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SAKARYA UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOTRANSFORMATION OF DEHYDROEPIANDROSTERONE BY *PENICILLIUM OLSONII*

MSc THESIS

Mena ALBAYATY

Chemistry Department

Biochemistry Program

DECEMBER 2023

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The thesis work titled "BIOTRANSFORMATION OF DEHYDROEPIANDROSTERONE BY *PENICILLIUM OLSONII*" prepared by Mena Albayaty was accepted by the following jury on 25/12 /2023 by unanimously/majority of votes as a MSc THESIS in Sakarya University Institute of Science and Technology, chemistry department.

Thesis Jury

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Mena Albayaty

TABLE OF CONTENTS

Page

ABBREVIATIONS

^{13}C NMR	: Carbon 13 Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR	: Proton Nuclear Magnetic Resonance Spectroscopy
DHEA	: Dehydroepiandrosterone
DMF	: Dimethylformamide
IR	: Infrared
TLC	: Thin Layer Chromatography
PDA	: Potato Dextrose Agar
ppm	: Part Per Million
rpm	: Revolution Per Minute

SYMBOLS

^{o}C	: Celsius degree
cm	: Centimeter
Δ	: Chemical shift difference
δ_C	: Chemical shift in ^{13}C NMR spectrum
δ_H	: Chemical shift in ^{1}H NMR spectrum
g	: Gram
J	: Interaction constant
lit	: Literature
mg	: Miligram
MHz	: MegaHertz
ml	: Milliliter
m	: Multiplet signal
s	: Singlet signal
pH	: Negative logarithm of hydrogen ion concentration
t	: Triplet signal

LIST OF TABLES

Page

LIST OF FIGURES

Page

BIOTRANSFORMATION OF DEHYDROEPIANDROSTERONE BY *PENICILLIUM OLSONII*

SUMMARY

Natural products not actively involved in creating and developing living things for the organisms they contain usually provide significantly better living conditions. Natural products are receiving increased attention because of their effects on other living things. Despite the fact that these compounds are numerous and have very different structures, due to some similarities in their biosynthesis, they are usually classified as terpenes, alkaloids, steroids, phenolic compounds, specialized carbohydrates, specialized peptides, polyketides, fatty acids, and fatty acid derivatives. One significant category of natural compounds is steroids. One of the most significant and well-known steroids is the cholesterol molecule that controls the fluidity of membranes in both humans and animals. Some substances with crucial roles, like vitamin D3, bile acids, and steroids, are biosynthesized from cholesterol. Cholesterol-derived steroid hormones are classified into five groups: progestogens (progestins), estrogens, androgens, androgens, mineralocorticoids, and glucocorticoids. The main circulating steroid usually classified as androgen is androgen, which encourages secondary sexual characteristics in males, such as dehydroepiandrosterone (DHEA).

Biotransformations are chemical reactions when enzymes operate on materials that are not their typical substrates. The biotransformation-related enzymes can function in various biological systems or as free, fixed molecules. The most often utilized biological systems for biotransformations include bacteria, yeasts, and molds. These microbes can be used for microbial biotransformations, which have numerous benefits over current chemical synthesis techniques. These microorganisms can be immobilized on appropriate surfaces or employed freely to carry out microbial biotransformations.

Due to the high regioselectivity and stereoselectivity of mold enzymes, microbial steroid biotransformations by molds are widely used to obtain many more important and functional compounds.

The aim of this study was to investigate how dehydroepiandrosterone **9**, also known as DHEA, is metabolized in *Penicillium olsonii* MRC 500780 isolate. For this purpose, fresh subcultures were periodically prepared for *Penicillium olsonii* MRC 500780 mold before the biotransformation experiment. Afterwards, the medium for the mold was prepared and distributed to the flasks and sterilized in an autoclave. The mold in the freshest subculture was inoculated into these flasks under sterile conditions, and the flasks were left to incubate for 3 days. DHEA **9** was added under sterile conditions to these flasks where enough mold grew and incubated for 5 days. After incubation, the medium was filtered, and possible metabolites were extracted to the organic phase by ethyl acetate extraction. The metabolites in the residue obtained by evaporation of the ethyl acetate extracts were separated by column chromatography. Structure determination of the metabolites was carried out by melting point determination, NMR

and IR spectroscopy. Structure determinations revealed that biotransformation of DHEA **9** by *Penicillium olsonii* MRC 500780 resulted in the compounds androst-4-ene-3,17-dione **5 (**8%**)**, 15α-hydroxyandrost-4-ene-3,17-dione **15 (**21%**)**, 3β,7β-dihydroxyandrost-5-ene-17-one **21 (**8%**)**, 3β,7α-dihydroxyandrost-5-ene-17-one **22 (**29%) and 15α,17β-dihydroxyandrost-4-ene-3-one 23 (6%).

DEHİDROEPİANDROSTERONUN *PENICILLIUM OLSONII* İLE BİYOTRANSFORMASYONU

ÖZET

Doğal ürünler, canlı organizmaların büyümesi ve çoğalması için gerekli olmayan maddelerdir. Diğer canlılar üzerindeki etkileri nedeniyle doğal ürünler canlılara fayda sağlamakta ve daha fazla ilgi çekmektedir. Terpenoidler, alkaloidler, steroidler, poliketidler, peptidler, fenilpropanoidler, özel amino asitler, özel karbonhidratlar ve yağ asitleri ve türevleri gibi gruplar doğal bileşikleri kategorize etmek için yaygın olarak kullanılmaktadır.

Biyotransformasyonlar, biyolojik sistemlerin ksenobiyotik maddeler üzerinde gerçekleştirebildiği kimyasal değişikliklerdir. Biyotransformasyonlar enzim içeren biyolojik sistemler ve serbest veya immobilize enzimler tarafından gerçekleştirilir. Biyotransformasyonlar için standart biyolojik sistemler olarak genellikle hücre kültürleri, doku kültürleri, organ kültürleri, mikrozomlar, mikroorganizmalar ve mikrop sporları kullanılır.

Canlılarda, enzimler aktivasyon enerjisini (EA) azaltarak neredeyse her reaksiyonu gerçekleştirir. Enzimler reaksiyonun dengeye ulaşması için gereken süreyi kısaltır, ancak süreç tarafından tüketilmez veya değiştirilmezler, reaksiyonun DG'sini veya denge konumunu da değiştirmezler. Uluslararası Biyokimya Birliği tarafından 3200'den fazla enzim kaydedilmiştir ve doğada 25.000 enzim olabileceği tahmin edilmektedir.

Oldukça etkili katalizörler oldukları için, enzimlerin kullanıcıları için belirli faydaları vardır. Örneğin, enzimatik reaksiyonların reaksiyon hızlarını 108-1010 kat artırılabilir ve nadir durumlarda bu 1012'yi bile geçebilir. Enzimler tamamen biyolojik olarak parçalanabilir olduklarından ve amino asitlerden oluştuklarından, çevre için kabul edilebilirdirler. Diğer kimyasal reaktiflerin çoğu çevre üzerinde olumsuz bir etkiye sahipken, enzimler genellikle ılımlı ortamlarda (pH 7, 30 °C ve 1 atm) çalışır. Sonuç olarak izomerizasyon, rasemizasyon, yeniden düzenlemeler ve parçalanma gibi belirli sorunlar azalır. Enzimler birbirleriyle uyumlu olduklarından, tipik olarak aynı veya benzer ortamlarda çalışırlar. Sonuç olarak, multienim sistemleri kullanılarak tek bir şişede birçok reaksiyon gerçekleştirilebilir. Çoğu enzim yüksek bir substrat toleransına sahipken, bazı enzimler amaçlanan işlevleriyle sınırlı değildir. Bu enzimler hem doğal hem de yapay çok çeşitli maddeleri kabul edebilmektedir. Enzimlerin katalizleyebileceği birçok farklı reaksiyon türü vardır ve pratik olarak bilinen herhangi bir reaksiyon bir enzim tarafından katalize edilebilir.

Enzimler kemoseçicilik, rejioseçicilik ve enantioseçicilik özelliklerine sahip moleküllerdir. Kemoseçici olduklarından, genellikle sadece belirli bir tür fonksiyonel grubu etkilerken diğer fonksiyonları değiştirmeden bırakırlar. Dolayısıyla enzimatik reaksiyonlar tipik olarak daha temiz bir kaliteye sahiptir. Rejiyoseçicilikleri sayesinde enzimler, aynı substrat molekülü içinde farklı kimyasal konumlarda bulunan fonksiyonel gruplar arasında ayrım yapabilmektedir. Enzimlerin karmaşık üç boyutlu

mimarisi bunu başarmalarını sağlayabilir. L-amino asitlerden türetilen kiral katalizörler oldukları için enzimler enantiyoselektiftir. Dolayısıyla enzimler bir substrat molekülü üzerindeki her türlü kiraliteyi tanıyabilir. Rasemik bir substrattaki her iki enantiyomer tipik olarak farklı oranlarda reaksiyona girerek kinetik bir reaksiyona neden olabilir ve prokiral bir substrat kiral bir ürüne dönüştürülebilir.

Bununla birlikte, enzim kullanmanın dezavantajları da vardır. Doğada bulunan bir tür enantiyomer bir enzimdir. Diğer tür enantiyomerik ürüne ihtiyaç duyulduğunda tam tersi stereokimyasal seçiciliğe sahip bir enzim gereklidir. Ancak bu çoğu zaman mümkün değildir. Enzimler özel çalışma koşulları gerektirir. Yüksek sıcaklıklar ve asidik pH bazı enzimleri engelleyebileceğinden, ılıman ortamlarda çalışmak zaman zaman sorunlu olabilir. Su, yüksek kaynama noktası ve yüksek buharlaşma ısısı nedeniyle enzimlerin bu çözücüde maksimum katalitik aktivitelerini sergilemelerine rağmen organik proseslerin çoğu için en az ideal çözücüdür. Buna ek olarak, organik moleküllerin çoğu sulu çözeltilerde zorlukla çözünür. Bu nedenle, enzimatik bir prosesin sulu bir ortamdan organik bir ortama geçirilmesi oldukça arzu edilen bir durumdur. Ancak, enzim denatürasyonu nedeniyle, bu durum katalitik aktivitede bir azalmaya neden olabilir. Enzimlerin dayandığı doğal kofaktörler son derece önemlidir. Enzimler, doğal olmayan substratları kabul etmedeki olağanüstü esnekliklerine rağmen, neredeyse tamamen NADH ve NADPH gibi doğal kofaktörlerine gereksinim duyarlar. Ne yazık ki, bu moleküller nispeten kararsız olduklarından, stokiyometrik seviyelerde kullanılamayacak kadar maliyetli olduklarından ve her ikisi de mümkün olmadığından, daha uygun fiyatlı sentetik muadilleriyle ikame edilemezler. Enzimler inhibisyon olaylarına karşı hassastır. Enzimlerin daha yüksek substrat ve/veya ürün konsantrasyonlarında çalışmayı durdurmasına neden olan madde veya ürün inhibisyonu, birçok enzimatik işlemi etkileyebilir. Ayrıca, bazı enzimler alerjik reaksiyonları tetikleyebilir, ancak bu risk onlara kimyasallar gibi davranarak ve kullanırken dikkatli olarak azaltılabilir.

Tipik olarak, biyotransformasyonlar bütün, bozulmamış mikroorganizmalar veya izole enzim sistemleri tarafından gerçekleştirilir. Yaklaşık 300 izole enzim sisteminin satışa sunulduğu tahmin edilmektedir. Gerekli enzim sistemlerinin çoğu membrana bağlı olduğundan ve ekstrakte edilmesi zor olduğundan, biyotransformasyonlar bütün hücre sistemleri olan mikroorganizmalar ile gerçekleştirirlir. Biyotransformasyonlar için kullanılan dört ana mikroorganizma türü; bakteriler, mayalar, küfler ve mikroalglerdir.

Mikroorganizmalar, spesifik olmayan enzim sistemleri aracılığıyla hem doğal hem de insan yapımı substratlar üzerinde çeşitli reaksiyonlar gerçekleştirir. Bu süreçler arasında mikrobiyal hidroksilasyonlar en yaygın ve tercih edilenidir. Mikrobiyal hidroksilasyon 1952 yılında kortikal steroidlerin sentezi ile ilgili önemli bir sorunun çözümüne yardımcı olduğunda, bu sürecin önemi ilk olarak fark edilmiştir. Pozisyonun diğer fonksiyonel gruplardan izole olması nedeniyle, C-11'e bir oksijen fonksiyonunun eklenmesi son derece zaman alıcı, pahalı ve zorlu bir prosedürdü. Rhizopus arrhizus bu sorunu mikrobiyal hidroksilasyon ile etkili bir şekilde çözdü. Bu mikrobiyal hidroksilasyondan sonra mikrobiyal biyotransformasyonlar popülerlik kazanmıştır. O zamandan beri, steroidler de dahil olmak üzere çeşitli substrat grupları mikrobiyal biyotransformasyonlar için yaygın olarak kullanılmaktadır. Mükemmel regio ve stereoseçicilikleri nedeniyle mikrobiyal steroid biyotransformasyonları, birçok önemli steroid hormon ve ilacın üretiminde yaygın bir kullanım alanı bulmuştur.

Son yıllarda çok sayıda mikrobiyal steroid biyotransformasyonu tanımlanmıştır. Mikrobiyal biyotransformasyonların verimliliğini artırmanın yanı sıra yeni, yararlı

mikroplar ve reaksiyonlar keşfetmek için hala tonlarca şaşırtıcı çaba sarf edilmektedir. Çok sayıda farklı mantar, 1952'de ilk mikrobiyal hidroksilasyonun tanımlanmasından bu yana biyotransformasyon reaksiyonları için sürekli olarak en çok araştırılan tam hücre sistemlerinden biri olmuştur. Çok sayıda steroid türü çeşitli mantarlar tarafından biyotransformasyona uğratılmıştır. Bu biyotransformasyonlardan mikrobiyal hidroksilasyonlar, Baeyer-Villiger oksidasyonları ve 5α-redüksiyonu dahil olmak üzere çeşitli ilgi çekici sonuçlar elde edilmiştir.

Bu çalışmada DHEA olarak da bilinen dehidroepiandrosteron 9, Penicillium olsonii MRC 500780 küfü ile 5 gün boyunca inkübe edilmiştir. Küf için 1 L damıtılmış su içinde besiyeri hazırlanmıştır. Besiyeri 250 mL'lik 10 erlenmeyer şişesine eşit olarak dağıtılmış ve bir otoklav ile sterilize edilmiştir. Bu şişelere fungus inoküle edilmiştir. Erlenler 25 ºC'de 3 gün boyunca çalkalayıcıda inkübe edilmiş ve DMF içindeki substrat bu erlenlere aseptik olarak eklenmiştir. Tüm şişeler aynı koşullar altında 5 gün daha inkübe edilmiştir. İnkübasyondan sonra, mantar miseli vakum altında süzülerek besiyerinden ayrılmıştır. Miselyum daha sonra etil asetat ile ekstrakte edilmiştir. Ekstraktlar susuz sodyum sülfat üzerinde kurutulmuş ve vakumda buharlaştırılarak kahverengi ve sakız kıvamında olan bir madde elde edilmiş ve daha sonra silika jel 60 üzerinde kromatografisi yapılmıştır. Kromatografi çalışmasından androst-4-ene-3,17-dion 5 (8%), 15α-hidroksiandrost-4-ene-3,17-dion 15(21%), 3β,7β-dihidroksiandrost-5-ene-17-on 21(8%), 3β,7α-dihidroksiandrost-5-ene-17-on 22 (29%) ve 15α,17β-dihidroksiandrost-4-ene-3-on 23(6%) bileşikleri elde edilmiştir. Bu bileşiklerin yapıları, başlangıç maddesinin erime noktası, NMR ve IR spektrumları ile kendilerine ait erime noktası, NMR ve IR spektrumları karşılaştırılarak ortaya çıkarılmıştır.

1. INTRODUCTION

Compounds that are observed in all living things and play a crucial role in their growth and development, such as amino acids, carbohydrates, fat, nucleotides, and organic acids, sugars are called primary metabolites. On the other hand, secondary metabolites, more commonly known as natural products, are a wide variety of organic compounds that are not essential in the development, growth, and reproduction of living things [1,2].

Natural products are found and isolated from different natural sources, such as plants, animals, microbes, insects, plant pathogens, endophytes, and marine organisms [3]. Natural products are generally divided into terpenoids, alkaloids, phenolic compounds, polyketides, fatty acids, specialized carbohydrates, specialized peptides, amino acids, and steroids [2, 4].

Steroids are lipophilic low molecular weight compounds that play several important physiological roles. The word steroid comes from the Greek word "stereos" meaning solid. Steroids are compounds with a cyclopentanoperhydrophenanthrene ring (sterane ring). This ring is made up of three cyclohexane rings, known as A, B, and C, and one cyclopentane ring known as D, fused with each other (Figure 1.1). Most steroid molecules contain methyl groups at C-10 and C-13, which are also known as C-18 and C-19, respectively. Steroids typically have hydroxyl or carbonyl groups in C-3 and C-17. Additionally, certain steroids have chains that are attached to carbon 17 on the D ring [5].

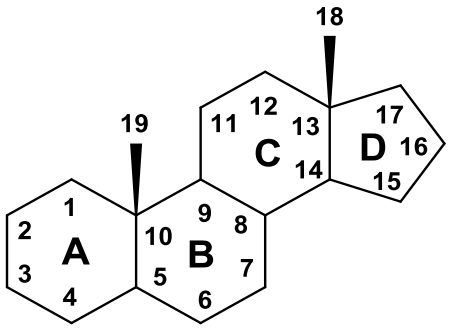

Figure 1.1. General steroid structure [4].

Steroids are a group of important organic compounds that contain a specific chemical structure. The most well-known steroids are sterols, which have aliphatic side chains consisting of 7, 8, or 9 carbon atoms at the 17^{th} carbon position and a hydroxyl group on the third carbon atom. The three most common sterols are cholesterol, stigmasterol, and ergosterol. Cholesterol is found in humans and other animals, while stigmasterol and ergosterol are found in plants and molds [6].

Cholesterol **1** is the most abundant steroid in the body and plays a crucial role in regulating the fluidity of cell membranes in both humans and animals. It is also an essential precursor for the biosynthesis of several other substances with significant functions, such as bile acids, vitamin D3, and steroid hormones. In fact, all mammalian steroids are derived from cholesterol [6, 7].

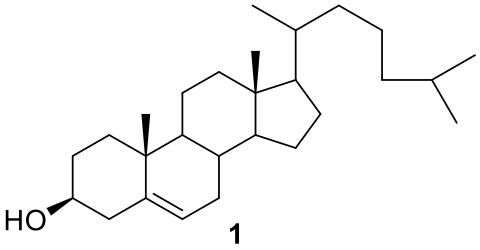

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Figure 1.2. Cholesterol structure [7].

Steroid hormones physiological and therapeutic roles as physiological process regulators are well known [8]. They also control a multitude of physiological and developmental processes from fetal life to adulthood [9]**.** According to their physiological action, there are five types of steroid hormones such as mineralocorticoids, glucocorticoids, progestogens, androgens and estrogens [8]. Aldosterone is the most significant physiological mineralocorticoid, and aldosterone's distinguishing feature is its ability to control fluid and electrolyte balance [10]. Glucocorticoids; which are known for their ability to mobilize carbohydrates, also have a wide role in regulating the metabolism of fat, glucose, and proteins [8]. Estrogens play a role in female reproduction, which produces secondary sexual features in women, together with many other biological systems, such as the immunological, circulatory, skeletal, and neuroendocrine systems [8, 11]. Progesterone plays a vital role in the reproductive system. It also helps maintain the early stage of pregnancy [8, 12]. Androgen causes secondary sexual characteristics in males [8, 13].

Steroid hormones are derivatives of cholesterol **1**. When the side chain of cholesterol **1** is removed, it is converted to pregnenolone **2**. Progesterone **3** is synthesized from the pregnenolone **2** in two separate reactions. The biosynthesis of androgens via pregnenolone **2** occurs in two different ways (Figure 1.3.), the Δ^4 pathway and the Δ^5 pathway [5].

The main pathway for androgen biosynthesis is the Δ^4 pathway. In this way, pregnenolone **2**, formed by shortening the side chain in the cholesterol **1** compound, is converted to 17α-hydroxyprogesterone **4** via progesterone **3**. As a result of the removal of the 17α-Hydroxyprogesterone **4** side chain, androst-4-en-3,17-dione **5**, also known as androstenedione, is synthesized. When androst-4-en-3,17-dione **5** is reduced at C-17, an active androgen known as testosterone **6** is synthesized. Testosterone **6** can be converted to dihydrotestosterone **7** by the enzyme 5α-reductase [5-7].

In the Δ^5 pathway, also known as the bypass, pregnenolone **2**, formed from cholesterol **1**, is converted to dehydroepiandrosterone **9**, also known as DHEA, via 17α-hydroxypregnenolone **8**. Dehydroepiandrosterone **9** can also be converted to testosterone **6** via androst-4-en-3,17-dione **5**. The end product of the Δ^4 pathway and Δ^5 pathways in the biosynthesis of androgens is androst-4-en-3,17-dione **5**, as can be seen in Figure 1.3. Androst-4-en-3,17-dione **5**, is also the starting material for estrogens such as estrone **10** and estriol **11**. Another estrogen, estradiol **12**, is synthesized from testosterone **6** [5-7].

DHEA **9**, which is a 3β-hydroxy-5-ene steroid, is a precursor to androgens and estrogens [14]. The level of the hormone peaks in early adulthood and then starts to decline at 70-80 years of age [15]. Most DHEA would be transformed into dehydroepiandrosterone sulfate, stored, and converted into certain hormones when needed because of its prolonged half-life in plasma. Being a type of significant pharmaceutical steroid, hydroxylation at various locations would display a variety of biological actions. For instance, hydroxylation at C-11α is necessary for anti-inflammatory effects, while hydroxylations at C-9α and C-16α are essential for glucocorticoid bioactivities [16].

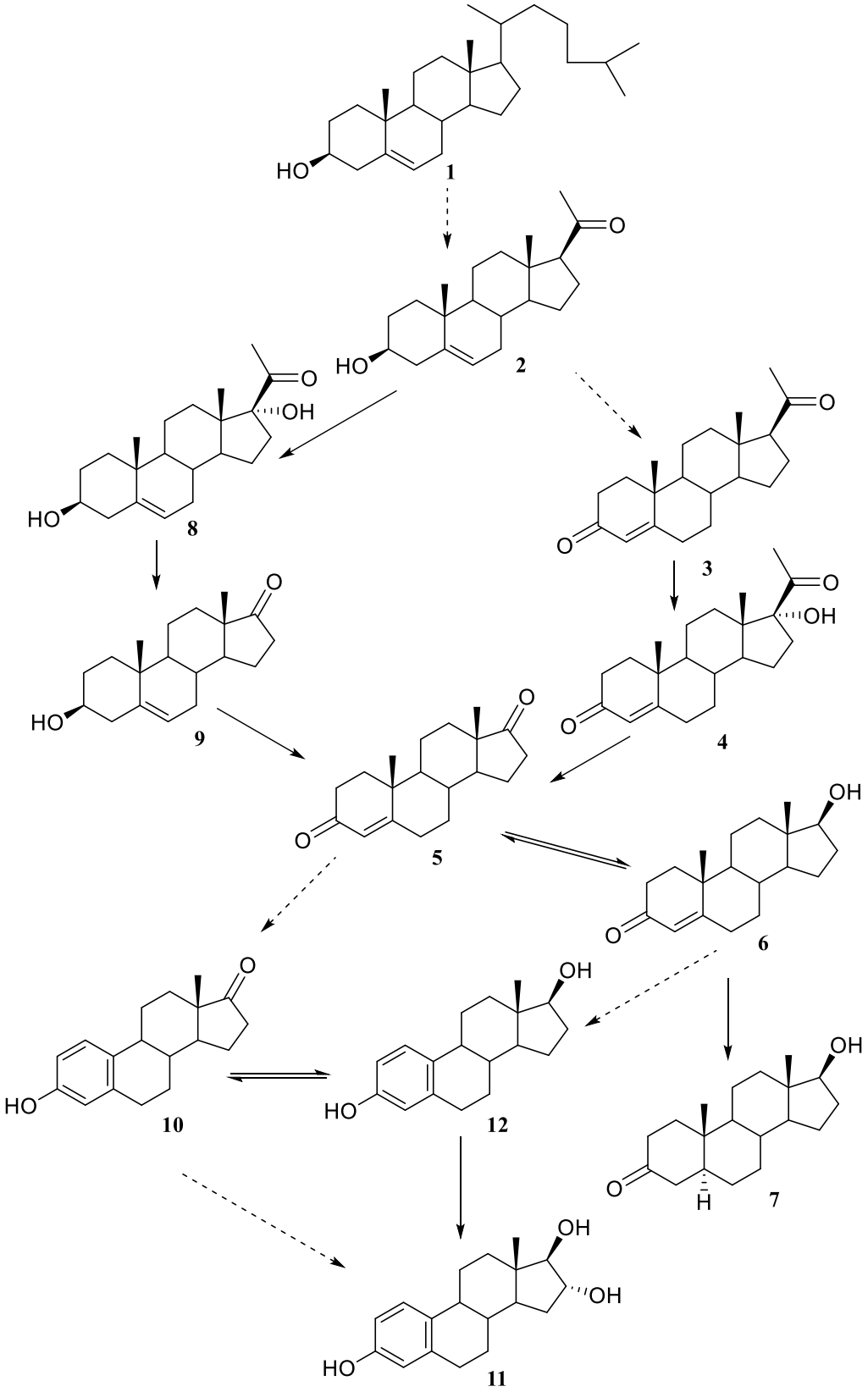


Figure1.3. Biosynthesis of androgens and estrogens [5, 6].

2. LITERATURE SURVEY

A substance or molecule that is foreign to a biological system and has a variety of impacts on it is referred to as a xenobiotic. It may be grouped as drugs, food additives, pesticides, hydrocarbons, environmental pollutants, carcinogens, and industrial chemicals. They are lipophilic in nature and absorb through the lungs and skin or gastrointestinal tract [5]. Biotransformation refers to the chemical modifications made to these xenobiotics by enzymes or cell, tissue, or organ cultures as well as by microbes or spores of microorganisms that contain enzymes [17].

2.1. Biotransformation

In order to form a molecule with greater polarity and chemically different product while maintaining the original carbon skeleton would be challenging. It can be obtained by biotransformation, which is a structural modification in a chemical compound in living cells, catalyzed by microorganisms or their enzymes, biotransformation has great potential to produce known products more efficiently [18].

Biotransformation catalyzes, inexpensively, the conversion of readily available precursors that cannot be transformed effectively by chemical or microbial methods into a more valuable end product, and also catalyzes the production of food metabolites, fine chemicals, and pharmaceuticals [19].

The biotechnological approach is used to produce secondary metabolites important for pharmaceutical use because of their different functions and biological activities, and such secondary compounds are glycosides, alkaloids, flavonoids, terpenoids, and volatile oils [19].

The natural transformation process is low, less productive, and nonspecific, unlike biotransformation which is a rapid and simple process that can reduce multistep chemical reactions, occurs in less time and money, react specifically, and only one step is efficient to obtain a good yield, there are no side reactions, the substrate could be natural or synthetic. Also, it can be carried out in mild conditions like neutral pH, ambient temperature (20-40 C^{o}) and at atmospheric pressure also, more than one

reaction can be achieved using cell cultures expressing a series of enzyme activities, and less environmental pollution [19]. Biotransformation deals with microbial (enzymatic) conversion of a substrate into a product by enzymatic reaction [20].

Nowadays, biotransformations utilize modified enzymes, semi-synthetic enzymes, and enzymes with an antibody structure or abzymes. The fact that the separation techniques used to isolate metabolites denature the enzyme and restrict its reutilization is one of the most significant issues with biotransformation investigations involving enzymes. Enzyme fixation has provided a solution to this issue [21]. After fixation, numerous enzymes and microorganisms can be constantly utilized in this manner. The limited solubility of most organic substrates in water presents another challenge for biotransformation research involving enzymes. Since it was demonstrated in 1985 that some enzymes, particularly lipases, might be employed in organic solvents, these biotransformations have used more frequently [4].

2.2. Enzymes, Advantages and Disadvantages

6 classifications indicate its group and sub-group, and they are; Oxidoreductases, which is an oxidation-reduction reaction, like the oxygenation of C-H, C-C, and C=C bonds, and removal of hydrogen atom equivalents. Transferase is the transfer reaction of functional groups, such as acyl, sugar, phosphoryl, aldehydic, and ketonic. Hydrolase reactions use water to break a chemical bond, as in C-N containing functions, esters, anhydrides, peptides, amides, and glycosides. Lyase reactions, in which functional groups are added or removed without hydrolysis, e.g., adding HX to double bonds as in C=C, C=N, and C=O. Isomerase reactions, in which a compound is converted to its isomer, such as cis-trans isomerization and C=C bond migration. Ligase reactions, in which new bonds are formed between carbon and another atom, for instance, the formation of phosphate ester bonds and C-O, C-S C-N, C-C [22]. The majority of beneficial biotransformation performed in organic synthesis is by the hydrolase class of enzyme. Oxidoreductase is the second, and the rest are low in use [22].

Enzymes have many advantages as biocatalysts. For example, using enzymes in organic synthesis provides many benefits by quickly performing their functions to about 10^8 –10^{10} faster than a reaction without enzyme [20]. Also, they need a very low amount of catalyst to be effective [20, 21]

They act under mild conditions of (20-40 ºC) temperature which minimize undesired side-reactions such as rearrangement, decomposition, isomerization, racemization, and pH of about 5-8 since enzymes are biomolecules of protein nature. Parameters such as pH and temperature can be changed very little to accelerate an enzymatic reaction [20, 21]. Additionally, they catalyze a wide range of reactions and also can promote reactions at the non-active side of the substrate. Because it can accept a large amount of substrate, it tolerates accepting a variety of non-natural substances [20, 21].

Enzymes are Chemo-regio selective; the final product tends to be cleaner and easier to remove impurities and undesirable side reactions because enzymes act on a single type of functional group. The other functionalities will remain unchanged due to its chemo-selectivity. Enzymes are also regio-selective because they can differentiate between the identical but located in different positions functional groups within the same substrate molecule because of their complex three-dimensional structure [21].

Because many organic compounds are insoluble in water, the most typical environment for enzymes to work is water. However, they also can work outside the aquas environment. In addition, they are environmentally acceptable since they are completely biodegradable, unlike heavy metals [20, 21].

Contrary to some heavy metals that act as catalysts and most reagents in classical synthesis processes, enzymes, the majority of which are protein in nature, are considered environmentally friendly because they can be degraded in nature [21].

Enzymes can efficiently catalyze reactions that are difficult or impossible to perform with other methods because enzymes are chiral biomolecules and also enantioselectivity, which means they only consist of L-amino acid therefore, they affect a prochiral substrate, converting it only into an enantiomer, and if enzymes only affect one of the enantiomers in a racemic mixture, separation of racemic mixtures also occurs [21].

There are numerous types of reactions in enzymes. Almost every synthetic process has an equivalent in the type of enzyme reaction. Alkane, alkene, aromatic compounds, alcohol, aldehyde, ketone, oxidation or reduction of sulfides and sulfoxides, carboxylation, decarboxylation, alkylation, and hydrolysis or synthesis of ester, ether, lactone, lactam, epoxide, acid anhydride, amide, and nitriles are reactions with enzymes. The Michael addition reaction, Diels-Alder reactions, acyloin and aldol

processes, halogenation and dehalogenation, isomerization, addition or removal of ammonia and hydrogen cyanide [21].

Because of the features mentioned above of enzymes, reactions that are difficult or impossible to achieve using organic synthesis methods can be easily carried out by enzymes. There are also some disadvantages to using enzymes. For instance, many of them need cofactors to carry out their reactions, which are expensive and unstable compounds [21].

Additionally, the use of enzymes may also cause some undesirable situations. For example, an enzyme has only one enantiomeric form, and an enzyme can only react with a particular enantiomer since there is no common method for biosynthesis of the other enantiomeric form [21].

Some enzymes must have their natural cofactors. Regarding enzymatic reactions, cofactors like NADH and NADPH must be in the reaction media and must be replaced. The main disadvantage of enzymatic reactions is that cofactors are typically very expensive, unstable substances and cannot be substituted with synthetic counterparts [21].

Although there are some dogmatic assumptions that enzymes only affect their substrates in their natural environment, most of them are expensive and very sensitive, also it can cause some allergic reactions [22].

2.3. Biotransformation Techniques

Generally, two methods are used for biotransformations: whole cell systems or isolated enzymes. The phrase "whole cell systems" typically refers to microorganisms as well as plant and animal cell, tissue, and organ cultures [21, 23]

It is often preferred to use whole-cell systems in biotransformation reactions. The fact that the majority of intracellular enzymes are unstable outside of the cell, that some proteolytic enzymes hydrolyze intracellular enzymes during homogenization, and that intracellular enzymes require cofactor 13 continuously and are isolated from one another are among the most significant causes of this, both expensive and complicated [23].

2.4. Microbial Biotransformation

A microbial biotransformation tool is used to obtain the chemical structure for natural products with complex structures such as steroids. It can efficiently synthesize the structures that are difficult to obtain by traditional methods [24].

Microorganisms have been used for thousands of years to produce fermented products such as beer, bread, cheese, and vinegar. The importance of the fermentation process was obvious during World War I, especially the acetone-butanol fermentation and the glycerol fermentation. Both acetone and glycerol were used to produce a reservoir to support the war, the development of fermentation, enzymatic processes, and bioconversion were observed after the war, which led to the yield of products with high marketing importance, such as vitamins, amino acid, vaccines, organic solvents, polysaccharide, and nucleotides [25].

Therefore, microorganisms have shown significant industrial importance because of their fermenting ability and the enormous variety of reactions they can carry out. The most important feature is the production of essential materials such as proteins, carbohydrate polymers, cells, and nucleic acids, which are large molecules or produce small molecules like primary and secondary metabolites [4, 25].

In addition, compared to plant and animal cells, microbial cells growth and development rate is significantly higher. This assures brief and quick biotransformations using microbial cells. Microbial cells are mechanically more stable than plant and animal cells because of their tiny size and strong cell walls. They also have an advantage in terms of environmental adaptation regarding growth methods. Furthermore, unlike plant and animal cells, microbial cells can metabolize various substrates [21].

Besides the importance of fermentation process production, microorganisms show exceedingly usage in the biotransformation process [21].

It is possible to modify microbes genetically. Higher yields of significant compounds previously acquired with low yields by genetic modification and other chemical processes carried out on microorganisms are possible. With genetic modifications, it is even feasible to bring about the necessary changes in crops [25].

One of the crucial features of microorganisms is that they can perform many different chemical reactions on many different synthetic and natural substrates attributed to the non-specific enzyme system of the microorganisms [21].

Most microbial biotransformation occurs under much milder conditions at room temperature and 1 atm pressure and is performed in a shorter time and more cheaply than conventional synthesis methods. While most reagents used in classical synthesis methods cause significant damage to the environment, microbial biotransformation is environmentally friendly [21].

Microorganisms are used in the biotransformation process as a whole cell system since the biotransformation process is preferred a whole cell system to avoid the problem of instability of many intracellular enzymes outside the cell and other issues such as their hydrolysis by the effect of some proteolytic enzymes during homogenization, the difficulty and damaging during purification process, the necessity of continuously renewal of their cofactors, and the high cost and difficulty of isolation process [22, 25].

The whole cell system is found in microbe, animals, and plant cells. Still, the microbial cell is preferred due to its higher growth and development rate, so the biotransformation process takes a shorter time, although because of the size of its cell walls are small, their cells are more mechanically stable than the plant and animal cells [21].

The ability of microorganisms to be genetically modified makes their use in biotechnology widespread. Thanks to genetic changes in microorganisms, essential products obtained with low yields by classical synthesis methods can be obtained with higher yields. It is even possible to provide specific changes on crops with genetic applications [25].

Microbial biotransformation reactions are enantioselective and regioselective, so it does not need to protect other functional groups on the substrate during microbial biotransformation. Also, due to the enantioselectivity, Molecules targeted by classical synthesis methods often result in racemic mixtures that are very difficult to separate. It is extremely important that only the targeted enantiomer can be obtained, especially in the synthesis of active pharmaceutical ingredients [20, 21].

There is no need for a functional group to be present near the site where biotransformation occurs. For example, microbial hydroxylations occur at sites far

from functional groups, microbial hydroxylation is among the most important and common microbial biotransformation processes, and different types and numbers of reactions can be performed in a single step [20, 21].

Microorganisms are very adaptable to a variety of environments. These characteristics allow for easy adaptation and application in various environments, including flasks and manufacturing fermenters [4, 21, 24].

The importance of microbial hydroxylation became visible in 1952 during the synthesis of an anti-inflammatory using cortical steroids. The insertion of an oxygen function at C-11 was a very long difficult and expensive process since that position was remote from other functional groups. This insertion was efficiently performed by a mold, namely "*Rhizopus arrhizus"* via microbial hydroxylation. As a result of discovering the mentioned reaction, progesterone **3** was converted to 11α-hydroxyprogesterone by microbial hydroxylation [20, 21, 26, 27]

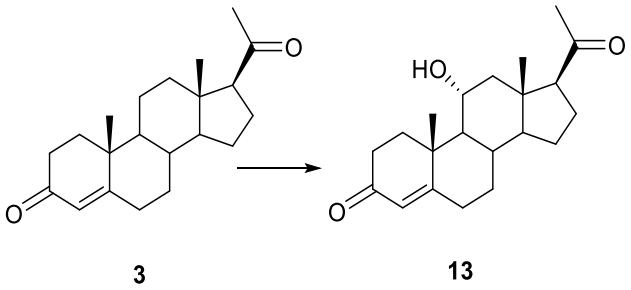


Figure 2.1. First microbial hydroxylation [21].

Therefore, various microorganisms, like molds, yeast, and Bactria, are used for microbial transformation, either freely or fixed to certain surfaces for evaluation as medicines and hormones. Numerous steroids are frequently employed as anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, pregestational, and anticancer drugs in addition to other applications [25, 27].

2.5. Steroid Biotransformation with Molds

Due to the high Regio and Stereoselectivity of biotransformation reactions of steroids, they are used widely in the production of important functional compounds such as drugs and hormones. This was discovered after the study of Murray and Peterson on identifying 11 α-hydroxylation of progesterone by a *Rhizopus* species in 1952. Since

then, the importance of microbial biotransformation of steroids in drug and hormone production has been known [28, 29].

Studies continue to activate existing microbial biotransformation further, identify new reactions and microorganisms and reactions that can be used, and isolate the microorganisms that can perform the desired structural transformation [29, 30].

Microbial steroid biotransformation has been carried out with many different molds. These studies resulted in interesting reactions such as microbial hydroxylations, Baeyer-Villiger oxidations, aromatization of the A ring, removal of side chains, oxidation of hydroxyl groups, reduction of ketone groups, microbial hydrogenations and dehydrogenations [20, 21, 28-31].

2.6. Biotransformation of DHEA 9 by Molds

Biotransformation of different steroids with *Penicillium* species with DHEA **9** generally resulted in Baeyer-Villiger oxidation, microbial hydroxylation, microbial hydrogenation, and dehydrogenation [16, 17, 24-27]. There are 9 studies in the literature on the incubation of DHEA **9** with *Penicillium* species [32-39].

Microbial transformation of dehydroepiandrosterone DHEA **9** with *P.griseopurpureum smith* (Figure 2.2.) yielded androst-4-ene-3,17-dione **5**, 17a-oxa-d-homo-androst-4-ene-3,17-dione **14**, 15α-hydroxyandrost-4-en-3,17-dione **15**, 15α-hydroxy-17a-oxa-d-homo-androst -4-ene-3,17-dione **16**, 14α-hydroxyandrost-4-en-3,17-dione **17** and 7α-hydroxyandrost-4-en-3,17-dione **18** [32]

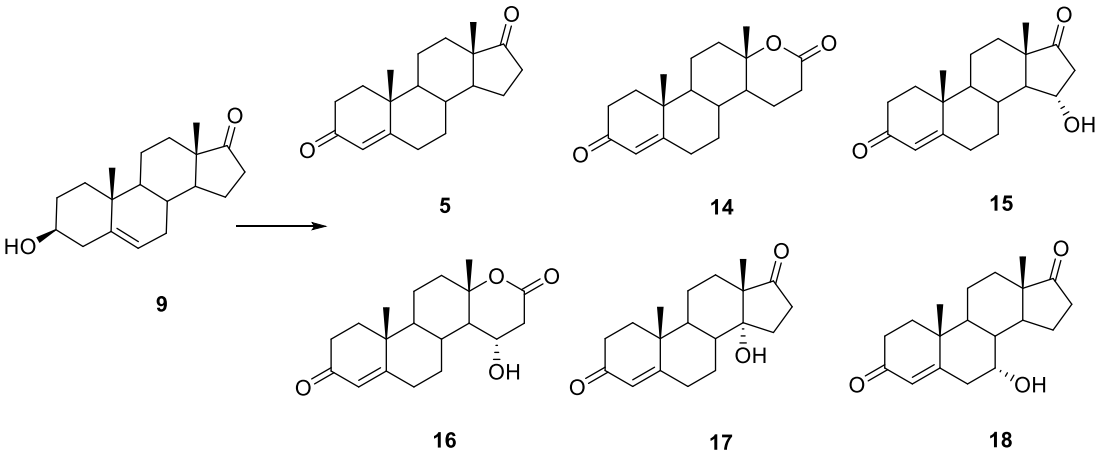


Figure 2.2. Biotransformation pathway of the substrate with *P.griseopurpureum* [32].

Incubation of DHEA **9** with *P. glabrum* (Figure 2.3) afforded androst-4-ene- 3,17-dione **5**, 17a-oxa-d-homo-androst-4-ene-3,17-dione **14**, 3β-hydroxy-17a-oxa-d-homo-androst-5-en-17-one **19** and 3β-hydroxy-17a-oxa-d-homo-5α-androstan-17-one **20** [32].

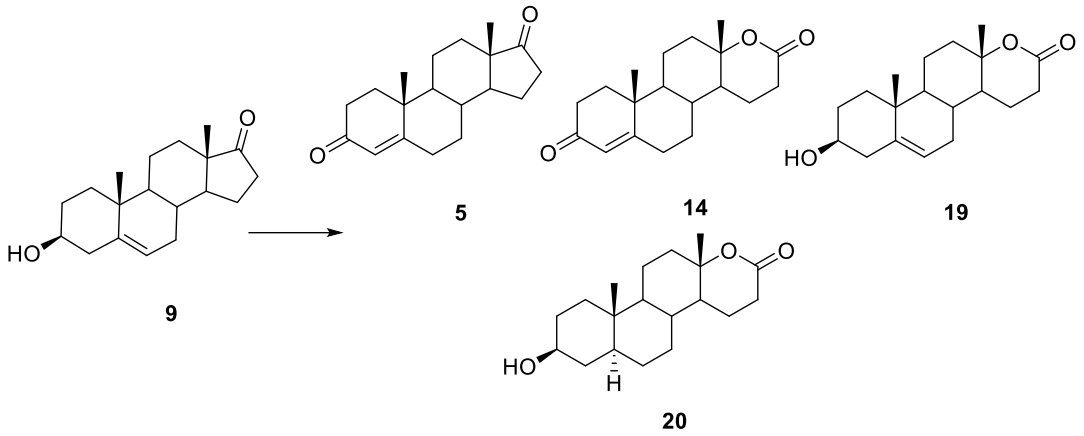


Figure 2.3. Biotransformation pathway of the substrate with *P. glabrum* [32].

Alina Swizdor found in her work with *P. lanosocoeruleum* KCH 3012 resulted in the conversion of the substrate DHEA to 17a-Oxa-d-homo-androst-4-ene-3,17-dione **14** (Figure 2.4.) [33].

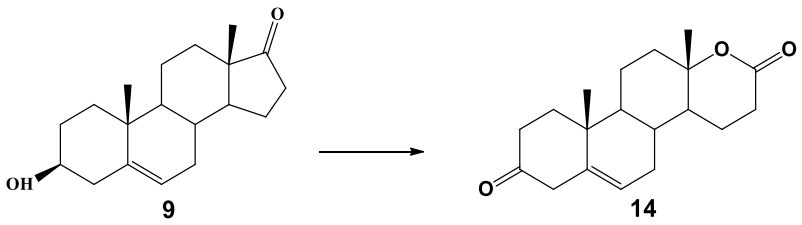


Figure 2.4. Biotransformation of substrate by *P. lanosocoeruleum* KCH 3012 [33].

Microbial biotransformation of DHEA **9** by *P. lilacinum* AM111 [34] and *P. vinaceum* AM110 [35] only afforded (Figure 2.5.) 3β-hydroxy-17a-oxa-d-homo-androst-5-en-17-one **19**.

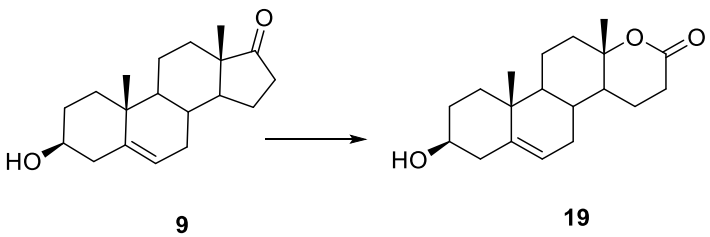


Figure 2.5. Biotransformation of the substrate with two *Penicillium* species [34, 35].

Biotransformation of the substrate by *P. commune* KCh W7 (Figure 2.5.) gave androst-4-en-3,17dione 5, 17a-oxa-d-homo-androst-4-ene-3,17-dione **14**, and of 3β-hydroxy-17a-oxa-d-homo-androst-5-en-17-one **19** [36].

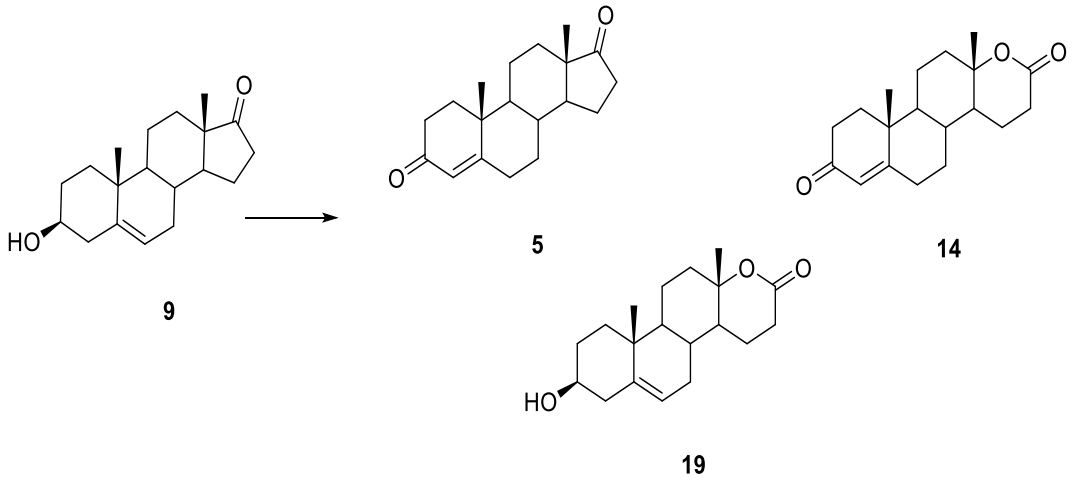


Figure 2.6. Biotransformation of the substrate with *P. commune* KCh W7 [36].

Incubation of DHEA **9** with *P. chrysogenum* KCh S4 [36] and *P. citreo-viride* A.C.C.C. 0402 [37] (Figure 2.6.) afforded androst-4-en-3,17dione (AD) **5** and 17a-oxa-d-homo-androst-4-ene-3,17-dione **14**.

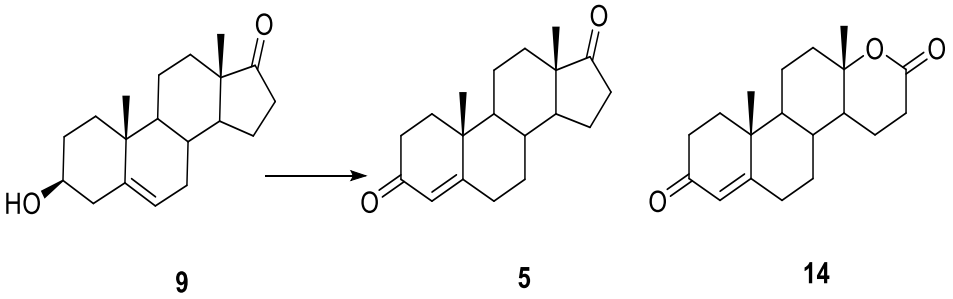
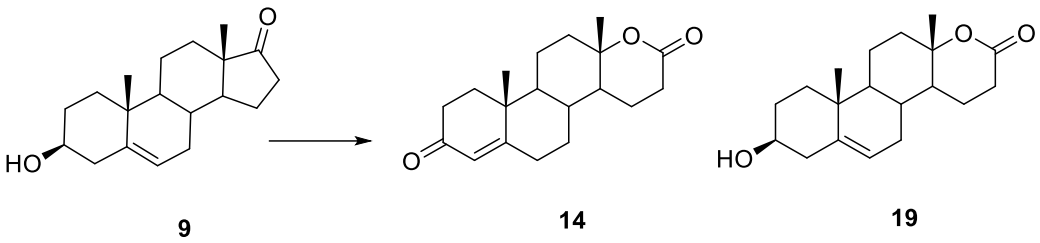


Figure 2.7. Incubation of the substrate with two *Penicillium species* [36, 37].

Microbial biotransformation of DHEA **9** by *P. simplicissimum* WY134-2 [38] and *P. camemberti* AM83 [39] (Figure 2.7) yielded 17a-oxa-d-homo-androst-4-ene-3,17-dione **14** and 3β-hydroxy-17a-oxa-d-homo-androst-5-en-17-one **19**.



9 14 19

Figure 2.8. Biotransformation of the substrate with two *Penicillium* isolates [38, 39].

2.7. The Aim of this Work

In this work, biotransformation of DHEA 9 by *Penicillium olsonii* MRC 500780 was carried out for 5 days to investigate its metabolism by this mold.

3. MATERIALS AND METHOD

3.1. General Information

In the biotransformation experiment, a Nüve 40 L autoclave was used to sanitize the media and glassware. The autoclave was set at 121 C for 20 minutes. The Nucleon brand's Class II Type biological safety cabinet (sterile cabinet) has been used for the biotransformation experiment's mold regeneration and incubations. Mold growth and biotransformation tests were conducted in a Gerhardt 500 Thermoshake shaker incubator. Using the Perkin Elmer SpectrumTwo spectrometer, infrared spectra were obtained. The Varian Mercury 300 NMR spectrometer, running at 300 MHz, was applied to collect ^{1}H NMR spectra. Tetramethylsilane standard was utilized to generate the internal signal and deuterochloroform as the solvent. Using deuterochloroform as a solvent, Varian Mercury 300 NMR spectrometer running at 75 MHz was used to get ^{13}C NMR spectra.

Thin layer chromatography (TLC) tests were performed after the biotransformation experiment and column chromatography study. TLC tests have been carried out using an ethyl acetate-hexane (1:1) solvent solution and 0.25 mm thick silica gel (Merck silica gel GF254). The steroids were made observable by immersing the steroids in TLC layers in p-anisaldehyde-sulfuric acid reagent and heating them for three minutes at 120°C. An Electrothermal IA 9200 melting point detector was applied to determine the melting point.

3.2. Experimental Studies

3.2.1. Preparation of agar slant media

The medium was prepared by boiling 11.7 g of potato dextrose agar (PDA) and 2.7 g of agar in 300 mL of distilled water. After being added to the halves of fifteen 22 mL pathological glass bottles of the Universal brand without cooling, it was autoclave sterilized at 121ºC for twenty minutes. Agar slant media were prepared once the molten

medium in these bottles was allowed to cool at an angle of around 45 degrees before freezing.

3.2.2. Mold culture preparation and refreshment

Some of the molds in the stock fungal culture were transferred under sterile conditions to 3 of the horizontal agar media to be allowed to multiply for 15 days at room temperature. Under sterile conditions, the most developed molds from the freshly created slanted agar cultures were transferred to fresh slanted agar media every two weeks. The biotransformation experiment employed the newest and fastest-growing mold that was produced after repeating this procedure twice.

3.2.3. Preparation of media for mold

The medium of *P.olsonii* mold was prepared by dissolving and mixing glucose (30g), and peptone (10g) in 1 L distilled water.

3.2.4. Biotransformation assay

Five 250 mL flasks containing the prepared medium was separated and autoclaved to ensure sterilization. These five flasks were inoculated with the freshest mold avaliable. They were then cultured for three days at 28 °C in a shaking incubator at 150 rpm. After the incubation period, the substrate (0.5 g) was dissolved in 5 mL of DMF and then aseptically transferred to the flasks in equal proportions. The flasks were incubated at 28°C at 150 rpm for five more days.

After the biotransformation experiment, a control flask had been employed. Only substrate and sterile media without any mold transfer were supplied in the control flask. In the control flask, every step of the biotransformation experiment was carried out exactly as it was done elsewhere. As no metabolites were detected during the extraction of TLC from the control flask, the outcomes of the biotransformation experiment were considered valid.

3.2.5. Separation of metabolites and structure determination

Following incubation, the mold medium was filtered out of the micelles as filtrate, and the micelles were subsequently washed with 500 mL of ethyl acetate. Three extractions of the filtrate components were made using one liter of ethyl acetate. Anhydrous

sodium sulfate was added to eliminate any potential water content in the extracts. An oily substance appeared after the extracts were taken out of the evaporator.

3.2.6. Purification of metabolites and determination of their structures

A TLC examination was performed for every biotransformation study to compare the substrate and the oily material produced. An adsorbent called silica gel 60 (Merck 107734, 230–400 mesh) extracts the steroids from the oily substance.

These substances were extracted from the column by eluting hexane with progressively higher concentrations of ethyl acetate. Increasing quantities of ethyl acetate in hexane were used as an eluent for two to three hours. Column chromatography separated the steroids obtained as a mixture from the column. The adsorbent used in the column is aluminum oxide 90 active neutral (Merck 101077, activity level I).

The structures of the separated compounds were determined by comparing the melting points, NMR, and FTIR spectra of each substance obtained with the starting materials.

Each biotransformation run was monitored with a control flask. Only uninoculated sterile media and one of the substrates were used for each control flask.

All procedures in the actual biotransformation studies were applied exactly to each control flask. Since no metabolites were observed when TLC was taken from these conical flasks, it was understood that the metabolites obtained from the actual biotransformation experiments were real biotransformation products. Additionally, mold growth in all flasks and changes in the appearance of the medium were monitored during the incubation.

4. EXPERIMENTAL FINDINGS

In this work, DHEA **9** was incubated with *P. olsonii* MRC 500780. The carbon skeleton of DHEA **9** was given in Figure 4.2.

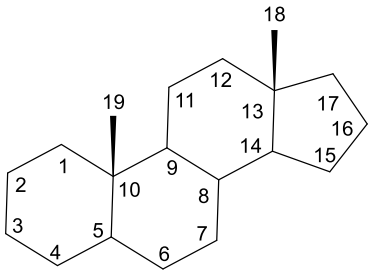


Figure 4.1. The carbon skeleton of the substrate [4].

The incubation of DHEA **9** with *P. olsonii* MRC 500780 for 5 days yielded a brown gum (1017 mg). This gum was then chromatographed on silica gel yielding unchanged substrate (87 mg), androst-4-ene-3,17-dione **5** (8%, 40 mg), 15α-hydroxyandrost-4-ene-3,17-dione **15** (21%, 110 mg), 3β,7β-dihydroxyandrost-5-ene-17-one **21** (8%, 42 mg), 3β,7α-dihydroxyandrost-5-ene-17-one **22** (29%, 153 mg) and 15α,17β-dihydroxyandrost-4-ene-3-one **23** (6%, 32 mg) (Figure 4.2.).

Androst-4-ene-3,17-dione **5** (8%, 40 mg)

m.p. : 163-164 °C, lit., 158-161 °C [41].

IR (ν_{max}/cm^{-1}): 1735, and 1670.

^{1}H NMR (300 MHz, $CDCl_3$): 0.91 (3H, s, 18-H), 1.21 (3H, s, 19-H), 5.75 (1H, s, 4-H).

^{13}C NMR (75 MHz, $CDCl_3$): See Table 4.1.

15α-Hydroxyandrost-4-ene-3,17-dione **15** (21%, 110 mg),

m.p. : 185-186 °C, lit., 178-180 °C [42].

IR (vmax/cm^{-1}): 3435, 1730 and 1670.

^{1}H NMR (300 MHz, $CDCl_3$): 0.96 (3H, s, 18-H), 1.23 (3H, s, 19-H), 4.41 (1H, m, 15β-H), 5.75 (1H, s, 4-H).

^{13}C NMR (75 MHz, $CDCl_3$): See Table 4.1.

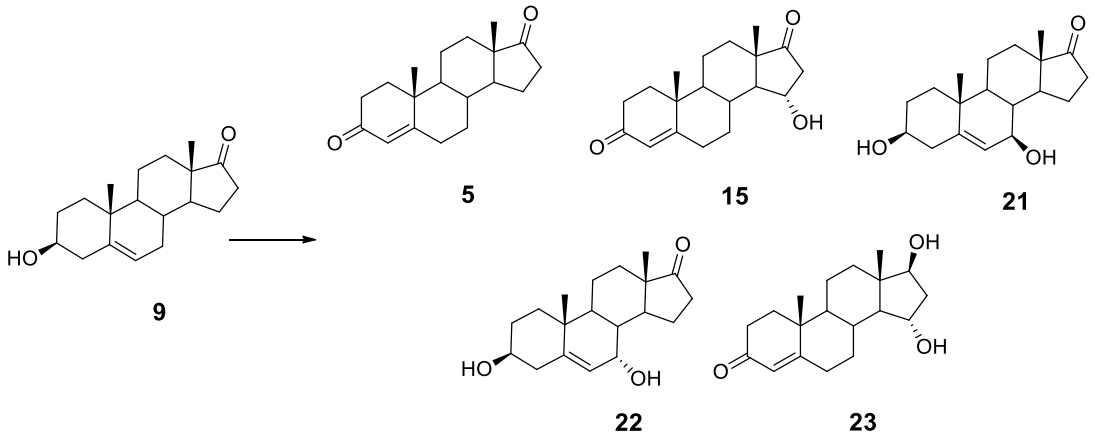


Figure 4.2. Incubation of the substrate with *P. olsonii* MRC 500780.

3β,7β-Dihydroxyandrost-5-ene-17-one **21** (8%, 42 mg),

m.p. : 211-212 °C, lit., 207 °C [43].

IR (v_{max}/cm^{-1}): 3250 and 1740.

^{1}H NMR (300 MHz, $CDCl_3$): 0.88 (3H, s, 18-H), 1.06 (3H, s, 19-H), 3.53 (1H, m, 3α-H), 3.92 (1H, m, 7α-H), 5.30 (1H, bs, 6-H).

^{13}C NMR (75 MHz, $CDCl_3$): See Table 4.1.

3β,7α-Dihydroxyandrost-5-ene-17-one **22** (29%, 153 mg)

m.p. : 201-202 °C, lit., 177 °C [43].

IR (v_{max}/cm^{-1}): 3360 and 1735.

^{1}H NMR (300 MHz, $CDCl_3$): 0.87 (3H, s, 18-H), 0.99 (3H, s, 19-H), 3.54 (1H, m, 3α-H), 3.95 (1H, m, 7β-H), 5.61 (1H, bs, 6-H).

^{13}C NMR (75 MHz, $CDCl_3$): See Table 4.1.

15α,17β-Dihydroxyandrost-4-ene-3-one **23** (6%, 32 mg).

m.p. : 97-98 °C, lit., 93-94 °C [44].

IR (v_{max}/cm^{-1}): 3390, 1655 and 1645.

^{1}H NMR (300 MHz, $CDCl_3$): 0.81 (3H, s, 18-H), 1.22 (3H, s, 19-H), 3.90 (1H, t, J = 8.5 Hz, 17α-H) 4.12 (1H, m, 15β-H), 5.75 (1H, s, 4-H).

^{13}C NMR (75 MHz, $CDCl_3$): See Table 4.1.

Table 4.1. ^{13}C NMR data for DHEA 9 and its metabolites.

C atom	**9**	**5**	**15**	**21**	**22**	**23**
1	37.12	35.61	35.37	36.82	36.86	35.65
2	31.48	33.75	33.60	31.40	30.96	33.85
3	71.47	199.25	199.72	71.19	70.98	199.75
4	42.11	123.98	123.42	41.57	42.46	123.67
5	140.97	170.36	171.26	143.64	146.34	171.35
6	120.83	32.44	32.51	125.44	123.43	32.75
7	31.35	31.17	31.05	72.79	64.14	32.13
8	31.42	35.03	35.17	40.38	37.40	35.28
9	50.13	53.70	53.50	48.16	41.81	53.84
10	36.57	38.53	38.49	36.61	37.09	38.63
11	20.29	20.19	19.97	20.34	19.97	20.49
12	30.71	30.78	31.61	31.16	31.10	36.57
13	47.49	47.38	50.16	47.75	47.06	44.25
14	51.67	50.72	56.91	51.12	44.83	58.36
15	21.82	21.61	69.69	24.16	21.81	72.38
16	35.80	35.72	46.05	35.96	35.73	42.48
17	221.28	220.26	216.59	221.33	221.43	78.62
18	13.49	13.58	15.06	13.55	13.19	12.54
19	19.38	17.25	17.24	19.14	18.18	17.46

5. RESULTS AND DISCUSSIONS

Incubation of DHEA **9** with *P. olsonii* MRC 500780 for 5 days gave five metabolites (Figure 5.1.).

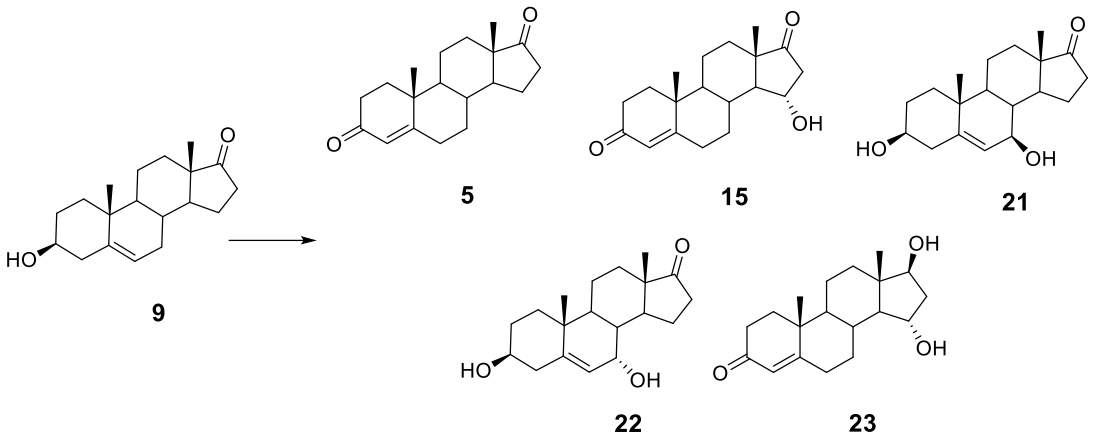


Figure 5.1. Incubation of the substrate with *P. olsonii* MRC 500780.

The first metabolite was identified as androst-4-ene-3,17-dione **5**. The ^{1}H NMR spectrum of **5** lacked the 3α-H resonance of **1** at δ_H 3.45 ppm (1H, m) and had significant downfield shifts for the olefinic proton signal ($\Delta\delta_H$ 0.41 ppm) and the 19-methyl group signal ($\Delta\delta_H$ 0.21 ppm) of **3**, showing that the 5-en-3β-hydroxyl moiety of **1** was changed into a 4-en-3-keto moiety.

The second metabolite was identified as 15α-hydroxyandrost-4-en-3,17-dione **15**. The ^{1}H NMR spectrum of **9** lacked the 3α-H resonance of starting material at δ_H 3.45 ppm (1H, m). It showed significant downfield shifts for the double bond signal ($\Delta\delta_H$ 0.41 ppm) and the 19-methyl group signal ($\Delta\delta_H$ 0.23 ppm), indicating that the 5-en-3β-hydroxyl moiety of **9** was converted into a 4-en-3-keto moiety. The metabolite had typical signals at δH 4.41 ppm (1H, m) and δC 69.69 ppm, suggesting the presence of a 15α-hydroxyl group [44,45]. The ^{13}C NMR spectrum of **15** had downfield shifts (Table 4.1.) for C-14 ($\Delta\delta_C$ 5.24 ppm) and C-16 ($\Delta\delta_C$ 10.25 ppm), further suggesting the presence of a 15α-hydroxyl group.

The third metabolite was identified as 3β,7β-dihydroxyandrost-5-en-17-one **21**. The NMR spectra of **21** had characteristic signals at δ_H 3.92 ppm (1H, m) and δ_C 72.79

ppm, indicating the presence of a 7β-hydroxyl group [44, 45]. The ^{13}C NMR spectrum of **21** had a downfield shift for C-8 ($\Delta\delta_C$ 8.96 ppm), whereas it showed a γ-gauche upfield shift for C-9 ($\Delta\delta_C$ 1.97 ppm), further indicating the presence of a 7β-hydroxyl group.

The fourth metabolite was identified as 3β,7α-dihydroxyandrost-5-en-17-one **22**. The NMR spectra of **22** showed distinctive signals [44, 45] at δ_H 3.95 ppm (1H, m) and δ_C 64.14 ppm, suggesting the presence of a 7α-hydroxyl group. The ^{13}C NMR spectrum of **22** showed a downfield shift for C-8 ($\Delta\delta_C$ 5.98 ppm), whereas it showed a γ-gauche upfield shift for C-9 ($\Delta\delta_C$ 8.32 ppm), further suggesting the presence of a 7α-hydroxyl group.

The fifth metabolite was identified as 15α,17β-dihydroxyandrost-4-en-3-one **23**. The ^1H NMR spectrum of **7** lacked the 3α-H resonance of **9** at δ_H 3.45 ppm (1H, m) and showed significant downfield shifts for the olefinic proton signal ($\Delta\delta_H$ 0.41 ppm) and the 19-methyl group signal ($\Delta\delta_H$ 0.22 ppm) of **9**, indicating at the 5-en-3β-hydroxyl moiety of **9** was changed into a 4-en-3-keto moiety. The NMR spectra of **23** had two unique signals at δ_H 3.90 ppm (1H, t, J = 8.5 Hz) and δ_H 4.12 ppm (1H, m), suggesting the presence of 17β- and 15α-hydroxyl groups [44], respectively. The ^{13}C NMR spectrum of **7** had two new signals at δ_C 72.38 and δ_C 78.62 ppm, further suggesting the presence of 15α- and 17β-hydroxyl groups, respectively [45]

As can be seen from Table 5.2. below, *P. olsonii* MRC 500780 mainly converted the 5-en-3β-hydroxyl moiety of **9** into a 4-en-3-keto moiety and then hydroxylated most of **9** at C-15α, accompanied by a minor reduction at C-17. In addition, *P. olsonii* MRC 500780 also hydroxylated some of **9** at C-7α and C-7β.

Table 5.1. Yields for metabolites.

Substrate	Metabolite	% Yield
DHEA **9**		
	Androst-4-ene-3,17-dione **5**	8
	15α-Hydroxyandrost-4-ene-3,17-dione **15**	21
	3β,7β-Dihydroxyandrost-5-ene-17-one **21**	8
	3β,7α-Dihydroxyandrost-5-ene-17-one **22**	29
	15α,17β-Dihydroxyandrost-4-ene-3-one **23**	6

The conversion of the 5-en-3β-hydroxyl moiety of **9** into a 4-en-3-keto moiety and hydroxylation at C-15α were previously carried out by some *Penicilium* species [32-39]. Hydroxylations at C-7α and C-7β and a reduction at C-17 on **9** were first carried out by *P. olsonii* MRC 500780, although these mentioned reactions are common for other fungi on **9** [16,17,24-27].

In short, it was shown that *P. olsonii* MRC 500780 mainly changed the 5-en-3β-hydroxyl moiety of **9** into a 4-en-3-keto moiety, then hydroxylated it at C-15α and reduced it at C-17 although it hydroxylated some of **9** at C-7α and C-7β. Our work on biotransformations of some other steroids by *P. olsonii* MRC 500780 and different molds is in progress.

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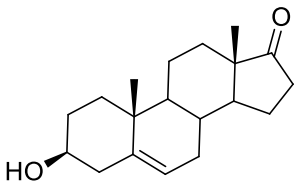
APPENDICES

APPENDIX A. NMR Spectra

APPENDIX A



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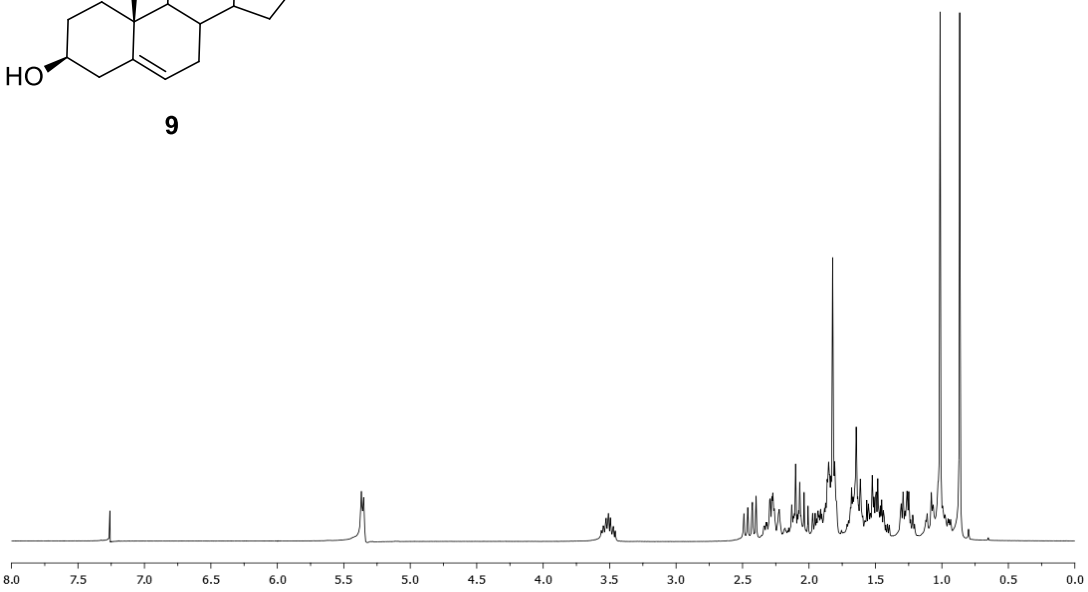


Figure A.1. ^{1}H NMR spectrum of **9**

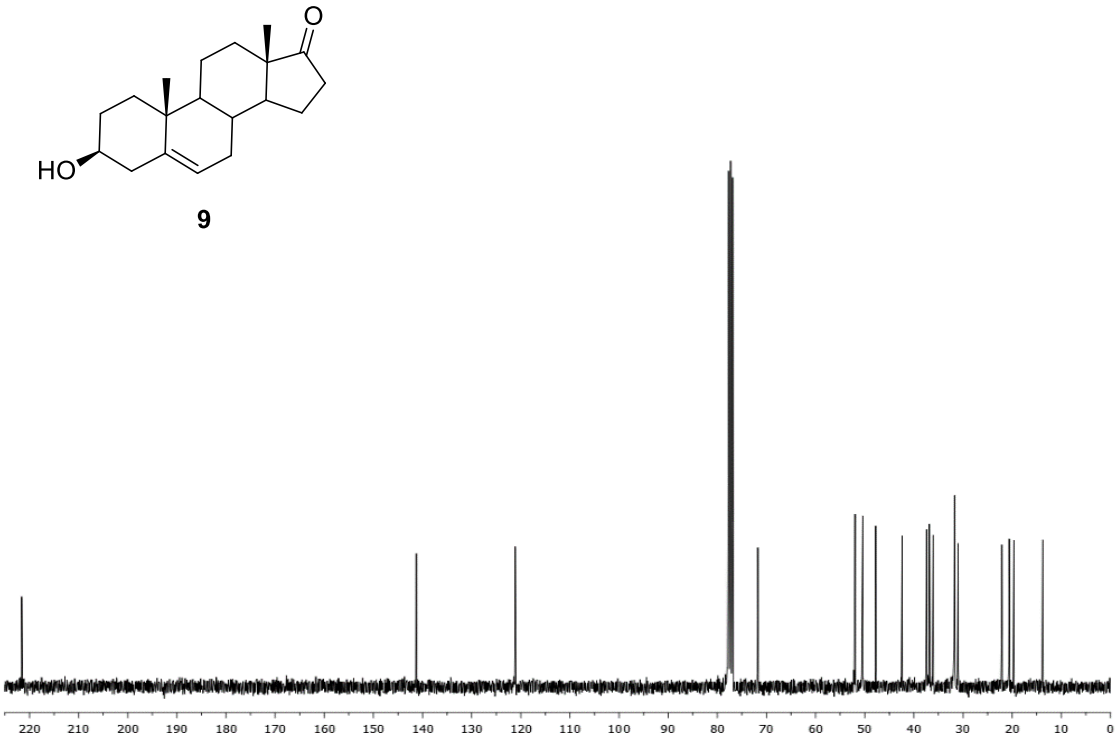


Figure A.2. ^{13}C NMR spectrum of **9**

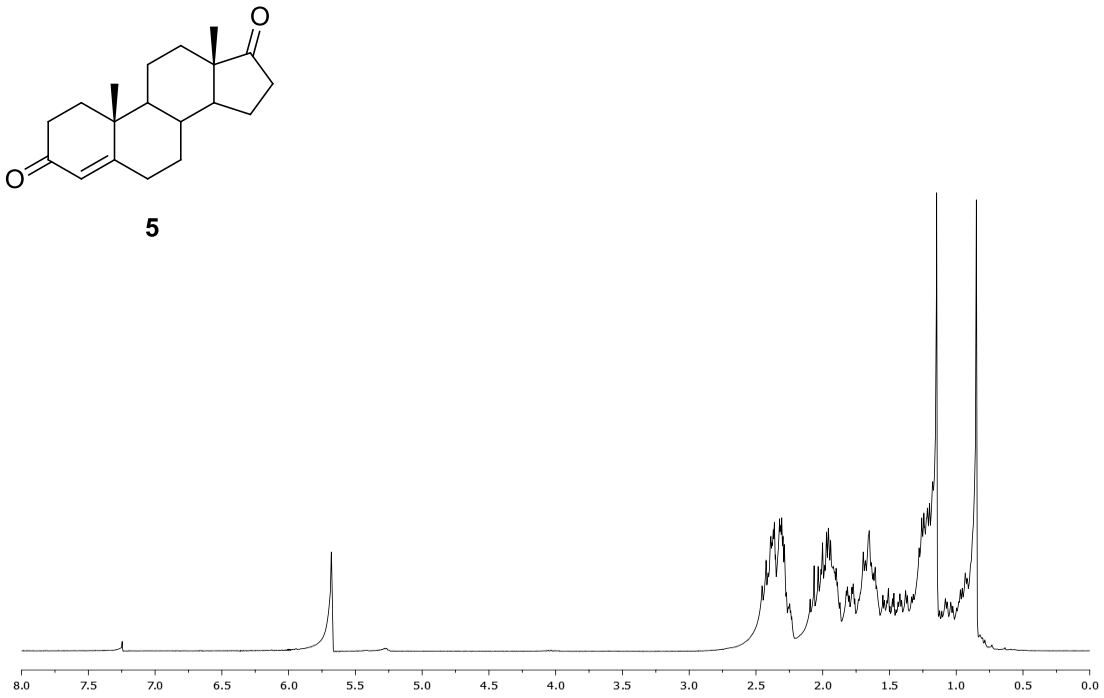


Figure A.3. ^{1}H NMR spectrum of **5**

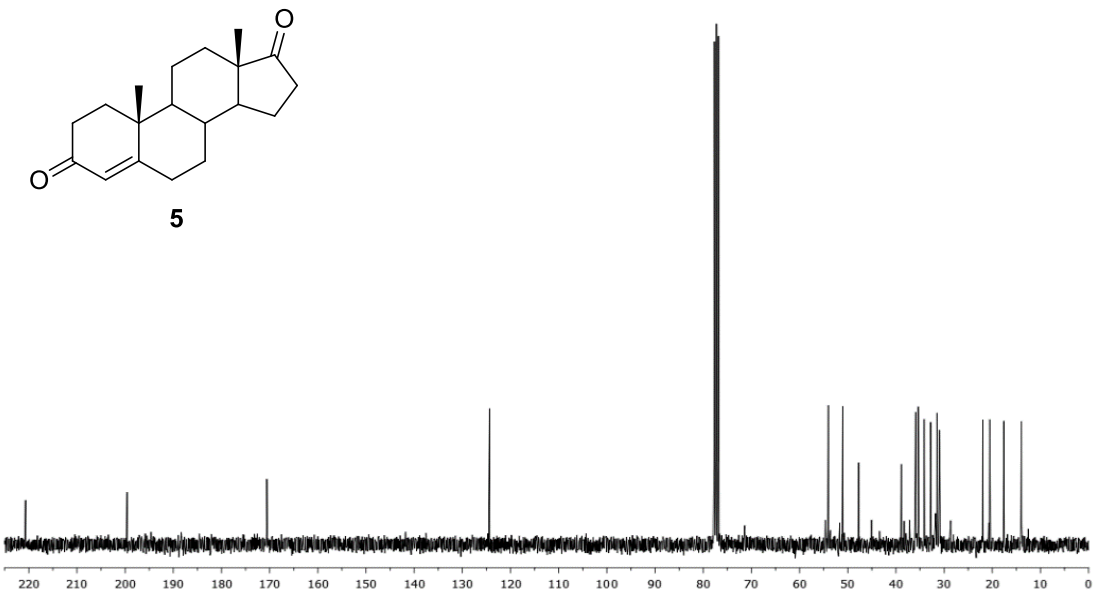


Figure A.4. ^{13}C NMR spectrum of **5**

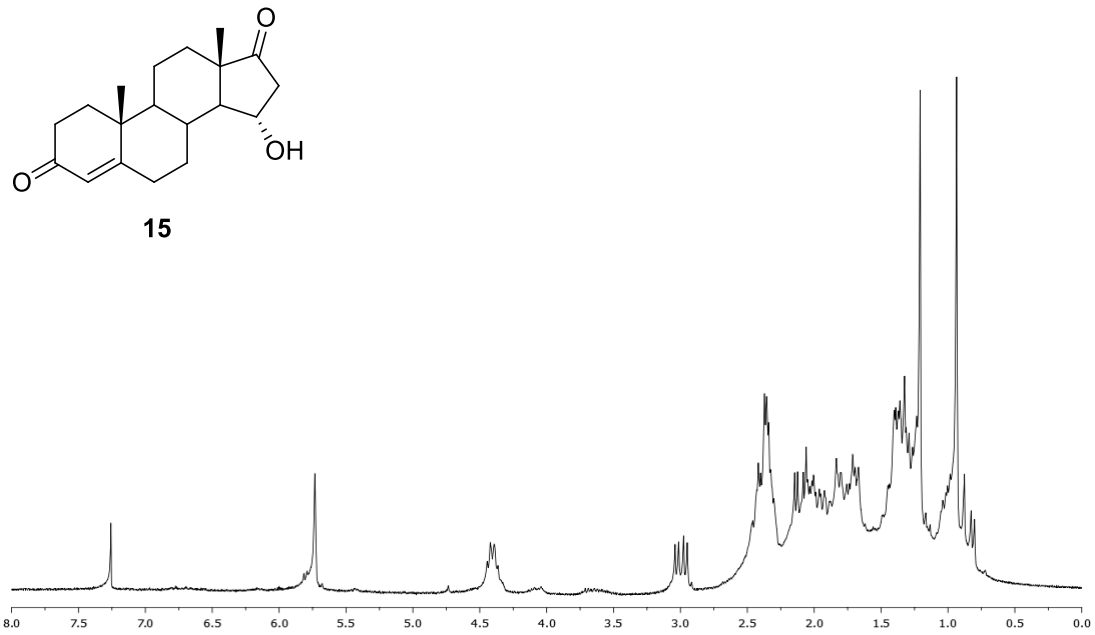


Figure A.5. ^{1}H NMR spectrum of **15**

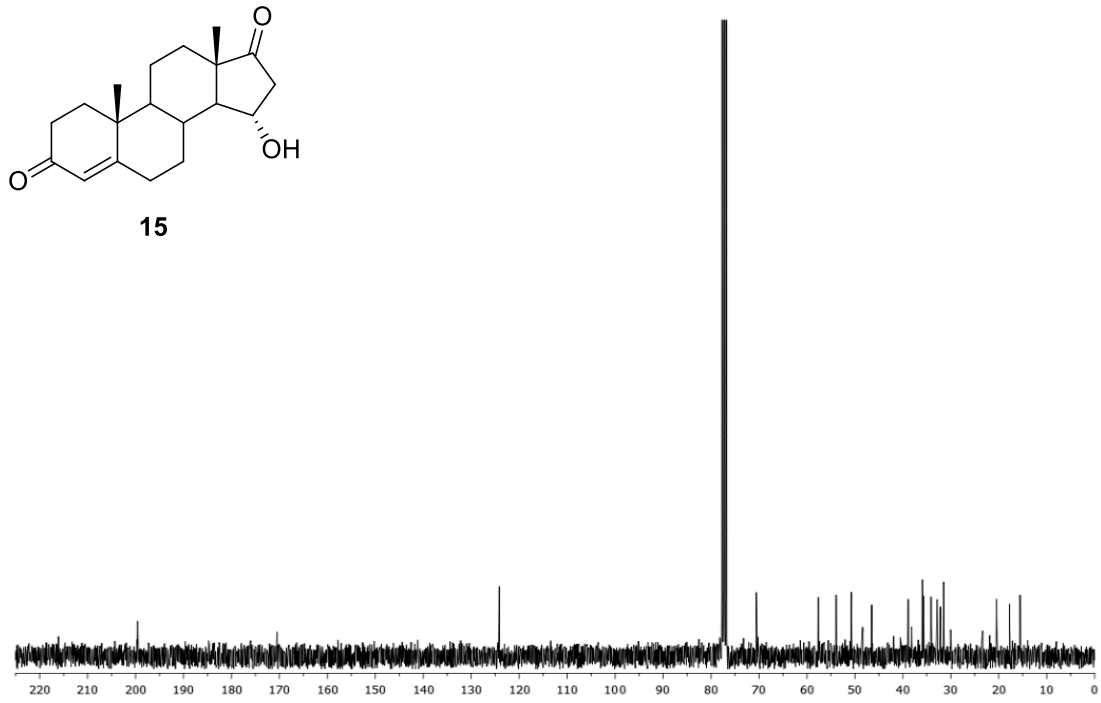


Figure A.6. ^{13}C NMR spectrum of **15**

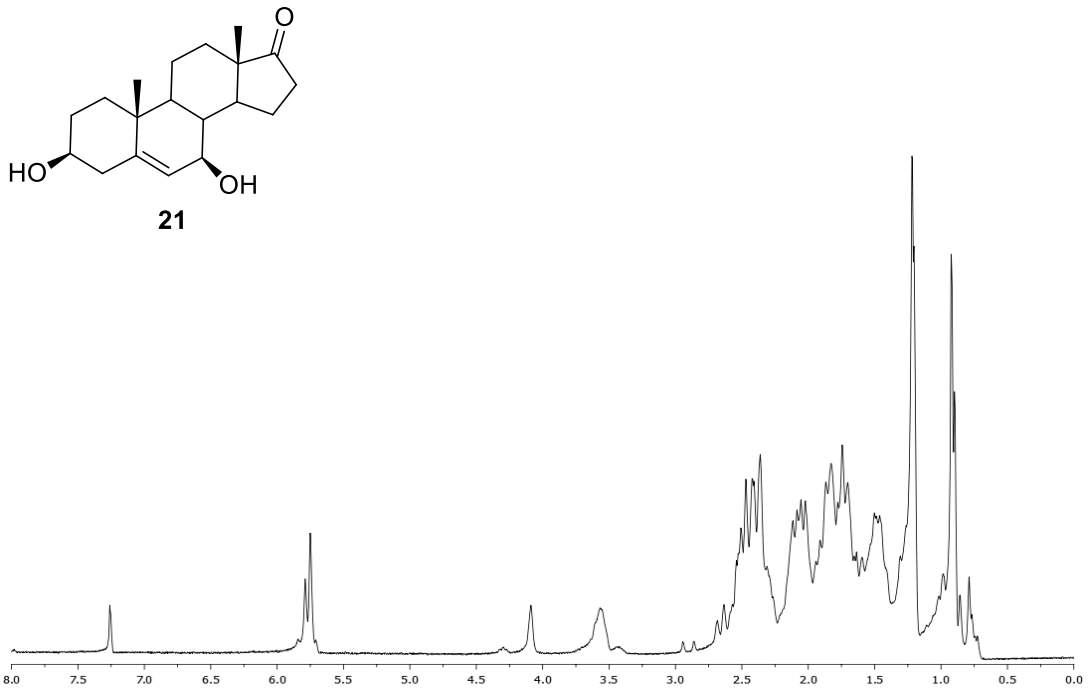


Figure A.7. ^{1}H NMR spectrum of **21**

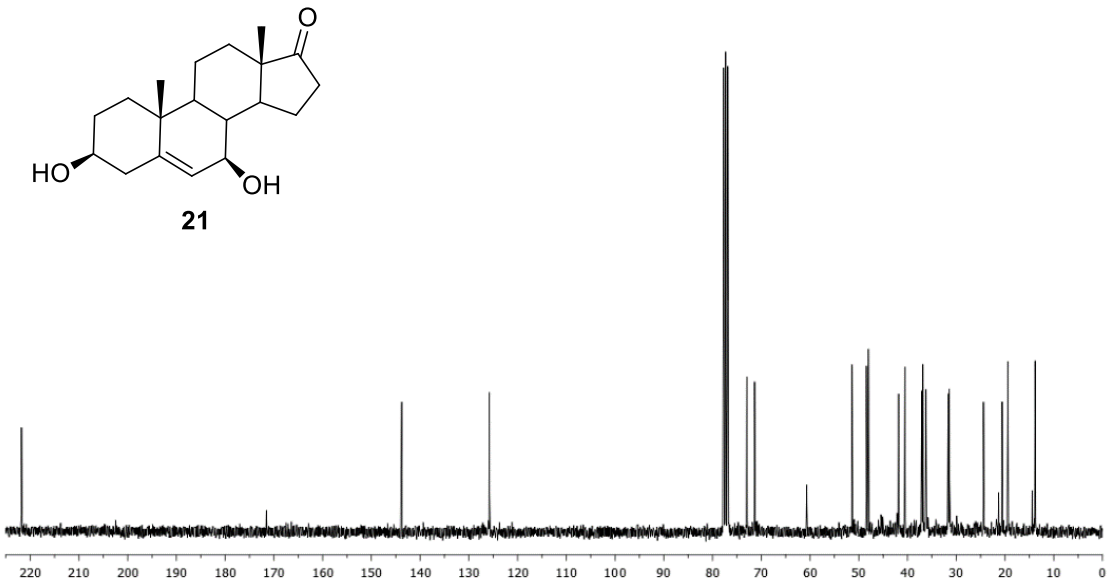


Figure A.8. ^{13}C NMR spectrum of **21**

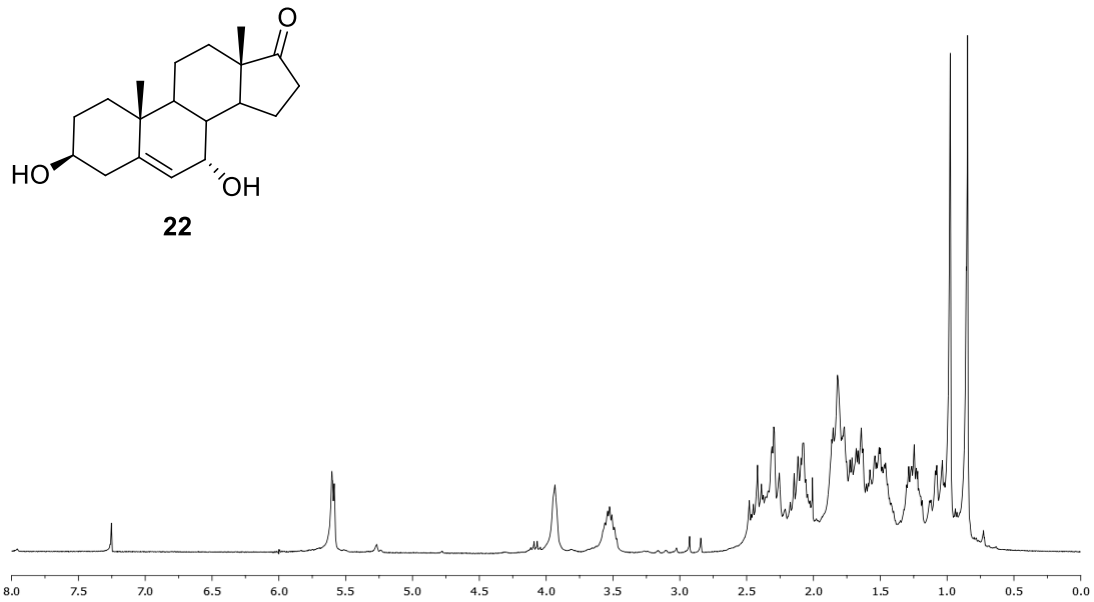


Figure A.9. ^{1}H NMR spectrum of **22**

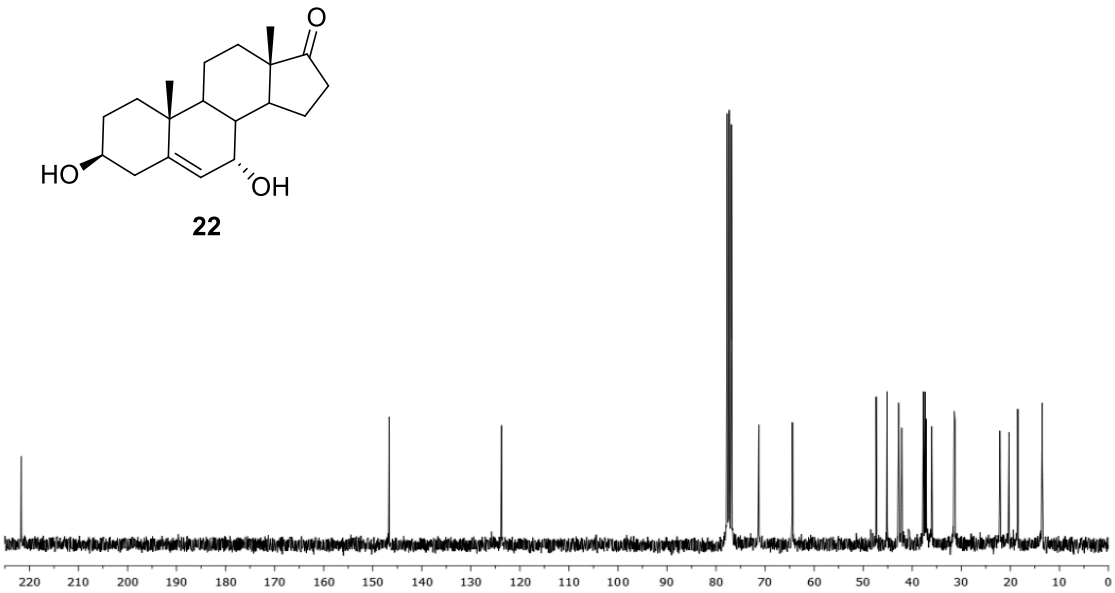
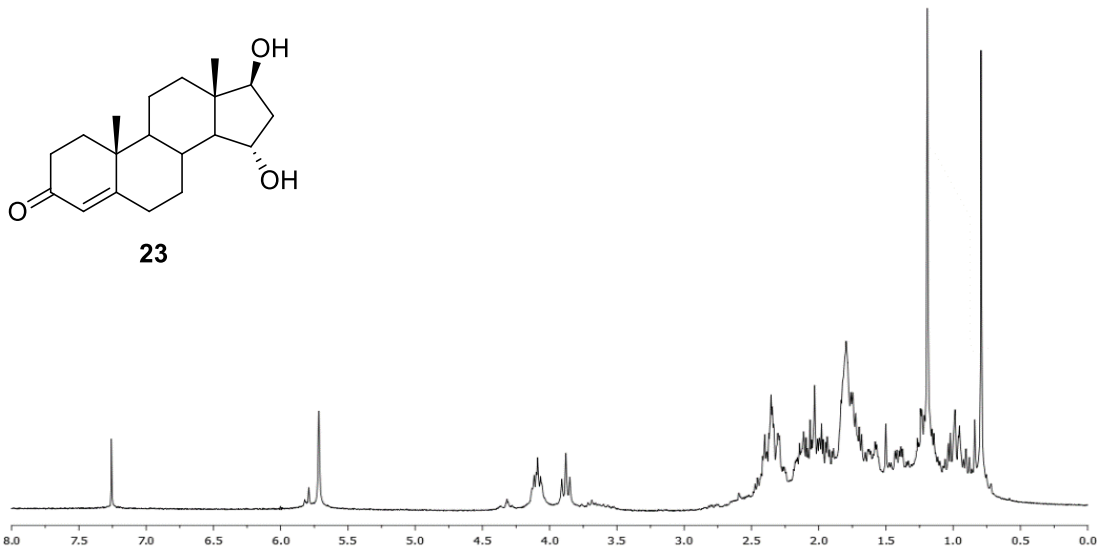
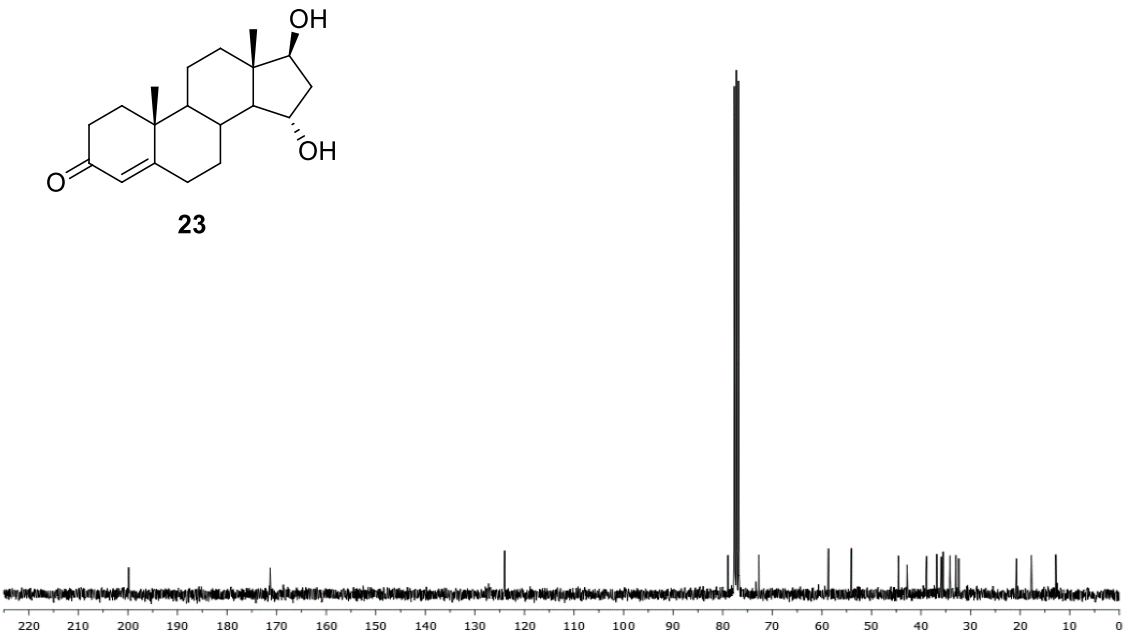


Figure A.10. ^{13}C NMR spectrum of **22**



23

Figure A.11. ^{1}H NMR spectrum of **23**



23

Figure A.12. ^{13}C NMR spectrum of **23**

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