AN INVESTIGATION OF THE NATURAL PRODUCTS COMPOSITION OF *Porphyra capensis* (A RED SEAWEED)

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WESTERN CAPE

BY

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A thesis submitted in partial fulfilment of the requirement for the award of the degree of Master of Science in the Faculty of Natural Sciences University of the Western Cape

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November 2017

ABSTRACT

Plants have been widely used in traditional medicine for a number of ailments, among which may be included infectious diseases such as colds, influenza, chicken pox, TB, etc. as well as lifestyle diseases such as diabetes and cancer. Seaweeds have also been shown to contribute to the maintenance of health through their nutritional and medicinal properties and recently, a great deal of interest has developed towards the isolation of bioactive compounds from marine sources due to their numerous health benefits. Furthermore, marine algae are valuable sources of structurally diverse metabolites with scientifically proven therapeutic claims.

Chemical constituents of red seaweed, *Porphyra capensis* was investigated in this present study along with subsequent brine shrimp lethality assay analysis of the crude extracts. The compounds isolated from the plant were from the hexane (6) and butanol (2) extracts. These compounds were all isolated and purified by various chromatographic techniques, namely silica gel chromatography, Sephadex LH-20 gel as well as C_{18} reversed phase silica gel.

The structures of the isolated compounds were analysed and characterised by NMR, GC-MS, ESI MS and FTIR spectroscopy. Eight compounds were isolated and identified as phytol, desmosterol, 9-eicosenoic acid, 5,8,11,14,17-eicosapentanoic acid, palmitic acid, methyl (E)-hexadec-9-enoate, glycerol and compound **1** (novel compound). All the compounds were isolated from *Porphyra capensis* for the first time. The hexane, butanol and methanol extracts were found to be non-toxic with the brine shrimp test LC_{50} value at least two times greater than µg/ml.

KEYWORDS: red algae; *Porphyra capensis*, brine shrimp lethality assay, chromatographic techniques, Sephadex LH-20, C^{18} reversed

DECLARATION

I, **Masande Nicholas Yalo** declare that the dissertation entitled **'An Investigation of Natural Product Composition of *Porphyra capensis* (a red seaweed)'** has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Signature: 

Date: ……01/12/2017……..

ACKNOWLEDGEMENTS

As I reach this stage in my life, my heart is occupied with joy and great love for my **Almighty God** who has constantly given me the strength to carry on even when I was walking in the darkest valley. *Glory be to **God**.*

During my Masters programme, I was privileged to have been encircled by people who have influenced me positively and who have contributed greatly towards my growth and maturity as a scientist. I am forever grateful to my supervisor **Prof. W.T. Mabusela** for his guidance, inspiration and support throughout my MSc studies at the University of the Western Cape. He did not only teach me how to do good science but also shared his friendship with me which made working with him a delightful experience. Thank you **Prof**, I have thoroughly enjoyed natural products chemistry and could not have wished for a better supervisor.

I am deeply indebted and very grateful with deep sincere appreciation to **Dr. Henry Mwangi** for his continuous help and guidance especially in the interpretation of the NMR data of the isolated compounds. **Mr Mthandazo Rondo** for his assistance with structural elucidation of one of the isolated compound, I greatly appreciate his input.

I thank the **National Research Foundation (NRF) of South Africa** for the financial support.

I am greatly thankful to **Miss Luveni Sonka** for sparing her precious time to help with issues and analysis related to the bioassay experiment.

I would like to thank all my colleagues and friends for providing a stimulating and fun environment. Their support and colleagueship meant a lot. Through my time in the lab I have had the greatest pleasure of working with them; **Mr. Ndikho Nako, Mr. Masixole Makhaba, Mr. Mkhuseli Koki, Mrs Zandile Mthembu.** Also, from outside our lab, support and friendship came from **Prof Martin Onani, Prof M. Ahmed, Dr Asanda Busa, Dr Popoola Olubenga, Dr Francis Ntumba Muya and Ninon Etsassala.**

Ultimately but not the least, none of this would have been possible without the love and patience of my family. I have been blessed with a very loving and supportive **family**. May **GOD** bless them in a mighty way in everything they do.

DEDICATION

I dedicate my dissertation work to my family for all of their continued love and support.

A special feeling of gratitude to my loving parents, **Harrison Mbonelwa** and **Monica Yalo** whose words of encouragement and push for tenacity ring in my ears till this day.

To My siblings **Nontobeko**, **Nwabisa**, **Sinalo** and **Sihle Yalo** who have been pillows, role models, catapults, cheerleading squad and sounding boards I have needed.

I also dedicate this dissertation to my two beautiful nieces **Inam** and **Lunje Yalo** who have made my life brighter every day since they were born.

Finally, it is dedicated to my late grandparents, **Leonard Mntwabantu** and **Nofundile Yalo.** I wish that you could be here to celebrate with me.

TABLE OF CONTENTS

CHAPTER 3: RESULTS AND DISCUSSION

CHAPTER 4: CONCLUSION AND RECOMMENDATIONS

LIST OF FIGURES

LIST OF TABLES AND SCHEMES

TABLES

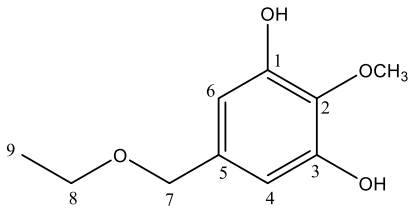
SCHEMES

LIST OF ABBREVIATIONS

1D NMR	one-dimensional nuclear magnetic resonance
2D NMR	two-dimensional nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon 13 nuclear magnetic resonance
br	broad signal
BSLA	brine shrimp lethality assay
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
CD_3OD	deuterated methanol
COSY	correlation spectroscopy
d	doublet
DCM	dichloromethane
dd	doublets of doublet
DEPT	distortionless enhancement by polarization transfer
DMSO	dimethyl sulfoxide
EPA	eicosapentanoic acid
ESI-MS	electrospray ionization mass spectrometry
et al.	*et altera* (and others)
EtOAc	ethyl acetate
FTIR	Fourier-transform infrared spectroscopy
g	gram
GC-MS	gas chromatography
H_2O	water
HMBC	heteronuclear multiple bond connectivity
HSQC	heteronuclear single quantum coherence
Hz	herz
IC_{50}	inhibitory concentration 50
J	coupling constant
L	liter
LC	liquid chromatography
LC_{50}	Lethal concentration
LC/MS	liquid chromatography-mass spectrometry

m	multiplet
MeOH	methanol
mg	milligram
mL	millilitre
*m*M	millimolar
MS	mass spectrometry
m/z	mass per charge
*µ*g	microgram
*µ*L	microliter
NMR	nuclear magnetic resonance
ppm	parts per million
PUFA	polyunsaturated fatty acid
q	quartet
s	singlet
t	triplet
TLC	thin layer chromatography
UV	ultra-violet

LIST OF ISOLATED COMPOUNDS



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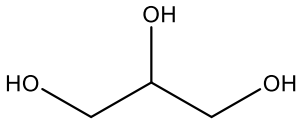

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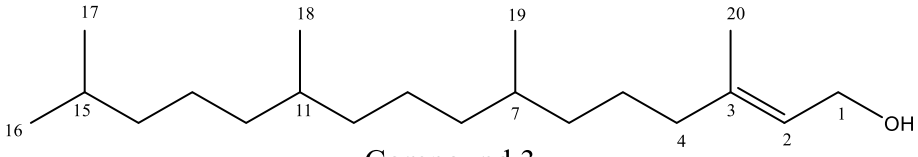

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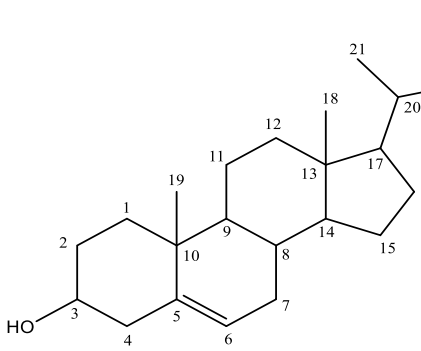

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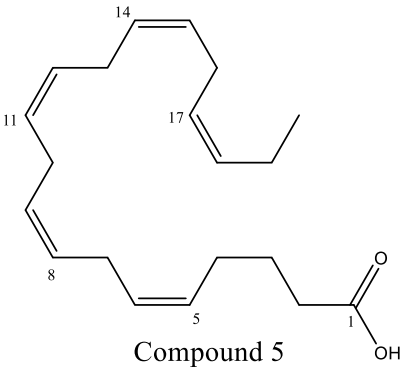

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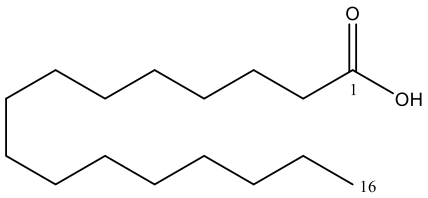

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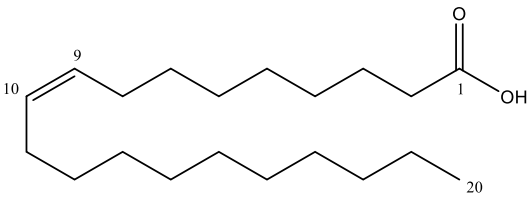

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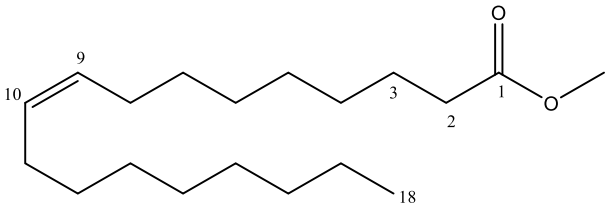


Compound 8

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction to seaweeds

Most people think of seaweeds simply as the plant-like stuff that washes up on the seashore. Seaweeds are simple marine plants that grow in the shallow waters at the edge of the world's ocean. The term seaweed includes macroscopic, multicellular, benthic marine algae that consist of members of brown, red and green algae (McHugh, 2003). They photosynthesize like terrestrial plants but differ morphologically and structurally. They have leaf-like structures (thalli) within which photosynthesis takes place but unlike terrestrial plants, they lack internal transport system made up of xylem and phloem vessels. Their thalli are dipped in seawater and that allows materials to move in and out of their tissues by diffusion.

They have a low content in lipids, but high concentration in polysaccharides, natural richness in minerals, polyunsaturated fatty acids and vitamins as well as bioactive molecules, which make them to be a good source of nutraceuticals (Rupe´rez and Saura-Calixto, 2001). They normally grow in the intertidal and also in the sub-tidal area up to a certain depth where very little photosynthetic light is available. They are divided into three groups on the basis of their colour and their photosynthetic and accessory pigments that they possess which in turn will reflect certain colours of light producing what appear to be green (Chlorophyta), brown (Phaeophyta) and red (Rhodophyta) sea plants. The brown colour of the brown seaweed result from the dominance of the xanthophyll pigments as for example fucoxanthin, that masks other pigments as chlorophyll-a and –b, β-carotene and other xanthophylls (zeaxanthin) (Kumar *et al*., 2008).

The green colour of the green algae is due to chlorophyll a and chlorophyll b. The green algae are the only ones which have cell walls consisting of starch. (Kumar *et al*., 2008).

The red colour of the red algae results from domination of the pigments phycoerythrin

and phycocyanin. Their walls are made of cellulose, agar and carrageenan (Kumar *et al*., 2008). Red algae are found in a variety of physical forms, including simple and branched filaments (Harbo, 1999). Seaweeds are also called macro-algae and that differentiates them from micro-algae (Cyanophyceae), which are microscopic in size, often unicellular, and are known as blue-green algae that sometimes grows and pollute streams and rivers. Naturally growing seaweeds are often referred to as wild seaweeds, in contrast to seaweeds that are cultivated or farmed (McHugh, 2003).

They are an excellent source of vitamins A, B_l, B_{12}, C, D and E, as well as minerals such as Ca, P, Na, K (Dhargalkar and Pereira, 2005).

Their abundance and distribution is influenced by availability of bedrocks for attachment, temperature, tidal amplitude, currents and wave action, nutrients, salinity, propagule availability and grazing pressure (Bustamante *et al*., 1997; Cervin *et al*., 2004; Stegenga *et al*., 1997), and there are approaximately about ten thousand known seaweed species world-wide (Braune and Guiry, 2011). They are harvested worldwide mainly for the extraction of chemical compounds that serves as thickening and gelling agents in foods and media use in medicinal and microbiological work.

1.2 Morphology of seaweeds

Seaweeds do not need support structures. Seaweeds have holdfasts instead of roots, which attach them to the sea floor. A holdfast is not necessary for water and nutrient uptake, but is acquired as an anchor for stability. The stalk or stem of seaweed is called as stipe and the function of the stipe is to give support to the rest of the plant for it to be able to stand firm. The structure of the stipe differs among sea weeds, they can be flexible, stiff, solid, gas-filled, very long (20 metres), short or completely absent (Druehl, 2000). Their leaves are called blades and the main function of the blades is to provide a large surface for the absorption of

sunlight. In some seaweed species the blades also provide support for the reproductive structures of the seaweed and some species have only one blade, which may be divided, while other species have numerous blades (Harbo, 1999).

Many types of seaweed have hollow, gas-filled structures called floats or pneumatocysts that provide help to keep the photosynthetic structures of the seaweed afloat so they are able to assimilate energy from the sun (Turner, 2003). Absorption of nutrients takes place directly through the walls of the thallus. The thallus may be simple filament of cells or columns of cells, a flattened membrane of one or several layers of cells or a complex set of organs including stipe holding photosynthetic frond and reproductive organs (Tudge, 2000).

1.3 Consumption of seaweeds

Although seaweeds are algae and not plants in the usual sense, people have always eaten them. They grow in all of the Earths coastal climatic zones, manifesting themselves in a wealth of species, colour, and shapes. In many countries in Asia, notably in China and Japan, seaweed products are important dietary resource, which makeup a substantial part of the total food intake. They have been consumed in Asia since ancient times, but only to a much lesser extent in the rest of the world (Chapman and Chapman, 1980; Indergaard and Ostgaard, 1991). Consumption of unprocessed dried seaweeds can yield many healing benefits. Many physical ailments in both humans and animals may be steadily alleviated with the simple addition of seaweeds to their respective diets. Although therapeutic seaweed constituents can be extracted as crude or pure isolates, chronic conditions usually demand patients eating the whole seaweed material and not just the extracts. Preference is that the seaweed should be eaten raw (Drum, 2008). Consequently, all essential minerals are provided by dietary seaweeds and no land plant comes close to seaweeds as sources of metabolically-required minerals (Bergner, 1997).

Seaweeds can provide minerals often absent from freshwater and food crops grown on mineral-depleted soils. In addition to their regular consumption, farming on food crops can use copious amounts of seaweeds for mulch and fertilizer. Their extracts may also be sprayed directly onto leaves for foliar feeding through the stomata. Invariably, algae are 20-50 % dry weight mineral (Nishizawa, 2002). This figure is estimated by burning off seaweed's organic material and weighing the remaining ash. The elements abundant in seaweeds include: potassium, sodium, calcium, magnesium, zinc, copper, chlorine, sulfur, phosphorous, vanadium, cobalt, manganese, selenium, bromine, iodine, arsenic, iron, and fluorine. The large brown seaweeds (kelps) tend to contain more minerals per unit weight than red seaweeds. Many human body organs require particular mineral elements as part(s) of their functional structure and an example is iron for hemoglobin in blood and iodine for thyroxine in the thyroid gland (Nishizawa, 2002).

1.4 Medicinal uses of seaweeds

Traditionally, seaweeds have been involved in the folk medicine for many years in India (300 BC), Egypt (1,550 BC), China (2,700 BC), and in Japan (13,000 - 300 BC) (NAAS, 2003; Tease, 2005). Their healing properties are believed to include everything from depressing cholesterol, shrinking goiters, treating cancer, dissolving tumors and cysts, detoxifying heavy metals, aiding in weight loss and reducing water retention. The ancient use of treating phlegm accumulation, goiter and cleansing heat is documented in the Chinese text in the Western Materia Media (Maina, 2014). The usage in the Western world took place later, where they used mucilage as the means of treating rashes and burns (Karleskint *et al*., 2010). In northern Europe seaweeds were utilised as food, fertilizers and feed before the industrialization between the years of 900 to around 1750 (Maehre *et al*., 2014). The use of seaweeds in human consumption has been very common mainly in Asian countries such as Japan, China

and Korea (Venugopal, 2008). The macroalgae are mainly used for food and for their content of phycocolloids, including alginate, agar and carrageenan's (Schiener *et al*., 2015; van den Hoek *et al*., 1995). Nevertheless, seaweed biomass has been utilised all over the world for numerous purposes; both in human- and animal food, as food additives, as fertilizers and as soil improvers (Fleurence, 1999; Schiener *et al*., 2015).

Seaweeds offer a wide range of therapeutic possibilities both internally and externally. Newer notable therapies also involve the treatment against obesity and benefits from antiviral, anti-cancer and antioxidants properties (Smit, 2004). A lot of studies done on seaweeds have revealed different active components, on the basis of the nature of ailment being treated. Literature outlines just how much work has been done on seaweeds all over the world, such as the identification of kainic acid which is produced by a red alga and which is a potent vermifuge (Moloney, 1998). The most important seaweed species in Japan are "Nori" (*Porphyra* spp.), Wakame (*Undaria pannatifida*) and Kombu (*Laminaria* spp.).

There are other types of seaweeds used topically in baths, facial treatments, body wraps, with claims of improvements in circulation of blood in the body, acne treatment, skin moisturizing, detoxification, purification, rejuvenating effects or exfoliation (De Roeck-Holtzhauer, 1991). The use of marine algae in this manner creates one aspect of therapy, a discipline grounded on the certainty that "The Sea washes away all of the ills of mankind," (Euripides, 480 BC). Additionally, many medical experts currently are using topical seaweed body therapies to promote skin beauty and health. Seaweed body treatments are believed to provide surface minerals that can diffuse into the skin, and they provide pleasurable and relaxing experiences when applied in the correct context. Habitually, cosmetic therapists have beliefs that marine algae can boost blood circulation, flush out toxins, arouse local metabolism in the skin, and assist in improving skin tone and smoothness, possibly by inducing a mild dermal brasion (De Roeck-Holtzhauer, 1991). In certain cosmetic products

such as lotions and creams, it is sometimes shown on the labels that the contents include "marine extract", "extract of alga", "seaweed extract" or something similar. This often means that one of the hydrocolloids extracted from seaweed has been added into the product. Carrageenan or alginate can improve the skin moisture retention properties of the product. Pastes of seaweed, made by cold grinding or freeze crushing, are used in thalassotherapy, where they are applied to the person's body and then warmed under infrared radiation and this treatment, in conjunction with seawater hydrotherapy, is said to provide relief for rheumatism and osteoporosis (McHugh, 2003). Some seaweed applications include acting as toners and moisturizers for the skin. Mainly, specific applications on body treatments include attempts to detoxify the body, enhance lymphatic drainage, treat cellulite and clear limb swelling (oedema) (De Roeck-Holtzhauer, 1991). In addition to the extensive topical use of seaweed, many types of macroalgae are treasured as dietary inclusions, supplements, or sources of pharmaceuticals (Maeda *et al*., 2005).

1.5 Classification of Algae

Algae in general form a polyphyletic group and the taxonomy of its various taxa is continuously being revised as more genetic and structural information is obtained in addition to established phenotypic characterization (Kavishe, 2015). The grouping of algae is divided into two kingdoms; the kingdom Eukaryota and the kingdom Prokaryota eubacteria. Prokaryota eubacteria comprises of two divisions, Cyanophyta (blue green algae) and Prochlorophyta.

The Eukaryota Kingdom is made up of the following divisions: Rhodophyta (red algae); Haptophyta; Heterokontophyta; Glaucophyta; Cryptophyta (cryptomonads); Dinophyta; Euglenophyta; Clorarachniophyta; and Chlorophyta (green algae). Taxonomic classification of various taxa currently includes molecular studies. Brodie and Lewis (2007); Brodie *et al*

.(1996); Broom *et al*. (1999); Broom *et al*. (2002); Jones *et al*. (2004); Kunimoto *et al*. (1999); Milstein and Oliveira (2005); Stiller and Waaland (1993) and Sutherland *et al*. (2011), established the taxonomic status of different *Porphyra* species by assessing their molecular phylogenetic relationships.

1.6 Rhodophya phylum (red algae)

The red algae, or Rhodophyta, are one of the oldest groups of eukaryotic algae, and also one of the largest, with about 5,000-6,000 species of mostly multicellular, marine algae, including many notable seaweeds (Chinnasamya *et al*., 2015). They form a distinct group characterized by attributes like eukaryotic cells without flagella and centrioles, using floridean polysaccharides as food reserves, with phycobiliproteins as accessory pigments giving them their red color, and with chloroplasts containing unstacked thylakoids (Campbell and Woelkerling, 1990).

They account for a vast majority of seaweed species that are currently known and represent the dominant group in terms of biodiversity in all seaweed floras of the world. Of all the macroalgae, Rhodophyta are the phylum for which the most substantial classification rearrangements have occurred in recent years. They have been divided into two subphyla (Cyanidiophytina and Rhodophytina), seven classes (Cyanidiophyceae, Bangiophyceae, Florideophyceae, Stylonematophyceae, Rhodellophyceae, Porphyridiophyceae and Compsopongonophyceae) and 33 orders (Guiry and Guiry, 2011; Saunders and Hommersand, 2004; Yoon *et al*., 2006).

Given their long and complex evolutionary history, they are characterised by a great genetic and morphological diversity, which is also reflected in the vast diversity of biological and secondary metabolites exhibited by these algae. Rhodophyta is rich in variety of secondary metabolites than green and brown algae. Red seaweed is the most important source of

biologically active metabolites in comparison to other algal. Majority of the bioactive constituents isolated from marine algae are classified chemically as aromatics, nitrogenheterocyclic, nitrosulphuric heterocyclic, brominated, sterols, dibutanoids, proteins, sulphated polysaccharides, and peptides (Kasanah *et al*., 2015).

1.7 *Porphyra* genus

Porphyra C. Kützing (Phylum: Rhodophyta, Class: Bangiophyceae, Order: Bangiales, Family: Bangiaceae) forms a polyphyletic clade with *Bangia* in the family Bangiaceae (Brodie *et al*., 1998; Broom *et al*., 2004; Muller *et al*., 2001; Nelson *et al*., 2006). The number of species comprised in the genus *Porphyra* is uncertain. Published estimated figure gives 272 different species (and infraspecific) in the database at present, of which 57 have been flagged as currently accepted taxonomically (Guiry and Guiry, 2017). On the other hand, new species continue to be recognized and described based on molecular analyses (Brodie *et al*., 2007; Lindstrom, 2008; Mols-Mortensen *et al*., 2012). Recent studies have shown mysterious species within the genus, which suggests that the diversity in the genus is even greater than previously thought (Brodie and Irvine, 1997; Broom *et al*., 2002). Most species *of Porphyra* are intertidal species, but a few species extend to the subtidal (e.g. *P. amplissima*, *P. miniata,* and *P. nereocystis;* Abbott and Hollenberg, 1976; Bird and McLachlan, 1992). Due to its usage as food, *Porphyra* is one of the most commercial seaweed (Blouin *et al*., 2011). *Porphyra* species have been frequently been used for studies of bioactivities; most of them refer to *Porphyra yezoensis*, the species mostly used for cultivation and consumption. The polysaccharide fractions of *Porphyra yezoensis*, have been shown to stimulate murine phagocytic functions in *vivo* and in *vitro* and the sulphate groups to stimulate macrophage activity (Yoshizawa *et al*., 1993).

Porphyra species are considered among the most nutritious seaweeds, containing vitamins, minerals, proteins, trace elements, fibre as well (Barsanti and Gualtieri, 2006; Dlaza, 2011; Kavishe, 2015). Some *Porphyra* species have been studied over the years by different authors and nutritionally characterized (Darcy-Vrillon, 1993; Lahaye, 1991; McDermid and Stuercke, 2003; Turner, 2003). In 1993, Darcy-Vrillon reported that *Porphyra* species have low levels of saturated fats. Purple laver "nori" contains a high percentage of Vitamin B12 (Watanabe *et al*., 1999). Turner (2003) carried out the nutritional analysis of some *Porphyra* species and addressed the high amounts of vitamins, mineral salts and proteins in their thalli. *Porphyra* proteins are made up of 17 types of amino acids (Sánchez-Machado *et al*., 2004) and this is comparable to soybean proteins. This genus is also rich in iron (Shaw and Liu, 2000) and other trace elements (Noda, 1993). Mumford and Miura (1988) reported that *Porphyra* contain high levels of Vitamin A (as beta-carotene), Vitamin B group vitamins and high levels of Vitamin C in fresh samples.

1.8 Species: *Porphyra capensis*

Porphyra capensis Kützing is one of the seaweed species occurring along the entire coast wherever rocks occur in the splash zone. It was first described by Kützing (1843) from samples collected from Cape of Good Hope, (Stegenga *et al*., 1997) and belongs to the order Bangiales, family Bangiaceae and class Bangiophyceae. It was listed as having economic potential (Molloy, 1990) but it is not utilized locally yet. *Porphyra capensis*, is a fast-growing, membranous seaweed that looks like crumpled black plastic when dried (Figure **1.1**). It is mainly distributed in the Southern part of Africa. It has been found by abalone farmers, in association with the Kelp *Ecklonia maxima* (Osbeck) Papenfuss, to be a valuable supplementary feed for the South African abalone *Haliotis midae* Linneaus.

Figure **1.1:** Photograph of *Porphyra capensis* (Source: www.seaweedafrica.org.)

Taxanomy:

Kingdom: *Plantae*

Phylum: *Rhodophyta*

Class: *Bangiophyceae*

Order: *Bangiales*

Family: *Bangiaceae*

Genus: *Porphyra*

Species: *Porphyra capensis.*

There has been reports that *Porphyra capensis* is found in locations other than southern Africa, and overall, the records are old, and recent evidence of the presence of *Porphyra capensis* from shores beyond southern Africa is lacking (Ramirez and Santelices, 1991; Silva *et* al., 1996*).* It shows great seasonality with highest densities in winter and summer (Griffin *et al*., 1999). A small-scale of food-market in SA and limited use of *Porphyra capensis* as

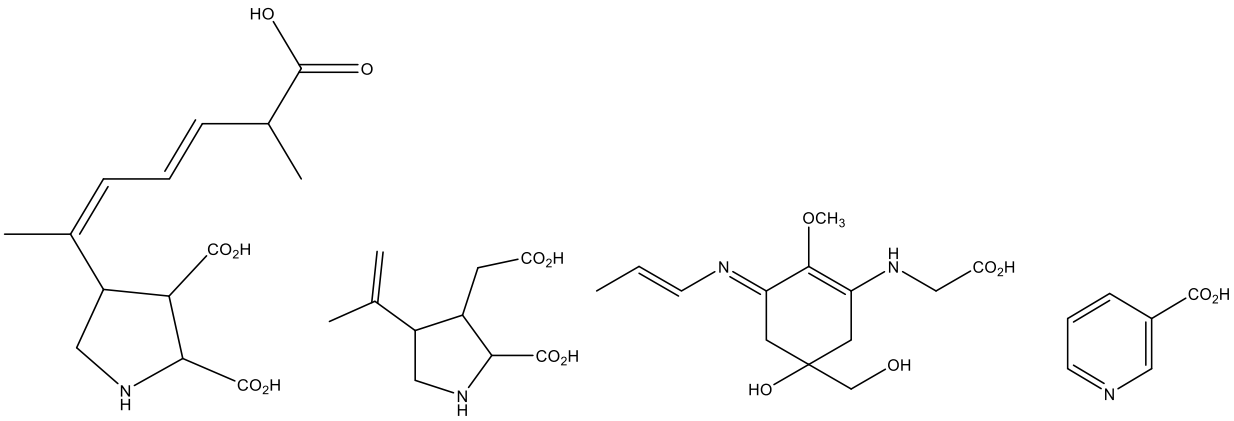
abalone feed (Troell *et al*., 2006) is responsible for localised harvesting of wild populations (Griffin *et al*., 1999).

1.9 Chemical studies on the species *Porphyra*

The chemistry of *Porphyra capensis* is not complete, because not much work has been conducted on this genus. However, several studies were undertaken on the structure and function of the polysaccharides isolated from different *Porphyra* species (Lahaye *et al*., 1985; Morrice *et al*., 1983; Rees and Conway 1962; Zang *et al*., 2003). Zang *et al* (2005) studied the structure of polysaccharide of *Porphyra capensis* and he concluded that it has a typical porphyran structure. Literature reveals that in Japan, the first compound to be isolated from one of the red algae *Chondria armata* was domoic acid (**9)** and it was named after the Japanese word for seaweed, "domoi" (Takemoto and Diago, 1958) and is produced by a large number of marine algae, including *Pseudo-nitzchia australis* and *Chondria armata* (Silvagni et al., 2005), and it has a structure similar to kainic acid (**10**) which is a potent vermifuge that has been produced by red algae such as *Digenea simplex* (Ramsdell and Zabka, 2008). Domoic acid has also been isolated from another red algae, *Alsidium corallinum*, by Impellizzeri *et al* (1975).

In addition to domoic acid, other alkaloids have been isolated from *Porphyra* species. Usijurene (**11**) a kind of mycosporine-glycine like amino acid was isolated from *Palmaria palmata* (a red seaweed) by Sekikana *et al* (1986). It was later reported by Nakayama *et al* (1999) from a *Porphyra yezoensis*, and that it has high antioxidant activity.

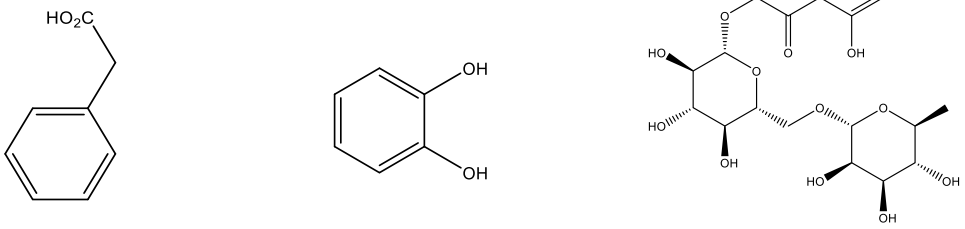
In 1961, Kanazawa found out that *Porphyra tenera* was rich in nicotinic acid (**12**) and it was later isolated from *Porphyra yezoensis* by Ding *et al* (2008), see figure **1.2**.



9 **10** **11** **12**

Figure **1.2:** Alkaloids isolated from *Porphyra*

In addition to alkaloids, polyphenols and related compounds have also been reported from red algae. Ding *et al* (2008) isolated phenylacetic acid (**13**) from *Porphyra yezoensis*. The phenylacetic acid was reported by (Hwang *et al*., 2001) and (Kim *et al*., 2004) to be active against bacteria and fungi. Kazlowska *et al* (2010) identified catechol (**14)** and rutin (**15**) by HPLC, MS and UV spectrometry, as the main bioactive phenolic compounds in a crude extract of *Porphyra dentata* that inhibited production of nitric oxide (NO) in lipopolysaccharide-stimulated macrophages through nuclear factor (NF-jB-dependent) inducible nitric oxide synthase (iNOS) gene transcription. Then the results also described the anti-inflammatory efficacy of *Porphyra dentata* and suggested a possible mechanism for the effect, see figure **1.3**.



13 **14** **15**

Figure **1.3:** Polyphenols from and related compounds from *Porphyra*

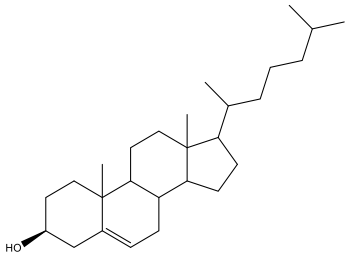
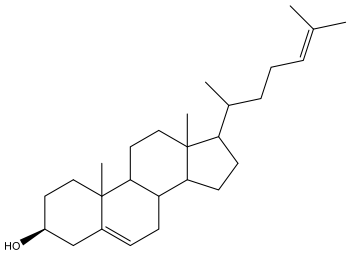
Other reported secondary metabolites from different *Porphyra* species are summarised in table **1.1:**

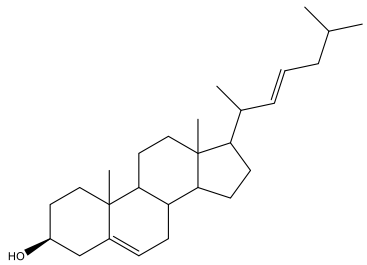
Table **1.1:** Selected secondary metabolites compounds from *Porphyra* species

Source	Activity	Reference
Mycosporine-like amino acids	Sun protective action	Bhatia *et al*., 2010; de la Coba *et al*., 2009
	Antioxidant	Tao *et al*., 2008
Phenolic compounds	Anti-inflammatory	Kazlowska *et al*., 2010
	Antioxidant	Zhao *et al*., 2006
Peptides	Antihypertensive activity	Liu *et al*., 2011; Yao *et al*., 2011 ;Yu *et al*., 2011
	Antioxidant	Yao and Tian, 2012; Yao *et al*., 2011, 2012
	Immunomodulation	Cian *et al*., 2012
Phycobiliproteins	Antiaging	Zhao and Tang, 2012
	Antioxidant	Fang *et al*., 2012; Zhao *et al*., 2012
	Tumor cell proliferation inhibiting	Fang *et al*., 2012
	Anti-inflammatory	Sakai *et al*., 2011
	Photodynamic effect	Wang *et al*., 2009
Polysaccharides		
Porphyran	Antioxidant	Zhang *et al*., 2003, 2009.; Zhao *et al*., 2006
	Anticancer	Kwon and Nam, 2006
	Immunomodulation	Bhatia *et al*., 2013 ; Ishihara *et al*., 2005
	Hypolipidemic effect	Inoue *et al*., 2009
	Glucose metabolism-modulation	Kitano *et al*., 2012

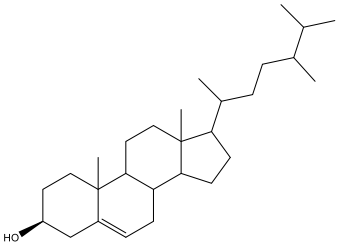
There is an extensive literature on the steroids of seaweed (table **1.2**). It has been stipulated that sterols have been demonstrated in almost all red seaweeds that have been studied. Seemingly they all have C_{27} sterols namely cholesterol (**16**), desmosterol (**4**), 22-dehydrocholesterol (**17**) that are always almost predominant. With the aid of the powerful analytical tools such as NMR, FTIR and GC-MS and the application of TLC and column chromatography it has been possible to isolate and identify sterols from mixtures. Sanchez-Machado *et al* (2004); Whittaker *et al* (2000), reported sterols identified in red seaweed such as campesterol (**18**), chalinasterol (**19**) which are not C_{27} sterols.

Table **1.2:** Sterols of Red Algae (Rhodophyta) (Patterson, 1971; Owings, R.M., 1976.)

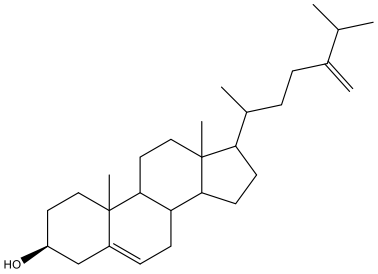
Sterol/compound	Plant source	Reference
	*Dilsea earnosa* *Tichocarpus crinirus* *Acanthopeltis japonica* *Rhodoglossum pulchrum*	Tsuda *et al*., 1957 Tsuda *et al*., 1958 Tsuda *et al*., 1958 Tsuda *et al*., 1958
(**16**) Cholesterol		
	*Porphyra purpura*	Gibsons *et al*., 1967
(**4**) Desmosterol		



(**17**) 22-dehydrocholesterol | *Hypnea japonica*
Porphyridium cruentum | Tsuda; K., *et al* 1960
Kanazawa, A., 1972



(**18**) Campesterol | *Rytiphlea tinctoria* | Devys *et al*., 1969



(**19**) chalinasterol | *Furce1laria fastigata* | Safe, 1974

Phytochemicals like phlorotannins present in red marine algae possess a number of biological properties including antiplasmin inhibition, detoxification of heavy metals, antibacterial effect, UV protection and chemoprevention against vascular risk factors (Kang *et al*., 2003).

1.10 Problem statement

Porphyra capensis is a member of the Rhodophyta family (red seaweed). In addition to serving nutritional purposes, some members of *Porphyra* genus are known to display anti-inflammatory, anti-mutagenic and anti-oxidant properties among others. One species, *Porphyra yezoensis*, has been reported to be efficient in the bioremediation of ocean waters through removal of excess inorganic nutrients. This research is being conducted in order to investigate the chemical composition of *Porphyra capensis* (red seaweed)

1.11 Justification

Seaweeds have been consumed in Asia since ancient times, and to a much lesser extent in the rest of the world. Since Africa has a range of macro-algae which have the potential for therapeutic applications in modern society, studies to understand their activity, efficiency and safety need to be pursued more vigorously. The relationships of structure and activity are important for the study. This research should reveal the main constituents which may be used as part of commercial products for the cosmeceutical and pharmaceutical industries.

1.12 Hypothesis

Seaweeds are considered a source of bioactive compounds as they produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities.

They produce polysaccharides, glycoproteins or other secondary metabolites with antimicrobial, anti-viral, antitumor and anticancer activities.

These compounds may remain bioactive following isolation from the algal source.

1.13 Objectives

1.13.1 General objectives

Preparation of crude extracts from red seaweed, both aqueous and organic solvent based; isolation and structural elucidation of secondary metabolites and test the organic extracts and isolates in bioassays.

1.13.2 Specific objectives

1. To perform extractions of crude extracts from red seaweed (*Porphyra capensis*), both aqueous and organic solvent based.
2. Carry out phytochemical screening of the crude extracts.
   a) Screening of crude organic extracts for various classes of compounds present.
3. To carry out fractionation of the organic extracts, towards isolation of pure compounds using various chromatographic techniques.
4. Characterization of the pure isolates.
   a) Pure compounds to be characterized with a combination of various spectroscopic techniques.
5. To carry out brine shrimp bioassay of the crude extracts

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and general methods.

2.1.1 Reagents

Solvents: *n*-hexane, dichloromethane, chloroform, ethyl acetate, butanol, methanol were purchased from Kimix, South Africa. Deuterated methanol, deuterated chloroform and acetonitrile and vanillin were purchased from Merck, South Africa.

2.1.2 General experimental methods

The experimental methods employed in the study included; NMR Spectroscopy, FTIR Spectroscopy, Mass Spectrometry, UV-lamp, Chromatography.

2.1.3 Solvent Evaporation

Solvent evaporation was performed on a Buchi Rotavapor RE 111, with the temperature of the water bath maintained at 45^0C.

2.1.4 Spectroscopy

2.1.4.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on Bruker-400 MHz NMR at the University of the Western Cape. Deuterated methanol (CD_3OD) and deuterated chloroform ($CDCl_3$) were used as solvents. The chemical shifts were expressed in δ (ppm), and coupling constants (*J*) in Hz.

2.1.4.2 Fourier Transform -Infrared Spectroscopy (FT-IR Spectroscopy)

IR spectra were recorded on a Perkin – Elmer paragon 1000 spectrometer that was corrected against an air background. Spectra were recorded using KBr pallets.

2.1.5 Chromatography

2.1.5.1 Column chromatography (CC)

Silica gel 60 (0.040-0.063mm) 230-400 mesh particle size (Merck) and sephadex LH-20 (Sigma Aldrich) were packed in glass columns (10-25mm diameter) for column chromatography. Size-exclusion chromatography was carried out using Sephadex® LH-20 (Pharmacia), eluting with ethanol and water

2.1.5.2 Thin layer chromatography (TLC)

Thin layer chromatography was carried out on pre-coated silica gel 60 F_{254} plates (Merck) with a 0.2 mm layer thickness. Visualisation of the TLC spots was carried out under UV light at 254nm and/or 366nm, and further detection of compounds was achieved by spraying with vanillin spray reagent (prepared by dissolving 15g of vanillin in 250ml ethanol followed by the addition of 2.5ml concentrated sulphuric acid). After spraying, the TLC plates were heated on a hot plate until spots became visible.

2.1.6 Mass Spectrometry (MS)

2.1.6.1 Gas chromatography Mass Spectrometry

GC-MS spectra were recorded using an Agilent technology gas chromatograph coupled to a model of 5977E MSO mass spectrometer at 250$^{\circ}C$ isothermal temperature equipped with flame ionisation detector (FID) on a DB 225 capillary column (30 m×0.25 mm×0.25 µm). Samples of 10 µl were injected into the GC analyser and the column temperature was

programmed at 225$^{\circ}C$, splitless injection and a flow rate set at 1.4 ml/min. Helium gas was used as the carrier gas. Analysis of the pure isolates was performed at the University of the Western Cape Chemistry department.

2.1.6.2 Electospray Ionozation Mass Spectrometry

ESI-MS spectra were recorded using Waters Synapt G2, ESI probe injected into a stream of acetonitrile, ESI positive, Cone Voltage 15. Analysis was performed at the University of Stellenbosch.

2.2 Red algae material and Extraction

2.2.1 Collection and preliminary treatments

A seaweed expect Prof Maneveld assisted us with the collection and identification. The alga material of *Porphyra capensis* was harvested at Bloubergstrand by hand, a suburb of Cape Town along the shores of Table Bay, 15 km due north of the city centre of Cape Town in December 2015 (Permit reference number: RES2015/35)./. The collected material was thoroughly washed with tap water to remove sand, epiphytes and salt on the surface of the sample. Then it was spread on a plastic to remove excess water (allow to dry) at room temperature. The dried material was ground into a powder prior to extraction.

2.2.2 Extraction

The fresh sliced bodies of the ground material (~1.00 Kg) were macerated with 80% MeOH for a period of about 48 hours. The extract was filtered using Whatman filter paper, the residue was washed with fresh MeOH (the process was repeated three times). The total extract was concentrated under vacuum to remove the methanol for freeze drying.

2.2.3 Preparation of crude extracts

The freeze dried material weighed 80.5 g and was suspended in water and partitioned successively with n-hexane (C_6H_{14}), dichloromethane (DCM), ethyl acetate (EtOAc) and butanol (BuOH). Each extract was concentrated to dryness under reduced pressure. Purification and isolation of natural products was achieved through one or a combination of chromatographic techniques.

2.3 Biological activity testing

2.3.1 Brine shrimp lethality bioassay

Procedure (Meyer et al., 1982)

2.3.1.1 Preparation of the simulated/sea water

40 grams of sea salt (nonionised NaCl) was weighed out, dissolved in 1 L of distilled water and then filtered to get a clear solution.

2.3.1.2 Hatching of the brine eggs

Brine shrimp eggs (50 mg) were weighed out and added to one side of a tank divided into two compartments, filled with simulated sea water and this side was covered with a foil. The tank was kept under constant aeration with an electrical air pump for 48 hours to allow the shrimp to hatch and mature as nauplii (larvae). Light was provided by a table lamp to attract the mature nauplii. The nauplii were used for the bioassay.

2.3.1.3 Preparation of the sample solutions

Clean test tubes were used for different concentrations of the test samples (one test tube for each concentration). Portions (20 mg) of methanol, n-hexane and butanol extracts were accurately weighed and dissolved in 2 mL DMSO (dimethyl sulfoxide) in a different test tube. Thus a concentration of 10 mg/mL was obtained which was used as a stock solution.

From this stock solution, 1000 µg/mL, 100 µg/mL and 10 µg/mL were prepared and were taken into different test tubes in triplicates respectively and each diluted with 5 mL simulated sea water so that the final concentration did not exceed 0.05% of DMSO. The nauplii (larvae) were collected with a Pasteur pipette after attracting them to one side of the tank with a light source. After 24 hours, the test tubes were observed using a magnifying glass and the numbers of the dead nauplii (larvae) were conveniently counted in each of the test tubes and were recorded as lethality data. From this data, the percentage mortality of nauplii was calculated for each concentration of the sample. The median lethal concentration (LC_{50}) of the test sample was calculated from the straight line of the graph.

2.4 Isolation and purification of compounds from *Porphyra capensis*.

2.4.1 Fractionation of the BuOH extract

The BuOH extract (7g) was pre-adsorbed on silica gel and fractionated on a column by gravity elution using the mixture of DCM elution as follows: 1L of 100%, then 1L volumes of mixtures with EtOAc in the following ratios (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100% EtOAc. Finally small portions of MeOH were introduced in the mixture with EtOAc up to 15% MeOH, (95:5), (90:10), (85:15). A total of 27 fractions were analysed by TLC using $CHCl_3$: MeOH: H_2O (200:52:6). Fractions with similar Rf values were pooled together. The combined fraction from vials 6-10 (70 mg) was dissolved in toluene-EtOAc (70:30) and loaded onto a flash column for further separation. The column was eluted with toluene-EtOAc (70:30) [500ml] and yielded a total of 16 fractions and those that had the same Rf value were pooled together and loaded on a flash column. Elution was with toluene-EtOAc (70:30) 250ml and this led to the isolation of compound **1** as a pale yellow amorphous powder (5 mg).

Fractions 16-21 were also combined (300 mg) and were dissolved in a mixture of $CHCl_3$: MeOH: H_2O (200: 56: 6) respectively and loaded on a flash column for further separation. A total of 16 subfractions was obtained and the ones with simiar Rf values were pooled together for further separation. Vial 6-8 (90 mg) were pooled together and dissolved in EtOH:H_2O (95:5) and subjected to Sephadex LH20 column chromatography for further separation. This led to the isolation of compound **2** (20 mg) as a colourless oil.

2.4.2 Fractionation of the hexane extract

The hexane extract (7.50g) was adsorbed on silica gel and fractionated on a column by gravity elution using the mixture of hexane elution as follows: 500ml of 100% hexane, then 250ml volumes of mixtures with EtOAc at the following ratios (95:5), (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100% EtOAc. Finally small volumes of MeOH were introduced in the mixture with EtOAc up to 15% MeOH (98.5:2.5), (95:5), (92.5:7.5), (90:10), (85:15). The column fractionation yielded 45 fractions and the collected fractions were analyzed by TLC using hexane-EtOAc (9.5:0.5, 8.5:1.5, 7:3). Fractions with similar Rf values were pooled together. The combined fraction from vials 2-4 (50mg) was loaded onto a silica gel column for further separation. The column was eluted with 500ml mixture of hexane-DCM (80:20) leading to the isolation of compound **6** as a white powder (14 mg).

Fraction 6-8 a yellow oil (210 mg) was dissolved in acetonitrile:methanol, loaded on a reversed phase column, the column eluted with solvent mixtures in the following ratios (100, 90:10, 80:20, 70:30, 60:40, 50:50, 100) which yielded compound **5** (30 mg)**, 7** (7 mg) and **8** (11 mg) respectively.

Fractions 14-18 were also combined (300 mg) and were dissolved in 50:50 hexane:DCM loaded on a flash column isocratic for further separation. The isocratic elution with 50:50

hexane:DCM solvent mixture led to the isolation of compound **3** (16 mg) as a yellow oil and compound **4** (11 mg) as a white solid respectively.

2.5 Physicochemical data of isolated compounds

2.5.1 Compound 1

Compound 1 was isolated as a pale yellow amorphous solid (5 mg), with the chemical formula found to be $C_{10}H_{14}O_4$, 198.22 g/mol. GC-MS data, m/z absent $[M]^+$, 186.0, 141.2, 78.1, 63.0, 45.0, 32.0. HRESIMS (positive ionization mode), m/z 199.0614 $[M+H]^+$. IR (υ): 3440, 3295, 3110, 2951, 1600, 1529, 1448, 1355, 11791, 1050, 990, 838, 782, 674 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, ppm): δ 6.29 (2H, *s*, H-4, H-6), δ 4.33 (2H, *d*, H-7), δ 3.70 (3H, *s*, OCH_3), δ 3.53 (2H, *q*, *J*= 7.08 Hz), δ 1.10 (3H, *t*, *J*= 7.06 Hz). ^{13}C-NMR ($CDCl_3$, 100 MHz, ppm), δ 150.2 (C-1, C-3), δ 137.3 (C-5), δ 134.5 (C-2), δ 105.9 (C-4, C-6), δ 63.7 (C-7), δ 59.4 (OCH_3), δ 56.9 (C-8), δ 17.0 (C-9).

2.5.2 1, 2, 3-propanetriol (2)

1,2,3-propanetriol was isolated as a colourless oil (20 mg), with the chemical formula found to be $C_3H_8O_3$. ^1H NMR (400 MHz, CD_3OD, ppm): δ 3.56 (1H, *m*, H-1), δ 3.50 (2H, *dd*, *J*= 4.78 Hz), δ 3.42 (2H, *dd*, *J*= 5.98 Hz). ^{13}C-NMR (100 MHz, CD_3OD, ppm), δ 72.9 (C-1), δ 63.5 (C-2, C-3).

2.5.3 Phytol (3)

Phytol was isolated as a yellow oil (16 mg); with the chemical formula found to be $C_{20}H_{40}O$, GC-MS, *m/z* 296.03 $[M+H]^+$. IR (υ): 3308, 2925, 2852, 1713, 1667, 1584, 1448, 1366, 1255, 1170, 994 cm^{-1}. ^1H-NMR (400 MHz, $CDCl_3$, ppm): *δ* 5.39 (1H, *t*, *J* = 6.38 Hz, H-2), *δ* 4.13 (*d*, 2H, *J* = 6.96, H-1), *δ* 1.99 (2H, *t*, *J*= 7.36, H-4), *δ* 1.65 (3H, *s*, H-20), *δ* 1.54 (1H, *m*, H-15), *δ* 1.36-1.13 (18H, br *m*, , H-5/H-14), *δ* 0.87 (6H, *d*, *J* = 6.44 *Hz*, H-16, H-17), *δ* 0.82-

0.81 (6H, *d*, *J* = 6.68 *Hz*, H-18, H-19). ^{13}C-NMR (400 MHz, $CDCl_3$, ppm): *δ* 140.4 (C-3), *δ* 123.1 (C-2), *δ* 59.4 (C-1), *δ* 39.9 (C-4), *δ* 39.4 (C-14), *δ* 37.4 (C-10), *δ* 37.4 (C-8), *δ* 37.4 (C-12), *δ* 36.7 (C-6), *δ* 32.8 (C-11), *δ* 32.7 (C-7), *δ* 28.0 (C15), *δ* 25.1 (C-5), *δ* 24.8 (C-13), *δ* 24.5 (C-9), *δ* 22.7 (C-17), *δ* 22.6 (C-16), *δ* 19.8 (C-19), *δ* 19.7 (C-18), *δ* 16.2 (C-20).

2.5.4 Cholesta-5,24-dien-3β-ol (desmosterol) (4)

Cholesta-5,24-dien-3β-ol was isolated as a white solid (11 mg); with the chemical formula found to be $C_{29}H_{48}O_1$; GC-MS data; *m*/*z* 384.3 $[M]^+$. IR (υ): 3385, 2930, 1690, 1452, 1370, 1050, 830, 734, 597 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) *δ* 1.84 (1H, *m*, H-1*ax*); *δ* 1.09 (1H, *m*, H-1*eq*); *δ* 1.83 (1H, *m*, H-2*ax*); *δ* 1.47 (1H, *m*, H-2*eq*); *δ* 3.55 (1H, *m*, H-3); *δ* 2.25 (1H, *m*, H-4*ax*); *δ* 2.18 (1H, *m*, H-4*eq*); *δ* 5.32 (1H, *dd*, *J*= 2.08 Hz, 5.24 Hz, H-6); *δ* 1.93 (1H, *m*, H-7*ax*); *δ* 1.54 (1H, *m*, H-7*eq*); *δ* 1.41 (1H, *m*, H-8); *δ* 0.91 (1H, *m*, H-9); *δ* 1.47 (1H, *m*, H-11*ax*); *δ* 1.42 (1H, *m*, H-11*eq*); *δ* 1.10 (1H, *m*, H-12*ax*); *δ* 2.01 (1H, *m*, H-12*eq*); *δ* 0.96 (1H, *m*, H-14); *δ* 1.08 (1H, *m*, H-15*ax*); *δ* 1.55 (1H, *m*, H-15*eq*); *δ* 1.82 (1H, *m*, H-16*ax*); *δ* 1.25 (1H, *m*, H-16*eq*); *δ* 1.08 (1H, *m*, H-17); *δ* 0.66 (3H, *s*,H-18); *δ* 0.98 (3H, *s*, H-19); *δ* 1.36 (1H, *m*, H-20); *δ* 0.92 (3H, *m*, H-21); *δ* 1.03 (2H, *m*, H-22); *δ* 1.36 (2H, *m*, H-23); *δ* 5.07 (1H, *tt*, *J*= 1.31 Hz, 7.08 Hz, *H*-24); *δ* 1.66 (3H,*s*, H-26); *δ* 1.58 (3H,*s*, H-27). ^{13}C NMR (100 MHz, $CDCl_3$, ppm); *δ* 37.3 (C-1); *δ* 31.7 (C-2); *δ* 71.8 (C-3); *δ* 42.3 (C-4); *δ* 140.8 (C-5); *δ* 121.7 (C-6); *δ* 31.9 (C-7); *δ* 31.9 (C-8); *δ* 50.1 (C-9); *δ* 36.5 (C-10); *δ* 21.1 (C-11); *δ* 39.8 (C-12); *δ* 42.4 (C-13); *δ* 56.8 (C-14); *δ* 24.3 (C-15); *δ* 28.2 (C-16); *δ* 56.1 (C-17); *δ* 11.9 (C-18); *δ* 19.4 (C-19); *δ* 35.6 (C-20); *δ* 18.6 (C-21); *δ* 36.1 (C-22); *δ* 24.7 (C-23); *δ* 125.2 (C-24); *δ* 131.0 (C-25); *δ* 25.7 (C-26); *δ* 17.9 (C-27)

2.5.5 5, 8, 11, 14, 17- eicosapentaenoic acid (5)

5, 8, 11, 14, 17- eicosapentaenoic acid was isolated as a yellow oil (30 mg); with the chemical formula found to be $C_{20}H_{32}O_2$, GC-MS data, m/z 302.3 $[M]^+$. IR (υ): 2920, 2851, 1701, 1452, 1296 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 5.35 (10H, *m*, H-5, H-6, H-8, H-

9, H-11, H-12, H-14, H-15, H-17, H-18), δ 2.81 (8H, *m*, H7, H-10, H-13, H-16), δ 2.35 (2H, *t*, *J* = 7.54 Hz, H-2), δ 2.09 (4H, *m*, H-4, H-19), δ 1.69 (2H, *p*, *J* = 7.4 Hz, H-3), δ 0.95 (3H, *t*, *J* = 7.54 Hz, H-20). ^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ 179.8 (C-1), δ 132.1 (C-18), 129.0 (C-8), 128.8 (C-6), 128.6 (C-15), 128.3 (C-12), 128.2 (C-8, C-9), 128.1 (C- 11), 127.9 (C-14), 127.0 (C-17), 33.4 (C-2), 26.5 (C-4), 25.6 (C-7, C-10, C-13, C-16), 24.5 (C-3), 20.6 (C-19), 14.3 (C-20).

2.5.6 Hexadecanoic acid (palmitic acid) (6)

Hexadecanoic acid was isolated as white flakes (14 mg), with the chemical formula found to be $C_{16}H_{32}O_2$, GC-MS data, m/z 256.3 $[M]^+$. IR (υ): 2920, 2851, 1701, 1452, 1296 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 0.86 (3H, *t*, *J*= 6.86 Hz, H-16), δ 1.26 (26 H, m, H-4 to H-15), δ 1.61 (2H, m, H-3), δ 2.32 (2H, *t*, *J*=7.52 Hz, H-2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 179.6 (C-1), 33.9 (C-2), δ 31.9 (C-14), δ 29.7 (C-8 to C-12), δ 29.6 (C-6, C-7), δ 29.4 (C-5), δ 29.3 (C-4), δ 29.1 (C-13), δ 24.7 (C-3), δ 22.7 (C-15), δ 14.1 (C-16).

2.5.7 (9*Z*)-9-Icosenoic acid (7)

(9*Z*)-9-Icosenoic acid was isolated as a jelly like yellow fat (7 mg), with the chemical formula found to be $C_{20}H_{38}O_2$, GC-MS data, m/z 310.3 $[M]^+$. IR (υ): 2921, 2868, 1705, 1449, 1294 cm^1. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 5.31 (2H, *m*, H-9, H-10), δ 2.32 (2H, *t*, *J* = 7.54 Hz, H-2), δ 1.99 (2H, *m*, H-8, H-11), δ 1.61 (p, *J* = 7.40 Hz, H-3), δ 1.23-1.28 (24H, *br s*, H-4/H-7, H-12/H-19), δ 0.86 (3H *t*, *J* = 6.84 Hz, H-20). ^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ 179.5 (C-1), δ 129.9 (C-9), δ 129.9 (C-10), δ 33.9 (C-2), δ 31.9 (C-18), δ 29.8-29.1 (C-4/C-7, C-12/C-17), δ 27.2(C-8, C-11), δ 24.7 (C- 3), δ 22.7 (C-19), δ 14.1 (C-20),

2.5.8 Methyl (Z)-octadec-9-enoate (8)

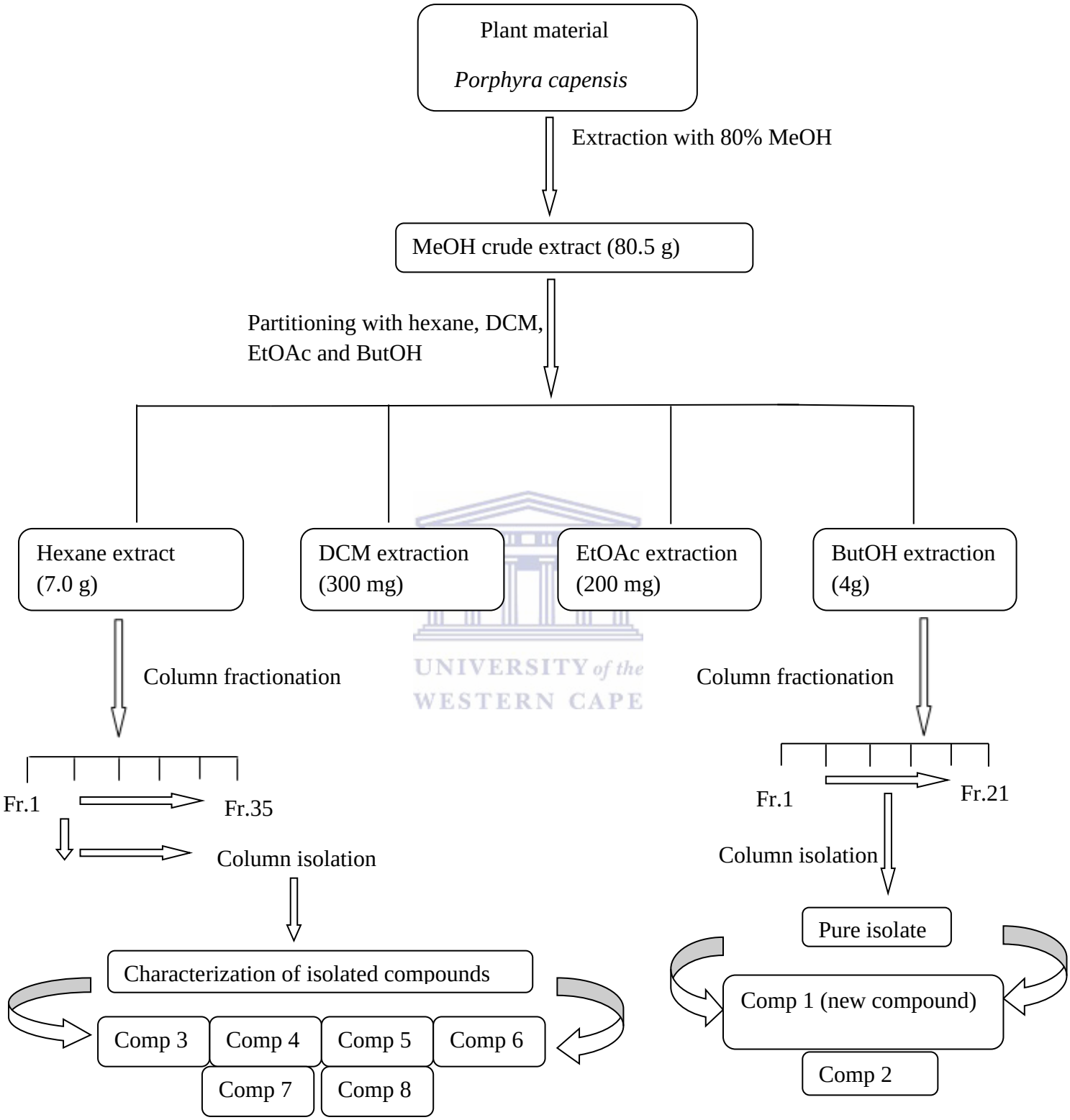
Methyl (Z)-octadec-9-enoate was isolated as a yellow oil (11 mg), with the chemical formula found to be $C_{19}H_{36}O_2$, GC-MS m/z 298.4 $[M+2H]^+$. IR (υ): 3464, 2930, 2861, 1744, 1457,

1360, 1180, 1022, 705 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, ppm,): δ 5.32 (*m*, 2H); δ 3.64 (*s*, 3H); δ 2.28 (*t*, 2H, *J*=7.34); δ 1.99 (*m*, 4H); δ 1.59 (*m*, 2H); δ 1.26-1.23 (*m*, 20H); δ 0.86 (*t*, 3H, *J*=6.82). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 174.4 (C-1); δ 129.9 (C-9/C-10); δ 51.5 (C-19); δ 34.1 (C-2); δ 31.9 (C-16); δ 29.6-29.0 (C-4/C-7, C-12/C-15); δ 27.2 (C-8/C-11); δ 24.9 (C-3); δ 22.7 (C-17), δ 14.1 (C-18).

CHAPTER 3

RESULTS AND DISCUSSION

Scheme 3.1: Isolation of natural products from *Porphyra capensis*



Eight compounds were isolated from *Porphyra capensis*. The extraction, isolation and purification of the eight compounds is summarized in scheme 3.1. It involves the extraction of the algae material with 80% methanol to give a crude extract. The crude extract was then suspended in water and partitioned using hexane, DCM, EtOAc and BuOH. Further purification of the hexane fraction using chromatographic techniques gave compounds **3**, **4**, **5**, **6**, **7** and **8**.

The BuOH fraction on the other hand was purified using column chromatography to give compounds **1** and **2**. The DCM and EtOAc fractions were not worked on due to time limitations and they were both in small quantities.

3.1: Structural elucidation and characterization of compounds

3.1.2 Compound **1**

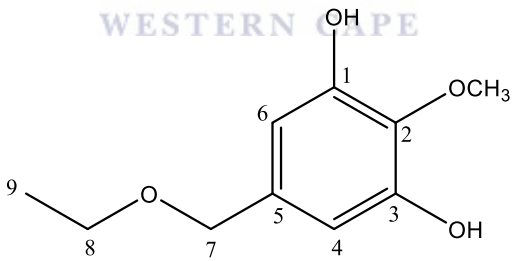


Figure **3.1:** Structure of **compound 1**

Compound **1** (Figure **3.1**) was isolated as a pale yellow amorphous solid by repeated column chromatography of the BuOH fraction. On TLC it showed an active spot under UV and a brown prominent spot was observed after spraying with vanillin. The molecular formula $C_{10}H_{14}O_4$ was established by the ion at *m/z* 186.0 g/mol $[M+3H- CH_3]^+$ in the GC-MS spectrum and the ESI-MS ion at *m/z* 199.0614 g/mol $[M+H]^+$. Theoretical mass of this

compound is 198.2158 g/mol. The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C and DEPT 135) and 2D-NMR experiments (HSQC, COSY and HMBC), (Table **3.1**), FTIR and MS data.

The FTIR spectrum of compound **1** in the range from 600 to 4000 cm^{-1} is shown in Figure **3.2**. The broad peaks in the region 3440, 3295 and 3110 cm^{-1} were assigned to –OH stretching vibration. The weak peak near 2951 cm^{-1} was attributed to aliphatic hydrocarbon stretching vibration assigned to –CH and $–CH_2$ groups. This band originates from –CH stretch vibration in aromatic methoxyl groups and in the methyl and methylene groups of side chains. The bands between 1500 cm^{-1} and 1600 cm^{-1} were due to aromatic ring C=C stretching vibrations. The bands observed from 1191 to 1050 cm^{-1} were the characteristic bands of C–O–C and aliphatic C–O, and bands in the range of 838–674 cm^{-1} were associated with C–H of benzene rings out of plane bending.

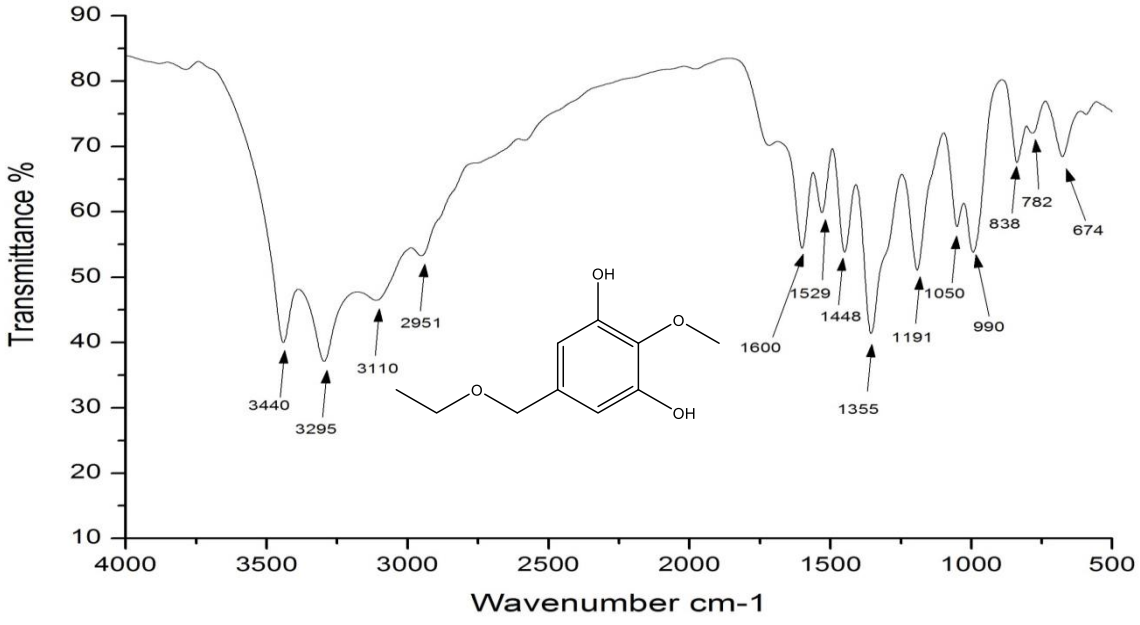


Figure **3.2**: FTIR of **compound 1**

The ^{1}H NMR spectrum (Figure **3.3**) analysis revealed five diagnostic signals. The presence of an intense singlet at δ 6.29 suggested the presence of two aromatic hydrogens in equivalent environment (*s*, H-4 and H-6, 2H) which also suggested the existence of a 1,2,3,5-tetrasubstituted benzene ring. One substituent must be an ethoxymethyl group due to the presence of the signals at δ 4.32 (2H, *s*, H-7), δ 3.54 (2H, *q*, *J* = 7.08 *Hz*, H-8) and δ 1.10 (3H, *t*, *J* = 7.06 *Hz*, H-9. Another deshielded singlet observed at δ 3.70 (3H, *s*, OCH_3) could be attributed to the three protons of the methoxyl group as one of the substituents of the attached to the benzene ring.

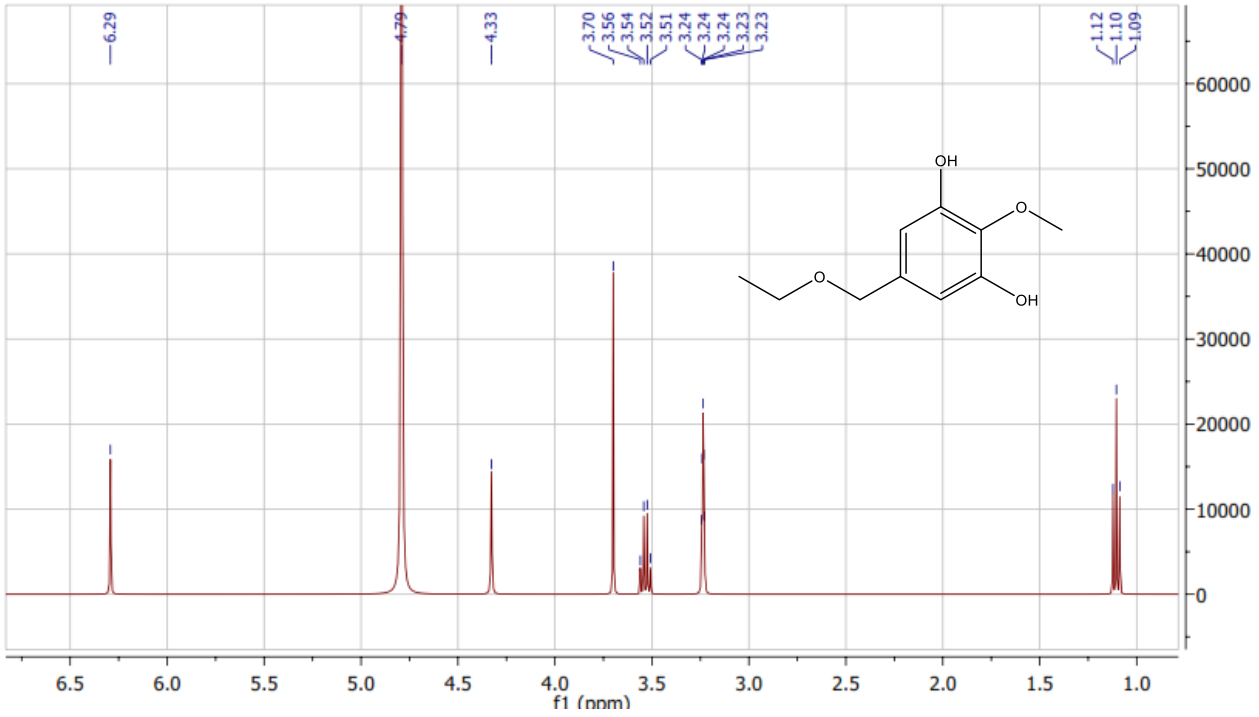


Figure **3.3**: ^{1}H NMR spectrum of **compound 1** in CD_3OD

The ^{13}C NMR and DEPT 135 spectra of compound **1** (Table **3.1**) exhibited the presence of ten carbon resonances, four substituted aromatic carbons at δ 150.2 (C-1, C-3), δ 137.3 (C-5) and δ 134.5 (C-2), two protonated aromatic carbons at δ 105.9 (C-4, C-6), two methylenes at δ 63.7 (C-7) and δ 56.9 (C-8), one methoxyl at δ 59.4 (OCH_3), and one methyl at δ 17.0 (C-9).

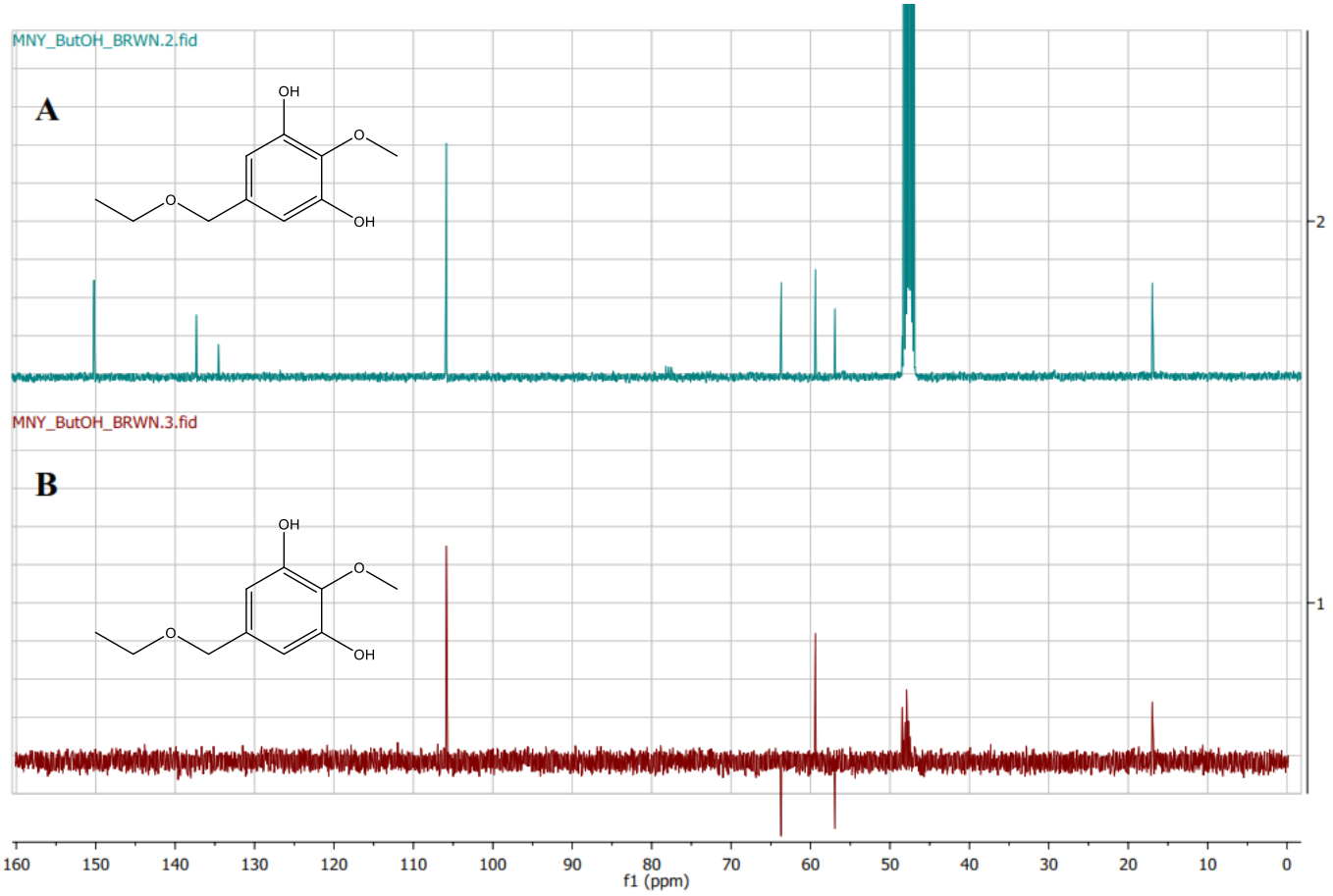


Figure **3.4**: Stacked ^{13}C and DEPT 135 spectra of **compound 1** in CD_3OD

A – ^{13}C NMR spectra of compound **1**
B – DEPT 135 of compound **1**

^{1}H-^{1}H COSY correlations were observed between the methyl protons at H-9 and the methylene protons resonating at H-2 suggesting the connection between the two, which is also supported by the respective *J* values. A much weaker four bond correlation was observed between H-7 protons and H-4, H-6 protons suggesting the linkage of the methylene group to the benzene ring.

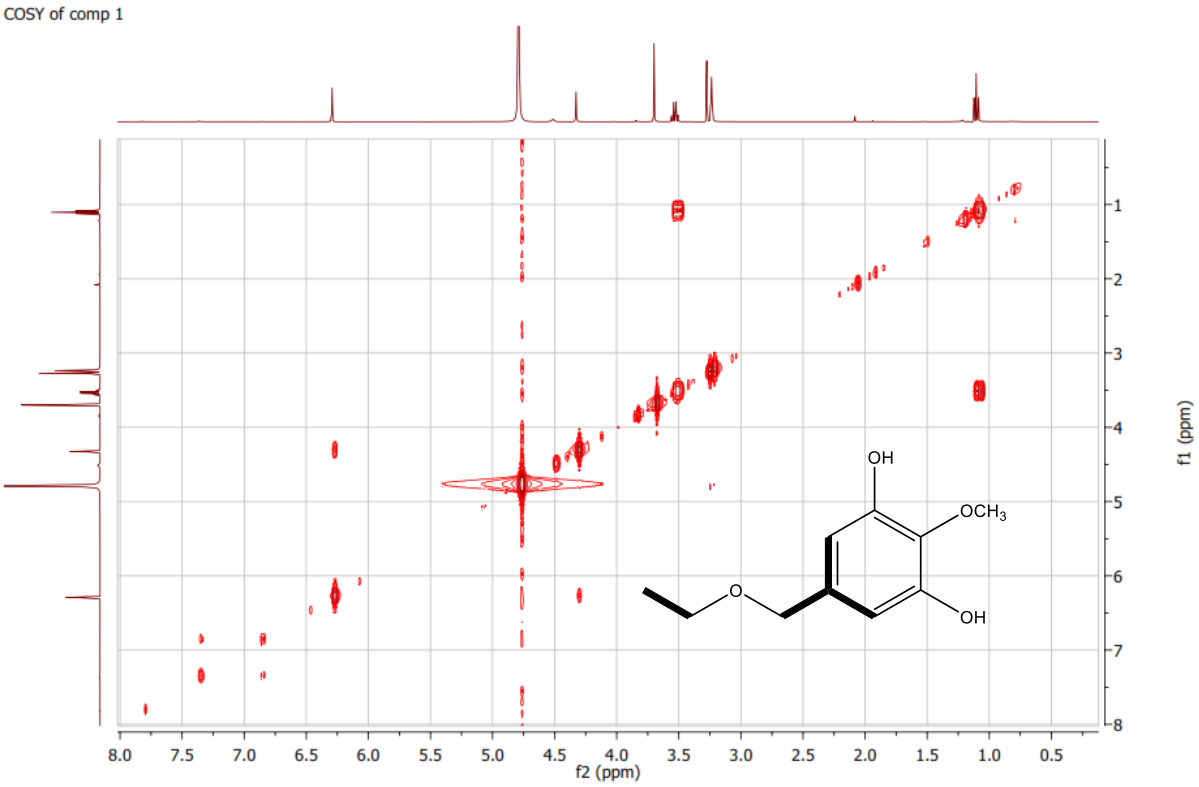


Figure **3.5**: COSY spectrum of **compound 1** in CD_3OD

HSQC experiment was used to assign proton signals to the corresponding carbon signals; the five HSQC correlations are well separated and are shown in Table **3.1**.

The HMBC spectrum (Figure **3.6**) was used to further confirm the structure of compound **1** and it was found to show the following correlations: H-4 to C-3, C-2, C-6 and C-7 and the same correlations were observed for H-6 to the same carbons suggesting that H-4 and H-6 are two equivalent protons. Other correlations observed include H-7 to C-4, C-6 and C-5 confirming the linkage of the methylene group to the benzene ring. The methoxyl group at δ 3.70 was positioned at C-2 in order to maintain symmetry in the molecule. The connectivity between C-8 and C-9 was supported by the HMBC correlation between H-8 to C-9 and H-9 to C-8

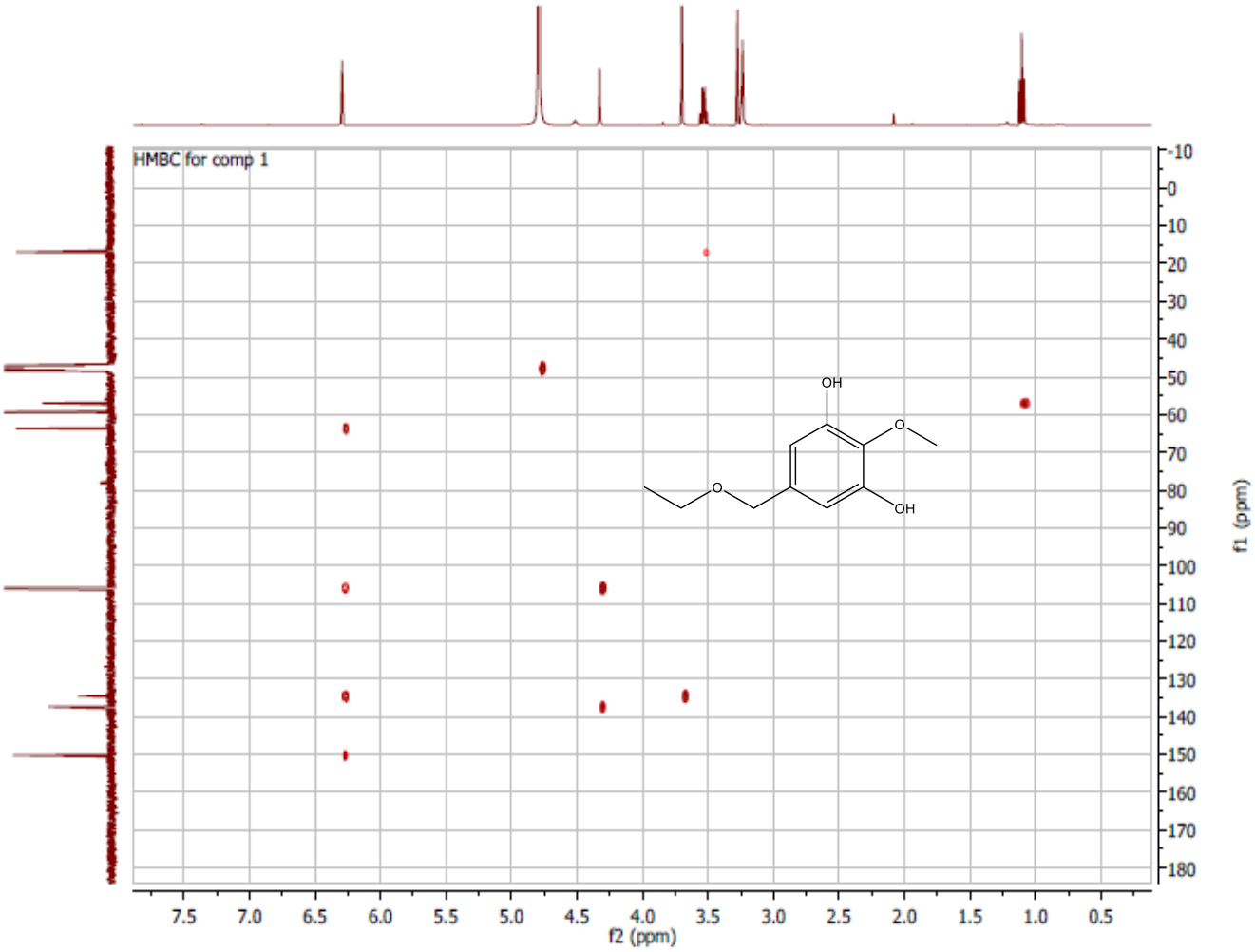


Figure **3.6**: HMBC spectrum of **compound 1** in CD_3OD

Table **3.1:** 1D and 2D NMR (CD_3OD) data for **compound 1**

Position	***δ* ^{13}C**	***δ* ^{1}H**	**DEPT**	**COSY**	**HSQC**	**HMBC**
1	150.2	-	-	-	-	-
2	134.5	-	-	-	-	-
3	150.2	-	-	-	-	-
4	105.9	6.29 (1H, *s*)	105.9	4.33	6.29	C-2, C-3, C-6, C-7
5	137.3	-	-	-	-	
6	105.9	6.29 (1H, *s*)	105.9	4.33	6.29	C-1, C-2, C-6, C-7
7	63.7	4.33 (2H, *s*)	63.7	6.29	4.33	C-4,C-5, C-6
8	56.9	3.53 (2H, *q*, *J*= 7.06 Hz)	56.9	1.10	3.53	C-9
9	17.0	1.10 (3H, *t*, $J_{,}$= 7.08 Hz)	17.0	3.53	1.10	C-8
OCH_3	59.3	3.70 (3H, *s*)	59.3	-	3.70	C-2

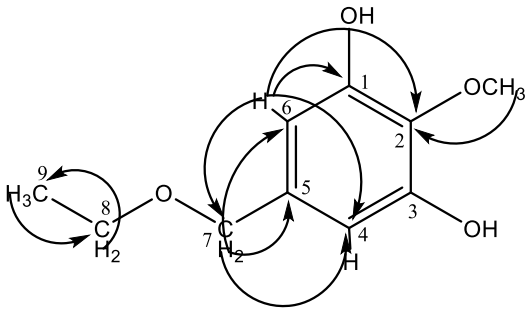
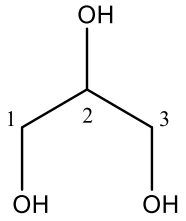


Figure **3.7:** HMBC correlations of **compound 1**

A database search provided no evidence for compound **1** as having been previously reported. As a pyrogallol derivative; the name of the compound is **5-(ethoxymethyl)-2-O-methylpyrogallol**.

3.1.2 Compound **2: 1,2,3-Propanetriol,**



Fifure **3.8:** Structure of **compound 2**

Compound **2** (Fifure **3.8**) was isolated from the BuOH extract as a colourless liquid (20 mg). Its chemical structure was elucidated by 1D ^{1}H, ^{13}C and DEPT (Table **3.2.2**). Its chemical formula was determined to be $C_3H_5(OH)_3$.

The ^{13}C NMR and DEPT135 spectrum showed two carbon signals resonating at δ 72.9 ppm and δ 63.5 ppm. The signal at δ 63.5 ppm was more intense than the other one

which suggests that it probably represents two carbon atoms (C-1 and C-3) and therefore C-2 could be associated with the signal at δ 72.9 ppm.

The ^{1}H NMR spectrum showed two doublet of doublets arising from two protons attached to the two terminal carbon atoms and a multiplet for the secondary proton. The two doublet of doublets are centered at δ 3.50 ppm (2H, *dd*, *J*= 4.78 Hz) and δ 3.42 ppm (2H, *dd*, *J*= 5.98 Hz) and the multiplet was observed at δ 3.56 ppm (1H, *m*, H-1). Theoretically, on the basis of the simple structure of compound **2** one would expect that the two protons on the terminal CH_2-OH groups would split into a doublet due to the hydrogen on the central carbon.

However, the pattern observed on the ^{1}H NMR spectrum is that of a doublet of doublets and this suggests that the two protons on each of the terminal CH_2-OH groups are not chemically equivalent. In addition, the ^{1}H NMR was run in a variety of deuterated solvents as well as high temperature (figure **3.7**), from which we observed the same splitting pattern.

Van Koningsveld (1970), did a conformational study of glycerol in order to estimate the conformer population of glycerol in D_2O solution. He demonstrated that the average environments of the terminal protons of glycerol can never be exactly identical by means of reference to the three most stable but different rotamers when the molecule is viewed along the C-1 – C-2 board of axis. The predominance of one or more of these rotamers places the two terminal H's in non-identical chemical environments and hence a chemical shift difference between the two protons can therefore be expected.

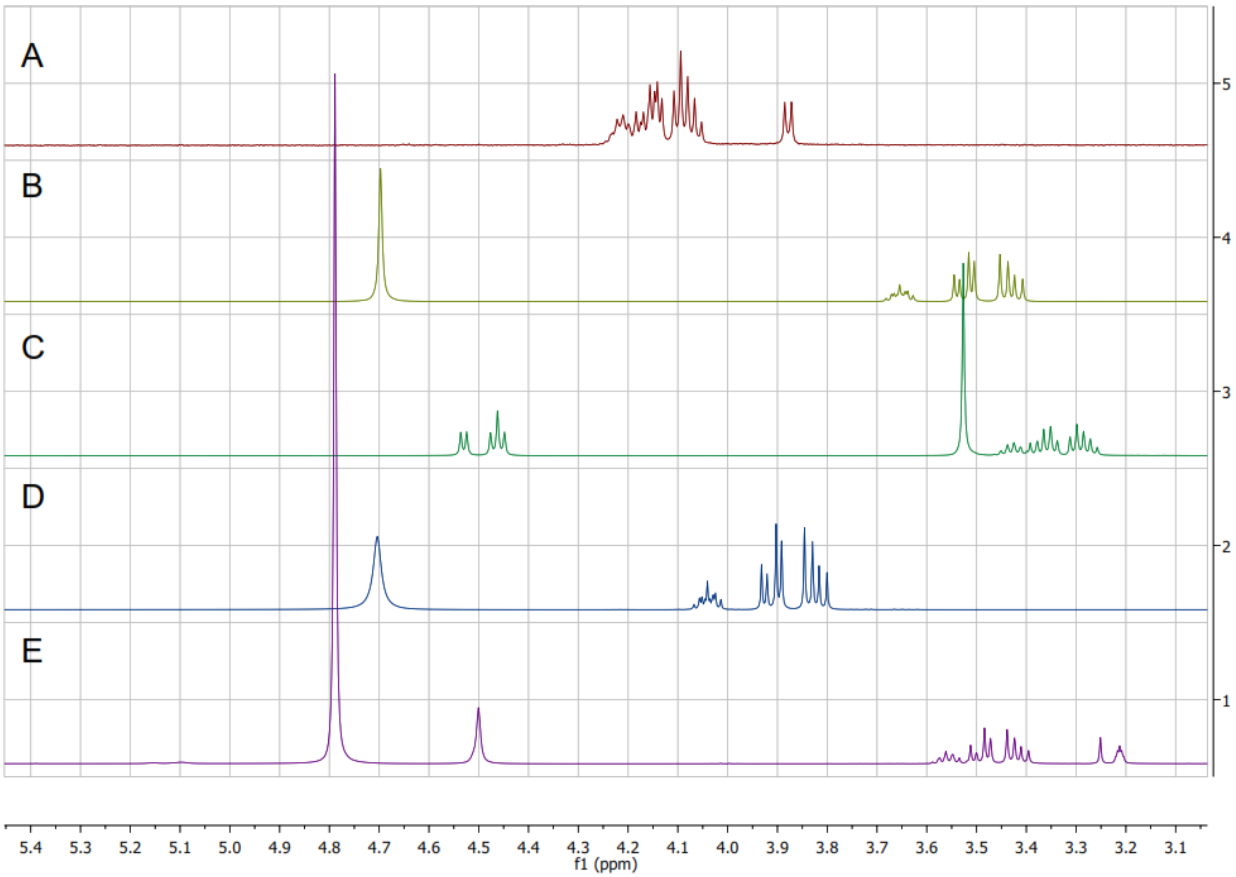


Figure **3.9**: Stacked ^{1}H NMR spectra of compound **2** in different solvents

A - Pure glycerol in $CDCl_3$, B – Pure glycerol in D_2O, C – Pure glycerol in DMSO-D_6
D – Pure glycerol in D_2O at 60°C, E – compound **2** in CD_3OD

From the spectral data we were able to conclude that the structure of compound **2** is glycerol. This compound has been isolated previously from many sources such as palm oil, soy, and coconut oil and as such, it has found many applications in the pharmaceutical, cosmetic, paint, food, tobacco, automotive, pulp and paper, leather and textile industries. It is also used as a feedstock for the production of various chemicals (Wang *et al*., 2001; da Silva *et al.*, 2009). Usov *et al* (2004) reported the identification of glycerol from a butanolic extract of brown seaweed *Colpomenia peregrine (*Sauv.) by GC-MS. Schweiger (1967) reported small amounts of glycerol being present in *Mycrocystis pyrifera* (a brown seaweed).

Table **3.2:** ^{1}H ^{13}C NMR (400 MHz, CD_3OD) data for **compound 2**

Position	δ ^{1}**H**	δ ^{13}**C**	**Glycerol**$_{(Lit)}$
1	3.50 (2H, *dd*, *J*= 4.78 Hz)	63.5	62.9
2	3.56 (*m*)	72.9	73.2
3	3.42 (2H, *dd*, *J*= 5.98 Hz)	63.5	62.9

Although glycerol may have been present in *Porphyra capensis* as its free form, it may also be degradation product of some conjugate compound. Glyceryl glycosides such as floridoside, isofloridoside, and a 3-floridosido a-n-mannopyranoside have been isolated from several red algae (Linderberg, 1955). Similar glycosides may be present originally in *Porphyra capensis* but may have been hydrolysed during the fractionation process.

3.1.3 Compound **3: Phytol**

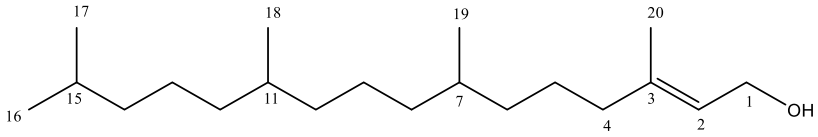


Figure **3.10:** Structure of **compound 3**

Column chomatography of the combined fractions of the hexane extract yielded 16 mg of compound **3** (Fifure **3.10**) as a yellow oil . The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C and DEPT 135) and 2D-NMR experiments (HSQC, COSY and HMBC), (Table **3.3**) and MS data. A molecular ion peak at *m*/*z* 296 (M+H)+ on GC-MS along with ^{1}H- and ^{13}C-NMR spectral data suggested its molecular formula to be $C_{20}H_{40}O$.

The ^{1}H NMR spectrum (Appendix 3a) showed resonances at δ 5.39 (1 H, *t*, *J* = 6.48 Hz) which indicated the presence of an olefinic proton (H-2) and the doublet at δ 4.13 (2H, *d*, *J* = 6.95 Hz) indicated the presence of an oxygenated allylic methylene group and was attributed to H-1. The presence of the triplet resonating at δ 1.99 (2H, *t*, *J* = 7.36 Hz) is due to the methylene protons adjacent to the double bond (H-4). A strong singlet at δ 1.65 (3H, *s*) that integrated for three protons, indicated a methyl group attached to an olefinic carbon and was attributed to H-20. The resonances in the shielded region at δ 0.81, 0.82 (6H, *d*, *J*= 6.68), δ 0.83 and δ 0.85 (6H, *d*, *J*= 6.42) were due to the four methyl protons H-18, H-19, H-16 and H-17 respectively. Other resonances in the shielded region δ 0.99-1.63 were attributed to the rest of methylene and methine protons in the molecule.

The ^{13}C NMR and DEPT 135 spectral data indicated resonances for twenty carbons, suggesting a diterpene. The ^{13}C NMR spectrum revealed the presence of olefinic carbons resonating at δ 140.4 (C-3) and δ 123.3 (C-2), three methine carbons at δ 28.0 (C-15), δ 32.7 (C-7), δ 32.8 (C-11), ten methylene carbons at δ 24.5 (C-9), δ 24.8 (C-13), δ 25.1 (C-5) , δ 36.7 (C-6), δ 37.3 (C-8), δ 37.4 (C-12), δ 37.4 (C-10), δ 39.4 (C-14), δ 39.9 (C-4), δ 59.4 (C-1) and five methyl carbons resonating at δ 16.2 (C-20), δ 19.7 (C-19), δ 19.8 (C-18), δ 22.6 (C-16), 22.7 (C-17).

^{1}H-^{1}H COSY experiment (Figure **3.11**) showed a long range (^{4}J) correlation between the methyl protons at (H-20) and the olefinic proton at δ 5.39 (H-2). A similar ^{1}H-^{1}H a long range (^{4}J) correlation was observed between the triplet resonating at δ 1.99 (H-4) with the olefinic proton at H-2 but this long range correlation is not observed between the methyl protons at H-20 and the methylene proton at H-4, therefore this implies that this long range correlation if influenced by a π bond between C-2 and C-3.

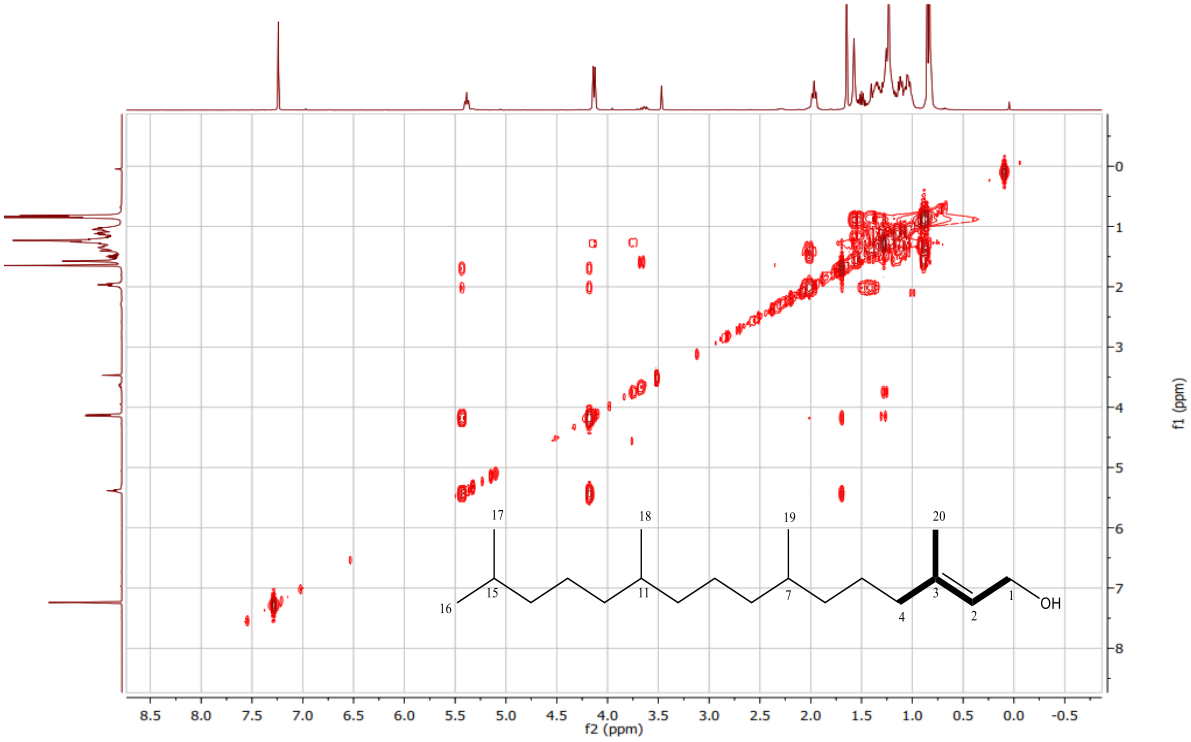


Figure **3.11**: COSY spectrum of compound **3** in $CDCl_3$

In the HMBC spectrum (appendix 3f), there was a correlation between the proton signal at H-20 and C-3, C-2 and C-4 which then confirmed the attachment of the methyl group to the olefinic carbon C-3. The chemical structure of compound **3** was finally confirmed by comparison with the previously reported data by similar ^{13}C NMR spectral data (Arigoni *et al.*, 1999). The compound was thus identified as **phytol**. Such compounds are used in the fragrance industry for the making cosmetics, shampoos, toilet soaps, household cleaners, and detergents. Phytol is widespread in nature, especially because it occurs ubiquitously as a component of chlorophyll (McGinty *et al.*, 2010; Vetter *et al.*, 2012). Phytol has also been isolated from other species of red algae such as *Gracilaria andersoniana* (Sims and Pettus, 1976) and *Aeodes orbitosa* (Mwangi, 2014).

Table **3.3:** ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) data for **compound 3**

Position	δ ^{13}**C**	δ ^{1}**H**	**COSY**	**HMBC**
1	59.4	4.13 (*d*, *J*= 6.96)	H-2, H-20	C-2, C-3
2	123.1	5.39 (*t*, *J*= 6.48 *Hz*)	H-1, H-4, H-20	C-4, C-20
3	140.4	-	-	-
4	39.9	1.99 (*t*, *J*= 7.36 *Hz*)	H-1, H-2	C-2, C-3, C-5, C-6, C-20
5	25.1	1.36 (*m*)		
6	36.7	1.19 (*m*)		
7	32.7	1.32 (*m*)		
8	37.4	1.18 (*m*)		
9	24.5	1.21 (*m*)		
10	37.4	1.17 (*m*)		
11	32.8	1.38 (*m*)		
12	37.4	1.15 (*m*)		
13	24.8	1.25 (*m*)		
14	39.4	1.13 (*m*)		
15	28.0	1.54 (*m*)		
16	22.6	0.87 (*d*, *J*= 6.44 *Hz*)		
17	22.7	0.87 (*d*, *J*= 6.44 *Hz*)		
18	19.8	0.82 (*d*, *J*= 6.68 *Hz*)		
19	19.7	0.81 (*d*, *J*= 6.68 *Hz*)		
20	16.2	1.65 (*s*)	H-1, H-2	C-2, C-3, C-4

3.1.4 Compound **4: Cholesta-5,24-dien-3β-ol (desmosterol)**

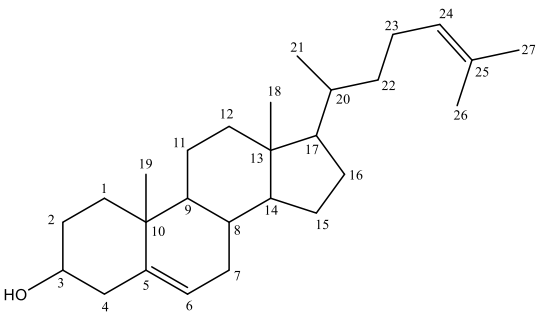


Figure **3.12:** Structure of **compound 4**

Column chromatography of the hexane extract yielded compound **4** (Figure **3.12**) as a white solid that gave a positive colour test for terpenes with the vanillin spray. This compound had a molecular formula $C_{27}H_{44}O$ as determined from its GC-MS data; m/z 384.4 $[M]^+$.

In the ^1H and spectrum (Appendix 4a) five upfield methyl signals were observed at δ 0.66 ppm (3H, *s*, Me-18), δ 0.91 ppm (3H, *dd*, *J*= 6.56 Hz, Me-21), δ 0.99 ppm (3H, *s*, Me-19), δ 1.58 ppm (3H, *s*, Me-27) and δ 1.66 ppm (3H, *s*, Me-26). A doublet of doublet appeared downfield in the olefinic region at δ 5.32 (1H, *dd*, *J*= 3.16 *Hz*, 2.08 *Hz*, H-6). The doublet of doublets splitting arises from the fact that the adjacent methylene protons at H-7 are non-equivalent, and hence they couple differently with H-6, thus giving a doublet of doublets. A multiplet was observed at δ 5.07 which was indicative of the olefin proton (H-24). The shielding of the olefinic proton at H-24 is due to the fact that it is on an aliphatic chain, therefore there's a free rotation which allows it for maximisation of the dihedral angle, unlike H-6 which is on the ring where there's rigidity. A multiplet was observed resonating at δ 3.50 (1H) due to the H-3.

The ^{13}C NMR and DEPT 135 experiments showed the presence of twenty seven carbon signals which indicated that the compound might possess a steroidal skeleton with a side chain. DEPT revealed the presence of twenty three carbon signals which have protons attached to them. Thus the other remaining four signals resonating at δ 140.7, δ 130.9, δ 42.4 and δ 37.2 could be attributed to quaternary carbons C-5, C-25, C-13 and C-10 respectively. Eight methine groups were observed at δ 125.2 (C-24), δ 121.7 (C-6), δ 71.8 (C-3), δ 56.8 (C-14), δ 56.1 (C-17), δ 50.1 (C-9), δ 35.6 (C-20) and 31.9 (C-8). The methylene carbon peaks appeared at δ 42.3 (C-4), δ 39.8 (C-12), δ 37.3 (C-1), δ 36.1 (C-22), δ 31.9 (C-7), δ 31.7 (C-

2), δ 28.2 (C-16), δ 24.7 (C-23), δ 24.3 (C-15) and δ 21.1 (C-11) while the five methyl carbons absorbed at δ 25.7 (C-26), δ 19.4 (C-19), δ 18.6 (C-21), δ 17.6 (C-27) and δ 11.9 (C-18). The chemical shift value for C-18 was found to be lower than C-19 due to γ-gauche interaction that increased the screening of the C-18 and therefore lowering chemical shift (Pateh *et al*, 2009).

^{1}H-^{1}H COSY long range (^{4}J) correlation was observed between the two methyl protons at δ 1.66 and δ 1.58 with the olefinic proton resonating at δ 5.07, this suggested linkage of the two methyl groups to the double bond. A correlation was observed between proton at δ 3.50 and the H-4*ax* proton at δ 2.26 and H-4*eq* at δ 2.21. A similar interaction was observed between δ 3.50 and H2*ax* protons at δ 1.82 and H-2*eq* at δ 1.49 and therefore the proton signal at δ 3.50 was attributed to H-3, and hence in the ^{13}C spectrum distinguishes itself by absorbing at δ 71.8 ppm which is the oxygenated region. HSQC correlations assisted in distinguishing the position of the hydroxyl group as the carbon signal at δ 71.8 (C-3) showed a correlation to the methine proton at δ 3.50 (H-3).

From the HMBC spectrum (Appendix 4f), correlations were observed between H-6 and C-7, C-8, C-10 and C-4. A similar interaction was observed between H-24 and C-26 and C-27. There was a correlation observed between H-18, C-13, C-17, and C-14 could be assigned to the structure as shown in (Figure **3.13**). The data obtained for compound **4** and upon comparison with literature data (Akihisa *et al*., 1988) confirmed the structure as **cholesta-5,24-dien-3β-ol (desmosterol).**

Table **3.4:** 1D and 2D ^{13}C NMR (400 MHz, $CDCl_3$) data for **compound 4**

Position	**δ ^{13}C**	**δ ^{1}H**	**COSY**	**HSQC**	**HMBC**
1	37.3	*ax*,1.09,*eq*1.84 (2H, *m*)	H-2*eq*	0.87, 1.12	C-2
2	31.7	*ax*,1.83,*eq*1.47 (2H, *m*)	H-1*eq*, H-3*ax*	1.88, 2.03	C-1, C-3
3	71.8	3.55 (1H, *m*)	H-2*ax*, H-2*eq*, H-4*ax*, H-4*eq*	3.55	C-2, C-4
4	42.3	*ax*,2.25,*eq*2.18 (2H, *m*)	H-3*ax*	2.25. 2.18	C-6, C-5, C-2, C-10
5	140.8	-		-	
6	121.7	5.32 (1H, *dd*, *J*= 3.16 *Hz*, 2.08 *Hz*)	H-7*eq*	5.32	C-4, C-8, C-10
7	31.9	*ax*,1.54,*eq*,1.93 (2H, *m*)	H-6	1., 1.54	C-6
8	31.9	1.41 (1H, *m*)		1.41	
9	50.1	0.91 (1H, *m*)		0.91	C-8, C-10
10	37.2	-		-	-
11	21.1	*ax*,1.42,*eq*,1.47 (2H, *m*)		1.42, 1.47	
12	39.8	*ax*,1.10,*eq*,1.99 (2H, *m*)		1.10, 1.99	C-13
13	42.4	-		-	-
14	56.8	0.96 (1H, *m*)		0.96	
15	24.3	*ax*,1.55,*eq*,1.05 (2H, *m*)		1.55, 1.05	
16	28.2	*ax*,1.82,*eq*,1.25 (2H, *m*)		1.82, 1.25	
17	56.1	1.08 (1H, *m*)		1.08	
18	11.9	0.66 (3H, *s*)		0.66	
19	19.4	0.98 (3H, *s*)		0.98	C-5, C-9
20	35.6	1.36 (1H, *m*)		1.36	C-21
21	18.6	0.91 (3H, *d*, *J*=6.6)		0.91	
22	36.1	*R*,1.01, *S*,*1*.39 (2H, *m*)		1.01, 1.39	
23	24.7	*R*,1.82,*S*,1.99 (2H, *m*)		1.82, 1.99	
24	125.2	5.07 (1H, *m*).	H-23, H-26, H-27	5.07	C-26, C-27
25	130.9	-	-	-	-
26	25.7	1.66 (3H, *s*)	H-24	1.66	C-24, C-25, C-27
27	17.6	1.58 (3H, *s*)	H-24	1.58	C-24, C-25, C-26

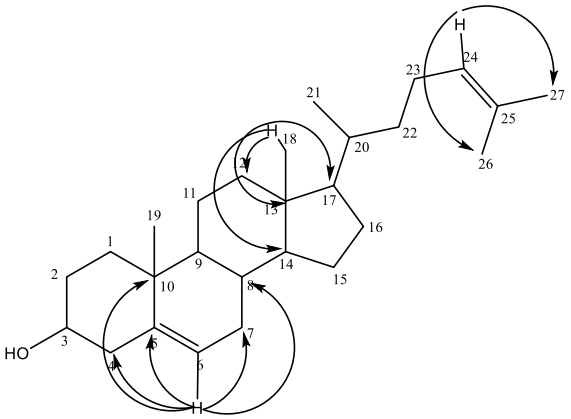


Figure **3.13:** HMBC correlations of **compound 4**

Desmosterol was first isolated from chick embryo (Stokes *et al*., 1956) and barnacle (Fagerlund and Idler., 1957) and some years later Idler *et al* (1970) isolated and identified desmosterol from the red seaweed *Rhodymenia palmata.* Gibbons *et al* (1967) reported desmosterol in four other species of red algae namely *Laurencia pinnatifida, Folysiphonia niffrescens, Porphyra purpurea, and dulse* (*Rhodymenia palmate*). Recent studies have identified desmosterol as a ligand of the liver x receptors (Lxrs) and an inhibitor of sterol biosynthesis.[3] Both Lxr isoforms α and β are present in brain and activation of Lxrs in brain can have multiple effects, which include increasing neurogenesis and reducing proliferating progenitors (Sacchetti *et al*., 2009) and it is also reported as important in the immune system because it suppresses inflammatory response genes (Spann *et al*., 2012)

A survey of the literature reveals that cholesterol commonly occurs in red algae as a major constituent with its biosynthetic precursor desmosterol also common (Bano *et al.* 1987, 1988; Chardon-Loriaux *et al.*,1976; Goodwin, 1974)

3.1.5 Compound **5: 5, 8, 11, 14, 17- eicosapentaenoic acid**

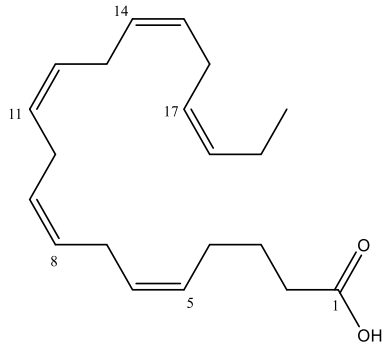


Figure **3.14:** Structure of **compound 5**

Compound **5** (Figure **3.14**) was isolated from the hexane extract as yellow oil (40 mg). The molecular formula of compound **5** was established as $C_{20}H_{30}O_2$ on the basis of GC-MS, m/z 302.3 and was calculated to be $C_{20}H_{30}O_2$. Analysis of the ^{1}H and ^{13}C spectra together with ^{1}H-^{1}H COSY and HMBC experiments, led to the assignment of all the ^{1}H and ^{13}C.

The ^{1}H NMR spectrum (appendix 5a) of compound **5** showed six important diagnostic proton signals. The broad multiplet at δ 5.26-5.42 which integrated for ten protons was assigned to the vinylic protons (10H, *m*, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15, H-17, and H-18). The signal at 2.81 (8H, *q*, H-7, H-10, H-13 and H-16) corresponds to the methylenic protons that are allylic to two double bonds hence the deshielding of the signal. Two triplets were observed at δ 2.34 (2H, *t, J*= 7.5 *Hz*, H-2) and δ 0.95 (3H, *t, J*= 7.54 *Hz*, H-20) which is a common characteristic of fatty acids. A multiplet resonating around δ 2.15-2.02 was attributed to the two allylic methylenic protons (4H, *m*, H-4 and H-19). The presence of a quintet at δ 1.69 (*p*, J = 7.40 *Hz*) is due to the split by four neighbouring proton from two neighbouring methylene groups which gives rise to a quintet.

^{13}C NMR signals indicated seventeen carbon atoms resonating at δ 179.8 (C-1), δ 132.1 (C-18), δ 129.0 (C-5), δ 128.8 (C-6), δ 128.6 (C-15), δ 128.3 (C-12), δ 128.2 (C-8 and C-9), δ 128.1 (C-11), δ 127.9 (C-14), δ 127.0 (C-17), δ 33.4 (C-2), δ 26.5 (C-4), δ 25.6 (C-10, C-13 and C16), δ 25.5 (C-7), δ 24.5 (C-3), δ 20.6 (C-20) and δ 14.3 (C-20). The high intensity signal at δ 128.2 is an indication of the presence of a pair of equivalent carbons (C-8 and C-9) and also at δ 25.6 the intensity signal implied that there were three equivalent carbon signals (C-10, C-13 and C16) (Aursand and Grasdalen., 1992).

^{1}H-^{1}H COSY NMR spectrum (appendix 5d) showed a cross peak between the olefin protons and a signal at δ 2.81 ppm which was already assigned to four methylene protons (H-7, H-10, H-13 and H-16) and at δ 2.15-2.02 ppm (H-19). The terminal methyl protons resonated at δ 0.95 ppm (H-20), and a ^{1}H-^{1}H COSY spectrum revealed a correlation between these protons and a methylene signal at δ 2.08 ppm (H-19). A triplet appeared at δ 2.35 ppm in the ^{1}H NMR spectrum which is characteristic of C-2 protons in a fatty acid. These protons showed a correlation with the quintet resonating at δ 1.69 ppm, which was readily assigned as H-3.

Table **3.5:** ^{1}H, ^{13}C and DEPT 135 NMR ($CDCl_3$) data for **compound 5**

Position	**δ ^{1}H**	**δ ^{13}C**	**COSY**	**HMBC**
1	-	179.8	-	-
2	2.34 (*t, J*= 7.5 *Hz*)	33.4	1.69 (p, *J* = 7.40 Hz)	C-1,C-3,C-4
3	1.69 (*p, J* = 7.40 *Hz*)	24.5	2.34 (*t, J*= 7.5 Hz)	C-1,C-2,C-4,C-6
4	2.08 (*m*)	26.5	5.26-5.42 (*m*)	C-2,C-3,C-6
5	5.26-5.42 (*m*)	129.0	2.08 (*m*)	-
6	5.26-5.42 (*m*)	128.8	2.08 (*m*)	-
7	2.81(*m*)	25.5	1.69 (p, *J* = 7.40 Hz)	C-8,C-9
8	5.26-5.42 (*m*)	128.2	2.81(*m*)	C-9
9	5.26-5.42 (*m*)	128.2	-	C-8
10	2.81(*m*)	25.6	5.26-5.42 (*m*)	-

11	5.26-5.42 (*m*)	128.1	2.81(*m*)	-
12	5.26-5.42 (*m*)	128.3	2.81(*m*)	-
13	2.81(*m*)	25.6	5.26-5.42 (*m*)	-
14	5.26-5.42 (*m*)	127.9	-	-
15	5.26-5.42 (*m*)	128.6	-	-
16	2.81(*m*)	25.6	-	C-18
17	5.26-5.42 (*m*)	127.0	-	-
18	5.26-5.42 (*m*)	132.1	-	C-20
19	2.05 (*m*)	20.6	0.95 (t, *J* = 7.54 Hz)	C-17C-18,C-20
20	0.95 (t, *J* = 7.54 Hz)	14.3	2.05 (*m*)	C-18,C-19

With the aid of these spectral data it could be deduced that compound **5** was an unsaturated fatty acid and after comparison with the literature data, compound **5** was identified as **5, 8, 11, 14, 17- eicosapentaenoic acid** (C_{20}:5ω3, EPA) (Fu *et al*., 2003). PUFAs, usually present as phospholipids, glycolipids or as free acids, are found in algal extracts and considered to be membrane components. Wood *et al* (1999) investigated the use of EPA as food additives in human nutrition as well as its effects on various inflammatory and autoimmune diseases (Borlak and Welch, 1994; Simopoulos, 2002). Red algae (e.g., *Porphyra* sp.) generally have high concentrations of eicosapentaenoic acid (C20:5, n-3, EPA) (Dawczynski *et al*., 2007).

3.1.6 Compound **6: Palmitic acid**

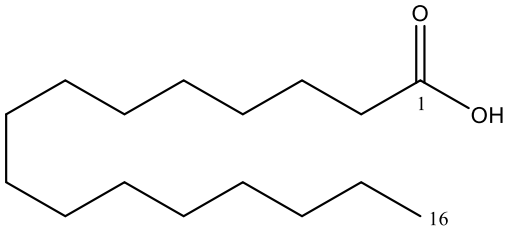


Figure **3.15:** Structure of **compound 6**

Compound **6** (Figure **3.15**) was isolated from the hexane fraction by repeated column chromatography and identified as **palmitic acid** by its 1D (^{1}H, ^{13}C and DEPT 135) (Table **3.6**), FTIR and MS data. It was isolated as a white flakey material and showed a molecular ion peak at *m/z* 256.3 $[M]^{+}$ on GC-MS. ^{1}H- NMR and ^{13}C-NMR spectral data showed its molecular formula to be $C_{16}H_{32}O_2$.

In the IR spectrum (appendix Eb) of compound **6**, a band at 2920 and 2851 cm^{-1} due to the saturated C-H bond and an intense band at 1701 cm^{-1} indicated the presence of carbonyl group of carboxylic acid and another absorption at 1452 cm^{-1} indicating bending of-CH_2- in compound.

The ^{1}H NMR spectrum (Appendix 6a) showed four aliphatic proton signals; a triplet signal at δ 2.34 ppm (2H, *t, J*=7.52.*Hz*) is attributed to the hydrogens adjacent to the carbonyl group which make them to be more deshielded (H-2). The spectrum also displays a multiplet signal at δ 1.61 ppm for the methylene group H-3 adjacent to H-2, a broad singlet resonating at δ 1.27 (H-4 to H-13) in chain methylenes and an intense triplet at δ 0.88 (*t, J*= 6.86 Hz) for methyl hydrogens at the end of the hydrocarbon chain (H-16).

Table **3.6:** ^{1}H and ^{13}C data for **compound 6**

Position	δ ^{1}**H ppm**	δ ^{13}**C ppm**	**DEPT 135**
1	-	179.6	-
2	2.31-2.34 (*t*, *J*= 7.52 Hz)	33.9	33.9
3	1.57-1.63 (*m*)	24.7	24.7
4	1.27-1.33 (*br s*)	29.7	29.7
5	1.27-1.33 (*br s*)	29.6	29.6
6 and 7	1.27-1.33 (*br s*)	29.4	29.4
8	1.27-1.33 (*br s*)	29.3	29.2
9-12	1.27-1.33 (*br s*)	29.7	29.7
13	1.27-1.33 (*br s*)	29.1	29.1
14	1.24 (*m*)	31.9	31.9
15	1.24 (*m*)	22.7	22.7
16	0.84-0.88 (*t*, *J*= 6.86 Hz)	14.1	14.1

The ^{13}C NMR and DEPT 135 spectrum showed the presence of sixteen carbon signals, consisting of a carbonyl group at δ 179.6 (C-1), and the DEPT 135 showed fourteen methylene carbon signals at δ 33.9 (C-2), 31.9 (C-14), 29.7(C-4) 29.7 (C-9), 29.7 (C-10), 29.7 (C-11), 29.7 (C-12), 29.6 (C-5), 29.4 (C-6), 29.4 (C-7), 29.3 (C-8), 29.1 (C-13), 24.7 (C-3) and 22.7 (C-15) and one methyl carbon at δ 14.1 (C-16). These spectral data suggested that compound **6** was a saturated fatty acid; it was thus concluded that compound is **palmitic acid**. Joshi *et al.* 2009 reported the isolation of palmitic acid from *Memecylon umbellatum* shrub or a small tree, belonging to the family Melastomataceae. According to the literature, the genus *Porphyra* has the highest amount of saturated fatty acid as palmitic acid (Colombo *et al*., 2006; Sanchez-Machado *et al*., 2004). Galbraith and Miller., (1973) reported that some long chain fatty acids such as linoleic acid possess antimicrobial activities. Furthermore **palmitic acid** has been assumed to be responsible for the antibacterial activity against Rhodobacteraceae bacterium R11A with an EC_{50} (Bazes *et al*., 2009). In 2002, Yff *et al*. isolated palmitic acid using bioassay guided fractionations from *Pentanisiaprunelloides* in

an effort to rationalise the use of this plant by indigenous healers in the treatment of inflammation and bacterial and viral infections.

3.1.7 Compound **7: (9*Z*)-9-ecosenoic acid**

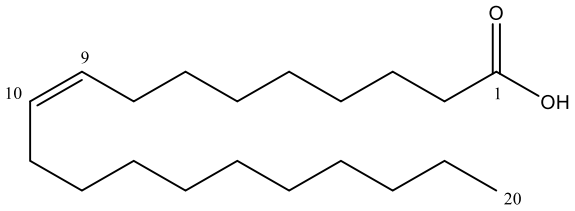


Figure **3.16:** Structure of **compound 7**

Compound **7** (Figure **3.16**) was isolated from the hexane extract as a jelly like yellow fat (7 mg). Its chemical structure was elucidated by 1D (^{1}H, ^{13}C and DEPPT 135), FTIR, and MS data. From the molecular ion peak 284.4 $[M+3H - CH_2CH_3]^+$, the molecular formula was calculated to be $C_{20}H_{38}O_2$. In the IR spectrum, the band at 2921 and 2864 $cm^{-1}$the C-H stretching of methyl and methylene groups. The presence of the absorption band near 1705 cm^{-1} is attributed to the carbonyl group, and the absorption band at 1449 cm^{-1} is due to the double bond. The ^{1}H NMR, ^{13}C NMR and DEPT-135 also aided to the elucidation of the structure of compound **7**.

Table **3.7:** 1D and 2D NMR data for **compound 7**

Position	δ ^{1}H	δ ^{13}C	COSY	HMBC
1	-	179.5	–	–
2	2.32 (*t, J*= 7.54 Hz)	33.9	1.61 (p, *J* = 7.40 Hz)	C-1, C-3, C-4
3	1.61 (p, *J* = 7.40 Hz)	24.7	2.32 (*t, J*= 7.54 Hz)	C-1, C-4, C-5
4	2.08 (m)		5.31-5.34 (*m*)	–
5-7	1.23-1.28 (*br s*)	29.8–22.7	1.61 (p, *J* = 7.40 Hz), 1.99 (*m*)	–
8	1.99 (*m*)	27.2	5.31-5.34 (*m*)	C-9, C-10
9	5.31-5.34 (*m*)	129.9	1.99 (*m*)	C-8, C-11
10	5.31-5.34 (*m*)	129.9	5.31-5.34 (*m*)	C-8, C-11
11	1.99 (*m*)	27.2		C-9, C-10
12-17	1.23-1.28 (*br s*)	29.8–22.7	1.61 (p, *J* = 7.40 Hz), 1.99 (*m*)	–
18	1.23 (*m*)	31.9		–
19	1.24 (*m*)	22.7		–
20	0.86 (t, *J* = 6.84 Hz)	14.1	1.24 (*m*)	C-18, C-19

^{1}H NMR spectrum (Appendix 7a) showed a multiplet at δ 5.32 (2H, *m*) indicating the presence of olefinic protons H-9 and H-10, at δ 2.32 (2H, *t, J*= 7.54 *Hz,*) there is a triplet indicating the presence of methylene which is bonded next to the carbonyl group and was attributed to H-2. At δ 1.99 (4H, *m*) there is a multiplet attributable to the methylene group that is adjacent to the C=C bond (H-8, H-11). The two peaks at δ 1.61 and 1.25 ppm are due to the protons of aliphatic methylene group. The triplet at δ 0.86 (*t, J* = 6.84 *Hz*) indicated the presence of protons of alkyl methyl group at the tail of the fatty acid chain (H-20).

The ^{13}C NMR spectrum (Appendix 7b) of compound **7** depicted a carbon signal in the carbonyl region resonating at δ 179.8 (C-1), two signals in the unsaturated carbon region at δ 129.9 and δ 129.9 were attributed to C-9 and C-10 respectively, methylene carbons in the

long hydrocarbon chain from δ 33.9 to 22.7 (C-2 to C-8, C-11 to C-19) and a methyl carbon at δ 14.1 (C-20).

From the ^{1}H-^{1}H COSY NMR spectrum (Appendix 7d) a correlation was observed between the olefinic protons at δ 5.34 ppm (H-9, H-10) and a signal at δ 1.99 ppm (H-8, H-11). A triplet appearing at δ 2.32 ppm in the ^{1}H NMR showed a ^{1}H-^{1}H correlation to a multiplet at δ 1.66 ppm, which was readily assigned as H-3. The terminal methyl protons resonated at δ 0.86 ppm (H-20), and a ^{1}H-^{1}H COSY spectrum revealed a connection between these protons and a methylene signal at δ 1.24 ppm (H-19).

HMBC spectrum (Appendix 7f) of compound **7** showed correlations between H-2 to C-1, C-3 and C-4, another interesting correlation was observed from the olefinic protons H-9 and H-10 to C-8 and C-11), the position of the double bond was assigned to C-9. The protons of the terminal carbon at H-20 showed a correlation with C-19 and C-18. On the basis of these spectral data it could be deduced that the structure of compound **7** is **9-eicosenoic acid**. Dawczynski *et al* (2007) detected high amounts of eicosenoic acid (C20:1; n-9) from another *Porphyra* sp; (*Porphyra tenera)*

3.1.8 Compound **8: Methyl (Z)-octadec-9-enoate**

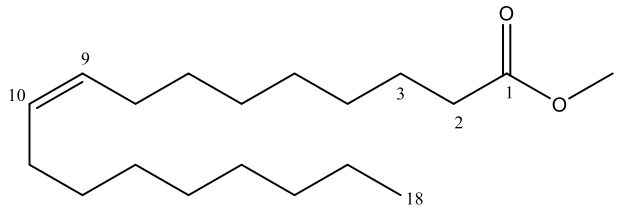


Figure **3.17:** Structure of **compound 8**

Column chromatography of fraction 5 of the hexane extract followed by reversed phased column chromatography yielded 11 mg of compound **8** (Figure **3.17**) as a yellow oil that gave a positive colour with vanillin spray. Its chemical structure was identified by its 1D (^{1}H, ^{13}C and DEPT 135), 2D NMR experiments (COSY, HSQC, and HMBC), FTIR, and MS data. Its molecular ion peak at appeared *m/z* 298.4 $[M+2H]^{+}$ in GC-MS along with ^{1}H- and ^{13}C-NMR spectral data showed its molecular formula to be $C_{19}H_{36}O_2$.

^{1}H NMR spectrum (Appendix 8a) showed a signal with chemical shift between δ 5.36-5.28 ppm (2H, *m*) which suggests the presence of olefinic protons and was attributed to H-9 and H-10, while the signal at δ 3.67 ppm represents the methyl group linked to the oxygen atom. The triplet at δ 2.28 ppm (2H, *t, J*= 7.34 Hz) represents the α-methylene protons and was attributed to H-2, while the multiplet between δ 2.00-1.96 ppm represents the methylene protons H-8, H-11. The broad singlet signal at δ 1.30 ppm is due to the protons of the backbone methylenes of long chain fatty acids and the triplet signal at δ 0.86 ppm (3H, *t*, 3H, *J*=6.82) is attributable to the terminal alkyl methyl group H-18.

^{13}C NMR spectrum (Appendix 8b) revealed a carboxylic group at δ 174.4 ppm and showed two olefinic carbons at δ 129.9 ppm. This was also supported by DEPT 135 spectrum which showed fourteen methylene carbons at δ 34.1, δ 31.9, δ 29.7, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 27.2, 25.0 and 22.7 ppm, while the peak observed at δ 51.5 ppm was attributed to the methoxyl carbon while the aliphatic methyl carbon is at δ 14.1.

In the HMBC experiment (Appendix 8f) it was observed there was a correlation between the protons at H-9 and H-10 to the C-8, C-11. A similar correlation was observed between the proton at H-2 and the carbons at C-1, C-3 and C-4. (Table **3.8**) while all other important HMBCs are shown in Figure **3.18**

Table **3.8:** 1D and 2D NMR data for **compound 8**

Position	δ ^{1}**H**	δ ^{13}**C**	**COSY**	**HMB C**
OCH_3	3.64 (*s*)	51.5	–	C-1
1	–	174.4	–	–
2	2.28 (*t*, *J*= 7.34 Hz)	34.1	1.61 (p, *J* = 7.40 Hz)	C-1, C-3, C-4
3	1.59 (p, *J* = 7.40 Hz)	25.0	2.32 (*t*, *J*= 7.54 Hz)	C-1, C-4, C-5
4-7	1.22-1.29 (*br s*)	29.8–22.7	–	–
8	1.99 (*m*)	27.2	5.31-5.34 (*m*)	C-9, C-10
9	5.28-5.36 (*m*)	129.9	1.99 (*m*)	C-8, C-11
10	5.28-5.36 (*m*)	129.9	1.99 (*m*)	C-8, C-11
11	1.99 (*m*)	27.2	5.31-5.34 (*m*)	C-9, C-10
12-15	1.23-1.28 (*br s*)	29.8–22.7	–	–
16	1.22-1.29 (*br s*)	31.9		–
17	1.22-1.29 (*br s*)	22.7		–
18	0.86 (*t*, 3H, *J*=6.82)	14.1	1.24 (*m*)	C-16, C-17

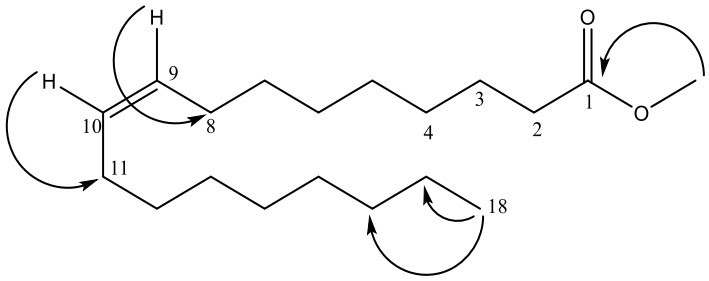


Figure **3.18:** HMBC correlations of **compound 8**

On the basis of the spectral data analysis and comparison with literature (Nurestri *et al*., 2009), the structure of **compound 8** was determined as **methyl (Z)-octadec-9-enoate**.

3.2 Biological evaluation of organic extracts

3.2.1 Brine shrimp lethality bioassay

The brine shrimp lethality assay (BSLA) is considered a useful tool for preliminary assessment of toxicity. In addition, the method is simple, rapid, economical and reproducible. A wide variety of biologically active chemical compounds are toxic to brine shrimp, in particular cytotoxic agents; the death of this organism when exposed to different concentrations of these compounds forms the basis of a toxicity test (Déciga-Campos *et al*., 2007). The percentage mortality vs. concentration was plotted. The regression equation displayed on the charts was used to calculate the LC_{50} values. Results are indicated in table format **(Table 3.9)**.

Table 3.9: Brine shrimp death indicated as % mortality at various concentrations.

	Brine Shrimp (%)					
Extracts	Time (h)	1000µg/mL	100µg/mL	10µg/mL	LC_{50} µg/mL	**Remarks**
methanol	24	2	4	1		
	24	3	2	2		**Inactive**
	24	3	1	2	3578.44	> 1000µg/mL
Average		**2.67**	**2.00**	**1.67**		
% Mortality		**26.7**	**20.0**	**16.7**		
n-hexane		1000µg/mL	100µg/mL	10µg/mL		
	24	2	1	0		
	24	5	5	0	2182.29	**Inactive**
	24	3	3	4		> 1000µg/mL
Average		**3.33**	**3.00**	**1.33**		
% Mortality		**33.3**	**30.0**	**13.3**		
Bu		1000µg/mL	100µg/mL	10µg/mL		
	24	5	2	1		
	24	3	2	2		**Inactive**
	24	2	2	2	2035.75	> 1000µg/mL
Average		**3.33**	**2**	**1.67**		
% Mortality		**33.3**	**20.0**	**16.7**		

From the results of the brine shrimp lethality bioassay (**Table 3.9**) showed that the MeOH, n-hexane and BuOH were inactive (LC_{50} > 1000 µg/mL). This may be due to the difference in the amount and kind of cytotoxic substances present in the extracts.

CHAPTER 4

CONLUSION AND FUTURE RECOMMENDATIONS

4.1 Conclusion

Since there is a lack of knowledge about alternative therapies and herbal medications, it was important to assess the phytochemistry and possibly identify compounds in *Porphyra capensis*. There are no literature reports on the phytochemistry of *Porphyra capensis* and thus this work is the first of its kind, which can be now be documented.

Subsequent to the investigations conducted in this study, a seaweed species *Porphyra capensis* was found to provide interesting results. A detailed investigation on the chemistry of the organic composition was conducted. This led to the isolation of both small and complex structures of the alga. Since bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still creates problems. Basically most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate them. The chromatographic technique used or employed in this study resulted in the isolation of eight compounds from *Porphyra capensis*. These compounds were characterized and identified as compound **1**, glycerol (**2**), phytol (**3**), desmosterol (**4**), eicosapentanoic acid (**5**) palmitic acid (**6**), 9-eicosenoic acid (**7**) and methyl (Z)-octadec-9-enoate (**8**). Further, the crude methanol, butanol and hexane extracts were subjected to the Brine shrimp test and found to be non-toxic with LC_{50} values above 2000 µg/ml.

These results are reported for the first time for this alga.

FUTURE RECOMMENDATIONS

4.2 Future recommendations

It is recommended that a reasonable amount of the material examined in this study must be used in future, so as to be able to obtain the available adequate amounts of the organic constituents in the plant of interest in; which in return will allow further relative biological studies on such isolated constituents.

Further research is necessary to investigate more on the chemical composition of the DCM and EtOAc extracts in addition to what has now been done on this study.

To investigate the effect of climate on the compounds produced produced by the algae by collecting the algae in different seasons and ascertaining if there is any changes in the composition of the compounds using HPLC as a method for seasonal profile of the compounds from the algae

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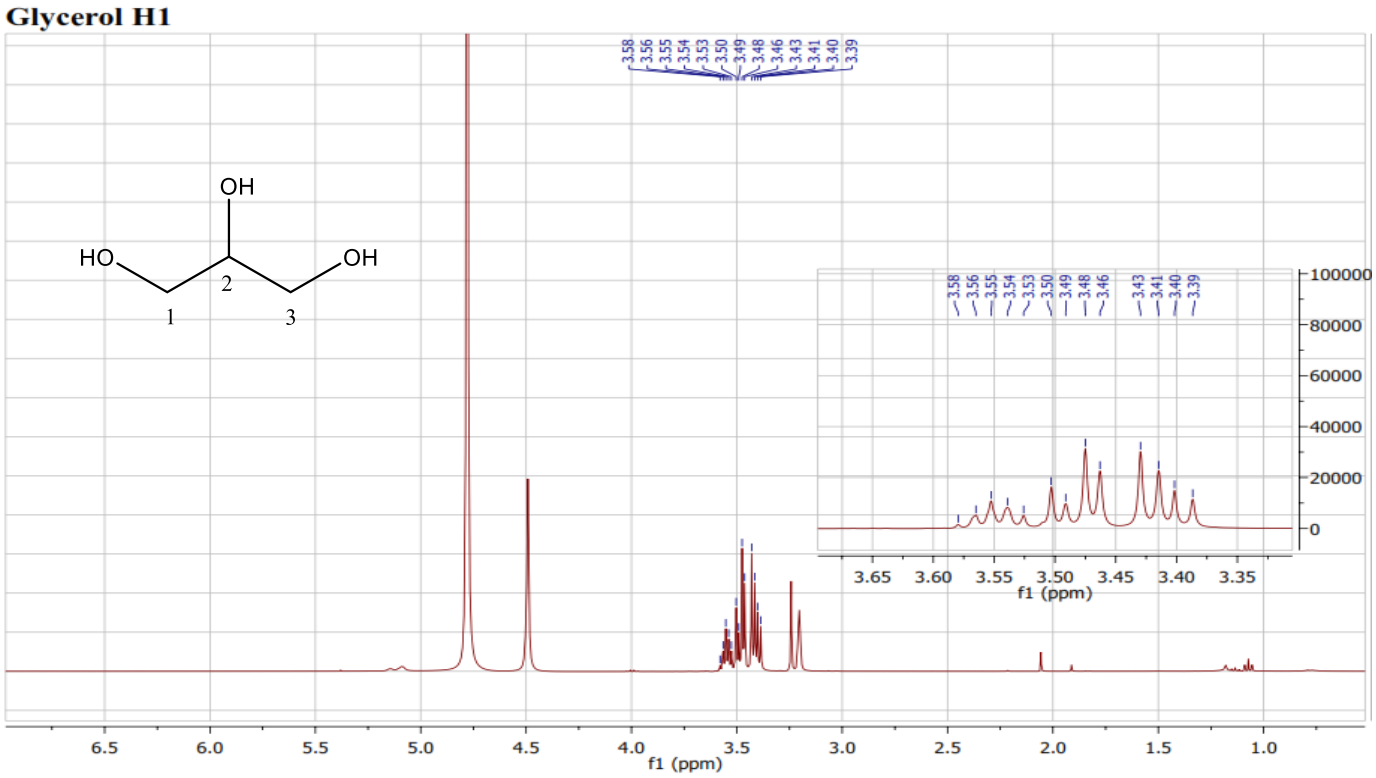
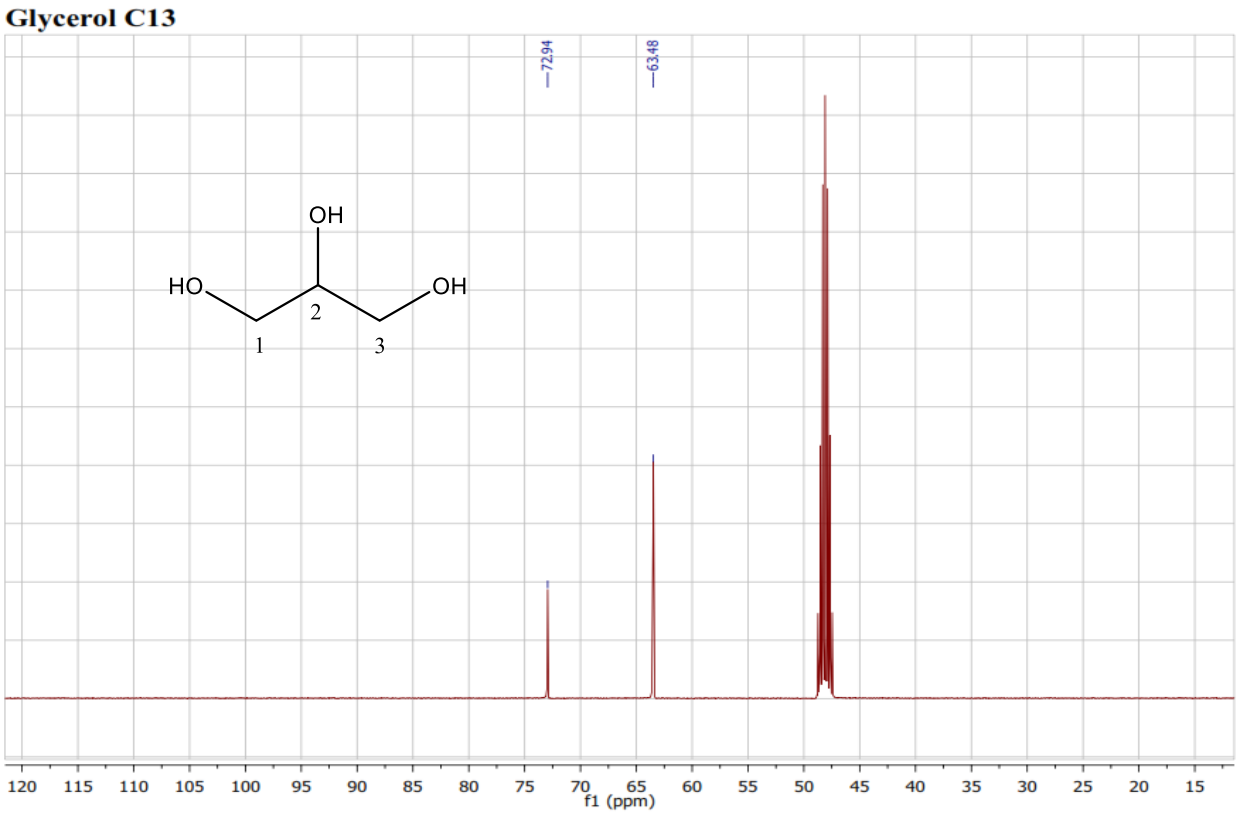
Appendix 1

NMR spectra of isolated compounds

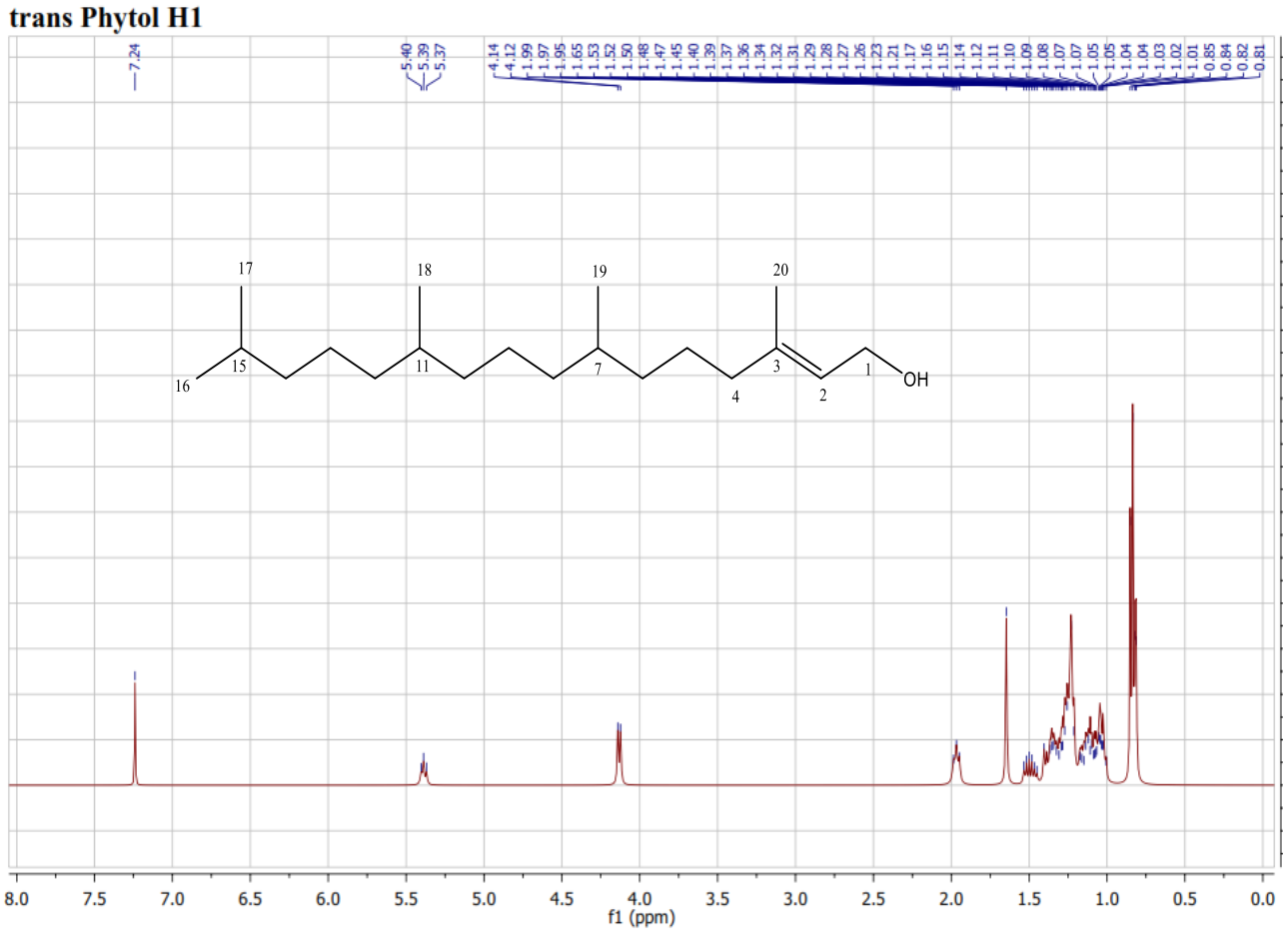
Appendix 2a**: ^{1}H NMR spectrum of glycerol in CD_3OD**



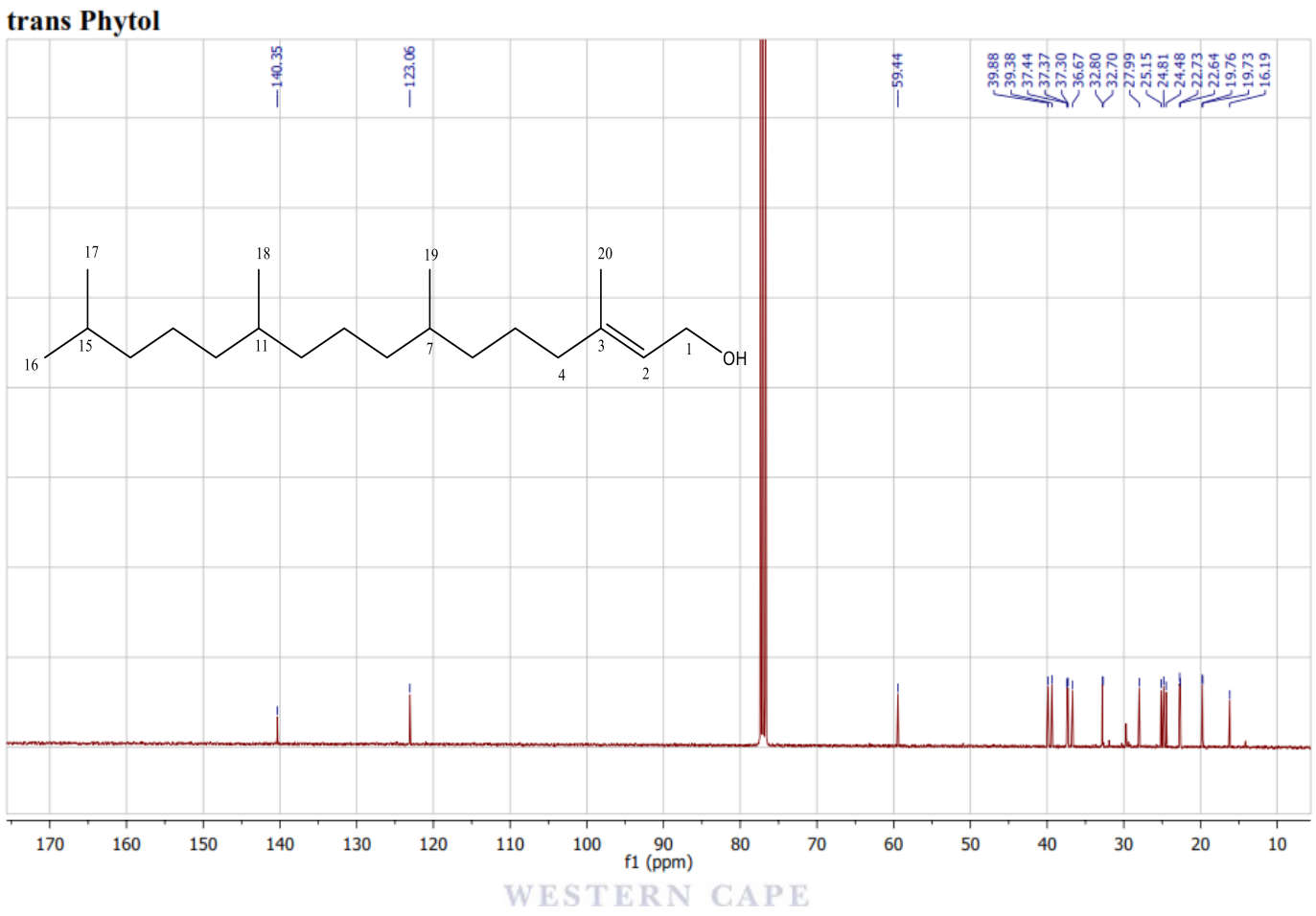
Appendix 2b**: ^{13}C NMR spectrum of glycerol in CD_3OD**



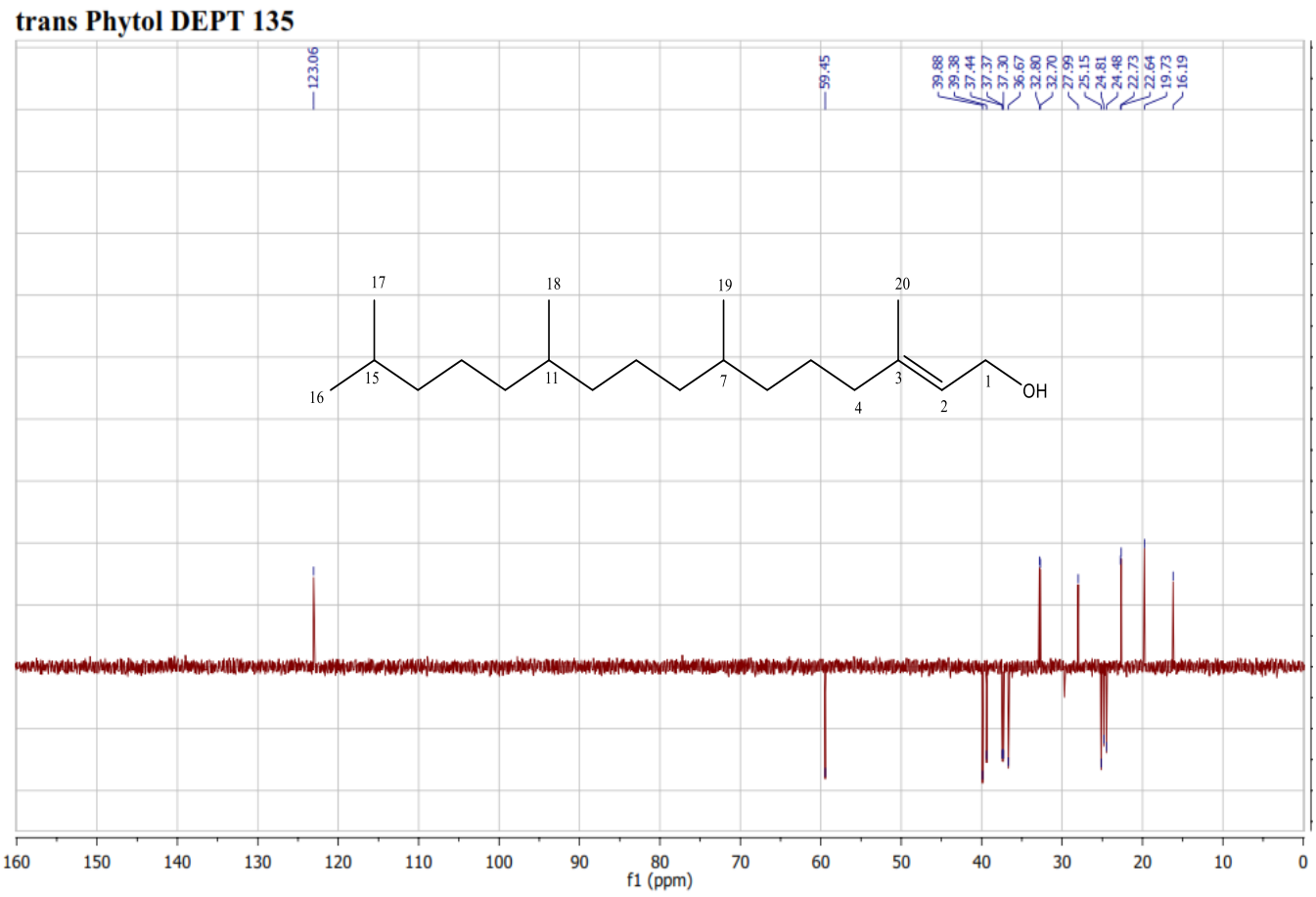
Appendix 3a**: ^{1}H NMR spectrum of Phytol in $CDCl_3$**



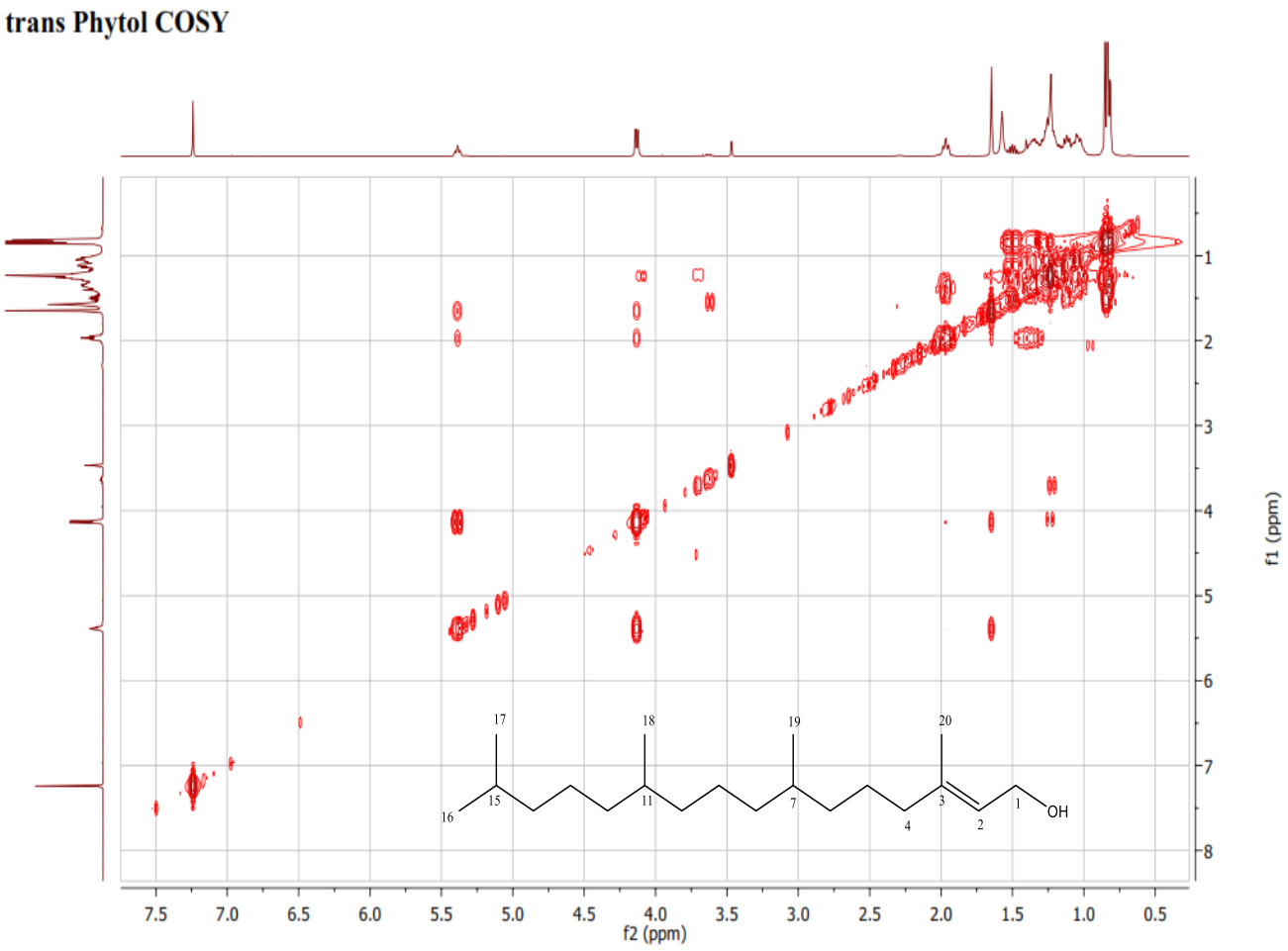
Appendix 3b**: ^{13}C spectrum of Phytol in $CDCl_3$**



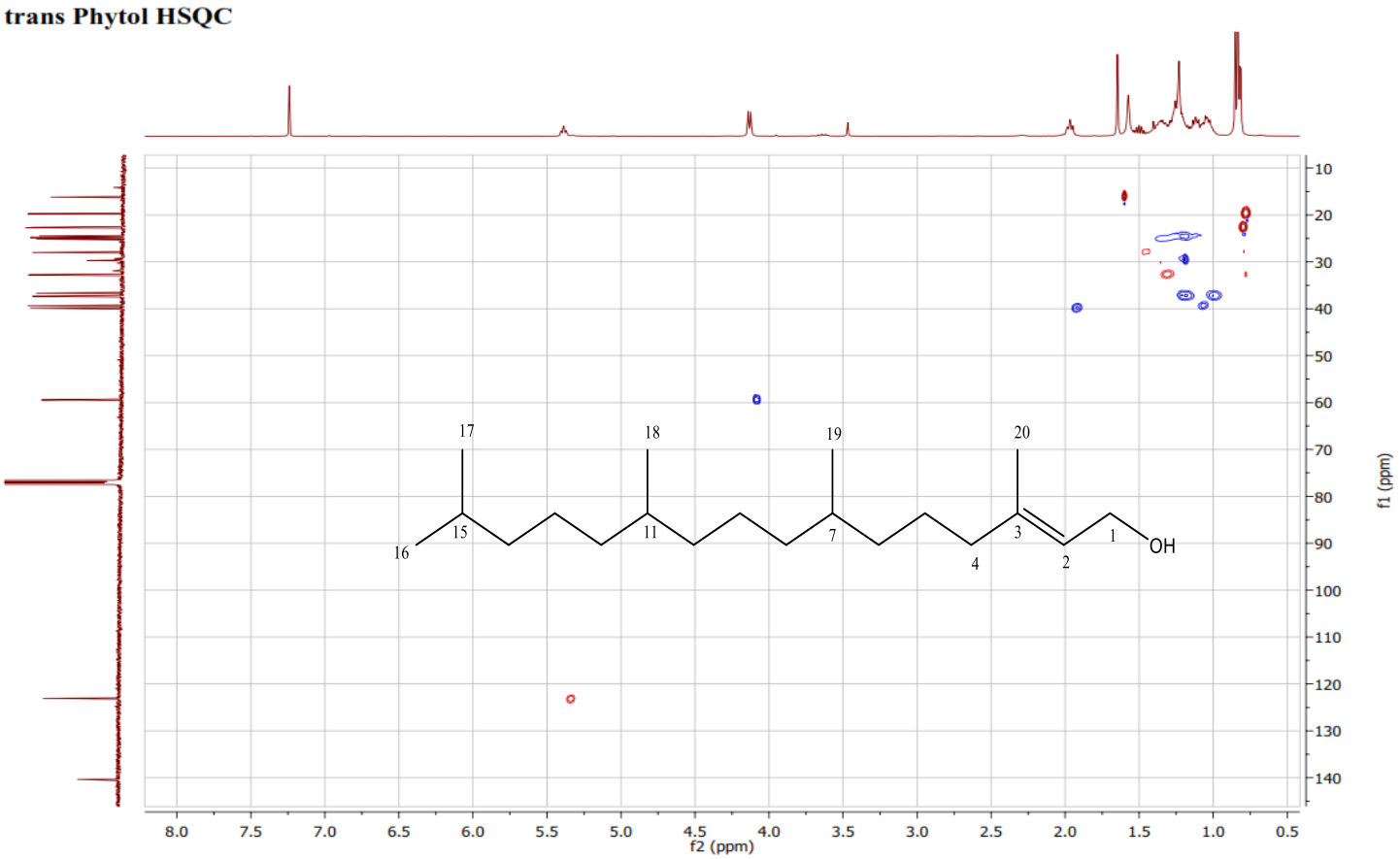
Appendix 3c**: DEPT 135 spectrum of Phytol in $CDCl_3$**



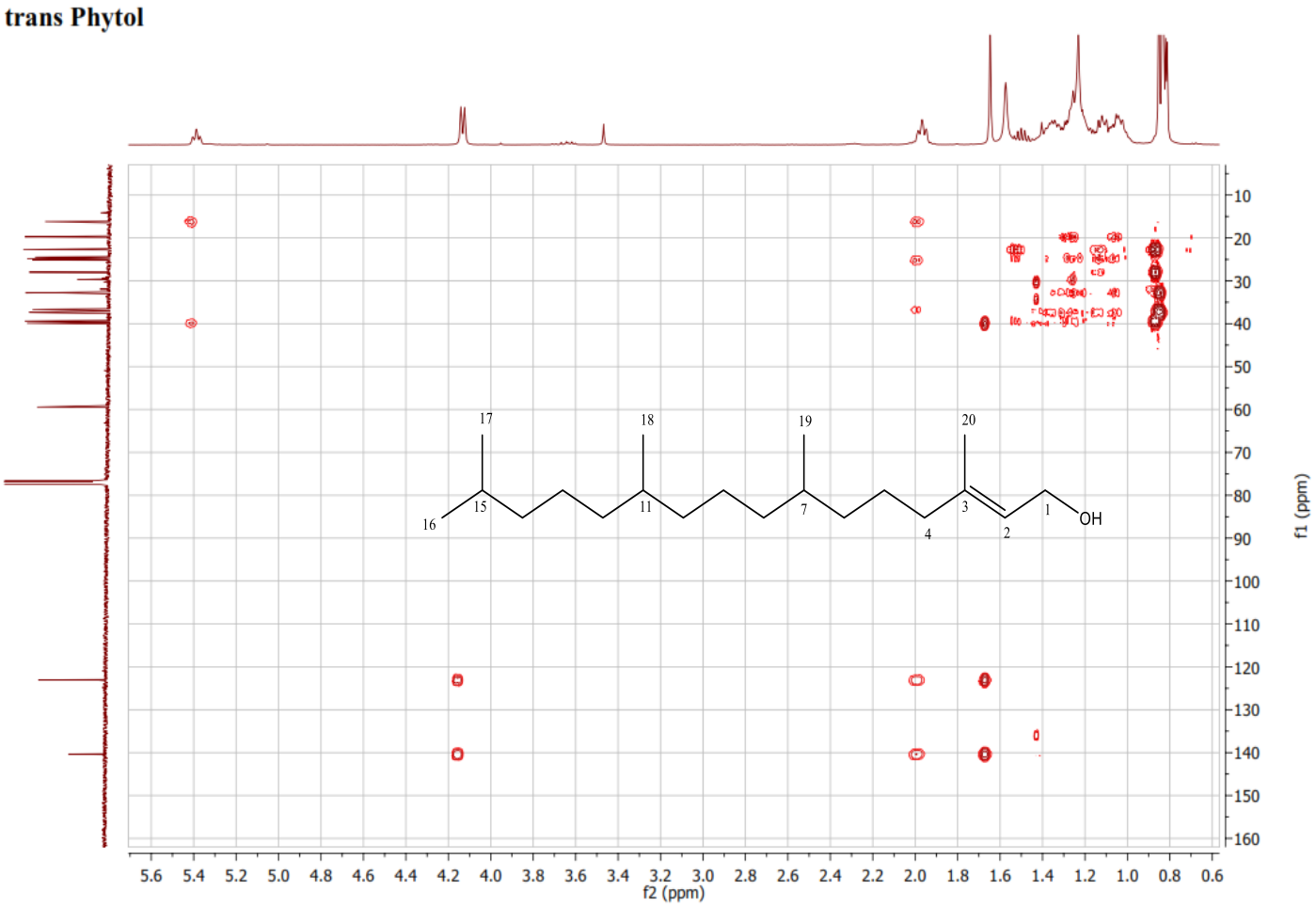
Appendix 3d**: COSY spectrum of Phytol in $CDCl_3$**



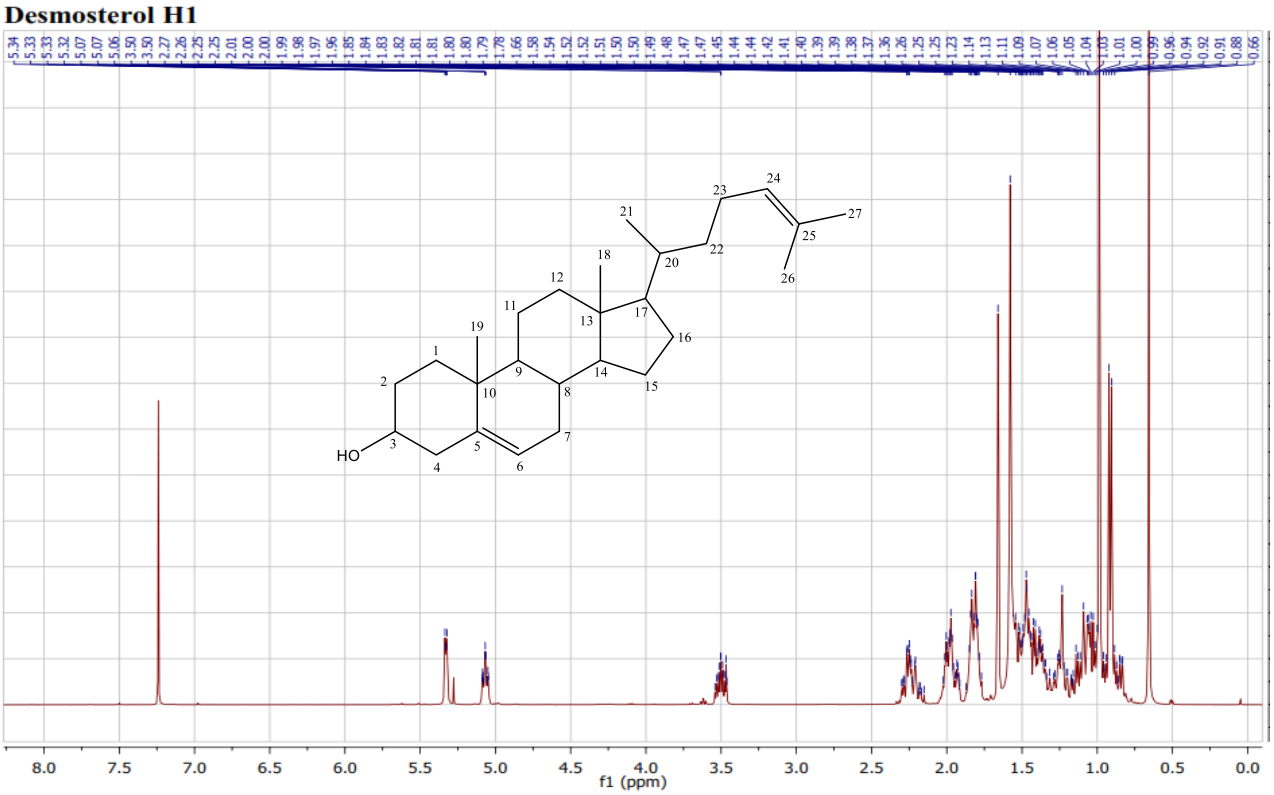
Appendix 3e**: HSQC spectrum of Phytol in $CDCl_3$**



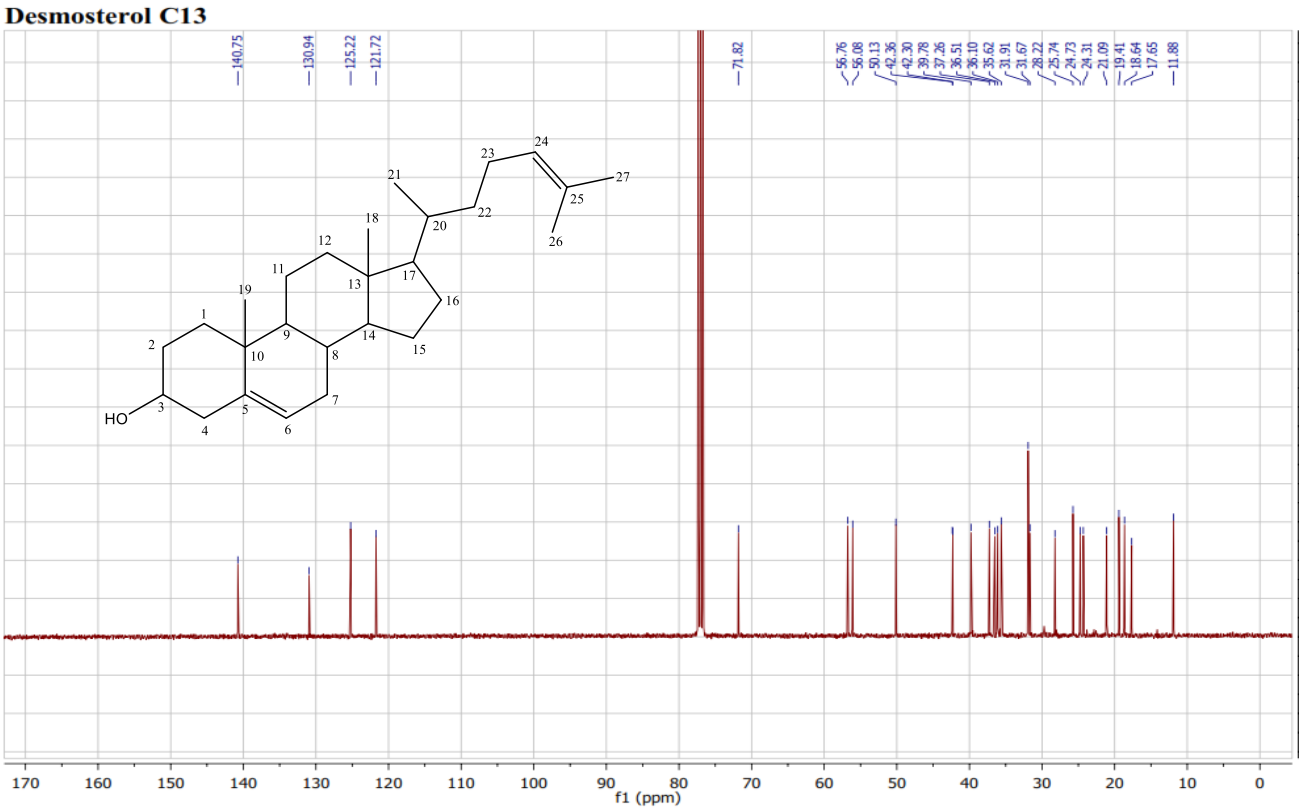
Appendix 3f**: HMBC spectrum of Phytol in $CDCl_3$**



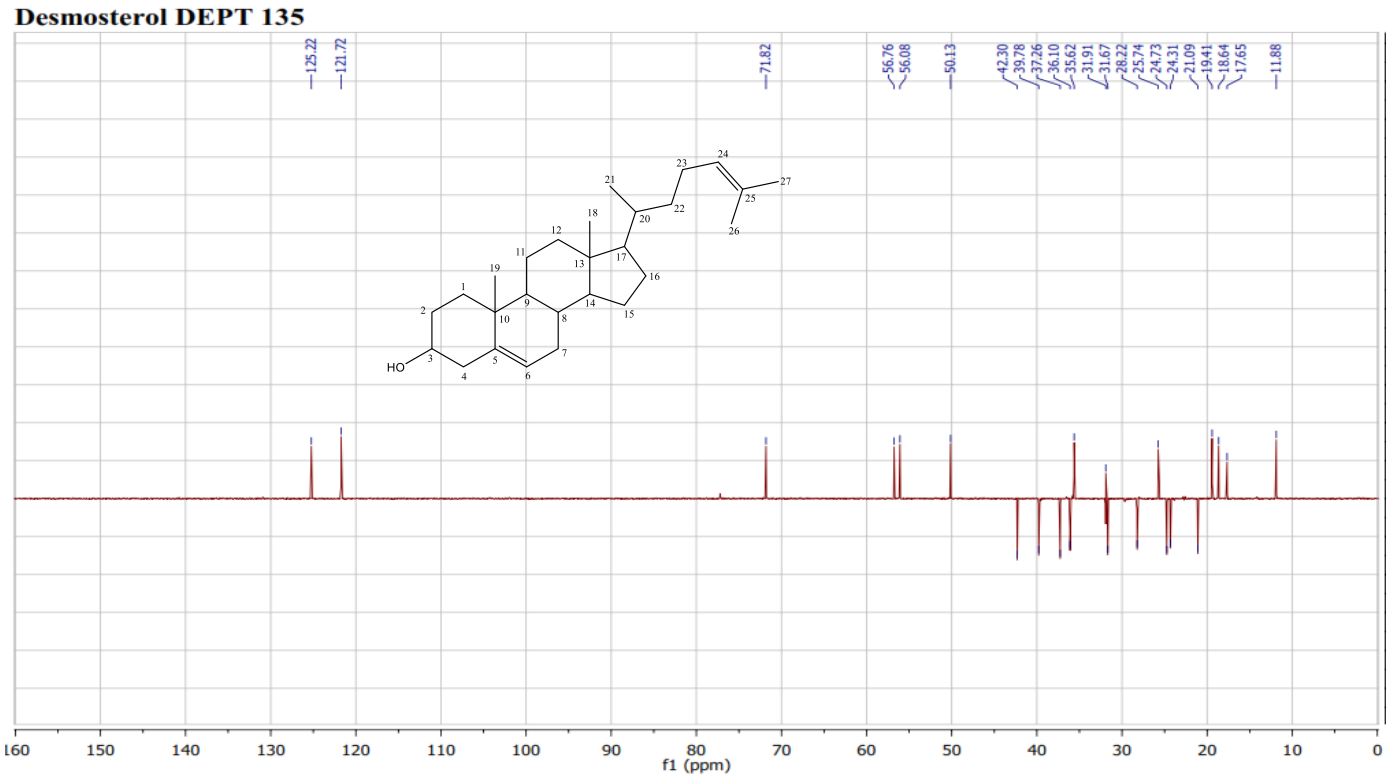
Appendix 4a**: ^{1}H spectrum of Desmosterol in $CDCl_3$**



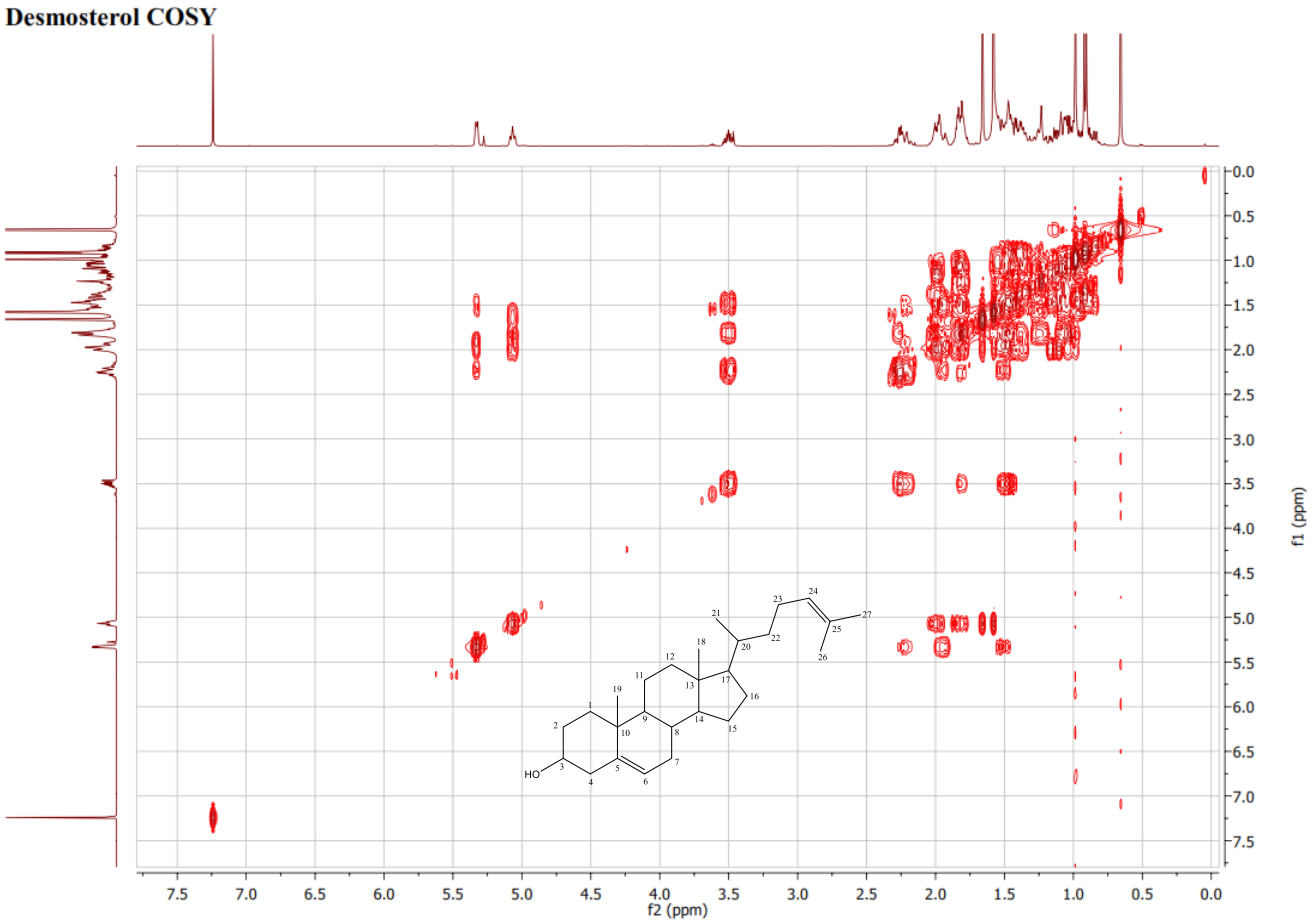
Appendix 4b**: ^{13}C spectrum of Desmosterol in $CDCl_3$**



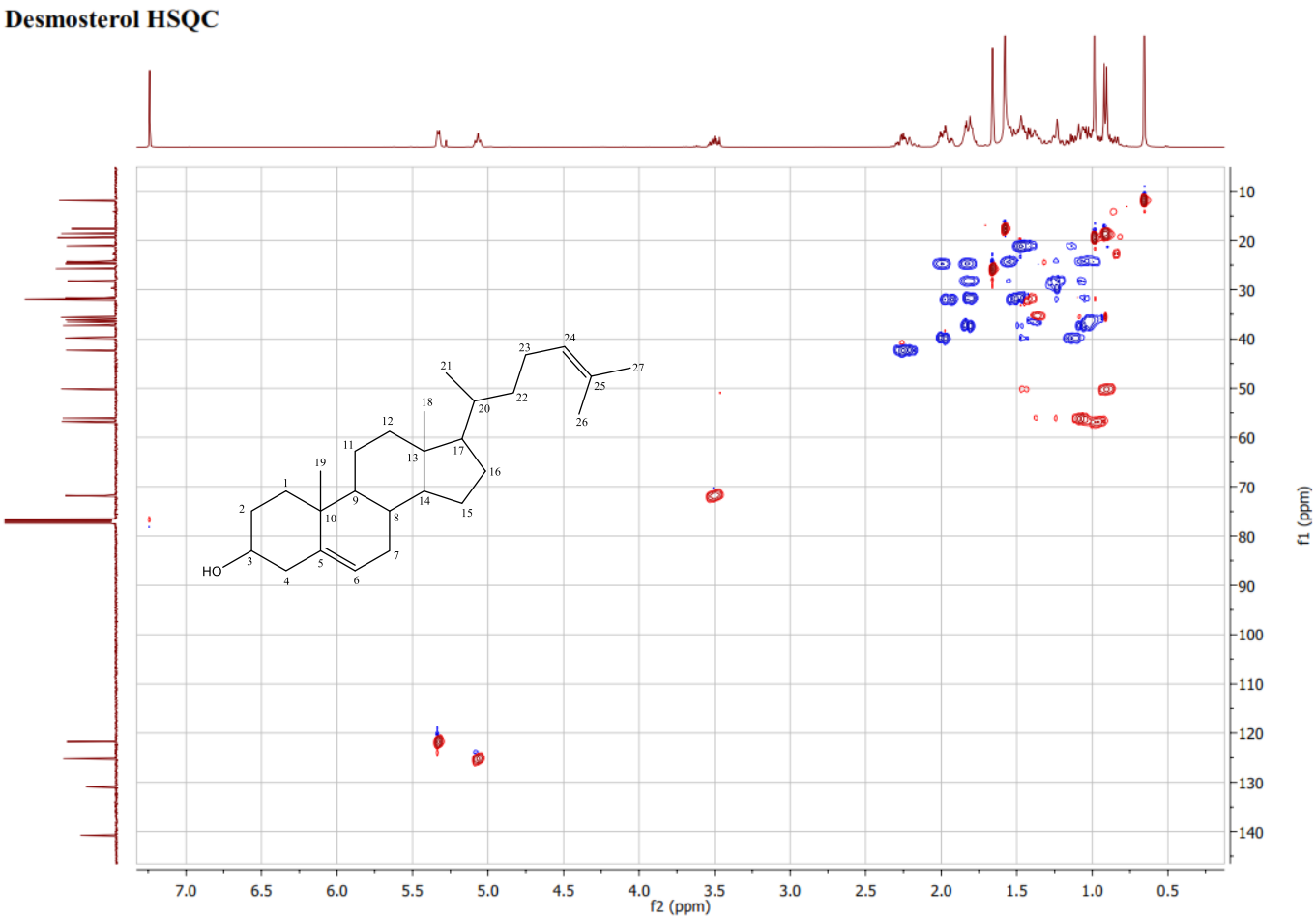
Appendix 4c**: DEPT 135 spectrum of Desmosterol in $CDCl_3$**



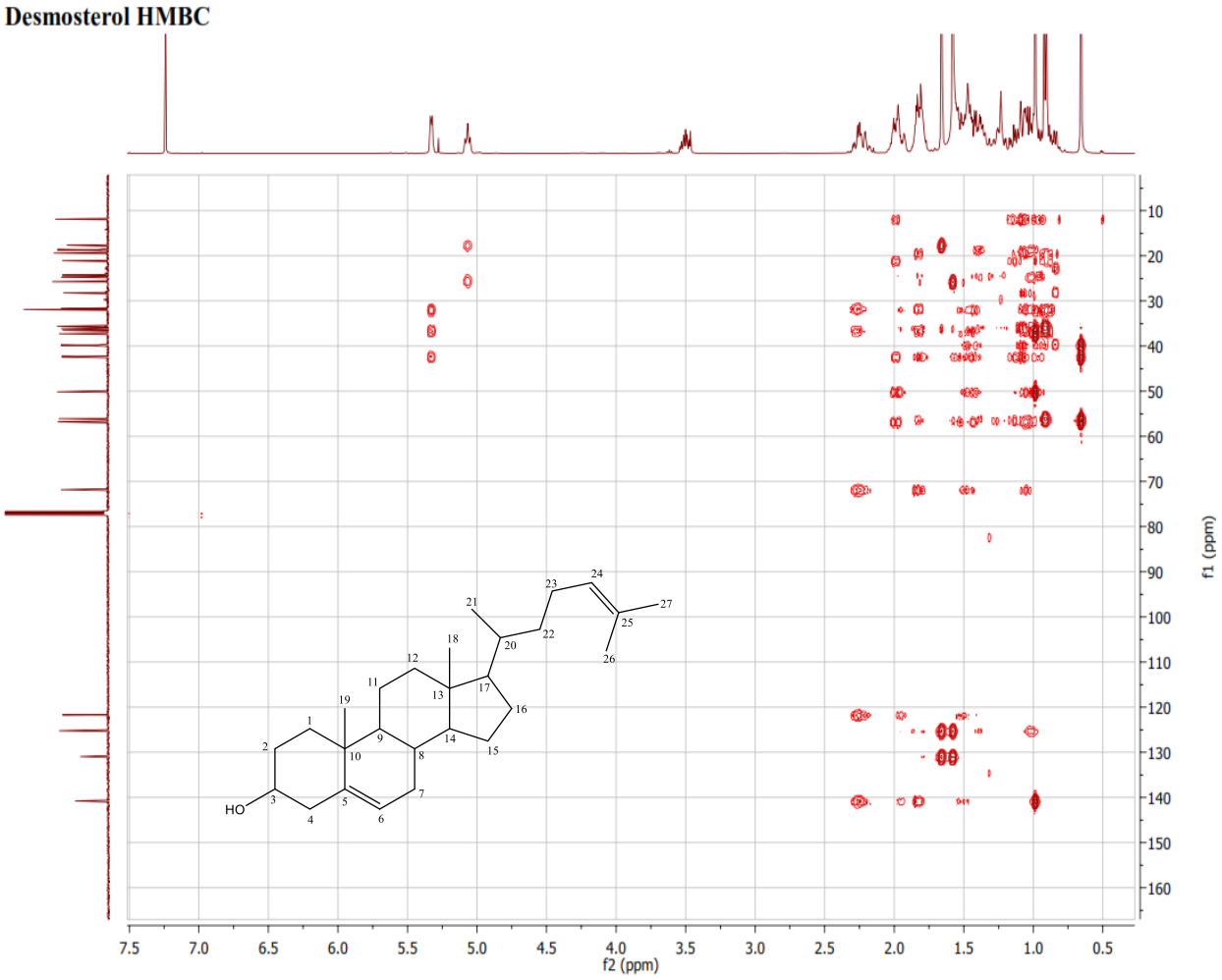
Appendix 4d**: COSY spectrum of Desmosterol in $CDCl_3$**



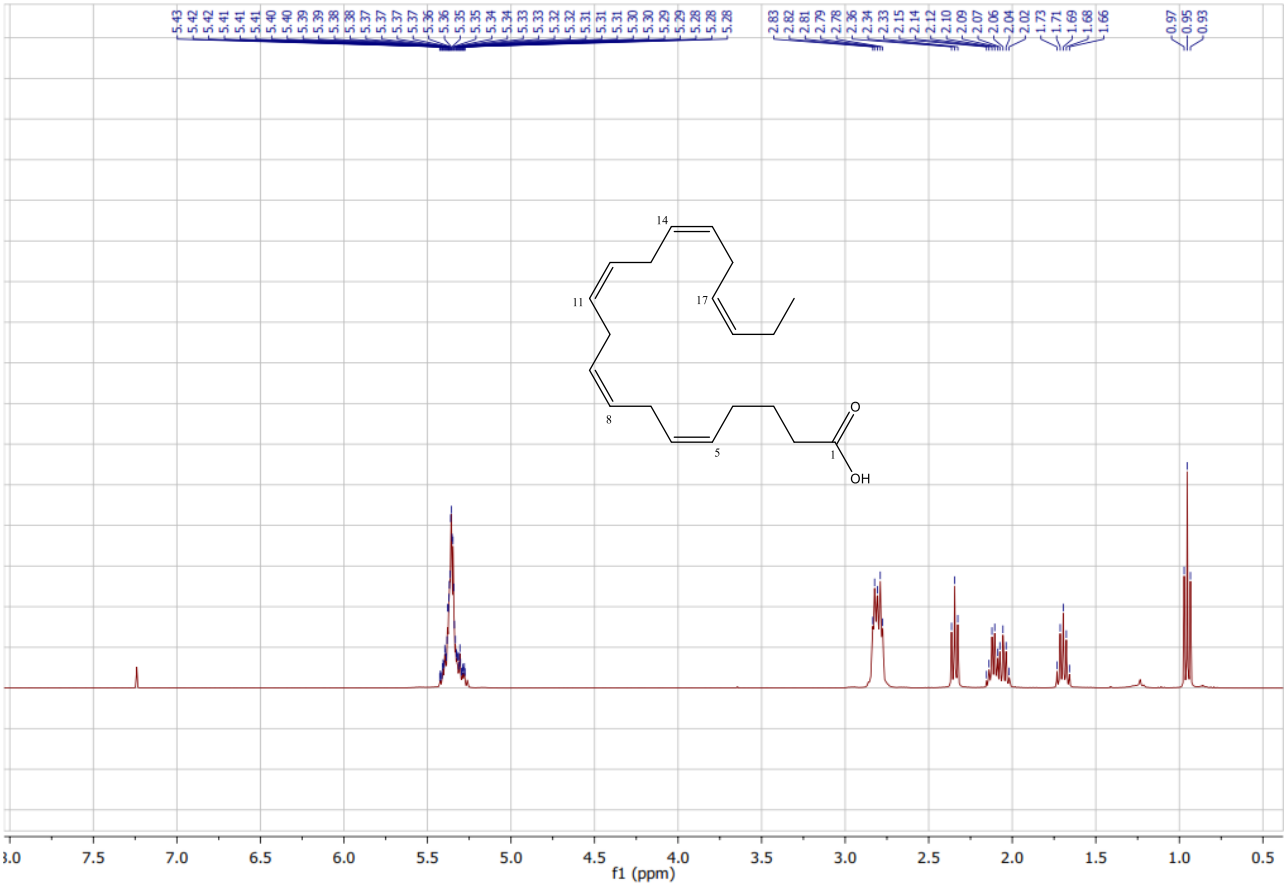
Appendix 4e**: HSQC spectrum of Desmosterol in $CDCl_3$**



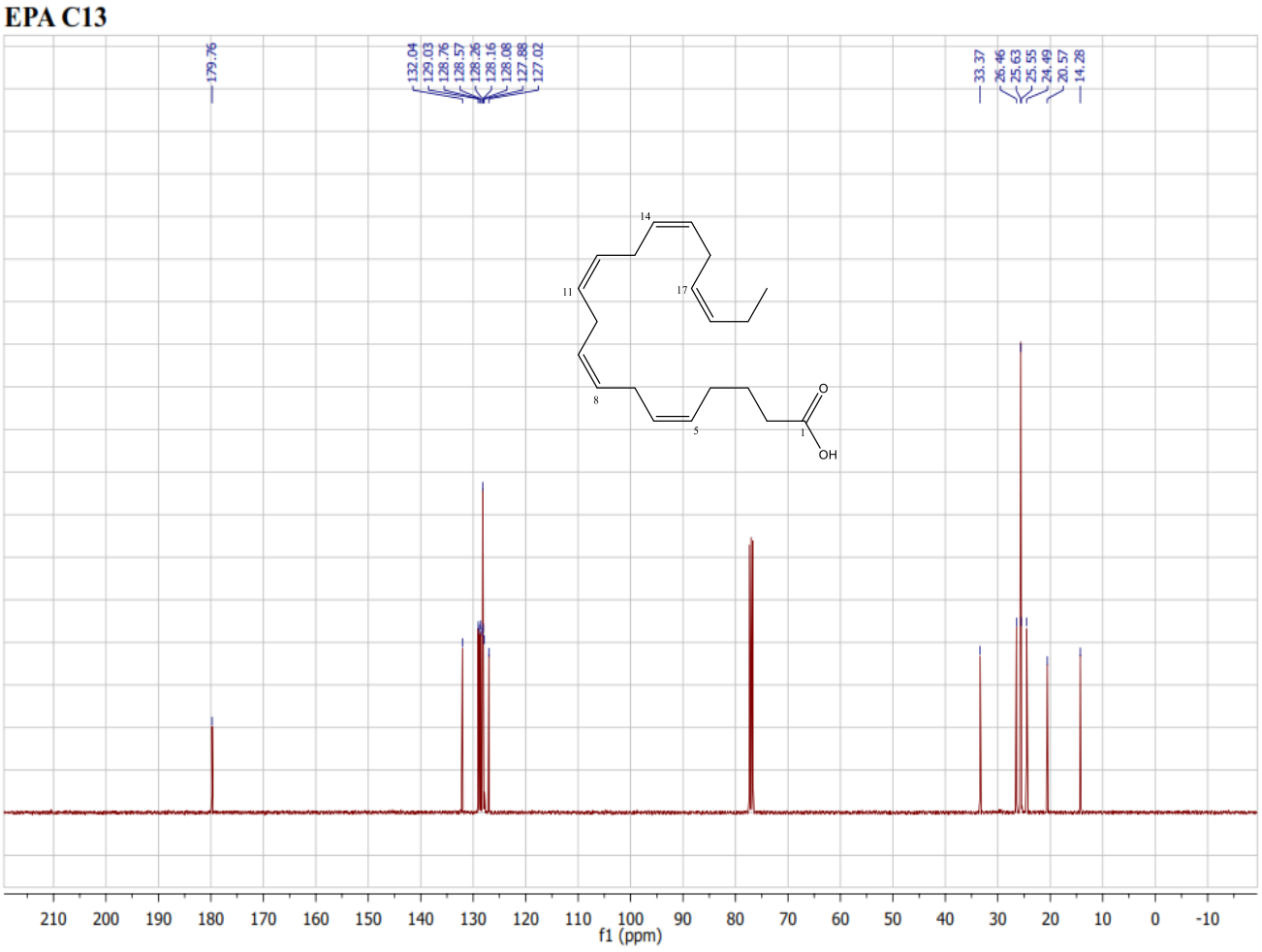
Appendix 4f**: HMBC spectrum of Desmosterol in $CDCl_3$**



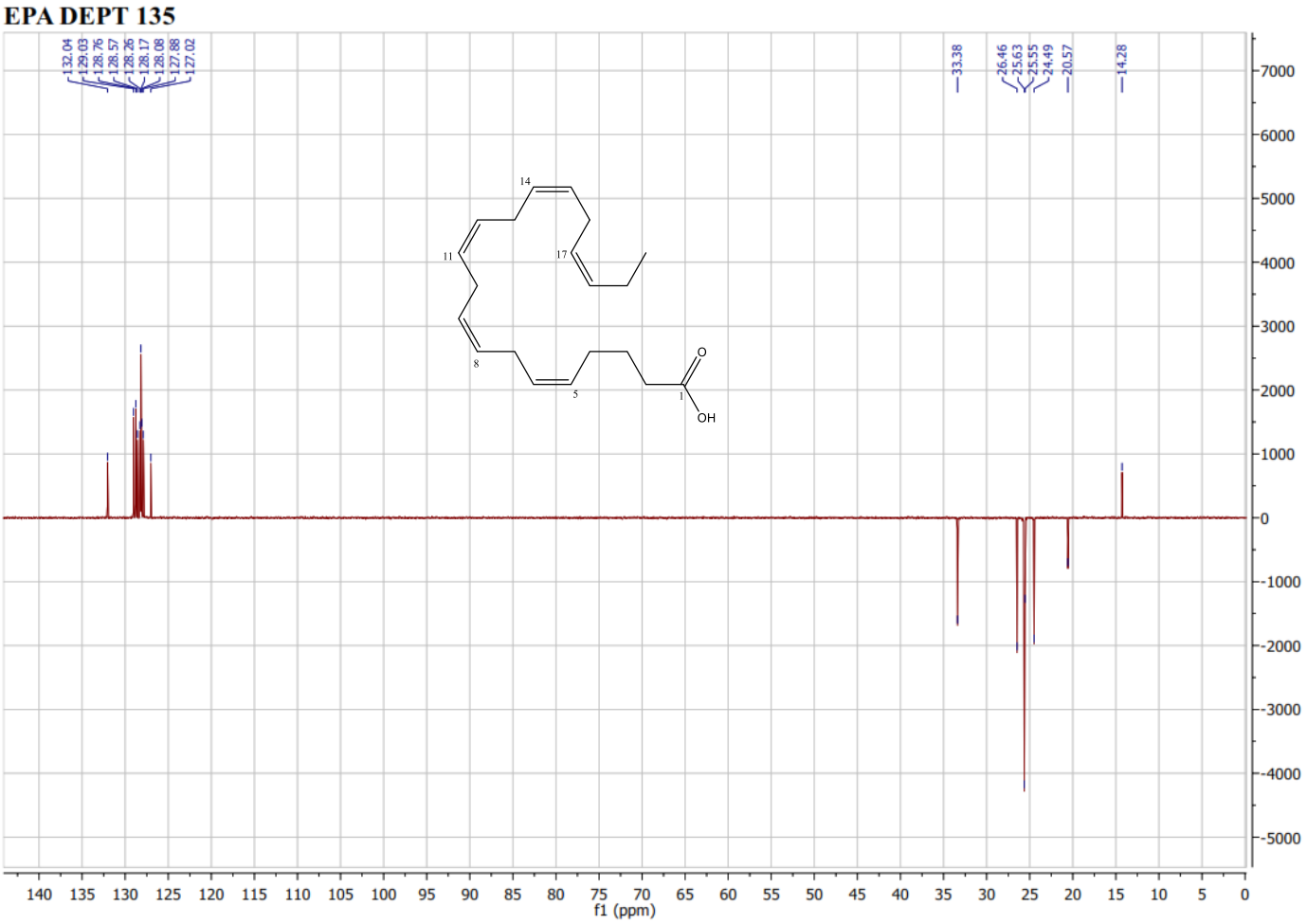
Appendix 5a: **^{1}H spectrum of 5, 8, 11, 14, 17- eicosapentaenoic acid in $CDCl_3$**



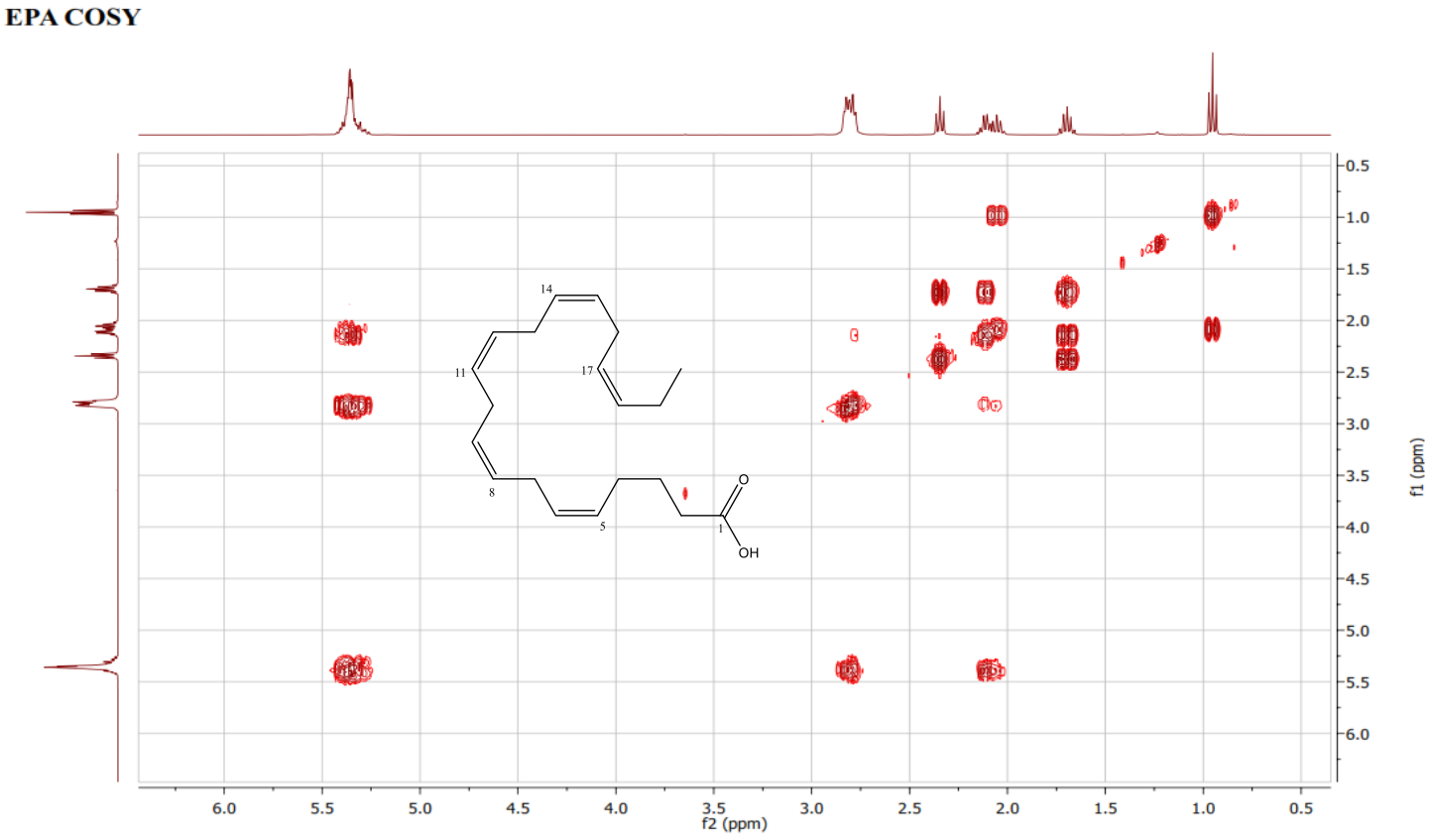
Appendix 5b**: ^{13}C spectrum of 5, 8, 11, 14, 17- eicosapentaenoic acid in $CDCl_3$**



Appendix 5e**: DEPT 135 spectrum of 5, 8, 11, 14, 17- eicosapentaenoic acid in $CDCl_3$**

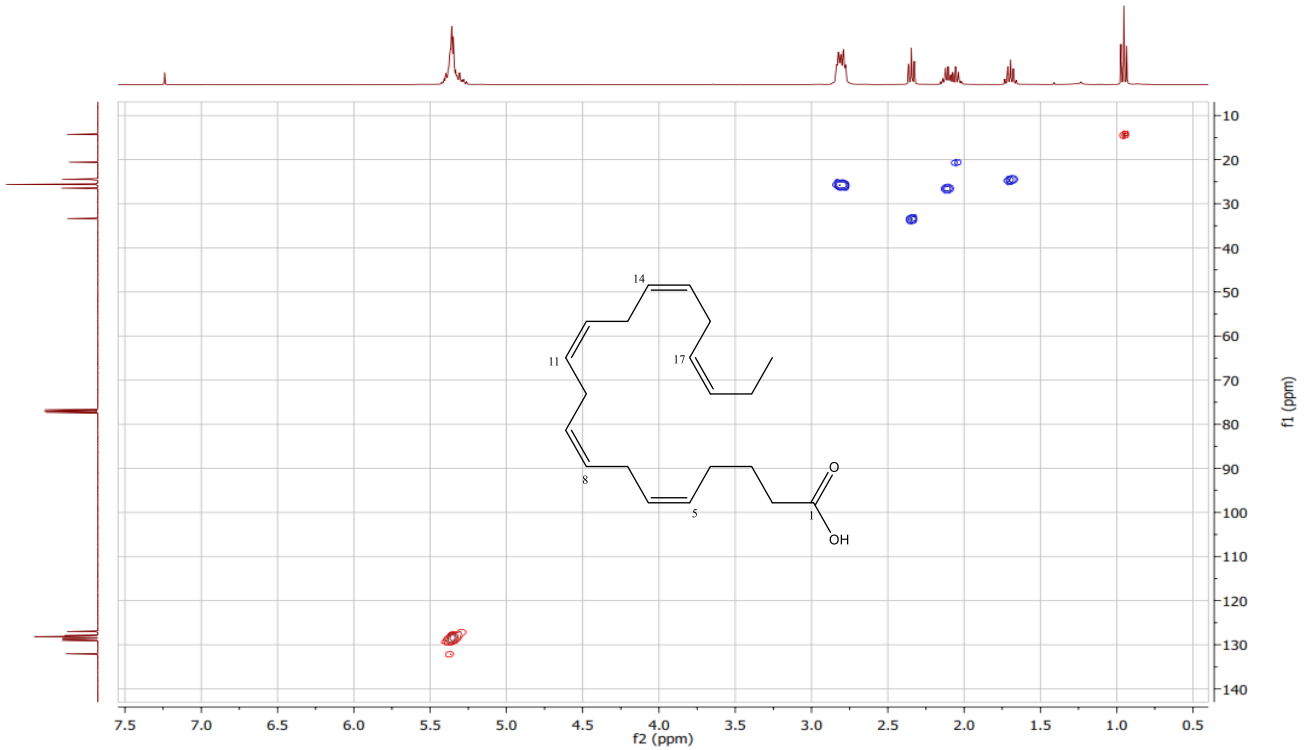


Appendix 5d**: COSY spectrum of 5, 8, 11, 14, 17- eicosapentaenoic acid in $CDCl_3$**



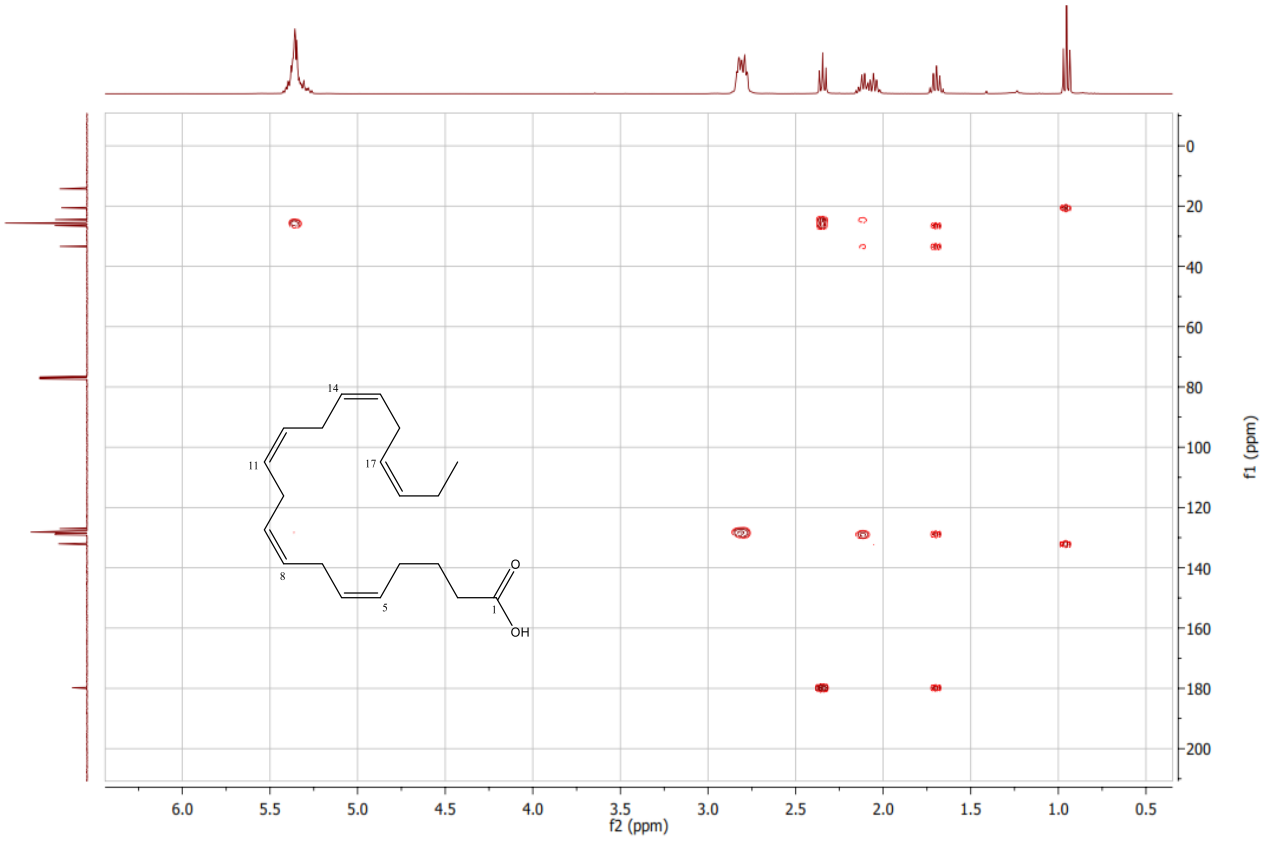
Appendix 5e**: HSQC spectrum of 5, 8, 11, 14, 17- eicosapentaenoic acid in $CDCl_3$**

EPA HSQC

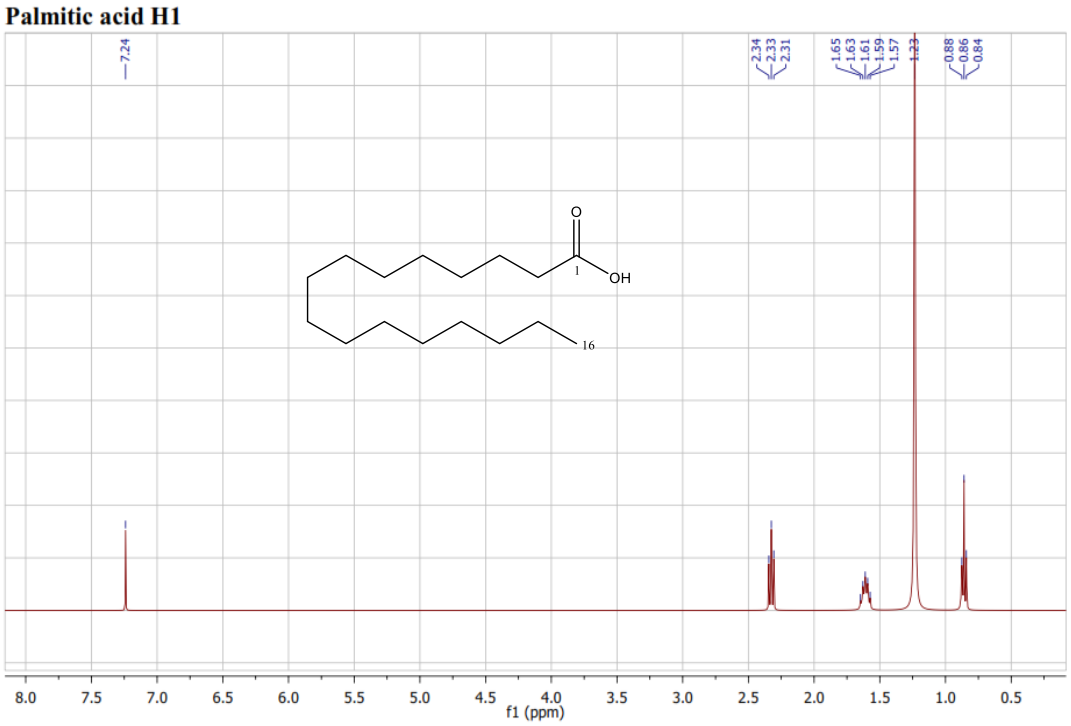


Appendix 5f**: HMBC spectrum of 5, 8, 11, 14, 17- eicosapentaenoic acid in $CDCl_3$**

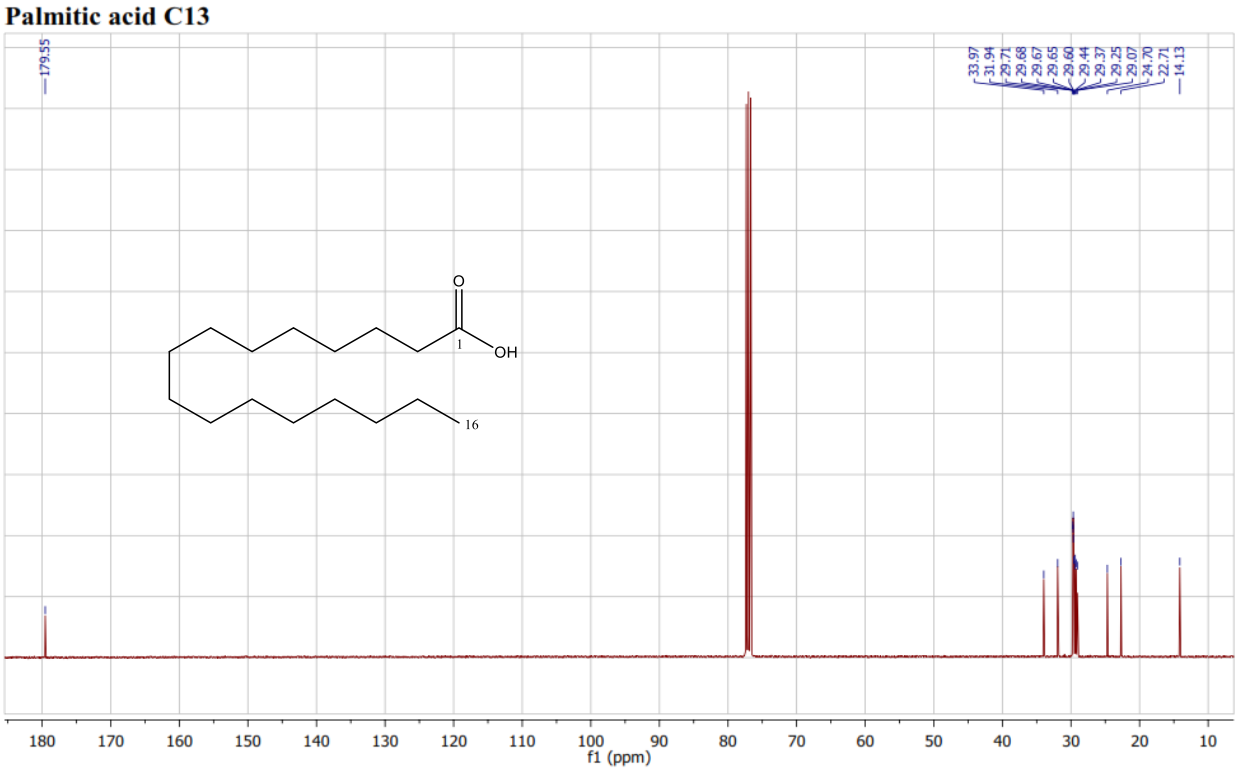
EPA HMBC



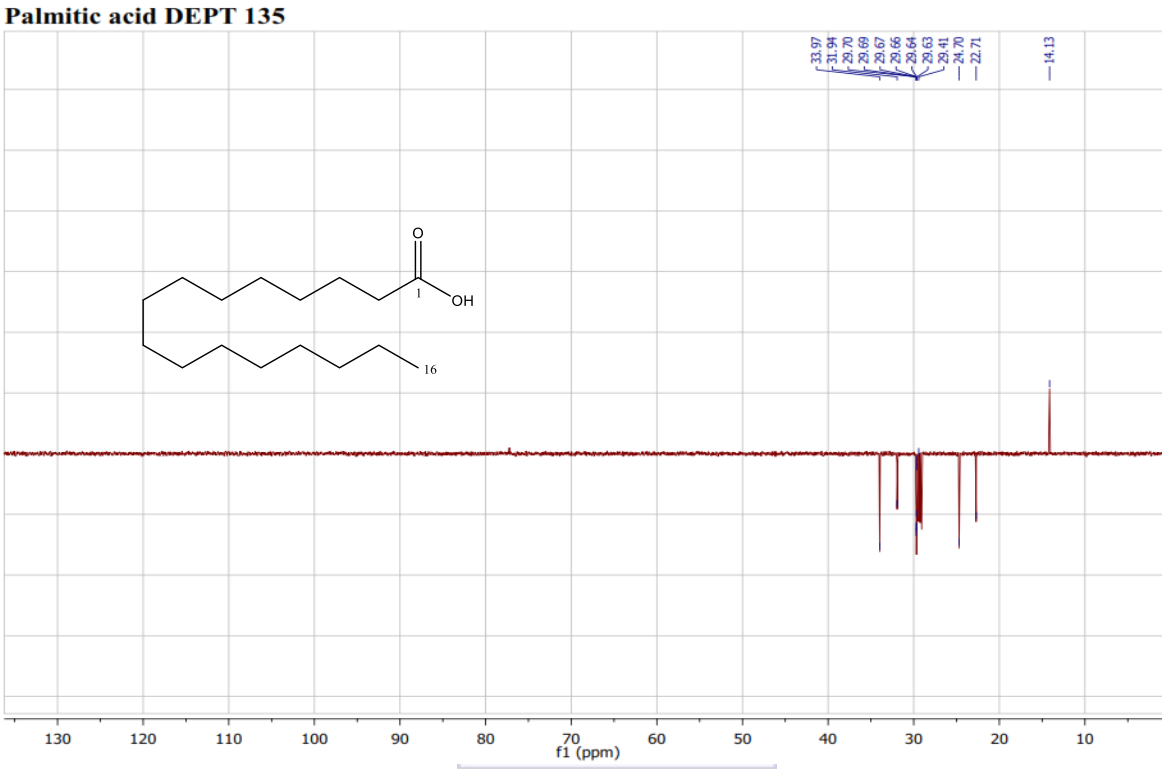
Appendix 6a**: ^{1}H spectrum of Palmitic acid in $CDCl_3$**



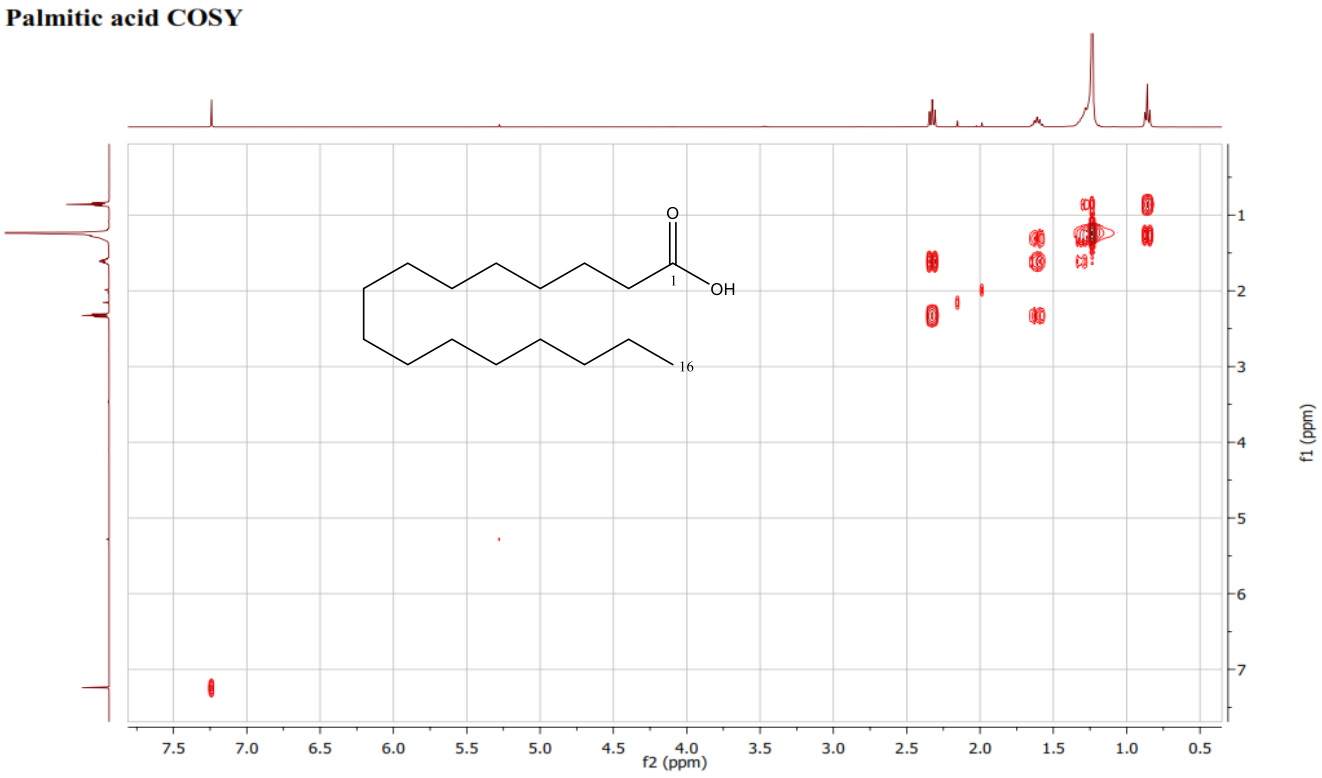
Appendix 6b**: ^{13}C spectrum of Palmitic acid in $CDCl_3$**



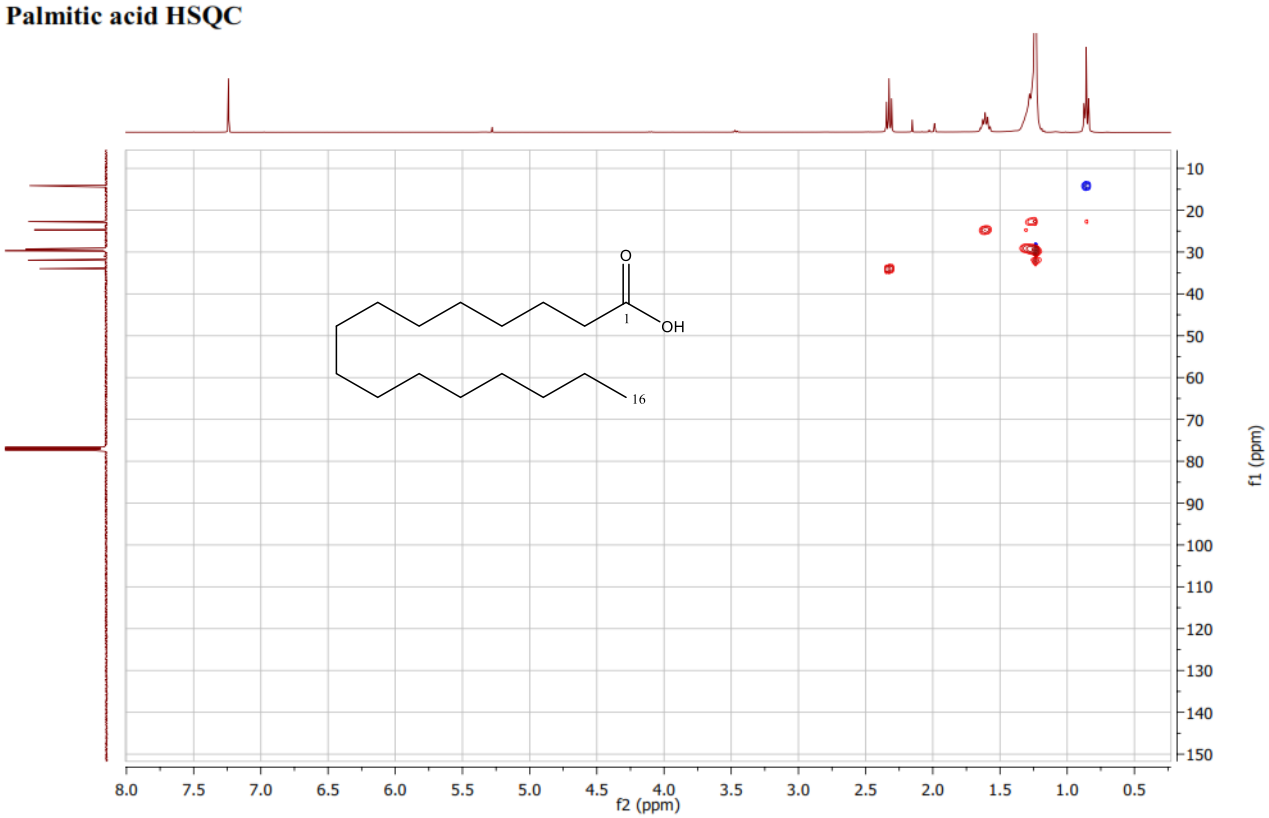
Appendix 6c**: DEPT 135 spectrum of Palmitic acid in $CDCl_3$**



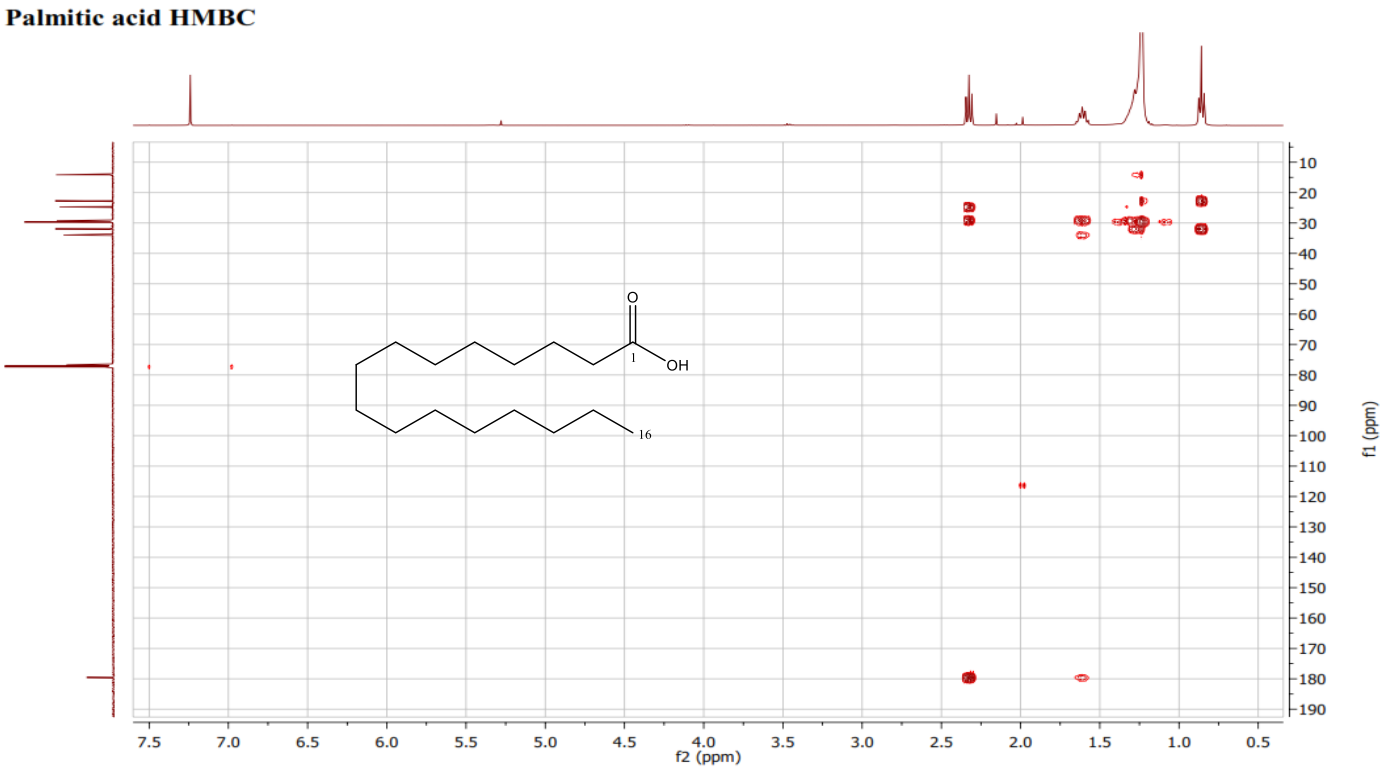
Appendix 6d**: COSY spectrum of Palmitic acid in $CDCl_3$**



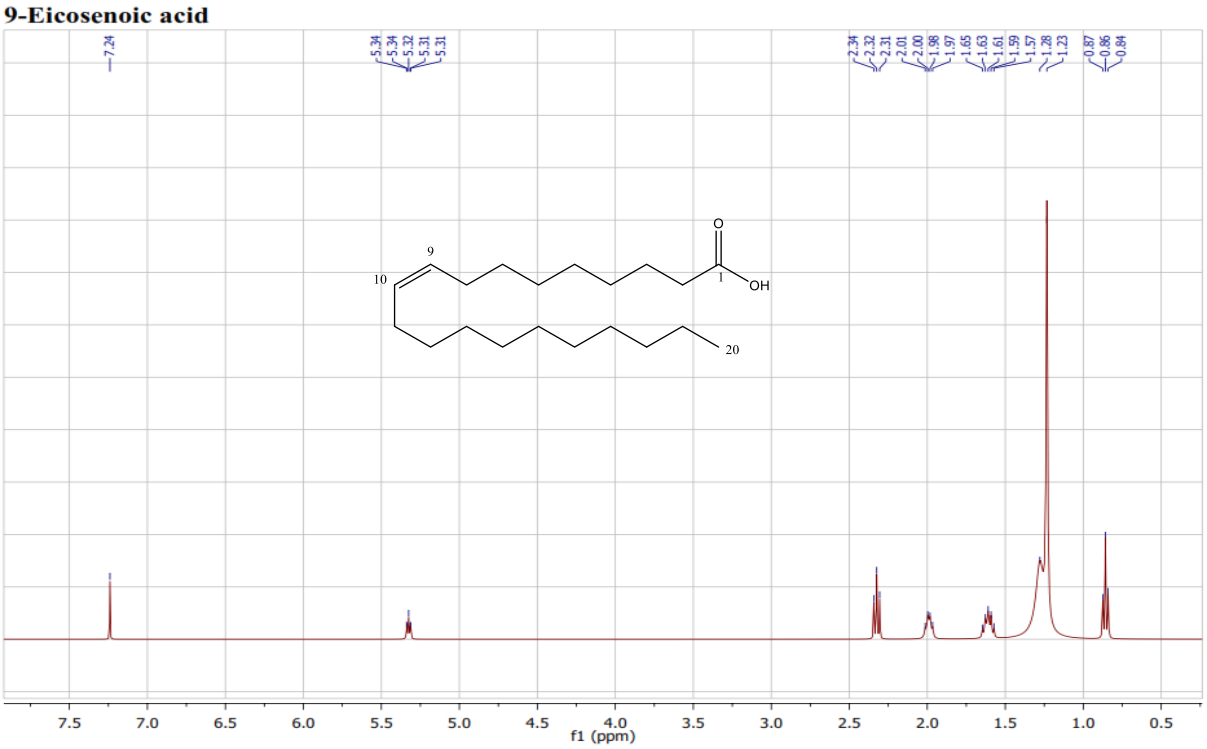
Appendix 6e**: HSQC spectrum of Palmitic acid in $CDCl_3$**



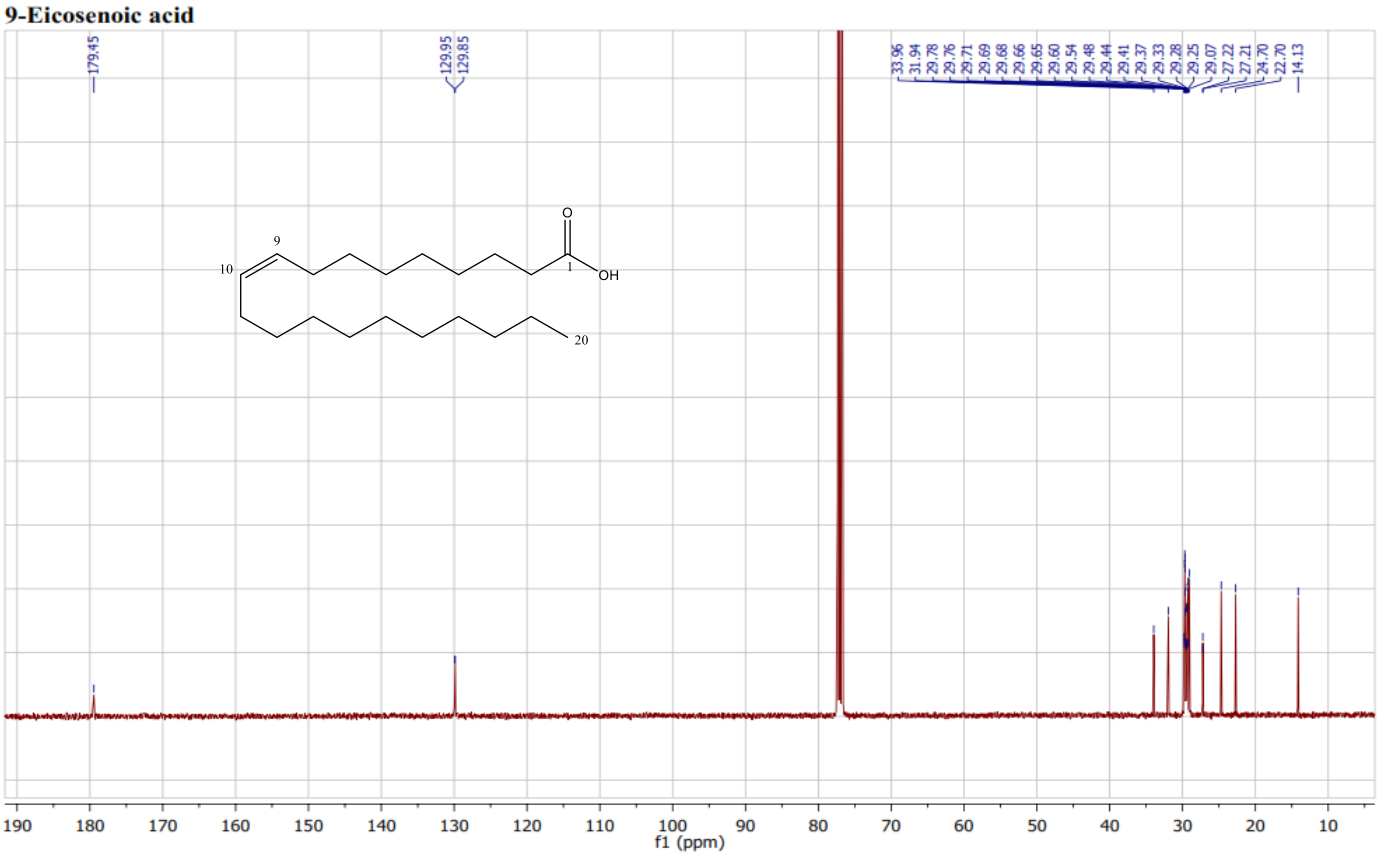
Appendix 6f**: HMBC spectrum of Palmitic acid in $CDCl_3$**



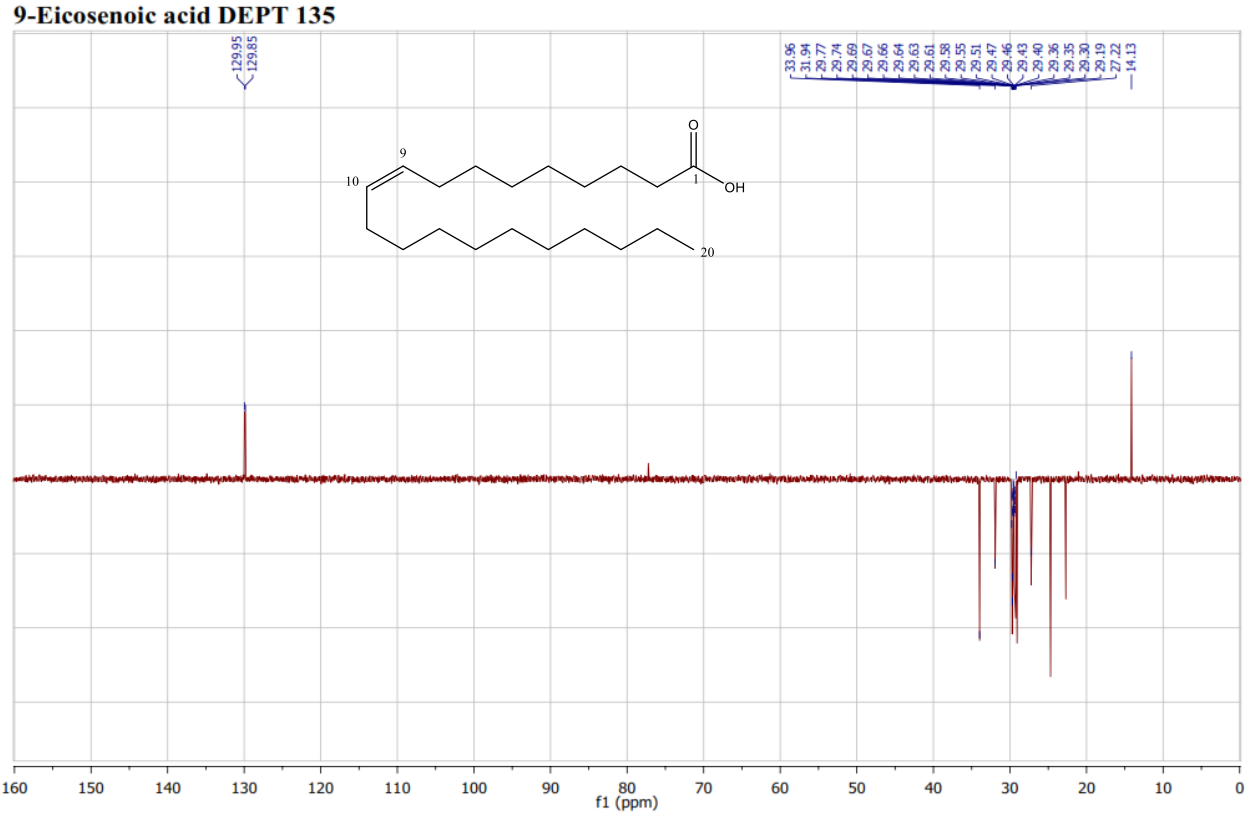
Appendix 7a**: ^{1}H spectrum of 9-eicosenoic acid in $CDCl_3$**



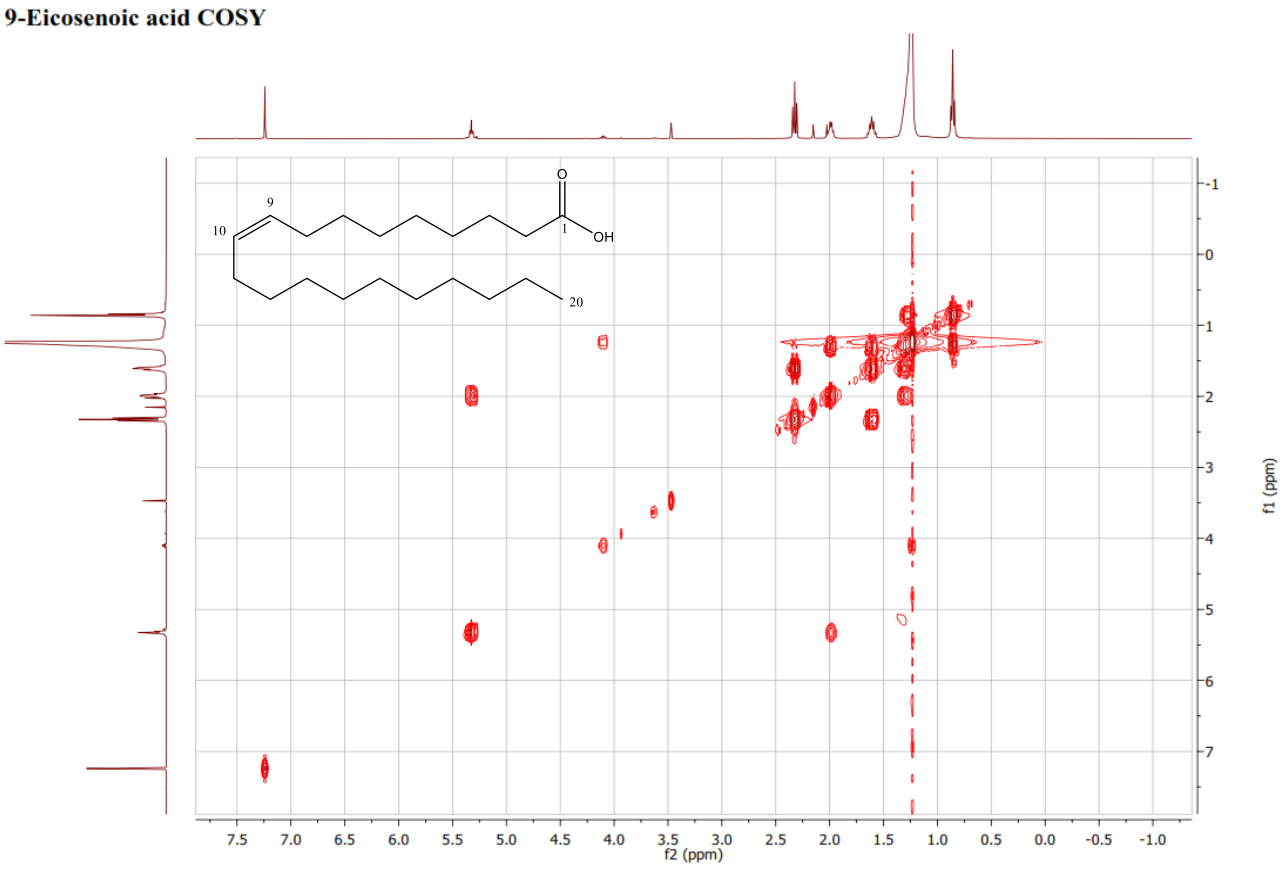
Appendix 7b**: ^{13}C spectrum of 9-eicosenoic acid in $CDCl_3$**



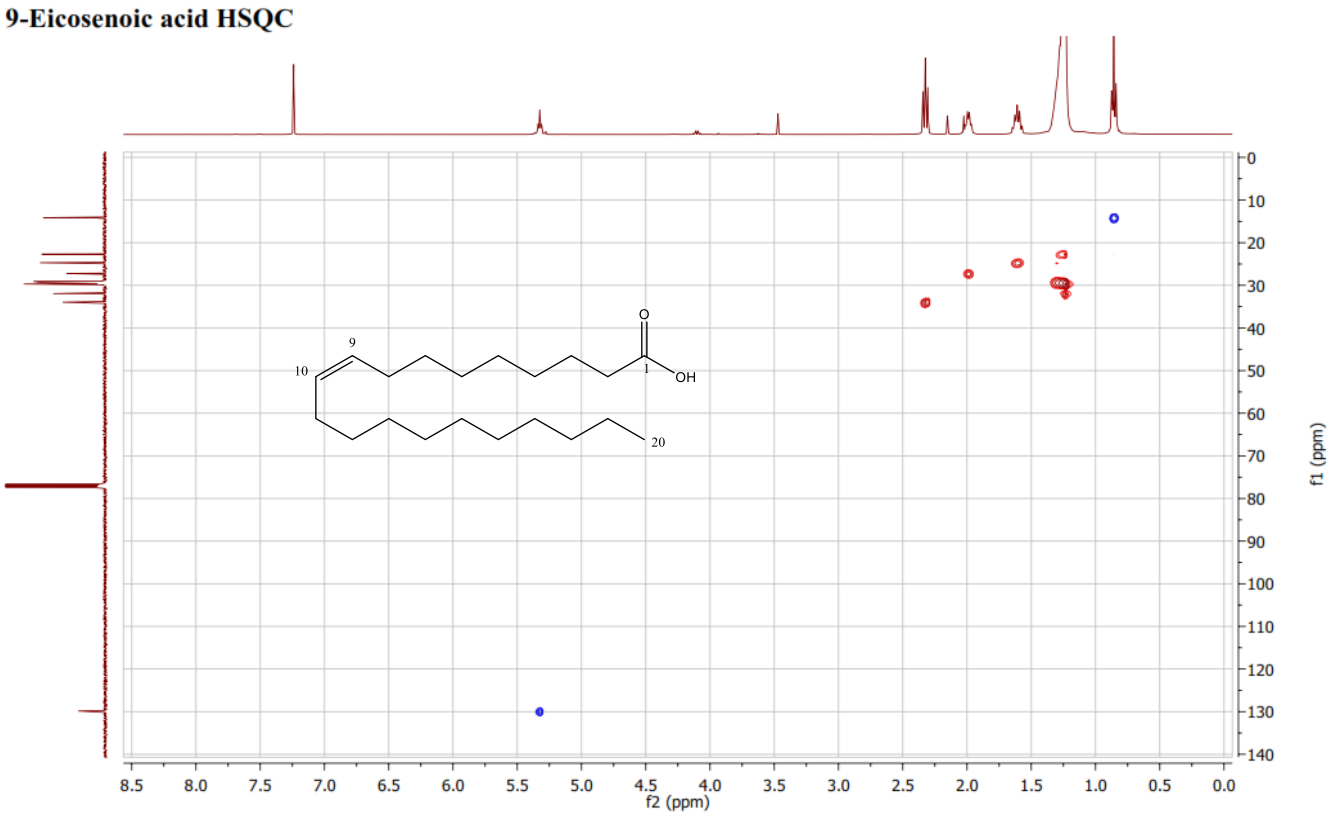
Appendix 7c**: DEPT 135 spectrum of 9-eicosenoic acid in $CDCl_3$**



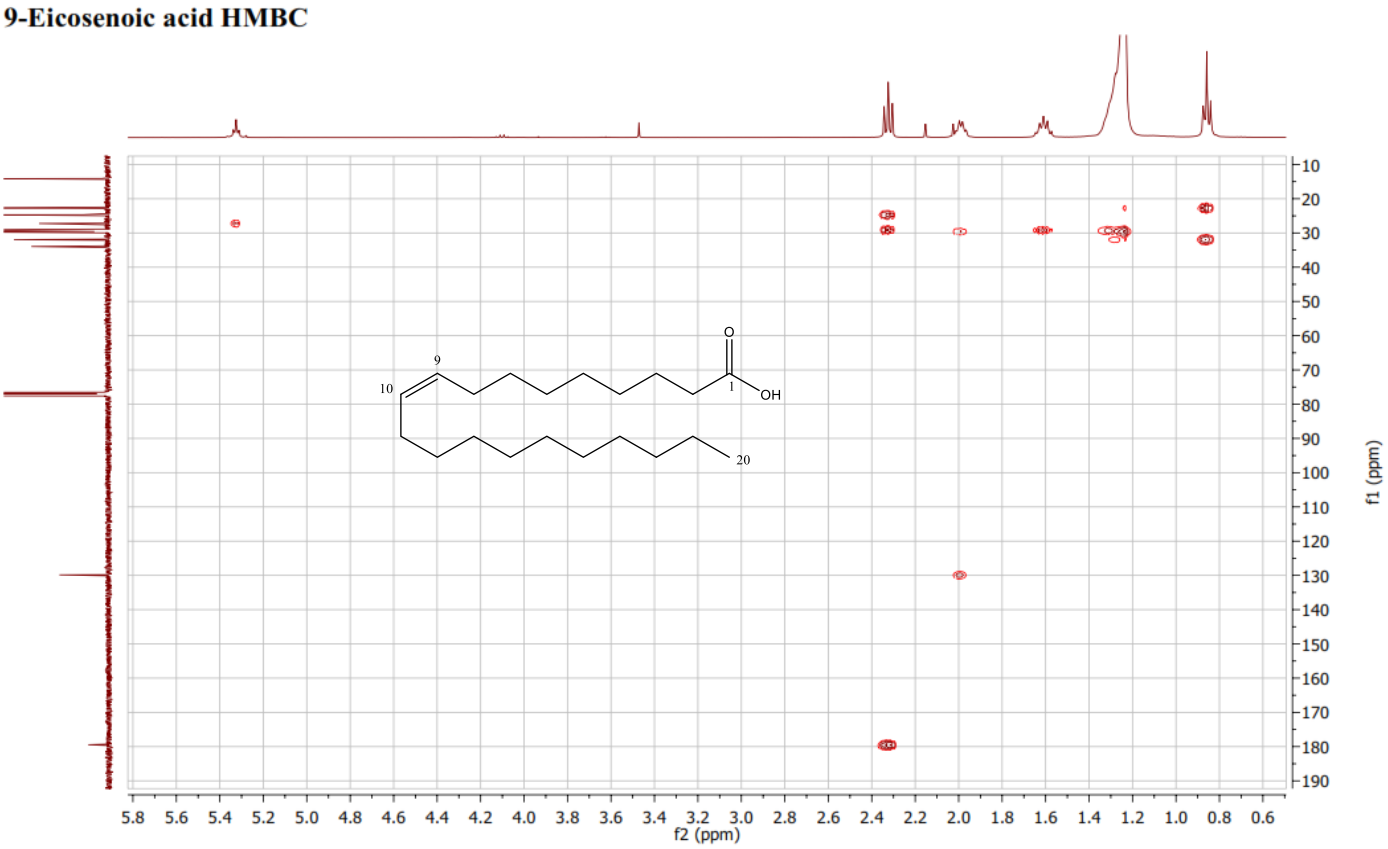
Appendix 7d**: COSY spectrum of 9-eicosenoic acid in $CDCl_3$**



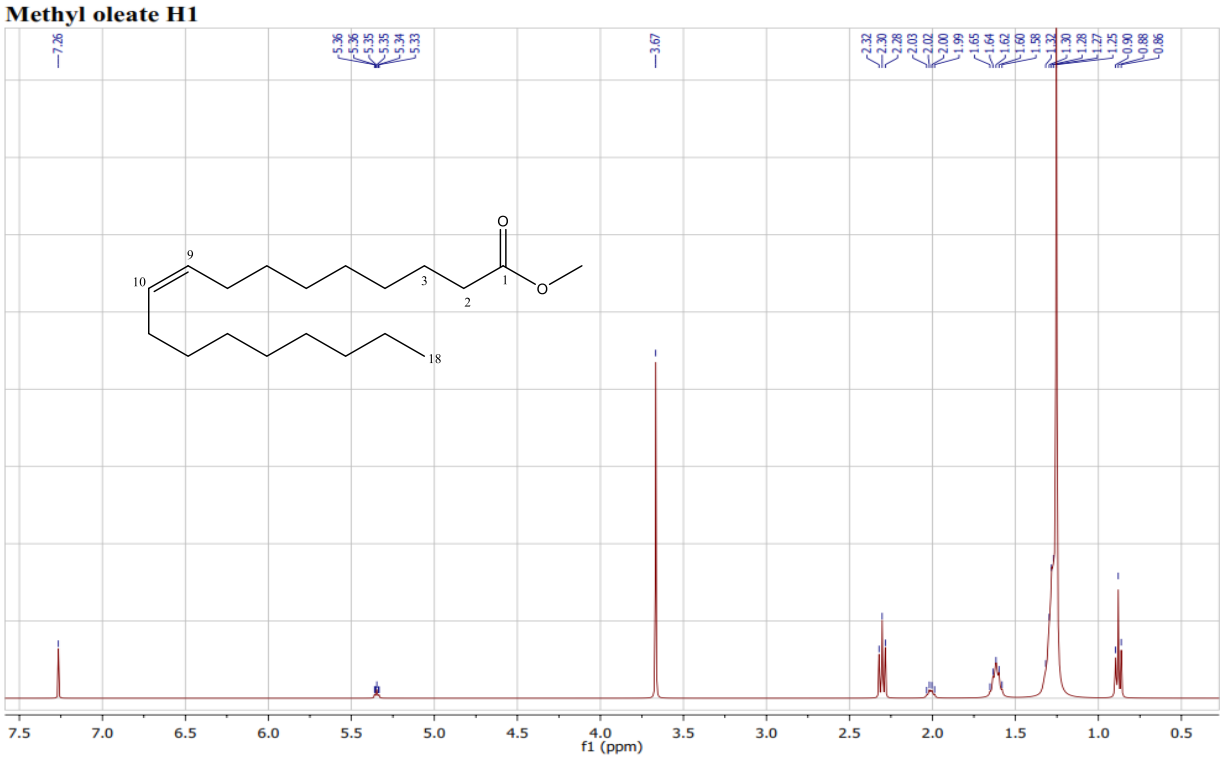
Appendix 7e**: HSQC spectrum of 9-eicosenoic acid in $CDCl_3$**



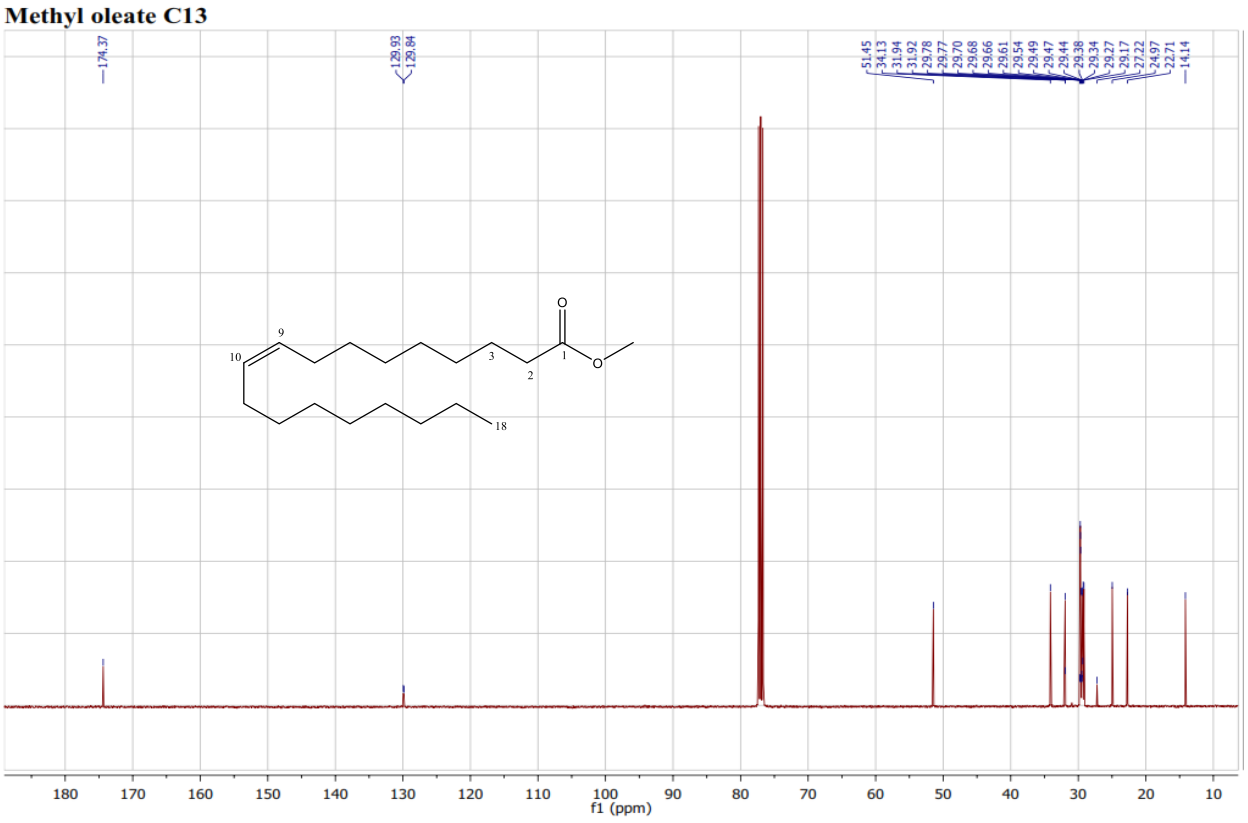
Appendix 7f**: HMBC spectrum of 9-eicosenoic acid in $CDCl_3$**



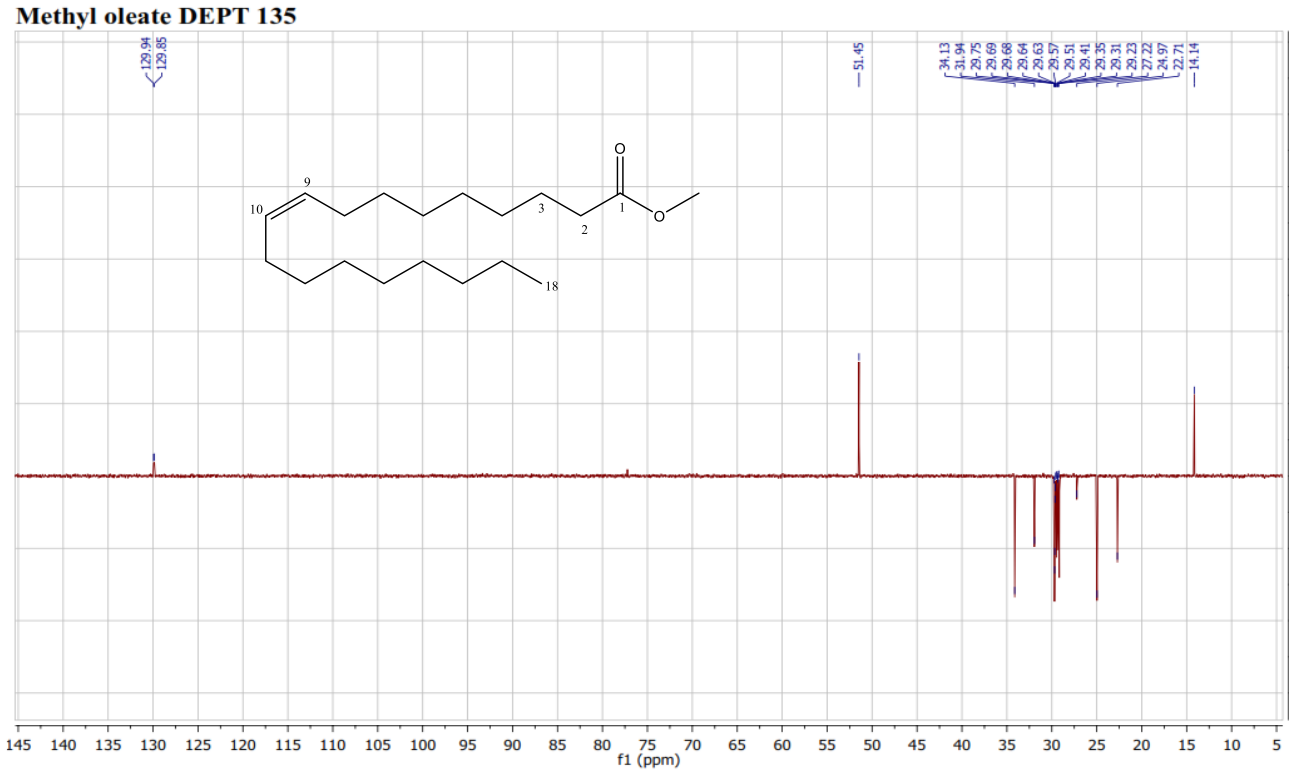
Appendix 8a**: ^{1}H spectrum of Methyl (Z)-octadec-9-enoate in $CDCl_3$**



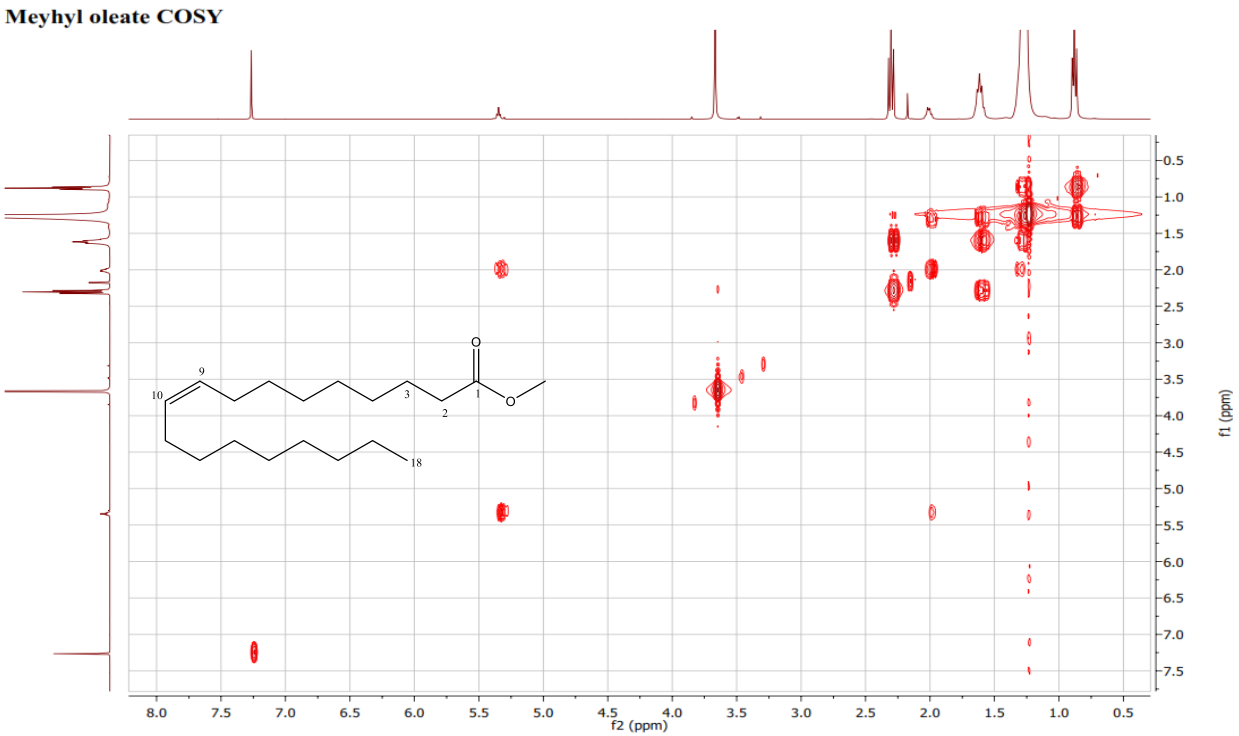
Appendix 8b**: ^{13}C spectrum of Methyl (Z)-octadec-9-enoate in $CDCl_3$**



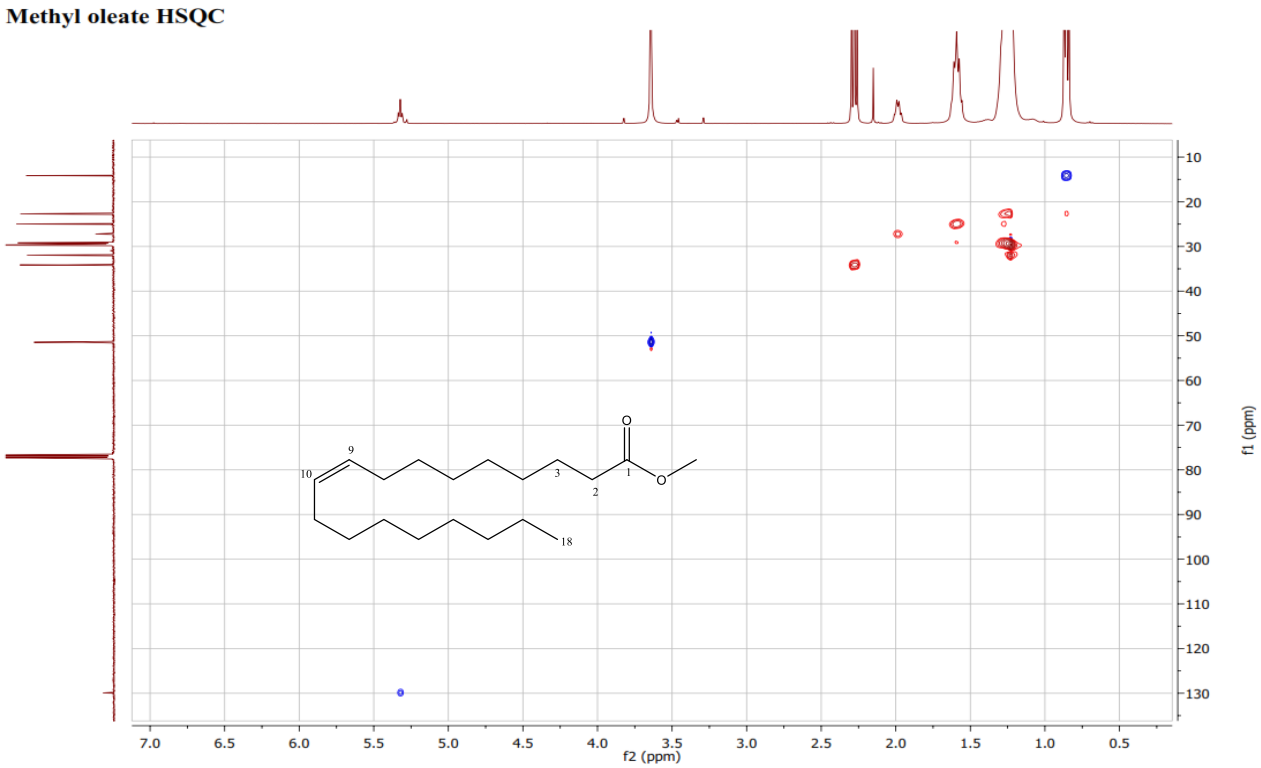
Appendix 8c**: DEPT 135 spectrum of Methyl (Z)-octadec-9-enoate in $CDCl_3$**



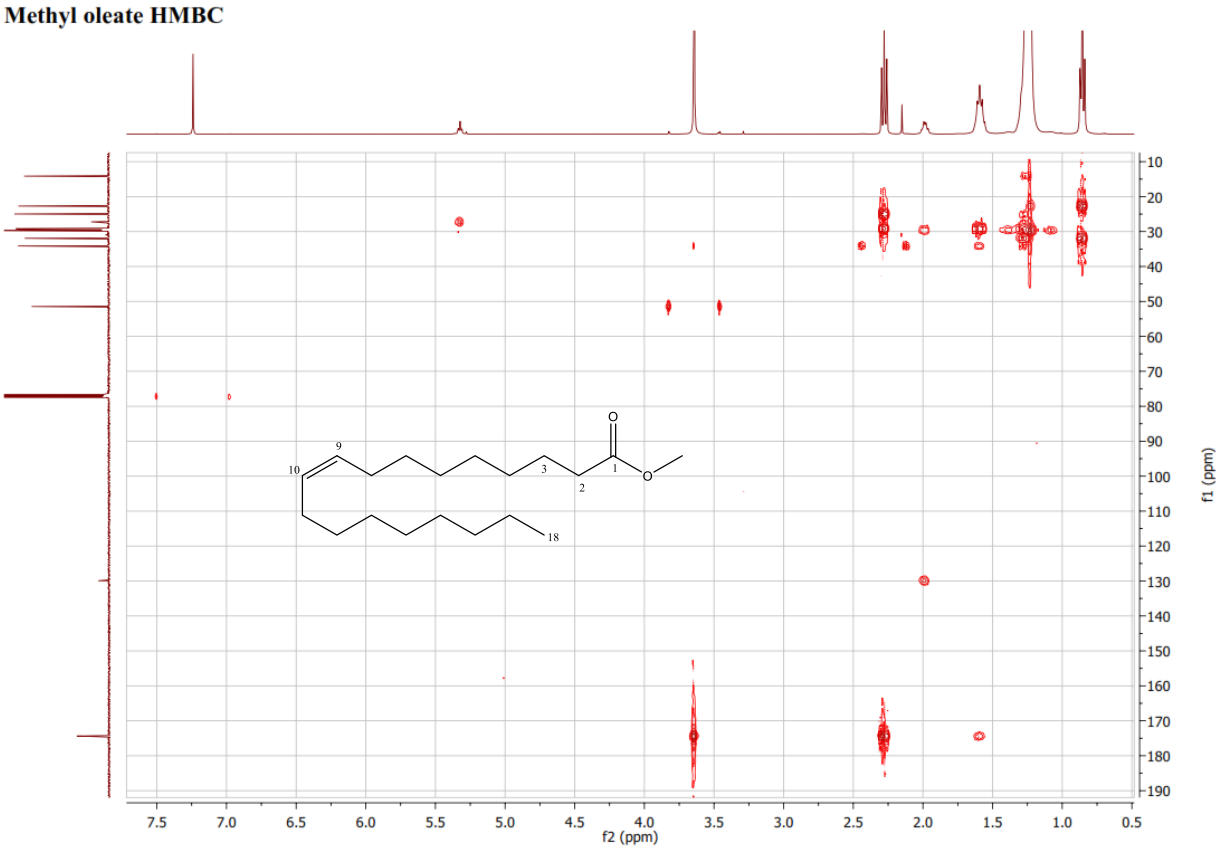
Appendix 8d**: COSY spectrum of Methyl (Z)-octadec-9-enoate in $CDCl_3$**



Appendix 8e**: HSQC spectrum of Methyl (Z)-octadec-9-enoate in $CDCl_3$**



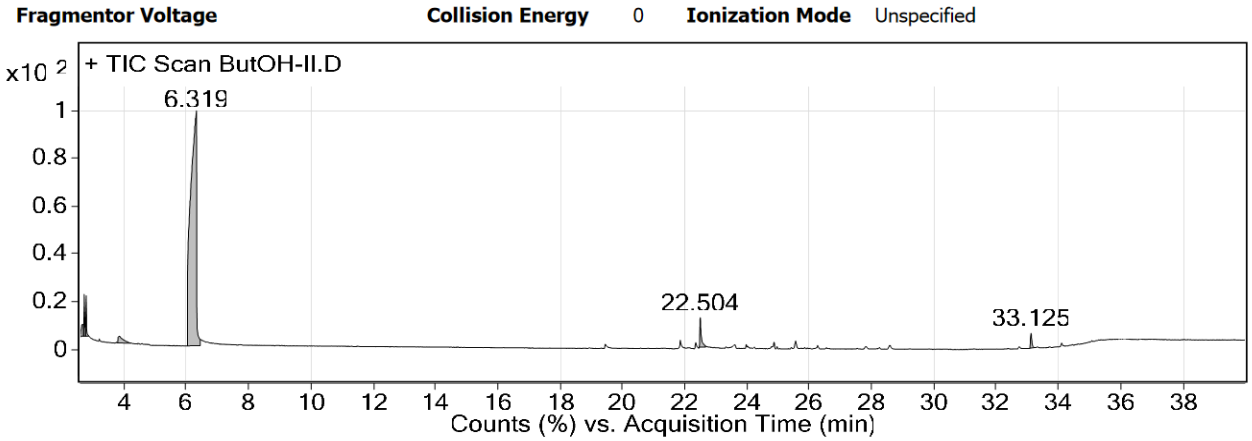
Appendix 8f**: HMBC spectrum of Methyl (Z)-octadec-9-enoate in $CDCl_3$**



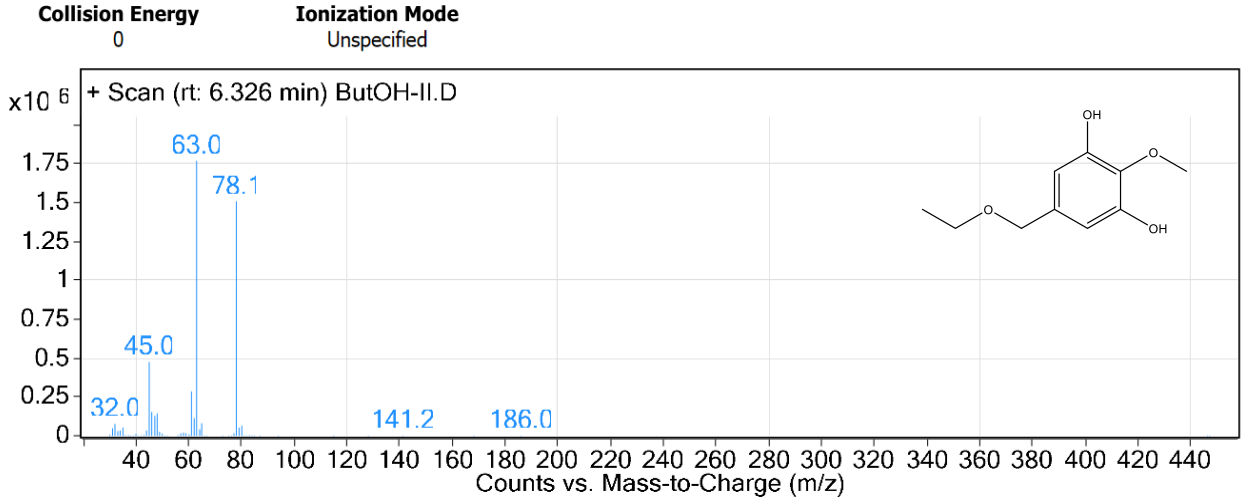
Appendix 2

GC-MS, ESI-MS and FTIR spectra of isolated compounds

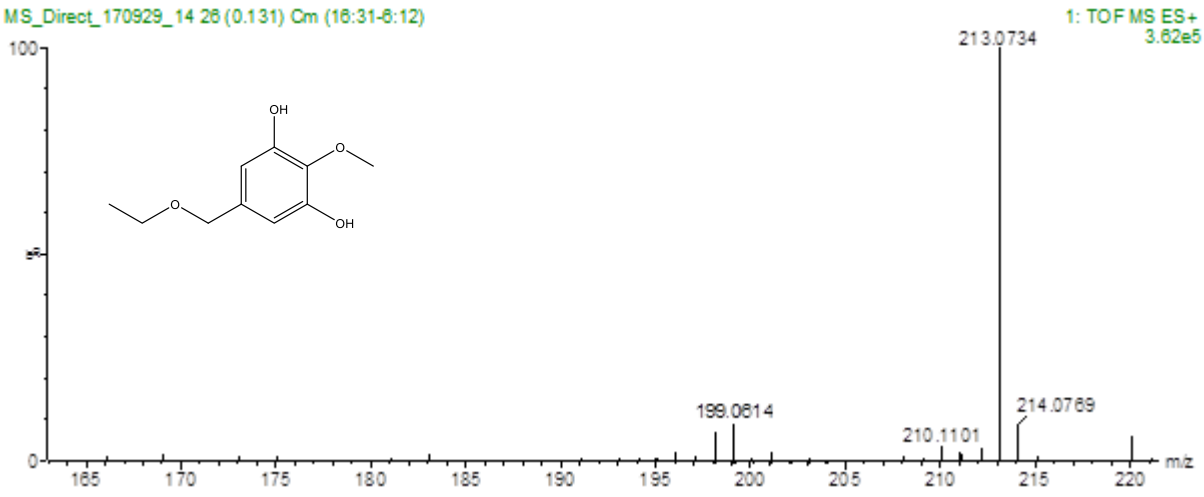
Appendix Ab**: GC-MS spectrum of compound 1 ($CHCl_3$)**



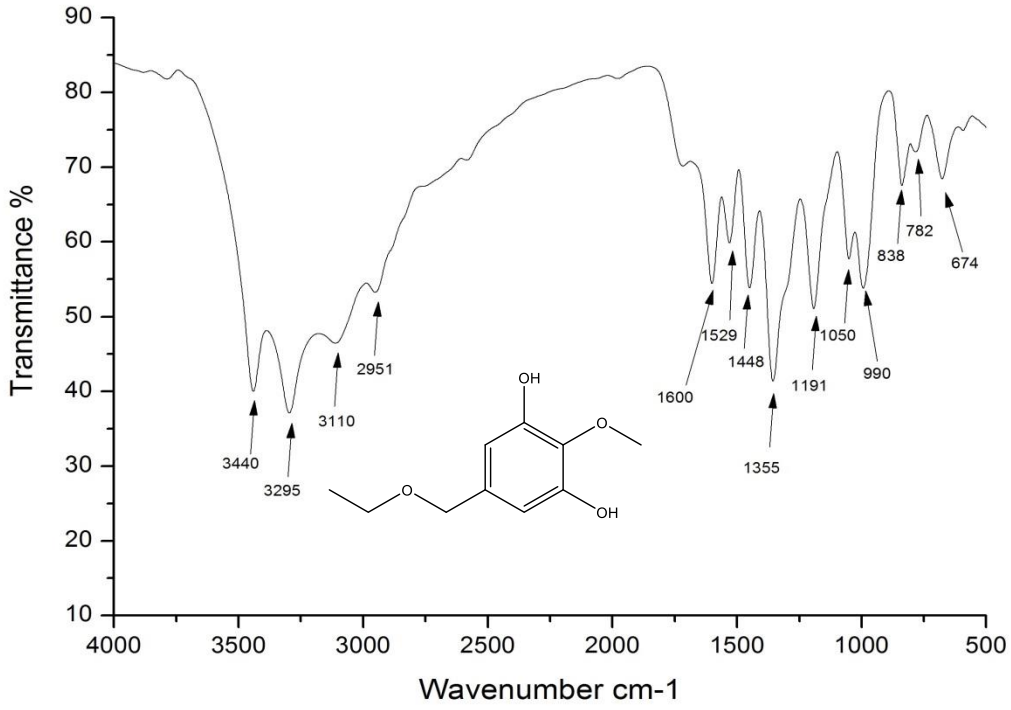
Appendix Ab**: GC-MS spectrum of compound 1 ($CHCl_3$)**



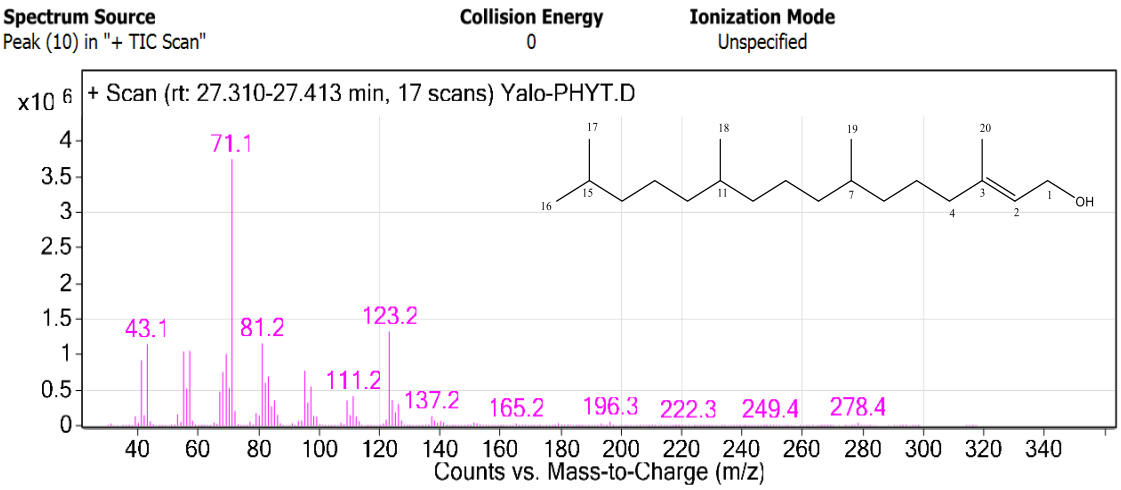
Appendix Ac **: ESI-MS spectrum of compound 1 (ACN)**



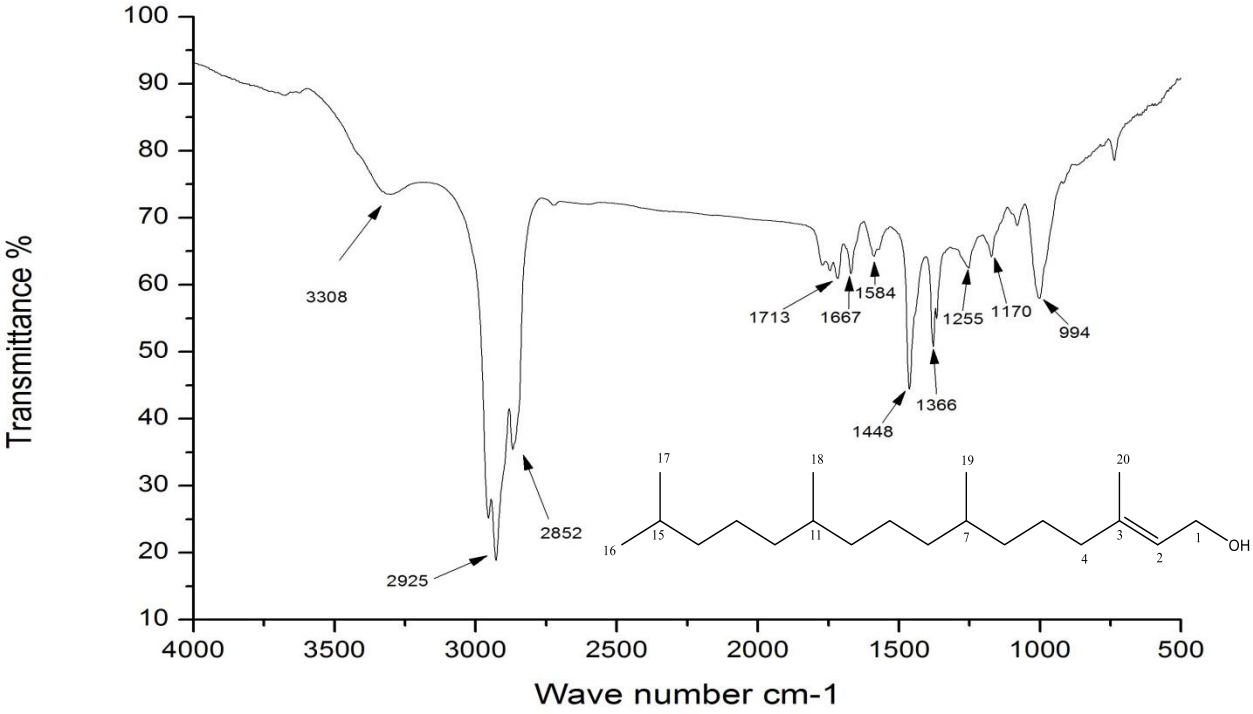
Appendix Ad**: FTIR spectrum compound 1 (KBr)**



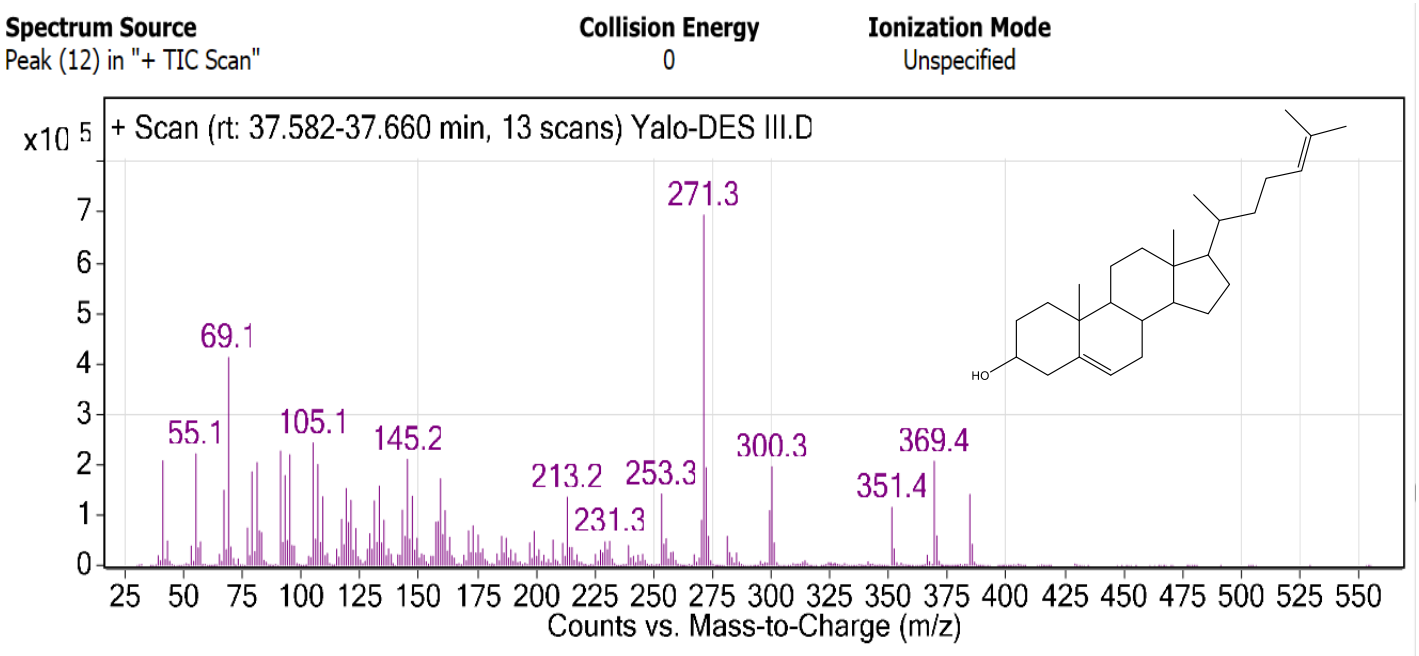
Appendix Bb**: GC-MS spectrum of Phytol ($CHCl_3$)**



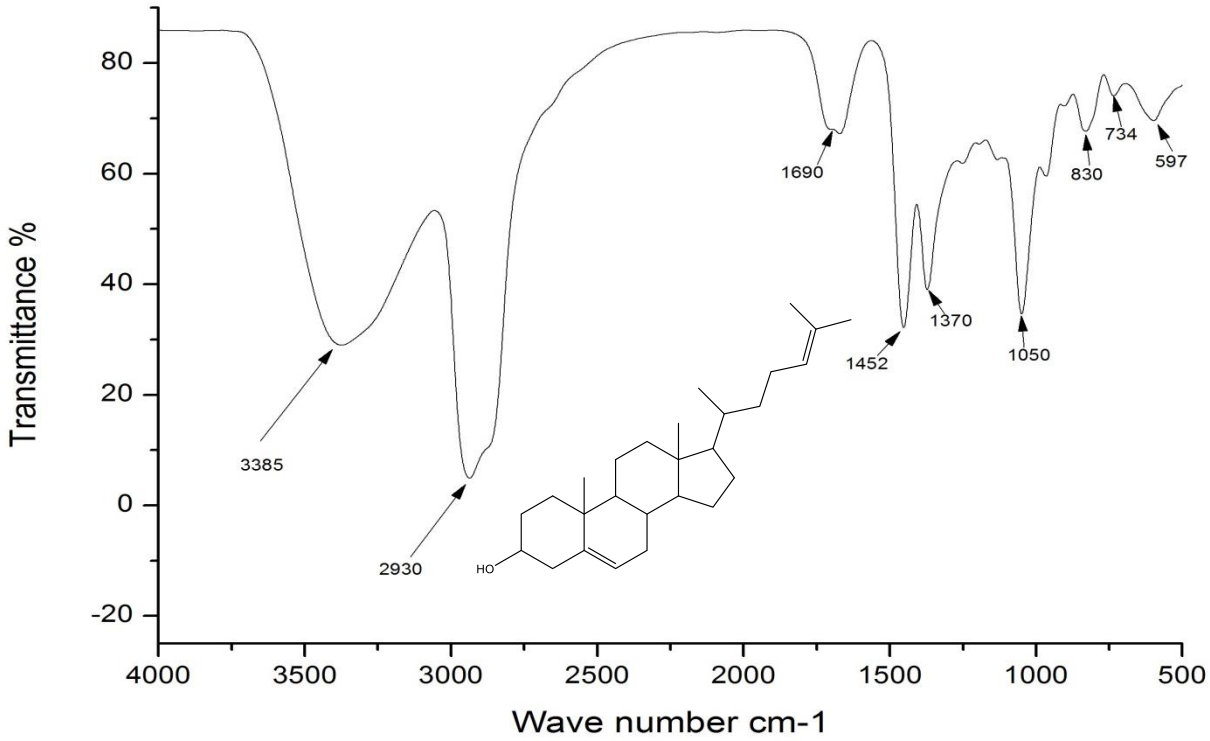
Appendix Bc**: FTIR spectrum of Phytol (KBr)**



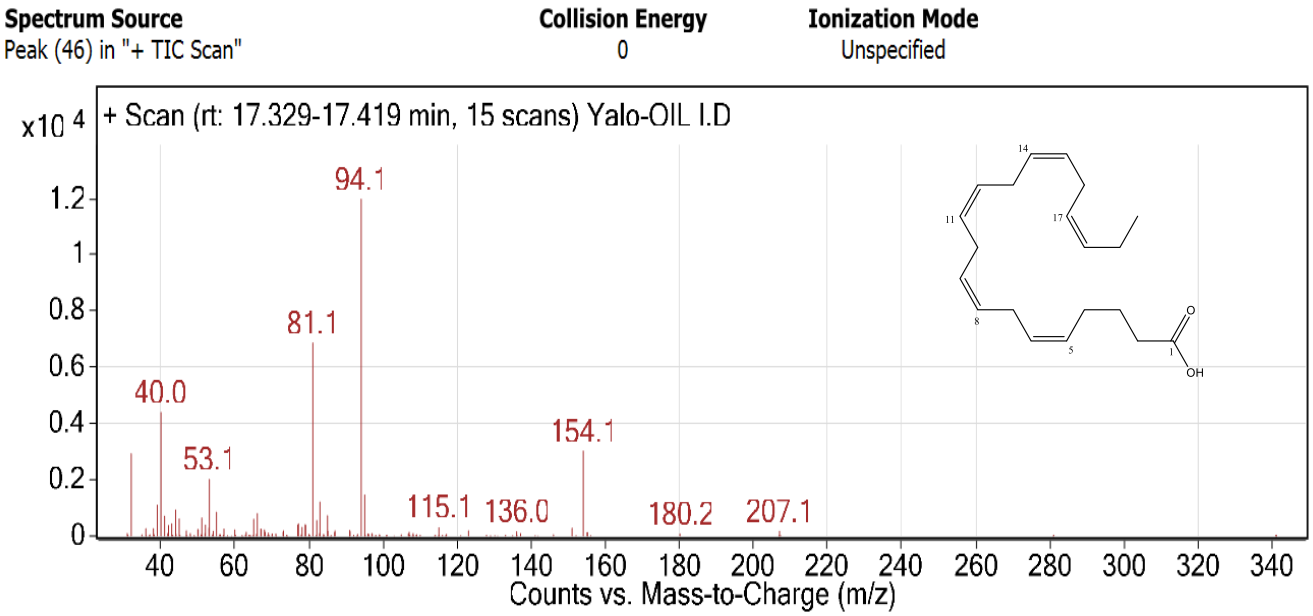
Appendix Ca**: GC-MS spectrum of Desmosterol ($CHCl_3$)**



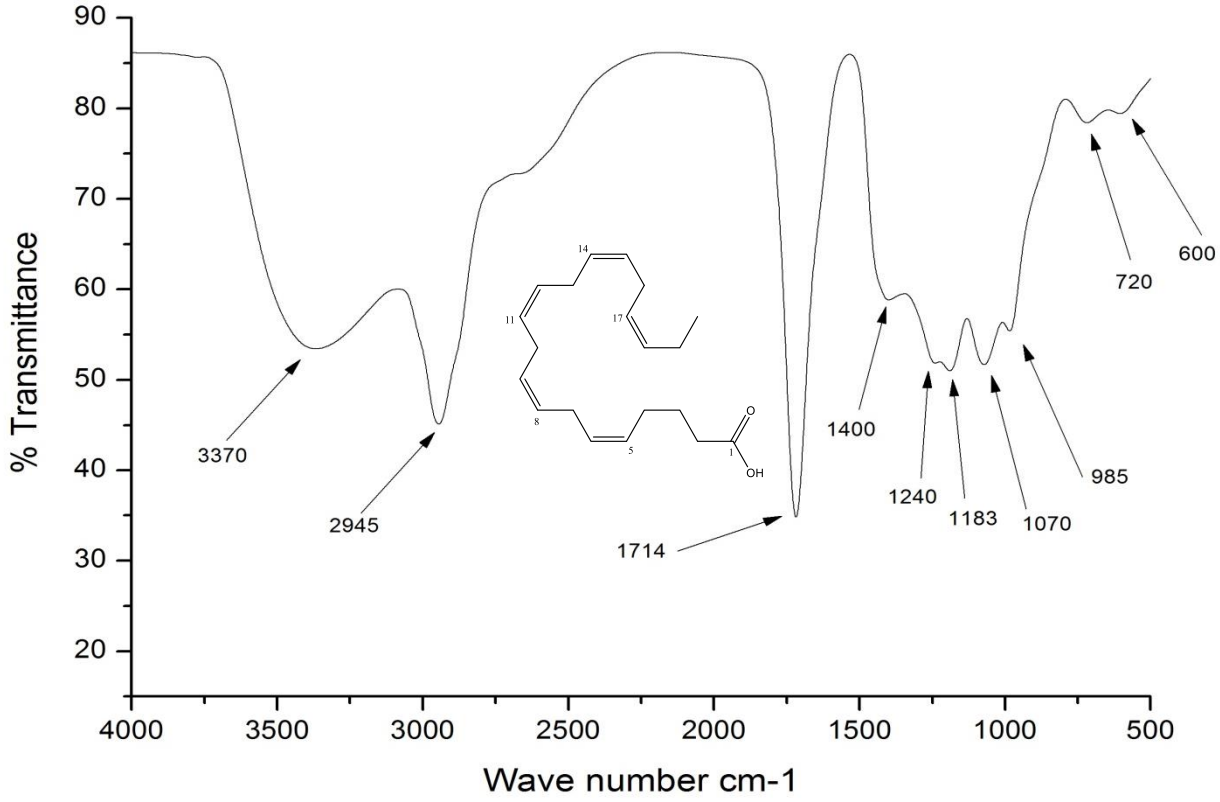
Appendix Cb**: FTIR spectrum of Desmosterol (KBr)**



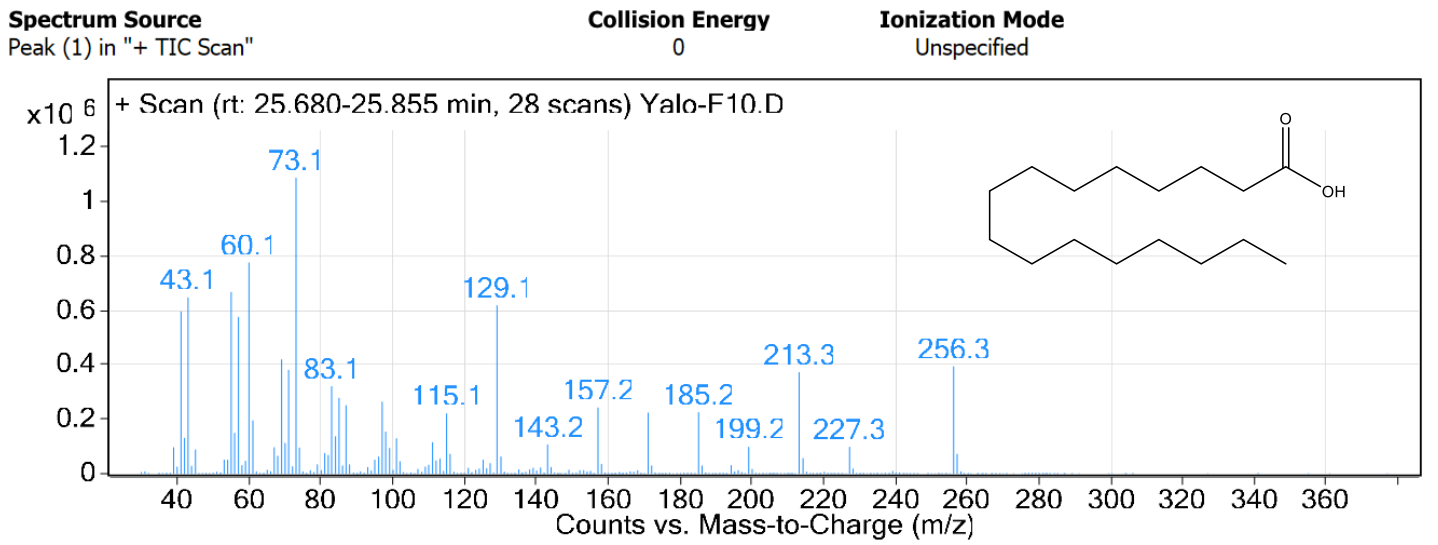
Appendix Da**: GC-MS spectrum of EPA ($CHCl_3$)**



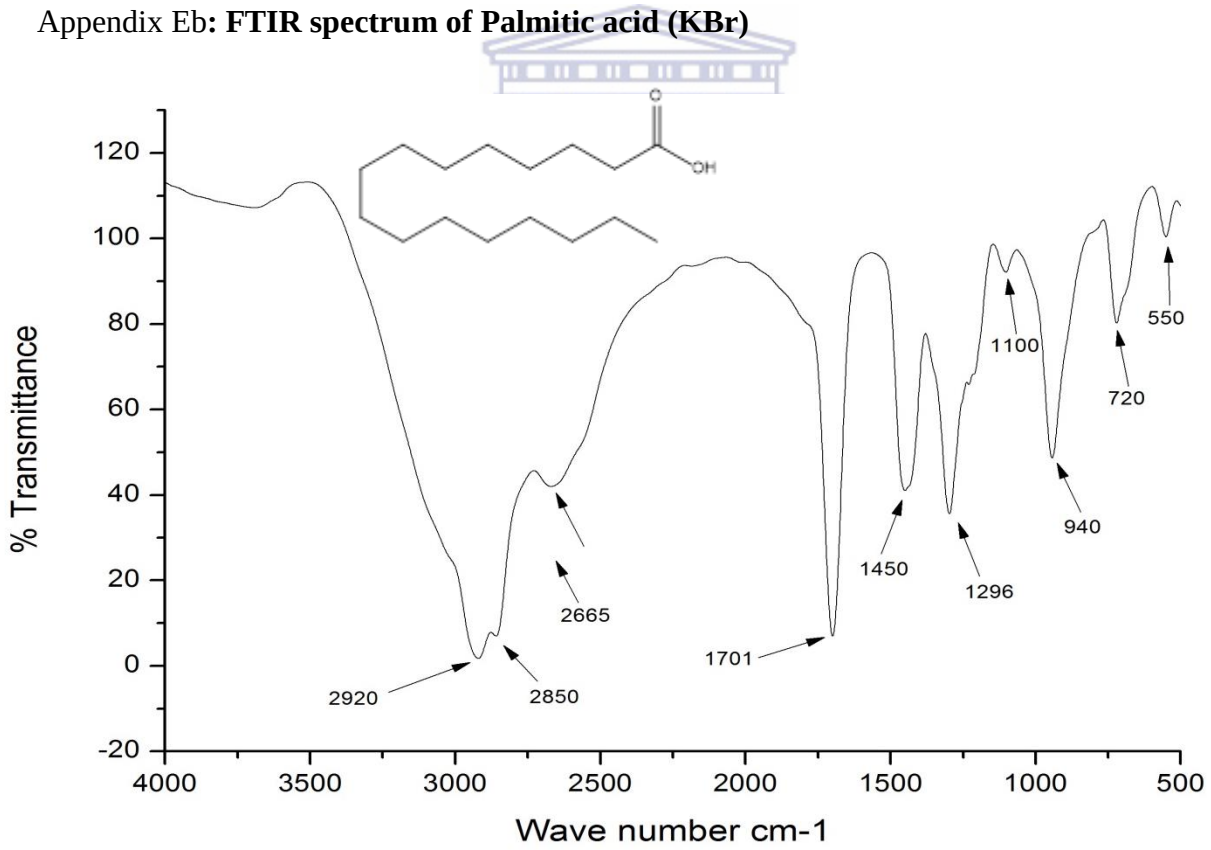
Appendix Db**: FTIR spectrum of EPA (KBr)**



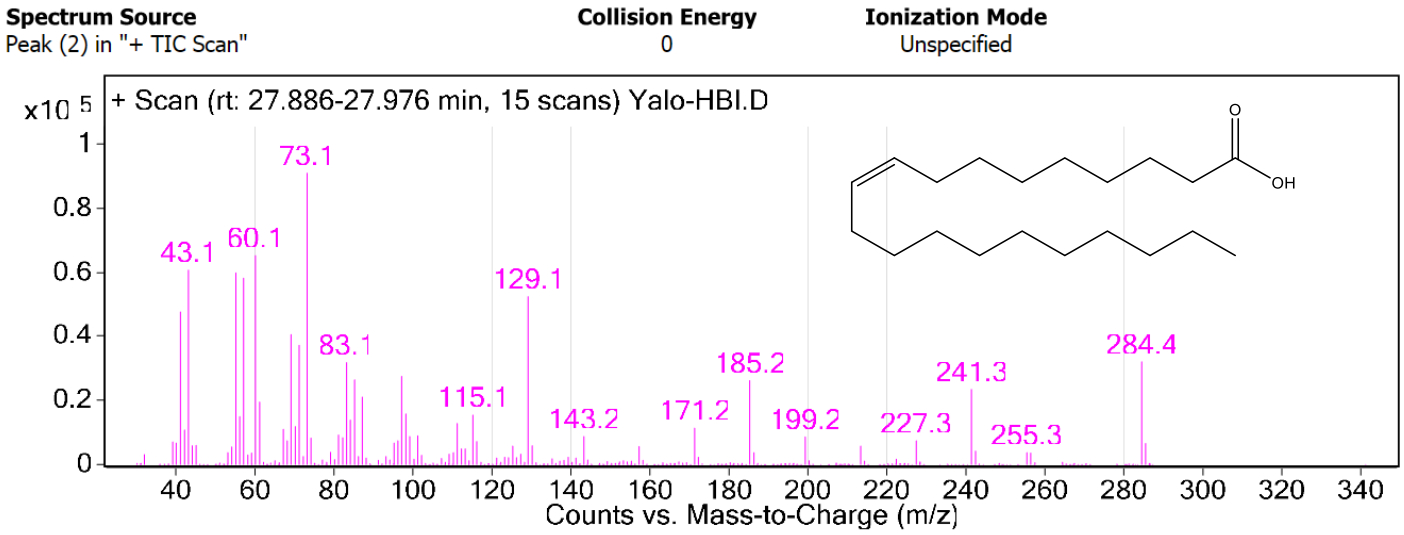
Appendix Ea**: GC-MS spectrum of Palmitic acid ($CHCl_3$)**



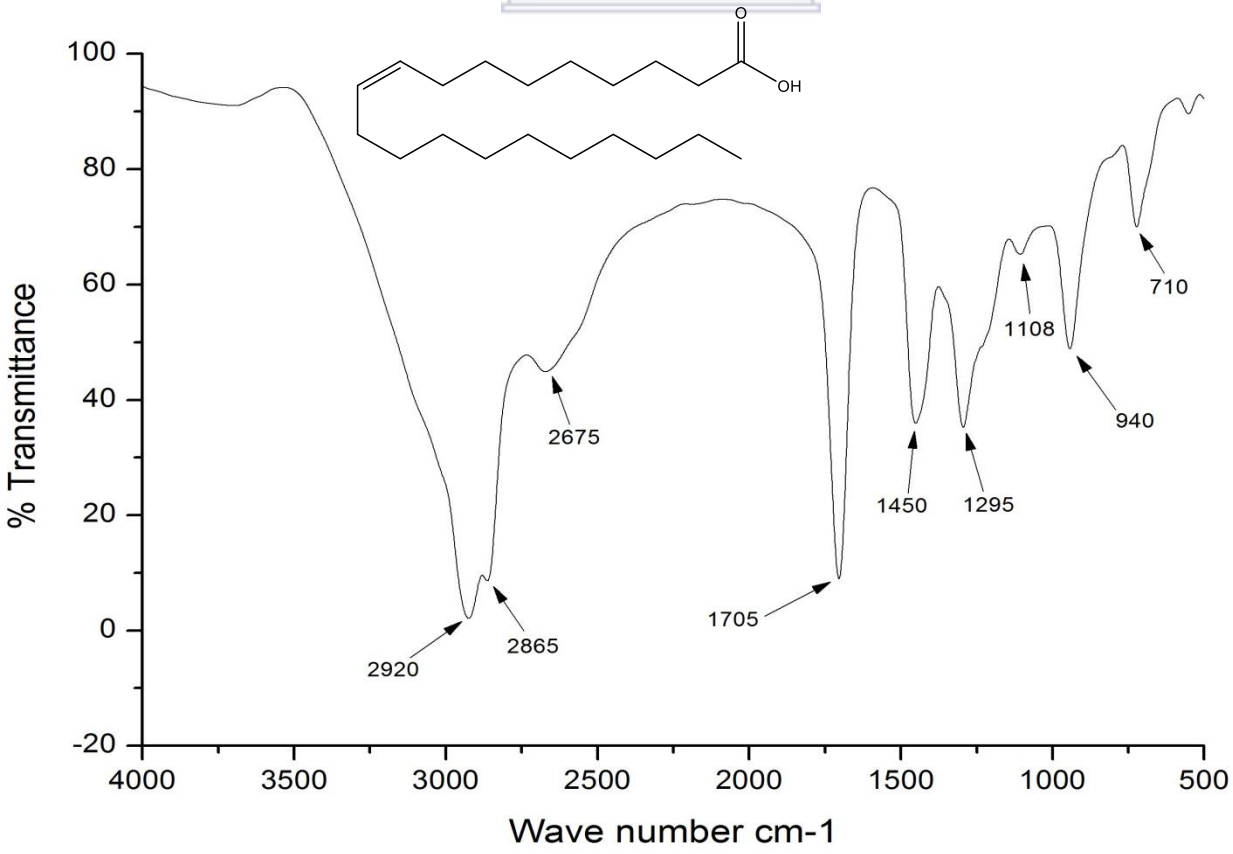
Appendix Eb**: FTIR spectrum of Palmitic acid (KBr)**



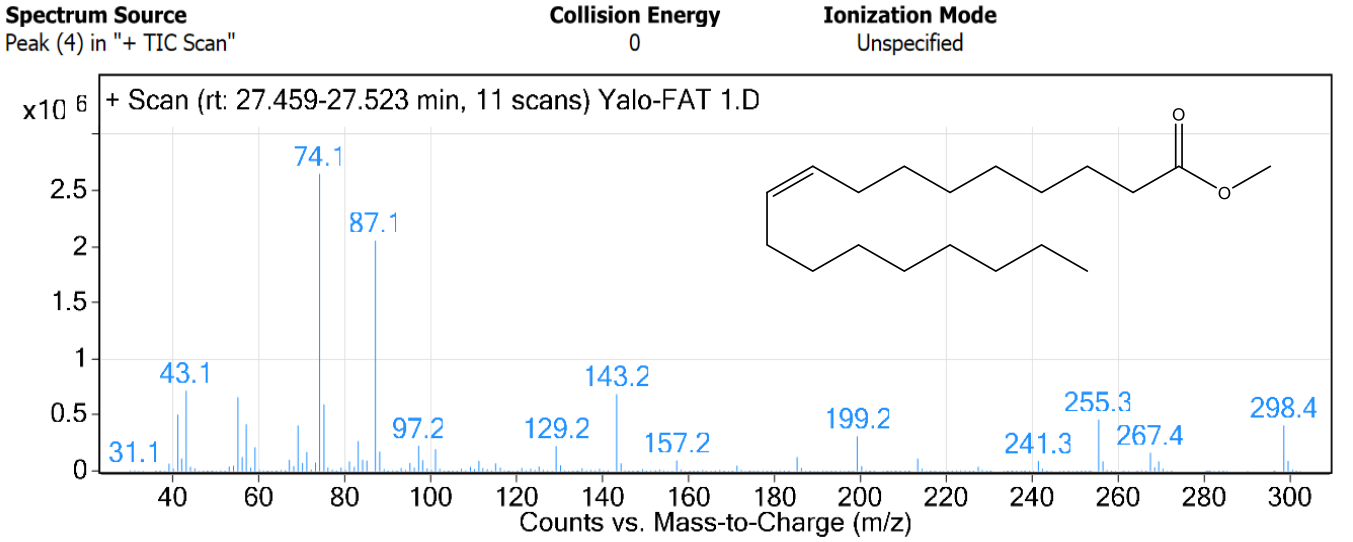
Appendix Fa**: GC-MS spectrum of 9-eicosenoic acid ($CHCl_3$)**



Appendix Fb**: FTIR spectrum of 9-eicosenoic acid (KBr)**



Appendix Ga**: GC-MS spectrum of Methyl oleate ($CHCl_3$)**



Appendix Gb**: FTIR spectrum of Methyl oleate (KBr)**