AMERICAN UNIVERSITY OF BEIRUT

MICROBIAL TRANSFORMATION OF ANTI-CANCER STEROID AND CYTOTOXICITY OF ITS METABOLITES AGAINST CANCER CELL LINES

by
DINA KAMAL FARRAN

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MICROBIAL TRANSFORMATION OF ANTI-CANCER STEROID EXEMESTANE AND CYTOTOXICITY OF ITS METABOLITES AGAINST CANCER CELL LINES

by

DINA KAMAL FARRAN

Approved by:

[Signature]

Dr. Elias Baydoun, Professor
Biology

Advisor

[Signature]

Dr. M. Iqbal Choudhary, Professor
International Center for Chemical and Biological Sciences, University of Karachi

Member of Committee

[Signature]

Dr. Colin Smith, Associate Professor
Biology

Member of Committee

Date of thesis defense: May 5, 2014

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AN ABSTRACT OF THE THESIS OF

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Title: Microbial Transformation of Anti-Cancer Steroid and Cytotoxicity of its Metabolites against cancer cell lines.

Microbial transformation of steroids has been extensively employed over the last decades for the production of novel drug analogues that are hardly synthesized by the classical chemical routes. Exemestane is a steroidal drug used to treat breast cancer by irreversibly binding to the aromatase enzyme responsible for the conversion of androgen to estrogen in postmenopausal women. Microbial transformation of exemestane (1) was investigated using the two fungal strains *Macrophomina phaseolina* and *Fusarium lini*. Biotransformation of the drug in *Fusarium lini* yielded only one metabolite 11α-hydroxy-6-methylene-androsta-1, 4-diene-3,17-dione (2); however it yielded three metabolites 16β, 17β-dihydroxy-6-methylene-androsta-1, 4-diene-3-one (3), 17β-hydroxy-6-methylene-androsta-1, 4-diene-3, 16-dione (4), and 17β-hydroxy-6-methylene-androsta-1, 4-diene-3-one (5) when performed in *Macrophomina phaseolina*. Metabolites (2), (3), and (4) are new compounds reported for the first time in this project, while metabolite (5) was previously described. Upon testing the metabolites against cancer cell lines of cervical and prostate origins, metabolite (2) was found to be moderately active.

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ABBREVIATIONS

ATCC	American Type Culture Collection
C13- NMR	Carbon Nuclear Magnetic Resonance
CC	Column Chromatography
COSY-NMR	Correlation Spectroscopy Nuclear Magnetic Resonance
CO2	Carbon dioxide
C	Carbon atom
°C	Degree Celsius
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
g	Gram
HeLa	Cervical cancer cell line
HMBC	Heteronuclear Multiple Bond Correlation
H1-NMR	Proton Nuclear Magnetic Resonance
HPLC	High-Performance Liquid Chromatography
IC_{50}	Half minimal inhibitory concentration
IR	Infrared
KH_2PO_4	Potassium Dihydrogen Phosphate
L	Liters
mL	Milliliters
MeOH	Methanol
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
µM	Micromole

µL	Microliter
NaCl	Sodium cloride
nm	Nanometers
NMR	Nuclear Magnetic Resonance
NOESY-NMR	Nuclear Overhauser Effect Spectroscopy Nuclear Magnetic Resonance
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
PC3	Prostate cancer cell line
PE	Petroleum Ether
SD	Standard Deviation
t_R	Retention time
TLC	Thin Layer Chromatography
UV	Ultraviolet

To My

Beloved Family

CHAPTER I

INTRODUCTION

A. Definition

Biotransformation is defined as the use of a living organism to change the chemical structure of a compound, usually to change one or two functional groups, and come up with new metabolites that resemble the parental drug but with few modifications. This process can be performed in fungi, bacteria, plants, human cells or any living organism that has a metabolism. Biotransformation could be done not only in whole cells but also using purified enzymes or crude ones. Sometimes, the use of a whole cell as a biocatalyst is the only choice especially when the enzymes of interest are membrane bound. Biotransformation in whole cells has many advantages over the use of pure enzymes such as handling, price, and time consumption. In addition, a whole cell contains all the cofactors required to carry out a cascade of reactions. However, this type of biotransformation can be limited by the toxicity of the substrate to the cell and the large number of byproducts due to undesired reactions. Over the last few years, there has been an increase in the production of high-value specialty chemicals through biotransformation using either isolated enzymes to catalyze single step transformation or whole cells to catalyze multi-step reactions. This increase is motivated by the fact that enzymes, especially the ones found in microorganisms, have the ability to catalyze a wide range of reactions with high levels of stereoselectivity, regioselectivity, and chemoselectivity. The selectivity and the specificity of the enzymes found in microorganisms make biotransformation an easier and faster approach towards the synthesis of new compounds as compared to the traditional chemical routes that are

much more expensive, time consuming and, often unable to modify compounds the same way microbial transformation does.

B. History

Very early on in history, without even being aware of neither the concept of biotransformation nor the existence of microorganisms, this process was used (along with fermentation) for the production of food and beverages. Among these practices was brewing which is the production of beer through steeping a starch source in water and then fermenting it with yeast. Another example, which goes back to the Egyptians, is leavening bread using yeast. However, the production of chemicals such as alcohols and organic acids through fermentation is relatively recent and the first reports in the literature only appeared in the second half of the 19th century, where lactic acid was probably the first optically active compound to be produced industrially by fermentation (Liese *et al.* 2006). Later on, it was discovered that microorganisms can modify compounds by simple well defined chemical reactions catalyzed by enzymes found in the living organism. Here came the concept of biotransformation. The main differences between biotransformation and fermentation are that first, there are several catalytic steps between the substrate and the product in fermentation while there is only few in a biotransformation. Second, the chemical structures of the substrate and the product resemble one another in biotransformation, but not necessarily in fermentation (Vasic-Racki 2006). Here is a summary of the progress done in the biotransformation field through history. In 1729, Pier Antonio Micheli's pioneering work on biotransformation of food and other organic matters led to the cultivation of microorganisms (Bull and Slater 1982). In 1814, Kirchhoff hydrolyzed starch to sugar using wheat extracts. Payen and Persoz similarly investigated the hydrolysis of starch to yield dextrin and sugar

using barley extracts in 1833. Berzelius, in 1835, worked on the hydrolysis of starch by diastase (Bornscheuer and Buchholz 2005). In 1858, Louis Pasteur specified the role of microorganisms in the fermentation of grape juice. In the late 19th century, Emil Fisher elaborated essential aspects of enzyme catalysis. His work revealed the specificity as a key characteristic of enzymes and consequently agreed with the "lock and key" model (Faber 2011). The presence of a living system was considered essential for fermentations up until Eduard Buchner showed that an enzyme can catalyze a chemical reaction without being necessarily associated with a living system in 1972. In 1916, an important progress in fermentology occurred when butanol was produced, and when acetone was industrially synthesized from molasses (Jones and Woods 1986). The enzymatic condensation of benzaldehyde with acetaldehyde, developed by Neuberg in 1921 to produce chiral acetoin, led to the commercial production of ephedrine alkaloid in 1934. In 1952, Murray and Peterson were the first to perform biotransformation in microbial strains; more particularly their work was done using a fungal species called *Rhizopu*s. Since then the field of microbial transformation has grown. In 1994, androst-4-en-3, 17-dione was biotransformed by *Mucor piriformis* (Madyastha 1994), *Aspergillus fumigates* (Garai and Mahato 1997*), curvularia lunata* (Faramarzi *et al.* 2008) and *Acremonium strictum* (Faramarzi *et al.* 2006). In 1976, Davis carried out the microbial transformation of pseudocodamine and d-tetrandrine in *Cunninghamella blakesleena* and *Streptomyces griesus* (Davis *et al. 1976*). *Curvularia lunata* was used for the microbial conversion of lapachol to dehydro-alpha-lapachone in 1979 (Oten *et al. 1979*). In the same year, *Aspergillus alliaceus* was used for the transformation of ellipticine (Chien *et al. 1979*). Biotransformation of laphacol, an antitumor agent, was conducted in *Cunninghamella echinulata* in 1981 (Otten *et al. 1981*). In 1983, rotenone and dihydrorotenone were biotransformed in *Cunninghamella blakesleena* (Sariaslani *et*

al. 1983). In the same year, another antitumor agent known as bouvardin was biotransformed by several fungal strains (Petroski *et al. 1983*). The microbial transformation of (+)adrenosterone was performed in *Fusarium lini, Cephalosporium aphidicola* and *Tricothecium roseum* in 2002 (Musharraf *et al. 2002*). Bioconversion of androst-1, 4-dien-3, 17-dione was also done in *Cephalosporium aphidicola* (Bhatti *et al. 2012*). In 2003, several fungal strains were used for the microbial transformation of Zaluzanin-D (Kumari *et al. 2003*). These studies are few among many similar researches done in the field of biotransformation.

When the catalytic activity of the enzymes as well as their key role in biotransformation became accepted and understood, enormous efforts were made to find ways to stabilize enzymes, one of which was enzyme immobilization. Two of the most important applications of enzyme immobilization were the isomerization of glucose and hydrolysis of penicillin using immobilized enzymes (Bhosale *et al. 1996,* Chong *et al.* 2004).

The introduction of modern techniques such as proteomics, genetic engineering and recombinant technology extended the field of biotransformation into new directions. Discovery of restriction enzymes in 1970s made it possible to cut genes at defined places (loci) and express them in other microorganisms to produce particular enzymes for large scale fermentations. The use of recombinant whole-cell systems for biotransformation is a field of research growing up rapidly. A genetically modified bacterial strain belonging to the genus *Erwinia,* encoded with a reductase from *Corynebacterium* was capable of converting D-glucose to 2-keto-L-gluconic acid, which is involved in the commercial synthesis of vitamin C (Sonoyama *et al. 1982*). Another important application of recombinant whole-cell biotransformation is the production of indigo, one of the world's largest selling textile dyes. Solvent resistant *E.*

coli, encoded with a multicomponent hydroxylase gene from *Acinetobacter* species, efficiently produced indigo from indole in a biphasic culture system (Doukyu *et al. 2003*). Similar work is currently performed in industries to produce chiral intermediates or products.

C. Types of Biotransformations

Biotransformations are divided into two main categories:

1. Biosynthetically–Directed Biotransformations

Biosynthetically–directed biotransformations that involve the transformation of a substrate that bears a structural relationship to a natural biosynthetic intermediate of the biocatalyst. In other words, it is the transformation of a substrate that resembles, structurally speaking, an intermediate found in the living organism. A known example is the production of higher plant gibberellins by microbial transformation of various hydroxylated *ent*-kaurenes.

2. Xenobiotic Biotransformations

Xenobiotic biotransformations involve the transformation of a substrate that is completely foreign to the biological system. Examples of xenobiotic biotransformations are the hydroxylation of steroids by microorganisms for the production of new analogues. Transformation of exemestane by fungal strains discussed in this project is one of these examples.

Biotransformation of xenobiotics could be divided into three main classes depending on the living organism used as biocatalyst:

- Biotransformation by animal cells

- Biotransformation by plant cells
- Biotransformation by microorganisms

a. Biotransformation by Animal Cells

Living organisms are continuously exposed to foreign substances or xenobiotics that could be either natural or synthetic. These chemicals are absorbed by the body through the lungs, gastrointestinal track, and skin. The physical property that makes these compounds easily absorbed is mainly their lipophilicity. However, their elimination is not easy and requires different properties of which is the solubility of the compounds in water. Here comes the role of biotransformation that converts foreign lipophylic compounds to water soluble ones in order to increase the rate of their excretion through the urine or feces (Parkinson 2001).

Without the process of biotransformation, the elimination of xenobiotics would take more time and therefore overwhelm the body and sometimes kill the living organism. The organs involved in biotransformation are the liver (where most of the biotransformation occurs due to the presence of a vast amount of enzymes), the lungs, the kidneys, the intestine (enterocytes, gut flora), and skin gonads. Foreign compounds exhibit different effects on the living organisms depending on their physiochemical properties. Some of them remain active or become active upon biotransformation in the body; others lose their function during this process, or become toxic. The ability or the potential of a living organism to biotransform xenobiotics depends on the extent to which the organism is exposed to foreign compounds. For instance, organisms feeding on a wide variety of food have a greater capacity to biotransform xenobiotics than organisms feeding only on plants, these in turn have a higher biotransforming potential than the ones feeding on a particular type of plants only. However, exposure is not the

only factor affecting the biotransformation potential. Genetics, enzymes, and stimulations by external compounds are all important factors that should be taken into consideration. In animal cells, biotransformation occurs mainly in the liver where most of the enzymes have broad specificity. Cytochrome P450 enzymes are examples of enzymes found in the liver and involved in the conversion of steroids to water soluble metabolites. The reactions catalysed by xenobiotic biotransforming enzymes are divided into two categories: phase I and phase II reactions.

- In phase I, xenobiotics are transformed from non-excretable to water soluble and excretable chemicals. Phase I reactions are mainly oxidative, reductive, and hydrolytic. These reactions mainly introduce a polar group (such as -OH, -SH, -NH2, -COOH) or expose an existing one. As a result, a small increase in the hydrophilicity of the compound occurs. Phase I products are directly eliminated or further processed by phase II reactions.

- In phase II, the reactions involve the conjugation of a small endogenous molecule to the compound, particularly to a functional group or to the group that has been introduced or exposed in phase I. These reactions are mainly acetylation, conjugation with amino acids, conjugation with glutathione, glucuronidation, methylation, and sulfonation. As a result, an increase in the hydrophilicity of the xenobiotic occurs, promoting its excretion. It is worth mentioning that not all the drugs undergo phase I biotransformation, some of them directly move to phase II where conjugation to an existing functional group occurs (Parkinson 2001).

Figure 1 summarizes the fate of xenobiotics in the liver.

Properties of human cytochrome P450s

- They are involved in phase I metabolism of almost 50% of drugs administered to humans as well as endogenous compounds such as steroids.

- All cytochrome P450s are hemoproteins.
- They are known to have a broad substrate specificity hence acting on different xenobiotics.
- They are flexible catalysts, catalysing various types of reactions especially the introduction of an oxygen atom to the compound.
- They generate hydroxylated products that are water soluble and could be easily excreted.
- They are mainly found in the liver but also in other tissues such as the brain, small intestine and lung.
- They generate carcinogenic or mutagenic products as a result of a bioactivation process.
- They have altered activity in diseased tissues, thus affecting the metabolism of drugs (Parkinson 2001).

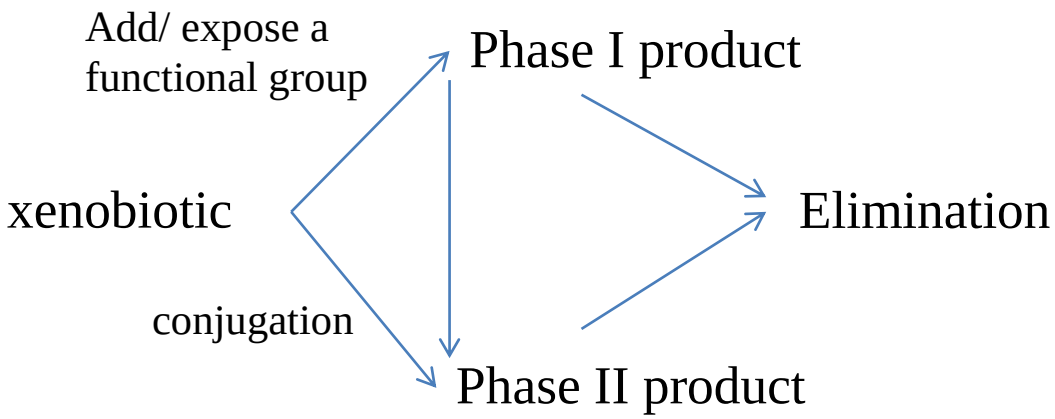


Fig. 1. The fate of xenobiotics in the liver

b. Biotransformation by Plant Cells

Plants are considered the "green liver" that cleans the environment from

various types of chemicals. This type of biotransformation is called phytoremediation and necessitates the ability of the plants to overcome the chemical and physical properties of xenobiotics, hence removing and decomposing environmental pollutants. For the foreign compound to enter the plant cell, it has first to pass through the plasma membrane. Hydrophobic compounds are passively diffused into the leaves through the waxy cuticle, while polar compounds undergo a systemic uptake ending up moving in the xylem or the phloem depending on the chemical and physical properties of the xenobiotic. The uptake relies also on passive diffusion, except when the compound is transported by proteins normally present for the translocation of essential nutrients or endogenous metabolites (Edwards *et al.* 2011).

The biotransformation of xenobiotics in plants resembles the mammalian one in terms of enzymes, proteins, DNA sequences, and metabolite patterns. Plants contain enzyme systems that resemble the P450 monooxygenases and glutathione transferases present in the liver in addition to other isoenzymes with specificity to xenobiotics and secondary plant substrates (Sandermann 1992).

Plant xenobiotic biotransformation is divided into three main phases which are activation, conjugation, and storage. The first two phases resemble the ones discussed in the liver earlier, while the last phase is specific to plants. Phase I involves the activation of the molecule by enzymes such as esterases to promote hydrolysis, cytochrome P450 or peroxidases to promote oxidation, and enzymes involved in reduction and hydroxylation. After activation, the molecule is either directly metabolized or conjugated to a carrier molecule in the second phase. The most common type of conjugations in plants phase II metabolism is conjugation to glutathione via glutathione-S-transferase enzymes. However, conjugation to sugar derivatives (such as galactose or glucose) or to malonyl derivatives is also possible. The conjuguates are then actively

translocated from the cytoplasm by trans-membrane ATP-dependent transporters. In phase III, biotransformed metabolites are not excreted like in the liver, instead, plants undergo internal compartmentation and storage in the vacuole when dealing with soluble conjuguates and in the cell wall when dealing with insoluble conjuguates (Van der lelie *et al.*). The compartmentation step is very critical in the detoxification of organic compounds since it takes away the conjugated metabolites from sites considered vulnerable in the cytosol. In addition, it further processes the conjuguates in the vacuolar matrix (Harms *et al.* 2000).

Therefore, there are two main differences between biotransformation in animals and plants. First, plants have a large variety of xenobiotic transforming enzymes as compared to animals due to the ability of plants to synthesize secondary metabolites. This gives the possibility to not only biotransform metabolites as xenobiotics but also to mistakenly consider them secondary metabolites and therefore activate endogenous enzymes to deal with them. Second, the fate of the conjuguated products is different in plants and animals as discussed earlier. In animals, conjuguated products are excreted which is unusual in plants that direct them to the vacuole for storage. This gives the metabolites the opportunity to undergo further biotransformation sometimes ending up with natural products (Edwards *et al.* 2011).

c. Biotransformation by Microorganisms

Biotransformation by microorganisms (such as fungi and bacteria) is commonly used for the production of high value chemicals. These organisms have the ability to biotransform natural products and xenobiotics. This characteristic makes microorganisms used in environmental biotechnology to restore ecological balance, and in industrial synthetic chemistry to produce new metabolites that could not be produced

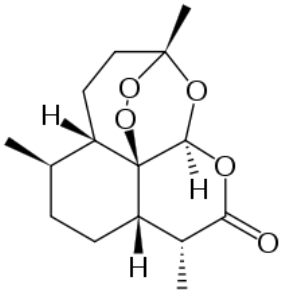
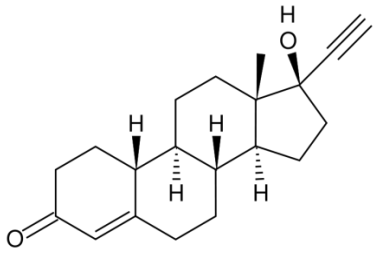
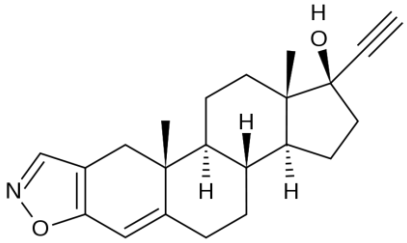
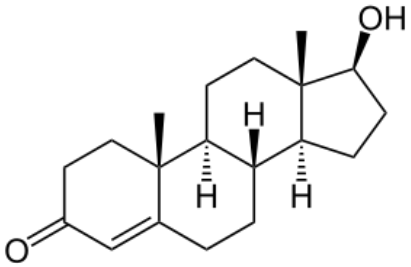
by the classical synthetic routes (Schmid *et al.*). Another characteristic of microbial biotransformation is its ability to mimic the mammalian metabolism of drugs. This enables the production of intermediates or metabolites in large amounts for toxicity studies, preclinical trials and even regulatory purposes. Although microorganisms do not produce same metabolites as mammals, they are still considered important models for studying the metabolism of xenobiotics. Here came the concept of "microbial models of mammalian metabolism" described by Smith and Rosazza (Gopishetty *et al.*).

Microorganisms have a high surface to volume ratio that acts on boosting the metabolic rate. They are also characterized by the presence of a large variety of enzymes resistant to heat, alkali, and acid, making them able to perform a wide range of stereo- and regioselective chemical reactions. In addition, they can easily adapt to a wide array of environments starting from nature to a laboratory flask where they grow on carbon and nitrogen sources. Microorganisms can be easily modified genetically to increase the yield of new metabolites as well as to change structures and activities. All these characteristics, along with the simple screening procedures and the diversity of species producing different enzymes that catalyze the same reactions, make microorganisms good models for biotransformation of xenobiotics (Barredo 2005).

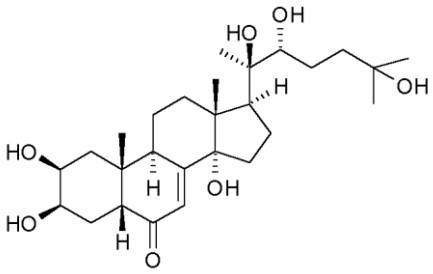
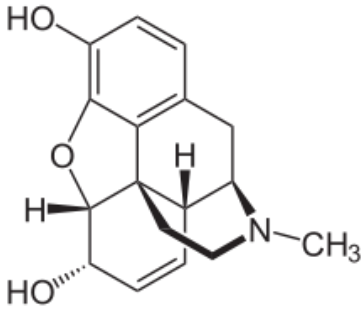
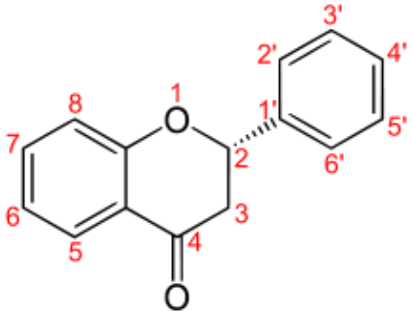
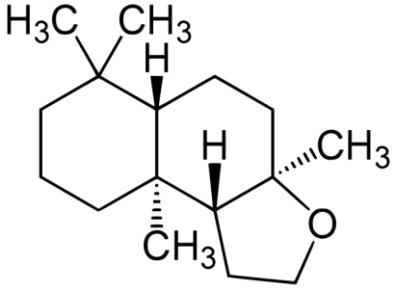
The different types of compounds fungi exploit for their growth gives them the ability to produce a wide range of enzymes. Approximately 60% of the enzymes used in industry nowadays are from fungal origins. These fungal enzymes are used in different applications such as bioremediation, hydrolyzing milk protein, baking, and fermenting coffee beans among many others. Almost all the fungal enzymes used in industry come from five genera which are *Rhizopus, Aspergillus, Penicillium, Humicola, Trichoderma* though the fungal world is very diverse and estimated to contain 1.5 million members. This raises the urgent need for the exploration of endophytes (Suryanarayanan 2012).

Microorganisms are known to biotransform different types of natural products, mainly alkaloids, terpenoids and steroids. Examples of microbial transformation are shown in Table 1.

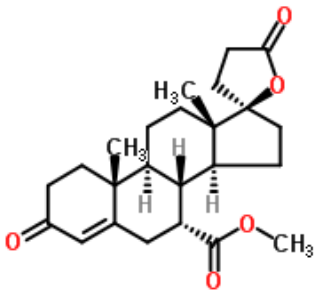
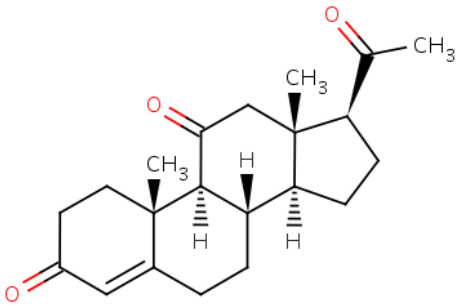
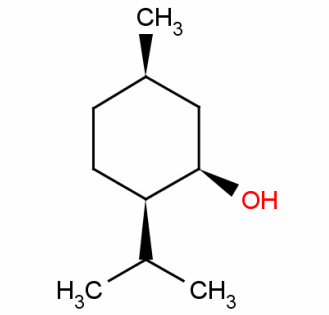
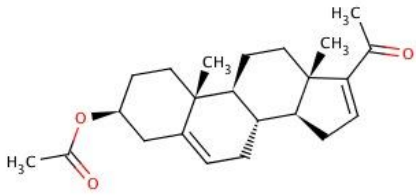
Table 1. Examples of xenobiotic biotransformations by microorganisms

Substrate	Microorganisms	References
 Artemisinin	*Aspergillus niger* *Cunninghamella echinulata*	Zhan *et al.* 2002
 Norethisterone	*Cephalosporium aphidicola*	Choudhary *et al.* 2004
 Danazol	*Aspergillus niger*, *Cephalosporium aphidicola*, *Fusarium lini*, *Bascillus cerus*	Choudhary *et al*. 2002
 Testosterone	*Aspergillus fumigatus* *Curvularia lunata*, *Pleurotus oestreatus* *Rhizopus stolonifer*, *Fusarium lini*	Mahato *et al*. 1984 Atta-ur-Rahman *et al*. 1998 Al Aboudi *et al*. 2008

"Table 1 – *Continued*"

Substrate	Microorganisms	References
 20-Hydroxyecdysone	*Curvularia lunata*	Changtam *et al.* 2006 and 2008
 Morphine	*Cylindrocrpon didymum*	Stabler *et al.* 2001
 Flavanone	*Aspergillus niger* *Penicillum chermesinium*	Suslowa *et al.* 2008
 Ambrox	*Macrophomina phaseolina*	Choudhary *et al.* 2012

"Table 1 – *Continued*"

Substrate	Microorganisms	References
 Mexrenon	*Beauveria bassiana* *Mortierella isabellina*	Preisig *et al.* 2003
 11-Ketoprogesterone	*Fusarium lini*	Choudhary *et al.* 2011
 (+)-isomenthol	*Fusarium lini* *Rhizopus stolonifer*	Choudhary *et al.* 2011
 16-Dehydropregnenolone	*Mucus racemosus*	Gea *et al.* 2008

- *Microbial transformation of alkaloids*

Alkaloids are group of compounds that contain a nitrogen atom. These compounds may be derived from various sources such as plants, microbes, and marine organisms. They are used as anticancer, analgesic, antimalarial, and in treatment of central nervous system disorders, hypertension, and Parkinson. Although alkaloids have a complex structure, microorganisms are able to biotransform them due to the presence of a large variety of enzymes in these biological systems. New molecular approaches such as genetic engineering will definitely remove any limitation to their use and will provide novel effective biocatalysists or improve existing ones (Rathbone *et al.* 2002).

One of the important pharmaceutical alkaloid compounds is opium-derived morphine and its derivatives. An example would be the biotransformation of thebaine a minor component of opium. Two major reasons make thebaine a good candidate for biotransformation studies; first Thebaine is of limited availability, second the N-demethylation of thebaine is considered a complicated chemical reaction that gives low yields. Biotransformation of thebain, performed in *Mucor piriformis,* yielded a major metabolite known as northebaine and proposed that the N-demethylation reaction did not occur by means of an N-oxide intermediate. The microorganism caused N-dealkylation of thebaine N-variants with good yields (Rathbone *et al.* 2002).

- *Microbial transformation of terpenoids*

Terpenoids, also known as isoprenoids, are considered the largest group of natural products. Different types of isoprenoids are found in microorganisms, plants and animals. These molecules give a variety of floral scents. They are made of isoprene units combined head to tail and named according to the number of isopentenyl units they include. A monoterpene is a terpenoid that contains two isopentenyl units; a sesquiterpene contains three units, a diterpene contains four units, a triterpene contains

six units, tetraterpenes contains eight units and finally any terpenoid that contains more than eight units is called polyterpene (Demyttenaere 2001). Essential oils are considered important sources of terpenoids. They include a mixture of terpenes, acids, ketones, aldehydes, esters and alcohols. Monoterpenes could be divided further to subgroups which are the acyclic, monocyclic, and bicyclic. Each subgroup contains terpene aldehyde, terpene ketone, and hydrocarbon terpenes (Demyttenaere 2001). The extraction of terpenes from plants is difficult and present many problems of which is the low yield; therefore an alternative would be the biotransformation of parental substrates using microorganisms to produce terpenoids. Since terpenoids are important fragrance molecules, their biotransformation would result in novel flavours called bioflavours (Demyttenaere 2001).

- *Microbial transformation of steroids*

Steroids are organic compounds that contain four cycloalkane rings. The core is constituted of seventeen carbon atoms that form three cyclohexane rings and one cyclopentane ring. Steroids are found in fungi, plants, and animals. Known examples of steroids are testosterone, estradiol, lipid cholesterol, and dexamethasone. Their activity depends on the functional groups bound to the core rings, their number, size and, position as well as the oxidation state of the rings (Donova *et al.* 2012).

Steroids are known to regulate cell proliferation, differentiation, and signal transduction pathways. Some of them act as signals between microorganisms and eukaryotic hosts, while others are considered allosteric modulators of neurotransmitter receptors. They are also used as therapy for treating cancer, inflammation, diabetes, metabolic disorders, obesity, hypertension, asthma, eczema, HIV, and cardiovascular problems among many other diseases. Steroidal pharmaceutical compounds along with antibiotics are considered the second largest category of compounds used as medicines

and among the most popular ones (Donova *et al.* 2012).

Microbial transformation of steroidal drugs is an effective tool for the production of active metabolites or intermediates by acting on positions that are sometimes hardly accessible by chemical agents. A good example would be the production of cortisone from deoxycholic acid. Using the classical chemical routes, the synthesis of cortisone required 31 chemical steps, however biotransformation of a simple precursor molecule by two fungal strains *Rhizopus arrhizus* and *Aspergillus niger* reduced the chemical steps to only few and brought down its price from $200 to only $1(Donova *et al.* 2012).

D. Exemestane

1. Structure

Exemestane, also known as aromacin, is a steroidal drug used to treat breast cancer in post-menopausal women. It's also described as 6-methylenandrosta-1,4-diene-3,17-dione and has $C_{20}H_{24}O_2$ as molecular formula (Figure 2). It is known to be soluble in methanol and N, N-dimethylformamide, but not in water.

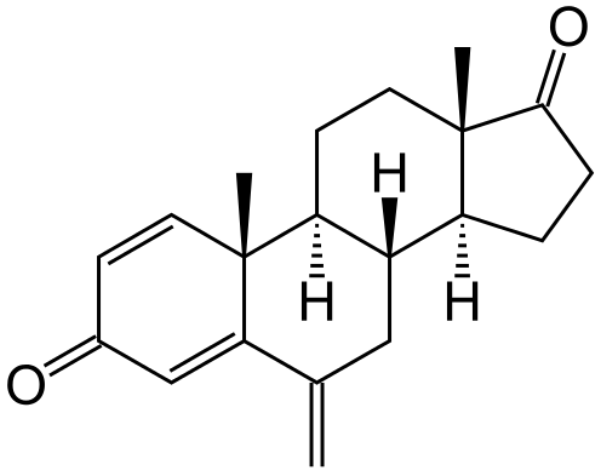


Fig. 2. Chemical structure of exemestane (1)

2. Function

Over the last forty years, tamoxifen, an estrogen receptor modulator (SERM), was the adjuvant treatment for early breast cancer. However, the side effects of this treatment led to the exploration of new compounds that are safer and more selective such as the aromatase inhibitors letrozole, anastrozole and exemestane (Robinson 2009). Some breast cancers require estrogen to grow; they have estrogen receptors and therefore are called ER-positive or estrogen-dependent. The principal source of estrogen in premenopausal women is the ovaries, while in postmenopausal women most of the estrogen is produced in peripheral tissues (fat tissues) by the conversion of androgen to estrogen via cytochrome p450 aromatase enzyme. By being structurally similar to the enzyme target, exemestane acts as a false substrate to the enzyme and is converted to an intermediate that binds irreversibly to the active site of aromatase causing its permanent inactivation; a process called "suicide inhibition." Therefore, exemestane is considered a steroidal aromatase inhibitor (Robinson 2009).Studies on exemestane showed that patients who were administered exemestane two to three years after having received tamoxifen treatment had more clinical benefits than those who continued receiving tamoxifen treatment for 5 years (Clemett *et al.* 2000).The advantage of taking exemestane appeared at the end of the 5^{th} year when the percentage of death or disease recurrence was reduced by 24% in case of treatment with exemestane while this percentage was only reduced by 3.3% in case of treatment with tamoxifen. However, the adverse effects of exemestane differ among patients and may include cardiovascular and skeletal disorders (Coombes *et al. 2007*). One of the most important drug metabolizing enzymes is the cytochrome P450 family. The expression and activity of these enzymes vary from an individual to another depending on the genetic (mutations, allelic forms) and environmental (food, medications, and diseases)

factors (Nagata *et al. 2001*).A study on the metabolism and excretion of exemestane in human showed that following administration of the radiolabeled drug, less than 1% of the initial dose was excreted unchanged, which means that it was extensively metabolized. The first step in the metabolism of exemestane occurs at position six where a methylene group gets oxidized, and at position 17 where a ketone group is reduced. This biotransformation occurs mainly in the liver by the isoenzyme cytochrome P-450 3A4 involved in the oxidation of the drug (Clemett *et al. 2000*).

Although exemestane lowers estrogen levels in postmenopausal women, it has no effect on the adrenal synthesis of corticosteroids or aldosterone. In addition, exemestane did not show any effect on other enzymes involved in the steroidogenic pathway (Deeks *et al. 2009*). Interestingly exemestane not only increases the testosterone level and lowers estrogen, but it also increases the levels of insulin-like growth factor (IGF) (Mrozek *et al. 2012*). The large reduction in estrogen levels combined with the rise in IGF makes exemestane an effective breast cancer medication.

E. Research Approach

The aim of this study is the production of new compounds from the biotransformation of exemestane. The choice of exemestane as a parental drug for the biotransformation process was not random; it was rather based on the significant importance of the drug in treating breast cancer and preventing the relapse of the disease. The biotransformation of the drug generates new metabolites that could be also active against cancer cell lines and therefore may become drug candidates.

We chose to do the biotransformation in fungi and not any other biological system because they are easy to manipulate, and grow fast. In addition, previous studies of biotransformation have shown successful attempt for the production of new

functional metabolites using fungi as biocatalysts. These living organisms are characterized by having a vast range of chemo-, regio-, and enantioselective enzymes. This feature makes biotransformation a powerful technique for the production of new compounds that are usually hard to synthesize by the classical chemical routes.

Specific aim 1:

We intended to find new metabolites through biotransformation of exemestane *(1)* by *Macrophomina phaseolina* and *Fusarium lini*. For this purpose we performed:

- Large scale fermentation
- Extraction of biotransformed metabolites
- Purification
- Structure determination

Specific aim 2:

We tested the activity of the newly generated metabolites against various cancer cell lines such as Hela and PC3. For this purpose we performed:

- MTT colorimetric assay to assess cell viability and to determine the cytotoxicity of the biotransformed products.

CHAPTER II

MATERIAL AND METHODS

A. Microbial Transformation

In a small scale trial, series of fungi were tested for their ability to biotransform the drug. This was done by preparing media for different strains of fungi, inoculating the spores into the media, feeding the fungi with the drug of interest, and incubating the flasks at room temperature for approximately 12-15 days. Biotransformation was checked on TLC plates at days 3, 7 and 12. New bands other than the one representing the parental drug exemestane appeared at different positions on the TLC depending on the functional group that has changed during the process of biotransformation. Fungi showing the most promising results were the ones used in the large scale. The aim of a small scale trial is first to choose the fungi (here *Fusarium lini* and *Macrophomina phaseolina*), second to determine the incubation time required so that the drug is fully biotransformed to new metabolites. In addition, the solvent system to be used for TLC throughout the whole project is determined in the small scale trial. This solvent mixture is chosen based on its polarity and its ability to give clear separate bands.

1. Culture Medium

Biotransformation was carried out using two fungal strains *Fusarium lini* and *Macrophomina phaseolina.* The fungi were obtained as a gift from the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. The fungi were grown in water based medium prepared by mixing glucose (40.0 g), glycerol (40.0 mL), peptone (20.0 g), yeast extract (20.0 g), KH_2PO_4 (20.0 g), and NaCl (20.0 g)

in distilled water (4.0 L). After preparation, media was distributed into 40 conical flasks (per fungus) and then the flasks were autoclaved at 121°C (Choudhary *et al.* 2012).

2. Inoculation

Spores of fungi grown on Sabouraud dextrose agar were inoculated into the autoclaved flasks containing media. The flasks were then incubated at room temperature on a shaker for three days for the fungi to grow.

3. Drug Feeding

The fungal strains were fed with the drug exemestane by dissolving 1g in 40 mL methanol and distributing it equally to the 40 flasks. Two control experiments were also conducted, in which the drug was only added to the media in one, and in the other fungi were grown in media without adding exemestane. All experimental flasks were then kept for fermentation on the shaker for 12 days.

4. Thin Layer Chromatography (TLC)

TLC is a technique used to separate compounds in a mixture. It is performed on sheets of glass or aluminum foil coated with a thin layer of silica gel referred to as the stationary phase. Thin-layer chromatography is used to monitor the progress of a reaction, identify compounds present in a mixture, and determine the purity of a substance. Compounds in a mixture travel at different rates depending on their attraction to the stationary phase and their solubility in the mobile phase. When silica gel, considered polar, is used as stationary phase polar compounds exhibit a strong interaction with the silica and dispel the mobile phase from the binding sites. As a result, less polar compounds move higher up the plate. On the other hand, the higher the

solubility of the compounds in the mobile phase, the higher they move across the TLC plate (Mangold 1964). The solvent used as mobile phase in this project was a mixture of 80% PE and 20% acetone.

5. Extraction

Fungal cultures from all 40 flasks were filtered and extracted with dichloromethane (DCM) CH_2Cl_2 by liquid-liquid extraction. DCM's volatility and ability to dissolve a wide range of organic compounds makes it a useful solvent for many chemical processes.

Liquid–liquid extraction is a method to separate compounds based on their solubilities in two immiscible liquids. In other words it is the extraction of a substance from one liquid to the other. The liquid rich in solutes is called the extract while the depleted solution is called the raffinate (Figure 3). We used around 12 L of dichloromethane (per fungus) for the extraction. Dichloromethane was then evaporated using the rotary evaporator and the obtained gum was analyzed by thin-layer chromatography that showed many bands representing the different metabolites resulting from the biotransformation of exemestane.

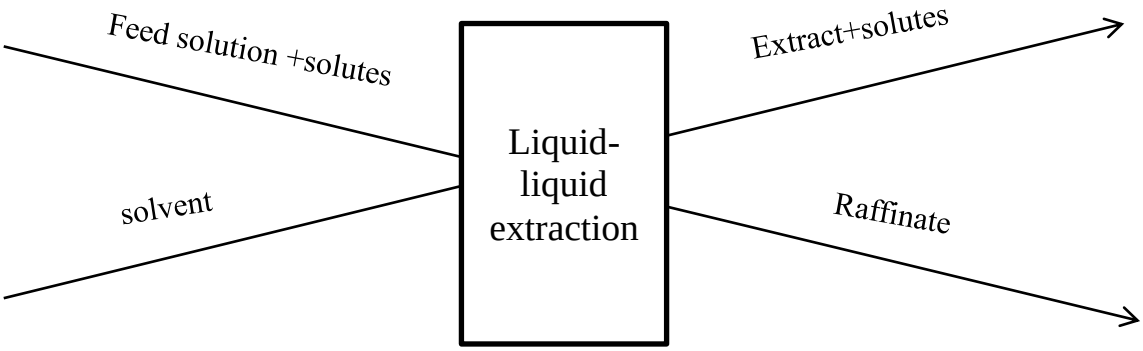


Fig. 3. Schematic representation of liquid-liquid extraction

6. Purification

a. Column Chromatography

The gum was fractionated using silica gel column chromatography in order to separate the metabolites from each other. The mobile phase was composed of petroleum ether and acetone, the same mixture used in thin layer chromatography (for both fungal strains). We started with 90% PE and 10% acetone and gradually increased the percentage of acetone 5% at a time. The eluted material for each specific percentage of petroleum ether and acetone was collected in a separate flask. TLC taken for all flasks showed one or two metabolites maximum in each flask. Some of the flasks had the same metabolite so we combined them in a single flask while those having two needed further purification (Choudhary *et al. 2012*).

b. High Performance Liquid Chromatography (HPLC)

In order to separate the metabolites present in the same flask and to further purify the samples, HPLC was performed. This technique relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts differently with the adsorbent material, causing different flow rates for the components and leading to the separation of the components as they flow out the column. The samples were subjected to repeated recycling HPLC reverse phase column H-80, L-80/ size exclusion column GS-320/1H, 2H. A mixture of 70 % MeOH and 30% water was used as mobile phase in reverse phase recycling HPLC, and methanol/chloroform in size exclusion HPLC. The flow rate was maintained at 4 mL/min, and peaks were monitored by UV 254 nm. The retention time (t_R) was then calculated according to their elution (Choudhary *et al. 2012*).

7. Nuclear Magnetic Resonance Spectroscopy

The final step is the identification of the chemical structure of the biotransformed metabolites and therefore the reactions achieved in the fungal systems. For this purpose, our samples were subjected to NMR analysis. This research technique relies on the nuclear magnetic resonance to determine the chemical and physical properties of atoms and the molecules in which they are found. It gives detailed information on the structure, the chemical environment of the molecule, the dynamics and the reaction state. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus gives access to details of the electronic structure of a molecule.

HPLC and NMR analysis were carried out in Pakistan in Dr Iqbal Choudhary's labs at the ICCBS (Choudhary *et al. 2012*).

B. Cell Viability Assay

1. Cell Lines

PC3 (prostate cancer) and Hela (cervical cancer) cell lines were used for testing the cytotoxicity of the biotransformed metabolites. These cell lines were purchased from the American Type Culture Collection (ATCC) for anticancer activity. Both cell lines (PC-3 and HeLa) were cultured in DMEM media supplemented with FBS (5%), pencillin (100 IU/mL) and streptomycin (100 mg/mL). The cells were grown in 5% CO2 humidified incubator at 37°C.

2. Cell Culture

Cells were split every three days at a ratio of 1:4, and the culture medium was changed every two days. The viability of the cells was determined by the trypan blue

dye exclusion method, and their number was counted using a hemocytometer. Cells were then seeded into 96-well plates at a density of 5.10^4 cells/mL and incubated for 24h in a 5% CO2 incubator at 37°C.

3. Cytotoxicity of the Metabolites

The cytotoxicity of the metabolites was determined using MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay that uses NAD(P)H-dependent cellular oxidoreductase enzymes, found in living cells, to reduce the tetrazolium dye to the purple formazan. After the 24h, cells were treated with different concentrations of metabolites (5-50 µM) and were incubated again. 48 h later, 200 µL of MTT (0.5 mg/mL) was introduced into each well and the plates were further incubated for 4 h. Finally, DMSO (100 µL) was added to the wells to dissolve formazan. The extent of MTT reduction to formazan was quantified by measuring the absorbance at 540 nm, using a micro plate reader (Spectra Max plus, Molecular Devices, CA, USA). The percent inhibition was calculated using the following formula:

% Inhibition = 100 – [(Absorbance of test compound-Absorbance of blank)/(Absorbance of control-Absorbance of blank)*100].

The IC_{50} is a value that indicates how much of the drug (exemestane) is needed to inhibit a given biological process by half (slowing the growth of cancer). In other words, it is the half minimal (50%) inhibitory concentration (IC) of a substance. By plotting the percent inhibition v/s log concentration an S shaped curve appears. The concentration at which the curve passes through the 50% inhibition level is the IC50 value (Sebaugh 2011). Figure 4 represents an outline for the techniques used in the production and identification of the biotransformed metabolites.

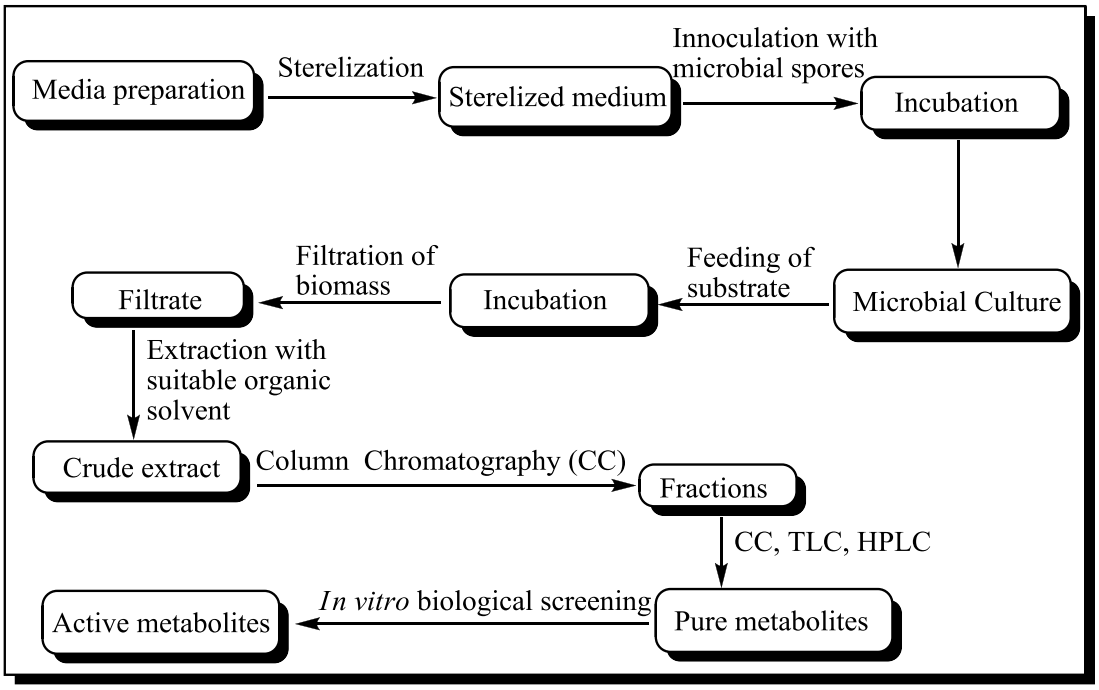


Fig. 4. Summary of the biotransformation and purification protocols

CHAPTER III

RESULTS AND DISCUSSION

Fungal steroid-transforming enzymes belong to different protein families; they are mainly membrane bound, and represent important biotechnological tools and therapeutic targets. These enzymes have been less intensively studied over the last few years because the whole fungal cell or cell fractions are being used as biocatalysts. Biotransformation in whole cells has many disadvantages since cells have a vast number of enzymes and the formation of by-products is very common. Thus, over-expression of the desired enzymes in the appropriate expression organisms would be a better approach, as this will provide higher yields and fewer by-products.

A. Biotransformation of Exemestane (1) by *Fusarium lini*

Fusarium lini is known to catalyze the oxidation at C-1, C-2, C-6, and C-11 of the steroidal skeleton (Al-Maruf *et al.* 2011). In this project, biotransformation of exemestane *(1)* using *Fusarium lini* yielded one metabolite which is 11α-Hydroxy-6-methylene-androsta-1,4-diene-3,17-dione *(2)* (Figure 5). The transformation occurred at C11 where a hydrogen atom was substituted by a hydroxyl group via a hydroxylase enzyme (hydroxylation reaction).

11α-Hydroxy-6-methylene-androsta-1,4-diene-3,17-dione (2): Amorphous material; $[\alpha]^{25}_{D}$: +81.4 (c = 0.096, MeOH); IR (KBr): νmax 3408, 1657 cm-1; UV(MeOH): λmax nm (log ε) 247 (3.78); H1- and C13- NMR see Tables 2 and 3.

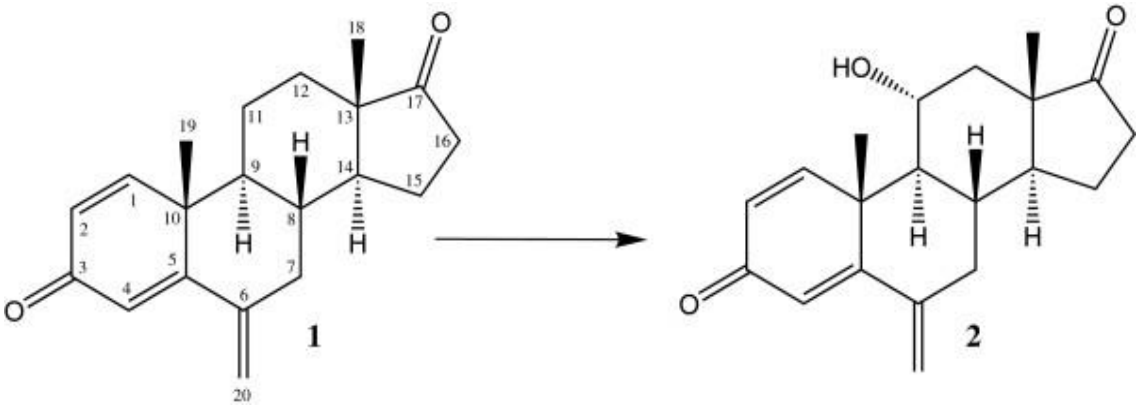


Fig. 5. Biotransformation of exemestane (1) by Fusarium lini yielded metabolite

Table 2. ^{13}C-NMR data of compounds 1–5 in ppm

COMPOUNDS					
Carbon	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[d]
1	155.0	154.9	158.1	157.6	158.2
2	128.0	128.0	127.8	127.9	127.7
3	185.8	185.8	188.7	188.6	188.7
4	122.9	122.9	122.7	122.8	122.7
5	167.9	167.9	171.6	171.2	171.7
6	147.1	147.0	147.7	147.3	147.8
7	39.87	32.1	41.4	41.2	41.3
8	36.0	39.6	36.6	35.9	37.2
9	50.9	48.6	51.9	51.4	51.7
10	44.3	44.3	45.6	45.5	45.6
11	22.63	71.5	23.0	23.2	23.6
12	32.0	32.0	38.1	36.6	37.5
13	48.1	48.1	43.7	43.5	44.2
14	51.3	50.8	48.2	45.4	51.8
15	22.3	22.3	35.9	36.6	24.3
16	35.8	39.7	70.6	217.7	30.5
17	218.8	218.0	81.7	86.8	82.1
18	13.9	14.5	12.5	11.9	11.6
19	20.1	20.1	20.1	20.1	20.1
20	112.2	112.2	112.6	112.9	112.4

[a] 125 MHz $(CD_3)_2CO$. [b] 150 MHz $(CD_3)_2CO$. [c] 125 MHz CD_3OD. [d] 75 MHz CD_3OD.

Table 3. ^{1}H-NMR data of compounds 1–5 in ppm, J in Hz

COMPOUNDS					
Carbon	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]
1	7.21 d (10.0)	7.21 d (10.5)	7.31 d (10.2)	7.34 d (10.5)	7.31 d (10.2)
2	6.14 dd (10.5, 2.0)	6.12 dd (10.5, 2.0)	6.21 dd (10.2, 1.8)	6.42, dd (10.5, 2.0)	6.22 dd (10.2, 1.8)
3	-	-	-	-	-
4	5.99, d (2.0)	6.00 d (2.0)	6.08 d (1.8)	6.11 d (2.0)	6.09 d (1.8)
5	-	-	-	-	-
6	-	-	-	-	-
7	2.69, 1.97 m	2.13, 1.28 m	2.56d (9.0), 1.87 m	2.57, 1.96 m	2.66, 1.82 m
8	2.03 m	1.98 m	1.86 m	1.98 m	1.83 m
9	1.34 m	1.62 m	1.35 m	1.48 m	1.05 m
10	-	-	-	-	-
11	1.92, 1.76 m	4.30 m	1.91, 1.31 m,	1.93, 1.84 m	1.75, 1.83 m
12	1.78, 1.28 m	2.13, 1.28 m	1.90, 1.16 m	2.01, 1.45 m	1.13, 1.92 m
13	-	-	-	-	-
14	1.43 m	1.37 m	0.93 m	1.63 m	1.27 m
15	1.99, 1.67 m	1.93, 1.80 m	2.20, 1.30 m	2.29, 1.95 m	1.65, 1.38 m
16	2.41, 1.95 m	2.66, 1.95 m	4.07 m	-	2.00, 1.51 m
17	-	-	3.30 d (7.5)	3.77 s	3.55 t (8.7)
18	0.92 s	0.99 s	0.99 s	0.81 s	0.81 s
19	1.19 s	1.18 s	1.18 s	1.21 s	1.17 s
20	5.03, 5.01 s	5.04, 5.02 s	5.02 t (1.9)	5.06, 5.04 s	4.99, 5.01 s

[a] 500 MHz $(CD_3)_2CO$. [b] 300 MHz CD_3OD.

The molecular formula $C_{20}H_{24}O_3$ [M+, m/z 312] of metabolite *2* was deduced from the HREI-MS (M+ m/z312.1705) (Appendix I, Figure 1), suggesting the addition of an oxygen to exemestane. The 1H-NMR spectral analysis of metabolite *2* displayed a downfield methine signal, as compared to the starting material exemestane, resonating at δ 4.30 (m, W1/2 = 15.6 Hz) (Table 3), while its respective carbon signal was at δ 71.5 in ^{13}C-NMR spectrum (Table 2). The HMBC spectrum displayed long-range couplings of the hydroxyl-bearing methine proton (δ 4.30) with C-9 (δ 48.6), C-10 (δ 44.3), and C-13 (δ 48.1), which suggested the position of the hydroxyl-bearing methine at C-11(Appendix I, Figure 3). H-11 also showed COSY cross peaks with H-9 (δ 1.62) and

H2-12 (δ 1.28, 2.13) (Appendix I, Figure 4). The stereochemical assignments were based on NOESY interactions between H-11 (δ 4.30), H-8 (δ 1.98), and Me-19 (δ 1.18) (Appendix I, Figure 5). H-11 was thus deduced as β-oriented. Metabolite *2* was finally identified as 11α-hydroxy-6-methyleneandrosta-1,4-diene-3,17-dione.

B. Biotransformation of Exemestane by *Macrophomina phaseolina*

Macrophomina phaseolina is previously known to catalyze the introduction of a double bond between C-1 and C-2, a hydroxyl group at C-6, C-15, C-16 and C-17, and a carbonyl group at C-17 of the steroidal skeleton (Choudhary *et al.* 2012). In this project, biotransformation of exemestane by *Macrophomina phaseolina* yielded three metabolites. In case of metabolite *3*, the transformation occurred at C16 and C17. At C 16 a hydrogen atom was substituted by a hydroxyl group via a hydroxylase enzyme and at C17 the carbonyl group was reduced to a hydroxyl group via a reductase enzyme. In case of metabolite *4*, the change also occurred at C16 and C17, where the carbonyl group at C17 was shifted to C16 by a mutase enzyme and substituted by a hydroxyl group via a hydroxylase. Concerning metabolite *5*, the change only occurred at C17 where the carbonyl group was reduced to a hydroxyl group by a reductase enzyme. Noteworthy, metabolites *2, 3* and *4* are new metabolites reported for the first time in this project; however, metabolite *5* was previously reported.

16β, 17β-Dihydroxy-6-methylene-androsta-1,4-diene-3-one (3): Amorphous material; $[\alpha]^{25}_{D}$: +181.6 (c =0.032, MeOH); IR (KBr): νmax 3388, 1658 cm^{-1}; UV(MeOH): λmax nm (log ε) 249 (4.03); H1- and ^{13}C-NMR:see Tables 2 and 3.

17β-Hydroxy-6-methylene-androsta-1,4-diene-3,16-dione (4): Amorphous material; $[\alpha]^{25}_{D}$: -56.0 (c = 0.043, MeOH); IR (KBr): νmax 3411, 1749, 1658 cm^{-1}; UV(MeOH): λ max nm (log ε) 247 (4.04); ^{1}H- and ^{13}C- NMR: see Tables 2 and 3.

17β-Hydroxy-6-methylene-androsta-1,4-diene-3-one (5): Amorphous material; $[\alpha]^{25}_{D}$: +174.5 (c = 0.046, MeOH); IR (KBr): νmax 3421, 1657, cm^{-1}; UV (MeOH): λ max nm (log ε) 248 (4.24);[1] H- and ^{13}C-NMR: see Tables 2 and 3.

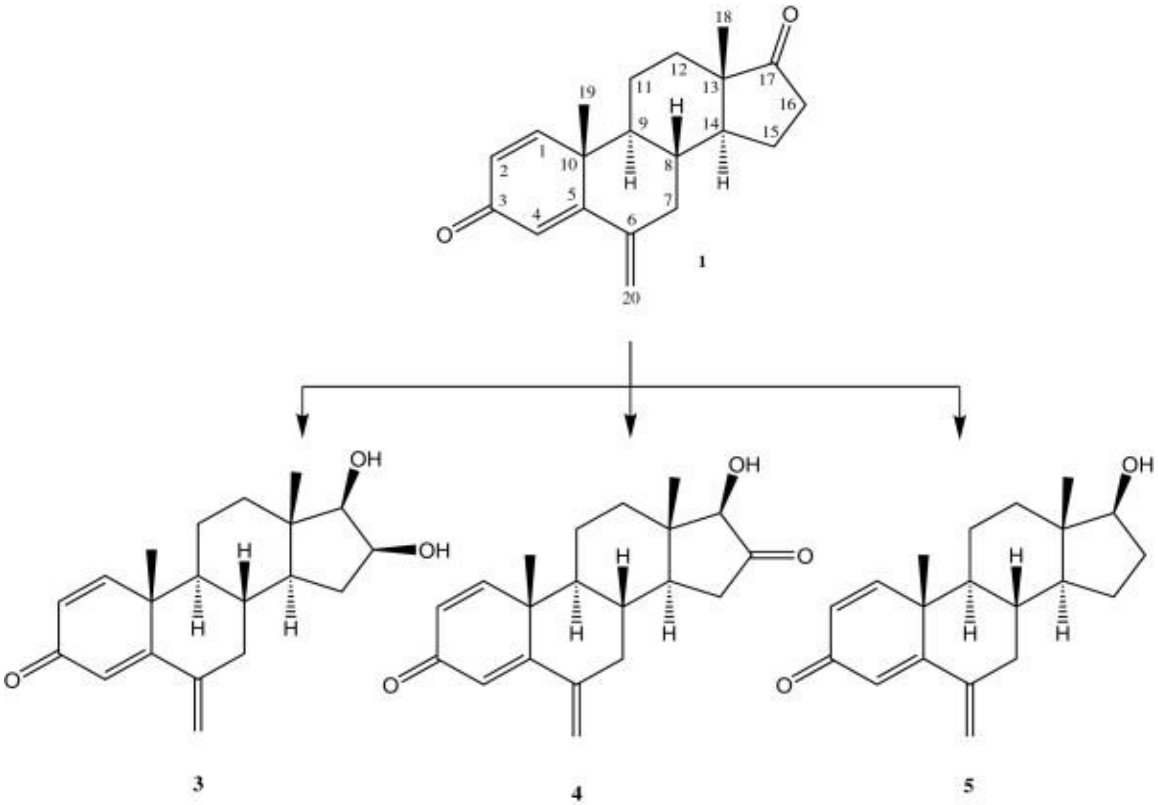


Fig. 6. Biotransformation of exemestane *(1)* by *Macrophomina phaseolina* yielded metabolites *3, 4* and *5*.

Biotransformation of exemestane by *Macrophomina phaseolina* yielded three metabolites (*3, 4* and *5*). The molecular composition of metabolite **(***3***)** $C_{20}H_{26}O_3$ was deduced from the HREI-MS analysis (M+ = m/z 314.1933, calcd 314.1882) (Appendix I, Figure 6). The ^{1}H-NMR spectra μm of this metabolite showed two hydroxyl-bearing methine proton peaks at δ 3.30 (d, J17,16 = 7.5 Hz, H-17) and 4.07 (m, W1/2 = 20.0 Hz) (Table 3). Its ^{13}C-NMR spectrum lacks signal for C-17 carbonyl, whereas new

methine carbon at δ 81.7 suggested the reduction of C-17 ketone into C-17 OH (Table 2). The proton geminal to the –OH group (δ 4.07) was correlated with C-13 (δ 43.7), C-14 (δ 48.2) and C-17 (δ 81.7) in the HMBC spectrum (Appendix I, Figure 8). The methine C-17 (δ 81.7) showed HMBC correlations with H-14 (δ 0.93, m) and H-18 (δ 0.99, s). Based on the above observations, the hydroxyl-bearing methine carbon was identified as C-16. The H-16 (δ 4.07) showed NOESY cross peaks with H-14 (δ 0.93), but no interaction with H-18 (δ 0.99) (Appendix I, Figure 10). Therefore the C-16 proton was assigned to be α-oriented. Metabolite *3* was thus identified as 16β, 17β-dihydroxy-6-methylene-androsta-1, 4-diene-3-one.

The Molecular formula $C_{20}H_{24}O_3$ of metabolite *4* (M+ m/z 312.1725, calcd 312.1720) was deduced from its HREI-MS (Appendix I, Figure 11). A distinct downfield methine proton signal appeared at δ 3.77 (br. s, W1/2 = 9.3 Hz) in the ^{1}H-NMR spectrum of this metabolite (Table 3). The ^{13}C-NMR spectrum showed a saturated ketone carbon signal at δ 217.7(Table 2). The rest of the spectrum was distinctly similar to metabolite *2*. The deshielded methine proton was HMBC correlated with this ketonic carbon, while its corresponding methine carbon at δ 86.8 showed HMBC correlations with H2-15 (δ 1.95, 2.29), and CH3-18 (δ 0.81)(Appendix I, Figure 13). These interactions, along with the appearance of a downfield proton (δ 3.77), indicated that the ketone at C-17 has been reduced into an –OH. Geminal H-17 (δ 3.77) showed NOESY correlations with H-14 (δ 1.63), indicating that it is axially (α-) oriented (Appendix I, Figure 15). The saturated ketone carbon (δ 217.7) was placed at C-16, based on the above mentioned HMBC correlations. The structure of metabolite *4* was finally identified as 17β-hydroxy-6-methylene-androsta-1, 4-diene-3, 16-dione.

Metabolite *5* has $C_{20}H_{26}O_2$ as molecular formula (HREI-MS, M+ m/z 298.1730, calcd 298.1733) (Appendix I, Figure 16). Based on ^{1}H- and ^{13}C-NMR

spectral data (Tables 2 and 3), compound *5* was identified as 17β-hydroxy-6-methyleneandrosta-1, 4-diene-3-one. It has been previously reported as a biotransformed product of exemestane mediated by an *in vitro* cytochrome P450 (Kamdem *et al.* 2011).

C. Cytotoxicity of the Metabolites against Cancer Cell Lines

The cytotoxicity of the metabolite is proportional to the number of viable cells able to reduce MTT to formazan. This means the darker the purple color, the less the cytotoxicity of the metabolite.

Compound 2 showed a moderate cytotoxicity against both cancer cell lines as compared to the standard drug, doxorubicin, and the parental drug exemestane with an IC50 of 16.8 ± 0.9 in Hela and 24.8 ± 0.7 μM in PC3 (Table 4). In both cell lines, the concentration of metabolite 2 is higher than that of doxorubicin (a standard drug used in cancer chemotherapy), however, still way below the concentration of exemestane needed to inhibit the growth of cancer by 50%. Compound 4 also exhibited a moderate activity against HeLa cell line with an IC50 of 37.2±0.8 (Table 4).

Table 4. In vitro cytotoxicity of compounds *1–5*

Compound Codes	**HeLa (Cervical cancer) (IC_{50}± S.D.) μM**	**PC-3 (Prostate cancer) (IC_{50} ±S.D.) μM**
1	>50	>50
2	16.8±0.9	24.8±0.7
3	>50	>50
4	37.2 ± 0.8	>50
5	>50	>50
Doxorubicin	**3.1 ± 0.2**	**0.9 ± 0.1**

D. Conclusion

Microbial transformation presents an important tool for the production of compounds that have the potential to be drug candidates and therefore help in the treatment of several diseases. The biotransformed products result from the action of fungal transforming enzymes on the parental drug. These enzymes are present in fungi mainly for detoxification and excretion of xenobiotics, to which the fungi are vulnerable. Biotransformation of exemestane using *fusarium lini* and *macrophomina phaseolina* yielded four metabolites; three of them are reported for the first time in this project while the last one was previously described. Upon testing the activity of the biotransformed metabolites against cancer cell lines only metabolite *2* showed moderate cytotoxicity. One of the important characteristics of an anti-cancer drug is being cytotoxic however; the cytotoxicity should be selective to cancerous cells and not to normal ones. Therefore, one of the future prospects would be to test the cytotoxicity of the biotransformed metabolites against normal cell lines. Another future approach would be to identify the enzymes acting on the parental drug exemestane and overexpressing them in the fungal strains in order to increase the yield and decrease the number of byproducts. Finally, the effect of the biotransformed metabolites on the aromatase enzyme could be further investigated to check if the biotransformed metabolites still exhibit the same inhibitory action on aromatase.

APPENDIX I

FIGURES

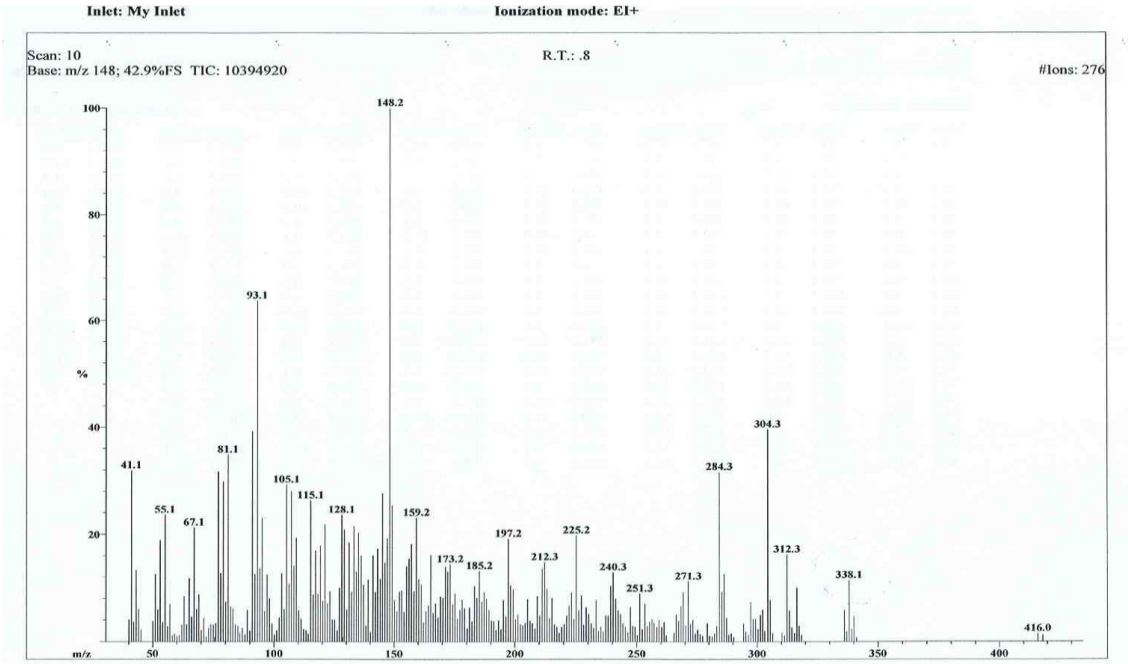


Fig. A1. Mass spectrum of metabolite *2*

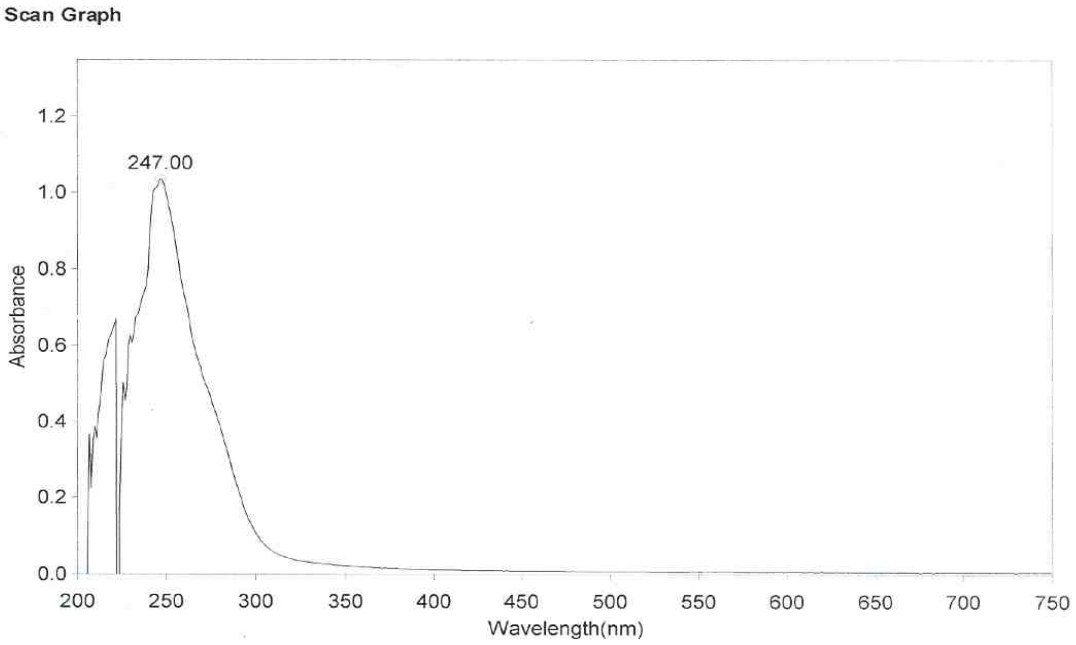


Fig. A2. UV spectrum of metabolite *2*

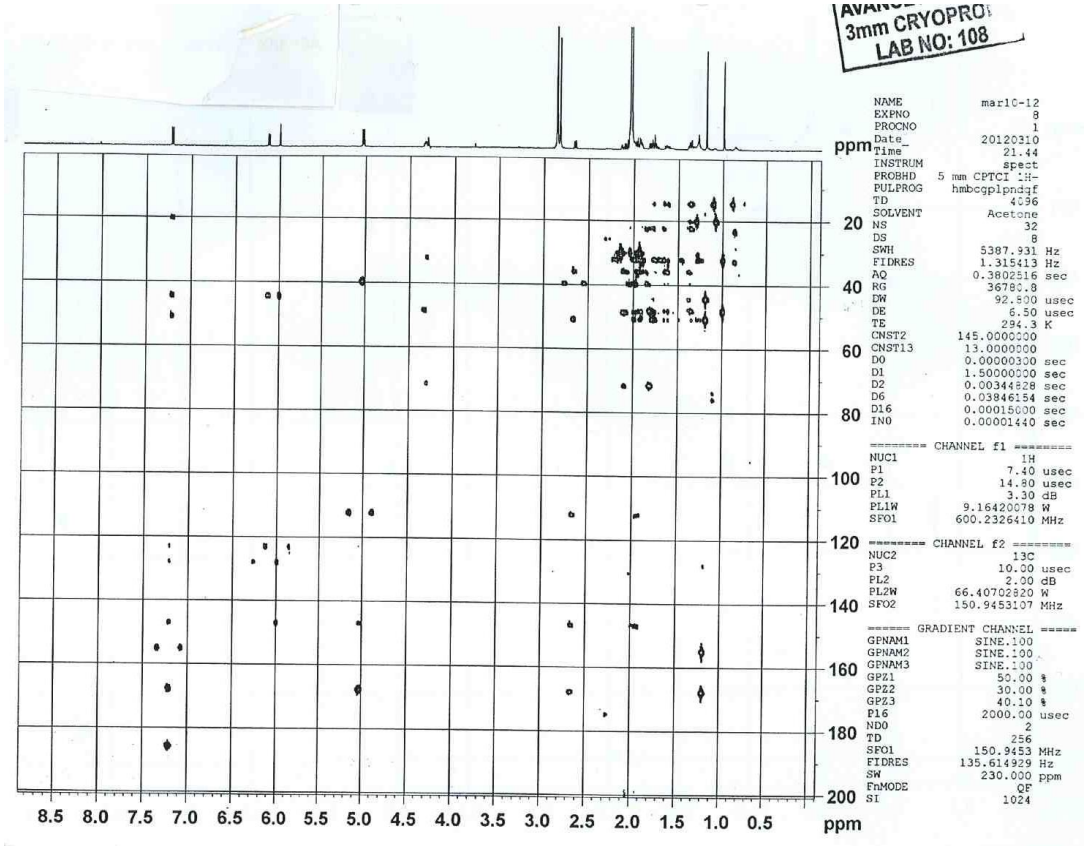


Fig. A3. HMBC spectrum of metabolite *2*

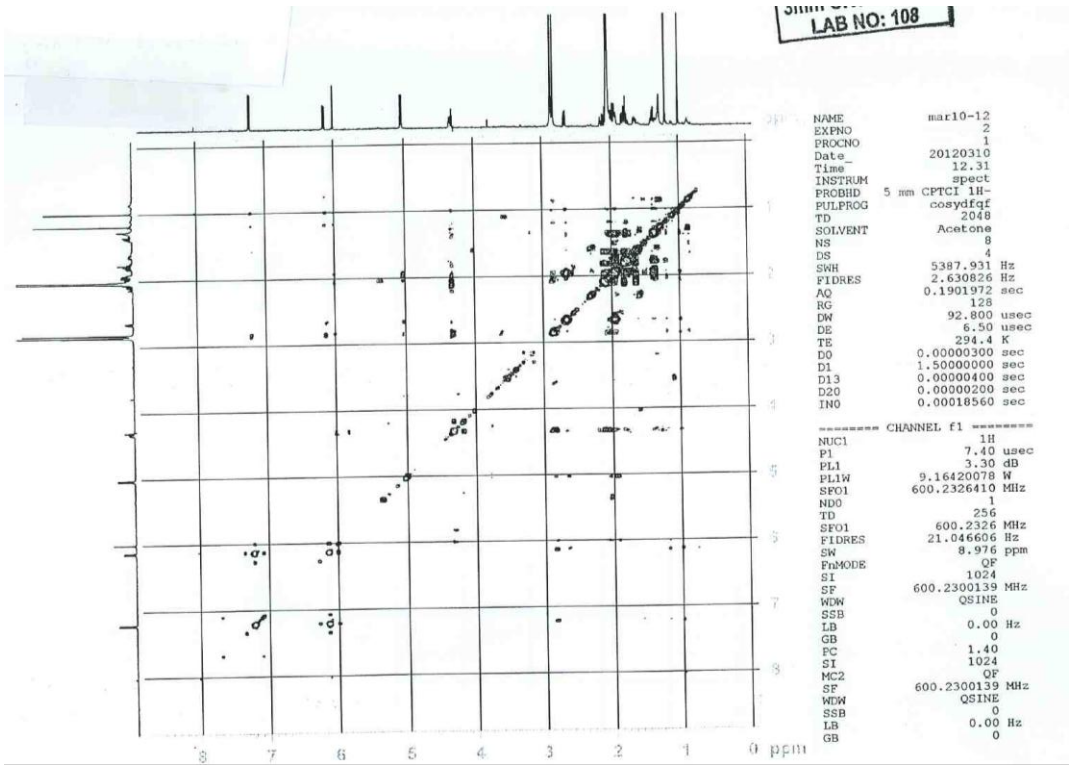


Fig. A4. COSY spectrum of metabolite *2*

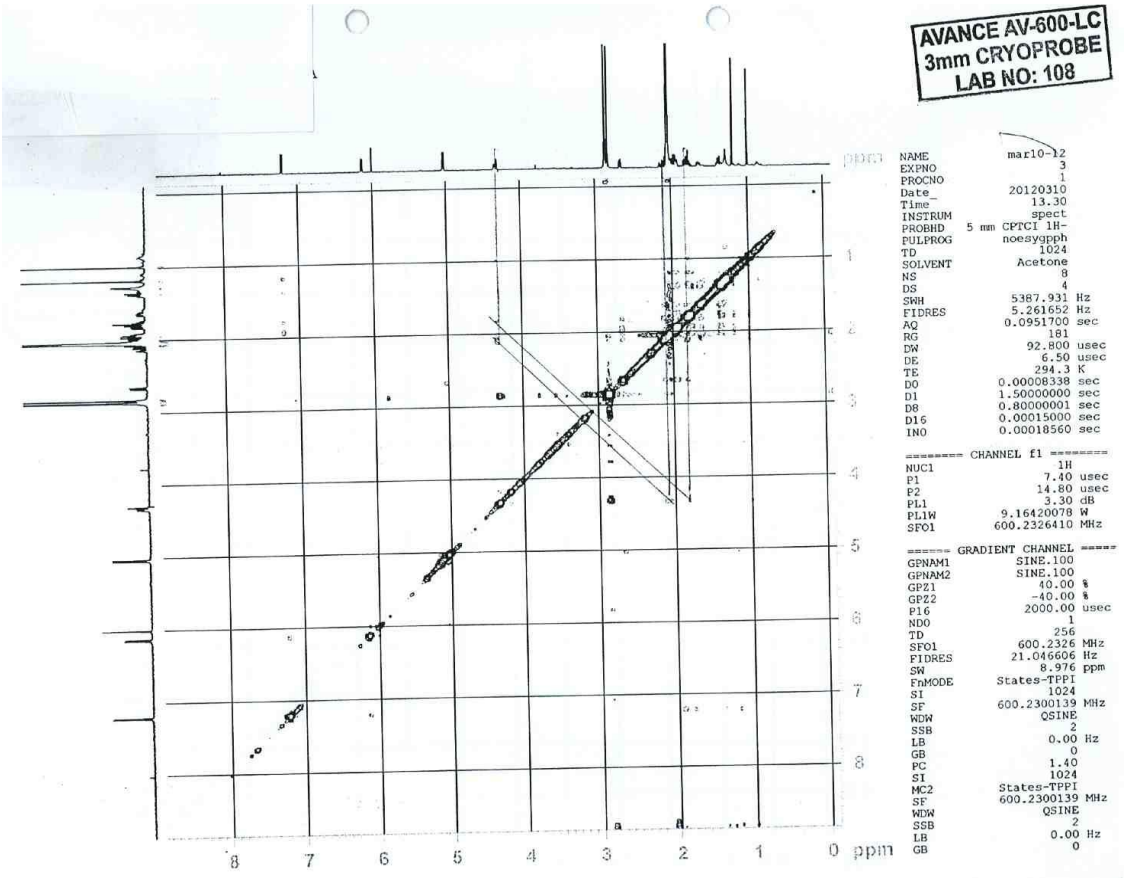


Fig. A5. NOESY spectrum of metabolite *2*

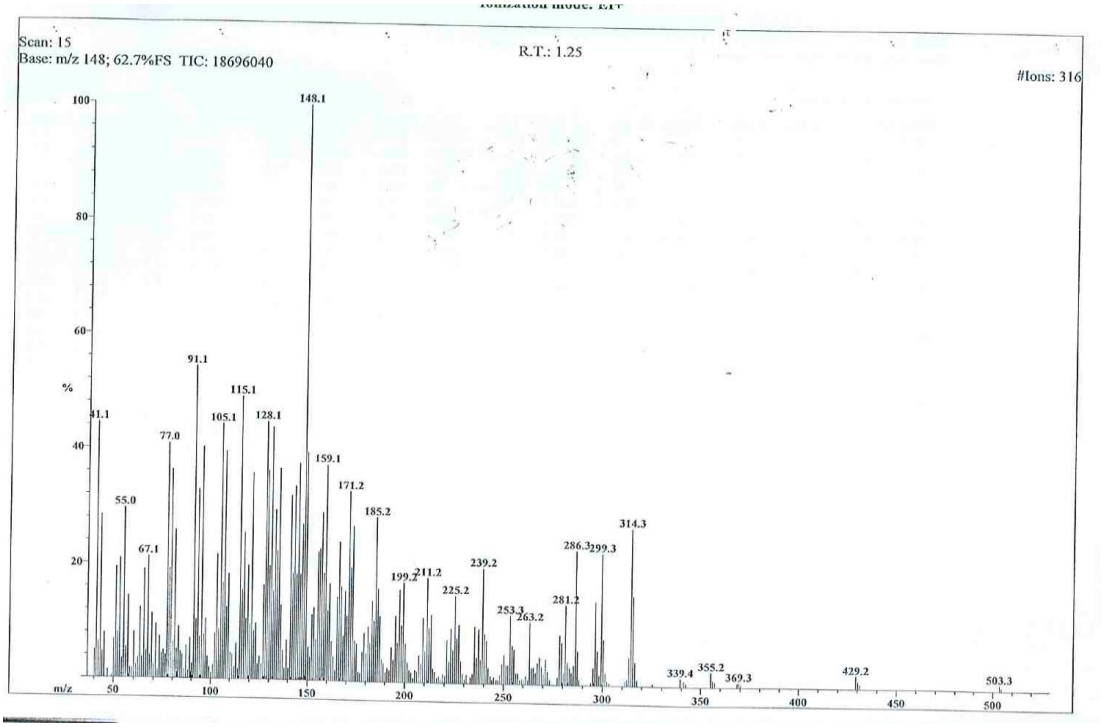


Fig. A6. Mass spectrum of metabolite *3*

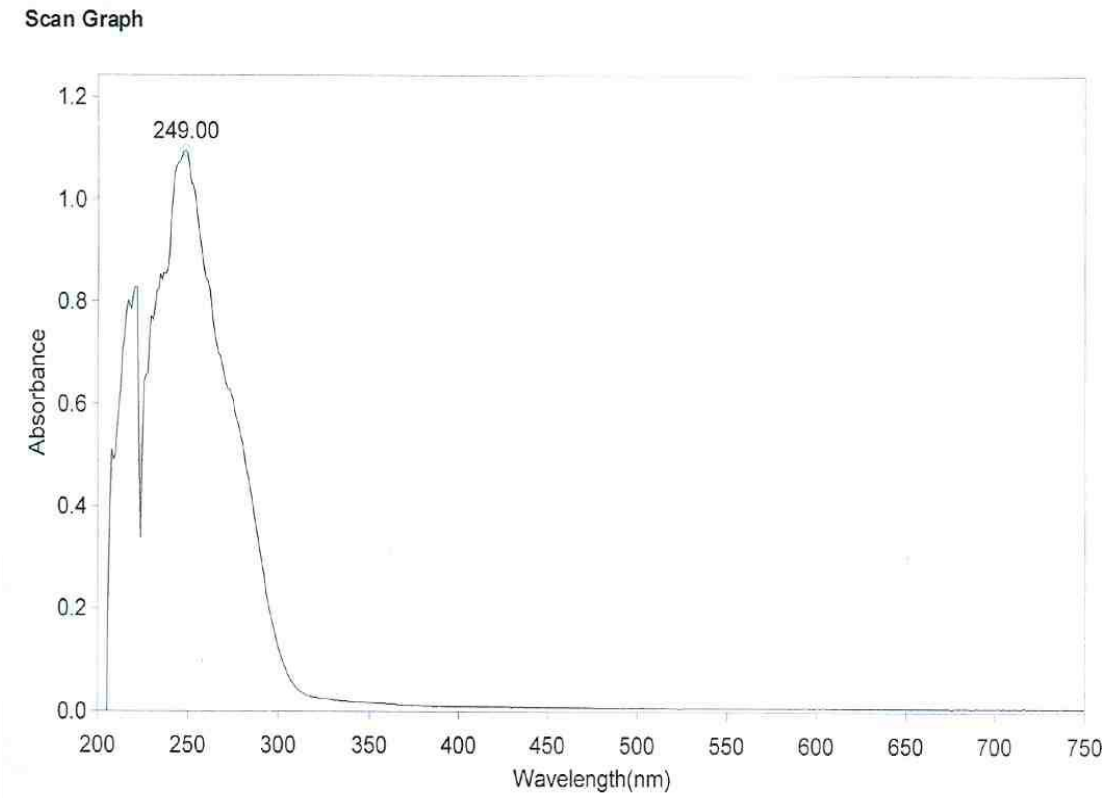


Fig. A7. UV spectrum of metabolite *3*

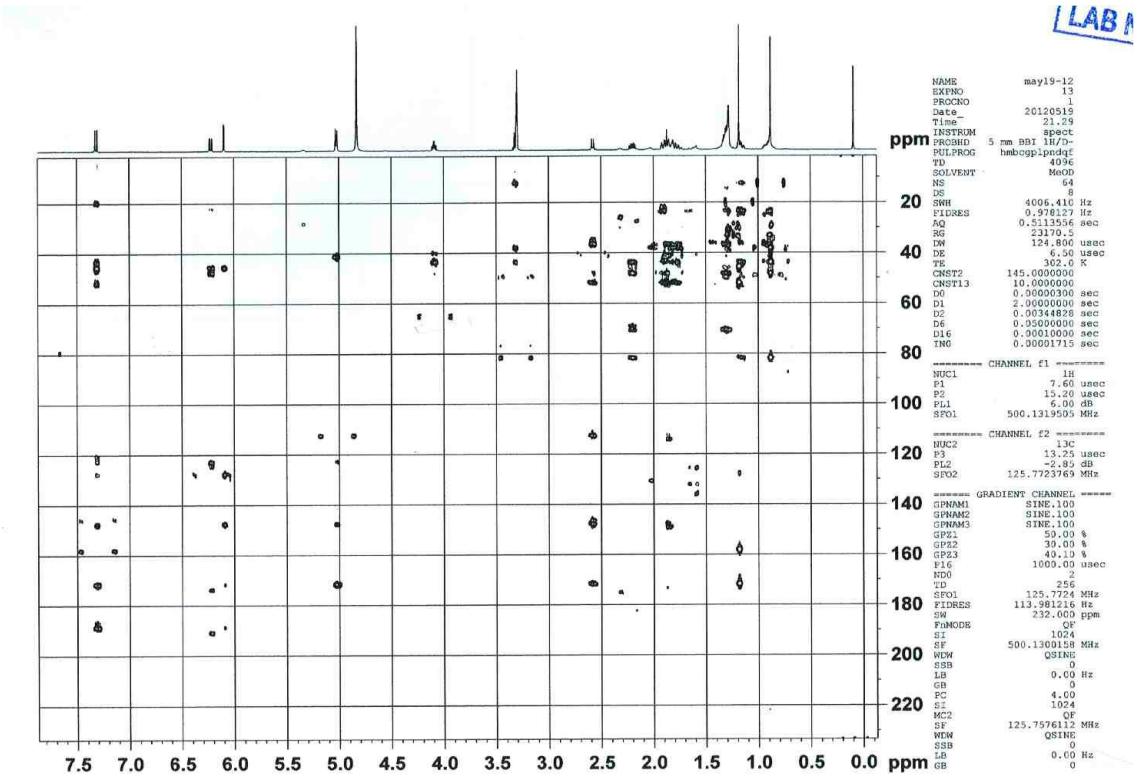


Fig. A8. HMBC spectrum of metabolite *3*

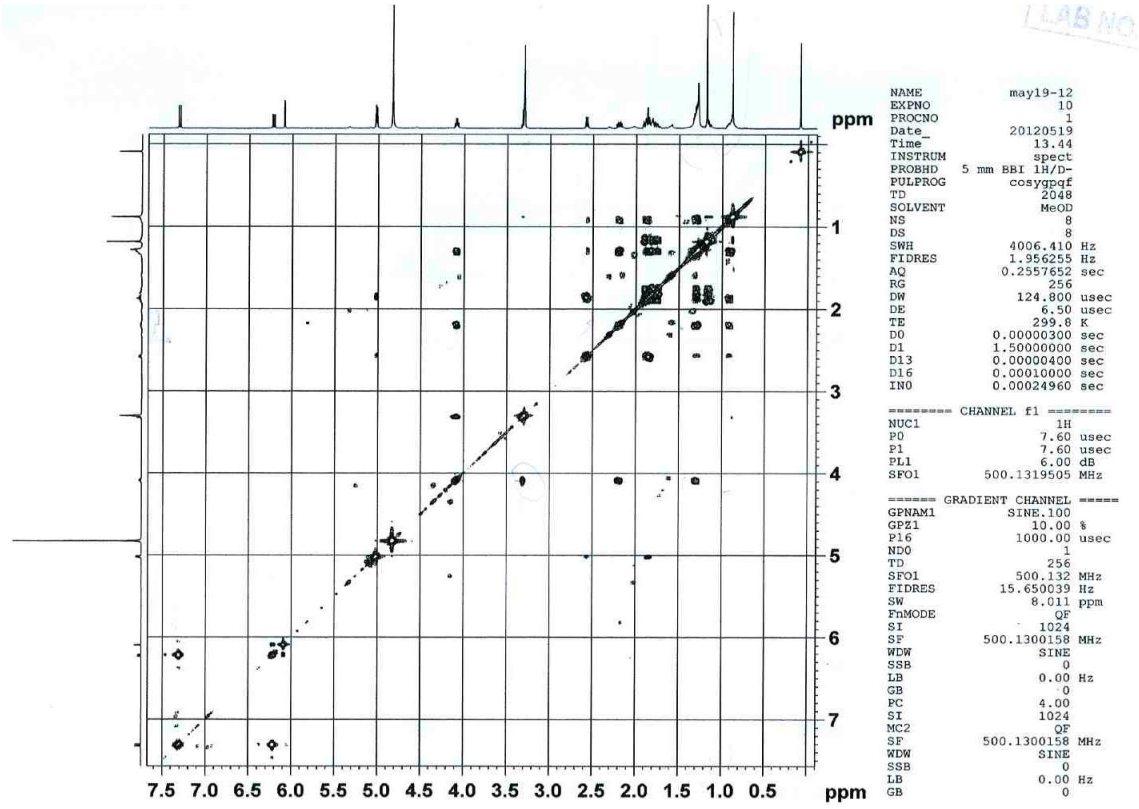


Fig. A9. COSY spectrum of metabolite *3*

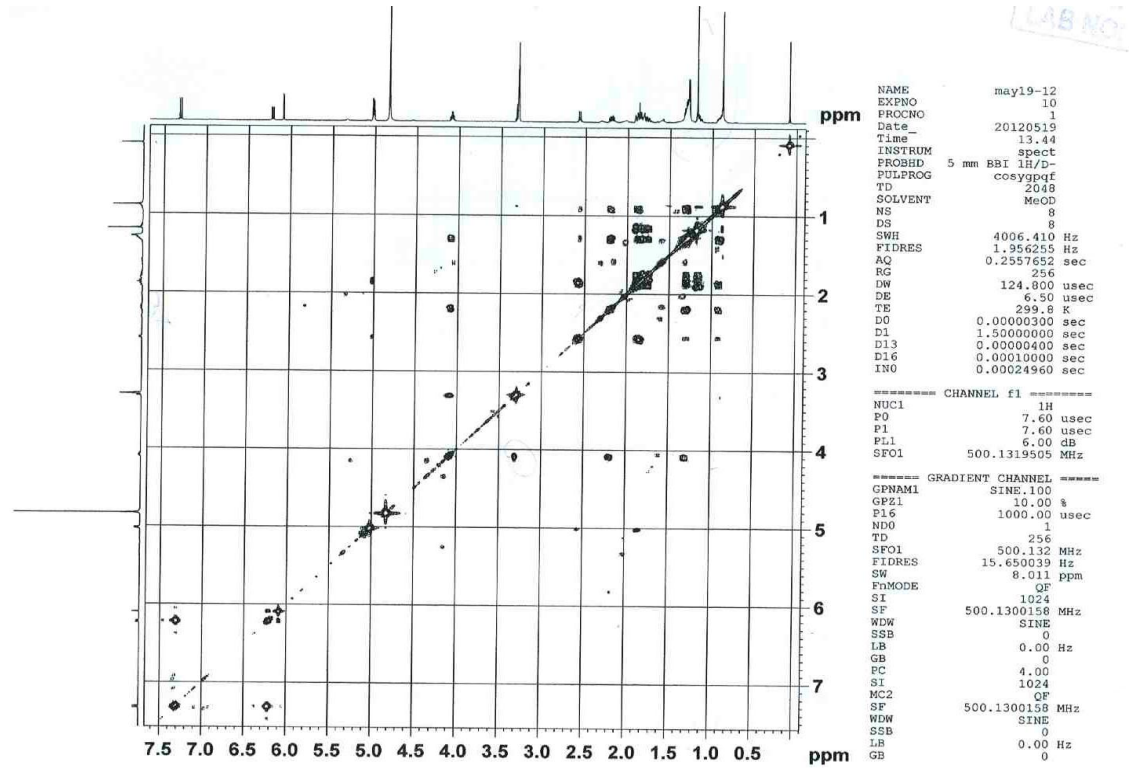


Fig. A10. NOESY spectrum of metabolite *3*

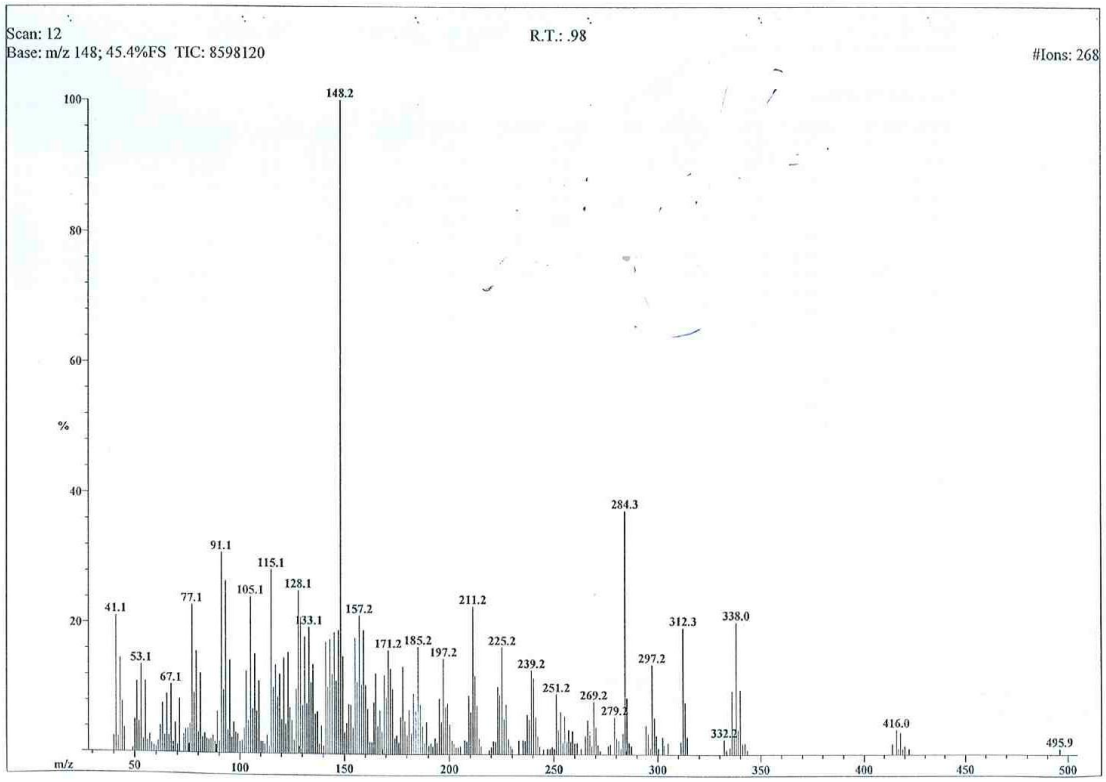


Fig. A11. Mass spectrum of metabolite *4*

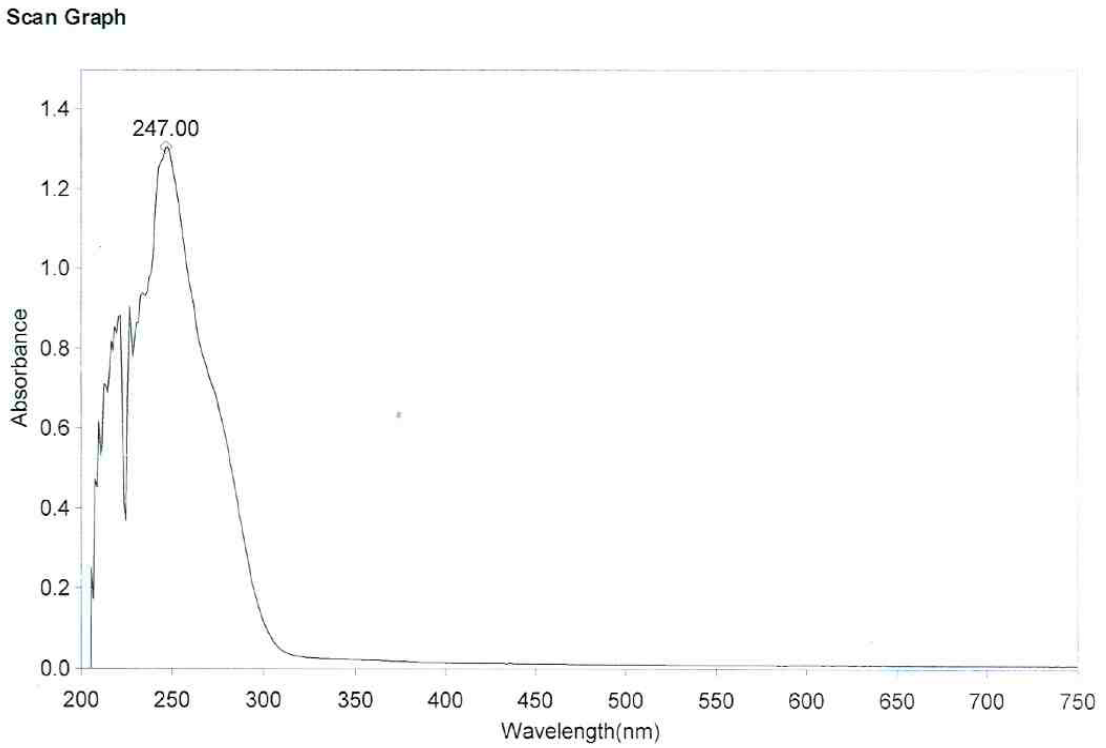


Fig. A12. UV spectrum of metabolite *4*

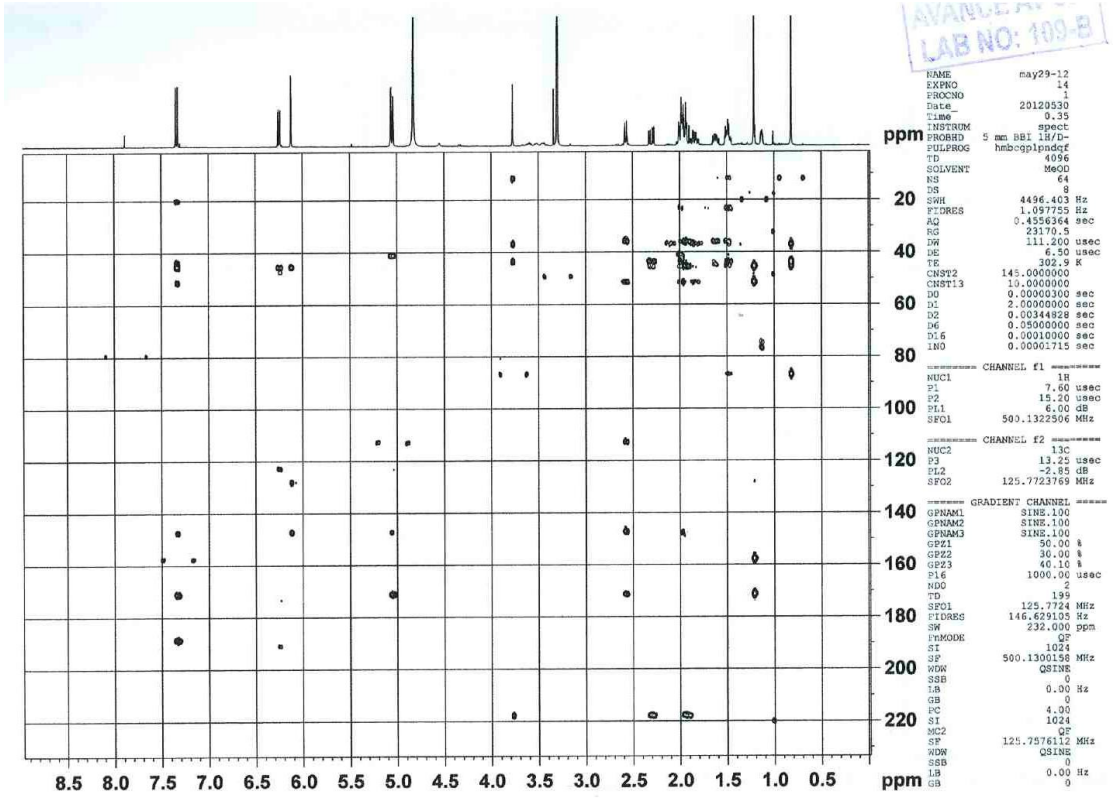


Fig. A13. HMBC spectrum of metabolite *4*

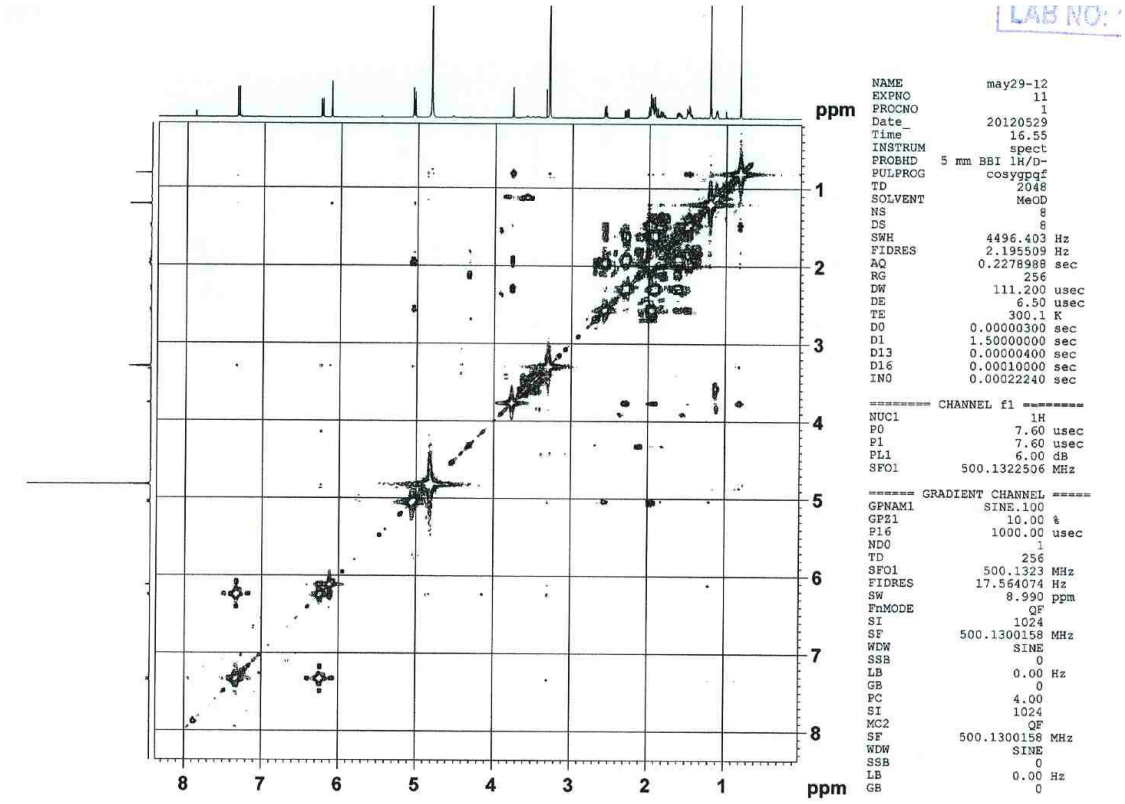


Fig. A14. COSY spectrum of metabolite *4*

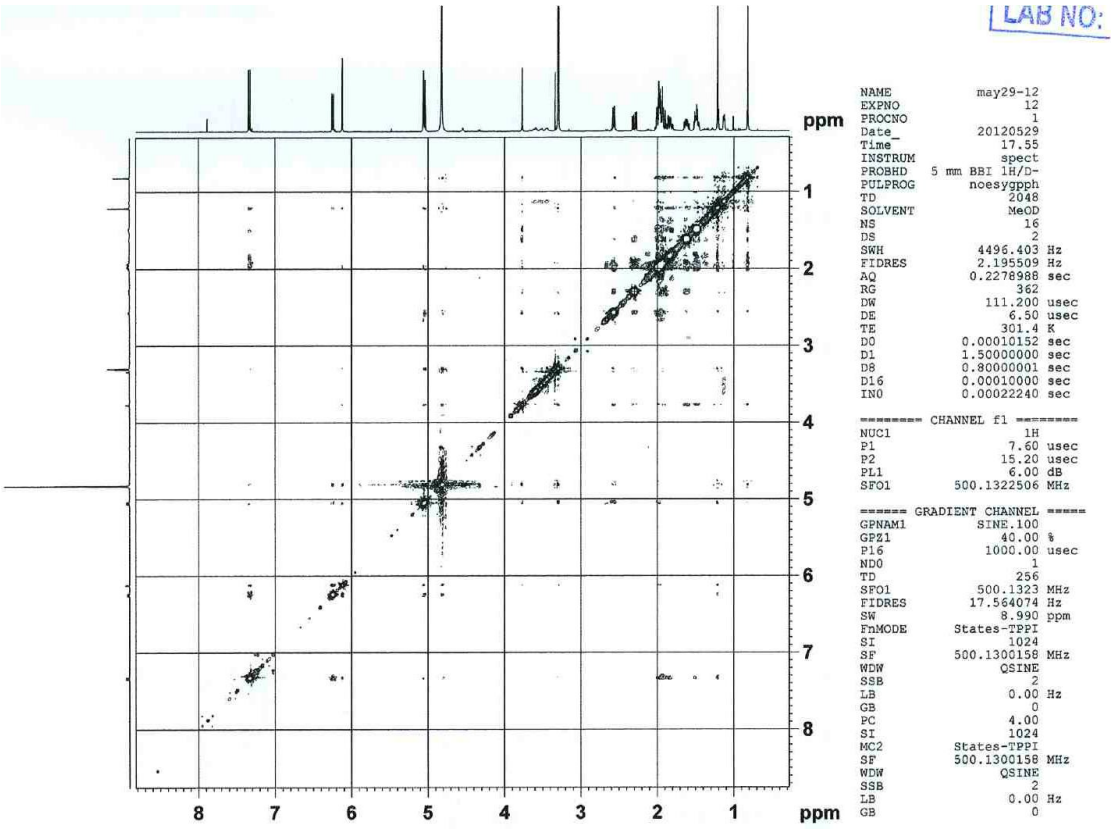


Fig. A15. NOESY spectrum of metabolite *4*

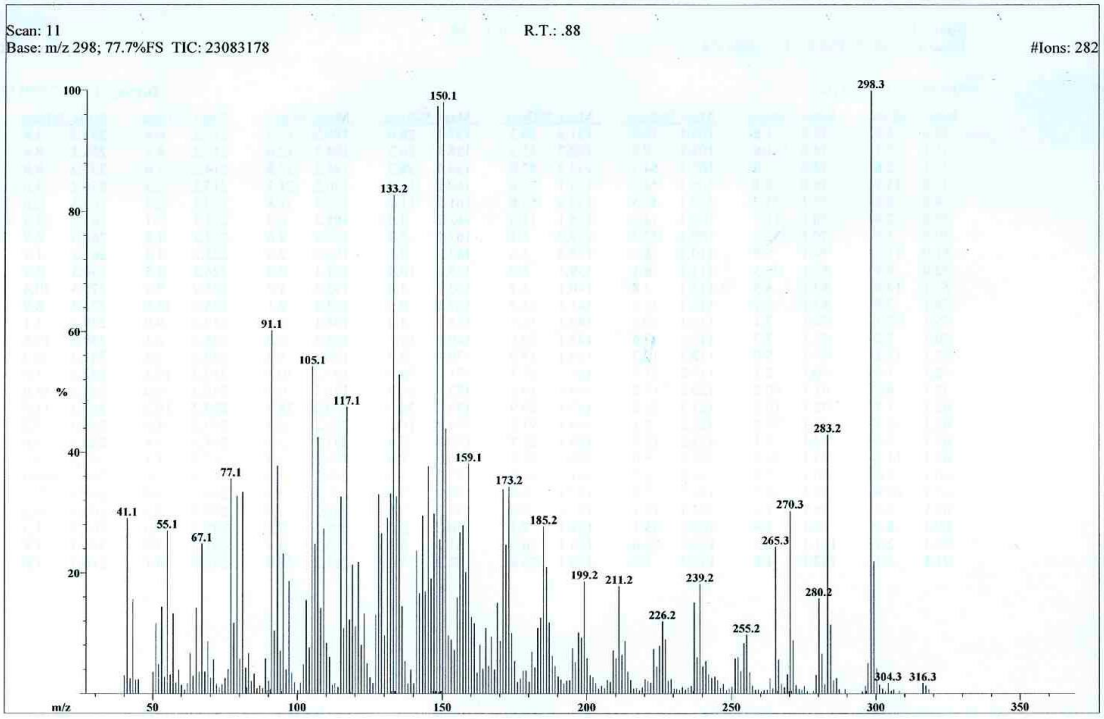


Fig. A16. Mass spectrum of metabolite *5*

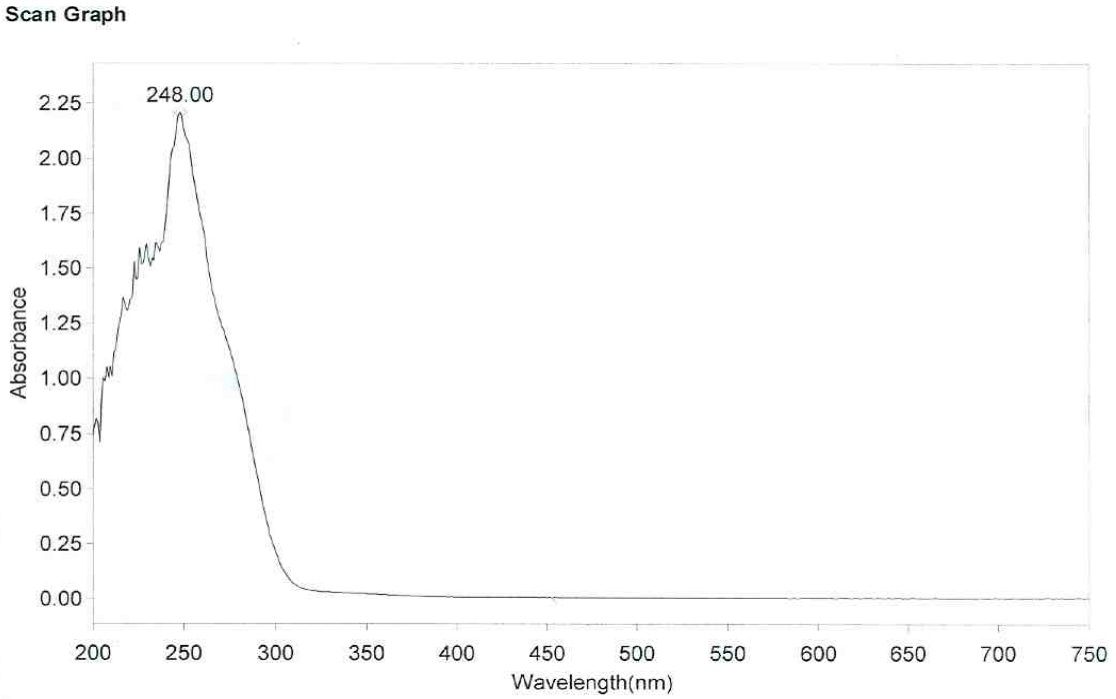


Fig. A17. UV spectrum of metabolite *5*

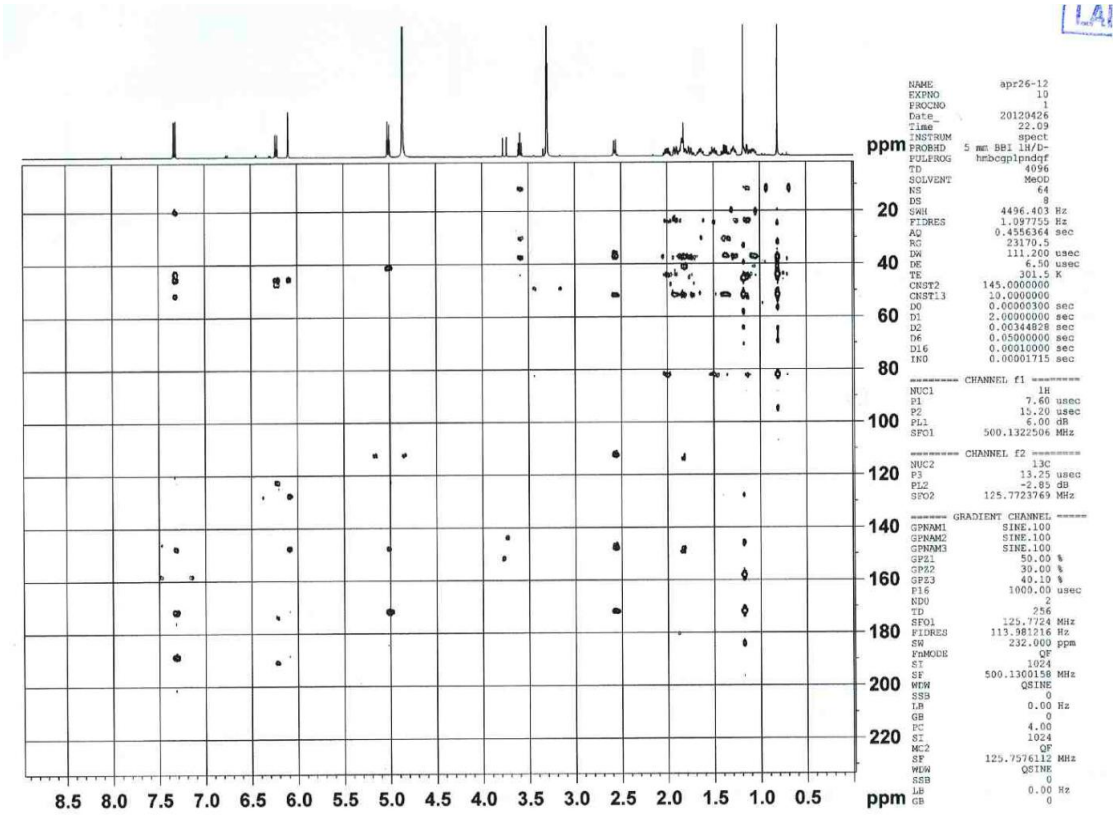


Fig. A18. HMBC spectrum of metabolite **5**

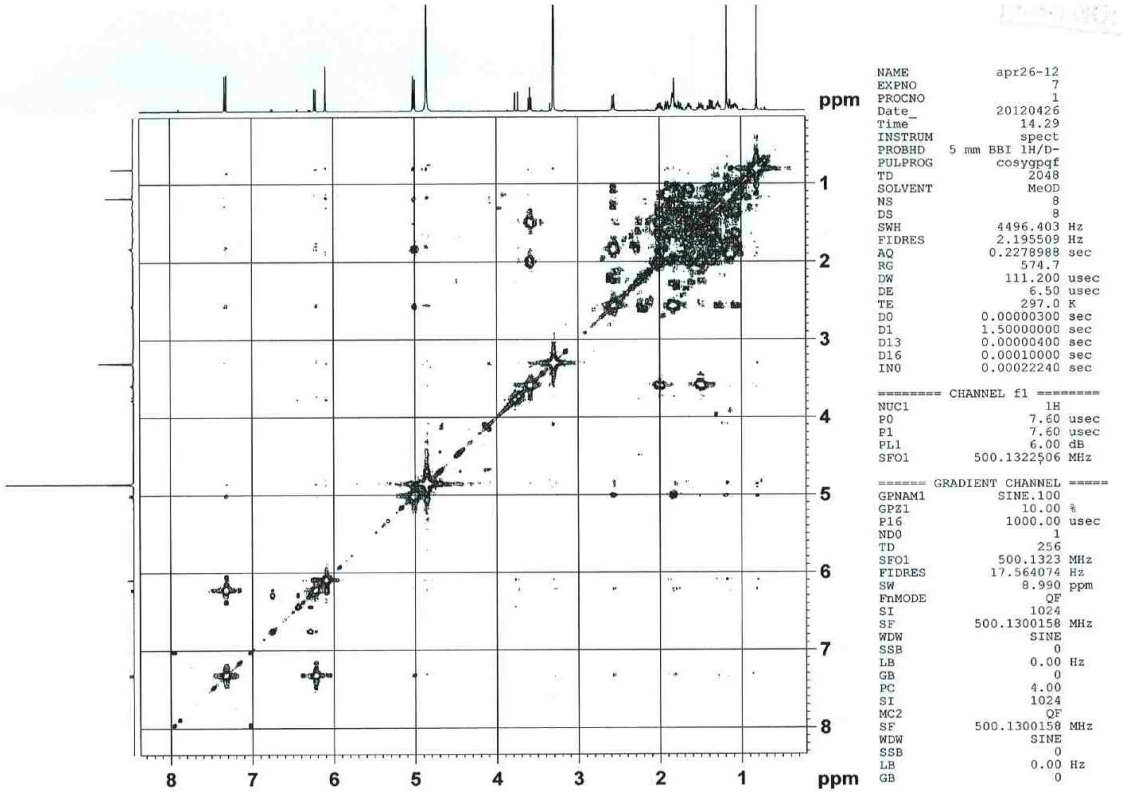


Fig. A19. COSY spectrum of metabolite *5*

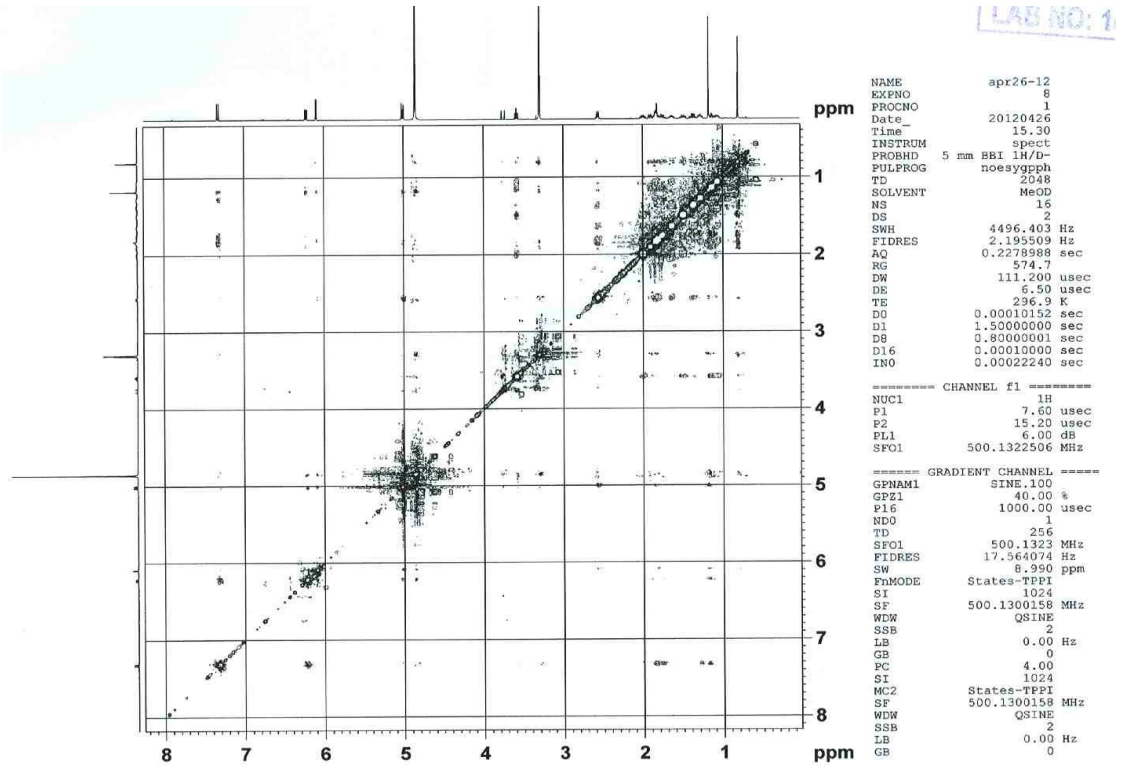


Fig. A20. NOESY spectrum of metabolite *5*

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