Identification of Steroidal Glycosides from South African Hyacinthaceae Species

Dissertation submitted in fulfillment of the requirement for the degree

Master of Science

by

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DECLARATION

The experimental work described in this dissertation was carried out in the School of Chemistry and Physics, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Fanie R. van Heerden.

I hereby declare that these studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where the use of published information from other authors has been made, it is duly acknowledged in the text.

Signed:…… [redacted] ……

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Signed:…….

Professor Fanie R. van Heerden (Supervisor)

ABSTRACT

Steroidal glycosides isolated from natural products have shown great bioactivity and anticancer potential. Previous studies showed that the *Ornithogalum* genus of Hyacinthaceae family is one of the sources of steroidal glycosides with vast structural diversity. Therefore, in search of cytotoxic steroidal glycosides from South African species, two *Ornithogalum* plant species were investigated. Phytochemical investigation of *Ornithogalum saundersiae* Baker bulbs afforded three undescribed steroidal glycosides namely 3-[(β-D-glucopyranosyl)oxy]-22-[α-L-rhamnopyranosyL-(1→2)-β-D-glucopyranosyl)oxy]-16,23-epoxy-23-(2-methyL-1-propenyl) cholestane triglycoside (**170**), (**171**), and (**172**). LC-MS-MS study conducted on bulb crude extract revealed a significant number of unidentified compounds. Liver cell viability assays on *O. saunderisae* crude extracts and isolated compounds reveal moderate cytotoxicity effects for **171** (IC_{50} = 25 µg/mL) and **172** (IC_{50} = 115 µg/mL). Negligible effects were shown for compound **170**. Phytochemical investigation of *Albuca batteniana* Hilliard & B.L.Burtt. resulted in the isolation of one previously known compound named 3-*O*-β-D-glucopyranosyL-β-sitosterol (**175**) exhibiting some cytotoxic effects on liver cells (IC_{50} =275 µg/mL). All the structures of isolates were elucidated by extensive spectroscopic analysis.

ACKNOWLEDGEMENTS

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To my loving parents and siblings, I appreciate your continued love and support. It is through them, I gained the strength to carry on.

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I would like to extend my appreciation to colleagues in the natural products research group and friends who have become family in the chemistry department. I will forever cherish the great moments together, shared knowledge, cheerful learning environment, and support during my time at the University of KwaZulu-Natal (UKZN).

Lastly, I am thankful for the financial support from National Research Fund (NRF).

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ABBREVIATIONS

°C	Degrees Celsius
ActD	Actinomycin D
AIDS	Acquired immunodeficiency syndrome
BLMs	Bleomycin
^{13}C	Carbon-13
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
$CHCl_3$	Chloroform
CPT-11	Irinotecan
CPT	Camptothecin
COSY	Homonuclear correlation spectroscopy
d	Doublet
DEPT	Distortionless enhancement by polarisation transfer
DNA	Deoxyribonucleic acid
EtOAc	Ethyl acetate
g	Gram(s)
G_2-M	Second growth phase of metastasis
^1H	Proton
h	Hour(s)
H_2O	Water
HepG2	Hepatoma G2 cells
HIV	Human immunodeficiency virus
HL-60	Human leukemia
HMBC	Heteronuclear multiple-bond correlation
HR-ESI-MS	High-resolution electrospray ionization – mass spectrometry
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single-quantum correlation
Hz	Hertz
IC_{50}	Half-maximal inhibitory concentration
IR	Infrared

LC-MS-MS	Liquid chromatography with tandem mass spectrometry
m	Multiple
M	Molar
microRNA	Small single-stranded non-coding ribonucleic acid
MeOH	Methanol
MMC	Mitomycin C
NMR	Nuclear magnetic resonance
OSW-1	17α-Hydroxy-16β-[(*O*-(2-*O*-*p*-methoxybenzoyL-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyL-α-L-arabinopyranosyl)oxy]cholest-5-en-22-one
PPM	Parts per million
PTX	Paclitaxel
q	Quartet
R_f	Retention factor
s	Singlet
SN-38	7-EthyL-10-hydroxycamptothecin
t	Triplet
TLC	Thin-layer chromatography
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1. BACKGROUND

Cancer is a collective term describing a group of diseases caused by the rapid replication of abnormal cells that grow beyond their normal boundaries. Clusters of these cells cause malignant tumours and neoplasms.[1] Malignant tumours may grow on almost any body part, including soft tissues, body fluids, and organs. The malignant tumours then replicate and spread to other parts of the body in a process called metastasizing, creating the global problem of cancer that we have faced since the dawn of recorded history.[2]

This deadly disease is currently the leading cause of death in developed countries and amongst the leading causes of death following heart diseases and infectious diseases such as HIV/AIDS, tuberculosis, and malaria in developing countries.[3] According to the World Health Organisation (WHO), nearly 10 million deaths worldwide were caused by cancer in 2020.[4] The numbers of reported cases for common cancers are:

- Breast cancer – 2.26 million
- Lung cancer – 2.21 million
- Colorectal cancer – 1.93 million
- Prostate cancer – 1.41 million
- Skin cancer (non-melanoma) – 1.20 million
- Stomach cancer – 1.09 million

The most common causes of death worldwide due to cancer are:

- Lung cancer – 1.8 million
- Colorectal cancer – 935 000
- Liver cancer – 830 000
- Stomach cancer – 769 000
- Breast cancer – 685 000

Cancer is mainly a genetic disease. Genetic changes in an individual's body affect how the body creates and multiplies cells, which may lead to cancer.[5] These changes may be inherited from one generation to the next but are also often influenced by the environment.[5] Exposure

to ionizing and ultraviolet radiations, chemical carcinogens such as tobacco smoke, biological carcinogens (viral infections), and food and water contaminants are amongst the leading environmental causes of cancer.[5]

There are more than 100 known types of cancer.[4] The type of cancer is classified according to the kind of genetic alterations that bring about cancer. The tumour is named after the tissue or organ it primarily grows on. In the process of metastasis, where cancer spreads to other parts of the body, the type of tumour remains the same as the original, and so does the name. Most cancers are carcinoma, sarcoma, leukemia, lymphoma, multiple myeloma, melanoma, brain, and spinal cord tumours.[3] These cancers are further described according to the body part the tumour invades, such as breast cancer, lung cancer, prostate cancer, etc.

At least 20% of malignant tumours are due to inflammation.[6] Inflammation is the normal protective response to any irritant or tissue repair in a living organism.[7] Inflammation is a positive response in the body. However, many environmental and lifestyle changes may cause the immune system to falsely trigger inflammation.[8] This happens when there is long-term oxidative stress (imbalances between the antioxidants produced and free radicals).

Animals and other living organisms produce antioxidants that protect cells against free radicals (reactive oxygen species) by chelating with catalytic metals, reacting with the free radicals, or acting as oxygen scavengers.[9] In that way, antioxidants prevent oxidative damage. Environmental changes, lifestyle, and diet may lead to the overproduction of reactive oxygen species. At the same time, the production of antioxidants remains the same, leading to reactive oxygen species overpowering the insufficient antioxidants.[9] The continuous generation of the reactive oxygen species without detoxification leads to oxidative stress that damages proteins, lipids, and DNA, thereby triggering chronic inflammation and causing several chronic diseases, including diabetes mellitus, cancer, arthritis, atherosclerosis, and neurodegenerative diseases.[10]

The fight against cancer led to the development of several compounds as anticancer drugs, also called antineoplastic or chemotherapeutic agents. The chemotherapeutic agents are used as single-drug therapy or in combination therapy, depending on the type of cancer being treated.[11] The different kinds of chemotherapeutic agents are alkylating agents (**Fig. 1.1**), which include cisplatin (**1**), chlorambucil (**2**), procarbazine (**3**), and carmustine (**4**);

antimetabolites (**Fig. 1.2**), including methotrexate (**5**), cytarabine (**6**), and gemcitabine (**7**); anti-microtubule agents (**Fig. 1.3**) such as vinblastine (**8**) and taxol (**9**); cytotoxic agents (**Fig. 1.4**) such as bleomycin (**10**) and mitomycin (**11**); and topoisomerase inhibitors (**Fig. 1.5**), including etoposide (**12**) and doxorubicin (**13**).[12,13]

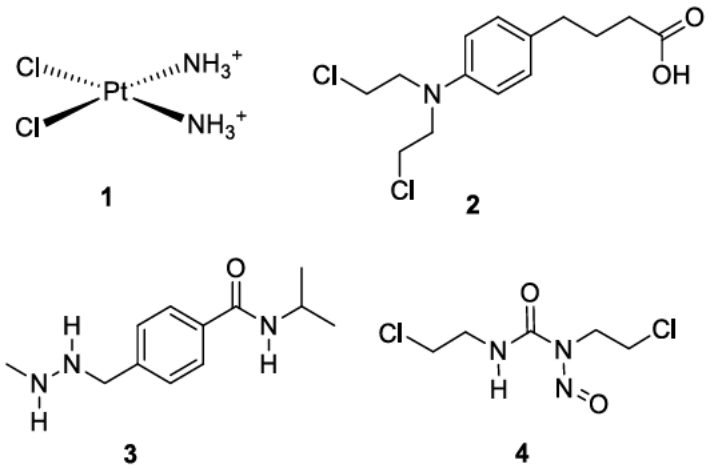


Figure 1.1. Structures of alkylating agents.

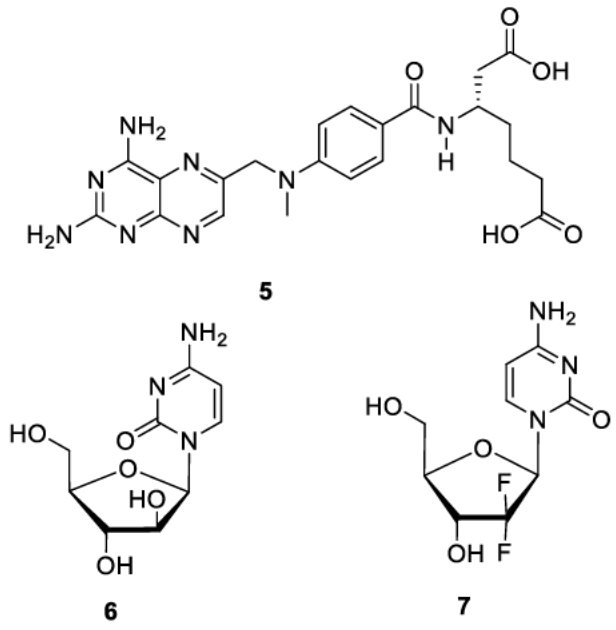


Figure 1.2: Structures of antimetabolites.

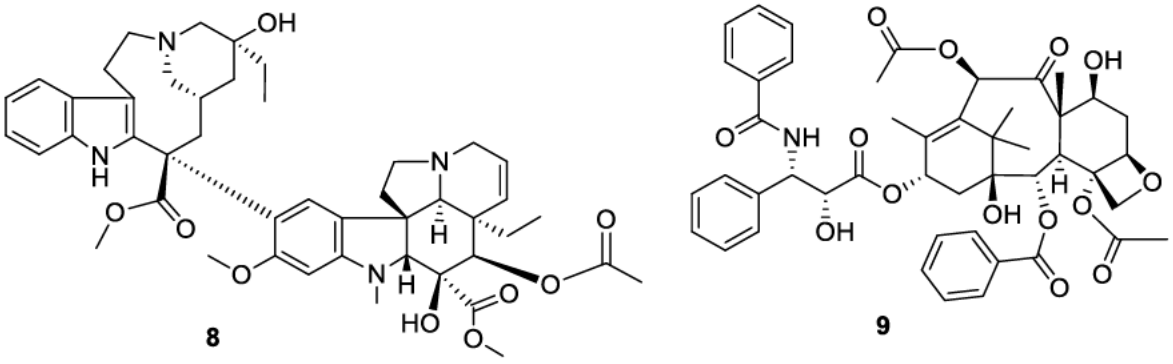


Figure 1.3. Structures of anti-microtubule agents.

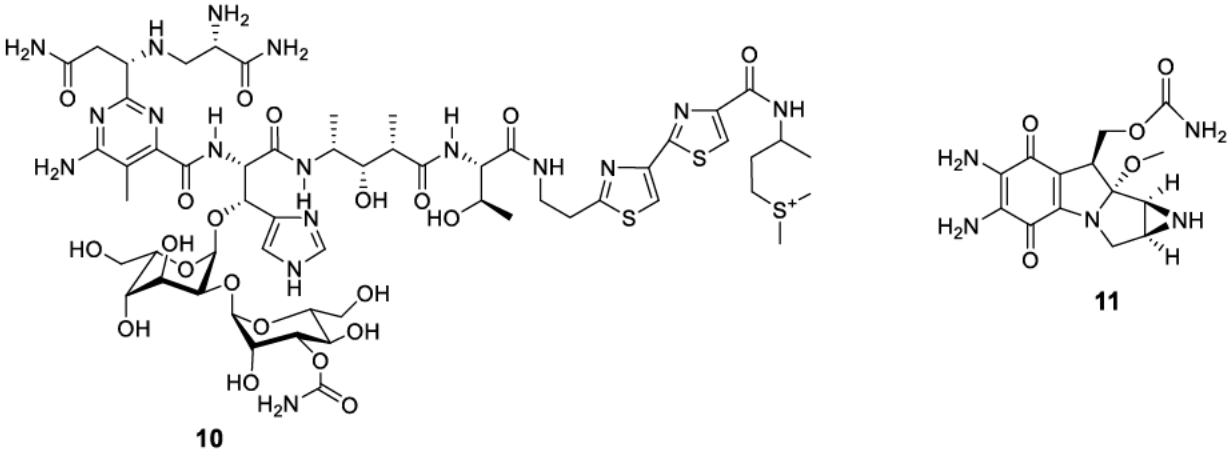


Figure 1.4: Structures of cytotoxic agents.

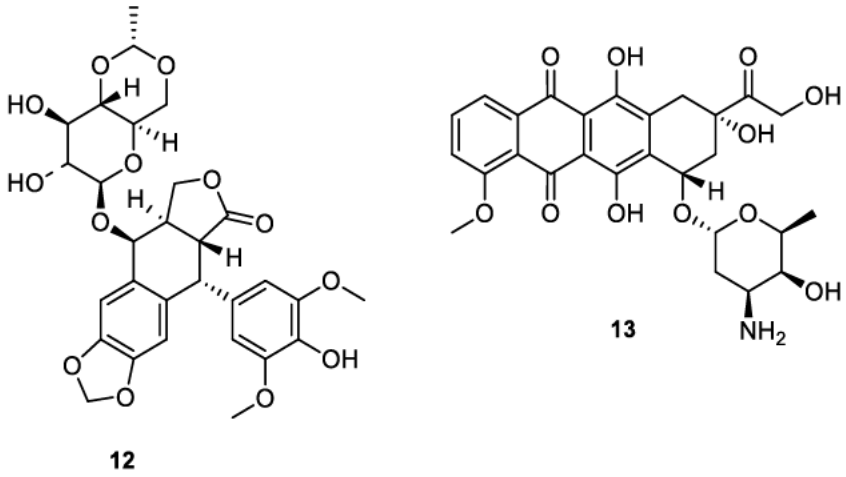


Figure 1.5. Structures of topoisomerase inhibitors.

Therapies different from chemotherapy are also used, e.g. surgery and immunotherapy, but these treatments are expensive. The majority of anticancer agents used do not accumulate at the tumour site and cause severe side effects such as hair loss, nausea, anemia, etc.[14]

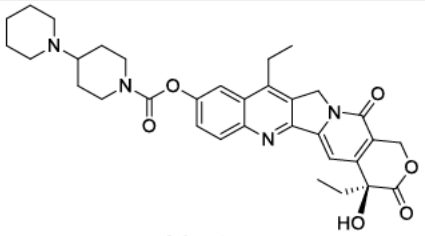
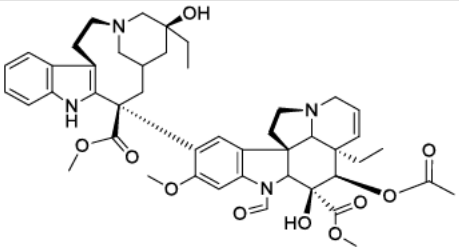
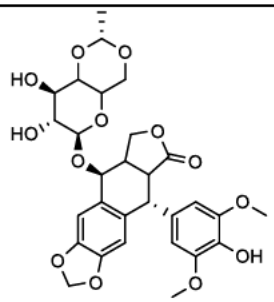
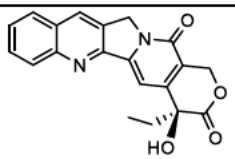
Considering the number of deaths cancer is responsible for, there is an urgent need for new and cheap anticancer agents. Many novel drugs are based on the isolation of compounds from natural organisms.[15] Natural products are often structurally complex compounds with multiple stereocentres, oxygen atoms, and ring systems that make them more target selective compared to synthetic compounds.[16]

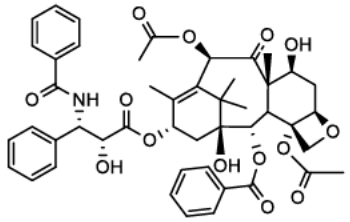
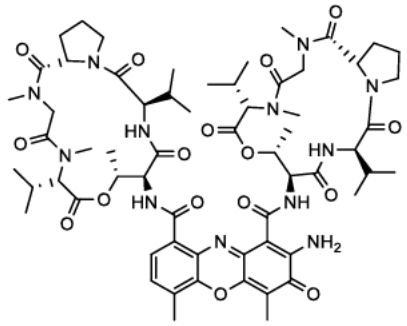
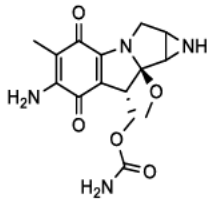
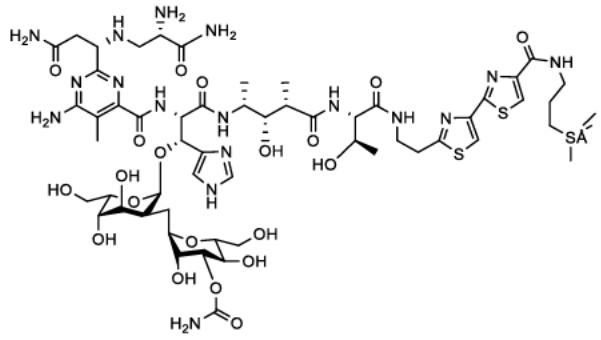
1.2. NATURAL PRODUCTS IN THE DEVELOPMENT OF CANCER TREATMENTS

Natural products refer to secondary metabolites in plants, microbes, and other living organisms.[17] Secondary metabolites are compounds generally specific to a species and are involved in the organism's survival. These organisms are usually active components in medicinal plants used in traditional medicine. Many secondary metabolites have been developed as pharmaceutical drugs due to the bioactivity of these compounds.[18] Newman and Cragg (2020) estimated that 64% of small molecule drugs that came onto the market in the United States of America during the timespan 1981-2019 are natural products or synthetic compounds based on natural products.[19] Newman and Cragg (2020) also reported that of the smalL-molecule antitumour agents developed from 1946 to 2019, 84% are natural products, natural product derivatives, synthetic natural product mimics, or natural product-inspired synthetic compounds. As such, natural products contributed significantly to developing anticancer drugs.[19] In **Table 1.1.**, some natural products currently used for cancer treatment are collated.

Vincristine, etoposide, paclitaxel, and irinotecan are some natural products isolated from plants that are still used in treating cancer.[20] Despite the promising potential of natural products, modern-day research has shifted focus to synthesizing small molecules and exploring synthetic compound libraries.[20,21] This shift was influenced by the difficulties in cultivating large amounts of natural product sources, isolating compounds, and developing these complex structures into drugs.[20]

Table 1.1. Natural products that are currently used for the treatment of cancer.

Secondary metabolites	Description
 Irinotecan Campto® or Camptosar®	Irinotecan (CPT-11) is a semi-synthetic analogue of the compound camptothecin isolated from *Camptotheca acuminate*. Irinotecan has shown great antitumour activity against human colon cancer cells and was approved in 1994 to treat cancer. The active irinotecan metabolite is 7-ethyL-10-hydroxycamptothecin (SN-38).[22,23] The mechanism of action is by interfering with the enzyme topoisomerase I, which controls the manipulation of the structure of DNA needed for replication. The interference results in breaks in normal double-stranded DNA and causes cell death.[22]
 Vincristine	Vincristine is an alkaloid isolated from the Madagascan plant *Vinca rosea* Linn. In the early 1960s.[24] It showed antitumour activity against advanced lymphosarcoma, reticulum cell sarcoma, Hodgkin's disease, breast cancer, bladder cancer, and various other sarcomas.[25] Vincristine was approved in 1963 for leukaemia treatment and is now also used to treat Hodgkin's disease, non-Hodgkin's lymphomas, breast cancer, small cell lung cancer, neuroblastoma, rhabdomyosarcoma.[26] The mechanism of action is by inhibiting tubulin polymerisation.[26]
 Etoposide	Etoposide is a semi-synthetic derivative of a toxin (podophyllotoxin) isolated from the American mayapple (*Podophyllum peltatum*) and was approved in 1983 to treat cancer.[27] Etoposide's mechanism of action is by binding to DNA topoisomerase II, thereby affecting different stages of cell metabolisms.[27]
 Camptothecin Hycamtin®	Camptothecin (CPT) was first isolated from a Chinese tree, *Camptotheca acuminate*, in 1958. The mechanism of action of CPT is by inhibiting DNA synthesis, causing cell death in the S-phase of the cell.[28] CPT was approved for treating ovarian, iqui-cell lung, and colorectal cancers and its analogue irinotecan.

 Paclitaxel Taxol®	Paclitaxel (PTX) was isolated from the bark of the Pacific yew tree, *Taxus brevifoli*, in 1971. It is also present in *T. baccata* and *T. cuspidate*. It was approved in 1992 and commercialized as an antineoplastic agent to treat ovarian cancer, Kaposi's sarcoma, breast cancer, advanced ovarian cancer, and microcytic lung malignant neoplasm. PTX is also used in the treatment of various other cancers, including prostate cancer, bladder cancer, cervical cancer, gastric cancer, head and neck cancer, endometrial malignancy, oesophageal cancer, brain oligodendroglioma, and testicular cancer.[29]
 Actinomycin D	Actinomycin D (ActD) is an antineoplastic agent isolated from the micro-organism *Streptomyces antibioticus*.[30] ActD was approved in 1964 for the treatment of several types of cancer.
 Mitomycin C	Mitomycin C (MCC) was first isolated from *Streptomyces caespotosus* by Japanese scientists in the 1950s, and mitomycin C was approved in the USA in 1974 for the treatment of bladder cancer. It has also shown activity against breast cancer, non-smalL-cell lung cancer, and head and neck cancer.[31] MMC is a highly active compound against a range of neoplastic diseases. However, it is now overlooked as the focus has shifted to new chemotherapeutic agents.
 Bleomycin	Bleomycin (BLMs) refers to a group of antibiotics derived from *Streptomyces verticillus*. Bleomycin displays activity against Lewis lung carcinoma.[32,33] Bleomycin is mainly used to treat brain tumours (glioblastomas and astrocytomas).

1.2.1 Steroidal glycosides as anticancer drugs

Recent studies on steroidal glycosides have led to the interest in these compounds by the pharmaceutical industry. Steroidal glycosides were discovered initially due to the poisonous nature of some plants from which they were isolated.[34] Naturally occurring plant glycosides are called saponins and are found in more than 100 plant families with about 150 different kinds of natural saponins that display anticancer activity.[35]

The aglycone of a saponin (soap-like compound) can be a triterpene or steroid (**Fig. 1.6**), and the glycone is usually an oligosaccharide.[36] There are 11 classes of aglycones in natural saponins, which are cucurbitanes, cycloartanes, dammaranes, hopanes, lanostanes, lupanes, oleananes, steroids, taraxasteranes, tirucallanes, and ursanes.[37] The presence of steroidal saponins was reported for Agavaceae, Dioscoreaceae, Liliaceae, Solanaceae, Scrophulariaceae, Amaryllidaceae, Leguminosae, and Rhamnaceae plant families.[38] A significant number of reviews have been written on the biological and pharmacological activities of steroidal saponins, including their cytotoxic and antitumour activity. Biological activities observed for saponins are influenced by slight differences in structures.[38]

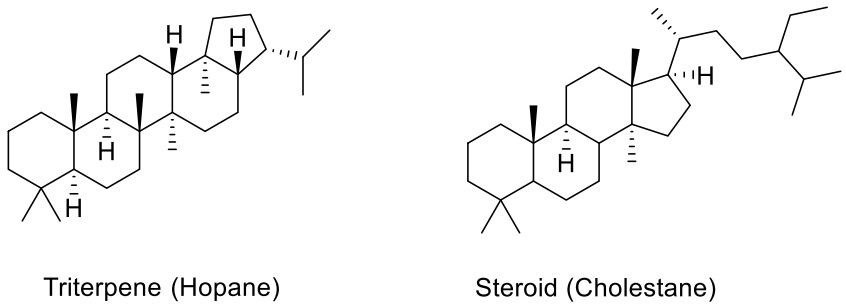


Figure 1.6. Some aglycone structures of saponins.

The structural changes of steroidal saponins that influence antitumour activities are:

- The sites of hydroxy groups on the aglycone
- The number of hydroxy groups on the aglycone
- The type of sugar linkage
- The lipophilicity of the sugar
- The number of sugar moieties
- The kind of sugar sequences.

Amongst the acylated glycosides, a steroidal cholestane diglycoside, 17α-hydroxy-16β-[(*O*-(2-*O*-*p*-methoxybenzoyL-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyL-α-L-arabinopyranosyl)oxy] cholest-5-en-22-one, named OSW-1 (**Figure 1.7**), showed great cytostatic activity against several malignant tumours.[39] OSW-1 was isolated from the bulb extract of a South African plant, *Ornithogalum saundersiae,* in 1992 by Japanese scientists and was identified as the main cytotoxic compound in the bulb in 1997 through a cytotoxicity-guided fractionation.[40] The cytotoxic-guided fractionation was conducted after highly potent cytotoxic activity against HL-60 human promyelocytic leukemia cells was observed for the *O. saundersiae* crude extract.[40,41]

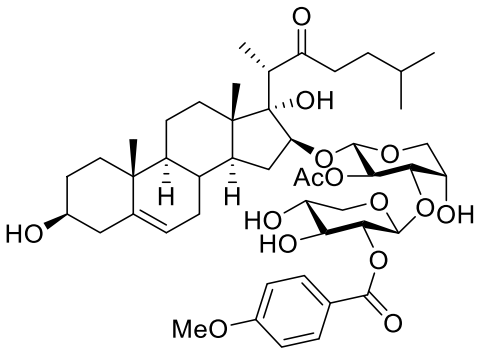


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Figure 1.7. Structure of OSW-1.

After that, OSW-1 received significant attention due to the cytotoxic activity against various cancer cell lines, including mouse mastrocarcinoma, human pulmonary adenocarcinoma, human pulmonary large cell carcinoma, human pulmonary squamous cell carcinoma, and leukemia cells.[39] Due to the complex structure of OSW-1, the key anticancer targets differ for different types of cancer cells with consistent activity with IC_{50} values in the nanomolar concentration range.[18] Different signalling pathways are followed to induce apoptosis.

OSW-1 has been proven to induce apoptosis in leukemia, breast cancer, colon cancer, pancreatic cancer, and cervical cancer. The most impressive feature of OSW-1 was that it showed less toxicity towards normal human fibroblasts while being about 10-100 times more potent to cancer cells than most clinical anticancer agents, including iquidate, cisplatin, camptothecin, mitomycin C, and taxol.[42]

The observed biological activities inspired the approaches to the synthesis of OSW-1 and its analogues.[34] The first chemists to synthesize OSW-1 were Fuch and Guo, who published the synthesis of the OSW-1 aglycone in 1998.[43] Yu and co-workers (1999) followed with the total synthesis of OSW-1 by coupling the cholestane aglycone with a disaccharide moiety.[43,44] Other chemists used different methodologies to synthesize OSW-1 and its analogues.[39] Further studies on *O. saundersiae*, *O. thyrsoides*, and *Galtonia candicans* (Hyacinthaceae) resulted in the isolation of different OSW-1 analogues.[41]

Although it is 30 years since the discovery of OSW-1, the specific mechanism of action, selective cytotoxicity to cancer cells, and pharmacokinetics are not completely understood.[18] Major advances have been made with studies on OSW-1 as a potential anticancer drug, and investigations are still ongoing.[18] The activity of OSW-1 against various cancer cell lines is outlined in **Table 1.2.**

Table 1.2. Results of *in vitro* experiments investigating the cytotoxic activity of OSW-1 against various cancer cell lines.[18]

Cell lines	**Efficacy (IC_{50} values)**	**Mechanism of action**
Adriamycin resistant P388 Camptothecin-resistant P388 Carcinoma Human normal pulmonary cell Human pulmonary adenocarcinoma Human pulmonary large cell carcinoma (Lu-65) Human pulmonary large cell carcinoma (Lu-99) Human pulmonary squamous cell Human leukaemia Mouse leukaemia Mouse microcarcinoma	0.88 nM 0.12 nM 0.18 nM 1720 nM 0.78 nM 0.23 nM 0.23 nM 0.30 nM 0.29 nM 0.15 nM 0.18 nM	Mechanism of action not published.[45]
Human leukaemia cells	0.19 nM	The mechanism of action is through damaging the structure and function of mitochondria leading to apoptosis.[46,47]
Human hepatocellular carcinoma cells Hep3B Human colon cancer cell HCT-116	IC_{50} values not published	Disruption of microRNAs is responsible for specific pathways in proliferation, apoptosis, cell adhesion, and migration. It induces apoptosis and necroptosis.[48,49,50]

Human ovarian cancer cell: SKOV-1 (monolayer) OVCAR-1 (monolayer) OVSAHO (monolayer) OVCAR-8 (monolayer) SKOV-3 (spheroids) OVCAR-8 (spheroids)	 4.0 $\pm$ 2.7 nM 2.2 $\pm$ 0.85 nM 1.8 $\pm$ 0.61 nM >1.000 nM 10 nM 100 nM	The mechanism of action is through antiproliferation in the absence of exogenously supplied cholesterol. [51]
Human breast cancer cell: MCF-7 T47D ZR-75-1 BT474 SKBR3 MDA-MB-231 MDA-MB-453 HCC-1937	 3.72 $\pm$ 0.78 nM 5.92 $\pm$ 1.21 nM 10.34 $\pm$ 0.07 nM 6.54 $\pm$ 1.14 nM 6.67 $\pm$ 0.13 nM 5.82 $\pm$ 2.35 nM 8.66 $\pm$ 0.19 nM 11.12 $\pm$ 4.42 nM	Inhibits tumour growth and metastasis by inducing apoptosis and reducing the expression of NFATc2.[52]
Human normal mammary epithelial cell	52.3 $\pm$ 8.72 nM	Mechanism of action not published.[53]
Human cervical cancer HeLa cells	IC_{50} values not published	OSW-1 causes breakage of the Golgi and activates TFE3, which triggers the apoptotic Golgi stress response.[53]
Human promyelocytic leukaemia cells	0.061 $\pm$ 0.0020 nM	The mechanism of action is through a iquidateal-independent signalling pathway. OSW-1 breaks down the DNA, activates caspase 3, and stops the G_2-M DNA damage checkpoint.[54]
Human lung adenocarcinoma cells	0.065 $\pm$ 0.018 nM	Mechanism of action not published [54]
Human colon cancer: LoVo SW480	35.5 $\pm$ 3.0 nM 69.9 $\pm$ 1.2 nM	Mechanisms of action are by inducing intrinsic apoptosis, increasing cellular calcium, changing mitochondrial membrane potential, disrupting mitochondrial morphology as well as releasing cytochrome C.[55]
Human normal colonic mucosal epithelial cells	159 $\pm$ 10.3 nM	Mechanism of action not published.[55]
Human leukaemia cells: HL-60 Raj K-562 KBM5 M1	 Average IC_{50} = 0.019 nM	Inhibits NCX1 and induces apoptosis with a iquidateal-mediated mechanism. [56]
Normal lymphocytes	Average IC_{50} = 1.64 nM	Mechanism of action not published.[56]

1.3. STUDY RATIONALE

The Hyacinthaceae is a large family with 700-900 species distributed through Africa, Europe, and Asia, but with the centre of biodiversity in sub-Saharan Africa. The subfamily Ornithogoloideae, with 200-300 species, is widely distributed in Africa and Eurasia. Within this family, *Ornithogalum* is the principal genus.[57] Several *Ornithogalum* L. species have traditional medicinal uses such as preventing and treating blood cholesterol, improving of heart function and stomach disorders, etc., with some of the species associated with livestock poisoning in southern Africa.[58] The extracts and compounds isolated from *Ornithogalum* (mainly steroidal glycosides and flavonoids) are structurally complex and highly bioactive. Steroidal glycosides isolated from *Ornithogalum* L. have displayed potent cytotoxic activity against various cancer cell lines.[34,58] From a phytochemistry perspective, most research on the genus *Ornithogalum* was performed on *O. saundersiae.*

Plants often display a diversity of chemotypes, i.e. the composition of the secondary metabolites is slightly different from one plant to another within the same species. Many South African plants are known to display this chemodiversity. *O. saundersiae* is used as a cut flower, and the bulbs are cultivated in many countries. However, bulbs used to produce cut flowers are genetically homogeneous and will be of the same chemotype. Most international research projects on *O. saundersiae* were performed on cut flower bulbs. However, a more diverse set of chemotypes are likely in South Africa, where the plants grow widely in the eastern parts of the country. Apart from *Ornithogalum*, there are other closely related genera, such as *Albuca*, on which limited phytochemical studies have been performed.

Further exploration of South African *Ornithogalum* species and species of related genera, such as *Albuca*, has the potential to produce cytotoxic steroid glycosides that might play a role in the fight against cancer. More phytochemical profiling of these genera will also add to the current data to better clarify the taxonomic classifications of *Ornithogalum*.[34]

1.4. AIM AND OBJECTIVES

Aim

This project aimed to identify and isolate steroidal glycosides from South African *Ornithogalum* and *Albuca* species that might be highly cytotoxic toward cancer cell lines and to investigate the LC-MS-MS profile of the crude extracts of these plants.

The specific objections were to:

- Collect plant material of *Ornithogalum* and *Albuca* species
- Extract the plant material with suitable solvents
- Analyse the crude extracts by LC-MS-MS
- Isolate steroidal glycosides from the complex mixtures
- Elucidate the structures of the compounds

1.5. OUTLINE OF THE DISSERTATION

This dissertation describes the identification and isolation of steroidal glycosides from two species, *Ornithogalum saundersiae,* and *Albuca batteniana*. It is comprised of five chapters. Chapter 1 investigates the background of this study, including the aim and objectives. Chapter 2 is a literature review on *Ornithogalum*, phytochemical investigations of the South African species of this genus, and traditional medicine based on *Ornithogalum*. Chapters 3 and 4 focus on the phytochemical investigations of *O. saundersiae* and *A. batteniana*, respectively. Chapter 6 evaluates cytotoxicity assays on the crude extracts and isolated compounds discussed in Chapters 3 and 4. General conclusions and future works are discussed in Chapter 6.

CHAPTER 2: LITERATURE REVIEW OF THE GENERA *ORNITHOGALUM* AND *GALTONIA*

2.1. INTRODUCTION

Globally, cancer is one of the leading diseases responsible for the deaths of millions of people each year. Many anticancer agents have been developed for different types of cancers, but most are extremely expensive or have various unmanageable toxic side effects.[59] There is a need for new and cheaper anticancer drugs with low toxicity to treat different kinds of cancerous tumours.

Natural products have played a pivotal role in the development of anticancer drugs. About 25% of the anticancer drugs approved between 1981 and 2019 were derived from natural products.[20] All cancer medicines are toxic to some extent, and some anticancer drugs were isolated from poisonous plants. In the search for new anticancer drugs, South African poisonous plants received significant attention as a source of bioactive steroidal glycosides.[40,41,60,61] This led to the isolation of the complex compound OSW-1 (**14**) from *Ornithogalum saunderisae* Baker in 1992.[39] OSW-1 displayed impressive cytotoxicity against leukemia HL-60 cell lines with IC_{50} values ranging between 0.0001 – 0.0003 μM.[62]

There is a need to explore more South African poisonous plants to extract the complex mixtures and isolate cytotoxic compounds. The Hyacinthaceae family is one of the largest plant families, with plants previously known to be toxic to animals.[58] These bulbous plants contain complex steroidal glycosides that might lead to the identification of additional highly active anticancer compounds.

This literature review focuses on botanical aspects of Hyacinthaceae, ethnopharmacology, and toxicity of the native South African species of the genus *Ornithogalum* L., along with cytotoxic activities associated with steroidal glycosides of the genera *Ornithogalum* and *Galtonia* Decne.

2.2. BOTANICAL ASPECTS OF THE HYACINTHACEAE

Hyacinthaceae (*sensu* APGll) is a plant family consisting of four subfamilies, Hyacinthoideae, Urgineoideae, Ornithogaloideae, and a small subfamily Oziroeoideae, with 70 genera and approximately 700-900 bulbous species, distributed mainly in Africa, Europe, and some parts of Asia, with one genus, *Oziroe*, in South America.[57,58,63,65] Hyacinthaceae is also regarded as the subfamily Scilloideae of Asparagaceae (*sensu* Lato), subsequently reducing the subfamilies of Hyacinthaceae to tribes of this family.[63,64]

Ornithogalum is the only genus belonging to the subfamily Ornithogoloideae*,* expanded by Manning et al. (2004) to accommodate the genera *Albuca*, *Dipcadi*, *Galtonia*, *Neopatersonia,* and *Pseudogaltonia* due to morphological similarities observed in phylogenetic studies based on 30 species conducted by Pfosser and Speta (1999).[38,63] Some similarities include fragrant flowers that close at night, green or white petals, and unique D-shaped seeds.[65] *Ornithogalum* plants can be identified by the thin-textured (can be thick) tepals with a narrow, vein-like median band and branched groups of veins.[38] However, further analysis of the taxonomic arrangements of Ornithogaloideae by Manning et al. (2009) led to the acceptance of three tribes, Albuceae, Dipcadie, and Ornithogaleae, with the genera *Dipcadi*, *Pseudogaltonia*, *Ornithogalum* and *Albuca*.[57] However, some publications still identify the four genera interchangeably as one genus. To date, continuous research is still ongoing on this genus and the Hyacinthaceae family.

2.3. ETHNOPHARMACOLOGY AND TOXICITY

2.3.1 Traditional medicine

Traditional medicine encompasses a set of past experiences indigenous to different cultural beliefs and backgrounds. The World Health Organization (WHO) defines traditional medicine as “the total knowledge, skills, and practices based on theories, beliefs, and experiences”. Traditional medicine is most common in African and Asian countries and is used for treating illnesses, prevention, and overall health maintenance.

Traditional medicine dates back to the stone Age and is still prevalent in current times as knowledge is passed from one generation to the next. Traditional medicine is commonly guided by traditional healers who rely primarily on herbal medicine and, in some cases,

animals and minerals. Many iqu-known traditional treatments are backed up by scientific data following a series of investigations but not all the knowledge is documented or openly shared globally. Analyses of these plants have led to discoveries of new drugs, such as vincristine and taxanes. However, the lack of thorough scientific investigations and poor regulations regarding the distribution of traditional medicine expose the recipients to dangers in the use of traditional medicine. Research has shown that some traditional medicines possess carcinogenic, mutagenic, and sometimes toxic properties unknown to healers and communities.

Traditional healers (*Sangoma* or *Inyanga* in IsiZulu) use a holistic approach in facilitating their practice. Traditional healing involves using herbs, remedies, and advice from the traditional healer. Spirituality is a vital component of traditional medicine; for this reason, African traditional healing forms a considerable part of African spirituality. About 80% of the South African population is estimated to use and trust traditional medicine as much as modern medicine. In some communities, traditional medicine is the preferred and easily accessible option. Almost every city in South Africa features outlets of herbal shops or wholesalers trading herbal medicines on a larger scale.

2.3.2. Historical medicinal uses of species from the *Ornithogalum* L. genus.

Several genera from *Ornithogalum* L. are used in traditional medicine; however, some have been reported to be poisonous to animals.[34] The genus is rich in saponins and steroidal glycosides, rendering the plants highly toxic. Some *Ornithogalum* species also display diverse biological activities, including antimicrobial, antioxidant, antitumor, and cytostatic activities. Traditional healers mainly use *Ornithogalum* species to treat diabetes, cardiac-related problems, hepatitis, and some types of cancer. Chinese traditional healers are reported to have been using *Ornithogalum* species for anti-inflammatory, anticancer, and antimicrobial bioactivities, even though *Ornithogalum* is found mainly in Europe and Southern Africa and not in China.[34]

2.4. PHYTOCHEMICAL PROFILE OF THE *ORNITHOGALUM* GENUS

2.4.1. *Ornithogalum* L.

Hyacinthaceae is a biodiverse family, rich with bioactive compounds, most of which are subfamily restricted with slight variations according to the geographical location in which they grow.[6] So far, compounds isolated and identified from the bulbs of the Hyacinthaceae species are mainly homoisoflavanones and spirocyclic nortriterpenoids from the Hyacinthoideae, bufadienolides from the Urgineoideae, and cardenolides and steroidal glycosides from the Ornithogaloideae subfamily.[58]

The Ornithogaloideae subfamily also features homoisoflavanoids, flavonoids, sterols, cholestane glycosides, cardenolides, and spirosterols.[39] The slight variations in the phytochemistry of the species are due to the geographical regions they are derived from; cholestane glycosides and spirosterols are present in African species, while cardenolides have been isolated from European species.[34] Livestock poisoning has drawn attention to the many toxic plants that are part of Hyacinthaceae. This has led to significant scientific attention towards this family, focusing mainly on the steroidal glycosides responsible for toxicity. Most of these toxic plants are found in the genus *Ornithogalum* (commonly known as Chinkerinchee and Star of Bethlehem).

Steroidal glycosides from *Ornithogalum* species are classified according to the aglycon framework.[39] The four aglycones of the steroidal glycosides where the steroid has 4 to 6 carbocyclic or *O*-heterocyclic rings are illustrated in **Figure. 2.1**. Cholestane glycosides are further divided into two groups, the common cholestane glycosides containing a 16,23-epoxy hexatomic ring and rearranged cholestane glycosides containing 24(23→22)-*abeo*-cholestane aglycon.[39]

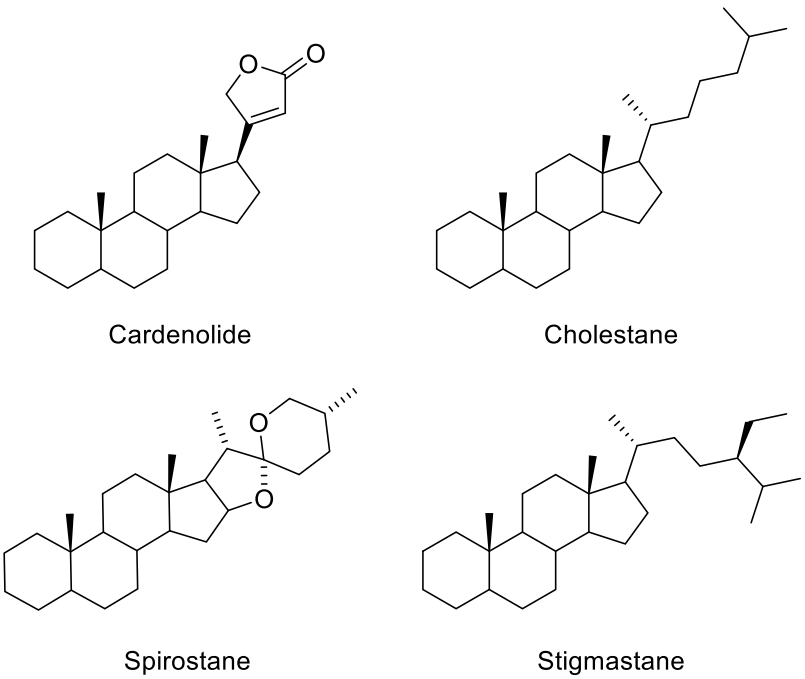
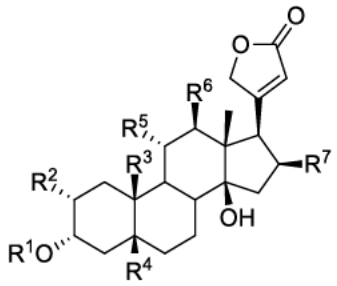


Figure 2.1. Structures of steroidal glycoside aglycones with steroids consisting of 4 to 6 carbocyclic or *O*-heterocyclic rings.

***Ornithogalum umbellatum* L.**

Scientific studies of *Ornithogalum* date back to the early 1950s, beginning with the Mediterranean native *Ornithogalum umbellatum* (commonly known as the Star of Bethlehem, Snowdrop) as one of the very first species to be investigated.[67,68] Convallatoxin (**15**) is the principal and main active compound in *O. umbellatum*, along with convalloside (**16**).[69] The cytotoxic activity of the cardenolide convallatoxin against Eagle's KB strain of human carcinoma cells is IC_{50} =0.002 μg mL^{-1}.[34,68] By structure-activity studies, it was found that the activity was enhanced by the presence of β-hydroxy groups at C-5 and C-14 and an aldehyde group at C-10. Additionally, the glycosidic portion was significant for the cytotoxic activity.[70] Further phytochemical studies on *O. umbellatum* led to the isolation of cardenolides **15**-**49** (**Fig. 2.2**), and two flavonoids, **50** and **51,** from the bulbs, while four flavonoids, **50**-**53**, have been isolated from the leaves to date (**Fig 2.3**).[58,70,71,72,73,74,75]



	R^1	R^2	R^3	R^4	R^5	R^6	R^7
15	α-L-Rhamnopyranosyl	H	CHO	OH	H	H	H
16	α-L-Rhamnopyranosyl-β-D-glucopyranosyl	H	CHO	OH	H	H	H
17	β-D-Glucopyranosyl-(1→4)-α-L-fucopyranosyl	H	OH	OH	H	H	H
18	α-L-Rhamnopyranosyl	H	CH_2OH	OH	H	H	H
19	6-Deoxy-β-D-gulopyranosyl	H	CHO	OH	H	H	H
20	α-L-Rhamnopyranosyl	H	CH_3	H	OH	H	H
21	α-L-Rhamnopyranosyl-β-D-glucopyranosyl	H	CH_3	H	OH	H	H
22	6-Deoxy-β-D-allopyranosyl	H	CHO	OH	H	H	H
23	β-D-Allopyranosyl	H	CHO	OH	H	H	H
24	3-Acetyl-β-digitopyranosyl-β-D-glucopyranosyl-α-L-rhamnopyranosyl	H	CHO	OH	H	H	H
25	α-L-Rhamnopyranosyl	H	CHO	OH	OH	H	H
26	6-Deoxy-β-D-allopyranosyl	H	CH_3	OH	H	H	H
27	6-Deoxy-β-D-allopyranosyl	H	CH_3	H	H	H	H
28	6-Deoxy-β-D-gulopyranosyl	H	CH_3	OH	OH	H	H
29	β-D-Allopyranosyl	H	CH_3	OH	OH	H	H
30	6-Deoxy-α-L-glucopyranosyl	H	CH_3	OH	H	H	H
31	α-L-Rhamnopyranosyl	H	CH_3	OH	H	H	H
32	β-D-Ribosyl	H	CH_3	OH	H	H	H
33	α-L-Arabinopyranosyl	H	CH_3	H	OH	H	H
34	6'-Deoxy-β-D-allopyranosyl-4'-β-D-xylo-pyranosyl-3''-β-D-apiofuranosyl	H	CHO	OH	H	H	H
35	α-L-Rhamnopyranosyl-4'-β-D-apiofuranosyl	H	CH_3	H	H	H	OH
36	β-D-Digitoxopyranosyl-4'-β-D-xylopyranosyl-3''-β-D-apiofuranosyl	H	CH_3	H	H	H	OAc
37	2'-Deoxy-β-D-allopyranosyl-4'-β-D-xylo-pyranosyl-3''-β-D-apiofuranosyl	H	CH_3	H	H	H	OAc
38	β-D-Digitoxopyranosyl-4'-β-D-xylo-pyranosyl-3''-β-D-apiofuranosyl	H	CH_3	H	H	OH	OAc
39	6-Deoxy-α-L-glucopyranosyl	H	CHO	OH	H	H	H
40	β-D-Allopyranosyl-β-D-xylosyl	H	CHO	OH	H	H	H
41	β-D-Digitoxopyranosyl-4'-glucopyranosyl	H	H	OH	H	H	H
42	α-L-Rhamnopyranosyl	H	CHO	OH	OH	H	H
43	6-Deoxy-α-L-glucopyranosyl	H	CHO	OH	OH	H	H
44	α-L-Rhamnopyranosyl-4'-β-D-apiofuranosyl	H	CH_3	H	OH	H	H
45	α-L-Rhamnopyranosyl-4'-α-L-L-rhamnopyranosyl	H	CH_3	H	OH	H	H
46	β-D-Digitoxopyranosyl-β-D-xylopyranosyl-α-L-rhamnopyranosyl	H	CH_3	H	OH	H	H
47	α-L-Rhamnopyranosyl	H	CH_3	OH	OH	H	H
48	β-D-Glucopyranosyl	H	CH_3	OH	OH	H	H
49	2-Deoxy-β-D-allopyranosyl	OH	CH_3	OH	OH	H	H

Figure 2.2. Cardenolide glycosides from *Ornithogalum umbellatum*.

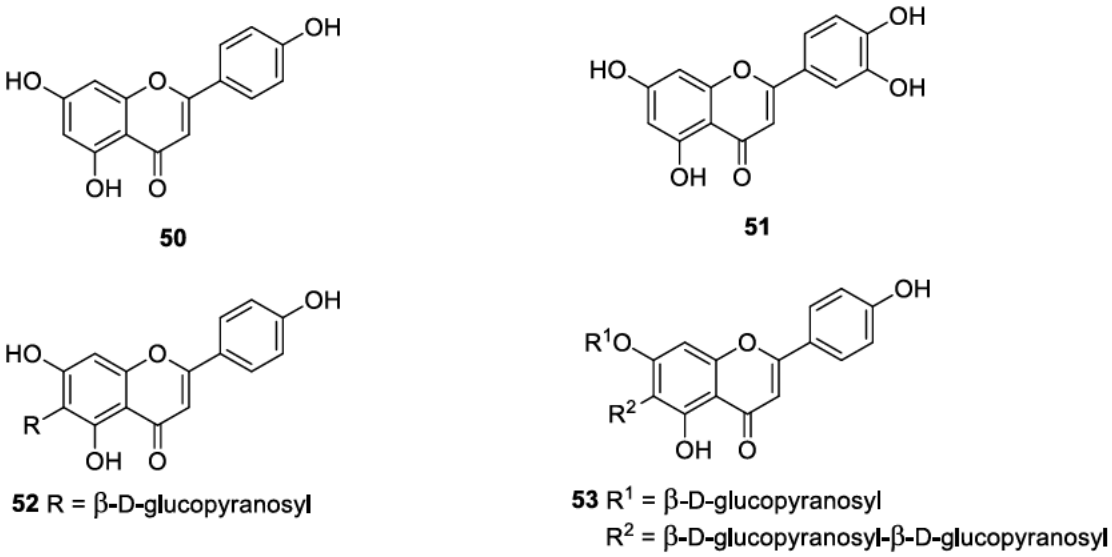


Figure 2.3. Flavonoids isolated from *Ornithogalum umbellatum*.

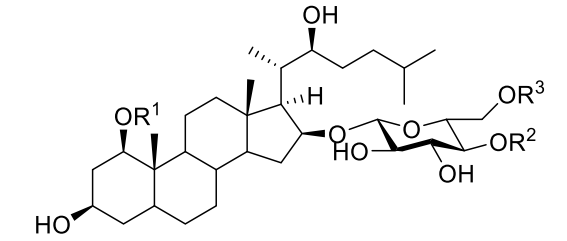
Other steroidal glycosides from *Ornithogalum* L. have been found in the South African natives *O. thyrsoides* and *O. saundersiae*, which are very poisonous species and have been associated with livestock poisoning.[34] Although no folkloric medicinal uses for these species have been recorded, they have been heavily investigated for their poisonous nature due to the presence of cholestane glycosides in *O. thyrsoides* (common names are African wonder flower and Cape lily) and *O. saundersiae*.[76]

In discussing the following species, the compounds are grouped and presented according to their respective classes.

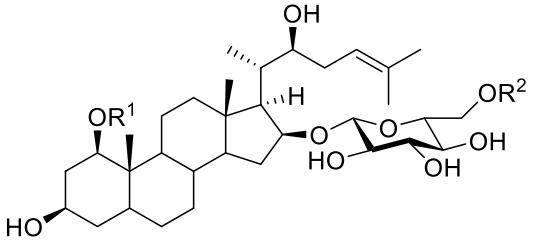
***Ornithogalum thyrsoides* Raf.**

O. thyrsoides, also referred to as *Eliokarmos thyrsoide* (Jacq.) Raf., is native to South Africa. The compounds isolated from this plant were cholestane glycosides (**54-80**) (**Fig 2.4**), spirostane glycosides (**81-94**) (**Fig 2.5**), and 22-hydroxy cholestane glycosides (**95-111**) (**Fig 2.6**). The earliest phytochemical investigations on this plant were on the methanolic bulb extract, resulting in the isolation of four cholestane bisdemosides (**54-57**) (**Fig 2.4**).[77] Compound **54** showed significant inhibitory activity on cyclic AMP phosphodiesterase with an IC_{50} = 15.3 × 10^{-5} M.[77] Kuroda et al. (2002) discovered the potent cytotoxic activity of the crude methanol extract of bulbs against leukemia HL-60 cells at IC_{50} = 0.79 mg mL^{-1}, and further isolated 12 bisdesmosidic cholestane glycosides (**58-69**, **80)** from the extracts.

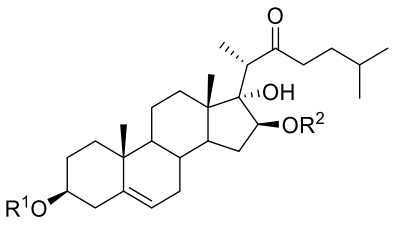
Nine of these compounds were novel (**58-66**)**,** and four were known (**67-69**, **80**). Impressive cytotoxic activity was observed for **58** and **80** with IC_{50} = 0.00016 and 0.00013 μg mL^{-1}, respectively.[62] Some cytotoxic activity was observed for the rest of the compounds, except for **59**, **62**, and **66**.[78] Additionally, nine spirocyclic glycosides (**81**-**85**, **87**-**90**), and seven cholestane glycosides (**95**-**100**, **107**) were isolated. The spirocyclic glycosides showed moderate cytotoxic activity against leukemia HL-60 cells with IC_{50} = 1.6 -5.3 μg mL^{-1} on average.[79,80] Four new polyoxygenated steroidal glycosides (**91**-**94**) were isolated from the bulbs of *O. thyrsoides*. However, the activities of these compounds were not reported.[80]



	R^1	R^2	R^3
54	α-L-Rhamnopyranosyl	H	H
55	α-L-Rhamnopyranosyl	H	Ac

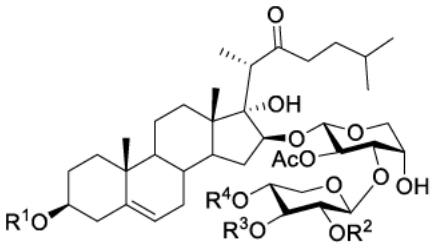


	R^1	R^2
56	α-L-Rhamnopyranosyl	H
57	α-L-Rhamnopyranosyl	Ac



80 R^1 = β-D-Glucopyranosyl, R^2 = α-L-Arabinopyranosyl

Figure 2.4. Cholestane glycosides from *Ornithogalum thyrsoides* Raf.



	R^1	R^2	R^3	R^4
58	β-D-Glucopyranosyl	TMB*	H	H
59	β-D-Glucopyranosyl-(1-6)-β-D-glucopyranosyl	H	H	H
60	β-D-Glucopyranosyl-(1-6)-β-D-glucopyranosyl	DMB*	H	H
62	β-D-Glucopyranosyl-(1-6)-β-D-glucopyranosyl	TMB	H	H
63	β-D-Glucopyranosyl-(1-4)-β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl	H	H	H
64	β-D-Glucopyranosyl-(1-4)-β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl	DMB	H	H
65	β-D-Glucopyranosyl-(1-4)-β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl	TMB	H	H
66	β-D-Glucopyranosyl-(1-4)-β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl	HMB*	H	H
67	β-D-Glucopyranosyl-(1-4)-β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl	H	DMB	H
68	β-D-Glucopyranosyl	H	H	H
69	β-D-Glucopyranosyl	DMB	H	H
70	H	H	H	H
14	H	PMB*	H	H
71	H	DMB	H	H
72	H	PHBz	H	H
73	H	TMBz	H	H
74	β-D-Glucopyranosyl	PHBz	H	H
75	H	CNM*	H	H
76	β-D-Glucopyranosyl	PMB	H	H
77	β-D-Glucopyranosyl	CNM	H	H
78	H	DMB	H	β-D-Glucopyranosyl
79	H	TMB	H	β-D-Glucopyranosyl

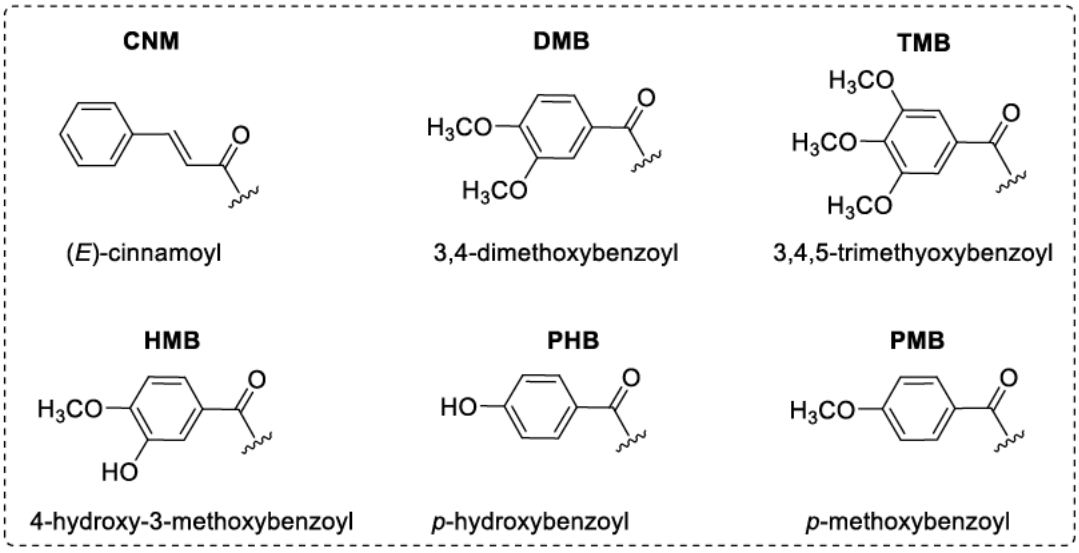
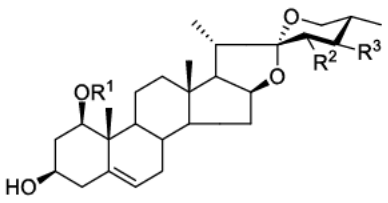
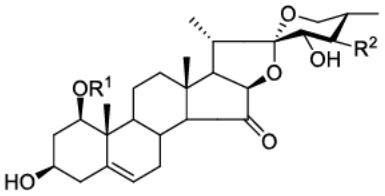


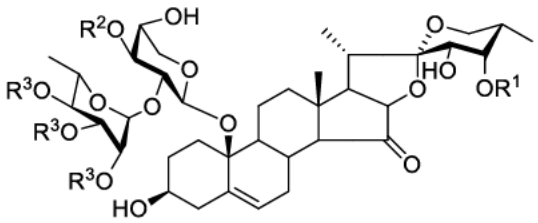
Figure 2.4 (cont.) Cholestane glycosides from *Ornithogalum thyrsoides* Raf.



	R^1	R^2	R^3
81	β-D-Glucopyranosyl	H	H
82	α-L-Rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	H	H
83	α-L-Rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)-α-L-arabinopyranosyl	H	H
84	α-L-Rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	H	OH
85	α-L-Rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)-α-L-arabinopyranosyl	H	OH
86	α-L-Rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)-α-L-arabinopyranosyl	OH	OH



	R^1	R^2
87	α-L-Rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	H
88	α-L-Rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	6-deoxy-β-D-Gulopyranosyl
89	α-L-Rhamnopyranosyl-(1→2)-O-[β-D-xylopyranosyl-(1→3)-α-L-arabinopyranosyl	6-deoxy-β-D-Gulopyranosyl
90	2,3,4-tri-O-acetyl-α-L-Rhamnopyranosyl-(1→2)-O-[β-D-xylopyranosyl-(1→3)-α-L-arabinopyranosyl	6-deoxy-β-D-Gulopyranosyl



	R^1	R^2	R^3
91	H	H	H
92	β-D-Gulomethylopyranosyl	H	H
93	β-D-Gulomethylopyranosyl	β-D-Xylopyranosyl	H
94	β-D-Gulomethylopyranosyl	β-D-Xylopyranosyl	Ac

Figure 2.5. Spirostane glycosides from *Ornithogalum thyrsoides* Raf.

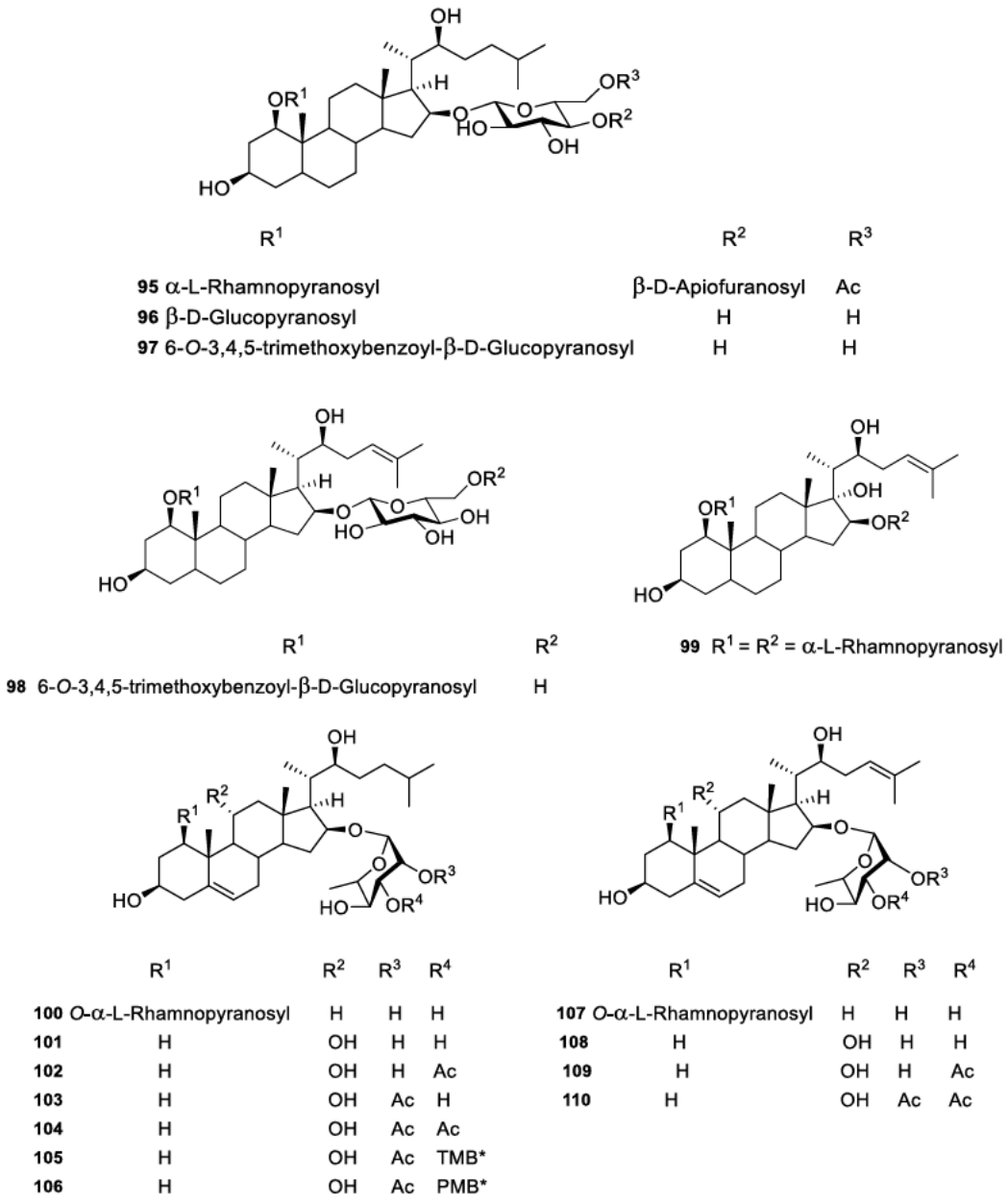


Figure 2.6. 22-Hydroxycholestane glycosides from *Ornithogalum* L. genus.

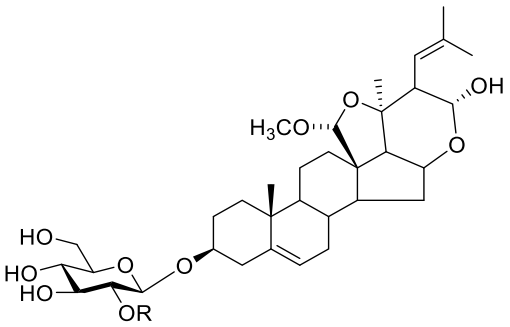
Ornithogalum saundersiae

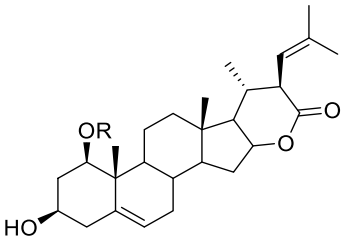
O. saundersiae (also known as *Galtonia saundersiae* (Baker)) is a species widely used for ornamental purposes and has no folkloric medicinal history.[62] It is found on the east coast of South Africa and Swaziland. *O. saundersiae* is cultivated internationally and sold for tens of millions of dollars annually at Dutch auctions.[39] The phytochemical studies on *O. saundersiae* began with the isolation of three acylated cholestane glycosides **14**, **70**, **71** from bulbs in 1992.[81] Both **14** and **71** showed inhibitory activity on cyclic AMP phosphodiesterase with IC_{50} values at 0.25 nM and 0.20 nM, respectively.[58] Kuroda et al. (2001) concluded that the

diglycoside moiety at C-16 combined with a carbonyl at C-22 was essential for the cytotoxic activity exhibited by **14** against various malignant tumour cells.[82]

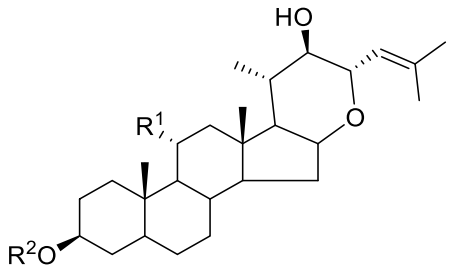
Within one decade of phytochemical investigations on *O. saundersiae* (1992 – 2002), twenty-five additional steroidal glycosides were identified and isolated.[34,58] Amongst them were six polyhydroxylated cholestane glycosides (**101**-**103**, **108**, **109**, **111**) (Compound **111** is formally known as saundersioside A), nine cholestane glycosides (**114**, **112**, **113**, **118**-**123**), where compounds **112**, **113**, and **118**-**123** are known as saundersiosides B – I, and ten rearranged cholestane glycosides (**110**, **75**-**79**, **104**-**106**, and **126**) (**Fig. 2.7 and 2.8)**. Compounds **102**, **109**, and **111** showed inhibitory activity on cyclic AMP phosphodiesterase with IC_{50} = 0.099 µM, IC_{50} = 0.109 µM, and 40% inhibition at 0.08 mg mL^{-1}, respectively.[61,62,83,84,85]

Steroidal glycosides **114**, **112**, **113**, **118**-**123**, and **126** were assayed for cytostatic activity on leukemia HL-60 and MOLT-4 cells. Cytotoxic activity against leukemia HL-60 cells was observed for **112**, **120**-**123**, and **126** (IC_{50} = 9.2 nM, 21 nM, 19 nM, 63 nM, 52 nM, and 20 nM, respectively).[84,86] Compounds **112**, **120**, and **126** (IC_{50} = 3.2 nM, 1.8 nM, and 4.2 nM, respectively) showed cytotoxic activity against MOLT-4 cells. The rearranged cholestane glycosides **101**, **104**-**106**, and **110** also showed cytotoxic activity on leukemia HL-60 cells (GI_{50} = 0.19 µM, 6.9 µM, 1.8 µM, 0.022 µM, and 0.80 µM, respectively).[60,84,87,88] Only compound **114** showed inhibitory activity (IC_{50} = 3.1 µM) towards the proliferation of peripheral blood lymphocytes. The rest of the steroidal glycosides showed no potent activity.[83]

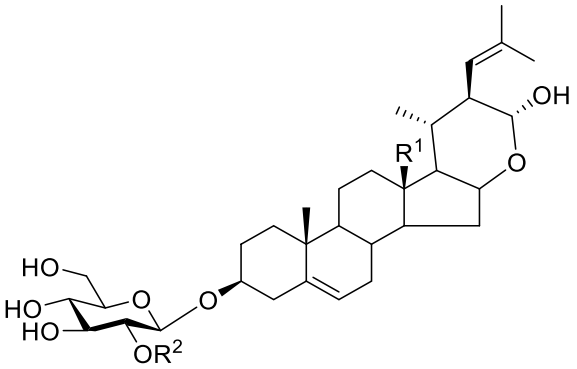




111 R = α-L-Rhamnopyranosyl-(1-2)-β-D-glucopyranosyl **113** R= β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl
112 R = 4-O-4-methoxybenzoyl-α-L-Rhamnopyranosyl

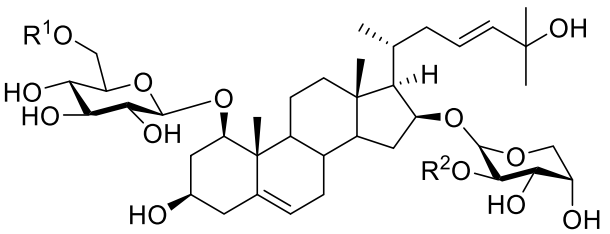


	R^1	R^2
114	H	α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl
115	H	β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl
116	OH	α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl
117	H	β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl



	R^1	R^2
118	CHO	α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl
119	CHO	α-L-Rhamnopyranosyl
120	CHO	4-*O*-(4-hydroxy-3-methoxybenzoyl)-α-L-Rhamnopyranosyl
121	CHO	4-*O*-4-methoxybenzoyl-α-L-Rhamnopyranosyl
122	CH_2OH	4-*O*-(4-hydroxy-3-methoxybenzoyl)-α-L-Rhamnopyranosyl
123	CH_2OH	4-*O*-4-methoxybenzoyl-α-L-Rhamnopyranosyl
124	CHO	α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl
125	COOH	α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl

Figure 2.7. Rearranged cholestane glycosides from *O. saundersiae*.



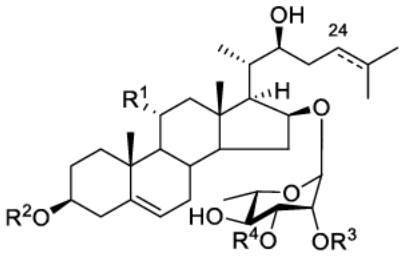
	R^1	R^2
126	H	3,4,5-trimethoxybenzoyl
127	β-D-Glucopyranosyl	TMBz

Figure 2.8. Cholestane glycosides from *O. saundersiae*.

The early 2000s showed no apparent new publications on the extraction and isolation of compounds from *O. saundersiae*. Iguchi et al. (2017, 2019) made a significant contribution to the research on this species by isolating twelve new cholestane rhamnosides (**133**-**145**), along with seven known steroidal glycosides in 2017, with three new OSW-1 analogues (**72**-**74**), one cholestane bisdemoside (**127**), one cholestane diglycoside (**115**), and four more cholestane glycosides (**146**-**149**) in 2019.[66,53]

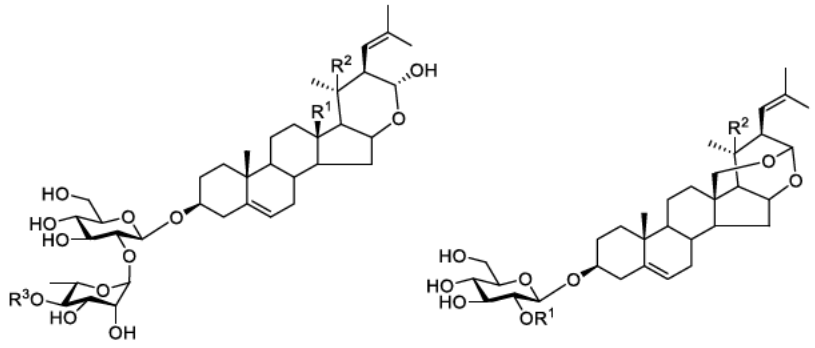
Compounds **133** and **138** yielded derivatives **132** and **139** upon treatment with 3% NaOMe in MeOH. All fourteen compounds were assayed for cytotoxic activity against HL-60 cells. Compounds **143**-**145** showed significant activity with IC_{50} values ranging between 0.05-0.16 µM, whereas the positive controls etoposide and cisplatin had IC_{50} values of 0.23 µM and 1.52 µM, respectively.[66]

Soon after, Chen et al. (2019) isolated eight new cholestane glycosides **128**-**131**, **116**, **117**, and **125** (osaundersioside A-H) and three known steroidal glycosides. Cytotoxic activity against five different cell lines (human colon cancer HCT-116, human hepatocyte carcinoma HepG2, human gastric carcinoma BGC-823, human non-smalL-cell lung cancer A549, and human breast carcinoma cell line MCF-7) was investigated for all the compounds.[89] Compound **130** showed significant cytotoxic activity against the MCF-7 cell lines with an IC_{50} value of 0.20 µM while the rest of the compounds were inactive. Compound **125** showed anti-inflammatory activity, inhibiting LPS-induced NO production in mouse peritoneal macrophages by 56.8% at 10 nM concentration.[89]



		R1	R2	R3	R4
128	Δ^{24}	OH	β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl	H	H
129	Δ^{24}	OH	α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl	H	H
130	Δ^{24}	H	α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl	H	H
131	Δ^{24}	H	β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl	H	H
132		OH	β-D-Glucopyranosyl	H	H
133		OH	β-D-Glucopyranosyl	Ac	H
134		OH	β-D-Glucopyranosyl	Ac	Ac
135		OH	β-D-Glucopyranosyl	Ac	PMBz
136		OH	β-D-Glucopyranosyl	Ac	TMBz
137	Δ^{24}	OH	H	Ac	H
138	Δ^{24}	OH	β-D-Glucopyranosyl	Ac	Ac
139	Δ^{24}	OH	β-D-Glucopyranosyl	H	H
140	Δ^{24}	OH	β-D-Glucopyranosyl	Ac	PMBz
141	Δ^{24}	OH	β-D-Glucopyranosyl	Ac	TMBz
142	Δ^{24}	OH	β-D-Glucopyranosyl-(1→2)-α-L-rhamnopyranosyl	Ac	Ac
143		H	β-D-Glucopyranosyl	Ac	Ac
144	Δ^{24}	H	H	H	H
145	Δ^{24}	OH	β-D-Glucopyranosyl	Ac	Ac

Figure 2.8 (cont). Cholestane glycosides from *O. saundersiae*.



	R1	R2	R3
146	CH_2OH	OH	PHBz
148	CH_2OH	OH	PMBz

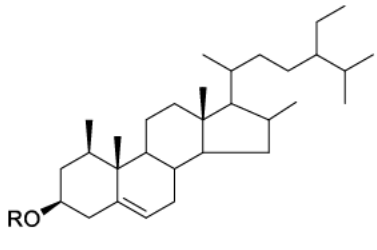
	R1	R2
148	α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside	H
149	*p*-hydroxybenzoyl-α-L-rhamnopyranoside	OH
150	α-L-rhamnopyranoside	H

Figure 2.9. Rearranged cholestane glycosides from *Ornithogalum* and *Galtonia* genera.

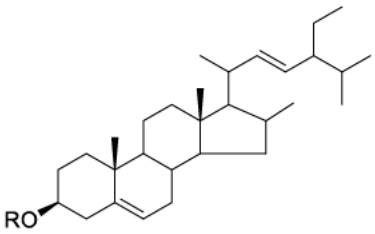
***Ornithogalum caudatum* Ait.**

Ornithogalum caudatum (now known as *Stellarioides longibracteata* (Jacq.) Speta or *Ornithogalum longibracteata* (Jacq.)) is another of the bulbous species on which a considerable number of publications have appeared.[58] It is used by the Zulus to treat diabetes and inflammation, whilst the Chinese use it to treat cancer, hepatitis, and parotitis.[58,90] Steroidal glycosides that have been isolated from *O. caudatum* include β-sitosterol (**151**), daucosterol (**152**), stigmasterol (**153**), and stigmasterol 3-*O*-β-D-glucopyranoside (**154**), and two spirosterols, caudaside A (**86**), and hecogenin (**155**).[91,92] The major class of secondary metabolites is flavonoids, with more than twenty known flavonoids isolated.[58,90,93]

None of the isolated compounds showed cytotoxic activity against mouse leukemia P388 and human pulmonary adenocarcinoma A-549 cells upon testing.[90] Aqueous bulb extracts showed some antitumour activity, and other extracts showed potential for the treatment of liver conditions.[94]

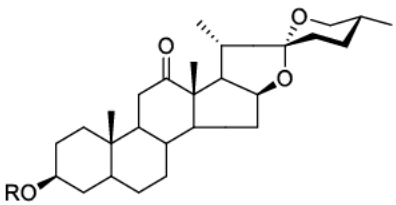


151 R = H
152 R = β-D-Glucopyranosyl



153 R = H
154 R = R = β-D-Glucopyranosyl

Figure 2.10. Stigmastane glycosides from the *Ornithogalum* L. genus.



155 R = β-D-Glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside

Figure 2.11. Spirostane glycosides from the *Galtonia* genus.

The species described above concludes the list of South African *Ornithogalum* L. species that contain many steroidal glycosides. Several steroidal glycosides isolated displayed great

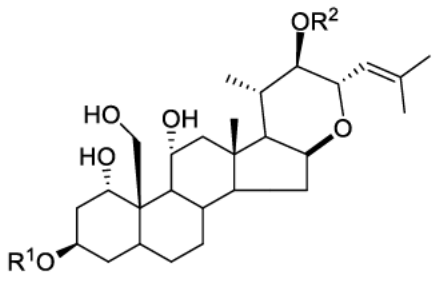
cytotoxic activities against various cancer cell lines. Apart from *O. caudatum*, no folkloric medicinal history is associated with these plants. *O. prasinum* and *O. toxicarium* are also native to South Africa, but no phytochemical investigations have been done on them.

2.4.2. *Galtonia* Decne.

In the continuous study of molecular phylogenetics of the sub-family Ornithogaloideae of the Hyacinthaceae family, several genera have been accepted as tribes, subgenera, and clades of *Ornithogalum* L.[63] However, two clades remain unresolved. One of the clades included *Eliokarmos*, *Ethesia*, and *Galtonia*.[63] Some *Ornithogalum* species were therefore named according to the abovementioned sub-clades and are still described as such in some articles. Consequently, it is important to investigate *Galtonia* species in phytochemical studies due to the taxonomic relationship with *Ornithogalum*.

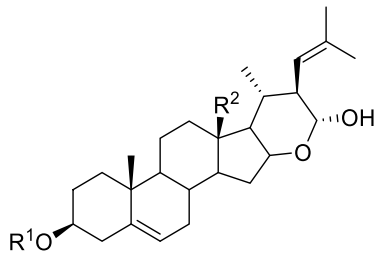
***Galtonia candicans* Decne.**

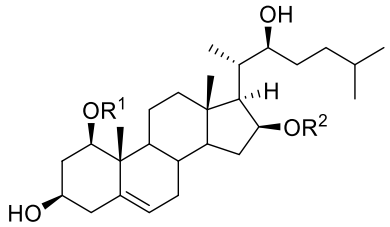
Galtonia Decne. Contain many bioactive compounds, and a considerable number of steroidal glycosides have been isolated from the South African species.[58] The methanol and butanol crude extracts of *Galtonia candicans* (Baker) Decne. Are active against a leukemia HL-60 cell line (methanol crude extract IC_{50} = 0.017 µM and butanol crude extract IC_{50} = 0.0056 µM). Kuroda and Mimaki isolated 14 steroidal glycosides from *G. candicans* in the early 2000s. Among the isolated steroidal glycosides were one hexacyclic rearranged cholestane diglycoside (**150**) (candicanoside A), one polyoxygenated cholestane diglycoside (**156**) (galtonioside A), six cholestane glycosides (**157**, **158**, **163-166**), and six cholestane bisdemosides (**159-162**, **168**, and **169**).[58,95,96,97]



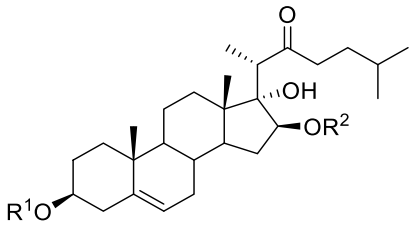
156 R^1 = 3,4,5-trimethoxybenzoyl ester,
R^2 = α-L-Arabinopyranosyl-(1→4)-β-D-glucopyranosyl

Figure 2.12. Structure of galtonioside A.



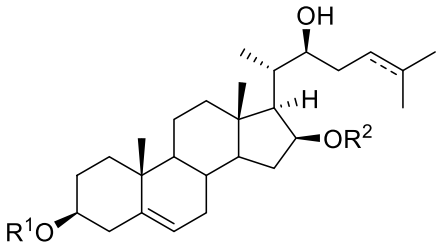


		R1	R2
157	R^1= α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl; R^2= CH_2OH		
158		β-D-Glucopyranosyl	β-D-Glucopyranosyl
159	Δ^{24}	β-D-Glucopyranosyl	β-D-Glucopyranosyl
160	Δ^{24}	β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl	β-D-Glucopyranosyl
161		β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl	β-D-Glucopyranosyl
162		α-L-Rhamnopyranosyl	β-D-Glucopyranosyl



	R1	R2
163	β-D-Glucopyranosyl	(2-*O*-(E)-cinnaamoyl-β-D-Xylopyranosyl)-(1→3)-2-*O*-acetyl-α-L-arabinopyranosyl
164	β-D-Glucopyranosyl	(2-*O*-3,4-dimethoxybenzoyl-β-D-Xylopyranosyl)-(1→3)-2-*O*-acetyl- α-L-arabinopyranosyl
165	β-D-Glucopyranosyl	β-D-Xylopyranosyl-(1→3)-2-*O*-acetyl- α-L-arabinopyranosyl
166	β-D-Glucopyranosyl	α-L-Arabinopyranosyl
167	H	α-L-Arabinopyranosyl

Figure 2.13. Structures of cholestane glycosides isolated from *Galtonia* genus.



		R1	R2
168	Δ^{24}	β-D-Glucopyranosyl	3-*O*-acetyl-α-L-Rhamnopyranosyl
169		β-D-Glucopyranosyl	3-*O*-acetyl- α-L-Arabinopyranosyl

Figure 2.14. Structures of cholestane bisdemosides isolated from *Galtonia* genus.

The isolated compounds were assayed for cytotoxic activity against a leukemia HL-60 cell line. Compounds **163**-**165** were the most active compounds (IC_{50} = 0.00012 µM, 0.00048 µM, 0.0024 µM, respectively), followed by candicanoside A (**150**), compound **166**, and galtonioside A (**156**) with IC_{50} = 0.032 µM, 0.053 µM, and IC_{50} = 0.057 µM, respectively.[95,97] Compound **169** also showed some activity at IC_{50} = 6.8 µM.[96] Activity was also observed against breast, CNS, and lung cancer lines.[58] There have not been any new developments relating to the isolation of steroidal glycosides from *G. candicans* since the early 2000s.

***Galtonia princeps* Decne.**

Only one cholestane glycoside (**162**) has been identified in *G*. *princeps* (Baker) Decne.[98] *G. viriflora* I.Verd. contains only monoglycosides and caffeic acids as major compounds.[99] Apart from *G. candicans*, *G*. *viridiflora*, and *G. princeps*, little information is available on the South African *Galtonia* species.

2.5. CONCLUSION

This review covers the phytochemical profiles and biological activities of four prominent *Ornithogalum* and two *Galtonia* species endemic to South Africa. The major compounds found were steroidal glycosides and flavonoids, which have contributed significantly to the biological activities associated with these plants. The extensive research dating back to the early 1950s was initiated by the observed poisonous properties of some plants causing sickness or death of livestock and folkloric ethnomedicinal uses of *Ornithogalum* species. Compounds, such as OSW-1 and its analogues, with potent cytotoxic activities towards cancer cell lines have been identified as lead compounds for the development of anticancer drugs. Phytochemical studies on the *Ornithogalum* genus have supported taxonomical studies on the Hyacinthaceae.

CHAPTER 3: IDENTIFICATION AND BIOACTIVITY OF STEROIDAL GLYCOSIDES FROM *ORNITHOGALUM SAUNDERSIAE*

3.1. INTRODUCTION

Ornithogoloideae is one of four subfamilies (including Hyacintiodeae, Urgineoideae, and Oziroeoideae) of Hyacinthaceae. Taxonomic classifications place the genera *Albuca*, *Dipcadi*, *Galtonia*, *Neopatersonia*, *Ornithogalum,* and *Pseudogaltonia* in Ornithogoloideae. Significant attention has been given to the genus *Ornithogalum* as a result of the highly bioactive compounds that were isolated from this genus. This genus is used in traditional medicine and is associated with livestock poisoning. *Ornithogalum* features ~200 bulbous species native to Europe, Africa, and Asia, with the majority of species endemic to Southern Africa, making Africa the primary source of biodiversity for the genus *Ornithogalum*. Some species are cultivated for ornamental purposes (*O. umbellatum, O. saundersiae, O. thyrsoides, O. nutans,* and *O. pyramidale*), and are popular in international trades as cut flowers.

Substantial phytochemical investigations of the genus *Ornithogalum* have focused on the European *O. umbellatum,* and the South African natives, *O. saundersiae,* and *O. thyrsoides*. Major classes of compounds isolated were steroidal glycosides and flavonoids, exhibiting remarkable cytotoxic bioactivities with unique mechanisms of action. Phytochemical investigations of this genus date back to the early 1950s, resulting in the discovery of many bioactive compounds. Natural product research has also evolved to accelerate investigations to meet the ever-shortening timelines of drug discovery. Bio-guided fractionation of crude extracts, Centrifugal Partition Chromatography (CPC), and Countercurrent Chromatography (CCC) are recent techniques gaining popularity among the researchers. Despite the development of modern techniques, there are still challenges in natural product chemistry. The use of hyphenated spectrometric and spectroscopic techniques offers unique de-replication tools in the search for new compounds. LC-NMR, LC-MS-MS, and GC-MS-MS can assist in the identification and structural elucidation of compounds without the need for prior isolations and purifications.

Liquid chromatography-tandem mass spectrometry (LC-MS-MS) is an analytical chemistry technique that uses the efficient fractionation power of liquid chromatography in

combination with the highly sensitive mass analysis capabilities of triple quad or quadrupole—time-of-flight (QTOF) mass spectrometers. This technique is used for the analysis of compounds present in an extract or mixture or for purification of compounds from crude extracts. The high separation power of LC, and the high degree of sensitivity and selectivity of MS based on unique mass/charge (*m/z*) transitions are important factors to consider for a successful analysis.

LC-MS-MS techniques became popular in the late 1990s as one of the most powerful and less time-consuming techniques used for non-volatile complex compounds to meet the shortening timelines in drug discovery. Natural products, being one of the common sources for drug discovery, makes it essential to consider LC-MS-MS methods for bio-guided fractionation and isolation of new and bioactive compounds. The raw data achieved from the tandem mass spectrometry experiments can be analyzed using many methods, depending on the user's scientific questions.

Molecular networking is one of the computational methods used to analyze tandem mass spectra of small molecules. It is a relatively new concept, introduced in 2012 for the analysis of live microbial colonies through the mapping of chemical diversity found in untargeted mass spectrometry. The concept was introduced in the field of microbiology and has since then provided a useful method for the elucidation and identification of structures of different compounds. The principle behind the molecular networking concept is the pairwise spectral alignment through a cosine spectral similarity algorithm, thereby discovering modified forms of peptides and proteins.

Structurally related compounds/molecules share similar fragment patterns when subjected to mass spectrometric fragmentation. Molecular networking makes use of this fact to derive the networks. In a spectral similarity search, the fragmentation spectra (MS^2) from ions at identical *m/z* values are compared, and further comparing (MS^2) spectra that are offset by the same *m/z* value difference as the precursor ion. During the spectral similarity search, every MS^2 spectrum in a dataset/raw data is compared against all the other MS^2 spectral data, creating a network of spectral relations. This creates the molecular network as the outcome of the input raw data.

Molecular networking was extended to analyze any spectral similarities of any set of tandem MS^2 spectra by bypassing the amino acid filtering function. This meant molecular networking was now possible for small molecules and natural products. A web-based platform called Global Natural Product Social Molecular Networking (GNPS) was curated in 2014 for easy accessibility to scientists globally. It was then published in the United States of America (USA) in 2016. Its reach has expanded to more than 150 countries since then and is used in the fields of biomedical science, environmental science, forensics, microbiology, and chemistry.

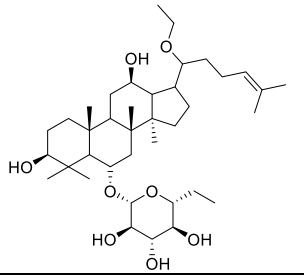
In this chapter, qualitative phytochemical investigations are reported, focusing on the identification and isolation of steroidal glycosides. The investigation started with LC-MS-MS analysis of the crude extract of the bulb of *O. saundersiae* to track the chemical profile of the extract and identify some compounds for isolation and structural determination.

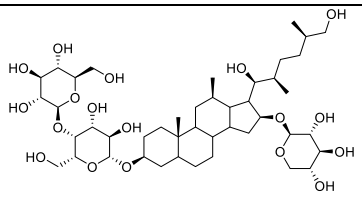
3.2. RESULTS AND DISCUSSION

3.2.1. LC-MS-MS analysis of the crude extract of *O. saundersiae*

A crude extract was prepared by soaking the dried, ground material of the bulb of *O. saundersiae* in MeOH. After removing the solvent, this extract was further partitioned with hexane, EtOAc, *n*-butanol, and H_2O. The EtOAc and *n*-butanol extracts were subjected to LC-MS-MS analysis. The results of this experiment are collated in **Table 3.1**.

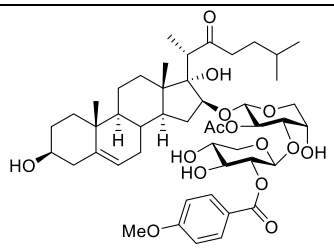
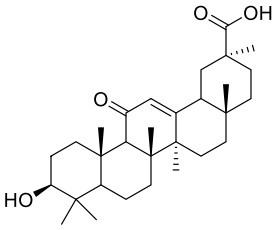
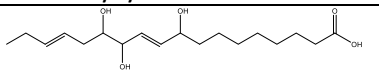
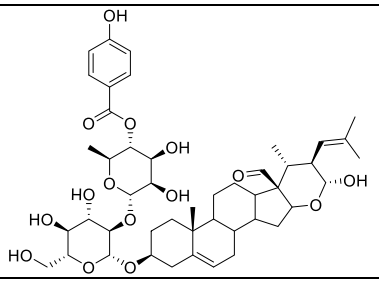
Table 3.1: LC-MS-MS data of compounds in *O. saundersiae* crude extracts (Positive ionization mode).

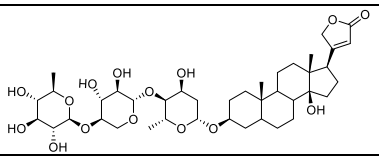
Crudes	$[M+H]^+$ (*m/z*)	$[M+Na]^+$ (*m/z*)	Molecular Formula	Error (ppm)	MS/MS	Proposed Compound
BuOH & EtOAc	665.4651	-	$C_{38}H_{65}O_9$	3.37	304.2613 563.5510 283.2824 585.5336 265.2525 379.2383 281.2475	

BuOH & EtOAc	938.5128	-	$C_{63}H_{70}O_7$	0.69	327.5128 309.0979 249.0764 903.4743 189.0766 415.3229 920.5034	No published data
BuOH & EtOAc	891.4941	-	$C_{44}H_{75}O_{18}$	-1.39	890.4908 277.2169 295.2262 249.0765 267.0870 277.2170 855.4523 873.4654 383.1344 346.2960	
BuOH & EtOAc	872.5776	-	$C_{54}H_{80}O_9$	-3.02	256.2635 362.2119 384.1935 871.5722 256.2633	No published data
BuOH & EtOAc	1052.5433	-	$C_{64}H_{76}O_{10}$	-0.52	332.3163 1017.5063 817.4746 295.2263 249.0763 267.0866 427.2832 429.2996 277.2174 411.2901 195.1379 429.3016 231.0874	No published data
BuOH & EtOAc	1052.5440	-	$C_{68}H_{76}O_{10}$	0.14	649.4318 1017.5084 870.5009 647.4186 267.0867 249.0759 267.0870	No published data
BuOH & EtOAc	916.4903	-	$C_{60}H_{68}O_8$	-1.22	827.4797 875.4445 231.0872 171.0665 712.4287 665.4267 171.0667 411.2902 429.2994	No published data

BuOH & EtOAc	871.5735	-	$C_{54}H_{79}O_9$	1.25	282.2782	No published data
BuOH & EtOAc	871.5766	-	$C_{47}H_{83}O_{14}$	-1.93	282.2796	No published data
BuOH & EtOAc	932.4854	-	$C_{60}H_{68}O_9$	-1.00	879.4372 399.3251 417.3368 189.0768	No published data
BuOH & EtOAc	980.7062	-	$C_{70}H_{92}O_3$	1.58	316.2851 383.1346 665.4260 399.3255 427.2847 295.2280 417.3380 647.4154 359.2978 429.2992 340.2850	No published data
BuOH & EtOAc	873.4639	-	$C_{47}H_{59}O_{15}$	0.29	344.3158 346.2954 679.4403 855.4526 316.2851 281.1024 417.3361 323.1129 415.3207 277.2168	No published data
BuOH & EtOAc	1214.5956	-	$C_{74}H_{86}O_{15}$	-0.88	348.3110 857.4321 839.4219 171.0658 267.0872 358.2939 249.0769 427.2826 279.2325	No published data
BuOH & EtOAc	400.1664	-	$C_{26}H_{24}O_4$	-2.65	284.2951 250.0861 384.1921	No published data
EtOAc	411.2904	-	$C_{27}H_{39}O_3$	1.17	314.2692 327.1080 360.3107 309.0986 281.1022 249.0767 277.2159 277.2176	No published data

					344.2795	
EtOAc	876.4754	-	$C_{61}H_{64}O_5$	0.03	330.3369 429.2999 411.2902 416.3244 171.0655 427.2844 327.1085 416.3240 647.4152 231.0860	No published data
EtOAc	1013.5110	-	$C_{54}H_{77}O_{18}$	0.01	302.3054 416.3242 323.1137 1048.5481 399.3260 267.0866 429.2992 416.3234 323.1138	No published data
EtOAc	1052.5446	-	$C_{68}H_{76}O_{10}$	0.71	316.3210 399.3265 427.2833 344.3149 344.3148 429.3000 383.1340	No published data
EtOAc	1342.8386	-	$C_{89}H_{114}O_{10}$	-1.94	231.0868 327.1086 415.3205 346.2959 295.2279 353.1251 277.2177 663.4114 1325.8127	No published data
EtOAc	839.4217	-	$C_{46}H_{63}O_{14}$	-0.10	332.2799 591.3536 267.0865 327.1102	No published data
EtOAc	870.5022	-	$C_{63}H_{66}O_3$	1.15	265.2536 458.3272 231.0870 399.3269 399.3259 283.2642	No published data

EtOAc	873.4639	-	$C_{47}H_{69}O_{15}$	0.29	360.3106 417.3356 342.3008 267.0877 383.1349 399.3267 462.3576 277.2168	 OSW-1
EtOAc	1310.8511	-	$C_{89}H_{114}O_8$	-0.21	277.2165 399.3271 1293.8229 171.0649 874.4958 417.3349 267.0873 1112.565 346.3311 249.0764 429.2995 647.4162	No published data
EtOAc	649.4323	-	$C_{37}H_{61}O_9$	1.14	324.2903	No published data
EtOAc	853.4375	-	$C_{47}H_{65}O_{14}$	0.43	323.1136 281.1022 853.4378 189.0758 277.2165 411.2904 353.1241 353.1239	No published data
EtOAc	469.3310	-	$C_{30}H_{45}O_4$	-1.67		 Glycyrrhetinic acid
BuOH & EtOAc	329.2320	-	$C_{18}H_{33}O_5$	-2.43	327.2165	 Trihydroxyoctadecadienoic acid
EtOAc	857.4332	-	$C_{46}H_{65}O_{15}$	1.00	855.4150	

EtOAc	783.4179	-	$C_{40}H_{63}O_{15}$	1.54	781.3979	
EtOAc	455.3148	-	$C_{29}H_{43}O_4$	-2.93		No published data
EtOAc	965.4708	-	$C_{74}H_{61}O$	-1.49	965.4698	No published data
BuOH & EtOAc	931.4871	-	$C_{71}H_{63}O$	-0.85		No published data
EtOAc	651.3743	-	$C_{35}H_{55}O_{11}$	-0.21		No published data
EtOAc	667.4021	-	$C_{43}H_{55}O_6$	3.35	667.4033	No published data
BuOH & EtOAc	623.3797	-	$C_{34}H_{55}O_{10}$	0.28	625.3943	No published data
BuOH & EtOAc	913.4587	-	$C_{49}H_{69}O_{16}$	0.15		No published data
BuOH & EtOAc	665.3875	-	$C_{36}H_{57}O_{11}$	-3.89	665.3866	No published data
BuOH & EtOAc	917.4535	-	$C_{48}H_{69}O_{17}$	0.03	917.4550 915.4381	No published data
EtOAc	977.4753	-	$C_{50}H_{73}O_{19}$	0.71		No published data
BuOH & EtOAc	1079.5074	-	$C_{72}H_{71}O_9$	-1.58	1079.5074	No published data
BuOH & EtOAc	871.4634	-	$C_{51}H_{67}O_{12}$	0.17		No published data
BuOH & EtOAc	691.4047	-	$C_{38}H_{59}O_{11}$	-1.50		No published data
BuOH & EtOAc	871.4634	-	$C_{51}H_{67}O_{12}$	0.17	871.4095 873.4234	No published data
BuOH & EtOAc	707.3973	-	$C_{45}H_{55}O_7$	3.56		No published data
EtOAc	1197.6904	-	$C_{91}H_{89}O$	-0.79		No published data

The **Table 3.1** above summarizes the results of untargeted LC-MS-MS analyses in positive ionization mode performed on the EtOAc and *n*-BuOH crude extracts of *O. saundersiae*. The resulting spectral data were processed with the Mzmine 2.51 platform to generate a list of all peaks with its parameters. The peaks were checked manually for identification by MS/MS

interpretation and reputable database search. Most of the identified compounds belong to the steroidal glycoside class and majority of the major compounds have not been reported. However, by analyzing ESI mass spectra data in the literature of complex steroid glycosides, it is clear that the molecular ions are not always observed. It is possible that many of the *m/z* values that are listed in **Table 3.1** are those of fragment ions and not molecular ions. To obtain better results, it will be necessary to optimize the ionization in the mass spectrometer.

3.2.2. Isolation of compounds 170, 171, and 172

In the analysis of *O. saundersiae* bulb extract, the *n*-butanol crude was fractionated by column chromatography (DCM-MeOH; 8:2, Column A), yielding the first set of fractions (**Scheme 3.1**). The combined fractions A60-101 were further purified on Sephadex® LH-20, affording a set of subfractions I of which E3-8 appeared cleaner, but in a small quantity. Semi-preparative HPLC on the E3-8 fraction yielded compound **170** (7 mg), referred to as compound H.

The second compound **171** (10 mg) was isolated as 2U4 following a sequential set of Sephadex® LH-20 columns, and finally preparative HPLC as shown in **Scheme 3.1**. A mixture of **171** and an isomer **172** was afforded as AB4 (24 mg) from A170-198 by column chromatography with a solvent system of $CHCl_3$-EtOAc-MeOH-H_2O (12:13:11:3). All attempts to separate the two isomers were unsuccessful.

TLC analysis was not useful in determining the purity of the compounds because all the compounds had similar R_f values and colour (purple spots) with the spray reagent. NMR played an important role in deducing the purity and identification of the isolates. Characterizations of the three compounds isolated from *O. saundersiae* are described below.

3.2.3. Characterization of compound 170

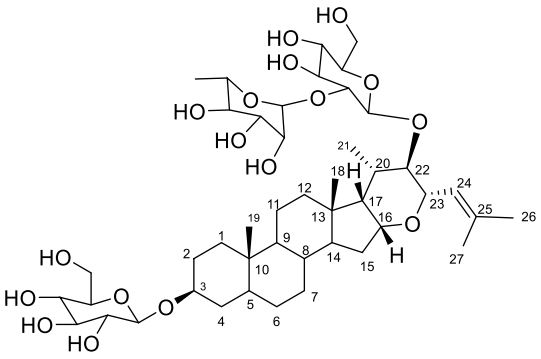


Figure 3.1: Structure of compound **170**

Compound **170** was isolated as an amorphous solid from *O. saundersiae* bulbs. The positive ion high-resolution electrospray mass spectrometry (HR-ESI(+)-TOF) showed an *m/z* 887.4984 $[M + H]^+$ in agreement with a molecular formula $C_{45}H_{74}O_{17}$ (calc. for $C_{45}H_{75}O_{17}$ 887.5004).

The ^{13}C NMR spectrum displayed a total of 45 carbons. In combination with the DEPT spectrum (**Figure 3.2**), these carbons were assigned as six methyl carbons, ten methylene carbons, twenty-five methine carbons, and four non-protonated carbons. Considering the structures of other compounds isolated from *O. saundersiae*, it was suspected that this compound might be a steroidal glycoside. The chemical shifts of three methine carbons observed at δ_c 102.8, 102.5, and 101.0 are characteristic of the anomeric carbons of monosaccharides. A steroid glycoside with three attached sugars to the steroid would account for the forty-five carbons observed in the molecular formula.

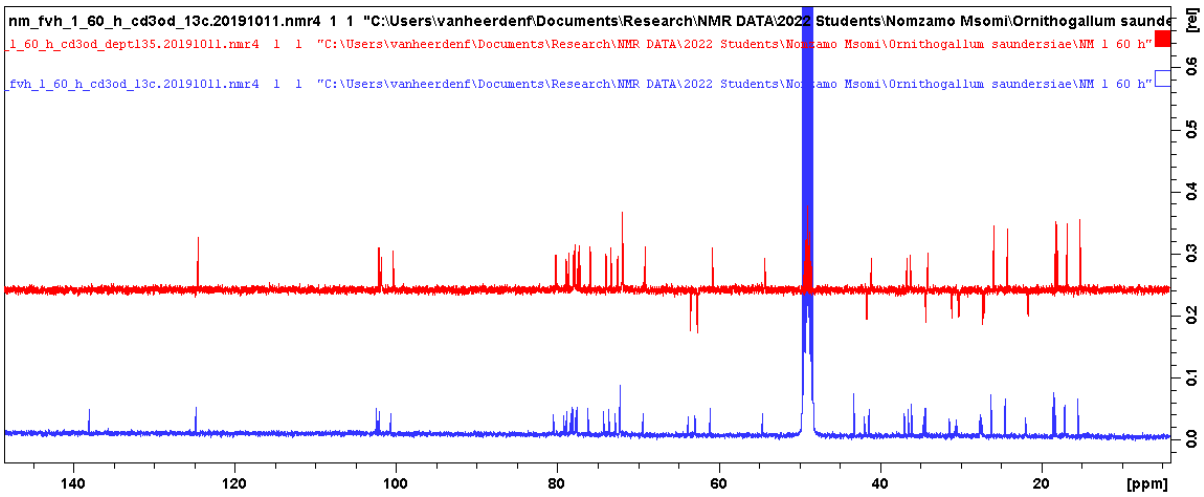


Figure 3.2 ^{13}C and DEPT NMR spectra of **170** (CD_3OD, 100 MHz).

In the ^{1}H NMR spectrum of **170** (**Figure 3.3**), six methyl signals were observed, two singlet methyls at δ_H 1.76 and 1.73, two doublets at δ_H 1.27 and 1.06, and two singlets at δ_H 1.00 and 0.90. The chemical shifts of the methyls resonating δ_H 1.76 and 1.73 are characteristic of methyl groups on a double bond. The chemical shifts of the two singlets at δ_H 1.00 and 0.90 agree with the reported chemical shifts for CH_3-19 and CH_3-18 in steroids.

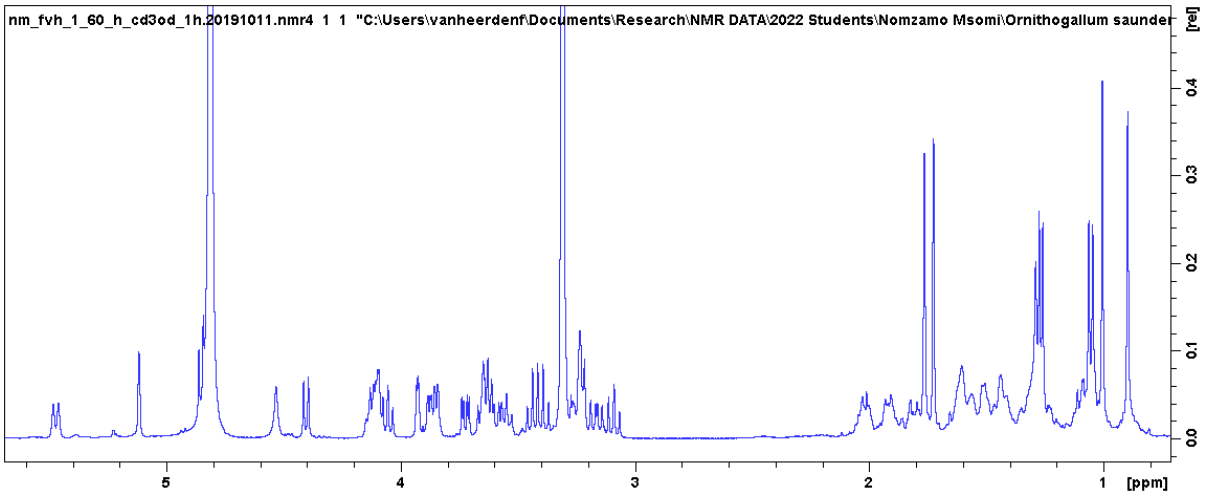


Figure 3.3 ^{1}H NMR spectrum of **170** (CD_3OD, 400 MHz).

HSQC δ_H/δ_C cross-peak-correlations (**Figure 3.4**) connected the proton resonating at δ_H 5.47 (br d, 1H, *J*=8.9 Hz) to the olefinic methine carbon at δ_C 125.2 (C-24) that is part of a trisubstituted alkene. Other important correlations allow us to assign the chemical shifts of the methyl groups.

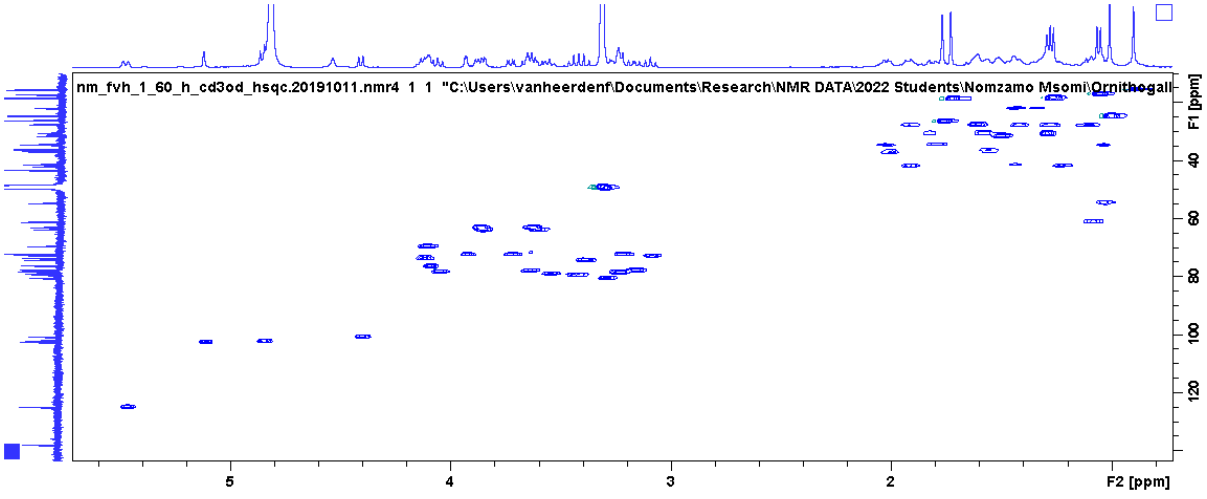


Figure 3.4 HSQC NMR spectrum of **170** (CD_3OD, 400 MHz).

The HMBC spectrum (**Figure 3.5**) was perhaps the most important tool in the structural elucidation of **170**. The methyl protons assigned to CH_3-18 correlated to four carbon signals, a methine resonating at δ_C 61.4 (C-17), a methine at δ_C 54.9 (C-14), a quaternary carbon at δ_C 43.6 (C-13), and a methylene carbon at δ_C 42.3 (C-12). Similarly, two- and three-bond correlations observed for the 19 methyl protons allowed the assignment of C-1, C-5, C-9, and C-10 resonances at δ_C 31.7, 37.4, 41.8, and 36.4, respectively. A doublet methyl proton signal (δ_H 1.06) also correlated to the resonance at δ_C 61.4, assigned to C-17. Therefore, the three proton signal at δ_H 1.06 was assigned to CH_3-21. This proton signal also correlated to a methine carbon at δ_C 34.7 (C -20) and a methine carbon at δ_C 77.9 (C-22). A chemical shift of δ_C 77.9 indicated that C-22 is bonded to an oxygen. The two sp^2 methyls resonating at δ_H 1.73 (δ_C 19.0) and 1.76 (δ_C 26.7) both correlated with the two olefinic carbons resonating at δ_C 125.2 and δ_C 138.3 and were assigned to CH_3-26 and CH_3-27. An expansion of the methyl region of the HMBC spectrum is given in **Figure 3.6**.

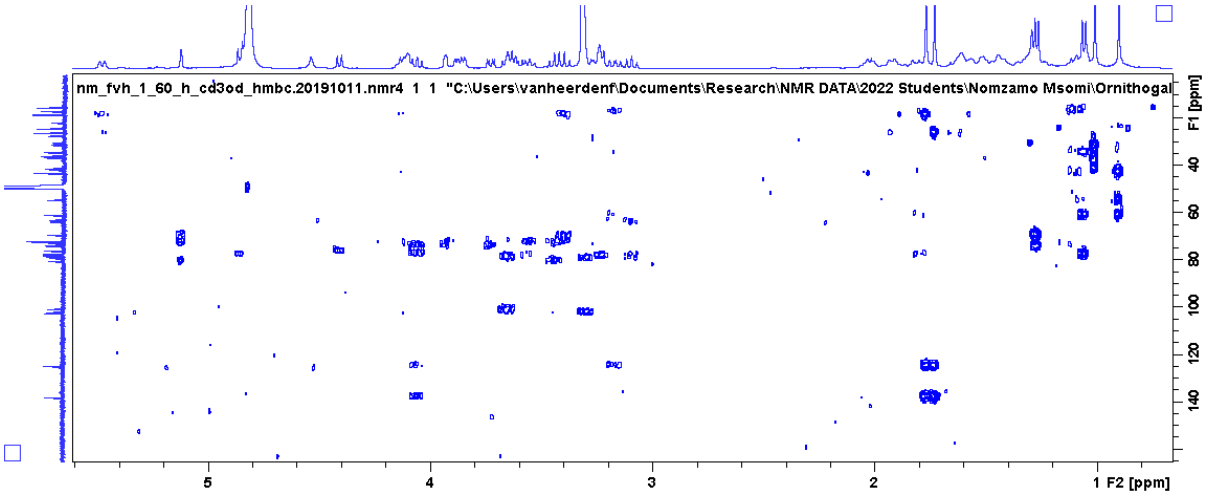


Figure 3.5 HMBC NMR spectrum of **170** (CD_3OD, 400 MHz).

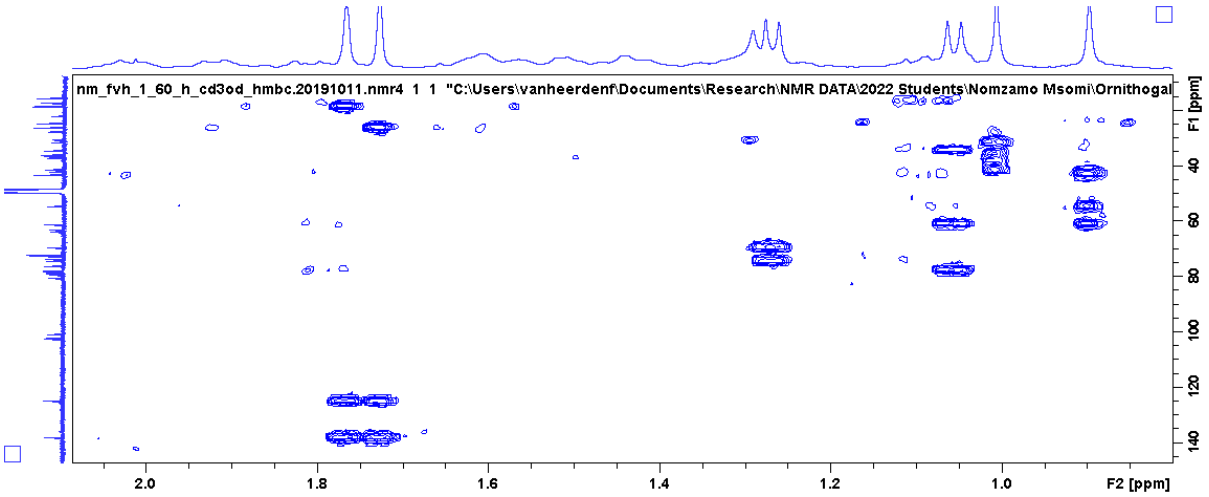


Figure 3.6 Expansion of HMBC NMR spectrum of **170** (CD_3OD, 400 MHz).

In the COSY NMR spectrum of **170** (**Figure 3.7**), a correlation was observed between H-24 (δ_H 5.47) and a proton resonating at δ_H 4.06 (H-23). In turn, this proton correlated to a proton resonating at δ_H 3.16 (H-22). The chemical shifts of H-22 and H-23 indicated that both these protons are attached to oxygen-bearing carbons.

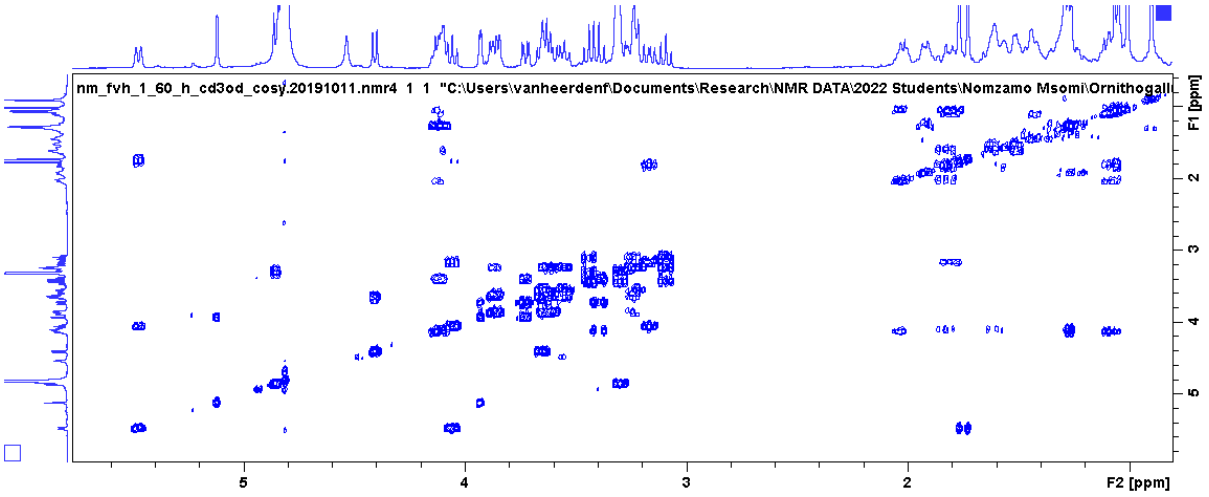


Figure 3.7 COSY NMR spectrum of **170** (CD_3OD, 400 MHz)

To unravel the carbohydrate structures, the anomeric protons and carbons were used as the starting points. The anomeric hydrogen of the first sugar (H-1) at δ_H 5.12 (d, *J*=1.6 Hz, δ_C 102.8) is coupled to the hydrogen resonating at δ_H 3.93 (dd, *J*=3.3 and 1.8 Hz, δ_C 72.6), which, in turn, is coupled to a doublet of doublets resonating at δ_H 3.72 (dd, *J*=9.9 and 3.6 Hz δ_C 72.6). The proton at δ_H 3.72 is coupled to a proton resonating at δ_H 4.12 (δ_C 69.8), which is obscured by three other proton signals but is coupled to the doublet methyl resonance at δ_H 1.27 (d, *J*=6.3

Hz, δ_C 18.7). The couplings observed for this monosaccharide indicated that H-1 and H-2 are both in equatorial positions and that H-3, H-4, and H-5 are all in axial positions. Therefore, this sugar was identified as rhamnose, which normally occurs as the L enantiomer in nature.

HMBC correlations observed at δ_H/δ_C 4.06 (H-23)/74.0 (C-16), and 4.14 (H-16)/78.5 (C-23) suggested a six-membered ring with an oxygen between C-16 and C-23. The coupling constants of H-22 and H-23 allowed the assignment of the relative configuration of this ring. H-22 appeared as a doublet of doublets with *J*=11.1 and 7.9 Hz, indicating that H-20, H-22, and H-23 are all in the axial position (**Figure 3.8**). The resonances of H-16 and H-17 overlapped with other protons and the coupling pattern could not be observed. The configuration of these two carbons were assigned the same as similar compounds in this plant.

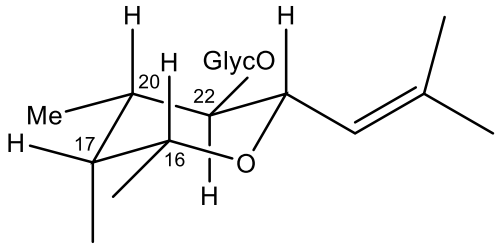


Figure 3.8 Relative configuration of heterocyclic ring of **170**.

The ^{1}H NMR spectrum of **170** contained three doublet signals likely to be anomeric protons of the glycosides at δ_H 5.12 (br d, *J*=1.6 Hz, H-1') for rhamnose 1, δ_H 4.85 (d, *J*=7.7 Hz, H-1'') for glucose 1, and δ_H 4.41 (d, *J*= 7.9 Hz, H-1''') for glucose 2. HSQC spectra assigned the anomeric carbons at δ_C 102.8, 102.5, and 101.0 for rhamnose 1, glucose 1, and glucose 2, respectively, thus providing evidence of three glycoside moieties present. HMBC correlations observed identified the attachment position of the sugar units on the aglycone. The observed correlations were δ_H/δ_C 3.30 (H-2'')/102.8 (C-1'), 3.16 (H-20)/102.5 (C-1''), and 4.10 (H-3)/101.0 (C-1'''), suggesting that rhamnose 1 is attached at position C-2'' of glucose 1, the rhamnose-(1→2)-glucose disaccharide moiety attached at C-22 of the aglycone, and glucose 2 is attached at C-3 of the aglycone.

In the identification of the types of sugars present, the coupling constants of anomeric protons were considered and an eq/eq splitting of *J*=1.6 Hz for H-1 of rhamnose, ax/ax splitting of *J*=7.7 Hz for glucose 1, and ax/ax splitting of *J*=7.9 Hz for glucose 2 were observed. Therefore, the configurations of glucosides moiety are α- and β- for L-rhamnose and D-

glucose, respectively. The assignment of all the ^{1}H and ^{13}C signals are collated in **Table 3.2**. All the HMBC correlations observed in the spectra are showed in **Figure 3.9**. Compound **170** was identified as a rearranged cholestane triglycoside, namely, 3-[(β-D-glucopyranosyl)oxy]-22-[α-L-rhamnopyranosyL-(1→2)-β-D-glucopyranosyl)oxy]-16,23-epoxy-23-(2-methyL-1-propenyl) cholestane triglycoside. The NMR data of this compound are in agreement with those published for related compounds previously isolated from *O. saundersiae*.[53,89] This compound has not been identified previously.

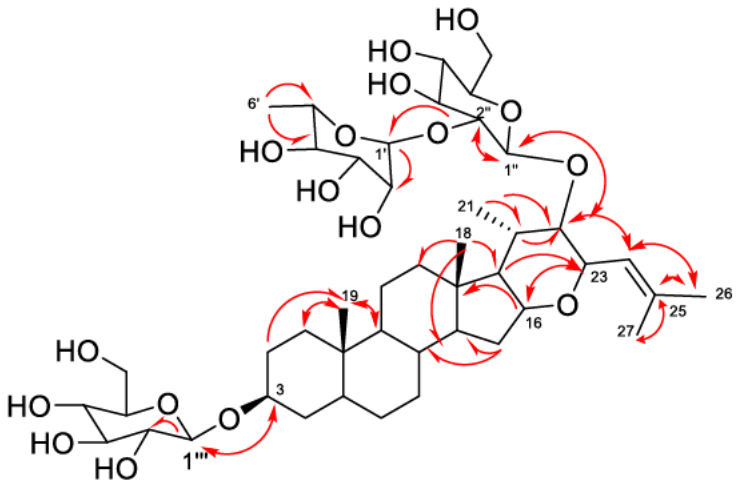


Figure 3.9 Major HMBC long-range ^{13}C-^{1}H coupling observed for compound **170**

Table 3.2. ^{1}H NMR, ^{13}C NMR, HMBC (CD_3OD) spectroscopic data of compound **170**:

Position	δ_H (ppm)	δ_C (ppm)	HMBC correlations
1	1.51 (2H, m)	31.7	19
2	1.62 (2H, s)	27.8	19
3	4.10 (1H)	76.6	1'''
4α	1.03 (1H, m)	35.0	
4β	2.03 (1H, m)		
5	2.00 (1H, m)	37.4	
6α	1.92 (1H, m)	27.9	
6β	1.43 (1H, m)		
7	1.10 (2H, m)	28.0	
8	1.56 (1H, m)	36.9	
9	1.43 (1H, m)	41.8	19
10	-	36.5	3, 4β
11α	1.33 (1H, m)	22.3	
11β	1.44 (1H, m)		
12α	1.23 (1H, m)	42.3	18
12β	1.91 (1H, m)		
13	-	43.6	16

14	1.04 (1H, m)	54.9	
15α	1.57 (1H, m)	30.9	8, 14
15β	1.33 (1H, m)		
16	4.14 (1H, m)	74.0	23
17	1.09 (1H, m)	61.4	18, 23
18	0.90 (3H, s)	15.9	12, 14, 17
19	1.00 (3H, s)	24.9	1, 9
20	1.81 (1H, m)	34.7	21, 22
21	1.06 (3H, d, *J*=6.5 Hz)	17.5	17, 20, 22
22	3.16 (1H, dd, *J*=11.1, 7.9 Hz)	77.9	23, 1'', 4''
23	4.06 (1H, t, *J*=8.4 Hz)	78.5	16, 22
24	5.47 (1H, br d, *J*=8.9 Hz)	125.2	22
25	-	138.3	26, 27
26	1.73 (3H, s)	19.0	24, 25
27	1.76 (3H, s)	26.7	25
Rha 1			
1'	5.12 (1H, d, *J*=1.6 Hz)	102,8	2''
2'	3.93 (1H, dd, *J*= 3.3, 1.8 Hz)	72.6	
3'	3.72 (1H, dd, *J*= 9.5, 3.4 Hz)	72.6	
4'	3.39 (1H, t, *J*=9.6 Hz)	74.6	
5'	4.12 (1H, obs[a])	69.8	
6'	1.27 (3H, d, *J*= 6.3 Hz)	18.7	4', 5'
Glc 1			
1''	4.85 (1H, d, *J*=7.7 Hz)	102.5	22, 2''
2''	3.30 (1H, t, obs[a])	80.8	1'
3''	3.44 (1H, t, *J*= 9.1 Hz)	79.5	
4''	3.09 (1H, t, *J*= 9.2 Hz)	74.6	
5''	3.24 (1H, m)	78.6	
6α''	3.85 (1H, dd, *J*= 12.4, 2.3 Hz)	64.2	
6β''			
Glc 2			
1'''	4.41 (1H, d, *J*= 7.9 Hz)	101.0	3
2'''	3.65 (1H, m)	78.1	3'''
3'''	3.88 (2H, dd, *J*= 5.0, 2.1 Hz)	63.3	
4'''	3.24 (1H, m)	78.7	
5'''	3.55 (1H, t, *J*= 8.6 Hz)	79.2	
6α'''	3.22 (1H, m)	72.6	
6β''	3.87 (1H, dd, *J*= 12.0, 1.5 Hz)		

*The assignments of peaks were made based on COSY, HSQC, and HMBC correlations. The chemical shifts are reported in δ (ppm) and coupling constants are reported in Hz.

[a]Signal obscured by other signals and coupling constants could not be determined.

3.3.3 Characterization of compound 171

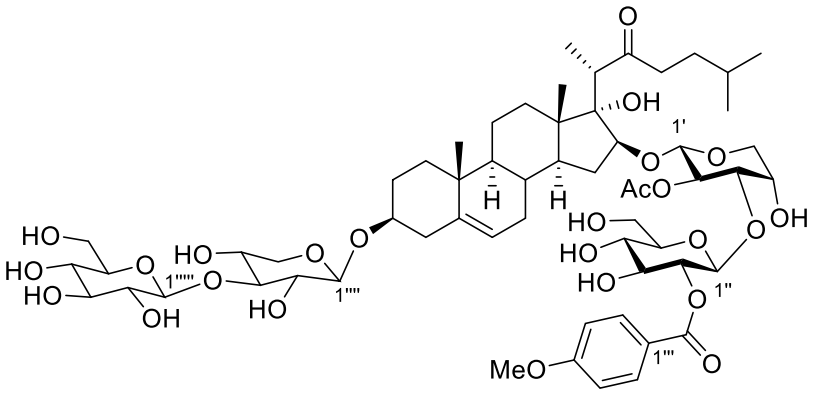


Figure 3.10. Structure of compound **171**.

Compound **171** was isolated as an amorphous solid from *O. saundersiae* bulbs. ^{1}H and ^{13}C spectrometric data displayed a total of 59 carbons (27 for the aglycone and 32 for four glycosides). The ^{1}H and ^{13}C NMR assignment of the aglycone of compound **171** confirmed a steroidal skeleton that was the same as the aglycone of the iqu-known compound OSW-1 (**14**). Thus, **14** and **171** display closely related ^{1}H- and ^{13}C-NMR data.

The ^{1}H NMR spectrum showed the presence of four anomeric protons for the glycosides at δ_H 4.16 (d, *J*=5.6 Hz, H-1'') for glucose A, δ_H 4.36 (d, *J*=8.0 Hz, H-1''''') for glucose B, δ_H 4.73 (d, *J*= 6.9 Hz, H-1') for arabinopyranose, and δ_H 4.38 (d, *J*=5.8 Hz, H-1'''') for xylopyranose, showing correlations in the HSQC spectrum with four carbon signals at δ_C 101.3, 104.9, 103.2, and 103.7, respectively. This was an indication of the presence of sugar moieties. HMBC correlations observed H-1'' of glucose A at δ_H 4.16 and C-3' of arabinopyranose at δ_C 78.0, and H-1' of arabinopyranose at δ_H 4.73 and C-16 of the aglycone at δ_C 89.3 established the presence of a (1→3) linked disaccharide moiety at position C-16 of the steroid. A similar pattern was observed for H-1''''' of glucose B at δ_H 4.36 and C-3'''' of xylopyranose at δ_C 78.0, and H-1'''' of xylopyranose at δ_H 4.38 and C-3 of the aglycone at δ_C 78.1, revealing a second (1→3) disaccharide moiety attached to C-3 of the steroid.

In the identification of the kind of sugar molecules present, the coupling constants of anomeric protons were considered and found to be ax/ax splitting of *J*=5.6 Hz for glucose, ax/ax splitting of *J*=8.0 Hz for glucose, ax/ax splitting of *J*=6.9 Hz for L-arabinose and ax/ax splitting of *J*=5.8 Hz for D-xylose. The coupling constants and comparison of the ^{13}C NMR

signals of the sugars with reference sugar units led to the configurations of glucosides moiety being α-D-galactose, β-D-glucose, α-L-arabinopyranose and β-D-xylopyranose.

The presence of the *p*-methoxybenzoyl and acetyl groups in the molecule was indicated by the ^{1}H NMR signals [δ_H 8.05 (2H, d, *J*=9.1 Hz); δ_H 7.02 (2H, d, *J*=8.9 Hz); δ_H 3.88 (3H, s)] and ^{13}C NMR signals [δ_C 123.6 I; δ_C 133.2 (CH) x 2; δ_C 114.8 (CH) x 2; 166.8 I; 165.4 I; 56.1 (CH_3)] for the *p*-methoxybenzoyl group, and ^{1}H NMR signals [δ_H 1.75 (3H, s)] and ^{13}C NMR signals [δ_C 170.7 I; 20.9 (CH_3)] for the acetyl group as can be seen in the HSQC spectrum.

The 27 carbons due to the aglycone were separated into five methyl, nine methylene, eight methine, and five quaternary carbon groups as derived from the ^{13}C NMR and DEPT spectra. The signals at δ_C 86.8 I, 142.0 I and 122.6 (CH), 89.3 (CH), 78.1 (CH), and 77.9 (CH) were assigned to a carbonyl carbon, a pair of olefinic carbons, and carbons bearing oxygens, respectively. The retrieved data was indicative of the fundamental structure of a cholestene derivative with one carbonyl group and three hydroxyl groups, two of which bear disaccharide iquida. The ^{1}H NMR displayed two tertiary methyl protons at δ_H 0.83 (s, C-18), and δ_H 1.03 (s, C-19), three secondary methyl protons at δ_H 1.10 (d, *J*=7.4 Hz, C-21), 0.81 (d, *J*=6.5 Hz, C-26), and 0.81 (d, *J*=6.5 Hz, C-27). The observed methyl group signals were correlated in the HSQC spectrum with signals at δ_C 12.1 (C-18), 19.8 (C-19), 13.9 (C-21), 23.0 (C-26), and 22.8 (C-27), respectively. The HMBC correlations at δ_H/ δ_C 0.84 (H-21)/77.9 (C-17) and δ_H/ δ_C 3.75 (H-16)/77.9 (C-17) indicated a tertiary hydroxy function at C-17. The presence of a quaternary carbon at δ_C 142.0 (C-5) and a methine at δ_C 122.6 (C-6) indicated the existence of a double bond between C-5 and C-6. The assignment of all the signals is outlined in **Table 3.3**.

Table 3.3. ^{1}H NMR, ^{13}C NMR, (CD_3OD) spectroscopic data of compound **171**:

Position	δ_H (ppm)	δ_C (ppm)
1α	1.09 (1H, m)	38.5
1β	1.85 (1H, m)	
2α	1.56 (1H, m)	30.8
2β	1.96 (1H, m)	
3	3.83 (1H, m)	78.1
4	2.27 (1H, m)	39.8
5	-	142.0
6	5.37 (1H, br s)	122.6
7	1.75 (2H, m)	33.5

8	1.54 (1H, m)	33.2
9	0.92 (1H, m)	51.2
10	-	37.8
11	1.54 (2H, m)	21.7
12	1.96 (2H, m)	33.0
13	-	47.3
14	1.62 (1H, m)	48.9
15α	1.33 (1H, m)	35.3
15β	2.16 (1H, m)	
16	3.75 (1H, m)	89.3
17	3.34 (m)	77.9
18	0.83 (3H, s)	13.9
19	1.03 (3H, s)	19.8
20	2.94 (1H, q, *J*=14.7, 7.4 Hz)	47.1
21	1.10 (3H, d, *J*=7.4 Hz)	12.1
22	-	86.8
23	1.24 (2H, m)	33.4
24	2.18 (2H, m)	40.1
25	1.34 (1H, m)	28.6
26	0.81 (3H, d, *J*= 6.5 Hz)	23.0
27	0.81 (3H, d, *J*= 6.5 Hz)	22.8
Xyl		
1’’’’	4.38 (1H, d, *J*=5.8 Hz)	103.7
2’’’’	3.22 (1H, m)	71.7
3’’’’	3.36 (1H, m)	78,0
4’’’’	3.16 (1H, t, *J*=8.9 Hz)	75.1
5’’’’	4.40 (1H, t, *J*=7.8 Hz)	102.7
Ara		
1’	4.73 (1H, d, *J*=6.9 Hz)	103.2
2’	5.01 (1H, t, *J*=7.6 Hz)	74.6
3’	3.83 (1H, d, *J*=4.7 Hz)	73.6
4’	3.48 (1H, br d, *J*=12.1 Hz)	77.0
5’	4.16 (1H, d, *J* = 5.7 Hz)	70.1
PMB		
1’’’	-	123.6
2’’’	8.05 (1H, d, *J*=9.1 Hz)	133.2 X 2
3’’’	7.02 (1H, d, *J*=8.9 Hz)	114.8 X 2
4’’’	-	166.8
5’’’	3.88 (3H, s)	56.1
6’’’	-	165.4
Glc A		
1’	4.16 (1H, d, *J*=5.6 Hz)	101.3
2’	4.86 (1H, d, *J*=1.8 Hz)	72.2

3’	3.79 (1H, m)	80.6
4’	3.50 (1H, m)	76.9
5’	3.32 (1H, m)	71.6
6’	3.59 (1H, m)	62.8
Glc B		
1’’’’’	4.36 (1H, d, *J*=8.0 Hz)	104.9
2’’’’’	3.21 (1H, m)	71.7
3’’’’’	3.34 (1H, m)	77.9
4’’’’’	3.21 (1H, m)	74.8
5’’’’’	3.57 (1H, m)	80.3
6’’’’’	3.77 (1H, m)	70.3
Ac		
1’’’’’’’’	-	170.7
2’’’’’’’	1.75 (3H, s)	20.9

*The assignments of peaks were made based on COSY, HSQC, and HMBC correlations. The chemical shifts are reported in δ (ppm) and coupling constants are reported in Hz

Compound **172** was isolated as a mixture with **171** and from the NMR data, it was clear that **172** is closely related to **171**. In the ^{1}H NMR spectrum, an unsubstituted benzoyl ring could be observed. However, we do not have enough data to propose a structure for compound **172**.

3.4 CONCLUSION

Despite the extraordinary efforts in phytochemical investigations on *O. saundersiae* for sourcing new and bioactive steroidal glycosides, there is still a significant number of compounds that have not been identified and reported. Advancements in LC-MS-MS techniques and data processing platforms can assist in the search for novel steroidal glycosides in the search for new anticancer agents. In this study, three new steroidal glycosides were isolated from the bulbs of *O. saundersiae* (**170**, **171**, and **172**). Compounds **171** and **172** showed structural similarities with the potent OSW-1 compound previously isolated from *O. saundersiae*.

3.5 EXPERIMENTAL

3.5.1 General experimental procedures

Plant material was weighed on an electronic analytical balance and the extractions were performed on an Infors AG CH-403 Bottmingeni orbital shaker at 25 °C. Analytical grade solvents were used for the extraction and purification. Prominent solvents used were

methanol (MeOH), *n*-butanol (BuOH), ethyl acetate (EtOAc), distilled water (H_2O), and chloroform ($CDCl_3$).

Column chromatography techniques comprised the use of glass columns of varying sizes depending on the amount of extract. The glass column was packed with silica gel (60 F_{254}, 40-63 µm, Merck) and the extract (separately adsorbed on a portion of silica gel) was loaded on a packed column.

Aluminium sheets (20 cm x 20 cm) pre-coated with Kieselgel 60 F_{254}, 0.25 mm (Merck) were used to carry out analytical thin-layer chromatography (TLC) to track the complexity of extracts and purity of compounds. Developed TLC plates were examined under ultraviolet (UV) light at two different wavelengths (λ 254 nm or λ 365 nm). Components undetectable under UV were further stained with an anisaldehyde stain reagent. The anisaldehyde stain reagent was prepared by mixing the reagents below in a 1 L volumetric flask and then placed in an ice bath to cool for 15 minutes and stored in a fridge (~4 °C).

- 85 mL of MeOH
- 10 mL of glacial acetic acid
- 2 mL of concentrated sulfuric acid
- 0.5 mL of *p*-anisaldehyde (98%)

A series of Sephadex® LH-20 (Fluka) gel filtrations were used to purify complex fractions retrieved through column chromatography. A Sephadex® LH-20 G slurry in MeOH was prepared and packed in an open glass column. Samples were prepared by dissolving it in a minimum volume of MeOH and the solution was then loaded in small amounts with a pipette on the open column. Analytical grade MeOH was used as the mobile phase.

Pure compounds were analyzed and isolated from small quantities of mixtures achieved by Sephadex® LH-20 column using semi-preparative High Performance Liquid Chromatography (HPLC). These fractionations were carried out on a SHIMADZU HPLC equipped with SIL-20A autosampler, SPD-M20A diode-array UV detector, LC-20AB P2000 pump, CBM-20A communication bus module, analytical LUNA 5µ C18 (250 x 4.60 mm) for sample analysis, and LUNA column 5µ C18 (250 x 10 mm) for semi-preparative HPLC. The column temperature was maintained at 21 °C and the columns were detected at a wavelength range of 210-450 nm.

The mobile phase consisted of a binary solvent system of a HPLC grade mixture of MeOH:I (4:3) + 0.1% formic acid (A) and ultra-pure water + 0.1% formic acid (B). An isocratic elution was used at a flow rate of 0.5 mL/min for analysis, and 1.8 mL/min for semi-preparative HLPC with a run time of 45 minutes. Each run was followed by a shutdown run for washing the column and restoring to initial conditions. Samples were dissolved in HPLC grade MeOH. For the analytical experiments, a concentration of 200 ppm was maintained, injecting 10 µL for each run. For semi-preparative HPLC experiments, samples were prepared at 4000 ppm concentration and injected at 300 µL for each run. All the sample solutions were filtered through 0.45 µm GHP (PALL, life sciences) membrane filter prior injection to remove any dust or solid impurities.

^{1}H and ^{13}C NMR spectral data of isolated compounds were recorded on a Bruker Avance lll 500 spectrometer, operating at 500 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, in CD_3OD solution at room temperature. The chemical shifts (δ) were determined relative to TMS (δ = 0 ppm) and recorded in parts per million (ppm) relative to the solvent peak ($CDCl_3$) δ_H 7.26 ppm and δ_C 77.02. The NMR experiments used for structural elucidation were ^{1}H, ^{13}C, COSY, DEPT-135, HSQC and HMBC. The coupling constants (*J*) are reported in Hertz (Hz). Multiplicities are abbreviated as s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), t (triplet), and m (multiplet).

For liquid chromatography-mass spectrometry (LC-MS) data of the isolated compounds, a SHIMADZU LCMS-2020 equipped with a Shim-pack GIST (3 µm C_{18}-HP, 4.6 x 150 mm) column and a quadrupole mass detector was used. Samples for the LC-MS were prepared at a concentration of ~200 ppm for extracts and ~10 ppm for pure compounds.

The LC-MS-MS analysis of EtOAc and BuOH crude extracts (bulb) were performed on a Waters Synapt G2 qTOF (Milford, USA) mass spectrometer linked to a Waters Acquity UPLC.

3.5.2 Collection of plant material

The bulb plant material of *O. saundersiae* was collected from Dr Elliot Ndlovu's farm (kwaMalulekoes herb farming) situated in Mooi River, KwaZulu-Natal province of South Africa. The plant was identified by Dr Elliot Ndlovu, a renowned herbalist and traditional healer practising as a *sangoma* and *inyanga*.

3.5.3 Extraction of plant material

The bulbs of *O. saundersiae* were chopped into smaller pieces and dried in a fume hood for 9 days, and then pulverized into a fine powder. The dried powder (200 g) was extracted with 1.5 L of MeOH. The mixture was extracted for 72 h on an electronic shaker. Thereafter, the mixture was filtered by gravitational filtration using a funnel and Whatman No. 1 filter paper and the filtrate was concentrated on a rotatory evaporator at 60 °C. The extract was dried further under high vacuum to yield 10.90 g of the crude bulb.

Methanol crude extract (10.90 g) was dissolved in 150 mL of distilled water and partitioned twice with hexane, EtOAc, and *n*-BuOH (150 mL in each partitioning). All the fractions were concentrated under vacuum on a rotatory evaporator. The masses of the extracts recovered for each material is summarised in **Table 3.5**.

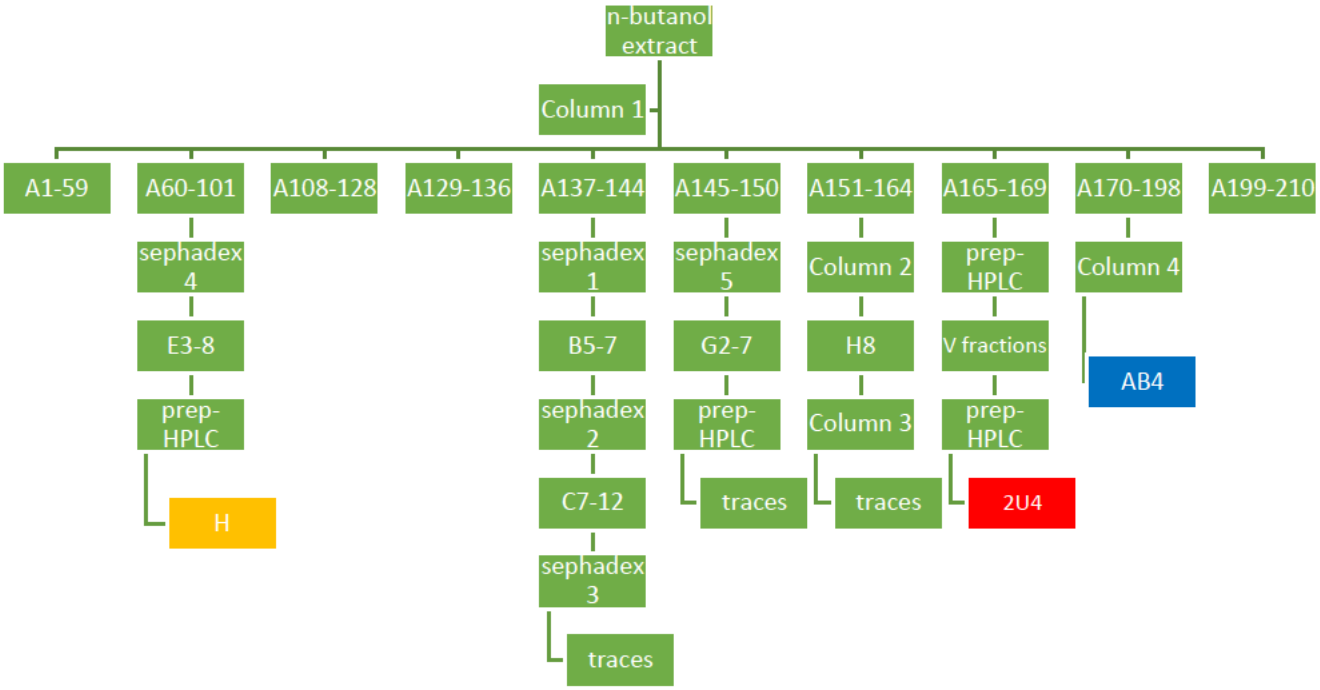
Table 3.5: Mass of fractions obtained from iquid-liquid fractionation of the *O. saundersiae* methanol extract.

Fractions	**Mass (g)**
Hexane	0.2447
Ethyl Acetate	2.3186
n-Butanol	5.2849
Water	1.2055

Further studies were carried on the *n*-butanol fraction.

3.5.4 Isolation of pure compounds

Pure compounds were isolated by sequential column chromatography, Sephadex® LH-20 gel filtrations and preparative HPLC methods as described in **Scheme 3.1**. The pure compounds indicated in orange, red and purple blocks were isolated using mixtures of $CDCl_3$, EtOAc, MeOH and H_2O in varying ratios.



Scheme 3.1: Schematic diagrams describing the reporting on all the experimental work done on crude extract and isolation of pure compounds from the n-butanol extract.

3.5.5 Physical and spectral data of the isolated compounds

3-[(β-D-Glucopyranosyl)oxy]-22-[α-L-rhamnopyranosyL-(1→2)-β-D-glucopyranosyl)oxy]-16,23-epoxy-23-(2-methyL-1-propenyl) cholestane triglycoside (**170**): amorphous solid; IR ν_{max} 3363, 2930, 1511, 1370, 1038, 768 cm^{-1} ; ^{1}H-NMR (CD_3OD , 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz), see **Table 3.1**; HR-ESI(+)-TOF (positive-ion mode) *m/z* 887.5004 $[M + H]^+$.

Cholestane glycoside (**171**): Amorphous solid; IR ν_{max} 3365, 2929, 1603, 1509, 1367, 1252, 1015, 769 cm^{-1} ; ^{1}H-NMR (CD_3OD , 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz), see **Table 3.2**.

Cholestane glycoside (**171**) + cholestane glycoside (**172**): amorphous solid; IR ν_{max} 3350, 2933, 1608, 1256, 1016, 849 cm^{-1}; ^{1}H-NMR (CD_3OD , 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz), see **Table 3.3**.

3.5.6 LC-MS-MS Analysis

The analysis of *O. saundersie* crude extracts (*n*-butanol and ethyl acetate extracts) were performed on a Synapt G2 qTOF from Waters (Milford, USA), a high quadrupole time-of-flight (qTOF) mass spectrometer with ESI ionization source. The Waters HSS T3 column, 2.1x150 mm was used. The mobile phase consisted of a water-0.1% formic acid solution as solvent A and HPLC grade Acetonitrile as solvent B. the gradient conditions were set at 100% for A and 0% for B at 0.50 min; 0% for A and 100% for B at 12 min; 100% for A and 0% for B at 13 min; and ending of at 100% for A and 0% for B at 15 min at a consistent flow rate of 0.400 mL/min. Data was acquired and reported in positive and negative ionization modes in the *m/z* range 100-2000. The mass spectral data was processed in Synapt_msE_processing_V1.

3.5.7 Global Natural Products Social Molecular Networking (GNPS)

LC-MS-MS raw spectral data obtained from the analysis of *n*-butanol and ethyl acetate crude extracts of *O. saundersiae* bulbs were analysed in molecular networks compiled on the GNPS web-based platform. Prior to the creation of networks, the MS^2 datasets were converted to mzML format with MS-Convert and thereafter uploaded on the GNPS platform through ProteoWizard application. The parameters for molecular network generation were set according to the following:

- The precursor ion mass tolerance of 1.0 Da
- The product ion tolerance of 0.5 Da
- A cosine score of 0.65

Other parameters were kept at the GNPS recommended parameters. The annotation of compounds was performed by manual interpretation of MS^2 spectra of each node and compared with the spectral databases offered by GNPS platform. The MS^2 molecular network is accessible at the GNPS website at the link:

http://massive.ucsd.edu/ProteoSAFe/

CHAPTER 4: PHYTOCHEMICAL INVESTIGATION OF *ALBUCA BATTENIANA* Hilliard & B.L.Burtt

4.1. INTRODUCTION

Albuca L. is a genus of flowering plants of perennial geophytes with bulbs that can be epigeal (growing partially above ground) or hypogeal (growing underground).[100] There are approximately 80 species globally, of which about 60 are found in southern Africa.[58] Some *Albuca* species are distributed in northern Africa and the Arabian Peninsula.[101]

In a recent paper on the taxonomy of the Ornithogoloideae subfamily, Manning et al. used DNA results to accommodate all the genera of this subfamily under one genus, *Ornithogalum* L. This classification includes *Albuca* and the genera *Dipcadi* (Dalz.) Baker, *Galtonia* Decne., *Neopatersonia* Schönland and *Pseudogaltonia* (Mast.) E.Phillips. However, based on morphology, other botanists did not accept all the changes in this classification and retained some of the older names of the genera, such as *Albuca*. *Albuca* species can be distinguished from those of *Ornithogulum* L. by the thick flowers, with white or yellow tepals with a green or brown band on the adaxial surface and simple veins ranging from 3-5 on each tepal.[38]

Albuca species are often used as traditional medicine for protective charms, protection against food poisoning, and the management and treatment of wounds, rheumatoid arthritis, and management of diabetes.[58,102] Because it is difficult to differentiate between the different *Albuca* species, the common names *uMaphipha intelezi* (IsiZulu) and *Inqwebeba* (IsiXhosa) are used for any *Albuca* species.[7] Despite its popularity among the Zulu and Xhosa traditional healers, limited information on the phytochemical profile of the *Albuca* genus is available. *A. iquidate* Dryand., *A. iquidat* (Thunb.) J.C.Manning & Goldblatt, and *A. setosa* Jacq. Are amongst the most researched species in this genus.

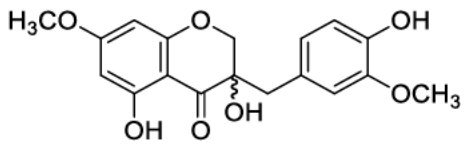
Folkloric usage of species of this genus resulted in the investigation of extracts for anti-inflammatory potential. In the following paragraphs, *Albuca species* on which phytochemical studies have been performed are discussed briefly.

***Albuca iquidat* (Thunb.) J.C.Manning & Goldblatt**

119. *iquidat*, commonly known as *Inqwebeba* in IsiXhosa, has been used by traditional healers for the management of diabetes mellitus, treatment of malignant tumours and as an aphrodisiac in the Eastern Cape, South Africa.[7,103] Qualitative phytochemical analysis of *A. iquidat* by Odeyemi et al. in 2017 revealed that flavonols, flavonoids, phenols, proanthocyanidins, saponins, and tannins are the major secondary metabolites present in the plant.[9] However, no specific compounds were isolated.

***Albuca iquidate* Dryand.**

119. *iquidate* Dryand. Is a plant with white flowers found mainly in the east coast region of South Africa. The IsiZulu name is *uMaphipha intelezi*, and it is used by the Zulu people and traditional healers as a protective charm and for the treatment of witchcraft-related food poisoning called *idliso*.[100,104] The bulbs of this plant can be found at the ethnomedicinal markets in Durban and Johannesburg (South Africa). Koorbanally et al. (2004) reported the first phytochemical study of *A. iquidate,* and one compound was isolated; a novel 3-hydroxy homoisoflavanoid (**173**)[100] (**Figure 4.1**). No biological assays on the extracts and compound **173** have been reported.



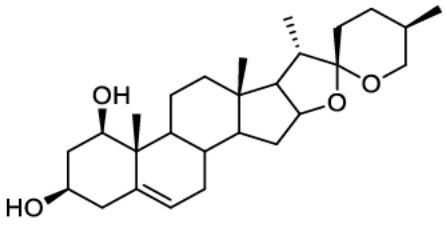
173

Figure 4.1. Homoisoflavanoid isolated from *A. iquidate*.

***Albuca nelsonii* N.E.Br.**

119. *nelsonii* N.E.Br. is a bulbous perennial plant that grows in clumps. Endemic to South Africa, this plant grows in the Gauteng, Eastern Cape, and KwaZulu-Natal regions and flowers from September to November. Zulu people call the plant *uMaphipha intelezi,* and the English common name is candelabrum lily. Phytochemical studies on *A. nelsonii* have been associated with only one steroidal sapogenin

aglycone, ruscogenin (**174**), isolated after acid treatment of the crude extract.[105] There is no scientific evidence of any biological activities of this compound to date.



174

Figure 4.2. Structure of ruscogenin (**174**).

Buwa and Staden (2005) investigated the antibacterial and antifungal activity of *A. nelsonii* bulbs and 12 other South African plants used against venereal diseases.[106] The aqueous, ethanolic, and ethyl acetate extracts of each plant were screened against *Bacillus subtilis* (ATCC No. 6051), *Escherichia coli* (ATCC No. 11775), *Klebsiella pneumoniae* (ATCC No. 13883), and *Staphylococcus aureus* (ATCC No.12600). Some of the extracts of *A. nelsonii* showed low antibacterial activity at a minimal inhibitory concentration (MIC) >12.5 mg/mL.[106]

***Albuca setosa* Jacq.**

119. *setosa* is commonly used by traditional healers for treating wounds, managing diabetes, and for ritual washing to protect against bad luck.[9,58,107] The species is endemic to southern Africa (South Africa, Lesotho, Swaziland, Botswana, Namibia, Mozambique, and Zimbabwe) and is considered one of the most traded and used species in South Africa, endangering this species.[102,108] No phytochemical studies have been published on this plant.

***Albuca batteniana* Hilliard & BL Burtt.**

Albuca batteniana is endemic to South Africa and is commonly found in the Western Cape and Eastern Cape, South Africa. It is grown as a garden flower and is widely known to Zulus and other ethnic groups for its good luck charm properties. Zulu people refer to plants with these properties as *Intelezi*.[109]

119. *batteniana* was also named *Ornithogalum batteniana* in some articles following the revision of the generic synopsis of Hyacinthaceae in sub-Saharan Africa by Manning et al. (2004), who classified this species under *Ornithogalum* L.[65] However,

it was re-instated as *Albuca* upon further molecular studies.[57] No phytochemical studies have been conducted on *A. batteniana*.

In this chapter, the phytochemical investigation of the bulbs of *A. batteniana* is described.

4.2. RESULTS AND DISCUSSION

4.2.1. Isolation of 3-*O*-β-D-glucopyranosyL-β-sitosterol (175)

Different approaches to isolate steroidal glycosides from *A. batteniana* were investigated in this study. The first experiments followed analytical methods used in the identification and isolation of steroidal glycosides from different *Ornithogalum* species.[53,89] This approach (described below) yielded no substantial results and no compounds could be isolated or identified (Pilot study 1). We then proceeded to apply a method used on *A. nelsonii* bulbs (Pilot study 2).[105] Below are the results and discussion of the two approaches.

Pilot study 1:

Fractions obtained by column chromatography were tracked by TLC. In all stages of the fractionation, major dark spots were observed on TLC that could possibly indicate the presence of carbohydrates. The most likely carbohydrates, glucose and sucrose, were analysed and compared by TLC ($CHCl_3$-EtOAc-MeOH-H_2O; 12:13:11:3). The results showed similar R_f values for both glucose and sucrose and components of extract as seen in **Figure 4.3.** We therefore conclude that glucose and sucrose are present as the major components in the crude extract of *A. Batteniana*.

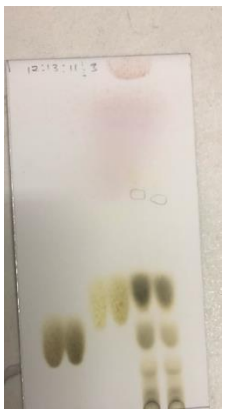


Figure 4.3. TLC plate showing the observed R_f values for glucose, sucrose, and *n*-butanol crude extract.

Other compounds with high R_f values on the TLC plates in the crude extract appeared only after staining and might be fatty acid derivatives or other non-polar compounds (**Figure 4.4**). The first pilot study was rendered unsuccessful for the identification and isolation of steroidal glycosides from *A. batteniana.*

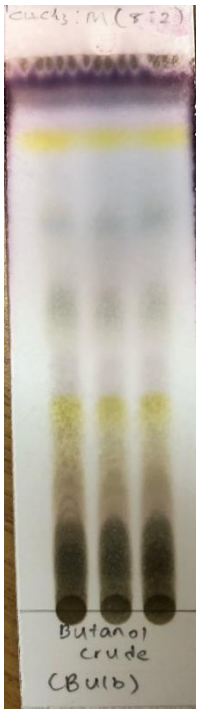


Figure 4.4. TLC plate showing different components in the *A. batteniana* crude extract.

It was decided to proceed with the method in pilot study 2, inspired by the isolation of ruscogenin (**174**) from *A. nelsonii*.[105] In this approach, the crude extract was treated with acid to hydrolise the complex glycosides to yield the major aglycone, ruscogenin.

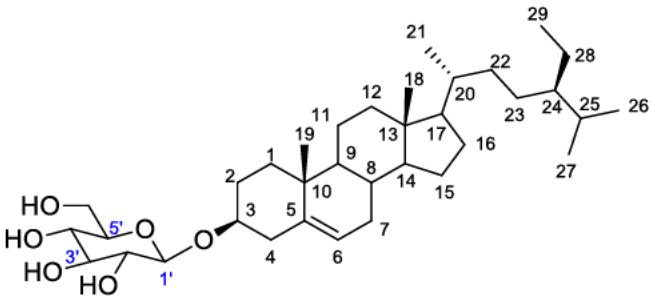
Pilot study 2:

The MeOH bulb extract of *A. batteniana* was subjected to a combination of reflux in acidic solution, extraction of the aqueous solution and a series of classical open column chromatographic methods to yield 3-*O*-β-D-glucopyranosyL-β-sitosterol (**175**) (fraction 4B, 12 mg).

4.2.2. Characterization of 3-*O*-β-D-glucopyranosyL-β-sitosterol (175)

3-*O*-β-D-GlucopyranosyL-β-sitosterol (**175**) was isolated and identified for the first time in *A. batteniana*. Compound **175** was identified on the basis of ^{1}H and ^{13}C NMR experiments, including HSQC and HMBC experiments, which were used to obtain unambiguous ^{1}H and ^{13}C

NMR assignments for the steroidal glycoside.



175

Figure 4.5. Structure of 3-*O*-β-D-glucopyranosyL-β-sitosterol.

3-*O*-β-D-Glucopyranosylsitosterol ($C_{35}H_{60}O_6$) was isolated as a white solid from the MeOH bulb extract of *A. batteniana*. The LR-MS spectrum (negative mode) revealed a chlorine adduct $[M+Cl]^-$ with *m/z*=611 as shown in **Plate 19**. This *m/z* value corresponds to a molecular formula of $C_{35}H_{60}O_6Cl$. The ^1H NMR spectrum (**Plate 20**) revealed two tertiary methyl groups at δ_H 0.69 (s, 3H, H-18), and δ_H 1.00 (s, 3H, H-19), three secondary methyl groups at δ_H 0.92 (d, 3H, *J*= 6.4 Hz, H-21), δ_H 0.81 (d, 3H, *J*=8.1 Hz, H-26), and δ_H 0.82 (d, 3H, *J*= 7.6 Hz, H-27), and one primary methyl group at δ_H 0.81 (t, 3H, *J*=7.2 Hz, H-29). The two singlet methyl resonances at δ_H 0.69 and δ_H 1.00 are characteristic of the resonances of H-18 and H-19, respectively, of a steroid. A doublet observed in the ^1H spectrum at δ_H 5.35 with a splitting of *J*=3.3 Hz represented the olefinic proton (H-6). An HSQC (**Plate 23**) cross-peak at δ_H/δ_C 5.35 (d, *J*=3.3 Hz)/122.7 (C-6), along with HMBC correlations (**Plate 24**) between δ_H 1.00 (H-19) and δ_C 141.6 (C-5), and δ_H 5.35 (H-6) and δ_C 141.6 (C-5) indicate that a $\Delta^{5,6}$ double bond is present. A multiplet signal observed downfield at δ_H 3.58 was assigned to H-3. The H-24 signal was also identified with the COSY and HMBC correlations, where both H-26 and H-27 correlated with C-24 at δ_C 47.0.

From the NMR data, it was clear that a carbohydrate was also present in the molecule. For the glycone, the ^1H NMR spectrum showed one anomeric proton resonating at δ_H 4.38 (d, *J*=8.2 Hz) with HMBC correlations between δ_H 4.38 (H-1') and δ_C 79.8 (C-3), indicating the carbohydrate moiety is attached to C-3 of the steroid. The large coupling constant of the anomeric proton (*J*=7.8 Hz) indicated that this proton is in the axial position. The COSY spectrum (**Plate 22**) was useful in connecting the resonances from the carbohydrate moiety

starting from the anomeric proton. The coupling constants of H-2 (δ_H 3.18), H-3 (δ_H 3.37), H-4 (δ_H 3.33), and H-5 (δ_H 3.27) indicated that all these protons are axial and, therefore, the sugar moiety is glucose. The ^{1}H-^{1}H correlations observed in a COSY experiment on glucose are illustrated in **Figure 4.6**. The long-range ^{13}C-^{1}H correlations observed in an HMBC experiment are illustrated in **Figure 4.7**. The ^{1}H and ^{13}C NMR data assigned for 3-*O*-β-D-glucopyranosyL-β-sitosterol is in agreement with reported data.[110,111]

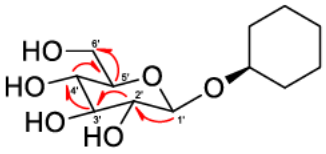


Figure 4.6. COSY ^{1}H-^{1}H correlations observed for 3-*O*-β-D-glucopyranosylsitosterol (**175**).

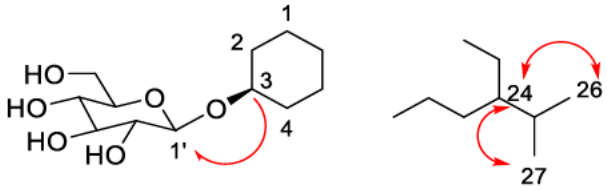


Figure 4.7. HMBC long-range ^{13}C-^{1}H correlations observed for 3-*O*-β-D-glucopyranosyL-β-sitosterol (**175**).

With the hydrolysis of the crude extract, it was expected that all glycosidic bonds will be hydrolysed and that the aglycone will be isolated. However, compound **175** was isolated as a steroidal glycoside. This indicates that the present glycosidic bond in 3-*O*-β-D-glucopyranosyL-β-sitosterol is strong.

3-*O*-β-D-GlucopyranosyL-β-sitosterol has been isolated from a large number of plant species. Reported biological activities associated with 3-*O*-β-D-glucopyranosyL-β-sitosterol are anti-inflammatory properties and anti-tumour activities (breast cancer).[112]

4.3. CONCLUSION

In conclusion, one steroidal glycoside, 3-*O*-β-D-glucopyranosyL-β-sitosterol, was isolated. This is the first report of the presence of this compound in *A. batteniana* and the *Albuca* genus. TLC evaluation of the extracts showed that there are significant amounts of the carbohydrates glucose and sucrose in the bulb extracts.

Table 4.1. ^{1}H NMR and ^{13}C NMR (CD_3OD-$CDCl_3$) spectroscopic data of **175**.

Position	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) (Literature)[111]
1_{ax}	1.08 (t, 1H, *J*= 9.9 Hz)	38.3	
1_{eq}	1.85 (m, 1H)		
2	1.18 (t, 2H, *J*= 6.1 Hz)	26.9	
3	3.58 (m, 1H)	79.8	3.54 (m, 1H)
4_{ax}	1.16 (m, 1H)	40.8	
4_{eq}	2.01 (m, 1H)		
5	-	141.6	
6	5.35 (d, 1H *J*= 3.3 Hz)	122.7	5.31 (s, 1H)
7	1.85 9m, 2H)	29.1	
8	0.92 (1H)	51.3	
9	0.92 (1H)	37.1	
10	-	37.6	
11	1.50 (2H)	21.9	
12_{ax}	2.27 (t, 1H, *J*= 11.6 Hz)	39.5	
12_{eq}	2.40 (1H)		
13	-	43.2	
14	1.13 (1H)	57.1	
15	1.33 (2H)	34.9	
16	2.58 (t, 2H, *J*= 6.4 Hz)	28.6	
17	1.37 (1H)	37.1	
18	0.69 (s, 3H)	12.4	
19	1.00 (s, 3H)	19.8	
20	1.02 (1H)	57.8	
21	0.92 (d, 3H, *J*= 6.4 Hz)	19.4	0.88 (d, 3H, J=6.3 Hz)
22	2.32 (2H)	34.9	
23	1.28 (2H)	23.9	
24	0.92 (d, 1H, *J*=6.4 Hz)	47.0	
25	1.65 (1H)	30.1	
26	0.81 (d, 3H, *J*=8.1 Hz)	19.4	0.79 (d, 3H, *J*=7.7 Hz)
27	0.82 (d, 3H, *J*= 7.6 Hz)	20.2	0.80 (d, 3H, *J*=7.0 Hz)
28	1.25 (2H)	23.5	
29	0.81 (t, 3H, *J*=7.20 Hz)	18.9	0.81 (t, 3H, *J*=6.6 Hz)
1’	4.38 (d, 1H, *J*= 7.8 Hz)	102.2	4.35 (d, 1H, *J*= 9.4 Hz)
2’	3.18 (t, 1H, *J*=8.3 Hz)	74.7	3.23 (m, 1H)
3’	3.37 (t, 1H, *J*=8.7 Hz)	77.7	3.38 (m, 1H)
4’	3.33 (t, 1H, *J*=8.2 Hz))	71.3	3.39 (m, 1H)
5’	3.27 (m, 1H)	77.4	3.30 (m, 1H)
6’	3.83 (dd, 2H, *J*= 12.0, 2.3 Hz)	62.5	3.68 (d, 1H, *J*=10.3 Hz)

*The assignments of peaks were made on the basis of COSY, HSQC, and HMBC correlations. The chemical shifts are reported in δ (ppm) and coupling constants are reported in Hz.

4.4. EXPERIMENTAL

4.4.1. General experimental procedures

In addition to the general experimental procedures given in Section 3.1, the following techniques were used specifically in the investigations of *A. batteniana*. ^{1}H and ^{13}C NMR spectral data of isolated compounds were recorded on a Bruker Avance lll 400 spectrometer, operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, in CD_3OD: $CDCl_3$ (1:1) solution at room temperature. Prominent solvents used for column chromatography and TLC were methanol (MeOH), *n*-butanol (BuOH), ethyl acetate (EtOAc), distilled water (H_2O), diethyl ether, and chloroform ($CHCl_3$).

4.4.2. Collection of plant material

The plant material of *A. batteniana* was collected from Prof van Heerden's garden, KwaZulu-Natal province of South Africa. The plant was identified by Ms Alison Young, curator of the botanical garden, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg campus. A voucher specimen (Accession number: NU0092222) was prepared and deposited at the University of KwaZulu-Natal Bews Herbarium (NU).

4.4.3. Extraction of plant material

The leaves and bulbs of *A. batteniana* were separately chopped into smaller pieces and dried in a fume hood for 7 days. The dried material was pulverized into fine powders. The dried bulb powder (88 g) was extracted with 900 mL of methanol and 43 g of the dried leaf powder was extracted with 700 mL methanol. The two mixtures were extracted for 48 h on an electronic shaker. The mixture was filtered by gravitational filtration using a funnel and Whatman No. 1 filter paper and the filtrate was concentrated on a rotatory evaporator at 60 °C. Subsequently, the extract was dried further under high vacuum to yield 17.54 g of the crude bulb extract and 4.89 g leaf extract. Further studies were continued on the bulb extract for both pilot studies.

<u>Pilot study 1:</u>

17.54 g Methanol crude extract was dissolved in 150 mL of distilled water and partitioned twice with each of the following solvents: hexane, ethyl acetate and *n*-butanol (150 mL in

each partition). All the fractions were concentrated under vacuum on a rotatory evaporator. The masses of the extracts recovered for each fraction are summarised in **Table 4.2**.

Table 4.2.: Mass of fractions obtained from iquid-liquid fractionation of the bulb extract.

Fractions	**Mass (g)**
Hexane	0.6080
Ethyl Acetate	0.3716
n-Butanol	0.9518
Water	15.4839

The majority of steroidal glycosides are known to reside in the *n*-BuOH fraction, thus further studies were carried out on the 0.95 grams extract. We experienced significant difficulties with finding suitable solvents for the extracts when running columns. A series of columns were run with solvent systems involving MeOH and $CHCl_3$ at varying ratios depending on the polarity of the extracts. However, it was not possible to isolate a pure compound.

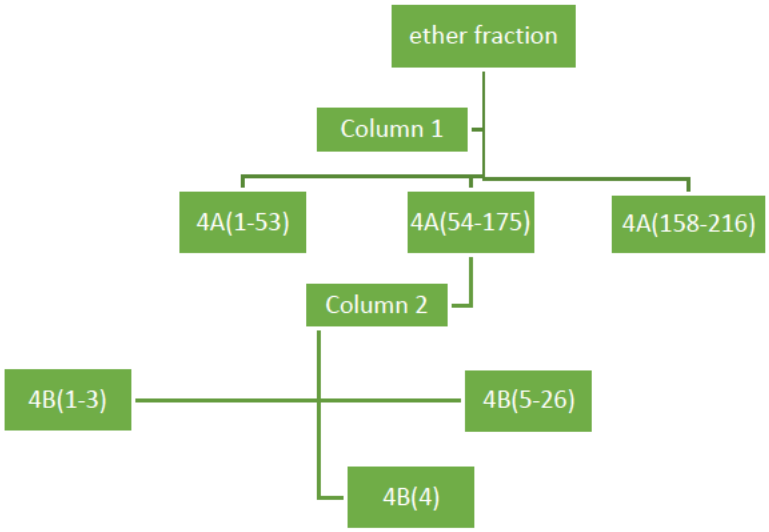
Pilot study 2:

The second extraction was conducted in a similar manner as Pilot study 1. The dried bulb powder (81 g, obtained in a second extraction of the plant material) was extracted with 830 mL of methanol and 43 g of the dried leaf powder was extracted with 400 mL MeOH. The two mixtures were extracted for 48 h on an electronic shaker. The mixture was filtered by gravitational filtration using a funnel and Whatman No. 1 filter paper and the filtrate was concentrated on a rotatory evaporator at 60 °C. Subsequently, the extract was dried further under high vacuum to yield 14.08 g of the crude bulb extract and 6.31 g leaf extract.

The crude extract (6.96 g) was refluxed for 5 hours in 50 mL MeOH containing a 32% HCl (2.5 mL) solution. The solution was then extracted three times with 150 mL diethyl ether (450 mL in total). The ether fraction was dried under vacuum with a rotatory evaporator to yield a mass of 4.87 g.

4.4.4. Isolation of 3-*O*-β-D-glucopyranosylsitosterol (175)

The ether extract was fractionated by column chromatography. The first column was run with a solvent system consisting of $CHCl_3$-EtOAc (8:2) and 216 fractions were collected. Fractions with a similar TLC profile were combined to yield 3 sub-frations. Fractions 4A54 – 4A157 were combined, then run on a second column (4B) with a solvent system of DCM-MeOH (9:1) that resulted in a set of 4B fractions. Compound **175** was obtained from this column as sample 4B4. The purity of the compound was confirmed by TLC, NMR, and LC-MS. A flow diagram of the isolation of compound **175** is given in **Scheme 4.1.**



Scheme 4.1 Schematic diagram describing the isolation of 3-*O*-β-D-glucopyranosyL-β-sitosterol (**175**) from the ether fraction of *A. batteniana*.

4.4.5. Physical and spectral data of the isolated 3-*O*-β-D-glucopyranosyL-β-sitosterol

3-*O*-β-D-GlucopyranosyL-β-sitosterol (**175**): Amorphous solid; IR ν_{max} 3263, 2914, 1641, 1023 cm^{-1} ; ^1H NMR ($CDCl_3$+CD_3OD , 400 MHz) and ^{13}C NMR ($CDCl_3$+CD_3OD, 100 MHz), see **Table 4.1**; HR-ESI(-)-TOF (negative-ion mode) *m/z* 611 $[M + Cl]^-$.

CHAPTER 5: CYTOTOXICITY ASSAYS OF CRUDE EXTRACTS AND ISOLATED COMPOUNDS OF *A. BATTENIANA AND O. SAUNDERSIAE*

5.1. INTRODUCTION

Japanese scientists have worked extensively on the genus *Ornithogalum*, specifically targeting bioactive extracts with medicinal potential. During this systematic survey, the ethanolic bulb extract of *O. saundersiae* showed potent cytotoxic activity against HL-60 human promyelocytic leukaemia cells. *O. saundersiae* is endemic to Southern Africa and is used as perennial garden plant that is cultivated mainly for ornamental purposes. It is endemic to Southern Africa and is largely exported to European countries as cut flowers. No folkloric medicinal history has been associated with this species.

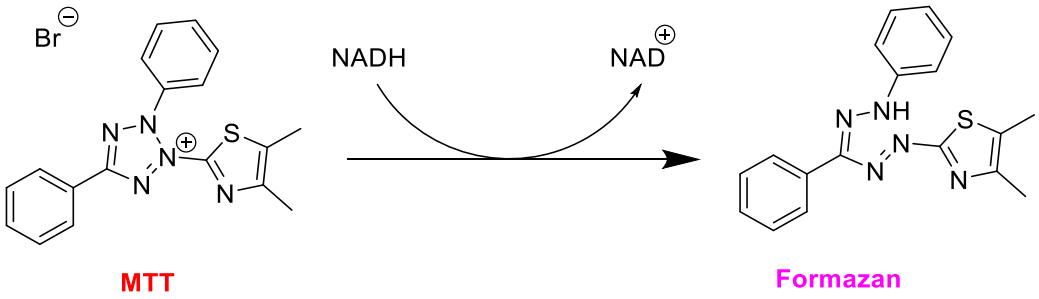
Studies on *O. saundersiae* led to the discovery of a highly potent saponin, OSW-1, isolated from the methanolic bulb extract by Kubo et al. (1992). OSW-1 was isolated as the main component of the extract and showed great cytostatic activity against a broad spectrum of malignant tumour cells such as: human pulmonary adenocarcinoma, human pulmonary squamous cell carcinoma, human pulmonary large cell carcinoma, leukaemia HL-60, and mouse mastrocarcinoma. Although the mechanism of action is not fully been understood, OSW-1 has been identified to be 10 to 100 times more potent than the current clinical anticancer treatments, with IC_{50} values ranging from 0.1 to 0.7 nM.

Several OSW-1 derivatives were later isolated from *O. saundersiae*, none matching the cytotoxicity of OSW-1. Upon evaluations of OSW-1 to identify the structural contributions to the high potency of the steroidal glycosides, it was found that the aromatic acid ester group attached at position C-16 of the steroid and the carbonyl group at C-22 were essential for the cytotoxic activity. In contrast, the common glucocylation at the C-3 hydroxy group showed no substantial effect on the activity. The discovery of OSW-1 helped emphasize the potential of natural products in the drug discovery field.

Plant species taxonomically related to *O. saundersiae* have also been investigated to find more potent compounds and OSW-1 analogues with less toxicity towards normal cells. Phytochemical studies on *O. thyrsoides* and *G. candicans* resulted in isolating more OSW-1

analogues with anticancer activities. However, none of these compounds exhibited cytotoxic activities so it can be considered as a new anticancer agent.

Cell viability is commonly measured with a colourimetric assay called the 3-(4,5-dimethylthiazoL-2-yl)-2,5-diphenyL-2H-tetrazolium bromide (MTT) assay. This tetrazolium dye can be reduced to its insoluble formazan by NAD(P)H-dependent cellular oxidoreductase enzymes under specific conditions (**Scheme 5.1**). The reduction of MTT (mitochondrial dehydrogenase) can only occur in viable cells resulting in the formation of a purple colour reflecting the number of viable cells. Increasing amounts of viable cells can be detected by increasing purple colouring, as seen in **Figure 5.1**.



Scheme 5.1. The reduction of MTT to an insoluble formazan.

Figure 5.1. A microtiter plate after an MTT assay.

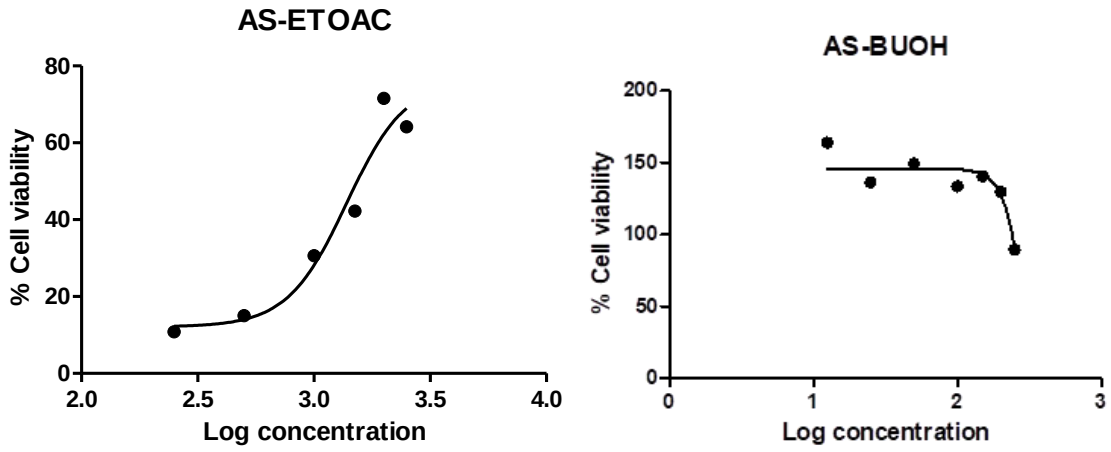
In this chapter, we investigate the effect of the *A. batteniana* and *O. saundersiae* crude extracts and the isolated compounds on a human liver cancer cell line (HepG2).

5.2. RESULTS AND DISCUSSION

Phytochemical investigations on two species from the genus *Ornithogalum* were conducted, resulting in the isolation of one compound (**175**) from *A. batteniana* and three compounds (**170**, **171**, and **172**) from *O. saundersiae*. The crude extracts and their respective compounds were evaluated in vitro for cytotoxic activities using liver cell lines (HepG2). Liver cells play an essential role in cellular metabolism and glucose homeostasis, thus being one of the most used cell lines for cytotoxicity evaluations. The results of cytotoxicity assays conducted for the two plants are outlined below.

Ornithogalum saundersiae

The crude extracts (EtOAc extract and n-BuOH extract) of *O. saundersiae* bulb were assayed. The EtOAc extract showed cytotoxic activity at low concentrations and a proliferative effect with increased concentrations, indicating the possibility of a carcinogen in the extract. The *n*-BuOH extract showed no cytotoxicity at low concentrations up to 250 µg/mL. This suggests that the extract is not cytotoxic and could be investigated for antibacterial or antiviral effects.

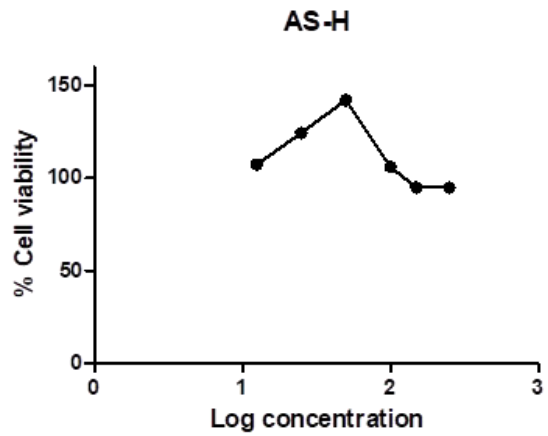


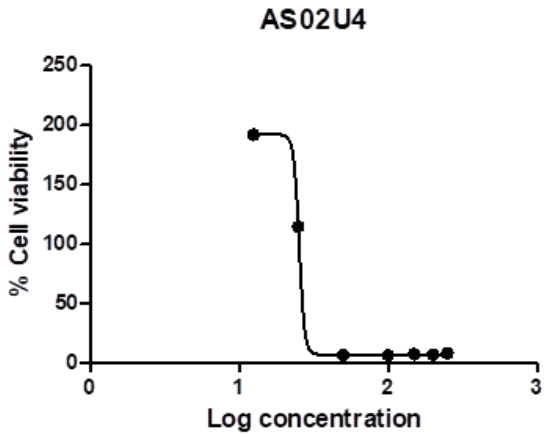
Key:

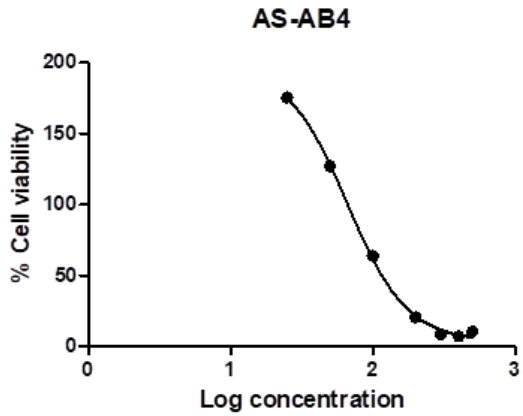
- AS-ETOAC: Ethyl acetate extract
- AS-BUOH: n-butanol extract

Figure 5.2. Assays for cytotoxicity of *O. saundersiae* crude extracts against human liver cancer cell line (HepG2).

Compounds **170**, **171**, and **172** isolated from the *n*-butanol extract were also assayed. **170** showed an increase in metabolic activity up to 50 µg/mL. A decline in metabolic activity was observed at this point but no cytotoxicity up to 250 µg/mL. This compound could be investigated for antibacterial or antiviral effect. The steroidal glycoside **171** also showed an initial increase in metabolic activity up to 25 µg/mL. Cytotoxic effect was observed above 25 µg/mL (IC_{50} = 25 µg/mL) and this compound could potentially be an anticancer agent but with a low safety margin (indicated by the steep curve). Assays of **172** showed a similar trend with initial increased metabolic activity up to 50 µg/mL and cytotoxic effects above 50 µg/mL (IC_{50} = 115 µg/mL). The activity observed for **171** and **171/172** mixture warrant further investigation of these two compounds.







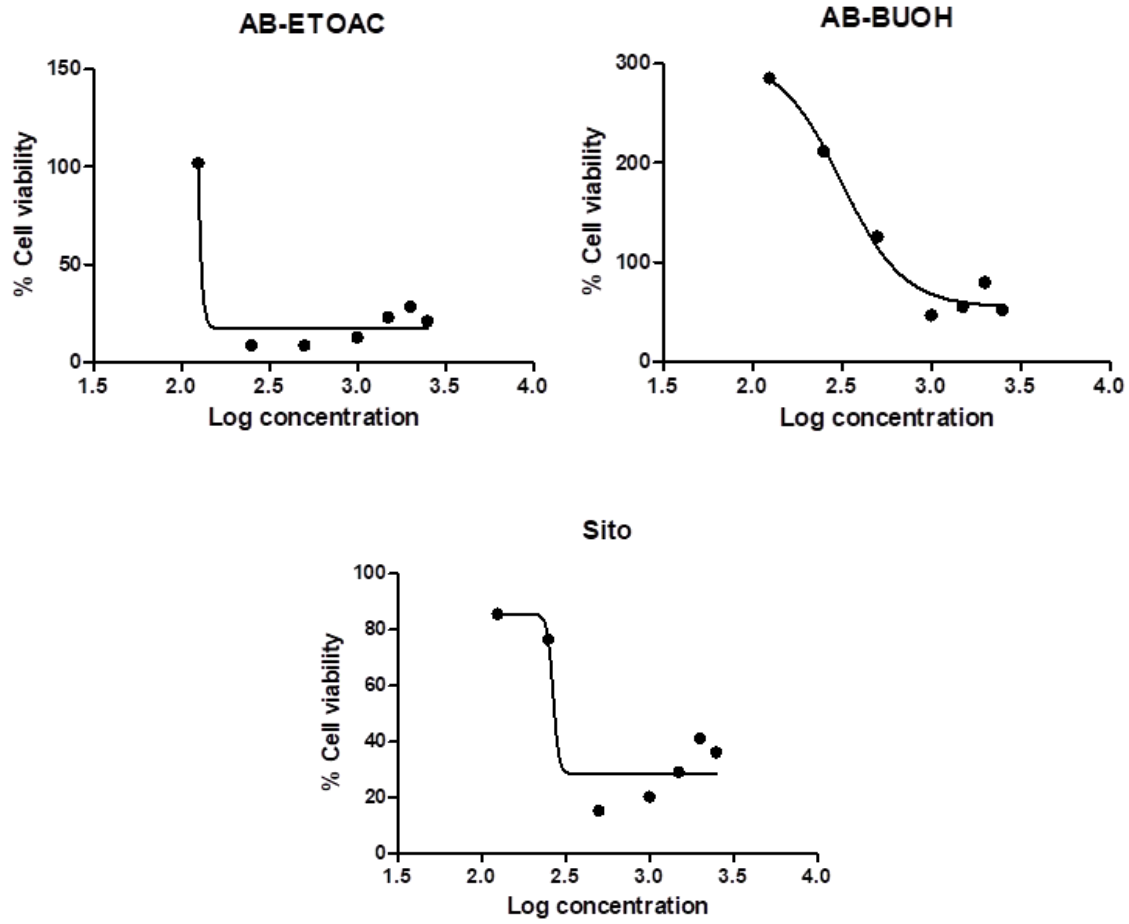
Key:

- AS-H: Compound **170**
- AS02U4: Compound **171**
- AS-AB4: Compound **172**

Figure 5.3. Assays for cytotoxicity of isolated compounds against human liver cancer cell line (HepG2).

Albuca batteniana

The crude extracts (EtOAc extract and n-BuOH extract) of *A. batteniana* bulb were assayed. The EtOAc extract and compound **175** showed no effect on cell viability and was safe up to 125 µg/mL (IC_{50} = 200 µg/mL) and 250 µg/mL (IC_{50} =275 µg/mL), respectively, in liver cells. Thereafter, a sharp decline in cell viability for liver cells can be seen. Both the EtOAc extract and compound **175** can be assayed for anticancer potential. The *n*-BuOH extract showed an increase in metabolic activity up to 500 µg/mL (IC_{50} = 316 µg/mL) and can be investigated for antibacterial and antiviral effects at this margin. From 500 µg/mL upwards, cytotoxic effects are observed and can be investigated for anticancer activity.



Key:

- AB-ETOAC: Ethyl acetate extract

- AB-BUOH: n-Butanol extract
- Sito: Compound **175**

Figure 5.4. Assays for cytotoxicity of *A. batteniana* crude extracts against human liver cancer cell lines (HepG2).

The ISO norm for this study was set at >75% cell viability for liver cells. Thus, extracts or compounds that retained >75% cell viability in accordance with the ISO norm are considered safe and could be explored for therapeutic effects such as antiviral and antibacterial. Samples yielding <50% cell viability are cytotoxic and can be considered for anticancer investigations. At the crude extract level, *A. batteniana* showed some cytotoxic effects whereas no apparent cytotoxic effects can be seen for *O. saundersiae* at crude level.

Compound **175** (3-*O*-β-D-glucopyranosyL-β-sitosterol) isolated from *A. batteniana* showed similar effects as the crude extracts. Previous studies carried out on **175** exhibited cytotoxic effects against hepatocellular cells (Huh7 and HepG2) via inducing apoptosis and activate caspase-3 and caspase-9 in the hepatocellular cells. From these results, **175** showed potential to be developed into therapeutic agents to treat liver cancer. In comparison to the highly potent OSW-1, **175** does not satisfy all the structural contributions bringing about potency.

The *O. Saundersiae* crude extracts were not cytotoxic to liver cells but the two isolated compounds (**171** and **172**) showed some cytotoxic effect. This data was not surprising considering the complexity of *O. saundersiae* extracts (refer to chapter 3 LC-MS-MS data), and no correlation can be made in terms of consistency between the bioactivity of crude extracts and isolated compounds. Compounds **171** and **172** are structural isomers of each other with a disaccharide attached at C-16 of the steroid and the carbonyl group at C-22 which were essential for OSW-1 potency.

5.3. EXPERIMENTAL

The (MTT) assays were carried out according to standard procedures by Dr Rene Myburg, a lecturer and researcher in the Department of Medicinal Biochemistry at the Howard college of UKZN.

5.4. CONCLUSION

This chapter presents successful in vitro assays of *A. batteniana* and *O.saundersiae* crude extracts, and isolated compounds against Liver cell line (HepG2). Minimal cytotoxic effects to HepG2 cells were observed for the *O. saundersiae* crude extracts and compound **170** in accordance with the ISO standard of cell viability >75%. Some cytotoxic effects were observed for *A. batteniana* crude extracts as well as compounds **171**, **172**, and **175**. Further investigations of anticancer effects of these extracts and compounds is recommended.

CHAPTER 6: CONCLUSION AND FUTURE WORK

The phytochemical investigation of medicinal plants has played an important role in drug discovery. Many of the medicines currently on the market as treatments for different illnesses are derived from natural products. *Ornithogalum* (Hyacinthacea) species are among the highly investigated species for their antitumour properties and has resulted in the identification of several bioactive compounds, one of them being the highly potent steroidal glycoside (OSW-1) isolated in 1992. This dissertation describes the phytochemistry of two South African plant species (*O. saundersiae* and *A. batteniana*) and investigate the bioactivity of the extracts and compounds isolated.

Upon examination of the bulb material of *O. saundersiae,* three novel steroidal glycosides were isolated (**170**, **171**, and **172**). All the purified structures were elucidated by using spectroscopic experiments. Cytotoxicity assays showed some cytotoxic effects on liver cells for **171** and **172**. The structural contributions responsible for the high potency of OSW-1 are also present in **171** and **172**. Therefore, bioassays for anticancer activity would be ideal for these two compounds.

Prior to this study, no phytochemical studies have been reported on *A. batteniana*. One compound was isolated from this plant and was identified to be 3-*O*-β-D-glucopyranosylsitosterol (**175**). 3-*O*-β-D-glucopyranosylsitosterol is a common steroidal glycoside in many plant families. The major components in this species were found to be sugars (glucose and sucrose) making it challenging to work with this plant in terms of the selection of solvents. The EtOAc and *n*-BuOH crude extracts showed moderate cytotoxic effects on liver cells.

Future work can include:

- Optimizing of the LC-MS-MS experiments so that the results can be used to create molecular networks for *O. suandersiae* crude extracts.
- The isolation of more compounds from *O. saundersiae* and *A. batteniana*.
- The determination of anticancer activities for the novel compounds.
- The semi-synthesis of isolated compounds to produce different analogues of steroidal glycosides

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APPENDIX

Page

Compound **170**:

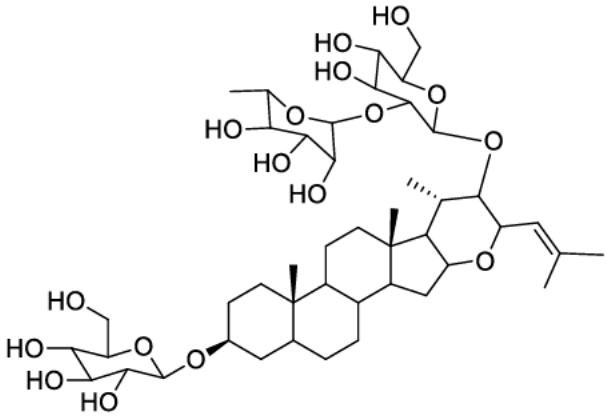
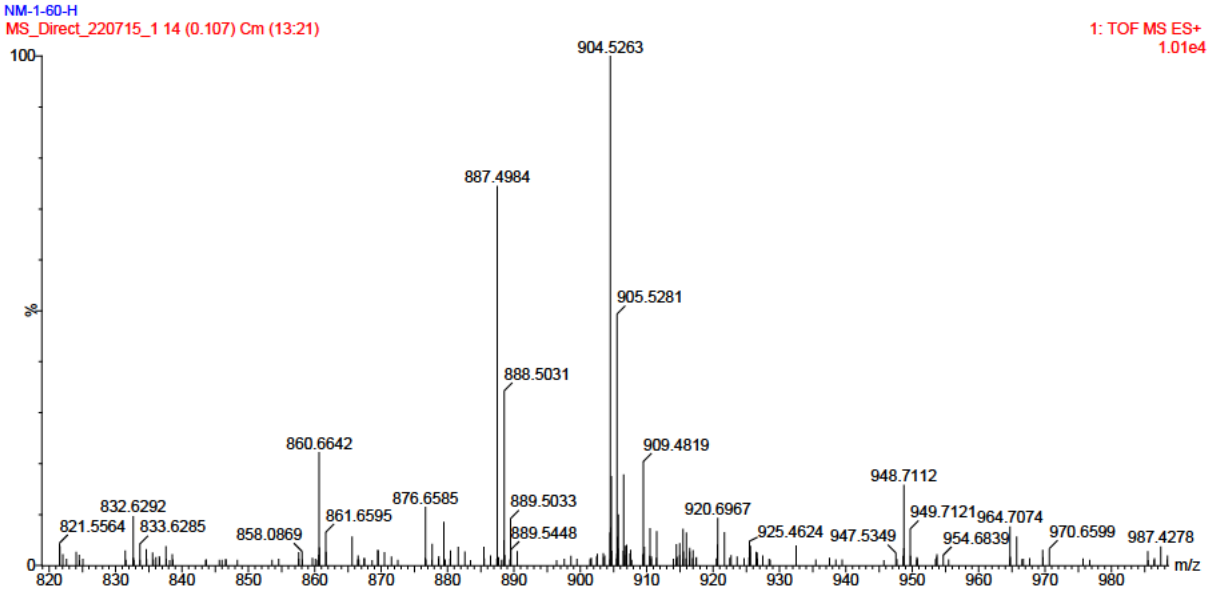


Plate 1. HR-ESI(-)-TOF spectrum of compound **170**.



Mass	Calc. mass	mDa	PPM	DBE	i-FIT (norm)	Formula
887.4984	887.5004	-2.0	-2.3	8.5	119.9	$C_{45}H_{75}O_{17}$

Plate 2. ^{1}H NMR spectrum of compound **170** (400 MHz, CD_3OD).

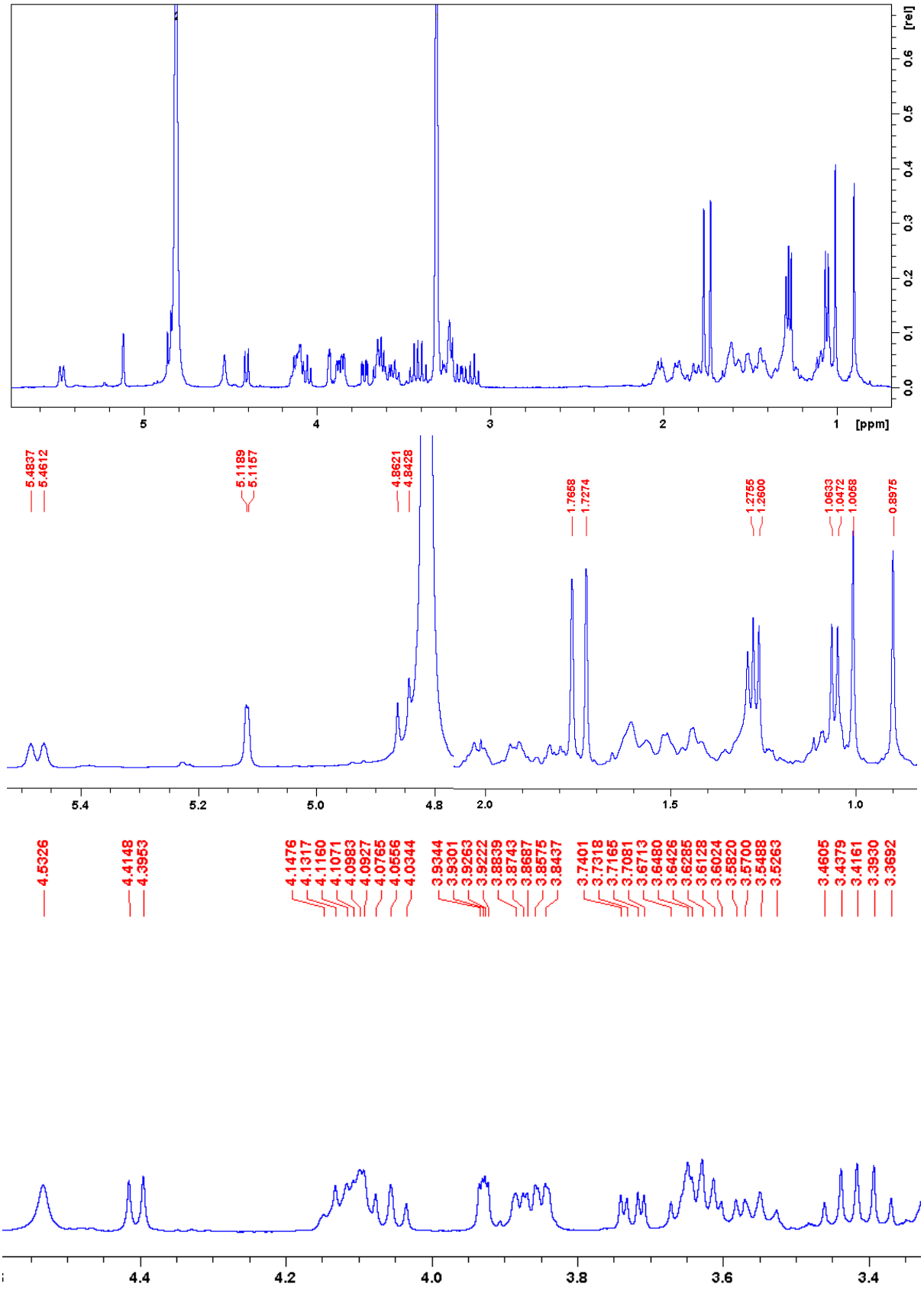


Plate 3. ^{13}C NMR and DEPT-135 NMR spectrum of compound **170** (100 MHz, CD_3OD).

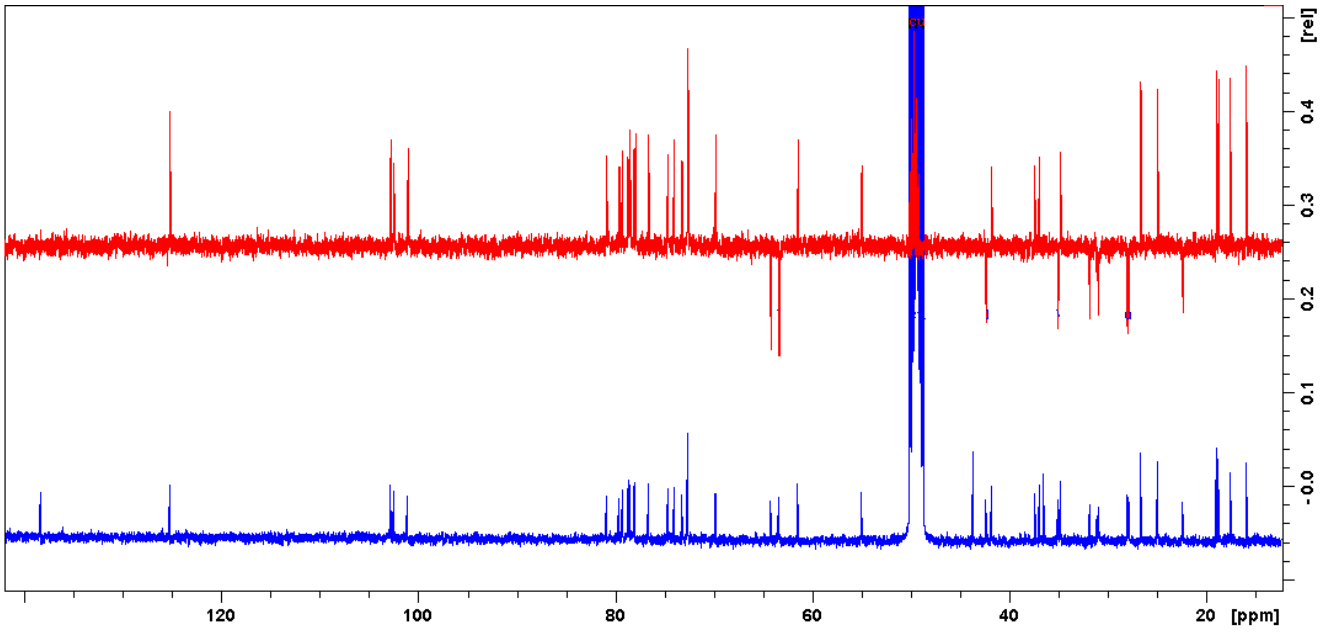


Plate 4. COSY spectrum of compound **170** (400 MHz, CD_3OD).

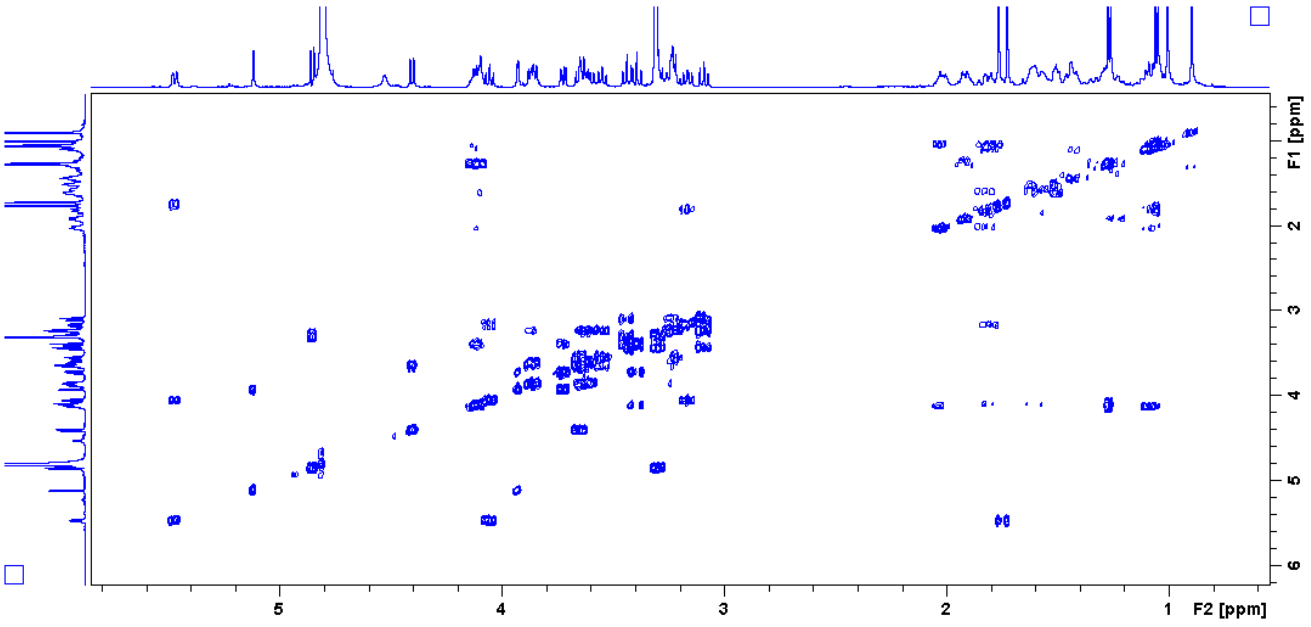


Plate 5. HSQC spectrum of compound **170** (400 MHz, 100 MHz, CD_3OD).

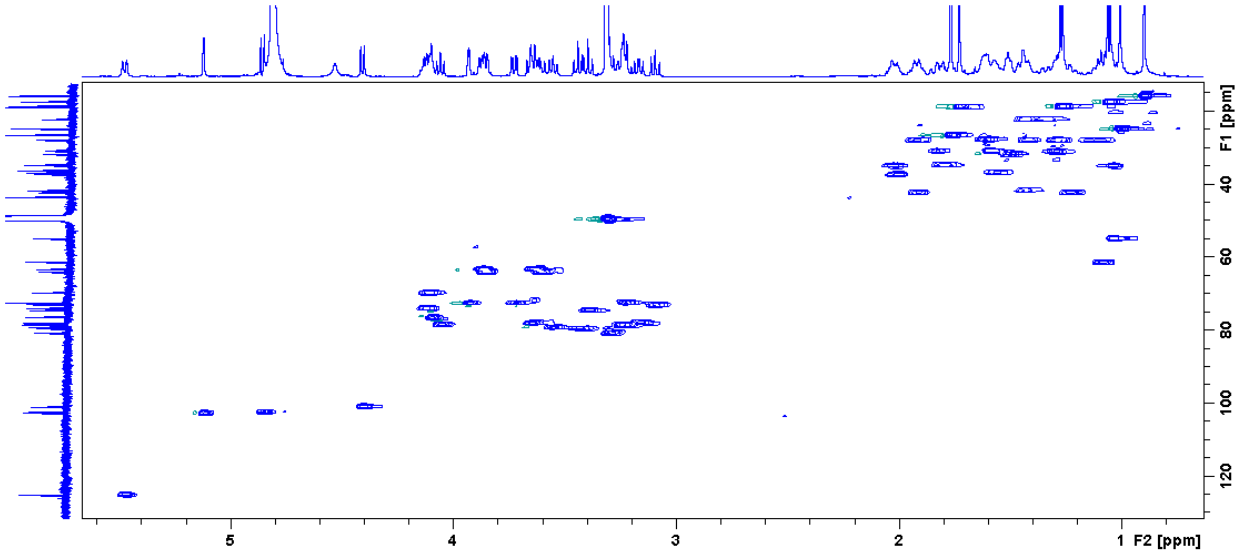
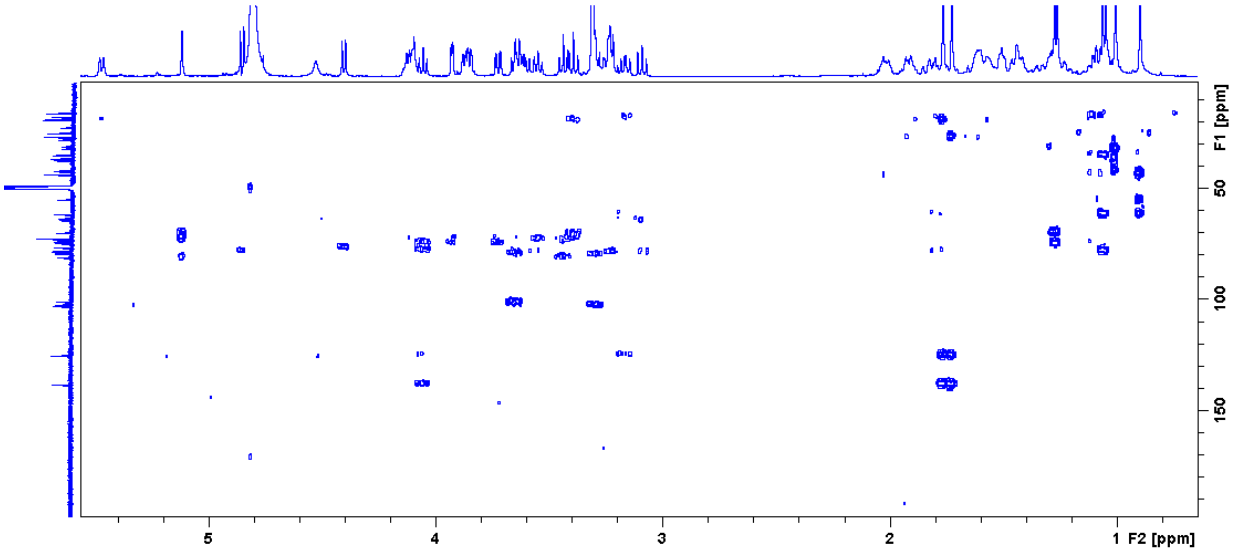


Plate 6. HMBC spectrum of compound **170** (400 MHz, 100 MHz, CD_3OD).



Compound **171**:

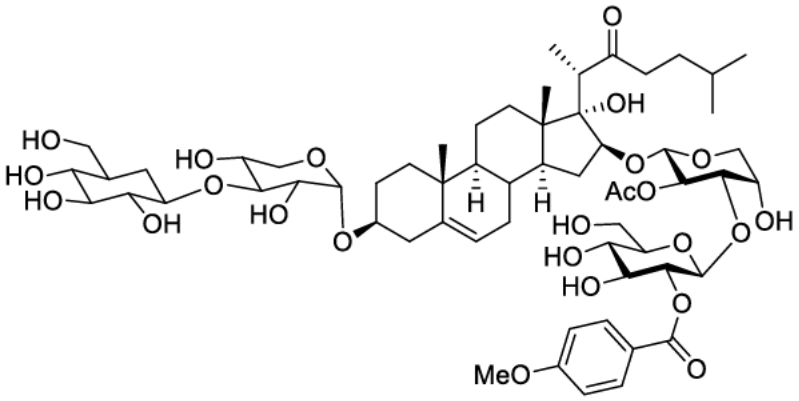


Plate 7. HR-ESI(-)-TOF spectrum of compound **171**.

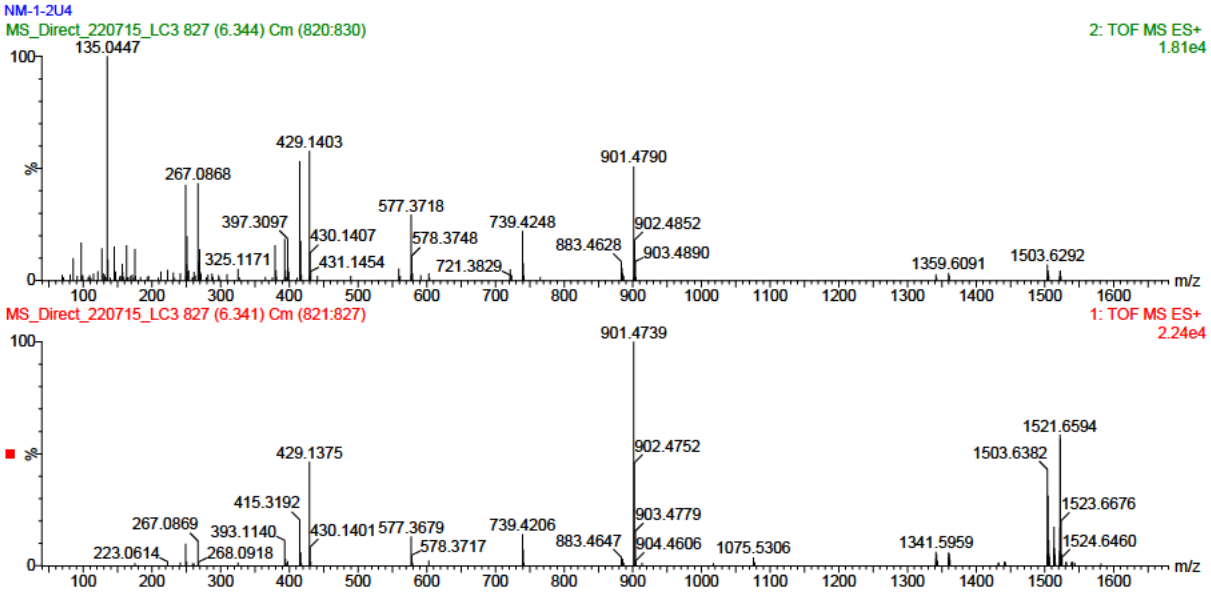


Plate 8. ^{1}H NMR spectrum of compound **171** (500 MHz, CD_3OD).

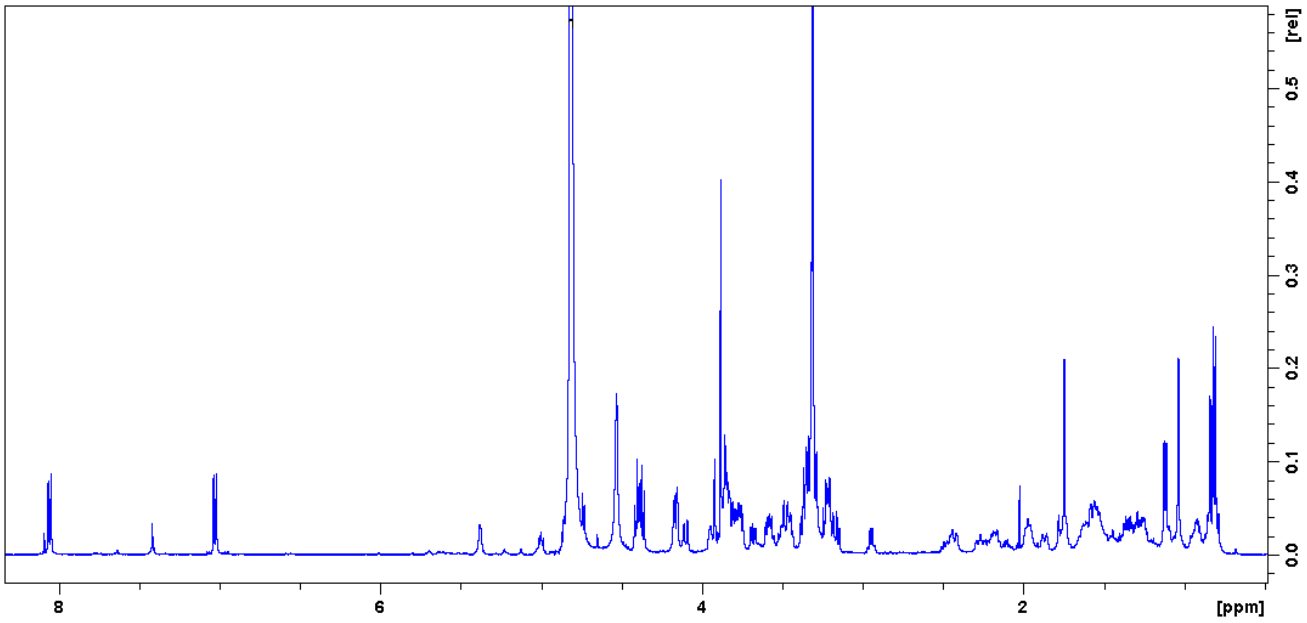


Plate 9. ^{13}C NMR and DEPT-135 NMR spectrum of compound **171** (100 MHz, CD_3OD).

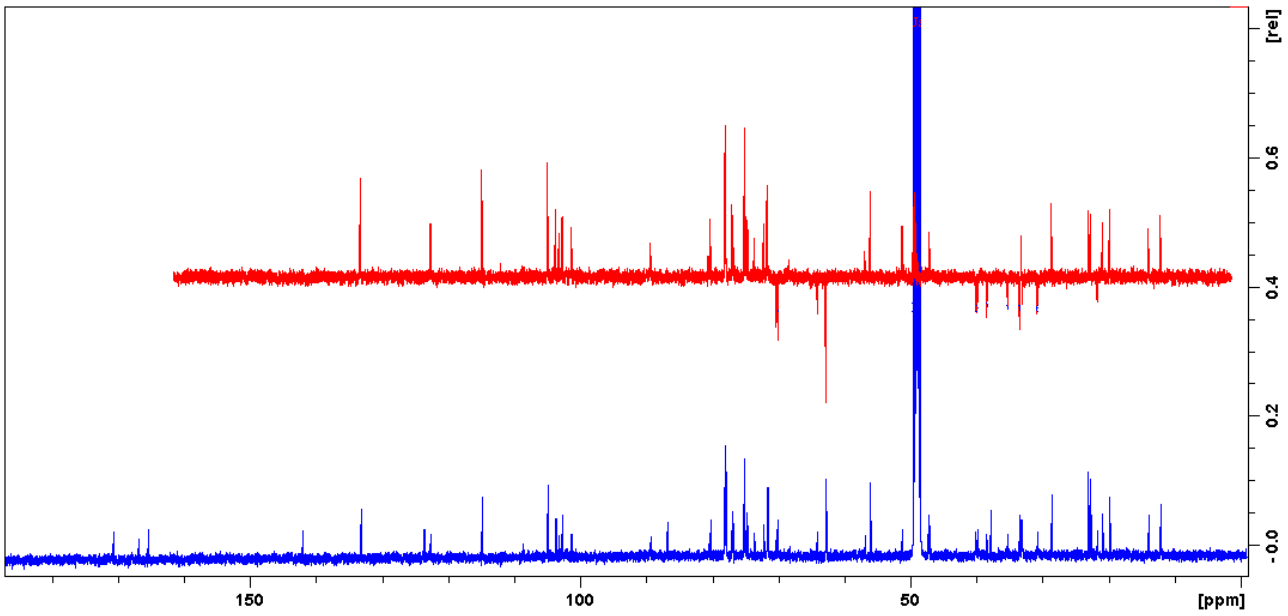


Plate 10. COSY spectrum of compound **171** (500 MHz, CD_3OD).

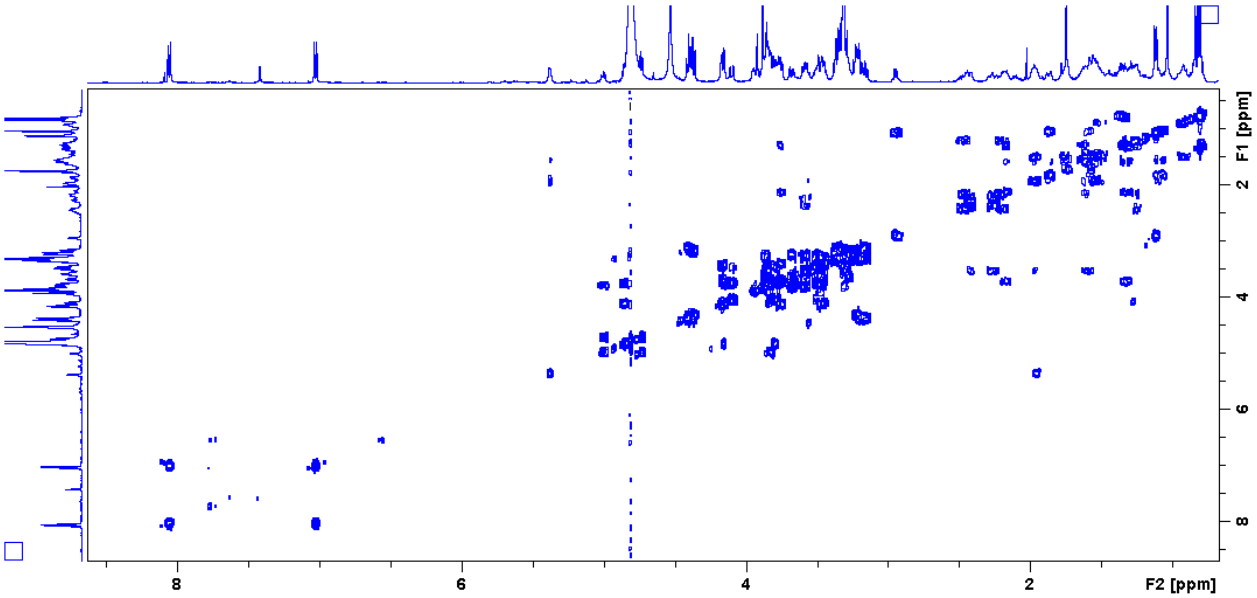


Plate 11. HSQC spectrum of compound **171** (500 MHz, 100 MHz, CD_3OD).

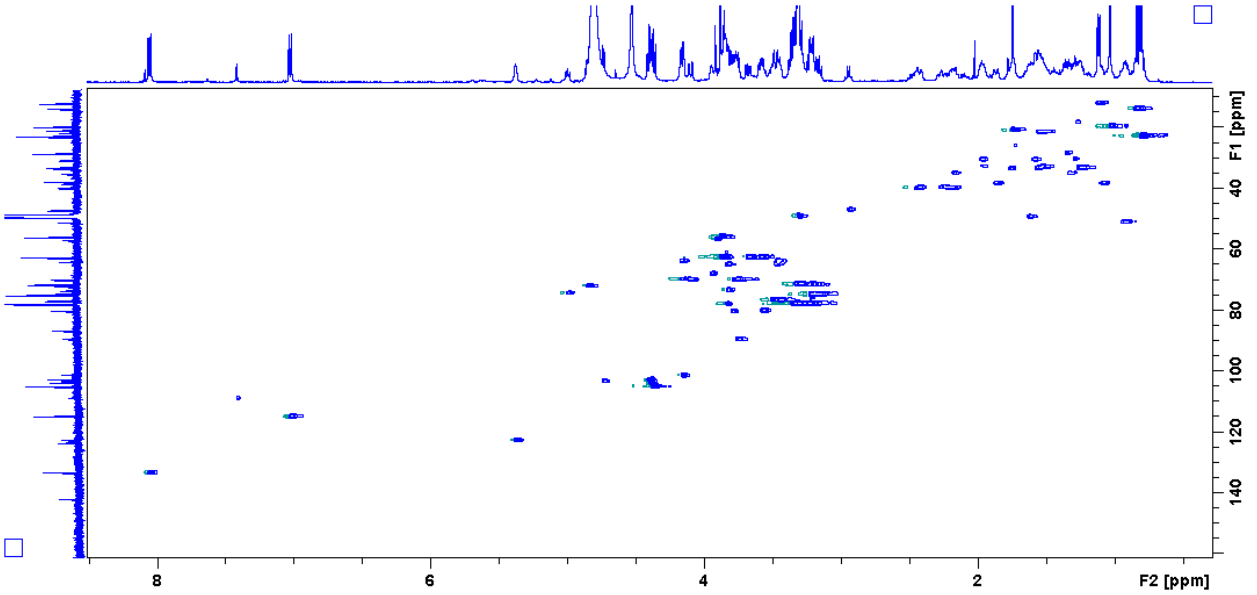
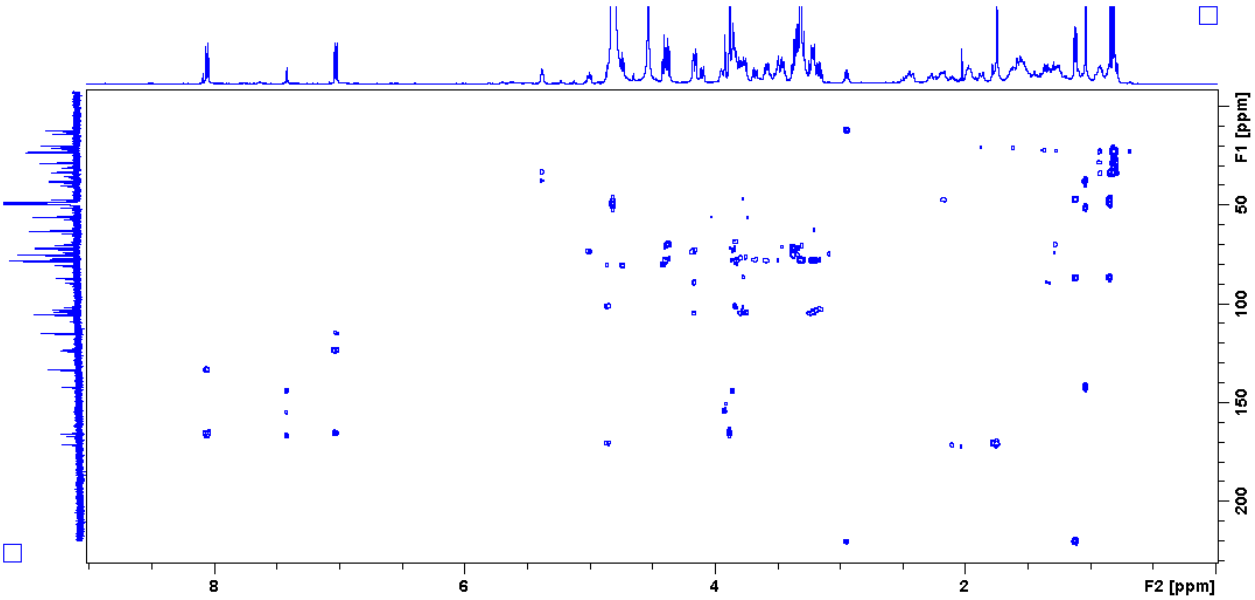


Plate 12. HMBC spectrum of compound **171** (500 MHz, 100 MHz, CD_3OD).



Compound **172**:

Plate 13. HR-ESI(-)-TOF spectrum of compound **172**.

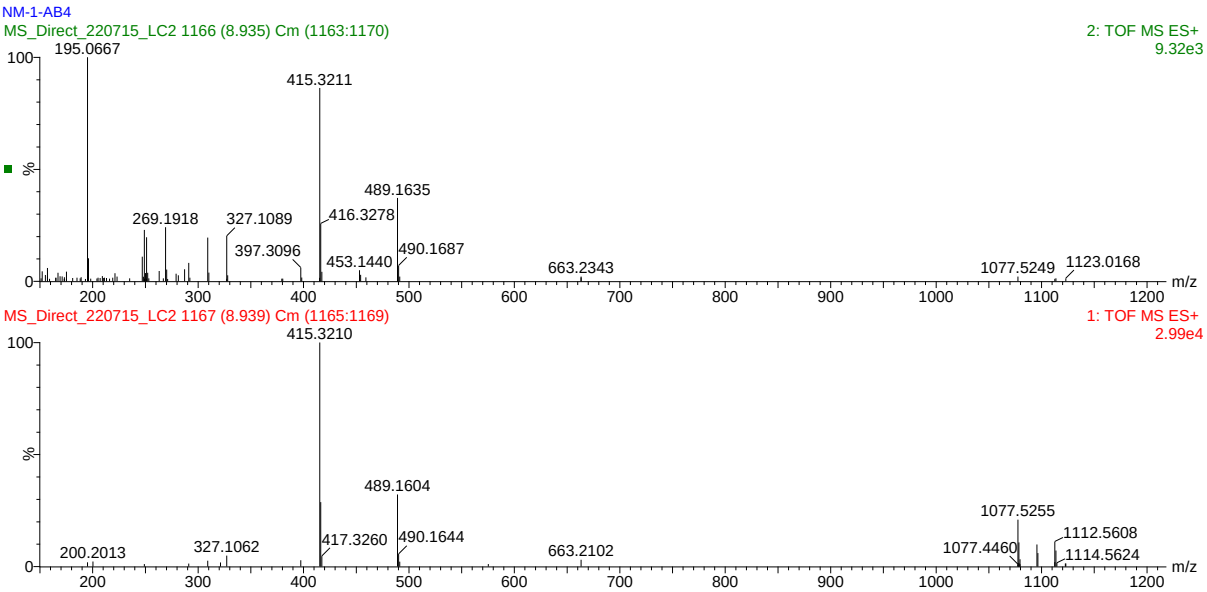


Plate 14. ^{1}H NMR spectrum of compound **172** (500 MHz, CD_3OD).

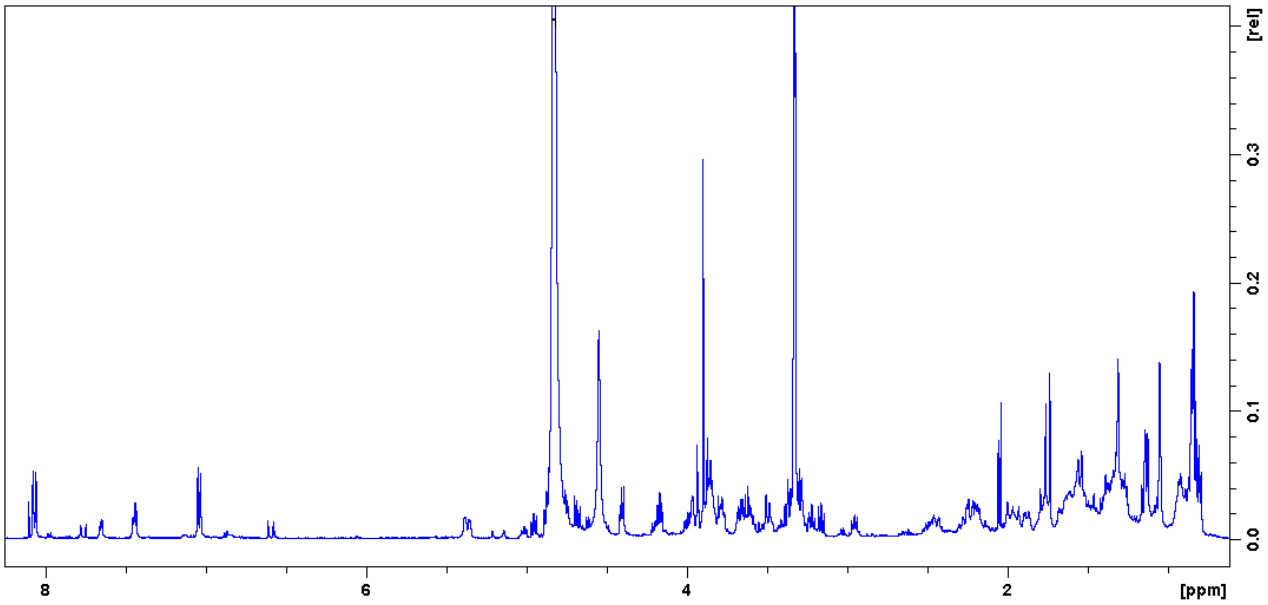


Plate 15. ^{13}C NMR and DEPT-135 NMR spectrum of compound **172** (100 MHz, CD_3OD).

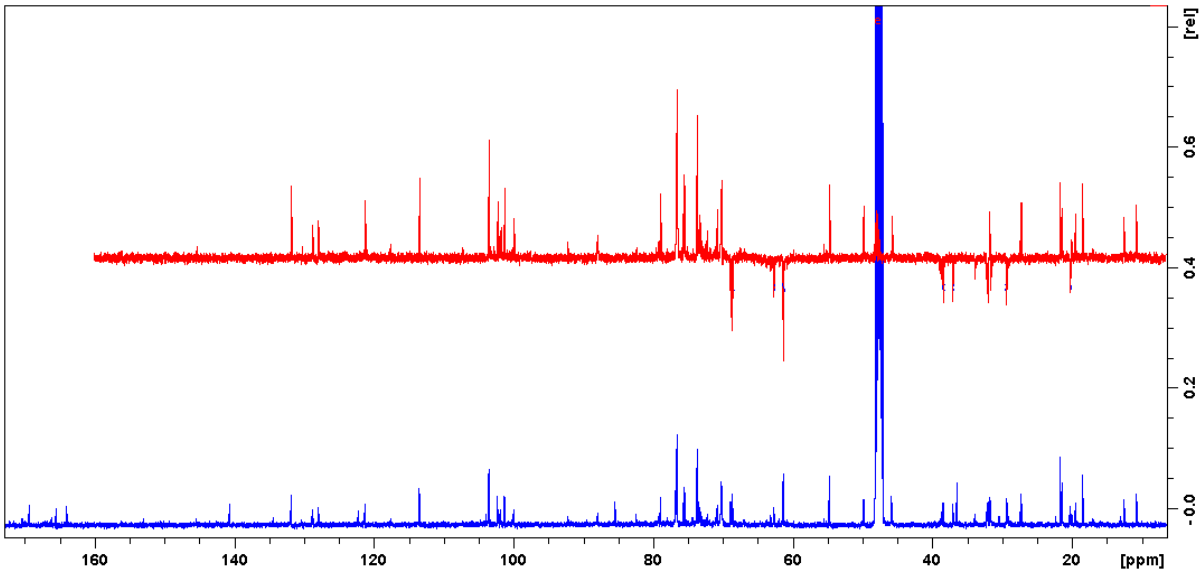


Plate 16. COSY spectrum of compound **172** (500 MHz, CD_3OD).

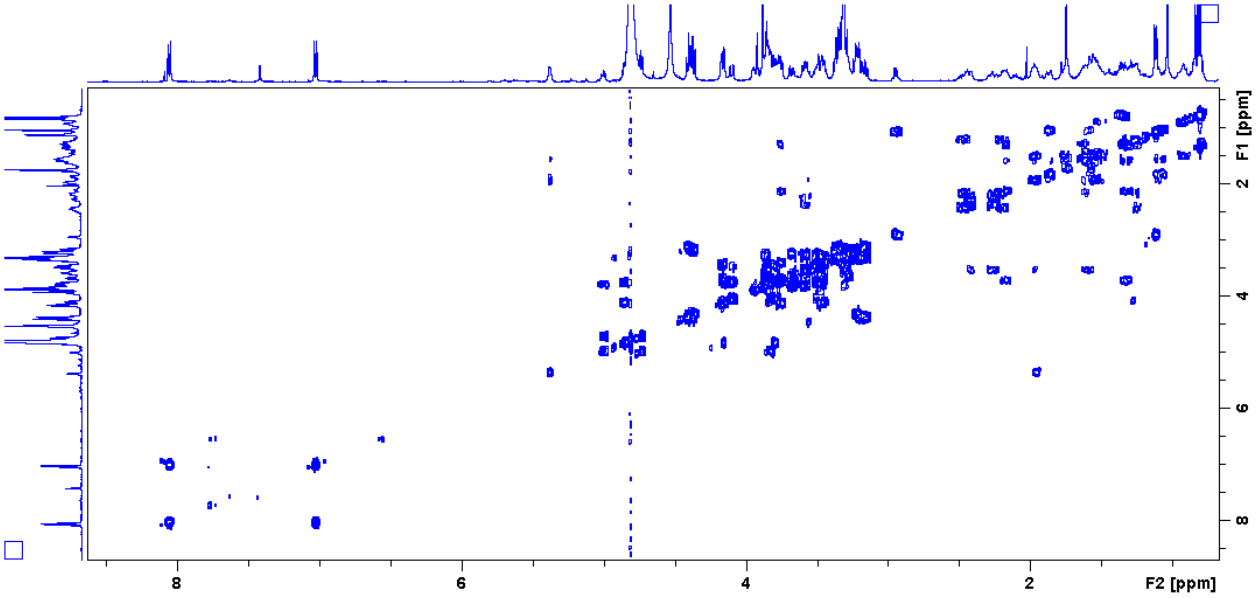


Plate 17. HSQC spectrum of compound **172** (500 MHz, 100 MHz, CD_3OD).

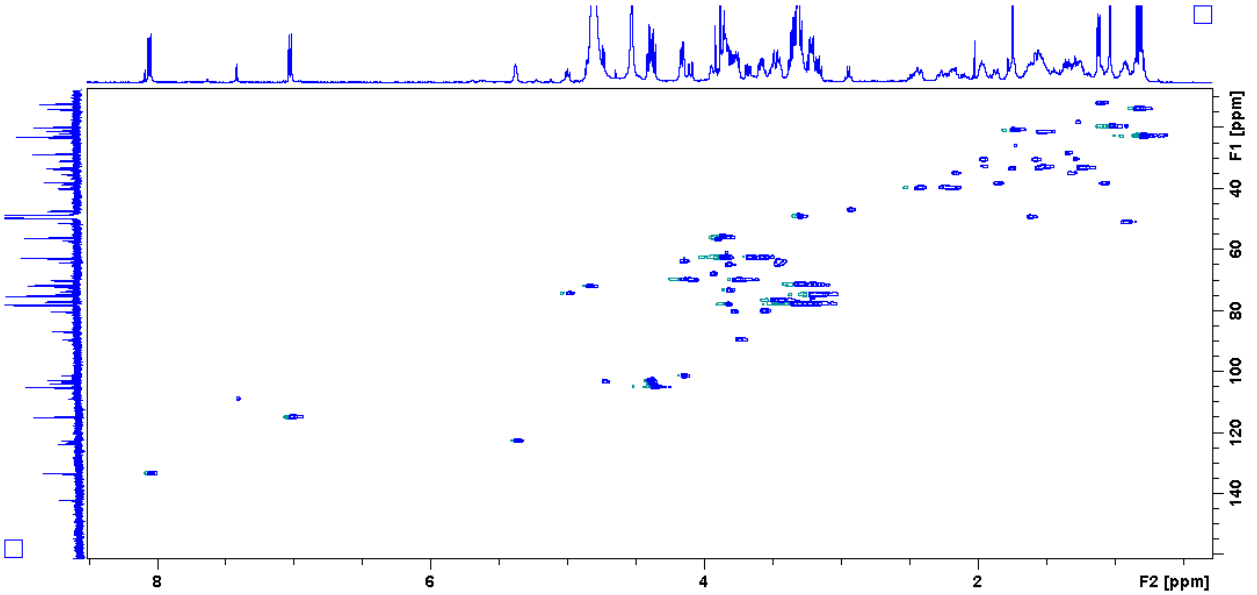
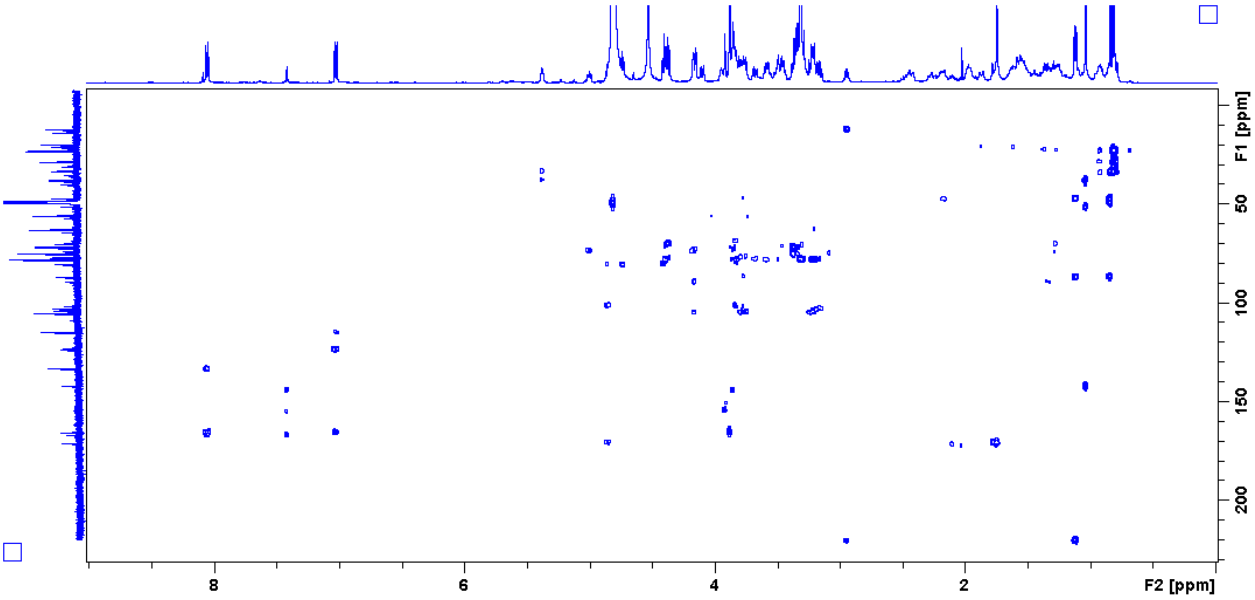


Plate 18. HMBC spectrum of compound **172** (500 MHz, 100 MHz, CD_3OD).



3-*O*-β-D-Glucopyranosylsitosterol (**175**):

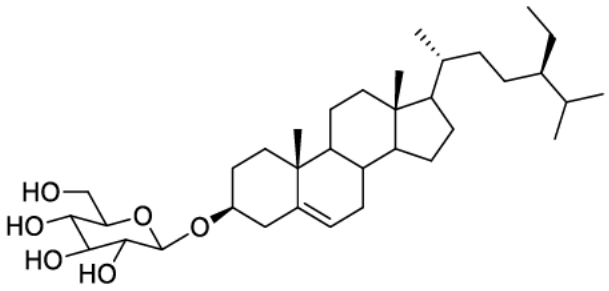


Plate 19. LR-ESI spectrum of 3-*O*-β-D-glucopyranosylsitosterol (**175**).

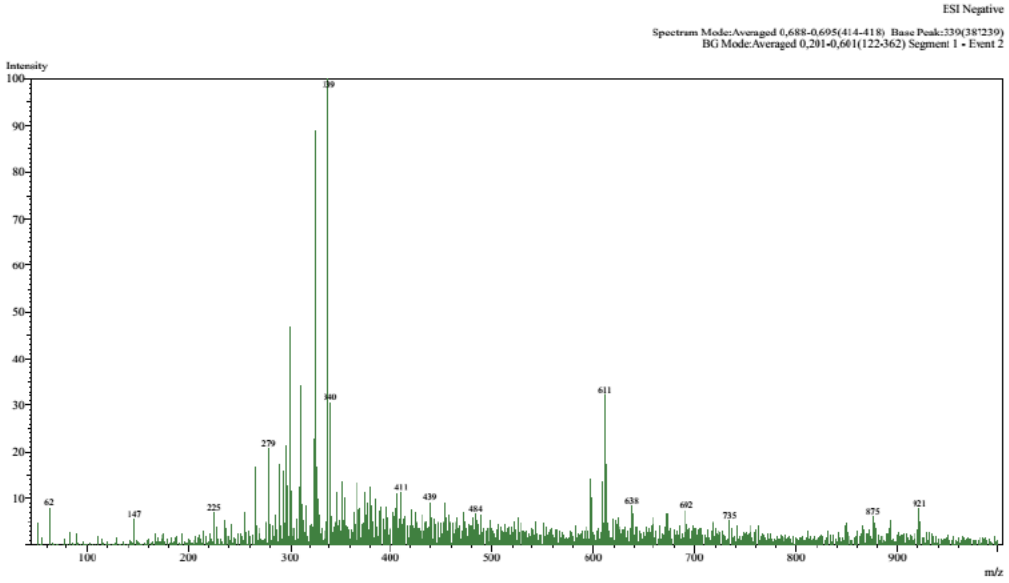


Plate 20. ^{1}H NMR spectrum of 3-*O*-β-D-glucopyranosylsitosterol (**175**) (400 MHz, $CDCl_3$+CD_3OD).

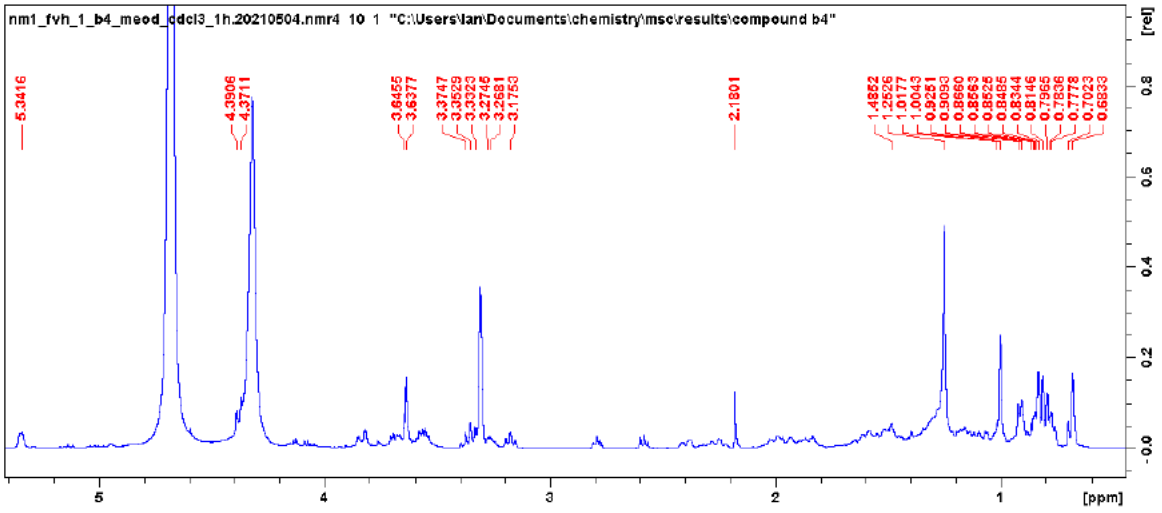


Plate 21. ^{13}C NMR and DEPT-135 NMR spectrum of 3-*O*-β-D-glucopyranosylsitosterol (**175**) (100 MHz, $CDCl_3$+CD_3OD).

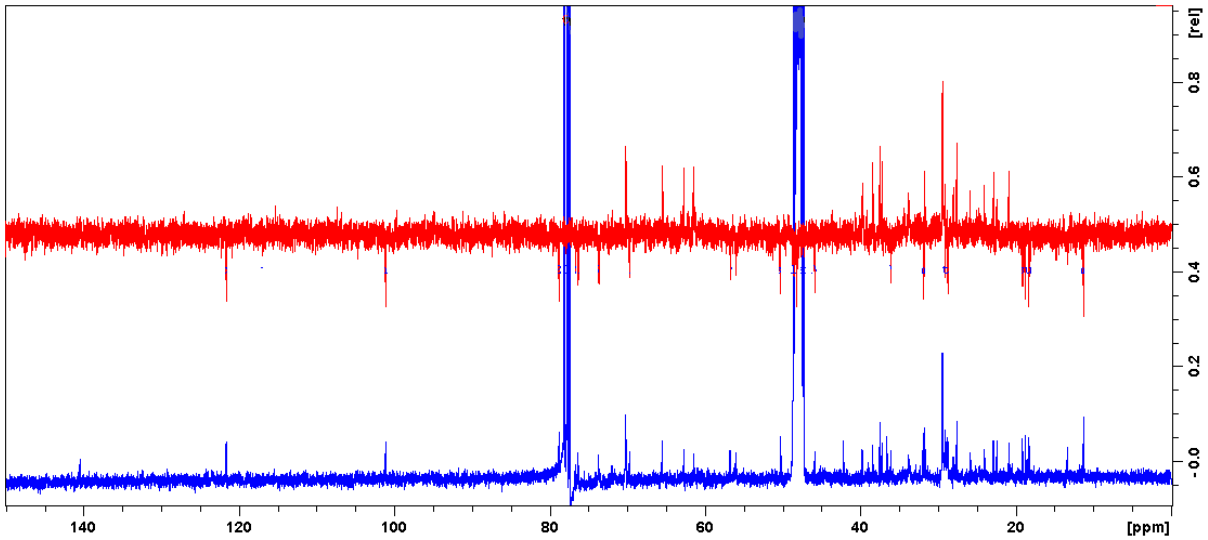


Plate 22. COSY spectrum of 3-*O*-β-D-glucopyranosylsitosterol (**175**) (400 MHz, $CDCl_3$+CD_3OD).

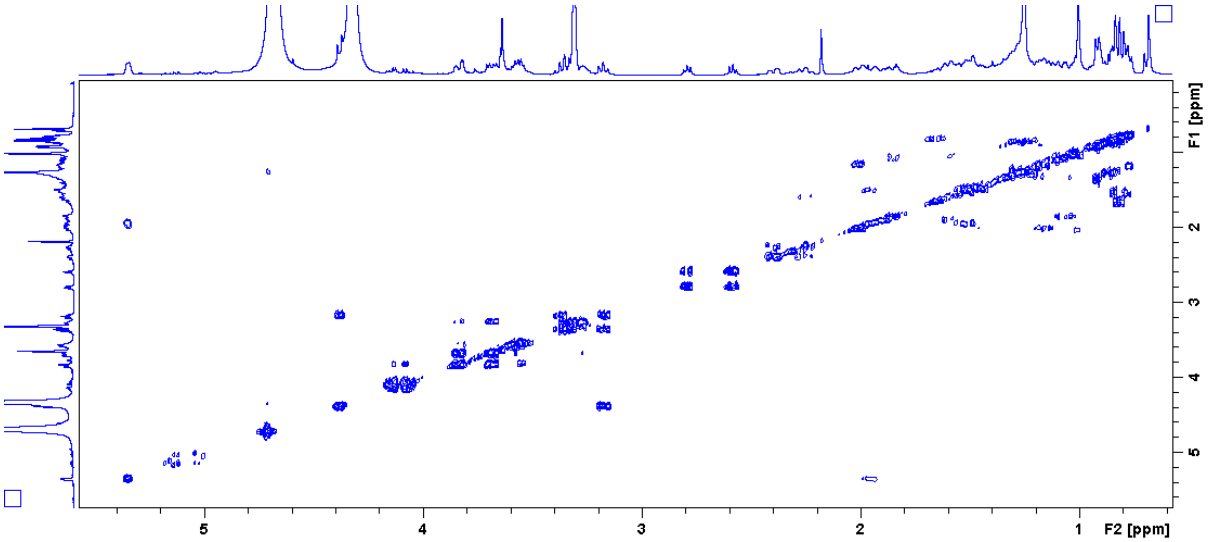


Plate 23. HSQC spectrum of 3-*O*-β-D-glucopyranosylsitosterol (**175**) (400 MHz, 100 MHz, $CDCl_3$+CD_3OD).

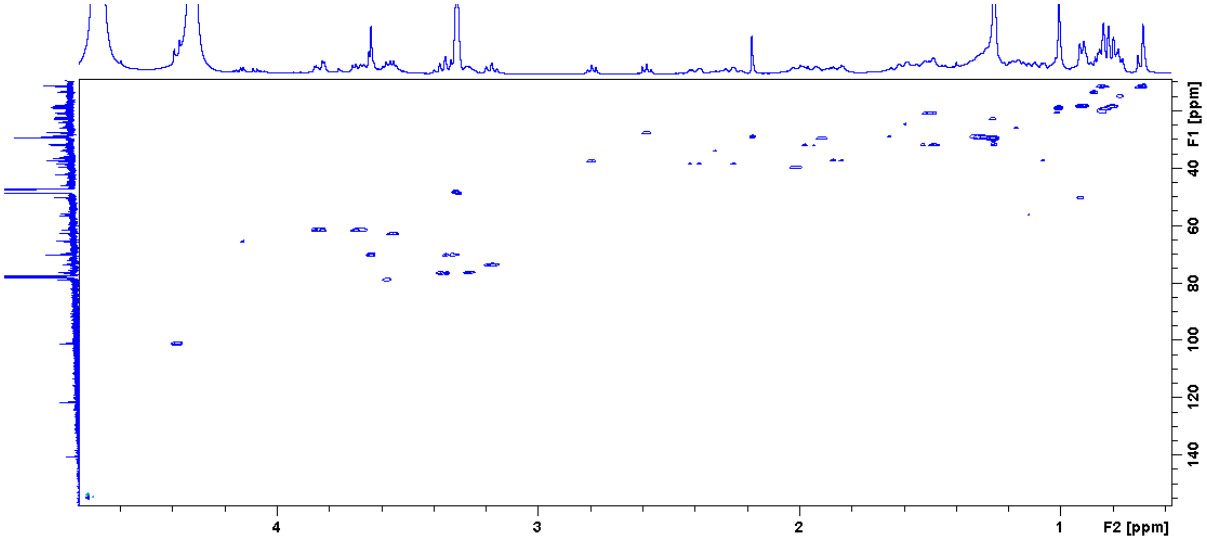


Plate 24. HMBC spectrum of 3-*O*-β-D-glucopyranosylsitosterol (**175**) (400 MHz, 100 MHz, $CDCl_3$+CD_3OD).