Investigation of the Phytochemistry and Biological Activity of Isoquinoline Alkaloids Isolated from the South African Medicinal Plants, *Cyrtanthus sanguineus* (Lindl.) Walp. and *Cyrtanthus obliquus* (L.f.) Ait

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Cyrtanthus sanguineus

A thesis submitted in fulfilment of the requirements for the degree ***Doctor of Philosophy in Pharmacology*** *to the University of Cape Town*

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DECLARATION

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I, **Natalie Dawn Brine**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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This thesis is presented for examination for the degree of Doctor of Philosophy.

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Date: 4/07/2001

PREFACE

The laboratory work described in this thesis was carried out between May 1998 and June 2001, and was part of a collaborative research agreement between the Pharmacology Division of the Department of Medicine at the Faculty of Health Sciences, of the University of Cape Town, South Africa, and the Department of Natural Products at the Faculty of Pharmacy, of the University of Barcelona, Spain. The isoquinoline alkaloids from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus* were isolated, and investigated for cytotoxicity and acetylcholinesterase inhibitory activity. Both plants belong to the Amaryllidaceae family and are widely used in traditional medicine in Southern Africa. Most of the structural elucidation experiments were performed at the University of Barcelona, in the Dept. of Natural Products, during my two visits to that department, from February to March 1999, and in September 2000.

This thesis has been divided into six chapters. Chapter 1 provides an introduction to the traditional medicine system of South Africa, followed by Chapter 2, which gives a background to the Amaryllidaceae family of plants, and reviews the literature on the isoquinoline alkaloids isolated from the Amaryllidaceae to date. Chapter 3 details the structural elucidation of the alkaloids from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*, including the interesting alkaloid, 11α-hydroxygalanthamine and the novel dinitrogenous alkaloid, obliquine. This chapter also covers the cytotoxicity screening performed on the isolated alkaloids, together with a discussion of the known biological and pharmacological activities of the alkaloids isolated in this investigation that have been previously isolated from other Amaryllidaceae species. Chapter 4 gives background information on galanthamine and its role in Alzheimer's disease therapy, and then details the investigation of the acetylcholinesterase inhibitory activity of 11α-hydroxygalanthamine. Full methodological details of all the experimental work are presented in Chapter 5, with the nuclear magnetic resonance spectra for all the alkaloids provided in Appendix 1. Chapter 6 provides a summary of the investigation and conclusions, together with suggestions for possible further research.

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I would like to thank the following people most sincerely for their valuable contribution towards the completion of this thesis and to my well being during the past four years:

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Thanks to Sylva Schwager of the Department of Medical Biochemistry, University of Cape Town, for donating the Chinese Hamster Ovarian cells original stock, and to Lester Davids of the Department of Medicine, University of Cape Town for donating the HepG2 cell line stock. I am grateful to both for their valuable advice on mammalian cell culturing.

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Thanks to Geoff Nichols for kind permission to reproduce his magnificent photograph of *Cyrtanthus sanguineus*, and to Kelly Irvin of the International Bulb Society for allowing me to use the wonderful photographs of Amaryllidaceae (including the *Cyrtanthus* photograph on the title page) from the Gallery of their website: www.bulbsociety.com. Thanks also to Dr Ben Van Wyk for permission to reproduce the photographs from his book "Medicinal Plants of South Africa" (Briza Publications, 1997). Thanks to Gordon and Breach Publishers, Reading, United Kingdom, for permission to reproduce the diagram: "Some Mechanisms and Intervention Sites at the Cholinergic Synapse".

I would like to thank my parents for their years of love, encouragement and support.

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ABSTRACT

The plants of the family Amaryllidaceae are widely distributed in Southern Africa and many have been used for centuries in the traditional medicine of the indigenous people of the region. As a result of their exploitation in traditional medicine, these plants have recently become the focus of pharmacological investigations, which have revealed that while many of them may be of pharmacological value, several of the plants are also highly toxic. This toxicity has been attributed to a group of isoquinoline alkaloids, which are exclusive to the Amaryllidaceae. Substantial progress has been made in the past few years in examining the South African Amaryllidaceae. The two South African species, *Cyrtanthus sanguineus* (Lindl.) Walp. and *Cyrtanthus obliquus* (L. f.) Ait were selected for this study from a literature search on Amaryllidaceae plants used in traditional medicine in South Africa. Both plants were considered to be potentially toxic due to their alkaloid content, and since neither of the two species had been previously examined phytochemically, they were a potential source of pharmacologically interesting compounds.

A combination of flash and preparative thin layer chromatography on the crude alkaloid extracts of the bulbs of both plants yielded 11α-hydroxygalanthamine, lycorine, lycoramine, 11-epihaemanthamine and haemanthidine from *Cyrtanthus sanguineus*, and further 11α-hydroxygalanthamine, the novel dinitrogenous alkaloid, obliquine, together with trisphaeridine, tazettine, narcissidine and 3-epimacronine from *Cyrtanthus obliquus*. The known alkaloids were identified from analysis of their ^{1}H NMR spectra and by direct comparison of their spectroscopic properties (MS, ^{1}H and ^{13}C NMR) with those reported in the literature. To the best of my knowledge, the isolation of 11-hydroxygalanthamine (habranthine) has been reported only twice previously and at that time the stereochemistry at C-11 was not conclusively determined, and the carbon resonances were not completely assigned. It was possible to resolve from coupling constants and 2-D NMR experiments that the hydroxyl is in the *pseudoaxial*-position on C-11 and to assign all ^{1}H and ^{13}C resonances.

11α-Hydroxygalanthamine is a derivative of galanthamine, which is a reversible inhibitor of acetylcholinesterase (AChE) with a competitive action. Recently, galanthamine (Reminyl™) has been approved for the treatment of Alzheimer's disease (AD), which is associated with a

deficit of cholinergic function in the brain, leading to progressive degeneration of memory and cognitive function. The binding of galanthamine to AChE arises from a number of moderate to weak interactions with the protein, including classical and non-classical hydrogen bonds. In light of this, it was proposed that the additional hydroxyl group on the C-11 of 11α-hydroxygalanthamine might render the alkaloid more active than galanthamine against AChE, by possibly facilitating the formation of an additional hydrogen bond with the active site of the enzyme molecule.

Using an adaptation of the method developed by Ellman (1961), 11α-hydroxygalanthamine was tested for its ability to inhibit acetylcholinesterase *in vitro*, in comparison to galanthamine and was found to inhibit the enzyme in a competitive, dose-dependent manner. There was, however, no marked improvement in the inhibitory action of 11α-hydroxygalanthamine over galanthamine, as reflected in the IC_{50} values (0.72 μM and 0.33 μM respectively). Wherever the quantity of material permitted, the isolated alkaloids were screened for cytotoxicity *in vitro* against a Chinese Hamster Ovarian (CHO) cell line and human heptoma (HepG2) cell line using the MTT assay (Mosmann, 1983). Lycorine showed moderate cytotoxicity against the CHO cells only (IC_{50} value 26 μg/ml), but not towards the HepG2 cells, whereas 11α-hydroxygalanthamine, obliquine, tazettine and narcissidine were not toxic to the CHO cell line or the HepG2 cell line (IC_{50} values >100μg/ml).

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Parts of the work presented in this thesis have been presented as posters at conferences or are currently being prepared for submission to journals for publication as follows:

Publications

An acetylcholinesterase inhibiting alkaloid from the South African *Cyrtanthus sanguineus*. **N. Brine**, W. Campbell, J. Bastida, C. Codina, F. Viladomat, A. Machocho and P. Smith. (under review)

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
AD	Alzheimer's disease
APOE	Apolipoprotein E
BSA	bovine serum albumin
BChE	butyrylcholinesterase
©	copyright
ca	approximately
ChAT	choline acetyltransferase
CHO	Chinese Hamster Ovarian (cells)
CNS	central nervous system
DMEM	Dulbecco's Modified Eagles' Medium
DMSO	dimethylsulphoxide
EIMS	electron impact mass spectroscopy
et al.	and all other authors
EtOAc	ethyl acetate
FAO	Food and Agricultural Organisation (of the United Nations)
FCS	foetal calf serum
g	gram(s)
h	hour(s)
HAMS-F$_{12}$	Ham's Nutrient Mixture F-12
Hex	hexane
HREIMS	high resolution electron impact mass spectroscopy
Hz	Hertz
IC$_{50}$	concentration of drug required to inhibit 50% of enzyme activity or 50% of cell viability
IR	infra red
J	chemical shift value
kg	kilogram
L	litre
λ	wavelength (nm)
M	molar
MeOH	methanol
min	minute(s)
ml	millilitre
mg	milligram
MS	mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
ng	nanogram
NH$_3$	ammonia
NMR	nuclear magnetic resonance
nm	nanometres
OR	optical rotation
PBS	phosphate-buffered saline
ppm	parts per million
Prep. TLC	preparative thin layer chromatography

®	registered trademark
rpm	revolutions per minute
S.E.M.	standard error of the mean
$T_{1/2}$	half-life
THA	9-amino-1,2,3,4-tetrahydrotacrine (tacrine)
TLC	thin layer chromatography
t_{max}	time to reach maximum blood drug level
™	registered trademark
TMS	trimethylsilane
μg	microgram
μl	microlitre
μmol	micromolar
UN	United Nations
UV	ultraviolet (radiation)
W.H.O.	World Health Organisation

Chapter 1

Introduction to Traditional Medicine in South Africa

1.1. Traditional Medicine: A Global Perspective

The term "traditional medicine" refers to the ways of protecting and restoring health that existed before the arrival of modern medicine. These approaches to health belong to the traditions of each country and have been handed down from generation to generation. It is estimated that more than 80% of the six billion (10^9) people in the world today live in the less developed countries, and that the majority of these people still rely chiefly on indigenous systems of medicine to satisfy their primary health care needs (Farnsworth 1994; Phillipson, 1994). For this segment of the world population, who very often live in abject poverty, and are unable to pay the cost of "Western" drugs, there is an urgent need to study traditional medicines.

The Alma-Ata Declaration at the International Conference on Primary Health Care in 1978 recommended that the governments of the world give high priority to the utilisation of traditional medicine practitioners, and incorporate proven traditional remedies into national drug policies and regulations, as part of the global strategy to achieve "Health for All by the Year 2000". To facilitate these aims the World Health Organisation (WHO) established a Programme for Traditional Medicine, with its headquarters in Geneva, medical officers in each of the global regional offices and subsequently up to thirty collaborating centres for traditional medicine in individual countries in Africa, the Americas, the Eastern Mediterranean, Europe, South-East Asia, and the Western Pacific. This programme has focused on the need to adequately train health workers with regard to traditional systems of healing and to encourage research in this area, emphasising the importance of regional and international collaboration.

1.2. Traditional Medicine in South Africa

Long before the southern tip of Africa became a recognised route to the East Indies by European explorers, members of the indigenous communities were identified as having a special competence to provide and maintain health care in the African populations (Edinburg, 1998). These traditional healers have flourished in the face of competition from modern medicine (Kale, 1995). Today, traditional medicine is used by between 60-80% of the South African population to meet their physical and psychological health needs (Van Wyk et al. 1997), most particularly in the rural areas where modern health care facilities are

Traditional Medical Practitioners (TMP) in the country (Edinburg, 1998), compared with approximately 30 000 general practitioners registered with The Health Professionals Council of South Africa. The ratio of healers to people in the KwaZulu-Natal province, for example, is 1:500 whilst the ratio of western medical doctors is 1:17 500. The traditional healers play a very prominent role in South African society (Veale et al., 1992). They are enshrined in the minds of the people and respected in their communities, and they are often opinion leaders (Kale, 1995).

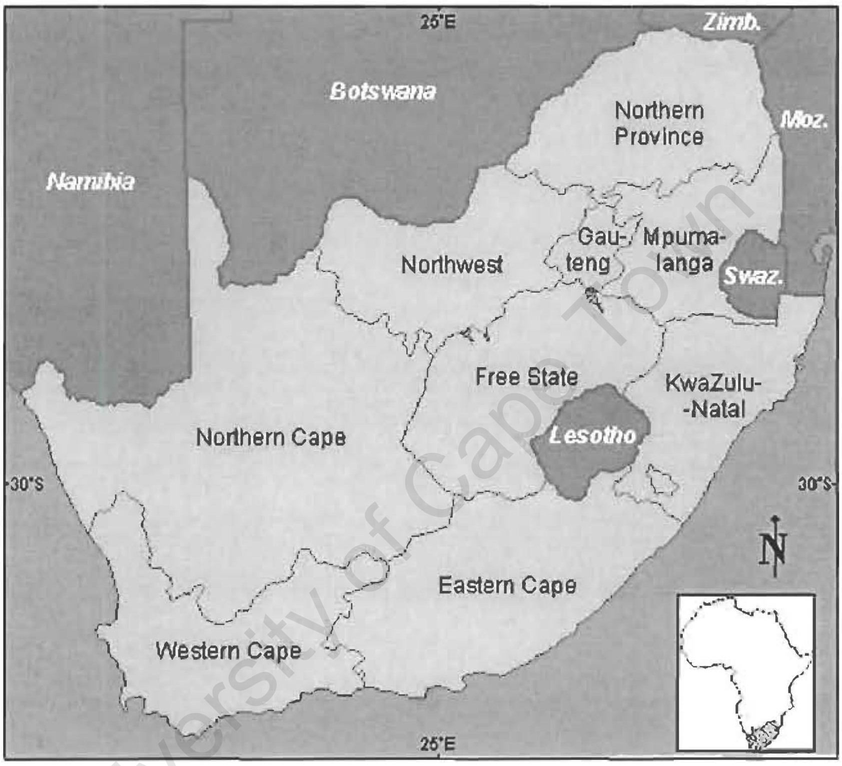


Figure 1.2.1. Map of the Provinces of South Africa

There are broadly three main types of traditional healers practising in South Africa. Firstly, there is the traditional doctor or herbalist, *inyanga* (Zulu; plural *izinyanga*), *ixwele* (Xhosa), *nqaka* (Sotho), *bossidokter* and *kruiedokter* (Western and Northern Cape). This is generally a male who uses herbal and other medicinal preparations for treating disease. Secondly, there is the *isangoma* (Zulu), *dingaka* (Sotho) or *amaggira* (Xhosa). *Isangomas* are diviners; they determine the cause of illness by using ancestral spirits, and they are usually female. The terms *inyanga* and *sangoma* used to refer exclusively to the herbalist and diviner respectively, but in modern times, the distinction has become blurred, with some healers practising both arts. The third type is the *umthandazi*, or faith healer who integrates Christian ritual and traditional practices. This latter group can be traced back to healers practising both arts. The third type is the *umthandazi*, or faith healer who integrates Christian ritual and traditional practices. This latter group can be traced back to the rise of the independent African church movement, which broke away from the more Western orientated missionary churches. They heal by prayer, by use of holy water or ash, or by touching a patient. In addition to these main groups, there are traditional birth attendants, prophets, spirit mediums, intuitives and dreamers, and traditional surgeons, who mainly perform circumcisions. Most of the elderly folk in rural areas have knowledge of herbal lore, and function as first-aid healers with a family repertoire of herbal remedies or *kruierate* (Kale, 1995; Van Wyk et al., 1997). The medicines are mostly taken orally, but are also given rectally, topically and by inhalation. Some medicines are placed in small incisions in the skin, to accelerate the onset of action (Edinburg, 1998).

In accordance with the World Health Organisation (WHO) policy to reassess traditional medicine as a potential health care resource in developing countries, the South African government aims to register traditional medicines and bring them under the vigilant control of the Medicines Control Council (MCC) inspectorate (Edinburg, 1998). In a move to legitimise traditional medicine, the South African parliament proposed in 1998 that a statutory council be set up to regulate the traditional healers, by looking into the registration of all qualifying traditional healers; promoting training, research and professionalism, and the creation of a traditional medicines database (Baleta, 1998). The Department of Health is currently establishing a National Reference Centre for Traditional Medicine to make use of the national source of knowledge and expertise, with the traditional healers forming an integral part of this structure. The benefits of recruiting the traditional healers into South Africa's primary health care are potentially enormous. In 1997, the national HIV/AIDS programme enlisted the services of three traditional healers as consultants to mobilise other traditional healers around the issue of sexually transmitted diseases and HIV/AIDS. In October 1998, the first South African traditional hospital was opened in Mpumalanga Province. The Samuel Traditional Hospital near KwaMhlanga has a staff of five different traditional doctors and two wards with 24 beds each.

1.3. The Use of Plants in Traditional Medicine

Traditional medicines are largely of plant origin, but may also contain minerals and animal materials (Joubert, 1990) or even human tissues, as well as agricultural and industrial chemicals (Stewart et al., 1998). The use of plants as medicines can be traced back over six thousand years to some of the earliest written records from civilisations in China, India and the Near East (Hamburger and Hostettman, 1991). During the course of history, experimentation has succeeded in distinguishing those plants that have beneficial effects from those that are toxic or merely non-effective. The WHO estimates that herbal and other plant-derived remedies are the most frequently used therapies worldwide, and that some 20 000 species of higher plants are used medicinally throughout the world today (Phillipson, 1994).

1.3.1. Plant-derived drugs

At least 25% of all prescription drugs dispensed worldwide today contain active compounds that were derived from higher plants (Farnsworth, 1994), with about 75% of these compounds being discovered through the investigation of traditional remedies (Gericke, 1993). This does not include those synthetic compounds that were derived via knowledge of natural product molecules (Farnsworth, 1994). Natural product molecules used clinically include the alkaloids, anthraquinones, iridoids, lignans, and mono-, sesqui-, di- and tri-terpenoids. The reductionism approach to drug discovery, whereby pure active compounds isolated from the plant replaced plant extracts, was initiated by the isolation of the opium poppy (*Papaver somniferum*) alkaloids such as morphine. Historically, many important drugs have been introduced this way, including: the antimalarial quinine from the *Cinchona* species; the cardiotonic glycosides, digitoxin, and later digoxin, from *Digitalis* (foxglove) species; the antispasmodic tropane alkaloids hyoscine and atropine from *Atropa belladona*, and the first tranquilliser, reserpine, from the Indian plant *Rauvolfia serpentina*. Salicin, which was originally isolated from the bark of the willow tree (*Salix* species), is the pharmacological ancestor of a family of drugs called salicylates, the best known of which is the world's most widely used drug, aspirin.

One aim of drug therapy in modern medicine is to selectively poison the cause of disease (Okpako, 1999). By their very nature poisons are biodynamic substances since they affect or are intended to affect the victim's body. Throughout the history of pharmacology, poisonous plants have therefore been major sources of drugs. Paracelsus highlighted this hazy distinction between poisons and medicines in the 15th century, when he declared, "solely the dose distinguishes that a thing is not poison". **Table 1.3.1.1** provides details of some of the drugs that were originally isolated from poisonous plants.

Recent years have witnessed a renewed interest in plants as sources of new pharmaceuticals in the Western world. This interest is being channelled into both the discovery of new biologically active molecules by the pharmaceutical industry, and the adoption of crude extracts of plants for self-medication by the general public (Houghton, 1995). In both these areas, considerable attention is being paid to investigating plants used traditionally for medicinal purposes. There are an estimated 250 000 higher plants species, and vast numbers of other living organisms in the world. Focussing on plants used for centuries in traditional medicine, rather than randomly screening plants is more likely to yield pharmacologically active compounds.

Indigenous peoples do not recognise all Western diseases. Disorders of the gastrointestinal tract, inflammation, skin infections and some viral diseases are more likely to be important to traditional healers, whilst diseases like cancer and cardiovascular complaints are less likely to be easily diagnosed by the healers. However, traditional remedies may indicate pharmacological activity for illnesses for which the biochemical mechanisms have yet to be discovered (Cox, 1994). Drug discovery from ethnobotanical leads can result in three different final outcomes:

(1) Unmodified natural plant products where ethnomedical use suggested clinical efficacy
(2) Unmodified natural products of which the therapeutic efficacy was unrelated to or only remotely suggested by indigenous plant use
(3) Modified natural or synthetic substances based on a natural product used in folk medicine.

Table 1.3.1.1. Pharmacologically useful compounds derived from poisonous plants

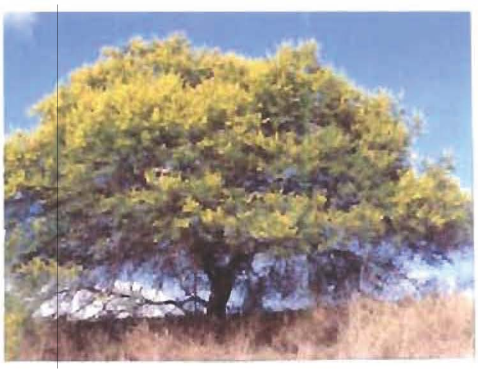
Compound	**Pharmacological activity**	**Plant source**	**Historical notes**
Atropine	Antidote for morphine & mushroom poisoning Suppresses mucous excretion- symptomatic relief of colds	*Atropa belladonna* *Datura stramonium*	Poison Religious use for inducing hallucinations
Emetine	Amoebicide, emetic, insect repellent	*Cephaelis ipecacuanha*	Poison
Colchicine	Antigout	*Colchicum autumnale*	Powerful poison
Digitoxin Digoxin	Cardiotonic Cardiotonic	*Digitalis purpurea* *Digitalis lanata*	An ointment for cleansing wounds and reducing swelling, and as a tea for use as an expectorant. Native Americans brewed the plant to treat swelling in the legs caused by heart problems.
Ergot alkaloids	Oxytocic - management of third stage labour, control of post-partum haemorrhage Vasoconstrictor - migraine relief	*Claviceps purpurea*	Infection of cereals esp. rye resulted in gangrene (St Anthony's fire), or convulsions and possible infertility
β-Erythroidine	Muscle relaxant	*Erythrina senegalensis*	Arrow poison
Morphine Noscapine	Analgesic Cough suppressant	*Papaver somniferum*	Opium used traditionally as an astringent, antispasmodic, hypnotic, sedative and narcotic
Ouabain	Cardiotonic	*Strophanthus gratus*	Dart poison used by African people
Physostigmine	Acetylcholinesterase inhibition - limited therapy for Alzheimer's disease	*Physostigma venenosum*	Ordeal poison to determine guilt

In the past 50 years, the advent of high-throughput, mechanism-based *in vitro* bioassays coupled with candidate plants derived from careful ethnopharmacological research has resulted in the discovery of exciting new pharmaceuticals (Cox, 1994). In the field of anticancer research, ethnopharmacology has yielded some important agents, the most outstanding examples being vinblastine and vincristine from the Madagascar periwinkle, *Catharanus roseus*, and etoposide, the semi-synthetic lignan, from the May-apple *Podophyllum peltatum*. More recently paclitaxel (taxol), a diterpenoid obtained from the Pacific yew, *Taxus brevifolia*, has been shown to be highly successful in clinical trials against advanced ovarian cancer (De Smet, 1997) and is currently undergoing clinical development. The people of China have a rich heritage of medicinal plant use, and modern investigations have verified the efficacy of many traditional uses. A noteworthy example is artemisinin, an antimalarial that was developed from *Artemisia annua*, which was documented as being used for centuries in Chinese traditional medicine to treat fevers and malaria. The soluble analogue artemether is now widely used and is recommended by the WHO as an antimalarial in areas where choloroquine-resistant strains of *Plasmodium falciparum* are prevalent. Considering that until recently only approximately 5 000 species of flowering plants of the world have been tested for therapeutic efficacy, there is little doubt that the scientific investigation of this largely untapped resource will yield interesting therapeutic initiatives.

1.3.2. Medicinal plants used in Southern Africa

Southern Africa has an incredible wealth of plant species, with well over 30 000 species of higher plants occurring in the sub-continent. South Africa is also the only country to have a complete plant kingdom entirely within its borders. The tiny Cape Flora Kingdom has almost 9 000 species, and is the most diverse temperate flora on earth, rivalling the tropical rainforests in terms of species richness. Approximately 3 000 species are utilised as medicines, with over 350 of these still commonly used and traded as medicinal plants. Many of these plants possess pharmacological properties, although only a small proportion of them has to date been investigated for their safety and efficacy (Van Wyk et al., 1997). A few examples of the medicinal plants of southern Africa are illustrated in **Plate 1.3.2.1**, with some details of their use and known properties given in **Table 1.3.2.1**.

Plate 1.3.2.1. Some common medicinal plants in South Africa


Acacia karroo


Artemisia afra


Cinnamomum camphora


Aloe ferox


Eucomis autumnalis


Hypoxis hemerocallidea


Protea repens


Harpagophytum procumbens


Ricinus communis

All photographs reproduced with kind permission from Van Wyk BE, Van Oudtshoorn B and Gericke N. **Medicinal Plants of South Africa.** Pretoria: Briza Publications; 1997.

Table 1.3.2.1. A selection of plants commonly used in traditional medicine in South Africa

Name	Plant parts	Medicinal uses	Known Pharmacology	Active components	References
Agathosma sp. Buchu	Leaves	Stomach complaints, rheumatism, kidney and urinary tract diseases	Antiseptic, diuretic	Essential oil compounds (diosphenol)	Van Wyk et al., 1997
Aloe ferox Mill. Bitter aloe	Leaves, roots	Laxative, arthritis, eczema, conjunctivitis, hypertension, stress	Laxative	Anthrone C-glucoside (aloin)	Watt & Breyer-Brandwijk, 1962, Van Wyk et al., 1997
	Leaf sap	Skin irritations, bruises, burns	Wound-healing	Glycoproteins	
Artemisia afra Jacq. ex Willd. African wormwood	Leaves, roots	Coughs, colds, influenza, fever, malaria, headache, earache, colic, intestinal worms	Decongestant Antibacterial	Essential oil	Hutchings, 1989
Eucomis autumnalis (Mill.) Chitt. subsp. *autumnalis* Pineapple flower	Bulb	Backache, fractures, urinary diseases, fevers, colic, stomach ache, syphilis To facilitate childbirth	Anti-inflammatory Antispasmodic Wound-healing	Homoiso-flavones, benzopyyrones, steroidal triterpenoids	Watt & Breyer-Brandwijk, 1962, Hutchings, 1996
Harpagophytum procumbens Devil's Claw	Secondary roots	Rheumatism and arthritis, general health tonic, digestive disorders	Analgesic Anti-arthritic	Iridoids, phytosterols Triterpenoids, flavonoid	Watt & Breyer-Brandwijk, 1962, Van Wyk et al., 1997
Hypoxis Hemerocallidea Fisch. & C.A. Mey. Inkomfe (Zulu)	Corm	Dizziness, insanity, bladder disorders, testicular cancer	Anti-cancer Anti-HIV Anti-inflammatory	Phytosterol glycosides	Hutchings, 1996 Van Wyk et al., 1997

1.3.3. The need for the conservation of medicinal plants

The recent commercialisation of traditional medicine is leading to the depletion of many species. It has been estimated that 60 000 species, nearly a quarter of the world's total, would become extinct by the year 2050, if present trends were to continue (Akerele, 1993). In September 2000, the Geneva-based World Conservation Union (IUCN) released the authoritative Red Data List of Threatened Species, which detailed 5 611 species of threatened plants, many of which are trees. In South Africa, there are growing concerns over the rapid destruction of traditional medicinal plant resources. Mander (1999) estimated that nearly 20 000 tonnes of plant materials are consumed annually in South Africa alone, with the current market in medicinal plants estimated to be US$35 million a year. More than 700 plant species are actively traded throughout South Africa for indigenous plant use. Rural women, one of the most economically marginalized groups in the country, harvest the plants to sell to traditional healers, resulting in a rapid depletion of resources. The Durban Metropolitan Area forms the hub of an active regional trade in medicinal plant harvesting, trade and consumption. There is such a burden on popular species that nine of the top ten medicinal plants traded in the KwaZulu-Natal Province are now RDListed (Scott-Shaw, 1999). Several plant species, such as *Siphonochilus aethiopicus* (wild ginger) and *Warburgia salutaris* (pepper-bark tree) have become extinct outside the protected areas of KwaZulu-Natal.

The demand for medicinal plants is likely to remain high in the future. An integrated national or provincial approach to the conservation of medicinal plants has yet to be properly implemented in South Africa. Despite this deficiency, there are various independent, socially and financially motivated projects underway; including a number of entrepreneurial ventures that grow selected species for the plant trade. Horticulturists have a definite role to play in the conservation of the flora of Southern Africa, and are making an important contribution to this by equipping traditional healers with appropriate propagation and cultivation skills, thus enabling the healers to grow the medicinal plants in their home environment. Horticultural training is being carried out by the Silver Glen Medicinal Plant Nursery in Chatsworth, Durban, which has also developed and published several propagation protocols (e.g. Crouch and Symmonds, 1999).

The Mpumalanga province is home to a vast range of medicinal plants, many of which are in danger of extinction due to over exploitation and destruction of the natural habitat. The Medicinal Flora Co-operative (MCF), an initiative aimed at finding a balance between the need to conserve the natural medicinal plant resources and the growing demand for plants from traditional healers, was launched in November 2000. The MCF will work to make medicinal plants of a high quality available for the market, thus lessening the pressure on wild plant populations.

1.4. Potential Toxicity of Traditional Herbal Remedies

Of the approximately 30 000 endogenous plant species in South Africa, about 300 can be considered poisonous to man (Rossouw, 1987). Almost an equal number of poisonous plants have been imported and are common in most domestic gardens. Watt and Breyer-Brandwijk (1962) list over 250 Xhosa or Amapondo names of medicinal plants, and of these, several families contain potentially toxic members, most notably the Amaryllidaceae, Hyacinthaceae, Colchicaceae and Eriospermaceae families. Toxic compounds found in these families include alkaloids, cardiotoxic glycosides, resins and steroid saponins (Hutchings and Terblanche, 1989). The function of these compounds in the plants is thought to be a protective, survival-ensuring one. **Table 1.4.1** lists some of the potentially toxic plants, which are commonly used in traditional medicine in South Africa today. Some of these plants are illustrated in **Plate 1.4.1**.

Plate 1.4.1. Some potentially toxic plants of South Africa

Acokanthera oppositifolia

Bowiea volubilis

Datura stramonium

Drimia robusta

Scilla natalensis

Strychnos henningsii

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Table 1.4.1. (a) Some potentially toxic medicinal plants used in South Africa

Name	Medicinal use(s)	Toxic Principle(s)	Features of poisoning	Fatalities (If any)	References
Acokanthera oppositifolia (Lam.) Codd Common poison bush	Headaches, snake bite, toothache, colds, anthrax, tapeworm	Cardiac glycosides	Heart failure	Reported deaths	Watt & Breyer-Brandwijk, 1962 Van Wyk et al., 1997
Bowiea volubilis Harv. ex Hook. f. Climbing potato	Headaches, oedema, purgative, sore eyes, sterility bladder complaints	Cardiac glycosides	Haemolysis, vomiting, diarrhoea, arrhythmia	Recorded human death	Hutchings & Terblanche, 1989 Vahrmeijer, 1981
Callilepis laureola DC Impila (Zulu)	Stomach complaints, tapeworm infestations, whooping cough, induce fertility	Atractyloside	Liver necrosis, hypoglycaemia, cardiac failure	Reported fatal liver necrosis after use	Bye and Dutton, 1991 Watson et al., 1979 Wainright et al., 1977
Datura stramonium L. Thorn apple	Asthma, aphrodisiac	Tropane alkaloids	Increased heart rate, eye muscle paralysis, reduced sweating and salivation, insomnia, hallucinations, mental confusion,		Watt & Breyer-Brandwijk, 1962 Van Wyk et al., 1997
Drimia robusta Bak. Isiklenama (Zulu)	Expectorant, emetic, diseases of bladder and uterus Used as arrow poisons	Calcium oxalate crystals	Highly irritant to skin		Watt & Breyer-Brandwijk, 1962

Table 1.4.1. (b) Some potentially toxic medicinal plants used in South Africa

Name	Medicinal use(s)	Toxic Principle(s)	Features of poisoning	Fatalities (If any)	References
Gloriosa superba Lindl. Flame Lily	Barrenness, sterility, aphrodisiac	Colchicine	Mucous membrane irritation, severe vomiting, diarrhoea, abdominal pain, respiratory failure	Recorded human and animal death	Watt & Breyer-Brandwijk, 1962
Scilla natalensis Planch. Inguduza (Zulu)	Enema , purgative, female infertility, internal tumours	Saponins	Dyspnoea in sheep	Death of sheep	Hutchings, 1996
Senecio sp.	Wound healing, blood purifiers Treat chest pains	Pyrrolizidine alkaloids	Liver damage, hepatic tumours	Death of stock	Vahrmeijer, 1981
Strychnos henningsii Gilg. Red bitterberry	Nausea and colic, purgative, rheumatic fever	Strychnos alkaloids	Stimulation of CNS		Watt & Breyer-Brandwijk, 1962
Urginia sp.	Stomach ailments, hypertension, diuretic, asthma	Cardiac glycosides	Gastrointestinal irritation, paralysis, heart failure	Recorded human deaths	Watt & Breyer-Brandwijk, 1962 Hutchings, 1996

1.4.1. Acute poisoning caused by traditional medicines

The field of poisoning caused by traditional remedies is relatively poorly studied. In developed countries such as the United States of America, poisoning by plants is almost invariably due to accidental ingestion of berries, seeds and mushrooms by infants (Lamminpaa and Kinos, 1996). In the developing world, the inappropriate use of traditional medicines has resulted in numerous fatalities, predominantly in children (Bye and Dutton, 1991). It is difficult to quantify the contribution of traditional medicines to the overall picture of acute poisoning in southern Africa as the available information is limited to case reports. Data collected for the period 1981-1985 from Ga-Rankuwa Hospital (Joubert, 1990) on 1306 patients admitted with acute poisoning, revealed that most poisonings were accidental, and that the most important causes of poisoning were kerosine (59%) and traditional medicines (15.8%). The major cause of mortality was traditional medicines, responsible for 51.7% of the deaths that occurred. The traditional healer was the main source of these medicines, whilst in the remaining cases, the remedy was either bought at an herbal shop or the patients themselves collected the medicines. More recently, an analysis of the Johannesburg forensic database for the years 1991-1995 (Stewart et al., 1999) revealed 206 cases in which a traditional remedy was either stated to be the cause of death or was found to be present in a case of poisoning with an unknown substance.

Poisoning with traditional medicines is serious because it is often fatal. The Ga-Rankuwa study revealed that 15.3% of the patients admitted due to poisoning with traditional medicines died compared with 4.6 % of all patients poisoned by other sources. Similar mortality statistics (13.0%) are reported from Zimbabwe (Nyazema, 1986). The significance of poisonings caused by traditional medicines in the South African population must be seen in context, as over 80% of acute poisonings in first world communities are due to orthodox medicines (Joubert and Mathibe, 1989). The causes and types of poisoning differ in different parts of the world and depend upon many different factors, such as education, socio-economic status, local beliefs, customs and demography (Kasilo and Nhachi, 1992).

The rural folk in many cases will identify and harvest their own herbal remedies from the veld. In urban areas, the people are able to purchase traditional medicines from herbalist shops. Poisonings can occur when a misidentified toxic plant is consumed instead of an intended safe plant (Huxtable, 1990) or if the toxicity of a correctly identified plant is unknown or ignored. The *Senecio* species are notoriously difficult to differentiate, even by experienced botanists, and as such, a toxic species might easily be inadvertently picked and used (Stewart et al., 1998). Toxicity in plants is known to vary with season, with the amount of toxin often being low in proportion to the high water content, as in the case of Amaryllidaceae (Hutchings and Terblanche, 1989). Often, the level of toxic compounds in a higher plant will increase just prior to flowering, and then drop again after seeding. The level of toxins in the plant can also be dependent on the length of the growing season and the type of soil in which the plant is growing, as well as stress factors such as drought that the plant endures during its life cycle. Traditional methods of preparation of plants for remedies include mixing, crushing, boiling, drying and burning (Edinburg, 1998). Heating would tend to destroy some of the toxins. Poisonous plants often have an offensive taste, which means that they are seldom ingested in harmful quantities. This bad taste does not however deter users of traditional herbal medicines from being poisoned.

Many patients keep their options open, and will consult western doctors as well as traditional healers, in which case there is the potential hazard of conventional drug interaction with the herbal remedy supplied by the traditional healer (D'Arcy, 1993). Herbal products can be adulterated, intentionally or otherwise (Huxtable, 1990). Specifically, contamination with pesticides occurs where plants grow in close proximity to commercial crops (Stewart et al., 1998). Urban drift in South Africa has led to a greater use of chemical constituents, such as liquid detergents, disinfectants and potassium permanganate, and the administration of such potent substances leads to adverse effects. There is an increasing awareness of dichromate toxicity after medical reports of its deliberate use in South Africa in traditional remedies (Wood et al., 1990; Michie et al., 1991). The mucosal irritation caused by potassium dichromate is exploited in its use in purgatives, most frequently administered in the form of enemas. The lethal oral dose of dichromate can be as little as 0.5 - 1.0 g. Another common cause of poisoning arises from the contamination of plants with moulds during wet periods or following inappropriate or prolonged storage (Stewart et al., 1998).

Although there are recorded cases where patients have been given a single lethally toxic dose, certain users of herbs are at a higher risk of intoxication, particularly chronic users, infants, foetuses, the elderly, the sick and the malnourished, and those on long-term medication. Babies are at special risk because they may accidentally be dosed with herbs at a higher level per kilogram bodyweight than adults would consume, and because babies lack the hepatic drug biotransformation and detoxification enzymes. Usually, dosages are estimated according to the size, weight and age, as well as the general constitution and condition of the patient. Children and older patients receive lower doses, or more diluted remedies than adults do (Edinburg, 1998) to avoid the dangers of over-dosing. Overdose is less likely with traditional remedies, as the medication is a crude extract of the herb, and as such contains small concentrations of active ingredients. If the mother is imbibing herbs, babies may also be exposed to plant toxins via the milk.

1.4.2. Clinical syndromes of traditional medicines poisoning

Generally, acute poisoning with traditional medicines presents with one of three major clinical syndromes (Joubert and Mathibe, 1989). Most commonly, there is a varying degree of gastrointestinal irritation, which affects either the upper or lower, or the whole gastrointestinal tract. Symptoms may vary from mild epigastric discomfort and nausea to severe vomiting with dehydration. Diarrhoea and even frank bleeding from the gastrointestinal tract may occur in severe cases. Many traditional medicines are purgatives administered as enemas, which can have adverse effects on the bowel. The second syndrome is hepatic or renal toxicity or both. Patients can develop hepatic or renal failure with chronic metabolic disturbances such as jaundice. The third syndrome is anticholinergic and involves the central nervous system. Patients can be confused and disorientated, and may hallucinate.

The major problem confronting the physician who has to deal with acute poisoning due to traditional medicines is the difficulty of making a diagnosis (Joubert and Mathibe, 1989). Since the patient who consults a traditional healer is already ill, it is difficult to determine whether symptoms are due to the original disease or the traditional remedy. The traditional medicine may adversely affect the original disease (McVann et al., 1992). On the other hand, patients might die from the original disease without the traditional medicine having contributed to the outcome. Solleder (1974) discusses this point with case histories. Botanical identification would be most important in providing the clinician with an idea of the possible toxin or toxins involved. Even in cases where a sample of the traditional remedy is obtained from patients or relatives, it is often impossible to make a positive identification of the toxin, since the remedy may consist of ground leaves or bark, a watery extract or might even be a mixture of several components (Joubert and Mathibe, 1989). Analytical methods, such as high-performance liquid chromatography, gas chromatography, mass spectrometry and immunoassays, can provide identification of the toxin in those isolated cases in which the history or symptoms give a clear lead. There is, however, still an urgent need for broad urine and serum toxicological screening methods to be developed (Stewart et al., 1998) to facilitate identification of the toxin and plan appropriate treatment. Usual management is supportive, and symptomatic, and includes measures such as adequate hydration, replacement of blood loss, glucose for hypoglycaemia, correction of electrolyte balances, and dialysis for acute renal failure (Joubert and Mathibe, 1989).

1.4.3. Traditional remedies used in infants and pregnant women

Plants used for infants, children or pregnant women are frequently collected and administered by the child's grandmother, who relies on traditional information, which has been passed on by word of mouth (Hutchings and Terblanche, 1989). Medicines for infants include purges and enemas thought necessary at birth and weaning for cleansing away of impurities believed to have been passed on by the mother. In the Transkei, these medicines are administered regularly to the newly born (sometimes at every second feed) for up to 3 months (Verdcourt and Trump, 1969). A study at Hlabisa Hospital, Kwazulu Natal (Moore and Moore, 1998) highlighted the association between administration of a traditional medicine enema and a distinct clinical syndrome amongst acutely ill children. Whilst the majority of enemas are given without incident, children struggling with an underlying illness may be unable to tolerate rectally administered traditional medicines. Further complications can arise from the frequent use of toxic products in chemical enemas. Traditional medicine enema constituents are usually organic, including specifically selected roots, bark, herbs and leaves. There is the danger of toxic components in certain traditional medicinal plants, such as the cardiac glycosides found in *Scilla natalensis* and *Scilla nervosus*, which are commonly used in enemas given to children (Hutchings and Terblanche, 1989; McVann et al., 1992).

It is estimated that approximately two-thirds of babies born worldwide are delivered by traditional birth attendants (Van Wyk and Gericke, 2000). Each healer or traditional midwife has their own repertoire of herbal remedies, depending on the availability of plants in the local environment. A wide range of plants are used to enhance fertility, regulate menstrual cycle, treat infection and pain, maintain pregnancy, tone the uterus, initiate and augment labour, expel a retained placenta and stimulate lactation. The chemistry, pharmacology and nutritional value of these plants are generally unknown. A literature survey by Veale et al., in 1992 revealed that 57 different plants are used during pregnancy and childbirth, of which 17 are known to be potentially poisonous. Some commonly used plants include *Agapanthus africanus*, *Clivia miniata*, *Gunnera perpensa*, *Pentanisia prunelloides*, *Rhoicissus tridentata* and *Triumfetta rhomboidea*. All of these plants have been shown to stimulate uterine contractions in the laboratory (Veale et al., 1989; Larsen et al., 1983; Kaido et al., 1997; Katsoulis et al., 1999).

The most common antenatal herbal medication taken by the Xhosa and Zulu during pregnancy is *isihlambezo* ("that which cleanses"), which is a decoction of different plants (Mabina et al., 1997). These remedies are prescribed to enhance foetal growth, strengthen the mother and the foetus, aid the normal physiological functioning of the mother, ensure an easy and uncomplicated delivery, and remove excess fluid from the abdomen (Gumede, 1978; Veale et al., 1992; Kaido et al., 1997). There are many different recipes for *isihlambezo*, and the ingredients are often a closely guarded secret. Pregnant women will obtain the recipe from the old women of the community, and failing that, a traditional healer can be consulted, or the ingredients purchased from an herbalist. The herbs are boiled in water for a long time, and the decoction kept in a covered pot, to be taken regularly by a pregnant woman. *Isihlambezo* plant mixtures are often given in the last trimester of pregnancy (Van Wyk and Gericke 2000). The preparations often include plants with oxytocic effects, and are used at low doses to strengthen and tone the uterine muscle in preparation for delivery. The *isihlambezo* can be increased in concentration at term to induce or augment labour (Veale et al., 1992). During the last trimester the foetus is least vulnerable to potentially harmful phytochemicals. In the first trimester, potentially toxic substances can disrupt embryo development, leading to abortion whereas in the second trimester, when organ development is taking place, potentially toxic substances can lead to congenital abnormalities (Van Wyk and Gericke, 2000). Certain childhood disorders, such as malnutrition, and congenital malformations, may be due to toxic or carcinogenic constituents present in herbal medicine taken during pregnancy (Mabina et al., 1997). Deaths from acute renal failure, fatal oesophageal strictures, and ruptured uteri, have been attributed to the use of herbal medicine (Bye and Dutton, 1991). Following the thalidomide tragedy (1960-1961), enormous amounts of money are now invested by the pharmaceutical industry into investigating the safety of drugs in pregnancy. Thalidomide was prescribed to pregnant women to ease their nausea during the first trimester. An estimated 10 000 babies were born with deformities, such as shortened or missing limbs, causing their feet or hands to be attached directly to their abdomens.

In conclusion, there is an urgent need to study the safety and efficacy of plants used in traditional medicine in Southern Africa and in the rest of the world. The identification of new compounds that have therapeutic value will contribute significantly towards the development of new pharmaceuticals, whilst toxicology studies on the medicinal plants will promote awareness of the dangers of inappropriate use of traditional medicines in the community. The research in this thesis focuses on two plants belonging to the Amaryllidaceae family, namely *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*. The Amaryllidaceae plants have been widely used in traditional medicine in Southern Africa for centuries. Pharmacological investigations have revealed that many of the plants have medicinal value, whilst some of the plants are also potentially toxic. The medicinal and toxic activities have been attributed to a group of isoquinoline alkaloids that are found exclusively in the Amaryllidaceae family of plants.

Chapter 2

The Amaryllidaceae Alkaloids

2.1. The Amaryllidaceae Family of Plants

The Amaryllidaceae is a widely distributed family of 59 genera and about 850 species (Meerow and Snijman, 1998). Although largely native to the tropics and subtropics, some species do occur naturally in temperate regions. There are pronounced centres of diversity in South America (28 genera), particularly in the Andean region, in Africa (19 genera), and in the Mediterranean (8 genera). All members of the Amaryllidaceae are perennial herbs, and all but three genera (*Clivia*, *Cryptostephanus* and *Scadoxus*) form tunicate bulbs, with these other three genera forming rhizomes. There are nine tribes in the family Amaryllidaceae, with Amaryllideae and Haemantheae being the two prominent tribes of Southern and Eastern Africa (Viladomat et al., 1997a). Amaryllideae consists of eleven recognised genera and a recent revised classification of the tribe recognises two monophyletic subtribes, Crininae (*Ammoncharis, Boophane, Crinum* and *Cybistetes*) and Amaryllidinae (*Amaryllis, Brunsvigia, Carpolyza, Crossyne, Hessea, Nerine* and *Strumaria*). Crininae is widespread in the tropical and temperate regions of sub-Saharan Africa, whereas Amaryllidinae is confined to the temperate regions of Southern Africa. The tribe Haemantheae consists of six genera (*Apodolirion, Clivia, Cyrtanthus, Gethyllis, Haemanthus* and *Scadoxus*), and has its centre of variation in southern Africa (Snijman et al., 1995). Fire ecology is important in the biology of some of the African genera: *Amaryllis*, *Haemanthus*, and *Hessea* in the South African fynbos region, and *Cyrtanthus* in grassland and savanna (Meerow and Snijman, 1998).

2.1.1. Traditional Uses of Amaryllidaceae in Africa

There are claims that crude preparations from the bulbs of Amaryllidaceae have been used in the treatment of tumours since the time of Hippocrates (Neuwinger, 1996). Many species of the Amaryllidaceae family have been used for at least two hundred years in the traditional medicine of the indigenous peoples of Southern Africa (Watt and Breyer-Brandwijk, 1962). A rare rock painting in Lesotho has been attributed to the San People, and is thought to depict a *Brunsvigia* species, whose use probably induced psychoactive effects (Loubser and Zietsman, 1994). The bulbs of the Amaryllidaceae plants are most frequently used, and most remedies, prepared from single plants, are administered as hot- or cold-water infusions. **Table 2.1.1** lists some of the traditional uses of Amaryllidaceae plants in Africa. Other than their use in traditional medicines, the Amaryllidaceae have little economic importance except as ornamentals. A large number of southern African Amaryllidaceae species in several genera are widely cultivated for their attractive flowers, most notably the species of *Boophane*, *Clivia*, *Crinum*, *Cyrtanthus*, *Hippeastrum*, *Nerine* and *Zephyranthes*. *Narcissus*, *Leucojum* and *Galanthus* are among the most important temperate-zone, spring-flowering bulbs in commerce (Meerow and Snijman, 1998). The genus *Narcissus*, which includes the popular daffodils, narcissi, and jonquils, has been extensively cultivated for over 300 years, as have a number of naturally occurring hybrids. Deliberate hybridisation was started in the 19th Century by English gardeners and has since been carried out on a large scale.

Plate 2.1. Photo gallery of some Amaryllidaceae species


Amaryllis belladonna


Brunsvigia radulosa


Clivia miniata


Cybistetes longifolia


Cyrtanthus spiralis


Galanthus nivalis


Narcissus jonquilla.


Nerine undulata


Scadoxus multiflorus

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Table 2.1.1. (a) Traditional uses of Amaryllidaceae in Africa

Plant Species	Part Used	Traditional Use (s)	Used By	Reference (s)
Amaryllis belladona L.	bulb	Treatment of cancer		Pettit et al., 1984
	flower	Antispasmodic		Watt and Breyer-Brandwijk, 1962
Ammocharis coranica (Ker-Gawl.) Herb	bulb	Treatment of afflictions caused by witchcraft	Zulu	Hutchings et al., 1996
		Paste to repair cracks in clay pots	Sotho	Watt and Breyer-Brandwijk, 1962
Apodolirion buchananii Bak.	bulb	Purgative/ enema for stomach ailments	Zulu	Hutchings et al., 1996
Boophane sp.	bulb	Topical treatment of leprosy, ulcers, febrile colds, asthma, coughs and wounds		Watt and Breyer-Brandwijk, 1962 Hutchings et al., 1996
Boophane disticha (L.f.)	bulb	Treatment of headaches, chest pains, bladder pains, muscle cramps	Zulu	Hutchings et al., 1996
	bulb	Administered to hysterical adolescent females	Zulu	Hutchings et al., 1996
	bulb	Outer dressings for circumcision wounds, burns, boils and abscesses	Xhosa, Sotho	Hutchings et al., 1996
	bulb	Arrow and rat poisons	Khoi, San	Hutchings et al., 1996
	bulb scale	Decoction administered as powerful sedative to violent, psychotic patients		Van Wyk and Gericke, 2000
		Diviners administer to patients to induce visual hallucinations		Van Wyk and Gericke, 2000
		To check bleeding and treat skin diseases	Zulu	Hutchings et al., 1996
	unspecified	To combat "red water" disease in cattle	Xhosa	Watt and Breyer-Brandwijk, 1962
	whole plant	Cultivated as protective charm	Zulu	Hutchings et al., 1996

Table 2.1.1. (b) Traditional uses of Amaryllidaceae in Africa

Plant Species	**Part Used**	**Traditional Use (s)**	**Used By**	**Reference (s)**
Brunsvigia sp.	leaves, roots	Infertility treatment in women	Zulu	Hutchings et al., 1996
Brunsvigia grandiflora Lindl.	bulb	Decoction for coughs and colds	Zulu	Watt and Breyer-Brandwijk, 1962
		Enema for renal and liver complaints	Zulu	Watt and Breyer-Brandwijk, 1962
	bulb scale	Dressing for circumcision wounds	Xhosa	Watt and Breyer-Brandwijk, 1962
Clivia miniata (Lindl.) Regel	aerial	To initiate labour and facilitate delivery in childbirth	Zulu	Veale et al., 1992
		To treat urinary tract infections and infertility	Xhosa	Hutchings et al., 1996
	leaves	To induce labour	Zulu	Veale et al., 1992
	root	Drunk to treat snakebite; applied to snakebite wound	Zulu	Hutchings et al., 1996
Clivia nobilis Lindl.	root	Protective sprinkling charms	Zulu	Hutchings et al., 1996
Crinum sp.	bulb	Mixed with pounded roots of *Gunnera perpensa* for treatment for urinary complaints and relief of pain from rheumatic fever	Zulu	Watt and Breyer-Brandwijk, 1962
	root	Part of decoction used as blood purifier for scrofula	Zulu	Watt and Breyer-Brandwijk, 1962
Crinum bulbispermum (Burm. F.) Milne-Redh. & Schweik.	bulb	Treatment of aching joints, rheumatism, varicose veins and backache	Zulu, Tswana	Hutchings et al., 1996
		Poultices for septic sores and abscesses	Zulu	Hutchings et al., 1996
	leaf	Infusion for rheumatic fever	Zulu	Hutchings et al., 1996
	unspecified	Part of infusion taken in pregnancy to ease delivery	Zulu	Hutchings et al., 1996

Table 2.1.1. (c) Traditional uses of Amaryllidaceae in Africa

Plant Species	Part Used	Traditional Use (s)	Used By	Reference (s)
Crinum bulbispermum (Burm. F.) Milne-Redh. & Schweik. (cont.)	unspecified	Treatment of colds, to induce lactation, and as protective charms	Sotho	Hutchings et al., 1996
Crinum delagoense Verdoorn	bulb	Taken to reduce swellings, and to treat urinary tract infections	Zulu	Hutchings et al., 1996
Crinum kirkii	bulb scale	Arrow and rat poison		Watt and Breyer-Brandwijk, 1962
Crinum macowanii Bak.	bulb	Treatment of sexually transmitted diseases and renal/urinary tract/bladder infections; as an emetic	Zulu	Hutchings et al., 1996
		Treatment of tuberculosis	Zulu	Van Wyk and Gericke, 2000
		To reduce fever, backaches	Xhosa	Hutchings et al., 1996
		To improve lactation		
		Treatment of itchy rashes, acne, boils and carbuncles		
Crinum moorei Hook. F.	bulb	Treatment of urinary tract infections and body swelling	Zulu	Hutchings et al., 1996
Cyrtanthus sp.	bulb	Sprinkling protective charms against storms and evil	Zulu	Hutchings et al., 1996
Cyrtanthus breviflorus Harv.	bulb	Treatment of roundworm and tapeworm	Zulu	Hutchings et al., 1996
Cyrtanthus obliquus (L.f.) Ait	bulb	Treatment of scrofula, chronic and tubercular coughs	Zulu	Watt and Breyer-Brandwijk, 1962
		Infusion to treat stomach ache		
	bulb scale	Applied externally to areas afflicted with leprosy		
		Snuff taken to relieve headaches		
		To aid union of fractures		

Table 2.1.1. (d) Traditional uses of Amaryllidaceae in Africa

Plant Species	Part Used	Traditional Use (s)	Used By	Reference (s)
Cyrtanthus sanguineus (Lindl.) Walp.	bulb	Taken during pregnancy to ensure healthy fetal growth and easy labour	Xhosa, Zulu	Veale et al., 1992
Gethyllis sp.	fruit	Remedy for colic, flatulence and indigestion	Khoi	Van Wyk and Gericke, 2000
Gethyllis linearis L. Bol.	fruit	Alcoholic tincture for digestive disturbances	Khoi	Watt and Breyer-Brandwijk, 1962
		To perfume rooms and linen	Afrikaans	Van Wyk and Gericke, 2000
Haemanthus sp.	whole plant	Topical treatment of leprosy, ulcers, febrile colds, asthma, coughs and wounds		Hutchings et al., 1996 Watt and Breyer-Brandwijk, 1962
Haemanthus albiflos Jacq.	bulb	Emetic	Zulu	Hutchings et al., 1996
		Treatment of chronic coughs	Xhosa	Hutchings et al., 1996
Haemanthus coccineus L.	leaf	Antiseptic for septic ulcers and anthrax pustules Drunk as diuretic in dropsy and as antiasthmatic	Afrikaans	Van Wyk and Gericke, 2000
Pancratium tenufolium	bulb	To induce visual hallucinations	San	Van Wyk and Gericke, 2000
Scadoxus muliflorus (Martyn) Raf.	rhizome	Applied over scarifications of breasts as galactagogue		
		Used in love charm emetics	Zulu	Hutchings et al., 1996
Scadoxus puniceus (L.) Friis & Nordal	rhizome & root	Treatment of headaches and as poultices		Hutchings et al., 1996
		Administered as antidote for suspected poisoning		
	unspecified	Emetic for coughs		Hutchings et al., 1996
		Taken during pregnancy to facilitate safe delivery		Veale et al., 1992
Zephyranthes candida (Lindl.) Herb.	leaves	Treatment for diabetes mellitus		Watt and Breyer-Brandwijk, 1962

2.1.2. Pharmacological and biological activities of Amaryllidaceae

As a result of their widespread exploitation in African traditional medicine, the Amaryllidaceae plants have become the focus of many pharmacological investigations in recent years. Some of the biological and pharmacological activites of African Amaryllidaceae are detailed in **Table 2.1.2.1**. Whilst many of the plants may be of pharmacological use, several of the plants are also potentially toxic, and adverse effects, and in severe cases, death, have been reported for both humans and animals. **Table 2.1.2.2** highlights some of the cases of Amaryllidaceae poisoning. The compounds responsible for the biological activities, including toxicity, are a group of isoquinoline alkaloids, which are unique to the Amaryllidaceae family. This group of alkaloids had been of minor pharmaceutical significance until recent years, when there was an enormous increase in interest due to the medical applications of galanthamine, an alkaloid isolated exclusively from the Amaryllidaceae species. Galanthamine is a potent inhibitor of acetylcholinesterase, and has application to Alzheimer's disease therapy. The major deficiency in Alzheimer's disease is acetylcholine, which plays an important role in memory function. Preventing the breakdown of acetylcholine in the synapse improves cholinergic neurotransmission. The role of galanthamine in the treatment of Alzheimer's disease is discussed in detail in **Chapter 4**.

Table 2.1.2.1. Pharmacological and biological activities of Amaryllidaceae

Plant species	Activity	Reference
Amaryllis belladona L.	Antifungal, antiyeast	
	Antiplasmodial against *Plasmodium gallinaceum* in chickens	Spencer et al., 1947
	Antineoplastic against murine P-388 lymphocytic leukemia *in vitro*	Charlson, 1980
	Cytotoxic against CA-9KB	Charlson, 1980
Boophane disticha (L. f.) Herb	Antiviral against rhinovirus strains	Beuscher et al., 1993
	Antihistaminic and adrenergic - causes smooth muscle of uterus to relax in guinea pigs	
Clivia miniata (Lindl.) Regel	Antiviral against poliomyelitis, measles and Coxsackie, Herpes 1 viruses *in vitro*	Leven et al., 1982
Crinum macowanii Bak.	Antiviral against flaviviruses (yellow fever and Japanese encephalitis)	Duri et al., 1994
Crinum moorei Hook. f.	Antifungal, antiyeast	Chaumont et al., 1978
Haemanthus albiflos Jacq.	Antiviral activity against Coxsackie B2, Echo virus 11, Poliovirus I, Rotavirus SA 11, Herpes Simplex 1 viruses	Husson et al., 1993
Scadoxus puniceus (L.) Friis & Nordal	Cytotoxic activity	Charlson, 1980
Zephyranthes grandiflora (Lindl.)	Antineoplastic against murine P-388 lymphocytic leukemia	Pettit et al., 1984

Table 2.1.2.2. Some Amaryllidaceae known to have caused human or animal poisoning

Plant species	**Features of poisoning**	**Fatalities (if any)**	**Reference**
Amaryllis belladonna L.	Respiratory paralysis Cardiac arrest	Reported animal deaths	Watt and Breyer-Brandwijk, 1962
Boophane disticha (L. f) Herb	Reported to have caused both acute and fatal poisoning following medicinal use Dizziness, visual disturbance, excitation or depression, stupor, coma	Recorded human and animal deaths	Watt and Breyer-Brandwijk, 1962; Hutchings and Terblanche, 1989
Clivia miniata (Lindl.) Regel	Salivation, vomiting, diarrhoea, depression of central nervous system		Veale et al., 1992
Clivia nobilis Lindl.	Vomiting	Suspected human death	Hutchings and Terblanche, 1989
Cyrtanthus breviflorus Harv.	Suspected cattle poisoning		Watt and Breyer-Brandwijk, 1962
Haemanthus sp.	Acute and fatal poisoning	Death in sheep	Viladomat et al., 1997(a)
Haemanthus multiflorus Martyn.	Dangerous swelling of the lips and tongue		Watt and Breyer-Brandwijk, 1962
Scadoxus puniceus (L.) Friis & Nordal	Dizziness, visual disturbances, excitation or depression of central nervous system	Reported human deaths	Veale et al., 1992

2.2. The Amaryllidaceae Alkaloids

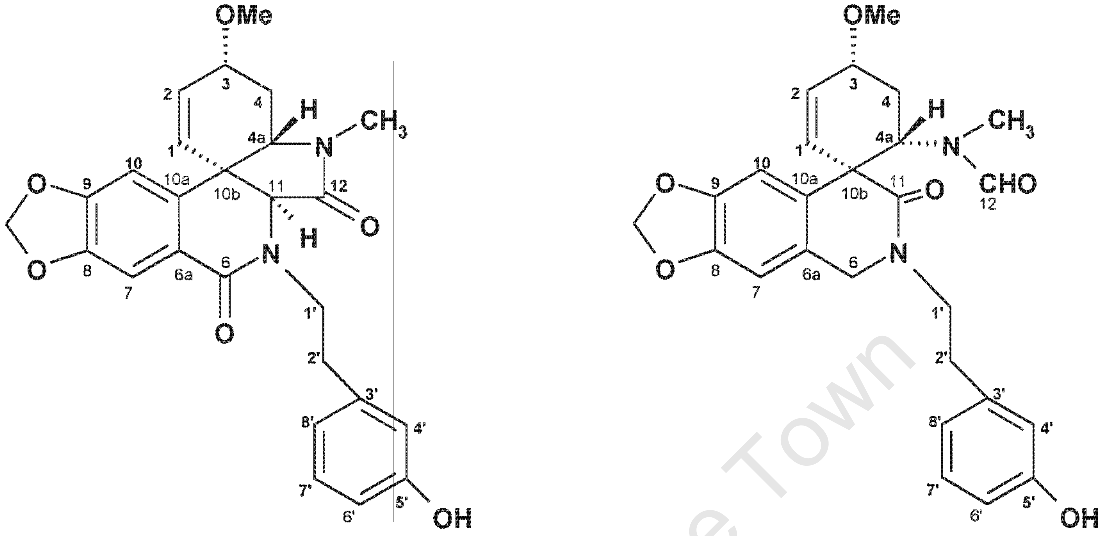
Alkaloids are compounds containing nitrogen, which is most often in a heterocyclic ring, that are common to about 15-20% of all vascular plants. The function of alkaloids in plants in not yet fully understood. It has been suggested that they are formed as metabolic by-products, but there is evidence to suggest that they may serve specific biological functions. In certain plants, the concentration of alkaloids increases just prior to seed formation and then drops off when the seed is ripe, suggesting that alkaloids may play a role in this process. The characteristic bitter taste of alkaloids and accompanying toxicity may help to repel insects and herbivores. In their pure form, most alkaloids are colourless, nonvolatile, crystalline solids that form an orange-coloured precipitate with Dragendorff's reagent (solution of potassium bismuth iodide). Alkaloids are often classified on the basis of their heterocyclic structure, with the principal classes of alkaloids being the pyrrolidine, pyridine, tropane, pyrrolizidine, isoquinoline, indole, quinoline, and the terpenoid and steroid alkaloids.

The isoquinoline alkaloids are found exclusively in the Amaryllidaceae, and it is unusual to find other types of alkaloids in Amaryllidaceae species. If present, they are always accompanied by typical Amaryllidaceae alkaloids. In fact, to date, only three alkaloids have been isolated from Amaryllidaceae that do not belong to the isoquinoline group, but to the mesembrane (Sceletium) type, typically found in the Aizoaceae family. The high water content of the Amaryllidaceae bulbs limits the total alkaloid content to a maximum of 1-2% (Hutchings, 1996).

The typical characteristics of the Amaryllidaceae alkaloids can be summarised as follows:

- A fundamental ring system composed of a C_6-C_1 and an N-C_2-C_6 building block derived from L-phenylalanine (L-phe) and L-tyrosine (L-tyr) respectively.
- The alkaloids are moderately weak bases (pKa value of 6-9) and form water-soluble salts.
- Most Amaryllidaceae alkaloids contain only one nitrogen atom, which is secondary, tertiary or quaternary, and the carbon content varies from 16-20 atoms. The notable exceptions to this are the dinitrogenous alkaloids, (+)-plicamine (**1**)

and (-) secoplicamine (**2**), which were first isolated from *Galanthus plicatus* subsp. *byzantinus* (Ünver et al., 1999), shown in **Figure 2.2.1**.



Plicamine (1)

Secoplicamine (2)

Figure 2.2.1. The structures of the dinitrogenous alkaloids plicamine (**1**) and secoplicamine (**2**)

The study of Amaryllidaceae alkaloids began with the isolation of lycorine from *Narcissus pseudonarcissus* by Gerrad in 1877. More than 200 species and varieties belonging to this plant family have to date been examined for alkaloids. Since the 1950's, substantial progress has been made in examining the South African Amaryllidaceae plants. By 1997, 110 different alkaloids had been isolated from 62 species, with several of these alkaloids having novel structures (Viladomat et al., 1997a). In the past 4 years further alkaloids have been isolated and identified from South African Amaryllidaceae.

2.2.1. Biosynthesis of the Amaryllidaceae alkaloids

The Amaryllidaceae alkaloids may be classified into nine principal skeletally homogenous subgroups, although there are several other alkaloids that are derivatives of the main structures. Representative alkaloids from each of these classes include norbelladine (**3**), lycorine (**4**), homolycorine (**5**), crinine (**6**), haemanthamine (**7**), tazettine (**8**), narciclasine (**9**), montanine (**10**), and galanthamine (**11**). The crinine-haemanthamine and lycorine- homolycorine series are most prominent amongst the southern African plants. When numbering the carbon atoms in the molecules, Ghosal's system (Ghosal et al., 1985) is generally used, since it has the following advantages:

- The aromatic ring A is always numbered in the same way.
- Ring C is always numbered in a clockwise direction, except for the homolycorine and augustamine type alkaloids, which have modified biosynthetic pathways.
- The benzylic position is always 6, with respect to the heteroatom.
- The vicinal position is always 12 with respect to the nitrogen in the pyrrolidine ring that is not involved in the ring fusion.

The biosynthesis of the Amaryllidaceae alkaloids is initiated by the formation of norbelladine (**3**) and its derivatives, which later undergo oxidative phenolic coupling, and subsequent transformation into the final alkaloid structures. Phenylalanine (L-Phe) and tyrosine (L-Tyr) are the precursors of norbelladine. L-Phe is the template for the C_6-C_1 fragment, corresponding to ring A and the benzylic position (C_6). The conversion of L-Phe to the C_6-C_1 unit proceeds through the loss of two carbon atoms from the side chain as well as the introduction of two or more oxygenated substituents into the aromatic ring, which occurs through cinnamic acids. The fragmentation of the cinnamic acid involves oxidation of the β-carbon to ketone or acid level, where the final product is protocatechuic aldehyde or its derivatives.

L-Tyr is the precursor of ring C, the two-carbon side chain (C-11 and C-12) and nitrogen, C_6-C_2-N of norbelladine (**3**). L-Tyr is not degraded further than tyramine before being incorporated into the Amaryllidaceae alkaloids, by the junction of the amine and the aldehyde, resulting in a Schiff's base, which is reduced to form (**3**)

(**Figure 2.2.1.1**). Ring A is then protected by methylation to *O*-methylnorbelladine (**12**) or related compounds, which then undergo intramolecular oxidative coupling of phenols (**Figure 2.2.1.2**).

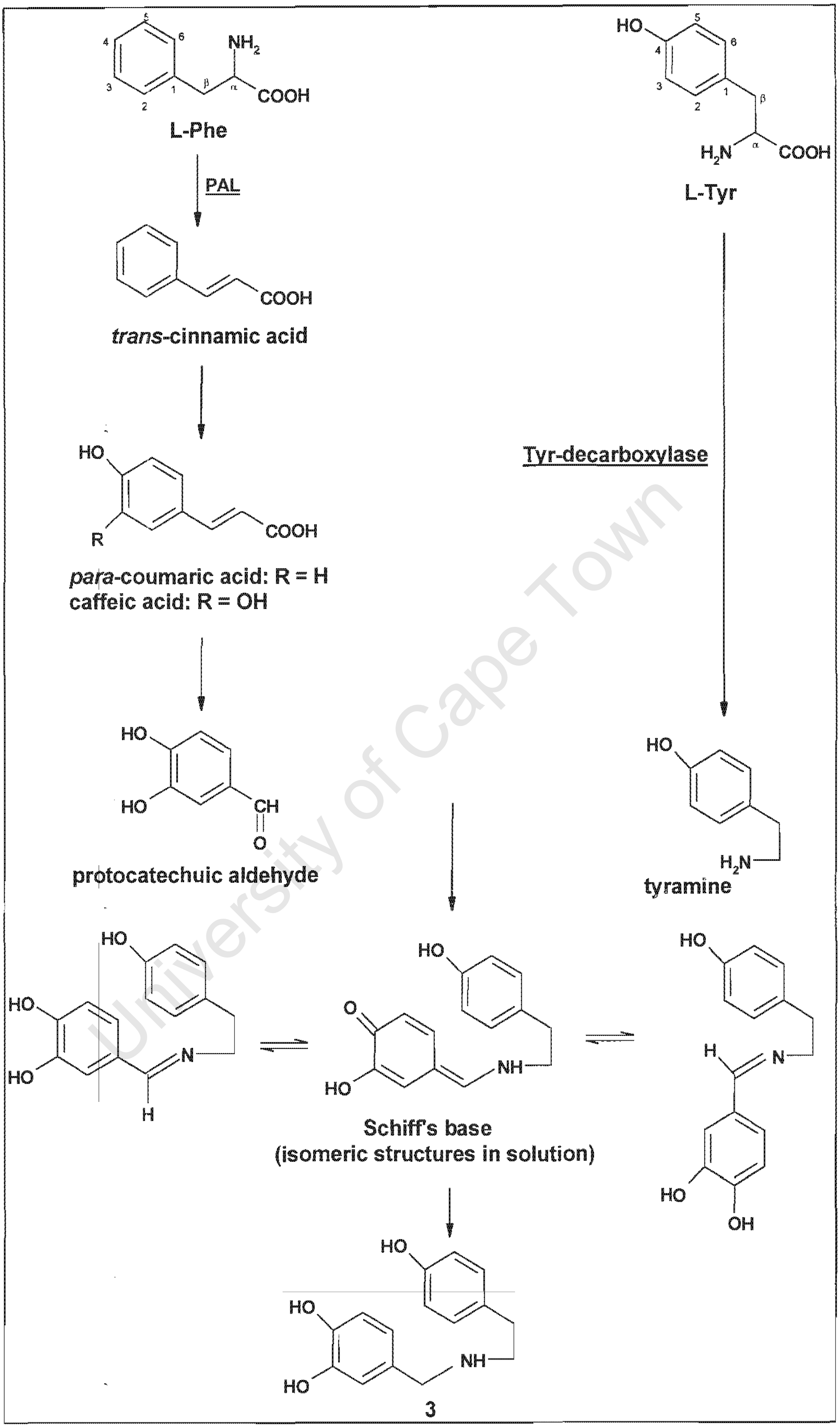


Figure 2.2.1.1. Biosynthetic pathway to norbelladine **(3)**

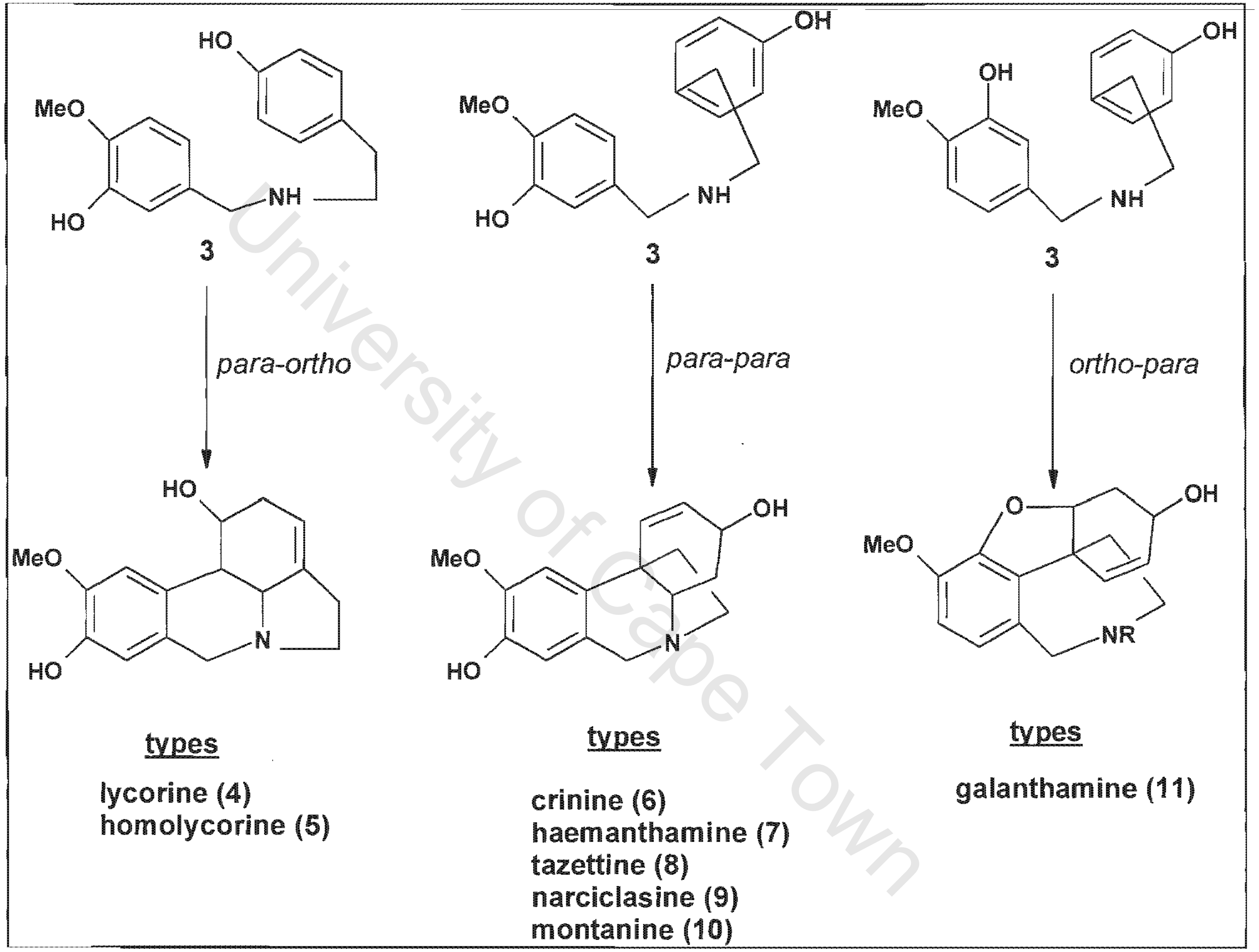


Figure 2.2.1.2. Oxidative phenyl-phenyl coupling in Amaryllidaceae alkaloids

2.2.1.1. *Para-ortho* oxidative phenolic coupling

Lycorine and Homolycorine type

These alkaloids are both derived from *para-ortho* phenolic oxidative coupling of *O*-methylnorbelladine (**12**). The lycorine type is the most abundant and important subgroup of the Amaryllidaceae alkaloids. Lycorine type alkaloids usually have oxygenated substituents at positions 1, 2, 8 and 9, and there is often a methylenedioxy group between positions 8 and 9. There is normally a double bond between C-3 and C-4, but this may occur between C-4 and C-11, as in the alkaloid narcissidine. The fusion between rings B and C is *trans* in all lycorine type alkaloids reported to date, with the exception of kirkine (**13**), where the fusion is *cis*. The homolycorine type alkaloids are directly derived from the lycorine type alkaloids. The pyran ring may be in the lactone, hemiacetal or ether cyclic form. The homolycorine type alkaloids have oxygenated substituents at positions 8 and 9, and at position 2 in a few cases. There is often a double bond between C-3 and C-4, and almost all reported alkaloids of this type have an NMe group. The fusion at rings B and C is normally *cis*, with H-4a and H-10b in a *trans*-diaxial orientation.

The conversion of *O*-methylnorbelladine into lycorine (**4**) proceeds via norpluviine (**14**). Hydroxylation at C-2 proceeds with an inversion of configuration, involving an epoxide. The ring is opened, and allylic rearrangement of the resulting alcohol occurs. Intramolecular oxidation of the methoxyl group at position 9 in norpluviine occurs later in the pathway to form the methylenedioxy group. The biosynthesis of homolycorine proceeds from *O*-methylnorbelladine and norpluviine, as in the case of lycorine. Later a different route is followed whereby benzylic oxidation of norpluvine takes place, followed by ring opening to form an amino aldehyde and then a hemiacetal. Methylation yields lycorenine (**15**), and subsequent oxidation results in homolycorine (**5**).

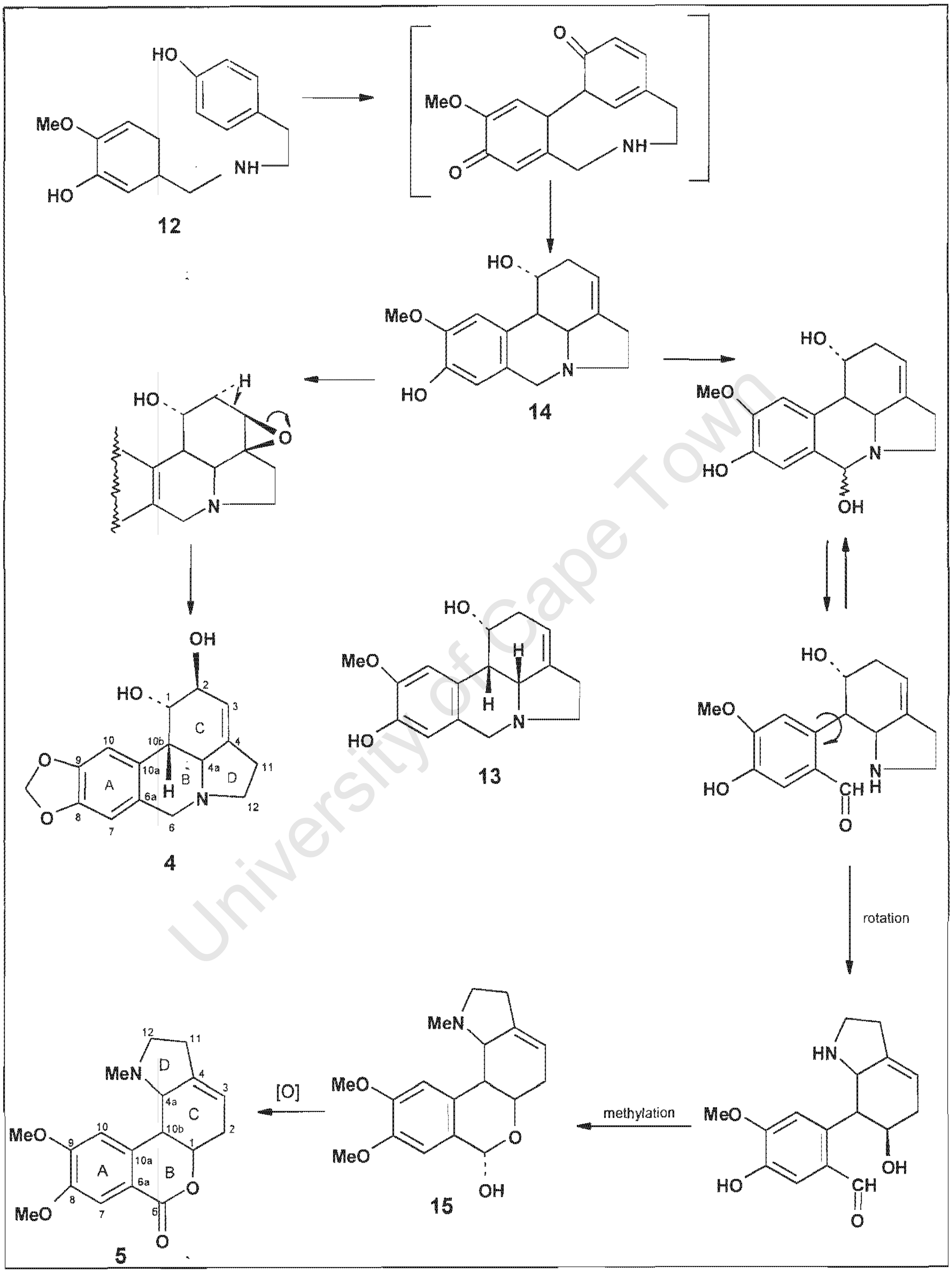


Figure 2.2.1.3. Biosynthesis of lycorine (**4**) and homolycorine (**5**)

2.2.1.2. *Para-para* oxidative phenolic coupling

Crinine and Haemanthamine types

The crinine and haemanthamine alkaloids are both derived from *O*-methylnorbelladine, as a result of *para-para* phenolic oxidative coupling (**Figure 2.2.1.4**). Crinine alkaloids possess an ethano bridge between the nitrogen atom and C-10b, specifically in the β-orientation, that is, above the plane of the skeleton. The fusion of rings B and C is typically *trans*, that is, H-4a is in the opposite orientation with respect to the bridge. There are oxygenated substituents at positions 3, 8 and 9 in some cases and in other cases at 6, 7 and 11. They normally have a double bond between positions 1 and 2, and the presence of a methylenedioxy group between positions 8 and 9 is common.

The haemanthamine type of alkaloid differs from the crinine type in that the ethano-bridge is in an α-orientation, that is, below the plane of the skeleton. They possess a double bond between C-1 and C-2 like the crinine types, and oxygenated substituents at positions 3, 8 and 9, and in other cases at 11. An oxygenated substitution at position 11 is more frequent in this subgroup than in the crinine type. Recently, cases of substitution at position 12 were reported in *Crinum delagoense* (Nair et al., 1998).

Tazettine type

Alkaloids in this subgroup all have a methylenedioxy group as the oxygenated substituent at position 8 and 9. There is typically a double bond between C-1 and C-2, and an oxygenated substituent at C-3. The NMe group is often present, with other substitutions at C-6 and C-11. The biosynthesis of the tazettine type alkaloids is thought to occur through the haemanthamine series. Haemanthamine (**7**) is irreversibly converted to haemanthidine (**16**) or epihaemanthamine (**17**) and later to tazettine (**8**). Transformation proceeds through the aldehyde form of the epimeric mixture, and after rotation and methylation, pretazettine (**18**) is formed (**Figure 2.2.1.4**).

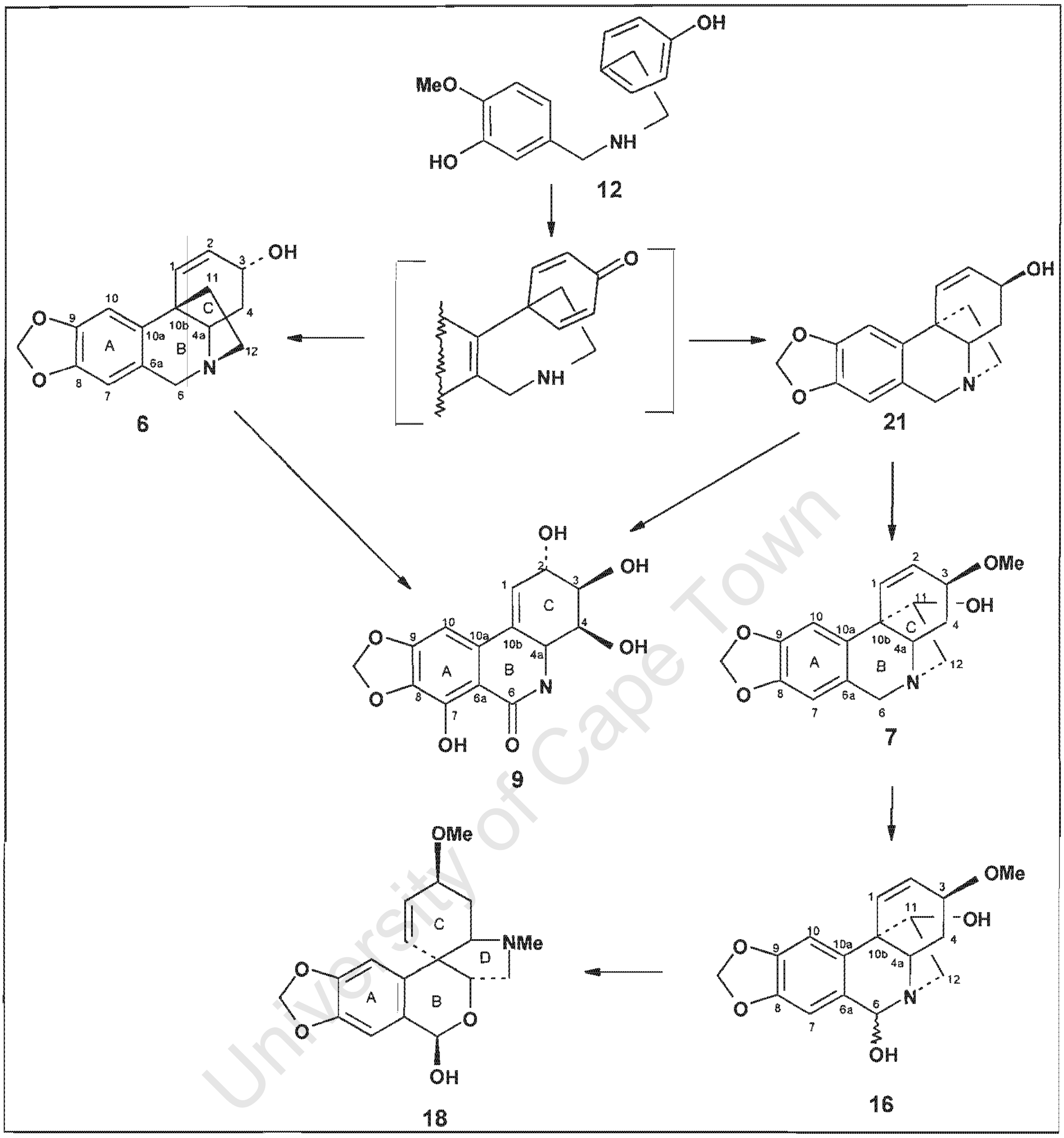


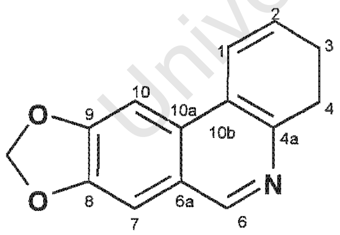
Figure 2.2.1.4. Biosynthesis of alkaloids by *para-para* oxidative phenolic coupling

Narciclasine type

This is a subgroup with very few reported alkaloids. All the alkaloids of this type have a methylenedioxy group between positions 8 and 9, with other substitutions at C-2, 3, 4 and 6. There is complete aromatization in trisphaeridine (**19**). The biosynthesis of the narciclasine type of alkaloids proceeds from the biosynthetic pathway of the crinine and haemanthamine type of alkaloids. *O*-Methylnorbelladine is incorporated into narciclasine (**9**) through *para-para* oxidative phenolic coupling, with retention of the methoxyl group of *O*-methylnorbelladine. The ethano-bridge is lost from the oxocrinine skeleton, passing through an intermediate with a *pseudoaxial* hydroxyl group at C-3.

Montanine type

Alkaloids of this type are quite rare and only a few have been reported. They have a methylenedioxy group between positions 8 and 9, and have a double bond between C-11a and C-1. The 5,11-methano bridge is in the α-orientation, and there are typically oxygenated substituents at positions 2 and 3, as in the case of montanine (**10**). The biosynthesis of these alkaloids is believed to proceed through 11-hydroxyvittatine (**20**), which is produced from vittatine (**21**), an intermediate in the biosynthesis of haemanthamine.



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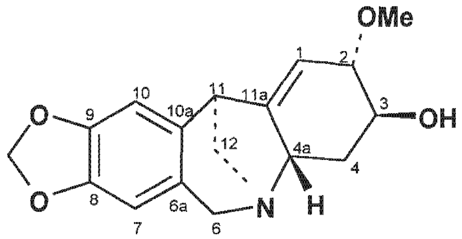

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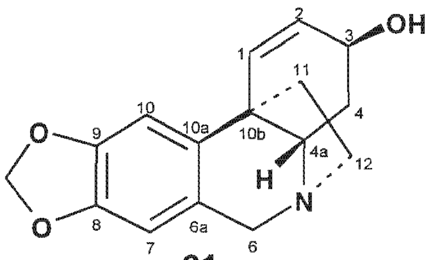

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Figure 2.2.1.5. The structures of trisphaeridine (**19**), montanine (**10**) and vittatine (**21**)

2.2.1.3. *Ortho-para* oxidative phenolic coupling

Galanthamine type

These alkaloids have the basic skeleton of dibenzofuran, and possess a seven-membered ring involving the nitrogen atom, with oxygenated substituents at position 3 and 9. The nitrogen atom is usually methylated, and there is frequently a double bond between C-4 and C-4a. Recent studies have shown that the biosynthesis of galanthamine (**11**) involves the phenol oxidative coupling of *O*-methylnorbelladine to a postulated dienone which undergoes spontaneous closure of the ether bridge to yield *N*-demethylnarwedine (**22**) giving norgalanthamine (**23**) after stereoselective reduction. In the final step of biosynthesis, norgalanthamine is *N*-methylated to galanthamine (Eichhorn et al., 1995), as shown in **Figure 2.2.1.6**.

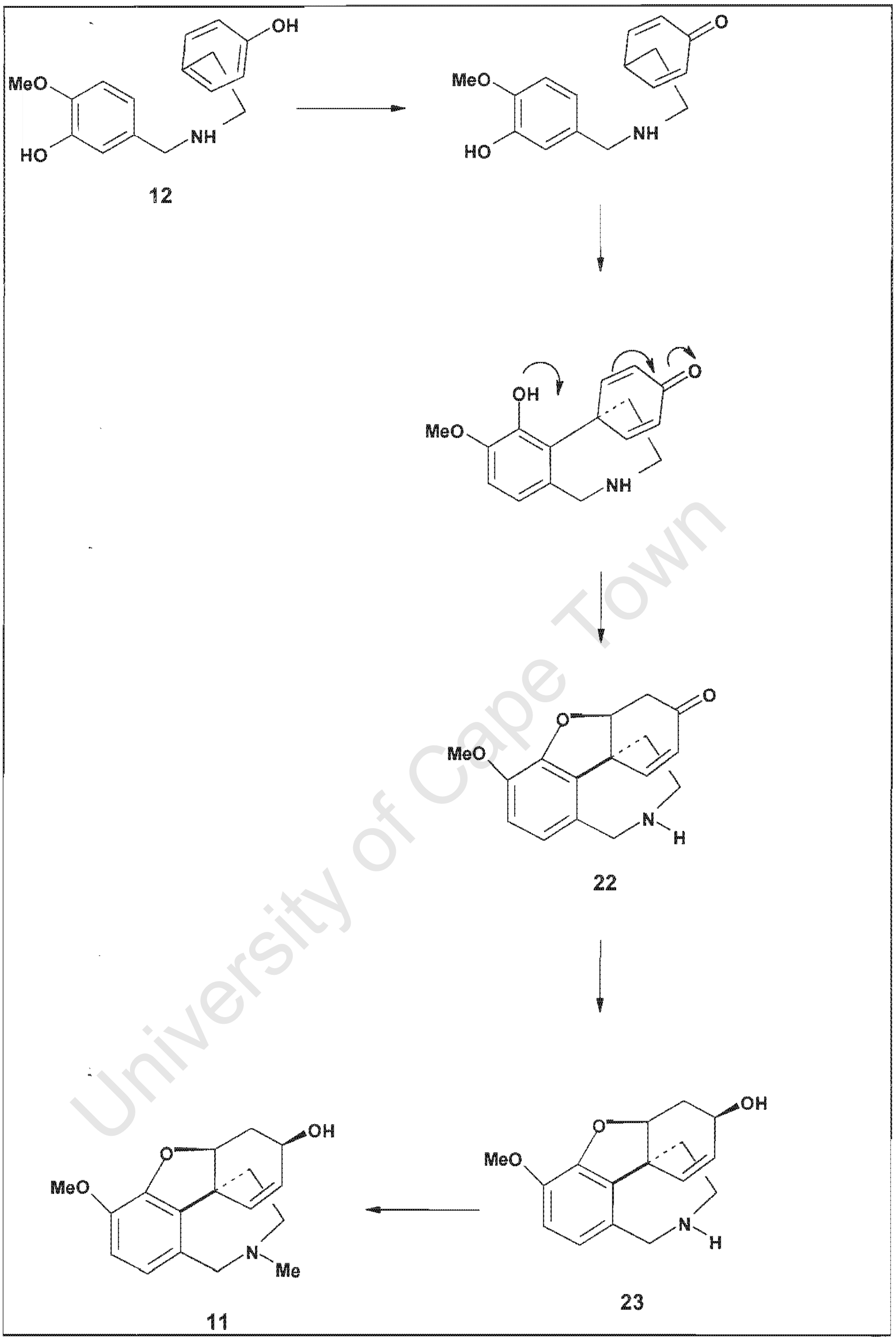
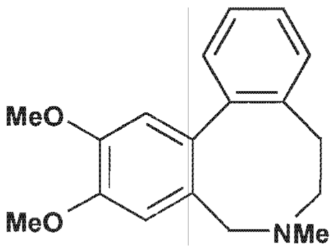


Figure 2.2.1.6. Proposed biosynthesis of galanthamine (**11**) by Eichhorn and Zenk, 1998.

Miscellaneous Amaryllidaceae alkaloids

There are other Amaryllidaceae alkaloids that cannot be classified in any of the above-mentioned subgroups. Some examples include buflavine (**24**) and 8-O-demethylbuflavine (**25**), which both possess an unusual eight membered B ring, and cherylline (**26**), which contains three rings like the narciclasine type, but without fusion between rings B and C.



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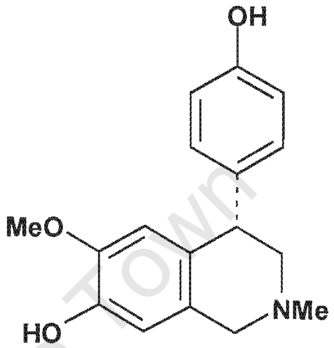

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Figure 2.2.1.7. The structures of buflavine (**24**) and cherylline (**26**)

2.3. Spectroscopy of Amaryllidaceae alkaloids

The three most important spectroscopic methods for determining the structures of Amaryllidaceae alkaloids are Proton Nuclear Magnetic Resonance (^{1}H NMR), Carbon^{13} Nuclear Magnetic Resonance (^{13}C NMR) and mass spectrometry (MS). A known alkaloid can usually be identified from the above data, either by direct comparison with authentic alkaloids isolated from other Amaryllidaceae, or else by careful comparison with literature data. Other spectroscopic data, including UV and IR, and physical properties, such as the melting point, optical rotation and CD curves have to be measured in order to identify the alkaloid structure completely.

2.3.1. Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) Spectroscopy is by far the most powerful spectroscopic technique for obtaining detailed structural information about organic compounds in solution. The number of each type of carbon and hydrogen atom in a molecule, as well as the immediate environment of each of these types of carbons and hydrogens can be determined. The advantage of NMR spectroscopy is that the sample is not destroyed, and can be completely recovered after the experiment. The first commercial NMR spectrometer was made in 1953. Today, 200 - 500 MHz instruments are now routinely used, with 600 and 800 MHz instruments also available. With the modern high-resolution instruments and techniques, sample amounts of less than 1 mg can produce well-defined spectra within reasonable time-spans.

The most widely utilised nuclei in 1-D NMR spectroscopy in organic chemistry are ^{1}H and ^{13}C. ^{1}H NMR spectroscopy gives important information about the different types of Amaryllidaceae alkaloids. Hawksworth *et al.* and Haugwitz *et al.* made several early contributions on homolycorine and crinane-haemanthamine type alkaloids in the 1960s. In the last ten years, the development of new 2-D NMR techniques, such as ^{1}H-^{1}H Homonuclear Shift Correlated Spectroscopy (COSY), Nuclear Overhauser Enhancement and Exchange Spectroscopy (NOESY), Rotating Frame Nuclear Overhauser Effect Spectroscopy (ROESY), Heteronuclear Multiple

Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) have facilitated the structural assignments and the settling of their stereochemistry.

^{1}H-^{1}H Homonuclear Shift Correlated Spectroscopy (COSY)

This is the most commonly used 2-D experiment, and yields two 1-D ^{1}H spectra which can be plotted at right angles to each other to give a COSY contour plot. The diagonal of this plot represents the ^{1}H NMR spectrum. Cross-peaks are observed for scalar coupled protons with significant coupling constants. It is therefore possible to trace a spin system and obtain sub-structures. ^{1}H-^{1}H COSY not only provides information for unambiguous ^{1}H NMR assignments, but also helps in ^{13}C NMR assignments via ^{1}H-^{13}C COSY spectrum. The shortcoming of COSY is that cross-peaks cannot be seen if the coupling constants between scalar coupled protons are too small (usually less than 1Hz). It is sometimes possible to observe long-range coupling (i.e. W coupling), meta-coupling (in an aromatic ring), and allylic coupling.

Nuclear Overhauser Enhancement and Exchange Spectroscopy (NOESY)

The NOESY experiment is a useful assignment aid that detects NOE's between nearby protons (3Å or closer) in a molecule, thus facilitating recognition of through space relationships. This is useful in confirming the stereochemistry in a complex molecule. In a 2D NOESY spectrum, cross-peaks are observed between proton pairs that are close in space. Deceptive results can be obtained from a NOESY spectrum if the choice of parameters is inappropriate.

Rotating Frame Nuclear Overhauser Effect Spectroscopy (ROESY)

The Rotating Frame NOESY (ROESY) experiment provides some advantages for small and medium-sized, as well as large molecules. Whereas the NOE decreases to zero and becomes negative as the mean correlation time for molecular rotation increases (large molecules move more slowly), in the rotating frame the maximum NOE remains positive and even increases from 50% to 67.5%. In addition to greater signal enhancement, the ROESY experiment also decreases spin diffusion, which is an advantage for larger molecules.

COSY, NOESY and ROESY are all homonuclear correlation techniques as they only show the relationship between protons in a molecule. There are heteronuclear correlation spectroscopic techniques available, which are discussed briefly as follows:

Heteronuclear Correlation Spectroscopy (HETCOR)

The ^{13}C - ^{1}H correlated spectroscopy provides connectivities between ^{13}C and ^{1}H signals, therefore allowing the number of hydrogen atoms attached to each individual ^{13}C atom to be deduced. In a HETCOR spectrum, cross-peaks are observed between the frequencies of the carbons and their directly attached protons, thus allowing information from one spectrum to help in the assignment of the other spectrum. In the axis where the ^{13}C spectrum is displayed, only the protonated carbon signals are detectable. The disadvantage of HETCOR is that the low natural abundance of ^{13}C diminishes sensitivity, and this technique has now become redundant. The Long-Range HETCOR experiment detects connectivities mediated by two- or three-bond couplings, therefore providing information about the connectivity patterns of protonated carbons and their neighbours.

Distortionless Enhancement by Polarization Transfer (DEPT)

This method is designed to produce decoupled carbon-13 NMR spectra with C-H multiplicity discrimination, providing the best way for distinguishing CH_3, CH_2 and CH groups in ^{13}C NMR spectra. The method relies on scalar coupling between the sensitive nucleus (^{1}H) and the less sensitive nucleus (^{13}C), and benefits from the transfer of a high degree of polarization from the sensitive nucleus to the insensitive nucleus, resulting in a significant increase in ^{13}C signal intensity.

Heteronuclear Multiple Quantum Coherence (HSQC)

HSQC is equivalent to HETCOR, and is an inverse-detection 2D NMR technique where heteronuclear chemical shift correlation is achieved via proton detection, therefore requiring a much smaller sample size to produce a useful spectrum within a shorter time. Both HETCOR and HSQC experiments result in a 2D data matrix, where the cross peaks show correlations between linked carbons and protons.

Heteronuclear Multiple Bond Correlations (HMBC)

This is the ^{1}H-detected alternative of Long-Range HETCOR. The contour plot of the 2D matrix achieved shows two-, three- or four-bond correlations between protons and carbons, which allows for determination of the linkage positions in a molecule.

2.3.2. Methods in NMR Spectroscopic Analysis

When elucidating structures, the following stages are generally involved:

1. The chemical shift data from the ^{1}H and ^{13}C NMR spectra enables the identification of certain functional groups present. In addition, the number of signals in the ^{13}C spectrum gives the total number of carbons present in the molecule.
2. The DEPT spectrum gives proton-carbon coupling patterns and therefore confirms the pattern of proton substitution.
3. The COSY spectrum aids ^{1}H NMR spectrum analysis, from which coupling information reveals through-bond connectivities between protons. Two- and three-bond couplings are mainly observed, with occasional four- or five-bond couplings. The magnitude of these couplings gives information on the relative spatial orientation of protons. Thus, the relationship between protonated carbons can be established.
4. The HSQC spectrum reveals one-bond proton-carbon connectivities which facilitates assignment of all the protonated carbons.
5. The HMBC spectrum gives information on two-, three- or four-bond couplings, and therefore confirms the relationships established by the ^{1}H and COSY spectra. This spectrum is particularly useful in establishing connectivities of protonated carbon atoms to quaternary centres, and the relationship between isolated ^{1}H-^{1}H spin systems in molecules.
6. The NOESY and ROESY spectra reveal through-space interactions present in a molecule, enabling the assignment of relative stereochemistry.

2.3.3. Proton Nuclear Magnetic Resonance of Amaryllidaceae alkaloids

Crinine - Haemanthamine type

The absolute configuration of these alkaloids is determined through the circular dichroism curves. Haemanthamine type alkaloids have the following characteristic features in their NMR spectra:

1. An AB system of 2 doublets that correspond to the benzylic protons of position 6 is observed.
2. The *para*-oriented aromatic protons produce two singlets in the range δ 6.4-7.0 ppm.
3. The pairs of alkaloids with a hydroxyl group at position 6, for example haemanthidine, appear as mixtures of epimers, which cannot be separated, even by HPLC.
4. There is a large coupling between proton H-4α and H-4a ($J_{4\alpha,\,4a}$ ~13 Hz) due to their *trans*-diaxial position.
5. When $CDCl_3$ is used as the solvent, the magnitude of the coupling constants between each olefinic proton (H-1 and H-2) and H-3 provides useful information about the configuration of the C-3 substituent. In those alkaloids in which the two-carbon bridge (C-11 and C-12) is *cis* to the substituent at C-3, an allylic coupling is seen between H-1 and H-3 ($J_{1,3}$ ~1-2 Hz), whilst H-2 shows a smaller coupling with H-3 ($J_{2,3}$ ~0-1.5 Hz). In the corresponding C-3 epimeric series, the opposite effect is observed, with a larger coupling between H-2 and H-3 ($J_{2,3}$ ~5 Hz), whilst the coupling between H-1 and H-3 is undetectable.
6. An additional coupling of H-2 with the equatorial H-4β, in a W-mechanism is frequently observed.

Tazettine type

In tazettine type alkaloids, the presence of an N-methyl group (δ 2.4-2.5 ppm) distinguishes them from the haemanthamine type, from which they proceed biosynthetically. The ^{1}H NMR spectrum always shows the signal corresponding to the methylenedioxy group.

Galanthamine type

The galanthamine types are the only Amaryllidaceae alkaloids that show *ortho*-coupling between both aromatic protons of ring A. The general traits of the ^{1}H NMR spectra of this type are:

1. An AB system of two doublets corresponding to the benzylic protons of position 6.
2. The two *ortho*-oriented aromatic protons produce two doublets with a coupling constant of $J_{7,8}$ ~8Hz.
3. The furan ring has a deshielding effect on H-1.
4. The alkaloids will typically have an N-methyl group but N-formyl and N-acetyl derivatives have been isolated.
5. The coupling constants of the olefinic protons H-4 and H-4a facilitate assignment of the substituent stereochemistry at C-3. When the coupling constant $J_{3,4}$ is ~5 Hz, the substituent is *pseudoaxial*, whilst if $J_{3,4}$ is ~0 Hz, this indicates that the substituent is *pseudoequatorial*.

Lycorine type

There have been extensive ^{1}H NMR studies on this group, and lycorine, as well as its main derivatives have been completely assigned. In the plant, lycorine is particularly vulnerable to the oxidation processes, giving several ring-C aromatized products. The main characteristics of the ^{1}H NMR spectra of the lycorine type are:

1. An AB system comprising two doublets that correspond to the benzylic protons of position 6.
2. Two singlets in the range δ 6.5-7.2 ppm corresponding to the *para*-oriented aromatic protons.
3. A coupling constant for $J_{4a,10b}$ of ~11Hz indicating a *trans* B/C ring junction, is typical of almost all lycorine-type alkaloids.
4. An olefinic proton around δ 5.5 ppm that is unique to the lycorine types.
5. The *cis*-lone pair of the nitrogen atom exerts a deshielding effect on the β-protons of positions 6 and 12 in relation to their α-homologues.

Homolycorine type

Lactone, hemiacetal or cyclic ether (unusual) alkaloids are included in this group. The general traits of the spectra for this type of alkaloid are as follows:

1. The majority of compounds contain a *cis* B/C ring junction, indicated by the small size of the coupling constant ($J_{1,10b}$).
2. The *para*-oriented aromatic protons result in two singlets. The deshielding effect of the *peri*-carbonyl group at C-6 on H-7 allows for differentiation between the H-7 and H-10 signals in lactone alkaloids.
3. The N-methyl group corresponds to a singlet in the range δ 2.0-2.2 ppm.
4. A vinylic proton around δ 5.5 ppm represents the C ring.
5. The *cis*-lone pair of the nitrogen atom causes greater deshielding of the H-12α than H-12β.
6. The substituent at position 6 is in the α-disposition, whilst the benzylic proton H-6β appears as a singlet between δ 5.0-6.0 ppm in the hemiacetal alkaloids.

2.3.4. ^{13}Carbon Nuclear Magnetic Resonance of Amaryllidaceae alkaloids

The carbon framework of the Amaryllidaceae alkaloids has been determined through extensive use of ^{13}C NMR spectroscopy. Assignments are made on the basis of chemical shifts and multiplicities of the signals (by the Distortionless Enhancement by Polarization Transfer (DEPT) experiment). The assignments can be corroborated by the use of 2D NMR techniques, such as Heteronuclear Multiple Quantum Correlation (HSQC) and Heteronuculear Multiple Bond Correlation (HMBC).

The ^{13}C NMR spectra of Amaryllidaceae alkaloids consist of two regions. The low-field (>90ppm) signals correspond to the carbonyl group, the olefinic and aromatic carbons, and those of the methylenedioxy group. The high-field region includes signals corresponding to the saturated carbon resonances. The N-methyl group is easily assigned by a quartet signal between 40-46ppm. The effect of substituents (OH, OMe, OAc) on the carbon resonances is important when determining the position of the functional groups.

Analysis of the spectra provides information on the following aspects:

1. The number of methine olefinic carbons.
2. Confirmation of the presence of a lactonic carbonyl group.
3. Confirmation of the presence of the nitrogen substituent and its nature.
4. Assignment of C-10b is made from a quaternary carbon signal in the chemical shift range of δ 42-50ppm.

2.3.5. Mass spectrometry of Amaryllidaceae alkaloids

There were extensive studies on the mass spectrometry of Amaryllidaceae alkaloids by electron impact in the 1960s and 1970s (Duffield et al., 1965). The fragmentation patterns in the Electronic Impact Mass Spectrometry (EIMS) of various skeletal types are fairly well documented and have considerable diagnostic value.

Crinine - Haemanthamine type

There are several factors that need to be considered for these types of alkaloids:

1. The mass spectra provide useful information about the relative configurations of the 5,10b-ethano bridge and the OMe at C-3. If both are on the same side (i.e. both α or β), there is a facile loss of ~~CH_3OH resulting in~~ a ~~very weak~~ molecular ion and a base peak at M^+ -32. If, however, they are on opposites sides, the molecular ion is very stable and is the base peak.
2. The aromatic ring plays an important role in the stabilization of the ions, and is retained in all high-mass fragments, whilst the nitrogen atom is usually lost.
3. The rupture of a bond β to the nitrogen atom initiates the fragmentation mechanism, which implies the opening of the C-11/C-12 bridge.
4. There are a relatively large number of nitrogen-free ions.

Galanthamine type

This type of alkaloid shows a characteristic intense molecular ion, as well as the [M-1] peak. Typically, there is elimination of the elements of ring B (including the nitrogen atom), and the breaking of ring C (with loss of a C_4H_6O) fragment. Similar behaviour is seen with the dihydro derivatives.

Lycorine type

In general, the molecular ion appears as quite an intense peak, and looses water, as well as C-1 and C-2 and their substituents. The ease at which this water is lost from the molecular ion depends greatly on the stereochemistry of the C-2 hydroxyl group. The loss of water does not occur in the acetyl derivatives.

Homolycorine type

In this alkaloid type, the cleavage of the labile bonds in ring C predominates, generating two fragments. The first fragment represents the pyrrolidine ring (including substituents in position 2) and the second fragment, which is less abundant, encompasses the aromatic lactone or hemilactone moeity. There is a low abundance of the molecular ion in all compounds with a double bond $\Delta^{3,4}$.

Tazettine type

In these alkaloids, minor variations in the stereochemistry can cause significant differences in the stereoisomers. In the mass spectrum of tazettine, which has a β configuration of the methoxy group at C-3, the dominant ion at *m/z* [M-84] results from C-ring fragmentation. Conversely, criwelline, which differs only in the configuration of the methoxyl group, generates a peak of low abundance at *m/z* [M-84]. Ions occur in both stereoisomers due to the loss of a methyl radical and then water from the molecular ion (Duffield et al., 1965).

Montanine type

The mass spectral fragments generated in these alkaloids are strongly influenced by the nature of the substituents at C-2 and C-3, as well as their configuration. All the alkaloids with a methoxy group give rise to an M-31 ion.

Chapter 3

Investigation of the Alkaloids from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*

3.1. The Genus *Cyrtanthus*

Cyrtanthus (tribe Haemantheae, sub tribe Cyrtanthinae) is a genus highly valued by horticulturalists for its beauty and variety of flower colour and form, and is southern Africa's most speciose genus of Amaryllidaceae (Snijman et al., 1995). Reid and Dyer list over 50 species in their 1984 review of Southern African *Cyrtanthus*. The centre of distribution is the southeastern Cape, which receives rainfall throughout the year. Other species occur in the winter rainfall area of the southern Cape, whilst a few species are found in the northern regions of South Africa, which receive summer rainfall (Reid and Dyer, 1984). A number of species occur in grassland, which is subject to annual spring burning. Other species are found in forested areas and several species occur in sandy coastal soils. Many *Cyrtanthus* species are very rare or have a limited distribution range. Through habitat destruction or vandalism, these species are becoming increasingly threatened. A new species, *Cyrtanthus wellandii*, was recently discovered in the Eastern Cape (Cowley, 2000). The species is rare, and has been assessed as belonging to the IUCN Red Data List category "Vulnerable D2".

The two South African species, *Cyrtanthus sanguineus* (Lindl.) Walp. and *Cyrtanthus obliquus* (L. f.) Ait were selected for this study from a literature search on Amaryllidaceae species used in traditional medicine in South Africa. Both plants were considered to be potentially toxic due to their alkaloid content, and since neither of the two species had been previously examined for alkaloids, they were a potential source of pharmacological interesting compounds. As detailed in Chapter 2, **Table 2.1.1(b)**, pregnant Xhosa and Zulu women take bulb infusions of *Cyrtanthus sanguineus*, from the fourth month of their pregnancy, to facilitate an easy labour, and ensure healthy foetal growth. (Veale et al., 1992; Hutchings et al., 1996). *Cyrtanthus obliquus* bulbs are one of the ingredients used in traditional medicine to treat chronic coughs and scrofula (Watt and Breyer-Brandwijk, 1962). A decoction of the bulb alone is taken for scrofulous coughs, and the dried bulb scales are taken as a snuff for the relief of headaches (Watt and Breyer-Brandwijk, 1962).

3.1.1. *Cyrtanthus sanguineus* (Lindl.) Walp.

Common names: Large Red Cyrtanthus, Inanda Lily, Kei Lily; Keilelie (Afrikaans); isilawu esimhlophe (Xhosa) (sanguineus-blood red) (Pooley, 1998). *C. sanguineus* reaches a height of about 300 mm and grows in clumps, on rock faces, near water and at the coast to an altitude of 600 m. The bulb is oval, ± 100 x 80 mm, with a long neck, which is mostly above ground. The leaves occur with the flowers and are ± 400 x 20 mm in size; green with a waxy bloom and reddish at the base. *C. sanguineus* is in flower from January to April, and the flowers are red, with lobes ± 50 x 17 mm, that are faintly scented and curl back, and generally occur in pairs. The *C. sanguineus* is a popular, attractive garden plant, which has been in cultivation since 1846, when it was imported into England from the Cape, and presented to the London Horticultural Society (Pooley, 1998, Reid and Dyer, 1984).

Plate 3.1.1. *Cyrtanthus sanguineus* by Geoff Nichols

3.1.2. *Cyrtanthus obliquus* (L. f.) Ait.

Common names: Giant Cyrtanthus, Knysnalelie (Afrikaans), umathunga (Zulu) (obliquus-slanting) (Pooley, 1998). A robust evergreen, that reaches a height of up to 600 mm, and grows in dry rocky grassland or thicket. The bulb is subglobos and can reach 100 mm in diameter, with the neck of the bulb above ground. The leaves grow in a fan, 200-600 x 30-60 mm, are blunt tipped and twisted, with the margins edged yellow-red. The plant flowers from August to February; the flowers occur in inflorescence, 6-12 at a time, and are hanging, with a hollow stem. The flowers are ± 70 x 25 mm, yellowish green and red in colour, with waxy bloom and lobes ± 25 x 15 mm, tipped green. *C. obliquus* is a hardy garden plant that has been cultivated in Europe for over 150 years (Pooley, 1998, Reid and Dyer, 1984).

Plate 3.1.2. *Cyrtanthus obliquus* © International Bulb Society, reproduced with kind permission from Kelly Irvin

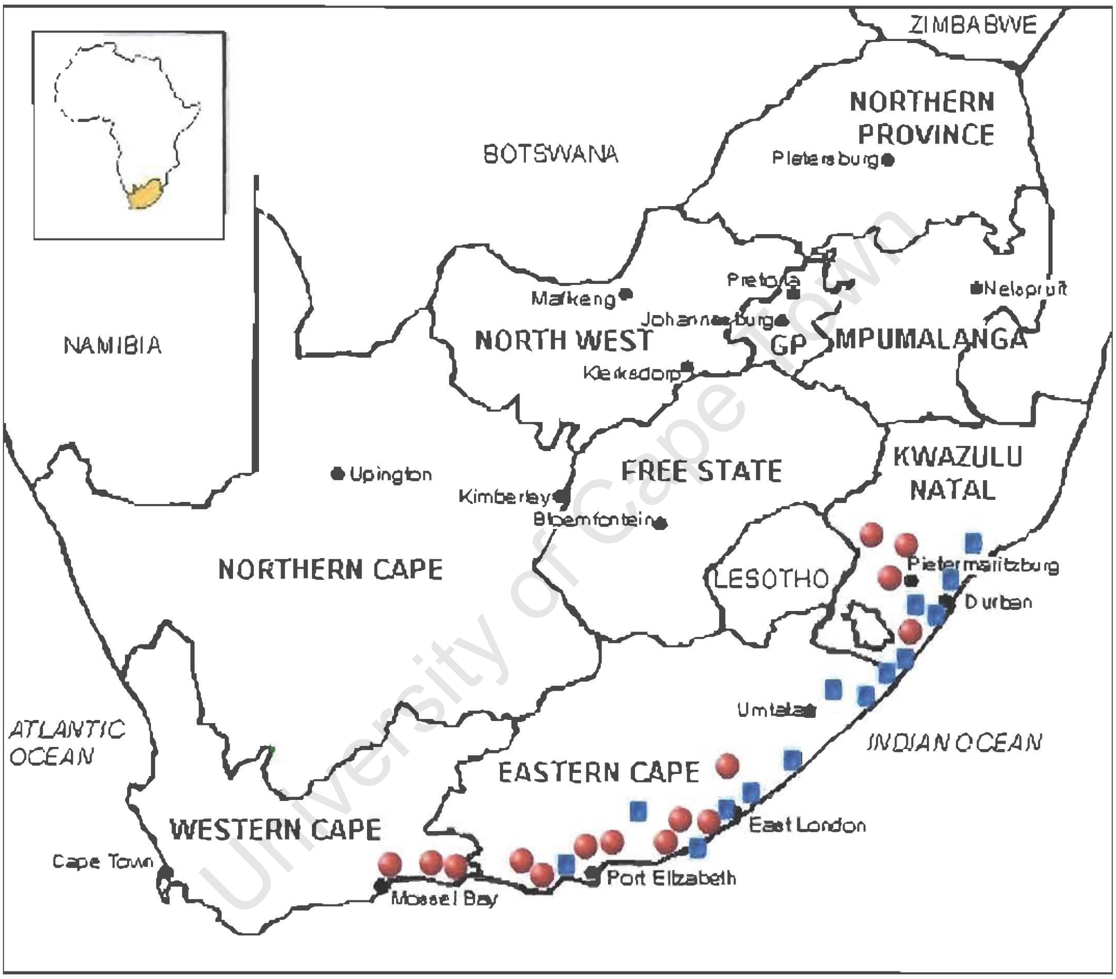
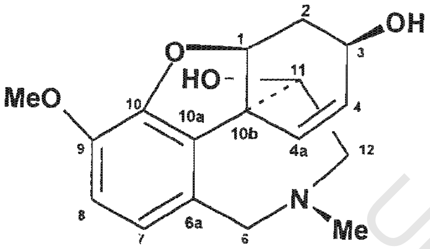
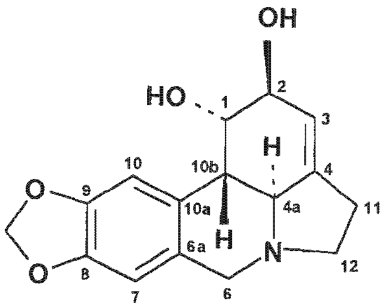


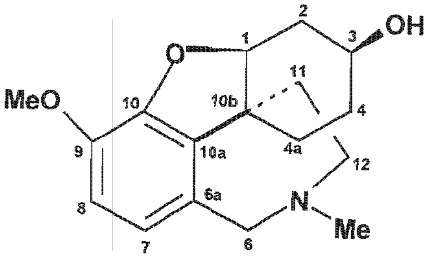
Figure 3.1.1. Distribution of *Cyrtanthus obliquus* (L.f.) Ait (red circles) and *Cyrtanthus sanguineus* (Lindl.) Walp. (blue squares) in South Africa.



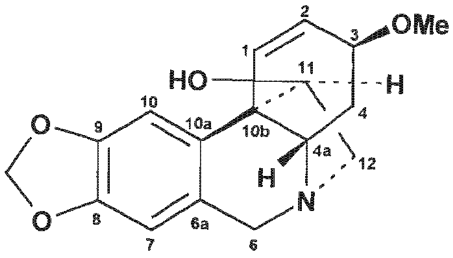
11α-Hydroxygalanthamine (27)



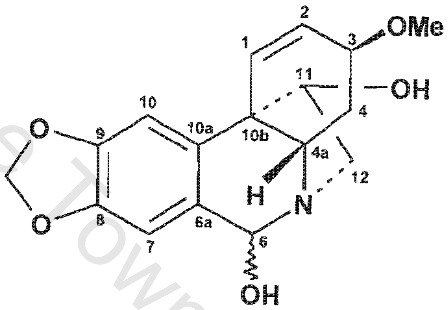
Lycorine (4)



Lycoramine (28)

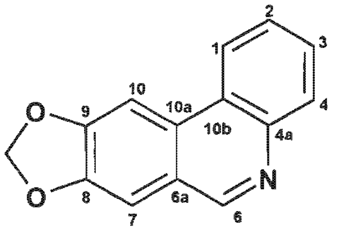


11-Epihaemanthamine (29)

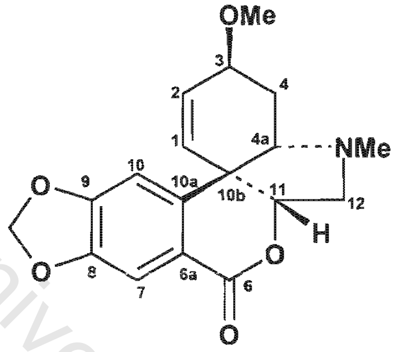


Haemanthidine (13)

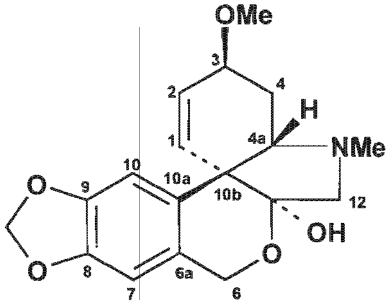
Figure 3.2.1. Alkaloids from *Cyrtanthus sanguineus*



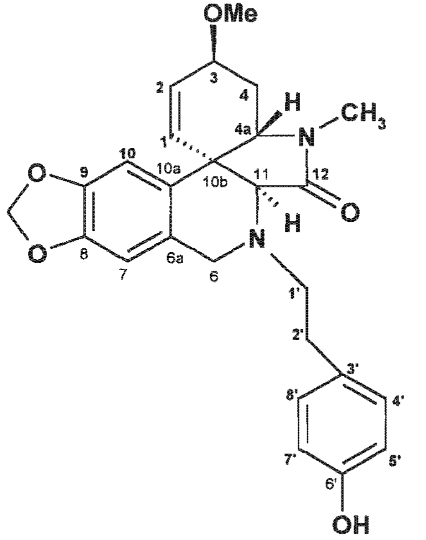
Trisphaeridine (19)



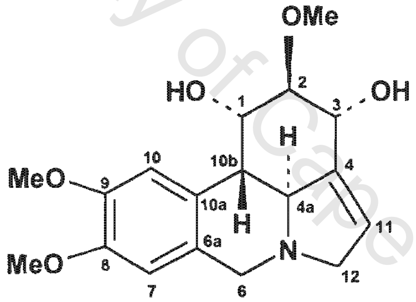
3-Epimacronine (31)



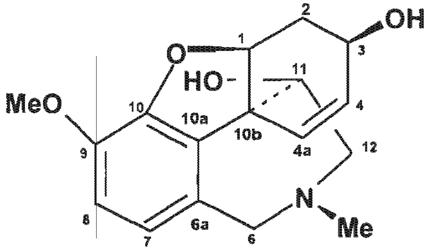
Tazettine (8)



Obliquine (30)



Narcissidine (32)



11α-Hydroxygalanthamine (27)

Figure 3.2.2. Alkaloids from *Cyrtanthus obliquus*

3.2.1. 11α-Hydroxygalanthamine (27)

To the best of my knowledge, the isolation of 11-hydroxygalanthamine has been reported only twice previously (Wildman and Brown, 1968; Vazquez Tato et al., 1988), and there has been no investigation of the biological activities of this alkaloid. Wildman and Brown (1968) reported the isolation of 11-hydroxygalanthamine from *Habranthus brachyandrus*, which they named habranthine, but they did not resolve the position of the hydroxyl group on C-11. Later, Vazquez Tato et al. (1988) isolated an alkaloid from *Pancratium maritimum* that they stated to be habranthine. These authors listed detailed ^{1}H NMR and incomplete ^{13}C NMR data for this alkaloid, but did not provide an explanation of how the assignments were made. H-12 (equivalent to H-11 in the revised numbering system) was shown to have the beta orientation, which would then place the hydroxyl group in the alpha orientation. However, their structural diagram of habranthine shows this hydroxyl group in the beta position. Hence the correct stereochemistry at C-11 still remained unclear.

In this investigation, complete assignment of ^{1}H and ^{13}C NMR data of 11α-hydroxygalanthamine was made possible by 2-D NMR experiments, which also provided evidence that the hydroxyl group is definitely in the *pseudoaxial* position on C-11 of the molecule. The ^{1}H-NMR spectrum of **27** was initially run in $CDCl_3$, which did not give clear resolution of the protons on C-11 and C-12, since these protons produced broad singlets. The compound was then re-run in CD_3OD, which gave a spectrum with H-11β, and H-12α and H-12β more clearly resolved. The protons of **27** were thus assigned on the basis of ^{1}H-NMR spectrum run in CD_3OD, with the exception of H-4 and H-4a, which were resolved from the ^{1}H-NMR spectrum in $CDCl_3$, as they coalesced in CD_3OD. The ^{1}H-NMR and ^{13}C-NMR data for 11α-hydroxygalanthamine isolated from *Cyrtanthus sanguineus* in $CDCl_3$ and CD_3OD is given in **Table 3.2.1.1** and the scalar and spatial correlations of the protons of **27** are given in **Table 3.2.1.2** respectively.

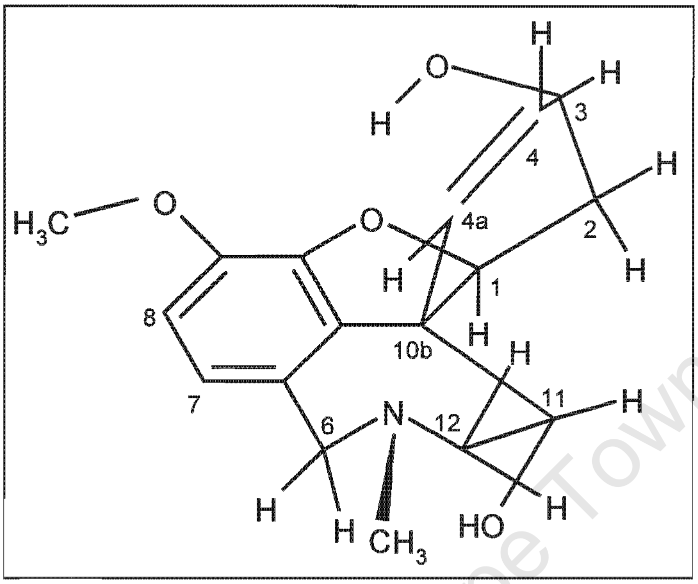


Figure 3.2.1.1. Structure of 11α-hydroxygalanthamine (**27**)

Table 3.2.1.1. ^{1}H NMR and ^{13}C NMR for 11α-hydroxygalanthamine (**27**). Assignments based on HSQC, DEPT and HMBC data.

Position	δH (JHz) in $CDCl_3$			δH (JHz) in CD_3OD			HSQC in CDCl3		HMBC
1		5.25	*brs*		5.20	*t* (3.8)	83.0	*d*	C-4a
2	α	2.00	*ddd* (13.8, 5.5, 2.6)	α	2.17	*ddd* (15.6, 5.3, 3.7)	29.6	*t*	
	β	2.57	*ddd* (13.8, 3.3, 1.5)	β	2.41	*m*			C-1, C-3, C-4, C-10b
3	α	4.04	*t* (4.5)		4.12	*dt* (2.8, 2.8)	61.4	*d*	C-1, C-2, C-4, C-4a
4		5.99	*ddd* (10.3, 5.0, 1.5)		5.96	*m*	129.6	*d*	C-2, C-3, C-10b
4a		5.72	*d* (10.3)		5.96	*m*	125.3	*d*	C-1, C-3, C-10a, C-10b
6	α	3.65	*d* (15.0)	α	3.59	*dd* (15.0, 1.9)	62.3	*t*	C-7, C-10a, C-12, NMe
	β	3.87	*d* (15.0)	β	3.87	*d* (15.0)			C-7, C-10a, C-12
6a							126.8	*s*	
7		6.56	*d* (8.0)		6.59	*d* (8.0)	121.3	*d*	C-6, C-8, C-9, C-10a
8		6.62	*d* (8.0)		6.71	*d* (8.0)	111.4	*d*	C-6a, C-7, C-9, C-10
9							144.0	*s*	
10							146.8	*s*	
10a							129.2	*s*	
10b							53.6	*s*	
11	β	3.49	*brs*	β	3.47	*dd* (5.0, 1.0)	67.1	*d*	C-4a, C-10b
12	α	3.18	*brs*	α	3.02	*ddd* (13.7, 5.0, 1.9)	60.7	*t*	C-10b, C-11
	β	3.18	*brs*	β	3.15	*dd* (13.7, 1.2)			C-6
NMe		2.59	*s*		2.51	*s*	48.5	*q*	C-6, C-12
OMe		3.76	*s*		3.80	*s*	55.8	*q*	C-9

Table 3.2.1.2. Scalar and spatial correlation of the protons of **27**

Proton		COSY	ROESY
1		H-2α, H-2β, H-3α, H-4a	H-2α, H-2β, H-11β
2	α	H-1, H-2β, H-3α	H-1, H-2β, H-3α
	β	H-1, H-2α, H-3α, H-4	H-1, H-2α, H-3α, H-4
3	α	H-1, H-2α, H-2β, H-4, H-4a	H-1, H-2α, H-2β, H-4
4		H-2β, H-3α, H-4a	H-2β, H-3α, H-4a
4a		H-1, H-3α, H-4	H-3α, H-4, H-6β, H-12β
6	α	H-6β, H-12α	H-6β, H-12α, NMe
	β	H-6α	H-6α, H-12β, NMe
7		H-8	H-8
8		H-7	H-7
11	β	H-12α	H-1, H-2α, H-12α, H-12β
12	α	H-6α, H-11β, H-12β	H-11β, H-12β, NMe
12	β	H-11β, H-12α	H-11β, H-12α, NMe
NMe			H-6α, H-6β, H-12α, H-12β
OMe			H-8

The molecular formula for 11α-hydroxygalanthamine should be $C_{17}H_{21}NO_4$. HREIMS provided *m*/z 303.14797 $[M]^+$, which is in accordance with the calculated molecular weight of 303.14706. An EIMS furnished *m*/z 303 $[M]^+$, 286 $[M\text{-}OH]^+$, 230, 213, 174, 115, 55. The IR spectrum showed absorption due to an aromatic hydroxyl group at 3395 cm^{-1}. Full IR data for **27** is given in Table 3.2.3.2.

The ^1H NMR spectrum of **27** in CD_3OD revealed that the aromatic protons at δ 6.71 and δ 6.59 consisted of an AB system of two *ortho*-coupled doublets (J = 8.0Hz), typical of a galanthamine type structure and these were assigned to H-8 and H-7 respectively. The ^1H NMR spectrum of **27** that was run in $CDCl_3$ showed two olefinic protons at δ 5.99 *ddd* (J = 10.3, 5.0, 1.5Hz) and δ 5.72 *d* (J = 10.3Hz), which were attributed to H-4 and H-4a, respectively. The magnitude of coupling constants for H-4 indicated a *pseudoequatorial* orientation for H-3, which resonated as a double triplet at δ4.12 (J = 2.8Hz). H-1 was identified as a triplet at δ5.20.

A *d* at δ3.87 and a *dd* at δ3.59 were assigned to the AB system of the benzylic protons at C-6. In the absence of coplanarity between the nitrogen non-bonding electrons and either H-6α or H-6β, the long range W coupling between H-12α and H-6α made it possible to distinguish between the two. The HSQC spectrum linked the H-11 chemical shift at δ3.47 *dd* (J = 5.0, 1.0 Hz) to a carbon resonance at δ67.1, which is consistent with the presence of an OH substituent at that position. The stereochemistry at C-11 was resolved from a consideration of the coupling constants between H-11 and H-12α and H-12β. From a model, the *pseudoaxial* OH results in dihedral angles between H-11β and H-12α and H-12β of approximately 90° and 45° respectively. For a *pseudoequatorial* OH these same angles for an H-11α are 180° and 45°. The measured J values (1.0 and 5.0 Hz) thus supported a *pseudoaxial* OH, which was confirmed by a correlation between the H-11β and H-1 and H-2α in the ROESY spectrum. The H-12α and H-12β resonated as a *ddd* at δ3.02 and a *dd* at δ3.15, and again the long range coupling to H-6α identified H-12α. The proton H-2α resonated at δ2.17 as a *ddd* (15.6, 5.3, 3.7 Hz) and 2β resonated at δ2.41 as a *m*. In addition further ROESY correlations were observed between H-4a and H-6β and H-12β. Finally the two singlets at δ3.80 and δ2.51 were assigned to O-Me and N-Me respectively.

The ^{13}C NMR and DEPT spectra identified 17 carbon resonances, made up of two methyl, three methylene, seven methine and five quaternary carbons. All protonated carbon resonances could be assigned from HSQC spectrum. HMBC correlations from H-8 to C-6a and C-10, H-7 to C-9 and C-10a, H-6α to C-10a, H-4a to C-10a and C-10b, H-2β to C-10b, H-4 to C-10b, H-11β to C-10b and H-12α to C-10b enabled the identication of the five quaternary carbons.

The identification of the 11α-hydroxygalanthamine that was isolated from *Cyrtanthus obliquus* was made possible by careful comparison of its ^{1}H NMR (500MHz) and ^{13}C-NMR data ($CDCl_3$) with that of the 11α-hydroxygalanthamine isolated from *Cyrtanthus sanguineus*. There were only minor differences seen between the spectra of the two samples. In the ^{1}H NMR spectra of the 11α-hydroxygalanthamine from *Cyrtanthus obliquus*, the H-6β was slightly less deshielded than the OMe group (δ3.75 and 3.82 respectively) whereas in the 11α-hydroxygalanthamine isolated from

Cyrtanthus sanguineus, H-6β is more deshielded than the OMe group (δ 3.83 and 3.80 respectively). In addition, the H-2β of 11α-hydroxygalanthamine from *Cyrtanthus obliquus* was more deshielded than that of the 11α-hydroxygalanthamine from *Cyrtanthus sanguineus* (δ 2.70 and 2.50 respectively). These small shifts in the NMR spectra can be attributed to variations in the quality of the solvents used for the spectroscopic experiments of the different samples. Further correlation was seen with the similar optical rotation readings, as given in **Table 3.2.3.1.**

3.2.2. Obliquine (30)

The isolation of the dinitrogenous alkaloid, designated as obliquine, is reported here for the first time. To the best of my knowledge, there have only been two dinitrogenous Amaryllidaceae alkaloids previously reported, namely plicamine (**1**) and secoplicamine (**2**), which were isolated from *Galanthus plicatus* subsp. *byzantinus* (Ünver et al., 1999). These compounds constitute a new subgroup of the Amaryllidaceae alkaloids, where a nitrogen atom replaces the oxygen atom in position 5 of a tazettine type of molecule, and that nitrogen atom is substituted by a pendant 6-hydroxyphenethyl moiety. Complete assignment of ^{1}H and ^{13}C NMR data of **30** was made possible by 2-D NMR experiments run in $CDCl_3$. Details are given in **Table 3.2.2.1** and **Table 3.2.2.2**.

Table 3.2.2.1. ^1H NMR, HSQC and HMBC data for obliquine (**30**) in $CDCl_3$. Carbon multiplicities were established by DEPT data.

Position		δH (JHz)		Correlated C-atom (HSQC)		HMBC
1		5.89	*d* (9.5)	136.5	*d*	C-2, C-3, C-4a, C-10a, C-10b, C-11
2		6.05	*dd* (9.5, 5.3)	124.3	*d*	C-1, C-3, C-4, C-10b
3	α	3.89	*m*	71.2	*d*	C-1, C-2, C-4a, OMe
4	α	1.49	*ddd* (13.5, 11.5, 3.0)	30.4	*t*	
	β	2.36	*dt* (13.5, 4.0)			C-2, C-3, C-4a, C-10b
4a		3.75	*dd* (11.5, 4.5)	61.6	*d*	C-10a, C-10b, C-11, C-12
6	α	3.67	*d* (15.3)	50.5	*t*	C-6a, C-7, C-10a, C-11, C-1'
	β	3.92	*d* (15.3)			C-6a, C-7, C-10a, C-11, C-1'
6a				129.1	*s*	
7		6.46	*s*	106.0	*d*	C-6, C-9, C-10a
8				146.8	*s*	
9				146.9	*s*	
10		6.75	*s*	108.4	*d*	C-6a, C-7, C-8, C-10a, C-10b
10a				130.5	*s*	
10b				44.4	*s*	
11		3.64	*s*	66.0	*d*	C-1, C-6, C-10a, C-10b, C-12, C-1'
12				172.3	*s*	
1'a		3.29	*dt* (12.5, 8.0)	56.8	*t*	C-6, C-11, C-2', C-3'
1'b		3.23	*dt* (12.5, 8.0)			C-6, C-11, C-2', C-3'
2'a		2.75	*t* (8.0)	33.8	*t*	C-1', C-3', C-4', C-8'
2'b		2.75	*t* (8.0)			C-1', C-3', C-4', C-8'
3'				132.0	*s*	
4', 8'		7.01	*d* (8.7) 2H	129.8	*d*	C-2'
5', 7'		6.73	*d* (8.7) 2H	115.2	*d*	C-3', C-6', C-4', C-8'
6'				154.3	*s*	
NMe		2.71	*s*	27.7	*q*	C-4a, C-12
OMe		3.45	*s*	56.5	*q*	C-3
OCH_2O		5.88	*s*	100.9	*t*	C-8, C-9

Table 3.2.2.2. Scalar and spatial correlation of the protons of **30**.

Proton		COSY	ROESY
1		H-2	H-2, H-11
2		H-1, H-3α	H-1, H-3α, OMe
3	α	H-2, H-4α, H-4β, OMe	H-2, H-4α, H-4β, OMe
4	α	H-3α, H-4β, H-4a	H-3α, H-4β, H-4a, H-11, NMe
	β	H-3α, H-4α, H-4a	H-3α, H-4α, H-4a, NMe
4a		H-4α, H-4β	H-4α, H-4β, H-10, NMe
6	α	H-6β, H-11	H-6β, H-7, H-11, H-1'a, H-1'b, H-2'a, H-2'b
	β	H-6α	H-6α, H-7, H-1'a, H-1'b, H-2'a, H-2'b
7			H-6α, H-6β
10			H-4a
11		NMe	H-1, H-4α, H-1'a, H-1'b, H-2'a, H-2'b
1'a		H-1'b, H-2'a, H-2'b	H-6α, H-6β, H-11, H-2'a, H-2'b, H-4', H-8'
1'b		H-1'a, H-2'a, H-2'b	H-6α, H-6β, H-11, H-2'a, H-2'b, H-4', H-8'
2'a		H-1'a, H-1'b, H-4', H-8'	H-6α, H-6β, H-11, H-1'a, H-1'b, H-4', H-8'
2'b		H-1'a, H-1'b, H-4', H-8'	H-6α, H-6β, H-11, H-1'a, H-1'b, H-4', H-8'
4', 8'		H-2a', H-2'b, H-5', H-7'	H-1'a, H-1'b, H-2'a, H-2'b, H-5', H-7'
5', 7'		H-4', H-8'	H-4', H-8'
NMe		H-11	H-4α, H-4β, H-4a
OMe		H-3α	H-2, H-3α
OCH_2O			

The molecular formula for an obliquine (**30**) structure would be $C_{26}H_{28}O_5N_2$. A HREIMS provided *m/z* 448.19980 [M^+], which is in accordance with the calculated molecular weight of 448.19982. The IR spectrum showed absorption due to an aromatic hydroxyl group at 3310 cm^{-1} and a lactam carbonyl group at 1672 cm^{-1}. Full IR data for **30** is given in Table 3.2.3.2.

Initial examination of the ^1H NMR spectrum of **30** showed the characteristic singlets associated with the *para*-orientated aromatic protons H-7 and H-10 at δ 6.46 and 6.75, respectively, which together with the methylenedioxy singlet at δ 5.88, established the substitution pattern of the aromatic A ring. The presence of an additional two doublets in the aromatic region (δ 7.00, δ 6.73), integrating for two hydrogens each, suggested the presence of a *para*-disubstituted benzene ring, which was determined to be the pendant phenethyl moiety. These doublets were assigned to the two pairs of *ortho*-coupled protons at 4' and 8', and at 5' and 7', respectively. The four aliphatic protons of this phenethyl moiety were distinguished as a *dt* at δ 3.29, a further *dt* at δ 3.23, and a separate triplet at δ 2.75. The two double triplets were assigned to the geminal protons on C-1', whilst the triplet was assigned to the geminal protons on C-2' of the phenethyl moiety. The ROESY experiment supported this assignment by providing spatial correlations for H-1'a and b, and H-2'a and b with the AB system of benzylic protons H-6α and H-6β, which resonated as two doublets at δ 3.67 and δ 3.92 respectively. H-6β was more deshielded due to its coplanarity with the nitrogen lone pair.

The C ring was characterised by a *d* at δ 5.89 and a *dd* at δ 6.05, which were assigned to the methine protons H-1 and H-2 respectively. A multiplet at δ 3.89 linked to a carbon resonating at δ 71.2 suggested that the methoxyl group was positioned at C-3, confirmation coming from a $^3J_{CH}$ HMBC contour with C-3 and ROESY correlations with H-2 and H-3. H-3 in turn, coupled in the COSY spectrum with the geminal protons H-4α (δ 1.49) and H-4β (δ 2.36). Ünver et al, 1999, assigned an alpha orientation to the OMe group in both plicamine and secoplicamine. I believe that in the obliquine structure the configuration is beta. For H-3α the dihedral angles between H-4α and H-3α and H-4α and H-4a are approximately 45° and 180° respectively. This is consistent with the calculated coupling constants for the H-4α *ddd* of 13.5, 11.5 and 3.0 Hz. An H-3β would result in two trans diaxial couplings. Assignments in this ring were completed by a *dd* at δ 3.75 attributed to H-4a.

The identification of ring D as a γ-lactam structure was confirmed by $^3J_{CH}$ HMBC correlations between the deshielded N-Me protons (δ 2.71) to both C-4a (δ 61.6) and the carbonyl group at C-12 (δ 172.3) and a $^2J_{CH}$ contour from H-11 to C-12. Key ROESY contours in this region of the structure were H-4a to H-10 and N-Me and H-11 to H-4α, H-1'a and 1'b and H-2'a and 2'b, confirming the beta orientation for H-4a and the alpha orientation for H-11.

3.2. Isolation and Identification of Alkaloids from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*

A combination of flash and preparative thin layer chromatography on the crude alkaloid extract of the bulbs of the plants yielded 11α-hydroxygalanthamine (75mg, **27**), lycorine (15 mg, **4**), lycoramine (5 mg, **28**), 11-epihaemanthamine (12 mg, **29**) and haemanthidine (5 mg, **13**) from *Cyrtanthus sanguineus*, and further 11α-hydroxygalanthamine (3 mg, **27**), the novel dinitrogenous alkaloid obliquine (9 mg, **30**), trisphaeridine (7 mg, **19**), tazettine (16mg, **8**), 3-epimacronine (5 mg, **31**) and narcissidine (23 mg, **32**) from *Cyrtanthus obliquus* respectively. The structures of all the alkaloids isolated from *Cyrtanthus sanguineus* are given in **Figure 3.2.1**, and the structures of the alkaloids isolated from *Cyrtanthus obliquus* are given in **Figure 3.2.2**. Full methodology details of the isolation and purification of the alkaloids are given in **Section 5.1.3**.

All protonated carbon resonances could be assigned from the HSQC spectrum, and the multiplicities were determined from the DEPT spectra. The ^{13}C-NMR and DEPT spectra confirmed the 26 carbon resonances, made up of two methyl, five methylene, eleven methine and eight quaternary carbon resonances. The HMBC's which identify all the quaternary carbons are from H-1 to C-10a and C-10b, H-4β to C-10b, H-4a to C-10a, C-10b and C-12, H-6α to C-6a and C-10a, H-6β to C-6a and C-10a, H-7 to C-9 and C-10a, H-10 to C-6a, C-8 and C-10b, H-11 to C-10a, C-10b and C-12, H-1'a and H-1'b to C-3', H-2'a and H-2'b to C-3', H-5', 7' to C-3' and C-6', NMe to C-12, and OCH_2O to C-8 and C-9 (**Table 3.2.2.1**). Further key ROESY correlations were from H-1 to H-11, H-6α to H-11, and H-6α and H-6β to H-1'a, H-1'b, H-2'a and H-2'b (**Table 3.2.2.2**).

3.2.2.1. Proposed biosynthetic pathway to obliquine (30)

The biosynthesis of obliquine, as proposed by Ünver et al, 1999, is probably an extension of the pathway producing the tazettine and crinine type of structures in the plant. As detailed in **Section 3.2**, tazettine (**8**) and 3-epimacronine (**31**) are both present in *Cyrtanthus obliquus*. Ring B of the tazettine molecule could be opened up to produce an aminoaldehyde (**33**), which can form a Schiff base with a molecule of tyramine. The structure can then undergo an intramolecular rearrangement, followed by ring closure with subsequent hydration. Allylic oxidation and oxidation of the carbinolamine, followed by methylation would generate obliquine (**30**).

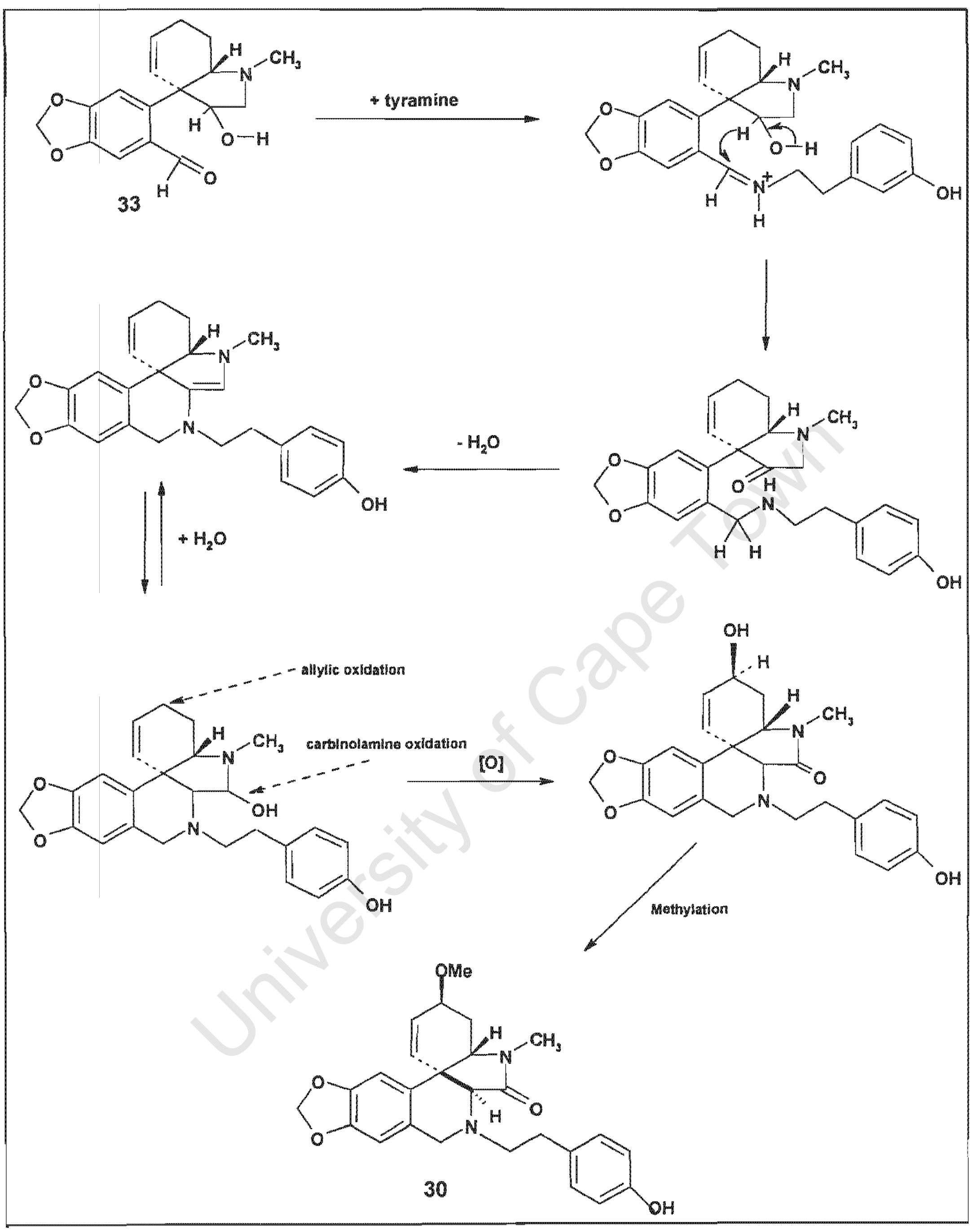


Figure 3.2.2.1.1. Proposed biosynthetic pathway to obliquine (**30**)

3.2.3. Discussion of the identification of the known alkaloids

The alkaloids 11-epihaemanthamine (**29**), haemanthidine (**13**), lycoramine (**28**), lycorine (**4**), 3-epimacronine (**31**), narcissidine (**32**), tazettine (**8**) and trisphaeridine (**19**) were identified by analysis of their ^{1}H NMR spectra, and by direct comparison of their spectroscopic properties (MS, IR, OR, m.p.'s, ^{1}H and ^{13}C NMR) with those given in the literature review by Bastida et al., 1998 and Viladomat et al., 1997. **Table 3.2.3.1** gives the chemical shift data for these alkaloids. Copies of all the spectra for all the alkaloids are given in **Appendix 1**. **Table 3.2.3.2** gives the MS, IR, OR and melting point data of all the alkaloids isolated from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*, including the data for 11α-hydroxygalanthamine (**27**) and obliquine (**30**).

11-Epihaemanthamine (29)

The A ring was characterised by the *para*-orientated aromatic protons at position 10 and 7, which produced two singlets at δ 6.84 and δ 6.49 respectively, and two doublets in the range δ 5.91-5.90, which were assigned to the protons of the methylenedioxy group. A doublet at δ 3.71 and a doublet at δ 4.34 were assigned to the AB system of the benzylic protons at C-6 of the B ring. The C ring was characterised by a *d* at δ 6.41 and *dd* at δ 6.38, which were assigned to the olefinic protons H-1 and H-2 respectively. A singlet at δ 3.48 was assigned to the OMe group at C-3, and a further multiplet at δ 4.40 was assigned to the methine proton on C-3. The geminal protons on C-4 were identified as a *ddd* at δ 2.14 and a *dd* at δ 1.90, which were assigned to H-4β and H-4α respectively. A *dd* at δ 3.22 was assigned to H-4a. A multiplet at δ 4.00 was assigned to H-11*exo*, and further multiplets at δ 3.44 and δ 3.49 were assigned to H-12*endo* and H-12*exo*, respectively. COSY correlations linked H-11*exo* to H-12*exo* and H-12*endo*, and H-11*exo* to H-4a. Since it was not possible to determine the stereostructure at C-11, since H-11*exo* resonated as a multiplet, the optical rotation reading ($[\alpha]_D^{20}$ -25.0°, lit. $[\alpha]_D^{20}$ -24.0°) was used to confirm that this alkaloid was 11-epihaemanthamine and not haemanthamine (lit. $[\alpha]_D^{20}$ +33.0°).

Haemanthidine (13)

The ^{1}H NMR spectrum of **13**, which was run in $CDCl_3$, showed a mixture of the two epimers. Two singlets were observed at δ 6.78 and δ 6.70, corresponding to the aromatic protons on C-7 and C-10 respectively, and two doublets in the range δ 5.87-5.84 were assigned to the methylenedioxy group protons. Two singlets at δ 5.83 and δ 5.82 were assigned to the proton on C-6, in the alpha and beta position respectively. Measurement of the heights of these singlet signals provided an estimation that the epimers are present in the ratio of *ca* 3 alpha: 2 beta. The olefinic protons of C-1 and C-2 of the C ring were identified as a *d* at δ 6.42 and a *dd* at δ 6.33 respectively, and a multiplet at δ 3.90 was assigned to H-3. A singlet at δ 3.42 was assigned to the protons of the OMe group on C-3. A *dd* at δ 3.20 was assigned to H-4a, whilst two multiplets at δ 2.30 and δ 2.18 were assigned to the geminal protons H-4α and H-4β, respectively. A large coupling was observed between H-4α and H-4a ($J_{4\alpha,4a}$ = 13.2Hz) indicative of their *trans*-diaxial position. A multiplet at δ 3.90 was assigned to H-11*endo*, with further multiplets at δ 4.32 and δ 3.19 assigned the H-12*endo* and H-12*exo*, respectively.

Lycoramine (28)

Two doublets at δ 6.72 and δ 6.62 were correlated to the *ortho*-oriented aromatic protons of position 8 and 7 respectively, indicative of a galanthamine type of alkaloid. The absence of resonances for olefinic protons confirmed that the double bond between C-4 and C-4a was not present, suggesting that that this alkaloid was a lycoramine type. Two doublets at δ 3.93 and δ 3.55 were assigned to the benzylic protons on C-6. Correlating with the spectral data generated for 11α-hydroxygalanthamine (**27**), in which H-6β was shown to be more deshielded than H-6α, it was possible to assign these doublets to H-6β and H-6α, respectively. A singlet at δ 3.85 and a singlet at δ 2.32 were assigned to the OMe and NMe group protons respectively. A triplet resonating at δ 3.10 and a doublet resonating at δ 3.00 were assigned to H-12α and H-12β, respectively. The protons on C-1 and C-3 resonated as multiplets at δ 4.30 and δ 4.10. A singlet at δ 2.45 was assigned to H-2α. It was not possible to distinguish between H-2β, the geminal protons on C-4, the geminal protons on C-4a and the geminal protons on C-11, as all these protons appeared as a shielded multiplet, in the range δ 1.6-2.0. Further evidence for the identification of this alkaloid was provided by the melting point and optical rotation data, which was in agreement with the literature (**Table 3.2.3.2**).

Lycorine (4)

Two singlets at δ 7.00 and δ 6.83 were assigned to the *para*-oriented aromatic protons of positions 10 and 7 respectively, and a singlet at δ 5.98 was assigned to the methylenedioxy group. Doublets at δ 4.45 and at δ 4.21 were assigned to the geminal protons H-6β and H-6α respectively. The C ring was characterised by three singlets at δ 5.79, δ 4.59 and δ 4.25, which were assigned to the methine protons H-3, H-1β and H-2α, respectively. The H-4a resonated as a *d* at δ 3.93. The geminal protons of C-11 resonated as a multiplet at δ 2.84. Further multiplets at δ 3.50 and δ 3.75 were assigned to H-12α and H-12β, respectively. COSY correlations were seen between H-11α and H-11β, between H-11α and β and H-12α and β, between H-4a and H-10b, and from H-1 to H-2 and H-3.

3-Epimacronine (31)

The aromatic protons of C-7 and C-10 resonated as two singlets at δ 7.52 and δ 6.75 respectively, and two doublets in the range δ 6.05-6.03 were assigned to the protons of the methylenedioxy group. The C ring was characterised by a *d* at δ 5.44 and a *dd* at δ 5.96 corresponding to the olefinic protons H-1 and H-2, respectively. A singlet at δ 3.43 was assigned to the protons of the OMe group on C-3, with a multiplet at δ 4.15 corresponding to H-3. It was not possible to distinguish between the geminal protons on C-4, which resonated as a multiplet at δ 1.70. H-4a resonated as a multiplet at δ 3.13. Ring D was identified from a singlet at δ 2.52, which was assigned to the protons of the NMe group. The geminal protons H-12α and H-12β were identified as a multiplet at δ 2.72 and a *dd* at δ 3.20, respectively. A further *dd* at δ 4.45 was assigned to H-11. Since it was not possible to distinguish between H-4α and H-4β, the coupling constants with H-3 could not be determined and used to confirm the configuration of the OMe group on C-3. It was thus not possible to confirm that this alkaloid was 3-epimacronine and not macronine. However, the optical rotation reading taken was $[\alpha]_D^{20}$ +271.9°, which is in agreement with the literature, whereas the literature value for macronine is $[\alpha]_D^{20}$ +413°.

Narcissidine (32)

Two singlets at δ 6.88 and δ 6.68 were assigned to the *para*-oriented aromatic protons of positions 10 and 7 respectively, whilst the protons of the two aromatic OMe groups on C-8 and C-9 resonated as singlets at δ 3.86 and δ 3.82. The geminal protons H-6β and H-6α resonated as doublets at δ 4.08 and at δ 3.58, respectively. A doublet at δ 2.70 was assigned to H-10b. The C ring was characterised by two multiplets at δ 4.67 and δ 4.66, which were assigned to H-1β and H-3β, respectively. The protons of the OMe group on C-2 resonated as a singlet at δ 3.44. H-2α was indistinguishable from H-4a, and the geminal protons H-12α and H-12β, since these protons all appeared together as a multiplet in the range δ 3.7-3.8. A *brs* at δ 5.58 was assigned to H-11. The ^{13}C-NMR and DEPT spectra identified 18 carbon resonances, made up of three methyl, two methylene, eight methine and five quaternary carbons.

Tazettine (8)

Two singlets at δ 6.50 and δ 6.85 were assigned to the aromatic protons of C-7 and C-10 respectively, and a further singlet at δ 5.90 was assigned to the protons of the methylenedioxy group. The benzylic protons of C-6 produced two pairs of doublets at δ 4.64 for H-6α and δ 4.96 for H-6β. The C ring was characterised by a two double triplets at δ 5.60 and δ 6.15 corresponding to the olefinic protons H-1 and H-2, respectively. A singlet at δ 3.47 was assigned to the protons of the OMe group on C-3, with a multiplet at δ 4.12 corresponding to H-3. The geminal protons H-4α and H-4β resonated as multiplets at δ 2.24 and δ 1.62 respectively, whilst the methine proton H-4a resonated as a multiplet at δ 2.86. Ring D was identified from a singlet at δ 2.40, which was assigned to the protons of the NMe group. The geminal protons H-12α and H-12β were identified as a *d* at δ 2.68 and a *d* at δ 3.30, respectively. Since H-3 resonated as a multiplet it was not possible to distinguish between tazettine and the epimeric criwelline. An optical rotation reading of $[\alpha]_D^{20}$ +126.9° was in agreement with the literature, whereas the literature reading for criwelline is $[\alpha]_D^{20}$ +278°.

Trisphaeridine (19)

Examination of the ^{1}H-NMR spectrum of **19**, which was run in $CDCl_3$, revealed that the chemical shift values for this alkaloid were typically very downfield and that there were fewer protons present, indicative of a structure that has complete aromatization. The most deshielded singlet at δ 9.03 was correlated to the H-6, which is characteristic of trisphaeridine. The A ring was characterised by two singlets at δ 7.28 and δ 7.86, which were assigned to the aromatic protons of C-7 and C-10 respectively, whilst a singlet at δ 6.11 was identified as the methylendioxy group. A *dd* at δ 8.32, a *ddd* at δ 7.57, a *ddd* at δ 7.63 and a *dd* at 8.07 were assigned to H-1, H-2, H-3 and H-4, respectively of the C ring. As expected, this alkaloid was not optically active.

Table 3.2.3.1(a) ^{1}H NMR data for *Cyrthanthus sanguineus* and *Cyrtanthus obliquus* alkaloids. Chemical shifts are reported in ppm (δ) from TMS.

Alkaloid	11-Epihaemanthamine	Haemanthidine	Lycoramine	Lycorine	3-Epimacronine	Narcissidine	Tazettine	Trisphaeridine
H-1	6.41 *d*	6.42 *d*	4.30 *m*	4.59 *s*	5.44 *d*	4.67 *m*	5.60 *dt*	8.32 *dd*
H-2	6.38 *dd*	6.33 *dd*	______	______	5.96 *dd*	______	6.15 *dt*	7.57 *ddd*
H-2α	______	______	2.45 *d*	4.25 *brs*	______	3.7-3.8 *m*	______	______
H-2β	______	______	1.6-2.0 *m*	______	______	______	______	______
H-3	4.40 *m*	3.90 *m*	4.10 *m*	5.79 *s*	4.15 *m*	4.66 *m*	4.12 *m*	7.63 *ddd*
H-4	______	______	______	______	______	______	______	8.07 *dd*
H-4α	1.90 *dd*	2.30 *m*	1.6-2.0 *m*	______	1.70 *m*	______	2.24 *m*	______
H-4β	2.14 *ddd*	2.18 *m*	1.6-2.0 *m*	______	1.70 *m*	______	1.62 *m*	______
H-4a	3.22 *dd*	3.20 *dd*	1.6-2.0 *m* (2H)	3.93 *d*	3.13 *d*	3.7-3.8 *m*	2.86 *m*	______
H-6	______	______	______	______	______	______	______	9.03 *s*
H-6α	3.71 *d*	5.83 *s*	3.55 *d*	4.21 *d*	______	3.58 *d*	4.64 *d*	______
H-6β	4.34 *d*	5.82 *s*	3.93 *d*	4.45 *d*	______	4.08 *d*	4.96 *d*	______
H-7	6.49 *s*	6.78 *s*	6.60 *d*	6.83 *s*	7.52 *s*	6.68 *s*	6.50 *s*	7.28 *s*
H-8	______	______	6.72 *d*	______	______	______	______	______
H-10	6.84 *s*	6.70 *s*	______	7.00 *s*	6.75 *s*	6.88 *s*	6.85 *s*	7.86 *s*
H-10b	______	______	______	2.99 *d*	______	2.70 *d*	______	______
H-11	______	______	______	______	4.45 *dd*	5.58 *brs*	______	______
H-11α	______	______	1.6-2.0 *m*	2.84 *m*	______	______	______	______
H-11β	______	______	1.6-2.0 *m*	2.84 *m*	______	______	______	______

Table 3.2.3.1(b) ^{1}H NMR data for *Cyrthanthus sanguineus* and *Cyrtanthus obliquus* alkaloids. Chemical shifts are reported in ppm (δ) from TMS.

Alkaloid	11-Epihaemanthamine	Haemanthidine	Lycoramine	Lycorine	3-Epimacronine	Narcissidine	Tazettine	Trisphaeridine
H-11endo	—	3.90 *m*	—	—	—	—	—	—
H-11exo	4.00 *m*	—	—	—	—	—	—	—
H-12α	—	—	3.10 *t*	3.50 *m*	2.72 *m*	3.7-3.8 *m*	2.68 *d*	—
H-12β	—	—	3.00 *d*	3.75 *m*	3.20 *dd*	3.7-3.8 *m*	3.30 *d*	—
H-12endo	3.44 *m*	4.32 *m*	—	—	—	—	—	—
H-12exo	3.49 *m*	3.19 *m*	—	—	—	—	—	—
OCH_2O	5.91-5.90 2*d*	5.87-5.84 2*d*	—	5.98 *s*	6.05-6.03 2*d*	—	5.90 *s*	6.11 *s*
OMe	—	—	—	—	—	3.86 *s*	—	—
OMe	—	—	—	—	—	3.82 *s*	—	—
OMe	3.48 *s*	3.42 *s*	3.85 *s*	—	3.43 *s*	3.44 *s*	3.47 *s*	—
NMe	—	—	2.32 *s*	—	2.52 *s*	—	2.40 *s*	—
Solvent	$CDCl_3$	$CDCl_3$	$CDCl_3$	CD_3OD	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$
MHz	300	200	200	200	200	200	300	300

Table 3.2.3.2. Data for the alkaloids isolated from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*

Alkaloid	MW	Molecular Formula	Melting pt / °C	Optical Rotation	Infra Red ν_{max} cm^{-1}
11α-Hydroxygalanthamine (*C. sanguineus*)	303	$C_{17}H_{21}NO_4$	193-194	$[\alpha]_D^{20}$ -51.9° ($CHCl_3$; *c* 0.07)	3450, 3395 (OH), 3010, 2918, 1615, 1500, 1278, 968 ($CHCl_3$)
11α-Hydroxygalanthamine (*C. obliquus*)	303	$C_{17}H_{21}NO_4$	192-194	$[\alpha]_D^{20}$ -61.5° (MeOH; *c* 0.2)	3452, 3395 (OH), 3014, 2918, 1618, 1500, 1275, 968 ($CHCl_3$)
Obliquine	448	$C_{26}H_{28}N_2O_5$	136-139	$[\alpha]_D^{20}$ -20.5° (MeOH; *c* 0.6)	3310 (OH), 3013, 2927, 2855, 2362, 1672 (lactam CO), 1513, 1482, 1238, 1037, 1039, 934, 833, 759 ($CHCl_3$)
11-Epihaemanthamine	301	$C_{17}H_{19}NO_4$	204-206	$[\alpha]_D^{20}$ -25.0° ($CHCl_3$; *c* 0.17)	3314 (OH), 2362, 1504, 1483, 1238, 1036, 935, 849, 757 ($CHCl_3$)
Haemanthidine	317	$C_{17}H_{19}NO_5$	178-180	$[\alpha]_D^{20}$ -14.9° ($CHCl_3$; *c* 0.27)	3316 (OH), 2362, 1510, 1483, 1240, 1036, 935, 839, 752 ($CHCl_3$)
Lycoramine	289	$C_{17}H_{23}NO_3$	113-116	$[\alpha]_D^{20}$ -78.0° (MeOH; *c* 0.5)	3394 (OH), 2362, 1574, 1282, 756 ($CHCl_3$)
Lycorine	287	$C_{16}H_{17}NO_4$	248-249	$[\alpha]_D^{20}$ -59.1° (EtOH; *c* 0.07)	3331 (OH), 2338, 1571, 1239, 937, 828 (CH_3OH)
3-Epimacronine	329	$C_{18}H_{19}NO_5$	125-129	$[\alpha]_D^{20}$ +271.9° ($CHCl_3$; *c* 0.05)	3328 (OH), 2341, 1569, 1472, 1233, 1082, 1028, 937, 810, 763 ($CHCl_3$)
Narcissidine	333	$C_{18}H_{23}NO_5$	201-204	$[\alpha]_D^{20}$ -27.5° ($CHCl_3$; *c* 0.17)	3250 (OH), 2338, 1574, 1513, 1115, 976, 758 ($CHCl_3$)
Tazettine	331	$C_{18}H_{21}NO_5$	200-203	$[\alpha]_D^{20}$ +126.9° ($CHCl_3$; *c* 0.25)	3348 (OH), 2361, 1589, 1482, 1247, 1084, 1038, 937, 826, 773 ($CHCl_3$)
Trisphaeridine	223	$C_{14}H_9NO_2$	129-131	Not optically active	2337, 1562, 941, 831, 768 ($CHCl_3$)

3.3. Cytotoxicity screening of *Cyrtanthus* alkaloids

Where sufficient quantities of alkaloid material permitted, the alkaloids isolated from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus* were evaluated *in vitro* for cytotoxicity, against mammalian Chinese Hamster Ovarian Cells (CHO) and human heptoma HepG2 cells. The CHO cell line is a homogeneous, rapidly proliferating, continuous cell line, which is easy to grow in a monolayer. Cytotoxicities were determined using a modification of the original MTT tetrazolium salt colorimetric assay described by Mosmann (1983). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay measures the metabolic viability of cells *in vitro*. Viable cells are able to reduce the water-soluble yellow coloured MTT to a water-insoluble purple-coloured formazan product. The amount of product formed is proportional to the metabolic activity and the number of cells in the test sample.

3.3.1. Results of cytotoxicity screening

Table 3.3.1. Evaluation of the cytotoxicity of some *Cyrtanthus* alkaloids against CHO cells and HepG2 cells by the MTT assay (n=3).

Alkaloid	**IC_{50} ± SEM (μg/ml)**	
	CHO cells	**HepG2 cells**
11α-Hydroxygalanthamine	> 100	> 100
Lycorine	26.55 ± 1.43	> 100
Narcissidine	> 100	> 100
Obliquine	> 100	> 100
Tazettine	> 100	> 100
Daunomycin	1.72 ± 0.38	1.07 ± 0.22

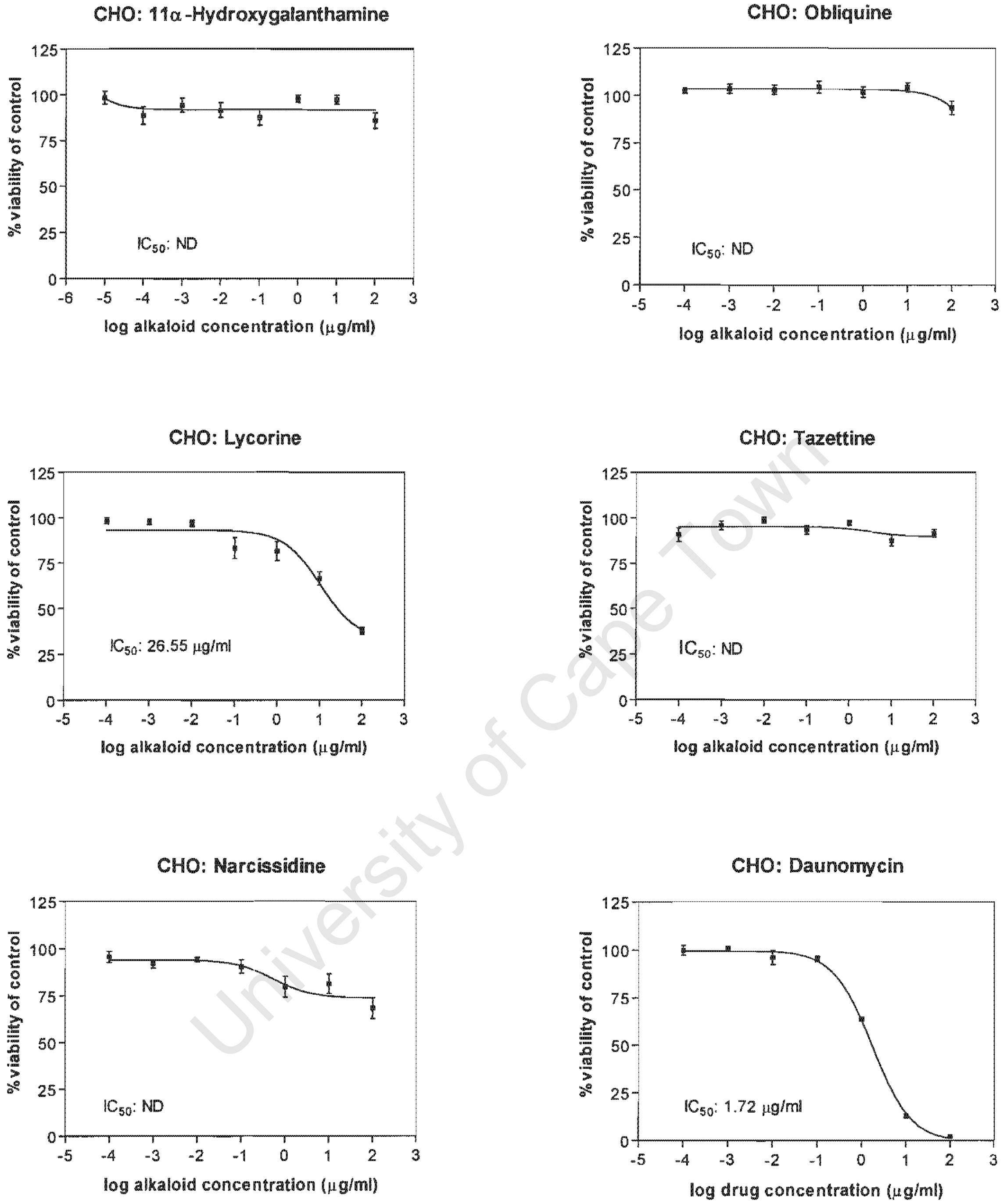


Figure 3.3.1.1. Cytotoxicity dose response curves for 11α-hydroxygalanthamine, obliquine, lycorine, tazettine, narcissidine and daunomycin, respectively against CHO cells (n=3). ND: not determinable

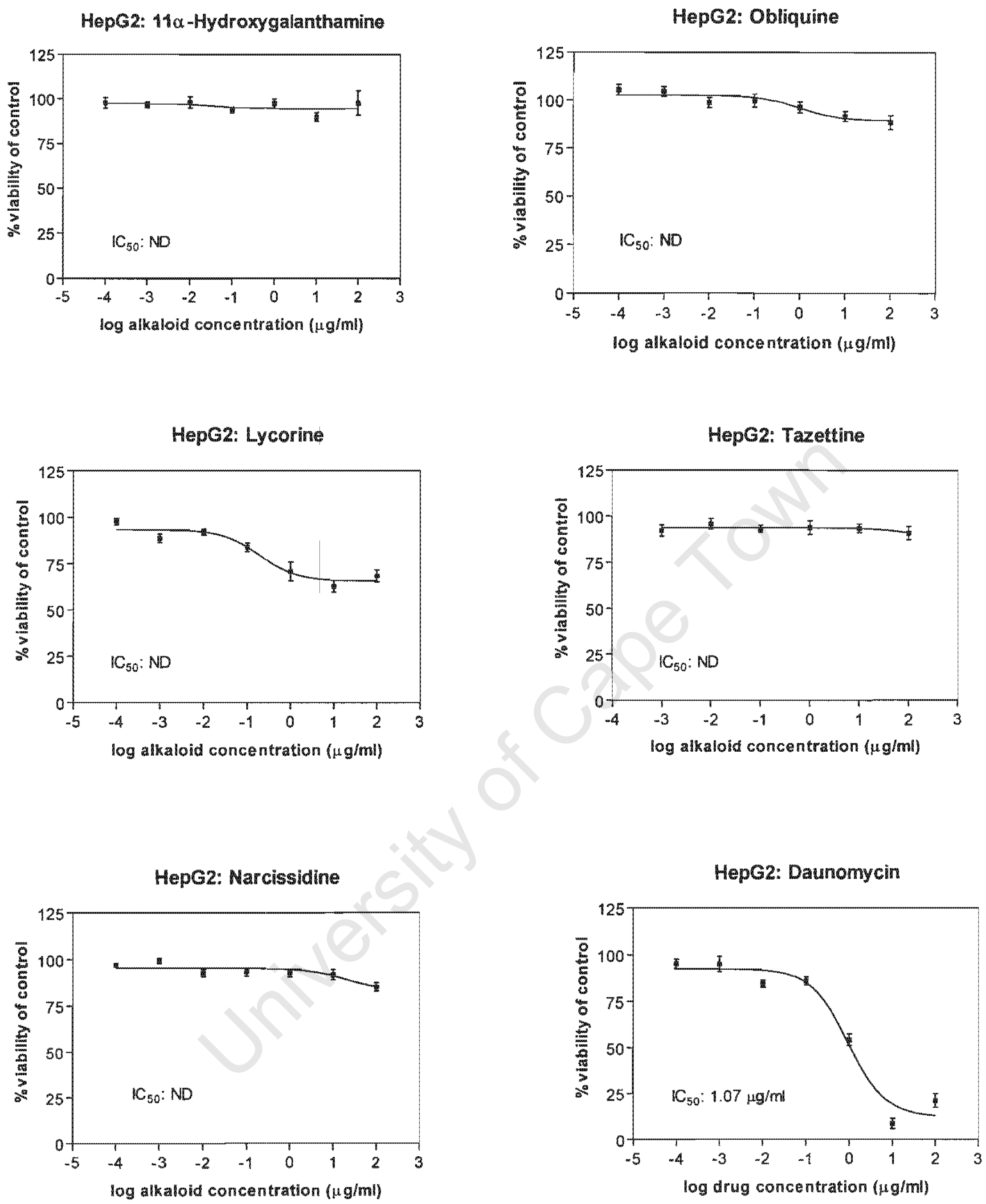


Figure 3.3.1.2. Cytotoxicity dose response curves for 11α-hydroxygalanthamine, obliquine, lycorine, tazettine, narcissidine and daunomycin, respectively against HepG2 cells (n=3). ND: not determinable

3.3.2. Discussion of the cytotoxicity screening results

A cytotoxicity study of 25 Amaryllidaceae alkaloids belonging to different skeletal types (Weniger et al., 1995) revealed that significant differences of activity could be observed with the different type of alkaloid skeleton tested, and from cell line to cell line used in the screening. In that study, tazettine was toxic to LMTK (murine alveolar non tumoral fibroblasts) cells (IC_{50} value 3.2 μg/ml), but not against HepG2 (human hepatoma) or Molt 4 (human lymphoid neoplasm) cells, whereas galanthamine showed selective toxicity against the LMTK cell line (IC_{50} value 0.5 μg/ml), and lycorine showed cytotoxicity against all three of the cell lines (IC_{50} values 0.4, 0.3 and 13 μg/ml against Molt 4, LMTK and HepG2, respectively).

The results from the *in vitro* cytotoxicity screening in this investigation revealed that 11α-hydroxygalanthamine, galanthamine, obliquine and tazettine are not toxic to the mammalian cell lines, CHO and HepG2, at concentrations greater than the pharmacological efficiency scale ($>10^{-2}$ M). Lycorine is moderately toxic to the CHO cells, with an IC_{50} value of 26.55 μg/ml, whilst narcissidine, which is also a lycorine-type of alkaloid, is not toxic at a concentration of up to 100 μg/ml, and neither lycorine nor narcissidine showed cytotoxicity to the HepG2 cells.

3.4. Reported biological and pharmacological activities of the known *Cyrtanthus* alkaloids

This section discusses the literature on the biological and pharmacological activities of the known alkaloids isolated from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*.

11-Epihaemanthamine (29)

A study on 18 Amaryllidaceae alkaloids (Jimenez et al., 1976) revealed that 11-epihaemanthamine inhibits HeLa cell growth at $<10^{-1}$ mM. The alkaloid also inhibits protein synthesis directed by endomyocarditis virus RNA in a S-30 ascites cell-free system, and is thought to inhibit protein synthesis in eukaryotic cells by inhibiting the peptide bond formation step.

Haemanthidine (13)

A report on the analgesic properties of several alkaloids showed that haemanthidine possesses greater activity than aspirin (Tanker et al., 1996). Haemanthidine has also shown significant anti-inflammatory action in mice (Çitoglu et al., 1998). It is cytotoxic *in vitro* against epidermoid carcinoma (A-431), nasopharyngeal carcinoma (KB), colon cancer (Co12), lung cancer (Lu1), melanoma (Me12) and breast cancer (ZR-75-1) cell lines, with significant activity against prostrate cancer (LNCaP), murine lymphocyte leukaemia (P-388) and sarcoma (HT) cell lines (Antoun et al., 1993).

Lycoramine (28)

Lycoramine is reported to produce acute poisoning of the digestive, respiratory, cardiovascular, neuromuscular and central nervous systems (Chao et al., 1965). It antagonises muscle paralysis induced by *d*-tubocurarine and also antagonises the ganglionic blockade, increasing the contraction response (Chao et al., 1965). Lycoramine is a weak inhibitor of plant ascorbic acid biosynthesis (Evidente et al., 1983). Animal studies showed that lycoramine has ten times stronger cholinesterase inhibitory activity than galanthamine *in vivo* (Tang et al., 1978). A study by Han et al. (1992) on the *in vitro* acetylcholinesterase (AChE) inhibitory action of various derivatives of galanthamine, reported 30% inhibition of AChE, compared to 93% inhibition by galanthamine. The AChE inhibitory activity of galanthamine is discussed in detail in **Chapter 4**.

Lycorine (4)

Lycorine occurs almost ubiquitously and exclusively in the Amaryllidaceae. Lycorine is a toxic principle and the alkaloid is reported to cause salivation, vomiting and diarrhoea when ingested in low doses, and paralysis and collapse at high doses (Van Wyk and Gericke, 2000). Most of the effects of lycorine on physiological processes have been ascribed to its inhibitory effect on ascorbic acid (AA) biosynthesis *in vivo* (Evidente et al., 1983). This ability to inhibit ascorbic acid biosynthesis has made lycorine a valuable tool for studying the AA-dependent reactions, such as cell division. Lycorine has been shown to inhibit cell division in rat fibroblasts (Weniger et al., 1995), to inhibit DNA synthesis (Ghosal et al., 1985) and to inhibit protein blocking peptide bond formation (Jimenez et al., 1975). Lycorine is also an inhibitor of cyanide-resistant respiration *in vivo*, as AA is needed for the synthesis of hydroxyproline-containing proteins, specifically utilised for the development of KCN-resistant respiration (Evidente et al., 1983). Lycorine is a respiratory stimulant (Abdalla et al., 1993), and relaxes isolated ephedrine-precontracted pulmonary artery. It also increases the contractility and rate of isolated perfused heart (Abdalla et al., 1993). These effects are mediated by stimulation of β-adrenergic receptors. A report on the analgesic properties of several alkaloids showed that lycorine possesses greater activity than aspirin (Tanker et al., 1996). The anti-inflammatory effects of lycorine have also been reported (Citoglu et al., 1998). Studies on the cholinergic effects of lycorine (Matsui 1962; Matsui et al., 1962) revealed that the alkaloid affects nerve-muscle transmission, and that it inhibits depressive effects on the central nervous system, causes mild hypotension, increases peripheral vasodilation and depresses cardiac movement.

Lycorine is a moderate antitumoural (Abdalla et al., 1993) and is cytotoxic against several cultured cell lines (Weniger et al., 1995; Lin et al., 1995). Lycorine's antiviral activity has been attributed to its inhibitory action on viral protein synthesis (Leven et al., 1983). It is active against several RNA and DNA viruses, including Herpes simplex type 1, poliomyelitis, coxsackie, Semliki Forest and measles viruses (Abdalla et al., 1993), but does not inhibit the activity of reverse transcriptase. It is responsible for the antiviral activity observed in crude extracts from the leaves and roots of *Clivia miniata* (Leven et al., 1982), where the alkaloid is typically present at levels of up to 0.4% of the dry weight (Van Wyk and Gericke, 2000). Lycorine is a weak protozoicide (Bastida et al., 1998), and inhibits plant growth by inhibiting protein synthesis (Ghosal et al., 1985). It acts to inhibit the plant cell cycle during interphase, which is AA dependent (Evidente et al., 1983), and has also been shown to inhibit germination of seeds and growth of roots (Ghosal et al., 1985). Lycorine-1-O-β-D-glucose has the reverse effect, and may also produce mitogenic activity in animal cells (Ghosal et al., 1985). Ungeremine, which is a natural metabolite of lycorine, is reported to be responsible, at least in part, for the growth-inhibitory and cytotoxic effects of lycorine (Ghosal et al., 1988). Certain bacteria transform lycorine into pancrissidine, which is less toxic than ungeremine.

3-Epimacronine (31)

3-Epimacronine has been shown to be weakly cytotoxic against human Molt 4 and murine LMTK cell lines (Weniger et al., 1995).

Narcissidine (32)

Narcissidine was screened for cytotoxicity against Hela cells *in vitro*, and was shown to be inactive (Jimenez et al., 1976).

Tazettine (8)

Tazettine is an artefact of the isolation procedure; the naturally occurring alkaloid is actually pretazettine (**34**). Pretazettine was found to inhibit HeLa cell growth as well as protein synthesis in eukaryotic cells (Jiménez et al., 1975). It was shown to be cytotoxic against fibroblastic LMTK and Molt 4 lymphoid cell lines (Weniger et al., 1995), and demonstrates a synergistic effect in combination with standard cytotoxic drugs (Suzuki et al., 1974). Pretazettine exhibited antiviral activity against the Rauscher leukaemia virus in mouse embryo cells (Suzuki et al., 1974), and against the *Herpes simplex* type 1 (Renard-Nozaki et al., 1989) and neurotropic RNA viruses (Zee-Cheng et al., 1978).

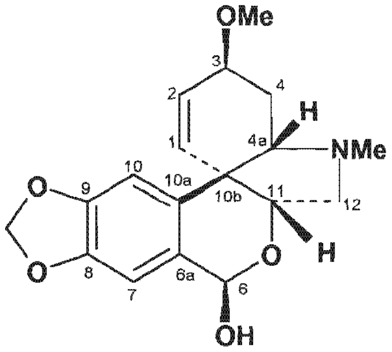


Figure 3.4.1. Structure of pretazettine (**34**).

The stereochemical rearrangement from pretazettine to tazettine appears to inactivate some of the biological activity of pretazettine (Furusawa et al., 1976). Tazettine is reported be cytotoxic against fibroblast cells (LMTK) (Weniger et al., 1995) and NIH-3T3 cell line (Furusawa et al., 1980). Animal studies have also shown that tazettine has antitumor activity (Furusawa et al., 1976). Tazettine inhibits ribosomal transpeptidase in plants (Kukhanova et al., 1983).

3.5. Discussion of the potential toxicity of *Cyrtanthus* species

From the evidence presented in the literature, together with the cytotoxicity screening data from this investigation, it can be said that *Cyrtanthus sanguineus* bulbs contain at least two potentially poisonous alkaloids, namely lycoramine and lycorine, whilst *Cyrtanthus obliquus* bulbs contain tazettine, which has also been reported to be moderately toxic (Weniger et al., 1995). This is a cause for concern, given the extensive use of both of the plant species in traditional medicine. The cholinergic effect of the 11α-hydroxygalanthamine, which is found in both species, also needs to be considered for its role in potential poisoning. The acetylcholinesterase inhibitory activity of 11α-hydroxygalanthamine is discussed in detail in **Chapter 4**.

The concentration of alkaloids present in the traditional remedies is likely to be low, since the traditional method of preparation involves steeping the chopped bulbs in water, to make an infusion. When considering the nature of the isoquinoline alkaloids, there are probably few of these compounds that would be present in an aqueous extract at neutral pH. When the alkaloids are extracted from the crude methanol extract using liquid phase extraction (**Section 5.1.3**), it is necessary to acidify the water in order to remove the alkaloids. Thin layer chromatography analysis of the aqueous extracts of the bulbs investigated in this study indicated that low concentrations of alkaloids are likely to be extracted when water is used as a solvent. However, it is difficult to obtain accurate information on the quantity of bulbs that would typically be used in the preparation of an infusion, how much is consumed, or for what length of time the remedy is taken. Repeated ingestion of a concentrated infusion may have an accumulative effect, particularly if the compounds are lipid soluble, and become deposited in the adipose tissue. This is particularly relevant in the case of *Cyrtanthus sanguineus*, which is taken by pregnant women for a course of approximately five months. The teratogenic effects of such a compound have not yet been investigated.

A further issue of concern is that modern day traditional healers may have access to organic solvents, such as alcohol, which they might incorporate into their repertoire of medicinal preparation techniques. In such a case there is the potential hazard of increased levels of alkaloids present in the remedies. Finally, the possible presence of further toxic compounds in the Amaryllidaceae plants such as crystals of calcium oxalate, which can cause skin irritation when the bulbs are handled, may increase the toxicity of the plants. In conclusion, the two species of *Cyrtanthus* that were investigated in this study are potentially toxic and as such, should be used as herbal remedies only with extreme caution.

3.6. Antiplasmodial screening of *Cyrtanthus* alkaloids

Previous investigations of Amaryllidaceae alkaloids for their antiplasmodial and cytotoxic activities have revealed that whilst some of the alkaloids have antiplasmodial activity, they are very often highly cytotoxic (Campbell et al., 1997), implying that the antiplasmodial activity of some of these alkaloids is general, nonspecific toxicity. It has been shown that among the 5-10b-ethanophenanthridine structures, crinamine, in which the ethano-bridge has the α-configuration, is the most active against the malaria parasite. In continuation of these investigations, narcissidine (**32**), tazettine (**8**), 11αhydroxygalanthamine (**27**) and obliquine (**30**) were screened for *in vitro* antiplasmodial activity against chloroquine-sensitive strains of *Plasmodium falciparum*, using the parasite lactate dehydrogenase assay (Makler and Hinrichs, 1993). The alkaloids were shown to be inactive up to a concentration of 25 µg/ml (results not shown). Cailean Clarkson and Donelly van Schalkwyk of the Malaria Research Group, at the Pharmacology Division, of the Department of Medicine, UCT, performed the testing.

3.7. Concluding Comments

The bulbs were supplied as horticultural specimens. This was necessary since the regulations of South Africa do not permit the harvesting of wild plants, and it also ensured that the bulbs supplied were authentic, as the supplier is an authority on Amaryllidaceae bulbs. However, there is a chance that cultivated bulbs do not contain as high levels of alkaloids as bulbs that have grown in the wild, since it is thought that the production of the alkaloids is accelerated in response to stress factors in the environment, such as drought. Cultivated bulbs, which are maintained in ideal growing conditions, would probably not experience such stress factors.

It would have been interesting to screen the aqueous extracts of the *Cyrtanthus* species for *in vitro* and perhaps *in vivo* cytotoxicity, to establish whether the traditional medicinal preparations of the bulbs produced toxic infusions. As detailed in **Section 5.1.3**, small quantities of aqueous extracts of the bulbs were prepared and analysed for alkaloid content using thin layer chromatography, which revealed that there were traces of alkaloid present in these extracts. Given the relative shortage of available plant material, these aqueous extracts were later subjected to liquid phase extraction in an attempt to acquire more alkaloids for phytochemical analysis, and the cytotoxicity screening of the aqueous extracts was unfortunately overlooked. This study did provide some indication of the potential toxicity of these *Cyrtanthus* species, however, by identifying some of the alkaloids present, such as lycorine, which has been shown to be highly toxic (Van Wyk and Gericke, 1997; Weniger et al., 1995). This study also provided some information on the activity of some of the alkaloids against Chinese Hamster Ovarian (CHO) cells and human heptoma (Hep G2) cells.

Chapter 4

Acetylcholinesterase Inhibition by 11α-Hydroxygalanthamine

4.1. The role of acetylcholinesterase inhibitors in the treatment of Alzheimer's disease

4.1.1. The epidemiology and etiology of Alzheimer's disease

The social and public health importance of dementia has been greatly increased by the unprecedented expansion in the world's elderly population. Statistics released by Alzheimer's disease International (ADI), show that there are currently nearly 18 million people with dementia in the world, and this figure is set to rise to 34 million by 2025. Dementia primarily affects elderly people, with the chances of having the condition rising sharply with age. Dementia is rare below the age of 60 years; between the ages of 65 and 70, 2% will have dementia, as compared to 20% among those over 80. Dementia is not, however, an inevitable consequence of old age and most 90 year olds, for example, do not have the condition. Although there have been few studies of the prevalence of dementia in the developing world, most evidence suggests that the prevalence rate is the same as in the developed world, which is 2-3% of those over the age of 60. In 1993, 200 million of the worlds' total of 356 million persons over 65 were living in developing countries. By 2020 seven of the ten countries with the largest elderly populations will be in the developing world.

Alzheimer's disease (AD) is the most common form of dementia and represents 40-60% of all late onset dementia disorders in Caucasian populations (Nordberg and Svensson, 1998). Within the industrial nations, Alzheimer's dementia is the fourth leading cause of death amongst the elderly, and women appear to be more susceptible. The prevalence of AD rises significantly with advancing age, with rates doubling approximately every 5 years over the age of 65. Dr Alois Alzheimer, a German neurologist, first described the clinical and pathologic symptoms of the disease, in 1907. Alzheimer's disease is associated with memory loss and other cognitive symptoms producing occupational and social disabilities.

Alzheimer's disease is characterised in the brain by the deposition of amyloid protein outside the neurone, resulting in the formation of plaques, and inside the neurone with neurofibrillary tangles, which are cytoskeletal components that affect the functioning of the neurone. The number of synapses is decreased in AD (Davis, 1999), and since the synapse is the unit of communication between cells, it is believed that the loss of synapses is reflected in dementia. Recently, researchers showed that when amyloid-β was used as a vaccine in a mouse model of Alzheimer's disease, plaque formation was reduced (Schenk et al., 1999) as the mice developed antibodies to the protein. Mice given the test vaccine performed markedly better than unvaccinated animals in memory tests. More recently, the link between plaque formation and brain dysfunction was confirmed in the Alzheimer mouse model (Janus et al., 2000; Morgan et al., 2000), providing hope that it may be possible to reverse dementia by simply disrupting the deposition of amyloid-β. Chen et al., (2000) corroborated the findings by differentiating the effects of the disease from those of ordinary ageing in mice. However, extrapolation of these results to human AD patients has still to be performed. Large-scale human clinical trials are shortly to be conducted by the Irish pharmaceutical company, Elan.

The pathologic changes of AD are most often seen in the temporal and parietal regions of the brain, with extension to the frontal cortex, resulting in characteristic deterioration of intellectual capacity in various domains, such as learning and memory, language abilities, reading and writing, and interaction with the environment. Personality changes are also common, leading to behavioural disturbances, and patients lose the ability to perform activities of daily living (Benzi and Moretti, 1998). In later stages, basic biological functions are disturbed and neurological alterations develop. The neurotransmitters that are the most often affected are those known to be involved in these processes, such as acetylcholine, which is eventually diminished in almost all AD patients. There is also evidence of a deficiency in corticotropin-releasing factor immunoreactivity and somatostatin immunoreactivity, indicating that the cells manufacturing these neuropeptides are dysfunctional and losing synapses. It is known that in patients with early-onset AD, norepinephrine and serotonin can be involved.

It is not yet known what initiates plaque formation or protein tangles in the brain of an AD patient. Risk factors currently considered to be positive include: a history of dementia in a first-degree relative, a history of Down's syndrome in a first-degree relative, a history of previous head injury with concussion, a history of hypothyroidism, and a previous history of depression (Henderson, 1993). In patients with well-documented genetic makeup, mutations in the gene associated with amyloid precursor protein, and in the genes associated with the proteins presenilin-1 and presenilin-2 seem to be highly associated with AD. There is also an association of an increased likelihood of AD with the ε 4-type variant of the gene that produces apolipoprotein E (*APOE*), when compared with the ε 3 or ε 2 variants of the gene (Frikke-Schmidt et al., 2001). It was previously thought that there was a link between AD and exposure to aluminium, but the evidence is still inconclusive. Some studies have found physical inactivity to be a risk factor, and that the risk may also be increased in elderly persons with little or no education. Finally, tobacco and analgesics may have a slight protective effect.

4.1.2. Cholinergic treatment strategies in Alzheimer's disease

Since the 1970s, highly consistent findings have clearly shown that some neurotransmitter systems are selectively altered in the Alzheimer's disease brain. The most dramatic abnormalities are those of the cholinergic system, providing the foundation for the "cholinergic hypothesis of Alzheimer's disease". This hypothesis formed the rationale for a symptomatic therapy of Alzheimer's disease aimed at potentiating central cholinergic function, in the hope that this would improve cognitive function. The main strategies that have been employed to manipulate cholinergic neurotransmission in AD patients are discussed briefly below.

4.1.2.1. Precursor loading

Choline is the immediate precursor of acetylcholine. Choline is sequestered from the extra synaptic space by a high affinity uptake mechanism and is then rapidly acetylated by choline acetyltransferase (ChAT) to acetylcholine. Neither the uptake mechanism nor ChAT activity is saturated at physiological choline concentrations. It has been shown that exogenously administered choline and phosphatidylcholine (lecithin) increase CNS acetylcholine concentrations (Cohen and Wurtman, 1976). However, attempts to improve cognition through precursor loading have proven unsuccessful in most AD patients (Winblad et al., 1993).

4.1.2.2. Release enhancers

Phosphatidylserine and 4-aminopyridine have been used in attempts to treat AD dementia through enhancing acetylcholine release. Slight improvement in cognitive function has been reported (Winblad et al., 1993). Phosphatidylserine has been shown to increase acetylcholine release from rat cortical slices, although the mechanism of action is as yet unknown. On the other hand, 4-aminopyridine acts to block potassium channels, thus prolonging depolarisation and thereby increasing neurotransmitter release.

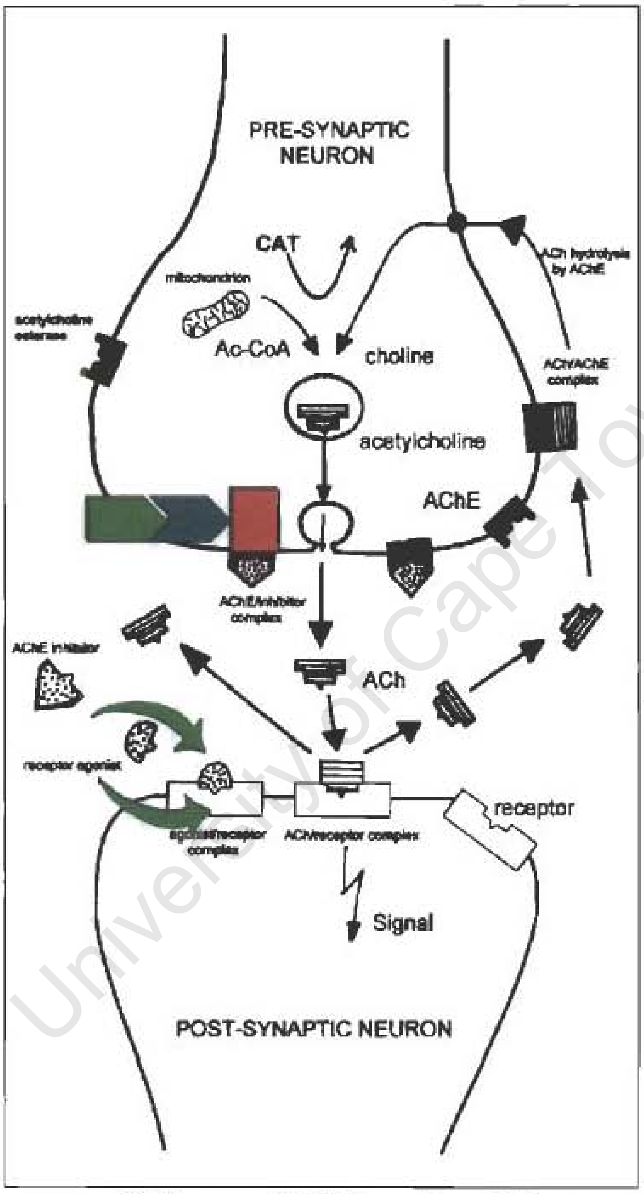


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Figure 4.1.2. Some mechanisms and intervention sites at the cholinergic synapse. Inhibiting acetylcholinesterase, which degrades the neurotransmitter can restore reduced intrasynaptic concentration of acetylcholine; or post-synaptic muscarinic receptors can be preserved by M1-agonists. *Reproduced with kind permission from Gordon and Breach Publishers, Reading, United Kingdom.*

4.1.2.3. Cholinergic agonists

Cholinergic agonists would be expected to have the advantage of directly activating postsynaptic target receptor sites. Unfortunately, most of these drugs are either short acting or do not efficiently cross the blood-brain barrier, and as such have either proven unsuccessful or given only slight improvement in cognitive function (Winblad et al., 1993).

4.1.2.4. Acetylcholinesterase inhibitors

Of the various possible strategies for enhancing cholinergic activity in the brain, acetylcholinesterase inhibition has been by far the most extensively used. The inhibitors of acetylcholinesterase should increase the efficiency of cholinergic transmission by preventing the hydrolysis of released acetylcholine, thus making more acetylcholine available at the cholinergic synapse. Like acetylcholine receptor agonists, acetylcholinesterase inhibitors are assumed to take advantage of the relative preservation of post-synaptic muscarinic receptors in Alzheimer's disease (Giacobini, 1998). A dysfunctional cholinergic system, as in AD, would benefit only if the acetylcholinesterase is inhibited to such a degree that the released acetylcholine can function post synaptically without affecting the pre-synaptic events necessary for renewed transmitter synthesis and release (Benzi and Moretti, 1998). Binding studies have shown that acetylcholinesterase inhibitors interact with both muscarinic (M_1, M_2) and nicotinic receptors (Wagstaff and McTavish, 1994). Clinical experience with cholinesterase inhibitors indicates that these compounds have positive effects on cognitive function, especially in attention and that long-term treatment also has effects on primary memory, episodic memory, visuospatial ability and psychomotor speed (Nordberg and Svensson, 1998). Unfortunately, few of the acetylcholinesterase inhibitors can pass into the brain and remain effective without severe side effects. Some of these acetylcholinsterase inhibitors are discussed in detail as follows.

4.1.2.4.1. Physostigmine (Synapton)

Physostigmine is a natural alkaloid that is a reversible inhibitor of acetylcholinesterase, with a short duration of action. Modest improvements of cognitive function in AD patients have been observed after treatment with physostigmine. Physostigmine has a short elimination half-life of 20-30min, a high plasma clearance of 1.5-2.8 L/min and a volume of distribution of 46.5 ± 19L. The short half-life is problematic as it causes fluctuations in blood levels and the need for frequent administration. Low plasma concentrations of 1-

2ng/ml have been obtained after oral administration of physostigmine, and the bioavailability varies between 11 and 37%. Considering these factors, the efficacy of use of this drug was limited in Alzheimer therapy (Johansson and Nordberg, 1993). A synthetic analogue, rivastigmine, was shown to be more suitable for the treatment of AD and was introduced into the UK in 1998 by Novartis.

4.1.2.4.2. Tacrine (Cognex®)

9-amino-1,2,3,4-tetrahydrotacrine (tacrine, THA) is a central-acting, reversible cholinesterase inhibitor. Tacrine was synthesised in 1945 by Albert and Gledhill, and was observed to be an AChE inhibitor in 1953 (Shaw and Bently). Tacrine was the first cholinesterase inhibitor to be approved for use in Alzheimer's disease (in the US in 1993 and in Europe in 1994). Its effects are mainly palliative although there may be some slowing or arresting of the disease course

Tacrine inhibits plasma cholinesterase and tissue acetylcholinesterase (AChE) with a higher affinity for butyrylcholinesterase (BChE) than AChE (Heilbronn, 1961). Tacrine has a longer elimination half-life than physostigmine, of 1.3 to 7 hours, which is possibly due to its larger volume of distribution. Pharmacokinetic investigations revealed that the oral bioavailability varies greatly between patients, in the range of approximately 17-37% (Johansson and Nordberg, 1993). Patients with a high bioavailability of the drug tended to improve more, but also had more liver enzyme elevations, than those with low bioavailability. Increased levels of serum alanine aminotransferase (ALT) have been observed in 49% of patients with Alzheimer's disease treated with tacrine. The hepatotoxic effects of tacrine have been reviewed by Watkins et al. (1994), who summarised data from 6 multicentre trials involving 2446 patients. Tacrine has been recently withdrawn from the market in several countries, following concern over associated adverse hepatic events.

Tacrine is metabolised in the liver by the cytochrome P450 (CYP) isoenzymes CYP1A2 and CYP2D6 and five metabolites have been found in serum and urine. The major metabolite is 1-hydroxytacrine that is found after both intravenous and oral administration of tacrine. The plasma concentration reached for this metabolite is between 5 to 10 times higher than for tacrine itself, and 1-hydroxytacrine has been reported to have the same elimination half-life as tacrine within some patients (Hartvig et al., 1991). 1-

Hydroxytacrine has also been given as a drug, called velnacrine (Hoescht-Roussel). The same liver enzyme abnormalities occur with velnacrine as with tacrine, and moreover there are some concerns about reports of lowered white cell counts associated with velnacrine (Whitehouse, 1993).

4.1.2.4.3. Donepezil (Aricept®)

Donepezil is a reversible, specific, piperidine acetylcholinesterase inhibitor that has a primarily non-competitive inhibitory action, but also shows some competitive characteristics. Pharmacokinetic studies on donepezil have shown that the t_{max} is 3 to 5 hours, with steady-state concentrations achieved following 15 days of treatment (Nordberg and Svensson, 1998). The elimination half-life has been estimated to be 70 to 80 hours and the mean apparent plasma clearance estimated at 0.13 L/h/kg. Donepezil shows cholinergic adverse effects with the most frequent effects being gastrointestinal and dizziness. No liver toxicity has been reported following up to 192 weeks of donepezil treatment (Rogers and Friedhoff, 1998), although 83% of 133 patients with mild to moderate AD experienced mild to transient adverse effects. The majority of these events were considered to be related to the patients' underlying Alzheimer's disease. In general, donepezil is far better tolerated and it is currently the drug of choice in AD therapy.

4.1.2.4.4. Huperzine A (HupA)

Huperzine A (HupA) is an alkaloid that was first extracted from *Huperzia serrata* (club moss) in 1948, and can now be synthesized in the laboratory. It is a potent reversible inhibitor of acetylcholinesterase. For centuries *Huperzia serrata* has been brewed as a tea in Chinese traditional medicine ("Chien Tseng Ta") for the treatment of many disorders, such as fever, inflammation, memory loss, schizophrenia and water retention (Shen, 1994). Huperzine has been shown to produce memory stimulation in animals and humans (Cheng and Tang, 1998). Clinical data from China revealed that 60% of patients with Alzheimer's disease under the active drug showed improved performance in memory and behaviour compared to 36% under placebo after 8 weeks of treatment. Furthermore, HupA may also have a neuroprotectant activity (Bai et al., 2000; Skolnick, 1997), and the drug has already been approved for use in Alzheimer Disease patients in China. HupA possesses a longer duration of action and higher therapeutic index than tacrine and donepezil, and the peripheral cholinergic side effects are minimal at therapeutic doses. There are few reported side effects, although there have been individual cases of nausea, vomiting, excessive salivation and tear production, and sweating. The details of these clinical trials are not well known in the West, as the documents are all written in Chinese. However, huperzine A continues to raise considerable interest in the West and many research projects are currently underway. An herbal extract of *Huperzia serrata* is already available in many countries.

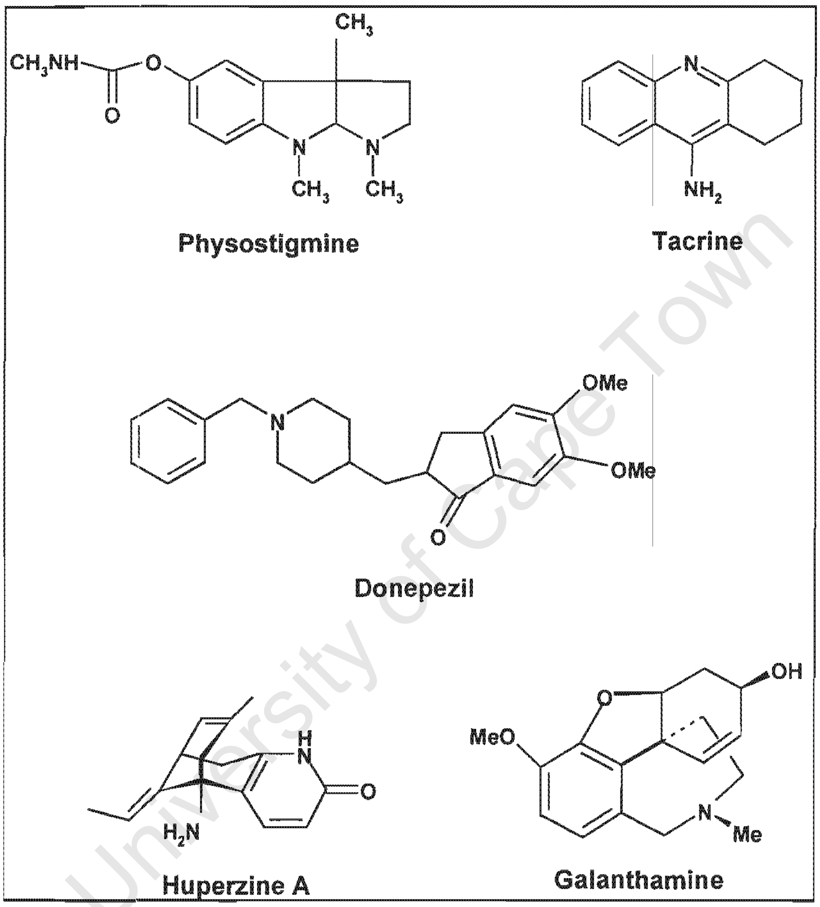


Figure 4.1.2.4. Chemical structures of acetylcholinesterase inhibitors physostigmine, tacrine, donepezil, huperzine A and galanthamine

4.1.2.4.5. Galanthamine (Galantamine, Reminyl™)

Galanthamine has been commercially available in some countries (primarily Eastern Europe) as a drug called Nivalin, for over 40 years for the reversal of neuromuscular blockade in anaesthetic practice and for the treatment of neurological conditions such as post-polio paralysis and myasthenia gravis (Fulton and Benfield, 1996). Galanthamine acts as a mild analeptic; it shows analgesic power as strong as morphine, and when applied in eye drops, it reduces the intraoccular pressure (Eichhorn et al., 1998). More recently, galanthamine has been invested for the treatment of Alzheimer's disease. The significance of galanthamine in the treatment of Alzheimer's disease was first proposed by Austrian researchers in 1986 as a better-tolerated alternative to the first generation compounds physostigmine and tacrine. The drug shows considerable promise, as it is hydrolysis-resistant, only moderately toxic and is more readily absorbed than physostigmine (Nordberg and Svensson, 1998). There is evidence that as well as inhibiting acetylcholinesterase, galanthamine also enhances the intrinsic action of acetylcholine on nicotinic receptors, probably through binding to an allosteric site of the receptor, stimulating the release of more acetylcholine.

Galanthamine is a reversible, selective inhibitor of acetylcholinesterase (AChE) with a competitive action. An *in vitro* study using erythrocytes from healthy volunteers determined that galanthamine is a selective inhibitor of acetylcholinesterase (Thomsen and Kewitz, 1990). The IC_{50} of galanthamine was 0.35μmol/L for acetylcholinesterase and 18.6μmol/L for butyrylcholinesterase. Although there is no direct evidence that butyrylcholinesterase activity is involved in cognitive function, these findings may be of clinical relevance as the inhibition of butyrylcholinesterase in conjunction with acetylcholinesterase may increase toxicity (Pacheco et al., 1995). Galanthamine was evaluated for effect on central cholinergic activity in samples of human post-mortem brain tissue (Thomsen et al., 1991). The IC_{50} values of galanthamine for acetylcholinesterase were 3.2μmol/L for the frontal cortex and 2.8μmol/L for the hippocampal region. Tacrine showed corresponding IC_{50} values of 1.0 and 1.1μmol/L. In the same study, similar acetylcholinesterase inhibitory activity for galanthamine was found in fresh brain cortex biopsies from patients undergoing brain tumour removal. Galanthamine was 10-fold less potent in inhibiting acetylcholinesterase from brain tissue than from erythrocytes in these patients (Thomsen et al., 1991). A study (Thomsen and Kewitz, 1990) on healthy volunteers revealed that the median maximum inhibition of erythrocyte acetylcholinesterase was 42% at approximately 1 hour after administration of a single 10mg oral dose of galanthamine. Enzyme activity returned to normal within 30 hours after administration of galanthamine. Sanguinine (6-*O*-demethylgalanthamine) is a metabolite of galanthamine formed by CYP2D6, and accounts for up to 20% of administered galanthamine (Bachus et al., 1999). Acetylcholine is also involved in the regulation of rapid eye movement (REM) sleep, a parameter that is altered in patients with Alzheimer's disease. In a study conducted on healthy volunteers, single 10mg or 15mg oral doses of galanthamine shortened REM latency, increased REM density and reduced the duration of slow wave sleep compared with placebo (Riemann et al., 1994).

Extensive Phase III trials in Europe and the United States concluded that galanthamine is effective and safe in Alzheimer Disease therapy (Raskind et al., 2000). After 6 months of treatment, galanthamine significantly improved cognition (including language, memory, attention and decision-making abilities) and global function, relative to placebo. Moreover, cognitive and daily functions, such as dressing, washing and feeding, were maintained for 12 months with a 24mg/day dose. Patients experienced an improvement in behavioural symptoms, including agitation, aggression, delusions and hallucinations. The clinical trials also showed that galanthamine is most effective if used early in the course of the disease, but that it is also effective later on in the disease course. The majority of adverse events were mild to moderate in severity and predominantly gastrointestinal, with nausea being the most commonly reported event with galanthamine. Other side effects reported included vomiting, dizziness, loss of appetite and weight loss, stomach cramps and pains, indigestion, diarrhoea, sleepiness, and urinary tract infection. Galanthamine is marketed as a hydrobromide salt under the name Reminyl™, and is being co-developed by Shire Pharmaceuticals and the Janssen Research Foundation (Johnson and Johnson's Belgian operation). It was launched as a product in the UK in September 2000, and in February 2001, the US Food and Drug Administration (FDA) issued approval of Reminyl™ for the treatment of Alzheimer's disease.

Galanthamine was first isolated in Russia in the 1940s from the bulbs of *Galanthus worownii* (Proskurnina and Areshknina, 1947) and structurally elucidated in Japan in the 1950s (Uyeo and Kobayashi, 1953). It can be found in related *Galanthus* species, such as *Galanthus nivalis* (common snowdrop), and many of the larger Amaryllidaceae species, including *Narcissus*. It has been suggested that a plant ("moly"), described by Homer in his epic poem, the *Odyssey*, which was used by Odysseus as an antidote against Circe's poisonous drugs, might have been *Galanthus nivalis* (Plaitakis and Duvoisin, 1983). This description of "moly" as an antidote may represent the oldest recorded use of an anticholinesterase to reverse central anticholinergic intoxication. In today's regulatory and commercial environment, drug development from purely botanical sources is generally not a viable long-term option, and this held true in particular for galanthamine. Traditionally the market had been served with plant extracts prepared from *Galanthus* cultures grown in Bulgaria, Turkey or the southwestern parts of the former Soviet Union. The galanthamine content of these botanical sources is about 0.1%. For botanical, climatic, and political reasons, supplies never exceeded 50kg and the price was correspondingly high and variable. Clearly this supply "bottleneck" needed to be removed before clinical development of galanthamine for Alzheimer's disease could be planned, with the annual requirements projected to reach the metric tonne scale once the drug was launched in significant markets. Galanthamine is a chiral compound with two asymmetric carbon atoms and four connected ring systems, two of them containing heteroatoms. Only the natural (-) galanthamine acts as an inhibitor of acetylcholinesterase whilst the (+) stereoisomer is inactive. Recently, the concise, scalable synthesis of (-) galanthamine was reported by Czollner et al. (1998) who have patented their Sanochemia process.

4.1.3. Structure of galanthamine related to activity

An *in vitro* structure-activity study (Han et al., 1992) conducted on synthetic analogues of galanthamine indicated that properly placed hydrophilic and lipophilic groups on galanthamine contribute to its effective binding to the AChE molecule. More recently, the X-ray crystal structure of galanthamine bound in the active site of *Torpedo californica* acetylcholinesterase (*Tc*AChE) was resolved (Greenblatt et al., 1999). It would appear that the binding of galanthamine to *Tc*AChE arises from a number of moderate to weak interactions with the protein, including classical and non-classical hydrogen bonds. The inhibitor binds at the base of the active site gorge of *Tc*AChE, interacting with both the choline-binding site (Trp-84) and the acyl-binding pocket (Phe-288, Phe-290). The tertiary amine appears to form a non-conventional hydrogen bond via its N-methyl group, to Asp-72, at the top of the gorge, whilst the hydroxyl group of galanthamine makes a strong hydrogen bond with Glu-199. The hydroxyl group also interacts with two water molecules.

Sanguinine (6-*O*-demethylgalanthamine), which has an extra hydroxyl group available for potential interaction with AChE, has been shown *in vitro* to be up to 10-fold more potent an inhibitor than galanthamine (Bores, 1996). In light of this, it was proposed that the additional hydroxyl group on the C-11 of 11α-hydroxygalanthamine (**27**), that was isolated from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*, might improve the binding affinity for AChE, by facilitating a possible extra hydrogen bond with the protein. The ability of 11α-hydroxygalanthamine (**27**) to inhibit AChE *in vitro* was investigated and compared to the inhibitory activity of galanthamine (**11**). The Department of Natural Products, of the University of Barcelona, donated the galanthamine used in this research.

4.2. Acetylcholinesterase Inhibition Assays

A photometric method for determining acetylcholinesterase activity *in vitro* was first described by Ellman et al. in 1961. Acetylcholinesterase hydrolyses acetylthiocholine to yield free thiocholine. The enzyme activity is measured by following the increase of yellow colour produced from thiocholine when it reacts with dithiobisnitrobenzoate ion. It is based on the coupling of these reactions:

Acetylthiocholine $\xrightarrow{\text{(AChE)}}$ thiocholine + acetate

Thiocholine + dithiobisnitrobenzoate → 5-thio-2-nitro-benzoic acid (yellow colour)

The inhibitory effect of 11α-hydroxygalanthamine (**27**) on erythrocyte acetycholinesterase *in vitro* was established, using an adaptation of the original photometric 96-well microtitre plate assay, as detailed in **Chapter 5.3**.

4.2.1. Results of AChE inhibition by 11α-hydroxygalanthamine

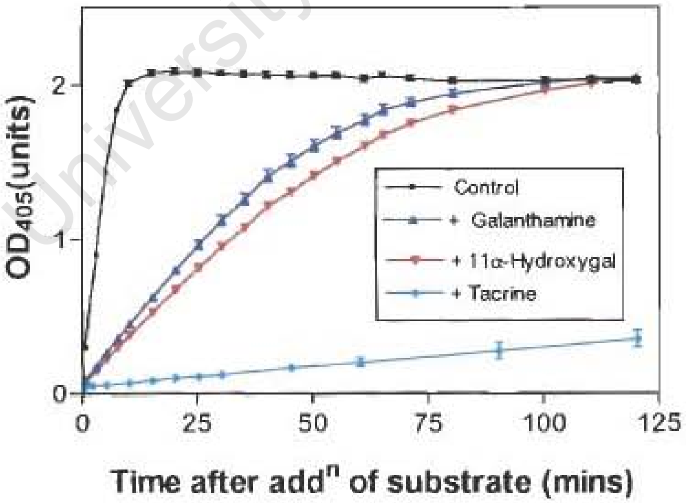


Figure 4.2.1.1. Acetylcholinesterase activity in the presence of galanthamine, 11α-hydroxygalanthamine (11α-hydroxygal) and tacrine, respectively [all 10^{-5} M] as a function of time (n=2)

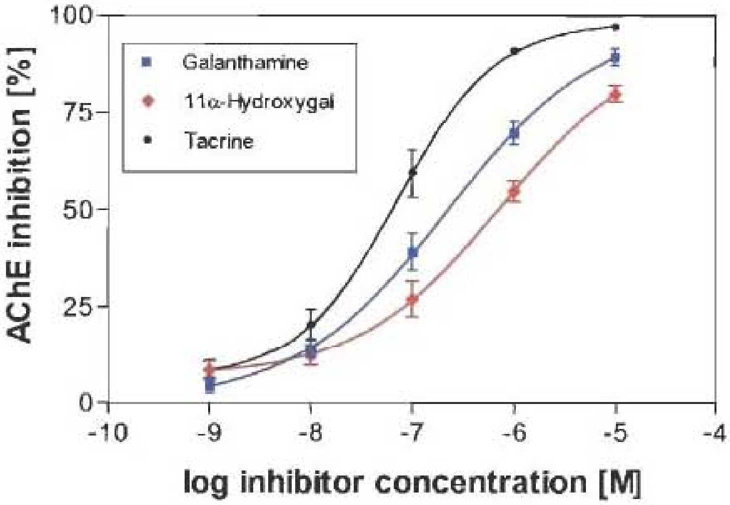


Figure 4.2.1.2. Dose response curves for the inhibition of erythrocyte AChE by galanthamine, 11α-hydroxygalanthamine (11α-hydroxygal) and tacrine, respectively (n=6).

Table 4.2.1.1. IC_{50} values for erythrocyte AChE inhibition (n=6)

Compound	**AChE Inhibition** IC_{50} ± SEM (μM)
Galanthamine	0.33 ± 1.32
11α-Hydroxygalanthamine	0.72 ± 1.15
Tacrine	0.10 ± 1.12

The AChE inhibitory activity of 11α-hydroxygalanthamine was demonstrated to be competitive, reversible inhibition (as shown in **Figure 4.2.1.1.**) and to be dose-dependent (**Figure 4.2.1.2.**) Statistical analysis of the mean IC_{50} values (concentration required to inhibit AChE by 50%) for galanthamine and 11α-hydroxygalanthamine showed that there is a significant difference between the mean IC_{50} values of galanthamine and 11α-hydroxygalanthamine, at 95% confidence limits ($p<0.05$). The IC_{50} value achieved for galanthamine (0.33 μM) is consistent with values previously reported for erythrocyte AChE inhibition (0.35 μM, Thomsen and Kewitz, 1990; Fulton and Benfield, 1996), whereas 11α-hydroxygalanthamine showed a reduced inhibitory action on AChE (0.72 μM). In conclusion, there was no improvement in the acetylcholinesterase inhibitory activity of 11α-hydroxygalanthamine over the activity of galanthamine. It is possible that the hydroxyl group in the *pseudoaxial* position on the C-11 of 11α-hydroxygalanthamine is spatially restricted from forming any additional hydrogen bond with the bridge-oxygen between C-10 and C-1. It is also possible that with the hydroxyl group projecting downwards, the 11α-hydroxygalanthamine is restricted from attaching as effectively as galanthamine onto the surface of the acetylcholinesterase.

4.2.2. Comparison of AChE inhibition by different alkaloid types

As detailed in **Chapter 2.2.1**, the Amaryllidacaeae alkaloids are classified into nine principal skeletally homogenous subgroups, with representative alkaloids from each of these classes including norbelladine (**3**), lycorine (**4**), homolycorine (**5**), crinine (**6**), haemanthamine (**7**), tazettine (**8**), narciclasine (**9**), montanine (**10**), and galanthamine (**11**). It was interesting to compare the AChE inhibitory activity of some of the different alkaloid types from the *Cyrtanthus* species, to determine the specificity of alkaloid structure for acetylcholinesterase inhibition. The alkaloids were prepared as stock solutions of 10^{-5} M, and the AChE inhibition assay performed in a 96-well microtitre plate as previously described.

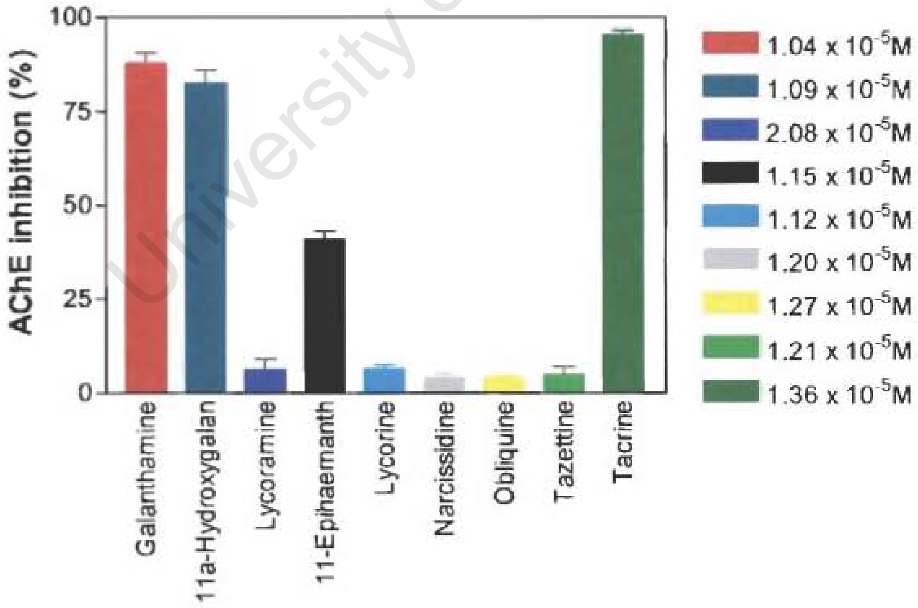


Figure 4.2.2.1. Comparison of acetylcholinesterase inhibition by some different *Cyrtanthus* alkaloid structural types, and tacrine (n=3).
[11α-hydroxygalan = 11α-hydroxygalanthamine; 11-epihaemanth = 11-epihaemanthamine]

The most potent AChE inhibition was achieved with the galanthamine type alkaloids, whilst the lycorine and tazettine types did not show much inhibition. Interestingly, lycoramine (1,2-dihydrogalanthamine) did not show much inhibition of AChE at a concentration of 10^{-5} M. There have been earlier reports of lycoramine producing a reduced inhibitory effect on AChE *in vitro* (Han et al, 1992). Lycoramine differs from galanthamine only by having saturation of the double bond between the C-4 and C-4a of the cyclohexene ring of galanthamine. When galanthamine binds to AChE, this double bond stacks against the π system of the indole ring of Trp-84 (Greenblatt et al., 1999), thus enhancing the binding affinity of the inhibitor.

Moderate inhibition (40%) was seen with 11-epihaemanthamine; however this activity is very short acting, and the inhibition is overcome within 5 minutes of substrate addition, whereas the galanthamine types exert AChE inhibition for up to 75 minutes after substrate addition (**Figure 4.2.1.1**; results not shown for 11-epihaemanthamine). It is possible that 11-epihaemanthamine is capable of competing with the substrate molecules and forming some transient hydrogen bonds with the hydrogen containing groups in the surface of the AChE active site, but that the inhibition produced is quickly overcome. It is noteworthy that lycorine, did not inhibit acetylcholinesterase. As discussed in **Chapter 3.4**, lycorine shows cholinergic action, and affects nerve-muscle transmission (Matsui et al., 1962). However, since lycorine does not inhibit acetylcholinesterase, this suggests that this enzyme may not be essential in the cholinergic effect.

4.3. Derivatization of 11α-Hydroxygalanthamine (27)

Systematic derivatization of galanthamine (Han et al., 1992) at the cyclohexene ring, tertiary amino, hydroxyl and methoxyl functions indicated that these structural features are essential for inhibition of acetylcholinesterase. The most potent galanthamine analogues, in descending order, were found to be *O*-demethylgalanthamine, *N*-benzylgalanthaminium bromide and *N*-methylgalanthaminium iodide respectively. These compounds showed reduced IC_{50} values (concentration required to inhibit 50% of acetylcholinesterase) when compared with galanthamine. In an effort to enhance the AChE inhibitory activity of 11α-hydroxygalanthamine, the derivatization of 11α-hydroxygalanthamine to *N*-Benzyl-11α-hydroxygalanthaminium bromide (**35**) and *N*-methyl-11α-hydroxygalanthaminium iodide (**36**) was attempted, based on the methodology described by Han et al., 1992. Both derivatization attempts, however, did not prove successful. A possible explanation for this failure is that with the hydroxyl group on C-11 of 11α-hydroxygalanthamine in the *pseudoaxial* position, there is steric hindrance that prevents the approaching reagent from reaching the non-bonding electrons on the nitrogen atom.

4.4. The Future of Alzheimer Disease Therapy

The acetylcholinesterase inhibitors are currently the only drugs marketed for the symptomatic treatment of cognition and behaviour in mild to moderate dementia. A number of other agents are reputed to enhance cognitive function, including anti-inflammatory compounds, antioxidants, oestrogens (in women), metabolic enhancers and neurotrophic agents. The antioxidants, such as vitamin E, bind to free radicals in the blood stream, and reduce the oxidative process, which may otherwise seriously damage cells.

The use of traditional medicinal plants in dementia therapy has been documented in many cultures of the world. One example is *Ginkgo biloba*, which is a tree of Chinese origin, now growing in many countries with temperate climates. The herb is perceived to have anti-aging properties, and is today widely sold as a herbal remedy in health food shops. Clinical trial data from a study by Le Bars et al. (1997) provided evidence for the beneficial effects of this herb in delaying or preventing the symptoms of neurodegeneration. The active substances are ginkgolides that interact with the cholinergic system, and have neuroprotective or regenerative activities (Bruno et al., 1993). In addition, the flavonoids present in *Ginkgo biloba* have an antioxidant effect.

There is widespread use of plant products by medical herbalists and aromatherapists in Europe to treat memory loss, and related conditions. Several plants from the Asteraceous and Lamiaceous families have long histories of use for restoring lost or declining cognitive function, most notably *Rosmarinus officinalis* (rosemary), *Salvia officinalis* (sage), *Artemisia absinthium* (wormwood) and *Melissa officinalis* (balm). Investigations were carried out to evaluate the human cholinergic receptor binding activity of extracts of these medicinal plants (Wake et al., 2000). Most of the plant extracts screened showed some nicotinic and muscarinic activity, although only some showed the dose-dependent receptor activity associated with genuine cholinergic activity. Further testing continues on extracts of these plants for other activities linked to improvement of cognitive function, including reduction of inflammation, oestrogenic activity and anti-oxidant effects, and some of the results are promising (Houghton, 2000). The oil of *Salvia* species showed cholinesterase inhibition, which has been attributed to some monoterpenes present in the oil, notably 1,8 cineole, camphor and α-pinene. *Melissa officinalis* oil did not produce significant cholinesterase inhibition, but did bind to oestrogen receptors, and also inhibited thromboxane synthesis, and monoterpenes were again shown to be responsible for this activity, in particular citral, geraniol and nerol. Several of the plant extracts had more than one effect suggesting that any clinical benefits observed might be due to a mixture of activities (Houghton, 2000). However, further *in vivo* testing and clinical studies are needed to substantiate claims of efficacy of these plant extracts.

4.5. Concluding Comments

In summary, extensive testing of 11α-hydroxygalanthamine (**27**) for *in vitro* inhibition of acetylcholinesterase showed that there is no increase in activity over that of galanthamine. Galanthamine is today readily available as it can be synthesised on a large scale (Czollner et al., 1998), so the use of derivatives such as 11α-hydroxygalanthamine as alternative sources of galanthamine may no longer be substantiated. A noteworthy point, however, is the high content of 11α-hydroxygalanthamine (**27**) in *Cyrtanthus sanguineus*, and the fact that the alkaloid is present in both species of *Cyrtanthus* examined in this investigation. When considering that the polarity of 11α-hydroxygalanthamine is probably higher than that of galanthamine due to the additional hydroxyl group, it can be predicted that 11α-hydroxygalanthamine is likely to be less effective than galanthamine at crossing the blood-brain barrier to reach the site of action in the brain. As discussed in **Section 4.1.2.4.5**, 6-*O*-demethylgalanthamine is a metabolite of galanthamine formed by cytochrome CYP2D6, and has been reported to be up to 10-fold more potent an inhibitor than galanthamine *in vitro* (Bores, 1996). The metabolism of 11α-hydroxygalanthamine might follow a similar pathway to that of galanthamine. However, it would be necessary to make use of animal models to investigate the efficacy of 11α-hydroxygalanthamine *in vivo*, and to ascertain the metabolite(s) of the alkaloid and the AChE inhibitory activity of such compound(s).

Assuming that 11α-hydroxygalanthamine, like galanthamine, enhances the intrinsic action of acetylcholine on nicotinic receptors, there is the possible application of 11α-hydroxygalanthamine to the treatment, or perhaps even prevention, of conditions requiring modulation of the nicotinic receptors, such as in the treatment of Parkinson's disease and as an aid to people trying to stop smoking cigarettes and other tobacco products. The modulating effect of 11α-hydroxygalanthamine on the nicotinic receptors *in vivo* would need to be investigated to substantiate the use of this alkaloid for such applications.

Chapter 5

Materials and Methods

5.1. Isolation and identification of *Cyrtanthus* alkaloids

5.1.1. General

Melting points (m.p.) were measured on a Gallenkamp (Sanyo) 350BM3.5 apparatus and are uncorrected. Optical rotations (OR) were taken on a Perkin-Elmer 241 Polarimeter. CD: Jasco J-700 Spectropolarimeter. EIMS were run on a Hewlett Packard 5989A Mass Spectrometer at 70 eV. ^{1}H, ^{13}C NMR, DEPT, COSY, HSQC, HMBC (60 and 110 ms) and ROESY (300 ms) spectra were recorded on a Varian VXR 500 (500MHz) or Varian VXR 400 in either $CDCl_3$ or CD_3OD, with TMS as internal standard. Some ^{1}H and ^{13}C NMR spectra were recorded on 400, 300 and 200 MHz Varian instruments. Chemical shifts are reported in units of δ (ppm) and coupling constants (J) are expressed in Hz.

5.1.2. Plant material

Mr Welland Cowley, horticulturist and owner of Cape Flora Nurseries, Port Elizabeth, South Africa, authenticated and supplied the bulbs of *Cyrtanthus sanguineus* (0.8 kg) and *Cyrtanthus obliquus* (1.1 kg).

5.1.3. Extraction and isolation of alkaloids

The two species of *Cyrtanthus* were extracted separately as follows. The fresh bulbs were finely chopped and exhaustively extracted by vigorous shaking with cold MeOH (5 x 2 litres) for 24 h, followed by Soxhlet extraction (2 litres) for 12 h. The hot and cold crude extracts were pooled and evaporated under reduced pressure. The residues were then dissolved in distilled water with sonication, and acidified with 10% hydrochloric acid to pH 3. The neutral material was removed by partitioning with diethyl ether (6 x 50 ml), to yield acidic extracts (1.23 g for *Cyrtanthus sanguineus* and 2.27 g for *Cyrtanthus obliquus* respectively). The remaining solutions were then basified with 10% ammonium solution to pH 8-9 and repartitioned with ethyl acetate (15 x 50 ml) and finally, with ethyl acetate: methanol (9:1) (3 x 50 ml). These basic extracts were combined and dried *in vacuo* to yield brown gummy residues (0.78 g for *Cyrtanthus sanguineus* and 1.56 g for *Cyrtanthus obliquus* respectively). The acidic and basic extracts of both plant species were analysed by thin layer chromatography (silica gel F_{254}; Merck) eluting with 10% methanol: ethyl acetate in an ammonia atmosphere Viewing the chromatogram spots under UV light (254/365 nm) and spraying with Dragendorff's reagent confirmed the presence of alkaloids in the basic extracts.

One bulb of each species was extracted separately in water, by shaking the chopped bulbs in cold water for 24 h, in an attempt to imitate the traditional method of preparation. This extract was concentrated by freeze-drying, and then analysed by thin layer chromatography as described previously. Faint staining with Dragendorff's reagent indicated the presence of alkaloids in very low concentrations.

5.1.3.1 Alkaloids from *Cyrtanthus sanguineus*

The basic extract was chromatographed by VLC (10 x 4.5 cm) on silica gel (10-40 μ, type H; Sigma), eluting with hexane, increasing the polarity with ethyl acetate and later up to ethyl acetate: methanol, at 200 ml per fraction. Full details of the VLC chromatography on *Cyrtanthus sanguineus* are given in **Table 5.1.3.1.1**. The fractions were then combined to give 4 main fractions containing alkaloids, on the basis of similar TLC profiles. Viewing the chromatogram spots under UV light (254/365 nm) and spraying with Dragendorff's reagent confirmed the presence of alkaloids in the fractions.

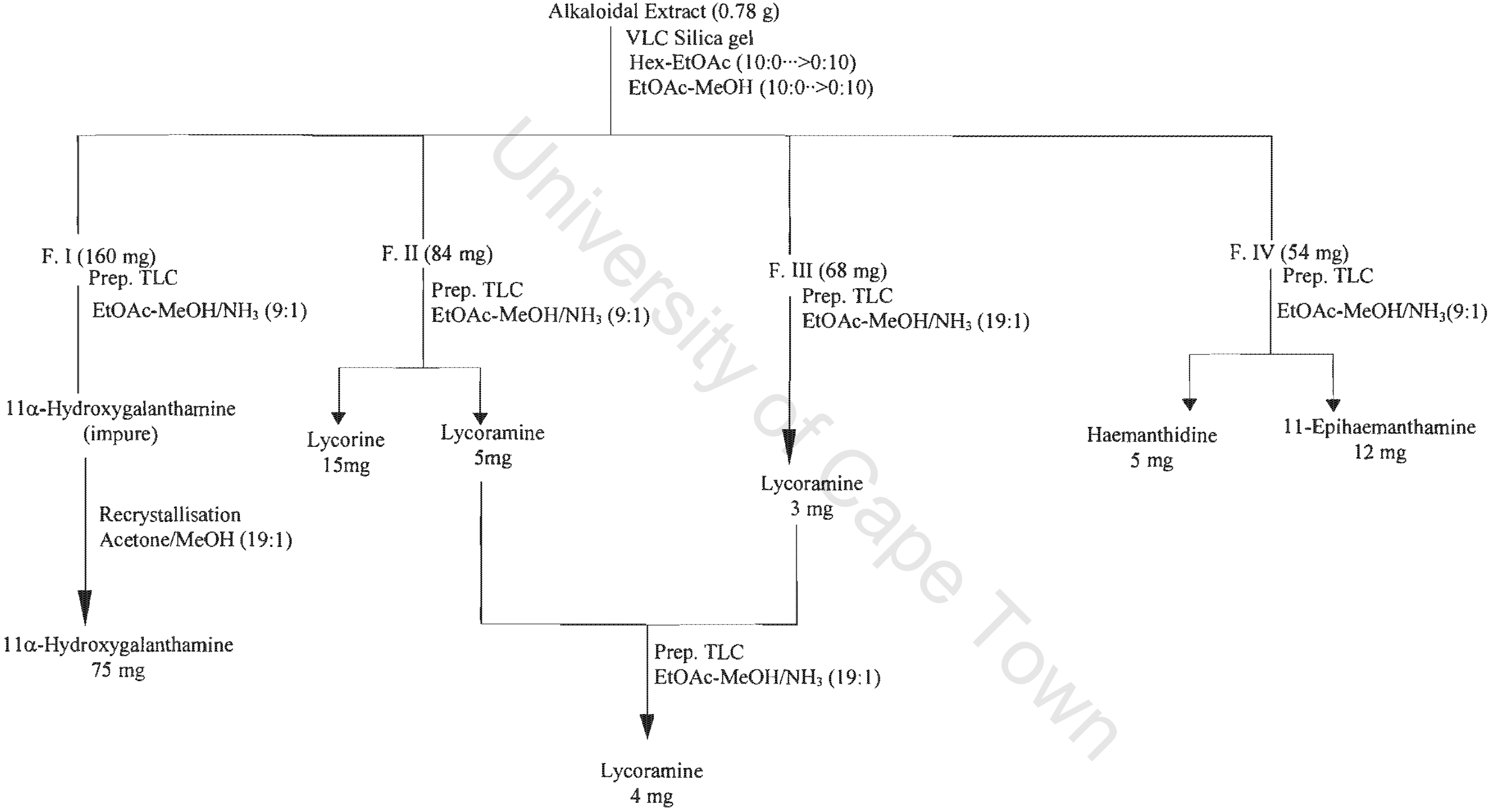
Fraction Alk1 (160 mg) was purified by preparative TLC using MeOH: EtOAc (1:9) in an NH_3 atmosphere, to yield 11α-hydroxygalanthamine (**27**, 75 mg) which crystallized from acetone: MeOH (19:1). Fraction Alk2 (84 mg) was separated by preparative TLC eluting with MeOH: EtOAc (1:9) in NH_3 atmosphere, to give 2 distinct alkaloid zones, which were recovered to yield lycorine (**4**, 15 mg) and lycoramine (**28**, 5 mg). Fraction Alk3 (68 mg) was subjected to preparative TLC, eluting with MeOH: EtOAc (1:19) in NH_3 atmosphere, and afforded more impure lycoramine (3 mg). This lycoramine was combined with that from fraction 2 and then further purified by preparative TLC with MeOH: EtOAc (1:19) in NH_3 atmosphere to yield a final 6 mg. Fraction Alk4 (54 mg) was purified using preparative TLC, eluting with MeOH: EtOAc (1:9) in NH_3 atmosphere to yield 11-epihaemanthamine (**29**, 12 mg) and the epimers of haemanthidine (**13**, 5 mg). The epimers were re-run on preparative TLC, eluting with EtOAc: MeOH (1:1) to obtain a purer product (3 mg). **Scheme 5.1.3.1** illustrates the isolation and purification of alkaloids from *Cyrtanthus sanguineus*.

Table 5.1.3.1.1. (a) VLC chromatography on *Cyrtanthus sanguineus*

VLC solvent system			**No. of fractions**	**TLC solvent system**	**Alkaloids present**	**R_f values**	**Combined fractions**
Hexane	**EtOAc**	**MeOH**					
100	0	0	10	Hex: EtOAc (1:1)	X		
90	10	0	10	Hex: EtOAc (1:1)	X		
80	20	0	10	Hex: EtOAc (1:1)	X		
70	30	0	20	Hex: EtOAc (1:1)	X		
60	40	0	20	Hex: EtOAc (1:4)	X		
50	50	0	20	Hex: EtOAc (1:4)	X		
40	60	0	20	EtOAc (100%)	X		
30	70	0	10	EtOAc: MeOH(9:1)	X		
20	80	0	10	EtOAc: MeOH(9:1)	X		
10	90	0	10	EtOAc: MeOH(9:1)	X		
0	100	0	90	EtOAc: MeOH(1:1)	✓	0.39	Alk 1
0	95	5	30	EtOAc: MeOH(1:1)	✓	0.39	Alk 1
0	90	10	20	EtOAc: MeOH(1:1)	✓	0.39	Alk 1
0	85	15	60	EtOAc: MeOH(1:1)	✓	0.35, 0.24	Alk 2
0	80	20	50	EtOAc: MeOH(1:1)	✓	0.35, 0.24	Alk 2
0	75	25	40	EtOAc: MeOH(1:1)	✓	0.35, 0.24	Alk 2

Table 5.1.3.1.1. (b) VLC chromatography on *Cyrtanthus sanguineus*

VLC solvent system			No. of fractions	TLC solvent system	Alkaloids present	R_f values	Combined fractions
Hexane	EtOAc	MeOH					
0	70	30	50	EtOAc: MeOH(1:1)	✓	0.22	Alk 3
0	60	40	30	EtOAc: MeOH(1:1)	✓	0.22	Alk 3
0	50	50	30	EtOAc: MeOH(1:9)	✓	0.16, 0.71	Alk 4
0	30	70	20	EtOAc: MeOH(1:9)	✓	0.16, 0.71	Alk 4
0	20	80	20	100% MeOH	✓	0.16, 0.71	Alk 4
0	10	90	20	100% MeOH	X		
0	0	100	20	100% MeOH	X		



Scheme 5.1.3.1. Fractionation of alkaloids from *Cyrtanthus sanguineus*

5.1.3.2. Alkaloids from *Cyrtanthus obliquus*

The basic extract was chromatographed by VLC (10 x 5 cm) on silica gel eluting with hexane, increasing the polarity with ethyl acetate and later up to ethyl acetate: methanol, at 200 ml per fraction. Full details of the VLC chromatography on *Cyrtanthus obliquus* are given in **Table 5.1.3.1.2**. Fractions were combined to give 6 main fractions containing alkaloids, on the basis of similar TLC profiles. Viewing the chromatogram spots under UV light (254/365 nm) and spraying with Dragendorff's reagent confirmed the presence of alkaloids in the fractions.

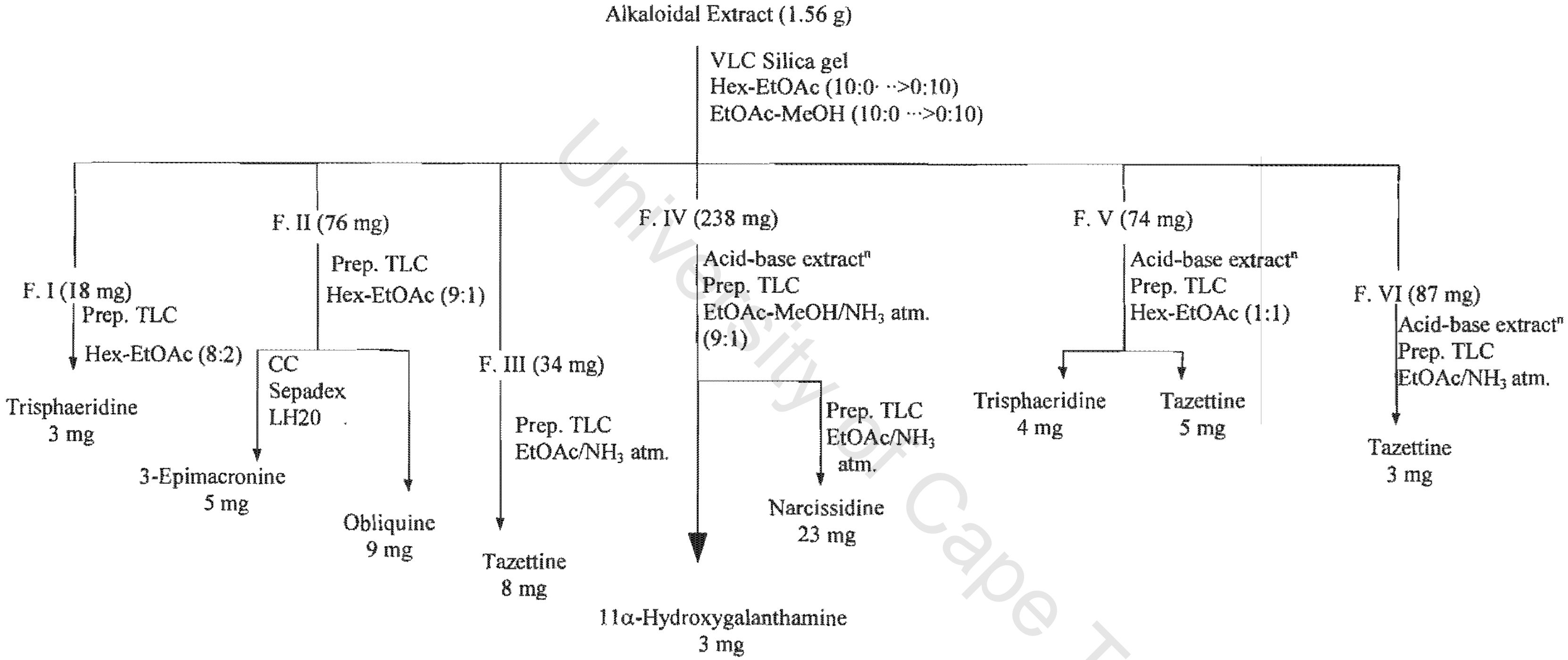
Fraction Alk1 (18 mg) was purified by preparative TLC, eluting with Hexane: EtOAc (8:2) to yield trisphaeridine (**19**, 3 mg). Fraction Alk2 (76 mg) was further fractionated by preparative TLC with Hexane: EtOAc (1:9) to give 2 further fractions. Fraction Alk2.1 yielded the novel alkaloid obliquine (**30**, 9 mg) Fraction Alk2.2 was cleaned up by column chromatography on Sephadex LH 20, and yielded 3-epimacronine (**31**, 6 mg). Fraction Alk3 (34 mg) was subjected to prep. TLC, eluting with 100% EtOAc in NH_3 atmosphere, and yielded brown crystals of tazettine (**8**, 8 mg). Fraction Alk4 (238 mg) was subjected to another round of acid-base extraction to yield a basic alkaloidal extract, which was purified by prep. TLC, in EtOAc: MeOH (1:1), resulting in 2 main alkaloid regions. Fraction Alk4.1 was purified by further prep. TLC in 100% EtOAc in NH_3 atmosphere, yielding narcissidine (**32**, 23 mg). Fraction Alk4.2 was identified as 11α-hydroxygalanthamine (**27**, 4 mg). Fraction Alk5 was cleaned up by a second acid-base extraction, yielding a basic alkaloidal extract, which was then fractionated by prep. TLC Hex: EtOAc (1:1), and yielded more trisphaeridine (**19**, 4 mg) and more tazettine (**8**, 5 mg). Fraction Alk6 was subjected to further acid-base extraction, and then purified by preparative TLC with 100% EtOAc in NH_3 atmosphere, resulting in the isolation of more tazettine (**8**, 3 mg). **Scheme 5.1.3.2** represents the isolation of alkaloids from *Cyrtanthus obliquus*.

Table 5.1.3.1.2. (a) VLC chromatography on *Cyrtanthus obliquus*

VLC solvent system			No. of fractions	TLC solvent system	Alkaloids present	R_f values	Combined fractions
Hexane	EtOAc	MeOH					
100	0	0	10	Hex: EtOAc (1:1)	X		
90	10	0	10	Hex: EtOAc (1:1)	X		
80	20	0	20	Hex: EtOAc (1:1)	✓	0.48	Alk 1
70	30	0	10	Hex: EtOAc (1:4)	✓	0.45, 0.33	Alk 2
60	40	0	10	Hex: EtOAc (1:4)	✓	0.45, 0.33	Alk 2
50	50	0	10	Hex: EtOAc (1:4)	✓	0.45, 0.33	Alk 2
40	60	0	20	EtOAc (100%)	✓	0.50	Alk 3
30	70	0	10	EtOAc: MeOH(9:1)	✓	0.53, 0.42	Alk 4
20	80	0	10	EtOAc: MeOH(9:1)	✓	0.53, 0.42	Alk 4
10	90	0	10	EtOAc: MeOH(9:1)	✓	0.53, 0.42	Alk 4
0	100	0	20	EtOAc: MeOH(9:1)	✓	0.53, 0.42	Alk 4
0	95	5	30	EtOAc: MeOH(4:1)	X		
0	90	10	20	EtOAc: MeOH(1:1)	X		
0	85	15	60	EtOAc: MeOH(1:1)	✓	0.47, 0.11	Alk 5

Table 5.1.3.1.2. (b) VLC chromatography on *Cyrtanthus obliquus*

VLC solvent system			**No. of fractions**	**TLC solvent system**	**Alkaloids present**	**R_f values**	**Combined fractions**
Hexane	**EtOAc**	**MeOH**					
0	80	20	20	EtOAc: MeOH(1:1)	X		
0	70	30	20	EtOAc: MeOH(1:4)	✓	0.56	Alk 6
0	60	40	50	EtOAc: MeOH(1:4)	✓	0.56	Alk 6
0	50	50	30	EtOAc: MeOH(1:4)	✓	0.56	Alk 6
0	40	60	30	EtOAc: MeOH(1:4)	X		
0	30	70	10	EtOAc: MeOH(1:9)	X		
0	20	80	10	100% MeOH	X		
0	10	90	10	100% MeOH	X		
0	0	100	10	100% MeOH	X		



Scheme 5.1.3.2. Fractionation of alkaloids from *Cyrtanthus obliquus*

5.2. Cell culture and cytotoxicity assay

Sylva Schwager of the Department of Medical Biochemistry, University of Cape Town donated the starting stock of the Chinese Hamster Ovarian (CHO) cell line, and Lester Davids of the Department of Medicine, University of Cape Town donated the starting stock of human heptoma (HepG2) cell line. The cells were then routinely cultured as adherent monolayers in 75 cm^2 culture flasks. Dulbeccos Eagles' Modified Medium (DMEM): Hams F-12 (1:1) supplemented with 10% heat-inactivated foetal calf serum (FCS), and gentamycin (0.04 μg/ml) was used as the culture medium for the CHO cells, and Dulbecco's MEM with Glutamax-1 (L-Alanyl-L-Glutamine) supplemented with 10% heat-inactivated FCS was used as the culture medium for the HepG2 cells. The culture medium reagents were all purchased from Highveld Biological, Johannesburg, South Africa, with the exception of the Dulbecco's MEM with Glutamax-1, which was purchased from Gibco (Life Technologies), Paisley, Scotland. The cells were incubated in a 5% $C0_2$ – 95% air humidified atmosphere at 37 °C. The culture medium was renewed every 2-3 days to remove cell debris and maintain an optimum pH of approximately 7.2. When the cells reached confluency, they were harvested from the culture flasks by the addition of 1% trpysin, then diluted in complete medium and subcultured into separate flasks. Stocks of the cell line were stored in 10% DMSO- 90% FCS in liquid nitrogen. The cells were allowed to undergo a maximum of ten subcultures, before fresh stocks were defrosted and the cell culture re-initiated.

For the MTT assay, the cells were adjusted to a concentration of 10^5/ml, and 100 μl/ well of cell suspension was seeded in 96 well culture plates (Costar) and incubated overnight at 37 °C in a 5% $C0_2$ – 95% air humidified atmosphere. Stock solutions of the alkaloids and the internal standard, Daunomycin (Daunorubicin) (Sigma, St Louis, USA) were prepared as 2 mg/ml, and serially diluted in culture medium in order to give a final test concentration range of 100 μg/ml to 1 ng/ml in the wells. After 48 hours of incubation at 37 °C, 25 μl of MTT (5 mg/ml, prepared in PBS and then filter-sterilised) was added to each well, and the microtitre plates incubated for a further 4 hours. The plates were then centrifuged at 2050 rpm for 10 minutes, and the MTT-medium supernatants carefully aspirated from the wells. Subsequently, 100 μl of DMSO was added to each well and the plates shaken gently for 5 minutes on a microtitre plate shaker to dissolve the formazan crystals. The absorbancies were determined at 540 nm on an automatic microtitre plate reader (Cambridge Technology). The MTT determination was performed in quadruplicate for each concentration of the different alkaloids, and the experiments performed on three separate occasions. Concentrations ≥ 100 µg/ml were not considered as toxic as this value corresponds to molar concentrations > 10^{-2}, which would be out of the pharmacological efficiency scale.

5.3. Acetylcholinesterase inhibition assay

The erythrocyte membranes were prepared according to the method of Heidrich and Leutner (1974). A volume of 20ml of red blood cells was washed 3 times, with an equal volume of ice-cold 5 mM sodium phospate/0.15 M sodium chloride (pH 8.0), and the cells were centrifuged at 20 000g for 15 minutes at 4°C in ice-cold 5 mM sodium phosphate (pH 8.0), until the erythrocyte membranes were white in appearance. The erythrocyte membranes were stored in aliquots of 1 ml in Eppendorff vials at -80°C.

It was necessary to determine the ideal quantity of erythrocyte membranes that was needed in the assay in order to ensure that the enzymatic reaction was in exponential phase at a feasible time point after addition of substrate to take sufficient readings. Aliquots of 10, 20, 30 and 40µl of erythrocyte membranes were transferred to a microtitre plate, and a volume of phosphate buffered saline (PBS) added to achieve a final volume of 100 µl in each well of the microtitre plate. The first column of the plate served as the blank, and consisted of 100 µl PBS only. The substrate was prepared fresh as 0.1 M *di*-sodium hydrogen phosphate / 0.5 mM 2,2'-dinitro-5,5'dithiobenzoic acid / 0.6 mM S-acetylthiocholine iodide (all Merck Laboratory Supplies, Johannesburg, South Africa), pH 7.5, and 100 µl was added to each well of the plate. Absorbancies were then read at λ = 405 nm in a microtitre plate reader (Cambridge Technology) over a time course of 1, 2, 5, 10, 15, 20, 25, 30, 45 and 60 minutes after addition of substrate. Based on the results of these experiments (**Figure 5.3.1.**), the decision was made to use an aliquot of 10 µl of erythrocyte membranes per well in the inhibition assays, and to take the average of readings at 2 minutes and 5 minutes after the addition of substrate.

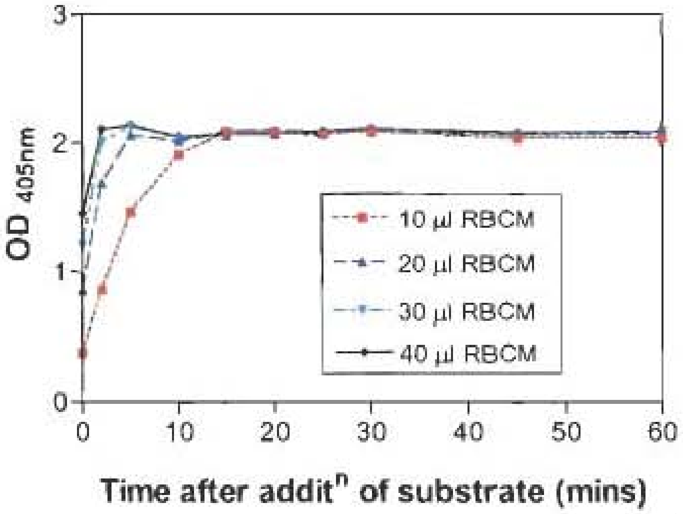


Figure 5.3.1. Acetylcholinesterase activity as a function of quantity of erythrocyte membranes and time after addition of substrate (n=2).

The amount of protein in the aliquots of erythrocyte membranes was determined by the BioRad™ assay using Bovine Serum Albumin (BSA) from Serovac (South Africa), and was found to be 0.981 µg/ml, i.e. 9.81 ng in the 10 µl aliquot.

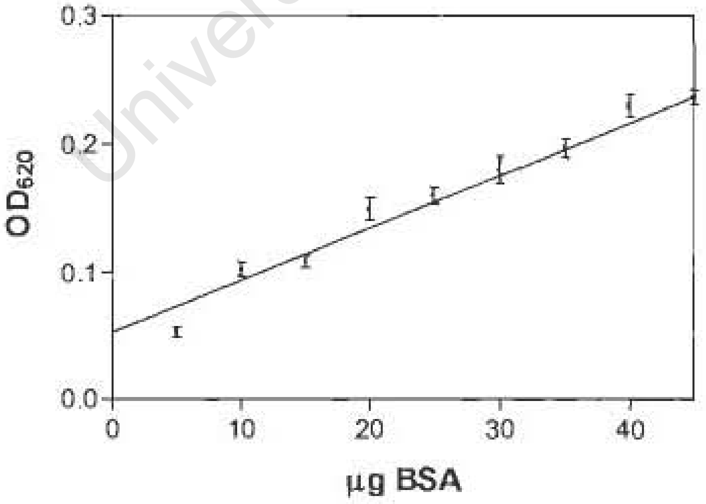


Figure 5.3.2. Biorad™ Protein Determination Assay Standard Curve ($n=3$, $r^2=0.9611$, at 95% confidence intervals)

Assuming the reaction follows first order enzyme kinetics, a maximum absorbance of 2.053 ± 0.0391 (n=3) was correlated with 0.124 μmoles of thiocholine product. Subsequent optical densities were converted to nmoles of thiocholine formed per minute.

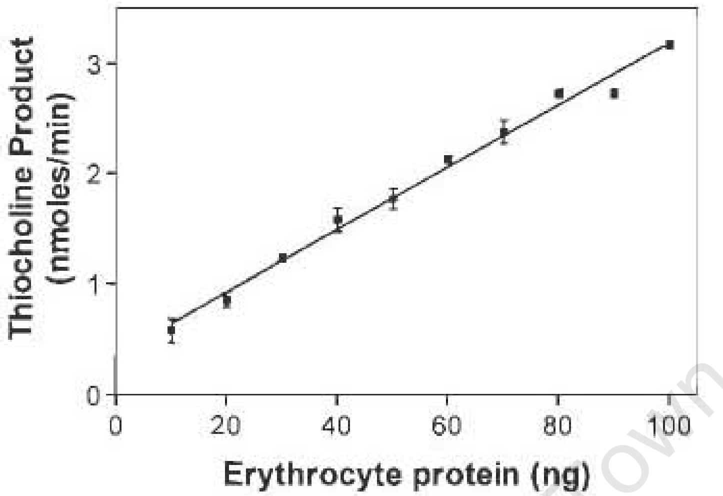


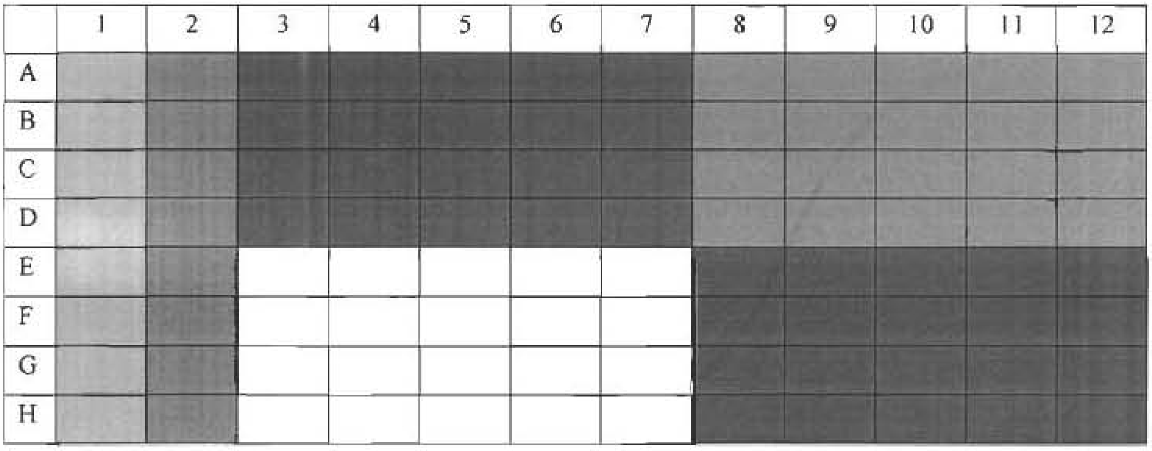
Figure 5.3.3. Plot of thiocholine production against amount of erythrocyte protein

Taking a standard reading at 5 minutes (OD_{405} = 1.438 ± 0.0103), which correlates to 0.0868 μmoles thiocholine product, the acetycholinesterase activity can be calculated as:

86.8 nmoles / 5 min / 9.8 ng protein = 1.77 nmoles / min / ng protein

For the acetycholinesterase inhibition assays, the alkaloids and the positive control, tacrine (Sigma, St Louis, USA) were dissolved in 100 μl of methanol and then serially diluted in PBS to the correct molarities. The compounds were initially screened for inhibition of erythrocyte AChE at 10^{-5} M, and thereafter, full dose response curves were obtained for a concentration range between 10^{-5} M and 10^{-9} M. The compounds and erythrocyte membranes were incubated at room temperature for 30 minutes prior to the addition of the freshly prepared substrate. The absorbencies at λ= 405nm were then determined using a microtitre plate reader (Cambridge Technology) at regular time intervals. The percentage AChE inhibition was calculated for each of the compounds in comparison to an internal methanol-phosphate buffer control. Experiments were performed in quadruplicate, and repeated at least 6 times.

The 96-well microtitre plate was set up as follows for the assay:



	1	2	3	4	5	6	7	8	9	10	11	12
A	░	▒	▓	▓	▓	▓	▓	▨	▨	▨	▨	▨
B	░	▒	▓	▓	▓	▓	▓	▨	▨	▨	▨	▨
C	░	▒	▓	▓	▓	▓	▓	▨	▨	▨	▨	▨
D	░	▒	▓	▓	▓	▓	▓	▨	▨	▨	▨	▨
E	░	▒	□	□	□	□	□	█	█	█	█	█
F	░	▒	□	□	□	□	□	█	█	█	█	█
G	░	▒	□	□	□	□	□	█	█	█	█	█
H	░	▒	□	□	□	□	□	█	█	█	█	█

Key:

░	Blank: 100 µl PBS only
▒	Control: 0.98 µg RBCM + 90 µl PBS/MeOH only
▓	Alk 1: 0.98 µg RBCM + 40 µl PBS + 50 µl Alk 1 (10^{-5} to 10^{-9} in Column 3-7)
▨	Alk 2: 0.98 µg RBCM + 40 µl PBS + 50 µl Alk 2 (10^{-5} to 10^{-9} in Column 8-12)
□	Alk 3: 0.98 µg RBCM + 40 µl PBS + 50 µl Alk 3 (10^{-5} to 10^{-9} in Column 3-7)
█	Standard: 0.98 µg RBCM + 40 µl PBS + 50 µl Tacrine (10^{-5} to 10^{-9} in Column 8-12)

Figure 5.3.4. Representation of microtitre plate for AChE assay

[RBCM: red blood cell membranes]

5.4. Derivatization of 11α-Hydroxygalanthamine

5.4.1. *N*-Benzyl-11α-Hydroxygalanthaminium bromide (35)

A solution of 11α-hydroxygalanthamine (25.0 mg, 0.0825 mmol in 20 ml dry ether) was prepared at room temperature, and placed in a sealed 100 ml round-bottomed flask, under an argon atmosphere. After 10 minutes, 0.02 ml (0.14 mmol) benzyl bromide (Merck) was added to the solution via a needle and the reaction mixture was stirred for 24 h. After this time, the mixture was filtered under vacuum, and the precipitate collected, dried and a ^{1}H NMR spectrum was run. This revealed that the derivatization was not successful, and that the 11α-hydroxygalanthamine remained unconverted. A second attempt using tetrahydrofuran (Merck) in place of the dry ether in order to dissolve the 11α-hydroxygalanthamine more effectively also proved unsuccessful.

5.4.2. *N*-Methyl-11α-Hydroxygalanthaminium iodide (36)

11α-Hydroxygalanthamine (20.0 mg, 0.066 mmol) was dissolved in 25 ml of tetrahydrofuran, and stirred in a 100 ml round bottomed flask, under an argon atmosphere, for 10 minutes at room temperature. Iodomethane (Merck) (0.10 ml, 1.62 mmol) was added to the solution, and the reaction mixture stirred for 12 h under an argon atmosphere. The resulting precipitate was removed by filtration under vacuum, and retained. The filtrate was dried and then the ^{1}H NMR spectra of both the precipitate and the dried filtrate again revealed that the derivatization had not been successful, and unconverted 11α-hydroxygalanthamine remained.

Chapter 6

Summary and Conclusions

6.1. Research Summary

The Amaryllidaceae family of plants are widely used in the traditional medicine of many people of the Southern African region. As a result, these plants have recently become the focus of pharmacological investigations into their medicinal and toxicological properties. Many of the plants have some promising pharmacological properties, such as antiviral, anticancer and antiplasmodial activity. However, many of the plants are also potentially toxic, and this toxicity, as well as the pharmacological activities, has been attributed to the isoquinoline alkaloids, which are found exclusively in this family of plants. Possibly the most interesting activity is the acetylcholinesterase inhibitory activity of galanthamine which has been developed as a symptomatic treatment for Alzheimer's disease. In the context of this interest in the potential medicinal application, as well as concern over the toxicity of these plants, two species of the Amaryllidaceae family were examined for their alkaloid content. The *Cyrtanthus sanguineus* and *Cyrtanthus obliquus* species were selected after an extensive literature search for Amaryllidaceae species that are commonly used in traditional medicine and that have not yet been examined for their alkaloid content.

The alkaloids 11α-hydroxygalanthamine, lycorine, lyoramine, 11-epihaemanthamine and the epimers of haemanthidine, were identified from *Cyrtanthus sanguineus*, and more 11α-hydroxygalanthamine, narcissidine, 3-epimacronine, tazettine, trisphaeridine, and the novel dinitrogenous alkaloid, denoted as obliquine, were isolated from *Cyrtanthus obliquus*. The isolation of 11-hydroxygalanthamine (habranthine) was reported by Wildman and Brown in 1968, and by Vazquez Tato et al., in 1988. However neither of these research teams provided conclusive evidence for the stereochemistry at C-11. In this investigation, complete assignment of ^{1}H and ^{13}C NMR data was made possible by 2-D NMR experiments, which also provided evidence that the hydroxyl group is in the *pseudoaxial* position on C-11 of the alkaloid. A noteworthy point is the high content of 11α-hydroxygalanthamine (**27**) in *Cyrtanthus sanguineus* (75 mg alkaloid from 0.8 kg of bulbs), and the fact that the alkaloid is present in both species of *Cyrtanthus* examined in this investigation.

Recently, the X-ray crystal structure of galanthamine bound in the active site of *Torpedo californica* acetylcholinesterase (*Tc*AChE) was resolved (Greenblatt et al., 1999). It would appear that the binding of galanthamine to *Tc*AChE arises from a number of moderate to weak interactions with the protein, including classical and non-classical hydrogen bonds. In light of this, it was proposed that the additional hydroxyl group on the C-11 of 11α-hydroxygalanthamine that was isolated from *Cyrtanthus sanguineus* and *Cyrtanthus obliquus*, might improve the binding affinity for AChE, by facilitating a possible extra hydrogen bond with the protein. The *in vitro* AChE inhibitory activity of 11α-hydroxygalanthamine was extensively investigated and compared to the inhibitory activity of galanthamine. 11α-Hydroxygalanthamine was shown to inhibit acetylcholinesterase in a dose-dependent, reversible, competitive manner, but the activity was slightly less than that of galanthamine (IC_{50} values 0.72 and 0.33 μM respectively. This reduction in activity was postulated to be the result of the hydroxyl group in the *pseudoaxial* position on the C-11 of 11α-hydroxygalanthamine being spatially restricted from forming any additional hydrogen bond with the bridge-oxygen between C-10 and C-1. It is also possible that with the hydroxyl group projecting downwards, the 11α-hydroxygalanthamine is restricted from attaching as effectively as galanthamine onto the surface of the acetylcholinesterase.

Wherever quantities of purified material permitted testing, the isolated alkaloids were screened for their cytotoxicity against two mammalian cell lines, namely the Chinese Hamster Ovarian (CHO) cell line and the human heptoma cell line (HepG2). Lycorine, which has been previously demonstrated to exhibit toxicity against a range of cell lines (Weniger et al., 1995) showed moderate cytotoxicity against the CHO cells (IC_{50} value 26 μg/ml). Notably, the 11α-hydroxygalanthamine did not show cytotoxicity against the CHO or HepG2 cells.

6.2. Research Prospects

This research demonstrated that the *in vitro* acetylcholinesterase inhibitory activity of 11α-hydroxygalanthamine is not greater than that of galanthamine. Before a decision could be reached as whether or not 11α-hydroxygalanthamine is worth pursuing as a possible acetylcholinesterase inhibitor, it would be necessary to use animal models to investigate the efficacy of 11α-hydroxygalanthamine *in vivo*, and to ascertain the metabolite(s) of the alkaloid and the AChE inhibitory activity of such compound(s). Considering that galanthamine is today readily available as it can be synthesised on a large scale (Czollner et al., 1998), the use of derivatives such as 11α-hydroxygalanthamine as alternative sources of galanthamine may no longer be justified, unless such derivatives were shown to be more active than galanthamine *in vivo*, or to be equally active and at the same time less toxic than galanthamine. Other possible medical applications of 11α-hydroxygalanthamine might involve the potential modulating effect on the nicotinic receptors that this alkaloid is likely to have, considering its similarity to galanthamine. Such applications might include the treatment and prevention of Parkinson's disease, and possibly aiding tobacco users trying to overcome their addiction to nicotine. The modulating effect of 11α-hydroxygalanthamine on the nicotinic receptors would need to be explored.

In terms of the phytochemical analysis of these two species of *Cyrtanthus*, an investigation using significantly larger quantities of plant material, and preferably plant material which has been harvested from the wild and has not been sourced commercially, might yield larger amounts of the alkaloids that were isolated in this study, thus facilitating further biological testing. Additional alkaloids, which may have interesting phytochemistry or biological activities, may also be isolated in this way.

Further *in vitro* cytotoxicity screening of aqueous extracts of the *Cyrtanthus* species and of the isolated alkaloids against numerous cell lines, as well as toxicology testing in animal models would enhance the limited toxicological knowledge currently available on these Amaryllidaceae plants.

6.3. Conclusions

This investigation of *Cyrtanthus sanguineus* and *Cyrtanthus obliquus* revealed that the isoquinoline alkaloids 11α-hydroxygalanthamine, 11-epihaemanthamine, haemanthidine, lycoramine and lycorine are present in *Cyrtanthus sanguineus*, and the alkaloids 11α-hydroxygalanthamine, narcissidine, 3-epimacronine, tazettine, trisphaeridine and the novel dinitrogenous alkaloid, obliquine, are found in *Cyrtanthus obliquus*.

Prior to this study, the stereochemistry at C-11 of 11-hydroxygalanthamine had not been clarified. Here it was possible to resolve from coupling constants and 2-D NMR experiments that the hydroxyl is in the *pseudoaxial*-position on C-11 and to fully assign all ^{1}H and ^{13}C resonances of 11α-hydroxygalanthamine.

The *in vitro* acetylcholinesterase inhibitory activity of 11α-hydroxygalanthamine was shown to be slightly less than that of galanthamine (IC_{50} values 0.72 μM and 0.33 μM, respectively). The IC_{50} value of galanthamine was consistent with that found in the literature. The slightly reduced inhibitory activity seen with 11α-hydroxygalanthamine might be the result of a reduction in the number of hydrogen bonds forming between the alkaloid and the surface of the acetylcholinesterase molecule.

The cytotoxicity screening results revealed that lycorine, which is present in *Cyrtanthus sanguineus*, is moderately toxic *in vitro* against the mammalian cell line CHO (IC_{50} value 26 μg/ml). Further evidence from the literature on the toxicity of this alkaloid, and that of lycoramine and tazettine, suggests that these plants should be used as traditional medicines only with extreme caution, and at low doses. The cytotoxicity screening also showed that 11α-hydroxygalanthamine is not toxic to the mammalian cell lines used in this study.

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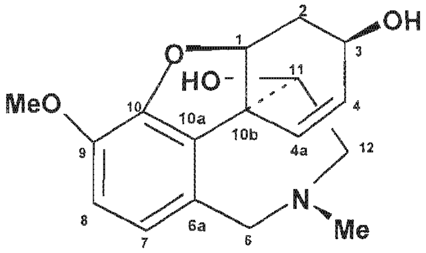
Appendix 1

NMR and Mass Spectroscopy Results

A1.1. 11α-Hydroxygalanthamine (27)



11α-Hydroxygalanthamine (27)

List of spectra:

- ^{1}H NMR (CD$_3$OD, 500MHz)
- ^{1}H NMR spectra expansions (CD$_3$OD, 400MHz)
- ^{1}H NMR (CDCl$_3$, 500MHz)
- ^{13}C NMR (CDCl$_3$, 200MHz)
- ^{13}C NMR DEPT (CDCl$_3$, 200MHz)
- COSY (CDCl$_3$, 500MHz; CD$_3$OD, 400MHz)
- NOESY (CD$_3$OD, 500MHz)
- ROESY (CDCl$_3$, 500MHz)
- HSQC (CDCl$_3$, 500MHz)
- HMBC (CDCl$_3$, 500MHz; CD$_3$OD, 400MHz)
- EIMS
- HREIMS

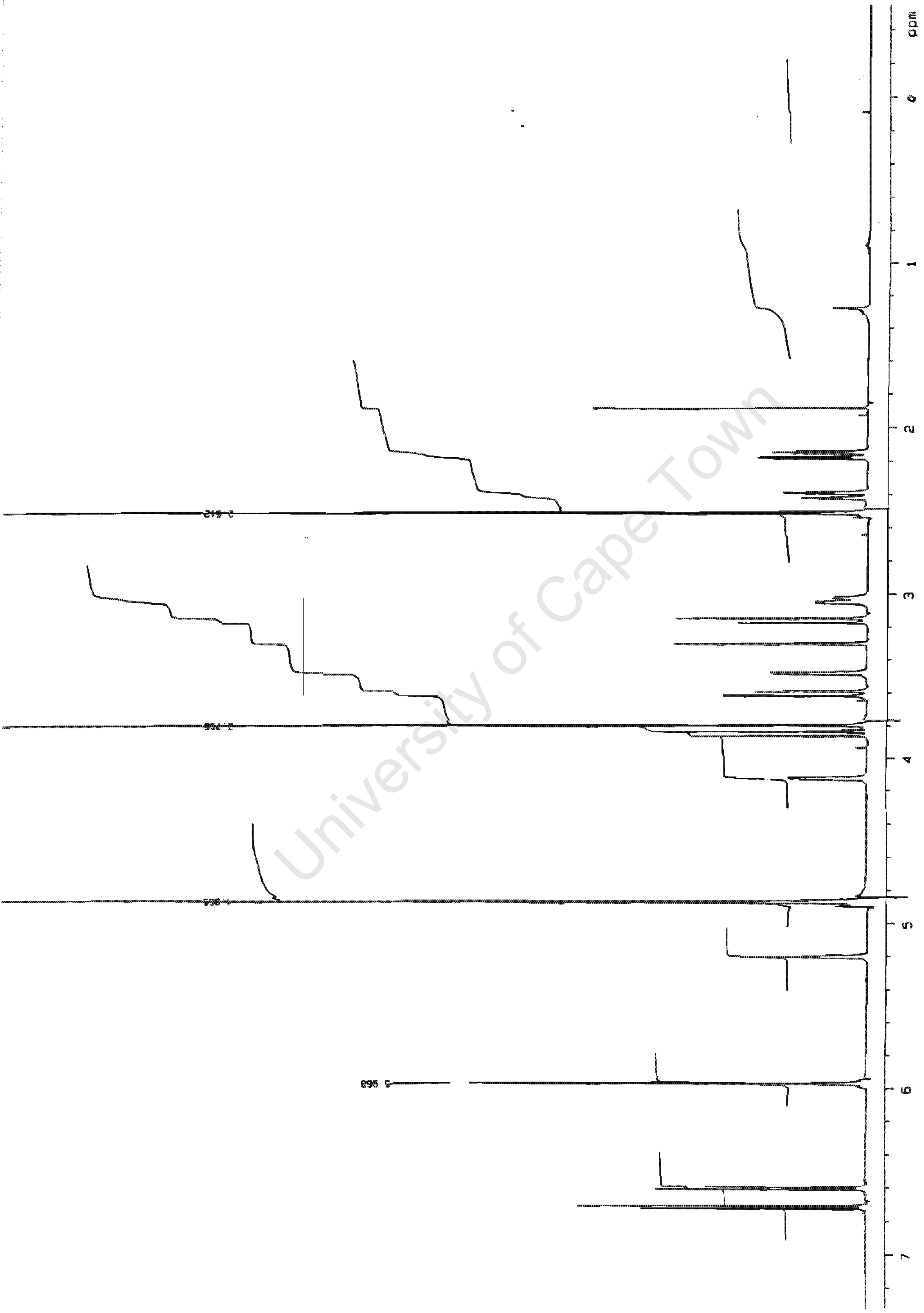


Figure A1.1.1. (a) ^{1}H NMR spectrum of 11α-hydroxygalanthamine in CD_3OD, 500MHz

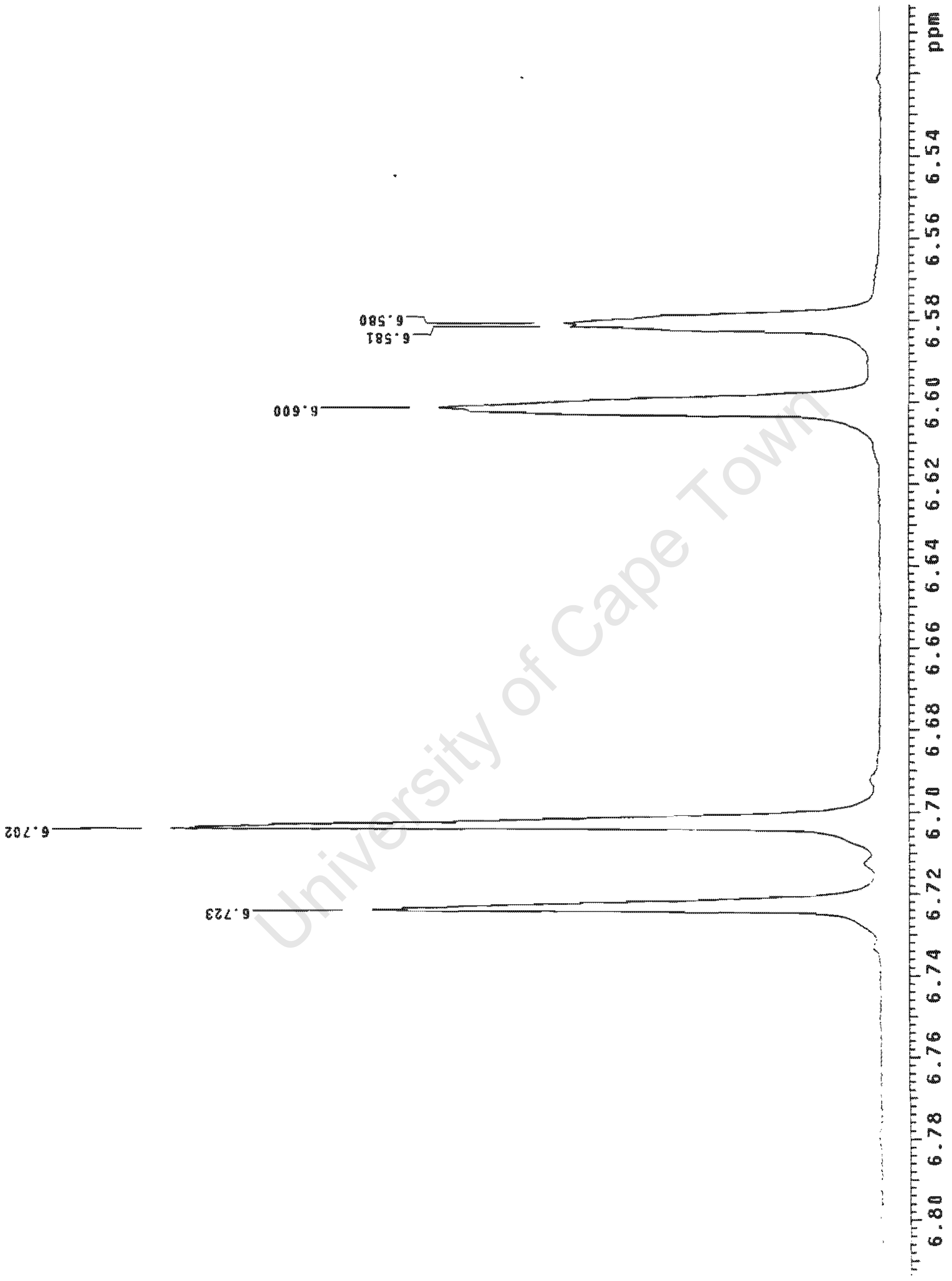


Figure A1.1.1. (b) Expansion of ^{1}H NMR spectrum of 11α-hydroxygalanthamine in CD_3OD, 400MHz

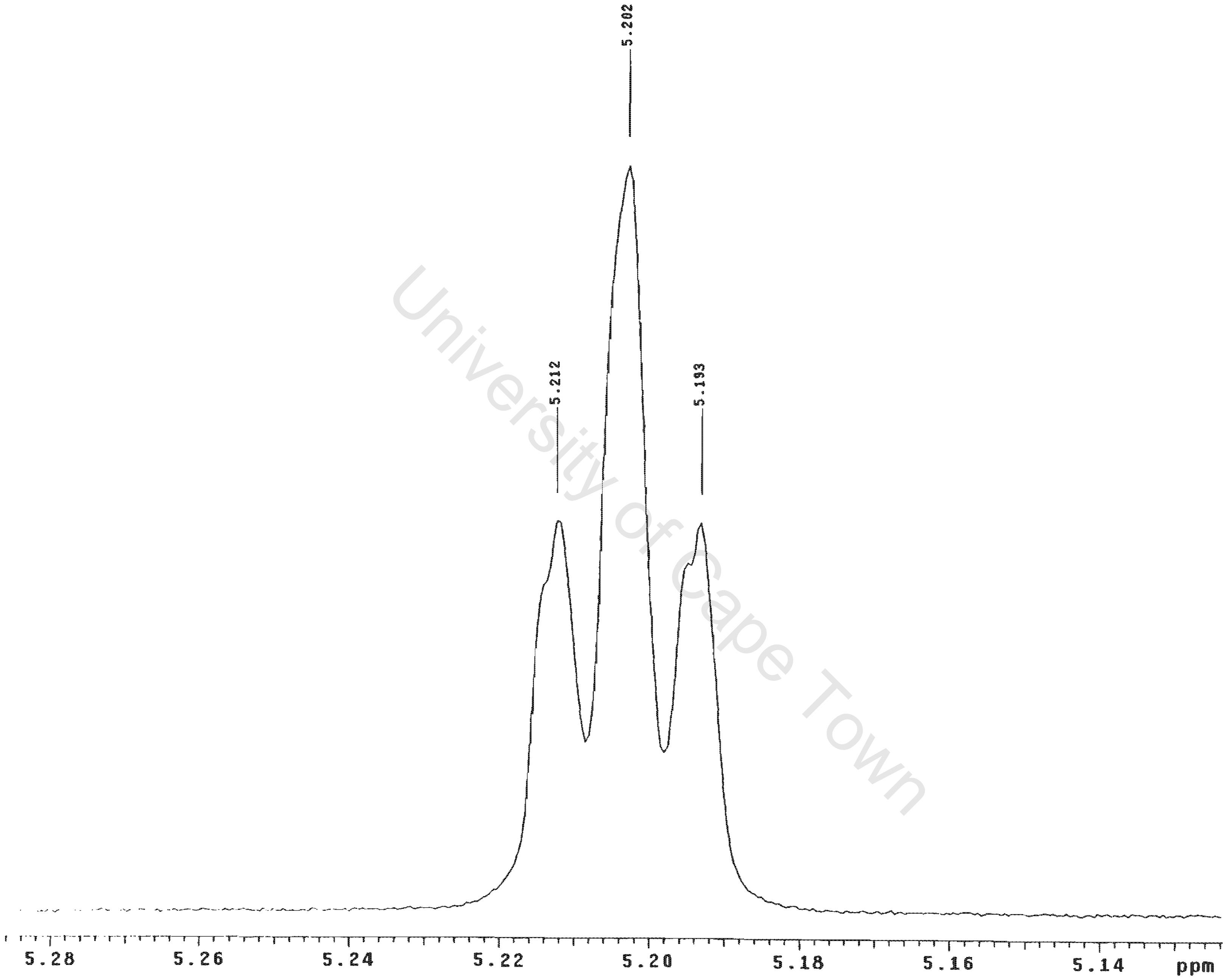


Figure A1.1.1. (c) Expansion of ^{1}H NMR spectrum of 11α-hydroxygalanthamine in CD_3OD, 400MHz

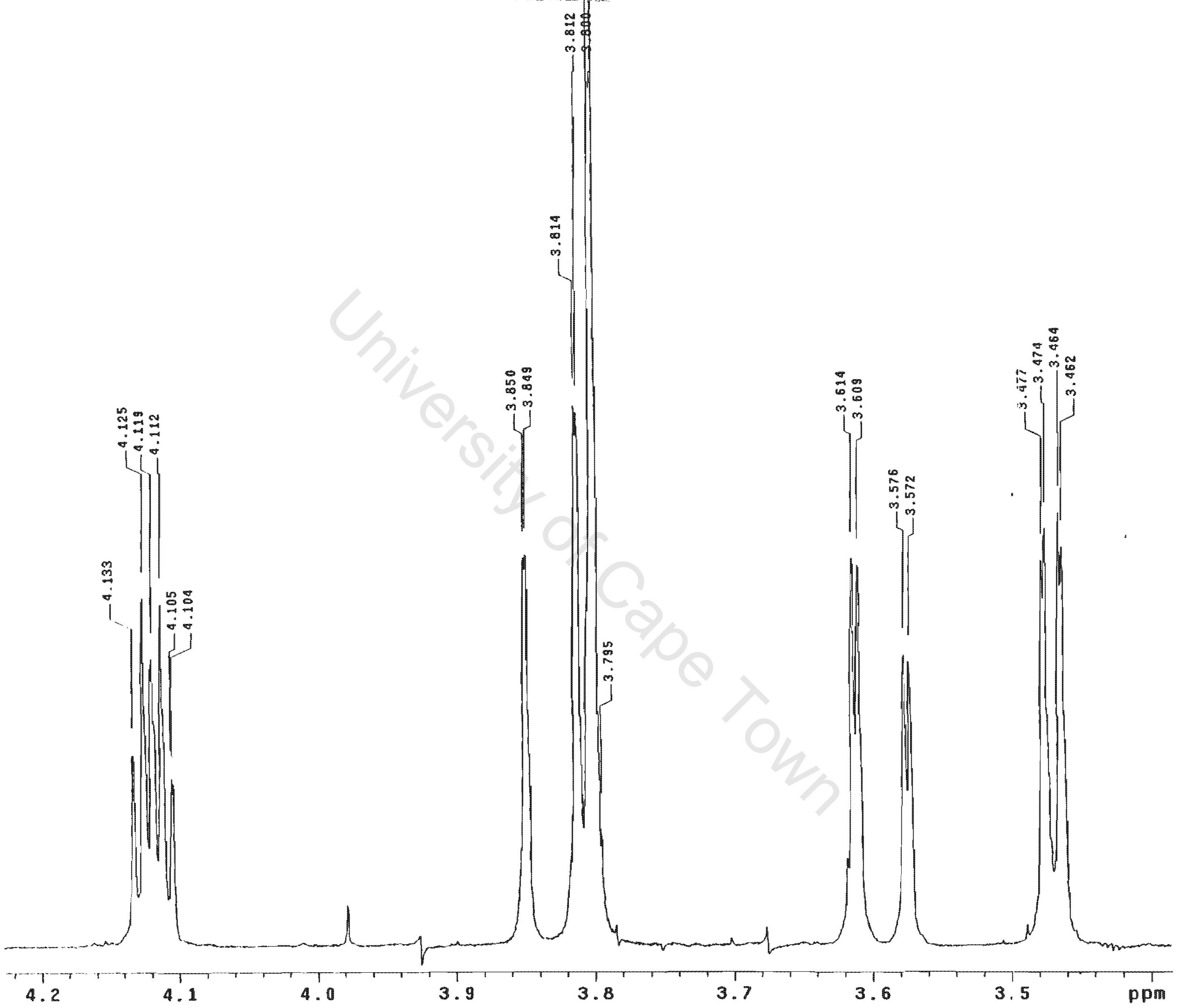


Figure A1.1.1. (d) Expansion of ^1H NMR spectrum of 11α-hydroxygalanthamine in CD_3OD, 400MHz

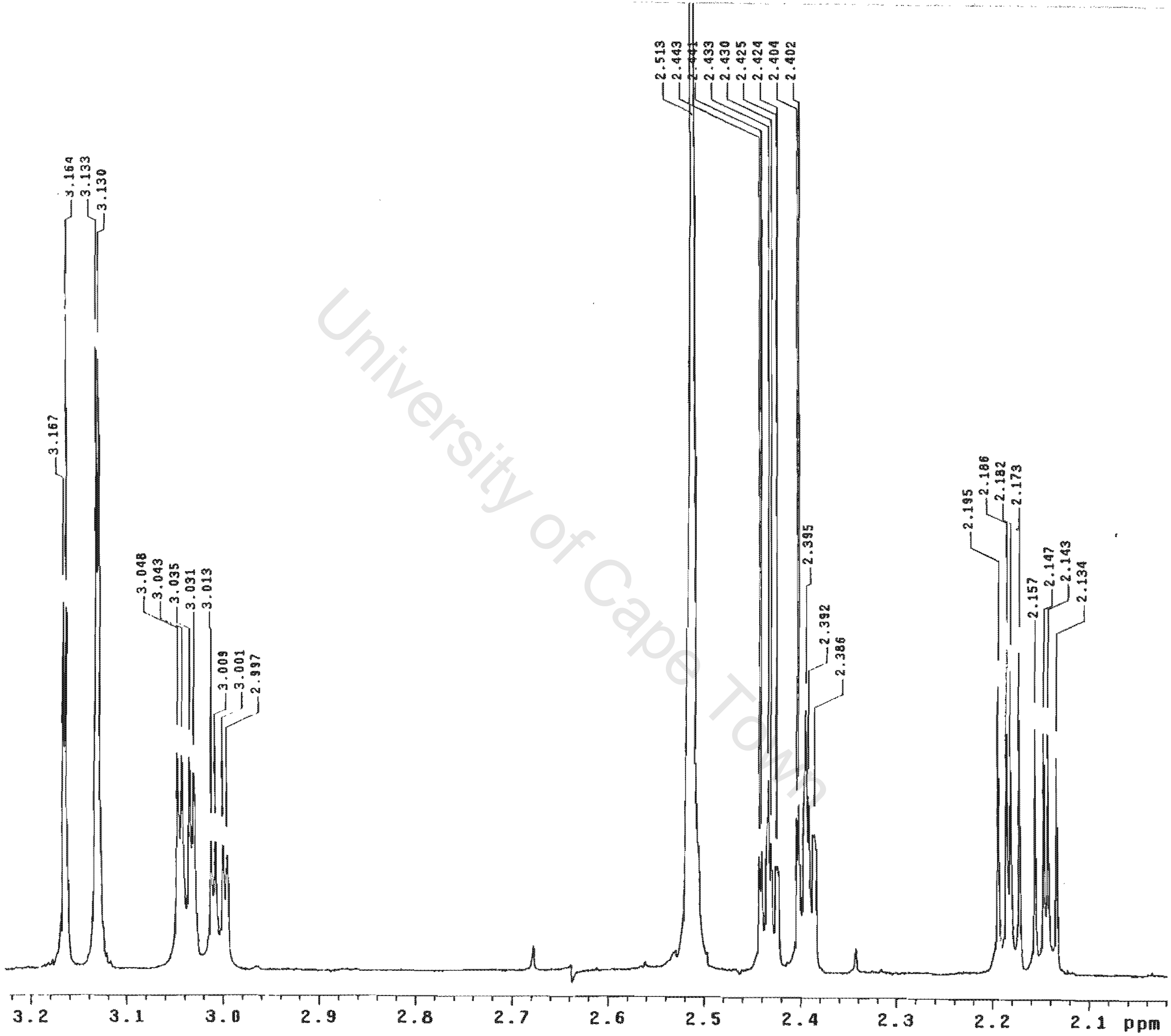


Figure A1.1.1. (e) Expansion of ^{1}H NMR spectrum of 11α-hydroxygalanthamine in CD_3OD, 400MHz

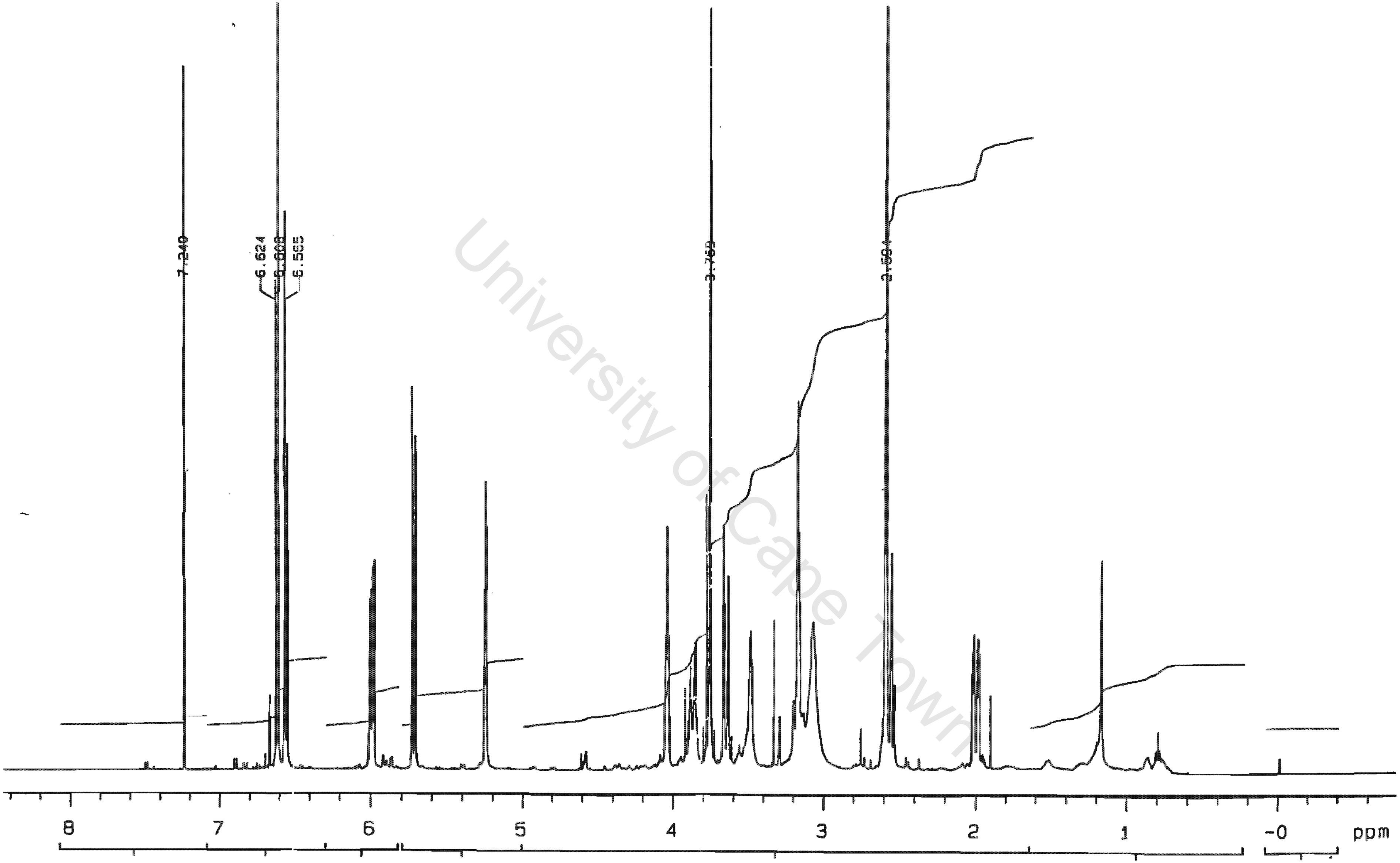


Figure A1.1.2. ^{1}H NMR spectrum of 11α-hydroxygalanthamine in $CDCl_3$, 500MHz

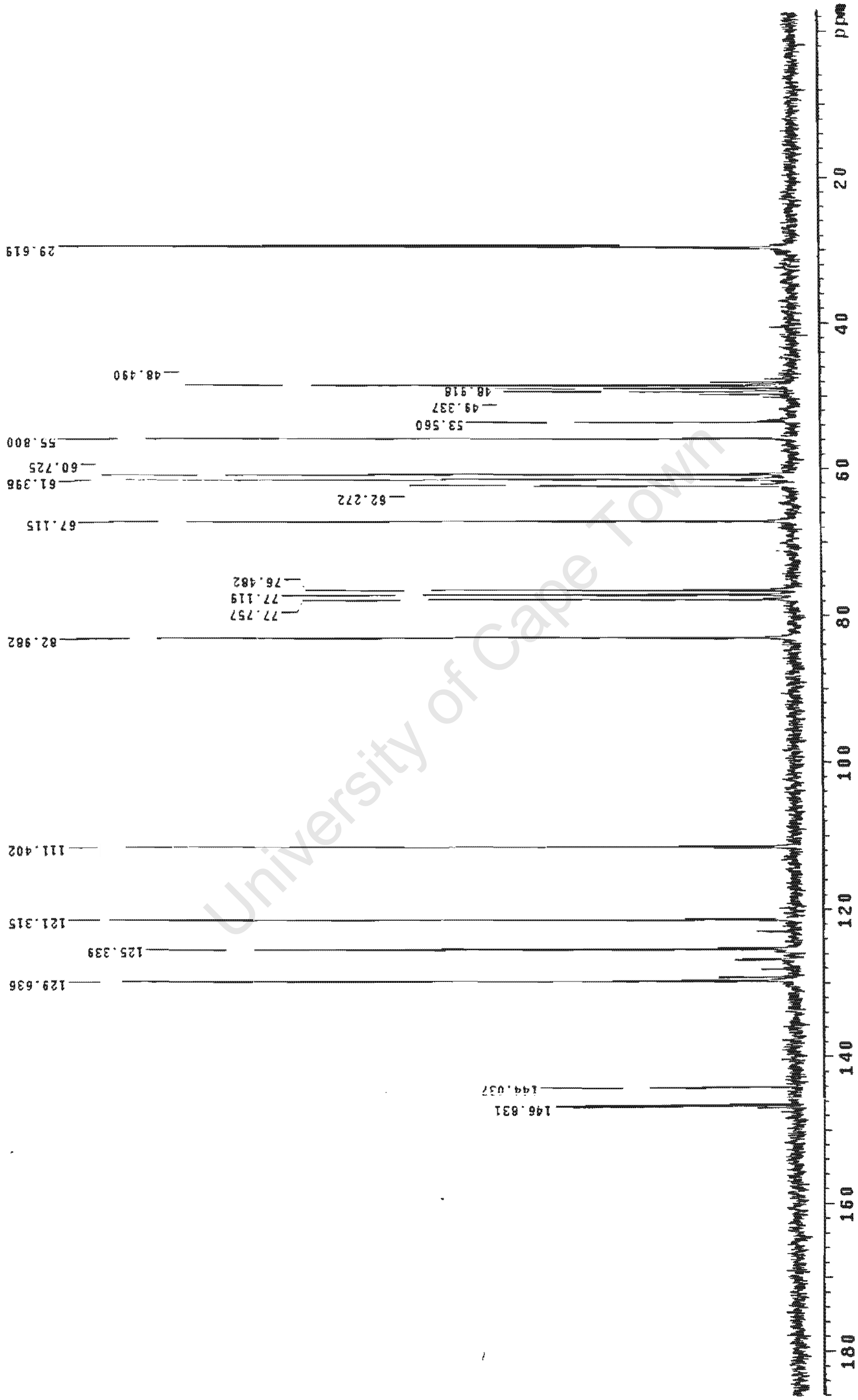


Figure A1.1.3. ^{13}C NMR spectrum of 11α-hydroxygalanthamine in CDCl$_3$, 200MHz

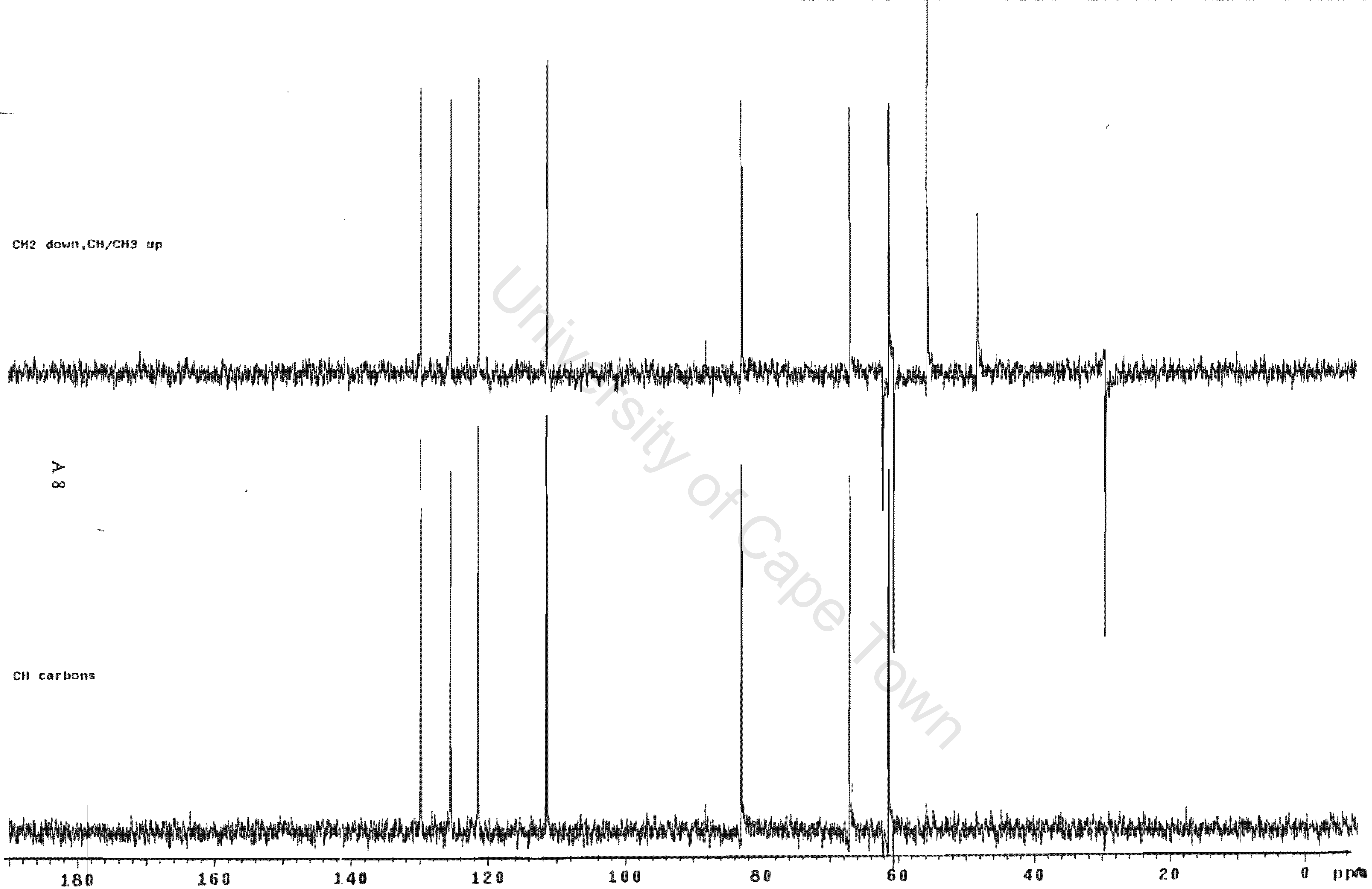
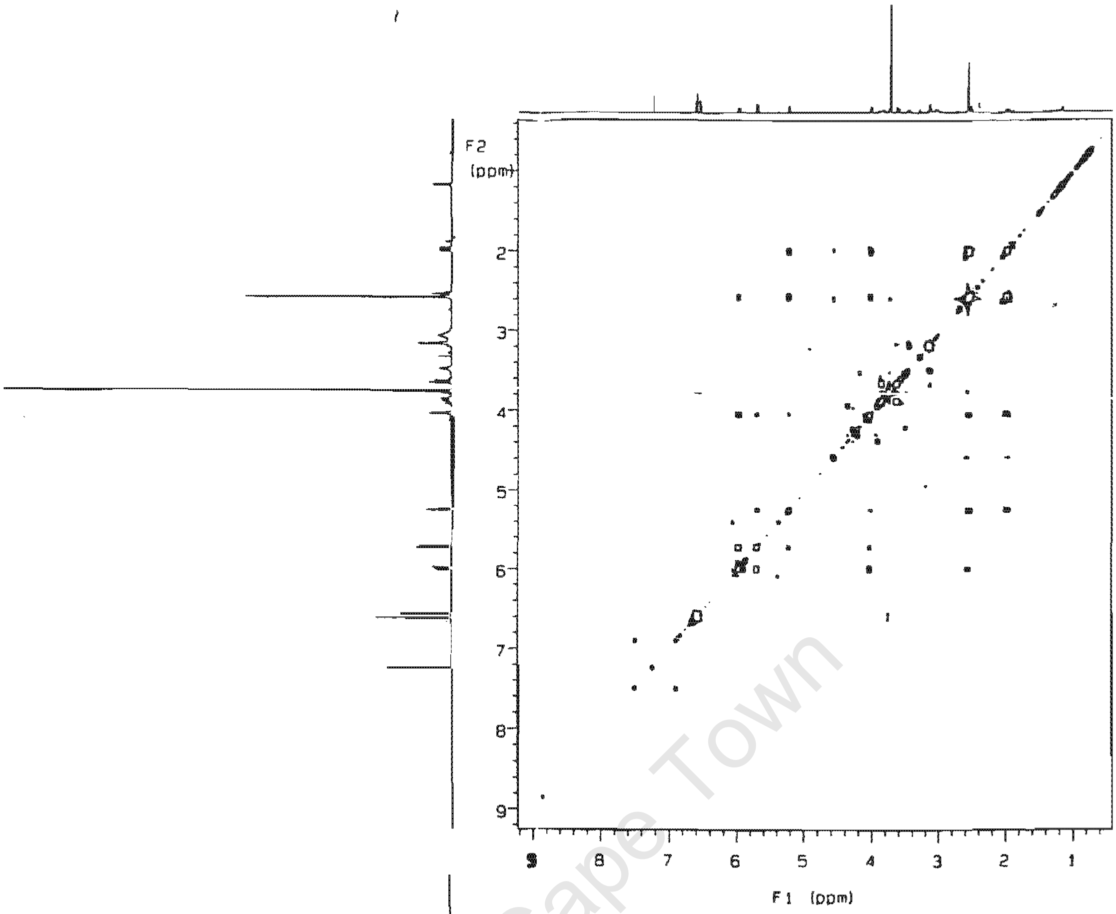


Figure A1.1.4. ^{13}C NMR DEPT spectrum of 11α-hydroxygalanthamine in $CDCl_3$, 200MHz



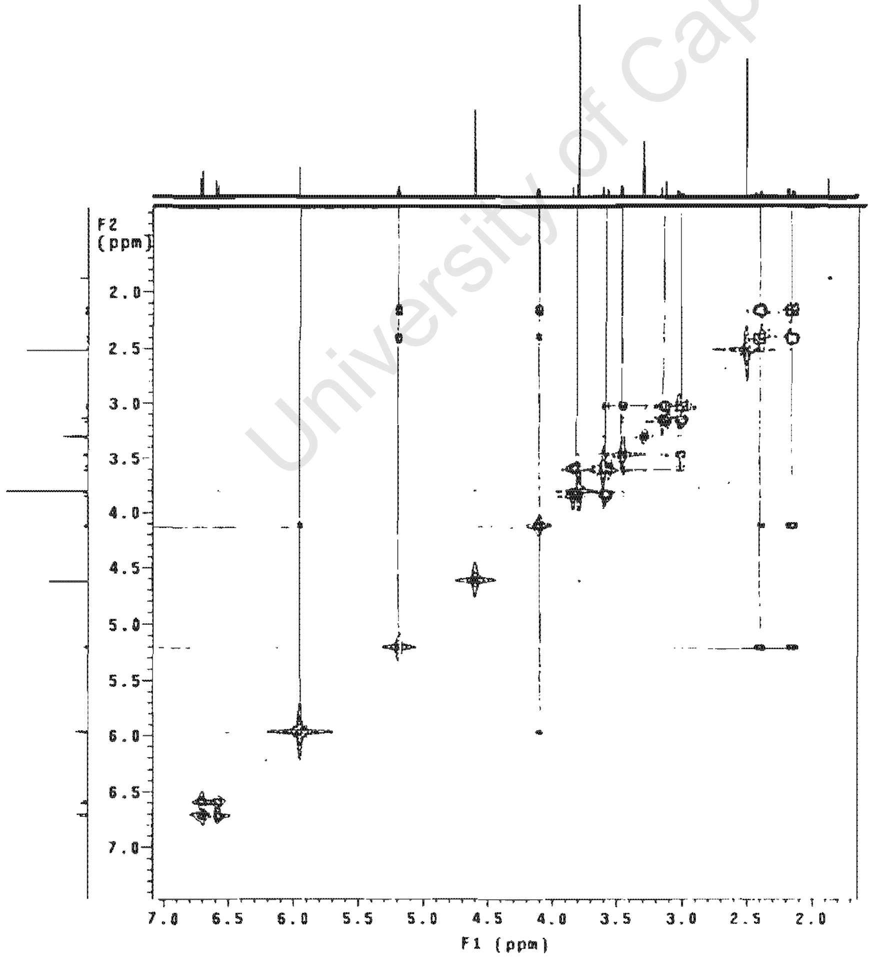


Figure A1.1.5. COSY spectrum of 11α-hydroxygalanthamine in $CDCl_3$, 500MHz (top) and in CD_3OD, 400MHz (bottom)

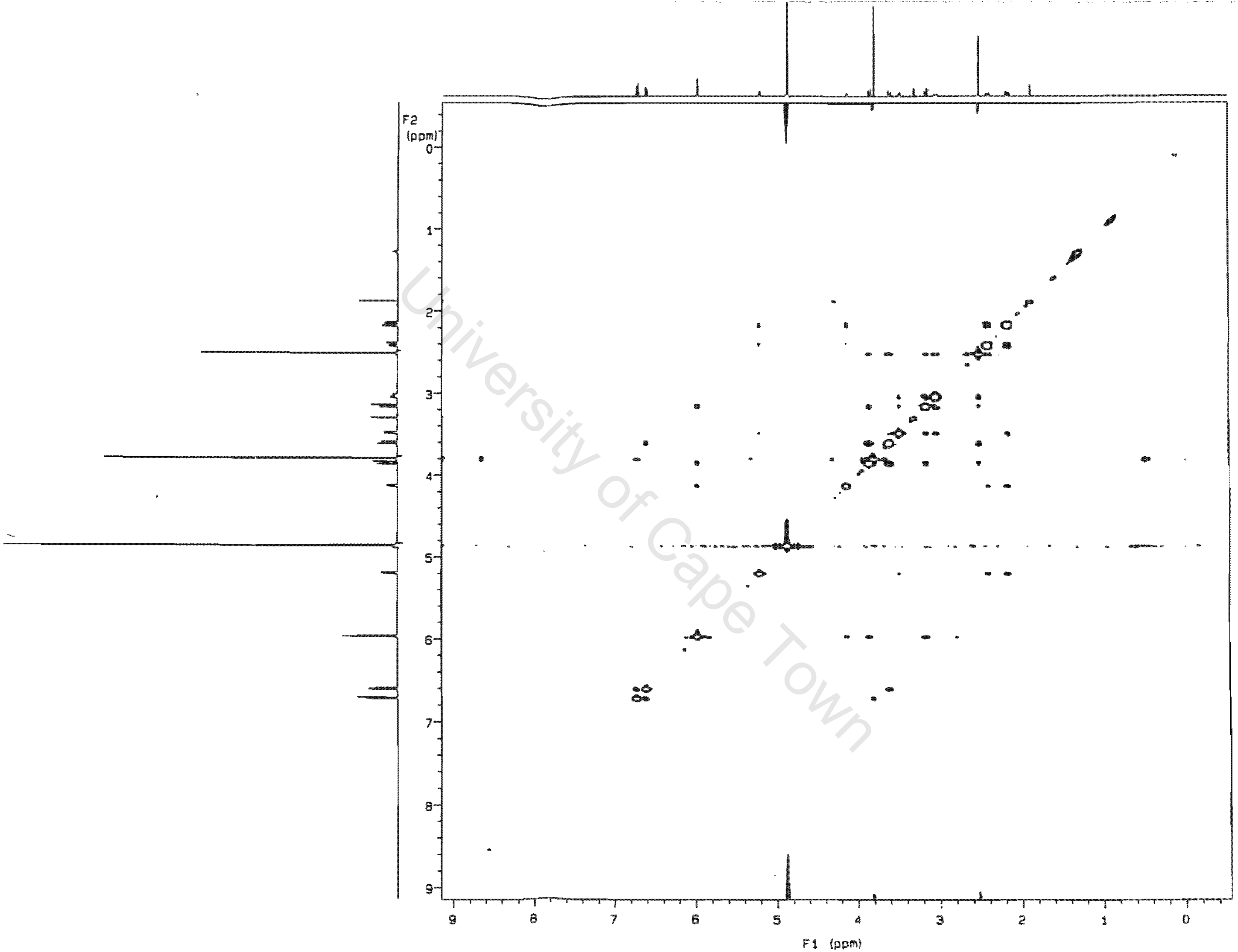


Figure A1.1.6. NOESY spectrum of 11α-hydroxygalanthamine in CD_3OD, 500MHz

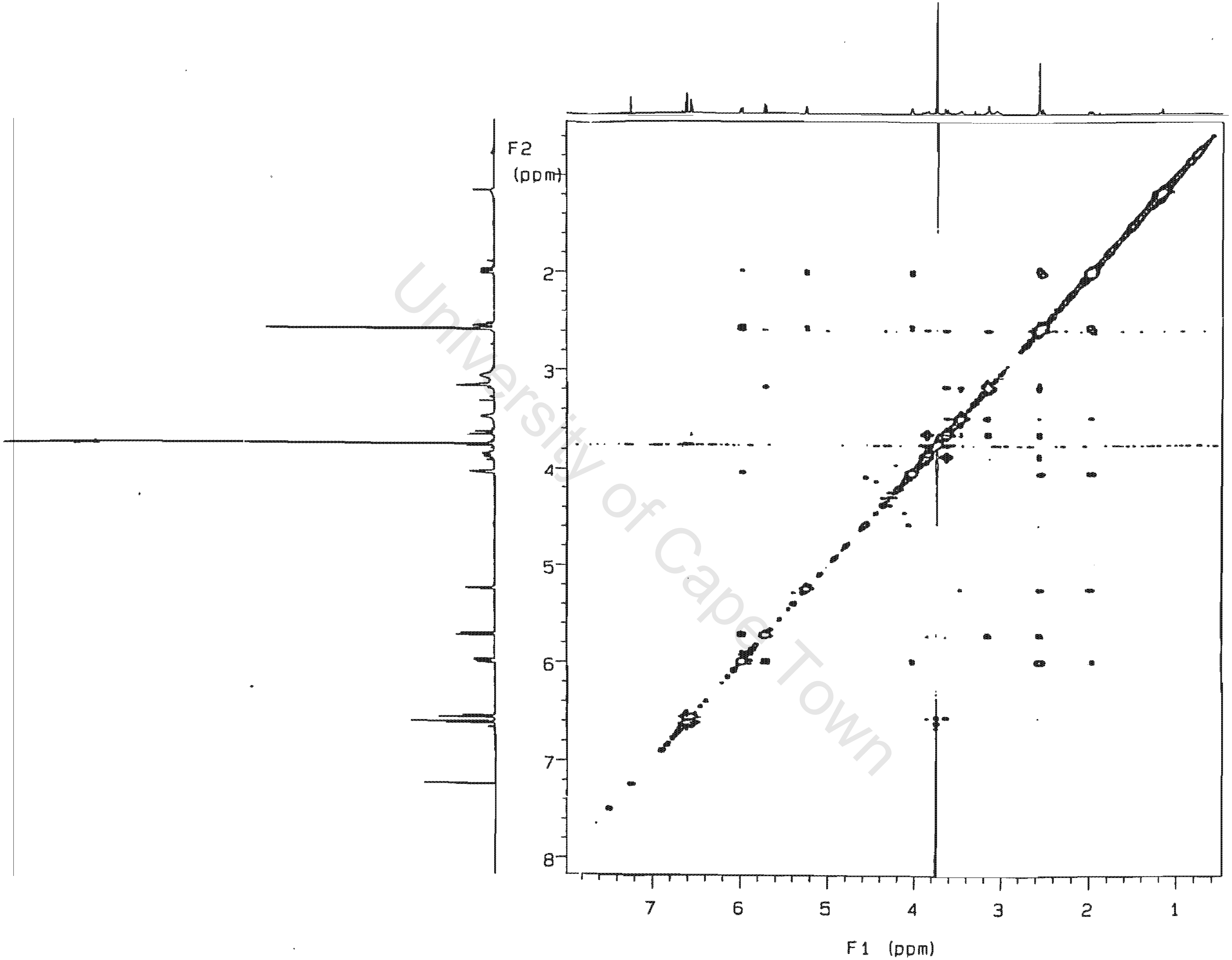


Figure A1.1.7. ROESY spectrum of 11α-hydroxygalanthamine in $CDCl_3$, 500MHz

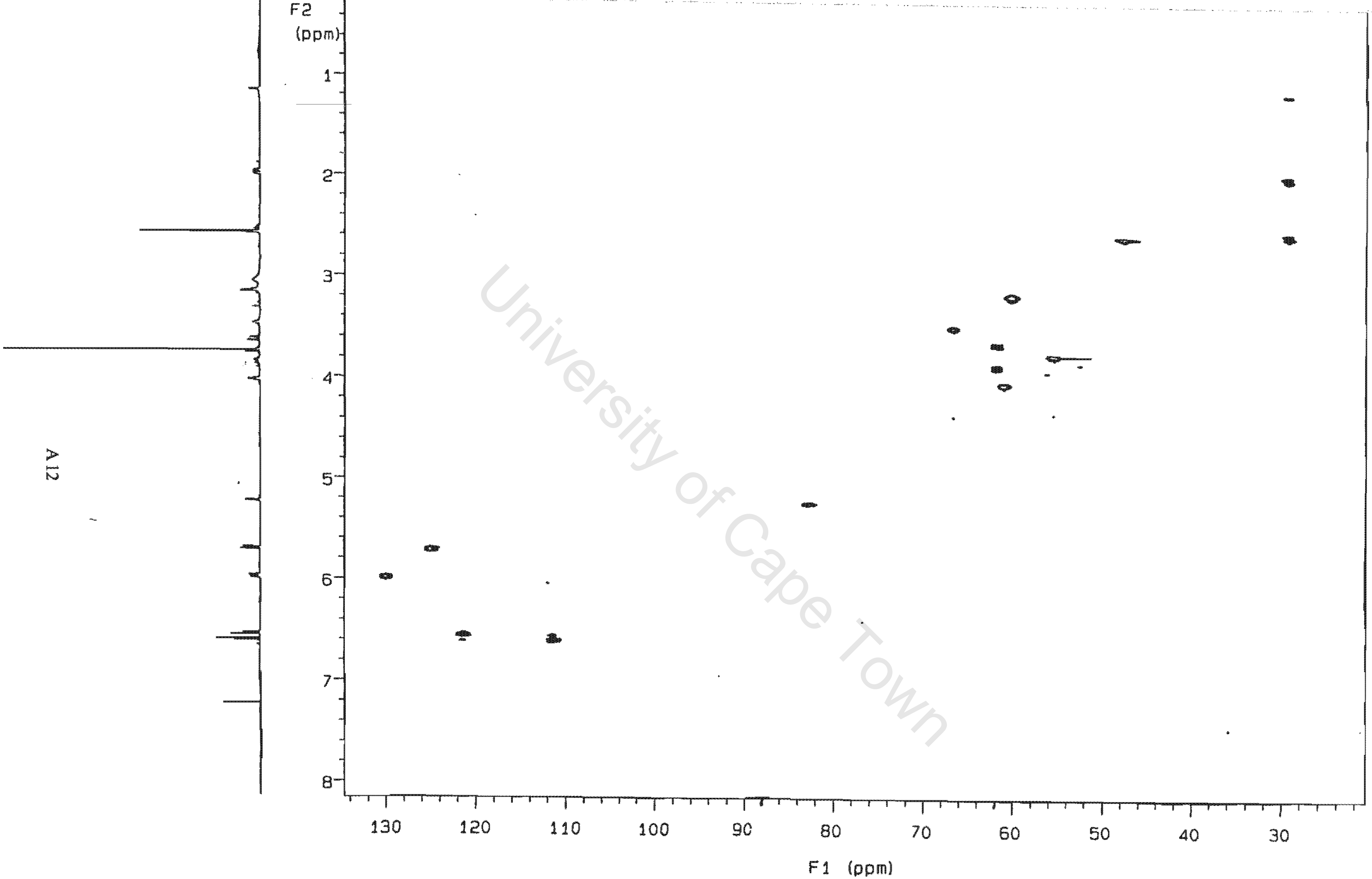


Figure A1.1.8. HSQC spectrum of 11α-hydroxygalanthamine in $CDCl_3$, 500MHz

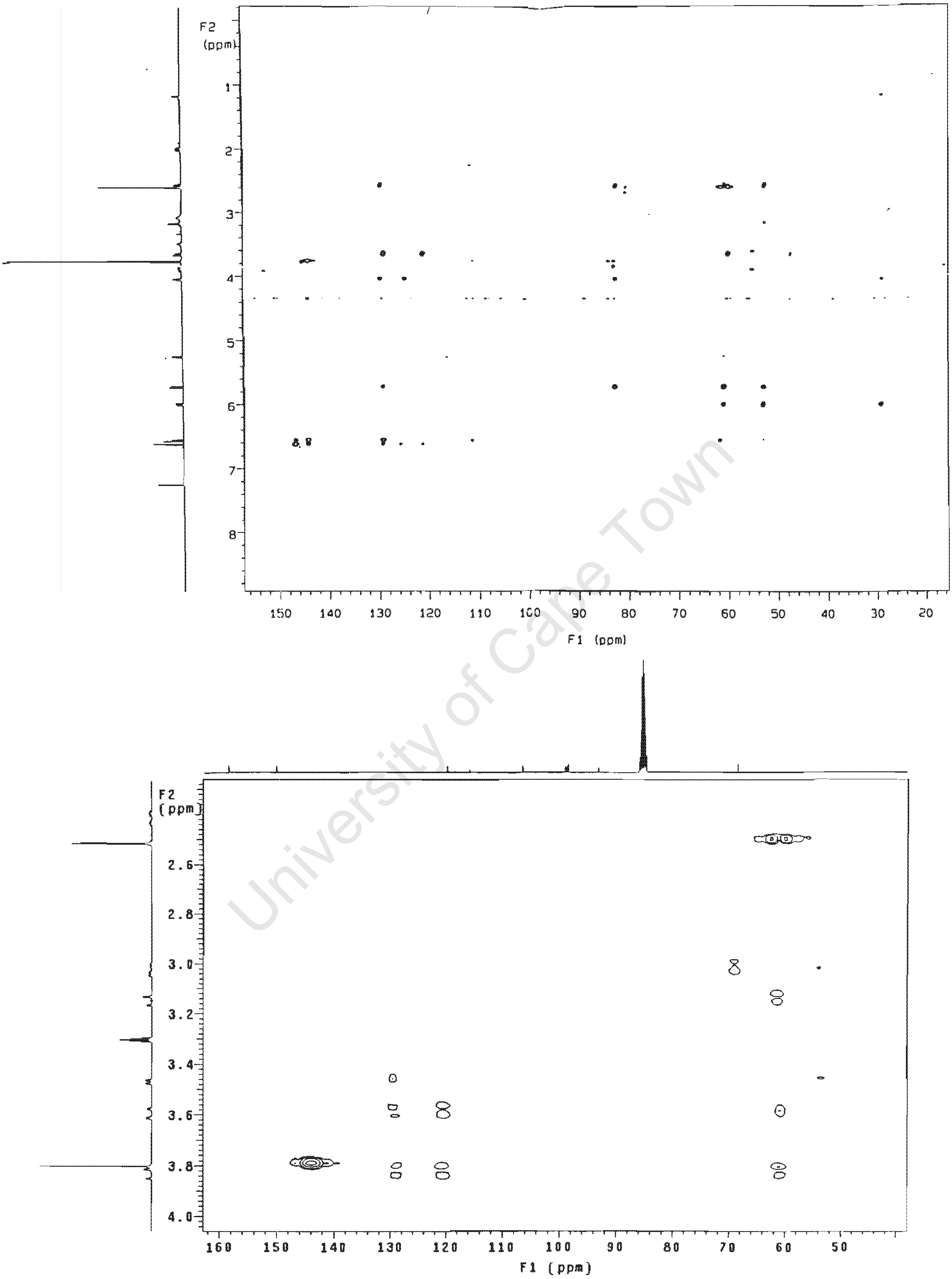


Figure A1.1.9. HMBC spectrum of 11α-hydroxygalanthamine in $CDCl_3$, 500MHz (top) and in CD_3OD, 400MHz (bottom)

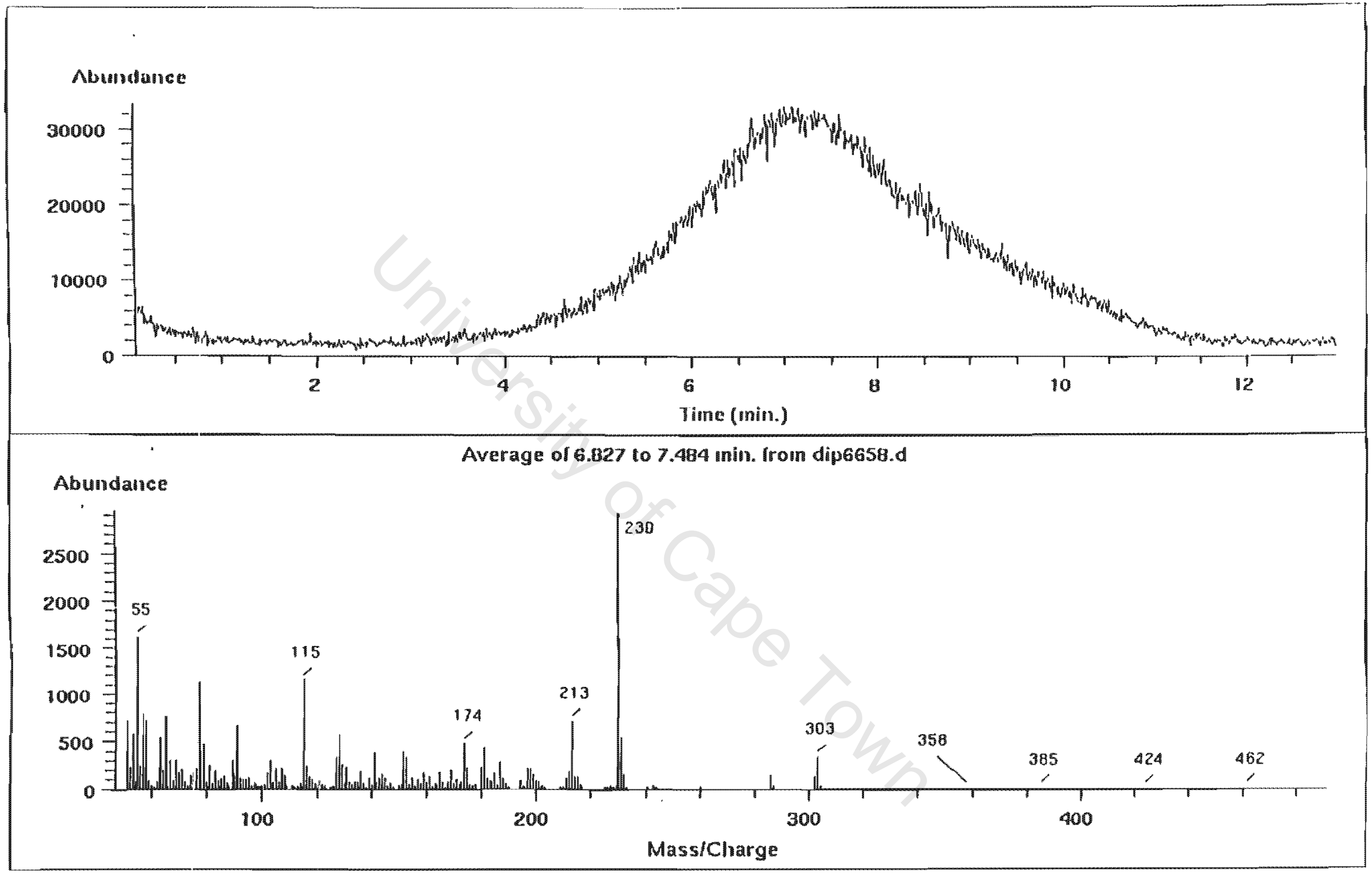


Figure A1.1.10. EIMS of 11α-hydroxygalanthamine

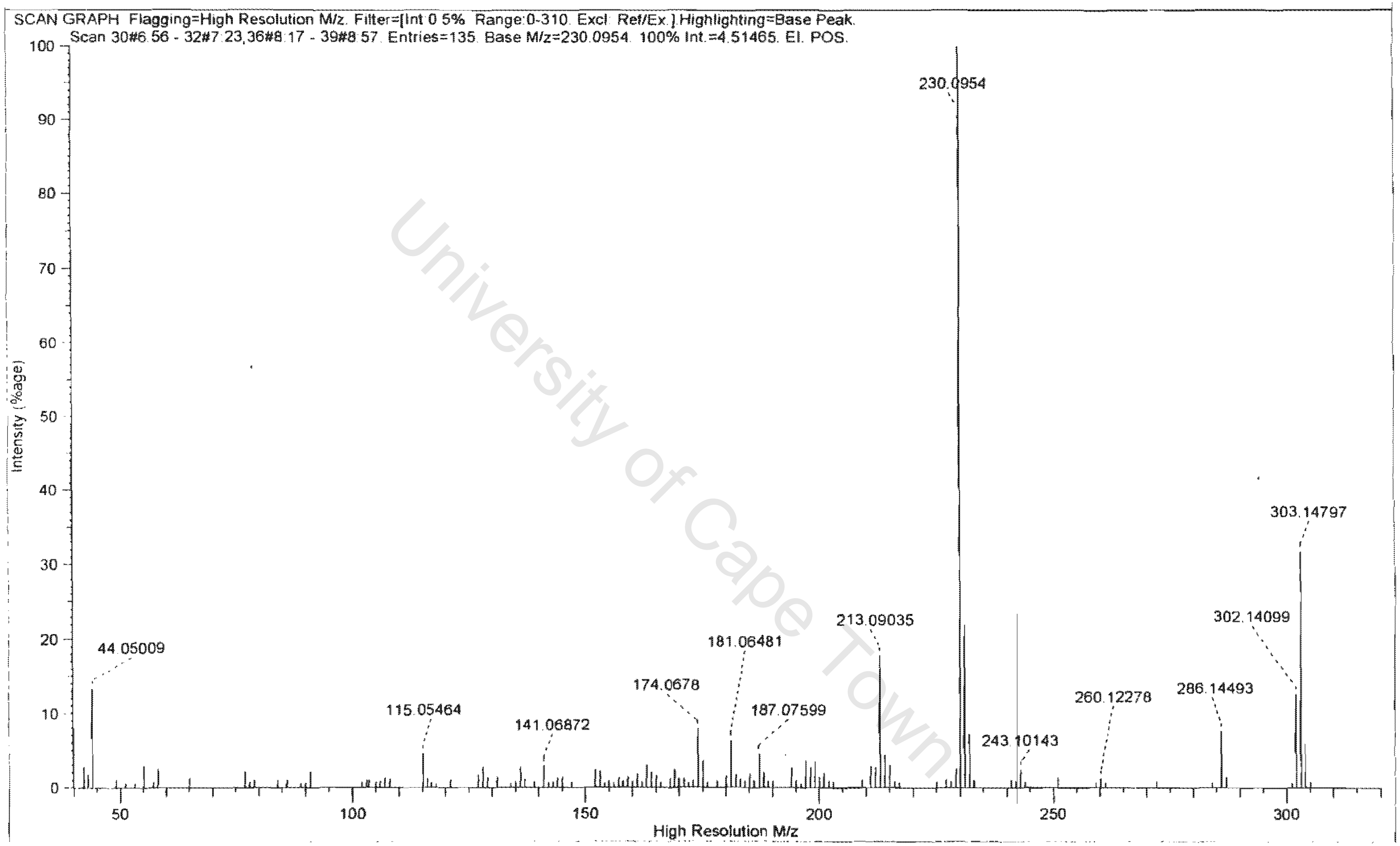


Figure A1.1.11. HREIMS of 11α-hydroxygalanthamine

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File Type       :  Hi-Res Data - Ctd
File Source     :  Acquired on MASPEC II system [II32/A002]
File Title      :  HYDGAL
Operator        :  Dr. P. Boshoff
Instrument      :  VG70-SEQ

Sort Field      :  High Resolution M/z (descending).
Scan Filter     :  Minimum Intensity= 3.50%
                   Mass Range= [0:304]

Selected Isotopes:
         Symbol   Min  Max  V'cy   Name
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              H     0   34    1    Hydrogen-1
              O     0    6    2    Oxygen-16
              N     0    1    3    Nitrogen-14

Allowable error = minimum of 20.0 ppm, 5.0 mmu.
Ring/Double Bond limits = [-0.5 : 100.0]

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  286.14493    7.50   286.14432    -2.1  -0.6   8.5  C17H20O3N
                      286.14164   -11.5  -3.3   4.0  C14H22O6

  232.09930    7.13   232.09737    -8.3  -1.9   7.5  C13H14O3N
                      232.09469   -19.9  -4.6   3.0  C10H16O6

  231.10014   21.78   231.10212     8.6   2.0   7.5  C14H15O3

  230.09540  100.00   230.09429    -4.8  -1.1   8.0  C14H14O3
                      230.09697     6.8   1.6  12.5  C17H12N

  214.09339    4.25           ----- No match found -----
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  197.06107    3.58   197.06025    -4.1  -0.8   9.5  C13H9O2

  187.07599    4.54   187.07590    -0.5  -0.1   7.5  C12H11O2

  181.06481    6.24   181.06534     2.9   0.5   9.5  C13H9O

  175.07452    3.55   175.07590     7.9   1.4   6.5  C11H11O2

  174.06780    7.87   174.06808     1.6   0.3   7.0  C11H10O2

  115.05464    4.50   115.05478     1.2   0.1   6.5  C9H7

   44.05009   13.32    44.05002    -1.5  -0.1   0.5  C2H6N


                              ***** End of Atomic Composition Report *****
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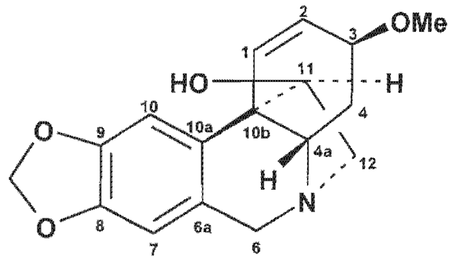
A1.2. 11-Epihaemanthamine (29)



11-Epihaemanthamine (29)

List of spectra:

- ^{1}H NMR ($CDCl_3$, 300MHz)
- COSY ($CDCl_3$, 300MHz)

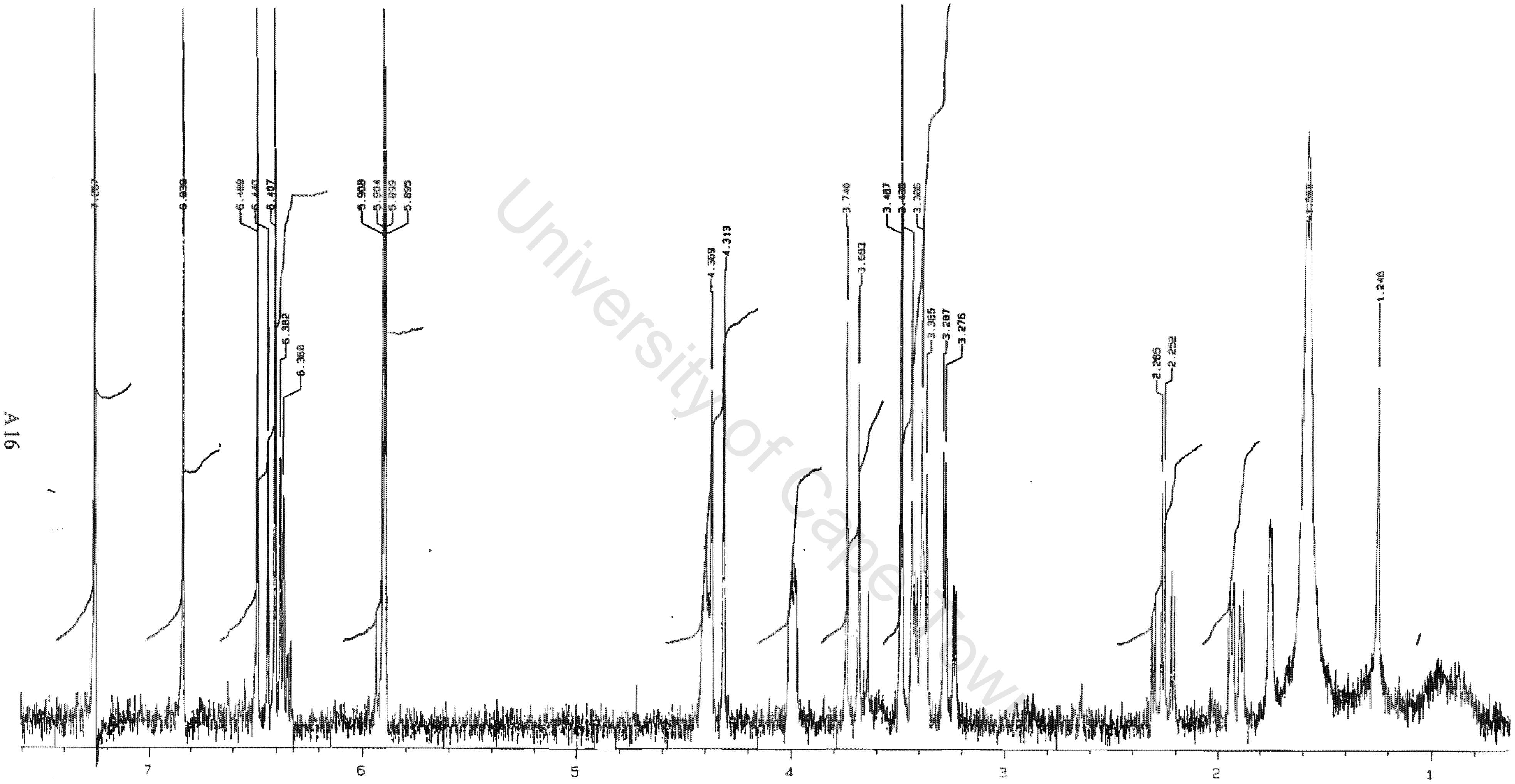


Figure A1.2.1. ^{1}H NMR spectrum of 11-epihaemanthamine in $CDCl_3$, 300MHz

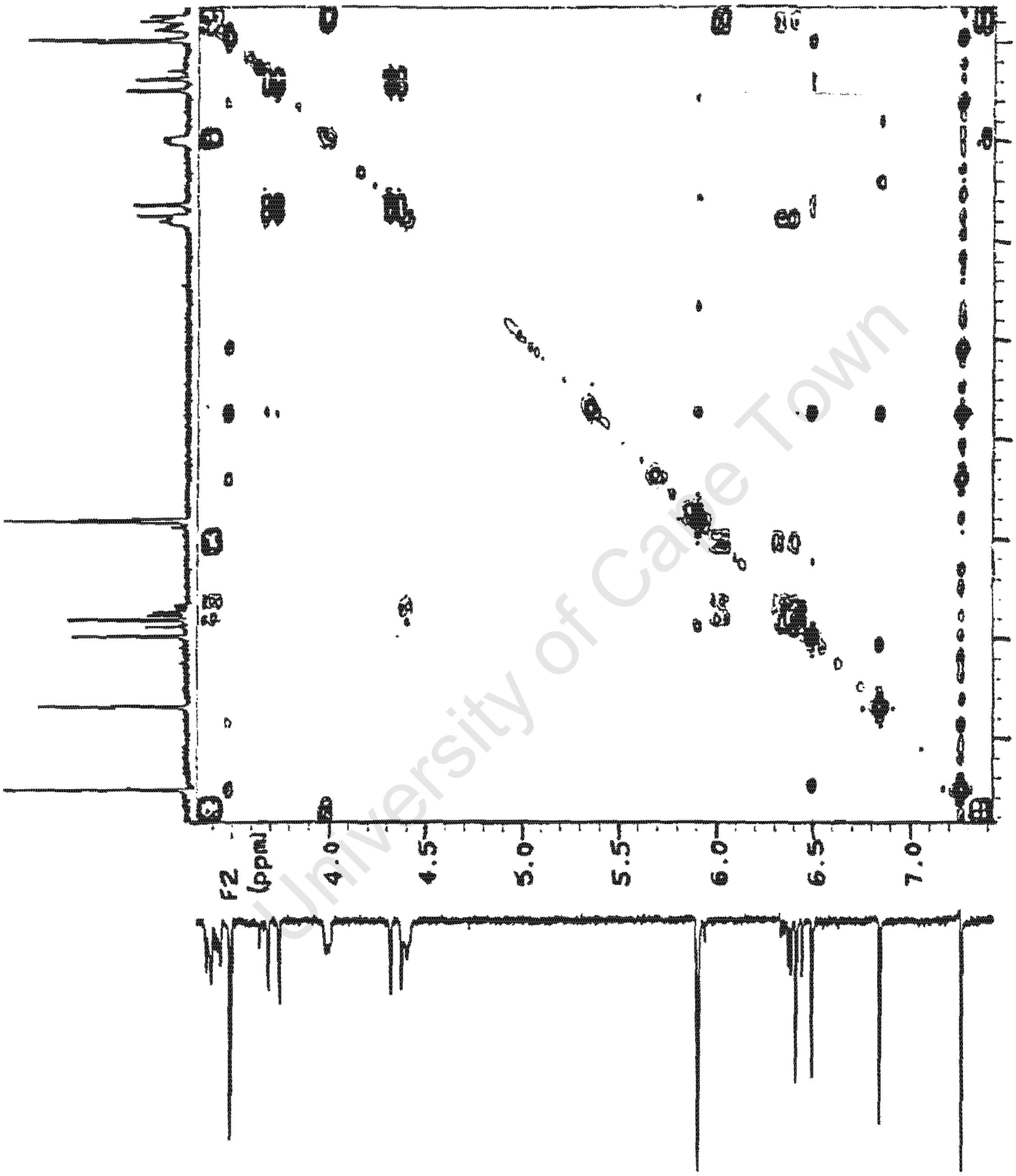
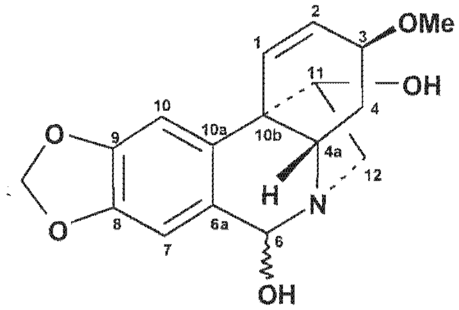


Figure A1.2.2. COSY spectrum of 11-epihaemanthamine in $CDCl_3$, 300MHz

A1.3. Haemanthidine (13)



Haemanthidine (13)

List of spectra:

- ^{1}H NMR ($CDCl_3$, 200MHz)
- EIMS

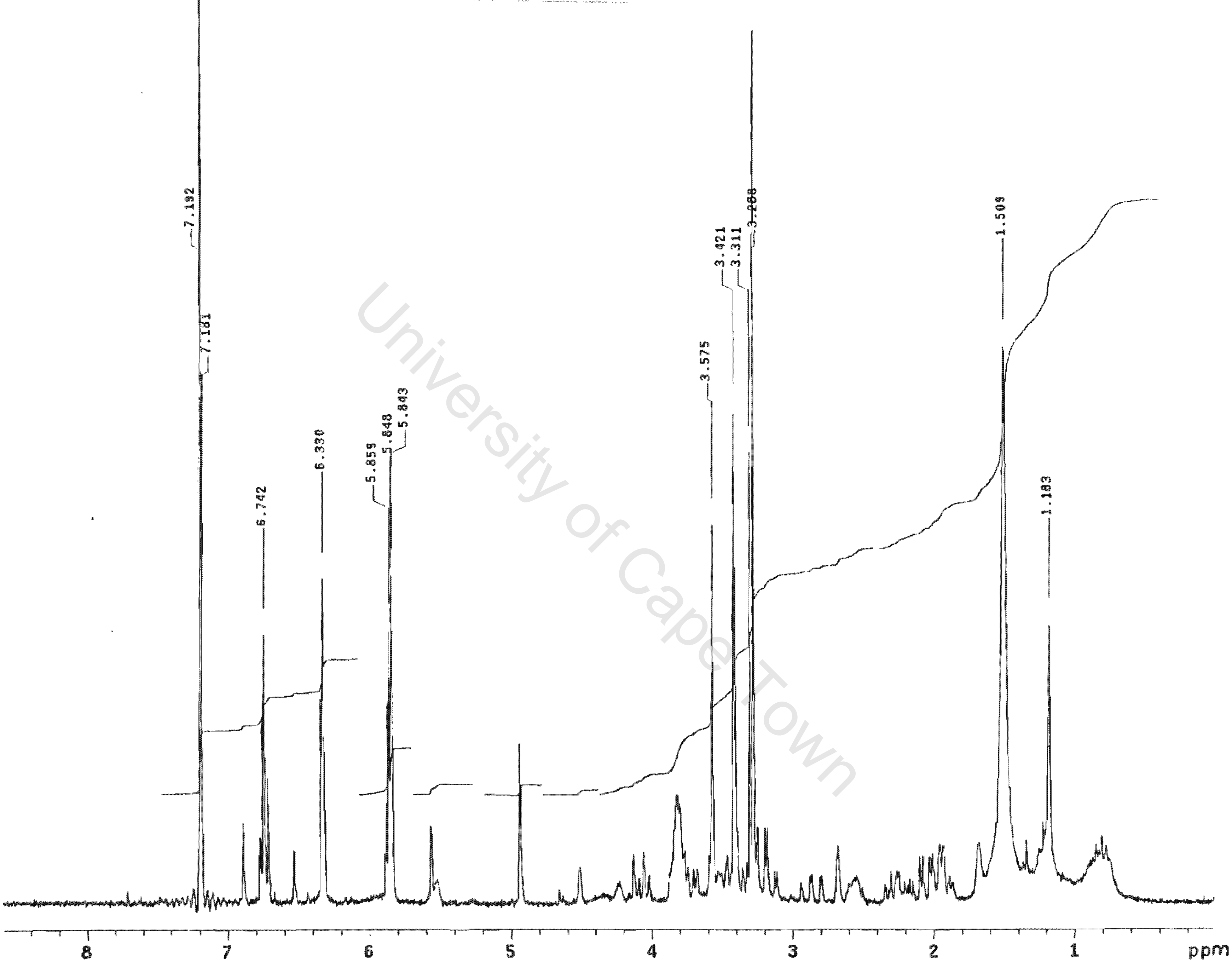


Figure A1.3.1. ^{1}H-NMR spectrum of haemanthidine in $CDCl_3$, 200MHz

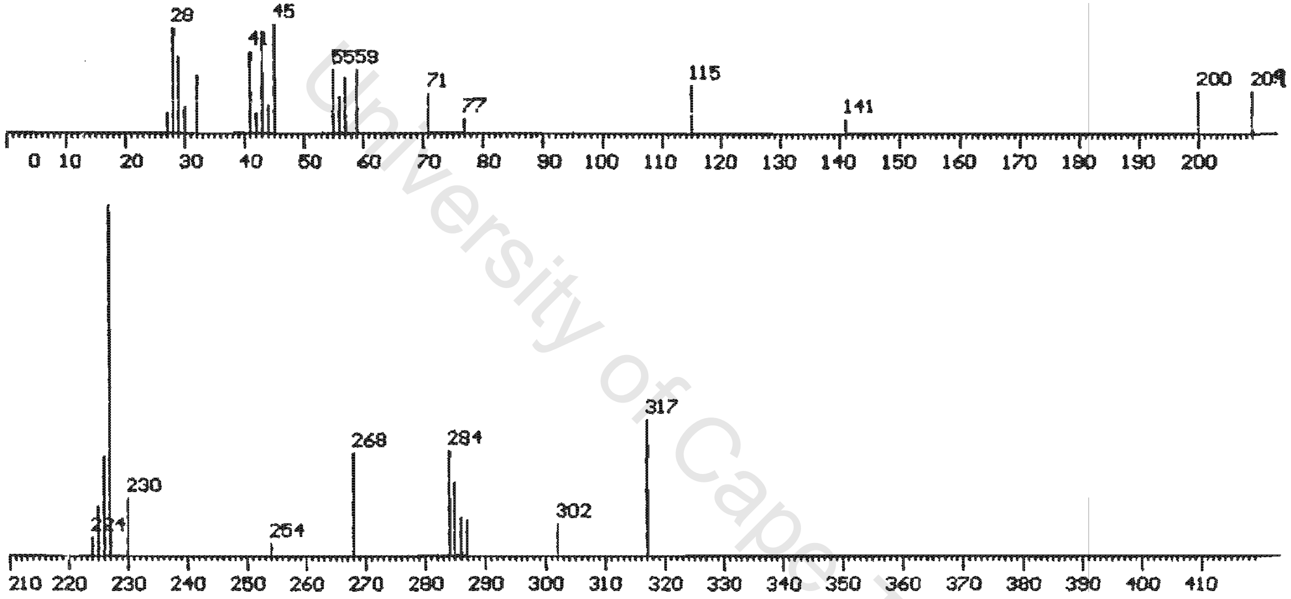
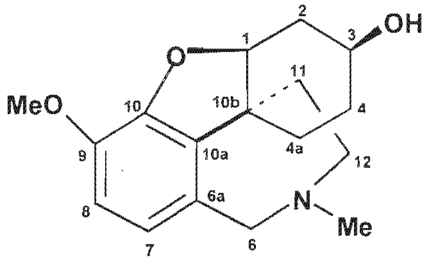


Figure A1.3.2. EIMS of haemanthidine

A1.4. Lycoramine (28)



Lycoramine (28)

List of spectra:

- ^{1}H NMR ($CDCl_3$, 400MHz)
- ^{13}C NMR (CD_3OD, 75MHz)
- EIMS

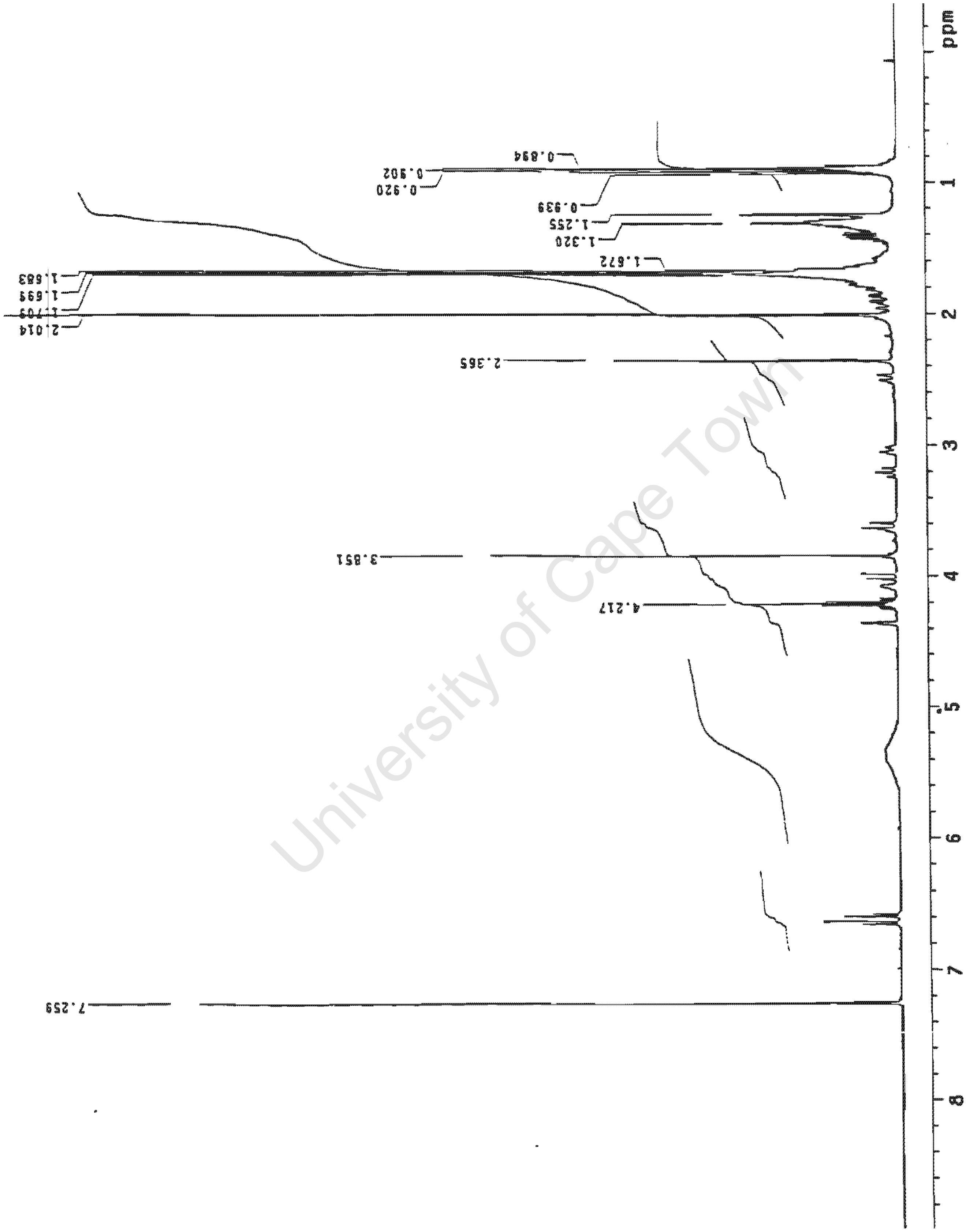


Figure A1.4.1. ^1H NMR spectrum of lycoramine ($CDCl_3$, 400MHz)

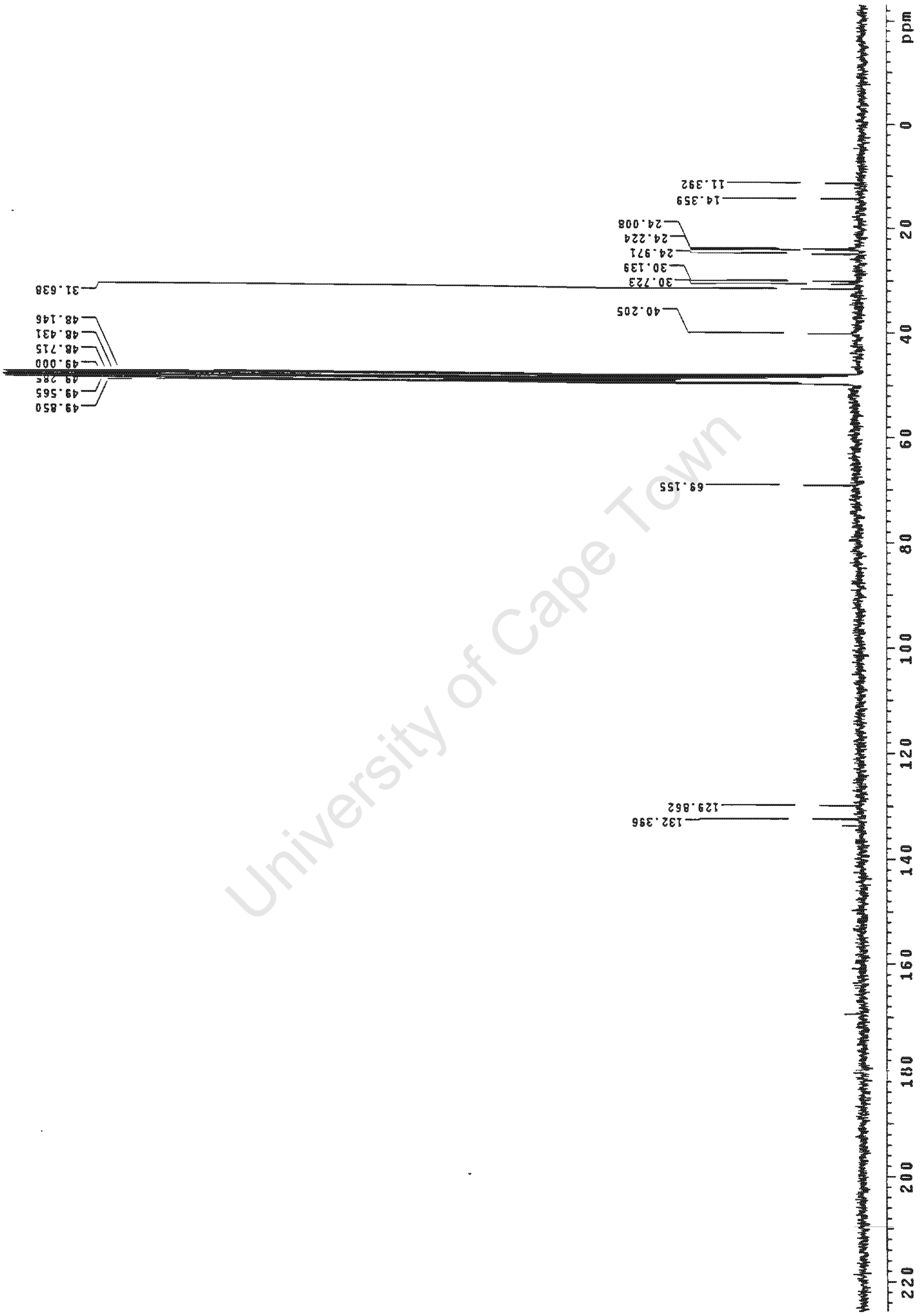


Figure A1.4.2. ^{13}C NMR spectrum of lycoramine (CD_3OD, 75MHz)

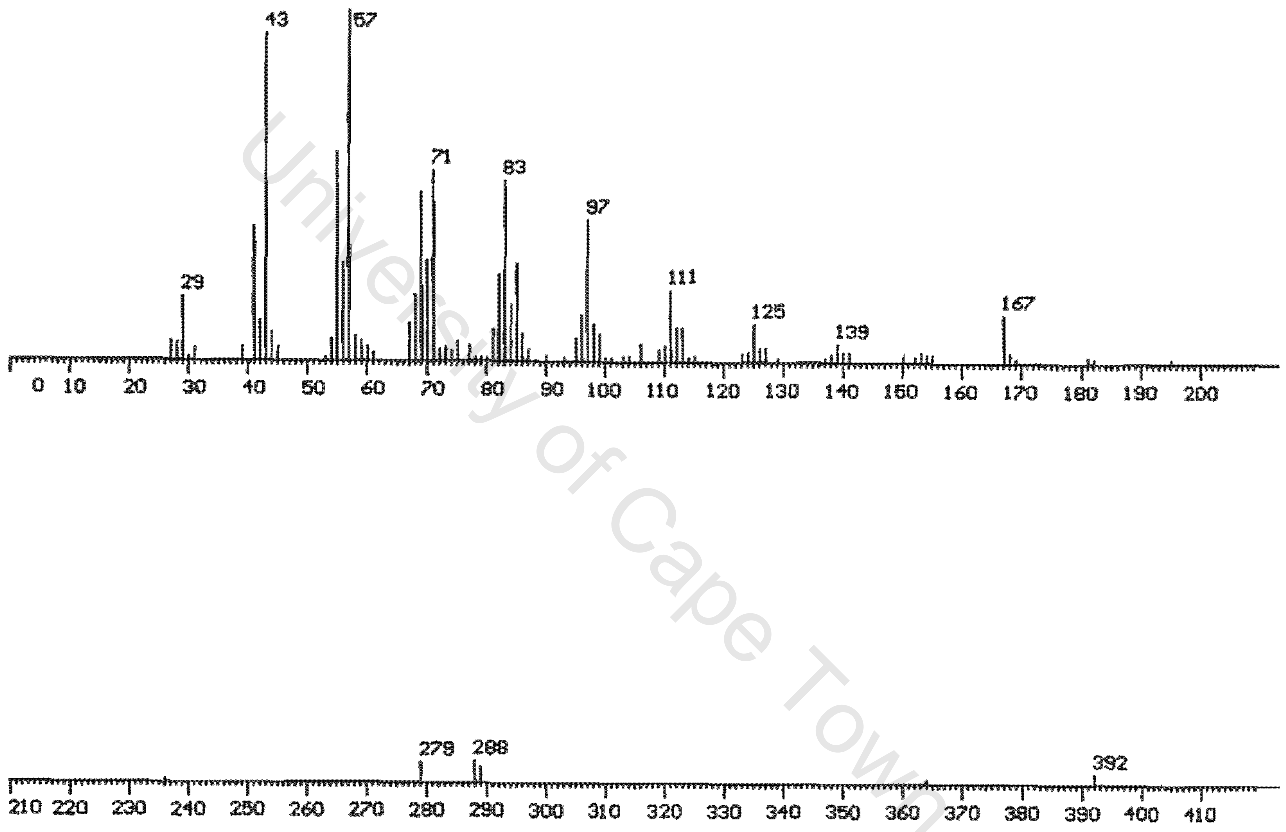
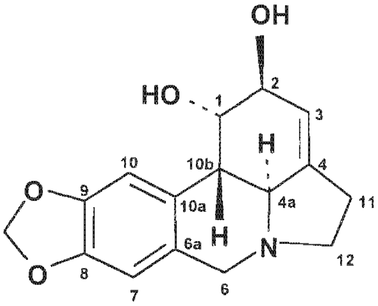


Figure A1.4.3. EIMS of lycoramine

A1.5. Lycorine (4)



Lycorine (4)

List of spectra:

- ^{1}H NMR (CD$_3$OD, 400MHz)
- COSY (CD$_3$OD, 400MHz)
- EIMS

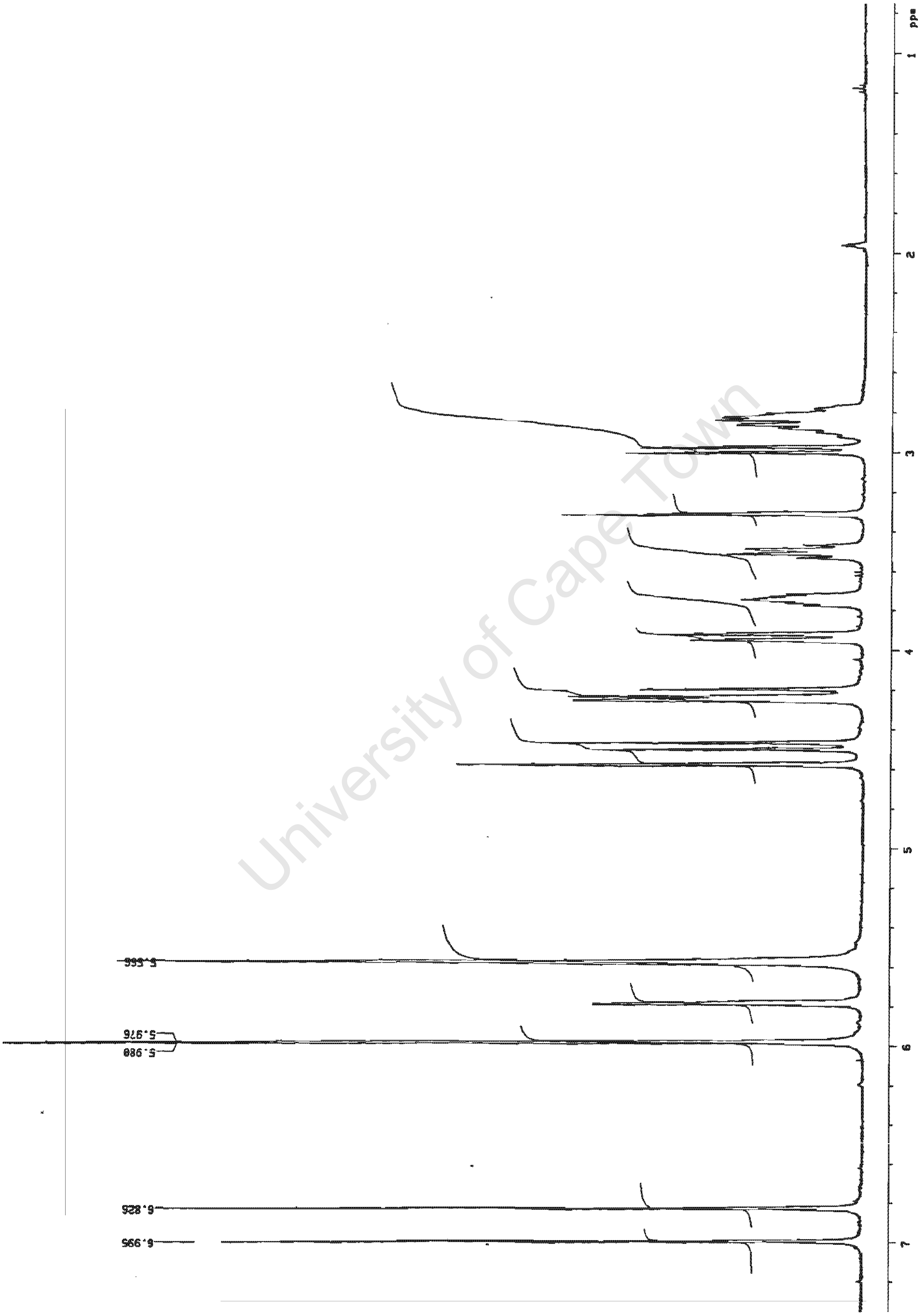


Figure A1.5.1. ^1H NMR spectrum of lycorine (CD_3OD, 400MHz)

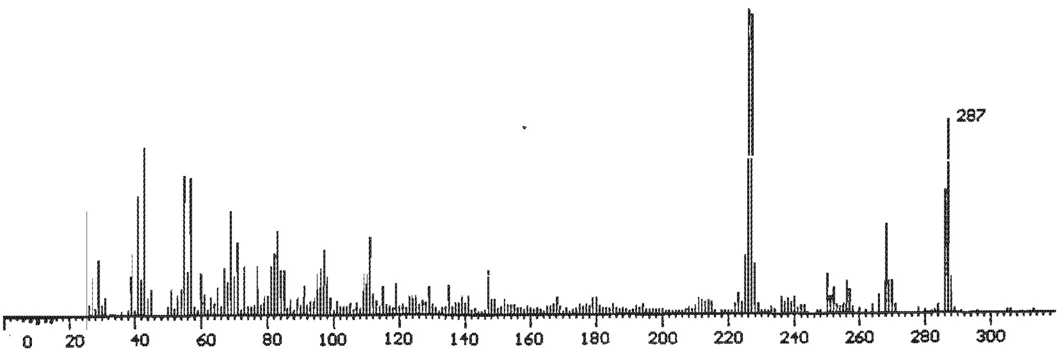


Figure A1.5.2. EIMS of lycorine

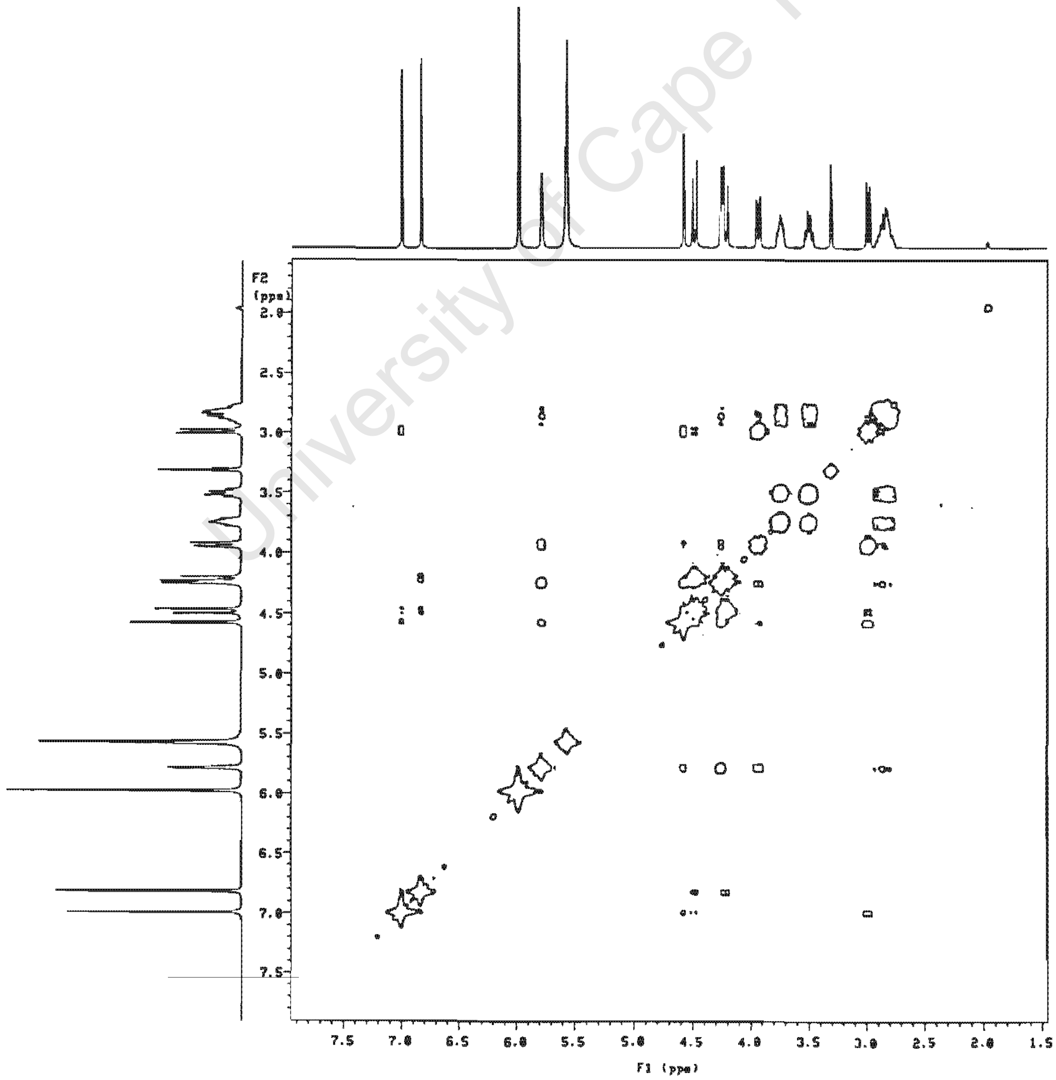
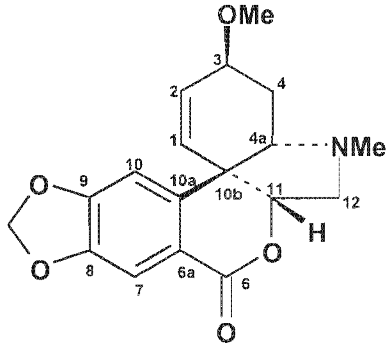


Figure A1.5.3. COSY spectrum of lycorine (CD_3OD, 400MHz)

A1.6. 3-Epimacronine (31)



3-Epimacronine (31)

List of spectra:

- ^{1}H-NMR ($CDCl_3$, 200MHz)
- EIMS

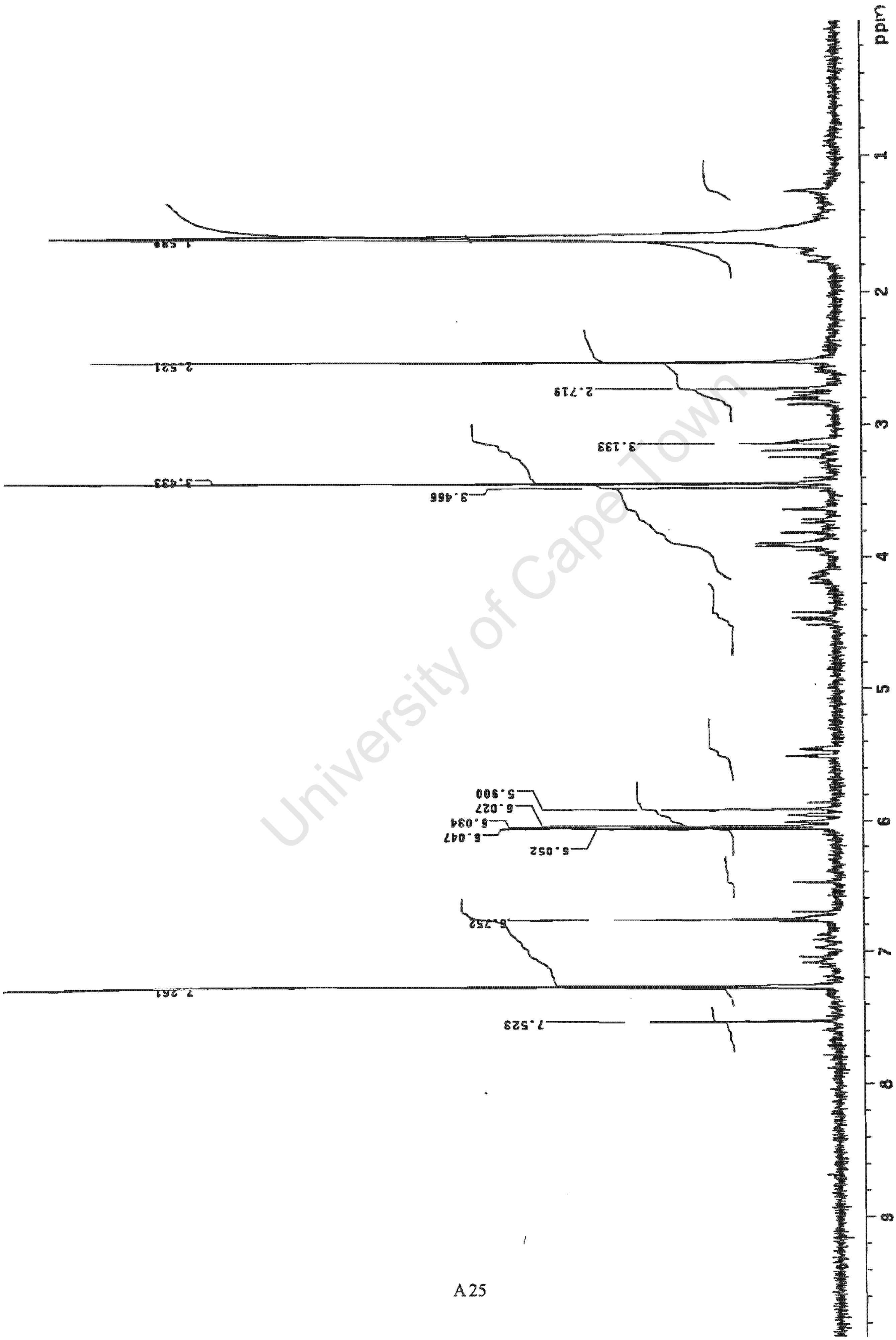


Figure A1.6.1. ^1H NMR spectrum of 3-epimacronine ($CDCl_3$, 200MHz)

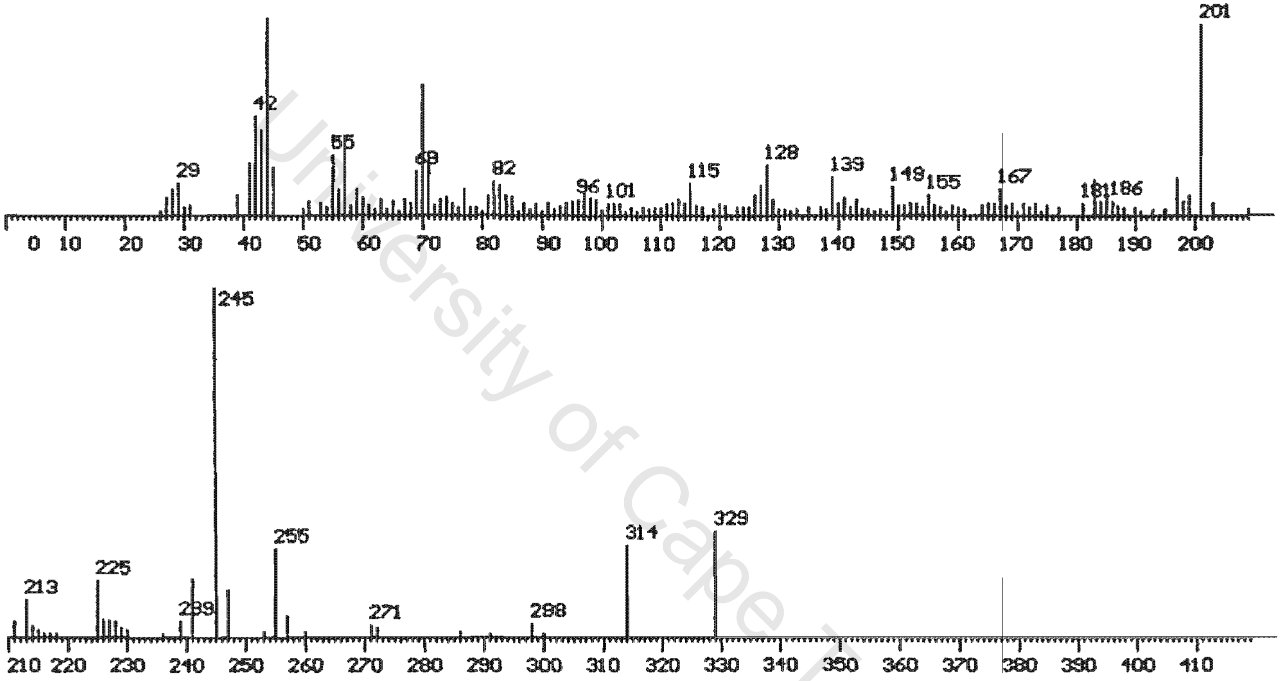
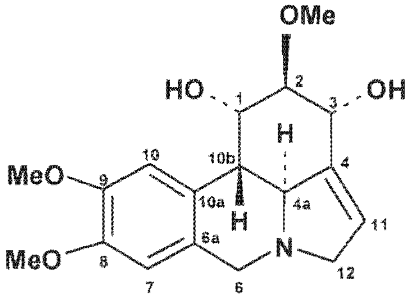


Figure A1.6.2. EIMS of 3-epimacronine

A1.7. Narcissidine (32)



Narcissidine (32)

List of spectra:

- ^{1}H NMR (CDCl$_3$, 200MHz)
- ^{13}C NMR (CDCl$_3$, 200MHz)
- ^{13}C NMR DEPT (CDCl$_3$, 200MHz)
- EIMS

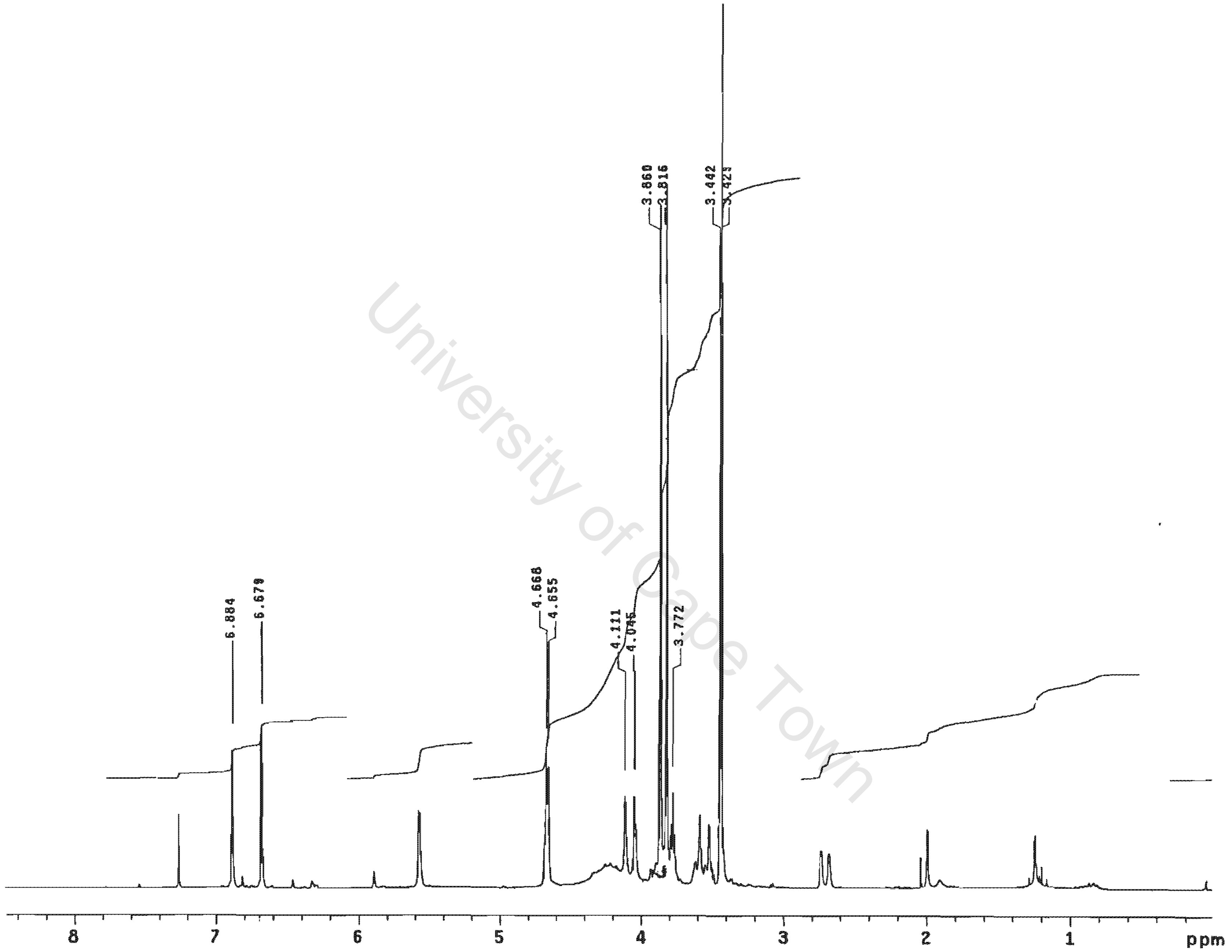


Figure A1.7.1. ^1H NMR spectrum of narcissidine in $CDCl_3$, 200MHz

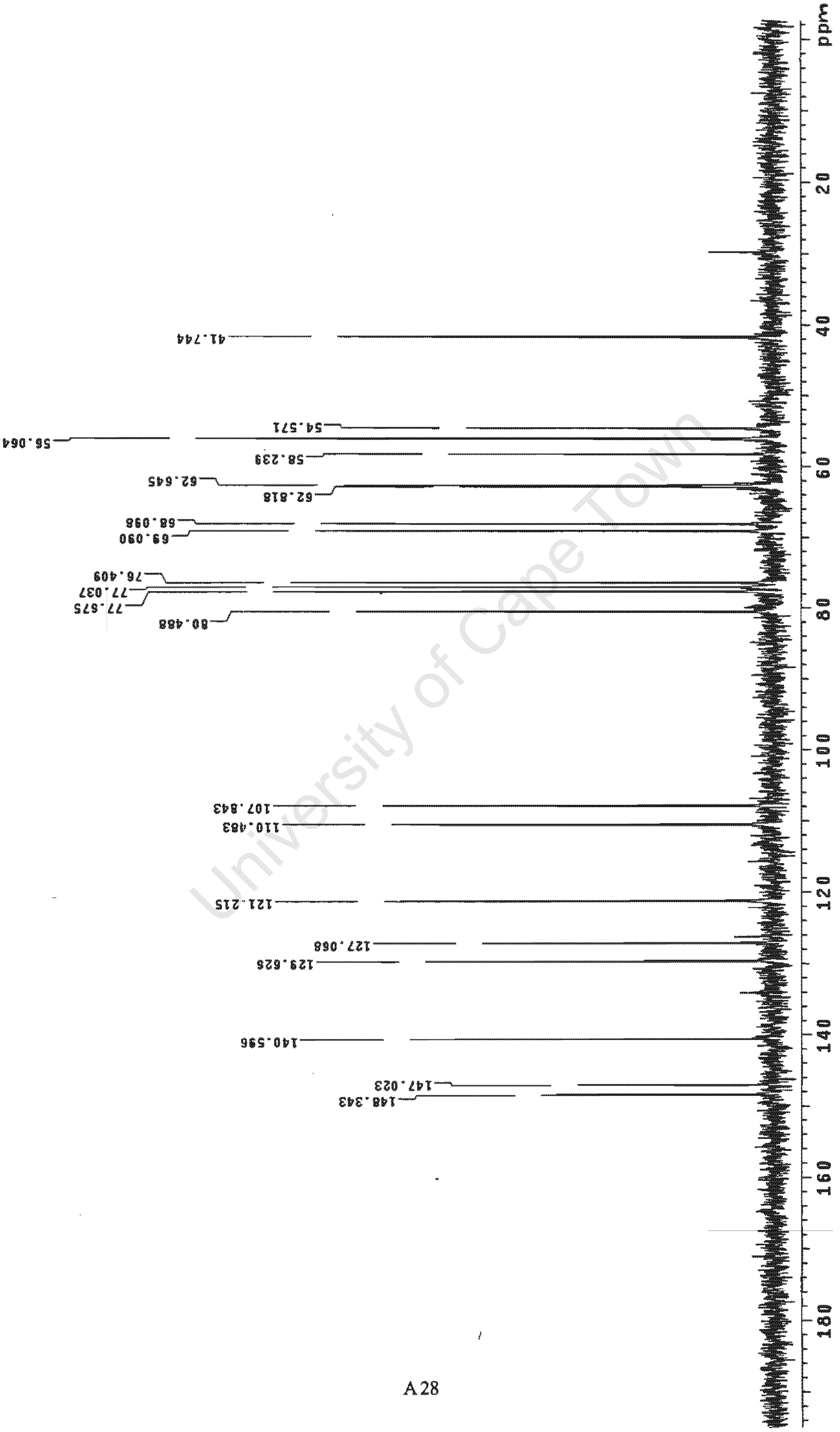


Figure A1.7.2. ^{13}C NMR spectrum of narcissidine in CDCl$_3$, 200MHz

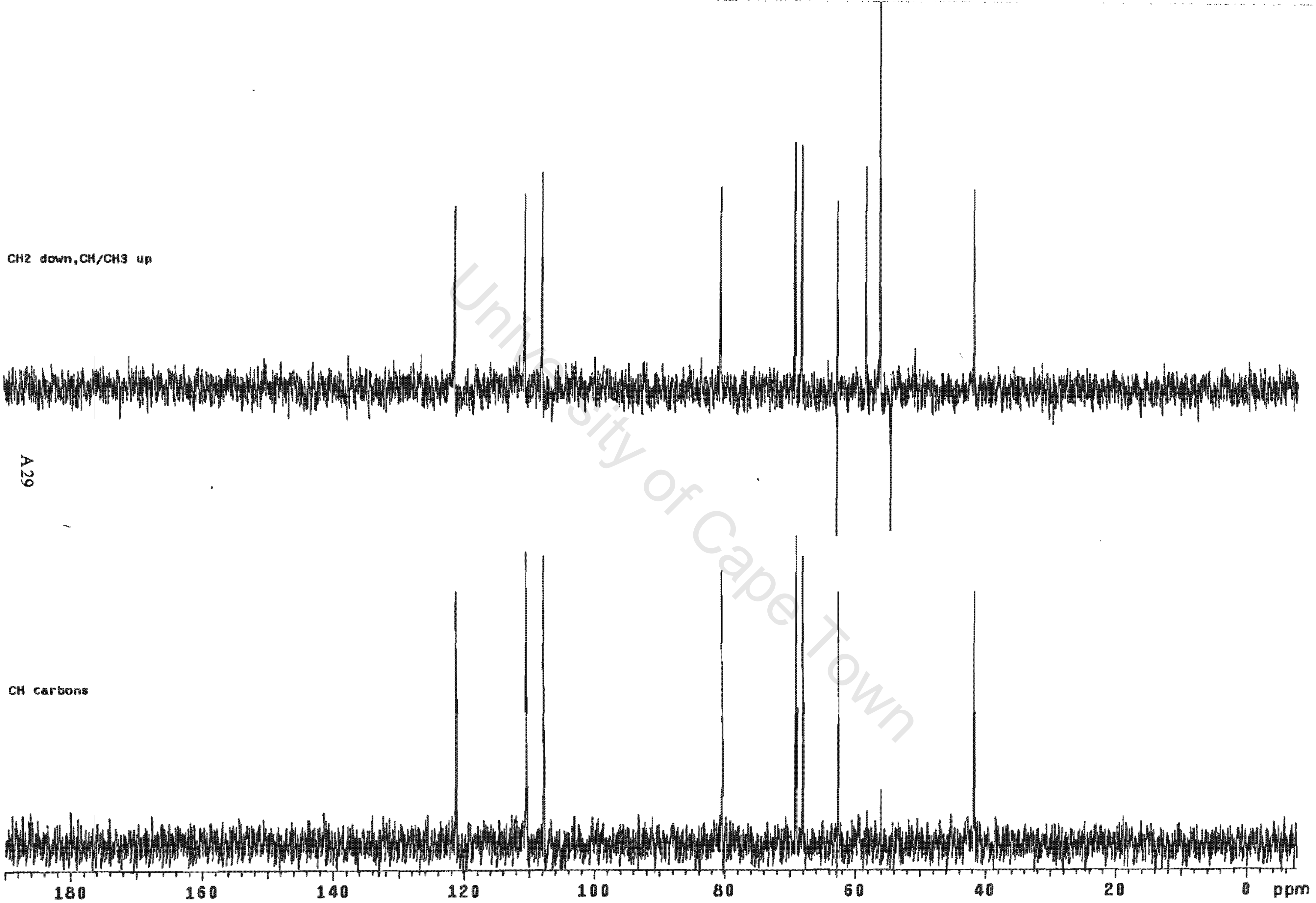


Figure A1.7.3. ^{13}C NMR DEPT spectrum of narcissidine in $CDCl_3$, 200MHz

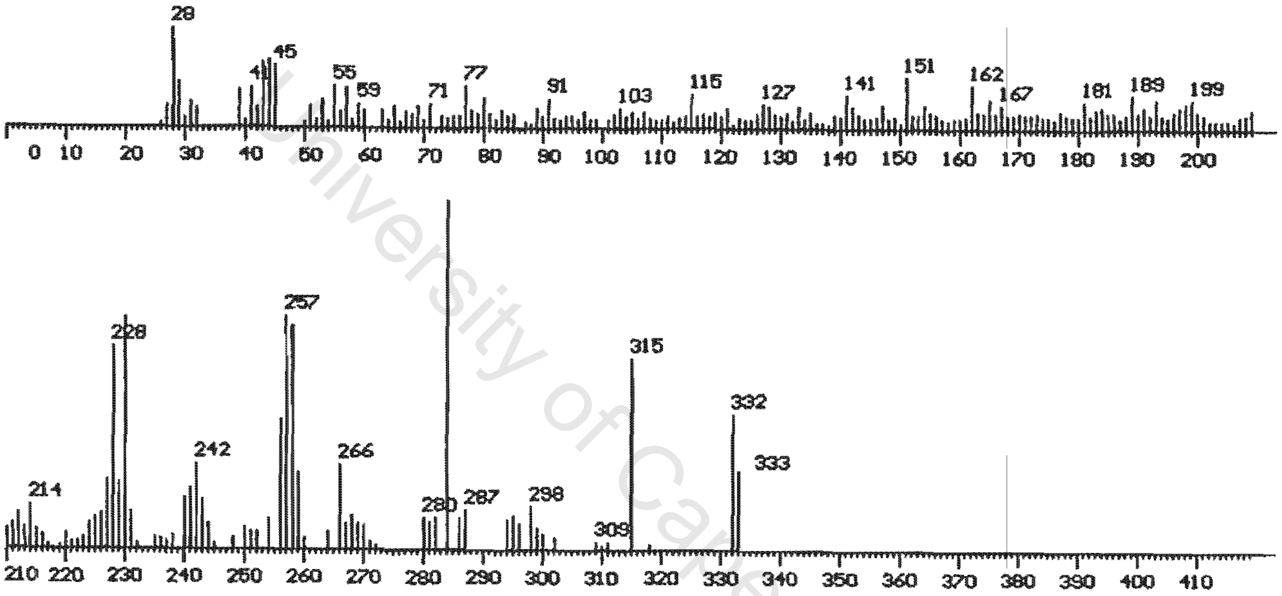
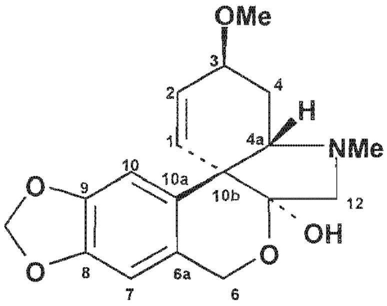


Figure A1.7.4. EIMS of narcissidine

A1.8. Tazettine (8)



Tazettine (8)

List of spectra:

- ^{1}H NMR ($CDCl_3$, 200MHz)
- EIMS

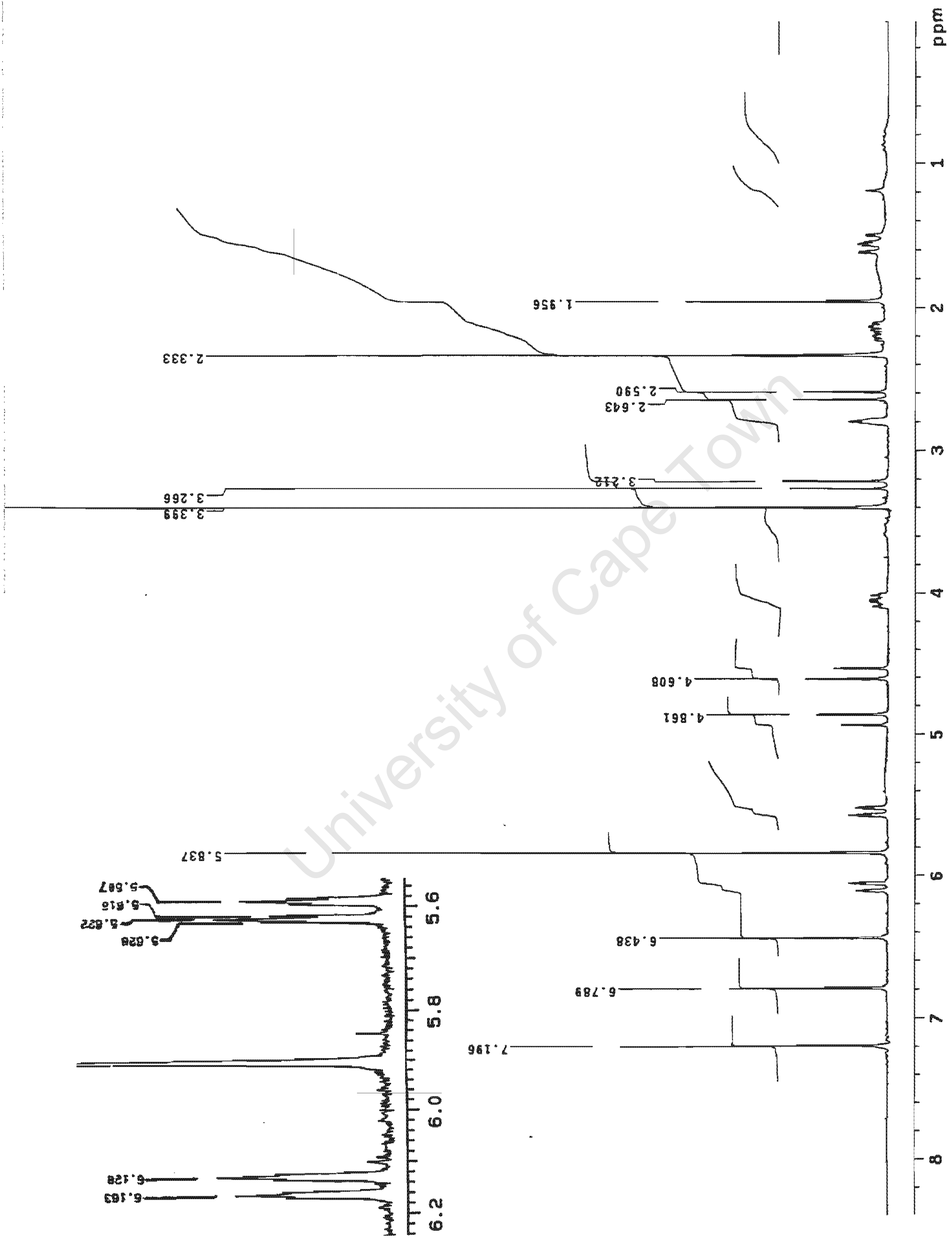


Figure A1.8.1. ^{1}H NMR spectrum of tazettine in $CDCl_3$, 200MHz

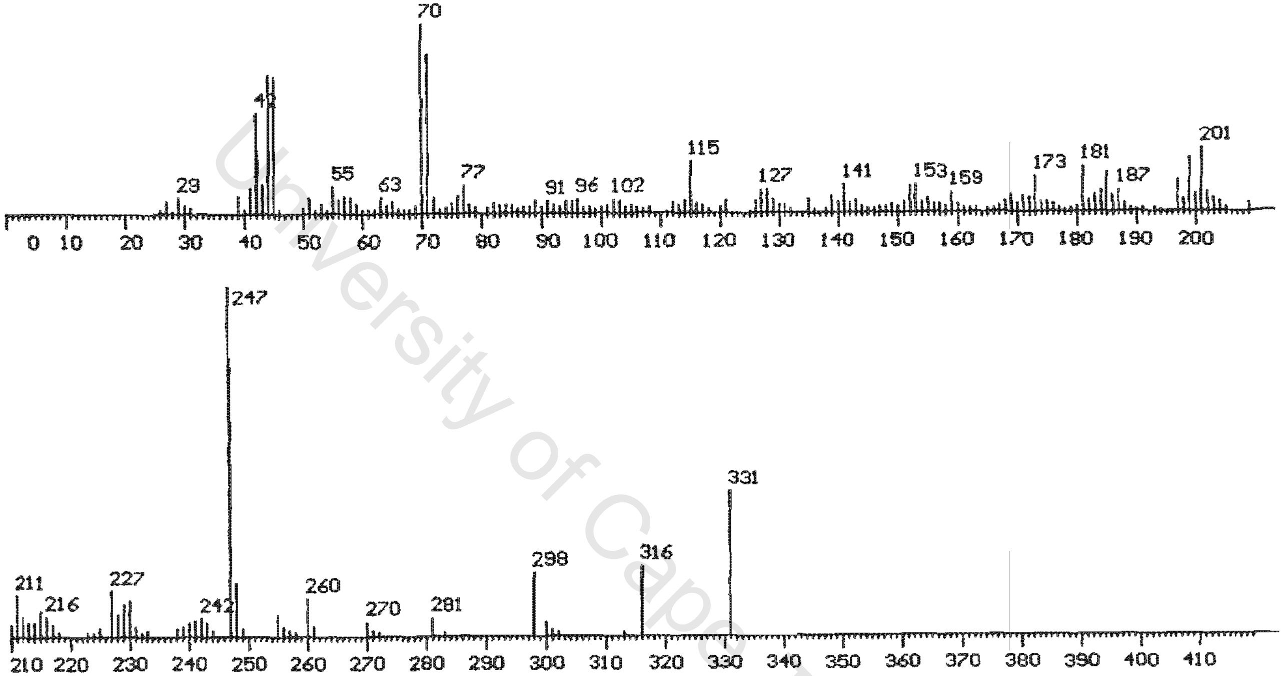
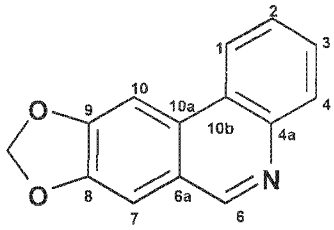


Figure A1.8.2. EIMS of tazettine

A1.9. Trisphaeridine (19)



Trisphaeridine (19)

List of spectra:

- ^{1}H NMR ($CDCl_3$, 300MHz)
- EIMS

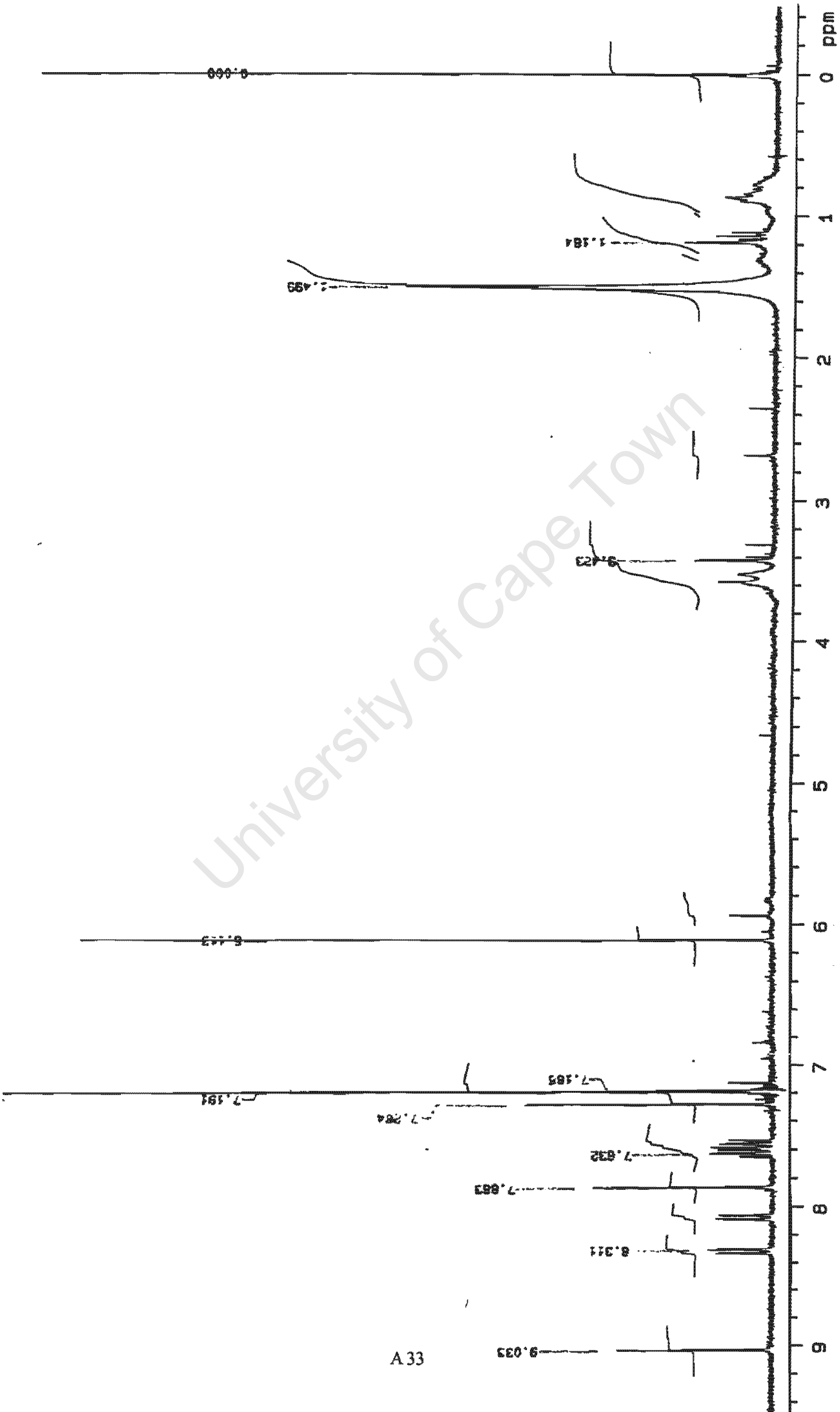


Figure A1.9.1. ^{1}H NMR spectrum of trisphaeridine in $CDCl_3$, 300MHz

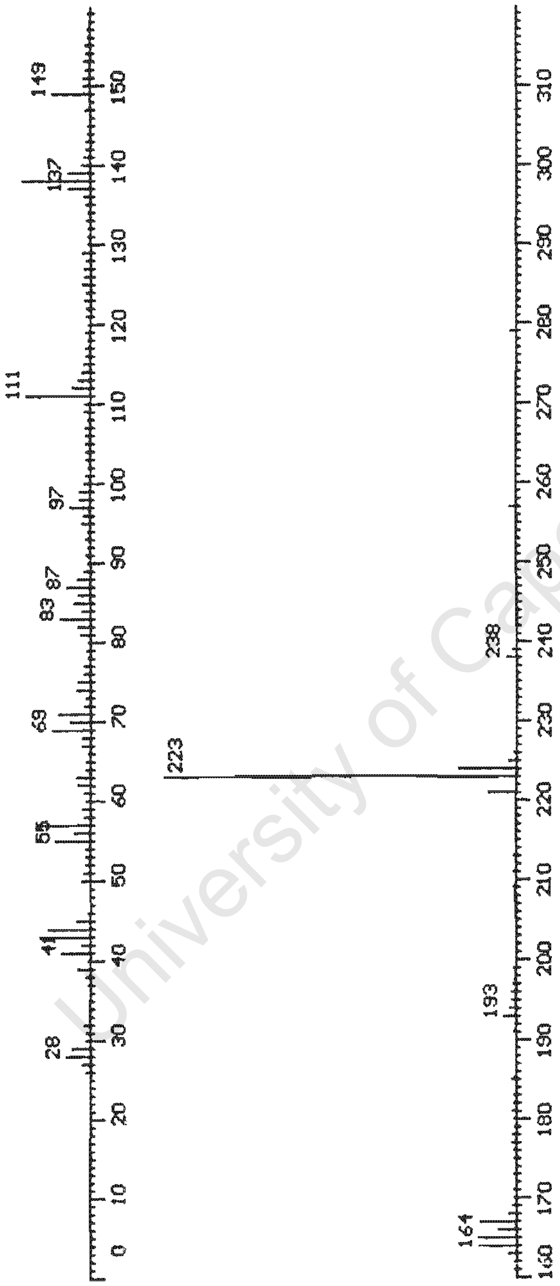
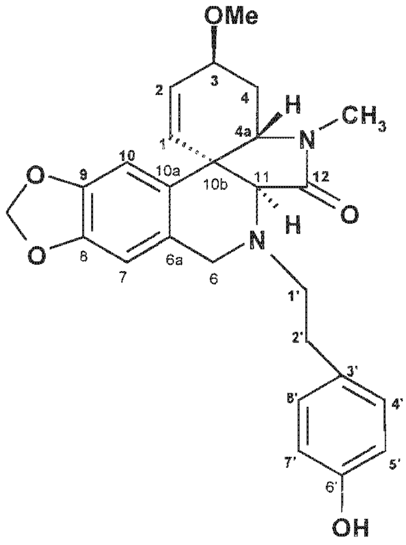


Figure A1.9.2. EIMS of trisphaeridine

A1.10. Obliquine (30)



Obliquine (30)

List of spectra:

- ^{1}H NMR (CDCl$_3$, 500MHz)
- ^{1}H NMR spectra expansions (CDCl$_3$, 500MHz)
- ^{13}C NMR (CDCl$_3$, 300MHz)
- ^{13}C NMR DEPT (CDCl$_3$, 300MHz)
- COSY (CDCl$_3$, 500MHz)
- ROESY (CDCl$_3$, 500MHz)
- HSQC (CDCl$_3$, 500MHz)
- HMBC (CDCl$_3$, 500MHz)
- HREIMS

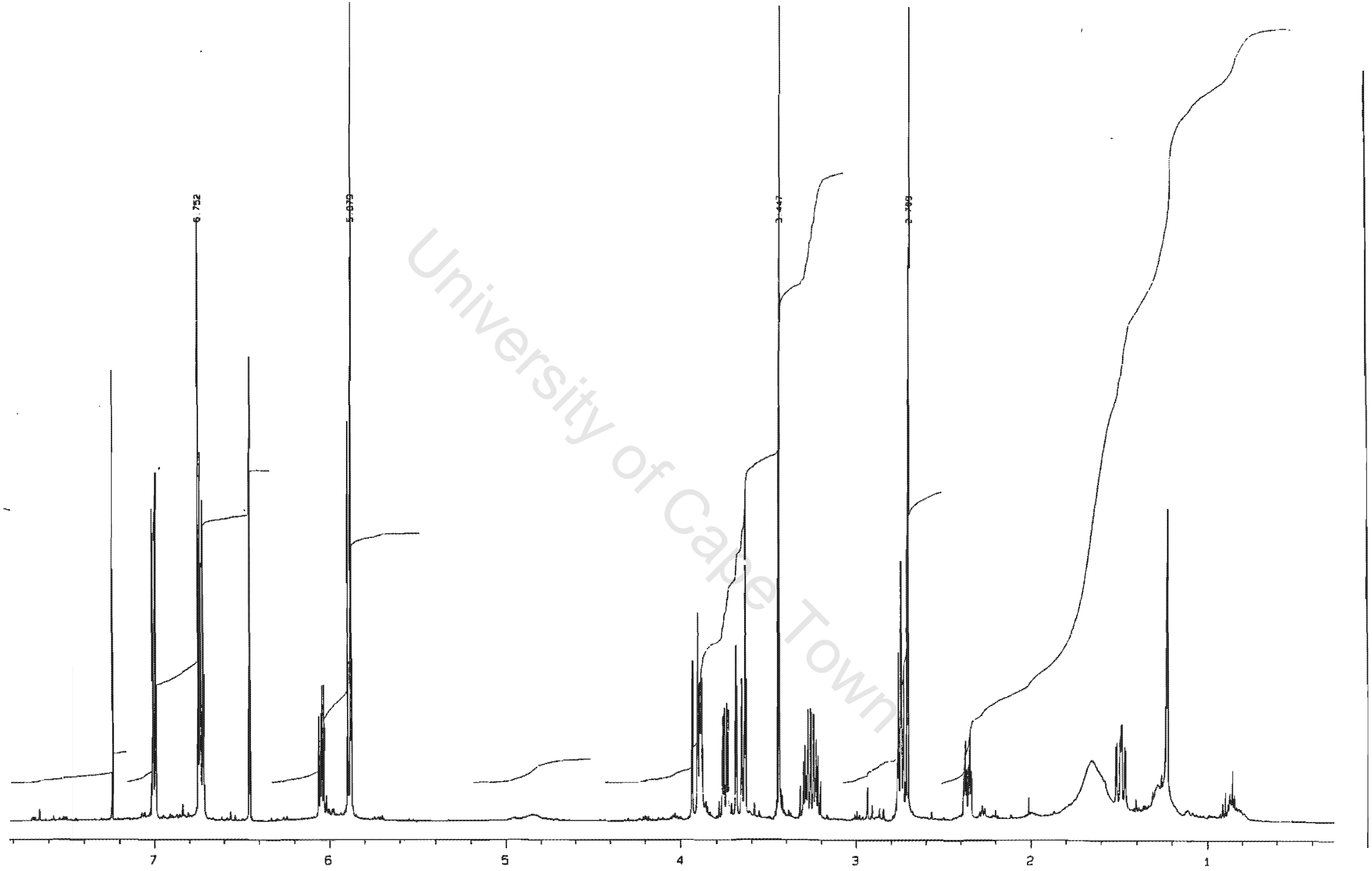


Figure A1.10.1. (a) ^{1}H NMR spectrum of obliquine in $CDCl_3$, 500MHz

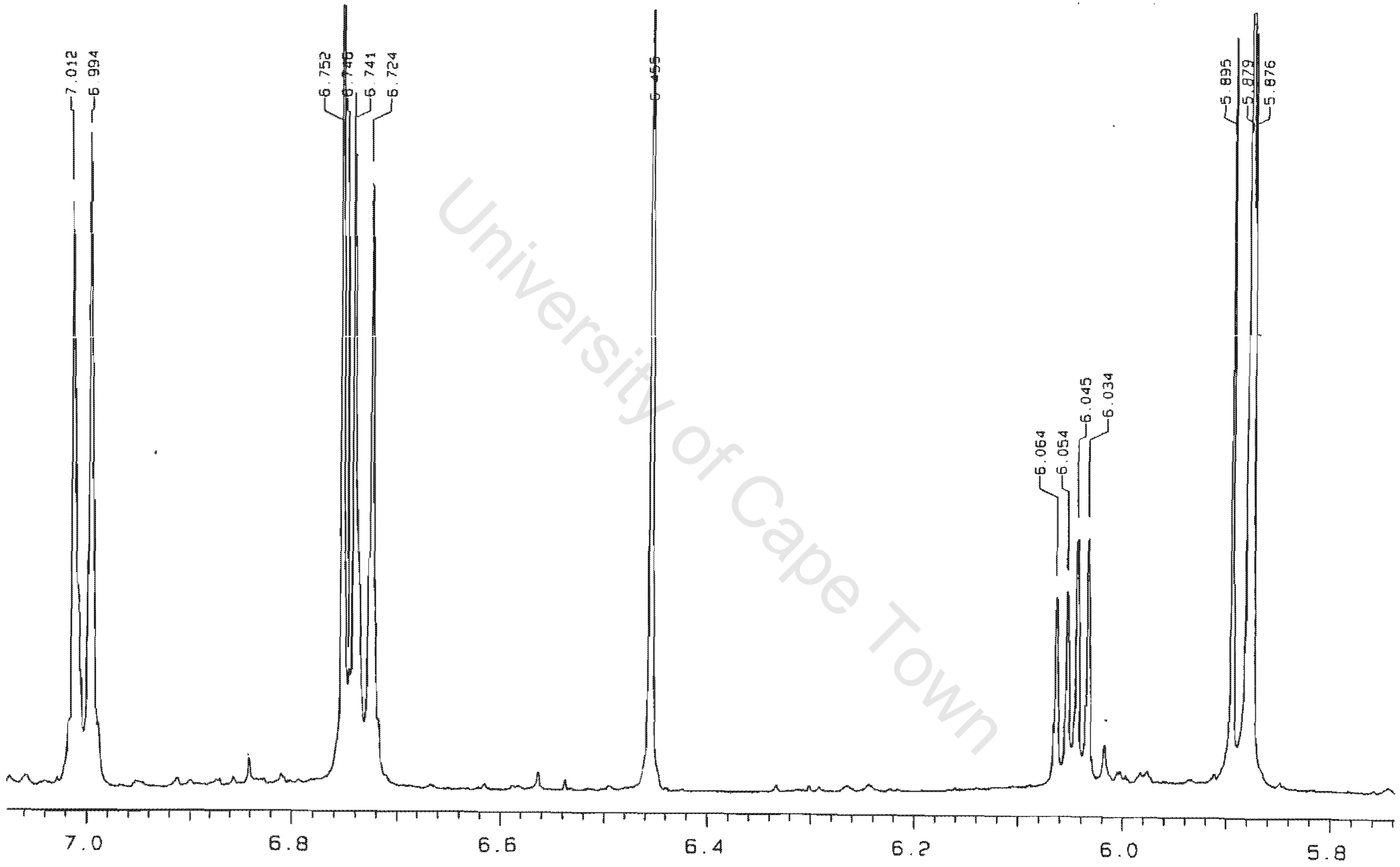


Figure A1.10.1. (b) Expanded ^1H NMR spectrum of obliquine in $CDCl_3$, 500MHz

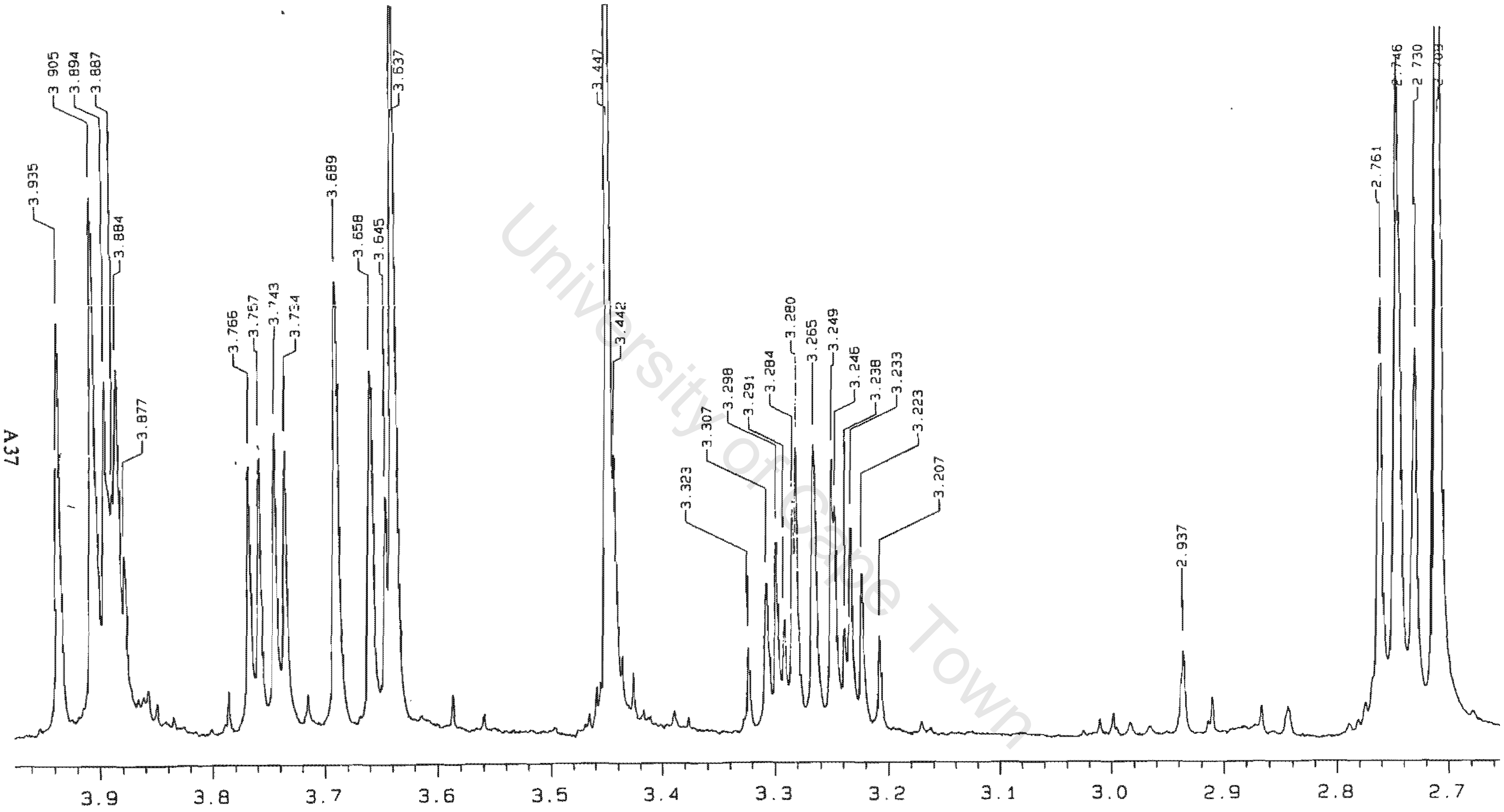


Figure A1.10.1. (c) Expanded ^{1}H NMR spectrum of obliquine in $CDCl_3$, 500MHz

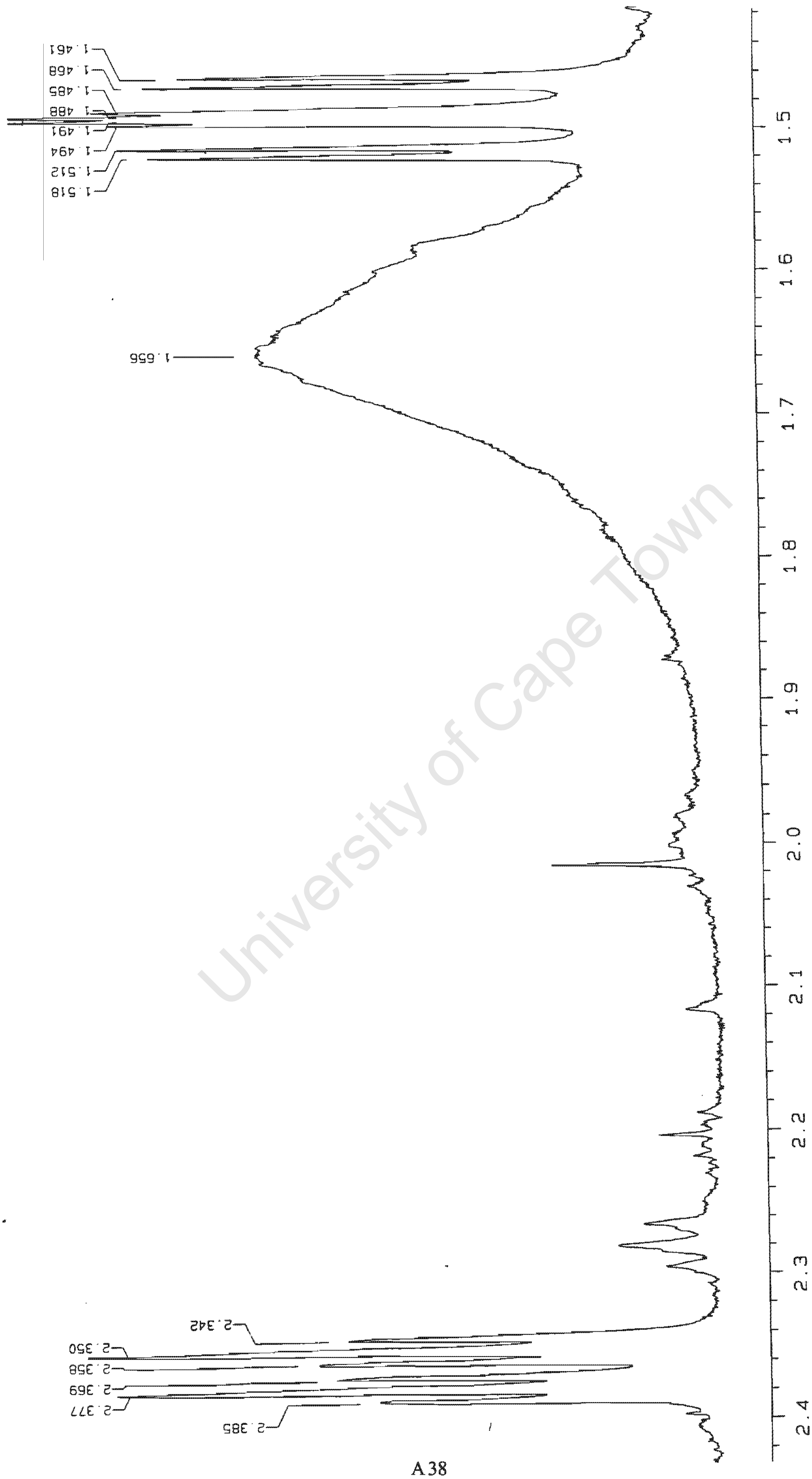


Figure A1.10.1. (d) Expanded ^1H NMR spectrum of obliquine in $CDCl_3$, 500MHz

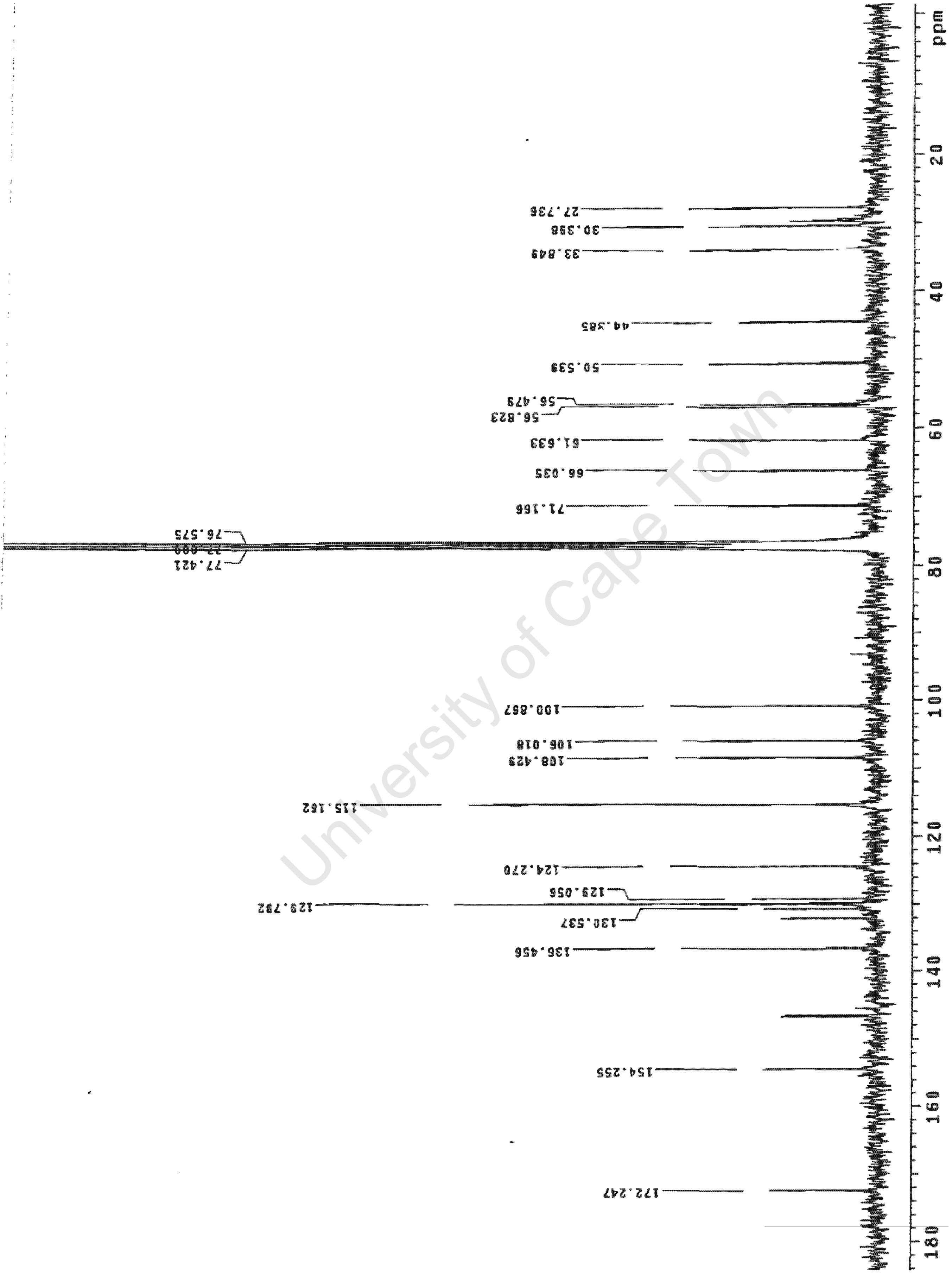


Figure A1.10.2. ^{13}C NMR spectrum of obliquine in $CDCl_3$, 300MHz

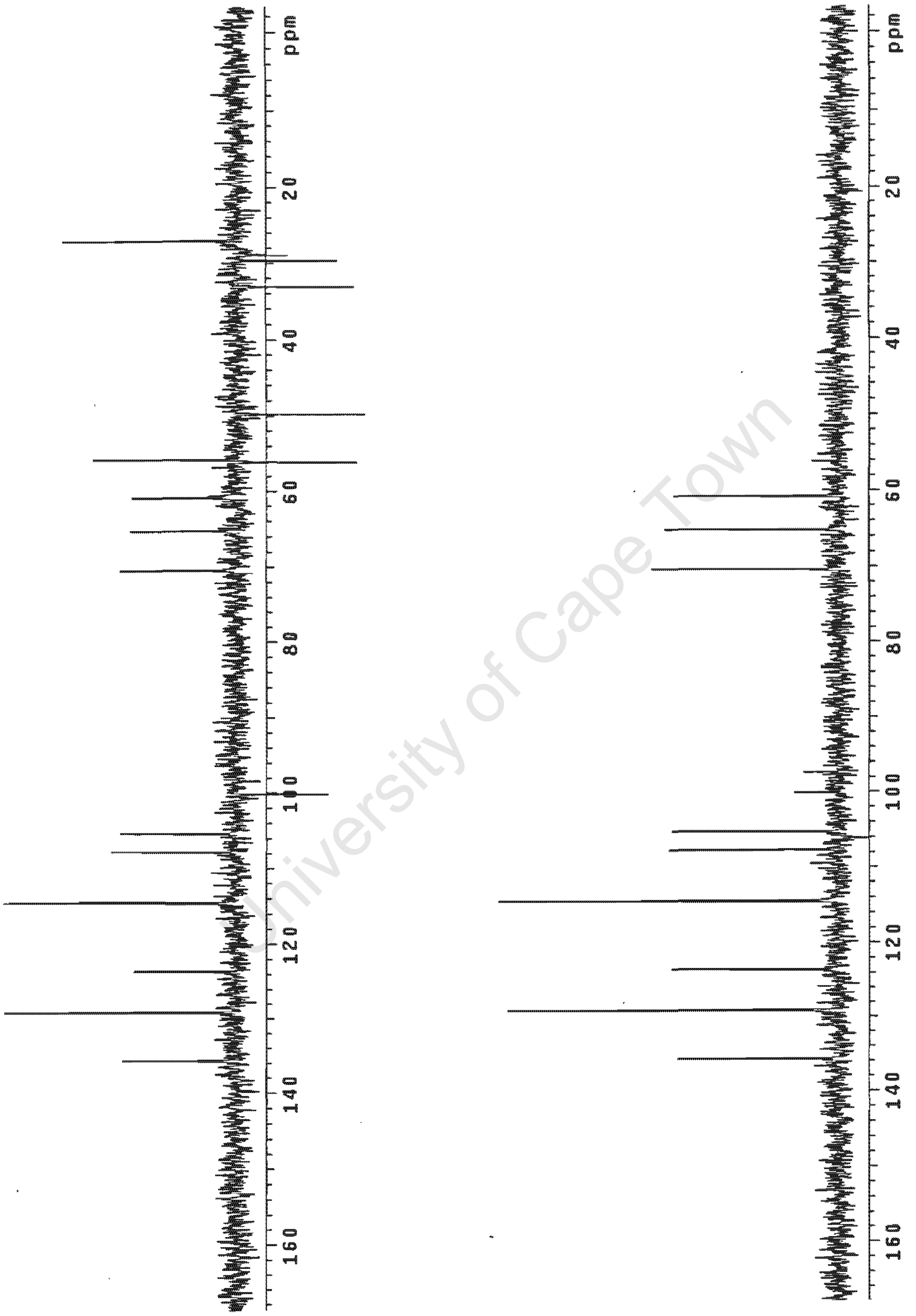


Figure A1.10.3. ^{13}C NMR DEPT spectrum of obliquine in $CDCl_3$, 300MHz

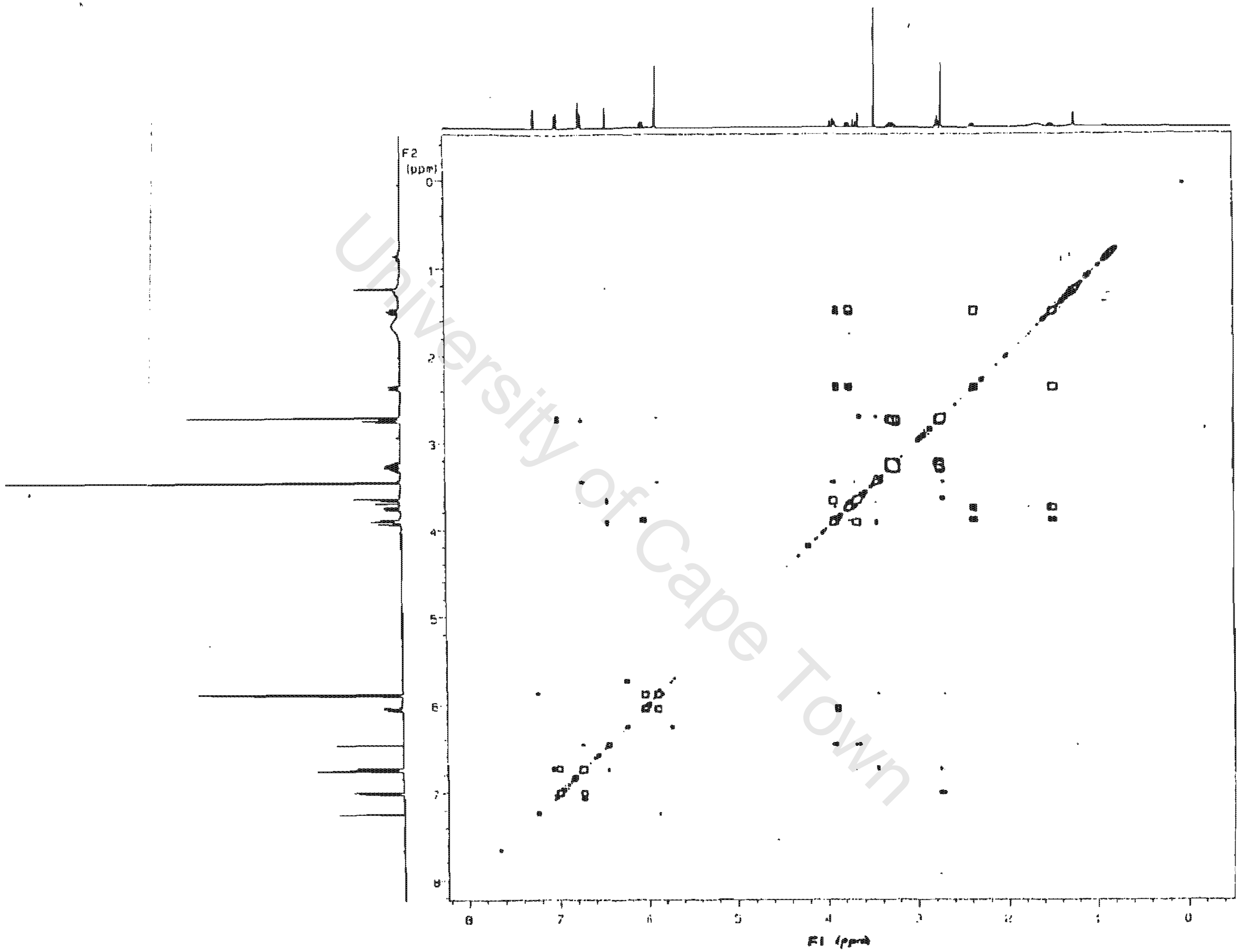


Figure A1.10.4. COSY spectrum of obliquine in $CDCl_3$, 500MHz

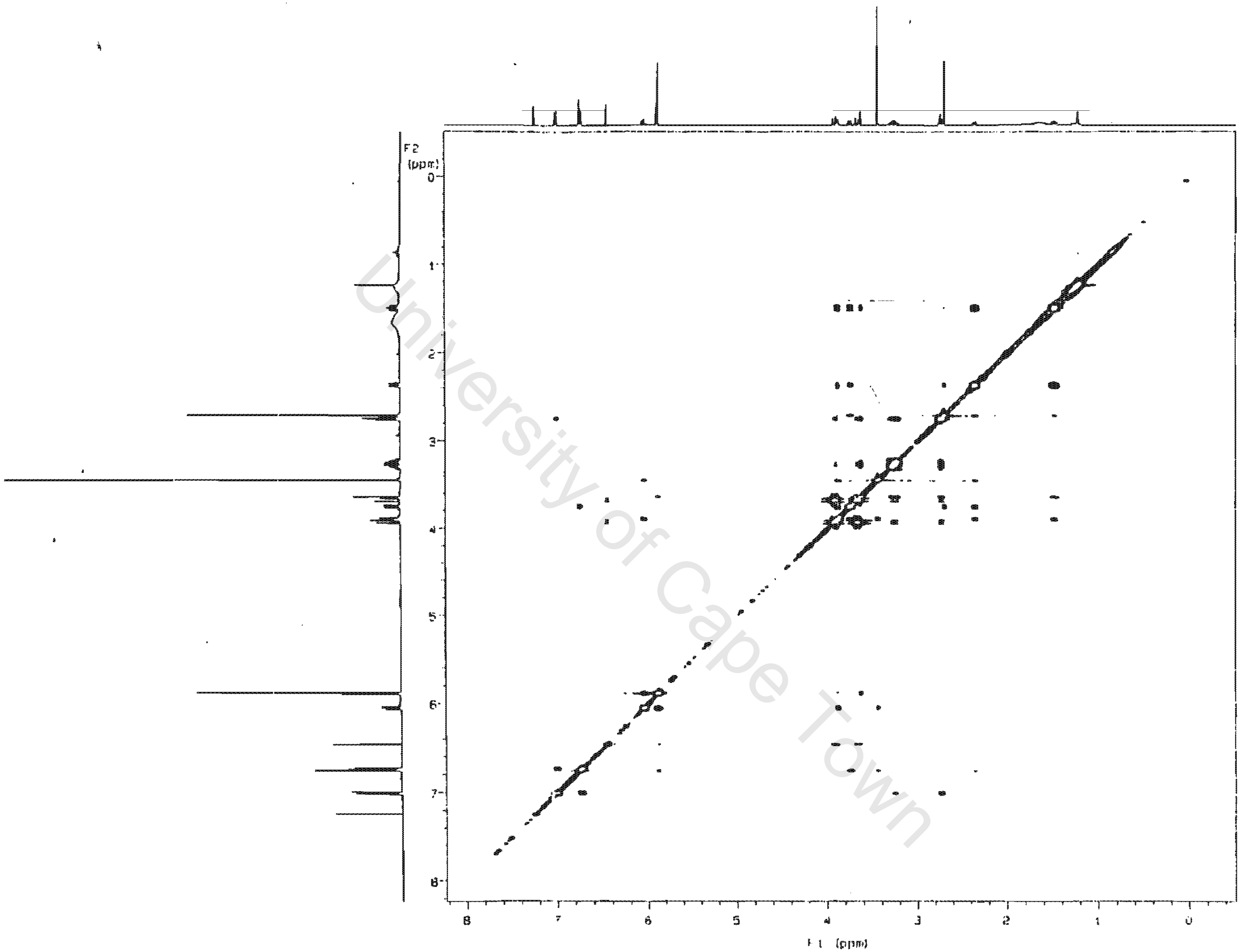


Figure A1.10.5. ROESY spectrum of obliquine in $CDCl_3$, 500MHz

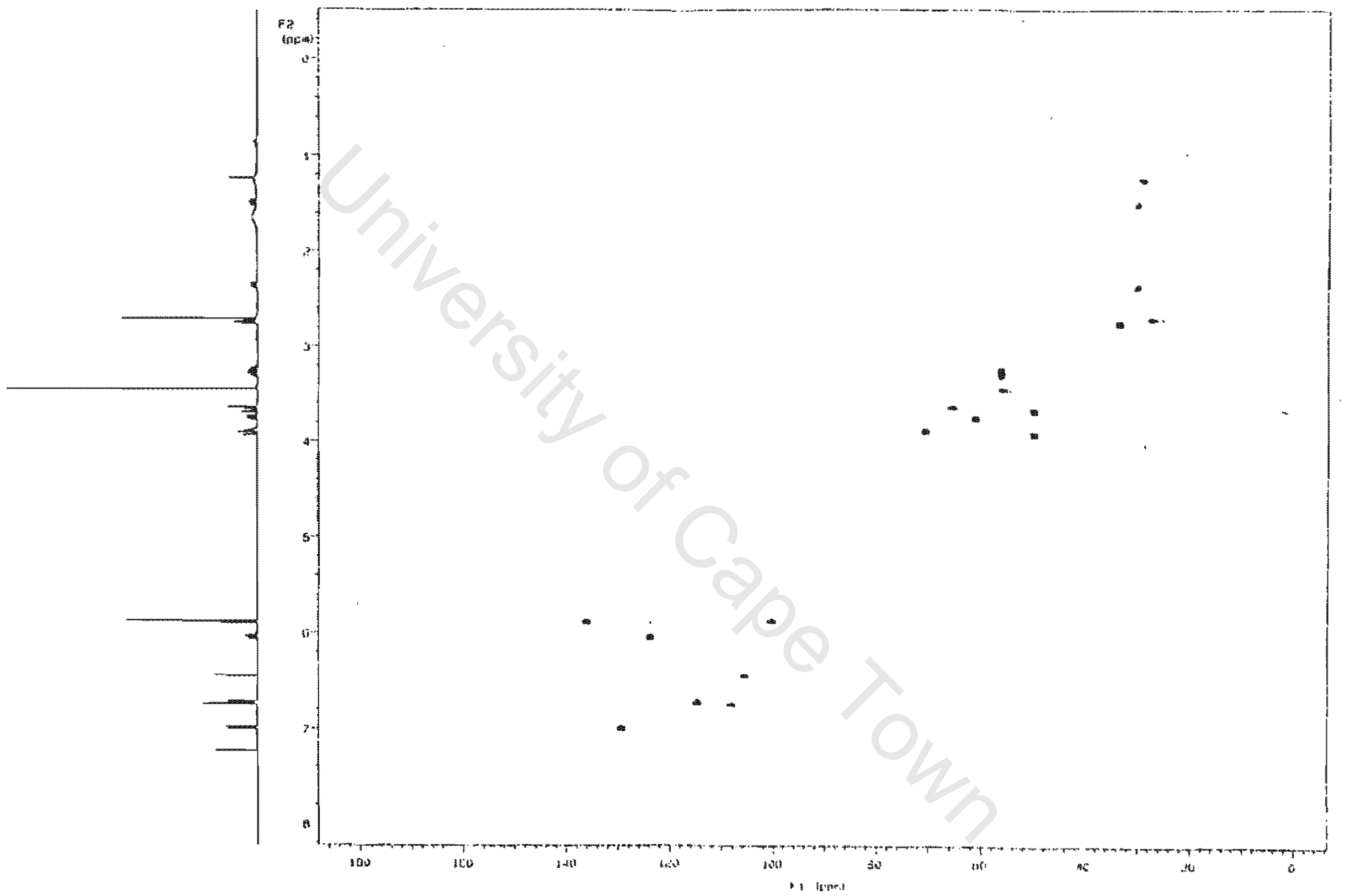


Figure A1.10.6. HSQC spectrum of obliquine in $CDCl_3$, 500MHz

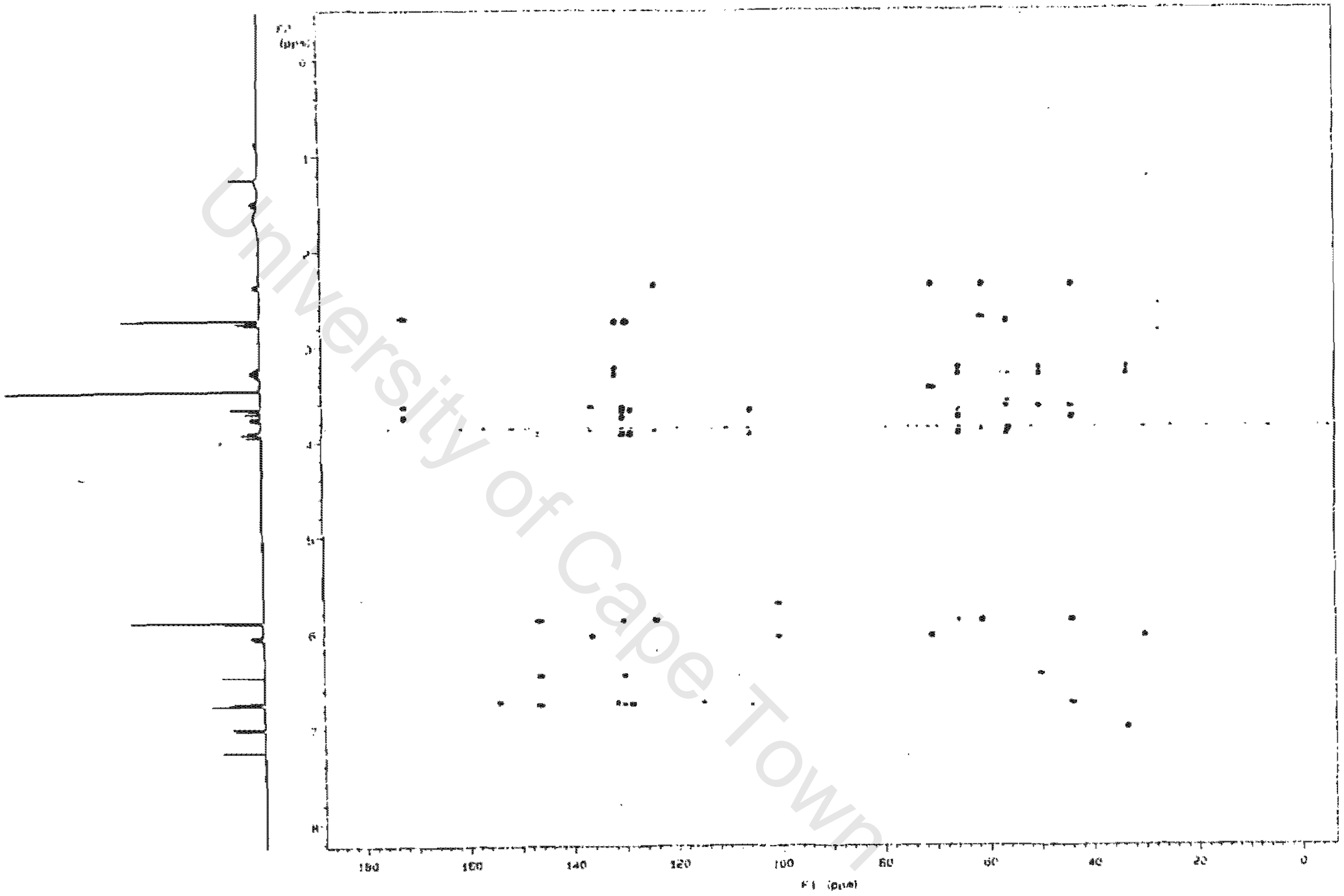


Figure A1.10.7. HMBC spectrum of obliquine in $CDCl_3$, 500MHz

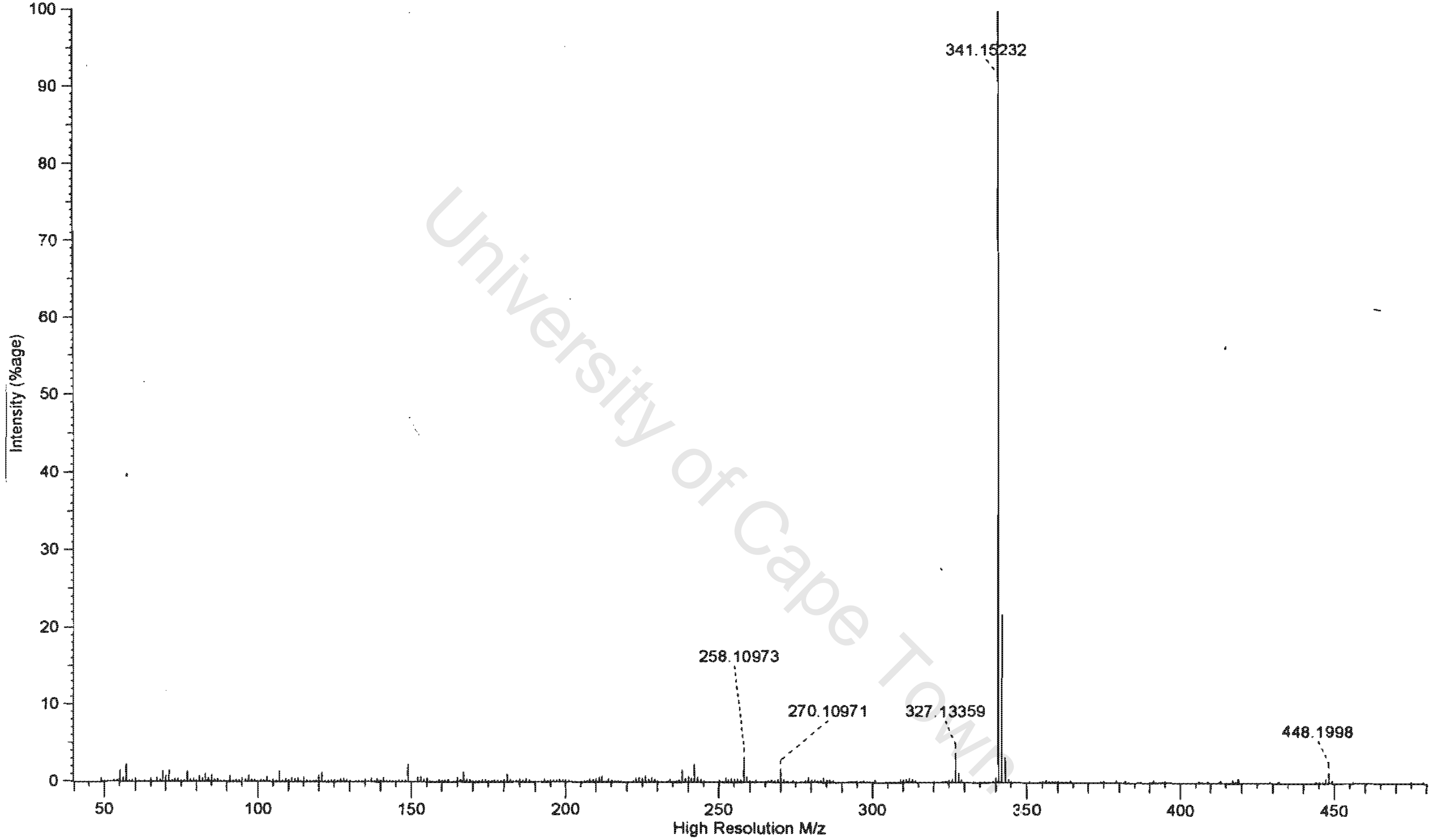


Figure A1.10.8. HREIMS of obliquine

ATOMIC COMPOSITION REPORT (MANUAL)

Selected Isotopes:

Symbol	Min	Max	V'cy	Name
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H	0	44	1	Hydrogen-1
O	0	8	2	Oxygen-16
N	0	3	3	Nitrogen-14

Allowable error = minimum of 20.0 ppm, 6.0 mmu.
Ring/Double Bond limits = [0.5 : 100.0]

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	448.19714	-5.9	-2.7	9.5	$C_{23}H_{30}O_8N$
	448.20250	6.0	2.7	18.5	$C_{29}H_{26}O_2N_3$
	448.20384	9.0	4.0	18.0	$C_{31}H_{28}O_3$
	448.19395	-13.1	-5.9	23.0	$C_{33}H_{24}N_2$
341.15232	341.15281	1.4	0.5	15.0	$C_{22}H_{19}ON_3$
	341.15416	5.4	1.8	14.5	$C_{24}H_{21}O_2$
	341.15013	-6.4	-2.2	10.5	$C_{19}H_{21}O_4N_2$
	341.14745	-14.3	-4.9	6.0	$C_{16}H_{23}O_7N$
327.13359	327.13448	2.7	0.9	10.5	$C_{18}H_{19}O_4N_2$
	327.13180	-5.5	-1.8	6.0	$C_{15}H_{21}O_7N$
	327.13716	10.9	3.6	15.0	$C_{21}H_{17}ON_3$
	327.13850	15.0	4.9	14.5	$C_{23}H_{19}O_2$
258.10973	258.11034	2.4	0.6	4.0	$C_{12}H_{18}O_6$
	258.10900	-2.8	-0.7	4.5	$C_{10}H_{16}O_5N_3$
	258.11302	12.7	3.3	8.5	$C_{15}H_{16}O_3N$

Selected Isotopes:

Symbol	Min	Max	V'cy	Name
C	0	40	4	Carbon-12
H	0	44	1	Hydrogen-1
O	0	8	2	Oxygen-16
N	0	3	3	Nitrogen-14

Allowable error = minimum of 20.0 ppm, 6.0 mmu.
Ring/Double Bond limits = [0.5 : 100.0]

Mass	Calculated	ppm	mmu	R/DB	Formula
417.18295	417.18411	2.8	1.2	19.0	$C_{28}H_{23}ON_3$
	417.18143	-3.6	-1.5	14.5	$C_{25}H_{25}O_4N_2$
	417.18546	6.0	2.5	18.5	$C_{30}H_{25}O_2$
	417.17875	-10.1	-4.2	10.0	$C_{22}H_{27}O_7N$
418.18064	418.18070	0.2	0.1	18.5	$C_{29}H_{24}O_2N$
	418.17802	-6.3	-2.6	14.0	$C_{26}H_{26}O_5$
	418.17668	-9.5	-4.0	14.5	$C_{24}H_{24}O_4N_3$
	418.18658	14.2	5.9	9.5	$C_{22}H_{28}O_7N$

***** End of Atomic Composition Report *****