# UNIVERSITY OF NAIROBI

# *IN VITRO AND IN SILICO* PHARMACOLOGIC EVALUATION OF THE ANTIRETROVIRAL ACTIVITY OF *CROTON* SPECIES

**BY**

**ERMIAS MERGIA TEREFE**

**U80/54741/2019**

**A Thesis Submitted for the Award of Doctor of Philosophy Degree in Molecular Pharmacology at the Department of Pharmacology and Pharmacognosy, University of Nairobi**

**2022**

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**Name of student**: Ermias Mergia Terefe

**Registration Number:** U80/54741/2019

**Faculty**: College of Health Sciences

**Department**: School of Pharmacy

**Section**: Pharmacology and Pharmacognosy

**Course Name**: Doctor of Philosophy in Molecular Pharmacology

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---------- Date -- **June 04, 2022**----------

**Ermias Mergia Terefe**
U80/54741/2019
Department of Pharmacology, University of Nairobi.

This PhD research work has been submitted with our approval as university supervisors.

---------- Date--09/06/2022---

**Prof. Faith Apolot Okalebo**
Department of Pharmacology and Pharmacognosy, University of Nairobi

---------- Date --------**13/06/2022**----------

**Prof. Solomon Derese**
Department of Chemistry, University of Nairobi

---------- Date----**13/06/2022**----------

**Dr. Joseph Muriuki**
Centre for Virus Research, Kenya Medical Research Institute

# DEDICATION

I dedicate my dissertation work to my parents, wife and children. A special feeling of gratitude to my loving father, Eng. Mergia Terefe, whose words of encouragement and push for tenacity ring in my ears. My mother, Tirunesh Wakshuma, for all her continued prayers and support.

# ACKNOWLEDGEMENTS

First of all, I thank the Almighty God for his grace and all the strength he gave me during my Ph.D. project. I am grateful to my wife Blen Lemma Balcha and children (Dunamis, Juliana and Chris) for their patience during late hours of work and all moments of frustration and happiness during this journey.

My Special appreciation goes to my supervisors Prof. Faith A. Okalebo, Prof. Solomon Derese, and Dr. Joseph Muriuki, for graciously providing good supervision, critical review, and commitment to shaping the Ph.D. project and guiding me through the whole program. I am grateful for your patience as you guided, supported, and encouraged me through my research work.

I acknowledge United States International University- Africa (USIU-A) Internal grant no. 10-2854, University of Nairobi (UON), Kenya Medical Research Institute (KEMRI), and the Institute of Primate Research (IPR) for their support toward the successful completion of the research work. I am grateful to UON-ERC for awarding me with the ethical clearance to carry out the research.

I thank Dr. Kipruto A Sinei and Dr. Margaret Oluka, Department of Pharmacology and Pharmacognosy, University of Nairobi, for providing needful support during my work. I also wish to thank to the research team at the Royal Botanic Gardens, Kew (United Kingdom) for assisting me in the NMR and MS analysis of the pure compounds.

Thank you to all my colleagues in the School of Pharmacy and Health Sciences, United States International University, for your support in my Ph.D. journey. It is impossible to mention individually all the persons who helped me one way or another; however, I would like to record my gratitude for all their assistance, both professional and personal.

# ABSTRACT

**Introduction**

The Human Immunodeficiency Virus (HIV) affects the body's defence mechanisms and leads to many opportunistic infections. Globally, more than half a million individuals lost their lives in 2020 due to this disease. Antiretroviral drugs have played a significant role in improving the quality of life of HIV-infected individuals. However, the side effects of these drugs, coupled with the virus's resistance to the various regimens, necessitate the search for potential new and effective antiretroviral medication.

**Objective**

The objective of this study was to evaluate the cytotoxicity and anti-HIV activity of crude and solvent fractions of *Croton macrostachyus*, *Croton megalocarpus,* and *Croton dichogamus* and to isolate pure compounds with potential antiretroviral activity.

**Methods**

Stem bark and leaves of *C. macrostachyus, C. megalocarpus,* and twigs of *C. dichogamus* are extracted using 1:1 v/v dichloromethane and methanol, followed by liquid-liquid partitioning using four different solvents. The crude and solvent fractions were tested for inhibition of laboratory-adapted HIV-$1_{IIIB}$ strain replication in the Human T-lymphocytic MT-4 cell line. In addition, using bioassay-guided fractionation, pure compounds with anti-HIV activity were isolated. Finally, the chemical structure of the new compounds was elucidated and mode of action predicted using in silico approach.

**Results**

The highest anti-HIV activity was observed from the ethyl acetate fractions of the bark of *C. megalocarpus* ($IC_{50}$ = 0.47 ± 0.12 µg/mL; selectivity index =346.3), the hexane fraction of the leaves of *C. macrostachyus* ($IC_{50}$ = 0.02 ±0.01µg/mL; SI of 9752.7) and the methanol fraction of *C. dicohgamus (*$IC_{50}$ =0.06 + 0.01 µg/ml; SI of 318.5). In addition, nine compounds were isolated from the ethyl acetate fraction of the bark of *C. megalocarpus* (ermiasolide A, B and C, 4H-α,7H-α,10α-eudesm-11-en-5β-ol, ermiasoid, lupeol, 11-acetoxy crotocascarin L, pinoresinol, and crotocascarin K), five compounds from the hexane fraction of the leaves of *C. macrostachyus* (2-methoxy benzyl benzoate, betulin, lupenone, lupeol acetate, lupeol) and four compounds from methanol fraction of the twigs of *C. dichogamus* (dihydroconiferyl acetate, crotocascarin ω, (4-hydroxy-3-methoxyphenyl)-propyl benzoate, β-oplopanone). Among these compounds, six of them (ermiasolide A, ermiasolide B, ermiasolide C, ermiasoid, 11-acetoxy

crotocascarin L and crotocascarin ω) were previously undescribed new compounds with anti-HIV activity. The new compounds displayed the highest anti-HIV activity by inhibiting more than 70% of viral replication with an $IC_{50}$ value of less than 0.04 µg/mL. The i*n vitro* assay showed that the bioactive compounds are more active in inhibiting more than 60 % of HIV-1 protease enzyme activity. In addition, molecular docking study showed that these compounds had strong binding with the HIV-1 PR at free binding energy of less than -7.00 kcal/mol and form hydrophobic and hydrogen bonding with several amino acids in the active site of the HIV protease enzyme and inhibit the binding of viral polypeptide to the catalytic domain of the enzyme.

**Conclusion**

The isolated pure compounds have displayed potent inhibition of HIV-1 protease enzyme activity due to the strong binding of the compounds in the enzyme's active site and the formation of several hydrogen bonds with key residues.

**Keywords:** HIV, MT-4 cells, *Croton macrostachyus*, *Croton megalocarpus*, *Croton dichogamus*, Cytotoxicity, AntiHIV activity

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# LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| ABC | Abacavir |
| ADMET | Absorption, Distribution, Metabolism, Excretion, and Toxicity |
| AIDS | Acquired Immune Deficiency Virus |
| ARV | Antiretroviral Drugs |
| AZT | Zidovudine |
| CDC | Center for Disease Control |
| COSY | Correlation Spectroscopy |
| DEPT | Distortionless Enhancement by Polarization Transfer |
| DMSO | Dimethyl sulfoxide |
| EACC | European Collection of Authenticated Cell Cultures |
| EBV | Epstiein-Barr virus |
| EFV | Efavirenz |
| ELISA | Enzyme Linked Immunosorbent Assay |
| HMBC | Heteronuclear Multi Bond Correlation Spectroscopy |
| HRMS | High Resolution Mass Spectrometry |
| HSQC | Heteronuclear Single Quantum Coherence Spectroscopy |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NACC | National AIDS Control Council |
| NVP | Nevirapine |
| PBS | Phosphate buffer saline |
| PMTCT | Prevention of Mother to child transmission |
| PrEP | Pre-exposure prophylaxis |
| TAM | Thymidine analogue mutations |
| TDF | Tenofovir |
| VMMC | Voluntary medical male circumcision |

# LIST OF APPENDICES

# PUBLICATIONS

1. **Terefe, E. M.,** Okalebo, F. A., Derese, S., Muriuki, J., Rotich, W., Mas-Claret, E., Sadgrove, N., Padilla-González, G. F., Prescott, T. A. K., Siddique, H., & Langat, M. K. (2022). Constituents of Croton megalocarpus with Potential Anti-HIV Activity. *Journal of Natural Products*. https://pubmed.ncbi.nlm.nih.gov/35709365/
2. **Terefe, E. M.,** Okalebo, F. A., Derese, S., Langat, M. K., Mas-Claret, E., Aljarba, N. H., Alkahtani, S., Batiha, G. E.-S., Ghosh, A., El-Masry, E. A., & Muriuki, J. (2022). In vitro anti-HIV and cytotoxic effects of pure compounds isolated from *Croton macrostachyus* Hochst. Ex Delile. *BMC Complementary Medicine and Therapies 2022 22:1*, *22*(1), 1–10. https://pubmed.ncbi.nlm.nih.gov/35705943/
3. **Terefe E. M.,** Okalebo F. A., Derese S., Muriuki J., Amuhaya E. K., Munyendo L. W., Janapati Y., Obila J., Mosweta J. (2022). Cytotoxicity and antiretroviral activity of the leaf and stem bark extracts of *Croton macrostachyus*. *Pharmaceutical Journal of Kenya*, *26*(1), 2022. https://psk.or.ke/journals/article-98
4. **Terefe, E. M.,** Okalebo, F. A., Derese, S., Batiha, G. E.-S., Youssef, A., Alorabi, M., & Muriuki, J. (2022). Cytotoxicity and anti-HIV activities of extracts of the twigs of Croton dichogamus Pax. *BMC Complementary Medicine and Therapies 2022 22:1*, *22*(1), 1–8. https://pubmed.ncbi.nlm.nih.gov/35216601/
5. **Terefe, E. M**.; Okalebo, F. A.; Derese, S.; Muriuki, J.; Batiha, G. E.-S. In Vitro Cytotoxicity and Anti-HIV Activity of Crude Extracts of *Croton Macrostachyus*, *Croton Megalocarpus* and *Croton Dichogamus*. *J. Exp. Pharmacol.* **2021**, *13*, 971–979. https://pubmed.ncbi.nlm.nih.gov/35221732/

# CHAPTER ONE

# INTRODUCTION

## 1.1. Background Information

Since the outbreak of the human immunodeficiency virus (HIV) pandemic, globally, more than 75.7 million people have been infected with HIV, resulting in more than 33 million death due to Acquired Immunodeficiency Syndrome (AIDS)-related diseases (UNAIDS, 2020). Currently, 38 million people live with the virus, out of which 54% (20.7 million) live in Southern and East African regions. In 2019, over 1.7 million people were infected by the virus (UNAIDS, 2020). In that year, the global death caused by the epidemic had reached more than half a million, with more than 300,000 of these deaths occurring in Southern and East Africa. Globally, among the 38 million people living with HIV, more than 10 million people did not have access to antiretroviral treatment as of the end of June 2020. Thus, more than 25% (5 million) of Southern and East African people do not have access to the treatment (UNAIDS, 2020).

Substantial advances have been made in antiretroviral therapy (ART) after the discovery of zidovudine in 1987 (McLeod & Hammer, 1992; Sperling, 1998a). At that time, zidovudine was prescribed as monotherapy for only patients with advanced, symptomatic disease as a five times daily dose. In mid-1996, it was discovered that antiretroviral drugs are far more effective when three or more are taken simultaneously (Sperling, 1998b). This combination therapy (comprising at least three ARV agents) has shown a significant decline in viral replication and improvement in patients' quality of life (Erik, 2010).

Although ART has contributed significantly to reducing death due to HIV, toxicities of the drugs, problems with adherence related to lifelong therapy, and drug resistance are challenging problems for the success of HIV care and treatment (Montessori *et al.*, 2004). In this study, we explored the anti-HIV activity of three plants from the genus *Croton*.

## 1.2. Statement of the problem

HIV/AIDS has a detrimental economic, psychosocial, and health-related impact on countries, households, and individuals around the world. As the number of people infected with HIV rises, medical treatment costs rise, and national resources shift to the HIV/AIDS program (World Health Organization, 2016). Furthermore, HIV-infected individuals are susceptible to a variety of opportunistic infections (OI), which can reduce their productivity and shorten their

lives and also co-administration of other drugs to treat OI's might result in drug interactions that can impair ARV therapy or induce serious side effects (Narayan *et al*., 2013).

The development of drug resistance has also posed a great challenge for treating clients, since many cases where patients are not responding even to second-line regimens. Moreover, this problem continues to be a danger to the worldwide struggle to end the epidemic by 2030 (UNAIDS, 2016). Hence, there is a need to discover novel drugs with antiretroviral activity. One of the systematic approaches to tackle these challenges is to discover new drugs by identifying bioactive antiretroviral compounds from natural products. *Croton* species have gained attention by providing different bioactive phytochemicals used to treat bacterial and viral infections (Saslis-Lagoudakis *et al*., 2012). This study evaluated the antiretroviral activity of *C. macrostachyus*, *C. megalocarpus*, and *C. dichogamus* using *in vitro* and *in silico* approaches.

## 1.3. Objectives

### 1.3.1. General Objective

The main objective of this research was to evaluate the i*n vitro* and *in silico* antiretroviral activity of *C. macrostachyus*, *C. megalocarpus,* and *C. dichogamus* against HIV.

### 1.3.2. Specific Objectives

The specific objectives of the research were to:

1. Evaluate the cytotoxicity and antiretroviral activity of crude extracts from *C. macrostachyus*, *C. megalocarpus,* and *C. dichogamus*
2. Evaluate the cytotoxicity and antiretroviral activity of solvent fractions of *C. macrostachyus*, *C. megalocarpus,* and *C. dichogamus*
3. Isolate pure compounds with a potential antiretroviral activity using bioassay-guided fractionation
4. Demonstrate the interaction of isolated pure compounds with HIV enzymes by using *in vitro* assays and *in silico* molecular docking approaches

## 1.4. Justification and significance of the study

There is a need to identify local alternatives, less expensive and less toxic drugs for the treatment of HIV. As herbs are the primary source of drugs, the search for new antiretroviral compounds can start from herbal medicines used by traditional healers as a remedy for HIV and related opportunistic infections. The plants included in this study, *C. macrostachyus, C.*

*megalocarpus, and C. dichogamus,* are used by herbal practitioners in Africa. These plants have promising antibacterial, antifungal, antiviral and anticancer effects (Singh, 2018; Obey *et al.*, 2016a; Al-Asmari *et al*., 2015).

Different parts of *C. macrostachyus, C. megalocarpus,* and *C. dichogamus* are used as herbal medicine by HIV patients for the treatment of pneumonia (Kamau *et al.*,2016), respiratory problems (Muthee *et al*., 2011; Njoroge *et al.*,2006), diabetes (Keter & Mutiso, 2012), wounds (Kamau *et al*., 2016), and as a purgative (Degu *et al*.,2016).

The *Croton* genus has gained the attention of many researchers for its potential source of bioactive compounds against HIV. Ravanelli *et al.,* (2016) have shown that alkaloids isolated from *C. echinocarpus* leaves have significant *In vitro* anti-HIV activity by inhibiting the HIV-1 reverse transcriptase enzyme. Tietjen *et al*., (2016b) reported that *C. megalobotrys* is widely used in Botswana to treat HIV, diarrhea, loss of appetite, and stomach problems.

Considering the wide medicinal use of these plants by HIV-positive patients, this research evaluated the effect of the crude and solvent fractions in inhibiting viral replication. It also investigated the anti-HIV activity of the isolated pure compounds in an i*n vitro* and *in silico* experiments. The findings of this study could lead to the development of new antiviral agents that are less toxic and more efficacious.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. Literature review on HIV

### 2.1.1. History of HIV Pandemic

In 1981, the first case of HIV/AIDS was identified (CDC, 1981). It was discovered two years later that HIV was the cause of the syndrome (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983; Gottlieb *et al.*, 1981*)*. HIV is the cause of the current epidemic (Heeney *et al*., 2006; Heuverswyn *et al*., 2006). HIV comprises four genetically distinct groups, namely M, N, O, and P (Sharp & Paul, 2011). Group M is further classified into nine subtypes/clades (A to D, F to H, J, and K) and is responsible for nearly all human infections worldwide (Sharp & Paul, 2011). In terms of antiretroviral medication, multiple studies demonstrate that patients infected with distinct clades of group M virus had similar responses to antiretroviral therapy. However, the diversity of the virus is a central problem in the design of an HIV vaccine (Walker & Burton, 2008).

### 2.1.2. HIV Epidemiology

Sexual intercourse is the main route for the transmission of HIV. Other transmission routes include blood transfusion, vertical transmission from mother to child, and percutaneous exposure (Capriotti, 2018; Fettig *et al*., 2014; Fox & Fidler, 2010; Gelmon *et al*., 2009; CDC, 2006). Due to ongoing transmission and new HIV infection, as shown in **Table 2.1-1,** in 2019, approximately 0.7 million people died, with 1.7 million new HIV infections (UNAIDS, 2020).

### 2.1.1. HIV-1 Structure and function

The human immune deficiency virus belongs to the family of retroviridae, the subfamily Lentivirinae and the genus Lentiviridae. It uses RNA as genetic material (Sharp & Paul, 2011). Viruses are simple in Structure and cannot replicate (Mcgilvray & Willis, 2004) by themselves and thus require the components of other cells for replication (require host cells to replicate). As shown in **Figure 2.1-1**, HIV has a spherical shape consisting of two single-stranded ribonucleic acids (RNAs) and three enzymes enclosed by an envelope (Sundquist & Kräusslich, 2012; Buchschacher, 2003).

Table 2.1-1: HIV Epidemiology in the world and Africa (UNAIDS, 2020)

| Key Indicators | Global data (millions) | Eastern & Southern Africa (millions) | Western & Central Africa (millions) | Middle East & Northern Africa |
|---|---|---|---|---|
| Adult and children Living with HIV | 38.0 [31.6– 44.5] | 20.7 [18.4–23.0] | 4.9 [3.9– 6.2] | 240,000 [170,000–400,000] |
| Adult and children newly infected with HIV | 1.7 [1.2– 2.2] | 0.73 [0.58–0.94] | 0.24[0.15–0.39] | 20,000 [11,000– 38,000] |
| Adult and child deaths due to AIDS | 0.69 [0.5– 0.97] | 0.3 [0.23– 0.39] | 0.14 [0.1–0.21] | 8000 [4900–14, 000] |

The HIV-1 genome comprises 9 genes that code for 15 viral proteins (Frankel & Young, 1998). The structural genes include *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope) (**Figure 2.1-2)**. Accessory genes include *vif*, *vpr*, *vpu*, and *nef*. They generally modulate host responses to the virus; two regulatory genes, *tat*, and *rev* regulate the replication cycle (Strebel, 2013; Strauss & Strauss, 2008).

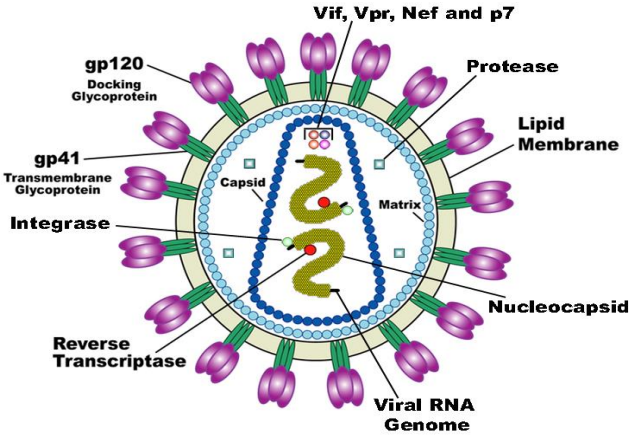



**Figure 2.1-1: HIV structure (Altmann, 2010)**

The polymerase gene (*pol*) encodes Pr160GagPol. Pr160GagPol is cleaved into protease (PR), reverse transcriptase (RT), and integrase (IN) by the viral protease (Freed, 2001). The *gag* gene produces four structural proteins: p24 (viral capsid), p6 and p7 (nucleocapsid proteins), and p17 (viral matrix) (Adamson & Freed, 2007; Freed, 1998).

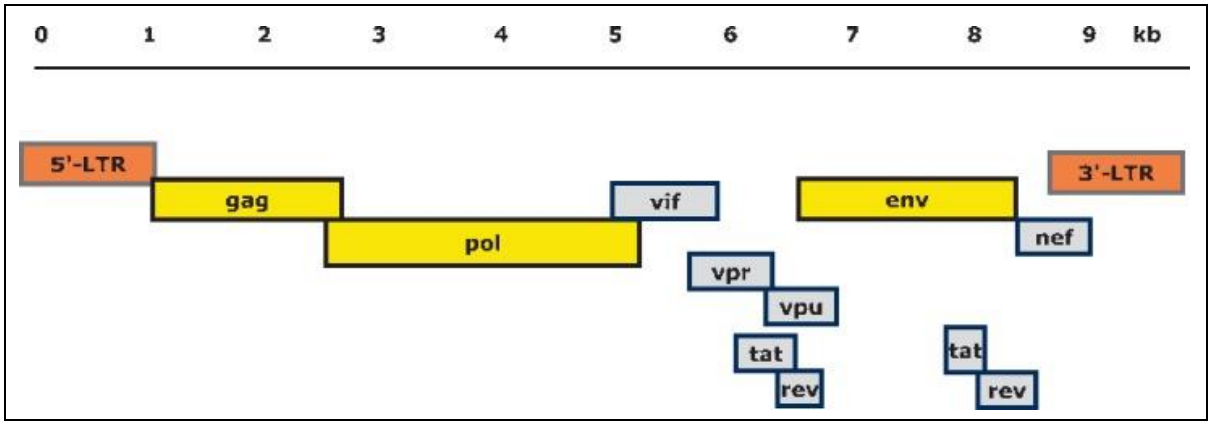



**Figure 2.1-2: Structure and organization of the HIV-1 genome**

(Rainer Seitz, 2016).

The *env* gene encodes gp160 polyprotein precursor. Sundquist & Kräusslich, (2012) and Buchschacher, (2003), found that the glycoproteins gp 120 and gp 41 are made from gp 160. The gp120 protrudes from the envelope's surface, making it easier to adhere to CD4. In addition, the viral lipid envelope surrounds an internal protein layer called the matrix (p17), which is derived from the *gag* polyprotein (p55) following proteolytic processing and is anchored to the internal surface of the virus envelope (Aloia *et al.*, 1993; Checkley *et al.*, 2011). As a result, targeting viral proteins and enzymes is the primary strategy adopted to develop ARVs currently used to treat HIV-1 infection.

### 2.1.2. HIV Lifecycle

#### 2.1.4.1. Attachment and Fusion

CD4 T lymphocytes and macrophages are primary targets of HIV infection (Douek *et al*., 2002). **Figure 2.1-3** shows that a conformational change on gp120 aids in binding the coreceptors, CCR5 or CXCR4 (Engelman & Cherepanov, 2012). This binding induces a conformational change on gp41 resulting in membrane fusion where the virus is internalized by endocytosis (Miyauchi *et al.*, 2009). Then, viral components are released to the cytosol of the host cell (Chan *et al*., 1997; Esté & Telenti, 2007).

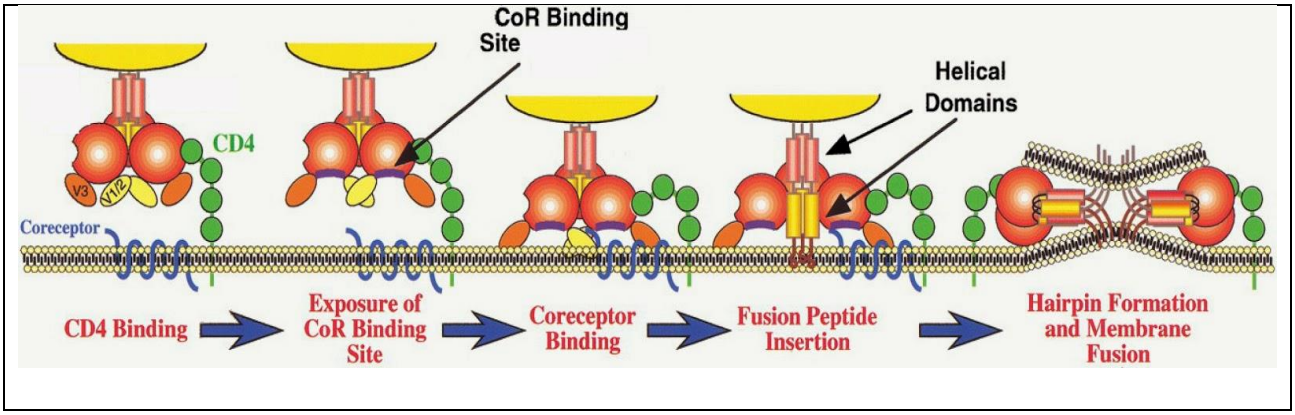



Figure 2.1-3: Attachment and Fusion of HIV

#### 2.1.4.2. Uncoating and Reverse Transcription

Inside the cytoplasm, HIV releases its RNA and enzymes (RT, IN, and PR) through a process termed uncoating ( Miyauchi *et al*., 2009; Fassati & Goff, 2001). Then, the RT enzyme performs the reverse transcription of viral RNA through its three domains/subunits in three consecutive steps, including the conversion of ssRNA to ssDNA (by reverse transcriptase domain), conversion of ssDNA to dsDNA (by polymerase domain), and final degradation of the prior RNA template (by RNase H domain) (Cimarelli & Darlix, 2014). The RT enzyme's lack of endonuclease activity has contributed to a high rate of mutation and is responsible for primary resistance to ARVs ( Schinazi *et al.*, 2013; Menéndez-Arias, 2009).

#### 2.1.4.3. Integration

As shown in **Figure 2.1-4**, integration takes place once the reverse transcription process is completed, and a preintegration complex (including the integrase, proteins, cellular proteins, and viral dsDNA) is formed in the cytosol and translocated to the nucleus with the aid of host proteins such as transportin-SR2 (Miller *et al.*, 1997). Then, dsDNA is integrated into the host chromosomal site with active transcription by the enzyme integrase (Wang *et al*., 2007).

#### 2.1.4.4. Transcription and replication

The host cell molecular machinery performs the transcription and replication of proviral DNA, DNA-dependent RNA polymerase II (pol II) **(Figure 2.1-4).** First, host pol II and viral transcription activator (tat) is involved in the transcription process (Carine, Sophie & Alessandro, 2013). Then, post-translational modifications are performed once the viral RNA is made (Karn & Stoltzfus, 2012). Finally, the enzyme protease cleaves *gag* and Gag-pol polyproteins, which facilitates the assembly and formation of mature infectious virions (Kohl *et al*., 1988).

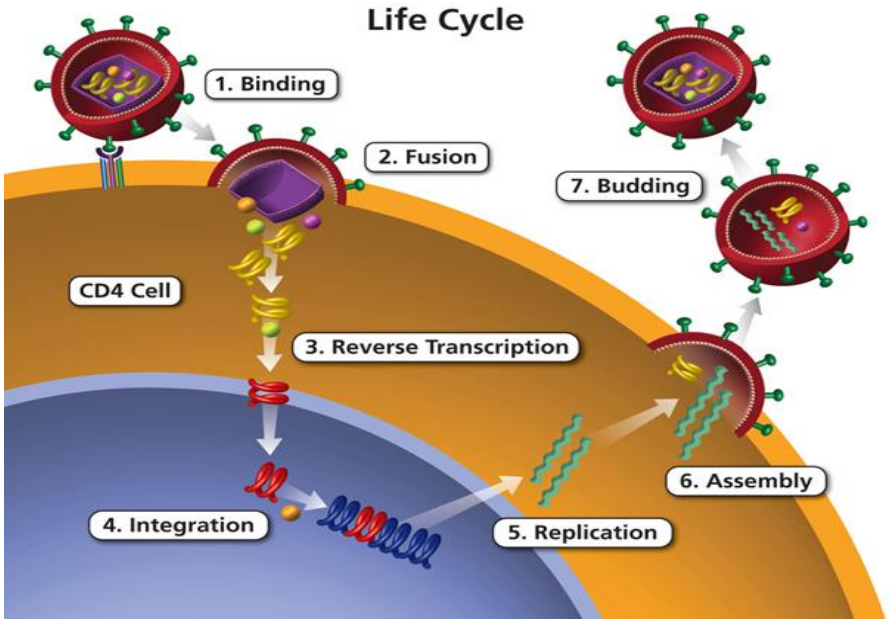



Figure 2.1-4: Life cycle of HIV
(AIDS info.nih.gov, 2018)

### 2.1.3. Pathogenesis and Natural History of HIV

Indirect and direct mechanisms of injury underlie the pathogenesis of HIV infection. Indirect injury is associated with immune suppression leading to opportunistic infections. HIV affects all elements of the immune system. CD4 depletion occurs through syncytium formation, apoptosis, and autoimmunity (Hazenberg *et al.*, 2003; Scheller & Jassoy, 2001).

The depletion of CD4 occurs due to the elimination of HIV-infected cells by virus-specific immune responses, loss of plasma membrane integrity because of viral budding, and interference with cellular RNA processing (Brenchley *et al*., 2004; Grossman *et al*., 2002). Furthermore, HIV indirectly causes a decrease in CD4 cell count by inducing cytomorphological changes (cytopathic effects), such as syncytium & plaque formation, cell transformation or lysis, apoptosis, and autoimmunity. Thus, not only does the virus destroy and disrupt the immune system, but it can also manipulate the immune system to its replicative advantage.

A decline in immune status parallels the decline in CD4 number and function, which limits the immune responses to intracellular infections and malignancy, resulting in the occurrence of different opportunistic infections (Bowen *et al.*, 2016; Capriotti, 2018; Gray & Cohn, 2013; Ismail & Lee, 2011; Miceli *et al.*, 2011; Tan *et al.*, 2012). HIV also causes direct injury to different body parts by attacking target cells. For example, it causes encephalopathy and peripheral neuropathy (Evans *et al*., 2011); it causes HIV-associated nephropathy (Wyatt

& Klotman, 2009). In addition, HIV causes cardiomyopathy (Barbaro, 2005), hypogonadism (Ashby *et al.*, 2014; Rochira & Guaraldi, 2014), dysmotility, and malabsorption (Nemechek *et al.*, 2000).

HIV infection follows the following stages of progression: primary HIV infection, asymptomatic stage/clinical latent stage, and AIDS. **Figure 2.1-5** shows that the initial stage following HIV infection is called primary HIV infection. Patients develop nonspecific 'flu-like symptoms during this stage, which can not be used to directly diagnose HIV infection because the symptoms are nonspecific. These symptoms include fever, fatigue, pharyngitis, lymphadenopathy, and rash (Maartens *et al.*, 2014; Winslow & Kerdel, 2015).

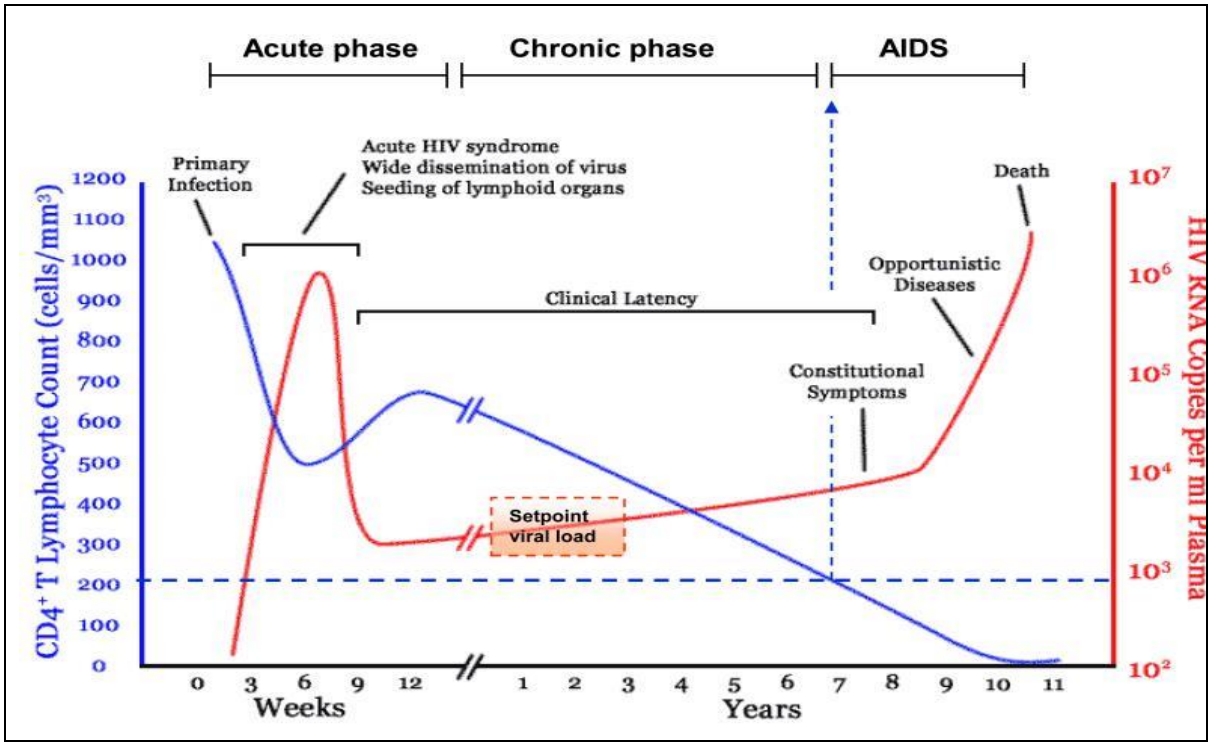


Figure 2.1-5: Stages of HIV infection (An & Winkler, 2010)

During primary infection, the patient could test negative for HIV-specific antibodies despite the presence of infection (Overbaugh & Morris, 2012). Thus, antibody testing should be repeated after 3 months. This stage is called the window period and is characterized by a very high viral load signifying extreme infectivity despite false-negative antibody results (Daar *et al.*, 2008; Rangsin *et al.*, 2004). Clinical management of patients during this time includes symptomatic treatment, counseling for risk reduction, and repeat antibody testing. Patients are most likely to transmit HIV during the early stage of infection (Herbert *et al.*, 2014). The second stage is termed the asymptomatic stage/latent infection. The infected individual may not show symptoms that could last up to 10 years until it presents with constitutional symptoms

defining AIDS. Then, the individual's immune system declines while the replication of the virus continues. Finally, after 8-10 years, various opportunistic infections manifest as AIDS (Melhuish & Lewthwaite, 2018; Sabin & Lundgren, 2013).

## 2.2. Molecular biology of retroviral enzymes

### 2.2.1. Reverse transcriptase (RT)

Reverse transcriptase is encoded by a gene called *pol*, which is found on the *gag* gene in all retroviruses (Götte *et al*., 1999). The viral protease cleaves a Gag-Pol polyprotein to generate HIV reverse transcriptase (Hughes, 2014). Reverse transcriptase in the form of a heterodimer consists of two subunits: p66 and p51. The p66 subunit is 560 amino acids long, while p51 comprises the first 440 amino acids of p66 (di Marzo Veronese *et al*., 1986; Lightfoote *et al*., 1986; Seniya *et al*., 2015).

RNase H and Polymerase are the two domains that make up the p66 subunit. Depending on the nucleic acid substrate, the active sites of RNase H and polymerase consist of 17 to 18 base pairs apart along with the nucleic acid (Jacobo-Molina *et al*., 1993; Sarafianos *et al*., 2001). The polymerization is proceeded by three aspartic acid residues (Asp-110, Asp-185, and Asp-186) that help in the position of two divalent metal ions ($Mg^{+2}$) (Götte *et al*., 1999; Hughes, 2014). In addition, the active site of RNase H contains four amino acid residues (Asp-443, Glu-478, Asp-498, and Asp-549) that help to place two $Mg^{+2}$ ions in a way that they do not form close contact with the RNA strand, that's why RNase H is unable to break the nucleotide (Gao *et al*., 1998; Ghosh *et al*., 1996; Powell *et al*., 1997).

The NNRTI pocket is composed of hydrophobic and hydrophilic residues. The hydrophobic residues are (Tyr-181, Tyr-188, Phe-227, Trp-229, Tyr-232, and Tyr-318 of p66) while the hydrophilic residues are (Lys-101, Lys-103, Ser-105, Asp-192, Glu-224 and His-235 of p66 and Glu-138 of p51) (Sluis-Cremer *et al*., 2004). Thus, the solvent-accessible entrance is formed by Leu-100, Lys-101, Lys-103, Val-179, Tyr-181, and Glu-138 (**Figure 2.2-1**).

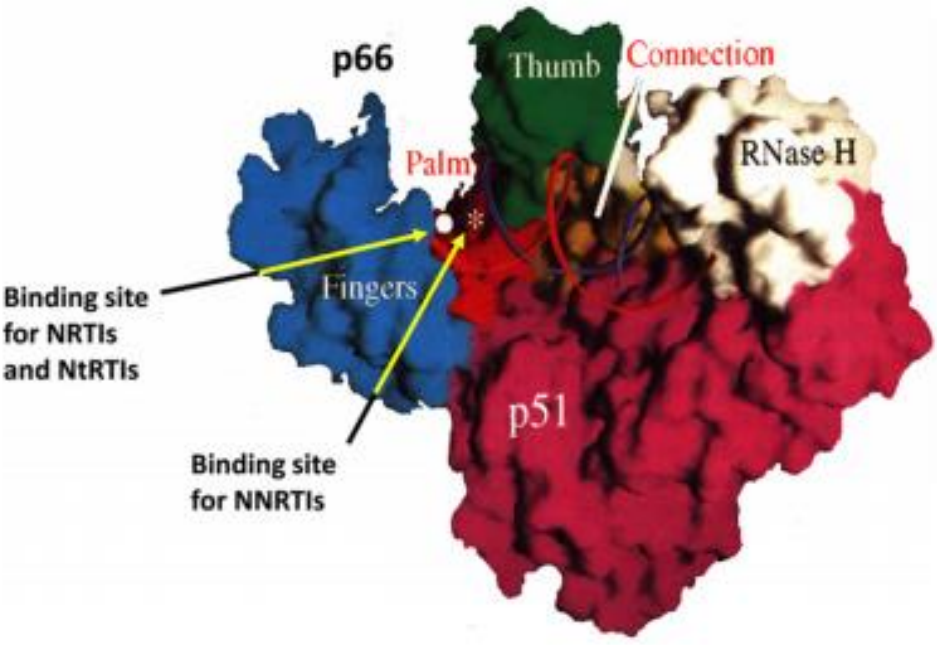



Figure 2.2-1: The NRTIs and NtRTIs binding sites with HIV reverse transcriptase along with the NNRTIs binding site (Jacobo-Molina *et al*., 1993)

### 2.2.2. HIV Integrase

HIV integrase is a polynucleotide transferase protein with a molecular weight of 32 kDa. As depicted in **Figure 2.2-2,** integrase is made up of 3 structural and functional domains: a zinc-binding domain (N-terminal), a catalytic domain (core), and a DNA-binding domain (C-terminal). The N-terminal (residues 1–50) contains a zinc-binding HHCC motif that promotes the production of tetramers, which are the enzyme's active form (Hajimahdi & Zarghi, 2016; Zheng *et al*., 1996). The C-terminal domain (CTD) binds to DNA in a nonspecific manner consisting of amino acids from 213 to 288. The catalytic domain (residues 50 to 212) has a highly conserved catalytic triad of DDE motif, which coordinates divalent metal ions ($Mn^{+2}$ or $Mg^{+2}$) and contains residues Asp-64, Asp-116, and Glu-152 (Kulkosky *et al*., 1992).

For activity, integrase, like most nucleotide transferase enzymes, needs two divalent metal ions bound in the active site (Quashie *et al*., 2012). Mutagenesis of these residues has been observed to fully eliminate all catalytic activity (Gerton *et al*., 1998). The phosphodiester cleavage and bond formation processes catalyzed during the integration sequence require these divalent metal ions. According to some research, these metal cofactors are divalent magnesium ions (Ding *et al*., 1995; Hsiou *et al*., 1996). The catalytic domain includes the enzyme active

site, although the zinc-binding and DNA binding domains are required for full functional activity (Engelman *et al*., 1993)

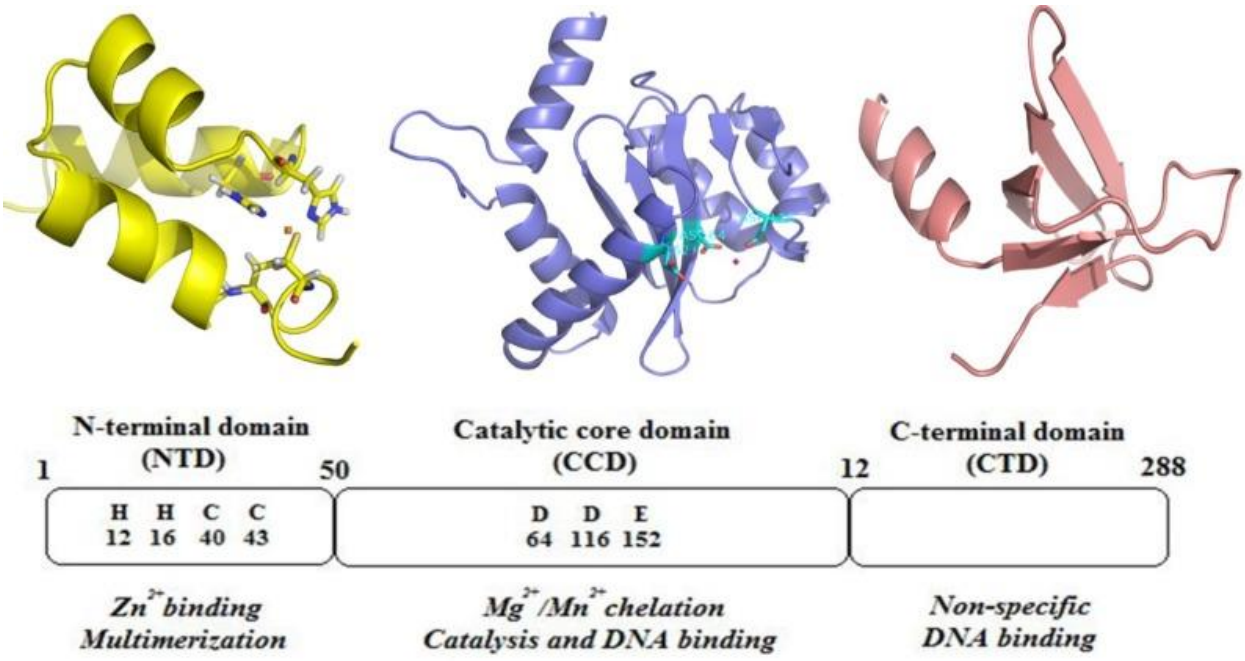



Figure 2.2-2: Structural domains of integrase IN (Hajimahdi & Zarghi, 2016)

### 2.2.3. HIV Protease

The HIV protease enzyme is a symmetrical dimer of two identical protein units, each consisting of 99 amino acids. Each subunit of the homodimer protein contains a catalytic aspartic acid Asp-25. Thus, the amino acid sequence of HIV protease is PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMSLPGRWKPKMIGGIGGFIKVR QYDQILIEICGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNF (Ishima *et al*., 2001). The amino acids Asp-25, Thr-26, and Gly-27 are located on the floor of the active site and provide a flap to act as the ceiling (Coop, 2019)**.** During virion maturation and retrovirus multiplication, the enzyme cleaves various peptide bonds in viral polypeptides, including gag, gag-pol, and polyproteins, but, crucially, it cleaves bonds between proline and an aromatic residue (phenylalanine or tyrosine) **(Figure 2.2-3).** As the viral infectivity depends on protease enzyme activity, it is a potential target for anti-AIDS drugs research (Lv *et al*., 2015).

The confirmation of the flexible region, which can be open, semi-open, or closed, helps the substrate enter the active site (Hornak *et al*., 2006). In addition, the intermolecular bonding between the two subunits helps to stabilize the subunits. Most of the protease interface is made up of the four anti-parallel beta-strands (Sluis-Cremer & Tachedjian, 2002). As shown in

**Figure 2.2-3**, the active site has eight binding subsites (S1, S2, S3, S4, S4′, S3′, S2′, and S1′) that bind to the P1, P2, P3, P4, P4′, P3′, P2′, and P1′ residues of an octa-peptide substrate**.** The Phe-Pro or Tyr-Pro sequences are recognized by HIV-1 protease as retrovirus-specific cleavage sites (Andersson *et al*., 2003).

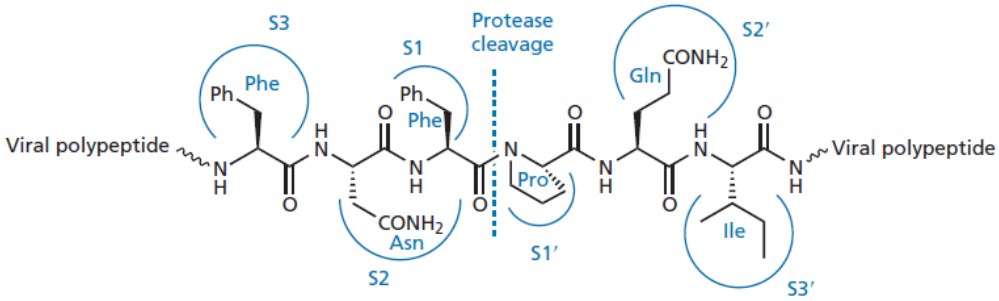



**Figure 2.2-3: Structure of the substrate and the binding subsites (Coop, 2019).**

The S1 subsite is relatively large and hydrophobic and contains Ile-50, Ile-84, Val-82, Thr-80, and Pro-81 (Figure 2.2-5). The S2 subsites are relatively small and hydrophobic compared with the S4 and S3 subsites and are predicted to accommodate smaller hydrophobic P2 residues (Bhhatarai & Garg, 2008). The S2 subsite contains residues like Asp-29, Asp-30, Ala-28, Ile-47, Gly-48, and Ile-50. The S3 subsite is usually large and, similar to S4, is partly exposed to solvent at the enzyme's surface. The S3 subsites of HIV-1 contain residues like Arg-8, Gly-48, Leu-23, Val-82, Thr 80, and Pro-81 (Thr-80’ and Pro-81’ are part of S3 and S1 subsites). The S4 subsite is close to the protein surface and is more hydrophobic **(Figure 2.2-4)**. The residues at subsite S4 are Asp-30, Met-46, Ile-47, Gly- 48, and Gln-58 (Babine & Bender, 1997; Bhhatarai & Garg, 2008).

The aspartic acids Asp-25 and Asp-25′ on the floor of the active site are involved in the catalytic mechanism (Coop, 2019). Most of the inhibitors are designed to imitate the substrate transition state of HIV protease. The inhibitor's OH group forms hydrogen bonds with the COOH group of Asp-25 and Asp-25′ in the protease active site residues. Therefore, the residues of HIV protease that interact with the inhibitors, such as Gly-27, Asp-29, Asp-30, and Gly-48, are highly conserved. The mutations that accumulate the drug resistance affect the structure of HIV protease, resulting in treatment failure (Lv *et al*., 2015).

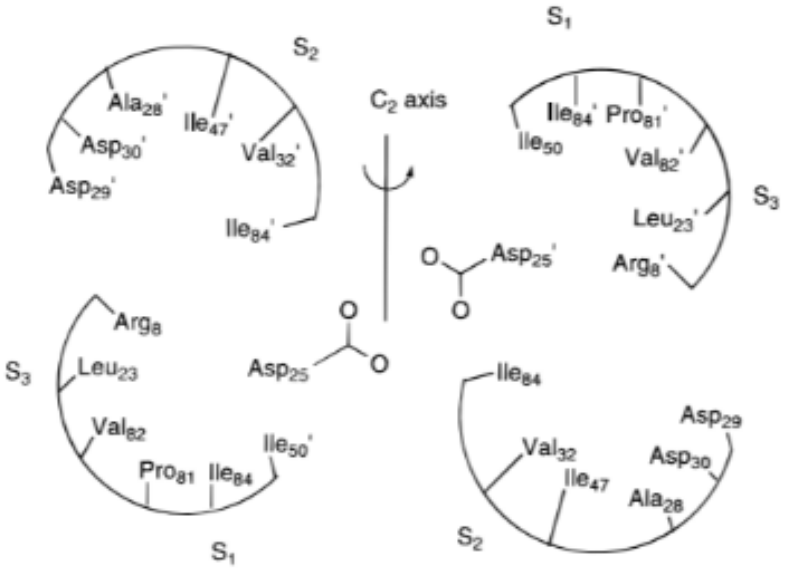



Figure 2.2-4: Amino acid residue that forms binding sites of HIV-1 protease

## 2.3. Antiretroviral drugs and resistance

Antiretroviral drugs can be classified as nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), and entry inhibitors. As depicted in **Figure 2.3-1,** antiretroviral drugs target the different stages of viral replication.

### 2.3.1. Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs)

Nucleoside/tide reverse transcriptase inhibitors (NRTIs) include zidovudine (AZT/ZDV), lamivudine (3TC), tenofovir (TDF) and abacavir (ABC) (Erik, 2010). The chemical structure of these drugs is depicted in **Figure 2.3-2** (Kakuda, 2000). All NRTIs are prodrugs that will be converted to active triphosphorylated forms by cellular enzymes (Kakuda, 2000), except TDF, which requires only two phosphorylation steps (Chapman *et al*., 2003). Bioactivation of NRTIs is carried out by several cellular enzymes (cytoplasmic or mitochondrial kinases and phosphotransferases), which sequentially transform the parent drug to monophosphate diphosphate, and finally, active triphosphate forms (Arts & Hazuda, 2012; Erik, 2010; Othumpangat & Noti, 2018). The pharmacologically active moiety for all NRTIs is an intracellular 5'-triphosphate metabolite. Active NRTI triphosphate competitively binds to the reverse transcriptase enzyme (DNA polymerase γ) active site and terminates the DNA chain elongation step (Arts & Hazuda, 2012; Cihlar & Ray, 2010; Kakuda, 2000).

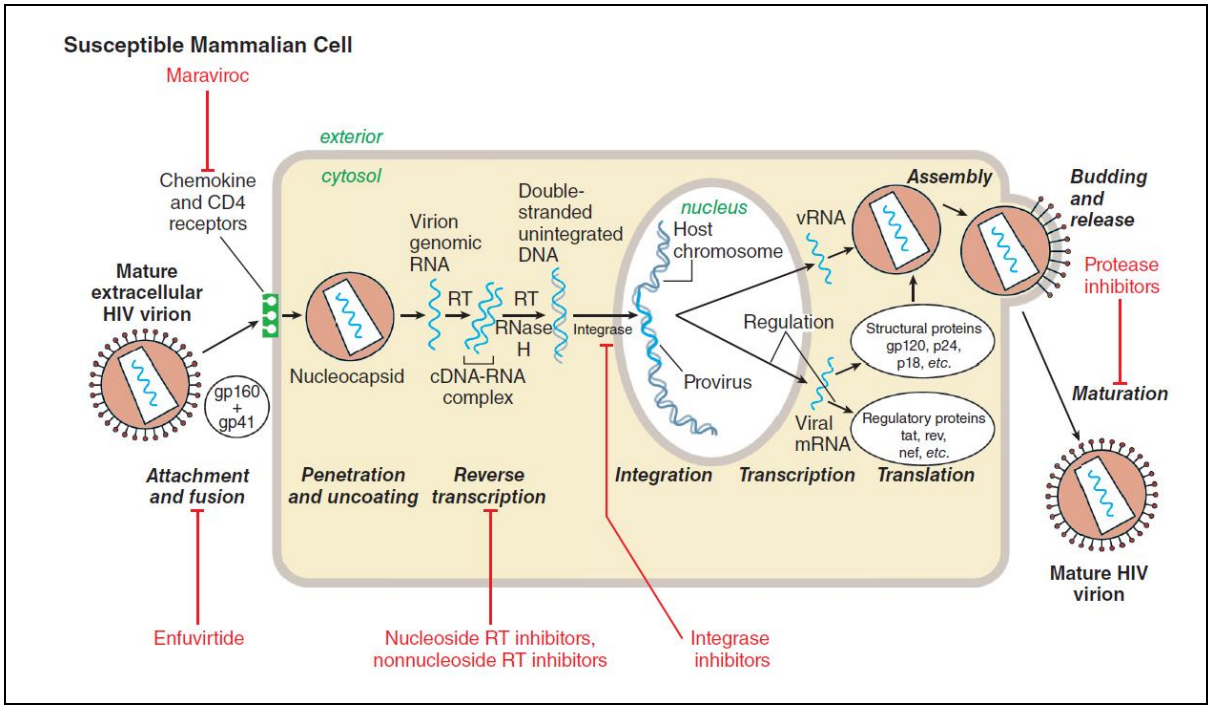



Figure 2.3-1: Life cycle of HIV and the site of action of antiretroviral drugs (Brunton *et al.*, 2017)

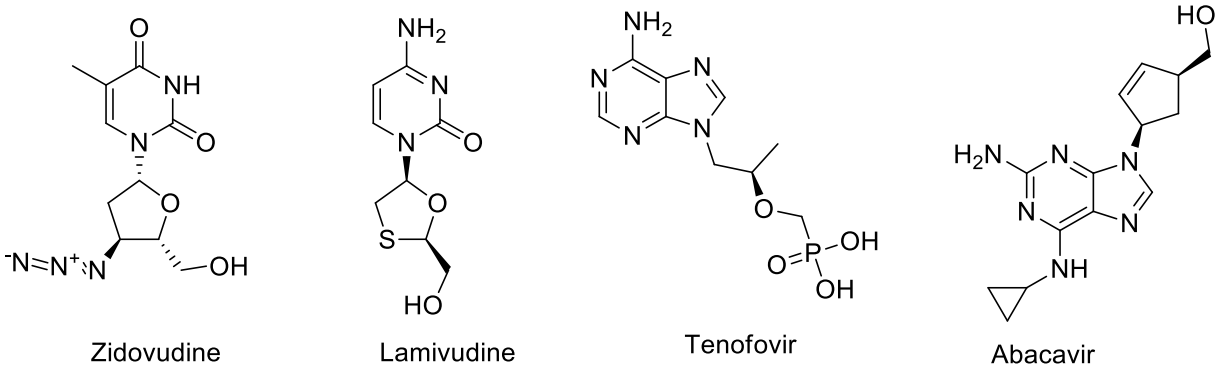



Figure 2.3-2: Chemical structures of nucleoside/tide reverse transcriptase inhibitors

The toxicities of NRTIs could be related to the binding of some drugs to mitochondrial DNA polymerase γ, which could lead to the inhibition of mitochondrial DNA synthesis and hence mitochondrial toxicity (Lee *et al.*, 2003). Those with low affinity for DNA polymerase γ, such as 3TC, FTC, and TDF, do not cause mitochondrial toxicity, such as peripheral neuropathy, pancreatitis, lipoatrophy, hepatic steatosis, and myopathy (Arts & Hazuda, 2012; Erik, 2004). Stavudine (d4T) and didanosine (ddI) are strongly associated with metabolic toxicity and long-term complications. Hence, countries have withdrawn both drugs from their national ARV medication list (World Health Organization, 2013). The other toxicities of

NRTIs include anemia, neutropenia, thrombocytopenia, lactic acidosis, and peripheral neuropathy (Foster & Lyall, 2008; Moyle, 2000; Russell *et al*., 2008). The lack of endonuclease activity contributes to a mutation during reverse transcription. As a result, HIV cannot correct incorporated nucleotides, which causes the translation of amino acids from the changed nucleotide sequence, resulting in the formation of proteins with different shapes and structures. Thymidine analogue mutations (TAMs) decrease susceptibility to ZDV, d4T, ABC, and TDF (Marcelin *et al*., 2004). Mutations selected by regimens lacking thymidine analogue (non-TAMs) include M184V alone, M184V + K65R, or L74V+ K65R, which causes intermediate resistance to TDF, ABC, 3TC, and FTC and increased susceptibility to ZDV (Inzaule *et al*., 2016; Miller, 2004). The M184V mutation is selected by lamivudine, and it reduces viral fitness (Gallant, 2006).

### 2.3.2. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) include nevirapine (NVP), efavirenz (EFV), etravirine, rilpivirine, and delavirdine (Arts & Hazuda, 2012). **Figure 2.3-3** shows the chemical structure of these drugs. They are non-competitive inhibitors of reverse transcriptase enzymes (Sluis-Cremer & Tachedjian, 2008), which bind to the hydrophobic pocket in the p66 subunit and cause conformational change in the enzyme (**Figure 2.3-4**) (Béthune, 2010).

One of the common toxicities of NNRTIs is rash, which starts to manifest in the first month of treatment. Severe rash and hepatotoxicity leading to treatment discontinuation are frequently noted with NVP. CNS toxicities are common even after the first dose of EFV (Béthune, 2010; Waters *et al.*, 2007).

Amino acid substitution type of mutations at the binding site of NNRTIs lead to resistance. A single point mutation (K103N) has been reported for NVP (Bacheler *et al*., 2000; Torti *et al*., 2001). Cross-resistance among NNRTIs is a major clinical concern, as both NVP and EFV are cornerstones of first-line regimens. This has driven the development of second-generation NNRTIs (etravirine) with an improved resistance profile, with the aim of offering treatment-experienced patients the chance to benefit from the convenient dosing and good tolerance profile of NNRTIs (Béthune, 2010; Minuto & Haubrich, 2008)

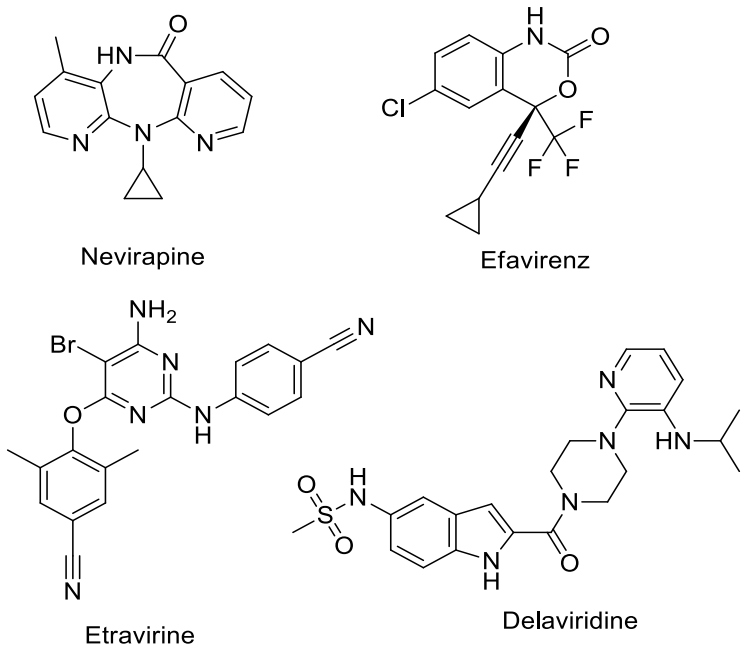



Figure 2.3-3: Chemical structures of Non-Nucleoside Reverse Transcriptase Inhibitors

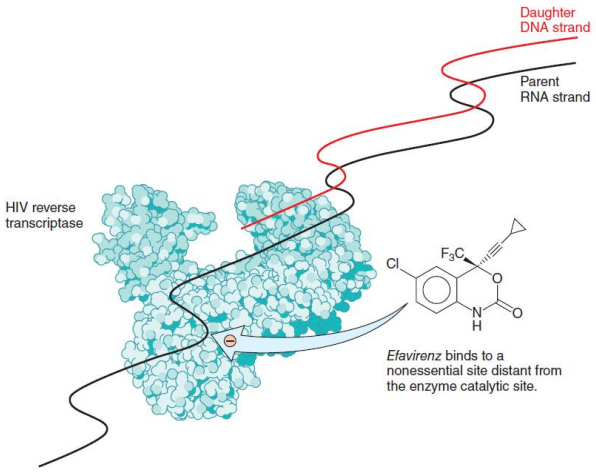



Figure 2.3-4: Molecular Target for Non-Nucleoside Reverse Transcriptase Inhibitors (Brunton *et al.*, 2017)

### 2.3.3. Integrase Inhibitors

Integrase inhibitors are also called integrase strand transfer inhibitors (InSTIs), as they target strand transfer reactions (Arts & Hazuda, 2012; McColl & Chen, 2010) and prevent the integration of dsDNA to the host chromosome (**Figure 2.3-5**). The integrase inhibitors available in clinical use include dolutegravir (DLV) and raltegravir (RAL) (McColl & Chen, 2010). **Figure 2.3-6** depicts their chemical Structure.

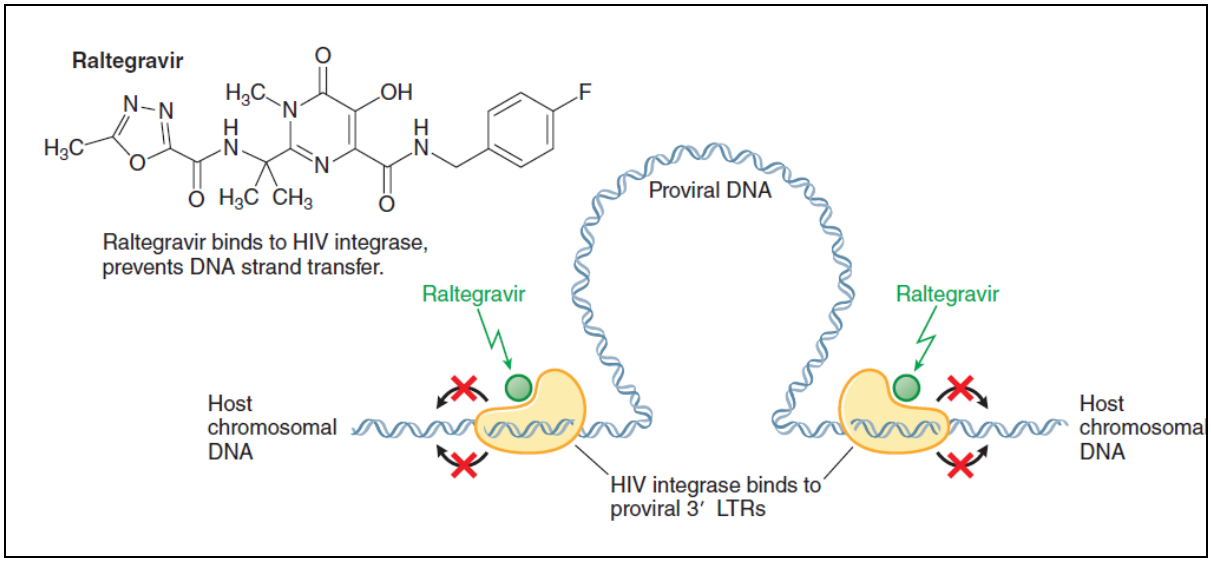



Figure 2.3-5: Mechanism of action of integrase inhibitors (Brunton *et al.*, 2017)

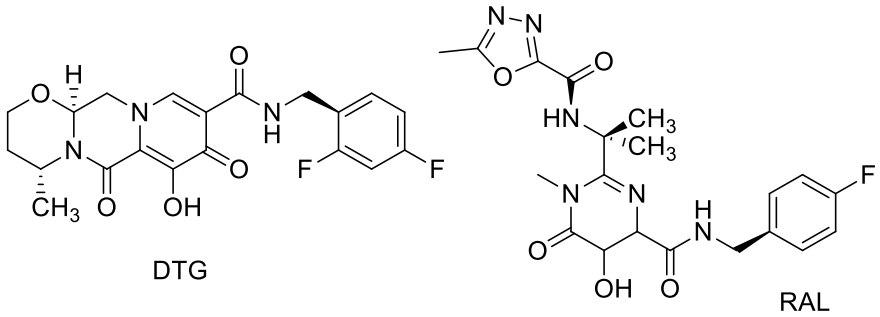



Figure 2.3-6: Chemical structures of integrase inhibitors

Common toxicities of integrase inhibitors include allergic reactions, headache, nausea, diarrhea, hyperglycemia, liver problems, trouble sleeping, tiredness, and changes in body fat (Lepik *et al.*, 2018). Mutations that cause the drug resistance to InSTIs are selected in the integrase-binding site near the amino acid residues essential for the proper functioning of $Mg^{2+}$ cofactors (Arts & Hazuda, 2012; Hare *et al.*, 2010; Hazuda *et al.*, 2000). Primary signature mutations in residues Y143, N155, or Q148 of the integrase gene have been identified to cause development of drug resistance against integrase inhibitors (Fransen *et al.*, 2009; McColl & Chen, 2010).

### 2.3.4. Protease and Maturation Inhibitors

Protease inhibitors include lopinavir (LPV/r), atazanavir (ATV/r)**,** darunavir, ritonavir (r), nelfinavir (NFV), indinavir, saquinavir-SGC, saquinavir-HGC, amprenavir, and fosamprenavir (Arts & Hazuda, 2012; Wensing *et al.*, 2010). **Figure 2.3-7** shows the chemical

structures of LPV and ATV. Protease inhibitors were designed using a computational molecular docking approach, where their Structure was driven by viral peptides, which are substrates of protease (Leung *et al.*, 2000; Wlodawer & Vondrasek, 1998). Protease inhibitors (PI) inhibit the formation of mature infectious virions (**Figure 2.3-8**). Except for nelfinavir, which failed to show increased bioavailability, "boosting" with ritonavir increases plasma concentrations (Hull & Montaner, 2011; Wensing *et al.*, 2010). Boosting indinavir resulted in high peak plasma levels, which caused nephrotoxicity, and as a result, it is not commonly utilized (Leth *et al.*, 2004; Voigt *et al.*, 2002).

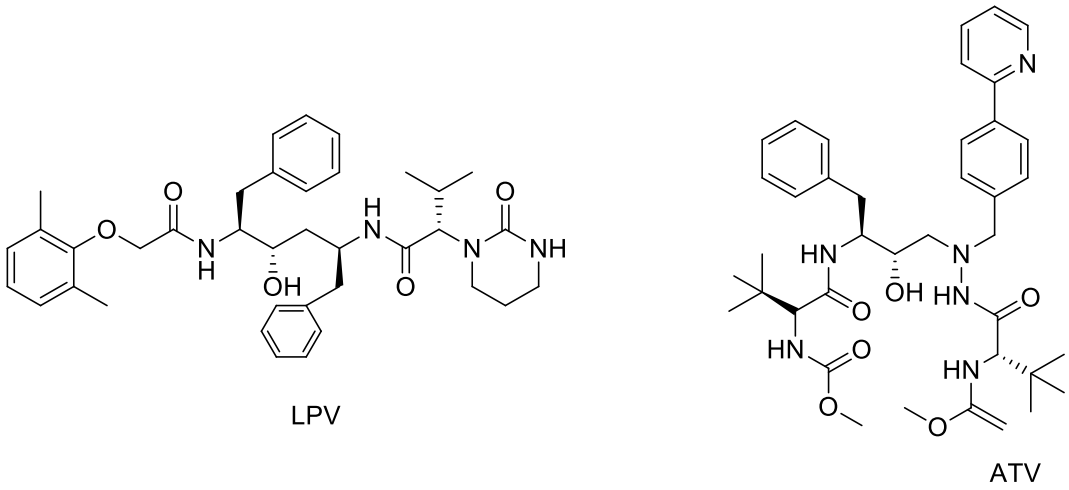



Figure 2.3-7: Chemical structures of protease inhibitors

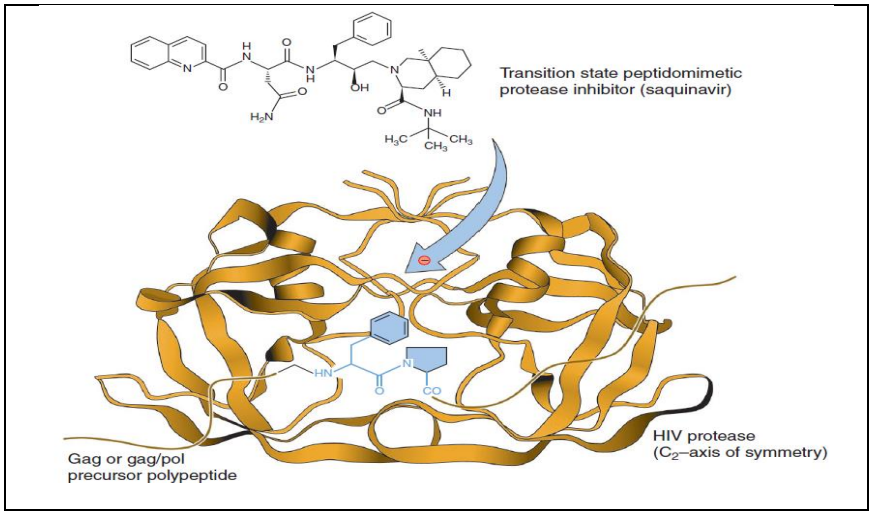



Figure 2.3-8: Mechanism of action of protease inhibitors

The toxicities of protease inhibitors include gastrointestinal intolerance, hepatitis, insulin resistance/diabetes, lipodystrophy, lipid abnormalities, bleeding, and electrocardiographic changes. The most common side effect of PIs is gastrointestinal

intolerance. Nausea and vomiting can be treatment-limiting (Boesecke & Cooper, 2008). These side effects were observed with all protease inhibitors but were most significant with complete dosing of ritonavir (600 mg twice daily), which is no longer used. All PIs can cause liver inflammation, although RTV has been more frequently associated with severe liver toxicity. Elevated liver enzymes can occur at any time during PI treatment (Herman & Easterbrook, 2001). Most protease inhibitors, except unboosted atazanavir, are associated with significant lipid abnormalities, such as hypertriglyceridemia and hypercholesterolemia. Lipodystrophy is a long-term complication of PI therapy, including metabolic (hyperglycemia and hyperlipidemia) and morphologic abnormalities (fat atrophy and fat deposition) (Busti *et al.*, 2004). Changes in body fat distribution have been reported in as many as 80% of patients receiving PIs (Reust, 2011).

Due to the modest size of the viral protease (11 kDa), resistance to protease inhibitors was thought to be unusual when they were first discovered (Arts & Hazuda, 2012). However, the protease gene is highly plastic, and resistance to all approved PIs has been documented, with polymorphisms found in 49 of the 99 codons that make up the protease gene and more than 20 alterations linked to drug resistance (Arts & Hazuda, 2012; Wensing *et al*., 2010). Furthermore, resistance to PIs occurs in stages, with a mutation in the substrate-binding cleft being the most common alteration (Miller, 2001). The enzyme's overall size increases as a result of a resistance-inducing mutation in the binding site. As a result, it decreases the inhibitor's capacity to bind, lowering the drug's susceptibility (Wensing *et al*., 2010). Protease inhibitors drug resistance can also be caused by mutations in the gag protein, a protease substrate (Clavel & Hance, 2004; Dam *et al*., 2009; Gupta *et al*., 2012).

### 2.3.5. Entry and Fusion Inhibitors

HIV-1 infection is a multi-step process involving interactions between viral envelope proteins and cell surface receptors and coreceptors. To promote fusion, HR1 and HR2 domains of gp-41 must interact with each other. The presence of a heterologous protein that can imitate these domains disrupts these viral protein domains' interactions and prevents fusion. Entry inhibitors inhibit the attachment of virus particles to receptors on target cells (Esté & Telenti, 2007). Drugs in this class are divided into fusion inhibitors and CCR5 antagonists. Fusion inhibitors prevent the viral and CD4 membranes from fusing (Tilton & Doms, 2010). In 2003, the first entrance inhibitor, enfuvirtide (T-20), a synthetic peptide corresponding to the two domains, was approved (Arts & Hazuda, 2012; Kilby *et al*., 1998; Lalezari *et al*., 2003; Tilton

& Doms, 2010). Enfuvirtide is a synthetic peptide that has a sequence that is identical to a portion of gp41's HR2 domain and competes with HR1 for binding (Arts & Hazuda, 2012; Kilby *et al*., 1998). However, its use has been restricted due to formulation difficulties and the development of drug resistance (Tilton & Doms, 2010; Wei *et al*., 2002).

The CCR5 antagonists bind to the receptor's transmembrane helices (Dragic *et al*., 2000), causing a conformational shift in the receptor that inhibits HIV-1 envelope recognition (Tilton & Doms, 2010). Vicriviroc, aplaviroc, and maraviroc are three CCR5 receptor antagonists demonstrated to inhibit HIV-1 in humans. However, since 2007, the FDA has only approved Maraviroc (MVC) as the only one (Arts & Hazuda, 2012; Tilton & Doms, 2010). Maraviroc binds to CCR5's hydrophobic transmembrane cavity, causing the chemokine receptor's shape to alter and prevent it from binding to gp120 (Arts & Hazuda, 2012; Dragic *et al*., 2000; Tilton & Doms, 2010). Because MVC targets a host cell protein, the development of drug resistance to it differs from that of other antiretrovirals. Tropism and switching are two possible resistance mechanisms. For example, most people with HIV-1 infection possess viruses that only use CCR5 as a coreceptor for infection in the early stages (Lobritz *et al*., 2010). However, as the infection proceeds, dual tropic viruses that employ the CXCR4 co-receptor begin to arise (Schuitemaker *et al*., 1992).

The CCR5 inhibitors can cause outgrowth of CXCR4 viruses and treatment failure in patients with a mixed population of circulating viruses, CCR5-tropic and CXCR4-tropic viruses (Lobritz *et al*., 2010). As a result, genotypic or phenotypic tropism testing is required before using CCR5 inhibitors and only individuals who have no CXCR4 tropic viruses are eligible for treatment with these medications (Kagan *et al*., 2012).

## 2.4. Discovery of New Antiretroviral Drugs

### 2.4.1. *In vitro* anti-HIV Assays

Natural products or isolated chemicals can be tested in human T-cell lines, including Jurkat, CEM-SS, MT4, H9, and PBMCs, for anti-HIV and cytotoxic activities using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or sodium 3-[1-(phenylamino)-carbonyl]- 3,4-tetrazolium bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) assays (Bahuguna *et al.*, 2017; Szucs *et al.*, 1988). The several assays that could be used to evaluate drug candidates' anti-HIV activity are listed in **Table 2.4-1.** The 50 percent cytotoxic concentration ($CC_{50}$), 50 percent effective concentration ($IC_{50}$), and selectivity index (SI), which is the ratio of $CC_{50}/EC_{50}$, can all be used to express the outcome. As a result, SI

represents the test material's antiviral activity as well as its ultimate toxicity. A high SI value suggests that the test substance is not harmful and has high antiviral activity. The i*n vitro* therapeutic index of drugs being investigated for further preclinical development investigations can be estimated using MTT or XTT (Pauwels *et al*., 1988).

Table 2.4-1: *In vitro* anti-HIV assays

| Target | Assays |
|---|---|
| HIV-1 Replication | MTT |
| | XTT |
| gp120/CD4 intereaction | gp120 ELISA |
| Reverse Transcriptase Enzyme | Radio-active method |
| | Non-radioactive ELISA |
| | RNase H activity |
| Integrase Enzyme | Radio-active method |
| | Non-radioactive ELISA |
| Protease | Flourometric method |
| | HPLC-based method |
| | Pepsin assay (indirect method) |

The effect of natural products or isolated compounds on reverse transcriptase enzymes can be evaluated by using tritium-labeled substrate & poly(rA). p(dT)12-18 as template primer or by using nonradioactive ELISA and/or by ribonuclease H activity (Ahn *et al*., 2004; Li *et al*., 2008; Świderek *et al*., 2012). The effect on integrase enzyme can be evaluated with recombinant HIV-Integrase by using radiolabeled oligonucleotide substrate (Lee-Huang *et al.,* 1995; Pommier & Neamati, 1999) or by using a non-radioactive ELISA kit (Ng *et al.,* 2002; Nutan *et al*., 2013). Activity against protease enzymes can be assayed using 2 direct methods (fluorometric or HPLC method) or an indirect method using pepsin enzyme as a substitute for HIV protease (Yust *et al*., 2004).

### 2.4.2. Computer-aided drug design and discovery (*in silico* analysis)

Computer-aided drug design and discovery (CADD) (*in silico*) research can speed up drug development by minimizing the need for costly laboratory work and clinical trials (Prieto-Martínez *et al*., 2019). For example, CADD is being used to find hits (active drug candidates), select leads (most likely candidates for further evaluation), and optimize leads that transform biologically active compounds into appropriate drugs by improving their pharmacokinetic properties (Gurung *et al*., 2021). Furthermore, computational methods aid in determining a compound's drug-likeness (Ghose *et al*., 1999; Lipinski *et al*., 2001; Veber *et al*., 2002).

Many biological processes, such as signal transmission, cell control, and molecular recognition, rely on macromolecular assemblies such as enzyme-substrate, drug-protein, drug-nucleic acid, protein-nucleic acid, and protein-protein interactions (Gurung *et al*., 2021; Prieto-Martínez *et al*., 2019). To understand the interaction mechanisms and devise therapeutic interventions, molecular docking is essential for determining the binding mode and affinity between constituent molecules in molecular recognition (Lv *et al*., 2015). Molecular docking is a computer-assisted method for determining the kind, strength, and direction of drug and receptor/target interactions (Kitchen *et al.*, 2004). Molecular docking studies can be performed using software such as GOLD from CCDC and GLIDE from Schrodinger, MOE, and Auto Dock Vina from Scripps Research Institute. The more negative the values, the better the binding free energy between the enzyme and the ligands (Granchi *et al*., 2015; Kumar *et al*., 2014; Seal *et al*., 2011).

Molecular docking can be used for three different purposes: first, for hit identification, where docking combined with a scoring function can be used to quickly screen large databases of potential drugs in silico to identify molecules that are likely to bind to the protein target of interest; second, for lead optimization, where docking can be used to predict where and in which relative orientation a ligand binds to a protein; and third, for lead optimization, where docking can be used to predict where and in which relative orientation Third, protein-ligand docking may be used to forecast contaminants that enzymes can degrade, which can then be used to build more potent and selective analogs; and finally, bioremediation, where protein-ligand docking can also be used to predict pollutants that enzymes can degrade.

We used the Molecular Operating Environment (MOE) 2015 docking tool in this research. This tool was chosen from among a variety of accessible resources for docking because of its user-friendly graphical interface. It provides a clear graphical representation of the data by displaying the locations and interactions of ligand and receptor-binding residues. The S-score is a numerical metric used in MOE to prioritize receptor–ligand-binding affinities for all possible binding geometries. The MOE is used in a variety of fields, including structure-based design, fragment-based design, pharmacophore discovery, medicinal chemistry applications, biologics applications, protein and antibody modeling, molecular modeling and simulations, cheminformatics, and QSAR, and the development and deployment of methods. The software calculates the S score by identifying salt bridges, hydrogen bonds, hydrophobic interactions, sulfur-LP, cation-, and solvent exposure. Based on the S score, inhibitor-receptor protein interactions are predicted (Clark & Labute, 2007).

## 2.5. Medicinal Plants with anti-HIV activity

It is estimated that there are more than 350,000 plant species in the world (Christenhusz & Byng, 2016). One-third of these species are found in Africa (Chingwaru *et al*., 2015). As explained in Section 1.1, 54% (20.7 million) of HIV-positive people live in Southern and East African regions, where more than 25% of them do not have access to antiretroviral therapy. As the HIV epidemic still affects many people's lives, there is a high demand for safe, effective, and affordable medicines for use in the treatment of HIV. In addition, with the emergence of drug-resistant HIV variants, there is a greater need to search for effective inhibitors of HIV. The lack of access to care and treatment still pushes the African community to rely on medicinal plants to treat various diseases, including opportunistic infections secondary to HIV. Despite the issue of access, many people are culturally inclined to visit traditional healers who use medicinal plants, as the use of different parts of plants for treating illness is part of African traditional medicine practice (Mahomoodally, 2013). Therefore, screening natural products offers a chance to find compounds that can inhibit the replication of HIV (Narayan *et al*., 2013).

Patients use various plants to treat HIV or opportunistic infections (Bessong & Obi, 2006; Cos *et al*., 2002). **Table 2.5-1** summarizes medicinal plants exhibiting anti-HIV activities. *In vitro* activity testing of several medicinal plants have shown that natural products exert their anti-HIV activity by different modes of action. Some of these plants include *Hypoxis hemerocallidea* and *Sutherlandia frutescens* (Maroyi, 2014), *Plectranthus barbatus*, *Siphonochilus natalensis* (Chingwaru *et al*., 2015; Maroyi, 2014), *Erythrina abyssinica* (Mohammed *et al*., 2012), *Cassia fistula* (Xu *et al*., 1996), *Bulbine alooides* (Klos *et al*., 2009) and *Dodonaea angustifolia* (Asres *et al*., 2001).

Burack *et al*., (1996) reported the anti-HIV activity of *Artemisia annua*, with an $IC_{50}$ of 2.0 µg/ml. In a related study, Gerenčer *et al*., (2006) demonstrated the antiviral and protective effects of *Cassia abbreviata* from the cytopathic effect of HIV. *Cassia abbreviata* displayed an $IC_{50}$ of 102.8 µg/mL. Asres & Bucar, (2005) reported that punicalagin, an ellagitannin isolated from the acetone fraction of *Combretum molle,* inhibits the replication of HIV with a selectivity index (SI) of 16. Justiprocumin B, an active compound isolated from *Justicia gendarussa,* has shown potent activity against HIV with $IC_{50}$ values in the range of 15–21 nM (Jiratchariyakul *et al*., 2001).

Table 2.5-1: List of some plant species exhibiting anti-HIV activities

| Ser. No. | Plant | Plant Part | Phytochemicals with Anti-HIV activity and their action | Reference |
|---|---|---|---|---|
| 1. | *Allanblackia stuhlmannii* (Engl.) | Whole plant | Benzophenone; *In vitro* anti HIV activity | (Fuller *et al*., 1999) |
| 2. | *Annona squamosa* L. | Fruit | Diterpenoids; $IC_{50}$= 0.8 µg/ml; SI > 5; Inhibits HIV-1 replication in H9 lymphocyte cells | (Wu *et al*., 1996) |
| 3. | *Aristolochia manshuriensis* Kom. | Stem | Oxoperezinone; Inhibit HIV-1 replication; $IC_{50}$= 17.5 µg/mL; SI= 1.43 | (Wu *et al*., 2003) |
| 4. | *Avicennia marina* var. rumphiana (Hallier f.) | Seed | Iridoid glycoside; $IC_{50}$ = 0.1 µg/ml; Restricts HIV-1 replication on the early stage of HIV infection. | (Behbahani, 2014) |
| 5. | *Calophyllum lanigerum* Miq. | Whole plant | Calanolide; *In vitro* anti-HIV activity | (Galinis *et al*., 1996) |
| 6. | *Cassine crocea* (Thunb.) C.Presl | Leaf | Glycoside; Potent *In vitro* anti-HIV activity. | (Prinsloo *et al*., 2010) |
| 7. | *Castanospermum austral* A. Cunn. & C. Fraser | Leaf | Alkaloid; *In vitro* anti-HIV activity | (Taylor *et al*., 2016) |
| 8. | *Celastrus hindsii* Benth. | Stem bark | Triterpene; anti-HIV replication activity in H9 lymphocyte cells; $IC_{50}$ =0.8 µg/ml. | (Kuo & Kuo, 1997) |
| 9. | *Detarium microcarpum* Guill. & Perr | Root | Flavonoids; *In vitro* anti-HIV activity | (Mahmood *et al*., 1993) |
| 10. | *Drymaria diandra* Blume | Stem bark | Alkaloid; Anti-HIV effects in H9 lymphocytes; $IC_{50}$ =0.699 µg/mL; SI of 20.6. | (Hsieh *et al*., 2004) |
| 11. | *Euphorbia erythradenia* Boiss. | Aerial part | Triterpene; *In vitro* anti-HIV activity | (Ayatollahi *et al*., 2016) |
| 12. | *Euphorbia neriifolia* L. | Stem bark | Diterpenoids; *In vitro* anti-HIV activity; $IC_{50}$ = 3.58; SI = 8.6 | (Zhao *et al*., 2014) |

| Ser. No. | Plant | Plant Part | Phytochemicals with Anti-HIV activity and their action | Reference |
|---|---|---|---|---|
| 13. | *Homalanthus nutans* (G. Forst.) Guill. | Stem bark | Prostratin; *In vitro* anti-HIV activity | (Cox, 1993) |
| 14. | *Ipomoea cairica* (L.) Sweet | Whole plant | Lignans; *In vitro* anti-HIV activity | (Schröder *et al*., 1990) |
| 15. | *Maytenus macrocarpa* (Ruiz & Pav.) Briq. | Stem bark | Triterpenes; anti-HIV activity in C8166 cells infected with HIV-$1_{MN}$ $IC_{50}$ = 1 µg/mL; SI= 35 | (Piacente *et al*., 2006) |
| 16. | *Mimusops elengi* L. | Bark | Saponin; *In vitro* anti-HIV activity | (Hayashi *et al*., 1995) |
| 17. | *Panax ginseng* C.A. Mey. | Root | Saponin; Inhibit HIV-1 replication; $IC_{50=}$13.4 µg/mL | (Zhang *et al*., 2002) |
| 18. | *Panax zingiberensis* C.Y. Wu & K.M. Feng | Rhizome | Zingibroside; Anti-HIV-1 activity $IC_{50}$ = 91.3 µM; $CC_{50}$ = 46.2 µM) | (Hasegawa *et al*., 1994) |
| 19. | *Rheum tanguticum* Maxim. ex Balf. | Leaf | Glycosides; Moderate anti-HIV activity | (Piacente *et al*., 1996) |
| 20. | *Tripterygium wilfordii* Hook. f. | Roots | Diterpene; anti-HIV replication activity in H9 lymphocyte cells; IC50 = 1 µg/ml | (Ke *et al*., 1992) |
| 21. | *Toddalia asiatica* (L.) Lam. | Root | Alkaloid; *In vitro* anti-HIV activity | (Rashid *et al*., 1995) |
| 22. | *Trigonostemon thyrsoideus* Stapf | Stem | Diterpenoid; Inhibit HIV-1 induced cytopathic effects; $IC_{50}$ = 2.19 µg/mL; TI >90. | (Zhang *et al*., 2010) |
| 23. | *Tripterygium wilfordii* Hook. f. | Roots | Sesquiterpene pyridine Alkaloids; potent anti-HIV activity; $IC_{50}$ = <0.10 µg/mL; TI >1000 | (Duan *et al*., 2000) |

## 2.6. The *Croton* genus

### 2.6.1. Botanical information on *Croton* genus

Members of the Euphorbiaceae family are found mainly in tropical Africa (Radcliffe-Smith, 2001). The family Euphorbiaceae is notable because they produce a milky sap (rich in secondary metabolites, mainly alkaloids and terpenoids) and comprises over 300 genera, of

8,000-9000 species (Wurdack & Davis, 2009). The family members have a diverse distribution, are subclassified into five subfamilies; including Acalyphoideae, Crotonoideae, Euphorbioideae, Oldfieldioideae, and Phyllanthoideae (Govaerts *et al*., 2000; Palgrave, 2002). Members of the Euphorbiaceae family have displayed anti-HIV activity in several experimental studies **(Table 2.6-1).** This study evaluates the antiretroviral efficacy of three African *Croton* species: *C. macrostachyus*, *C. megalocarpus*, and *C. dichogamus.*

The name "*Croton*" is a Greek word referring to thick, smooth seeds, a common feature of most *Croton* species. The genus *Croton* belongs to the *Crotonoideae* subfamily. The genus consists of 1300 trees, shrubs or small trees, with variously stellate hairy or lepidote scales distributed worldwide in the tropics and subtropics (Wurdack & Davis, 2009; Caruzo *et al.*, 2011 and Berry *et al.*, 2005). *Croton* species are widely distributed in warm tropical regions like tropical America, India, and Africa. In addition, a wide diversity of *Croton* plants is reported in Madagascar, West Indies, and southern Brazil. The leaves of *Croton* plants are alternate, sometimes opposite, rarely whorled, simple, and usually have two glands at the top of the petiole. Contact with some of these plant leaves can cause dermatitis. Their fruits occur as three-lobed capsules, while seeds of others are reported to be tumor promoters (Palgrave, 2002 and Mabberley, 2008).

Table 2.6-1: Plants from the *Croton genus* that have displayed anti-HIV activity

| **Ser. No.** | **Plant** | **Plant Part** | **Reference** |
|---|---|---|---|
| 1. | *Croton billbergianus Müll. Arg.* | Trunk | (Matsuse *et al*., 1998) |
| 2. | *Croton gratissimus Burch.* | Leaf | (Ali *et al*., 2002) |
| 3. | *Croton tiglium L.* | Seed | (El-Mekkawy *et al*., 2000) |
| 4. | *Croton zambesicus Müll. Arg.* | Seed | (Ali *et al*., 2002; Hussein *et al*., 1999) |
| 5. | *Croton tiglium* | Seed | (El-Mekkawy *et al*., 1998, 2000) |
| 6. | *Croton megalobotryus* | Bark | (Tietjen *et al*., 2016) |

### 2.6.2. Ethnomedicinal uses of *Croton* genus

The common ethnomedicinal uses of the different *Croton* species are summarized in **Table 2.6-2**.

Table 2.6-2: Ethnomedicinal uses of the *Croton* genus

| Scientific Name (Other names, region) | Reported use | References |
|---|---|---|
| *Croton alienus* (Kenya) | Antimicrobial activities | (Ndunda *et al.,* 2013) |
| | Antileishmanial activities | |
| | Antiplasmodial activities | |
| *Croton arboreous Millsp.* ("Cascarillo", Mexico) | Anti-inflammatory | (Aguilar-Guadarrama & Rios, 2004) |
| *Croton bonplandianum* | Antioxidant activity | (Keerthana *et al*., 2013) |
| | Antimicrobial activity | (Jeeshna *et al.,* 2011) (Burgos *et al*., 2015; Khanra *et al*., 2016) |
| | Wound healing | (Divya *et al.,* 2011) |
| | Antidiabetic | (Qaisar *et al.,* 2014) |
| *Croton californicus Müller Arg.* (California, U.S.A.) | Pain reliever for rheumatism. | (Williams *et al*., 2001, 2005) |
| *Croton cajucara Benth.* ("Sacaca", Peru and Brazil) | Antioxidant activity- reduces hepatic oxidative stress | (Rodrigues *et al*., 2012) |
| | Antimicrobial Activity | (Alviano *et al*., 2005; Freires *et al*., 2015) |
| | Antiprotozoal-Antileishmanial activity | (Lima *et al*., 2015; Rosa *et al*., 2003) |
| | Anti-ulcer activity | (Brito *et al*., 1998; De Paula *et al*., 2008) |
| | Antinociceptive activity | (Campos *et al*., 2002; Rodrigues *et al*., 2012; Rosa *et al*., 2003) |
| | Antihyperlipidemic activity | (Bighetti *et al.,* 2004) |
| *Croton celtidifolius Baill.* ("Sangue-de- adave", Brazil) | Anti-inflammatory and antioxidant | (Nardi *et al*., 2003, 2007) |
| | Antinociceptive effect | (DalBó *et al*., 2005, 2006; Nardi *et al*., 2006) |
| | Antiplatelet activity | (Neiva *et al*., 2008) |
| | Central nervous system activity, Neuroprotective activity | (Assis *et al*., 2014; Moreira *et al*., 2010) |
| | Anticancer activity | (Biscaro *et al*., 2013) |
| | Cardioprotective activity | (Hort *et al*., 2012) |
| *Croton draco Cham. & Schltdl* (Mexico and Central America) | Cough, flu, diarrhea, and stomach ulcers, and wound healing, anti-septic | (Murillo *et al.,* 2001) |

| Scientific Name (Other names, region) | Reported use | References |
|---|---|---|
| *Croton eluteria Bennett.*, Cascarilla", *Syn. C. eluteria* (L.) Wright, West Indies, and Northern South America-Bahama Island) | Bronchitis, diarrhea, and dysentery<br>Antipyretic and Antimalarial | (Vigor *et al.*, 2001) |
| *Croton echinocarpus (Brazil)* | Anti HIV activity | (Ravanelli *et al.*, 2016) |
| *Croton kongensis Gagnep.* (*"*Plao Ngeon" or "Plau Noi"; Thailand; China) | Dysmenorrhoea | (Thongtan *et al.*, 2003) |
| | Antimycobacterial activity | |
| *Croton lechleri* (one of the "sangre-de-drago" plants; Ecuador and Peru) | Wound healing, purgative and tonic | (Cai *et al*., 1991, 1993) |
| | Anticancer and Antioxidant activity | (Alonso-Castro *et al*., 2012; Lopes *et al*., 2004; Montopoli *et al*., 2012; Pieters *et al*., 1993; Rossi *et al*., 2003) |
| | Antisecretory/Antiulcer and Antidiarrheal activity | (Froldi *et al*., 2009; Tamariz Ortiz *et al*., 2013; Tradtrantip *et al*., 2010) |
| | Immunomodulatory activity | (Risco *et al*., 2003) |
| | Anti-inflammatory activity | (Perdue *et al.*, 1979) |
| *Croton macrostachys Hochst. ex* A. Rich *ex* Delile (Syn. *C. macrostachys* var. *mollissimus* Chiov.; (Madagascar, Somali, Sudan, Eritrea, Kenya, Angola Guinea, Liberia, Malawi, Zambia, and Zimbabwe) | Antidiabetic, purgative | (Obey *et al*., 2016b; Salatino *et al*., 2007) |
| | Antimicrobial activity | (Kalayou *et al*., 2012; Tene *et al*., 2009; Voukeng *et al*., 2016) |
| | Antimalarial activity | (Bantie *et al.*, 2014) |
| | Ant mycobacterial activity | |
| | Antidiabetic | (Arika *et al*., 2015) |
| | Anticancer activity | (Morris Kupchan *et al.*, 1969) |
| | Purgative and anti-inflammatory activity | (Mazzanti *et al*., 1987) |
| *Croton malambo Karsten* ("Palomatias", "Torco"; Venezuela and Colombia) | Antinociceptive and anti-inflammatory | (Suárez *et al*., 2003) |
| | Antibacterial activity | (Suárez *et al.*, 2008) |
| | Antioxidant activity | (Colorado *et al.*, 2010) |
| *Croton megalobotrys* (Somalia, Kenya, Uganda, the Democratic Republic of the Congo, Rwanda, Burundi, Tanzania, Malawi, Zambia, and Mozambique.) | Antibacterial activity | (Selowa *et al.*, 2010) |
| | Anti-HIV Activity | (Tietjen *et al*., 2016) |
| | Sexually transmitted illness | (Ndubani & Höjer, 1999; Tietjen *et al*., 2018) |
| | Anti-plasmodial and Antioxidant | (Abosi & Majinda, 2015) |
| *Croton megalocarpus* | Antioxidant activity | (Kivevele *et al.*, 2011) |
| | Antibacterial activity | (Kariuki *et al.*, 2014) |

| Scientific Name (Other names, region) | Reported use | References |
|---|---|---|
| (Kenya Congo and Mozambique, Malawi and Zimbabwe) | | |
| *Croton nepetaefolius Baill.*, ("Marmeleirovermelho". Brazil) | Antimicrobial and insecticidal activity | (Araújo *et al*., 2006; Carneiro *et al*., 2011; Gomes *et al*., 2013; Morais *et al*., 2006; Sá *et al*., 2012) |
| | Antifungal activity | (Fontenelle *et al*., 2008) |
| | Antispasmodic activity | (Magalhaes *et al*., 2004) |
| | Antinociceptive activity | (Abdon *et al.,* 2002) |
| | Antioxidant activity | (Selene *et al.,* 2006) |
| | Cardiovascular – vasorelaxant activity | (Lahlou *et al.*, 1999) |
| | Antihypertensive activity | (Lahlou *et al*., 2000) |
| *Croton oblongifolius Roxb.*, | Tonic, against flat worms, to treat dysmenorrhoea, as purgative, dyspepsia and dysentery. Chronic enlargement of the liver | (Ahmed *et al*., 2002; Ngamrojnavanich *et al*., 2003; Singh *et al*., 1999; Sommit *et al*., 2003) |
| *Croton palanostigma* Klotzsch (Peru) | Wound healing and intestinal inflammation. | (Miller *et al*., 2012; Porras-Reyes *et al*., 1993) |
| | Anticancer activity | (Miller *et al*., 2007; Sandoval *et al*., 2002) |
| *Croton roxburghii NP.* (India) | Antidote in snake poisoning and for infertility, fever and wounds. | (Gupta *et al.*, 2004) |
| | Antibacterial and Antioxidant | (Mahbubur *et al.*, 2014; Panda, *et al.,* 2010; Rath *et al*., 2011) |
| | Antifungal activity | (Sujogya *et al.*, 2010) |
| | Antimicrobial, anthelmintic, and antiviral activity | |
| *Croton sublyratus Kurz* ("Plau noi"; South-Eastern Asian Countries and Thailand) | Anti-helminthic and to treat dermatological problems | (Tansakul & De-Eknamkul, 1998; Vongchareonsathit & De-Eknamkul, 1998)s |
| | Antiulcer | (Kitazawa *et al*., 1979) |
| *Croton schiedeanus* ("Almizclillo, Columbia) | Antihypertensive activity | (Guerrero *et al.,* 2001a, 2001b; Guerrero *et al*., 2002; Guerrero *et al.*, 2004) |
| *Croton sylvaticus* (Syn. *C. verdickii* De Wild; *C. oxypetalus* Mull. Arg. and *C. stuhlmannii* Pax | Antimicrobial, anti-inflammatory, antioxidant, larvicidal | (Maroyi, 2017e) |
| | Antimicrobial activity | (Ndunda *et al*., 2013) |
| | Antileishmanial activity | |
| | Antiplasmodial activity | |

| Scientific Name (Other names, region) | Reported use | References |
|---|---|---|
| (Ethiopia, Northern parts of Africa, South Africa, Angola) | | |
| *Croton tiglium* L. (Asia) | Laxative | (Tsai *et al*., 2004) |
| | Intestinal disorder | (Wang *et al*., 2008) |
| | Antioxidant activity | (Saikia & Upadhyaya, 2011) |
| | Antidermatophyte activity | (Chien *et al.*, 2016) |
| | Anticancer activity | (Kupchan *et al*., 1976) |
| | AntiHIV activity | (El-Mekkawy *et al*., 2000) |
| *Croton tonkinensis* Gagnep.("Kho sam Bac Bo"; Vietnam ) | Stomach ache, abscesses, dyspepsia, impetigo, and gastric/duodenal ulcers. | (Giang *et al*., 2003) |
| | Anti-tubercular activity | (Jang *et al*., 2016) |
| | Anti-inflammatory activity | (Kuo *et al*., 2013) |
| | Antibacterial activity | (Giang *et al.*, 2006) |
| *Croton urucurana* | Wound-healing and treating rheumatism | (Orlandi-Mattos *et al*., 2002) |
| | Antiulcer activity | (Wolff Cordeiro *et al*., 2012) |
| | Antibacterial activity | (Oliveira *et al*., 2008; Peres *et al*., 1997; Simionatto *et al*., 2007) |
| | Antifungal activity | (Gurgel *et al.*, 2005) |
| | Antiinflammatory and antinociceptive activity | (Cordeiro *et al*., 2016) |
| *Croton zehntneri Pax. et Hoffm.* "Canelade-cunhã"; Brazil) | Central nervous system effects- Antidepressant and anti-anxiety activity | (Bernardi *et al.*, 1991; Giorgi *et al.*, 1991; Lazarini *et al.*, 2000) |
| | Anthelmintic activity | (Camurça-Vasconcelos *et al*., 2007) |
| | Adjuvant in antibiotic therapy | (Coutinho *et al*., 2010) |
| | Wound healing | (Cavalcanti *et al*., 2012; Malveira Cavalcanti *et al*., 2012) |
| | Antifungal activity | (Fontenelle *et al*., 2008) |
| | Antinociceptive activity | (Oliveira *et al.*, 2001) |
| | Antimicrobial activity | (Andrade *et al*., 2015; Donati *et al*., 2015) |
| | Antimalarial activity | (Mota *et al*., 2012) |
| | Gastro protective activity, Hepatoprotective activity | (Andrelina *et al*., 2013; Lima *et al*., 2008) |
| *Croton zambesicus* (Syn.*C. amabilis* Muell.Arg.; Originally a Guineo-Congolese species but now Widespread in Tropical Africa) | Fever, dysentery and convulsions, anti-hypertensive, anti-microbial (urinary infections) | (Block *et al*., 2002; Ngadjui *et al*., 2002) |
| | Prevention of cardiovascular diseases | (Robert *et al.*, 2010) |

| Scientific Name (Other names, region) | Reported use | References |
|---|---|---|
| | Antimicrobial activity | (Abo *et al*., 1999; Okokon & Nwafor, 2010b) |
| | Antiplasmodial activity | (Okokon & Nwafor, 2009a) |
| | Antiproliferative, antioxidant, and antibacterial activities | (Yagi *et al.*, 2016) |
| | Antiulcer and anticonvulsant activity | (Okokon & Nwafor, 2009b) |
| | Anti-inflammatory, analgesic, and antipyretic activities | (Okokon & Nwafor, 2010) |
| | Nephroprotective effect | (Okokon *et al.*, 2011) |

### 2.6.3. The pharmacological activities of the genus *Croton*

The antibacterial effect of *Croton lechleri* against gram-positive *Staphylococcus aureus* was reported by Chen *et al*., (1994), who isolated 2,4,6-trimethoxyphenol, 1,3,5-trimethoxybenze-ne, crolechinic acid and korberins A and B from the blood-red sap of the plant. Alviano *et al.* (2005) reported the antimicrobial activity of linalool, a monoterpene isolated from *C. cajucara* against *S. aureus, Streptococcus mutans*, gram-negative anaerobic *Porphyromonas gengivalis* and *Candida albicans* (Alviano *et al.*, 2005). In another study, Gurgel *et al*., (2005) demonstrated the antifungal activity of *C. urucurana* against *Tricophyton tonsurans, Epidermophyton floccossum, Tricophyton rubrum, Microsporum canis* and *Tricophyton mentagrophytes* (Gurgel *et al*., 2005), which could be due to the tannin compounds, gallocatechin and epigallocatechin (Gurgel *et al.*, 2005).

Different researchers have reported the antiviral activity of the proanthocyanidin tannin. Tannins have shown efficacy against herpes, influenza, and hepatitis viruses (Barnard *et al.*, 1993; Chen *et al*., 1994; Jones, 2003; Orozco-Topete *et al*., 1997; Ubillas *et al*., 1994; Wyde *et al*., 1993). Similarly, the works of Orozco-Topete *et al*., (1997) have shown the anti-HIV activity of the same compound. In addition, the alkaloidal compound taspin, isolated from different *Croton* species, has been shown to inhibit the reverse transcriptase enzyme of HIV (Sethi, 1977). The phorbol esters 12-*O*-acetylphorbol-13-decanoate, 12-Odecadienoylphorbol-13-(2-methylbutyrate and tetradecanoylphorbol- 13-acetate (TPA) isolated from the seeds of *C. tiglium* have shown anti-HIV activity (El-Mekkawy *et al*., 1998, 2000). Furthermore, the derivative of phorbol esters, 12-*O*-acetylphorbol-13-decanoate, has shown potential anti-HIV activity as an inhibitor of HIV-1 proliferation (Masuda & Harada, 1993; Nakamura, 2004).

The aqueous extracts *C. cuneatus* (Pereira *et al.,* 1999), *C. malambo* (Suárez *et al*., 2003), and *C. celtidifolius* (Nardi *et al*., 2003) have antinociceptive and anti-inflammatory effects. The anti-inflammatory effects of volatile oils of *C. zehntneri* (Oliveira *et al*., 2001), *C. cajucara* (Bighetti *et al.,* 1999), *C. nepetaefolius* (Abdon *et al*., 2002), and *C. sonderianus* (Santos *et al.,* 2005) have been reported. In another study, Risco *et al*., (2003) demonstrated that two phytochemicals, taspine or proanthocyanidins, isolated from *Croton* species possess anti-inflammatory effects. In an earlier study by Perdue *et al*., (1979), the alkaloid taspine was reported to be a potential anti-inflammatory compound. The anti-inflammatory effect of taspine is attributed to the inhibition of sensory neuron activation (Miller *et al.,* 2001).

The following phytochemicals isolated from different *Croton* species possess anti-inflammatory efficacy: sesquiterpenes (5α,10β-4(15)-eudesmen-1β,6β- diol, spathulenol, 5α,10β-3-eudesmen-1β,6α diol and the diterpenoid junceic acid, cajucarinolide, and proanthocyanidins (Aguilar-Guadarrama & Rios, 2004; Carvalho *et al*., 1996; DalBó *et al*., 2005; Maciel *et al*., 2000; Yoshitatsu *et al.,* 1992).

The antitumor effects of *C. lechleri* (Chen *et al*., 1994) and *C. draco* (Gupta *et al*., 1996) have been reported. Ethnobotanical and pharmacological efficacy of *C. lechleri* for the treatment of cancer has been reported by many investigators (Cai *et al.,* 1993; Graham *et al.,* 2000; Pieters *et al.,* 1993). Taspine, an alkaloid isolated from *C. lechleri,* has antiproliferative efficacy against the leukemia cell line (Montopoli *et al*., 2012; Rossi *et al*., 2003). In another study, the antitumor activity of *Croton palanostigma* was reported by Sandoval *et al*., (2002). Trans-dehydrocrotonin, isolated from the bark of *C. cajucara,* has shown immunomodulatory and antitumor activity (Melo *et al.,* 2004) by inducing apoptosis and inhibiting cell differentiation (Maristella *et al.,* 2004; Maristella *et al.,* 2003).

Terpenoids isolated from *C. oblongifolius* have shown antitumor activity (Roengsumran *et al*., 2001, 2002, 2004; Sommit *et al*., 2003). In a related study, Kawai *et al*., (2005) demonstrated that the acyclic diterpenoid plauntol, isolated from *C. sublyratus,* exhibits antitumor effects by inhibiting angiogenesis (Kawai *et al*., 2005). Wound healing activity, antihemorrhagic activity, myorelaxant, antispasmodic and antihypertensive activities of the *Croton* genus have also been reported (Salatino *et al*., 2007).

### 2.6.4. *Croton macrostachyus* Hochst. ex Delile

#### 2.6.4.1. Description of *Croton macrostachyus*

*C. macrostachyus* is a medium-sized tall tree or shrub that grows up to 30 meters (Edwards and Tadesse, 1995; Umberto, 2012). It is commonly known as a "broad-leaved *Croton*" or "rush foil". It has various local/vernacular names, including "*bisana*" in Ethiopia, "*msinduzi, mutundu*" in Kenya (Dubale *et al.,* 2015; Lulekal *et al.,* 2013; Maroyi, 2017a; Teklehaymanot, 2009; Teklehaymanot *et al.*, 2007; Umberto, 2012). *C. macrostachyus* has a wide distribution in Africa, where in Kenya, it is widely found in the Karura Forest (Naturgucker., 2018).

#### 2.6.4.2. Ethnomedicinal Uses of *Croton macrostachyus*

*C. macrostachyus* is used as a remedy for a variety of illnesses. The plant possesses various medicinal properties (Maroyi, 2017c) and treats constipation in Ethiopia, Cameroon, Rwanda, Kenya, Tanzania, Somalia, and Uganda. Usually, the decoction, macerated leaf, stem bark, or root is used (Ahmed *et al.,* 2013; Mazzanti *et al*., 1987; Pascaline & Charles, 2011). In Kenya, the leaf and root decoction is used by many patients, including HIV-infected patients, as a cure for cough, back pain, bleeding, skin diseases, warts, pneumonia, and wounds (Jeruto *et al.,* 2008; Kareru *et al.,* 2007; Obey *et al*., 2016b; Okello *et al.,* 2010; Pascaline *et al*., 2010).

In Kenya, *C. macrostachyus* bark juice, leaf, and root decoction are used as remedy for backache, bleeding, cancer, colds, cough, diarrhea, dysmenorrhoea, east coast fever, malaria, measles, obesity, pneumonia, ringworm, skin diseases, typhoid, warts, and wounds (Agisho *et al*., 2014; Bekele & Reddy, 2015; Mesfin *et al*., 2009). The leaves of *C. macrostachyus* are used by farmers in Kenya as biological pest control when mixed with tobacco (*Nicotiana tobacuum* L.) and boiled overnight (Edwards and Tadesse, 1995). In addition, roots of *Cucumis ficifolius* are often used in combination with *C. macrostachyus* bark as a remedy for abdominal and stomach pain (Teklay *et al.,* 2013). Similarly, *Allium sativum* is given with *C. macrostachyus* to treat malaria (Bekele & Reddy, 2015; Mesfin *et al*., 2009).

### 2.6.4.3. Phytochemistry of *C. macrostachyus*

**Table 2.6-3** summarizes the compounds previously isolated from *C. macrostachyus.*

Table 2.6-3: Phytochemicals isolated from *C. macrostachyus*

| Ser. No. | Name of compound | Part of the plant | Reference |
|---|---|---|---|
| 1. | Sitosterol palmitate | Twigs | (Tala *et al*., 2013a) |
| 2. | Sitosterol | Stem bark and twigs | (Maroyi, 2017b; Tala *et al*., 2013a) |
| 3. | Stigmasterol | Stem bark and twigs | (Addae-Mensah *et al.*, 1992; Tala *et al*., 2013) |
| 4. | Betulin | Stem bark and twigs | (Addae-Mensah *et al*.,, 1992; Maroyi, 2017b; Tala *et al*., 2013; Tene *et al*., 2009) |
| 5. | Lupeol | Stem bark and twigs | (Addae-Mensah *et al.*,1992; Tala *et al.*, 2013; Tene *et al.*, 2009) |
| 6. | 3$\beta$-Acetoxy taraxer-14-en-28-oic acid | Roots | (Kapingu *et al*., 2000) |
| 7. | Lupenone | Twigs | (Tala *et al*., 2013a) |
| 8. | Betulinic acid | Twigs | |
| 9. | Zeroin | Twigs | |
| 10. | 28-*O*-Acetylbetulin | Twigs | |
| 11. | Lupeol acetate | Twigs | |
| 12. | Neoclerodan-5,10-en-19,6$\beta$;20,12-diolide | Roots | (Kapingu *et al*., 2000) |
| 13. | (+) Hardwickiic acid | Stem bark and twigs | (Addae-Mensah *et al*., 1989; Kapingu *et al*., 2000) |
| 14. | 12-Oxo-hardwickiic acid | Stem bark and twigs | |
| 15. | Crotomacrine | Fruits | (Tane *et al*., 2004) |
| 16. | Floridolide A | Stem bark | |
| 17. | Trachyloban-19-oic acid | Roots | (Kapingu *et al*., 2000) |
| 18. | Trachyloban-18-oic acid | Roots | |
| 19. | Crotomachlin | Stem bark and twigs | (Addae-Mensah *et al*., 1989) |
| 20. | Crotepoxide | fruits | (Ndunda, 2014) (Morris *et al*., 1969) |
| 21. | Lichexanthone | Twigs | (Tala *et al*., 2013a) |
| 22. | Methyl 2,4-dihydroxy-3,6-dimethylbenzoate | Twigs | |
| 23. | Methyl gallate | Twigs | |
| 24. | Benzoic acid | Twigs | |

#### 2.6.4.4. Pharmacological effects of extracts of *C. macrostachyus*

Antibacterial effects of *C. macrostachyus* against *N. gonorrhea* (Tefera *et al.,* 2012), *B. cereus, E. coli* and *P. aeruginosa* (Cyrus *et al.,* 2008) and *S. pyogenes* (Taye *et al.,* 2011) have been reported. In a related study, Obey *et al*., (2016) reported that the ethyl acetate extract of stem bark of *C. macrostachyus* has good antibacterial activity against *E. coli, S. typhi, K. pneumoniae, E. aerogenes,* and *L. monocytogenes.* Taye *et al*., (2011) demonstrated the antibacterial activity of methanol leaf extract against *S. pyogenes* with a minimum bacterial concentration (MBC) value of 7.81 mg/mL.

The antimycobacterial activity of *C. macrostachyus* in an *in vitro* experimental study was reported by Gemechu *et al.,* (2013). Semenya & Maroyi, (2013) also demonstrated the antimycobacterial activity of methanolic leaf extracts of *C. macrostachyus* with minimum inhibitory concentration (MIC) values ranging from 12.5 to 100 $\mu$g/mL. This study demonstrated that *C. macrostachyus* has potential as an herbal medicine in the treatment and management of tuberculosis, a leading cause of death in sub-Saharan Africa (Semenya & Maroyi, 2013).

Antimicrobial and antifungal effects of *C. macrostachyus* extracts have been reported by Desta, (1993) and Taniguchi & Kubo, (1993). The isolated diterpenoid 12-oxo-hardwickic acid has shown efficacy against *Candida albicans* (Tene *et al*., 2009). Ngo Bum *et al*., (2012) reported that decoctions of *C. macrostachyus* possess anticonvulsant effects (Lulekal *et al.,* 2008). The antimalarial efficacy of leaf and stem bark extracts of *C. macrostachyus* has been reported by Karunamoorthi & Ilango, (2010) and Owuor *et al*., (2012). Bantie *et al*., (2014) demonstrated a chemoprotective effect against malaria.

The anthelmintic efficacy of seed extracts of *C. macrostachyus* has been reported by Eguale *et al.,* (2007). Kamanyi *et al*., (2009) demonstrated that extracts of stem bark of *C. macrostachyus* exhibited anti-inflammatory activity in experimental mouse models of inflammation (Kamanyi *et al.,* 2009). Similar findings were also reported by Nguelefack *et al.,* (2015).

Methanolic leaf extract of *C. macrostachyus* showed antioxidant activity with an $IC_{50}$ value of 0.11 mg/mL. The documented antioxidant activities of *C. macrostachyus* leaf extracts were probably due to flavonoids and phenols that havebeen isolated from fruits, leaves, and roots (Amuamuta *et al*., 2015; Degu *et al*., 2016; Eguale *et al*., 2007). Flavonoids and phenolic compounds found in plants have antioxidant properties (Miguel *et al*., 2014).

### 2.6.5. *Croton megalocarpus* Hutch.

#### 2.6.5.1. Description of *C. megalocarpus*

*C. megalocarpus* (family Euphorbiaceae) is a medium-sized tree or shrub that grows up to 35 meters tall (Thijssen, 1996). It is commonly planted as a shade tree (Maroyi, 2017e; Thijssen, 1996). The species name "*megalocarpus*" refers to the species' relatively large fruits (Hyde, *et al.*, 2012; Hyde, 2013). *C. megalocarpus* is commonly known by various local/vernacular names including '*mbali"* in East Africa, "*msuduzi*" in Kenya (Bussmann, 2006; Fratkin, 1996; Kipkore *et al.*, 2014; Kiringe, 2006), "*umunege*" in Rwanda, and "*umuraangara*" in Tanzania: (Ibrahim & Ibrahim, 1998; Johns *et al.*, 1994; Minja, 1994; Umberto 2012). *C. megalocarpus* is widely distributed in Africa (Maroyi, 2017e; Thijssen, 1996). It is considered one of the vital medicinal plants in Kenya (Njoroge *et al*., 2010). It is found in Laikipia County and is traded in traditional herbal medicine ("*muthi*") markets in Thika and Nairobi, Kenya (Njoroge, 2012).

#### 2.6.5.2. Ethnomedicinal Uses of *C. megalocarpus*

*C. megalocarpus* is mainly used to treat respiratory problems (Muthee *et al*., 2011; Grace *et al.*, 2006), fever (Ichikawa, 1987; Johns *et al*., 1994; Nanyingi *et al*., 2008), and wounds (Kamau *et al.*, 2016; Nanyingi *et al.*, 2008). In Kenya, *C. megalocarpus* is used in the treatment of constipation (Kipkore *et al.*, 2014) and as an herbal medicine for backache, chest problems (Bussmann, 2006; Kiringe, 2006), malaria (Bussmann, 2006; Cyrus *et al*., 2008; Fratkin, 1996) and stomach ache (Bussmann, 2006; Fratkin, 1996; Kiringe, 2006).

The bark decoction is used for arthritis (Pascaline *et al*., 2010), colds (Fratkin, 1996), cough, diarrhea (Gakuubi & Wanzala, 2012; Nanyingi *et al*., 2008), fever (Ichikawa, 1987; Johns *et al*., 1994; Nanyingi *et al*., 2008), wounds (Kamau *et al*., 2016; Nanyingi *et al*., 2008), and vomiting induction (Kiringe, 2006). In addition, the leaf and root decoction of *C. megalocarpus* is used by HIV patients for the treatment of pneumonia (Kamau *et al*., 2016), respiratory problems (Muthee *et al*., 2011), wounds, and diabetes (Keter & Mutiso, 2012).

### 2.6.5.3. Phytochemistry of *C. megalocarpus*

**Table 2.6-4** summarizes the compounds previously isolated from *C. megalocarpus.*

Table 2.6-4: Phytochemicals isolated from *C. megalocarpus*

| Ser. No | Compound name | Part of the plant | Reference |
|---|---|---|---|
| 1. | Betulin | Stem bark | (Addae-Mensah *et al*., 1992; Maroyi, 2017b; Tala *et al*., 2013; Tene *et al*., 2009) |
| 2. | 3-β-O-Acetoacetyl lupeol | Stem bark | |
| 3. | Aleuritolic acid | Stem bark | (Aldhaher *et al*., 2017; Langat *et al*., 2012) |
| 4. | Lupeol | Stem bark | (Barbosa *et al*., 2003; Burns *et al*., 2000; Stephane *et al*., 2019) |
| 5. | Chiromodine | Stem bark | (Addae-Mensah *et al*., 1989; Markó *et al*., 1999) |
| 6. | Epoxychiromodine | Stem bark | |
| 7. | Crotonolide E | Root | (Alqahtani, 2015; Liu *et al*., 2014) |
| 8. | Furocrotinsulolide A | Root | (Alqahtani, 2015; Graikou *et al*., 2005) |
| 9. | 3β,4β:15,16-diepoxy-13(16),14-ent-clerodadien-17,12S-olide | Root | (Alqahtani, 2015) |
| 10. | 3β,4β:15,16-diepoxy-13(16),14-ent-clerodadiene | Root | (Alqahtani, 2015; Harinantenaina & Asakawa, 2007; Roengsumran *et al*., 2001) |
| 11. | *Trans*-annonene | Root | (Alqahtani, 2015; McCrindle *et al*., 1976) |
| 12. | Crotohalimaneic acid | Root | (Alqahtani, 2015; Ndunda B., 2014; Roengsumran *et al*., 2004) |
| 13. | 7,13-abietadien-2-one | Root | (Alqahtani, 2015) |
| 14. | 7,13-abietadien-2-ol | Root | |

### 2.6.5.4. Pharmacological effects of extracts of C. megalocarpus

The antibacterial efficacy of *C. megalocarpus* against *B. subtilis* and *S. aureus* has been documented by Matu & Van Staden, (2003). Kariuki *et al*., (2014) demonstrated that aqueous extracts of *C. megalocarpus* have antibacterial activity against *P. aeruginosa, E. coli, K. pneumoniae* and *S. aureus.* The phytochemicals betulin, lupeol, lauric acid, and palmitoleic acid are responsible for the antibacterial activities of *C. megalocarpus* (Duric *et al.*, 2013; Ouattara *et al.*, 1997).

Lall *et al.*, (2006) demonstrated the antifungal activities of β-sitosterol, betulin, and lupeol against *Aspergillus flavus, Aspergillus niger, Cladosporium cladosporioides,* and *Phytophthora* spp. Similarly, Nisar *et al*., (2013) reported the antifungal effects of betulin and lupeol against *Aspergillus flavus, Aspergillus niger, Candida albicans, Candida glaberata,* and

*Microsporum caris.* Matu & Van Staden, (2003) and other workers demonstrated the anti-inflammatory effect of *C. megalocarpus*, which is attributed to phytochemicals such as sitosterol (Nirmal *et al.*, 2012), betulin (Lin *et al*., 2009), lupeol (Geetha & Varalakshmi, 1998), linoleic acid and linolenic acid (G. Zhao *et al*., 2005), which have cyclooxygenase enzyme inhibition potential. Others have also reported the antinociceptive effects of linoleic acid, lupeol, and oleic acid *(*Lima *et al.*, 2013*;* Gichui, 2016*;* Mota *et al.*, 2015). Wambugu & Waweru, (2016) documented the wound healing activity of extracts of *C. megalocarpus*, where phytochemicals such as betulin, lupeol (Ebeling *et al*., 2014), and linolenic acid (Lewinska *et al.*, 2015) are responsible for this activity.

### 2.6.6. *Croton dichogamus* Pax

#### 2.6.6.1. Description of *C. dichogamus*

*C. dichogamus* Pax (Euphorbiaceae) grows as a shrub or tree in Ethiopia, Kenya, Madagascar, Mozambique, Tanzania, and Somalia (Govaerts, 1999). It grows up to 7.5 meters tall or more but is usually only 2 - 5 meters tall.

#### 2.6.6.2. Ethnomedicinal uses of *C. dichogamus*

In East African countries, decoctions of leaves, roots, and whole plants are used to treat fever, chest ailments, stomach diseases, tuberculosis, impotence, and malaria (Jeruto *et al*., 2008; Kokwaro, 1976). In Tanzania**,** the roots of *C. dichogamus* are milled and then mixed with porridge for the treatment of tuberculosis because the shrub is believed to be efficient in the management of respiratory ailments. The leaves are also used as a tonic, antimalarial and nutritional supplement. Patients inhale the smoke of burnt leaves to provide relief from fever (Hedberg *et al*., 1983).

#### 2.6.6.3. Phytochemistry of *C. dichogamus*

**Table 2.6-5** summarizes the compounds previously isolated from the stem bark of *C. dichogamus.*

Table 2.6-5: Phytochemicals isolated from *C. dichogamus*

| Ser. No | Name of compound | Plant part | Reference |
|---|---|---|---|
| 1. | Aleuritolic acid | Root | (Aldhaher *et al*., 2017; Langat *et al*., 2012) |
| 2. | Crotonolide E | Root | (Alqahtani, 2015; Liu *et al*., 2014) |
| 3. | Furocrotinsulolide A | Root | (Alqahtani, 2015; Graikou *et al*., 2005) |

| Ser. No | Name of compound | Plant part | Reference |
|---|---|---|---|
| 4. | 3β,4β,15,16-diepoxy-13(16),14-clerodadiene | Root | (Aldhaher *et al*., 2017; Harinantenaina *et al*., 2006) |
| 5. | 3β,4β,15,16-diepoxy-13(16),14-*ent*-clerodadiene-17,(12S)-olide | Root | (Aldhaher *et al*., 2017; Pudhom & Sommit, 2011) |
| 6. | 15,16-epoxy13(16),14-*ent*-clerodadien-3-one (*trans*-cascarillone) | Root | (Aldhaher *et al*., 2017) |
| 7. | 15,16-Epoxy-5,13(16),14-*ent*-halimatriene-3-ol | Root | |
| 8. | 15,16-epoxy4(18),13(16),14-*ent*-clerodatrien-3a-ol (gbaninol) | Root | |
| 9. | 15,16-epoxy-3β-hydroxy-5(10),13(16),14-*ent*-halimatriene-17,(12*S*)-olide | Root | |
| 10. | 15,16-epoxy-3α-hydroxy-4(18),13(16),14-*ent*-clerodatrien-17,(12S)-olide (crotonolide F) | Root | (Aldhaher *et al*., 2017; Liu *et al*., 2014) |
| 11. | Crotoxide A | Leaves | (Jogia *et al*., 1989) |
| 12. | Crotoxide B | Leaves | |
| 13. | Crotohaumanoxide | Root | (Aldhaher *et al*., 2017; Li *et al*., 2010; Tchissambou *et al*., 1990) |
| 14. | Crotodichogamoin A | Root | (Aldhaher *et al*., 2017) |
| 15. | Crotodichogamoin B | Root | |
| 16. | Crothalimene A | Root | |
| 17. | Crothalimene B | Root | |
| 18. | Depressin | Root | (Aldhaher *et al*., 2017; Li *et al*., 2010) |
| 19. | Cadalene | Root | (Aldhaher *et al*., 2017) |
| 20. | 6α-methoxy-patchoulan-4-ene (6a-methoxycyperene) | Root | (Aldhaher *et al*., 2017; Barreto *et al*., 2013) |
| 21. | 4-patchoulen-3-one (cyperotundone) | Root | (Aldhaher *et al*., 2017) |
| 22. | 4-patchoulene (cyperene) | Root | |
| 23. | 1(6),7,9-cadinatriene-4α,5β-diol (4α,5β-corocalanediol) | Root | |
| 24. | 1,3,5-cadinatriene-(7R,10S)-diol (10-epi-maninsigin D) | Root | |

#### 2.6.6.4. Pharmacological effects of extracts of *C. dichogamus*

Magadula, (2012) demonstrated the antimycobacterial activity of an ethanolic root extract of *C. dichogamus* against *M. indicus pranii* and *M. madagascariense indicus*, giving a minimum inhibitory concentration (MIC) of 1.25 mg/ml (Magadula, 2012). Studies have shown that *C. dichogamus* has insecticidal activity against *Anopheles gambie* and is

extensively used to treat malaria in lake basins (Omara, 2020). In addition, the presence of a very high concentration of terpenoids in *C. dichogamus* has been linked to its antimicrobial properties (Magadula, 2012).

A recent study on the phytochemical constituents of most croton species pointed out that the presence of clerodane diterpenoids in these species results in higher anti-inflammatory activity than the standard drug used in the study (Somteds *et al*., 2019). The high level of saponins in *C. dichogamus* crude stem extracts (Johns *et al*., 1999) was confirmed to lower cholesterol levels in humans and animals by preventing endogenous and exogenous cholesterol absorption, thus lowering serum cholesterol (Thompson, 1993).

# CHAPTER THREE

# METHODOLOGY

## 3.1. Materials

Commercial silica gel (100−200, 200−300, and 300−400 mesh; Qingdao, China) was used for column chromatography (CC). Sephadex LH-20 (Amersham Biosciences) was also used for CC. All solvents used for column chromatography were of analytical grade (Shanghai Chemical Reagents Co., Ltd.). Analytical TLC using precoated aluminum-backed plates (silica gel 60 $F_{254}$, Merck) were used. Spots were detected on TLC under UV light at 254 or 365 nm, followed by spraying with 1% vanillin-sulfuric acid spray reagent and warming. Extraction and CC was performed at the research laboratory of United States International University, Kenya.

1D and 2D NMR spectra were recorded in $CDCl_3$ on a 400 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts (δ) were expressed in ppm and were referenced against the solvent resonances at $\delta_H$ 7.26 and $\delta_C$ 77.23 ppm for $^1H$ and $^{13}C$ NMR for $CDCl_3$. Structural assignments of the new compounds were made with additional information from 1H-1H COSY, HSQC, NOESY, and HMBC experiments. Mass spectra were recorded on a GC-MS Bruker MicroToF Mass Spectrometer by direct injection using a Bruker Bioapex-FTMS with electrospray ionization. The spectroscopic analysis was performed at the Jodrell Laboratory, Royal Botanic Gardens Kew (UK).

For the *in vitro* experiment, human T-lymphocyte MT-4 cells (ARP-120) and Human immunodeficiency virus type 1 (HIV-$1_{IIIB}$) strain were obtained from the National Institute of Health (NIH) HIV Reagent Program. The *in vitro* cytotoxicity and anti HIV activity tests were carried out in the institute of primate research (IPR) and Kenya medical research institute (KEMRI), Kenya.

## 3.2.Photochemical study of the medicinal plants

### 3.2.1. Collection of the plant specimen and ethical consideration

The leaves and stem bark of *C. macrostachyus* and *C. megalocarpus* were collected from a USIU botanical garden in June 2020. The twigs of *C. dichogamus* were collected from Mwala Constituency, Machakos County, Kenya, in August 2020 (**Figure 3.2-1 to 3.2-3**). The collection of these medicinal plants was performed after obtaining the required ethical approval from the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee

(KNH-UON ERC), approval number KNH/ERC/A/154 and research permit from the National commission for science, technology and innovation (NACOSTI), permit number NACOSTI/P/22/15579. Expert botanist and taxonomist performed taxonomic identification, and voucher specimen TEREFE E./044, TEREFE E./045 and TEREFE E./046 were deposited for *C. megalocarpus*, *C. macrostachyus*, and *C. dichogamus* respectively at the United States International University herbarium for future reference. After collection, the leaves were thoroughly washed and dried in the shade. Then, the dried leaves were pulverized using a mortar and pestle at the medicinal plants laboratory of United States International University Africa (USIU-A).

### 3.2.2. Preparation of Crude extract

The powdered plant materials were separately extracted with 1:1 $CH_2Cl_2$:$CH_3OH$ solvent using the cold maceration technique. Maceration was continued for 7 days with frequent agitation in an orbital shaker, and the extract was filtered. Extraction was repeated three times, and the filtrates of all portions were pooled. Finally, the extracts were concentrated using rotavapor at 30ºC to obtain dry extracts. The extract was weighed and packed in a glass vial and stored in a desiccator over silica gel until use. The percentage yield was calculated using equation 1.

$$\textbf{\textit{Percentage yield}} = \frac{\textbf{\textit{Mass (gm) of extract obtained}}}{\textbf{\textit{Mass (gm) of dried plant}}} \textbf{\textit{X}}\ \mathbf{100} \quad \text{... ...Equation 1}$$

A B C

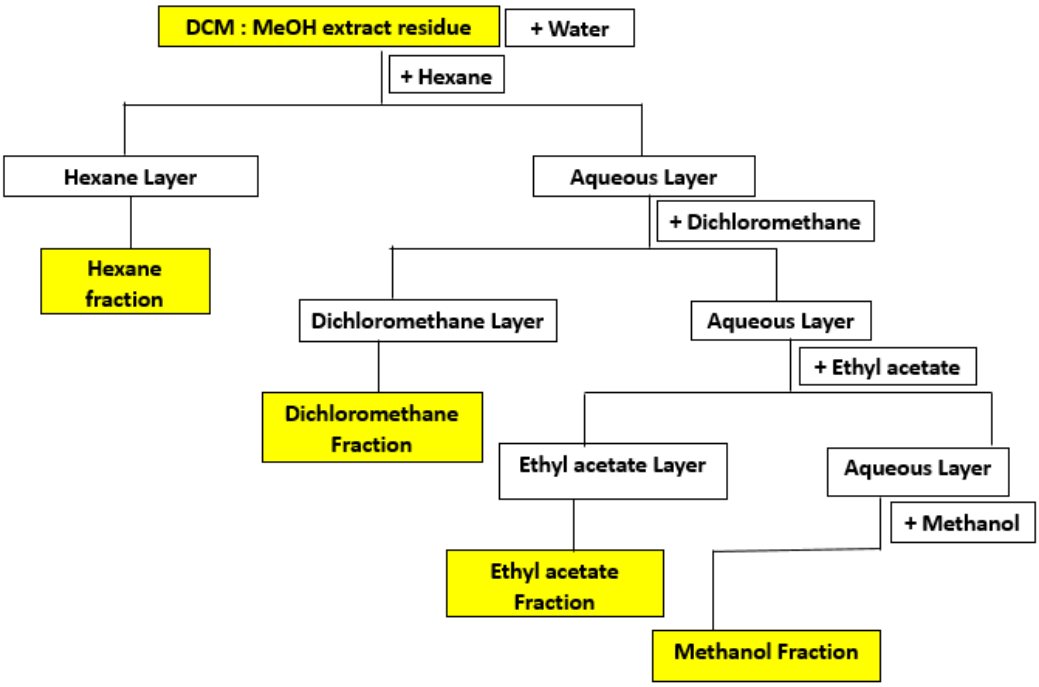
Figure 3.2-1: A, *C. macrostachyus*; B, *C. megalocarpus*; C, *C. dichogamus*

Figure 3.2-2: Collection of stem bark of *C. megalocarpus*

Figure 3.2-3: Crude and liquid-liquid extraction of the plant extracts

### 3.2.3. Liquid-Liquid separation of the crude extracts

The dried crude mass 1:1 $CH_2Cl_2$:$CH_3OH$ extracts of *C. megalocarpus, C. macrostachyus,* and *C. dichogamus* were separately dissolved in distilled water (200 mL) and successively partitioned using different solvents of increasing polarity (n-hexane, dichloromethane, ethyl acetate, and methanol) in separatory funnels, as shown schematically in **Scheme 3.2-1.** The different solvent fractions were concentrated under reduced pressure using a rotary evaporator, and the resulting product was dried in an oven at 30°C. The dried fractions were then transferred into separate vials and stored in a desiccator for further use.




Scheme 3.2-1: Flow chart of liquid-liquid extraction of plant extracts

### 3.2.4. Chromatographic technique and isolation of pure compound

The different fractions of the three plants were subjected to column chromatography using 60-120 mesh silica gel and eluted successively with varying concentrations of ethyl acetate and n-hexane (E: H). Bioassay-guided fractionation was performed on the partitions showing the highest antiretroviral activity. Each fraction was then evaluated for antiretroviral activity, and the fraction with the highest activity was further subjected to open column chromatography on 200-400 mesh silica gel (**Figure 3.2-4 to 3.2-5**). Compound purity was determined using thin-layer chromatography (TLC) on precoated aluminium-backed plates (silica gel 60 $F_{254,}$ Merck), and compounds were visualized using UV radiation at 254 nm, followed by an anisaldehyde spray reagent (1 percent p-anisaldehyde:2 percent $H_2SO_4$: 97 percent cold MeOH) and heating. Final purifications were carried out in selected solvent systems using preparative thin layer chromatography (Merck 818133) and gravity column chromatography (Merck Art. 9385), which used a 2 cm diameter column packed with silica gel.

A B

Figure 3.2-4: Column chromatography of the studied plant fractions


Figure 3.2-5: Examples of the isolated pure compounds

### 3.2.5. Structural elucidation of isolated compounds

The isolated pure compounds were characterized by nuclear magnetic resonance (NMR) and High-resolution mass spectrometry (HRMS) at the Jodrell Laboratory, Royal Botanic Gardens Kew (UK). A Perkin-Elmer Frontier/Spotlight 200 spectrometer was used to record FTIR spectra. In addition, 1D and 2D NMR spectra in $CDCl_3$ were collected at room temperature using 400 MHz Bruker AVANCE NMR equipment. Chemical shifts (δ) were measured in ppm and compared to the solvent resonances for $^{1}H$ and $^{13}C$ NMR for $CDCl_3$ at $\delta_H$ 7.26 and C 77.23 ppm.

The chemicals were analysed for HRMS using a Vanquish UHPLC system (Thermo Scientific, Waltham, MA, USA) linked to a 100 Hz photodiode array detector (PDA) and a Thermo Scientific Orbitrap Fusion Tribrid (Thermo Scientific) high-resolution tandem mass spectrometer. Chromatographic separation (5 µL) was performed on a Luna C18 column (150 mm × 3 mm i.d., 3 µm, Phenomenex, Torrance, CA, USA) using a mobile phase gradient of 0:90:10 to 90:0:10 (MeOH (A): water (C): acetonitrile + 1% formic acid (D)) over 60 min. Then, 90% A was held for 10 minutes and returned to initial conditions over 5 minutes at 30ºC (flow rate: 400 µL/min). UV detection done between 210 and 550 nm. Mass spectrometry detection was performed in positive and negative ionization modes using the full scan and data dependent $MS^2$ and $MS^3$ acquisition modes.

Total ion current (TIC) chromatograms were obtained over the range of 125–1800 *m/z* using spray voltages of +3.5 kV and −2.5 kV for the positive and negative ionization modes, respectively. Four different scan events were recorded for each ionization mode as follows: 1) full scan; 2) $MS^2$ of the most intense ion in scan event 1; 3) $MS^3$ of the most intense ion in scan event 2; and 4) $MS^3$ of the second most intense ion in scan event 2. Additional parameters for the mass spectrometer included: full scan resolution, 60,000 FWHM; capillary temperature, 350ºC; ion transfer tube temperature, 325ºC; RF lens (%), 50; automatic gain control (AGC) target, 4.0e5 (Full scan) and 1.0e4 ($MS^n$); intensity threshold, 1.0e4; CID collision energy, 35; activation Q, 0.25; and isolation window (*m/z*), 4. Nitrogen was used as the drying, nebulizer, and fragmentation gas.

### 3.3. *In vitro* assay for antiretroviral activity

#### 3.3.1. Preparation of extracts and test compounds

Since most plant extracts and purified compounds are readily soluble in DMSO, (Cos *et al*., 2009), the concentrated dried extracts were dissolved in 1% v/v dimethylsulfoxide (DMSO) and stored in 2 ml cryovials at 4°C until use. Each extract and pure compound were reconstituted to obtain a stock solution of 4 mg/ml. The stock solutions were filtered through a 0.22 µm membrane filter (Sigma, South Africa) and stored at 4°C until further use (Rege *et al*., 2010).

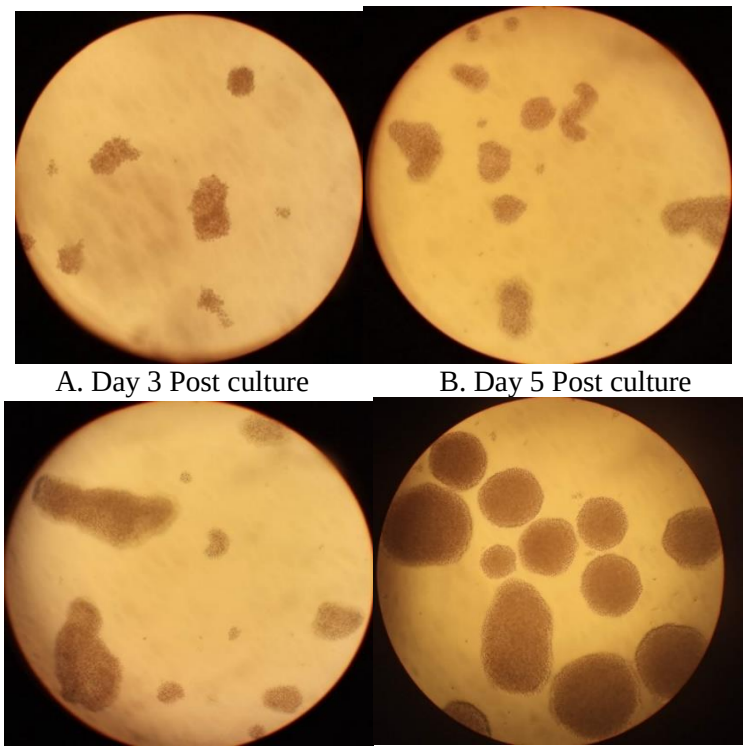
#### 3.3.2. Cell culture, maintenance, and viability test

Since the discovery of the Human Immunodeficiency Virus (HIV), much effort has been devoted to finding susceptible, highly permissive cell lines and developing reliable assays for detecting the multiplication of the virus. The first isolates of HIV were from mononuclear cells in the peripheral blood of patients with Acquired Immunodeficiency Syndrome (AIDS), and the first successful transmission of HIV to an established T-cell line, H9, was achieved by Popovic *et al*. (Popovic *et al*., 1984; Szucs *et al*., 1988). Other T-cell lines were found to be susceptible to HIV infection. Each of these cell lines had one thing in common: the presence of a CD4 epitope on the surface of the cell to which the envelope protein of HIV could bind (Szucs *et al*., 1988).

Harada *et al.*, (1985) reported that a lymphocyte cell line, MT-4, was highly susceptible to HIV infection and typically grew in clusters. Without a virus, these cell aggregate to form into clusters after dissociation by pipetting within 2 to 3 hours. After superinfection with HIV, rapid induction and release of HIV antigens were observed, accompanied by a marked cytopathogenic effect (Harada and Yamamoto, 1985; Szucs *et al*., 1988). Moreover, many scholars have used the MT-4 cell line to evaluate the anti-HIV activity of various compounds (Asres and Bucar, 2005; Weislow *et al*., 1989).

In this study, human T-lymphocyte MT-4 cells (ARP-120) were obtained from the National Institute of Health (NIH) HIV Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH; donated by Dr. Douglas Richman. Human T-lymphocytic MT-4 cells express CD4, CXCR4, and CCR5 and are used for cytotoxicity inhibition assays for antiviral drugs (Harada *et al*., 1985; Larder *et al*., 1989; Pauwels *et al*., 1987). The vials containing frozen MT-4 cells were retrieved from the liquid nitrogen tank and rapidly thawed (Gao *et al*., 2009).

In a tissue culture laminar flow hood, the exterior of the vials was decontaminated by spraying 70% ethanol. Then, the vial was opened and resuspended in 10 mL complete culture medium (CCM) containing RPMI-1640 with 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 100 IU of penicillin/mL, 100 µg of streptomycin/mL, 1 ng of recombinant interleukin-2 (Invitrogen)/ml, and 2 µg of phytohemagglutinin/mL as described previously by Gao *et al*., (2009). The CCM was sterilized with a 0.22 µm membrane filter before use. The cultures were incubated at 37ºC in a humidified incubator with atmospheric conditions of 5% $CO_2$. Every 3-5 days, the cultures were centrifuged, pelleted, and resuspended in 5-10 mL RPMI 1640 supplemented with 10% FBS. The culture flasks were routinely examined for changes using an inverted microscope (**Figure 3.3-1**), which determined the frequency of media changes. The cell count and viability were measured by the trypan blue dye exclusion technique (Miyoshi *et al*., 1982).



A. Day 3 Post culture B. Day 5 Post culture

C. Day 8 Post culture D. Day 14 Post culture

Figure 3.3-1: MT-4 cell culture

The dye exclusion test was used to determine the number of viable cells present in the cell suspension. This method is based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not (Strober, 2015). Briefly, an aliquot of MT-4 cell suspension was centrifuged for 5 min at 1500 rpm, and the supernatant was discarded. The cell pellet was then resuspended in a 1 ml complete medium. Ten microliters of 0.4% trypan blue was mixed with 10 µL of MT-4

cell suspension and incubated for 3 minutes at room temperature. The percentage of cell viability was determined using a hemocytometer. The number of viable cells of a cell suspension, which was mixed with 0.2% Trypan Blue (Biowhittaker, Wakersville) (v/v 1:1), was determined. A drop of the mixture was applied to a hemocytometer. This mixture was spread across a grid by capillary action. Only the viable (translucent) cells that lay within or touched the left or top boundary were counted. The percentage cell viability was determined using equation **(2)**. The number of viable cells per ml in the original sample was calculated using equation **(3)**.

$$\%\ \boldsymbol{cell\ viability} = \frac{\boldsymbol{Total\ viable\ cells\ (unstained)}}{\boldsymbol{Total\ cells\ (Viable+dead)}}\ \boldsymbol{x\ 100} \quad \text{Eq. 2}$$

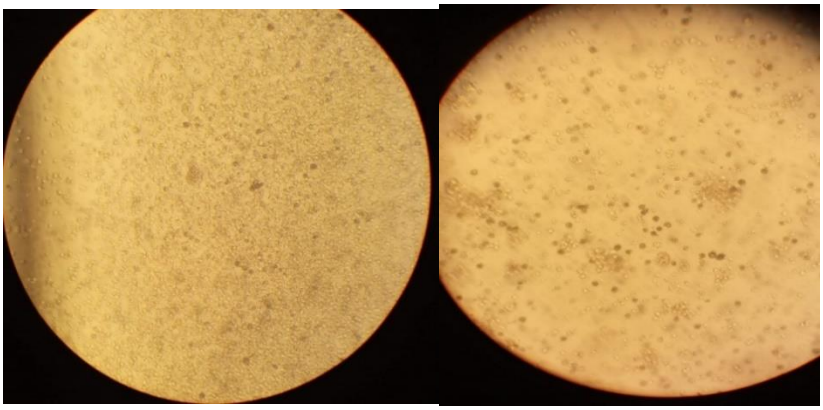
$$\boldsymbol{Number\ of\ viable\ cells\ per\ ml} = \boldsymbol{Average\ No.\ of\ viable\ cells\ per\ square\ x\ Dilution\ factor\ x\ 10^4} \quad \text{Eq. 3}$$

After the MT-4 cells were incubated over a 3-day period, they were then subcultured in RPMI, and stock cultures were stored at -70ºC in a freezer until required. Cell maintenance was performed according to standard protocols Miyoshi *et al*., (1982). All cell culture procedures were carried out in a Class III biological safety cabinet. The unit was swabbed/sterilized with 70% ethanol before and after each use.

### 3.3.3. Preparation of the virus and viral infectivity test

Human immunodeficiency virus type 1 (HIV-$1_{IIIB}$) strain was obtained from the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH. Dr. Robert Gallo donated human immunodeficiency virus-1 IIIB (ARP-398). HIV-$1_{IIIB}$ is highly capable of replicating in human T cell lines and appears to be well adapted for i*n vitro* culture in T cells (Popovic *et al*., 1984).

The frozen HIV-$1_{IIIB}$ vials were thawed by immersion in room temperature water, and the vial was swirled until viruses were thawed. Then, 400 µl of the virus was transferred to 75 $cm^2$ tissue culture flasks (T75) containing 10 mL of 1 x $10^5$ pelleted MT4 cells/mL, vortexed, and incubated at 37°C for 1 hr for virus adsorption. After 1 hr, 20 ml of RPMI-1640 was added gently and mixed with vortexing. The cells were then pelleted by centrifuging at 1500 rpm for 5 min. The pelleted cells were then washed and resuspended in 30 ml RPMI 1640 with L-glutamine and 20% fetal bovine serum in a T75 flask, incubated at 37ºC and monitored for the development of cytopathic effects (CPEs).



A. CPE at Day 3 B. CPE at Day 6

C. CPE at Day 10

Figure 3.3-2: Development of cytopathic effects in MT-4 cells

MT-4 cells inoculated with HIV showed markedly different cell growth and viability patterns than mock-infected cells. The number of viable cells rapidly decreased at 24 hours post-infection, and by day 4, only 2-4% of the infected MT-4 cells were viable. In contrast, the mock-infected cells grew well, reaching a plateau between days 2 and 4. After 4 days, the viability of these cells began to decline appreciably. The infected cells displayed cytopathic effects (CPE), including became round, lost their surface characteristics, became refractile, and diminished in size. By day 3, many infected cells developed balloon-like, cytoplasmic swelling **(Figure 3.3-2).** The dose of the virus influenced the number of viable cells and the time course of appearance of these cytopathic effects. These observations agreed with previous reports of many scholars who used the MT-4 cell line to evaluate the anti-HIV activity of various compounds (Asres and Bucar, 2005; Harada and Yamamoto, 1985; Szucs *et al*., 1988; Weislow *et al*., 1989).

The virus infectivity test was performed to determine the infectious titer of the virus, which can cause cytopathic effects (CPE) in tissue culture over a reasonable period of 3 to 10

days while cells in culture remain viable (Gao *et al*., 2009; Reed and Muench, 1938). In addition, this procedure was performed to quantify the amount of virus required to produce a cytopathic effect in 50% of cell culture replicates ($TCID_{50}$) (Pauwels *et al*., 1988). CPE induced by the virus was observed under a microscope after 3–4 days. The $TCID_{50}$ was then calculated as described by Reed & Muench, (1938) and Ramakrishnan, (2016). For the anti-HIV activity testing, 640 µL of HIV-$1_{IIIB}$ virus at 1.26 $X10^8$ $TCID_{50}$/ml was used to infect 1 $x10^8$ cell/ml. All procedures were carried out following biosafety guidelines defined in the BMBL, NIH-CDC HHS Publication No. (CDC) 21-1112 (CDC, 2009).

### 3.3.4. Cytotoxicity assay

#### 3.3.4.1. Cytotoxicity test using Human T-Lymphocyte MT-4 cells

A cytotoxicity test was conducted to evaluate the cytotoxicity of the plant extracts by measuring cell death caused by the plant extracts. The assay was conducted using an MTT colorimetric assay as described by Mosmann (1983) and Pauwels *et al*., (1988). The MTT assay is based on the reduction of the yellow-colored tetrazolium salt MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by NAD(P)H-dependent cellular oxidoreductase enzymes (Berridge *et al*., 2005) to an insoluble dark-blue colored formazan that can be measured spectrophotometrically (Mosmann, 1983) (**Figure 3.3-3).** Formazan production indicates the number of viable cells; therefore, an increase or decrease in cellular viability results in a change in the amount of formazan formed, which indicates the degree of cellular cytotoxicity ($CC_{50}$) caused by the plant extract.

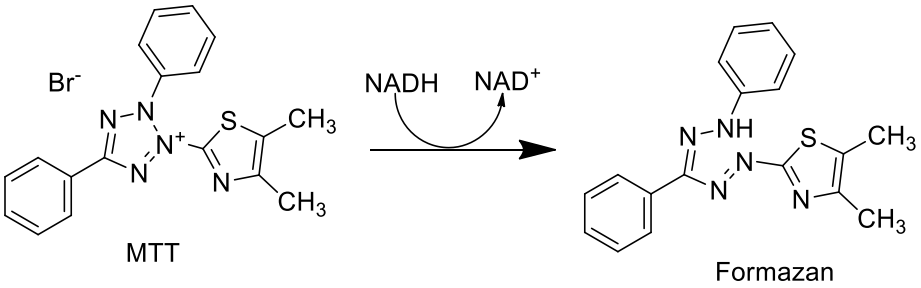



Figure 3.3-3: Conversion of MTT to Formazan by oxidoreductase enzymes

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in PBS to obtain a final concentration of 5 mg/ml and filtered to sterilize and remove insoluble residue (ACTG Laboratory Technologist Committee, 2004; Szucs *et al*., 1988). The assay was carried out in 96-well, flat-bottomed microtiter plates. To each well, 200 µL of MT-4 cells ($1\times10^5$ cells) in growth media was added. The plates were preincubated for 24 h at 37ºC to

allow stabilization. Then, 50 µL of the test compounds (at a concentration of 4 mg/ml) were added to the first column of the well. With a multichannel pipette, 50 µL was transferred (in triplicate) from the wells labeled 1 to wells labeled 2, and such transfers were continued (serial dilution), moving from left to right, changing tips prior to mixing contents of the next column of wells. Finally, 50 µL was discarded from the wells in column 12. Different concentrations (800 – $8.192\times10^5$ µg/mL) of test compounds were prepared through serial dilution. Each dilution was tested in triplicate. The negative control (NC) wells contained 50 µL of MT-4 cells in 0.5% DMSO (Weislow *et al*., 1989). Positive control (zidovudine, tenofovir, abacavir and nevirapine) drugs were also added in triplicate.

A 96-well microtiter plate containing the test compounds and positive and negative controls was incubated at 37°C in a humidified atmosphere of 5% $CO_2$ for 5 days. After incubation, 20 µl of MTT reagent (5 mg/ml MTT in phosphate-buffered saline) was added to each test well and control well. The plate was further incubated at 37°C in a $CO_2$ incubator for 4 hours.

After 4 hours of incubation, 100 µl of DMSO was added to dissolve the dark-blue formazan crystals from surviving cells (Bahuguna *et al*., 2017). After the formazan crystals were dissolved completely, the resulting optical density (OD) readings were measured relative to the controls on an ELISA plate reader at 570 nm at a reference wavelength of 620 nm (Szucs *et al*., 1988). First, the percentage viability was determined using the formula in equation **(4).**

$$\%\ \boldsymbol{cell\ viability} = \frac{\boldsymbol{Absorbance\ of\ treated\ cells}}{\boldsymbol{Absorbance\ of\ negative\ control}}\ \boldsymbol{X100} \text{ … … … … … ..Eq. 4}$$

The 50% cellular cytotoxicity concentration ($CC_{50}$) was defined as the concentration of the test compound that reduced the absorbance of the negative control by 50%. A dose-response curve was plotted to enable the calculation of the concentrations that reduced the number of viable cells by 50% ($CC_{50}$). The concentration that determined cell viability above 80% ($CC_{20}$) was chosen as the maximum non-toxic concentration (MNTC). The maximum concentration that caused cytotoxic effects were calculated by using the formula in equation **5.**

$$\%\ \boldsymbol{viability\ inhibition}\ (\boldsymbol{Emax_c}) = \frac{\boldsymbol{Abs.of-ve\ control - Abs.of\ treated\ wells}}{\boldsymbol{Abs.of-ve\ control}}\ \boldsymbol{x\ 100} \text{ … … ..Eq. 5}$$

### 3.3.4.2. FM-55-M1 cell viability assay

FM-55-M1 cell line is a tumor cell line from primary melanomas. FM-55-M1 cells (ECACC, UK) were maintained in a humidified incubator with 5% $CO_2$ at 37°C in RPMI-1640

media with L-glutamine supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were detached with trypsin, centrifuged at 200 RCF before resuspension in culture media, and seeded into transparent 96-well plates at 10,000 cells per well. Test compounds were serially diluted in DMSO before transferring into cell plates keeping DMSO at a constant concentration of 0.5% v/v. Cells were incubated with compounds for 72 hours before the addition of CellTiter 96 reagent (Promega, UK), and the absorbance was read at 490 nm in a Tecan Infinite M200 plate reader. Cell viability was determined relative to no compound controls.

### 3.3.5. Anti-HIV activity test

#### 3.3.5.1. Inhibition of Cytopathic effect of HIV

The effects of the test compounds in preventing cytopathic effects that occur as a result of HIV-1 replication were evaluated by MTT colorimetric assay. MT-4 cells suspended at 1 X $10^8$ cells/ml were infected with 640 µL of HIV-$1_{IIIB}$ virus at 1.26 X$10^8$ $TCID_{50}$/ml as described in section 3.3.4.1. After infection, 200 µL HIV-infected MT-4 cells (1 X$10^5$ cells/well) in growth media were added to each well. The plates were preincubated for 24 h at 37ºC to allow stabilization. Then, 50 µL of the test compounds (at a concentration of 4 mg/mL) were added to the first column of the well. With a multichannel pipette, 50 µL was transferred (in triplicate) from the wells labeled 1 to wells labeled 2. Such transfers were continued (serial dilution), moving from left to right, changing tips prior to mixing contents of the next column of wells. Finally, 50 µL was discarded from the wells in column 12. Different concentrations (800 to 8.192×$10^5$ µg/mL) of test compounds were prepared through serial dilution. Each dilution was tested in triplicates. The microtiter plates were incubated at 37ºC in a 5% $CO_2$ incubator for 5 days. Two negative controls, infected untreated cells, and uninfected untreated (mock) cells, and four positive controls (zidovudine, tenofovir, abacavir, and nevirapine) were also included. After 5 days of incubation, cell viability was determined by the MTT assay described in section 3.3.4.1 (Gustafson *et al*., 2004; Pauwels *et al*., 1988).

A dose-response curve was plotted to calculate the concentrations that reduced viral replication by 50% ($IC_{50}$) (Gustafson *et al*., 2004; Pauwels *et al*., 1988; Weislow *et al*., 1989). The effective inhibitory concentration at 50% ($IC_{50}$) is defined as the concentration of the test compound that achieves 50% protection in infected cultures. The efficacy of the test compounds, was determined by calculating the viral inhibition rate ($Emax_{AV}$) using equation **6**, as previously described by (Betancur-Galvis *et al*., 2002; Uğur *et al*., 2017).

$$\textbf{\textit{Viral inhibition rate}}\ (\textbf{\textit{Emax}}_{\textbf{\textit{AV}}}) = \frac{(OD_T)_{HIV} - (OD_C)_{HIV}}{(OD_C)_{mock} - (OD_C)_{HIV}}\ \boldsymbol{X\ 100} \ldots\ldots\ldots \text{Eq. 6}$$

Where, $(OD_T)_{HIV}$ is the optical density measured at a given concentration of the test compound in HIV-infected cells; $(OD_C)_{HIV}$ is the optical density measured for the negative control infected untreated cells; $(OD_C)_{mock}$ is the optical density measured for the negative control uninfected untreated cells. The selectivity index (SI) of the test compounds was calculated using equation **7**, as the ratio of 50% cytotoxic concentration ($CC_{50}$) to 50% effective concentration ($IC_{50}$). Thus, SI reflects both the antiviral activity and eventual toxicity of the test compounds. Thus, a high SI value indicates low toxicity of the test compound and high activity against the virus.

$$\textbf{Selectivity index (SI)} = \frac{\textbf{50\% cytotoxic concentration (CC50)}}{\textbf{50\% Inhibitory concentration (IC50)}} \ldots\ldots\ldots \text{Eq. 7}$$

### 3.3.5.2. *In vitro* reverse transcriptase enzyme inhibition assay

The HIV-1 reverse transcriptase inhibition assay was conducted i*n vitro* using the EnzChek® Reverse transcriptase assay kit (E-22064) provided by Molecular Probes (Eugene, Oregon, United States) following the manufacturer's instructions. Reverse transcriptase was purchased from Promega (Madison, Wisconsin, United States) (GoScript™ Reverse Transcriptase: catalogue number A5004), and enzyme dilution buffer was purchased from Thermo Fisher (Waltham, Massachusetts, United States) (catalogue number B19). Assays were performed in 96-well microtiter plate. GoScript™ RT was supplied at a concentration of 160 u/µL but was diluted to 53.3 u/µL in enzyme dilution buffer and frozen as 10 aliquots in volumes of 150 µL at −80ºC. A preliminary run was conducted using titrants of RT to determine the ideal starting concentration, using a ladder approach with a starting concentration of 26.7 u/µL. A final enzyme concentration of 1.1 u/µL was used. Frozen stock solution was diluted to 3.4 u/µL in enzyme dilution buffer to obtain the aliquot concentration to give the final concentration of 1.1 u/µL after the reactants were combined. The assay was initiated by annealing the template and primer. A volume of 5 µL of the poly(A) ribonucleotide template was mixed with 5 µL of oligo $d(t)_{16}$ primer and allowed to anneal for an hour at room temperature. After annealing, a 10 µL volume of primer was diluted by adding 1.99 mL of polymerization buffer. This final solution was used as the reaction mixture. The reaction was commenced by combining 20 µL of the reaction mixture and 30 µL of the pure compound and thereafter adding 10 µL of the enzyme at 3.4 u/µL. The reaction was allowed to continue for 1 hour at 25ºC and terminated with the addition of 30 µL of 15 mM EDTA. Then

180 µL of PicoGreen® dsDNA quantitation reagent solution was added to the wells and 5 minutes were allowed to pass before reading the fluorescence intensity, using 480 nm as the excitation wavelength and 520 nm as the emission wavelength. The pure compounds were prepared by dissolving to a 20 mg/mL concentration in dimethyl sulfoxide and diluting in nuclease-free water. A starting concentration of 50 µg/mL was used during initial testing, so the starting aliquot concentration was 100 µg/mL to ensure a final concentration of 50 µg/mL. A final concentration of 10 µg/mL of the pure compounds was used for the *in vitro* enzyme assay. All compounds were assayed in triplicate.

#### 3.3.5.3. Protease enzyme inhibition assay

The pure compounds were evaluated using an HIV-1 Protease Inhibitor Screening Kit (Fluorometric) (ab211106, Abcam, United Kingdom) at a concentration of 10 µg/mL as previously described by Aldhaher *et al*., (2017). Pepstatin (1 mM) and DMSO (1%, v/v) were used as a positive and negative control, respectively. Then, the HIV-1 protease fluorescent substrate was added, and fluorescence was measured (excitation/emission = 330/450 nm) in kinetic mode for 90 min at 37ºC using a PerkinElmer EnSpire plate reader. The assay was performed according to the manufacturer's instructions (Ab211106 HIV-1 Protease Inhibitor Screening Kit (Fluorometric), 2020).

### 3.3.6. Statistical analysis

The $CC_{50}$ and $IC_{50}$ values were calculated with GraphPad Prism v9 using the equation for sigmoidal dose-response (variable slope). Statistical significance in the comparison between control drugs and extract cytotoxicity and antiviral activity parameters ($CC_{50}$, $Emax_C$, $IC_{50}$, and $Emax_{AV}$) were determined by one-way ANOVA followed by Dunnett's post hoc tests. A difference was considered significant when $p < 0.05$.

## 3.4. *In silico* antiretroviral activity

For the computational study, Chem draw software version 19.1 was used for drawing the chemical structures, while MOE 2015 software was used for the molecular docking studies. To visualize the protein-ligand interactions, discovery Studio Visualizer 3.1 Studio (BIOVIA, 2017), LigPlot$^+$ (Laskowski & Swindells, 2011), and protein-ligand interaction profiler web-based tool (Adasme *et al*., 2021) was used (Dassault Systèmes BIOVIA, 2018).

Molecular docking studies were performed on MOE2015 software package using the following files download from Protein Data Bank: HIV-1 RT in complex with known inhibitor Nevirapine (PDB ID: 1JLB), multidrug HIV-1 protease in complex with Atazanavir (3EL9),

wild-type HIV protease in complex with Darunavir (4LL3), and wild-type HIV-1 protease in complex with Lopinavir (6DJ1).

The proteins were prepared by first removing all water molecules, and in the case of proteases also the phosphate, formate and chloride ions present in the PDB file. Then, hydrogens were added, and the structures were protonated. For the ligands, they were energy minimized using force field MMFF94x on MOE2015.

To validate the docking protocol used, known inhibitors were removed from their corresponding binding pockets and redocked. The Root Mean Square Deviation (RMSD) value from the known co-crystallized conformation was < 3 Å in all cases. Compounds were then docked using MOE2015 with triangle matcher, scoring by London dG, 30 poses as placement method and rigid receptor, GBVI/WSA dG 5 poses as refinement method in all targets. The lowest scoring affinity pose in each ligand was used to study the ligand interactions.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

This research investigated the cytotoxicity and anti-HIV activity of crude extracts, solvent fractions, and isolated pure compounds from three Kenyan *Croton* plants. Furthermore, the mode of action of pure compounds was predicted using computational methods. The results of this study are presented and discussed below.

## 4.1. Yield for plant extraction

As shown in **Table 4.1-1.**, *C. megalocarpus* has a higher percentage yield than the other crude extracts. **Table 4.1-2** presents the yields after liquid-liquid extraction.

Table 4.1-1: Yields of extracts obtained from *Croton* Species

| **Plant species** | **Part extracted** | **Mass (gm) (1:1 DCM: MeOH)** | **Percentage yield (%w/w)** |
|---|---|---|---|
| *C. macrostachyus* | Leaf | 420 gm | 12.6% |
| | Stem Bark | 482 gm | 9.5% |
| *C. megalocarpus* | Leaf | 350 gm | 17.5% |
| | Stem Bark | 330 gm | 6.5% |
| *C. dichogamus* | Twigs | 450 gm | 9.0% |

Table 4.1-2: Weight of liquid/liquid partition of the crude extracts

| **Plant species** | **Part extracted** | **Weight of partitioned** | | | |
|---|---|---|---|---|---|
| | | **Hexane fraction** | **DCM Fraction** | **Ethyl acetate** | **Methanol fraction** |
| *C. macrostachyus* | Leaf | 67 gm (30%) | 104 gm (46.6%) | 38 gm (17%) | 14 gm (6.3%) |
| | Stem bark | 50 gm (28.6%) | 22 gm (12.6%) | 28 gm (16%) | 75 gm (42.9%) |
| *C. megalocarpus* | Leaf | 60 gm (22.5%) | 100 gm (37.5%) | 22 gm (8.2%) | 85 gm (31.8%) |
| | Stem bark | 11 gm (0.08%) | 80 gm (56.3%) | 40 gm (28.2%) | 11 gm (7.7%) |
| *C. dichogamus* | Twigs | 34 gm (15.8%) | 97 gm (45.1%) | 4 gm (1.9%) | 80 gm (37.2%) |

### 4.2. Cytotoxicity and antiretroviral activity of the crude extracts of *C. macrostachyus, C. megalocarpus,* and *C. dichogamus*

The cytotoxicity and anti-HIV-1 activity of the crude extracts (1:1 $CH_2Cl_2$:$CH_3OH$) of the three plants are summarized in **Table 4.2-1**. The antiviral assay results indicated that, the crude leaf extracts of *C. megalocarpus* (ELC) and crude twig part extracts of *C. dichogamus* (CDC) extracts have the highest anti-HIV activity. The CDC displayed highest anti-HIV activity ($Emax_{AV}$ = 73.7%) with wider selectivity index (SI) of 3116.0. The potency of CDC ($IC_{50}$ = 0.001 ± 0.00 µg/mL), is similar to AZT and its efficacy is comparable to NVP and better than ABC. Though the crude bark extract of *C. megalocarpus* (EC) has the best efficacy ($Emax_{AV}$ = 85.9 %) of the extracts, it was the least potent with an $IC_{50}$ value of 3.73 ± 1.20 µg/mL and also displayed narrow selectivity index (SI + 0.9) indicating its cytotoxicity (**Table 4.2-1**). All the tested extracts have shown cytotoxiciy as compared to approved drugs with $Emax_C$ greater than 36.8%. The crude (1:1 v/v DCM: Methanol) bark extract of *C. macrostachyus* (AC) was highly cytotoxic as compared to other extracts by inhibiting cell growth by 49.9 % with $CC_{50}$ value of 45.9 ± 0.12 µg/mL. The crude leaf extract of *C. macrostachyus* (ALC) caused cytotoxicity at lowest $CC_{50}$ value of 0.008 ± 0.00 µg/mL, comparable to AZT and NVP. Among the FDA approved antiretroviral drugs, Tenofovir was the least cytotoxic with a low $Emax_C$ and high $CC_{50}$. NVP was cytotoxic of the approved drugs.

**Table 4.2-1: Cytotoxicity and anti-HIV activities of crude extracts of three *C.* species**

| Materials | Cytotoxicity | | | Antiviral activity | | SI |
|---|---|---|---|---|---|---|
| | MNTC (µg/mL) | $CC_{50}$ (µg/mL) | $Emax_C$ (%) | $IC_{50}$ (µg/mL) | $Emax_{AV}$ (%) | |
| **AZT** | 0.38 ± 0.19 | 0.53 ± 0.29 | 36.28 ± 0.83 | 0.002 ± 0.00 | 83.5 ± 0.57 | 279.4 |
| **TDF** | 4.92 ± 0.71 | 6.73 ± 0.24 | 13.17 ± 0.43 | 0.04 ± 0.01 | 80.55 ± 0.46 | 176.5 |
| **ABC** | 0.18 ± 0.03 | 0.26 ± 0.00 | 17.83 ± 0.57 | 0.05 ± 0.031 | 58.67 ± 0.43 | 5.0 |
| **NVP** | 0.57 ± 0.0 | 0.82 ± 0.0 | 39.13 ± 0.65 | 0.24 ± 0.09 | 72.53 ± 0.47 | 3.5 |
| **EC** | 0.79 ± 0.62 | 3.27 ± 0.12 | 43.63 ± 0.05 | 3.73 ± 1.20 | 85.89 ± 0.85 | 0.9 |
| **ELC** | 20.56 ± 0.00 | 27.7 ± 0.65 | 36.77 ± 0.62 | 0.05 ± 0.03 | 74.65 ± 0.06 | 571.3 |
| **AC** | 8.33 ± 0.43 | 45.9 ± 0.12 | 49.96 ± 0.21 | 1.56 ± 0.39 | 74.36 ± 0.74 | 29.4 |
| **ALC** | 0.006 ± 0.00 | 0.008 ± 0.00 | 37.42 ± 0.44 | 0.05 ± 0.01 | 73.80 ± 0.89 | 0.2 |
| **CDC** | 3.35 ± 0.62 | 4.70 ± 0.26 | 42.37 ± 0.71 | 0.001 ± 0.00 | 73.74 ± 0.48 | 3116.0 |

Results are shown as means ± S.E. M (n=3)

AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; EC, *C. megalocarpus* bark extract; ELC, *C. megalocarpus* leaf extract; AC, *C. macrostachyus* bark extract, ALC, *C. macrostachyus* leaf extract; CDC, *C. dichogamus* aerial part extract; MNTC, Maximum non-toxic concentration; $CC_{50}$, 50% cytotoxic concentration; $Emax_C$, Maximum cytotoxic effect %; $IC_{50}$, 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

## 4.3. Evaluation of cytotoxicity and antiretroviral activity of solvent fractions of *C. macrostachyus, C. megalocarpus,* and *C. dichogamus*

### 4.3.1. Cytotoxicity and anti-HIV activity of solvent fractions of *C. megalocarpus*

As depicted in **Table 4.3-1,** the hexane (EH) and ethyl acetate (EE) fractions of the bark showed the highest $CC_{50}$ values of 201.6 ± 0.95 and 162.4 ± 0.65 µg/mL, respectively. However, despite having a high $CC_{50}$ value, the EH fraction displayed the highest cytotoxic effect by inhibiting more than 79% cell viability. Similarly, the ethyl acetate fraction of the leaf (ELE) showed the highest cytotoxicity by inhibiting 80.3% of cell viability. In comparison, the methanol fraction of the leaves (ELM) and the ethyl acetate fraction of the bark (EE) showed minor cytotoxic effects than the other extracts tested. As depicted in **Figure 4.3-1,** the $CC_{50}$ of the EH, EE, and ELE fractions of *C. megalocarpus* was significantly ($P < 0.001$) higher than that of the control drugs, which indicates that it requires higher concentrations of the fractions for cytotoxic effects to appear. In addition, despite having close or higher $CC_{50}$ values, which may indicate the safety of the tested substances, all the tested extracts had higher cytotoxic effects ($Emax_C$s) than the control drugs, indicating their possible high toxic effects with increasing concentration levels.

**Table 4.3-1: Cytotoxicity and anti-HIV activities of solvent fractions of *C. megalocarpus***

| Materials | Cytotoxicity | | Antiviral activity | | | SI |
|---|---|---|---|---|---|---|
| | MNTC (µg/mL) | $CC_{50}$ (µg/mL) | $Emax_C$ (%) | $IC_{50}$ (µg/mL) | $Emax_{AV}$ (%) | |
| AZT | 0.38 ± 0.19 | 0.53 ± 0.29 | 36.28 ± 0.83 | 0.002 ± 0.00 | 83.5 ± 0.57 | 279.4 |
| TDF | 4.92 ± 0.71 | 6.73 ± 0.24 | 13.17 ± 0.43 | 0.04 ± 0.01 | 80.55 ± 0.46 | 176.5 |
| ABC | 0.18 ± 0.03 | 0.26 ± 0.00 | 17.83 ± 0.57 | 0.05 ± 0.031 | 58.67 ± 0.43 | 5.0 |
| NVP | 0.57 ± 0.0 | 0.82 ± 0.0 | 39.13 ± 0.65 | 0.24 ± 0.09 | 72.53 ± 0.47 | 3.5 |
| EH | 80.66 ± 0.55 | 201.6 ± 0.95 | 79.09 ± 0.91 | 0.26 ± 0.00 | 89.00 ± 0.01 | 780.5 |
| ED | 0.16 ± 0.05 | 5.07 ± 0.65 | 78.03 ± 0.98 | 0.04 ± 0.02 | 80.94 ± 0.29 | 139.4 |
| EE | 134.1 ± 0.05 | 162.4 ± 0.65 | 48.93 ± 0.05 | 0.47 ± 0.12 | 88.01 ± 0.00 | 346.3 |
| EM | 5.57 ± 0.69 | 25.2 ± 0.05 | 59.81 ± 0.25 | 0.46 ± 0.11 | 93.00 ± 0.00 | 55.2 |
| ELH | 3.48 ± 0.36 | 4.66 ± 0.41 | 31.49 ± 0.44 | 0.002 ± 0.00 | 72.93 ± 0.85 | 2972.0 |
| ELD | 0.81 ± 0.39 | 1.17 ± 0.151 | 36.64 ± 0.26 | 0.001 ± 0.00 | 82.87 ± 0.04 | 616.4 |
| ELE | 47.62 ± 0.25 | 148.3 ± 0.65 | 80.3 ± 0.71 | 12.71 ± 0.13 | 95.63 ± 0.38 | 11.7 |
| ELM | 0.18 ± 0.001 | 0.29 ± 0.09 | 30.02 ± 0.99 | 0.001 ± 0.00 | 93.40 ± 0.6 | 186.3 |

Results are shown as means ± S.E. M (n=3)

AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; EH, hexane fraction of *C. megalocarpus* bark extract; ED, dichloromethane fraction of *C. megalocarpus* bark extract; EE, ethylacetate fraction of *C. megalocarpus* bark extract; EM, methanol fraction of *C. megalocarpus* bark extract; ELH, hexane fraction of *C. megalocarpus* leaf extract; ELD, dichloromethane fraction of *C. megalocarpus* leaf extract; ELE, ethylacetate fraction of *C. megalocarpus* leaf extract; ELM, methanol fraction of *C. megalocarpus* leaf extract; MNTC, maximum nontoxic concentration; $CC_{50}$, 50% cytotoxic concentration; $Emax_C$, maximum cytotoxic effect %; $IC_{50}$, 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

As shown in **Table 4.3-1**, all the tested solvent fractions showed inhibition of cytopathic effects at an $IC_{50}$ value lower than the maximum non-toxic concentration (MNTC)**.** Comparing the effect of the fractions in preventing CPE with the reference drugs, all the extracts showed similar efficacy to that of the control drugs. They showed non-significantly different $Emax_{AV}$ values to that of the control drugs **(Figure 4.3-2)**. The solvent fractions tested also showed $IC_{50}$ values not significantly different from that of the control drugs, except for extract ELE, which displayed lower potency, as it showed a significantly higher $IC_{50}$ value. The ethyl acetate (ELE) and methanol (ELM) fractions of the leaves prevented CPE by 95.63% and 93.4%, respectively. Similarly, the hexane fraction of the bark (EH) showed an $Emax_{AV}$ value of 89.0 %. Despite their highest anti-HIV activity, these fractions displayed higher cytotoxicity. However, the bark's ethyl acetate (EE) fraction showed the highest anti-HIV activity ($Emax_{AV}$= 88.01 %) at an $IC_{50}$ value of 0.47 ± 0.12 µg/mL with a selectivity index of 346.3. This is comparable with the activity if AZT and TDF. Putting the cytotoxicity and anti-HIV activity all together, the EE fraction was found to be the safest and active fraction of *C. megalocarpus.*

#### 4.3.1.1.Discussion

The cytotoxicity and anti-HIV activity of solvent fractions of *C. megalocarpus* was tested against laboratory-adapted strains of HIV ($HIV\text{-}1_{IIIB}$) in human T-lymphocytic MT-4 cells to find possible anti-HIV drugs from natural sources. Medicinal properties of *C. megalocarpus* bark, leaves, and roots have been reported to treat or manage various human and animal diseases across the species' distributional area. Maroyi, (2017a) examined and documented 41 ethnomedicinal uses of *C. megalocarpus* in the literature, with Kenya accounting for 87.5 percent of the ethnomedicinal uses.

*C. megalocarpus* is used to cure a variety of ailments, including respiratory difficulties (Muthee *et al*., 2011), fever (Ichikawa, 1987; Timothy Johns *et al*., 1994; Nanyingi *et al*., 2008), and wounds (Kamau *et al*., 2016; Nanyingi *et al*., 2008). For example, *C. megalocarpus* is used in Kenya to treat constipation (Kipkore *et al*., 2014) as well as backache, chest difficulties (Bussmann, 2006; Kiringe, 2006), malaria (Bussmann, 2006; Cyrus *et al*., 2008; Fratkin, 1996), and stomach ache (Bussmann, 2006; Fratkin, 1996; Kiringe, 2006). In addition, HIV-infected patients utilize *C. megalocarpus* leaf and root decoctions to treat pneumonia, respiratory difficulties, wounds, and diabetes (Keter & Mutiso, 2012). Four solvent fractions of *C. megalocarpus* leaf and stem bark were tested for their capacity to inhibit HIV-1

replication in this study. In addition, the MTT test was used to assess the toxicity of these extracts in human T-lymphocytic MT-4 cells.

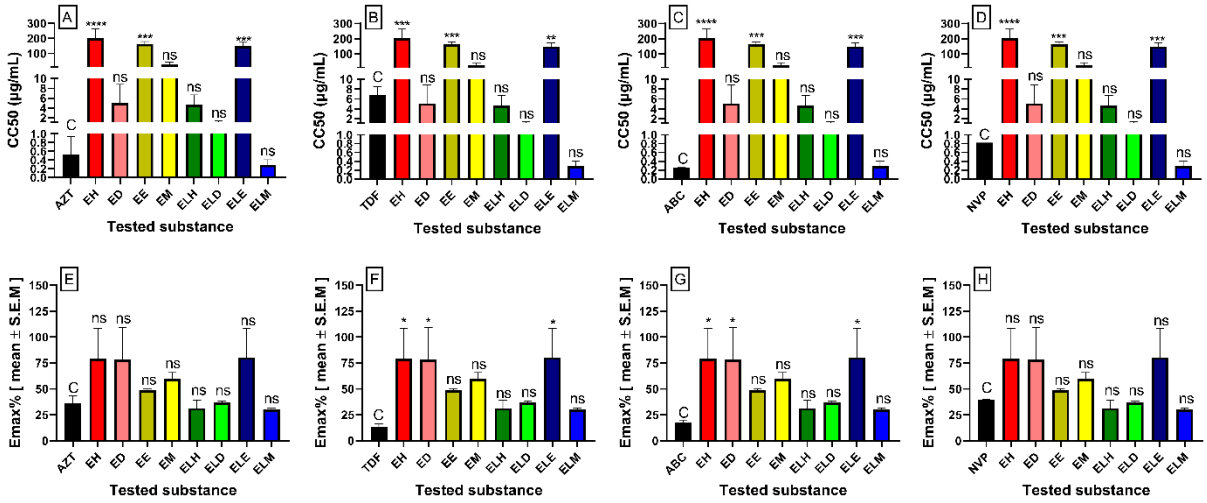



Figure 4.3-1: Cytotoxicity of solvent fractions of *C. megalocarpus*

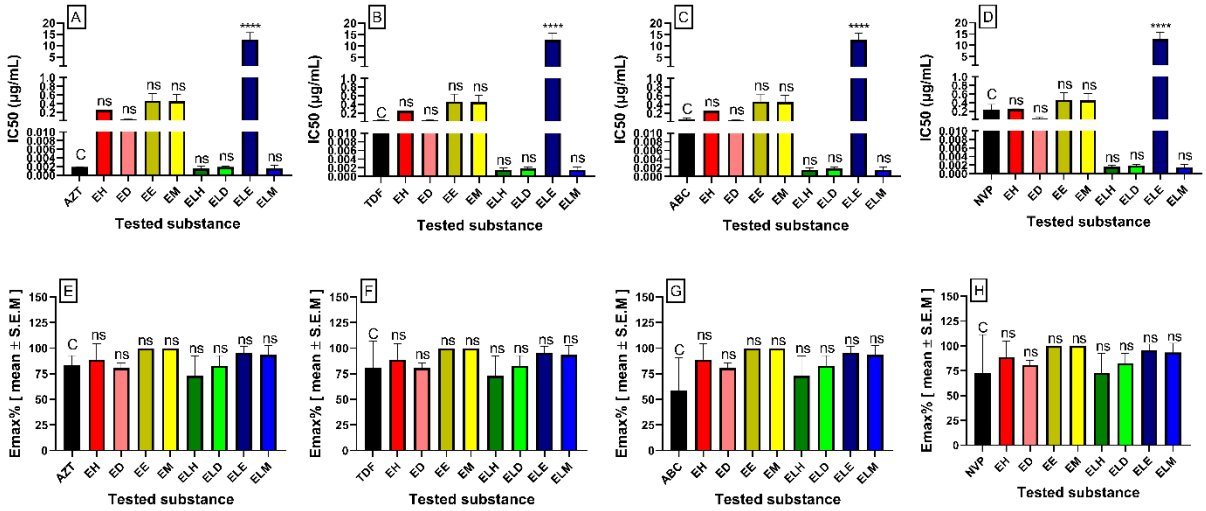



Figure 4.3-2: Anti-HIV activity of solvent fractions of three *C. megalocarpus*

The results are expressed as the mean of three independent experiments ± S.E.M. C, control; AC, *C. macrostachyus* bark extract; EH, Hexane fraction of *C. megalocarpus* bark extract; ED, Dichloromethane fraction of *C. megalocarpus* bark extract; EE, Ethylacetate fraction of *C. megalocarpus* bark extract; EM, methanol fraction of *C. megalocarpus* bark extract; ELH, Hexane fraction of *C. megalocarpus* leaf extract; ELD, Dichloromethane fraction of *C. megalocarpus* leaf extract; ELE, Ethylacetate fraction of *C. megalocarpus* leaf extract; ELM, methanol fraction of *C. megalocarpus* leaf extract; ns; not significant, *Denotes p-value < 0.05, ** Denotes p-value <0.01, *** Denotes p-value < 0.001.

The hexane (EH) and dichloromethane (ED) bark fractions had the most cytotoxicity, blocking 78-80 percent of the cell growth. The leaf's methanol fraction (ELM), on the other hand, showed the least cytotoxicity, inhibiting 30% of cell growth compared to the ethyl acetate fraction of the leaves (ELE), which inhibited cell viability by 80%. Cytotoxic chemicals

produce irreversible alterations in organelles and membrane transport systems and changes in cellular structures and activities.

The bark's ethyl acetate (EE) fractions had the highest efficacy in inhibiting the virus's cytopathic effect, followed by the leaves' ethyl acetate (ELE) and methanol fractions (ELM), which inhibited CPE by 95.63 percent and 93.4 percent, respectively. The bark fractions have shown to be less cytotoxic and more effective at reducing CPE than the leaf fractions. The EE and leaf dichloromethane (ELD) fractions had higher SI values of 346.3 and 616.4, respectively. Only extract ELE had lesser antiviral efficacy (EmaxAV = 72.93%) in this experiment while keeping a high selectivity index value (SI=2972). The selectivity index represents the test drugs' antiviral efficacy as well as their eventual toxicity. A high SI value suggests that the test substance is not harmful and has high antiviral activity. With an $IC_{50}$ value comparable to that of the control medicines ($IC_{50}$= 0.47, SI=346.3), the ethyl acetate fraction of the bark showed potential anti-HIV efficacy. Similarly, with SI values of 55.2 and 186.3, the methanol fractions of both the bark and leaf extracts respectively demonstrated excellent anti-HIV activity, preventing more than 80 percent of CPE in virus-infected cells. These findings corroborate those of a prior study on the same plant's pharmacologic effects. According to Matu & Van Staden, (2003), the methanol extract of *C. megalocarpus* has the strongest anti-inflammatory effect due to cyclooxygenase enzyme inhibition.

The terpenoids found in the solvent fractions may be responsible for the fractions' anti-HIV efficacy. Betulin, a triterpenoid isolated from the stem bark of *C. megalocarpus*, was shown to have antiviral effects in cell cultures infected with herpes simplex type I, influenza FPV/Rostock, and ECHO 6 viruses in a prior study (Pavlova *et al*., 2003). Lupeol, a triterpenoid derived from the same plant, has shown anti-HSV action *In vitro* (M. K. Parvez *et al*., 2019). Several terpenoids extracted from plants have been shown to have anti-HIV action in previous research. Celasdin-B, a triterpene isolated from *Calophyllum lanigerum*, had an $EC_{50}$ of 0.8 μg/ml and inhibited HIV replication in H9 lymphocyte cells (Y. H. Kuo & Kuo, 1997). In addition, Eurifoloids E and F, diterpenoids derived from *Euphorbia neriifolia*, were found to have considerable anti-HIV activity in a separate investigation, with $EC_{50}$ values of 3.58 0.31 (SI = 8.6) and 7.40 0.94 μM (SI = 10.3), respectively (Zhao *et al*., 2014). Trigonothyrins A-C, highly functionalized daphnane diterpenoids isolated from the stems of *Trigonostemon thyrsoideum*, have shown to suppress HIV-1-induced cytopathic effects in another investigation, with an $IC_{50}$ value of 2.19 μg/mL and SI more than 90 (Zhang *et al*., 2010).

### 4.3.2. Cytotoxicity and anti-HIV activity of solvent fractions of *C. macrostachyus*

**Table 4.3-2** summarize the cytotoxicity and anti-HIV activity of four solvent fractions of *C. macrostachyus* leaf and stem bark. At $CC_{50}$ values of 0.25 ± 0.07 µg/mL and 0.58 ± 0.05 µg/mL, respectively, the methanol (AM) and hexane (AH) fractions of *C. macrostachyus* bark were shown to be cytotoxic, decreasing cell growth ($Emax_C$) by more than 49%. The ethyl acetate component of the bark (AE) was more cytotoxic, reducing cell viability by 63%. With an $Emax_C$ value of 29.33 percent, the dichloromethane fraction (AD) exhibited reduced cytotoxicity. With a $CC_{50}$ value of 173.5 µg/mL, the hexane fraction of the leaves (ALH) demonstrated the most negligible cytotoxicity, followed by the ethyl acetate fraction of the leaves (ALE) with a $CC_{50}$ value of 165.9 µg/mL. Finally, the leaf's methanol fraction (ALM) had the maximum cytotoxicity, with a $CC_{50}$ of 6.28 ± 0.51 µg/mL and a 54.47 percent inhibition of cell viability. The cytotoxic effect of the examined extracts and control medicines on the MT4 cell line was determined using the MTT assay and extracts AE, AM, and ALM were shown to have significantly higher cytotoxicity ($P < 0.01$) than TDF and ABC (**Figure 4.3-3**). However, most of the extracts examined had a larger maximum cytotoxic effect (Emaxc) than the control medicines, indicating that they could have more harmful effects at higher concentrations.

With an $IC_{50}$ value of 0.04 ± 0.00 µg/mL, the dichloromethane fraction of the bark had better anti-HIV activity. Similarly, the ethyl acetate fraction of the bark (AE) reduced the cytopathic effect (CPE) by 86.53 percent with an $IC_{50}$ value of 3.69 ± 0.11 µg/mL, which is substantially lower than the maximum non-toxic concentration (141.9 ± 0.25 µg/mL), yielding selectivity index of 46.6. The hexane fraction of the bark (AH) prevented 90.8 percent of cell death caused by CPE while being less potent ($IC_{50}$= 8.09 ± 0.55 µg/mL). The anti-HIV activity of the methanol fraction (AM) was lower ($Emax_{AV}$= 67.91%; SI= 0.5). (**Table 4.3-2**).

With $IC_{50}$ values of 0.02 ± 0.01 µg/mL, the hexane fraction of the leaves had the strongest anti-HIV activity, blocking 83 percent of the virus-induced cytopathic impact, yielding the highest selectivity index (SI) of 9752. The dichloromethane fraction of the leaf, on the other hand, had a reduced efficiency in suppressing viral reproduction, with an $IC_{50}$ of 5.37 ± 0.13 µg/mL and a SI of 0.5. The antiviral activity of the tested fractions revealed that the control medications and the tested fractions had antiviral efficacy that was nearly identical, with $Emax_{AV}$ values that were non-significantly different (**Figure 4.3-4**). Furthermore, with the exception of extracts AH and ALD, which revealed lesser potency as seen by the

significantly ($P < 0.01$) greater $IC_{50}$ value, most of the fractions had $IC_{50}$ values that were not statistically different from those of the control medicines, showing comparable potency.

Table 4.3-2: Cytotoxicity and anti-HIV-1 activities of solvent fractions of *C. macrostachyus*

| Materials | Cytotoxicity | | | Antiviral activity | | SI |
|---|---|---|---|---|---|---|
| | MNTC (µg/mL) | $CC_{50}$ (µg/mL) | $Emax_C$ (%) | $IC_{50}$ (µg/mL) | $Emax_{AV}$ (%) | |
| **AZT** | 0.38 ± 0.19 | 0.53 ± 0.29 | 36.28 ± 0.83 | 0.002 ± 0.00 | 83.5 ± 0.57 | 279.4 |
| **TDF** | 4.92 ± 0.71 | 6.73 ± 0.24 | 13.17 ± 0.43 | 0.04 ± 0.01 | 80.55 ± 0.46 | 176.5 |
| **ABC** | 0.18 ± 0.03 | 0.26 ± 0.00 | 17.83 ± 0.57 | 0.05 ± 0.031 | 58.67 ± 0.43 | 5.0 |
| **NVP** | 0.57 ± 0.0 | 0.82 ± 0.0 | 39.13 ± 0.65 | 0.24 ± 0.09 | 72.53 ± 0.47 | 3.5 |
| **AH** | 0.18 ± 0.09 | 0.25 ± 0.07 | 49.11 ± 0.16 | 8.09 ± 0.55 | 90.8 ± 0.88 | 0.03 |
| **AD** | 0.99 ± 0.26 | 1.13 ± 0.16 | 29.33 ± 0.18 | 0.04 ± 0.00 | 87.61 ± 0.56 | 26.2 |
| **AE** | 141.9 ± 0.25 | 171.8 ± 0.1 | 63.05 ± 0.32 | 3.69 ± 0.11 | 86.53 ± 0.71 | 46.6 |
| **AM** | 0.08 ± 0.07 | 0.58 ± 0.05 | 52.74 ± 0.44 | 1.15 ± 0.03 | 67.91 ± 0.55 | 0.5 |
| **ALH** | 142.2 ± 0.4 | 173.5 ± 0.8 | 39.44 ± 0.65 | 0.02 ± 0.01 | 82.78 ± 0.67 | 9752.7 |
| **ALD** | 1.328 ± 0.66 | 2.47 ± 1.37 | 29.64 ± 0.54 | 5.37 ± 0.13 | 76.45 ± 0.14 | 0.5 |
| **ALE** | 100.9 ± 0.51 | 165.9 ± 0.65 | 42.89 ± 0.84 | 0.05 ± 0.03 | 83.81 ± 0.01 | 3186.7 |
| **ALM** | 7.92 ± 0.35 | 6.28 ± 0.51 | 54.47 ± 0.06 | 0.05 ± 0.02 | 77.04 ± 0.37 | 138.6 |

Results are shown as mean ± S.E. M (n=3)

AZT, zidovudine; TDF, tenofovir; ABC, abacavir; NVP, nevirapine; AH, hexane fraction of *C. macrostachyus* bark extract; AD, dichloromethane fraction of *C. macrostachyus* bark extract; AE, ethylacetate fraction of *C. macrostachyus* bark extract; AM, methanol fraction of *C. macrostachyus* bark extract; ALH, hexane fraction of *C. macrostachyus* leaf extract; ALD, dichloromethane fraction of *C. macrostachyus* leaf extract; ALE, ethylacetate fraction of *C. macrostachyus* leaf extract; ALM, methanol fraction of *C. macrostachyus* leaf extract; MNTC, maximum nontoxic concentration; $CC_{50}$, 50% cytotoxic concentration; $Emax_{C,}$ maximum cytotoxic effect %; $IC_{50,}$ 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

#### 4.3.2.1.Discussion

Ethnobotanical and ethnopharmacological studies have confirmed the traditional usage of *C. macrostachyus* in treating various infectious disorders. HIV, hepatitis, amoebiasis, epilepsy, anthrax, jaundice, leprosy, malaria, cancer, ringworm, and skin illnesses are all treated with *C. macrostachyus* (Agisho *et al*., 2014; Focho *et al*., 2009; Tefera *et al*., 2012). Leaf and root decoction of the leaves are used to treat cough, back pain, bleeding, skin problems, warts, pneumonia, and wounds (Kareru *et al*., 2007; Obey *et al*., 2016a; Okello *et al*., 2010b; Pascaline *et al*., 2010). In addition, *C. macrostachyus* has also been utilized in the past to treat viral infections. In Ethiopia, for example, a bark decoction of *C. macrostachyus* mixed with *Juniperus procera* leaves, *Eragrostis tef* (Zucc.) stems, and roots of *Cyphostemma cyphopetalum* is used as a rabies herbal treatment (Maroyi, 2017d). Based on this information, we tested the cytotoxicity and anti-HIV activity of solvent fractions of *C. macrostachyus* in human T-lymphocytic MT-4 cells against laboratory-adapted strains of HIV (HIV-1IIIB).

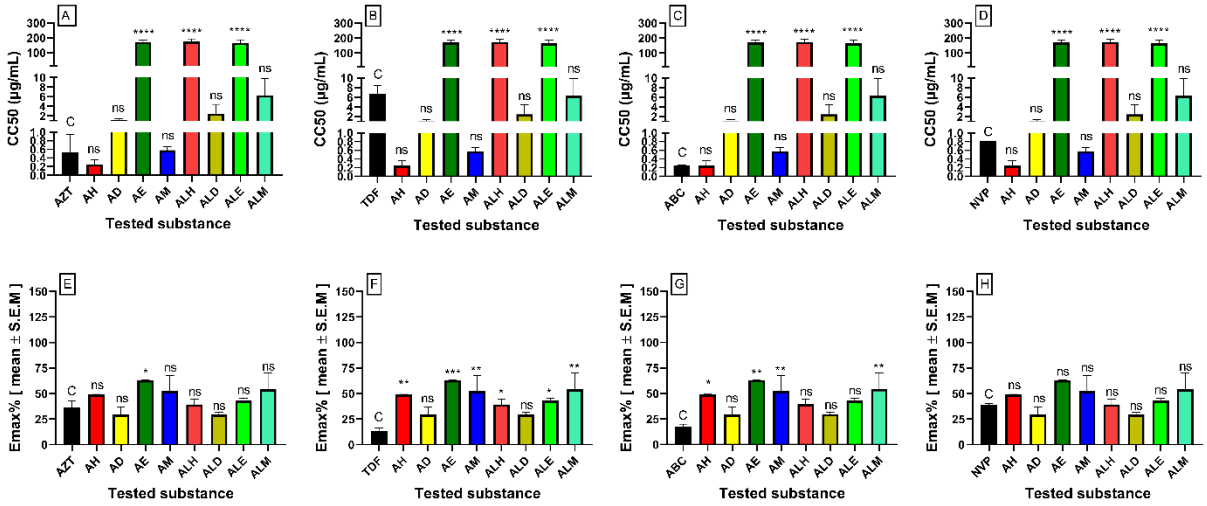



Figure 4.3-3: Cytotoxic effect for solvent fractions for *C. macrostachyus*

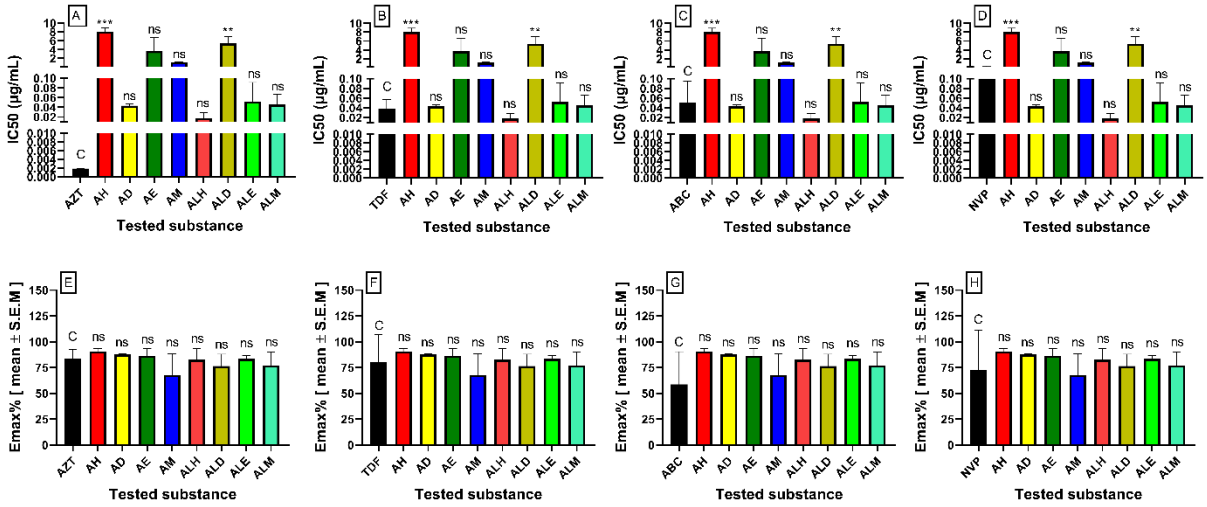



Figure 4.3-4: Anti-HIV activity of solvent fractions of *C. macrostachyus*

The results are expressed as the mean of three independent experiments ± S.E.M. C, control; AH, hexane fraction of *C. macrostachyus* bark extract; AD, dichloromethane fraction of *C. macrostachyus* bark extract; AE, ethylacetate fraction of *C. macrostachyus* bark extract; AM, methanol fraction of *C. macrostachyus* bark extract; ALH, hexane fraction of *C. macrostachyus* leaf extract; ALD, dichloromethane fraction of *C. macrostachyus* leaf extract; ALE, ethylacetate fraction of *C. macrostachyus* leaf extract; ALM, methanol fraction of *C. macrostachyus* leaf extract; ns; not significant, *Denotes p value < 0.05, ** Denotes p value <0.01, *** Denotes p value < 0.001.

The $CC_{50}$ values of AE, ALH, and ALE were significantly higher than the control medicines, indicating that larger concentrations of these fractions are necessary for cytotoxic effects to manifest (**Figure 4.3-3**). ALH showed promise by suppressing 82.78 percent of the virus-induced cytopathic effect at a concentration substantially lower than the highest cytotoxic concentration ($IC_{50}$= 0.02 ± 0.01 µg/mL; MNTC=142.2 ± 0.4 µg/mL; SI of 9752.7), demonstrating the fraction's efficacy and safety. Similarly, the leaves' ethyl acetate fraction

inhibited 83.81 percent CPE ($IC_{50}$=0.05 ± 0.03 µg/mL; MNTC=100.9 ± 0.51 µg/mL; SI of 3186.7). The selectivity index represents the test drugs' antiviral efficacy as well as their eventual toxicity. A high SI value suggests that the test substance is not harmful and has high antiviral activity.

Pentacyclic triterpenoids in the hexane and ethyl acetate leaf fractions could be responsible for the antiviral activity ($Emax_{AV}$ > 80.8 percent). Betulin, betulinic acid, lupeol, and lupeol-acetate were among the triterpenoids identified previously from *C. macrostachyus* (Addae-Mensah *et al*.,, 1992; Maroyi, 2017b; Tala *et al*., 2013; Tene *et al*., 2009). Gutiérrez-Nicolás *et al*., (2012), found that lupeol acetate reduces viral entrance into CD4 cells, while betulinic acid inhibits HIV-1 RT (Esposito *et al*., 2013). The activity of the hexane and ethyl acetate fractions of the leaves of *C. macrostachyus* could be attributed to the phytochemical constituents.

### 4.2.3. Cytotoxicity and anti-HIV activity of solvent fractions of *C. dichogamus*

**Table 4.3-3**, summarize the cytotoxicity and anti-HIV activity of solvent fractions of *C. dichogamus*. The CC50 value for the methanol fraction was 19.58 ± 0.79 µg/mL, indicating that cytotoxic effects require greater concentrations of the fraction. Furthermore, the dichloromethane fraction was linked to a considerably ($P < 0.01$) higher maximum cytotoxic effect, inhibiting more than 60% of cell viability ($Emax_C$=66.22 percent), implying that higher doses could lead to more deleterious effects (**Figure 4.3-5**).

The methanol fraction of *C. dicohgamus* had high anti-HIV activity ($Emax_{AV}$=90.83 percent), at a much lower concentration ($IC_{50}$ = 0.06 ± 0.01 µg/mL) than the maximum non-toxic concentration (MNTC=15.4 µg/mL), indicating the extract's safety (**Table 4.3-3**). The antiviral effect of the studied extracts was comparable to that of the control medicines, as shown in **Figure 4.3-6**. There was no significant difference in their viral-induced CPE inhibition, $IC_{50}$ nor $Emax_{AV}$ values.

Table 4.3-3: Cytotoxicity and anti-HIV-1 activities of solvent fractions of *C. dichogamus*

| Materials | Cytotoxicity | | | Antiviral activity | | SI |
|---|---|---|---|---|---|---|
| | MNTC (µg/mL) | $CC_{50}$ (µg/mL) | $Emax_C$ (%) | $IC_{50}$ (µg/mL) | $Emax_{AV}$ (%) | |
| **AZT** | 0.38 ± 0.19 | 0.53 ± 0.29 | 36.28 ± 0.83 | 0.002 ± 0.00 | 83.5 ± 0.57 | 279.4 |
| **TDF** | 4.92 ± 0.71 | 6.73 ± 0.24 | 13.17 ± 0.43 | 0.04 ± 0.01 | 80.55 ± 0.46 | 176.5 |
| **ABC** | 0.18 ± 0.03 | 0.26 ± 0.00 | 17.83 ± 0.57 | 0.05 ± 0.031 | 58.67 ± 0.43 | 5.0 |
| **NVP** | 0.57 ± 0.0 | 0.82 ± 0.0 | 39.13 ± 0.65 | 0.24 ± 0.09 | 72.53 ± 0.47 | 3.5 |
| **CDD** | 49.7 ± 0.61 | 167 ± 0.18 | 66.22 ± 0.18 | 0.03 ± 0.02 | 81.3 ± 0.23 | 5491.61 |
| **CDE** | 0.84 ± 0.08 | 0.99 ± 0.2 | 42.38 ± 0.95 | 0.55 ± 0.27 | 77.89 ± 0.99 | 1.8 |
| **CDH** | 0.02 ± 0.01 | 0.04 ± 0.02 | 12.24 ± 0.37 | 0.03 ± 0.01 | 77.59 ± 3.52 | 0.9 |
| **CDM** | 15.4 ± 0.45 | 19.58 ± 0.79 | 42.2 ± 0.615 | 0.06 ± 0.01 | 90.83 ± 0.18 | 318.5 |

Results are shown as mean ± S.E. M (n=3)

AZT, zidovudine; TDF, tenofovir; ABC, abacavir; NVP, nevirapine; CDH, hexane fraction of *C. dichogamus* twig extract; CDD, dichloromethane fraction of *C. dichogamus* twig extract; CDE, ethylacetate fraction of *C. dichogamus* twig extract; CDM, methanol fraction of *C. dichogamus* twig extract; MNTC, maximum nontoxic concentration; $CC_{50}$, 50% cytotoxic concentration; $Emax_C$, maximum cytotoxic effect %; $IC_{50}$, 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

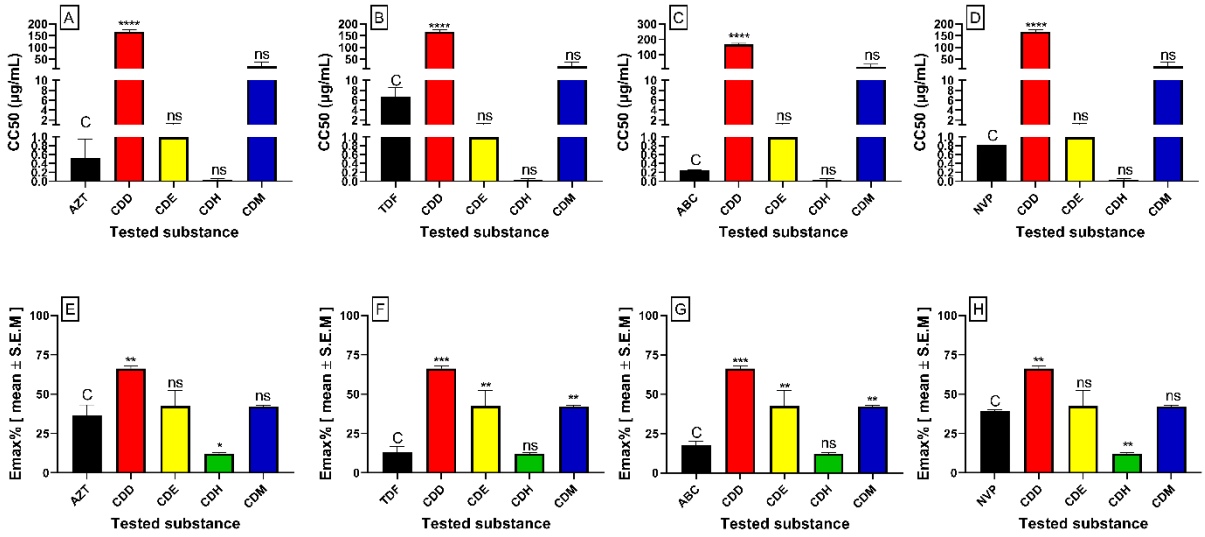



Figure 4.3-5: Cytotoxic effect of solvent fractions of *C. dichogamus*

The results are expressed as the mean of three independent experiments ± S.E.M. C, control; CDH, hexane fraction of *C. dichogamus* twig extract; CDD, dichloromethane fraction of *C. dichogamus* twig extract; CDE, ethylacetate fraction of *C. dichogamus* twig extract; CDM, methanol fraction of *C. dichogamus* twig extract; ns, not significant, *Denotes p value < 0.05, ** Denotes p value <0.01, *** Denotes p value < 0.001.

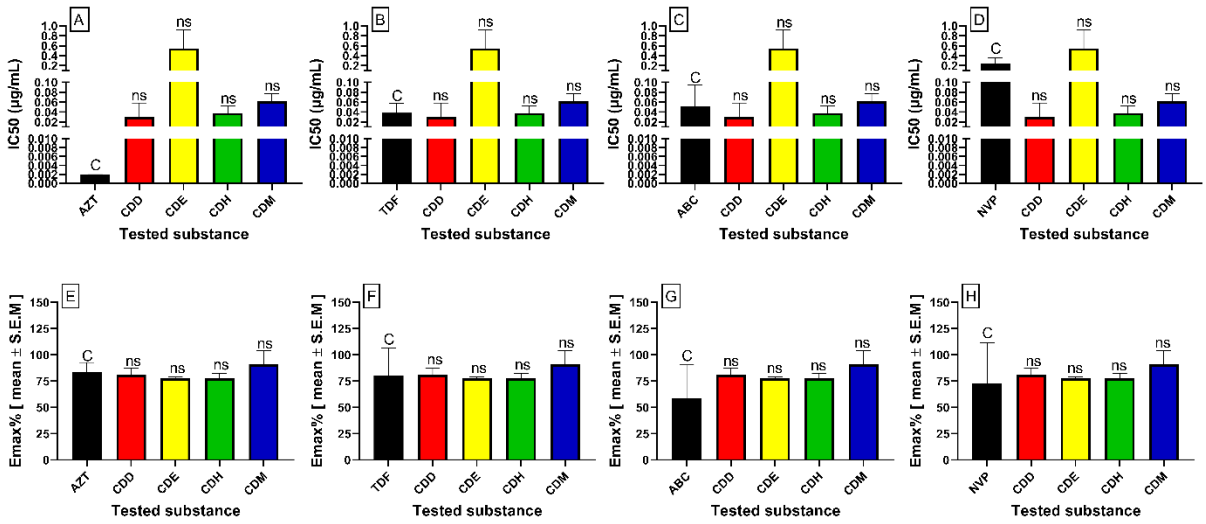



Figure 4.3-6: Anti-HIV activity of solvent fractions of *C. dichogamus*

The results are expressed as the mean of three independent experiments ± S.E.M. C, control; CDH, hexane fraction of *C. dichogamus* twig extract; CDD, dichloromethane fraction of *C. dichogamus* twig extract; CDE, ethylacetate fraction of *C. dichogamus* twig extract; CDM, methanol fraction of *C. dichogamus* twig extract; ns, not significant, *Denotes p value < 0.05, ** Denotes p value <0.01, *** Denotes p value < 0.001.

### 4.2.3.1. Discussion

According to our findings, the two solvent fractions (CDD and CDM) of *C. dichogamus* twig portions had $CC_{50}$ values greater than the control medicines, implying that larger fraction concentrations are necessary for cytotoxic effects. Furthermore, the methanol fraction was found to be less toxic ($Emax_C$ = 42.2%), whereas the dichloromethane fraction was found to be toxic, inhibiting cell viability by more than 60%.

Our findings on the plant's cytotoxicity are consistent with those of Aldhaher *et al*., (2017), Shang *et al*., (2013), and Pudhom & Sommit, (2011). For example, at a dose of 100 mM, Aldhaher *et al*., (2017) found 10-epi-Maninsigin D, a diterpenoid isolated from the n-hexane fraction of the root of *C. dichogamus*, reduced the viability of Caco-2 human colon cancer cell lines by 43%. Using the MTT method, maninsigin D was cytotoxic to HL-60, A-549, SW-480, SMMC-7721, and MCF-7 human cell lines with $IC_{50}$ values > 40 mM (Shang *et al*., 2013). In comparison to the other fractions, the methanol fraction had the best antiviral activity, inhibiting 90% of the virus's cytopathic effect on the cell line ($IC_{50}$= 0.06 µg/mL). One or more terpenoids, saponins, or other bioactive chemicals may be responsible for the methanol extract's ability to block the virus's cytopathic effect.

## 4.4. Isolation and characterization of compounds with a potential antiretreoviral activity using bioassay-guided fractionation

### 4.4.1. *Croton megalocarpus*

Based on the anti-HIV activity findings of the solvent fractions from *C. megalocarpus,* the ethyl acetate fraction from the stem bark of *C. megalocarpus* was subjected to column chromatography to yield nine pure bioactive compounds (**Table 4.4-1).** The chemical structure of these compounds is depicted in **Figure 4.4-1**. In this study we report the anti HIV activity of three new (previously undescribed) eudesmane type sequiterpenes and two new crotofolane diterpenoids isolated from *C. megalocarpus.*

Table 4.4-1: Compounds isolated from the stem bark of *C. megalocarpus*

| Ser No. | Name | Code |
|---|---|---|
| **Eudesmane type sequiterpenes** | | |
| 1. | 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A)* | **E12** |
| 2. | 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B)* | **E22** |
| 3. | 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (ermiasolide C) * | **E17** |
| 4. | 4H-α,7H-α,10α-eudesm-11-en-5β-ol | **E5** |
| **Crotofolane diterpenoids** | | |
| 5. | 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid)* | **E37** |
| 6. | 11-acetoxy crotocascarin L* | **E24** |
| 7. | Crotocascarin K | **E23** |
| **Other pure compounds** | | |
| 8. | 3β-hydroxylup-20(29)-ene (lupeol) | **E19** |
| 9. | Pinoresinol | **E2** |

*new or previously undescribed compounds

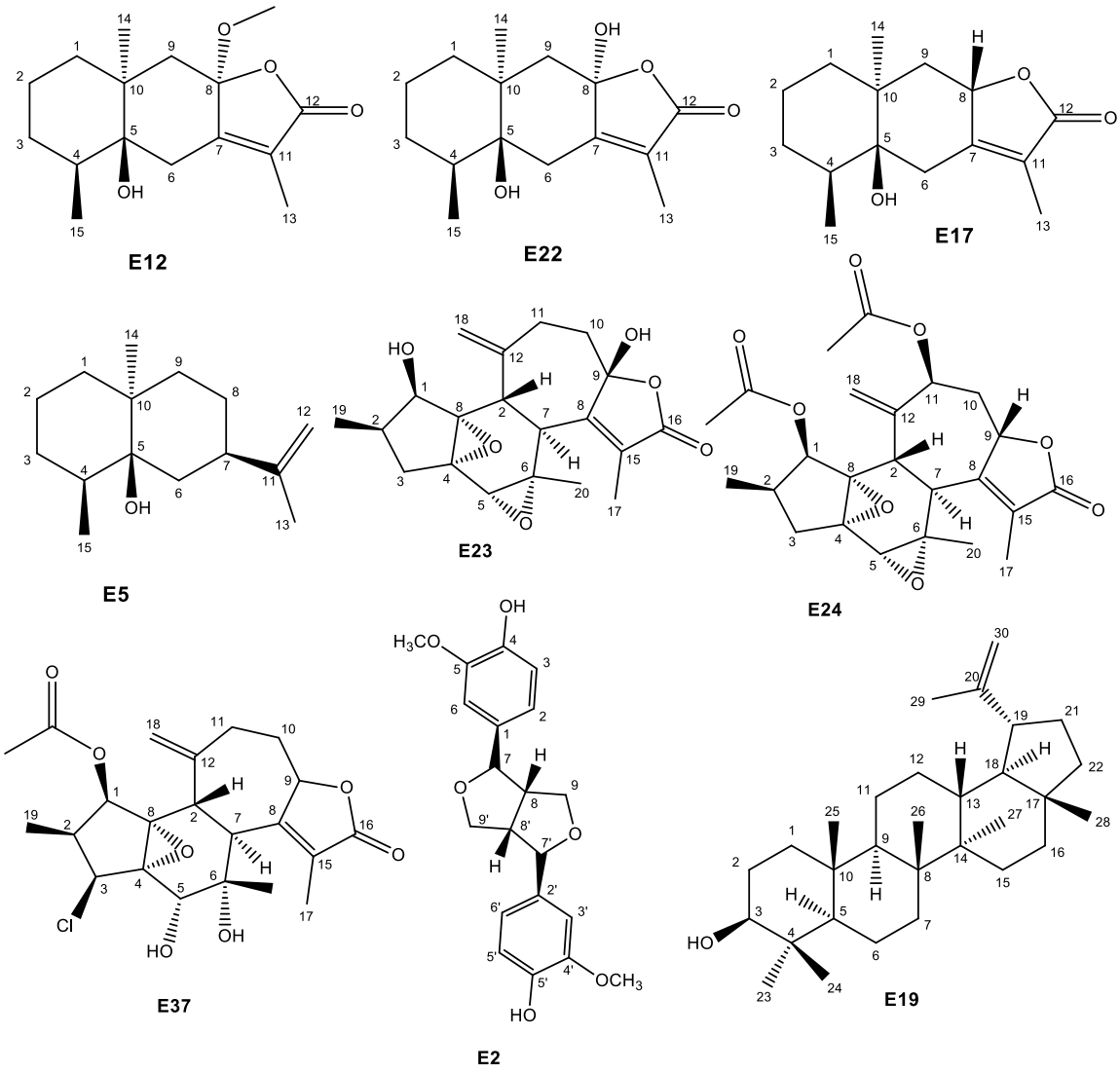
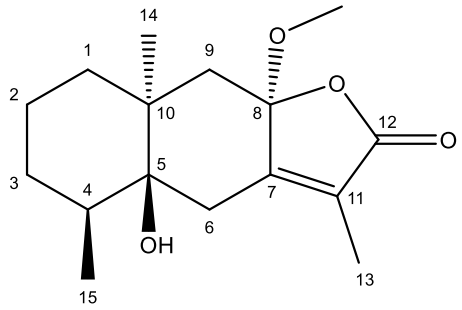



Figure 4.4-1: Pure compounds isolated from *C. megalocarpus*

### 4.4.1.1. Chemistry of eudesmane type diterpenoids isolated from *C. megalocarpus*

#### 4.4.1.1.1. E12: 5β-Hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (Ermiasolide A)




**E12**

5β-Hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (compound **E12)** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a brown oil. The HRMS spectrum **(Appendix 1)** showed a molecular ion peak at *m/z* $[M+H]^+$ 281.1744 for $C_{16}H_{22}O_4$ (calc. $C_{16}H_{24}O_4$ + H, *m/z* 281.1747). The $^1$H NMR spectrum **(Table 4.4-2 and 4.4-3)** of this compound showed four methyl proton resonances at $\delta_H$ 3.16 (s) for a methoxy group, 1.85 (d, *J*=1.3), 1.24 (s), and 0.90 (d, *J*=6.7) (**Appendix 2)**. The $^{13}$C, DEPT, and HSQCDEPT NMR spectra **(Appendix 3-5)** supported 16 carbon resonances, including a carbonyl resonance at $\delta_C$ 172.0, two double bond resonances at 156.8 and 127.7, an anomeric methine carbon at 106.5, an oxygenated carbon resonance at 77.8, a methoxylated carbon at 50.4, and carbon resonances for 3 methyls, 5 methylenes, and 1 methine. The above information suggested that compound **E12** was a methoxylated sesquiterpenoid. The use of 2D NMR spectra, HMBC, and COSY **(Appendix 6-7)** suggested that an allylic carbon resonance at 1.85 was part of an α,β-unsaturated carbonyl system with carbonyl carbon resonance at 172 and double bond carbon resonances at 156.8 and 127.7. A carbon resonance at 106.5 was assigned to an acetal group. It was observed in the HMBC spectrum to correlate with the methoxy group proton resonance and methylene proton resonances assignable to $H_2$-6 and $H_2$-9. In addition, $H_2$-6 proton resonances showed correlations in the HMBC spectrum with C-4, C-5, C-7, C-10, and C-11, whereas $H_2$-9 showed correlations in the HMBC spectrum with C-1, C-5, C-7, C-8, and C-14. C-4 was observed to be a methine carbon resonance, which corresponded with a proton resonance at $\delta_H$ 1.96 (m) for H-4. The H-4 proton resonance was coupled with a methyl doublet proton at 0.90 (d, *J*=6.7), attributable to H-15. In addition, H-4 was coupled to proton resonances for $H_2$-3, which were coupled to $H_2$-2 in the COSY spectrum. $H_2$-2 was also coupled

to $H_2$-1 in the COSY spectrum. The above data suggested that compound E12 was a methoxylated sesquiterpenoid that was determined to be a eudesmane class.

The NOESY spectrum **(Appendix 8)** of compound **E12**, acquired using DMSO, showed that the methoxy groups H-4 and 3H-14 were on the same face of the molecule, whereas 5-OH and 3H-15 were on the other face of the molecule. The HRMS data $[M+H]^+$ = 280.1744 supported the proposed structure; hence, **E12** was determined to be undescribed 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide, trivially named **ermiasolide-A**.

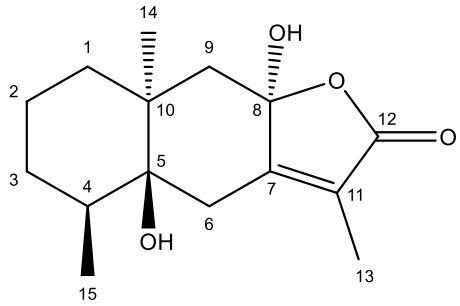
Table 4.4-2: NMR data for 5β-Hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide **(**ermiasolide A, E12) in $CDCl_3$

| **No.** | **$^{13}$C NMR** | **$^{1}$H NMR** | **HMBC (H→C)** | **COSY** | **NOESY** |
|---|---|---|---|---|---|
| 1α | 34.3 | 1.52 | 2, 10 | 1β, 2α, 2β | 1β, 2α, 2β |
| 1β | | 1.14 | 3 | 1α, 2α, 2β | 1α, 2α, 2β |
| 2α | 20.0 | 1.67 m | x | 1α, 1β, 2β | 1α, 1β, 2β |
| 2β | | 1.59 m | x | 1α, 1β, 2α | 1α, 1β, 2α |
| 3α | 30.1 | 1.48 m | 2 | 2α, 2β, 3β | 2α, 2β, 3β |
| 3β | | 1.30 m | 2 | 2α, 2β, 3α | 2α, 2β, 3α |
| 4 | 34.1 | 1.96 m | 2, 5, 10 | 14, 3α, 3β | 14 |
| 5 | 77.8 | - | - | - | - |
| 6α | 32.0 | 2.71 d, 13.9 | 5, 7, 8, 10, 11 | 6β | $OCH_3$ |
| 6β | | 2.27 dd, 1.4, 13.9 | 5, 7, 11 | 6α,13 | 14, 15, $OCH_3$ |
| 7 | 156.8 | - | - | - | - |
| 8 | 106.5 | - | - | - | - |
| 9α | 45.8 | 1.96 d, 13.7 | 7, 8, 10, 11 | 9β | 9β,14, 15 |
| 9β | | 1.82 d, 13.7 | 8, 7, 10 | 9α | 9α |
| 10 | 38.9 | - | - | - | - |
| 11 | 127.7 | - | - | - | - |
| 12 | 172.0 | - | - | - | - |
| 13 | 8.6 | 1.85 d, 1.3 | 7, 11, 12 | 6β | $OCH_3$ |
| 14 | 21.0 | 1.24 s | 1, 5, 9, 10 | - | 4, 6α, $OCH_3$ |
| 15 | 15.3 | 0.90 d, 6.7 | 3,4,5 | 4 | 6β |
| $OCH_3$ | 50.4 | 3.16 s | 8 | - | 6α, 6β, 13, 14 |

Table 4.4-3: NMR data for 5β-Hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide **(**ermiasolide A, E12) in DMSO

| **No.** | **$^{13}$C NMR** | **$^{1}$H NMR** | **HMBC (H→C)** | **COSY** | **NOESY** |
|---|---|---|---|---|---|
| 1α | 33.4 | 1.60 m | 2, 10 | 1β, 2α, 2β | 1β, 2α, 2β |
| 1β | | 0.94 m | 3 | 1α, 2α, 2β | 1α, 2α, 2β |
| 2α | 19.7 | 1.57 m | x | 1α, 1β, 2β | 1α, 1β, 2β |
| 2β | | 1.37 m | x | 1α, 1β, 2α | 1α, 1β, 2α |
| 3α | 29.4 | 1.29 m | 2 | 2α, 2β, 3β | 2α, 2β, 3β |
| 3β | | 1.29 m | 2 | 2α, 2β, 3α | 2α, 2β, 3α |
| 4 | 33.8 | 1.84 m | 2, 5, 10 | 14, 3α, 3β | 14 |
| 5 | 76.4 | - | - | - | - |
| 6α | 31.7 | 2.66 d, 13.9 | 7, 8, 10, 11 | 6β | 15, $OCH_3$ |
| 6β | | 2.22 dd, 1.4, 13.9 | 7, 11 | 6α,13 | 14, 15, $OCH_3$ |
| 7 | 157.1 | - | - | - | - |
| 8 | 106.1 | - | - | - | - |
| 9α | 45.2 | 1.82 m | 5, 7, 8, 10 | 9β | 9β,14, 15 |
| 9β | | 1.82 m | 8, 7, 10 | 9α | 9α |
| 10 | 38.3 | - | - | - | - |
| 11 | 126.4 | - | - | - | - |
| 12 | 171.3 | - | - | - | - |
| 13 | 8.2 | 1.76 s | 7, 11, 12 | 6β | $OCH_3$, |
| 14 | 20.6 | 1.14 s | 1, 5, 9, 10 | - | 4, 6β |
| 15 | 15.1 | 0.83 d, 6.7 | 3,4,5 | 4 | $OCH_3$, |
| $OCH_3$ | 49.7 | 3.03 s | 8 | - | 6α, 6β, 13, 14, |
| 5-OH | - | 3.86 s | 4, 5, 6, 10 | | 2β, 3β, 4, 6β, 13, 15 |

#### 4.4.1.1.2. E22: 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B)




**E22**

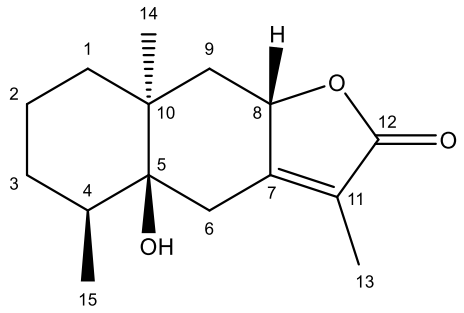
5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (compound **E22)** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a yellowish oil. The HRMS spectrum **(Appendix 9)** of compound **E22** showed a molecular ion peak at *m/z* $[M+H]^+$ 267.1589 for $C_{16}H_{22}O_4$ (calc. $C_{15}H_{22}O_4$ + H, *m/z* 267.1591). The $^1$H NMR spectrum **(Table 4.4-4)** of this compound showed three methyl proton resonances at $\delta_H$ 1.78 (s), 1.29 (s), and

0.89 (d, *J*=6.7) and missing the resonance for a methoxy group that was observed in compound **E12 (Appendix 10)**. The $^{13}C$, DEPT, and HSQCDEPT NMR spectra **(Appendix 10-16)** supported 15 carbon resonances, including a carbonyl resonance at $\delta_C$ 173.0, two double bond resonances at 158.9 and 125.3, a hemiketal methine carbon at 104.1, an oxygenated carbon resonance at 78.0, and carbon resonances for 3 methyls, 5 methylenes, and 1 methine. The $^{13}C$ and DEPT NMR spectra were missing the methoxylated carbon resonance as in compound **E12**. The 2D spectra for compound **E22** were similar to those of compound **E12**. Compound **E22** was determined to be an undescribed derivative of compound **E12**, 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide, trivially named **ermiasolide B**.

Table 4.4-4: NMR data for 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B, E22) in $CDCl_3$

| **No.** | **$^{13}C$ NMR** | **$^{1}H$ NMR** | **HMBC (H→C)** | **COSY** | **NOESY** |
|---|---|---|---|---|---|
| 1α | 34.2 | 1.55 m | 2, 10 | 1β, 2α, 2β | 1β, 2α, 2β |
| 1β | | 1.14 m | 3 | 1α, 2α, 2β | 1α, 2α, 2β |
| 2α | 20.0 | 1.68 m | x | 1α, 1β, 2β | 1α, 1β, 2β |
| 2β | | 1.49 m | x | 1α, 1β, 2α | 1α, 1β, 2α |
| 3α | 30.0 | 1.48 m | 2 | 2α, 2β, 3β | 2α, 2β, 3β |
| 3β | | 1.27 m | 2 | 2α, 2β, 3α | 2α, 2β, 3α |
| 4 | 34.1 | 1.94 m | 2, 5, 10, 15 | 14, 3α, 3β | 14 |
| 5 | 78.0 | - | - | - | - |
| 6α | 31.8 | 2.71 d, 13.9 | 5, 7, 8, 10, 11 | 6β | 15, $OCH_3$, |
| 6β | | 2.43 d, 13.9 | 5, 7, 11 | 6α,13 | 14, 15 |
| 7 | 158.9 | - | - | - | - |
| 8 | 104.1 | - | - | - | - |
| 9α | 47.0 | 1.90 m | 5, 7, 8, 10, 14 | 9β | 9β,14, 15 |
| 9β | | 1.88 m | 5, 7, 8, 10, 14 | 9α | 9α |
| 10 | 38.9 | - | - | - | - |
| 11 | 125.3 | - | - | - | - |
| 12 | 173.0 | - | - | - | - |
| 13 | 8.4 | 1.78 s | 7, 11, 12 | 6β | |
| 14 | 15.2 | 0.89 d, 6.7 | 1, 5, 9, 10 | - | 4, 6β |
| 15 | 21.2 | 1.29 s | 3,4,5 | 4 | |
| 5-OH | | | | | |
| 8-OH | | | | | |

#### 4.4.1.1.3. E17: 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (ermiasolide C)



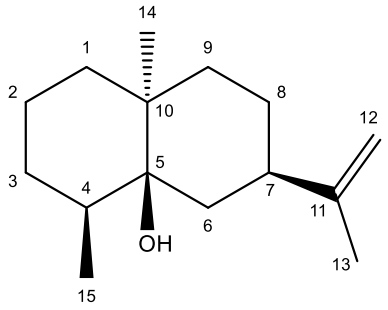

**E17**

5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (compound **E17)** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a yellowish oil. Compound **E17** was determined to be an undescribed 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide, an 8-H derivative of compounds **E22** and **E12**. The HRMS spectrum **(Appendix 17)** of compound **E17** showed a molecular ion peak at *m/z* $[M+H]^+$ 251.1641 for $C_{15}H_{22}O_3$ (calc. $C_{15}H_{22}O_3$ + H, *m/z* 251.1642). The $^1$H NMR spectrum **(Table 4.4-5)** of this compound showed three methyl proton resonances at $\delta_H$ 1.80 (s), 0.90 (d), and 0.82 (s) and missing the resonance for a methoxy group that was observed in compound **E12 (Appendix 18)**. The $^{13}$C, DEPT, and HSQCDEPT NMR spectra **(Appendix 19-24)** supported 15 carbon resonances, including a carbonyl resonance at $\delta_C$ 175.4, two double bond resonances at 161.4 and 121.5, an oxygenated carbon resonance at 76.5, and carbon resonances for 3 methyls, 5 methylenes, and 2 methines. The $^{13}$C and DEPT NMR spectra were missing the methoxylated carbon resonance, the hemiketal methine carbon at 104.1 as in compound **E12**, and only had one oxygenated carbon when compared to **E22**. The 2D spectra for compound **E17** were similar to those of compounds **E12** and **E22**. Compound **E17** was determined to be an undescribed derivative of compound **E12** and **E22**, 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide, trivially named **ermiasolide C**.

Table 4.4-5: NMR data for 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (ermiasolide C, E17) in $CDCl_3$

| No. | $^{13}$C NMR | $^{1}$H NMR | HMBC (H→C) | COSY | NOESY |
|---|---|---|---|---|---|
| 1α | 36.9 | 1.50 m | 10 | 1β, 2α, 2β | 1β, 2α, 2β |
| 1β | | 1.34 m | 2,3 | 1α, 2α, 2β | 1α, 2α, 2β |
| 2α | 21.2 | 1.67 m | x | 1α, 1β, 2β | 1α, 1β, 2β |
| 2β | | 1.59 m | x | 1α, 1β, 2α | 1α, 1β, 2α |
| 3α | 30.2 | 1.50 m | 2 | 2α, 2β, 3β | 2α, 2β, 3β |
| 3β | | 1.23 m | 2 | 2α, 2β, 3α | 2α, 2β, 3α |
| 4 | 36.2 | 1.81 m | 2, 5, 10, 15 | 14, 3α, 3β | 14 |
| 5 | 76.5 | - | - | - | - |
| 6α | 35.4 | 2.55 m | 5, 7, 8, 10, 11 | x | 15 |
| 6β | | 2.55 m | 5, 7, 8, 10, 11 | x | 14, 15 |
| 7 | 161.4 | - | - | - | - |
| 8 | 78.0 | 5.44 m | - | - | - |
| 9α | 43.2 | 2.24 d | 1,7, 8, 14 | 9β | 9β,14, 15 |
| 9β | | 1.39 d | 1,7, 8, 14 | 9α | 9α |
| 10 | 38.5 | - | - | - | - |
| 11 | 121.5 | - | - | - | - |
| 12 | 175.4 | - | - | - | - |
| 13 | 8.6 | 1.80 s | 7, 11, 12 | 6β | |
| 14 | 14.8 | 0.93 d | 1, 5, 9, 10 | - | 4, 6β |
| 15 | 23.9 | 0.82 s | 3,4,5 | 4 | |
| 5-OH | | | | | |

#### 4.4.1.1.4. E5: 4H-α,7H-α,10α-eudesm-11-en-5β-ol




**E5**

4H-α,7H-α,10α-eudesm-11-en-5β-ol (compound **E5)** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a yellowish oil. Compound **E5** was determined to be the known 4H-α,7H-α,10α-eudesm-11-en-5β-ol previously isolated from orange juice (Näf *et al.*, 2011). The HRMS spectrum **(Appendix 25)** of compound **E5** showed a molecular ion peak at *m/z* $[M+H]^+$ 221.1889 for $C_{15}H_{24}O$ (calc. $C_{15}H_{24}O$ + H, *m/z* 221.1900).

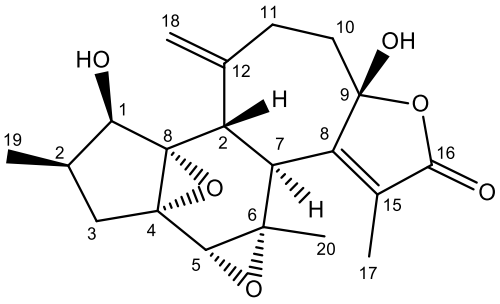
The $^{1}$H NMR spectrum **(Table 4.4-6)** of this compound showed three methyl proton resonances at 1.73 (s), 1.03 (s), and 0.79 (d, *J*=6.7) **(Appendix 26)**. The $^{13}$C, DEPT, and HSQCDEPT NMR spectra **(Appendix 27-32)** supported 15 carbon resonances, including two double bond resonances at 150.8 and 108.4, an oxygenated carbon resonance at 75.1, and carbon resonances for 3 methyls, 6 methylenes, and 2 methines. The $^{1}$H and $^{13}$C NMR spectra were similar to those of the known 4H-α,7H-α,10α-eudesm-11-en-5β-ol previously isolated from orange juice **(Table 4.4-6)** (Näf *et al*., 2011).

Table 4.4-6: NMR data for 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5) in $CDCl_3$

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR*** | **$^{1}$H NMR** | **$^{1}$H NMR*** | **HMBC (H→C)** | **COSY** | **NOESY** |
|---|---|---|---|---|---|---|---|
| 1α | 34.8 | 35.0 | 1.58 | | x | 1β, 2α | |
| 1β | | | 1.34 | | x | 1α | |
| 2α | 20.8 | 20.7 | 1.65 m | | x | 1α, 1β, 2β | 7 |
| 2β | | | 1.47 m | | x | 2α | |
| 3α | 30.5 | 30.4 | 1.34 m | | 5 | 4 | |
| 3β | | | 1.42 m | | 5 | 4 | 15 |
| 4 | 34.5 | 34.3 | 1.72 m | | 3 | 3α, 3β, 15 | |
| 5 | 75.1 | 74.8 | - | | | - | |
| 6α | 26.2 | 26.0 | 1.54 m | | 11 | 7 | |
| 6β | | | 1.54 m | | 11 | 7 | |
| 7 | 40.3 | 40.1 | 2.41 m | 2.41 m | x | 6α, 6β, 8α 8β | 2α, 8α |
| 8α | 35.8 | 35.70 | 1.57 m | | 11 | 7 | 7 |
| 8β | | | 1.34 m | | 11 | 7 | |
| 9α | 35.2 | 34.6 | 1.57 m | | x | 9β | |
| 9β | | | 1.07 m | | x | 9α | |
| 10 | 37.1 | 36.9 | - | | - | - | |
| 11 | 150.8 | 150.7 | - | | - | - | |
| 12A | 108.6 | 108.4 | 4.72 s | 4.72 s | 7, 13 | 12B, 13 | 12B, 13 |
| 12B | | | 4.69 s | 4.70s | 7, 13 | 12A, 13 | 12A, 13 |
| 13 | 20.6 | 20.9 | 1.73 s | 1.74 s | 7, 12, 14 | 12A, 12B | 12A, 12B, 15 |
| 14 | 21.1 | 20.4 | 1.03 s | 1.03 s | 1, 5, 9, 10 | 4 | 3β, 4, 13 |
| 15 | 15.1 | 14.9 | 0.79 d 6.7 | 0.80 d 6.7 | 3, 4, 5 | - | 13 |
| 5-OH | | | | | | - | |

*****(Näf *et al*., 2011)

#### 4.4.1.1.5. E23: Crotocascarin K




**E23**

Compound **E23** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a yellowish oil. Compound **E23** was determined to be the known crotocascarin K previously isolated from *C. cascarilloides*. The HRESIMS **(Appendix 33)** of compound **E23** showed a molecular ion peak at *m/z* 361.1643 $[M+H]^+$ (calcd. for $C_{20}H_{24}O_6$ + H, *m/z* 361.1646). The $^1$H-NMR spectrum **(Table 4.4-7)** showed three methyl resonances, two oxygenated methine resonances, and exocyclic methylene protons resonated **(Appendix 34)**. In contrast, the $^{13}$C NMR spectrum showed 20 carbon resonances, which showed a close resemblance to those of the previously reported crotocascarin K. The $^{13}$C NMR, DEPT, HSQCDEPT, HMBC, COSY and NOESY spectrum of **E23** is depicted in **Appendix 35-40.**

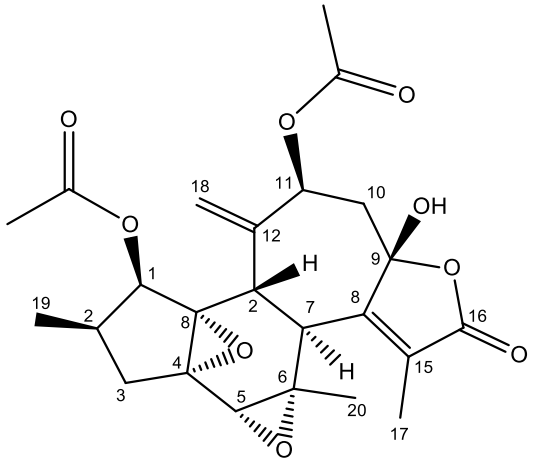
Table 4.4-7: NMR data for Crotocascarin K (E23) in $CDCl_3$

| No. | $^{13}$C NMR | $^{13}$C NMR* | $^1$H NMR | $^1$H NMR* |
|---|---|---|---|---|
| 1 | 73.6 | 73.7 | 4.20 m | 4.18 d 5.1 |
| 2 | 33.5 | 33.7 | 2.01 m | 2.04 m |
| 3α | 36.1 | 36.2 | 2.39 m | 2.39 dd 13.5, 7 |
| 3β | | | 1.65 m | 1.68 dd 13.5, 10.2 |
| 4 | 60.2 | 60.4 | - | - |
| 5 | 58.0 | 58.2 | 3.17 s | 3.11 s |
| 6 | 56.4 | 56.6 | - | - |
| 7 | 43.9 | 44.1 | 3.01 dd 1, 12.6 | 2.97dd 12.6, 1.1 |
| 8 | 158.8 | 159.7 | - | - |
| 9 | 107.2 | 108.0 | - | - |
| 10α | 41.9 | 41.8 | 2.45 m | 2.53 m |
| 10β | | | 1.61 m | 1.56 |
| 11α | 34.9 | 35.2 | 2.46 m | 2.41 m |
| 11β | | | 2.35 m | 2.33 ddd 14.8, 13.4, 6.4 |
| 12 | 147.3 | 147.7 | - | - |
| 13 | 39.3 | 39.3 | 3.32 d 12.6 | 3.35 d 12.6 |
| 14 | 69.9 | 70.0 | - | - |
| 15 | 130.5 | 130.1 | - | - |

| | | | | |
|---|---|---|---|---|
| 16 | 170.5 | 171.3 | - | - |
| 17 | 9.6 | 9.6 | 1.91 d 1 | 1.89 d 0.9 |
| 18A | 114.6 | 114.4 | 5.07 br s | 5.06 s |
| 18B | | | 5.06 br s | 5.04 s |
| 19 | 12.1 | 12.2 | 1.03 d 7.2 | 1.03 d 7.2 |
| 20 | 20.2 | 20.3 | 1.15 s | 1.14 s |

*(Kawakami *et al*., 2015)

#### 4.4.1.1.6. E24: 11β-acetoxy crotocascarin L




**E24**

Compound **E24** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a yellowish oil. Compound **E24** showed a molecular ion peak at *m/z* 445.1854 $[M+H]^+$ (calcd. for $C_{24}H_{28}O_8$ + H, *m/z* 445.1857), which gave 11 degrees of unsaturation **(Appendix 41).** The IR spectrum showed the presence of an α,β-unsaturated lactone at 1746 $cm^{-1}$. The $^1H$ and $^{13}C$ NMR spectra **(Table 4.4-8)** were similar to those of the known crotocascarin L, previously isolated from *C. cascarilloides* (Kawakami *et al*., 2016), except for the presence of an acetoxy group due to a proton methyl resonance at 2.12 (s) and carbon resonances at $\delta_C$ 169.1 and 21.2 for an acetoxy group **(Appendix 42-43)**. The COSY, HMBC, and NOESY spectra **(Appendix 44-48)** supported placement of the acetoxy group at the C-11 position. Notably, the exocyclic proton resonances at $\delta_H$ 5.32 (s) and 5.30 (s) showed a correlation in the HMBC spectrum with an oxygenated carbon resonance at 74.6, which corresponded to a proton resonance at 5.59 (t, 3.4). This proton resonance further showed a correlation with a carbonyl carbon resonance of an acetoxy group at 169.1, hence the assignment of the acetoxy group to the C-11 position.

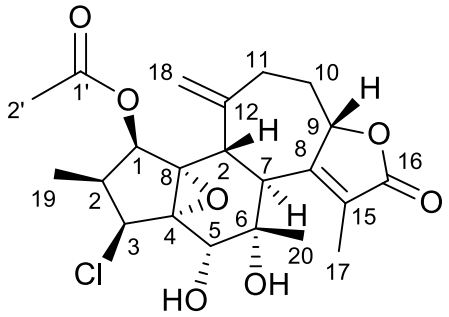
Table 4.4-8: NMR data for 11β-acetoxy crotocascarin L (E24)

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR*** | **$^{1}$H NMR** | **$^{1}$H NMR*** | **COSY** | **NOESY** |
|---|---|---|---|---|---|---|
| 1 | 74.8 | 74.9 | 5.46 d 5.2 | 5.47 d 6 | 2 | 2,11 |
| 2 | 33.9 | 33.4 | 2.19 m | 2.05 m | 1,3α,3β,19 | 1,7 |
| 3α | 36.8 | 36.9 | 2.46 dd 6.5, 12.9 | 2.41 dd 14, 7 | 2,3β | 7 |
| 3β | | | | 1.65 dd 14, 10 | 2,3α | 13,19 |
| 4 | 60.8 | 60.5 | - | - | - | - |
| 5 | 57.7 | 57.8 | 3.21 s | 3.16 s | 20 | 17,20 |
| 6 | 56.2 | 56.4 | - | - | - | - |
| 7 | 44.2 | 44.1 | 3.17 s | 2.99 dq 13, 1 | 13,17 | 2,3α,10α,13,20 |
| 8 | 161.1 | 158.9 | | - | - | - |
| 9 | 78.2 | 107.8 | 5.03 br d 10.5 | - | - | - |
| 10α | 42.3 | 42.0 | 2.85 dt 3.7, 14.1 | 2.46 o | 10β,11 | 7 |
| 10β | | | 1.39 m | 1.59 ddd 13, 13, 5 | 10α,11 | 13 |
| 11α | 74.6 | 35.0 | 5.59 t 3.4 | 2.46 o | 10α,10β,11 | 1,7 |
| 11β | | | | 2.11 ddd 13, 13, 5 | - | - |
| 12 | 143.9 | 146.6 | - | - | - | - |
| 13 | 33.6 | 39.6 | 3.16 s | 3.05 d 13 | 7 | 10β,19 |
| 14 | 68.6 | 68.8 | - | - | - | - |
| 15 | 128.9 | 130.7 | - | - | - | - |
| 16 | 173.1 | 170.4 | - | - | - | - |
| 17 | 9.9 | 9.7 | 1.92 0.9 | 1.91 s | 7 | 5 |
| 18A | 118.9 | 115.1 | 5.32 s | 5.08 s | 18B | 18B |
| 18B | | | 5.30 s | 5.06 s | 18A | 18A |
| 19 | 12.7 | 12.5 | 0.90 d 7.1 | 0.92 d 7 | 2 | 3β,13 |
| 20 | 19.6 | 20.2 | 1.11 s | 1.18 s | 5 | 5,7 |
| 1-$CH_3$ | 21.1 | 20.6 | 2.10 s | 2.11 s | - | x |
| 1-CO | 169.3 | 169.5 | - | - | - | - |
| 11-$CH_3$ | 21.2 | x | 2.12 s | | - | x |
| 11-CO | 169.1 | x | - | - | - | - |
| 9-OH | | | | 3.43 br s | - | - |

*(Kawakami *et al.*, 2016)

#### 4.4.1.1.7. E37: 1β-Acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid)

Compound **E37** was trivially named ermiasoid, a rare example of a chlorinated crotofolane diterpenoid.




**E37**

1β-Acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (**E37**) gave a molecular formula of $C_{22}H_{27}ClO_7$, with nine degrees of unsaturation, as determined by (+)-HRESIMS analysis, which displayed an ion peak at *m/z* 439.1514 $[M+H]^+$ (calcd for $C_{22}H_{27}ClO_8$ + H, *m/z* 439.1518). The IR spectrum exhibited the presence of an α,β-unsaturated lactone (1747 $cm^{-1}$) and hydroxy groups (3436 $cm^{-1}$). The $^1H$ NMR spectrum ($CDCl_3$, **Table 4.4-9**) gave resonances for two singlet methyls ($\delta_H$ 1.62 s; 1.93 s), two doublet methyls ($\delta_H$ 1.19 d, 7.7 Hz; 1.83 d, 1.7 Hz), two olefinic protons ($\delta_H$ 5.32, s and 4.82, s), three oxygenated methine protons ($\delta_H$ 5.37 t, 5.8 Hz; 5.10 d, 8.2 Hz; 3.55 d 7.3 Hz), a halomethine proton ($\delta_H$ 3.61 s), two pairs of methylene protons ($\delta_H$ 2.32 m, and 1.61 (m); 2.31, m and 2.31, m), three methine protons ($\delta_H$ 2.81 m; 3.73 d, 11.9 Hz; 2.85 d, 11.9 Hz), and a proton of a hydroxy group ($\delta_H$ 3.96 s). The $^{13}C$ NMR spectrum ($CDCl_3$, **Table 4.4-9**), together with DEPT results, showed 22 carbon resonances including two carbonyl carbon resonances at $\delta_C$ 173.7 and, 172.5, four alkene resonances at $\delta_C$ 160.2, 144.9, 129.5 and 113.9, six oxygenated carbon resonances at $\delta_C$ 80.9, 78.0, 75.6, 81.9, 82.7 and 66.3, a halogenated carbon resonance at $\delta_C$ 66.8, four methyl carbon resonances, two methylene carbon resonances, and three methine carbon resonances (**Appendix 49-57)**. The use of the COSY and HMBC spectra indicated **E37** to be a derivative of crotocascarin L, previously reported from the Japanese *C. cascarilloides*, with de-hydroxylation at C-9, chlorination at the C-3 position, with the epoxy group opening to form a diol at C-5 and C-6 (Kawakami *et al.*, 2016). The two carbonyls and two double bonds, accounted for four of the nine degrees of unsaturation, and suggested that **E37** is pentacyclic. A doublet methyl group $\delta_H$ 1.19 (d, 7.7 Hz), found in the five-membered ring A, was evident in the $^1H$ and $^1H$-$^1H$ COSY spectrum and assignable as $CH_3$-19. This methyl group proton resonance was coupled to the

methine resonances of H-2 ($\delta_H$ 2.81, m) in the COSY spectrum, and in turn, H-2 was coupled to an oxygenated methine proton resonance at $\delta_H$ 5.10 (d, 8.2 Hz), either at the H-1 or the H-3 position. A correlation in the HMBC spectrum between this oxygenated doublet methyl with a typical C-13 methine carbon resonance at $\delta_C$ 44.0, allowed for its assignment as H-1 (Kawakami *et al*., 2016). The corresponding H-13 methine proton resonance was assigned using the HSQC spectrum ($\delta_H$ 2.85, d, $J$ = 11.9 Hz). The H-1 resonance gave a correlation in the HMBC spectrum with a carbonyl carbon of the acetoxy group, and hence an acetate group was placed at the C-1 position. The C-12(18) exocyclic double bond, was present as in previously reported crotocascarins, as shown by two singlet H-18 proton resonances $\delta_H$ 5.32 and $\delta_H$ 4.82, which corresponded to a methylene carbon resonance at $\delta_C$ 113.9 in the HSQC spectrum (Kawakami *et al*., 2016). These two resonances exhibited correlations in the HMBC spectrum with a methylene carbon resonance at $\delta_C$ 35.1 that was assigned to C-11. The two H-11 resonances were overlapped at $\delta_H$ 2.31 and coupled in the COSY spectrum with the methylene protons at $\delta_H$ 2.32 and $\delta_H$ 1.16 for $H_2$-10, which, in turn, were coupled to a deshielded oxygenated proton resonance at $\delta_H$ 5.37 (t, 5.8 Hz) for H-9. The corresponding C-9 resonance occurred at $\delta_C$ 82.7, and gave an HMBC correlation with a methine proton resonance at $\delta_H$ 3.73 (d, 11.9 Hz) for H-7. H-7 and H-13 were seen to be coupled in the COSY spectrum, with a $J$ value of 11.9 Hz, suggesting a *trans* configuration at the junction. Also, H-7 had correlations in the HMBC spectrum between the carbon resonances at $\delta_C$ 78.0 (C-5), 75.6 (C-6), 129.5 (C-8) and 160.2 (C-15), with the latter two typical of an α,β-unsaturated lactone ring formed from the isopropyl group of a crotofolane derivative (Kawakami *et al.*, 2016). The $CH_3$-17 resonance ($\delta_H$ 1.83, d, 1.7 Hz) displayed correlations in the HMBC spectrum between the carbon resonances at $\delta_C$ 173.7 (C-16), 160.2 (C-15) and 129.5 (C-8). A singlet methyl group proton resonance at $\delta_H$ 1.62 afforded a correlation in the HMBC spectrum with C-7, and hence was assigned as $CH_3$-20. This resonance also gave correlations in the HMBC spectrum with a fully substituted carbon resonance at $\delta_C$ 75.6 (C-6) and a methine carbon resonance at 78.0 (C-5). The carbon resonances were deshielded compared to typical epoxide carbon resonances in reported crotofolanes such as crotocascarin L, (Kawakami *et al*., 2016) consistent with hydroxy groups being present at C-5 and C-6. The resonance present at $\delta_H$ 3.55 (s) was assigned as H-5 using the HSQC spectrum, and it showed correlations in the HMBC spectrum between the fully substituted carbon resonances at $\delta_C$ 81.9 (C-4), 66.3 (C-14), and a methine carbon resonance at $\delta_C$ 66.8 that was determined to be attached to a Cl atom. A chlorinated carbon resonance present at C-3 in the diterpenoid, laevinoid, has been previously reported by Wang

*et al.*, (Wang *et al.*, 2013) and the chemical shift was identical to that observed for 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L. The HRMS of this compound showed a protonated molecular peak $[M+H]^+$ at *m/z* 439.1514 and a characteristic isotope at *m/z* 441.1491 [M+2] with a relative abundance of ca 32%, indicating the presence of a chlorine atom in the structure. The accurate mass value of this compound was consistent with the molecular formula, $C_{22}H_{27}O_7Cl$ (error = 0.68 ppm), while any non-chlorinated alternative was inconsistent with the MS (high error values) and NMR data. The NOESY spectrum **(Figure 4.4-2)** showed that H-5, H-9, H-13, 3H-19 and 3H-20 were on the same of the molecule tentatively assigned as β, whereas H-1, H-2, H-3 and H-7 were assigned as α. This compound was determined to be an undescribed monochlorinated crotofolane trivially named ermiasoid.

Table 4.4-9: NMR data for 1β-Acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin (Ermiasoid) (E37)

| **No.** | **$^{13}$C NMR** | **$^{1}$H NMR** | **HMBC (H→C)** | **COSY** | **NOESY** |
|---|---|---|---|---|---|
| 1 | 80.9 | 5.10 d 8.2 | 2, 14, 19 | 2 | 2, 13 |
| 2 | 33.5 | 2.81 m | 3, 4, 14 | 1, 19 | 1, 7 |
| 3 | 66.8 | 3.61 s | 1, 2 | x | 19 |
| 4 | 81.9 | - | - | - | - |
| 5 | 78.0 | 3.55 s | 3, 4, 6, 7, 14 | x | 20 |
| 6 | 75.6 | - | - | - | - |
| 7 | 44.5 | 3.73 d 11.9 | 8,9, 13, 15, 16 | x | 20 |
| 8 | 129.5 | - | - | - | - |
| 9 | 82.7 | 5.37 t 5.8 | x | 10α, 10β, 17 | 13 |
| 10α | 34.3 | 2.32 m | 9, 12 | 9, 11α, 11β | 10β, 11α, 11β |
| 10β | | 1.61 m | 9, 12 | 9, 11α, 11β | 10α, 11α, 11β |
| 11α | 35.1 | 2.31 m | x | 10α, 10β, 11β | 11β, 18A, 18B |
| 11β | | 2.31 m | x | 10α, 10β, 11α | 11α, 18A, 18B |
| 12 | 144.9 | - | - | - | - |
| 13 | 44.0 | 2.85 d 11.9 | 7, 11, 12, 18 | 7 | 1, 9, 19, 20 |
| 14 | 66.3 | - | - | - | - |
| 15 | 160.2 | - | - | - | - |
| 16 | 173.7 | - | - | - | - |
| 17 | 10.5 | 1.83 d 1.7 | 8, 15, 16 | 9 | **7** |
| 18A | 113.9 | 5.32 s | 3, 11 | 18B | 11α, 11β, 18B |
| 18B | | 4.82 s | 3, 11 | 18A | 11α, 11β, 18A |
| 19 | 10.4 | 1.19 d 7.7 | 1, 2, 3 | 2 | 3, 13, 1-$CH_3$ |
| 20 | 27.5 | 1.62 s | 5, 6, 7 | - | 5, 8, 13 |
| 1-$CH_3$ | 20.6 | 1.93 s | C=O | - | 19 |
| 1-CO | 172.5 | - | - | - | - |

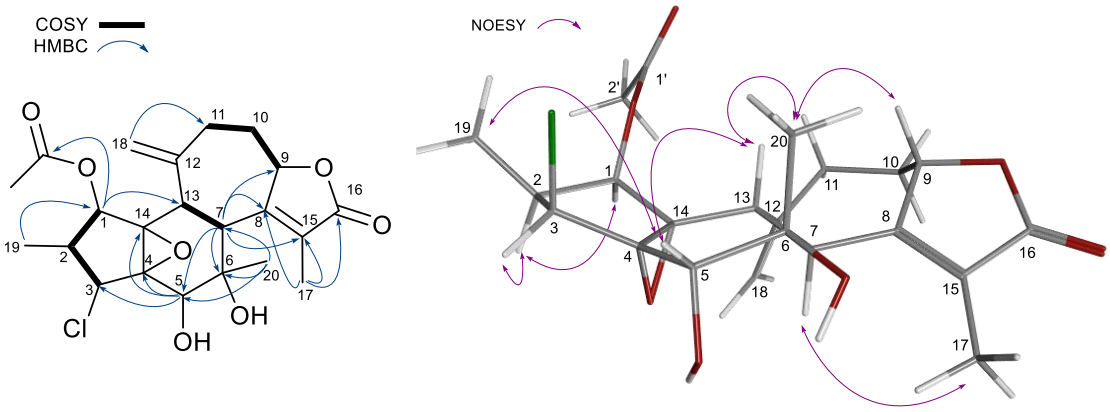
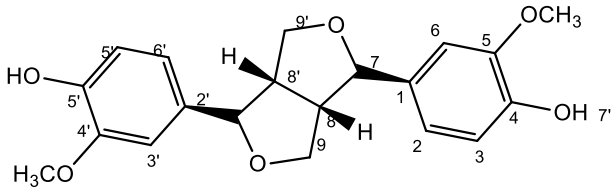



Figure 4.4-2: Key COSY, HMBC and NOESY correlations observed for 1β-Acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin (Ermiasoid) (E37)

#### 4.4.1.1.8. E2: Pinoresinol




**E2**

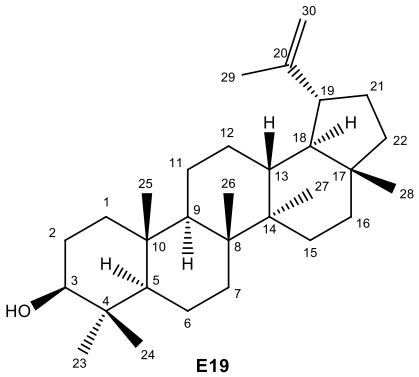
Compound **E2** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a yellowish oil. The $^{1}$H NMR spectrum **(Table 4.4-10)** showed three peaks of aromatic protons at $\delta_H$ 6.89 (1H, s), 6.83 (1H, dd, J = 8.0, 2.3 Hz) and 6.91 (1H, d, J = 8.0 Hz), suggesting that the aromatic ring has three substituted positions. The oxymethine proton was evident at 4.74 (d, 4.2) ppm as a doublet, and the methylene groups bearing oxygen atoms at 4.26 and 3.88 ppm were two doublets of the doublet. A methine proton was observed at 3.10 ppm (multiplet), and a methoxy group was confirmed at 3.92 ppm (singlet). The data of compound **E2** were consistent with previously isolated pinoresinol (Trinh *et al*., 2007). The HSQC, HMBC and COSY spectrum of **E2** is depicted in **Appendix 59-61.**

Table 4.4-10: NMR data for Pinoresinol (E2)

| No. | $^1$H NMR | $^1$H NMR* |
|---|---|---|
| 1/1’ | - | - |
| 2/2’ | 6.89 s | 6.89 (2H, d, 1.5) |
| 3/3’ | - | - |
| 4/4’ | - | - |
| 5/5’ | 6.91 d 8.0 | 6.88 (2H, d, 8.0) |
| 6/6’ | 6.83 dd 2.3, 8.0 | 6.82 (2H, dd, 1.5, 8.0) |
| 7/7’ | 4.74 d (4.2) | 4.73 (2H, d, 5.0) |
| 8/8’ | 3.10 m | 3.10 (2H, m) |
| 9A/9A’ | 4.26 dd 6.7, 10.0 | 4.24 (2H, dd, 7.0, 9.5) |
| 9B/9B’ | 3.88 dd 4.5, 10.0 | 3.88 (2H, dd, 4.0, 9.5) |
| 3-$OCH_3$/3’-$OCH_3$ | 3.92 s | 3.90 (3H, s) |

*(Trinh *et al*., 2007)

#### 4.4.1.1.9. E19: 3β-Hydroxylup-20(29)-ene (lupeol)



E19

Compound **E19** was isolated from the ethyl acetate soluble extract of the stem bark of *C. megalocarpus* as a white solid and identified as the known lupeol, which has been isolated previously from many sources (Ahmad & Atta-ur-rahman, 1994; Burns *et al*., 2000). The $^1$H NMR spectrum **(Table 4.4-10)** of compound E19 showed the presence of seven methyl group proton resonances at $\delta_H$ 0.75, 0.78, 0.82, 0.92, 0.94, 0.94 and 1.67, which corresponded to the carbon resonances at $\delta_C$ 19.5, 18.2, 14.7, 16.2, 16.3, 15.6, and 28.2 in the $^{13}$C NMR spectrum and HSQCDEPT spectrum **(Appendix 62-63).** The $^1$H NMR spectrum showed a proton resonance at $\delta_H$ 3.18 (1H, dd, *J*=11.1, J=4.9 Hz), which was seen to correspond to a carbon resonance at 79.2 in the HSQCDEPT spectrum. This proton resonance and the H-5 proton resonance at 0.69 (d *J*=9.1 Hz) were seen to correlate in the HMBC spectrum with the 3H-23 and 3H-24 methyl group resonances at 0.96 and 0.76. The HMBC spectrum **(Appendix 93-97)** showed a correlation between the downfield methyl group resonance at 1.67 and the two nonequivalent methylene proton resonances at 4.68 and 4.56, which were ascribed to H-29A

and H-29B. Compound **E19** was identified as the known 3β-hydroxylup-20(29)-ene, commonly known as lupeol, which has been evaluated for its anti-inflammatory and antiangiogenic activities. A literature search revealed $^{13}$C NMR chemical shifts similar to those of compound **E19** for lupeol.

Table 4.4-11: NMR data for lupeol (E19) in $CDCl_3$

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR*** | **$^{1}$H NMR** | **$^{1}$H NMR*** |
|---|---|---|---|---|
| 1α | 38.9 | 38.7 | 1.66 m | 1.65 m |
| 1β | | | 0.92 m | 0.91 m |
| 2α | 27.6 | 27.4 | 1.59 m | 1.60 m |
| 2β | | | 1.66 m | 1.65 m |
| 3 | 79.2 | 79.0 | 3.18 dd (11.1, 4.9) | 3.18 dd (11.4, 4.9) |
| 4 | 39.0 | 38.8 | | - |
| 5 | 55.5 | 55.3 | 0.68 d (9.2) | 0.69 d (9.3) |
| 6α | 18.5 | 18.3 | 1.50 m | 1.51 m |
| 6β | | | 1.39 m | 1.39 m |
| 7α | 34.5 | 34.3 | 1.38 m | 1.38 m |
| 7β | | | 1.38 m | 1.38 m |
| 8 | 41.0 | 40.8 | - | - |
| 9 | 50.6 | 50.4 | 1.28 m | 1.27 m |
| 10 | 37.4 | 37.2 | - | - |
| 11α | 21.1 | 20.9 | 1.41m | 1.41 m |
| 11β | | | 1.20 m | 1.21 m |
| 12α | 25.3 | 25.2 | 1.07 m | 1.06 m |
| 12β | | | 1.66 m | 1.67 m |
| 13 | 38.9 | 38.3 | 1.64 m | 1.64 m |
| 14 | 43.2 | 42.9 | - | - |
| 15α | 28.2 | 27.5 | 1.01 m | 1.00 m |
| 15β | | | 1.69 m | 1.67 m |
| 16α | 35.8 | 35.6 | 1.39 m | 1.38 m |
| 16β | | | 1.49nm | 1.47 m |
| 17 | 43.2 | 43.0 | - | - |
| 18 | 48.5 | 48.0 | 1.36 m | 1.35 m |
| 19 | 48.2 | 47.9 | 2.37 m | 2.38 m |
| 20 | 151.3 | 151.0 | - | - |
| 21α | 30.0 | 29.9 | 1.33 m | 1.32 m |
| 21β | | | 1.92 m | 1.92 m |
| 22α | 40.2 | 40.0 | 1.21 m | 1.19 m |
| 22β | | | 1.39 m | 1.38 m |
| 23 | 28.2 | 28.0 | 0.98 s | 0.97 s |
| 24 | 15.6 | 15.4 | 0.75 s | 0.77 s |
| 25 | 16.3 | 16.1 | 0.82 s | 0.83 s |
| 26 | 16.2 | 16.0 | 1.02 s | 1.02 s |
| 27 | 14.7 | 14.6 | 0.97 s | 0.98 s |
| 28 | 18.2 | 18.0 | 0.79 s | 0.79 s |
| 29A | 109.5 | 109.3 | 4.56 br m | 4.56 br s |
| 29B | | | 4.68 d (2.3) | 4.68 br s |
| 30 | 19.5 | 19.3 | 1.67 s | 1.69 s |

*(Jamila *et al*., 2014)

#### 4.4.1.2. Cytotoxicity and anti-HIV activity of pure compounds isolated from *C. megalocarpus*

The cytotoxic and antiviral activity findings for the pure compounds isolated from *C. megalocarpus* and the control drugs are summarized in **Table 4.4-12.** In the MTT cytotoxic assay using human T-lymphocytic MT-4 cells, the pure compounds isolated from *C. megalocarpus* showed significantly ($p < 0.001$) higher $CC_{50}$ values than the control drugs **(Figure 4.4-3)**. Although the maximum cytotoxic effect ($Emax_c$) was higher (>60%), the compounds (except E2 and E5) inhibited viral replication at much lower concentrations ($IC_{50}$) than the maximum noncytotoxic concentration (MNTC), indicating the safety of the compounds (**Table 4.4-12)**. Furthermore, a cytotoxicity test performed on FM-55-M1 human melanoma cells revealed the $CC_{50}$ value of these compounds to be above 50 µM, which indicates that these compounds could be considered safe as they need high concentrations to exert cytotoxic effects. Among the tested compounds, E12, ermiasolide A, displayed the highest anti-HIV activity by inhibiting HIV-induced CPE by 93% at an $IC_{50}$ value of 0.002 µg/mL. Similarly, E17, E5, and E23 inhibited viral replication by 86, 83.9, and 83.3%, respectively (**Table 4.4-12).**

Table 4.4-12: Cytotoxicity and anti-HIV-1 activities of pure compounds isolated from *C. megalocarpus*

| Materials | Cytotoxicity | | | Antiviral activity | | SI |
|---|---|---|---|---|---|---|
| | MNTC (µg/mL) | $CC_{50}$ (µg/mL) | $Emax_C$ (%) | $IC_{50}$ (µg/mL) | $Emax_{AV}$ (%) | |
| **AZT** | 0.38 ± 0.19 | 0.53 ± 0.29 | 36.28 ± 0.83 | 0.002 ± 0.00 | 83.5 ± 0.57 | 279.4 |
| **TDF** | 4.92 ± 0.71 | 6.73 ± 0.24 | 13.17 ± 0.43 | 0.04 ± 0.01 | 80.55 ± 0.46 | 176.5 |
| **ABC** | 0.18 ± 0.03 | 0.26 ± 0.00 | 17.83 ± 0.57 | 0.05 ± 0.031 | 58.67 ± 0.43 | 5.0 |
| **NVP** | 0.57 ± 0.0 | 0.82 ± 0.0 | 39.13 ± 0.65 | 0.24 ± 0.09 | 72.53 ± 0.47 | 3.5 |
| **E2** | 1.01 ± 0.03 | 16.14 ± 0.41 | 65.91 ± 0.41 | 0.02 ± 0.01 | 63.06 ± 0.58 | 959.0 |
| **E5** | 8.8 ± 0.69 | 79.09 ± 0.49 | 69.00 ± 0.77 | 0.033 ± 0.01 | 83.91 ± 0.39 | 2380.8 |
| **E12** | 41.84 ± 0.11 | 96.77 ± 0.44 | 62.21 ± 0.67 | 0.002 ± 0.00 | 93.4 ± 0.60 | 39872.3 |
| **E17** | 64.93 ± 0.26 | 148.1 ± 0.05 | 65.00 ± 0.01 | 0.044 ± 0.01 | 77.46 ± 0.93 | 3384.4 |
| **E19** | 75.75 ± 0.74 | 141.8 ± 0.7 | 70.28 ± 0.75 | 0.047 ± 0.02 | 77.01 ± 0.38 | 3048.2 |
| **E22** | 4.973 ± 0.25 | 38.44 ± 0.63 | 81.59 ± 0.41 | 0.002 ± 0.00 | 69.51 ± 0.26 | 25339.5 |
| **E23** | 150.9 ± 0.55 | 183.7 ± 0.65 | 53.18 ± 0.57 | 0.002 ± 0.00 | 83.3 ± 0.97 | 89741.1 |
| **E37** | 45.73 ± 0.53 | 91.46 ± 0.21 | 64.4 ± 0.94 | 0.012 ± 0.00 | 86.64 ± 0.69 | 7423.7 |

Results are shown as means ± S.E. M (n=3)

AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; E2, Pinoresinol; E5, 4H-α,7H-α,10α-eudesm-11-en-5β-ol; E12, ermiasolide A; E17, ermiasolide C; E19, lupeol; E22, ermiasolide B; E23, Crotocascarin K; E37, ermiasoid; MNTC, Maximum nontoxic concentration; $CC_{50}$, 50% cytotoxic concentration; $Emax_{C,}$ Maximum cytotoxic effect %; $IC_{50,}$ 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

The pure compounds (E12, E22, and E23) showed $IC_{50}$ and $Emax_{AV}$ values approximately similar to AZT's, indicating their potential antiviral efficacy (**Figure 4.4-4**). In addition, these compounds displayed higher potency than TDF, ABC, and NVP. Only E2 (pinoresinol) showed lower antiviral efficacy ($Emax_{AV}$ = 63.06%). As depicted in **Figure 4.4-5**, the pure compounds displayed concentration-dependent inhibition of virus-induced CPE, as the % CPE inhibition increased with increasing concentrations of the pure compounds.

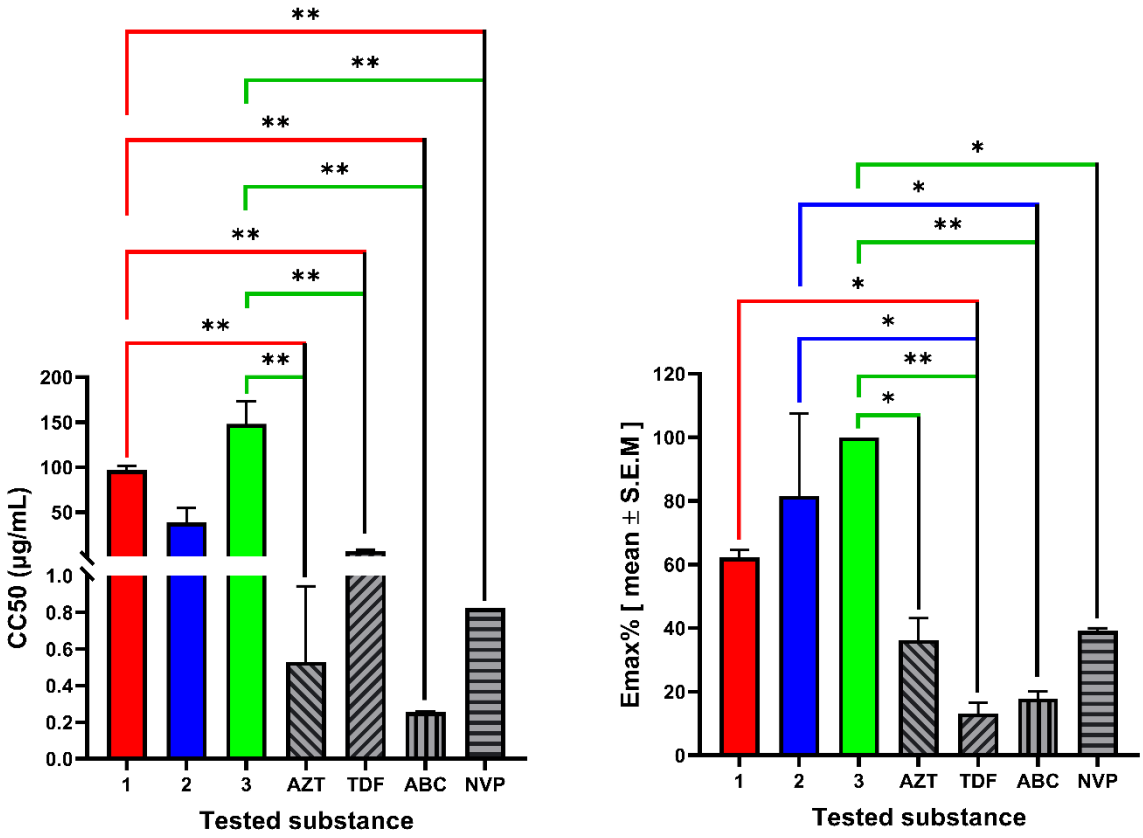



Figure 4.4-3: Cytotoxicity of ermiasolides isolated from *C. megalocarpus*

The results are expressed as the mean of three independent experiments ± S.E.M. 1, ermiasolide A; 2, ermiasolide B; 3, ermiasolide C; AZT, zidovudine, ABC, abacavir, NVP, nevirapine; TDF, tenofovir; C, control; ns; not significant, *Denotes p value < 0.05, ** Denotes p value <0.01, *** Denotes p value < 0.001.

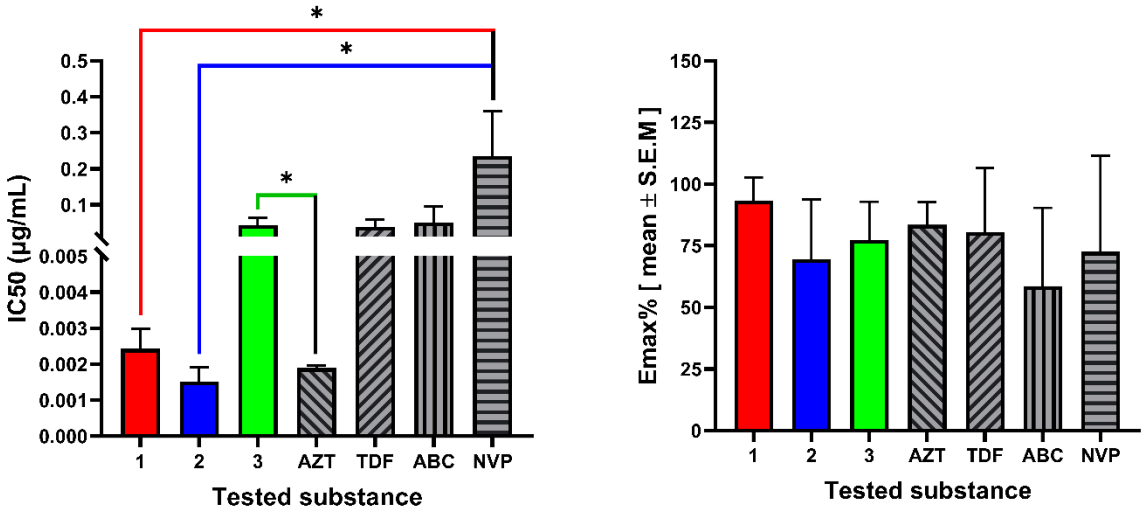



Figure 4.4-4: Anti-HIV activity of ermiasolides from *C. megalocarpus*

The results are expressed as the mean of three independent experiments ± S.E.M. AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; 1, ermiasolide A; 1, ermiasolide B; 3, ermiasolide C. *Denotes p value <0.05

The demonstrated highest anti-HIV activity of Ermiasolide A (E12) and Ermiasolide B (E22) could be due to presence of hydrogen bond forming methoxy and hydroxyl groups in C-8 of the structures, respectively. These important functional groups were missing in Ermiasolide C (E17) which could have contributed to its lower antiviral efficacy ($Emax_{AV}$= 77.46 %). Furthermore, the presence of methoxy group in E12 at C-8, contributes to its highest anti-HIV activity ($Emax_{AV}$= 93%) as compared to E22 which has –OH group at C-8. This difference could be due to the role of the methoxy group in forming stable hydrophobic interactions at the receptor site as depicted in the *in silico* study (**Figure 4.5-5**). Previous studies have confirmed the role of hydrophobic interactions as driving force of ligand–protein intereaction (Gurung *et al*., 2021)

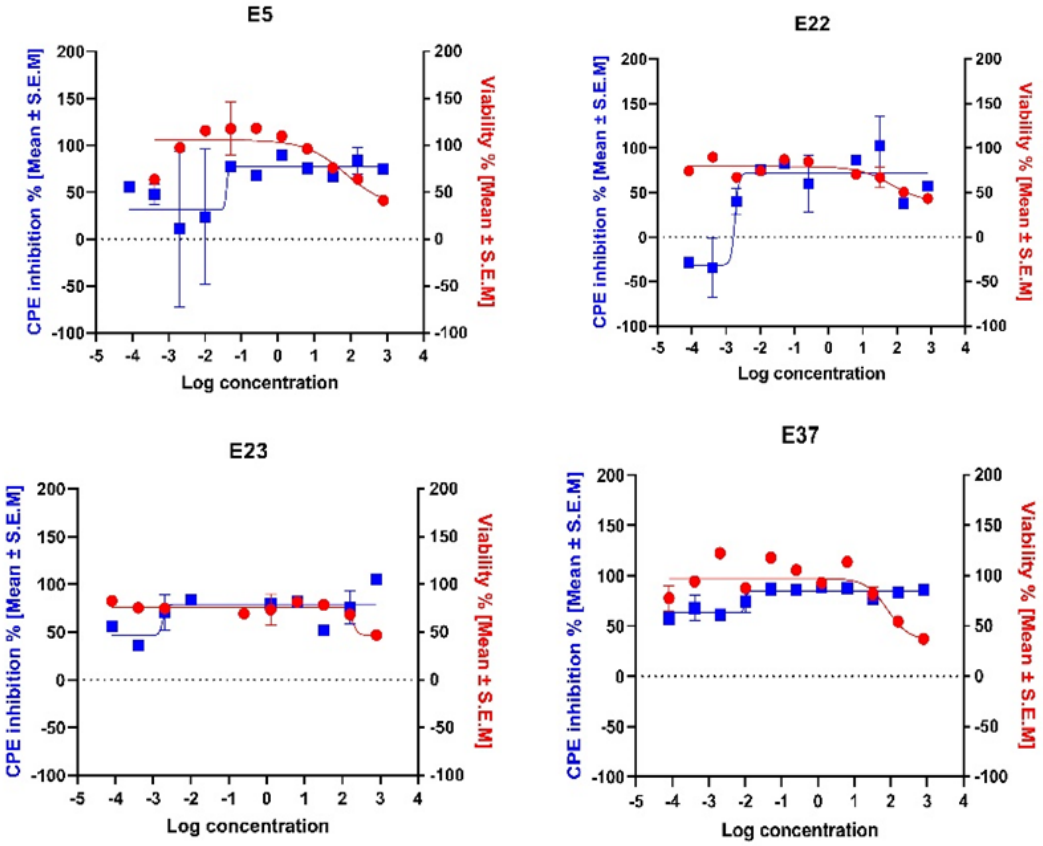



Figure 4.4-5: Concentration-response curves analysis for the anti-HIV activity of pure compounds isolated from *C. megalocarpus.*

Results presented in the curves are means ± S.E. M of three independent experiments. Cell viability % (red line) and the inhibition % of the virus-induced cytopathic effect (blue line) associated with control drugs and the tested extracts at the concentration level (800 – $8.192\times10^5$ µg/mL). E5, 4H-α,7H-α,10α-eudesm-11-en-5β-ol; E22, ermiasolide B; E23, Crotocascarin K; E37, ermiasoid

### 4.4.1.3. Discussion

In this study we report the anti HIV activity of three new eudesmane type sequiterpenes and two new crotofolane diterpenoids isolated from *C. megalocarpus.*

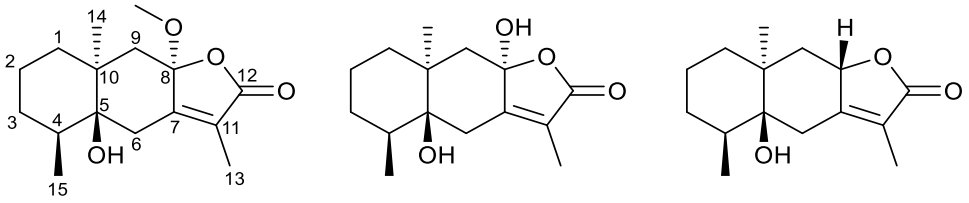
1. **Eudesmane type sesquitepenes**

Sesquiterpene lactones are a subclass of terpenoids, which is a large group of compounds found in plants. Sesquiterpene lactones play an important role in plant defense in nature, acting as antibacterials, antivirals, antifungals, and insecticides (Tuasha *et al*., 2022). Sesquiterpene lactones have piqued the interest of researchers in recent years, primarily due to their cytotoxic and anticancer properties (Matejić *et al.,* 2014). Among isolated sesquiterpene lactones, eudesmane type sesquiterpenoids have displayed cytotoxic and potential antiviral

activity. Zhu *et al* (Zhu *et al*., 2010) have demonstrated cytotoxic activity of three eudesmane lactones isolated from *Ajania przewalskii* Poljakov (Asteraceae).

A number of sesquiterpenes have previously been isolated from *Croton* species. β-caryophyllene from *C. aubrevillei, C. geayi*; caryophyllene oxide, γ-cadinene and α-cadinene from *C. geayi* (Radulović *et al*., 2006); blumenol A from *C. pedicellatus* (Langat *et al*., 2018a); crocrassins A, crocrassins B (Ghosh *et al.*, 2013), cracroson H (Qiu *et al.*, 2018), 6S-hydroxy-cyperenoic acid, crassifterpenoid A (Tian *et al*., 2019) from *C. crassifolius,* and patchoulenone (Aye *et al*., 2019) from *C. oblongifolius.* Eudesmane type sesquiterpenoids were not previously reported from *Croton* species and makes this research fundamental.

In this research, we demonstrated the anti-HIV activity of three new or previously undescribed eudesmane type sesquiterpenes, 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (**E12**), 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (**E22**), and 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (**E17**) isolated from the stem bark of *C. megalocarpus* (Figure 4.4-6). As depicted in **Table 4.4-12**, E12, E22 and E17 inhibited HIV-1 replication with $IC_{50}$ values of 0.002 µg/mL, 0.002 µg/mL and 0.044 µg/mL, respectively.




**E12** **E22** **E17**

Figure 4.4-6: Eudesmane type sesquiterpenes isolated from *C. megalocarpus*

A molecular docking study **Table 4.5-2** and **4.5-3** revealed strong binding affinity of these compounds with HIV-1 protease enzyme (3EL9) compared with the HIV-1 reverse transcriptase**.** Docking studies indicated that the predicted free energy of binding obtained for ermiasolides against HIV-1 reverse transcriptase were higher (ΔG = -3.555 kcal/mol for **E17**, ΔG = -2.839 kcal/mol for **E22,** ΔG = -2.317 kcal/mol for **E12** and ΔG = -1.550 kcal/mol for **E5**) compared to the known inhibitor nevirapine (ΔG = -7.679 kcal/mol). Lower predicted energies of binding were obtained against HIV-1 protease (ΔG = -6.067 kcal/mol for **E12**, ΔG = -5.994 kcal/mol for **E22,** ΔG = -5.850 kcal/mol for **E17** and ΔG = -5.750 kcal/mol for **E5**), although in both cases they were higher than that obtained for the positive control atazanavir (ΔG = -11.49 kcal/mol) (**Table 4.5-3**). However, the results obtained indicated that the

mechanism of action leading to the observed anti-HIV activities is through the HIV-1 protease inhibition, which agreed with the *in vitro* assay (**Table 4.5-1**).

The high anti-HIV activity of Ermiasolide A (E12) and Ermiasolide B (E22) could be due to presence of hydrogen bond forming methoxy and hydroxyl groups in C-8 of the structures, respectively. These important functional groups were missing in Ermiasolide C (E17) which could have contributed to its lower antiviral efficacy ($Emax_{AV}$= 77.46 %). Furthermore, the presence of methoxy group in E12 at C-8, could contribute to its higher anti-HIV activity ($Emax_{AV}$= 93%) compared to **E22** which presents a hydroxy group at C-8 **(Figure 4.4-6).** This difference could be due to the role of the methoxy group in forming stable hydrophobic interactions at the enzyme active site as depicted in the *in silico* study (**Figure 4.5-1 and 4.5-2**). Previous studies have confirmed the role of hydrophobic interactions as driving force of ligand–protein interaction (Gurung *et al*., 2021). The results obtained could indicate that the mechanism of action leading to the observed anti-HIV activities is through HIV-1 protease inhibition, which agrees with the data obtained in the *in vitro* assay. Figure 4.4-7 and 4.4-8 depict the interactions of the ermiasolides with HIV enzymes.

**2. Crotofolane diterpenoids**

In 1976, a crotofolane diterpenoid, crotofolin A, was reported from a Jamaican plant, *Croton corylifolius* Lam. (Euphorbiaceae) and, since then, despite several studies on the chemistry of *Croton* species, crotofolane-type diterpenoids have been reported on rarely (Burke *et al*., 1979). Crotofolanes have fused 5-, 6- and 7-membered rings, and are biosynthesized from cembranes via casbane and lathyrane through cross annular cyclization (Kawakami *et al*., 2015). In total, approximately 38 crotofolanes have been reported, and all are restricted to the genus *Croton*. The previous seven *Croton* species that have yielded crotofolanes are *C. argyrophyllus* (Filho *et al.*, 2013). *C. caracasanus* (Chávez *et al.*, 2013), *C. cascarilloides* (Gao *et al*., 2018), *C. corylifolius* (Burke *et al.*, 1979), *C. dichogamus* (Aldhaher *et al.*, 2017), *C. haumanianus* (Kawakami *et al.*, 2016) and *C. insularis* (Maslovskaya *et al.*, 2014). Thus far, *C. megalocarpus* has only yielded clerodane and abietane diterpenoids (Zeng & Zhan, 2019). Chlorinated natural products have been observed only rarely, and the few that have been reported have diverse activities (Langat *et al*., 2020). Chlorinated compounds are rare in the genus *Croton*, with only one chlorinated diterpenoid, laevinoid, reported from *Croton laevigatus* (Wang *et al*., 2013).

In this research, we reported three crotofolane diterpenoids including previously undescribed 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (E37) and 11β-

acetoxycrotocascarin L (**E24**), and known compound, crotocascarin K (E23) (**Figure 4.4-7**). As depicted in **Table 4.4-12** and **Figure 4.4-8**, E37 and E23 inhibited HIV-1 replication with $IC_{50}$ values of 0.012 µg/mL and 0.002 µg/mL, respectively.

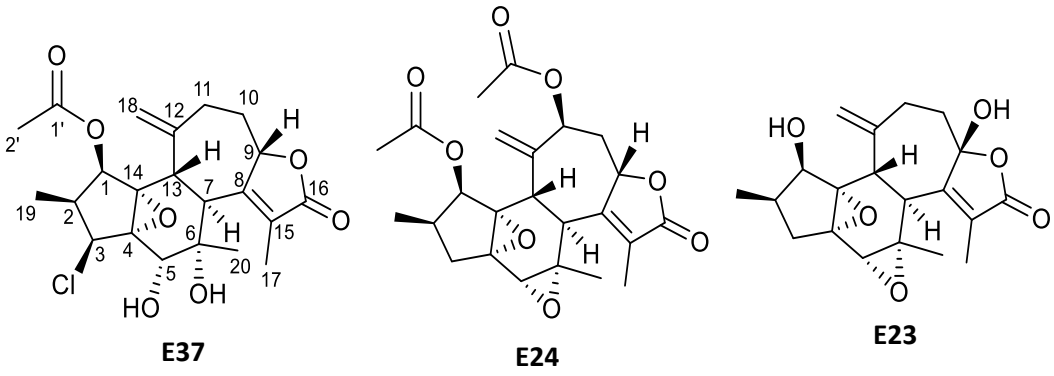



Figure 4.4-7: Crotofolane diterpenoids isolated from *C. megalocarpus*

Docking studies indicated that the predicted free energy of binding obtained for both compounds against HIV-1 reverse transcriptase were higher (ΔG = -1.24 kcal/mol for **E37**, ΔG = -4.68 kcal/mol for **E23**) compared to the known inhibitor nevirapine (ΔG = -7.679 kcal/mol). Lower predicted energies of binding were obtained against HIV-1 protease (ΔG = -7.326 kcal/mol for E37, ΔG = -7.115 kcal/mol for **E23**), although in both cases they were higher than that obtained for the positive control atazanavir (ΔG = -11.49 kcal/mol) (**Table 4.5-3**). However, the results obtained indicated that the mechanism of action leading to the observed anti-HIV activities is through the HIV-1 protease inhibition, which agreed with the *in vitro* assay.

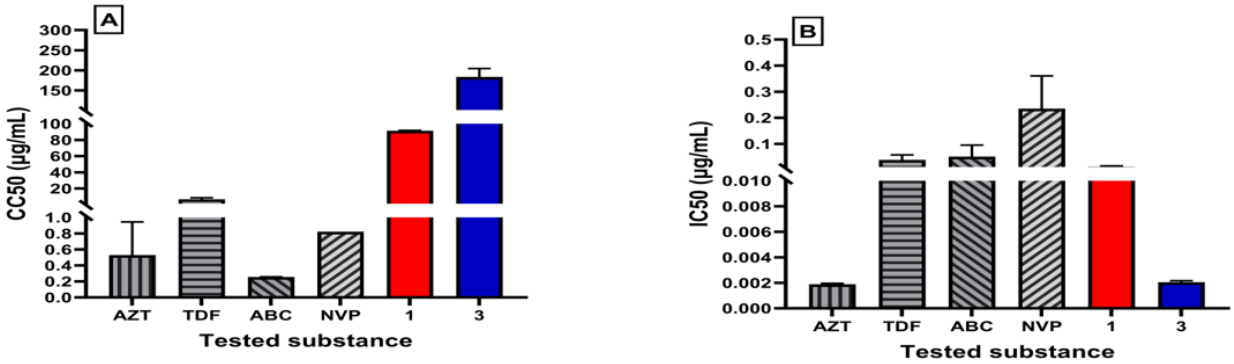



Figure 4.4-8:**.** Cytotoxicity $IC_{50}$ [A] and antiviral activity $IC_{50}$ [B] values of the control drugs and the tested compounds E37 (1) and E23 (3). Results expressed are mean of three independent experiments ± S.E.M.

### 4.4.2. *Croton macrostachyus*

Based on the findings from the anti-HIV activity of the solvent fractions from *C. macrostachyus*, the hexane fraction of the leaves of *C. macrostachyus* was subjected to column chromatography to yield seven pure compounds **(Table 4.4-4).** The chemical structure of these compounds is depicted in **Figure 4.4-9**.

Table 4.4-13: Compounds isolated from the stem bark of *C. macrostachyus*

| Ser. No. | Name | Code |
|---|---|---|
| **1.** | 2-Methoxy benzyl benzoate | **A5** |
| **2.** | Betulin | **A19** |
| **3.** | Lupenone | **A4b** |
| **4.** | Lupeol acetate | **A11** |
| **5.** | 3β-hydroxylup-20(29)-ene (lupeol) | **A7** |
| **6.** | Stigmasterol | **A15** |
| **7.** | Sitosterol | **A16** |

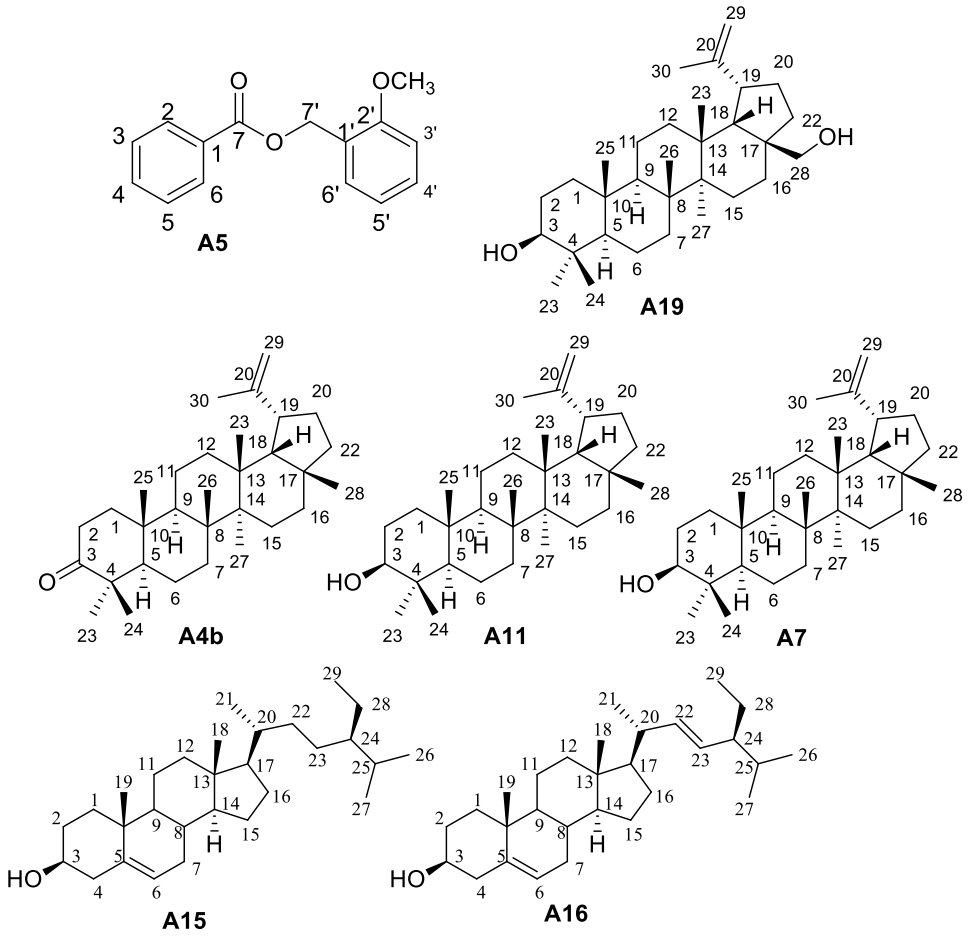
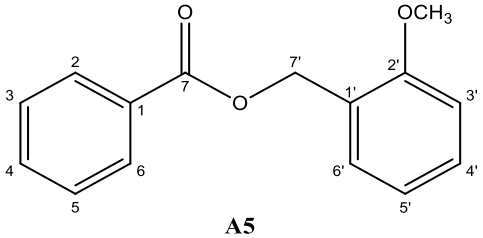



Figure 4.4-9: Pure compounds isolated from *C. macrostachyus*

### 4.4.2.1. Chemistry of pure compounds isolated from *C. macrostachyus*

#### 4.4.2.1.1. A5: 2-Methoxy benzyl benzoate




**A5**

2-Methoxy benzyl benzoate (compound **A5)** was isolated from the hexane soluble extract of the leaf of *C. macrostachyus* as a clear oil. The $^{1}$H-NMR spectrum **(Table 4.4-14)** of **A5** showed one aromatic methoxy (3.79, s), one carbinol methylene (5.45, s), and nine aromatic protons (6.89-8.02) **(Appendix 64)**. The *J* coupling pattern of the five aromatic protons showed the presence of one monosubstituted phenyl ring. The $^{13}$C NMR spectrum **(Appendix 65)** indicated the presence of 15 carbons, including one carbonyl (8 166.8), one methoxy (56.7), one carbinol methylene (62.4), and two aromatic rings. These data led to identifying **A5** as a 2-methoxybenzyl benzoate, which was previously reported (Asakawa *et al*., 1996; Nkunya, 2004; Van Kiem *et al*., 2005a). The DEPT, HSQCDEPT, HMBC, and COSY spectrum of **A5** is depicted in **Appendix 66-69**.

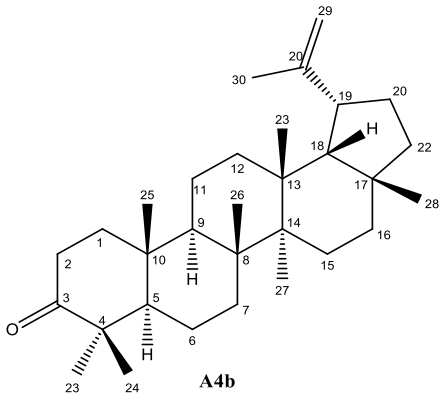
Table 4.4-14: NMR data for 2-Methoxy benzyl benzoate (A5) in $CDCl_3$

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR*** | **$^{1}$H NMR** | **$^{1}$H NMR*** |
|---|---|---|---|---|
| 1 | 130.6 | 130.9 | - | - |
| 2 | 129.9 CH | 129.8 | 8.02 dd 8.5, 1.7 | 8.13 dd 8.4, 2.1 |
| 3 | 129.6 CH | 130.1 | 7.37 m | 7.45 ddd 8.4, 8.0, 1.7 |
| 4 | 133.1 CH | 133.2 | 7.48 ddd 8.5, 1.7, 1.4 | 7.58 dd 8.0, 2.1 |
| 5 | 129.7 CH | 130.1 | 7.25 dd 1.8, 8.1 | 7.45 ddd 8.4, 8.0, 1.7 |
| 6 | 129.9 CH | 129.8 | 8.02 dd 8.5, 1.7 | 8.13 dd 8.4, 2.1 |
| 7 | 166.8 | 167.0 | | - |
| 1’ | 124.6 | 124.9 | - | - |
| 2’ | 157.7 | 158.0 | - | - |
| 3’ | 110.7 CH | 110.9 | 6.89 d 8.1 | 6.98 dd 8.0, 1.8 |
| 4’ | 128.5 CH | 129.8 | 7.37 m | 7.35 dd 8.0, 7.9, 2.0 |
| 5’ | 120.6 CH | 120.9 | 6.90 dd 1, 7.5 | 7.01 ddd 7.9, 7.5, 1.8 |

| | | | | |
|---|---|---|---|---|
| 6’ | 128.5 CH | 128.7 | 7.37 m | 7.45 dd 7.5, 2.0 |
| 7’ | 62.4 $CH_2$ | 62.6 | 5.36 s | 5.45 s |
| $OCH_3$ | 56.7 $CH_3$ | 55.9 | 3.79 s | 3.89 s |

*(Van Kiem *et al*., 2005)

#### 4.4.1.1.1. A4b: Lupenone



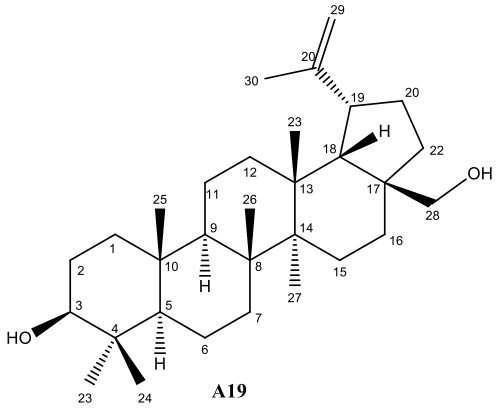

Lupenone (compound **A4b)** was isolated from the hexane soluble extract of the leaf of *C. macrostachyus* as a white solid and identified as the widely known lupane triterpenoid lupenone. The $^{13}$C NMR spectrum **(Table 4.4-15)** showed thirty carbon resonances**.** Two double bond carbon resonances at 109.6 and 151.6 and a carbonyl carbon resonance at δ 218.6 in conjunction with the molecular formula suggested that compound **A4b** was a pentacyclic triterpenoid. Analysis of the $^{1}$H NMR, 13C NMR, and DEPT spectra showed that compound **A4b** possessed seven methyl proton resonances at 1.07, 1.02, 0.93, 1.07, 1.68, 0.96, and 0.79, ten methylene groups, which included the characteristic lupane type-triterpenoid terminal double bond proton resonances at 4.69 (br s *W*1/2 = 5.10 Hz) and 4.57 (br s *W*1/2 = 5.32 Hz), assignable to the two H-29 protons and five methine groups **(Appendix 70-76).** The ketone carbon resonance, observed at δ 218.6, was ascribed to C-3 on biogenetic grounds (Carpenter *et al*., 1980). A search in the literature showed that the $^{13}$C NMR chemical shifts for compound **A4b** and those of the common compound lupenone (Carpenter *et al*., 1980) isolated previously from *Poa huecu* Par., *Madhuca butyracea*, *Mangifera indica*, and many other sources were the same (Ahmad & Atta-ur-rahman, 1994). Lupenone is known to have a broad spectrum of biological activities. It is reported to be active against both gram-positive (*Bacillus subtilis*) and gram-negative (*Pseudomonas pyocyanee*) bacteria (Kim, 2001). Lupenone has also been found to show antifungal activities against *Saccharomyces cerevisiae* and *Microsporum gypseum* (Kim, 2001; Mubiu *et al*., 2017). Lupenone is also known to show antioxidative activity on lipid peroxidation by 6.4% (Ahmad & Atta-ur-rahman, 1994; F. Xu *et al*., 2018).

Table 4.4-15: NMR data for Lupenone (A4b) in $CDCl_3$

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR*** | **$^{1}$H NMR** | **$^{1}$H NMR*** |
|---|---|---|---|---|
| 1α | 40.0 | 39.6 | 1.92 m | 1.90 m |
| 1β | | | 1.42 m | 1.42 m |
| 2α | 34.2 | 34.1 | 2.50 m- | 2.48 m |
| 2β | | | 2.48 m | 2.42 m |
| 3 | 217.8 | 217.9 | - | - |
| 4 | 47.4 | 47.3 | | - |
| 5 | 54.9 | 54.9 | 1.33 m | 1.34 m |
| 6α | 19.7 | 19.2 | 1.51 m | 1.47 m |
| 6β | | | 1.51 m | 1.47 m |
| 7α | 33.6 | 33.5 | 1.45 m | 1.43 m |
| 7β | | | 1.45 m | 1.43 m |
| 8 | 40.8 | 40.7 | - | - |
| 9 | 49.8 | 49.7 | 1.39 m | 1.39 m |
| 10 | 36.7 | 36.8 | - | - |
| 11α | 21.5 | 21.4 | 1.42 m | 1.43 m |
| 11β | | | 1.30 m | 1.43 m |
| 12α | 25.2 | 25.1 | 1.70 m | 1.69 m |
| 12β | | | 1.12 m | 1.10 m |
| 13 | 38.1 | 38.1 | 1.70 m | 1.68 m |
| 14 | 42.8 | 42.8 | - | - |
| 15α | 27.4 | 27.4 | 1.69 m | 1.68 m |
| 15β | | | 1.04 m | 1.02 m |
| 16α | 35.5 | 35.5 | 1.51 m | 1.50 m |
| 16β | | | 1.43 m | 1.41 m |
| 17 | 42.9 | 42.9 | - | - |
| 18 | 48.2 | 48.2 | 1.39 m | 1.39 m |
| 19 | 47.9 | 47.9 | 2.40 m | 2.39 m |
| 20 | 150.3 | 150.8 | - | - |
| 21α | 29.8 | 29.6 | 1.95 m | 1.93 m |
| 21β | | | 1.29 m | 1.27 m |
| 22α | 39.6 | 39.4 | 1.43 m | 1.42 m |
| 22β | | | 1.23 m | 1.21 m |
| 23 | 26.7 | 26.6 | 1.07 s | 1.07 s |
| 24 | 21.1 | 21.0 | 1.02 s | 1.02 s |
| 25 | 16.0 | 15.9 | 1.07 s | 1.07 s |
| 26 | 15.8 | 15.7 | 0.93 s | 0.93 s |
| 27 | 14.5 | 14.4 | 0.96 s | 0.96 s |
| 28 | 18.0 | 17.9 | 0.79 s | 0.79 s |
| 29A | 109.4 | 109.3 | 4.69 d 2.0 | 4.69 br s |
| 29B | | | 4.57 s | 4.57 br s |
| 30 | 19.3 | 19.6 | 1.61 s | 1.68 s |

*(Carpenter *et al.*, 1980)

#### 4.4.1.1.2. A19 – Betulin



A19

Betulin (compound **A19)** was isolated from the hexane soluble extract of the leaf of *C. macrostachyus* as a white solid and identified as the known betulin, which has been isolated previously from *C. macrostachyus* (Tala *et al*., 2013b). The $^{1}$H NMR spectrum **(Table 4.4-16)** of compound **A19** showed the presence of seven methyl group proton resonances at 0.76, 0.81, 0.96, 0.99, 1.01 and 1.68, which corresponded to the carbon resonances at 15.3, 16.0, 27.9, 14.7, 15.9 and 19.0 in the $^{13}$C NMR spectrum and HSQCDEPT spectrum. The $^{1}$H NMR spectrum **(Appendix 77)** showed a proton resonance at 3.19 (1H, dd, *J*=10.8, J=4.9 Hz), which was seen to correspond to a carbon resonance at 79.2 in the HSQCDEPT spectrum **(Appendix 78-80).** This proton resonance and the H-5 proton resonance at 0.68 (d *J*=9.4 Hz) were seen to correlate in the HMBC spectrum with the 3H-23 and 3H-24 methyl group resonances at 0.96 and 0.76. The HMBC spectrum **(Appendix 81-83)** showed a correlation between the downfield methyl group resonance at 1.67 and the two nonequivalent methylene proton resonances at 4.67 dd 2.1 and 4.57 (m), which were ascribed to H-29A and H-29B. In addition, two nonequivalent methylene proton resonances at 3.80 dd 1.6, 11.0 and 3.33 (d, 11.0) were ascribed to H-28A and H-28B. Compound **A19** was identified as the known betulin.

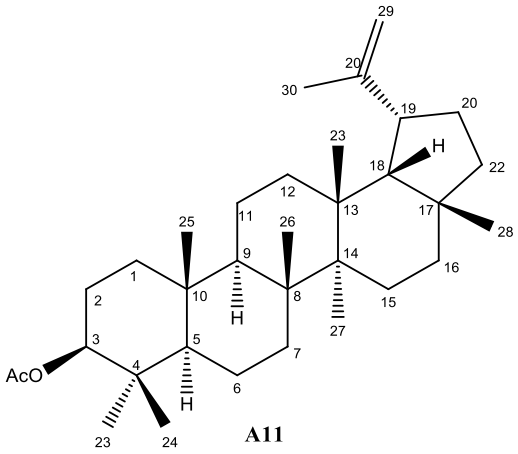
Table 4.4-16: NMR data for Betulin (A19) in $CDCl_3$

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR*** | **$^{1}$H NMR** | **$^{1}$H NMR*** |
|---|---|---|---|---|
| 1α | 38.9 | 38.6 | 1.69 m | 1.65 dd 12.6, 3.6 |
| 1β | | | 0.88 m | 0.89 dd 12.6, 3.2 |
| 2α | 27.2 | 27.0 | 1.72 m | 1.58 m |
| 2β | | | 1.08 m | 1.58 m |
| 3 | 79.2 | 78.8 | 3.19 dd 4.9, 10.8 | 3.18 dd 8.0, 3.2 |
| 4 | 39.1 | 38.8 | - | - |
| 5 | 55.5 | 55.2 | 0.68 d 9.4 | 0.68 d, 18.0 |
| 6α | 18.5 | 18.2 | 1.55 m | 1.50 m |
| 6β | | | 1.42 m | 1.38 m |

| | | | | |
|---|---|---|---|---|
| 7α | 34.4 | 34.2 | 1.41 m | 1.37 m |
| 7β | | | 1.41 m | 1.37 m |
| 8 | 41.1 | 40.8 | - | - |
| 9 | 50.9 | 50.3 | 1.32 m | 1.29 m |
| 10 | 37.3 | 37.1 | - | - |
| 11α | 21.0 | 20.8 | 1.42 m | 1.40 m |
| 11β | | | 1.24 m | 1.19 m |
| 12α | 25.4 | 25.1 | 1.64 m | 1.62 m |
| 12β | | | 1.06 m | 1.05 m |
| 13 | 37.5 | 37.2 | 1.67 m | 1.63 m |
| 14 | 42.9 | 42.6 | - | - |
| 15α | 27.2 | 27.0 | 1.60 m | 1.69 m |
| 15β | | | 1.06 m | 1.08 m |
| 16α | 29.4 | 29.1 | 1.96 m | 1.89 m |
| 16β | | | 1.26 m | 1.28 m |
| 17 | 48.0 | 47.7 | | - |
| 18 | 48.9 | 48.7 | 1.57 m | 1.58 m |
| 19 | 48.0 | 47.6 | 2.37, m | 2.36, m |
| 20 | 150.7 | 150.3 | - | - |
| 21α | 29.9 | 29.7 | 1.96 m | 1.98 m |
| 21β | | | 1.43 m | 1.29 m |
| 22α | 34.2 | 33.9 | 1.86 m | 1.85 m |
| 22β | | | 1.06 m | 1.04 m |
| 23 | 28.2 | 27.9 | 0.96 s | 0.92 s |
| 24 | 15.6 | 15.3 | 0.76 s | 0.74 s |
| 25 | 16.3 | 16.0 | 0.81 | 0.79 s |
| 26 | 16.2 | 15.9 | 1.01 s | 0.99 s |
| 27 | 15.0 | 14.7 | 0.99 s | 0.94 s |
| 28 | 60.7 | 60.8 | 3.80 dd 1.6, 11.0 | 3.75 d 9.0 |
| | | | 3.33 d 11.0 | 3.30 d 9.0 |
| 29A | 109.9 | 109.7 | 4.67 d 2.1 | 4.65 brs |
| 29B | | | 4.57 m | 4.55 brs |
| 30 | 19.3 | 19.0 | 1.68 s | 1.68 s |

*(Jamila *et al*., 2014)

#### 4.4.1.1.3. A11: Lupeol acetate



A11

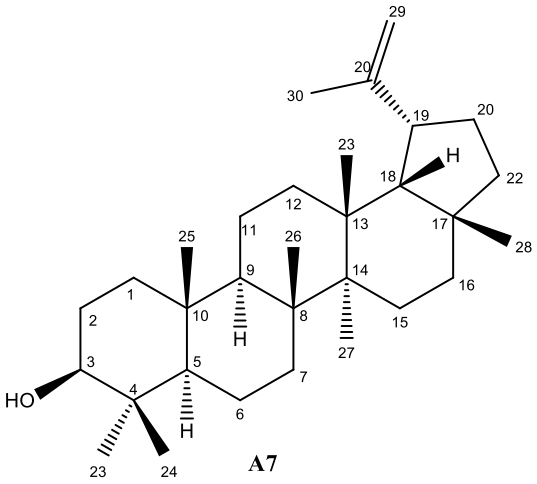
Lupeol acetate (Compound **A11)** was isolated as a white powder from the leaf of *C. macrostachyus* and identified as 3β-acetoxylup-20(29)-ene, commonly called lupeol acetate. The LREIMS spectrum for compound **A11** gave a molecular ion peak at *m/z* 468, which indicated a molecular formula of $C_{32}H_{52}O_2$. The FTIR spectrum displayed an absorption band at 1732 cm-1 that was attributed to a carbonyl group stretch and absorption bands at 1640, 1246, and 880 cm-1 that were ascribed to terminal double bond stretches. The $^{13}C$ NMR spectrum **(Appendix 84-85)** showed thirty-two carbon resonances for compound **A11**. The presence of a carbonyl carbon resonance at 171.2 and two double bond carbon resonances at 109.6 and 151.2 in conjunction with the molecular formula suggested that compound **A11** was a pentacyclic acetylated triterpenoid. The $^{1}H$ NMR spectrum **(Table 4.4-17)** displayed seven methyl proton resonances at 1.68 (s), 0.78 (s), 0.94 (s), 0.83 (s), 0.85 (s), 1.02 (s), and 0.84 (s) and an acetate methyl group proton resonance at δ 2.04 (s) (Ahmad & Atta-ur-rahman, 1994). Furthermore, the terminal double bond proton resonances at 4.68 (br s *W*1/2 = 6.09 Hz) and δ 4.57 (br s *W*1/2 = 6.62 Hz) were characteristic of the terminal double bond of a lupane-type triterpenoid (Ahmad & Atta-ur-rahman, 1994). The two proton resonances at δ 4.68 (br s) and δ 4.57 (br s) were ascribed to the two H-29 protons. Comparing the $^{13}C$ NMR chemical shifts with those found in the literature confirmed that compound **A11** was the known 3β-acetoxylup-20(29)-ene. 3β-Acetoxylup-20(29)-ene has been previously shown to demonstrate anti-arthitic effects in complete Freund's adjuvant (CFA)-induced arthritis rats (Kweifio-Okai & Carroll, 1993). The DEPT, HSQCDEPT, HMBC, COSY and NOSEY spectra of A11 is depicted in **Appendix 86-90.**

Table 4.4-17: NMR data for Lupeol acetate (A11) in $CDCl_3$

| No. | $^{13}$C NMR | $^{13}$C NMR* | $^{1}$H NMR | $^{1}$H NMR* |
|---|---|---|---|---|
| 1α | 38.3 | 38.4 | 1.64 m | 1.69 m |
| 1β | | | 0.98 m | 0.98 m |
| 2α | 23.6 | 23.7 | 1.58 m | 1.60 m |
| 2β | | | 1.58 m | 1.60 m |
| 3 | 80.9 | 81.0 | 4.47 dd 5.6, 10.5 | 4.47 dd 4.4, 12.8 |
| 4 | 37.9 | 37.8 | = | - |
| 5 | 55.3 | 55.4 | 0.79 m | 0.76 dd 10.8, 5.8 |
| 6α | 18.1 | 18.2 | 1.49 m | 1.50 m |
| 6β | | | 1.36 m | 1.40 m |
| 7α | 34.1 | 33.6 | 1.36 m | 1.38 m |
| 7β | | | 1.36 m | 1.38 m |
| 8 | 40.7 | 40.9 | - | - |
| 9 | 50.2 | 50.4 | 1.29 m | 1.30 m |
| 10 | 37.0 | 37.1 | - | - |
| 11α | 20.8 | 21.0 | 1.39 m | 1.40 m |
| 11β | | | 1.20 m | 1.22 m |
| 12α | 25.0 | 25.1 | 1.65 m | 1.68 m |
| 12β | | | 1.05 m | 1.07 m |
| 13 | 38.0 | 38.1 | 1.65 m | 1.68 m |
| 14 | 42.7 | 42.9 | - | - |
| 15α | 27.3 | 27.5 | 1.65 m | 1.68 m |
| 15β | | | 1.35 m | 1.38 m |
| 16α | 35.5 | 35.6 | 1.46 m | 1.48 m |
| 16β | | | 1.35 m | 1.38 m |
| 17 | 42.9 | 43.0 | - | - |
| 18 | 47.9 | 48.0 | 1.37 m | 1.39 m |
| 19 | 48.2 | 48.3 | 2.37 dd 11.0, 5.5 | 2.33 dt 11.1, 5.6 |
| 20 | 150.9 | 150.9 | - | - |
| 21α | 29.7 | 29.9 | 1.70 m | 1.82 m |
| 21β | | | 1.92 m | 1.93 m |
| 22α | 39.9 | 40.0 | 1.36 m | 1.38 m |
| 22β | | | 1.19 m | 1.20 m |
| 23 | 27.8 | 28.0 | 0.85 s | 0.82 s |
| 24 | 16.4 | 16.5 | 0.84 s | 0.82 s |
| 25 | 16.1 | 16.2 | 0.83 s | 0.82 s |
| 26 | 15.8 | 16.0 | 1.02 s | 1.00 s |
| 27 | 14.4 | 14.5 | 0.95 s | 0.91 s |
| 28 | 17.9 | 18.0 | 0.78 s | 0.81 s |
| 29A | 109.2 | 109.4 | 4.68 d 2.3 | 4.69 s |
| 29B | | | 4.56 m | 4.57 s |
| 30 | 19.2 | 19.3 | 1.68 s | 1.68 s |
| 1’ | 170.9 | 171.3 | - | - |
| 2’ | 21.2 | 21.7 | 2.04 s | 2.04 s |

*(Jamila *et al*., 2014)

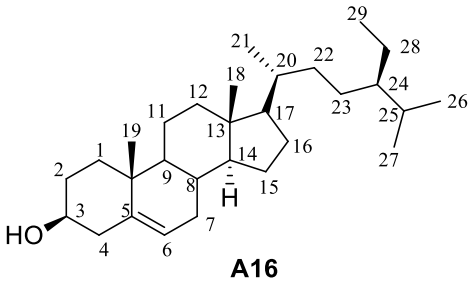
#### 4.4.1.1.4. A7 - 3β-hydroxylup-20(29)-ene (lupeol)




**A7**

Lupeol (compound **A7)** was isolated from the hexane soluble extract of the leaf of *C. macrostachyus* as a white solid and was found to be the common lupeol. Furthermore, the $^{1}H$ NMR and $^{13}C$ NMR spectra for compound **A7** were the same as those of compound **E19** previously isolated from *C. megalocarpus*. Therefore, the structural elucidation description of this compound was the same as that of compound **E19,** which was also isolated from *C. megalocarpus*. The $^{1}H$ NMR, $^{13}C$ NMR, DEPT, HSQCDEPT, HMBC, COSY and NOESY spectra of A17 are depicted in **Appendix 91-97.**

#### 4.4.1.1.5. A16: Sitosterol




**A16**

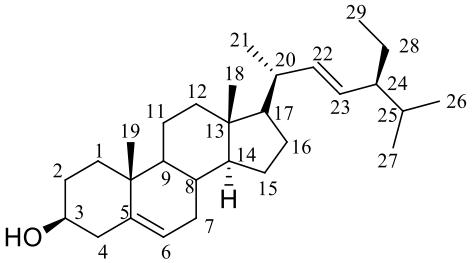
Sitosterol (compound **A16)** was isolated from the hexane soluble extract of the leaf of *C. macrostachyus* as a white solid and was identified as the known phytosterol sitosterol. The $^{1}H$ NMR spectrum **(Appendix 98)** displayed proton resonances at δ = 5.35 (m), assigned to H-6. The oxymethine proton resonance at 3.52 m was assigned as H-3. The $^{13}C$ NMR data of compound **A16** were compared to those reported by Xin Zhang *et al*., (2006) for sitosterol and were identical. Compound **A16** was therefore identified as the widely known sitosterol **(Table 4.4-18).**

Table 4.4-18: NMR data for Sitosterol (A16) in $CDCl_3$

| No. | $^{13}$C NMR | $^{13}$C NMR* | $^{1}$H NMR | $^{1}$H NMR* |
|---|---|---|---|---|
| 1α | 37.3 | 37.5 | 1.85 | - |
| 1β | | | 1.09 | - |
| 2α | 31.7 | 31.9 | 1.84 m | - |
| 2β | | | 1.89 m | - |
| 3 | 71.8 | 72.0 | 3.55 m | 3.52 m |
| 4α | 42.3 | 42.5 | 2.25 m | - |
| 4β | | | 2.29 m | - |
| 5 | 140.8 | 140.9 | - | - |
| 6 | 121.8 | 121.9 | 5.37 *d*, *J* =5.5 | 5.35 d, *J* =4.9 |
| 7α | 32.0 | 32.1 | 1.98 m | - |
| 7β | | | 1.48 m | - |
| 8 | 31.5 | 31.9 | 1.46 m | - |
| 9 | 50.3 | 50.4 | 0.92 m | - |
| 10 | 36.5 | 36.7 | - | - |
| 11α | 21.1 | 21.3 | 1.48 m | - |
| 11β | | | 1.38 m | - |
| 12α | 39.8 | 40.0 | 1.98 m | - |
| 12β | | | 1.15 m | - |
| 13 | 40.1 | 40.1 | - | - |
| 14 | 56.8 | 57.0 | 0.99 m | - |
| 15α | 24.3 | 24.5 | 1.59 m | - |
| 15β | | | 1.07 m | - |
| 16α | 28.4 | 28.5 | 1.85 m | - |
| 16β | 28.4 | 28.5 | 1.24 m | - |
| 17 | 56.1 | 56.3 | 1.10 m | - |
| 18 | 12.0 | 12.0 | 0.70 s | 0.68 s |
| 19 | 19.4 | 19.3 | 1.03 s | 1.01 s |
| 20 | 36.2 | 36.4 | 1.35 m | - |
| 21 | 19.0 | 19.0 | 0.95 d, *J* =6.5 | 0.92 s |
| 22α | 33.8 | 34.1 | 1.33 m | - |
| 22β | | | 1.02 m | - |
| 23α | 26.1 | 26.4 | 1.18 m | - |
| 23β | | | 1.14 m | - |
| 24 | 45.8 | 46.1 | 0.92 m | - |
| 25 | 28.4 | 28.5 | 1.66 m | - |
| 26 | 18.8 | 19.2 | 0.87 m | 0.81 d, *J* =6.1 |
| 27 | 19.9 | 19.6 | 0.88 m | 0.83 d, *J* =6.6 |
| 28α | 23.4 | 23.3 | 1.27 m | - |
| 28β | | | 1.22 m | - |
| 29 | 11.9 | 12.4 | 0.85 *t*, *J* =7.1 | 0.84 t, *J* =7.4 |

*(Xin Zhang *et al*., 2006)

#### 4.4.1.1.6. A15: Stigmasterol




**A15**

Stigmasterol (compound **A15)** was isolated from the hexane soluble extract of the leaf of *C. macrostachys* as a white solid and was identified as the known phytosterol stigmasterol. The $^{1}$H NMR spectrum **(Appendix 98)** displayed proton resonances at 5.13 (dd, *J* = 8.5,15.2 Hz), 4.99 (dd, *J* = 8.5, 15.2 Hz), 5.32 (d, *J* = 5.0 Hz) for double bond proton resonances. An oxymethine proton resonance at δ 3.49 (m) was also observed for compound **A15**. The $^{13}$C NMR spectrum for compound **A15** showed 29 carbon resonances, which were comparable to those reported by De-Eknamkul & Potduang, (2003) for stigmasterol **(Table 4.4-19).** Compound **A15** was therefore identified as the known stigmasterol, which is very common in the Plant Kingdom.

Table 4.4-19: NMR data for Stigmasterol (A15) in $CDCl_3$

| No | $^{13}$C NMR | $^{13}$C NMR* | $^{1}$H NMR | $^{1}$H NMR* |
|---|---|---|---|---|
| 1α | 37.2 | 37.7 | 1.07 dd, *J* =13.2 | - |
| 1β | | | 1.85 d, *J* =13.2 | - |
| 2α | 31.6 | 32.1 | 1.81 m | - |
| 2β | | | 1.49 m | - |
| 3 | 71.8 | 72.0 | 3.51 m | - |
| 4α | 42.3 | 42.7 | 2.26 d, *J*=13.2 | - |
| 4β | | | | - |
| 5 | 140.7 | 140.8 | - | - |
| 6 | 121.7 | 121.8 | 5.34 d, *J* =5.4 | 5.35 m |
| 7α | 31.9 | 32.3 | 1.99 m | - |
| 7β | | | 1.98 m | - |
| 8 | 31.6 | 32.2 | 1.48 m | - |
| 9 | 50.1 | 50.9 | 0.92 m | - |
| 10 | 36.5 | 36.9 | - | - |
| 11α | 21.1 | 21.5 | 1.54 m | - |
| 11β | | | 1.53 m | - |
| 12α | 40.5 | 40.1 | 1.98 m | - |
| 12β | | | 1.15 m | - |
| 13 | 42.2 | 42.6 | - | - |
| 14 | 56.8 | 57.3 | 0.99 m | - |

| | | | | |
|---|---|---|---|---|
| 15α | 24.3 | 24.7 | 1.54 m | - |
| 15β | | | 1.07 m | - |
| 16α | 29.3 | 29.4 | 1.71 m | - |
| 16β | | | 1.24 m | - |
| 17 | 56.1 | 56.4 | 1.15 m | - |
| 18 | 12.2 | 12.2 | 0.69 m | 0.70 s |
| 19 | 19.7 | 19.6 | 1.01 m | 1.01 s |
| 20 | 40.5 | 40.9 | 2.07 m | - |
| 21 | 21.2 | 21.4 | 1.07 d, *J* =6.6 | 1.02 d, *J* =6.6 |
| 22 | 138.3 | 138.8 | 5.17 d, *J* = 15.8 | 5.16 dd, *J* = 15.4, 8.4 |
| 23 | 129.2 | 129.6 | 5.04 d, *J* =15.0 | 5.02 dd, *J* =15.4, 8.8 |
| 24 | 51.2 | 51.7 | 1.56 m | - |
| 25 | 33.9 | 32.3 | 1.48 m | - |
| 26 | 19.3 | 19.2 | 0.81 m | 0.85 d, *J*=6.6 |
| 27 | 21.1 | 21.3 | 0.83 m | 0.80 d, *J*=6.6 |
| 28α | 25.4 | 25.8 | 1.43 m | - |
| 28β | | | - | - |
| 29 | 12.0 | 12.5 | 0.80 t, *J* =7.2 | 0.80 t, *J*=7.7 |

*(Blunt & Stothers, 1977)

#### 4.4.1.2. Cytotoxicity and anti-HIV activity of pure compounds isolated from *C. macrostachyus*

The cytotoxic and antiviral activity findings for the pure compounds isolated from *C. macrostachyus* and the control drugs are summarized in **Table 4.4-20.** Among the three isolated compounds from *C. macrostachyus*, A4b (lupenone) displayed the highest $CC_{50}$ value of 32.46 ± 0.7 µg/mL **(Table 4.4-20).** Furthermore, a comparison between A4b, A11, and A19 and the control drugs showed that all three compounds had significantly ($p < 0.001$) higher $CC_{50}$ values than AZT, ABC, and NVP, which indicates their safety and that high concentration levels are required to exert cytotoxic effects **(Figure 4.4-10)**. In addition, the maximum cytotoxic effect ($Emax_c$) of the compounds was not significantly different from the cytotoxic effect of AZT and NVP.

Betulin (E19) observed the highest anti-HIV activity, which inhibited virus-induced CPE by 76% with an $IC_{50}$ value of 0.002 ± 0.04 µg/mL, which is much lower than the maximum nontoxic concentration (MNTC), which also indicates the safety and efficacy of the compound. Furthermore, all three compounds displayed anti-HIV activity at significantly ($p < 0.05$) lower $IC_{50}$ values than TDF and NVP **(Figure 4.4-11),** indicating their higher potency**.**

In addition, the three compounds displayed significantly ($p < 0.05$) higher inhibition of viral-induced CPE ($Emax_{AV}$) than ABC. The antiviral activity of the tested compounds

($Emax_{AV}$) showed that both the control drugs (except ABC) and the tested compounds had approximately similar antiviral efficacy, as they showed non-significantly different $Emax_{AV}$ values **(Figure 4.4-11).** Furthermore, the results showed that the tested compounds had a higher selectivity index, indicating their efficacy at lower cytotoxic effects. As depicted in **Figure 4.4-12**, the pure compounds (A4b, A11, and A19) displayed concentration-dependent inhibition of virus-induced CPE, as the % CPE inhibition increased with increasing concentrations of the pure compounds.

Our finding on the anti-HIV activity of these pure compounds is in agreement with previous reports. A report by Chaniad *et al*., (2019) explained the efficacy of betulin (E19) as a potent anti-HIV compound with an $IC_{50}$ value of $17.7 \pm 0.6$ μM. Similarly, Esposito *et al*., (2017) reported that lupeol acetate and lupeol inhibited HIV-1 RT-associated RNase H function with $IC_{50}$ values of 63 and 11.6 μM, respectively.

Table 4.4-20: Cytotoxicity and anti-HIV activities of pure compounds isolated from *C. macrostachyus*

| Materials | Cytotoxicity | | | Antiviral activity | | SI |
|---|---|---|---|---|---|---|
| | MNTC (μg/mL) | $CC_{50}$ (μg/mL) | $Emax_C$ (%) | $IC_{50}$ (μg/mL) | $Emax_{AV}$ (%) | |
| **AZT** | 0.38 ± 0.19 | 0.53 ± 0.29 | 36.28 ± 0.83 | 0.002 ± 0.00 | 83.5 ± 0.57 | 279.4 |
| **TDF** | 4.92 ± 0.71 | 6.73 ± 0.24 | 13.17 ± 0.43 | 0.04 ± 0.01 | 80.55 ± 0.46 | 176.5 |
| **ABC** | 0.18 ± 0.03 | 0.26 ± 0.00 | 17.83 ± 0.57 | 0.05 ± 0.031 | 58.67 ± 0.43 | 5.0 |
| **NVP** | 0.57 ± 0.0 | 0.82 ± 0.0 | 39.13 ± 0.65 | 0.24 ± 0.09 | 72.53 ± 0.47 | 3.5 |
| **A5** | 0.002 ± 0.00 | 0.001 ± 0.00 | 39.1 ± 2.22 | 0.25 ± 0.02 | 53.22 ± 3.345 | 0.0073 |
| **A4b** | 14.17 ± 0.94 | 28.83 ± 0.54 | 38.53 ± 0.69 | 0.002 ± 0.001 | 64.74 ± 0.52 | 14084.0 |
| **A11** | 16.01 ± 0.64 | 32.46 ± 0.7 | 55.91 ± 0.93 | 0.002 ± 0.00 | 75.8 ± 0.59 | 15097.7 |
| **A19** | 16.54 ± 0.35 | 31.74 ± 0.55 | 55.13 ± 0.15 | 0.002 ± 0.04 | 76.17 ± 0.02 | 15551.2 |
| **A7** | 75.75 ± 0.74 | 141.8 ± 0.7 | 70.28 ± 0.75 | 0.047 ± 0.02 | 77.01 ± 0.38 | 3048.2 |
| **A15** | 4.70 ± 3.43 | 12.38 ± 10.88 | 43.71 ± 2.02 | 0.14 ± 0.04 | 76.77 ± 23.24 | 86.5 |
| **A16** | 4.41 ± 0.04 | 5.83 ± 0.20 | 42.74 ± 0.09 | 5.58 ± 0.231 | 84.65 ± 0.54 | 1.0 |

Results are shown as mean ± S.E.M (n=3).

AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; A5, 2-methoxy benzyl benzoate; A4b, lupenone; A11, lupeol acetate; A19, betulin; A7, lupeol; A16, sitosterol; A15, stigmasterol; MNTC, Maximum nontoxic concentration; $CC_{50}$, 50% cytotoxic concentration; $Emax_C$, Maximum cytotoxic effect %; $IC_{50}$, 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

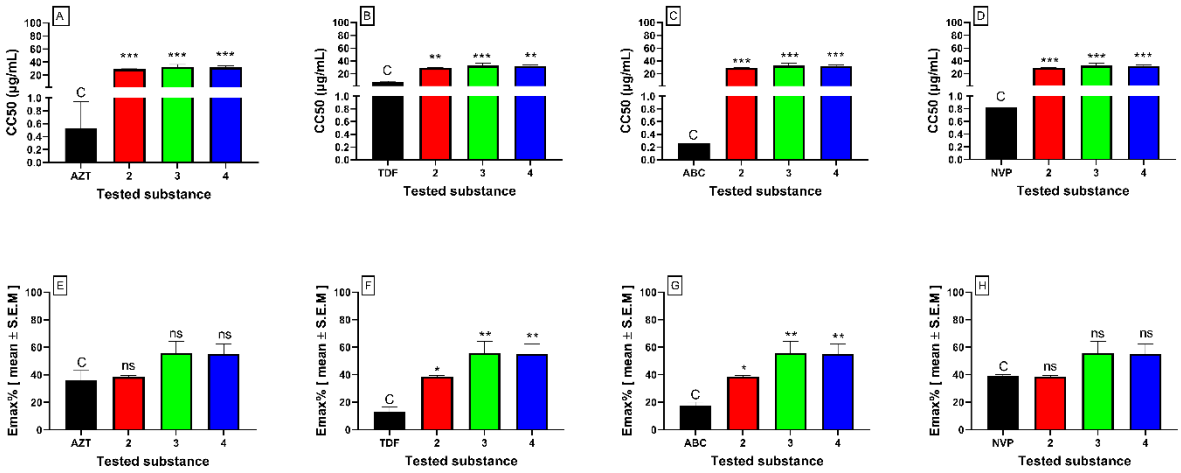



Figure 4.4-10: Cytotoxicity of pure compounds isolated from *C. macrostachyus*

The results are expressed as the mean of three independent experiments ± S.E.M. AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; Lupenone (2); Lupeol acetate (3); Betulin (4); CC50, 50% cytotoxic concentration; $Emax_C$, Maximum cytotoxic effect; C; control, ns, not significant, *Denotes p value < 0.05; **Denotes p value < 0.01, ***Denotes p value < 0.001.

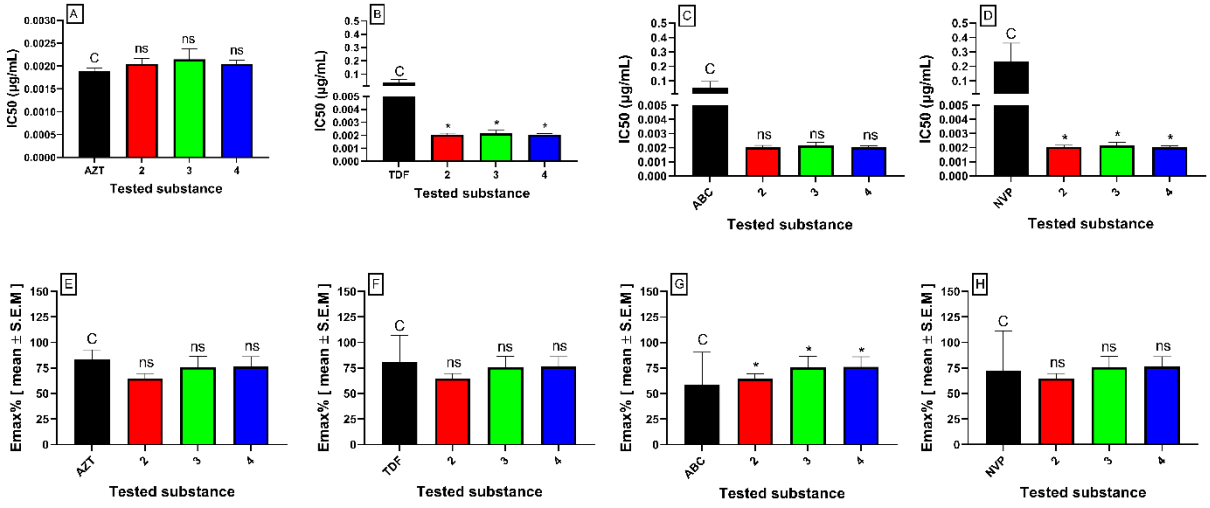



Figure 4.4-11: Anti-HIV activity of pure compounds isolated from *C. macrostachyus*

The results are expressed as the mean of three independent experiments ± S.E.M. AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; Lupenone (2); Lupeol acetate (**3**); Betulin (4); CC50, 50% cytotoxic concentration; $Emax_C$, Maximum cytotoxic effect; C; control, ns, not significant, *Denotes p value < 0.05; **Denotes p value < 0.01, ***Denotes p value < 0.001.

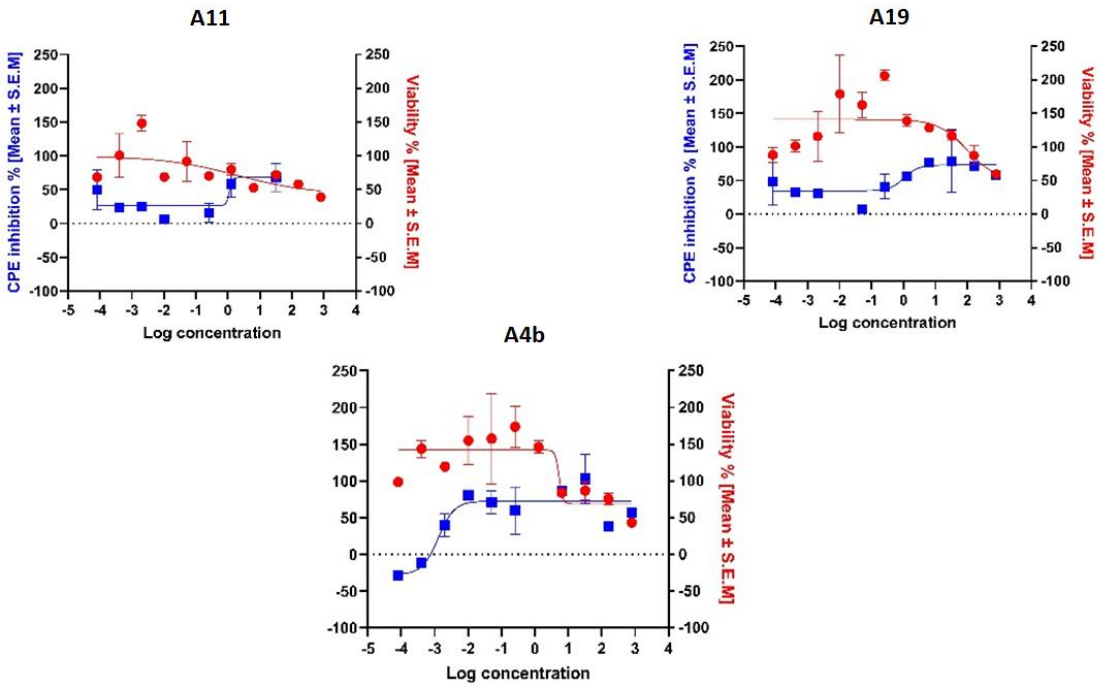



Figure 4.4-12: Concentration-response curve analysis for the anti-HIV activity of pure compounds isolated from *C. macrostachyus*

Results presented in the curves are means ± S.E. M of three independent experiments. Cell viability % (red line) and the inhibition % of the virus-induced cytopathic effect (blue line) associated with control drugs and the tested extracts at the concentration level (800 – $8.192\times10^5$ µg/mL); A4b, Lupenone; A11, Lupeol acetate; A19, Betulin

### 4.4.2. *Croton dichogamus*

Based on the findings from the anti-HIV activity of the solvent fractions from *C. dichogamus*, the methanol fraction of the twigs of *C. dichogamus* was subjected to column chromatography to yield six pure compounds (**Table 4.4-21**). The chemical structure of these compounds is depicted in **Figure 4.4-13**.

Table 4.4-21: Compounds isolated from the twigs of *C. dichogamus*

| Ser. No. | Name | Code |
|---|---|---|
| **1.** | Dihydroconiferyl acetate | **CD1a** |
| **2.** | (4-Hydroxy-3-methoxyphenyl)-propyl benzoate | **CD1b** |
| **3.** | Crotocascarin ω | **CD12a** |
| **4.** | β-Oplopanone | **CD12b** |
| **5.** | 3β-hydroxylup-20(29)-ene (lupeol) | **CD4** |
| **6.** | Stigmasterol | **CD6a** |
| **7.** | Sitosterol | **CD6b** |

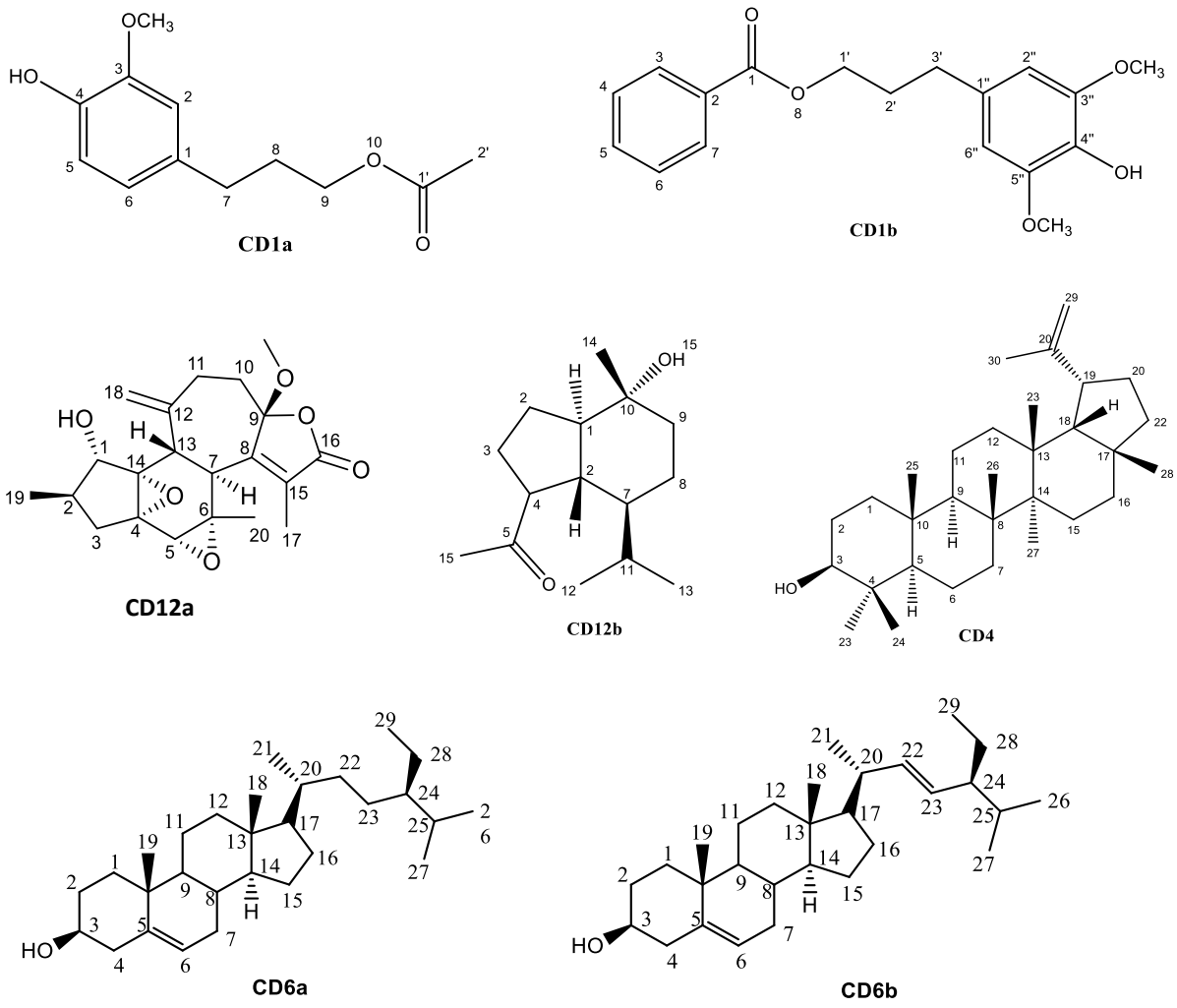
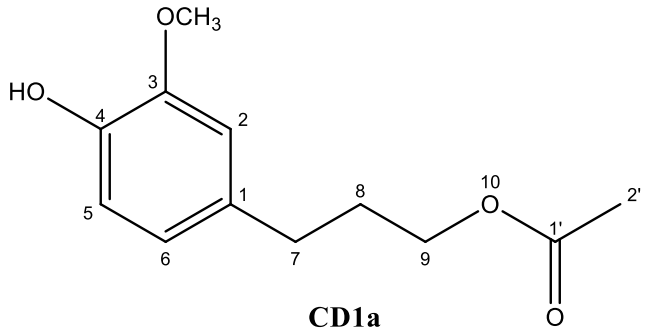



Figure 4.4-13: Pure compounds isolated from *C. dichogamus*

### 4.4.2.1. Chemistry of compounds isolated from *C. dichogamus*

#### 4.4.2.1.1. CD1a: Dihydroconiferyl acetate




**CD1a**

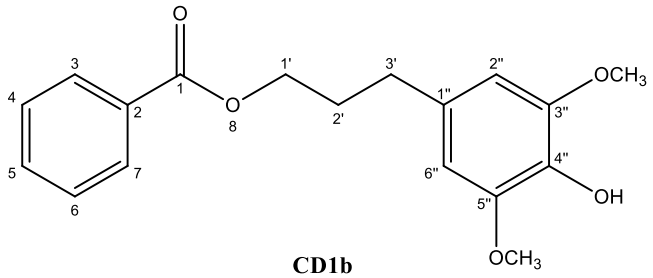
Dihydroconiferyl acetate (compound **CD1a)** was isolated from the methanol extract of the twigs of *C. dichogamus* as a white solid and identified as the known dihydroconiferyl acetate. The $^{1}$H NMR spectrum **(Table 4.4-22)** showed resonances at 2.73 (m, H-8), 2.63 (m, H-7), 4.34 (t, 6.7, H-9), 3.82 (3H, s, methyl protons of methoxy group), 6.67 (s, H-6), 6.67 (d, 8:9 Hz, H-5), and 6.76 (d, 8,9, H-2) **(Appendix 99)**. The $^{13}$C NMR spectrum **(Appendix 100-105)** showed resonances at 31.5 (C-7), 31.5 (C-8), 56.0 (methyl carbon of methoxy group), 64.5 (C-9), 111.1 (C-2), 115.5 (C-5), 121.1 (C-6), 133.13 (C-1), 144.4 (C-4), and 146.6 (C-3). This is the first report of the isolation of dihydroconiferyl acetate from *C. dichogamus.*

Table 4.4-22: NMR data for dihydroconiferyl acetate (CD1a) in $CDCl_3$

| No. | $^{13}$C NMR | $^{13}$C NMR* | $^{1}$H NMR | $^{1}$H NMR* |
|---|---|---|---|---|
| 1 | 133.3 | 134.1 | - | - |
| 2 | 111.1 | 113.1 | 6.67 s | 6.75 d 1.7 |
| 3 | 146.6 | 148.9 | - | - |
| 4 | 144.0 | 145.7 | - | - |
| 5 | 115.5 | 116.1 | 6.76 d 8.9 | 6.69 d 8.0 |
| 6 | 121.1 | 121.8 | 6.67 d 8.9 | 6.60 dd 1.7, 8.0 |
| 7 | 32.0 | 32.6 | 2.63 m | 2.59 t 7.6 |
| 8 | 31.5 | 31.7 | 2.73 m | 1.90 ttd 6.5, 7.6, 14.9 |
| 9 | 64.5 | 65.0 | 4.34 t 6.7 | 4.04 t 6.5 |
| 1’ | 171.5 | 173.1 | - | - |
| 2’ | 21.2 | 20.8 | - | 2.02, s |
| $OCH_3$ | 56.0 | 56.3 | 3.82 s | 3.82 s |

*(Kondo *et al*., 2007)

#### 4.4.2.1.2. CD1b: (4-Hydroxy-3-methoxyphenyl)-propyl benzoate




**CD1b**

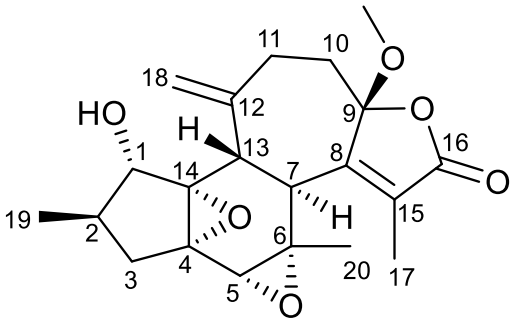
(4-Hydroxy-3-methoxyphenyl)-propyl benzoate (compound **CD1b)** was isolated from the methanol extract of the twigs of *C. dichogamus* as a white solid and identified as the known 3'-(4''-hydroxy-3'',5''-dimethoxyphenyl)-propyl benzoate. The $^{1}$H NMR spectral data **(Table 4.4-23)** showed the presence of two benzene rings. The $^{1}$H NMR spectral data **(Appendix 99)** showed three methylene groups due to resonances at 4.10, 2.71 and 2.05 bridging between benzoate and aromatic rings and two methoxyls at d 3.87 (each 3H, s) groups on the aromatic ring. The $^{13}$C NMR spectrum **(Appendix 100-105)** showed the signal of carbonyl ester at d 166.9. All data are consistent with the structure of 3'-(4''-hydroxy-3'',5''-dimethoxyphenyl)-propyl benzoate. This is the first report of the isolation of compound **CD1b** from *C. dichogamus.*

Table 4.4-23: NMR data for (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b) in $CDCl_3$

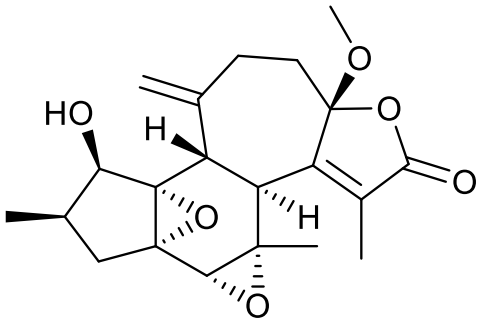
| No. | $^{13}$C NMR | $^{13}$C NMR* | $^{1}$H NMR | $^{1}$H NMR* |
|---|---|---|---|---|
| 1 | 166.9 | 166.8 | - | - |
| 2 | 130.5 | 130.3 | - | - |
| 3 | 129.6 | 129.5 | 8.04 dd 8.4, 1.4 | 8.04 dd 8.6, 1.1 |
| 4 | 128.5 | 128.4 | 7.44 t 7.3 | 7.45 tt 7.5, 1.6 |
| 5 | 133.1 | 132.9 | 7.55 t | 7.57 tt 7.5, 1.1 |
| 6 | 128.5 | 128.4 | 7.44 t | 7.45 tt 7.5, 1.6 |
| 7 | 129.6 | 129.5 | 8.04 dd 8.4, 1.4 | 8.04 dd 8.6, 1.1 |
| 1' | 64.1 | 64.3 | 4.10 t 6.7 | 4.33 t, 6.5 |
| 2' | 30.7 | 30.5 | 2.05 m | 2.07 m |
| 3' | 31.5 | 31.3 | 2.71 t | 2.72 br t |
| 1'' | 133.1 | 133.2 | - | - |
| 2'' | 129.5 | 129.5 | 7.08 d 8.9 | 7.08 d 8.4 |
| 3'' | 114.5 | 115.3 | 6.83 d 8.9 | 6.77 d 8.4 |
| 4'' | 154.3 | 153.9 | - | - |
| 5'' | 114.5 | 115.3 | 6.83 d 8.9 | 6.77 d 8.4 |
| 6'' | 129.7 | 129.5 | 7.08 d 8.9 | 7.08 d 8.4 |
| 4''-OH | - | - | | 5.15 br s |
| 3''-$OCH_3$ | | | 3.87 | |
| 5''-$OCH_3$ | | | 3.87 | |

*****(Athikomkulchai *et al*., 2006)

#### 4.4.2.1.3. CD12a: Crotocascarin ω




CD12a




1a

Crotocascarin ω **(c**ompound **CD12a)** was isolated from the methanol-soluble fraction of a 1:1 $CH_2Cl_2$:$CH_3OH$ extract of twigs of *C. dichogamus* and identified as an undescribed epimer of the known crotocascarin M (**1a**) previously isolated from *Croton cascarilloides* (**Figure 1**). Therefore, the compound was assigned as crotocascarin ω. Compound (**CD12a**) has a molecular formula of $C_{21}H_{26}O_6$, with nine degrees of unsaturation, as determined from (+)-HRESIMS analysis, which displayed an ion peak at *m/z* 375.1799 $[M+H]^+$ (calcd for $C_{21}H_{26}O_6$ + H, *m/z* 375.1807). The IR spectrum exhibited the presence of an α,β-unsaturated lactone (1733 $cm^{-1}$) and hydroxy groups (3429 $cm^1$). The $^1$H-NMR spectrum (**Table 4.4-24**) showed four methyl resonances, including a doublet resonance, downfield resonances for the methyls of a methoxy, and an acetoxy and an allylic methyl resonance, a hemiketal resonance, five oxygenated methine resonances, and exocyclic methylene proton resonances. In contrast, the $^{13}$C NMR spectrum showed 21 carbon resonances, including a hemiketal carbon resonance at 109.6 **(Appendix 106-111)**. The use of HMBC and COSY showed that compound **CD12a** was an isomer of the known crotocascarin M (Kawakami *et al*. 2016) (**Figure 4.4-14**), with differences in the $^{13}$C NMR chemical shifts for C-1 (75.7 for compound **CD12a** and 73.7 for crotocascarin M), C-3 (33.3 for compound **1** and 36.3 for crotocascarin M), C-4 (62.9 for compound **CD12a** and 60.3 for crotocascarin M), C-5 (56.2.7 for compound **1** and 58.2 for crotocascarin M), C-6 (63.3 for compound **CD12a** and 56.6 for crotocascarin M), C-11 (33.1 for compound **CD12a** and 34.9 for crotocascarin M), C-12 (145.8 for compound **CD12a** and 147.7 for crotocascarin M), (45.8 for compound **CD12a** and 39.5 for crotocascarin M) and C-14 (73.3 for compound **CD12a** and 70.7 for crotocascarin M). In addition, $^1$H NMR showed differences for H-5, H-7, 2H-10, 2H-11, H-3, 3H-17, 2H-18, 3H-19, and 1-OH between compound **CD12a** and crotocascarin M. The NOESY spectrum showed that H-1, H-5, H-13,

9-$OCH_3$, 3H-19, and 3H-20 were on one face, whereas 1-OH and H-7 were on the other face (**Figure 2**). The data described above supported the assignment of **CD12a** as a 1α-hydroxy derivative of the known crotocascarin M (**1a**) named crotocascarin ω (Kawakami *et al*. 2016; Kawakami *et al*. 2015).

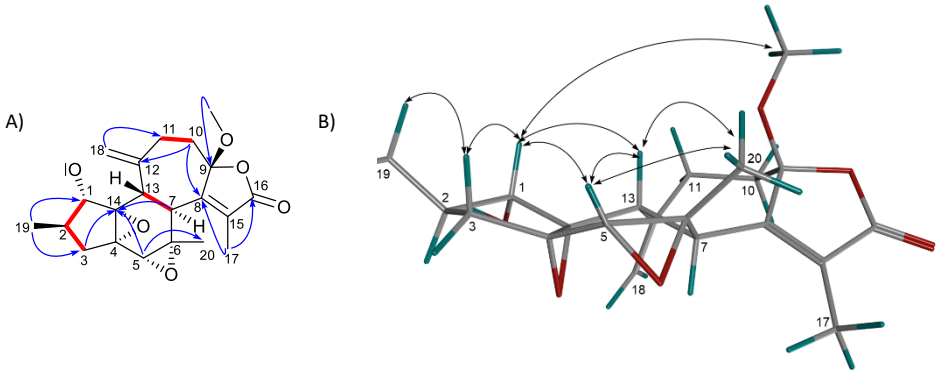


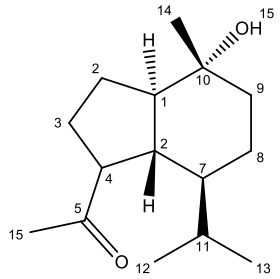

Figure 4.4-14: (A): Key COSY (red bold bonds) and HMBC (blue arrows) correlations for compound **CD12a** (B): Key NOESY (double headed arrows) correlations observed for compound **CD12a**

Table 4.4-24: NMR table for Crotocascarin ω (CD12a)

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR*** | **$^{1}$H NMR** | **$^{1}$H NMR*** | **HMBC** | **COSY** | **NOESY** |
|---|---|---|---|---|---|---|---|
| 1 | 75.7 | 73.7 | 4.16 dd 4.6 4.6 | 4.19 dd 5, 5 | 4 | 2,1-OH | 1-OH,9-$OCH_3$ |
| 2 | 33.5 | 33.6 | 1.95 m | 2.05 m | X | 1,3α, 3β | 19 |
| 3α | 33.3 | 36.3 | 2.18 dd 11.5, 6.1 | 2.41 dd 14, 7 | 1,3,14 | 1,3β | 1,3β |
| 3β | | | 1.68 dd 11.5, 13.7 | 1.65 dd 14, 10 | X | 1,3α | 1,3α |
| 4 | 63.3 | 60.3 | - | - | - | - | - |
| 5 | 56.2 | 58.2 | 3.15 d 0.6 | 3.10 s | 6, 14, 20 | 20 | 1,19 |
| 6 | 62.9 | 56.6 | - | - | - | - | - |
| 7 | 45.8 | 44.1 | 2.96 dd 1.4, 12.6 | 2.97 dq 13, 1 | 7,8,14 | 13,17 | 1,5,20 |
| 8 | 159.4 | 159.7 | - | - | - | - | - |
| 9 | 109.6 | 109.7 | - | - | - | - | - |
| 10α | 35.8 | 35.8 | 2.65 m | 2.81 ddd 14,7, 7 | 8,9 | 10β,11α,11β | 10β,11α,11β |
| 10β | | | 1.61 | 1.37 ddd 14, 14, 4 | 12 | 10α,11α,11β | 10α, ,11α,11β |
| 11α | 33.1 | 34.9 | 2.33 m | 2.45 ddd 14, 4, 4 | 11 | 10α,10β,11β | 10α,10β,11β |
| 11β | | | 2.27 m | 2.15 ddd 14, 14, 4 | 2 | 10α,10β,11α | 10α,10β,11α |
| 12 | 145.8 | 147.7 | - | - | - | - | - |
| 13 | 44.5 | 39.5 | 2.91 d 12.6 | 3.26 d 13 | 7 | 7 | 1,9-$OCH_3$ |
| 14 | 73.3 | 70.7 | - | - | - | - | - |
| 15 | 129.0 | 129.8 | - | - | - | - | - |
| 16 | 171.1 | 170.9 | - | - | - | - | - |
| 17 | 10.1 | 9.6 | 1.94 d 1.3 | 1.89 d 1.3 | 8,15,16 | 7 | x |
| 18A | 114.6 | 114.6 | 5.28 br s | 5.07 s | 2, 11 | 18B | 1 |
| B | | | 5.16 br s | 5.05 br s | 2, 11 | 18A | 11α,11β |
| 19 | 12.2 | 12.2 | 0.98 d 7.1 | 1.03 d 7.0 | 1,5 | 1 | 5,20 |
| 20 | 20.1 | 20.3 | 1.16 s | 1.12 s | 5,6,13 | 5 | 1-OH,19 |
| 9-$OCH_3$ | 51.9 | 51.6 | 3.40 s | 3.43 br s | 9 | - | 1 |
| 1-OH | | | 1.29 d 4.9 | 1.47 d 5 | 14 | 1 | 1,19 |

*(Kawakami *et al.*, 2016)

#### 4.4.2.1.4. CD12b: *β-Oplopanone*




**CD12b**

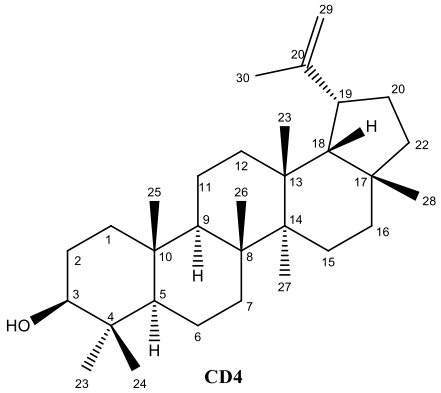
*β-Oplopanone* (compound **CD12b)** was isolated from the methanol extract of the twigs of *C. dichogamus*. The $^{1}$H NMR spectrum **(Appendix 112)** of compound **CD12b** showed four methyl group resonances, including downfield resonances of 2.19 (s), 1.20 (s), 0.90 d (6.9) and 0.68 d (6.9), four methine resonances, and four methylene resonances. The $^{13}$C NMR spectrum **(Appendix 113)** showed 15 carbon resonances, including one ketone carbon resonance at 211.1. The $^{1}$H and $^{13}$C NMR data were consistent with those of the known *β-oplopanone previously isolated from Magnolia fargesii* **(Table 4.4-25)** (Jung *et al*., 1997). The DEPT, HSQCDEPT, HMBC, COSY, NOESY of CD12b is depicted in **Appendix 114-118**. This is the first report of the isolation of *β-oplopanone* from *C. dichogamus.*

Table 4.4-25: NMR table for CD13: *β*-Oplopanone (CD12b)

| **No.** | **$^{13}$C NMR** | **$^{13}$C NMR** | **$^{1}$H NMR*** | **$^{1}$H NMR*** |
|---|---|---|---|---|
| 1 | 57.3 | 56.95 | 1.46 m | 1.45 m |
| 2α | 25.5 | 25.27 | 1.80 m | 1.83 m |
| 2β | | | 1.41 m | 1.42 m |
| 3α | 28.9 | 28.57 | 1.95 m | 1.97 m |
| 3β | | | 1.57 m | 1.58 m |
| 4 | 56.0 | 55.69 | 2.64 m | 2.65 ddd 5.4, 5.7, 10.5 |
| 5 | 211.6 | 212.3 | - | - |
| 6 | 47.0 | 46.68 | 1.80 m | 1.82 m |
| 7 | 49.7 | 49.37 | 1.09 m | 1.08 m |
| 8α | 23.3 | 22.96 | 1.59 m | 1.60 m |
| 8β | | | 1.08 m | 1.10 m |
| 9α | 42.3 | 42.2 | 1.79 m | 1.80 m |
| 9β | | | 1.37 m | 1.38 m |
| 10 | 73.3 | 72.96 | - | - |
| 11 | 29.7 | 29.47 | 1.46 m | 1.45 m |
| 12 | 22.2 | 21.90 | 0.90 d 6.9 | 0.88 d 7.2 |
| 13 | 15.8 | 15.8 | 0.68 d 6.9 | 0.69 d 7.2 |
| 14 | 20.6 | 20.26 | 1.20 s | 1.20 s |
| 15 | 29.8 | 31.5 | 2.19 s | 2.19 s |

*****(Jung *et al*., 1997)

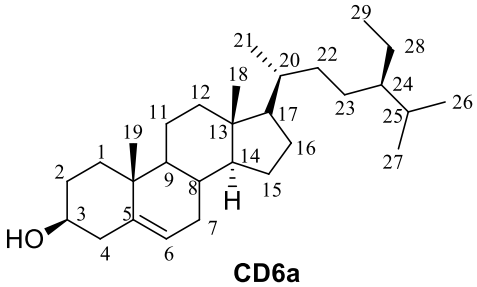
#### 4.4.2.1.5. CD4 - Lupeol




**CD4**

Lupeol (compound **CD4)** was isolated from the methanol soluble extract of the leaf of *C. dichogamus* as a white solid and was found to be the common lupeol. Furthermore, the $^{1}$H NMR and $^{13}$C NMR spectra for compound **CD4** were the same as those of compound **E19 (Appendix 119)** and **A7,** isolated from *C. megalocarpus* and *C. macrostachyus*. Therefore, the structural elucidation description of this compound was the same as that of compound **E19** and **A7**.

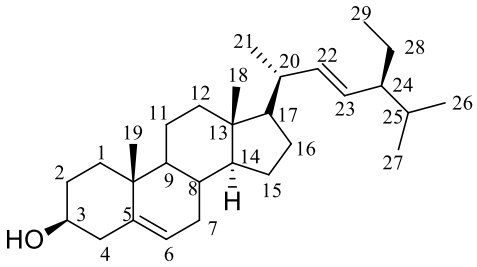
#### 4.4.2.1.6. CD6a: Sitosterol




**CD6a**

Sitosterol (compound **CD6a)** was isolated from the methanol soluble extract of the twigs of *C. dichogamus* as a white solid and was identified as the known phytosterol sitosterol. Furthermore, the $^{1}$H NMR **(Appendix 120)** and $^{13}$C NMR spectra for compound **CD6a** were the same as those of compound **A16 (Appendix 98)** previously isolated from *C. macrostachyus*. Therefore, the structural elucidation description of this compound was the same as that of compound **A16,** which was also isolated from *C. macrostachyus*.

#### 4.4.2.1.7. CD6b: Stigmasterol




**CD6b**

Stigmasterol (compound **CD6b)** was isolated from the methanol soluble extract of the twigs of *C. dichogamus* as a white solid and was identified as the known phytosterol stigmasterol. Furthermore, the $^{1}$H NMR **(Appendix 120)** and $^{13}$C NMR spectra for compound **CD6b** were the same as those of compound **A15 (Appendix 98)** previously isolated from *C. macrostachyus*. Therefore, the structural elucidation description of this compound was the same as that of compound **A15,** which was also isolated from *C. macrostachyus*.

### 4.4.2.2. Cytotoxicity and anti-HIV activity of pure compounds isolated from *C. dichogamus*

The cytotoxic and antiviral activity findings for the compounds isolated from *C. dichogamus* and the control drugs are summarized in **Table 4.4-26**. Comparison between CD12a and CD12b, and the control drugs regarding the cytotoxic effect showed that both compounds had significantly ($p < 0.01$) higher maximum cytotoxic effect ($Emax_c$) when compared with the control drugs TDF and ABC. Although the three compounds had a high maximum cytotoxic effect ($Emax_c$), they also had a very high $CC_{50,}$ which indicates that high concentration levels are required to exert cytotoxic effects (**Figure 4.4-15**). The antiviral activity of the tested compounds ($Emax_{AV}$) showed that they had approximately similar antiviral efficacy as the control ones since they showed non-significantly different $Emax_{AV}$ values. As depicted in **Figure 4.4-16**, the $IC_{50}$ values of the compounds were not significantly different from those of the control drugs, indicating their comparable potency.

Table 4.4-26: Cytotoxicity and anti-HIV activities of pure compounds isolated from *C. dichogamus*

| Materials | Cytotoxicity | | | Antiviral activity | | SI |
|---|---|---|---|---|---|---|
| | MNTC (µg/mL) | $CC_{50}$ (µg/mL) | $Emax_C$ (%) | $IC_{50}$ (µg/mL) | $Emax_{AV}$ (%) | |
| **AZT** | 0.38 ± 0.19 | 0.53 ± 0.29 | 36.28 ± 0.83 | 0.002 ± 0.00 | 83.5 ± 0.57 | 279.4 |
| **TDF** | 4.92 ± 0.71 | 6.73 ± 0.24 | 13.17 ± 0.43 | 0.04 ± 0.01 | 80.55 ± 0.46 | 176.5 |
| **ABC** | 0.18 ± 0.03 | 0.26 ± 0.00 | 17.83 ± 0.57 | 0.05 ± 0.031 | 58.67 ± 0.43 | 5.0 |
| **NVP** | 0.57 ± 0.0 | 0.82 ± 0.0 | 39.13 ± 0.65 | 0.24 ± 0.09 | 72.53 ± 0.47 | 3.5 |
| **CDM** | 15.4 ± 0.45 | 19.58 ± 0.79 | 42.2 ± 0.62 | 0.06 ± 0.01 | 90.83 ± 0.18 | 318.5 |
| **CD1a** | 46.97 ± 0.52 | 164.5 ± 0.25 | 66.54 ± 1.055 | 0.039 ± 0.01 | 80.94 ± 0.28 | 4205.01 |
| **CD12a** | 16.36±0.29 | 31.46±0.51 | 55.03±5.11 | 0.002±0.01 | 76.19±0.01 | 15429.1 |
| **CD12b** | 475.4 ± 0.3 | 138.7 ± 0.35 | 76.03 ± 0.005 | 0.168 ± 0.045 | 68.72 ± 0.05 | 824.1 |

Results are shown as means ± S.E.M (n=3)
AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; CD1a, Dihydroconiferyl acetate; CD12a, Crotocascarin ω; CD12b, β-Oplopanone; MNTC, Maximum nontoxic concentration; $CC_{50}$, 50% cytotoxic concentration; $Emax_C$, Maximum cytotoxic effect %; $IC_{50}$, 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

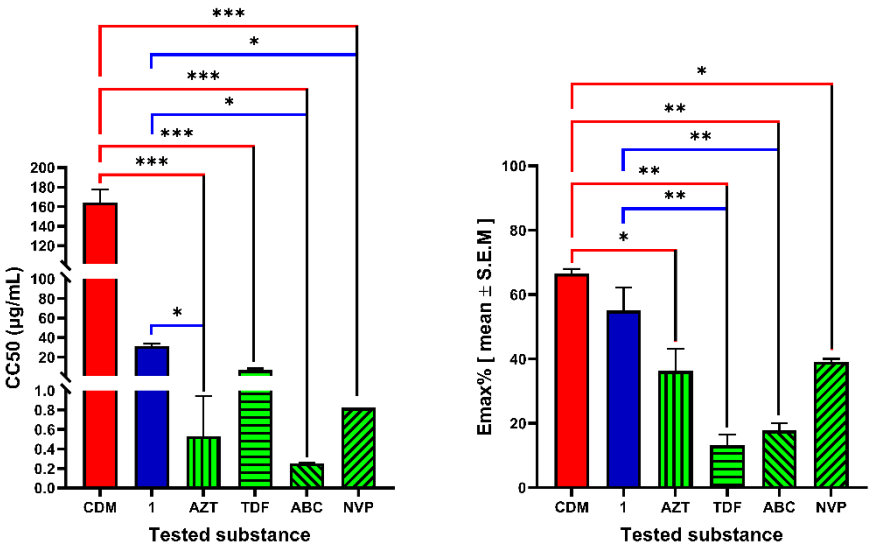



Figure 4.4-15 Cytotoxicity of Crotocascarin ω (CD12a) (1)
Cytotoxicity of crotocascarin ω and methanol-soluble extract (CDM) of the 1:1 CH2Cl2:CH3OH extract of the twigs of C. dichogamus. *Denotes p value < 0.05; **Denotes p value < 0.01, ***Denotes p value < 0.001.

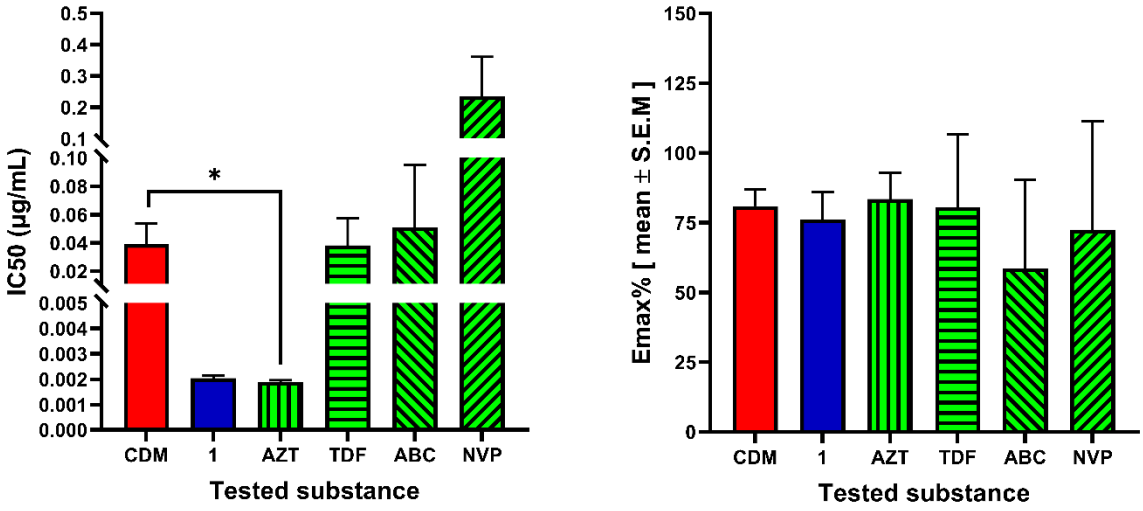



Figure 4.4-16 Anti-HIV activity effects of Crotocascarin ω (1)

Anti HIV effect of crotocascarin ω and methanol-soluble extract (CDM) of the 1:1 CH2Cl2:CH3OH extract of the twigs of *C. dichogamus*. *Denotes p value < 0.05.

### 4.4.2.3. Discussion

To propose a mode-of-action of crotocascarin ω (CD12a), in silico inhibition modelling assays were carried out using HIV reverse transcriptase and HIV protease. Docking studies were performed on HIV-1 RT in complex with known inhibitor nevirapine (PDB ID 1JLB) as well as HIV-1 PR in complex with known antiviral atazanavir (PDB ID: 3EL9) using MOE2015 software. The predicted free energy of binding obtained for **CD12a** against HIV-1 RT was higher (ΔG -1.38 kcal/mol) compared to the known inhibitor nevirapine (ΔG -7.60 kcal/mol), due to the lack of the crucial π-H interaction shown by Nevirapine. The main interaction contributing to the binding was the hydrogen bond between hydroxy group at C-1 and Cys181 (**Figure 4.4-17**). For HIV-PR, the predicted free energy of binding exhibited by compound 1 was ΔG -6.25 kcal/mol, which was also higher compared to the positive control atazanavir (ΔG -11.49 kcal/mol). The main interaction contributing to the binding was the hydrogen bonding between one of the epoxide oxygens and the backbone N-H of Asp29(B). Asp29(B) also shows a key hydrogen bonding interaction with one of the amide carbonyls of Atazanavir (**Figure 4.4-18**). With these results it can be hypothesized that the potential anti-HIV activity of crotocascarin ω could be caused by HIV-1 PR inhibition.

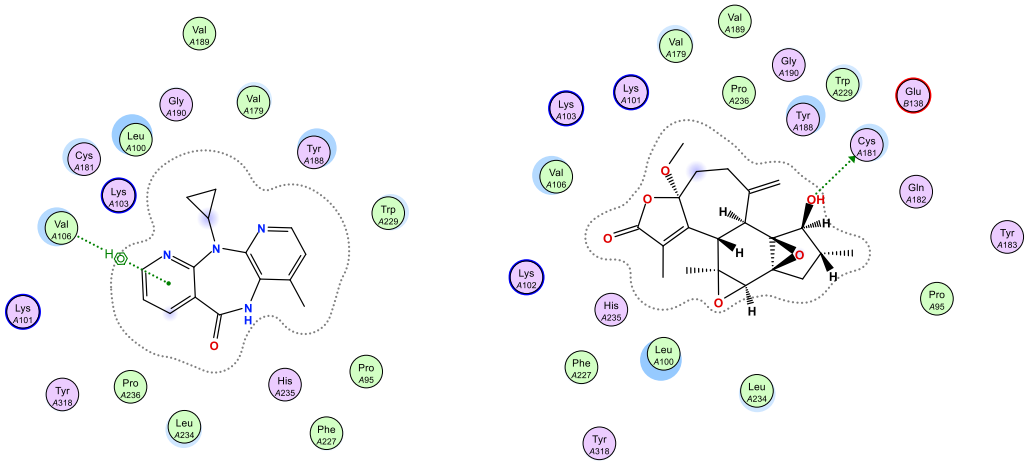


Figure 4.4-17**.** Ligand-protein intereaction of Nevirapine and crotocascarin ω (CD12a) with HIV-1 RT (PDB ID: 1JLB)

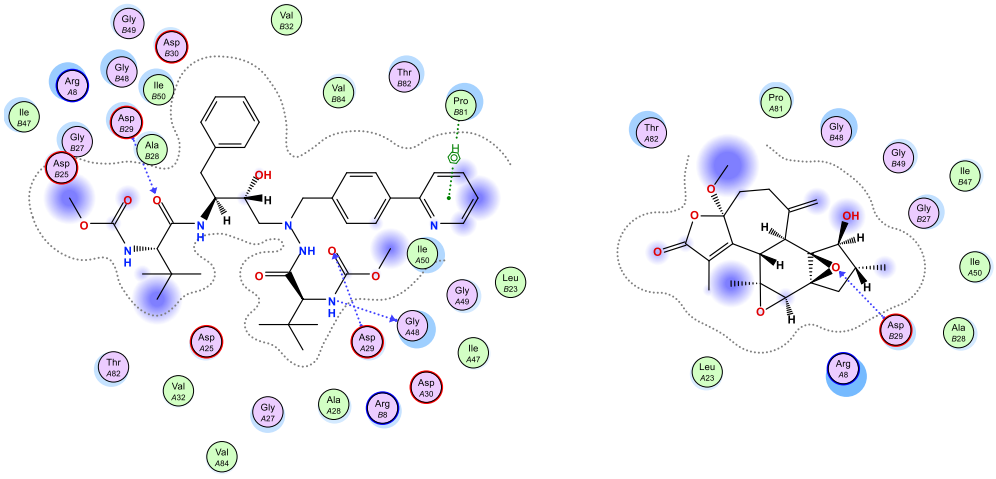


Figure 4.4-18**.** Ligand-protein interactions of atazanavir and crotocascarin ω (CD12a) with HIV-1 PR (PDB ID: 3EL9)

## 4.5. Investigation of the *in vitro* and *in silico* interaction of the isolated compounds against HIV enzymes

### 4.5.1. *In vitro* assays on the effect of pure compounds on retroviral enzymes

To investigate the mode of action of the pure compounds, cell-free enzyme inhibition assays were carried out using HIV reverse transcriptase and HIV protease kit (*Ab211106 HIV-1 Protease Inhibitor Screening Kit (Fluorometric)*, 2020) as described previously (Rotich *et al*., 2021). At 10 µg/mL, none of the compounds inhibited reverse transcriptase activity; however, at the same concentration, compounds isolated from *C. megalocarpus* E22, E12 and E17 inhibited HIV-1 protease by 93.3, 87.2, and 82.4%, respectively (**Table 4.5-1**).

Table 4.5-1 HIV-1 Protease enzyme inhibition activity of compounds isolated from *C. megalocarpus*

| Code | Name of compound | Concentration µg/mL | % inhibition |
|---|---|---|---|
| E37 | Ermiasoid | 10 | 63.3 |
| E5 | 4H-α,7H-α,10α-eudesm-11-en-5β-ol | 10 | 94.1 |
| E12 | 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A) | 10 | 87.2 |
| E17 | 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (ermiasolide C) | 10 | 82.4 |
| E22 | 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) | 10 | 93.3 |
| E24 | 11-acetoxy crotocascarin L | 10 | 81.5 |
| E23 | Crotocascarin K | 10 | 75.9 |
| Control (Protease Inhibitor) | Pepstatin | n.d | 100.0 |

### 4.5.2. *In silico* anti-HIV activity of pure compounds

#### 4.5.2.1. Molecular docking study

A molecular docking study was conducted to predict the binding geometries of the compounds with HIV-1 Reverse Transcriptase and HIV-1 Protease. **Table 4.5-2** and **4.5-3** summarize the docking results for these compounds and FDA-approved antiretroviral drugs. The predicted free energy of binding obtained for the pure compounds against HIV-1 RT (PDB ID: 1JLB) was higher than the known inhibitor nevirapine (ΔG -7.679 kcal/mol). Similarly, the compounds analyzed also gave higher predicted binding energies than the known inhibitor Atazanavir when the docking was performed on HIV-1 PR (PDB ID: 3EL9). The docked

compounds displayed strong binding affinity with HIV-1 protease enzyme (3EL9) compared with the HIV-1 reverse transcriptase**.** The results obtained could indicate that the mechanism of action leading to the observed anti-HIV activity is through HIV-1 protease inhibition, which agrees with the results obtained *in vitro*.

Table 4.5-2: Molecular docking analysis of against HIV-1 RT in complex with Nevirapine (PDB ID: 1JLB)

| **Code** | **Name of compounds** | **Free energy of binding (ΔG) kcal/mol** |
|---|---|---|
| NVP | Nevirapine | -7.679 |
| E17 | 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (ermiasolide C) | -3.555 |
| E22 | 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) | -2.839 |
| E12 | 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A) | -2.317 |
| E5 | 4H-α,7H-α,10α-eudesm-11-en-5β-ol | -1.550 |
| CD12a | Crotocascarin ω | -1.38 |
| E23 | Crotocascarin K | -1.297 |
| E2 | Pinoresinol | -1.295 |
| E24 | 11-acetoxy crotocascarin L | 0.611 |
| E37 | 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) | 3.378 |

Table 4.5-3: Molecular docking analysis of against HIV-1 PR in complex with Atazanavir (3EL9)

| **Code** | **Name of Comound** | **Free energy of binding (ΔG) kcal/mol** |
|---|---|---|
| ATV | Atazanavir | -11.49 |
| E24 | 11-acetoxy crotocascarin L | -7.985 |
| E37 | 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (ermiasoid) | -7.326 |
| E23 | Crotocascarin K | -7.115 |
| E2 | Pinoresinol | -7.078 |
| CD12a | Crotocascarin ω | -6.25 |
| E12 | 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A) | -6.067 |
| E22 | 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) | -5.994 |
| E17 | 5β,8H-β-hydroxy eudesm-7(11)-en-12,8-olide (ermiasolide C) | -5.850 |
| E5 | 4H-α,7H-α,10α-eudesm-11-en-5β-ol | -5.750 |

#### 4.5.2.2. Ligand-Protein interaction

To better understand the mode of protease enzyme inhibition of the compounds, ligand-protein interactions were visualized. The interactions of the pure compounds with the HIV-1 protease enzyme demonstrate that the ligands interact with most of the enzyme's active site residues. The residues were discussed in section 2.2.3 and are also summarized in **Table 4.5-4.** Among the intermolecular interactions, hydrophobic, hydrogen, alkyl, pi-sigma, salt bridges, and halogen bonds were involved in ligand-protein interactions **(Table 4.5-5).**

Table 4.5-4: Amino acids in the binding site of HIV-1 protease enzyme

| **Binding site** | **Active site residues** |
|---|---|
| Catalytic site | Asp-25, Thr-26, Gly-27 |
| S1 and S1’ subsite | Ile-50, Ile-84, Pro-81, Thr-80, Val-82 |
| S2 and S2’ subsite | Asp-29, Asp-30, Ala-28, Ile-47, Gly-48, Ile-50 |
| S3 and S3’subsite | Arg-8, Gly-48, Leu-23, Val-82, Thr-80, Pro-81 |
| S4 and S4’ subsite | Asp-30, Met-46, Ile-47, Gly-48, Gln-58 |

Table 4.5-5: Ligand-protein interaction profile for compounds docked with HIV-1 PR (3EL9)

| Ser. No. | Name of compound | Hydrophobic interactions | Hydrogen bond Interactions | Pi cation interactions | Salt bridges | Halogen bond |
|---|---|---|---|---|---|---|
| 1. | Atazanavir | **S1**- Ile-50 (3.52 Å), Ile-50′ (3.72 Å), Pro-81′ (3.81 Å)<br>**S2**- Ala-28′ (3.59 Å), Ile-47 (3.60 Å),<br>**S3**- Leu-23 (3.64 Å),<br>Thr-82 (3.47 Å), Thr-82′ (3.56 Å) | Catalytic site- Asp-25 (3.07 Å),<br>Gly-27 (2.86 Å)<br>**S1**- Ile-50 (3.88 Å)<br>**S2**- Asp-29 (3.69 Å), Gly-48 (3.27 Å)<br>**S3**- Arg-8 (3.72 Å), Arg-8′ (3.26 Å), | Arg-8′ (3.84 Å) | - | |
| 2. | 11-acetoxy crotocascarin L (E24) | **S1**- Ile-50 (3.52 Å), Ile 50 (3.78 Å)<br>**S2**- Asp 30 (3.57 Å), Ile-47 (3.84 Å),<br>**S3**- Arg-8′ (3.99 Å), Leu-23′ (3.77 Å), | - | | Arg-8 (3.82 Å) | |
| 3. | Ermiasoid (E37) | **S1**- Ile-50 (3.34 Å), Ile-50 (3.71 Å),<br>**S3**- Leu 23′ (3.09 Å), Thr-82′ (3.84 Å) | **Catalytic site** -Gly-27′ (3.23 Å),<br>**S1**- Ile 50 (3.45 Å), Ile-50′ (3.75 Å)<br>**S2**- Asp-29′ (3.38 Å), Asp-30′ (3.04 Å), | | | Asp 25 (3.23) |
| 4. | Crotocascarin K (E23) | **S2**- Asp-30′ (3.78 Å)<br>**S3**- Leu-23 (3.75 Å), Thr-82 (3.63 Å), Val-84 (3.68 Å) | **Catalytic site**- Asp-25 (2.67 Å), Gly-27′ (3.45 Å),<br>**S2**- Asp-29′ (3.16 Å) | | | |
| 5. | Pinoresinol (E2) | **S2**- Ala-28′ (3.57 Å) | **S1**- Ile 50 (3.29 Å), Ile 50′ (4.04 Å)<br>**S2**-Asp-29 (3.68 Å), Asp-29′ (3.84 Å), Asp-30 (4.01 Å), | | | |
| 6. | Ermiasolide A (E12) | **S1**- Ile-50 (3.70 Å), Ile-50′ (3.66 Å),<br>**S2**-Ala 28 (3.67 Å),<br>Thr-82′ (3.81 Å) | Thr-82′ (3.62 Å) | | | |
| 7. | Ermiasolide B (E22) | **S1**- Ile-50 (3.78 Å), Ile-50′ (3.84 Å),<br>**S2**-Ala 28 (3.75 Å),<br>Thr-82′ (3.95 Å) | **Catalytic site** - Asp-25 (2.82 Å),<br>**S1**- Ile 50 (4.04 Å),<br>Thr-82′ (3.55 Å) | | | |
| 8. | Ermiasolide C (E17) | **S1**- Ile-50 (3.48 Å),<br>Val-84′ (3.89 Å), | **Catalytic site**- Asp-25′ (3.09 Å) | | | |
| 9. | 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5) | **S1**- Ile-50′ (3.51 Å)<br>**S2**-Ala-28 (3.93 Å),<br>Val-32 (3.59 Å), | **S1**- Ile 50 **(3.95 Å),** Ile 50′ (3.68 Å) | | | |

Residues within the active site are colored red.

**1. Interaction of 11β-acetoxy crotocascarin L (E24) with Protease (PDB ID: 3EL9)**

A molecular docking study showed that 11β-acetoxy crotocascarin L forms strong binding to the receptor with an estimated free binding energy of -7.985 kcal/mol making it superior in binding efficiency compared to all docked compounds **(Table 4.5-3).** E24 interacts with key residues in S1, S2, and S3 subsites of the HIV-PR enzyme. E24 forms hydrophobic interactions between E24 and Ile-50 (3.52 Å), Ile 50 (3.78 Å), Asp 30 (3.57 Å), Ile-47 (3.84 Å), Arg-8′ (3.99 Å), and Leu-23′ (3.77 Å), at the active site of the protease enzyme. Even though E24 is not binding to the catalytic domain of the enzyme, these hydrophobic interactions could interfere with the binding of the viral polypeptide to the active site of the protease by causing conformational change on the enzyme active site and later can cause inhibition of viral replication. Hydrophobic interactions are the main driving force in drug-receptor interactions (Ferreira De Freitas & Schapira, 2017a).

Furthermore, the C-11 acetoxy group forms a salt bridge with Arg-8 (3.82 Å), contributing to ligand binding (Segala *et al*., 2016). The methyl groups at C-6 and C-15 of E24 were involved in alkyl interactions with Ile-47, Ile-50, and Val-32 **(Figure 4.5-1).** These interactions involve charge transfer and help intercalate the compound in the receptor's binding site (3EL9).

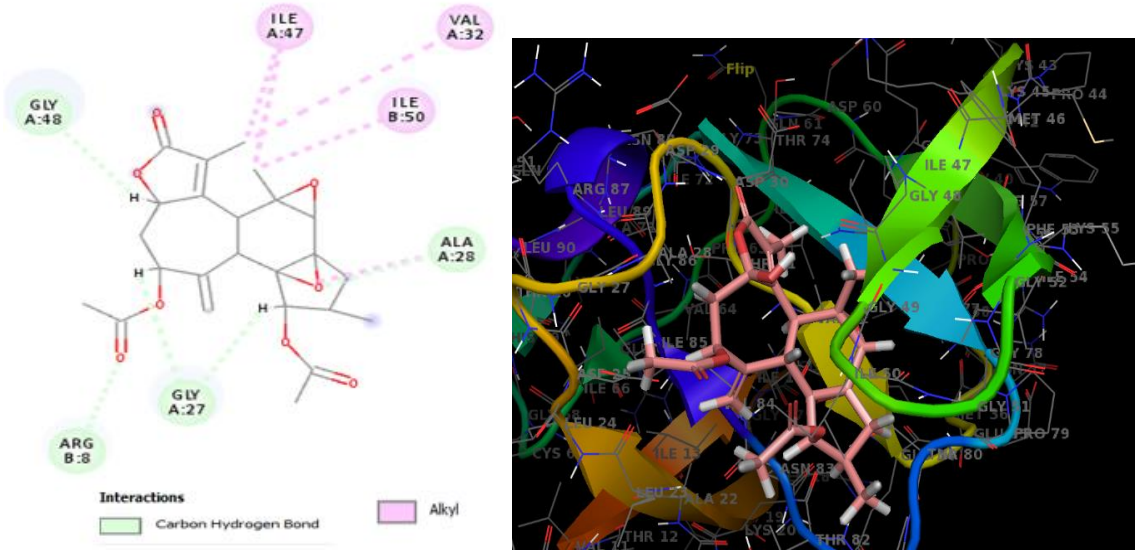



Figure 4.5-1: 2D and 3D visualization of the interaction of 11β-Acetoxy crotocascarin L (E24) with HIV-1 PR (3EL9)

**2. Interaction of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37) with protease enzyme (PDB ID: 3EL9)**

Ermiasoid (E37) is a mon-chlorinated crotofolane diterpenoid isolated from the bark of *C. megalocarpus*. It has displayed promising anti-HIV activity by inhibiting more than 86 % of HIV-induced cytopathic effects in MT-4 cells at a lower concentration of 28 nM. Even though the compound was not active against the reverse transcriptase enzyme, it has displayed activity against the protease enzyme. Ermiasoid binds well with 3EL9 ligand-binding site with a free binding energy of -7.326 kcal/mol making it the second most active molecule to inhibit protease enzyme (3EL9 **(Table 4.5-3).**

Ermiasoid (E37) forms three hydrophobic and five hydrogen bond interactions with residues in the active site of the HIV-PR enzyme. Table 4.5-5 shows that hydrophobic interactions were formed with Ile-50, Leu-23, and Thr-82 residues in S1 and S3 subsites. These interactions introduce stabilizing charges responsible for ligand binding efficiency in the receptor's binding site (3EL9). In addition, the hydroxyl groups at C-5 and C-6 contribute to the H-bonding with Gly-27 and Ile-50, respectively. This finding is exciting as compounds with dihydroxy ethylene groups are reported to have protease inhibitor activity (Coop, 2019). The epoxy group of E37 is also involved in the hydrogen bond interactions with Ile-50 and Ile-50′ in S1 and S1’ subsites, respectively. Similarly, the carbonyl group at C-16 forms hydrogen bonding with the Asp-29’ in the S2’ sub-site of the enzyme. Ermiasoid forms a halogen bond with Asp-25, key catalytic residue for HIV-PR **(Figure 4.5-2).** Halogen bonds play a key role in membrane permeability, affinity with proteins, metabolic stability of compounds (Ferreira De Freitas & Schapira, 2017b) and also can form specific molecular interactions that contribute to the recognition of ligands by proteins (Scholfield *et al*., 2013). These interactions interfere binding of the viral polypeptide with the protease enzyme and reduce or inhibit the HIV-PR enzyme's catalytic function, hence contributing to the anti-HIV activity of ermiasoid.

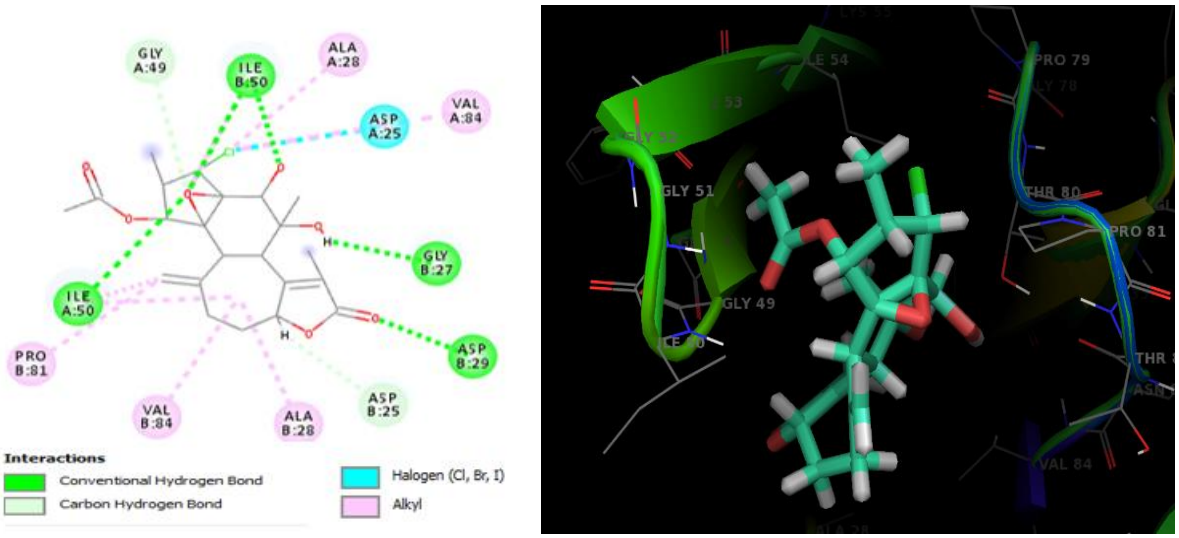



Figure 4.5-2: 2D and 3D visualization of the interaction of Ermiasoid (E37) with HIV-1 PR (3EL9)

**3. Interaction of crotocascarin K (E23) with Protease enzyme (PDB ID: 3EL9)**

Crotocascarin K is a crotofolane diterpenoid isolated from the ethyl acetate fraction of *C. megalocarpus*. The *in vitro* assay has displayed strong anti-HIV activity by inhibiting viral replication by 83.3 % with an $IC_{50}$ value of 0.002 µg/mL. In addition, it has shown protease inhibitor activity by inhibiting 76 % of the HIV-1 PR enzyme *in vitro* activity. As depicted in **Table 4.5-3,** the binding energy of the interaction of crotocascarin K with 3EL9 was -7.115 kcal/mol making it the 3rd most active compound with the ability to inhibit the protease enzyme.

Crotocascarin K's high affinity for the enzyme could be attributed due to its ability to form two hydrophobic and three hydrogen bond interactions with residues in the active site of the HIV-PR enzyme. The hydrophobic interactions were formed with Asp-30′ and Leu-23 at S2′ and S3 subsites of the protease enzyme **(Table 4.5-5).** The methyl group at C-15, and the cyclopentane ring form an alkyl interaction with Ile-47′, Ala -28′, Ile-50and Ile-50′ **(Figure 4.5-3).** These interactions help in stabilizing the compound in the binding site of the receptor (3EL9).

The β-hydroxyl group at C-9 of E23 forms hydrogen bond interactions with Asp-25. Similarly, the β-hydroxyl group at C-1 forms a hydrogen bond with Gly-27′ and Asp-29′ at S2′ subsite of the enzyme. The formation of hydrogen bonds with the residues at the catalytic region (Asp-25 and Gly-27) will impair the enzyme's activity. In biological complexes, hydrogen bonds are the most common directed intermolecular interactions, and they contribute significantly to the specificity of molecular recognition

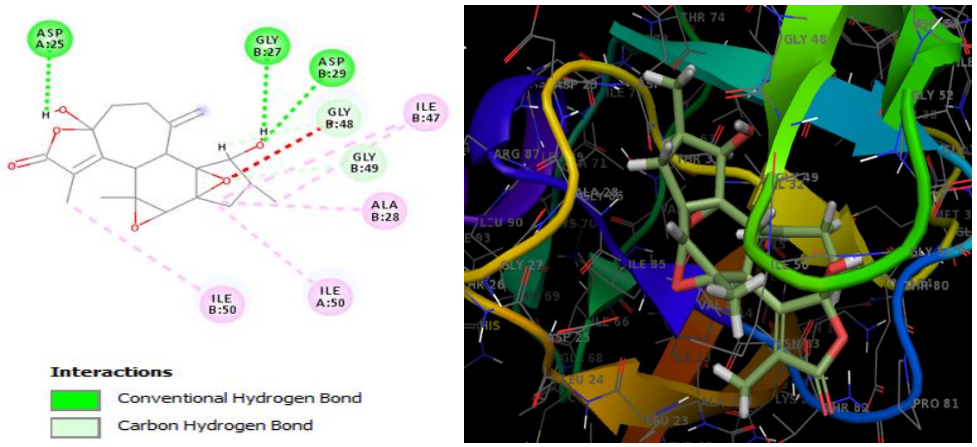



Figure 4.5-3: 2D and 3D visualization of interactions of crotocascarin K (E23) with HIV-1 PR (3EL9)

**4. Interaction of Pinoresinol (E2) with Protease enzyme (PDB ID: 3EL9)**

Pinoresinol binds to the protein satisfactorily with an estimated free binding energy of -7.078 kcal/mol making it the fourth most active drug with the ability to inhibit protease enzyme (3EL9). Table 4.5-4 shows that Pinoresinol (E2) forms five hydrogen bond interactions with HIV-PR (3EL9). The methoxy group at C-4' contributes to the hydrogen bond interaction with Asp-29, while the –OCH3 group at C-5 plays a role in van der Waals interactions with Asp-30 in the S2 subsite of the enzyme. The two aromatic rings of pinoresinol form hydrophobic interaction with Ala-28 in the S2 subsite of the HIV-1 PR enzyme (**Figure 4.5-4**). This interaction stabilizes the compound in the enzyme's binding site and renders the substrate's binding to the enzyme. In addition, the oxygen in the tetrahydrofuran moiety is involved in hydrogen bonding with Ile-50 in both S1 and S1' sub-sites of the enzyme.

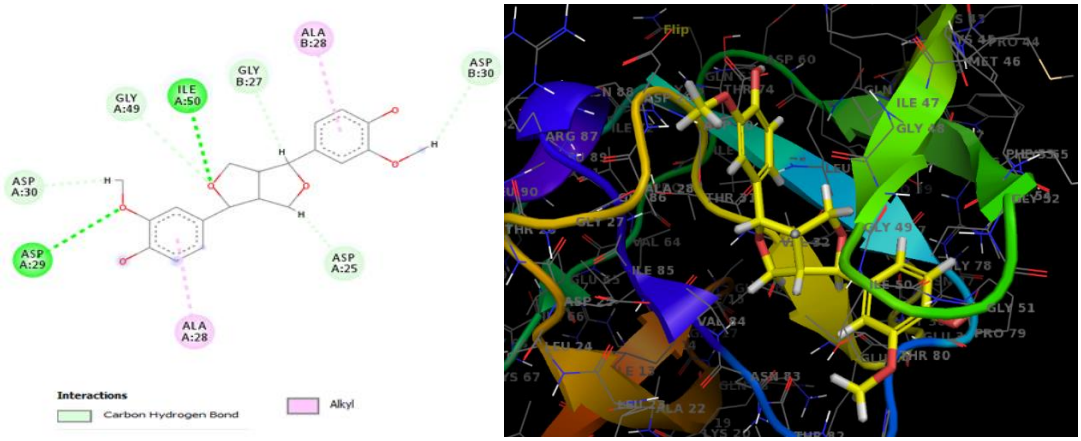



Figure 4.5-4: 2D and 3D visualization of the interaction of pinoresinol (E2) with HIV-1 PR (3EL9)

**5. Interaction of 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12, 8-olide (ermiasolide A) with HIV-1 Protease enzyme (PDB ID: 3EL9)**

Ermiasolide A binds to the protease enzyme with an estimated free binding energy of -6.067kcal/mol making it the fifth most active drug. Ermiasolide A (**E12**) forms 3 hydrophobic interactions in the S1 and S2 subsites of the protease enzyme **(Table 4.5-5).** The methyl group at C-4 and the two cyclohexane rings are involved in hydrophobic interactions with Ala-28, Ile-50, and Val-84 **(Figure 4.5-5).** Furthermore, the methoxy group at C-8 contributes in hydrophobic interaction and in stabilizing the compound within the binding pocket. In addition, E12 forms hydrogen bonding with Thr-80 and Thr-82. The binding of E12 with Thr-82 is similar to that seen in Atazanavir and E37**,** suggesting this amino acid is embedded within the hydrophobic pocket of the receptor and plays a role in molecular recognition.

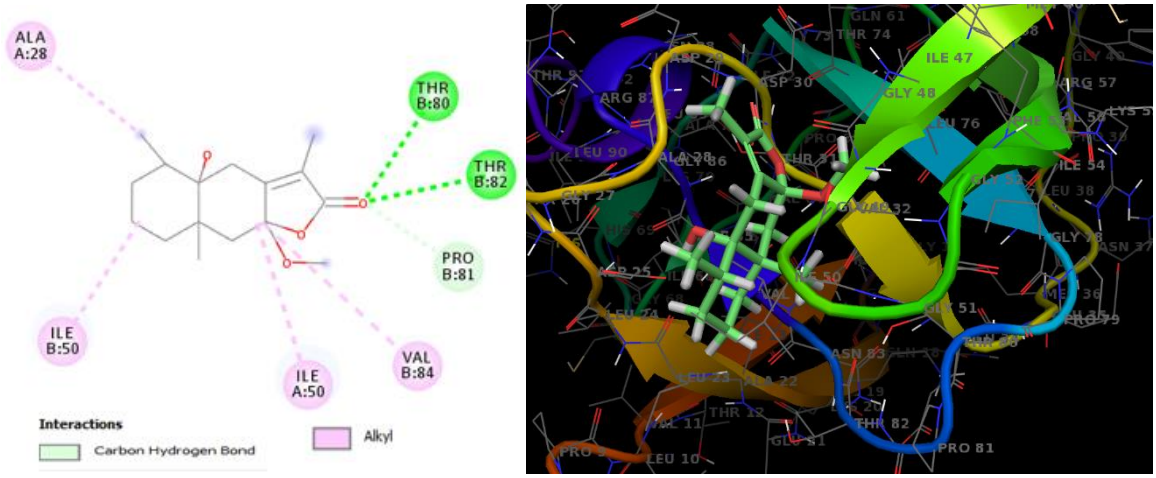



Figure 4.5-5: 2D and 3D visualization of the interactions of ermiasolide A (E12) with HIV-1 PR (3EL9)

# CHAPTER FIVE

## CONCLUSION AND RECOMMENDATIONS

This research reports the chemistry, anti-HIV activity, and mode of action of five new previously undescribed compounds isolated from three Kenyan *Croton* species.

1. The crude (1:1 v/v dichloromethane: methanol) extracts of *C. macrostachyus*. *C. megalocarpus* and *C. dichogamus* showed varying degrees of anti-HIV activity. The crude extract of *C. dichogamus* displayed the highest anti-HIV activity ($Emax_{AV}$= 73.7 percent).
2. Among the four solvent fractions (hexane, dichloromethane, ethyl acetate, and methanol) tested, the methanol fraction of the twigs of *C. dicohgamus* displayed the highest anti-HIV activity ($Emax_{AV}$=90.8 %), followed by the ethyl acetate fraction of the bark of *C. megalocarpus* ($Emax_{AV}$= 88.0 %) and the hexane fraction of the leaves of *C. macrostachyus* ($Emax_{AV}$= 83 %).
3. Using bioassay-guided fractionation, 19 known and 6 new previously undescribed compounds were isolated and characterized from the three *Croton* species. Four new compounds with anti-HIV activity were isolated from *C. megalocarpus,* while two new compounds were from *C. dichogamus*. Twenty-one of the isolated compounds have displayed anti-HIV activity by inhibiting more than 75 % of HIV-induced cytopathic effects on the Human T-lymphocytic cell line.
4. Among the isolated compounds, four eudesmane type sesquiterpenes, Ermiasolide A, B, C, 4H-α,7H-α,10α-eudesm-11-en-5β-ol, and three crotofolane diterpenoids, ermiasoid, crotocascarin K and crotocascarin ω displayed the highest protease inhibitor efficacy. In addition, these compounds displayed the lowest predicted binding energy (strong affinity) against HIV-1 PR ($\Delta G < -5.0$ kcal/mol) in an *in silico* study. Mechanistic studies showed that the isolated pure compounds form hydrophobic interactions with several residues in the binding pocket and hydrogen bonding with the key residues in the catalytic domain of the HIV-1 protease, including ASP 25 and GLY 27, GLY 48.

## Recommendations

I. *In silico* quantitative structure-activity relationship (QSAR) studies should be conducted to analyze the responsible functional groups for the observed anti-HIV activity.

II. Since most of the compounds isolated in this study had a higher cytotoxicity effect, investigation of these compounds for their anticancer effect should be carried out.

III. Anti-HIV activity of the pure compounds should also be determined in an *in vivo* animal model.

IV. Studies involving the synthesis of the bioactive compounds should be conducted to perform additional *in vitro* and *in vivo* studies.

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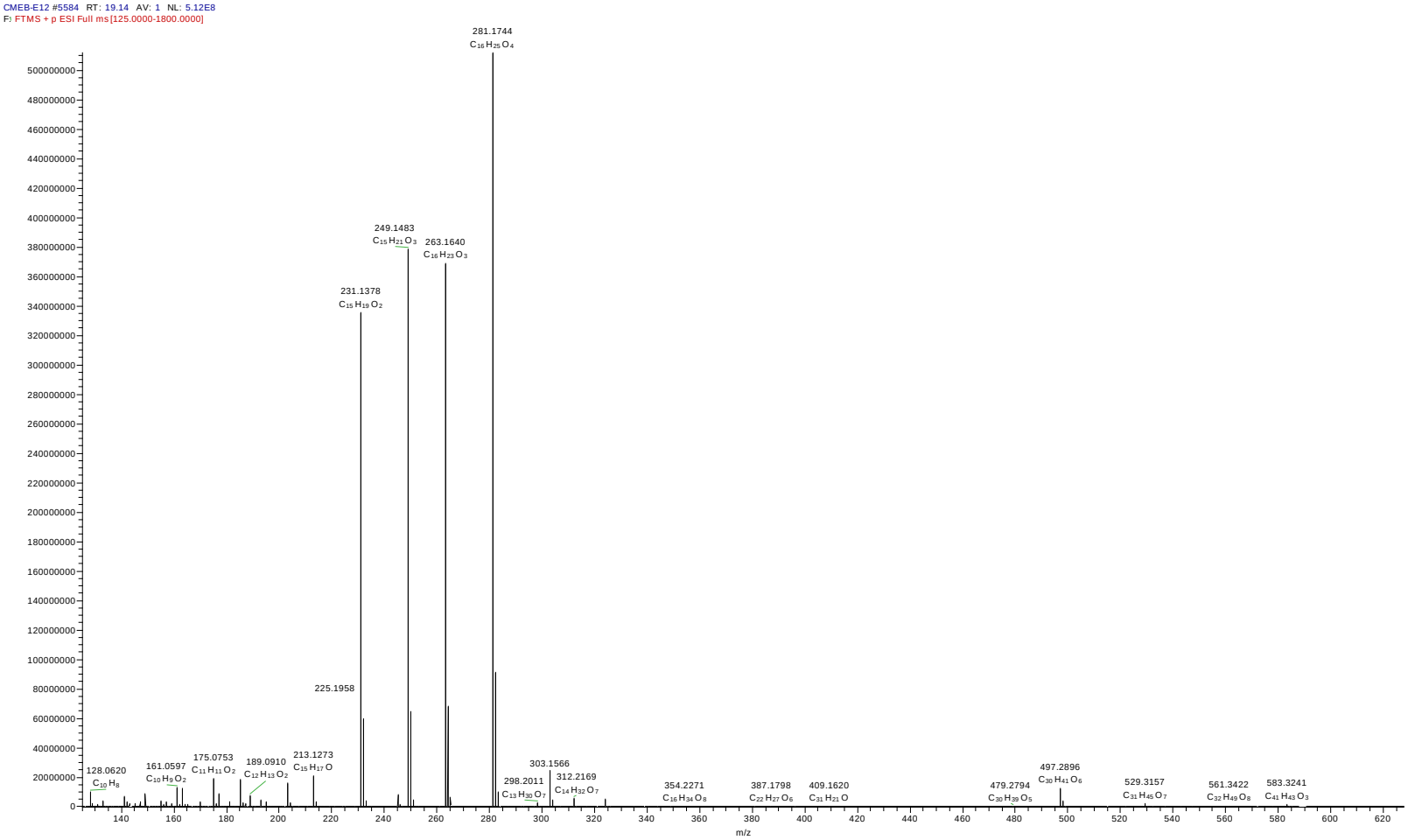
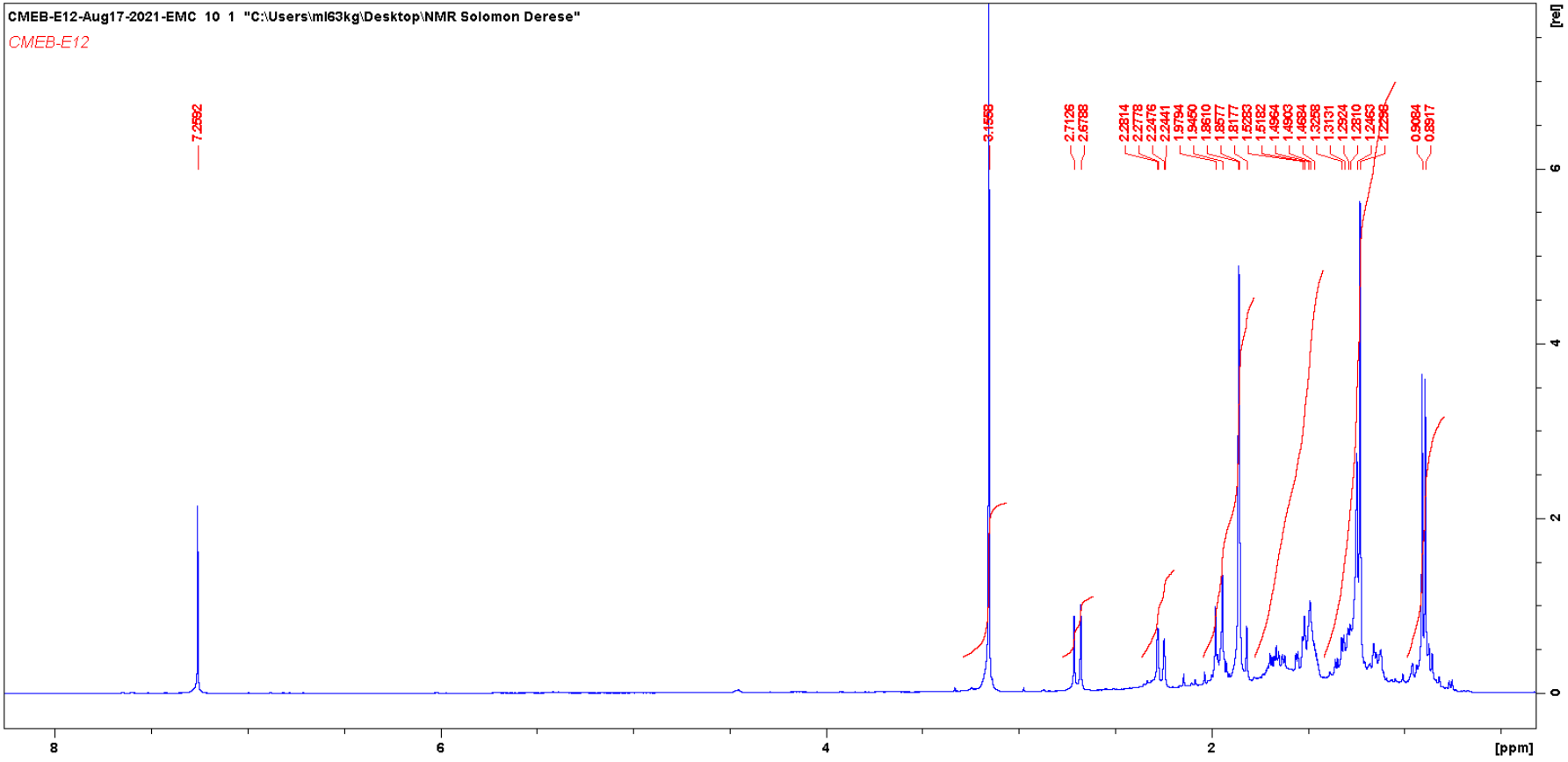
# APPENDICES

Appendix 1 Mass Spectrum of 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12, 8-olide (ermiasolide A) (E12)

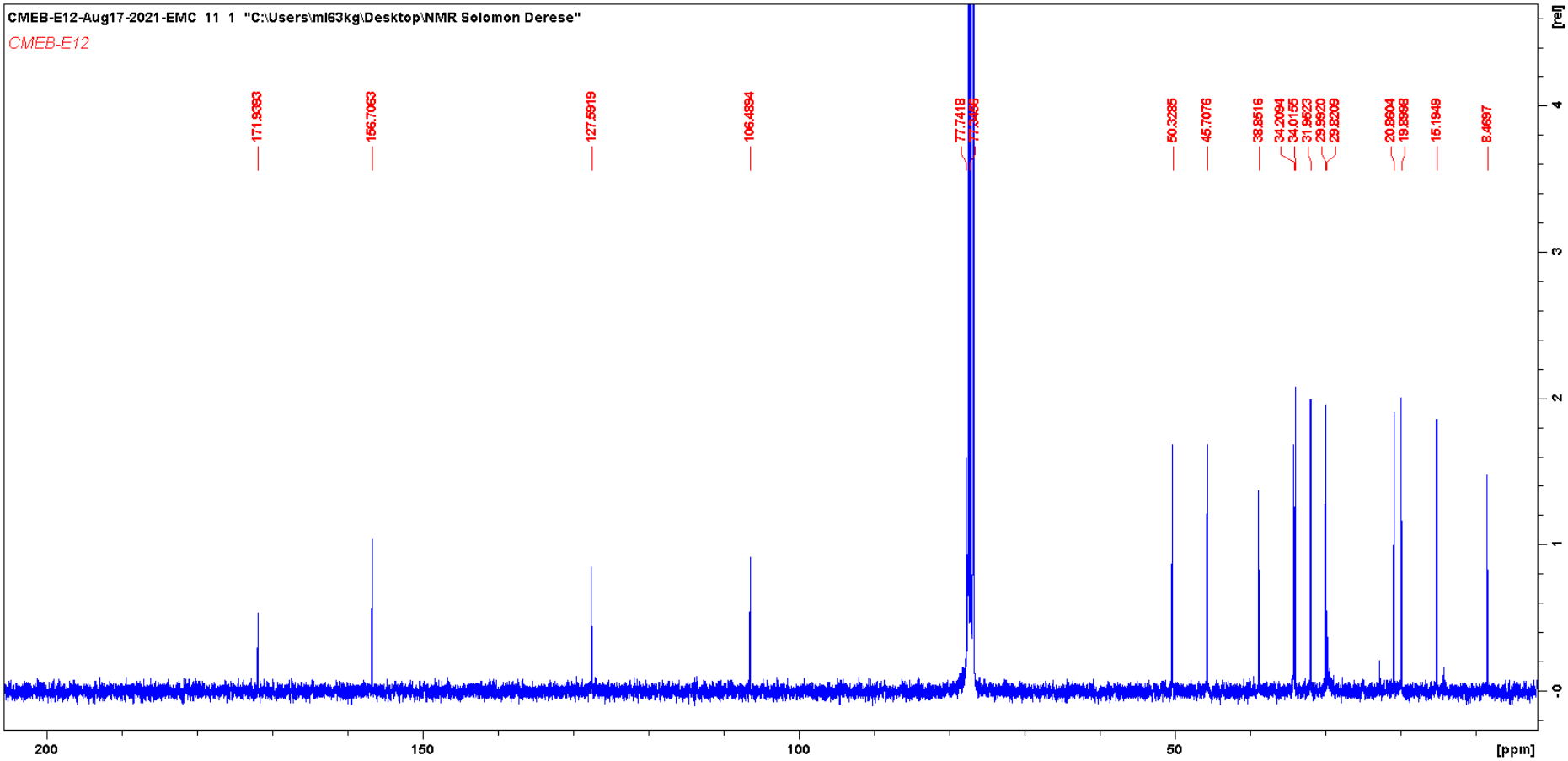



HRESIMS *m/z* 281.1744 $[M+H]^+$ (calcd. for $C_{16}H_{24}O_4$ + H, *m/z* 281.1747)

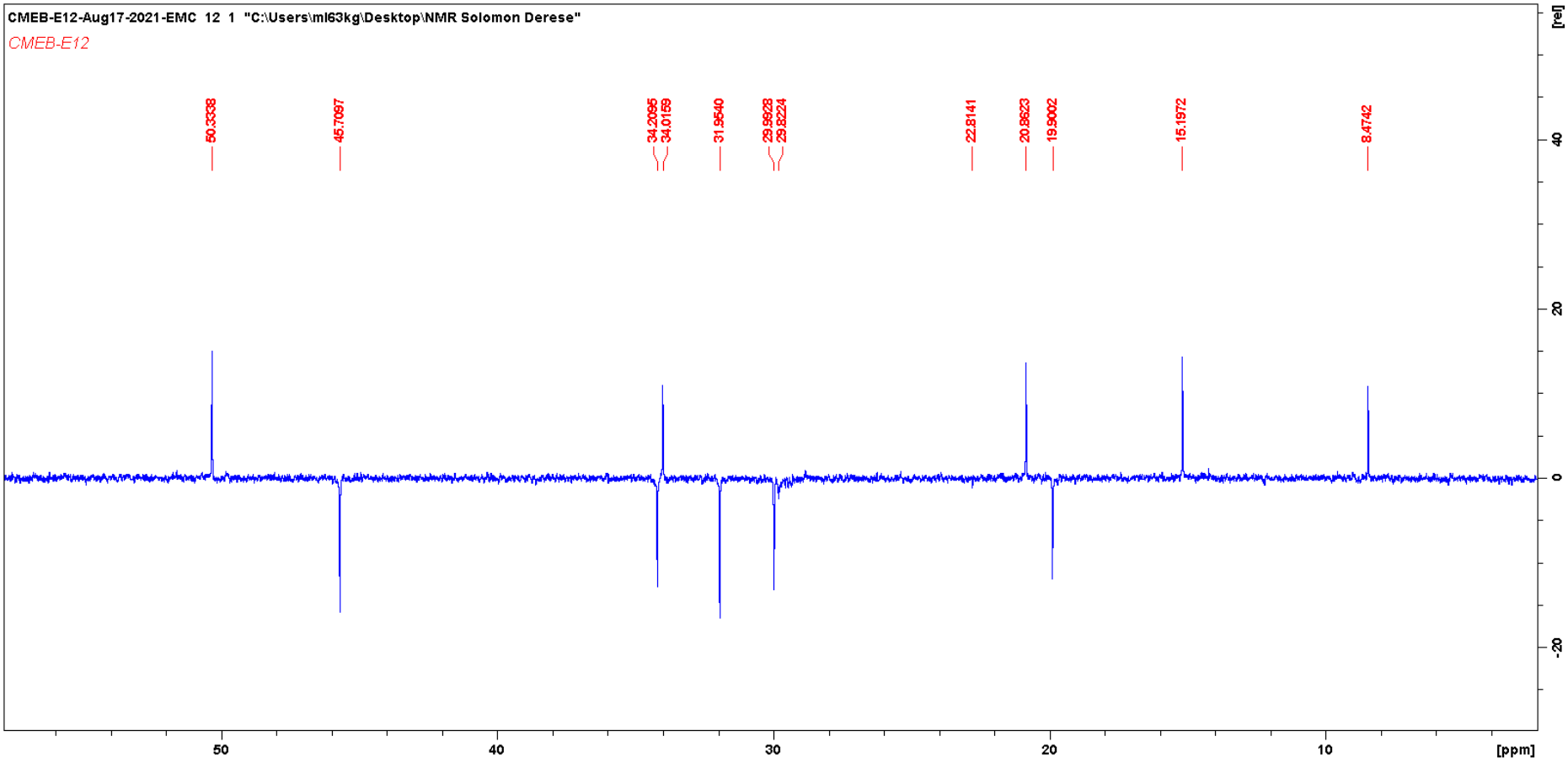
Appendix 2 $^{1}$H NMR Spectra for 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A) (E12)



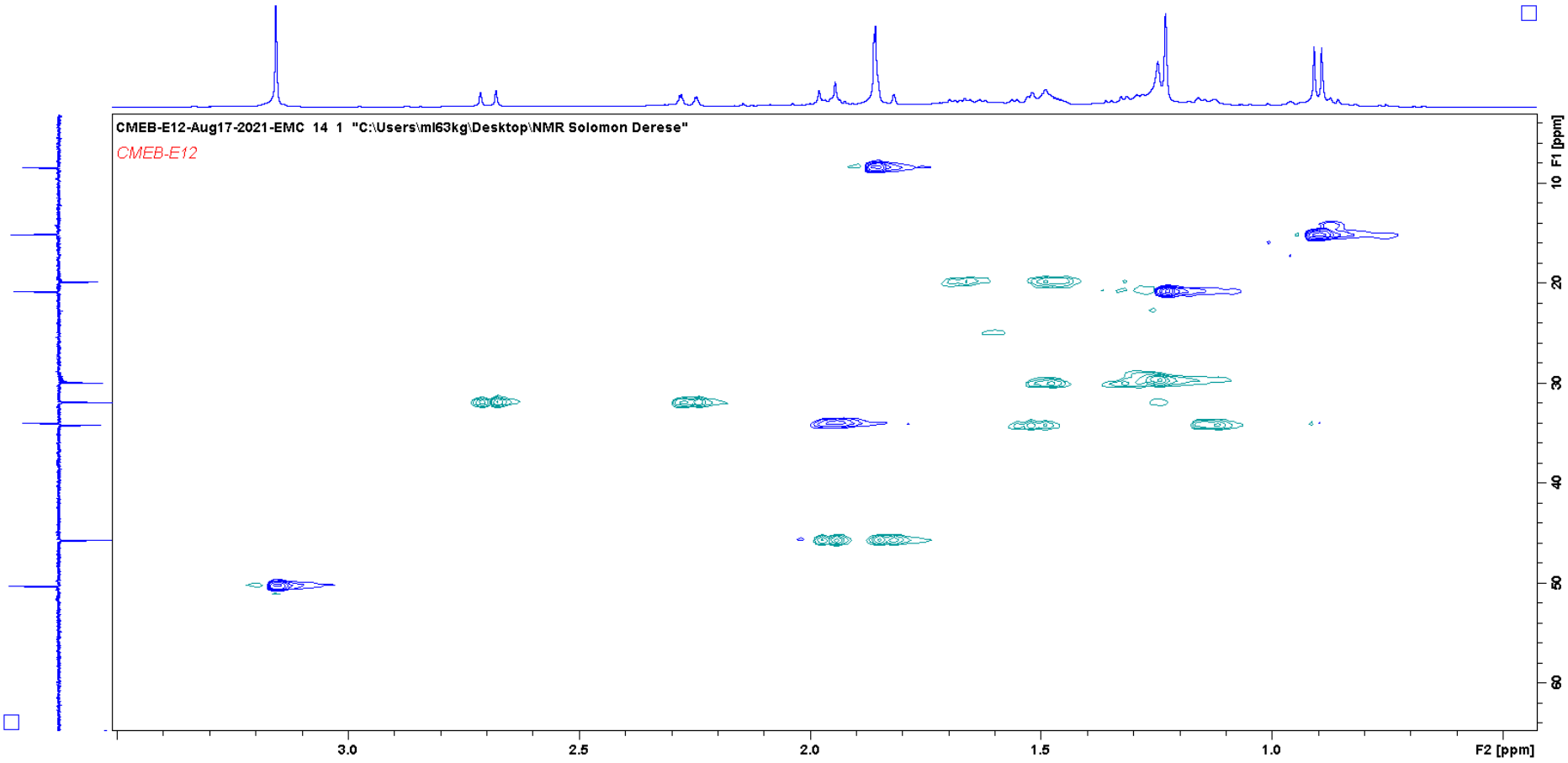
Appendix 3 $^{13}$C NMR Spectra for 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12, 8-olide (ermiasolide A) (E12)



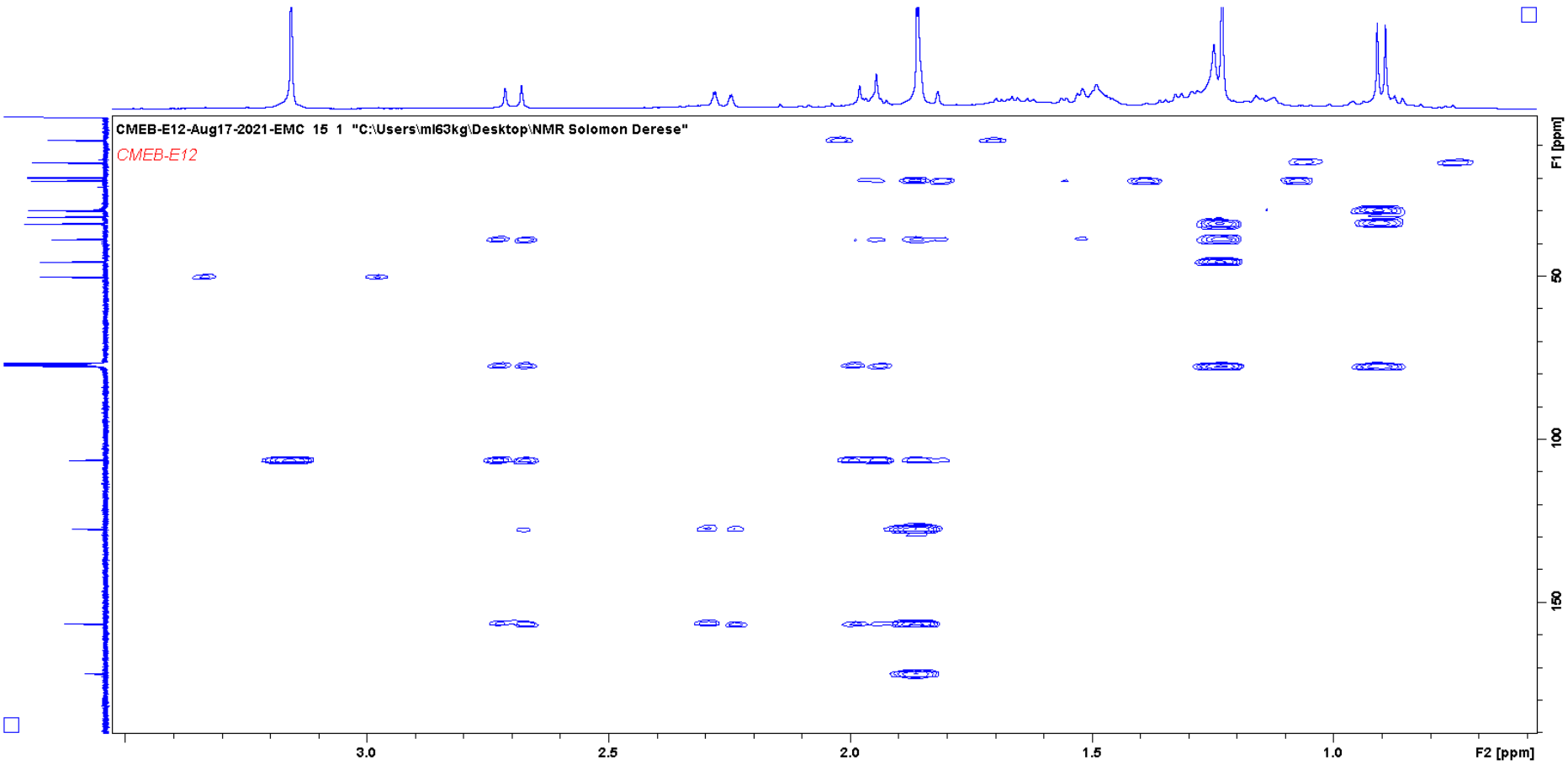
Appendix 4 DEPT Spectrum for 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12, 8-olide (ermiasolide A) (E12)



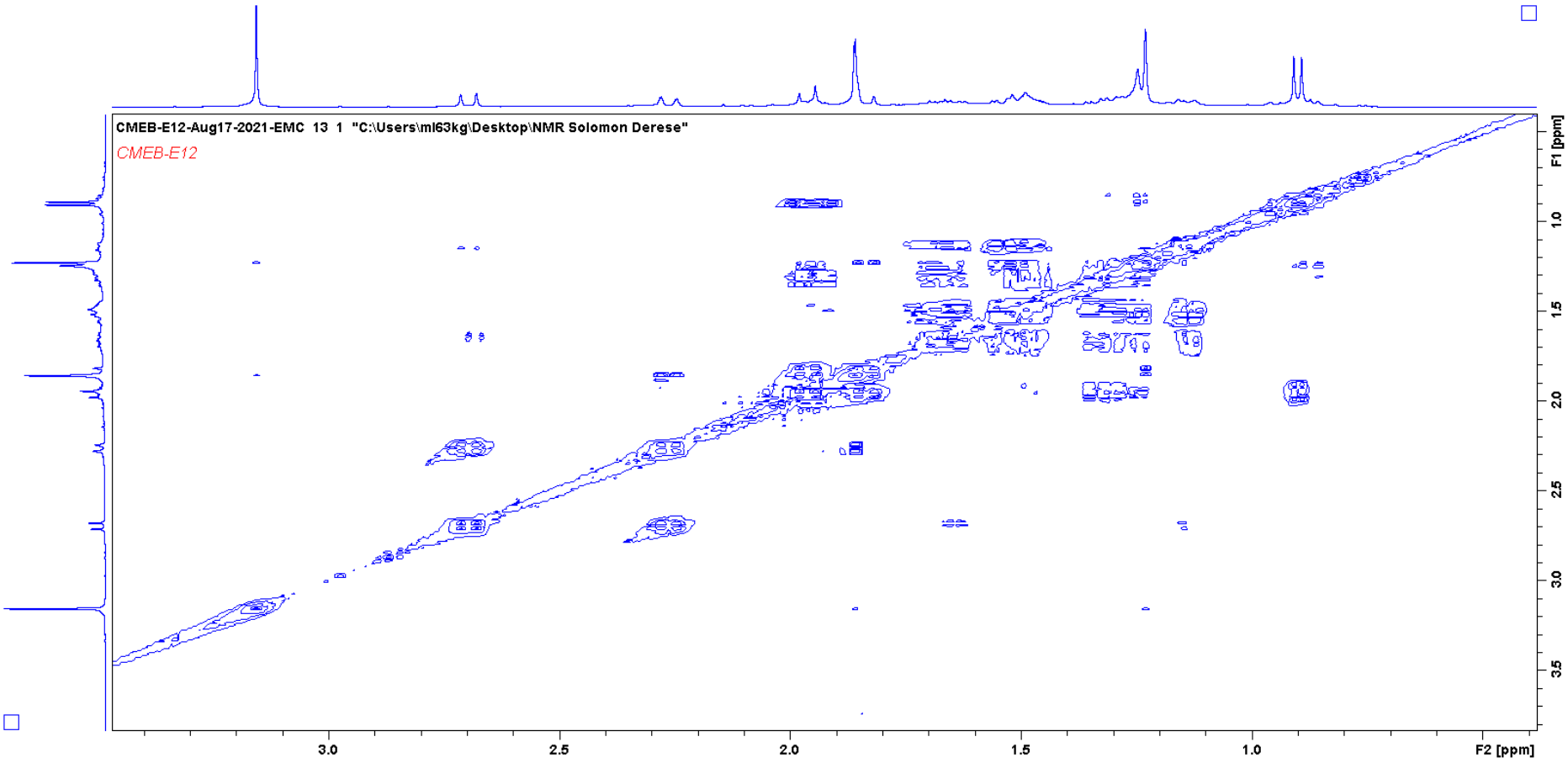
Appendix 5 HSQCDEPT Spectra for 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A) (E12)



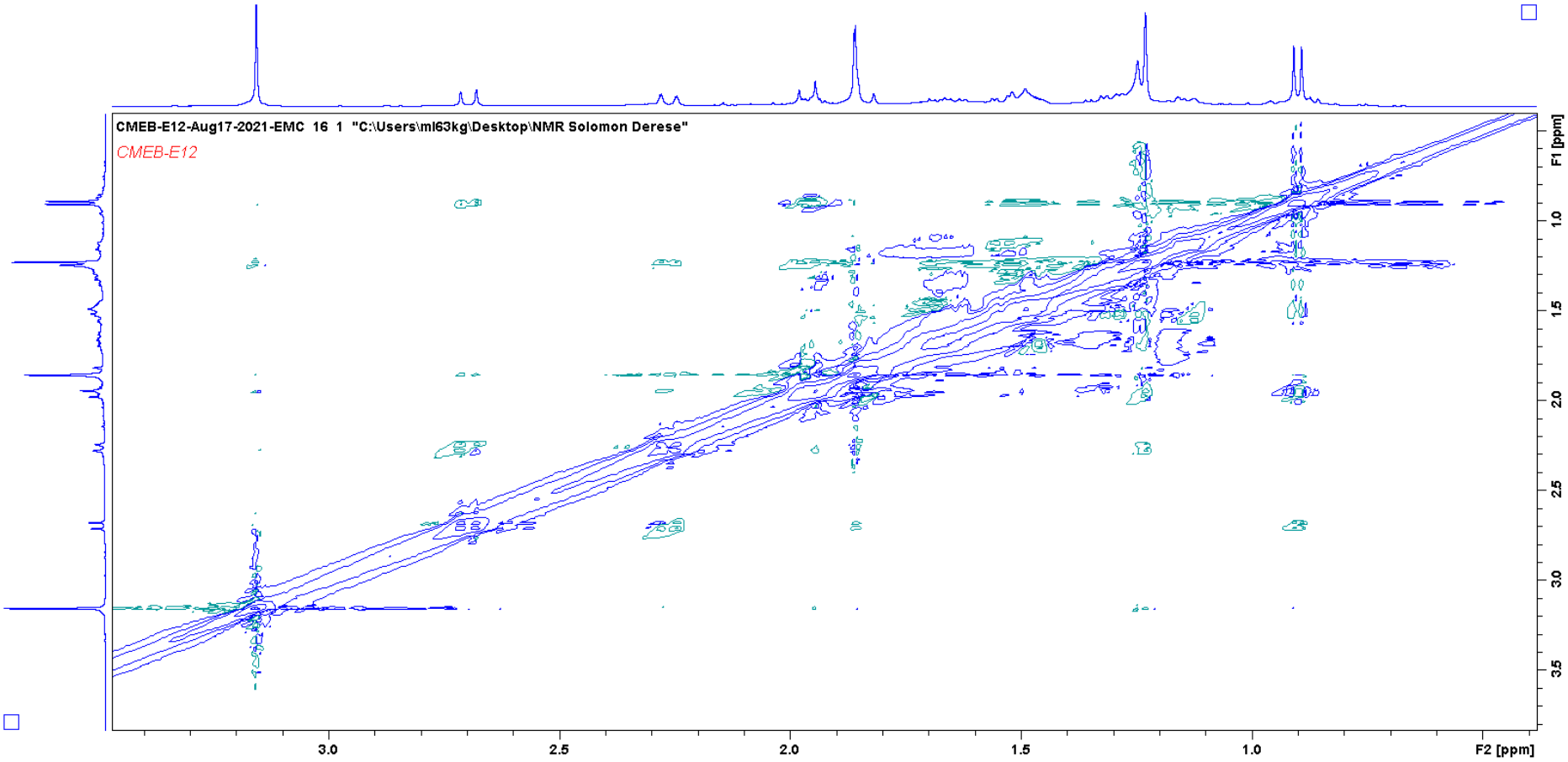
Appendix 6 HMBC Spectrum for 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12, 8-olide (ermiasolide A) (E12)



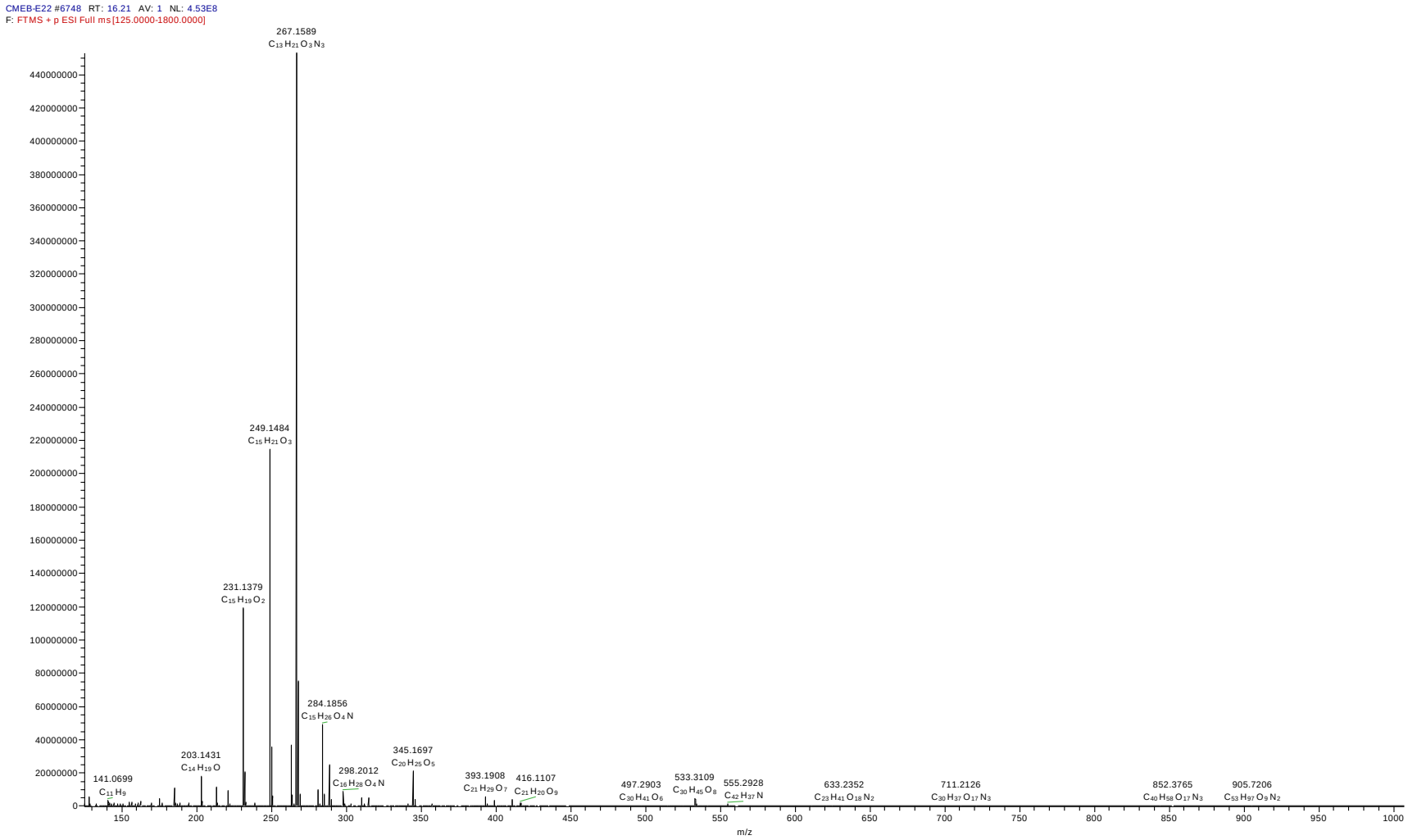
Appendix 7 COSY Spectrum for 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A) (E12)



Appendix 8 NOSEY Spectrum for 5β-hydroxy-8α-methoxy eudesm-7(11)-en-12,8-olide (ermiasolide A) (E12)

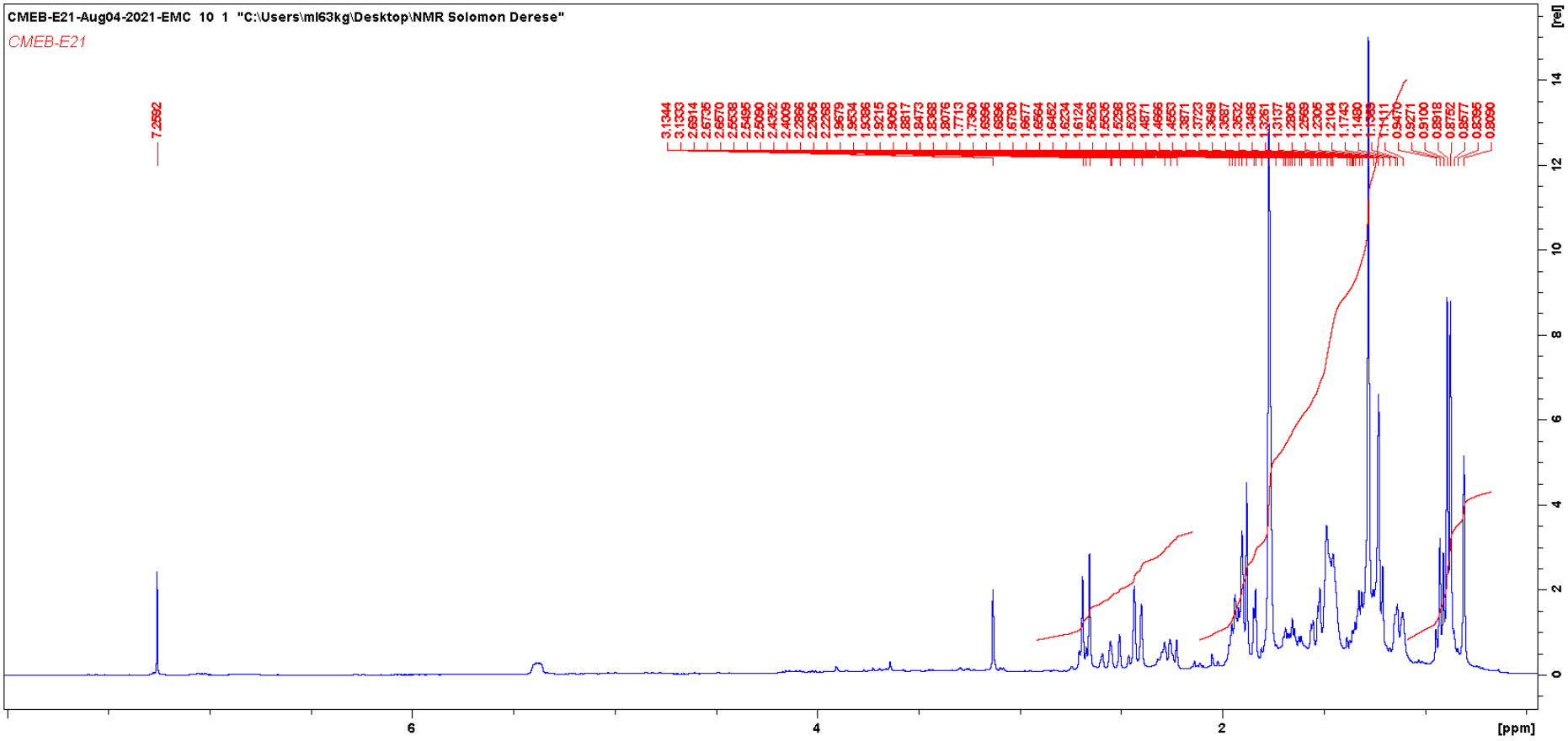


Appendix 9 Mass Spectrum of 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)

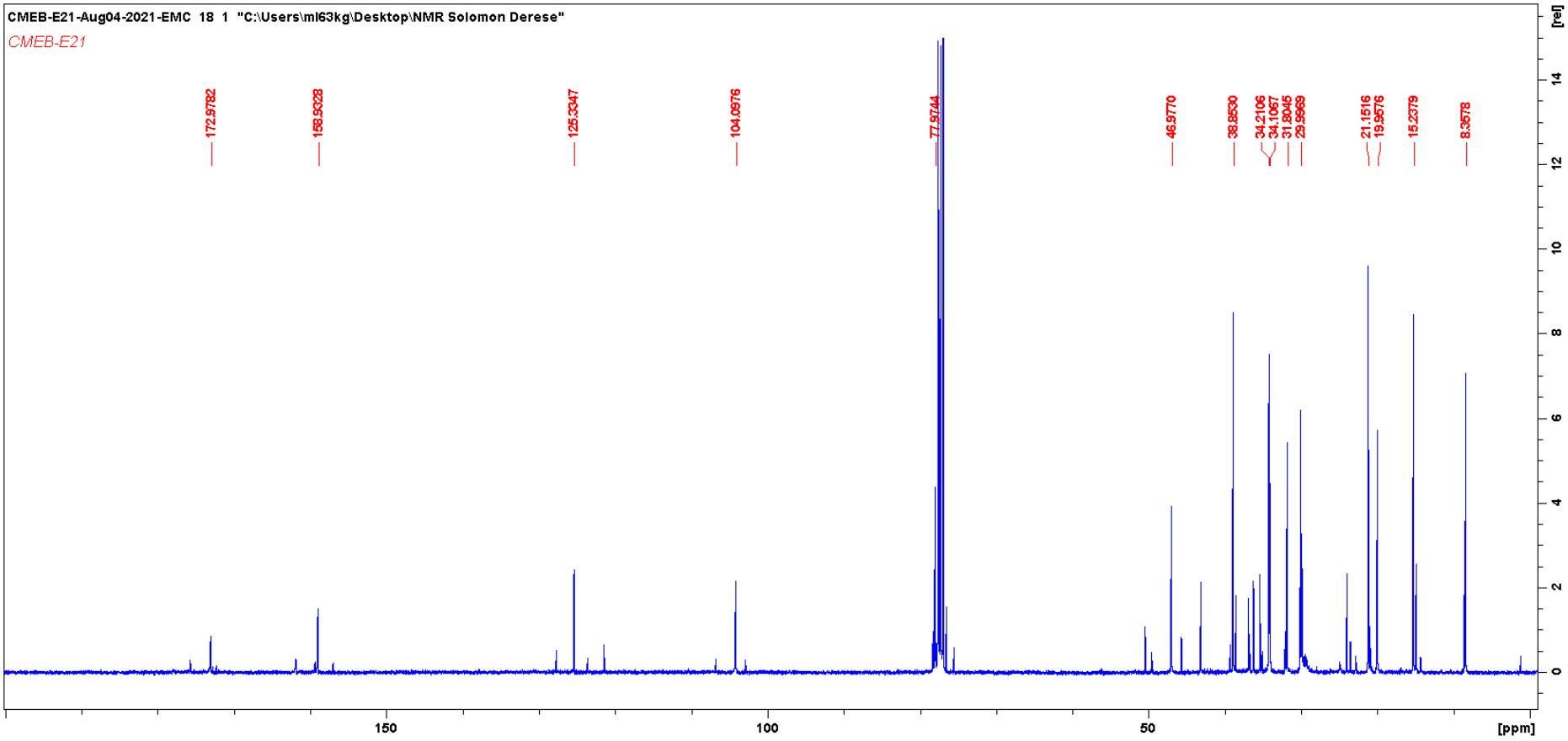



HRESIMS *m/z* 267.1589 $[M+H]^+$ (calcd. for $C_{15}H_{22}O_4$ + H, *m/z* 267.1591)

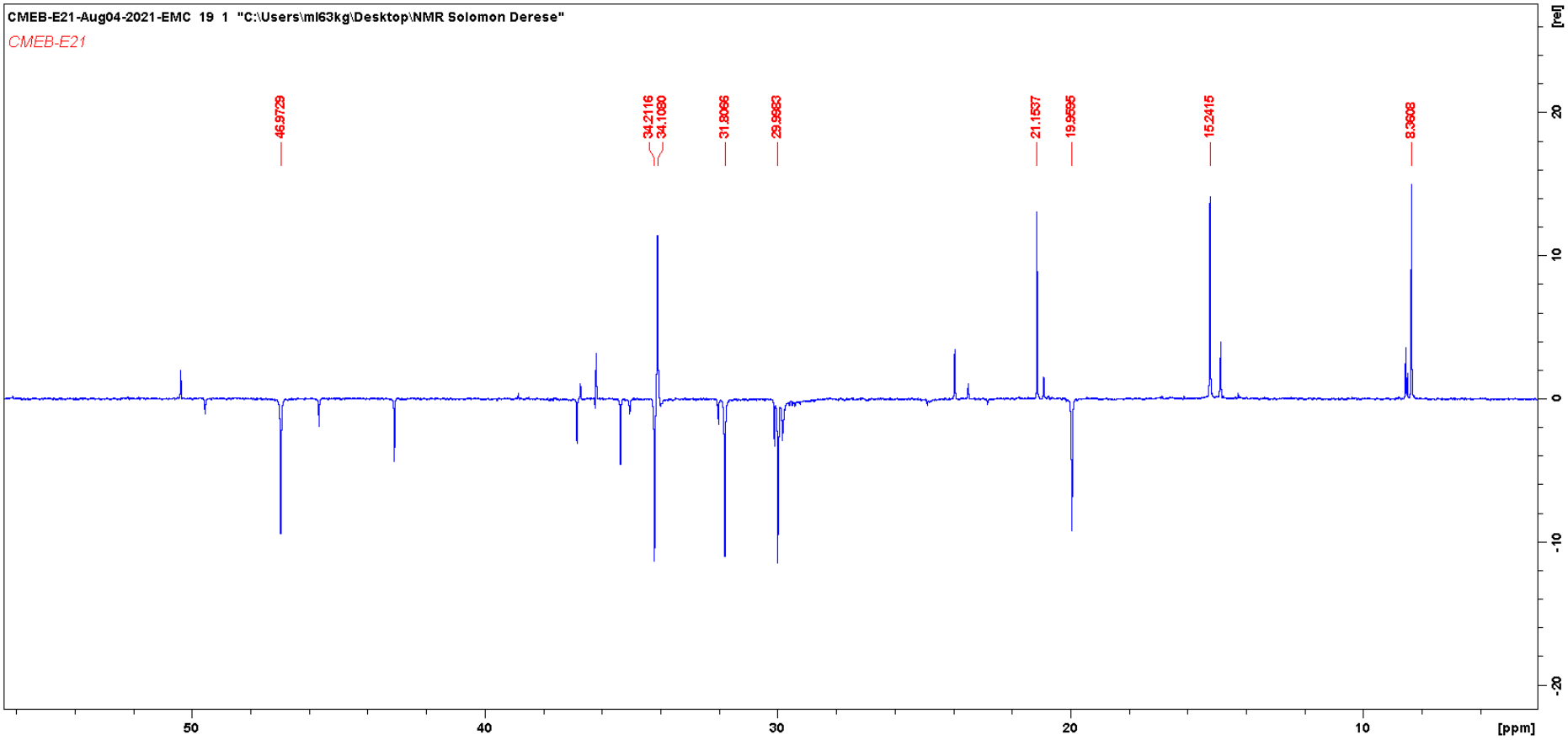
Appendix 10 $^{1}$H NMR spectra of 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)



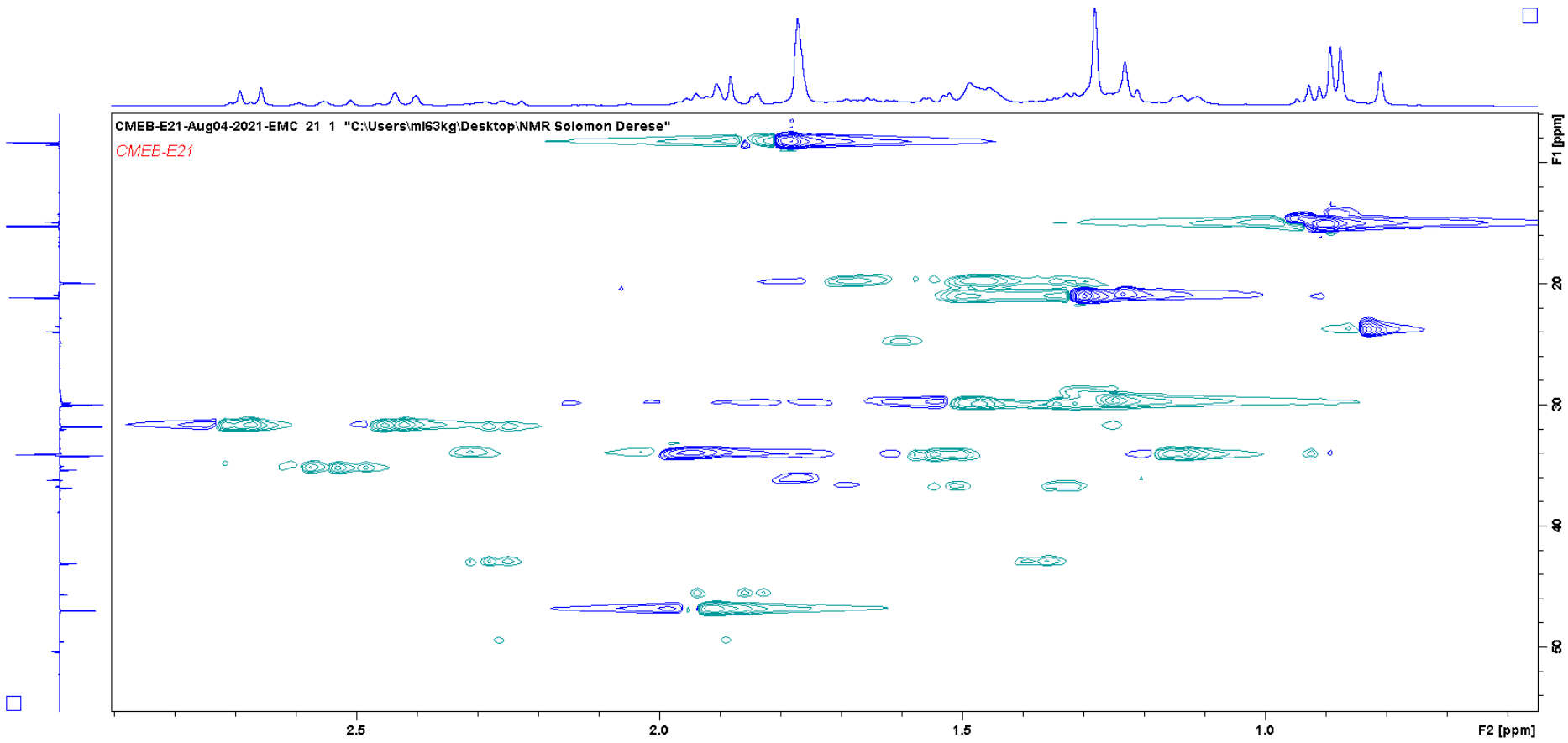
Appendix 11 $^{13}C$ NMR spectra of 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)



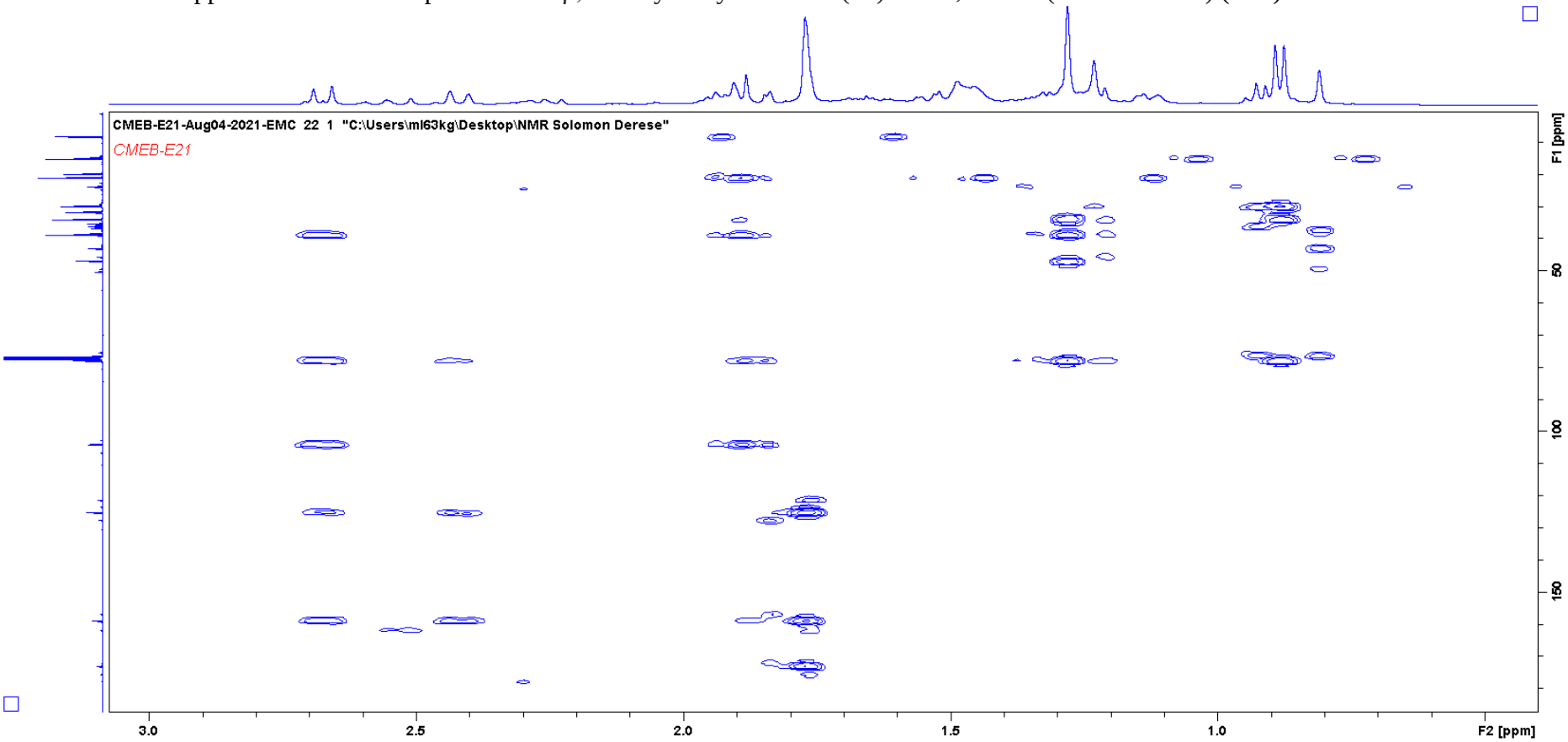
Appendix 12 DEPT spectrum of 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)



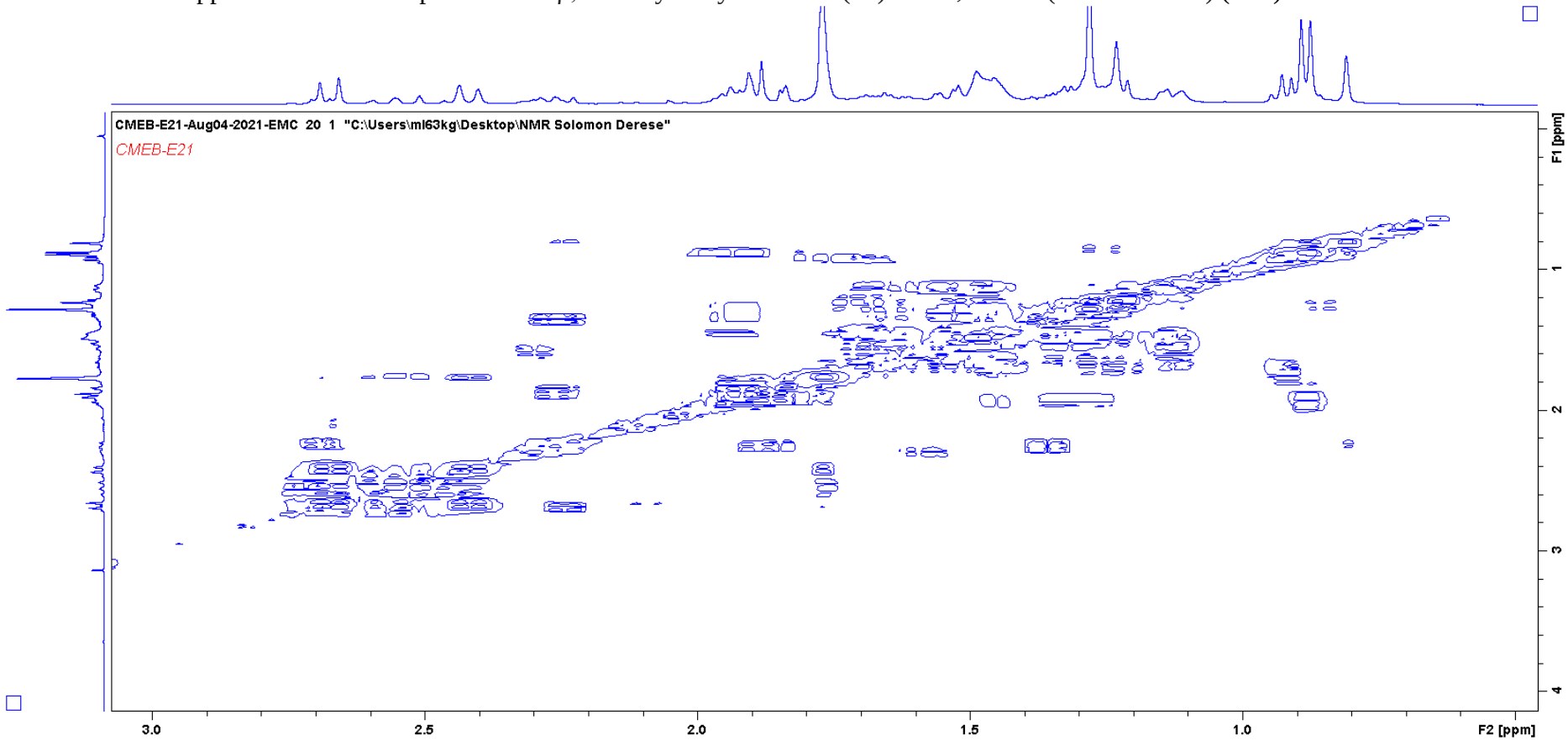
Appendix 13 HSQCDEPT spectrum of 5β, 8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)



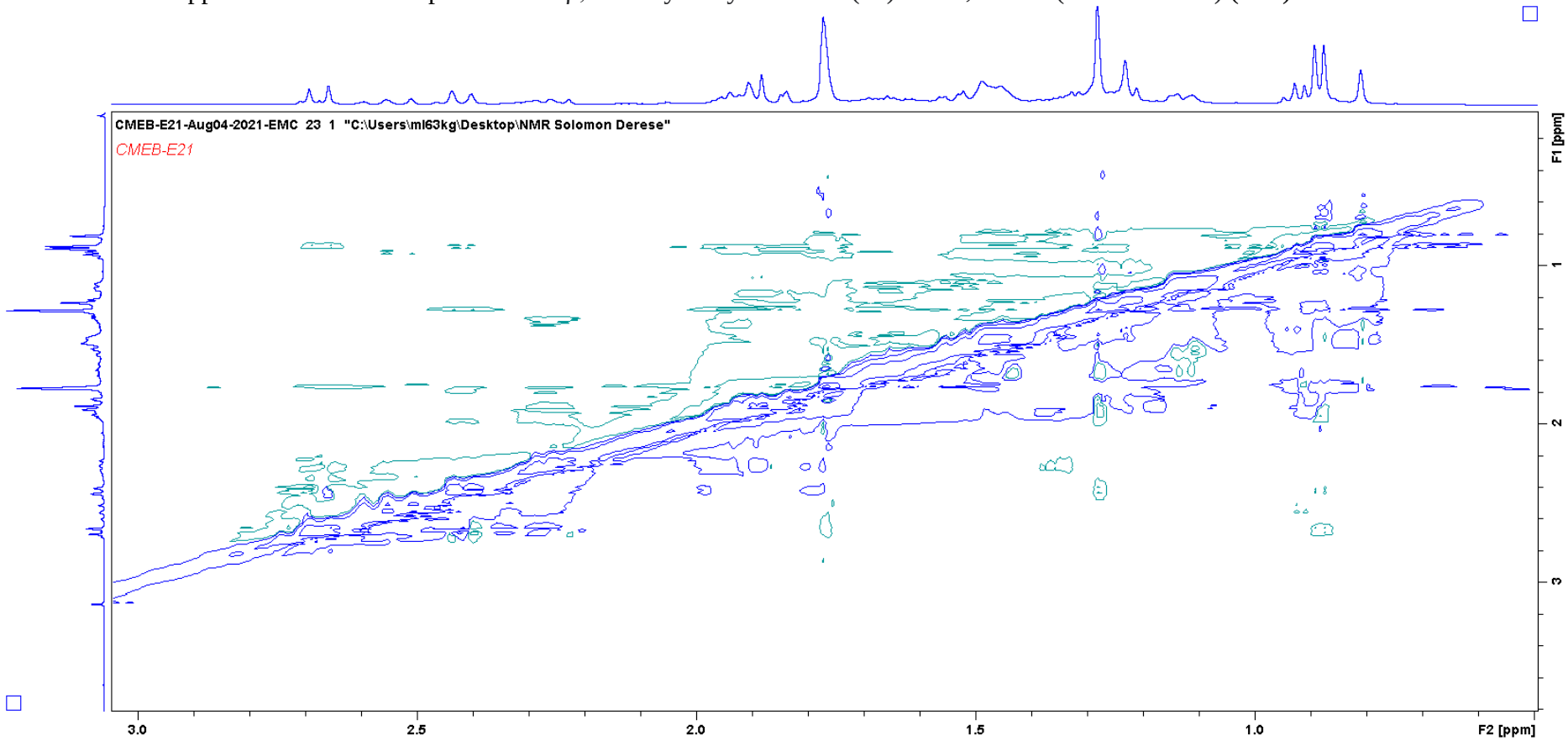
Appendix 14 HMBC spectrum of 5β,8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)



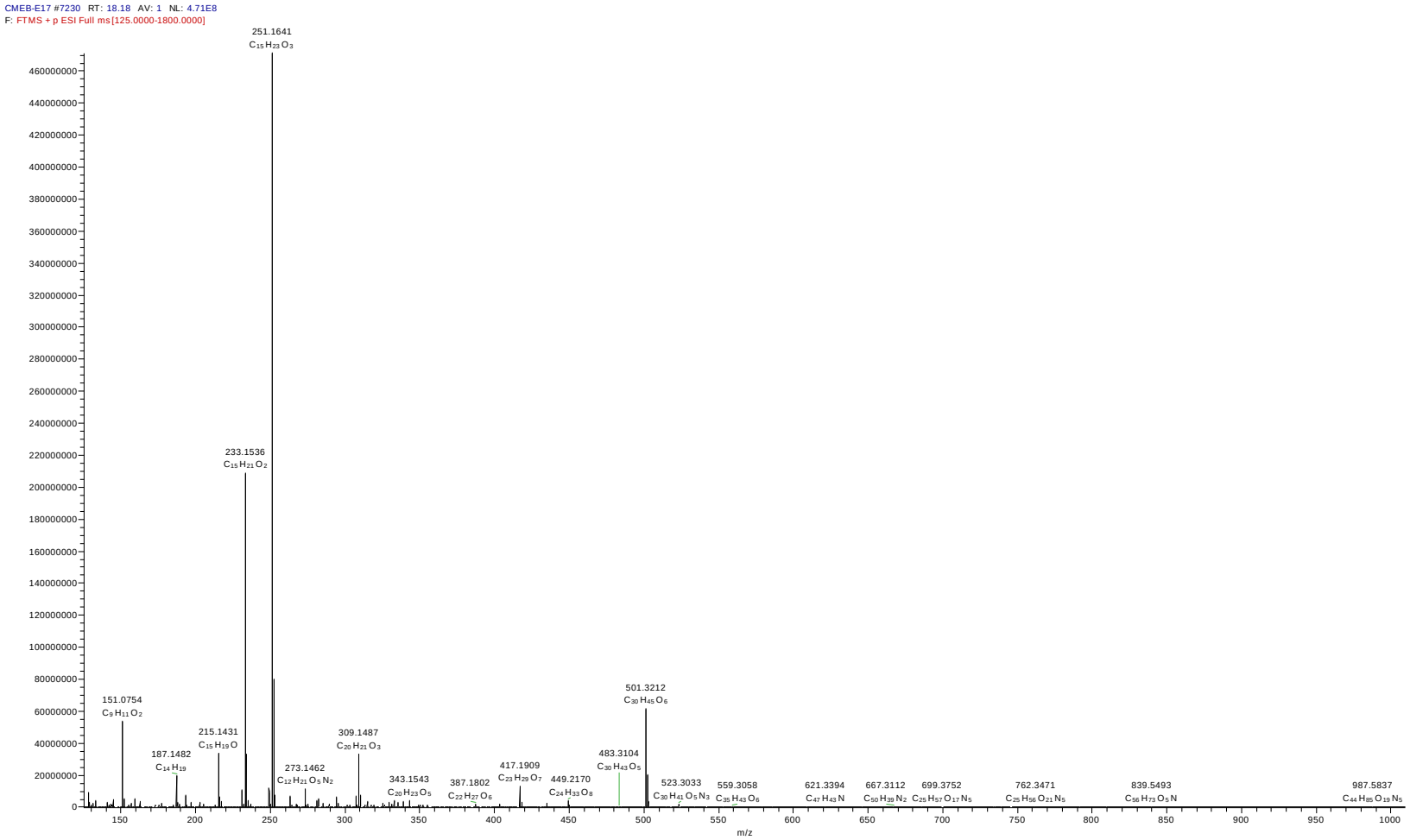
Appendix 15 COSY spectrum of 5β, 8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)



Appendix 16 NOESY spectrum of 5β, 8α-dihydroxy eudesm-7(11)-en-12,8-olide (ermiasolide B) (E22)

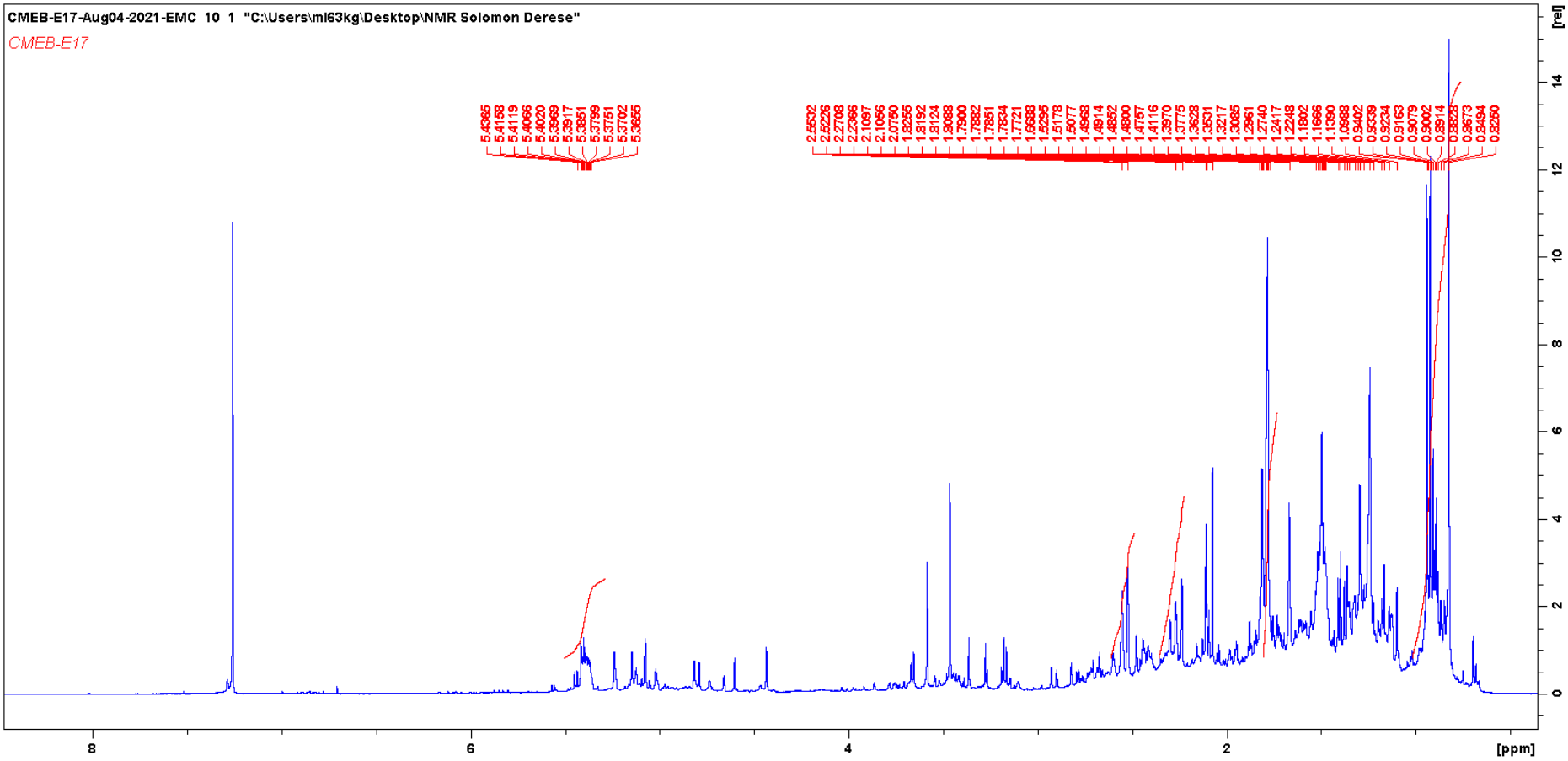


Appendix 17 Mass spectrum of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)

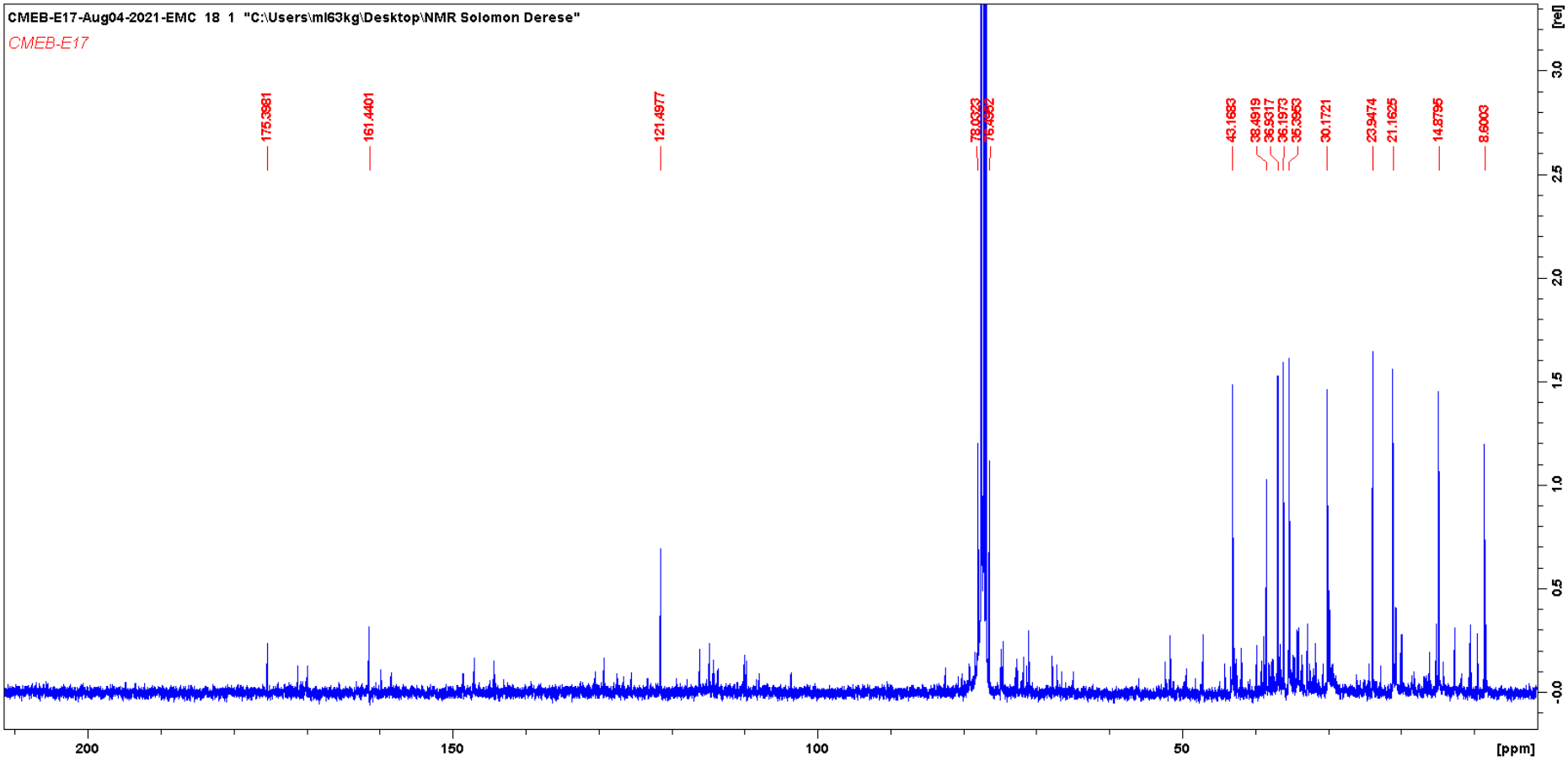



HRESIMS *m/z* 251.1641 $[M+H]^+$ (calcd. for $C_{15}H_{22}O_3$ + H, *m/z* 251.1642)

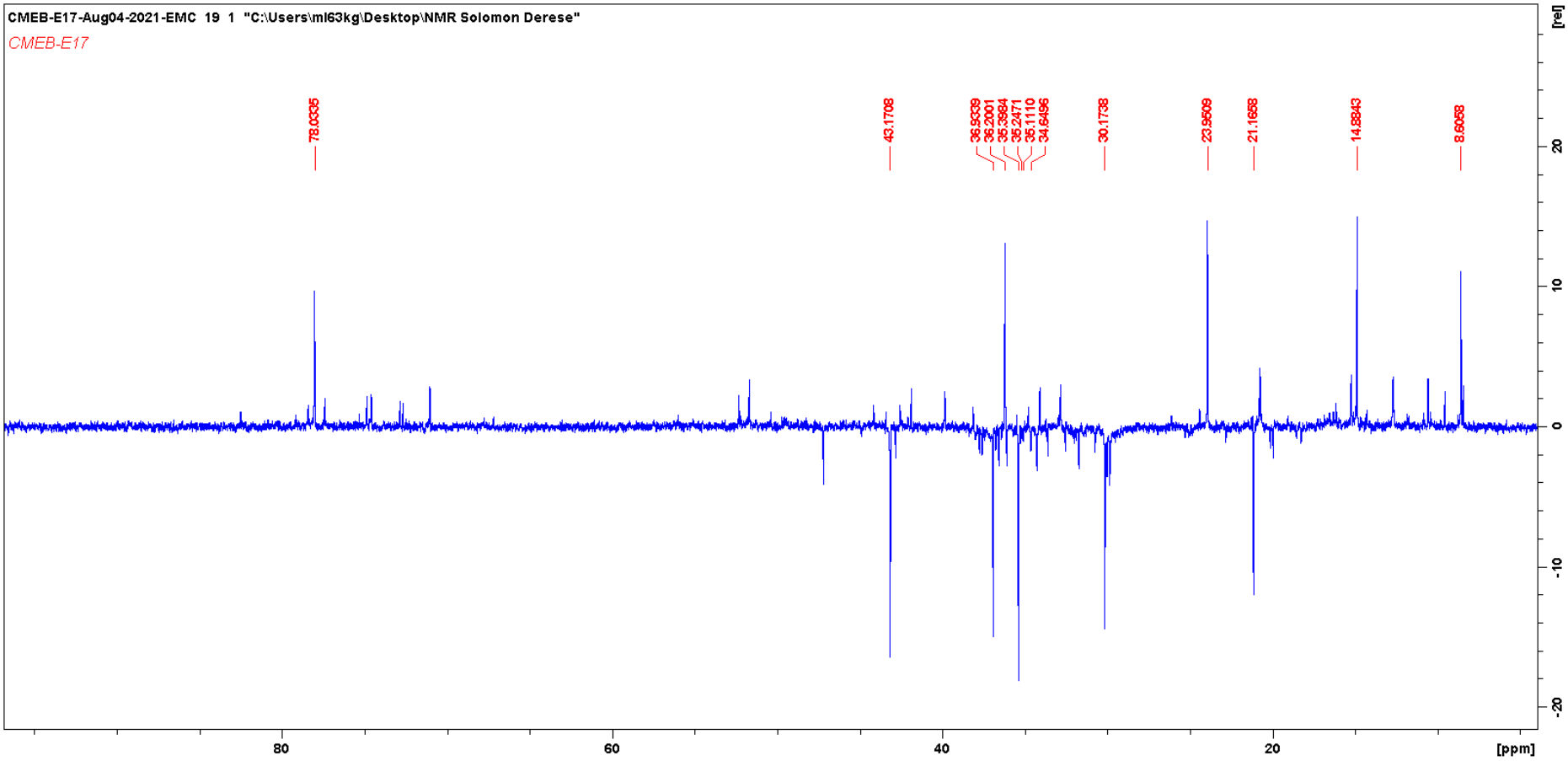
Appendix 18 $^{1}$H NMR spectra of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)



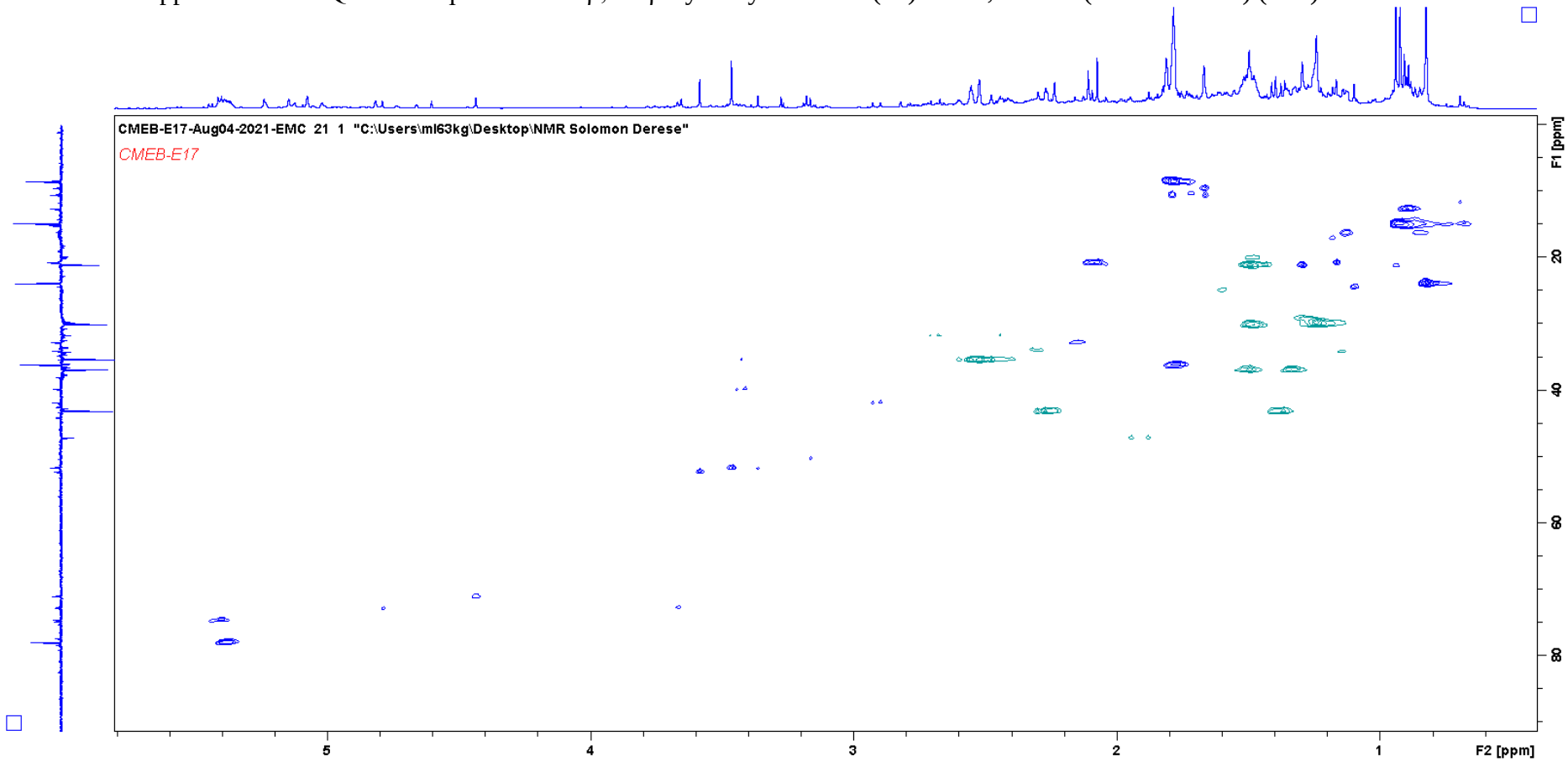
Appendix 19 $^{13}$C NMR spectra of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)



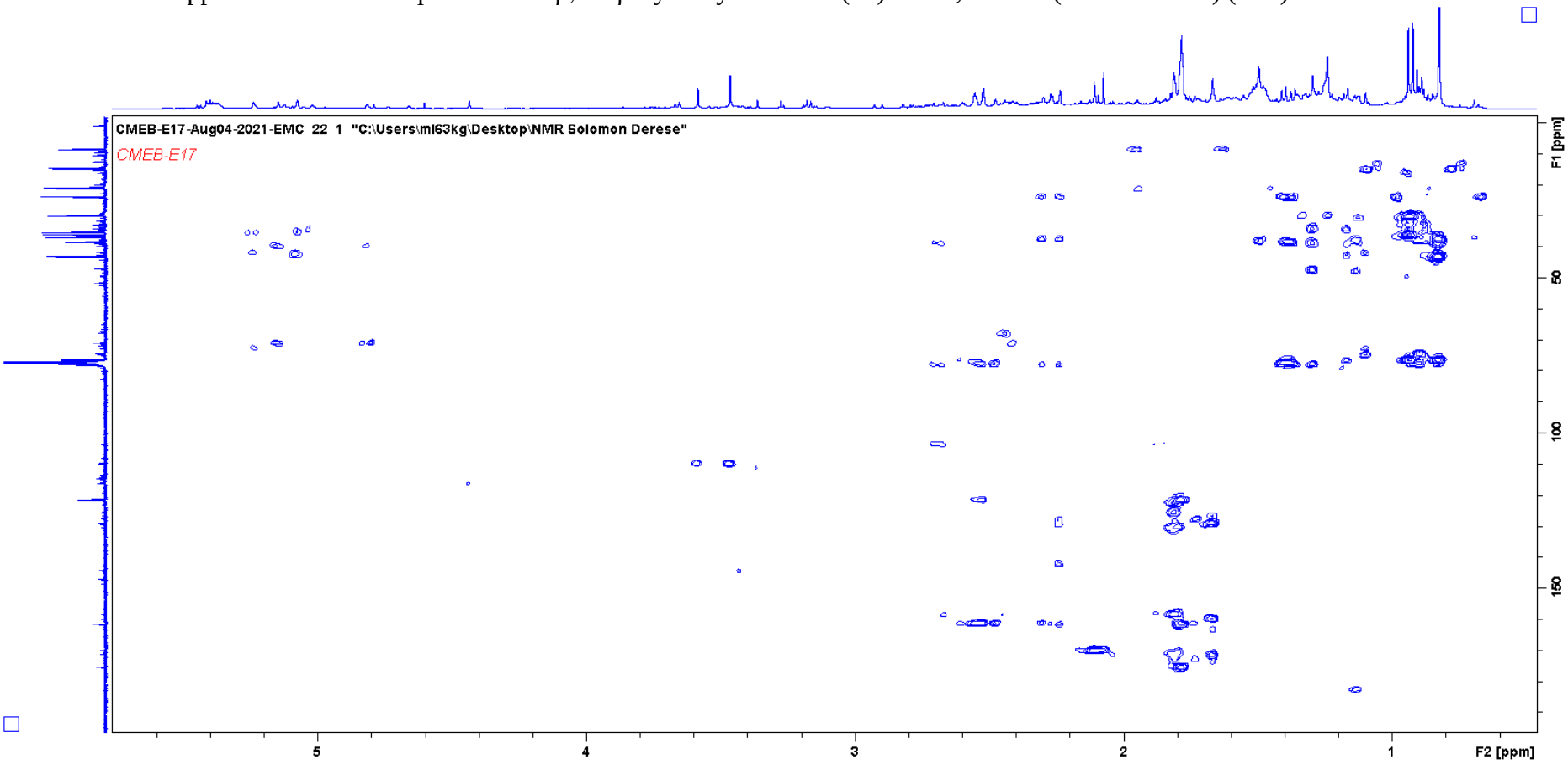
Appendix 20 DEPT spectrum of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)



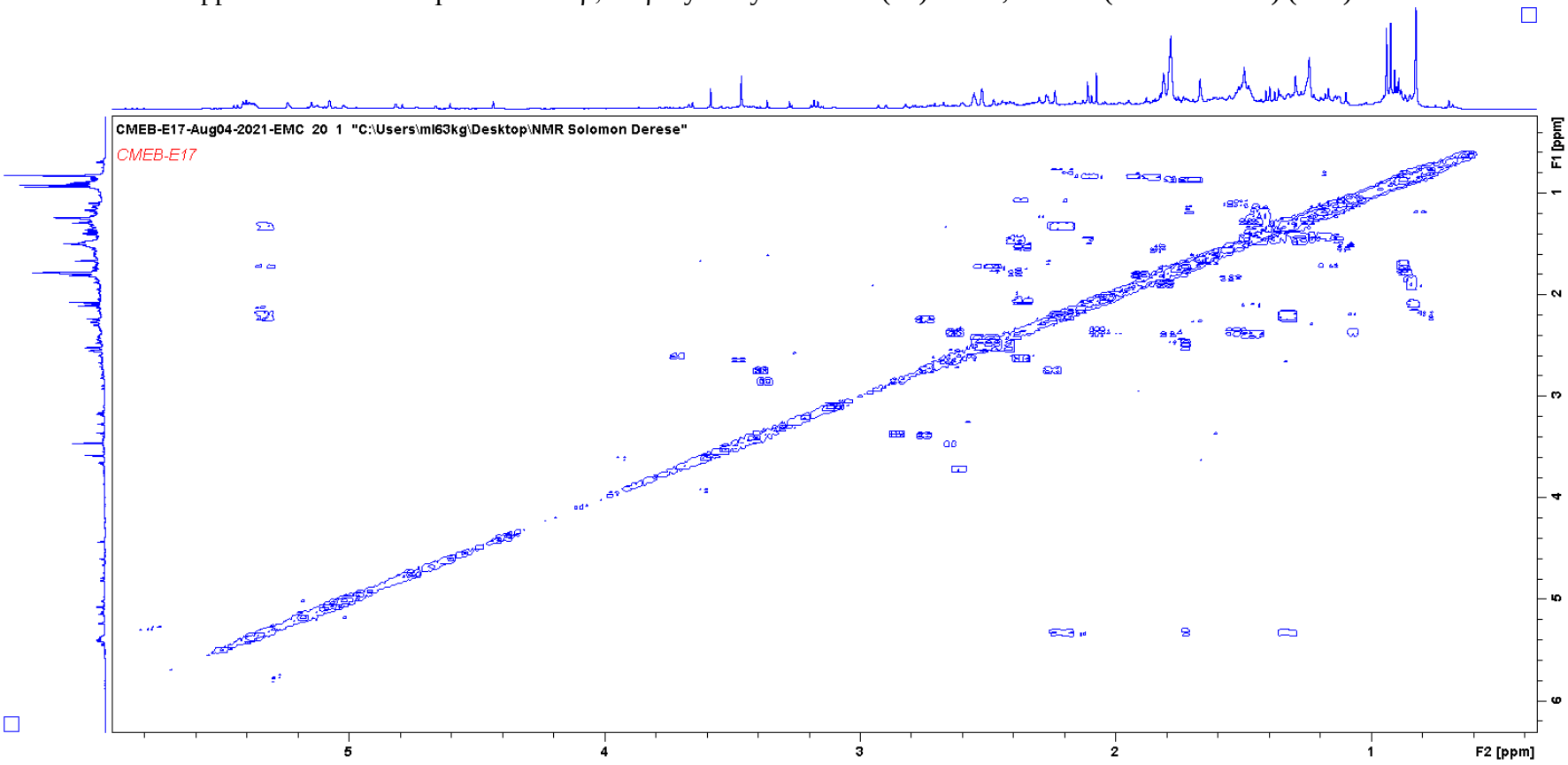
Appendix 21 HSQCDEPT spectrum of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)



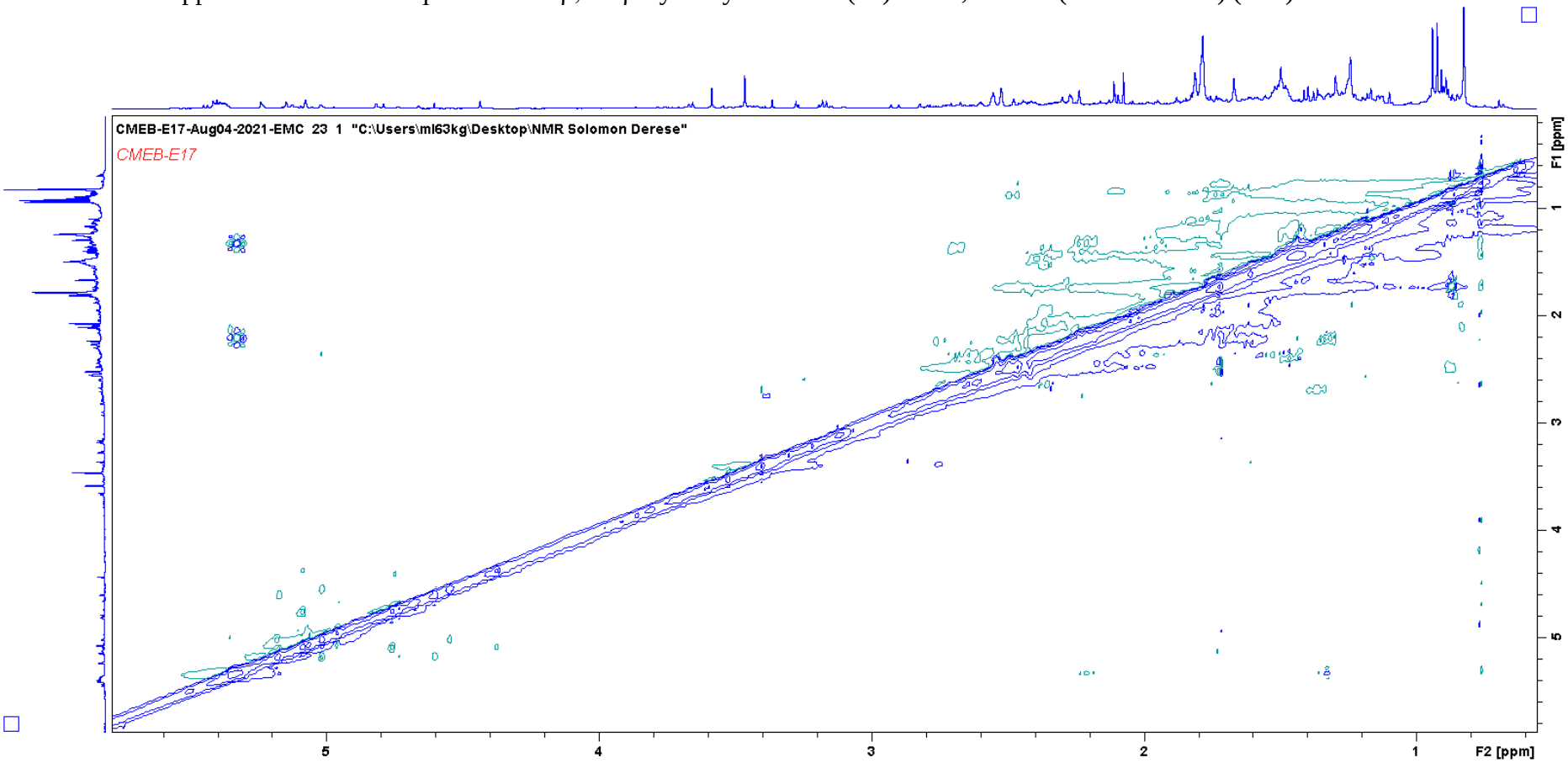
Appendix 22 HMBC spectrum of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)



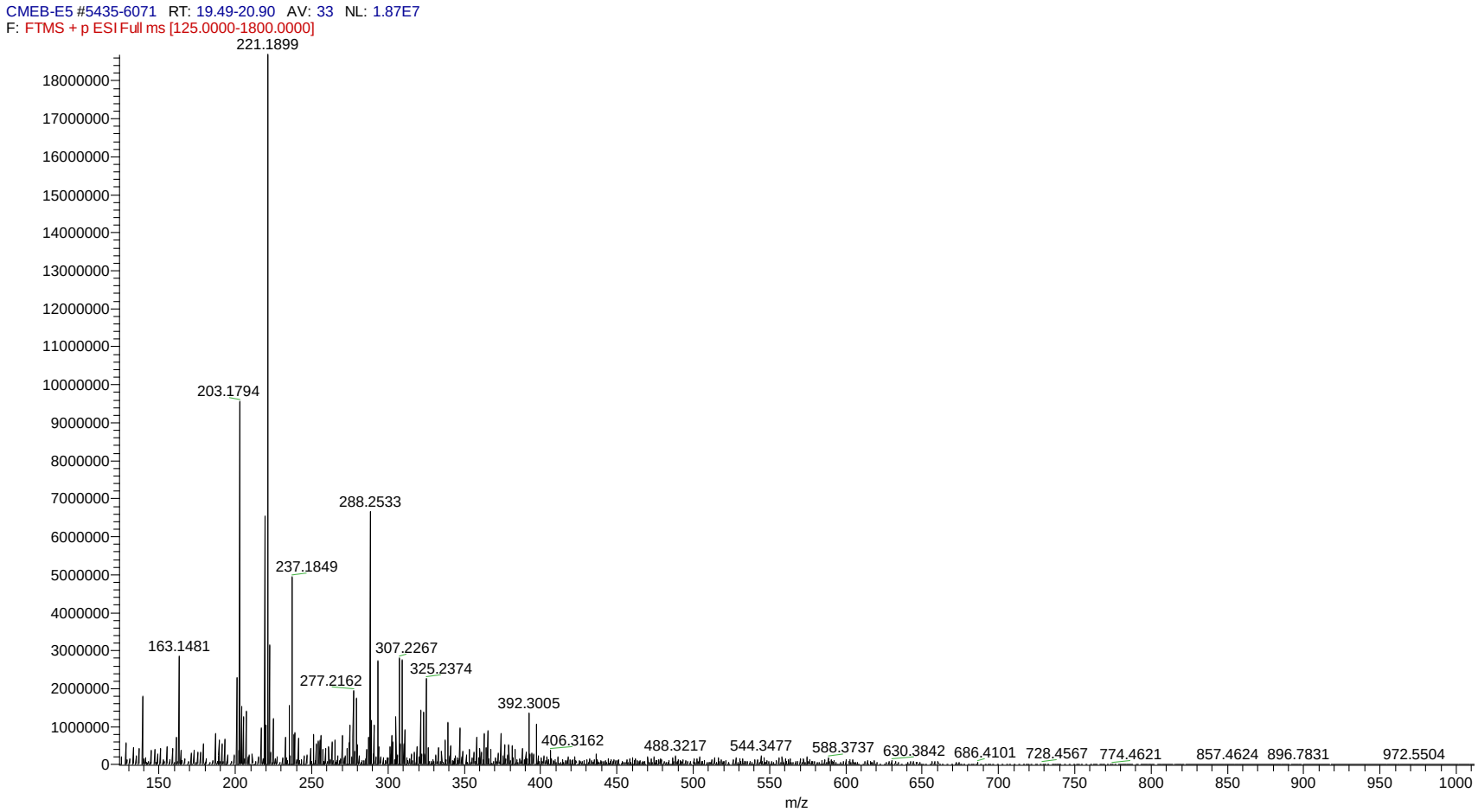
Appendix 23 COSY spectrum of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)



Appendix 24 NOESY spectrum of 5β,8H-β-hydroxy eudesm-7(11)-en-12, 8-olide (ermiasolide C) (E17)

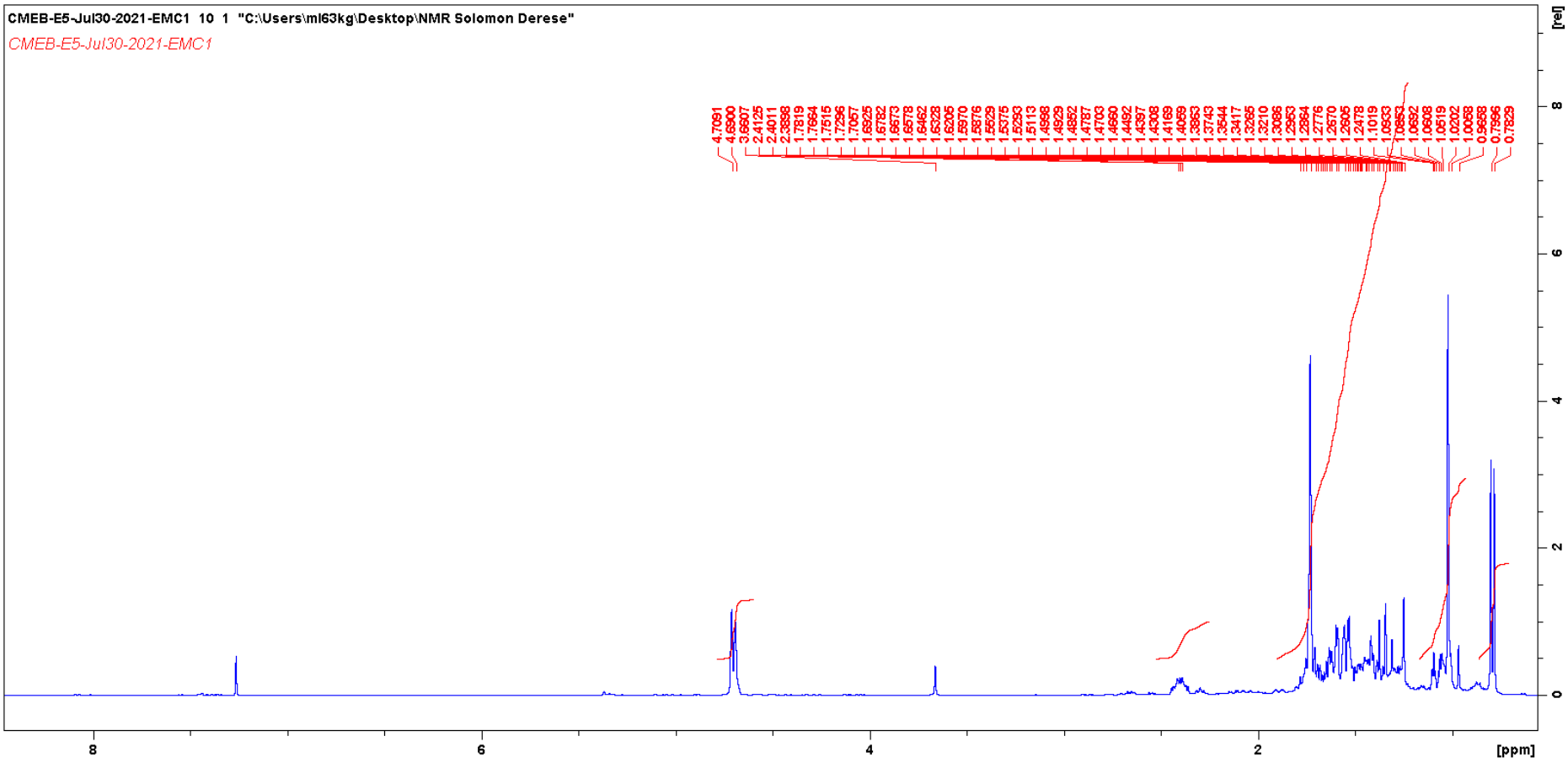


Appendix 25 Mass spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)

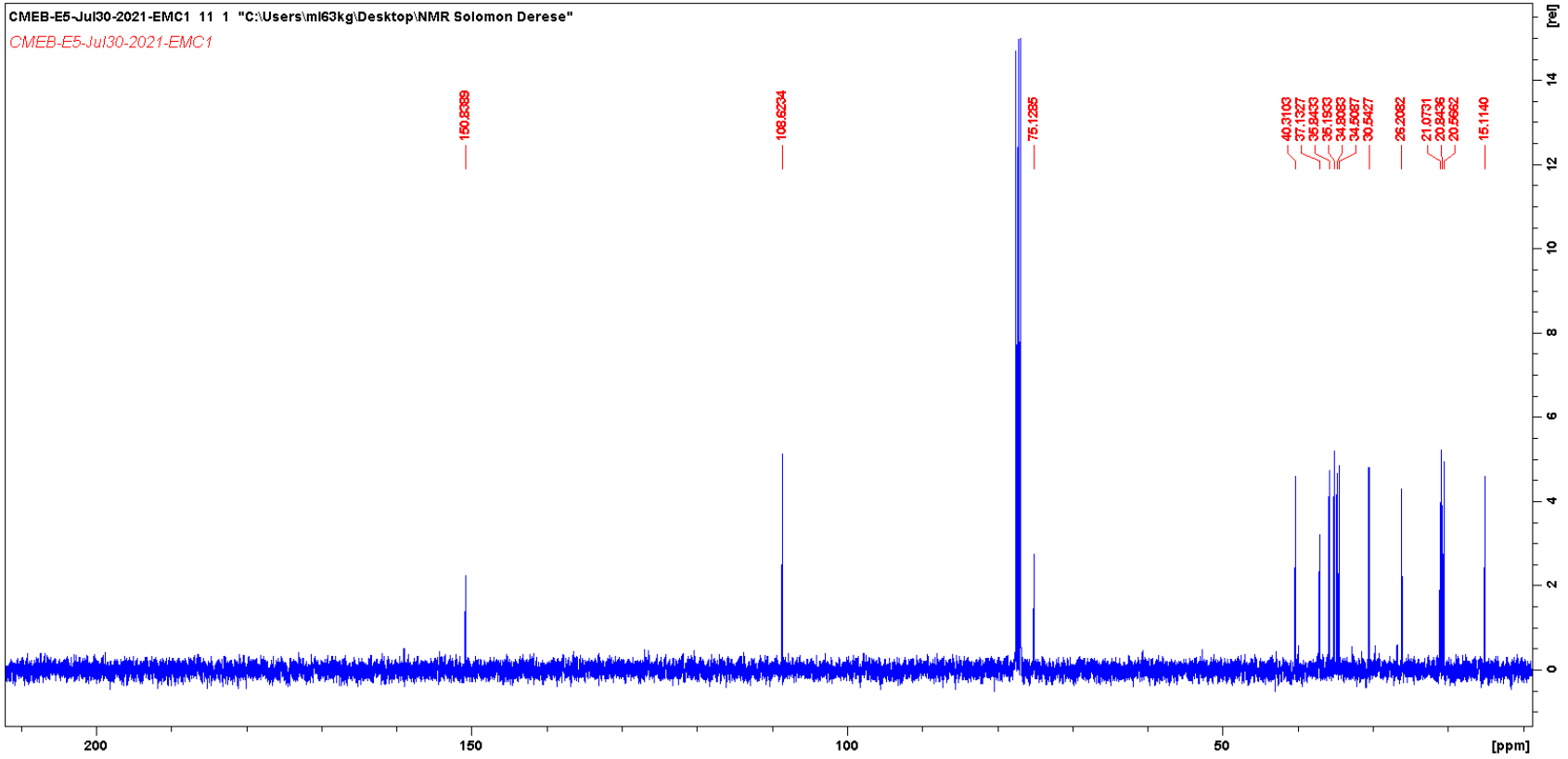



HRESIMS *m/z* 221.1899 $[M+H]^+$ (calcd. for $C_{15}H_{24}O$ + H, *m/z* 221.1900)

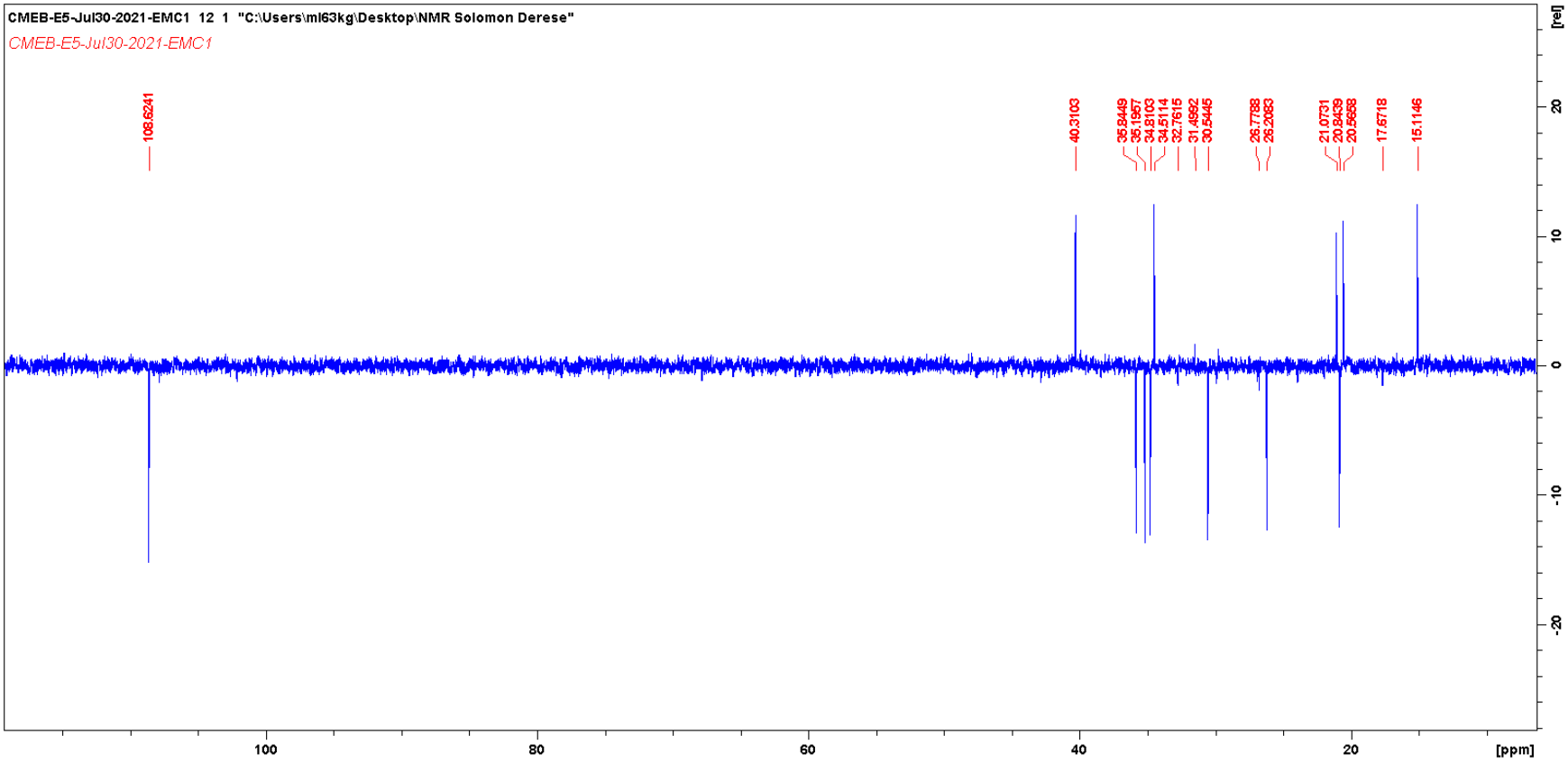
Appendix 26 $^{1}$H NMR spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)



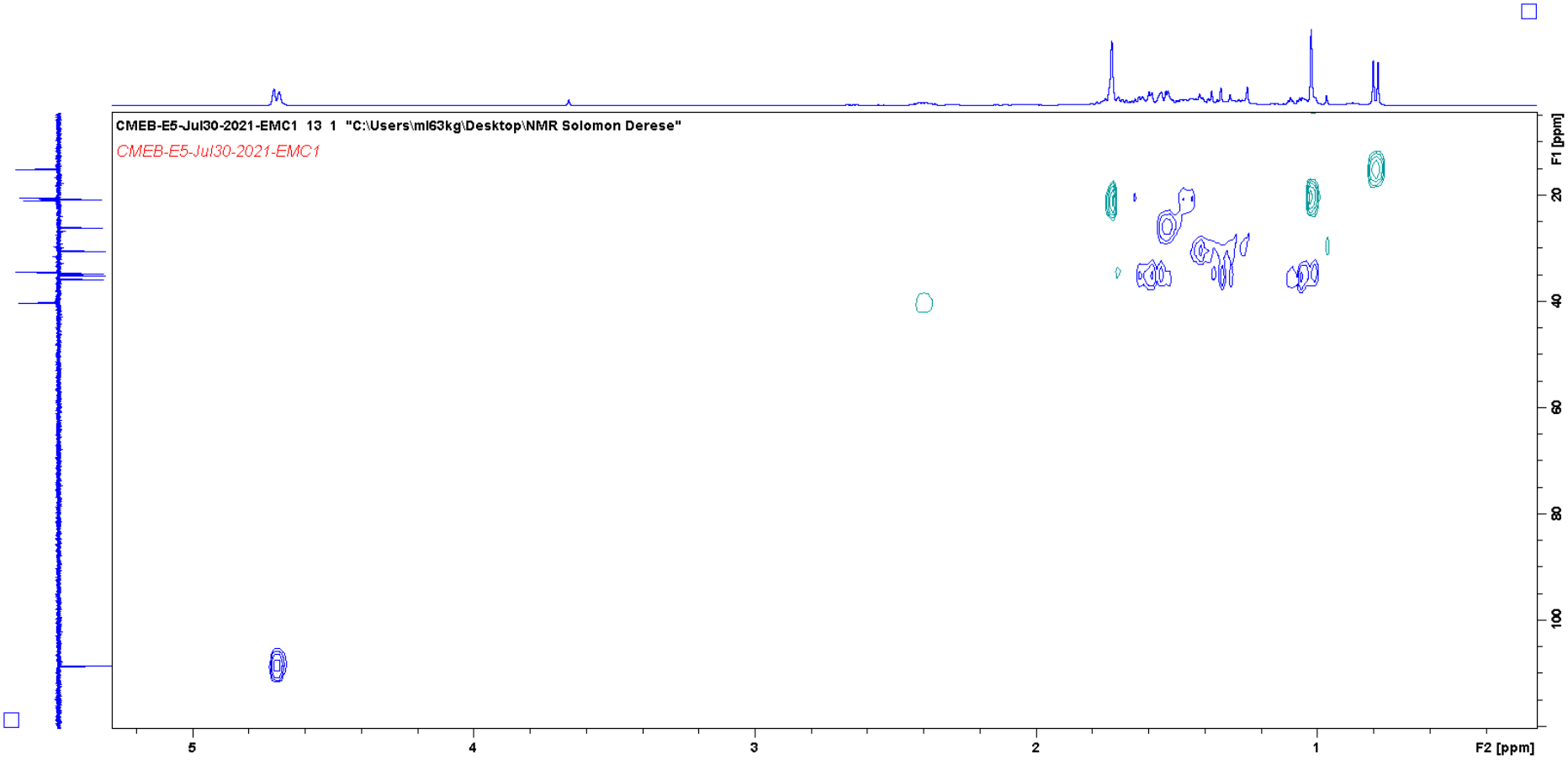
Appendix 27 $^{13}$C NMR spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)



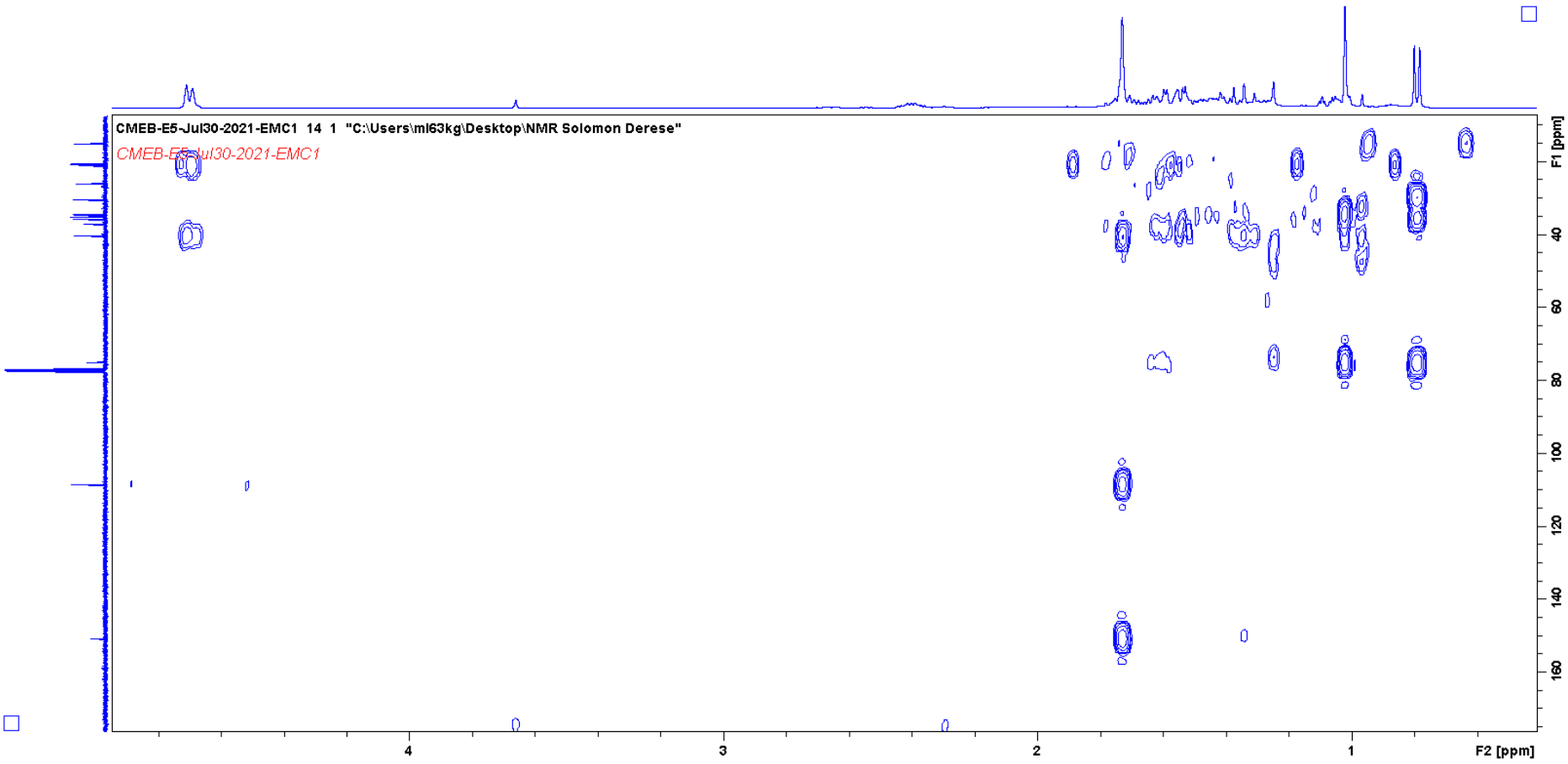
Appendix 28 DEPT spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)



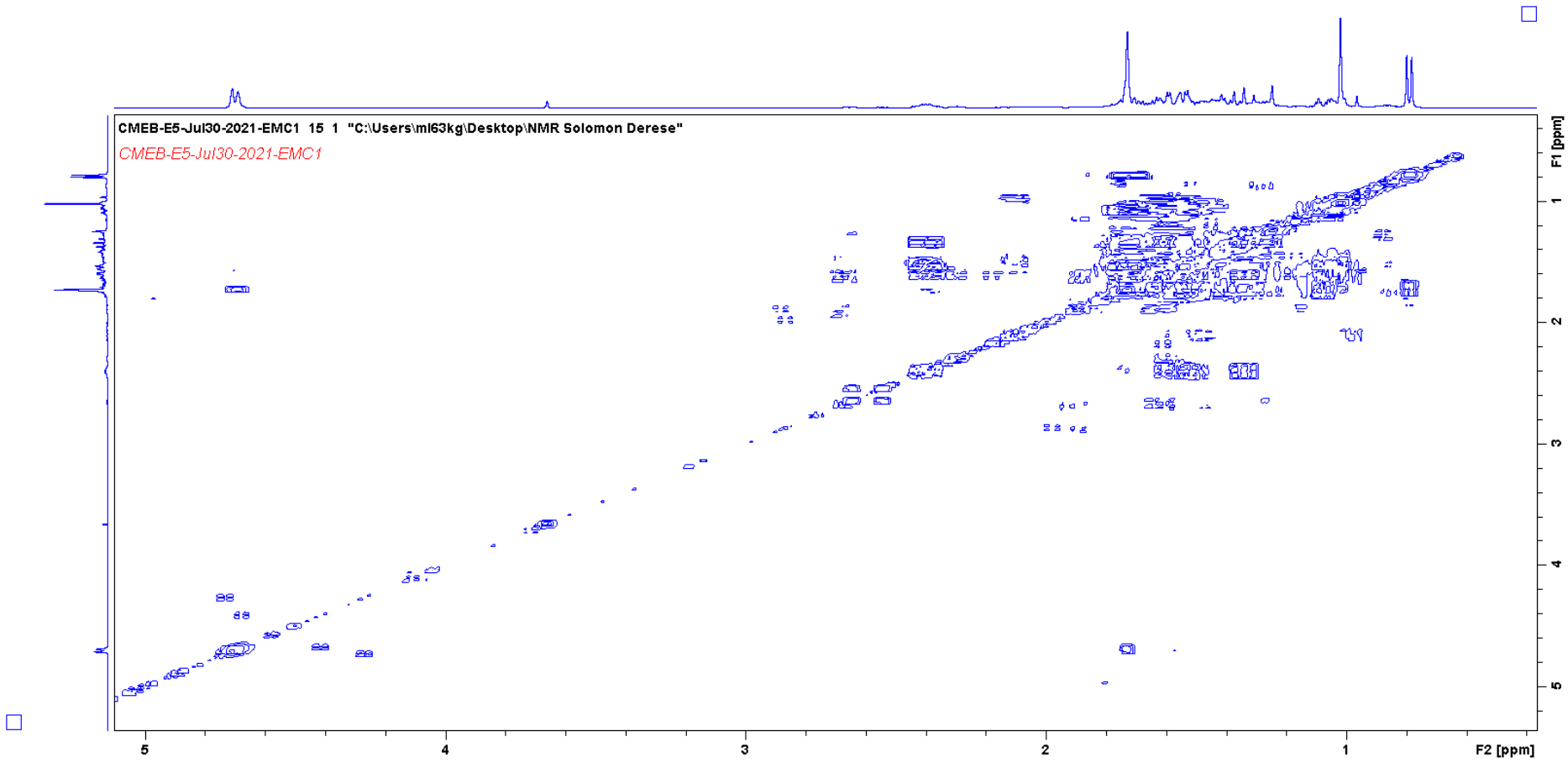
Appendix 29 HSQCDEPT spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)



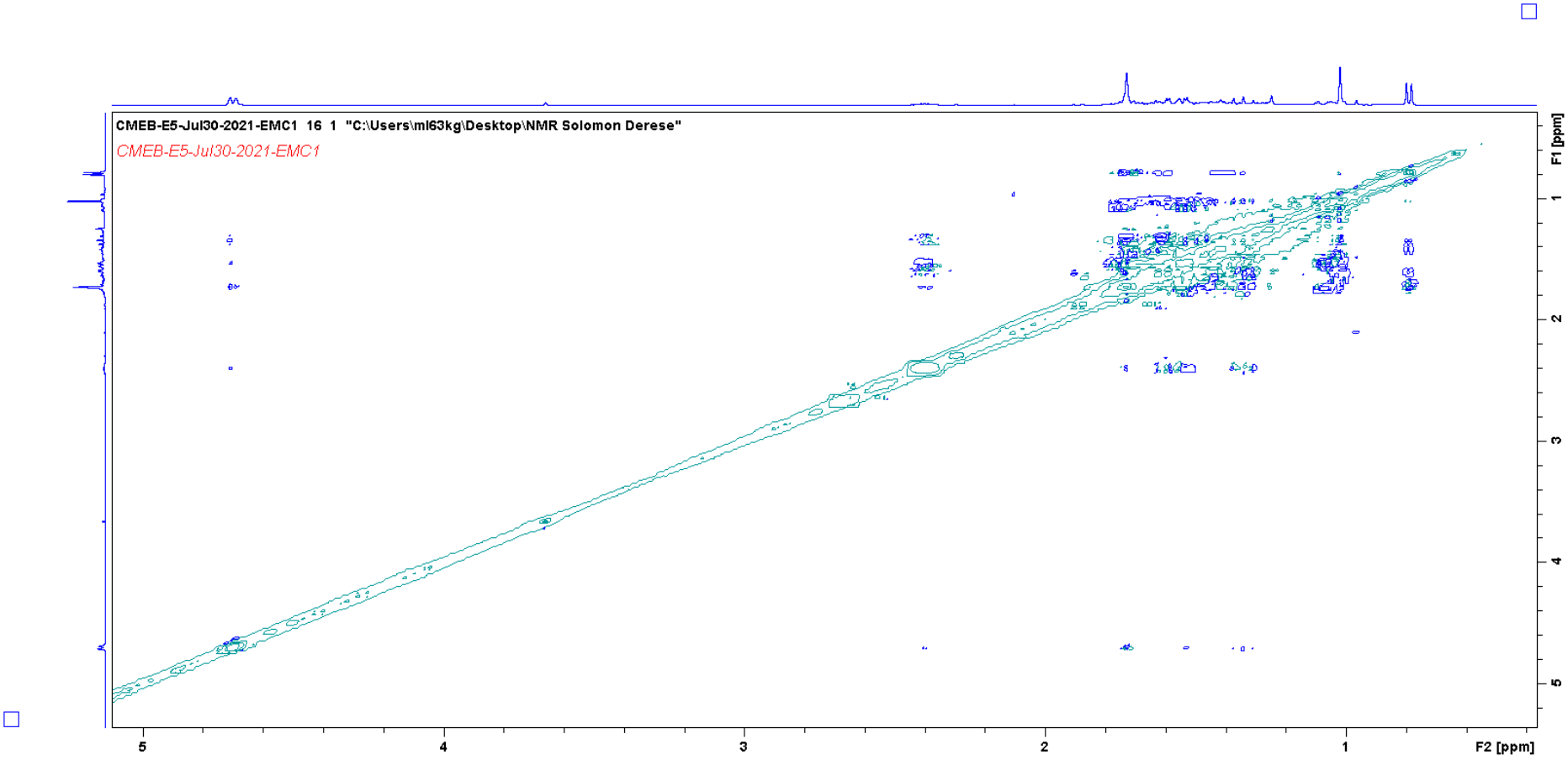
Appendix 30 HMBC spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)



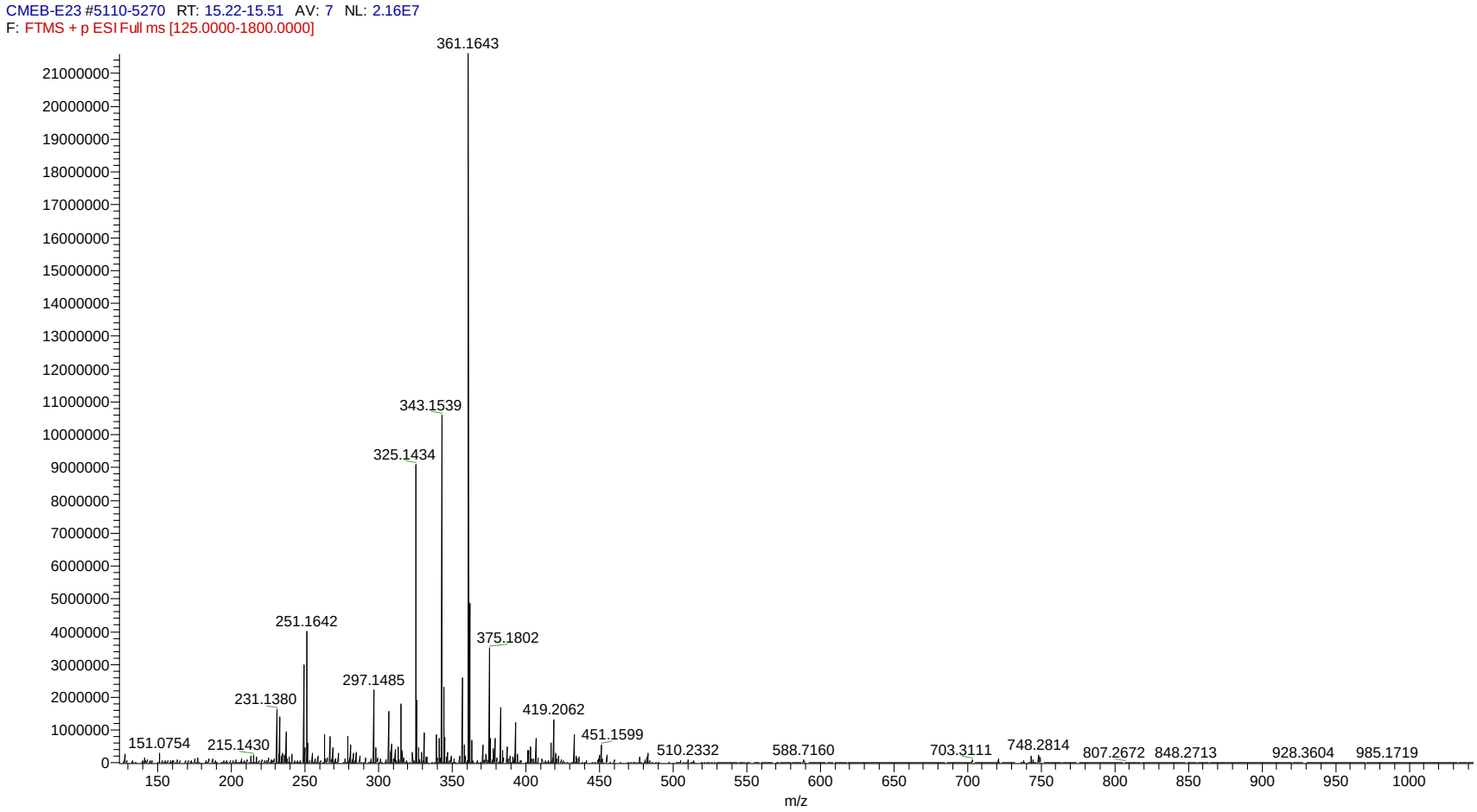
Appendix 31 COSY spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)



Appendix 32 NOSEY spectrum of 4H-α,7H-α,10α-eudesm-11-en-5β-ol (E5)

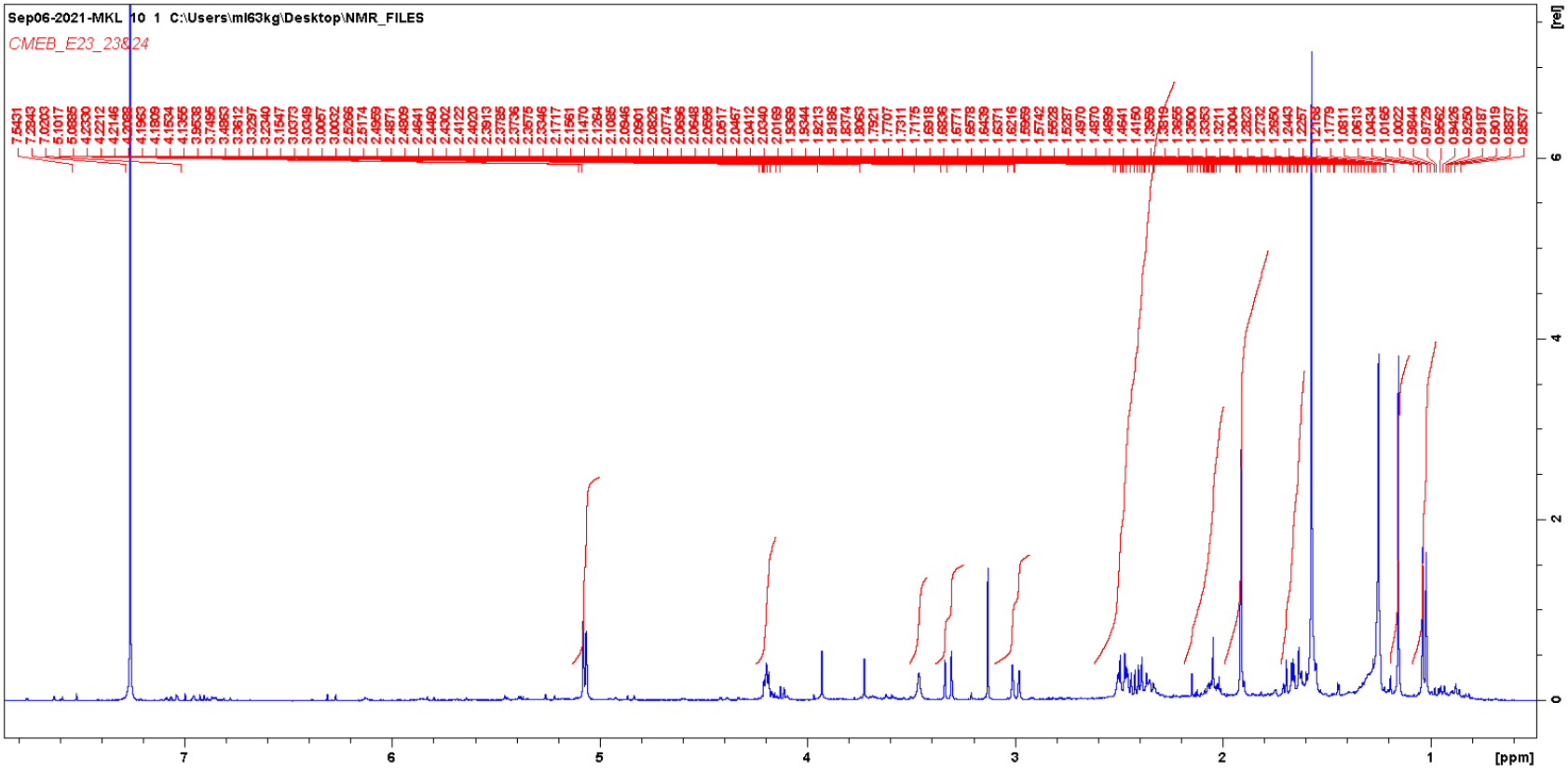


Appendix 33 Mass spectrum of Crotocascarin K (E23)

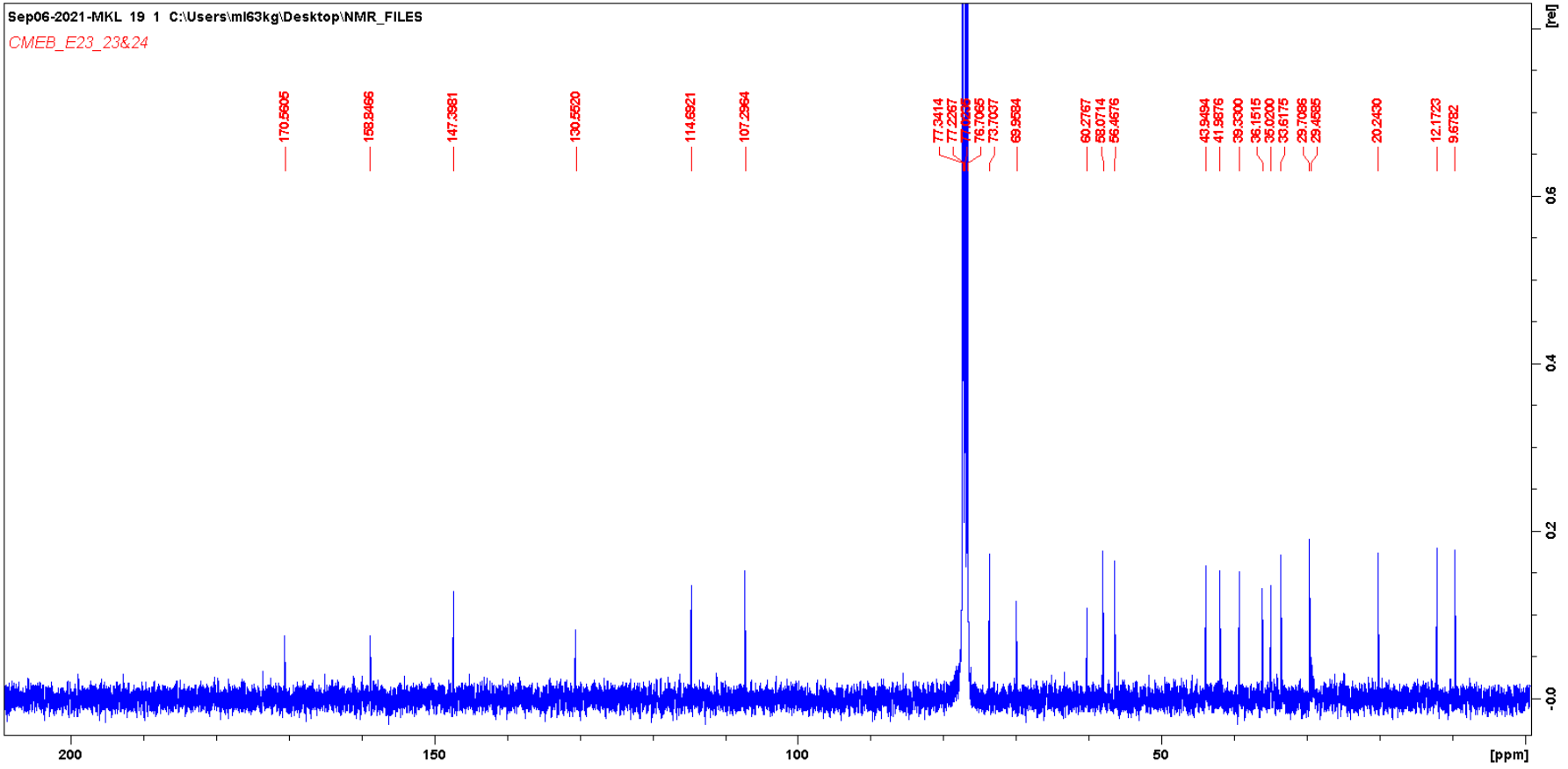



HRESIMS *m/z* 361.1643 $[M+H]^+$ (calcd. for $C_{20}H_{24}O_6$ + H, *m/z* 361.1646)

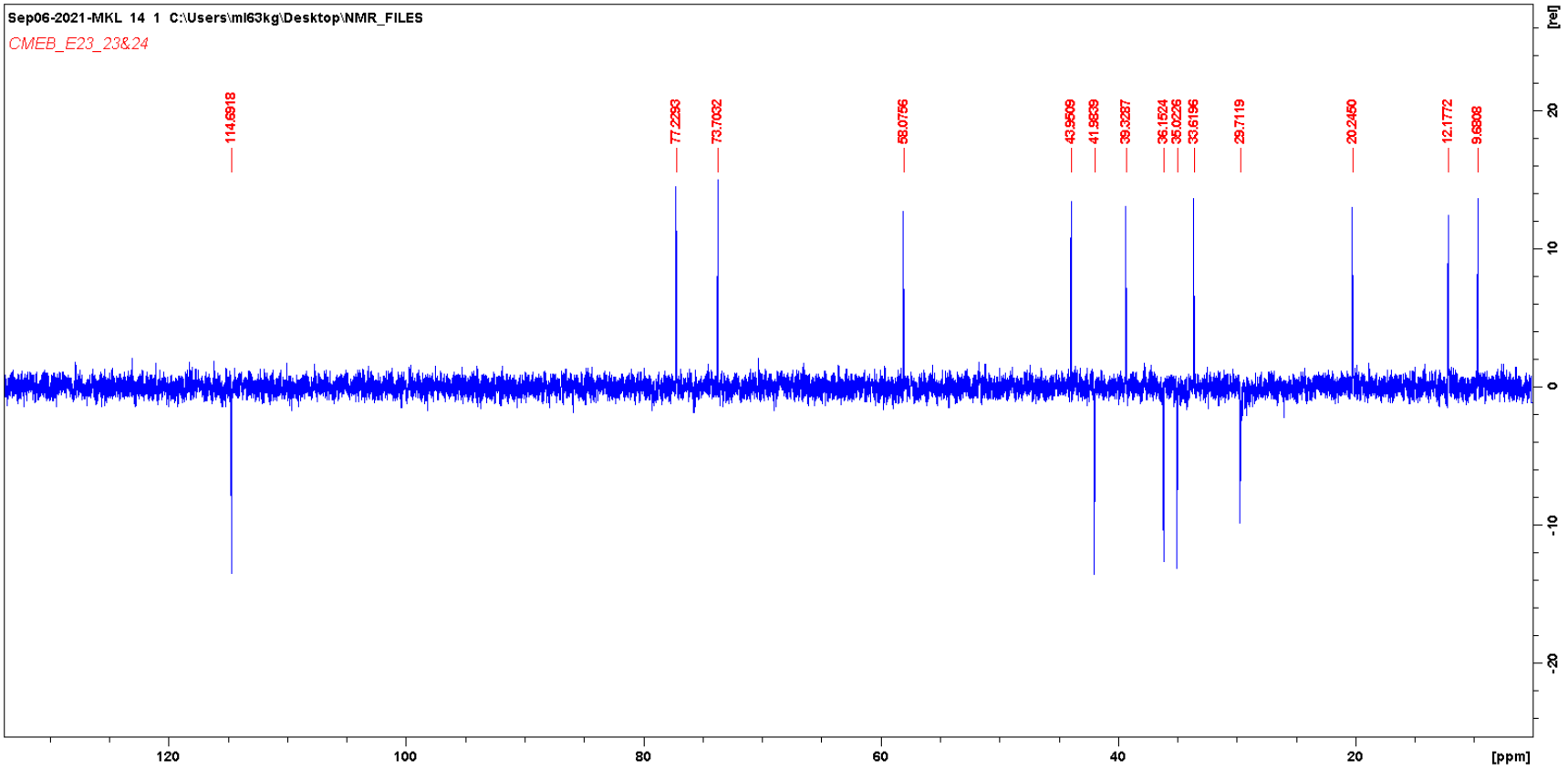
Appendix 34 $^{1}$H NMR spectrum of Crotocascarin K (E23)



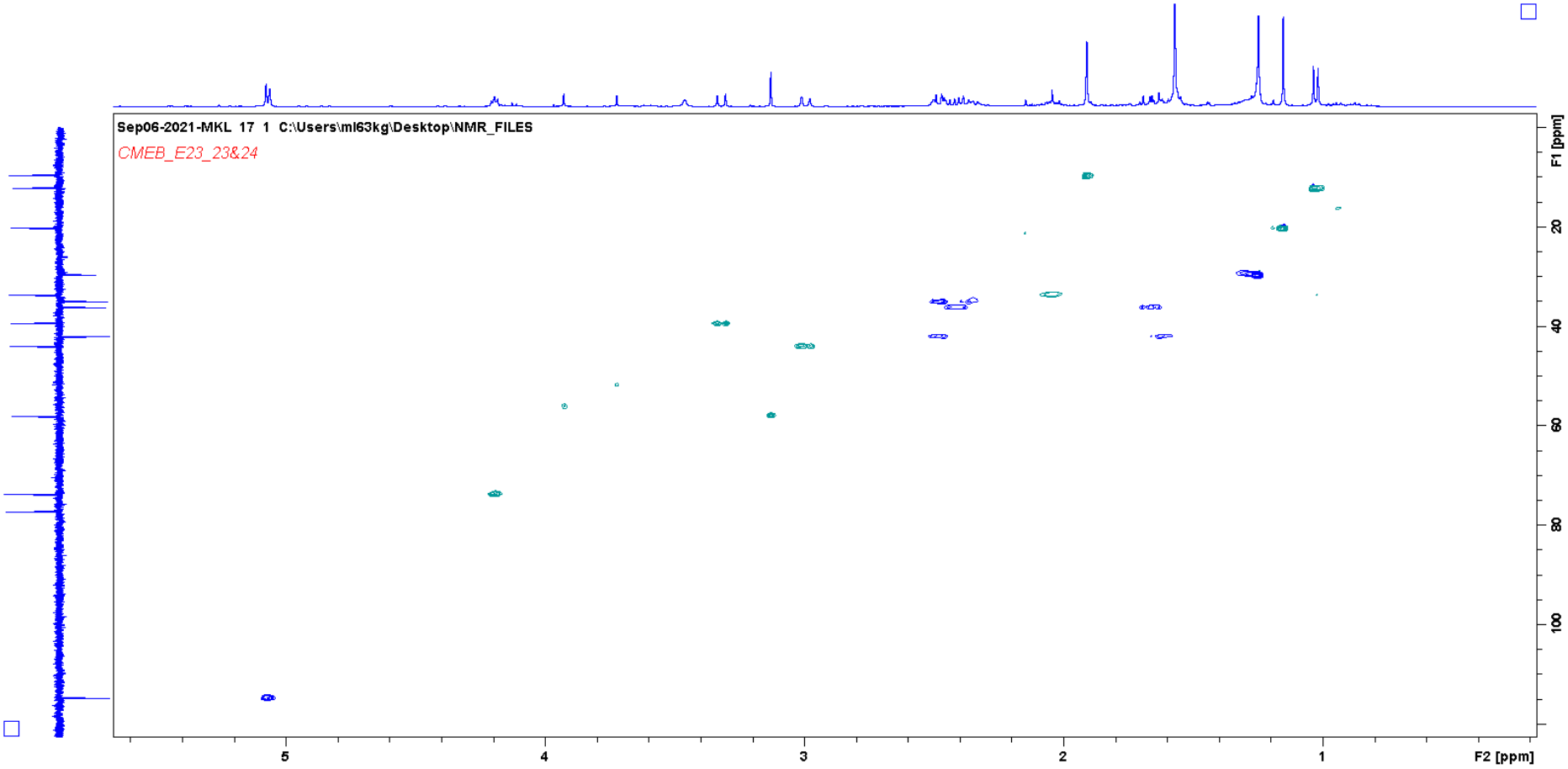
Appendix 35 $^{13}$C NMR spectrum of Crotocascarin K (E23)



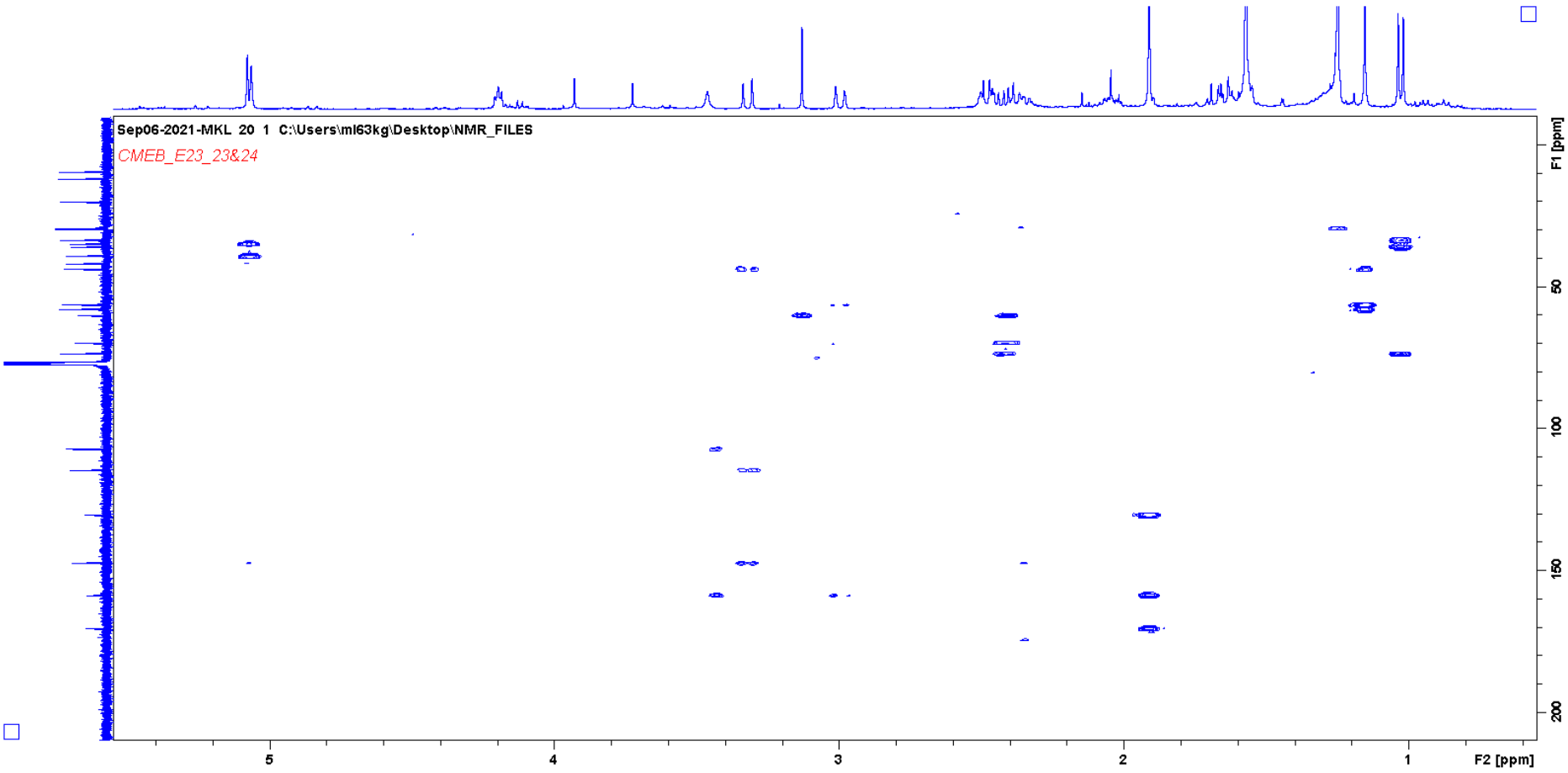
Appendix 36 DEPT spectrum of Crotocascarin K (E23)



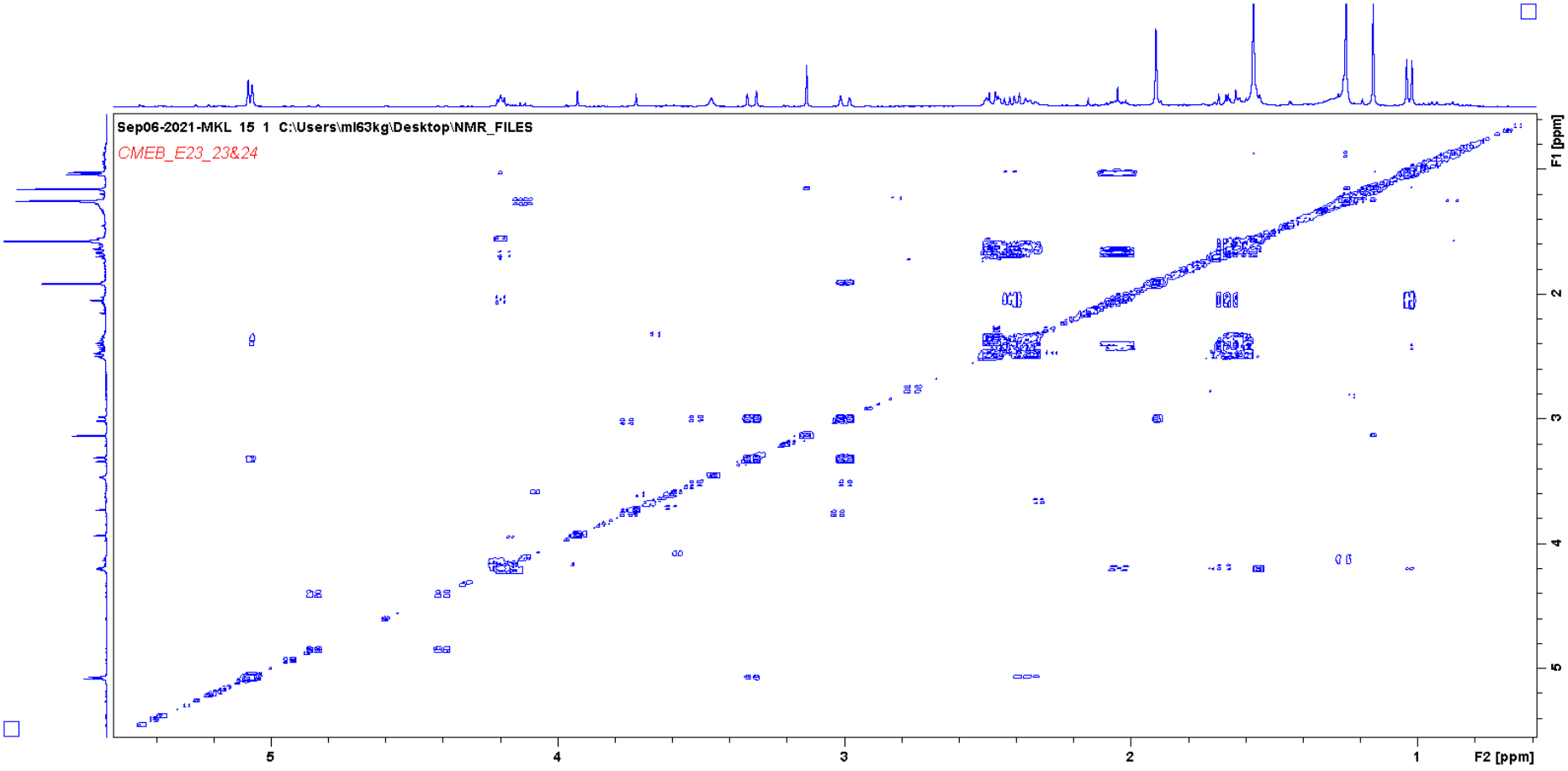
Appendix 37 HSQCDEPT spectrum of Crotocascarin K (E23)



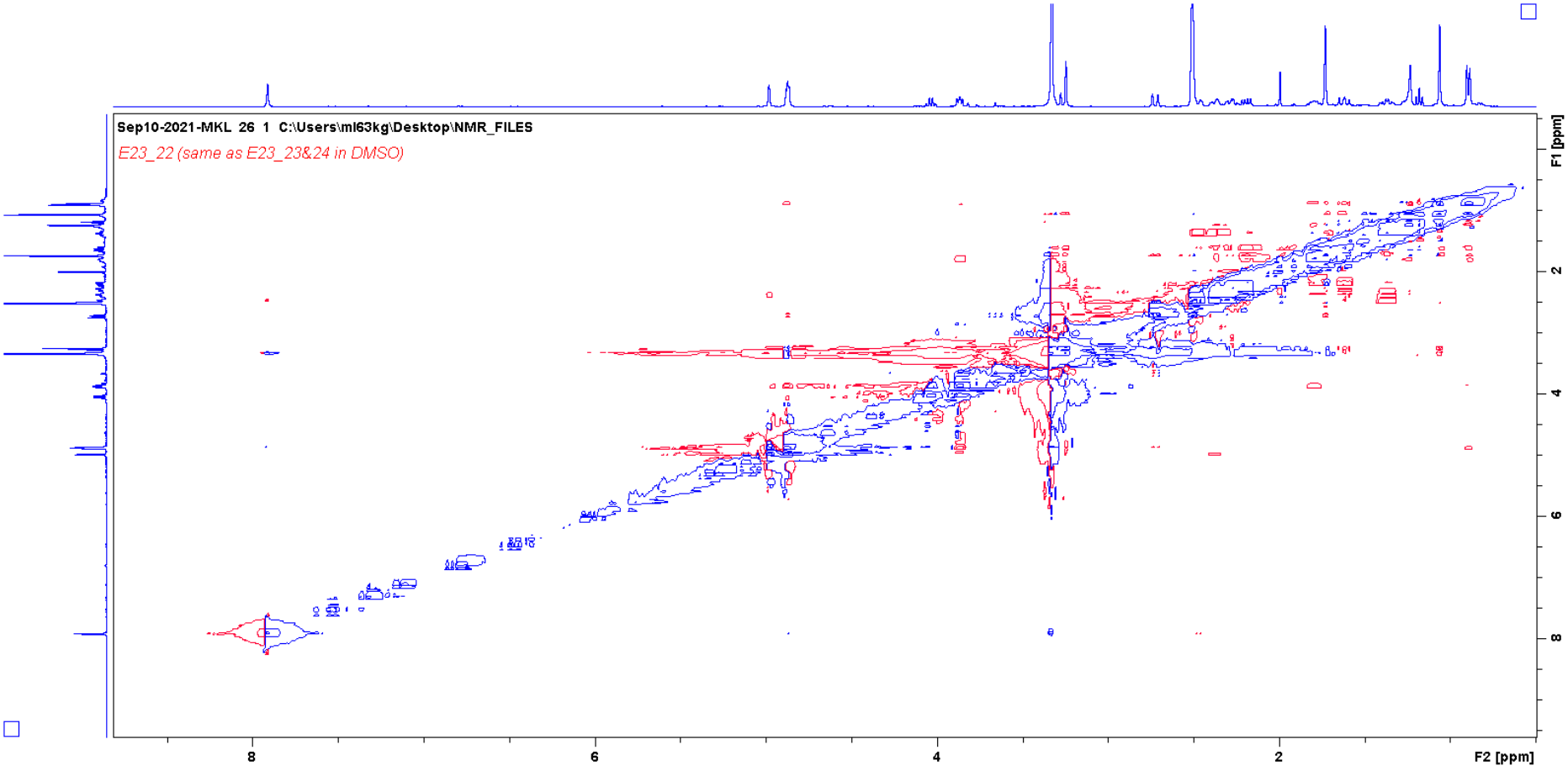
Appendix 38 HMBC spectrum of Crotocascarin K (E23)



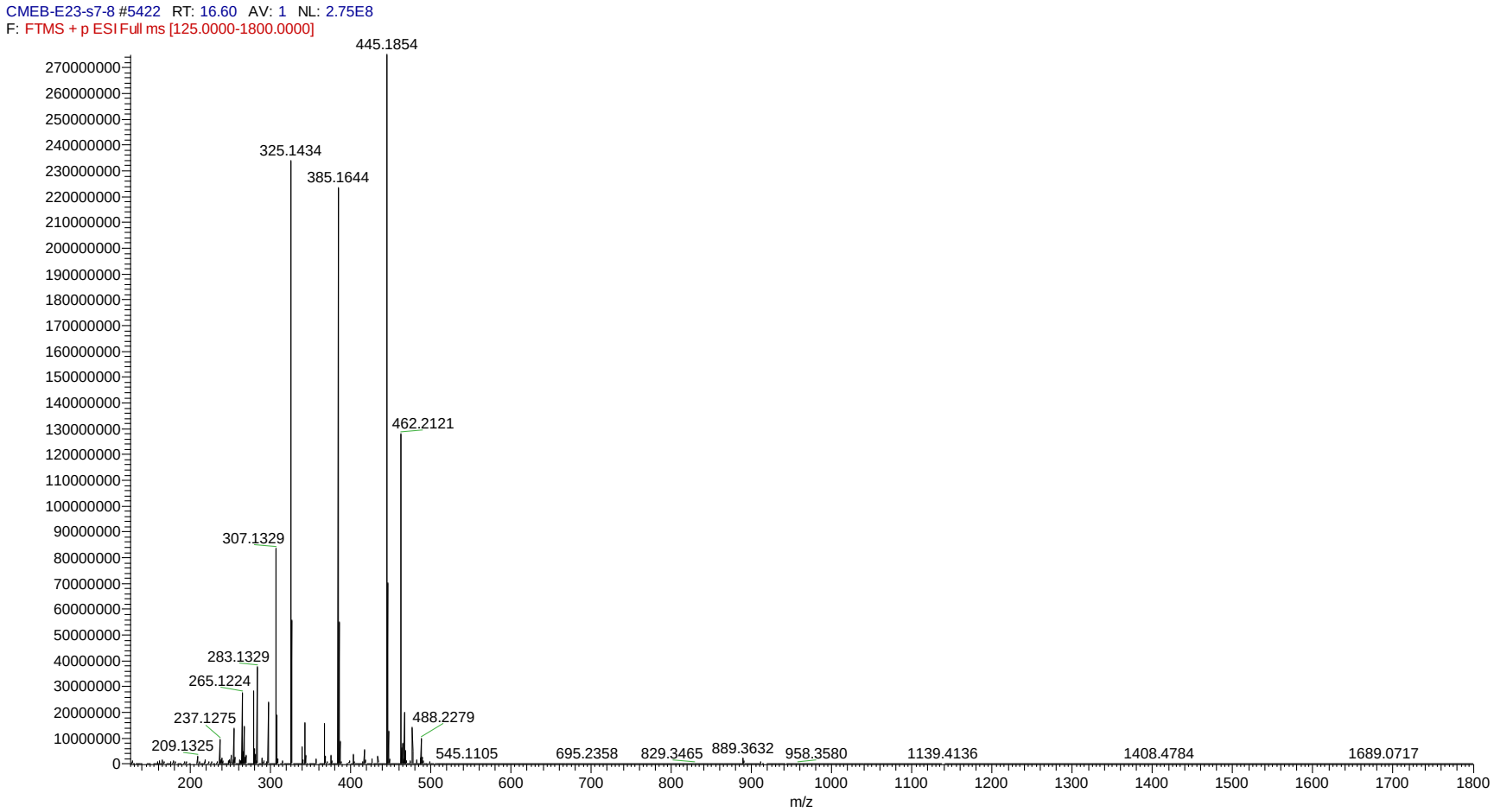
Appendix 39 COSY spectrum of Crotocascarin K (E23)



Appendix 40 NOESY spectrum of Crotocascarin K (E23)

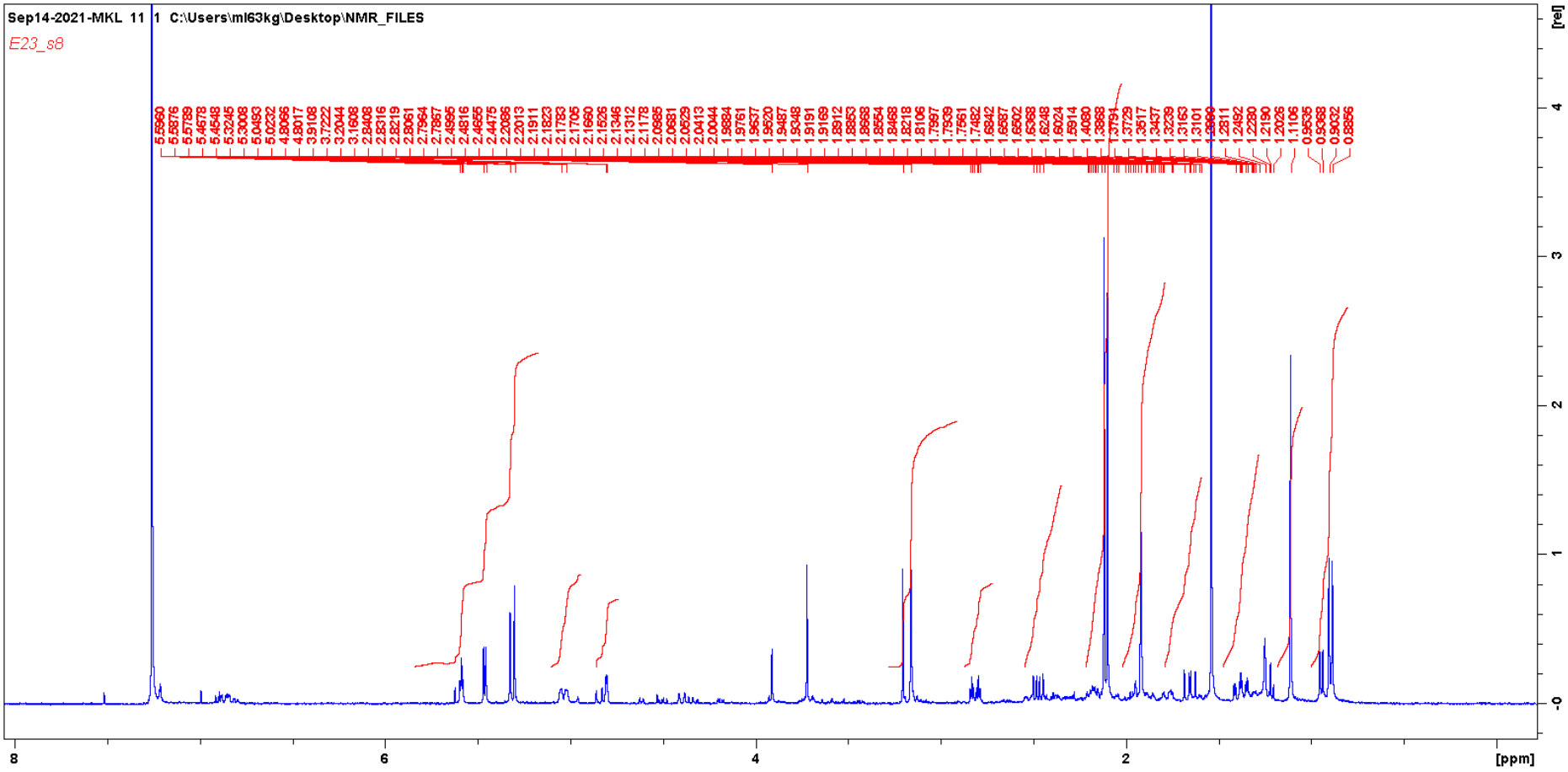


Appendix 41 Mass spectrum of 11-acetoxy crotocascarin L (E24)

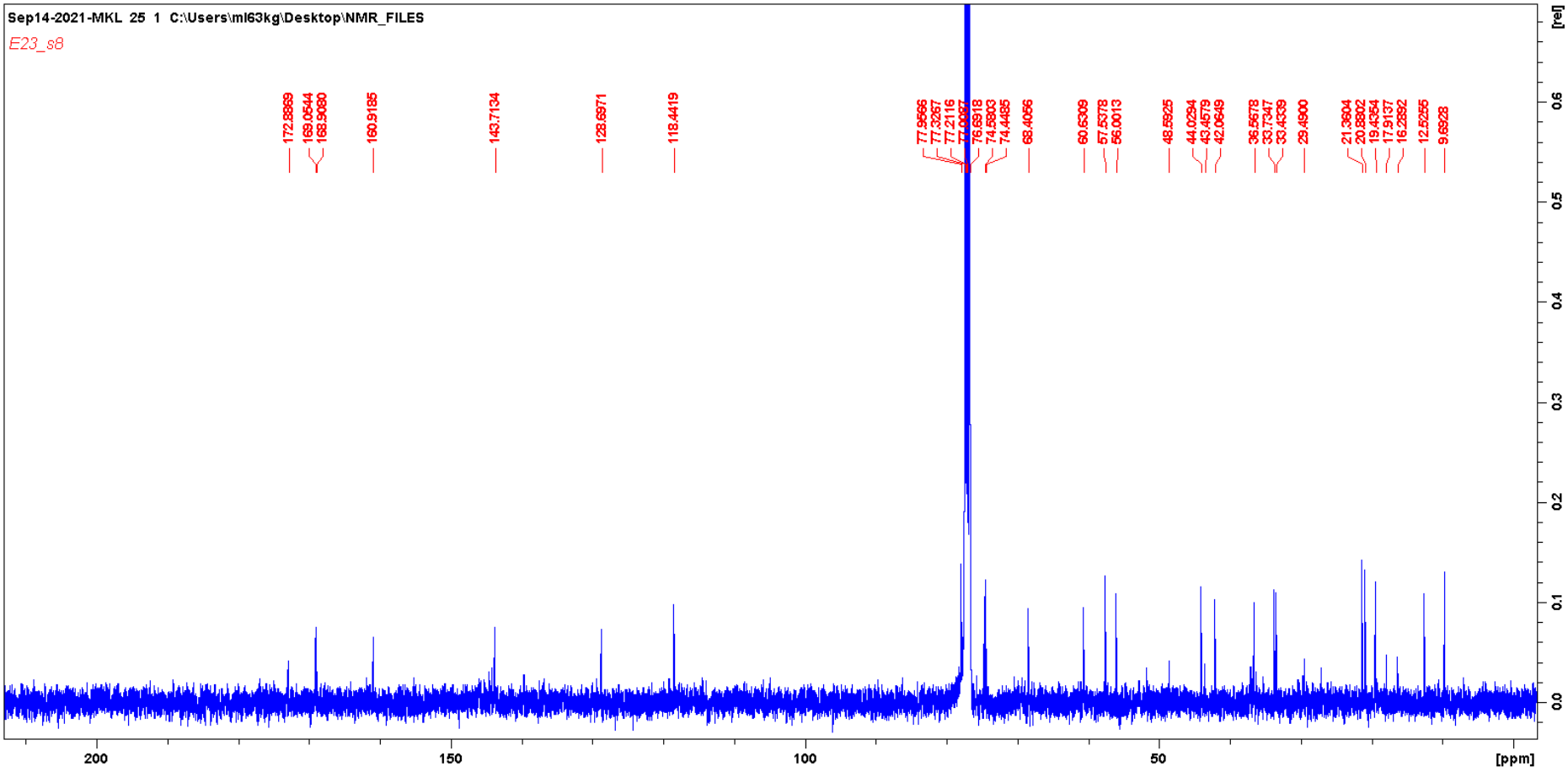



HRESIMS *m/z* 445.1854 $[M+H]^+$ (calcd. for $C_{24}H_{28}O_8$ + H, *m/z* 445.1857)

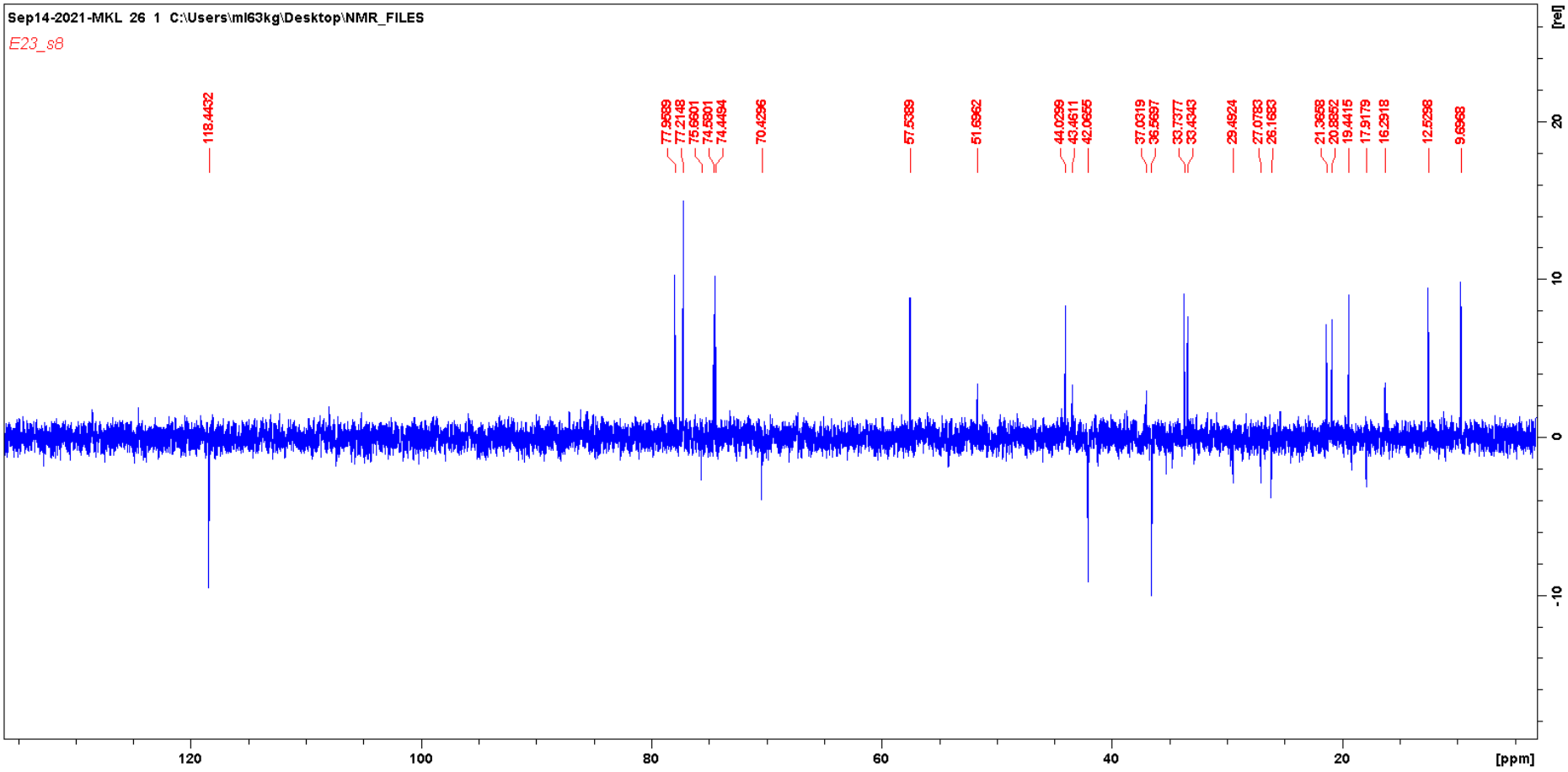
Appendix 42 $^{1}$H NMR spectrum of 11-acetoxy crotocascarin L (E24)



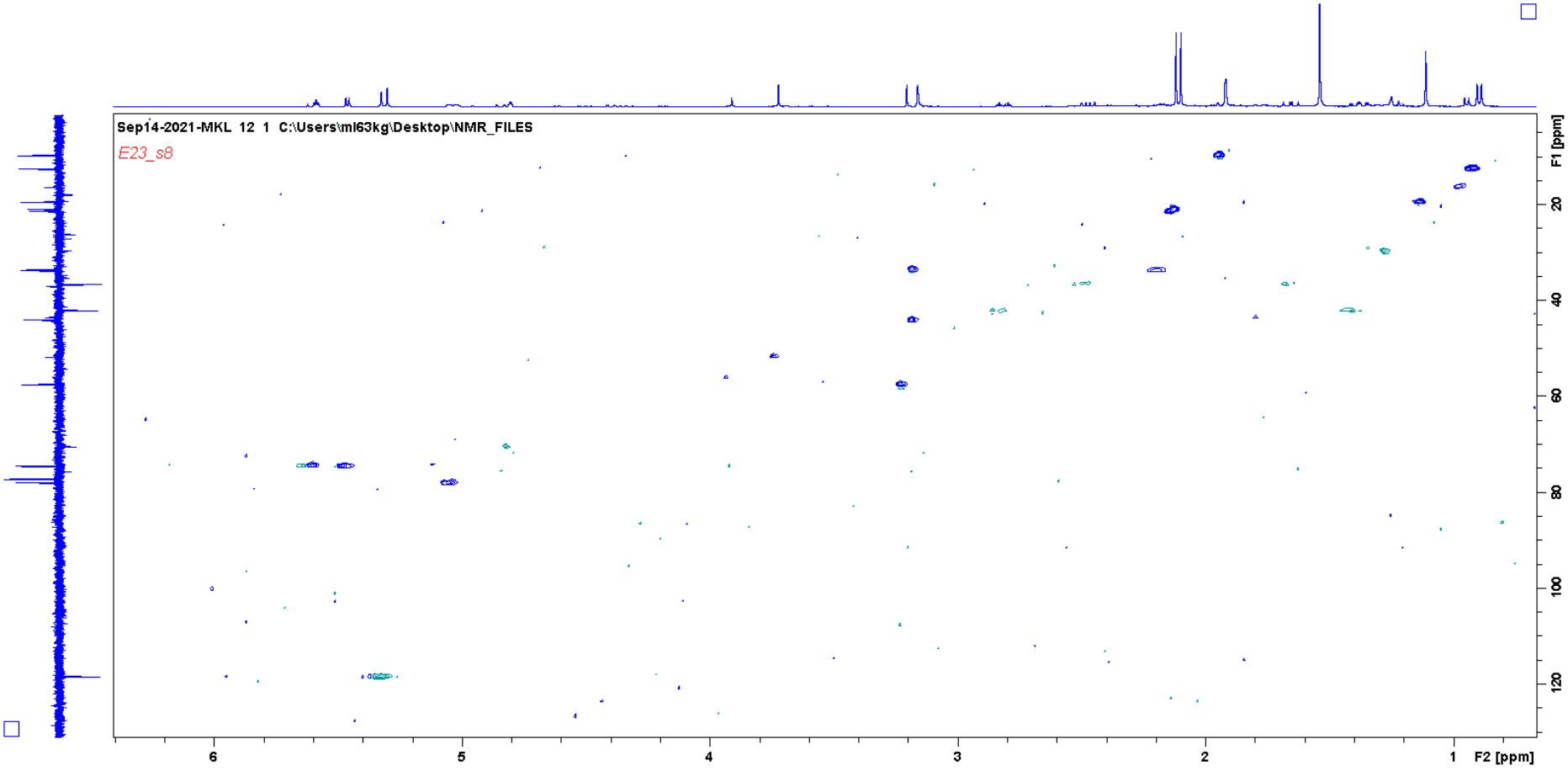
Appendix 43 $^{13}$C NMR spectrum of 11-acetoxy crotocascarin L (E24)



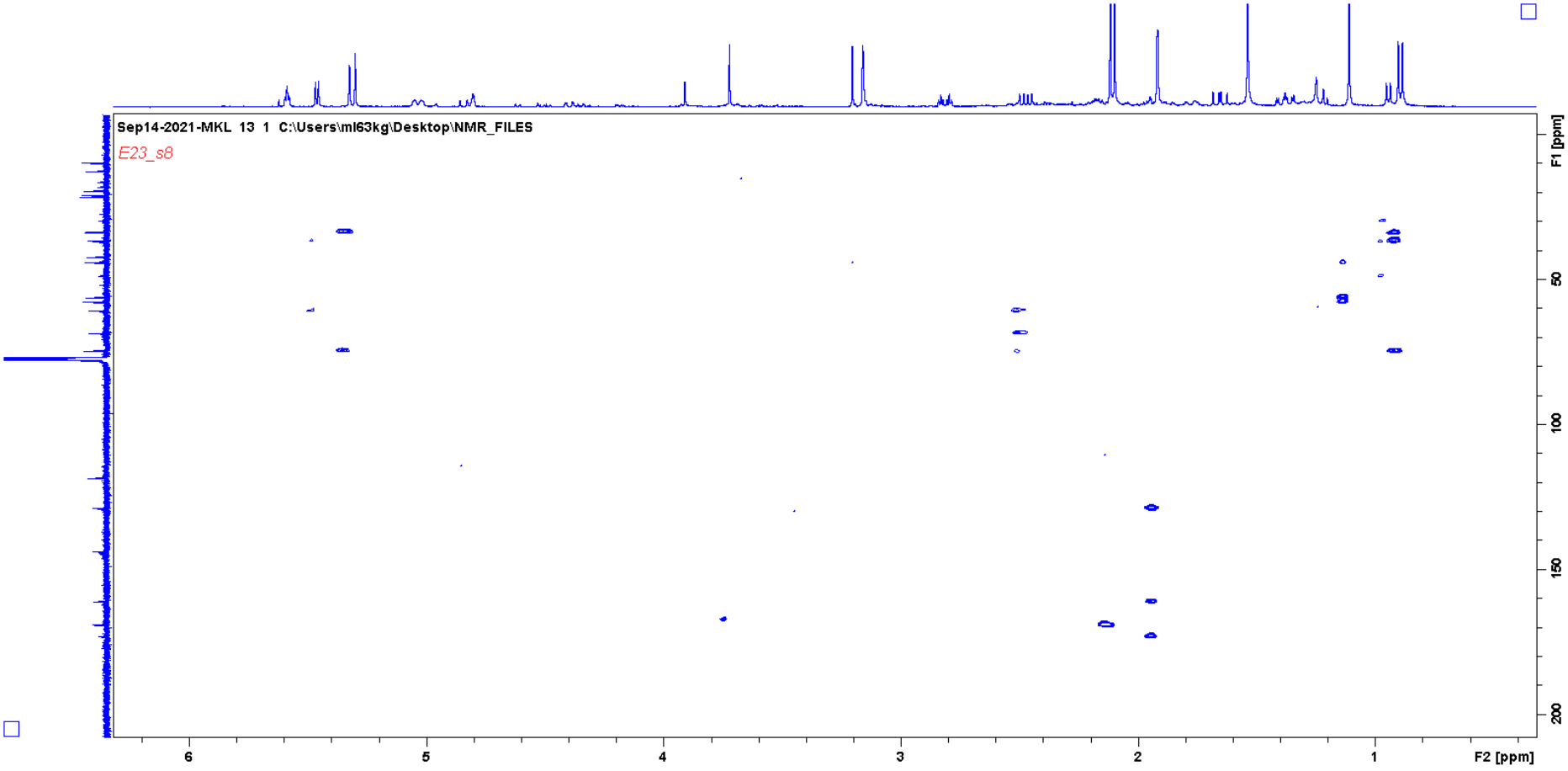
Appendix 44 DEPT spectrum of 11-acetoxy crotocascarin L (E24)



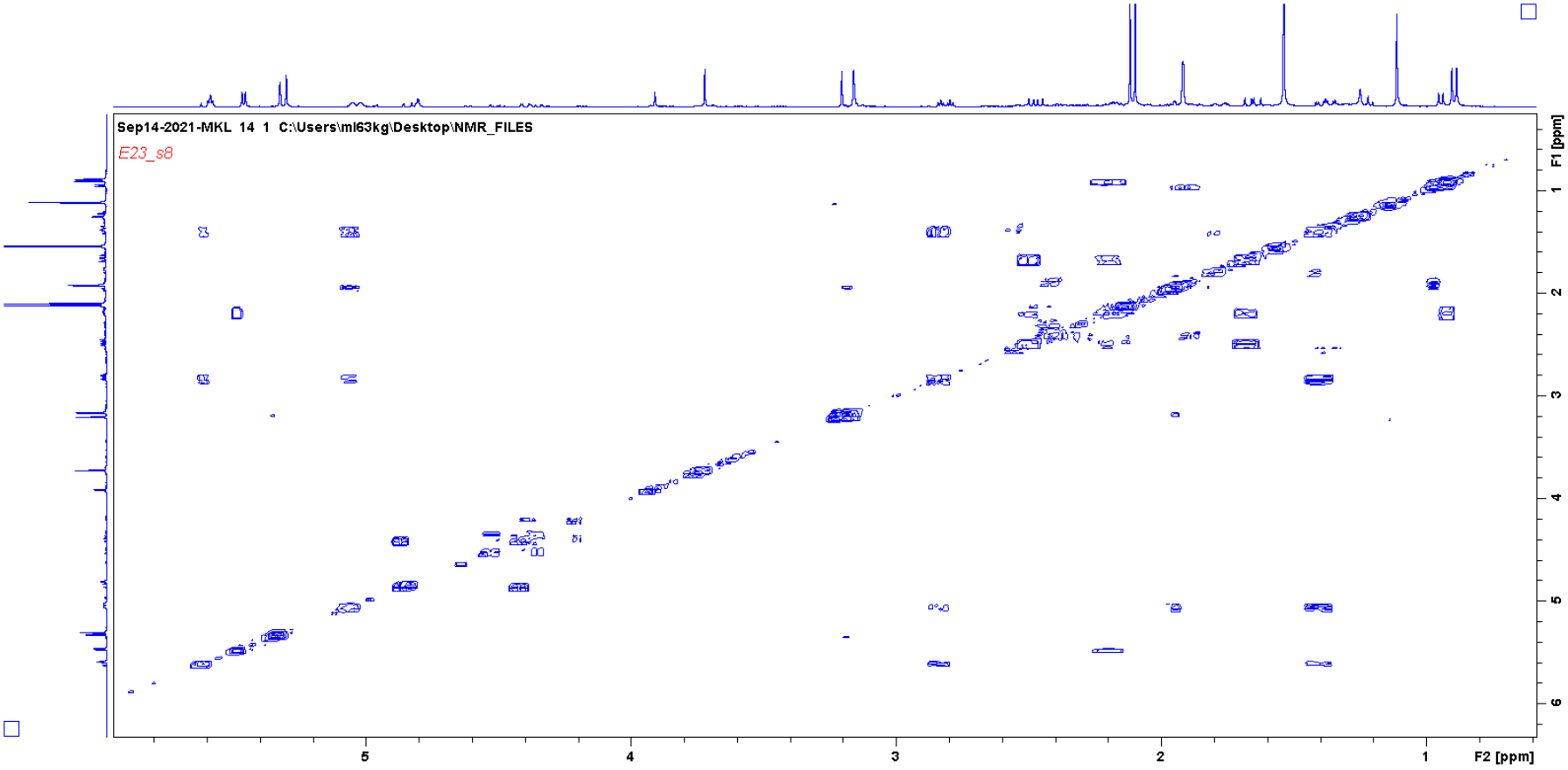
Appendix 45 HSQCDEPT spectrum of 11-acetoxy crotocascarin L (E24)



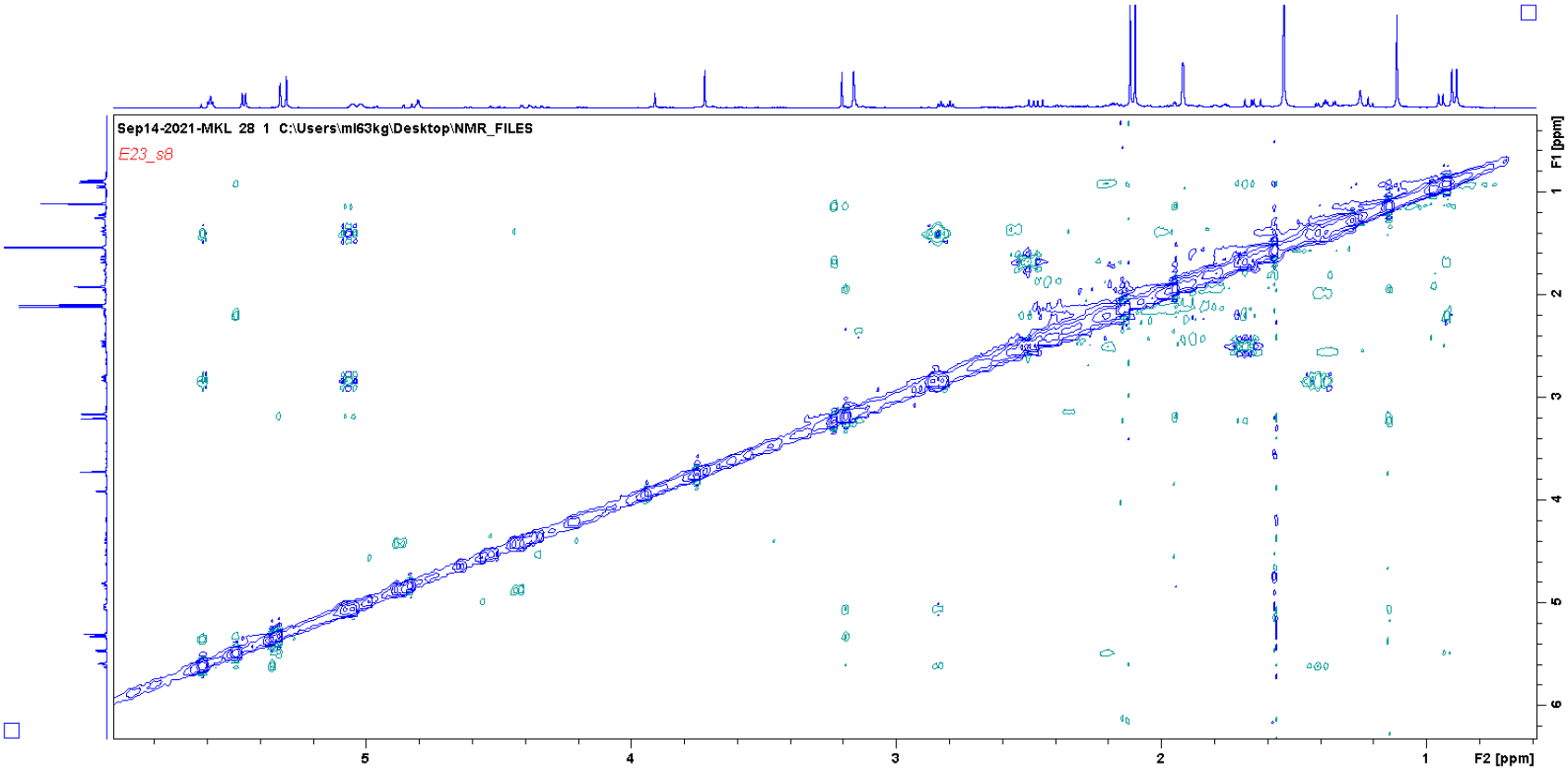
Appendix 46 HMBC spectrum of 11-acetoxy crotocascarin L (E24)



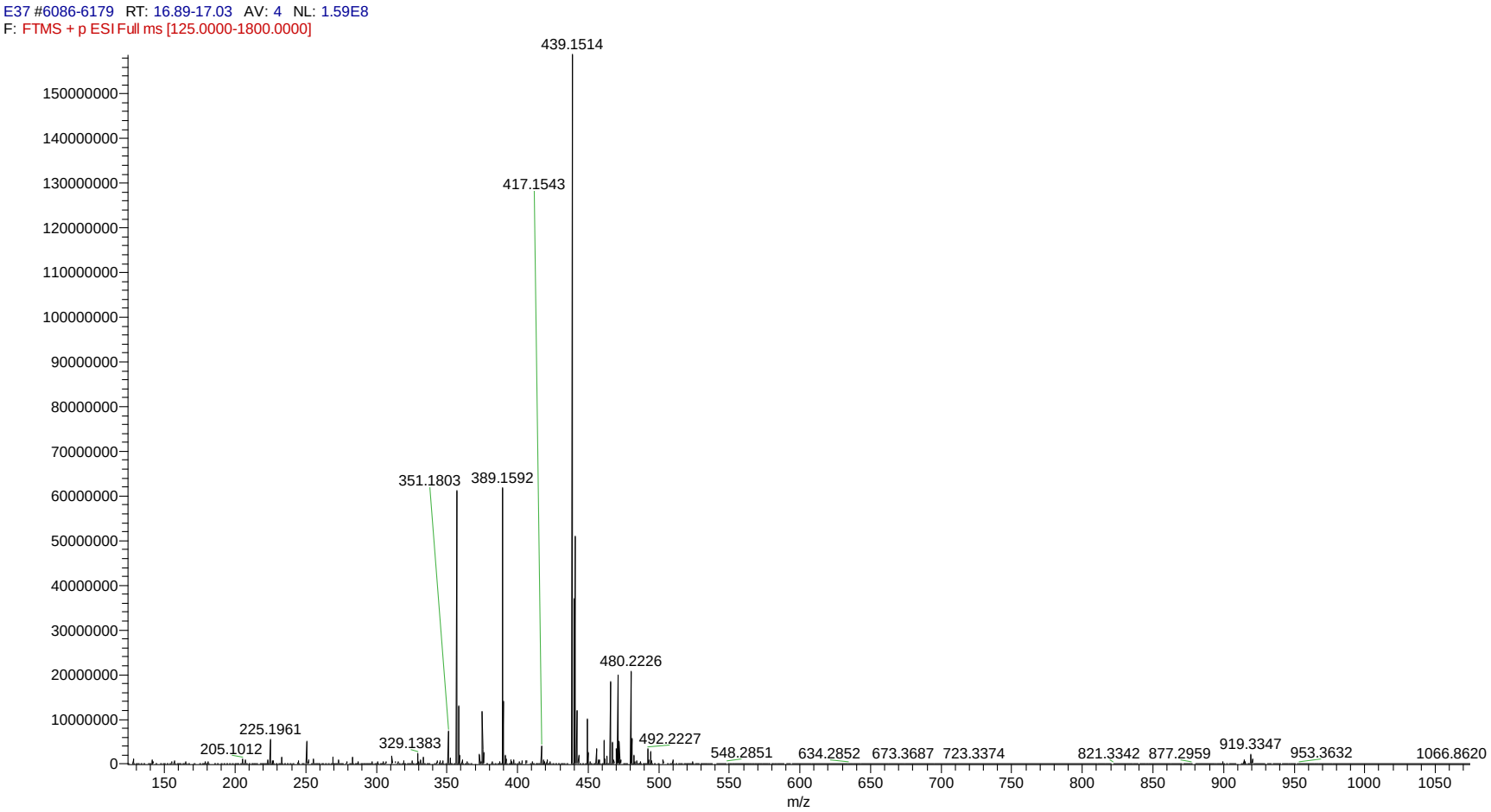
Appendix 47 COSY spectrum of 11-acetoxy crotocascarin L (E24)



Appendix 48 NOESY spectrum of 11-acetoxy crotocascarin L (E24)

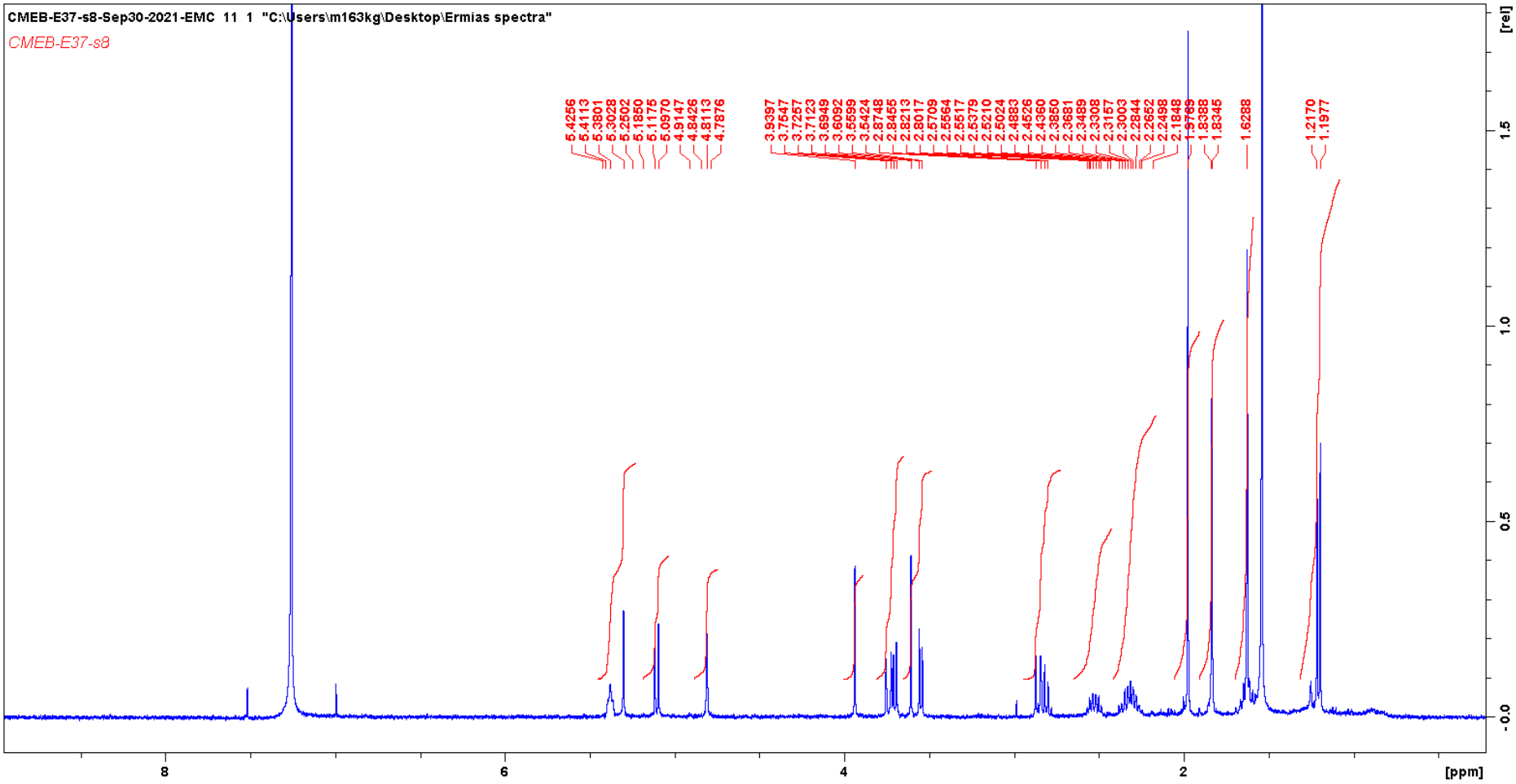


Appendix 49 Mass spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)

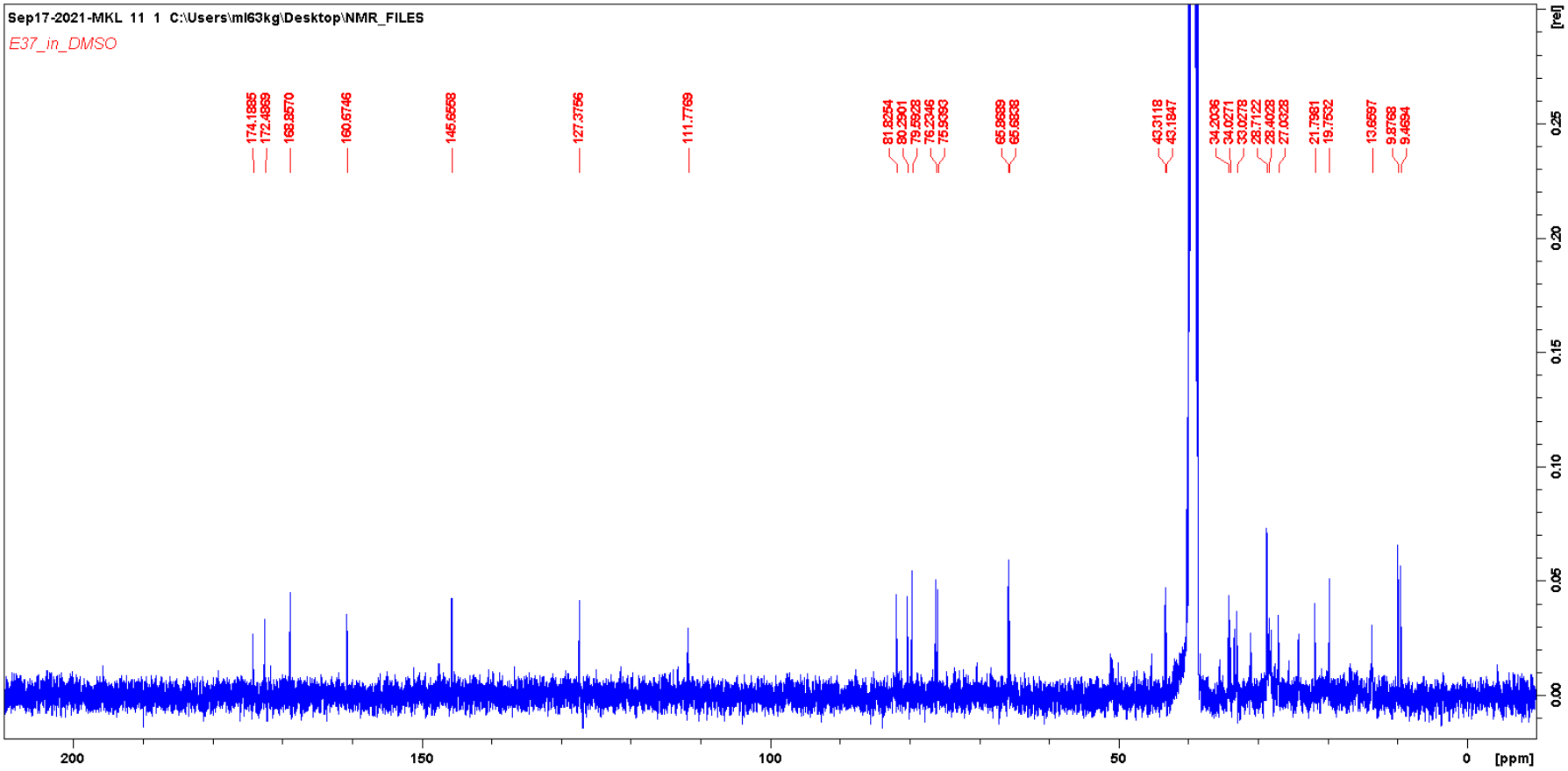



HRESIMS *m/z* 439.1514 $[M+H]^+$ (calcd. for $C_{22}H_{27}ClO_8$ + H, *m/z* 439.1518)

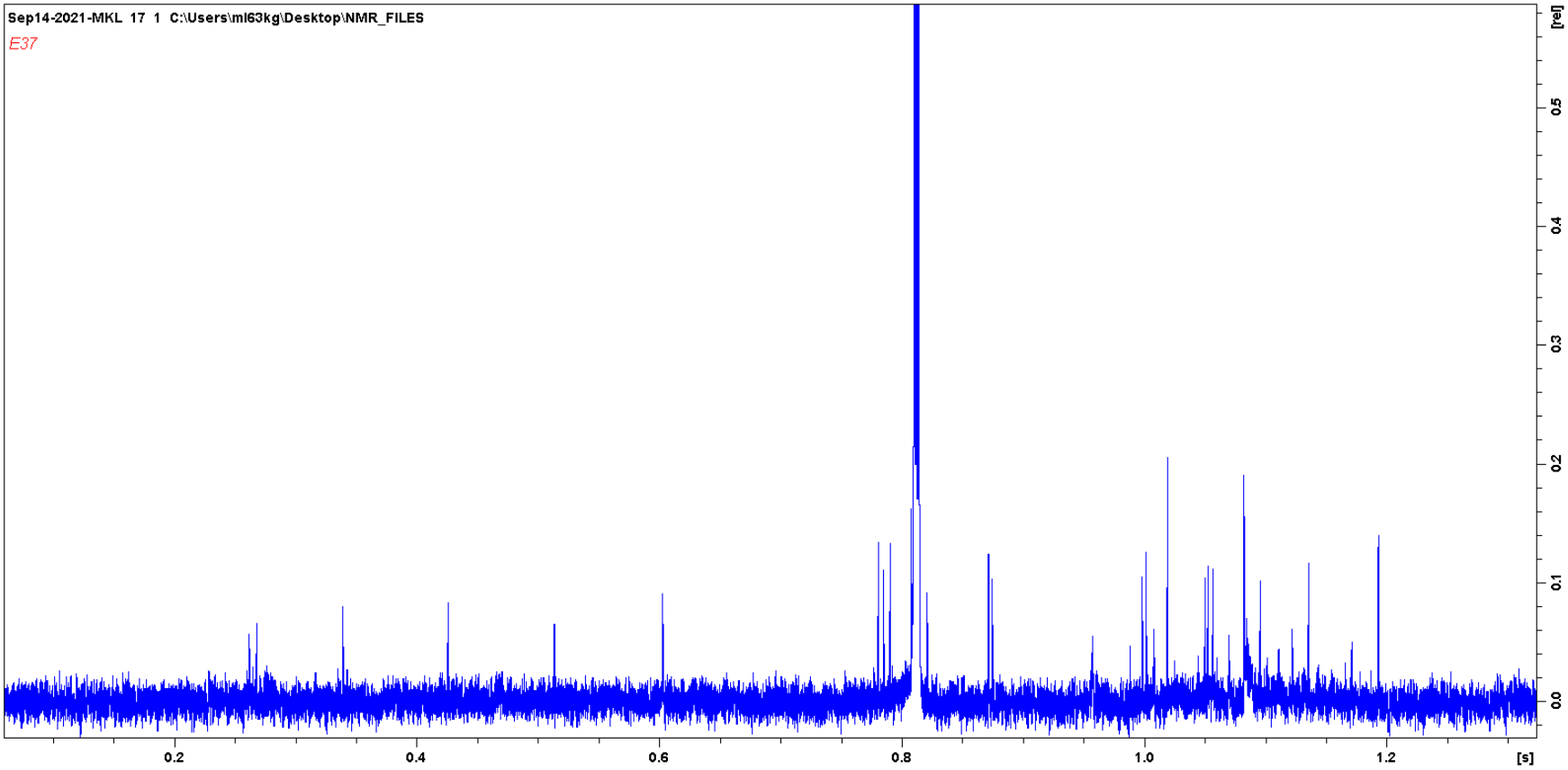
Appendix 50 $^{1}$H NMR spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



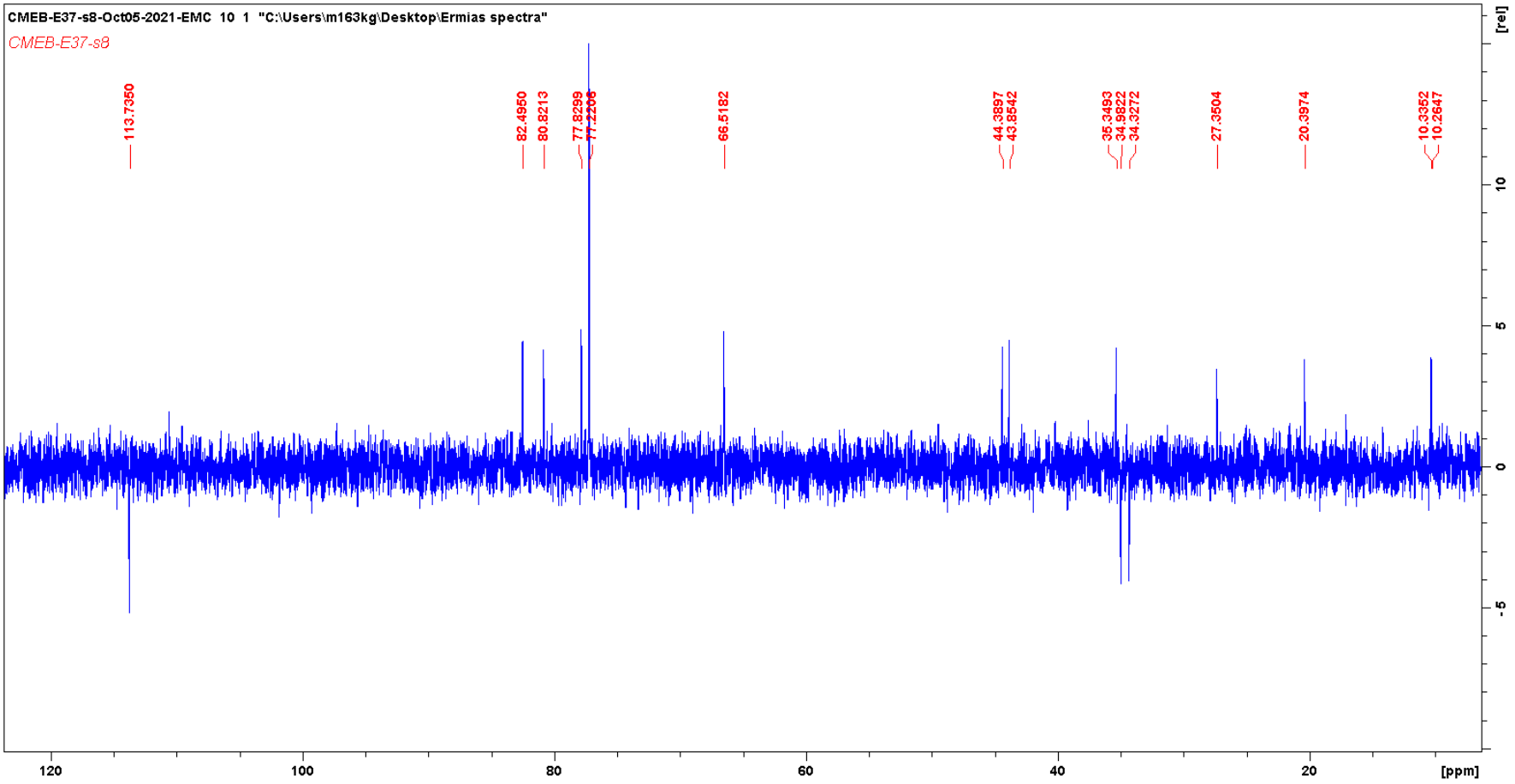
Appendix 51 $^{13}$C NMR spectrum in DMSO of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



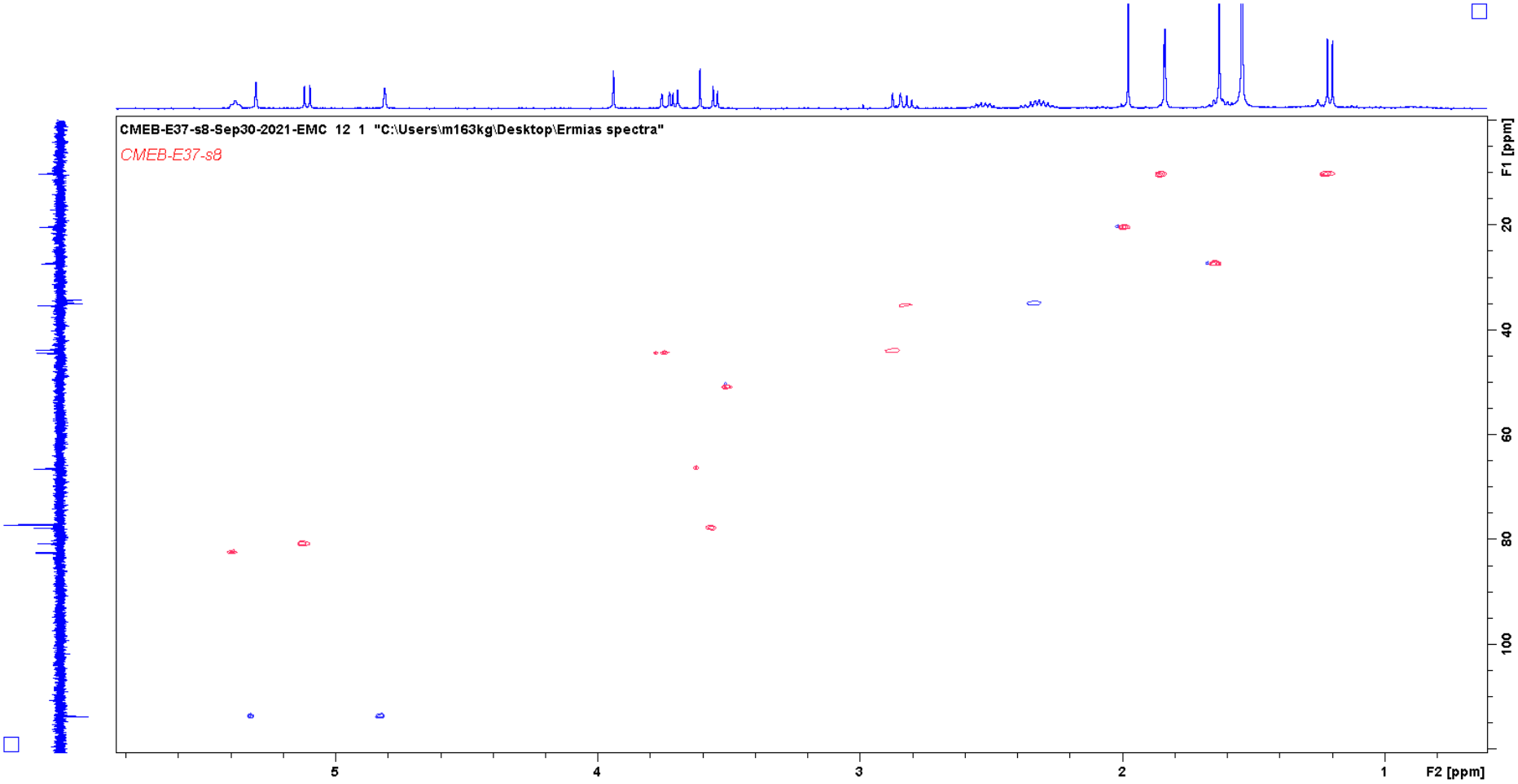
Appendix 52 $^{13}$C NMR spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



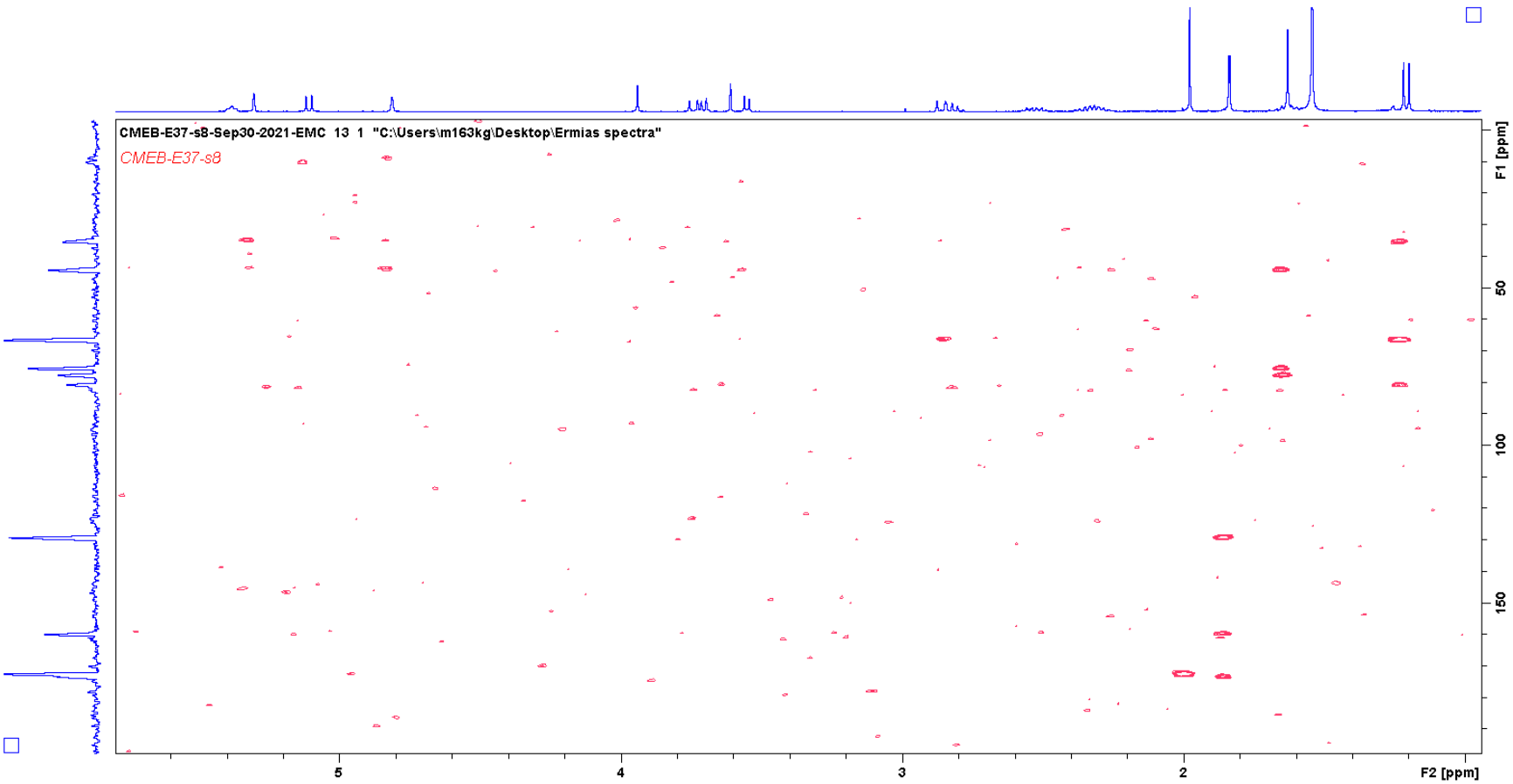
Appendix 53 DEPT spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



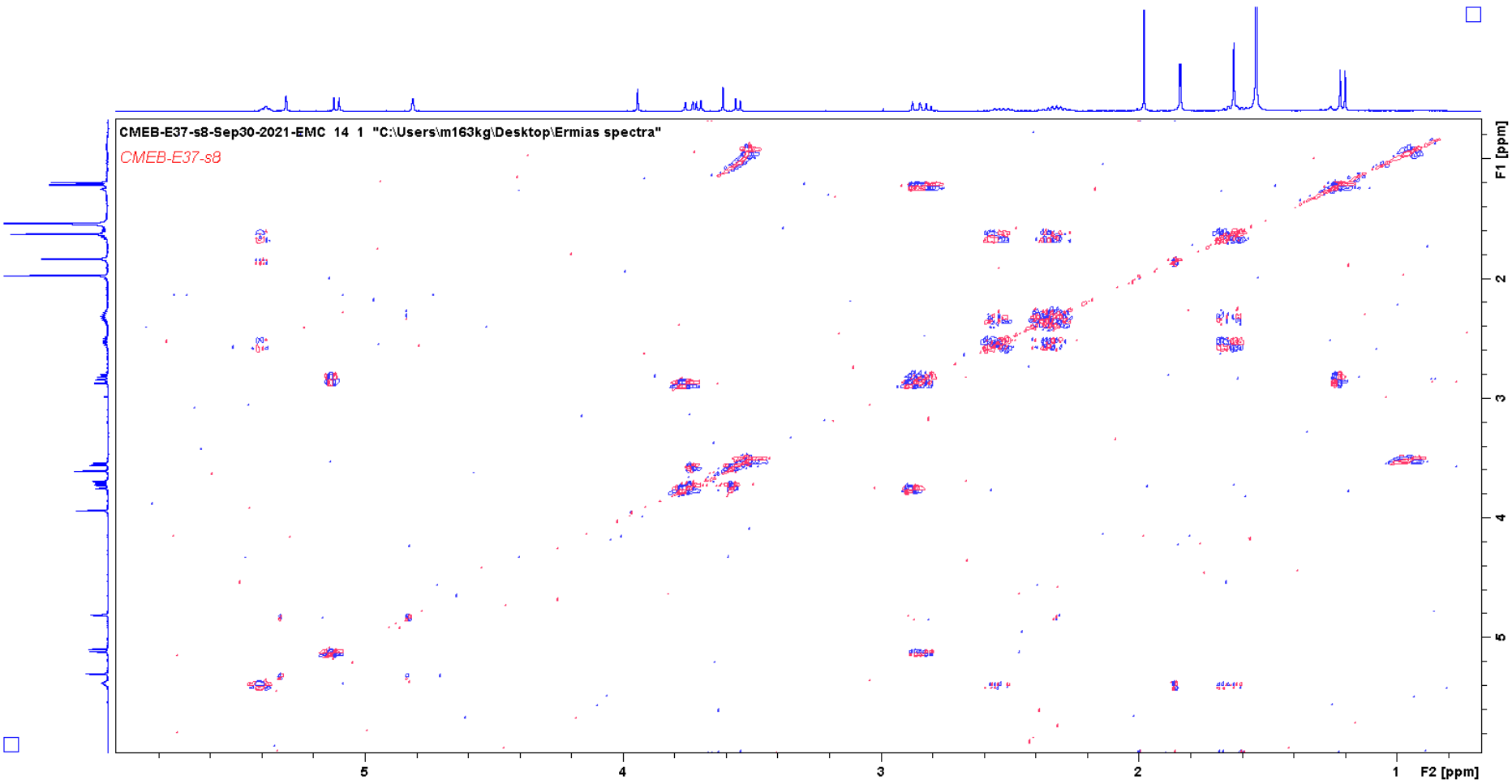
Appendix 54 HSQCDEPT spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



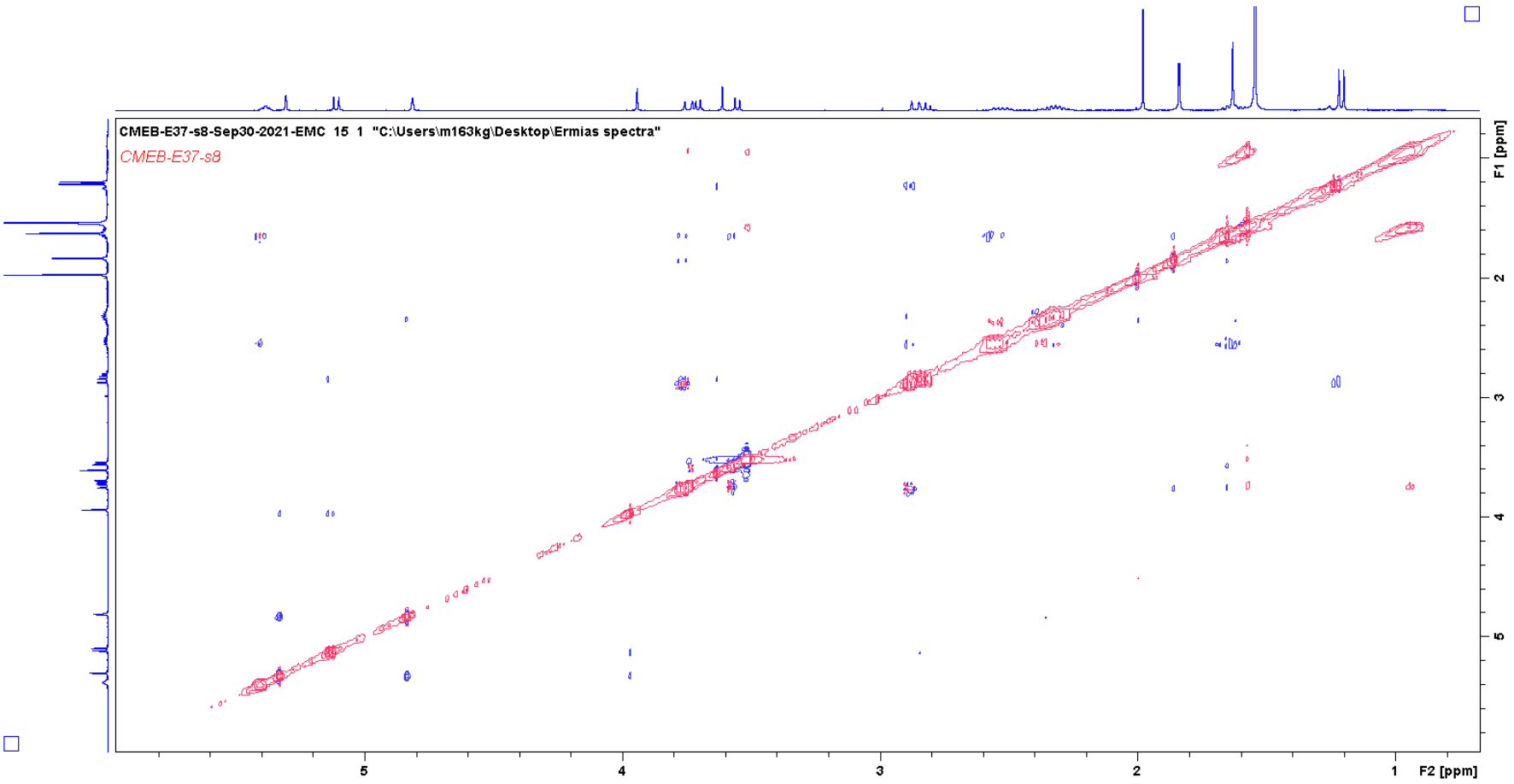
Appendix 55 HMBC spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



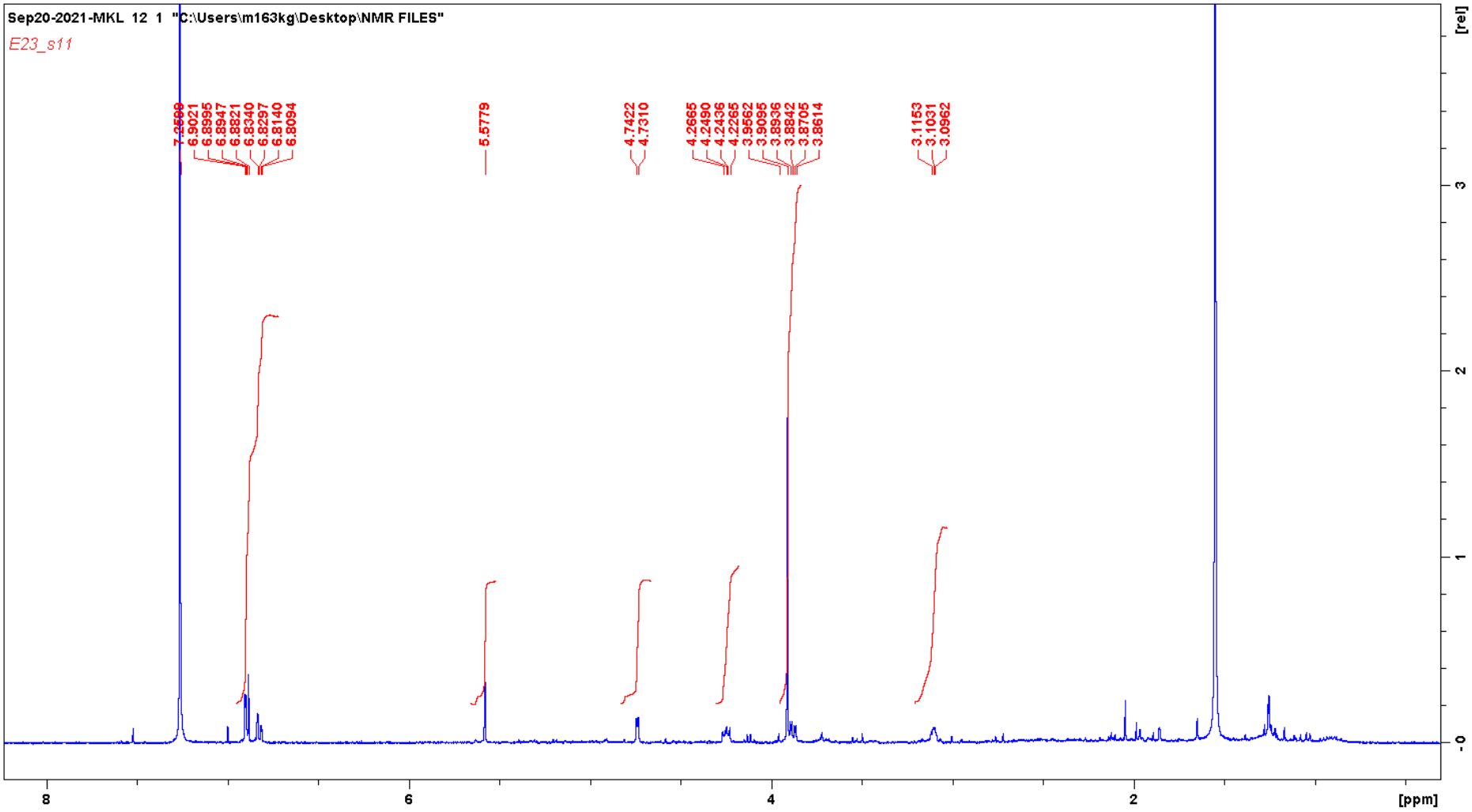
Appendix 56 COSY spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



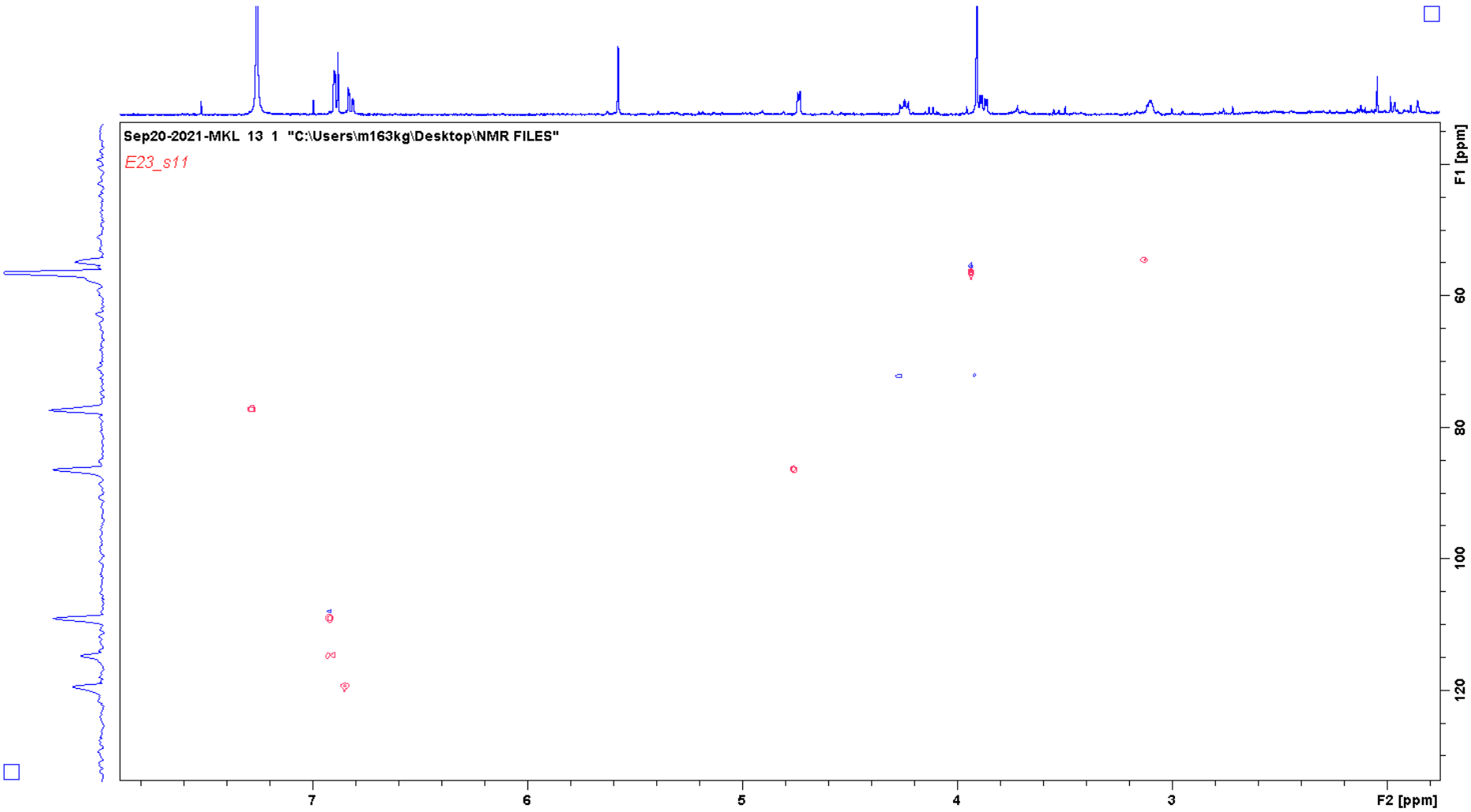
Appendix 57 NOESY spectrum of 1β-acetoxy-3β-chloro-5α,6α-dihydroxycrotocascarin L (Ermiasoid) (E37)



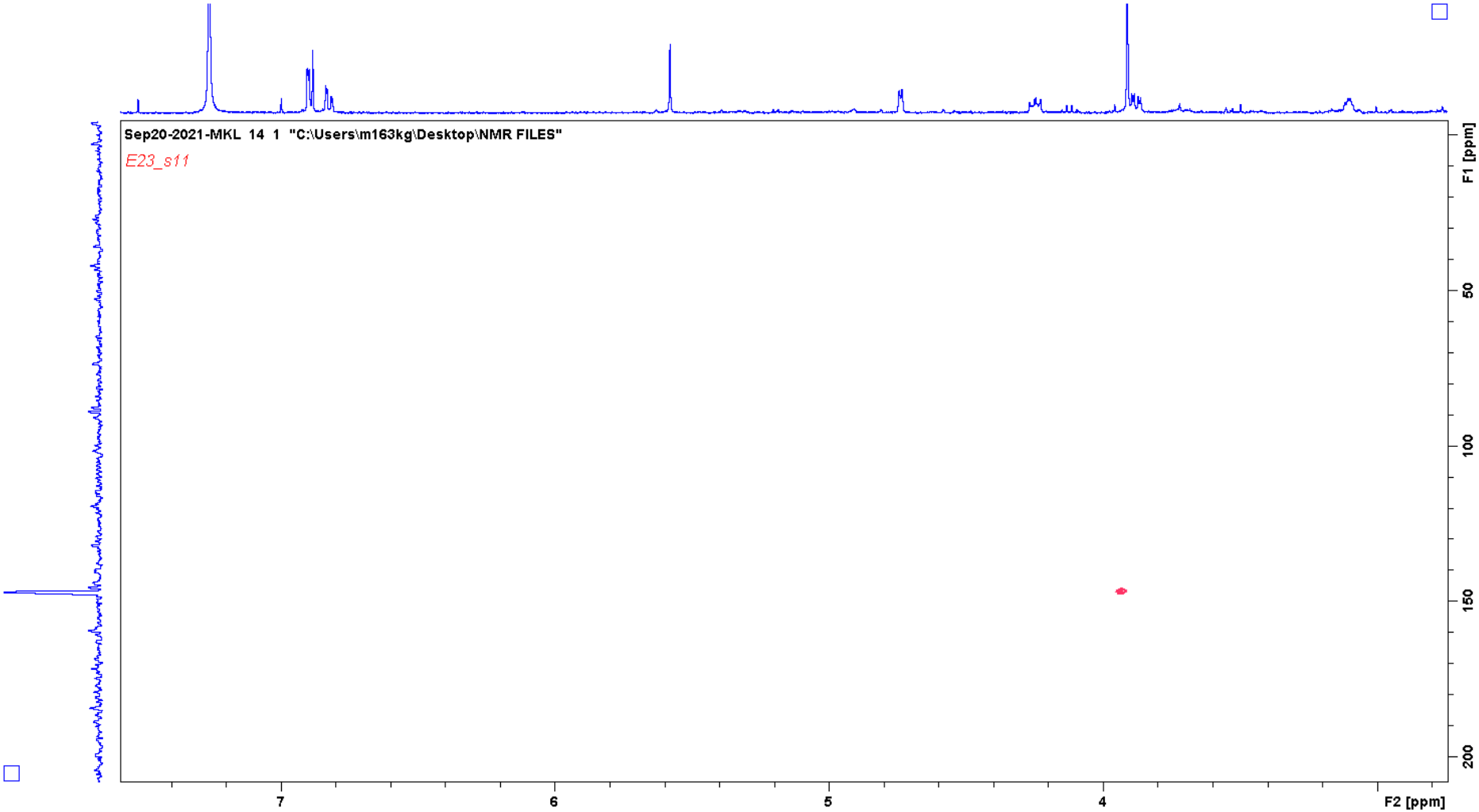
Appendix 58 $^{1}$H NMR spectra of Pinoresinol (E2)



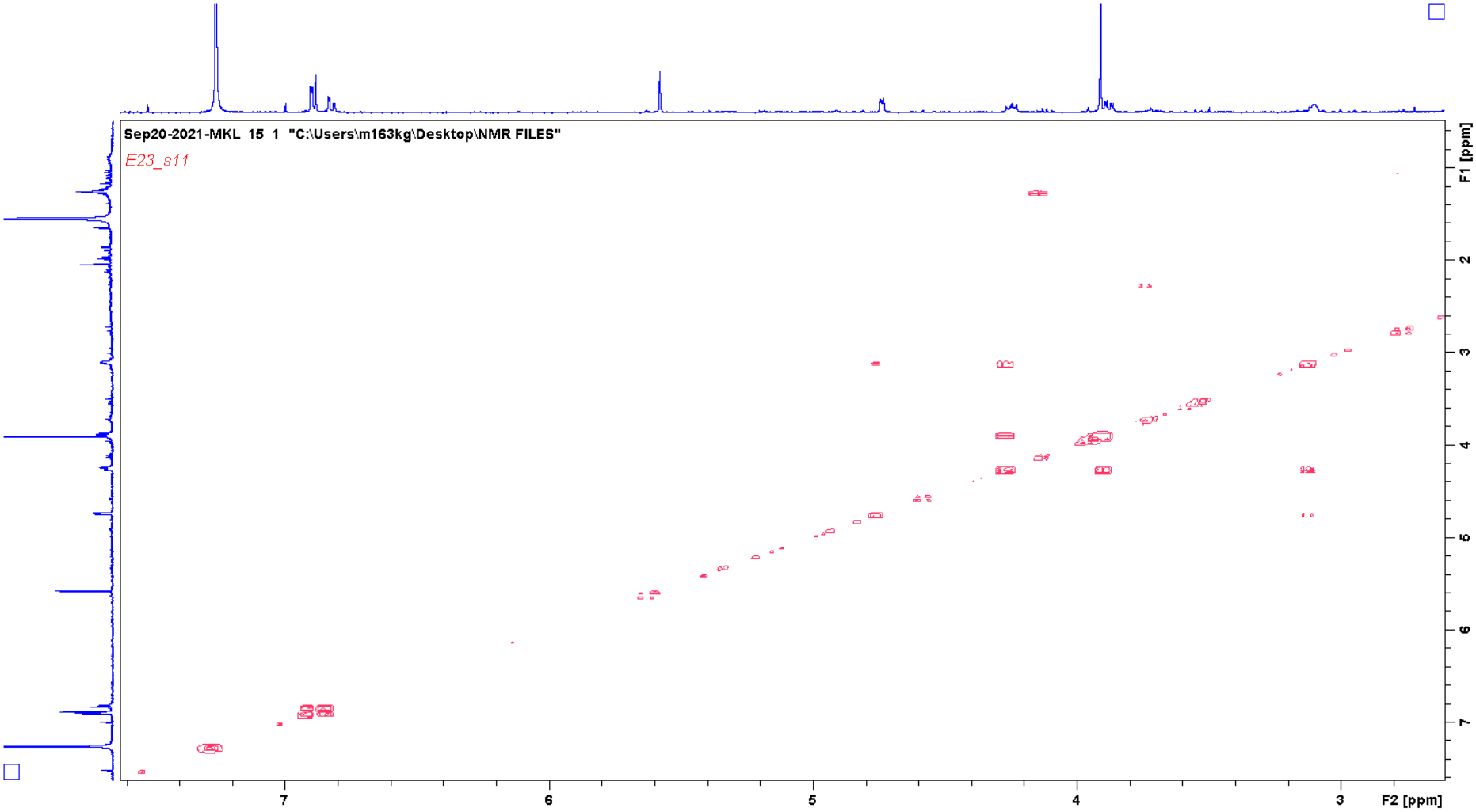
Appendix 59 HSQC spectrum of Pinoresinol (E2)



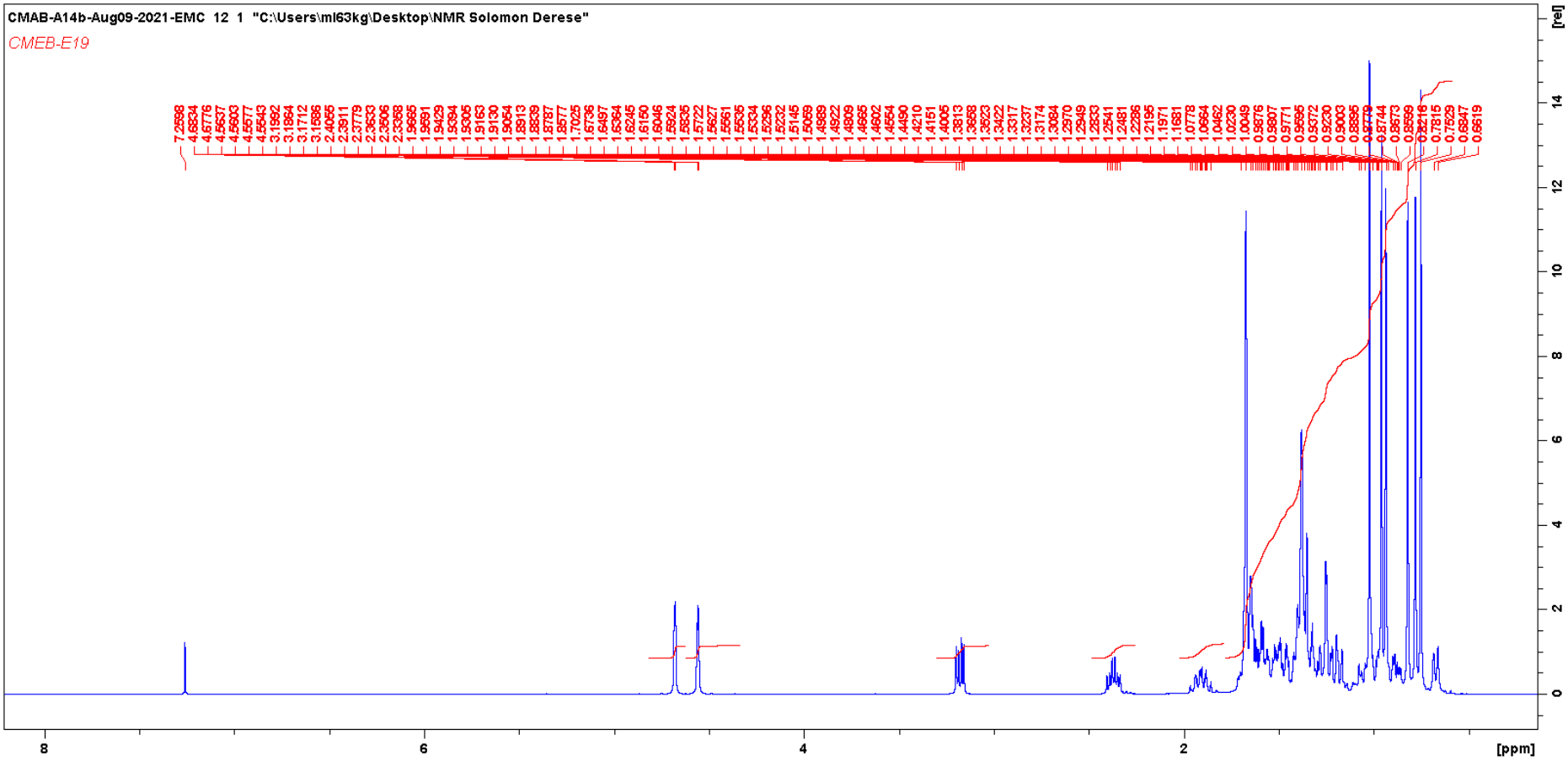
Appendix 60 HMBC spectrum of Pinoresinol (E2)



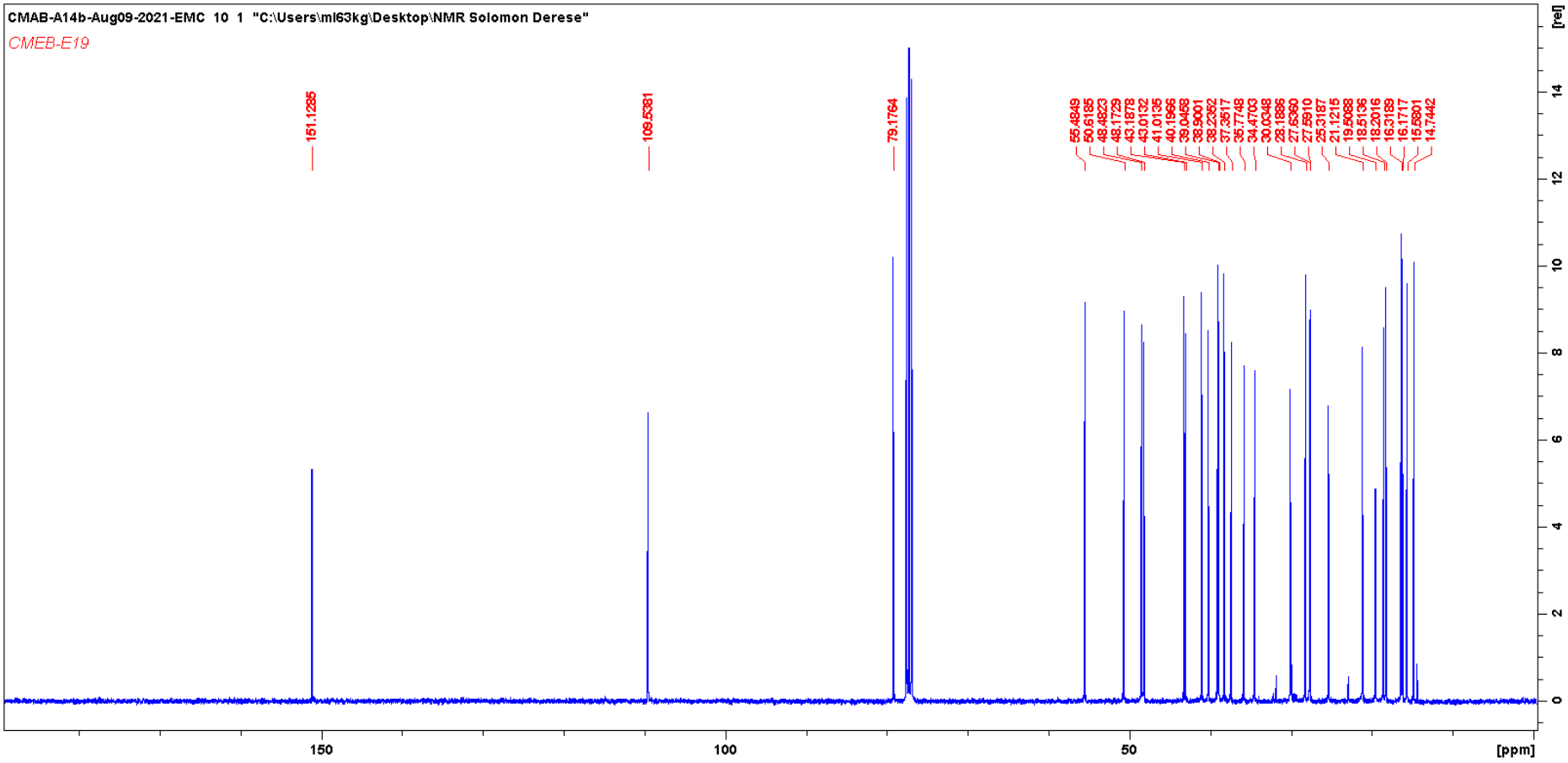
Appendix 61 COSY spectrum of Pinoresinol (E2)



Appendix 62 $^{1}$H NMR spectra of 3β-Hydroxylup-20(29)-ene (lupeol) (E19)

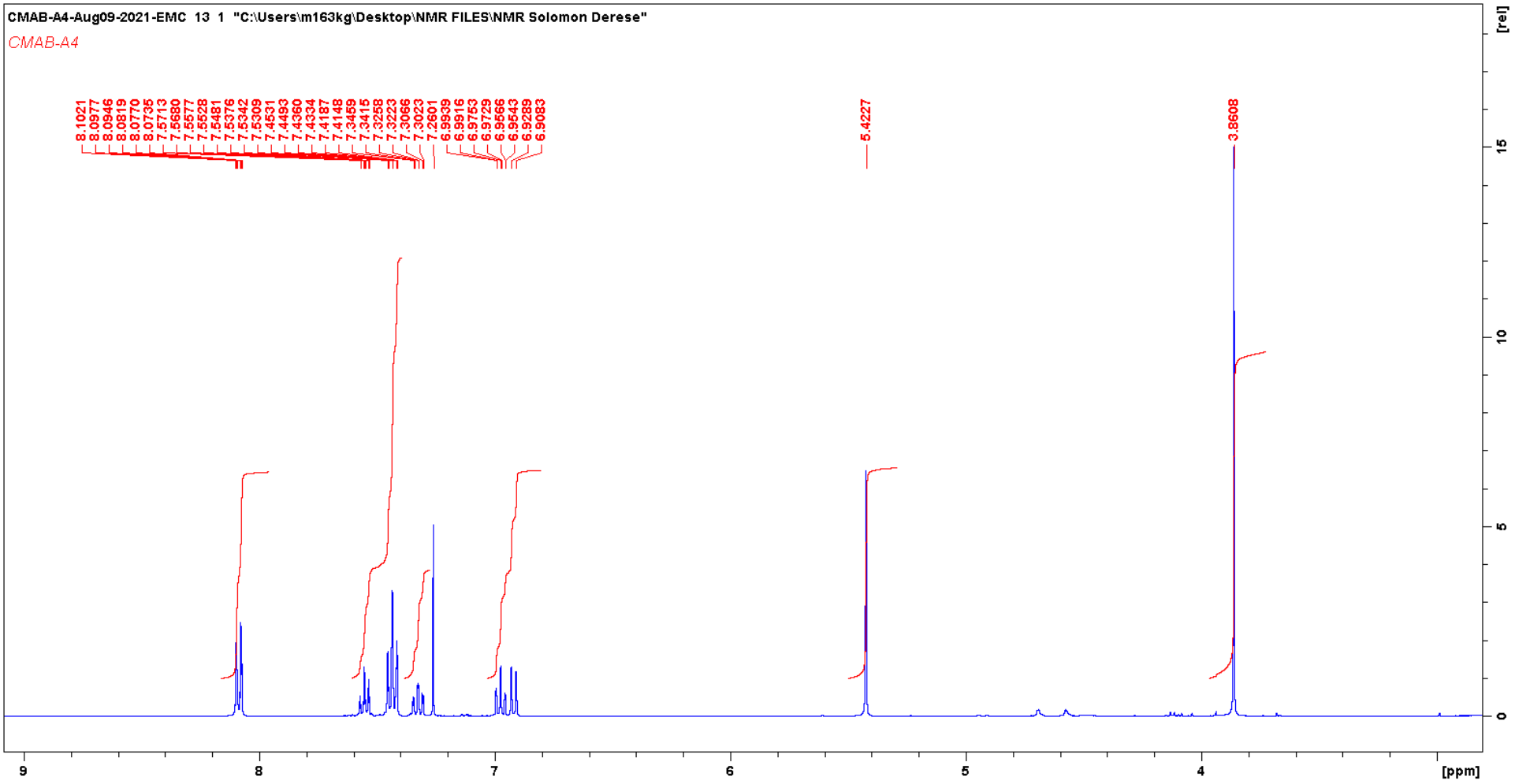


Appendix 63 $^{13}$C NMR spectra of 3β-Hydroxylup-20(29)-ene (lupeol) (E19)

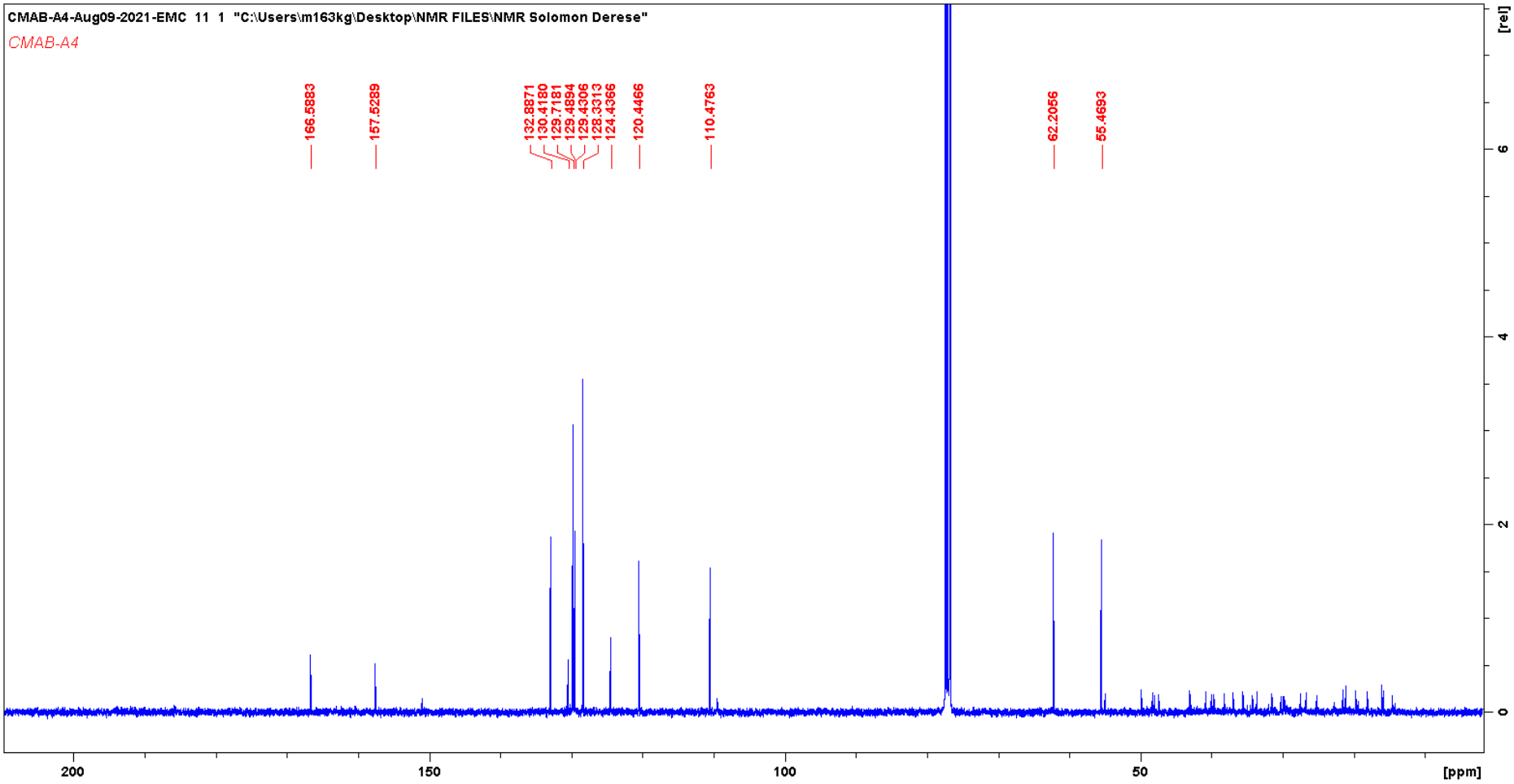



*For DEPT, HSQCDEPT, COSY, HMBC, NOESY of lupeol see appendix 93-97

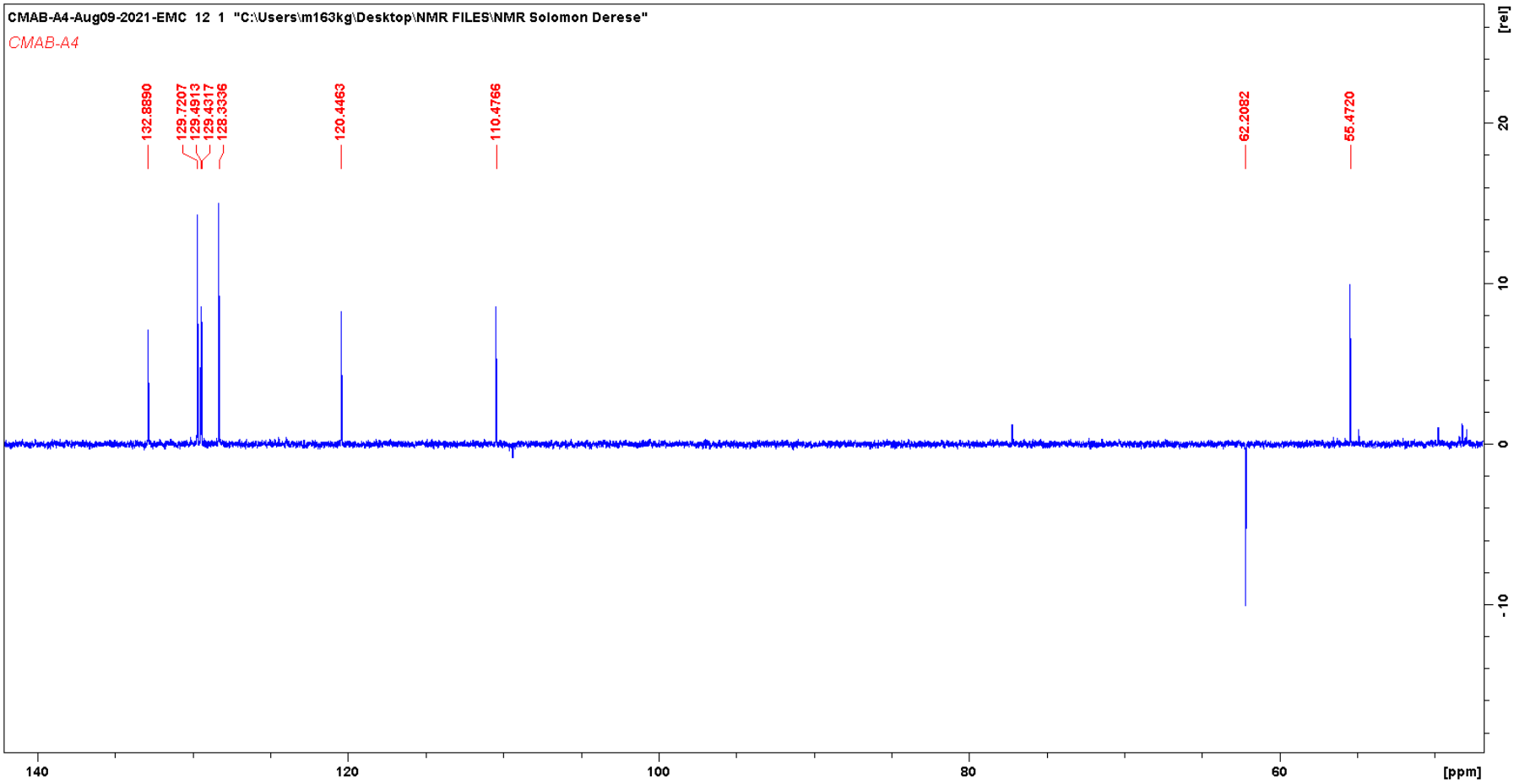
Appendix 64 $^{1}$H NMR spectra of 2-Methoxy benzyl benzoate (A5)



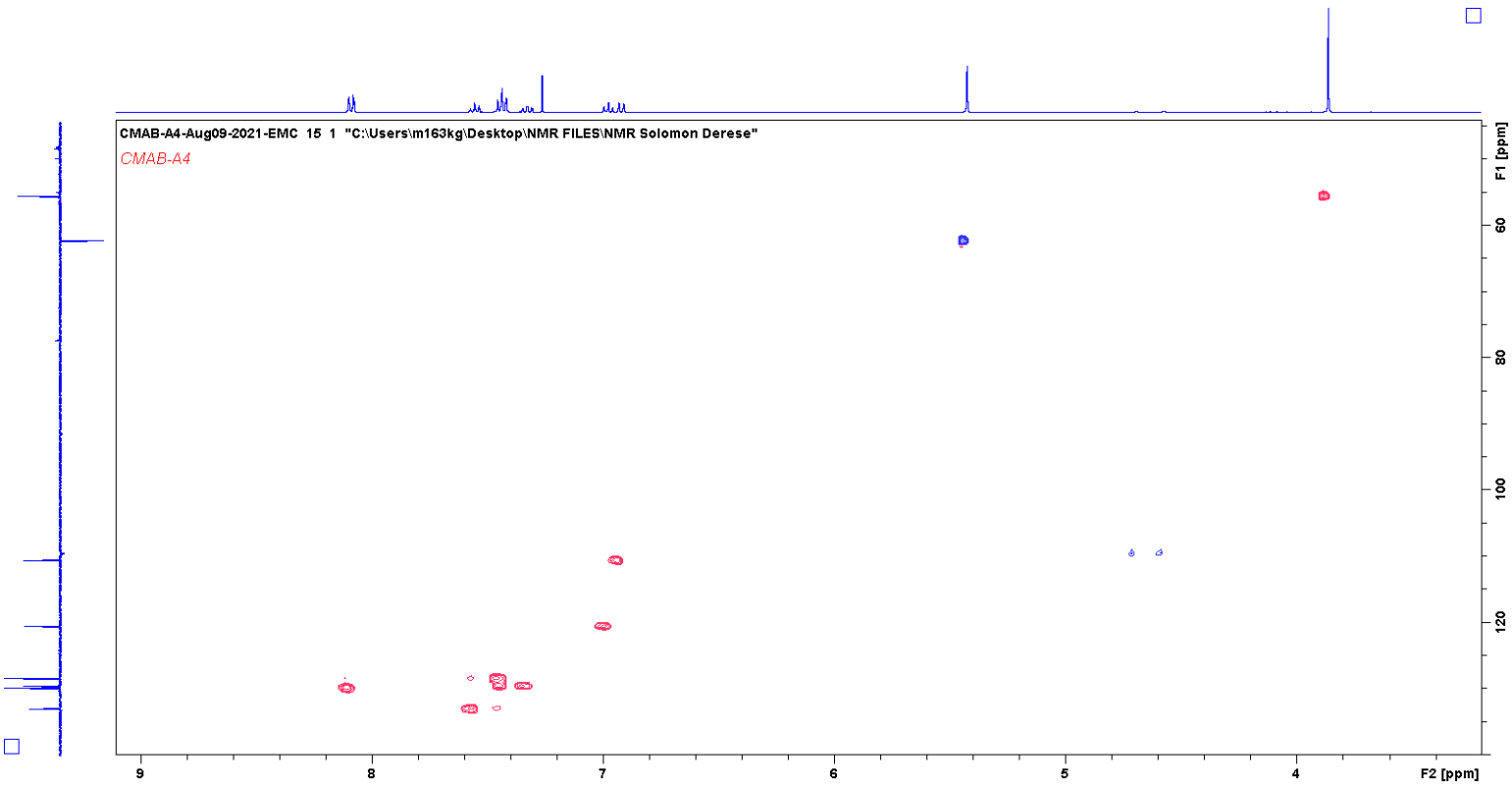
Appendix 65 $^{13}$C NMR spectra of 2-Methoxy benzyl benzoate (A5)



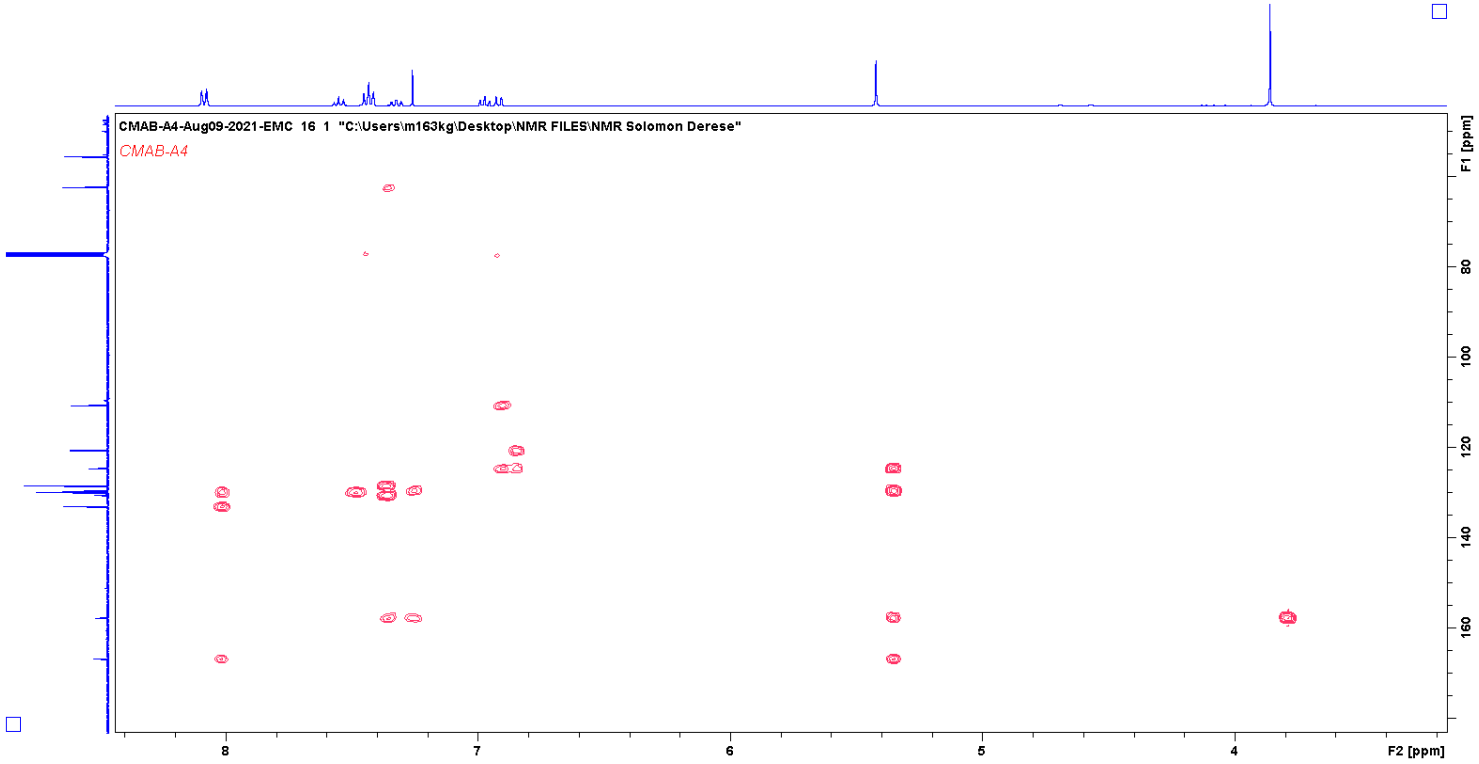
Appendix 66 DEPT spectrum of 2-Methoxy benzyl benzoate (A5)



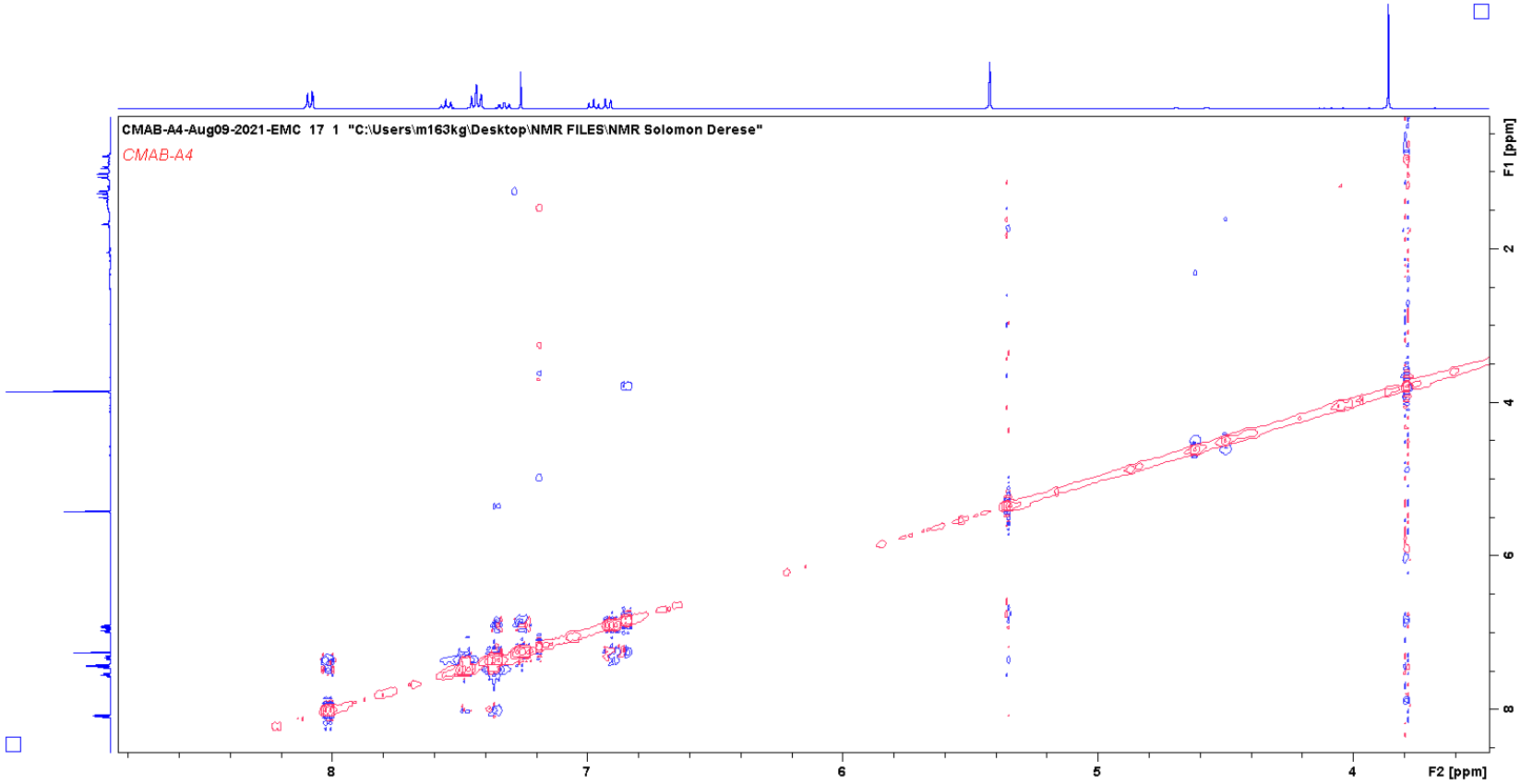
Appendix 67 HSQCDEPT spectrum of 2-Methoxy benzyl benzoate (A5)



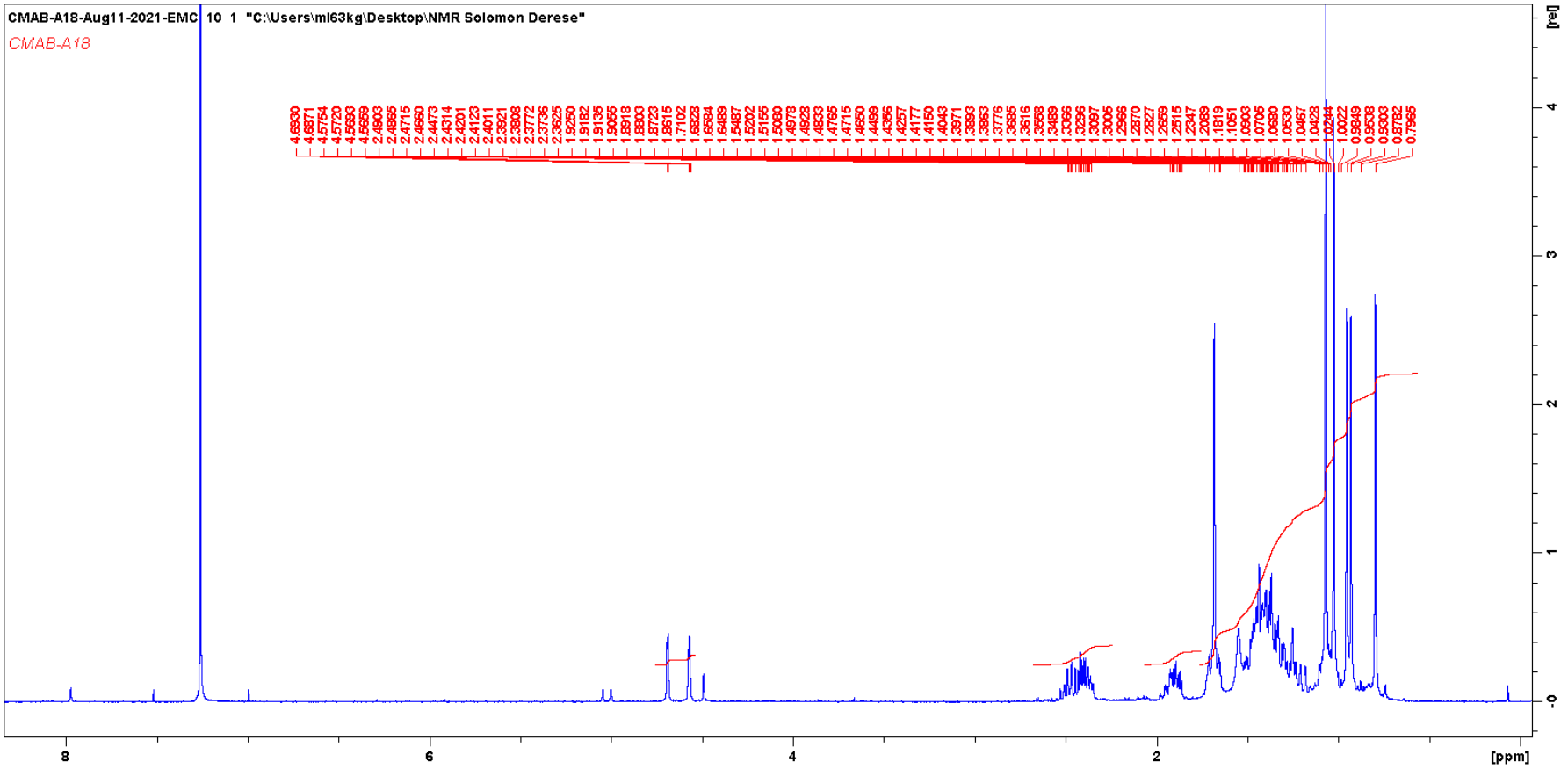
Appendix 68 HMBC spectrum of 2-Methoxy benzyl benzoate (A5)



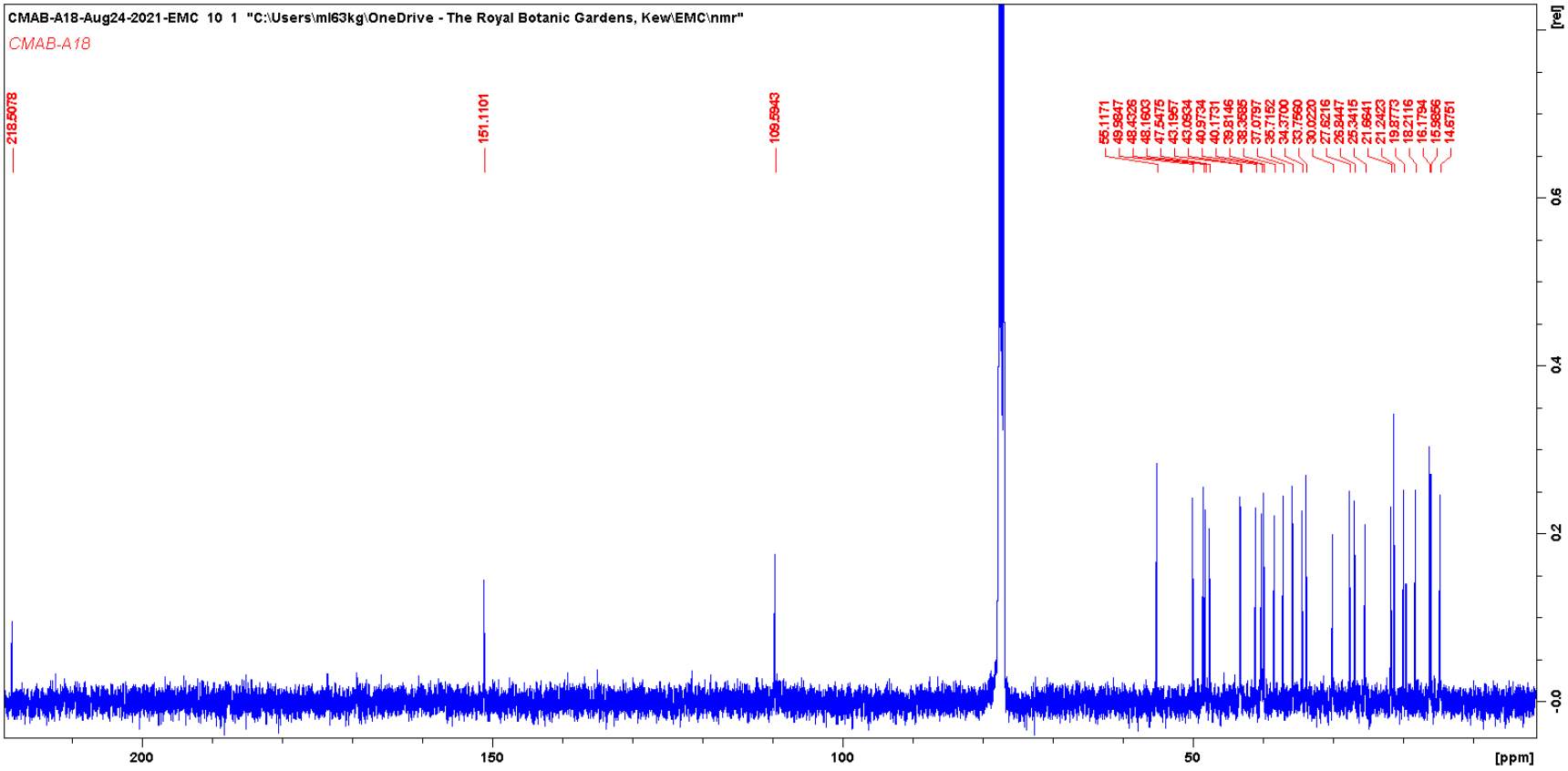
Appendix 69 COSY spectrum of 2-Methoxy benzyl benzoate (A5)



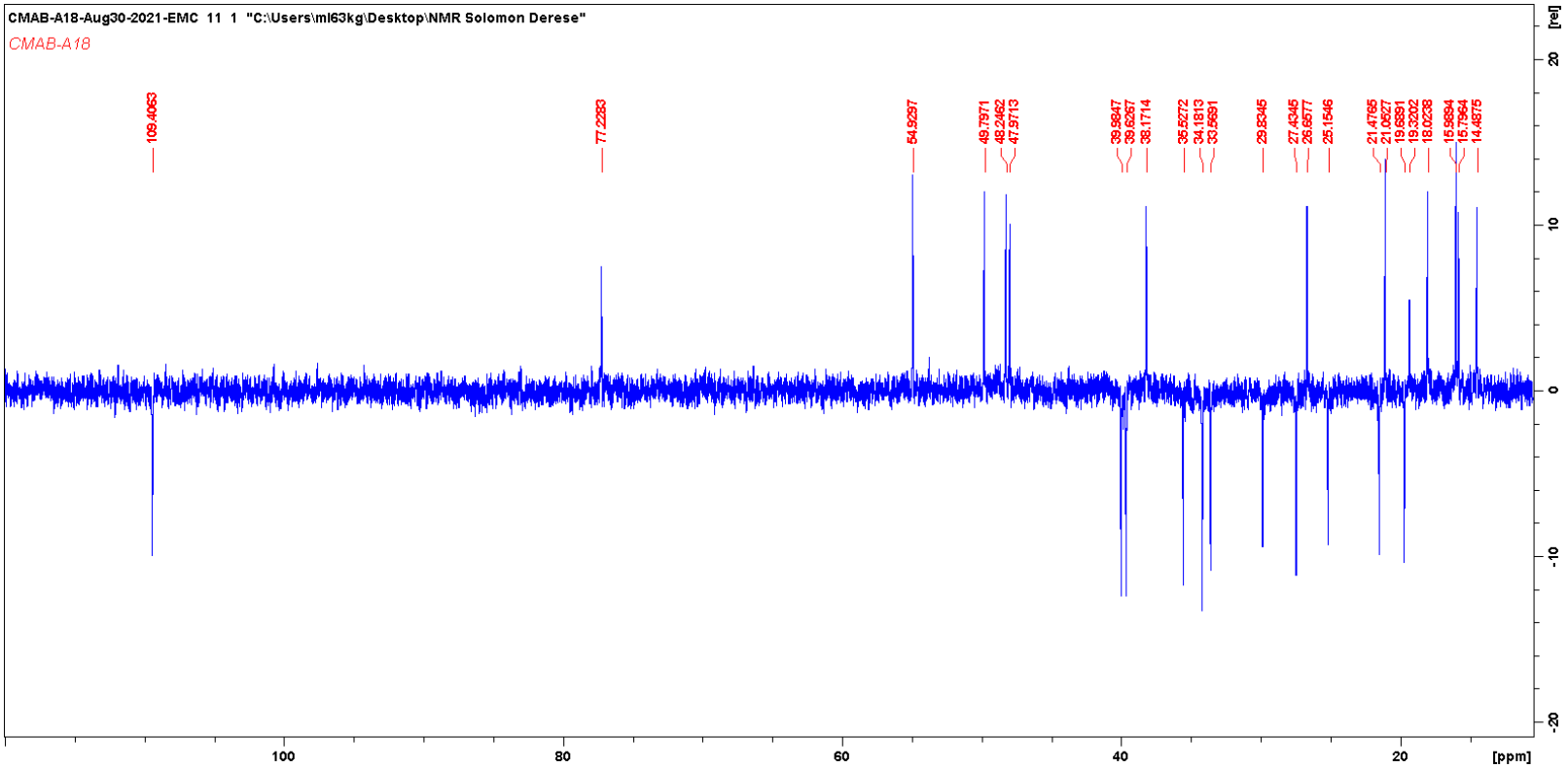
Appendix 70 $^{1}$H NMR spectrum of Lupenone (A4b)



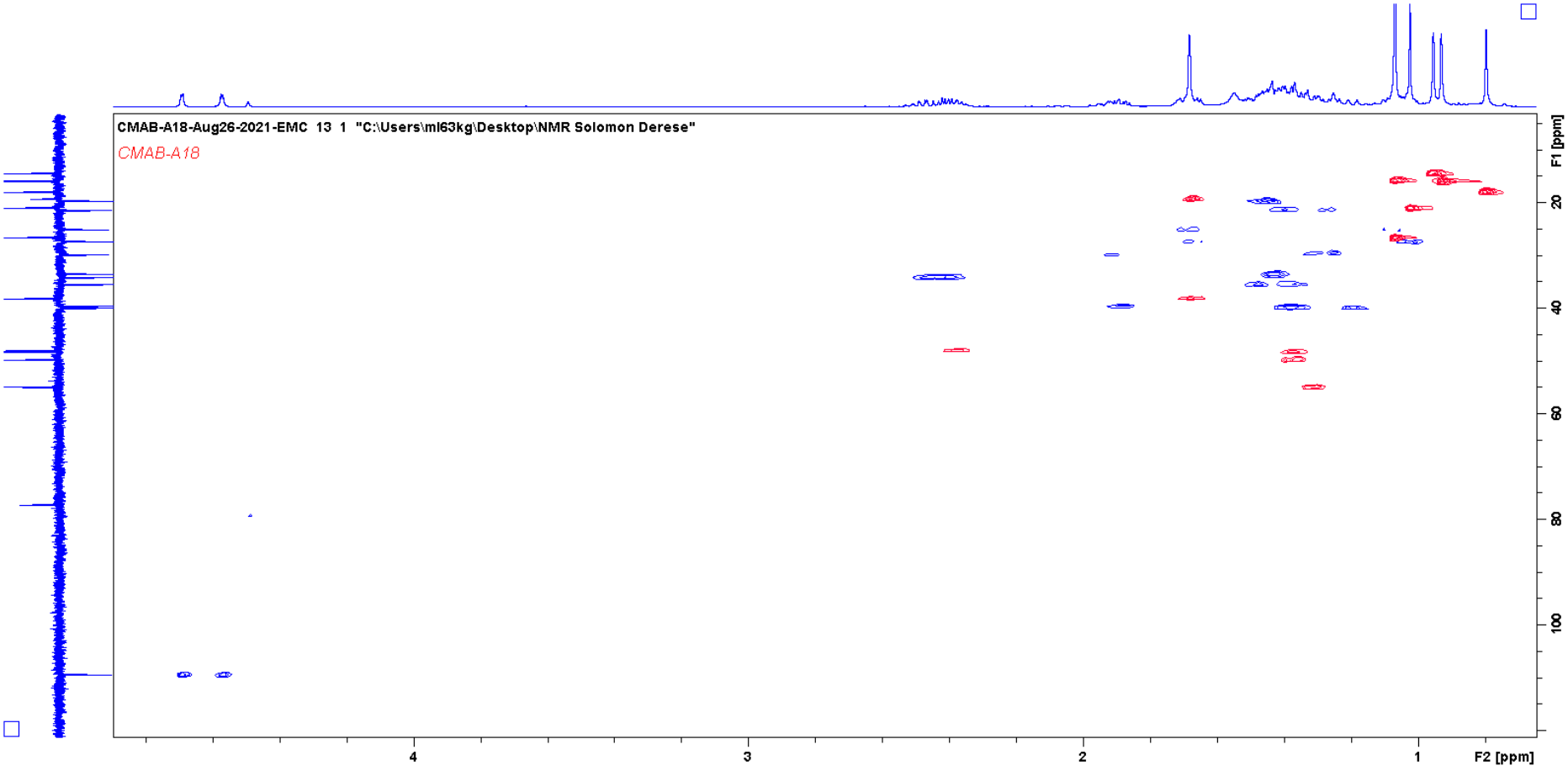
Appendix 71 $^{13}$C NMR spectrum of Lupenone (A4b)



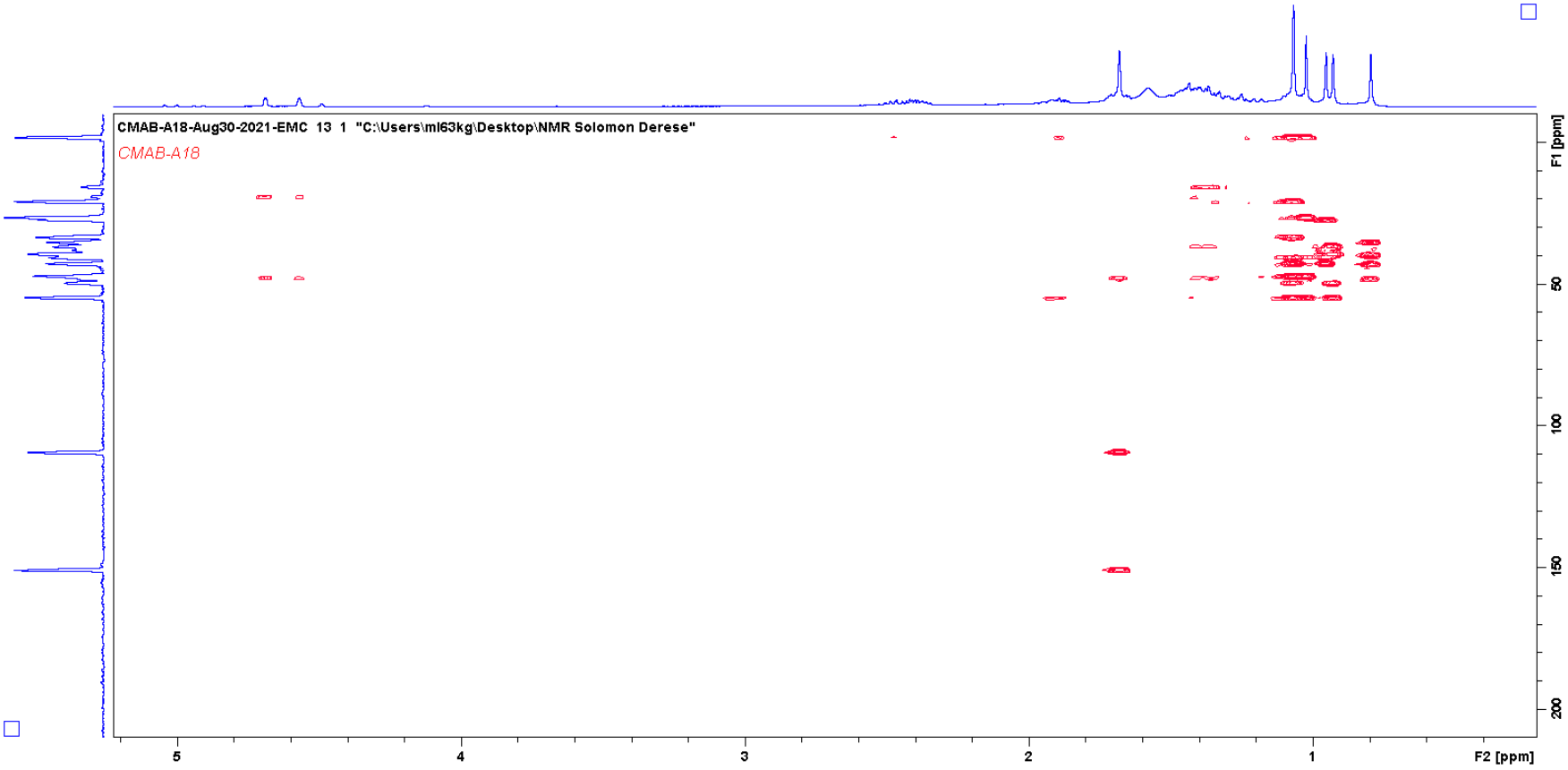
Appendix 72 DEPT spectrum of Lupenone (A4b)



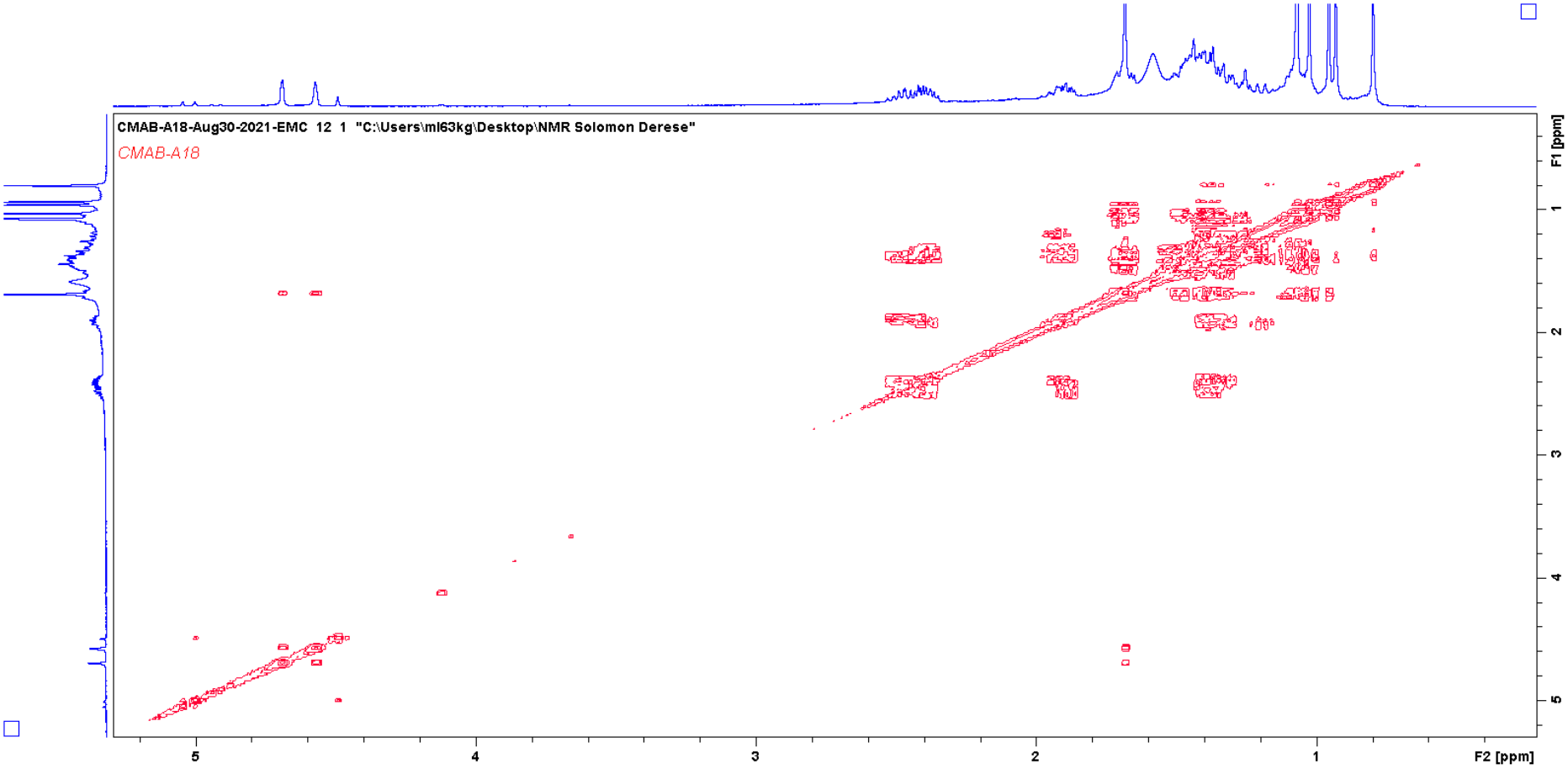
Appendix 73 HSQCDEPT spectrum of Lupenone (A4b)



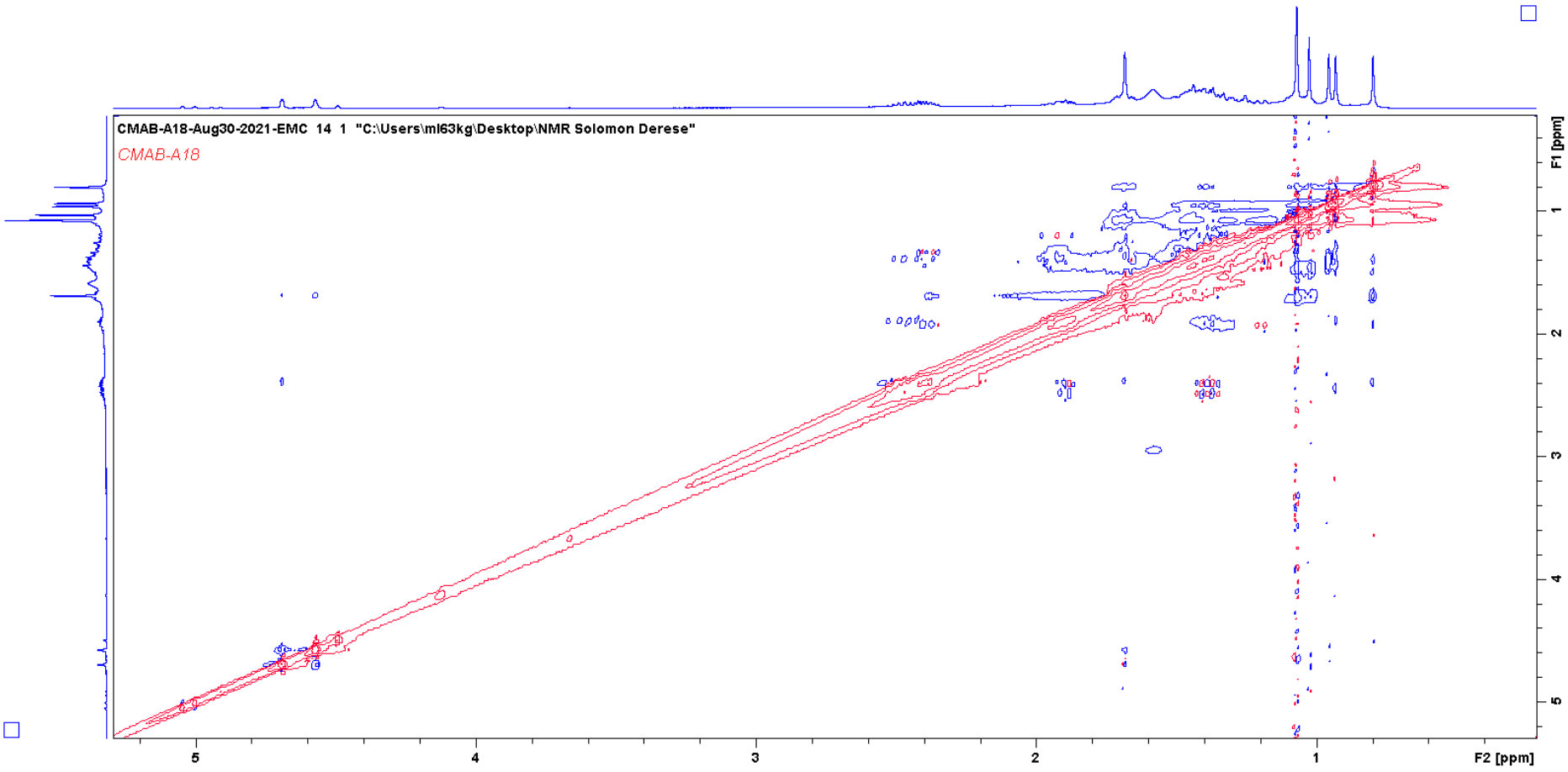
Appendix 74 HMBC spectrum of Lupenone (A4b)



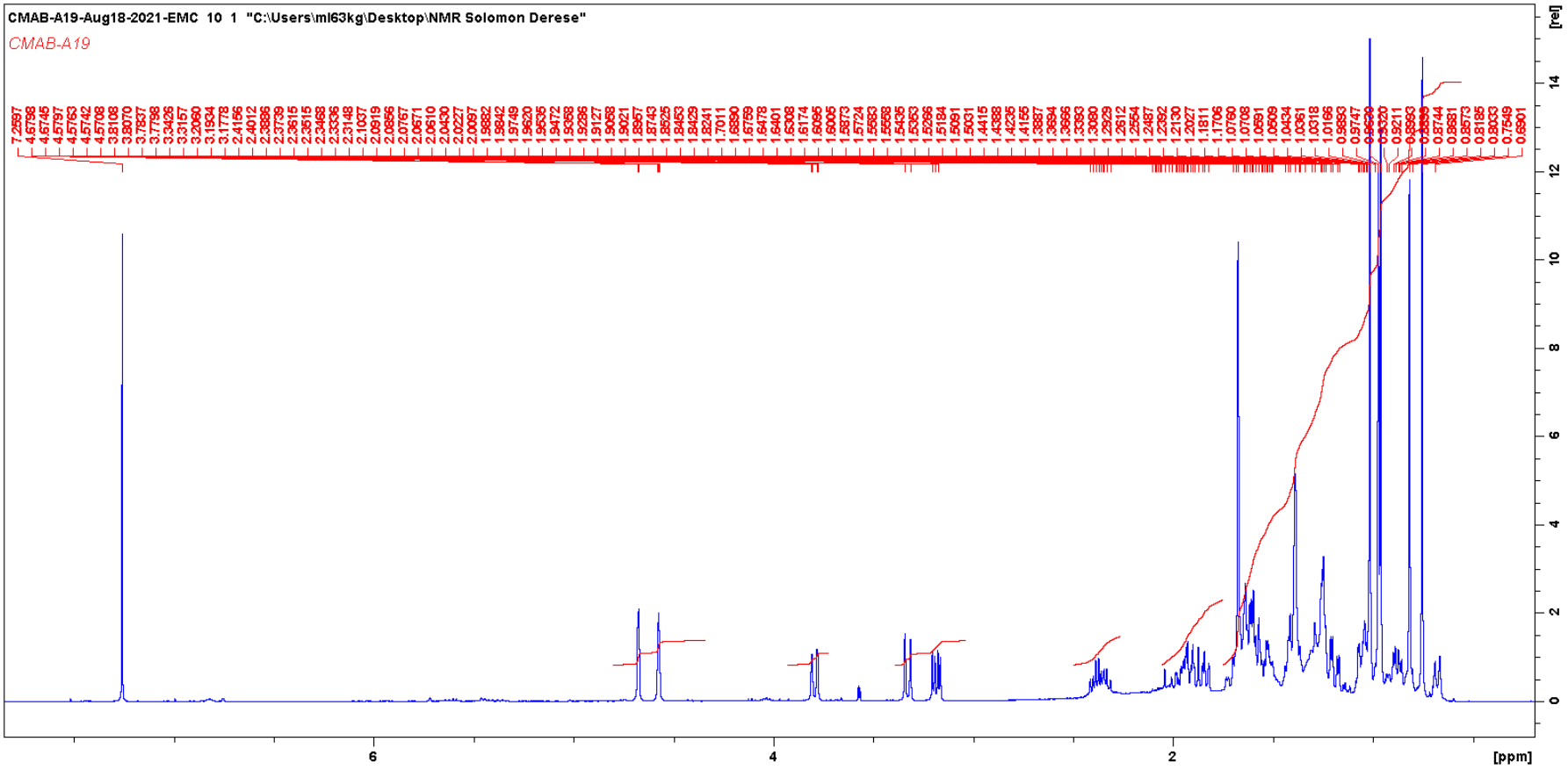
Appendix 75 COSY spectrum of Lupenone (A4b)



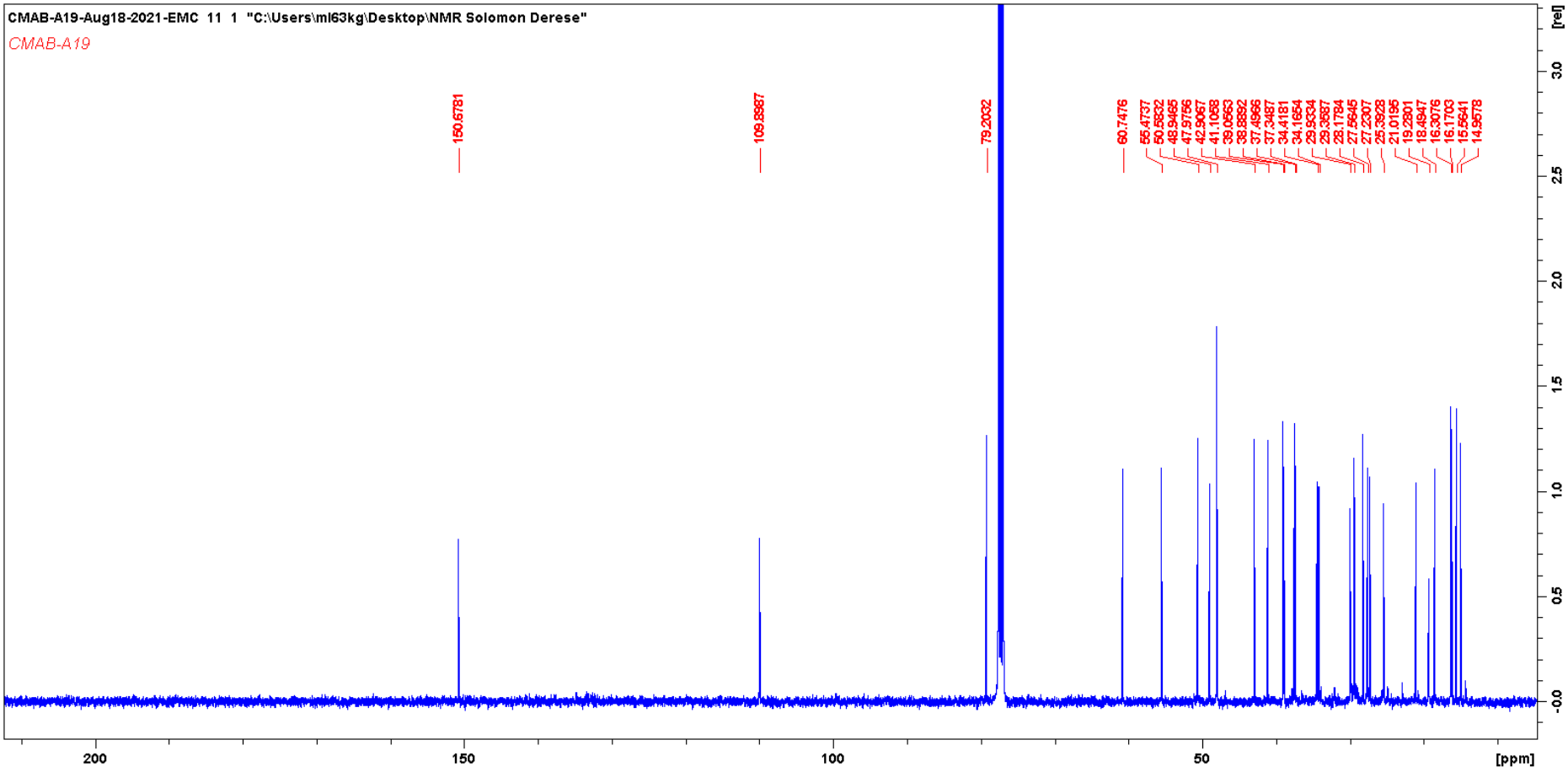
Appendix 76 NOESY spectrum of Lupenone (A4b)



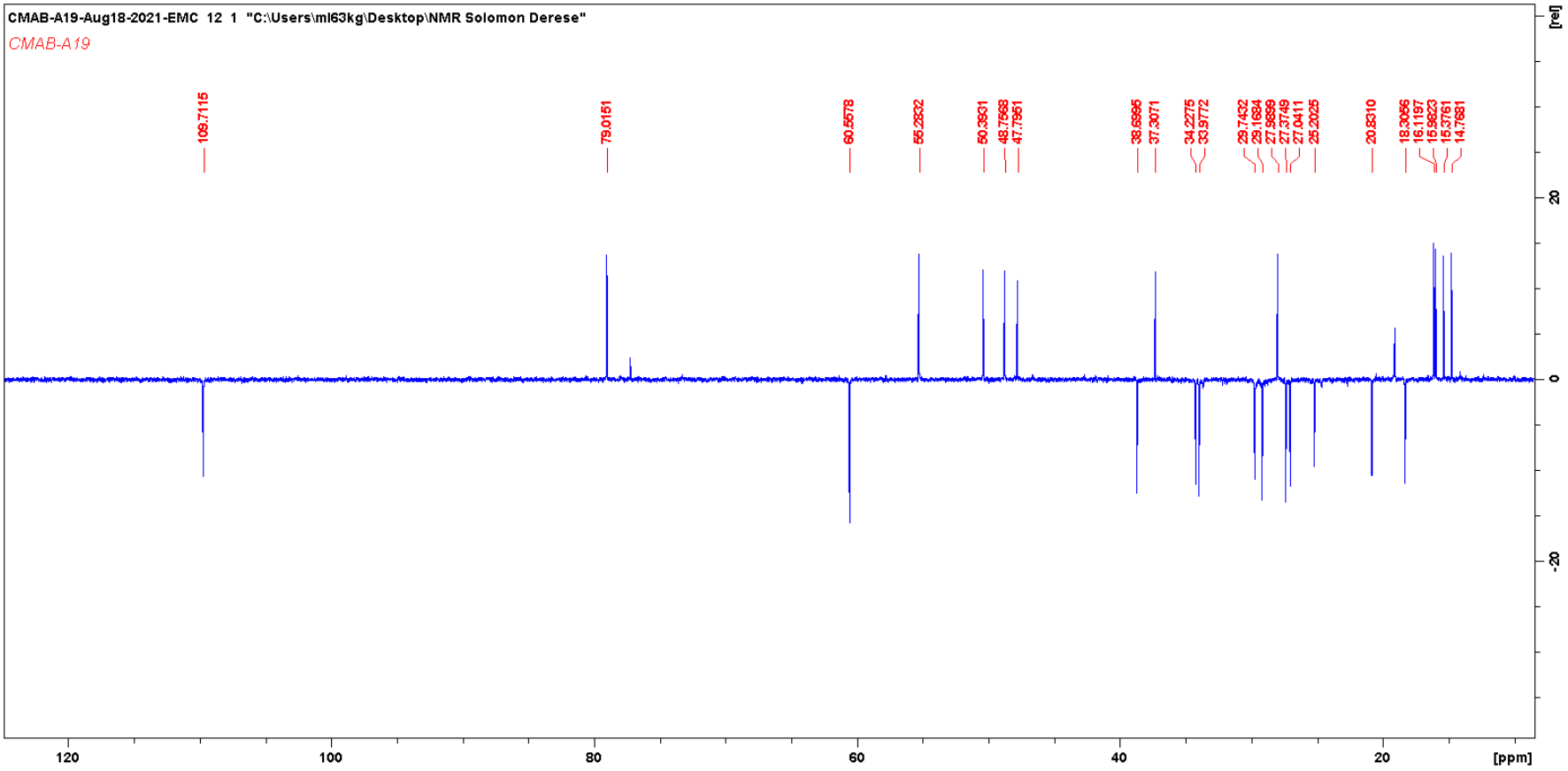
Appendix 77 $^{1}$H NMR spectrum of Betulin (A19)



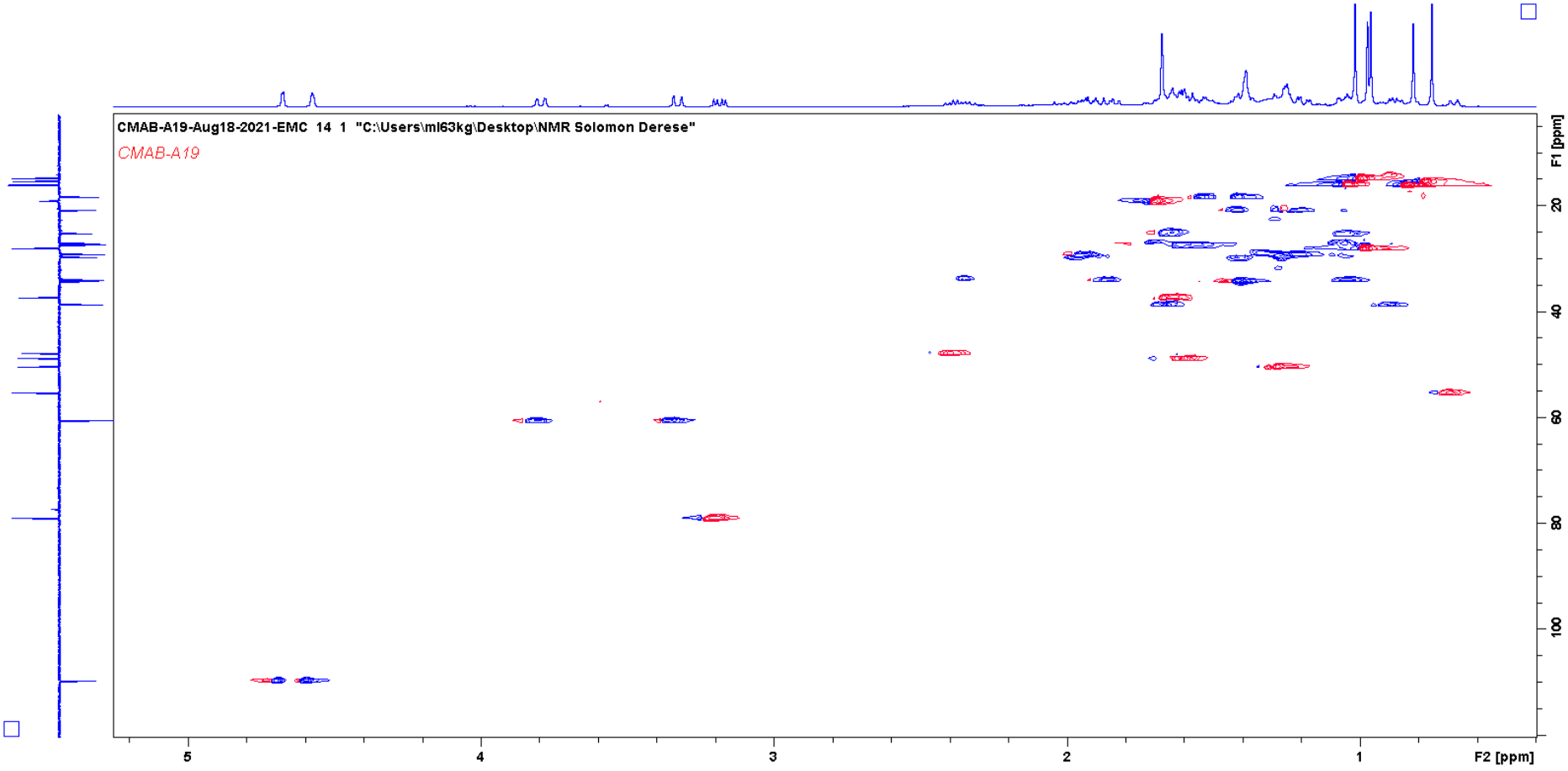
Appendix 78 $^{13}C$ NMR spectrum of Betulin (A19)



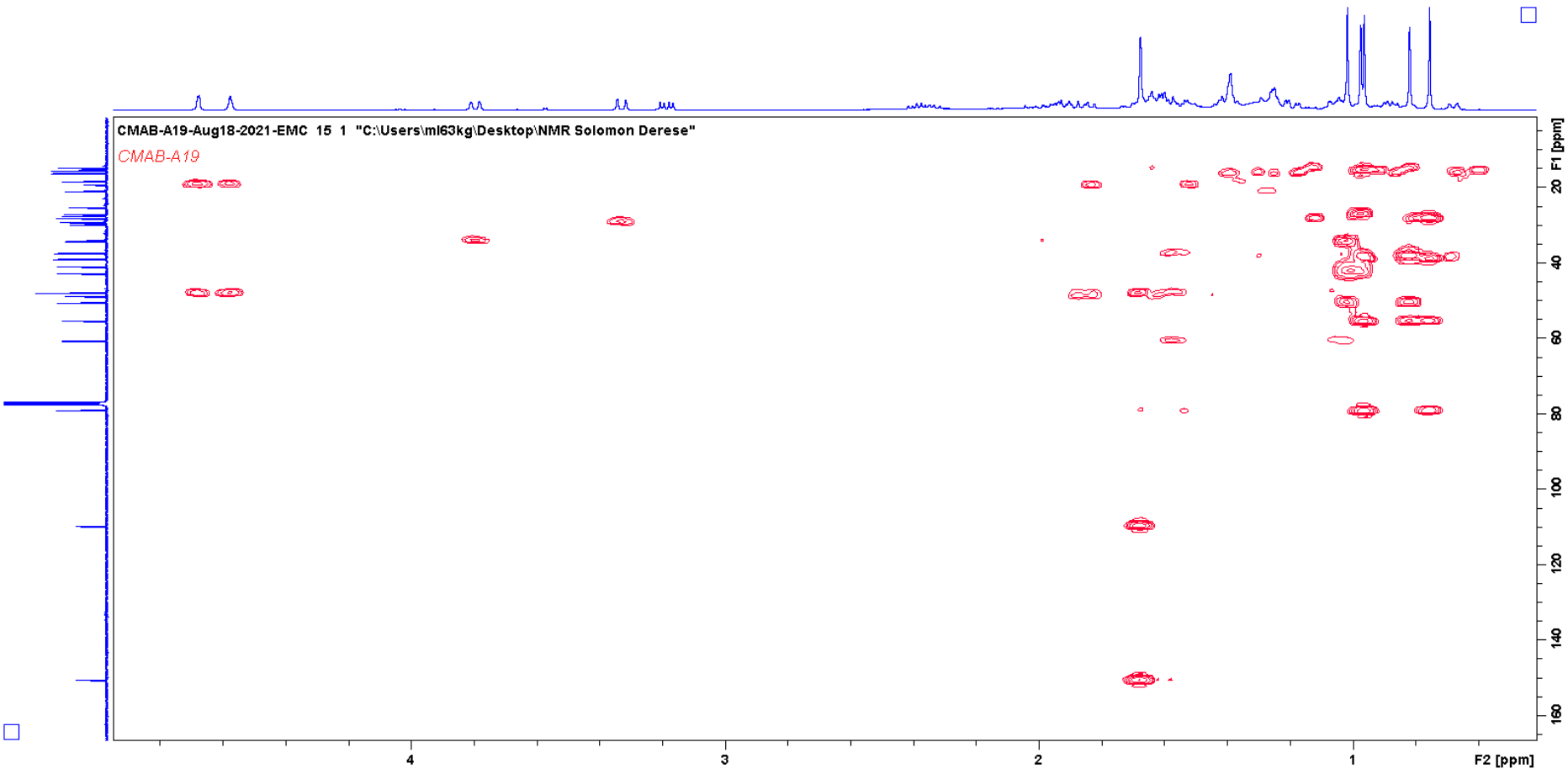
Appendix 79 DEPT spectrum of Betulin (A19)



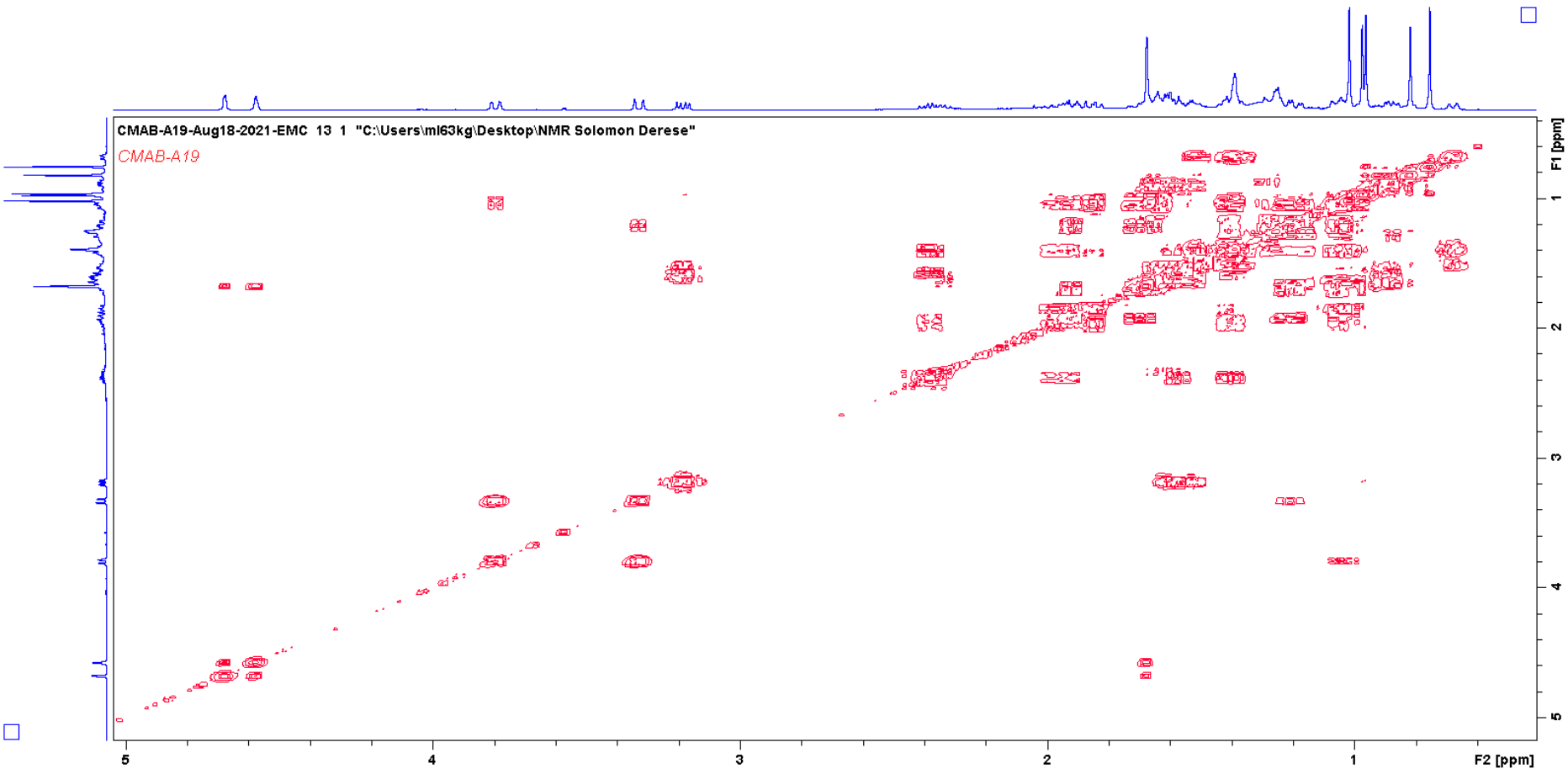
Appendix 80 HSQCDEPT spectrum of Betulin (A19)



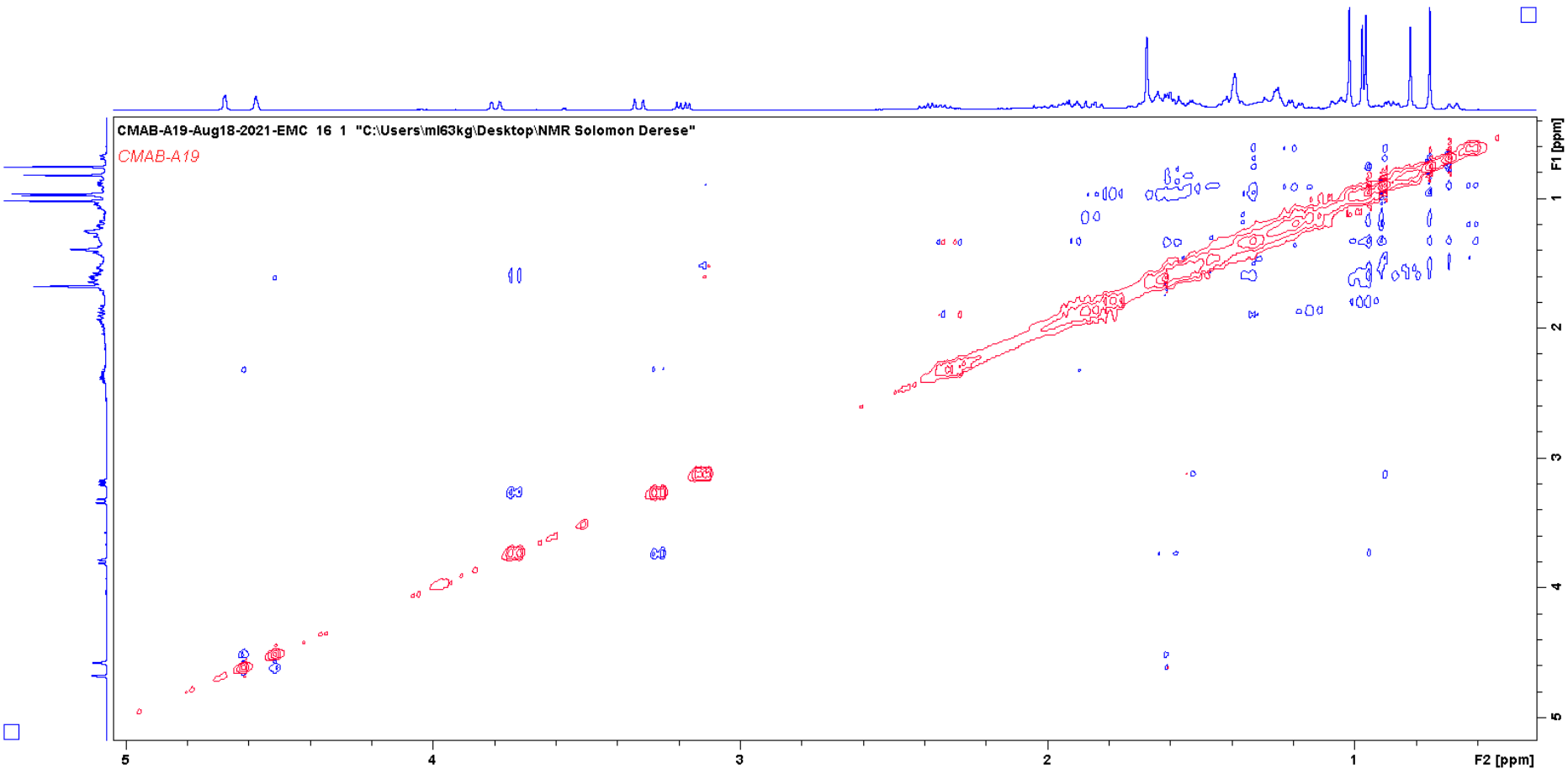
Appendix 81 HMBC spectrum of Betulin (A19)



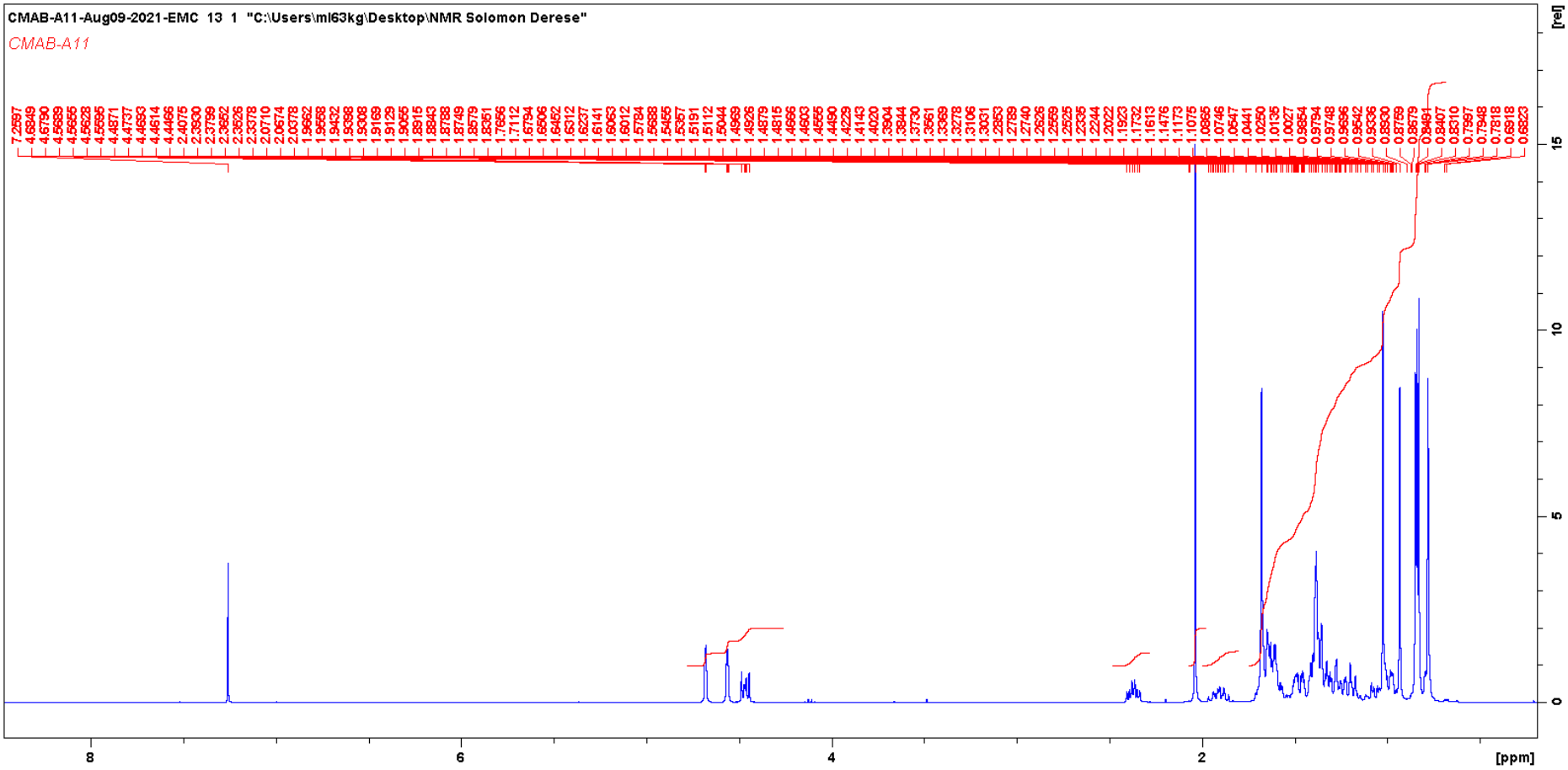
Appendix 82 COSY spectrum of Betulin (A19)



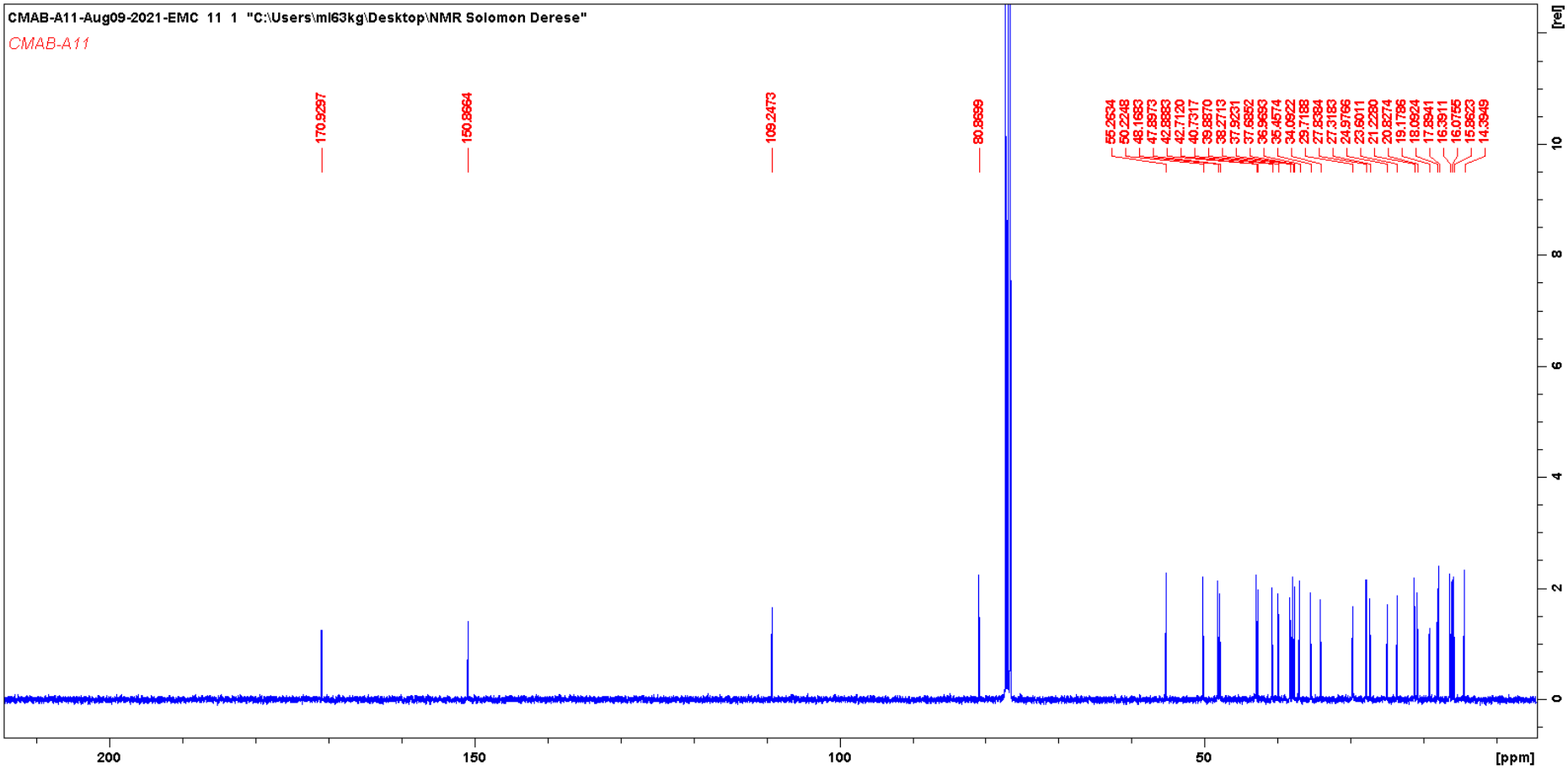
Appendix 83 NOSEY spectrum of Betulin (A19)



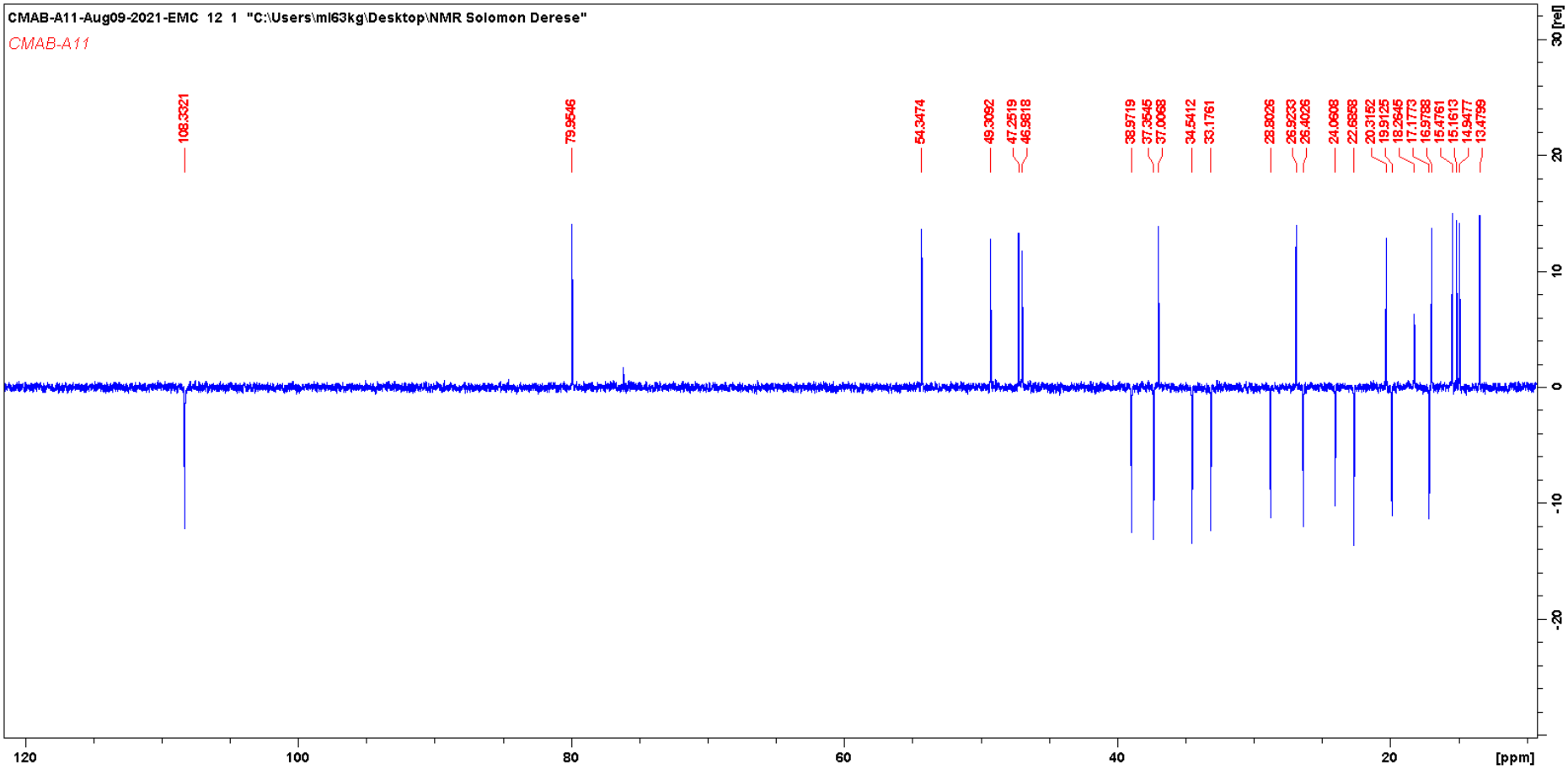
Appendix 84 $^{1}$H NMR spectrum of Lupeol acetate (A11)



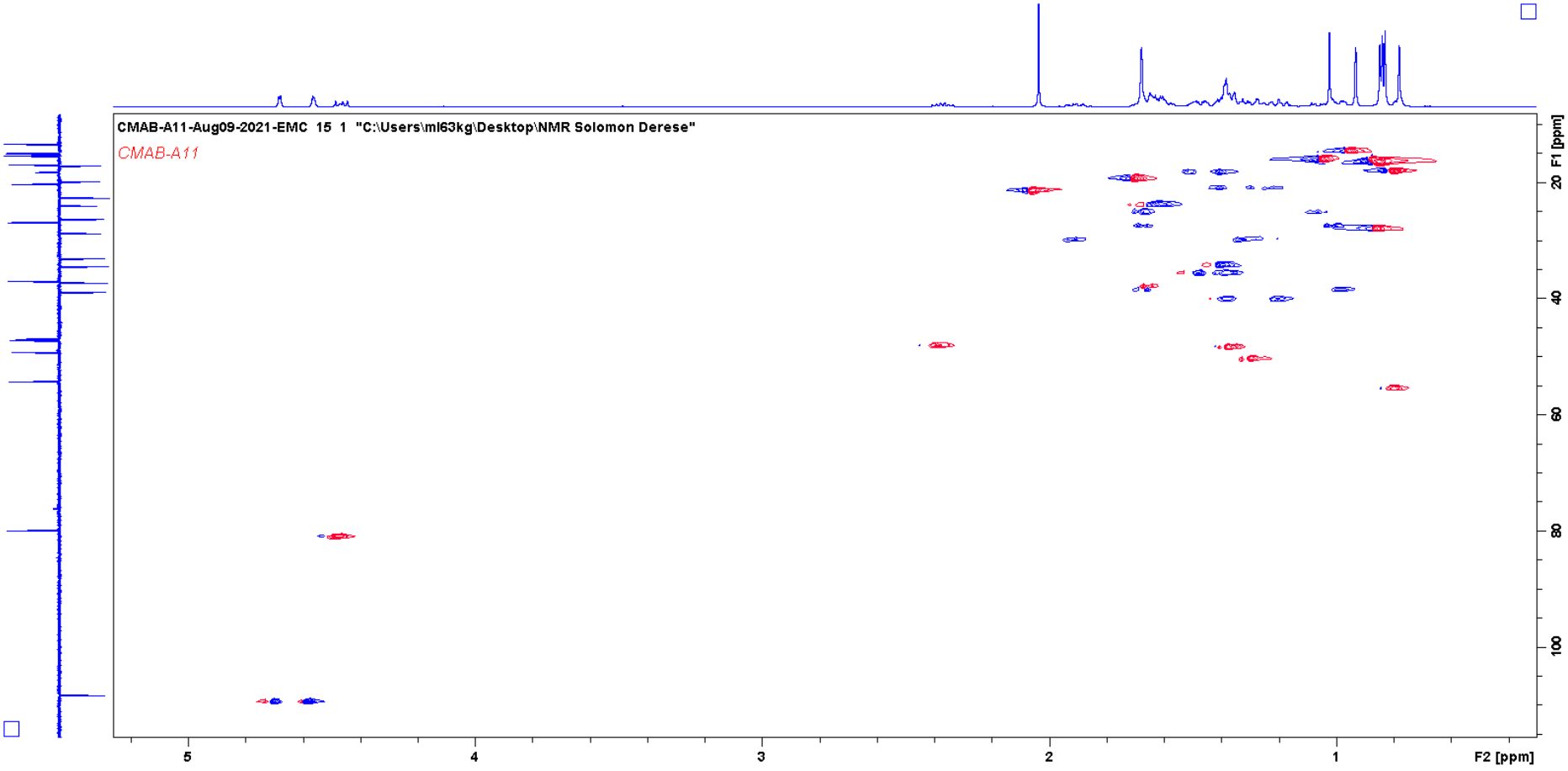
Appendix 85 $^{13}$C NMR spectrum of Lupeol acetate (A11)



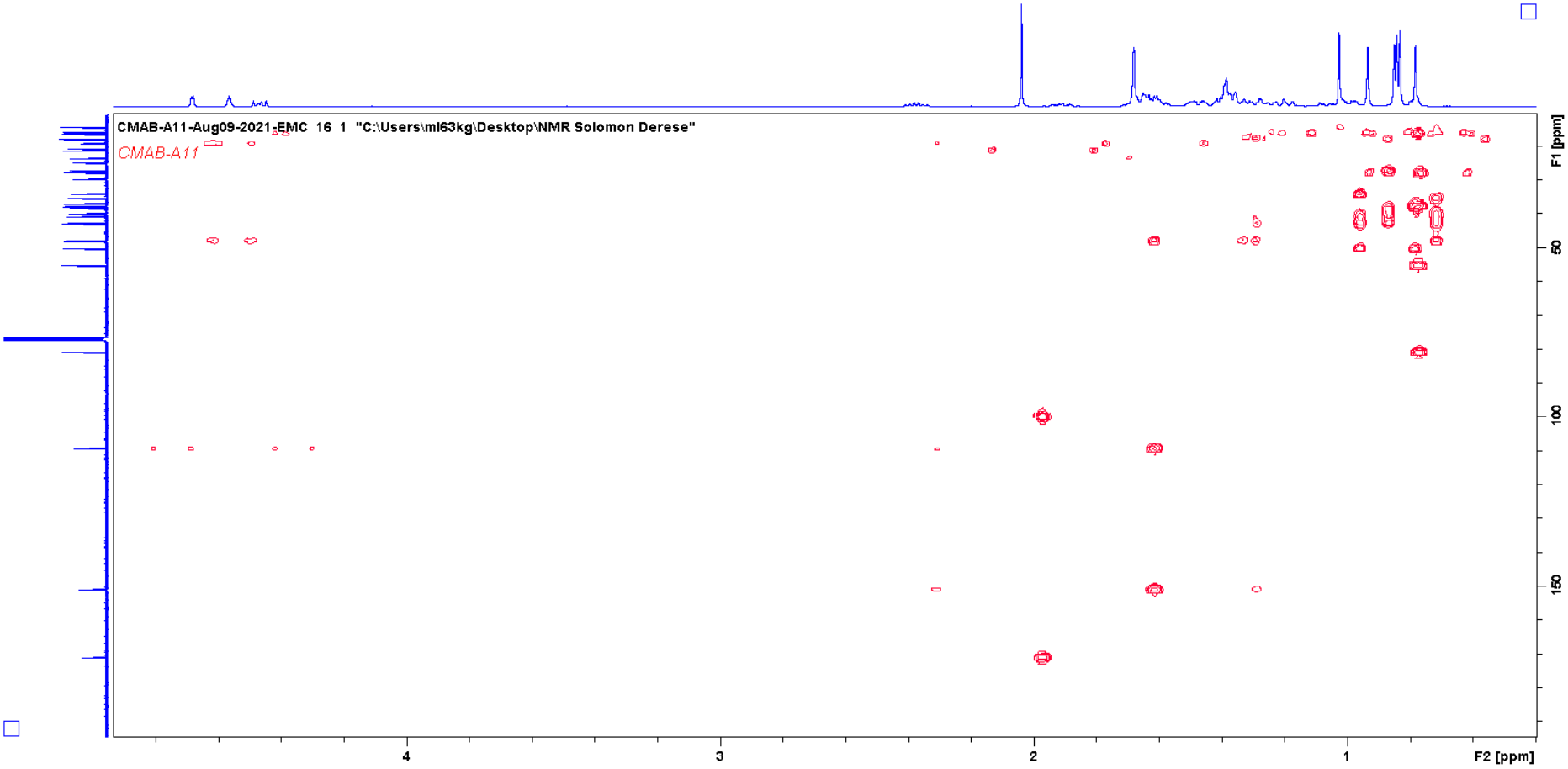
Appendix 86 DEPT spectrum of Lupeol acetate (A11)



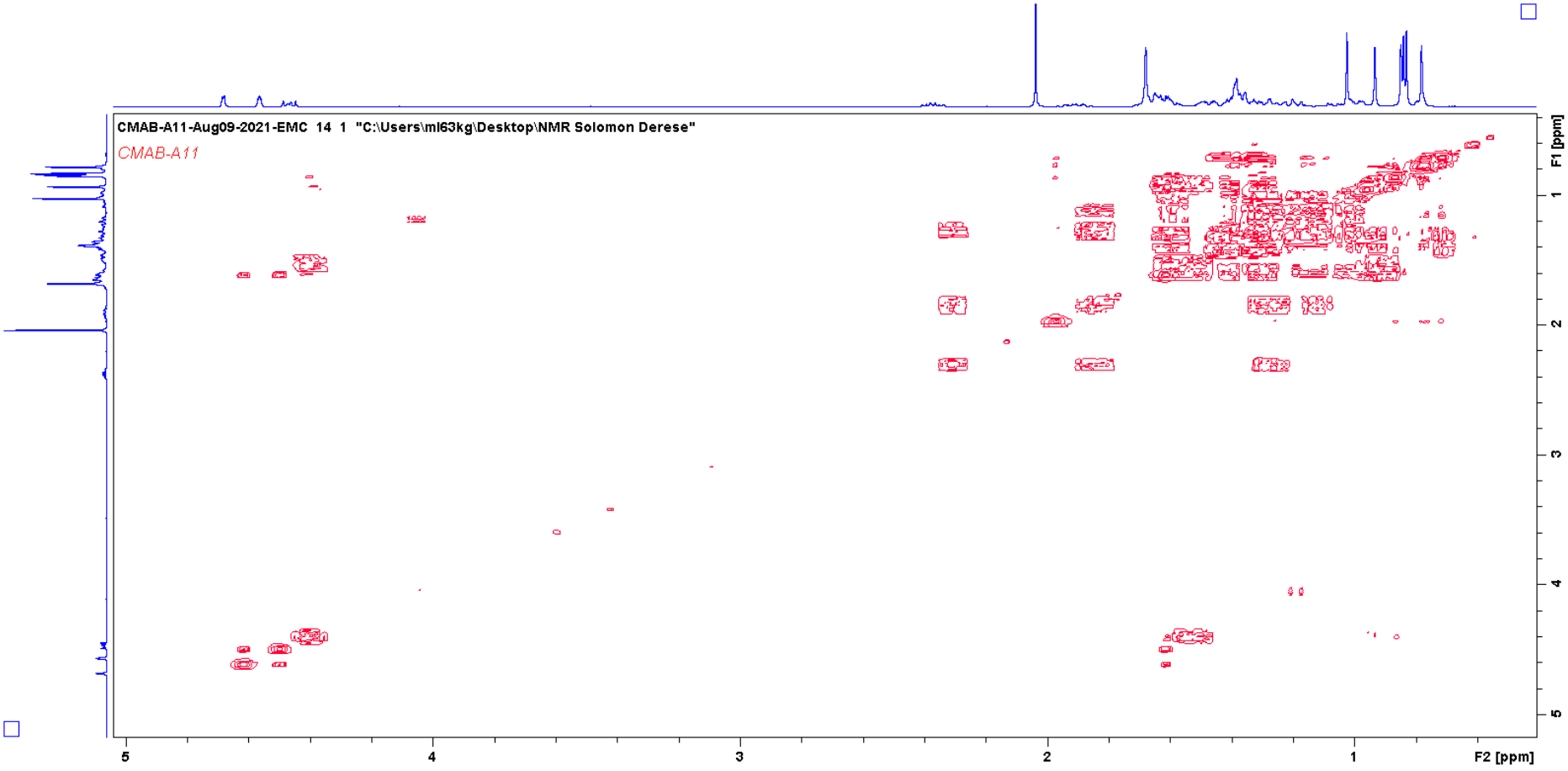
Appendix 87 HSQCDEPT spectrum of Lupeol acetate (A11)



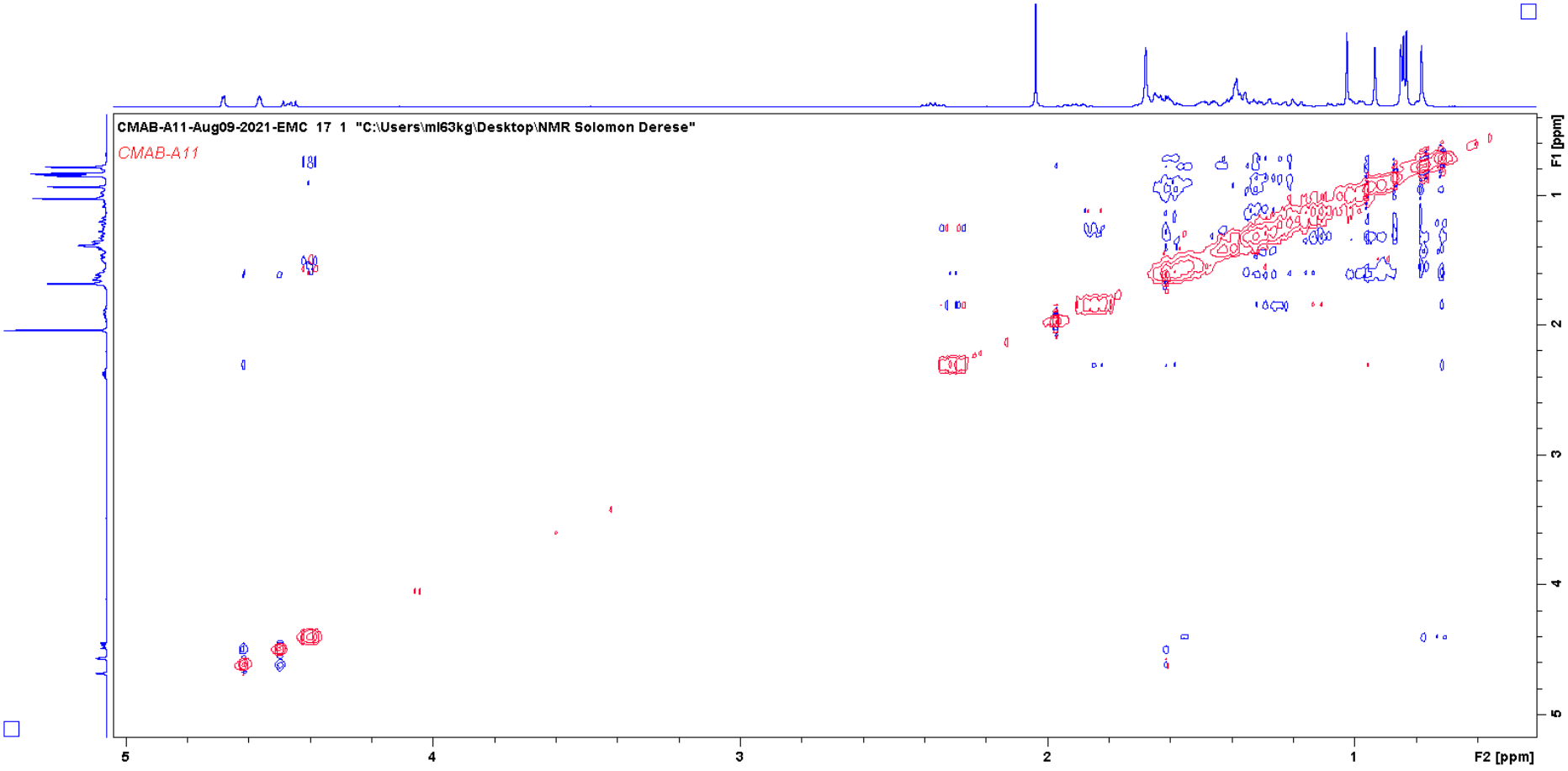
Appendix 88 HMBC spectrum of Lupeol acetate (A11)



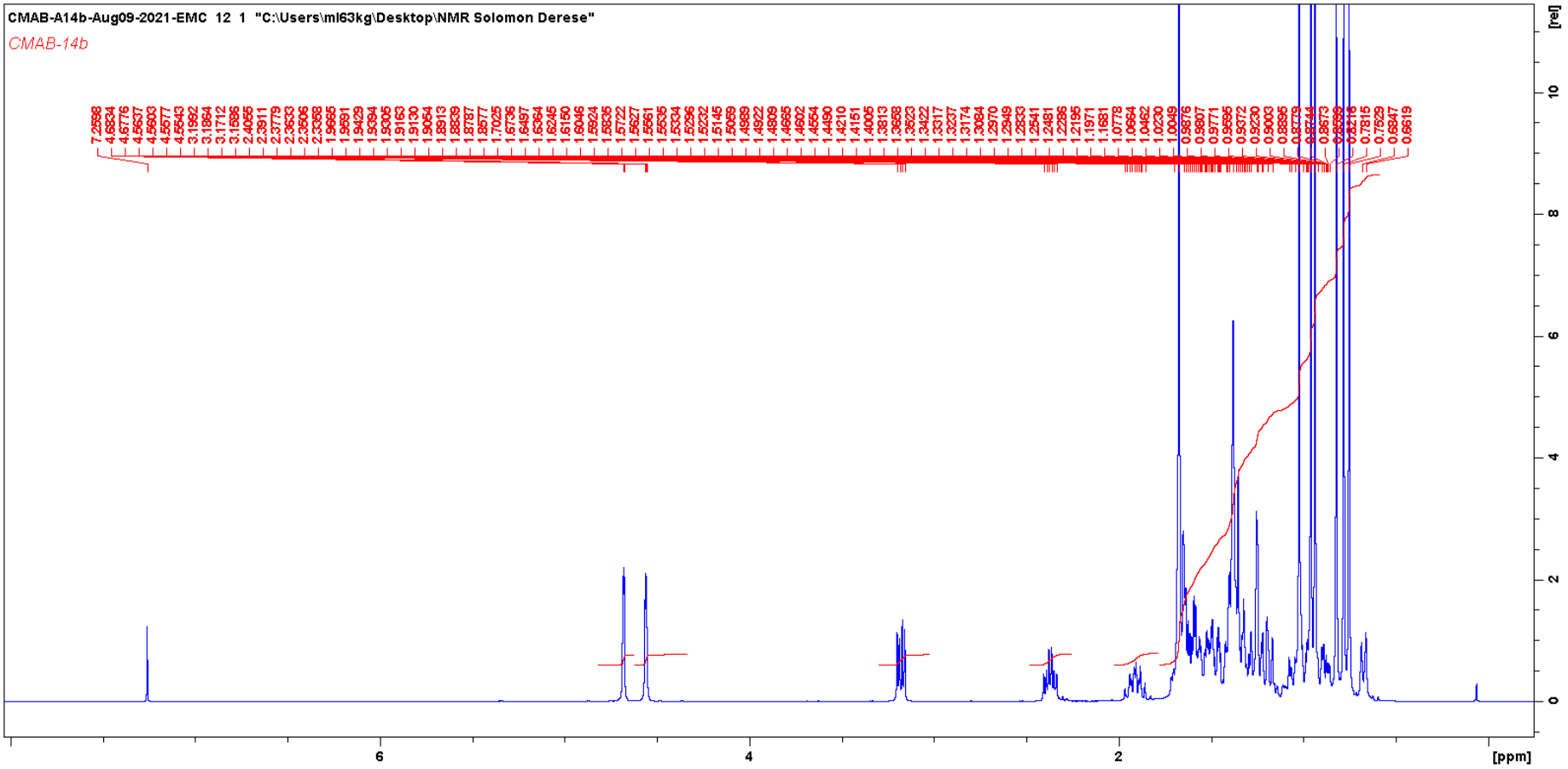
Appendix 89 COSY spectrum of Lupeol acetate (A11)



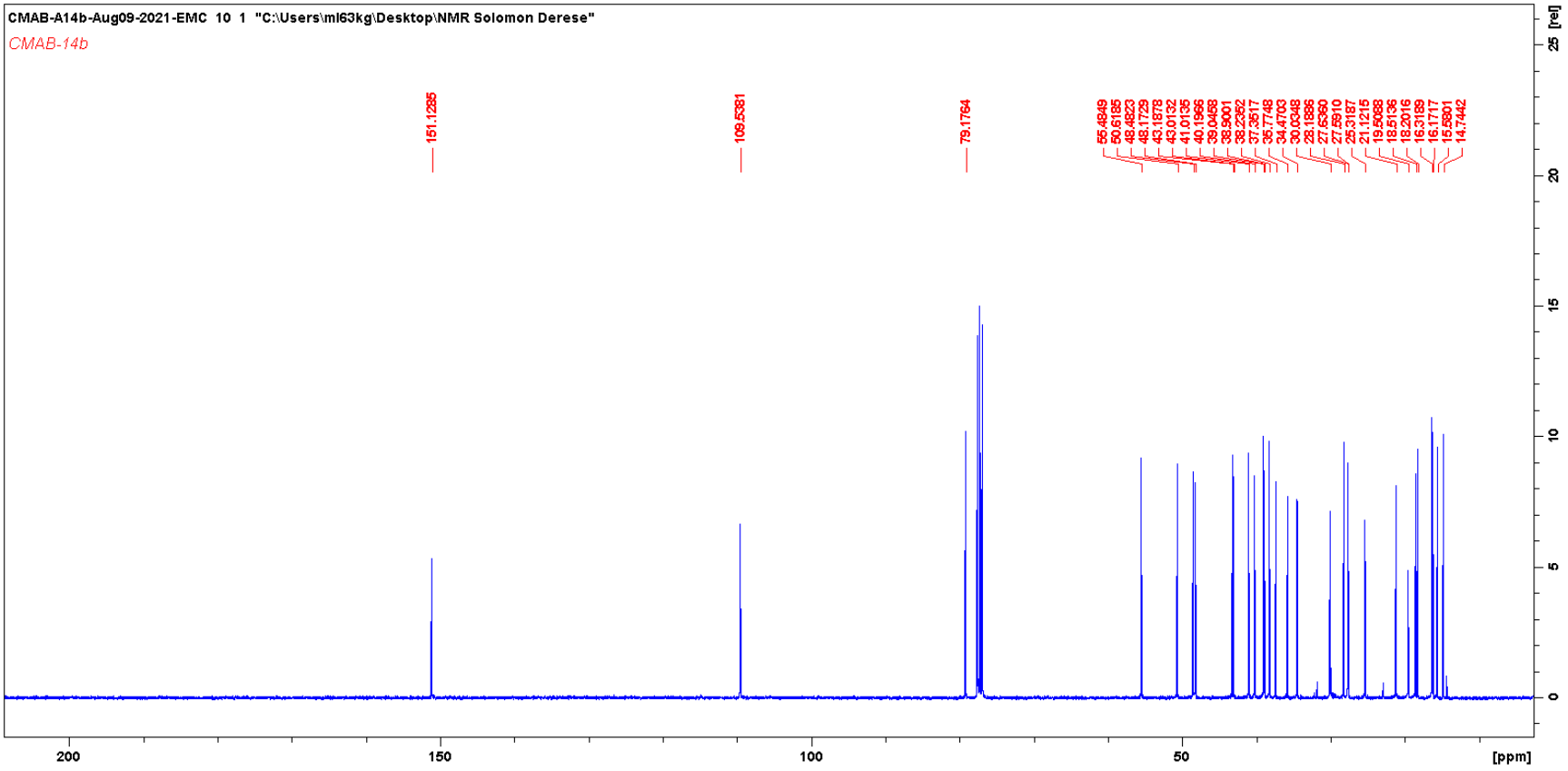
Appendix 90 NOSEY spectrum of Lupeol acetate (A11)



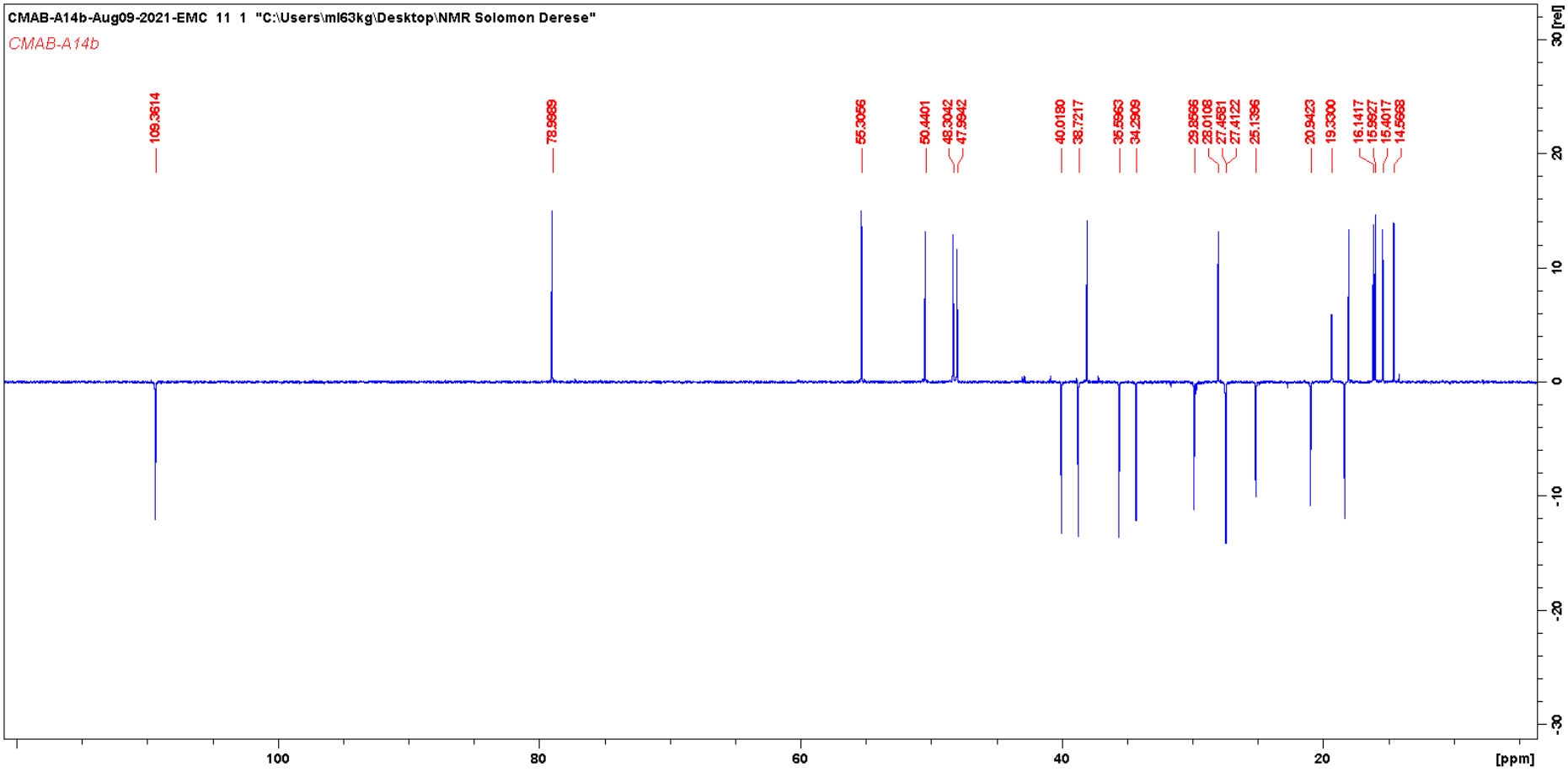
Appendix 91 $^{1}$H NMR spectrum of Lupeol (A7)



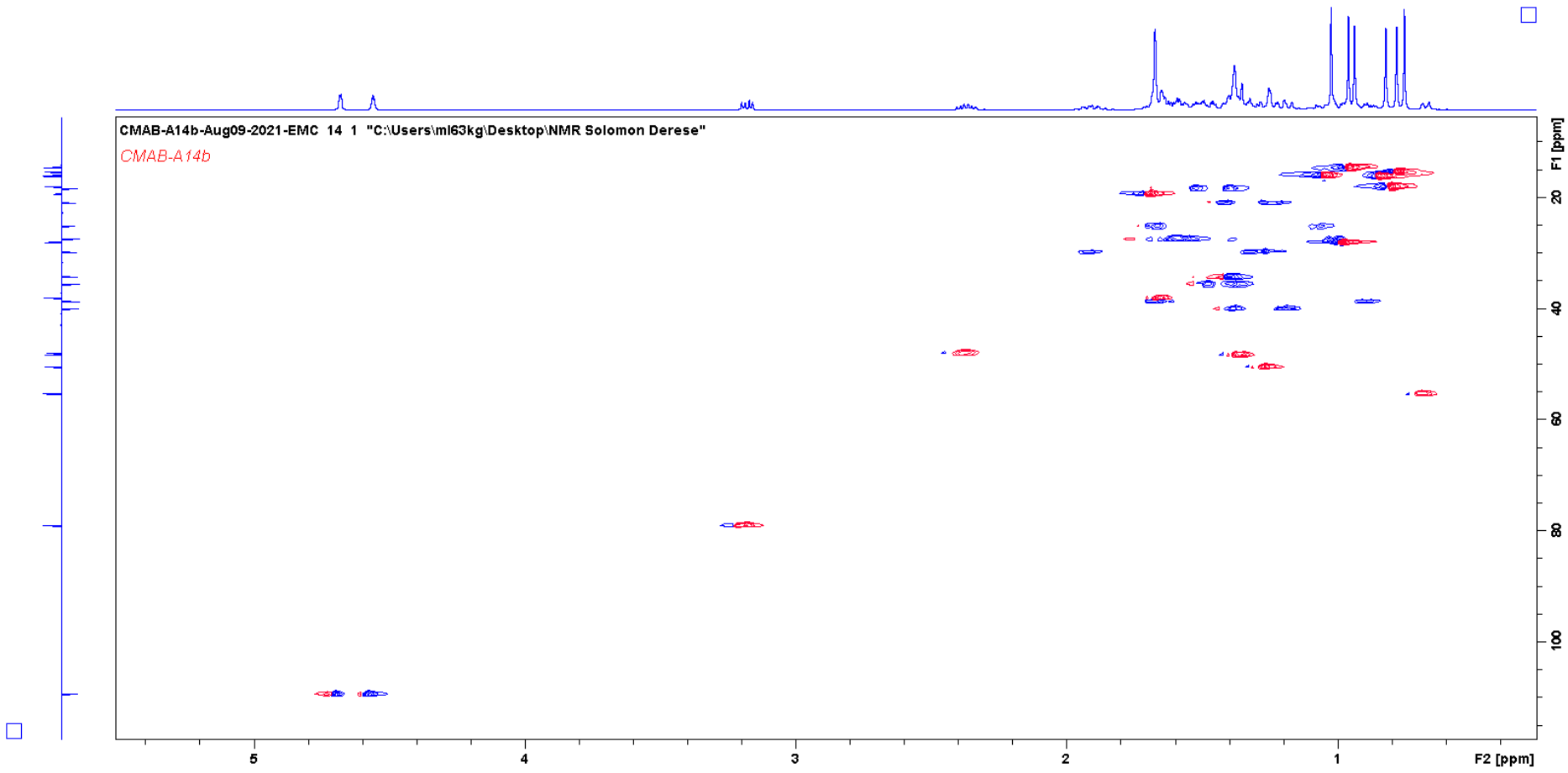
Appendix 92 $^{13}$C NMR spectrum of Lupeol (A7)



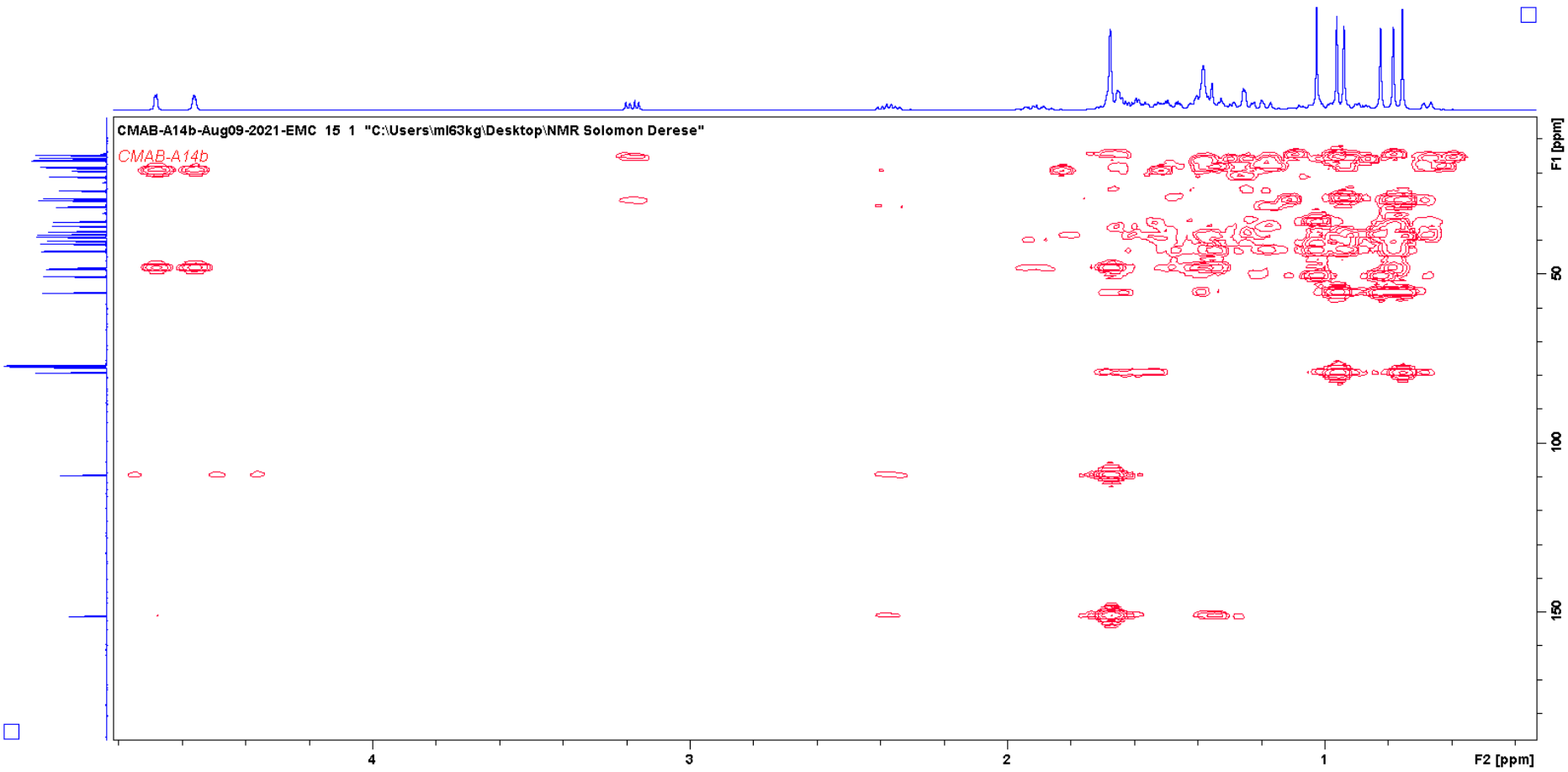
Appendix 93 DEPT spectrum of Lupeol (A7)



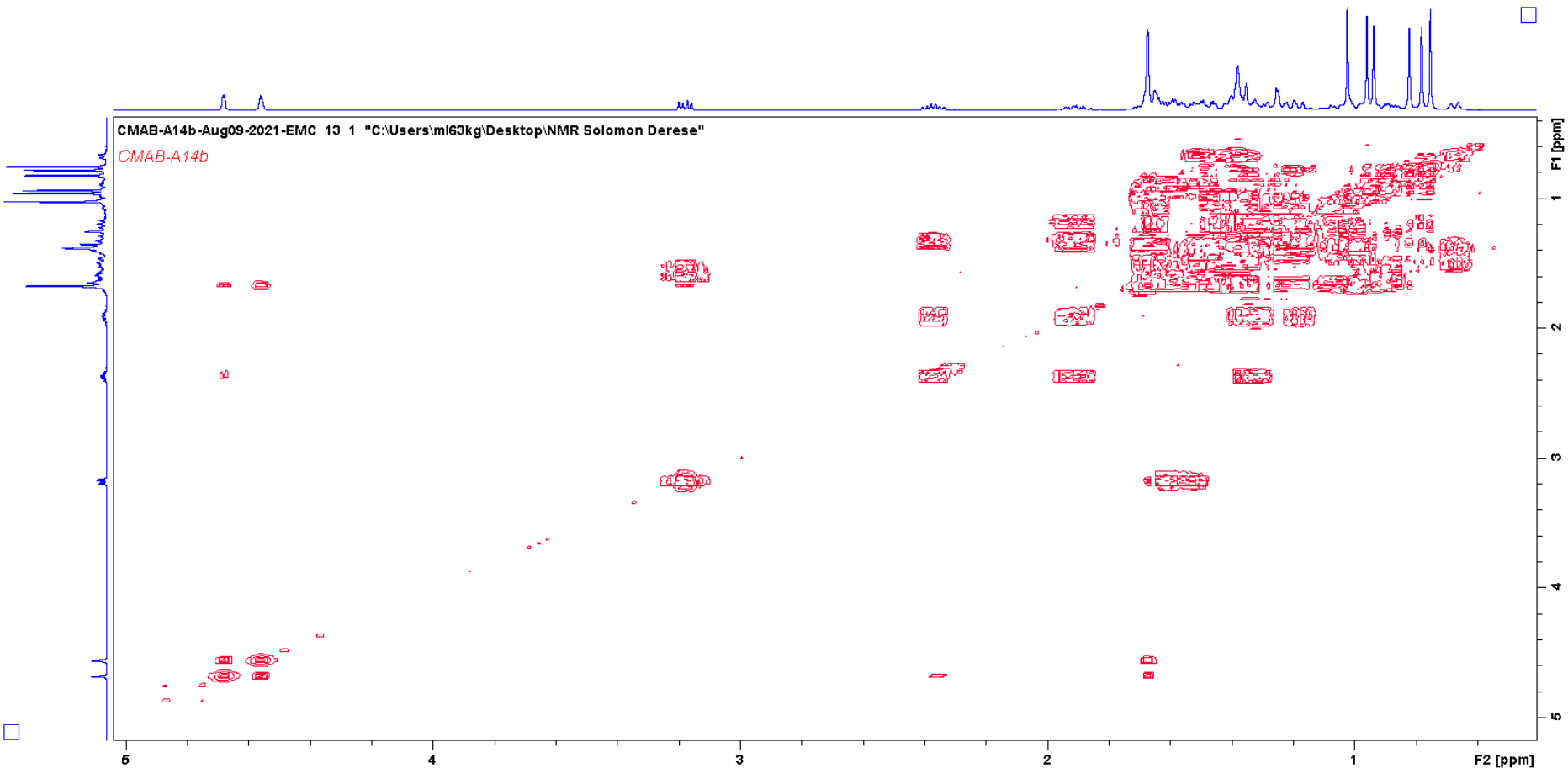
Appendix 94  HSQCDEPT spectrum of Lupeol (A7)



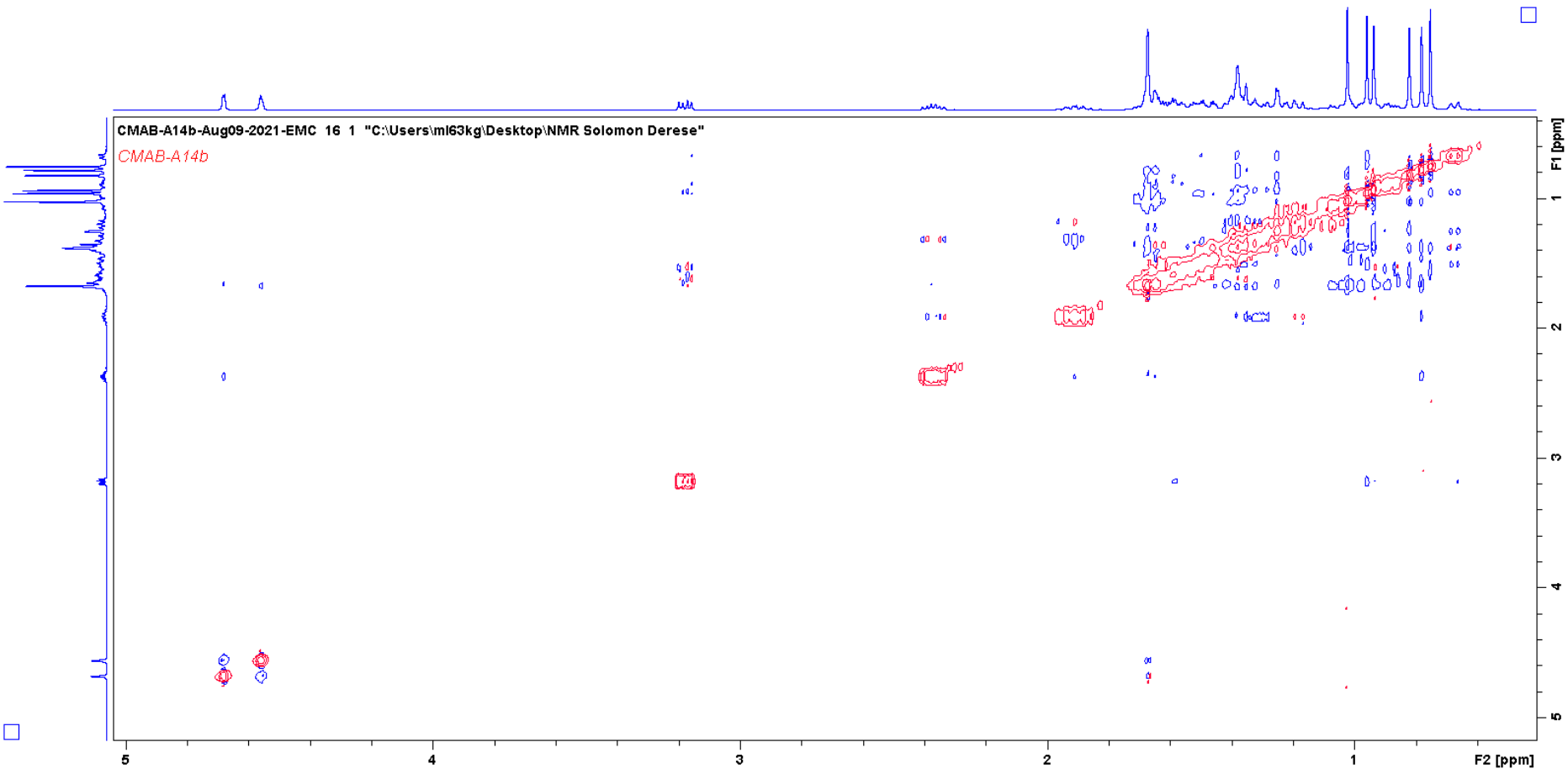
Appendix 95  HMBC spectrum of Lupeol (A7)



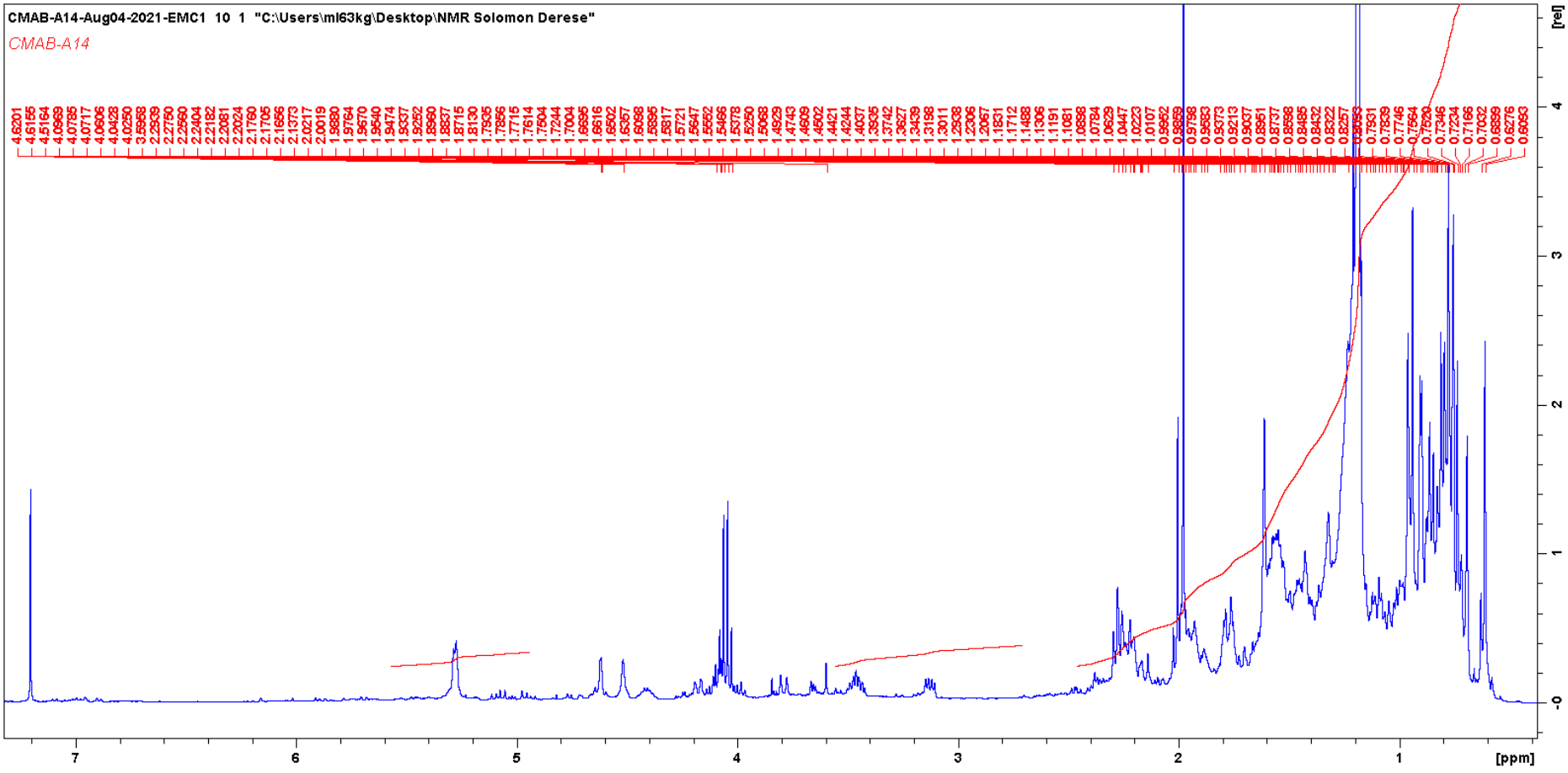
Appendix 96 COSY spectrum of Lupeol (A7)



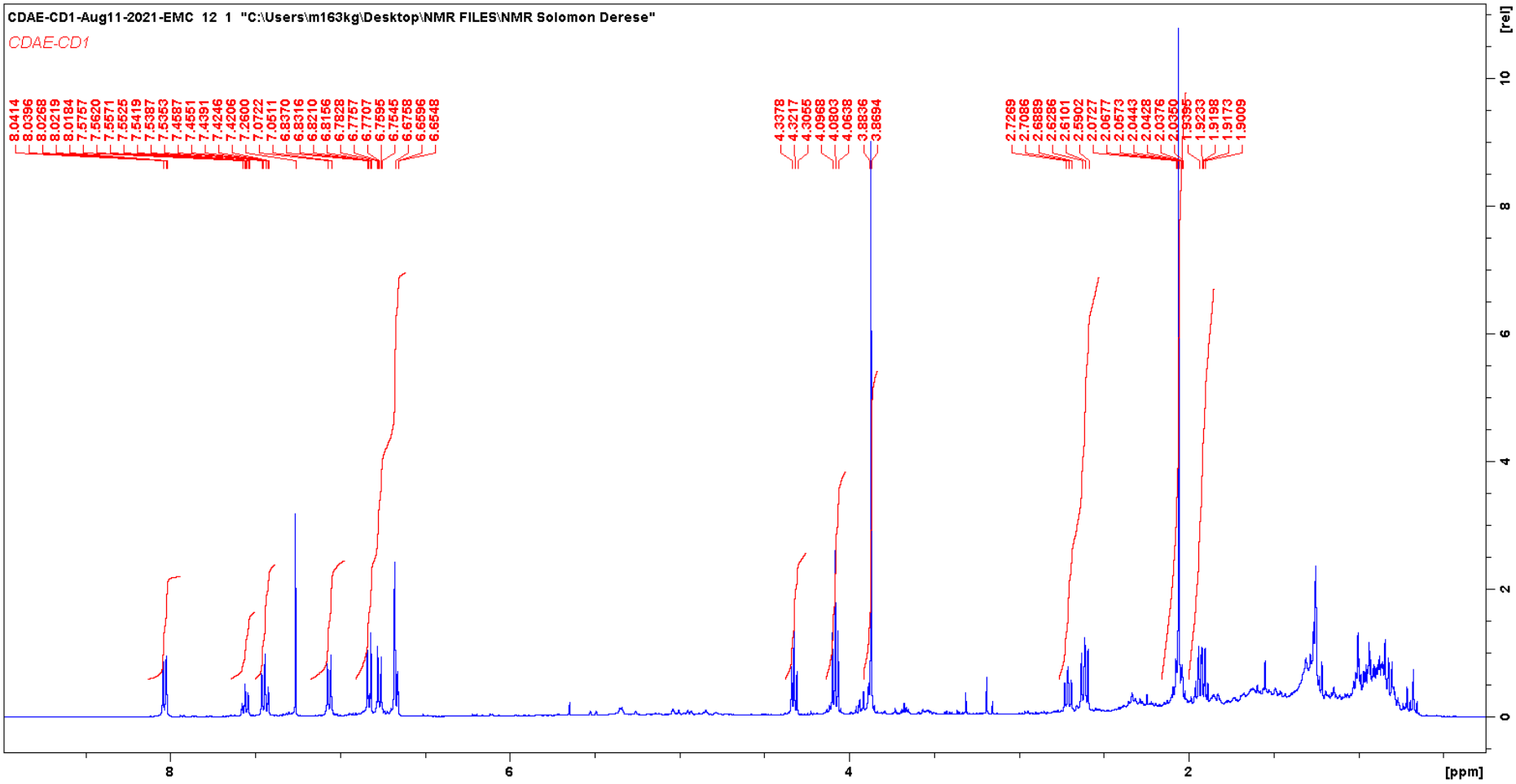
Appendix 97  NOESY spectrum of Lupeol (A7)



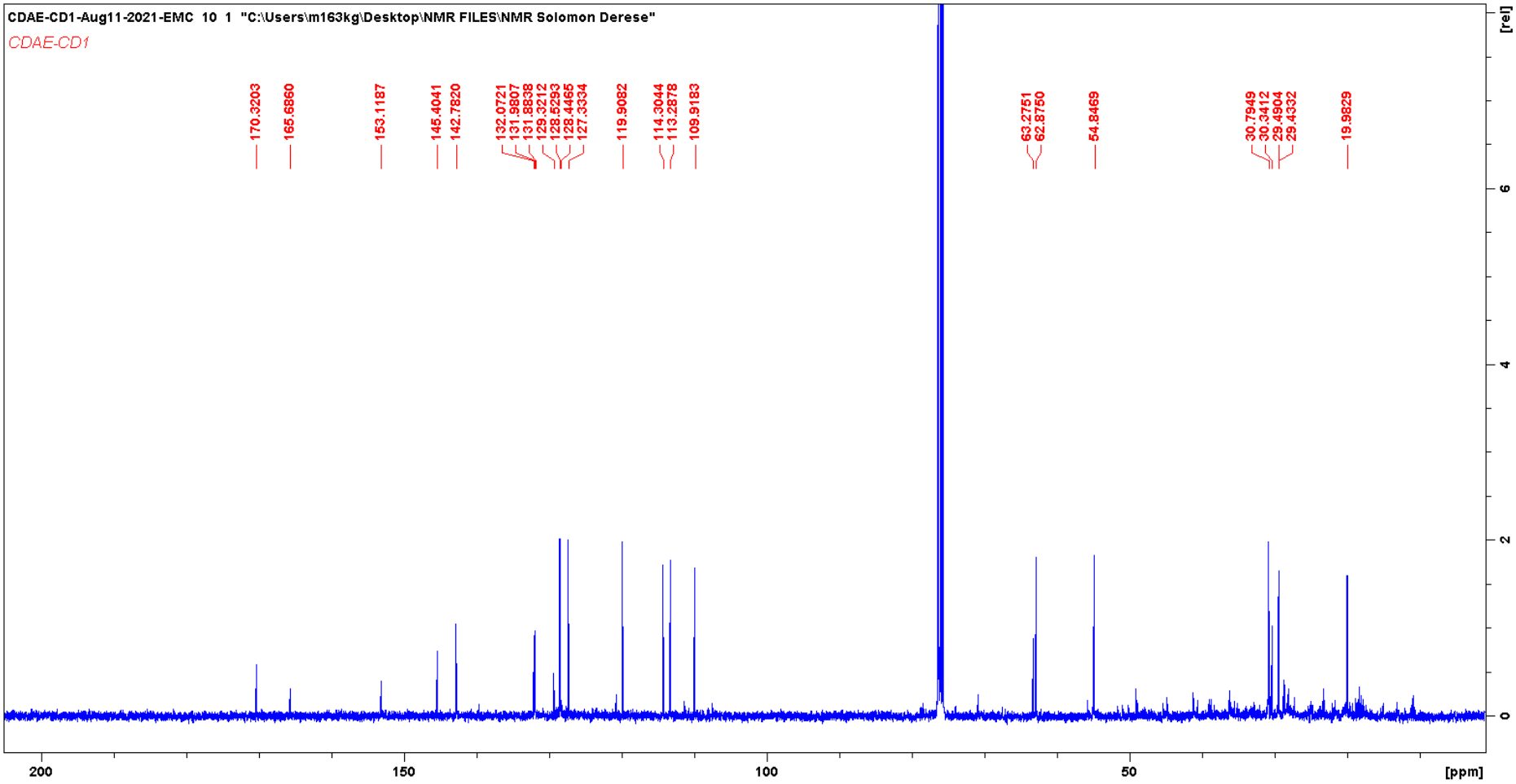
Appendix 98 $^{1}$H NMR Spectra for Sitosterol (A15) and Stigmasterol (A16)



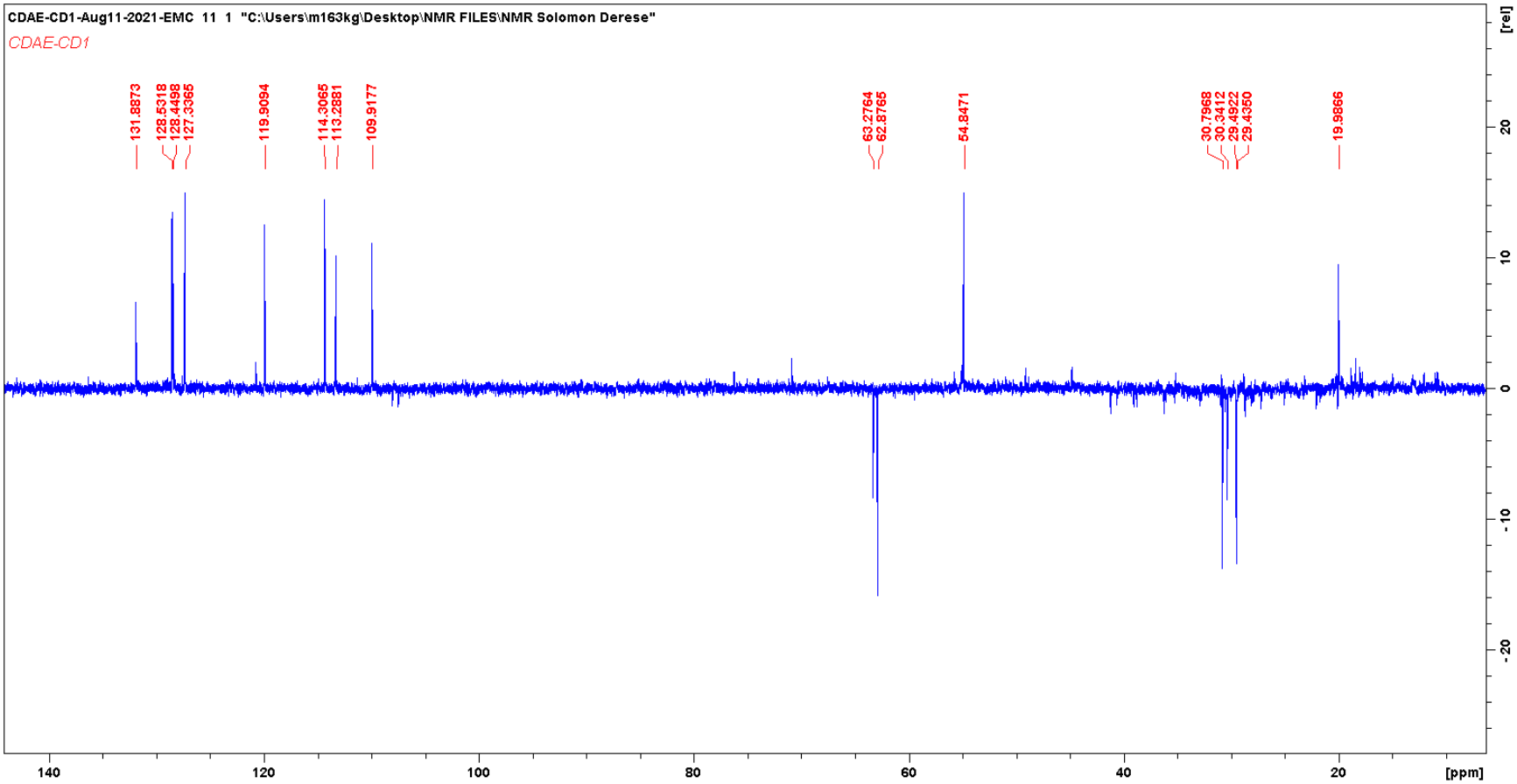
Appendix 99 $^{1}$H NMR Spectra for CD1 (mixture of Dihydroconiferyl acetate (CD1a) and (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b)



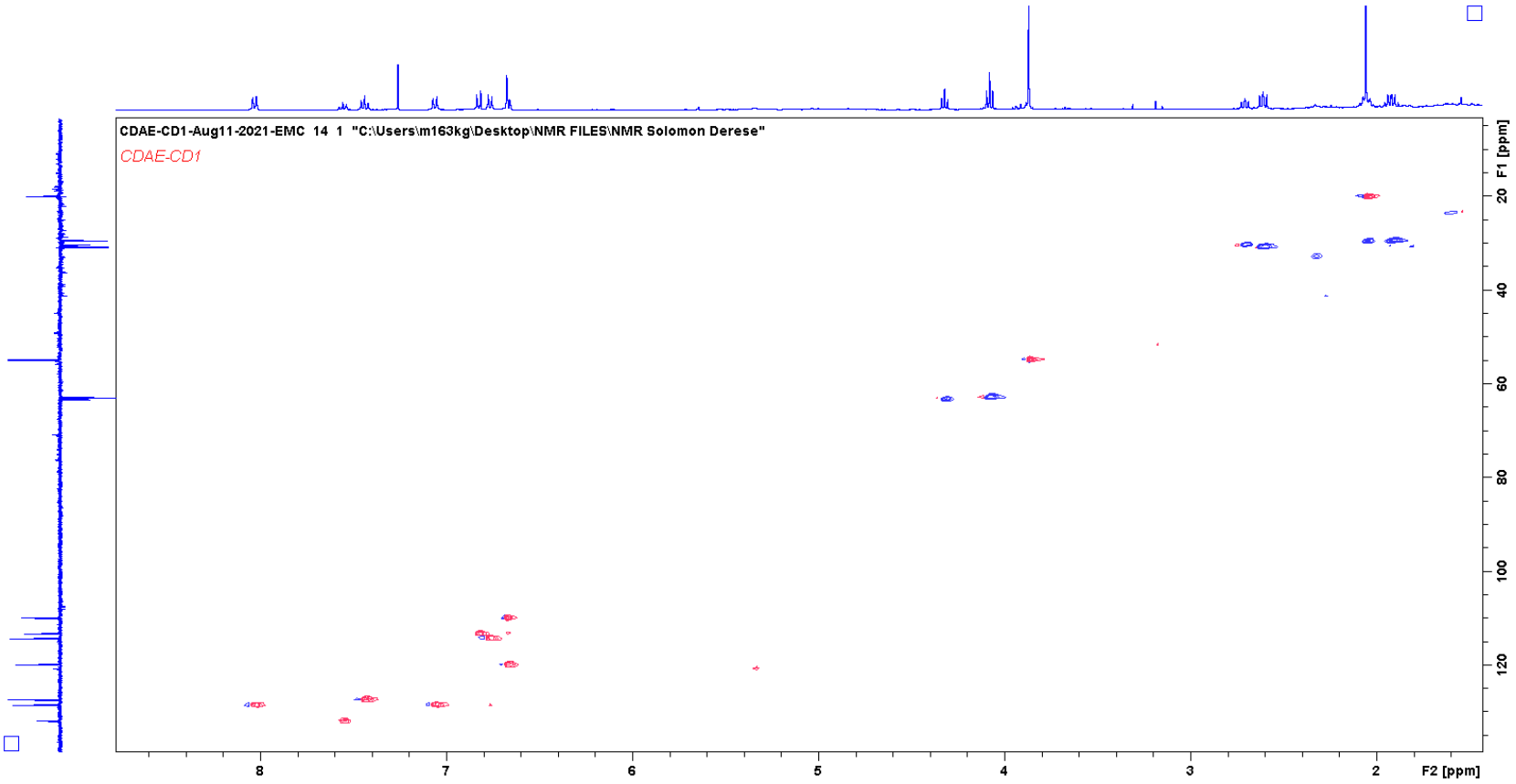
Appendix 100 $^{13}C$ NMR Spectra for CD1 (mixture of Dihydroconiferyl acetate (CD1a) and (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b)



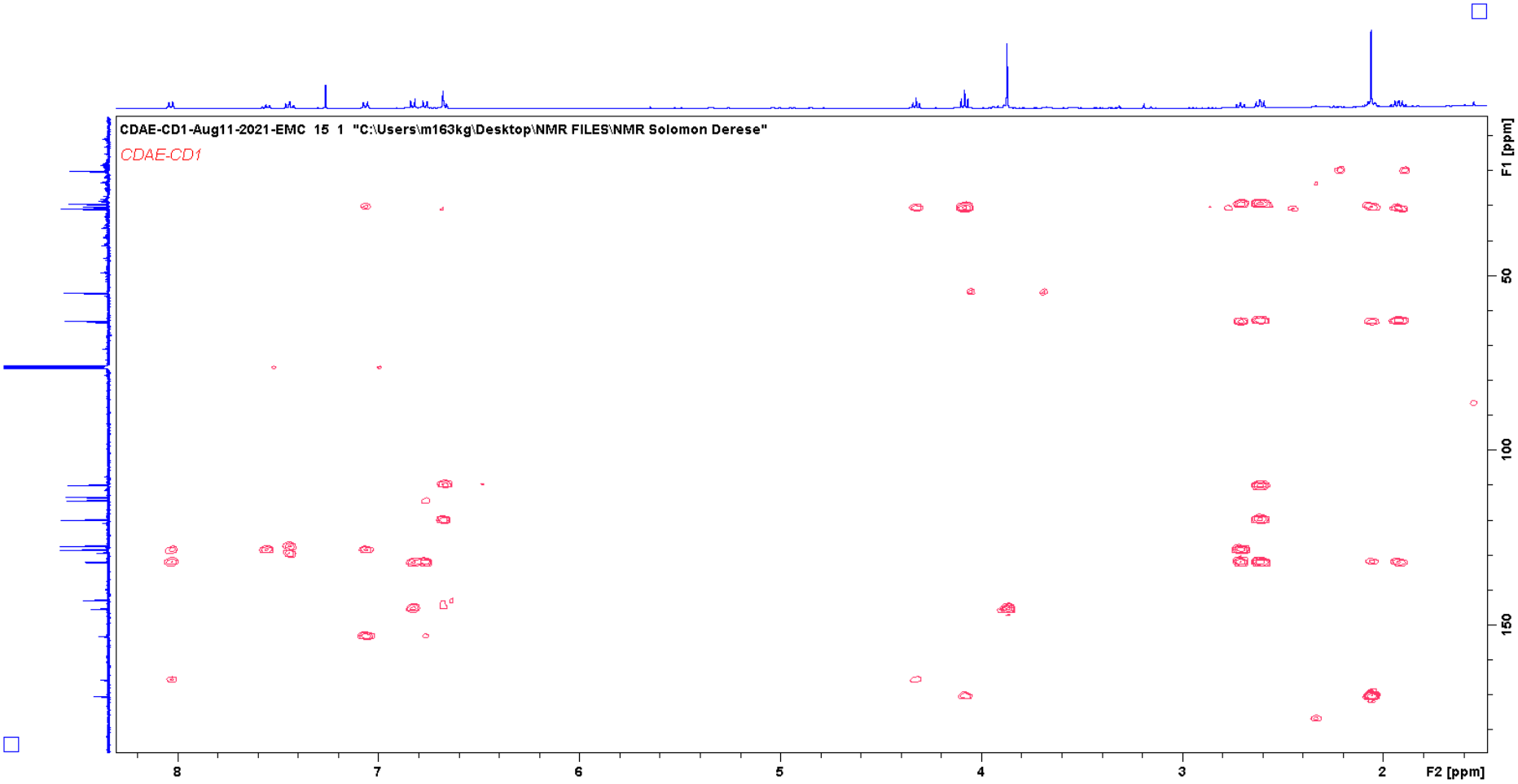
Appendix 101 DEPT Spectra for CD1 (mixture of Dihydroconiferyl acetate (CD1a) and (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b)



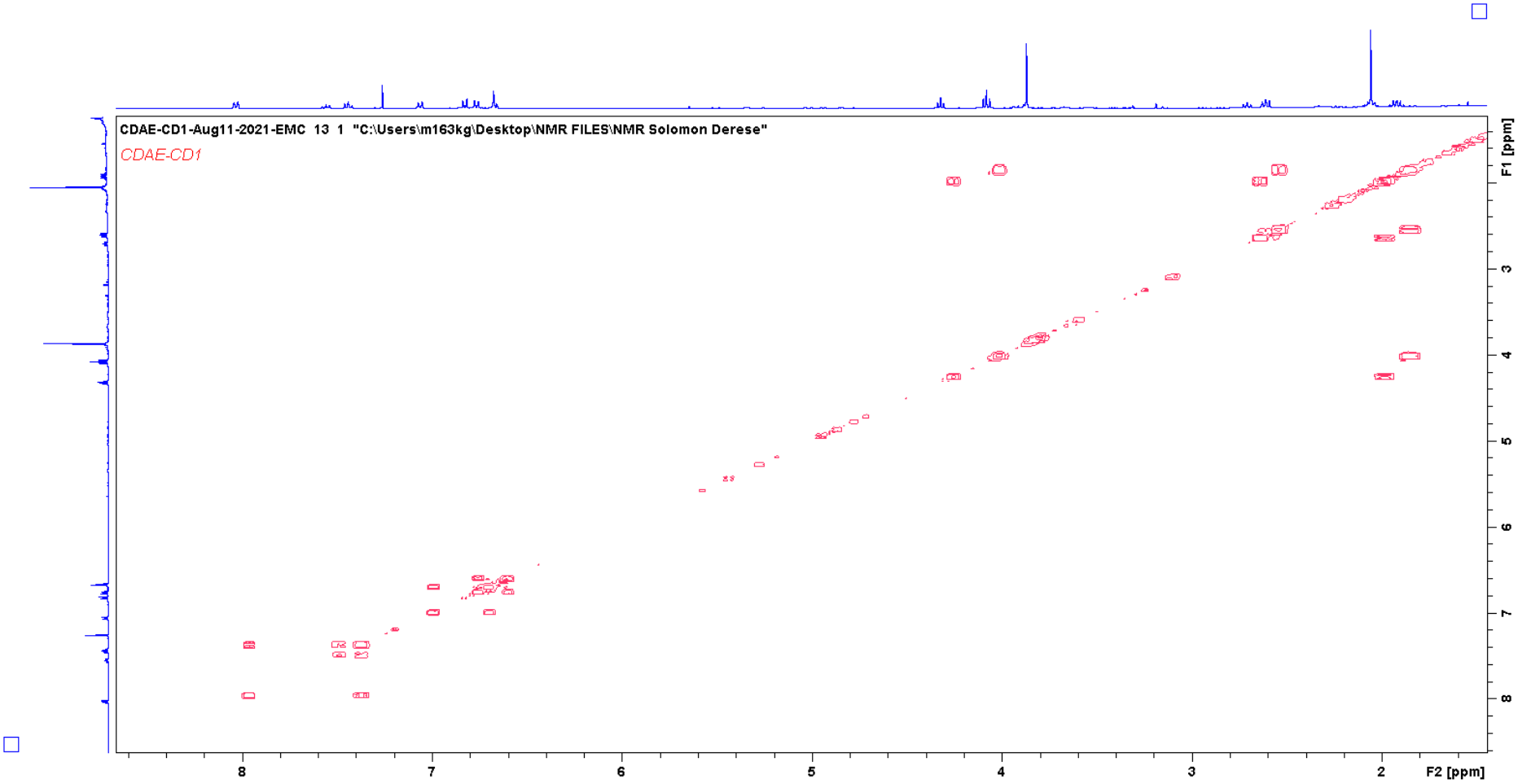
Appendix 102 HSQCDEPT Spectra for CD1 (mixture of Dihydroconiferyl acetate (CD1a) and (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b)



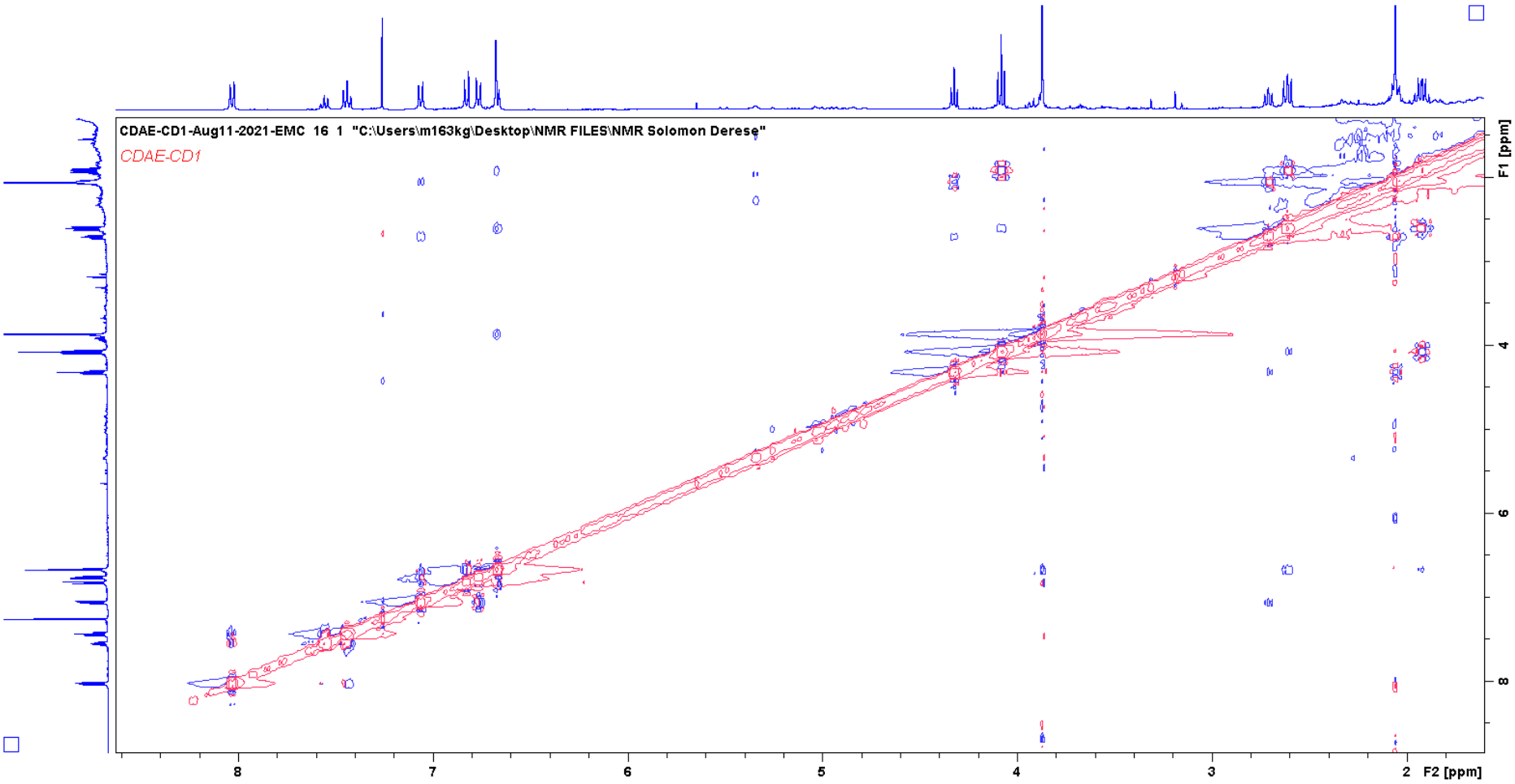
Appendix 103 HMBC Spectra for CD1 (mixture of Dihydroconiferyl acetate (CD1a) and (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b)



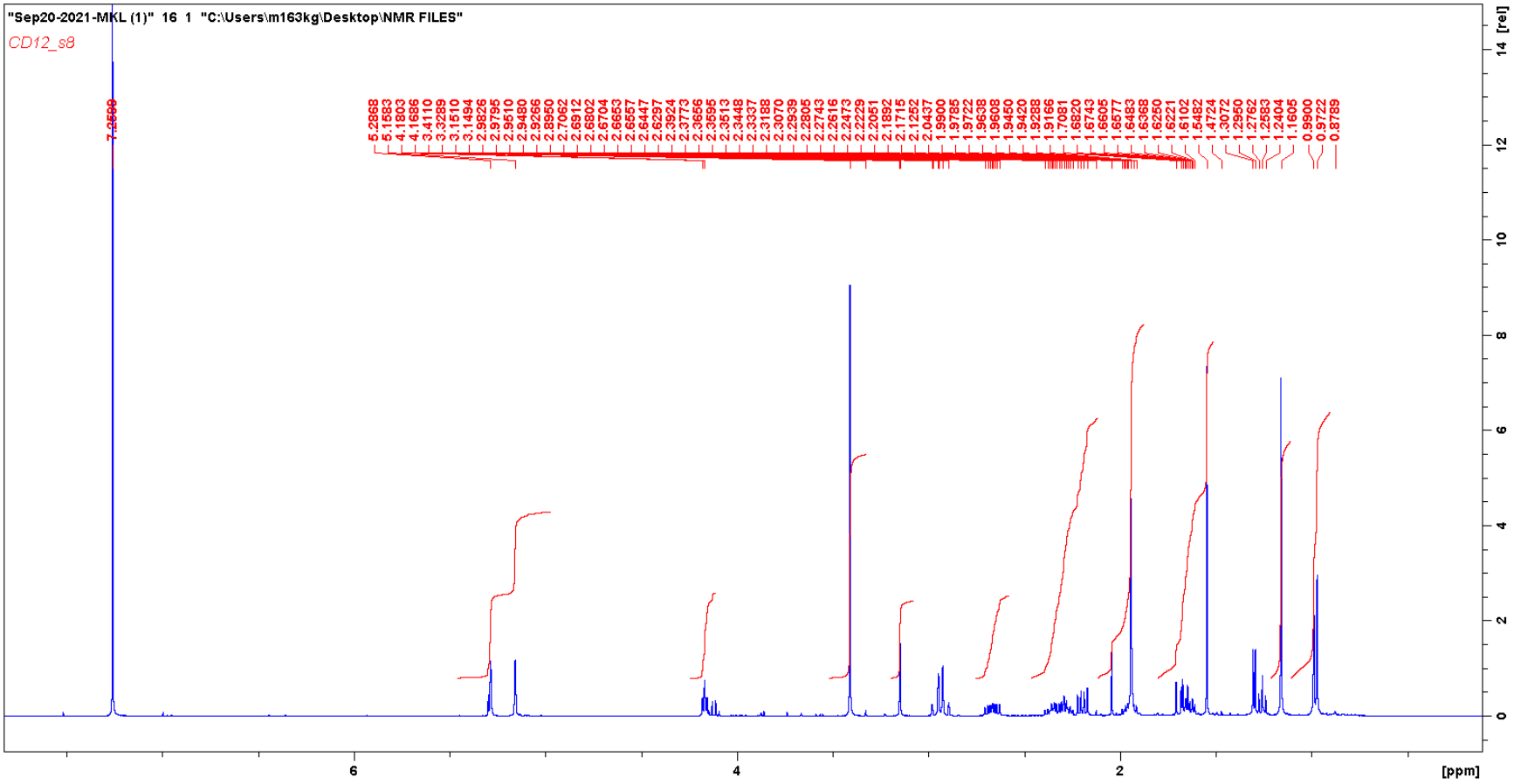
Appendix 104 COSY Spectra for CD1 (mixture of Dihydroconiferyl acetate (CD1a) and (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b)



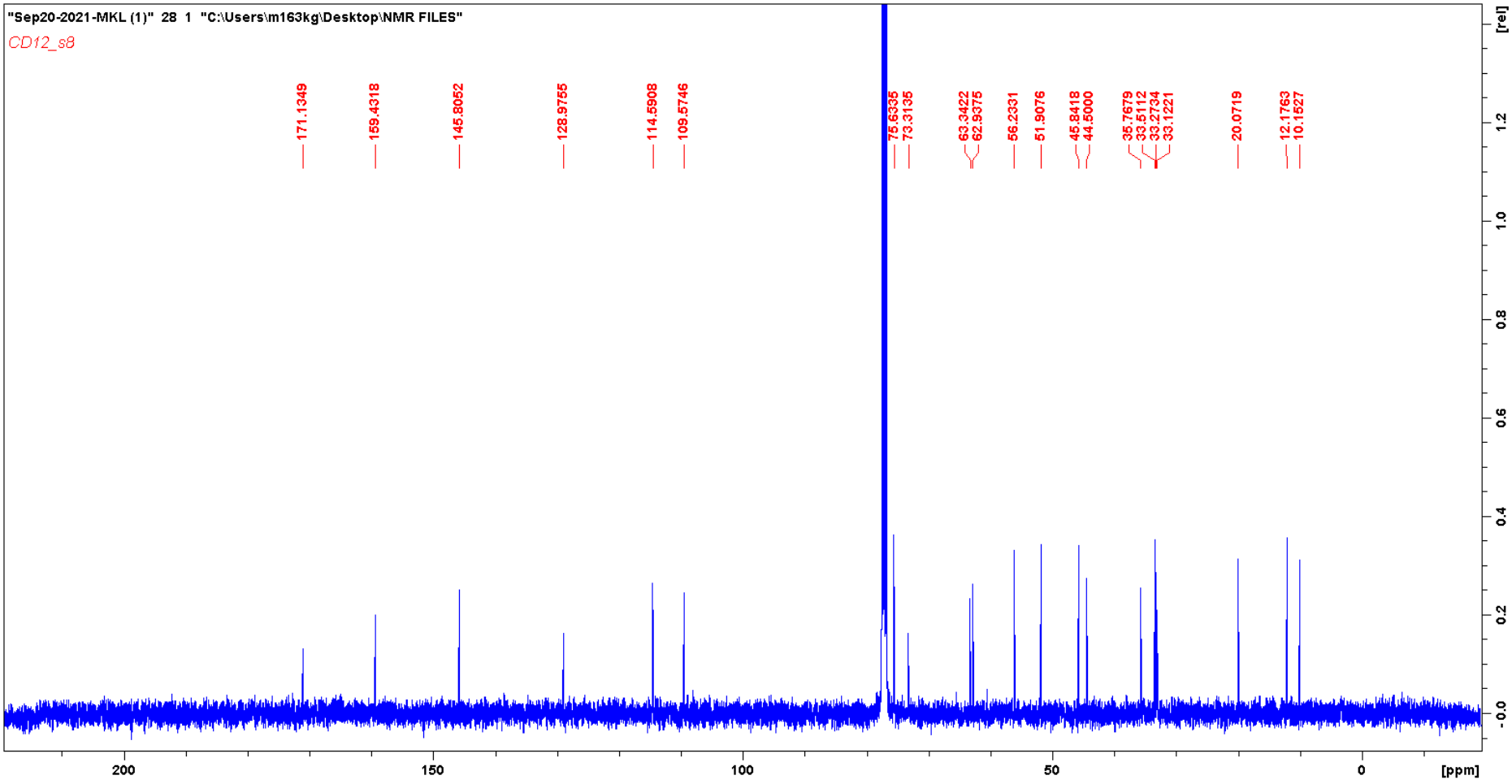
Appendix 105 NOESY Spectra for CD1 (mixture of Dihydroconiferyl acetate (CD1a) and (4-Hydroxy-3-methoxyphenyl)-propyl benzoate (CD1b)



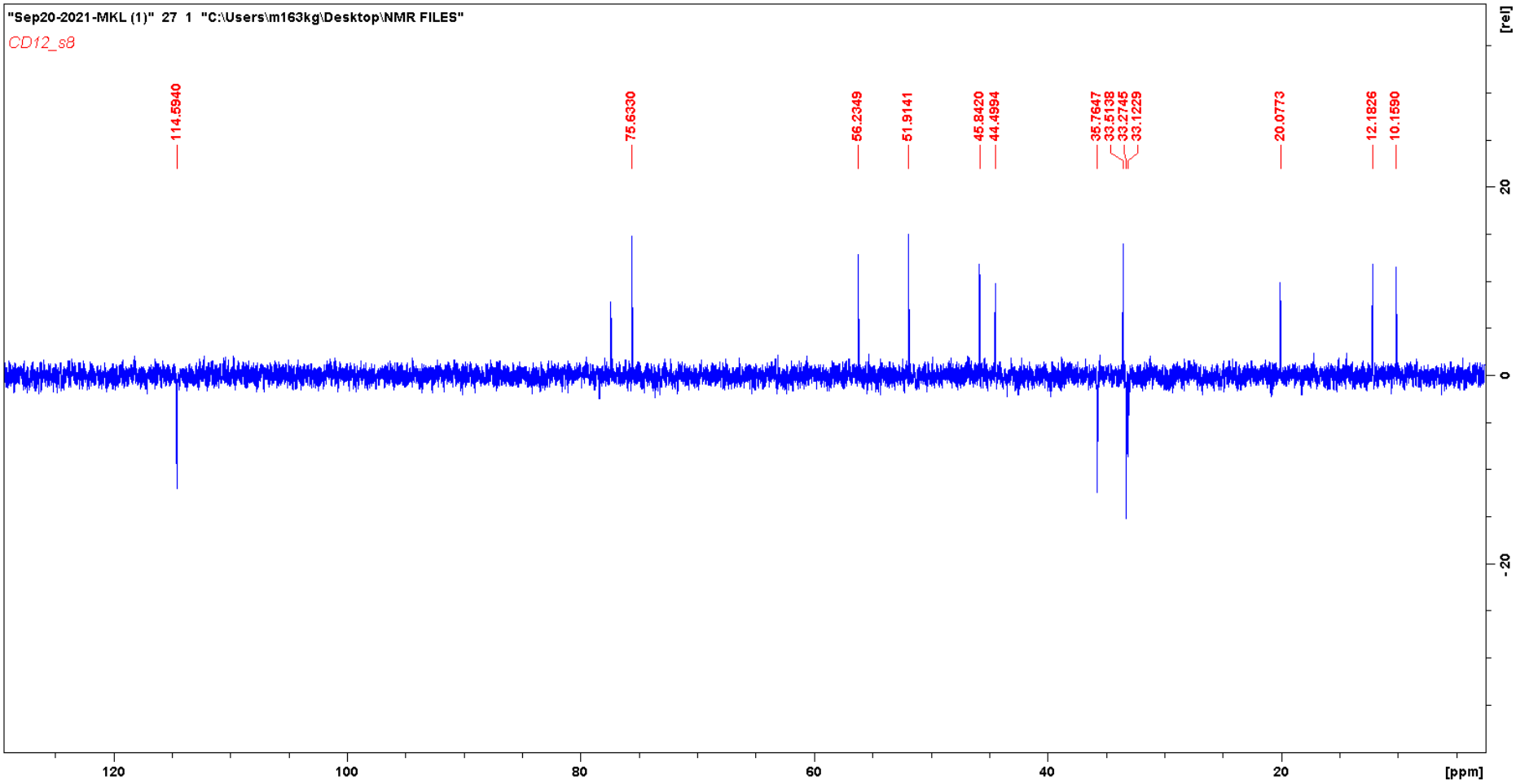
Appendix 106 $^{1}$H NMR Spectra for crotocascarin ω (CD12a)



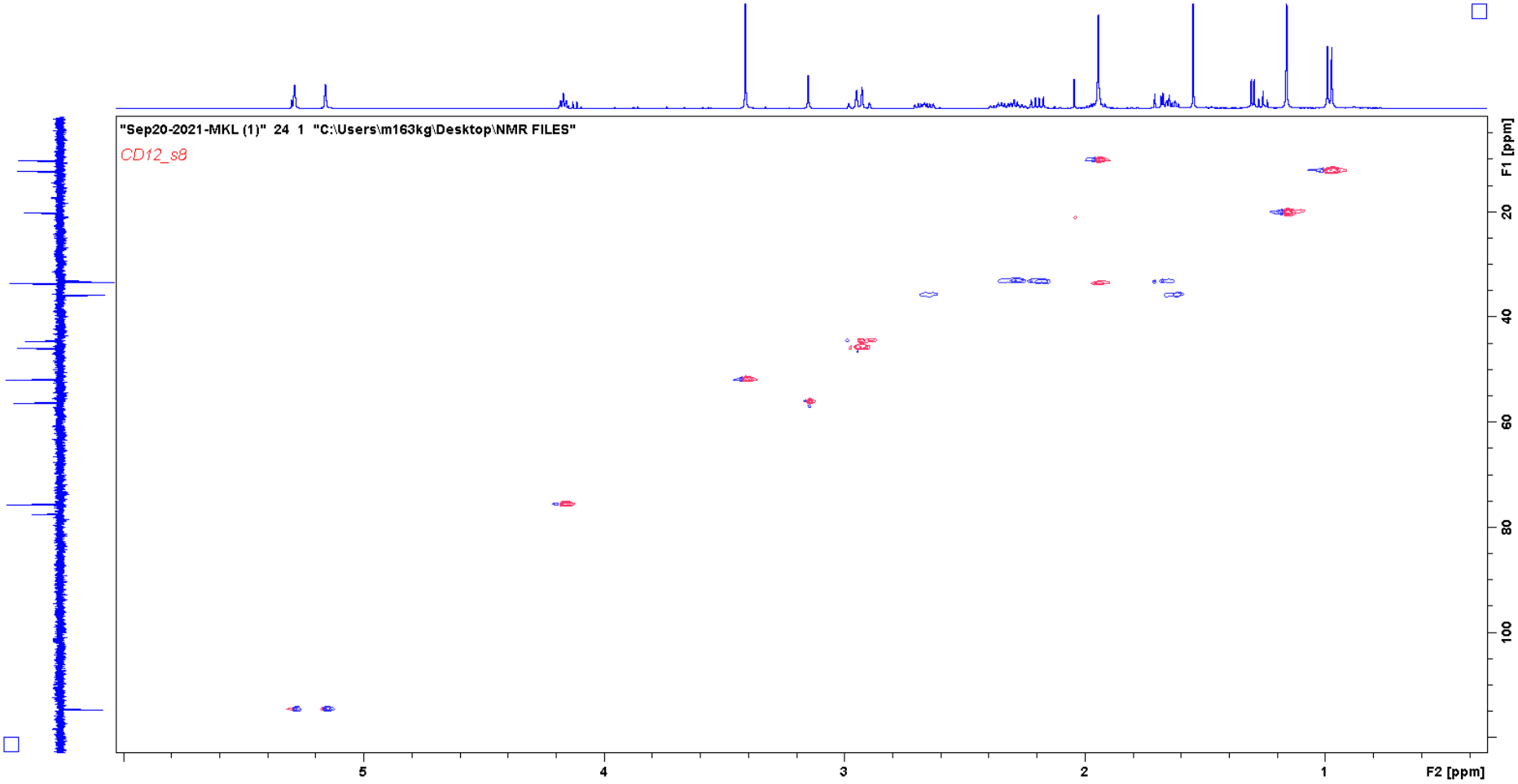
Appendix 107 $^{13}$C NMR Spectra for crotocascarin ω (CD12a)



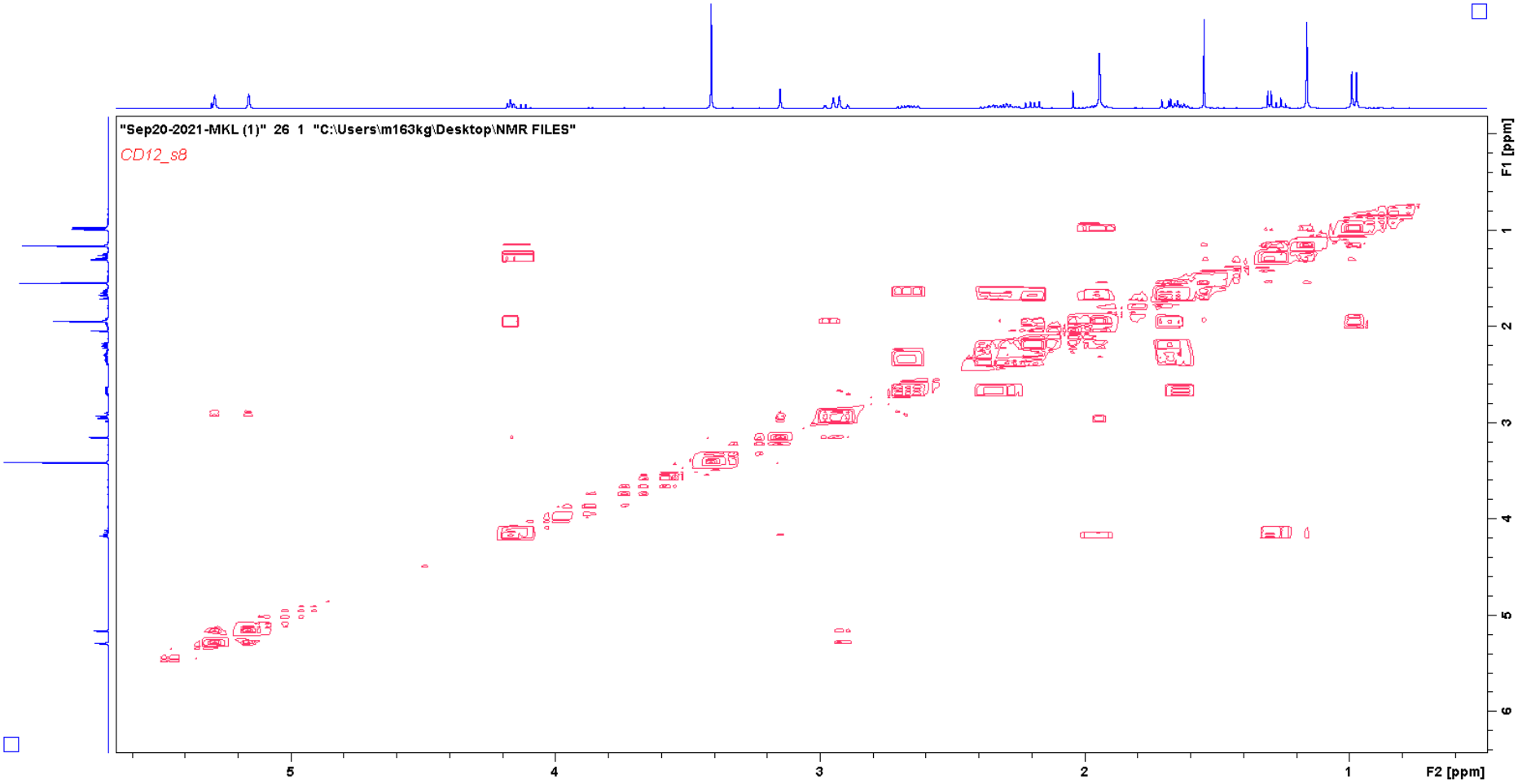
Appendix 108 DEPT Spectra for crotocascarin ω (CD12a)



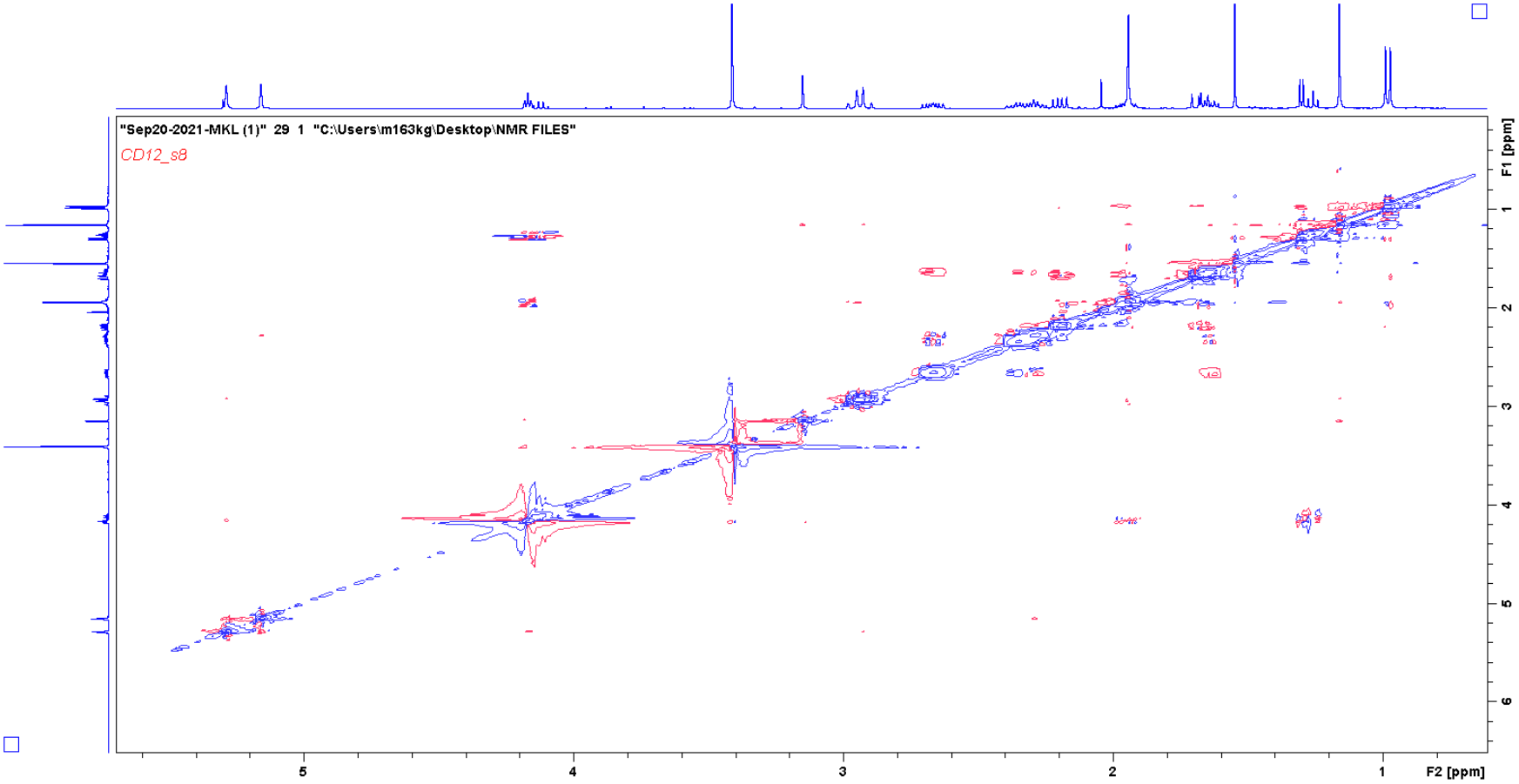
Appendix 109 HSQCDEPT Spectra for crotocascarin ω (CD12a)



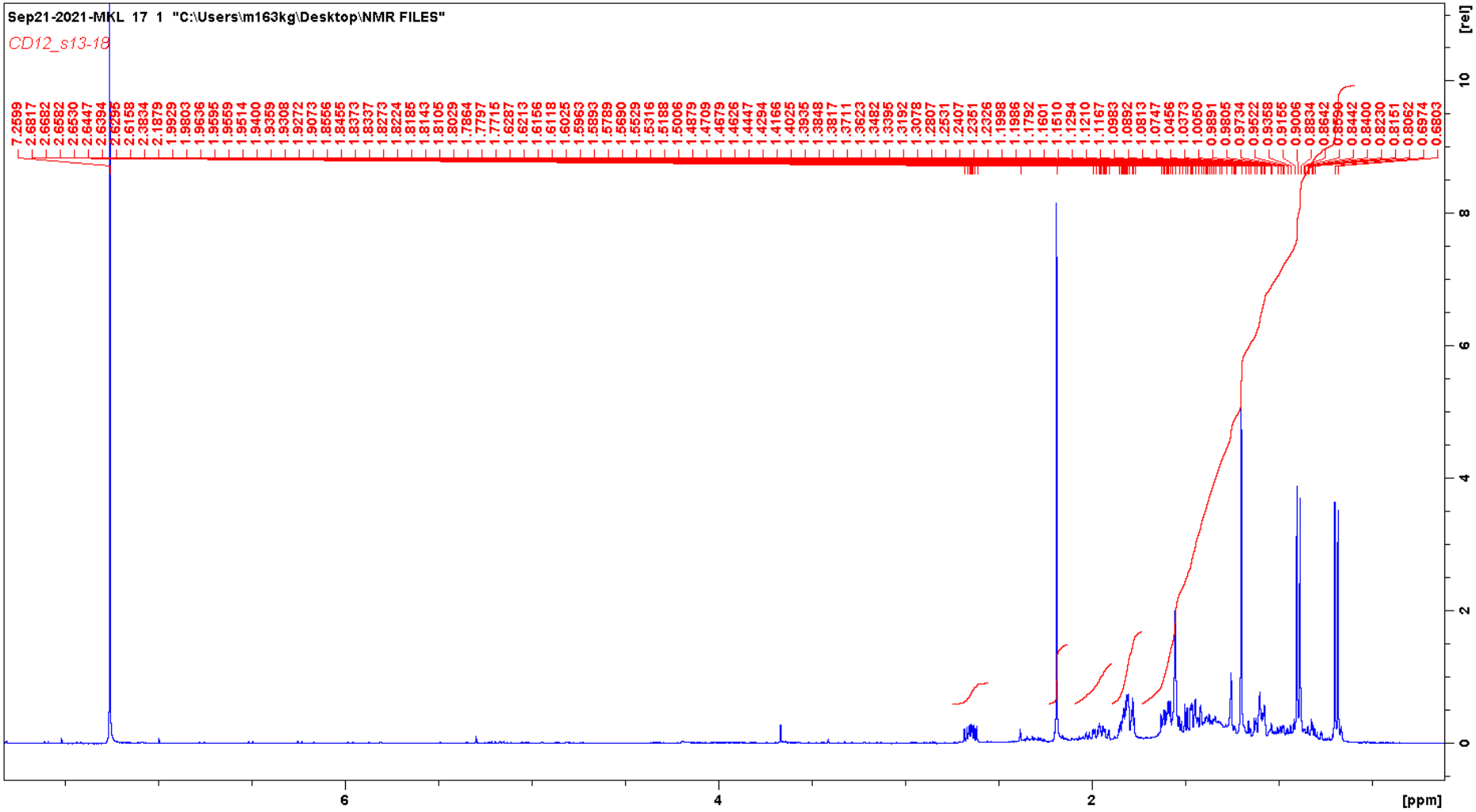
Appendix 110 COSY Spectra for crotocascarin ω (CD12a)



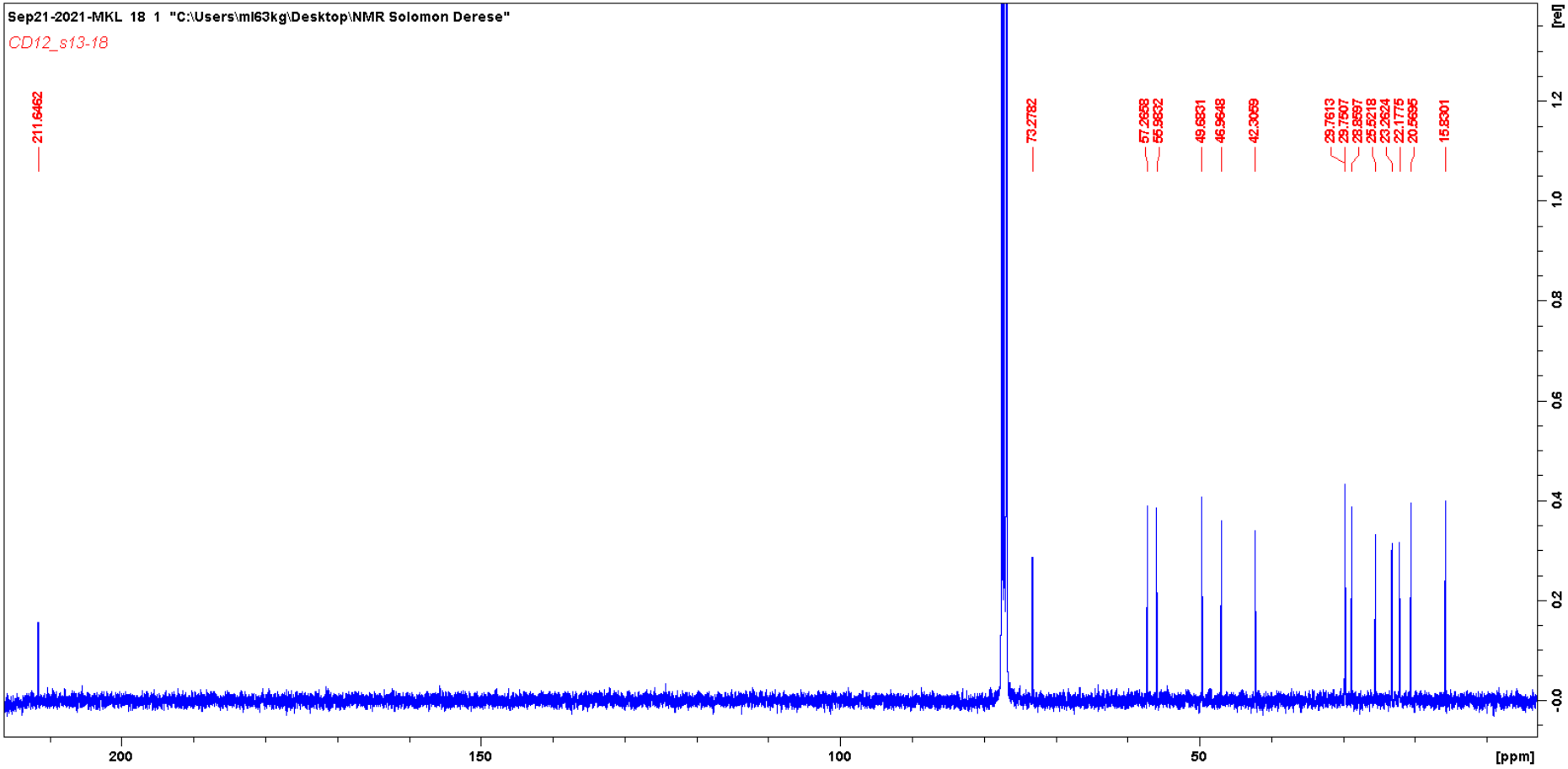
Appendix 111 NOESY Spectra for crotocascarin ω (CD12a)



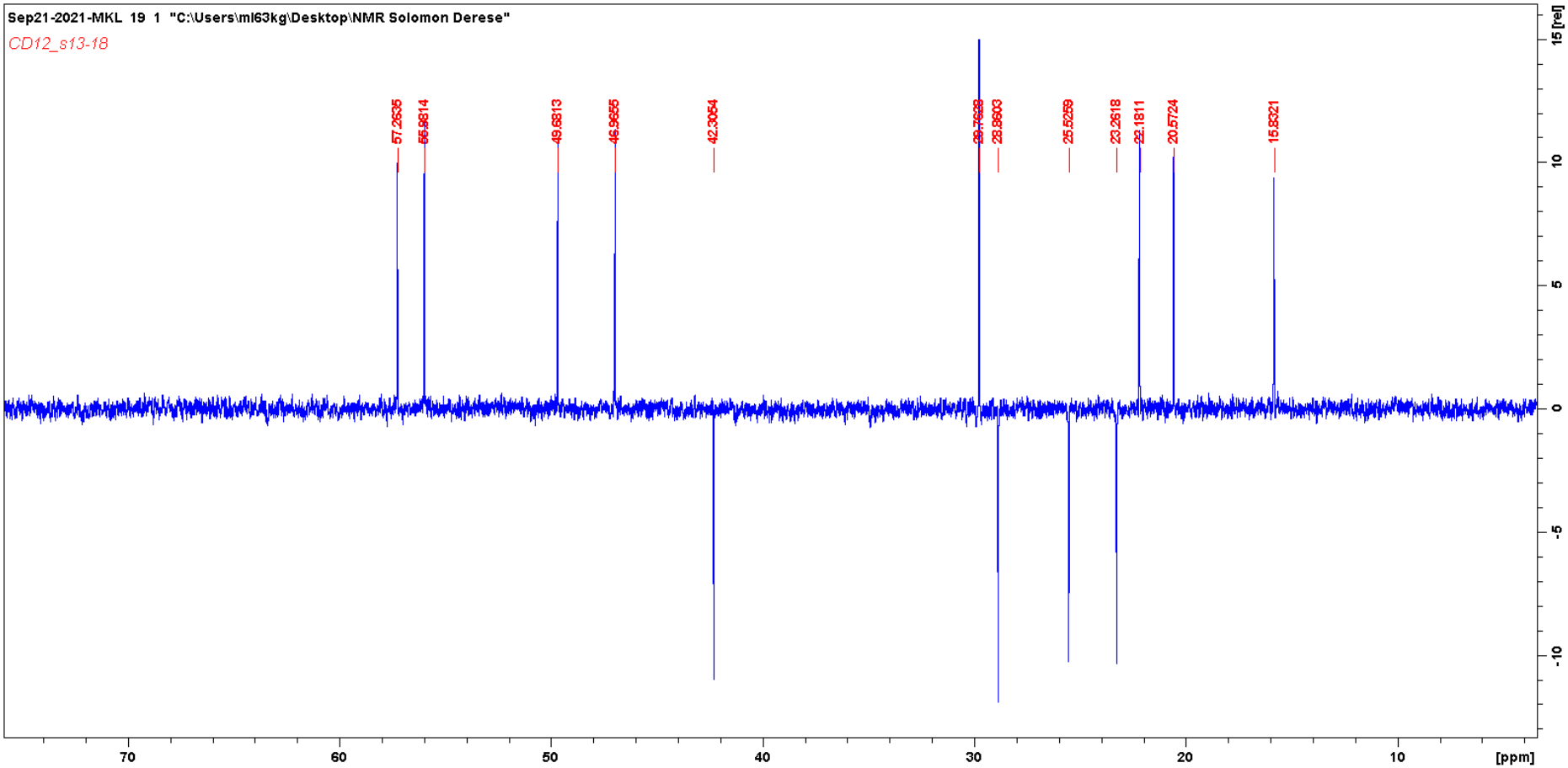
Appendix 112 $^{1}$H NMR Spectra for β-Oplopanone (CD12b)



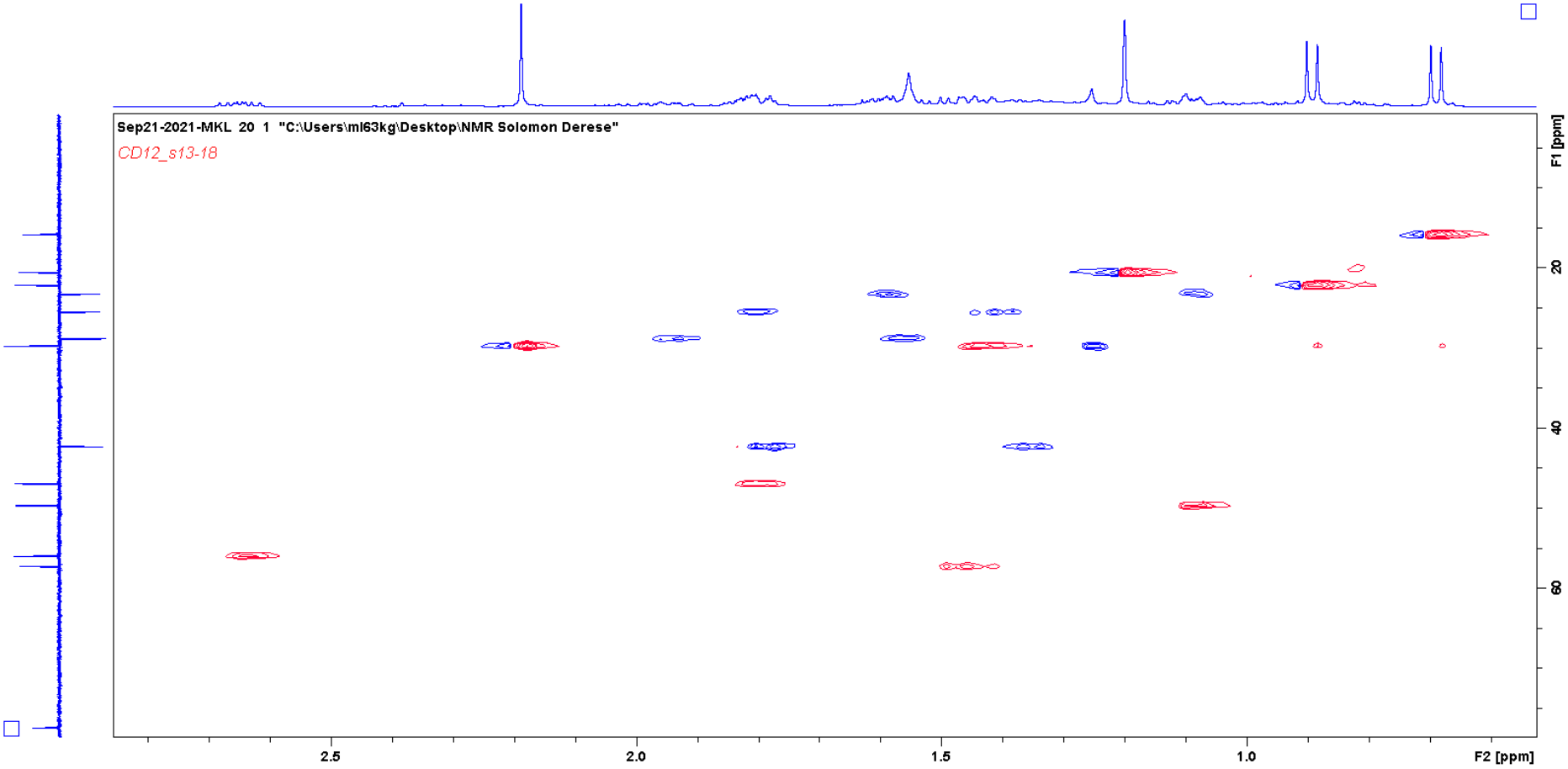
Appendix 113 $^{13}$C NMR Spectra for β-Oplopanone (CD12b)



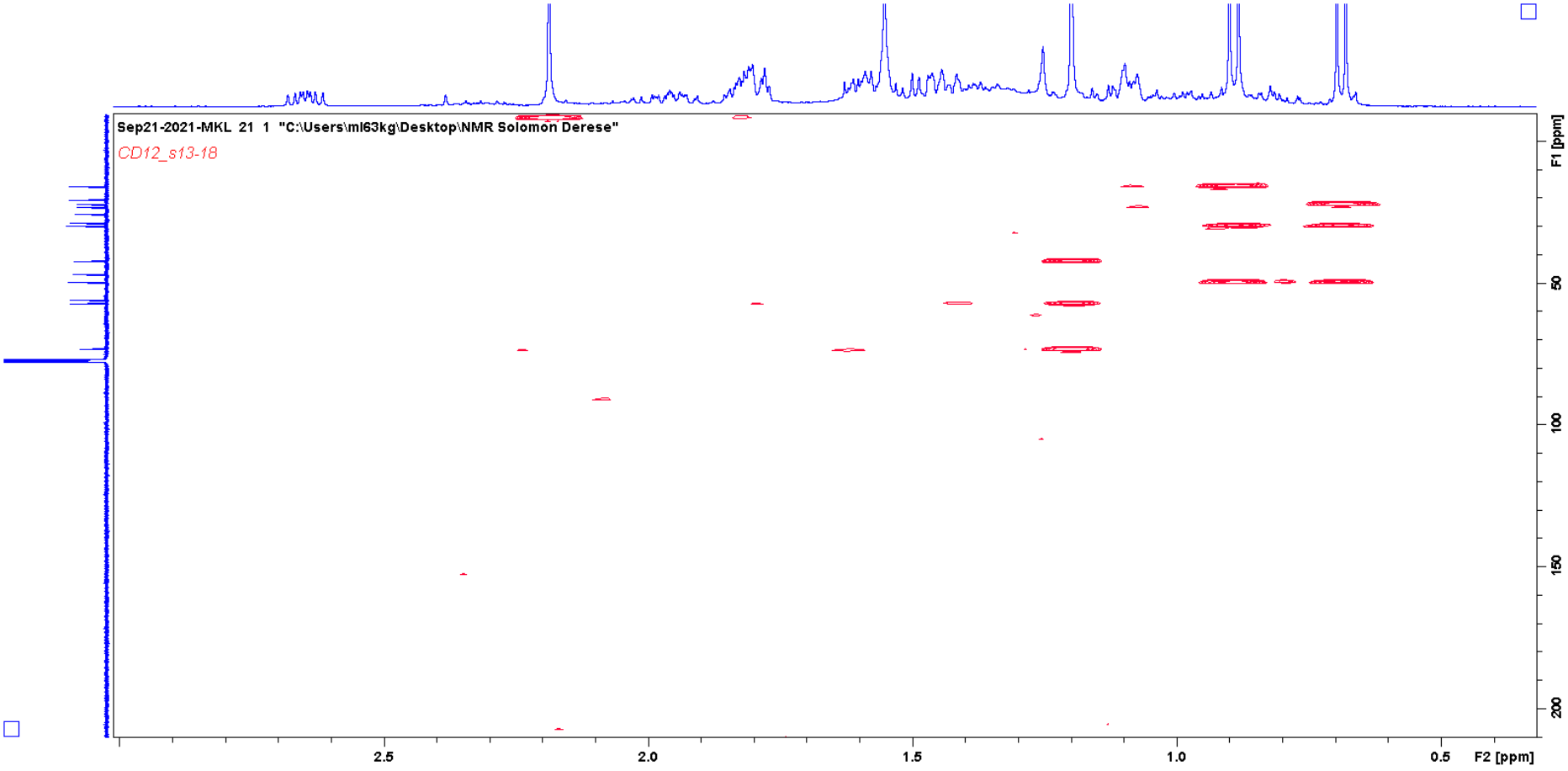
Appendix 114 DEPT Spectra for β-Oplopanone (CD12b)



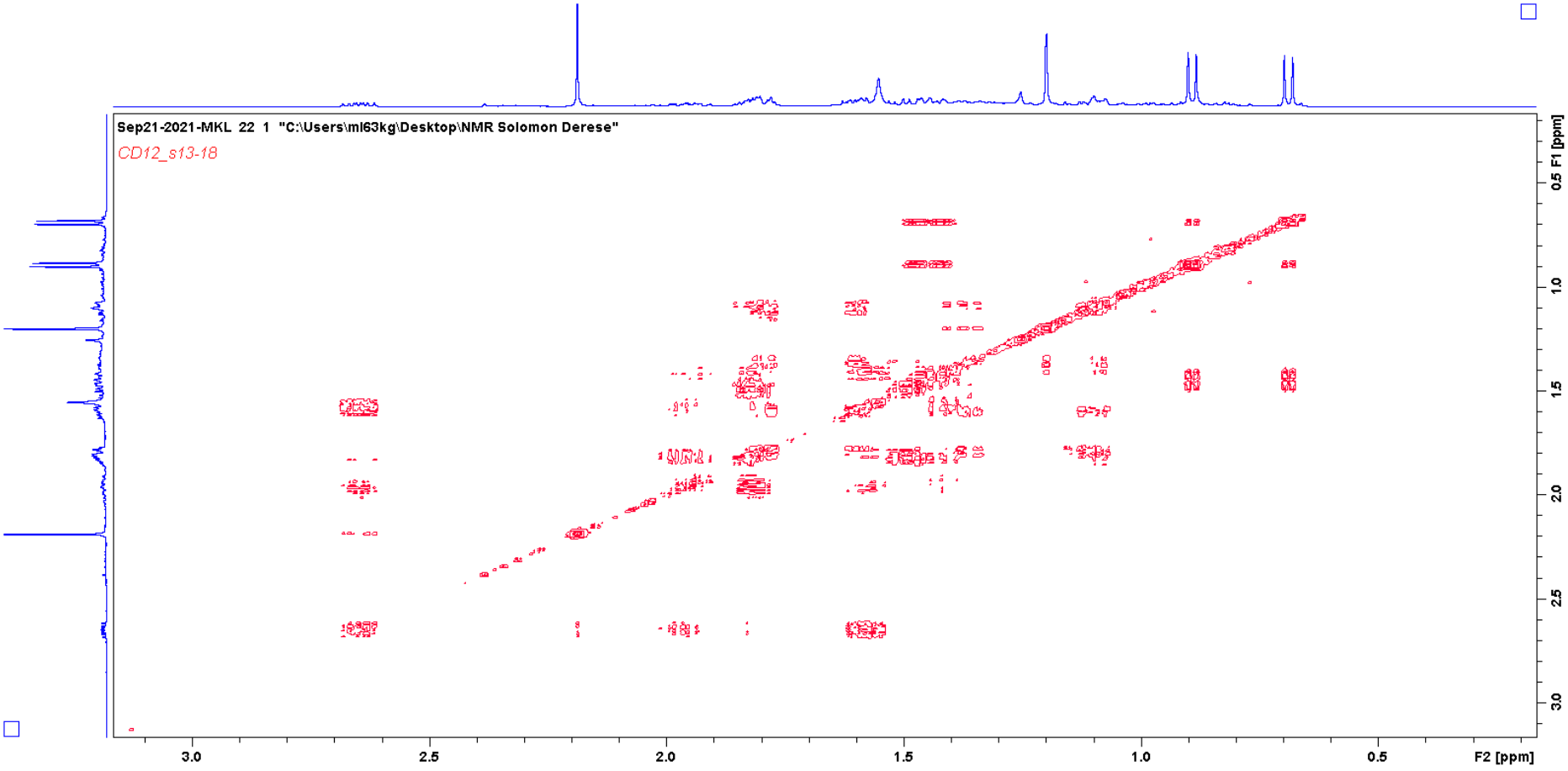
Appendix 115 HSQCDEPT Spectra for β-Oplopanone (CD12b)



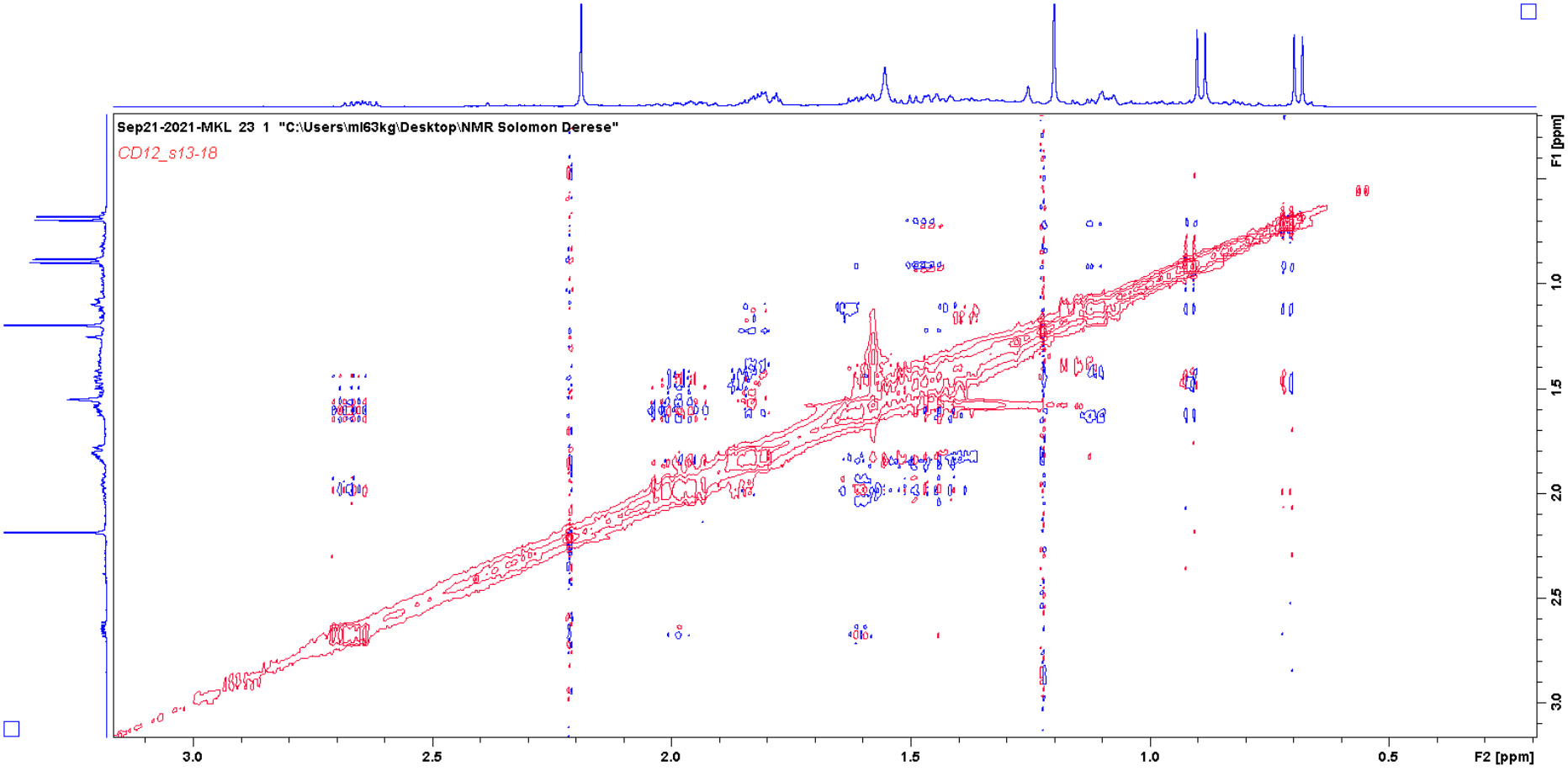
Appendix 116  HMBC Spectra for β-Oplopanone (CD12b)



Appendix 117 COSY Spectra for β-Oplopanone (CD12b)



Appendix 118 NOESY Spectra for β-Oplopanone (CD12b)



Appendix 119 $^{1}$H NMR CD6a and CD6b (Sitosterol and stigmasterol)