Cardiovascular effects of (13*S*)-9α, 13α-epoxylabda-6β(19), 15(14)diol dilactone, a diterpenoid isolated from the organic extract of *Leonotis leonurus* leaves, in anaesthetized normotensive rats

OBIKEZE KENECHUKWU CHIBUZO

Thesis submitted in fulfillment of the requirements for the degree of Philosophiae doctor in Pharmacology, School of Pharmacy, University of the Western Cape.

Supervisor: Prof. Pierre Mugabo

Co-supervisor: Prof. Ivan Green

AUGUST 2009

ABSTRACT

Cardiovascular effects of (13S)-9α, 13α-epoxylabda-6β(19), 15(14)diol dilactone, a diterpenoid isolated from the organic extract of *Leonotis leonurus* leaves, in anaesthetized normotensive rats"

K. C. Obikeze

Plants used in traditional medicines have served as sources of some of the drug compounds used in medicines today, and could still serve as leads for the development of new drugs to treat existing chronic diseases such as hypertension. This study was aimed at the isolation and identification of a cardio-active compound from *L. leonurus*, a plant commonly used in traditional medicines in South Africa for the treatment of hypertension and other cardiac problems. The possible mechanisms by which the isolated compound produced its effect on the cardiovascular system were explored using the anaesthetized normotensive rat model.

Fractionation of the organic extracts of the leaves led to the isolation of a novel diterpene, (13*S*)-9α, 13α-epoxylabda-6β(19),15(14)diol dilactone (EDD) whose structure was elucidated using infra red (IR), nuclear magnetic resonance (NMR), mass spectroscopy (MS), and X-ray diffraction analysis. In anaesthetized normotensive male Wistar rats, EDD (0.5 mg/kg – 5.0 mg/kg; IV) produced slight non-significant decreases in systolic pressure (SP), diastolic pressure (DP), and mean arterial pressure (MAP) with the lower (0.5 mg/kg – 2.0 mg/kg) doses, while significant increases in SP, DP and MAP occurred with the higher (3.0 mg/kg – 5.0 mg/kg) doses. All doses of EDD administered also produced significant decreases in heart rate (HR).

Prazosin and reserpine pre-treatment abolished the vasoconstrictive effect of EDD, suggesting an indirect vasoconstrictive effect for EDD via the release of catecholamines.

Atenolol pre-treatment led to increases in the negative chronotropic effect of EDD, while the positive chronotropic effect of dobutamine was significantly decreased by EDD, suggesting the involvement of the β_1 adrenoceptor in the negative chronotropic effect of EDD. In animals pre-treated with verapamil, a cardio-selective Ca^{2+} channel blocker, no significant changes in HR occurred with all EDD doses, but HR values were significantly lower than those obtained with EDD in non pre-treated animals.

The results of this study indicate that (13*S*)-9α, 13α-epoxylabda-6β(19),15(14)diol dilactone, a novel dilactone diterpene isolated from the leaves of *L. leonurus* has an effect on the cardiovascular system. EDD exhibits a dual effect on the cardiovascular system by producing a vasoconstrictive effect accompanied by bradycardia. The vasoconstrictive effect of EDD is probably due to the release of catecholamines, while the negative chronotropic effect is probably due to β_1 adrenoceptor antagonism. Further studies are however required to fully determine the mechanism by which EDD produces its cardiovascular effects.

KEY WORDS

Plants

Leonotis leonurus

Diterpenes

(13*S*)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone (EDD)

Anaesthetized normotensive rat

Cardiovascular effect

Negative chronotropic effect

Vasoconstriction

Blood pressure

Heart rate

DECLARATION

I declare that *Cardiovascular effects of (13S)-9α, 13α-epoxylabda-6β(19), 15(14)diol dilactone, a diterpenoid isolated from the organic extract of Leonotis leonurus leaves, in anaesthetized normotensive rats* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: **Obikeze, kenechukwu chibuzo**

Signed: _____

Date: **12/08/09**

DEDICATION

Dedicated ….

To God strengthens me to do all things.

To the ones whose love and prayers keep me going.

To Nosipho, Dad, Mum, Obinna, Uzoamaka, and Nnanna.

ACKNOWLEDGEMENTS

I am grateful to Jean McKenzie for all her guidance with the structure elucidation experiments of this study and to the late Mr. Yusuf Alexander and Mr. Vinesh Jeaven for their technical support.

CONTENTS

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER FOUR

RESULTS

LIST OF TABLES AND FIGURES

LIST OF APPENDIXES

TABLE OF ABBREVIATIONS

BP	Blood pressure
CC	Column chromatography
CHF	Congestive heart failure
COSY	Correlation spectroscopy
DP	Diastolic pressure
GC-MS	Gas chromatography – mass spectrometry
GLC	Gas-liquid chromatography
HECTOR	Heteronuclear correlation
HMBC	Heteronuclear multiple-bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
HR	Heart rate
HSQC	Heteronuclear single quantum correlation,
IR	Infra red
LC-MS	Liquid chromatography - mass spectrometry
LC-NMR	Liquid chromatography - nuclear magnetic resonance
MAP	Mean arterial pressure
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
SP	Systolic pressure
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy

CHAPTER ONE

Introduction

There is no generally accepted date as to when man first began using plants as a source of medicine, but it is well accepted that plants have for a long time played a dual role as food and medicine. Early practitioners probably observed by trial and error or by pure accident the beneficial and harmful properties of certain plants and this may have sparked an interest in the medicinal properties of plants that endures to this day. Evidence of the extensive use of plants as drugs can be found in ancient literature originating from China, India, and even in Egyptian hieroglyphics (Farnsworth, 1990; Pemberton, 1999; Mahady, 2002; Patwardhan, 2005). Since most ancient African societies had no written language, important traditional medicinal recipes were preserved in oral traditions and examples abound in the folklore of all African societies (Williamson et al., 1996). The use of plants as medicines has remained through the ages, especially in traditional societies in Africa, South America and Asia where traditional medicine remains an integral part of their culture.

Traditional medicinal practices evolved from the fusion of cultural practices with the use of plants, minerals and animals as medicines, and can be defined as the totality of knowledge and practices that are based on an indigenous belief system, which are used for the maintenance of health and the prevention or complete elimination of physical and/or mental disorders (Tabuti et al., 2003). Early medications started out as decoctions, poultices, ointments, or solutions of plants, animal parts and minerals. Twentieth century advances in medicine such as the discovery of penicillin and morphine led to the decline of

the use of traditional medicines and the disappearance of many of the traditional indigenous medicinal practices. As a consequence a great number of these remedies have disappeared with time, although some still presently find use for the treatment of diseases by traditional medicine practitioners of all cultures. In present times, despite the abundance of synthetic and semi-synthetic drugs, a significant proportion of the population of developing countries still depend on traditional medicines for their health care needs. The World Health Organization (WHO) estimates that 60% of the world's population is dependent on traditional medicines for their primary health care needs, with the figure rising to about 80% of the population in developing countries (Farnsworth, 1990; Zhang, 2000; Mulholland, 2005). Interestingly about 15-20 million of the traditional medicine users in developing countries reside in Southern Africa alone (Duncan et al., 1999). South Africa is a country with a large biodiversity of over 30 000 higher plant species of which approximately 3000 are presently used as medicines by an estimated 27 million people (Mander et al., 1997). In addition to this, it is estimated that 80% of the population consult traditional healers first for their health problems (Duke, 2001). It is therefore no surprise that as of 1996, an estimated 20 000 tonnes of over 700 medicinal plant species were traded yearly in South Africa (Mander et al., 1997). In recent times, the persistence of chronic diseases such as asthma, heart diseases, and diabetes, the rising HIV epidemic, coupled with the rising cost of medicines has led to a renewed interest in the use of traditional medicines in societies where traditionally they were used and also in western countries that had previously seen little demand for traditional medicines.

In recent years, an increase in the prevalence of ‘diseases of lifestyle’ such as diabetes and cardiovascular disorders has been noted in developing countries (Reddy et al., 2006). With respect to cardiovascular diseases, these changes have led to an increased disease burden on public health facilities as well as an increase in mortality and morbidity (Tabuti et al., 2003). Though there are many medicines in use for the management and treatment of these diseases, the search for better treatment regimes for hypertension and other cardiovascular diseases continues. In developing countries a large number of the individuals suffering from these diseases are not able to afford the existing medicines and so depend on cheap and readily available traditional medicines for treatment or cure. With the recent increase in research interest in traditional medicines, a lot of the research into traditional medicine has been focused on their use in the treatment of cardiovascular diseases. A large number of the plants used in traditional medicines are claimed to be effective in treating ‘heart problems’, though some claims are more specific for cardiovascular conditions such as congestive heart failure (CHF), systolic hypertension, angina pectoris, arteriosclerosis, cerebrovascular insufficiency, venous insufficiency and cardiac arrhythmias (Mashour et al., 1998; van Wyk et al., 2000).Various researchers have documented the extensive use of plants as diuretics, cardiotonics, antihemorrhagics, antioxidants, and as vasodilators in the treatment of cardiovascular diseases (van Wyk et al., 2000). Examples of such plants include *Ginko biloba* for the treatment of heart diseases, *Stephania tetandra* for the treatment of hypertension, *Lingusticum walliichii* as a circulatory stimulant and an antihypertensive drug, and *Uncaria rhynchophylla* for hypertension (Mashour et al., 1998; Mahady, 2002). In South Africa *Leonotis leonurus* has been used extensively in traditional medicine for the treatment of cardiovascular diseases (van Wyk et al., 2000).

The use of plant medicines has evolved with time, from the use of simple preparations of plant parts, to the use of pure chemical compounds isolated from plants (Noumi et al., 1999). In the 19th and 20th centuries, the isolation of very important chemicals like morphine, quinine, reserpine and penicillin from plants initiated a sustained period of bioprospecting, which only gradually declined with the rise of the petrochemical industry and the development of synthetic and semi-synthetic drugs (Manitto et al., 1981). Presently most of the drugs used in therapy are synthetic in origin, but approximately 25% are or were originally derived from plants and are used as either pure chemicals or as they appear in nature (decoctions or extracts of leaves, barks, fruits, flowers, and roots) (Kutchan, 1995). Plants have also served as templates from which synthetic drugs have been designed. Tropicamide derived from atropine, an alkaloid obtained from *Atropa belladonna*, chloroquine derived from quinine, procaine and tetracaine derived from cocaine obtained from coca leaves are some well known examples. Though the trend of drug discovery has shifted further away from plants as more and more drugs are synthetic in origin, the therapeutic potential of plants still abounds. A recent resurgence in bioprospecting has been fueled by the therapeutic use of plants in situations where conventional therapy has had little effect such as the use of *Silybum marianum* to prevent liver damage from the death cap mushroom and infectious hepatitis, the use of immune stimulants from coneflower for viral infections, artemisinin from *Artemisia annua* to treat drug-resistant malaria and the controversial use of *Cannabis sativa* to treat pain and nausea in cancer and cancer chemotherapy (Kutchan, 1995). The demand for new drugs for the treatment of diseases such as cancer, diabetes, hypertension and AIDS has also contributed to the resurgence in research into the pharmacological effects of traditional medicines.

Presently there are about 119 plant-derived chemical compounds of known structure in use as drugs. Interestingly only 90 species of the 250 000 species of higher plants on the planet contribute to these plant-derived chemical compounds in present use (Cox, 1990). Since plants contain a wide variety of complex organic molecules, they are presently the target of many researchers looking for new bioactive molecules or molecules to serve as lead compounds in pharmaceutical drug design (Rates, 2001).

The WHO has long encouraged the promotion and development of traditional medicines, especially in developing countries where there has been a sustained practice of traditional medicines (WHO, 2002). South Africa has been one of the countries on the forefront of this move as evidenced in recent moves to incorporate traditional medicines into the healthcare system (Department of Health, 2008). In addition to this, a driving force to the resurgence of traditional medicine has been the move by many nations to protect and preserve indigenous knowledge systems and floral biodiversity. In South Africa, the government has promoted research into traditional medicines, both to preserve indigenous knowledge and the essential validation for their use (Department of Health, 2008). Furthermore the increase in the concurrent use of traditional and orthodox medicines has increased the need for information on traditional medicinal plants in order to accurately predict potentially harmful interactions (Rates, 2001). The increase in reliance on traditional medicines, the rejuvenation of interest in plant medicines as a potent source of new drugs for chronic diseases and the need for a pharmacologic profile of plants in use as traditional medicines have lead to a recent increase in research into the pharmacologic effects of plants that are used in traditional medicine. With only about 350 of the commonly used and traded plant

species having undergone chemical investigations, there is still a large pool of plant based chemicals that could potentially deliver the next major new drug (Soejarto, 1996; Mander et al., 1997). This study focused on *L. leonurus*, a plant widely used in traditional medicine in South Africa to treat cardiovascular diseases.

The second chapter of this thesis gives an ethnobotanical description of *Leonotis leonurus.* It also examines the various studies on the composition, pharmacologic and in particular, cardiovascular effects of extracts or compounds isolated from the plant. It also describes various methods used in the extraction and characterization of compounds from plants. The various methods used in cardiovascular research are examined and reasons for choosing the method used in the study are given. Finally the motivation for the study, the hypothesis and the objectives of the study are stated. Chapter three lists the materials and describes the methods used in the study. The methods used in the extraction and characterization of the isolated compound and the cardiovascular model used to evaluate its effects are described. Chapter four contains illustrations and a description of the results obtained. It describes results of the extraction process as well as results of nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy, X-ray crystallography and mass spectroscopy (MS) experiments carried out on the isolated compound. It also contains a description of the results obtained with the administration of the isolated compound and other drugs in the anaesthetized normotensive rat model. Chapter five contains a discussion of the results obtained. It also contains the conclusions derived from the study and recommendations.

CHAPTER TWO

Literature review

Cardiovascular diseases such as hypertension and congestive heart failure (CHF) place a great burden on health services. The possibility of traditional medicines relieving some of this burden has fueled investigations to validate the therapeutic or pharmacologic effects of plants used in traditional medicines to treat cardiovascular diseases. To evaluate the cardiovascular properties of these plants, various methods and pharmacologic models designed to mimic the cardiovascular system have been used. The choice of model usually depends on the specific cardiovascular property of the plant being studied, as most of the plants are described for use for a wide range of cardiovascular diseases ranging from hypertension and congestive heart failure to arteriosclerosis and cerebrovascular insufficiency (Sofowora, 1982; van Wyk et al., 2000). While some researchers have focused on validating the therapeutic claims of the plants by studying them in the form that they are used in traditional medicines, others have attempted to isolate the compounds responsible for the cardiovascular effects of the plants.

In this chapter, the different types of plant extracts and compounds that have been shown to exhibit cardiovascular effects will be discussed. A description of *L. leonurus*, the plant that is the focus of this study is given, and its traditional use and reports from previous investigations on extracts of the plant are discussed. Some of the methods employed in the isolation and identification of compounds from plants and some *in vivo* and *in vitro* models used in the evaluation of cardiovascular activity will also be discussed. Finally, a

description of the cardiovascular system and its regulatory mechanisms as well as the mechanisms by which drugs can alter cardiovascular activity are discussed.

2.1 Plant and plant compounds with cardiovascular effects

All traditional medicines include recipes of aqueous decoctions of various plant parts such as the leaves, stems, roots, barks, and fruits, and for this reason most investigations on the cardiovascular activity of plants have focused on the aqueous extracts. In studies of the cardiovascular properties of the leaves of *Eremophilia alternofilia*, a traditional Aboriginal medicinal plant, the aqueous extract was reported to mediate an initial, but transient, positive inotropic effect followed by an immediate negative inotropic effect accompanied by an increase in heart rate and coronary perfusion on isolated rat hearts perfused by Langendorff perfusion (Pennacchio et al., 1995). The aqueous extract of the bark of *Terminalia arjuna* produced positive chronotropic and inotropic effects in studies on the isolated frog heart, while in anaesthetized dogs the aqueous extract of the dried bulbs of *Allium sativum* produced diuretic and natriuretic effects (Chopra et al., 1958; Pantoja et al., 1991; Pantoja et al., 1996). Extracts of *Agapanthus africanus*, *Agave Americana*, *Adenopodia spicata*, *Clausena anisata*, *Dietes iridioides*, *Stangeria eriopus* and *Tulbaghia violacea* were shown by Duncan and co-workers (1999) to inhibit angiotensin converting enzyme (ACE) in assays of plants commonly used in South Africa to treat hypertension. Organic extracts of plants have also been studied for cardiovascular effects, with Jager and co-workers (1996) reporting cyclooxygenase enzyme inhibition with ethanol extracts of *L. leonurus.* Cometa and co-workers (2001) reported reversible increases in blood pressure with methanolic and butanolic extracts of *Curculigo pilosa* in anaesthetized normotensive

rats while methanol extracts of the leaves of *Brillantaisia nitens* exhibited vasorelaxant properties in strips of rat aorta (Dimo et al., 2006).

Pure compounds isolated from plants have also been studied for their cardiovascular effects, with the digitalis glycoside digoxin probably the best known example. In one of the early experiments to determine the cardiovascular effects of plant compounds, a glycoside isolated from the bark of *Terminalia arjuna* was found to increase the force of contraction of the frog heart and also to raise blood pressure (Dwivedi, 2007). Tetrandrine, an alkaloid extract of *S. tetrandra*, was reported to induce and sustain hypotensive effects in rats for more than 48 hours, with effects similar to those of verapamil, a calcium ion channel antagonist also used in the study (Mashour et al., 1998). The alkaloids harmine, harmaline and harmalol, were found to decrease heart rate in normotensive dogs and isolated rat hearts, and increased developed pressure, peak aortic flow and myocardial contractile force in isolated perfused rat hearts (Aarons et al., 1977). Ulubelen in 2003 reported antihypertensive activity with two diterpenoids from *Salvia syriaca*; ferruginol and 3β-hydroxystigmast-5-en-7-one. Diterpene kaurenoids isolated from *Alepidea amatymbica* and *Xylopia aethiopica* were reported to reduce blood pressure and heart rate in anaesthetized rats, dilate coronary vessels, and also exhibit a diuretic effect comparable to that of hydrochlorothiazide (Somova et al., 2001). The *neo*-clerodane diterpenoids, (12*R*)-12-hydroxycascarillone and 5-hydroxy-*cis*-dehydrocrotonin isolated from *Croton schiedeanus* were reported to produce a vasorelaxant effect in isolated aorta rings, while trans-dehydrocrotonin isolated from *Croton cajucara* induced hypotension and bradycardia in anaesthetized normotensive rats (Guerrero et al., 2004; Silva et al., 2005). The literature so far indicates that plant extracts and compounds so far investigated mostly exhibit

hypotensive effects either by reducing heart rate or dilating blood vessels or a combination of both. This does not indicate that plants cannot exhibit hypertensive effects, but rather is an indication that most investigations are guided by the traditional uses of the plants, hence the predominance of hypotensive effects. Interestingly some compounds isolated from plants have exhibited a dual effect on the cardiovascular system. Labd-8 (17)-en-15-oic acid and labdane-302, two terpenes isolated from *Xylopia langsdorffianna* produced both hypotension and tachycardia in anaesthetized normotensive rats (de Oliveira et al., 2006; Lahlou et al., 2007). Visnagin, isolated from the fruits of *Ammi visnaga*, a plant used for the treatment of angina pectoris, was found to lower blood pressure without any effect on the heart rate in anaesthetized male Wistar rats by Duarte and co-workers (2000). In addition to the phytochemical classes such as alkaloids and terpenes mentioned above, other phytochemical classes such as glycosides are known to exhibit cardiovascular effects, and flavonoids have been documented to either have a direct effect on the cardiovascular system, or to be cardio protective through their lipid lowering actions (Dryskog et al., 2005; Narender et al., 2006).

2.2 *Leonotis leonurus*

Leonotis leonurus R. BR (Laminaceae) is one of ten species that derive their genus name - leonotis from the Greek words "leon" (a lion) and "otis" (an ear), describing the typical lion ear shaped flowers of the plants. The plant is indigenous to the Southern tip of Africa, and is usually found at forest margins, on rocky hillsides, riverbanks and in tall grasslands of the Eastern Cape, Western Cape, Kwazulu-Natal and Mpumalanga provinces of South Africa (Batten, 1986; van Wyk et al., 2000). The plant is a shrub growing between two and

five meters in height. It has a thick wooden base, pale brown branches and long, hairy and narrow leaves with serrated upper edges arranged opposite each other on the stems. The bright orange flowers are tubular in shape, and are arranged in circles along branch ends, and the fruit consists of four little nutlets seated at the base of the calyx tube (Adamson and Salter 1950; van Wyk et al., 2000). Locally the plant is known in the various languages as wilde dagga (Afrikaans), wild dagga (English), umunyane (Isizulu), lebake (Sotho) and umfincafincane (Isixhosa) (Watt and Breyer-Brandwijk 1962; van Wyk et al., 2000).

Figure 2.1: *Leonotis leonurus* R. BR (Laminaceae)

In going with the traditional use of most shrubs, most documented evidence of the use of *Leonotis leonurus* in traditional medicine indicates the use of the leaves, stems, flowers and fruits, administered as decoctions, inhalations, infusions, tonics, or poultices depending on the illness (Hutchings et al., 1996). In most communities, the plant is used in traditional

medicine for a wide variety of illnesses ranging from minor ailments like bites, stings and boils to major conditions like hypertension, tuberculosis, and epilepsy (Hutchings et al., 1996; Noumi et al., 1999; Duke, 2001; Ososki et al., 2002). In certain communities however its use is more specific; for example it is reported to be used for the treatment of menstrual disorders in the Dominican Republic, for hypertension by the Bafa in Cameroon, as a strong purgative and an enemagogue by the Hottentots of South Africa, and to relieve hemorrhoids by the Zulus (Watt and Breyer-Brandwijk 1962). The dried leaves of the plant have been reported to possess mild hallucinogenic effects when smoked, and the leaves were also smoked to relieve epileptic fits by the Hottentots of South Africa (van Wyk et al., 2000).

Phytochemical analysis of the leaves and stems of *L. leonurus* have shown that the plant contains alkaloids, flavonoids, resins, tannins, terpenoids, saponins, and quinines (Watt and Breyer-Brandwijk 1962; Laonigro et al., 1979; Bienvenu et al., 2002). Phytochemical studies on the plant have led to the isolation of a number of compounds from the stems and leaves of the plant. The compounds so far isolated from the plant include leonurine a mildly psychoactive alkaloid, marrubin a labdane type lactone that has also been isolated from *Marrubium vulgare* and is thought to be an artifact of premarrubin produced during extraction, marloth a dark green resin that is also thought to be responsible for the plant's narcotic properties, leonitin a terpenoid also found in *Leonotis ocymifolia*, 1,2,3-trihydroxy-3,7,11,15-tetramethylhexadecan-1-yl-palmitate, a diterpene ester, two labdane terpenoids known as compounds X and Y, and leonurun (figure 2.2) (Kaplan and Rivett 1968; Rivett 1964; Habtemariam et al., 1994; Hutchings et al., 1996; van Wyk et al., 2000; Duke, 2001; McKenzie et al., 2006; Agnihotri et al., 2009). Most of the compounds so far

isolated from the plant have been terpenoids, and the terpenoids so far isolated from *L. leonurus* have closely related structures usually with the differences being the substituent on C-20 and stereoisomerism around C-9, C-13 (see figure 2.2). Other compounds however, are quite dissimilar in their structures (figure 2.2). It has been noted that the Leonotis species is very rich in terpenoids, especially the diterpenoid lactones, and some researchers have also noted possible geographical differences in the phytochemical constituents of *L. leonurus* (Laonigro et al., 1979; McKenzie et al., 2006).

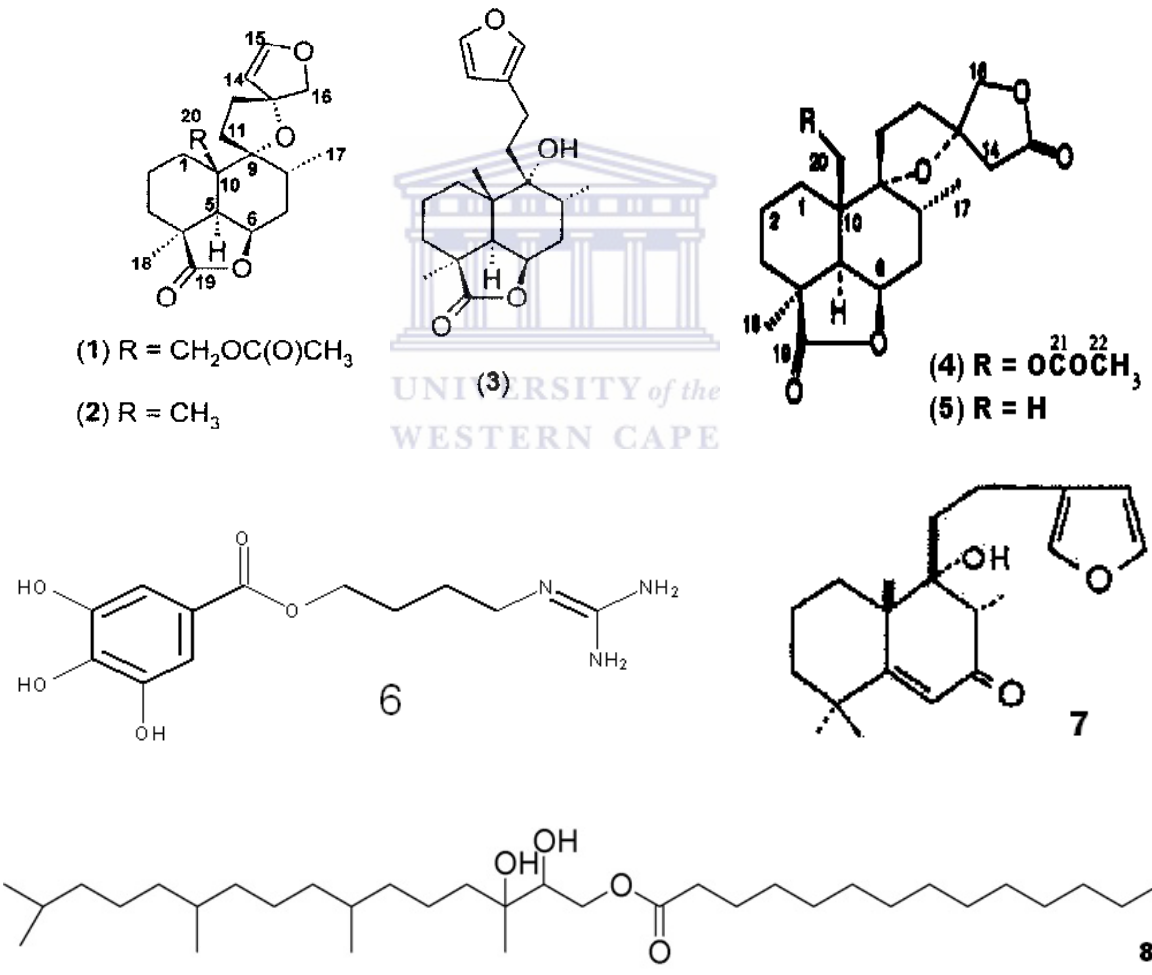


Figure 2.2: Structures of leonurun (1), premarrubiin (2), marrubiin (3), leonitin (4), compound X (5), leonurine (6), compound Y (7) and 1,2,3- trihydroxy-3,7,11,15-tetramethylhexadecan-1-yl-palmitate (8).

Attempts have been made by various researchers to validate the claims made in traditional medicine of the therapeutic use of *L. leonurus*. An aqueous extract of the leaves was tested for anticonvulsant properties by Bienvenu and co-workers (2002) and found to be effective in protecting mice against seizures induced by pentylenetetrazole, picrotoxin and N-methyl-DL-aspartic acid. Extracts of the leaves and stems have also shown antibacterial activity *in vitro* (Kamatou et al., 2006). Organic extracts of the leaves were reported by Jager and co-workers (1996), investigating the use of the plant traditionally for treating headaches and inflammation, to be a good inhibitor of cyclooxegenase (COX) enzyme. Various studies have investigated the cardiovascular effects of different extracts of the plant with mixed results. Njagi (2001) reported no significant effect on both blood pressure and heart rate in anaesthetized normotensive Wistar rats with a 1 mg/ml infusion of the aqueous extract, while Ojewole (2003) on the other hand reported decreases in blood pressure and heart rate in anaesthetized, normal and spontaneously hypertensive rats. The differences may be explained as dose related since Ojewole administered 22 – 800 mg/kg doses of the extract, doses far greater than the 1mg/ml dose used by Njagi. In another study however Obikeze (2005) administered 0.5 – 7.0 mg/kg of the aqueous extract to anaesthetized normotensive rats and observed increases in blood pressure and decreases in heart rate, a result strikingly different from that reported by both Njagi and Ojewole. Obikeze (2005) also reported an increase in blood pressure and heart rate with a chromatographic fraction of the methanol extract of the leaves. *In vitro* studies on the plant also yielded conflicting results, with Mugabo and co-workers (2002) reporting a positive chronotropic and inotropic effect on isolated Langendorff perfused male Wistar rat hearts with the aqueous extract, while Ojewole (2003) reported the aqueous extract to relax

vascular smooth muscle contractions induced by bath-applied noradrenaline (0.1-10 M), and potassium (5- 40 mM) in strips of the descending aorta and portal vein. Despite the number of compounds so far isolated from the plant, none have so far been evaluated for pharmacologic activity. Though extracts of the plant have been studied for cardiovascular activity, a definitive mechanism of action for the cardiovascular effects of the extracts or pure compounds so far isolated from the plant is yet to be elucidated. The literature revealed that a variety of plant derived compounds exhibit varying effects on the cardiovascular system and plants belonging to the Leonotis spp contain a diverse mix of these compounds, though especially rich in terpenoids. Since terpenoids have been shown to exhibit cardiovascular effects, they could be wholly or partly responsible for the cardiovascular effects observed with aqueous and organic extracts of *L. leonurus* (David et al., 1998; Somova et al., 2001; Ulubelen, 2003; Guerrero et al., 2004; Silva et al., 2005; de Oliveira et al., 2006; Lahlou et al., 2007).

2.3 Isolation of plant compounds

Plants contain a mixture of chemical compounds belonging to the different phytochemical classes, with different solubility profiles in aqueous and non-aqueous solvents. For the isolation of plant compounds, typically the fresh plant is immersed in a suitable solvent and the resulting extracts are utilised, but alternatively fresh plant parts could be dried under stringent conditions and then extracted with a solvent. Plant parts could also be ground up to help break down cells and increase the degree of extraction. Solvent extraction is usually the first step in isolating chemical compounds from a plant. Typical solvents used include water, ethanol, methanol and hexane, and usually the organic solvents are the more

favoured if an exhaustive extraction is desired. Since plants typically contain a large number of chemical compounds, the investigation of plants for possible useful chemical compounds usually follows a systematic plan (Harborne, 1973). The classic process is to continuously extract the dried and powdered material in a soxhlet apparatus with a range of solvents followed by concentration of the resulting extracts. Bioassay guided fractionation of the residues of the extracts is usually used when investigators are prospecting for compounds that could be useful in specific diseases (Harborne, 1998; Pieters and Vlietinck 2005). In bioassay guided fractionation, the crude extracts of the plant material are tested for the desired biological activity and the active fraction then fractionated. Fractions obtained are evaluated for biological activity and those active fractions are pooled and refractionated and retested. This process is repeated until pure compounds with the desired bioactivity are finally found (Luo et al., 2008; Ngueyem et al., 2008). Bioassay guided fractionation has the advantage of producing pure compounds that have the particular desired bioactivity. Investigations are normally focused on a particular disease with the idea that compounds isolated could eventually be useful in treating the disease, or serve as leads for other useful synthetic compounds. On the downside, the cumbersome cycle of fractionation and assaying makes this process time consuming. It has also been noted that a lot of the effects of crude plant extracts are the sum of the effects of individual compounds found within the extract, and thus a degree of caution must of necessity be adopted in bioassay guided fractionation since it may not yield pure compounds with significant bioactivity even if the original extracts showed significant activity (Pieters and Vlietinck 2005). Fractionation of the crude plant extract could either be done using the different physical properties of solvents or using physical separation techniques such as

chromatography which is by far the more common technique. Fractionation on a chromatographic column is effected using solvent mixtures that selectively separate compounds according to their different phytochemical classes (Harborne, 1998). The advantage of this technique is that it is a quick and simpler method for identifying and isolating only compounds that belong to the specific phytochemical class of interest. Since most plants contain more than one compound belonging to the same phytochemical class, further fractionation is often required to separate and isolate each as a single pure compound. The fractionation process could also provide compounds that do not necessarily belong to the desired phytochemical class such as lipids and these would naturally have to be separated from the desired compounds for evaluation. After the initial extraction process of the plant the residue has to be chromatographed in order to separate each of the components in the extract for subjection to the identification process. Separation is carried out mainly using one or a combination of the following chromatographic techniques; column chromatography (CC), thin layer chromatography (TLC), gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC). The choice of separation technique depends largely on the solubility profile and volatility of the compounds to be separated. Generally CC and TLC are preferred for water and lipid-soluble compounds, while GLC is preferred for volatile compounds and HPLC for non-volatile compounds such as terpenoids, phenolics, alkaloids and sugars (Harborne, 1998). Chromatographic separation is based on the differences in the partition coefficients of compounds within a mixture when they are allowed to partition between a stationary phase and a migrating solvent. Compounds highly soluble in the migrating solvent are transported faster while those less soluble are slower moving, with the most soluble compounds eluting

first and the least soluble last. In TLC, the stationary phase comprises of a thin layer of absorbent material such as silica gel, polyvinylpyrrolidone, polyamide, and ion exchange resins, on a supportive base such as glass or aluminium. Separation takes place in a tank with the atmosphere saturated with the solvent, and the mobile phase is allowed to run up the plate starting just below the level of the mixture to be separated (Miller, 1975). TLC is a relatively cheap, fast and effective means for separating mixtures, but it is not very effective for separating compounds with similar chemical characteristics, or for very complex mixtures. TLC is also only useful for separating very small quantities of mixtures, though preparative TLC with a thicker absorbent layer could be used to separate larger quantities. Column chromatography satisfies this need for separating large quantities of materials. Silica gel packed into a cylindrical column acts as the stationary phase, while the mobile phase is used to elute the material packed at the top of the column. The mobile phase could either be moved by gravity or a low pressure pump. Column chromatography unfortunately suffers the same problem as TLC as it is sometimes unable to separate out quantities of pure compounds from a complex mixture. HPLC overcomes some of the problems associated with column chromatography. In HPLC, the stationary phase is bonded to a porous polymer in a narrow-bore column made of stainless steel and the mobile phase passed through at high pressures (Hamilton and Sewell 1982). Unlike column chromatography, HPLC systems are usually computerised, allowing for a programmed change in the concentration of the constituents of the mobile phase, stringent monitoring and control of the chromatographic environment and also incorporation of various detectors to detect separated samples. HPLC systems are highly sensitive to impurities and are unsuitable for separating most crude plant extracts, usually requiring some pre-

treatment to remove most of the bulk impurities such as tannins that would otherwise clog the columns (Harborne, 1998). Unfortunately HPLC systems which are only designed to separate microgram quantities of samples at a time, are complex to operate and expensive compared to TLC and CC. GLC is similar to HPLC with the exception that the sample passes through a heated column as a gas, and an inert gas such as nitrogen or argon acts as the mobile phase. GLC, primarily only suitable for volatile compounds, is both expensive to run (Miller, 1975). One of the last stages of the isolation of the almost pure compound is re-crystallization. In a saturated and hot solution, pure crystalline matrixes of the compound form spontaneously, and this behaviour is taken advantage of to produce pure crystals of the isolated molecule in a suitable organic solvent.

In practice, most researchers use a combination of these and other extraction and separation methods to isolate pure compounds from plants. In the current study solvent extraction, CC and TLC were utilised in the isolation of a pure diterpene from the leaves of *L. leonurus*.

2.4 Identification of compounds

Compounds isolated from plants could reasonably be expected to play a role as precursors for new bioactive molecules or as drugs themselves. However, before they can be utilized, they have to be properly identified and their properties fully evaluated. There are many methods available for the identification of chemical compounds present in a plant, but basically they all depend on the fact that all chemicals have unique physicochemical properties. Physical identification methods include melting point, R_f values from TLC, optical rotation and X-ray crystallography, while ultra violet (UV), infra red (IR), nuclear

magnetic resonance (NMR) and mass spectroscopy (MS) identify the structures of the compounds using their spectral characteristics.

Every compound possesses a unique melting point and this could be used to compare an unknown compound against possible reference compounds. It is however important to note that even small quantities of impurities could alter the melting point of a compound and so melting point alone cannot be used to identify compounds but rather as one of many tools. R_f values from TLC experiments can also be used in very few instances for the identification of a compound on a comparative basis only and never as a confirmation. R_f values are measured as the distance migrated by the compound on the stationary phase relative to the distance migrated by the solvent phase. Experiments are usually done on a TLC plate with a reference compound and the sample compound on the same plate, and exact R_f values could be an indication that they are similar compounds. R_f values are not definite as two compounds with very similar chemical structures may have the same R_f value. The UV spectra also contribute a limited role in the structural identification of a compound, but also have limitations similar to the other methods mentioned above. For a new compound, elucidation of the structure is vital to determining its novelty. The following methods are routinely used in structure elucidation.

Infrared (IR) measurements are made between 4000 and 667 cm^{-1} in the electromagnetic spectrum. In IR spectroscopy, vibrations of individual bonds or functional groups within the compound under investigation appear as spectral bands or peaks above 1200 cm^{-1} that are distinct for each such bond or functional group. Below 1200 cm^{-1}spectral bands indicate vibration of the whole molecule. An advantage of IR spectroscopy is that spectra are simple to interpret and could be used to either determine functional groups contained in

the compound or compare its spectrum to other known compounds for identification (Skoog and West 1971; Harborne, 1998).

NMR spectroscopy works on the principle that certain atomic nuclei have spin and magnetic moment properties and exposure of these nuclei to a magnetic field would lead to a splitting of their energy levels. This molecular environment then influences absorption of radiation by the nuclei and the extent of absorption can be correlated with the molecular structure of the compound (Skoog and West 1971). NMR can only be used for compounds containing nuclei with an odd atomic number since these nuclei have a residual spin and include atoms such as ^{13}C, ^{15}N and ^{1}H. Traditionally NMR is measured in a liquid state, with the compound dissolved in a solvent such as carbon tetrachloride or deuterated chloroform. NMR spectroscopy is usually done in 2 dimensions (2D) and among the different types of NMR analysis are: COSY, HMQC, HSQC, HMBC, HECTOR, TOCSY, NOESY and ROESY with each giving a different amount of structural information for an unambiguous structural assignment. Thus correlation spectroscopy (COSY) measures the frequency of those protons which are coupled to each other as off diagonal peaks that then correlate to their chemical shifts, and is used to analyse proton (^{1}H) spectra (Keeler, 2006). Heteronuclear multiple quantum coherence (HMQC) measures ^{13}C nuclei shifts that are correlated to the protons attached to them also as diagonal peaks on two separate frequency co-ordinates. Heteronuclear single quantum correlation (HSQC) spectra usually indicate single bond correlations between ^{13}C and a proton, heteronuclear multiple-bond correlation (HMBC) spectra indicate all long range ^{13}C – ^{1}H or ^{15}N – ^{1}H couplings in the compound, and heteronuclear correlation (HECTOR) effects the same results as ^{13}C-^{1}H correlation spectra. Total correlation spectroscopy (TOCSY) is similar to COSY in that it observes

protons only, but in addition also indicates protons connected by an unbroken chain of couplings. Nuclear overhauser effect spectroscopy (NOESY) is quite similar to COSY, but while COSY indicates which protons are in close proximity due to their coupling through bonding electrons, NOESY indicates which protons are located in close proximity to each other in the compound through non-bonding through space effects viz., interactions of small magnetic moments between protons near each other (Keeler, 2006). The NMR spectrometer comprises of a powerful magnet that supplies the magnetic field, a radio-frequency source that supplies a plane-polarized beam perpendicular to the path of the magnetic field, and a signal detector surrounding the sample and at right angles to the radio-frequency source to detect radio-frequency signals produced by the resonating nuclei (Skoog and West 1971). To elucidate the structure of a compound using NMR, the pure compound in a solution is introduced into the instrument and the different spectra mentioned above obtained. Analysis of the spectra then builds up a composite picture of the compound. NMR spectroscopy is one of the most powerful tools for the elucidation of the structure of a compound, and like mass spectroscopy (MS) requires only a small quantity of the sample. The downside to NMR spectroscopy is that it is expensive, requires expertise to operate and can only be applied to pure samples.

X-rays are part of the electromagnetic spectrum between gamma-rays and UV light, usually produced when a high velocity stream of electrons impact a metal target in an evacuated tube. X-rays are high energy, short wavelength beams suited to probing the structural arrangement of atoms and molecules (Harley and Wiberley 1954).Three X-ray methods are used in analytical chemistry to determine the chemical composition of a compound: X-ray powder-diffraction, X-ray fluorescent and X-ray absorption analysis. X-

ray fluorescent and absorption analysis depend on the absorption and fluorescent properties of a particular element and so are useful to identify elements, while X-ray diffraction analysis is able to determine the actual three dimensional structure of a compound. For complex compounds that are crystalline in nature, X-ray diffraction patterns are characteristic for both the form of the crystal and the spacing of the layers of the crystal lattice and hence its universal use in solving structural problems. In X-ray crystallography the integrated intensities of the diffraction peaks are used to reconstruct the electron density map within a unit cell of the crystal to give a structural picture of the crystalline compound. For structure elucidation, the single crystal diffractometer and a computer programme are used, while powder diffractions are suitable for compounds whose fingerprint is already known.

In the identification and structural analysis of complex compounds, mass spectrometry has proven to be a powerful and versatile tool, perhaps as important and powerful as infrared or NMR spectroscopy. The basic principle of the mass spectrometer is that the impact of an electron stream on a given molecule produces a group of positive particles whose mass distribution is characteristic for the given molecule (Skoog and West 1971). In other words each molecule produces a particular unique fingerprint of its fragments. Samples undergoing mass spectroscopy experiments are first ionized using electrospray ionization (ESI) to allow for maximum impact with the electron stream. ESI is a liquid-phase ion source and ionizes the sample at atmospheric pressure before the ions are transferred into the mass spectrometer. In the mass spectrometer, positive ions are separated from negative ions and accelerated by means of a potential difference. Accelerated ions are then subjected to a strong magnetic field on a curved path which serves to focus ions of a particular mass

to charge ratio (*m/z*) on a collector electrode. Varying the magnetic field changes the ions collected and spectra produced for the compound from which it may then be identified. Mass spectrometry is a very powerful tool for structural identification and analysis, but it is a complex and expensive equipment to operate. Its advantage over NMR is that most mass spectral data are easier to interpret, and also it provides the molecular weight of the compound (Skoog and West 1971).

Hyphenated systems combining the chromatographic separation techniques described above and analytical instruments like NMR and MS are becoming very popular. Examples include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-nuclear magnetic resonance (LC-NMR) systems (Kite et al., 2003). These systems have the advantage of rapid and effective separation and identification of very complex mixtures, but are unfortunately still very expensive. For this study, NMR, IR, MS and X-ray crystallography were used to elucidate the structure of the diterpene isolated from the leaves of *L. leonurus*.

2.5 Models in cardiovascular research

Methods in cardiovascular research can broadly be classified as *in vivo* or *in vitro* depending on whether whole live animals, cells, tissues or organs are used. Some of the earliest experiments on the effects of compounds on the cardiovascular system were carried out in the late 19th century mostly using isolated frog hearts (Skrzypiec-Spring et al., 2007). Presently rats, mice, guinea pigs, rabbits and dogs are the preferred test subjects because as mammals, their cardiovascular systems are much more similar to that of humans than those

of amphibians. For *in vivo* experiments, rats and mice are cheaper to breed than dogs, and are more robust than guinea pigs and rabbits. Rats unlike mice are big enough to enable easy handling during experiments, and unlike rabbits are very stable under anaesthesia (Livius et al., 2000). Examples of animal models used for *in vivo* cardiovascular experiments include the normotensive, spontaneously hypertensive, 2-Kidney 1-clip hypertensive, DOCA/salt hypertensive and salt-loaded hypertensive models. The normotensive rat model is ideal for pharmacologic screening where the actual cardiovascular effect of the substance is unknown because it allows for both hypertensive and hypotensive effects to be observed. To test for antihypertensive effects, a hypertensive model such as the spontaneously hypertensive, 2-Kidney 1-clip hypertensive, DOCA/salt hypertensive or salt-loaded hypertensive model is used. *In vitro* methods on the other hand involve the use of isolated organs, tissue or cells usually in an environment that simulates the natural physiological environment. Examples of preparations of parts of organs include rabbit ear artery, rat mesenteric artery, rat atrium, and rat or rabbit aorta preparations, while an example of the isolated perfused organ preparation for *in vitro* experiments is the isolated perfused heart system (Langendorff or working heart perfused) (Gross, 1994; Consolini and Sarubbio 2002). Cellular models for cardiovascular research include the loose patch clamp technique (Ruff, 1996; Zhang and Tan 1997). *In vitro* methods are generally used to study the effects of test substances on individual organs or parts of organs and help give a clearer idea of the actions of the test substances on the organ or cell in question. The models are usually preferred when elucidating the mechanism of action of test substances, but do not always give an accurate picture of the effect in a whole animal.

For the present work the *in vivo* experimental model was chosen because it is easier to extrapolate results from this model to humans. The model is also able to measure the effect of test substances within the complex interactions of the nervous system as would occur in clinical practice, unlike isolated organ, tissue or cell models. The anaesthetized normotensive rat model was chosen since the cardiovascular effect of the test compound was unknown.

The anaesthetized normotensive rat model allows the researcher to record changes in blood pressure and heart rate in intact animals, in response to administered drugs. The rat is anaesthetized with sodium pentobarbitone, injected intra-peritoneally, and the trachea exposed and cannulated for artificial respiration. To record changes in heart rate and blood pressure, the carotid artery, the abdominal aorta or the femoral artery is exposed and cannulated. The cannula is connected to a pressure transducer and thence to a suitable instrument to record changes in blood pressure and heart rate. Drug substances are infused via a cannula inserted into either the jugular or femoral vein (figure 2.3).

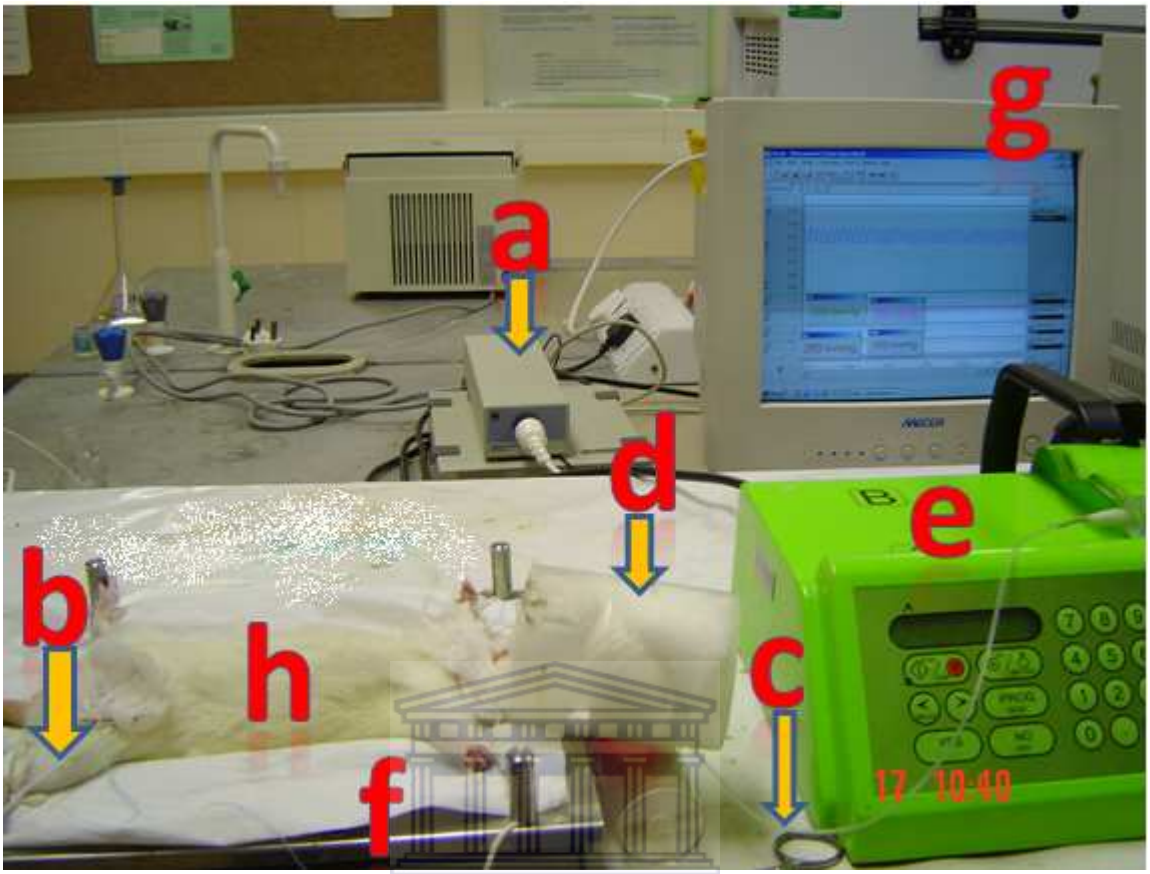


Figure 2.3: The anaesthetized normotensive rat model (a= BP transducer; b= arterial catheter; c= venous catheter; d= Oxygen mask; e= syringe pump; f= small animal operating table; g= computer running chart 5 software; h= male Wistar rat).

One of the advantages of the anaesthetized normotensive rat model is that the experiment could run for more than 24 hours if the animals is suitably prepared and kept under anaesthesia (Hearse and Sutherland 1999). The anaesthetized normotensive rat model records changes in heart rate, systolic pressure, diastolic pressure and mean arterial pressure. Normal ranges are between 116mmHg – 145mmHg (systolic), 76mmHg – 97mmHg (diastolic), 103mmHg – 129mmHg (mean arterial pressure) and 296 – 388pbm (heart rate) for a 300g rat (Livius et al., 2000). Male animals are preferred to female animals, and young rats are used instead of old ones. This is because in the older rats, the

responsiveness of the heart to stimulation is reduced, the walls of the arteries are less elastic and as with humans, older rats tend to be spontaneously hypertensive (Hearse and Sutherland 1999).

2.6 The cardiovascular system

The cardiovascular system comprises of the heart – a four-chambered muscular pump that pumps blood around the body and a network of blood vessels made up of arteries, veins, arterioles, venules and capillaries. This complex system is designed to deliver nutrients to the organs, tissues and cells of the body and to remove metabolic waste to their sites of excretion. The heart is auto-rhythmic in that the rhythmic pumping action is controlled by impulses generated within the heart itself, with certain muscle cells within the heart specially adapted to spontaneously generate and rapidly transmit the electrical impulses that produce the cycles of contraction and relaxation. Two nodes or clusters of these cells are located within the walls of the right atrium: the sinoatrial (SA) node and the atrioventricular (AV) node (Rang et al., 2007). The SA node acts as the pacemaker of the heart, while the AV node conducts generated impulses rapidly to the left and right ventricles. In myocardial cells within the ventricles, the impulse causes a rapid depolarization of the cell membrane due to the rapid influx of Na^+ through voltage gated channels. This is followed by a gradual influx of Ca^{2+} leading to a sustained depolarization and then repolarization by efflux of K^+. The presence of the slow inward calcium current distinguishes cardiac cells from neuronal cells and enables the rhythmic contraction of the ventricles (Fozzard, 2002). Contraction of the ventricles drives blood from the heart into the systemic circulation. Apart from the intrinsic rhythmicity of the heart dictated by the

pacemaker, the heart is also subject to regulation through neural control via the sympathetic and parasympathetic nervous systems and hormonal control through the release of adrenaline and noradrenaline from the adrenal medulla (Hoffman, 1998a).

Sympathetic control of cardiac rhythm is through post ganglionic nerve fibers that innervate the SA and AV nodes, coronary vessels, atria and ventricular myocardium. Sympathetic stimulation leads to the release of noradrenaline, the neurotransmitter in the postganglionic synapse and its agonist effect on β_1-adrenergic receptors on the myocardial cell surface leads to an increase in heart rate (a positive chronotropic effect) and force of contraction (a positive inotropic effect). β_1 receptors in the heart mediate neural and hormonal regulation of the heart. Stimulation of these receptors with an agonist drug would lead to a positive chronotropic and inotropic effect, while inhibition of these receptors with an antagonist would lead to a negative chronotropic and inotropic effect. Parasympathetic control of cardiac rhythm is via the vagus nerve which also innervates the SA and AV nodes, coronary vessels, atria and ventricular myocardium. Parasympathetic stimulation releases acetylcholine which acts on M_2 receptors and results in a decrease in activity of the SA node, impulse conduction through the AV node, atrial contractility and force of ventricular contraction. The net effect is a negative chronotropic and (to a lesser degree) a negative inotropic effect. Sympathetic stimulation can increase cardiac output by 50% - 100% of the resting values, while parasympathetic stimulation can decrease it by 10% - 20%. The resting heart rate is controlled by a balance of sympathetic and hormonal stimulation and the opposing parasympathetic vagal tone (Hoffman, 1998a).

Blood vessels that carry blood from the heart to tissues and cells are called arteries. Arteries are hollow tubes surrounded by contractile cells that contract to narrow and relax to increase the lumen size through which blood flows. Blood pressure (BP) measures the force that blood flowing through the arteries exerts on the walls of the arteries. BP rises and falls with contraction and relaxation of the ventricles, with the maximum pressure known as systolic pressure (SP) coinciding with ventricular contraction and the minimum pressure known as diastolic pressure (DP) coinciding with ventricular relaxation. The force exerted by blood on the vessel walls depends on the quantity of blood flowing through the vessels – determined by cardiac output (CO) and the resistance of the vessel walls to the stretching effect of the pressure – peripheral vascular resistance (PVR). The relationship between BP, CO and PVR can be expressed as:

$$BP = CO \times PVR$$

CO is the volume of blood pumped by the heart into the systemic circulation per unit of time and is dependent on heart rate (HR) and the volume of blood pumped out into systemic circulation with each contraction of the ventricles – stroke volume (SV). The relationship between CO, SV and HR is expressed as:

$$CO = SV \times HR$$

As can be inferred from the above equations, PVR plays a huge role in determining blood pressure. One aspect of blood pressure not yet mentioned is the effect of the venous system. The walls of veins are not as muscular as those of arteries and so do not contribute a lot to total PVR. Venous return - the flow of blood back to the heart, however has a direct effect on stroke volume, which then determines CO. Venous pooling reduces venous return and so reduces stroke volume, eventually leading to a reduction in blood pressure as seen

from the above equations. Dilatation of the veins would lead to a pooling of blood in the larger veins, leading to a decrease in preload. A significant decrease in preload would lead to a decrease in force of contraction (because of a decrease in the stretching of the ventricular walls), stroke volume and cardiac output (Seeley et al., 2003). Constriction of the veins would lead to an increase in blood flow to the heart, leading to an increase in preload. The increase in preload then leads to an increase in force of contraction, stroke volume and cardiac output.

The contractile walls of arteries are innervated by sympathetic nerves and contain α_1 and β_2 receptors which in addition to α_2 receptors found in presynaptic end plates mediate sympathetic regulation of the peripheral vascular resistance. Adrenaline and noradrenaline, the neurotransmitters in the sympathetic nervous system act on these receptors to control vascular resistance in the arteries. Activation of α_1 receptors by noradrenaline leads to vasoconstriction via an increase in intracellular Ca^{2+} in smooth muscle cells. Stimulation of α_2 and β_2 receptors leads to vasodilatation, with α_2 receptor stimulation inhibiting the release of neurotransmitters for the synapse. As can be seem from the above, blood pressure is determined by a complex interaction between the sympathetic and parasympathetic nervous system and mediated by β_1 and M_2 receptors in the heart controlling heart rate and cardiac output, and α_1, α_2 and β_2 receptors in the arteries mediating peripheral vascular resistance. Changes in blood pressure reflect changes in one or more of these parameters, and the cardiovascular effect of any compound is determined by its agonist or antagonist effect on one, some or all of these receptors (Benowitz, 1998).

2.7 Drugs acting on the cardiovascular system

As can be inferred from the above discussion, drugs can also alter the normal functioning of the cardiovascular system through their effects as agonists or antagonists on the sympathetic and parasympathetic nervous system innervating the heart and blood vessels. The drugs could be broadly classified as chronotropic, inotropic and vasoactive agents.

Chronotropic agents act on the myocardium to increase or decrease the heart rate – a positive or negative effect. The increase in heart rate (positive chronotropic effect) is achieved either by an agonist effect on β_1 receptors in the heart, or by a decrease in vagal tone, while the decrease in heart rate (negative chronotropic effect) is achieved by either an inhibitory effect on β_1 receptors in the heart, or by blockade of Ca^{2+} channels in cardiac myocytes or by increasing vagal tone. Positive chronotropic agents that act via an agonistic effect on β_1 receptors include adrenaline, noradrenaline, isoproterenol and dobutamine, while atropine antagonizes the effect of the parasympathetic neurotransmitter acetylcholine. Negative chronotropic agents acting via $\beta_1$blockade include β blockers like propranolol and atenolol, while verapamil, nifedipine and diltiazem block Ca^{2+} channels by preventing the opening of voltage-gated L-type calcium channels (Rang et al., 2007).

Inotropic agents alter the contractility of the myocardium to either increase (a positive effect) or decrease (a negative effect) the force of ventricular contraction and in so doing stroke volume. Contractility of the heart is dependent on intracellular Ca^{2+}, and the entry of Ca^{2+} into cardiac cells is dependent on the opening of voltage gated Ca^{2+} channels and intracellular Na^{+} concentration. Positive inotropic agents increase intracellular Ca^{2+} and include adrenaline, noradrenaline, dobutamine, cardiac glycosides, dopamine and isoproterenol. Negative inotropic agents prevent the entry of Ca^{2+} into myocardial cells and

include the Ca^{2+} channel blockers mentioned above, and the β blockers propanolol and atenolol. As is evident from the above, some drugs possess both chronotropic and inotropic effects on the heart.

As previously mentioned, peripheral vascular resistance plays a huge role in determining blood pressure. Certain agents called vasoactive agents could cause the constriction or dilatation of blood vessels by either acting on α_1, α_2, and β_2 receptors or altering the release of the neurotransmitter noradrenaline into synaptic cleft. Phenylephrine and methoxamine would act as agonists on α_1 receptors and cause vasoconstriction consequently increasing the blood pressure, while on the other hand α_1 receptor antagonists such as prazosin and α_2 receptor agonists such as clonidine would produce vasodilatation. Reserpine which depletes vascular stores of the catecholamines adrenaline and noradrenaline, and methyldopa which prevents catecholamine synthesis would also produce vasodilatation. For non-selective alpha receptor (α_1 and α_2) antagonists, the vasodilatation produced by α_1 blockade is accompanied by reflex tachycardia due to the increased release of the neurotransmitter noradrenaline (Rang et al., 2007). Reflex tachycardia could also be due to intrinsic mechanisms monitoring blood pressure such as the baroreceptor reflex. Certain pressure receptors found around the aortic arch detect increases in blood pressure and via an increase or decrease in parasympathetic activity in the heart decrease or increase the heart rate. This mechanism is meant to keep blood pressure within normal limits, but when drugs are used to change cardiac parameters, could alter the results of the drugs' effect on the cardiovascular system.

Drugs acting on the cardiovascular system would produce the observed effects through any of the mechanisms mentioned above. With novel compounds exhibiting cardiovascular

activity, the mechanism of action could be the result of interactions with any of the receptors listed above, or could involve cellular processes such as the opening or closing of ion channels or the synthesis or inhibition of certain chemical mediators like cyclic adenosine monophosphate (cAMP).

The increase in reliance on traditional medicines, the rejuvenation of interest in plant medicines as a potential source of new drug compounds and the need for the pharmacologic analysis of plants in use as traditional medicines have lead to increased research on plants used in traditional medicine. Despite recent interest in plant medicines, there are still a large number of plants used in traditional medicines that are yet to be scientifically studied. The traditional use of a plant could serve as a pointer to possible therapeutic effects of compounds isolated from it, and these plants could yield novel compounds that could be useful as new drugs, or precursors in the development of new drugs for the management or cure of diseases. *L. leonurus* has widespread use in traditional medicine for treating cardiovascular diseases, and extracts of the plant have been reported to produce cardiovascular effects. Since *L. leonurus* contains compounds belonging to phytochemical classes that exhibit cardiovascular effects, it could yield individual compounds with cardiovascular effects. If such cardioactive compounds exist in the extracts of the plant, then an understanding of the mechanism of action of the effect of the compounds may bring us closer to a new drug for the treatment of cardiovascular diseases.

HYPOTHESIS

The plant *L. leonurus* used in traditional medicine for the treatment of cardiovascular diseases contains pure compound(s) that on their own possess cardiovascular effect.

AIMS AND OBJECTIVES

This study aims to:

- Isolate and characterize one compound from *L. leonurus* that exhibits cardiovascular effects.
- Determine the possible mechanism(s) by which the isolated compound exhibits its cardiovascular effect.

CHAPTER THREE

Materials and methods

Equipment and chemicals used in the study are listed in this chapter. Also described are the different methods employed in the isolation, structure elucidation and *in vivo* assessment of the compound.

3.1 Materials used in extraction and identification

3.1.1 Chemicals

a) Hexane (b.p. 65-70^{o}C) (Kimix, Cape Town, South Africa)

b) Ethyl acetate (distilled prior to use) (Kimix, Cape Town, South Africa)

c) Silica gel (35-60 mesh and 70-230 mesh) (Sigma-Aldrich, Steinheim, Germany)

d) Methanol (Sigma-Aldrich, Steinheim, Germany)

e) Concentrated HCl (Kimix, Cape Town, South Africa)

f) Chloroform (Kimix, Cape Town, South Africa)

g) Ammonia (Kimix, Cape Town, South Africa)

h) Anhydrous magnesium sulphate (Kimix, Cape Town, South Africa)

i) Acetic acid glacial (Kimix, Cape Town, South Africa)

j) Trichloromethane –d ($CDCl_3$) (Merck, Johannesburg, South Africa)

k) Nujol IR grade (Merck, Johannesburg, South Africa)

l) Acetonitrile (Merck, Johannesburg, South Africa)

m) Distilled water

3.1.2 Equipment

I) Equipment used in extraction

a) Soxhlet apparatus

b) Heating mantle

c) Grinder

d) TLC aluminum sheets Silica gel 60 F_{254}; 20 x 20cm (Merck, Johannesburg, SA)

e) Low pressure column chromatography system: Glass column; Minipuls 3 peristaltic pump; UV detector 112; fraction collector FC203B (all Gilson, Middleton, USA)

f) Rotovapour (Bibbly Sterilin, England)

II) Equipment used in identification

a) Bellingham & Stanley ADP 220 polarimeter (Tunbridge Wells, England)

b) Perkin-Elmer Paragon 1000 PC IR spectrometer (Waltham, MA, USA)

c) Varian Unity*Inova* 600 NMR spectrometer (Palo Alto, CA, USA)

d) Waters API X-TOF Ultima mass spectrometer (Milford, MA, USA)

e) Gallenkamp melting point equipment (Leicestershire, England)

f) Siemens SMART diffractometer (Madison, WI, USA)

3.2 Materials used in the anaesthetized normotensive rat model

3.2.1 Drugs and Chemicals

a) Atenolol (Astra Zeneca, Sunninghill, South Africa)

b) Prazosin (Sigma-Aldrich, Steinheim, Germany)

c) Dobutamine (Intramed, Porth Elizabeth, South Africa)

d) Reserpine (Sigma-Aldrich, Steinheim, Germany)

e) Verapamil (Sigma-Aldrich, Steinheim, Germany)

f) Sodium pentobarbitone (Kyrn Laboratories, Johannesburg, South Africa)

g) Heparin (Intramed, Porth Elizabeth, South Africa)

h) Tween 80 (Sigma-Aldrich, Steinheim, Germany)

i) K-Y jelly (Johnson & Johnson, Midrand, South Africa)

j) Normal saline

3.2.2 Equipment

a) Small animal operating table (BioScience, Cape Town, South Africa)

b) Blood pressure transducer (AD instruments, Bella Vista, Australia)

c) Power Lab 4/20T (AD instruments, Bella Vista, Australia)

d) Chart 5 for windows (AD instruments, Bella Vista, Australia)

d) BP amplifier (AD instruments, Bella Vista, Australia)

e) Syringe pump (Ascor AP22, CA, USA)

f) Oxygen mask

3.2.3 Animals

Male Wistar rats aged between 3 and 4 months were used to assess the cardiovascular effects of the isolated compound. Rats were used because they provide a better compromise between size and heart rate than rabbits which although larger in size, have a higher resting heart rate. In spite of the fact that some researchers have suggested that there are no gender specific differences in cardiovascular parameters for the rats, only male Wistar rats were used to ensure uniformity (Livius et al., 2000). The age range of 3-4 months was chosen because that appears to be the optimal age for male Wistar rats. Older rats have lower heart rates and a reduced sensitivity to cardioactive substances, probably due to loss of elasticity in the arteries which occurs with age (Livius et al., 2000).

3.3 Methods

3.3.1 Extraction

The stems and leaves of *Leonotis leonurus* were collected from Montague Botanic Gardens, Cape Town, South Africa, and a voucher specimen (No. 6859) deposited at the herbarium of the University of the Western Cape. The fresh leaves of the plant were washed with distilled water, dried at 30°C in a ventilated oven for 72 hours, and ground into a fine powder. The soxhlet apparatus was used to extract 183 g of the ground leaves in methanol over a period of 24 hours and the resulting extract was filtered through a filter paper to remove small solid particles. The rotovapor was used to remove excess solvent *in vacuo* to afford a thick dark brown residue. Distilled water was added to the residue and the

resulting solution was acidified using concentrated HCl, and exhaustively extracted with chloroform. The acidified solution was then basified with concentrated ammonia, and sequentially exhaustively extracted with ethyl acetate followed by chloroform. Analytical TLC chromatography of the three extracts on aluminum backed TLC plates containing F_{254} dye using a mobile phase made of a mixture of ethyl acetate: hexane (2:3) was carried out to determine if the extracts contained similar fractions as indicated by similar TLC profiles. The three extracts were then combined and dried with anhydrous magnesium sulphate, and after filtration, the solvent was removed under reduced pressure.

The resulting residue was fractionated using low pressure column chromatography with silica gel (70–230 mesh grade) as the stationary phase and ethyl acetate: hexane (1:4) as eluent. Fractions as identified by the UV recorder were collected using a fraction collector and those displaying a similar TLC profile were combined to yield a total of 7 fractions. The fraction containing the greatest number of compounds (fraction 3) was then re-chromatographed on the low-pressure column chromatography column using ethyl acetate: hexane: acetic acid as the eluent in the following sequential combinations:

i) Ethyl acetate: Hexane: Acetic acid (3:2:1)

ii) Ethyl acetate: Hexane: Acetic acid (3:2:0.5)

iii) Ethyl acetate: Hexane: Acetic acid (3:1:0.5)

The pure bands as indicated on the UV recorder were collected using the fraction collector, and further purified using preparative TLC plates with a mixture of ethyl acetate: hexane: methanol (3:1:0.5) and 0.5% glacial acetic acid as mobile phase. The separated bands were then scrapped off the TLC plates, dissolved in the mobile phase and silica gel filtered off. The solvent was then removed using the rotovapor and the residue re-dissolved in

chloroform and allowed to evaporate very slowly to deposit pure crystals (1.53 g) of the diterpenoid: (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone.

3.3.2 Physical properties of the isolated diterpenoid.

Melting point

Melting point was determined using the Gallenkamp melting point apparatus. The average reading from four experiments was taken as the melting point of the compound.

Optical rotation

Optical rotation of a sample of the isolated diterpene to light (wavelength of the D line of a sodium source) at 19^{o}C was measured using a Bellingham & Stanley ADP 220 polarimeter.

Infra red (IR) spectroscopy

The sample was made up with nujol and IR spectra recorded on a Perkin-Elmer Paragon 1000 PC spectrometer that was corrected against an air background.

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC, HMBC, DEPT and NOESY NMR spectra were collected on a Varian Unity*Inova* 600 NMR spectrometer (Palo Alto, CA, USA) at 25 °C. ^{1}H and ^{13}C NMR spectra expressed in δ (ppm) were referenced to tetramethylsilane (TMS).

X-ray crystallography

Single-crystal X-ray diffraction analysis of the compound was carried out using the Siemens SMART diffractometer (Madison, WI, USA) at room temperature using graphite monochromated M_o Kα radiation.

Mass spectroscopy (MS)

An ESI high impact mass spectrum of the compound dissolved in acetonitrile was obtained on the Waters API X-TOF Ultima LC-MS equipment at 350^oC.

3.3.3 Anaesthetized normotensive rat model

Healthy male Wistar rats aged between 3 and 4 months, and within the weight range between 250 g and 400 g were used for the *in vivo* study of the compound. The animals were weighed and anaesthetized with sodium pentobarbitone (40 mg/kg) injected by intra-peritoneal route. Anaesthetized animals were then transferred to a small animal operating table and restrained. A midline incision was made in the throat region to access the trachea, and an incision made into the trachea. A lubricated hollow tracheal tube was carefully inserted into the trachea through the incision and secured in place with a knot. The tracheal tube was cleared of fluids, and an oxygen mask placed over the head and throat region to facilitate breathing.

The external jugular vein was then exposed, excess tissue carefully cleaned off it and clamped at the end proximal to the heart with a bulldog clamp to prevent blood loss. The exposed section was then filled with blood by gently massaging the head, and tied off at the

end proximal to the head. An incision was made into the vein, and a catheter lubricated with K-Y jelly and filled with a 10% heparin solution was carefully inserted into the vein and secured in place. The lower abdominal region was opened and the femoral artery carefully separated from the accompanying vein, nerve and tissue. The artery was tied off at the distal end and clamped with the bulldog at the proximal end. An incision was made into the artery between the tied end and the clamp, and a lubricated catheter filled with a 10% heparin solution carefully inserted and secured with a knot. The bulldog clamp was then carefully removed to allow for blood flow into the catheter. Care was taken to make sure that the arterial catheter did not block the branch-off of the renal artery from the aorta. Incisions were cleaned, sutured using catgut sutures, covered with gauze and kept moist for the duration of the experiment. A temperature probe was inserted into the rectum to monitor the animal's temperature throughout the experiment. Adjustments to body temperature were done by either increasing or decreasing the heating on the small animal operating table to keep rectal temperature at 37.3±0.5^{o}C. After surgery, animals were allowed a 30 minute recovery period before the commencement of experiments. During the recovery period, blood pressure calibrations were done on the chart 5 software and recording of parameters started. After the 30 minute stabilization period, animals whose blood pressure and heart rate readings were beyond the normal ranges (systolic pressure 116mmHg – 145mmHg; diastolic pressure 76mmHg – 97mmHg; mean arterial pressure 103mmHg – 129mmHg; and heart rate 296 – 388bpm) were excluded from the experiments (Livius et al., 2000). A syringe pump was used to infuse drugs at a constant rate (0.3 ml/min) through the venous cannula. A sufficient recovery period between

individual doses was allowed to let cardiovascular parameters return to their baseline values.

3.3.4 Drugs administered

To determine the doses of the drugs to be used, animals were divided into 6 groups, with each group of 8 animals receiving one of the drugs listed below.

a) Group I [(13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone]: Dose-response curve (0.5 – 5.0 mg/kg).

b) Group II (Reserpine): Dose-response curve (0.01 – 1.0 mg/kg).

c) Group III (Prazosin): Dose-response curve (0.5 – 10.0 mg/kg).

d) Group IV (Atenolol): Dose-response curve (0.5 - 3.0 mg).

e) Group V (Dobutamine): Dose-response curve (30 – 90 µg/kg)

f) Group VI (Verapamil): Dose-response curve (0.25 – 5.0 mg/kg)

To elucidate the mechanisms via which (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone produced its effect, animals were divided into 5 groups, with each group consisting of 8 animals. The following protocols were used to infuse the isolated diterpene and standard drugs combinations for each group.

a) Group VII: Reserpine (0.5 mg/kg) then (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone (0.5 mg/kg – 5.0 mg/kg).

   A baseline effect of the compound was first determined and then reserpine (0.5 mg/kg) administered. A time period (6 hours) was allowed for sufficient depletion of vascular stores of catecholamines, and the isolated compound re-administered.

b) Group VIII: Prazosin (7.0 mg/kg) then (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone (0.5 mg/kg – 5.0 mg/kg).

   Each dose of the isolated compound was administered immediately after the administration of prazosin.

c) Group IX: Atenolol (2.0 mg/kg) and (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone (0.5 mg/kg – 5.0 mg/kg).

   Each dose of the isolated compound was administered 12-15 minutes after the administration of atenolol.

d) Group X: Dobutamine (75 µg/kg) and (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone (0.5 mg/kg – 5.0 mg/kg).

   Each dose of the isolated compound was administered immediately after the administration of dobutamine.

e) Group XI: Verapamil (4.0 mg/kg) and (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone (0.5 mg/kg – 5.0 mg/kg).

   Each dose of the isolated compound was administered immediately after the administration of verapamil.

The effects of the compound and the standard drugs on systolic pressure (SP), diastolic pressure (DP), mean arterial pressure (MAP) and heart rate (HR) were evaluated.

3.4 Statistical analysis

The means of actual values for systolic pressure (SP), diastolic pressure (DP), mean arterial pressure (MAP) and heart rate (HR) from the experiments are reported. The difference between the baseline value of the parameters assessed and the maximum response obtained with the administration of (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone or standard drugs was calculated and expressed as a percentage of the baseline value (percentage change). The percentage change in the value of the parameters obtained with the administration of (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone following the administration of standard drugs was also calculated and expressed as a percentage of maximum response obtained with the standard drugs. Percentage change of the difference between the maximum response obtained with the administration of (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone alone and the maximum response obtained with the administration of (13*S*)-9α, 13α-epoxylabda-6(19),15(14)diol dilactone in animals pre-treated with standard drugs was calculated. Mean change was calculated and statistically analyzed using the student's t test for significant difference ($p<0.05$).

3.5 Ethical considerations

The animals were allowed free access to food and water and all experiments were conducted in accordance with regulations as stipulated by the University of Western Cape concerning animal experiments.

CHAPTER FOUR

Results

The results of the structure elucidation experiments of the tested compound isolated from *Leonotis leonurus* are presented in this chapter. Cardiovascular effects of the compound in anaesthetized normotensive male Wistar rats, as well as the results of experiments in animals pre-treated with the following standard drugs: reserpine, atenolol, prazosin,

dobutamine, and verapamil are also presented. Dose-response curves for all standard drugs administered are also presented here.

Following the extraction and fractionation processes described in the previous chapter, crystallization from chloroform yielded 1.53 g of a colourless, crystalline compound with melting point 162.9 – 164.2^{o}C and optical rotation $[\alpha]^{19}_{D}$ +14.1 (*c* 0.78, $CHCl_3$) (Obikeze et al., 2008). The isolated yield for the compound was 0.83%, and TLC on aluminium backed plates containing F_{254} dye with an ethyl acetate: hexane: (3:2) combination as mobile phase yielded a single spot with an R_f value of 0.53.

4 .1 Structure elucidation

The infrared spectrum of the isolated compound as a Nujol mull indicated strong bands at 3626, 2909, 1770, 1462, 1374, 1196, 1100, 994, and 911 cm^{-1} (Appendix I). ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC, HMBC, DEPT and NOESY NMR spectra collected on a Varian Unity*Inova* 600 NMR spectrometer at 25°C were used in structure elucidation. Data from the ^{1}H and ^{13}C spectra is given below, while appendixes II – XXXV contain the full NMR spectra of the compound.

^{1}H NMR ($CDCl_3$, 600MHz): δ 4.70 (1H, dd, *J*=4.6, 5.7 Hz, H-6), 4.38 (1H, ddd, *J*=6.0, 7.2, 9.0 Hz, H-15a), 4.20 (1H, ddd, *J*=6.3, 7.2, 9.0 Hz, H-15b), 2.46 (1H, ddd, *J*=6.3, 7.2, 13.3 Hz, H-16a), 2.27 (3H, m, H-11a, H-12a, H-16b) , 2.17 (3H,m,H-5, H-7eq, H-8), 2.09 (3H, m, H-1eq, H-3eq, H-12b), 1.90 (1H, ddd, *J* = 4.2, 12.2, 18.3 Hz, H-11b), 1.73 (1H, m, H-2eq), 1.65 (1H, m, H-7ax), 1.56 (1H, m, H-2ax), 1.45 (1H, dt, *J* = 5.1, 14.4 Hz, H-3ax),

1.28 (3H, s, H-18), 1.19 (1H, dt, *J*=8.2, 13.2 Hz, H-1ax), 1.05 (3H, s, H-20) and 0.91 (3H, d, *J* = 6.1 Hz, H-17).

^{13}C NMR ($CDCl_3$, 150 MHz): δ 183.8 (C, C-19), 171.4 (C, C-14), 93.5 (C, C-9), 83.6 (C, C-13), 76.1 (CH, C-6), 65.0 (CH_2, C-15), 45.8 (CH, C-5), 44.1 (C, C-4), 39.3 (C, C-10), 37.3 (CH_2, C-16), 34.3 (CH_2, C-12), 32.2 (CH_2, C-7), 31.5 (CH, C-8), 29.3 (CH_2, C-11), 28.3 (CH_2, C-3), 27.8 (CH_2, C-1), 23.8 (CH_3, C-20), 23.1 (CH_3, C-18), 17.9 (CH_2, C-2) and 17.5 (CH_3, C-17).

The three dimensional (3D) X-ray crystallography structure of the compound obtained from X-ray diffraction spectroscopy using the Siemens SMART diffractometer is given in Figure 4.1. High-resolution electron impact mass spectroscopy (HREIMS) on a Waters API X-TOF Ultima indicated a $[M^+H]^+$ ion at *m/z* 349.2009, which correlates with the molecular formula $C_{20}H_{29}O_5$ (calculated for $C_{20}H_{29}O_5$: 349.2015) (appendix XXXVI). Following convention, the compound was named (13*S*)-9α, 13α-epoxylabda-6β(19),15(14) diol dilactone (EDD) and figure 4.2 shows its chemical structure and numbering.

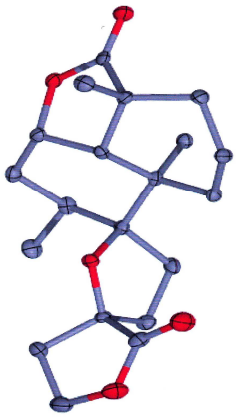


Figure 4.1: 3D structure of (13*S*)-9*α*, 13*α*-epoxylabda-6β(19),15(14)diol dilactone.

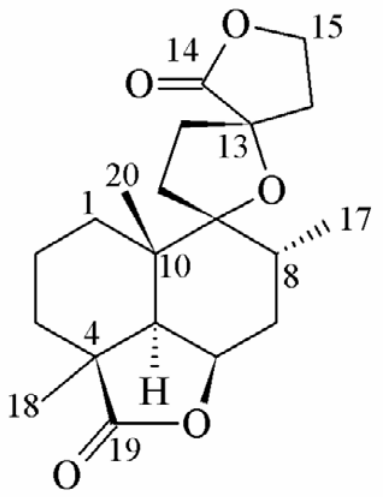


Figure 4.2: Chemical structure of (13*S*)-9*α*, 13*α*-epoxylabda-6β(19),15(14)diol dilactone (Obikeze et al., 2008).

Scalar couplings measured in the ^{1}H NMR spectrum and also from correlations observed in the 2D NOESY spectrum are shown below in figure 4.3.

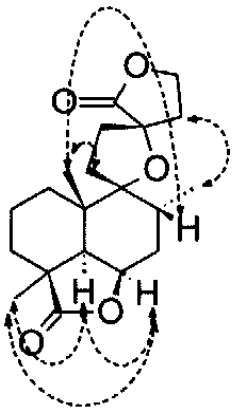


Figure 4.3: NOE correlations for (13*S*)-9*α*, 13*α*-epoxylabda-6β(19),15(14)diol dilactone.

4 .2 Results of cardiovascular experiments

The anaesthetized normotensive rat model was used to study the effects of all the drugs administered IV on the following cardiovascular parameters: systolic pressure (SP), diastolic pressure (DP), mean arterial pressure (MAP) and heart rate (HR). The cardiovascular effect of EDD was determined using 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 3.0 mg/kg, 4.0 mg/kg and 5.0 mg/kg doses of the compound. Doses above 5.0 mg/kg resulted in the death of most animals and so were discontinued. Dose-response curves of the following standard drugs: reserpine (0.01 – 1.0 mg/kg), prazosin (0.5 – 10.0 mg/kg), atenolol (0.5 – 3.0 mg/kg), dobutamine (30 – 90 µg/kg) and verapamil (0.25 – 5.0 mg/kg) were also obtained using the same cardiovascular model. For each standard drug administered, the dose which elicited 80% of the maximum response obtained on the dose-response curves was used to pre-treat animals before administration of EDD.

Results obtained for the different substances administered are presented below in the form of graphs and tables. The graphs give the actual baseline blood pressure and heart rate readings of the values obtained after administration of the standard drugs and EDD, while the percentage change in these parameters with the administration of EDD in pre-treated and non pre-treated animals is also presented in the tables. Tables showing the percentage of the difference in the values of the parameters for EDD administered in pre-treated animals compared to the values of the parameters for EDD administered in non pre-treated animals are presented. However the percentage change in the parameters assessed for dose-response experiments compared with the standard drugs is not presented because this is not the focus of the present study.

4.2.1 Dose-response for the cardiovascular effects of (13*S*)-9α, 13α-epoxylabda-6β(19),15(14)diol dilactone (EDD)

4.2.1.1 Effect on systolic pressure (SP)

Figure 4.4 shows the effect of EDD (0.5 mg/kg – 5.0 mg/kg) on SP. EDD at the lower doses (0.5 mg/kg – 2.0 mg/kg) produced slight decreases in SP, which were neither dose-dependent nor statistically significant when compared to the control readings of 128.0mmHg ± 4.8. With the higher doses (3.0 mg/kg – 5.0 mg/kg) EDD produced statistically significant, dose-dependent increases in SP when compared to the control. The 3.0 mg/kg dose induced a 29.7% increase in SP (from 128.0mmHg ± 4.8 to 166.0mmHg ± 2.6), the 4.0 mg/kg induced a 34% increase in SP (from 128.0mHg ± 4.8 to 171.5mmHg ± 7.8), and the highest dose induced a 35.8% increase in SP (from 128.0mmHg ± 4.8 to 173.0mmHg ± 5.1) (figure 4.4 and table 1).

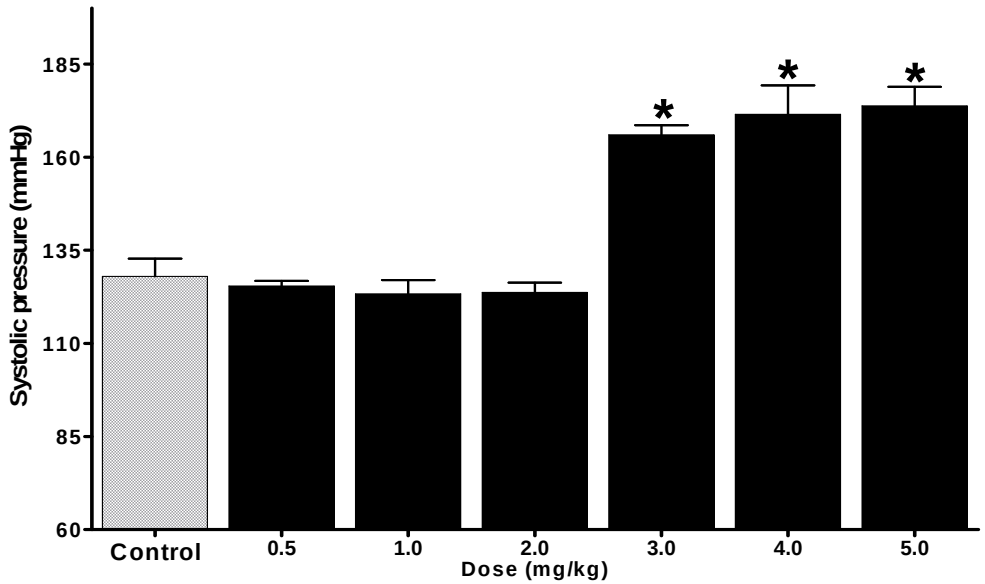


Figure 4.4: Effect of (13*S*)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone on systolic pressure.

*** indicates statistical significance.**

4.2.1.2 Effect on diastolic pressure (DP)

The lower doses of EDD induced dose-dependent decreases in DP (from 78.0mmHg ± 4.7 to: 75.6mmHg ± 1.7; 73.1mmHg ± 3.8; and 72.9mmHg ± 4.1 for the 0.5 mg/kg; 1.0 mg/kg and 2.0 mg/kg doses respectively), which were not statistically significant when compared to the control (78.0mmHg ± 4.7) (figure 4.5). The higher doses of EDD however induced dose-dependent statistically significant increases in DP. The 3.0 mg/kg dose increased DP by 37.2% (from 8.0mmHg ± 4.7 to 107mmHg ± 4.8), the 4.0 mg/kg dose by 49.6% (from 78.0mmHg ± 4.7 to 116.7mmHg ± 9.1) and the 5.0 mg/kg dose by 51% (from 78.0mmHg ± 4.7 to 117.8mmHg ± 7.1) (figure 4.5 and table 1).

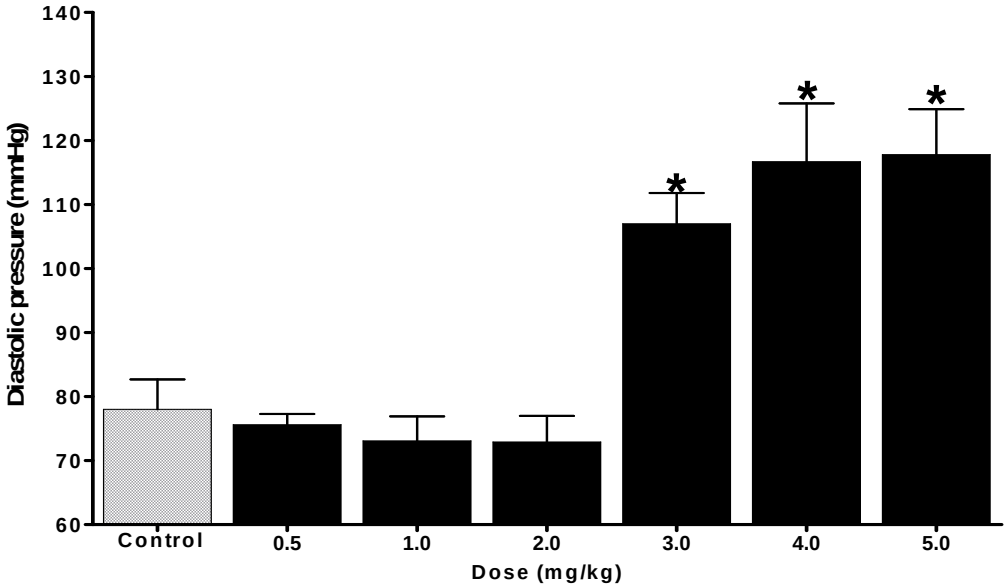


Figure 4.5: Effect of (13*S*)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone on diastolic pressure.

*** indicates statistical significance.**

4.2.1.3 Effect on mean arterial pressure (MAP)

Changes to MAP with the administration of EDD are presented in figure 4.6 below. The lower doses produced slight decreases in MAP which were neither dose-dependent nor statistically significant when compared to the control reading of 111.3mmHg ± 3.8. With the higher doses of EDD, dose-dependent and statistically significant increases in MAP occurred. A 31.5% increase in MAP occurred with the 3.0 mg/kg dose (from 111.3mmHg ± 3.8 to 146.3mmHg ± 4.6), while a 37.7% increase occurred with the 4.0 mg/kg dose (from 111.3mmHg ± 3.8 to 153.2mmHg ± 8.2) and a 39.4% increase occurred with the 5.0 mg/kg dose (from 111.3mmHg ± 3.8 to 155.1mmHg ± 5.7) (figure 4.6 and table 1).

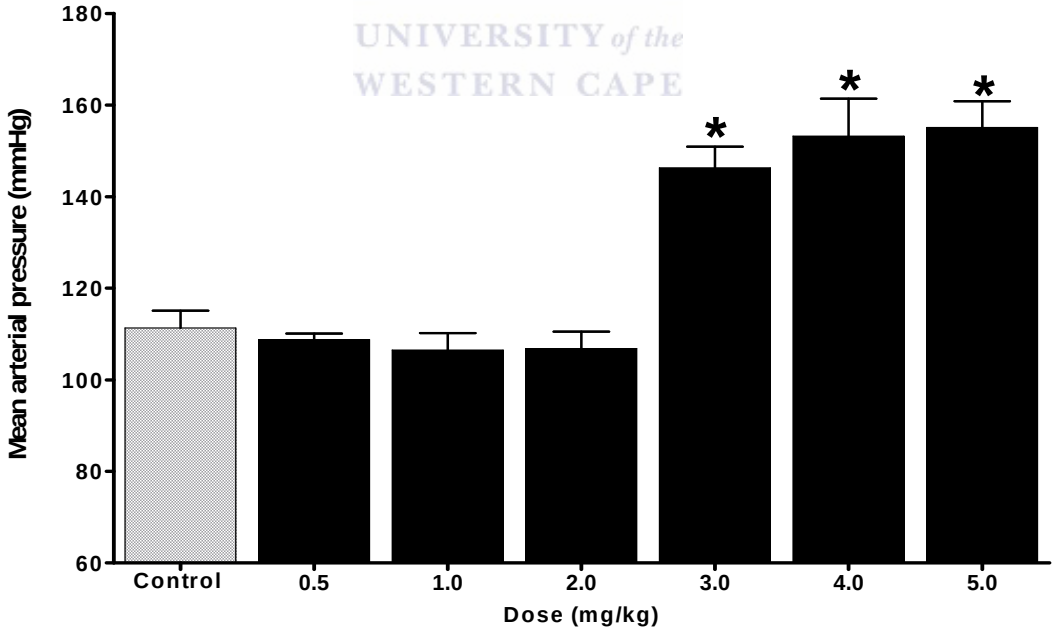


Figure 4.6: Effect of (13*S*)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone on mean arterial pressure.

*** Indicates statistical significance.**

4.2.1.4 Effect on heart rate (HR)

All doses of EDD administered produced dose-dependent decreases in HR (figure 4.7). The decreases produced ranged from a 10% decrease (from 368.4bpm ± 6.5 to 331.4bpm ± 6.1) with the 0.5 mg/kg dose to a 22.6% decrease (from 368.4bpm ± 6.5 to 285.2bpm ± 14.4) with the 5.0 mg/kg dose (table 1). All decreases were statistically significant when compared to the control reading of 368.4bpm ± 4.5.

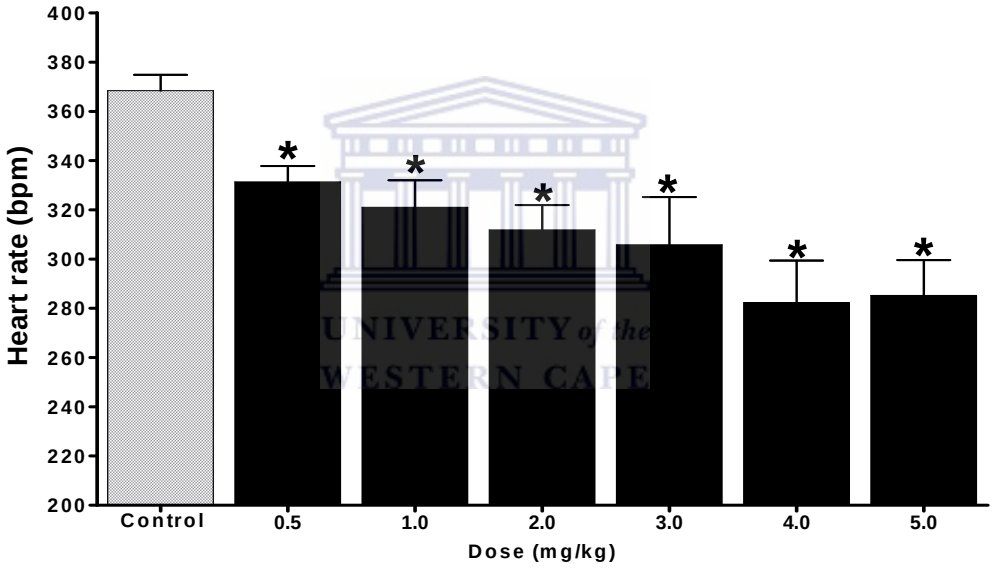


Figure 4.7: Effect of (13*S*)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone on heart rate.

*** indicates statistical significance.**

Table 1: Percentage change ± SEM (% Δ ± SEM) for changes in SP, DP, MAP and HR after administration of EDD (0.5 mg/kg – 5.0 mg/kg). n=8.

% Δ ± SEM

Dose (mg/kg)	SP	DP	MAP	HR
0.5	-2.0 ± 1.44	-3.1 ± 1.79	-2.3 ± 1.39	-10.0 ± 6.40 *
1.0	-3.7 ± 3.77	-6.3 ± 3.84	-4.3 ± 3.73	-12.9 ± 11.54 *
2.0	-3.4 ± 2.62	-6.5 ± 4.12	-4.0 ± 3.78	-15.3 ± 10.26 *
3.0	29.7 ± 2.67 *	37.2 ± 4.88 *	31.5 ± 4.62 *	-17.0 ± 19.41 *
4.0	34.0 ± 7.81*	49.6 ± 9.18 *	37.7 ± 8.25 *	-23.4 ± 17.18 *
5.0	35.8 ± 5.1 *	51.0 ± 7.12 *	39.4 ± 5.71 *	-22.6 ± 14.40 *

* indicates statistical significance when compared to the control.

4.2.2 Dose-response for the cardiovascular effects of standard drugs

4.2.2.1 Effect on SP, DP and MAP

Reserpine

Figure 4.8 below shows the effect of reserpine administered in doses between 0.01 mg/kg and 1.00 mg/kg on blood pressure (BP). All doses administered induced significant dose-dependent decreases in SP when compared to control (127.3mmHg ± 6.0). The lowest dose (0.01 mg/kg) produced the least decrease of 29.1% (from 127.3mmHg ± 6.0 to 90.3mmHg ± 4.95), while the highest dose (1.00 mg/kg) produced the greatest decrease of 38.2% (from 127.3mmHg ± 6.0 to 78.7mmHg ± 2.34) in SP. Reserpine also produced statistically significant decreases in DP with all the doses administered. The lowest dose induced the least decrease of 26.7% (from 82.3mmHg ± 9.4 to 60.3mmHg ± 1.42), while the greatest decrease in DP of 44.9% (from 82.3mmHg ± 9.4 to 45.3mmHg ± 2.5) was produced by the 0.5 mg/kg dose. Dose-dependent decreases in MAP which were significant with all EDD doses administered occurred (figure 4.8).

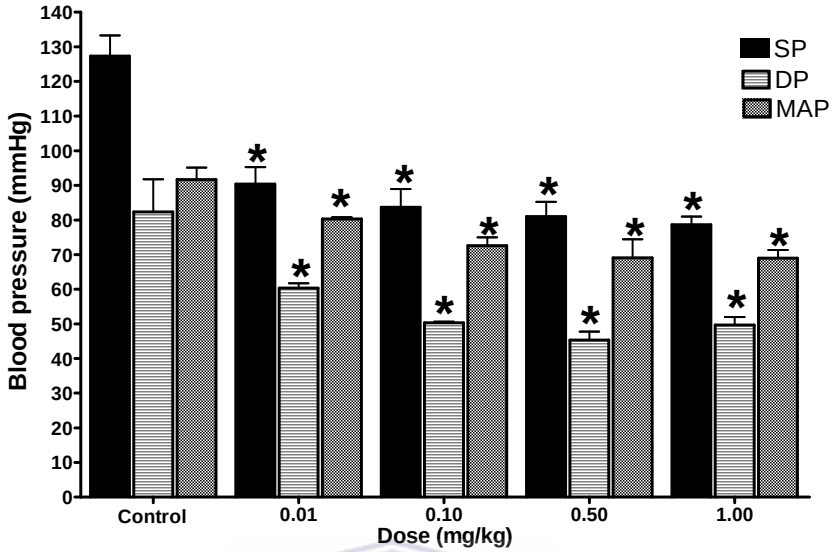


Figure 4.8: Dose-response graph of the effect of reserpine on systolic pressure, diastolic pressure and mean arterial pressure.

***Indicates statistical significance.**

Atenolol

The effect of atenolol administered in the dose range of 0.5 mg/kg to 3.0 mg/kg is shown below in figure 4.9. Atenolol produced dose-dependent decreases in SP with the lowest dose producing the least decrease of 13.7% (from 131.9mmHg ± 1.0 to 113.9 mmHg ± 1.03) and the highest dose producing the greatest decrease in SP of 47.1% (from 131.9mmHg ± 1.0 to 69.8mmHg ± 4.01). All the doses produced statistically significant decreases in SP when compared to control (131.9mmHg ± 1.03). Significant dose-dependent decreases in DP were also recorded with atenolol, ranging from a 10.9% decrease (from 73.7mmHg ± 0.4 to 65.7mmHg ± 0.3) with the lowest dose to a 32.7% decrease (from 73.7mmHg ± 0.4 to 49.6mmHg ± 4.8) with the highest dose (figure 4.9).

Significant dose-dependent decreases in MAP also occurred with all doses of atenolol administered, ranging from a 13.1% decrease (from 112.5mmHg ± 2.6 to 97.8mmHg ± 2.5) with the lowest dose to a 44% decrease (from 112.5mmHg ± 2.6 to 63.0mmHg ± 1.5) with the highest dose (figure 4.9).

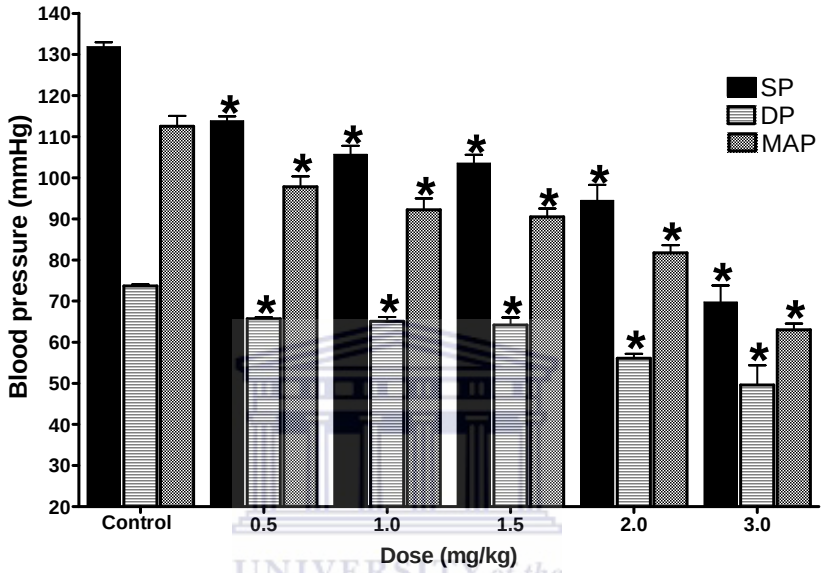


Figure 4.9: Dose-response graph of the effect of atenolol on systolic pressure, diastolic pressure and mean arterial pressure.

*** indicates statistical significance.**

Prazosin

Figure 4.10 below illustrates the effect of prazosin (0.5 mg/kg – 10.0 mg/kg) on SP, DP and MAP. All doses administered induced significant dose-dependent decreases in SP when compared to the control (128.3mmHg ± 1.0). The lowest dose produced a 9.8% decrease (from 128.3mmHg ± 1.0 to 115.1mmHg ± 1.0), while the highest dose produced a 28% decrease in SP (from 128.3mmHg ± 1.0 to 91.1mmHg ± 5.07). Significant dose-

dependent decreases in DP ranging from a 9.5% decrease (from 76.0mmHg ± 0.3 to 65.9mmHg ± 0.3) to a 36.4% decrease (from 76.0mmHg ± 0.3 to 46.2mmHg ± 3.0) with the lowest and highest doses respectively also occurred. As expected statistically significant dose-dependent decreases in MAP also occurred with prazosin administration. A 9.6% decrease occurred (from 108.3mmHg ± 0.7 to 100.3mmHg ± 0.6) with the smallest dose and a 34.3% decrease occurred (from 108.3mmHg ± 0.7 to 76.2mmHg ± 4.3) with the largest dose when compared to the control (108.3mmHg ± 0. 7) (figure 4.10).

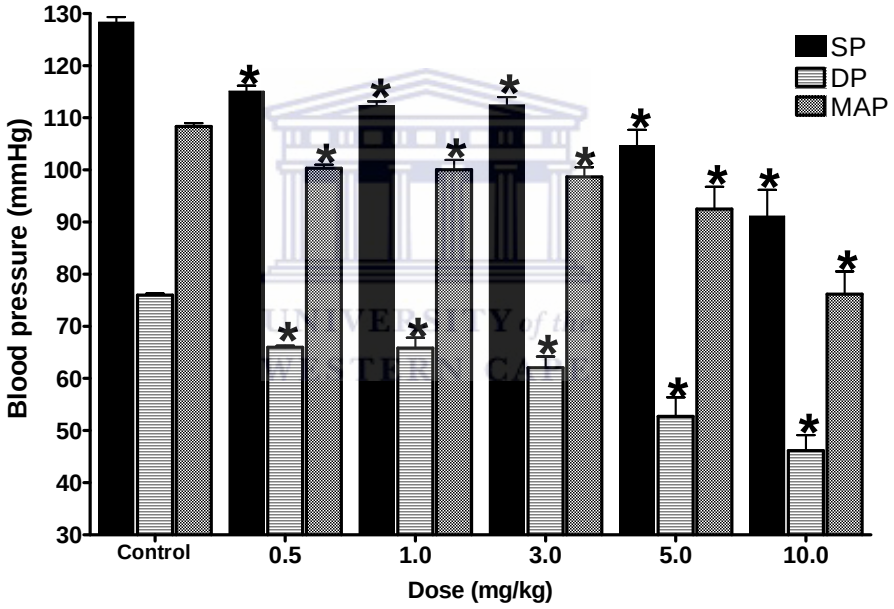


Figure 4.10: Dose-response graph of the effect of prazosin on systolic pressure, diastolic pressure and mean arterial pressure.

*** indicates statistical significance.**

Dobutamine

The effect of dobutamine administered in the dose range of 30 µg/kg - 90 µg/kg on SP, DP and MAP is shown below in figure 4.11. Dobutamine produced dose-dependent increases in SP which were only significant with the 75 µg/kg and 90 µg/kg doses. The 75 µg/kg dose produced a 8.8% increase in SP (from 128.0mmHg ± 3.2 to 139.2mmHg ± 1.8) and the 90 µg/kg dose produced a 12.4% increase in SP (from 128.0mmHg ± 3.2 to 143.9mmHg ± 4.8). Non-significant increases in DP were observed with the 30 µg/kg and 45 µg/kg doses, but non-significant decreases in DP occurred with the administration of all other doses (figure 4.11). Non-significant dose-dependent increases in MAP were observed with the least increase of 1.8% (from 110.5mmHg ± 5.2 to 112.5mmHg ± 9.2) occurring with the lowest dose and the highest increase of 8.5% (from 110.5mmHg ± 5.2 to 119.9mmHg ± 11.7) occurring with the greatest dose (figure 4.11).

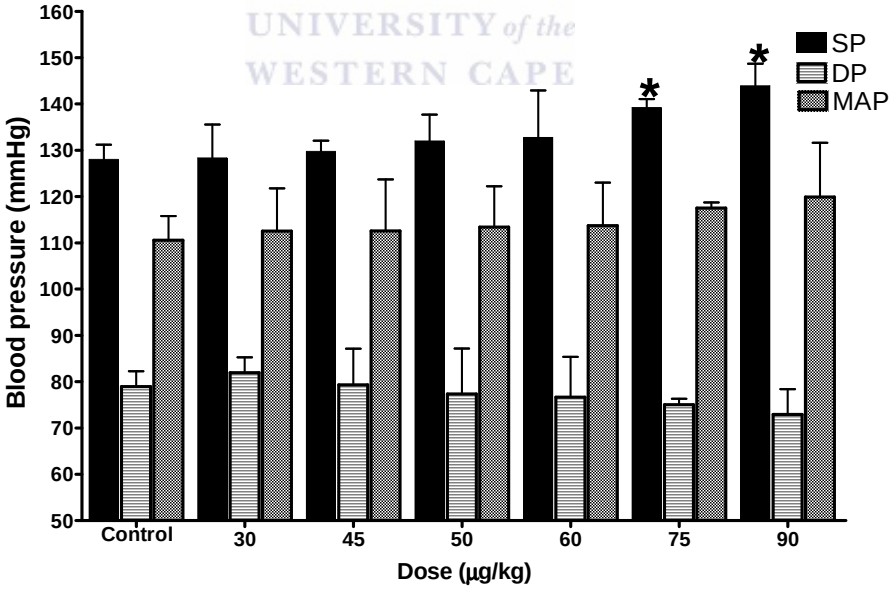


Figure 4.11: Dose-response graph of the effect of dobutamine on systolic pressure, diastolic pressure and mean arterial pressure.

*** indicates statistical significance.**

Verapamil

Figure 4.12 illustrates the effect of verapamil (0.25 mg/kg to 5.0 mg/kg) on SP, DP and MAP. Verapamil produced dose-dependent decreases in SP, the least decrease of 8.6% (from 127.3mmHg ± 6.0 to 117.3 mmHg ± 3.9) and the greatest decrease of 44.1% (from 127.3mmHg ± 6.0 to72.0mmHg ± 5.8) occurring with the lowest and highest doses respectively, significant when compared to control (127.3mmHg ± 6.0) only with doses above 0.5 mg/kg (figure 4.12). Dose-dependent decreases in DP were observed for all doses administered, with a 15.8% decrease (from 82.3mmHg ± 9.4 to 69.3mmHg ± 4.9) occurring with the lowest dose and a 59.8% decrease (from 82.3mmHg ± 9.4 to 33.1mmHg ± 3.9) occurring with the highest dose. Apart from the lowest dose (0.25 mg/kg) decreases in DP were all statistically significant. Dose-dependent decreases in MAP were also observed for all doses administered, with a 9.8% decrease (from 112.3mmHg ± 3.5 to 101.3mmHg ± 1.9) occurring with the lowest dose and a 47.4% decrease (from 112.3mmHg ± 3.5 to 59.1mmHg ± 4.0) occurring with the highest dose. All decreases in MAP were statistically significant except with the 0.25 mg/kg dose (figure 4.12).

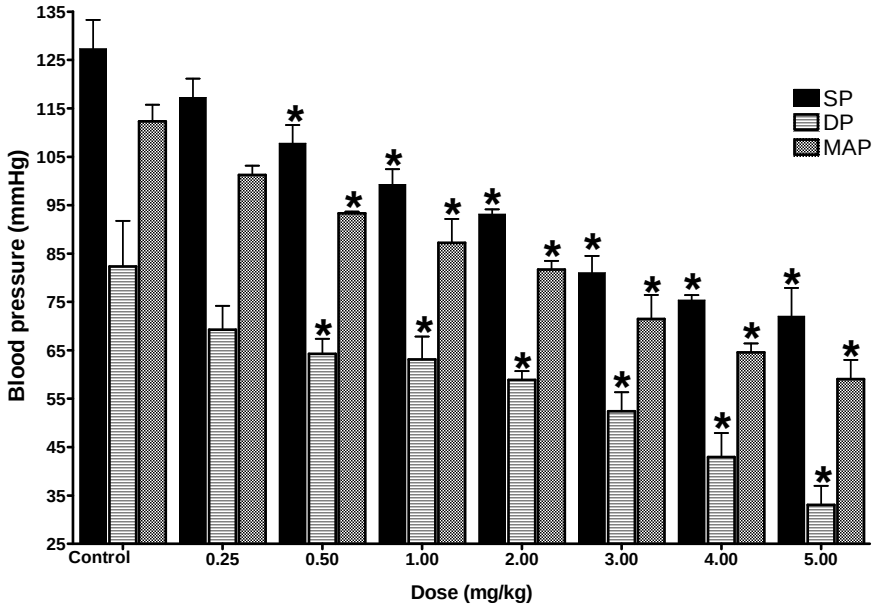


Figure 4.12: Dose-response graph of the effect of verapamil on systolic pressure, diastolic pressure and mean arterial pressure.

*** indicates statistical significance.**

4.2.2.3 Effect on heart rate (HR)

Reserpine

The effect of reserpine administered in the dose range of 0.01 mg/kg to 1.0 mg/kg on HR is shown below in figure 4.13. Dose-dependent decreases in HR occurred with all doses administered, ranging from a 8.7% decrease (from 368.7bpm ± 12.9 to 336.7bpm ± 7.2) with the lowest dose to a 27.8% decrease (from 368.7bpm ± 12.9 to 266bpm ± 2.95) with the highest dose. The decrease in HR was statistically significant for doses above 0.01 mg/kg (figure 4.13).

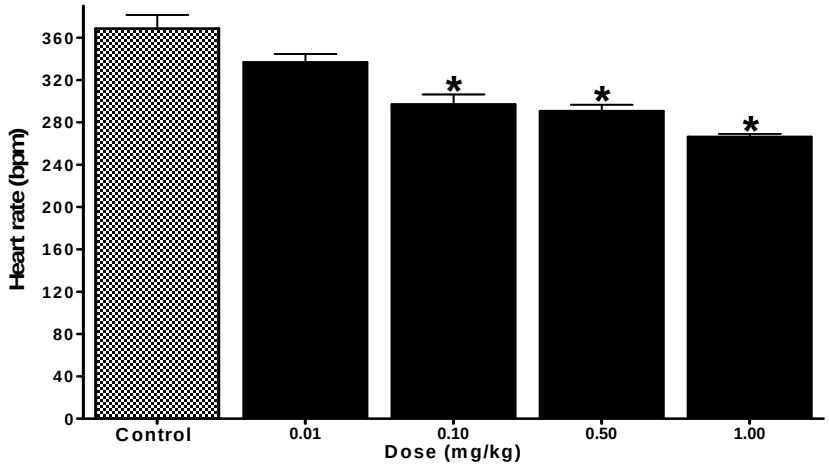


Figure 4.13: Dose-response graph of the effect of reserpine on heart rate.
*** indicates statistical significance.**

Atenolol

Figure 4.14 illustrates the effect of the different doses of atenolol on HR. Atenolol produced significant dose-dependent decreases in HR for all doses administered ranging from a 16.5% decrease (from 364.4bpm ± 3.8 to 304.4bpm ± 3.7) with the lowest dose of 0.5 mg/kg to a 52.6% decrease (from 364.4bpm ± 3.8 to 172.7bpm ± 4.6) with the highest dose (figure 4.14).

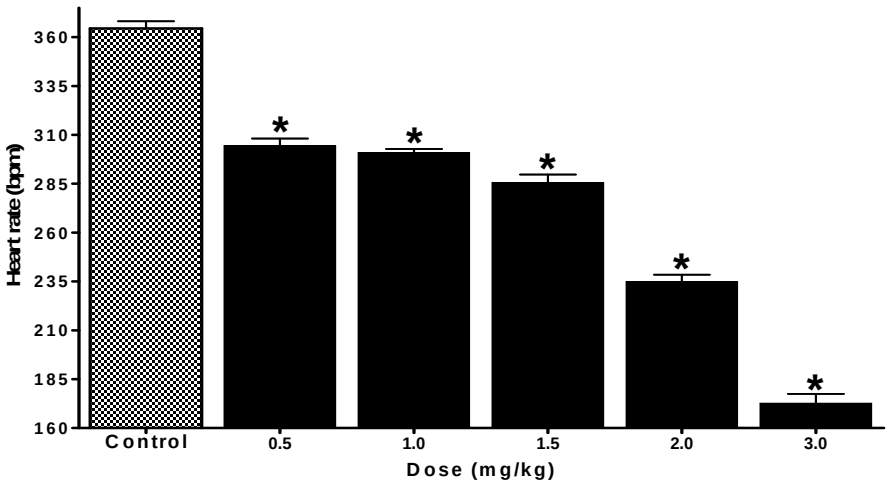


Figure 4.14: Dose-response graph of the effect of atenolol on heart rate.
*** indicates statistical significance.**

Prazosin

The effect of prazosin administered in the dose range of 0.5 mg/kg to 10.0 mg/kg on HR is shown below in figure 4.15. Prazosin produced dose-dependent increases in HR with the lowest dose increasing HR by 3% (from 371.1bpm ± 5.3 to 375.1bpm ± 5.3) and the highest dose increasing HR by 20.3% (from 371.1bpm ± 5.3 to 438.0bpm ± 16.3). Statistically significant increases when compared to control (371.1bpm ± 5.3) only occurred with the 5.0 mg/kg and 10.0 mg/kg doses (figure 4.15).

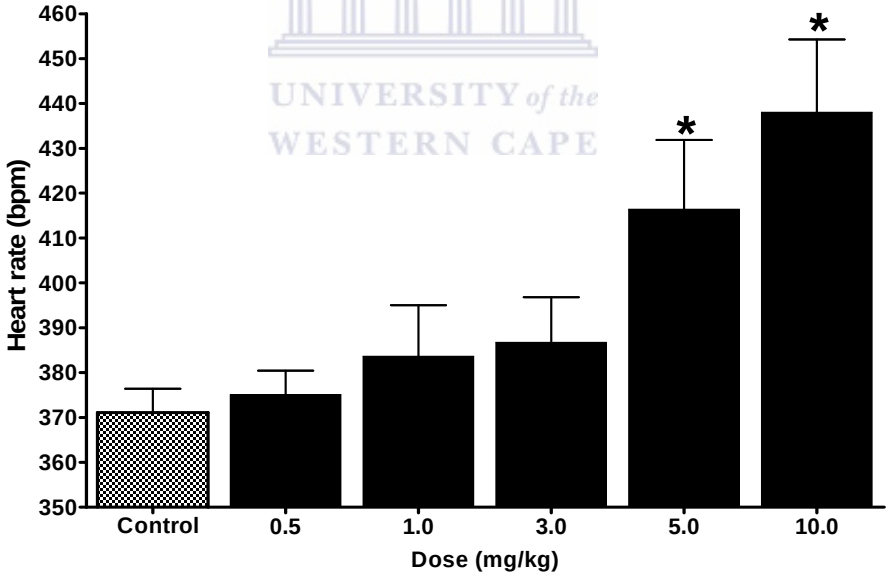


Figure 4.15: Dose-response graph of the effect of prazosin on heart rate.
*** indicates statistical significance.**

Dobutamine

Dobutamine administered in the dose range of 30 µg/kg to 90 µg/kg produced dose-dependent increases in HR (figure 4.16). The lowest dose increased HR by 1.1% (from 368.6bpm ± 6.3 to 372.6bpm ± 6.2) while the highest dose increased HR by 22.1% (from 368.6bpm ± 6.3 to 450.1bpm ± 13.3). With the lowest dose, the increase in HR was non-significant, while for all other doses statistically significant increases when compared to the control value of 368.6bpm ± 6.3 occurred (figure 4.16).

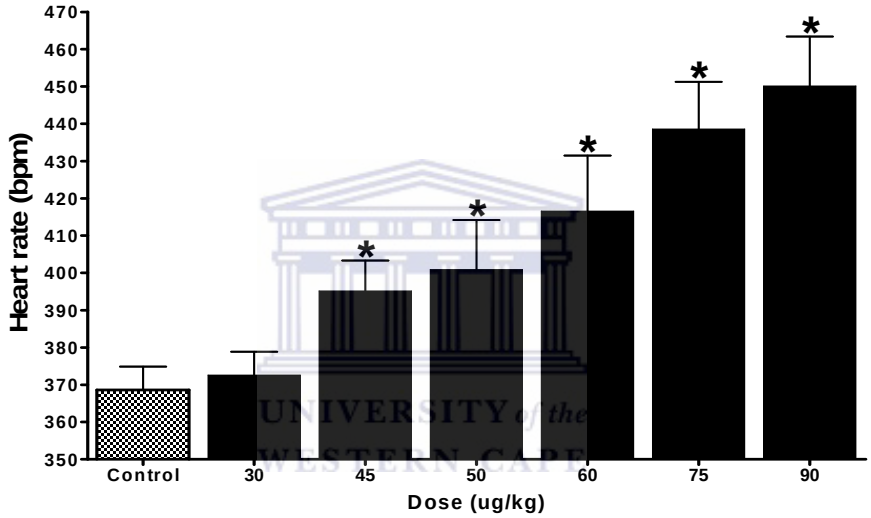


Figure 4.16: Dose-response graph of the effect of dobutamine on heart rate.
*** indicates statistical significance.**

Verapamil

Verapamil administered in the dose range of 0.25 mg/kg to 5.0 mg/kg produced dose-dependent decreases in HR (figure 4.17). The lowest dose decreased HR by 5.6% (from 368.3 ± 8.3 to 366.3bpm ± 3.3) while the highest dose decreased HR by 35.3% (from 369.3 ± 8.3 to 257.0bpm ± 4.0). With the lowest dose, the decrease in HR was non-significant,

while for all other doses statistically significant decreases when compared to the control value of 368.3bpm ± 8.3 occurred (figure 4.17).

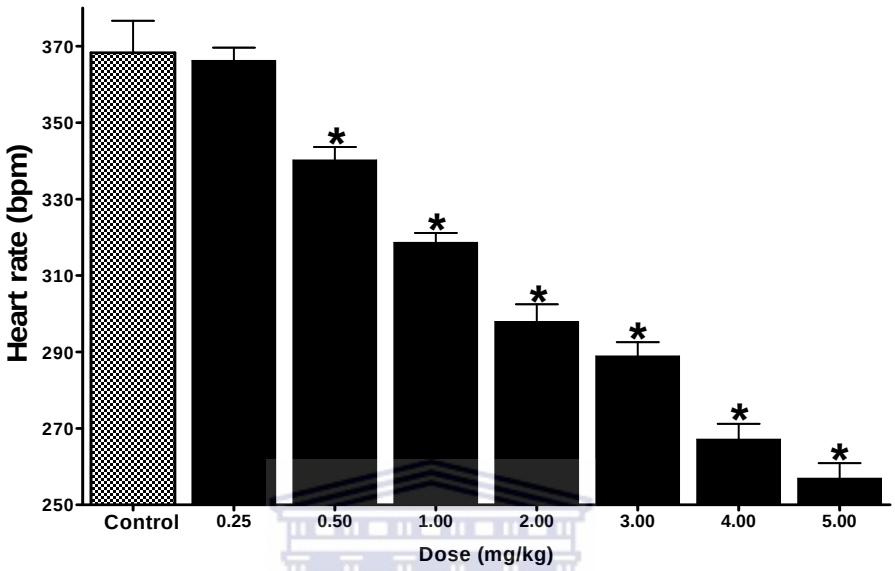


Figure 4.17: Dose-response graph of the effect of verapamil on heart rate. * indicates statistical significance.

4.2.3 Cardiovascular effects of EDD in the presence of standard drugs

4.2.3.1 Systolic pressure (SP)

Reserpine

Figure 4.18 and tables 2 and 3 below illustrate the effect of EDD on SP in reserpinized animals. Reserpine pre-treatment decreased SP by 36.4% (from 127.3mmHg ± 6.0 control to 81.0mmHg ± 4.2). Administration of EDD afterwards led to significant dose-dependent increases in SP with the 0.5 mg/kg to 3.0 mg/kg doses of EDD. The greatest increase of 50.6% (from 81.0mmHg ± 4.2 to 122.0mmHg ± 2.5) occurred with the lowest dose, while

the least increase of 19.8% (from 81.0mmHg ± 4.2 to 105.9mmHg ± 7.0) occurred with the 3.0 mg/kg dose. The 4.0 mg/kg and 5.0 mg/kg doses of EDD produced non-significant, dose-dependent decreases in SP (when compared to reserpine alone) (table 2).

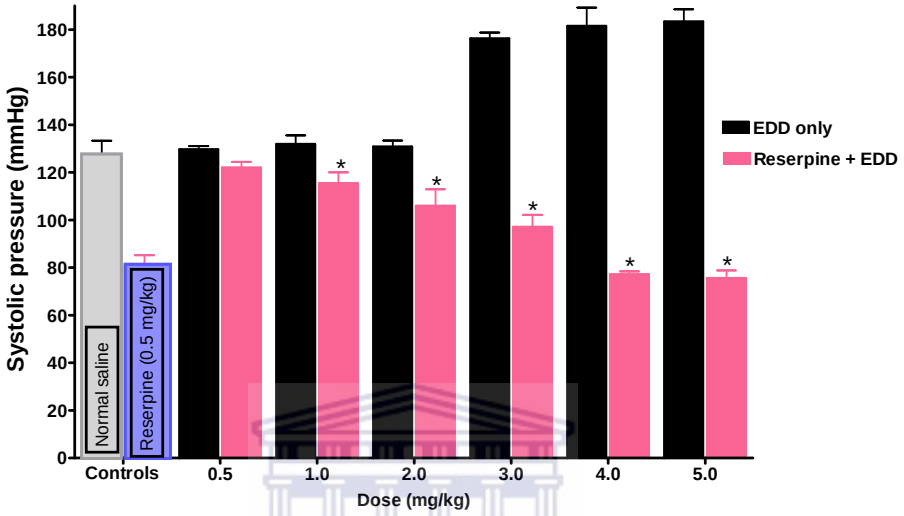


Figure 4.18: Effect of EDD on systolic pressure in animals pre-treated with reserpine. * indicates statistical significance when compared to EDD administered alone.

When compared with the SP values obtained with EDD administration in non-reserpinized animals, dose-dependent statistically significant decreases in SP occurred with the administration of EDD doses greater than 0.5 mg/kg in reserpinized animals (figure 4.18 and table 3). The least decrease in SP of 5.9% (from 129.6mmHg ± 1.4 in non reserpinized animals to 122.0mmHg ± 2.5 in reserpinised animals) occurred with the 0.5 mg/kg dose which was non-significant, while the greatest decrease of 58.8% (from 183.4mmHg ± 5.2 in non reserpinised animals to 75.6mmHg ± 3.2 in reserpinised animals) occurred with the largest dose (table 3).

Table 2: Percentage change ± SEM (% Δ ± SEM) for changes in SP, DP, MAP and HR after administration of EDD (0.5 mg/kg – 5.0 mg/kg) in animals pretreated with reserpine (0.5 mg/kg). n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	50.6 ± 1.23 *	65.3 ± 0.69 *	53.8 ± 1.32 *	12.7 ± 8.35
1.0	42.5 ± 1.69 *	61.2 ± 2.54 *	46.6 ± 3.21 *	7.4 ± 3.45
2.0	30.7 ± 0.58 *	50.4 ± 2.96 *	37.2 ± 1.67 *	1.5 ± 2.58
3.0	19.8 ± 2.52 *	60.9 ± 1.35 *	28.7 ± 1.35 *	-0.5 ± 4.21
4.0	-4.8 ± 1.98	55.0 ± 1.86 *	8.3 ± 1.86 *	-7.3 ± 3.56
5.0	-6.7 ± 2.63	46.8 ± 2.98 *	8.9 ± 2.98 *	-8.9 ± 4.25

* indicates statistical significance when compared to reserpine (0.5 mg/kg).

Table 3: Percentage change ± SEM (% Δ ± SEM) for SP, DP, MAP and HR with EDD administration in reserpinised animals compared to EDD administration in non-reserpinised animals. n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	-5.0 ± 3.45	-3.3 ± 3.68	-5.3 ± 2.63	-5.3 ± 6.04
1.0	-12.5 ± 2.58 *	-6.3 ± 1.23	-11.1 ± 2.54 *	-5.9 ± 11.25
2.0	-19.0 ± 4.21 *	-5.4 ± 1.69	-15.9 ± 2.96 *	-7.1 ± 6.89
3.0	-45.0 ± 3.56 *	-23.7 ± 0.58 *	-40.4 ± 1.35 *	-7.3 ± 12.31
4.0	-57.5 ± 4.25 *	-30.6 ± 2.52 *	-51.6 ± 1.86 *	-8.6 ± 16.24
5.0	-58.8 ± 0.65 *	-39.1 ± 1.98 *	-52.6 ± 2.98 *	-9.0 ± 9.34

* indicates statistical significance when compared to EDD administered alone.

Atenolol

EDD was administered after administration of atenolol (2 mg/kg), and the effects on SP are shown in figure 4.19 and tables 4 and 5 below. Atenolol pre-treatment decreased SP by 28.4% (from 131.9mmHg ± 1.0 to 94.5mmHg ± 3.8), and EDD administration afterwards produced increases in SP with all the doses. The increases were non-significant with the 0.5 mg/kg – 2.0 mg/kg doses, but were significant with all other EDD doses when compared to atenolol (table 4).

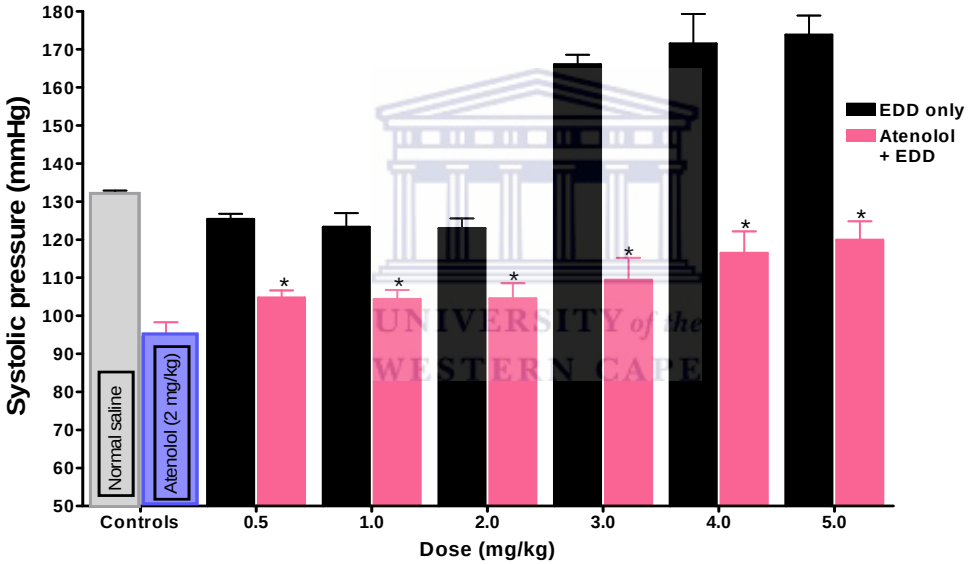


Figure 4.19: Effect of EDD on systolic pressure in animals pre-treated with atenolol. * indicates statistical significance when compared to EDD administered alone.

When compared with the SP values obtained with EDD administration in animals not pre-treated, significant decreases in SP occurred for all EDD doses in animals pre-treated with atenolol (figure 4.19 and table 5). The least difference of 15.0% (from 123.0mmHg ± 2.6 in animals not pre-treated to 104.5mmHg ± 4.0 in pre-treated animals) occurred with the 2.0

mg/kg dose while the greatest decrease in SP of 34.1% (from 166.0mmHg ± 2.6 in animals not pre-treated to 109.4mmHg ± 5.8 in pre-treated animals) occurred with the 3.0 mg/kg dose (table 5).

Table 4: Percentage change ± SEM (% Δ ± SEM) for changes in SP, DP, MAP and HR after administration of EDD (0.5 mg/kg – 5.0 mg/kg) in animals pre-treated with atenolol (2 mg/kg). n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	10.8 ± 2.56	16.0 ± 0.35 *	12.0 ± 4.45 *	-2.4 ± 5.82
1.0	10.4 ± 1.30	16.0 ± 1.70 *	11.7 ± 4.34 *	-5.2 ± 5.35
2.0	10.6 ± 3.70	15.5 ± 3.80 *	11.7 ± 4.38 *	-10.8 ± 2.84 *
3.0	15.8 ± 3.78 *	21.3 ± 4.13 *	17.0 ± 4.38 *	-14.9 ± 5.38 *
4.0	23.2 ± 4.68 *	25.1 ± 4.80 *	23.7 ± 2.49 *	-19.2 ± 2.03 *
5.0	26.9 ± 4.23 *	29.1 ± 3.83 *	27.4 ± 3.49 *	-23.1 ± 4.24 *

* indicates statistical significance when compared to atenolol (2.0 mg/kg).

Table 5: Percentage change ± SEM (% Δ ± SEM) for SP, DP, MAP and HR with EDD administration in animals pre-treated with atenolol compared to EDD administration in non pre-treated animals. n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	-16.5 ± 1.92 *	-13.9 ± 1.40	-15.9 ± 3.78 *	-30.9 ± 3.58 *
1.0	-15.4 ± 4.45 *	-11.0 ± 3.70	-14.3 ± 1.93 *	-30.6 ± 3.75 *
2.0	-15.0 ± 4.34 *	-11.0 ± 2.60	-14.1 ± 2.49 *	-32.8 ± 2.45 *
3.0	-34.1 ± 4.38 *	-36.4 ± 2.63 *	-34.7 ± 4.02 *	-34.6 ± 4.15 *
4.0	-32.1 ± 4.38 *	-39.9 ± 7.80 *	-34.1 ± 5.83 *	-32.8 ± 6.03 *
5.0	-31.0 ± 2.49 *	-38.5 ± 5.12 *	-32.9 ± 5.72 *	-36.6 ± 5.83 *

* indicates statistical significance when compared to EDD administered alone.

Prazosin

EDD was administered after pre-treatment with prazosin (7.0 mg/kg), and the effects on SP are shown in figure 4.20 and tables 6 and 7 below. Prazosin pre-treatment decreased SP by 25.4% (from 128.3mmHg ± 1.0 to 95.5mmHg ± 3.4), and with the administration of EDD slight non-significant non dose-dependent changes in SP occurred. Increases occurred with the 0.5 mg/kg and 2.0 mg/kg doses while decreases occurred with all other doses, but all were not significant when compared to the prazosin control (figure 4.20 and table 6).

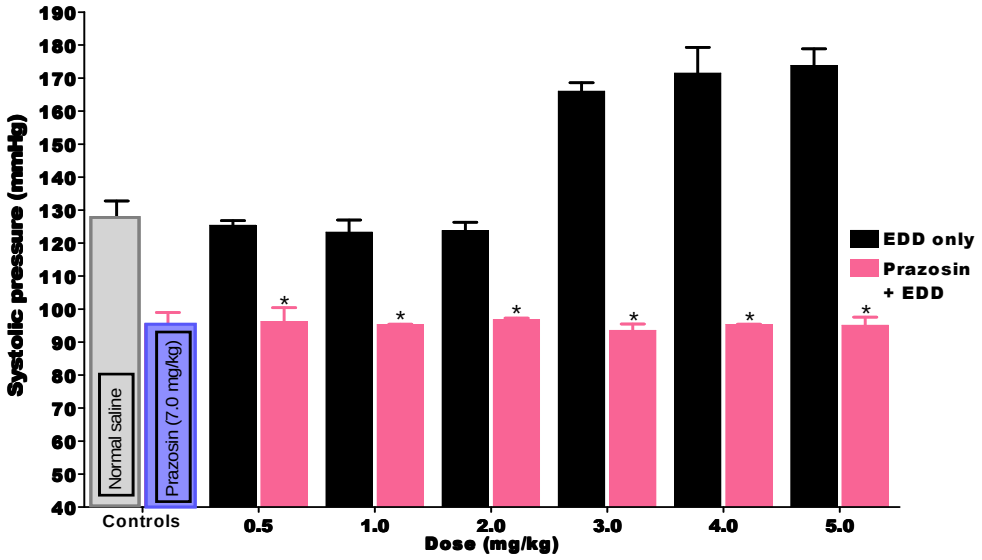


Figure 4.20: Effect of EDD on systolic pressure in animals pre-treated with prazosin. * indicates statistical significance when compared to EDD administered alone.

When compared with the SP values obtained with EDD administration in animals not pre-treated, significant decreases in SP occurred for all EDD doses in animals pre-treated with prazosin. The least decrease of 21.7% (from 123.7mmHg ± 2.6 in animals not pre-treated to 96.8mmHg ± 0.4 in pre-treated animals) occurred with the 2.0 mg/kg dose, while the greatest decrease of 45.3% (from 173.8mmHg ± 5.1 in animals not pre-treated to 95.1mmHg ± 2.4 in pre-treated animals) occurred with the 5.0 mg/kg dose (table 7).

Table 6: Percentage change ± SEM (% Δ ± SEM) for changes in SP, DP, MAP and HR after administration of EDD (0.5 mg/kg – 5.0 mg/kg) in animals pre-treated with prazosin (7 mg/kg). n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	0.7 ± 1.43	-0.4 ± 1.77	1.03 ± 3.82	-11.9 ± 4.67 *
1.0	-0.2 ± 3.72	0.8 ± 2.35	0.0 ± 1.31	-12.5 ± 1.71 *
2.0	1.4 ± 2.61	-2.4 ± 4.65	0.5 ± 3.70	-12.9 ± 3.82 *
3.0	-2.1 ± 2.63	-4.1 ± 4.10	-3.0 ± 3.80	-13.7 ± 4.12 *
4.0	-0.2 ± 2.28	4.4 ± 3.82	0.9 ± 0.40	-14.7 ± 4.80 *
5.0	-0.4 ± 0.20	1.4 ± 1.23	-0.0 ± 2.28	-18.2 ± 3.74 *

* indicates statistical significance when compared to prazosin (7.0 mg/kg).

Table 7: Percentage change ± SEM (% Δ ± SEM) for SP, DP, MAP and HR with EDD administration in animals pre-treated with prazosin compared to EDD administration in non pre-treated animals. n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	-23.3 ± 3.43 *	-26.3 ± 2.57 *	-23.7 ± 2.35 *	4.5 ± 2.25 *
1.0	-22.7 ± 4.25 *	-22.9 ± 2.32 *	-22.7 ± 4.65 *	7.2 ± 0.11 *
2.0	-21.7 ± 0.08 *	-25.1 ± 0.20 *	-22.5 ± 4.10 *	9.9 ± 0.27 *
3.0	-43.7 ± 0.43 *	-49.9 ± 0.40 *	-45.4 ± 3.80 *	11.0 ± 2.28 *
4.0	-44.4 ± 1.95 *	-50.0 ± 2.28 *	-45.8 ± 1.23 *	18.8 ± 0.20 *
5.0	-45.3 ± 0.06 *	-51.9 ± 0.49 *	-46.9 ± 3.55 *	12.8 ± 1.67 *

* indicates statistical significance when compared to EDD administered alone.

Dobutamine

EDD was administered after pre-treatment with dobutamine (75 µg/kg), and the effects on SP are shown in figure 4.21 and tables 8 and 9 below. Dobutamine pre-treatment increased SP by 8.8% (from 128.0mmHg ± 3.2 to 139.2mmHg ± 1.8), and with the administration of EDD further dose-dependent increases in SP occurred, which were significant with the higher (3.0 mg/kg – 5.0 mg/kg) doses (figure 4.21 and table 8).

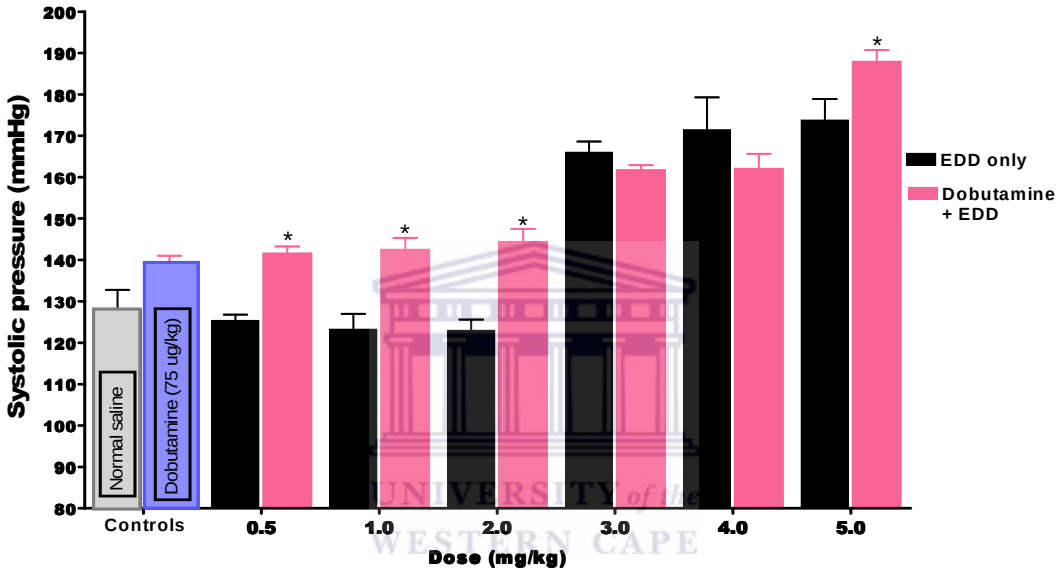


Figure 4.21: Effect of EDD on systolic pressure in animals pre-treated with dobutamine.
*** indicates statistical significance when compared to EDD administered alone.**

When compared with the SP values obtained with EDD administration in animals not pre-treated with dobutamine, significant increases in SP occurred with the 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 5.0 mg/kg doses in pre-treated animals (figure 4.21 and table 9). The least increase of 8% (from 173.8mmHg ± 5.1 in animals not pre-treated to 187.8mmHg ± 3.0 in pre-treated animals) occurred with the 5.0 mg/kg dose, while the greatest increase of 17.3% (from 123.0mmHg ± 2.6 in animals not pre-treated to 144.3mmHg ± 3.2 in pre-

treated animals) occurred with the 2.0 mg/kg dose. Non-significant decreases occurred with the 3.0 mg/kg and 4.0 mg/kg doses in animals pre-treated with dobutamine (table 9).

Table 8: Percentage change ± SEM (% Δ ± SEM) for changes in SP, DP, MAP and HR after administration of EDD (0.5 mg/kg – 5.0 mg/kg) in animals pre-treated with dobutamine (75 µg/kg). n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	1.6 ± 1.31	-0.8 ± 1.24	1.4 ± 1.41	-16.0 ± 3.80 *
1.0	2.2 ± 1.51	4.8 ± 1.58	3.1 ± 3.71	-18.7 ± 1.34 *
2.0	3.6 ± 2.59	-2.2 ± 2.81	2.7 ± 2.62	-19.5 ± 3.72 *
3.0	16.1 ± 3.77 *	53.6 ± 3.24 *	24.4 ± 2.67 *	-20.6 ± 3.78 *
4.0	16.3 ± 1.87 *	69.8 ± 1.22 *	28.0 ± 7.87 *	-20.8 ± 4.60 *
5.0	34.9 ± 2.19 *	75.6 ± 3.13 *	43.9 ± 1.69 *	-21.4 ± 8.21 *

* indicates statistical significance when compared to dobutamine (75 µg/kg).

Table 9: Percentage change ± SEM (% Δ ± SEM) for SP, DP, MAP and HR with EDD administration in animals pre-treated with dobutamine compared to EDD administration in non pre-treated animals. n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	12.8 ± 1.24 *	-1.5 ± 4.67	9.5 ± 1.31 *	11.2 ± 1.40 *
1.0	15.5 ± 1.58 *	7.6 ± 1.70	13.7 ± 1.51 *	11.1 ± 3.71 *
2.0	17.3 ± 2.81 *	0.7 ± 3.80	13.5 ± 2.59 *	13.2 ± 2.67 *
3.0	-2.6 ± 3.24	7.7 ± 4.10	-0.1 ± 3.77	13.9 ± 2.68 *
4.0	-5.6 ± 1.22	9.2 ± 4.88	-1.8 ± 1.87	23.1 ± 7.80 *
5.0	8.0 ± 3.13 *	11.9 ± 3.83 *	9.0 ± 2.19 *	20.9 ± 5.10 *

* indicates statistical significance when compared to EDD administered alone.

Verapamil

EDD was administered after pre-treatment with verapamil (4.0 mg/kg), and the effects on SP are shown in figure 4.22 and tables 10 and 11 below. Verapamil pre-treatment decreased SP by 41.1% (from 127.3mmHg ± 6.0 to 75.4mmHg ± 1.0), and with the administration of EDD a slight non-significant increase in SP occurred with the 0.5 mg/kg dose, while with all other doses significant dose-dependent increases in SP occurred (figure 4.22 and table 10).

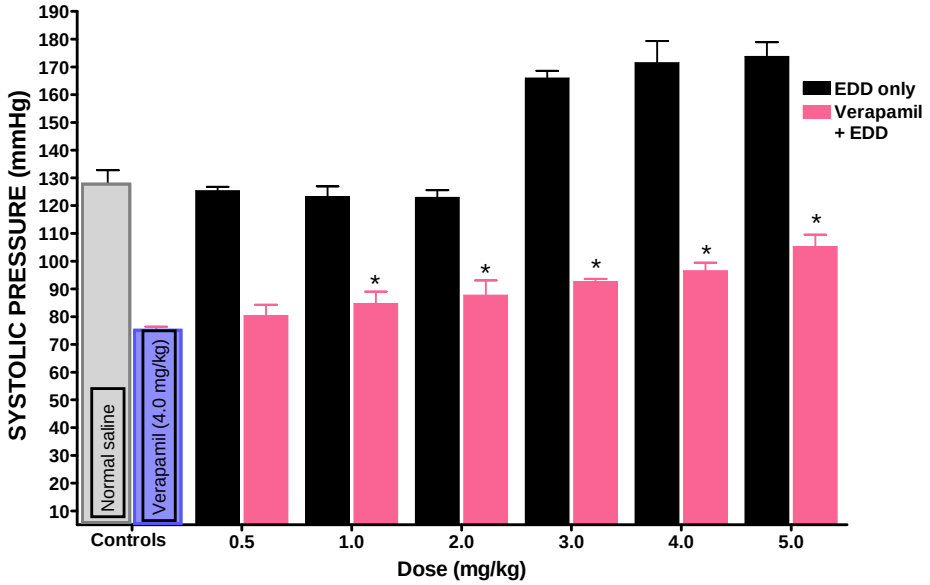


Figure 4.22: Effect of EDD on systolic pressure in animals pre-treated with verapamil. * indicates statistical significance when compared to EDD administered alone.

When compared to animals that received no pre-treatment, significant decreases in SP occurred for all EDD doses in animals pre-treated with verapamil. The least decrease of 35.9% (from 125.4mmHg ± 1.4 in animals not pre-treated to 80.4mmHg ± 3.9 in pre-treated animals) occurred with the lowest dose, while the greatest decrease of 62.4% (from 173.8mmHg ± 5.1 in animals not pre-treated to 105.3mmHg ± 4.3 in pre-treated animals) occurred with the highest dose (figure 4.22 and table 11).

Table 10: Percentage change ± SEM (% Δ ± SEM) for changes in SP, DP, MAP and HR after administration of EDD (0.5 mg/kg – 5.0 mg/kg) in animals pre-treated with verapamil (4.0 mg/kg). n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	6.7 ± 1.70	16.3 ± 2.32 *	8.8 ± 4.93	5.7 ± 1.87
1.0	12.5 ± 3.80 *	24.6 ± 2.01 *	15.2 ± 2.32 *	1.8 ± 3.04
2.0	16.4 ± 4.10 *	26.4 ± 4.01 *	18.6 ± 2.10 *	-1.6 ± 1.94
3.0	23.0 ± 1.04 *	36.3 ± 2.28 *	25.9 ± 4.01 *	-3.9 ± 7.92
4.0	28.1 ± 3.94 *	48.4 ± 4.90 *	32.6 ± 2.28 *	-5.2 ± 3.48
5.0	39.7 ± 4.23 *	59.7 ± 1.68 *	44.1 ± 4.94 *	-7.9 ± 2.34

* indicates statistical significance when compared to verapamil (4.0 mg/kg).

Table 11: Percentage change ± SEM (% Δ ± SEM) for SP, DP, MAP and HR with EDD administration in animals pre-treated with verapamil compared to EDD administration in non pre-treated animals. n=8.

% Δ ± SEM				
EDD (mg/kg)	**SP**	**DP**	**MAP**	**HR**
0.5	-35.9 ± 1.40 *	-33.9 ± 3.80 *	-35.4 ± 1.87 *	-14.8 ± 3.70 *
1.0	-39.3 ± 3.70 *	-40.4 ± 1.30 *	-39.6 ± 3.04 *	-15.3 ± 2.60 *
2.0	-40.1 ± 2.60 *	-42.0 ± 3.70 *	-40.5 ± 1.94 *	-15.7 ± 2.60 *
3.0	-56.2 ± 2.60 *	-64.0 ± 3.40 *	-58.1 ± 1.87 *	-16.0 ± 7.28 *
4.0	-60.6 ± 3.48 *	-71.1 ± 4.68 *	-63.3 ± 3.04 *	-10.3 ± 5.10 *
5.0	-62.4 ± 2.34 *	-74.0 ± 1.49 *	-65.4 ± 1.94 *	-13.7 ± 4.93 *

* indicates statistical significance when compared to EDD administered alone.

4.2.3.2 Diastolic pressure (DP)

Reserpine

Figure 4.23 below illustrates the effect of EDD on diastolic pressure in reserpinised animals. Reserpine pre-treatment decreased DP by 44.9% (from 82.3mmHg ± 9.4 to 45.3mmHg ± 2.5), and with the administration of EDD, significant dose-dependent increases in DP occurred (table 2). The greatest increase of 65.3% (from 45.3mmHg ± 2.5 to 75.0mmHg ± 1.2) occurred with the lowest dose, while the least increase of 46.8% (from 45.3mmHg ± 2.5 to 66.6mmHg ± 2.6) occurred with the highest dose (figure 4.23 and table 2).

When compared to non reserpinised animals, dose-dependent decreases in DP occurred for all EDD doses in reserpinised animals, but were significant with only the 3.0 mg/kg, 4.0 mg/kg and 5.0 mg/kg doses. The least decrease of 3.3% (from 77.5mmHg ± 1.7 in non-reserpinised to 75.0mmHg ± 1.2 in reserpinised animals) occurred with the lowest dose, while the greatest decrease of 39.1% (from 109.4mmHg ± 7.1 in non-reserpinised to 66.6mmHg ± 2.6 in reserpinised animals) occurred with the highest dose (figure 4.23 and table 3).

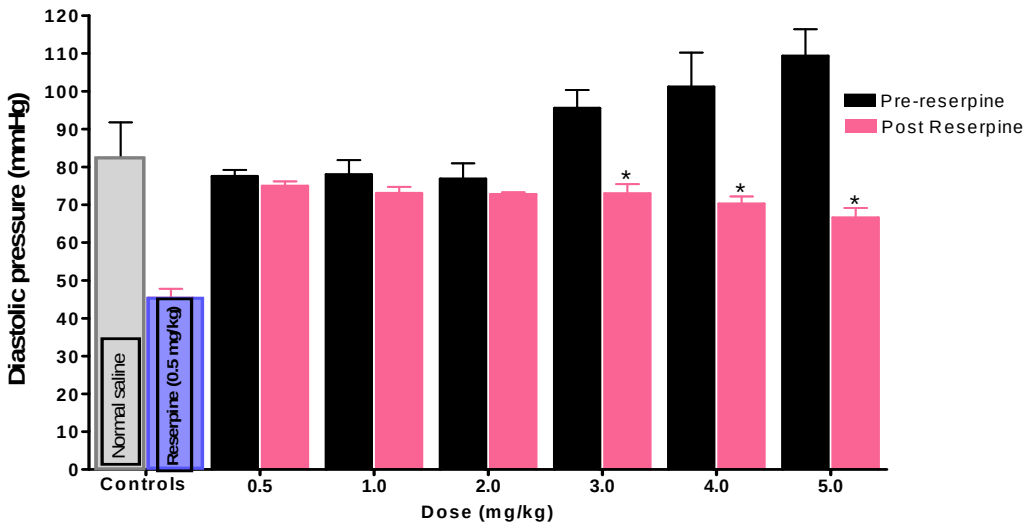


Figure 4.23: Effect of EDD on diastolic pressure in animals pre-treated with reserpine.
*** indicates statistical significance when compared to EDD administered alone.**

Atenolol

The effects of EDD administered after pre-treatment with atenolol (2 mg/kg) on DP are shown in figure 4.24 below. Atenolol pre-treatment decreased DP by 23.9% (from 73.7mmHg ± 0.4 to 56.1 mmHg ± 1.1), and with the administration of EDD increases in DP occurred with all the doses (table 4).

When compared with the DP values obtained with EDD administration in animals not receiving atenolol pre-treatment, decreases in DP occurred for all EDD doses in pre-treated animals, but were statistically significant with the 3.0 mg/kg – 5.0 mg/kg doses (figure 4.24 and table 5). The least decrease of 11% (from 73.1mmHg ± 3.8 in animals not pre-treated to 65.1mmHg ± 5.4 in pre-treated animals) occurred with the 1.0 mg/kg dose, while the

greatest decrease of 39.9% (from 116.7mmHg ± 9.1 in animals not pre-treated to 70.2mmHg ± 2.5 in pre-treated animals) occurred with the 4.0 mg/kg dose (table 5).

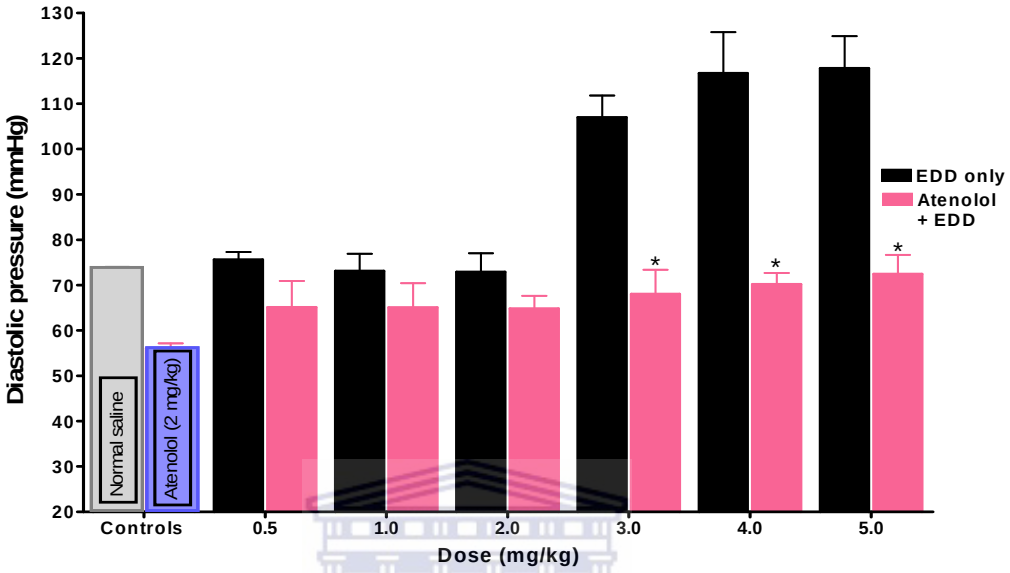


Figure 4.24: Effect of EDD on diastolic pressure in animals pre-treated with atenolol. * indicates statistical significance when compared to EDD administered alone.

Prazosin

EDD was administered after pre-treatment with prazosin (7.0 mg/kg), and the effects on DP are shown in figure 4.25 below. Prazosin pre-treatment decreased DP by 28.3% (from 76.0mmHg ± 0.3 to 55.9mmHg ± 2.6), and with the administration of EDD, slight non-significant non dose-dependent changes in DP occurred. Increases occurred with the 1.0 mg/kg, 4.0 mg/kg and 5.0 mg/kg doses while decreases occurred with all other doses, but all were not significant when compared to the prazosin control (figure 4.25 and table 6).

When compared with the DP values obtained with EDD administration in animals not receiving prazosin pre-treatment, significant decreases in DP occurred for all EDD doses in animals pre-treated with prazosin. The least decrease of 22.9% (from 73.1mmHg ± 3.8 in animals not pre-treated to 82.3mmHg ± 0.1 in pre-treated animals) occurred with the 1.0 mg/kg dose, while the greatest decrease of 51.9% (from 117.8mmHg ± 7.1 in animals not pre-treated to 56.7mmHg ± 0.2 in pre-treated animals) occurred with the 5.0 mg/kg dose (figure 4.25 and table 7).

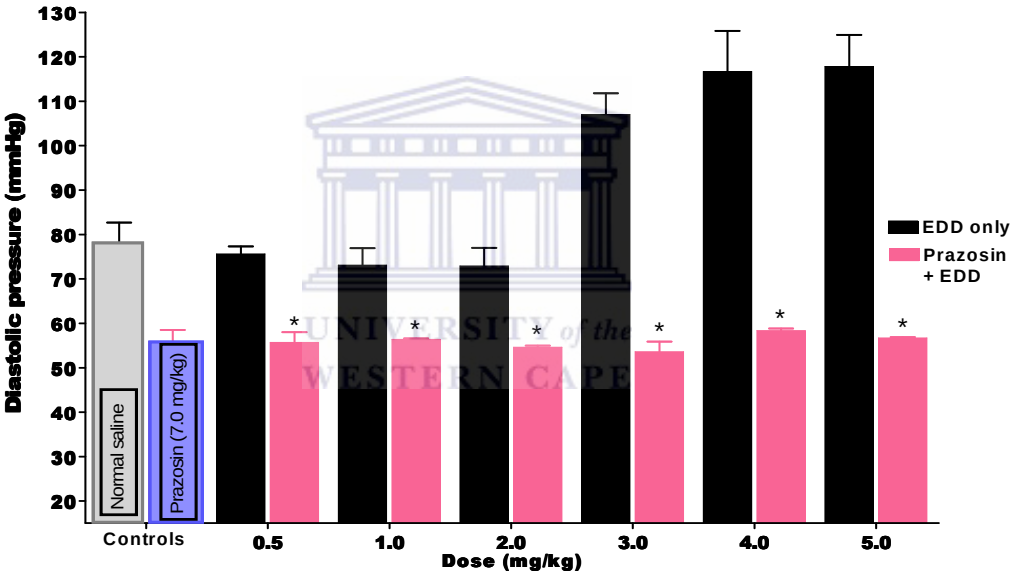


Figure 4.25: Effect of EDD on diastolic pressure in animals pre-treated with prazosin. * indicates statistical significance when compared to EDD administered alone.

Dobutamine

EDD was administered after pre-treatment with dobutamine (75 µg/kg), and the effects on DP are shown in figure 4.26 below. Dobutamine pre-treatment decreased DP by 5.1%

(from 79.0mmHg ± 3.3 to 75.0mmHg ± 1.3), and with the administration of EDD increases in DP occurred with the 1.0 mg/kg, 3.0 mg/kg, 4.0 mg/kg and 5.0 mg/kg, which were statistically significant with the higher (3.0 mg/kg – 5.0 mg/kg) doses. Non significant decreases in DP occurred with the 0.5 mg/kg and 2.0 mg/kg doses (figure 4.26 and table 8).

When compared with the DP values obtained with EDD administration in animals not receiving dobutamine pre-treatment, increases in DP occurred with the 1.0 mg/kg - 5.0 mg/kg doses in animals pre-treated with dobutamine. A 0.7% increase (from 72.9mmHg ± 4.1 in animals not pre-treated to 73.4mmHg ± 3.8 in pre-treated animals) occurred with the 2.0 mg/kg dose which was the least increase. Statistically significant increases occurred only with the 5.0 mg/kg dose with an 11.9% increase (from 117.8mmHg ± 7.1 in animals not pre-treated to 131.8mmHg ± 1.7 in pre-treated animals). A slight non-significant decrease occurred with the 0.5 mg/kg dose (figure 4.26 and table 9).

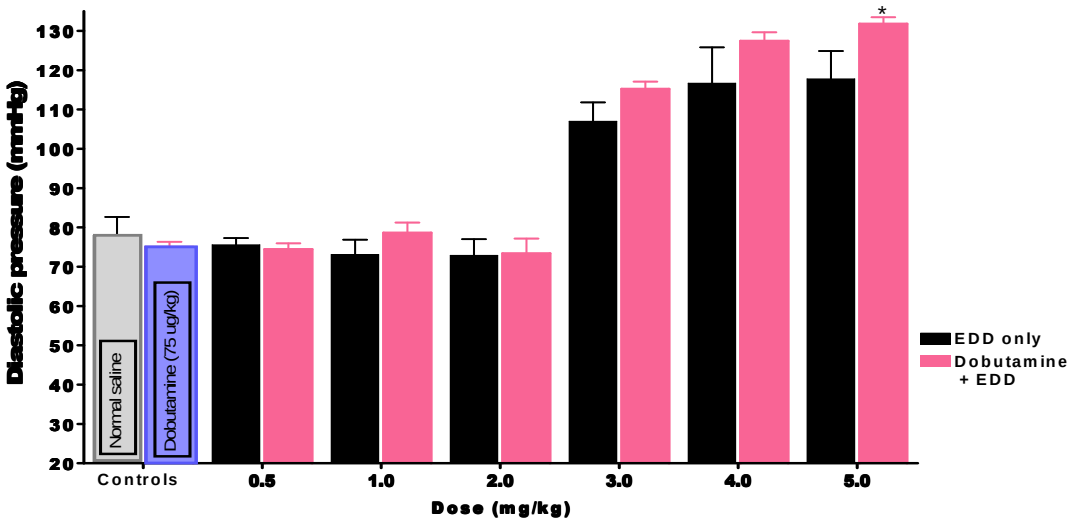


Figure 4.26: Effect of EDD on diastolic pressure in animals pre-treated with dobutamine.
*** indicates statistical significance when compared to EDD administered alone.**

Verapamil

EDD was administered after pre-treatment with verapamil (4.0 mg/kg), and the effects on DP are shown in figure 4.27 below. Verapamil pre-treatment decreased DP by 44.9% (from 78.0mmHg ± 4.7 to 43.0mmHg ± 4.9), and with the administration of EDD, dose-dependent increases occurred with all doses (table 10).

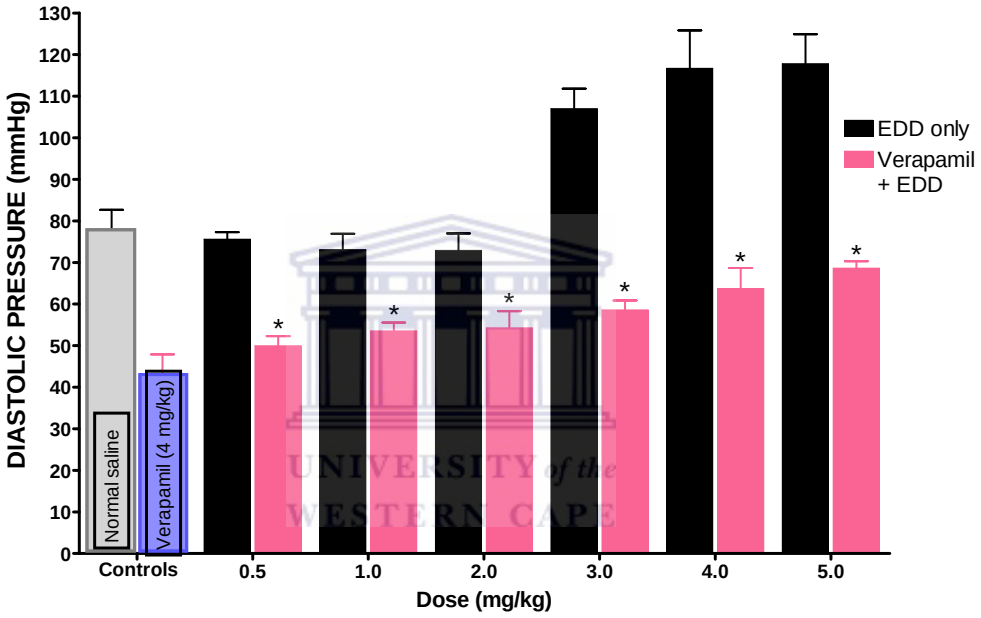


Figure 4.27: Effect of EDD on diastolic pressure in animals pre-treated with verapamil.
*** indicates statistical significance when compared to EDD administered alone.**

When compared with the DP values obtained with EDD administration in animals that received no verapamil pre-treatment, significant dose-dependent decreases in DP occurred for all EDD doses in animals pre-treated with verapamil. The least decrease of 33.9% (from 75.6mmHg ± 1.7 in animals not pre-treated to 50.0mmHg ± 2.3 in pre-treated animals) occurred with the smallest dose, while the greatest decrease of 74% (from 117.8mmHg ±

7.1 in animals not pre-treated to 68.6mmHg ± 1.7 in pre-treated animals) occurred with the 5.0 mg/kg dose (table 11).

4.2.3.3 Mean arterial pressure (MAP)

Reserpine

Figure 4.28 below illustrates the effect of EDD on MAP in reserpinized animals. Reserpine pre-treatment decreased MAP by 38.5% (from 112.3mmHg ± 3.5 to 69.1mmHg ± 5.3), and with the administration of EDD dose-dependent increases in MAP occurred with all doses of EDD. The highest increase of 53.8% (from 69.1mmHg ± 5.3 to 106.3mmHg ± 0.7) occurred with the smallest dose, while the smallest increase of 8.3% (from 69.1mmHg ± 5.3 to 74.8mmHg ± 1.4) occurred with the 4.0 mg/kg dose (figure 4.28 and table 2).

When compared with the MAP values obtained with EDD administration in non-reserpinised animals, dose-dependent decreases in MAP occurred in reserpinised animals which were significant for all doses above 0.5 mg/kg (figure 4.28 and table 3).

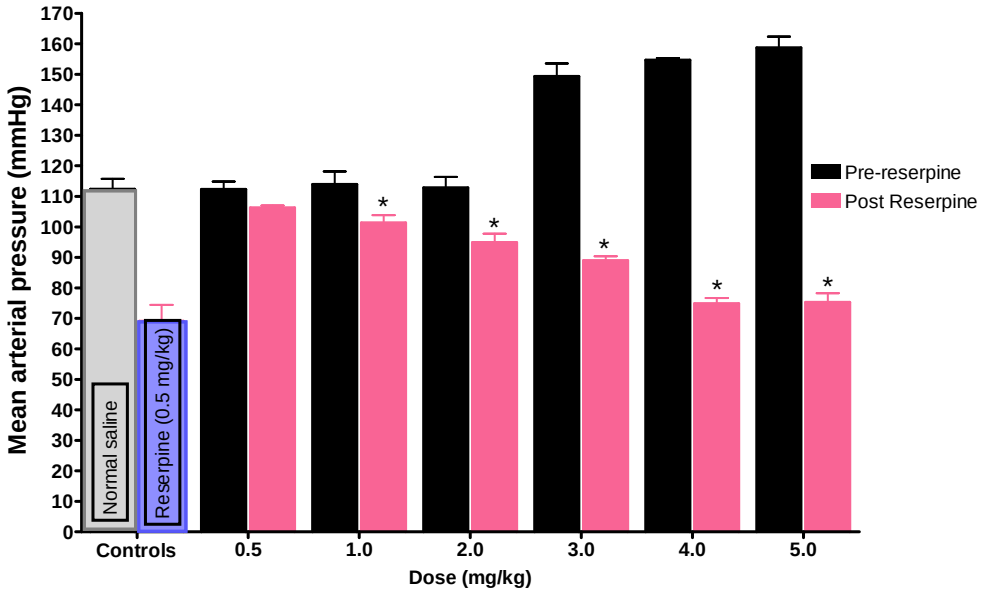


Figure 4.28: Effect of EDD on mean arterial pressure in animals pre-treated with reserpine.
*** indicates statistical significance when compared to EDD administered alone.**

Atenolol

The effects of EDD administered after pre-treatment with atenolol (2 mg/kg) on MAP are illustrated below in figure 4.29. Atenolol pre-treatment decreased MAP by 23.4% (from 112.5mmHg ± 2.6 to 81.7mmHg ± 1.9), and with the administration of EDD significant dose-dependent increases in MAP occurred (table 4).

When compared with the MAP values obtained with EDD administration in animals not pre-treated with atenolol, all doses produced significantly lower increases to MAP (table 5). The least difference of 14.1% (from 106.3mmHg ± 3.7 in animals not pre-treated to 91.3mmHg ± 4.4 in pre-treated animals) occurred with the 2.0 mg/kg dose, while the greatest difference of 34.7% (from 146.3mmHg ± 4.6 in animals not pre-treated to

68.0mmHg ± 5.4 in pre-treated animals) occurred with the 3.0 mg/kg dose (figure 4.29 and table 5).

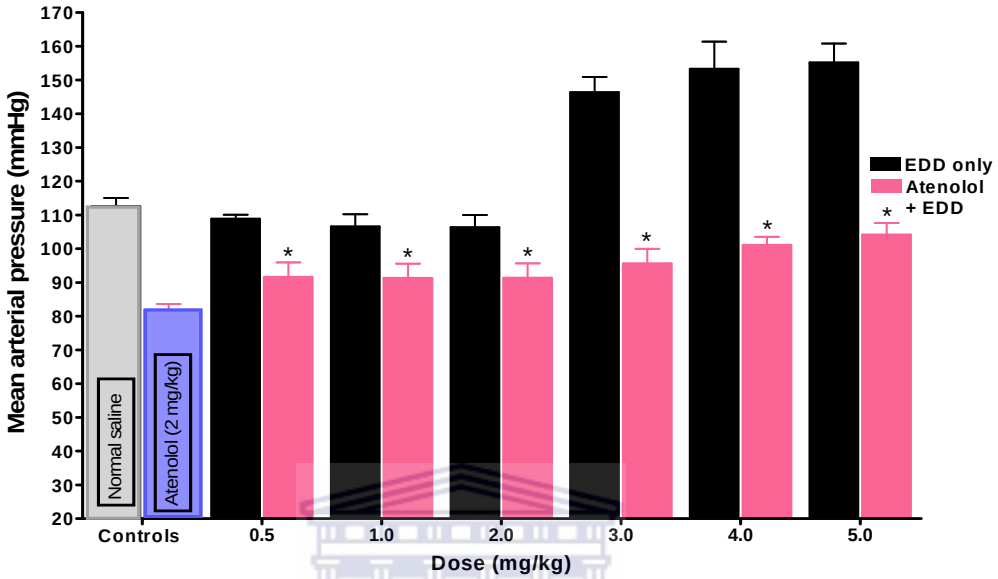


Figure 4.29: Effect of EDD on mean arterial pressure in animals pre-treated with atenolol.
*** indicates statistical significance when compared to EDD administered alone.**

Prazosin

EDD was administered after pre-treatment with prazosin (7.0 mg/kg), and the effects on MAP are shown in figure 4.30 below. Prazosin pre-treatment decreased MAP by 23.2% (from 108.3mmHg ± 0.7 to 83.2mmHg ± 4.5), and with the administration of EDD slight non-significant non dose-dependent changes in MAP occurred. Increases occurred with the 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 4.0 mg/kg doses while decreases occurred with all other doses, but all were not significant when compared to the prazosin control (table 6).

When compared with the MAP values obtained with EDD administration in animals not receiving prazosin pre-treatment, significant decreases in MAP occurred for all EDD doses

in animals pre-treated with prazosin (table 7). The least difference of 22.5% (from 106.8mmHg ± 3.7 in animals not pre-treated to 82.8mmHg ± 0.3 in pre-treated animals) occurred with the 2.0 mg/kg dose, while the greatest difference of 46.9% (from 155.1mmHg ± 5.7 in animals not pre-treated to 82.3mmHg ± 1.7 in pre-treated animals) occurred with the 5.0 mg/kg dose (figure 4.30 and table 7).

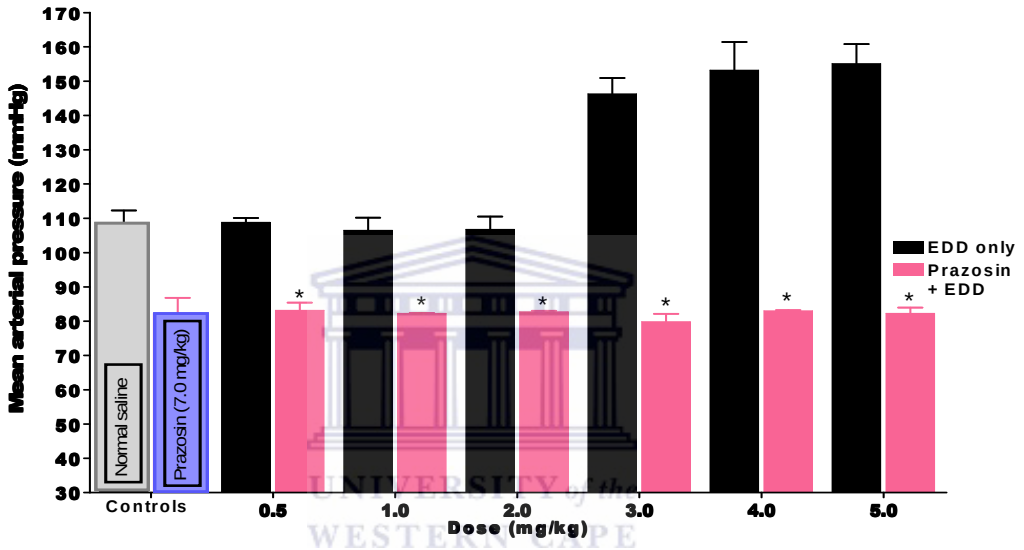


Figure 4.30: Effect of EDD on mean arterial pressure in animals pre-treated with prazosin. * indicates statistical significance when compared to EDD administered alone.

Dobutamine

EDD was administered after pre-treatment with dobutamine (75 µg/kg), and the effects on MAP are shown in figure 4.31 below. Dobutamine pre-treatment increased MAP by 6.3% (from 110.5mmHg ± 5.2 to 117.5mmHg ± 1.2), and with the administration of EDD further dose-dependent increases in MAP occurred which were significant with the 3.0 mg/kg, 4.0 mg/kg and 5.0 mg/kg doses (table 8). When compared with the MAP values obtained with

EDD administration in animals not receiving dobutamine pre-treatment, statistically significant increases in MAP occurred with the 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 5.0 mg/kg doses, while slight non-significant decreases occurred with the 3.0 mg/kg and 4.0 mg/kg doses in pre-treated animals (figure 4.31 and table 9).

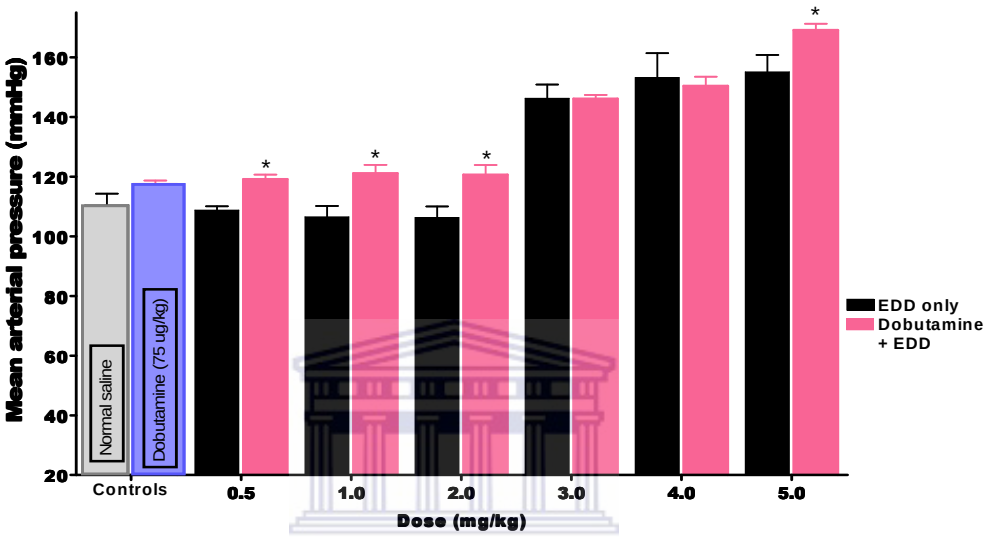


Figure 4.31: Effect of EDD on mean arterial pressure in animals pre-treated with dobutamine.
*** indicates statistical significance when compared to EDD administered alone.**

Verapamil

EDD was administered after pre-treatment with verapamil (4.0 mg/kg), and the effects on MAP are shown in figure 4.32 below. Verapamil pre-treatment decreased MAP by 37.6% (from 103.4mmHg ± 3.8 to 64.6mmHg ± 1.9), and with the administration of EDD dose-dependent increases in MAP occurred which were statistically significant with doses above 0.5 mg/kg (table 10).

When compared with the MAP values obtained with EDD administration in animals not pre-treated with verapamil, significant decreases in MAP occurred for all EDD doses in animals pre-treated with verapamil (table 11). The least difference of 35.4% (from 108.8mmHg ± 1.3 in animals not pre-treated to 70.3mmHg ± 3.0 in pre-treated animals) occurred with the lowest dose, while the greatest difference of 65.4% (from 155.1mmHg ± 5.7 in animals not pre-treated to 93.1mmHg ± 5.2 in pre-treated animals) occurred with the 5.0 mg/kg dose (figure 4.32 and table 11).

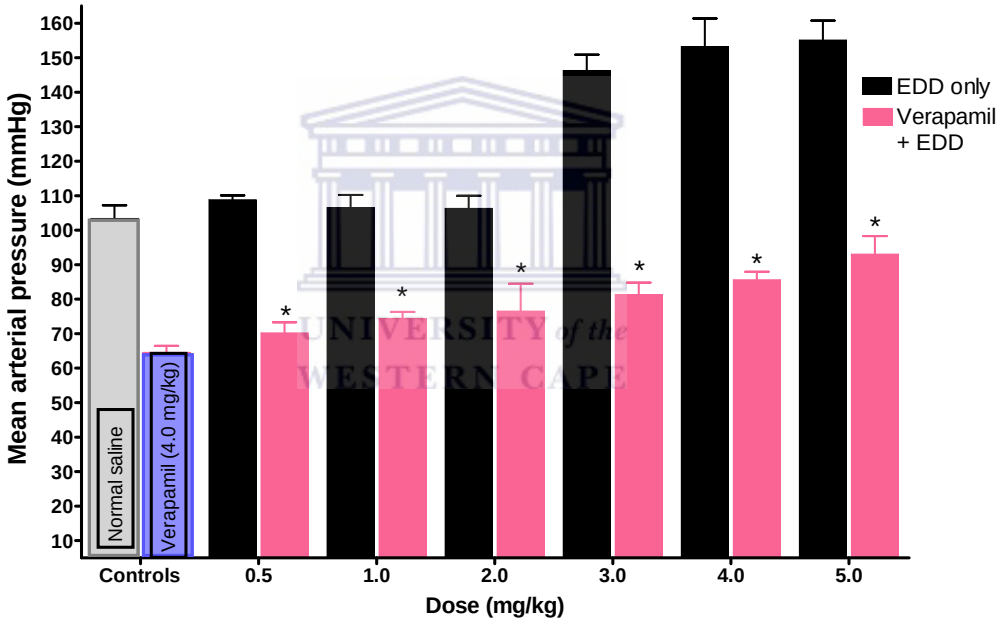


Figure 4.32: Effect of EDD on mean arterial pressure in animals pre-treated with verapamil.
*** indicates statistical significance when compared to EDD administered alone.**

4.2.3.4 Heart rate (HR)

Reserpine

Figure 4.33 below illustrates the effect of EDD on HR in reserpinized animals. Reserpine pre-treatment decreased HR by 21.2% (from 368.7bpm ± 12.9 to 290.7bpm ± 6.0), and with the administration of EDD increases in HR occurred with the 0.5 mg/kg – 2.0 mg/kg doses, while dose-dependent decreases occurred with the 3.0 mg/kg – 5.0 mg/kg doses (table 2). The highest increase of 12.7% (from 290.7bpm ± 6.0 to 327.5bpm ± 11.3) occurred with the smallest dose, while the smallest increase of 1.5% (from 290.7bpm ± 6.0 to 295.0bpm ± 12.3) occurred with the 2.0 mg/kg dose (figure 4.33 and table 2).

When compared with the HR values obtained with EDD administration in non-reserpinised animals, non-significant decreases in HR occurred with all doses administered in reserpinised animals (figure 4.33 and table 3).

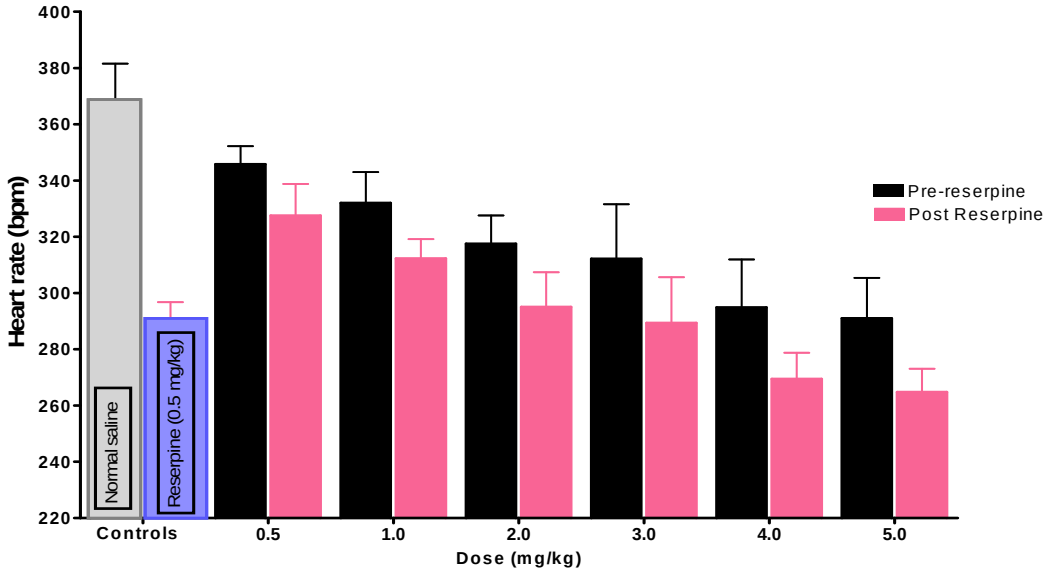


Figure 4.33: Effect of EDD on heart rate in animals pre-treated with reserpine.
*** indicates statistical significance when compared to EDD administered alone.**

Atenolol

EDD was administered after pre-treatment with atenolol (2 mg/kg), and the effects on HR are shown in figure 4.34 below. Atenolol pre-treatment decreased HR by 36.3% (from 364.4bpm ± 3.8 to 234.9bpm ± 3.6), and with the administration of EDD further dose-dependent decreases in HR occurred with all the doses (statistically significant with the 2.0 mg/kg – 5.0 mg/kg doses) (table 4).

When compared with the HR values obtained with EDD administration in animals not pre-treated with atenolol, statistically significant decreases in HR occurred with all doses administered in pre-treated animals (table 5). The least difference of 30.6% (from 321bpm ± 11.0 in animals not pre-treated to 222.7bpm ± 2.5 in pre-treated animals) occurred with the 1.0 mg/kg dose, while the greatest difference of 36.6% (from 285.2bpm ± 14.4 in animals not pre-treated to 180.7bpm ± 4.7 in pre-treated animals) occurred with the 5.0 mg/kg dose (figure 4.34 and table 5).

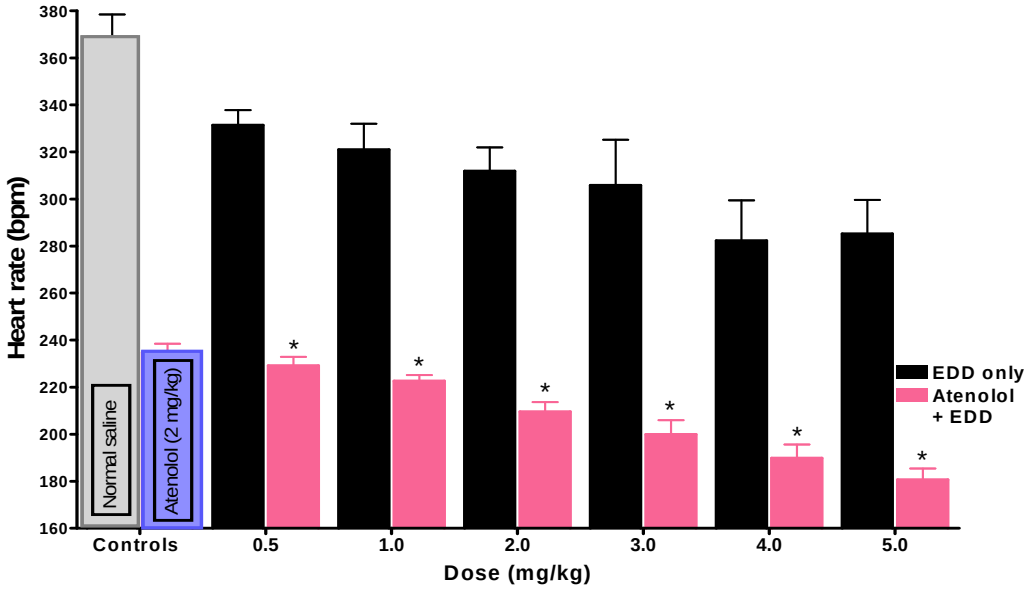


Figure 4.34: Effect of EDD on heart rate in animals pre-treated with atenolol.
*** indicates statistical significance when compared to EDD administered alone.**

Prazosin

EDD was administered after pre-treatment with prazosin (7.0 mg/kg), and the effects on HR are shown in figure 4.35 below. Prazosin pre-treatment increased HR by 6.7% (from 368.4bpm ± 6.5 to 393.2bpm ± 3.4), and with the administration of EDD significant dose-dependent decreases in HR occurred (table 6).

When compared with the HR values obtained with EDD administration in animals that received no prazosin pre-treatment, significant increases in HR occurred for all EDD doses in animals pre-treated with prazosin (table 7). The least difference of 4.5% (from 331.4bpm ± 6.4 in animals not pre-treated to 346.4bpm ± 4.7 in pre-treated animals) occurred with the lowest dose, while the greatest difference of 18.8% (from 282.3bpm ± 17.1 in animals not

pre-treated to 335.4 ± 3.6 in pre-treated animals) occurred with the 4.0 mg/kg dose (figure 4.35 and table 7).

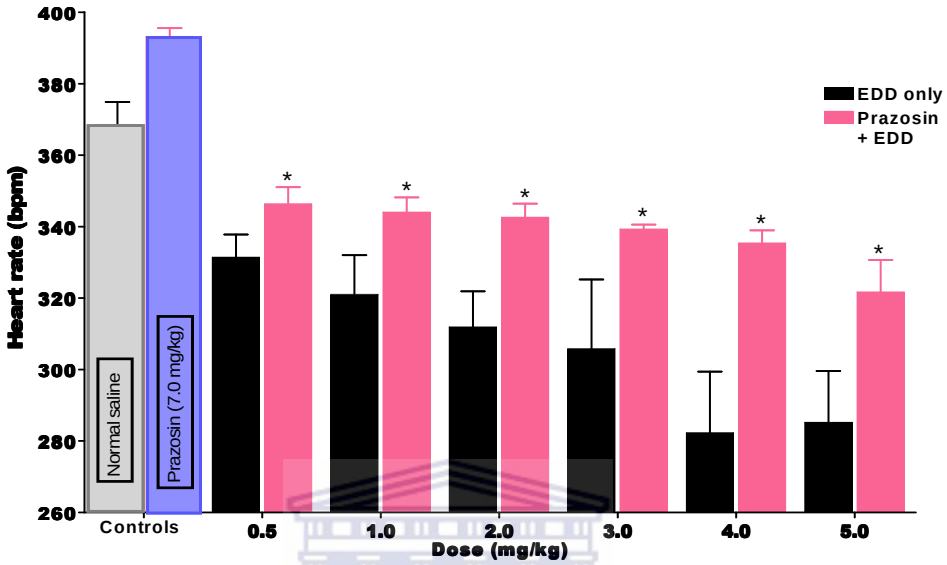


Figure 4.35: Effect of EDD on heart rate in animals pre-treated with prazosin. * indicates statistical significance when compared to EDD administered alone.

Dobutamine

EDD was administered after pre-treatment with dobutamine (75 µg/kg), and the effects on HR are shown in figure 4.36 below. Dobutamine pre-treatment increased HR by 19.1% (from 368.6bpm ± 6.3 to 438.6bpm ± 12.6), and with the administration of EDD significant dose-dependent decreases in HR occurred (table 8).

When compared with the HR values obtained with EDD administration in animals not pre-treated with dobutamine, statistically significant increases in HR occurred in pre-treated animals with all doses administered (table 9). The least difference of 11.1% (from

321.0bpm ± 11.0 in animals not pre-treated to 356.6bpm ± 2.8 in pre-treated animals) occurred with the 1.0 mg/kg dose, while the greatest difference of 23.1% (from 282.3bpm ± 17.1 in animals not pre-treated to 347.5bpm ± 1.6 in pre-treated animals) occurred with the 4.0 mg/kg dose (figure 4.36 and table 9).

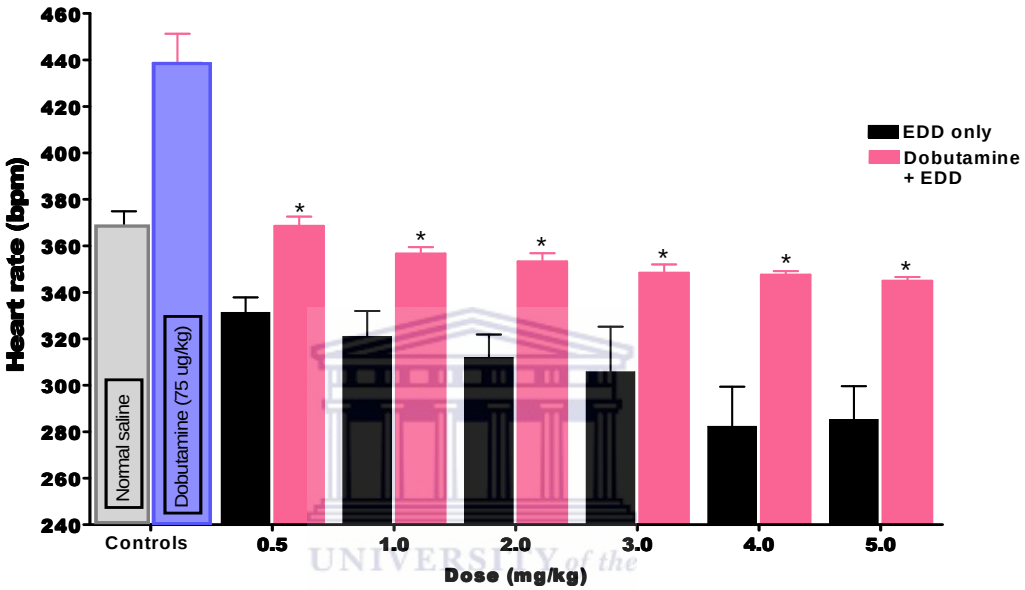


Figure 4.36: Effect of EDD on heart rate in animals pre-treated with dobutamine. * indicates statistical significance when compared to EDD administered alone.

Verapamil

EDD was administered after pre-treatment with verapamil (4.0 mg/kg), and the effects on HR are shown in figure 4.37 below. Verapamil pre-treatment decreased HR by 27.5% (from 368.4bpm ± 6.5 to 267.2bpm ± 4.0), and with the administration of EDD dose-dependent increases occurred with the 0.5 mg/kg and 1.0 mg/kg doses, while dose-

dependent decreases occurred with the other doses (2.0 mg/kg – 5.0 mg/kg) (figure 4.37). All changes to HR were however non-statistically significant (table 10).

When compared with the HR values obtained with EDD administration in animals not pre-treated with verapamil, significant decreases in HR occurred for all EDD doses in animals pre-treated with verapamil (table 11). The least difference of 10.3% (from 282.3bpm ± 17.1 in animals not pre-treated to 253.3bpm ± 2.4 in pre-treated animals) occurred with the 4.0 mg/kg dose, while the greatest difference of 16% (from 305.8bpm ± 19.4 in animals not pre-treated to 256.8bpm ± 1.1 in pre-treated animals) occurred with the 3.0 mg/kg dose (figure 4.37 and table 11).

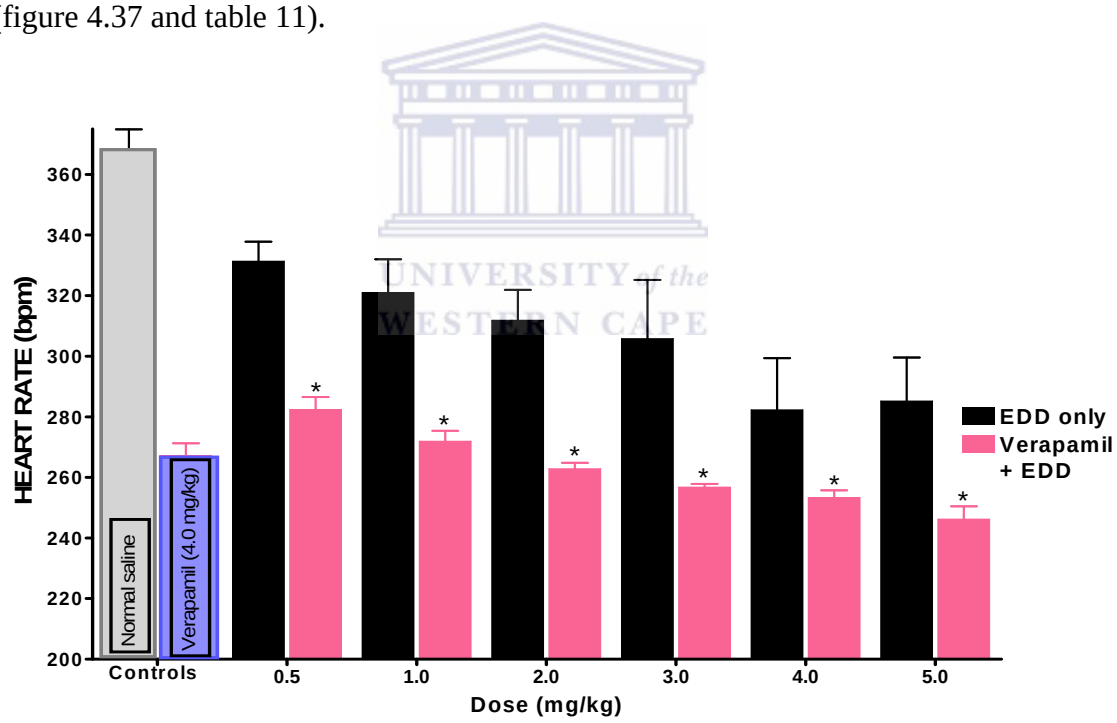


Figure 4.37: Effect of EDD on heart rate in animals pre-treated with verapamil. * indicates statistical significance when compared to EDD administered alone.

CHAPTER FIVE

Discussion

5.1 Structure elucidation

Fractionation of the organic extracts of the leaves of *L. leonurus* yielded colourless needles of a new diterpenoid compound named (13*S*)-9α, 13α-epoxylabda-6β(19),15(14)diol dilactone (EDD).

Diastereotopic protons in the δ 1 to 2.5 ppm region and the doublet of doublets at δ 4.70 ppm region of the ^{1}H NMR spectrum of EDD indicated that the new molecule was likely to be a diterpenoid with similarities to those previously isolated from the plant (see appendixes II-IX) (Rivett, 1964; Kaplan and Rivett 1968; Habtemariam et al., 1994; McKenzie et al., 2006; Agnihotri et al., 2009). The ^{13}C NMR spectrum of the compound indicated the presence of 20 unique carbon atoms and the DEPT spectrum indicated the presence of three CH_3, eight CH_2 and three CH groups. The distinctive chemical shifts of two of the remaining six quaternary carbons at δ 83.6 and 93.5 ppm indicated the likely presence of two-spiro fused five-membered rings. Chemical shifts at δ 183.8 and 171.4 ppm indicated the presence of two carbonyl moieties (see appendixes X-XII).

The spectral data presented above suggested a number of similarities with another diterpenoid simply known as compound X, that had previously been isolated from *L. leonurus* and this assisted in the deduction of the structure of this new diterpenoid (Kaplan and Rivett 1968). From the HSQC, HMBC and COSY NMR spectra the presence of ethano carbons at C-15 and C-16 in EDD (coupling seen in the ^{1}H and COSY NMR spectra in appendixes II - IX and XII – XVI respectively) as opposed to compound X which has two isolated CH_2 groups at C-14 and C-16, helped confirm that EDD and compound X are

positional isomers of one another (see figure 5.1). The main structural difference between the two compounds is that the lactone carbonyl group in EDD is at the C-14 position while in compound X, the lactone carbonyl group is at the C-15 position (figure 5.1).

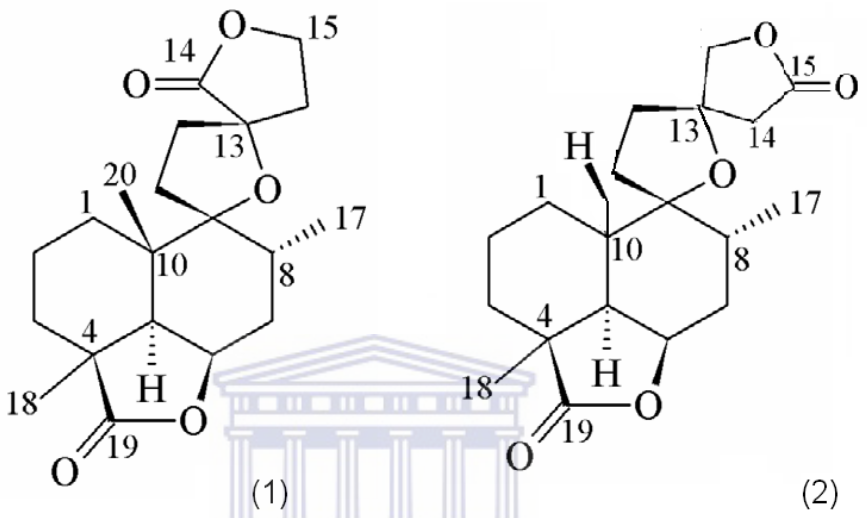


Figure 5.1: Positional isomerism between EDD (1) and compound X (2).

The IR spectra of EDD showed absorptions at: 2909 cm^{-1} and 1374 cm^{-1} due to the saturated C - H bond; 1770 cm^{-1} and 1100 cm^{-1} due to double and single bond character of the ester C = O bond; 1462 cm^{-1} due to the saturated C - H stretching frequencies; 1196 cm^{-1} due to O – C = O bond stretching and 911 cm^{-1} due to C - O cyclic bonds (see appendix I). The high-resolution electron impact mass spectrum (HREIMS) indicated a $[M^{+}H]^{+}$ ion at *m/z* 349.2009, which correlates with the molecular formula $C_{20}H_{29}O_5$ (calculated for $C_{20}H_{29}O_5$ at 349.2015), and X-ray crystallography confirmed the structure of the compound (see figure 4.2 and appendix XXXVI). An extensive search of the

literature and databases of chemical structures indicated that this is the first time the diterpenoid (13*S*)-9α, 13α-epoxylabda-6β(19),15(14)diol dilactone has been isolated from *L. leonurus* or any other plant.

Despite the abundance of terpenoids, especially the diterpenoid lactones in the *Leonotis* species, it is surprisingly rewarding to our group that this is the first time this compound has been isolated from the plant. Geographical differences in the constituents of *L. leonurus* have been reported by some researchers as a possible reason for the isolation of compounds which had not been previously isolated from the plant (Laonigro et al., 1979; McKenzie et al., 2006). This could explain the isolation of the novel compound EDD from the plant, as leonurun, another novel compound isolated from *L. leonurus* harvested from the same geographical location as the plants used in this study, has been reported by Mckenzie and co-workers (2006). As noted earlier, most of the compounds so far isolated from the plant have been diterpenoids with closely related structures (see figure 2.2). The relative stereochemistry of EDD is consistent with that of other diterpenoids so far isolated from *L. leonurus* as evidenced from the scalar couplings measured in the ^{1}H NMR spectrum and also from correlations observed in the 2D NOESY spectrum collected (see Figure 4. 3) (Rivett, 1964; Kaplan and Rivett 1968; Laonigro et al., 1979; Kruger and Rivett 1988; McKenzie et al., 2006; Obikeze et al., 2008).

5.2 *In vivo* cardiovascular effects of EDD

The diterpenoid (13*S*)-9α, 13α-epoxylabda-6β(19),15(14)diol dilactone (EDD) isolated from the methanol extracts of the leaves of *L. leonurus* was administered intravenously to

anaesthetized normotensive male Wistar rats in order to evaluate its cardiovascular effect. The lower doses (0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg) of EDD seemed to have little or no effect on blood pressure (slight non-significant decreases in SP, DP and MAP) (see figures 4.4 – 4.6 and table 1), while the administration of the higher doses (3.0 mg/kg, 4.0 mg/kg and 5.0 mg/kg) of EDD induced dose-dependent statistically significant increases in BP (see figures 4.4 – 4.6 and table 1). The decreases in BP with the lower doses of EDD though non-significant, were similar to the effects of the aqueous extract as reported by Ojewole (2003). The increases in BP observed with the higher doses on the other hand were similar to those reported by Obikeze (2005) using the crude aqueous extract of *L. leonurus* leaves in the same animal model and also using a fraction of the methanol extract in the isolated perfused rat heart model. The observed dose-dependent effects of EDD on BP were similar to those that had been previously reported with other compounds (Church and Hodgson 2000). All doses of EDD administered also induced significant dose-dependent decreases in HR, an effect quite similar to that observed with other diterpenoids (see figure 4.7 and table 1) (Somova et al., 2001; Silva et al., 2005). The results seem to suggest that EDD exhibits a dual effect in the cardiovascular system of the rats: a negative chronotropic effect and a vasoconstrictive effect. None of the diterpenoids so far isolated from *L. leonurus* have been assayed for possible cardiovascular activity, but diterpenoids isolated from other plants have been reported to exhibit various effects on the cardiovascular system such as vasorelaxation, bradycardia, tachycardia and diuresis (Somova et al., 2001; Guerrero et al., 2004; Silva et al., 2005; de Oliveira et al., 2006; Lahlou et al., 2007).

The decreases in SP, DP and MAP observed with the lower EDD doses though only slight and non-significant were similar to the effects reported by Mugabo *et al* (2002)and Ojewole (2003) using the crude aqueous extracts of *L. leonurus* in anaesthetized normotensive rats. This effect was also similar to the vasorelaxant effects that have been reported with other diterpenoids isolated from plants (Dubey et al., 1981; Somova et al., 2001; Oliveira et al., 2003; Guerrero et al., 2004; Ambrosio et al., 2006; Lahlou et al., 2007). Vasoconstrictive effects similar to those observed with the higher EDD doses have also been reported with other plant extracts such as pilosidine isolated from *Curculigo pilosa*, but are yet to be reported with diterpenoids (Kwan et al., 1999; Cometa et al., 2001; Chen et al., 2004). The dose dependent decreases in HR produced by all EDD doses on the other hand were similar to the effects of the aqueous extract of *L. leonurus* as reported by both Obikeze (2005) and Ojewole (2003) in anaesthetized normotensive rats, and also with some diterpenoids isolated from other plants as reported by Somova and co-workers (2001) and Silva and co-workers (2005). Interestingly, the opposite effect (a positive chronotropic effect) was reported by Mugabo and co-workers (2002) with the crude aqueous extract of *L. leonurus* in Langendorff perfused isolated rat hearts and by Obikeze (2005) with a fraction of the methanol extract of the leaves of *L. leonurus* administered to anaesthetized normotensive rats.

Intricate relationships exist between the various components making up the cardiovascular system as seen in the equations below:

Blood Pressure (BP) = Cardiac Output (CO) x Peripheral Vascular Resistance (PVR)

and

Cardiac Output (CO) = Heart Rate (HR) x Stroke Volume (SV)

The interplay of the various components of the above equations would influence the resultant cardiovascular effects of any administered compounds. A direct relationship exists between BP and HR, with an increase in HR resulting in an increase in BP and vice versa. With EDD administration, especially with the higher doses, decreases in HR coincided with increases in BP, suggesting that the compound may have a dual effect on the cardiovascular system: acting independently on the heart to produce a reduction in HR and at the same time increasing PVR to produce an increase in BP. Methoxamine (a selective α-adrenoceptor agonist) produces an increase in BP via vasoconstriction that is accompanied by bradycardia due to the activation of the baroreceptor reflex, and this effect is similar to that observed with the higher doses of EDD (Hoffman, 1998a). Activation of the baroreceptor reflex may be responsible for the decrease in HR that is seen accompanying the increase in BP with the higher EDD doses, but this does not however explain the decrease in HR that accompanied the slight decrease in BP observed with the lower EDD doses. Biphasic effects similar to the one that was observed with the administration of EDD have also been reported with other diterpenoids isolated from other plants, but these have mainly been in the form of tachycardia accompanying a hypotensive effect (Aceret et al., 1996; de Oliviera et al 2006; Lahlou et al., 2007). The decrease in BP at the lower doses may however be due to a vasodilatory effect, which has been reported with many other diterpenes, but the significant increases in BP that occurred with the higher EDD doses make a vasodilatory effect highly unlikely (Somova et al., 2001; Silva et al., 2005). The decreases in BP with the lower doses may be the result of a greater negative chronotropic effect overcoming the weaker vasoconstrictor effect elicited at these doses.

The decrease in HR observed with EDD administration is probably not the result of the activation of the baroreceptor reflex, but rather the result of a direct negative chronotropic effect on the heart by the compound. Thus, if baroreceptor reflex activation is not entirely responsible for the negative chronotropic effect of the lower doses of EDD, and BP is elevated while HR is decreased at the higher doses of EDD, then it is probable that EDD exhibits a dual effect on the cardiovascular system viz., an increase in BP due to a vasoconstrictor effect and a decrease in HR due to a negative chronotropic effect.

Increases in BP could occur either through a direct vasoconstrictor effect or an indirect vasoconstrictor effect (Ruffolo and Hieble 1995; Hoffman, 1998a). A direct vasoconstrictor effect is mediated by an agonistic effect on α_1 receptors found in contractile tissue of the arteries, while an indirect vasoconstrictor effect is mediated via the release of catecholamines into the synaptic space (Ruffolo and Hieble 1994; Hoffman, 1998a). Reserpine depletes vascular stores of catecholamines and the pre-treatment of the animals with this drug would decrease the vasoconstrictive effect of EDD, if this effect was mediated via the release of catecholamines at the synapse (Chen and Chan 1989; Consolini and Sarrubbio 2002). EDD administration in animals pre-treated with reserpine led to dose-dependent decreases in BP when compared to the EDD effect in non-reserpinized animals (see figures 4.18, 4.23, 4.28 and table 3). The decreases observed were statistically significant at the higher doses administered and BP values were even lower than the values in the control group. The administration of reserpine led to the elimination of the vasoconstrictive effect produced by EDD, indicating that EDD may produce its vasoconstrictive effect via the release of catecholamines (Korsziak and Story 1994; Consolini and Sarubbio 2002). No significant changes in HR occurred in reserpinized

animals compared to their non-reserpinized counterparts and this indicates that EDD does not produce its negative chronotropic effect by preventing the release of catecholamines.

The results from reserpinized animals gives a strong indication that EDD has a vasodilatory effect, and the possibility of this being the result of an agonistic effect on α_1 receptors is further explored. The administration of EDD in animals pre-treated with prazosin, an α_1 adrenoceptor blocker, produced no significant changes in BP. Compared to non pre-treated animals BP values in pre-treated animals were however significantly reduced (see figure 4.20, 4.25, 4.30 and table 7). The fact that pre-treatment with prazosin, an α_1 adrenoceptor blocker, abolished the vasoconstrictive effect of EDD suggests that its effect on BP is mediated by α_1 receptors (Frew et al., 1994). HR values were significantly higher in animals pre-treated with prazosin compared to those without prazosin pre-treatment as a result of reflex tachycardia occurring with prazosin administration (see figure 4.35, table 7) (Shah and Yadav 2008). The results obtained in animals pre-treated with reserpine and prazosin suggests that EDD produces an indirect vasoconstrictive effect in the animals via the release of catecholamines. This indirect vasoconstrictive mechanism has been reported as the mechanism for the effect of 1-α-naphthylmethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline and the crude venom gland extract of *Steatoda capensis* on BP (Korszniak and Story 1994; Lee et al., 1994).

EDD produced a negative chronotropic effect on the heart that could be due to β_1 adrenoceptor blockade, Ca^{2+} channel blockade, or Na^{+}/K^{+} pump blockade. Various diterpenoids have been reported to affect the heart via these mechanisms (Toya et al., 1998; Silva et al., 2005; De Oliveira et al., 2006). Atenolol produces a negative chronotropic effect via β_1 adrenoceptor blockade and administration with another negative chronotropic

agent should produce an additive effect (Pearson et al., 1989). EDD administration in animals pre-treated with atenolol led to further dose dependent decreases in HR (see figure 4.34 and table 5). The results however are not conclusive on the mechanism of action of EDD as the additive effect on HR could also be due to Ca^{2+} channel and/or Na^{+}/K^{+} pump blockade (Summers and McMartin 1993). The results however give a clear indication that EDD does have a negative chronotropic effect on the heart. Interestingly there were increases in BP with the administration of EDD in animals pre-treated with atenolol, but these values were significantly lower than the values obtained with the administration of EDD in non pre-treated animals. This once more suggests a dual effect for EDD since increases in BP occurred even in the presence of the combined negative chronotropic effects of atenolol and EDD. Animals were pre-treated with dobutamine, a β_1 adrenoceptor agonist, prior to the administration of EDD to determine if the negative chronotropic effect of EDD was the result of β_1 adrenoceptor blockade. Administration of a β_1 adrenoceptor agonist leads to an increase in HR, an effect that would be reversed by a β_1 adrenoceptor antagonist administered afterwards (Hoffman, 1998b). Dobutamine as expected produced an increase in HR in the animals and the administration of EDD in those animals pre-treated with dobutamine lead to significant decreases in HR (see figure 4.36, table 9). The decrease in HR with the administration of EDD in animals pre-treated with dobutamine was similar to the effect reported with atenolol and CGP 20712A, both β_1 adrenoceptor antagonists, and this confirms the negative chronotropic effect of EDD (Juan-Fita et al., 2005). The negative chronotropic effect of EDD could be the result of competitive β_1 adrenoceptor antagonism or the result of Ca^{2+} channel blockade (Bakheet et al., 1999; Somova et al., 2001). SP values with the lower doses of EDD in animals pre-treated with

dobutamine were higher than those in non pre-treated animals. This could be due to the higher HR values at those doses in the pre-treated animals. In animals pre-treated with dobutamine, administration of the lower EDD doses led to slight increases in BP, despite the decreases in HR that occurred. It is important to note that although HR was decreased with the administration of all EDD doses in dobutamine pre-treated animals, the HR values were significantly higher than those obtained in animals receiving only EDD. Since dobutamine is known to induce increases in BP mostly via the increase in cardiac output produced by its β_1 adrenoceptor agonist effect on the heart (a positive chronotropic and inotropic effect), the significant increases in BP noted here with the administration of EDD in animals pre-treated with dobutamine points to a synergistic effect on BP supporting the suggestion that EDD has a dual effect (Calvin, 1989).

To further explore the mechanism by which EDD produced its negative chronotropic effect on the heart, EDD was administered to animals pre-treated with a cardio-selective Ca^{2+} channel blocker verapamil which produces decreases in HR by selective blockade of Ca^{2+} channels in cardiac myocytes (Wakabayashi et al., 1995). Decreases in HR only occurred with EDD doses above 1.0 mg/kg in animals pre-treated with verapamil, but verapamil pre-treatment produced significant additive negative chronotropic effects with all EDD doses (see figure 4.37 and table 11). If EDD produced its negative chronotropic effect via a different mechanism from Ca^{2+} channel blockade, then additive negative chronotropic effects would be expected for all EDD doses in animals pre-treated with a Ca^{2+} channel blocker. The results indicate however that no significant decreases in HR occurred with the administration of EDD in animals pre-treated with verapamil. Calcium channel blockade has been identified as the mechanism of action of the cardiovascular effects of many plant

compounds including diterpenoids (Bakheet et al., 1999; Somova et al., 2001; Consolini and Sarubbio 2002; Ambrosio et al., 2006; de Oliveira et al., 2006). Calcium channel blockade does not however only occur with the Ca^{2+} channels in the heart, but blockade of Ca^{2+} channels found in the contractile tissues of arteries would also produce vasodilatation (Fozzard, 2002). EDD on its own did not produce decreases in BP, but rather our results indicate that increases in BP occurred with EDD administration due to an indirect α_1 agonistic effect. Hence the vasoconstrictive and negative chronotropic effect of EDD can only be attributed to Ca^{2+} channel blockade if the Ca^{2+} channel effect was selective for myocardial Ca^{2+} channels only. β_1 adrenoceptor agonists produce a positive chronotropic and inotropic effect by increasing intracellular Ca^{2+} in cardiac cells, and β_1 adrenoreceptor blockade would prevent the increase of intracellular Ca^{2+}, an effect similar to that produced by Ca^{2+} channel blockade. The implication is that the negative chronotropic effect of EDD is most likely due to β_1 adrenoceptor antagonism.

The results from this study are not however conclusive on the precise mechanism for the action of EDD and consequently, further experiments using the loose patch clamp technique to determine the effect of EDD on the Na^+/K^+ pump and isolated organ experiments to determine its effect on myocardial and arterial Ca^{2+} channels and its vasoconstrictor effect would be required to confirm the mechanism of action of EDD.

Limitations

The scope of this study was limited by the lack of sufficient funds and access to the equipment needed to carry out some of the additional experiments listed above.

Conclusion

A novel diterpenoid; (13*S*)-9α, 13α-epoxylabda-6β(19),15(14)diol dilactone (EDD) was isolated from the methanol extracts of the leaves of *L. leonurus* using fractionation techniques. The chemical formula as well as the structure of the compound was determined using IR, NMR, MS and X-ray diffraction techniques. EDD was evaluated for its cardiovascular effects on the anaesthetized normotensive rat model using male Wister rats and was found to exhibit a dual effect on the cardiovascular system. EDD elevated BP and decreased HR in the animals. Using pre-treatment with standard cardio-active drugs as a lead, the increase in BP elicited by EDD was the result of vasoconstriction probably via the release of catecholamines, while its negative chronotropic effect was probably due to β_1 adrenoceptor antagonism. Further experiments are however needed to confirm these postulations.

REFERENCES

Aarons, D.H., Rossi, G.V. & Orzechowski, R.F. (1977). Cardiovascular actions of three Harmala alkaloids: Harmaine, harmaline and harmalol. *Journal of Pharmaceutical Sciences*, 66: 1244-1248.

Aceret, T.L., Brown, L., Miller, B.J., Coll, J.C. & Sammarco, P.W. (1996). Cardiac and vascular responses of isolated rat tissues treated with diterpenes from *Sinularia flexibilis* (Coelenterata: Octocorallia). *Toxicon*, 34: 1165-1171.

Adamson, R.S. & Salter, T.M. (1950). *Flora of Cape Peninsula*. Cape Town: Juta and Co Ltd.

Agnihotri, V.K., Elsohly, H.N., Smillie, T.J., Khan, I.A. & Walker, L.A. (2009). Constituents of *Leonotis leonurus* flowering tops. *Phytochemistry Letters*, in press, doi:10.1016/j.phytol.2009.02.001.

Ambrosio, S.R., Trapelli, C.R., da Costa, F.B. & de Oliveira, A.M. (2006). Kuarane and pimarane-type diterpenes from the *Viguiera* species inhibit vascular smooth muscle contractility. *Life Sciences*, 79: 925-933.

Bakheet, D.M., El Tahir, K.E.H., Al-Sayed, M.I., El-Obeid, H.A. & Al-Rashood, K.A. (1999). Studies on the cardiovascular depressant effects of N-Ethyl- and N-Benzyl-1,2-diphenyl-ethanolamines in the rat Elucidation of the mechanisms of action. *General Pharmacology*, 33: 17–22.

Batten, A. (1986). *Flowers of Southern Africa*. Johannesburg: Frandsen publishers.

Benowitz, N.L. (1998). Antihypertensive agents. Katzung, B.G. (ed.), *Basic & Clinical pharmacology*, 7th ed. Connecticut: Apnge: 153-178.

Bienvenu, E., Amabeoku, G.J., Eagles, P.K., Scott, G. & Springfield, E.P. (2002). Anticonvulsant activity of aqueous extract of *Leonotis leonurus*. *Phytomedicine*, 9(3): 217-223.

Calvin, J.E. (1989). Right ventricular afterload mismatch during acute pulmonary hypertension an its treatment with dobutamine: A pressure segment length analysis in a canine model. *Journal of critical care*, 4: 239-250.

Chen, C.H. & Chan, S.H.H. (1989). Involvement of postsynaptic α_2-adrenoceptors and guanine nucleotide-binding protein in guanabenz-induced cardiovascular suppressant effects in the rat. *Neuroscience letters*, 105: 183-188.

Chen, B.H., Islam, M.W., Radhakrishnan, R., Wahab, S.A. & Naji, M.A. (2004). Influence of aqueous extract from *Neurada procumbens* L on blood pressure of rats. *Journal of ethnopharmacology*, 90: 191-194.

Chopra, R.N., Chopra, I.C., Handa, K.I. & Kapur, L.D. (1958). Terninalia arjuna W & A (Combretaceae). Chopra, R.N., Chopra, I.C., Handa, K.I. & Kapur, I.D. (eds.), *Chopra's indigenous drugs of India*, 1st ed. Calcutta: UN Dhur & Sons: 422-424.

Church, J.E. & Hodgson, W.C. (2000). Dose-dependent cardiovascular and neuromuscular effects of stonefish (*Synanceja trachynis*) venom. *Toxicon*, 38: 391-407.

Cometa, M.F., Palazzino, G., Galeffi, C. & Palmery, M. (2001). Studies on vasoconstrictor activity of *Curculigo pilosa* extracts and of its isolated compounds. *Il Farmaco*, 56: 353-356.

Consolini, A.E. & Sarubbio, M.G. (2002). Pharmacological effects of *Eugenia uniflora* (Myrtaceae) aqueous crude extract on rat's heart. *Journal of ethnopharmacology*, 81: 57-63.

Cox, P.A. (1990). Ethnopharmacology and the search for new drugs. Chadwick, D.J & Marsh, J. (eds.), *Bioactive compounds from plants. CIBA foundation symposium 154.* Chichester: Wiley: 40-55.

David, J.P., David, J.M., Yang, S.W. & Cordell, G.A. (1998). A bis-labdenic diterpene from *Moldenhawera nutans*. *Phytochemistry*, 50(3): 443-447.

Dawei, W., Yuping L., Wei, L. & Huwei, L. (2004). Separation methods for anti-bacterial and
anti-rheumatism agents in plant medicines. *Journal of Chromatography* B, 812(1-2): 101-117.

de Oliveira, A.P., Furtado, F.F., da Silva, M.S., Tavares, J.F., Mafra, R.A., Araujo, D.A.M., Cruz, J.S. & de Medeiros, I.A. (2006). Calcium channel blockade as a target for the cardiovascular effects induced by the 8 (17), 12*E*, 14-labdatrien-18-oic acid (labdane-302). *Vascular Pharmacology*, 44(5): 338-344.

Department of Health. (2008). *Draft policy on African traditional medicine for South Africa*. Pretoria: Department of Health. [Online]. Available http://www.doh.gov.za/docs/policy-f.html

Dimo, T.B., Mtopi, O.S., Nguelefack, T.B., Kamtchouing, P., Zapfack, L., Asongalem, E.A. & Dongo, E. (2006). Vasorelaxant effects of *Brillantaisia nitens* Lindau (Acanthaceae) extracts on isolated rat vascular smooth muscle. *Journal of ethnopharmacology*, 111: 104-109.

Duarte, J., Torres, A.I. & Zarzueelo, A. (2000). Cardiovascular effects of visnagin on rats. *Planta Medica*, 66: 35-39.

Dubey, M.P., Srimal, R.C., Nityanand, S. & Dhawan, B.N. (1981). Pharmacological studies on coleonol, a hypotensive diterpene from *Coleus forskohlii*. *Journal of ethnopharmacology*, 3(1): 1-13.

Duke, J.A. (2001). *Handbook of medicinal herbs*. Boca Raton: CRC press.

Duncan, A.C., Jager, A.K. & van Staden, J. (1999). Screening of Zulu medicinal plants for angiotensin converting enzyme (ACE) inhibitors. *Journal of ethnopharmacology*, 68(1): 63-70.

Dwivedi, S. (2007). *Terminalia arjuna* Wight & Arn. – A useful drug for cardiovascular disorders. *Journal of ethnopharmacology*, 114: 114-129.

Dyrskog, S.E.U., Jeppesen, P.B., Colombo, M., Abudula, R. & Hermansen, K. (2005). Preventive effects of a soy-based diet supplemented with stevioside on the development of the metabolic syndrome and type 2 diabetes in Zucker diabetic fatty rats. *Metabolism*, 54(9): 1181-1188.

Farnsworth, N.R. (1990). The role of ethnopharmacology in drug development. Chadwick, D.J & Marsh, J. (eds.), *Bioactive compounds from plants*. *CIBA foundation symposium 154*. Chichester: Wiley: 2-21.

Fozzard, H.A. (2002). Cardiac sodium and calcium channels: a history of excitatory currents. *Cardiovascular research*, 55: 1-8.

Frew, R., Hamilton, M.G. & Lundy, P.M. (1994). Identification of noradrenaline in venom from the funnel-web spider *Hololena curta*. *Toxicon*, 32(4): 511-515.

Gross, D.R. (1994). *Animal models in cardiovascular research*. Boston: Springer.

Guerrero, M.F., Puebla, P., Carron, R., Martin, M.L. & San Roman, L.R. (2004). Vasorelaxant effect of new *neo*-clerodane diterpenoids isolated from *Croton schiedeanus*. *Journal of ethnopharmacology*, 94: 85-89.

Habtemariam, S., Gray, A.I. & Waterman, P.G. (1994). Diterpenes from the leaves of *Leonotis ocymifolia* var. Raineriana. *Journal of natural products*, 57(11): 1570-1574.

Hamilton, R.J. & Sewell, P.A. (1982). *Introduction to high performance liquid chromatography* (2nd ed.). London: Chapman and Hall.

Harborne, J.B. (1973). *Phytochemical methods*. New York: Chapman and Hall.

Harborne, J.B. (1998). *Phytochemical methods: a guide to modern techniques of plant analysis*. London: Chapman and Hall.

Harley, J.H. & Wiberley, S.E. (1954). *Instrumental analysis*. Chichester: John Wiley & Sons Ltd.

Hearse, D.J. & Sutherland, F.J. (1999). Experimental models for the study of cardiovascular function and disease. *Pharmacological research*, 41(6): 597-603.

Hoffman, B.B. (1998a). Adrenoceptor-activating and other sympathomimetic drugs. Katzung, B.G. (ed.), *Basic & Clinical pharmacology, 7th ed.* Connecticut: Apnge: 118-135.

Hoffman, B.B. (1998b). Adrenoceptor antagonist drugs. Katzung, B.G. (ed.), *Basic & Clinical pharmacology, 7th ed.* Connecticut: Apnge: 136-151.

Hutchings, A., Scott, A.H. & Lewis, G. (1996). *Zulu medicinal plants, an inventory*. Pietermaritzburg: University of Natal press.

Jager, A.K., Hutchings, A. & van Staden, J. (1996). Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. *Journal of ethnopharmacology*, 52(2); 95-100.

Juan-Fita, M.J., Vargas, M.L. & Hernandez, J. (2005). The phosphodiesterase 3 inhibitor cilostamide enhances inotropic responses to glucagon but not to dobutamine in rat ventricular myocardium. *European journal of pharmacology*, 512: 207-213.

Kamatou, G.P.P., Viljoen, A.M., van Vuuren, S.F. & van Zyl, R.L. (2006). *In vitro* evidence of antimicrobial synergy between *Salvia chamelaeagnea* and *Leonotis leonurus*. *South African journal of Botany*, 72(4): 634-636.

Kaplan, E.R. & Rivett, E.A. (1968). The structures of compounds X and Y, two labdane diterpenoids from *Leonotis leonurus*. *Journal of Chemical society* (C), 262-266.

Keeler, J. (2006). *Understanding NMR spectroscopy*. Chichester: John Wiley & Sons Ltd.

Kite, G.C., Veitch, N.C., Grayer, R.J. & Simmonds, M.S.J. (2003). The use of hyphenated techniques in comparative phytochemical studies of legumes. *Biochemical systematic and ecology*, 31(8): 813-843.

Korszniak, N.V. & Story, D.F. (1994). Effects of the venom of the Theridiid spider, *Steatoda capensis* Hann, on autonomic transmission in rat isolated atria and caudal artery. *Toxicon*, 32(1): 85-96.

Kruger, G.J. & Rivett, D.E.A. (1988). Diterpenoids of Leonotis species. Part 7. The crystal and molecular structure of compound X, a labdane from *L. leonurus*. *South African journal of chemistry*, 41(3): 124-125.

Kutchan, M.T. (1995). Alkaloid biosynthesis – The basis for metabolic engineering of medicinal plants. *The plant cell*, 7: 1059-1070.

Kwan, C.Y., Ma, F.M. & Hui, S.C.G. (1999). Inhibition of endothelium-dependent vascular relaxation by tetradrine. *Life sciences*, 64(25): 2391-2400.

Lahlou, S., de Barros Correia, C.A., do Santos, M.V., David, J.M., David, J.P. Duarte, G.P. & Magalhaes, P.J.C. (2007). Mechanisms underlying the cardiovascular effects of a

labdenic diterpene isolated from *Moldenhawera nutans* in normotensive rats. *Vascular pharmacology*, 46(1): 60-66.

Laonigro, G., Lanzetta, R., Parrilli, M., Adinolfi, M. & Mangoni, L. (1979). The configuration of the diterpene spiroethers from *Marrubium vulgare* and from *Leonotis leonurus*. *Gazzella chimica Italiana*, 109: 145-150.

Lee, Y.S., Kim, C.H., Yun-Choi, H.S. & Chang, K.C. (1994). Cardiovascular effect of a naphthylmethyl substituted tetrahydroisoquinoline, YS49, in rat and rabbit. *Life sciences*, 55(21): 415-420.

Livius, V.U., Kilo, J., Luscher, T. & Gassmann, M. (2000). Circulation. The handbook of experimental animals. The laboratory rat. *Georg J Krinke*, 17: 345-355.

Luo, P., Zhang, Z., Yi, T., Zhang, H., Liu, X. & Mo, Z. (2008). Anti-inflammatory activity of the extracts and fractions from *Erigeron multiradiatus* through bioassay-guided procedures. *Journal of ethnopharmacology*, 119(2): 232-237.

Mahady, G.B. (2002). *Ginkgo Biloba* for the prevention and treatment of cardiovascular disease: A review of the literature. *The journal of cardiovascular nursing*, 16(4): 21-32.

Mander, J., Quinn, N.W. & Mander, M. (1997). *Trade in wildlife medicinal in South Africa*. Investigational report No. 157, Institute of natural resources, Pietermaritzburg. Unpublished paper.

Manitto, P. & Sammes, P.G. (1981). *Biosynthesis of natural products*. London: Ellis Horwood.

Mashour, N.H., Lin. G.I. & Frishman, W.H. (1998). Herbal medicine for the treatment of cardiovascular disease: Clinical considerations. *Archives of internal medicine*, 158(20): 2225-2234.

McKenzie, J.M., Green, I.R. & Mugabo, P. (2006). Leonurun, a novel labdane diterpenoid from *Leonotis leonurus. South African journal of chemistry*, 59: 114-116.

Miller, J.M. (1975). *Separation methods in chemical analysis*. Chichester: John Wiley.

Mugabo, P., Njagi, A., Dietrich, D.L. & Syce, J. (2002). Cardiovascular effects of *Leonotis leonurus* in the normotensive rat. *Revista de fitoterapia*, 2(1).

Mulholland, D.A. (2005). The future of ethnopharmacology. A southern African perspective. *Journal of ethnopharmacology*, 100: 124-126.

Narender, T., Khaliq, T., Puri, A. & Chander, R. (2006). Antidyslipidemic activity of furano-flavonoids isolated from *Indigofera tinctoria. Bioorganic & medicinal chemistry letter*, 16(13): 3411-3414.

Ngueyem, T.A., Brusotti, G., Marrubini, G., Grisoli, P., Dacarro, C., Vidari, G., Vita Finzi, P. & Caccialanza, G. (2008). Validation of use of a traditional remedy from *Bridelia grandis* (Pierre ex Hutch) stem back against oral streptococci. *Journal of ethnopharmacology*, 120(1): 13-16.

Njagi, A. (2001). *Leonotis leonurus effect on blood pressure and cardiac function*. Honours report, University of the Western Cape. Unpublished paper.

Normann, H., Snyman, I. & Cohen., M. (1996). *Indigenous knowledge and its uses*. Pretoria: Human sciences research council publishers.

Noumi, E., Houngue, F. & Lontsi, D. (1999). Traditional medicines in primary health care: plants used for the treatment of hypertension in Bafia, Cameroon. *Fitoterapia*, 70: 134-139.

Obikeze, K.C. (2005). *Cardiovascular effects of Leonotis leonurus extracts in normotensive rats and in isolated perfused rat heart*. Unpublished Master's thesis. Bellville: University of the Western Cape.

Obikeze, K.C., McKenzie, J.M., Green, I.R. & Mugabo, P. (2008). Characterization and cardiovascular effects of (13*S*)-9, 13-epoxylabda-6(19),15(14)diol dilactone, a diterpenoid isolated from *Leonotis leonurus*. *South African journal of chemistry*, 61: 119-122.

Ojewole, J.A. O. (2003). Hypotensive effect of *Leonotis leonurus* aqueous leaf extract in rats. *American journal of hypertension*, 16(5): A40.

Oliveira, A.P., Furtado, F.F., Guedes, D.N. & Medeiros, L.A. (2003). *Cardiovascular effects induced by 8 (17), 12E, 14-labdatrien-18-oic acid (labdane-302)*. Delivered at the XXXV Brazilian Pharmacology and experimental therapeutics society annual meeting, Buenos Aires, Unpublished paper.

Ososki, A.L., Lohr, P., Reiff, M., Balick, M.J., Kronenberg, F., Fugh-Berman, A. & O'Connor, B. (2002). Ethnobotanical literature survey of medicinal plants in the Dominican Republic used for women's health conditions. *Journal of ethnopharmacology*, 79: 285–298.

Pantoja, C.V., Chiang, L.C.H., Norris, B.C. & Concha, J.B. (1991). Diuretic, natriuretic and hypotensive effects produced by *Allium Sativum* (garlic) in anaethetised dogs. *Journal of ethnopharmacology*, 31: 325-331.

Pantoja, C.V., Norris, B.C. & Contreras, C.M. (1996). Diuretic and natriuretic effects of chromatigraphically purified fraction of garlic (*Allium sativum*). *Journal of ethnopharmacology*, 52: 101-105.

Patwardhan, B. (2005). Ethnopharmacology and drug discovery. *Journal of ethnopharmacology*, 100: 50-52.

Pearson, A.A., Gaffney, T.E., Walle, T. & Privitera, P.J. (1989). A stereoselective central hypotensive action of atenolol. *Journal of Pharmacological experiment and therapy*, 250: 759-763.

Pemberton, R.W. (1999). Insects and other arthropods used as drugs in Korean traditional medicine. *Journal of ethnopharmacology*, 65: 207-216.

Pennacchio, M., Alexander, E., Ghisalberti, E.L. & Richmond, G.S. (1995). Cardioactive effects of *Eremophila alternifolia* extracts. *Journal of ethnopharmacology*, 47: 91-95.

Pieters, L. & Vlietinck, A.J. (2005). Bioguided isolation of pharmacologically active plant components, still a valuable strategy for the finding of new lead compounds? *Journal of ethnopharmacology*, 100: 57–60.

Rates, S.M.K. (2001). Plants as source of drugs. *Toxicon*, 39: 603-613.

Rang, H.P., Dale, M.M., Ritter, J.M. & Flower, R.J. (2007). *Rang and Dale's pharmacology*. Philadelphia: Churchill Livingstone.

Reddy, K.S., Naik, N. & Prabhakaran, D. (2006). Hypertension in the developing world: a consequence of progress. *Curr Cardiol Rep*, 8(6): 399-404.

Rivett, D.E.A. (1964). The islation of Marrubiin from *Leonotis leonurus* R.Br. *Journal of chemical society*, 1857-1858.

Ruff, R.L. (1996). Sodium channel slow inactivation and the distribution of sodium channels on skeletal muscle fibres enable the performance properties of different skeletal muscle fibre types. *Acta physiologica Scandinavica*, 156(3): 159-168.

Ruffolo, R.R. & Hieble, J.P. (1994). α-adrenoceptors. *Pharmacology & therapeutics*, 61(1-2): 1-64.

Ruffolo, R.R. & Hieble, J.P. (1995). New concepts in α-adrenoceptor pharmacology. *Pharmacological research*, 31(1): 153.

Seeley, R.R., Stephens, T.D. & Tate, P. (2003). *Anatomy & Physiology*. Boston: McGraw-Hill.

Shah, A.K. & Yadav, M.R. (2008). Recent advances in selective α_1-adrenoreceptor antagonists as antihypertensive agents. *Bioorganic & medicinal chemistry*, 16(9): 4759-4800.

Silva, R.M., Oliveira, F.A., Cunha, K.M.A., Maia, J.L., Maciel, M.A.M., Pinto, A.C., Nascimento, N.R.F., Santos, F.A. & Rao, V.S.N. (2005). Cardiovascular effects of *trans*-dehydrocrotonin, a diterpene from *Croton cajucara* in rats. *Vascular pharmacology*, 43: 11-18.

Skoog, D.A. & West, D.M. (1971). *Principles of instrumental analysis*. New York: Holt, Rinehart and Winston inc.

Skrzypiec-Spring, M., Grotthus, B., Szelag, A. & Schulz, R. (2007). Isolated heart perfusion according to Langendorff – still viable in the new millennium. *Journal of pharmacological and toxicological methods*, 55: 113-126.

Soejarto, D.D. (1996). Biodiversity prospecting and benefit-sharing: Perspective fro the field. *Journal of ethnopharmacology*, 51: 1-15.

Sofowora, A. (1982). *Medicinal Plants and traditional Medicine in Africa*. Chichester: John Wiley.

Somova, L.I., Shode, F.O., Moodley, K. & Govender, Y. (2001). Cardiovascular and diuretic activity of kaurene derivatives of *Xylopia aethiopica* and *Alepidea amatymbica*. *Journal of ethnopharmacology*, 77(2): 165-174.

Summers, R.J. & McMartin, L.R. (1993). Adrenoceptors and their second messenger systems. *Journal of neurochemistry*, 60: 10-23.

Tabuti, J.R.S., Dhillion, S.S. & Lye, K.A. (2003). Traditional medicine in Balamoji country, Uganda: its practitioners, users and viability. *Journal of ethnopharmacology*, 85: 119-129.

Toya, Y., Schwencke, C. & Ishikawa, Y. (1998). Forskolin derivatives with increased selectivity for cardiac adenylyl cyclase. *Journal of molecular and cellular cardiology*, 30: 97-108.

Ulubelen, U. (2003). Cardioactive and antibacterial terpenoids from some Saliva species. *Phytochemistry*, 64(2): 395-399.

van Wyk, B., van Outshoorn, B. & Gericke, N. (2000). *Medicinal plants of South Africa*. Cape Town: Briza publications.

Wakabayashi, I., Sakamoto, K. & Hatake, K. (1995). Inhibitory effects of cadmium ion on extracellular Ca^{2+}-independent contraction of rat aorta. *European Journal of Pharmacology and pharmacology*, 293: 133-140.

Watt, J.M. & Breyer-Brandwijk, M.G. (1962). *Medicinal and poisonous plants of Southern Africa*. Edinburg: E & S Livingstone.

Williamson, E.M., Okpako, D.T & Evans, J.F. (1996). *Pharmacological methods in phytotherapy research volume 1: Selection, preparation and pharmacological evaluation of plant material*. Chichester: John Wiley & sons.

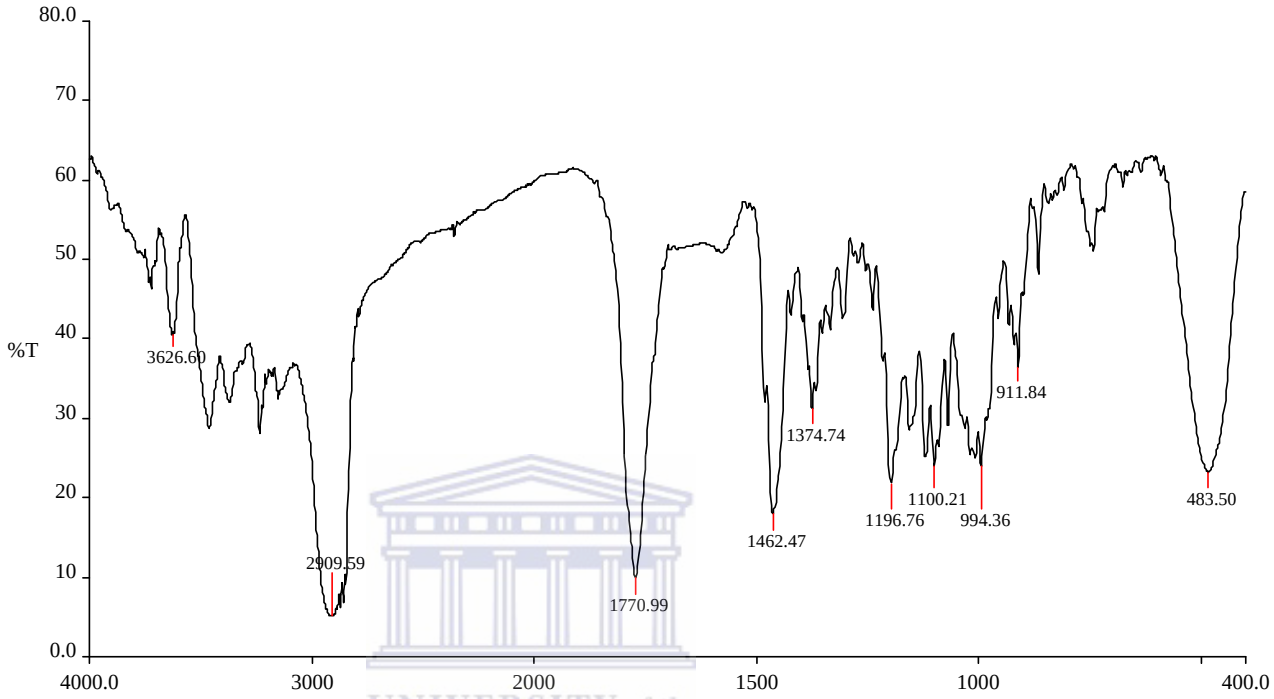
World Health Organization (2002). *WHO traditional medicine strategy 2002-2005*. Geneva: World Health organization.

Zhang, X. (2000). *General guidelines for methodologies on research and evaluation of traditional medicine*. Geneva: World Health organization.

Zhang, C.Y., & Tan, B.K.H. (1997). Mechanisms of cardiovascular activity of *Andrographis paniculata* in the anaesthetized rat. *Journal of ethnopharmacology*, 56: 97-101.

APPENDIXES

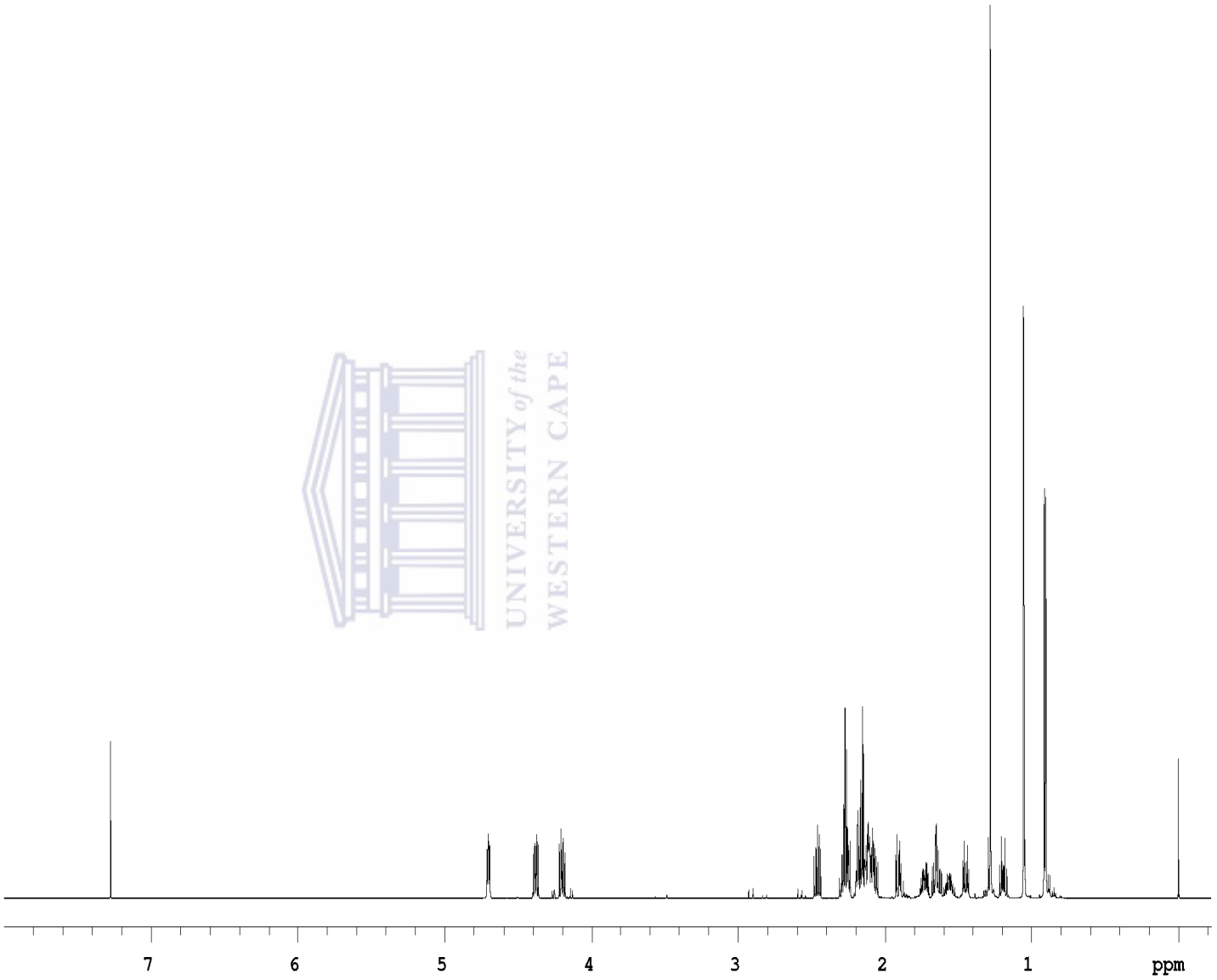
APPENDIX I: IR Spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

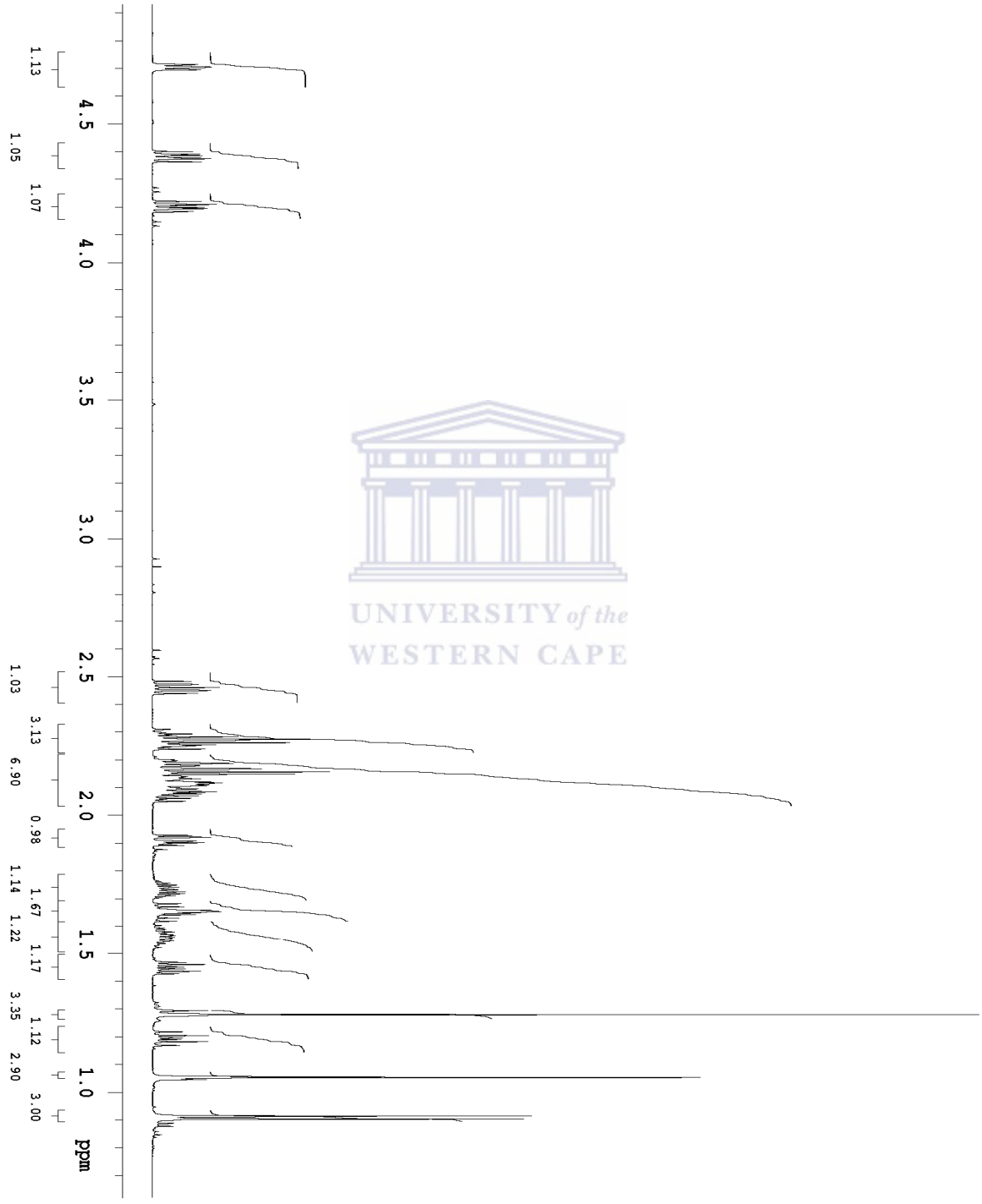


APPENDIX II: ^{1}H spectra for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

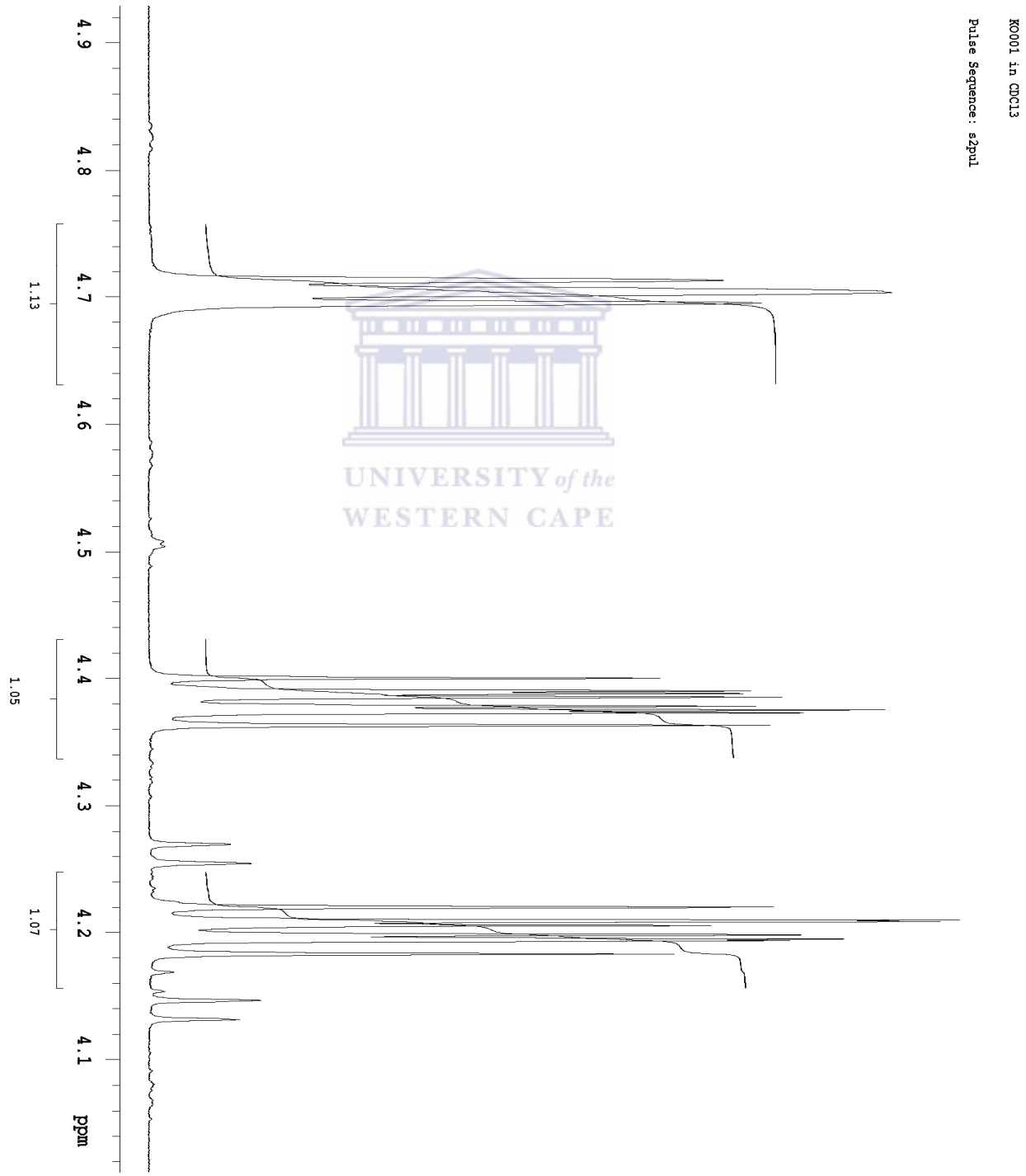
Pulse Sequence: s2pul



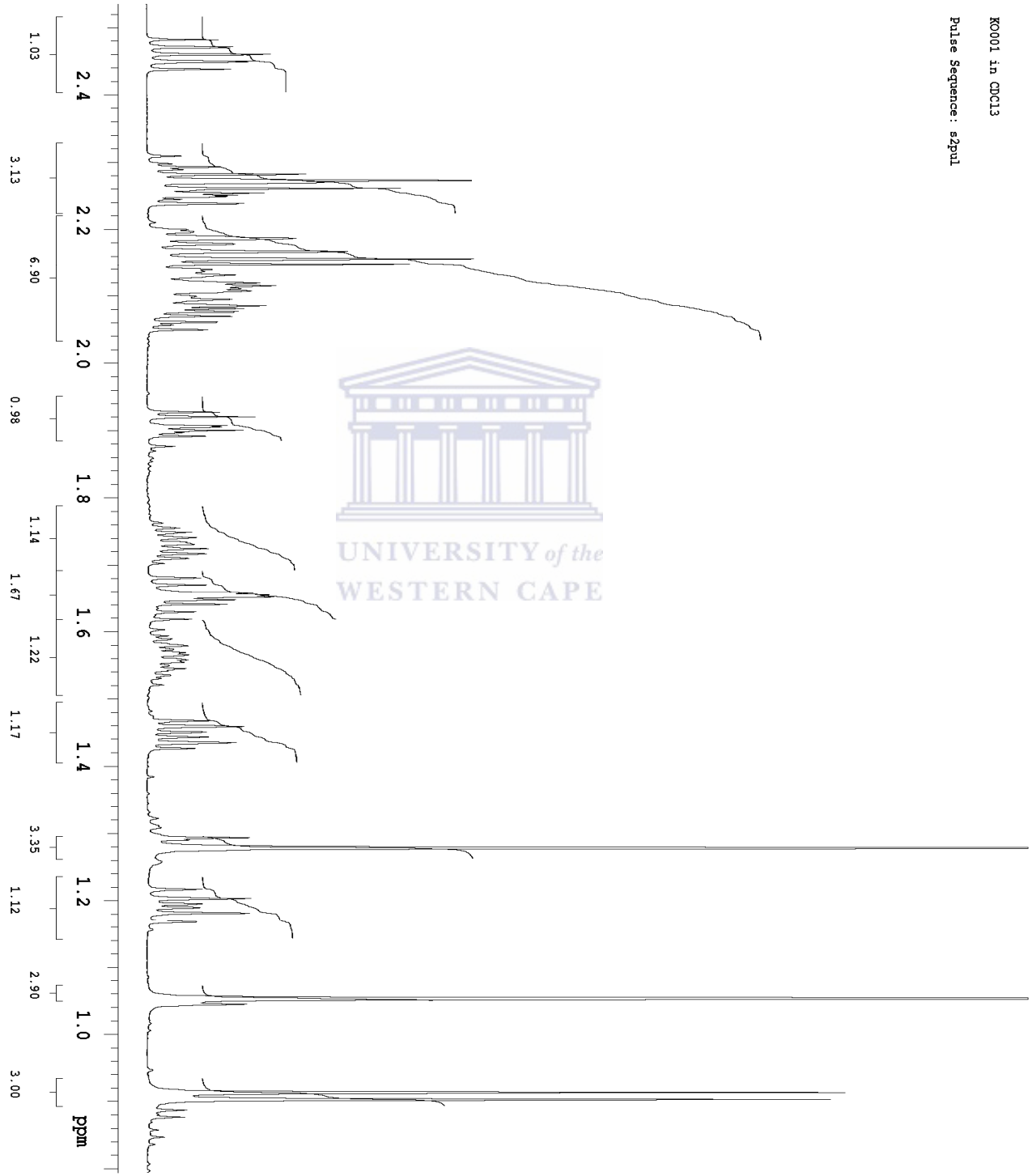


APPENDIX III: ^{1}H spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

APPENDIX IV: ^{1}H spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



APPENDIX V: ^{1}H Spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

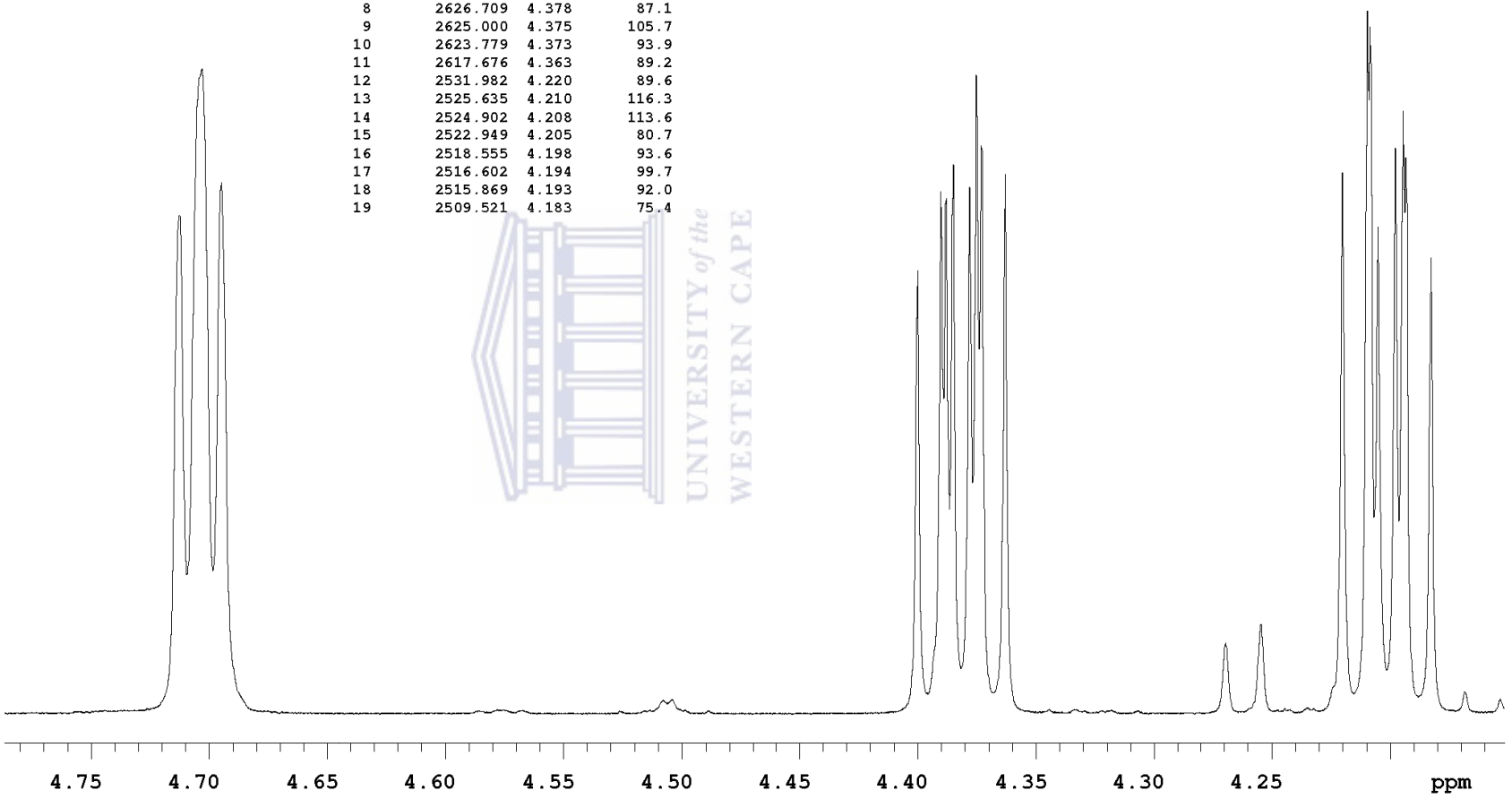


UNIVERSITY of the
WESTERN CAPE

KO001 in CDCl3

Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT
1	2827.637	4.713	82.4
2	2821.777	4.703	106.7
3	2816.895	4.695	87.9
4	2639.893	4.400	73.4
5	2634.033	4.390	86.4
6	2632.568	4.388	85.3
7	2630.859	4.385	90.9
8	2626.709	4.378	87.1
9	2625.000	4.375	105.7
10	2623.779	4.373	93.9
11	2617.676	4.363	89.2
12	2531.982	4.220	89.6
13	2525.635	4.210	116.3
14	2524.902	4.208	113.6
15	2522.949	4.205	80.7
16	2518.555	4.198	93.6
17	2516.602	4.194	99.7
18	2515.869	4.193	92.0
19	2509.521	4.183	75.4



APPENDIX VII: ^{1}H spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

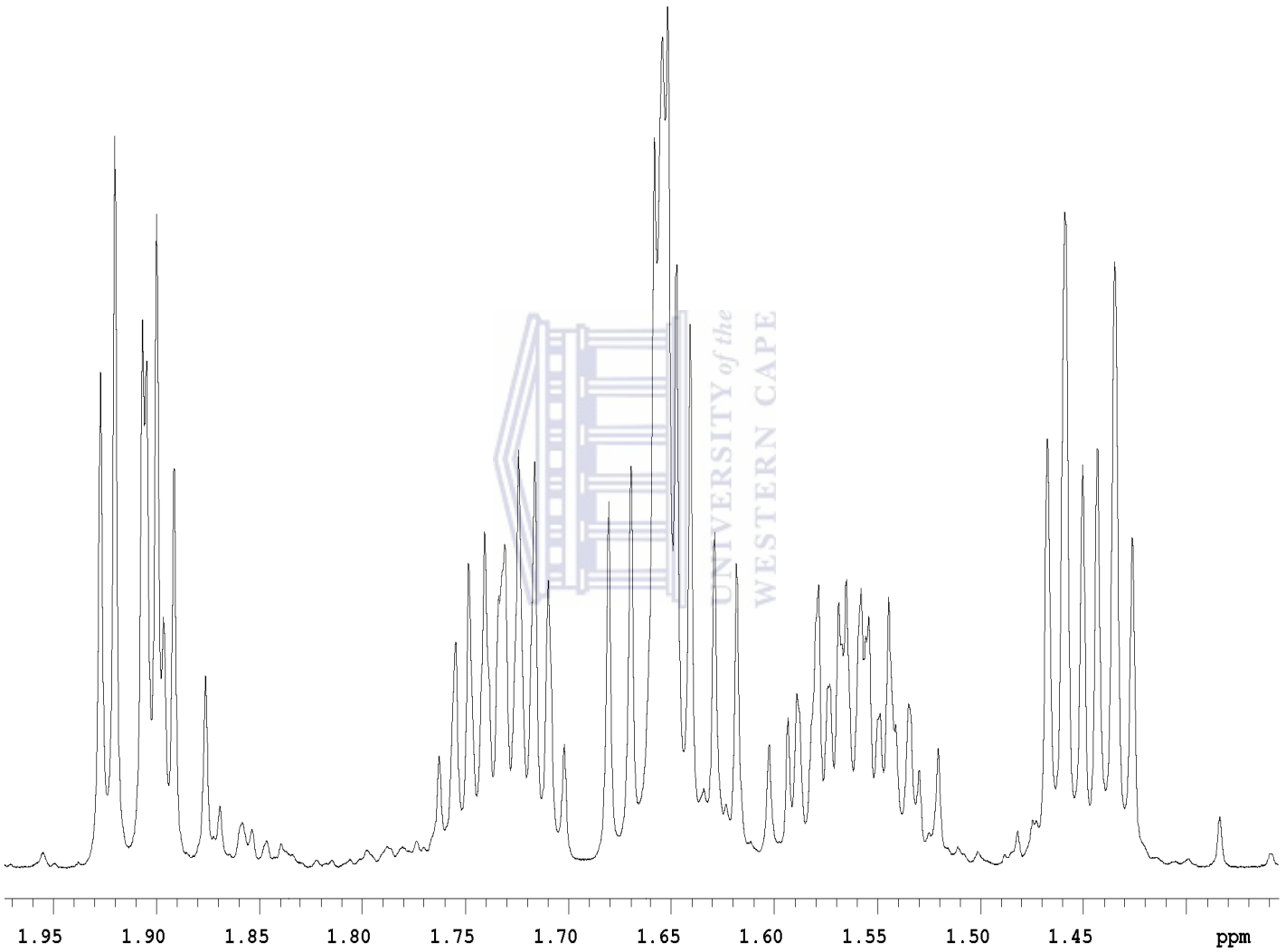
Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT	INDEX	FREQUENCY	PPM	HEIGHT
1	1489.258	2.482	33.7	30	1281.738	2.136	27.4
2	1482.910	2.472	40.7	31	1278.564	2.131	42.2
3	1481.934	2.470	39.5	32	1271.484	2.119	53.4
4	1476.074	2.460	58.4	33	1269.043	2.115	61.1
5	1469.971	2.450	51.1	34	1266.602	2.111	44.7
6	1468.994	2.448	48.1	35	1264.404	2.107	49.6
7	1462.646	2.438	39.7	36	1262.939	2.105	35.8
8	1385.254	2.309	16.2	37	1259.521	2.099	23.8
9	1378.662	2.298	12.1	38	1256.836	2.095	40.5
10	1375.488	2.293	34.9	39	1250.977	2.085	56.4
11	1373.291	2.289	17.5	40	1248.535	2.081	45.8
12	1369.141	2.282	75.0	41	1245.850	2.076	43.2
13	1363.770	2.273	153.0	42	1241.699	2.070	40.4
14	1362.061	2.270	84.9	43	1236.572	2.061	33.7
15	1356.445	2.261	119.7	44	1233.887	2.057	13.0
16	1352.295	2.254	55.6	45	1229.736	2.050	28.9
17	1350.342	2.251	42.8				
18	1349.121	2.249	38.1				
19	1346.680	2.245	16.8				
20	1343.018	2.238	45.9				
21	1319.824	2.200	19.6				
22	1317.871	2.197	22.2				
23	1312.012	2.187	70.5				
24	1307.129	2.179	41.4				
25	1306.641	2.178	41.7				
26	1300.049	2.167	94.9				
27	1293.457	2.156	154.0				
28	1288.818	2.148	123.8				
29	1283.691	2.140	31.1				

APPENDIX VIII: ^{1}H spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

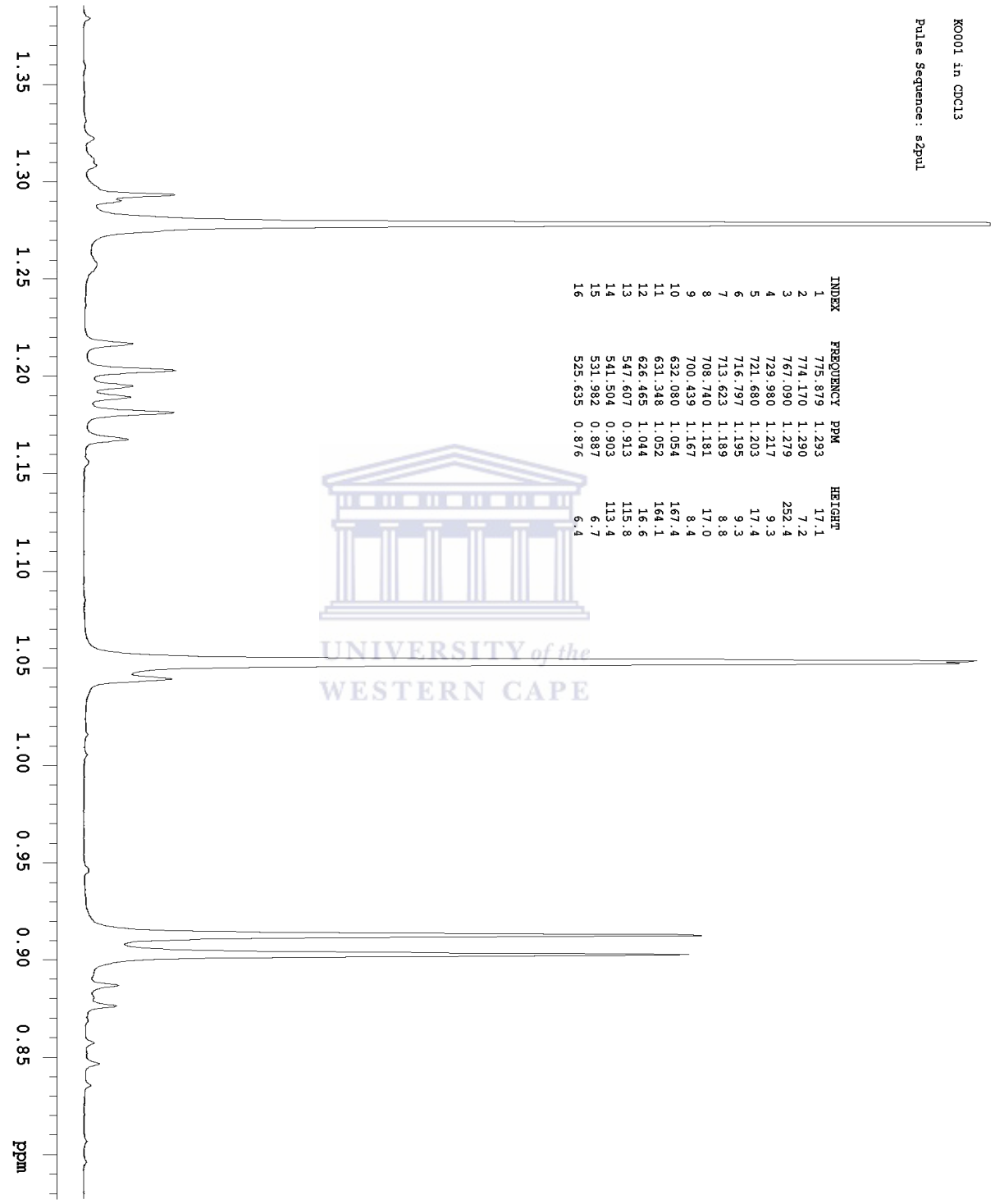
Pulse Sequence: s2pul



KO001 in CDCl3

Pulse Sequence: s2pul

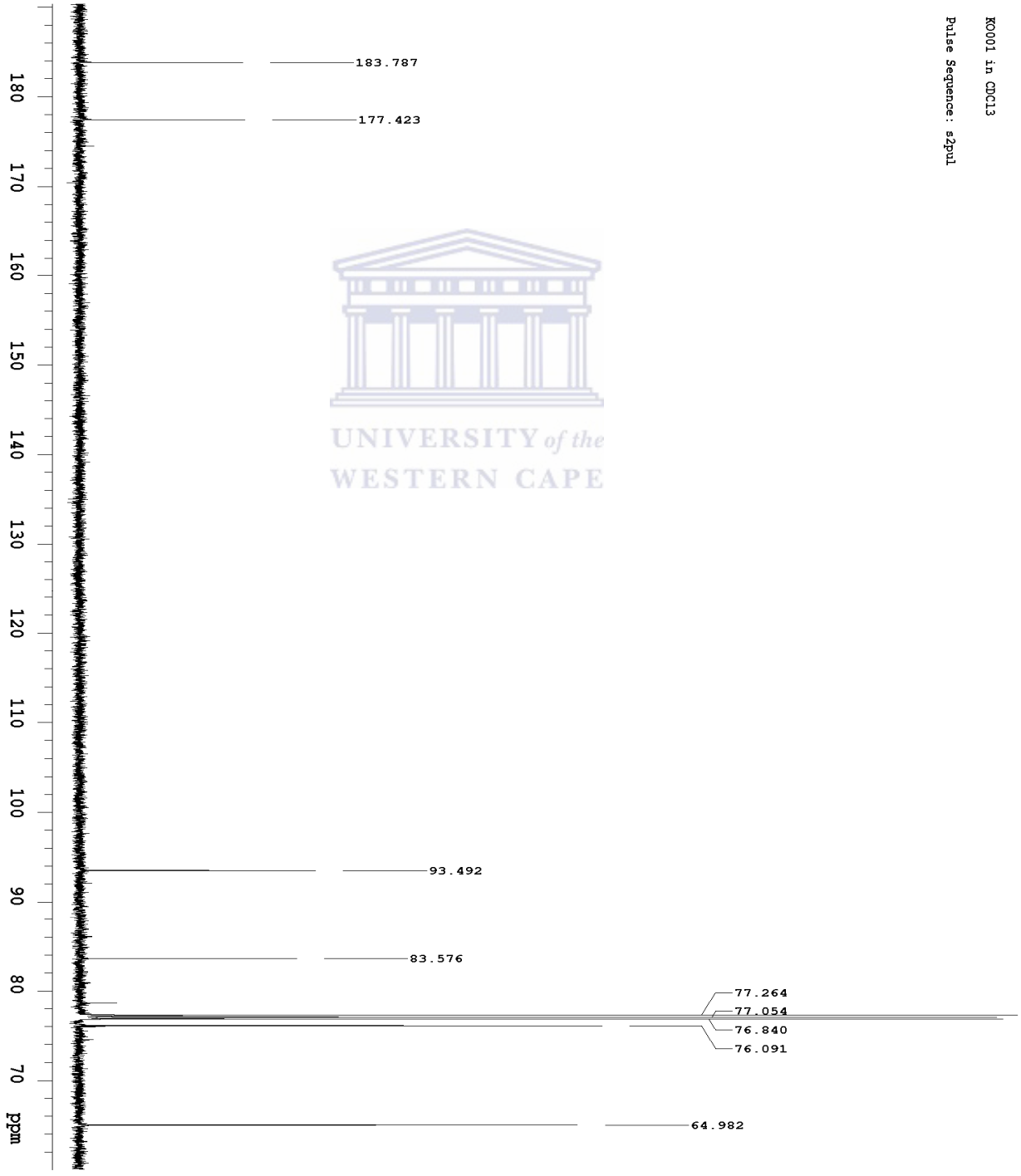
INDEX	FREQUENCY	PPM	HEIGHT	INDEX	FREQUENCY	PPM	HEIGHT
1	1156.250	1.927	84.2	30	956.055	1.593	25.7
2	1152.100	1.920	124.2	31	953.369	1.589	29.8
3	1144.043	1.907	93.2	32	947.021	1.578	48.3
4	1142.822	1.905	86.2	33	943.848	1.573	31.4
5	1139.893	1.900	111.1	34	941.162	1.569	45.2
6	1137.939	1.897	42.8	35	938.965	1.565	49.2
7	1134.766	1.891	67.9	36	934.814	1.558	47.7
8	1125.732	1.876	32.8	37	933.350	1.556	39.7
9	1057.617	1.763	19.3	38	932.617	1.554	42.9
10	1052.734	1.755	38.5	39	929.199	1.549	26.6
11	1049.072	1.749	51.9	40	926.758	1.545	46.1
12	1044.434	1.741	57.2	41	924.805	1.541	24.6
13	1040.283	1.734	46.4	42	920.898	1.535	28.2
14	1038.574	1.731	55.1	43	917.725	1.530	16.7
15	1034.424	1.724	71.0	44	912.354	1.521	20.5
16	1029.785	1.716	69.1	45	880.371	1.467	73.0
17	1025.879	1.710	49.0	46	875.244	1.459	111.4
18	1021.240	1.702	21.4	47	870.117	1.450	68.6
19	1008.301	1.681	62.4	48	865.967	1.443	71.3
20	1001.953	1.670	68.5	49	860.840	1.435	102.9
21	994.873	1.658	124.0	50	855.713	1.426	56.2
22	992.432	1.654	141.0				
23	990.967	1.652	146.2				
24	988.281	1.647	102.6				
25	984.375	1.641	92.4				
26	980.469	1.634	13.8				
27	977.295	1.629	57.1				
28	970.947	1.618	51.9				
29	961.426	1.602	21.2				



APPENDIX IX: ^{1}H spectrum of (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

APPENDIX X: ^{13}C Spectral data for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

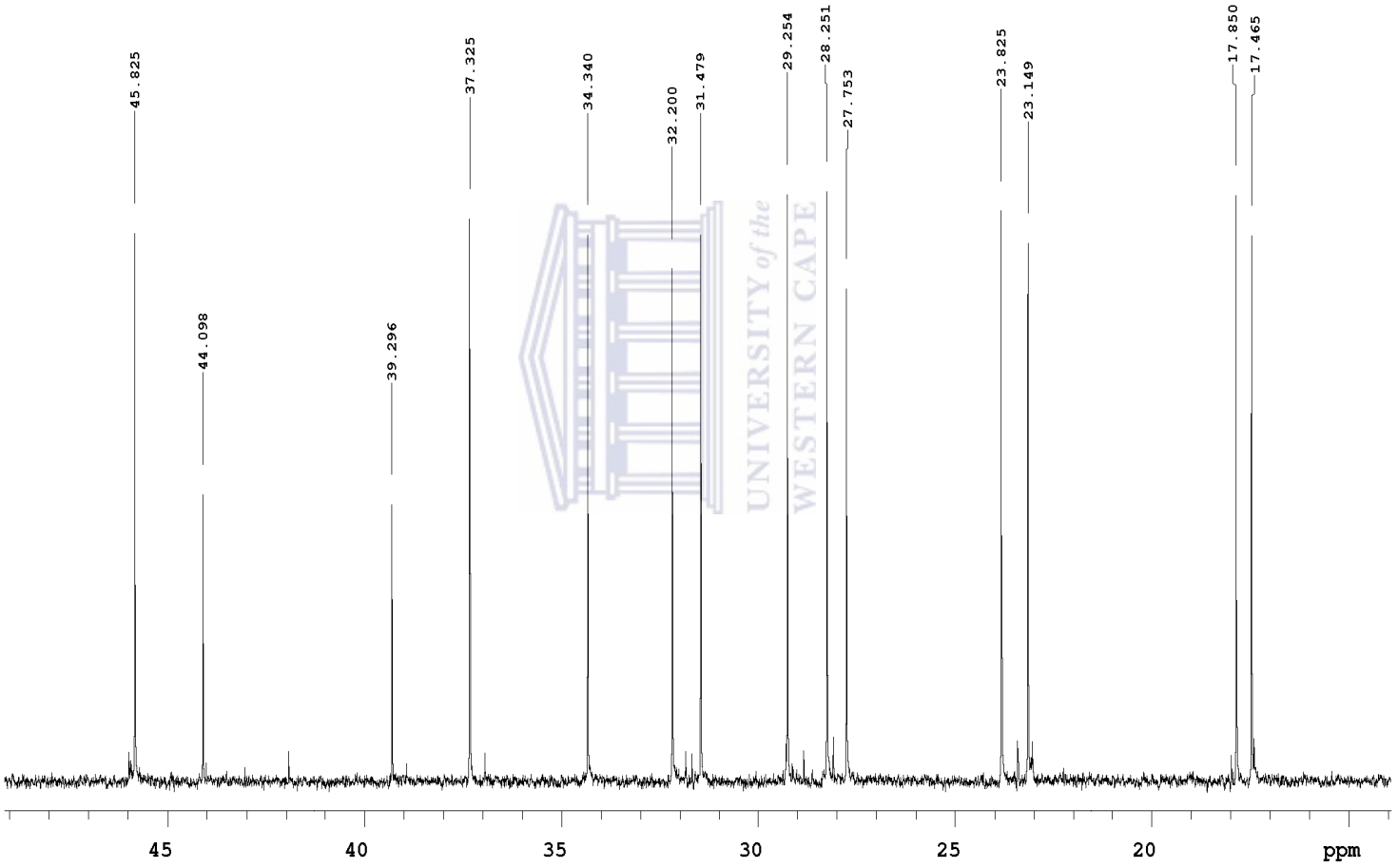
APPENDIX XI: ^{13}C spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



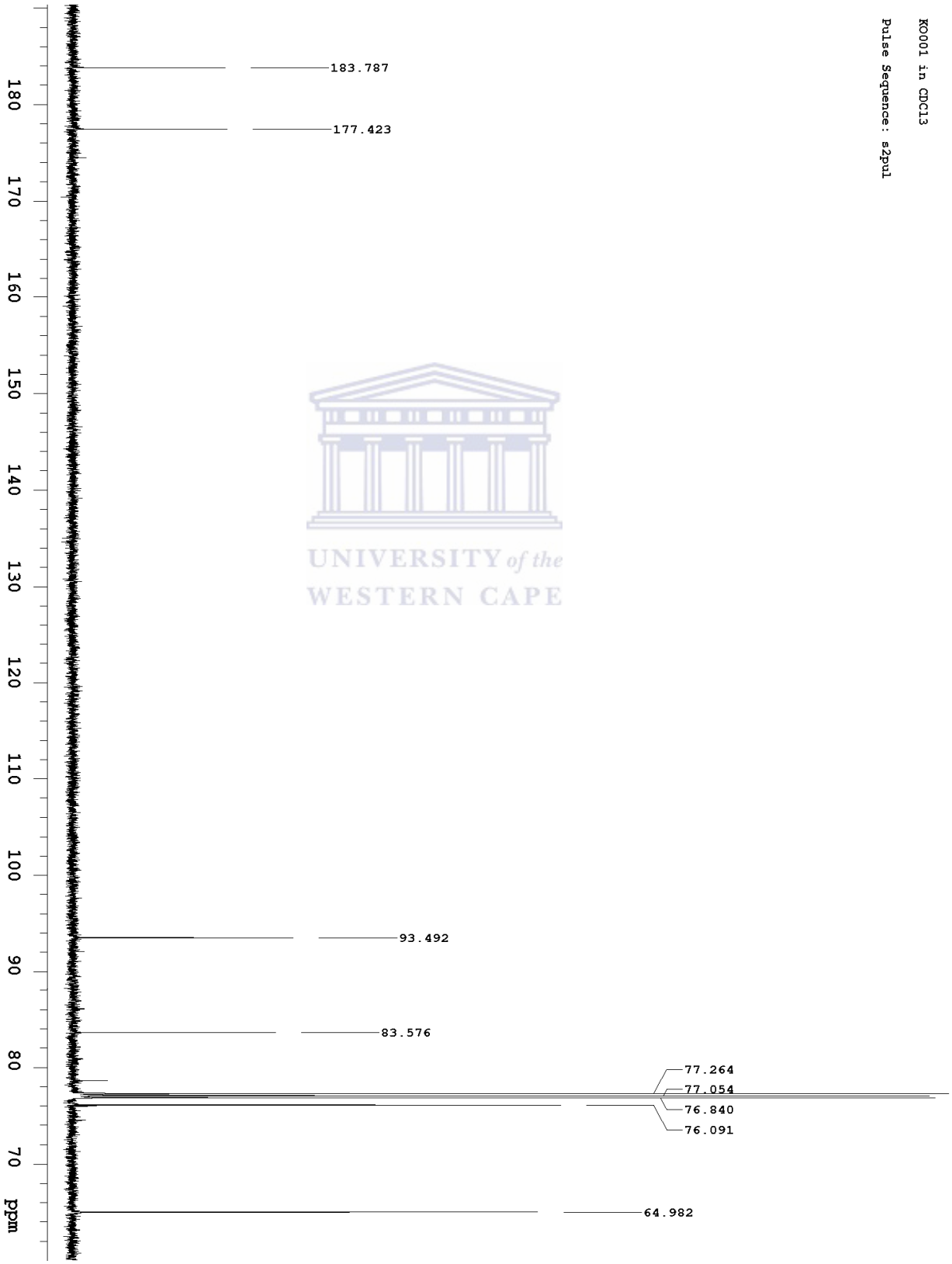
UNIVERSITY of the
WESTERN CAPE

KO001 in CDCl3

Pulse Sequence: s2pul



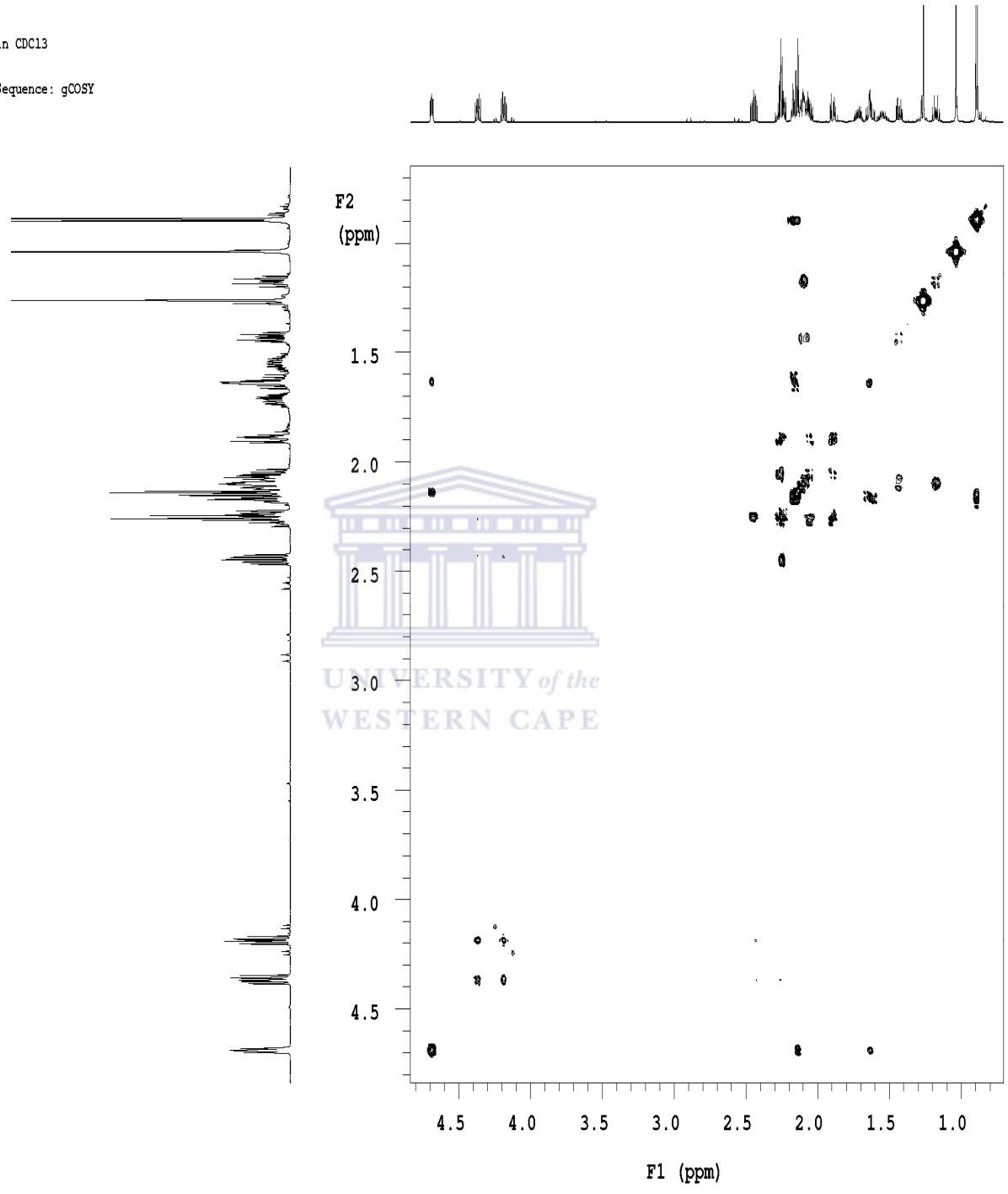
APPENDIX XII: ^{13}C spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



APPENDIX XIII: gCOSY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

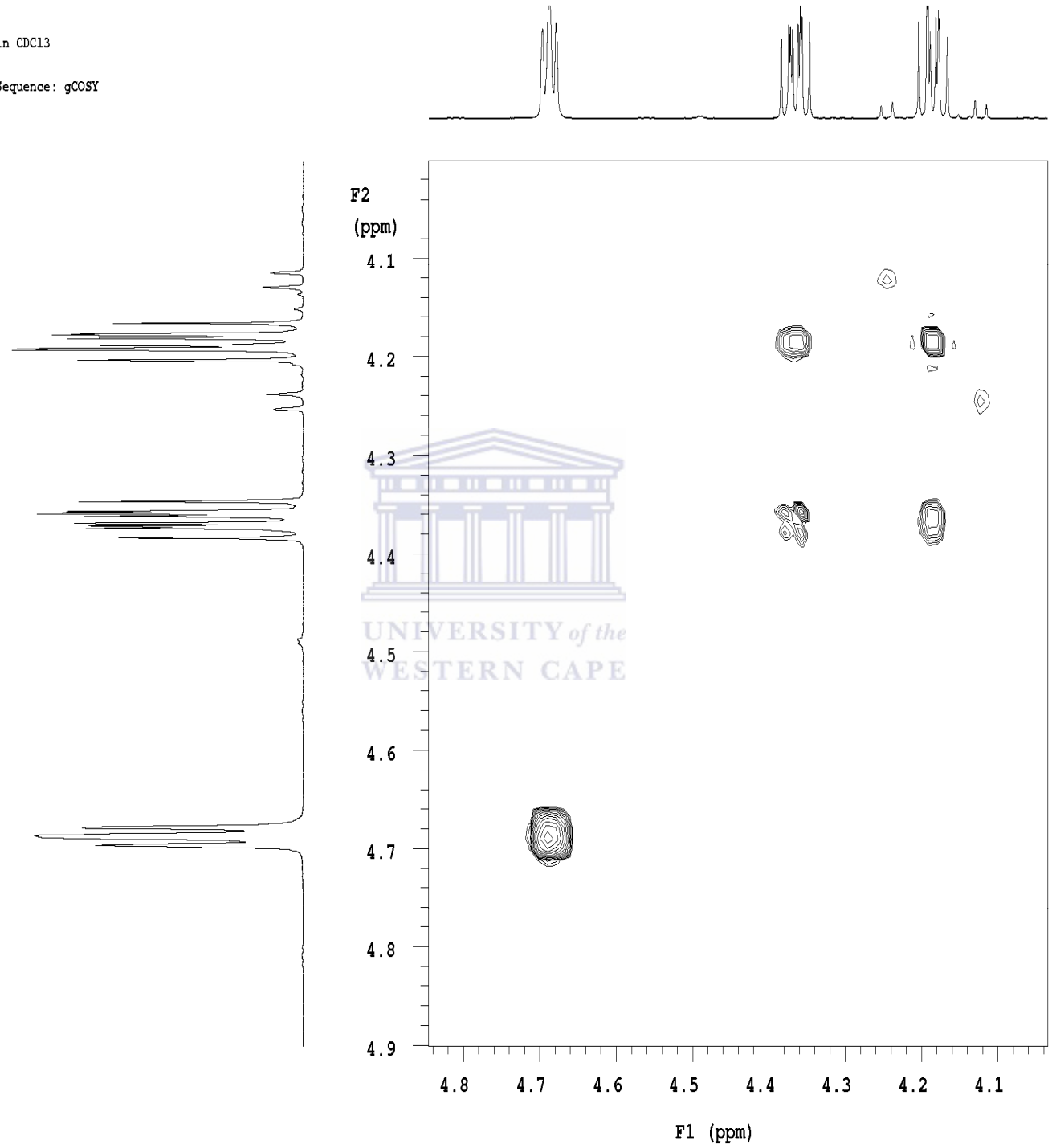
Pulse Sequence: gCOSY



APPENDIX XIV: gCOSY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

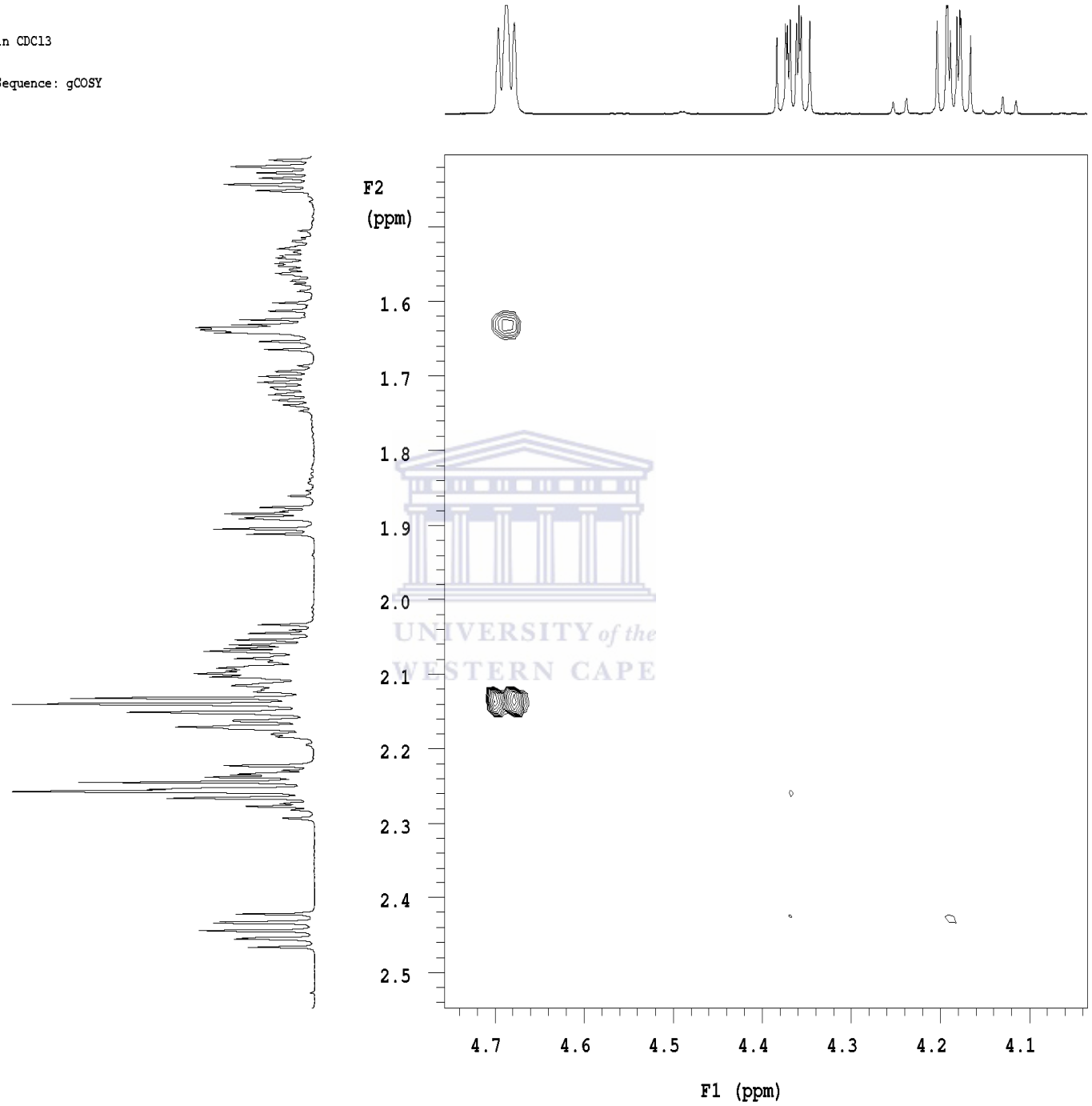
Pulse Sequence: gCOSY



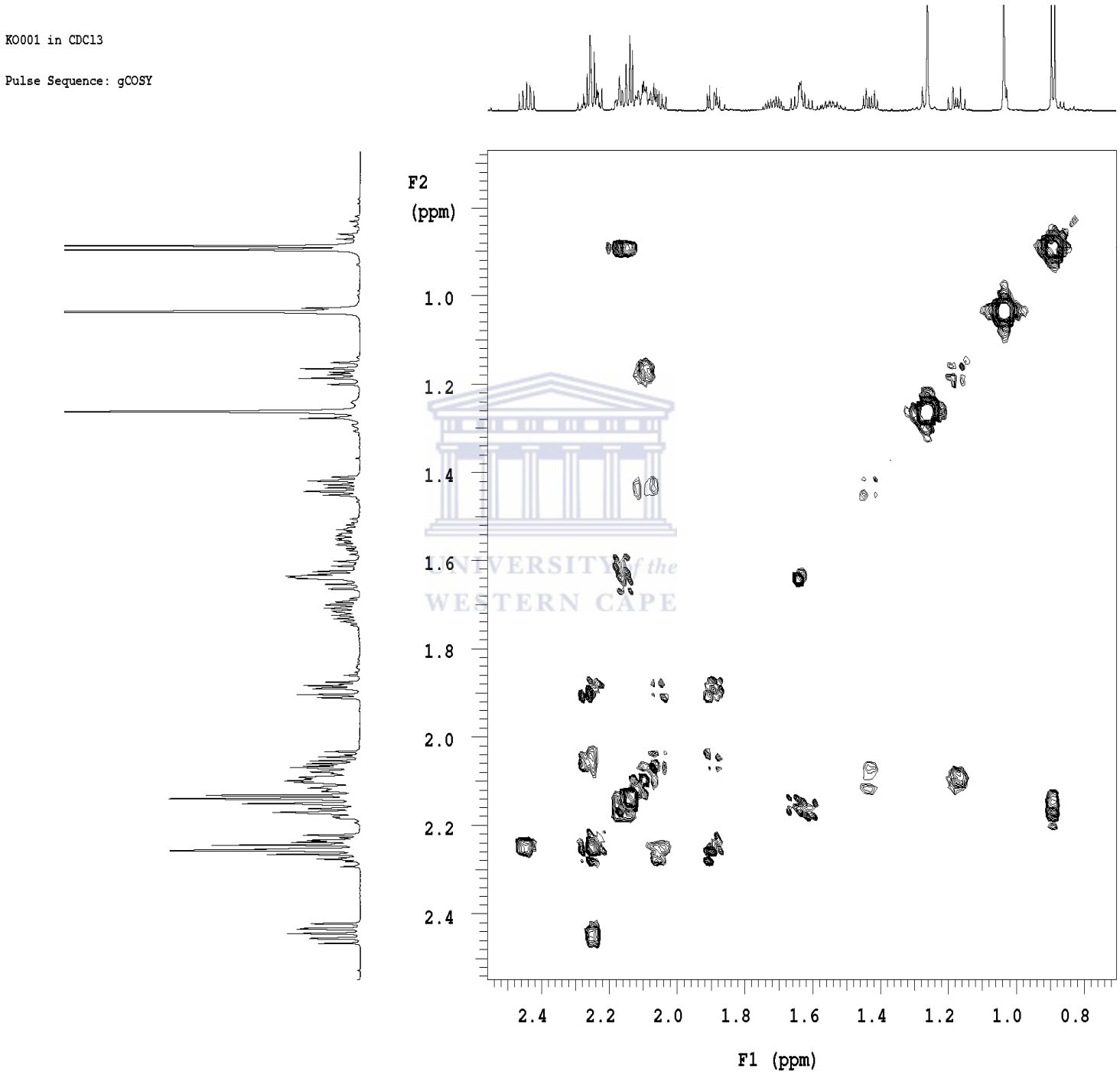
APPENDIX XV: gCOSY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

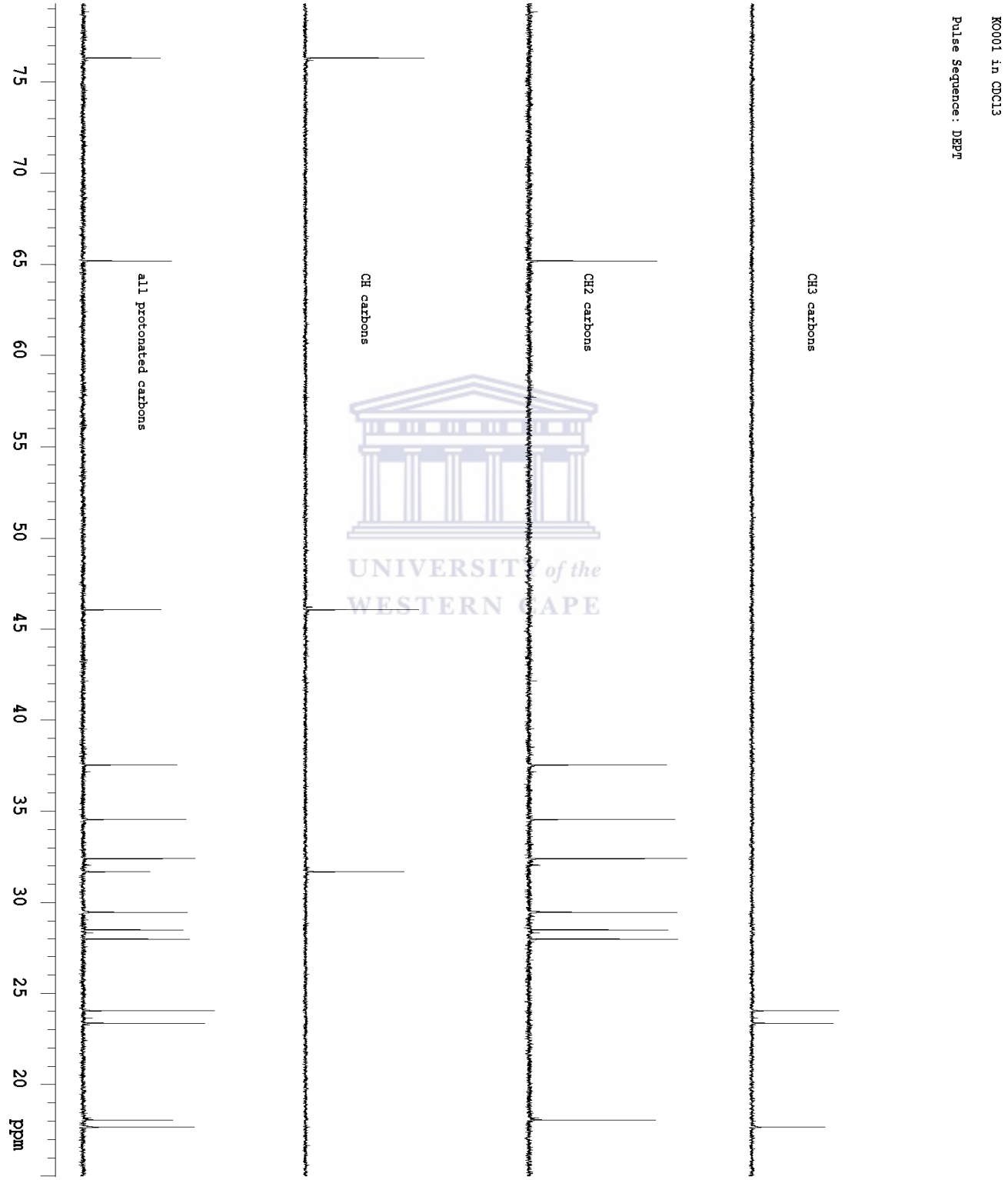
Pulse Sequence: gCOSY



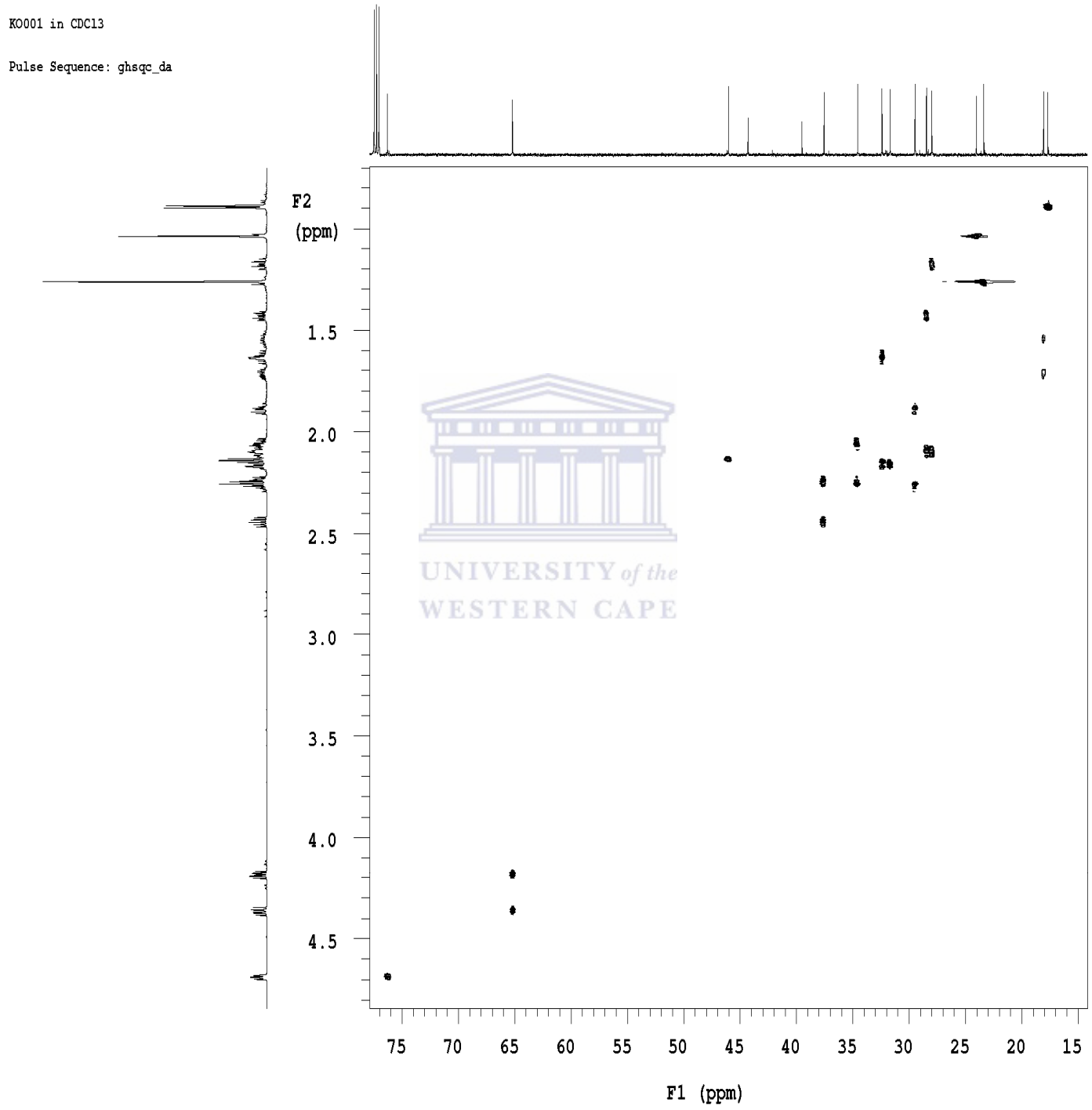
APPENDIX XVI: gCOSY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



APPENDIX XVII: DEPT spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



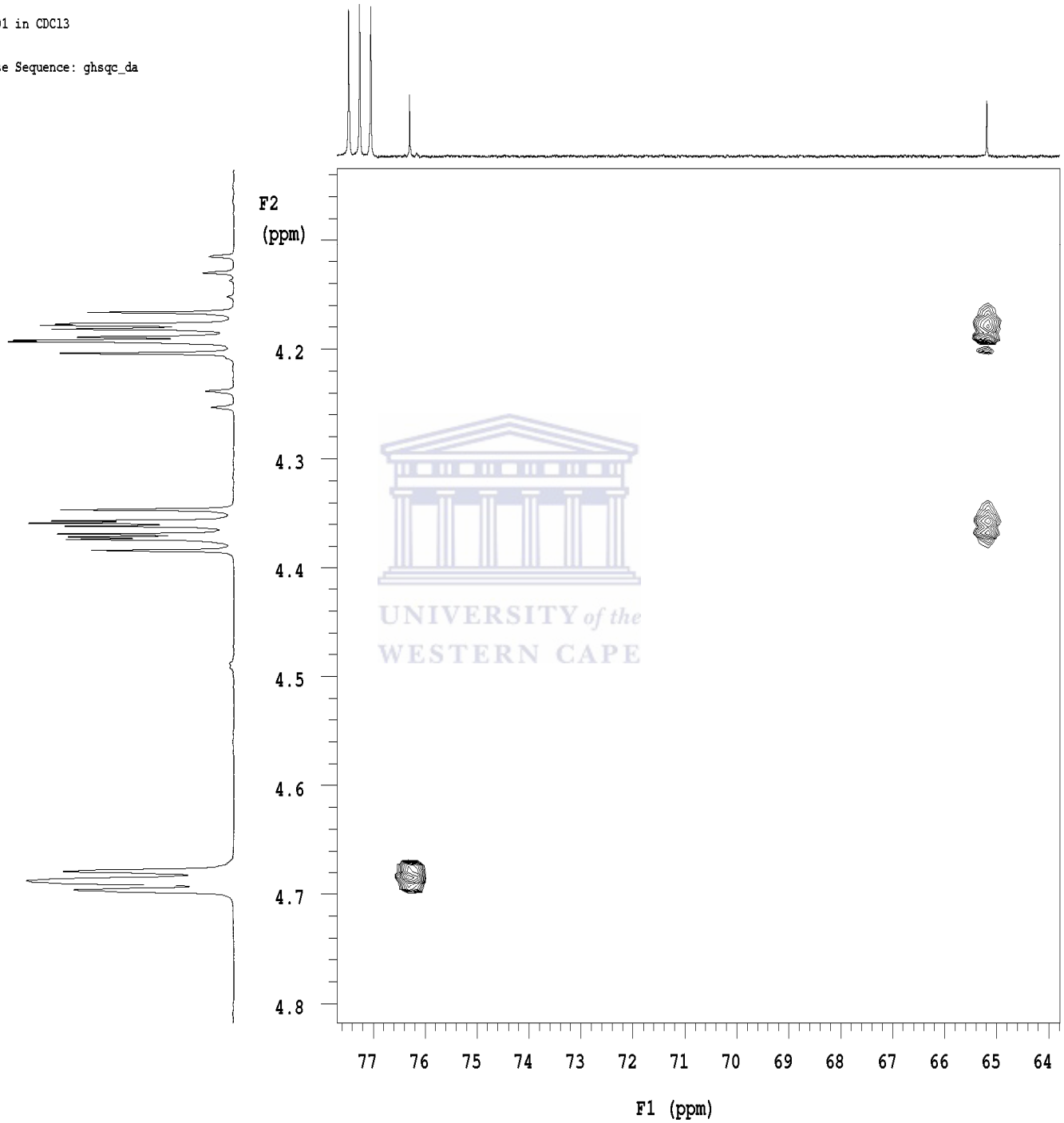
APPENDIX XVIII: gHSQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



APPENDIX XIX: gHSQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

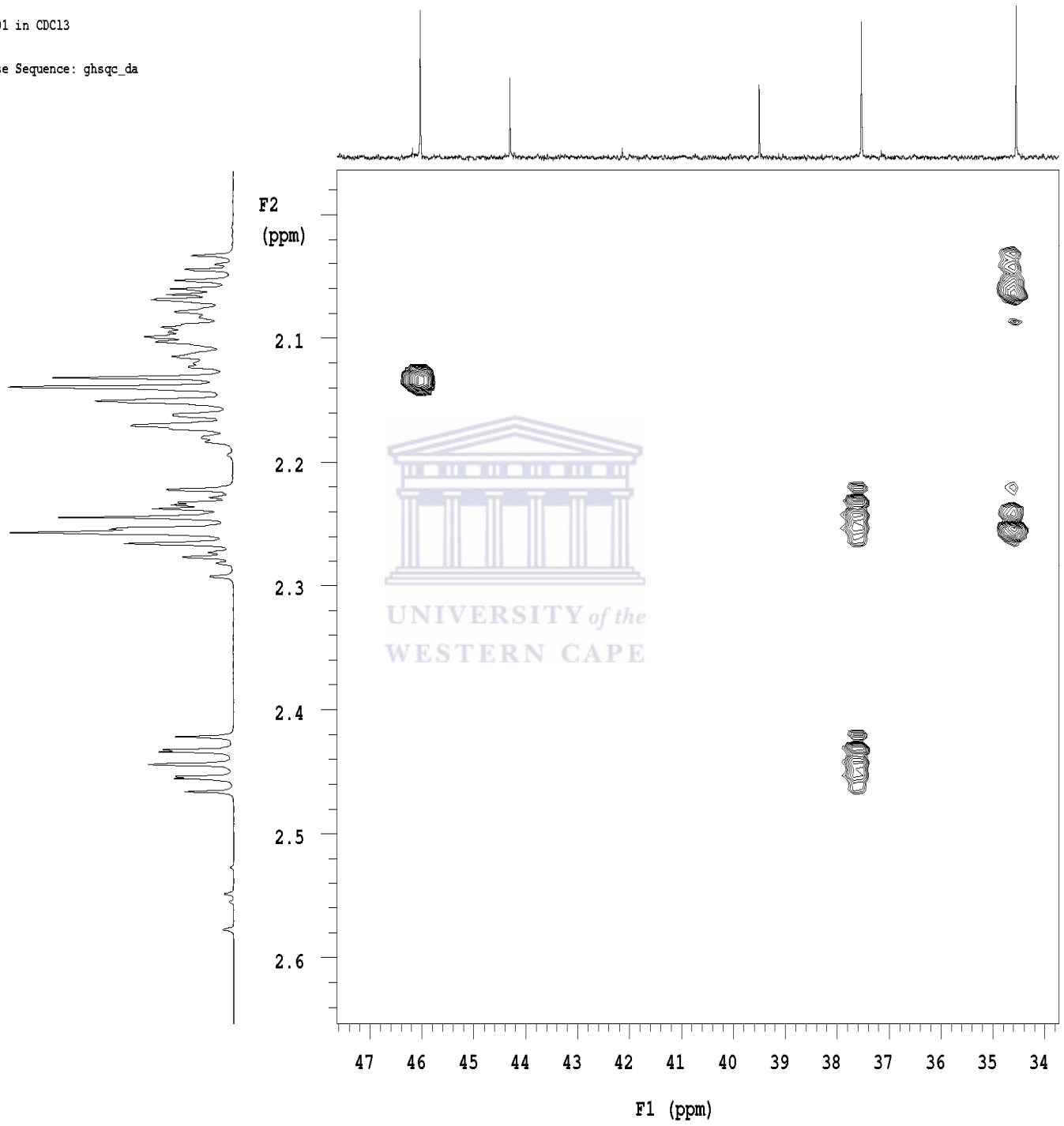
Pulse Sequence: ghsqc_da



APPENDIX XX: gHSQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

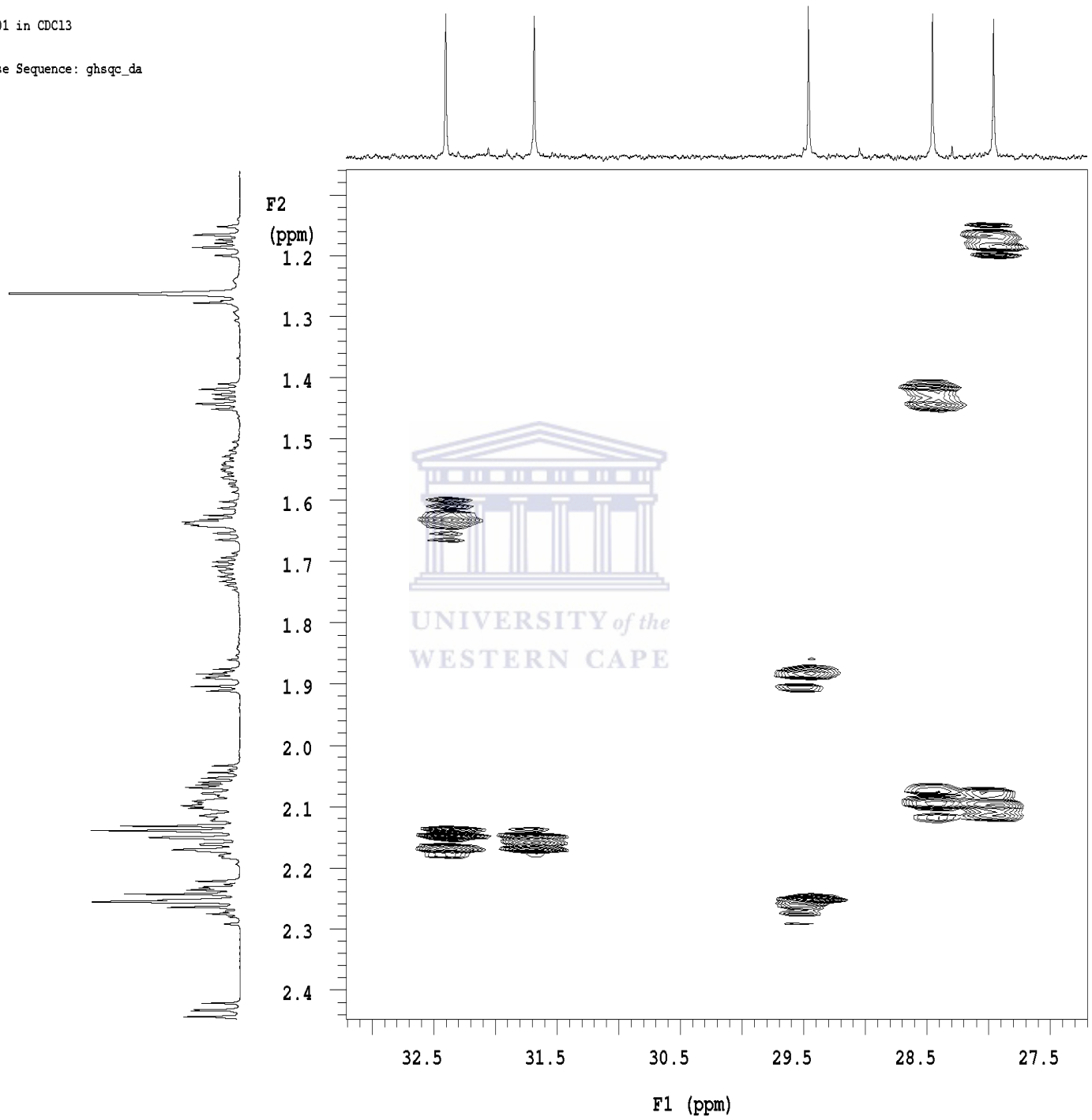
Pulse Sequence: ghsqc_da



APPENDIX XXI: gHSQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

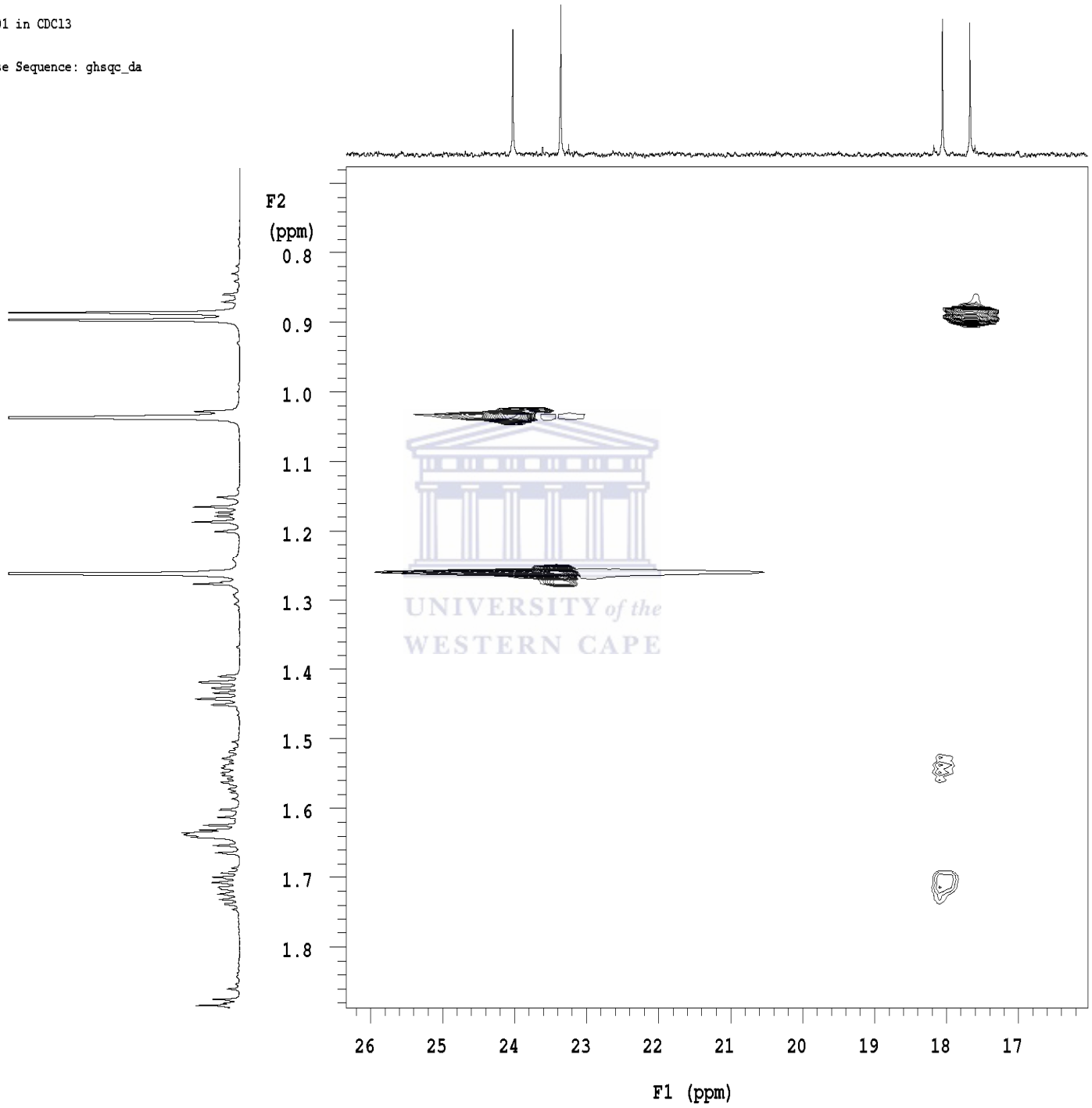
Pulse Sequence: ghsqc_da



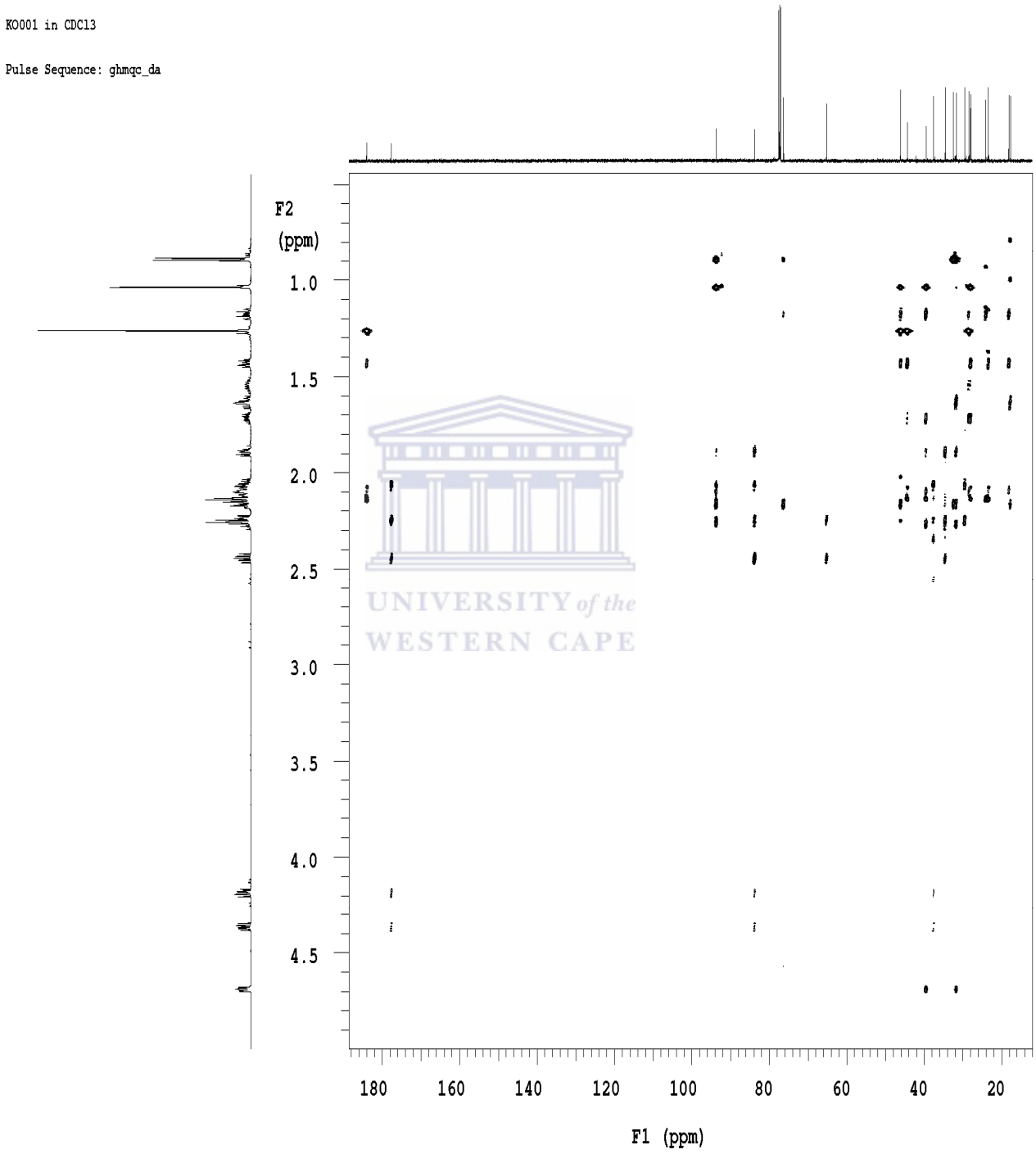
APPENDIX XXII: gHSQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

KO001 in CDCl3

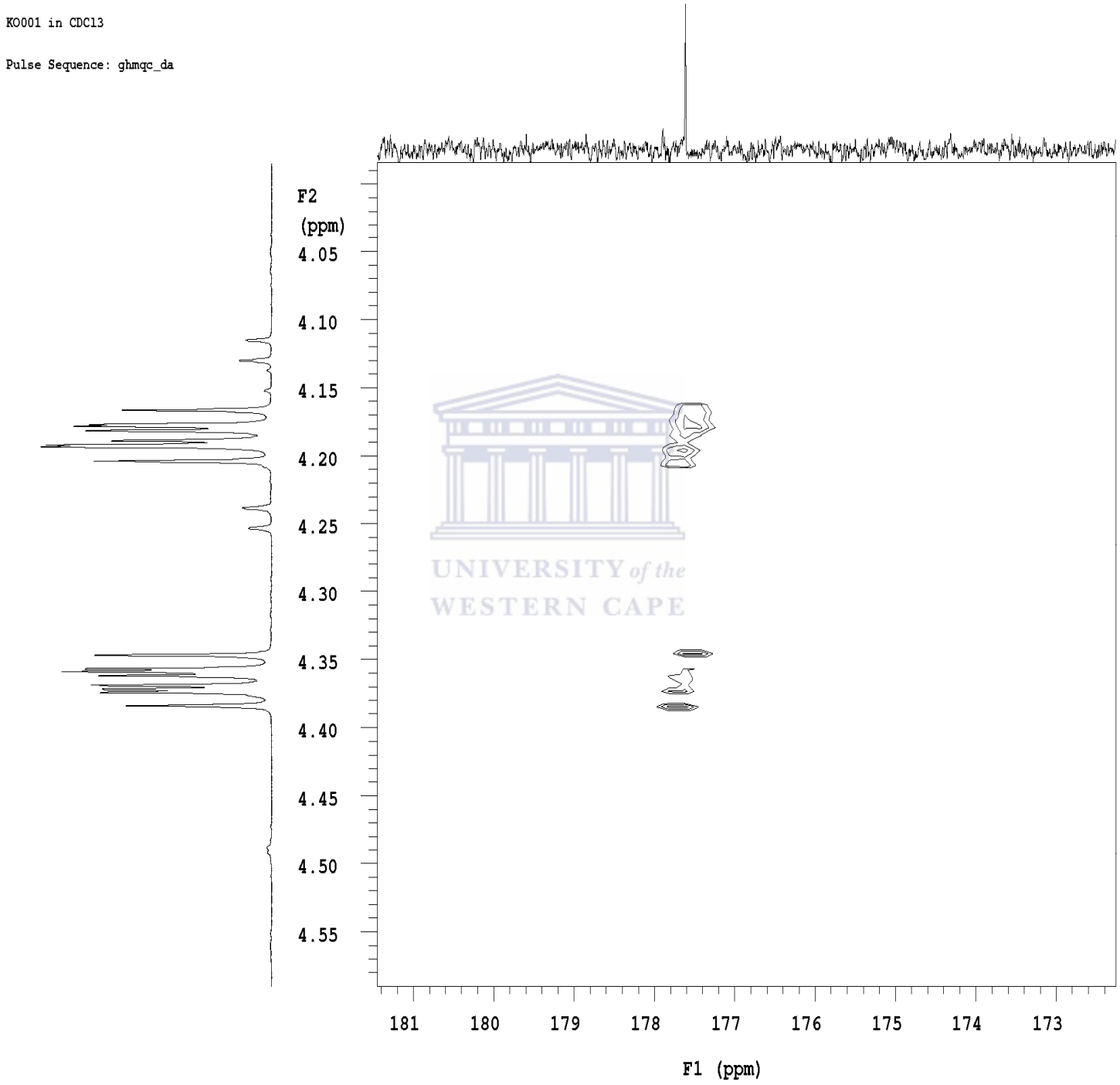
Pulse Sequence: ghsqc_da



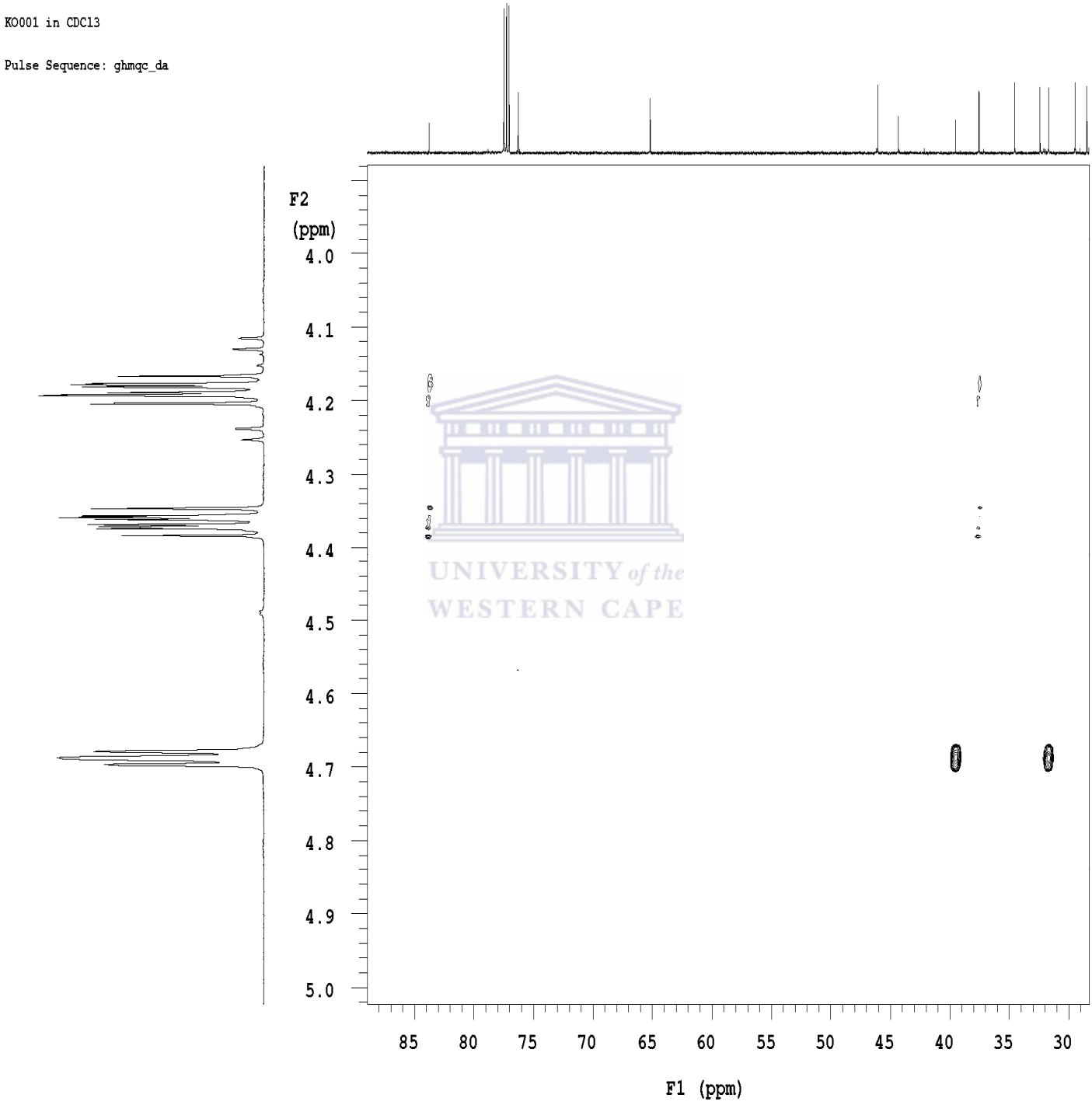
APPENDIX XXIII: gHSMQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



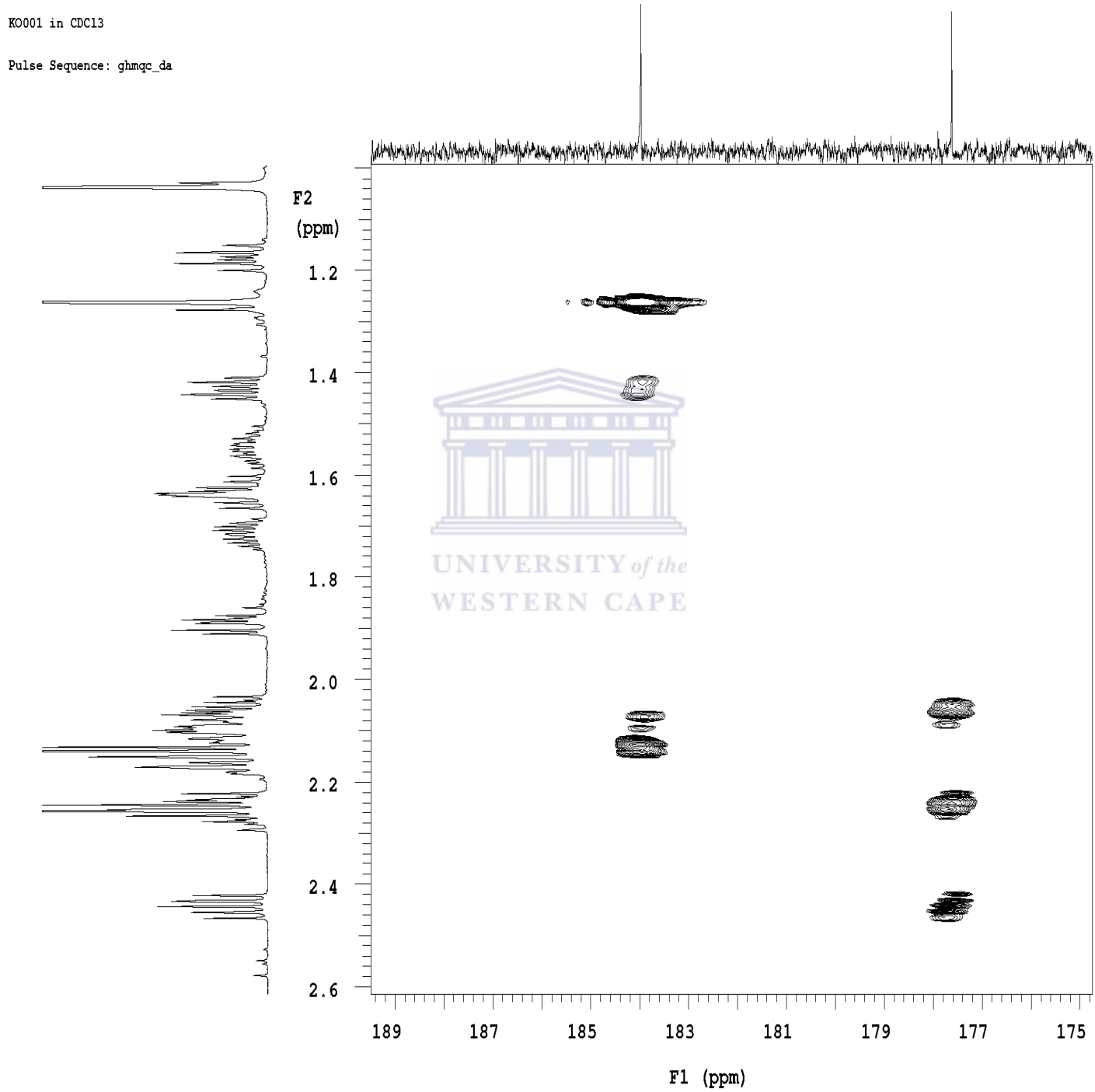
APPENDIX XXIV: gHSMQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



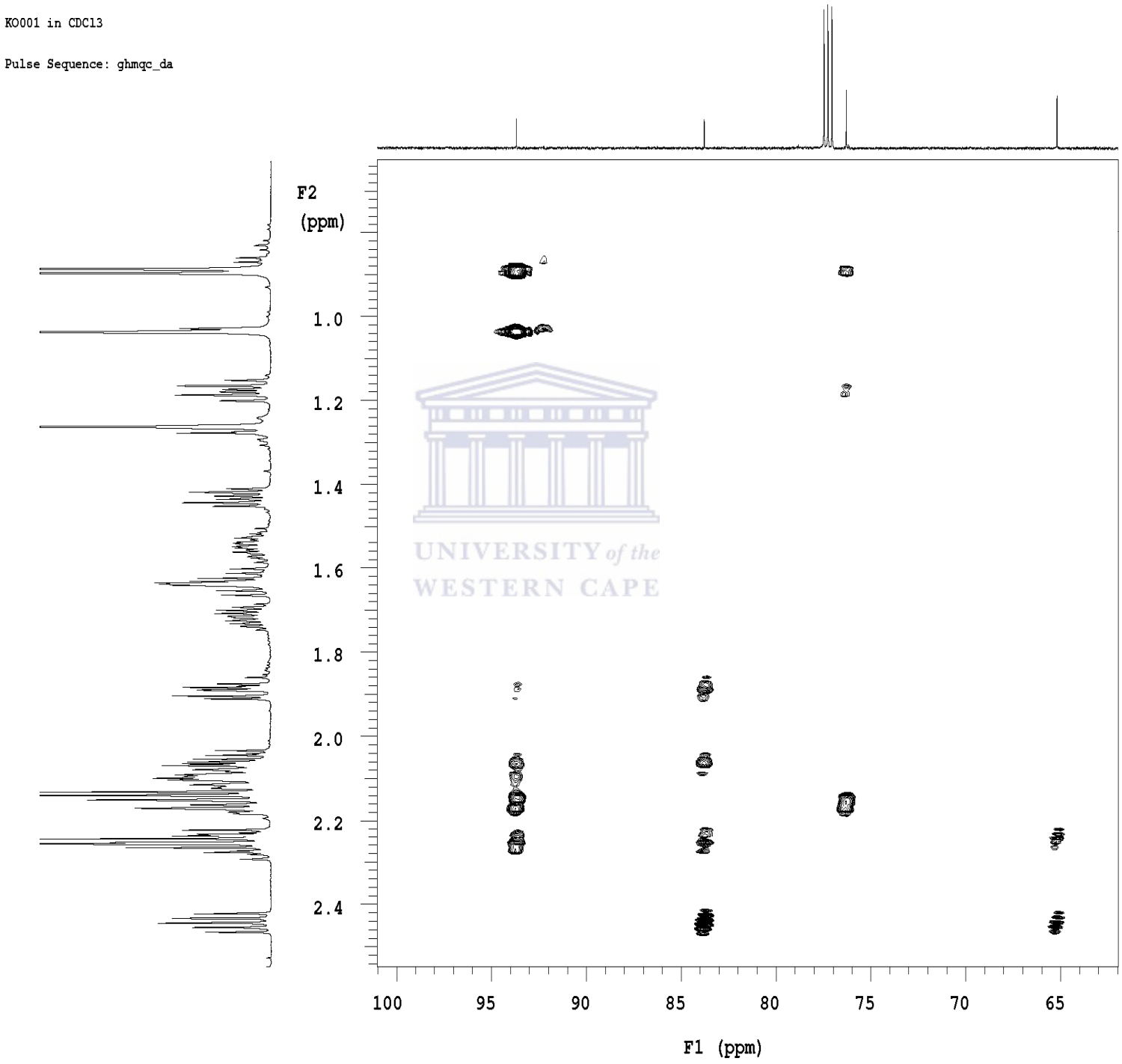
APPENDIX XXV: gHSMQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



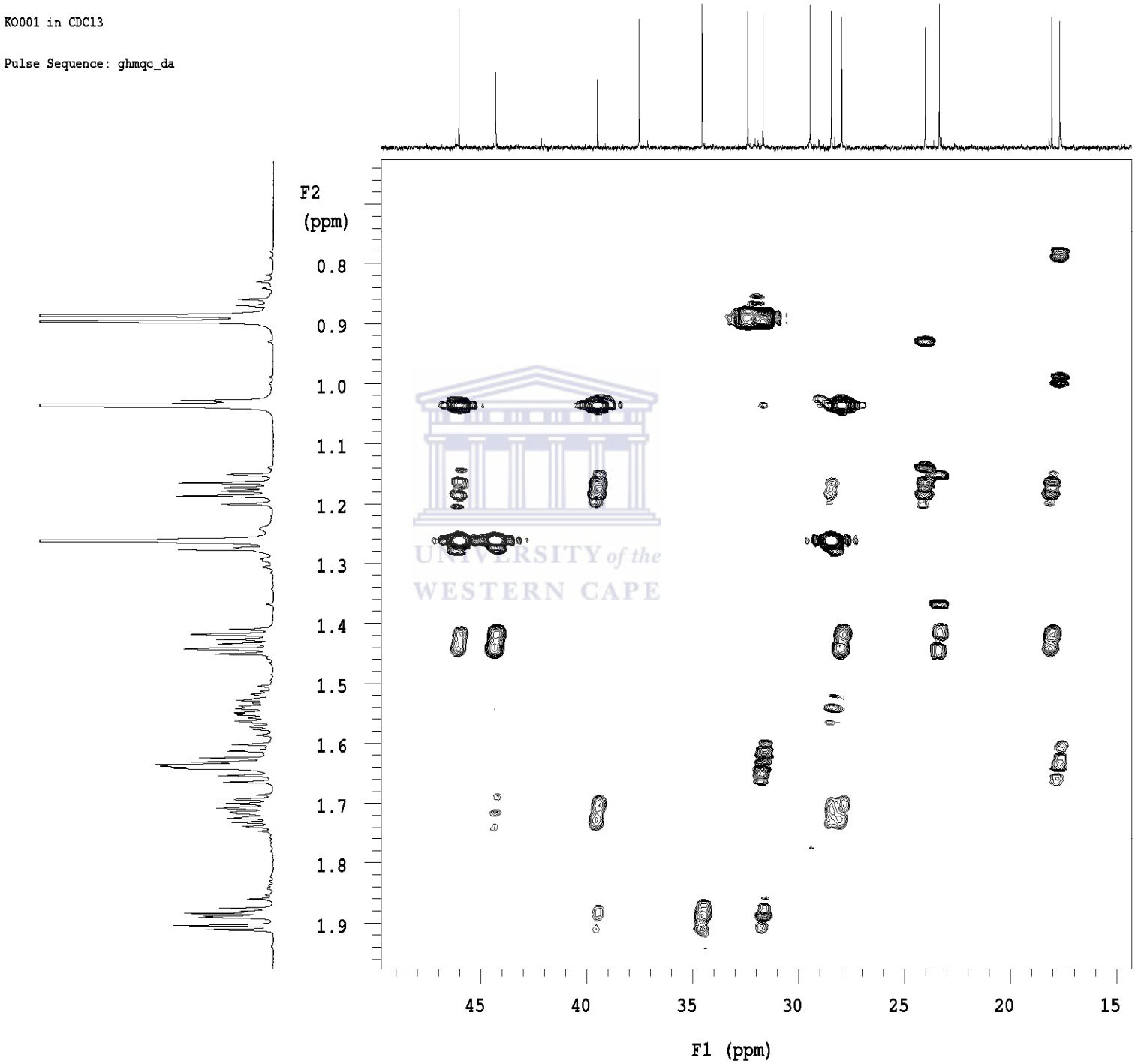
APPENDIX XXVI: gHSMQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



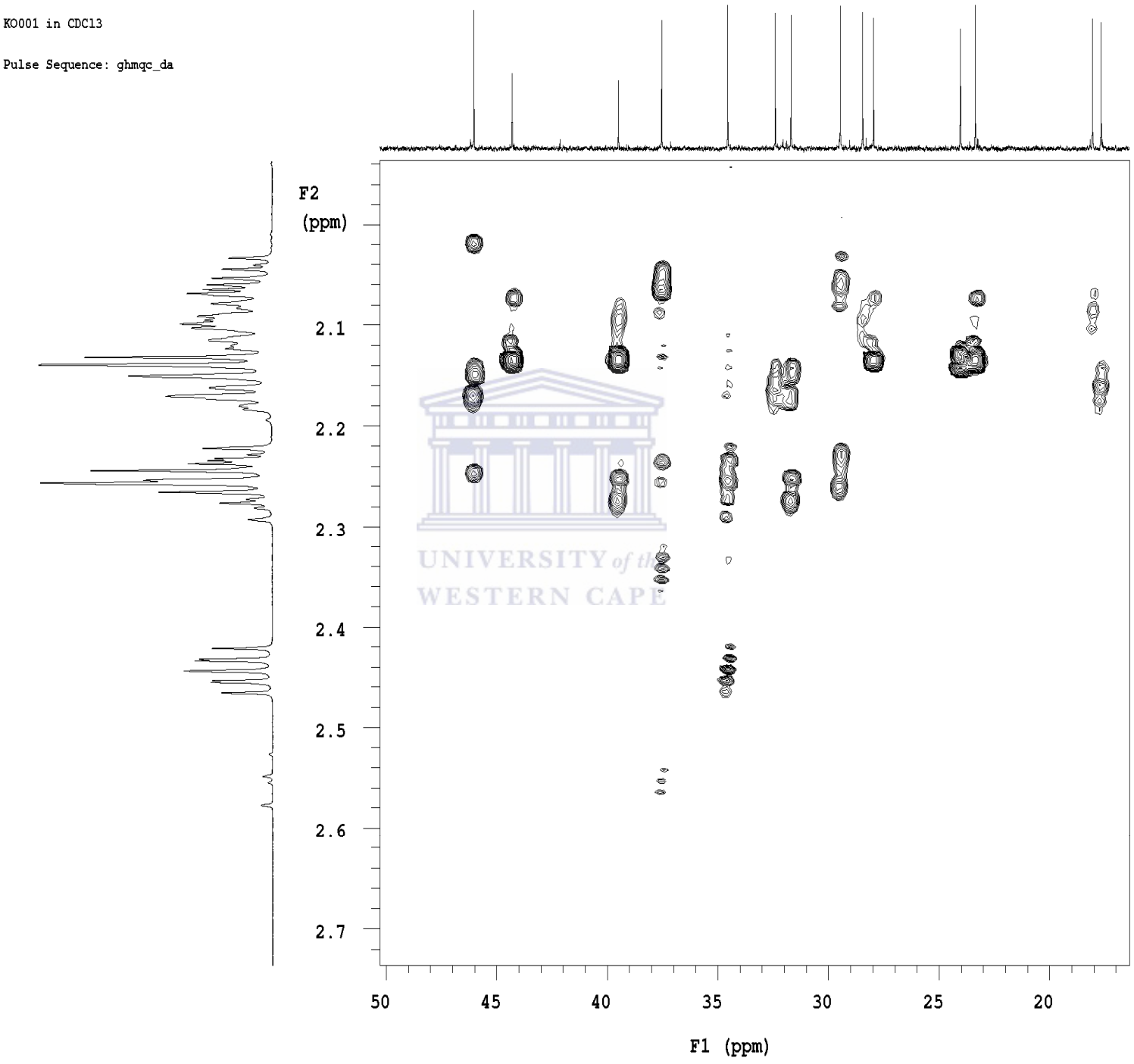
APPENDIX XXVII: gHSMQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



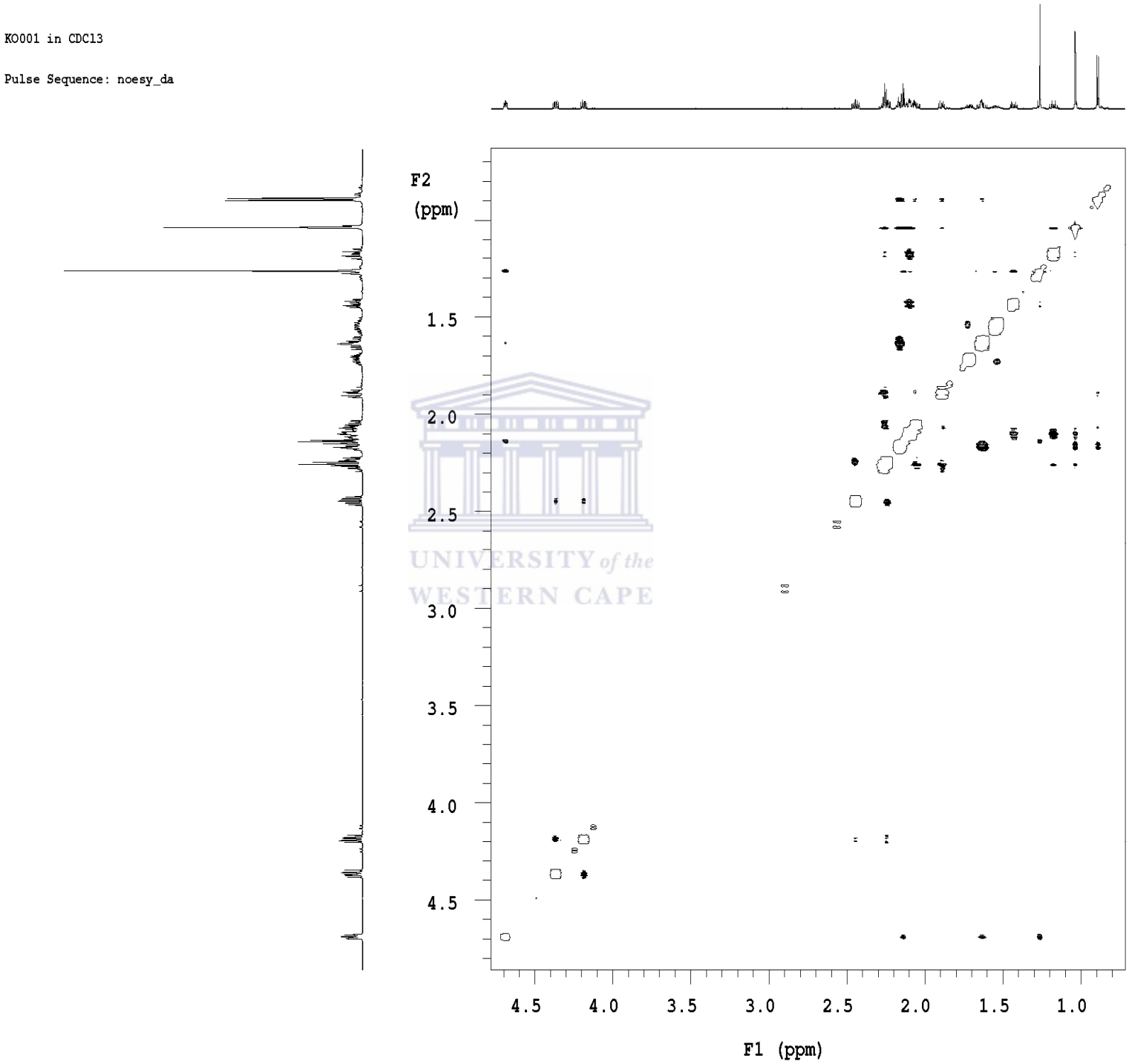
APPENDIX XXVIII: gHSMQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



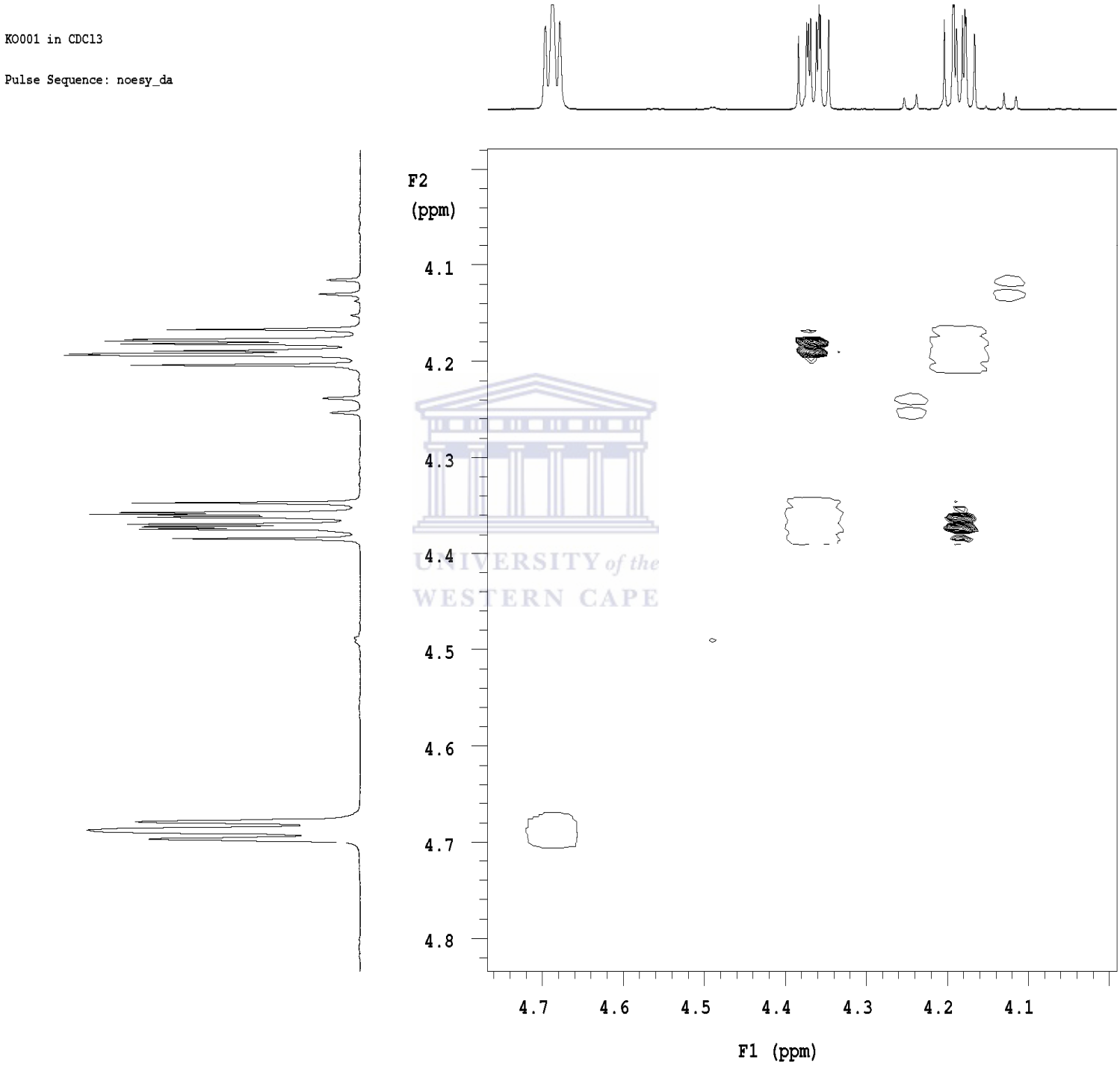
APPENDIX XXIX: gHSMQC spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



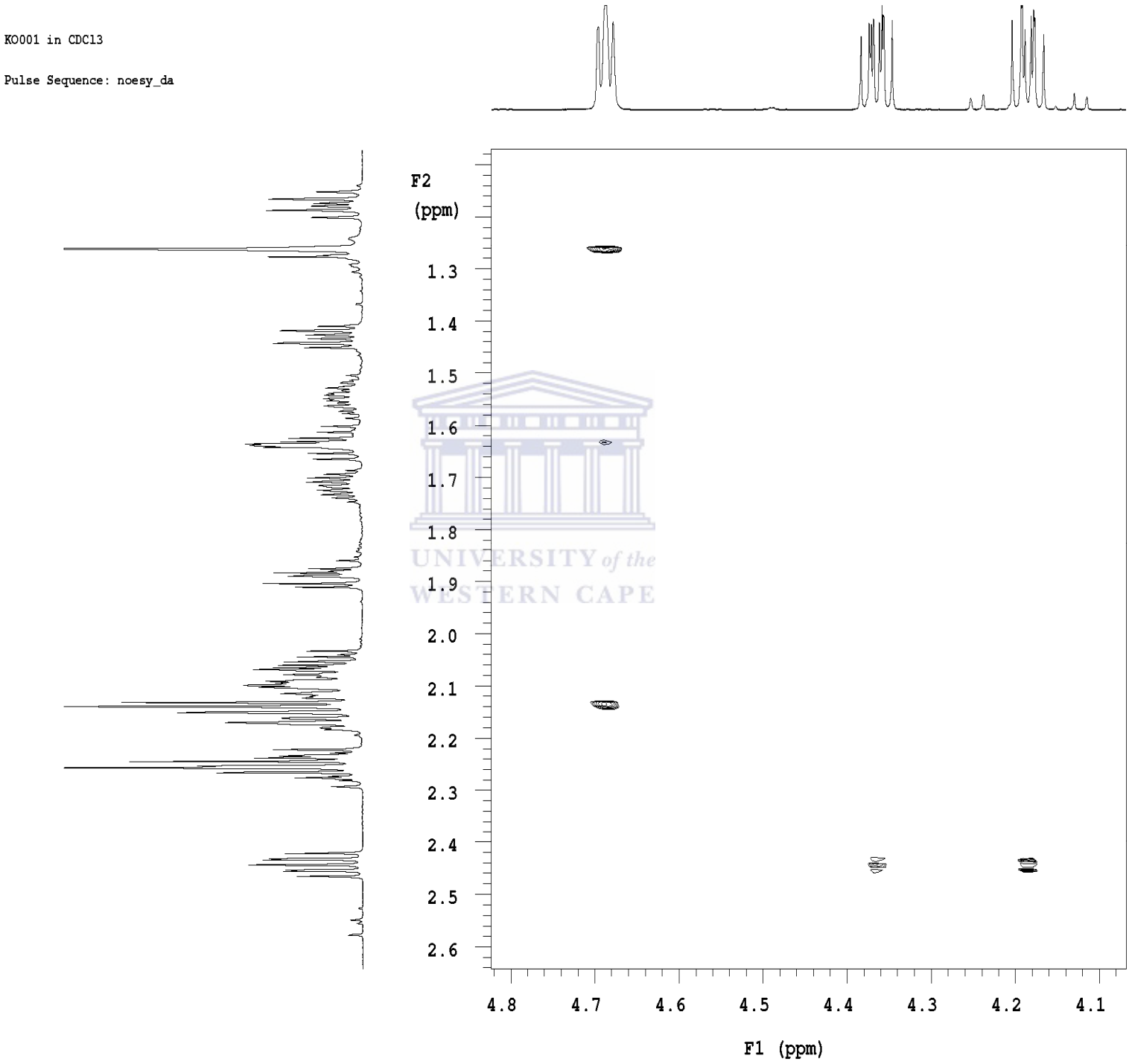
APPENDIX XXX: NOESY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



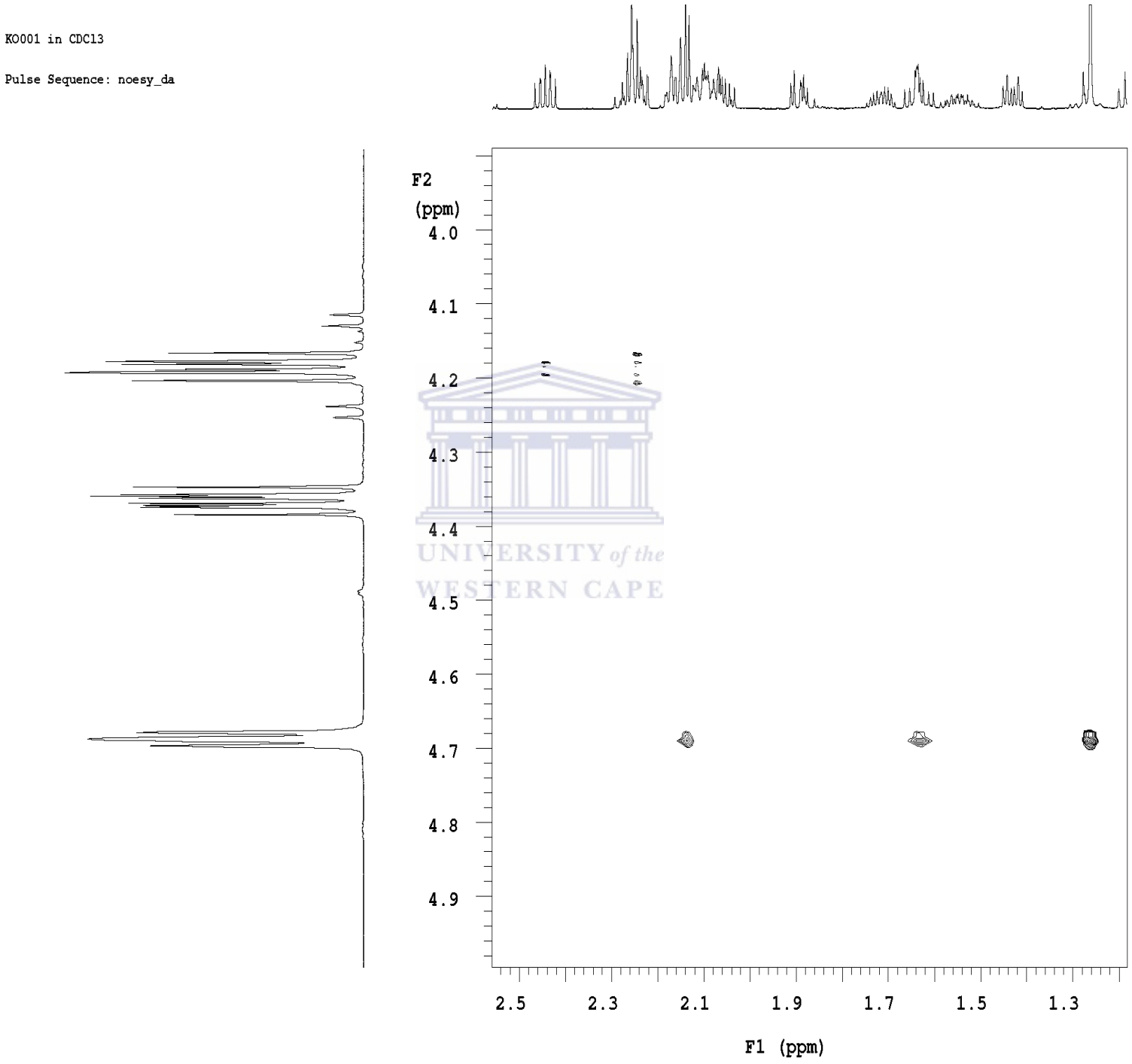
APPENDIX XXXI: NOESY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



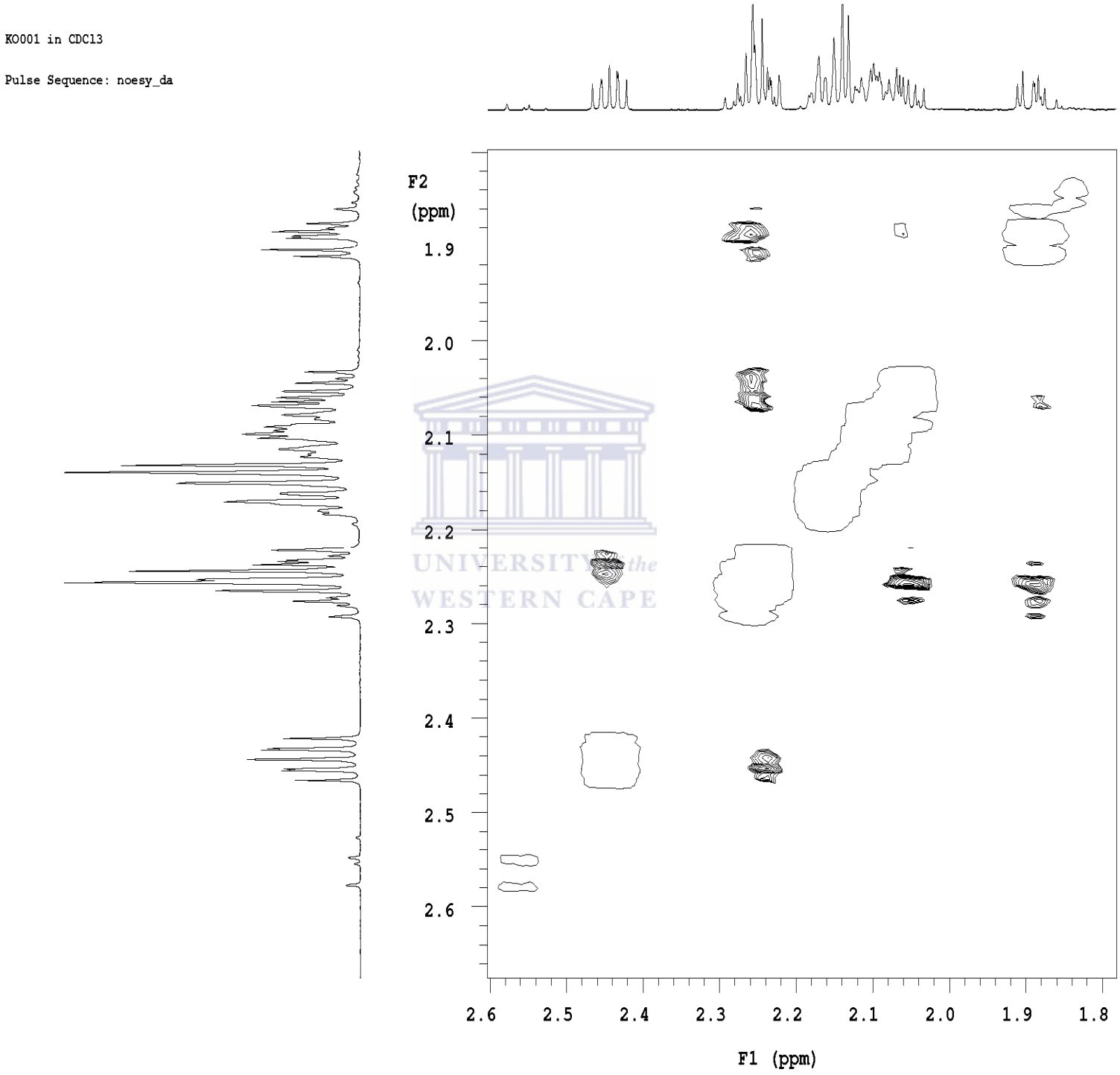
APPENDIX XXXII: NOESY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



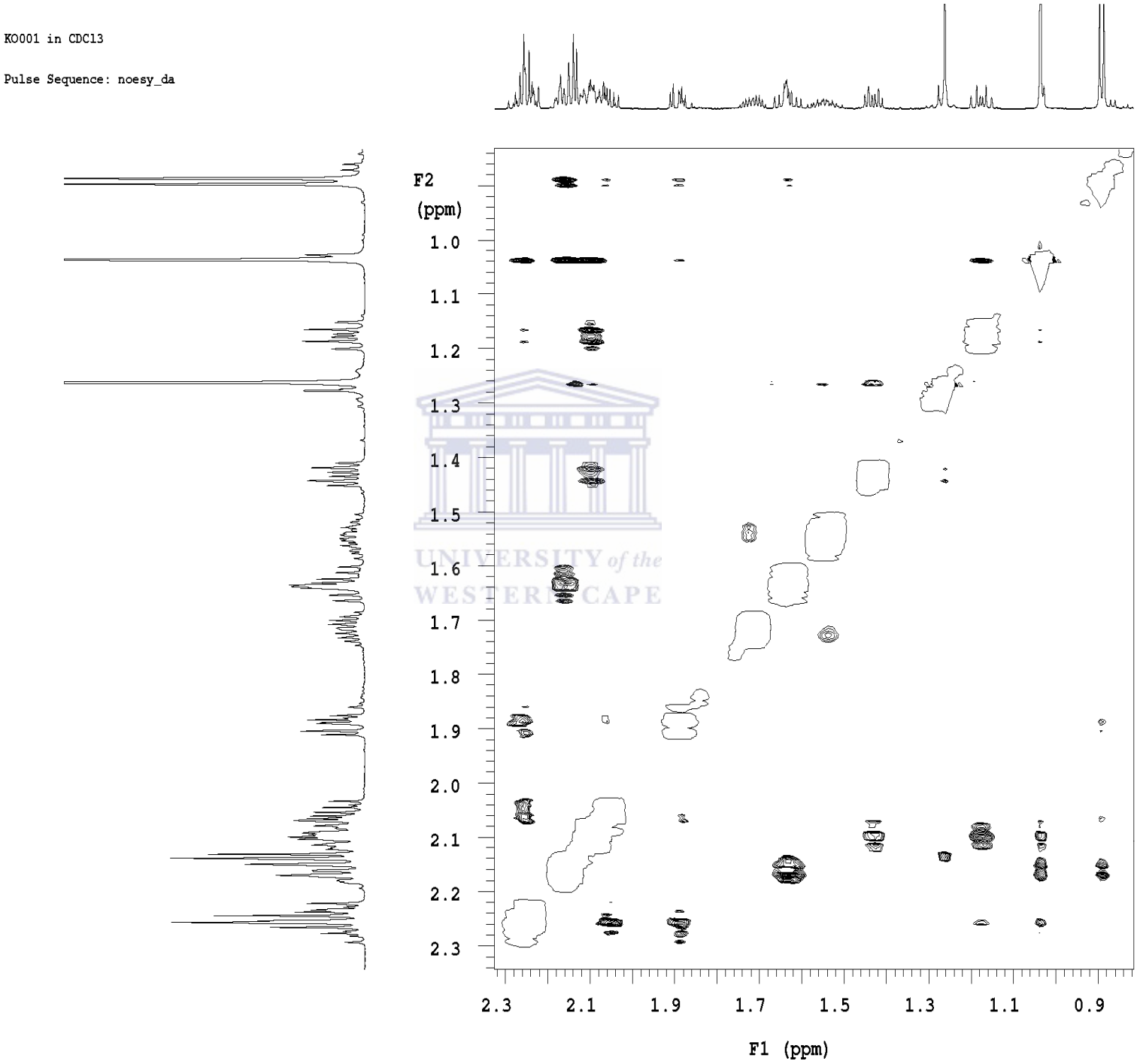
APPENDIX XXXIII: NOESY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



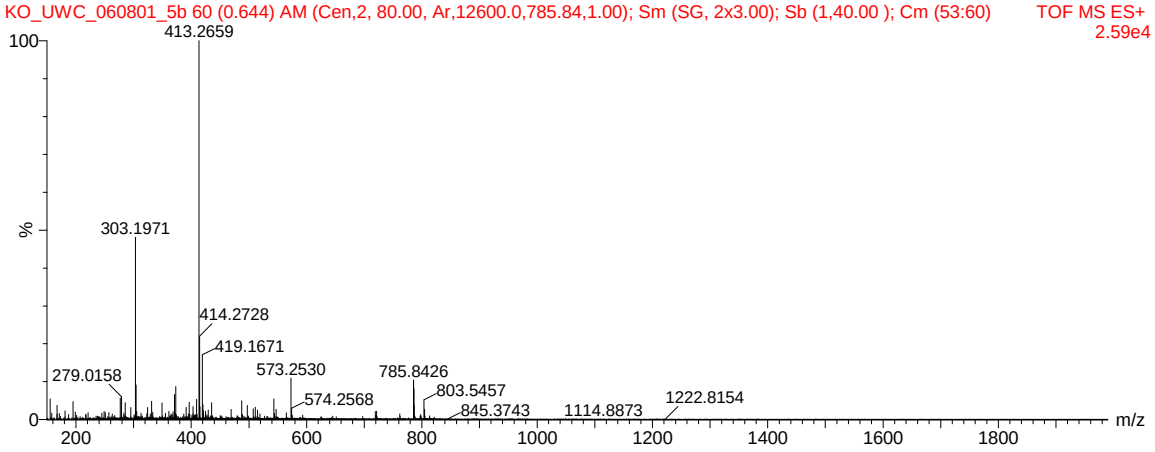
APPENDIX XXXIV: NOESY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

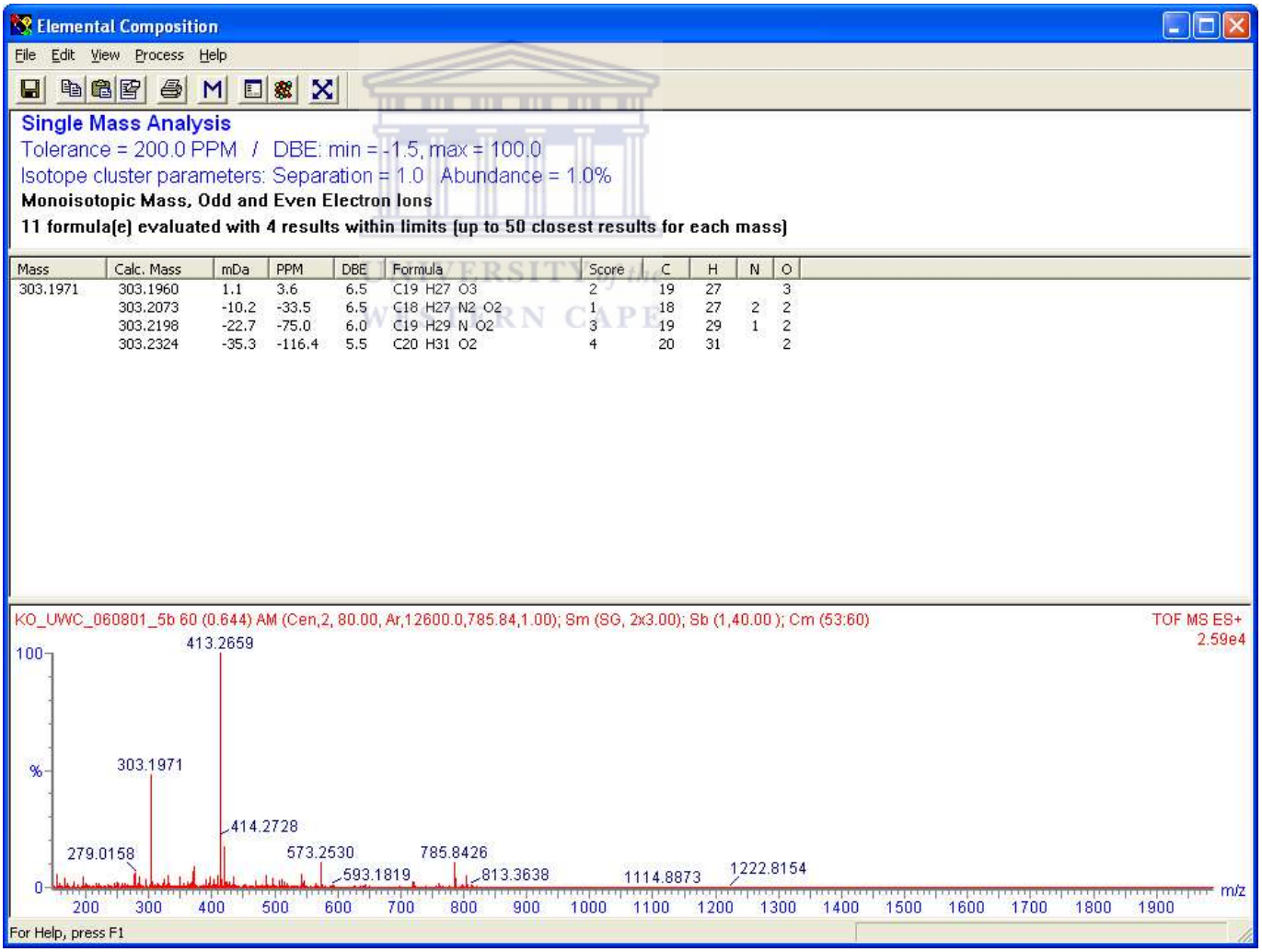


APPENDIX XXXV: NOESY spectrum for (13S)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone



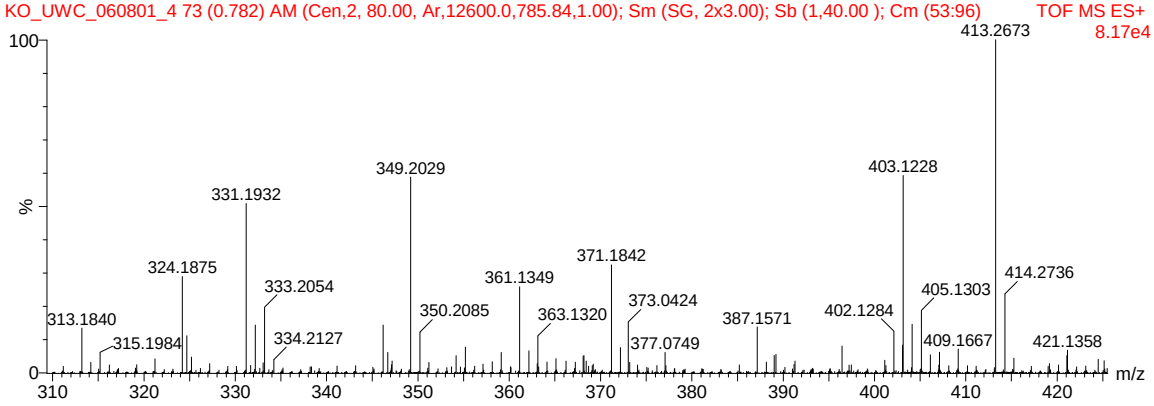
APPENDIX XXXVI: MS spectra for (13*S*)-9α,13α-epoxylabda-6β(19),15(14)diol dilactone

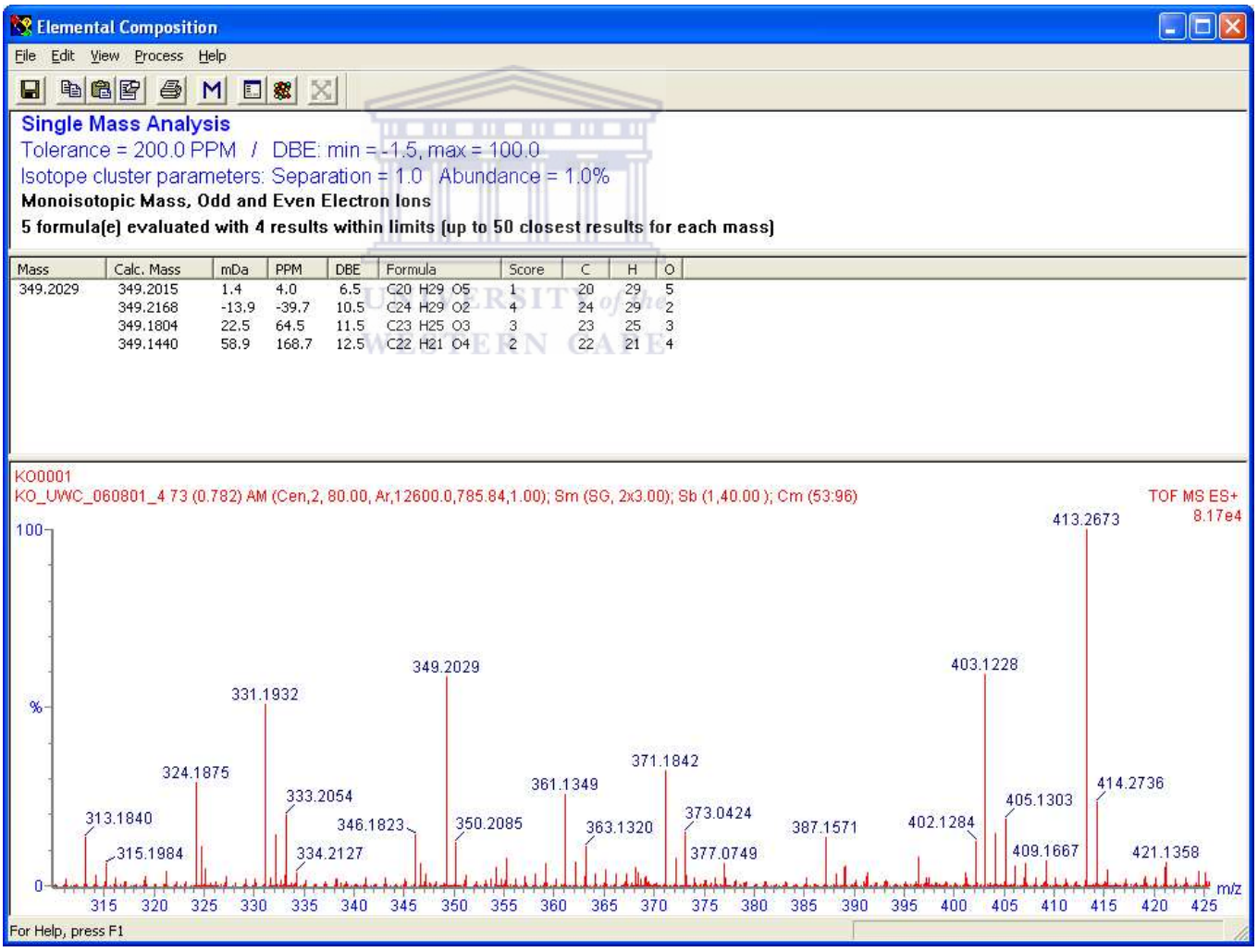


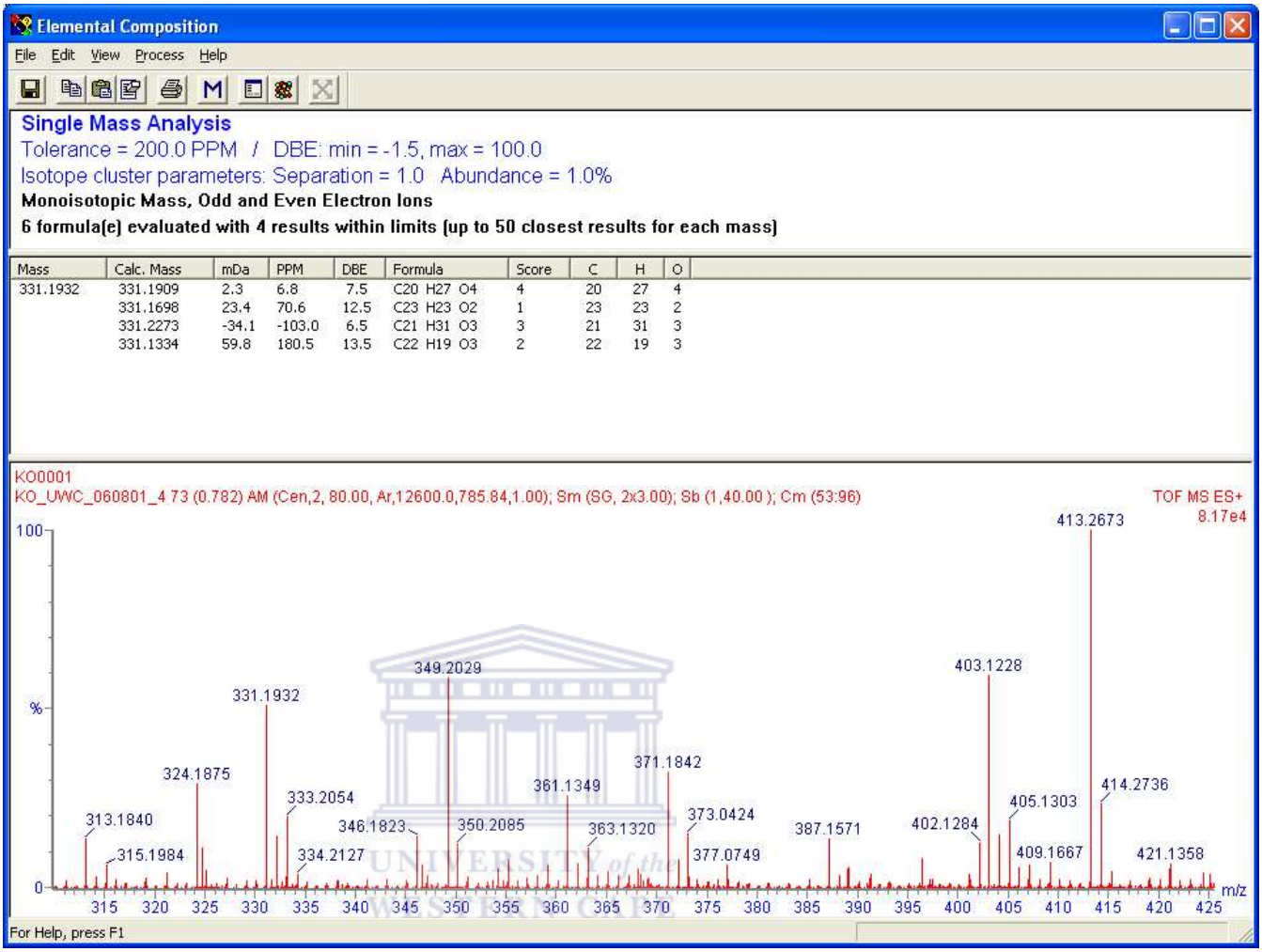


KO0001

KO_UWC_060801_4 73 (0.782) AM (Cen,2, 80.00, Ar,12600.0,785.84,1.00); Sm (SG, 2x3.00); Sb (1,40.00); Cm (53:96) TOF MS ES+ 8.17e4






Elemental Composition
File Edit View Process Help
Single Mass Analysis
Tolerance = 200.0 PPM / DBE: min = -1.5, max = 100.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%
Monoisotopic Mass, Odd and Even Electron Ions
6 formula(e) evaluated with 4 results within limits (up to 50 closest results for each mass)
Mass Calc. Mass mDa PPM DBE Formula Score C H O
331.1932 331.1909 2.3 6.8 7.5 C20 H27 O4 4 20 27 4
331.1698 23.4 70.6 12.5 C23 H23 O2 1 23 23 2
331.2273 -34.1 -103.0 6.5 C21 H31 O3 3 21 31 3
331.1334 59.8 180.5 13.5 C22 H19 O3 2 22 19 3
KO0001
KO_UWC_060801_4 73 (0.782) AM (Cen,2, 80.00, Ar,12600.0,785.84,1.00); Sm (SG, 2x3.00); Sb (1,40.00); Cm (53:96)
TOF MS ES+
8.17e4
100
%
0
413.2673
403.1228
349.2029
331.1932
371.1842
324.1875
361.1349
414.2736
405.1303
333.2054
373.0424
313.1840
346.1823
350.2085
363.1320
387.1571
402.1284
315.1984
334.2127
377.0749
409.1667
421.1358
m/z
315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425
For Help, press F1