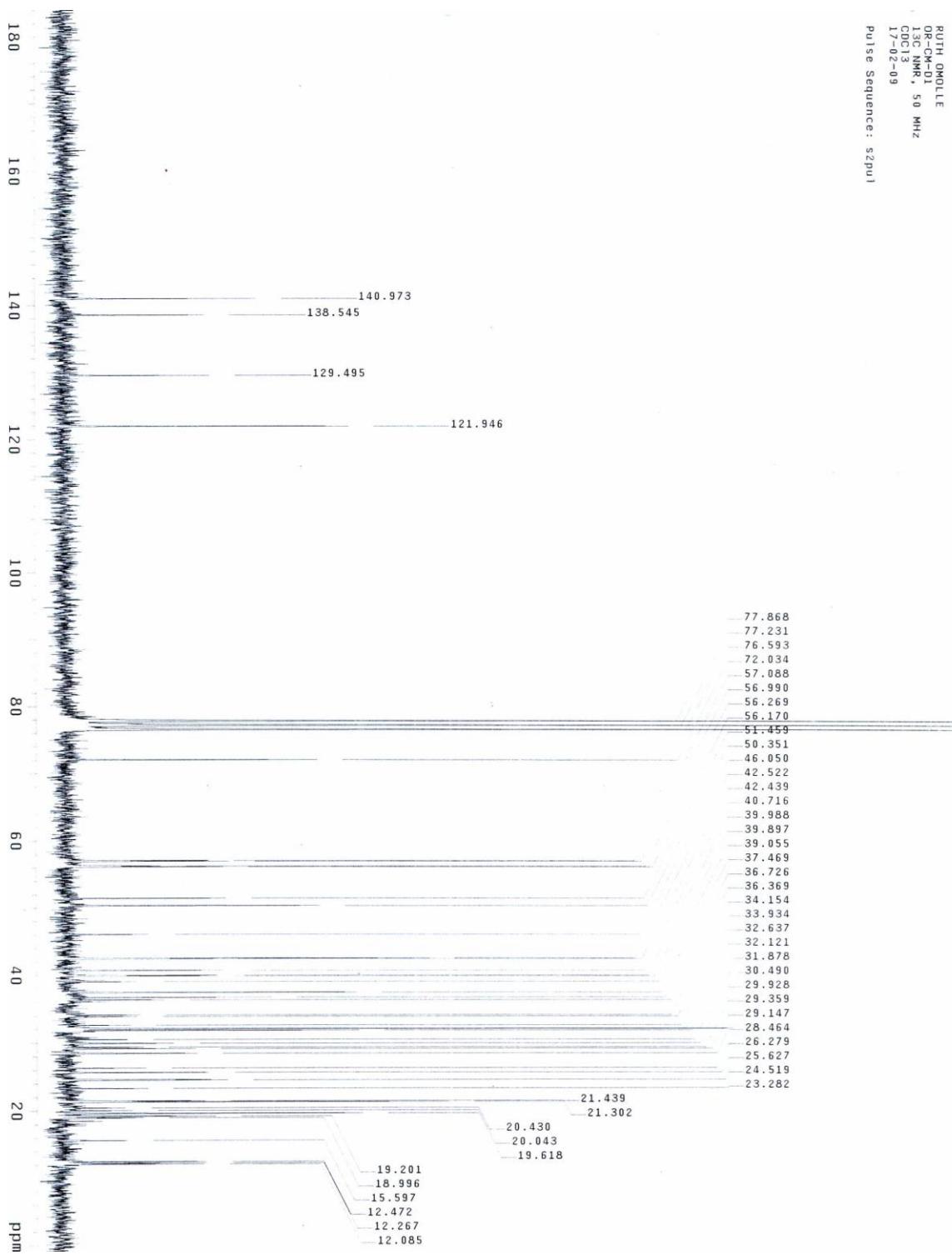
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APPENDICES

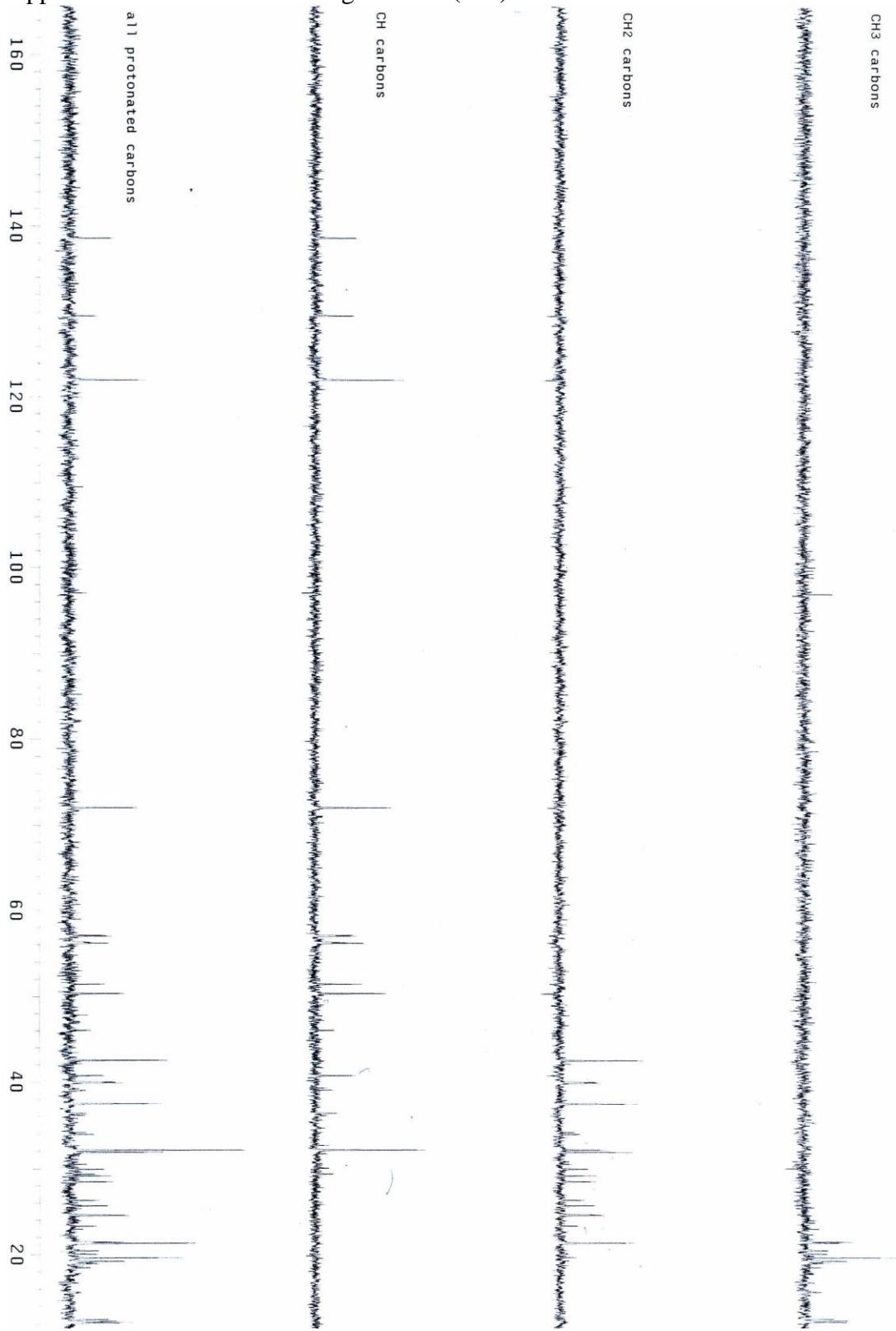
Appendix 1a: ^1H NMR (200 MHz, CDCl_3) spectrum for stigmasterol (**204**)



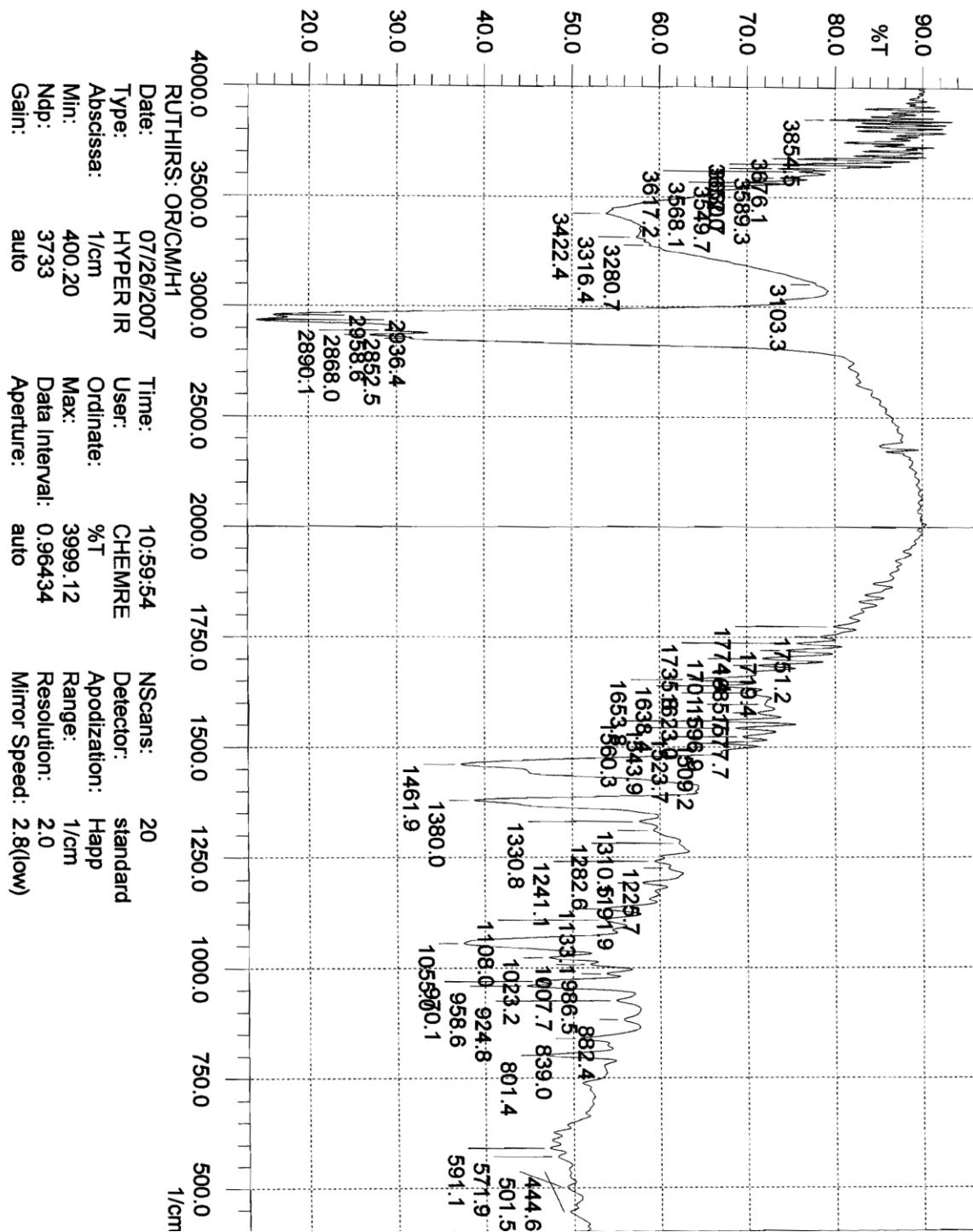
Appendix 1b: ^{13}C NMR (200 MHz, CDCl_3) spectrum for stigmasterol (**204**)



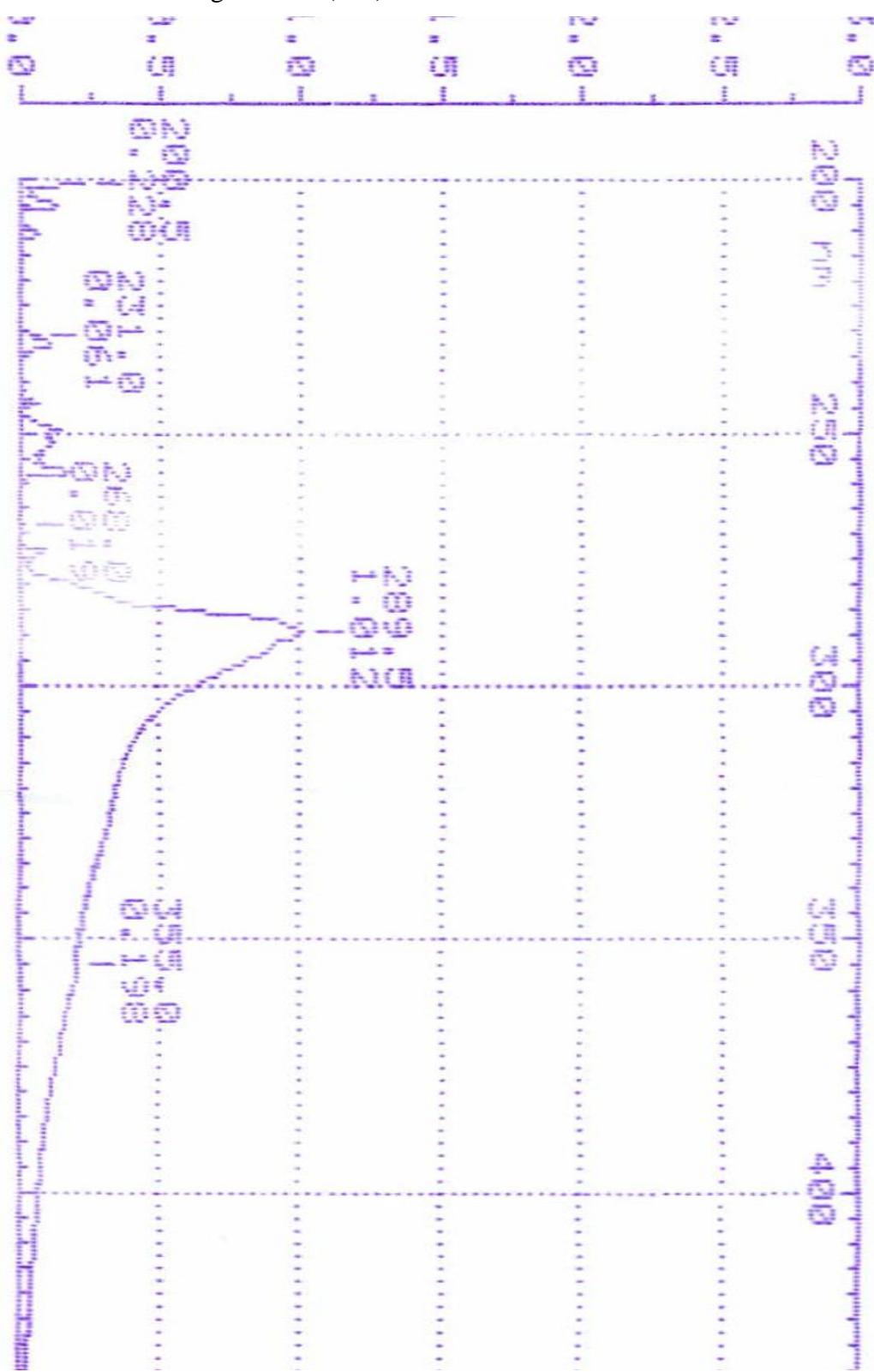
Appendix 1c: DEPT data for stigmasterol (204)



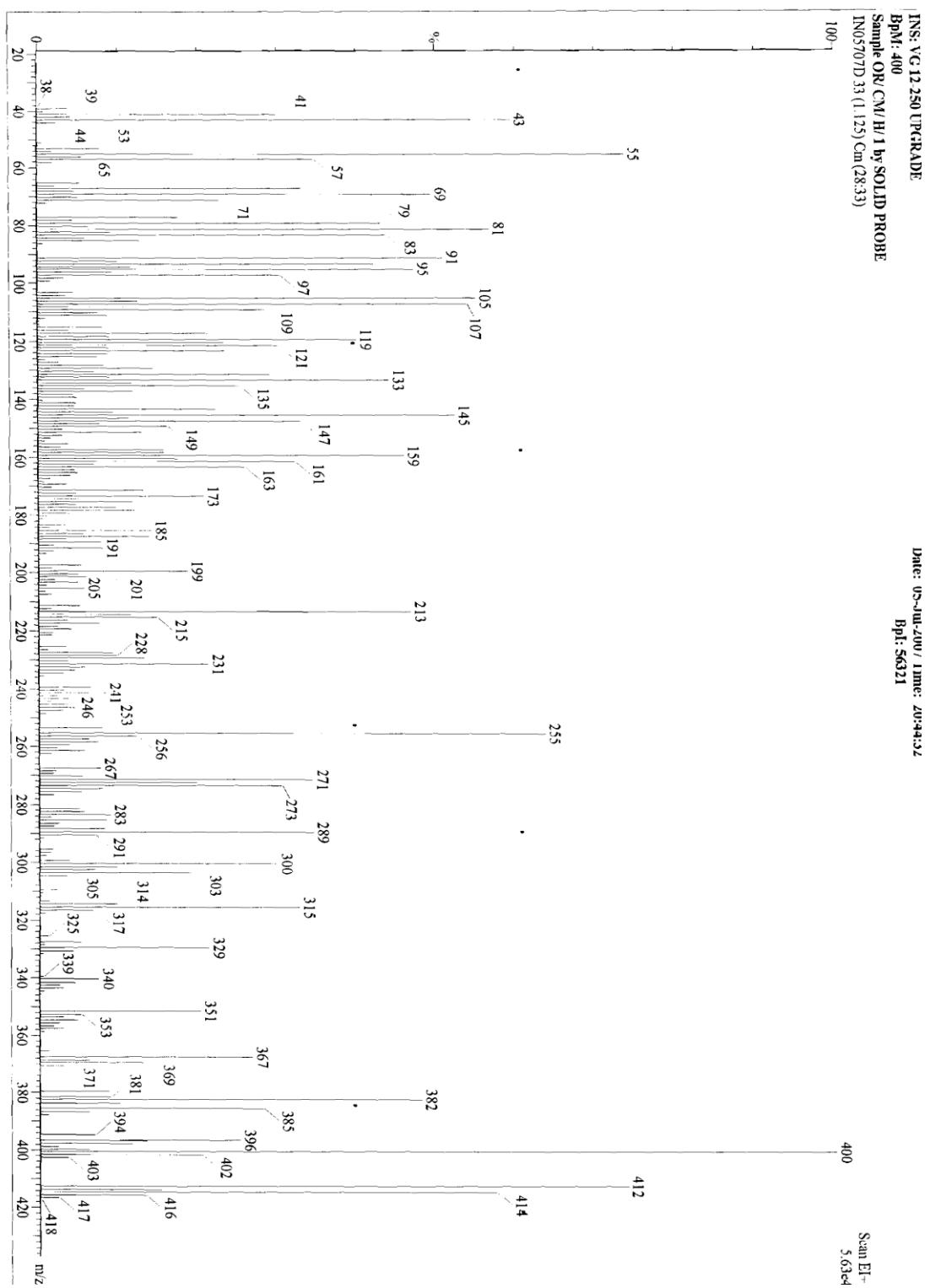
Appendix 1d: IR spectrum for stigmasterol (204)



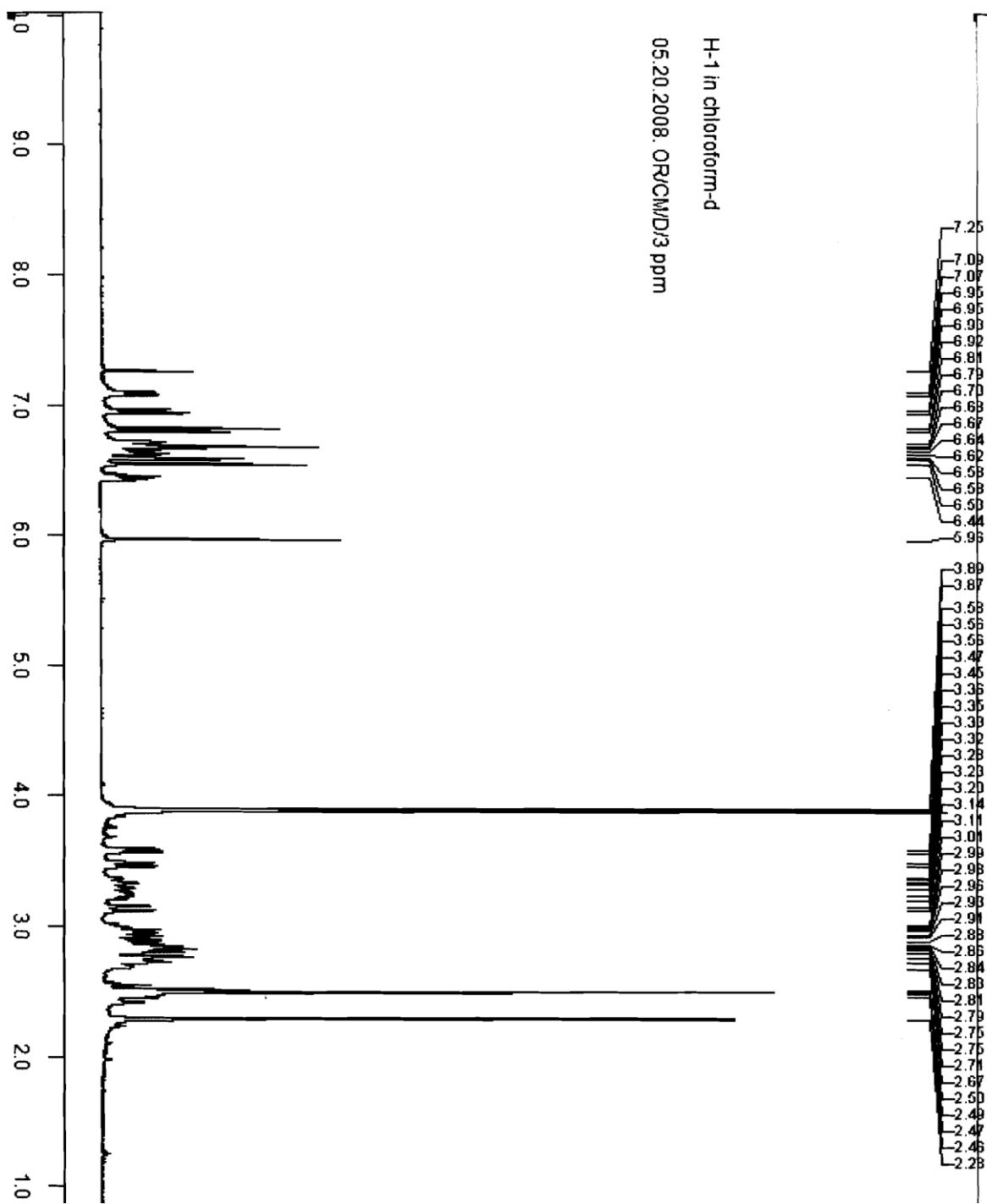
Appendix 1e: UV for stigmasterol (204)



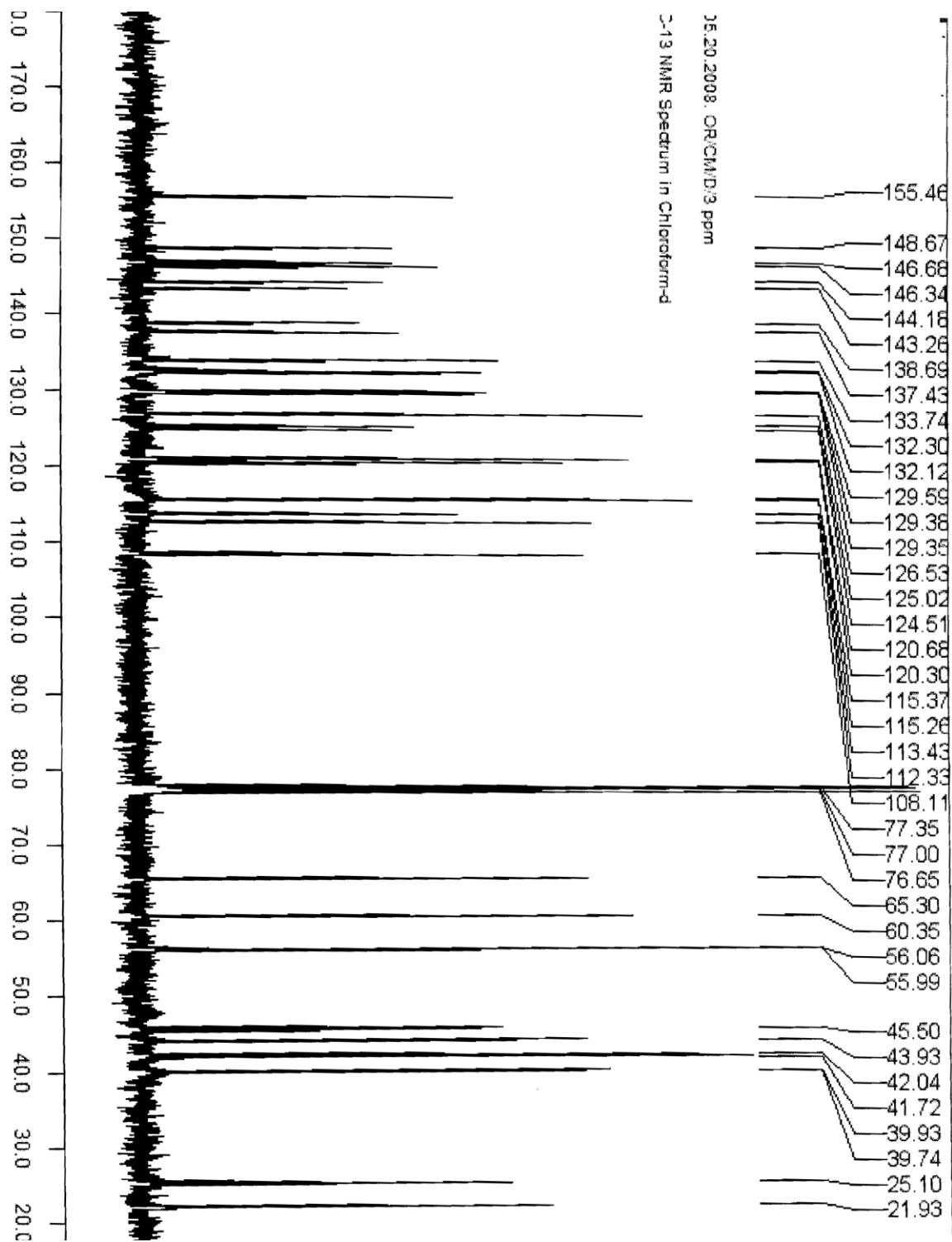
Appendix 1f: Mass spectrum for stigmasterol (**204**)



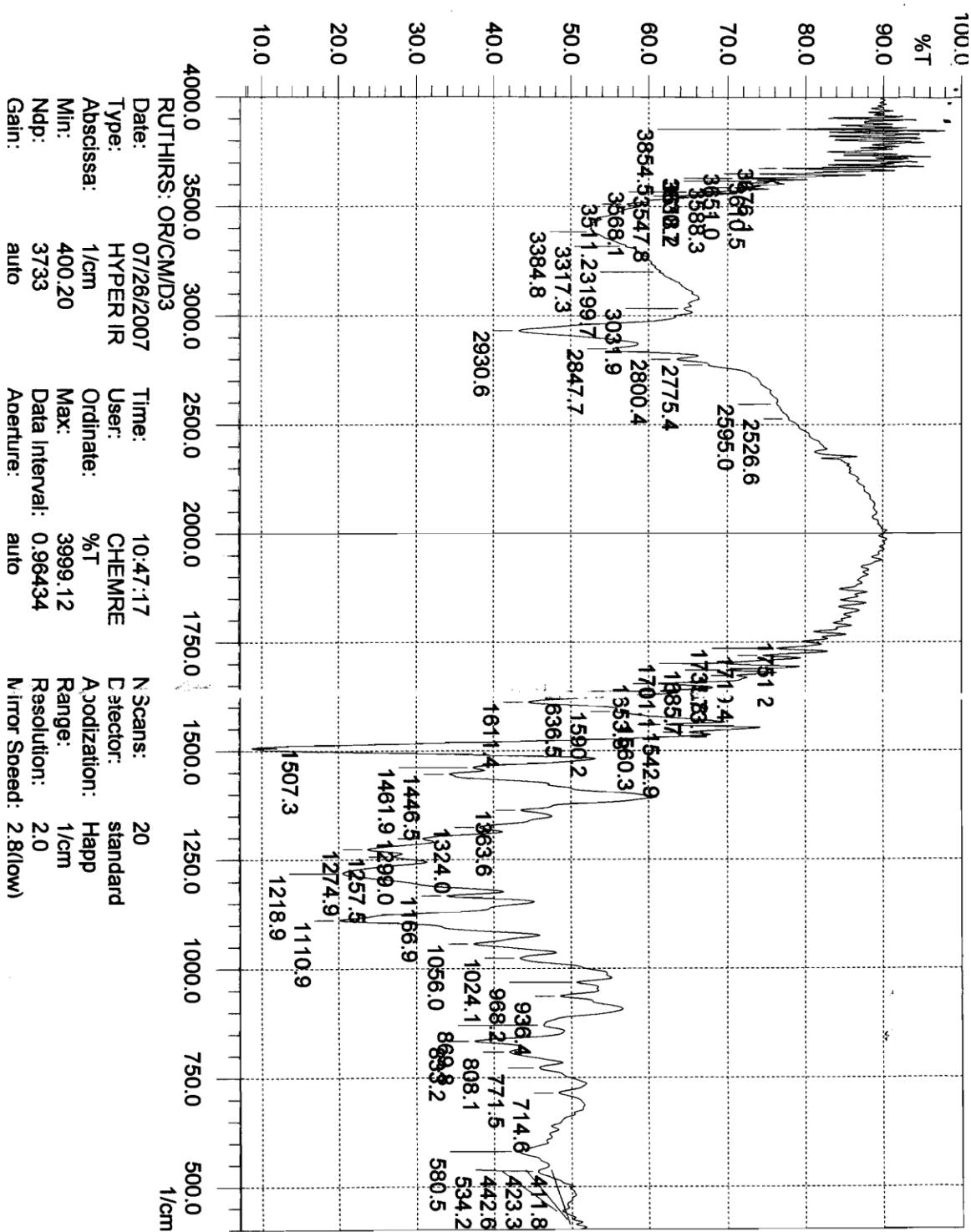
Appendix 2a: ^1H NMR (400 MHz, CDCl_3) spectrum of (-)-curine (**205**)



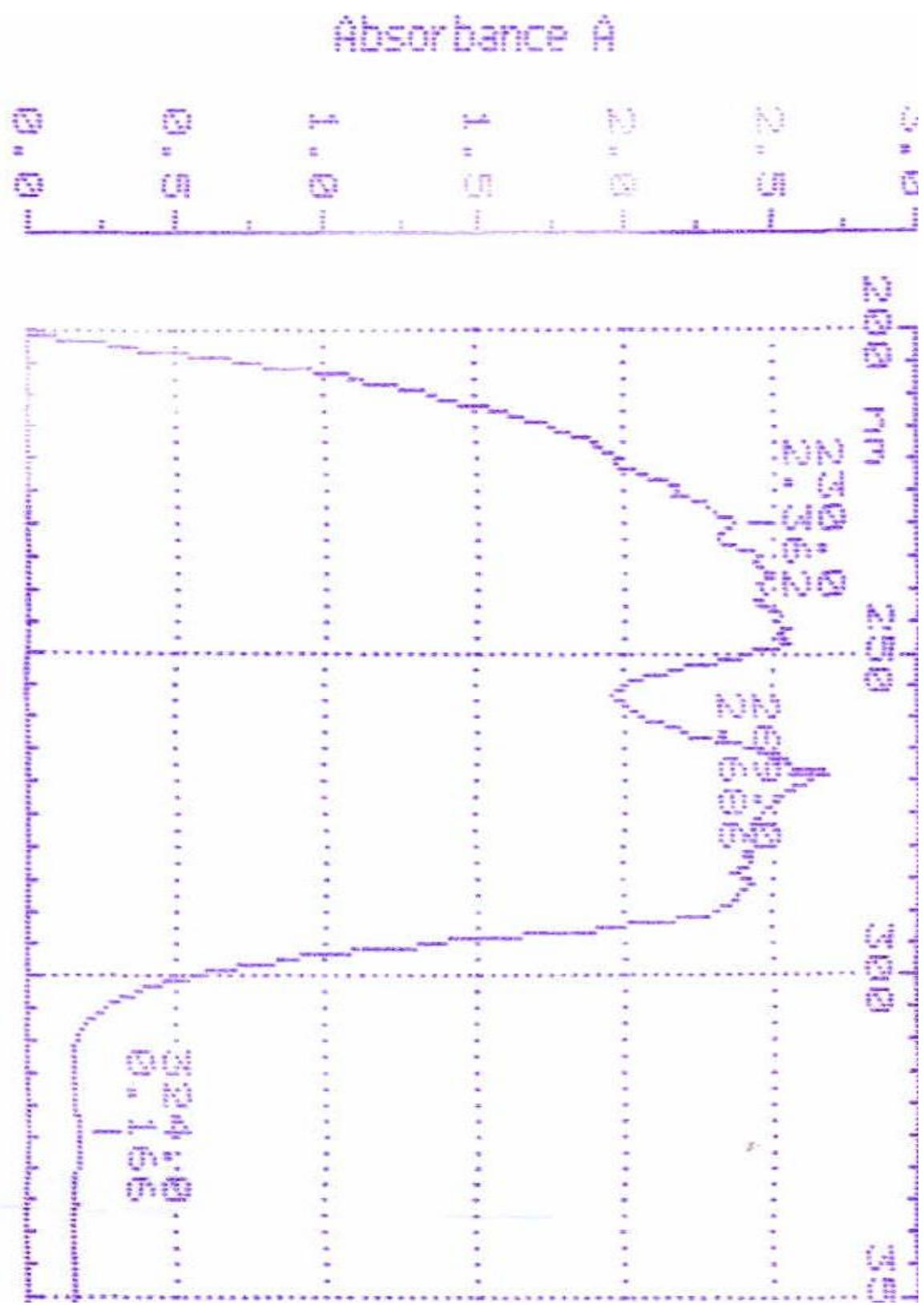
Appendix 2b: ^{13}C NMR (400 MHz, CDCl_3) spectrum of (-)-curine (**205**)



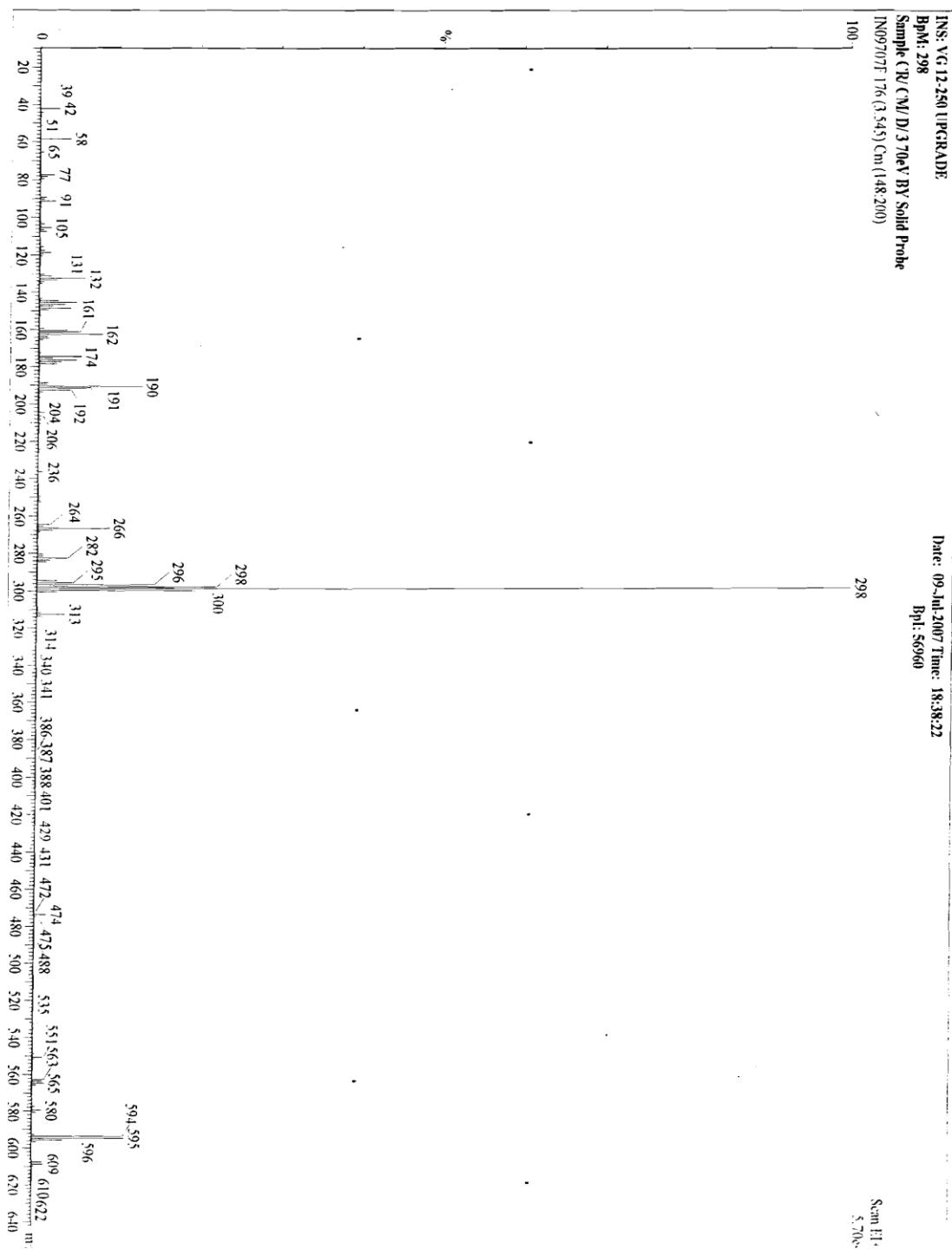
Appendix 2c: IR spectrum of (-)-curine (**205**)



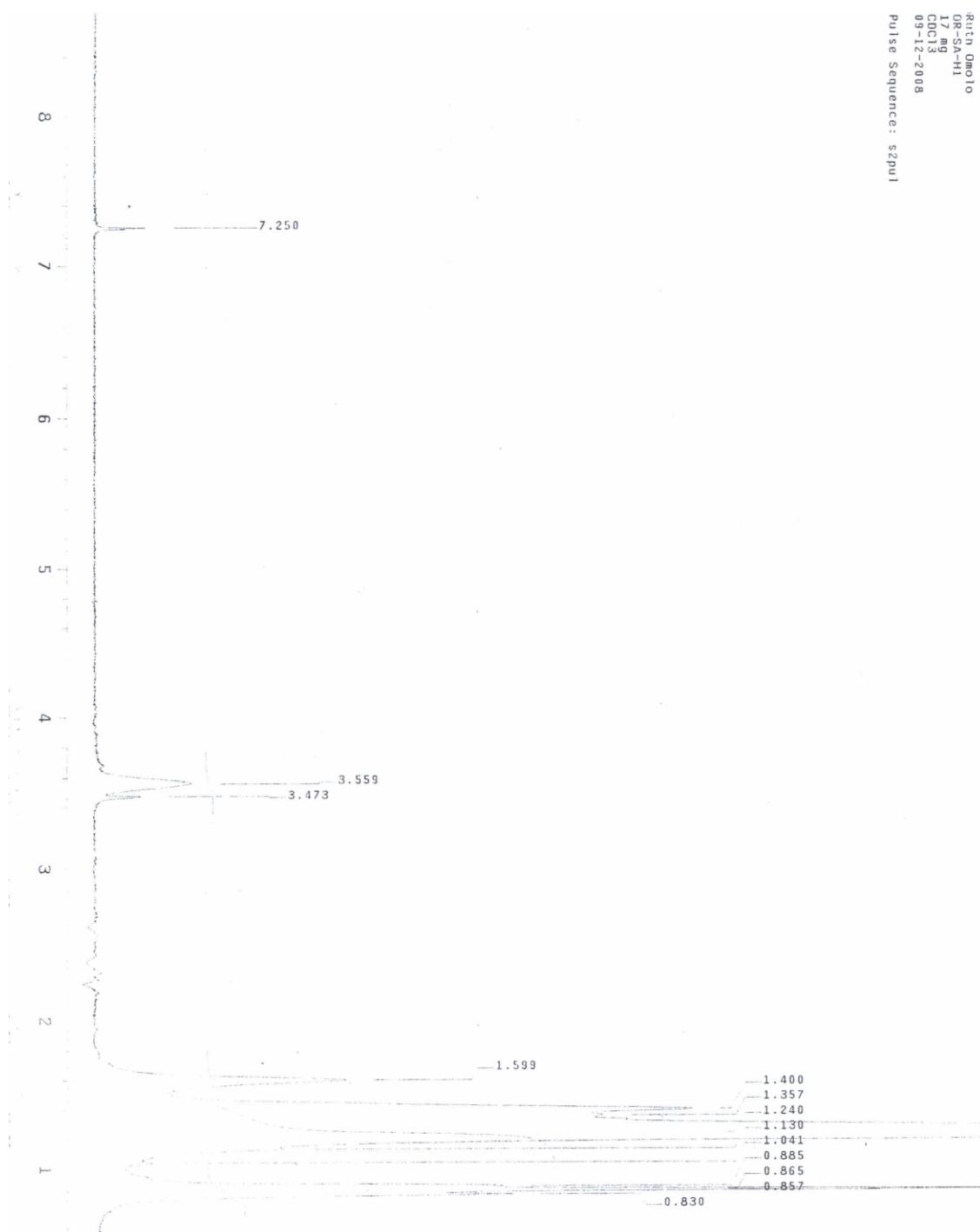
Appendix 2d: UV spectrum of (-)-curine (**205**)



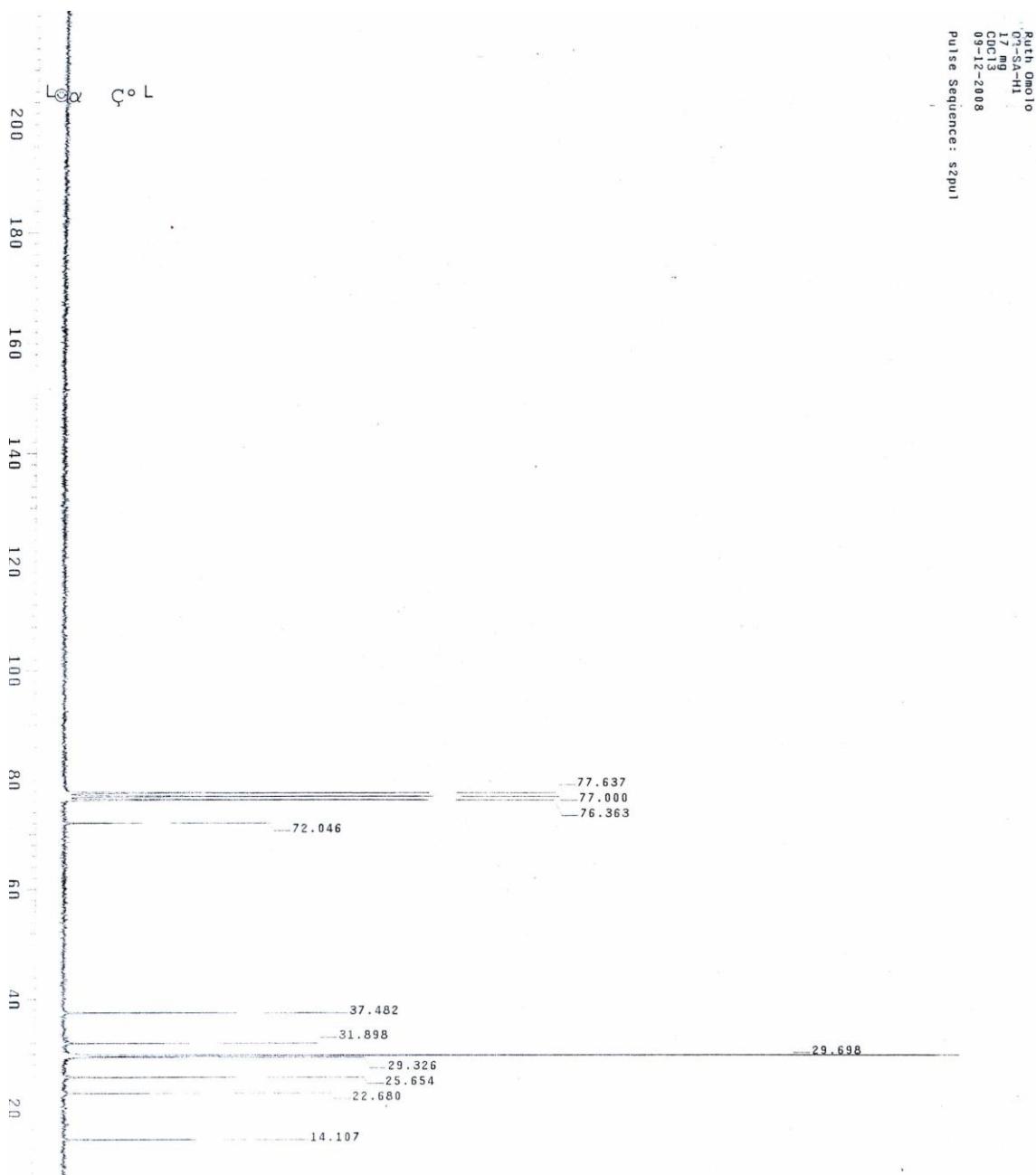
Appendix 2e: Mass spectrum of curine (**205**)



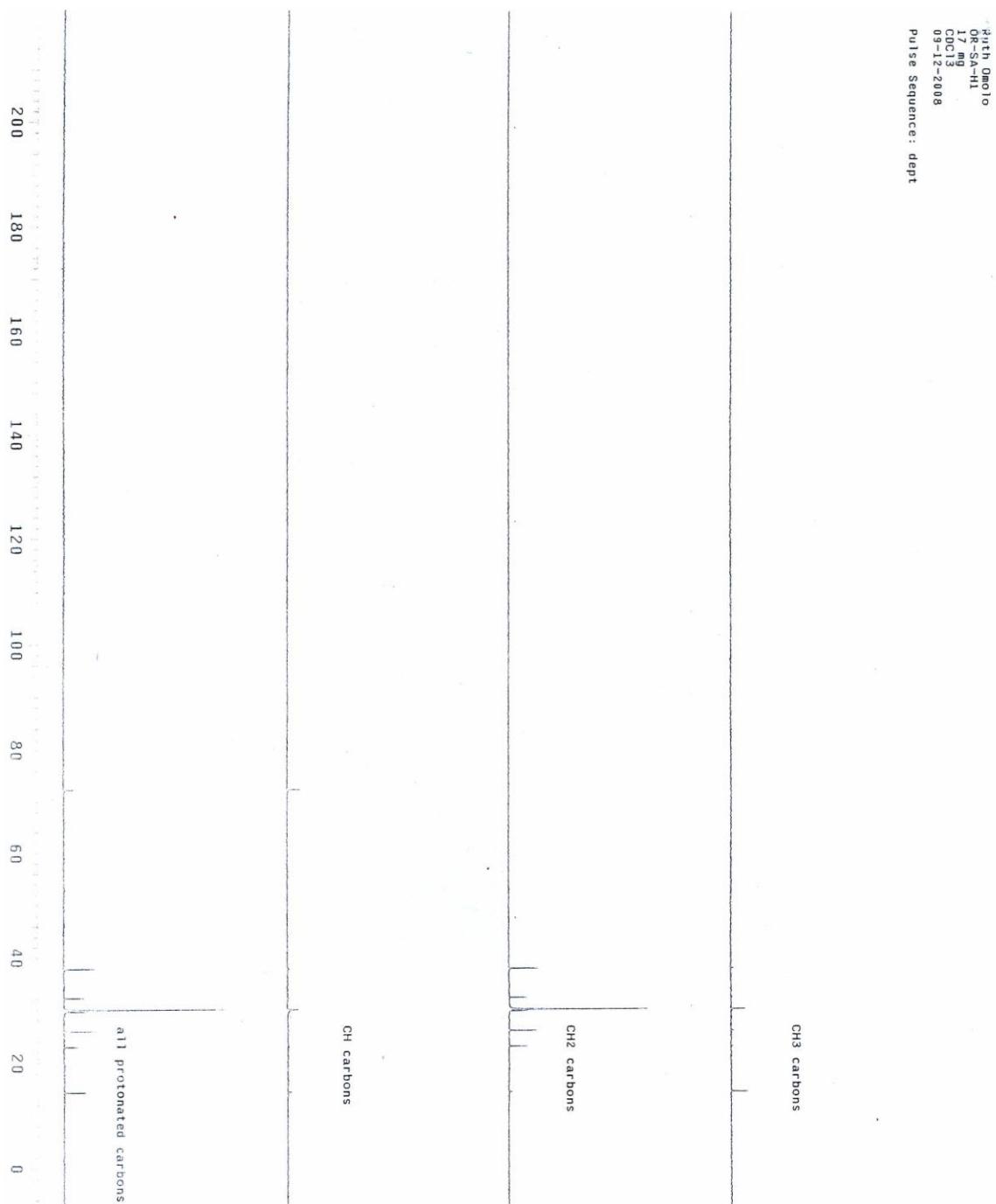
Appendix 3a: ^1H NMR (200 MHz, CDCl_3) spectrum of (+)-nonacosan-10-ol (**206**)



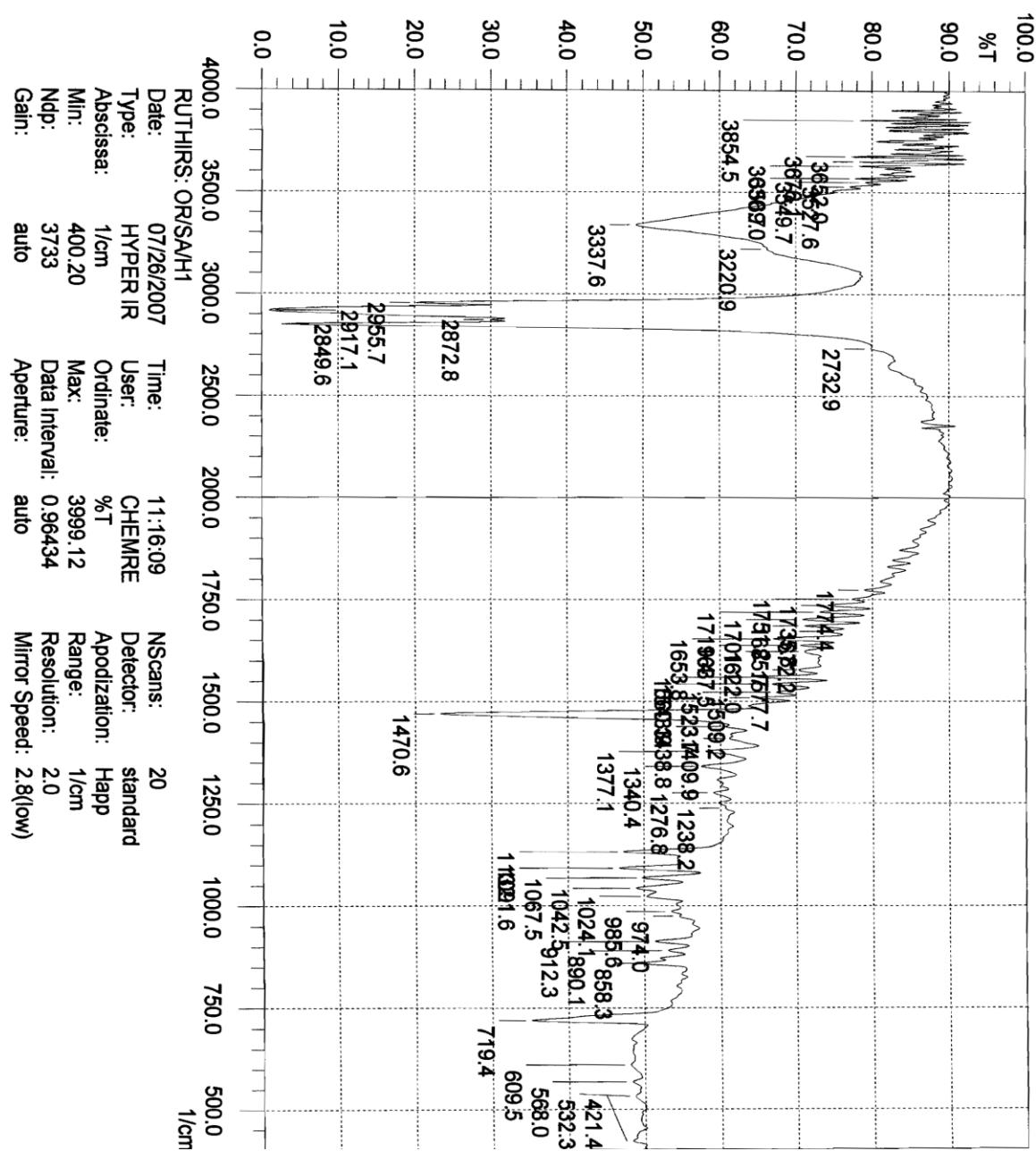
Appendix 3b: ^{13}C NMR (200 MHz, CDCl_3) spectrum of (+)-nonacosan-10-ol (**206**)



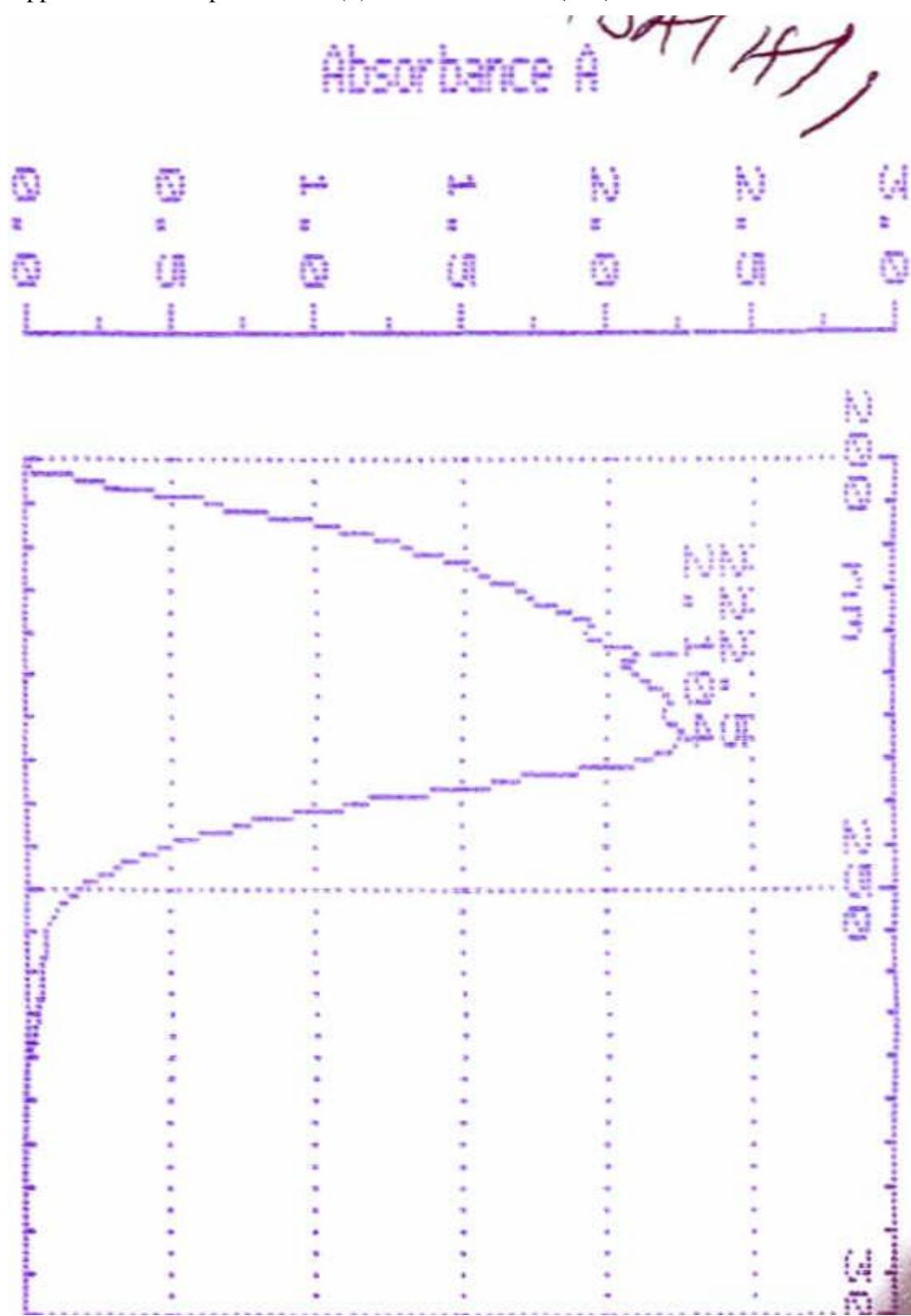
Appendix 3c: DEPT spectrum for (+)-nonacosan-10-ol (**206**)



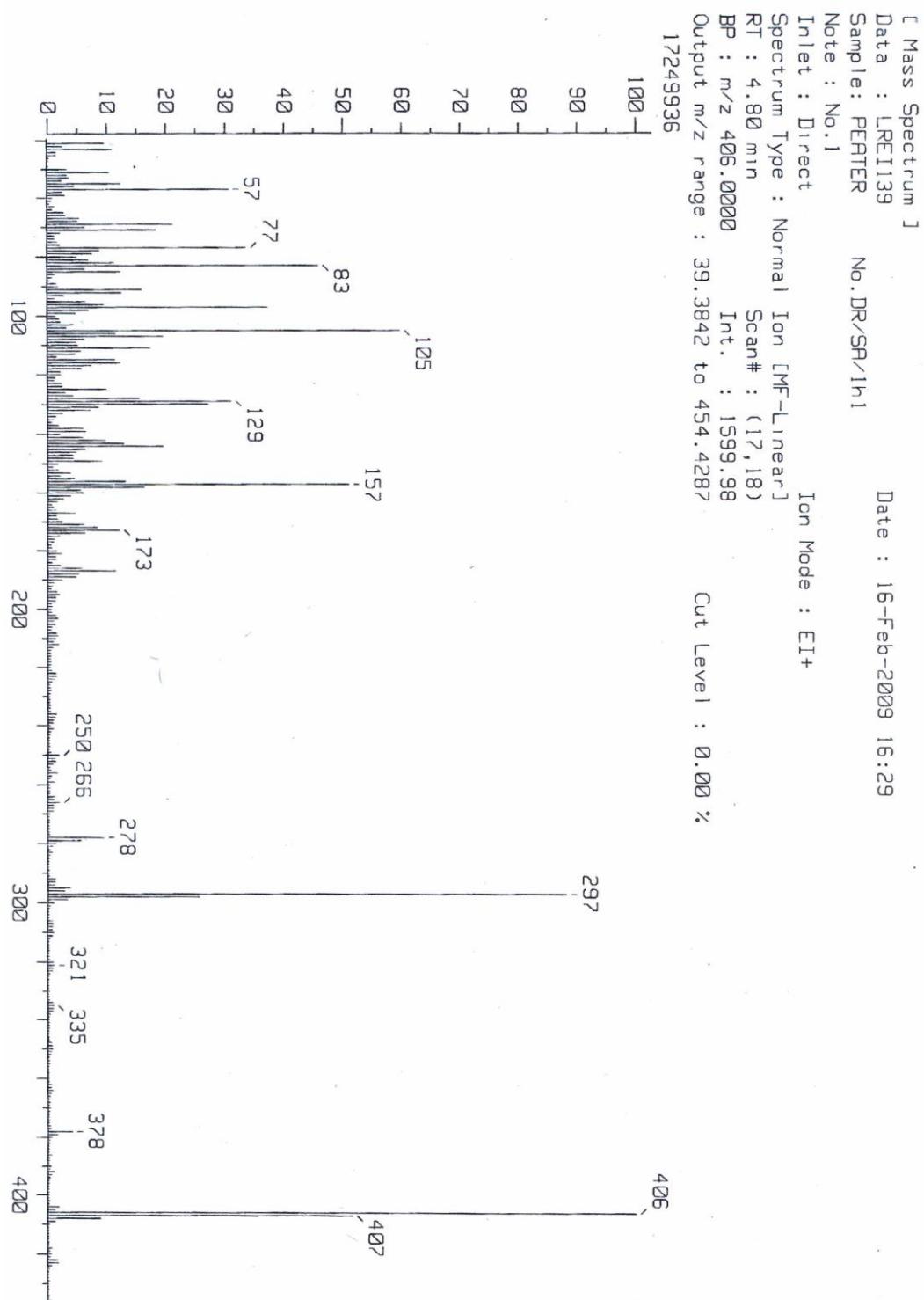
Appendix 3d: IR spectrum for (+)-nonacosan-10-ol (**206**)



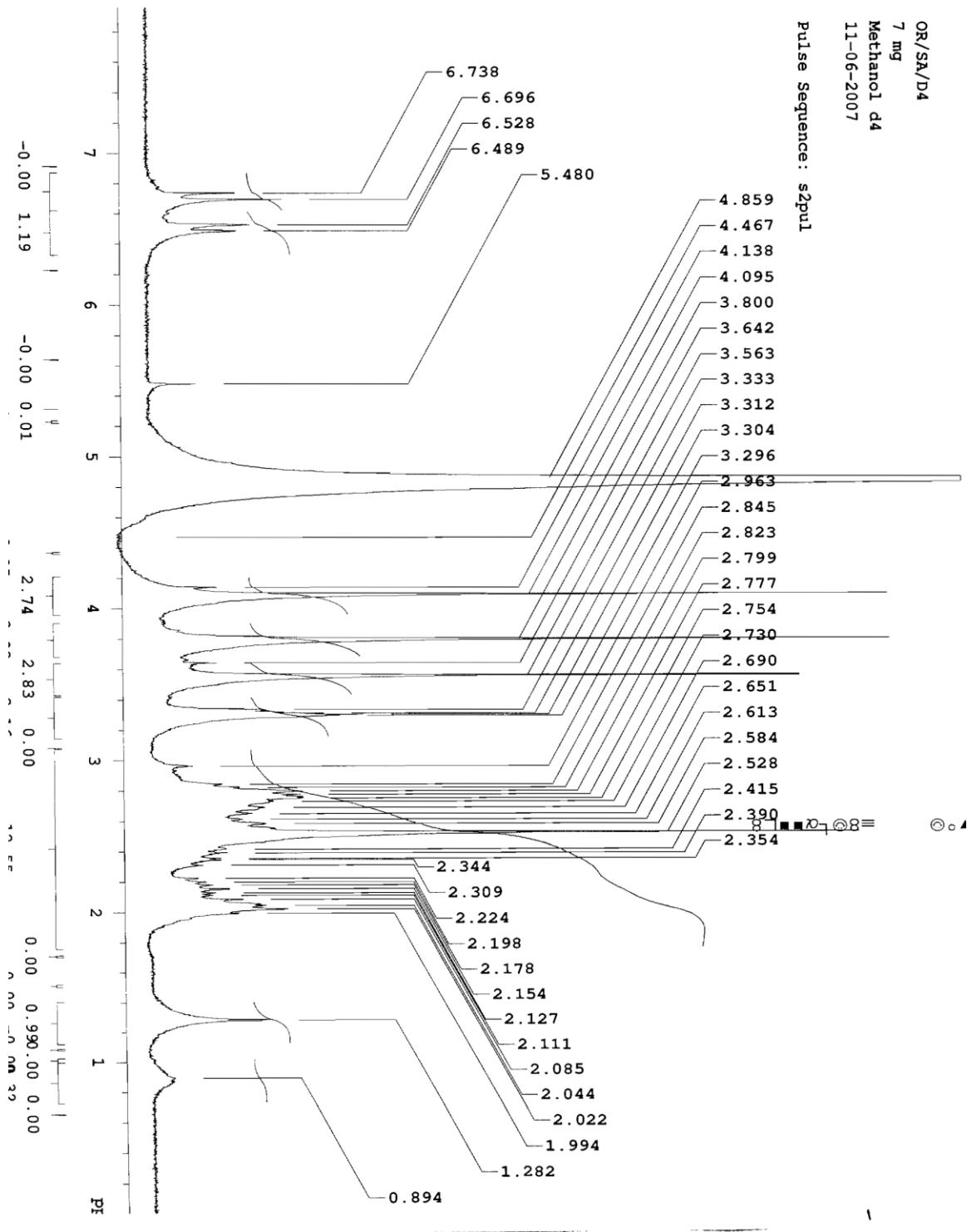
Appendix 3e: UV spectrum for (+)-nonacosan-10-ol (**206**)



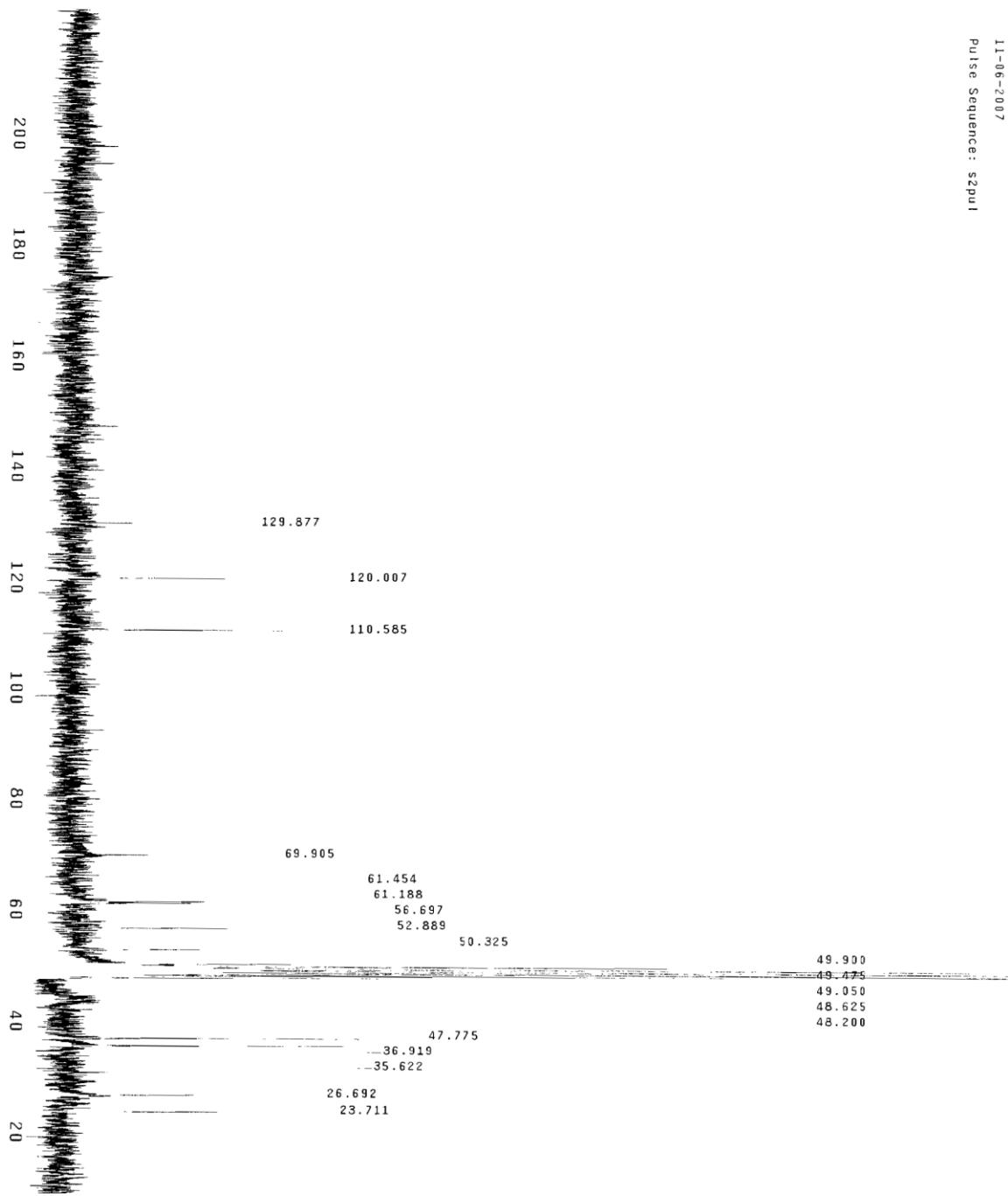
Appendix 3f: Mass spectrum for (+)-nonacosan-10-ol (**206**)



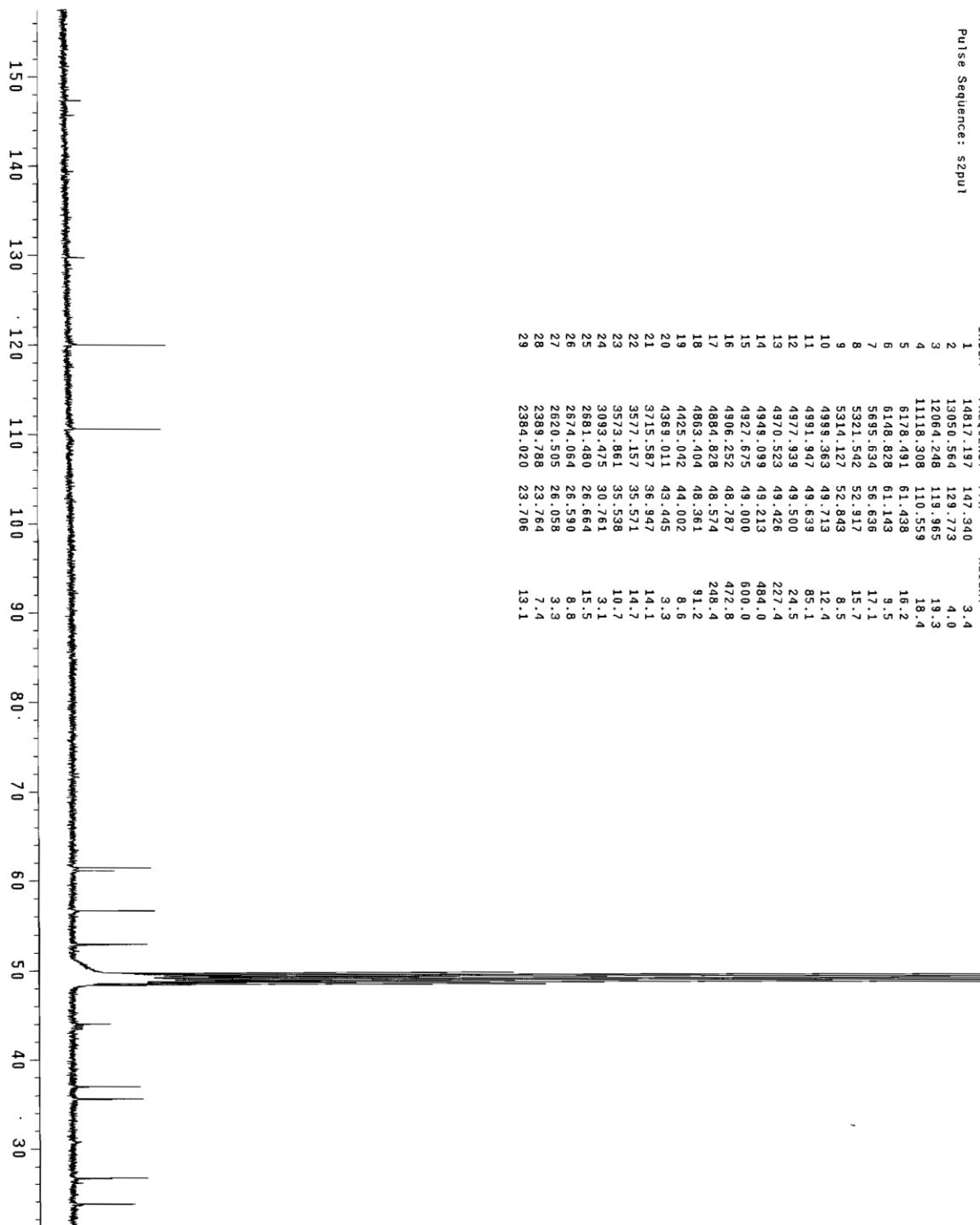
Appendix 4a: ^1H NMR (200 MHz, CD_3OD) spectrum of (-)-5-oxoaknadinine (**207**)



Appendix 4b: ^{13}C NMR (200 MHz, CD_3OD) spectrum of (-)-5-oxoaknadinine (**207**)

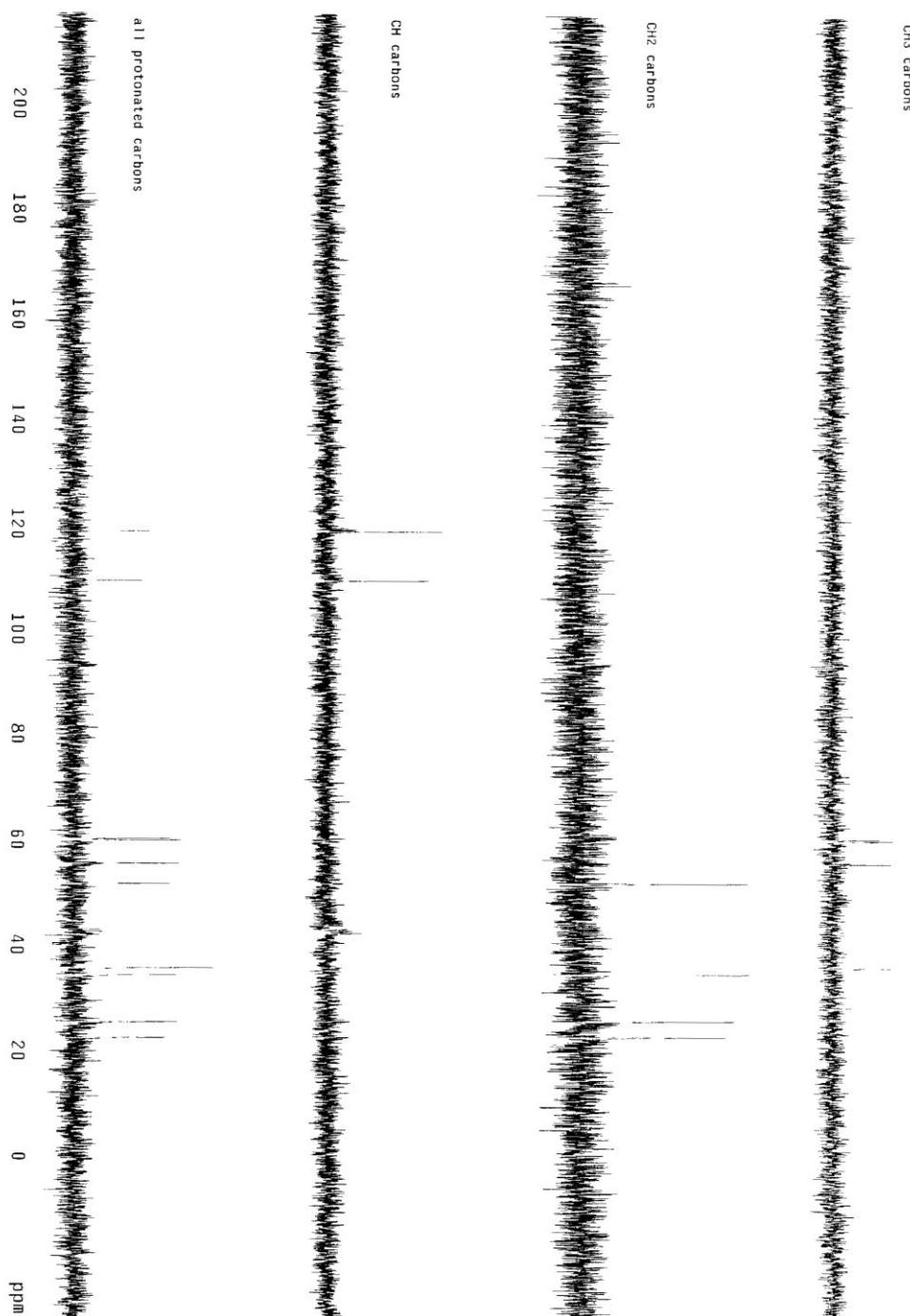


Appendix 4c: ^{13}C NMR (400 MHz, CD_3OD) spectrum of (-)-5-oxoaknadinine (**207**)

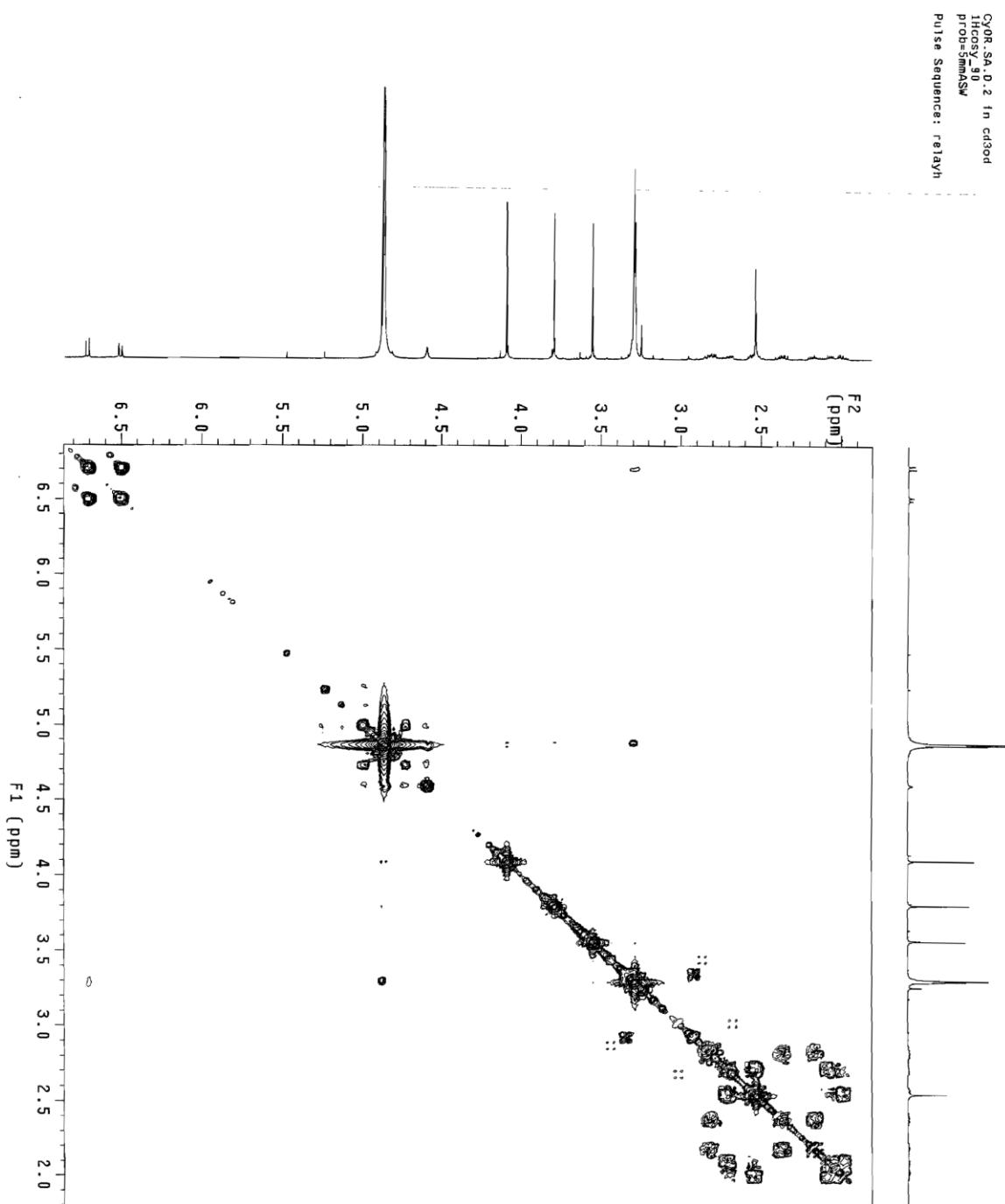


1.1 - 6.0 δ ppm
Pulse Sequence: dept

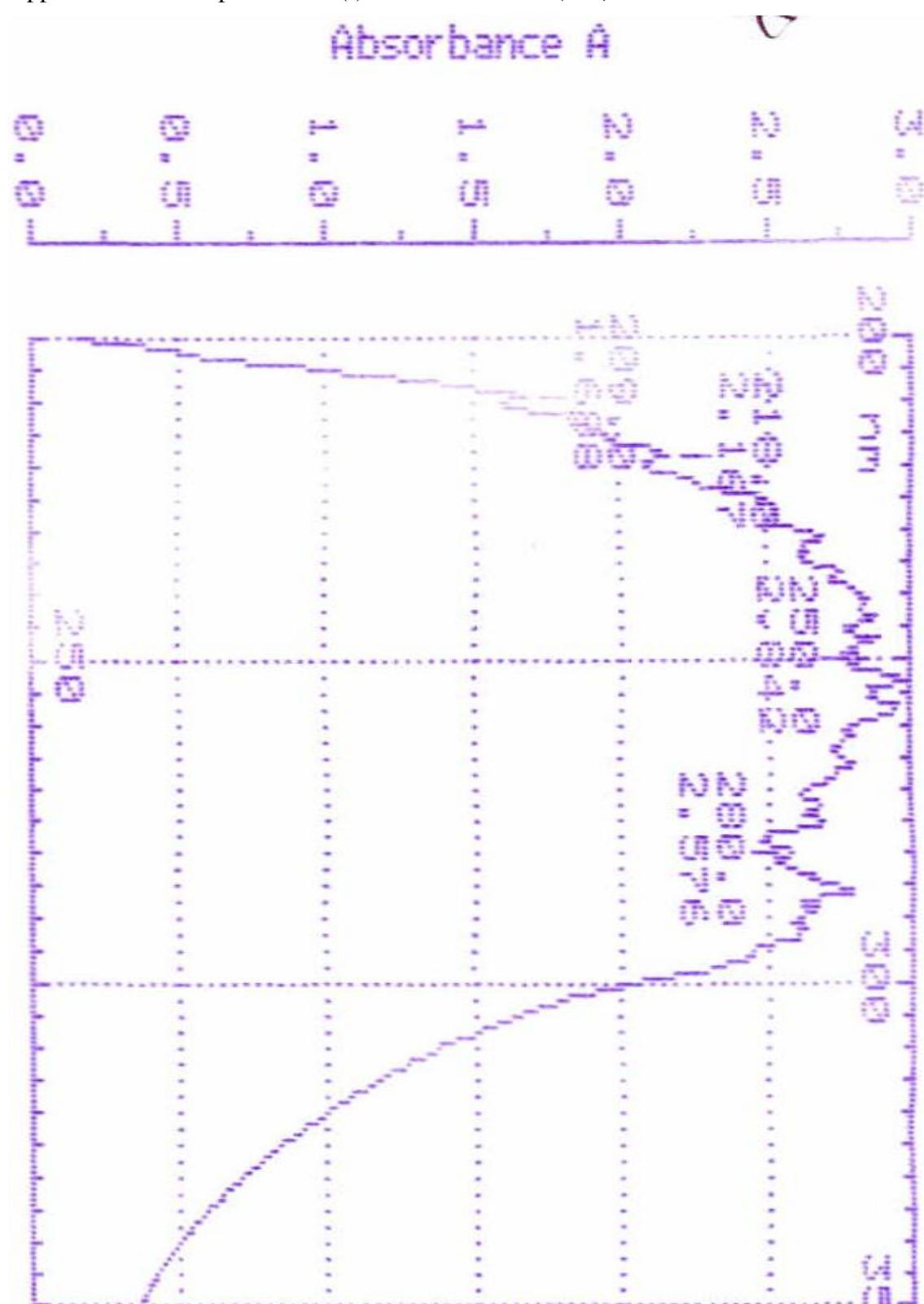
Appendix 4d: DEPT spectrum of (-)-5-oxoaknadinine (**207**)



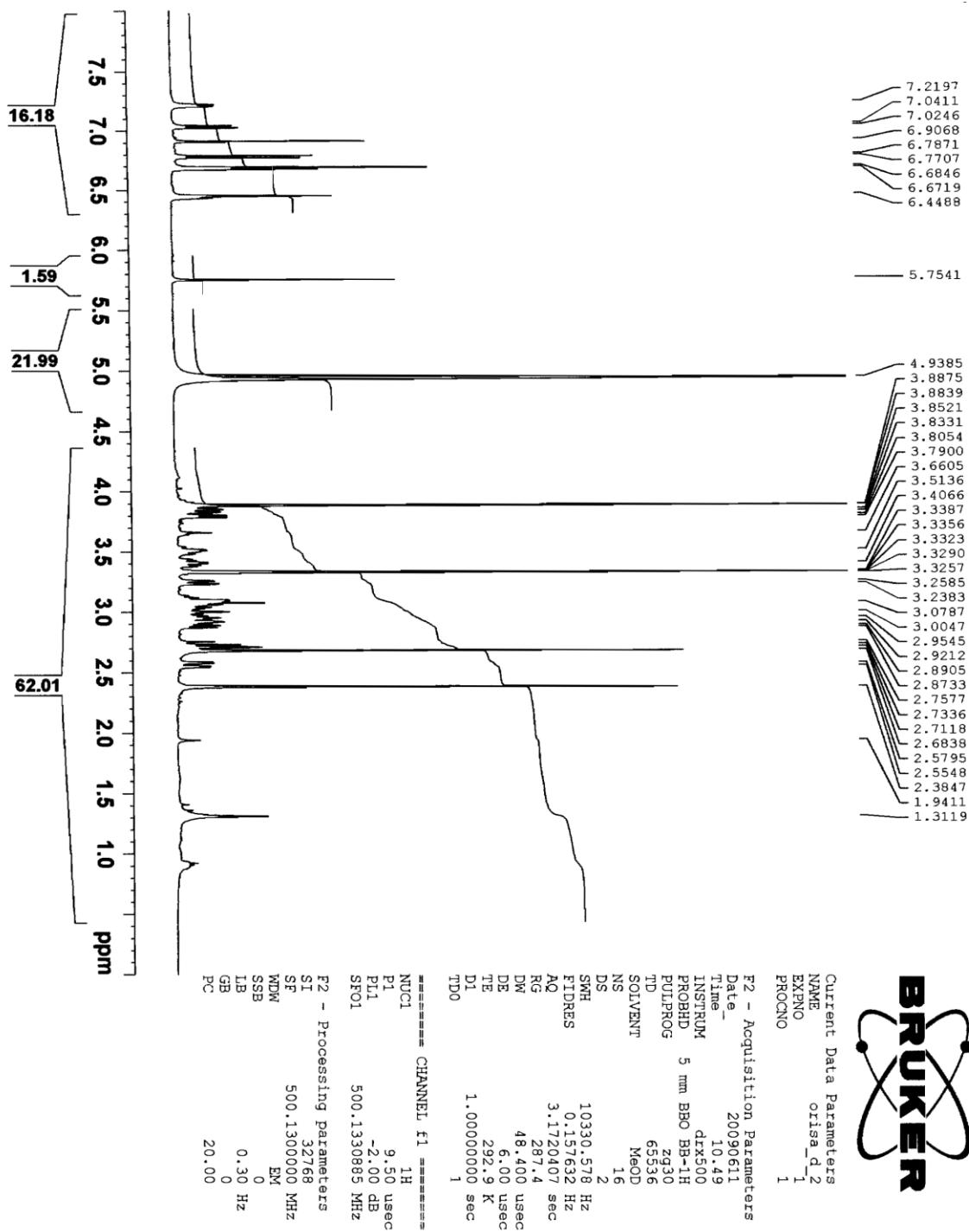
Appendix 4e: COSY spectrum of (-)-5-oxoaknadinine (**207**)



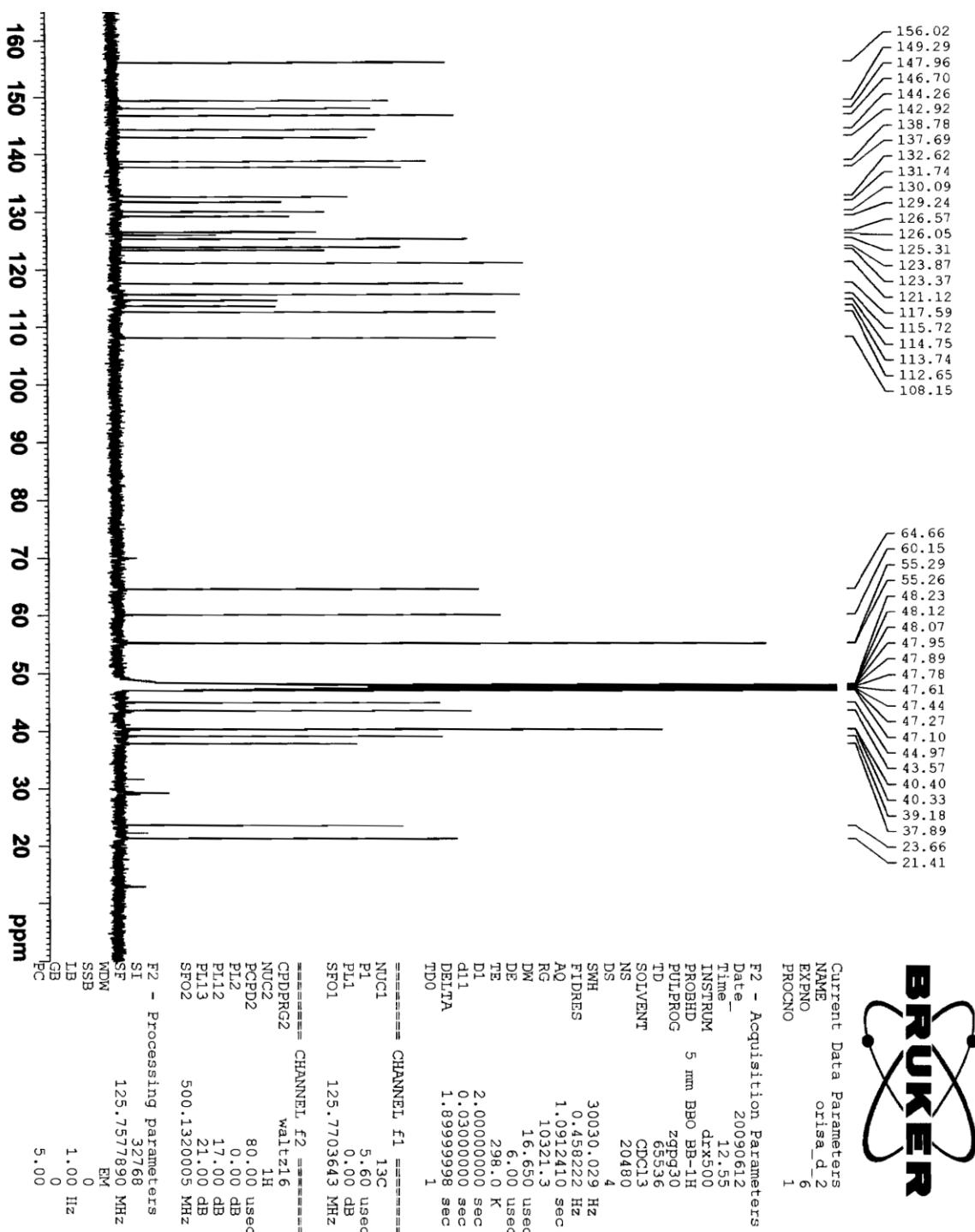
Appendix 4f: UV spectrum of (-)-5-oxoaknadinine (**207**)



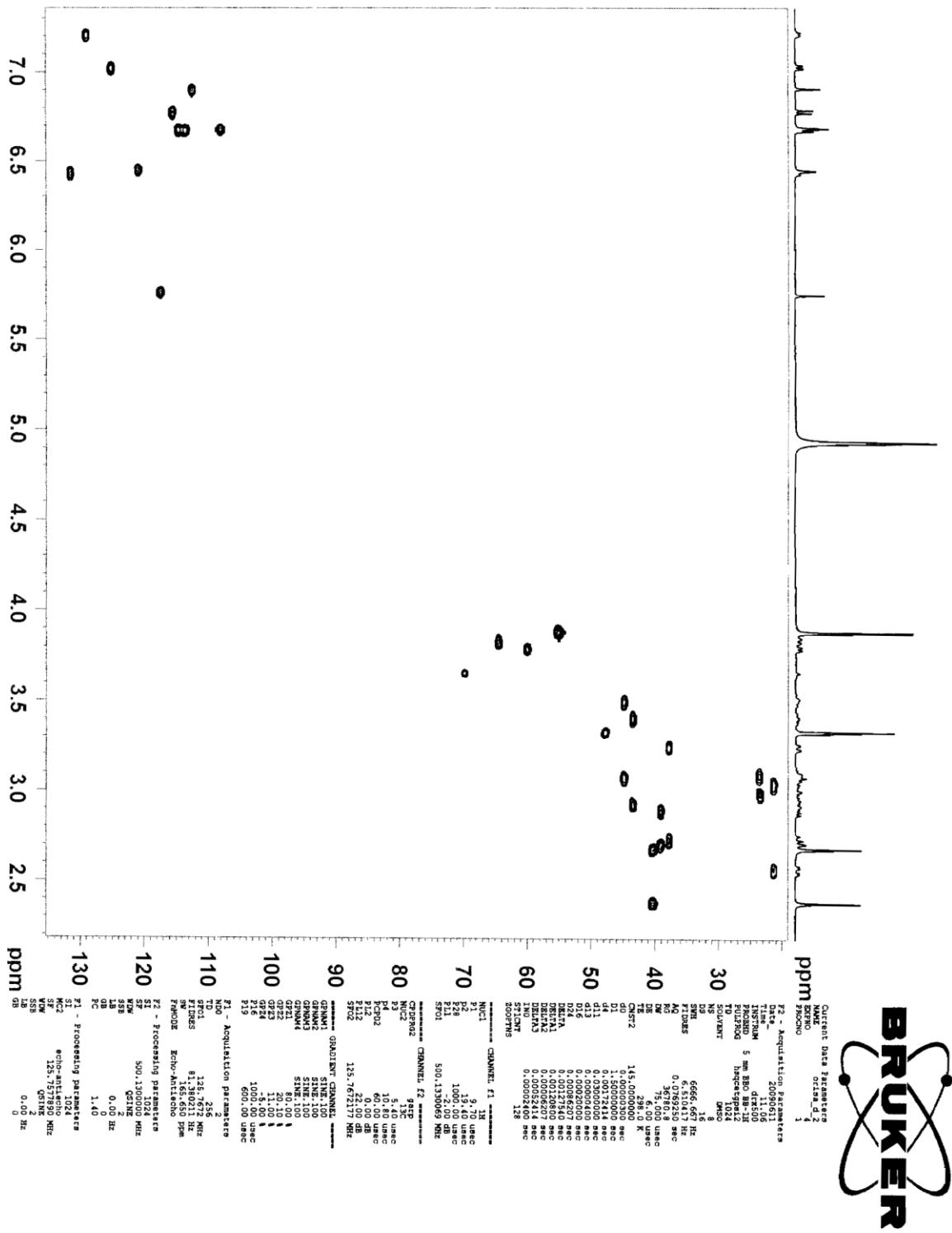
Appendix 5a: ^1H NMR (500 MHz, CD_3OD) spectrum of (-)-pseudourine (**208**)



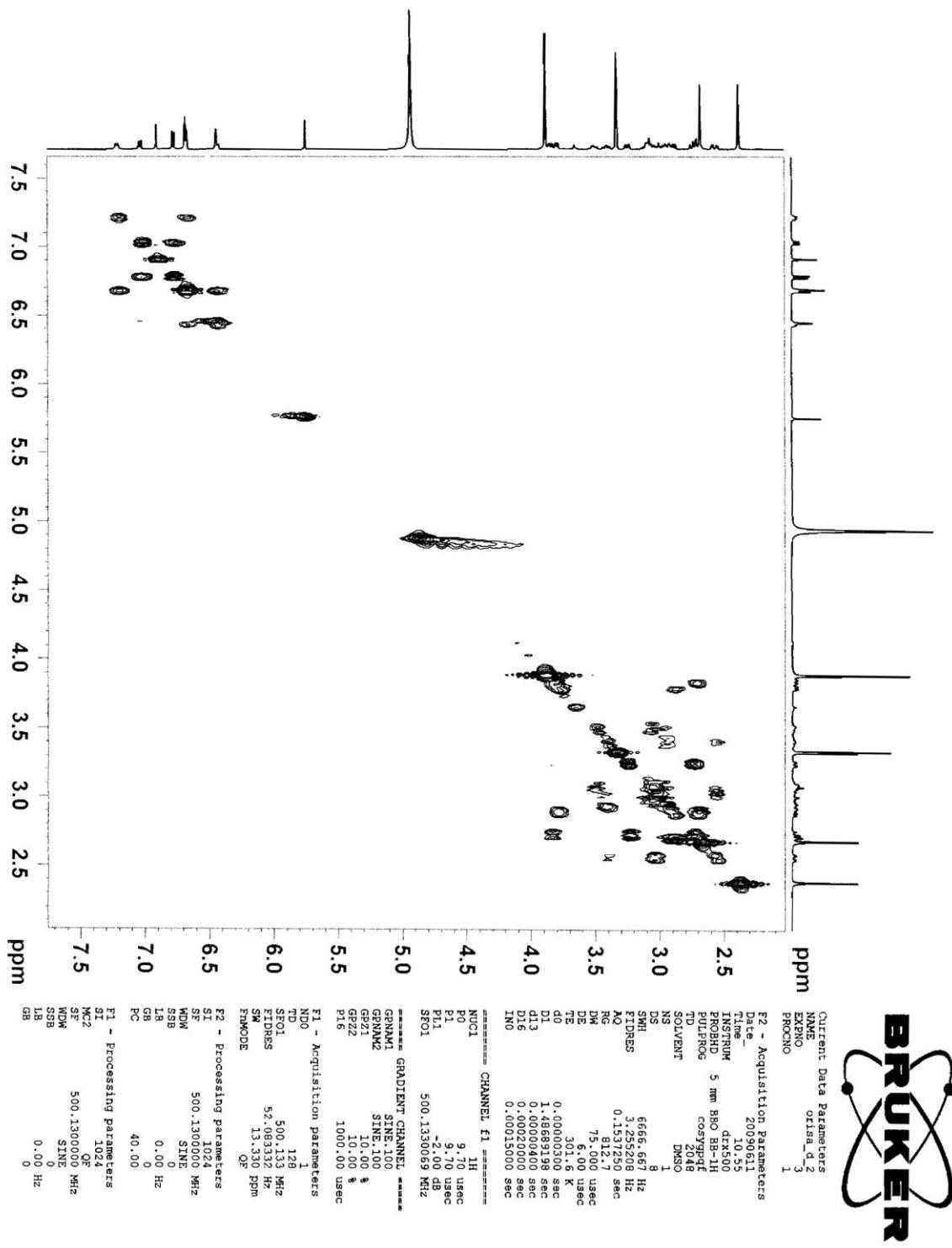
Appendix 5b: ^{13}C NMR (500 MHz, CD_3OD) spectrum of (-)-pseudourine (**208**)



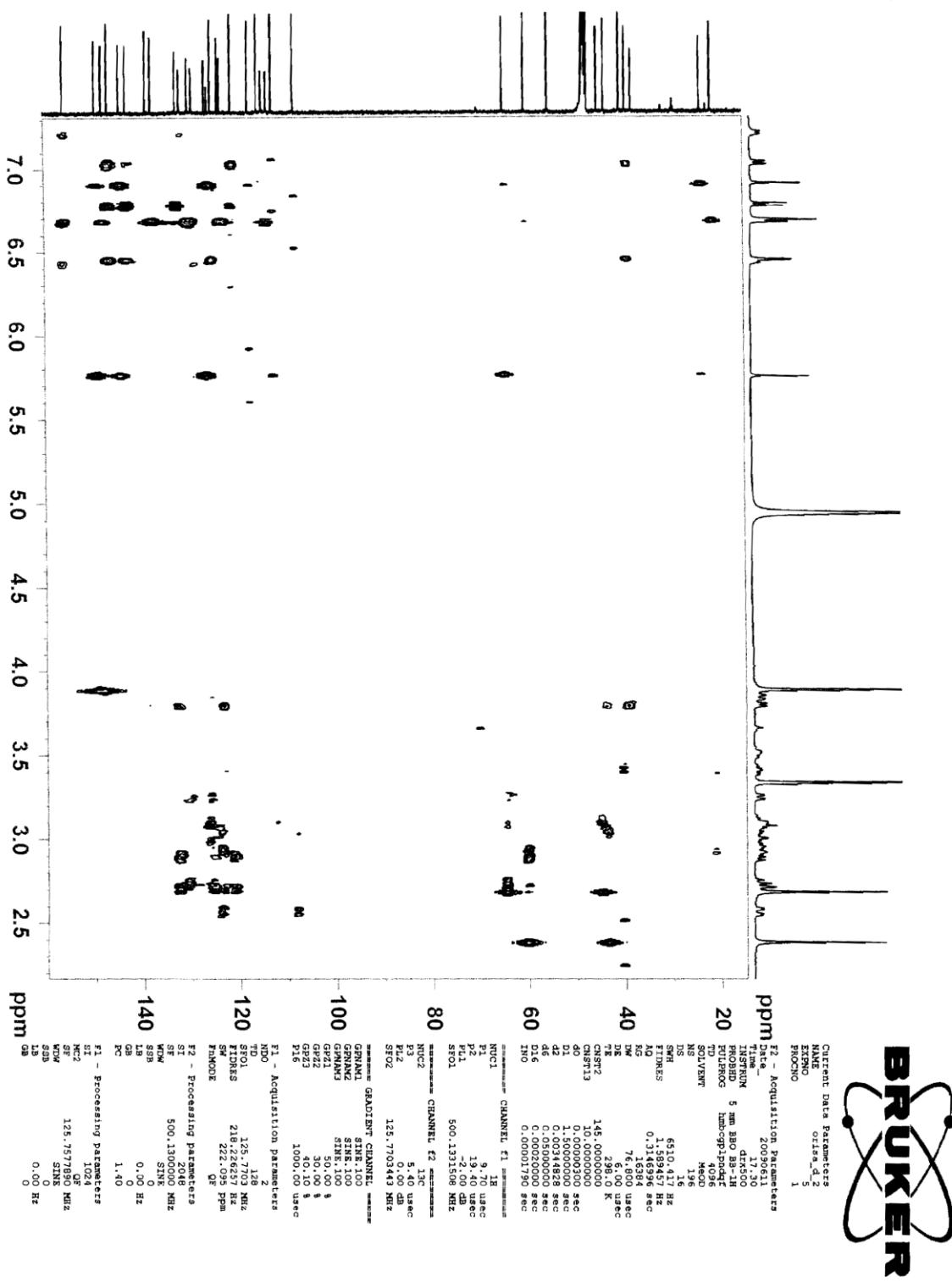
Appendix 5c: HMQC spectrum of (-)-pseudourine (**208**)



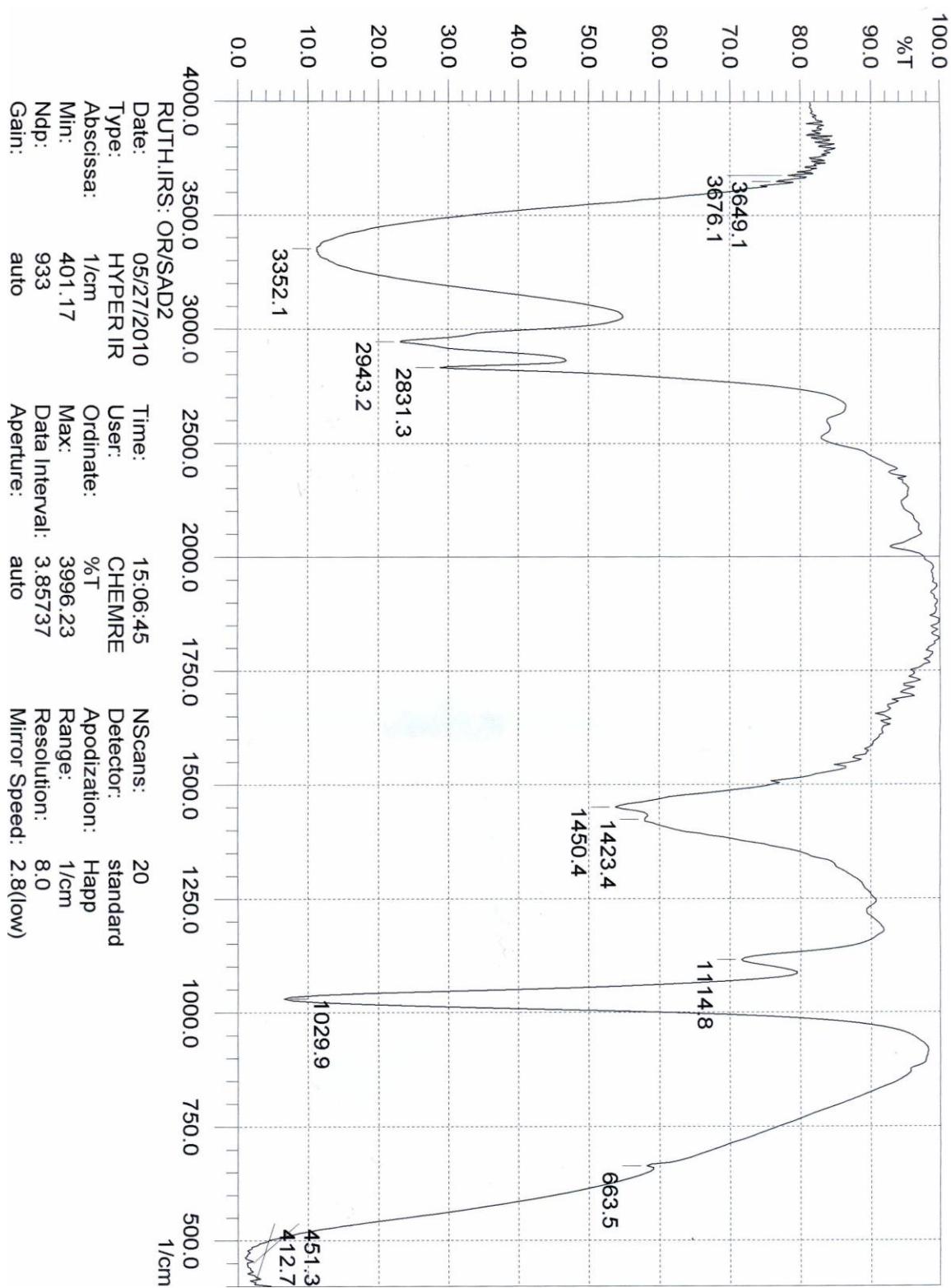
Appendix 5d: COSY spectrum of (-)-pseudourine (**208**)



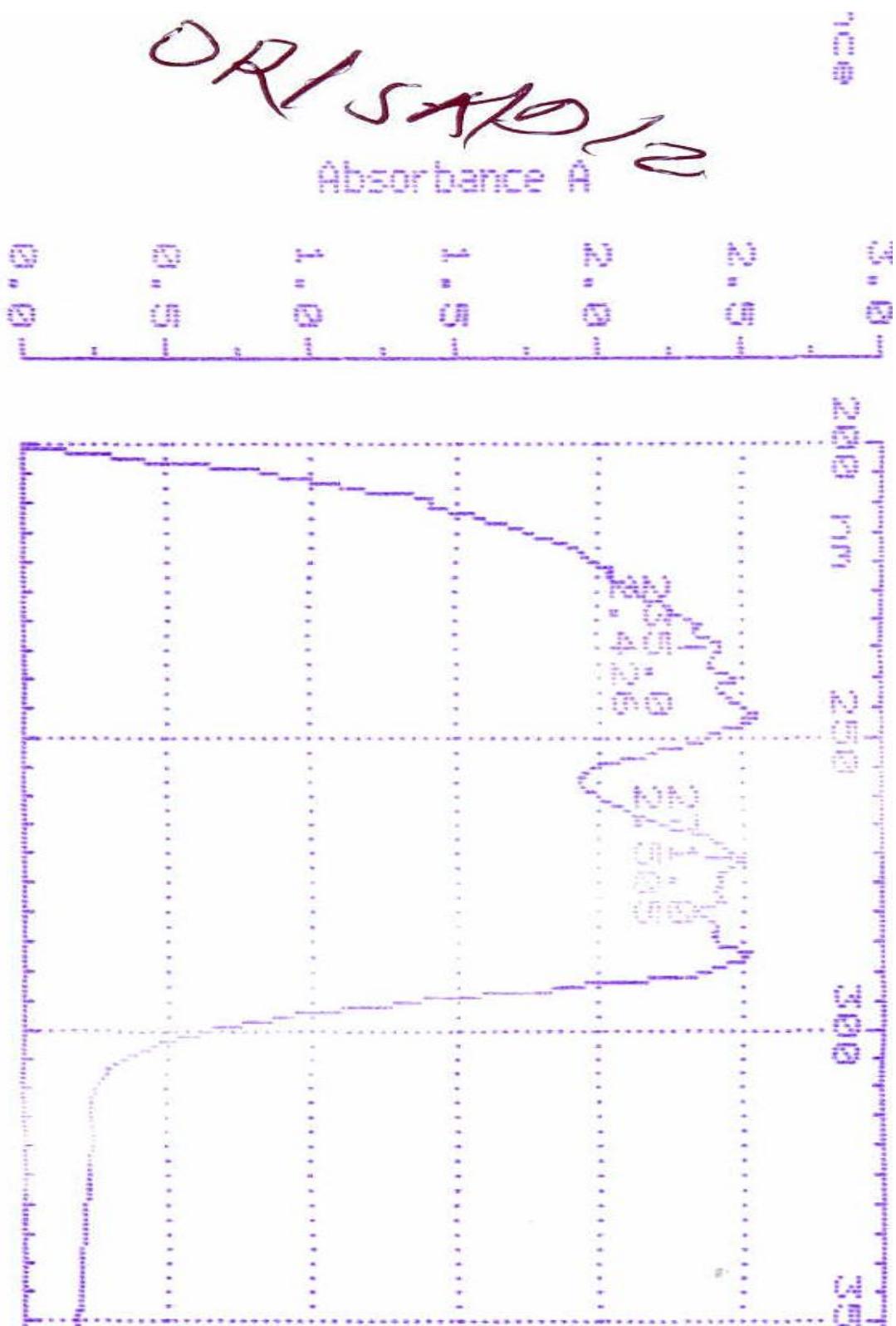
Appendix 5e: HMBC spectrum of (-)-pseudourine (**208**)



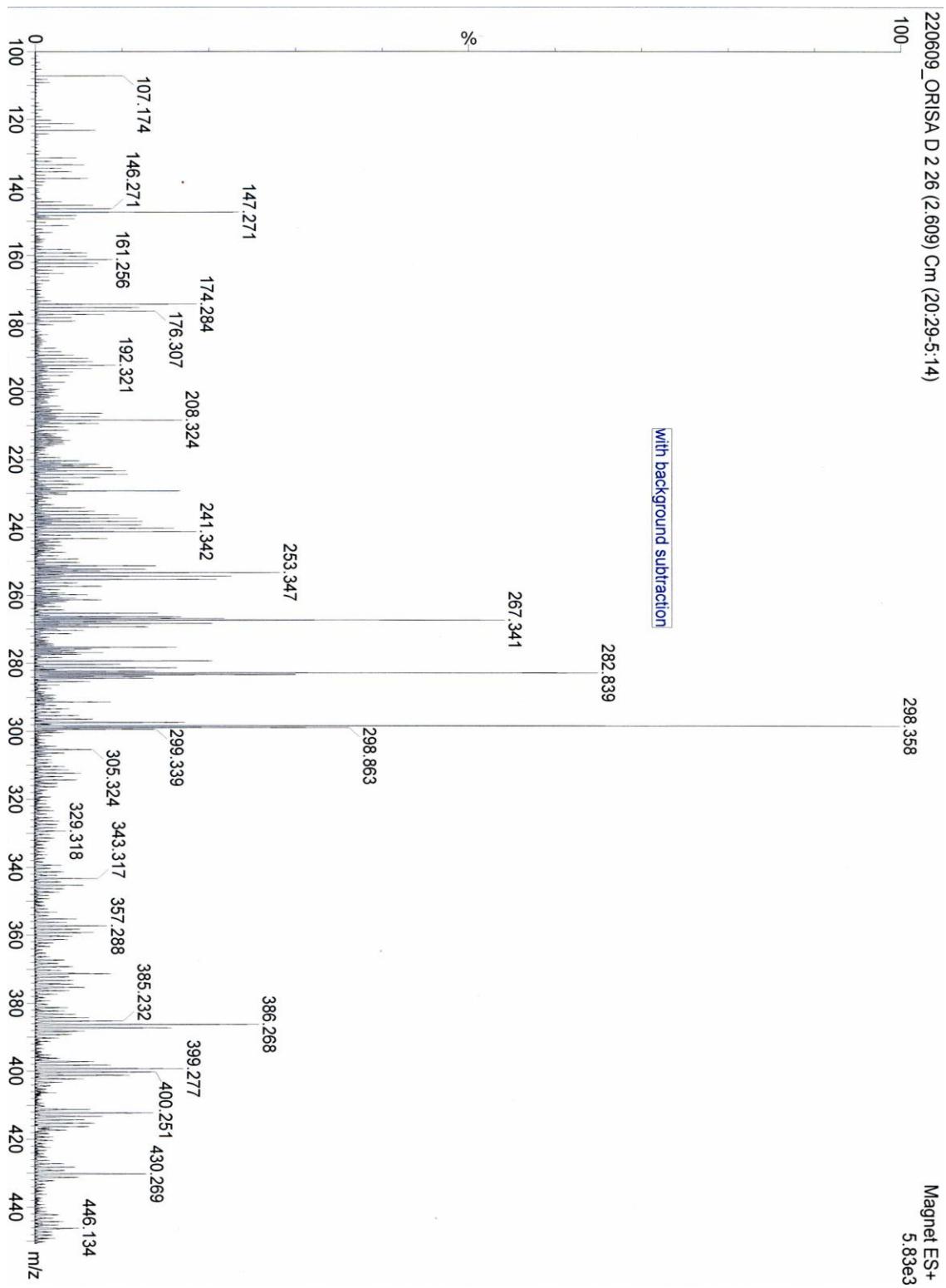
Appendix 5f: IR spectrum of (-)-pseudocurine (**208**)



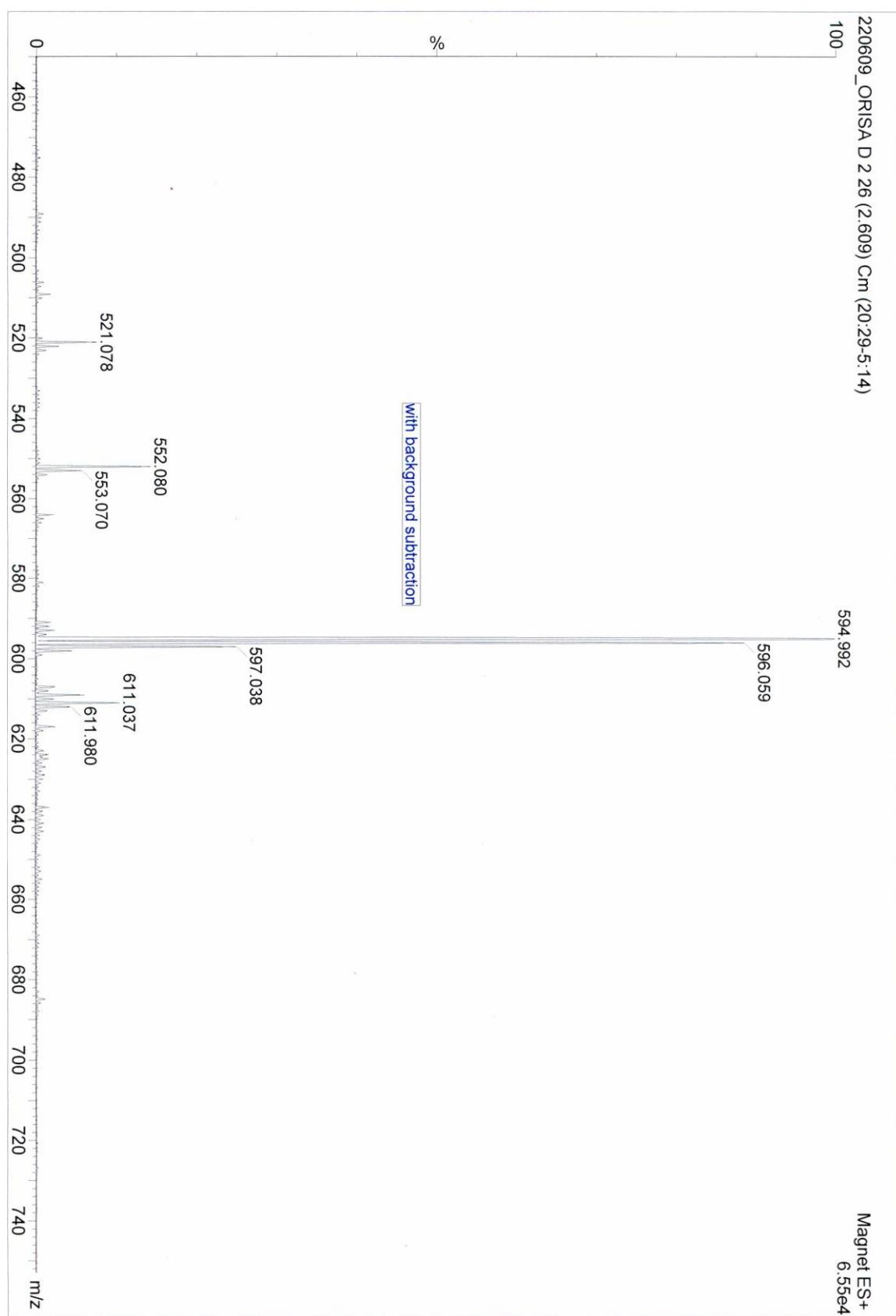
Appendix 5g: UV spectrum of (-)-pseudourine (**208**)



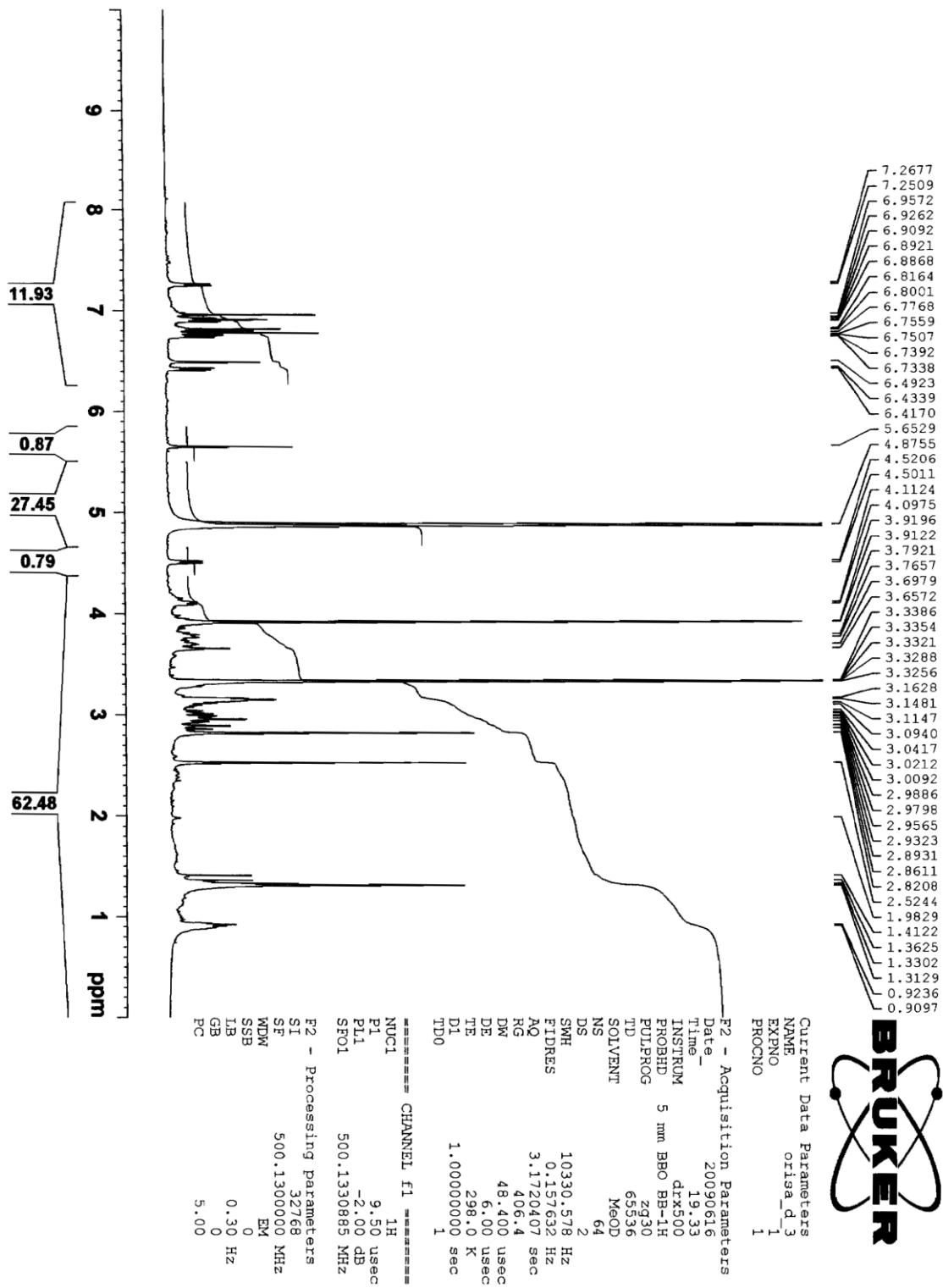
Appendix 5h: Mass spectrum of (-)-pseudourine (**208**)



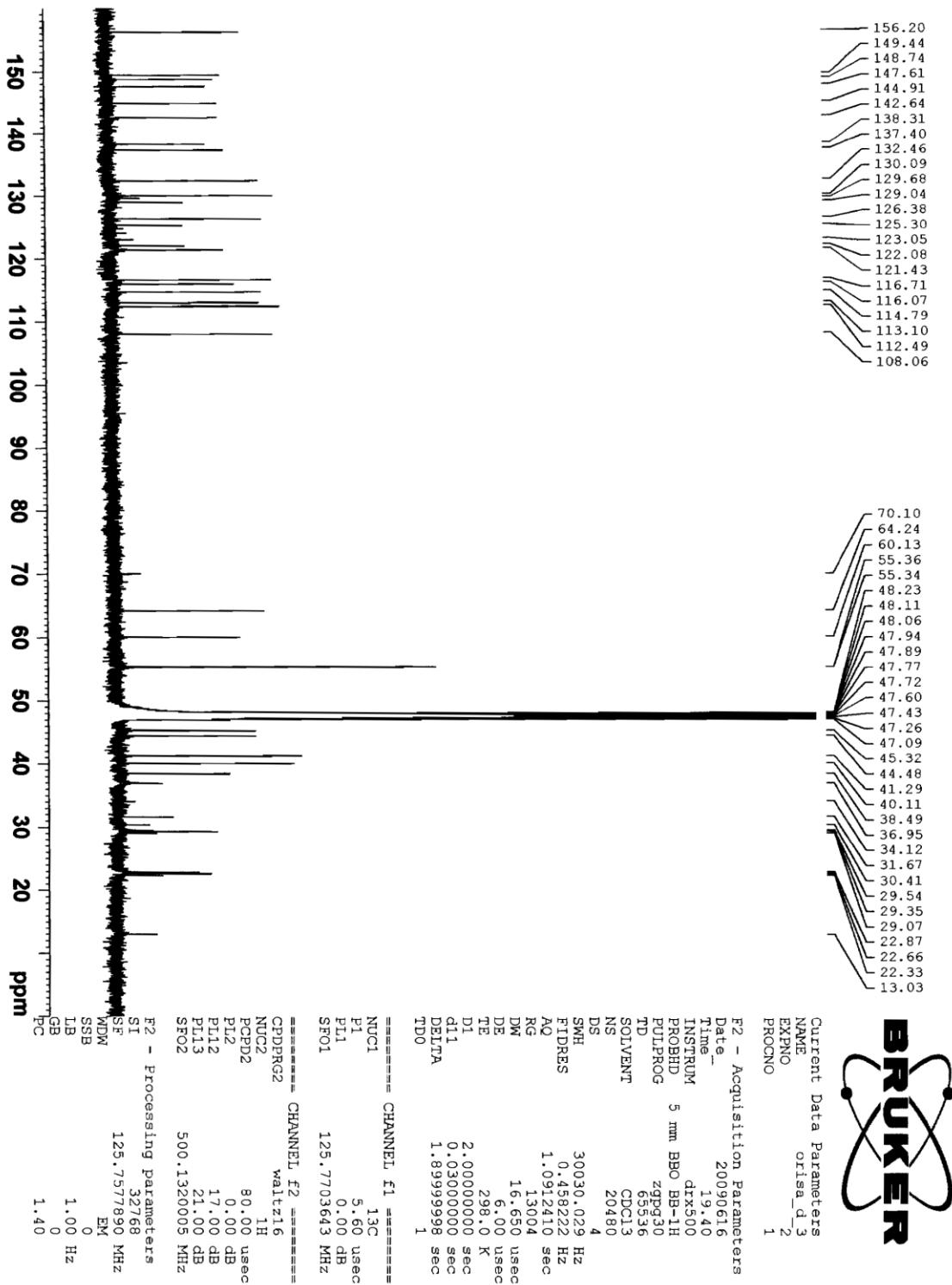
Appendix 5i: Mass spectrum of (-)-pseudourine (**208**)

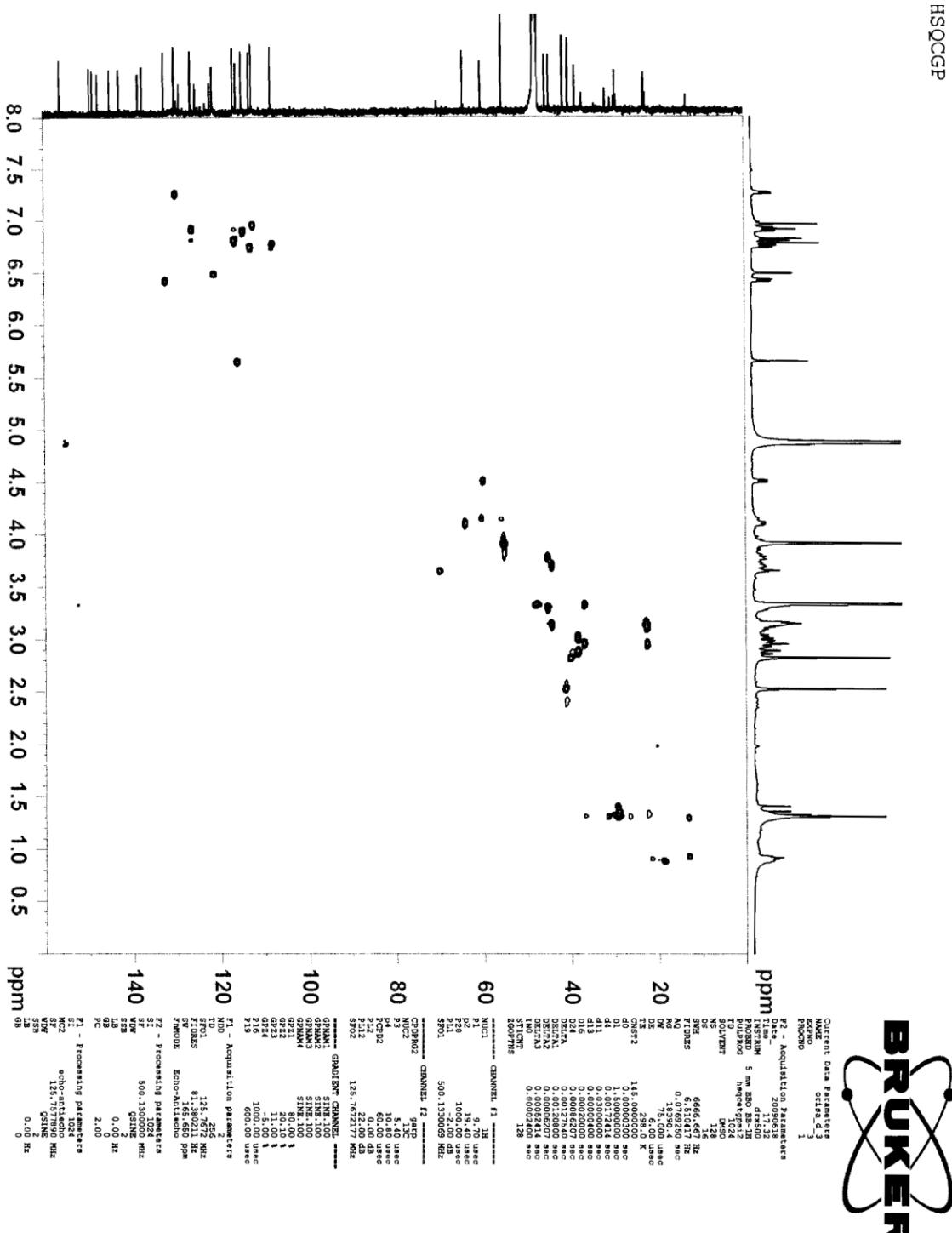


Appendix 6a: ^1H NMR (500 MHz, CD_3OD) spectrum of (-)-isocurine (**209**)

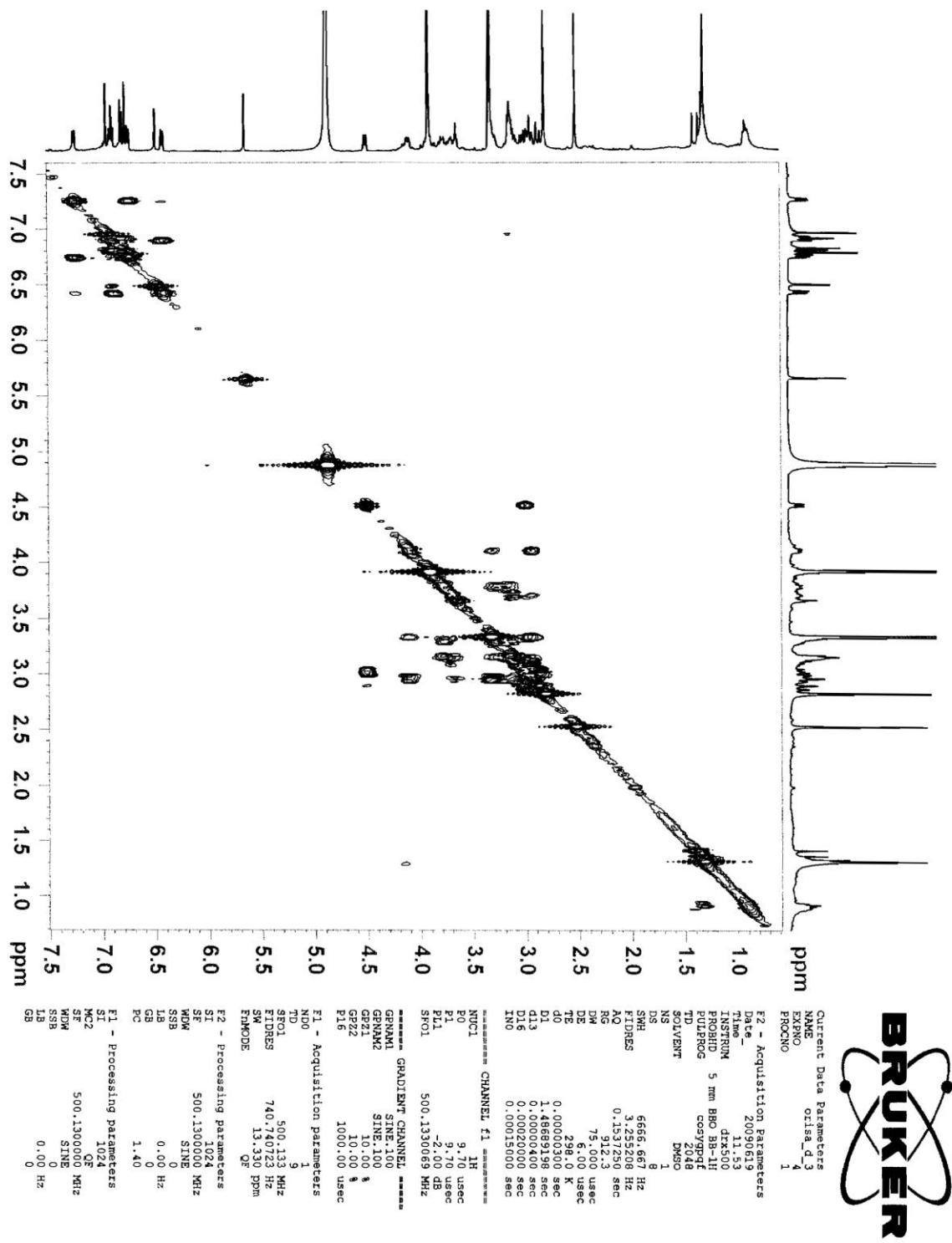


Appendix 6c: ^{13}C NMR (500 MHz, CD_3OD) spectrum of (-)-isouridine (**209**)

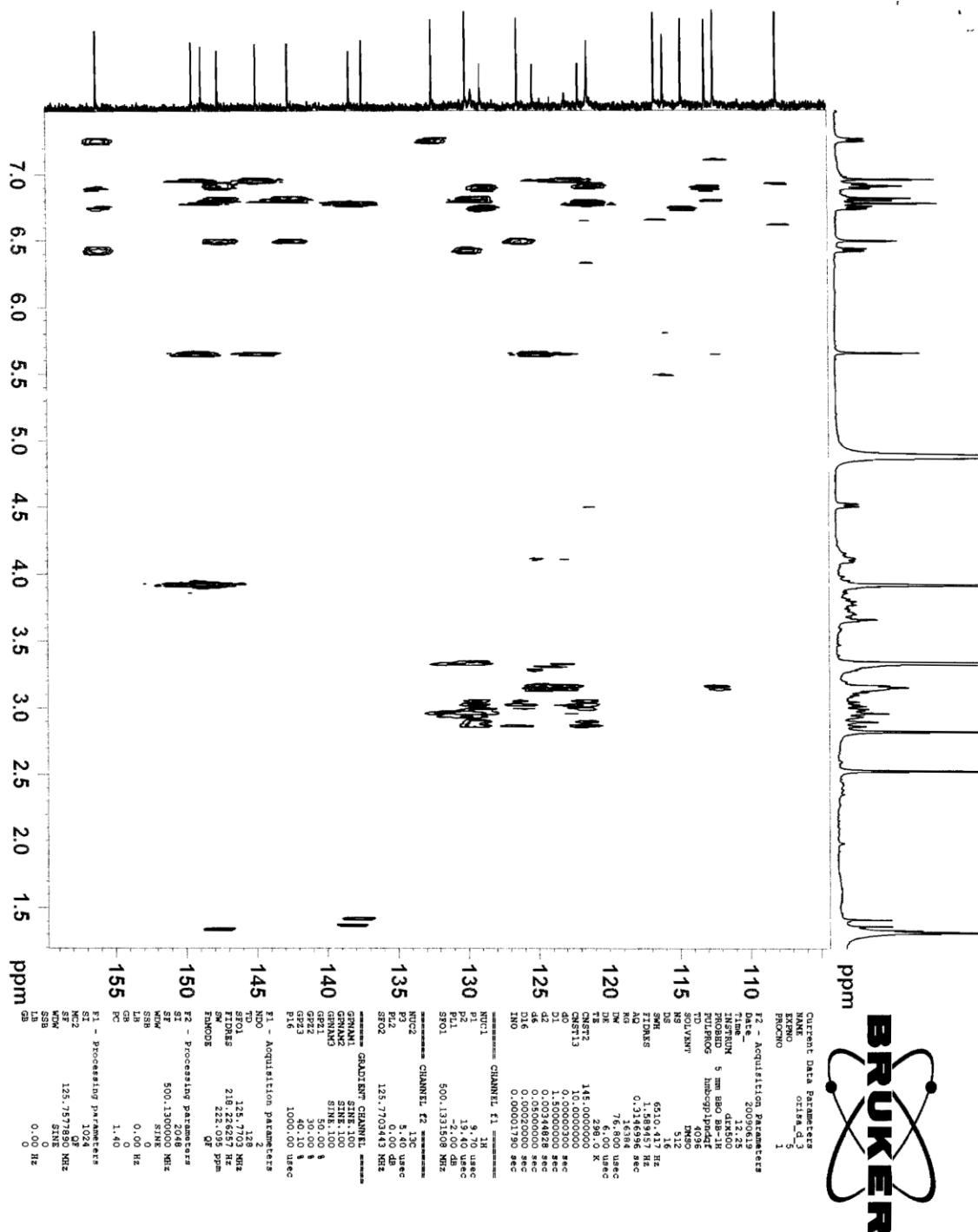


Appendix 6c: HMQC spectrum of (-)-isourine (**209**)

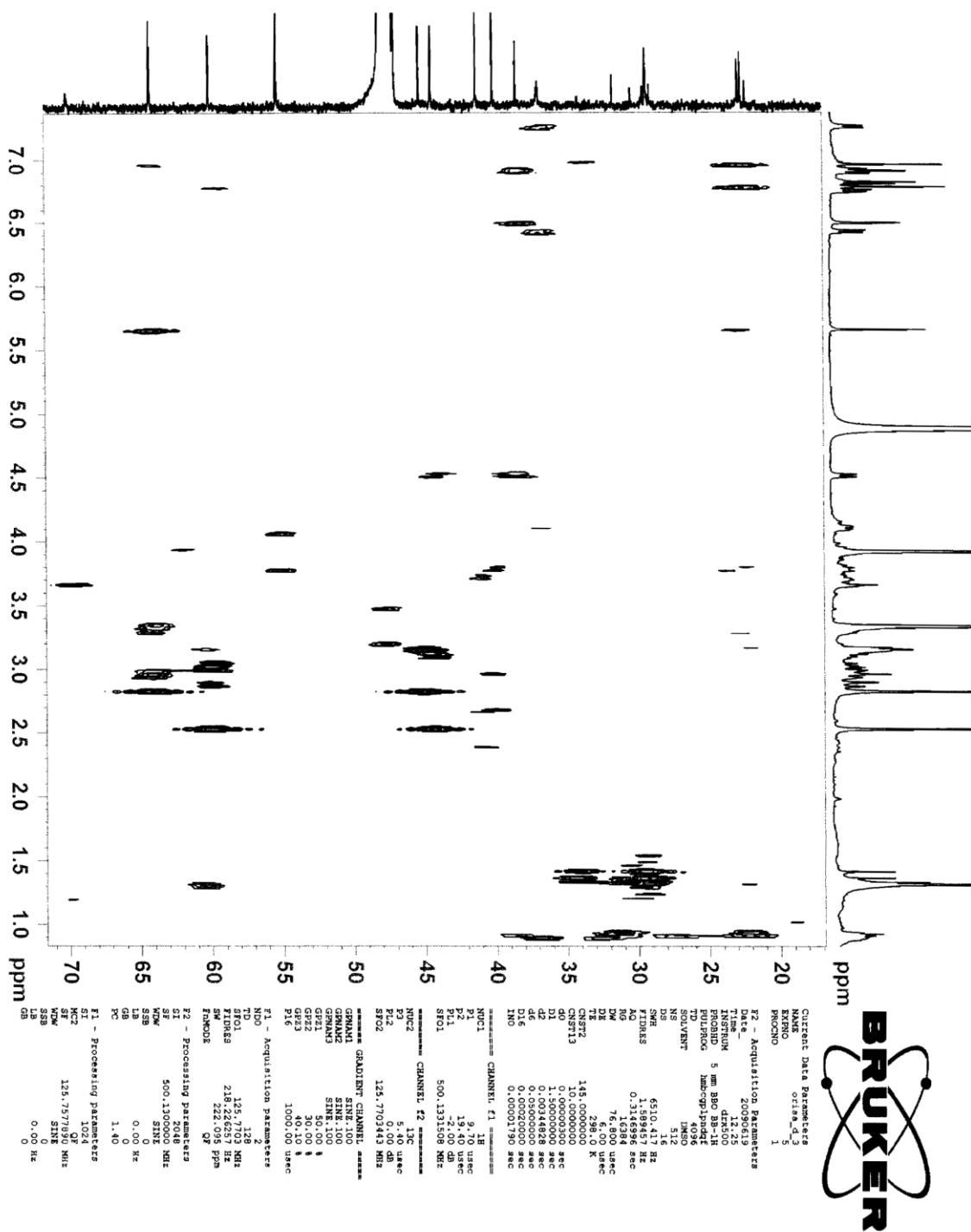
Appendix 6d: COSY spectrum of (-)-isocurine (**209**)



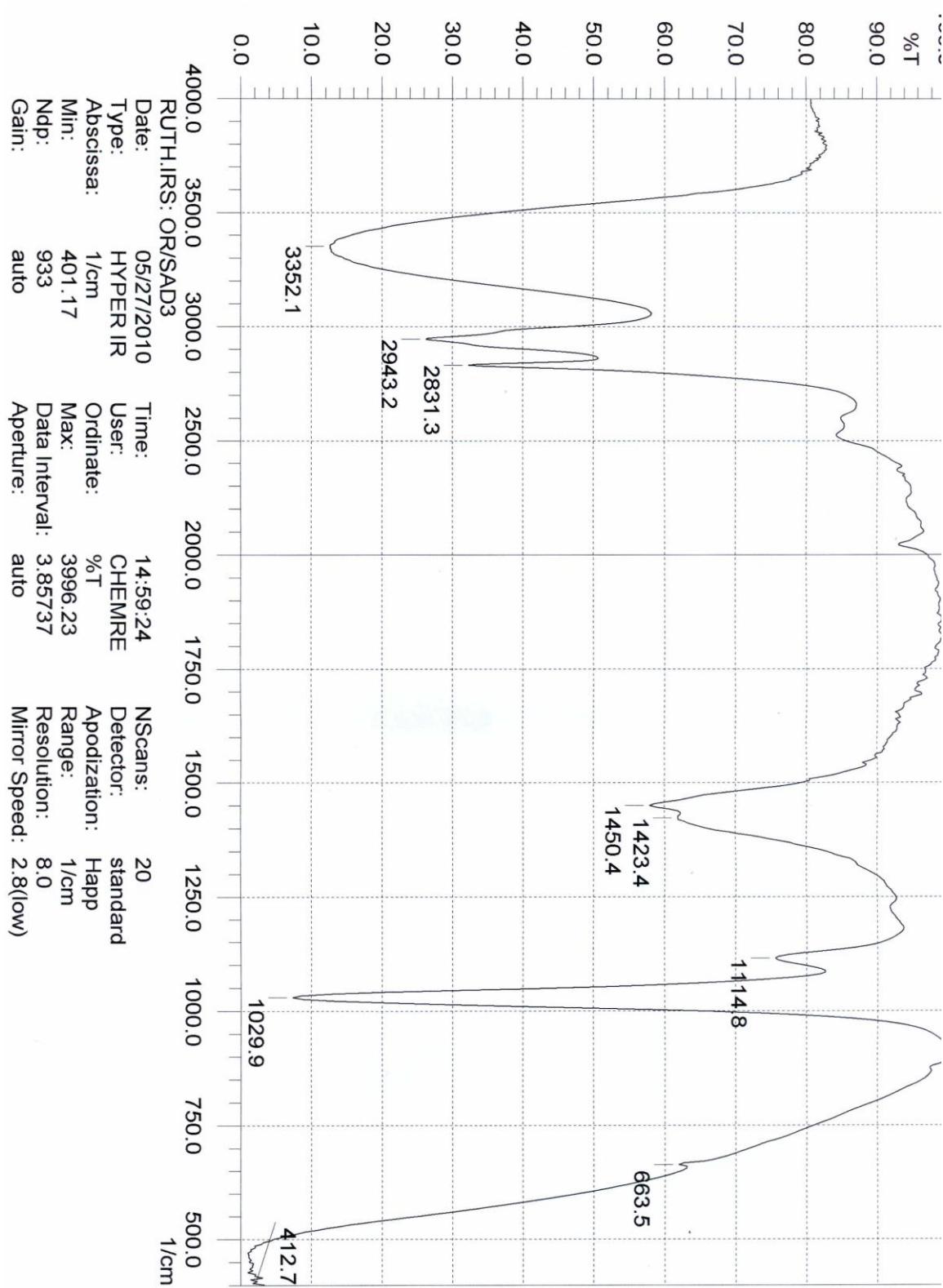
Appendix 6e: HMBC spectrum of (-)-isourine (**209**)



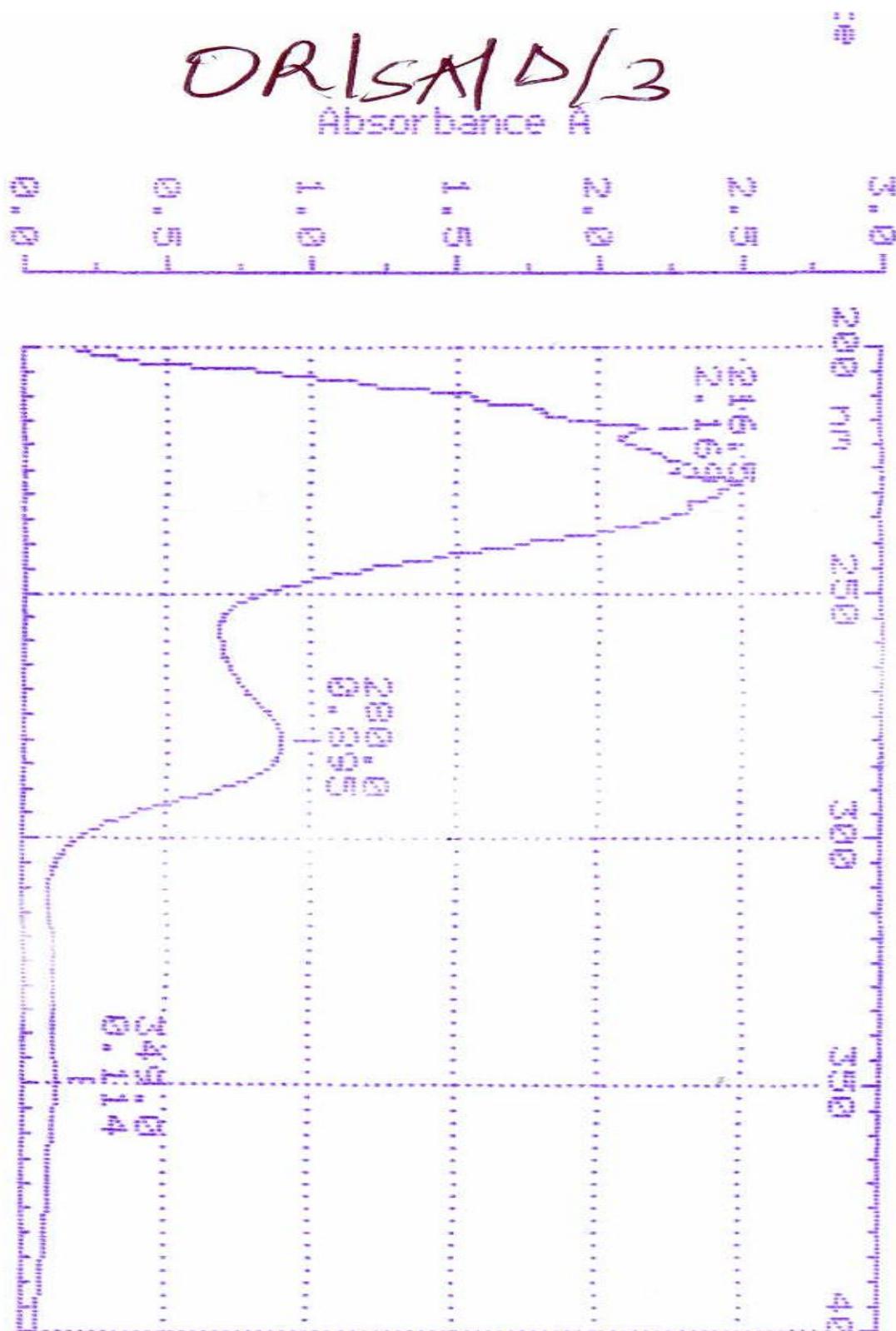
Appendix 6f: HMBC spectrum of (-)-isourine (**209**)



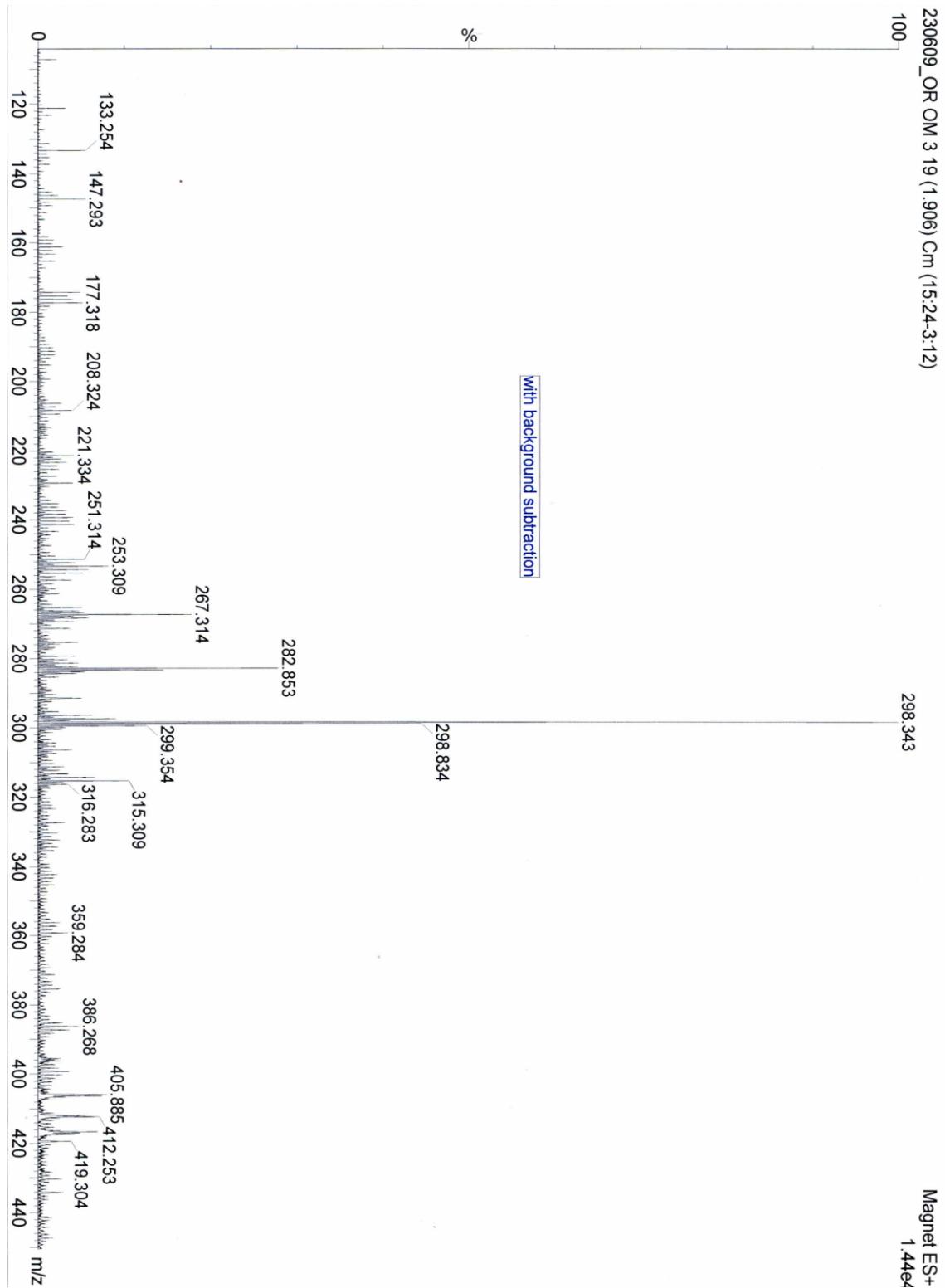
Appendix 6g: IR spectrum of (-)-isocurine (**209**)



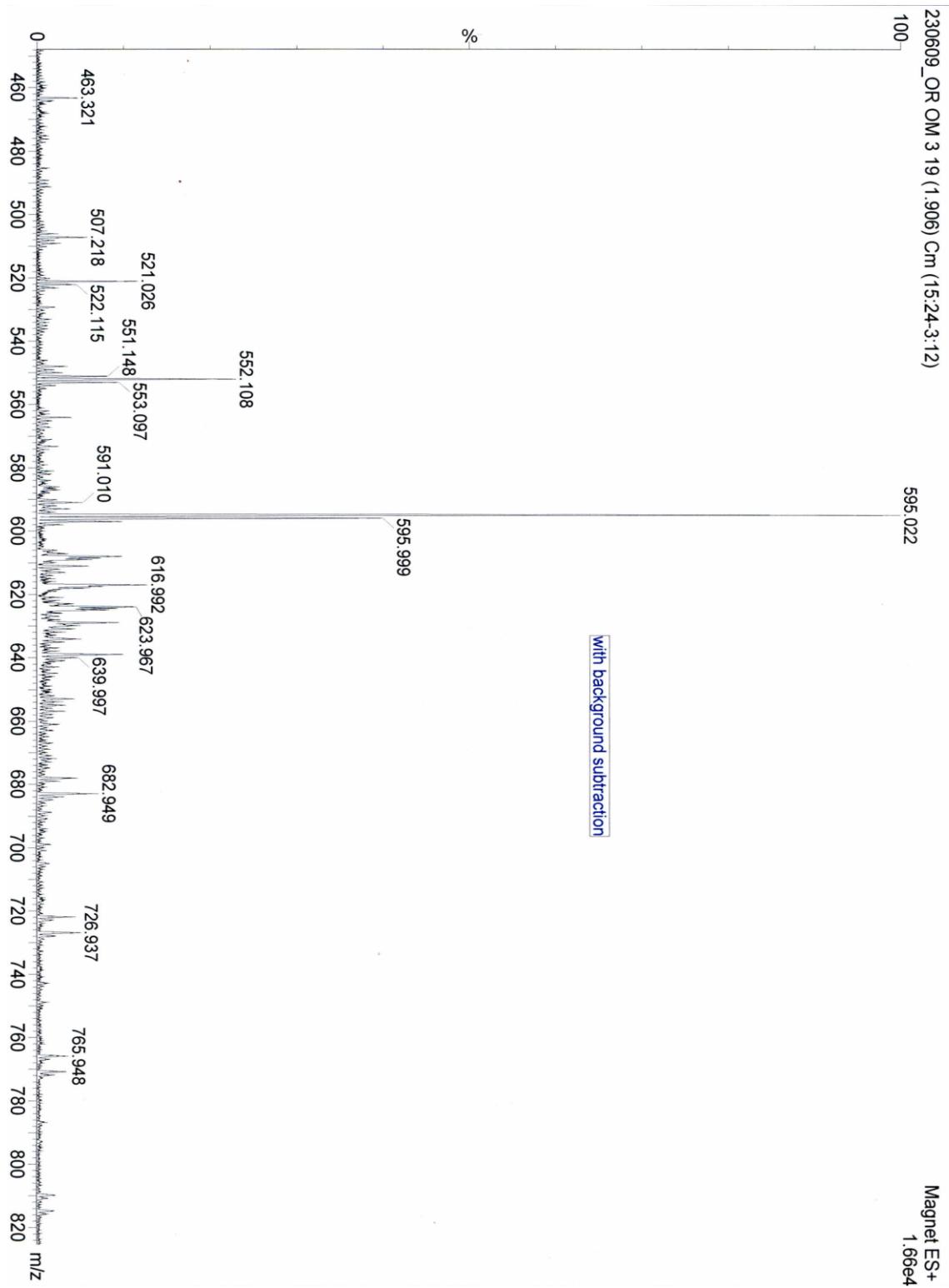
Appendix 6h: UV spectrum of (-)-isocurine (**209**)



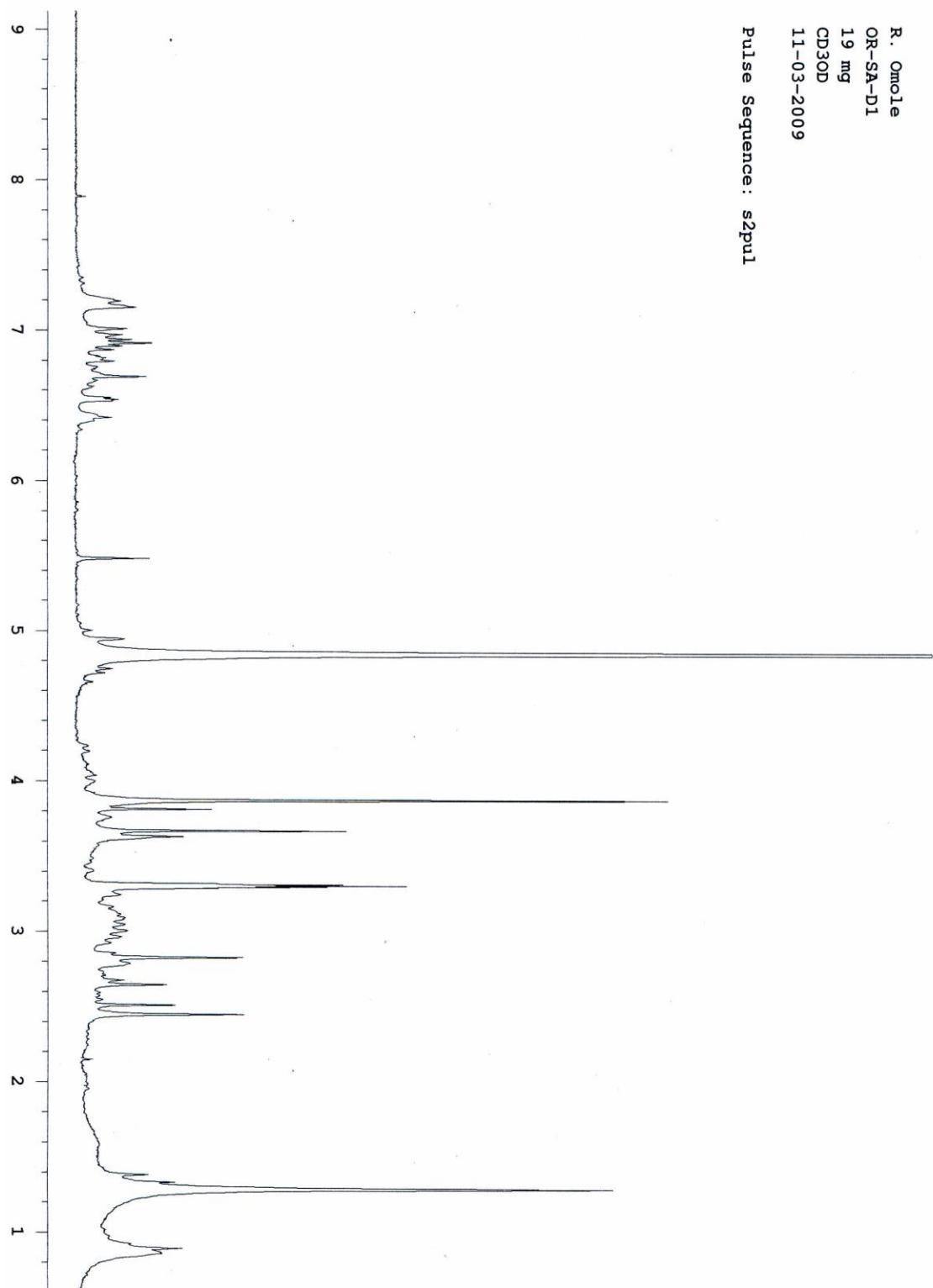
Appendix 6i: Mass spectrum of (-)-isocurine (**209**)



Appendix 6j: Mass spectrum of (-)-isocurine (**209**)



Appendix 7a: ^1H NMR (200 MHz, CD_3OD) spectrum of OR/SA/D1



Appendix 7b: ^{13}C NMR (200 MHz, CD_3OD) spectrum of OR/SA/D1

