T.R.
SAKARYA UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOTRANSFORMATION OF PREGNENOLONE BY *PENICILLIUM OLSONII*

MSc THESIS

Atheer AL-SAMARRAI

Chemistry Department

Chemistry Program

JANUARY 2024

T.R.
SAKARYA UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOTRANSFORMATION OF PREGNENOLONE BY *PENICILLIUM OLSONII*

MSc THESIS

Atheer AL-SAMARRAI

Chemistry Department

Chemistry Program

Thesis Advisor: Prof. Dr. Kudret YILDIRIM

JANUARY 2024

The thesis work titled "BIOTRANSFORMATION OF PREGNENOLONE BY *PENICILLIUM OLSONII*" prepared by Atheer Al-Sammarrai was accepted by the following jury on 2024/01/04 by unanimously as a MSc Thesis in Sakarya University, Institute of Science and Technology, Chemistry Department.

Thesis Jury

Head of Jury :	**Prof. Dr. İsmail KIRAN** Eskişehir Osmangazi University	.....
Jury Member :	**Prof. Dr. Kudret YILDIRIM** Sakarya University	.....
Jury Member :	**Prof. Dr. Gülnur ARABAC** Sakarya University	.....

DECLARATION OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I declare that the thesis work titled "BIOTRANSFORMATION OF PREGNENOLONE BY *PENICILLIUM OLSONII*" which I have prepared in accordance with the Regulation on Graduate Educa tion and Training of Sakarya University Institute of Science and Technology and the Scientific Research and Publication Ethics Directive of Higher Education Institutions that the thesis is my own, original work; that I acted inaccordance with the above-mentioned regulations and directives at all stages of my work, that I did not take the innovations and results contained in the thesis from elsewhere, that I duly cited the works I used in the thesis as references, that I did not submit this thesis to another scientific committee for academic purposes and title,and that I did not submit this thesis to another scientific committee for academic purposes and title, and that a report was obtained in accordance with the criteria determined by the Institute using the plagiarism software program subscribed by Sakarya University in accordance with Articles 9/2 and 22/2 of the Graduate Education and Training Regulation published in the Official Gazette dated 20.04. 2016, dated 20.04. I declare that in accordance with Articles 9/2 and 22/2 of the Regulation on Graduate Education and Training published in the Official Gazette dated 20.04. 2016, a report has been obtained per the criteria determined by the Institute using the plagiarism software program subscribed by Sakarya University. I accept all kinds of legal responsibility that may arise in a situation contrary to this statement I have made regarding my work.

01/07/2024

Atheer Al-SAMARRAI

To my father and mother, and to those who deserve love ....

ACKNOWLEDGMENTS

My sincere gratitude goes out to my supervisor, Prof. Dr. Kudret YILDIRIM, whose knowledge and experience have been priceless to me. He supervised the study with extreme care and patience, never once withholding his support. I am deeply grateful for his time and effort in helping me to complete this thesis.

I would also like to give special thanks to Assistant Professor Dr. Ali KURU for his guidance and advice through all the stages of my study, which played a significant role in the success of this thesis.

I want to express my gratitude to the faculty members and research assistants of Sakarya University, Faculty of Science, Department of Chemistry, and my laboratory friends, who supported me in every subject I needed during my graduate education.

Finally, I would like to thank my family for their unwavering support throughout my life and for helping me get to where I am today.

Atheer Al-SAMARRAİ

TABLE OF CONTENTS

Page

ABBREVIATIONS

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

SYMBOLS

^{o}C	**:** Celsius degree
cm	**:** Centimeter
Δ	**:** Chemical shift difference
δC	**:** Chemical shift in the ^{13}C NMR spectrum
δH	**:** Chemical shift in ^{1}H NMR spectrum
g	**:** Gram
J	**:** Interaction constant
lit.	**:** Literature
mg	**:** Miligram
MHz	**:** MegaHertz
mL	**:** Mililitre
m	**:** Multiplet
s	**:** Singlet
pH	**:** The negative logarithm of hydrogen ion concentration
t	**:** Triplet signal

LIST OF TABLES

Page

LIST OF FIGURES

Page

BIOTRANSFORMATION OF PREGNENOLONE BY *PENICILLIUM OLSONII*

SUMMARY

Natural products that are not directly involved in the growth and development of living organisms often provide better living conditions for the organisms in which they are found. These compounds are particularly highly regarded because of their effects on other living organisms. Although natural products have many different structures, they are generally grouped as terpenes, alkaloids, steroids, phenolic compounds, specialized carbohydrates, specialized peptides, polyketides, fatty acids and fatty acid derivatives due to some common structural characters in their biosynthesis. Steroids are one of the most important groups of natural products. Cholesterol is a very important steroid that regulates the fluidity of membranes in animals and humans. Cholesterol is the starting material for some important compounds such as steroid hormones, bile acids and vitamin D_3. Steroid hormones are classified into 5 groups: progestagens (progestins), estrogens, androgens, glucocorticoids and mineralocorticoids. Cholesterol is converted into progesterone, which is the starting material for other steroid hormones, over pregnenolone by shortening its side chain through several reactions. Pregnenolone is a important steroid, which is the starting material for progesterone.

Biotransformations are the chemical changes that occur on xenobiotics by biological systems such as microorganisms, microorganism spores, microsomes and cell, tissue and organ cultures containing enzymes or enzymes. Microbial biotransformations with microorganisms are mostly applied with microorganism groups such as molds, microbial algae, yeasts and bacteria. Today, the production of many important chemicals, such as hormones and drugs, is usually carried out by microbial biotransformations instead of classical synthesis methods.

In this work, biotransformation of pregnenolone was carried out with *Penicillium olsonii* MRC 500780. The medium for *Penicillium olsonii* MRC 500780 was distributed in flasks and sterilized in autoclave. After the mold was inoculated into these flasks under sterile conditions, the flasks were left to incubation for 3 days. Then pregnenolone was added under sterile conditions and incubated for another 5 days. After incubation, steroids in the filtered medium were extracted with ethyl acetate. The steroids in the residue obtained by evaporation of the extracts were separated by column chromatography. Incubation of the substrate with *Penicillium olsonii* MRC 500780 afforded progesterone and 15α-hydroxypregn-4-ene-3,20-dione. The structure determinations of the metabolites were carried out by comparing the melting points, NMR and IR spectra of the substrate with those of metabolites and the subsrate.

PREGNENOLONUN *PENICILLIUM OLSONII* İLE BİYOTRANSFORMASYONU

ÖZET

Tüm canlılar yaşamları boyunca doğal substratları olmayan çeşitli kimyasal maddelerle karşılaşırlar ve bunlar ksenobiyotik olarak adlandırılır. Enzimlerin veya enzim içeren biyolojik sistemlerin ksenobiyotikler üzerinde meydana getirdiği kimyasal değişikliklere biyotransformasyon denir.

Enzimler canlı organizmalardaki hemen hemen tüm reaksiyonları aktivasyon enerjisini (EA) düşürerek gerçekleştirirler. Enzimler reaksiyon dengesine ulaşma süresini azaltmalarına rağmen, reaksiyon tarafından tüketilmezler.

Enzimler çok etkili katalizörler olduklarından ve kullanıcıları için bazı avantajlar sağladıklarından, örneğin, enzimatik bir reaksiyon için reaksiyon hızı 108-1010 kat hızlanır ve bu 1012 değerini bile aşabilir. Enzimler amino asitlerden yapıldıkları ve tamamen parçalanabildikleri için çevresel olarak kabul edilebilirdir. Diğer kimyasal reaktiflerin çoğu çevresel sorunlara neden olsa da, enzimler genellikle ılımlı koşullar altında (yaklaşık pH 7, 30 °C ve 1 atm) hareket eder ve bu da izomerizasyon, rasemizasyon, yeniden düzenlemeler, ayrışma gibi bazı sorunları en aza indirir. Enzimler birbirleriyle uyumlu olduklarından, enzimler genellikle aynı veya benzer koşullar altında çalışırlar. Bu nedenle, çok enzimli sistemler kullanılarak bir ortamda birden fazla reaksiyon gerçekleştirilebilir. Bazı enzimler sadece belli bileşikler ile etkileşirken bazı enzimler yüksek substrat teloransı gösterirler ve çok çeşitli doğal veya doğal olmayan bileşikler ile etkileşebilir. Enzimler geniş bir reaksiyon spektrumu gösterir ve neredeyse bilinen her reaksiyona eşdeğer bir enzimatik reaksiyon vardır.

Enzimler kemoselektif, regioselektif ve enantioselektif moleküllerdir. Enzimler kemoselektif oldukları için genellikle sadece tek bir tür fonksiyonel grup üzerinde etkili olduklarından ve diğer fonksiyonlar değişmeden kaldığından, enzimatik reaksiyonlar genellikle daha temiz olma eğilimindedir. Enzimler regioselektif olduklarından, aynı substrat molekülünün farklı bölgelerinde kimyasal olarak bulunan fonksiyonel gruplar arasında ayrım yapabilirler. Enzimler, karmaşık üç boyutlu yapıları nedeniyle bu avantajlara sahip olabilirler. Enzimler enantiyoselektif oldukları ve sadece L- amino asitlerden oluştukları için kiral katalizörlerdir. Bu nedenle, substrat molekülü üzerindeki her türlü kiralite enzimler tarafından algılanır. Enzimin etkisi ile prokiral bir substrat, kiral bir ürüne dönüşebilir ve rasemik bir substrattaki her iki enantiyomer ile genellikle farklı oranlarda reaksiyona girerek kinetik bir resulosyona neden olabilir.

Biyotransformasyonlar genellikle ya izole enzim sistemleri ya da mikroorganizmalar tarafından gerçekleştirilir. Ticari olarak mevcut 300'den fazla izole enzim sistemi olduğu düşünülmektedir. İlgili birçok enzim sistemi membrana bağlı olduğundan ve izole edilmesi zor olduğundan, biyotransformasyonlar için genellikle mikroorganizmalar kullanılır. Biyotransformasyonlar için genellikle kullanılan yaygın mikroorganizma grupları küfler, mayalar, bakteriler ve mikroalglerdir.

Mikroorganizmalarla gerçekleştirilen biyotransformasyonlar mikrobiyal biyotransformasyonlar olarak adlandırılır. Mikrobiyal biyotransformasyonlar, çevre dostu olmaları, daha hızlı, daha ucuz olmaları ve erlenden fabrika fermentörüne kadar çeşitli ortamlarda gerçekleştirilebilmeleri gibi önemli avantajları nedeniyle biyotransformasyonlar için çoğunlukla mikroorganizmalar kullanılmaktadır. Mikrobiyal biyotransformasyonlar için mikroorganizmalar serbest olarak veya uygun yüzeylere immobilize edilerek kullanılabilmektedir. Mikrobiyal biyotransformasyonlar çoğunlukla küfler, mikrobiyal algler, mayalar ve bakteriler ile uygulanmaktadır. Küfler ve bakteriler gibi mikroorganizmalar, spesifik olmayan enzimlerini kullanarak birçok doğal veya sentetik substratta çok farklı kimyasal değişiklikler üretebilirler. Mikrobiyal biyotransformasyonlar sitokrom P-450 enzimleri tarafından gerçekleştirilir ve en önemli mikrobiyal biyotransformasyonlardan biri mikrobiyal hidroksilasyondur. Mikrobiyal hidroksilasyon reaksiyonu ilk olarak 1952 yılında gözlemlenmiştir. Bazı kortikosterodların ilaç olarak sentezi sırasında, klasik kimyasal yöntemlerle fonksiyonel gruplardan uzak bir konuma oksijen fonksiyonu yerleştirmek çok uzun ve pahalı bir süreçti. Bu sorunun Rhizopus arrhizus küfü aracılığıyla söz konusu pozisyonda bir hidroksilasyonla ortadan kaldırılması, tüm dikkatleri mikrobiyal biyotransformasyonlara yöneltti. Bu keşif ve mikrobiyal hidroksilasyonun öneminin anlaşılmasının ardından, birçok farklı mikroorganizma ile birçok farklı kimyasal türünün biyotransformasyonları gerçekleştirilmiştir.

Tüm canlılarda bulunan, büyüme ve gelişmelerinde doğrudan rol almayan organik bileşiklere ikincil metabolitler veya doğal ürünler denir. Bu kimyasallar genellikle içinde bulundukları organizmalar için daha iyi yaşam koşulları sağlar. Doğal ürünler, özellikle diğer canlılar üzerindeki etkileri nedeniyle çok dikkat çeken bileşiklerdir. Doğal ürünler tüm canlılarda bulunmasına rağmen, daha çok bitkiler, mikroorganizmalar, mantarlar ve böceklerde gözlenir. Doğal ürünler çok sayıda ve çok farklı yapıda olmalarına rağmen, biyosentezlerindeki bazı ortak benzerlikler nedeniyle genellikle terpenler, alkaloidler, steroidler, fenolik bileşikler, özelleşmiş karbonhidratlar, özelleşmiş peptitler, poliketitler, yağ asitleri ve yağ asidi türevleri olarak gruplandırılırlar.

Steroidler doğal ürünlerin en önemli gruplarından biridir. C-3'de bir hidroksil grubu ile D halkasındai farklı uzunluklara ve yapılara sahip alifatik yan zincirleri içeren steroidler steroller olarak adlandırılır. Mantarlardaki ergosterol, bitkilerdeki stigmasterol, hayvanlar ve insanlardaki kolesterol en yaygın ve en iyi bilinen sterollerdir.

Kolesterol hayvan ve insan membranlarının akışkanlığını düzenleyen önemli bir moleküldür. Kolesterol biyosentezi oldukça karmaşıktır ve 3 aşamada öetlenebilir. İlk aşamada önce 3 asetil koenzim A bileşiğinden once mevalonat sentezlenir ve daha sonra izoentenil difosfat olarak da bilinen izopentenil pirofosfata dönüştürülür. İkinci aşamada izopentenil pirofosfat bileşiği önce dimetilalil pirofosfata izomerize edilir. Dimetilallil pirofosfatın bir izopentenil pirofosfat ile kondensasyonu sonucunda bir geranil pirofosfat oluşur. Geranil pirofosfatın başka bir izopentenil pirofosfat ile kondenzasyonu farnesil pirofosfat oluşumu ile sonuçlanır. İki adet farnesil pirofosfatın bir indirgeme reaksiyonunda kondenzasyonu sonucunda bir triterpen ve diğer triterpenlerin başlangıç maddesi olan skualen oluşur. Son adımda, skualenin epoksidasyonu ile skualen epoksit oluşur ve bu bileşiğin daha sonra halkalaşması ilk halkalı bileşik olarak lanosterol oluşumuna yol açar. Lanosterol ise daha sonar 19 reaksiyon üzerinden kolesterol bileşiğine dönüştürülür.

Kolesterol aynı zamanda safra asitleri, D3 vitamini ve steroid hormonlar gibi bazı çok önemli bileşiklerin başlangıç maddesidir. Kolesterol, yan zinciri kısaltılarak pregnenolon üzerinden diğer steroid hormonların başlangıç maddesi olan progesterone dönüştürülür. Pregnenolon, progesteron için başlangıç materyali olan önemli bir steroitdir. Steroid hormonlar; glukokortikoidler, mineralokortikoidler, progestajenler (progestinler), östrojenler ve androjenler olmak üzere 5 gruba ayrılır. Glukokortikoidler ve mineralokortikoidler kortikosteroidler olarak bilinirken, androjenler ve östrojenler ve progestajenler cinsiyet hormonları olarak bilinir.

Eşey hormonlarının temel işlevleri üreme organlarının büyümesini ve gelişmesini, ikincil eşey özellikleri ve üreme döngüsünü düzenlemektir. Bazı eşey hormonlarının güçlü anabolik etkileri de vardır; kemik, kas ve deri gibi birçok dokunun gelişimini teşvik eder ve metabolizmayı korurlar. Progestagenlerin en yaygın ve aktif temsilcisi olan progesteron, doğal bir steroid hormondur ve aynı zamanda memelilerde diğer steroid hormonların sentezlendiği bir ara bileşiktir. Androjenler erkek omurgalılarda, östrojenler ise dişi omurgalılarda aktif olan eşey hormonlarıdır. Östrojenler ise androjenlerden sentezlenir.

Bu çalışmada pregnenolon *Penicillium olsonii* MRC 500780 ile 5 gün boyunca inkübe edildi. Bir litre besiyeri hazırlandı ve 250 mL'lik 10 erlene eşit olarak dağıtıldı. Erlenlerdeki besiyeri daha sonra otoklavda sterilize edildikten sonra bu erlenlere P. olsonii inoküle edildi. Erlenler 28 ºC'de 3 gün boyunca çalkalayıcıda inkübe edildikten sonar DMF içindeki substrat aseptik olarak erlenlere eklendi. Tüm erlenler 5 gün daha inkübe edildikten sonar küf miselleri vakum altında gerçekleştirilen filtrasyon ile besiyerinden ayrıldı. Miseller etil asetat ile yıkandıktan sonar filtrat etil asetat ile ekstrakte edildi. Ekstraktlar susuz sodyum sülfat ile kurutulup evaporasyon ile uçurulduğunda yağımsı kahverengi bir madde elde edildi. Bu yağımsı madde daha sonra silika jel 60 üzerinde kolon kromatografisine maruz bırakıldı. Kromatografi çalışmasından progesteron ve 15α-hidroksipregn-4-en-3,20- dion metabolitleri elde edidi. Bu metabolitlerin yapıları substrata ait erime noktası, NMR ve IR spektrumlarının metabolitlere ait erime noktaları, NMR ve IR spektrumlarının karşılaştırılması ile belirlendi.

1. INTRODUCTION

Organic compounds in living organisms are generally analyzed in three different groups. In the first group, there are compounds such as amino acids and monosaccharides, which are elements of primary metabolism that directly affect the growth and development of all living things and are called primary metabolites. The second group includes high molecular weight molecules (biopolymers) such as proteins, cellulose and lignins. The last group includes secondary metabolites, which are elements of secondary metabolism that do not have a direct effect on the growth and development of living organisms. Compounds in this last group are also known as natural products. Although natural products are not directly involved in the growth and development of living organisms, they usually provide some advantages to the organisms that contain them. In addition, natural products are often compounds that stand out for their effects on other living things. Although natural products are found in every living things, they are generally more common in microorganisms, fungi, plants and insects [1-3].

Although natural products have different structures and are numerous, they are generally divided into groups such as terpenes, alkaloids, steroids, phenolic compounds, specialized carbohydrates, specialized peptides, polyketides, fatty acids and fatty acid derivatives due to some similarities in their biosynthesis [1-3].

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 cyclopentane rings (rings A, B and C) and one cyclohexane ring (ring 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. Some steroids carry double bonds in the A or B rings [4-5].

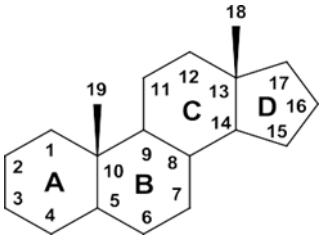


Figure 1.1. Cyclopentanoperhydrophenanthrene ring [4-5].

Steroids carrying a hydroxyl group at C-3 and different aliphatic side chains ttached to D rings are known as sterols. Ergosterol in fungi, stigmasterol in plants and cholesterol 1 in animals and humans are the most common and best known examples of sterols [4-5].

Cholesterol 1 is an important molecule regulating the fluidity of animal and human membranes. The biosynthesis of cholesterol 1 is long and complex and can be analyzed in 3 steps. In the first step, mevalonate is first synthesized from 3 acetyl coenzyme A, which is then converted to isopentenyl pyrophosphate, also known as isoentenyl diphosphate. In the second step, the isopentenyl pyrophosphate is first isomerized to dimethylallyl pyrophosphate. As a result of the condensation of dimethylallyl pyrophosphate with an isopentenyl pyrophosphate, one geranyl pyrophosphate is formed. Condensation of geranyl pyrophosphate with another isopentenyl pyrophosphate results in the formation of farnesyl pyrophosphate. As a result of the condensation of two farnesyl pyrophosphates in a reduction reaction squalene is formed. Squalene is a triterpene and the starting material of other triterpenes. In the final step, squalene epoxide is formed by the epoxidation of squalene and the subsequent ringing of this compound leads to the formation of lanosterol as the first cyclic compound. Lanosterol is converted into cholesterol compound through 19 reactions [4-5].

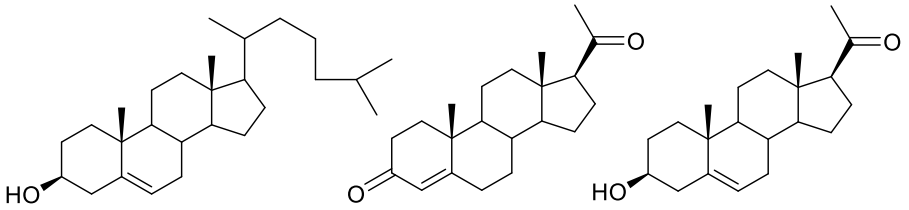
Cholesterol 1 is the starting material for some important compounds such as bile acids, vitamin D3, and steroid hormones. Due to their hydrophobic and hydrophilic nature, bile acids derived from cholesterol 1 play important roles in the digestion and absorption of lipids in the small intestine. Bile acids are also important in the prevention of stone formation in the gallbladder and excess cholesterol 1 in the body is removed by bile acids [4-5].

Vitamin D3 is produced by the spontaneous isomerization of 7-dehydrocholesterol after its cleavage from the B-ring by solar UV radiation under the skin. Vitamin D3 is

converted to calcitriol by hydroxylation in the liver and kidney. Calcitriol is involved in the regulation of calcium and phosphorus metabolism together with calcitonin and parathormone [4-5].

Steroid hormones are divided into five groups: glucocorticoids, mineralocorticoids, progestagens (progestins), estrogens and androgens. The most important representative of glucocorticoids is the hormone cortisol and glucocorticoids have an effect on carbohydrate, lipid and protein metabolism. Glucocorticoids also have important anti-inflammatory and immunosuppressive properties. The most important representative of mineralocorticoids is the hormone aldosterone and mineralocorticoids are involved in the regulation of water and mineral metabolism. Progestagens, androgens and estrogens are also called sex hormones. The main functions of sex hormones are the regulation of the growth and development of reproductive organs, secondary sexual characteristics and the reproductive cycle. Progesterone 2, which is also known as pregn-4-ene-3,20-dione, is the most common and active representative of progestagens. Progesterone 2 is both a natural steroid hormone and an intermediate compound from which other steroid hormones in mammals are synthesized. In humans, progesterone 2 is involved in the preparation of the endometrium for pregnancy. The release of progesterone 2 at the beginning of pregnancy protects the mother and the developing fetus by preventing the start of a new reproductive cycle. Androgens act in male vertebrates, whereas estrogens act in female vertebrates. Androgens are the starting materials from which estrogens are synthesized. In the body, androgens are synthesized mostly in the testes (testes) and occasionally in the adrenal cortex [4-5].

Cholesterol 1 is converted into pregnenolone 3 by shortening its side chain through several reactions. Pregnenolone 3, which is also known as 3β-hydroxypregn-5-ene-20-one, is a steroid hormone and a steroid that is the starting material for the compound progesterone [4-5]. The structures of cholesterol 1 and its derivatives progesterone 2 and pregnenolone 3 are given in Figure 1.2.



1 **2** **3**

Figure 1.2. Structures of some important steroids [4-5]

2. LITERATURE SURVEY

2.1. Biotransformations

Throughout their lives, living organisms interact with chemicals that are foreign to them. These chemicals are called xenobiotics. The chemical changes on xenobiotics by enzymes or enymes within biological systems such as microorganisms, microorganism spores, microsomes, cell, tissue and organ cultures are called biotransformations [6]. The oxidation of ethanol to acetic acid by bacteria in vinegar production and the conversion of sugars to ethanol by brewer's yeast are among the first recorded examples of biotransformation in history. Most of the enzymes used in biotransformations are purified from biological sources, while the rest are still only commercially available [6, 7].

Although there are some dogmatic preconceptions that enzymes only affect their substrates in their natural environment and that they are expensive and highly sensitive, this is not the case for many enzymes [7].

Enzymes provide many benefits to their users because they perform their functions very quickly. Reactions involving enzymes can take place 108-1010 times faster than reactions without enzymes [7].

Enzymes, which are of protein nature, are considered to be environmentally friendly because they are completely degradable in nature, unlike some heavy metals that function as catalysts and many reagents in classical synthesis processes [7].

Since enzymes mostly catalyze under mild conditions where the temperature is between 20-40 °C and the pH is in the range of 5-8, they rarely give undesirable side reactions such as racemization, degradation, conversion and isomerization that result from the application of known synthesis methods [7].

Some enzymes can perform chemical changes in many natural or synthetic compounds due to their broad substrate spectrum [7].

In multienzyme systems, enzymes acting under similar or identical conditions can carry out reactions in metabolic pathways in the same environments [7].

Since enzymes can carry out a large number and different types of reactions, there is an enzymatic reaction corresponding to almost every synthetic reaction [7].

Enzymes are regioselective and stereoselective due to their complex three-dimensional structure and can even separate functional groups of different parts of their substrates. In addition to this, enzymes are also chemoselective, which affect only a certain functional group and not others. This feature prevents the formation of by-products [7].

Enzymes are also enantioselective chiral biomolecules as they contain only L-amino acids. Therefore, enzymes can convert a prochiral substrate into an enantiomer by acting on it. Enzymes can also separate racemic mixtures by affecting only one of the enantiomers in a racemic mixture. With these advantages, enzymes can easily perform reactions that are difficult or impossible to perform with other methods [7].

However, the use of enzymes can also cause some undesirable situations. For example, since there is only one enantiomeric form of an enzyme, this enzyme can only react with a specific enantiomer [7].

Some enzymes are very sensitive to changes in parameters such as temperature and pH that affect their activity. For example, since enzymes are proteins, parameters such as temperature and pH can be changed slightly to accelerate an enzymatic reaction because they can cause denaturation of the enzyme [7].

Although water is the most active medium for enzymes, many organic compounds are hardly soluble in water. Performing an enzymatic reaction in an organic solvent may result in denaturation of the enzymes, which have protein nature, resulting in loss of activity [7].

When there are excessive amounts of substrates or products in the reaction medium, enzymes can be subjected to inhibition, which is characterized by loss of activation [7].

Most enzymes require some specific molecules called cofactors to catalyze their reactions and in order for such enzymes to be functional, cofactors must be present in the reaction medium and constantly replenished. The major disadvantages are that cofactors are unstable and expensive compounds and some synthetic equivalents can not be used in their place [7].

Enzymes can cause some allergic reactions. These allergic reactions can be avoided by using enzymes as carefully as other chemicals [7].

2.2. Microbial Biotransformations

Purified enzymes or whole-cell systems are usually used for biotransformations. As whole-cell systems, microorganisms and cell, tissue and organ cultures of plant or animal origin are often used [7].

Purification processes for enzymes are quite expensive and difficult, and enzymes can be damaged during purification. In addition to this, as most enzymes are unstable outside the cell, cofactors must be provided and constantly renewed. Scientists to generally prefer whole-cell systems for biotransformations due to the problems above [7].

Biotransformation studies involving whole-cell systems are often applied with microbial cells. Since the growth and development rate of microbial cells is higher than other cells, biotransformation studies using microorganisms can be carried out in a shorter time. Microbial cells are much more mechanically stable than other cells as they are smaller in size and contain durable cell walls. The fact that microbial cells adapt to their environment much more easily is a feature that benefits their users. In addition to this, microbial cells can perform chemical changes on a much larger number and different types of substrates than the cells of other living organisms [7].

Microbial biotransformations have become important elements of biotechnology due to their many advantages over known synthesis methods [6-7]. Since microbial cells can be genetically modified, their use in biotechnology is increasing [8].

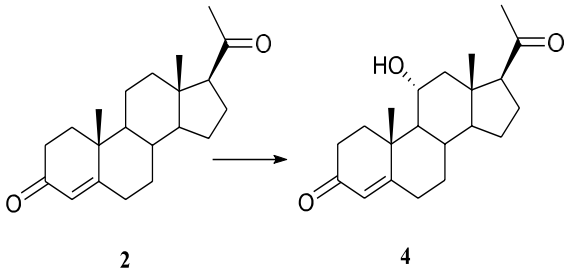
Microbial cells contain non-specific enzymes that can make many different chemical changes on a large number and different types of natural or synthetic substrates [6-7].

Unlike other known synthetic methods, microbial biotransformations can generally be performed under very mild conditions such as 1 atm pressure and room temperature. Microbial biotransformations are considered to be environmentally friendly while most reagents used other known synthetic methods cause significant irreversible damage to our environment [6, 7].

Microbial biotransformations can be carried out at lower costs and in shorter times compared to other known synthetic methods [6-7].

Microbial biotransformations usually do not require a specific functional group to be present near the site of the biotransformation [6, 7]. For example, microbial hydroxylations occur at sites far from functional groups [6].

Microbial hydroxylations are among the most valuable and widespread microbial biotransformation reactions [6, 7]. The importance of microbial hydroxylations was first recognized in 1952 during the synthesis of cortical steroids [9]. The addition of an oxygen function to the C-11 position of these steroids, which has no functional groups in the immediate vicinity, was a very expensive and time-consuming process with the methods available at that time. Solving this problem in a single step with the mold Rhizopus arrhizus made microbial biotransformations very popular in a very short time [6]. In this reaction, progesterone 2 was converted into 11α-hydroxyprogesterone 4 (Figure 2.1).



2 4

Figure 2.1. First microbial hydroxylation [9].

Following the discovery and understanding of the importance of microbial hydroxylation, biotransformations of many different groups of chemical substances have been realized with many different microorganisms. Nowadays, the production of important chemical substances such as hormones and drugs is generally realized by microbial biotransformations instead of classical synthesis methods [10].

2.3. Steroid Biotransformations by Molds

Steroid biotransformations by molds have been applied in the production of many important drugs due to the high regioselectivity and stereoselectivity of fungal enzymes [10-14]. Enourmous efforts have been made to get known microbial

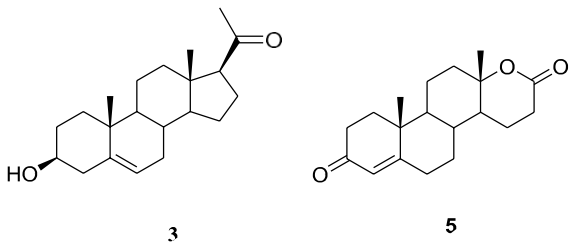
biotransformations with higher yields and to identify new reactions and microorganisms [10].

Microbial steroid biotransformations have been carried out with many molds and these biotransformations have yielded interesting results 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 [10-14].

One of the most widely distributed and well-known fungi, *Penicillium* can be found in a wide variety of territories, including soil, plants, air, indoor spaces, and different food items. It is widely distributed and significantly affects human existence economically. As its primary purpose in nature is the breakdown of organic materials, certain species produce a wide variety of mycotoxins and cause disastrous rots as pre- and postharvest infections on food crops. Certain *Penicillium* species have beneficial effects as well. For example, the food sector uses certain species to make fermented sausages and specialty cheeses like Roquefort and Camembert. Because of their capacity to degrade, species are being tested to see whether they can produce novel enzymes. Its greatest contribution to medicine and source of renown is the discovery of penicillin, which transformed the way bacterial illnesses are treated. Since then, a large number of additional extrolites with a variety of uses have been found, supporting the idea that *Penicillium* or one of its products has an impact on every human [15].

Biotransformation of different steroids with *Penicillium* species with pregnenolone 3 generally resulted in Baeyer-Villiger oxidation, microbial hydroxylation, microbial hydrogenation, and dehydrogenation [10-14]. There are some studies in the literature on the incubation of 3 with *Penicillium* species [16-20].

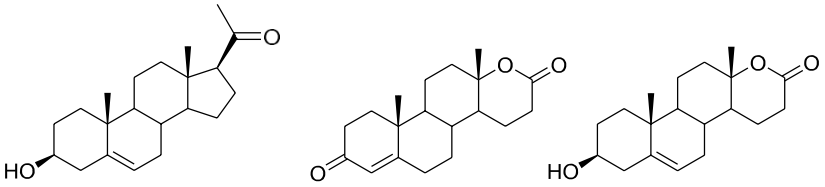
Microbial biotransformation of pregnenolone 3 with P. vinaceum AM110 (Figure 2.2) only yielded 17a-Oxa-d-homo-androst-4-ene-3,17-dione 5 [16].



3 5

Figure 2.2. Incubation of 3 with P. vinaceum AM110 [16].

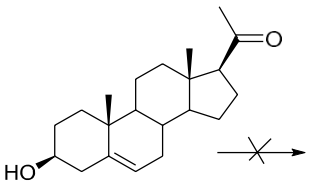
Incubation of pregnenolone 3 with P. lanosocoeruleum KCH 3012 [17], P. simplicissimum WY134-2 [18] and P. camemberti AM83 [19] afforded 17a-Oxa-d-homo-androst-4-ene-3,17-dione 5 and 3β-hydroxy-17a-oxa-d-homo-androst-5-en-17-one 6 (Figure 2.3).



3 5 6

Figure 2.3. Incubation of 3 with some *Penicillium* species [17-19].

Microbial biotransformation of pregnenolone 3 with P. citreo-viride (Figure 2.4) only yielded the unreacted substrate [20].



3

Figure 2.4. Incubation of 3 with P. citreo-viride [20].

2.4. The Aim of this Work

In this work, biotransformation of pregnenolone 3 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

The media and glassware to be used in the experiments were sterilized in an autoclave (Nüve OT 40 L) at 121 ºC for 20 minutes. Nucleon brand Class II Type sterile cabinet was used for the addition of molds to the slopes and for the addition of molds and substrates to the sterilized flasks. A shaker (Gerhardt THO 500 Laboshake) was used for incubations. Infrared spectra were taken with a Perkin Elmer Spectrum Two spectrometer. 1H NMR spectra were taken by a Varian Mercury 300 NMR spectrometer at 300 MHz using deuterochloroform (CDCl3) as solvent and tetramethylsilane standard as on internal signal. 13C NMR spectra were taken by a Varian Mercury 300 NMR spectrometer at 75 MHz using deuterochloroform as solvent. Melting points of steroids were measured with an Electrothermal IA 9200 melting point apparatus and not repeated.

The biotransformation experiment and column chromatography were monitored by thin layer chromatography (TLC). TLC technique has been carried out using an ethyl acetate-hexane (1:1) solvent solution and 0.25 mm thick silica gel plates (Merck silica gel GF254). The steroids were monitored by immersing the steroids in TLC plates in p-anisaldehyde-sulfuric acid reagent and heating them for 3 minutes at 120°C.

P. olsonii MRC 500780 isolate used in the study was obtained from the Culture Collection of TUBITAK, Marmara Research Center, Food Technology and Research Institute.

Pregnenolone 3 was obtained from Sigma-Aldrich. PDA and agar used for the preparation of microbial slope cultures, all chemicals used for mold media and all solvents used in the studies were obtained from Merck.

3.2. Preparation of Agar Slunts

The medium for microbial slope cultures was prepared by boiling 11.7 g of potato dextrose agar (PDA) and 2.7 g of agar in 300 mL of distilled water. The medium was

added to the test tubes up to half of their capacity without cooling and the mouths of the tubes were covered with sufficient amount of cotton wool. The tubes were wrapped with aluminum foil and sterilized in autoclave at 121 ºC for 20 minutes. The liquified medium in the tubes was allowed to cool at an average inclination of 45 degrees before solidification and sterile agar slunts were prepared.

3.3. Preparation and Replenishment of Agar Slunts

The mold obtained from the culture collection was added to 3 sterile Agar Slunts under sterile conditions and allowed to grow at room temperature. The best-developed molds in these agar slunts were transferred to new sterile agar slunts under sterile conditions every 2 weeks. By repeating the same process 2 times, the freshest and best-developed mold available was used for the biotransformation experiment.

3.4. Preparation of the Medium for *P. olsonii*

The medium for P.olsonii was prepared by dissolving and mixing glucose (15 g) and peptone (5 g) in 0.5 L distilled water [21].

3.5. Biotransformation experiment

The medium prepared for *P.olsonii* was divided into 5 separate 250 mL flasks and sterilized in the autoclave. *P.olsonii* was added to these flasks under sterile conditions and incubated in a shaking incubator (150 rpm) at 28 °C for 3 days. After incubation, the substrate (0.5 g) was dissolved in DMF (5 mL) under sterile conditions and transferred to the flasks in equal volumes. The flasks were then incubated at 25°C for another 5 days (150 rpm).

The biotransformation experiment was monitored by means of a control flask. A control flask was prepared by adding substrate to a flask containing only sterile medium. All the same procedures in the main biotransformation experiment were carried out in the control flask. After these procedures, the results of the biotransformation study were considered as acceptable as no metabolites were observed when TLC was taken from the control flask.

3.6. Separation and Structure Determination of Metabolites

After incubation, the medium was filtered from the mycelia by filtration and the mycelia were washed with some ethyl acetate (250 mL). Steroids in the filtrate were extracted 3 times with ethyl acetate (0.5 L). Anhydrous sodium sulfate was added to the collected extracts and the extracts were dehydrated. An oily substance was then obtained by evaporating the extracts in an evaporator. A TLC work was performed comparing this oily substance with the substrate. Based on the results of the TLC work, steroids in the oily substance were separated by column chromatography on silica gel 60 (Merck 107734, 230-400 mesh). Column chromatography was performed with increasing concentrations of ethyl acetate in n-hexane as eluent. Structure determinations were performed by comparing the melting point, NMR and IR spectra of each steroid with those of the substrate.

4. EXPERIMENTAL FINDINGS

In this work, pregnenolone 3 was incubated with *P. olsonii* MRC 500780. The carbon skeleton of pregnenolone 3 was given in Figure 4.1.

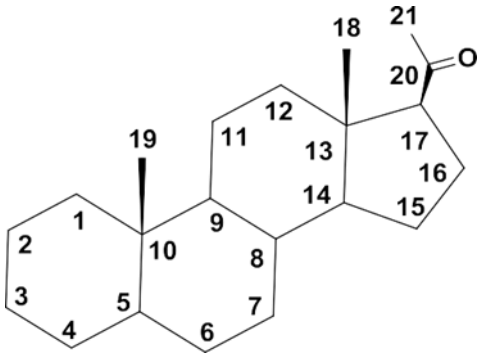


Figure 4.1. The carbon skeleton of the substrate.

The incubation of pregnenolone 3 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 (94 mg), pregn-4-ene-3,20-dione 2 (10%, 49 mg) and 15α-hydroxypregn-4-ene-3,20-dione 7 (58%, 304 mg) (Figure 4.2).

Pregn-4-ene-3,20-dione **2** (10%, 49 mg)

m.p. : 119-120 °C, lit., 114-118 °C [22].

IR (ν_{max}/cm^{-1}): 2920, 1700 and 1665.

^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (3H, s, 18-H), 1.21 (3H, s, 19-H), 2.14 (3H, s, 21-H),

2.54 (1H, t, J = 8.5 Hz, 17α-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 the substrate and its metabolites.

C atom	**3**	**2**	**7**
1	37.04	35.42	35.19
2	31.25	33.68	33.78
3	71.22	199.45	199.60
4	41.94	123.63	123.63
5	140.71	171.08	171.14
6	120.97	32.55	32.66
7	31.53	31.60	31.84
8	31.60	35.25	34.99
9	49.69	53.35	53.62
10	36.28	38.34	38.50
11	20.84	20.76	20.50
12	38.56	38.37	38.74
13	43.78	43.71	44.37
14	56.64	55.73	62.53
15	22.54	24.12	73.06
16	24.27	22.54	35.10
17	63.43	63.23	60.75
18	13.01	13.12	14.48
19	19.19	17.12	17.35
20	209.55	209.31	208.49
21	31.38	31.38	31.44

15α-Hydroxypregn-4-ene-3,20-dione **7** (58%, 304 mg).

m.p. : 235-236 °C, lit., 237-238 °C [23].

IR (v_{max}/cm^{-1}): 3490, 2950, 1730 and 1695.

^{1}H NMR (300 MHz, CDCl3): 0.72 (3H, s, 18-H), 1.22 (3H, s, 19-H), 2.13 (3H, s, 21-H), 2.82 (1H, m, 17α-H), 4.11 (1H, m, 15β-H), 5.74 (1H, s, 4-H)

^{13}C NMR (75 MHz, CDCl3): See Table 4.1.

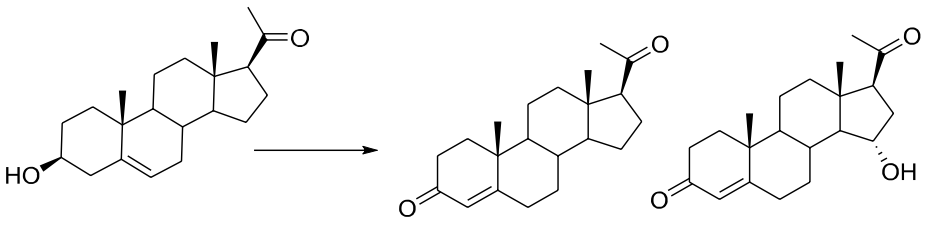
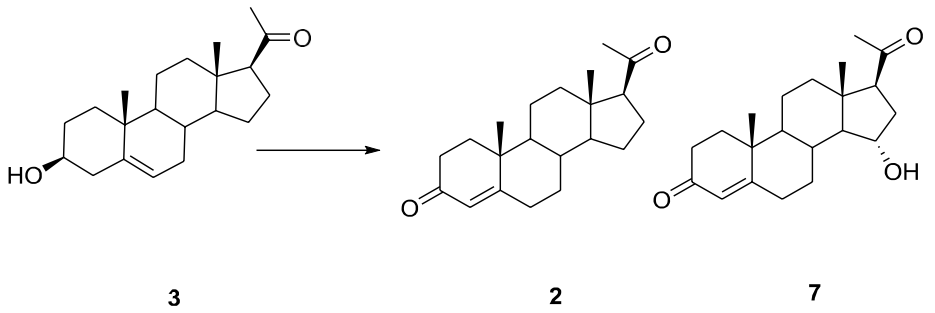


Figure 4.2. Incubation of 3 with *P. olsonii* MRC 500780.

5. RESULTS AND DISCUSSIONS

Incubation of pregnenolone **3** with *P. olsonii* MRC 500780 for 5 days gave two metabolites (Figure 5.1.).



3 **2** **7**

Figure 5.1. Incubation of 3 with P. *olsonii* MRC 500780.

The first metabolite was identified as pregn-4-ene-3,20-dione **2**. The ^{1}H NMR spectrum of **2** lacked the 3α-H resonance of **3** at δ_H 3.51 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 **3** was changed into a 4-en-3-keto moiety.

The second metabolite was identified as 15α-hydroxypregn-4-ene-3,20-dione **7**. The ^{1}H NMR spectrum of **7** lacked the 3α-H resonance of starting material at δ_H 3.51 ppm (1H, m). It showed significant downfield shifts for the double bond signal ($\Delta\delta_H$

0.40 ppm) and the 19-methyl group signal ($\Delta\delta_H$ 0.22 ppm), indicating that the 5-en-3β-hydroxyl moiety of **3** was converted into a 4-en-3-keto moiety. The metabolite showed typical signals [24,25] at δ_H 4.11 ppm (1H, m) and δ_C 73.06 ppm, suggesting the presence of a 15α-hydroxyl group. The ^{13}C NMR spectrum of **7** had downfield shifts (Table 5.1) for C-14 ($\Delta\delta_C$ 5.89 ppm) and C-16 ($\Delta\delta_C$ 10.83 ppm), further suggesting the presence of a 15α-hydroxyl group.

Table 5.1. ^{13}C NMR data for the substrate and its metabolites.

C atom	**3**	**2**	**7**
1	37.04	35.42	35.19
2	31.25	33.68	33.78
3	71.22	199.45	199.60
4	41.94	123.63	123.63
5	140.71	171.08	171.14
6	120.97	32.55	32.66
7	31.53	31.60	31.84
8	31.60	35.25	34.99
9	49.69	53.35	53.62
10	36.28	38.34	38.50
11	20.84	20.76	20.50
12	38.56	38.37	38.74
13	43.78	43.71	44.37
14	56.64	55.73	62.53
15	22.54	24.12	73.06
16	24.27	22.54	35.10
17	63.43	63.23	60.75
18	13.01	13.12	14.48
19	19.19	17.12	17.35
20	209.55	209.31	208.49
21	31.38	31.38	31.44

As can be seen from Table 5.2. below, *P. olsonii* MRC 500780 mainly converted the 5-en-3β-hydroxyl moiety of 3 into a 4-en-3-keto moiety and hydroxylated most of 3 at C-15α position.

Table 5.2. Yields for metabolites.

Substrate	Metabolite	% Yield
Pregnenolone **3**		
	Preg-4-ene-3,20-dione **2**	10
	15α-Hydroxypregn-4-ene-3,20-dione **7**	58

Reports on the conversion of the 5-en-3β-hydroxyl moiety of 3 into a 4-en-3-keto moiety and hydroxylation at C-15α are very common by most fungi [10-14]. Reports on the conversion of the 5-en-3β-hydroxyl moiety of 3 into a 4-en-3-keto moiety by *Penicillium* species were very common [10-14,16-19] whilst hydroxylation at C-15α on 3 was first carried out by *P. olsonii* MRC 500780.

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

REFERENCES

[1] Hanson, J. R. 2003. Natural Products: The Secondary Metabolites, The Royal Society of Chemistry, Cambridge, 1-2.

[2] Mann, J. 1994. Chemical Aspects of Biosynthesis, First edition, Oxford University Pres. New York. 2-4.

[3] Clayden, J., Greeves, N., Warren, S., Wothers, P. 2001. Organic Chemistry. First edition. Oxford University Pres. Oxford. 1413-1414.

[4] Onat, T., Emerk, K., Sözmen, E. Y. 2002. İnsan Biyokimyası, Palme Yayıncılık, Ankara, 481-659

[5] Keha, E. E., Küfrevioğlu, Ö. İ. 2005. Biyokimya, Dördüncü Baskı, Aktif Yayınevi, Erzurum, 185-188.

[6] Hanson, J.R. 1995. An Introduction to Biotransformations in Organic Chemistry. W.H. Freeman Spektrum, 1-62. New York.

[7] Faber, K. 2003. Biotransformations in Organic Chemistry, Fifth Edition. Springer- Verlag. Berlin. 1-407.

[8] Demain A.L. 2000. Small Bugs. Big Business: The Economic Power of the Microbe, Biotechnology Advances, 18, 499-514.

[9] Peterson, D.H., Murray, H.C., Epstein, S.H., Reineke, L.M., Weintraub, A., Meister, P.D., Leigh, H.M. 1952. Microbiological Oxygenation of Steroids, I. Introduction of Oxygen at Carbon-11 of Progesterone. J. Am. Chem. Soc., 74, 5933–5936.

[10] Nassiri-Koopaei N., Faramarzi M.A. 2015. Recent developments in the fungal transformation of steroids. Biocatalysis and Biotransformation, 33,1- 28.

[11] Bhatti H,N., Khera R.A. 2012. Biological transformations of steroidal compounds: a review. Steroids. 77, 1267-1290.

[12] Donova, M.V., Egorova, O.V., Microbial Steroid Tranformation: Current State and Prospects, Applied Microbiology and Biotechnology, 94, 1423–1447, 2012.

[13] Fernandes, P., Cruz, A., Angelov, B., Pinheiro, H. M., Cabral, J. M. S., Microbial Conversion of Steroids Compounds: Recent Developments, Enzyme and Microbial Technology, 32, 688–705, 2003.

[14] Mahato, S. B., Garai, S., Advances in Microbial Steroid Biotransformation, Steroids, 62, 332–345, 1997.

[15] Visagie, C.M., Houbraken, Frisvad, J. C J., Hong, S-B., Klaassen, C.H.W., Perrone, G., Seifert K. A., Varga, J., Yaguchi T., Samson, R. A. Identification and nomenclature of the genus *Penicillium*, Studies in Mycology, 78: 343–371, 2014.

[16] Panek, A., Łyczko, P., Świzdor, A. (2020). Microbial modifications of androstane and androstene steroids by *Penicillium vinaceum*. Molecules, 25(18), 4226.

[17] Swizdor, A. (2013). Baeyer-villager oxidation of some C19 steroids by *Penicillium lanosocoeruleum*. Molecules, 18(11), 13812–13822.

[18] Yang, B., Wang, Y., Chen, X., Feng, J., Wu, Q., Zhu, D., Ma, Y. (2014). Biotransformations of steroids to testololactone by a multifunctional strain *Penicillium simplicissimum* WY134-2. Tetrahedron, 70(1), 41-46.

[19] Kołek, T., Szpineter, A., Świzdor, A. (2009). Studies on Baeyer–Villiger oxidation of steroids: DHEA and pregnenolone D-lactonization pathways in *Penicillium camemberti* AM83. Steroids, 74(10-11), 859-862.

[20] Liu, H. M., Li, H., Shan, L., & Wu, J. (2006). Synthesis of steroidal lactone by *Penicillium citreo-viride*. Steroids, 71(11-12), 931-934.

[21] Bartmańska, A., Dmochowska-Gładysz, J., & Huszcza, E. (2005). Steroids' transformations in *Penicillium notatum* culture. Steroids, 70(3), 193-198.

[22] Numazawa, M., Nagaoka, M., Kunitama, Y., Synthetic transformations using iodotrimethylsilane: regiospecific deoxygenation of the dihydroxyacetone moiety at C-17 of corticoid steroids, Journal of the Chemical Society. Chemical communications, 1984, 1, 31 – 32.

[23] Peart, P. C., McCook, K. P., Russell, F. A., Reynolds, W. F., Reese, P. B. (2011). Hydroxylation of steroids by *Fusarium oxysporum, Exophiala jeanselmei* and *Ceratocystis paradoxa*. Steroids, 76(12), 1317-1330.

[24] Kirk, D.N., Toms, H.C., Douglas, C., White, K.A., Smith, K.E., Latif, S., and Hubbard, R.W.P. 1990. A survey of the high-field 1H NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds, Journal of the Chemical Society, Perkin Transactions 2, 1567-1594.

[25] Blunt, J.W., and Stothers, J.B. 1977. 13C NMR spectra of steroids a survey and commentary. Organic Magnetic Resonance, 9, 439-464.

APPENDICES

APPENDIX A. NMR Spectra

APPENDIX A

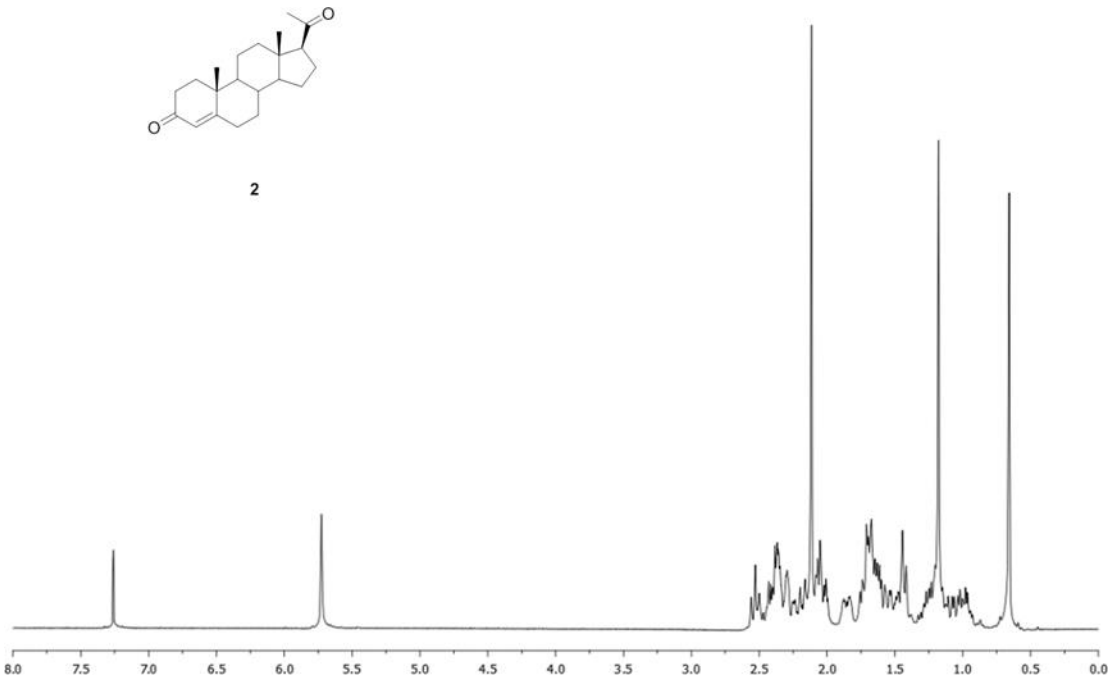


Figure A.1. ^{1}H NMR spectrum of compound **2**.

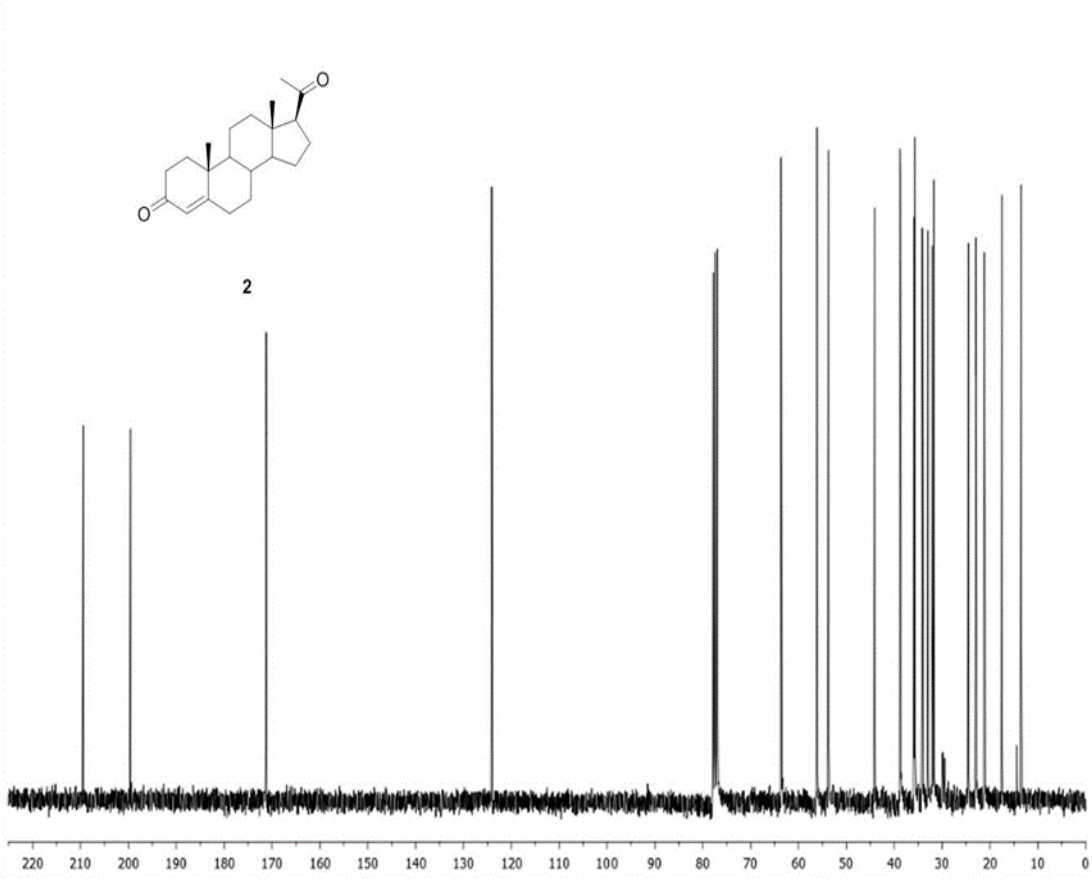


Figure A.2. ^{13}C NMR spectrum of compound 2

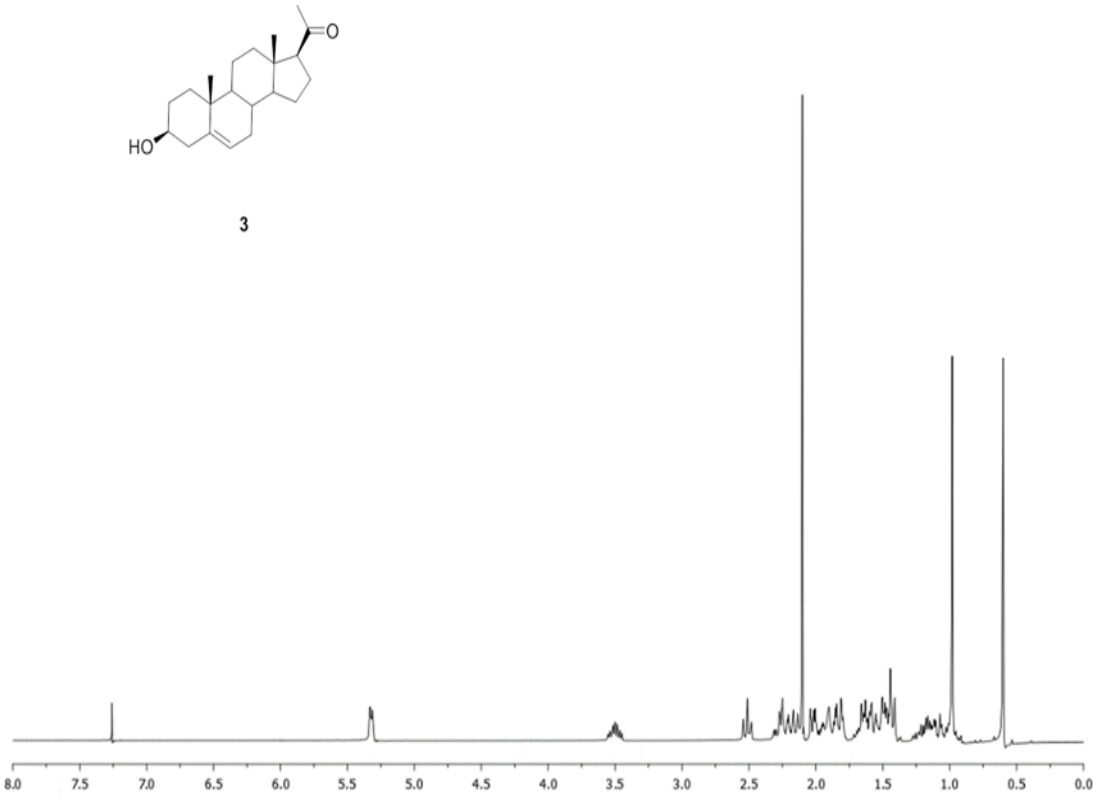


Figure A.3. ^{1}H NMR spectrum of compound **3**

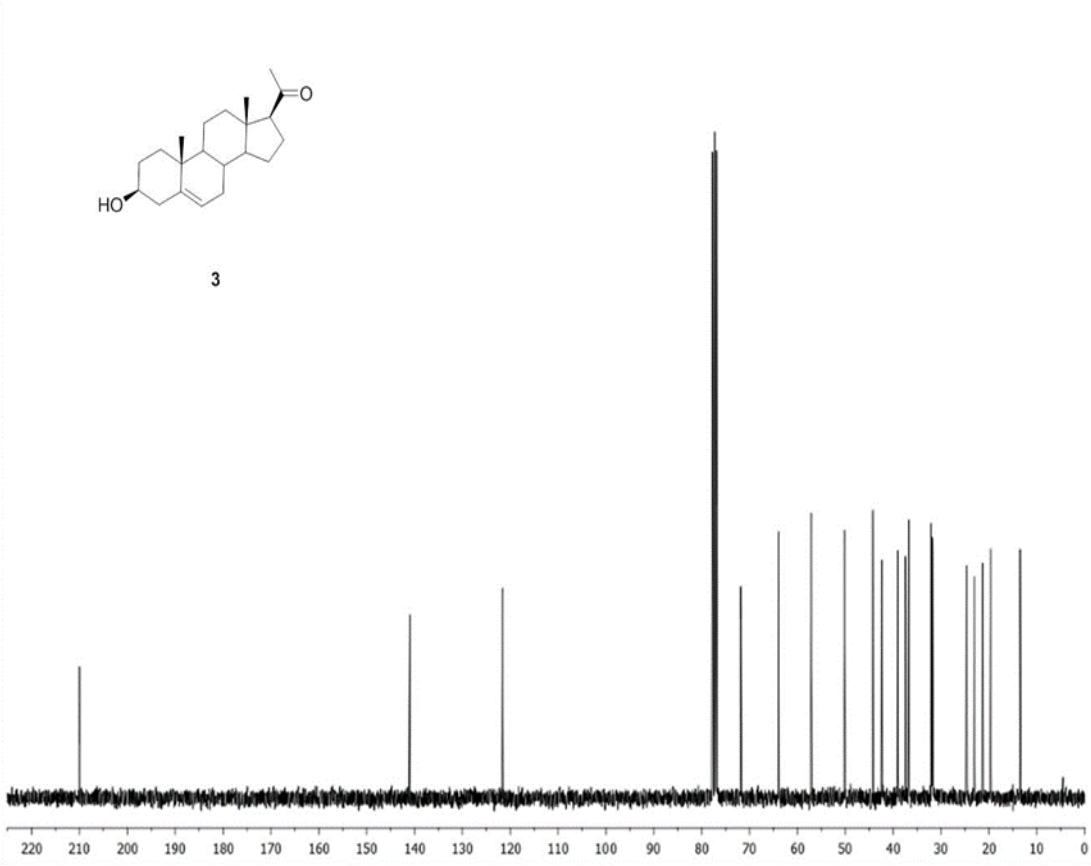


Figure A.4. ^{13}C NMR spectrum of compound **3**

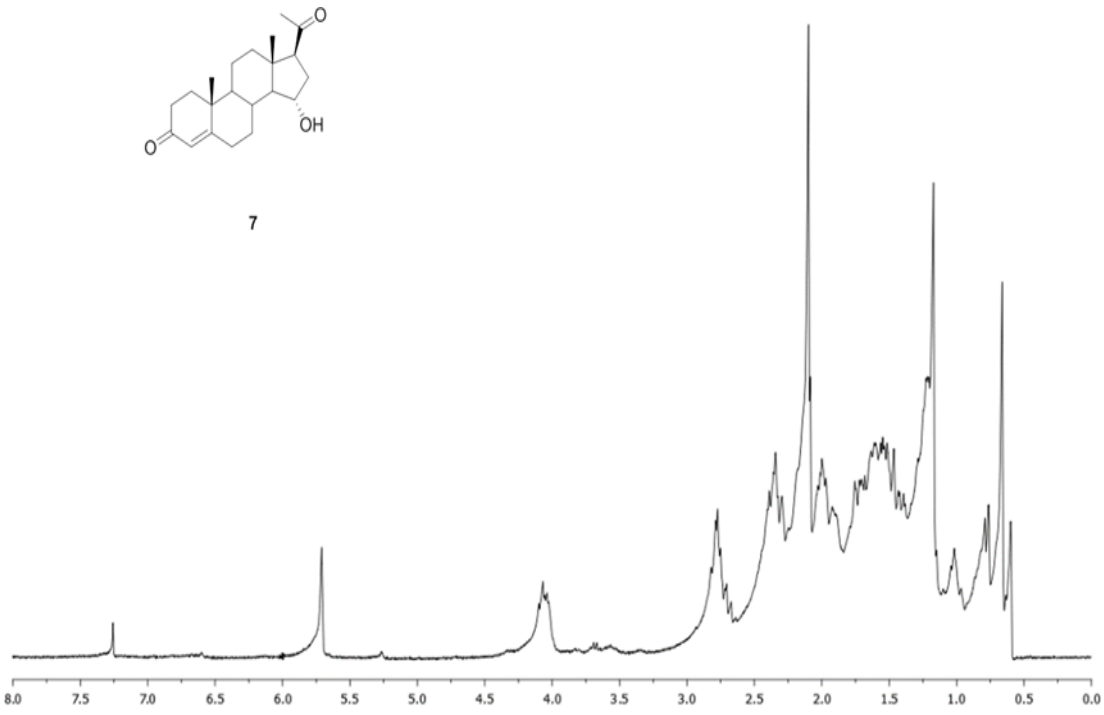


Figure A.5. ^{1}H NMR spectrum of compound **7**

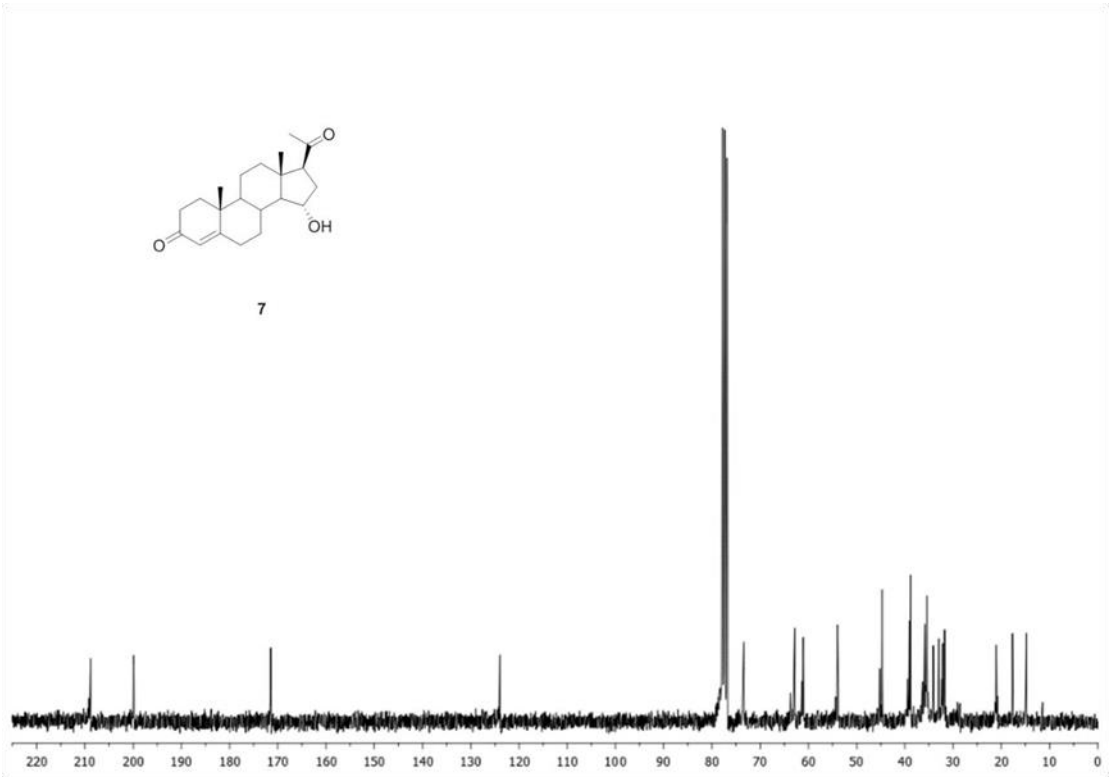


Figure A.6. ^{13}C NMR spectrum of compound **7**

CURRICULUM VITAE

Name Surname : Atheer Al-SAMMARRAİ

EDUCATION:

- **Undergraduate** : University of Samarra, College of Applied Sciences, Applied Chemistry department , Samarra, Iraq, (2016-2020).
- **Graduate** : Sakarya University, Institute of Science, Chemistry program (in progress)

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- International Oral Presentation
  (Mena Albayaty, Atheer Al-Samarrai, Halil İbrahim Yilmaz, Ali Kuru, Kudret Yildirim, Microbial conversion of dehydroepiandrosterone and pregnenolone by two *Penicillium* species. 6th International Eurasian Biological and Chemical Science Conference, 241, Ankara, Turkey).