ANTI-CANCER AND ANTI-INFLAMMATORY SECONDARY METABOLITES OF *Fagaropsis angolensis* (ENGL.) H.M. GARDNER

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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

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DEDICATION

This piece of work is dedicated to my dear parents Mr. Patrick Mukavi Kisilu and Mrs. Cecilia Munaa Mukavi for your prayers, support and encouragement throughout this journey.

To my sisters; Elizabeth and Faith, all my family members and my late grandmother, without your unwavering support, counsel and motivation I would not have made it this far. My sincere gratitude goes to you all.

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ABBREVIATIONS/SYMBOLS AND ACRONYM

^{13}C NMR	Carbon 13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
2D NMR	Two Dimensional Nuclear Magnetic Resonance
Δ	Delta values
ACS	American Cancer Society
CC	Column Chromatography
CD$_3$OD	Deuterated methanol
COSY	Correlation Spectroscopy
D	Doublet
Dd	Doublet of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl Sulphoxide
ESI-HRMS	Electrospray Ionization High-resolution Mass Spectrum
EtOAc	Ethylacetate
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GLOBOCAN	Global Burden of Cancer
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
IC$_{50}$	50% inhibitory concentration
IR	Infrared
IARC	International Agency for Research on Cancer
J	Coupling constant
LMIC	Low-and Middle-income Countries
LPS	Lipopolysaccharide
M	Multiplet

MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MHz	Mega Hertz
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spestroscopy
PBMC	Peripheral Blood Mononuclear Cells
RPMI	Roswell Park Memorial Institute Medium
S	Singlet
SBS	School of Biological Sciences
T	Triplet
TB	Tuberculosis
TLC	Thin Layer Chromatography
TNF-α	Tumor Necrosis Factor-alpha
UV	Ultraviolet
WHO	World Health Organization

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ABSTRACT

Cancer has become a key public health affliction worldwide. Recent studies have shown that genetic factors cause only 5–10% of all human cancers, while the rest are caused by lifestyle. Epidemiological and clinical studies have shown that chronic inflammatory diseases predispose individuals to various types of cancer. An estimated 20 % of all cancer related deaths globally arise from primary infections and inflammation. Current treatments for cancer include radiotherapy, chemotherapy, surgery, hormone, immune and targeted therapies. However, the efficacy of these treatments is constrained by their unexpected detrimental effects on other non-target tissues, development of multi-drug resistant cancer cell lines and high cost. Medicinal plants are increasingly attracting attention of researchers as a source of complementary and alternative therapies to mitigate cancer morbidity and mortality. Plants have been reported to contain bioactive phytochemicals with anti-infective properties against chronic diseases, including inflammation and cancer. In the current study the air-dried roots bark and leaves of *Fagaropsis angolensis* were pulverized into fine powders. The plant materials were then exhaustively extracted with 50% CH_3OH in CH_2Cl_2 (v/v) at room temperature by cold solvent percolation. Separation was carried out using column chromatography on silica gel as the stationary matrix and eluted with gradients of *n*-hexane, EtOAc and MeOH. The resultant fractions were then purified using finer silica gel by recurrent column chromatography, Sephadex LH 20 and Chromatotron to afford a total of five compounds including two new norhopane derivatives; 3β,6β,22-trihydroxy-7β,11α-di[(4-hydroxybenzoyl)oxy]-21αH-24-norhopa-4(23)-ene (**42**) and 3β,6β,22-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-21αH-24-norhopa-4(23)-ene (**43**) together with the known norhopane, 3β,6β,11α-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene (**44**) and a norneohopane, (21α-*H*)-24-norneohopa-4(23), 22(29)-diene-3β,6β,7β-triol 7-caffeate (**30**) from the root bark. The leaves afforded a reported flavone, tachrosin (**45**). Their structure elucidation was achieved by detailed 1D and 2D NMR, HRESI-MS, FT-IR and UV spectra for the newly described compounds and by comparison of these data with those of correlated compounds in the published literature. Resazurin reduction assay was used to evaluate the cytotoxicity of compound **46**, with doxorubicin as reference anticancer drug. Compound **46** displayed minimal activity since the cell viability was more than 70 % against drug sensitive CCRF-CEM. Owing to the risk associated with chronic inflammation to initiation, promotion and progression of carcinogenesis, compounds **42 – 45** were assessed for their anti-inflammatory activity by quantifying the levels of cytokines Interleukin-1β (IL-1β), Interleukin-2 (IL-2), Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Tumor necrosis factor-alpha (TNF-α) in lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMCs). All tested compounds decreased secretion of IL-1β and TNF-α. Compounds **43** and **45** clearly decreased secretion of IL-2, GM-CSF and TNF-α in comparison with the reference drug ibuprofen. The findings from this study revealed that *F. angolensis* contains significant amounts of hopane-type triterpenoid derivatives with potential to downregulate pro-inflammatory biomarkers and further provide a scientific rationale for using the plant in Kenyan folk medicine as anti-pain solution.

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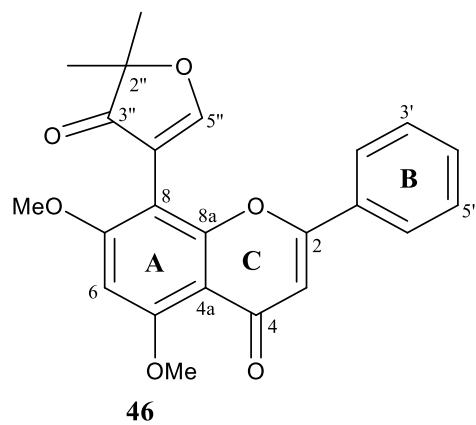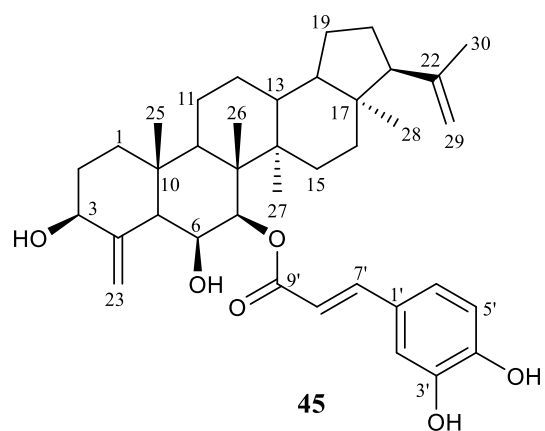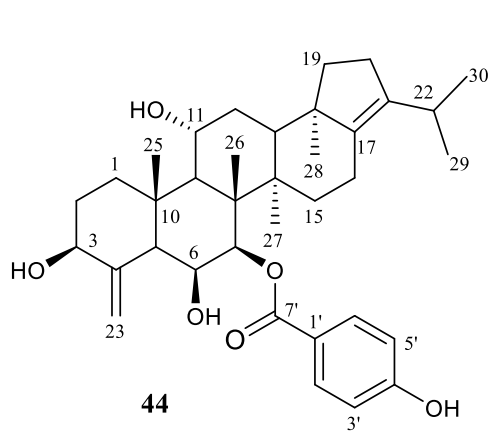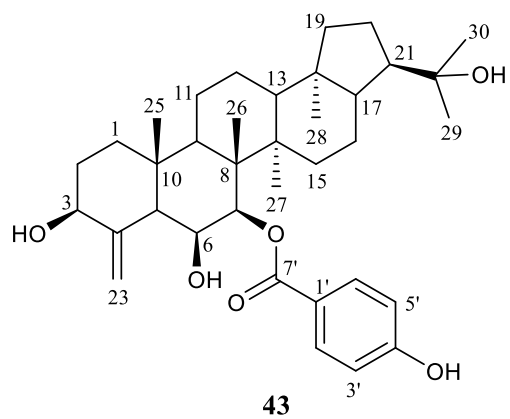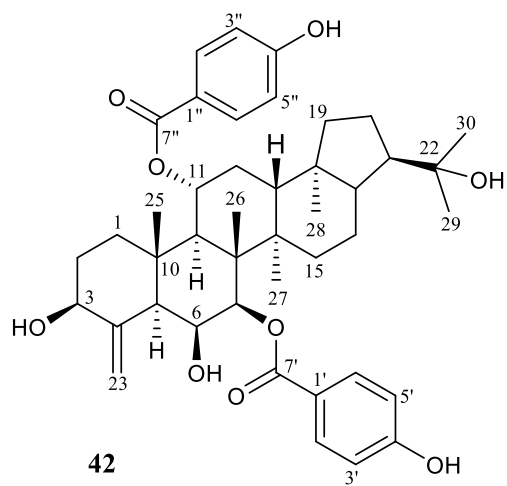
CHAPTER ONE
INTRODUCTION

1.1 Background information

Cancer is a universal name for a broad group of illnesses characterized by unrestrained cell division and propagation of abnormal cells (Hejmadi, 2009). In cancerous cells, division and proliferation is overwhelming leading to tumors that may invade other tissues and organs of the body (Anand *et al*., 2008). Inflammation, on the other hand, is a defensive response of tissues to harmful stimuli like injury, cell death, degeneration and infection with microbes such as bacteria, viruses and fungi (Azab *et al*., 2016). Recent epidemiological and clinical studies have demonstrated that chronic inflammation is a major risk factor for about one-third of all cancer states (Hsu *et al*., 2010).

The use of plants in folk medicine for prevention and management of a wide variety of diseases has been in existence for a long time (Lukhoba *et al*., 2006). Plants with medicinal value contain bioactive compounds some of which have been used as drugs or precursors for synthetic analogues with improved bioactivity (Odeleye, 2010). Some examples of plant phytochemicals (**Figure 1.1**) that have been used in medicine since time immemorial include morphine (**1**), a potent pain medication which was first isolated from opium poppy seeds in 1805 (Pacifici, 2016). Others include the plant hormone salicylic acid (**2**) first reported by Hippocrates in the 4th century B.C as a bitter powder from willow tree bark and used to ease pain among women during child birth (Klessig & Malamy, 1994). The alkaloid, quinine (**3**), sequestered from the bark of cinchona tree, has been used since 1600s to treat malaria (Achan *et al*., 2011). In recent past, development of modern separation techniques and pharmacological testing methods has rejuvenated the interest in herbal medicine leading to discovery of new clinically important chemotherapeutics (Muiva *et al*., 2014).

In Kenya, different communities rely on herbal medicine for management of various diseases/disorders including cancer owing to limited accessibility and/or affordability of pharmaceutical drugs (Muthee *et al*., 2011). However, many of these medicinal plants including *Fagaropsis angolensis* have relatively very few or no reported scientific studies regarding their use in folk medicine. As a result, their effectiveness and safety profiles have not been established. The objective of the current study is

thus, to isolate pure compounds from *F. angolensis* root bark and evaluate their *in-vitro* anticancer activities.
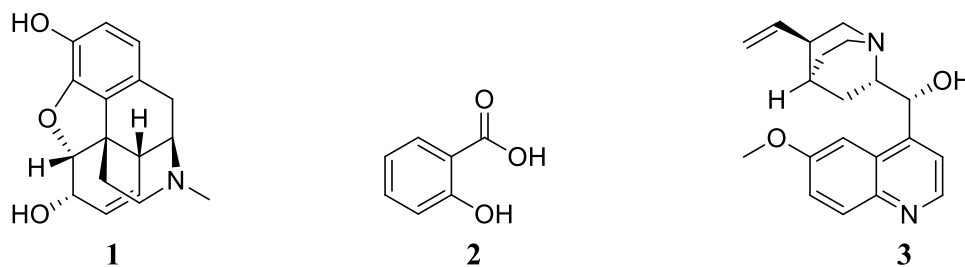
Figure 1.1: Chemical structures of some effective phyto-constituents since ancient times

1.1.1 Chronic inflammation and cancer

The link between inflammation and cancer has been attributed to chronic inflammation. The findings of epidemiological and clinical studies have shown that inflammatory diseases predispose individuals to various types of cancer including; colorectal, stomach, liver, cervical, gastric, bladder, esophageal, ovarian and prostate cancer (Mantovani *et al*., 2008). An estimated 15-20 % of all cancer related deaths globally arise from primary infections and inflammation, simply as part of the host adaptive immune responses (Coussens & Werb, 2002).

Particularly, excessive and protracted over-expression of pro-inflammatory mediators, including TNF-α, IL-1β, GM-CSF and IL-6 has been implicated as an important contributor to initiation, promotion and progression of tumorigenesis (Garcıa-Lafuente *et al*, 2009; Venancio *et al*., 2016). Previous *in vitro* and *in vivo* studies have shown that plant derived compounds have potential bioactivities against these pro-inflammatory mediators, thereby suppressing carcinogenesis (Patočka, 2003; Garcıa-Lafuente *et al*., 2009).

1.1.2 Cancer mortality in the World

By the year 2018, cancer was the second leading cause of mortality worldwide, causing 9.6 million deaths annually (WHO, 2018). About one in six deaths globally is as a result of cancer with approximately 70 % prevalence in developing countries (WHO, 2018). Lung cancer accounts for the highest mortality (1.76 million deaths), followed by colorectal (862 000 deaths), stomach (783 000 deaths), liver (782 000 deaths) and breast (627 000 deaths) (WHO, 2018). The prevalence of cancer is on the

rise due to elderly population, in addition to exposure to risk factors such as tobacco use, obesity, chronic inflammation, alcohol use, infectious agents, radiation and increased urbanization (Torre *et al*., 2015). The most often detected and leading causes of cancer mortality, both in developing and developed countries are lung and breast cancer in men and women respectively, (Torre *et al*., 2015). In the recent past, the worldwide cancer burden has shifted to low-and middle-income countries (LMIC), accounting for approximately 57% of cases and 65% of cancer deaths globally and this is attributed to population growth, aging and increasing incidence of cancer risk factors (Ferlay *et al*., 2012).

1.1.3   Cancer mortality in Africa

Cancer has become an emerging threat to public health in Africa. While cancer is presently not the top cause of mortality in sub-Saharan Africa, data suggest that the cancer burden is on the rise and will become a major problem in the first quarter of the 21st century (Morhason-bello *et al*., 2013). According to the International Agency for Research on Cancer (IARC) figures, nearly 645,000 cases and 456,000 deaths were reported in 2012 in Africa (GLOBOCAN, 2012). Additionally, cancers such as prostate, lung, and breast are detected at much higher incidences than in the past due to changes in lifestyle associated with urbanization and economic development (Jemal *et al*., 2012). Based on the GLOBOCAN figures for 2012, cervical cancer was the second most detected cancer, with the highest prevalence and death rates recorded in Eastern and West Africa (Vaccarella *et al*., 2017).

1.1.4   Cancer mortality in Kenya

In Kenya, cancer ranks the third chief cause of mortality after infectious diseases and heart related ailments resulting to approximately 7% of the total mortality annually. The prevalence of cancer is estimated at roughly 28,000 cases with over 22,000 deaths annually (Topazian *et al*., 2016).

The most frequently diagnosed cancers in men and women are prostrate and breast cancer respectively (Korir *et al*., 2015). Breast and cervical cancers account for nearly 44% of all cancer cases in women. Esophageal cancer is common in both sexes, and has a strangely irregular geographical dissemination, resulting in critical endemic regions in many countries (Korir *et al*., 2015). Previous studies implicated western

Kenya as a region with high risk for cancer of esophagus and exceptional to patients aged 30 years or younger (Dawsey *et al*., 2010; Parker *et al*., 2010). This has been attributed to frequent exposure to carcinogenic levels of alcohol and acetaldehyde due to high consumption of fermented *mursik* milk (Nieminen *et al*., 2012). In Africa, Kenya is among countries with highest prevalence of breast cancer and mortality rates have increased significantly in recent past (Sawe *et al*., 2016).

1.2 Statement of the problem

Current conventional cancer and chronic inflammation treatment modalities are very expensive, toxic, and less effective and majority of patients suffer from multidrug resistance (Clarke *et al*., 2005). This has necessitated studies to investigate potential sources of novel, less toxic and more efficacious chemopreventive agents, and plants have been considered an important part of ethnopharmacological approach to cancer and chronic inflammation treatment. There is also an urgent necessity to carry out phytochemical studies to ascertain the efficacy and cytotoxicity of herbal plants already in use. Among Coastal and Eastern Kenyan communities, *F. angolensis* is used traditionally for treatment and management of various diseases including cancer (Kareru *et al*., 2007; Jeruto *et al*., 2010). However, its bioactive constituents are relatively understudied and thus, there is need to carry out phytochemical studies on the plant to provide a scientific justification for its use in cancer management. This study therefore, focused on determining anti-cancer and anti-inflammatory constituents of *F. angolensis* used in traditional medicine.

1.3 Justification of the study

Recent studies on global burden of cancer have demonstrated that cancer accounts for more deaths than HIV, TB and Malaria combined and 70% of the worldwide burden is in developing countries like Kenya (Center *et al*., 2011). In Kenya, many rural communities depend on herbal medicine to manage a number of diseases including cancer and chronic inflammation (Kareru *et al*., 2007). *F. angolensis* is a medicinal plant used traditionally in cancer management, albeit there is limited scientific information on its safety, effectiveness and the bioactive constituents responsible for anticancer activity.

Previous screening of methanolic root stem extract which showed significant toxicity

on colon cancer cell lines (IC$_{50}$ 8.33 ± 1.42 µg/mL) compared to the reference drug doxorubicin (IC$_{50}$ 19.00 ± 9.00 µg/mL) provided motivation for further research (Yiaile *et al*., 2017). Therefore, phytochemical investigation of this plant with aim of isolating and characterizing anticancer and anti-inflammatory compounds to further justify its conservation and use in cancer management has been undertaken. The *in-vitro* anti-cancer and anti-inflammatory compounds will serve as lead agents for development of affordable, efficient and safe therapeutic drugs.

1.4 Hypothesis

Fagaropsis angolensis does not contain stable anti-cancer and anti-inflammatory secondary metabolites that can be isolated and characterized

1.5 Objectives

1.5.1 General objective

To isolate and characterize secondary metabolites from the root bark and leaves of *F. angolensis* with *in-vitro* anti-cancer and anti-inflammatory activities

1.5.2 Specific objectives

1. To isolate pure compounds from solvent extracts of the root bark and leaves of *F. angolensis*

2. To characterize the structures of the isolated compounds using spectroscopic and spectrometric techniques

3. To determine *in-vitro* anti-cancer and anti-inflammatory activities of pure compounds isolated from *F. angolensis*

CHAPTER TWO

LITERATURE REVIEW

2.1 Use of plants in medicine

Plants have been used for management of various ailments since ancient times. Over 50% of first line drugs in clinical use have been derived from natural sources including plants (Bijauliya *et al*., 2017). Folk medicine plays an integral role in management of protracted and life-threatening diseases and more than 80% of the global population use folk medicine for their main health care (Ayob *et al*., 2014). It is important to note that geographical distribution determines the abundance, accessibility and usage of these medicinal plants by native communities (Ochwang'i *et al*., 2014). Due to the massive pool of novel, less toxic and more effective bioactive constituents, ethnopharmacological studies are critical to discover new molecules that can serve as leads for drug development (Kiplimo, 2016).

The World Health Organization (WHO) approximates that more than 66% of the communities residing in low-and middle-income (LMIC) countries rely on folk medicine from plants for their health care necessities (WHO, 2008). This is ascribed to the fact that plant-based preparations are fairly cheap and easily accessible (Amin & Mousa, 2007; WHO, 2008; Ramawat & Goyal, 2008). Such herbal plant remedies comprise of blends of different plant parts and approaches of preparation, offering diverse biological activities which are attributed to the synergistic properties of the bioactive constituents present (Darshan & Doreswamy, 2004). In some occasions, traditional medicines are combined with conventional drugs to achieve a therapeutic effect (Kipkore *et al*., 2014).

2.2 Anti-inflammatory activity of natural products

The practice of using plants or plant-based products as a remedy to manage various ailments including inflammatory disorders in folk medicine is known since antiquity and is well established in most cultures (Azab *et al*., 2016).

Anti-inflammatory activity is one of the most reported biological activities of plant derived natural products. Ethnopharmacological activity-based research into plant compounds has provided immense contributions to the lengthy process of drug

development (Chekalina *et al*., 2018). However, despite the enormous progress in the development of numerous anti-inflammatory treatments, the safety and effectiveness of conventional anti-inflammatory drugs is over-shadowed by their undesired side effects (Maione *et al*., 2016). In the last decades, phytochemical and pharmacological studies have led to characterization of a variety of bioactive compounds (**Figure 2.1; Table 2.1**) with promising anti-inflammatory activities (Maione *et al*., 2016).

Table 2.1: Anti-inflammatory compounds isolated from plants

Compound	Plant source	Part(s) used	Reference
Kaempferol (**4**)	*Cassia renigera*	Flowers	Tang *et al*., 2015
Quercetin (**5**)	*Hypericum perforatum*	Leaves	Chekalina *et al*., 2018
Apigenin (**6**)	*Citrus sinensis*	Fruits	
Fisetin (**7**)	*Hymenaea courbaril*	Xylem sap	
Chrysin (**8**)	*Oroxylum indicum*	Stem barks	Rani *et al*., 2016
Luteolin (**9**)	*Stachys lavandulifolia*	Aerial parts	Lv *et al*., 2011
Formosumone A (**10**)	*Cratoxylum formosum*	Leaves	Xiong *et al*., 2014
Ursolic acid (**11**)	*Eriobotrya japonica*	Leaves	Kang *et al*., 2008
Oleanolic acid (**12**)	*Syzygium aromaticum*	Leaves	Azab *et al*., 2016
Maslinic acid (**13**)	*Olea europaea*	Fruits	Huang *et al*., 2011
Cucurbitacin B (**14**)	*Cucurbita andreana*	Fruits	Azab *et al*., 2016
Schisantherin A (**15**)	*Schisandra sphenanthera*	Fruits	Ci *et al*., 2010
Cimiracemate A (**16**)	*Cimicifuga racemosa*	Rhizomes	Yang *et al*., 2009
Honokiol (**17**)	*Magnolia abovata*	Stem bark	Munroe *et al*., 2007
Chlorojanerin (**18**)	*Saussurea heteromalla*	Whole plant	Saklani *et al*., 2012
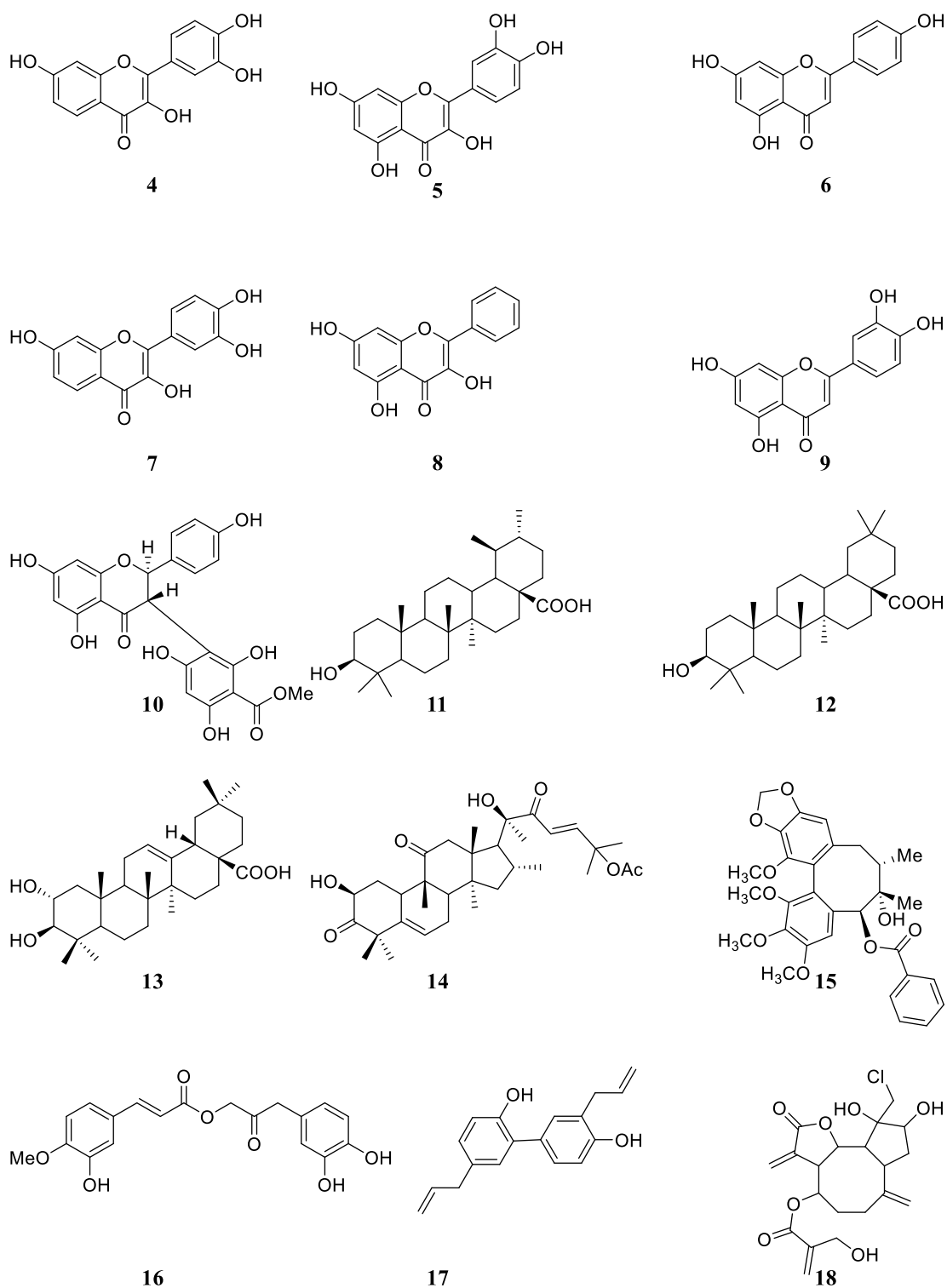
Figure 2.1: Anti-inflammatory compounds isolated from plants

2.3 Medicinal plants as a source of anti-cancer drugs

Since ancient times, plants have afforded an enormous pool of agents that have been

used in medicine, pharmacy and biology (Gordaliza, 2007). Over 60% of first line anticancer drugs in clinical use are derived from natural sources, including plants (Cragg *et al*., 2005; Newman *et al*., 2003). An estimated 35000 plant species have been shown to possess anticancer potencies and are being used in management of cancer (Kaur *et al*., 2015). A number of remarkable novel compounds (**Figure 2.1**) are in clinical use owing to their selective properties against cancer-related molecular targets (Cragg & Newman, 2005). Some of these include paclitaxel (**19**), docetaxel (**20**) vincristine (**21**), vinblastine (**22**), topotecan (**23**), flavopiridol (**24**) , etoposide (**25**) and irinotecan (**26**) (Sisodiya, 2013).
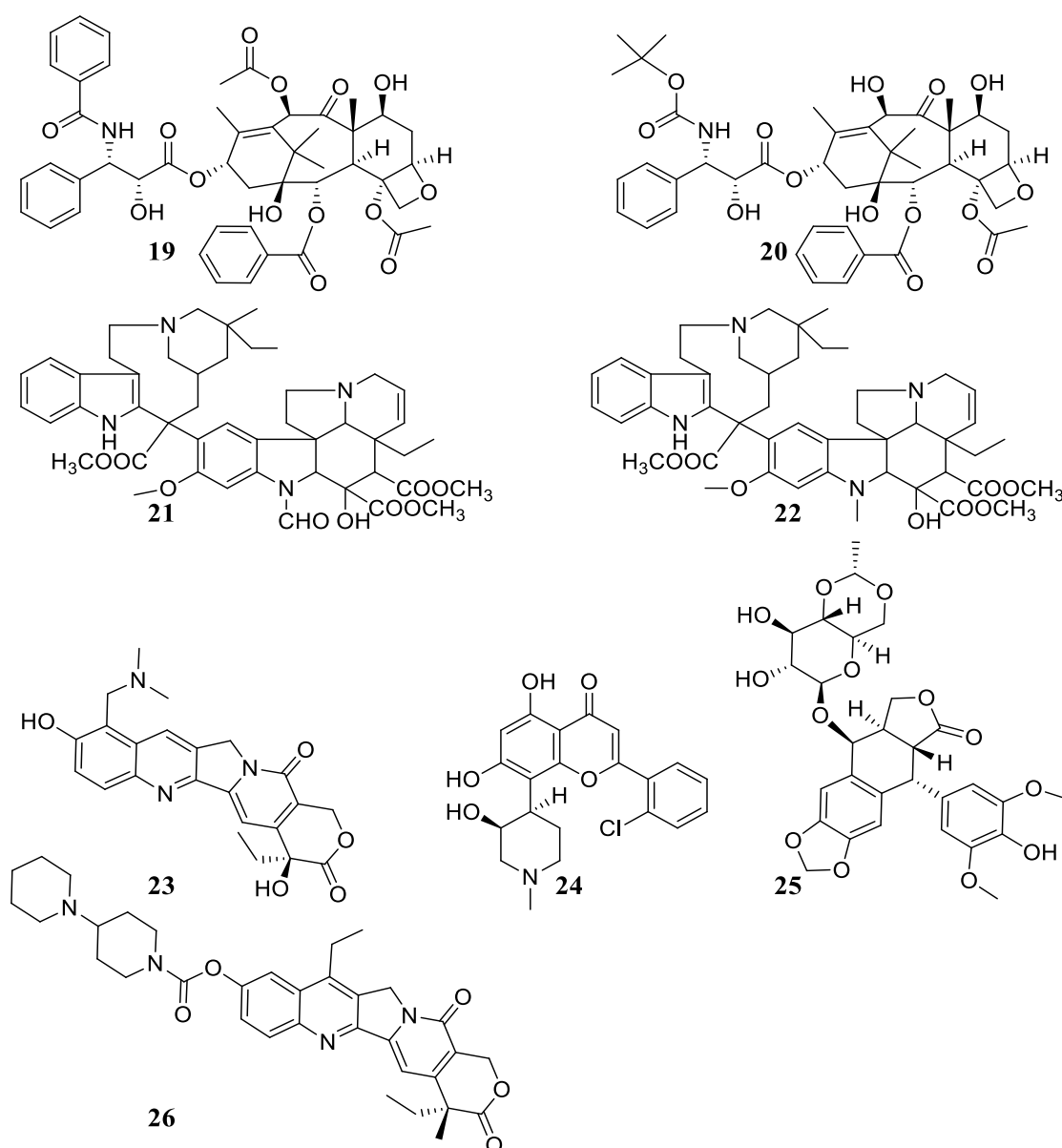
Figure 2.2: Plant based anticancer compounds in clinical development

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2.4 Rutaceae family

Rutaceae family contains approximately 160 genera and 1730 species of shrubs, herbs and trees, usually placed in the order sapindales distributed in dry, evergreen forest and throughout tropical regions of the world (Supabphol & Tangjitjareonkun, 2014) The main genera in the family are *Citrus*, *Zanthoxylum* and *Agathosma* (Tamokou *et al*., 2017). Recent phytochemical studies of plants from the family Rutaceae have shown diverse class of compounds such as alkaloids, flavonoids, triterpenoids and coumarins (Yiaile *et al*., 2018).

2.5 *Fagaropsis angolensis*

Fagaropsis angolensis is a deciduous tree that can grow to a height of 24 m, with pinkish grey bark, which is slightly rough and is sometimes covered with corky outgrowths. Its leaves are opposite, imparipinnate and bear 5 to 11 elliptic leaflets, which are glabrous, except for the midrib, and pitted with gland-dots primarily near the margin. The flowers are greenish yellow in terminal pinnacles that are 3 to 12 cm in length and bear fruits that are 6 to 8mm in diameter and purple round berry when ripe (Dalitz *et al*., 2011). It occurs in dry, evergreen forest and throughout tropical regions, distributed in Kenya, Ethiopia, DR Congo, Uganda, Rwanda, Tanzania, Zambia, Malawi, Mozambique and Zimbabwe (Eggeling, 1952; Waterman & Khalid, 1981).

(Photo taken by Vaderament-A Nchiozem-Ngnitedem)

Figure 2.3: Aerial part of *F. angolensis* (Engl.) H.M. Gardner

2.6 Ethnomedicinal uses of some *Fagaropsis* species

The leaf and root decoctions of *F. angolensis* have been used in folk medicine for management of malaria, back joint aches and cancer (Kareru *et al*., 2007; Jeruto *et al*., 2010). In Eastern Kenya, *F. hildebrandtii* (Engl.) Milne is used in management of chronic joint pains. A glass of leaves', roots' and shoots' concoction is taken orally, twice a day until the patient recovers (Wambugu *et al*., 2011). *F. angolensis* has also been used by the Coastal and Central Kenyan communities for management of malaria. The leaf decoction is taken orally, one cup three times every day for 3-4 days (Nguta *et al.*, 2010). In Southern Ethiopia and South-Western Uganda the leaves, fruits and bark decoctions of *F. angolensis* are used ethno medically in treatment and management of stomachache, rheumatisim, diarrhea, cough, cancer, stabbing pain and snake bite (Lacroix *et al*., 2011; Kewessa *et al*., 2015).

2.7 Biological activity of *Fagaropsis* species

Aqueous, methanolic and ethyl acetate extracts of *F. angolensis* stem and root barks have demonstrated antiplasmodial activities against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* as well as anti-cancer activities against human tumor cell lines (Kirira *et al*., 2006; Lacroix *et al*., 2011). The ethanolic extract from the stem bark of *F. angolensis* has been previously shown to exhibit selective *in-vitro* inhibitory activities against *Staphylococcus aureus* and *Candida albicans* with MIC of 64 and 32 µg/mL, respectively (Kuglerova *et al*., 2011). The whole root extract of *F. angolensis* displayed moderate *in vitro* inhibitory effects against throat cancer cell lines with IC_{50} value of 10.05 ± 2.15 µg/mL compared to 2.5 ± 0.5 µg/mL of the reference drug doxorubicin. Conversely, the methanolic root bark extract exhibited significant toxicity against colon cancer cell lines (IC_{50} 8.33 ± 1.42 µg/mL) compared to that of the reference drug doxorubicin (IC_{50} 19.00 ± 9.00 µg/mL) (Yiaile *et al*., 2017). Previous studies by Muia and colleagues on the *n*-hexane and aqueous root bark extracts of *F. angolensis*, revealed acute oral toxicity at >2000 mg/Kg in mice (Muia *et al*., 2020).

2.8 Phytochemical information on the genus *Fagaropsis*

Although the phytochemical work on this genus is very scanty, previous studies carried out on some *Fagaropsis* species have revealed presence of limonoids and alkaloids as the dominant secondary metabolites as well as sterols and ester

derivatives (Waterman & Khalid, 1981; Blaise *et al*., 1985; Bettarini *et al*., 1993; Boustie *et al*., 1995; Mudalungu *et al*; 2013).

2.8.1   Limonoids from the genus *Fagaropsis*

Limonoids are modified highly oxygenated triterpenoid derivatives of limonin, a bitter tetranortriterpenoid isolated from *citrus* fruits and are widely confined in Meliaceae and Rutaceae families. They are stereochemically homogeneous compounds containing or derivative of a 4,4,8-trimethyl-17 furanylsteroid backbone and are synthesized through terpenoids biosynthetic pathway (Tan & Luo, 2011). Limonoids found in this genus are largely restricted to the substitution of A and B rings and have been studied for their chemotaxonomic and commercial importance (Boustie *et al*., 1990; Roy & Saraf, 2006). Some of the limonoids isolated from the species of this genus are outlined in **Table 2.1** below.

Table 2.2: Some limonoids from the genus *Fagaropsis*

Compounds	Plant source	Plant part	Reference
Fagaropsine (**27**)	*F. glabra*	Trunk bark	Boustie *et al*., 1995
Isofraxinellone (**28**)	,,	,,	Blaise *et al*., 1985
Fraxinellone (**29**)	,,	,,	,,
Fraxinellonone (**30**)	,,	,,	Boustie *et al*., 1990
Rutoevin (**31**)	*F. angolensis*	Stem bark	Waterman & Khalid, 1981
Limonin diosphenol (**32**)			
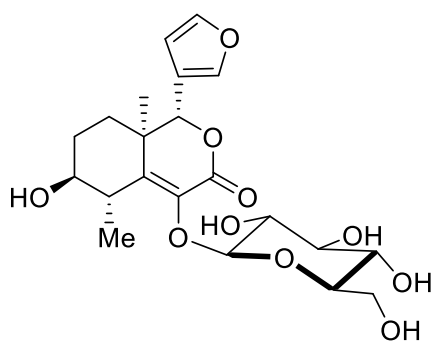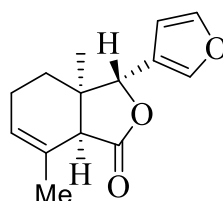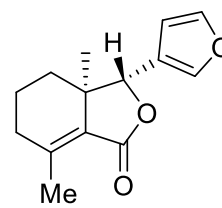
27   **28**   **29**
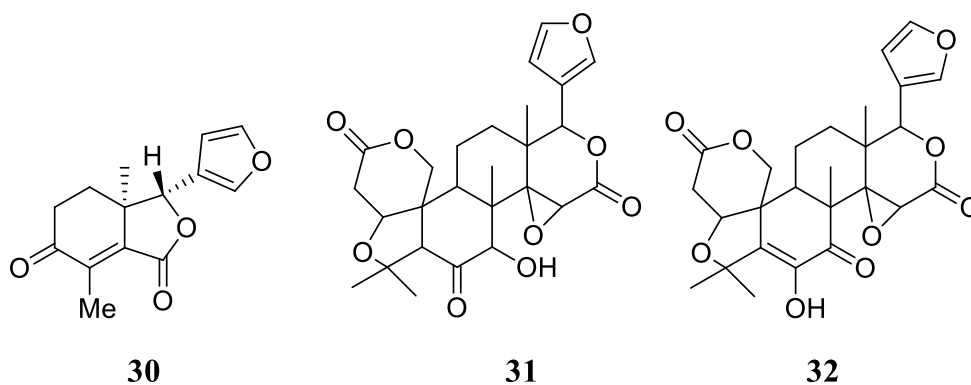
Figure 2.4: Limonoids from the genus *Fagaropsis*

2.8.2 Alkaloids from the genus *Fagaropsis*

Alkaloids are nitrogen containing compounds, which are characteristically alkaline owing to existence of heterocyclic ring comprising of a nitrogen atom (Matsuura & Fett-Neto, 2015). Two classes of alkaloids have been previously reported from the genus *Fagaropsis* namely benzophenanthridines and indole alkaloids (Waterman & Khalid, 1981; Khalid & Waterman, 1985; Bettarini *et al*., 1993). To date, over 100 benzophenanthridines have been isolated from plants according to Han *et al*. (2016) among which only four are reported from this genus. Some of the alkaloids previously reported from the genus *Fagaropsis* are indicated in **Table 2.2** below.

Table 2.3: Benzophenanthridine alkaloids from the genus *Fagaropsis*

Compound	Plant source	Plant part	Reference
6-acetonyl-dihydrochelerythrine (**33**)	*F. angolensis*	Stem bark	Waterman & Khalid, 1981
6-acetonyl-dihydrosanguinarine (**34**)	,,	,,	,,
6-acetonyl-dihydronitidine (**35**)	,,	,,	,,
6-hydroxymethyldihydronitidine (**36**)	,,	,,	Khalid & Waterman, 1985

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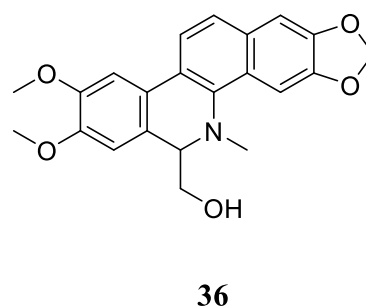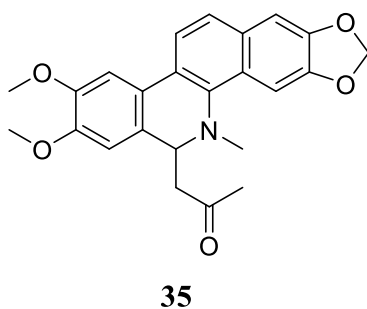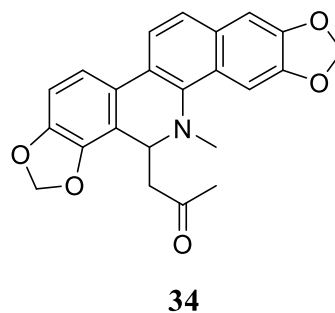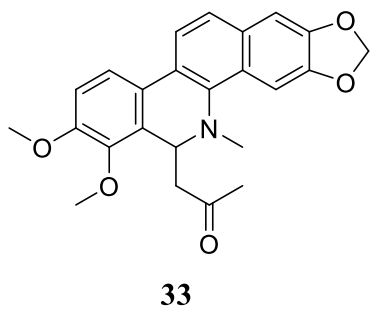
In addition to benzophenanthridines, two tryptophan-derivatives such as canthin-6-one (**37**) and 5-methoxycanthin-6-one (**38**) have also been reported from *F. angolensis* (Bettarini *et al*., 1993).

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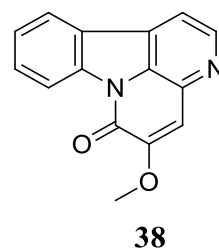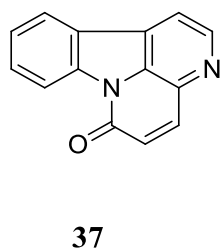
Other phytochemicals which have been identified from *Fagaropsis* species include phenanthrene carboxylic acid derivative (**39**), methylheneicosane ester derivative (**40**) and hexyl-9,10-dihydroxydec-5-enoate (**41**) (Mudalungu *et al*., 2013).
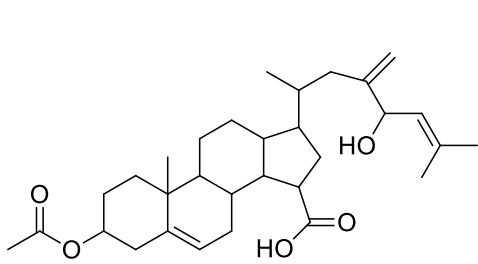
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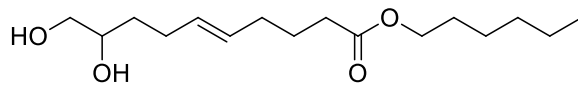
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CHAPTER THREE

MATERIALS AND METHODS

3.1 General

1D and 2D NMR experiments were recorded at 600 MHz (^{1}H) and 150 MHz (^{13}C) respectively, on a Bruker Avance III spectrometer using standard pulse sequences and referenced to residual solvent peaks. IR analyses were performed on a Bruker-Tensor 27 FT-IR Spectrometer using a diffuse reflection apparatus (cricket, Harrick Scientific). HRESIMS experiments were conducted on a LTQ-Orbitrap mass spectrometer (Thermo Scientific, USA) furnished with a HESI-II source. Optical rotation was performed in Kruss Optronic Polarimeter P8000-T. For column chromatography, Merk Silica gel 60 (0.063-0.200mm) and Sephadex LH–20 (25–100 µm, Amersham Biosciences) were used as stationary phases. TLC was performed on pre-coated silica gel 60 plates (230-400 mesh, Merck Grade, Darmstadt, Germany). Compounds on TLC were visualized under UV light at 254 or 365 nm and further by spraying with H_2SO_4–water (0.5:9.5, v/v) and warming.

3.2 Plant material collection

The root bark and leaves of *F. angolensis* were collected from the Coastal region of Kenya at Mrima Hill forest, Kwale County (S 04º 29′18.7′′ E 039º15′19.9′′) in May 2018. The plant identification and authentication was done by Mr. Patrick C. Mutiso, a taxonomist in the School of Biological Sciences Herbarium, University of Nairobi and a sample specimen preserved under Voucher number (NNA 2018/007).

3.3 Extraction and isolation of compounds from *Fagaropsis angolensis*

The root bark (4.25 kg) and leaves (1.15 kg) of *F. angolensis* were each air-dried, ground into fine powder and exhaustively extracted by cold percolation with 50% CH_3OH in CH_2Cl_2 (v/v) at room temperature for 48 hrs. The extracts were combined and filtered using a Büchner funnel and the solvent was removed under reduced pressure using a rotary evaporator (R-100 Büchi, Switzerland). The root bark yielded a brown crude extract (170.56 g) while the leaves afforded a dark green crude extract (75.12 g) which translated to 4.02% and 6.53%, respectively of the powdered material. The root bark extract (170.56 g) was adsorbed onto equal amount of silica gel then separation was carried out using column chromatography on silica gel as the

stationary matrix with 10% ethyl acetate (EtOAc) in *n*-hexane as the mobile phase initially. Elution was done in order of increasing polarity of the solvent system by increasing the polar solvent, from 10%, 20%, 30%, 40%, 50% EtOAc up to neat EtOAc and then using mixtures of 10%, 20%, 30% and 40% of MeOH in EtOAc. This resulted in 505 fractions of 100 mL each, which were combined based on their TLC profiles to only 8 fractions (Fr. 18A-Fr. 18H). Fraction Fr.18E of the main column afforded pink amorphous powder which were filtered in vacuo using a Büchner funnel and washed repeatedly with 20% EtOAc in *n*-hexane to give compound **45** (12.0 mg). Fraction Fr. 18H was further subjected to column chromatography using silica gel as the stationary phase eluting with gradients of 10% up to 40% MeOH in CH_2Cl_2 to give a semi-pure fraction (90.0 mg). This minor fraction was finally purified through a Chromatotron with 5% CH_3OH in CH_2Cl_2 eluent to afford compound **42** (2.32 mg). Fractions 18F and 18G were further combined to yield 8.15g and separated on silica gel column with 15% EtOAc in *n*-hexane then purified through Chromatotron to afford compound **43** (6.21 mg) and **44** (4.56 mg).

The leaves extract (75.12 g) was adsorbed onto equal amount of silica gel then subjected to silica gel column chromatography eluting with gradients of *n*-hexane/EtOAc (from 9:1, 8:2, 7:3, 1:1 and 0:10)) and EtOAc/MeOH MeOH (from 10:0, 9.5:0.5, 7:3, 1:1 and 0:10) as mobile phases resulting in 250 fractions of 100 mL each and further combined based on their TLC profiles into 5 main fractions (Fr. 14A-14E). Fr. 14C (*n*-hexane-EtOAc (7:3)) was further separated on silica gel column with gradients of *n*-hexane-EtOAc (from 9:1 to 0:10) to yield compound **46** (5.03 mg).

3.4 Biological activities

3.4.1 Resazurin Reduction Assay

Resazurin reduction assay (O'Brien *et al*., 2000) was carried to evaluate the anti-cancer potency of compound **46** against drug-sensitive CCRF-CEM leukemia cells. The assay is based on reduction of the oxidized non-fluorescent blue dye, resazurin, to the pink highly fluorescent resorufin by metabolically viable cells. Non-viable cells quickly loose the metabolic ability to reduce resazurin and, therefore, produce no fluorescent signal. Briefly, adherent cells were detached by treatment with 0.25% trypsin/EDTA and an aliquot of 1×10^4 cells was placed in each well of a 96-well cell

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culture plate in a total volume of 200 μL. Cells were allowed to attach overnight and then treated with different concentrations of the test samples. For suspension cells, aliquots of 2×104 cells per well were seeded in 96-well plates in a total volume of 100 μL. The test samples were immediately added in varying concentrations in additional 100 μL of culture medium to obtain a total volume of 200 μL/well. After 48h, 20 μL 0.01% w/v in double-distilled water (ddH$_2$O) were added to each well and the plates were incubated at 37 °C for 4h. Fluorescence was measured using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done at least two times, with three replicate each. The cell viability was evaluated based on a comparison with untreated cells.

3.4.2 Anti-inflammatory Assay

The anti-inflammatory experiments for compounds **42 – 45** were performed at Pharmacelsus, Saarbrücken, Germany. The human peripheral blood mononuclear cells (PBMCs) were obtained from blood ethically collected from four healthy donors with the ethnicity Caucasian (male, 41 years old), African-American (male, 31 years old), African American-Hispanic (male, 29 years old) and Caucasian (male, 32 years old). The pure compounds and the positive control ibuprofen were dissolved in dimethyl sulfoxide (DMSO) to achieve 20 mM stock solutions. Lipopolysaccharide (LPS) was dissolved in cell culture medium at a concentration of 1 mg/mL. The pure compounds and the positive control ibuprofen were used in a concentration of 100 μM. The final concentration of DMSO in all samples was 0.5% and all samples were co-incubated with 10 μg/mL LPS. The PBMCs are the main source of cytokines within the circulating blood. Due to the small amounts of cytokines released by PBMC into the supernatant, a bead-based assay (ProcartaPlex, Luminex) was used to quantify the four cytokines in parallel within a 50 μL sample using appropriate calibration standards. Human cryopreserved PBMC were thawed according to the manufacturer's instructions. Four vials of cells from different donors were pooled. Cells were washed, resuspended in RPMI 1640 containing 10% FBS, plated in 96-well round bottom plates at 100,000 PBMC / well and exposed to the test items at the concentrations specified above. Therefore, dilutions of test items were prepared in a 96-well plate and transferred to the PBMC containing wells. The cells were incubated for 24 hours at 37 °C and 5% CO$_2$. Then, plates were centrifuged for 3 min at 350 gyrations without brake and cell-free supernatant was collected and forwarded to

cytokine bead-array assay. The latter was conducted according to manufacturer's instructions and read in a MagPix reader. For the dose-response relationship, absolute concentrations were calculated by the MagPix software using two separate calibration series as provided by the manufacturer. As negative control, cells were incubated only with cell culture medium. As a positive control for inflammation, cells were incubated with 10 μg/mL LPS and as positive control for anti-inflammation, cells were co-incubated with 10 μg/mL LPS and 100 μM ibuprofen.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Secondary metabolites isolated from *Fagaropsis angolensis*

Chemical investigation of the root bark and leaves of *Fagaropsis angolensis* led to isolation of a total of five compounds. The roots bark extract afforded four compounds including two new norhopane derivatives; 3β,6β,22-trihydroxy-7β,11α-di[(4-hydroxybenzoyl)oxy]-21αH-24-norhopa-4(23)-ene (**42**) and 3β,6β,22-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-21αH-24-norhopa-4(23)-ene (**43**) along with the known norhopane, 3β,6β,11α-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene (**44**) and a norneohopane (21αH)-24-norneohopa-4(23), 22(29)-diene-3β,6β,7β-triol 7-caffeate (**45**). The leaves extract yielded a known flavone, tachrosin (**46**). Their spectroscopic and spectrometric data are discussed below.

4.1.1   3β,6β,22-trihydroxy-7β,11α-di[(4-hydroxybenzoyl)oxy]-21αH-24-norhopa-4(23)-ene (42)

Compound **42** was obtained as white amorphous solids with α_D^{22} = -19.0, (*c*. 0.03, MeOH). Its positive mode HRESI mass spectrum (Appendix **1K**) revealed a molecular adduct ion [M+Na]$^+$ at *m/z* 739.3812 corresponding to the molecular formula $C_{43}H_{56}O_9$ (calcd. 739.3822 for $C_{43}H_{56}O_9Na$). Its UV spectrum (Appendix **1I**) showed absorption maximum at λ$_{max}$ 259 nm while its FT-IR spectrum (Appendix **1J**) revealed characteristic absorption bands at 3411 cm^{-1} for hydroxy groups, 2971 cm^{-1} for methyl groups and 1690 cm^{-1} for ester carbonyl groups. The NMR data of **42** (Table **4.1**; Appendix **1A; 1B**) showed resonances of two *para*-disubstituted hydroxybenzene moieties attached at δ$_H$/δ$_C$ 5.21/76.3 and δ$_H$/δ$_C$ 5.77/74.2, each bearing an ester carbonyl at δ$_C$ 167.5 (C-7′) and 167.2 (C-7″) respectively. The ^{1}H and ^{13}C NMR spectra also showed a set of signals with AA′BB′ spin system with resonances at δ$_H$/δ$_C$ 7.97/133.1 (2H, *d*, *J* = 8.8 Hz, H-2′/6′), 7.91/132.9 (2H, *d*, *J* = 8.8 Hz, H-2″/6″), 6.87/116.3 (2H, *d*, *J* = 8.8 Hz, H-3′/5′) and 6.87/116.1 (2H, *d*, *J* = 8.8 Hz, H-3″/5″). Six tertiary methyls at δ$_H$/δ$_C$ 1.13 (3H, *s*, H-25)/16.8, 1.83 (3H, *s*, H-26)/13.8, 1.20 (3H, *s*, H-27)/18.3, 0.75 (3H, *s*, H-28)/15.5, 1.14 (3H, *s*, H-29)/29.7 and 1.12 (3H, *s*, H-30)/26.0; an exomethylene group at δ$_H$ 5.42 (1H, *d*, *J* = 1.8 Hz), 5.21 (1H, *m*) and δ$_C$ 106.2 (C-23); four oxymethines at δ$_H$/δ$_C$ 3.94 (1H, *m*)/73.6 (C-3),

4.35 (1H, *dd*, *J* = 3.8, 1.9 Hz)/71.6 (C-6), 5.21 (1H, *d, J*= 3.8 Hz)/76.3 (C-7) and 5.77 (1H, *td*, *J* = 11.3, 5.5 Hz)/74.2 (C-11) were also evidenced as part of the NMR data of **42**.

The initial analysis of these data set suggested that compound **42** was similar to 3β,6β-dihydroxy-7β,11α-di[(4-hydroxybenzoyl)oxy]-21α*H*-24-norhopa-4(23),22(29)-diene (Stevenson *et al*., 2016) previously isolated from *Zanha africana* root bark. The main difference was the presence of an additional tertiary oxygenated carbon at δ_C 73.9 (C-22) in compound **42**. Thus, **42** was suggested to be a 24-norhopane derivative with an isopropanolyl moiety at C-22 and an olefin at C-4/C-23. Both *p*-salicylate moieties were placed on the hopane backbone based on the interactions evidenced in the HMBC spectrum (Appendix **1F**) of **42** from H-7 (δ_H 5.21) to the carbonyl carbon at C-7′ (δ_C 167.5), the tertiary methyl carbon at C-26 (δ_C 13.8) and from H-11 (δ_H 5.77) to the carbonyl at C-7″ (δ_C 167.2) together with the methine carbon at C-9 (δ_C 53.0). This was further supported by the ^{1}H-^{1}H COSY spectrum (Appendix **1D**) which showed $J_{1H,1H}$ correlations of H-7 (δ_C 5.21, *d, J* = 3.8 Hz) with the oxymethine proton at H-6 (δ_H 4.35, *dd, J* = 3.8, 1.9 Hz) and H-11 (δ_H 5.77, *td, J* = 11.3, 5.5 Hz) with the methine proton at H-9 (δ_H 2.17, *d, J* = 11.3 Hz) respectively. In addition, the placement of the hydroxyl group at C-3, C-6 and C-22 in **42** was confirmed from the HMBC cross peaks between H-3 (δ_H 3.94) with C-1 (δ_C 44.6), C-2 (δ_C 36.7), C-4 (δ_C 151.1), C-5 (δ_C 51.0); H-6 (δ_H 4.35) with C-7 (δ_C 76.3), C-8 (δ_C 50.0), C-10 (δ_C 43.6) and between H-29/H-30 (δ_H 1.14/1.12) with C-21 (δ_C 51.9) and C-22 (δ_C 73.9), respectively (Nguyen *et al*., 2019; Stevenson *et al*., 2016).

The relative stereochemistry of **42** was established using NOESY spectrum (Appendix **1G**; **1H**), coupling constants between aliphatic protons and biosynthetical overview of hopane triterpenoids. The hopane-type triterpene is derived from an all-chair cyclization of the squalene precursor (Volkman, 2005). The *β*-orientation of the 3-OH group was confirmed by the characteristic coupling constants of H-3 (*dd, J* = 11.9, 5.2 Hz) (Jayasinghe *et al*., 2001) and the NOESY correlations between H-3 and H-6. The NOESY spectrum also confirmed the relative configuration of the stereocenters at C-6, C-7 and C-21 as indicated by the correlations between H-6 with H-7 and H-6 with H-5; H-7 with H$_3$-27; and H-21 with H$_3$-28 respectively. Proton H-9 showed an axial-axial coupling with $J_{9,11}$ =11.3Hz suggesting that H-11 was *β*-

oriented. Thus, compound **42** was newly characterized as 3β,6β,22-trihydroxy-7β,11α-di[(4-hydroxybenzoyl)oxy]-21αH-24-norhopa-4(23)-ene.

Table 4.1: NMR (600 and 150 MHz, CD₃OD) spectroscopic data of compound **42**

C-position	δ_C	δ_H (*m, J* in *Hz*)	HMBC (^2J, ^3J)
1	44.6	2.34 *m*, 1.42 *m*	-
2	36.7	1.74 *m*, 1.33 *m*	-
3	73.6	3.94 *m*	C-1, 2, 4, 5
4	151.1	-	-
5	51.0	1.89 *m*	-
6	71.6	4.35 *dd* (3.8, 1.9)	C-10, 8, 7
7	76.3	5.21 *d* (3.8)	C-26, 7′
8	50.0	-	-
9	53.0	2.17 *d* (11.3)	C-26, 25, 10, 1, 8, 11
10	43.6	-	-
11	74.2	5.77 *td* (11.3, 5.5)	C-9, 7″
12	33.5	1.84 *m*	-
13	49.5	1.79 *m*	C-28, 27, 14
14	45.7	-	-
15	40.5	1.66 *m*, 1.04 *m*	-
16	26.0	1.66 *m*, 1.31 *m*	-
17	52.9	1.08 *m*	-
18	47.6	-	-
19	40.8	1.45 *m*, 1.05 *m*	-
20	25.7	1.72 *m*, 1.38 *m*	-
21	51.9	1.72 *m*	-
22	73.9	-	-
23	106.2	5.42 *d* (1.8), 5.21 *m*	C-5, 4, 3
25-Me	16.8	1.13 *s*	C-10, 1
26-Me	13.8	1.83 *s*	C-14, 8, 9, 7
27-Me	18.3	1.20 *s*	C-15, 14, 13, 8
28-Me	15.5	0.75 *s*	C-19, 18, 17, 13
29-Me	29.7	1.14 *s*	C-30, 22, 21

30-Me	26.0	1.12 *s*	C-29, 22, 21
7-O-(4-OHBz)		-	-
7′	167.5	-	-
1′	123.1	-	-
2′,6′	133.1	7.97 *d* (8.8)	C-2′/6′, 4′, 7′
3′,5′	116.3	6.87 *d* (8.8)	C-3′/5′, 1′, 4′
4′	163.5	-	-
11-O-(4-OHBz)		-	-
7″	167.2	-	-
1″	122.8	-	-
2″,6″	132.9	7.91 *d* (8.8)	C-2″/6″, 4″, 7″
3″,5″	116.1	6.87 *d* (8.8)	C-3″/5″, 1″, 4″
4″	163.6	-	-

4.1.2   3β,6β,22-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-21α*H*-24-norhopa-4(23)-ene (43)

Compound **43** was isolated as white amorphous solids with α_D^{22} = + 23.1, (*c* 0.13, MeOH). Its molecular formula $C_{36}H_{52}O_6$ was determined by its positive mode HRESI mass spectrum (Appendix **2J**) showing a sodiated adduct [M+Na]$^+$ at *m/z* 603.3658 (calcd. 603.3662 for $C_{36}H_{52}O_6Na$). Its UV spectrum (Appendix **2H**) indicated absorption maximum at λ_{max} 276 nm and its FT-IR spectrum (Appendix **2I**) showed characteristic absorption bands at 3411 cm^{-1} for hydroxy stretching, 2970 cm^{-1} for methyl stretching and 1691 cm^{-1} for ester carbonyl groups.

The spectral data of **43** (Table **4.2**) was rather identical to that of **42** except that **43** had only one *p*-salicylate moiety attached at δ_H/δ_C 5.19/77.1 (C-7′) bearing an ester carbonyl at δC 167.7. This was demonstrated by the significant upfield shifts experienced by C-11 ($\Delta\delta$ -51.7ppm) and C-12 ($\Delta\delta$ -8.4ppm) respectively. Therefore, **43** was also identified as a 24-norhopane derivative with a 2-hydroxy-2-propyl group at C-21 and an olefin at C-4/C-23. The HMBC spectrum (Appendix **2F**) showed interactions from H-7 to C-1′ (δC 167.7) highlighting the position of the benzoyl group on the hopane core. The ^{1}H-^{1}H COSY spectrum (Appendix **2D**) showed vicinal relationship between the two oxymethine protons H-7 (δ_H 5.19) and H-6 (δ_H 4.38), in addition to vinylic correlations between the exomethylene protons H$_2$-23 (δ_H 5.36/5.21) to the oxymethine proton H-3 (δ_H 3.94).

The multiplicities, coupling constants and the NOESY correlations (Appendix **2G**) for H-3, H-6 and H-21 were identical for **42** and **43** confirming that the configurations of

these atoms were conserved between the two compounds. As expected the *β*-orientation of 7-(4-hydroxybenzoyl) oxy group was indicated by the NOESY correlations between H-7 with H$_3$-27 and H-7 with H-5. Therefore, the structure of compound **43** was newly characterized as 3β,6β,22-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-21*αH*-24-norhopa-4(23)-ene.

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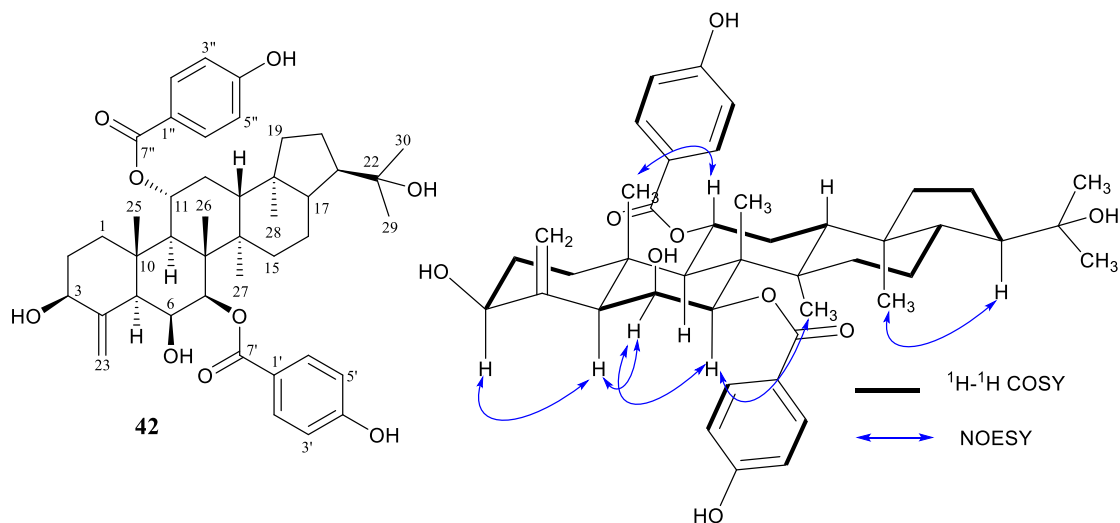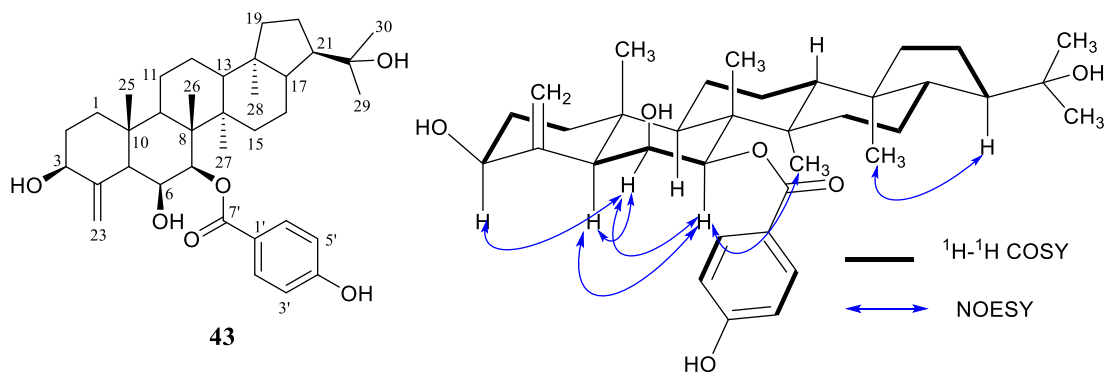**Table 4.2:** NMR (600 and 150 MHz, CD$_3$OD) spectroscopic data of compound **43**

C-position	δ_C	δ_H (*m, J* in *Hz*)	HMBC (^2J, ^3J)
1	41.6	1.83 *m*, 1.17 *m*	-
2	33.1	1.90 *m*, 1.50 *m*	-
3	74.0	3.94 *m*	C-4
4	151.0	-	-
5	51.5	1.75 *m*	-
6	71.9	4.38 *dd* (3.8, 2.0)	C-10, 8, 7, 5, 4
7	77.1	5.19 *d* (3.8)	C-26, 7′, 14, 8
8	48.0	-	-
9	50.6	1.62 *m*	-
10	39.7	-	-
11	22.5	1.77 *m*, 1.61 *m*	-
12	25.1	1.72 *m*, 1.50 *m*	-
13	49.6	1.58 *m*	-
14	45.0	-	-
15	36.9	1.70 *m*, 1.01 *m*	-
16	24.5	1.73 *m*, 1.51 *m*	-
17	52.8	1.04 *m*	-
18	46.0	-	-
19	41.0	1.50 *m*, 1.01 *m*	-
20	25.6	1.70 *m*, 1.50 *m*	-
21	52.0	1.75 *m*	-
22	73.9	-	-
23	105.8	5.36 *m*, 5.21 *m*	C-5, 4, 3
25-Me	16.6	1.06 *s*	C-10, 1, 9
26-Me	13.0	1.69 *s*	C-14, 8, 9, 7
27-Me	17.9	1.09 *s*	C-15, 14, 13, 8
28-Me	15.8	0.73 *s*	C-19, 18, 17, 13

29-Me	29.7	1.13 *s*	C-30, 22, 21
30-Me	26.1	1.11 *s*	C-29, 22, 21
7-O-(4-OHBz)	-	-	-
7′	167.7	-	-
1′	123.2	-	-
2′,6′	133.1	7.96 *d* (8.8)	C-3′/5′, 2′/6′, 4′,7′
3′,5′	116.1	6.86 *d* (8.8)	C-3′/5′, 1′, 4′
4′	163.5	-	-

4.1.3   3β,6β,11α-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23), 17(21)-diene (44)

Compound **44** was isolated as white amorphous powder which was UV$_{254}$ active. The spot of this compound was also visible upon exposure to iodine vapor and further on spraying with H_2SO_4–water (0.5:9.5, v/v). Its molecular formula $C_{36}H_{52}O_6$ was determined from its positive mode HRESI mass spectrum (Appendix **3H**) which showed a molecular adduct ion [M+Na]$^+$ at *m/z* 601.3503 (calcd. 603.3505, for $C_{36}H_{50}O_6Na$). The ^1H NMR spectrum of **44** (Appendix **3A**; Table **4.3**) clearly showed presence of six methyl groups at δ_H 1.26 (3H, *d*, *J* = 4.1, H-26), 1.63 (3H, *s*, H-26), 1.21 (3H, *s*, H-27), 0.90 (3H, *s*, H-28), 0.89 (3H, *s*, H-29), and 0.99 (3H, *d*, *J* = 6.9, H-30). Four oxymethine protons at δ_H 3.93 (1H, *m*), 4.16 (1H, *dt*, *J* = 10.8, 5.4), 4.32 (1H, *dd*, *J* = 3.6, 2.0) and 5.15 (1H, *d*, *J* = 3.6) , one exomethylene group at δ_H 5.41 (1H, *d*, *J* = 1.9), 5.22 (1H, *m*), in addition to signals at δ_H 6.87 (2H, *d*, *J* = 8.8) and 7.95 (2H, *d*, *J* = 8.8), assignable to four aromatic protons of an AA′BB′ system.

The ^{13}C NMR data (Table **4.3**; Appendix **3B**) and HSQC spectrum (Appendix **3E**) displayed resonances of 34 carbons characteristic of a triterpenoid. Analysis of DEPT spectrum (Appendix **3C**) of **44** revealed six methyl carbons at δ_C 13.5 (C-26), 16.1 (C-27), 16.6 (C-25), 18.9 (C-28), 21.6 (C-29) and 22.2 (C-30), eight methylene carbons at δ_C 21.1 (C-16), 28.2 (C-20), 33.6 (C-2), 35.7 (C-15), 36.7 (C-12), 42.7 (C-19), 43.6 (C-1) and 105.9 (C-23), twelve methine carbons at δ_C 27.5 (C-22), 49.6 (C-13), 51.7 (C-5), 55.5 (C-9), 71.0 (C-11), 72.0 (C-6), 73.9 (C-3), 77.1 (C-7), 116.2 (C-3′/5′) and 133.1 (C-2′/6′) and ten quaternary carbons at δ_C 41.2 (C10), 44.7 (C-14), 49.8 (C-8), 50.8 (C-18), 123.1 (1′), 137.6 (C-21), 140.2 (C-17) 151.4 (C-4), 163.6 (C-4′) and 167.5 (C-7′) . The resonances at δ_C 137.6 (C-21) and 140.2 (C-17) confirmed that the E-ring was a fused cyclopentene while δ_C 167.5 was associated with the ester carbonyl at C-7′.

The ^{1}H-^{1}H COSY spectrum (Appendix **3D**) revealed key vicinal correlations between two oxymethine protons H-7 (δ_H 5.15) and H-6 (δ_H 4.32) as well as vinylic correlations between the exomethylene protons H$_2$ (δ_H 5.41/5.22) and the oxymethine proton H-3 (δ_H 3.93). Further, placement of the *p*-salicylate group at C-7 was confirmed from the HMBC correlations (Appendix **3F**) between the oxymethine proton H-7 (δ_H 5.15) and the ester carbonyl C-7' (δ_C 167.5). In addition, the isopropyl group was located at C-21 on the basis of HMBC correlations observed between the *gem*-dimethyl protons δ_H 0.89 (H$_3$-29) and 0.99 (H$_3$-30) and the quaternary carbon at C-21 (δ_C 137.6). Analysis of the multiplicities, coupling constants and correlations observed from NOESY spectrum (appendix **3G**) for H-3, H-6, H-7 and H-11 were identical for **42** and **44** confirming that the configurations of these atoms were conserved between the two compounds. Thus, based on the aforementioned data and the comparisons with published literature values, compound **44** was identified as a 24-norhopane derivative, 3β,6β,11α-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene (Stevenson *et al*., 2016) previously characterized from *Zanha africana* root bark.
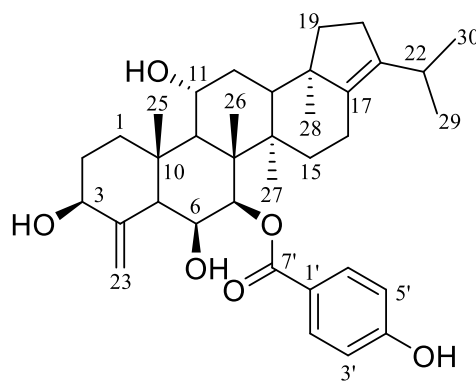
44

Table 4.3: NMR (600 and 150 MHz, CD$_3$OD) spectroscopic data of compound **44**

C-position	δ_C	δ_H (*m, J* in *Hz*)	HMBC (^2J, ^3J)
1	43.6	2.91 *m*, 1.23 *m*	C- 2, 3, 10, 25
2	33.6	1.83 *m*, 1.48 *m*	-
3	73.9	3.93 *m*	C- 4
4	151.5	-	-
5	51.7	1.81 *m*	C- 3, 4, 10, 26
6	72.0	4.32 *dd* (3.6, 2.0)	C- 5, 7, 8, 10
7	77.1	5.15 *d* (3.6)	C- 7', 8, 14, 26
8	49.8	-	-

9	55.5	1.73 *m*	C- 1, 8, 10, 11
10	41.2	-	-
11	71.0	4.16 *dt* (10.8, 5.4)	C- 8, 9, 11
12	36.7	1.75 *m*, 1.58 *m*	C- 11, 13
13	49.6	1.59 *m*	-
14	44.7	-	-
15	35.7	1.45 *m*, 1.18 *m*	-
16	21.1	2.12 *m*, 1.89 *m*	C- 14, 15
17	140.2	-	-
18	50.8	-	-
19	42.7	1.72 *m*, 1.40 *m*	-
20	28.2	2.23 *m*, 2.17 *m*	C- 17, 19, 21
21	137.6	-	-
22	27.5	2.59 *m*	-
23	105.9	5.41 *d* (1.9), 5.22 *m*	C- 3, 4, 5
25-Me	16.6	1.26 *d* (4.1)	C- 1, 5, 9, 10
26-Me	13.5	1.63 *s*	C- 7, 8, 9, 14
27-Me	16.1	1.21 *s*	C- 8, 14, 15
28-Me	18.9	0.90 *s*	C- 17, 18, 19
29-Me	21.6	0.89 *s*	C- 21, 22, 30
30-Me	22.2	0.99 *d* (6.9)	C- 21, 22, 29
7-O-(4-OHBz)		-	-
7′	167.5	-	-
1′	123.1	-	-
2′,6′	133.1	7.95 *d* (8.8)	C- 3′, 4′, 5′, 7′
3′,5′	116.2	6.87 *d* (8.8	C- 1′, 4′
4′	163.6	-	-

4.1.4 (21*αH*)-24-norneohopa-4(23), 22(29)-diene-3β,6β,7β-triol 7-caffeate (45)

Compound **45** was isolated as a pink amorphous powder which was active on UV$_{254}$. Its molecular weight was determined by its positive mode HRESI mass spectrum (Appendix **4H**) showing a molecular adduct ion [M+Na]$^+$ at *m/z* 627.3656 (calcd. 627.3662 for C$_{38}$H$_{52}$O$_6$Na) which was consistent with the proposed molecular formula C$_{38}$H$_{52}$O$_6$. The ^{1}H-NMR spectrum of **45** (Appendix **4A**) displayed characteristic peaks for five tertiary methyl proton singlets at δ_H 0.91 (3H, *s*, H-25), 1.49 (3H, *s*, H-26), 0.97 (3H, *s*, H-27), 0.64 (3H, *s*, H-28), and 1.61(3H, *s*, H-30). Two exomethylene groups at δ_H 5.24 (1H, *s*,), 5.10 (1H, *s*)/H-23 and 4.63 (2H, *m*, H-29), three oxymethines at δ_H 3.76 (1H, dd, *J* = 11.6 and 5.6 Hz), 4.11 (1H, *s*) and 4.92 (1H, d, *J* = 3.9 Hz) together with signals assignable to a 6,7 dihydrocinnamoyl moiety. A resonance for an isopropenyl group, was observed at δ_H 4.63 (2H, *m*) and assigned to C-29 based on vinylic correlations between H$_2$-29 and H-21 (δ_H 2.16, *m*) in the ^{1}H-H COSY spectrum (Appendix **4D**).

The ^{13}C NMR data of **45** (Table **4.4**; Appendix **4B**) aided by DEPT and HSQC experiments (Appendix **4C; 4E**) displayed 38 signals characteristic of triterpenoids (the signal at δ_C 44.3 being an overlapping of C-14 and C-17). Further analysis of the ^{13}C NMR and DEPT data of **45** disclosed eight signals at δ_C 166.7, 148.7, 146.1, 144.9, 126.1, 121.7, 116.3, 115.9 and 115.0 which were assigned to a *trans*-caffeoyl moiety (Li *et al.*, 2003). Moreover, in agreement with ^{1}H NMR data, three characteristic signals for oxymethine carbons resonating at δ_C 75.2, 71.9 and 70.2 together with two exomethylene groups at δ_C 105.3, 151.0, 110.4 and 147.7 were observed. Based on the above spectroscopic data and the published literature, compound **45** was characterized as a norneohopane caffeate, (21α*H*)-24-*nor*-neohopa-4(23), 22(29)-diene-3β,6β,7β-triol 7-caffeate, previously isolated from *Filicium decipiens* stem bark (Jayasinghe *et al.*, 2001).
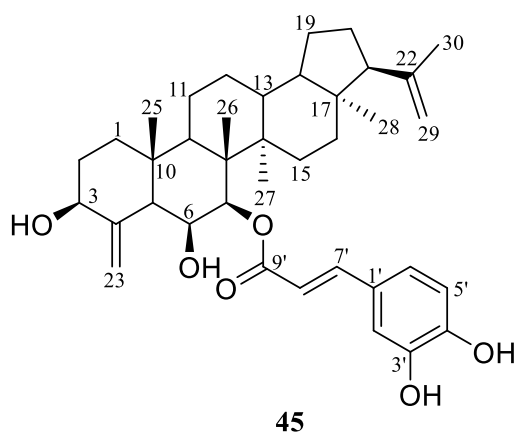

45

Table 4.4: NMR (600 and 150 MHz, C_2D_6OS) spectroscopic data of compound **45**

C-position	δ_C	δ_H (*m, J* in *Hz*)	HMBC (^2J, ^3J)
1	40.3	1.47 *m*, 1.08 *m*	C- 10, 25
2	32.8	1.73 *m*, 1.30 *m*	-
3	71.9	3.76 *dd* (11.6, 5.6)	C-5
4	151.0	-	-
5	49.8	1.66 *m*	C-4, 10, 25
6	70.2	4.11 *s*	C-5
7	75.2	4.92 *d* (3.9)	C-8, 9′, 14, 26
8	46.5	-	-
9	49.0	1.45 *m*	C-10
10	38.8	-	-
11	21.5	1.77 *m*, 1.09 *m*	-
12	21.4	1.65 *m*, 1.46 *m*	-
13	48.1	1.47 *m*	C-27
14	44.3	-	-

15	35.2	1.52 *m*, 0.96 *m*	-
16	40.6	1.64 *m*, 1.08 *m*	-
17	44.3	-	-
18	53.5	0.95 *m*	-
19	24.3	1.39 *m*	-
20	27.0	1.77 *m*, 1.36 *m*	-
21	47.9	2.16 *m*	C- 18, 20, 22, 29, 30
22	147.7	-	-
23	105.3	5.24 *s*, 5.10 *s*	C- 3, 18
25-Me	16.3	0.91 *s*	C- 5, 9, 10
26-Me	12.6	1.49 *s*	C- 7, 8, 14
27-Me	17.5	0.97 *s*	C- 8, 14, 15
28-Me	15.4	0.64 *s*	C- 16, 18, 17, 21
29	110.4	4.63 *m*	C- 21, 30
30-Me	20.0	1.61 *s*	C- 21, 22, 29
Trans-caffeoyl moiety)	-	-	-
1′	126.1	-	-
2′	115.0	7.03 *d* (2.1)	C- 3′, 4′, 6′, 7′
3′	146.1	-	-
4′	148.7	-	-
5′	116.3	6.75 *d* (8.2)	C- 1′, 3′, 4′
6′	121.7	6.98 *dd* (8.4, 2.1)	C- 2′, 4′, 7′
7′	144.9	7.45 *d* (15.7)	C- 1′, 2′, 6′, 9′
8′	115.9	6.24 *d* (15.7)	C- 1′, 9′
9′	166.7	-	-

4.1.5 Tachrosin (46)

Compound **46** was isolated from the CH_2Cl_2/MeOH (1:1) extract of the leaves of *F. angolensis* as a yellow paste. The compound on TLC showed a blue fluorescent colour under UV_{254} light and a yellow coloration upon spraying with H_2SO_4–water (0.5:9.5, v/v). Its positive mode HRESI mass spectrum (Appendix **5G**) showed a quasi-molecular ion peak at *m/z* 393.1334 (calcd. 393.1338 for $C_{23}H_{21}O_6$) consisted with the molecular formula $C_{23}H_{20}O_6$. The ^1H NMR data (Appendix **5A**) which showed a resonance at δ_H 6.70 (1H, *s*, H-3) along with ^{13}C NMR (Appendix **5B**) resonances at δ_C 163.6 (C-2), 108.9 (C-3) and 180.2 (C-4) indicated that compound **46** was a flavone derivative (Smalberger *et al.*, 1971; Muiva, 2017). The sharp singlet at δ_H 6.75 which was assigned to H-6 (δ_C 93.4) based on its HMBC correlations (Table **4.5**, Appendix **5E**) with δ_C 109.2 (C-4a), 162.7 (C-5), 163.9 (C-7), and 99.3 (C-8) indicated that ring A was tri-substituted. The NMR data further revealed presence of two methoxy groups at δ_H 4.04 (δ_C 56.8) and δ_H 4.01 (δ_C 57.0). The HMBC cross peaks depicted from δ_H 4.04 to C-5 (δ_C 162.7) and δ_H 4.01 to C-7 (δ_C 163.9) supported

the placement of these methoxy groups in ring A (Salame *et al.*, 2012).

Presence of a 3-furanone ring substituent at C-8 was evidenced by resonances at δ_H 8.64 (1H, *s*, H-5″) and 1.55 (6H, *s, gem*-dimethyl groups); δ_C 207.4 (C-3″), 110.6 (C-4″), 179.3(C-5″), 89.5 (C-2″) and 23.3 (for the *gem*-dimethyl groups) (Muiva *et al.*, 2014). The downfield shift at δ_H 8.64 for H-5″ suggested an olefinic proton attached to oxygen and is part of an *α,β*-unsaturated carbonyl system in the 3-furanone ring (Smalberger *et al.*, 1971;Salame *et al.*, 2012). The NMR resonances at δ_H/δ_C 127.5/7.82 (2H, *m*, H-2′/6′), 130.0/7.50 (2H, *m*, H-3′/5′) and 132.8/7.55 (1H, *m*, H-4′) revealed that ring B was not substituted. Thus, compound **46** was identified as tachrosin previously reported from the leaves and stems of *Tephrosia polvstachyoides* (Smalberger *et al.*, 1971). This is therefore, the first report of its isolation from *Fagaropsis* genus.
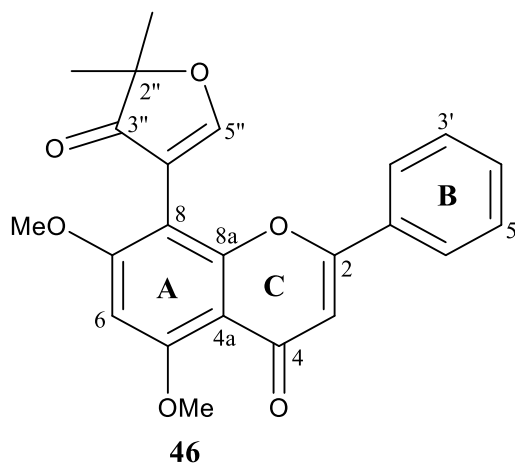

46

Table 4.5: NMR (600 and 150 MHz, CD₃OD) spectroscopic data of compound **46**

C-position	δ_C	δ_H (*m, J* in *Hz*)	HMBC (^2J, ^3J)
2	163.6	-	-
3	108.9	6.70 *s*	C- 4, 4a
4	180.2	-	-
4a	109.2	-	-
5	162.7	-	-
6	93.4	6.75 *s*	C- 4a, 5, 7, 8
7	163.9	-	-
8	99.3	-	-
8a	158.1	-	-
1′	132.7	-	-
2′/6	127.5	7.82 *m*	C- 2, 4′
3′/5′	130.0	7.50 *m*	C- 4′
4′	132.8	7.55 *m*	C- 2′, 6′
2″	89.5	-	-

3″	207.4	-	-
4″	110.6	-	-
5″	179.3	8.64 *s*	C- 2″, 3″, 4″
CH$_3$O-5	56.8	4.04 *s*	C- 5
CH$_3$O-7	57.0	4.01 *s*	C- 7
Me$_2$-2″	23.3	1.55 *s*	C- 2″, 3″

4.2 Biological activity

4.2.1 Cytotoxicity

Compound **46** was screened to evaluate its cytotoxic potency against drug-sensitive CCRF-CEM leukemia cells using resazurin reduction assay with doxorubicin as the reference anticancer drug (Table **4.6** and Figure **4.1**). In the resazurin reduction assay used, pure compounds are considered active when they cause cell inhibition of more than 70% at 10 µM (Nyaboke *et al*., 2018). Based on this criterion, compound **46** exhibited minimal activity since it showed cell viability of about 92.49% (7.51% inhibition) at 10 µM compared to doxorubicin which exhibited 100% inhibition at the same concentration.

Table 4.6: Anticancer results of compound **46** and doxorubicin against CCRF-CEM (mean ± SD, n = 3)

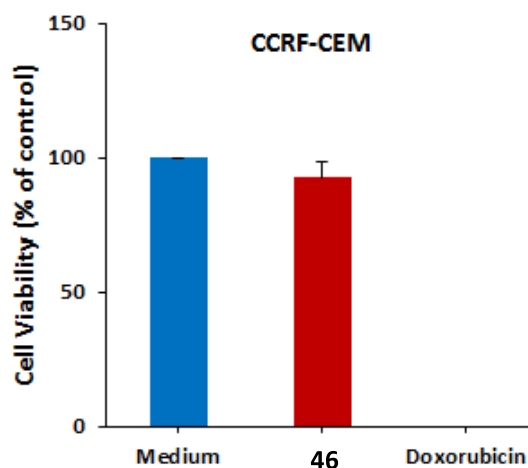|	Cell Viability (% of control)	Cell Inhibition (% of control)
Samples (10 µM)	CCRF-CEM	CCRF-CEM
Medium	100.00 ± 0.00	0.00 ± 0.00
Compound **46**	92.49 ± 6.28	7.51 ± 6.28
Doxorubicin	0.00 ± 0.00	100.00 ± 0.00

Figure 4.1: % Cell viability of compound **46** and doxorubicin against CCRF-CEM

4.2.2 Anti-inflammatory

To determine the anti-inflammatory activity of compounds **42 – 45** isolated from *F. angolensis* root bark, the levels of pro-inflammatory cytokines IL-1β, IL-2, GM-CSF and TNF-α in LPS-stimulated PBMCs was evaluated. The assay is based on the fact that when inflammation occurs, many cytokines are produced and released from PBMCs as part of the immune response. This situation can be replicated *in vitro* by incubating PBMCs with bacterial LPS, a major structural component of the outer wall of gram-negative bacteria, and considered to be a potent initiator of inflammatory responses (O'Bryan *et al.*, 2000). Ibuprofen was used as the reference anti-inflammatory drug.

As shown in **Table 4.7** and **Figure 4.2**, after incubation of PBMCs with LPS the release of the cytokines IL-1β, GM-CSF and TNF-α were increased compared to the medium control. Upon co-incubating the cells with bacterial LPS and ibuprofen, the release of these pro-inflammatory cytokines was decreased to 21.97-77.40% in comparison with LPS control. However, ibuprofen did not show an effect on the release of IL-2. As shown in **Tables 4.8; 4.9** and **Figure 4.3**, all tested compounds showed a decreased release of IL-1β from 35.93-99.71% compared to LPS control. At 100 µM, compounds **43 – 45** resulted to a decreased production of IL-2 (43.46-72.61% of LPS control) while **42** showed an increased release of the cytokine (111.94%) in comparison with the LPS control. All the tested items inhibited production of GM-CSF except for compound **42** which increased GM-SCF secretion to 162.24%. The production of TNF-α was significantly reduced by all test items

compared to the LPS control (16.09-46.17%). The decrease was in the similar range than it was for ibuprofen (77.40% of LPS control). The potential of these compounds to downregulate expression of pro-inflammatory biomarkers may contribute to the anti-cancer chemopreventive effects of *F. angolensis*.

These preliminary anti-inflammatory results allowed molecular structure-activity relationship for the three related norhopanes, **42 – 44**. It is clear that compound **43** (16.09 – 43.46 % of LPS control) exhibited the highest inhibition potencies against all cytokines followed by **44** (21.92 – 73.00 % of LPS control) and lastly **42** (46.17 – 162.24 % of LPS control). Compounds **42** and **43** have similar substitution pattern except presence of an additional *para*-hydroxybenzoate moiety at C-11 in **42**. The presence of this additional moiety seemed to contribute to significant decrease in cytokine inhibition activity of **42** compared to **43**. However, it is not clear whether it is the substitution of the *p*-salicylate and the isopropanolyl moieties by a hydroxyl and isopropyl at C-11 and C-22 positions respectively or the presence of an olefinic group at C-17/C-21 that is contributing to higher anti-inflammatory activity in **44** (21.92 – 73.00 % of LPS control) compared to **42** (46.17–162.24% of the LPS control).

Table 4.7: Results of controls (mean ± SD, n = 3)

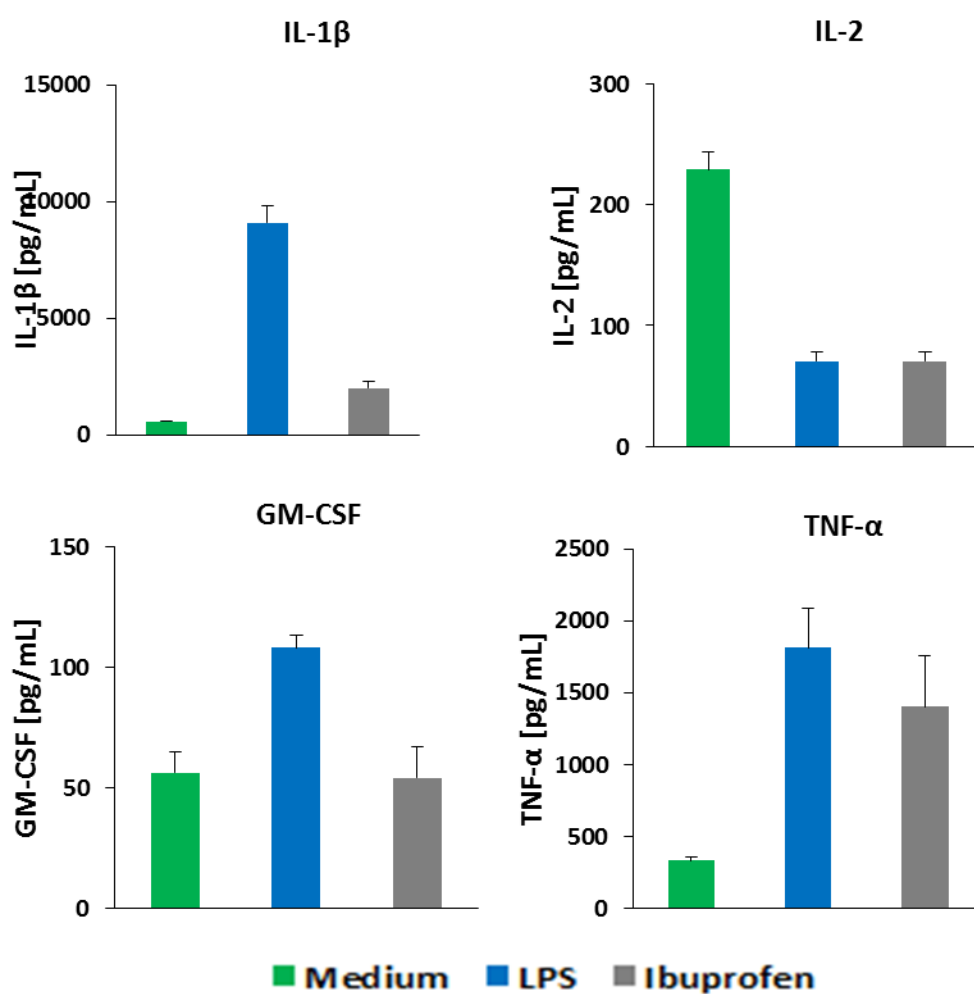| | | Cytokine release [pg/mL] | | | |
		IL-1β	IL-2	GM-CSF	TNF-α
Controls					
Medium	Mean	568.68	229.25	56.33	334.79
	SD	26.22	14.03	8.87	19.97
LPS	Mean	9080.11	70.45	108.06	1815.02
	SD	712.46	7.28	5.24	271.69
Ibuprofen	Mean	1995.27	70.45	54.25	1404.79
	SD	287.26	7.28	12.69	357.71
Ibuprofen	% of LPS control	21.97	100.00	50.21	77.40

Figure 4.2: Results of controls (mean ± SD, n = 3)

Table 4.8: Results of pure compounds (mean ± SD, n = 3)

		Cytokine release [pg/mL]			
		IL-1β	IL-2	GM-CSF	TNF-α
Compound (100 µM)					
42	Mean	9053.65	78.87	175.31	838.07
	SD	2079.7	0.00	63.90	312.52
43	Mean	3262.36	30.62	39.07	291.99
	SD	134.24	0.00	7.76	60.56
44	Mean	6150.29	51.16	78.89	397.78
	SD	1104.94	0.00	0.00	59.16
45	Mean	5994.72	40.89	10.95	394.19
	SD	1601.90	14.53	2.87	74.00
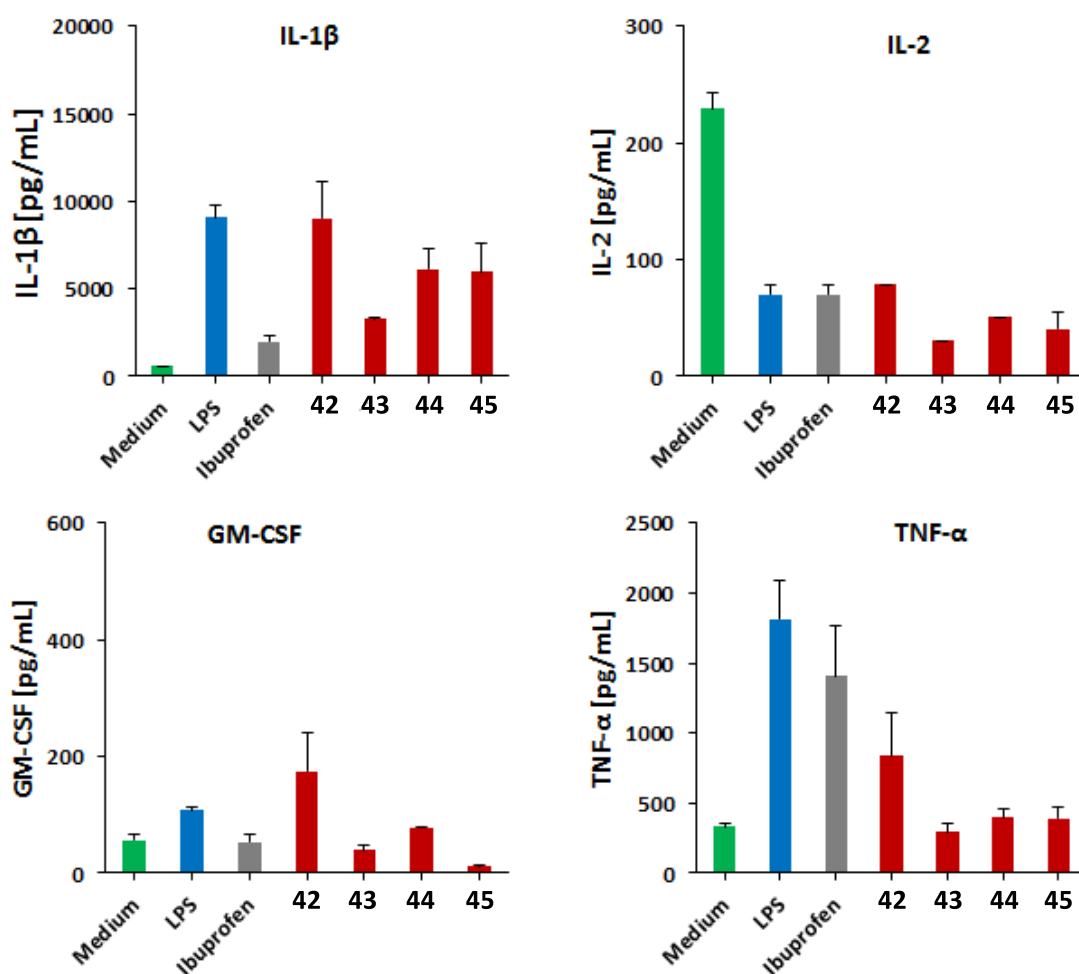
Figure 4.3: Results of cytokine release of PBMCs after incubation with test items (n = 3)

Table 4.9: Percentage of Cytokine Release compared to LPS Control

| Compound (100 µM) | cytokine release [% of LPS control] | | | |
	IL-1β	IL-2	GM-CSF	TNF-α
Ibuprofen	21.97	100.00	50.21	77.40
42	99.71	111.94	162.24	46.17
43	35.93	43.46	36.15	16.09
44	67.73	72.61	73.00	21.92
45	66.02	58.04	10.14	21.72

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this study, phytochemical investigation of dichloromethane: methanol (1:1) roots bark and leaves extracts of *Fagaropsis angolensis* (Engl.) H.M.Gardner was conducted. A total of five compounds were isolated and characterized, of which two were found to be new. The roots bark afforded two new norhopane triterpenoids, 3β,6β,22-trihydroxy-7β,11α-di[(4-hydroxybenzoyl)oxy]-21α*H*-24-norhopa-4(23)-ene (**42**) and 3β,6β,22-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-21α*H*-24-norhopa- 4(23)-ene (**43**) along with a previously reported norhopane, 3β,6β,11α-trihydroxy-7β-[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene (**44**) and a norneohopane (21α*H*)-24-*nor*-neohopa-4(23),22(29)-diene-3β,6β,7β-triol 7-caffeate (**45**). The leaves extract yielded one known flavone, tachrosin (**46**).

Using resazurin reduction assay with doxorubicin as the reference drug, compound **46** displayed minimal activity against drug sensitive CCRF-CEM. Compounds **42 – 45** were evaluated for anti-inflammatory activity. All individual compounds exhibited anti-inflammatory effect against the tested cytokines in comparison with the LPS control. Compounds **43** and **45** caused the significant decrease of the production of IL-2, GM-CSF and TNF-α compared to the reference drug ibuprofen.

5.2 Recommendations for further studies

1. The roots bark and leaves of *Fagaropsis* should be investigated further using modern separation techniques HPLC and prep-HPLC to comprehensively isolate most of the minor phyto-constituents.

2. Re-isolation and structure modification of the reported compounds to obtain synthetic analogues with more potent bioactivity.

3. The isolated compounds should be subjected to antimicrobial assays to determine their potential as antibiotics.

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Appendix 1A: ^{1}H NMR spectrum (600 MHz, CD$_3$OD) of compound 42
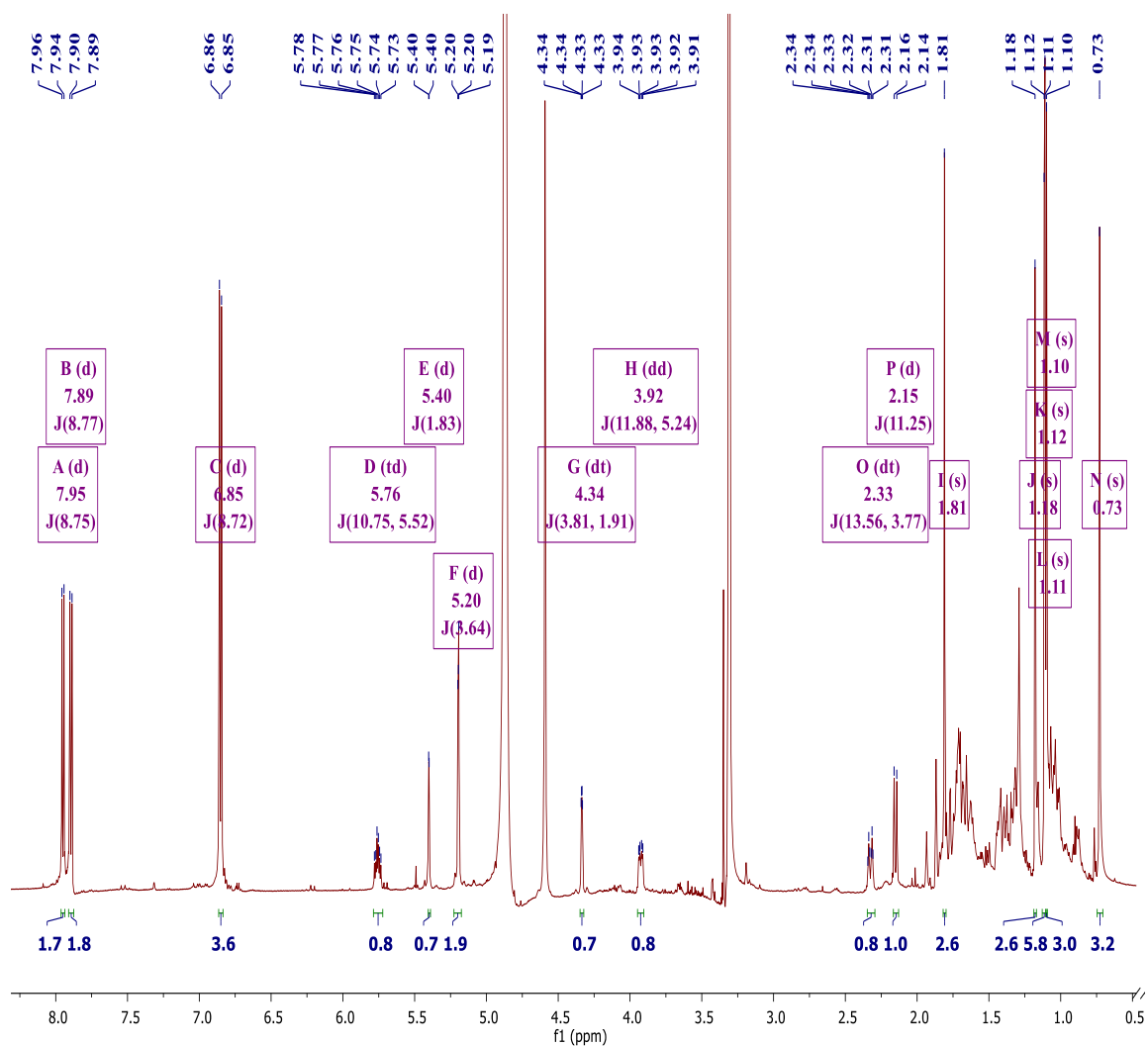
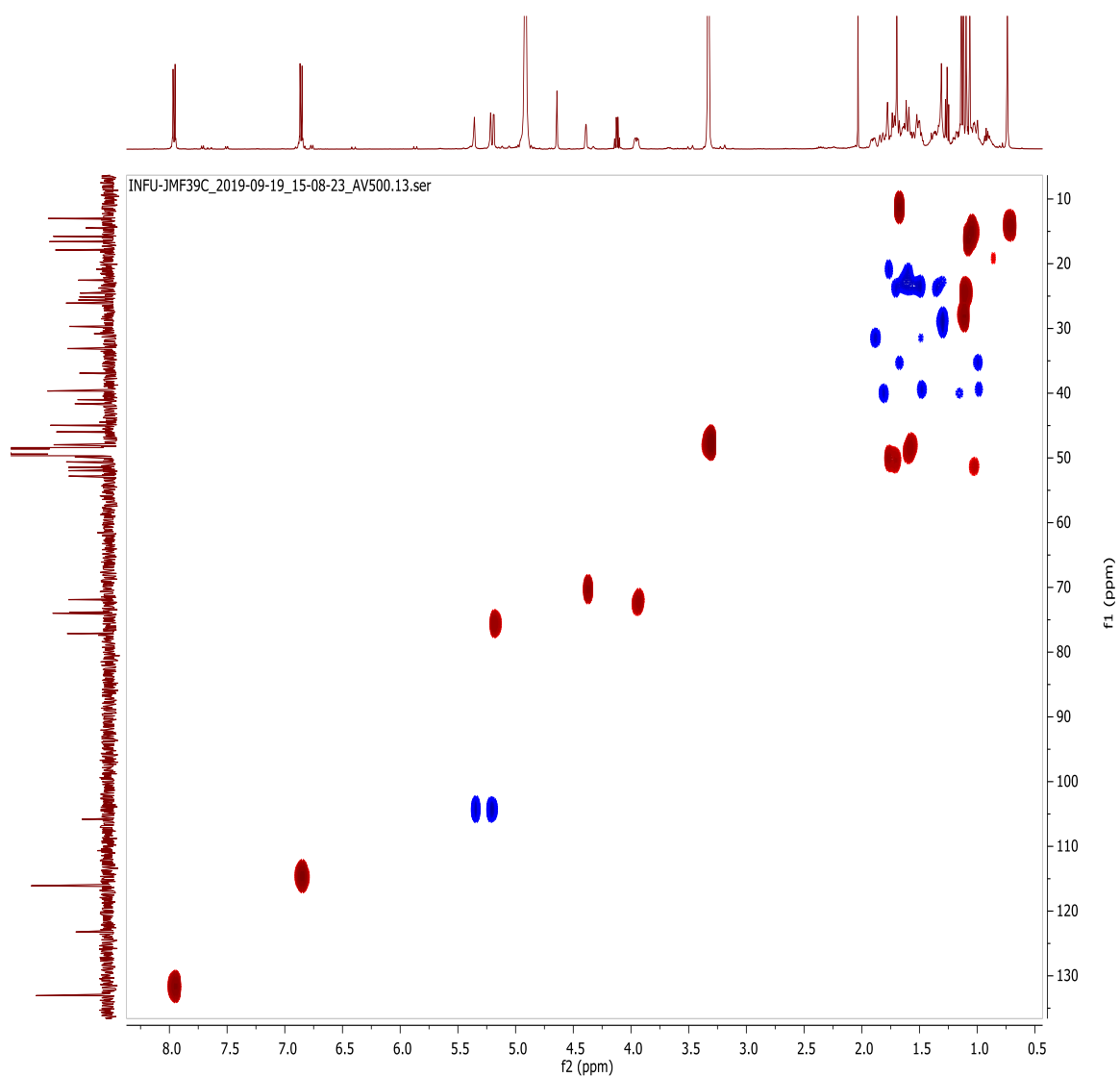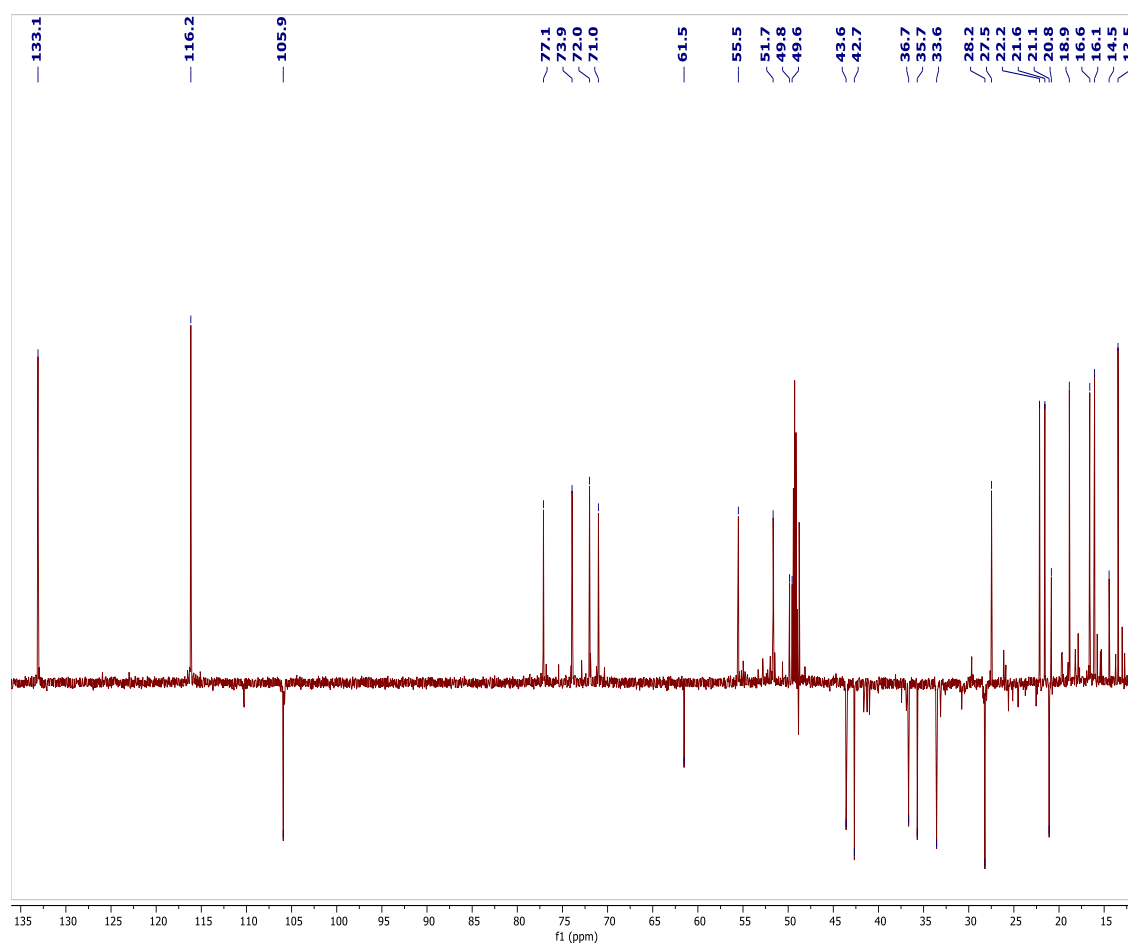**Appendix 1B: ¹³C NMR spectrum (150 MHz, CD₃OD) of compound 42**

46
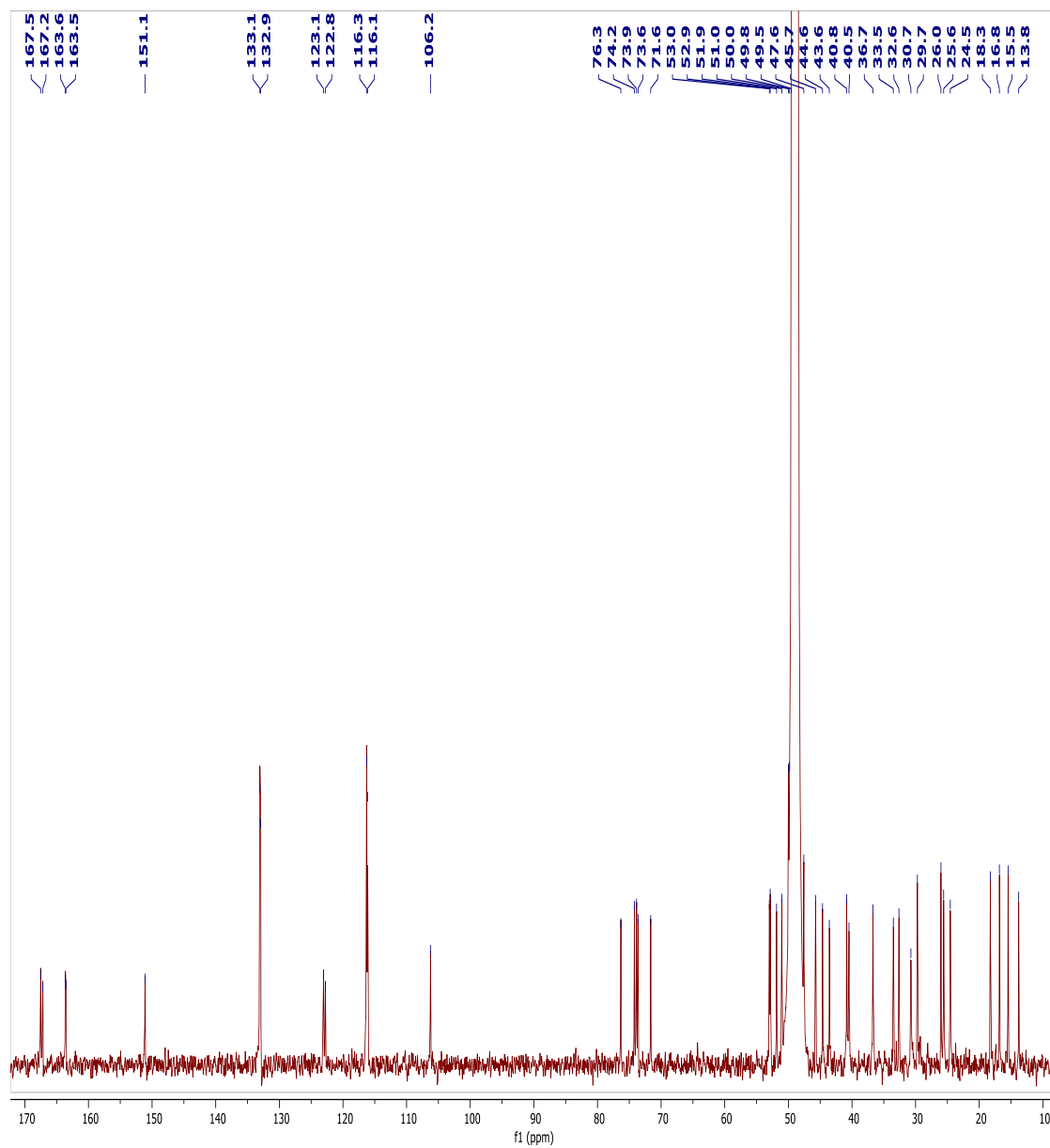
Appendix 1C: DEPT spectrum (CD₃OD) of compound 42

INFU-JMF-25C_2019-06-06_14-13-21_AV600.12.fid — C13 DEPT135 — z_C13DEPT135 MeOD /NMR-Daten INFU 44

INFU-JMF-25C_2019-06-07_13-39-36_AV600.11.ser
gCOSY
z_gCOSY MeOD /NMR-Daten INFU 38
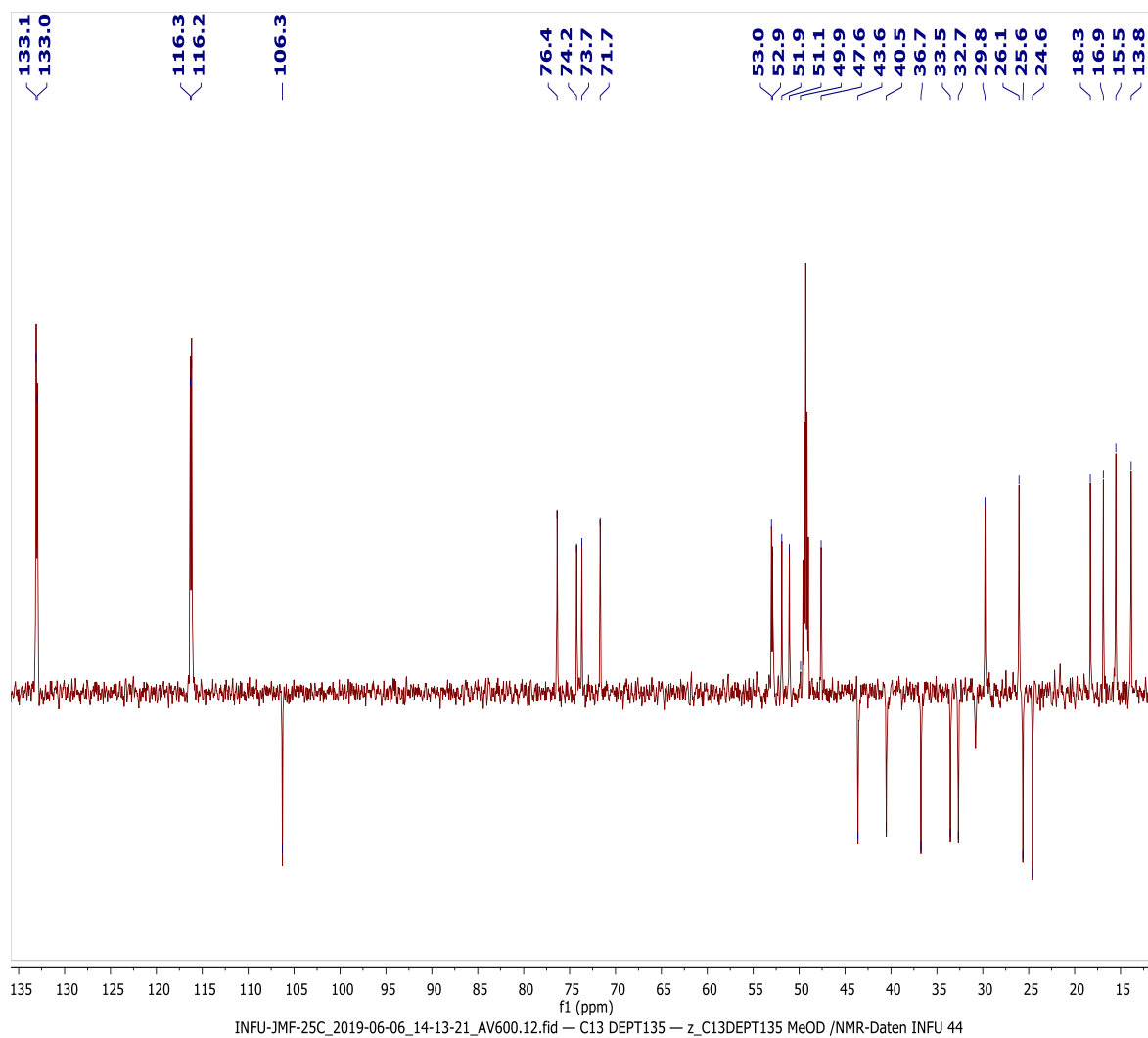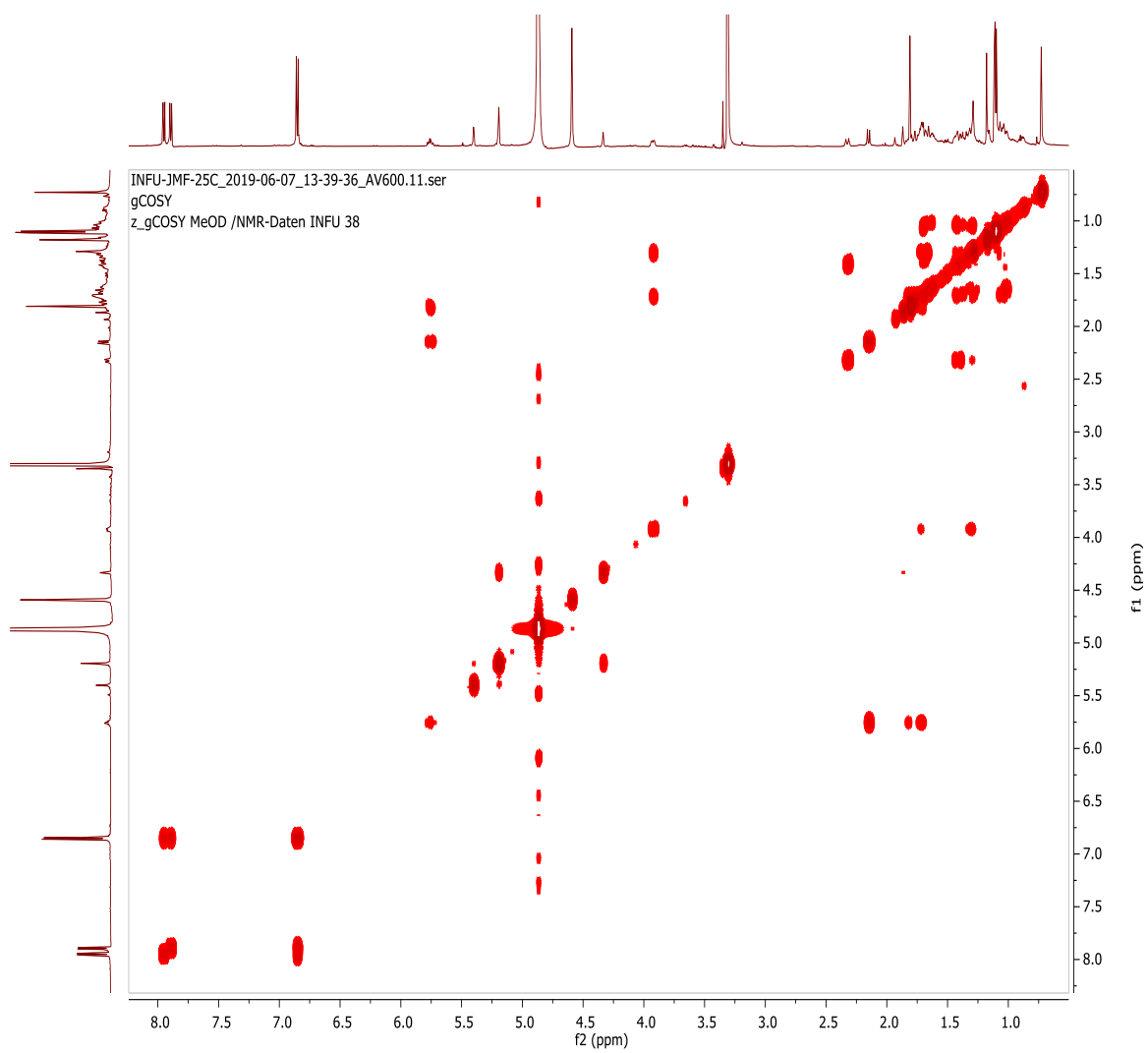
Appendix 1E: HSQC spectrum (CD₃OD) of compound 42

INFU-JMF-25C_2019-06-07_13-39-36_AV600.12.ser
gHSQC
z_gHSQC_adiab MeOD /NMR-Daten INFU 38
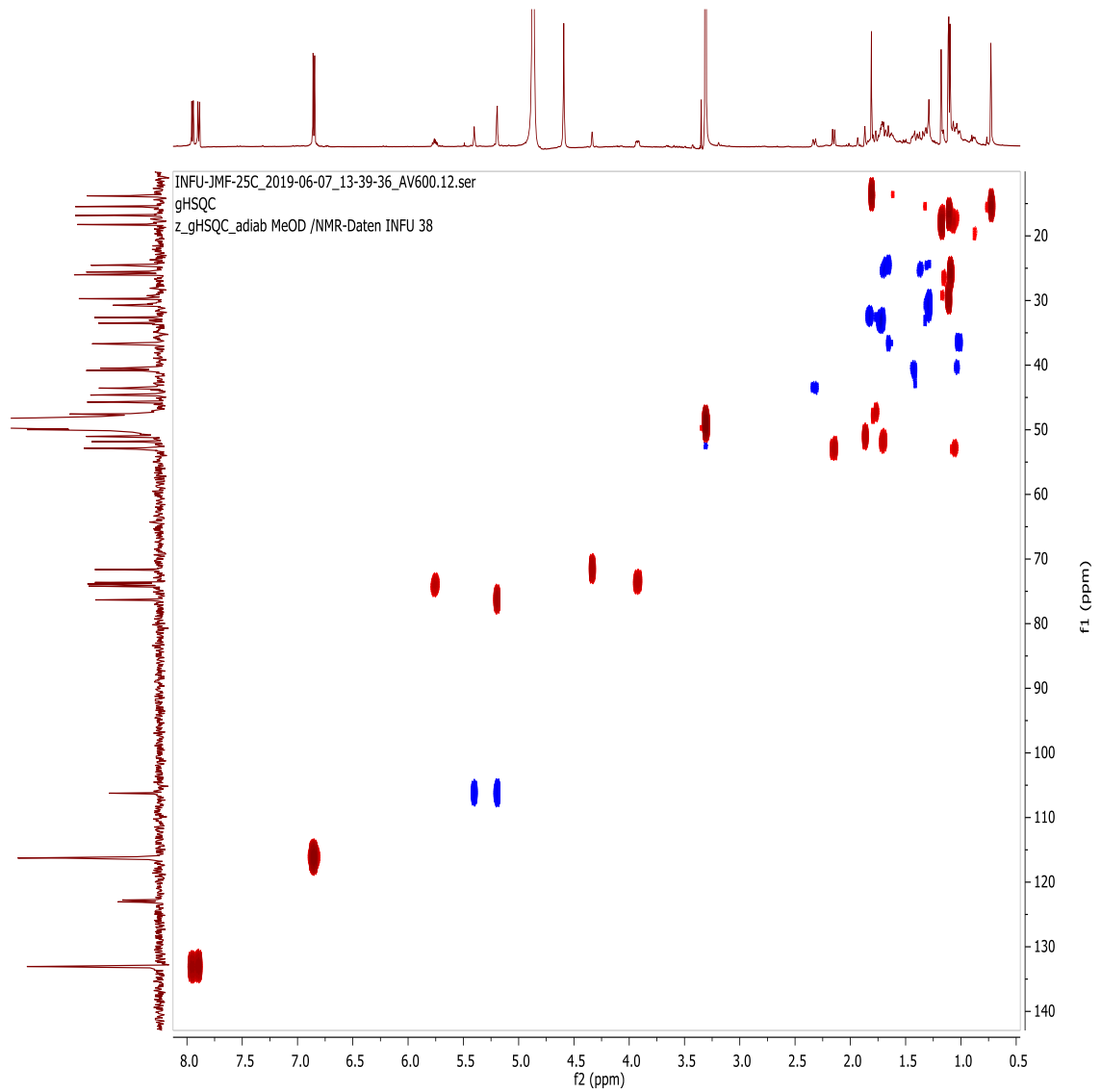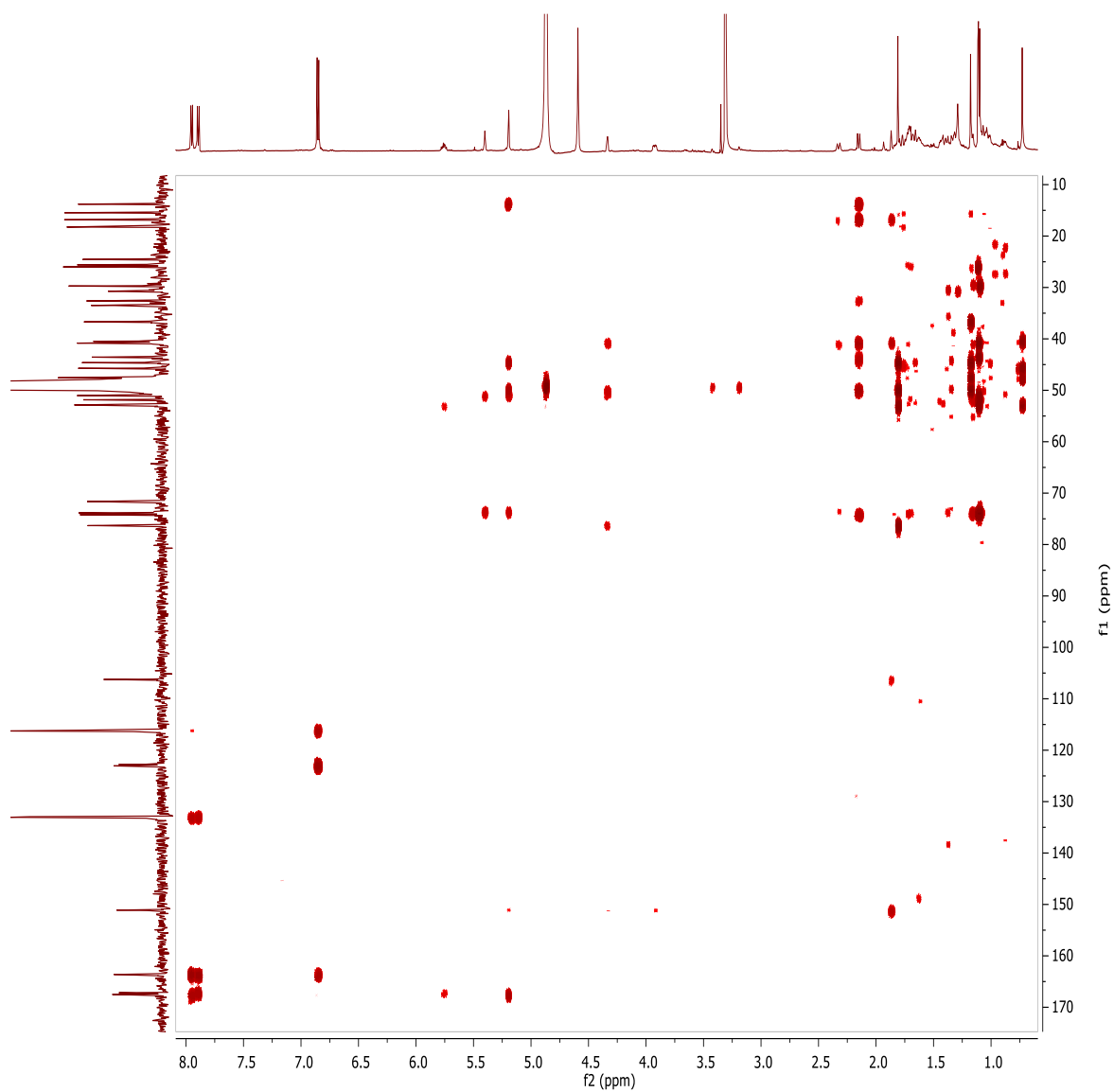
Appendix 1G: NOESY spectrum (CD₃OD) of compound 42

INFU-JMF25c_2019-11-15_14-35-53_AV600/11 — NOESYGPPH — — z_gNOESY MeOD /NMR-Daten INFU 20
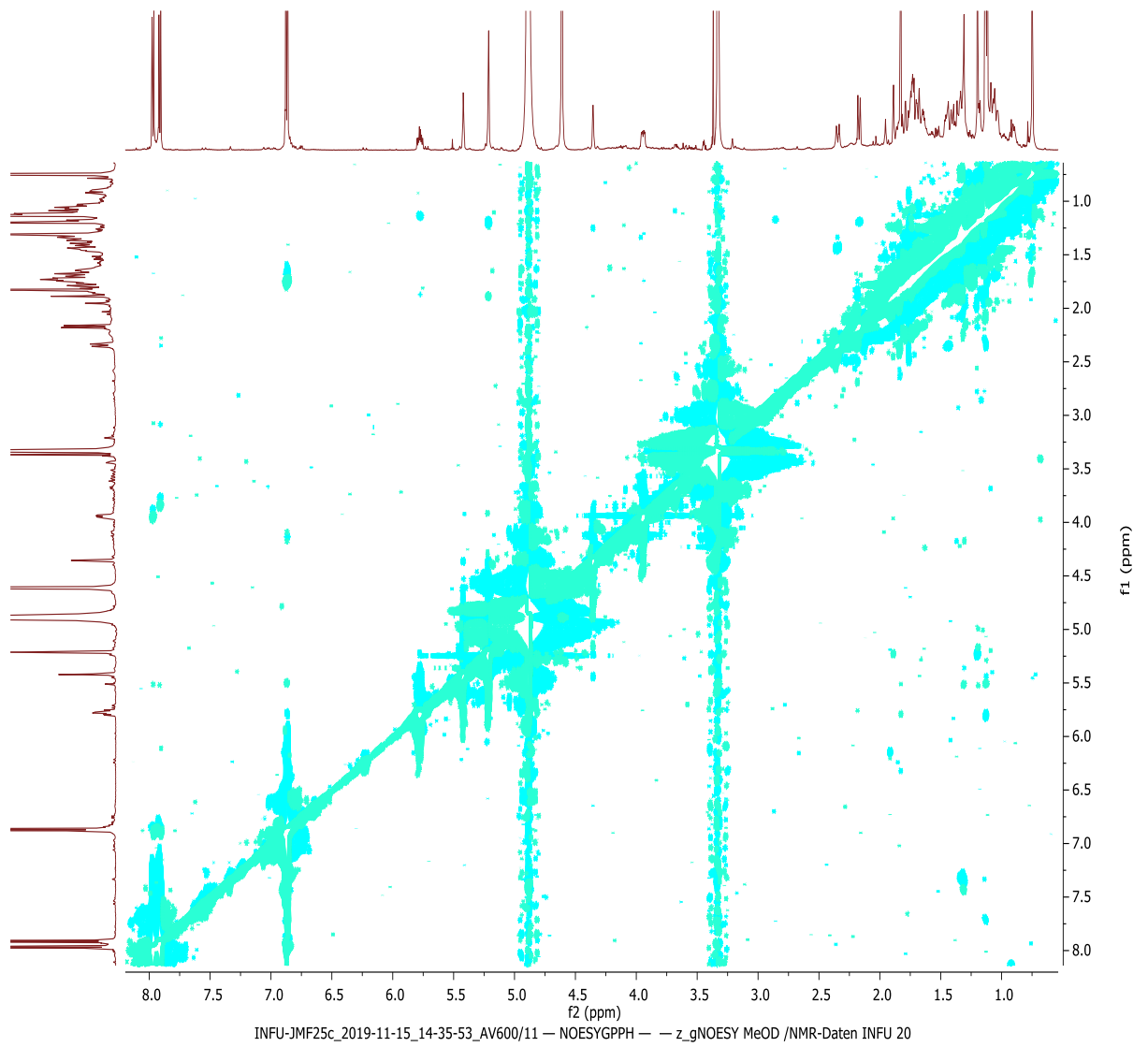
Appendix 1H: Enlarged NOESY spectrum (CD₃OD) of compound 42

INFU-JMF25c_2019-11-15_14-35-53_AV600/11 — NOESYGPPH — — z_gNOESY MeOD /NMR-Daten INFU 20
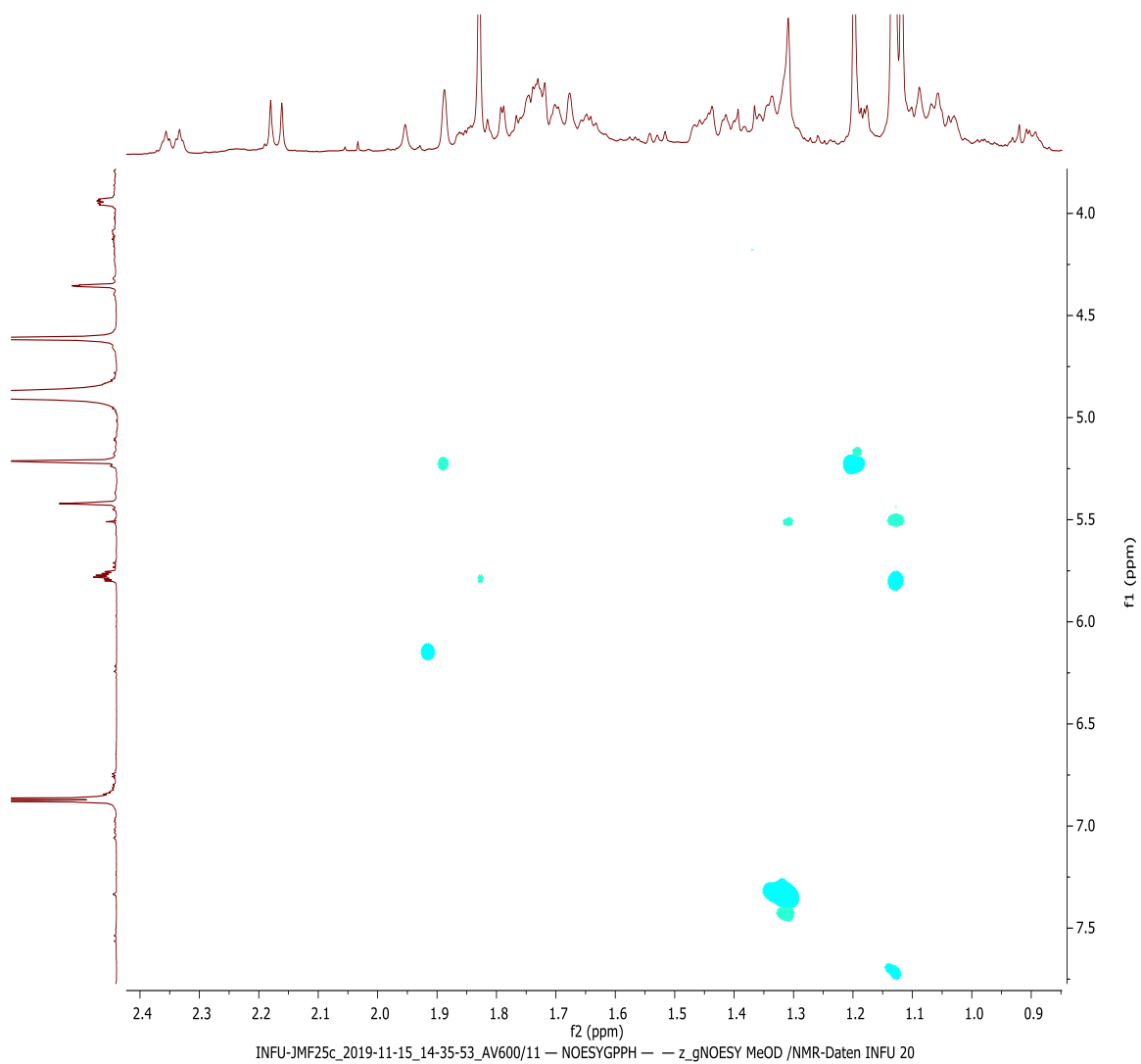
Appendix 1I: LC-UV spectrum of compound 42

259.0000
$C_{14} H_4 O_4 Na$
-0.1798 mmu

330.0000
$C_{15} H_6 O_9$
-0.6332 mmu

wavelength (nm)
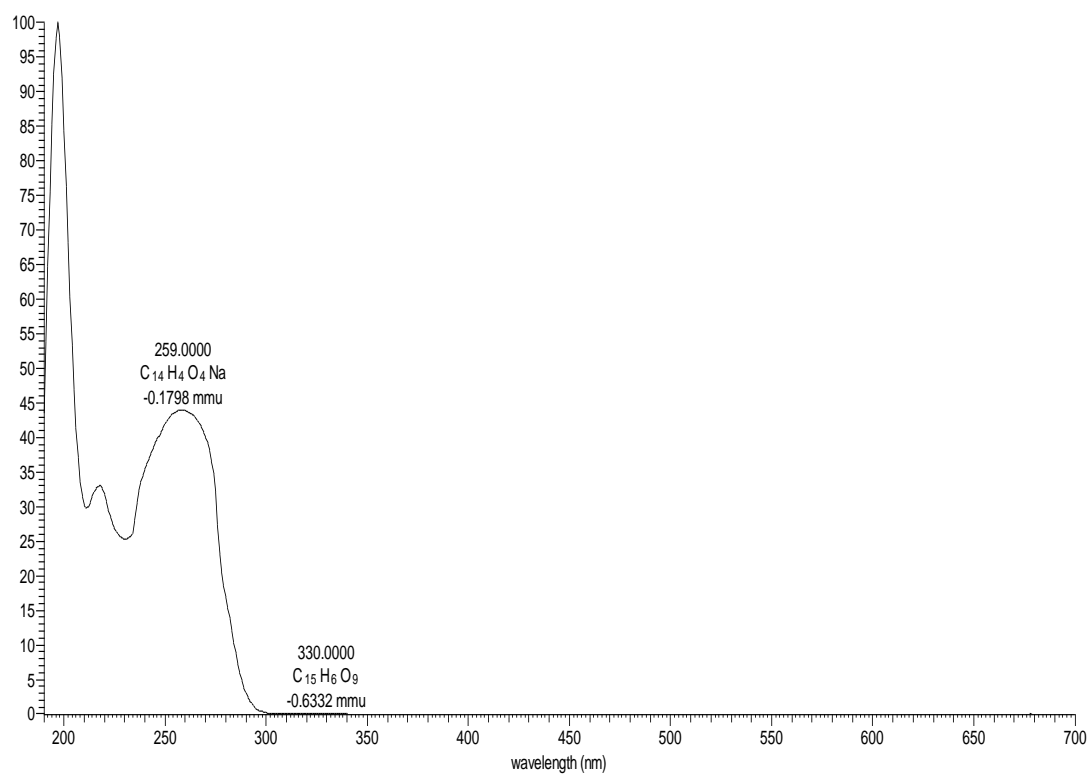
Appendix 1J: IR spectrum of compound 42
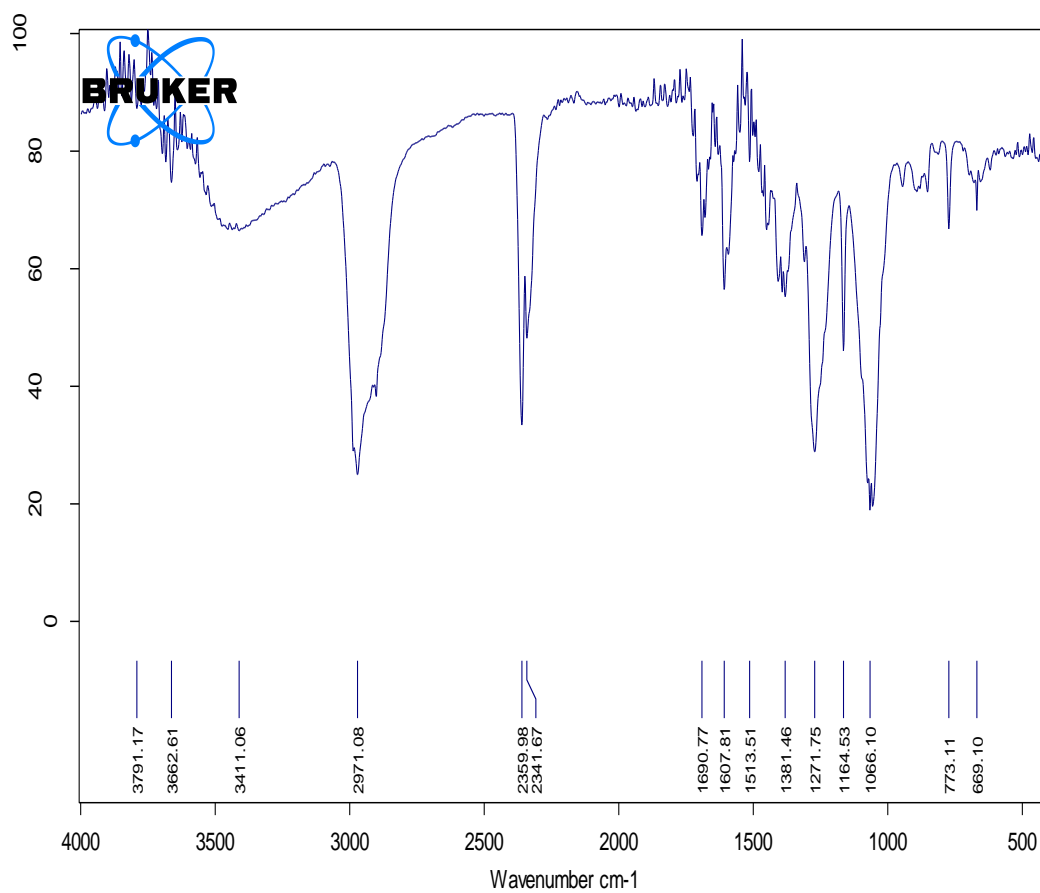
Appendix 1K: Mass spectrum of compound 42
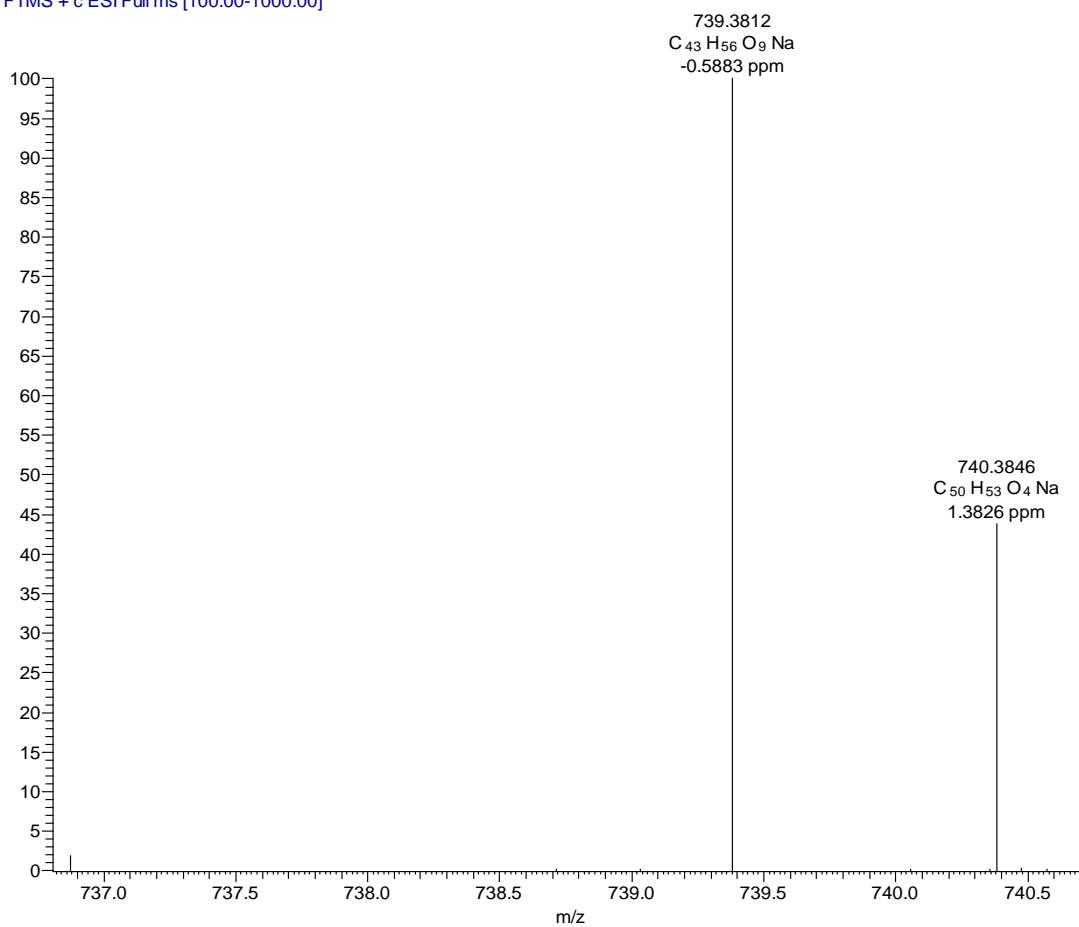
Appendix 2A: ^{1}H NMR spectrum (600 MHz, CD$_3$OD) of compound 43
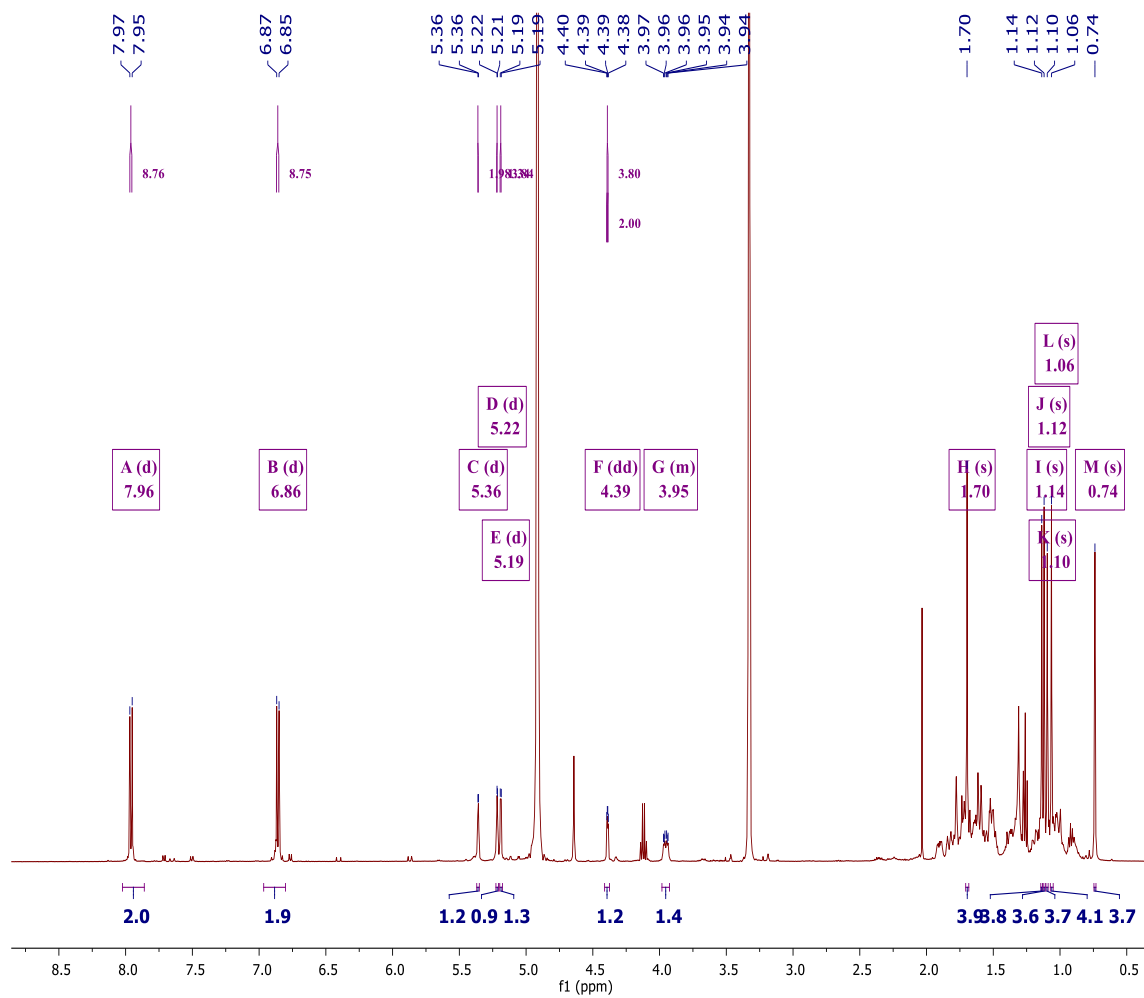
Appendix 2B: ^{13}C NMR spectrum (150 MHz, CD₃OD) of compound 43

INFU-JMF39C_2019-09-19_15-08-23_AV500.11.fid —
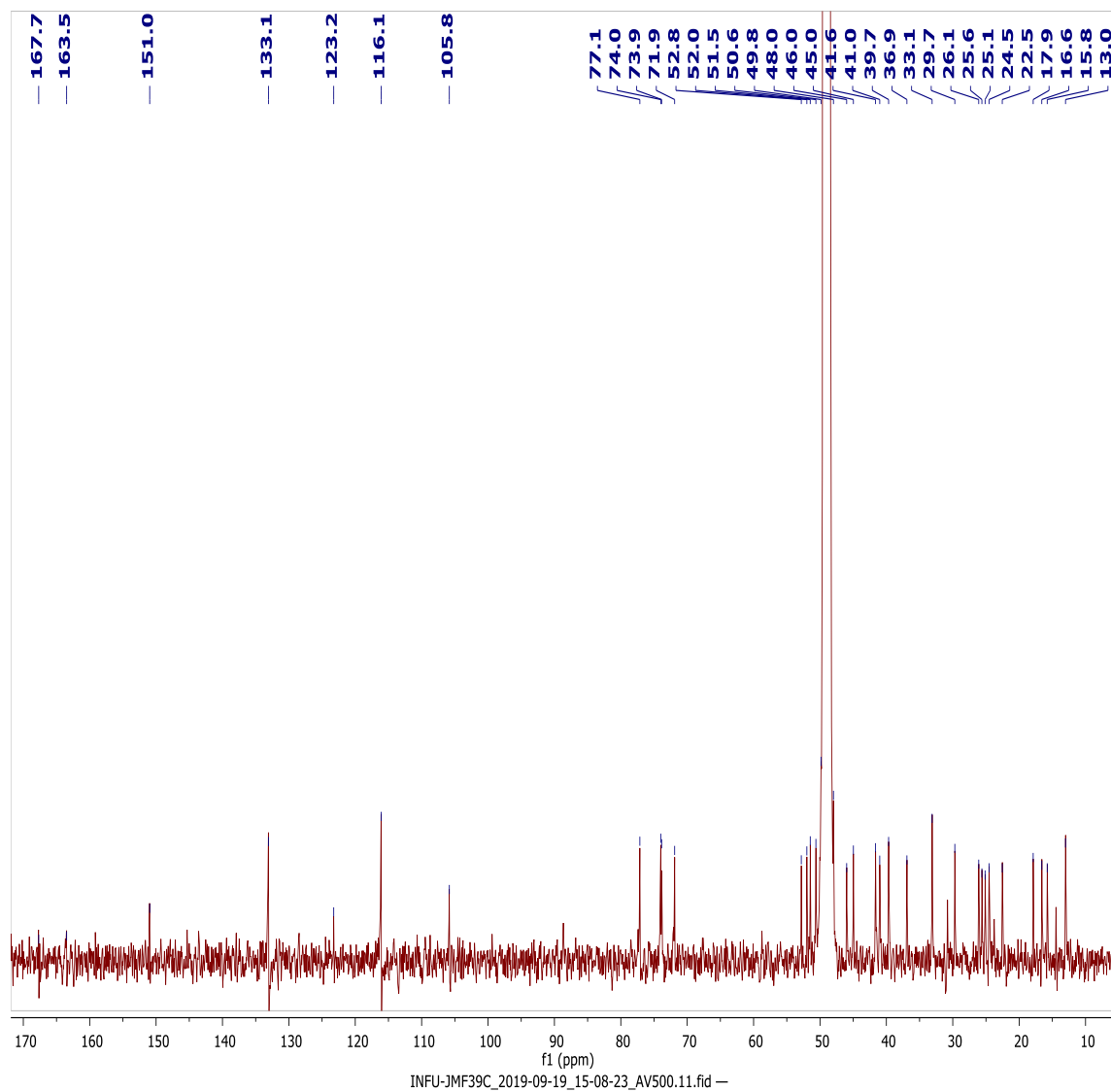
Appendix 2C: DEPT spectrum (CD₃OD) of compound 43

INFU-JMF39c_2019-10-18_11-25-48_AV600.12.fid — C13 DEPT135 — z_C13DEPT135 MeOD /NMR-Daten INFU 17
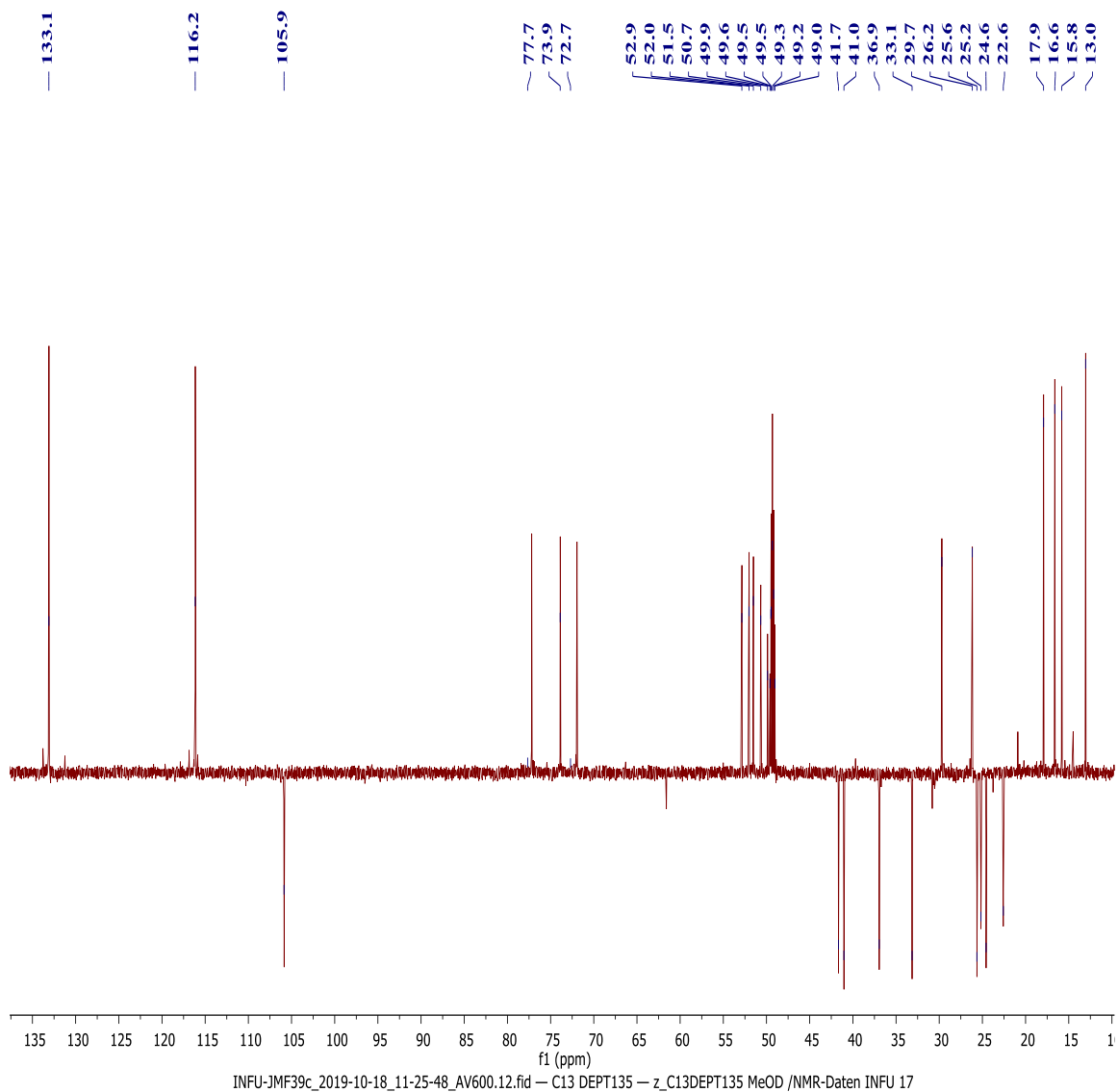
Appendix 2D: ^1H-^1H COSY spectrum (CD₃OD) of compound 43

INFU-JMF39C_2019-09-19_15-08-23_AV500.12.ser
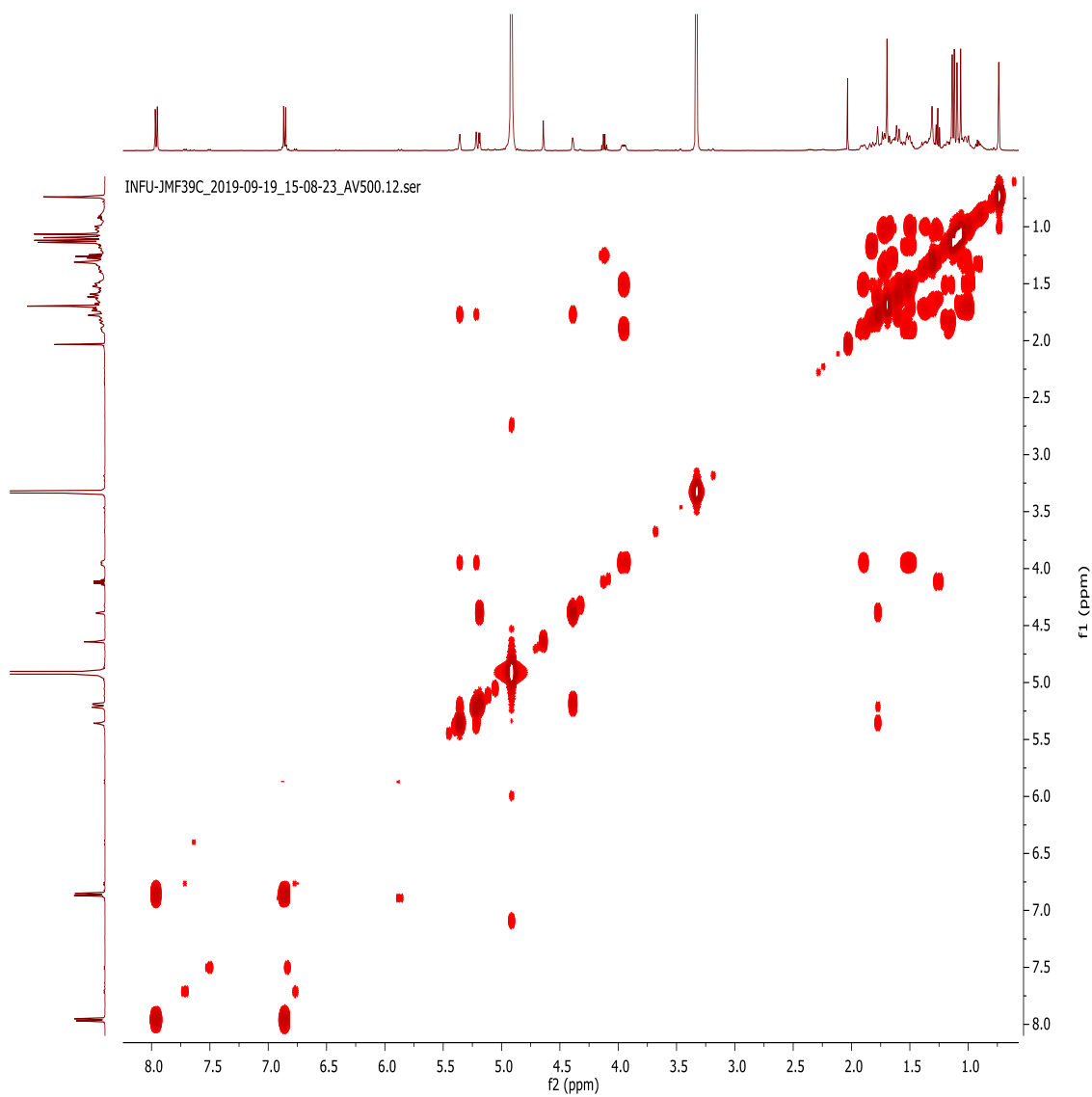
Appendix 2E: HSQC spectrum (CD₃OD) of compound 43

INFU-JMF39C_2019-09-19_15-08-23_AV500.13.ser

INFU-JMF39C_2019-09-19_15-08-23_AV500.14.ser
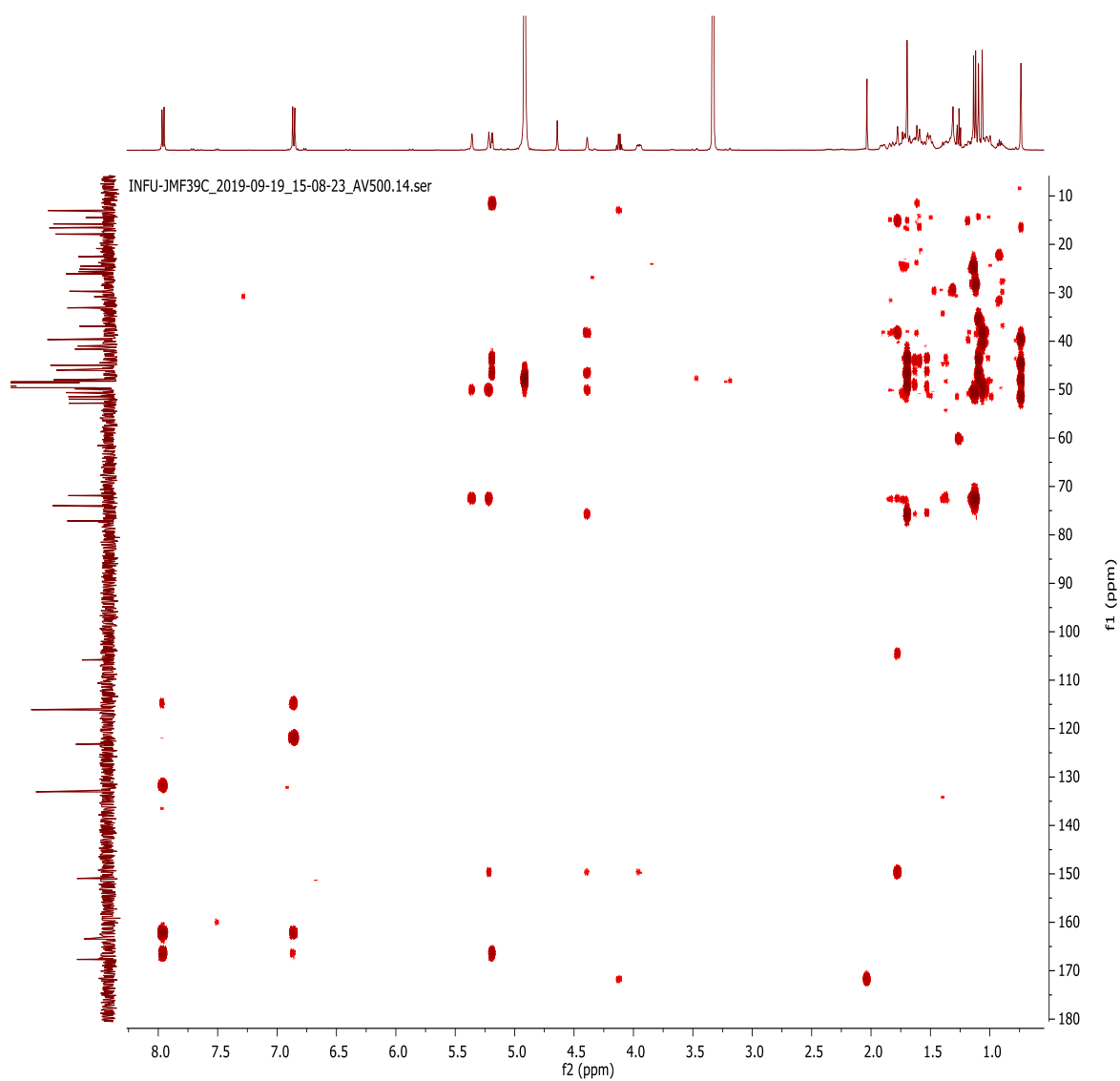
Appendix 2G: NOESY spectrum (CD₃OD) of compound 43
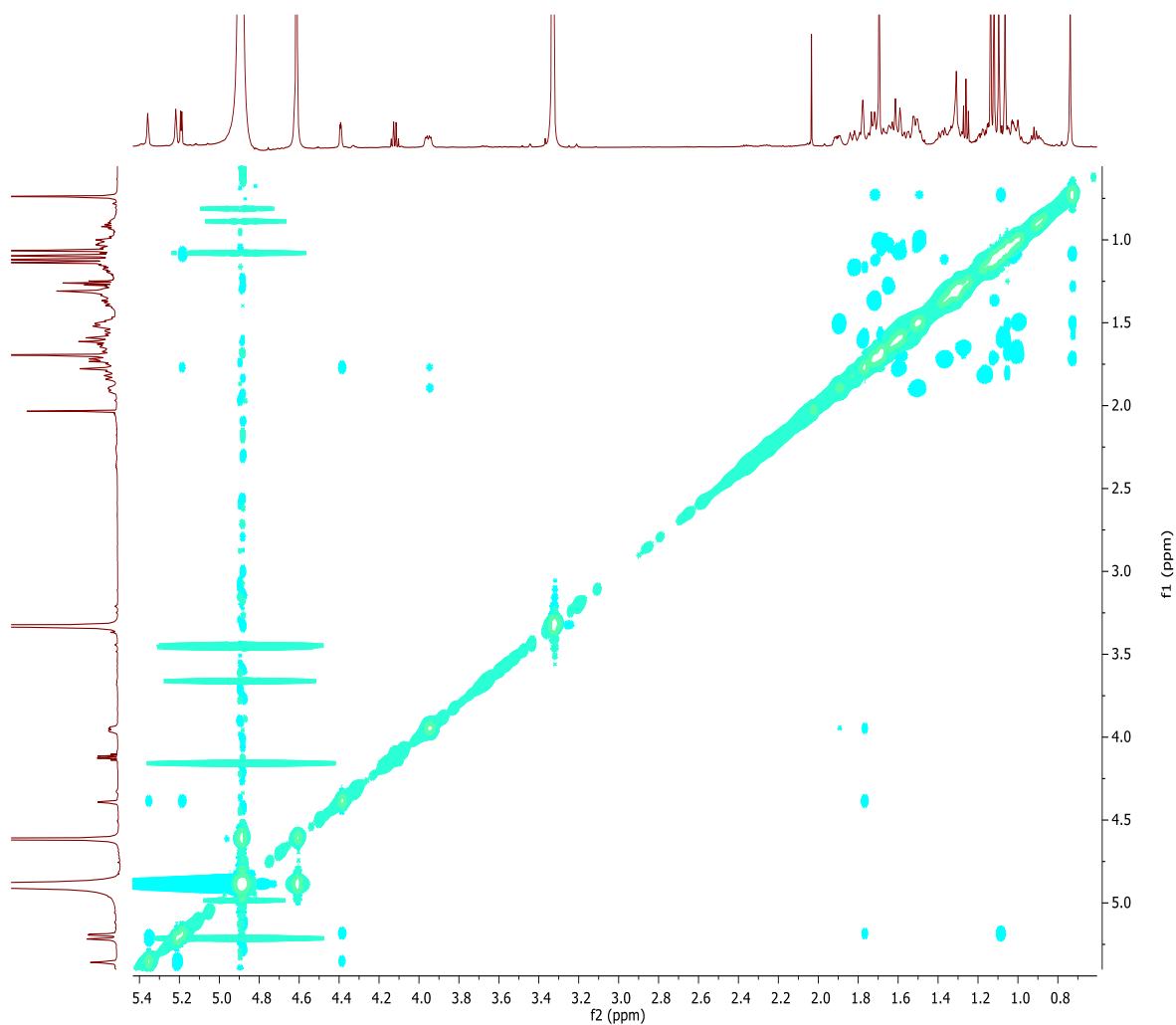
Appendix 2H: LC-UV spectrum of compound 43

230.0000
$C_{15} H_2 O_3$
0.1545 mmu

276.0000
C_{23}
0.5484 mmu

wavelength (nm)

63

Appendix 2I: IR spectrum of compound 43
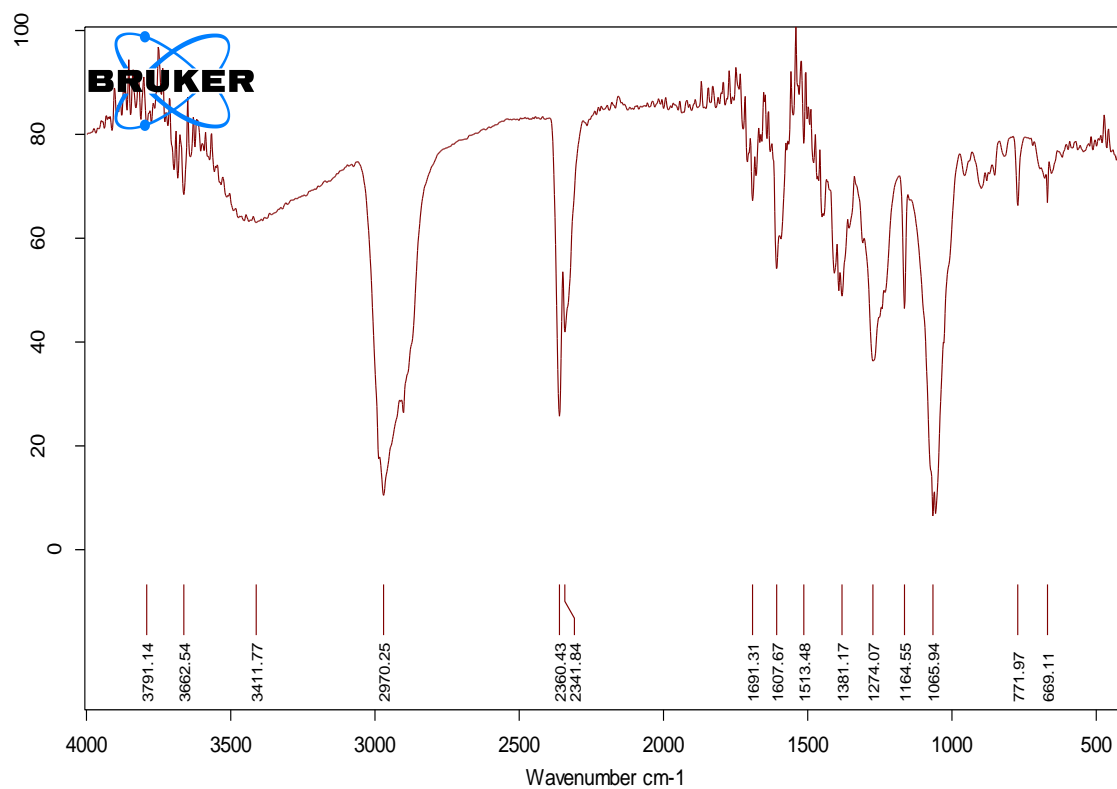
Appendix 2J: Mass spectrum of compound 43

JMF39C #917-933 RT: 26.51-26.79 AV: 9 NL: 5.17E5
T: FTMS + c ESI Full ms [100.00-1000.00]

603.3658
$C_{36}H_{52}O_6Na$
0.3147 ppm

604.3691
$C_{45}H_{48}O$
-1.3812 ppm

598.4101
$C_{33}H_{58}O_9$
4.2937 ppm

600.3571
$C_{40}H_{49}O_3Na$
-0.4665 ppm

605.3723
$C_{27}H_{57}O_{14}$
-3.2384 ppm

m/z

65
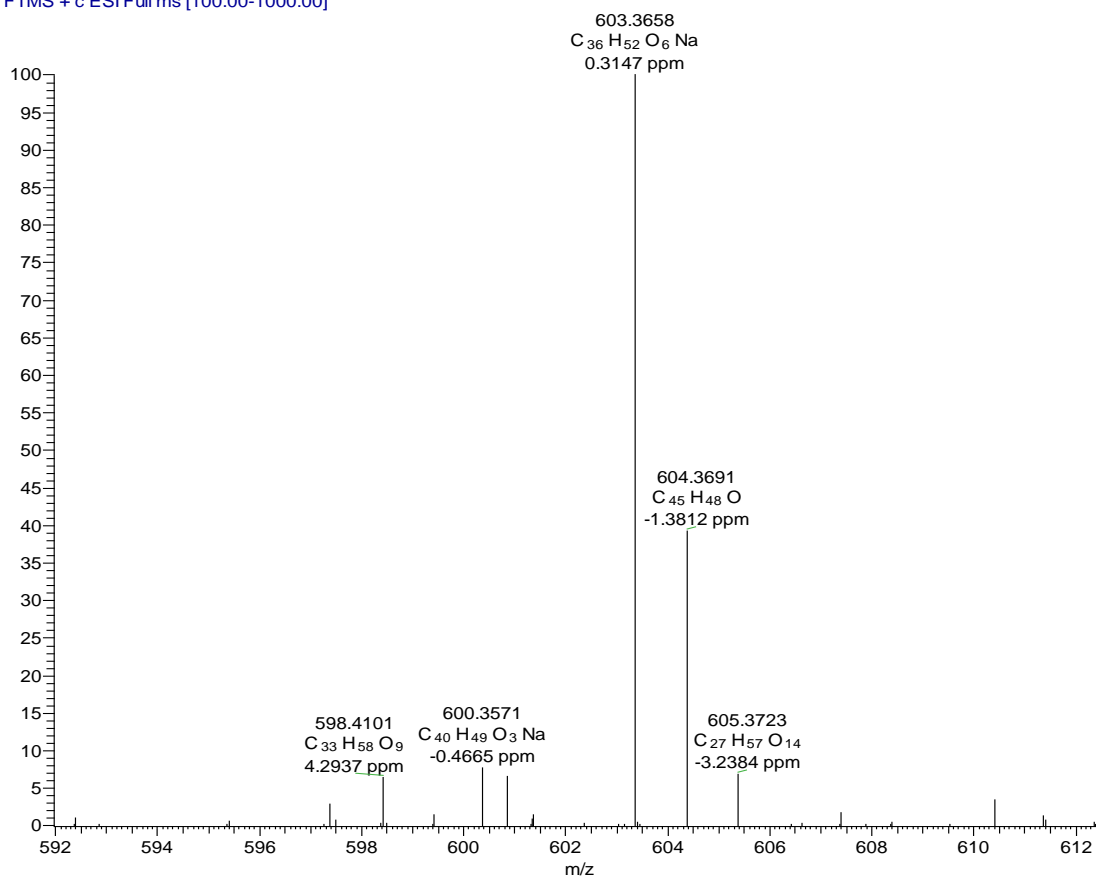
Appendix 3A: ¹H NMR spectrum (600 MHz, CD₃OD) of compound 44
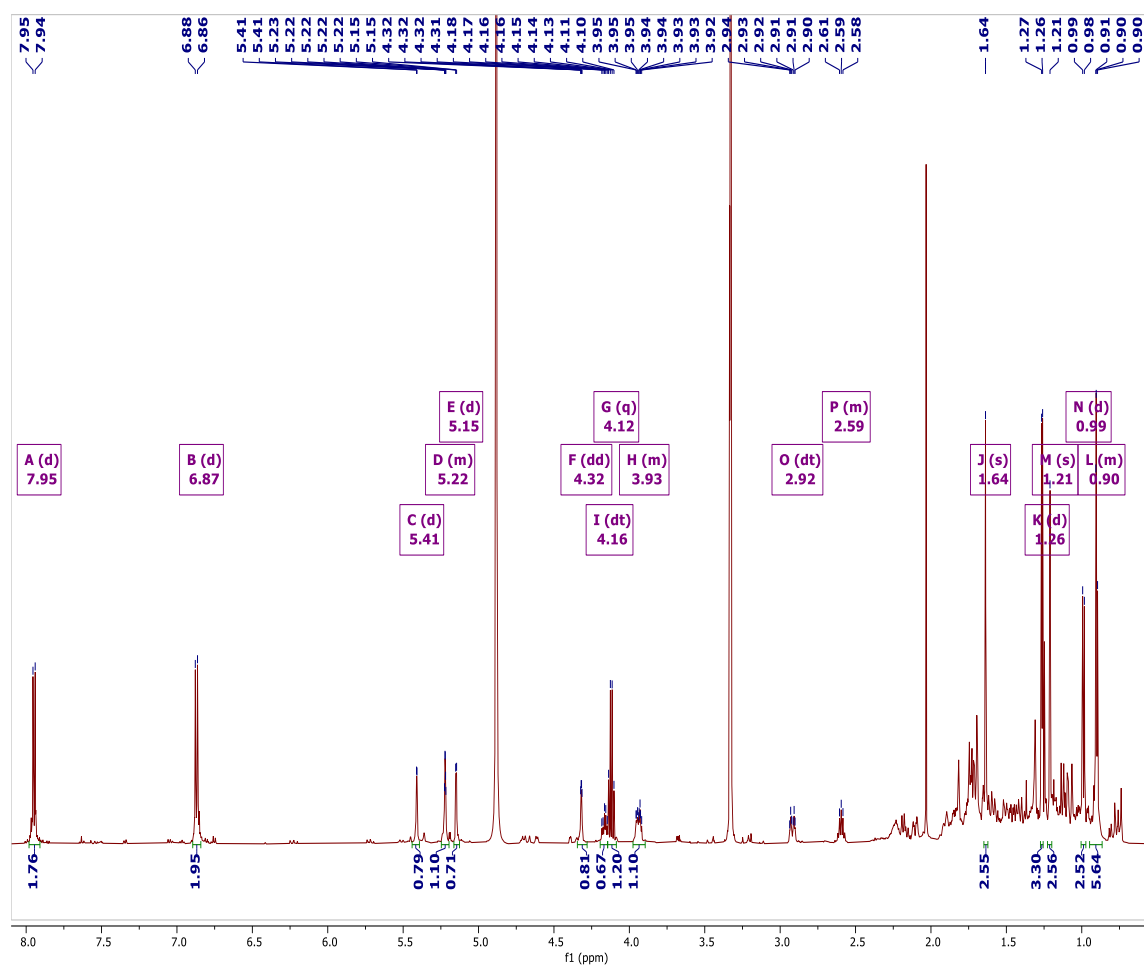
Appendix 3B: ^{13}C NMR spectrum (150 MHz, CD$_3$OD) of compound 44
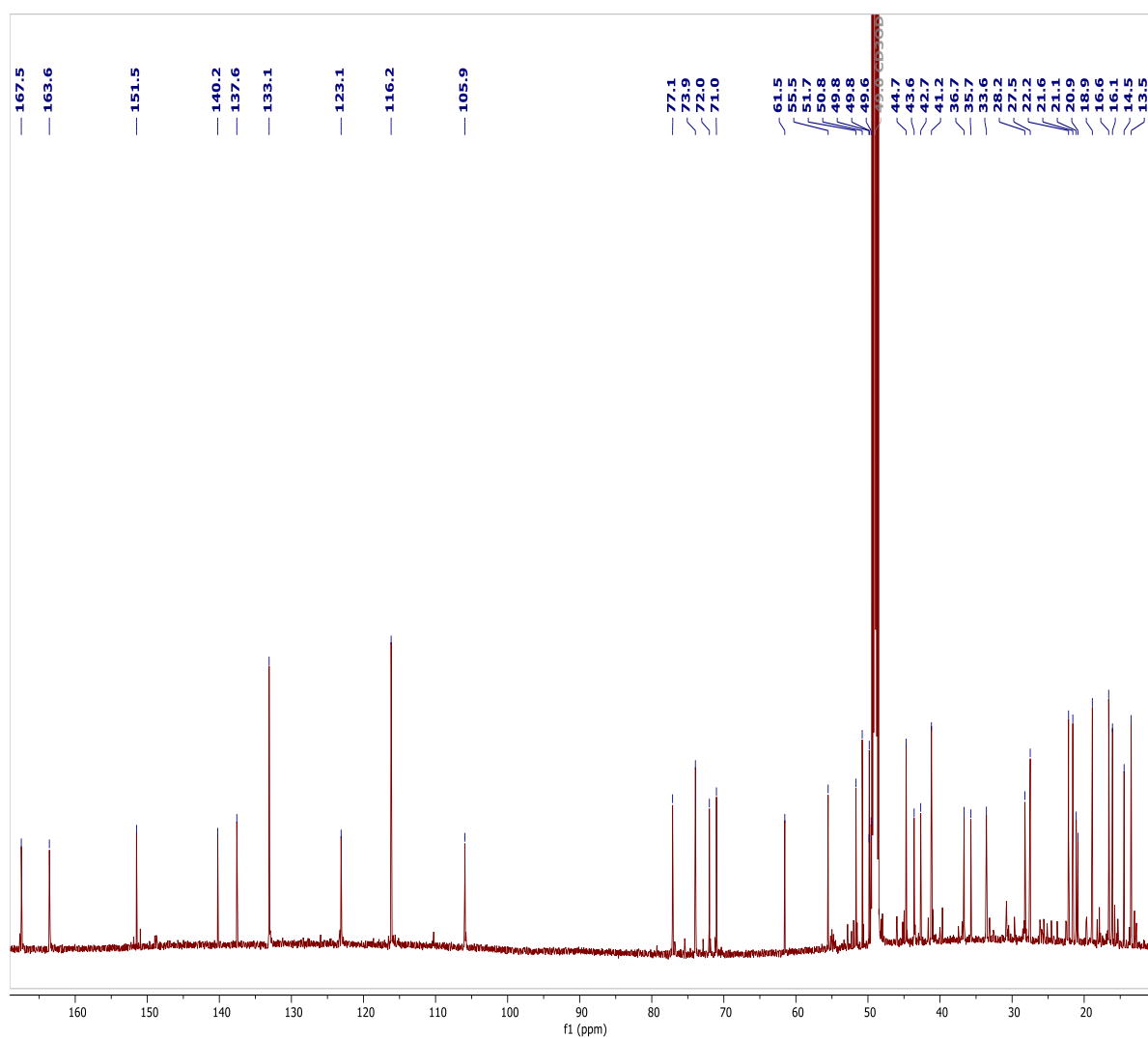
Appendix 3C: DEPT spectrum (CD₃OD) of compound 44

Appendix 3D: ^{1}H-^{1}H COSY spectrum (CD$_3$OD) of compound 44

INFU-JMF-39A_2019-09-20_15-16-42_AV600.13.ser
gCOSY
z_gCOSY MeOD /NMR-Daten INFU 17
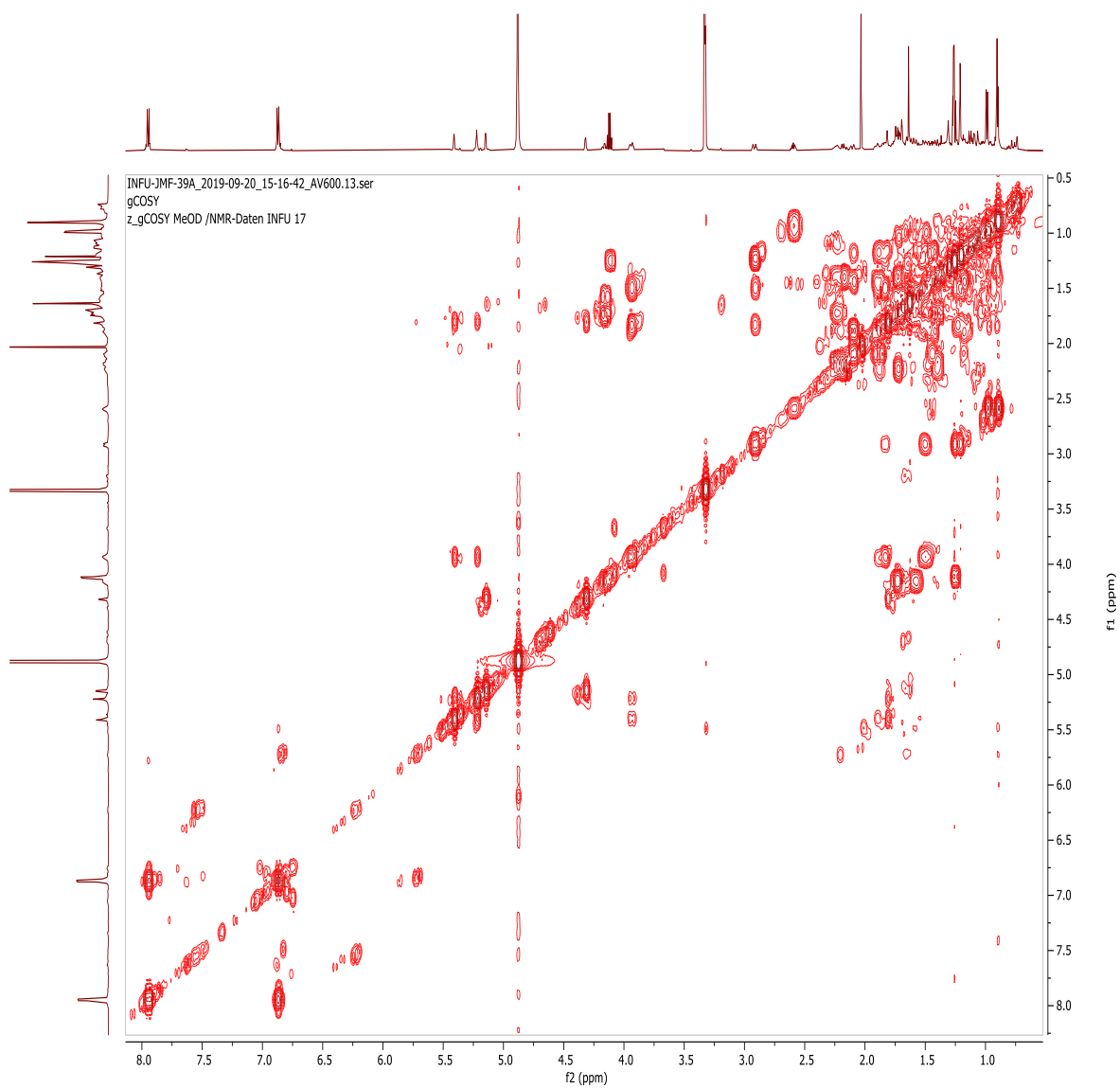
Appendix 3E: HSQC spectrum (CD₃OD) of compound 44

INFU-JMF-39A_2019-09-20_15-16-42_AV600.15.ser
gHSQC
z_gHSQC_adiab MeOD /NMR-Daten INFU 17

INFU-JMF-39A_2019-09-20_15-16-42_AV600.16.ser
HMBCGPND

z_gHMBC MeOD /NMR-Daten INFU 17
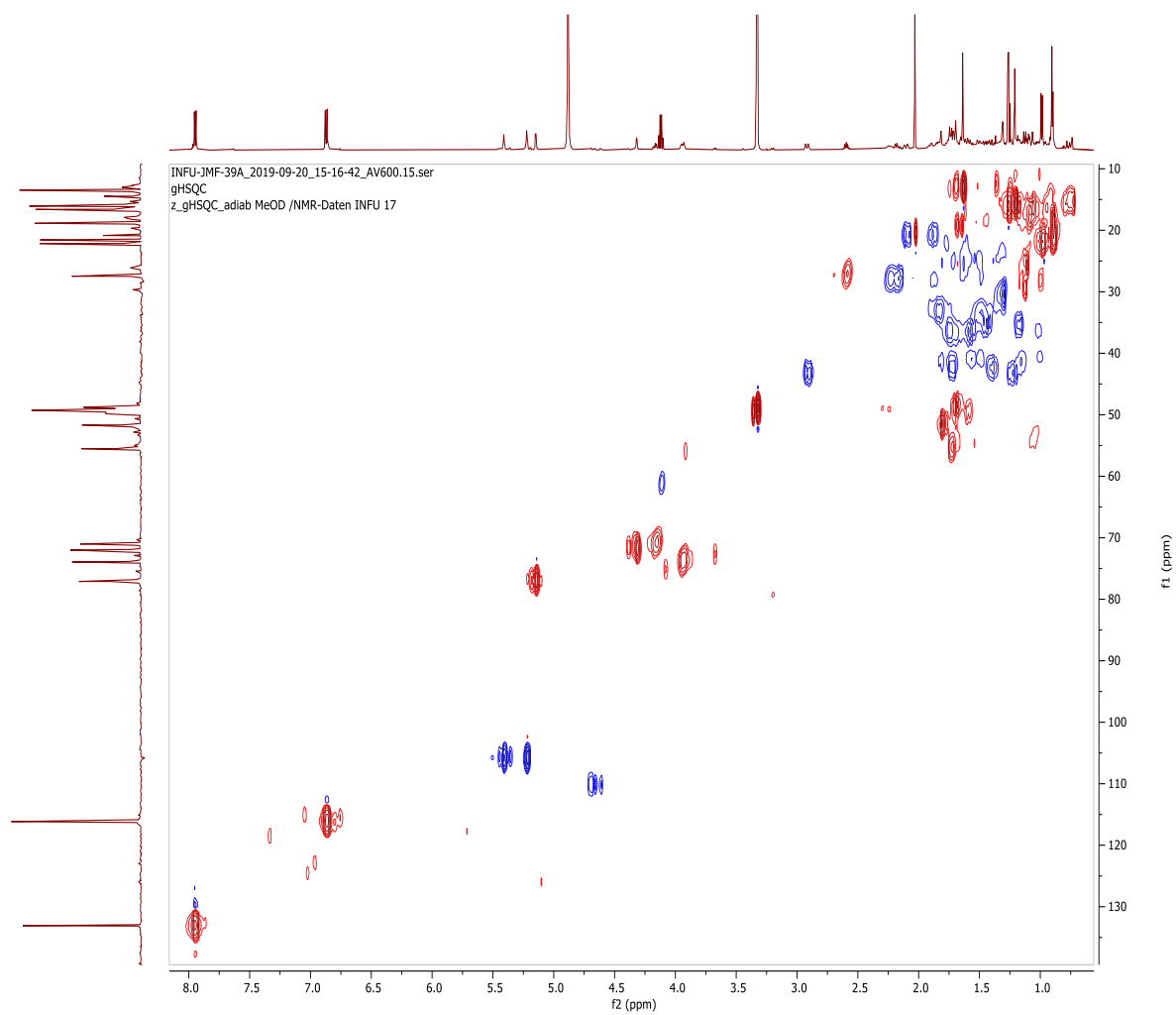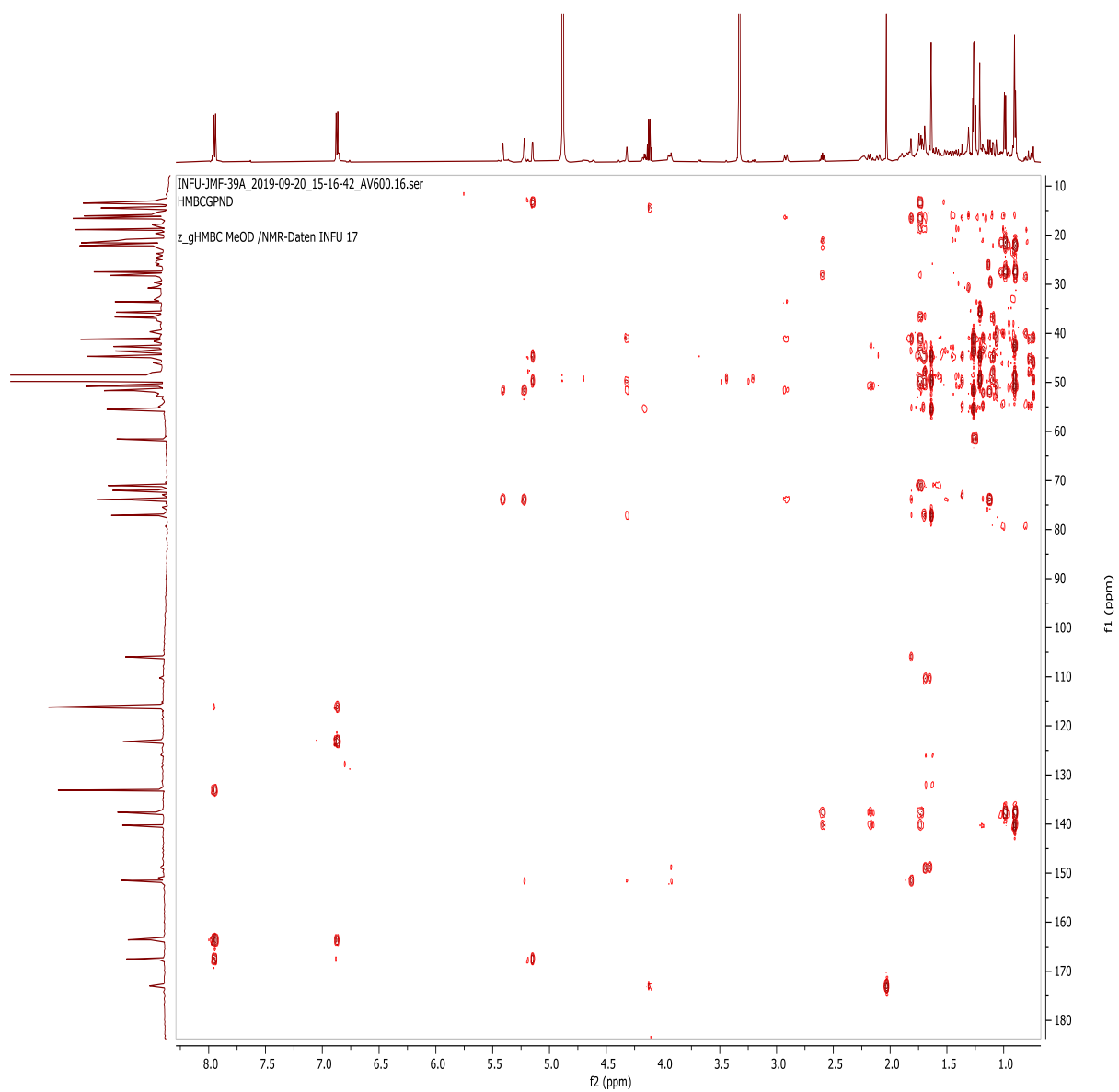
Appendix 3G: NOESY spectrum (CD₃OD) of compound 44

INFU-JMF-39A_2019-09-20_15-16-42_AV600.14.ser
NOESYGPPH

z_gNOESY MeOD /NMR-Daten INFU 17
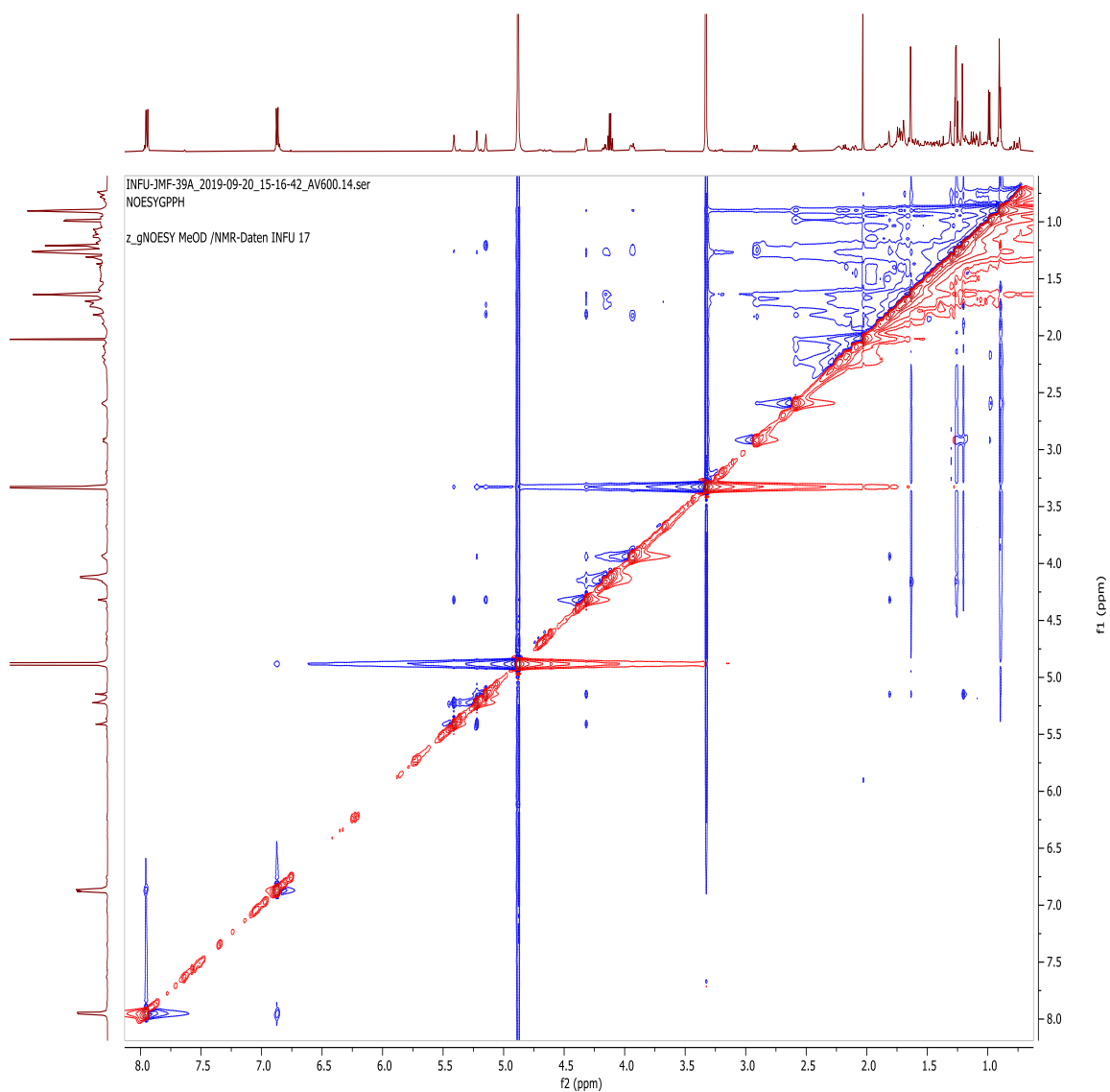
Appendix 3H: Mass spectrum of compound 44
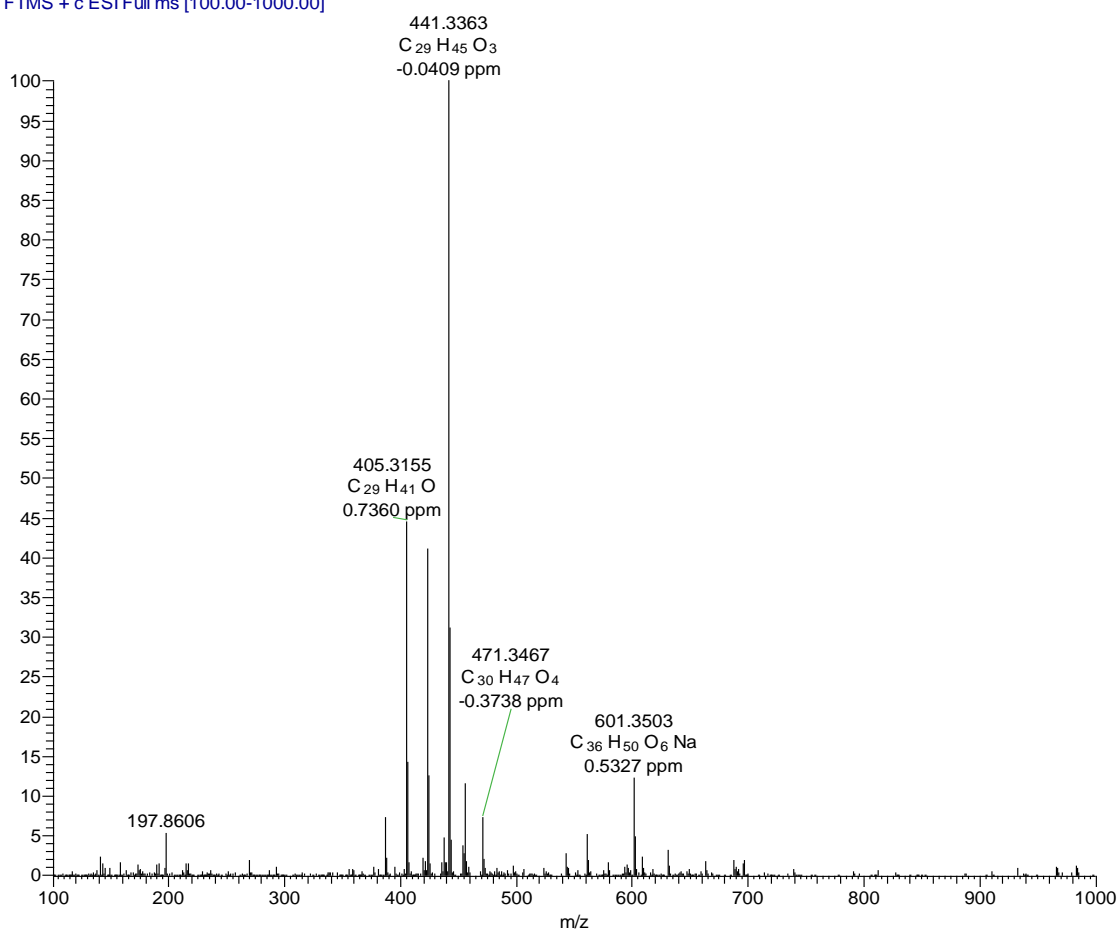
Appendix 4A: ^{1}H NMR spectrum (600 MHz, DMSO-d_6) of compound 45
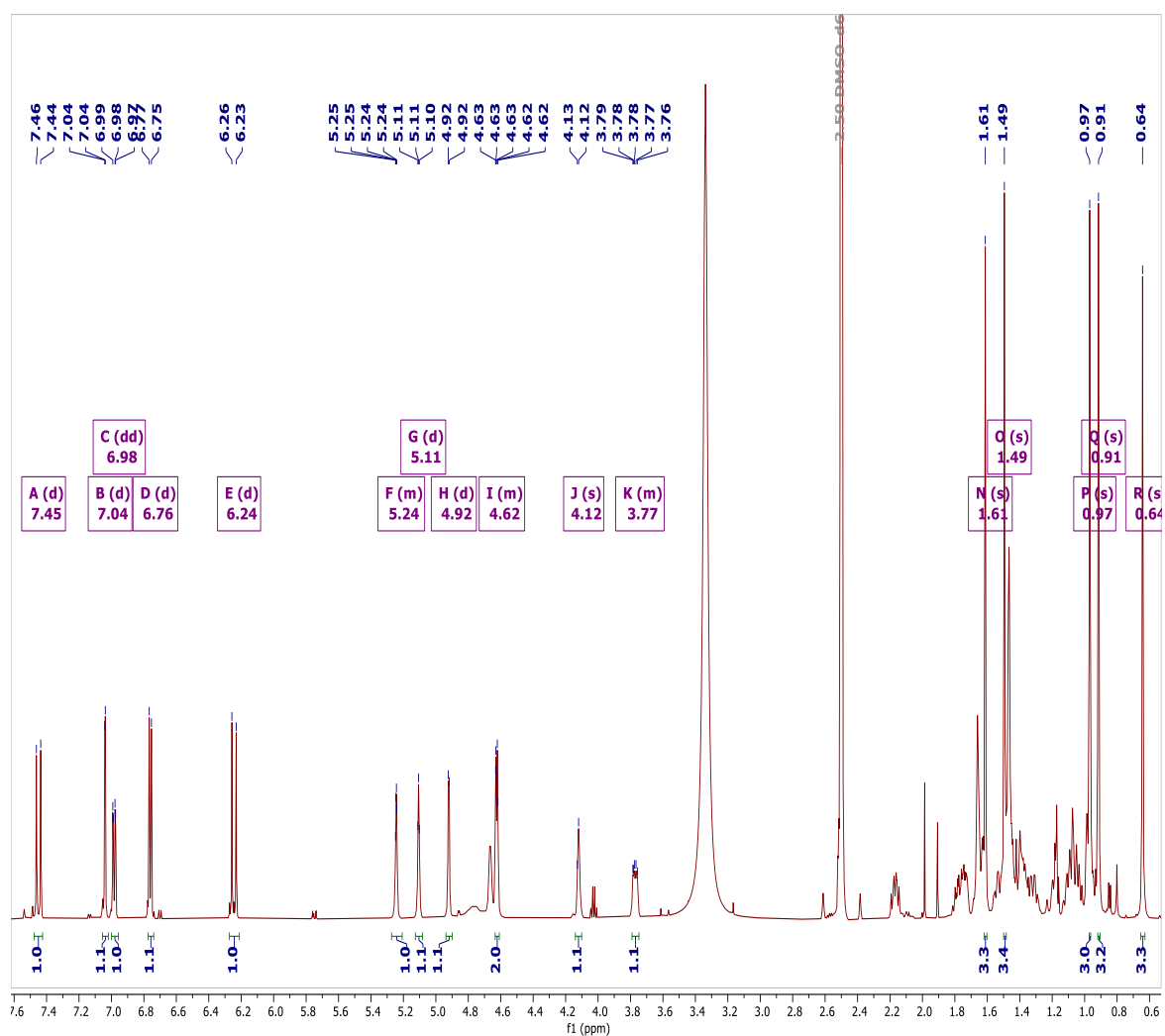
Appendix 4B: ^{13}C NMR spectrum (150 MHz, DMSO-d_6) of compound 45
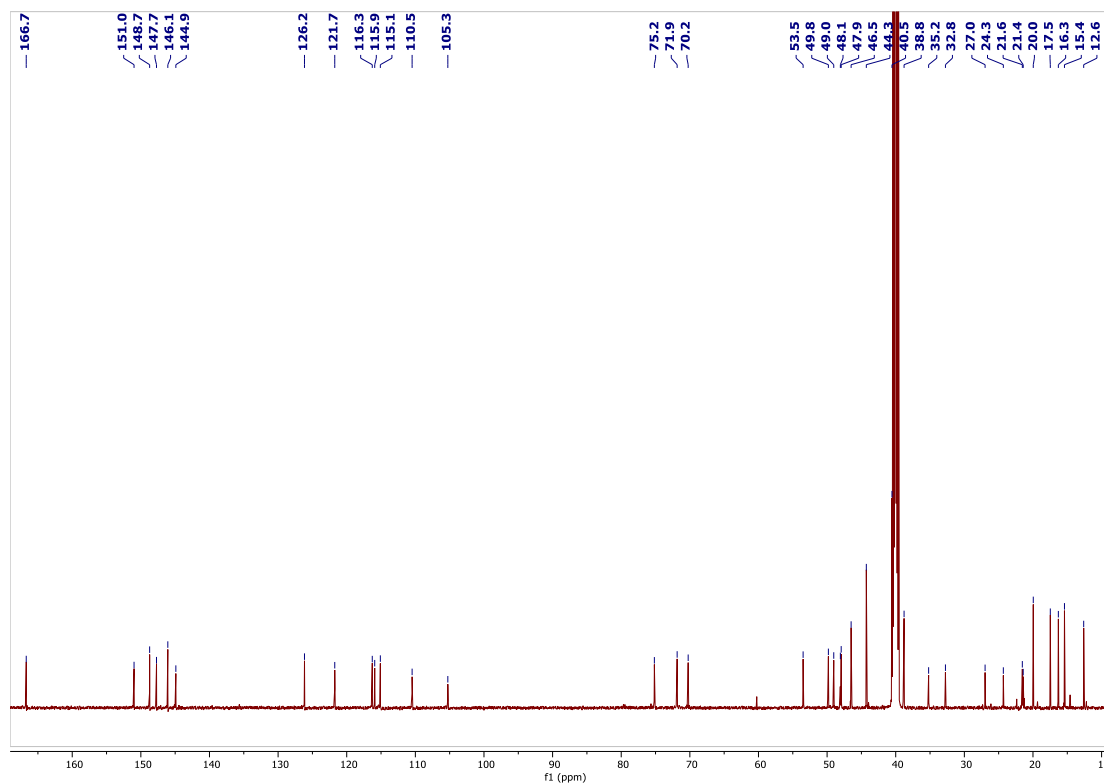
Appendix 4C: DEPT spectrum (DMSO-d_6) of compound 45
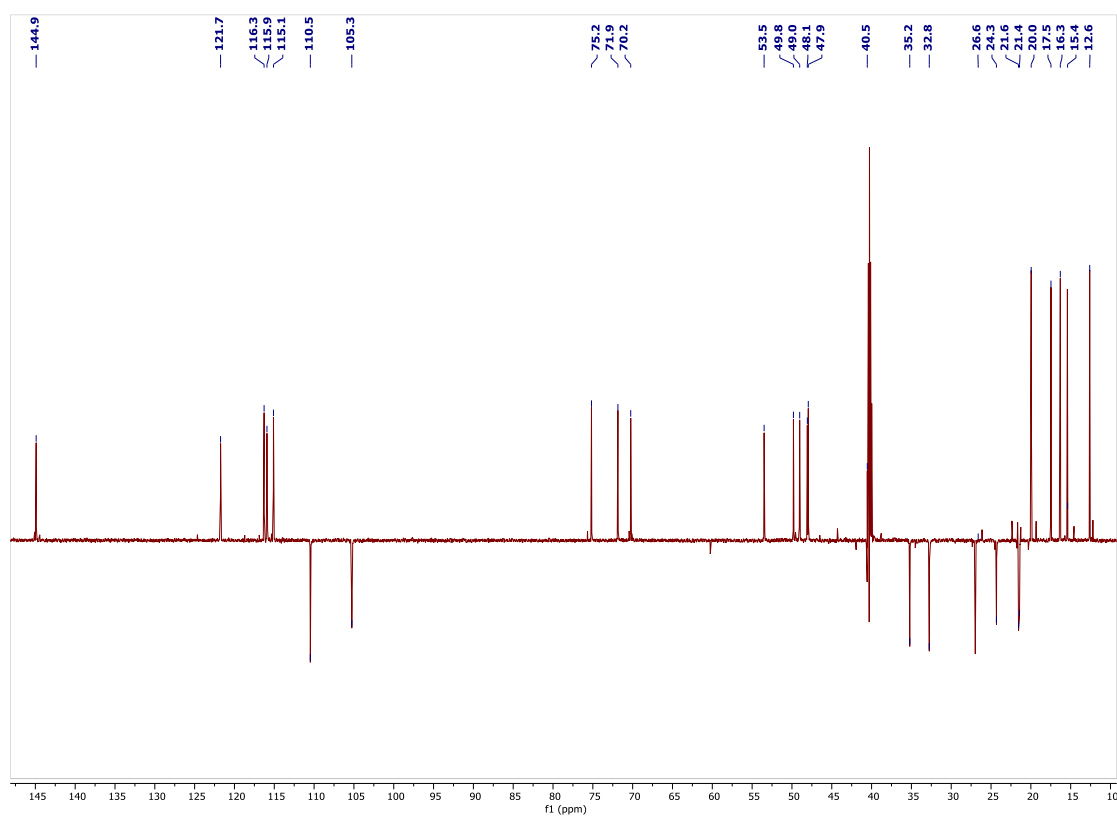
Appendix 4D: ^{1}H-^{1}H COSY spectrum (DMSO-d_6) of compound 45
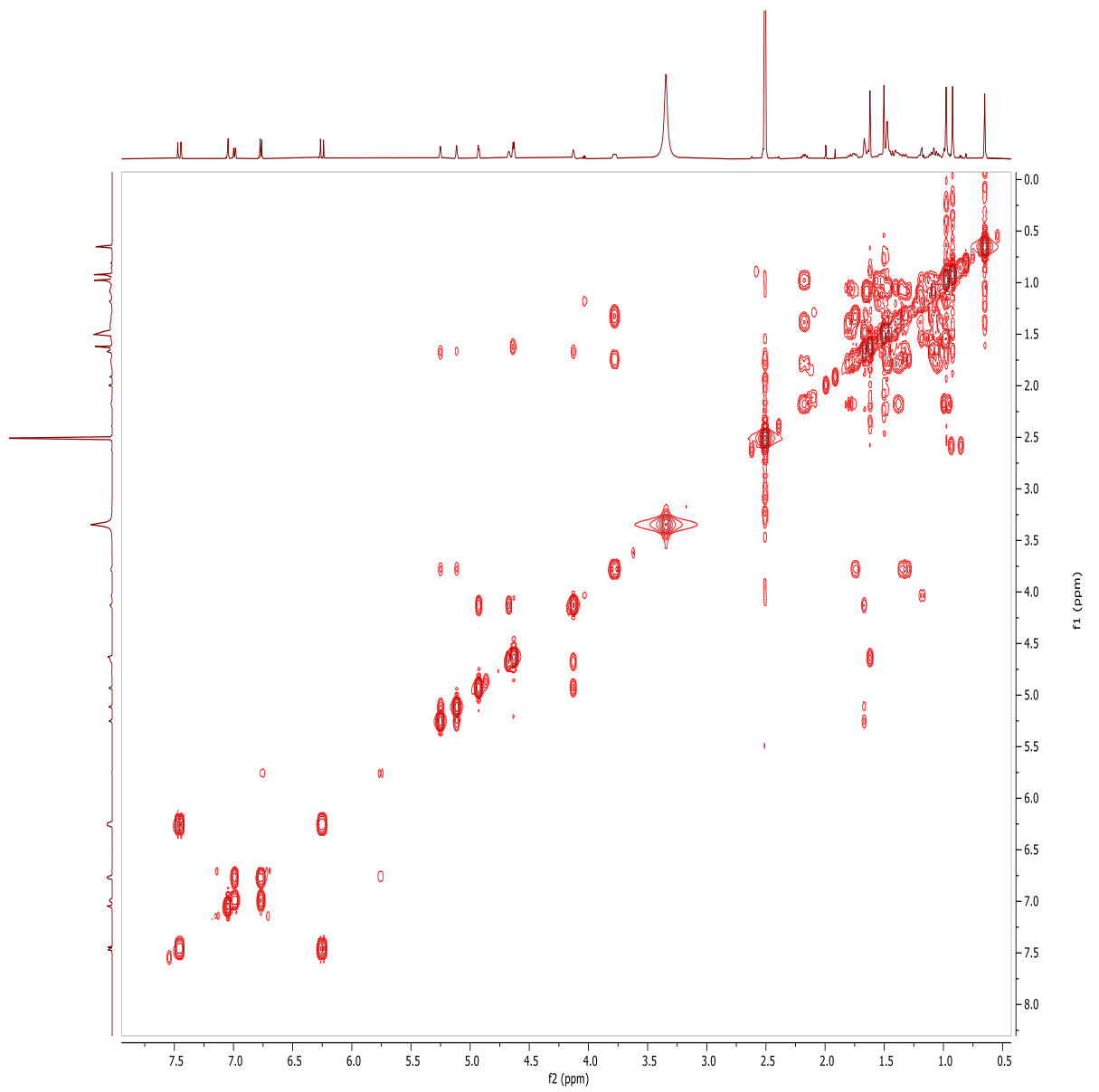
Appendix 4E: HSQC spectrum (DMSO-d_6) of compound 45

INFU-JMF-9A_2019-06-04_15-50-42_AV600/14
gHSQC
z_gHSQC_adiab DMSO /NMR-Daten INFU 37
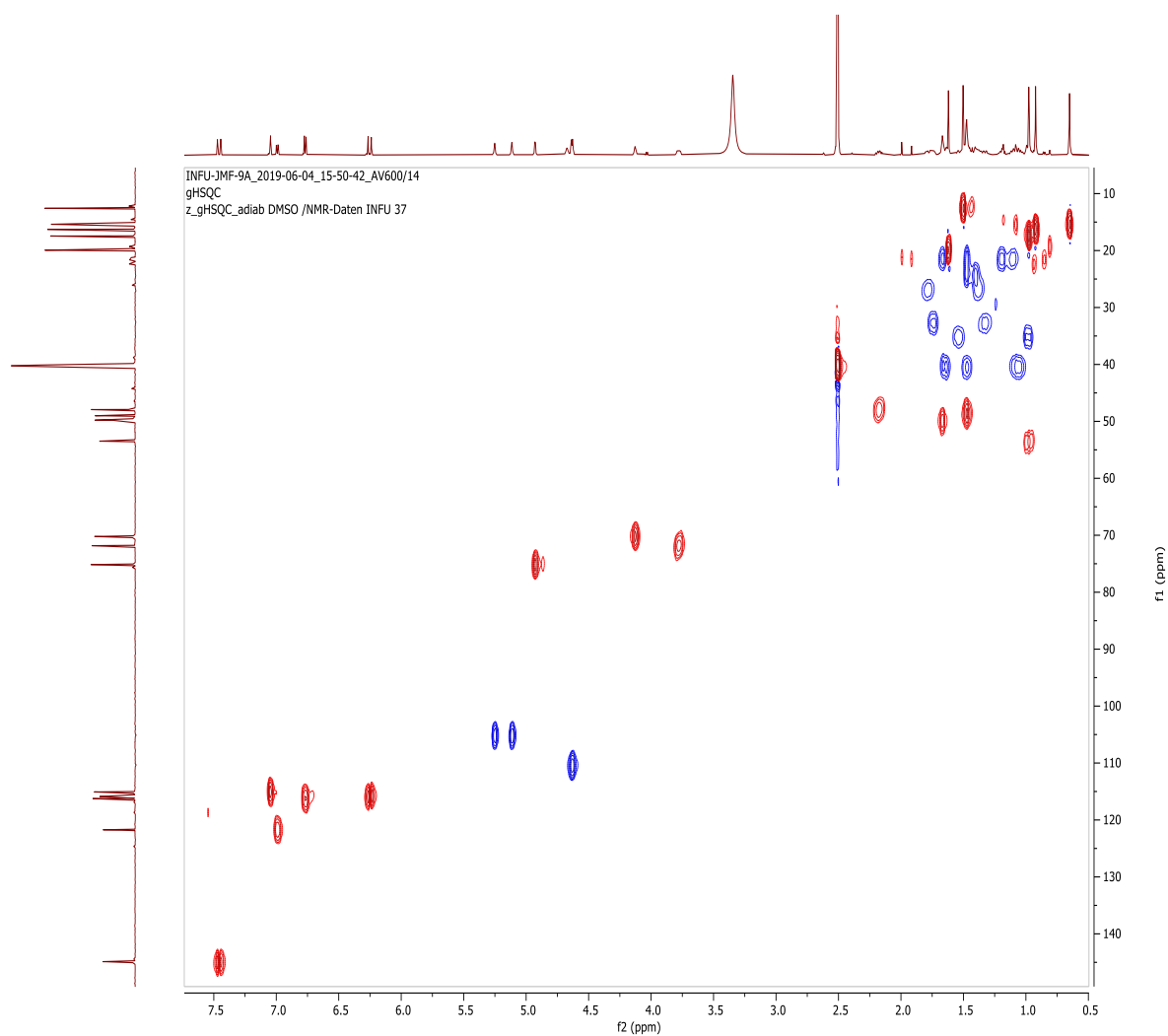
Appendix 4F: HMBC spectrum (DMSO-d_6) of compound 45

INFU-JMF-9A_2019-06-04_15-50-42_AV600/13
NOESYGPPH

z_gNOESY DMSO /NMR-Daten INFU 37
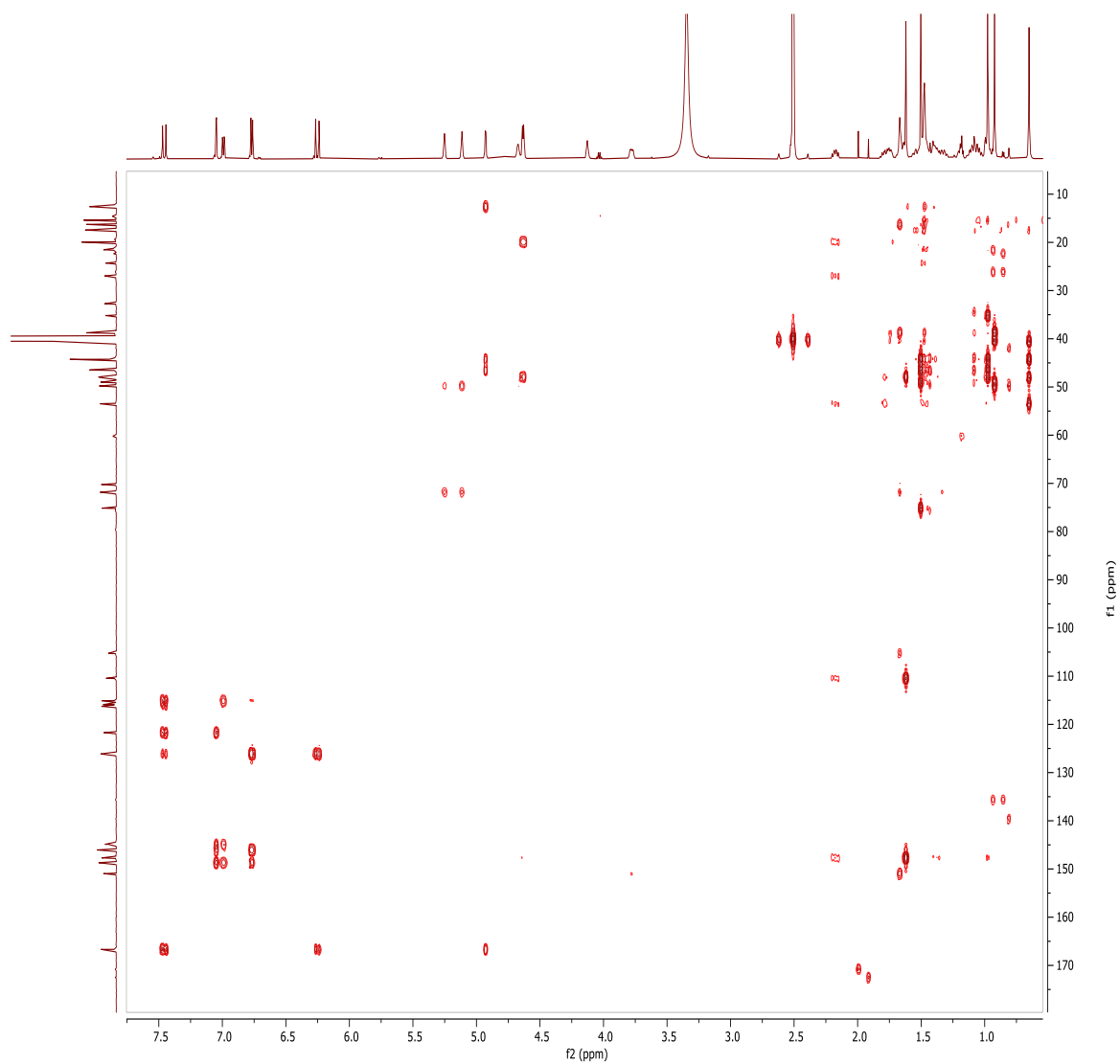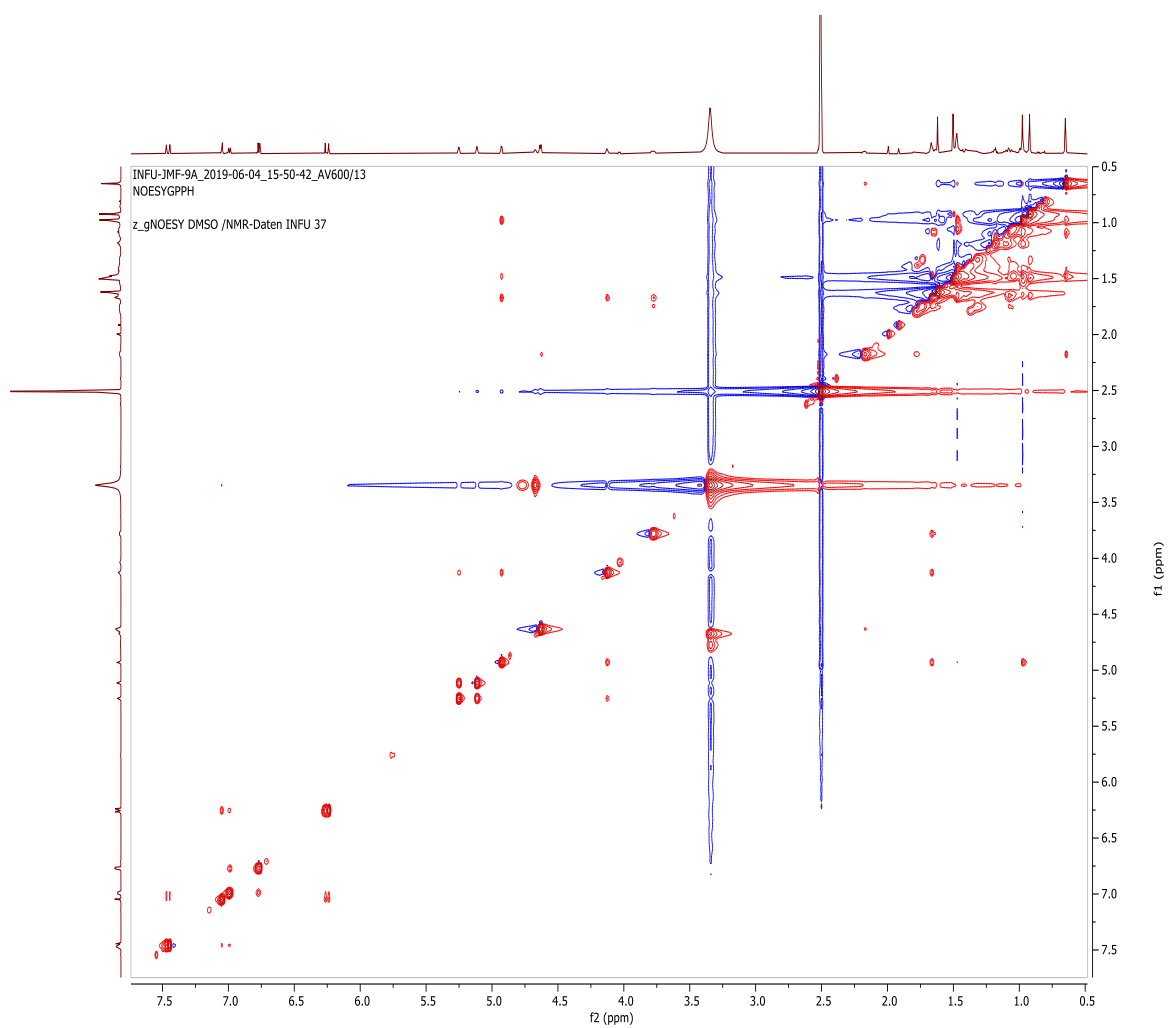
Appendix 4H: Mass spectrum of compound 45

JMF9A #1706-1716 RT: 29.15-29.29 AV: 11 NL: 1.88E5
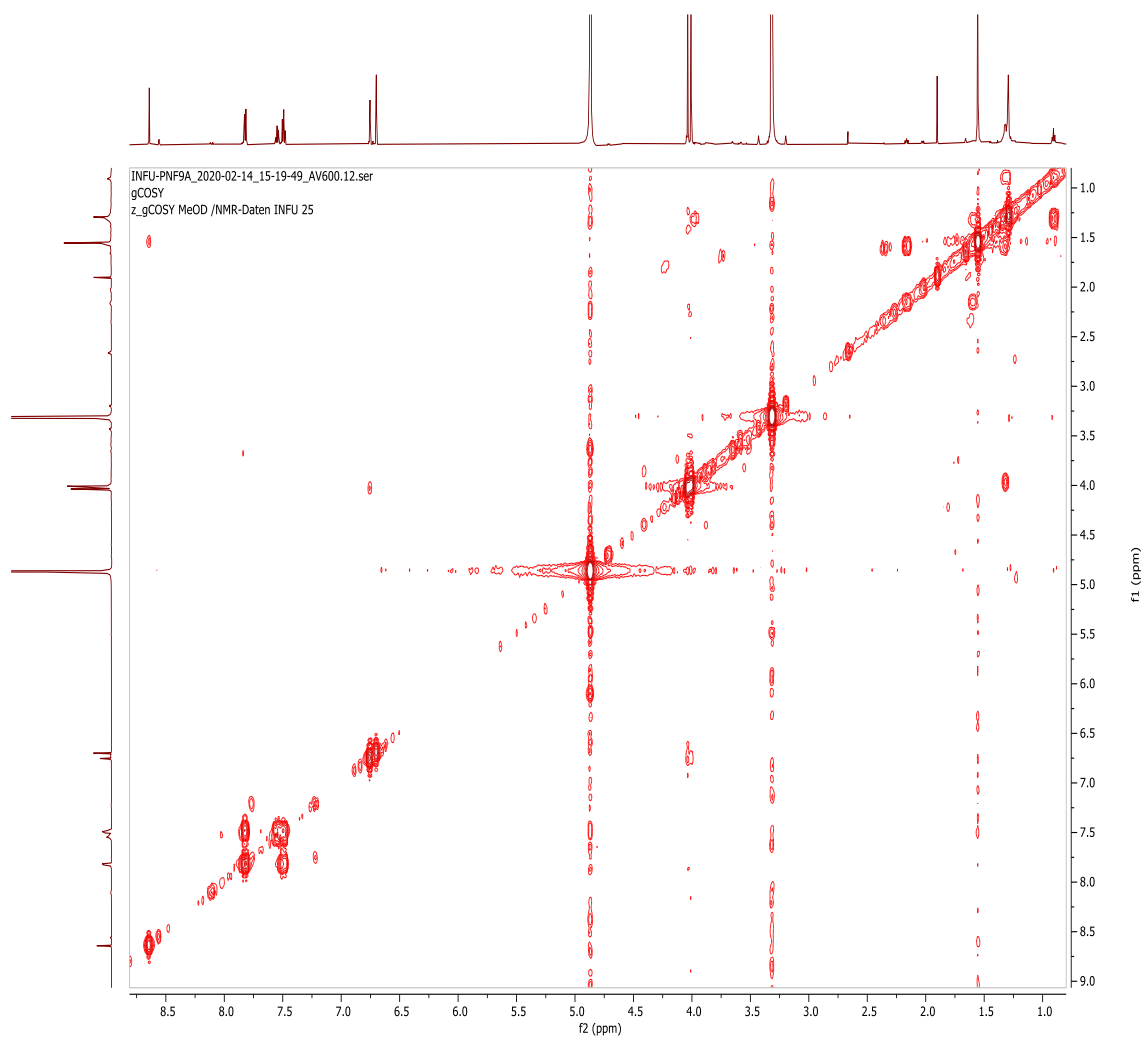T: FTMS + c ESI Full ms [100.00-1000.00]

627.3656
$C_{38}H_{52}O_6Na$
-0.0479 ppm

569.3625
$C_{38}H_{49}O_4$
-0.0008 ppm

605.3835
$C_{38}H_{53}O_6$
-0.2460 ppm

587.3730
$C_{38}H_{51}O_5$
-0.1530 ppm

636.4258
$C_{36}H_{60}O_9$
4.0443 ppm

501.3759
$C_{26}H_{54}O_7Na$
-0.6035 ppm

540.4468
$C_{36}H_{60}O_3$
-12.6908 ppm

563.5514

m/z

81
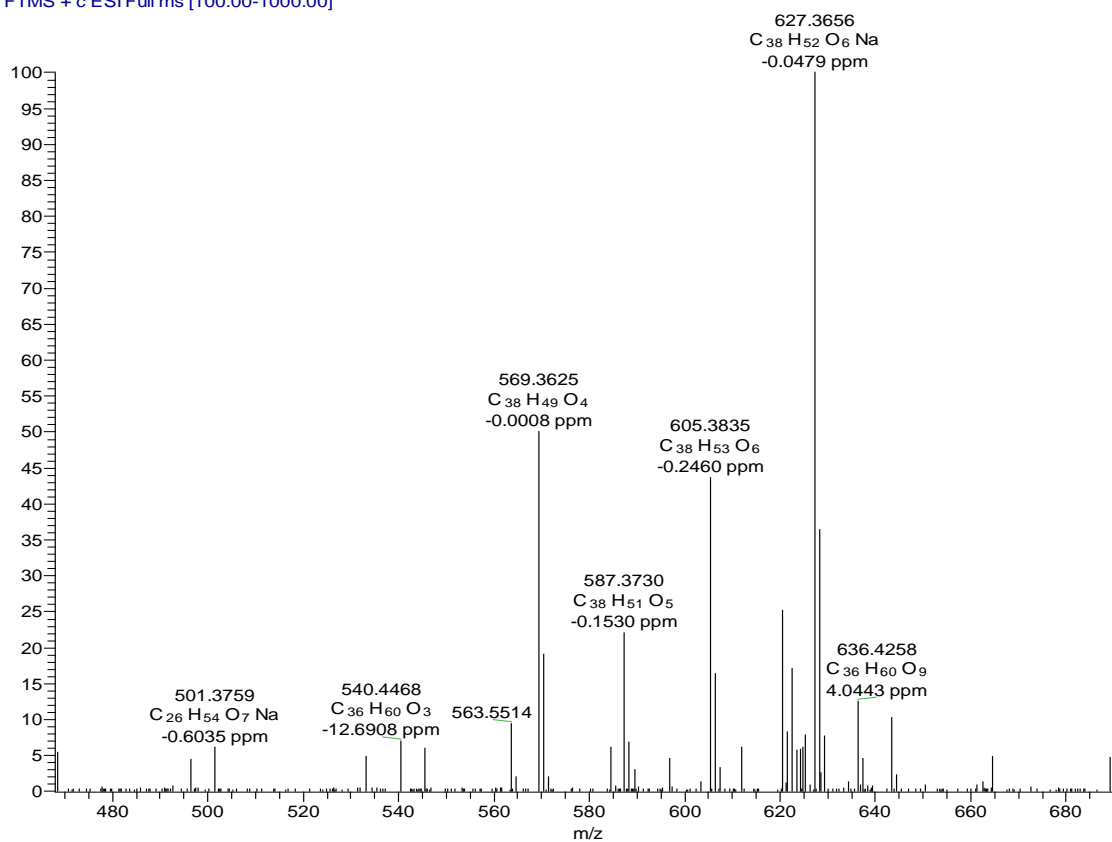
Appendix 5A: ¹H NMR spectrum (600 MHz, CD₃OD) of compound 46
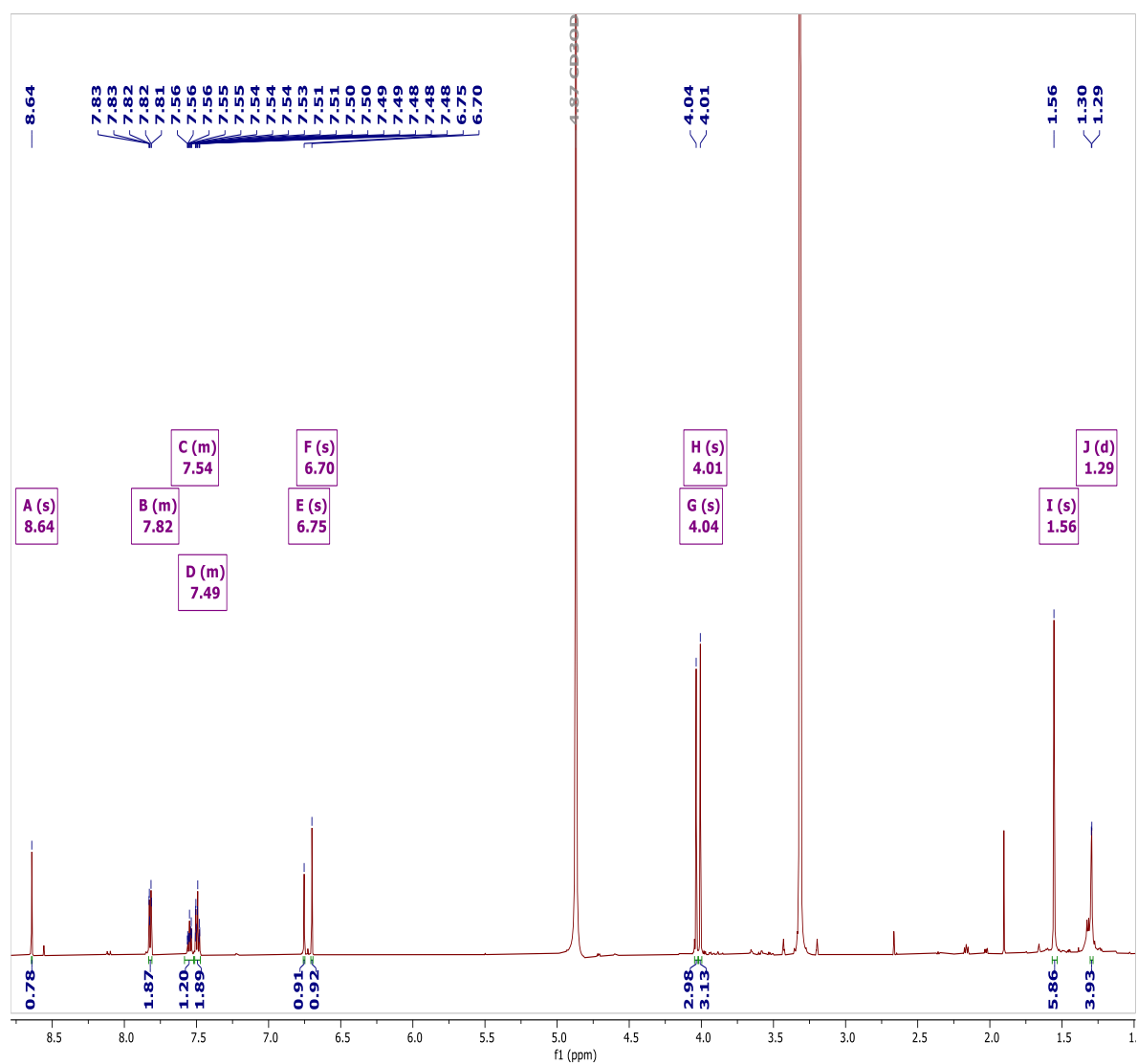
Appendix 5B: ^{13}C NMR spectrum (600 MHz, CD$_3$OD) of compound 46
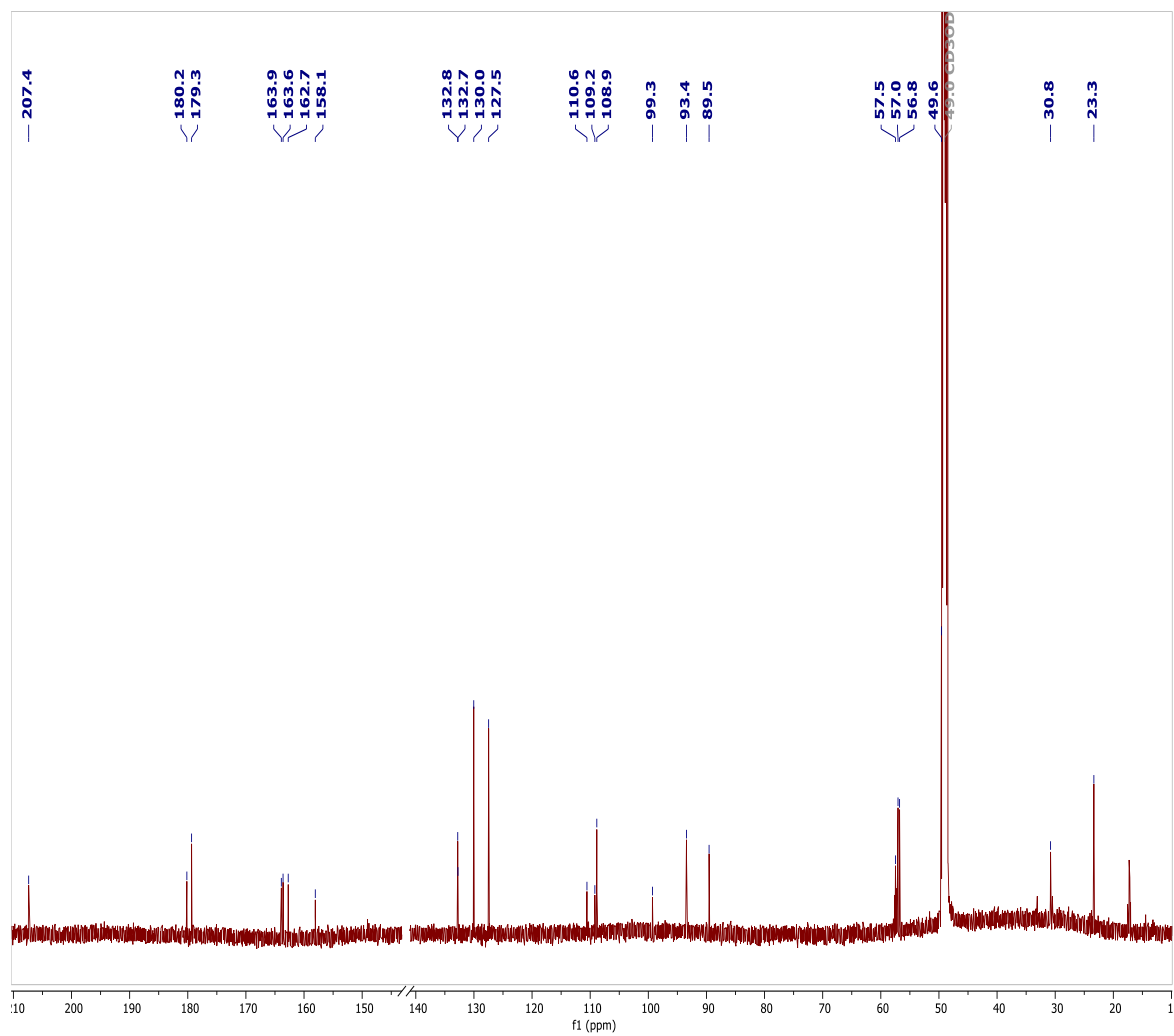
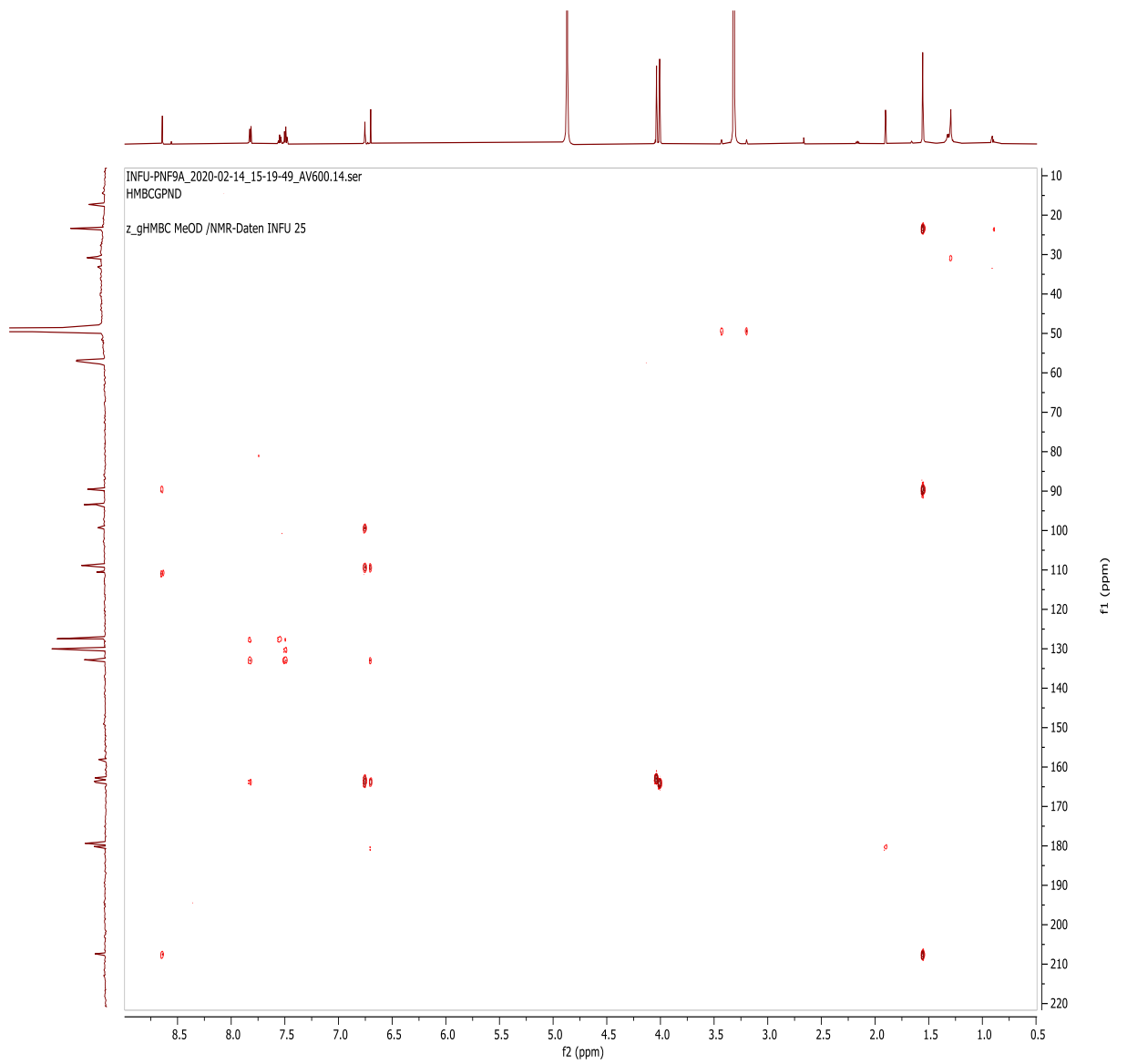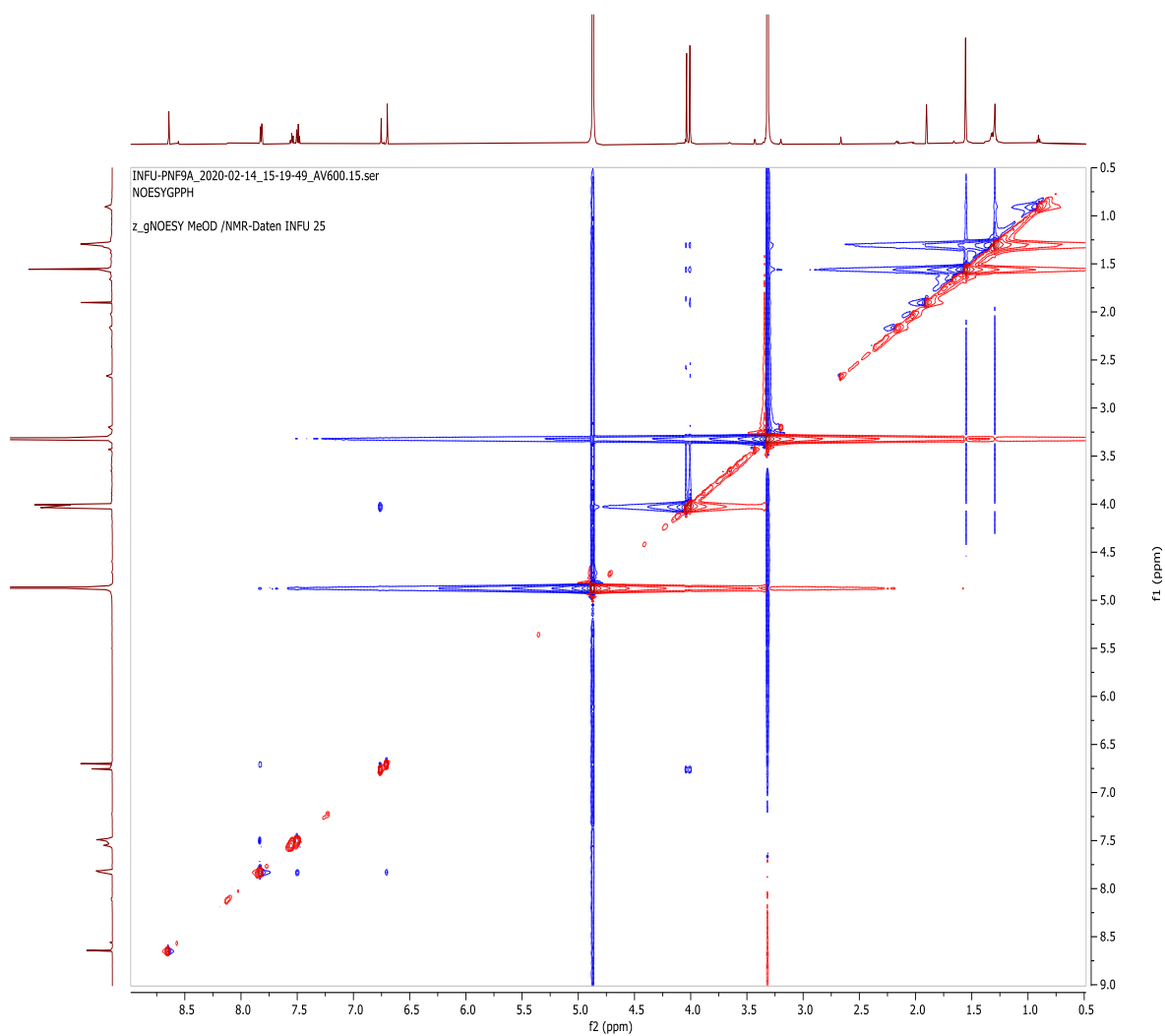**Appendix 5C: ¹H-¹H COSY spectrum (CD₃OD) of compound 46**

INFU-PNF9A_2020-02-14_15-19-49_AV600.12.ser
gCOSY
z_gCOSY MeOD /NMR-Daten INFU 25

Appendix 5D: HSQC spectrum (CD₃OD) of compound 46

INFU-PNF9A_2020-02-14_15-19-49_AV600.13.ser
gHSQC
z_gHSQC_adiab MeOD /NMR-Daten INFU 25
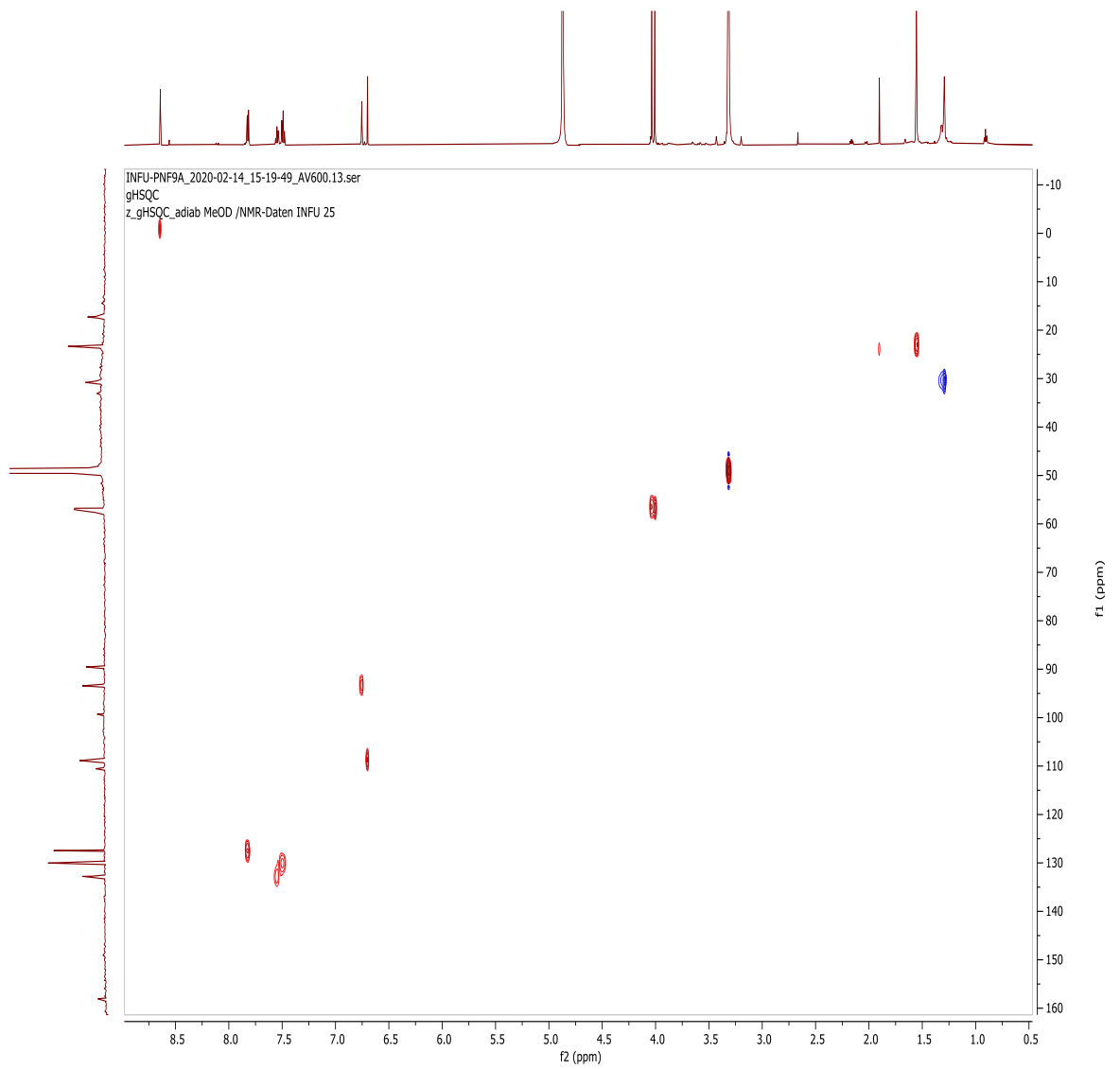
Appendix 5E: HMBC spectrum (CD₃OD) of compound 46

INFU-PNF9A_2020-02-14_15-19-49_AV600.14.ser
HMBCGPND

z_gHMBC MeOD /NMR-Daten INFU 25

Appendix 5F: NOESY spectrum (CD₃OD) of compound 46

INFU-PNF9A_2020-02-14_15-19-49_AV600.15.ser
NOESYGPPH

z_gNOESY MeOD /NMR-Daten INFU 25

Appendix 5G: Mass spectrum of compound 46

393.1334
$C_{23}H_{21}O_6$
0.2949 ppm
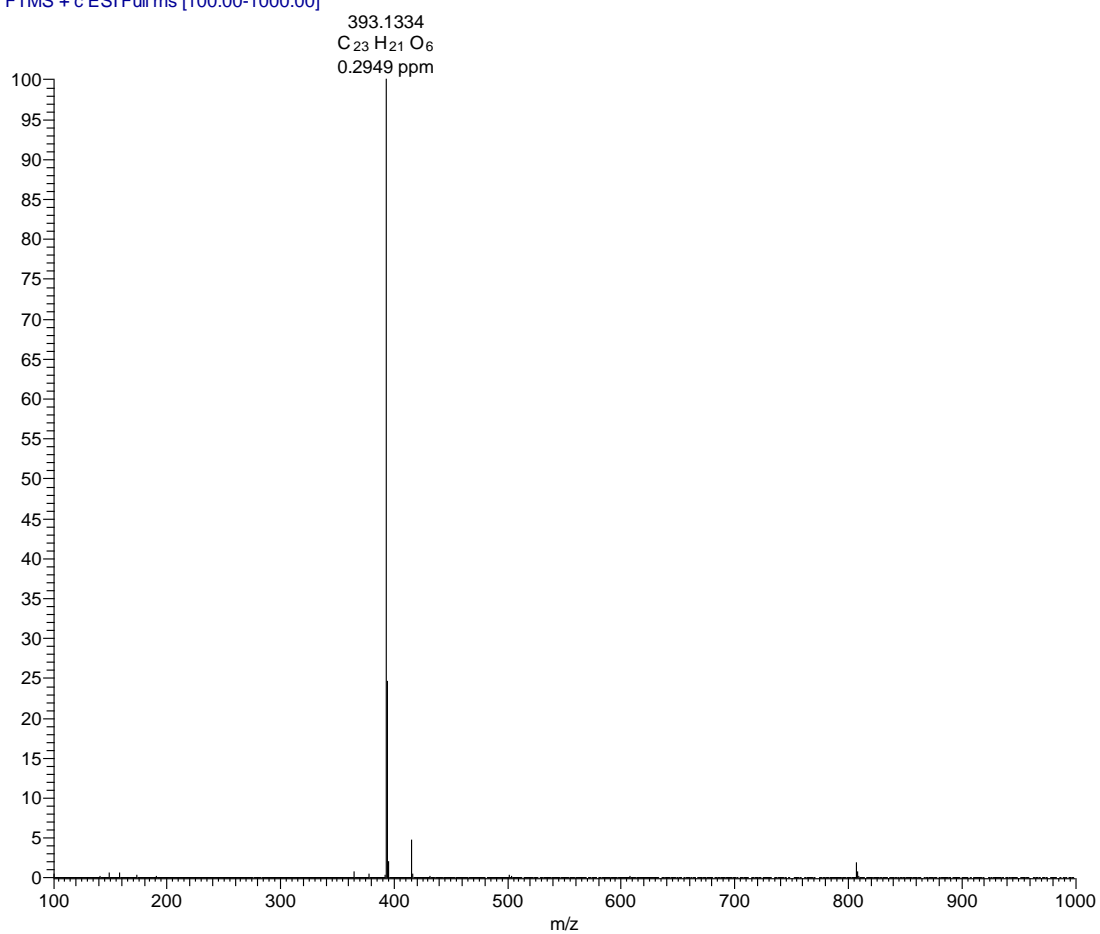
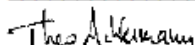**Appendix 6A: Certificate of analysis**

SIGMA-ALDRICH®

Certificate of Analysis

Product Name:
Lipopolysaccharides from Escherichia coli O111:B4 – γ-irradiated, BioXtra, suitable for cell culture

Product Number:	L4391
Batch Number:	067M4036V
Brand:	SIGMA
Storage Temperature:	Store at 2 - 8 ℃
Quality Release Date:	26 APR 2017
Recommended Retest Date:	APR 2023

Test	Specification	Result
Appearance (Form)	Lyophilized Powder	Lyophilized Powder
Appearance (Colour)	White to White w/ Yellow Cast	Off-White
Solubility (Solvent)	Water	Water
Solubility (Conc)	4.90 - 5.10 mg/ml	5.00 mg/ml
Solubility (Turbidity)	Slightly Hazy to Turbid	Hazy
Solubility (Color)	Colorless to Faint Yellow	Colorless
Protein Content (Method)	Lowry-TCA	Lowry-TCA
Prot. Content (% Protein)	$\leq$ 1.00 %	0.69 %
Potency (Sample EU/mg)	$\geq$ 500000 EU/mg	> 1800000 EU/mg
Gamma-Irrad.(Gamma srterilization)	Pass	Pass
Gamma-Irrad.(Measured Dose)	Min. 2.5 Mrad (25KGy)	Min. 2.5 Mrad (25KGy)
Vial Size (Solid/Vial)	1.00 - 1.00 mg	1.00 mg
Vial Content (Actual Content)	1.00 - 1.20	1.06
Vial Content (Overpack)	$\leq$ 20.00 %	6.00 %
Storage Conditions	Desiccated	Desiccated

Theo Ackermann

Theo Ackermann PhD MScEng CQM
Manager, Quality and Regulatory Affairs
Jerusalem, Israel IL

Version Number: 1   Page 1 of 1

Appendix 6B: Certificate of analysis

Certificate of Analysis

Product Name:
Ibuprofen - ≥98% (GC)

Product Number:	**I4883**
Batch Number:	**SLBR3566V**
Brand:	SIGMA
CAS Number:	15687-27-1
MDL Number:	MFCD00010393
Formula:	C13H18O2
Formula Weight:	206.28 g/mol
Quality Release Date:	12 MAY 2016
Recommended Retest Date:	MAY 2022
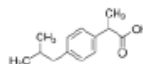
Test	Specification	Result
Appearance (Color)	White to Faint Yellow	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless to Faint Yellow	Colorless
Solubility (Turbidity) 50 mg/mL, EtOH	Clear	Clear
Titration with NaOH	> 98 %	98 %
Purity (GC)	> 98 %	100 %

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

Version Number: 1   Page 1 of 1

Appendix 6C: Certificate of analysis
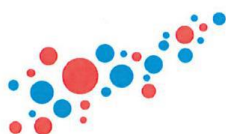
PRODUCT DATA SHEET – ePBMC® Uncharacterized Cryopreserved Human PBMC

Catalog No.:	CTL-UP1
Product name:	ePBMC® - Uncharacterized Cryopreserved Human PBMC
Size:	>10x10^6 cells / vial
Description:	Human PBMC (Peripheral Blood Mononuclear Cells) isolated from leukopacks and frozen in CTL-CryoABC™ serum-free freezing medium. These leukopacks were ethically collected from healthy donors with no risk of breaching privacy. Tested negative for HBsAg, HBcAb, HCV, HTLV I/II and STS by serology; as well as HIV I, HCV and WNV by NAT (nucleic acid testing)
Performance:	T cell functionality by ELISPOT equivalent to fresh cells
Applications:	PBMCs are suited for T cell monitoring in ELISPOT, ELISA, cytokine bead arrays, tetramer/ pentamer, and cytokine capture assays or any assay that requires live functional PBMC
Recommended test concentration:	Investigators are advised to determine optimal concentrations for individual applications. CTL recommends of 100,000 to 800,000 cells / well concentration for ELISPOT
Stability and Storage:	Cryopreserved cells are shipped in a dry cryoshipper, and should be unpacked immediately upon receipt. Short-term storage of cells (24h) at -80°C is acceptable, but should be minimized to ensure maximum stability. For long-term storage, cryopreserved cells should be stored in liquid nitrogen. Thawed samples must be used immediately and have a finite life span in culture. Avoid repeated freeze-thaw cycles!
Long-term Storage:	-169°C to -196°C (must be on liquid nitrogen (LN2) vapor)
Thawing:	Thaw protocol included
Usage:	**FOR RESEARCH USE ONLY! Not intended for direct therapeutic or diagnostic use in humans or animals, or for use in in vitro diagnostic procedures!**
Characterization results:	PBMCs from 4 donors, 1 vial each to Pharmacelsus GmbH, Dr. Tanja Wolf

Sample ID#	HHU20190624	HHU20190904	HHU20191003	HHU20191009	x
Ethnicity	Caucasian	African/American	African American/Hispanic	Caucasian	---
Age	41	31	29	32	---
Gender	Male	Male	Male	Male	---
ABO/Rh	AB/Pos	B/Pos	A/Pos	0/Pos	---

SFC- Spot Forming Cells

RI _____   Jan. 17, 2020

CTL Representative   Date

C.T.L., 20521 Chagrin Blvd., Shaker Heights, Ohio 44122 USA
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