Microbial Hydroxylation of Steroids: Characterization of Steroid Hydroxylase

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MRS. SWATI PRAMOD KOLET

UNDER THE GUIDANCE OF

DR. H. V. THULASIRAM

DIVISION OF ORGANIC CHEMISTRY

CSIR-NATIONAL CHEMICAL LABORATORY

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राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा रोड, पुणे - 411 008. भारत
NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411008. India

Dr. H. V. Thulasiram
Principal Scientist,
Chemical Biology Unit,
Division of Organic Chemistry,
CSIR-National Chemical Laboratory,
Pune- 411008.

+91 20 2590 2478
hv.thulasiram@ncl.res.in

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled ***"Microbial Hydroxylation of Steroids: Characterization of Steroid Hydroxylase"*** which is being submitted to the ***University of Pune*** for the award of ***Doctor of Philosophy*** in ***Chemistry*** by ***Mrs. Swati P. Kolet*** was carried out by the candidate under my supervision at the CSIR-National Chemical Laboratory, Pune. Such materials as obtained from other sources have been duly acknowledged in the thesis.

December 2013
Pune

Dr. H. V. Thulasiram
(Research Supervisor)

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled ***"Microbial Hydroxylation of Steroids: Characterization of Steroid Hydroxylase"*** submitted for the award of degree of ***Doctor of Philosophy*** in ***Chemistry*** to the University of Pune has not been submitted by me to any other university or institution. This work was carried out by me at the CSIR-National Chemical Laboratory, Pune, India. Such materials as obtained from the other sources have been duly acknowledged in the thesis.

Mrs. Swati P. Kolet **December 2013**
(Research student)
Chemical Biology Unit,
Organic Chemistry Division,
CSIR-National Chemical Laboratory,
Pune- 411 008.

Dedicated to

my family . . .

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Swati Kolet

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Abbreviations

Å	Angstrom
ACN	Acetonitrile
AcOH	Acetic acid
aq.	Aqueous
Calcd.	Calulated
Cat.	Catalytic/catalyst
$CDCl_3$	Deuterated Chloroform
CD_3OD	Deuterated Methanol
Conc.	Concentrated
Cong.	Conjugated
c	Concentration
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
DCM	Dichloromethane
DI	Deionized
DTT	Dithiothreitol
1D	One Dimensional
2D	Two Dimensional
d	Doublet
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
EtOAc	Ethyl acetate
ESI+	Electrospray ionization positive mode
g	Gram
× g	Gravitational force
h	Hours
HIV	Human Immuno Difficiency Virus
HPLC	High Performance Liquid Chromatography
Hz	Hertz
HRMS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple-Bond Correlation spectroscopy
IR	Infra red
J	Coupling constant
KCl	Potassium Chloride
L	Liter
LC-MS	Liquid Chromatography-Mass Spectrometry
MALDI-TOF	Matrix Assisted Laser Desorption-Time-Of-Flight
MeOH	Methanol
MF	Molecular formula
mg	Milligram
MHz	Megahertz

min	Minutes
M	Mole
µL	Microliter
µM	Micromolar
mL	Milliliter
mM	Millimolar
mmol	Millimoles
m.p.	Melting point
MS	Mass spectrometry
MW	Molecular weight
Me_4Si	Tetramethyl silane
m	Multiplet
m/z	Mass to Charge ratio
NCIM	National collection of industrial microorganisms
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NADH	Nicotinamide adenine dinucleotide reduced
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NAD^+	Nicotinamide adenine dinucleotide
Obs.	Observed
ORTEP	Oak Ridge Thermal Ellipsoid Plot Program
ppm	Parts per million
Pet-ether	Petroleum ether
PMSF	Phenylmethanesulfonyl fluoride
R_t	Retention time
R_f	Retention factor
RP	Reverse phase
rt	Room temperature
s	Second
SKF-525A	Proadifen
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Tris-HCl	Trisaminomethane hydrochloride
t	Triplet
UV-Vis	Ultraviolet-Visible
δ	Chemical shift in ppm
$[\alpha]_D$	Optical rotation
ºC	Degree Celsius
%	Percentage

Abstract

The thesis entitled **"Microbial Hydroxylation of Steroids: Characterization of Steroid Hydroxylase"** has been divided into four chapters

Chapter 1: Introduction and literature review

The relevant literature explaining the biotransformation with a particular emphasis on fungi mediated hydroxylation on hormonal steroids and the enzymes involved there in.

Chapter 2: Materials and Methods

The materials, experimental conditions and equipments used in the study.

Chapter 3: Biocatalyst mediated hydroxylation of steroids by *Mucor sp* (*M881*)

This chapter is divided into three sections

Section 3.1: The transformation of 3-one-4-ene steroids such as progesterone, testosterone, 17α-methyltestosterone, 4-androstene-3,17-dione, 19-nortestosterone using fungal strain *M881*.

Section 3.2: The transformation of 3β-hydroxy steroids such as epiandrosterone and dehydroisoandrosterone using *M881*.

Section 3.3: The immobilization of fungal strain *M881* using calcium alginate and the application of immobilized fungal culture for transformation of progesterone.

Chapter 4: *In vivo* and *in vitro* investigations of 14α-hydroxylase activity in *Mucor hiemalis*

This chapter is divided into two sections

Section 4.1: The transformation of progesterone and testosterone using fungal strain *Mucor hiemalis*. Transformation pathways were deduced using isolated metabolites as substrates and time course experiments.

Section 4.2: Cell free preparation of *Mucor hiemalis* for 14α-hydroxylase activity. The standardization of experimental conditions for preparation of microsomal suspension of *M. hiemalis* and hydroxylase assay

Chapter 1: Introduction and literature review

This chapter presents a brief discussion on chemical and biological importance of the steroidal compounds. The relevant literature explaining the biotransformation with a particular emphasis on fungi mediated hydroxylation on hormonal steroids and the enzymes involved there in is discussed.

Chapter 2: Materials and methods

This chapter deals with the experimental condition used to study the biotransformation of steroids using the fungal strains belonging to genera *Mucor* (*M881* and *M. hiemalis*). All analytical methods including TLC, column chromatography, GC, GC-MS, HPLC for the isolation and purification of metabolites are discussed. The spectroscopic techniques used for the identification of metabolites such as 1D, 2D NMR, FT-IR, UV-Visible spectroscopy and single X-ray crystalographic studies are included in this chapter. Experiemnts carried out for standerdization of different paramenters to obtained active crude lysate with 14α-hydroxylase activity from *M. heimalis* is presented.

Chapter 3: Biocatalyst mediated hydroxylation of steroids by *Mucor sp.* (*M881*)

In the present chapter the transformation of different steroids such as progesterone (**1**), testosterone (**2**), 17α-methyltestosterone (**3**), 4-androstene-3,17-dione (**4**), 19-nortestosterone (**5**), epiandrosterone (**6**) and dehydroisoandrosterone (**7**) using fungal strains is described. Fungal strains from different genera such as *Rhizopus*, *Mucor*, *Neurospora*, *Aspergillus Cunninghamella* were screened for the transformation of steroids. From the TLC and HPLC analyses, the fungal system belonging to the genera of *Mucor* (abbreviated as *M881*) [from NCIM, Pune catalogue no. 881] carried out quantitative conversion and was selected for the transformation of studied steroids.

3.1 Biotransformation of 3-one-4-ene steroids using *M881*

3.1.1 Transformation of progesterone (1)

The large scale fermentation of progesterone (**1**) with *M881* and purification of metabolites furnish three metabolites, 11α-hydroxyprogesterone (**1a**), 6β,11α-dihydroxyprogesterone (**1b**), 6β-hydroxypregn-4-ene-3,11,20-trione (**1c**). Time

course experiment carried out using **1** (0.7 g/L) revealed that during early stages of incubation (two days) nearly 35% of **1** was transformed into metabolites **1a**, **1b** and **1c**. By prolonging the incubation period to 12 days, the transformation was increased to 98% with the formation of 6β,11α-dihydroxyprogesterone (**1b**) as a major metabolite (96%) (Fig. 1).

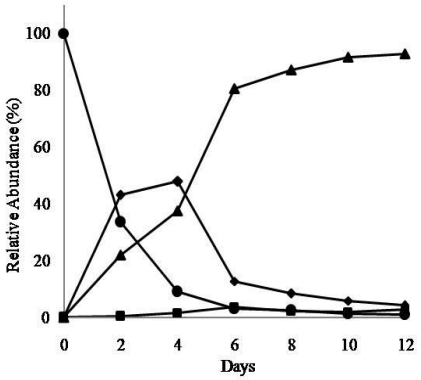
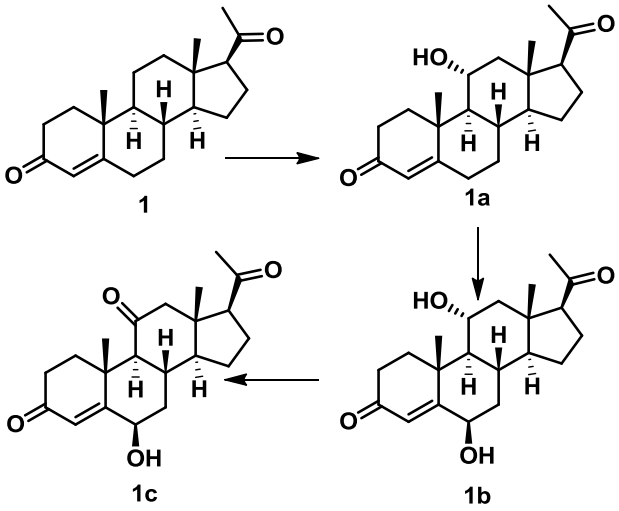


Figure 1: Time course study of progesterone (**1**) transformation: (●) progesterone (**1**), (♦) 11α-hydroxyprogesterone (**1a**), (▲) 6β, 11α-dihydroxyprogesterone (**1b**), (■) 6β-hydroxypregn-4-ene-3,11,20-trione (**1c**).



Scheme 1: Transformation of progesterone (**1**) by fungal strain *M881*

M881 has initiated the transformation of **1** by hydroxylation at 11α- position to form **1a** which further undergoes one more hydroxylation at 6β-position to form **1b**. This was further confirmed by incubating **1a** with *M881*, which transformed into **1b**. Small amount of **1b** was converted into **1c** as the microorganism oxidizes 11α-hydroxyl group on **1b**. Based on these results, the biotransformation pathway of progesterone by *M881* was deduced (Scheme 1).

3.1.2 Transformation of testosterone (2)

Transformation of **2** was carried out by incubating with *M881* culture for six days leads to the formation of 6β-hydroxytestosterone (**2a**), 11α-hydroxytestosterone (**2b**) and 6β,11α-dihydroxytestosterone (**2c**) metabolites. The optimum concentration for the transformation was determined from substrate concentration studies and it was found to be 1 g/L. Time-course experiments of **2** (1 g/L) (Fig. 2) indicated that after twelve days of incubation, **2c** was found to be the major metabolite (84%). The biotransformation pathway for **2** was confirmed by incubating 6β-hydroxytestosterone (**2a**) and 11α-hydroxytestosterone (**2b**) with *M881*(Scheme 2).

3.1.3 Transformation of 17α-methyltestosterone (3)

The fungal strain *M881,* transformed **3** into three metabolites which were identified

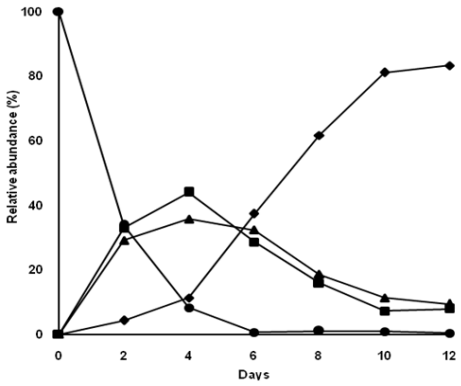
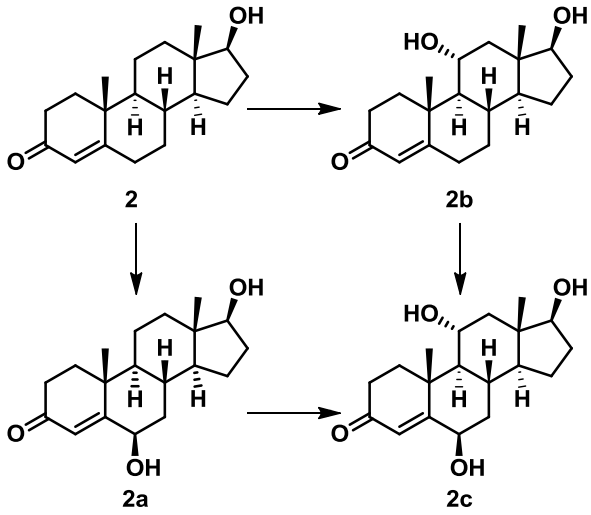


Figure 2: Time course study of testosterone (**2**) transformation in shake flask: (●) Testosterone (**2**), (■) 6β-hydroxytestosterone (**2a**), (▲) 11α-hydroxytestosterone (**2b**), (♦) 6β,11α-dihydroxytestosterone (**2c**).



Scheme 2: Transformation of testosterone (**2**) by fungal strain *M881***.**

as 11α-hydroxy-17α-methyltestosterone (**3a**), 6β-hydroxy-17α-methyltestosterone (**3b**) and 6β,11α-dihydroxy-17α-methyltestosterone (**3c**). From the time course study (0.7 g/L), with prolonged incubation, increase in the concentration of **3c** was observed (Fig. 3). At the end of twelve days of incubation, 90% of **3** was converted into **3c** as a major metabolite (Scheme 3).

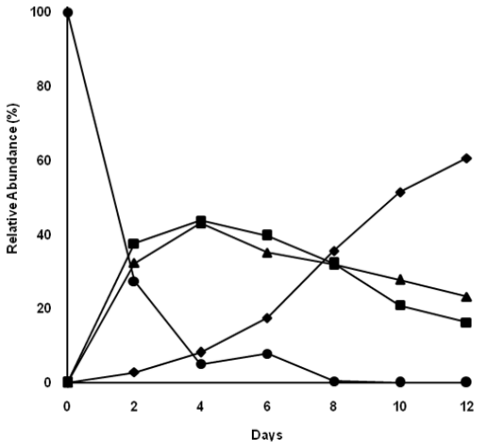
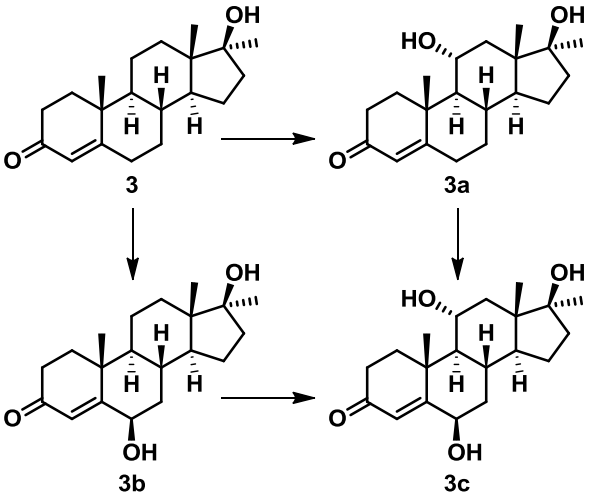


Figure 3: Time course study of 17α-methyltestosterone transformation: (●) 17α-methyltestosterone (**3**), (▲) 11α-hydroxy-17α-methyltestosterone (**3a**), (■) 6β-hydroxy-17α-methyltestosterone (**3b**), (♦) 6β,11α-dihydroxy-17α-methyltestosterone (**3c**).



Scheme 3: Transformation of 17α-methyltestosterone (**3**) by fungal strain *M881*.

3.1.4 Transformation of 4-androstene-3,17-dione (4)

TLC and HPLC analyses of the crude extract (1.63 g) obtained after the transformation of 4-androstene-3,17-dione (**4**) with *M881*, indicated the presence of

four metabolites as 6β-hydroxy-4-androstene-3,17-dione **(4a),** 11α-hydroxy-4-androstene-3,17-dione (**4b**), 6β,11α-dihydroxy-4-androstene-3,17-dione (**4c**), 7β-hydroxy-4-androstene-3,17- dione (**4d**). Time-course for the transformation of **4** indicated that on prolong incubation with *M881* the percentage of

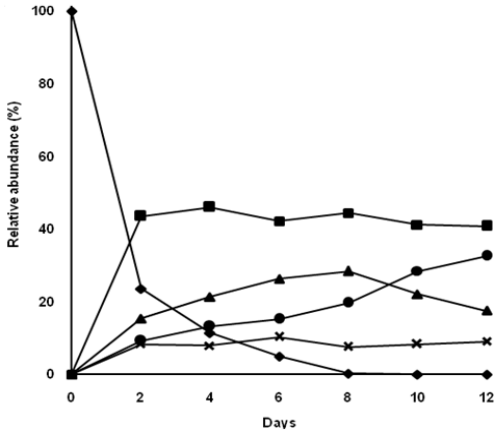
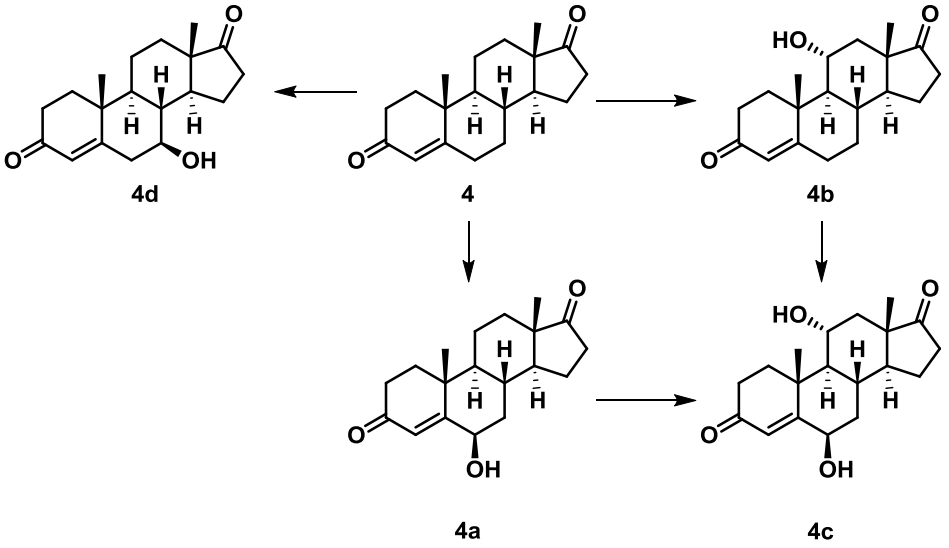


Figure 4: Time course study of 4-androstene-3,17-dione (**4**) transformation: (♦) 4-androstene-3,17-dione (**4**), (▲) 6β-hydroxy-4-androstene-3,17-dione (**4a**), (■) 11α-hydroxy-4-androstene-3,17-dione (**4b**), (●) 6β,11α-dihydroxy-4-androstene-3,17-dione (**4c**), (x) 7β-hydroxy-4-androstene-3,17-dione (**4d**)

6β,11α-dihydroxy metabolite was increased with decrease in the percentage of both 6β- and 11α-hydroxy metabolites (**4a** and **4b**) (Scheme 5).



Scheme 4: Transformation of 4-androstene-3,17-dione (**4**) by fungal strain *M881*.

3.1.5 Transformation of 19-nortestosterone (5)

Transformation of **5** (0.7 g/L) with *M881* resulted in the production of two hydroxy derivatives which were identified from spectral data analyses as 6β-hydroxy-19-nortestosterone (**5a**), 10β-hydroxy-19-nortestosterone (**5b**). Time course experiment for the transformation of 19-nortestosterone (**5**) (Fig. 5) explained that the fungal strain *M881* transformed 19-nortestosterone (**5**) into **5a** and **5b**. By prolonging the incubation period to 12 days, the transformation of **5** was increased to 97% with proportionate increase in the level of **5a** and **5b**. However, the formation of

dihydroxy derivative was not observed as seen with substrate **1** to **4** (Scheme 5).

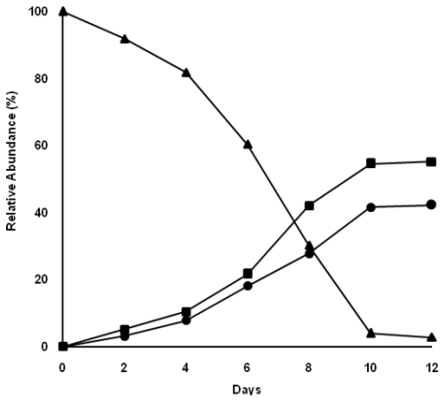
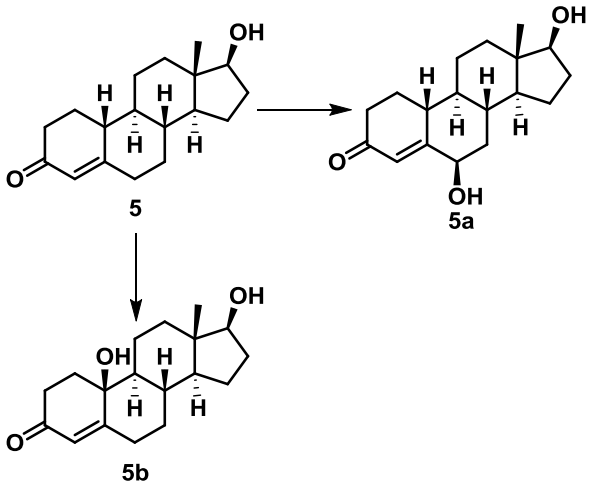


Figure 5: Time course study of 19-nortestosterone transformation: (▲) 19-nortestosterone (**5**), (●) 6β-hydroxy-19-nortestosterone (**5a**), (■) 10β-hydroxy-19-nortestosterone (**5b**)



Scheme 5: Transformation of 19-nortestosterone (**5**) by fungal strain *M881*.

3.2 Studies on large scale fermentation

Scale-up study was carried out in shake flasks (600 mL media) containing progesterone (**1**) (0.7 g/L) or testosterone (**2**) (1.0 g/L) as substrate. Each flask was inoculated with

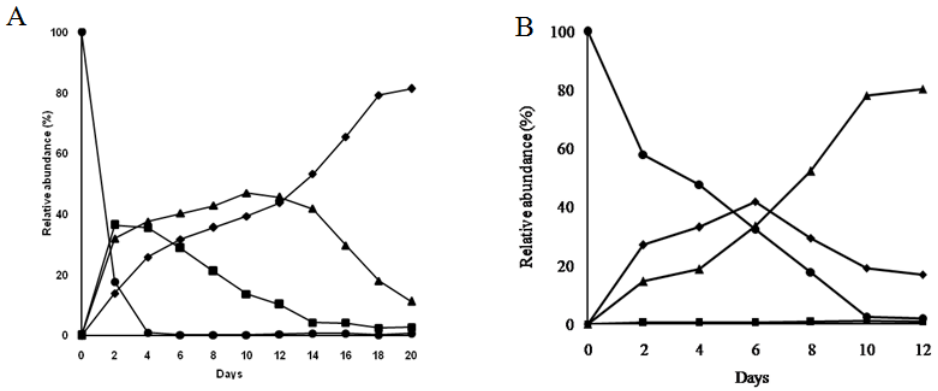


Figure 6: Time course study in fermentor: **A)** Testosterone (**2**) transformation :(●) Testosterone (**2**), (■) 6β-hydroxytestosterone (**2a**), (▲) 11α-hydroxytestosterone (**2b**), (♦) 6β,11α-dihydroxytestosterone (**2c**). **B) P**rogesterone (**1**) transformation in fermentor: (●) progesterone (**1**), (♦) 11α-hydroxyprogesterone (**1a**), (▲) 6β, 11α-dihydroxyprogesterone (**1b**), (■) 6β-hydroxypregn-4-ene-3,11,20-trione (**1c**).

M881 culture and incubated on a rotary shaker. On alternate days the aliquots were drawn aseptically from flasks, extracted and analyzed by HPLC. These results revealed that, the fungal system quantitatively converted **1** and **2** into corresponding dihydroxy metabolites in 10 days of incubation. Similar results were obtained when fermentor was used with 5000 mL of CzapekDox media containing **1** (0.7 g/L) or **2** (1.0 g/L) (Fig. 6).

Thus, the fungal system, *M881* can be used for the large-scale production of 6β,11α-dihydroxy derivatives of **1**, **2**, **3** and **4** with fine tuning of the fermentation conditions.

3.3: Biotransformation of 3β-hydroxy steroids using *M881*

3.3.1: Transformation of epiandrosterone (6)

The fermentation of epiandrosterone (**6**) using *M881* revealed that organism can transform the substrate **6** into one major metabolite. From the spectral data analyses, the metabolite was identified as 7β-hydroxy-epiandrosterone (**6a**). Substrate concentration experiments revealed that *M881* could able to transform epiandrosterone (**6**) into single product, **6a** in highly efficient manner at the concentration of 1.0 g/L. (Scheme 6) The time course experiments with 1.0 g/L concentration revealed that after 4 days of incubation over 90% of epiandrosterone (**6**) was converted into 7β-hydroxy-epiandrosterone (**6a**) (Fig. 7).

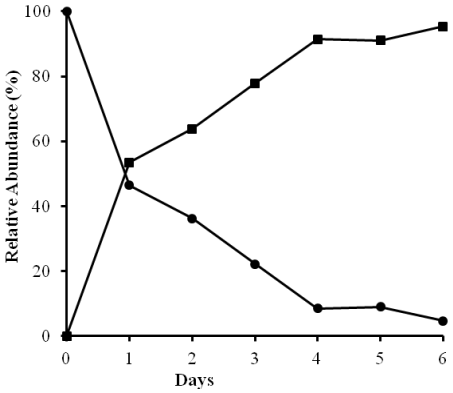
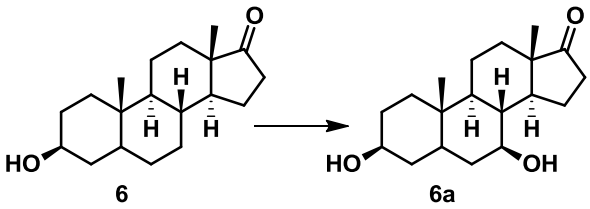


Figure 7: Time course study of epiandrosterone (**6**) transformation: (●) epiandrosterone (**6**), (■) 7β-hydroxy epiandrosterone (**6a**)



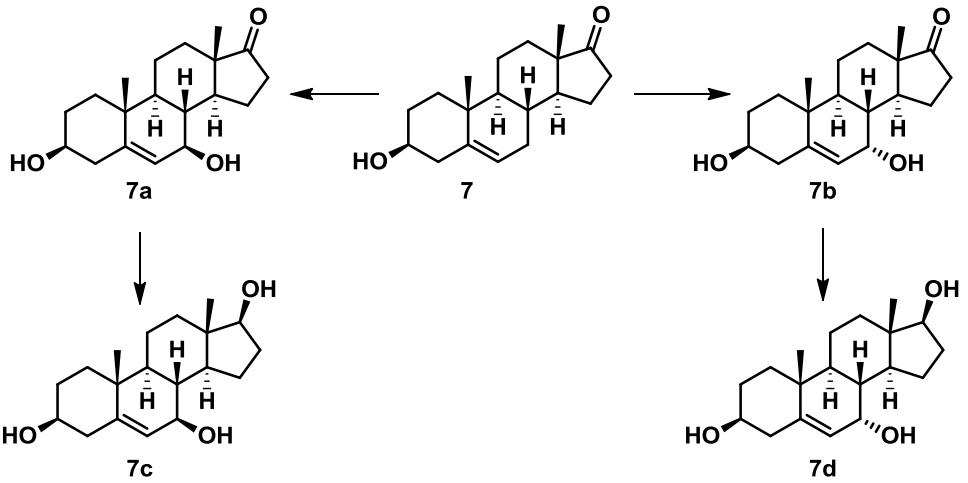
Scheme 6: Transformation of epiandrosterone (**6**) by fungal strain *M881*

3.3.2: Transformation of dehydroisoandrosterone (7)

When dehydroisoandrosterone (**7**) (0.7 g/L) was used as a substrate, the fungal system *M881* very efficiently transformed it into four metabolites which were purified and identified as 7β-hydroxy-dehydroisoandrosterone (**7a**), 7α-hydroxy-dehydroisoandrosterone (**7b**), 3β,7β,17β-trihydroxyandrost-5-ene (**7c**), 3β,7α,17β-trihydroxyandrost-5-ene (**7d**) (Scheme 7).

3.4 Transformation of steroid by immobilized mycelia of *M881*

This section describes the studies on the immobilization of 36 h grown mycelia of *M881* into calcium alginate. The immobilized mycelia transformed the added progesterone into corresponding hydroxylated metabolites. The HPLC analysis showed conversion



Scheme 7: Transformation of dehydroisoandrosterone (**7**) into hydroxy derivatives by fungal strain *M881*

of progesterone (**1**) into two metabolites 11α-hydroxyprogestrone (**1a**) and 6β,11α-dihydroxyprogestrone (**1b**). After six days of incubation 70% conversion of progesterone (**1**) into hydroxylated metabolites was observed. (Fig 8)

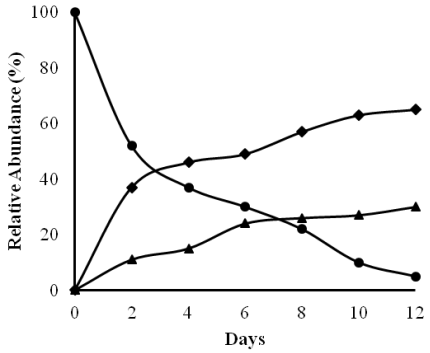
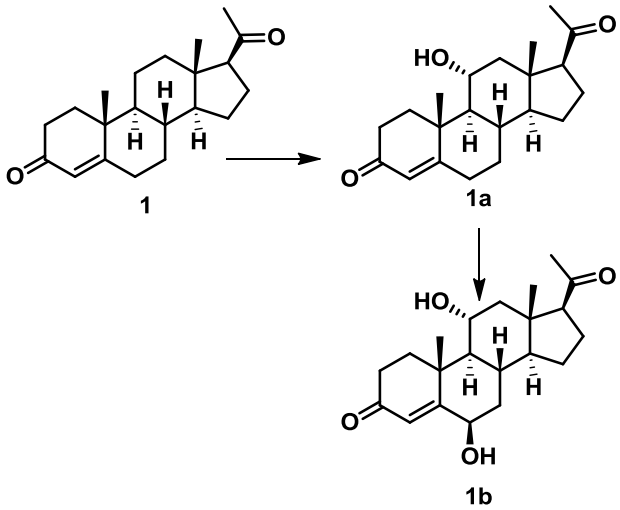


Figure 8: Time course study of progesterone (**1**) transformation with alginate immobilized *M881:* (●) progesterone (**1**), (♦) 11α-hydroxyprogesterone (**1a**), (▲) 6β, 11α-dihydroxyprogesterone (**1b**)



Scheme 8: Transformation of progesterone (**1**) with alginate immobilized *M881*

3.5 Summary and Conclusion

In Conclusion, the versatile fungal strain *Mucor* sp. (*M881*) could able to carry out the regio- and stereo- specific hydroxylation at C-6β and C-11α position on progesterone (**1**), testosterone (**2**) and 17α-methyltestosterone (**3**) and 4-androstene-3,17-dione (**4**) to form corresponding 6β,11α-dihydroxy derivatives (**1b, 2c, 3c** and **4c**) as a major metabolites. The fungal strain, *M881* efficiently hydroxylate 3β-hydroxy steroids (**6** and **7**) at 7α/β postions. The immobilized *M881* culture also found to be capable in hydroxylating steroids.

Chapter 4: *In vivo* and *in vitro* investigations of 14α-hydroxylase activity in *Mucor hiemalis*

Steroids with C-14 hydroxyl group are cardio-active which makes them highly valuable from pharmacological aspects. 14β-hydroxy derivatives of steroids are important enhancers in contractility of isolated cardiac muscle. In this chapter, ability of fungal strain *Mucor hiemalis* was explored to hydroxylate progesterone **(1)** and testosterone (**2**) at C-14α position.

4.1 Biotransformation of 3-one-4-ene steroids using *M. hiemalis*

4.1.1: Transformation of progesterone (1)

After incubation of progesterone with 36 h grown culture of *M. hiemalis* for five days yielded a crude extract which was subjected to column chromatography. The three hydroxylated metabolites obtained in pure form were characterised by various spectral data analyses and identified as 14α-hydroxyprogesterone (**1d**), 7α,14α-dihydroxyprogesterone (**1e**), 6β,14α-dihydroxyprogesterone (**1f**). Time-course experiments (Fig. 9) indicated that *M. hiemalis* could able to transform over 83% of progesterone (**1**) into 14α-hydroxyprogesterone (**1d**), 7α,14α-dihydroxyprogesterone (**1e**), 6β,14α-dihydroxyprogesterone (**1f**) metabolites at the end of three days of incubation period. The relative percentage of metabolites were remained unchanged even after prolonging incubation beyond four days. Incubation of 14α-hydroxyprogesterone (**1d**) with *M. hiemalis* yielded **1e** and **1f**. These results

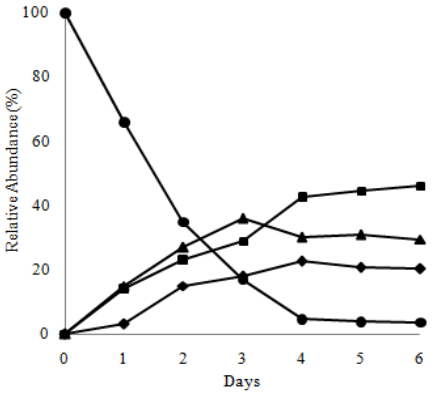
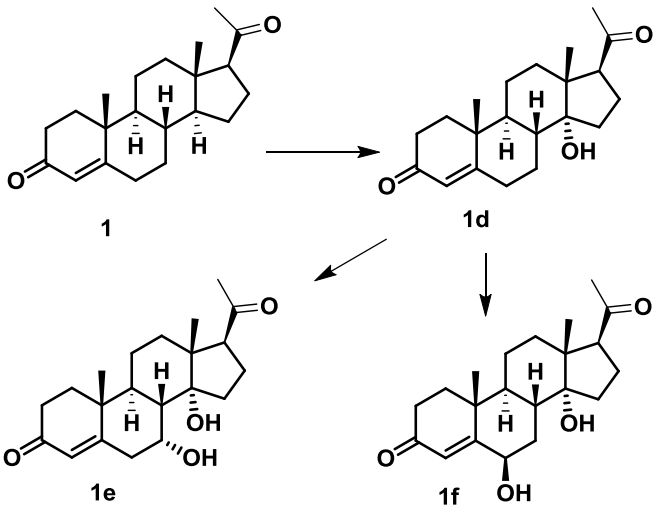


Figure 9: Time course study of Progesterone (**1**) transformation with *M. hiemalis*: (●) progesterone (**1**), (▲) 14α-hydroxyprogesterone (**1d**), (■) 7α,14α-dihydroxyprogesterone (**1e**), (♦) 6β,14α-dihydroxyprogesterone (**1f**).



Scheme 9: Transformation of progesterone (**1**) by *Mucor hiemalis*

indicated that, the fungal system carries out hydroxylation at 14α-poisition which will be further undergo hydroxylation at 6β - or 7α positions to yield **1e** and **1f** (Scheme 9).

4.1.2: Biotransformation of testosterone (2)

The large scale fermentation of testosterone (**2**) with *M. hiemalis* and purification of metabolites obtained after six days incubation furnished four hydroxy metabolites. These metabolites were identified as 14α-hydroxytestosteone (**2d**), 7α,14α-dihydroxytestosterone (**2e**), 6β,14α-dihydroxytestosterone (**2f**), 14α-hydroxy-4-androstene-3,17-dione (**2g**) based on various spectral studies and comparing the spectral data with earlier reports. Time course experiments carried out with **2** revealed that during early stages of incubation (one day) the 14α-hydroxytestosterone (**2d**) was the major metabolite which was converted in to all other three metabolites **2e**, **2f** and **2g** after prolonging the incubation (Fig.10). After four days of incubation no major change in the level of metabolites in fermentation medium was observed. Further when **2d** was incubated with *M. hiemalis*, subsequent hydroxylation was occurred at 6β- or 7β-position to form **2e** and **2f** and oxidation of 17β-hydroxyl group of **2d** also observed. The studies clearly indicates that *M. hiemalis* could able to carry out hydroxylation at 14α-position in very efficient manner to form **2d** which further under goes hydroxylation or oxidation leading to the formation of **2e**, **2f** and **2g.** Based on these

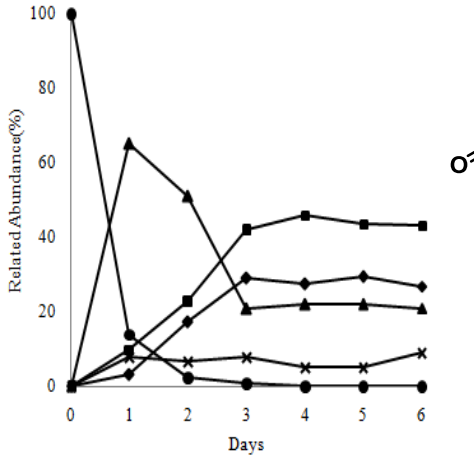
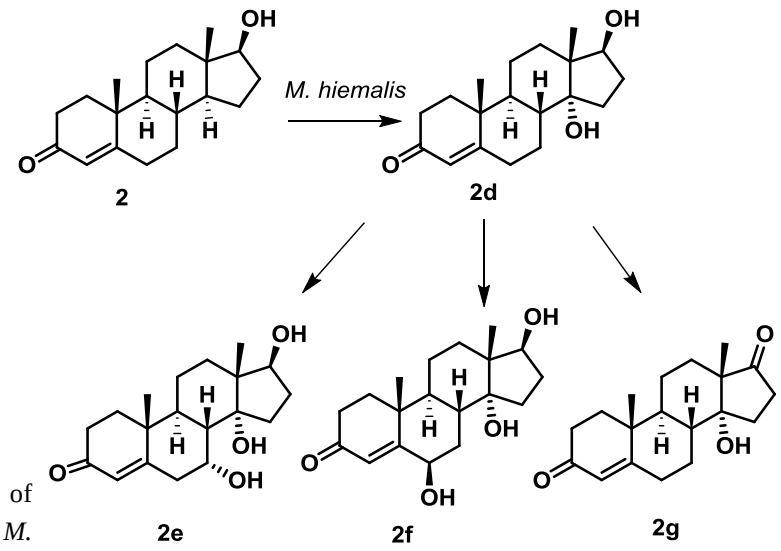


Figure 10: Time course study of Testosterone (**2**) transformation with *M. hiemalis*: (●) testosterone (**2**), (▲) 14α-hydroxytestosteone (**2d**), (■) 7α,14α-dihydroxytestosterone (**2e**), (♦) 6β,14α-dihydroxytestosterone (**2f**), (x)14α-hydroxy-4-androstene-3,17-dione (**2g**)



Scheme 10: Transformation of testosterone (**2**) by *Mucor hiemalis*

observations, the biotransformation pathway of testosterone (**2**) by *M. hiemalis* was established (Scheme 10).

4.2 Standardization of active microsomal suspension preparation from *M. hiemalis*

In this section, different key parameters guiding the activity of enzyme in cell free extract of *M. hiemalis* such as substrate for induction, pH of extraction buffer, co-factors, storage conditions etc were standardized to obtain optimum *in vitro* 14α-hydroxylase activity.

4.2.1 Localization of 14α-hydroxylase system in *M. hiemalis*

The hydroxylase activity observed for samples obtained after centrifugation of crude lysate at various gravitational forces (x g) revealed that all the components of 14α-hydroxylase system were membrane bound proteins (Fig. 11) as complete hydroxylase activities observed were from microsomal pellet obtained at 1,20,000 x g.

Hydroxylase assays were carried out in presence of NADPH or NADPH generating system indicated the formation of 14α-hydroxylated product **1d** in presence of NADPH however there was no 14α-hydroxylase activities observed in presence of NADH. Further, the formation of **1d** was increased to three fold when NADPH generation system was used instead of isolated NADPH as cofactor. These observations indicated that 14α-hydroxylase in *M. hiemalis* is NADPH dependent hydroxylase system and NADPH generating system is an ideal source of co-factor for maximum

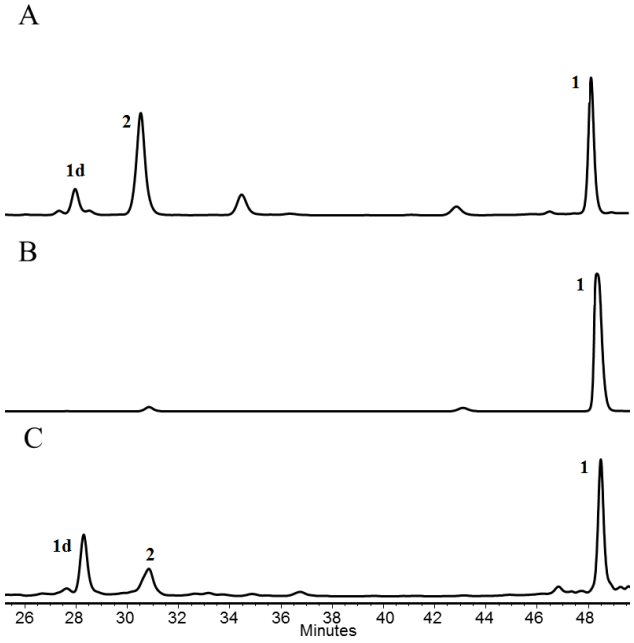


Figure. 11: LC-MS analysis of hydroxylase assays with (A) 10250xg suspension, (B) 120000xg supernatant, (C) microsomal suspension. – Testosterone (**2**) used for induction, Progesterone (**1**) substrate for hydroxylase assay, (**1d**) 14α-hydroxyprogesterone product formed after assay.

hydroxylase activity . Addition of PMSF to extraction buffer at the concentration of 2.5 mM, found to be ideal condition for retaining the maximum hydroxylase activities during cell free hydroxylase studies.

4.2.2 Induction with different substrates

On induction of enzyme with different substrates (Table 1), maximum activity in microsomal pellet was obtained when progesterone used as inducer with 12 h induction period. Induction with testosterone, 17α-methyltestosterone and estradiol resulted in microsomal suspension which showed the 14α-hydroxylase activities when progesterone was used as substrate (Table 1). However, there was no hydoxylase

Table 1: Effect of different inducers on hydroxylase activity in microsomal suspension

No.	Inducer (15 mg/100 mL for 12 h)	% relative activity
1	Progesterone	100%
2	Testosterone	65%
3	17α-methyltestosterone	59%
4	Estradiol	44%
5	Androsterone	No activity
6	Cholesterol	No activity
7	Phenabarbitol	No activity
8	Uninduced	No activity

activity observed with microsomal pellet obtained from the mycelia induced with phenobarbital, androsterone and cholesterol (Table 1). Further no 14α-hydroxylase activity was observed with uninduced mycelial microsomal preparations. These results indicate that 14α-hydroxylase system in *M. hiemalis* is inducible and the level induction is depending on the inducer being used.

4.2.3: Optimization of pH and incubation temperature

Screening the extraction buffers with range of pH from 7.2 to 8.7, revealed that the extraction buffer of pH 8.2, yielded a microsomal suspension with maximum

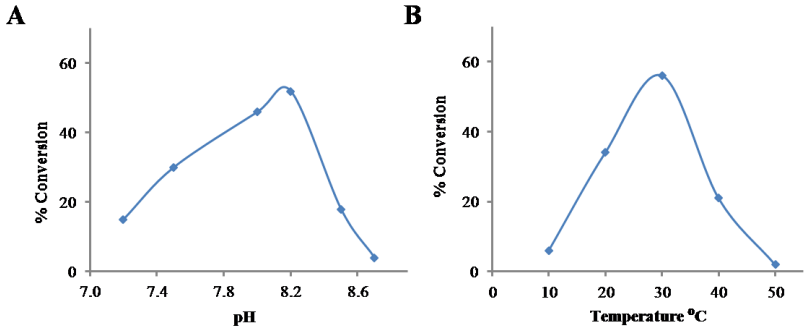


Figure 12: Effect of (**A**) pH and (**B**) temperature on 14α-hydroxylase activity

hydroxylase activity (Fig 12A). Hydroxylase assay carried out at various incubation temperatures in the range of 10 ºC to 50 ºC indicated that 30 ºC is the optimum incubation temperature for 14α-hydroxylase activity (Fig. 12B).

4.2.4: Effect of inhibitors

When the hydroxylase assay carried out in the presence of different known cytochrome P450 inhibitors (Fig. 13) such as SKF-525A, carbon monoxide, cytochrome c, N-methylmaleimide and ketocanazol, significant inhibition in the 14α-hydroxylase activity was observed. Loss of activity up to 70-75% was observed when the assay was carried out in presence of SKF-525A (1 mM) and cytochrome c (50 µM). Similarly, drastic

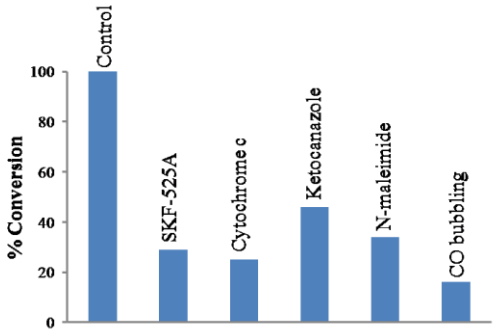


Figure 13: Effect of different inhibitors on 14α-hydroxylase activity

reduction in the hydroxylase activity was observed when the assay mixture was bubbled with carbon monoxide (for 30 sec with 1-2 bubble per sec). Inhibition of 14α-hydroxylase activity from *M. hiemalis* by known cytochrome P450 inhibitors indicated that the hydroxylase system belongs to cytochrome P450 family.

4.3 Summary and Conclusion

In conclusion, the fungal system *M. hiemalis* could able to carryout the efficient hydroxylation at 14α-position on two hormonal steroids, progesterone (**1**) and testosterone (**2**) to form corresponding 14α-hydroxy metabolites which further transformed into 6β/7α, 14α-dihydroxy derivatives on extended incubation. The biotransformation pathway of **1** and **2** by *M. hiemalis* was deduced based on the time course and resting cell experiments using substrates and isolated metabolites. The hydroxylase assays with microsomal suspension prepared from *M. hiemalis* with varying experimental parameters such as inducer, pH, temperature etc. outlined the optimum conditions required to obtain maximum 14α-hydroxylase activity *in vitro*.

Chapter 1: Introduction and Literature Review

1.1: Steroids

Steroids (Greek, stereos = solid) are naturally occurring solid alcohols, prevalent terpenoid lipids in animals and plants. The basic skeleton of steroids consists of three cyclohexanes and one cyclopentane in a fused ring system arranged in the form of a perhydrocyclopentanophenanthrene. A perhydrophenanthrene (rings A, B, and C) is the completely saturated derivative of phenanthrene (Fig. 1.1 A). The vast diversity of the natural and synthetic members of this class depends on variation in the side chain substitution (primarily at C-17), degree of unsaturation, nature of oxidation and the stereochemical relationship at the ring junctions[1]. The polycyclic hydrocarbon known as 5α-cholestane represents the example of basic steroid skeleton (Fig. 1.1 B and C).

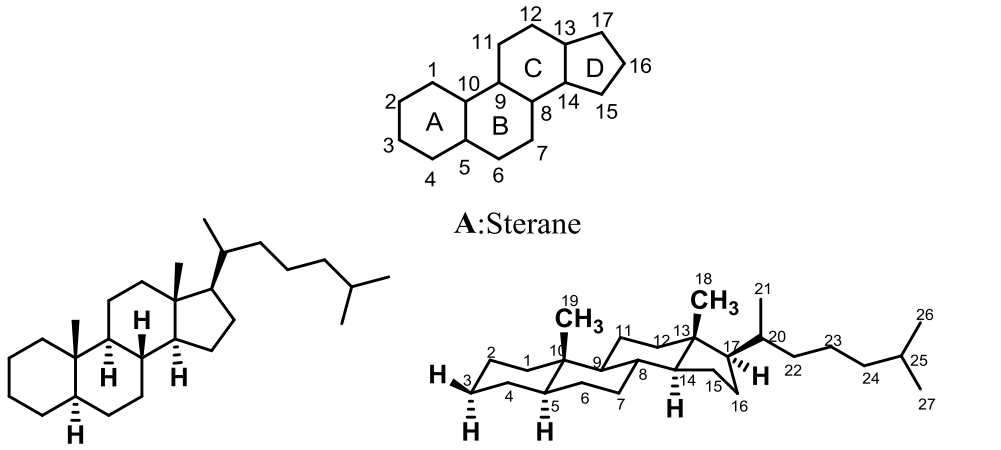


Figure 1.1: Basic steroid skeleton

The term "cholestane" refers a group of steroids with 27 carbons that includes a side chain of eight carbons at 17-position. Numbering begins in ring A at C-1 and proceeds around rings A and B to C-10, then into ring C beginning with C-11 around rings C and D to C-17 as in Fig 1.1. The angular methyl groups at positions 18 and 19 are β and have an axial orientation. Similarly, the configurations of the 8, 9 and 14 hydrogen are β, α and α, respectively. The side chain at C-17 is in β orientation with respect to the cyclopentane D ring. All the ring junctions, A/B fused ring, B/C fused ring, and C/D fused ring have *trans* (diequatorial) stereochemistry. In the case of steroids having double bonds at different positions, the symbol Δ is often use to designate a carbon–carbon double bond (C═C), for example if double bond is present between 4 and 5 positions, the compound is referred to as a Δ^4-steroid and if the C═C is between positions 5 and 10, the compound is designated a $\Delta^{5(10)}$ steroid. Asymmetric centers in side chains are denoted preferentially with the *R* and *S* notations.

Naturally occurring steroids are classified into different classes depending upon the number of carbon atoms[2] (Fig. 1.2) as cholestanes (e.g. cholesterol) with 27 carbons, cholanes (e.g. cholic acid) with 24 carbons, pregnanes (e.g. progesterone) with 21 carbons, androstane (e.g. testosterone) with 17 carbons and estrane (e. g. estradiol) with 16 carbons. Taxonomically steroids are classified from their source in nature[3] as plant steroids (sterols e.g. phytosterols, diosgenine, brassinosteroids), insect steroids (e.g. ecdysteroids), animal steroids (e.g different sex hormones: androgens, estrogen,

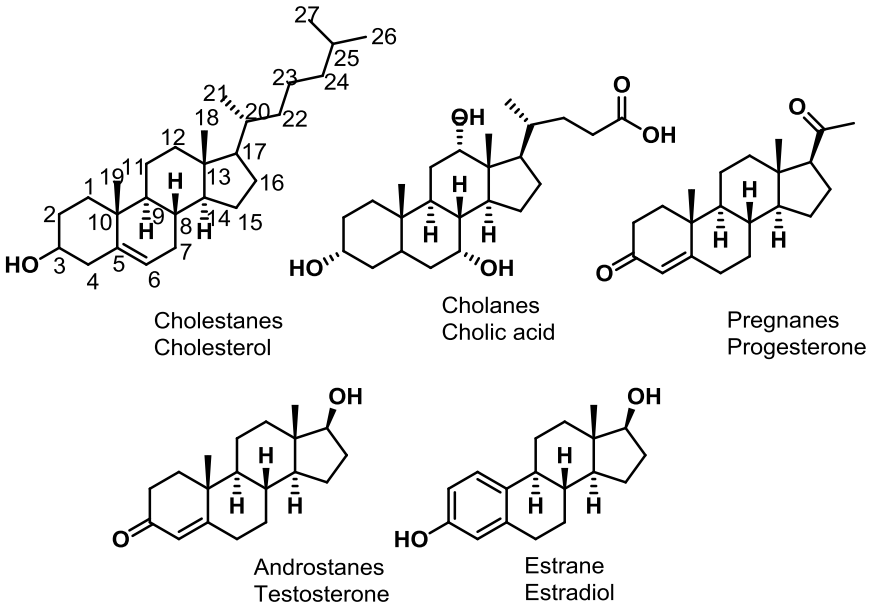


Figure 1.2: Classification of steroids based on carbon numbers

bile acids, cholesterol) and fungal steroids (e.g. ergosterols). Steroids are biologically important molecules and their activities are influenced by type, regio- and stereo-positions of the functional groups attached to the basic skeleton. For example, corticosteroid with 11β-hydroxy functional group shows anti-inflammatory activity[4], 14β-hydroxy pregnanes are cardioactive molecules[5] whereas its epimer with 14α-hydroxy functional group is antigonadotropic.[6] The activity of steroids is related to their association with respective receptors which regulates gene expression. Wide array of steroid derivatives are available in market for different therapeutic applications (Fig. 1.3) such as anti-inflammatory, immunosuppressive, anabolic and contraceptive agent[7-13]. They have been used in the treatment of breast and prostate cancer[14], coronary heart disease, HIV infection[15], Type II diabetes[10] and fungal infections.[16] Last few decades new class of steroids, known as a neurosteroids, for example, 17β-estradiol, 7β-androsterone, alphoxolone, ganaxolone[17,18] etc. has attracted attention of researchers and pharmaceutical companies. Such steroids causes neuronal excitability by interacting

with cell surface and are found as memory enhancers, inducers of endocrine response to stress[17], anticonvulsants[19] and anti-depressives. Their role in reducing spinal cord damage, DNA and myelin repair[9] made them important players in drug market. Because of such diverse activities, there is always a thirsting demand for new steroidal entities in pharmaceutical field.

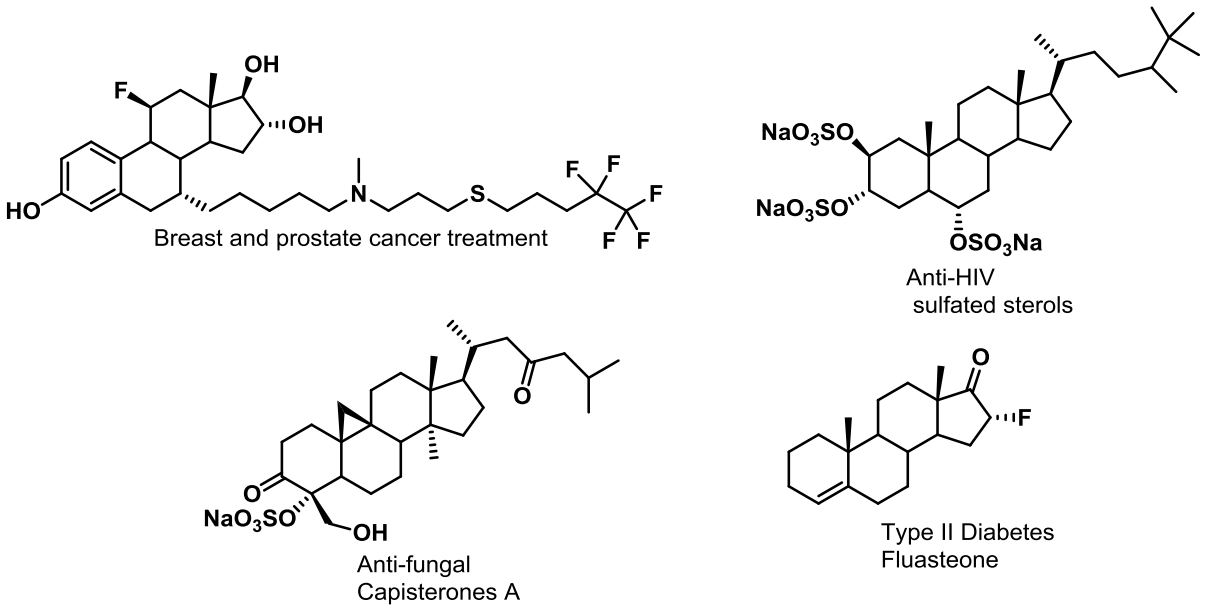


Figure 1.3: Examples of pharmaceutical steroidal drugs (API)

Because of complex structure with several chiral centers and inactive carbons in the basic skeleton of steroids, their synthesis/modifications is difficult or will be tedious process with many steps of protection and deprotection through the traditional chemical synthesis. On the other hand, Biocatalysts, considered as nature's catalysts known to carry out efficient functionalization at remote or inactive carbons under mild conditions. In recent years, the most striking and significant development in the field of synthetic chemistry has been the application of biocatalysts along with chemical reactions. Biocatalysts display a far greater specificity than the conventional organic reagents. Whole cell microorganisms are increasingly being utilized as tool in the hands of organic chemists.

1.2: Biotransformation

The process of structural modifications assisted by enzymes, plant cells, animal cells or microorganisms is collectively termed as biotransformation. The art of fermentation/biotransformation, defined in the broadest sense as the chemical transformation of organic compounds with the help of biocatalysts, is an old process. In almost every civilization, right from the prehistoric times, man has practiced the

fermentation of fruit, grain and milk to obtain intoxicating and nourishing dietary factors. The ability of yeast to make alcohol in the form of beer was known to the Sumarians and the Babylonians before 6000 B.C. Much later by about 4000 B.C., the Egyptians discovered the leaven bread. References to the production of wine, another product of fermentation, has been found in excavations at Lachish and other sites (3000 B.C) [20]. By the 14th century A.D., the distillation of alcoholic spirits from fermented grain, a practice thought to have originated in China or the Middle East began to appear in many parts of the world.

Existence of microbes was recognized in the 17th century by Dutch microscopist Anton Van Leewenhoek (1677). By 1857, a sufficient understanding had been developed to provide the necessary background for the work of Louis Pasteur on the fermentation of sugar to lactic acid and ethanol. Pasteur discovered that all fermentative processes are the result of microbial activity and the individual microbial species are responsible for discrete chemical alteration of selected substrates[20,21]. Later by 1896, Bertrand carried out extensive studies of the oxidative process, on a series of polyhydric alcohols by using *Acetobacter xylinum* species[22,23,24]. The next major developments in the field arose from the finding of Linther and von Liebig[25] that fermenting yeast reduced furfuraldehyde to the alcohol.

Biotransformation is a biological process in which organic compounds are modified to their derivatives with the help of microorganisms as whole cells or isolated enzymes. Microbial cells are preferred more as compared to animal cells or plant cells[26] due to the following reasons:

- **Surface-volume ratio:** The microorganisms have high surface-volume ratio as compared to the plant or animal cell culture.
- **Growth Rate:** The microorganism have high growth rate as compared to the plant or animal cell culture thus the transformation using the cell culture is less time consuming.
- **Sterility:** Sterility is an important factor that should be taken care of. In case of plant or animal cell culture it is difficult to maintain sterility as compared to the transformation using microorganism.
- **Metabolism Rate:** The microorganisms possess high rate of metabolism for the efficient transformation of the substrate added as compared to the plant or animal culture.

Microbial transformations are classified into two classes- biosynthetic transformations which are the part of various routine physiological processes in living cells and other class of transformations where substrates are not the primary requirements of biological systems. Both the transformations have applications in different fields such as fine chemicals, neutraceutical and pharmaceutical industries etc. Microbial transformation is an effective tool for the preparation of compounds, which may be otherwise difficult to prepare by conventional synthetic methods. Microorganism grows in aqueous environment which makes the biotransformation process economical and green. Microbial diversity in nature with their metabolic flexibilities can provide spectrum of enzymes for different reactions and vast array of compounds. Being enzymatic, the microbial transformations can be advantageous in the cases where traditional synthetic routes are tedious or not possible.

1.2.1: Advantages of microbial transformation[27]

- A microbial transformation requires mild conditions such as aqueous environment, physiological pH ranging from 5 to 7, ambient temperature etc which are of particular interest for the sensitive and highly reactive substrates. This minimizes the problems of undesired side reactions such as decomposition, isomerization, racemization and rearrangement.
- Enzymes involved in transformation can carry out functionalization on chemically inactive sites in the molecules which are either impossible or time consuming by chemical synthetic routes.
- High regio- and stereo-selectivity can be obtained using microbial transformation, often with 100% ee which otherwise may require costly chiral reagents.
- Several reactions can be combined in one fermentation step and programmed to occur in a specific sequence in a suitable microorganism with a number of appropriate enzyme systems can be used.
- As enzymes involved in the transformation are very specific, chance of side reactions are very less.
- More often, biocatalyst mediated preparation of an organic compound is cheaper than to synthesize chemically.
- Above all, the process mediated by biocatalysts are green and ecofriendly and waste generated in the biotransformation is biodegradable or minimum environmental pollution unlike process mediated by chemical reagents.

1.2.2: Difficulties of microbial transformation[27] and way out

- Most of the organic compounds are insoluble in water which limits the availability of substrates to the microbes or enzymes for the transformation in aqueous environment. It can be overcome by using biphasic transformation, cyclodextrin or ionic liquids as carriers for organic compounds in water.
- Enzyme involves in the transformation are usually substrate specific which limits the generalization of transformation process for range of compounds. Screening of large number of microorganisms from different environmental conditions can increase the chances of finding the suitable biocatalyst for variety of compounds.
- Many biocatalytic reactions are prone to substrate or product inhibition. Use of advance techniques like continuous flow reactors can sometimes be useful to tackle the limitation.

Thus a combination of microbial transformations and chemical reactions can be exploited for the partial as well as total synthesis of organic compounds.

1.2.3: Earlier reports and current status for Microbial transformation

Biotransformation processes is well known for several thousand years. Our ancestors in earlier days have used microbial transformation for ethanol production from fermentable sugars when concept of microorganisms was not evolved. Yeast mediated transformations in particular, have been extensively used since thousand years of mankind for the production of bread, dairy products and alcoholic beverages. The biotransformation of ethanol to acetic acid (vinegar) by *Acetobactor* was probably the first true biotransformation process applied in an industrial manner[28].

Microbial transformation offers many advantages over the conventional chemical methods. In 1921, yeast cells assisted microbial reaction for the stereospecific preparation of D-(-)-ephedrine was described. Yeast cell has catalyzed the condensation of benzaldehyde with acetaldehyde to form optically active L-1-hydroxy-1-phenyl-2-propane which was then chemically converted into D-(-)-ephedrine. This was the first example of successful combination of chemical and microbial transformation. The research efforts in this field were triggered again in 1952 when Murray and Peterson patented the process of 11α-hydroxylation of progesterone by a *Rhizopus* species[29], a decisive step in the development of the practical synthesis of steroids with useful biological activity. Since then biotransformation has attracted the special attention for the synthesis of different biologically important complex molecules.

Thousands of microbial transformations involving different types of reactions with organic compounds and natural products have now become known, some of them have proved to be very useful for synthetic organic chemistry. Enzymatic reactions (biotransformation) are often unique in their potential to produce fine chemicals in high optical purity which has explored in many fields for the production of more complex molecules, as a result of which many new drugs contain one or more chiral centers are marketed as a single enantiomer. Although there is increase in awareness on biocatalysis and many more new biocatalysts available on a daily basis, in most of the synthetic laboratories, usage of biocatalysts is not extended beyond the few simple hydrolases for esterification or kinetic resolutions.

1.2.4: Practical aspects of microbial conversions

- **Organism:** For microbial transformation, the primary requirement is selection of microorganism having the enzyme for specific conversion which can be done by random screening or enrichment procedure. Random screening consists of addition of the substrate to a culture of a large number of microorganisms and after a given incubation period, the medium is analyzed for formation of the product. If a particular organism brings about the desired transformation, the organism is selected for further investigation. In enrichment procedure, large amount of substrate is added to the soil samples together with water and additional nutrients. Organism, which is capable of utilizing substrate, grows in the medium and these organisms are isolated and used for the degration/transformation studies. In some cases, it may be advantageous to use a mixture of two or more microorganisms (mixed culture consortium) for conversion of a particular substrate.
- **Sterilization:** Sterility is necessary because contamination can suppress the desired reaction, induce the formation of faulty conversion products or cause total substrate breakdown. Fermentation media is sterilized by autoclaving.
- **Aeration and stirring:** For efficient microbial transformation, oxygen should be placed in intimate contact with cellular structure. Sterile air can be obtained by passing air through filters. Stirring or orbital shaking increases the efficiency of the transformation.
- **Diffusion:** For the transformation, which occurs within the cell, the solubility of the substrate in medium and its rate of diffusion are rate-limiting steps. Emulsifiers such a Tween, water miscible solvents with low toxicity (ethanol,

acetone, DMF, DMSO), cyclodextrin or biphasic ionic liquids may help to solubilize hydrophobic substrates.

- **Elimination of side reactions:** Side reactions can produce unwanted products and reduce yield of desired product which may complicate the process of purification. It can be prevented to some extent by controlling heat or pH of the medium. Isolation of enzyme and its engineering will be more practical solution to get single product in transformation process.
- **Product isolation:** The end product of transformation reactions are usually extra cellular and may occur in either dissolved form or suspended form. Depending on the solubility, the final transformation product may be recovered by precipitation, adsorption or ion exchangers or extraction with appropriate solvents. The transformation broth can be subjected for distillation to obtain volatile products.

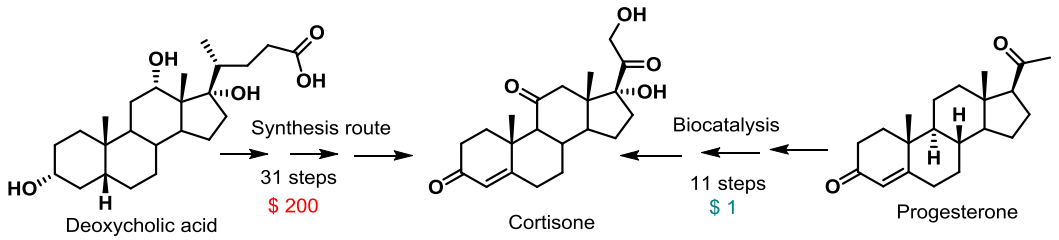
1.2.5: Comparison between enzyme and whole cell systems

The biotransformation can be carry out using whole cell or isolated enzyme, the preference among which is dependent on different factors such as the type of reaction[27], the requirement of co-factors or the scale in which the biotransformation has to be performed. Each approach has its own advantages and disadvantages. Many enzymatic systems are now commercially available or are relatively easy to isolate, they can be stable, easy to use and often produce single product. However, for some reactions where cofactors are used or the need to regenerate the cofactor can limit the use of pure enzymes. Whole cells do not have this disadvantage and are comparatively more stable. But the biotransformation with whole cells some time resulted in side reactions and formation of more than one product is likely.

1.3: Microbial transformation of steroids

The steroid modifications catalyzed by microbial cells represent an engrained research area in biotechnology and large scale industrial processes. The importance of steroid microbial transformation was triggered first time in 1952 when Murray and Peterson of Upjohn Company patented the process of 1lα-hydroxylation of progesterone by a *Rhizopus* species[29], which had reduced 31 chemical reaction steps for the synthesis of cortisone to 11 steps and brought down the cortisone manufacturing cost from 200$ (1949) to 1$ (1979)[30] (Scheme 1.1). Since then microbial steroid transformation has become a powerful tool for the synthesis of novel steroidal drugs, steroid active

pharmaceutical ingredients and key intermediates.



Scheme 1.1: Biocatalyst mediated synthesis of cortisone

The microbial transformations are convenient for bioconversion of complex steroidal molecules at specific positions which otherwise requires complicated and multi-step chemical reactions. Many times the chemical derivatization of steroids accompanies protection of groups and their subsequent regeneration, which lowers the overall yield and makes the process more expensive. Chemical synthesis also requires the use of toxic reagents which are hazardous for health and constitute a serious environmental disposal problem. On the other hand microbial steroid conversions are performed in aqueous environment, at ambient temperature and pH range and thus can provide an efficient alternative to the chemical synthesis. Bioconversion of steroids in combination to chemical synthesis thus can become a key approach for the large scale production of biologically active complex steroid molecules[31]. (Table 1.1) Microorganisms are known to carry out various functionalizations on steroid skeletons[32] (Scheme 1.2)

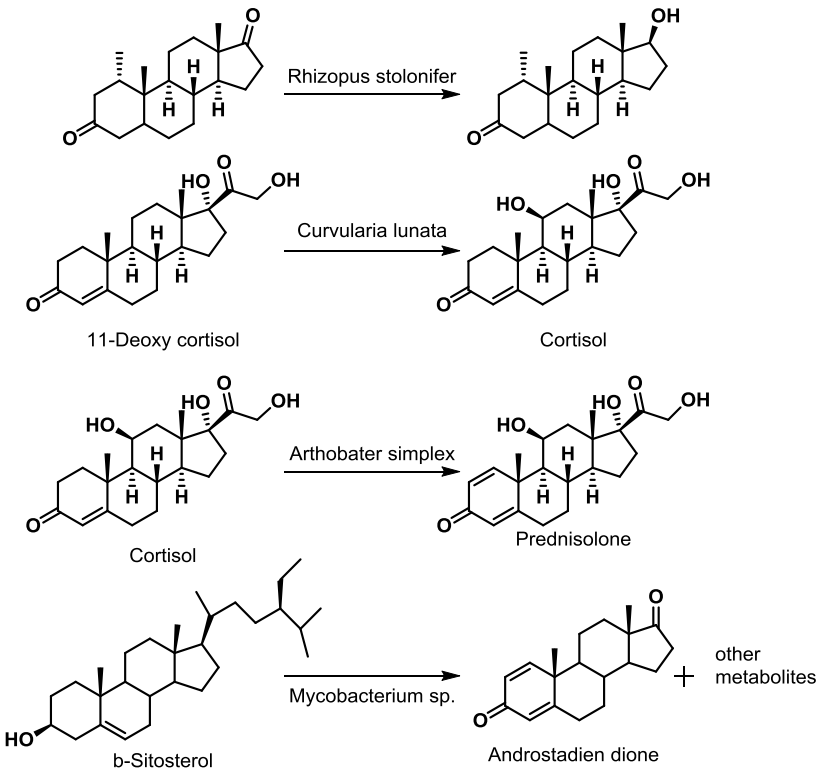
Table 1.1: Some steroid transformations of commercial importance

Reaction	**Substrate to Product**	**Microorganism**	**Some inductrial producers**
11α-hydroxylation	Progesterone to 11α-hydroxyprogesterone	*Rhizopus nigricans*	The Upjohn Company
11β-hydroxylation	11-Dehydroxy cortisol to cortisol	*Curvularia lunata*	Pfizer, Inc.; Gist-brocades
16α-hydroxylation	9α-fluorocortisol to 9α-fluoro-16α-hydroxycortisol	*Streptomyces rosechromogenus*	E.R. Squibb and Sons; Lederle Laboratories
1-Dehydrogenation	Cortisol to prednisolone	*Arthrobacter simplex*	Schering Corporation
1-Dehydrogenation, Side-chain cleavage, and ring D expansion	Progesterone to 1-dehydrotestolactone	*Septomyxa affinis*	The Upjohn Company
Side-chain cleavage,	β-sitosterol to androstadienedione and/or androstenedione	*Cylindrocarpan radicicola*	G.D. Searle and Company

- **Hydroxylation:** Introduction of hydroxyl group in regio- and stereo-selective manner is the most common and important bioconversion for steroid functionalization. Hydroxylation is a common consequence of cytochrome P450 catalysed transformations. The oxygen atom in the hydroxyl group is derived from molecular oxygen (gaseous). Many microorganisms have the capability to introduce hydroxyl groups at various carbon atoms of the steroid molecules, in which fungi are the most efficient hydroxylating systems. The hydroxylation at the 11-position of progesterone was one of the first hydroxylation described.
- **Reduction:** Different microorganisms can carry out reduction of carbonyl and carbon-carbon double bonds in the steroid molecules into single optical isomer.
- **Side chain cleavage:** Synthesis of many steroidal hormones by side chain cleavage of phytosterols such as sitosterol, cholesterol along with some sapogenins such as diosgenin are well established transformations in pharmaceutical industries.
- **Baryer-villiger oxidation:** Reactions of these types are mostly involved in steroid core during degradation by microorganism.
- **Double bond formation:** Dehydrogenation with simultaneous introduction of a double bond has been reported for all four rings of the steroid nucleus. Oxidation of 3β-hydroxy group to keto group with concomitant formation of Δ 5 to Δ 4 is well established bioconversion in the synthesis of steroidal hormones with 4-ene-3-one functionality in ring A.
- **Ring A aromatization:** The microbial aromatization of suitable steroid substrates can lead to ring A aromatic compounds, particularly the estrogens which constitutes an important ingredient in oral contraceptives drugs and play important role in the replacement therapy for menopause treatment.
- **Steroid degradation:** Complete degradation of steroids is mostly studied because of potential environmental risk due to presence of steroids in waste.

1.3.1: Microbial hydroxylations of steroids

Hydroxylation of steroids has very high potential to get library of biologically important molecules, intermediates and its derivatives to offer steroid nucleus suitable for pharmaceutical applications. Hydroxylation mainly changes polarity of compounds which in turn affects toxicity, uptake and excretion of compounds from the cell. Hydroxylation also plays an important role in detoxification of steroid molecules in living systems. Hydroxylated steroids often express a higher biological activity as



Scheme 1.2: Steroid transformation with different microorganisms

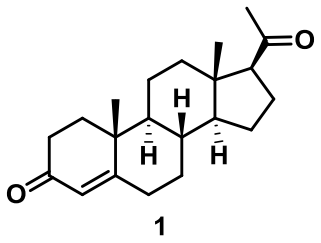
compared to their less polar non-hydroxylated analogs, for example, 7α-hydroxydehydroepiandrosterone exhibited several fold higher immunoprotective properties than non-hydroxylated dehydroepiandrosterone.[33] Because of complex structure, it is a troublesome task to introduce hydroxyl group at specific position on a steroid moiety. Microbial hydroxylation is one of the well-established routes to get diverse array of hydroxylated steroids. Microorganisms which can introduce hydroxyl group on basic steroid skeleton in positions from C-1 to C-21 are well documented and their ability of regio- and stereo-selective hydroxylation has found diverse applications in steroid industry.

The regio- and stereo-selective hydroxylation with particular organism mainly depends on the nature, type and positions of functional groups present on the steroidal nucleus. However, there are few organisms which can carry out hydroxylation at specific position for a range of steroidal molecules.

1.3.2: Microbial hydroxylation of steroidal hormones

1.3.2.1: Progesterone (1) transformation: Progesterone (**1**) is a C-21 steroid hormone which belongs to the pregnane class of steroids. It is a female sex hormone, involved in the regulation of menstrual cycle and commonly called as P4. Progesterone has the ability to reduce damage from spinal cord injury and repairing of myelin following

nerve lesion.[9] The hydroxyl derivatives of progesterone are found to have potent activities than parent compound. For example 14β-hydroxy progesterone is cardioactive and its C-3 gylcoside derivative show strong interaction with cardiac glycoside receptor of heart muscle.[34] The hydroxylation of progesterone using different microorganisms is well documented. The transformation of progesterone with *Mucor piriformis* yielded 14α-hydroxyprogesterone as major metabolites along with traces of 6β,14α-, 7α,14α-, 7β,14α-dihydroxy progesterone derivatives. Incubation of progesterone with *Curvularia lunata* hydroxylated progesterone specifically at 15β-position whereas other cultures like *Aspergillus phoenicis* and *Aspergillus fumigatus* also gave few dihydroxy progesterone derivatives. The filamentous fungi *Rhizopus nigricans, Aspergillus ochraceus, Aspergillus fumigatus, Rhizopus arrhizus* efficiently produced 11α-hydroxyprogesterone, this process was scaled up to industrial level. The transformation of progesterone with different organism is summarized in the Table 1.2 which reveals that different microorganism can be used to get spectrum of hydroxylated, biologically important progesterone derivatives.



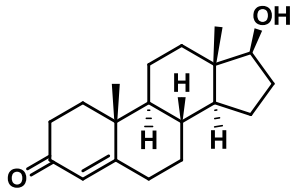
1

Table 1.2: Microbial hydroxylation of progesterone (**1**)

2β-hydroxylation	Cyanidiophyceac emersonii[35], Scemedesmus capricortum[35]
6β-hydroxylation	*Mucor piriformis*[36], *Acremonium strictum*[37], *Cephalosporium aphidicola*[38], *Cyanidiophyceac emersonii*[35] , *Muriella aurantiaca*[35], *Galdieria sulphuraria*[35], *Scemedesmus capricortum*[35], *Mucor griseocyanus*[39], *Apiacrea chrysosperma*[40], *Aspergillus nidulance*[41], *Botryosphaerica obtusa*[42], *Syncephalastrum racemosum*[43], *Caldariella acidophila*[44], *Rhizopus arrhizus*[45] , *Rhizopus oryzae*[46]
6α-hydroxylation	*Caldariella acidophila*[44],
7β-hydroxylation	*Mucor piriformis*[36], *Acremonium strictum*[37], *Aspergillus fumigatus*[47], *Botryosphaerica obtusa*[42],
7α-hydroxylation	*Mucor piriformis*[36], *Cochliobolus lunata*[48], *Mucor griseocyanus*[49], *Curvularia clavata*[50], *Pseudomonas blaksleeanus*[51]
9α-hydroxylation	*Cyanidiophyceac emersonii*[35], *Apiacrea chrysosperma*[40], *Botryosphaerica obtusa*[42]
11β-hydroxylation	*Cochliobolus lunata*[48], *Cochliobolus specifer*[52], *Aspergillus nidulance*[41], *Aspergillus niger*[53]
11α-hydroxylation	*Rhizopus nigricans*[54], *Aspergillus ochraceus*[55], *Acremonium strictum*[37], *Aspergillus fumigatus*[47], *Cochliobolus specifer*[52], *Cephalosporium aphidicola*[38], *Rhizopus arrhizus*[56], *Botryosphaerica obtusa*[57], *Aspergillus phoenicis*[58], *Aspergillus niger*[53], *Mucor racemosus*[59]
12β-hydroxylation	*Cephalosporium aphidicola*[38]
12α-hydroxylation	*Mucor racemesus*[57]
14α-hydroxylation	*Mucor piriformis*[36], *Mortierella isobellina*[60], *Cochliobolus lunata*[48], *Cyanidiophyceac emersonii*[35], *Mucor griseocyanus*[39], *Apiacrea chrysosperma*[40], *Curvularia clavata*[50], *Mucor racemosus*[59], *Mucor hiemalis*[61], *Mucor plumbeus*[62], *Mucor parasiticus*[63]

15β-hydroxylation	*Acremonium strictum*[37], *Aspergillus fumigatus*[47], *Apiacrea chrysosperma*[40], *Nigrospora sphaerica*[57], *Botryosphaerica obtusa*[42], *Pseudomonas blaksleeanus*[51], *Aspergillus phoenicis*[58], *Curvularza lunata*[50]
15α-hydroxylation	*Muriella aurantiaca*[35], *Apiacrea chrysosperma*[40], *Sepedonium ampullosporum*[64]
16α-hydroxylation	*Cyanidiophyceac emersonii*[35], *Sepedonium ampullosporum*[64], *Streptomyces coriofaciens*[65],
17α-hydroxylation	*Acremonium strictum*[37], *Cochliobolus specifer*[52], *Sepedonium ampullosporum*[64], *Rhizopus nigricans*[54], *Curvularia lunata*[50]
21-hydroxylation	*Cochliobolus specifer*[52], *Aspergillus nidulance*[41]

1.3.2.2: Testosterone (2) transformation: Testosterone (**2**) is a C-19 anabolic steroid and male sex hormone responsible for the development of secondary male characteristics. It is an anabolic steroid and plays important role in the treatment of Diabetes[10], promoting the growth of muscle and bones.[11] The hydroxylation of testosterone is important because of its physiological role in metabolism and also in the improvement of the catabolic states[66]. In microbial system testosterone was activelytransformed by *M. griseocynus* and *M. piriformis* into 14α-hydroxytestosterone and 14α-hydroxy-4-androstene-3,17-dione as major metabolites. Microbial transformation of testosterone by *Aspergillus ochraceus* and few *Rhizopus sp.* afforded 11α-hydroxytestosterone. In literature, many organisms have been reported to carry out hydroxylation of testosterone from C2 to C16 positions as mentioned in the Table 1.3.



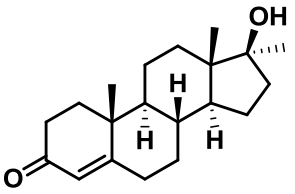
2

Table 1.3: Microbial hydroxylation of testosterone (**2**)

2β-hydroxylation	*Gnomonia fructicola*[67], *Whetzelinia sclerotiorum*[62]
6β-hydroxylation	*Fusarium oxysporum*[68] , *Curvularia lunata*[69], *Geobacillus kaustophilus*[70] *Whetzelinia sclerotiorum*[62], *Phanerochaete chrysosporium*[62] , *Absidia glauca*[71], *Thamnostylum piriforme*[63], *Phycomyces blakesleeanus*[72], *Fusarium culmorum*[73], *Rhizopus nigricans*[74], *R. arrhizus*[74], *R. reflexus*[74] , *Botryosphaerica obtusa*[75] , *Giberella fujikuroi*[76] , *Rhizomucor tauricus*[77], *Fusarium oxysporum*[78]
6α-hydroxylation	*Curvularia lunata*[69], *Trichoderma hamatum*[79]
7α-hydroxylation	*Didymosphaeria igniaria*[80], *Absidia glauca*[71], *Phycomyces blakesleeanus*[72], *Botrytis cinerea*[81]
9α-hydroxylation	*R. equi*[82]
11β-hydroxylation	*curvularia lunata*[83]
11α-hydroxylation	*Trichoderma hamatum*[79], *Beauveria bassiana*[84], *Rhizopus oryzae*[46], *Aspergillus ochaceous*[85], *Rhizopus nigricans*[74], *R. arrhizus*[74], *R. reflexus*[74], *Rhizomucor tauricus*[77], *Rhizopus stolonifer*[86], *Fusarium lini*[86]
12β-hydroxylation	*Fusarium oxysporum*[78]
14α-hydroxylation	*Curvularia lunata*[69], *Mucor plumbeus*[62], *Absidia coerulea*[87], *Mucor griseocyanus*[63], *Mucor piriformis*[88], *Aspergillus wentii*[89]
15β-hydroxylation	*Fusarium culmorum*[73], *Aspergillus fumigatus*[90]

15α-hydroxylation	*Fusarium oxysporum*[78] , *Fusarium lini*[86]
16α-hydroxylation	*Whetzelinia sclerotiorum*[62], *Wojnowicia graminis*[91]
16β-hydroxylation	*Whetzelinia sclerotiorum*[62], *Wojnowicia graminis*[91]

1.3.2.3: 17α-methyltestosterone (3) transformation: Like testosterone, 17α-methyltestosterone (**3**) is an anabolic steroid, useful in the treatment of male with testosterone deficiency and breast cancer in females. It is a potent inhibitor of aromatase activity[12] and also known to stimulate growth and weight. Hydroxyl derivatives of 17α-methyltestosterone are as active as parent compound[10]. As microbial reactions are unique for their hydroxylation, it can be used to get different 17α-methyltestosterone hydroxyl derivatives. Microbial transformation of 17α-methyltestosterone with *Rhizopus sp*. mainly gives 11α-hydroxy metabolites. The pattern of 17α-methyltestosterone transformation by *M. griseocynus* and *M. piriformis* is similar to that of testosterone where, 14α-hydroxylation is the major activity. Transformation of 17α-methyltestosterone is summarized in the Table 1.4 which indicates the choice of microorganism for the required transformation.

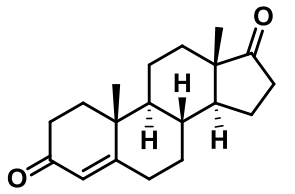


3

Table 1.4: Microbial hydroxylation of 17α-methyltestosterone (**3**)

6β-hydroxylation	*Fusarium culmorum*[92], *Cephalosporium aphidicola*[93], *Rhizopus nigricans*[74], *R. arrhizus*[74], *and R. reflexus*[74]
7α-hydroxylation	*Botrytis cinerea*[81] , *M. racemosus*[94] , *Absidia coerulea*[87], *Mucor griseocyanus*[95]
11β-hydroxylation	*Absidia coerulea*[87]
11α-hydroxylation	*Fusarium culmorum*[92] , *Trichoderma hamatum*[79], *Rhizopus nigricans*[74], *R. arrhizus*[74], *R. reflexus*[74]
12β-hydroxylation	*Fusarium culmorum*[92]
12α-hydroxylation	*Mucor racemosus*[94]
14α-hydroxylation	*Cephalosporium aphidicola*[93]
15α-hydroxylation	*Penicillium notatum*[96], *Mucor racemosus*[94], *Fusarium culmorum*[92]

1.3.2.4: 4-androstene-3,17-dione (4) transformation: 4-androstene-3,17-dione (**4**) belongs to the C-19 androgen class of steroid. It is important hormonal metabolite in different tissues and known to take part in metabolism of drugs.[13] It is a common precursor of male and female sex hormone. The 14α-hydroxy derivative of 4-androstene-3,17-dione are cardioactive and reputed aromatase inhibitor.[97] Earlier reports for hydroxylation of 4-androstene-3,17-dione are included in the Table 1.5, which indicate that only



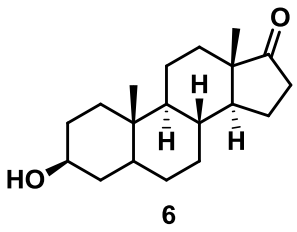
4

few fungal systems such as *Neurospora crassa*, *M. racemosus* could be able to carry out hydroxylation at 6β- and 7α-positons whereas others such as *Rhizopus* and *Aspergillus sp*. selectivity hydroxylates at 11α-position.

Table 1.5: Microbial hydroxylation of 4-androstene-3,17-dione (**4**)

1α-hydroxylation	*Penicillium sp*[98]
6β-hydroxylation	*Acremonium strictum*[37], *Aspergillus fumigatus*[99], *Botryosphaerica obtusa*[75], *Neurospora crassa*[100], *M. racemosus*[101], *Rhizopus nigricans*[74], *R. arrhizus*[74], *R. reflexus*[74] ,*Thamnostylum piriforme*[63]
7β-hydroxylation	*Acremonium strictum*[37], *Aspergillus fumigatus*[99], *Botryosphaerica obtusa*[75]
7α-hydroxylation	*Mucor piriformis*[102], *Acremonium strictum*[37], *Neurospora crassa*[100], *M. racemosus*[101], *Thamnostylum piriforme*[63]
9α-hydroxylation	*Neurospora crassa*[100], *Bordetella sp.*[103], *R. erythropolis*[104]
11α-hydroxylation	*Acremonium strictum*[37], *Aspergillus fumigatus*[99], *Cunnmghamella elegans*[105], *Beauveria bassiana*[106], *Rhizopus nigricans*[74], *R. arrhizus*[74]*R. reflexus*[74]
14α-hydroxylation	*Curvularia lunata*[107], *Acremonium strictum*[37], *Mucor piriformis*[102], *Cunninghamella elegans*[105], *Neurospora crassa*[100], *M. racemosus*[101], *Gongronella butleri*[108], *Thamnostylum piriforme*[63], *Mucor griseocyanus*[95]
14β-hydroxylation	*Mucor piriformis* [102]
15α-hydroxylation	*C. lunata*[107], *Fusarium lini*[86]

1.3.2.5: Trans-androsterone (6) transformation: Androsterone (**6**) have anticonvulsant property[109] and its hydroxyl derivatives were shown to exert anti-glucocorticoid and neuroprotective activity[18]. In literature, few microbial species such as *Rhizopus* sp. are reported which have the ability to convert androsterone to 7β-hydroxyandrosterone. Many other microorganisms are also known to have the ability to hydroxylate androsterone at various positions (Table 1.6).



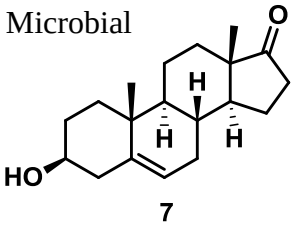
6

Table 1.6: Microbial hydroxylation of trans-androsterone (**6**)

1α-hydroxylation	*Penicillium sp.*[110]
7β-hydroxylation	*R. arrhizus*[111], *R. circinnans*[111]
7α-hydroxylation	*Mortierella isabellina*[112], *Fusarium moniliforme, Rhizopus nigricans*
9α-hydroxylation	*Mortierella isabellina*[112], *Absidia regnieri*[113], *Diaporthecelastrina*[114]
11β-hydroxylation	*Aspergillus tamarii*[115]
11α-hydroxylation	*Mortierella isabellina*[112], *Aspergillus wentii*[116], *Cephalosporium aphidicola* [117] , *Aspergillus ochraceus*[118]

1.3.2.6: Trans-dehydroandrosterone (7) transformation: Similar to androsterone, dehydroepiandrosterone (**7**) is endogenous and most abundant steroid hormone in

human. It is used in the treatment of various age related diseases. Microbial hydroxylations of this hormone 7 is well studied (Table 1.7).

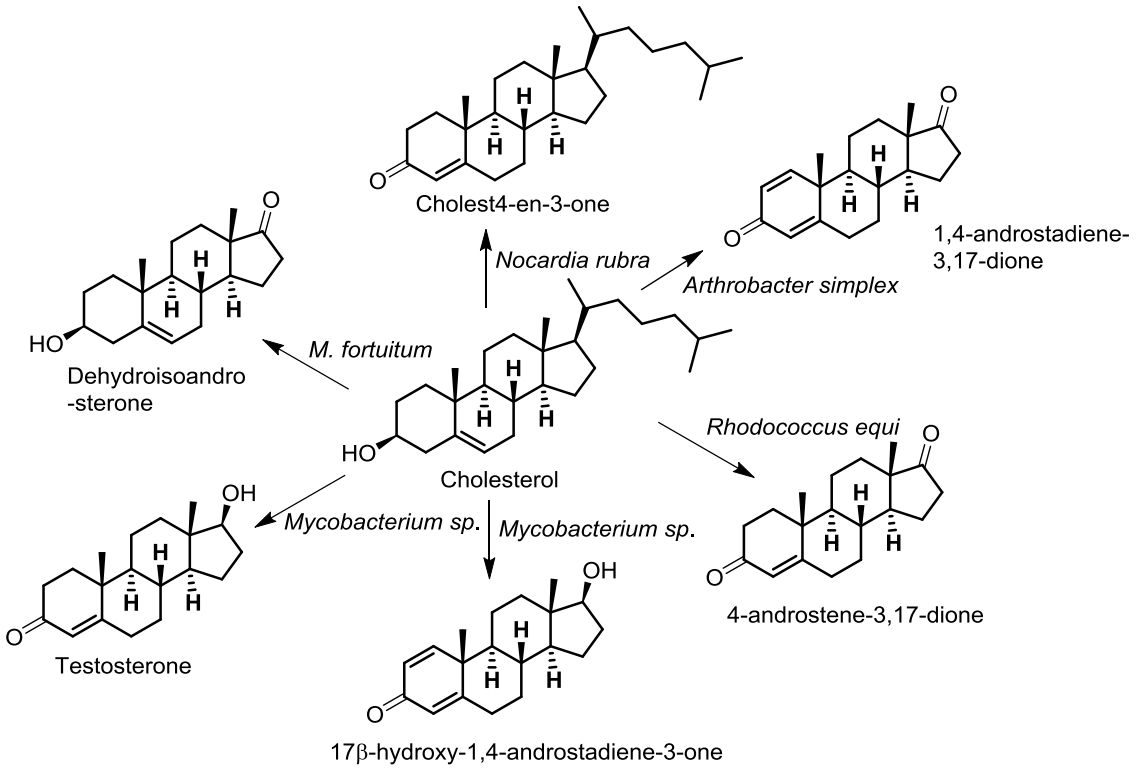


7

Table 1.7: Microbial hydroxylation of trans-dehydroandrosterone (**7**)

1α-hydroxylation	*Penicillium sp*[119].
4β-hydroxylation	*Cephalosporium aphidicola*[120]
7β-hydroxylation	*Aspergillus wentii*[116], *Botryodiplodia malorum*[121], *Mucor racemosus*[122], *Absidia orchidis*[123], *Fusarium oxysporum*[68]
7α-hydroxylation	*Aspergillus wentii*[116], *Penicillium griseopurpureum*[124], *Curvularia lunata*[6] , *Gibberella zeae*[125], *Colletotrichum lini*[121], *Mucor racemosus*[122], *Rhizopus stolonifer*[126], *Absidia orchidis*[123], *Fusarium oxysporum*[68], *Gelasinospora retispora*[127], *P. chrysosporium*[62], *M. plumbeus*[62]
11β-hydroxylation	*Rhizopus stolonifer*[126]
11α-hydroxylation	*Rhizopus arrhizus*[128], *Gelasinospora retispora*[127]
14α-hydroxylation	*Penicillium griseopurpureum*[124], *Curvularia lunata*[6]
15α-hydroxylation	*Penicillium griseopurpureum*[124], *Colletotrichum lini*[121], *Fusarium oxysporum*[68]
16β-hydroxylation	*Pleurotus ostreatus*[129]

1.3.2.7: Cholesterol (8) transformation and side chain cleavages: Microbial degradation of the C-17 saturated side chain of widely available phytosterols such as cholesterol; sitosterol etc for the preparation of androstanes is a well studied transformation. Different androstanes such as 4-androstene-3,17-dione, 1,4-



Scheme 1.3: Microbial transformation and side chain cleavage of cholesterol (**8**)

androstadiene-3,17-dione, testosterone, androsterone, have been obtained by fermentation of cholesterol with various strains such as *Arthrobacter amplex* [130], *Mycobacterium fortuitum* [131], *M. mucosum, M. lactrcola* [132] and *Rhodococcus terrae* [133].

1.3.3: Methods for the enhancement of steroid microbial conversion

Despite substantial developments in microbial steroid transformation, few aspects still need attention for rapid industrialization of steroid transformation processes. Major difficulties in microbial steroid conversions are solubility of the steroids in aqueous medium, substrate and product toxicity to the biocatalysts and recovery of metabolites.The solubility of steroids in aqueous medium is in the range of 1 to 100 µM.[134] It limits the availability of substrate to the whole cells resulting in low substrate concentration in the bioconversion process. Being solid in nature, addition of powdered steroids results in the formation of clumps in the fermentation media. It can be overcome by dissolving steroids into water miscible organic solvents. But the concentration of organic solvents should not be more than 5% which may affect microbial efficiency. Few methods for substrate addition based on steroid suspensions in emulsifying agents such as Tween-80[135], Triton X-100[136], polyethylene glycol, silicon, vegetable oil etc are renowned. Addition of inclusion complexes of steroids with cyclodextrins is an effective method to reduce hydrophobic nature and increase dissolved substrate concentration in the fermentation media[102]. The result of this method depends on the chemical nature of cyclodextrin used. The uptake of steroid molecules inside the cell is controlled by cell membrane permeability which can be modulated using different reagents like D,L-norleucine, glycine, lectin, antibiotics[137,138].

The toxicity of added substrate or metabolites in transformation process is another factor which effects overall performance of steroid microbial conversion. The easiest way to overcome this drawback of transformation is the selection of strain through intense screening for microbial conversions which can tolerate high concentration of substrate.[139] The toxicity of substrate as well as product can also be reduced by using some additives in the fermentation media. For example, amberlite XAD 7 resin is used as additive to absorb 4-androstene-3,17-dione and 1,4-androst-diene-3,17-dione formed during mycobacterium catalyzed phytosterol bioconversion.[140] Immobilization of cells in different media such as polyvinyl alcohol, calcium alginate, polyacrylamide gel[141] etc can be used to reduce toxicity of steroids thus also permits recycling of biomass. Separation of metabolites formed in the fermentation media from

biomass is also easier with immobilized culture. But it suffers from drawback of low productivity and extra processing is required for immobilization of cells[83].

1.4: Steroid hydroxylases

Microbial hydroxylation introduces a hydroxyl group at the desired position of a steroid molecule. Fungal hydroxylations can replace a series of separate chemical reactions which are not economical, or which can even be impossible otherwise. As discussed earlier, a large number of steroidal substrates have been investigated for the hydroxylation and fungi can hydroxylate steroids at almost all positions

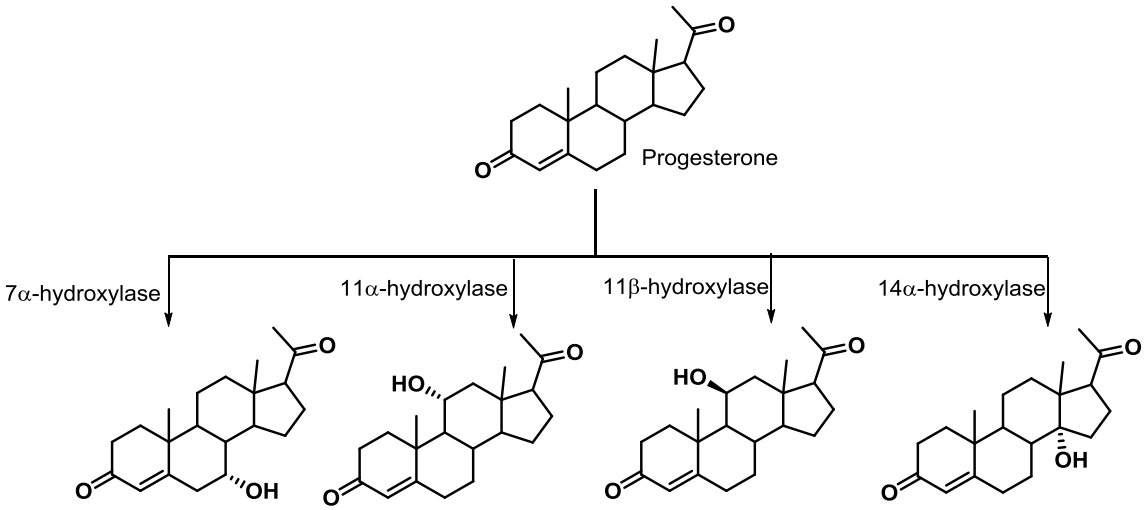


Figure 1.4: The hydroxylation of steroids catalyzed by 7α-, 11α -, 11β- and 14α -hydroxylases

Microbial steroid hydroxylation reactions are catalyzed by the enzymes collectively called as steroid hydroxylases or monooxygenases (Fig. 1.4). These enzymes have the ability to incorporate oxygen atom into substrate from the atmospheric molecular oxygen. These hydroxyalases are multicomponent enzymes and belong to Cytochrome P450 family. The fungal hydroxylases are mainly inducible with substrates and are often localized on the endoplasmic reticulum membrane. The number of monohydroxylated products and the positions of hydroxyl group insertion depend on the geometry of the steroid molecules and are generally possible at four binding positions (normal, reverse, inverted-normal, and inverted-reverse). It was confirmed experimentally, from the evidence of hydroxylation of 3β-hydroxy-17α-oxa-D-homo-5α-androstan-17-one at four different carbons (11β-, 6β-, 7β-, 11α-) corresponding to different binding positions[142]. In recent years, the microbial steroid hydroxylases that have been isolated includes a 7α- and 15β-hydroxylases of *Phycomyces blakesleeanus*[51], a 14α-hydroxylase of *Mucor piriformis*[88], an inducible 11α-hydroxylase from *Nocardia*

crassa[143], a 9α-hydroxylase from *Rhodococcus*[144], a 11β- hydroxylase and 14α-hydroxylase from *Cochliobolus lunatus*[145] and Cunninghamella Blakesleeana[146], a 11α-hydroxylase from *Nectria haematococca*[147] and *Rhizopus sp.*, a 7α-hydroxylase from *Fusarium moniliforme*[148], and a 15β-hydroxylase from *Penicillium raistricki*.[149]

1.4.1: Cytochrome P450 monooxygenase

There is a broad interest in the P450s because of the significance of these enzymes in a wide variety of disciplines, ranging from medical genetics to inorganic chemistry. Cytochrome P450 monooxygenase system represents one of the largest superfamily (Fig. 1.5)[150] of enzymes. Cytochrome P450 system with a cellular chromophore, was first named in 1961, because of the spectral properties of the enzymes. Cytochrome P450 is the collective name for a distinct group of protoheme-containing proteins, which show a soret absorption band at around 450 nm, in the carbon monoxide-difference spectrum of dithionite-reduced samples [151], where carbon monoxide binds and acts as ligand to the heme iron. The systematic nomenclature for these enzymes has been derived

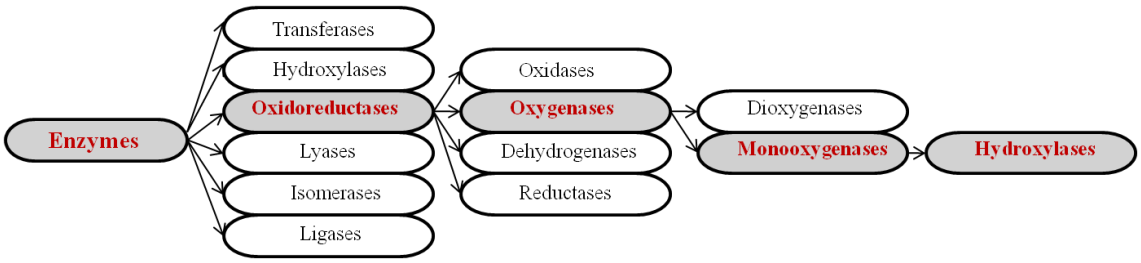


Figure 1.5: Assignment of Cytochromes P450 to enzyme groups. P450 are associated with the dark grey colored subdivisions

based on structural homology.[152] Cytochrome P450 proteins are conveniently arranged into the families and subfamilies on the basis of percentage amino acid sequence identity (Fig. 1.6). The enzymes that share 40% identity are assigned to a particular family designated by an Arabic numeral, whereas those sharing 55% identity make up a particular subfamily designated by a letter. For example, the sterol 27-hydroxylase enzyme and the vitamin D3 24-hydroxylase enzyme are both assigned to the CYP27 family because they share 40% sequence identity. Sterol 27-hydroxylase is further assigned to the CYP27A subfamily, and vitamin D3 24-hydroxylase assigned to CYP27B, because their protein sequences are <55% identical. At present, 267 families with more than 5000 genes are notified.

The cytochrome P450 proteins are heme-type multicomponent enzymes, which are broadly dispersed among prokaryotes and eukaryotes. Cytochrome P450 enzymes

(CYPs or P450s) are heme containing monooxygenases which were recognized and defined as a distinct class of hemoproteins over 50 years ago[153]. Heme is a prosthetic group linked to the apoprotein via a conserved cysteine consisting of an iron ion coordinated with four nitrogen atoms of the porphyrin. These enzyme catalyze a wide variety of oxidation reactions including the activation of sp^3 hybridized carbon atoms, epoxidation of C=C double bonds, aromatic hydroxylation, N-oxidation, deamination and dehalogenation, as well as N-, O-, S- dealkylation. Their ability to catalyze the

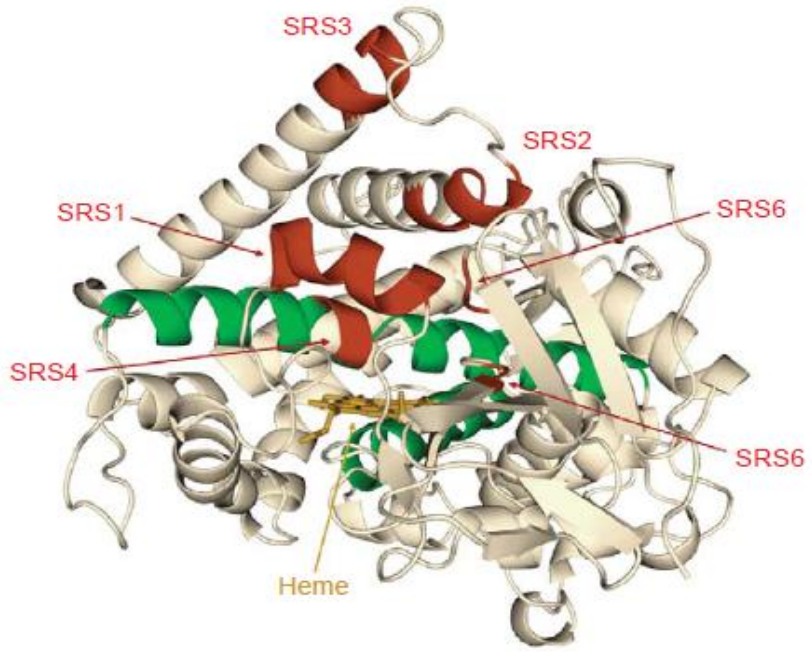


Figure 1.6: Model of the conserved tertiary structure of P450 monooxygenase. The protoporphyrin IX (heme) is coloured orange, the substrate recognition site (SRS1-SRS6) is coloured red, and the heme coordinating I and L helices are shown in green. The model was generated using PyMol (http://pymol.sourseforge.net/) from the crystal structure1jpz of P450-BM3

regio-, chemo- and stereospecific oxidation of a vast number of substrates reflects their biological roles and makes them important candidates for the biotechnological applications.

The general reaction catalyzed by cytochrome P450 enzyme is-

$$RH + O_2 + NADPH / NADH + H^+ \rightarrow ROH + H_2O + NADP^+ / NAD^+$$

In all cases, the multicomponent cytochrome P450 monooxygenase requires two electrons that are ultimately supplied by reduced nicotinamide cofactors (NADH or NADPH). The P450 system has been classified into two main classes (although other small subclasses are there from III-X but are very rarely observed) depending upon the ways by which the required electrons are transported to the heme center, the class I mitochondrial/bacterial type and the class II microsomal type.[154] (Fig. 1.7)

Class I P450 includes most bacterial cytochrome P450 systems and the mitochondrial P450 systems from eukaryotes. The P450 systems in this class consist of three separate proteins: a FAD-containing reductase, which transfers electrons from cofactors NADH or NADPH to the second component of the system, a ferredoxin, which in turn reduces the cytochrome P450. In bacteria, all three proteins are soluble whereas in eukaryotes, ferredoxin is the only soluble protein of the mitochondrial matrix, the reductase and cytochrome P450 are membrane associated and membrane-bound to the inner mitochondrial membrane, respectively.[155]

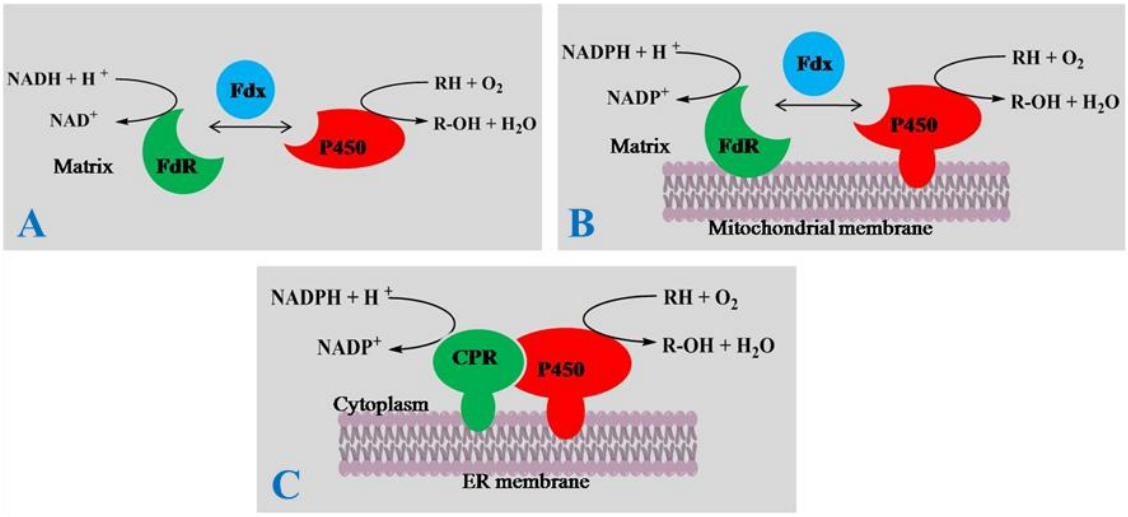


Figure 1.7: Schematic organization of different cytochrome P450 systems. (A) Class I, bacterial system; (B) Class I, mitochondrial system; (C) Class II microsomal system

Class II cytochromes P450 are found in the endoplasmic reticulum of eukaryots. CYP system in this class contains two integral membrane proteins (Fig 1.7), the cytochrome P450 and the NADPH cytochrome P450 reductase (CPR; EC 1.6.2.4). In these, a cytochrome P450 reductase contains C-terminal region with tightly bound flavin cofactors (FAD and FMN) that accepts electrons from NADH / NADPH. The small N-terminal portion binds both with the membrane and the P450 monooxygenase. The flow of electron is from NADH or NADPH to FAD, then to FMN and finally to the P450 monooxygenase.

1.4.2: Mechanism of cytochrome P450 catalyzed microbial hydroxylation

The mechanism for the hydroxylation by cytochrome P450 system has been particularly well studied in the case of cytochrome $P450_{cam}$ by Griffin and Peterson[156]. The resting enzyme is in the ferric state, has a thiolate proximal ligand and the distal ligand is usually a water molecule. The catalytic cycle of cytochrome P450[157] is triggered, as the substrate molecule enters into the active site and displaces the axial water molecule (**A**

Fig 1.9). Substrate binding to P450 is normally a fast process. The substrates of P450s are typically lipophilic and readily bind to the hydrophobic binding pocket in the active site of the P450s. This is resulted in the displacement of the iron from the original position in the porphyrin (**B**, Fig 1.8, 1.9) which causes a shift in the redox potential of the heme iron atom by up to 300 mV and makes the heme a better electron sink and triggers an electron transfer from the reductase protein, this electron-transfer event then initiates the cycle. Class II P450s receive electrons from NADPH via CPR, whereas the electrons from NADH or NADPH are sequentially transferred to ferredoxin reductase, ferredoxin and P450 in class I P450s. An electron transfer from the reductase causes the reduction of the iron(III) center to the ferrous state (**C**, Fig. 1.9).

The reduced form of CYP (intermediate **C** in Fig. 1.9) is an extremely efficient reducing agent with iron(II) porphyrin complex. Triplet dioxygen reacts with ferrous P450 to produce a stable dioxygen adduct. One electron from the iron(II) center and one from the triplet oxygen pair create an iron(III)- oxygen bond. This oxygen-iron complex (intermediate **D** in Fig. 1.9) is relatively stable but can dissociate to an iron(III) and superoxide anion. The second reduction step is the rate-determining step in many P450s. This relatively slow step generates a negatively charged iron(III)-peroxo complex (intermediate **E** in Fig. 1.9) which is probably quickly protonated at this stage to generate the iron(III)- hydroperoxo complex **E′**. Threonine residue at the active site in P450 plays a key role in the protonation of the iron-peroxo intermediate **E**. The protonation of the terminal oxygen atom of intermediate **E** is not going to change the oxidation state of the iron center which is already III. The first protonation produces

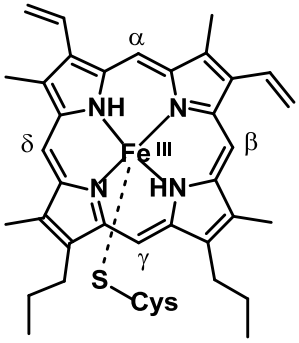


Figure 1.8: an iron-(III) protoporphyrin-IX linked with a proximal cysteine ligand

a P450-FeIII-OOH intermediate which can behave as a nucleophile. The second protonation is necessary to produce an electrophilic high-valent iron- (V)-oxo species (intermediate **F** in Fig. 1.9) with subsequent elimination of a water molecule. In this second protonation step, the formal oxidation state of the iron center increases from III to V with the formation of the iron-oxo species **F**. This iron-oxo intermediate is highly

unstable. At the end of the cycle, the oxygen insertion occurs into the substrate through abstraction of one hydrogen atom from the substrate to give a radical intermediate (R•) followed by oxygen rebound to form C–OH[158] as shown in Fig 1.10. Here Tyrosine comes into play, which together with the product complex forms a polar environment which causes the re-entrance of bulk water molecules into the pocket and releases the hydroxylated product from the enzyme.

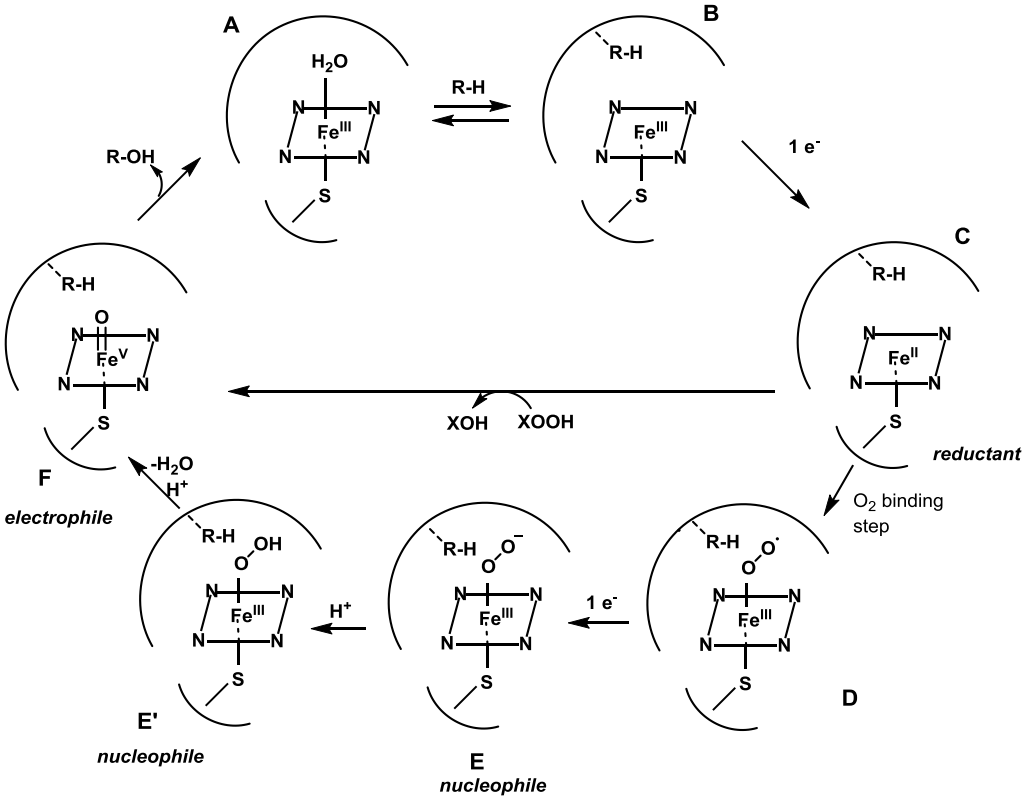


Figure 1.9: The currently accepted mechanism of P450 enzymes

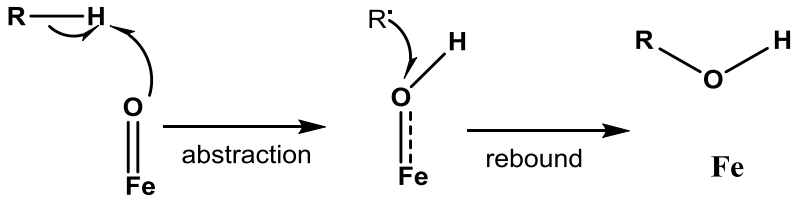


Figure 1.10: Hydrogen abstraction –oxygen rebound mechnism

1.4.3: Applications of P450 enzymes

The diverse activities of P450 monooxygenases have potential applications in different field of science. A well-established commercial application of P450 is the biotransformation of progesterone to cortisone by *Rhizopus sp*. The P450 enzyme also find application in the treatment of hazardous chemical waste due to their potential for bioremediation and biodegradation of organic compounds such as industrial dyes,

pesticide, insecticide etc. and the demand for highly active enzymes that are capable of degrading environmental waste is growing constantly.

1.4.4: Limitation of enzyme application

Although cytochrome P450 systems represent a promising alternatives, a number of limitations have restricted their use in synthesis and in industrial applications. These include substrate specificity, the need for a complex system of cofactors, incompatibility with organic solvents, low activity, poor stability and efforts for the identification and purification of enzyme. Several research groups have attempted to overcome these drawbacks using approaches such as mutagenesis, chemical modifications, conditions engineering, immobilization and used activity based protein profiling in the purification step. Recombinant versions of these enzymes with fused reductase domain, is a growing research topic.

Immobilization on various supports has proven very useful to enhance the stability of cytochrome enzyme by entrapment in calcium alginate or in polyacrylamide or by adsorption on cyanogen bromide activated Sepharose 4B.[159,160] The need for the expensive cofactor NAD(P)H and a redox partner is a notable obstacle for the use of isolated P450s in biotechnology. Use of NADPH generating system is very common approach where Glucose-6-phosphate dehydrogenase uses Glucose-6-phosphate as substrate and converts $NAD(P)^+$ to NAD(P)H *in situ*. This NADPH will be available for cytochrome catalyzed hydroxylation reaction, $NAD(P)^+$ formed in the reaction will be recycled. The use of chemicals to directly replace one or more cofactors is yet another alternative to facilitate the use of P450 enzymes in synthesis. Strong oxidants that directly oxidize the Fe(II) of P450s to an Fe-oxo species e.g. sodium periodate, sodium chlorite, iodozobenzene and peracids have proven useful to hydroxylate various substrates, yet have a high potential to generate side reactions.[160,161] Site-directed mutagenesis have been applied very successfully to stabilize and/or solubilize proteins of different P450 systems, especially membrane-bound microsomal P450s.[162] Different techniques have been evolved such as gel permeation, ion-exchange chromatography for the purification of proteins.

1.5: Immobilization of fungal cells

Immobilization of cells or pure enzyme is one of the promising methods to overcome few limitations of biotransformation such as contamination, tedious extraction of metabolites, less stability of cells and pure enzyme etc. Thus, the immobilization has

enormous importance from an industrial point of view. Many methods namely adsorption, covalent bonding, cross-linking, entrapment and encapsulation are widely used for immobilization[163].

- **Adsorption:** This was the first example of cell immobilization[164]. The adsorption phenomenon is based on electrostatic interactions (van der Walls forces) between the charged support and microbial cells. All glasses or ceramic supports are comprised of varying proportions of oxides of alumina, silica, magnesium, zirconium, etc. which involves in bond formation between the cell and the support.
- **Covalent bonding:** The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (crosslinking) agent[165]. For covalent linking, chemical modification of the surface is necessary.
- **Cross linking:** Microbial cells can be immobilized by cross-linking each other with bi- or multifunctional reagents such as glutaraldehyde.[166] The chemicals used for cross-linking are toxic which limits the applicability of this process.
- **Entrapment:** The most extensively studied method in cell immobilization is the entrapment of microbial cells in polymer matrices. The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. Among the above matrices, polyacrylamide and alginate have been widely used.

Table 1.8: Transformation of steroids by immobilized fungal strains

Subtrate	Reaction	Organism	Support Material
Cortexolone	11β-hydroxylation	*Curvularia lunata*	Ca-Alginate[167], Photo-crosslinked resin[168]
Progesterone	11α-hydroxylation	*Rhizopus nigricans*	Agar[169]
		Rhizopus stolonifer	Photo-crosslinked resin[170]
		Aspergillus ochaceus	Polyacrylamide[171]
		Aspergillus phoenicus	Ca-Alginate[172]
Androsterone	15α-hydroxylation	*Aspergillus phoenicus*	Ca-Alginate[172]
	Dehydrogenation	*Rhizopus sp.*	Ca-Alginate[172], Polyacrylmide[172]
		Aspergillus sp.	Ca-Alginate[172], Polyacrylmide[172]

Use of immobilized-cell technology for the biotransformation of steroids has been well explored.[173] Several immobilized fungal cell systems have been studied for steroid hydroxylation processes are summarized in the Table 1.8

1.5.1: Advantages and Disadvantages of Immobilized Cells

Immobilized living cell systems offers several advantages over conventional suspended-cell cultures[174]:

- The physico-chemical interactions between carriers and cells often give rise to increased stability of the entrapped cells which may, in turn, lead to increased cell productivity.
- The re-use of immobilized cells is possible, which makes repeated or continuous operations possible in various types of reactors.
- Wash-out of the cells can be avoided with immobilization, even at high dilution rates.
- The immobilized cells can be easily separated from the reaction system, making the separation and extraction of product from biomass easier.

However, there are few disadvantages in the use of immobilized cells:

- The yields of products may sometime be lowered due deficiency of nutrients
- Different incubation condition may result in the formation of unwanted side-reaction products.

1.6: Scope and Significance of the Present Work

Steroids and their hydroxy derivatives are one of the major classes of terpenoids and widely distributed in animal and plant kingdom. They are used as pharmaceutically important compounds and are key regulators of various biological functions in the cell. Modifications of complex molecules by hydroxylation are very important tool to get library of biologically important molecules. Most ancient and well studied route of chemical synthesis for hydroxylation of steroids involves use of hazardous chemicals. The regio- and stereo- specific hydroxylation of steroids can be achieved in mild conditions using suitable microbial systems. Although, fungal mediated transformations of steroids are well documented, only few fungal species were studied for scale-up and large scale production of hydroxyl derivatives of hormonal steroids. Thus there is a need to establish a biotransformation process for the large scale production of commercially important steroid hydroxyl derivatives. As filamentous fungi are known for their ability to hydroxylate steroids in regio and stereo-selective manner, we have screened and

utilized few fungal systems for the efficient and quantitative transformation of 4-ene-3-one and androstane steroids.

The thesis describes transformation of hormonal steroids using fungal systems belong to the genera of *Mucor*. The quantitative biotransformation of various steroids with 4-ene-3-one functionality such as progesterone, testosterone, 17α-methyltestosterone, 4-androstene-3,17-dione into their respective 6β-,11α-dihydroxy derivatives were studied using a fungal strain *Mucor sp.* (*M881*). The scale up studies at laboratory level up to table top fermentor (5 L capacity) with progesterone and testosterone as substrates for the production of corresponding 6β-,11α-dihydroxy derivatives has been demonstrated. Furthermore, the biotransformation of testosterone and progesterone using a fungal system *Mucor hiemalis* was studied, which efficiently carried out hydroxylation at 14α-, 7α/6β position to form corresponding 7α/6β, 14α-dihydroxy derivatives. Cell free studies indicated that 14α-hydroxylase system was NADPH dependent, inducible, membrane bound and belong to cytochrome P450 family.

***Chapter 2*: Materials and Methods**

This chapter describes the materials used in the study, the maintenance and propagation of the fungal systems, fermentation procedure, and isolation and characterization of biotransformation products.

***Chapter 3*: Biocatalyst mediated hydroxylation of steroids by *Mucor sp* (*M881*)**

This chapter is divided into three sections

Section 3.1: This section describes the transformation of 3-one-4-ene steroids using fungal strain *M881* and biotransformation pathways for these compounds was deduced. Fermentor experiments for large scale production of hydroxylated steroids is presented.

Section 3.2: This section describes the transformation of 3β-hydroxy steroids such as epiandrosterone and dehydroisoandrosterone using *M881*.

Section 3.3: This section describes the resting cell experiments and immobilization of

fungal strain *M881* using calcium alginate and the application of immobilized fungal culture for transformation of progesterone.

Chapter 4*: *In vivo* and *In vitro* investigations of 14α-hydroxylase activity in *Mucor hiemalis

This chapter is divided into two sections

Section 4.1: This section describes the transformation of progesterone and testosterone

using fungal strain *Mucor hiemalis*. Transformation pathways were deduced using isolated metabolites as substrates and time course experiments.

Section 4.2: This section describes cell free preparation of *Mucor hiemalis* for 14α-hydroxylase activity. The standardization of experimental conditions for preparation of microsomal suspension of *M. hiemalis* and hydroxylase assay is presented.

1.7: References

(1) Morgan, B. P.; Moynihan, M. S. *Kirk-Othmer Encycl. Chem. Tech., John Wiley & Sons: New York* **1997**, *22*, 851-921.

(2) Edgren, R. A.; Stanczyk, F. Z. *Contraception* **1999**, *60*, 313-313.

(3) Donova, M. V.; Egorova, O. V. *Applied Microbiology and Biotechnology*, *94*, 1423-1447.

(4) Barnes, P. J.; Adcock, I. *Trends Pharmacol. Sci.* **1993**, *14*, 436-441.

(5) Templeton, J. F.; Kumar, V. P. S.; Bose, D.; Smyth, D. D.; Kim, R. S.; Labella, F. S. *Can. J. Physiol. Pharm.* **1988**, *66*, 1420-1424.

(6) Yaderets, V. V.; Andryushina, V. A.; Voishvillo, N. E.; Stytsenko, T. S.; Zeinalov, O. A. *Pharm. Chem. J.* **2009**, *43*, 55-58.

(7) Al-Awadi, S.; Afzal, M.; Oommen, S. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 48-52.

(8) Mahato, S. B.; Garai, S. *Steroids* **1997**, *62*, 332-345.

(9) Koenig, H. L.; Schumacher, M.; Ferzaz, B.; Dothi, A. N.; Ressouches, A.; Guennoun, R.; Jungtestas, I.; Robel, P.; Akwa, Y.; Baulieu, E. E. *Science* **1995**, *268*, 1500-1503.

(10) Traish, A. M.; Saad, F.; Guay, A. *J. Androl.* **2009**, *30*, 23-32.

(11) Powers, M. L.; Florini, J. R. *Endocrinol.* **1975**, *97*, 1043-1047.

(12) Mor, G.; Eliza, M.; Song, J.; Wiita, B.; Chen, S.; Naftolin, F. *J. Steroid Biochem.* **2001**, *79*, 239-246.

(13) Kliewer, S. A.; Moore, J. T.; Wade, L.; Staudinger, J. L.; Watson, M. A.; Jones, S. A.; McKee, D. D.; Oliver, B. B.; Willson, T. M.; Zetterstrom, R. H.; Perlmann, T.; Lehmann, J. M. *Cell* **1998**, *92*, 73-82.

(14) Chlebowski, R. T.; Herrold, J.; Ali, I.; Oktay, E.; Chlebowski, J. S.; Ponce, A. T.; Heber, D.; Block, J. B. *Cancer* **1986**, *58*, 183-186.

(15) Tan, K.; Roda, R.; Ostrow, L.; McArthur, J.; Nath, A. *Neurology* **2009**, *72*, 1458-1464.

(16) Chung, S. K.; Ryoo, C. H.; Yang, H. W.; Shim, J. Y.; Kang, M. G.; Lee, K. W.; Kang, H. I. *Tetrahedron* **1998**, *54*, 15899-15914.

(17) Mellon, S. H.; Griffin, L. D. *Trends Endocrin. Met.* **2002**, *13*, 35-43.

(18) Dudas, B.; Hanin, I.; Rose, M.; Wulfert, E. *Neurobiol. Dis.* **2004**, *15*, 262-268.

(19) Kokate, T. G.; Svensson, B. E.; Rogawski, M. A. *J. Pharmacol. Exp. Ther.* **1994**, *270*, 1223-1229.

(20) Wolley, L. *The New American Library, New York* **1965**, *Vol I*, 234.

(21) Vallery-Radot, L. D.; "Pasteur Fermentation Centennial"; Chas.Pifzer and Co., I., Newyork **1957**, p 4.

(22) Bertrand, G. *Compt.Rend* **1896**, *122*, 900.

(23) Bertrand, G. *Ann.Chim.Phys.* **1904**, *8*, 3.

(24) Bertrand, G. *Bull.Soch.Chim.France* **1896**, *15*, 627.

(25) Linther, C. J., von Leibig, H. J., ,, , *Z.Physiol.Chem.,I.* **1911**, *72*, 449.

(26) Robert, S. M.; Turner, N. J.; Willetts, A. J.; Turner, M. K. **1995**, First Edition, 39-40.

(27) Faber, K. **2003**, *5th Edition.*

(28) Leresche, J. E.; Meyer, H. P. *Org. Process Res. Dev.* **2006**, *10*, 572-580.

(29) Hogg, J. A. *Steroids* **1992**, *57*, 593-616.

(30) Carballeira, J. D.; Quezada, M. A.; Hoyos, P.; Simeo, Y.; Hernaiz, M. J.; Alcantara, A. R.; Sinisterra, J. V. *Biotechnol Adv* **2009**, *27*, 686-714.

(31) Sebek, O. K.; Perlman, D. *Microbial Technology: Fermentation Technolgy, New York: Academic Press* **2006**, 483-496. .

(32) Donova, M. V.; Egorova, O. V. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1423-1447.

(33) Janeczko, T.; Dmochowska-Gladysz, J.; Kostrzewa-Suslow, E.; Bialonska, A.; Ciunik, Z. *Steroids* **2009**, *74*, 657-661.
(34) Templeton, J. F.; Ling, Y. Z.; Zeglam, T. H.; Labella, F. S. *J. Med. Chem.* **1993**, *36*, 42-45.
(35) Pollio, A.; Pinto, G.; Dellagreca, M.; Demaio, A.; Fiorentino, A.; Previtera, L. *Phytochemistry* **1994**, *37*, 1269-1272.
(36) Madyastha, K. M. *P. Indian A. S.-Chem. Sci.* **1994**, *106*, 1203-1212.
(37) Yoshihama, M. *Yukijirushi Nyugyo Kenkyusho Hokoku* **1993**, *99*, 1-70.
(38) Farooq, A.; Hanson, J. R.; Iqbal, Z. *Phytochemistry* **1994**, *37*, 723-726.
(39) Templeton, J. F.; Kumar, V. P. S.; Marat, K.; Kim, R. S.; Labella, F. S.; Cote, D. *J. Nat. Prod.* **1987**, *50*, 463-467.
(40) Smith, K. E.; Latif, S.; Kirk, D. N.; White, K. A. *J. Steroid Biochem.* **1988**, *31*, 83-89.
(41) El-Refai, A. H. a. G., K. M. *Egypt. J. Microbial.* **1988**, *22*, 327.
(42) Smith, K. E.; Latif, S.; Kirk, D. N.; White, K. A. *J. Steroid Biochem.* **1989**, *33*, 271-276.
(43) Sen, R.; Samanta, T. B. *J. Steroid Biochem.* **1981**, *14*, 307-309.
(44) De Rosa, M. G., A.; Sodano, G.; Trabucco, A. *Experientia* **1981**, *37*, 541.
(45) Peterson, D. H.; Murray, H. C.; Eppstein, S. H.; Reineke, L. M.; Weintraub, A.; Meister, P. D.; Leigh, H. M. *J. Am. Chem. Soc.* **1952**, *74*, 5933-5936.
(46) Petric, S.; Hakki, T.; Bernhardt, R.; Zigon, D.; Cresnar, B. *J. Biotech.* **2010**, *150*, 428-437.
(47) Smith, K. E.; Ahmed, F.; Williams, R. A. D.; Kelly, S. L. *J. Steroid Biochem.* **1994**, *49*, 93-100.
(48) Vitas, M.; Smith, K.; Rozman, D.; Komel, R. *J. Steroid Biochem.* **1994**, *49*, 87-92.
(49) Dtugonski, J.; Bartnicka, K.; Chojecka, V.; Sedlaczek, L. *World J. Microb. Biot.* **1992**, *8*, 500-504.
(50) Jain, V. M., J.; Ratko, M. *Steroids* **1990**, *55*, 17.
(51) Smith, K. E.; Latif, S. A.; Kirk, D. N. *J. Steroid Biochem.* **1991**, *38*, 249-256.
(52) Atta, F. M.; El-kady, I. A. *Bull. Fac. Sci. Assiut. Univ.* **1994**, *B23*, 83-91.
(53) Abdel-Fattah, A. F.; El-Refu, A. H.; Sallam, L. A. R.; Badawl, M. A. *Microbial. Espanola.* **1978**, *30*, 59.
(54) Kim, M. H.; Kim, M. N. *Misaengmul. Hakhoechi.* **1991**, *29*, 111-116.
(55) Dutta, T. K.; Samanta, T. B. *J. Gen. Appl. Microbiol.* **1992**, *38*, 283-288.
(56) Morrin, M.; Ward, O. P. *Biotechnol. Lett.* **1989**, *11*, 319-324.
(57) Smith, K. E.; Latif, S.; Kirk, D. N. *J. Steroid Biochem.* **1989**, *33*, 927-934.
(58) Kim, M. N.; Ergan, F.; Dhulster, P.; Atrat, P.; Gellf, G.; Thomas, D. *Biotechnology Letters* **1982**, *4*, 233-238.
(59) Hamdy, A. A. *Egypt. J. Microbial.* **1999**, *34*, 167-179.
(60) Holland, H. L.; Poddar, S.; Tripet, B. *J. Ind. Microbiol.* **1992**, *10*, 195-197.
(61) Antoniou, T. N. H.; Curran, B. P. G.; Smith, K. E. *Microbiology-Uk* **1994**, *140*, 1633-1640.
(62) Lamm, A. S.; Chen, A. R. M.; Reynolds, W. F.; Reese, P. B. *Steroids* **2007**, *72*, 713-722.
(63) Hu, S. H.; Genain, G.; Azerad, R. *Steroids* **1995**, *60*, 337-352.
(64) Smith, K. E.; White, K. A.; Kirk, D. N. *J. Steroid Biochem.* **1989**, *33*, 81-87.
(65) Bihari, V.; Joshi, A. K.; Khan, A. W.; Basu, S. K. *J. Microb. Biotechnol.* **1988**, *3*, 45.
(66) Jayyosi, Z.; Cooper, K. O.; Thomas, P. E. *Arch. Biochem. Biophys.* **1992**, *298*, 265-270.
(67) Holland, H. L.; Brown, F. M.; Chenchaiah, P. C.; Chernishenko, M. J.; Khan, S. H.; Rao, J. A. *Can. J. Microbiol.* **1989**, *67*, 268-274.

(68) Peart, P. C.; McCook, K. P.; Russell, F. A.; Reynolds, W. F.; Reese, P. B. *Steroids* **2011**, *76*, 1317-1330.
(69) Kollerov, V. V.; Shutov, A. A.; Fokina, V. V.; Sukhodol'skaya, G. V.; Gulevskaya, S. A.; Donova, M. V. *Appl. Biochem. Biotechnol.* **2010**, *46*, 198-205.
(70) Al-Tamimi, S.; Al-Awadi, S.; Oommen, S.; Afzal, M. *Int. J. Food Sci. Nutr.* **2010**, *61*, 78-86.
(71) Huszcza, E.; Dmochowska-Gladysz, J. *J. Basic. Microb.* **2003**, *43*, 113-120.
(72) Smith, K. E.; Latif, S.; Kirk, D. N. *J. Steroid Biochem.* **1989**, *32*, 445-451.
(73) Kolek, T.; Swizdor, A. *J. Steroid Biochem.* **1998**, *67*, 63-69.
(74) Eppstein, S. H.; Meister, P. D.; Leigh, H. M.; Peterson, D. H.; Murray, H. C.; Reineke, L. M.; Weintraub, A. *J. Am. Chem. Soc.* **1954**, *76*, 3174-3179.
(75) Smith, K. E.; Latif, S.; Kirk, D. N. *J. Steroid Biochem.* **1990**, *35*, 115-120.
(76) Chatterjee, T. K.; Basak, A.; Dasgupta, C.; Barua, A. K.; Sen, K. *J. Gen. Appl. Microbiol.* **1979**, *25*, 339-341.
(77) Hunter, A. C.; Mills, P. W.; Dedi, C.; Dodd, H. T. *J. Steroid Biochem.* **2008**, *108*, 155-163.
(78) Wilson, M. R.; Gallimore, W. A.; Reese, P. B. *Steroids* **1999**, *64*, 834-843.
(79) Bartmanska, A.; Dmochowska-Gladysz, J. *Enzyme Microb. Tech.* **2007**, *40*, 1615-1621.
(80) Janeczko, T.; Swizdor, A.; Dmochowska-Gladysz, J.; Bialonska, A.; Ciunik, Z.; Kostrzewa-Suslow, E. *J. Mol. Catal. B-Enzym.* **2012**, *82*, 24-31.
(81) Huszcza, E.; Dmochowska-Gladysz, J. *Phytochemistry* **2003**, *62*, 155-158.
(82) Kim, Y. U.; Han, J.; Lee, S. S.; Shimizu, K.; Tsutsumi, Y.; Kondo, R. *Arch. Pharm.* **2007**, *340*, 209-214.
(83) Andrushina, V. A.; Druzhinina, A. V.; Yaderets, V. V.; Stitsenko, T. S.; Voishvillo, N. E. *Appl. Biochem. Biotechnol.* **2011**, *47*, 42-48.
(84) Huszcza, E.; Dmochowska-Gładysz, J.; Bartmańska, A. *Z Naturforsch C.* **2005**, *60*, 103-108.
(85) Sehgal, S. N.; Singh, K.; Vezina, C. *Can. J. Microbiol.* **1968**, *14*, 529-&.
(86) Al-Aboudi, A.; Mohammad, M. Y.; Musharraf, S. G.; Choudhary, M. I.; Atta ur, R. *Nat. Prod. Res.* **2008**, *22*, 1498-1509.
(87) Brzezowska, E.; DmochowskaGladysz, J.; Kolek, T. *J. Steroid Biochem.* **1996**, *57*, 357-362.
(88) Madyashtha, K. M.; Joseph, T. *J. Steroid Biochem.* **1993**, *45*, 563-569.
(89) Yildirim, K.; Kupcu, I.; Gulsan, F. *Z. Naturforsch. C*, *65*, 688-692.
(90) Mahato, S. B.; Mukherjee, A. *J. Steroid Biochem.* **1984**, *21*, 341-342.
(91) Herzog, H. L.; Gentles, M. J.; Basch, A.; Coscarelli, W.; Zeitz, M. E. A.; Charney, W. *J. Org. Chem.* **1960**, *25*, 2177-2182.
(92) Swizdor, A.; Kolek, T. *Steroids* **2005**, *70*, 817-824.
(93) Hanson, J. R.; Nasir, H.; Parvez, A. *Phytochemistry* **1996**, *42*, 411-415.
(94) Torshabi, M.; Badiee, M.; Faramarzi, M. A.; Rastegar, H.; Forootanfar, H.; Mohit, E. *Chem. Nat. Compd.* **2011**, *47*, 59-63.
(95) Singh, K.; Sehgal, S. N.; Vezina, C. *Can. J. Microbiol.* **1967**, *13*, 1271-&.
(96) Bartmanska, A.; Dmochowska-Gladysz, J.; Huszcza, E. *Steroids* **2005**, *70*, 193-198.
(97) Kitawaki, J.; Kim, T.; Kanno, H.; Noguchi, T.; Yamamoto, T.; Okada, H. *J. Steroid Biochem.* **1993**, *44*, 667-670.
(98) Dodson, R. M.; Goldkamp, A. H.; Muir, R. D. *J. Am. Chem. Soc.* **1960**, *82*, 4026-4033.
(99) Banerjee, S.; Mukherjee, E.; Mahato, S. B. *J. Chem. Res.* **1993**, 236-237.
(100) Faramarzi, M. A.; Agheinejad, M.; Yazdi, M. T.; Amini, M.; Hajarolasuadi, N. *Steroids* **2008**, *73*, 13-18.

(101) Faramarzi, M. A.; Badiee, M.; Yazdi, M. T.; Amini, M.; Torshabi, M. *J. Mol. Catal. B-Enzym.* **2008**, *50*, 7-12.
(102) Schlosser, D.; Irrgang, S.; Schmauder, H. P. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 16-20.
(103) Lin, Y. L.; Song, X.; Fu, J.; Lin, J. Q.; Qu, Y. B. *J. Chem. Technol. Biot.* **2009**, *84*, 789-793.
(104) Rodina, N. V.; Andryushina, V. A.; Stytsenko, T. S.; Turova, T. P.; Baslerov, R. V.; Panteleeva, A. N.; Voishvillo, N. E. *Appl. Biochem. Microbiol.* **2009**, *45*, 395-400.
(105) Crabb, T. A.; Dawson, P. J.; Williams, R. O. *J. Chem. Soc. Perkin T.1* **1980**, 2535-2541.
(106) Xiong, Z. G.; Wei, Q.; Chen, H. M.; Chen, S. W.; Xu, W. J.; Qiu, G. F.; Liang, S. C.; Hu, X. M. *Steroids* **2006**, *71*, 979-983.
(107) Choudhary, M. I.; Sultan, S.; Khan, M. T. H.; Yasin, A.; Shaheen, F.; Atta Ur, R. *Nat. Prod. Res.* **2004**, *18*, 529-535.
(108) Kollerov, V. V.; Shuttov, A. A.; Fokina, V. V.; Sukhodol'skaya, G. V.; Donova, M. V. *J. Mol. Catal. B-Enzym.* **2008**, *55*, 61-68.
(109) Kaminski, R. M.; Marini, H.; Kim, W. J.; Rogawski, M. A. *Epilepsia* **2005**, *46*, 819-827.
(110) Noguchi, S.; Fukushim.Dk *J. Org. Chem.* **1965**, *30*, 3552-&.
(111) Bell, A. M.; Clark, I. M.; Denny, W. A.; Jones, E. R. H.; Meakins, G. D.; Muller, W. E.; Richards, E. E. *J. Chem. Soc. Perkin T.1* **1973**, 2131-2136.
(112) Kolek, T.; Milecka, N.; Swizdor, A.; Panek, A.; Bialonska, A. *Org. Biomol. Chem.* **2011**, *9*, 5414-5422.
(113) Bell, A. M.; Jones, E. R. H.; Meakins, G. D.; Miners, J. O.; Wilkins, A. L. *J. Chem. Soc. Perkin T.1* **1975**, 2040-2043.
(114) Bell, A. M.; Boul, A. D.; Jones, E. R. H.; Meakins, G. D.; Miners, J. O.; Wilkins, A. L. *J. Chem. Soc. Perkin T.1* **1975**, 1364-1366.
(115) Brannon, D. R.; Parrish, F. W.; Wiley, B. J.; Long, L. *J. Org. Chem.* **1967**, *32*, 1521-&.
(116) Yildirim, K. *Collect. Czech. Chem. C.* **2010**, *75*, 1273-1281.
(117) Bensasson, C. M.; Hanson, J. R.; Hunter, A. C. *Phytochemistry* **1998**, *49*, 2355-2358.
(118) Bell, A. M.; Chambers, V. E.; Jones, E. R. H.; Meakins, G. D.; Muller, W. E.; Pragnell, J. *J. Chem. Soc. Perkin T.1* **1974**, 312-317.
(119) Herzog, H. L.; Gentles, M. J.; Hershberg, E. B.; Carvajal, F.; Sutter, D.; Charney, W.; Schaffner, C. P. *J. Am. Chem. Soc.* **1957**, *79*, 3921-3922.
(120) Atta Ur, R.; Choudhary, M. I.; Asif, F.; Farooq, A.; Yaqoob, M. *Nat. Prod. Lett.* **2000**, *14*, 217-224.
(121) Romano, A.; Romano, D.; Ragg, E.; Costantino, F.; Lenna, R.; Gandolfi, R.; Molinari, F. *Steroids* **2006**, *71*, 429-434.
(122) Li, H. P.; Liu, H. M.; Ge, W. Z.; Huang, L. H.; Shan, L. H. *Steroids* **2005**, *70*, 970-973.
(123) Schwarz, V.; Protiva, J. *Folia Microbiol.* **1974**, *19*, 156-163.
(124) Huang, L. H.; Li, J. A.; Xu, G.; Zhang, X. H.; Wang, Y. G.; Yin, Y. L.; Liu, H. M. *Steroids* **2010**, *75*, 1039-1046.
(125) Lobastova, T. G.; Khomutov, S. M.; Vasijeva, L. L.; Lapitskaya, M. A.; Pivnitsky, K. K.; Donova, M. V. *Steroids* **2009**, *74*, 233-237.
(126) Choudhary, M. I.; Shah, S. A. A.; Musharraf, S. G.; Shaheen, F.; Atta Ur, R. *Nat. Prod. Res.* **2003**, *17*, 215-220.
(127) Koshimura, M.; Utsukihara, T.; Hara, A.; Mizobuchi, S.; Horiuchi, C. A.; Kuniyoshi, M. *J. Mol. Catal. B-Enzym.* **2010**, *67*, 72-77.
(128) Holland, H. L.; Diakow, P. R. P. *Can. J. Chem.* **1979**, *57*, 436-440.

(129) Thoa, H. K.; Sasek, V.; Budesinsky, M.; Jablonsky, I.; Eignerova, L.; Chan, N. G.; Prochazka, Z. *Collect. Czech. Chem. C.* **1978**, *43*, 336-343.
(130) Lee, C. Y.; Chen, C. D.; Liu, W. H. *Appl. Microbiol. Biotechnol.* **1993**, *38*, 447-452.
(131) Liu, W. H.; Kuo, C. W.; Wu, K. L.; Lee, C. Y.; Hsu, W. Y. *J. Ind. Microbiol.* **1994**, *13*, 167-171.
(132) Lee, C. Y.; Liu, W. H. *Appl. Microbiol. Biotechnol.* **1992**, *36*, 598-603.
(133) Kreit, J.; Lefebvre, G.; Germain, P. *J. Biotech.* **1994**, *33*, 271-282.
(134) Goetschel, R.; Bar, R. *Enzyme Microb. Tech.* **1992**, *14*, 462-469.
(135) Szykula, J.; Hebda, C.; Orpiszewski, J.; Saganska, K. *Biotechnol. Lett.* **1991**, *13*, 917-922.
(136) Vasudevan, P. T.; Zhou, T. *Biotechnol. Bioeng.* **1997**, *53*, 391-396.
(137) Rumijowska-Galewicz, A.; Ziolkowski, A.; Korycka-Machala, M.; Sedlaczek, L. *World J. Microb. Biot.* **2000**, *16*, 237-244.
(138) Korycka-Machala, M.; Ziolkowski, A.; Rumijowska-Galewicz, A.; Lisowska, K.; Sedlaczek, L. *Microbiol-Sgm* **2001**, *147*, 2769-2781.
(139) Malaviya, A.; Gomes, J. *Bioresour. Technol.* **2008**, *99*, 6725-6737.
(140) Huang, C. L.; Chen, Y. R.; Liu, W. H. *Enzyme Microb. Tech.* **2006**, *39*, 296-300.
(141) Houng, J. Y.; Chiang, W. P.; Chen, K. C.; Tiu, C. *Enzyme Microb. Tech.* **1994**, *16*, 485-491.
(142) Hunter, A. C.; Elsorn, J.; Ross, L.; Barrett, R. *BBA-Mol. Cell Biol. L.* **2006**, *1761*, 360-366.
(143) Nega, E.; Grunwaldt, G. *J. Basic Microb.* **1997**, *37*, 139-145.
(144) Mutafov, S.; Angelova, B.; Avramova, T.; Boyadjieva, L.; Dimova, I. *Process Biochem.* **1997**, *32*, 585-589.
(145) Lu, W. Y.; Du, L. X.; Wang, M.; Jia, X. Q.; Wen, J. P.; Huang, Y. P.; Guo, Y. W.; Gong, W.; Bao, H. K.; Yang, J.; Sun, B. *Appl. Biochem. Biotechnol.* **2007**, *142*, 17-28.
(146) Bhosale, S.; Saratale, G.; Govindwar, S. *J. Basic Microb.* **2006**, *46*, 444-448.
(147) Ahmed, F.; Williams, R. A. D.; Smith, K. E. *J. Steroid Biochem.* **1996**, *58*, 337-349.
(148) Cotillon, A. C.; Doostzadeh, J.; Morfin, R. *J. Steroid Biochem.* **1997**, *62*, 467-475.
(149) Irrgang, S.; Schlosser, D.; Fritsche, W. *J. Steroid Biochem.* **1997**, *60*, 339-346.
(150) Degtyarenko, K. N.; Archakov, A. I. *Febs Letters* **1993**, *332*, 1-8.
(151) Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2379-2384.
(152) Nelson, D. R.; Koymans, L.; Kamataki, T.; Stegeman, J. J.; Feyereisen, R.; Waxman, D. J.; Waterman, M. R.; Gotoh, O.; Coon, M. J.; Estabrook, R. W.; Gunsalus, I. C.; Nebert, D. W. *Pharmacogenetics* **1996**, *6*, 1-42.
(153) Klingenberg, M. *Arch. Biochem. Biophys.* **1958**, *75*, 376-386.
(154) Hannemann, F.; Bichet, A.; Ewen, K. M.; Bernhardt, R. *BBA-Gen. Subjects* **2007**, *1770*, 330-344.
(155) Bernhardt, R. *Rev. Physiol. Bioch. P.* **1996**, *127*, 137-221.
(156) Griffin, B. W.; Peterson, J. A. *Biochemistry* **1972**, *11*, 4740-4747.
(157) Meunier, B.; de Visser, S. P.; Shaik, S. *Chem. Rev.* **2004**, *104*, 3947-3980.
(158) Hamdane, D.; Zhang, H. M.; Hollenberg, P. *Photosynth. Res.* **2008**, *98*, 657-666.
(159) King, D. J.; Azari, M. R.; Wiseman, A. *Method Enzymol.* **1988**, *137*, 675-686.
(160) Taylor, M.; Lamb, D. C.; Cannell, R. J. P.; Dawson, M. J.; Kelly, S. L. *Biochem. Bioph. Res. Co.* **2000**, *279*, 708-711.
(161) Hrycay, E. G.; Gustafsson, J. A.; Ingelmansundberg, M.; Ernster, L. *Eur. J. Biochem.* **1976**, *61*, 43-52.
(162) Bernhardt, R. *J. Biotech.* **2006**, *124*, 128-145.
(163) Ramakrishna, S. V.; Prakasham, R. S. *Curr. Sci. India* **1999**, *77*, 87-100.

(164) Hattori, T.; Furusaka, C. *J. Biochem.* **1960**, *48*, 831-837.
(165) Navarro, J. M.; Durand, G. *Eur. J. Appl. Microbiol.* **1977**, *4*, 243-254.
(166) Chibata, I.; Tosa, T.; Sato, T. **1974**, *Japanese Patent No 74,25,189*.
(167) Ohlson, S.; Flygare, S.; Larsson, P. O.; Mosbach, K. *Eur. J. Appl. Microbiol.* **1980**, *10*, 1-9.
(168) Sonomoto, K.; Hoq, M.; Tanaka, A.; Fukui, S. *J. Ferment. Technol.* **1981**, *59*, 465-469.
(169) Maddox, I. S.; Dunnill, P.; Lilly, M. D. *Biotechnol. Bioeng.* **1981**, *23*, 345-354.
(170) Sonomoto, K.; Nomura, K.; Tanaka, A.; Fukui, S. *Eur. J. Appl. Microbiol.* **1982**, *16*, 57-62.
(171) Bihari, V.; Goswami, P. P.; Rizvi, S. H. M.; Khan, A. W.; Basu, S. K.; Vora, V. C. *Biotechnol. Bioeng.* **1984**, *26*, 1403-1408.
(172) Kim, M. N.; Ergan, F.; Dhulster, P.; Atrat, P.; Gellf, G.; Thomas, D. *Biotechnol. Lett.* **1982**, *4*, 233-238.
(173) Mahato, S. B.; Mukherjee, A. *Phytochemistry* **1984**, *23*, 2131-2154.
(174) Baklashova, T. G.; Koshcheenko, K. A.; Skryabin, G. K. *Appl. Microbiol. Biotechnol.* **1984**, *19*, 217-223.

Chapter 2: Materials and Methods

Introduction

This chapter describes materials and techniques routinely practiced in the maintenance and propagation of fungal cultures. Detail procedures for fermentation using suspended fungal cells, extraction and characterization of metabolites, immobilization of fungal cells and preparation of crude cell free extract using fungal culture are described.

2.1: Materials

2.1.1: Chemicals

Media ingredients, salts and acids were purchased from Merck (India) and Himedia, Mumbai. Analytical grade solvents were procured from Merck (India). Steroids such as progesterone (**1**), testosterone (**2**), 17α-methyltestosterone (**3**), 4-androstene-3,17-dione (**4**),19-nortestosterone (**5**), trans-androsterone (**6**), trans-dihydroandrosterone (**7**) were obtained from Sigma-Aldrich.

2.1.2: Microorganisms

Microorganisms employed in the present study were either isolated from soil or obtained from National Collection of Industrial Microorganisms (NCIM), NCL, Pune, and MTCC, IMTECH, Chandigarh. All the fungal cultures were maintained and propagated on potato dextrose agar (PDA) slants and preserved at 4 ºC as reported earlier[1]. During retrieval/propagation, purity of each culture was monitored by plating it on PDA in a sterile environment followed by incubation at 30 ºC. After specific growth period, the cultures were analyzed microscopically to confirm the purity.

2.1.3: Maintenance and propagation of the organisms

The stock cultures were maintained on Czapek-Dox agar or Potato Dextrose agar slants.

Potato Dextrose Agar [PDA]: The composition and preparation of PDA medium is as follows.

Potato	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1000 ml
pH	7

The potatoes were cut into small cubes, boiled until it became soft, filtered through cheesecloth, the filtrate was then made up to 1000 ml volume. To this dextrose and agar was added, pH was adjusted to 7, prior to adding agar. The mixture was boiled to melt agar. Aliquots (8 mL) of the hot homogenous solution were distributed into test tube (20

ml size) which were then plugged with cotton and autoclaved at 15 psi for 20 min. The tubes were kept in slant position while hot and allowed to solidify. To prepare solid base agar flasks, 100 mL of the homogenous solution were dispensed into 500 mL Erlenmeyer flask, plugged and autoclaved. The flasks were then kept on a flat surface while hot and allowed to solidify.

Czapek-Dox-Agar Slants: 2.0 g of agar was weighed into 100 ml of modified Czapek-Dox medium and the slants prepared as described above. The solidified slants were kept at 30 ^{o}C for a day to check for any possible contamination. The slants (/flasks) thus prepared were inoculated under aseptic conditions from the stock culture and incubated at 30 ^{o}C for 5 days to ensure good sporulation. The slants were then stored at 4 °C till further use.

Growth of the Microorganisms

Sterile water (5 mL) was added to a sporulated slant and the spores were loosened by scraping the surface gently with an inoculation loop. 1 ml of this spore suspension (2.78 x10^{8} spore count/mL) was added to each 500 mL Erlenmeyer flask containing 100 ml of sterile modified Czapek-Dox medium. Different constituents of the modified Czapek Dox media (Table 2.1) were supplemented in 1 liter de-ionized water and pH of the media was adjusted to 5.8 using 1 M K_2HPO_4. The flasks thus inoculated were kept on a rotary shaker (220 rpm) at 30 ^{o}C for required period of incubation.

Table 2.1: Composition of modified Czapek Dox media

Ingredient	Quantity per liter
Dextrose	30.0 g
Sodium nitrate	3.0 g
Di-potassium hydrogen phosphate	1.0 g
Potassium chloride	0.5 g
Magnesium sulphate	0.5 g
Yeast extract	0.5 g
Corn steep liquor	7.0 mL
Ferrous sulphate	10.0 mg

The constituents except $FeSO_4.7H_20$ (which was added after the pH was adjusted), were dissolved in double distilled water and the volume made upto 1000 mL. The pH was maintained at 5.8. The medium was distributed to each of 500 mL Erlenmeyer flasks (100 Ll per flask). The flasks were plugged with cotton and autoclaved at 15 psi for 20 min. The flasks were left at room temperature for 1 day before use to check for possible contamination.

2.2: Fermentation Conditions

2.2.1: Inoculation of fermentation media with fungal culture

Sterile water (5 mL) was added to a well-sporulated slant and the spores were loosened by scraping the surface gently. Sterile PDA slants were streaked with the loop containing spores, incubated at 30 ºC till well sporulation was observed and stored at 4 ºC for further use. One mL of above spore suspension (~$2.8x10^8$ spores count/mL) was added to 500 mL Erlenmeyer flasks containing 100 mL of sterile modified Czapek Dox medium. Complete inoculation procedure was carried out in a bio-safety cabinet under sterile environmental conditions. The flasks were then incubated at 30 ºC on a rotary shaker (200 rpm) for 36 h.

2.2.2: Screening of fungal strains for biotransformation of steroids

Fungal strains from different genera such as *Aspergillus*, *Rhizopus*, *Neurospora*, *Cunninghamella*, *Mucor* (soil isolated as well as procured from NCIM, NCL, Pune and MTCC, IMTECH, Chandigarh) were screened for useful transformations on steroids. 500 mL Erlenmeyer flasks containing 100 mL modified Czapek Dox media were inoculated using one mL of spore suspension as described above. After 36 h growth period, the cultures were supplemented with steroid substrates (Fig. 2.1) (20 mg, dissolved in 0.2 mL of tetrahydrofuran) and incubation was continued for next five days

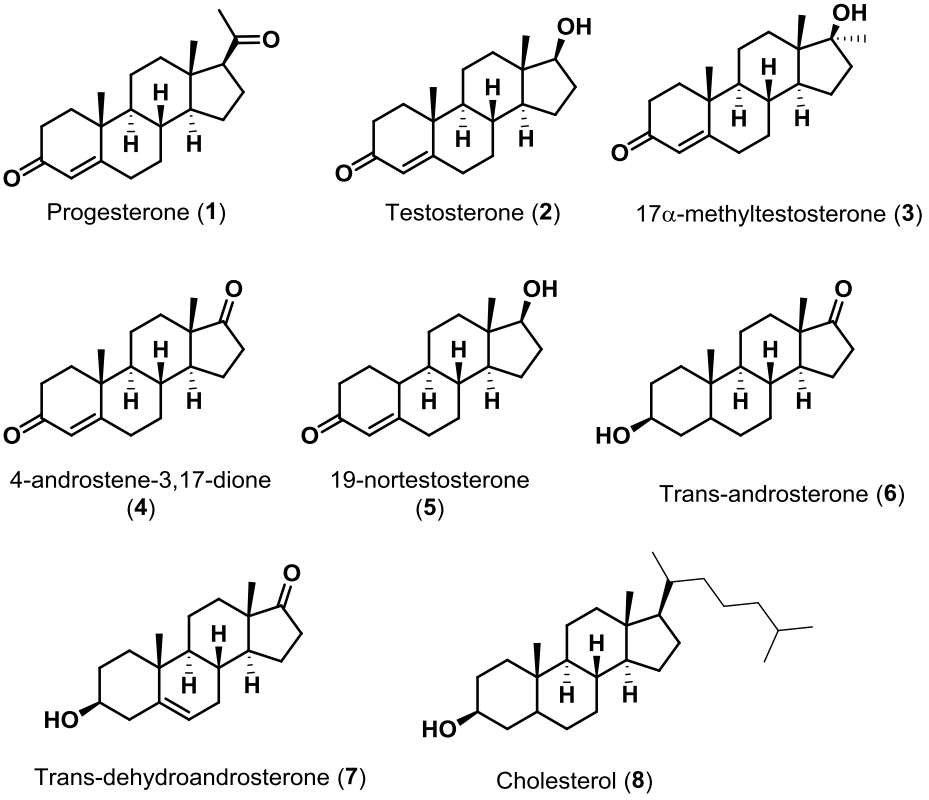


Figure 2.1: Different steroid substrates used for the biotransformation

at 30 ºC on rotary shaker. After this incubation period, mycelia were separated from fermentation media by filtration through muslin cloth. Both fermentation media and mycelia were separately extracted three times with equal volume of ethyl acetate (100 mL × 3) and the extracts were analyzed by TLC, HPLC and LC-MS for possible transformation. These studies indicated that, the fungal systems belonging to genera of *Mucor* were shown to exhibit efficient transformation of the steroids and were therefore chosen for subsequent transformation studies.

2.2.3: Substrate concentration study

In substrate concentration studies, a varied concentration of substrates (steroids) ranging from lesser to higher (20-200 mg) was added to different flasks containing 36 h grown cultures of *Mucor sp*. in 100 mL media. Flasks were then incubated at 30 ºC on rotary shaker (200 rpm) for six days. At the end of the incubation period, mycelia were separated from fermentation media by filtration through muslin cloth. Both fermentation media and mycelia were separately extracted twice with equal volume of ethyl acetate (100 mL × 3) and crude extracts were analyzed by TLC and HPLC. The concentration of the substrate at which complete bioconversion observed was further used for time course study and large-scale fermentation.

2.2.4: Time-course experiment

In time course experiments, fungal cultures were grown as explained earlier, and incubated at 30 ºC on rotary shaker (200 rpm). Then, the optimum concentration of substrate standardized from substrate concentration study, was asceptically added to 36 h grown culture and incubation was continued. At every specific interval of incubation period, aliquots were drawn for extraction and analysis of the metabolites, until 12 days. The metabolites formed were extracted in ethyl acetate and monitored by TLC and HPLC analyses. The extent of bioconversion was determined on the basis of area under the peak for respective metabolite. All analyses were carried out under identical conditions.

2.2.5: Resting cell experiment

The flasks containing sterile medium were inoculated with fungal strain and incubated at 30 °C on a rotary shaker (200 rpm) as already described. After 36 h growth, mycelia were filtered through muslin cloth and the mycelia obtained were washed repeatedly, first with distilled water and later with phosphate buffer (0.1 M, pH 7.2). Mycelia (3 gm) wet weight was suspended in 50 mL phosphate buffer (0.1 M, pH 7.2)

supplemented with 0.2% dextrose. Substrate (20 mg in 0.2 mL THF) was added to the flasks and incubated at 30 ºC on a rotary shaker (200 rpm) for 24 h. After the incubation period, filtered mycelia and broth were extracted separately with ethyl acetate (50 mL × 3) and analyzed by TLC and HPLC.

2.2.6: Large scale fermentation with *M881*

Fermentation was performed in 3L sterile modified Czapek Dox media[2] distributed equally in 30 flasks of 500 mL volume. Spore suspension (2.5 mL) from fresh and well grown *M881* culture (NCIM Cat no. 881) maintained on a PDA slants was added to all the flasks which were then incubated at 30 °C on a rotary shaker at 200 rpm for 36 h. After this growth period, pH of the fermentation media was adjusted to ~7.0 using sterile 1M K_2HPO_4. Substrate 70 mg (**1** / **3** / **4** / **5** / **7**) or 100 mg (**2** / **6**), dissolved in 0.2 mL tetrahydrofuran (THF) was added to every flask and the incubation was continued for 6 days. Two different control sets were maintained in all experiments, first, substrate control which was composed of sterile medium with substrate and was incubated under identical conditions without the culture and second, culture control which consisted of culture incubated under identical conditions but without substrate.

Substrate concentration studies were carried out using varied concentrations of substrates (0.3 g/L to 1.2 g/L) in fermentation medium. In time course experiments, incubations were carried out with 0.7 g/L substrate concentration (**1** / **3** / **4** / **5** / **7**) or 1.0 g/L (**2** / **6**) for 1 to 12 days and the level of transformation was monitored after every alternate day of incubation by TLC and HPLC analyses

2.2.7: Large scale fermentation with *M. hiemalis*

Fermentation procedures used with *M. hiemalis* (NCIM Cat no. 873) were identical to those practiced for *M881*. In brief, 2L media distributed uniformly in 20 flasks of 500 mL capacity was inoculated with spore suspension from fresh and well grown culture of *M. hiemalis* propagated on PDA slants and was incubated at 30 °C on a rotary shaker at 200 rpm for 36 h. After this growth period, 70 mg substrate (**1** / **2**) in 0.2 mL tetrahydrofuran (THF) was added to each flask and the incubation was continued for five days. Two different control sets were maintained in all experiments, first, substrate control which was composed of sterile medium with substrate and was incubated under identical conditions without the culture and second, culture control which consisted of culture incubated under identical conditions but without substrate. Time course studies were carried out with 0.7 g/L substrate concentration in fermentation media containing

36 h grown culture of *M. hiemalis*. Each flask was extracted after every 24 h until 6 days and the extent of transformation was monitored by HPLC at regular intervals.

2.2.8: Scale-up and Fermentor studies with *M881*

To standardize the scale-up of fermentation conditions for preparative scale production of hydroxylated steroids using *M881*, transformations were performed in three different volumes (100 mL, 600 mL, 5000 mL) of modified Czapek Dox media. Time course experiments were performed in shake flasks with 100 mL and 600 mL media and in 5 L fermentation media with 7 L capacity table-top fermentor (New Brunswick Scientific). Shake flask experiments were carried out as previously explained. In large scale fermentation studies, 5 L media contained in a fermentor vessel was inoculated with 100 mL of 24 h grown culture of *M881* and was incubated at 30 ºC for 24 h with following controlled parameters: rotary agitation- 200 rpm; dissolved oxygen- (D.O.) 60-80% (of saturation), airflow- 2 SLPM (Standard L per min). After the incubation period, substrate (3.5 g of **1** or 5 g of **2**) dissolved in 20 mL of THF was added to the fermentation media and incubation was continued. Aliquots were drawn aseptically at every 48 h, extracted with ethyl acetate and the extent of transformation was monitored by HPLC and LC-MS analyses.

2.2.9: Immobilization of *M881* cells in calcium alginate

M881 cells were immobilized in calcium alginate using a standardized protocol (Fig. 2.2) and the beads obtained were used for transformation of progesterone (**1**). For time course experiment, 30 mg of **1** was added to six flasks containing 12 g of immobilized beads suspended in 50 mL DI water supplemented with 2% glucose and the reaction mixture was incubated at 30 ºC on a rotary shaker at 200 rpm. On every alternate day, one flask was withdrawn and beads were separated from the broth by filtration. Metabolites were extracted in ethyl acetate and analyzed by TLC and HPLC.

2.3: Extraction of metabolites

At the end of incubation period, contents from all flasks were pooled and filtered. The metabolites formed after transformation were extracted from broth and mycelia using ethyl acetate (3 L x 4 for broth and 500 mL x 4 for mycelia in large scale fermentation). As observed from TLC and HPLC analyses, the metabolites present in both extracts were found to be same were therefore pooled. The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure on a rotary

evaporator. The crude residue obtained was subjected to column chromatography for purification of individual metabolites.

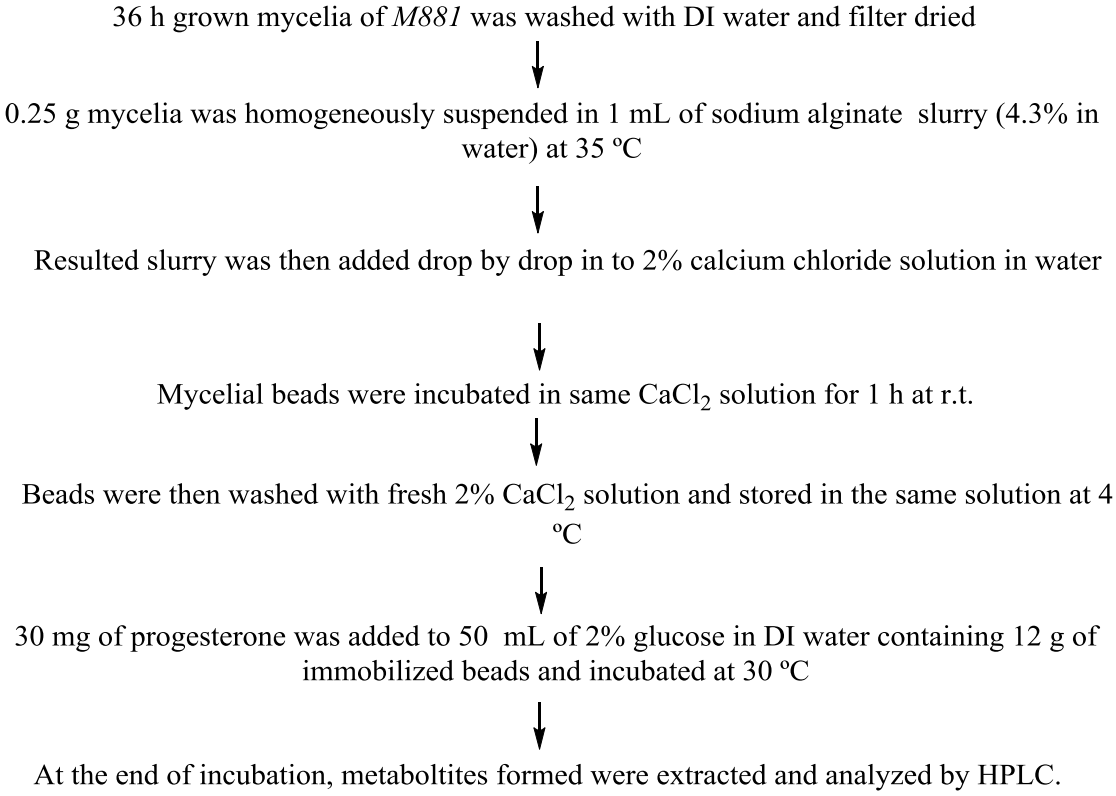


Figure 2.2: Schematic procedure for the immobilization of *M881* cells

2.4: Standardization of cell free preparation in *M. hiemalis*

A general scheme shown in figure 2.3 was used for the cell free study of steroids' transformation with *M. hiemalis*. Hydroxylase system in *M. hiemalis* was induced by 0.3 mM testosterone (dissolved in DMSO) (when progesterone was the substrate in hydroxylase assay) or 0.3 mM progesterone (dissolved in DMSO) (when testosterone was the substrate in hydroxylase assay) in 100 mL of 24 h grown culture. The culture was incubated under identical fermentation conditions for 12 h, as described. Induced mycelia were harvested by filtration, washed three times with saline (0.5% NaCl) and finally with 0.1 M Tris-HCl buffer (pH 8.2) to remove adhering testosterone and its metabolites. Washed mycelia was dried by pressing between filter papers and suspended into pre-cooled extraction buffer (4 mL/g of mycelia) composed of glucose (0.25 M), KCl (10 mM), EDTA (10 mM), glycerol (20%, v/v), DTT (5 mM), PMSF (2.5 mM) in Tris-HCl buffer (0.1 M, pH 8.2). Subsequently, all the operations were carried out at 4 °C unless otherwise mentioned. The mycelial suspension was pulverized in bead beater in presence of 0.5 mm glass beads (4 mL/g of mycelia). The homogenate was centrifuged using Beckman Avanti centrifuge at 3000 × g for 10 min to remove glass

beads. The supernatant obtained was again centrifuged at 10250 × g for 30 min to remove the cell debris. The cell free extract was collected and further centrifuged on Thermo Ultra-100 centrifuge at 120000 × g for 90 min. The pellet thus obtained was

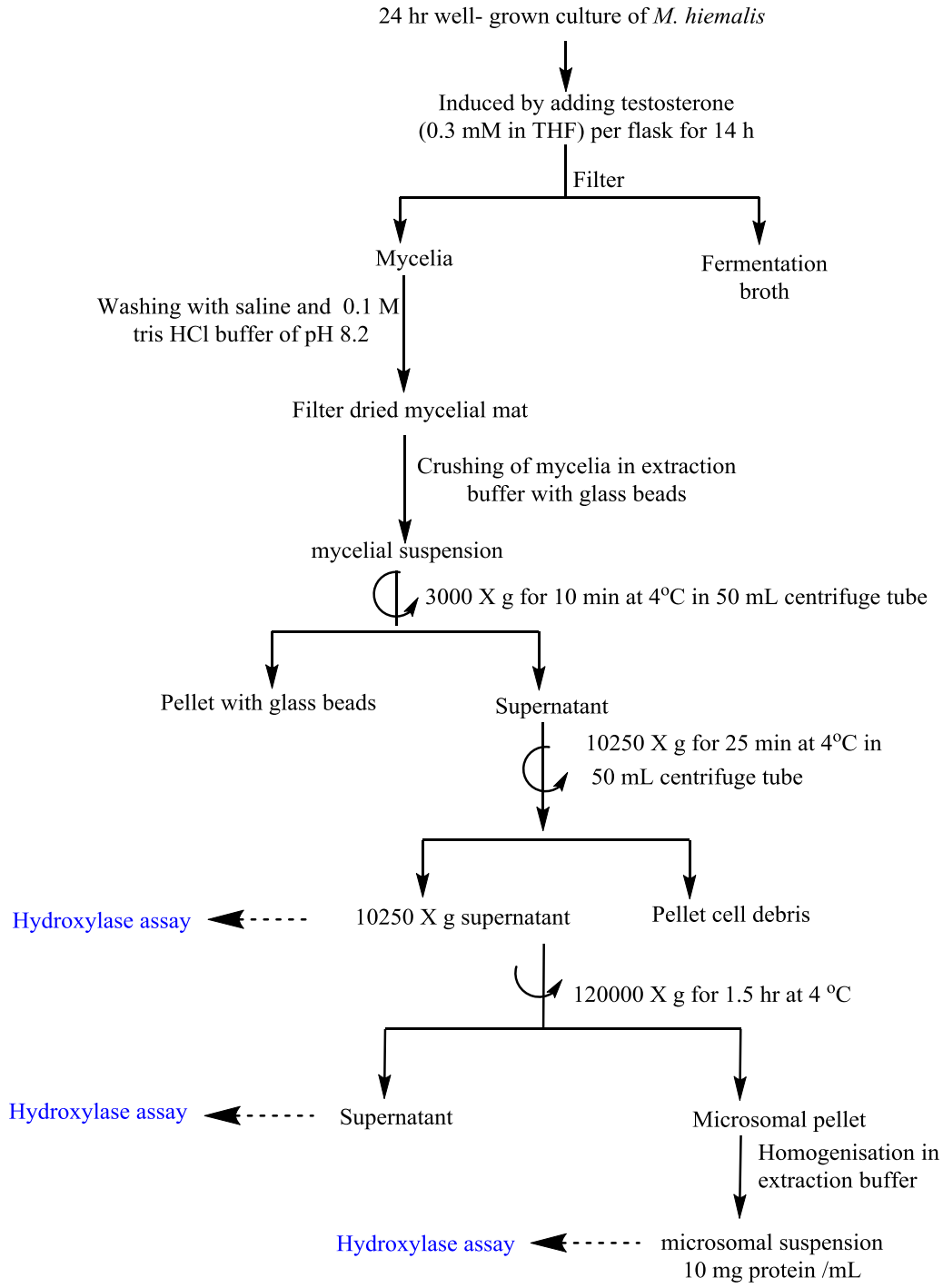


Figure 2.3: Protocol for the preparation of active microsomal suspension.

designated as microsomal fraction. It was washed by suspending it homogeneously into extraction buffer and subjected to ultra centrifugation as described in figure 2.3. Washed microsomes were re-suspended in extraction buffer at a concentration of about 10 mg

protein/ mL. Protein concentration in the assay samples were estimated using Bradford method[3].

2.4.1: Assay and incubation conditions

For hydroxylase assay, the reaction mixture consisted of 500 µL of protein (10 mg/mL), 500 µL of freshly prepared NADPH (using NADPH generating system consisting of 2 mM $NADP^+$, 20 mM Glucose-6-phosphate, 1U of Glucose-6-phosphate dehydrogenase, 10 mM $MgCl_2$ in Tris-HCl buffer of pH 8.2) and 0.3 mM progesterone (**1**) (10 µL of 10 mg/mL solution in DMSO) in a total volume of 1 mL. The assay mixture contained in a 5 mL glass tube was incubated under aerobic condition for 6 h at 30 °C on a water bath 200 rpm. The reaction was terminated by the addition of 1 mL of ethyl acetate with vortexing. Reaction mixture was extracted with ethyl acetate (1 mL X 3) and the enzymatic product(s) formed were subsequently analyzed by HPLC and LC-MS.

2.4.2: Induction of 14α-hydroxylase with different inducers

To analyze the effect of different inducers such as progesterone, testosterone, 17α-methyltestosterone, androsterone, 4-androstene-3,17-dione, cholesterol, phenobarbital on 14α-hydroxylase activity with **1** as a substrate in the hydroxylase assay, separate sets of experiments were designed under identical conditions. Microsomal suspension was prepared separately for each inducer and the hydroxylase assay was performed. Assay samples were analyzed by HPLC for percentage conversion of **1** to 14α-hydroxyprogesterone (**1d**).

2.4.3: Optimization of pH and incubation temperature

To evaluate the effect of pH on hydroxylase activity, Tris-base extraction buffers of different pH (pH 7.0, 7.5, 7.7, 8.0, 8.2, 8.5 and 8.7) were employed during cell lysis step for the preparation of microsomal suspension. Hydroxylase assay with each sample was carried out at the pH same as that of extraction buffer. The assay samples were then extracted and analyzed by HPLC.

To examine the effect of temperature on enzyme activity, hydroxylase assay samples were incubated at different temperatures ranging from 10 ºC to 50 ºC with an increment of 10 ºC. The reaction mixture was preincubated for reaching the required temperature and the reaction was initiated by adding NADPH generating system and incubated for 6 h on a metabolic shaker. At the end of incubation, the reaction mixtures were extracted with ethyl acetate and analyzed by HPLC as described above.

2.4.4: Inhibition study of 14α-hydroxylase from *M. hiemalis*

In the inhibition study of hydroxylase system, microsomal suspension was incubated with varied concentrations of common cytochrome P450 inhibitors for 5 min at room temperature and then hydroxylase assay was carried out as mentioned above. Carbon monoxide inhibition was studied by bubbling CO gas into 1 mL of microsomal preparation for 30 sec (1-2 bubbles per sec) followed by hydroxyalse assay. In control experiment N_2 gas was bubbled instead of CO gas.

2.4.5: Spectral analysis of 14α-hydroxylase from *M. hiemalis*

Cytochrome P450 contents were estimated according to the method of Omura and Sato[4]. Microsomal protein (2 mg/mL) was taken in Tris-HCl buffer (0.01 M, pH 7.4) containing EDTA (1 mM) and KCl (0.15 M). The microsomal suspension was reduced with dithionite and baseline correction was made. Carbon monoxide was bubbled into the sample cuvette for 30 sec (1-2 bubbles per sec) and spectrum recorded. An extinction coefficient of 91 cm^{-1} mM^{-1} for the difference between 450 nm and 490 nm was used to determine the amount of cytochrome P450.

2.5: Chromatographic procedure

2.5.1: Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is an important technique for separation of mixtures of organic compounds. In TLC, components of the mixture are partitioned between an adsorbent (the stationary phase, usually silica gel, SiO_2) and a solvent (the mobile phase) which flows through the adsorbent. Thin layer chromatography **(**TLC) was performed on pre-coated silica gel 60-F254 plates (0.25 mm) procured from Merck and compounds were visualized by spraying with a solution of 3.2% anisaldehyde, 2.8% sulfuric acid, 2% acetic acid in ethanol followed by heating. Two solvent systems were used for the analysis of different steroids

- Solvent I: 5% methanol in CH_2Cl_2 for progesterone (**1**) and 4-androstene-3,17-dione (**4**).
- Solvent II: 10% methanol in CH_2Cl_2 for testosterone (**2**), 17α-methyltestosterone (**3**), 19-nortestosterone (**5**), epiandrosterone (**6**) and dehydroepiandrosterone (**7**)

2.5.2: Silica-gel Column chromatography

A silica-gel slurry packed in a glass column was used for the purification of metabolites. In the silica-gel column chromatography stationary phase used was silica (230-400 mesh) and the mobile phase used was mixture of methanol and dichloromethane (0 to

10% of methanol in CH_2Cl_2). Less polar metabolites were eluted first from the column than more polar compounds. The crude residual mixture was loaded on silica gel (230-400 mesh) packed in the glass column. The metabolites were eluted from the column by gradient elution, with different percent of methanol in CH_2Cl_2. 25 mL of fractions were collected and analyzed by TLC. The fractions with pure compound were concentrated to furnish the metabolites.

2.5.3: High performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) analyses of metabolites were performed on Waters 600A HPLC instrument. X-bridge C_{18} column (analytical - 4.6 × 100 mm, 5 μm and Semi-preparative – 9.1 × 100 mm, 5 μm) was employed for separation of metabolites. The metabolites were eluted from the column using methanol/water as a mobile phase with 1 mL/min flow rate. The signals corresponding to metabolites were obtained with the help of in-built dual wavelength UV detector set at 245 nm wavelength. For analytical separation, metabolite mixture was dissolved in methanol and 20 μL of solution was injected on to the X-bridge C_{18} column (analytical - 4.6 × 100 mm). Different gradients were used for the metabolites of different substrates (Table 2.2). For preparative purification of metabolites, X-bridge C_{18} column (19 × 100 mm) was used with methanol/water isocratic elution (30% to 50% of methanol in water) at flow rate of 15 mL/min.

Table 2.2: HPLC gradient elution programs (1 mL/min flow rate, 245 nm)

Progesterone (**1**)			Testosterone (**2**) and 17α-methyltestosterone (**3**)			4-androstene-3,17-dione (**4**) and 19-nortestosterone (**5**)		
Time	% water	% ACN	Time	% water	% ACN	Time	% water	% ACN
0.1	95.0	5.0	0.1	95.0	5.0	0.1	95.0	5.0
8.0	80.0	20.0	6.0	80.0	20.0	6.0	80.0	20.0
15.0	70.0	30.0	20.0	70.0	30.0	30.0	75.0	25.0
30.0	60.0	40.0	35.0	60.0	40.0	40.0	60.0	40.0
35.0	50.0	50.0	37.0	50.0	50.0	45.0	20.0	80.0
37.0	40.0	60.0	40.0	20.0	80.0	53.0	95.0	5.0
38.0	20.0	80.0	45.0	95.0	5.0	60.0	95.0	5.0
45.0	95.0	5.0	-	-	-	-	-	-

2.5.4: Ultra pressure liquid chromatography-Mass spectrometry (UPLC-MS)

Waters make Acquity SQD was used for the mass analysis of the biotransformation products and hydroxylase assay samples. The instrument was equipped with X-bridge C_{18} column (4.5 × 50 mm, 3.5 μm), PDA detector and SQ detector with ESI mode. For the analysis of assays as well as biotransformation samples, gradient elution program

was used (Table 2.3) along with 0.3 mL/min flow rate. The PDA detector was set in the range of 200 – 400 nm and mass was scanned in the range from 100 to 500 amu.

Table 2.3: UPLC gradient elution programs (0.3 mL/min flow rate, 200-400 nm, 100-500 mass range)

Time (min)	0.0	0.5	6.0	9.0	16.0	19.0	20.0
% water	90.0	90.0	40.0	40.0	10.0	90.0	90.0
% ACN	10.0	10.0	60.0	60.0	90.0	10.0	10.0

2.5.5: High resolution Mass spectrometry (HRMS)

The high resolution mass spectra of metabolites were obtained on Waters make Q-Exactive, Waters make QTOF (SYNAPT-HDMS) and Q Exactive Orbitrap (Thermo Scientific).

2.5.6: Gas chromatography (GC)

GC analyses were carried out on an Agilent 7890 instrument equipped with a hydrogen flame ionization detector and HP-5 capillary column (30 m X 0.32 mm X 0.25 µm, J & W Scientific). Nitrogen was used as carrier gas at a flow rate of 0.8 mL/min. Initially, the column temperature was maintained at 150 °C, followed by a temperature gradient from 150 °C to 250 °C at 7 °C/min and held constant for 30 min at 250 °C, then raised to a temperature of 280 °C at 15 °C/min and maintained for 3 min. The injector and detector temperatures were maintained at 300 °C and operated in split mode (split ratio 10:1).

2.6: Characterization of metabolites

Different spectroscopic techniques and X-ray diffraction study was used for the identification of the metabolites formed in the biotransformation process.

2.6.1: Spectroscopic techniques

^{1}H and ^{13}C NMR spectra were recorded in CD_3OD or $CDCl_3$on Bruker AC-400 at 400.13 and 100.03 MHz or on a Bruker DRX-500 spectrometer at 500.13 and 125.78 MHz respectively. Chemical shifts are reported in parts per million with respect to TMS (tetramethylsilane) as internal standard. NMR spectra of the compounds were analyzed using ACD/NMR processor software. IR spectra were recorded on Schimadzu 8400 series FTIR instrument and values are reported in cm^{-1}. The solution of metabolites in chloroform was used to make thin film of compound in the NaOH plates. UV spectrums of the compounds (solution in methanol) were recorded on Cary-300 instrument.

Optical rotations ($[\alpha]_D$) were recorded using Jasco, P-1020 polarimeter and are reported in deg/dm and the concentration (c) is given in g per 100 mL in the specified solvent.

2.6.2: X-ray diffraction analysis

X-ray intensity data measurements of the compounds **1b** and **2c** were carried out on a Bruker SMART APEX I CCD diffractometer with graphite-monochromatized (MoK_α= 0.71073Å) radiation at room temperature. The X-ray generator was operated at 50 kV and 30 mA. Data were collected with ω scan width of 0.3° at different settings of φ (0°, 90°, 180° and 270°) keeping the sample-to-detector distance fixed at 6.145 cm and the detector position (2θ) fixed at -28°. The X-ray data collection was monitored by SMART program[5].

All the data were corrected for Lorentzian, polarization and absorption effects using SAINT and SADABS programs[5] (Bruker, 2003). SHELX-97 was used for structure solution and full matrix least-squares refinement on F^2 [6] ORTEPs were generated using Mercury-3.0 program[7].

2.7: References

(1) Thulasiram, H. V.; Madyastha, K. M. *Can. J. Microbiol.* **2005**, *51*, 447-454.
(2) Haldar, S.; Kolet, S. P.; Thulasiram, H. V. *Green Chemistry* **2013**, *15*, 1311-1317.
(3) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248-254.
(4) Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370-2377.
(5) Bruker (2003). SADABS (Version 2.05), S. V., SAINT (Version 6.45) and SHELXTL (Version 6.14). Bruker AXS Inc., Madison, Wisconsin, USA.
(6) Sheldrick, G. M. *Acta. Crystallogr. A* **2008**, *64*, 112-122.
(7) Macrae, C. F.; Edgington, P. R.; McCabe, P.; Pidcock, E.; Shields, G. P.; Taylor, R.; Towler, M.; van De Streek, J. *J. Appl. Crystallogr.* **2006**, *39*, 453-457.

Chapter 3: Biocatalyst Mediated Hydroxylation of Steroids by *Mucor sp.* (*M881*)

Introduction

The importance of microbial steroid transformations has attracted attention of researchers since 1952 with the identification of 11α-hydroxylation activity of a *Rhizopus* species.[1] This invention lead to reduction in cost of production of important steroidal drug cortisone from $200 to $1 during 1970s.[2] Steroid compounds and their derivatives are involved in various biologically process and used as anti-inflammatory, immunosuppressive, diuretic, anabolic and contraceptive agents.[3-9] The use of chemical synthesis routes in the preparation of steroid derivatives is limited because of their complex structure. It often requires complicated, multi-step schemes, subsequent protection and de-protection steps which results in the overall low yield of the process. Further, chemical synthesis involves the use of hazardous reagents and byproducts increasing the difficulties in disposal. Microbial steroid conversions require mild reaction conditions and are performed in aqueous environment, at ambient temperature, pH range and thus can provide an efficient alternative to the chemical synthesis. Bioconversion can be done at the positions of steroid molecules hardly available for chemical agents, in regio- and stereo-specific manner. Several chemical reaction steps thus can be replaced by one biotransformation step. The ability of various microorganisms in derivatizing different pharmaceutically important steroid molecules is now well recognized and applications of biotransformation in the partial synthesis of steroidal molecules are well studied.

The hydroxylation of steroids is an important reaction as it increases the polarity of compounds, which in turn affects toxicity, uptake, and excretion of compounds from the cell.[10-12] It also plays an important role in the detoxification of steroid molecules in living systems. Microbial hydroxylation reactions are unique in their diversity and it offers access to inaccessible sites on steroid molecules. The hydroxylations are possibly the most widespread type of steroid bioconversions and different microorganisms capable of hydroxylating steroids on positions from C1 to C21 are well documented. As hydroxyl derivatives of steroids are important ingredients of various pharmaceutically important preparations, the development of efficient transformation methods for their large-scale production has attracted the attention of many researchers. As filamentous fungi are known for their ability to hydroxylate steroids in regio- and stereo-selective manner,[13-16] we have screened various fungal strains for their ability to hydroxylate different biologically important

steroid molecules such as hormonal steroids, anabolic steroids etc.

In the present study, the transformation of different steroids such as progesterone (**1**), testosterone (**2**), 17α-methyltestosterone (**3**), 4-androstene-3,17-dione (**4**), 19-nortestosterone (**5**), epiandrosterone (**6**) and dehydroepiandrosterone (**7**) with different fungal strains were studied. About 20 different fungal strains were screened for the transformation of steroids. From the TLC and HPLC analyses, the fungal system belonging to the genera of *Mucor* (abbreviated as *M881*) [from National Collection of Industrial Microorganisms (NCIM), Pune catalogue no. 881] carried out efficient conversion and was selected for the transformation of studied steroids.

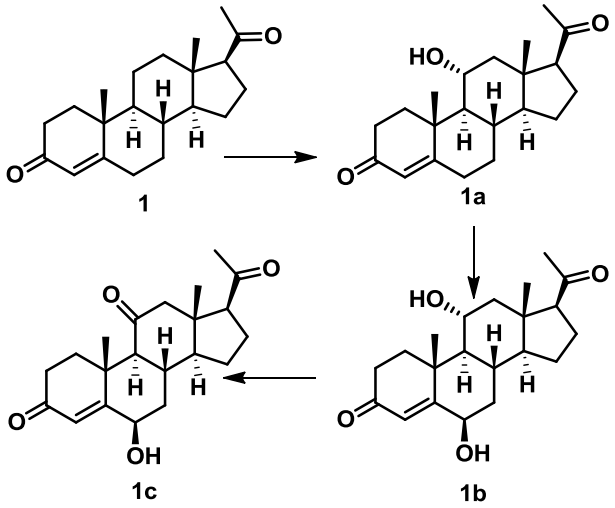
3.1: Biotransformation of 3-one-4-ene steroids using *M881*

To investigate the biotransformation of steroidal enones (**1**), (**2**), (**3**), (**4**) and (**5**), 20 different fungal systems were screened (as described in materials and methods, chapter 2). The TLC, HPLC and LC-MS analyses of the crude extracts obtained after incubating the presently studied steroids for five days, with different fungal strains revealed that the fungal strain *M881* efficiently transformed all enones into corresponding hydroxylated metabolites. All other fungal systems did not show any noticeable transformation hence, *M881* was selected for biotransformation study of (**1**), (**2**), (**3**), (**4**) and (**5**). From the substrate concentration study of (**1**) in the range from 0.3 to 1.2 g/L of fermentation media, it was observed that, when substrate concentration was more than 0.7 g/L (1.0 g/L for testosterone transformation), high amount of unconsumed substrate left in the fermentation media after 6 days of incubation. Thus efficient transformation, in the required time was observed with 0.7 g/L (1.0 g/L for testosterone transformation) substrate concentration, which was then used for large-scale biotransformation and time-course experiments for all five studied steroids.

3.1.1: Transformation of progesterone (1)

A batch of 30 flasks was inoculated with *M881* and the substrate **1** (0.7 g/L) was added after 36 h of incubation. At the end of six days of incubation period, the contents of all flasks were pooled and processed as described in materials and methods (Chapter 2). The crude extract (1.87 g) upon TLC and HPLC analyses indicated the presence of one major and two minor metabolites along with small amount of substrate **1**. The crude extract was subjected to column chromatography on silica gel (230-400 mesh, 50 g) and elution of metabolites was carried out with

methanol/dichloromethane (CH_2Cl_2) gradient mixture. Eluting the column with 2% methanol in CH_2Cl_2 yielded unmetabolized substrate **1** (38 mg, R_f: 0.83 system: I; R_t: 41 min,). Further, elution of the column with 3% methanol in dichloromethane yielded a fraction (1.01 g) containing three metabolites (R_f: 0.41, 0.40, 0.29 system: I; R_t: 26.3 min, 19.5 min, 16.8 min respectively), which were purified by preparative HPLC (117 mg, 54 mg, 741 mg respectively).



Scheme 3.1: Transformation of progesterone (**1**) by *M881*

The HRMS spectrum of compound with R_f: 0.41 (system: I) showed $[M+Na]^+$ peak at *m/z* 353.2065 ($C_{21}H_{30}O_3Na$) indicating the introduction of a hydroxy group to the parent structure (**1**). In the UV spectrum, absorption at 239 nm indicated presence of enone functional group in the compound **1a**. The IR analysis of compound **1a** showed two carbonyl absorption bands at 1701 (CO), 1658 (conj. CO) cm^{-1}similar to progesterone (**1**) and additional hydroxyl absorption band at 3433 cm^{-1}. The ^1H NMR spectrum (CD_3OD, 400 MHz) of compound **1a** showed a new downfield signal for oxygen-bearing methine proton at δ 3.97 (1H, dt, *J* =4.6, 10.7 Hz,) which indicated the introduction of a hydroxyl group on methylene carbon. The singlets at δ 0.69, 1.35 and 2.14 were corresponding to C-18, C-19 and C-21 tertiary methyl groups, respectively. The singlet observed at δ 5.72 corrosponding to C-4 proton indicated that enone functionality was intact in the metabolites as in the substrate **1**. In the ^{13}C NMR spectrum (CD_3OD, 100 MHz) of compound the presence of signals for three methyl, seven methylene, six methine and five quaternary carbons confirmed that the new hydroxylation has occurred on methylene carbon. The signals at δ 34.9 and 124.6 corresponding to C-2 and C-4 carbons remain unchanged showing that hydroxylation has not occurred in ring A.

The signals for C-16 and C-6 carbons were unchange as in the substrate **1** which revealed that newly introduced hydroxyl group was not present in ring B and D of the metabolite. The downfield shift of signal for C-11 carbon from δ 21.7 in the substrate **1** to δ 69.3 and also of C-9 and C-12 carbons in ^{13}C NMR spectra confirmed the insertion of a hydroxy group in progesterone at C-11 position. The stereochemistry of the newly introduced hydroxyl group at C-11 was deduced to be α on the basis of coupling constants and NOESY correlations of H-18β (δ = 0.69) and H-19β (δ = 1.35) with H-11 (δ = 3.97). From the melting point (165-166 ºC), optical rotation (+177, *c* = 1.8 in MeOH) and spectral data, the metabolite was identified as 11α-hydroxyprogesterone (**1a**) and the spectral data for **1a** matched well with the earlier report.[17]

The molecular formula for compound **1b** with R_f: 0.29 (system: I) was deduced as $C_{21}H_{30}O_4$ from HRMS analysis which showed $[M+Na]^+$ ion peak at *m/z* 369.2038, indicating the introduction of two hydroxy group in the substrate **1**. The UV absorbance at 239 nm and signals at 3445 (OH), 1703 (CO) and 1651 (conj. CO) in IR indicated the presence of hydroxyl, carbonyl and enone groups, respectively in the metabolite **1b**. The ^1H NMR spectrum of compound showed two new downfield signal for oxygen-bearing methine protons at δ 3.97 (1H, dt, J =4.6, 10.7 Hz,) and 4.22 (1H, t) indicating introduction of hydroxyl group at methylene carbons. The signals for all three methyl protons were unchange as in the substrate but the signal for C-4 proton showed down field shift from δ 5.71 to 5.74 indicating the presence of one hydroxyl group at C-6 carbon. In ^{13}C NMR spectrum of compound **1b**, signals for C-2 and C-16 carbons were unchanged as in the substrate which ruled out the possibility of hydroxylation in ring A and D. Two new signals for methine carbons were observed at δ 73.8 and δ 69.3 with diappearance of signals for methylene C-6 and C-11 carbons at δ 34.7 and δ 21.7, respectively. Further, the downfield shift of signals for C-12 and C-9 carbons was oberved which confirmed the positions of newly introduced hydroxyl groups at C-6 and C-11 positions. The NOESY correlations of C-11 proton with C-18 methyl and C-19 methyl protons further confirmed the hydroxyl group to be α in nature. The stereochemistry of the C-6 hydroxyl group was deduced to be β on the basis of the NOESY interaction between H-6 and H-14α proton (δ = 1.35). From X-ray diffraction data of metabolite (**1b**) (CCDC reference number = CCDC 922747 and SD) it was identified as 6β,11α-dihydroxyprogesterone (Fig. 3.1). The melting point (213-214 ºC), optical rotation

(+24 *c* = 0.68 in MeOH) and spectral data for the compound was in good agreement with the earlier report.[18]

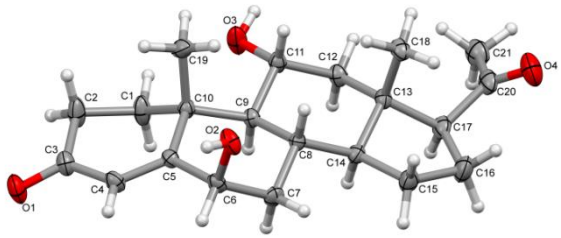


Figure 3.1: X-ray diffraction data of **1b**

UV spectrum of the compound **1c** with R_f: 0.40 (system: I) showed absorbance at 239 nm indicated presence of enone functionality. The IR spectrum exhibited absorbance at 3417, 1736, 1642 cm^{-1} corrosponding to hydroxyl, carbonyl and conjugated carbonyl groups respectively. The HRMS for the metabolite showed a peak at *m/z* 367.1885 $[M+Na]^+$ indicating that the molecular formula was $C_{21}H_{28}O_4$ with increrase in 30 mass units. The ^1H NMR spectrum showed appearance of new downfield narrow triplet at δ 4.28 (1H, t) indicating the introduction of a hydroxy group at methylene carbon. The downfield shift of signal for C-4 proton from δ 5.71 to 5.79 revealed that the hydroxylation was occurred at C-6 position. The singlets at δ 0.64, 1.58 and 2.11 were corresponded to C-19, C-18 and C-21 tertiary methyl groups, respectively. The ^{13}C NMR spectra of metabolite **1c** revealed the presence of three methyl, six methylene, six methine and six quaternary carbons. In the spectrum two new signals at δ 210.99, δ 73.76 and disappearance of signals at δ 21.7, 34.7 characteristics for C-11 and C-6 carbon were observed along with downfield shift of signal for C-12 carbon from δ 39.7 to δ 57.3, confirmed introduction of hydroxyl group at C-6 position and oxo group at C-11 position. The stereochemistry of the hydroxyl group was confirmed from NOESY correlation of C-6 proton with H-14α. From these observations the metabolite was identified as 6β-hydroxypregn-4-ene-3,11,20-trione (**1c**). Melting point and optical rotation of the compound was observed as 204-206 ºC and +107 (*c* = 1.8 in MeOH) respectively.

Substrate concentration studies indicated that the organism could transform

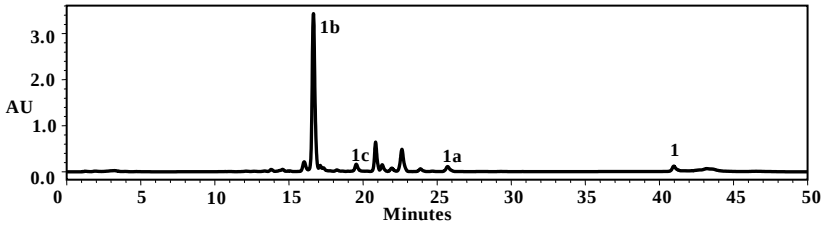


Figure 3.2: The HPLC analysis of progesterone (**1**) transformation with *M881* after 8 d

progesterone (**1**) into **1a**, **1b**, and **1c** in efficient manner at the concentration of 0.7 g/L. Increase in the substrate concentration decreased the level of metabolites

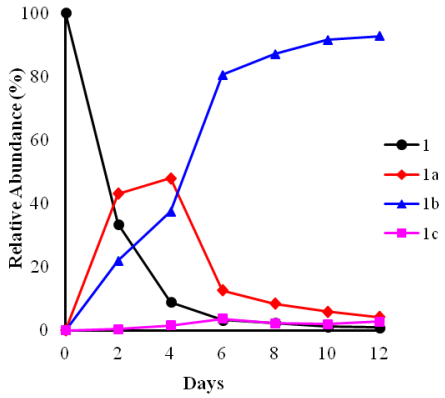


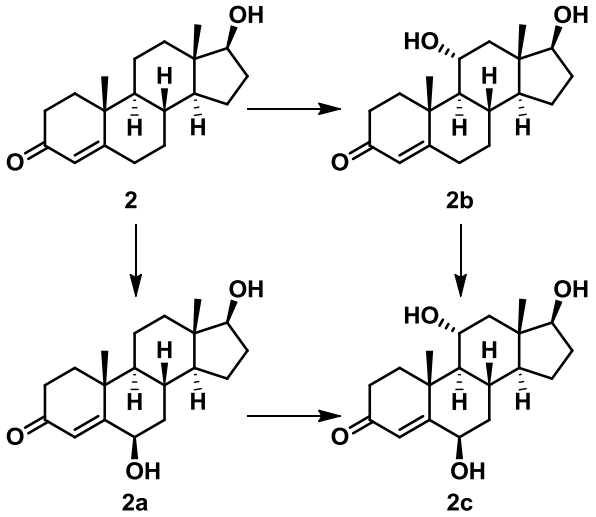
Figure 3.3: Time course study of progesterone (**1**) transformation: (●) progesterone (**1**), (♦) 11α-hydroxyprogesterone (**1a**), (▲) 6β, 11α-dihydroxyprogesterone (**1b**), (■) 6β-hydroxy pregn-4-ene-3,11,20-trione (**1c**).

formation. Time course experiment carried out using **1** revealed that during early stages of incubation (two days) nearly 35% of **1** was transformed into metabolites **1a**, **1b** and **1c**. By prolonging the incubation period to 12 days, the transformation was increased to 98% with the formation of 6β,11α-dihydroxyprogesterone (**1b**) as a major metabolite (96%) (Fig. 3.2 and 3.3). *M881* has initiated the transformation of **1** by hydroxylation at 11α- position to form **1a** which further undergoes one more hydroxylation at 6β-position to form **1b**. This was further confirmed by incubating **1a** with *M881*, which transformed into **1b**. Moreover, the formation of 6β-hydroxyprogesterone was not observed by HPLC and LC-MS analyses. Small amount of **1b** was converted into **1c** as the microorganism oxidizes 11α-hydroxyl group on **1b**. Based on these results, the biotransformation pathway of progesterone by *M881* was deduced (Scheme 3.1).

3.1.2: Transformation of testosterone (2)

Transformation of **2** (1.0 g/L) was carried out using 30 flasks as described in materials and methods (Chapter 2). The crude extract (2.3 g) obtained after 6 days of incubation was subjected to HPLC and LC-MS analyses, which indicated the presence of three metabolites along with trace amount of unmetabolised substrate **2**. The metabolites were purified over silica gel column (230-400 mesh, 38 g) chromatography by eluting with gradient mixture of methanol in dichloromethane. Elution with 2% methanol in CH_2Cl_2 yielded a fraction containing substrate **2** (27 mg R_f: 0.79 system: II; R_t: 30.2 min). Other three metabolites were subsequently

eluted with 2.5, 2.8 and 3.1% methanol in CH_2Cl_2 (369 mg, 251 mg, 546 mg, R_f: 0.60, 0.44, 0.32 system II; R_t: 16 min, 19 min, 10.5 min, respectively). From HRMS, IR, ^1H, ^{13}C NMR spectral data analyses and X-ray diffraction data, the compounds were characterized.



Scheme 3.2: Transformation of testosterone (**2**) by *M881*.

The HRMS spectrum of compound **2a** with R_f 0.60, showed peak at *m/z* 327.1932 ($[M+Na]^+$) indicating the introduction of a hydroxyl group on testosterone (**2**) with molecular formula $C_{19}H_{28}O_3$. The compound showed UV absorbance at 239 nm which indicated presence of unsaturated carbonyl functionality in the molecule. In the IR spectrum of compound absorption bands at 3429, 1664 cm^{-1}corresponding to hydroxyl and conjugated carbonyl groups were observed. The ^1H NMR spectrum (CD_3OD, 400 MHz) of compound **2a** showed a new downfield signal for oxygen-bearing methine proton at δ 4.26 (1H, t), indicated the introduction of a hydroxyl group on methylene carbon. The downfield shift of signal for C-4 proton from δ 5.74 to 5.78 indicated introduction of a hydroxyl group at C-6 position. The singlets at δ 0.81 and 1.39 were assigned to C-18 and C-19 methyl protons. Similar to substrate **2**, the signal for oxygen bearing C-17 methine proton at δ 3.61 (1H, t) was observed. From the signals in the ^{13}C NMR spectrum (CD_3OD, 100 MHz) of compound the presence of two methyl, seven methylene, six methine and four quaternary carbons was established. The δ values for C-2, C-11 and C-16 were similar to that of substrate indicating absence of hydroxyl group in rings A, C and D. The shift of signal from δ 34.7 for C-6 carbon to δ 73.6 in ^{13}C NMR revealed that the hydroxylation was occurred at C-6 position. It was further confirmed by downfield shift of signal for C-7 carbon and gauche upfield shift of C-8 carbon in the

metabolite **2a** compared to the substrate **2**. By comparing the melting point (213-214 ºC) and optical rotation (+24 *c* = 0.68, MeOH) of the compound with the earlier report,[19] the hydroxylation was confirmed at C-6β position and the compound was identified as 6β-hydroxytestosterone (**2a**).

For compound **2b** with R_f 0.44, the HRMS spectrum showed $[M+H]^+$ at *m/z* 305.2106, which established the introduction of a hydroxyl group on testosterone with increase in mass by 16 units in agreement with the molecular formula $C_{19}H_{28}O_3$. The UV absorbance at 239 nm and characteristic bands at 3406, 1658 cm^{-1} in IR spectrum indicated presence of hydroxyl and conjugated carbonyl functional groups in the metabolites **2b**. The appearance of new downfield signal for oxygen bearing methine proton at δ 3.99 (1H, dt, J =4.6, 10.7 Hz) in ^1H NMR indicated introduction of hydroxyl group on methylene carbon. The signals at δ 0.81, 1.36 and 5.71 were assigned to the C-18, C-19 and C-4 protons, respectively. The triplet at δ 3.59 (1H, t) indicated the presence of C-17β hydroxyl group in the metabolites as in the substrate **2**. In ^{13}C NMR spectrum of the compound, the new downfield signal at δ 69.4 was observed and signal at δ 21.7 corresponding to C-11 carbon disappeared. The signals for C-2, C-6 and C-16 were observed at δ values similar to substrate showed introduction of hydroxyl group in ring C. Further, the downfield shift of signal for C-12 carbon confirmed the introduction of hydroxyl group at C-11α position. The melting point and optical rotation of compound was recorded as 177-178 ºC and +86 (*c* = 0.62 in MeOH), respectively. The position and stereochemistry of hydroxyl group was confirmed by comparing the NMR data with **1a** and the earlier report.[12] From all above observations the metabolite was identified as 11α-hydroxytestosterone (**2b**).

The molecular formula of compound **2c** eluted at the end of the column with R_f 0.32 was deduced as $C_{19}H_{28}O_4$ from the HRMS data with *m/z* 343.1857 for $[M+Na]^+$, indicating an increase in mass by 32 units and introduction of two hydroxyl groups in testosterone (**2**). The UV spectrum of compound showed absorbance at 239 nm and IR spectrum showed bands at 3400 and 1658 cm^{-1}, which

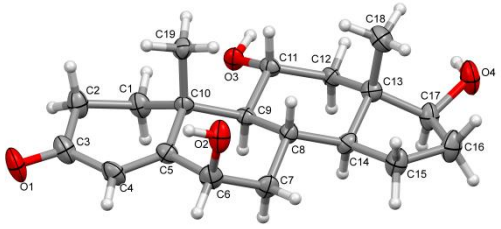


Figure 3.4: X-ray diffraction data of **2c**

are characteristic for the presence of hydroxyl and unsaturated carbonyl groups. Two new downfield signals at δ 4.00 (1H, dt, *J* =4.6, 10.7 Hz) and δ 4.25 (1H, t) were observed in ^{1}H NMR spectrum of the metabolite indicated introduction of two hydroxyl group. In the ^{13}C NMR spectrum of metabolite **2c**, signals were observed for two methyl, six methylene, seven methine and four quaternary carbons. Two new downfield signals for methine carbons observed at δ 73.8 and 69.4 confirmed the introduction of hydroxyl groups at methylene carbons. Signals for C-2 and C-16 carbons were same as that of substrate which indicated that hydroxylation occurred at B and C rings. The signals for C-6 (δ 34.7) and C-11 (δ 21.7) carbons disappeared which confirmed the presence of hydroxyl groups at C-6 and C-11 carbons. From the melting point, optical rotation, coupling constants in ^{1}H NMR spectrum and X-ray diffraction data of compound **2c** (Fig. 3.4), the stereochemistry of two newly introduced hydroxyl groups was deduced as C-6β and C-11α. The compound was identified as 6β,11α-dihydroxytestosterone (**2c**) and is in agreement with the reported data.[19]

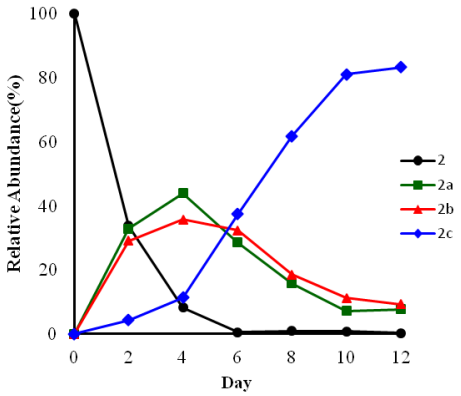


Figure 3.5: Time course study of Testosterone (**2**) transformation: **(●)** Testosterone (**2**), **(■)** 6β-hydroxytestosterone (**2a**), (▲) 11α-hydroxytestosterone (**2b**), **(♦)** 6β,11α-dihydroxytestosterone (**2c**).

The optimum concentration (1 g/L) for the transformation was determined from substrate concentration studies. Time-course experiments (Fig. 3.5) indicated that *M881* could transform 66% of testosterone (**2**) into 6β-hydroxytestosterone (**2a**), 11α-hydroxytestosterone (**2b**) and 6β,11α-dihydroxytestosterone (**2c**) at the end of 48 h incubation. After twelve days of incubation, **2c** was found as major metabolite (84%) (Fig. 3.6). The biotransformation pathway for **2** (Scheme 3.2) was confirmed by incubating 6β- hydroxytestosterone (**2a**) and 11α-hydroxytestosterone (**2b**) with *M881*. It was observed that both metabolites **2a** and **2b** were transformed into dihydroxy metabolite **2c**.

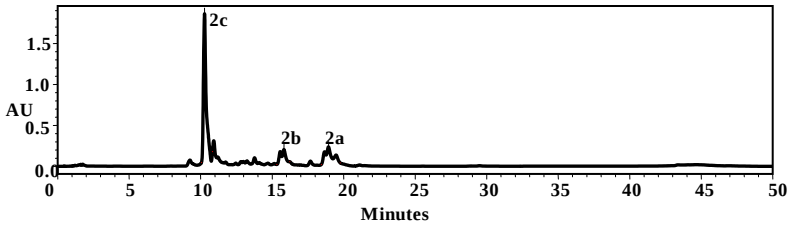
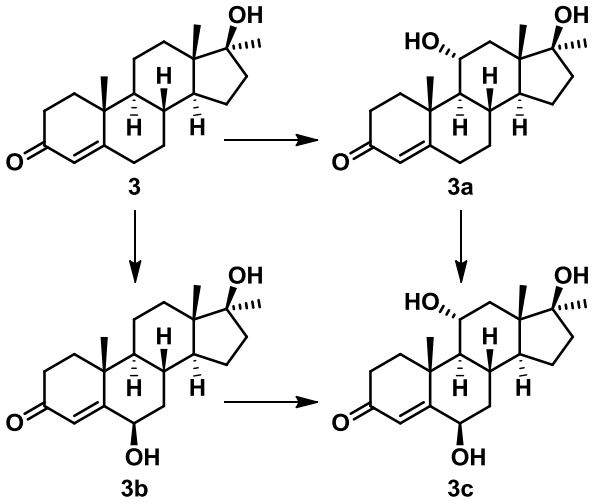


Figure 3.6: The HPLC analysis of testosterone **(2)** transformation with *M881* after 10 d

3.1.3: Transformation of 17α-methyltestosterone (3)

For the purification of metabolites, transformation of **3** (0.7 g/L) was carried using 30 flasks inoculated with *M881* as described in materials and methods (chapter 2). The TLC, HPLC and LC-MS analyses of crude extract (1.79 g) obtained after six days of incubation showed the presence of three metabolites along with trace amount of unmetabolised substrate **3**. The metabolites were purified over silica gel column (230-400 mesh, 44 g) chromatography with gradient mixture of methanol in CH_2Cl_2 as mobile phase. Elution of the column with different % of methanol in CH_2Cl_2 yielded fractions containing pure compounds with R_f values 0.81, 0.65, 0.41 and 0.35 (system: II), respectively. The identification of metabolites was done based on the spectral data analyses (HRMS, IR, ^1H and ^{13}C NMR).



Scheme 3.3: Transformation of 17α-methyltestosterone (**3**) by *M881*.

The compound **3a** having R_f 0.65 exhibited $[M+Na]^+$ ion peak at *m/z* 341.2087 in the HRMS spectra corresponding to molecular formula $C_{20}H_{30}O_3$, indicating introduction of one hydroxyl group in the substrate (**3**). The compound showed UV absorbance at 239 nm and IR absorption bands at 3412 and 1653 cm^{-1} indicating the presence of hydroxyl and conjugated carbonyl group in the metabolite **3a**. In ^1H NMR spectrum new signal for oxygen bearing proton was observed at δ 4.01 (1H, dt, J =4.6, 10.7 Hz) confirming the introduction of a hydroxyl group on

methylene carbon. The singlets at δ 0.92, 1.21 and 1.36 were assigned to three methyl protons at C-18, C-17, C-19 carbons. Also the signal at δ 5.71 for C-4 proton was unchanged as in the substrate. In ^{13}C NMR spectrum signals were observed for three methyl, seven methylene, five methine and five quaternary carbons. The signals at δ 34.9 and 124.6 corresponding to C-2 and C-4 carbons were unchanged which shows that hydroxylation have not occurred in ring A. The signals for C-16 and C-6 carbons were at same δ values as in the substrate **3** which revealed that newly introduced hydroxyl group was not present in ring B and D of the metabolite. The downfield shift of signal for C-11 carbon from δ 21.7 in the substrate **3** to δ 69.8 and of C-9 and C-12 carbons confirmed the insertion of a hydroxyl group at C-11 position. The stereochemistry of the newly introduced hydroxyl group at C-11 was deduced to be α on the basis of coupling constants which were 4.6, 10.7 Hz. The melting point (151-152 ºC) and optical rotation +61 (*c* =1.37 in MeOH) of compound agreed well with the earlier report.[20] The pattern of NMR data was similar to that of 11α-hydroxytestosterone (**2b**) which confirmed that compound was 11α-hydroxy-17α-methyltestosterone (**3a**).

The UV spectrum of the metabolite **3b** (R_f 0.41) showed absorbance at 239 nm indicating the presence of unsaturated carbonyl group which was confirmed from IR spectra with absorption bands at 1653 cm^{-1} and 3425 cm^{-1} which indicates the introduction of hydroxyl group. The HRMS spectrum of compound showed peak at *m/z* 319.1932 ([M+H]$^+$) corresponding to increase in 16 mass units and a hydroxylation on 17α-methyltestosterone (**3**) with molecular formula $C_{20}H_{30}O_3$. In the ^{1}H NMR spectrum of metabolite, new downfield signal for oxygen bearing methine proton at δ 4.26 (1H, t) was observed revealed that hydroxylation was occurred at methylene carbon. The downfield shift of signal from δ 5.67 to 5.78 for C-4 proton and that of C-19 methyl protons from δ 1.14 to 1.40 indicated presence of hydroxyl group at C-6 position. The signals for C-18, C-17, C-19 methyl protons were unchanged as in the substrate **3**. In ^{13}C NMR spectrum signals were observed for three methyl, seven methylene, five methine and five quaternary carbons. The presence of signals at δ 35.1, 21.7 and 24.2 for C-2, C-11 and C-15 carbons, respectively, eliminated the possibility of hydroxylation at methylene carbons in ring A, C, D. The downfield shift of signals for C-19 methyl carbon and C-4 carbon from δ 17.7 and 124.1 to δ 19.7 and 126.7, respectively confirmed the introduction of hydroxyl group at C-6 position. From the coupling constant for C-6 proton signal in

^{1}H NMR spectrum and by comparing the melting point 246-247 ºC and optical rotation +5 (*c* = 1.30 in MeOH) of compound **3b** with the reported data,[21] the compound was identified as 6β-hydroxy-17α-methyltestosterone (**3b**).

The molecular formula of compound **3c** with R_f 0.35 was $C_{20}H_{30}O_4$, as evidenced from HRMS which showed $[M+Na]^+$ ion peak at 357.3063 and suggested incorporation of two oxygen atoms into parent compound. In the UV spectrum absorption at 239 nm indicated presence of unsaturated carbonyl functional group in the compound **3c**. The IR analysis of compound **3c** showed carbonyl absorption bands at 1666 cm^{-1}similar to 17α-methyltestosterone (**3**) in addition to hydroxyl absorption band at 3423 cm^{-1}. The ^{1}H NMR spectrum (CD_3OD, 400 MHz) of compound **3c** showed two new downfield signals at δ 4.04 (1H, dt, J =4.6, 10.7 Hz), 4.25 (1H, t), indicating the introduction of hydroxyl groups on methylene carbons. The signal for C-4 proton showed downfield shift, indicated that the hydroxylation has occurred at C-6 position. In the ^{13}C NMR spectrum (CD_3OD, 100 MHz) of the compound, the presence of signals for three methyl, six methylene, six methine and five quaternary carbons confirmed that the hydroxylations have occurred on methylene carbons. Two new signals were appeared at δ 73.8 and 69.8 whereas signals at δ 33.0 and 21.1 characteristic for C-6 and C-11 carbons in the starting compound **3** were missing. Further, downfield shift of signals for C-12, C-7 carbons and gauche upfield shift for C-8 carbon confirmed the introduction of hydroxyl group at C-6 and C-11 carbons. The stereochemistry of hydroxyl groups were deduced from coupling constant (*J* =4.6, 10.7 Hz for C-11 proton), melting point (264-265 ºC) and optical rotation (+ 6.36 *c* = 1.71 in MeOH) of compound **3c** with

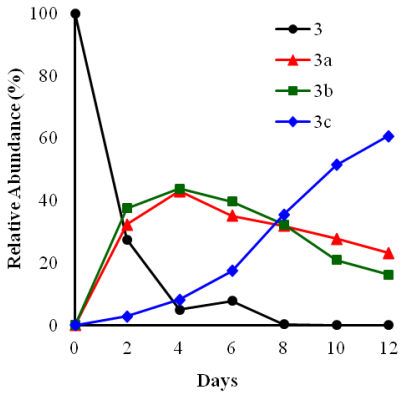


Figure 3.7: Time course study of 17α-methyltestosterone transformation: (●) 17α-methyltestosterone (**3**), (▲) 11α-hydroxy-17α-methyltestosterone (**3a**), (■) 6β-hydroxy-17α-methyltestosterone (**3b**), (♦) 6β,11α-dihydroxy-17α-methyltestosterone (**3c**).

that of earlier report[22] and the compound was identified as 6β,11α-dihydroxy-17α-methyltestosterone (**3c**).

From time course experiments (Fig 3.7) (concentration 0.7 g/L), it was observed that the fungal system can convert 17α-methyltestosterone (**3**) into 11α-hydroxy-17α-methyltestosterone (**3a**), 6β-hydroxy-17α-methyltestosterone (**3b**) and 6β,11α-dihydroxy-17α-methyltestosterone (**3c**) (Scheme 3.3) (Fig. 3.8). However, with prolonged incubation, increase in the concentration of **3c** was observed. At the end of twelve days of incubation, 90% of **3** was converted into **3c** as a major metabolite.

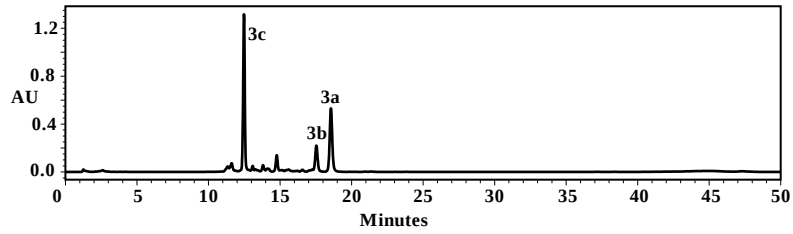
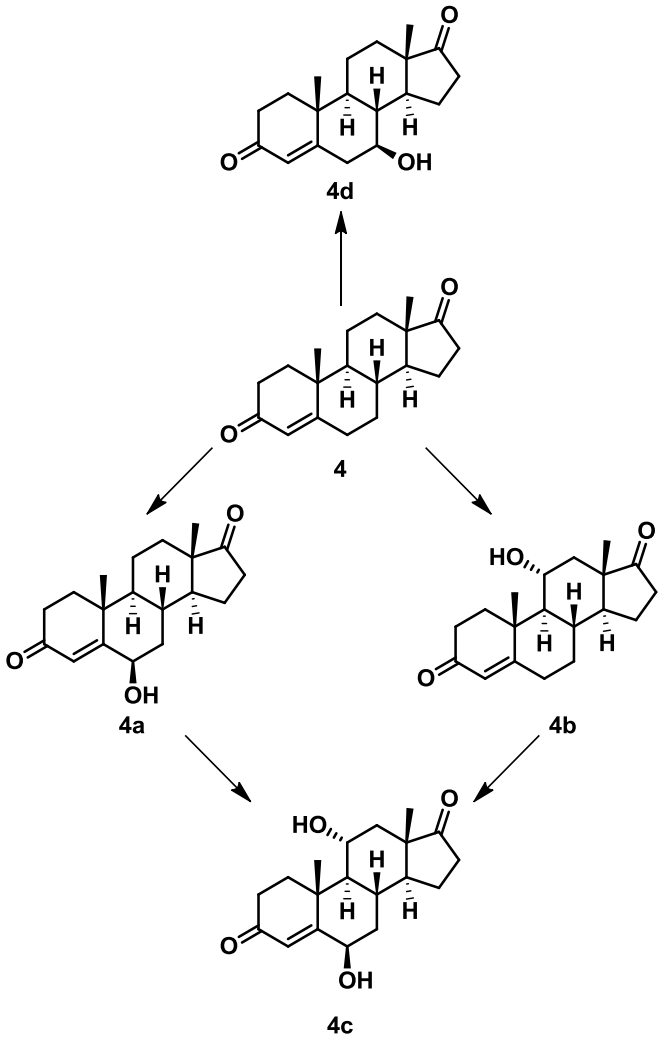


Figure 3.8: The HPLC analysis of 17α-methyltestosterone **(3)** transformation with *M881* after 10 d

3.1.4: Transformation of 4-androstene-3,17-dione (4)

TLC and HPLC analyses of the crude extract (1.63 g) obtained after processing a batch of 30 flasks for the transformation of 4-androstene-3,17-dione (**4**), inoculated with *M881*, as described in materials and methods (chapter 2), indicated the presence of four metabolites along with small amount of substrate **4**. The metabolites [57 mg (**4**), 338 mg, 472 mg, 198 mg, and 391 mg, R_f: 0.82 (**4**), 0.64, 0.58, 0.56, and 0.32 system: I, R_t: 46.2 (**4**), 26.1 min, 24.8 min, 21 min, and 12.5 min, respectively] were purified over silica gel (230-400 mesh, 44 g) column chromatography by eluting with methanol/dichloromethane (CH_2Cl_2) gradient mixture. By evaluating the spectral data and comparing data with earlier reports the metabolites were identified.

The HRMS spectrum of compound **4a** with R_f: 0.64 (system: I) showed $[M+H]^+$ peak at *m/z* 303.1945 ($C_{19}H_{26}O_3$) indicating the introduction of a hydroxyl group to the parent structure (**4**). In the UV spectrum absorption at 239 nm indicated presence of unsaturated carbonyl functional group in the compound **4a**. The IR analysis of compound **4a** showed three absorption bands at 3409 (OH), 1727 (CO) and 1651 (conj. CO) cm^{-1}. The ^1H NMR spectrum (CD_3OD, 400 MHz) of compound **4a** showed a new downfield signal for oxygen-bearing methine proton at δ 4.34 (1H, t) which indicated the introduction of a hydroxyl group on methylene carbon. The singlets at δ 0.97 and 1.44 were corresponded to C-18 and C-19 tertiary methyl



Scheme 3.4: Transformation of 4-androstene-3,17-dione (**4**) by *M881*.

groups, respectively. The downfield shift for singlet at δ 5.83 relative to substrate **4** corresponding to C-4 proton indicated that hydroxylation was occurred at C-6 position. In the ^{13}C NMR spectrum (CD_3OD, 100 MHz) of compound the signals at δ 35.0, 21.3 and 22.7 corresponding to C-2, C-11 and C-15 carbons were unchanged showed that hydroxylation was not occurred in ring A, C and D, respectively. The introduction of new downfield signal at δ 73.4 with disappearance of signal for C-6 methylene carbon at δ 32.5 indicated that hydroxylation has occurred at C-6 position. It was further supported by downfield shift of signals for C-7 carbon and gauche up-field shift of C-8 carbon. The stereochemistry of newly introduced hydroxyl group was determined by comparing the spectral data with that of **2a** and the melting point, optical rotation of compound **4a** with the earlier report[3] and the compound was characterized as 6β-hydroxy-4-androstene-3,17-dione (**4a**).

For compound **4b** with R_f 0.58, the HRMS spectrum showed $[M+Na]^+$ at *m/z* 325.1779, which established the introduction of a hydroxyl group on 4-androstene-

3,17-dione with increase in mass by 16 units in agreement with the formula $C_{19}H_{26}O_3$. The UV absorbance at 239 nm and characteristic bands at 3412, 1653 cm^{-1} in IR spectrum indicated presence of hydroxyl and conjugated carbonyl functional groups in the metabolites **4b**. The appearance of new downfield signal for oxygen bearing methine proton at δ 4.01 (1H, dt, J =4.6, 10.7 Hz) in ^{1}H NMR indicated introduction of hydroxyl group on methylene carbon. The signals at δ 0.95, 1.38 and 5.73 were assigned to the C-18, C-19 and C-4 protons, respectively. In ^{13}C NMR spectrum of the compound, signals were observed corresponding to two methyl, seven methylene, five methine and five quaternary carbons. The new downfield signal at δ 69.1 was observed and signal at δ 21.4 corresponding to C-11 carbon has disappeared. The signals for C-2, C-6 and C-16 were observed at δ values similar to substrate showed that newly introduced hydroxyl group was present in ring C. In addition the downfield shift of signal for C-12 carbon from δ 33.7 to 43.5 confirmed the introduction of hydroxyl group at C-11 position in the substrate (**4**). The melting point and optical rotation of compound were 215-216 ºC and +152 (*c* = 0.71 in MeOH), respectively. The position and stereochemistry of hydroxyl group was confirmed as α from coupling constant of newly introduced hydroxyl group and by comparing NMR data with **1a** and earlier report.[23] The compound was characterized as 11α-hydroxy-4-androstene-3,17-dione (**4b**)

The molecular formula for compound **4c** with R_f: 0.32 was deduced as $C_{19}H_{26}O_4$ from HRMS analysis which showed $[M+Na]^+$ ion peak at *m/z* 341.1710, indicating introduction of two hydroxyl group in the substrate **4**. The UV absorbance at 239 nm and signals at 3383 (OH), 1740 (CO) and 1668 (conj. CO) cm^{-1} indicated the presence of hydroxyl, carbonyl and conjugated carbonyl groups respectively in the metabolite **4c**. The ^{1}H NMR spectrum of compound showed two new downfield signals for oxygen-bearing methine protons at δ 4.05 (1H, dt, J =4.6, 10.7 Hz,) and 4.31 (1H, t) indicating introduction of hydroxyl group at methylene carbons. The signals for all three methyl protons were unchanged as in the substrate but the signal for C-4 proton showed down field shift from δ 5.73 to 5.81 indicating the presence of one hydroxyl group at C-6 carbon. In ^{13}C NMR spectrum of the compound **4c**, signals for C-2 and C-16 carbons were unchanged as in the substrate which ruled out the possibility of hydroxylation in ring A and D. Two new signals for methine carbons were observed at δ 73.3 and 69.1 with disappearance of signals for methylene C-6 and C-11 carbons at δ 32.5 and 21.4, respectively. Furthermore the

downfield shift of signals for C-12 and C-9 carbons was observed which confirmed the positions of newly introduced hydroxyl groups at C-6 and C-11 positions. From the coupling constants for both newly introduced hydroxyl groups, melting point and optical rotation of metabolite stereochemistry of C-6 and C-11 hydroxyl group was deduced as β- and α- respectively. It was further confirmed by comparing ^{1}H and ^{13}C NMR spectral data of compound with that of 6β,11α-dihydroxytestosterone (**2c**) and earlier report.[23] From the above evidences the compound was identified as 6β,11α-dihydroxy-4-androstene-3,17-dione (**4c**).

UV spectrum of the compound **4d** showed absorbance at 239 nm indicating the presence of enone functionality. The IR spectrum exhibited an absorption at 3383, 1740, 1668 cm^{-1} corrosponding to hydroxyl, saturated ketone and α, β unsaturated ketone respectively. The HRMS spectrum showed molecular ion peak at *m/z* 325.1800 $[M+Na]^{+}$ corresponding to molecular formula $C_{19}H_{26}O_3$, indicating the introduction of one hydroxyl group in the parent compound **4**. In the ^{1}H NMR spectrum (CD_3OD, 400 MHz) of **4d** the appearance of one new multiplet at δ 3.45 (1H) compared to parent compound confirmed the introduction of a hydroxyl group at methylene carbon. By comparing the signals in ^{13}C NMR spectrum for metabolite **4d** and substrate **4** the position of newly introduced hydroxyl group was confirmed. The signals for two carbonyl carbons at δ 210.5 and 199.5 confirmed the presence of ketone and unsaturated ketone in the metabolite **4d**. The unchanged signals at δ 35.5, 21.5 and δ 20.0 for C-1, C-11 and C-16 carbons, respectively in the spectrum indicated that hydroxylation has occurred in ring B. The downfield shift of signal for C-8 carbon from δ 36.6 to 43.8 confirmed the introduction of hydroxyl group at C-7

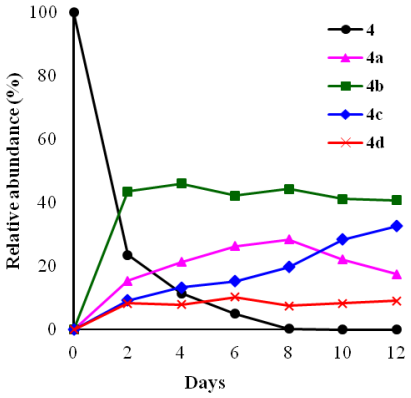


Figure 3.9: Time course study of 4-androstene-3,17-dione (**4**) transformation: (♦) 4-androstene-3,17-dione (**4**), (▲) 6β-hydroxy-4-androstene-3,17-dione **(4a),** (■) 11α-hydroxy-4-androstene-3,17-dione (**4b**), (●) 6β,11α-dihydroxy-4-androstene-3,17-dione (**4c**), (**x**) 7β-hydroxy-4-androstene-3,17-dione (**4d**)

position.The melting point and optical rotation of the compound obtained was 215-217 ºC and +129 (*c* = 1.1 in MeOH). Further, by comparing the data with earlier report[24], the compound was identified as 7β-hydroxy-4-androstene-3,17-dione (**4d**).

Time-course for the transformation of 4-androstene-3,17-dione (**4**) (Fig. 3.9) revealed that in the early stages of incubation nearly 50% of **4** was transformed into four metabolites (**4a** to **4d**) with 11α-hydroxy-4-androstene-3,17-dione (**4b**) as major metabolite (Fig. 3.10). It was observed that on prolong incubation percentage of 6β,11α-dihydroxy metabolite increased with decrease in the percentage of both 6β- and 11α-hydroxy metabolites (**4a** and **4b**). (Scheme 3.4)

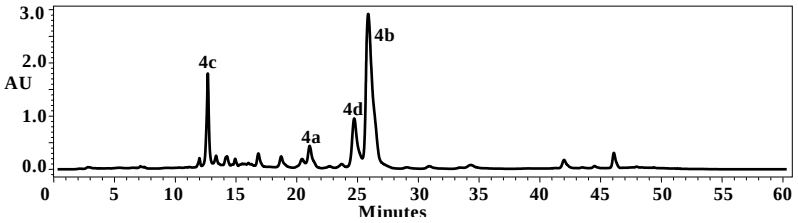
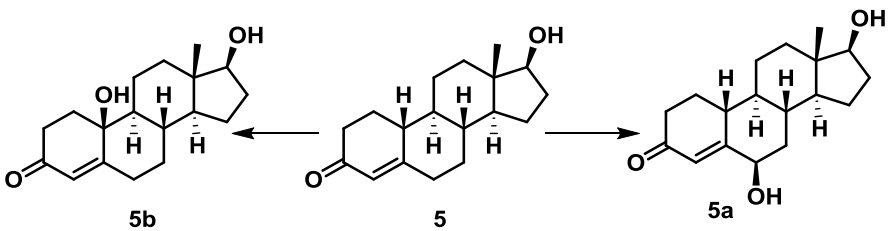


Figure 3.10: The HPLC analysis of 4-androstene-3,17-dione (**4**) transformation with *M881* after 12 d

3.1.5: Transformation of 19-nortestosterone (5)

Transformation of **5** (0.7 g/L) was carried out using 30 flasks inoculated with *M881* as described in materials and methods (chapter 2). The crude extract (1.6 g) on TLC, HPLC and LC-MS analyses indicated the presence of two metabolites with unmetabolized substrate **5**. The metabolites were purified over silica gel column (230-400 mesh, 38 g) chromatography. Elution of the column with 1% of methanol in CH_2Cl_2 yielded a fraction containing substrate **5** (608 mg R_f: 0.81, R_t: 22.8 min). Other two metabolites were then eluted as a mixture with 1.5% of methanol in CH_2Cl_2. Two metabolites were then purified using preparative HPLC (326 mg, 289 mg, R_f: 0.64, 0.64 system: II; R_t: 10.1 min, 11.8 min respectively) and were characterized from spectral data analyses.



Scheme 3.5: Transformation of 19-nortestosterone (**5**) by *M881*

The HRMS of the compound **5a** with R_t 10.1 min (Fig 3.12) displayed the molecular ion peak at *m/z* 313.1778 $[M+Na]^+$ corresponding to molecular formula

$C_{18}H_{26}O_3$ indicating the increase in mass by 16 units and introduction of one hydroxyl group to the parent steroid **5**. In the UV spectrum of compound **5a**, absorbance was observed at 239 nm which revealed the presence of α,β-unsaturated carbonyl group. The IR spectrum showed two absorption band at 3429, 1664 cm^{-1} corresponding to the presence of –OH and -C=C-C=O functional groups in the isolated metabolite. In the ^1H NMR spectrum one downfield signal was observed for the oxygen bearing methine protons at δ 4.29 (1H, t) indicating hydroxylation at methylene carbon. The signal at δ 3.59 was unchanged as in the substrate revealed presence of C-17β hydroxyl group. The downfield shift of signal for C-4 proton from δ 5.80 to 5.86 revealed the introduction of hydroxyl group at C-6 position. In ^{13}C NMR spectrum signals were observed for one methyl, seven methylene, seven methine and three quaternary carbons. The presence of signals at δ 37.1, 27.2 and 30.6 for C-2, C-11 and C-16 carbons, respectively as in the case of starting compound **5** eliminated the possibility of hydroxylation at methylene carbons in ring A, C, D. The downfield shift of signal for C-7 carbon from δ 32.0 to 39.1 and gauche up-field shift of C-8 carbon confirmed introduction of hydroxyl group at C-6 position. By comparing the spectral data, melting point and optical rotation of compound **5a** with earlier reported data[25], the metabolite was found as 6β-hydroxy-19-nortestosterone (**5a**).

The compound **5b** collected at R_t 11.8 min in the HPLC (Fig 3.12) purification, exhibited ($[M+Na]^+$) ion peak at *m/z* 313.1761 in the HRMS spectra corresponding to molecular formula $C_{18}H_{26}O_3$, representing introduction of a hydroxyl group in the substrate **5**. The compound exhibit absorbance at 239 nm in the UV spectrum and peaks at 3429, 1664 cm^{-1} in IR spectrum indicated presence of hydroxyl and conjugated carbonyl groups. The absence of any new signal in the range from δ 3.3 to 4.5 compare to substrate **5** in ^1H NMR spectrum revealed that hydroxylation occurred at methine carbon. The signal for C-18 methyl protons was unchanged, which ruled out the possibility of hydroxylation at C-14 methine carbon. In ^{13}C NMR new signal for quaternary carbon appeared at δ 70.9 and the signal at δ 43.8 disappeared. There was no change in the signal for C-7 and C-11 carbon but downfield shift of signal for C-1 carbon from δ 27.2 to 32.7 observed indicated that hydroxylation was occurred at C-10 methine carbon. By comparing the melting point (205-206 ºC) and optical rotation (+71 *c* = 1.02 in MeOH) of metabolite **5b** with the earlier report,[25] the compound was identified as 10β-hydroxy-19-nortestosterone

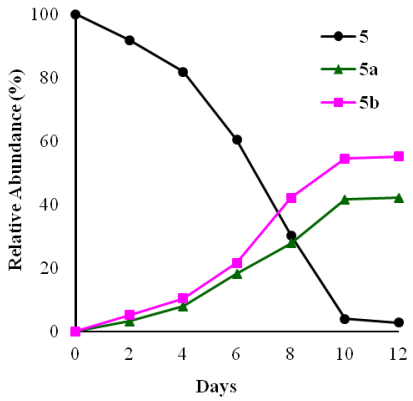


Figure 3.11: Time course study of 19-nortestosterone transformation: (▲) 19-nortestosterone (**5**), (●) 6β-hydroxy-19-nortestosterone (**5a**), (■) 10β-hydroxy-19-nortestosterone (**5b**)

(5b) (Scheme 3.5).

Time course experiment for the transformation of 19-nortestosterone (**5**) (Fig 3.11) explained that fungal strain *M881* transformed 19-nortestosterone (**5**) into **5a** and **5b**. By prolonging the incubation period to 12 days, the transformation of **5** was increased to 97% with proportionate increase in the level of **5a** and **5b** (Fig 3.12). However, formation of dihydroxy derivative was not observed as seen with **1** to **4**

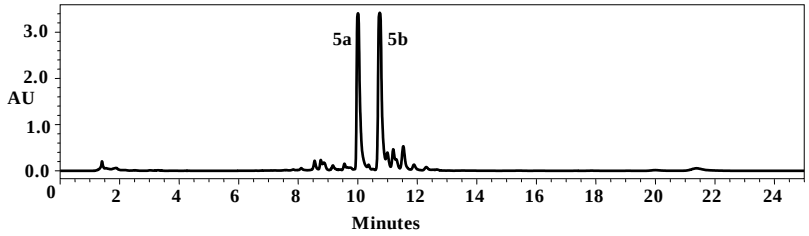


Figure 3.12: The HPLC analysis of 19-nortestosterone (**5**) transformation with *M881* after 10 d

3.2: Studies on large scale fermentation

Scale-up studies were carried out in shake flasks (600 mL media) containing progesterone (**1**) (0.7 g/L) or testosterone (**2**) (1.0 g/L) as substrate and as described in materials and methods (chapter 2). Each flask was inoculated with *M881* culture and incubated on a rotary shaker. On alternate days the aliquots were drawn aseptically from flask, extracted and analyzed by HPLC and LC-MS. These results revealed that, the fungal system quantitatively converted **1** and **2** into corresponding dihydroxy metabolites in 10 days of incubation. Similar results were obtained when fermentor was used with 5000 mL of CzapekDox media containing **1** (0.7 g/L) or **2**(1.0 g/L) (Fig. 3.13). Thus, the fungal system, *M881* can be used for the large-scale production of 6β,11α-dihydroxy derivatives of **1**, **2**, **3**, and **4** with fine tuning of the fermentation conditions**.**

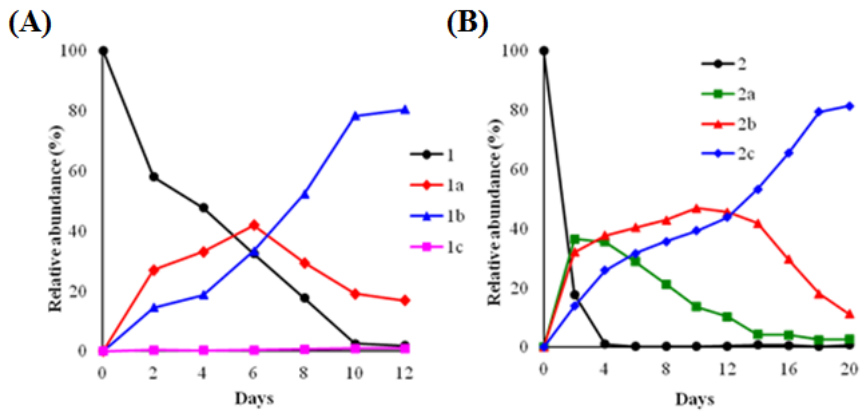


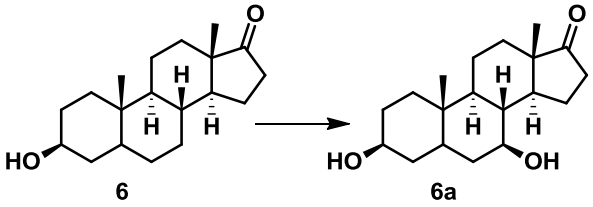
Figure 3.13: Time course study in fermentor **A:** Progesterone (**1**) transformation: (●) progesterone (**1**), (♦) 11α-hydroxyprogesterone (**1a**), (▲) 6β, 11α-dihydroxyprogesterone (**1b**), (■) 6β-hydroxy pregn-4-ene-3,11,20-trione (**1c**). **B:** Testosterone (**2**) transformation :**(●)** Testosterone (**2**), **(■)** 6β-hydroxytestosterone (**2a**), (▲) 11α-hydroxytestosterone (**2b**), **(♦)** 6β,11α-dihydroxytestosterone (**2c**).

3.3: Biotransformation of 3β-hydroxy steroids using *M881*

Anabolic steroids such as epiandrosterone (also called as trans-androsterone or isoandrosterone) and dehydroisoandrosterone (also known as trans-dehydroandrosterone or dehydroepiandrosterone) are collectively termed as 3β-hydroxy steroids. These steroids are synthesized in the different parts of body and involves in various metabolic processes. The production of dehydroepiandrosterone (**7**) in the brain of human and rat were reported[26,27] and considered as "neurosteroid".[28,29] The 7α-hydroxylation of 3β-hydroxysteroids by cytochrome P4507B1 in the human liver and brain is well studied.[30] The hydroxylated derivative of 3β-hydroxy steroids was shown to exert anti-glucocorticoid[31] and neuroprotective effects. Thus there is craving demand of a new method for the production of their hydroxyl derivatives on industrial scale. As fungal culture has the potential to produce the hydroxyl derivatives of steroids, we have screened wide range of fungal strains for the efficient hydroxylation of 3β-hydroxysteroids such as androsterone and dehydroisoandrosterone. Out of the screened cultures, the fungal strain *M881* was chosen for further study which efficiently transformed trans-androsterone and dehydroisoandrosterone. From the substrate concentration study of **6** and **7** in the range from 0.3 to 1.2 g/L of fermentation media, it was observed that, when substrate concentration was more than 1 g/L, high amount of unconsumed substrate left in the fermentation media after 6 days of incubation. This concentration was then used to study the transformation of **6** and **7** with *M881*.

3.3.1: Transformation of epiandrosterone (6)

For the large scale purification of metabolites obtained after transformation of epiandrostrone (**6**) with *M881*, fermentation was carried out in the batch of 30 flasks, as described in materials and methods (chapter 2) with 1 g/L substrate concentration. After 6 days of incubation, metabolites formed were extracted using ethyl acetate. The TLC analysis of crude extract obtained (3.71 g) showed only one major metabolite which was purified by column chromatography (230-400 mesh, 40 g) and elution with 2% of methanol in dichloromethane (1.86 g, R_f: 0.49, system II) (Scheme 3.6).



Scheme 3.6 Transformation of epiandrosterone (**6)** by *M881*

The HRMS spectrum of purified metabolite **6a** showed the $[M+Na]^+$ at *m/z* 329.2095, consistent with the addition of 16 mass units to the parent compound **6** in agreement with the molecular formula $C_{19}H_{30}O_3$, representing the introduction of a hydroxyl group in the parent compound. The IR spectrum showed characteristic absorption at 3455 and 1726 cm^{-1} for hydroxyl group and saturated ketone, respectively. In the ^1H NMR spectrum, one new signal was observed for oxygen bearing methine proton at δ 3.6 (1H, m) indicating the introduction of hydroxyl group at methylene carbon. The signals at δ 3.48 (1H, m), 0.89 (3H, s) and 0.87 (3H, s) were assigned to H-3α, H-18, H-19 protons. In the ^{13}C NMR spectrum of metabolite **6a,** chemical shifts were assigned by comparing the data with that of **6**. The appearance of signals for two methyl, eight methylene, six methine and three quaternary carbons indicated introduction of a hydroxyl group on methylene carbon. The signals at δ 221.4 and 70.9 indicated presence of 17-carbonyl and oxygen bearing C-3 carbons in the metabolite **6a**. The signals at δ 31.4, 24.9 and 20.7

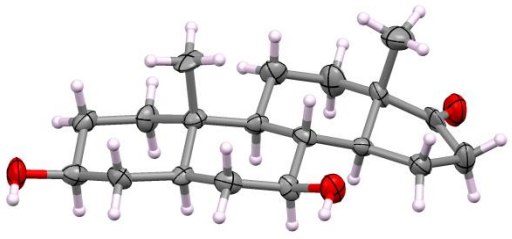


Figure 3.14. X-ray diffraction data of 7β-hydroxy-epiandrosterone (**6a**)

corresponding to C-2, C-15 and C-11 carbons, respectively remained unchanged which showed that hydroxylation was not occured in the ring A, C or D. The shift of signal for C-7 carbon from δ 31.5 to 74.8 and downfield shift of signal for C-8 carbon indicated the hydroxylation at C-7 position. The position and stereochemistry of newly incorporated hydroxyl group was confirmed from the X-ray diffraction analysis (Fig. 3.14) and the metabolite was identified as 7β-hydroxy-epiandrosterone (**6a**). The data was matched well with the earlier report.[32] Substrate concentration experiments revealed that *M881* could able transform epiandrosterone (**6**) into single product, **6a** in highly efficient manner at the concentration of 1.0 g/L.

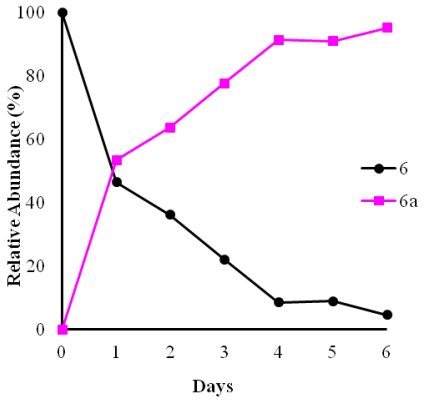


Figure 3.15: Time course study of epiandrosterone (**6**) transformation: (●) epiandrosterone (**6**), (■) 7β-hydroxy-epiandrosterone (**6a**)

The time course experiments with 1.0 g/L concentration revealed that after 4 days of incubation over 90% of epiandrosterone (**6**) was converted into 7β-hydroxy-epiandrosterone (**6a**) (Scheme 3.6, Fig. 3.15).

3.3.2: Transformation of dehydroepiandrosterone (7)

For the purification of metabolites, transformation of **7** (1 g/L) was carried out using 30 flasks inoculated with *M881* as described in materials and methods (chapter 2). The TLC and LC-MS analyses of crude extract (3.19 g) obtained after six days of incubation showed the presence of four metabolites. The metabolites were purified over silica gel column (230-400 mesh, 49 g) chromatography with gradient mixture of methanol in CH_2Cl_2 as mobile phase. Elution of the column with different % of methanol in CH_2Cl_2 (from 1% to 3%) yielded fractions containing pure compounds with R_f values 0.64, 0.63, 0.57, 0.56 (system: II), respectively. The identification of metabolites (Scheme 3.7) was done based on the spectral data analyses (HRMS, IR, ^1H and ^{13}C NMR).

UV spectrum of the compound with R_f 0.64 (**7a**) did not show any absorbance beyond 220 nm which indicated the absence of any chromophoric group. The IR spectrum exhibited absorption at 3308, 1727, 1668 cm^{-1} corrosponding to hydroxyl, saturated ketone and carbon - carbon double bond functional group. The HRMS spectrum showed molecular ion peak at *m/z* 327.1942 $[M+Na]^+$ corresponding to molecular formula $C_{19}H_{28}O_3$, indicating introduction of a hydroxyl group on the substrate **7**. In the ^1H NMR spectrum of **7a** the appearance of new multiplet at δ 3.88 (1H) confirmed the hydroxylation at methylene carbon. A multiplet at δ 3.44 indicated the presence of 3β-hydroxy group as in the substrate and the singlets at δ 0.9 and δ 1.1 were corresponded to C-19 and C-18 tertiary methyl group, respectively. The ^{13}C NMR spectra revealed the presence of two methyl, seven methylene, six methine and four quaternary carbons indicating that the new hydroxylation was occurred on methylene carbon. The signal for 17-carbonyl carbon at 224.5 was present as in the substrate revealed that carbonyl group was intact in the metabolite. On comparing with the substrate spectral data, methylene signals at δ 36.9 and 32.5 were assigned to C-16 and C-12 carbons, whereas C-1 and C-2 carbons resonated at δ 38.1 and 32.5. Signals at δ 25.2 and 21.5 was unchanged showed that hydroxylation has not occurred in ring C and D of the parent compound. The shift of signal from δ 31.5 for C-7 carbon in substrate to 73.3 and downfield shift of signal for C-8 carbon indicated that hydroxylation was occurred at C-7 position. The stereochemistry of the newly introduced hydroxyl group was deduced by comparing the spectral data, melting point (213-214 ˚C) and optical rotation (+59, *c* = 1.3 in $CHCl_3$) of metabolite **7a** with the reported data[33] and the metabolite was identified as 7β-hydroxy-dehydroepiandrosterone (**7a**).

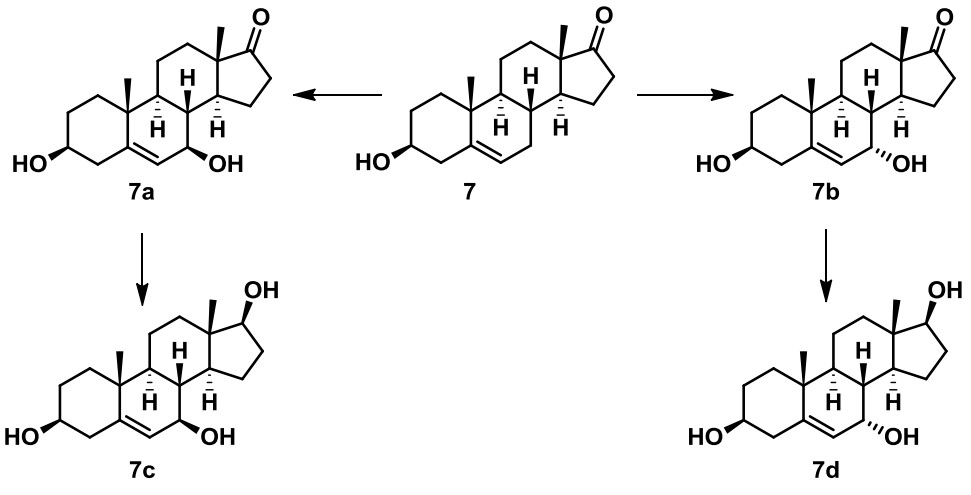
The HRMS of the compound with R_f 0.63 (**7b**) displayed the molecular ion at *m/z* 327.1931 $[M+Na]^+$ corresponding to molecular formula $C_{19}H_{28}O_3$ indicating the introduction of a hydroxyl group to the parent steroid **7**. The IR spectrum showed three absorption band at 3385, 1721, 1600 cm^{-1} corresponding to the presence of –OH, -C=O, -C=C- functional groups in the isolated metabolite. The ^1H NMR spectrum of metabolite was distinctly similar to that of substrate except one new multiplet at δ 3.91 corresponding to one proton indicating a hydroxylation at methylene carbon. It showed a multiplet at δ 3.48 and two singlets at δ 0.89 and 1.03 representing no change in other functional groups during transformation. The ^{13}C NMR spectra of **7b** constituted signals for two methyl, seven methylene, six methine

and four quaternary carbons confirming the new hydroxylation on methylene carbon. The signals for two methyl groups appeared at δ 18.7 (C-19) and 13.7 (C-18) whereas signals at δ 22.7 for C-15 and δ 21.2 for C-11 eliminated the possibility for hydroxylation in ring C and D. No significant change in δ values for C-1, C-2 carbons (δ 38.0 and 32.4 respectively) was observed. The downfield shift of signal at δ 31.5 for C-7 carbon to 64.8 confirmed the position of newly introduced hydroxyl group at C-7. The optical rotation of metabolite in chloroform was -63 (*c* = 1.7) which established the presence of 7α-OH functional group. The spectral data, melting point (182-183 ºC) and optical rotation of metabolites was in good agreement with the reported data[33] and the metabolites was identified as 7α-hydroxy-dehydroepiandrosterone (**7b**).

IR spectrum of the compound with R_f 0.57 (**7c**), exhibited absorption at only 3289 cm^{-1}. The absence of signal in the region from 1700 cm^{-1} to 1750 cm^{-1} indicated reduction of C-17 carbonyl group in the parent compound. The HRMS data of metabolite (**7c**) exhibited the molecular ion peak at 329.2084 $[M+Na]^+$ corresponding to the molecular formula $C_{19}H_{30}O_3$. It showed increase of 18 mass units indicating reduction of one double bond with simultaneous hydroxylation of the substrate **7**. In the ^1H NMR spectrum, three downfield signals were observed for the oxygen bearing methine protons at δ 3.42 (1H, m), 3.56 (1H, t, J=8.32) and 3.73 (1H, m). The multiplet at δ 3.42 and two singlets at δ 0.75 and 1.08 indicated presences 3β-hydroxy and two tertiary methyl groups, respectively. The triplet at δ 3.56 is typical for methine proton with 17β-OH functional group indicated reduction of 17-carbonyl group. The multiplet at δ 3.73 showed introduction of one hydroxyl group into parent compound. The ^{13}C NMR spectrum of metabolite **7c** showed two new methine signals at δ 82.3 and 74.0 and the characteristic signal for the 17-carbonyl group was disappeared which confirmed that 17-carbonyl group was reduced. By comparing with reported data the signal at δ 82.3 was assigned to the C-17 carbon. The signals at δ 72.1, 19.5 and 11.6 in the spectra were assigned for C-3, C-19 and C-18 methyl groups as in the substrate which ruled out the possibility of hydroxylation in ring A. The presence of signals at δ 21.9 and 26.6 characteristic for C-11 and C-15 carbons indicated hydroxylation has not occurred in ring C and D. Downfield shift of signal for C-7 and C-8 carbons from δ 31.5 and 36.23 in parent compound to δ 74.0 and 41.2 respectively confirmed the hydroxylation at C-7 position. From the optical rotation of compound in chloroform which was +51 (*c* =

0.71) and melting point 231-233 ºC stereochemistry of compound was confirmed and the metabolite was identified as 3β,7β,17β-trihydroxyandrost-5-ene (**7c**) which matched well with the reported data.[34]

The molecular formula of metabolite eluted in the last with R_f 0.56 (**7d**) was deduced as $C_{19}H_{30}O_3$ based on the molecular ion peak at *m/z* 329.3089 $[M+Na]^+$ in its HRMS spectrum which showed increase in the mass by 18 units compared to the substrate. The IR spectrum of metabolite **7d** showed the presence of hydroxyl group and double bond. In the UV spectrum no absorption was observed indicating absence of chromophoric group in the compound **7d**. In the ^{1}H NMR spectrum of compound two new signal were appeared for oxygen bearing methine proton at δ 3.60 (1H, t), 3.76 (1H, m) along with signals at δ 3.47 (1H, m), 1.01 (3H) and 0.75 (3H, s) which were assigned for 3α-H and two methyl groups (H-18, H-19) respectively. The triplet at δ 3.60 with coupling constant 8.4 Hz corresponded to a proton at C-17 with β-OH functional group indicating reduction of 17-carbonyl group. The multiplet at δ 3.76 revealed the introduction of one new hydroxyl group in the substrate. In the ^{13}C NMR spectrum of the compound, signal at δ 224.0 disappeared when compared to substrate similar to metabolite **7c** indicated reduction of 17-carbonyl group. The spectrum showed signals for two methyl, seven methylene, seven methine, three quaternary carbons and three signals for oxygen bearing methine carbons. The signal at δ 82.5 and 72.0 were assigned to C-17 and C-3 carbons respectively. Decrease in the number of methylene carbon relative to substrate indicated that the new hydroxylation occurred on methylene carbon. Signals for C-3, C-11, C-15, C-18 and C-19 (δ 72.0, δ 21.5, δ 24.2, δ 11.4 and δ 18.7 respectively) was unchanged compared to parent compound **7** which excluded the



Scheme 3.7: Transformation of dehydroepiandrosterone (**7**) by *M881*

option of hydroxylation in A, C and D rings. Thus the newly arrived signal at δ 65.4 was assigned to C-7 carbon indicating that the hydroxylation occurred at C-7 position. By considering the melting point (263.-265˚C) and optical rotation (-37, *c* = 0.35, $CHCl_3$) of compound **7d**, the metabolite was identified as 3β,7α,17β-trihydroxyandrost-5-ene (**7d**) and the data was in well agreement with the reported data[34].

3.4: Transformation of steroid by immobilized mycelia of *M881*

Entrapment of cells or pure enzyme in different agent is a technique well established to overcome few limitations of biotransformation using suspended culture or pure enzyme. Agents such as polyacrylamide, polyurethane and calcium alginate gels have been employed for this technique. The biotransformation using immobilized viable cells has few advantages over mycelia suspended in the fermentation media which include the easy separation and reuse of the cells, high cell concentrations, flexibility in reactor design and operation as well as the stabilization of several cell functions.

The cells of *M881* were trapped by dripping a suspension of fungal spores in aqueous sodium alginate into a stirred solution of chilled calcium chloride. The monovalent sodium ions were replaced by the divalent calcium ion, which cross-links the polysaccharide, resulting in spontaneous polymerization to form spherical. Progesterone was used as a representative substrate to study the effect of immobilization of *M881* cell in calcium alginate on the transformation. The time dependent variation in the progesterone transformation by the cells suspended and entrapped in calcium alginate was investigated.

3.4.1: Transformation of progesterone (1) with immobilized mycelia of *M881*

30 mg of progesterone (**1**) was added to 50 mL of 2% solution of glucose in water containing 12 gm of mycelial beads immobilized in calcium alginate pellets as described in materials and methods (chapter 2) and incubated for six days. After six days of incubation, metabolites formed were extracted and analyzed by TLC and

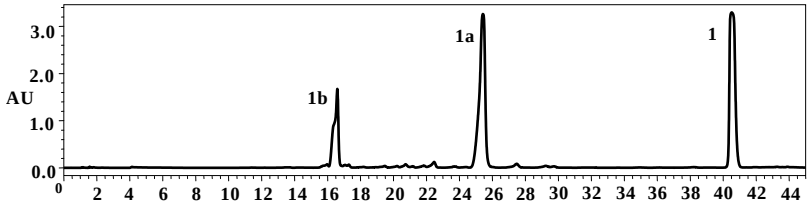
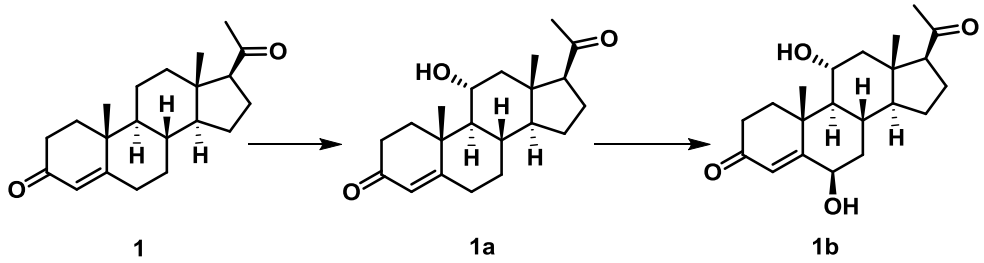


Figure 3.16: Transformation of progesterone with immobilized cells of *M881*

HPLC. The HPLC analysis showed conversion of progesterone (**1**) into two metabolites 11α-hydroxyprogestrone (**1a**) and 6β,11α-dihydroxyprogestrone (**1b**) (Scheme 3.8). After six days of incubation 70% conversion of progesterone (**1**) into hydroxylated metabolites was observed (Fig. 3.16).

Time course experiment carried out using **1** as substrate with the immobilized mycelia revealed that, after 12 days of incubation, 11α-hydroxyprogestrone (**1a**) was major a metabolite, with only 5% unconsumed progesterone **1** left out in the fermentation flask (Fig. 3.16). With immobilized mycelia, the percentation of dihydroxy derivative **1b** has increased only upto 30% even after increase in the incubation period to 12 days, which was found to be different than the transformation with suspended mycelia, where **1b** obtained as a major metabolite.



1 **1a** **1b**

Scheme 3.8: Transformation of progesterone (**1**) by *M881* immobilized in calcium alginate

The study carried out to check the stability of immobilized beads at 4 °C, as discused in the materials and methods (Chapter 2) indicated that there was no change in the transformation activity of immobilized beads even after one month storage at 4 °C.

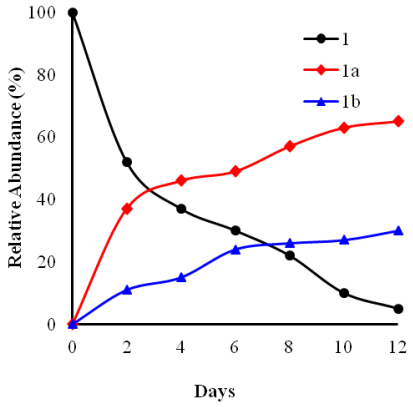


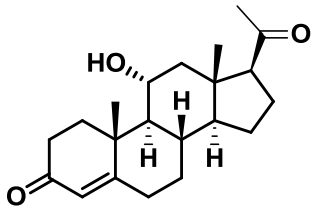
Figure 3.17: Time course study of progesterone (**1**) transformation using calcium alginate immobilized cells of *M881*: (●) progesterone (**1**), (♦) 11α-hydroxyprogesterone (**1a**), (▲) 6β, 11α-dihydroxyprogesterone (**1b**)

3.5: Summary and Conclusion

In Conclusion, the versatile fungal strain *Mucor* sp. (*M881*) could able to carry out the regio- and stereo- specific hydroxylation at 6β,11α-positions in steroids with 4-ene-3-one functionality. *M881* efficiently carried out hydroxylation at C-6β and C-11α position on progesterone (**1**), testosterone (**2**), 17α-methyltestosterone (**3**) and 4-androstene-3,17-dione (**4**) to form corresponding 6β,11α-dihydroxy derivatives **1b, 2c, 3c** and **4c** as a major metabolites. However the fungal system failed to carryout hydroxylation at 11α position when 19-nortestosterone (**5**) was used as a substrate indicates that the methyl group at C10 position is necessary for the 11α-hydroxylation by *M881*. The large scale fermentation studies in 5 L fermentor with progesterone (**1**) and testosterone (**2**) clearly indicates that this fungal system can be used for large scale asymmetric synthesis of 6β,11α-dihydroxy derivatives of **1**, **2**, **3** and **4**. The fungal strain, *M881* efficiently hydroxylate epiandrosterone (**6**) one major metabolite 7β-hydroxy-epiandrosterone (**6a**), whereas dehydroepiandrosterone (**7**) was hydroxylated at both 7α/β positions. The transformation of progesterone (**1**) with immobilized *M881* mycelia revealed that the immobilized culture can be used instead of suspended cells for hydroxylation of steroids

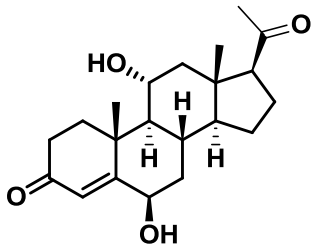
3.6: Spectral data

11α-hydroxyprogesterone (1a)



Colorless crystals (crystallized from chloroform/methanol), mp: 165 ºC, $[\alpha]_D^{22}$: +177.86 (c=1.8, MeOH), λ_{max} (MeOH): 245 nm; IR ν_{max} (cm^{-1}): 3433 (OH), 1701 (CO), 1658 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.69 (3H, s, H-18), 1.35 (3H, s, H-19), 2.14 (3H, s, H-21), 3.96 (1H, dt, J =4.6, 10.7 Hz, H-11β), 5.72 (1H, s, H-4); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): δ 211.6 (C-20), 203.0 (C-3), 175.3 (C-5), 124.7 (C-4), 69.3 (C-11), 64.3 (C-17), 60.0 (C-10), 56.5 (C-14), 50.9 (C-12), 45.2 (C-13), 41.4 (C-9), 38.6 (C-1), 36.2 (C-8), 34.9 (C-2), 34.7 (C-6), 33.0 (C-7), 31.4 (C-21), 25.2 (C-15), 23.8 (C-16), 18.6 (C-19), 14.7 (C-18); HRMS: *m/z* 353.2065 $[M+Na]^+$ [calcd-*m/z* 353.2093].

6β,11α-dihydroxyprogesterone (1b)

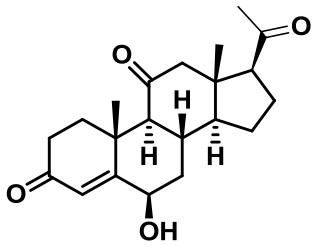


Colorless crystals (crystallized from ethyl acetate); m.p: 231-232 ºC, $[\alpha]_D^{22}$: +148 (c=0.35 MeOH), λ_{max} (MeOH) : 245 nm; IR ν_{max} (cm^{-1}): 3444 (OH), 1703 (CO), 1651 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.67 (3H, s, H-18), 1.46 (3H, s, H-19), 2.10 (3H, s, H-21), 3.97 (1H, dt, J =4.6, 10.7 Hz, H-11β), 4.22 (1H, t, H-6α), 5.74 (1H, s, H-4); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): δ 211.7 (C-20), 203.6 (C-3), 171.8 (C-5), 127.2 (C-4), 73.8 (C-6), 69.3 (C-11), 64.3 (C-17), 60.0 (C-10), 56.4 (C-14), 50.8 (C-12), 45.3 (C-13), 40.6 (C-7), 40.3 (C-9), 38.9 (C-1), 35.2 (C-2), 31.4 (C-21), 29.7 (C-8), 25.2 (C-15), 23.8 (C-16), 20.3 (C-19), 14.7 (C-18); HRMS: *m/z* 369.2038 $[M+Na]^+$ [calcd- *m/z* 369.2042].

X-ray Crystal data of 1**b.** CCDC reference number = CCDC 922747 $C_{21}H_{30}O_4$, M=346.45, colorless block crystal, 0.23 x 0.18 x 0.13 mm^3, monoclinic, space group $P2_1$, a =7.5635(15), b=11.522(2), c=10.837(2)Å, β =96.781(3)°, V = 937.9(3) $Å^3$, Z = 2, T = 297(2) K, $2\theta_{max}$=52.00°, D_{calc} (g cm^{-3}) = 1.227, $F(000)$ =

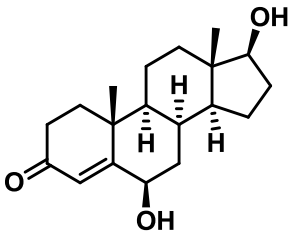
376, μ (mm^{-1}) = 0.083, 5883 reflections collected, 1939 unique reflections (R_{int}=0.0194), 1895 observed ($I > 2\sigma(I)$) reflections, multi-scan absorption correction, T_{min} = 0.9713, T_{max} = 0.9897, 237 refined parameters, S = 1.17, $R1$=0.0430, $wR2$=0.1074 (all data R = 0.0437, $wR2$ = 0.1080), maximum and minimum residual electron densities; $\Delta\rho_{max}$ = 0.173, $\Delta\rho_{min}$= -0.190 ($eÅ^{-3}$).

6β-hydroxypregn-4-ene-3,11,20-trione (1c)



Colorless crystals (crystallized from chloroform/methanol), mp: 204 ºC, $[\alpha]_D^{22}$: +107.55 (c=1.8, MeOH), λ_{max} (MeOH): 245 nm; IR ν_{max} (cm^{-1}): 3446 (OH), 1703 (CO), 1651 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.64 (3H, s, H-18), 1.58 (3H, s, H-19), 2.11 (3H, s, H-21), 4.28 (1H, t, H-6α), 5.79 (1H, s, H-4); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): δ 211.0 (C-20), 210.8 (C-11), 203.6 (C-3), 171.8(C-5), 127.2 (C-4), 73.8 (C-6), 63.2 (C-17), 62.8 (C- 10), 57.3 (C-12), 55.1 (C-14), 47.9 (C-13), 40.0 (C-7), 39.1 (C-9), 37.1 (C-1), 34.8 (C-2), 32.3 (C-21), 31.3 (C-8), 24.9 (C-15), 24.4 (C-16), 19.3 (C-19), 14.7 (C-18); HRMS: *m/z* 367.1885 $[M+Na]^+$ [calcd- *m/z* 367.1886].

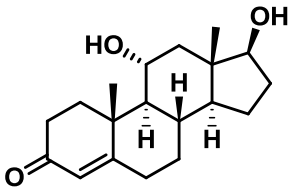
6β-hydroxytestosterone (2a)



Colorless crystals (crystallized in chloroform), mp: 213-214 ºC, $[\alpha]_D^{22}$: +24.52 (c= 0.68 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3429 (OH), 1664 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si) δ 0.81 (3H, s, H-18), 1.39 (3H, s, H-19), 4.26 (1H, t, H-6α), 5.78 (1H, s, H-4), 3.59 (1H, t, H-17α); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): δ 203.2 (C-3), 171.6 (C-5), 126.6 (C-4), 81.3 (C-17), 73.6 (C-6), 55.3 (C-9), 51.7 (C-14), 44.0 (C-13), 39.4 (C-12), 39.3 (C-10), 38.3 (C-7), 37.7 (C-1), 35.0 (C-

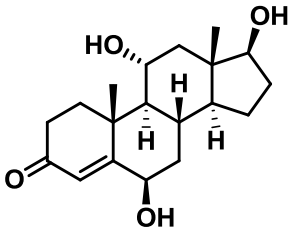
2), 31.1 (C-16), 30.6 (C-8), 24.2 (C-15), 21.7 (C-11), 19.7 (C-19), 11.6 (C-18); HRMS: *m/z* 327.1932 $[M+Na]^+$ [calcd- *m/z* 327.1936].

11α-hydroxytestosterone (2b)



Colorless crystals (crystallized from chloroform) mp: 177-178 ºC, $[\alpha]_D^{22}$: +86.68 (c= 0.62 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3406 (OH), 1658 (conj. CO); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.81 (3H, s, H-18), 1.36 (3H, s, H-19), 3.99 (1H, dt, J = 4.6, 10.7 Hz, H-11β), 3.62 (1H, t, H-17α), 5.71 (1H, s, H-4); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): δ 203.0 (C-3), 175.6 (C-5), 124.6 (C-4), 81.3 (C-17), 69.4 (C-11), 60.3 (C-10), 51.1 (C-12), 49.1 (C-14), 44.6 (C-13), 41.5 (C-9), 38.6 (C-1), 36.6 (C-8), 34.9 (C-2), 34.8 (C-6), 32.6 (C-7), 30.7 (C-16), 24.1 (C-15), 18.7 (C-19), 12.7 (C-18); HRMS: *m/z* 305.2106 $[M+H]^+$ [calcd- *m/z* 305.2116].

6β, 11α-dihydroxytestosterone (2c)

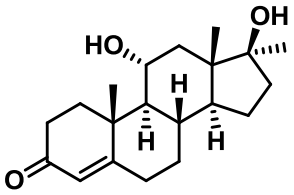


Colorless crystals (crystallized from chloroform); mp: 225-226 ºC, $[\alpha]_D^{22}$: +17.64 (c= 0.82 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3400 (OH), 1658 (conj. CO); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.83 (3H, s, H-18), 1.51 (3H, s, H-19), 4.00 (1H, dt, J = 4.6, 10.7 Hz, H-11β), 4.25 (1H, t, H-6α), 5.77 (1H, s, H-4); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): δ 203.6 (C-3), 171.9 (C-5), 127.2 (C-4), 81.9 (C-17), 73.8 (C-6), 69.4 (C-11), 60.3 (C-10), 51.0 (C-12), 49.0 (C-14), 44.7 (C-13), 40.7 (C-9), 40.3 (C-7), 38.5 (C-1), 35.2 (C-2), 30.2 (C-8), 30.0 (C-16), 24.1 (C-15), 20.4 (C-19), 12.7 (C-18); HRMS: *m/z* 343.1857 $[M+Na]^+$ [calcd- *m/z* 343.1886].

X-ray Crystal data of **2c.** CCDC reference number = CCDC 922257 $C_{19}H_{28}O_4$, 1.83(H_2O), M= 353.44, colorless plate, 0.29 x 0.15 x 0.11 mm^3, orthorhombic, space group $C222_1$, a = 13.996(3), b= 22.787(3), c= 36.436(6) Å, V = 11621(3) $Å^3$, Z = 24, T = 297(2) K, $2\theta_{max}$=50.00°, D_{calc} (g cm^{-3}) = 1.212, $F(000)$ =

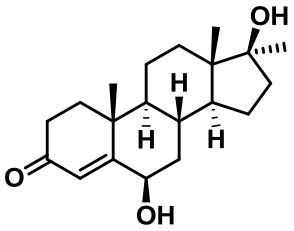
4616, μ (mm^{-1}) = 0.088, 56665 reflections collected, 10238 unique reflections (R_{int}=0.0670), 7630 observed ($I > 2\sigma(I)$) reflections multi-scan absorption correction, T_{min} = 0.9745, T_{max} = 0.9905, 727 refined parameters, S = 1.107, $R1$=0.0682, $wR2$=0.1475 (all data R = 0.0949, $wR2$ = 0.1596, maximum and minimum residual electron densities; $\Delta\rho_{max}$ = 0.331, $\Delta\rho_{min}$= -0.170 ($eÅ^{-3}$).

11α-hydroxy-17α-methyltestosterone (3a)



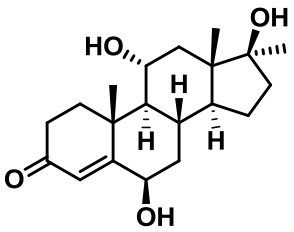
Colorless crystals (crystallized from CH_2Cl_2/Methanol), mp: 151-152 ºC, $[\alpha]_D^{22}$: +61.12 ($c_=$1.37 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3412 (OH), 1653 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.92 (3H, s, H-18), 1.36 (3H, s, H-19), 1.21 (3H, s, 17α-CH_3) 4.01 (1H, dt, J =4.6, 10.7 Hz, H-11β), 5.71 (1H, s, H-4); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 203.0 (C-3), 175.6 (C-5), 124.6 (C-4), 81.7 (C-17), 69.8 (C-11), 60.2 (C-9), 50.9 (C-14), 47.3 (C-10), 44.3 (C-12), 41.5 (C-13), 39.2 (C-16), 38.6 (C-1), 37.5 (C-8), 34.9 (C-2), 34.8 (C-6), 32.8 (C-7), 26.1 (C-20), 24.2 (C-15), 18.7 (C-19), 15.7 (C-18); HRMS: *m/z* 341.2087 $[M+Na]^+$ [calcd-*m/z* 341.2093]

6β-hydroxy-17α-methyltestosterone (3b)



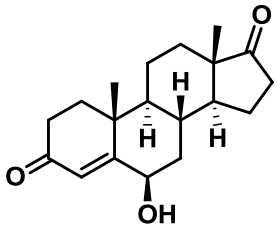
Colorless crystals (crystallized from CH_2Cl_2/methanol), mp: 246-247 ºC, $[\alpha]_D^{22}$: +5.09 ($c_=$ 1.30 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3425 (OH), 1653 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.92 (3H, s, H-18), 1.40 (3H, s, H-19), 1.19 (3H, s, 17α-CH_3), 4.26 (1H, t, H-6α), 5.78 (1H, s, H-4); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 203.2 (C-3), 171.6 (C-5), 126.7 (C-4), 82.2 (C-17), 73.6 (C-6), 55.2 (C-9), 51.5 (C-14), 46.8 (C-13), 39.5 (C-16), 39.4 (C-10), 39.2 (C-7), 38.7 (C-1), 35.1 (C-2), 32.7 (C-12), 31.9 (C-8), 26.0 (C-20), 24.2 (C-15), 21.8 (C-11), 19.7 (C-19), 14.6 (C-18); HRMS: *m/z* 341.208 $[M+Na]^+$ [calcd- *m/z* 341.2093].

6β,11α-dihydroxy-17α-methyltestosterone (3c)



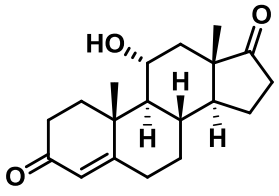
Colorless crystals (crystallized from chloroform methanol); mp: 264-265 ºC, $[\alpha]_D^{22}$: + 6.36 (c=1.71 in MeOH), λ_{max} (MeOH) 239 nm; IR ν_{max} (cm^{-1}): 3423 (OH),1666 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.94 (3H, s, H-18), 1.52 (3H, s, H-19), 1.22 (3H, s, 17α-CH_3), 4.04 (1H, dt, J =4.6, 10.7 Hz, H-11β), 4.25 (1H, t, H-6α), 5.78 (1H, s, H-4); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 203.7 (C-3), 172.0 (C-5), 127.5 (C-4), 81.7 (C-17), 73.8 (C-6), 69.8 (C-11), 60.3 (C-10), 50.8 (C-14), 47.5 (C-10), 44.3 (C-12), 40.8 (C-13), 40.3 (C-7), 39.2 (C-16),38.7 (C-1), 35.2 (C-2), 30.8 (C-8), 26.1 (C-20), 24.1 (C-15), 20.4 (C-19), 15.7 (C-18); HRMS: *m/z* 357.2063 $[M+Na]^+$ [calcd- *m/z* 357.2042].

6β-hydroxy-4-androstene-3,17-dione (4a)



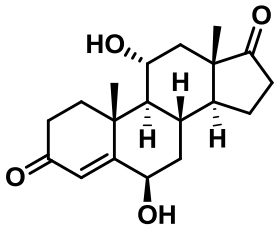
Colorless crystals (crystallized from CH_2Cl_2 / methanol); mp.: 190-192 ºC, $[\alpha]_D^{22}$: + 112 (c= 0.8 in MeOH), λ_{max}(MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3409 (OH), 1727 (CO), 1657 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.97 (3H, s, H-18), 1.44 (3H, s, H-19), 4.34 (1H, t, H-6α), 5.83 (1H, s, H-4); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 223.5 (C-17), 203.0 (C-3), 171.1 (C-5), 126.9 (C-4), 73.4 (C-6), 55.1 (C-9), 51.1 (C-14), 49.1 (C-13), 39.4 (C-10), 38.5 (C-16), 38.3 (C-1), 36.6 (C-7), 35.0 (C-2),32.5 (C-12), 30.7 (C-8), 22.7 (C-15), 21.3 (C-11), 19.7 (C-19), 14.1 (C-18); HRMS: *m/z* 303.1945 $[M+H]^+$ [calcd- *m/z* 303.1960].

11α-hydroxy-4-androstene-3,17-dione (4b)



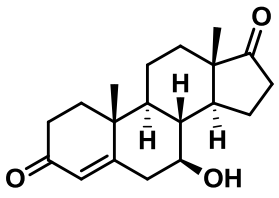
Colorless crystals (crystallized from CH_2Cl_2 / methanol); mp: 215 ºC, $[\alpha]_D^{22}$: + 152 (c= 0.71 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3412 (OH), 1653 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.95 (3H, s, H-18), 1.38 (3H, s, H-19), 4.01 (1H, dt, J =4.6, 10.7 Hz, H-11β), 5.73 (1H, s, H-4); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 221.8 (C-17), 203.0 (C-3), 175.1 (C-5), 124.8 (C-4), 69.1 (C-11), 60.2 (C-9), 51.3 (C-14), 49.1 (C-13), 43.5 (C-12), 41.5 (C-10), 38.5 (C-16), 36.6 (C-1), 35.7 (C-8), 34.9 (C-2), 34.6 (C-6), 31.7 (C-7), 22.6 (C-15), 18.7 (C-19), 14.9 (C-18); HRMS: *m/z* 325.1779 $[M+Na]^+$ [calcd- *m/z* 325.1780].

6β,11α-dihydroxy-4-androstene-3,17-dione (4c)



Colorless crystals (crystallized from CH_2Cl_2 / methanol); mp: 260-262 ºC, $[\alpha]_D^{22}$: + 70 (c= 1.1 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3383 (OH), 1740 (CO), 1668 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.97 (3H, s, H-18), 1.53 (3H, s, H-19), 4.05 (1H, dt, J =4.6, 10.7 Hz, H-11β), 4.31 (1H, t, H-6α), 5.81 (1H, s, H-4); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 222.1 (C-17), 203.7 (C-3), 171.6 (C-5), 127.3 (C-4), 73.6 (C-6), 69.1 (C-11), 60.2 (C-9), 51.1 (C-14), 49.0 (C-13), 43.4 (C-12), 40.7 (C-10), 40.2 (C-7), 37.5 (C-16), 36.7 (C-1), 35.2 (C-2), 29.4 (C-8), 22.6 (C-15), 20.4 (C-19), 14.9 (C-18); HRMS: *m/z* 341.171 $[M+Na]^+$ [calcd- *m/z* 341.1729].

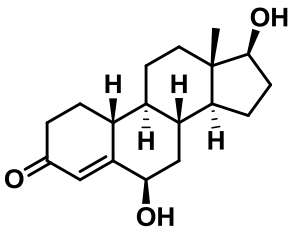
7β-hydroxy-4-androstene-3,17-dione (4d)



Colorless crystals (crystallized from CH_2Cl_2 /methanol); mp: 215-217 ºC, $[\alpha]_D^{22}$: + 129 (c= 1.1 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3417 (OH), 1736 (CO), 1642 (conj. CO); ^{1}H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.94 (3H, s, H-18), 1.27 (3H, s, H-19), 3.45 (1H, dt, H-7α), 5.75 (1H, s, H-4); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 223.5 (C-17), 202.1 (C-3), 171.2 (C-5), 125.0 (C-4), 75.1 (C-7), 52.1 (C-9), 51.8 (C-14), 49.1 (C-13), 43.8 (C-8), 43.5 (C-6), 39.4 (C-10), 36.8 (C-

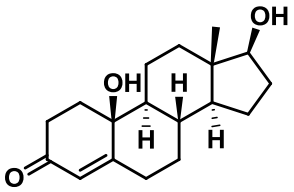
16), 36.6 (C-1), 34.7 (C-2), 32.4 (C-12), 25.9 (C-15), 21.4 (C-11), 17.6 (C-19), 14.3 (C-18); HRMS: *m/z* 325.18 $[M+Na]^+$ [calcd- *m/z* 325.1778].

6β-hydroxy-19-nortestosterone (5a)



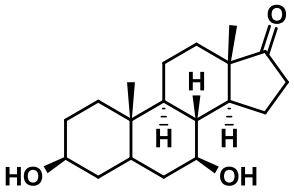
Colorless crystals (crystallized from CH_2Cl_2/methanol), mp: 206-211 ºC, $[\alpha]_D^{22}$: -65.16 (c= 0.92 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3429 (OH), 1664 (conj. CO); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.82 (3H, s, H-18), 4.29 (1H, t, H-6α), 5.86 (1H, d, H-4 J=1.83Hz), 3.59 (1H, t, H-17α), ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 203.3 (C-3), 168.8 (C-5), 125.6 (C-4), 82.3 (C-17), 72.5 (C-6), 50.9 (C-9), 50.9 (C-14), 44.4 (C-13), 39.5 (C-10), 39.1 (C-7), 37.7 (C-12), 37.1 (C-2), 27.2 (C-1), 34.9 (C-8), 30.6 (C-16), 24.0 (C-15), 27.2 (C-11), 11.6 (C-18); HRMS: *m/z* 313.1778 $[M+Na]^+$ [calcd- *m/z* 313.1780].

10β-hydroxy-19-nortestosterone (5b)



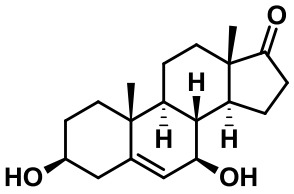
Colorless crystals (crystallized from CH_2Cl_2/methanol), mp: 205-206 ºC, $[\alpha]_D^{22}$: +71.05 ($c_=$ 1.02 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3429 (OH), 1664 (conj. CO); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.80 (3H, s, H-18), 5.73 (1H, d, H-4 J=1.83Hz), 3.58 (1H, t, H-17α); ^{13}C NMR (100 MHz; CD_3OD; Me_4Si): δ 202.3 (C-3), 168.7 (C-5), 124.7 (C-4), 82.3 (C-17), 70.9 (C-10), 54.5 (C-9), 51.4 (C-14), 44.0 (C-13), 37.5 (C-12), 36.5 (C-8), 34.5 (C-6), 34.5 (C-7), 33.2 (C-2), 32.7 (C-1), 30.6 (C-16), 24.3 (C-15), 21.2 (C-11), 11.5 (C-18); HRMS: *m/z* 313.1761 $[M+Na]^+$ [calcd- *m/z* 313.1780].

7β-hydroxy-epiandrosterone (6a)



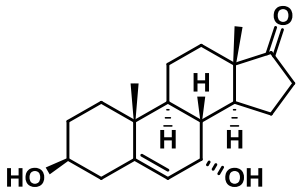
Colorless crystals (crystallized from $CHCl_3$/methanol), mp: 239-241 ºC, $[\alpha]_D^{22}$: +45 ($c_=$ 1.7 in $CHCl_3$), λ_{max} (MeOH): 210 nm; IR ν_{max} (cm^{-1}): 3455 (OH), 1726 (CO); ^1H NMR (400 MHz, $CDCl_3$, Me_4Si): δ 0.87 (3H, s, H-19), 0.89 (3H, s, H-18), 3.48 (1H, m, H-3β), 3.60 (1H, m, H-7β); ^{13}C NMR (100 MHz, $CDCl_3$, Me_4Si): δ 221.4 (C-17), 74.8 (C-7), 70.9 (C-3), 52.5 (C-14), 51.0 (C-9), 48.3 (C-13), 42.9 and 42.0 (C-5 and C-8), 38.8 (C-16), 37.6 (C-1), 36.8 and 36.0 (C-4 and C-6), 35.1 (C-10), 31.5 and 31.4 (C-12 and C-2), 24.9 (C-15), 20.7 (C-11), 14.0 (C-19), 12.4 (C-18); HRMS: *m/z* 329.2095 $[M+Na]^+$ [calcd- *m/z* 329.2093].

7β-hydroxy-dehydroepiandrosterone (7a)



Colorless crystals (crystallized from $CHCl_3$/methanol), mp: 213-214 ºC, $[\alpha]_D^{22}$: +59 ($c_=$ 1.3 in $CHCl_3$), λ_{max} (MeOH): 210 nm; IR ν_{max} (cm^{-1}): 3308 (OH), 1727 (C=O), 1668 (C=C); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.90 (3H, s, H-19), 1.10 (3H, s, H-18), 3.44 (1H, m, H-3β), 3.88 (1H, m, H-7α), 5.29 (1H, s, H-6); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): 224.2 (C-17), 144.2 (C-5), 127.2 (C-6), 73.3 (C-7), 72.0 (C-3), 52.6 (C-14), 49.9 (C-9), 49.0 (C-13), 42.5 (C-4), 40.8 (C-8), 38.1 (C-1), 37.8 (C-10), 36.9 (C-16), 32.5 (C-12), 32.2 (C-2), 25.2 (C-15), 21.5 (C-11), 19.5 (C-19), 14.0 (C-18); HRMS: *m/z* 327.1942 $[M+Na]^+$ [calcd- *m/z* 327.1936].

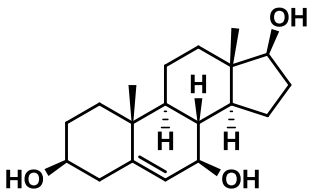
7α-hydroxy-dehydroepiandrosterone (7b)



Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 182-183 ºC, $[\alpha]_D^{22}$: -63 ($c_=$ 1.7 in $CHCl_3$), λ_{max} (MeOH): 210 nm; IR ν_{max} (cm^{-1}): 3385 (OH), 1721 (C=O), 1659 (C=C); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.89 (3H, s, H-19), 1.03 (3H, s, H-18), 3.48 (1H, m, H-3β), 3.91 (1H, m, H-7β), 5.58 (1H, d, J=4.86 Hz , H-6); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): 223.8 (C-17), 146.8 (C-5), 124.6 (C-6), 71.9 (C-3), 64.8 (C-7), 48.4 (C-13), 45.3 (C-14), 43.7 (C-9), 42.9 (C-4), 38.6 (C-8 and C-

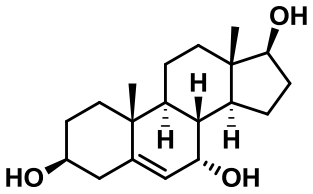
10), 38.0 (C-1), 36.6 (C-16), 32.4 (C-12), 32.1 (C-2), 22.7 (C-15), 21.2 (C-11), 18.7 (C-19), 13.7 (C-18); HRMS: *m/z* 327.1931 $[M+Na]^+$ [cal- *m/z* 327.1936].

3β,7β,17β-trihydroxyandrost-5-ene (7c)



Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 231-233 ºC, $[\alpha]_D^{22}$: +51 ($c_=$ 0.7 in $CHCl_3$), λ_{max} (MeOH): 210 nm; IR ν_{max} (cm^{-1}): 3289 (OH), 1651 (C=C); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.75 (3H, s, H-19), 1.08 (3H, s, H-18), 3.42 (1H, m, H-3β), 3.56 (1H, t, J=8.32, H-17α), 3.73 (1H, m, H-7α), 5.25 (1H, s, H-6); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): 144.1 (C-5), 127.4 (C-6), 82.3 (C-17), 74.0 (C-7), 72.1 (C-3), 52.3 (C-14), 50.1 (C-9), 44.23 (C-13), 42.6 (C-4), 41.2 (C-8), 38.3 (C-1), 37.8 (C-10), 37.7 (C-16), 32.3 (C-2), 30.8 (C-12), 26.6 (C-15), 21.9 (C-11), 19.5 (C-19), 11.6 (C-18); HRMS: *m/z* 329.2084 $[M+Na]^+$ [calcd- *m/z* 329.2093].

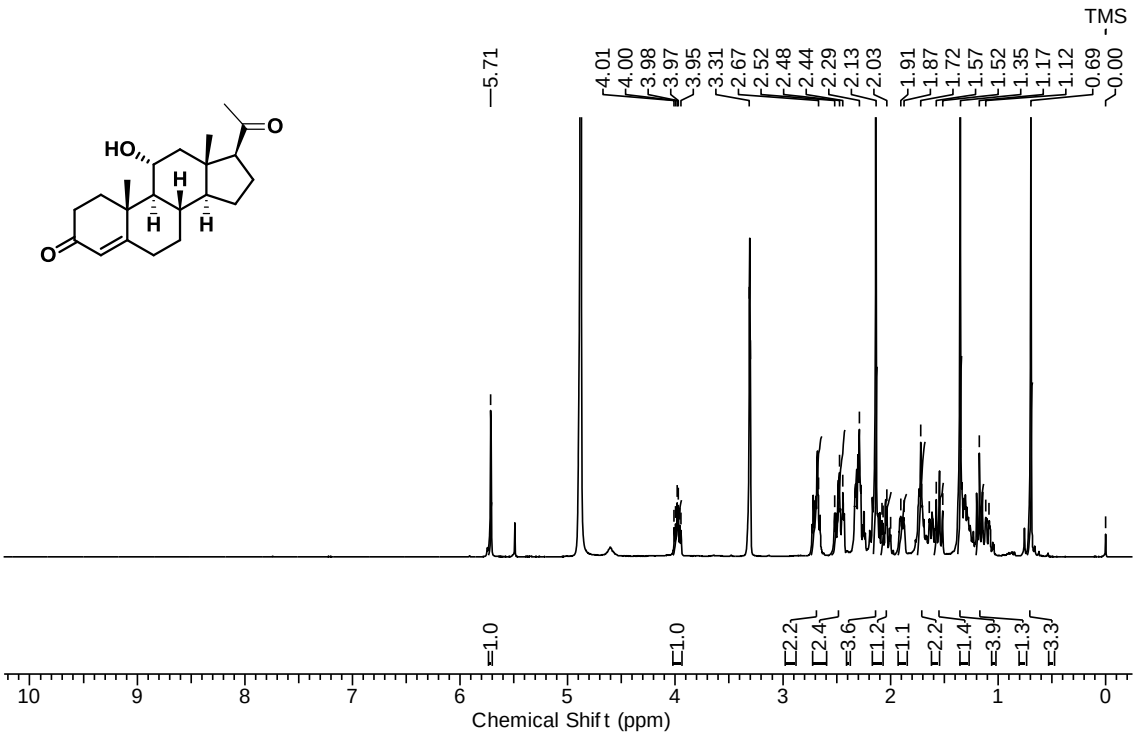
3β,7α,17β-trihydroxyandrost-5-ene (7d)



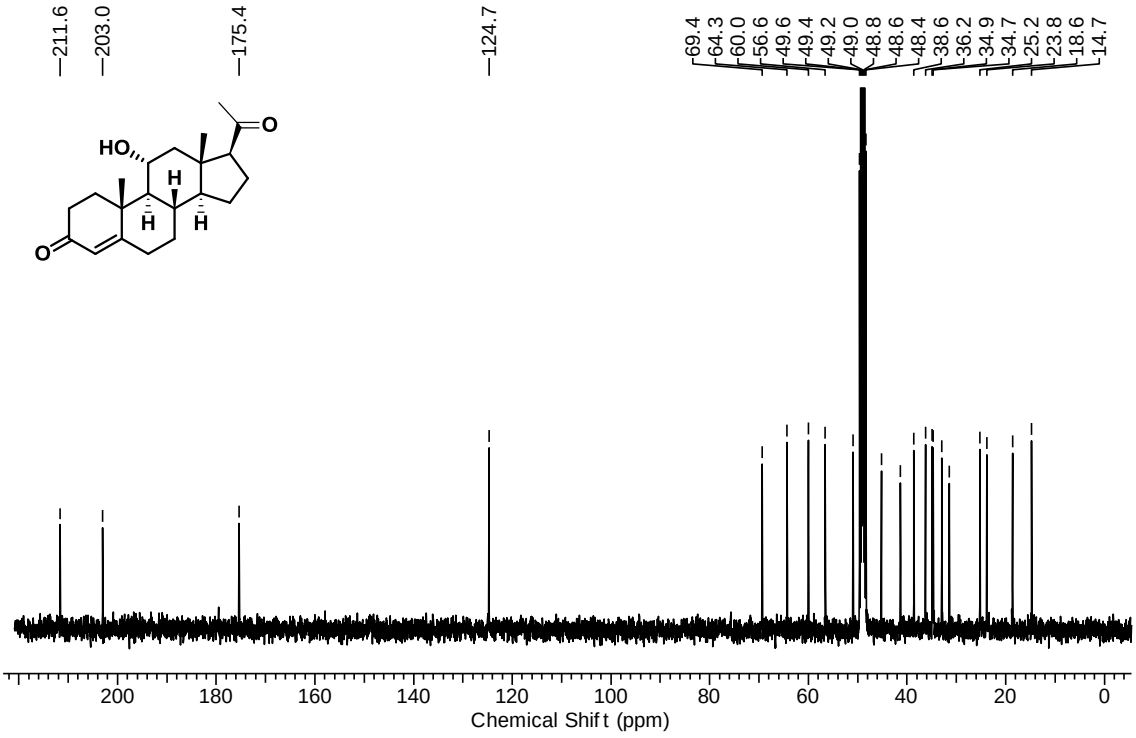
Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 263-265 ºC, $[\alpha]_D^{22}$: -3 ($c_=$ 0.35 in $CHCl_3$), λ_{max} (MeOH): 210 nm; IR ν_{max} (cm^{-1}): 3305 (OH), 1659 (C=C); ^1H NMR (400 MHz, CD_3OD, Me_4Si): δ 0.75 (3H, s, H-19), 1.01 (3H, s, H-18), 3.47 (1H, m, H-3β), 3.60 (1H, t, J=8.37, H-17α), 3.76 (1H, m, H-7β), 5.55 (1H, d, J=4.82 Hz, H-6); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): 146.7 (C-5), 124.8 (C-6), 82.5 (C-17), 72.0 (C-3), 65.4 (C-7), 45.3 (C-14), 43.6 (C-9 and C-13), 42.9 (C-4), 39.1 (C-8), 38.5 (C-10), 38.1 (C-1), 37.5 (C-16), 32.1 (C-2), 30.6 (C-12), 24.2 (C-15), 21.5 (C-11), 18.7 (C-19), 11.4 (C-18); HRMS: *m/z* 329.2089 $[M+Na]^+$ [calcd- *m/z* 329.2093].

3.7: Selected spectra

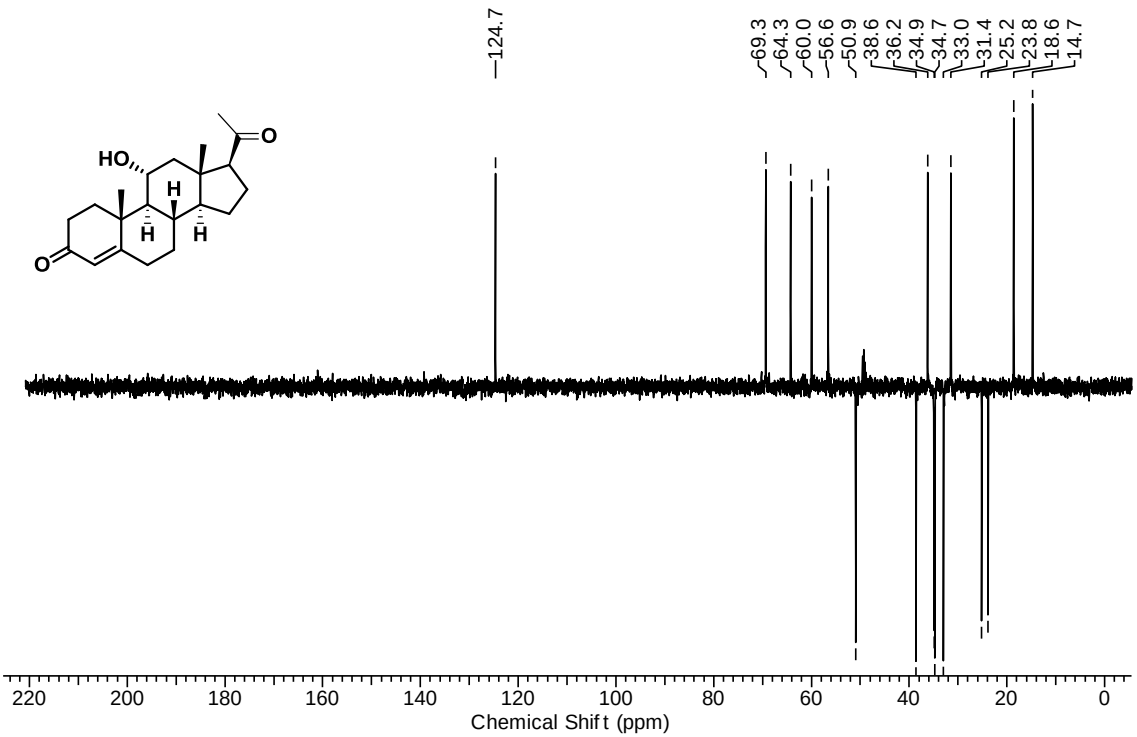
11α-hydroxyprogesterone (1a)



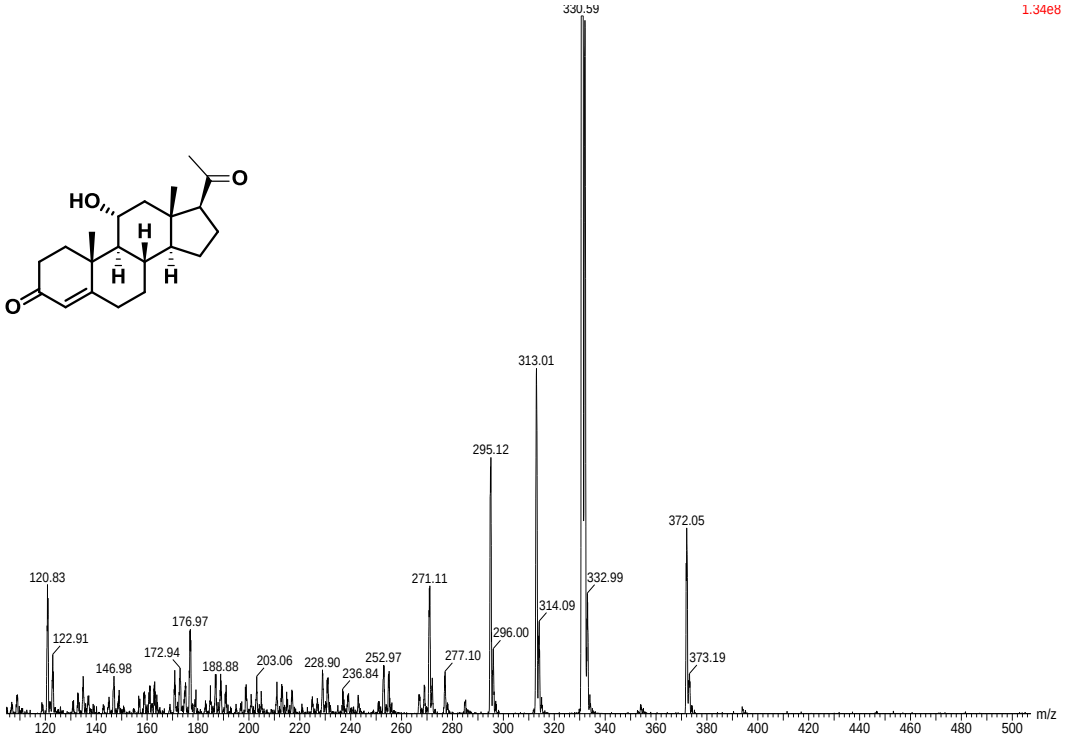
^{1}H NMR spectrum of **1a** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **1a** in CD_3OD at 100 MHz

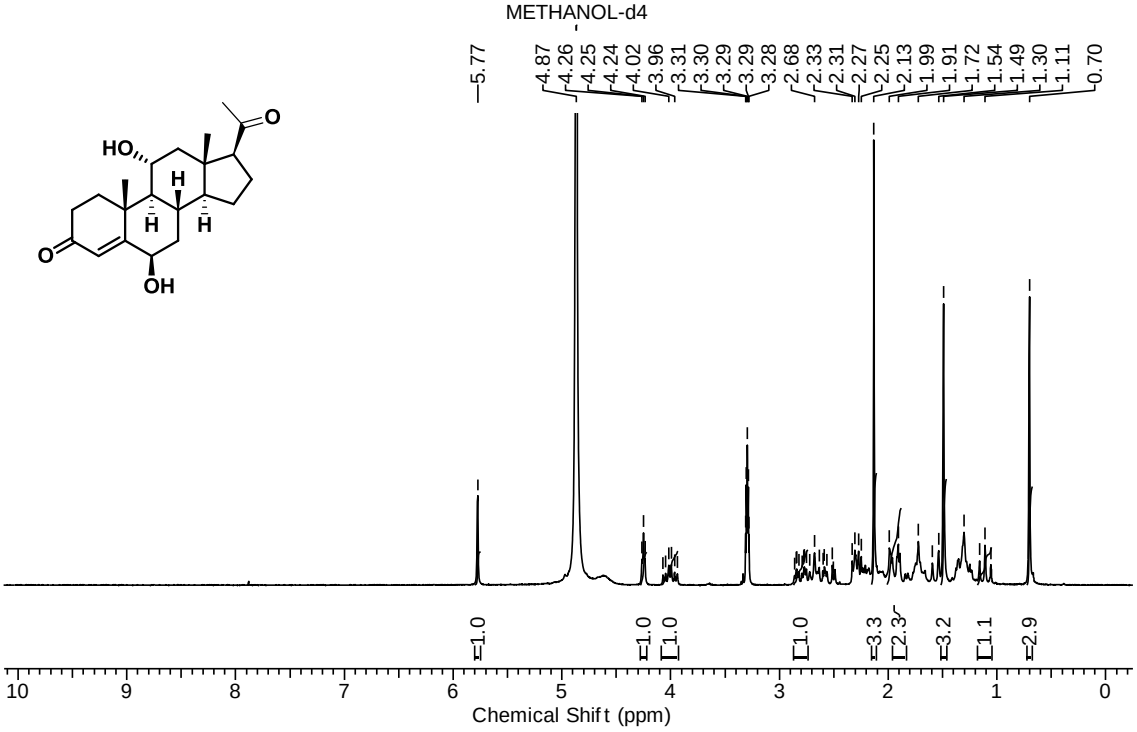


DEPT NMR spectrum of **1a** in CD_3OD at 100 MHz

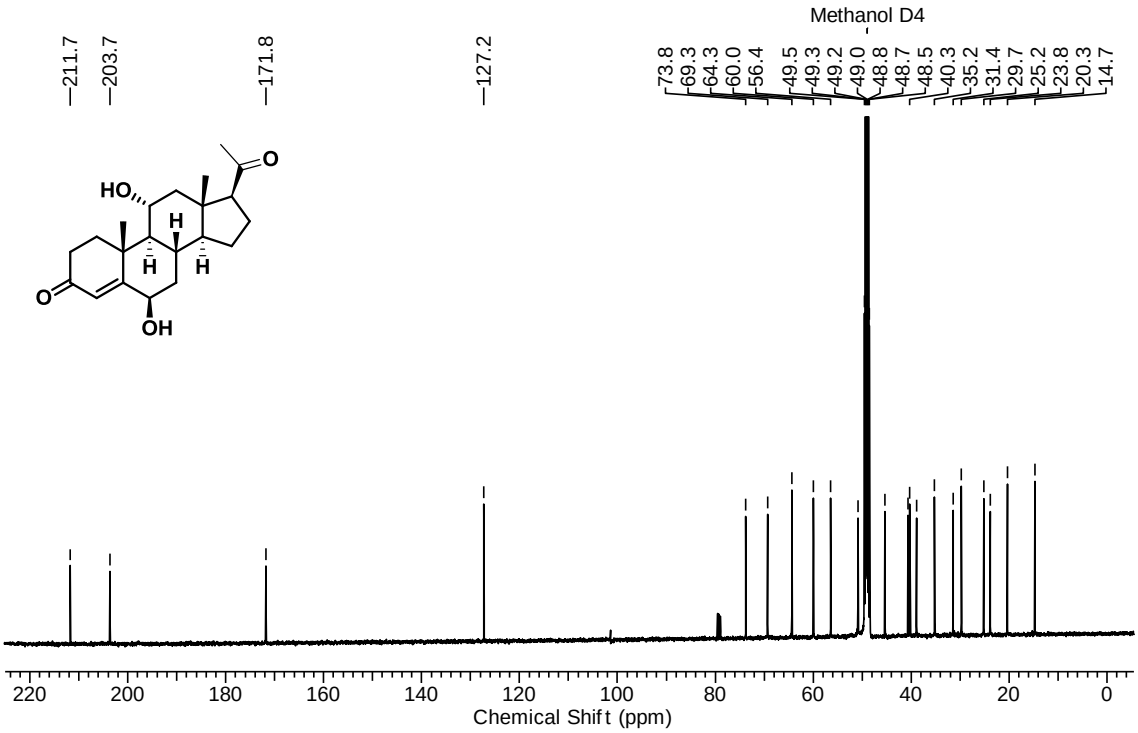


Mass spectrum of **1a** (ESI+ mode)

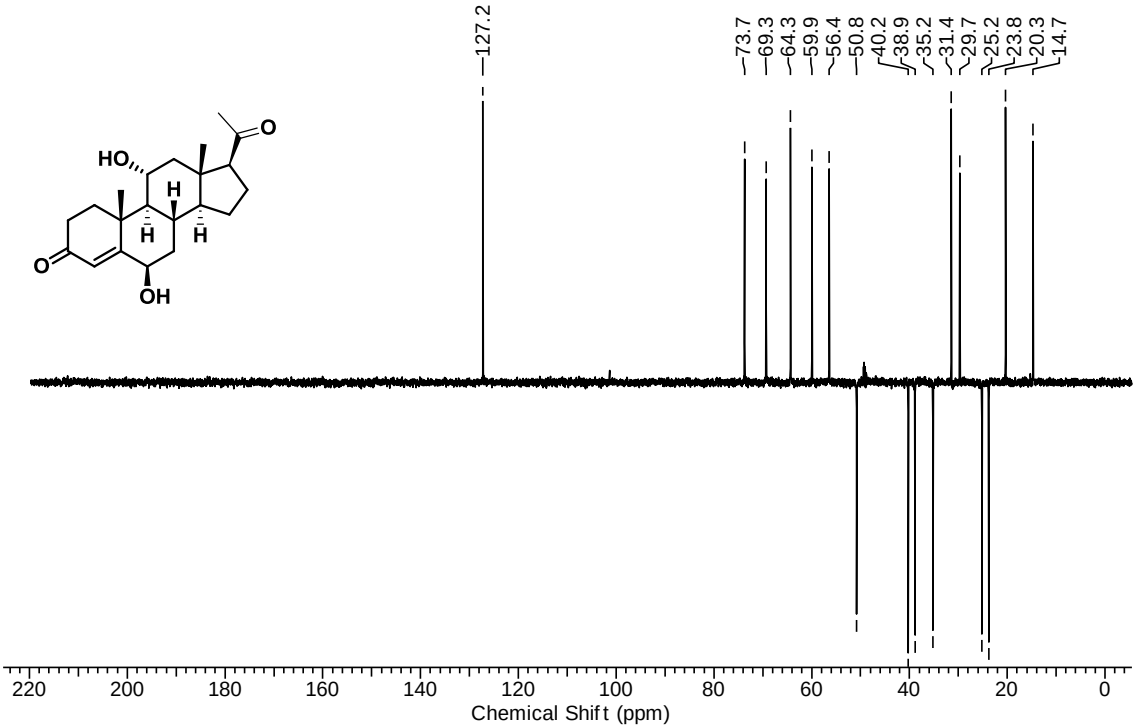
6β, 11α-dihydroxyprogesterone (1b)



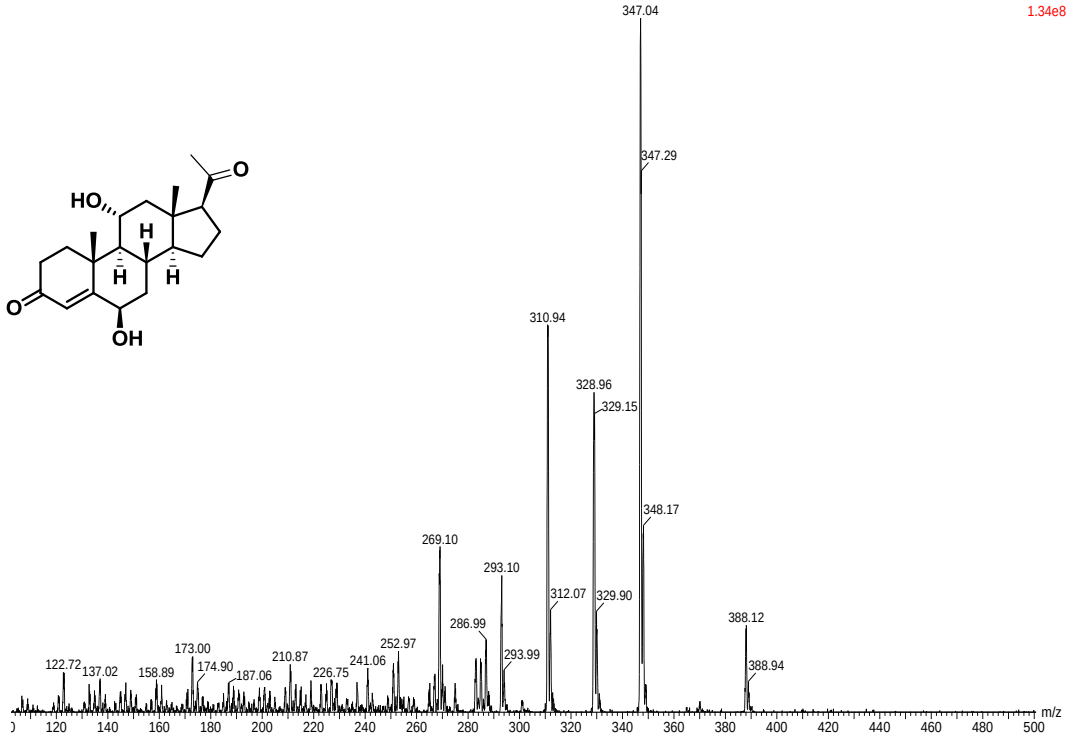
^{1}H NMR spectrum of **1b** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **1b** in CD_3OD at 100 MHz.

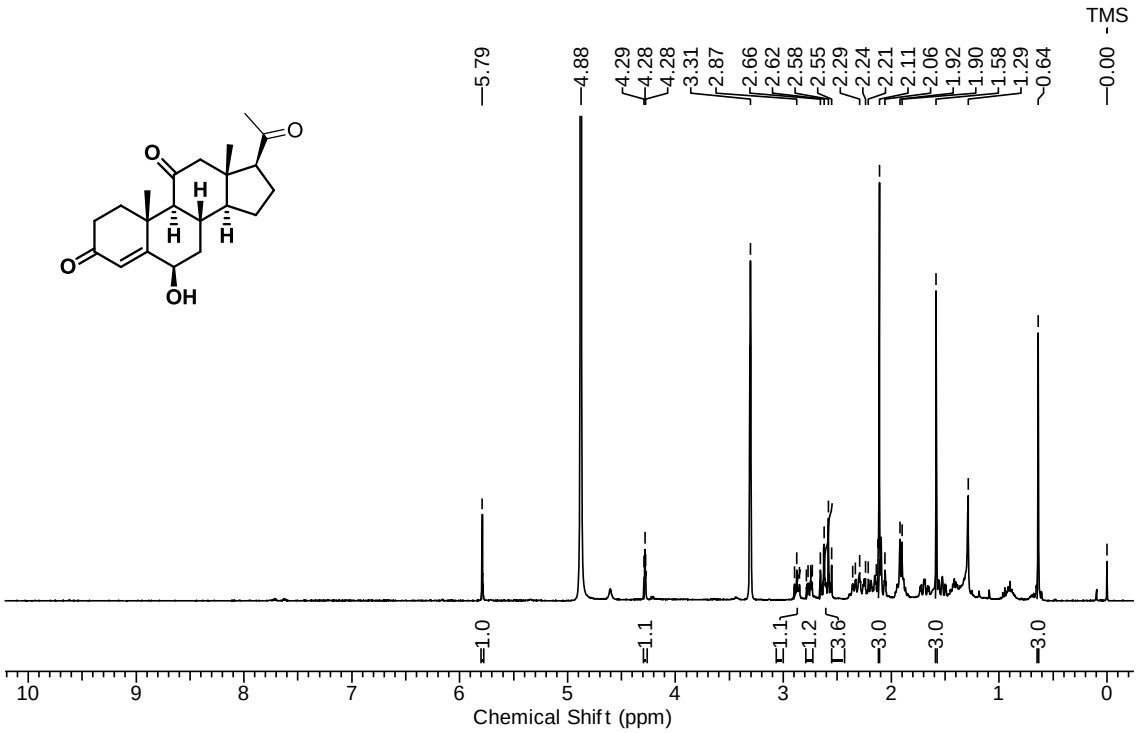


DEPT NMR spectrum of **1b** in CD_3OD at 100 MHz.

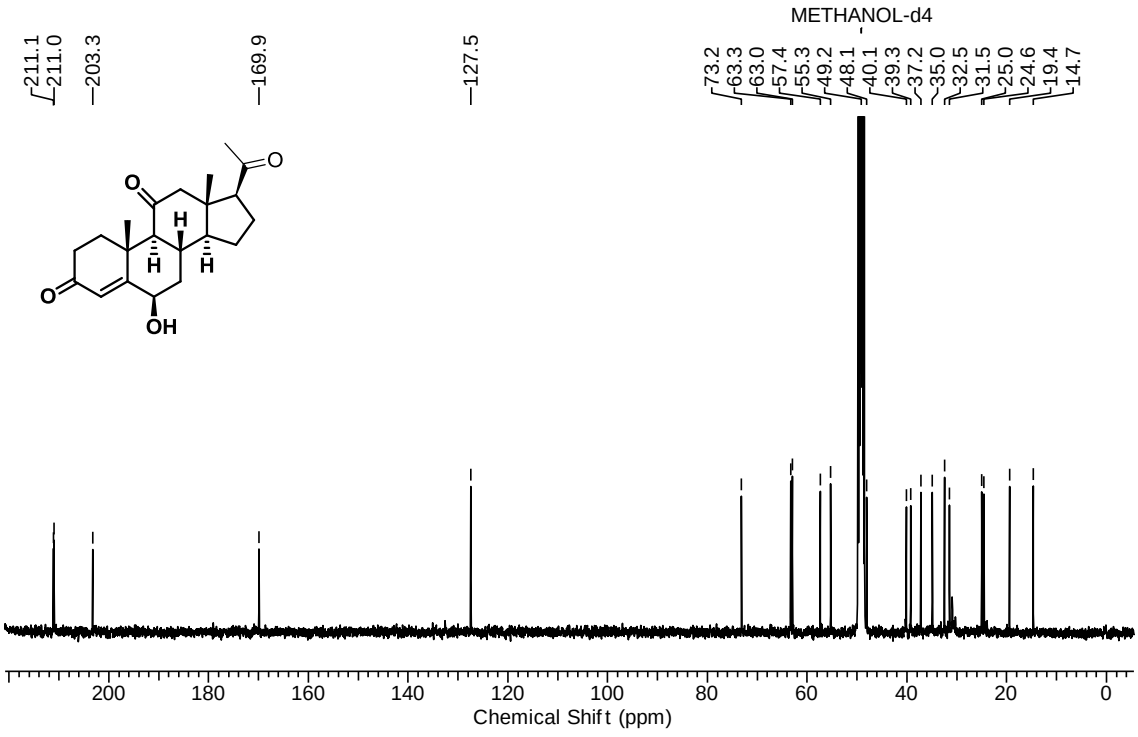


Mass spectrum of **1b** (ESI+ mode)

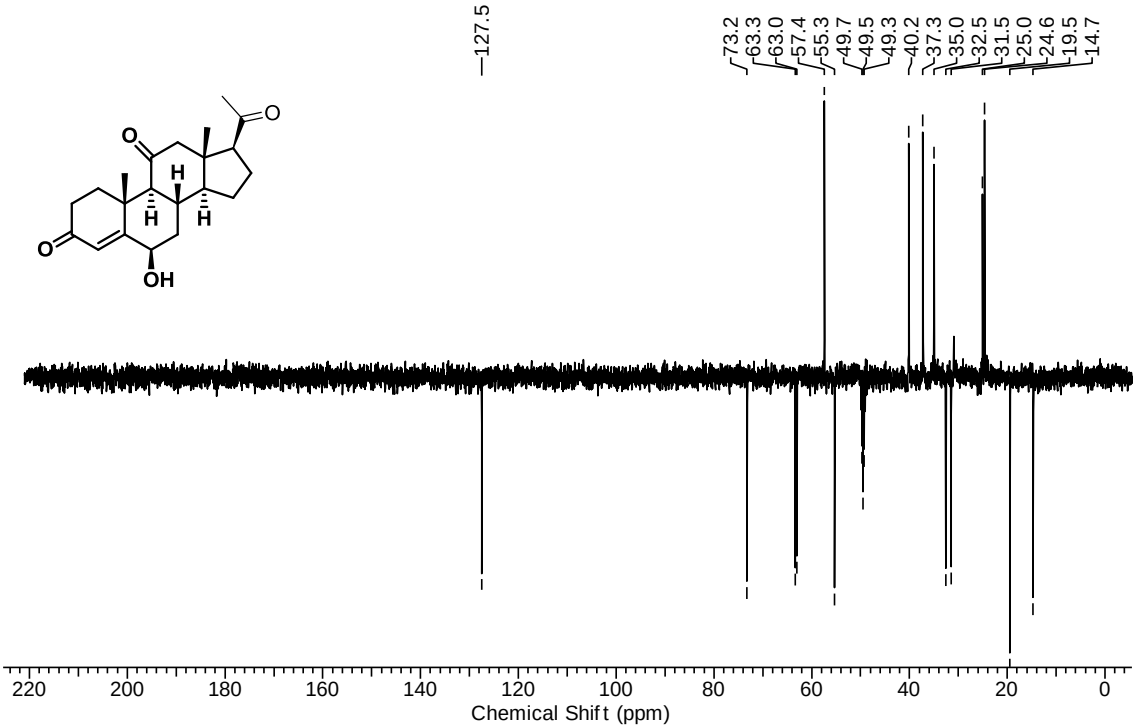
6β-hydroxypregn-4-ene-3,11,20 trione (1c)



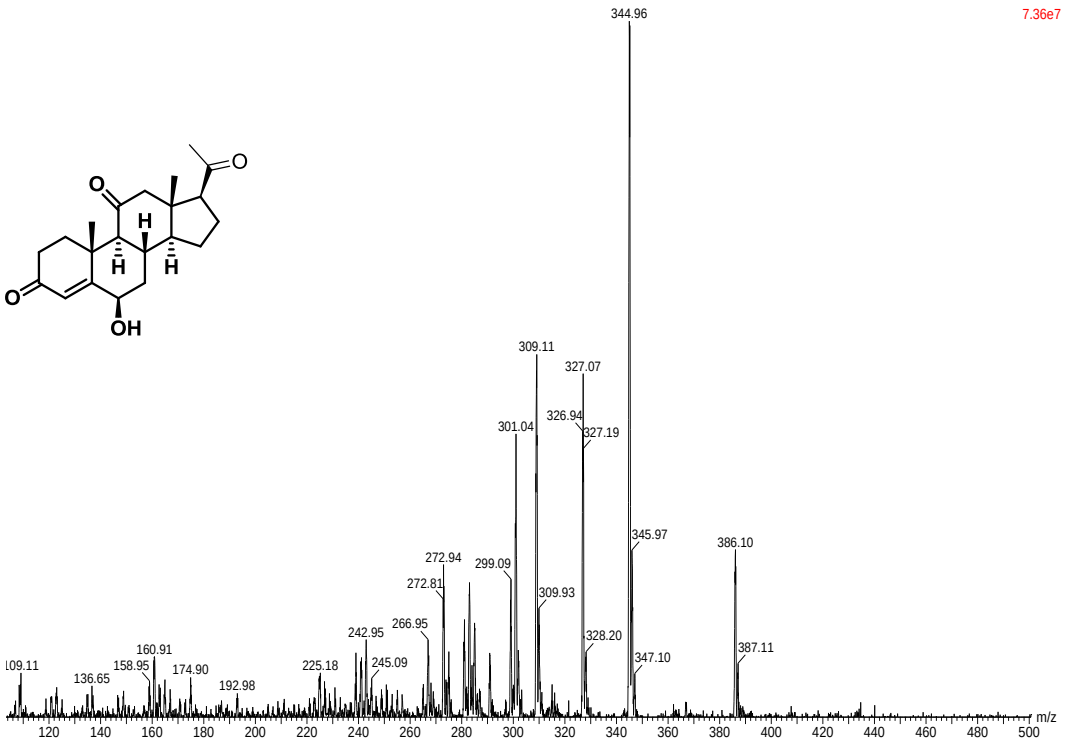
^{1}H NMR spectrum of **1c** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **1c** in CD_3OD at 100 MHz.

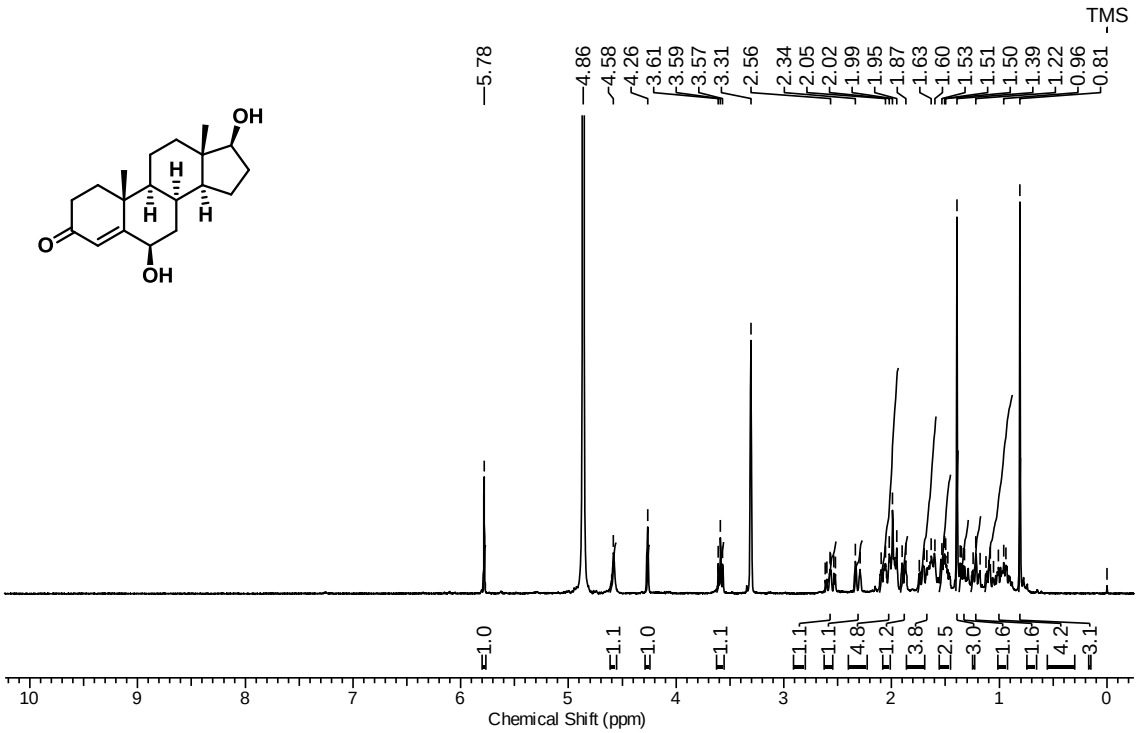


DEPT NMR spectrum of **1c** in CD_3OD at 100 MHz.

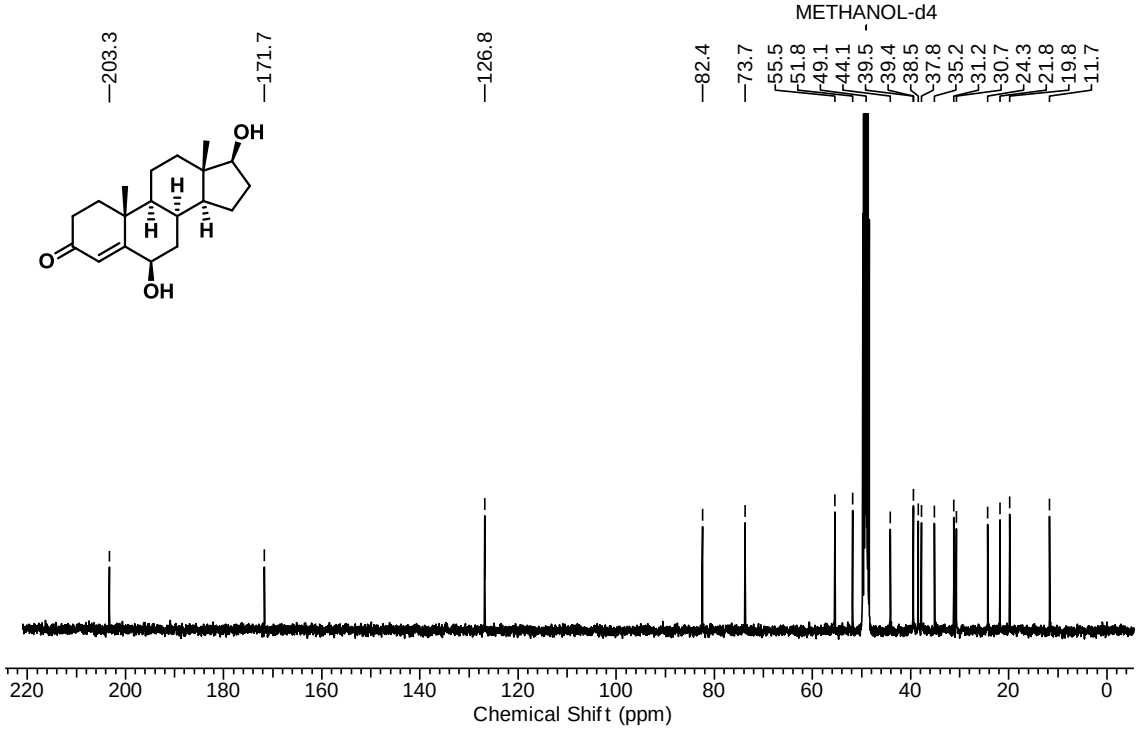


Mass spectrum of **1c** (ESI+ mode)

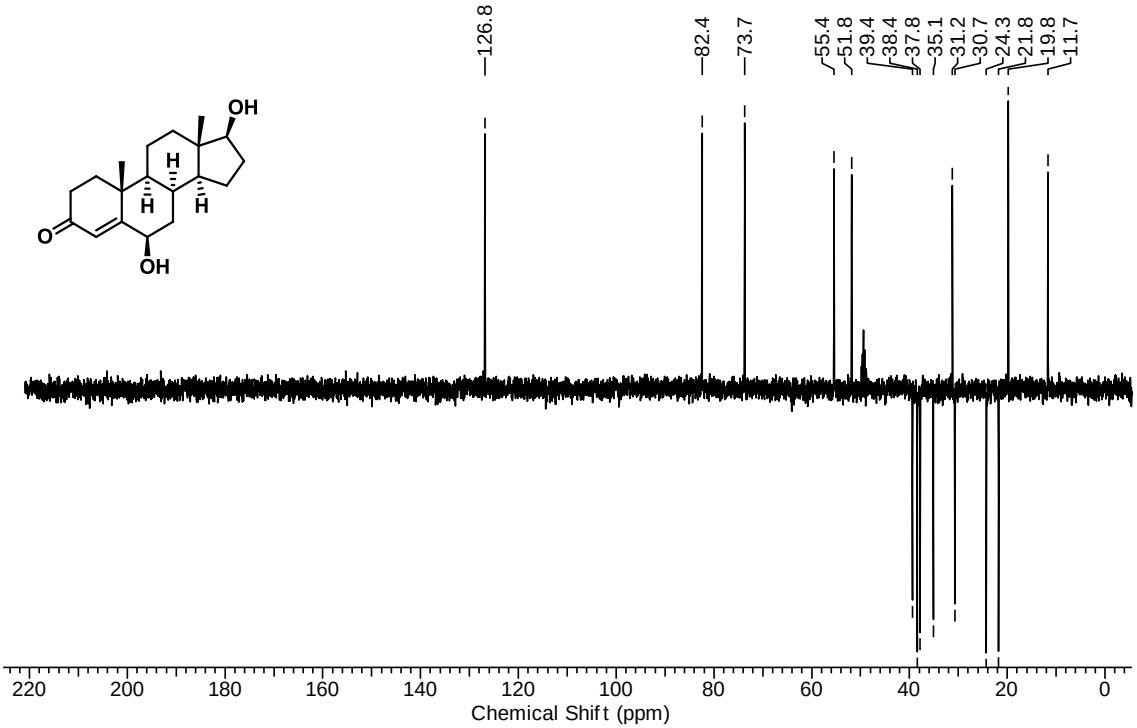
6β-hydroxytestosterone (2a)



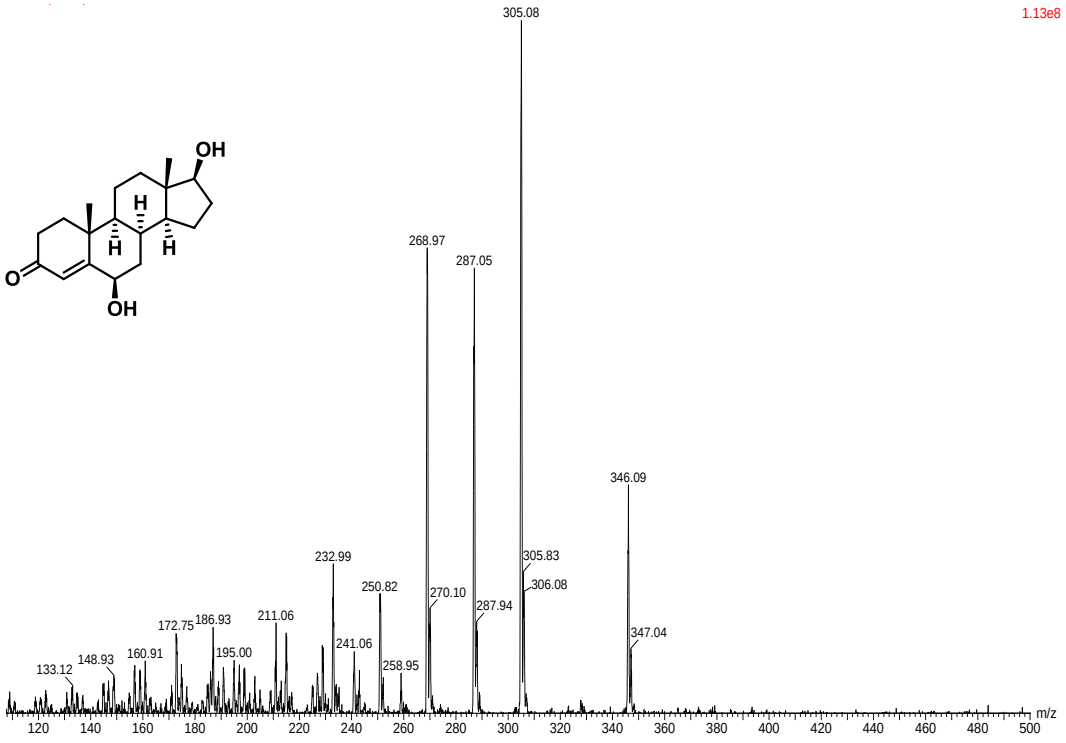
^{1}H NMR spectrum of **2a** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **2a** in CD_3OD at 100 MHz.

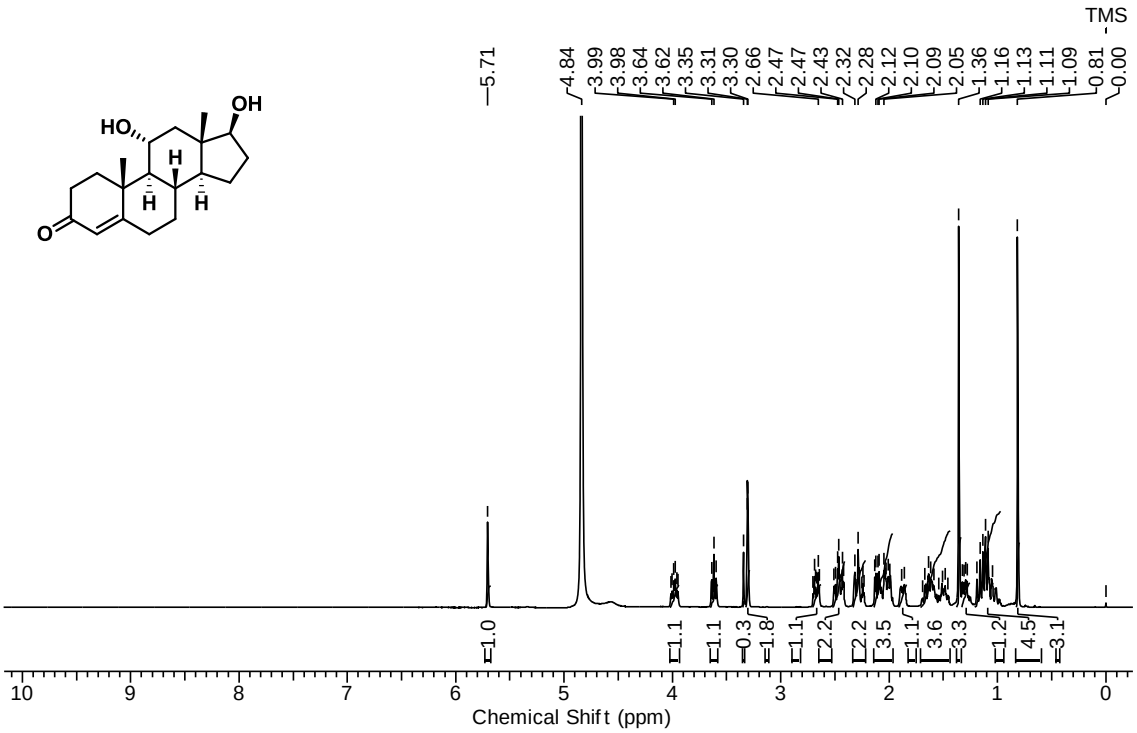


DEPT NMR spectrum of **2a** in CD_3OD at 100 MHz.

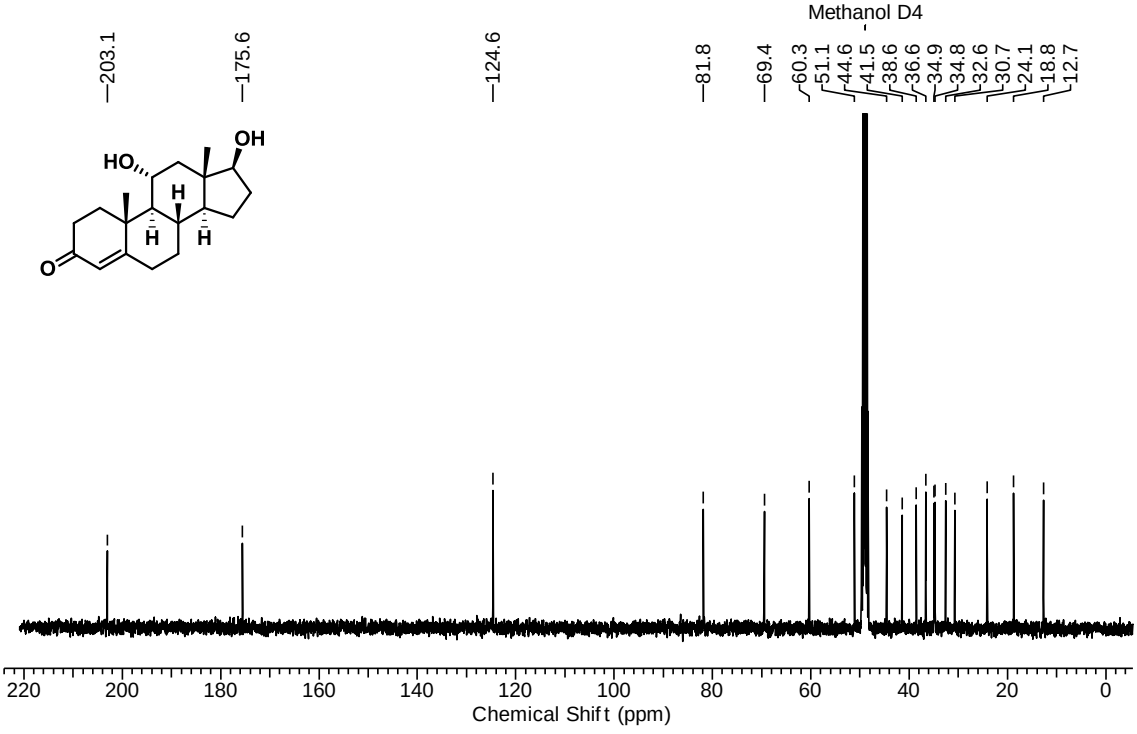


Mass spectrum of **2a** (ESI+ mode)

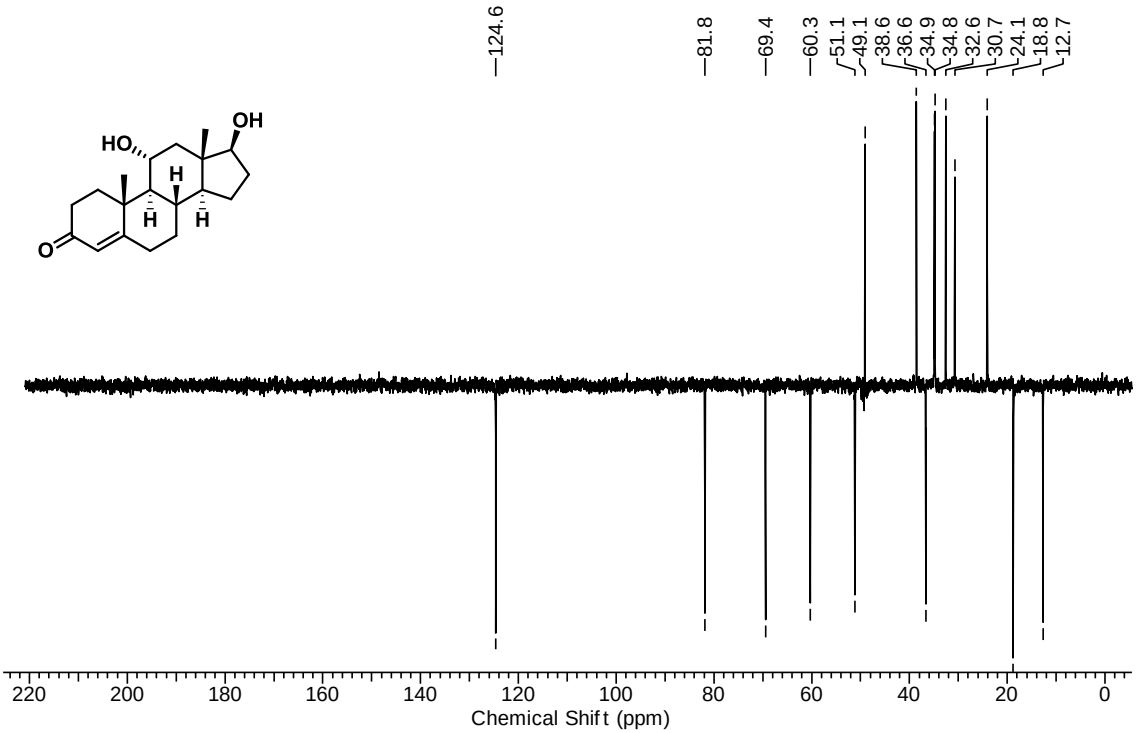
11α-hydroxytestosterone (2b)



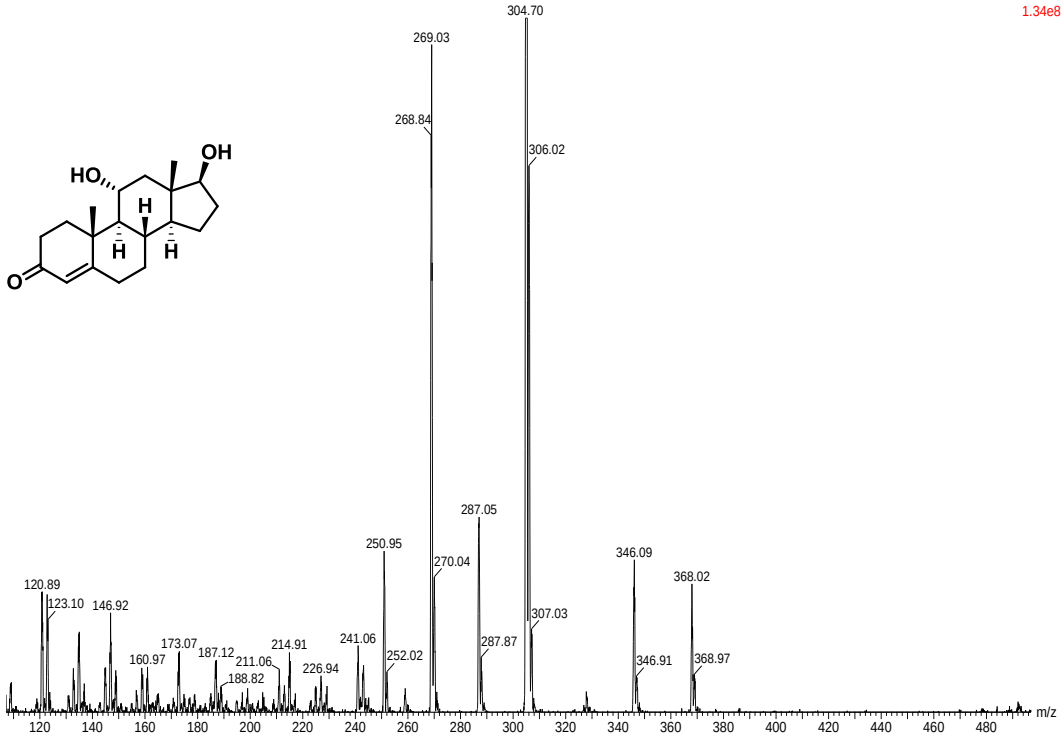
^{1}H NMR spectrum of **2b** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **2b** in CD_3OD at 100 MHz.

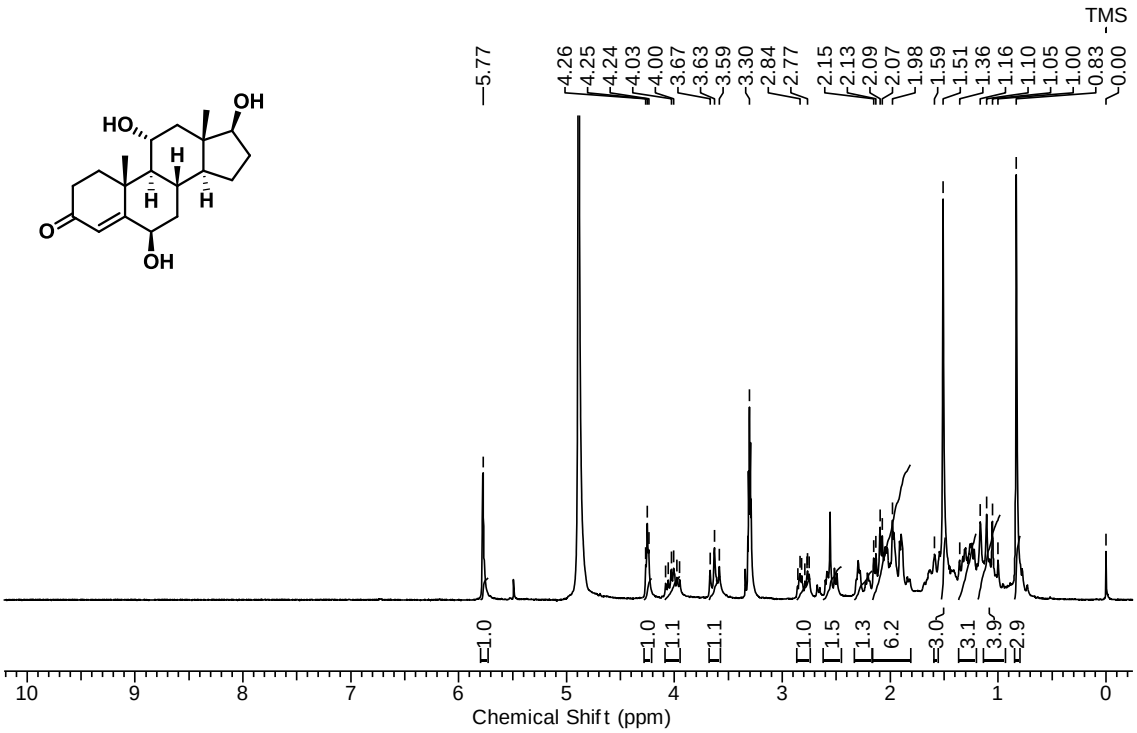


DEPT NMR spectrum of **2b** in CD_3OD at 100 MHz.

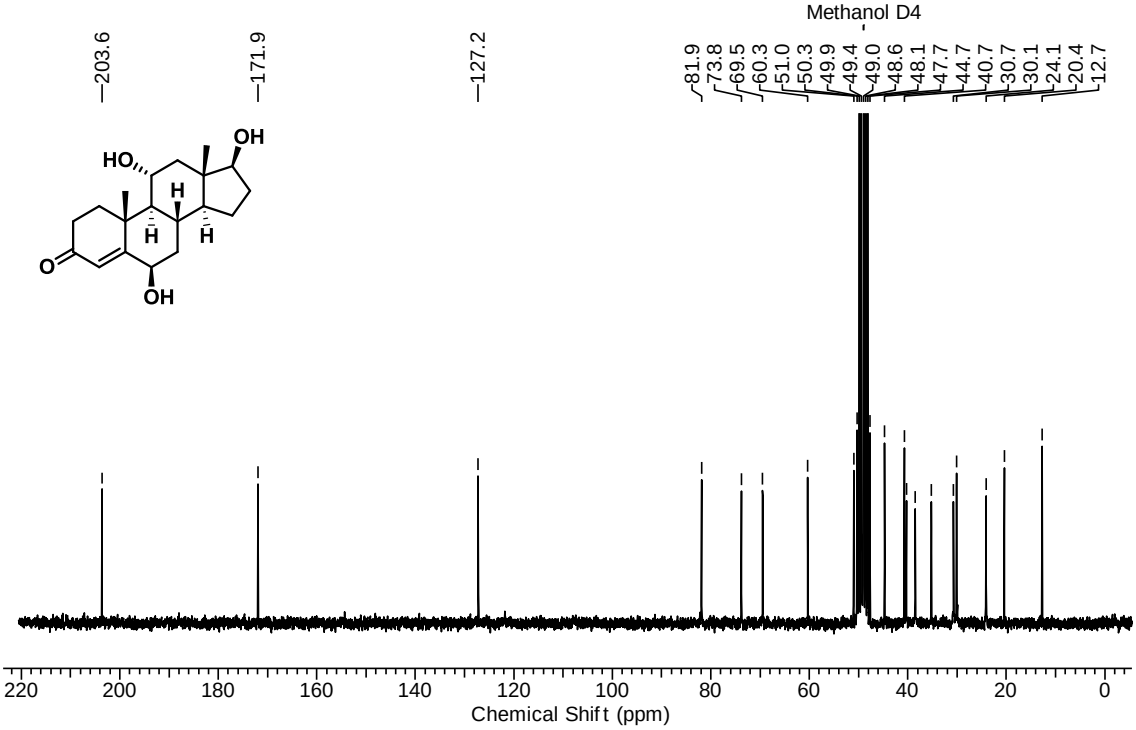


Mass spectrum of **2b** (ESI+ mode)

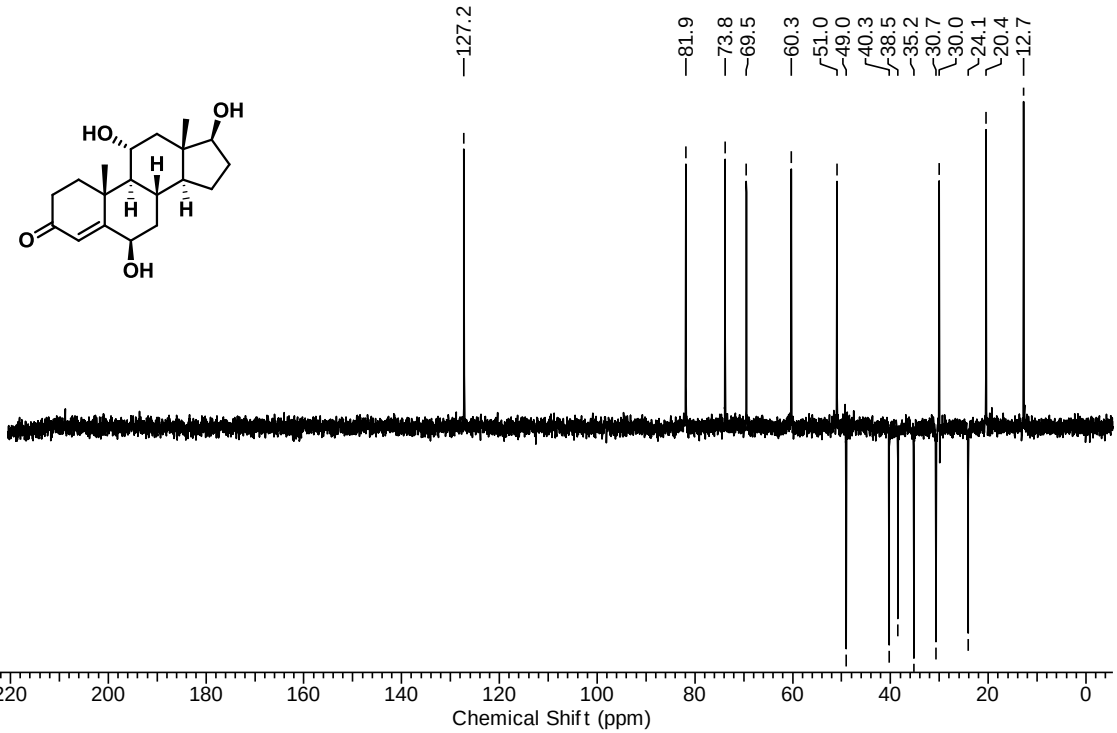
6β, 11α-dihydroxytestosterone (2c)



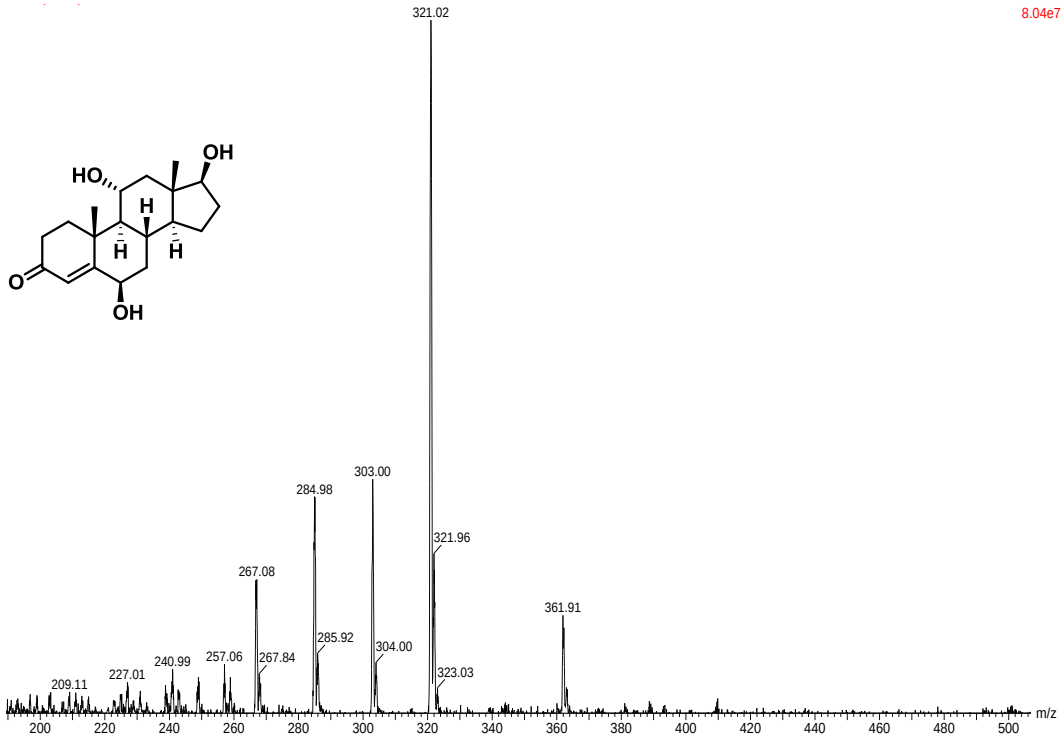
^{1}H NMR spectrum of **2c** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **2c** in CD_3OD at 100 MHz.

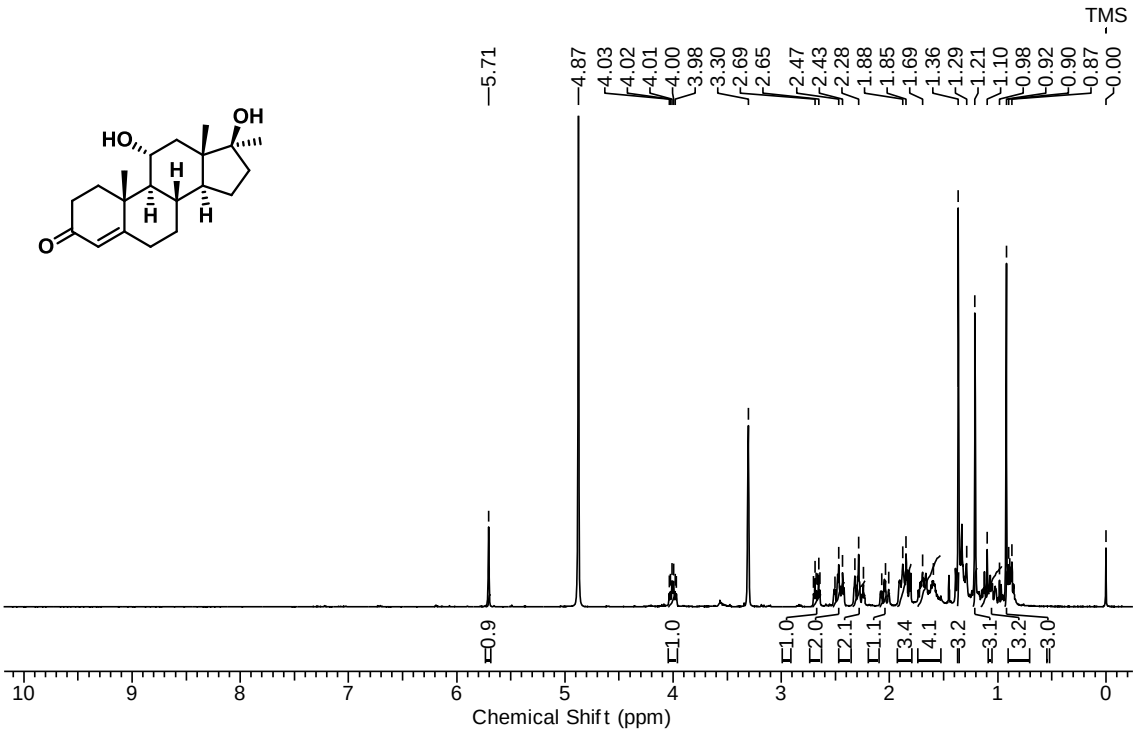


DEPT NMR spectrum of **2c** in CD_3OD at 100 MHz.

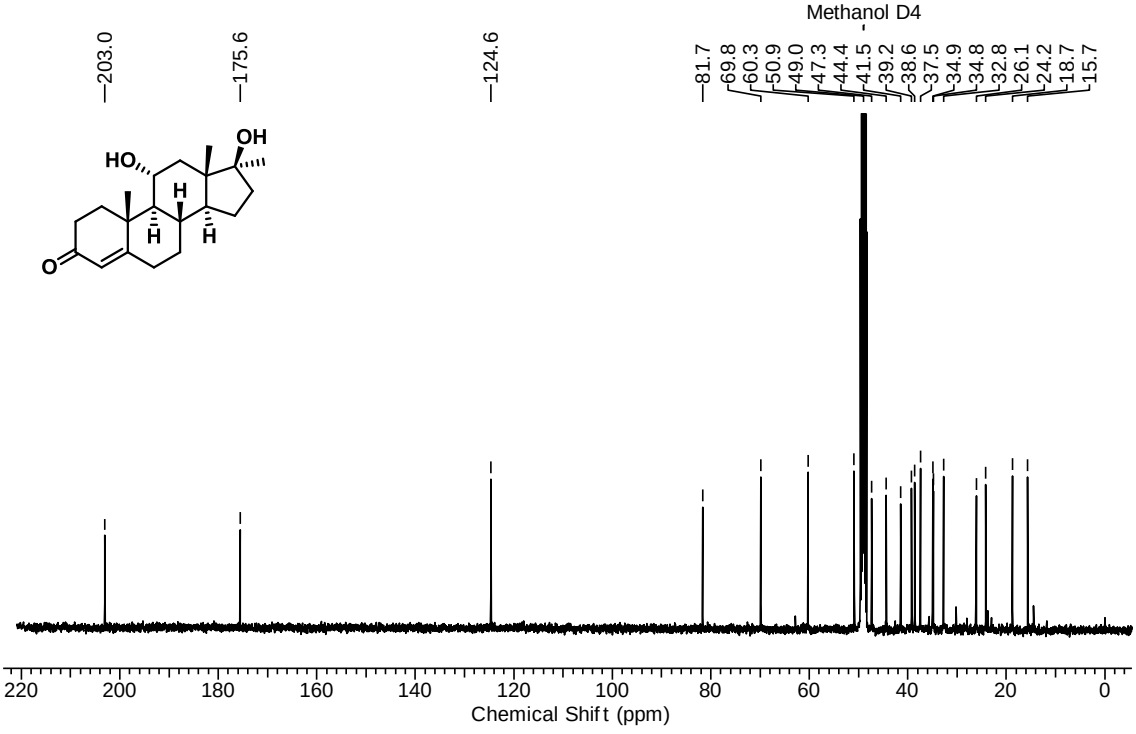


Mass spectrum of **2c** (ESI+ mode)

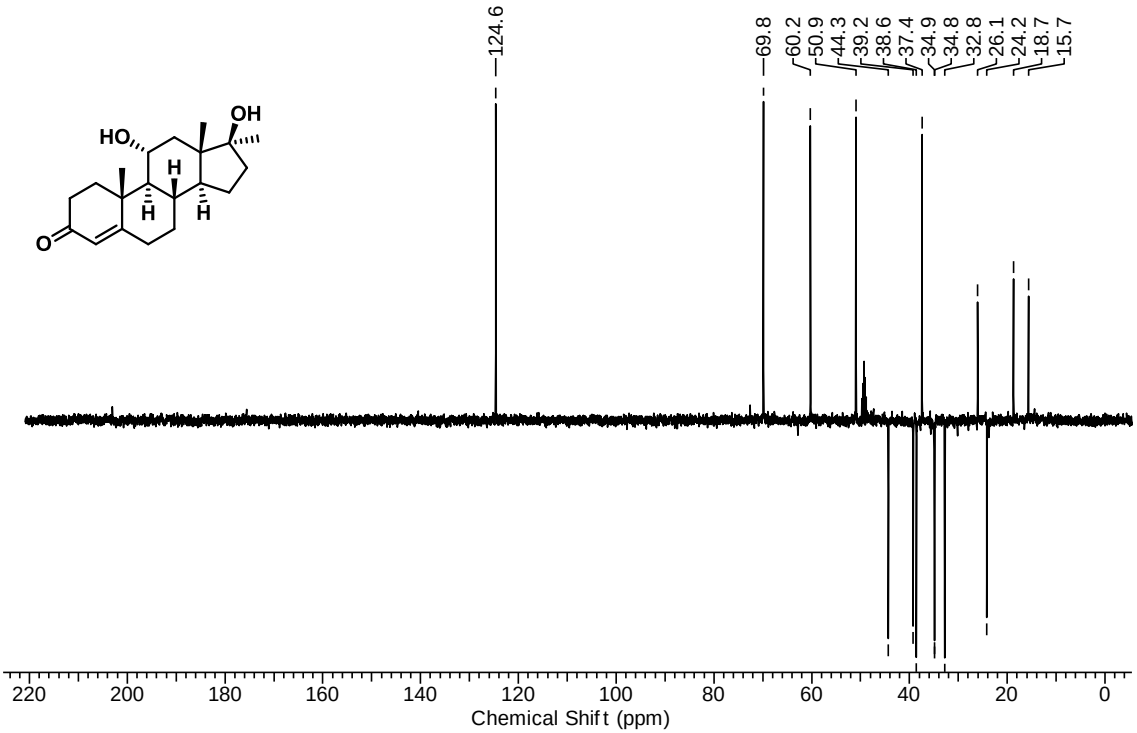
11α-hydroxy-17α-methyltestosterone (3a)



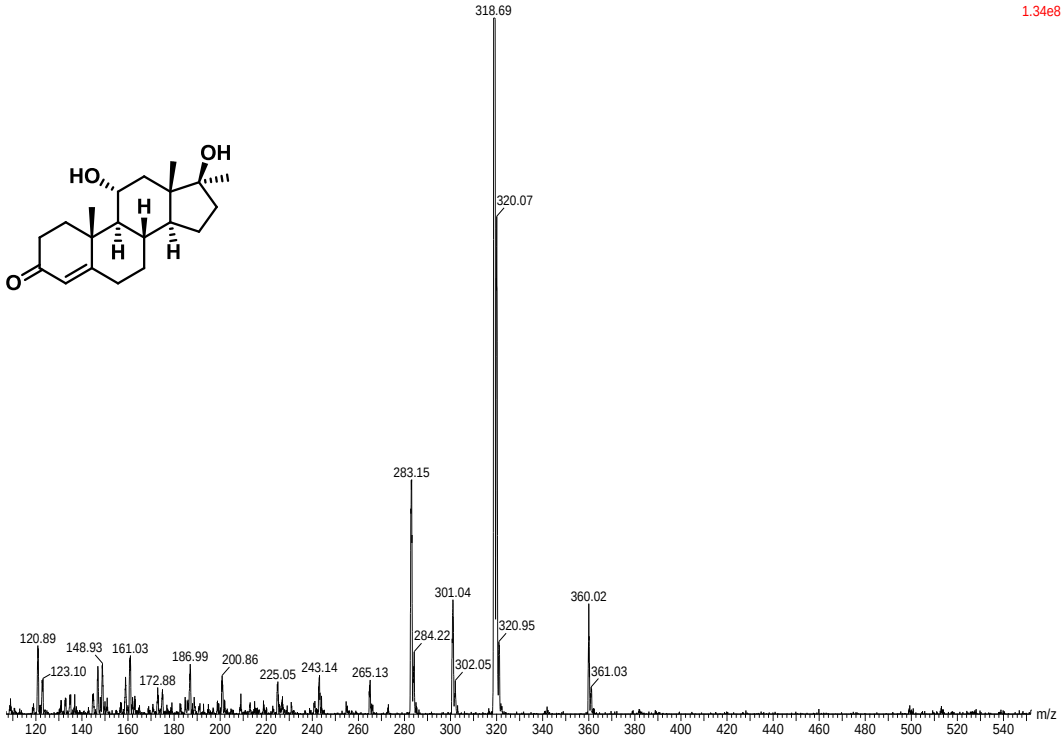
^{1}H NMR spectrum of **3a** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **3a** in CD_3OD at 100 MHz.

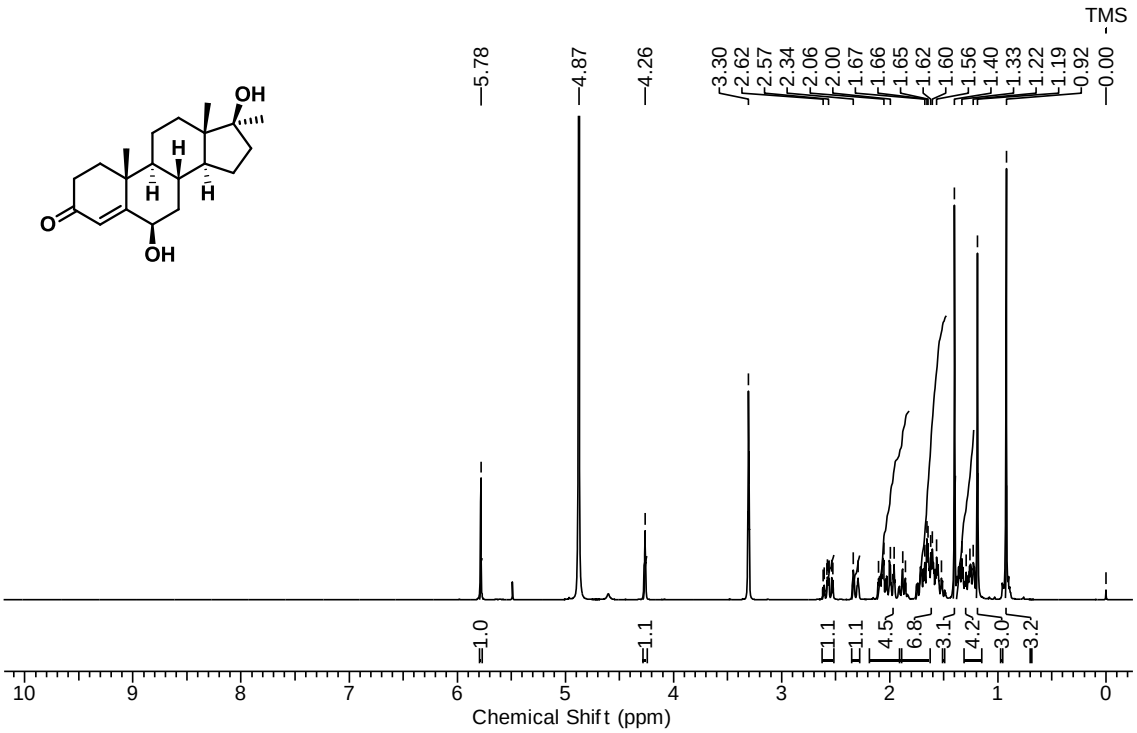


DEPT NMR spectrum of **3a** in CD_3OD at 100 MHz.

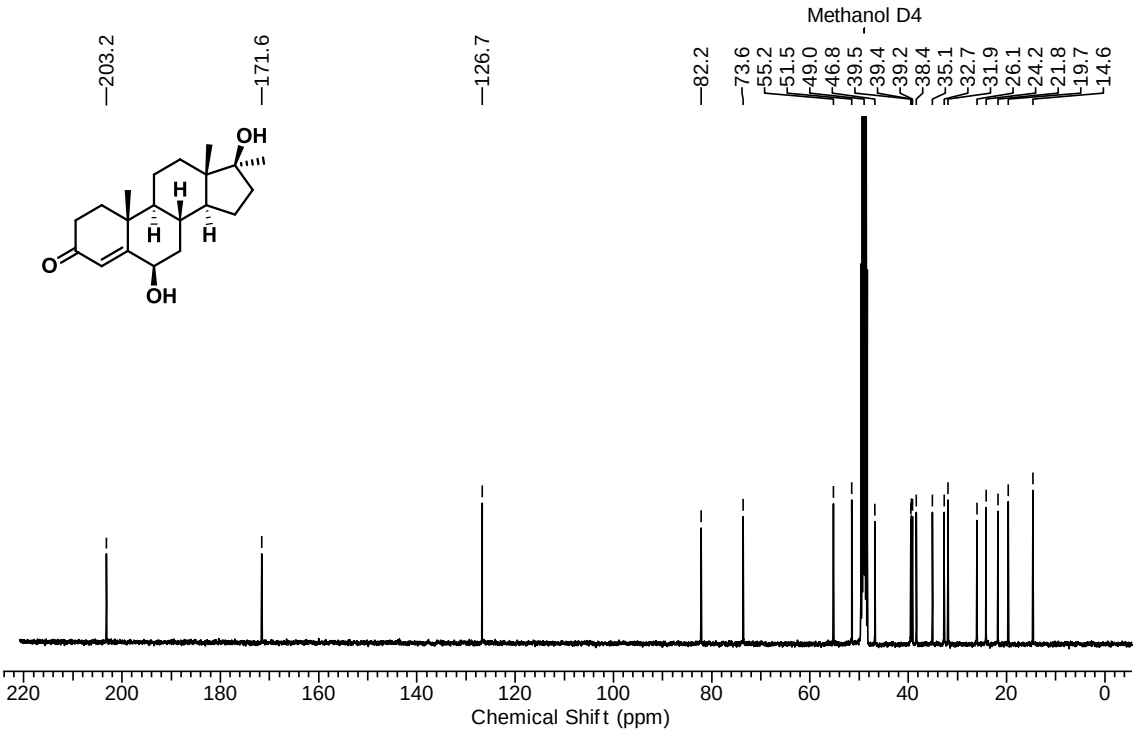


Mass spectrum of **3a** (ESI+ mode)

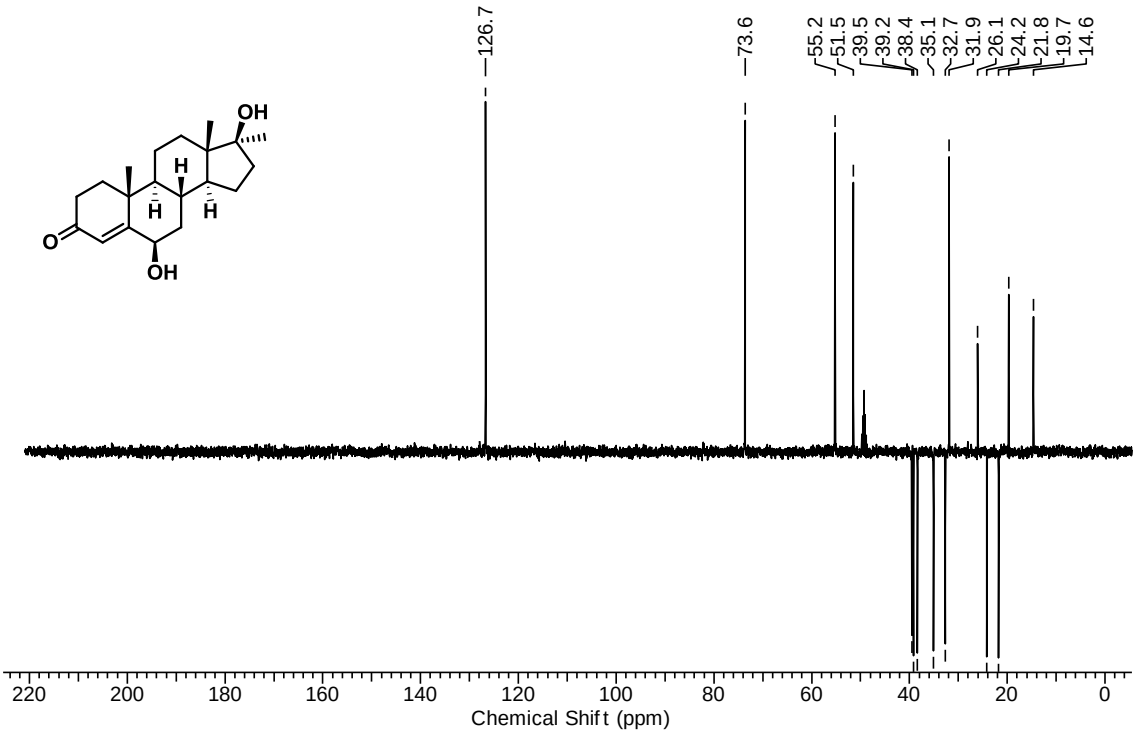
6β-hydroxy-17α-methyltestosterone (3b)



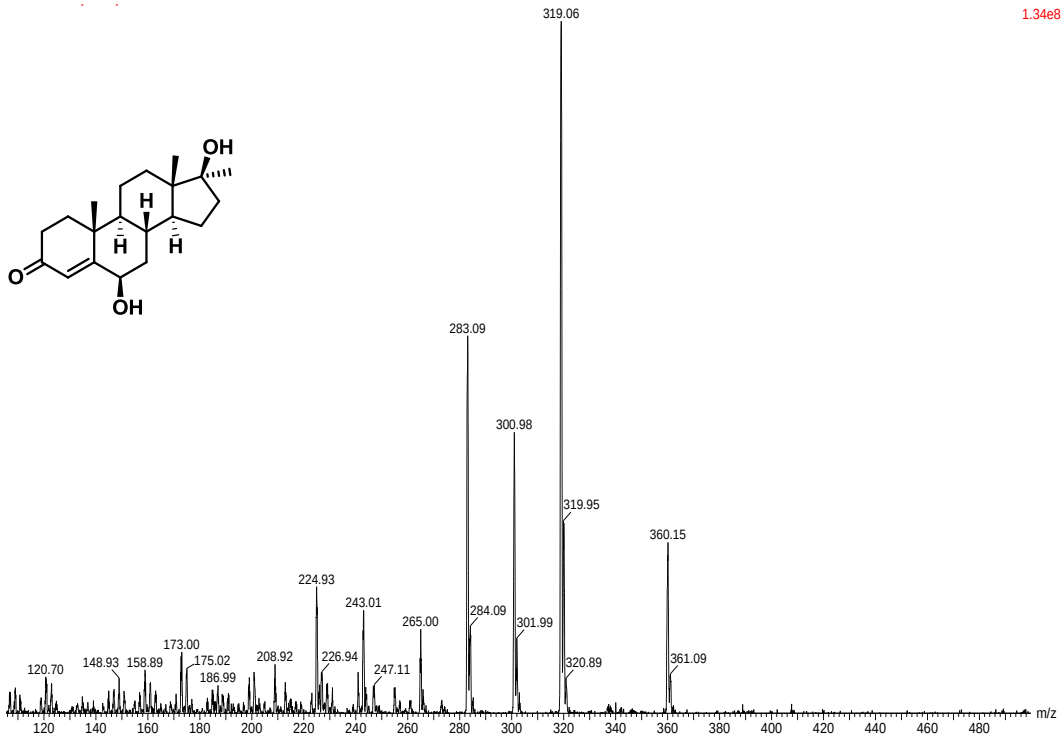
^{1}H NMR spectrum of **3b** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **3b** in CD_3OD at 100 MHz.

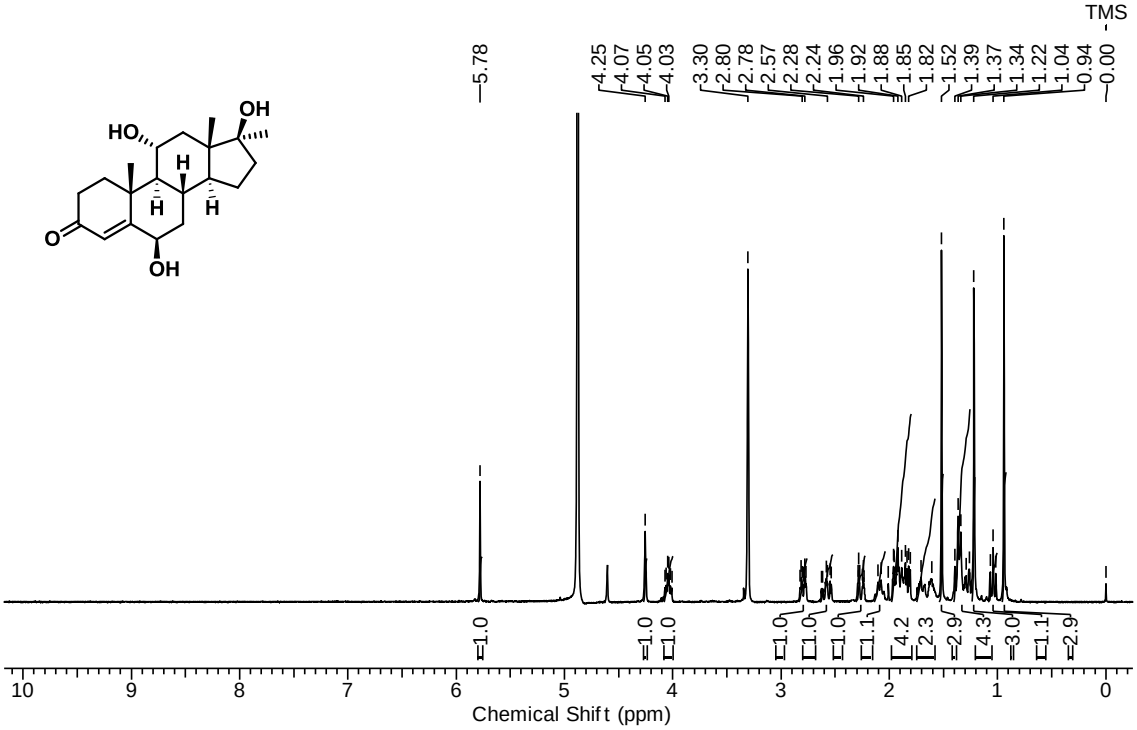


DEPT NMR spectrum of **3b** in CD_3OD at 100 MHz.

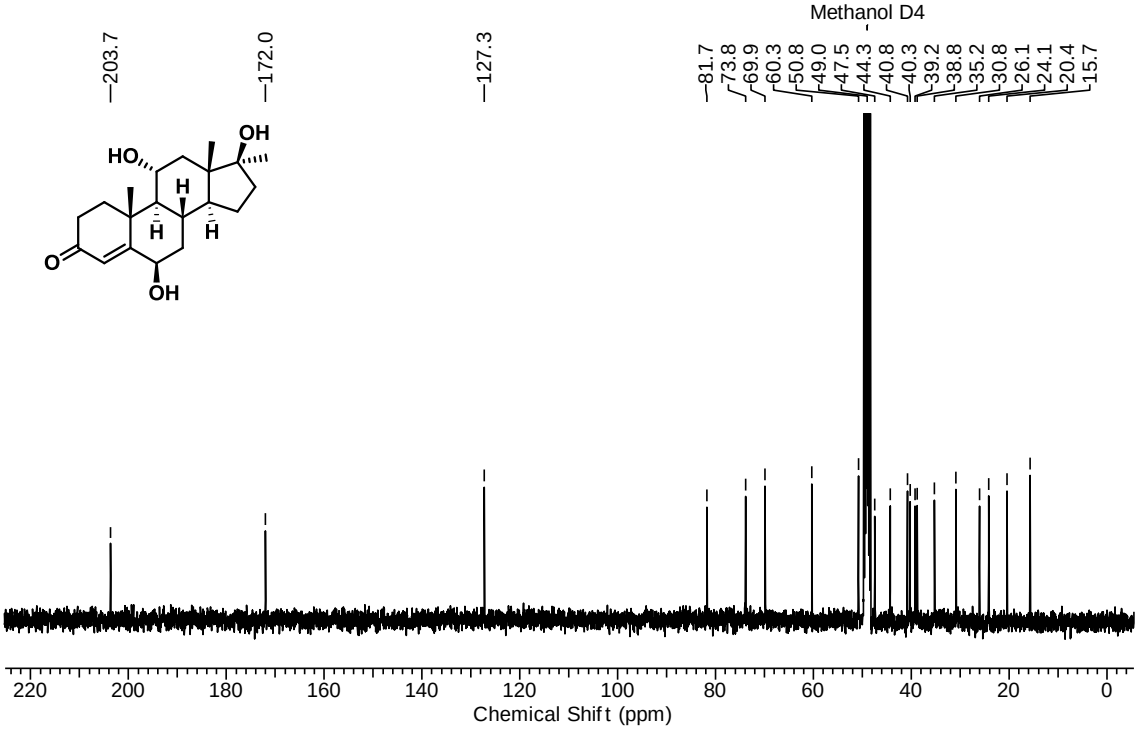


Mass spectrum of **3b** (ESI+ mode)

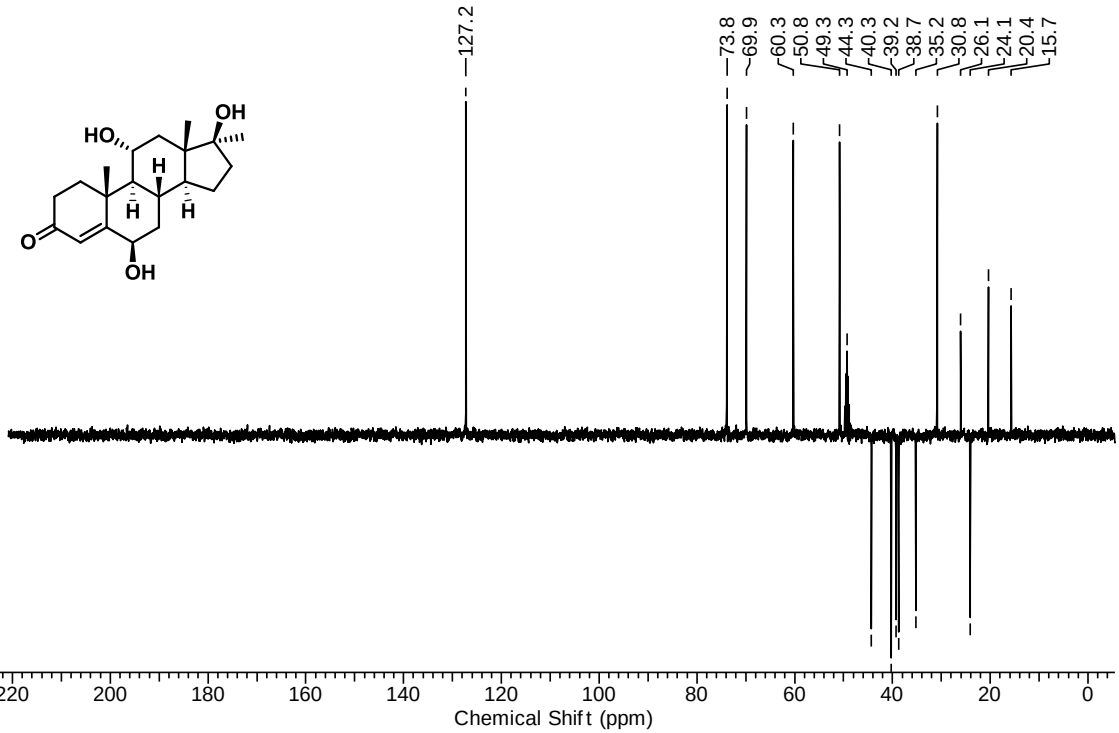
6β,11α-dihydroxy-17α-methyltestosterone (3c)



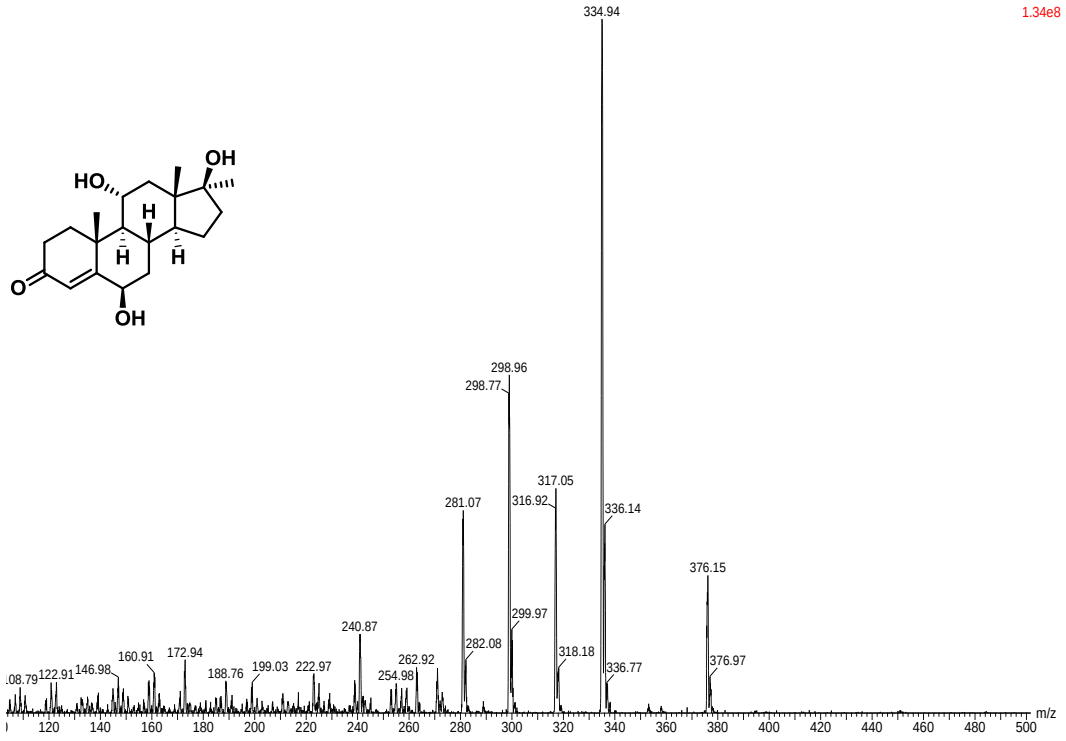
^{1}H NMR spectrum of **3c**in CD_3OD at 400 MHz



^{13}C NMR spectrum of **3c** in CD_3OD at 100 MHz.

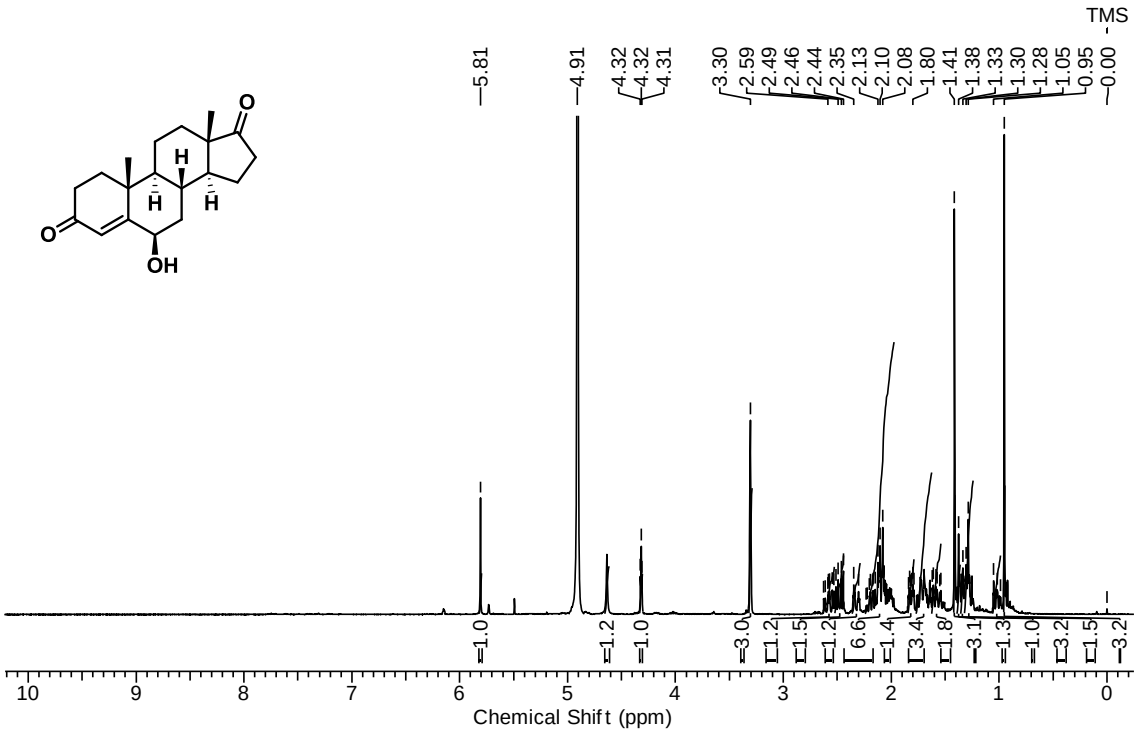


DEPT NMR spectrum of **3c** in CD_3OD at 100 MHz.

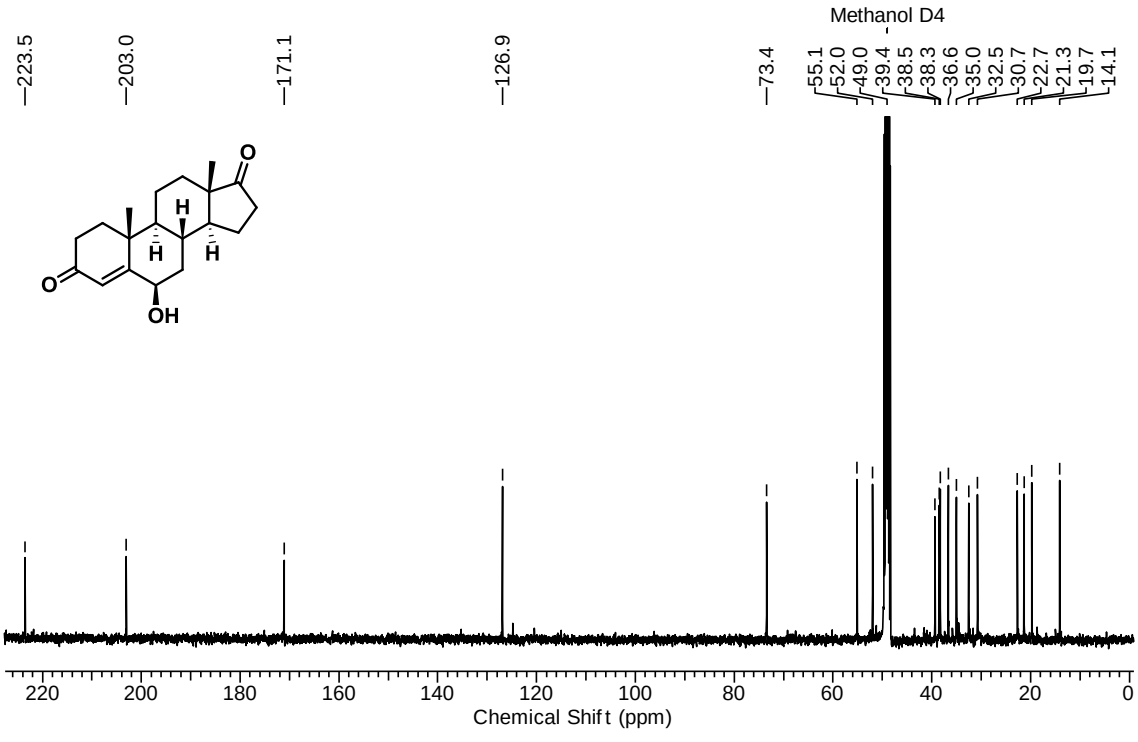


Mass spectrum of **3c** (ESI+ mode)

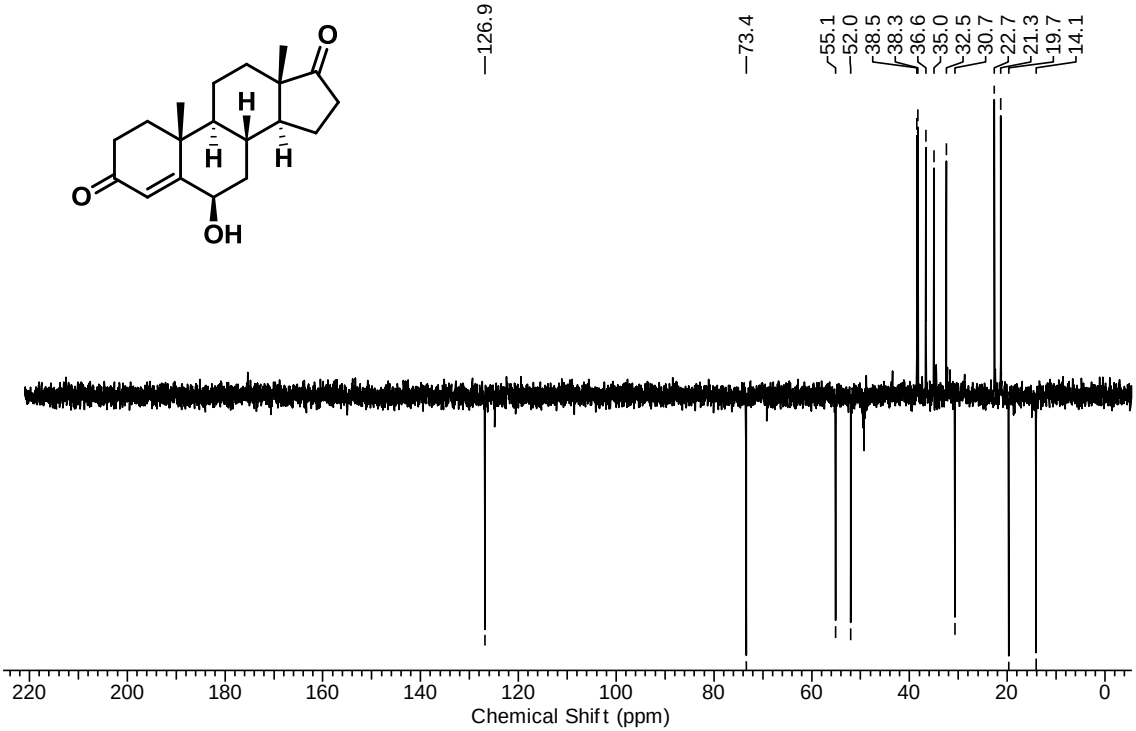
6β-hydroxy-4-androstene-3,17-dione (4a)



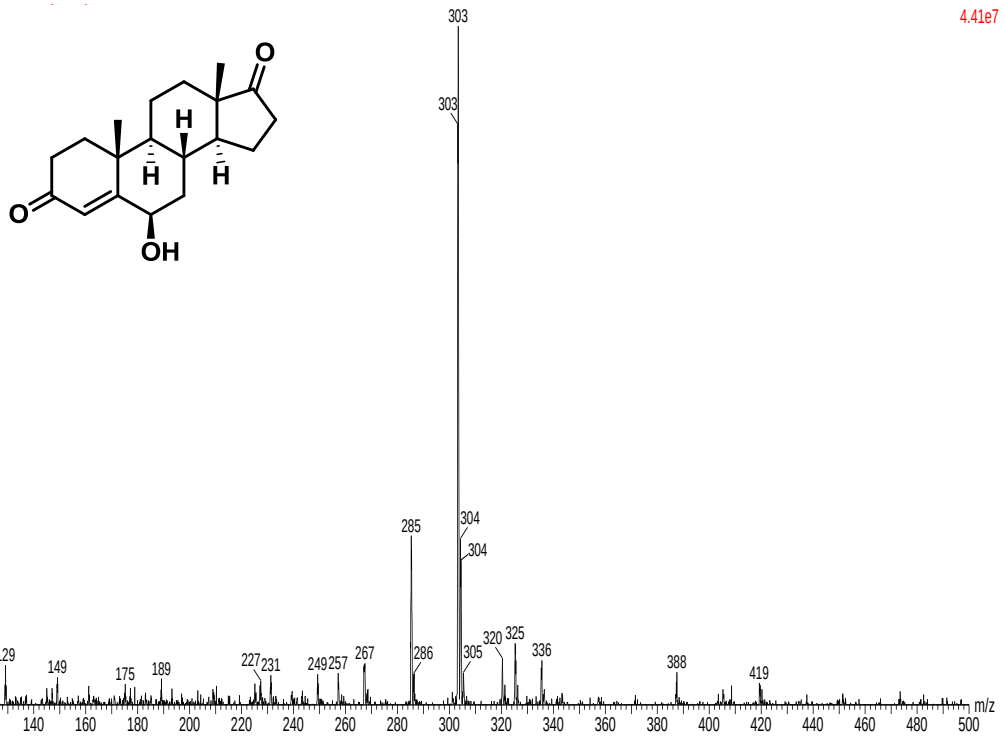
^{1}H NMR spectrum of **4a** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **4a** in CD_3OD at 100 MHz.

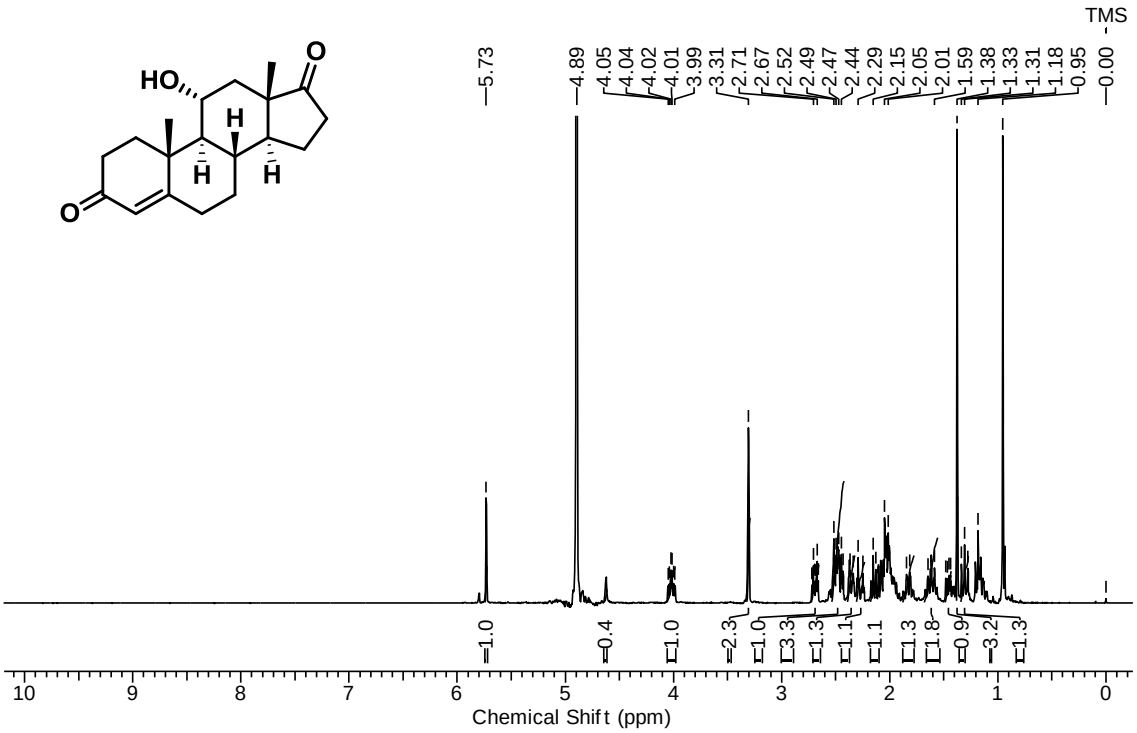


DEPT NMR spectrum of **4a** in CD_3OD at 100 MHz.

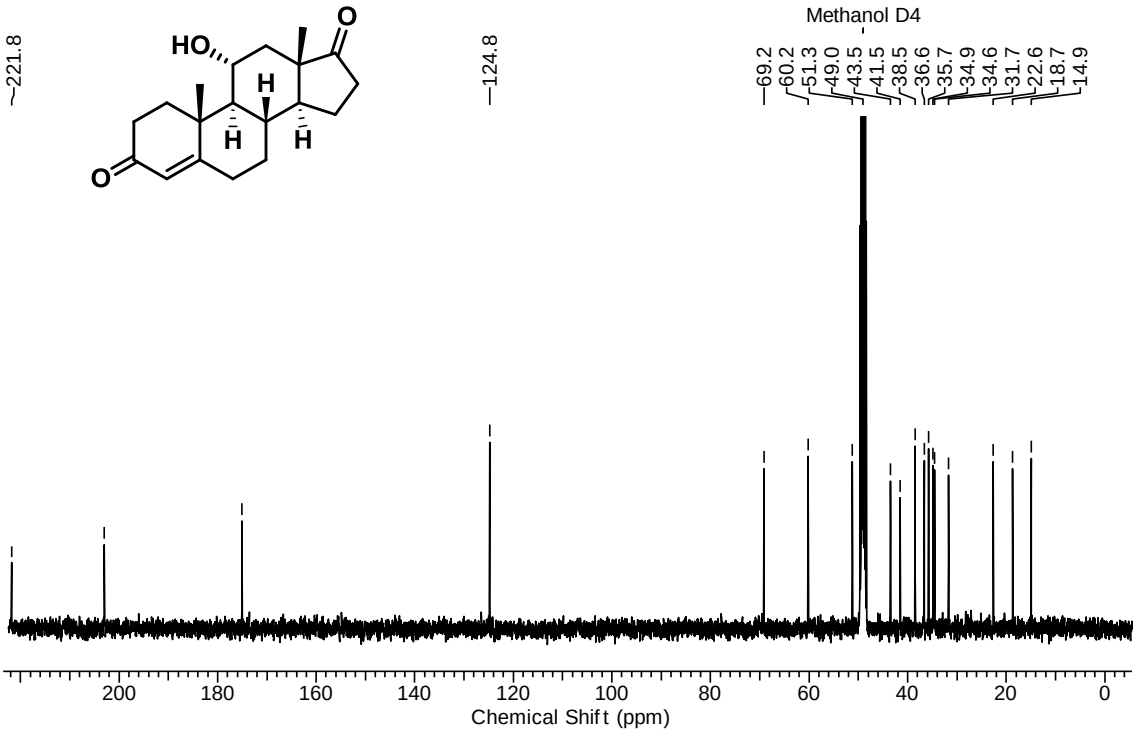


Mass spectrum of **4a** (ESI+ mode)

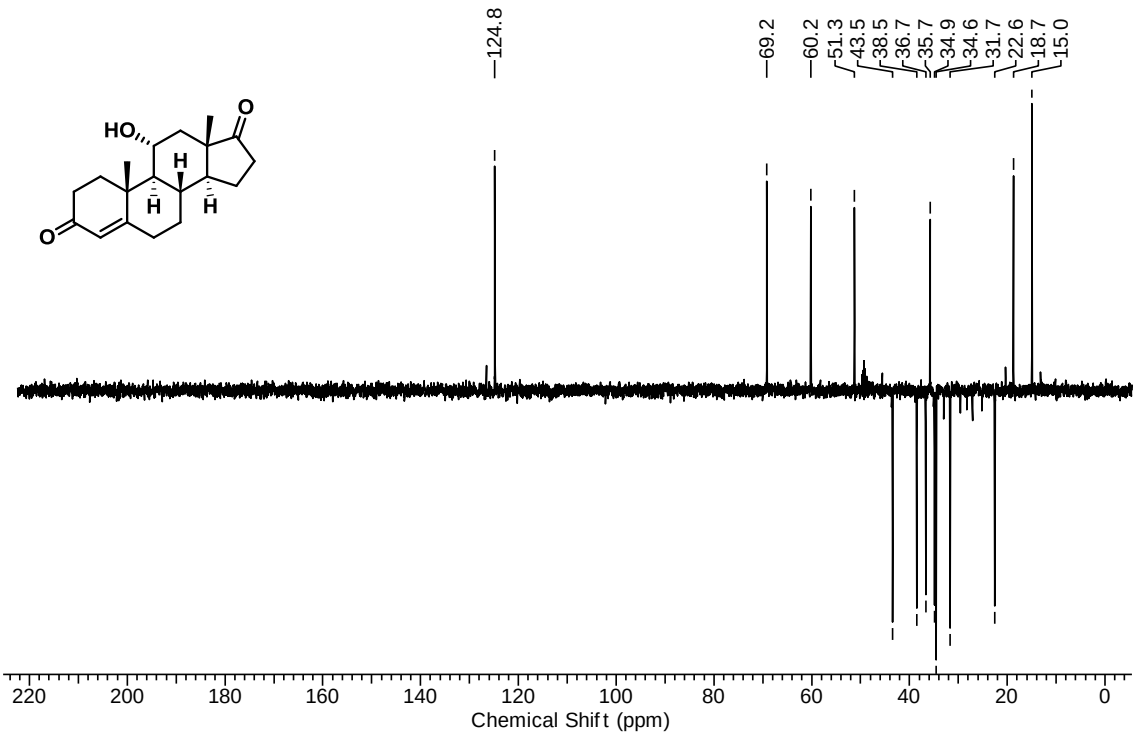
11α-hydroxy-4-androstene-3,17-dione (4b)



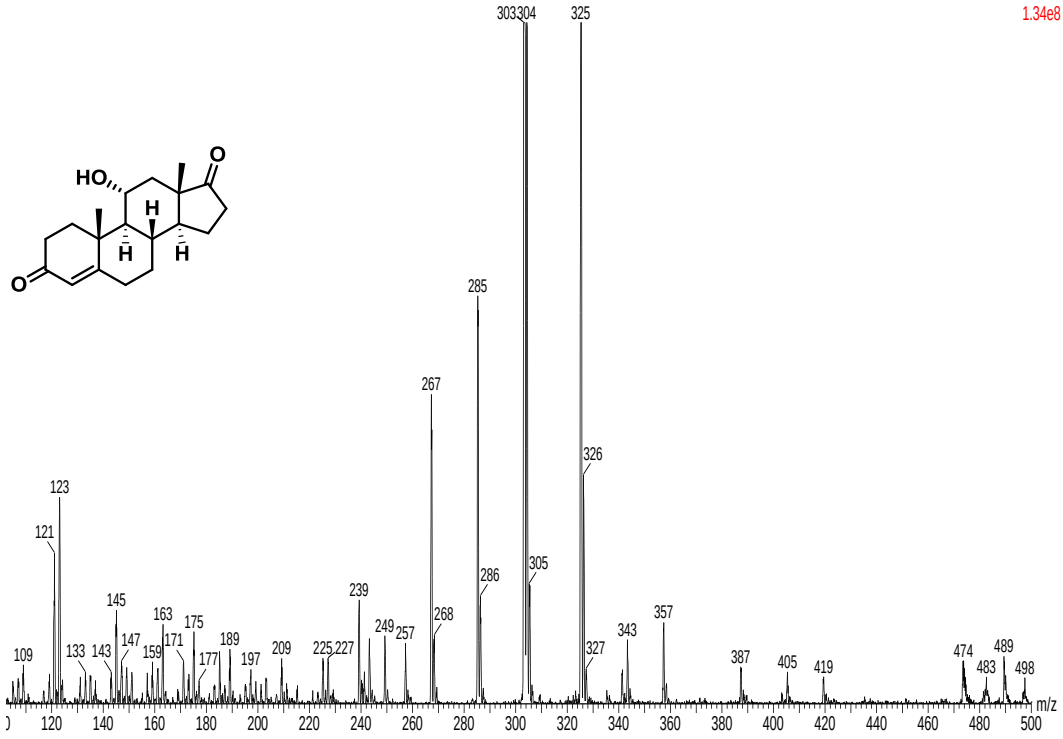
^{1}H NMR spectrum of **4b** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **4b** in CD_3OD at 100 MHz.

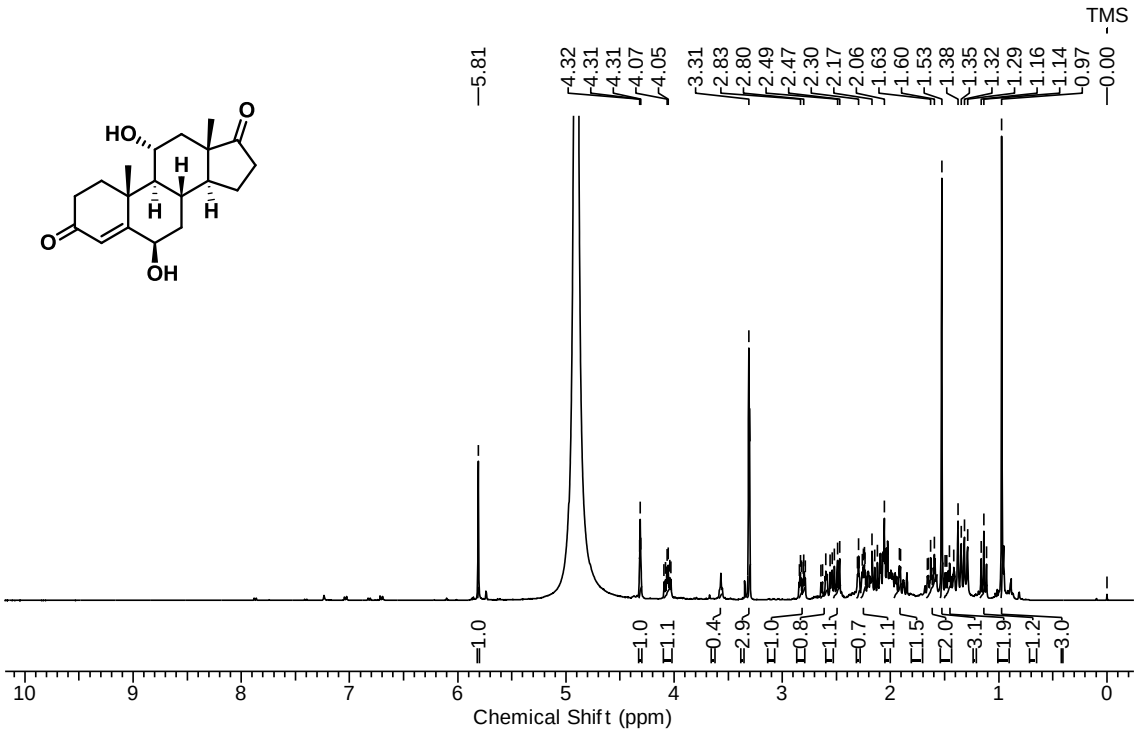


DEPT NMR spectrum of **4b** in CD_3OD at 100 MHz.

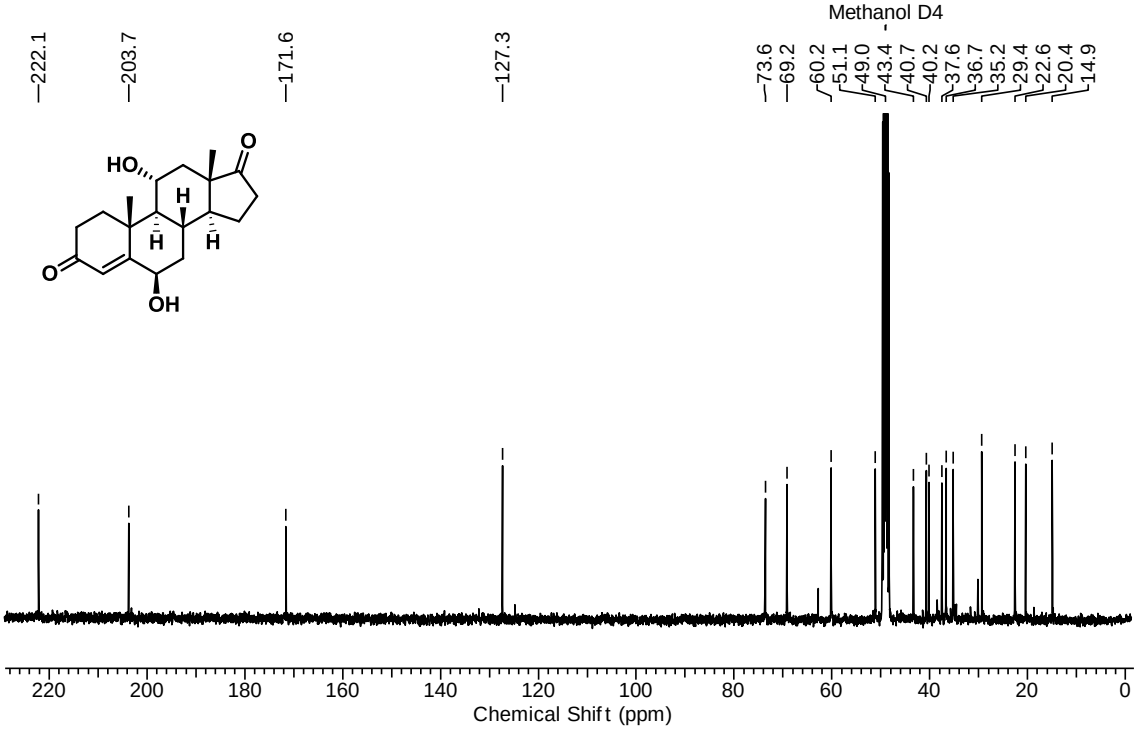


Mass spectrum of **4b** (ESI+ mode)

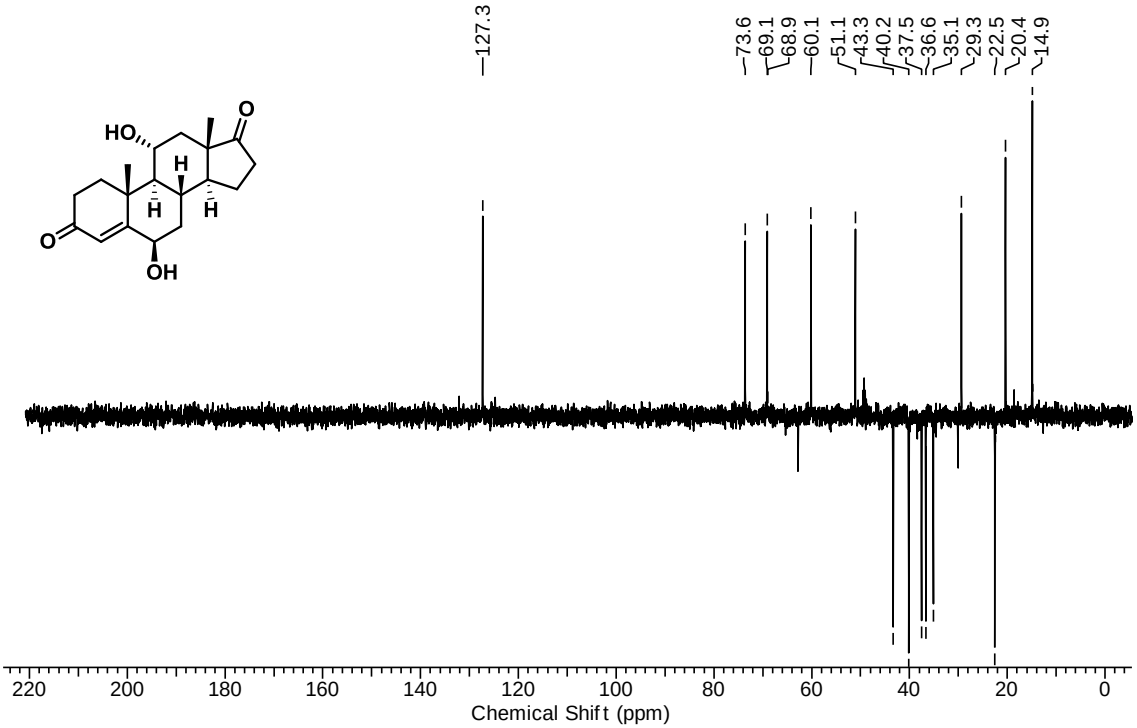
6β,11α-dihydroxy-4-androstene-3,17-dione (4c)



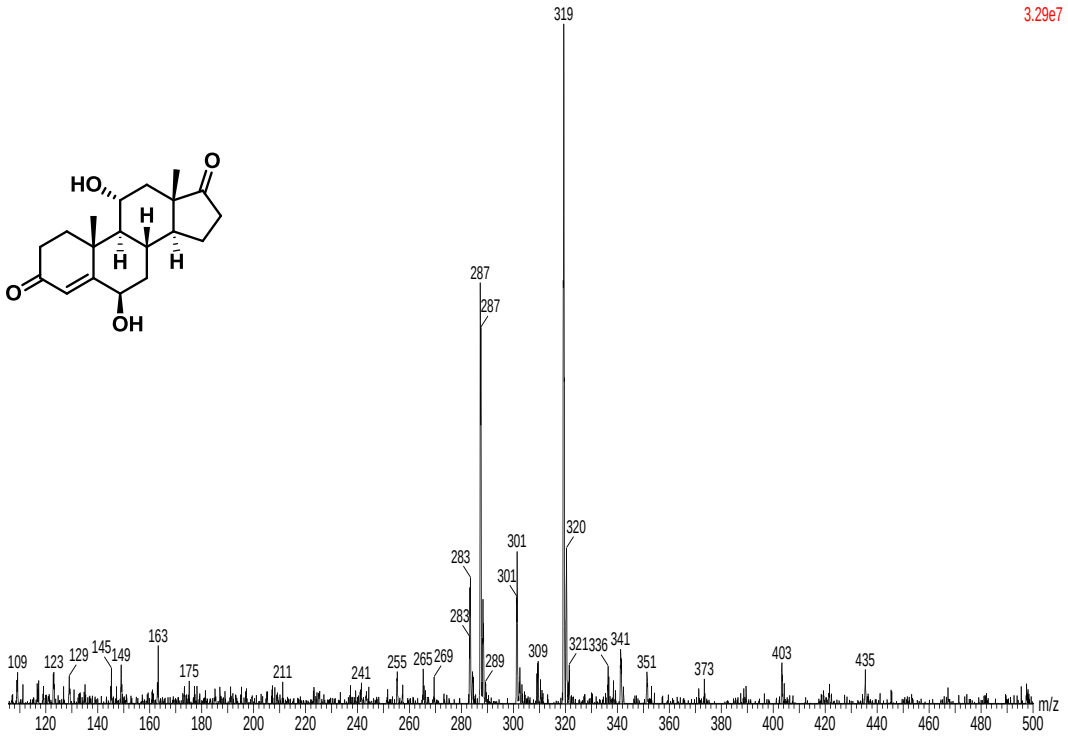
^{1}H NMR spectrum of **4c** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **4c** in CD_3OD at 100 MHz.

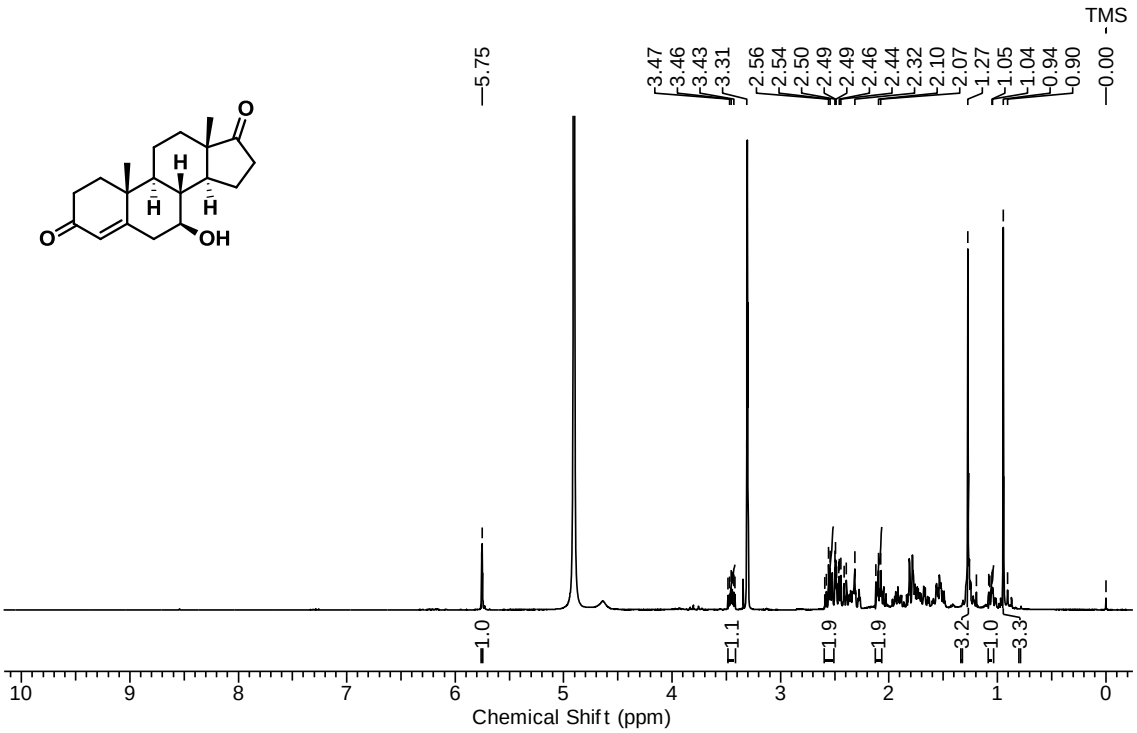


DEPT NMR spectrum of **4c** in CD_3OD at 100 MHz.

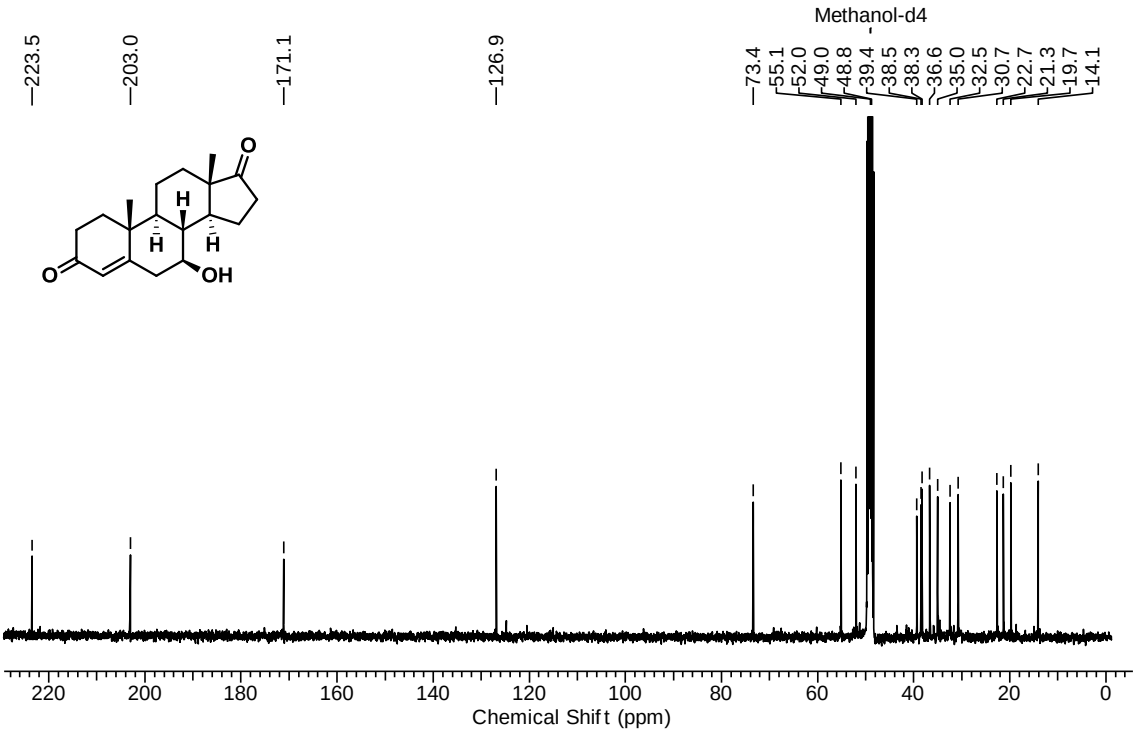


Mass spectrum of **4c** (ESI+ mode)

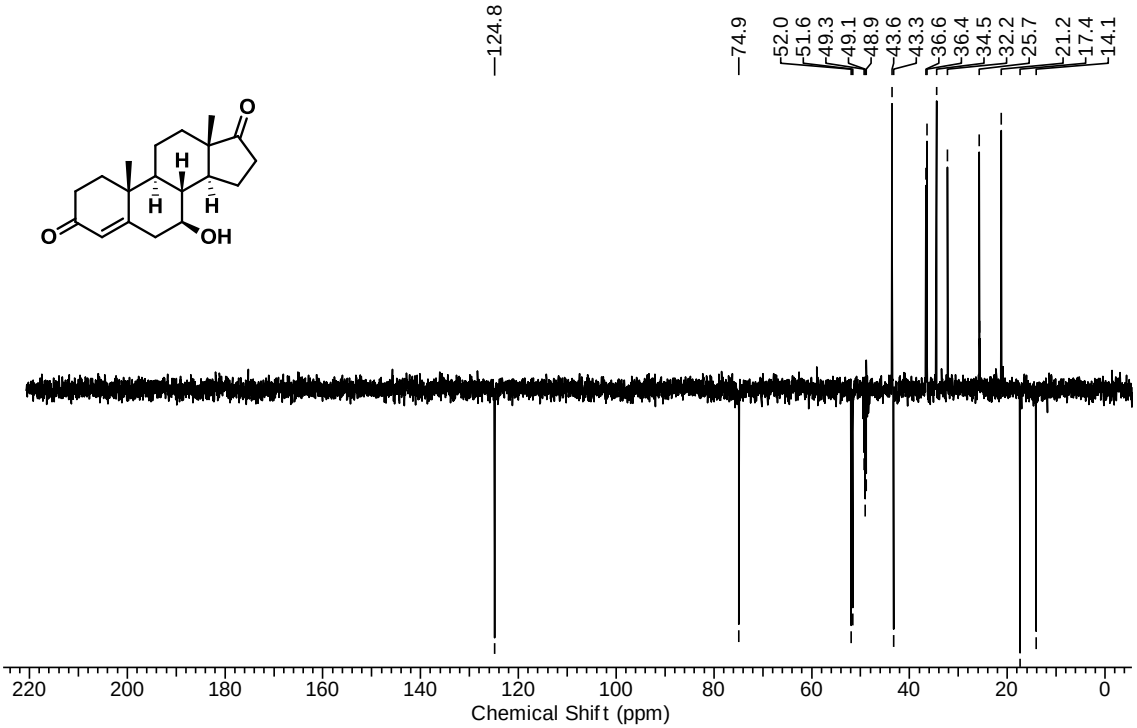
7β-hydroxy-4-androstene-3,17-dione (4d)



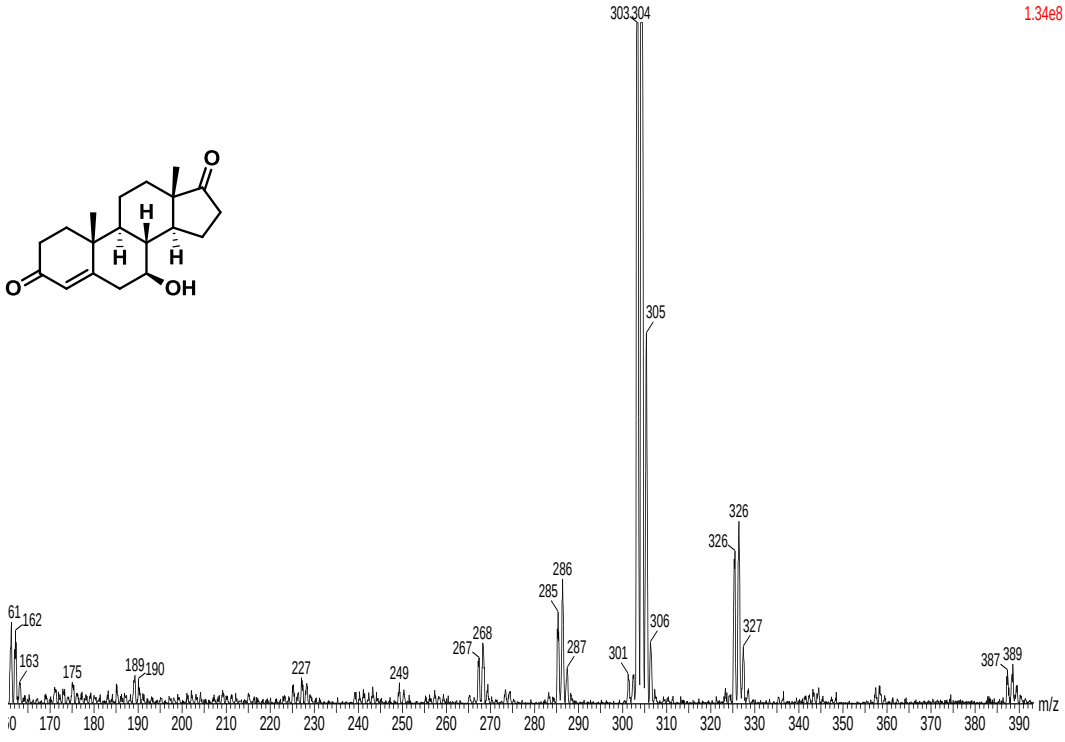
^{1}H NMR spectrum of **4d** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **4d** in CD_3OD at 100 MHz.

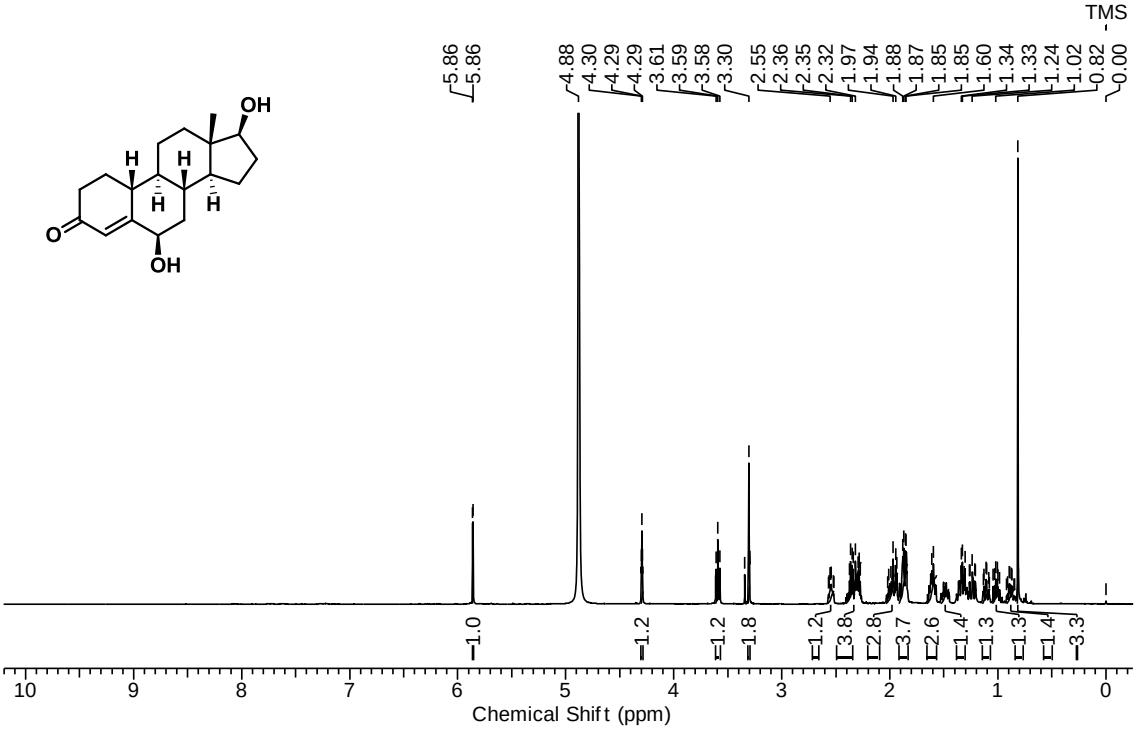


DEPT NMR spectrum of **4d** in CD_3OD at 100 MHz.

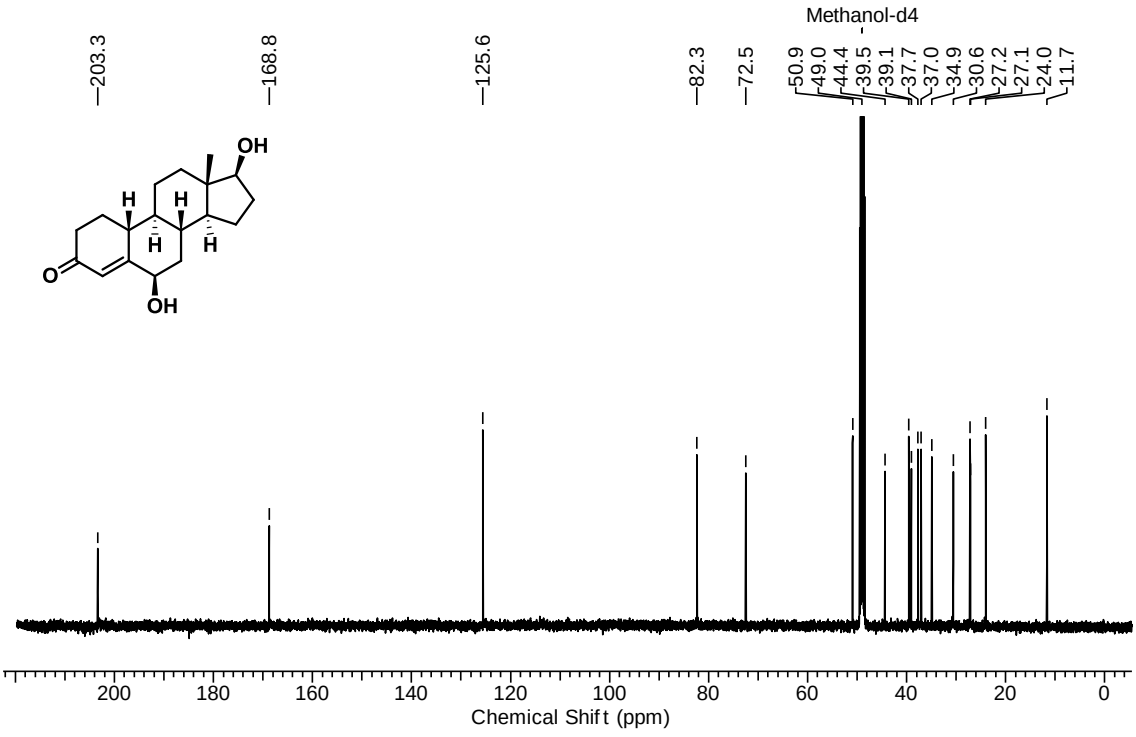


Mass spectrum of **4d** (ESI+ mode)

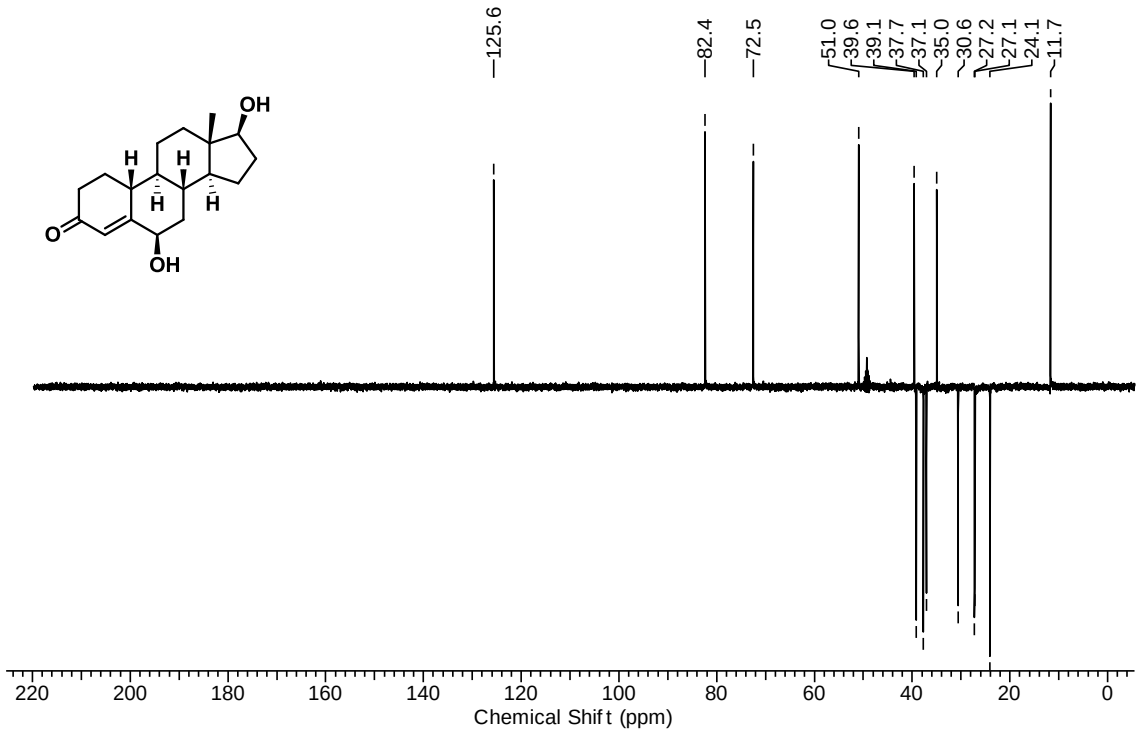
6β-hydroxy-19-nortestosterone (5a)



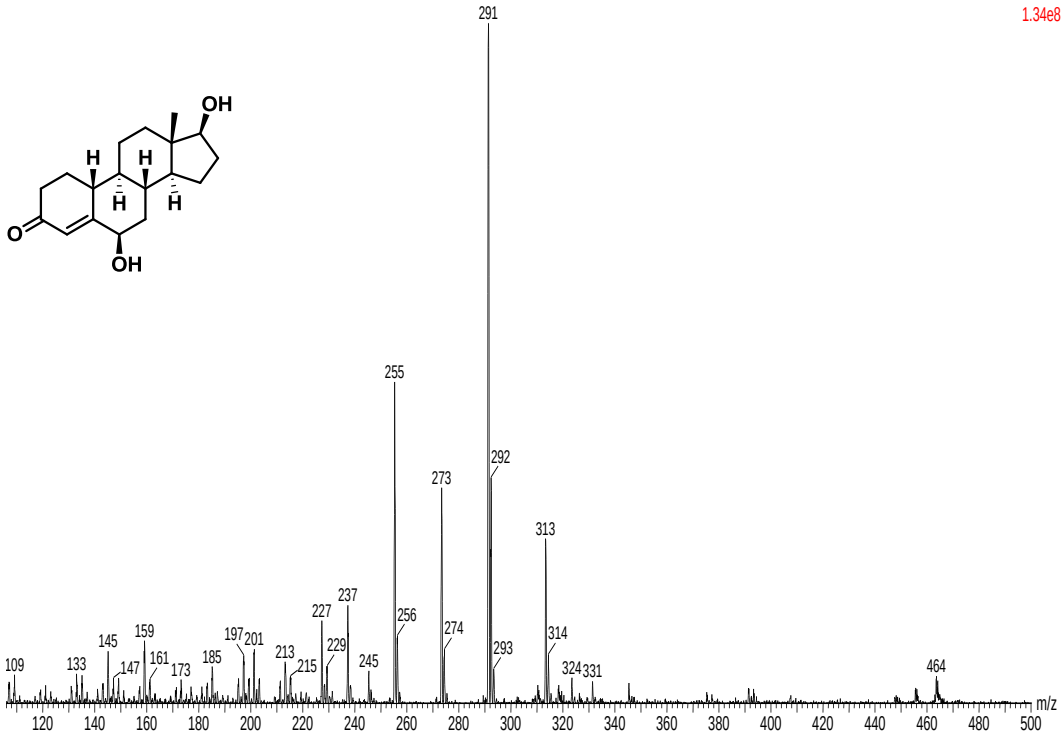
^{1}H NMR spectrum of **5a** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **5a** in CD_3OD at 100 MHz.

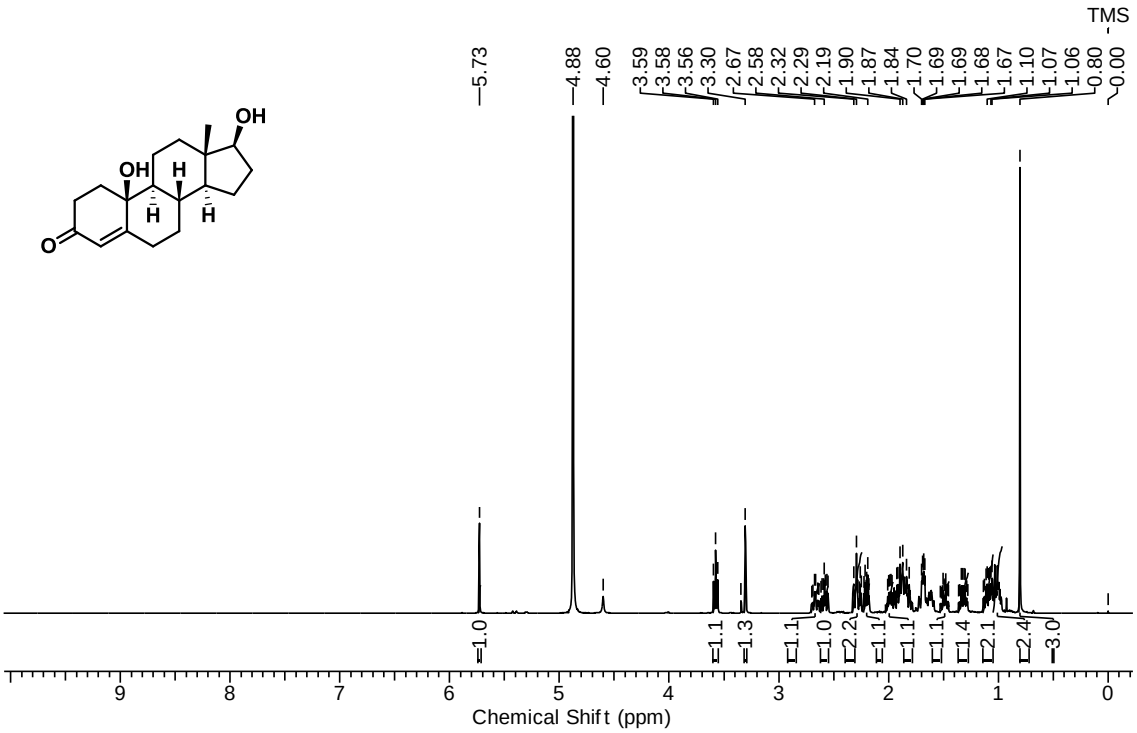


DEPT NMR spectrum of **5a** in CD_3OD at 100 MHz.

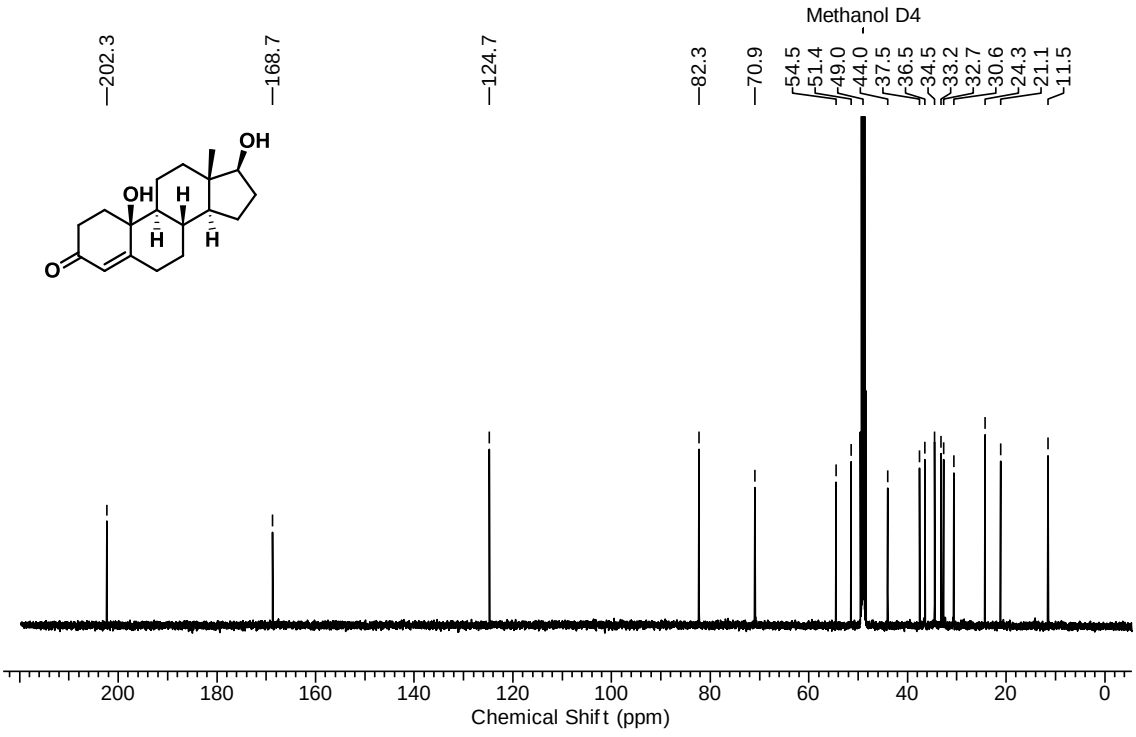


Mass spectrum of **5a** (ESI+ mode)

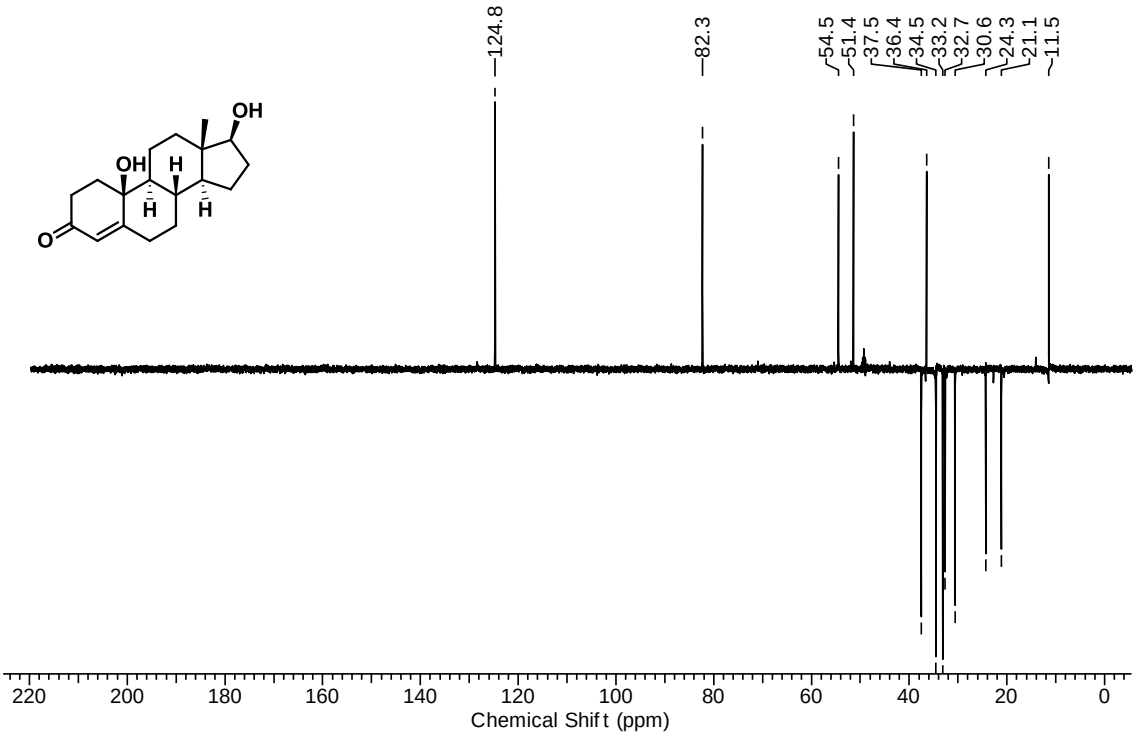
10β-hydroxy-19-nortestosterone (5b)



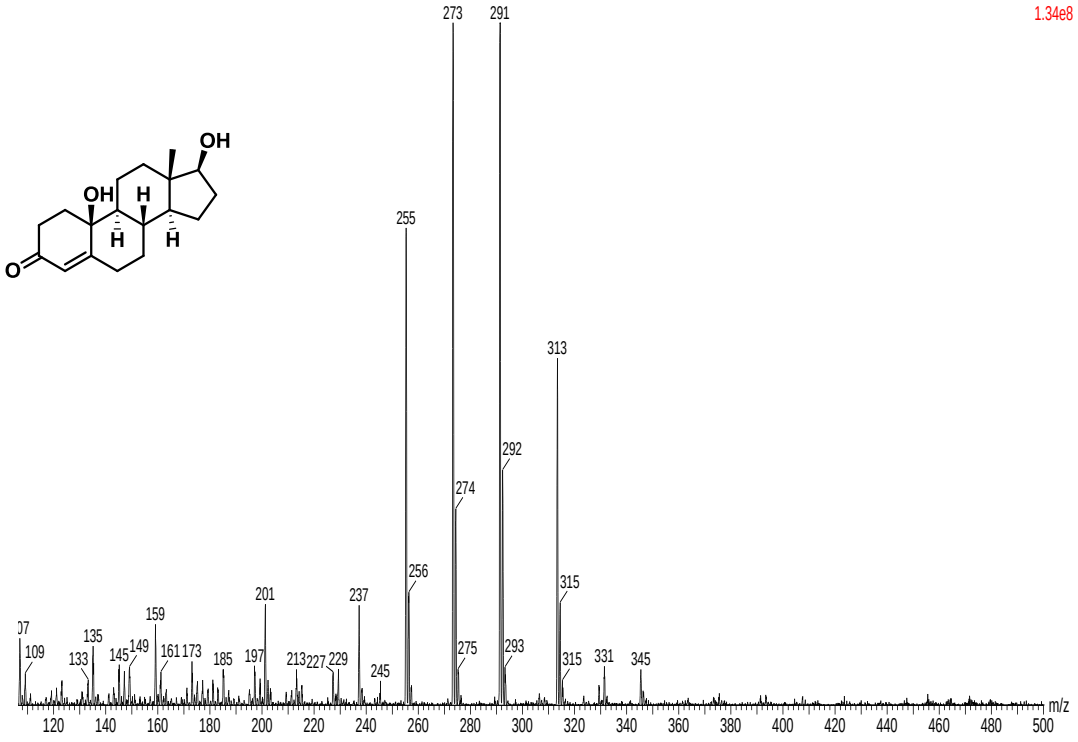
^{1}H NMR spectrum of **5b** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **5b** in CD_3OD at 100 MHz.

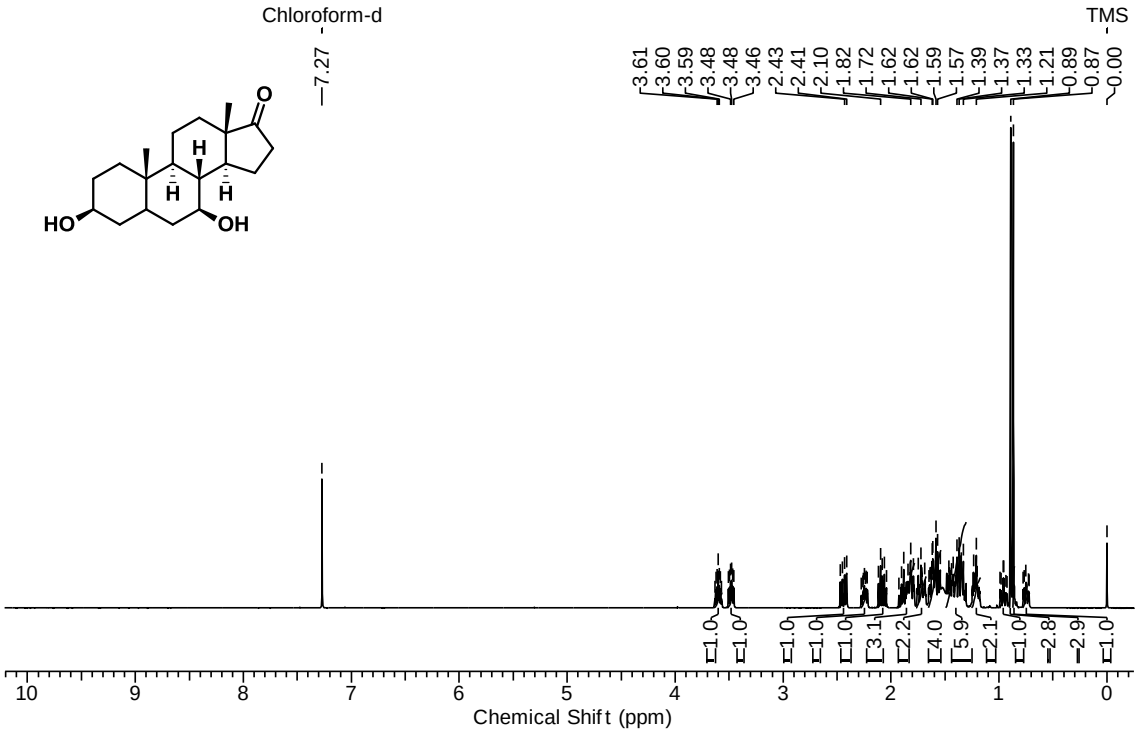


DEPT NMR spectrum of **5b** in CD_3OD at 100 MHz.

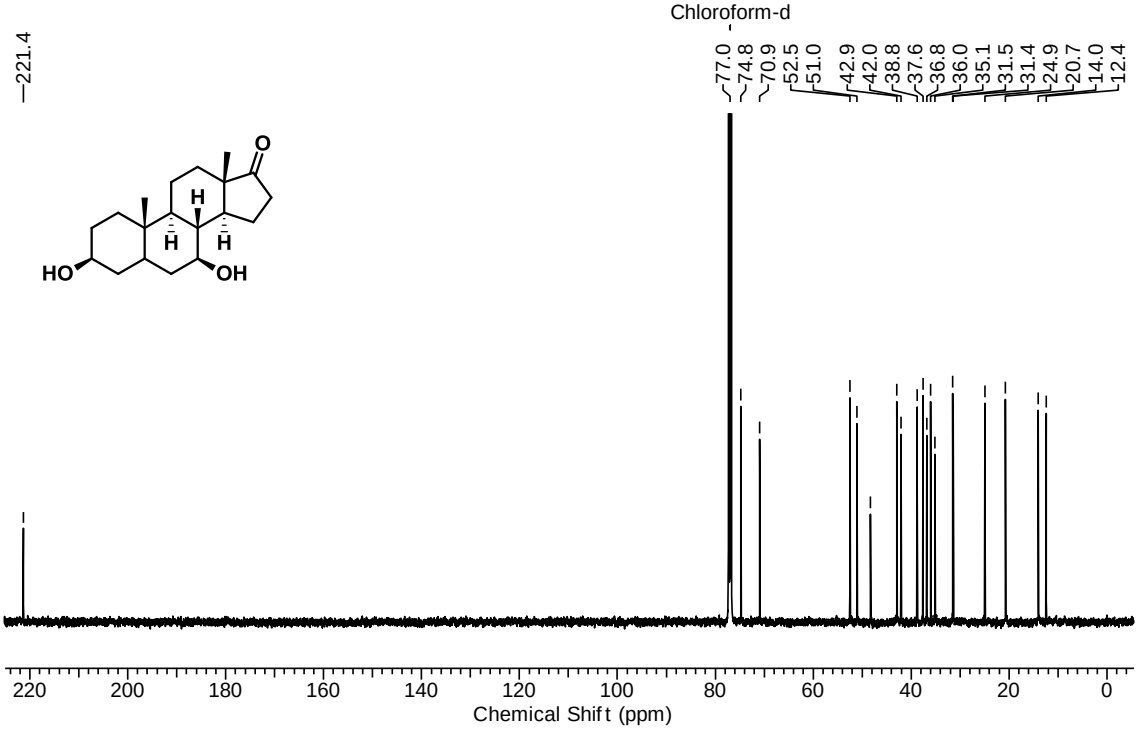


Mass spectrum of **5b** (ESI+ mode)

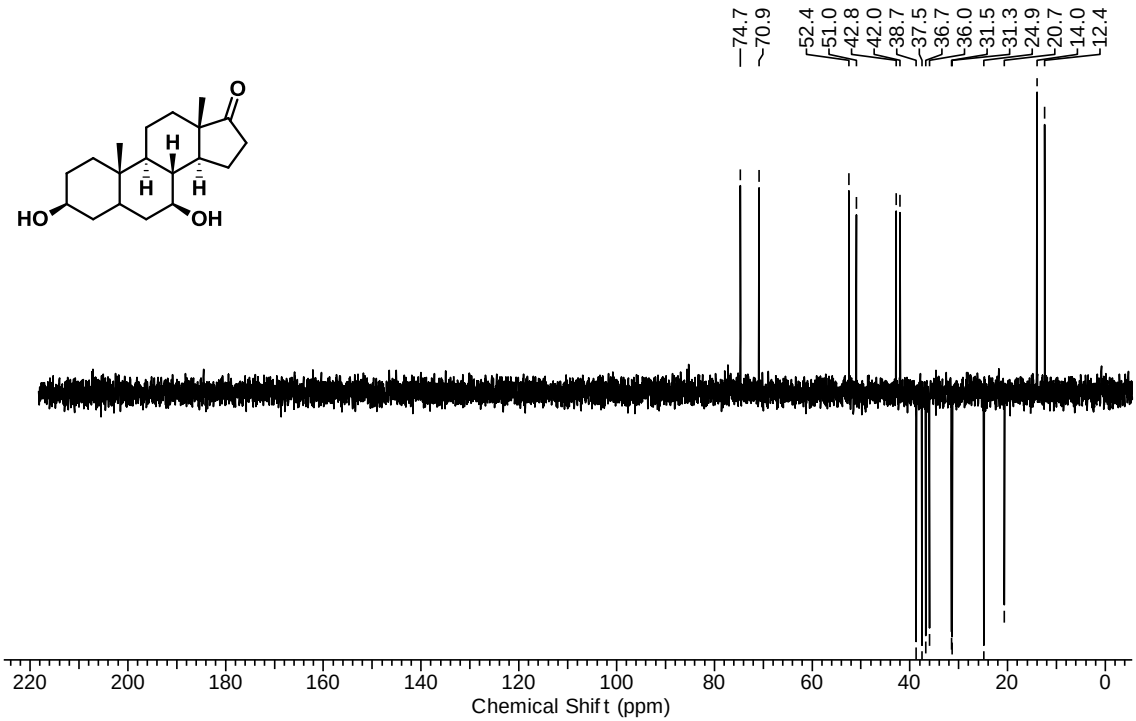
7β-hydroxy-epiandrosterone (6a)



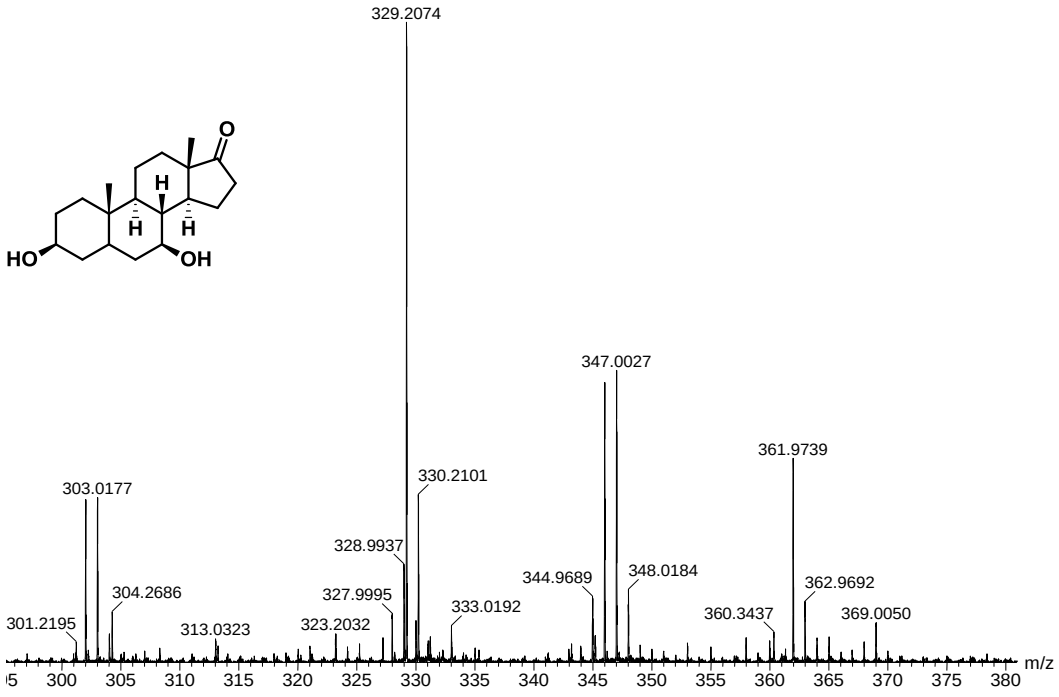
^{1}H NMR spectrum of **6a** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **6a** in CD_3OD at 100 MHz.

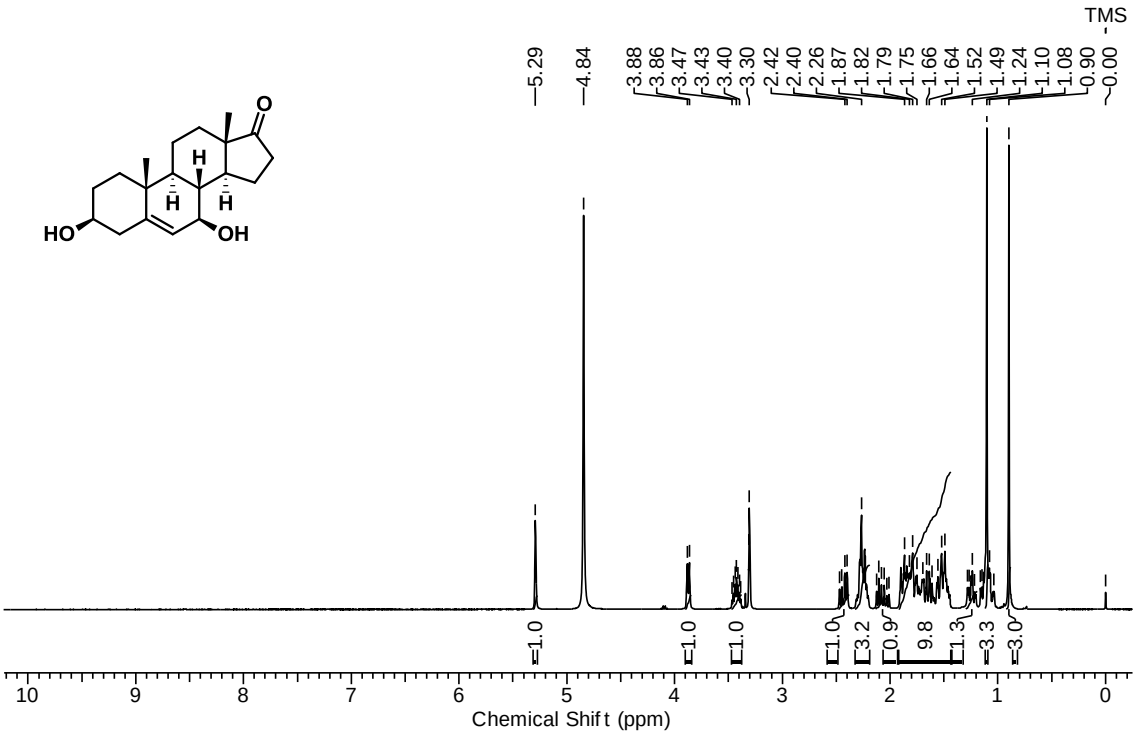


DEPT NMR spectrum of **6a** in CD_3OD at 100 MHz.

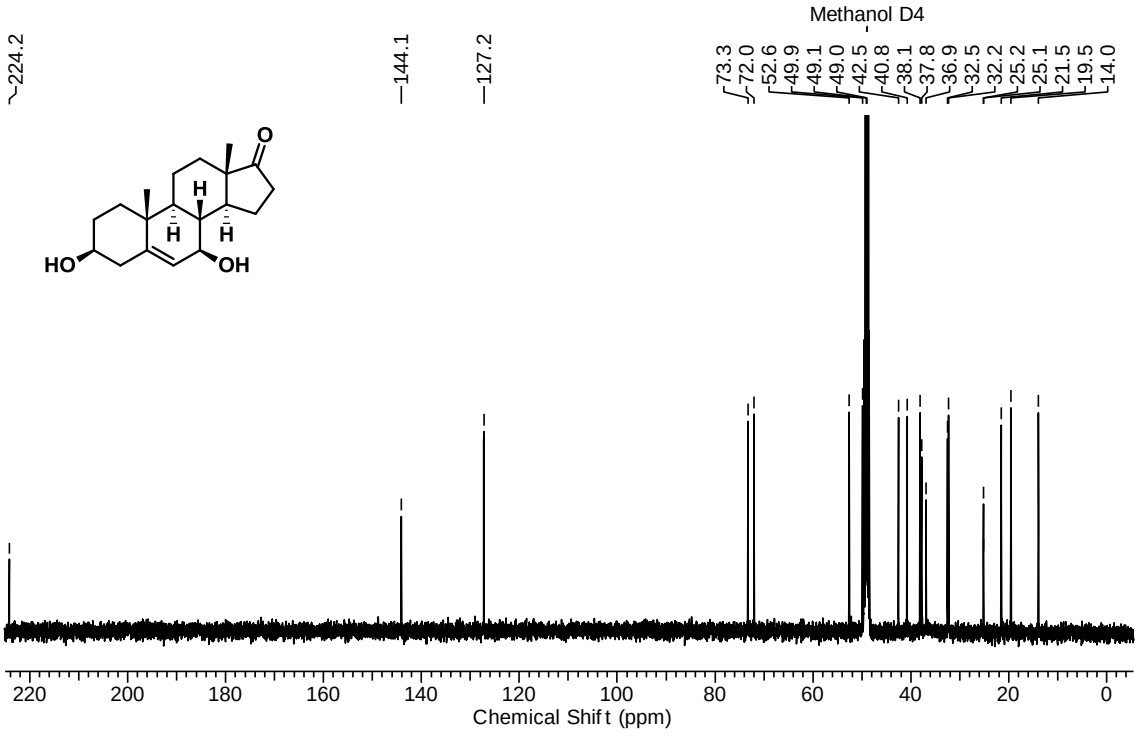


Mass spectrum of **6a** (ESI+ mode)

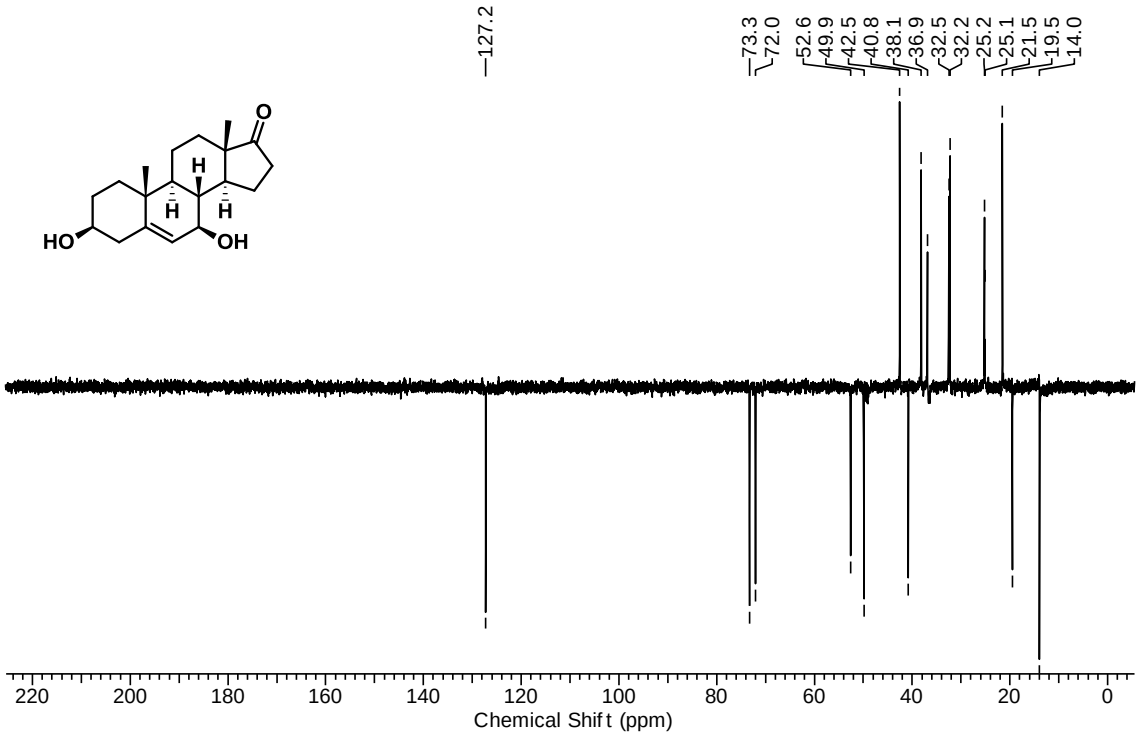
7β-hydroxy-dehydroepiandrosterone (7a)



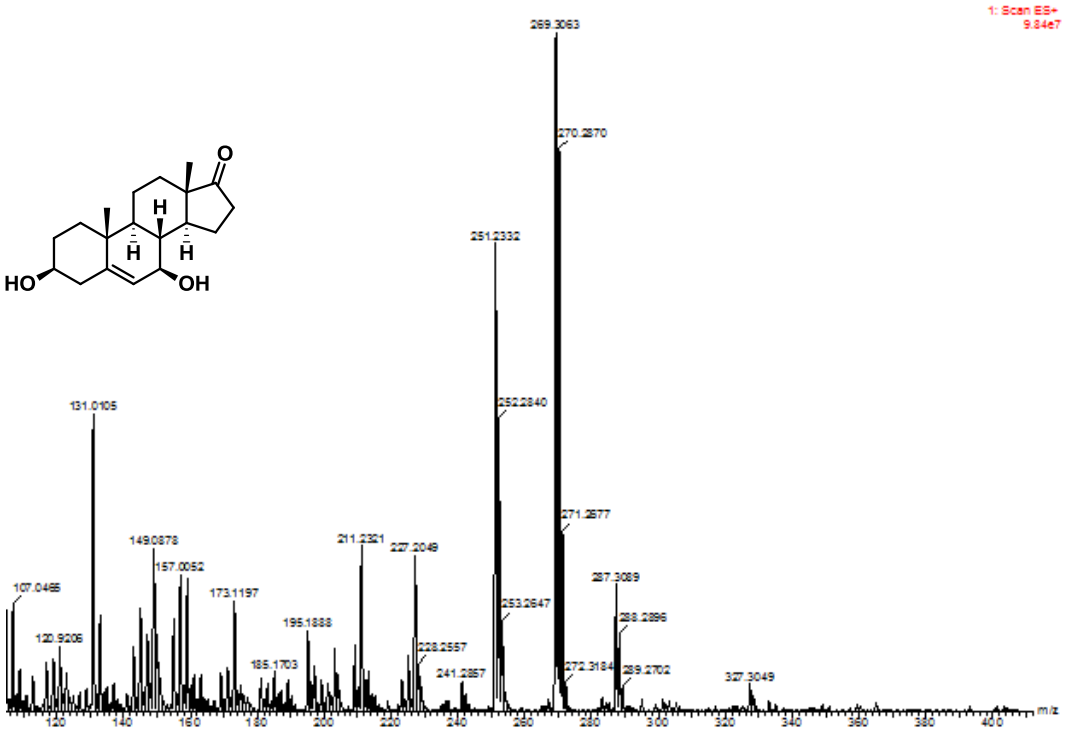
^{1}H NMR spectrum of **7a** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **7a** in CD_3OD at 100 MHz.

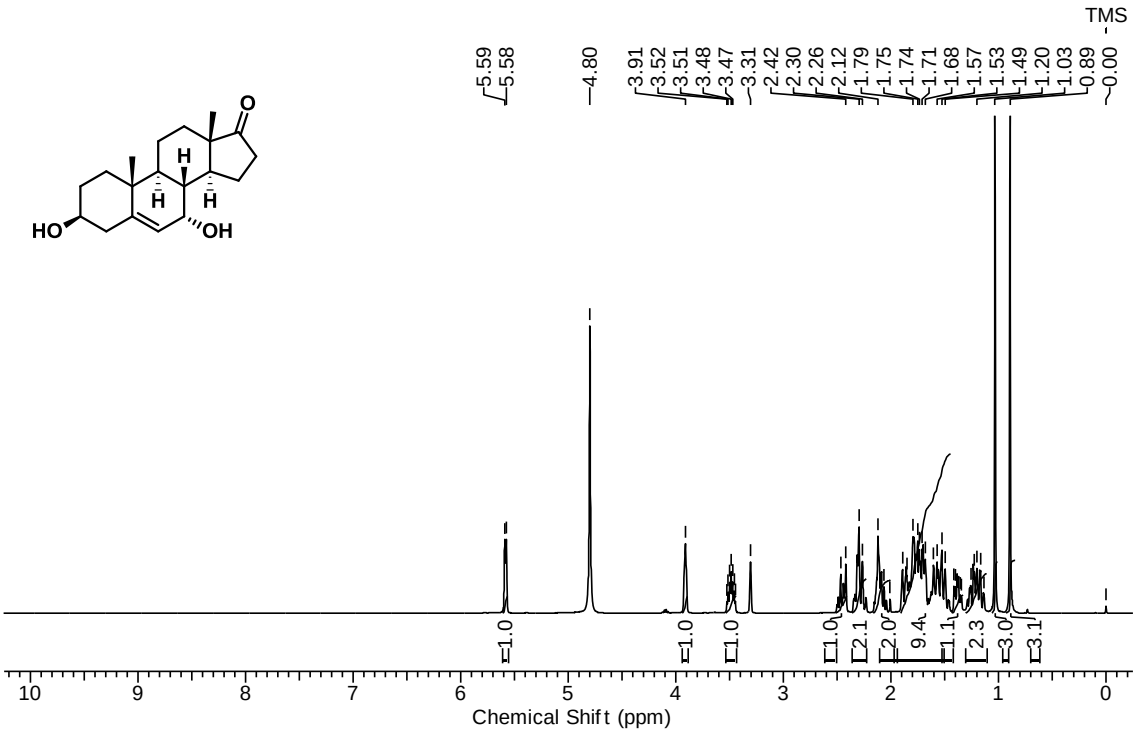


DEPT NMR spectrum of **7a** in CD_3OD at 100 MHz.

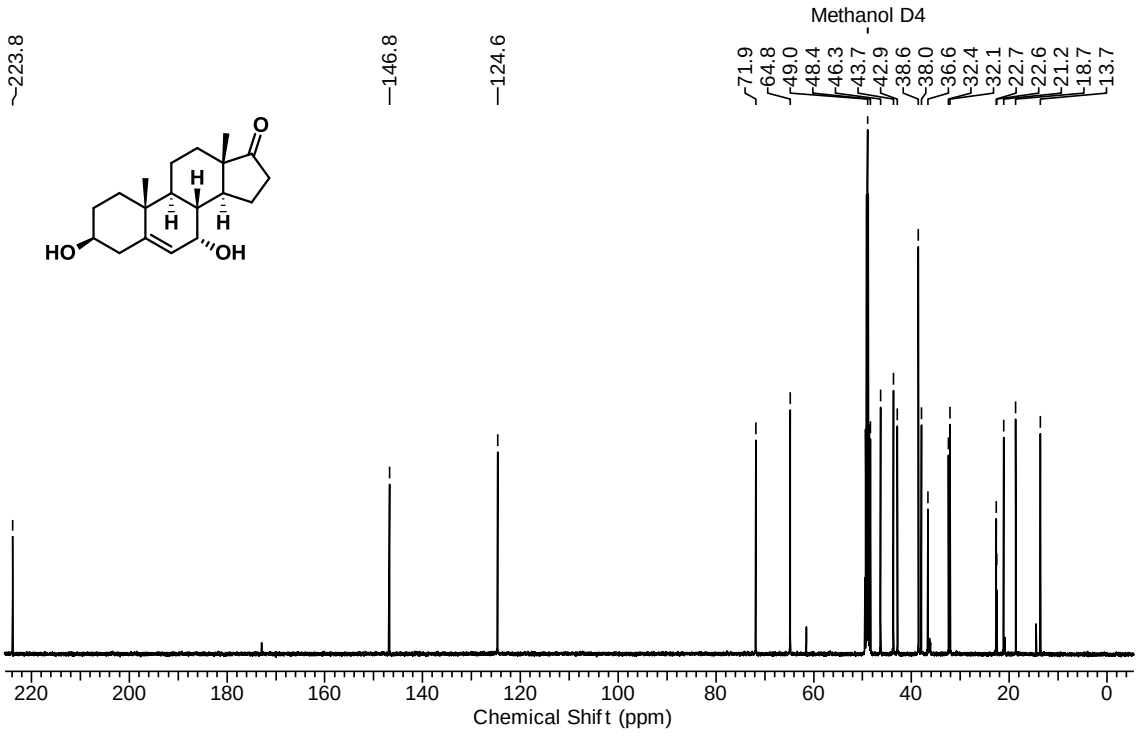


Mass spectrum of **7a** (ESI+ mode)

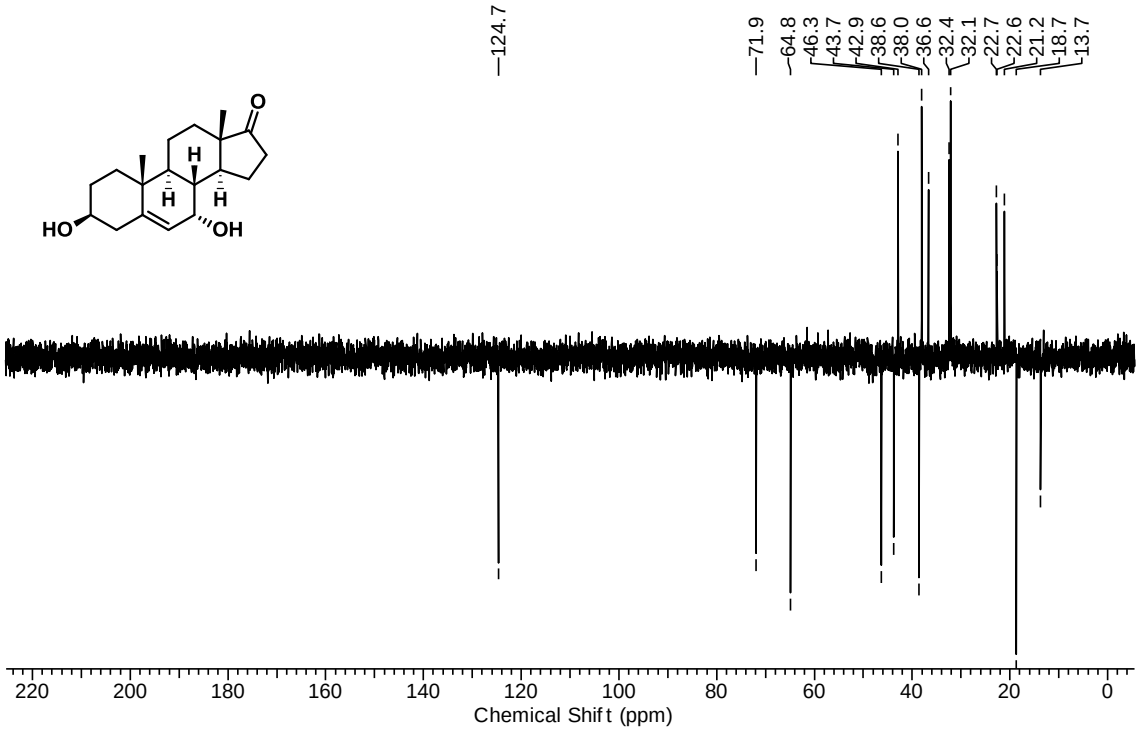
7α-hydroxy-dehydroepiandrosterone (7b)



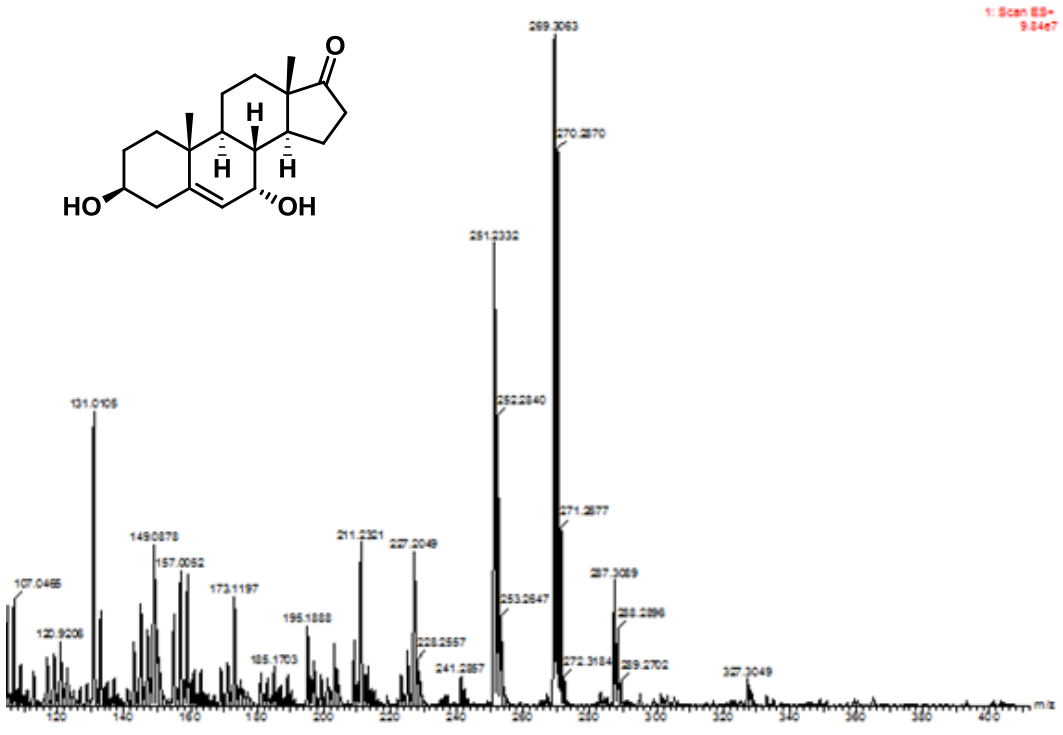
^{1}H NMR spectrum of **7b** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **7b** in CD_3OD at 100 MHz.

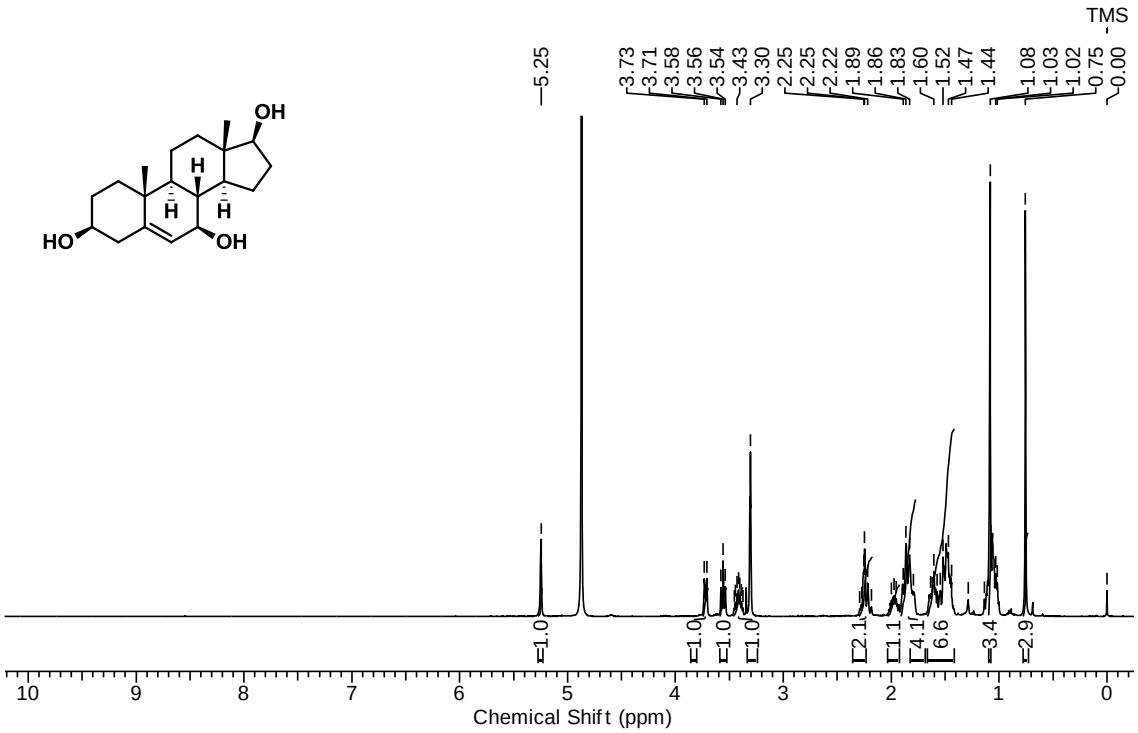


DEPT NMR spectrum of **7b** in CD_3OD at 100 MHz.

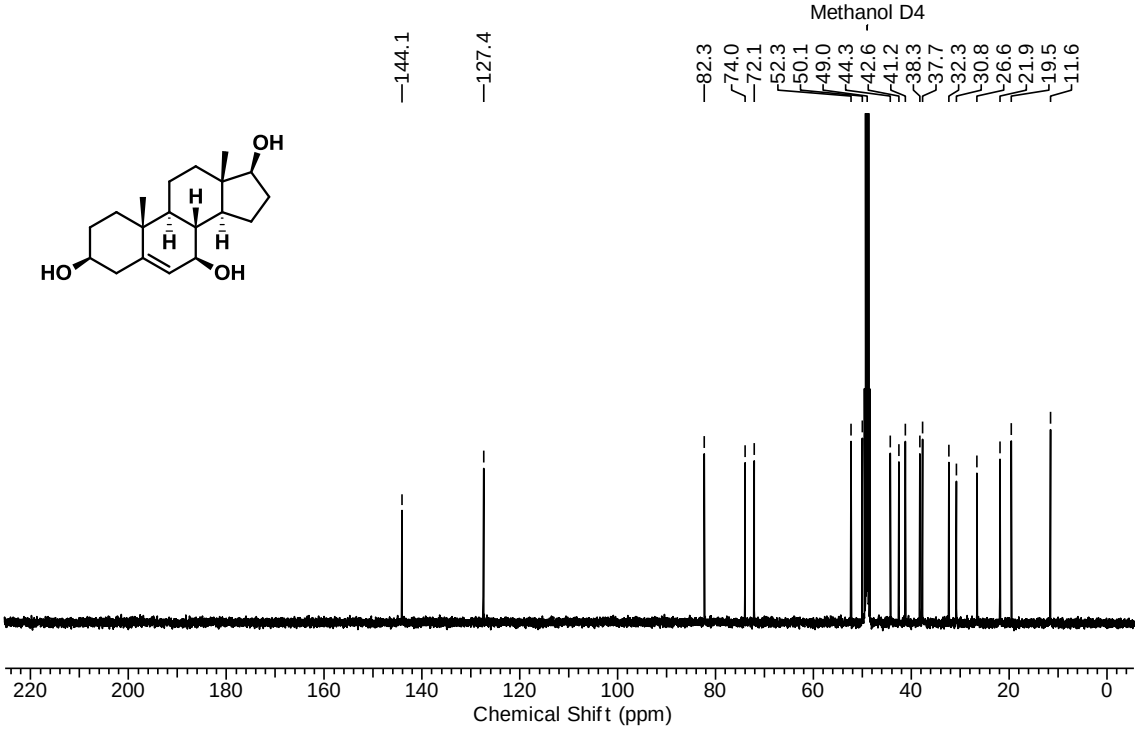


Mass spectrum of **7b** (ESI+ mode)

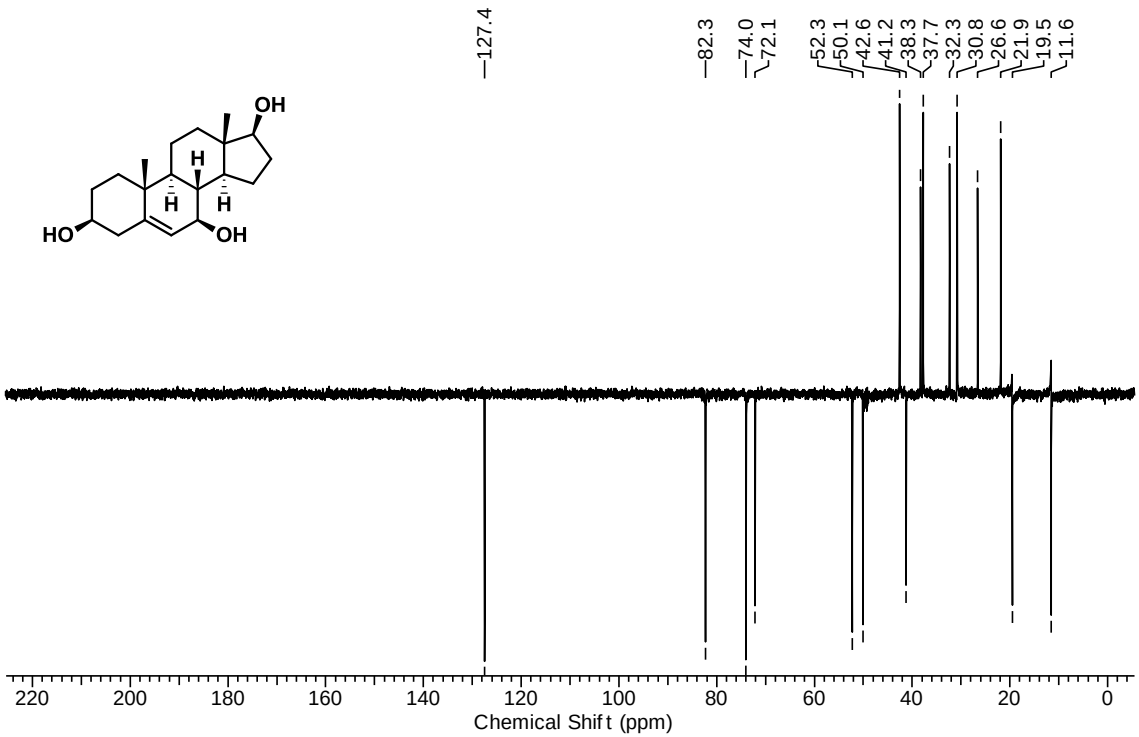
3β,7β,17β-trihydroxyandrost-5-ene (7c)



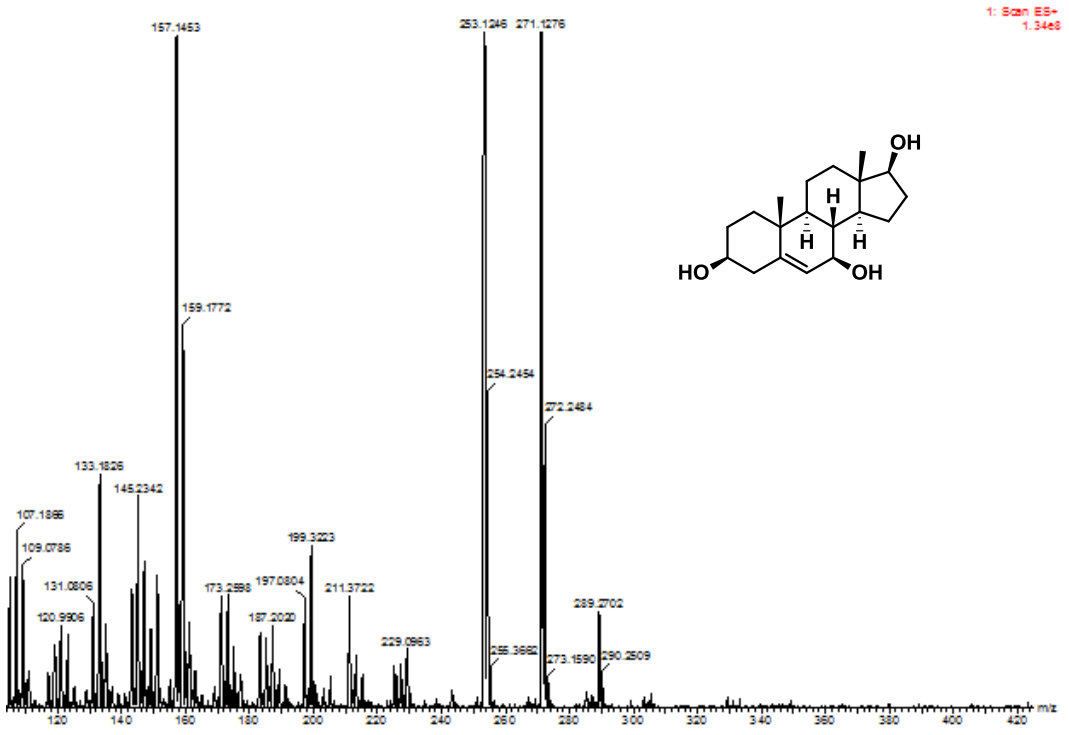
^{1}H NMR spectrum of **7c** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **7c** in CD_3OD at 100 MHz.

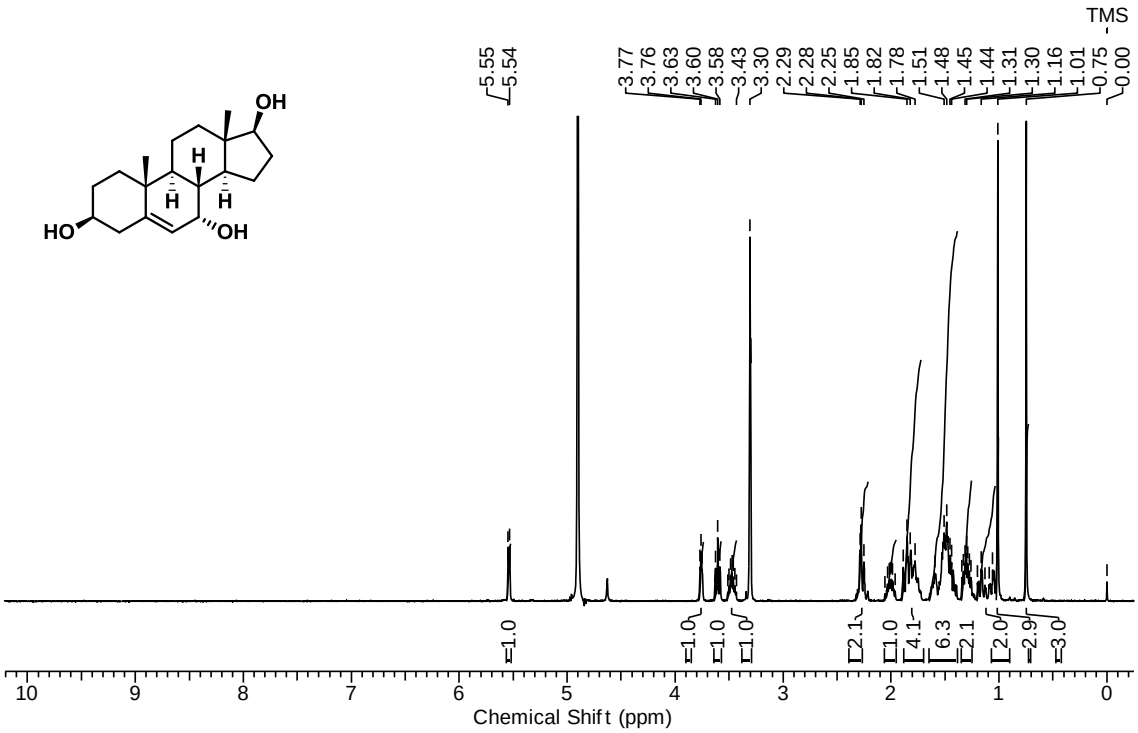


DEPT NMR spectrum of **7c** in CD_3OD at 100 MHz.

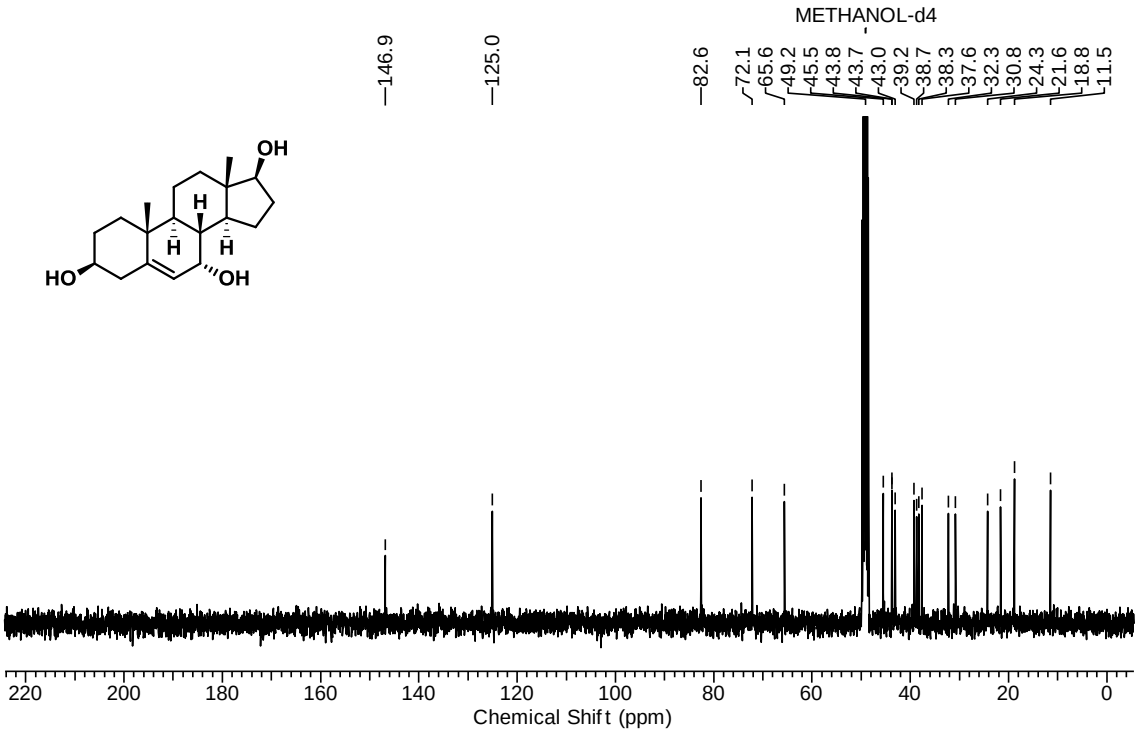


Mass spectrum of **7c** (ESI+ mode)

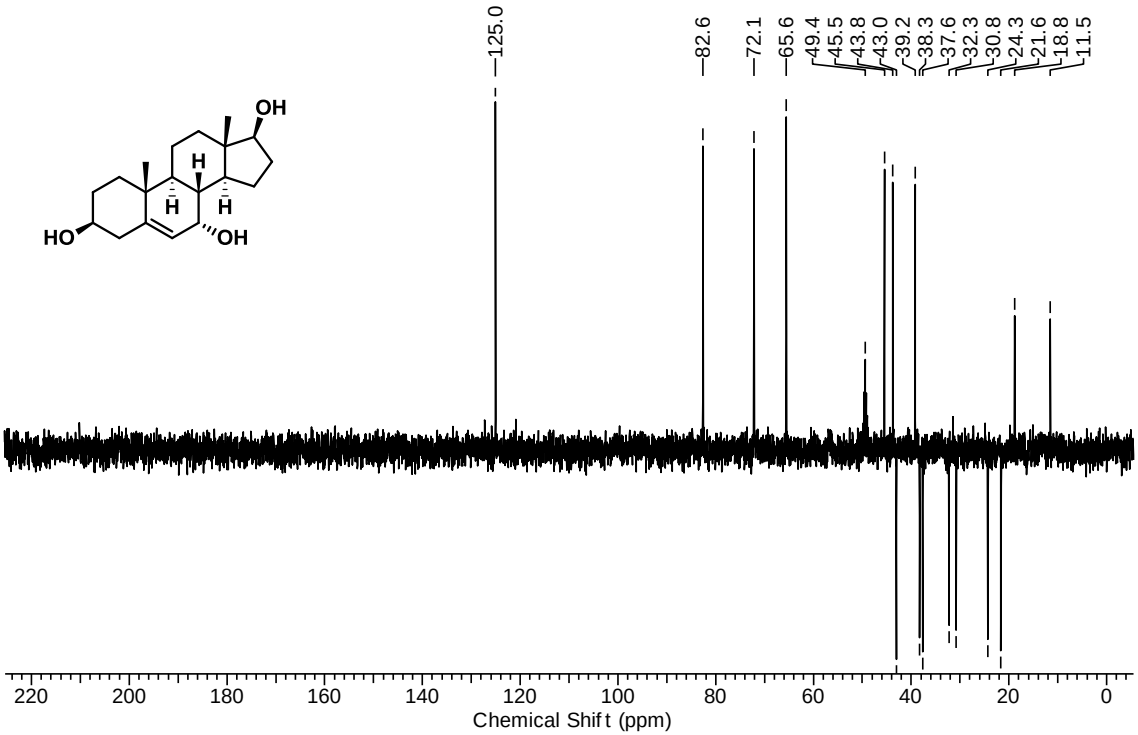
3β,7α,17β-trihydroxyandrost-5-ene (7d)



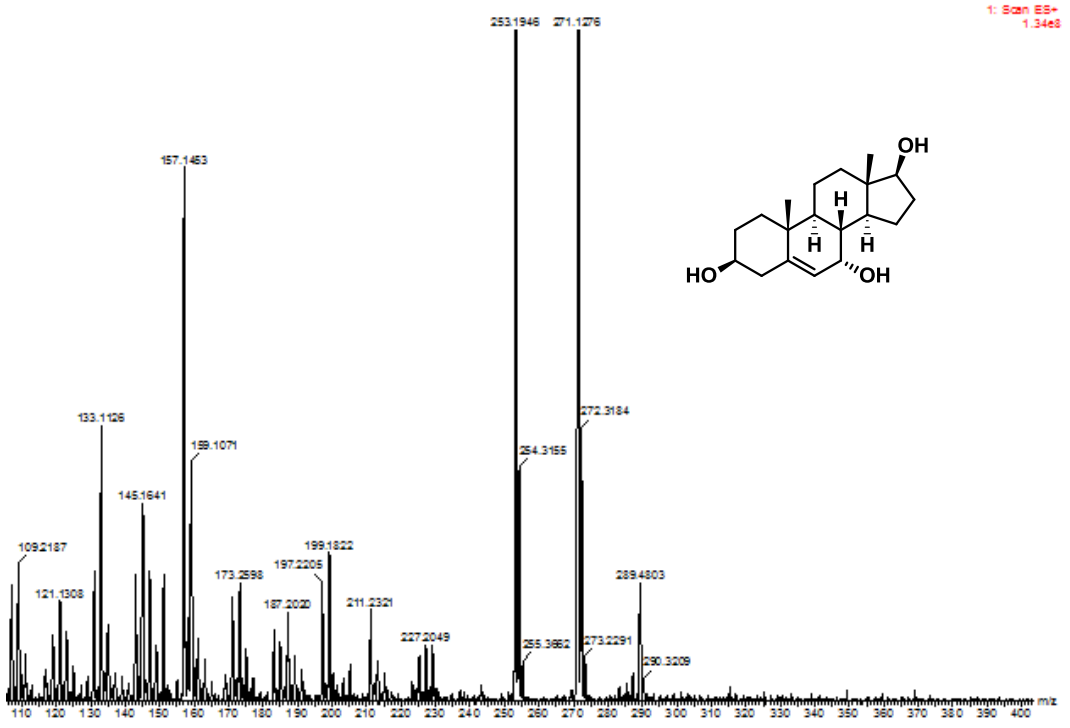
^{1}H NMR spectrum of **7d** in CD_3OD at 400 MHz



^{13}C NMR spectrum of **7d** in CD_3OD at 100 MHz.



DEPT NMR spectrum of **7d** in CD_3OD at 100 MHz.



Mass spectrum of **7d** (ESI+ mode)

3.8: References

(1) Hogg, J. A. *Steroids* **1992**, *57*, 593-616.

(2) Carballeira, J. D.; Quezada, M. A.; Hoyos, P.; Simeo, Y.; Hernaiz, M. J.; Alcantara, A. R.; Sinisterra, J. V. *Biotechnol. Adv.* **2009**, *27*, 686-714.

(3) Al-Awadi, S.; Afzal, M.; Oommen, S. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 48-52.

(4) Mahato, S. B.; Garai, S. *Steroids* **1997**, *62*, 332-345.

(5) Koenig, H. L.; Schumacher, M.; Ferzaz, B.; Dothi, A. N.; Ressouches, A.; Guennoun, R.; Jungtestas, I.; Robel, P.; Akwa, Y.; Baulieu, E. E. *Science* **1995**, *268*, 1500-1503.

(6) Traish, A. M.; Saad, F.; Guay, A. *J. Androl.* **2009**, *30*, 23-32.

(7) Powers, M. L.; Florini, J. R. *Endocrinology* **1975**, *97*, 1043-1047.

(8) Mor, G.; Eliza, M.; Song, J.; Wiita, B.; Chen, S.; Naftolin, F. *J. Steroid Biochem.* **2001**, *79*, 239-246.

(9) Kliewer, S. A.; Moore, J. T.; Wade, L.; Staudinger, J. L.; Watson, M. A.; Jones, S. A.; McKee, D. D.; Oliver, B. B.; Willson, T. M.; Zetterstrom, R. H.; Perlmann, T.; Lehmann, J. M. *Cell* **1998**, *92*, 73-82.

(10) Ashley, R. L.; Arreguin-Arevalo, J. A.; Nett, T. M. *Reprod. Biol. Endocrin.* **2009**, *7*.

(11) Spratt, D. I. *Best Prac. Res. Cl. En.* **2001**, *15*, 479-494.

(12) Jayyosi, Z.; Cooper, K. O.; Thomas, P. E. *Arch. Biochem. Biophys.* **1992**, *298*, 265-270.

(13) Hu, S. H.; Genain, G.; Azerad, R. *Steroids* **1995**, *60*, 337-352.

(14) Madyastha, K. M. *P. Indian A. S.-Chem. Sci.* **1994**, *106*, 1203-1212.

(15) Elmonem, A.; Elrefai, H.; Sallam, L. A.; Elkady, I. A. *J. Gen. Appl. Microbiol.* **1970**, *16*, 137-142.

(16) Peterson, D. H.; Murray, H. C.; Eppstein, S. H.; Reineke, L. M.; Weintraub, A.; Meister, P. D.; Leigh, H. M. *J. Am. Chem. Soc.* **1952**, *74*, 5933-5936.

(17) Farooq, A.; Hanson, J. R.; Iqbal, Z. *Phytochemistry* **1994**, *37*, 723-726.

(18) Al-Awadi, S.; Afzal, M.; Oommen, S. *Biocatal. Biotransfor.* **2005**, *23*, 323-328.

(19) Ye, M.; Guo, D. *J. Biotech.* **2005**, *117*, 253-262.

(20) Protiva, J.; Schwarz, V.; Martinko.J *Folia Microbiol.* **1968**, *13*, 139-145.

(21) Fernandes, P.; Cruz, A.; Angelova, B.; Pinheiro, H. M.; Cabral, J. M. S. *Enzyme. Microb. Tech.* **2003**, *32*, 688-705.

(22) Hanson, J. R.; Nasir, H.; Parvez, A. *Phytochemistry* **1996**, *42*, 411-415.

(23) Xiong, Z. G.; Wei, Q.; Chen, H. M.; Chen, S. W.; Xu, W. J.; Qiu, G. F.; Liang, S. C.; Hu, X. M. *Steroids* **2006**, *71*, 979-983.

(24) Holland, H. L.; Thomas, E. M. *Can. J. Chem.* **1982**, *60*, 160-164.

(25) Huszcza, E.; Dmochowska-Gladysz, J. *J. Basic. Microb.* **2003**, *43*, 113-120.

(26) Corpechot, C.; Robel, P.; Axelson, M.; Sjovall, J.; Baulieu, E. E. *P. Natl. Acad. Sci-Bol* **1981**, *78*, 4704-4707.

(27) Brown, R. C.; Cascio, T.; Papadopoulos, V. *J. Neurochem.* **2000**, *74*, 847-859.

(28) Lanthier, A.; Patwardhan, V. V. *J. Steroid Biochem.* **1986**, *25*, 445-449.

(29) Weill-Engerer, S.; David, J. P.; Sazdovitch, V.; Liere, P.; Eychenne, B.; Pianos, A.; Schumacher, M.; Delacourte, A.; Baulieu, E. E.; Akwa, Y. *J. Clin. Endocr. Metab.* **2002**, *87*, 5138-5143.

(30) Kim, S. B.; Chalbot, S.; Pompon, D.; Jo, D. H.; Morfin, R. *J. Steroid Biochem.* **2004**, *92*, 383-389.

(31) Kalimi, M.; Shafagoj, Y.; Loria, R.; Padgett, D.; Regelson, W. *Mol. Cell. Biochem.* **1994**, *131*, 99-104.

(32) Ricco, C.; Revial, G.; Ferroud, C.; Hennebert, O.; Morfin, R. *Steroids*, *76*, 28-30.
(33) Li, H. P.; Liu, H. M.; Ge, W. Z.; Huang, L. H.; Shan, L. H. *Steroids* **2005**, *70*, 970-973.
(34) Li, H. P.; Yu, P.; Zhang, H. J.; Liu, H. M. *Chinese J. Chem.* **2008**, *26*, 1666-1668.

Chapter4: *In vivo* and *In vitro* Investigations of 14α-hydroxylase Activity in *Mucor hiemalis*

Introduction

Hydroxysteroids have been implicated in many physiological conditions and are major constituents of pharmaceutical preparations being utilized in the treatment of various diseases[1-3]. In specific, steroids with C-14 hydroxyl group are cardio-active which makes them highly valuable from pharmacological aspects. 14β-hydroxy derivatives of steroids are important enhancer in contractility of isolated cardiac muscle[4]. 14α-hydroxy derivatives of androstane and pregnane possess high gestagen, contraceptive and carcinolytic activities[5]. Proligestone (14α-hydroxypregnane) is well known for its antigonadotropic activity[5] whereas, 14α-hydroxy-4-androstene-3,17-dione is a reputed aromatase inhibitor[6]. Despite of such diverse biological activities very less efforts has been made for the synthesis of 14α- and 14β-hydroxy derivatives of steroids. Tertiary nature of C-14 carbon intricate the utility of conventional chemical synthetic methods for synthesizing 14-hydroxy derivatives of steroids. Previously reported synthetic strategies for direct hydroxylation, selectively at 14α- and 14β-positions are restricted to low yields and less selectivity.[7,8] However, enzymatic transformation can play a crucial role to hydroxylate on the remote carbons of steroidal skeleton associated with high regio- and stereo-selectivity. Microbial hydroxylation has found interesting application in the production of new steroid derivatives. A very few fungal strains such as *Mucro racemoseus*, *Mucor piriformis*, *Mucor griseocyanus*, *Actinomucor elegans*, *Thamnostylum piriforme* are known to hydroxylate at 14α- position of steroidal structure [9-11] which can easily be converted to cardioactive 14β-hydroxy steroids using well-known synthetic methods.[12]

Although whole-cell biocatalysis is the method of choice for the preparative hydroxylation of steroids, it has few drawbacks, such as cells have a vast number of enzymes thus the formation of by-products is very common. The study of isolated steroid hydroxylase enzymes, continues to attract attention as mean to identify and characterize responsible genes, cloning and overexpression of the desired enzymes which would be a better approach, as this will provide higher yields and fewer by-products. In recent years, the number of microbial steroid hydroxylases have been isolated, it includes the 7α- and 15β-hydroxylase of *Phycomyces blakesleeanus* [13] the 14α-hydroxylase of *Mucor piriformis*[14], an inducible 11α-hydroxylase from *Nocardia crassa*[15] and *Rhizopus oryzae*[16], a 9α-hydroxylase from *Rhodococcus*[17], the 11β-hydroxylase and 14α-hydroxylase from *Cochliobolus lunatus*[18,19], an 11α-hydroxylase from *Nectria haematococca*[20] and *A. ochaceous*[21], a 7α-hydroxylase from *Fusarium*

moniliforme[22], and the 15β-hydroxylase from *Penicillium raistricki*.[23]

Most of the hydroxylases system from fungal origin responsible for the steroidal hydroxylations, belongs to cytochrome P450 family and known as microsomal bound monooxygenase systems. It is strictly a multicomponent system involving more than one enzyme according to electron transport chain. The substrate conversion based, co-factors assisted hydroxylase assay is the only approach to check the status of active enzymes. Therefore, it is essential to get active crude cell lysate and microsomal suspension for substrate transformation based hydroxylase assay. Although, preparation of active crude lysate and purification of different steroid hydroxylases such as 7α-, 9α-, 11β-, and 11α-hydroxylase [24-27] are well studied, very limited progress is documented on 14α-hydroxylase. [28]

4.1: Biotransformation of 3-one-4-ene steroids using *M. hiemalis*

Smith and co-worker have studied the regulation of progesterone 14α-hydroxylase in whole cell of *M. hiemalis* using different transcription and translation inhibitors.[29] In the present study, ability of fungal strain *Mucor hiemalis* is explored both at the whole cell and cell free level to hydroxylate testosterone and progesterone at 14α-position. The isolation of metabolites and time dependent evaluation of biotransformation revealed the possible metabolic pathway of studied steroids. Further, the factors effecting 14α-hydroxylase activity in the crude cell lysate and microsomal suspension have been investigated.

4.1.1: Transformation of progesterone (**1**)

M. hiemalis mediated transformation was carried out in large-scale with an aim of isolation and characterization of the metabolites. Transformation of **1** (0.7 g/L) was carried out using 20 flasks as described in materials and methods (Chapter 2). The crude extract (2.3 g) obtained after five days of incubation was subjected to TLC, HPLC and LC-MS analyses, which indicated the presence of three metabolites along with trace amount of unmetabolised substrate **1** (Fig.4.1). The metabolites were purified over silica gel column (230-400 mesh, 50 g) chromatography by eluting with gradient mixture of methanol in dichloromethane. Elution with 0.8% methanol in CH_2Cl_2 yielded a fraction containing substrate **1** (69 mg, R_f: 0.79 system: II; R_t: 45.5 min). Other three metabolites were subsequently eluted by gradually increasing the % of methanol in CH_2Cl_2 (249 mg, 480 mg, 125 mg, R_f: 0.45, 0.36, 0.32 system II; R_t: 21 min, 16.5 min, 13 min, respectively). The compounds were characterized from HRMS, ^{1}H, ^{13}C NMR

spectral data analyses.

The molecular formula of compound **1d** with R_f 0.45 was $C_{21}H_{30}O_3$, as evidenced from HRMS which showed $[M+Na]^+$ ion peak at 353.2087 (calcd-353.2093) and suggested incorporation of one oxygen into parent compound. In the UV spectrum

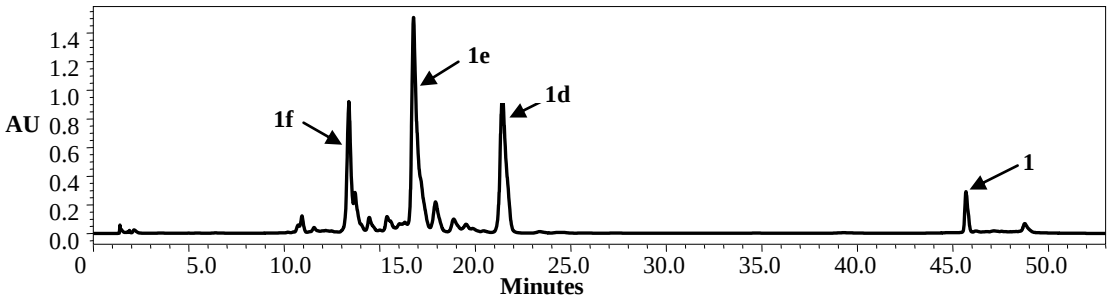


Figure 4.1: The HPLC analysis of progesterone (**1**) transformation with *M. hiemalis* after 5 days.

absorption at 239 nm indicated presence of enone functional group in the compound **1d**. The IR analysis of compound **1d** showed two carbonyl absorption bands at 1713 and 1651 cm^{-1} similar to progesterone (**1**) with additional hydroxyl absorption band at 3368 cm^{-1}. In the ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of compound the presence of signals for three methyl, eight methylene, four methine and six quaternary carbons indicated the new hydroxylation on methine carbon. The ^1H NMR spectrum ($CDCl_3$, 500 MHz) of compound **1d** showed downfield shift in the signal for C-18 methyl protons from δ 0.64 (3H, s) to 0.78 (3H, s) and that of C-17 methine proton from δ 2.54 (1H, t, *J*=9.0 Hz) as in progesterone **1** to δ 3.22 (1H, t, *J*=8.84 Hz). Also, new signal for oxygen bearing proton was not observed in the spectrum, Which confirmed that hydroxylation was occurred at C-14 tertiary carbon. Further, the position and stereochemistry of newly introduced hydroxyl group was confirmed by comparing the melting point (195-196 ºC), optical rotation (195, *c* = 2.16 in chloroform) and spectral data with earlier reports[30,31] and the metabolite **1d** was identified as 14α-hydroxyprogesterone.

UV spectrum of the compound with R_t 0.36 (**1e**) showed absorbance at 239 nm indicating the presence of enone functionality. The IR spectrum exhibited an absorption at 3447, 1705, 1651 cm^{-1} corrosponding to hydroxyl, saturated ketone and α, β unsaturated ketone, respectively. The HRMS spectrum showed molecular ion peak at *m/z* 369.2033 $[M+Na]^+$ corresponding to molecular formula $C_{21}H_{30}O_4$ (calcd. 369.2042 for $[M+Na]^+$), indicating introduction of two hydroxyl groups in the parent compound **1**. In the ^1H NMR spectrum ($CDCl_3$, 500 MHz) of **1e** the appearance of only one new multiplet at δ 4.31 (1H) compared to parent compound was observed which indicated the introduction of one hydroxyl group at methine carbon and another at methylene

carbon. Similar to the metabolite **1d** downfield shift of signal for C-17 proton was observed indicating introduction of one hydroxyl group at C-14 carbon. The position of newly introduced hydroxyl groups in metabolite **1e** were further confirmed from the analysis of ^{13}C NMR spectrum. The signals for two carbonyl carbons at δ 210.5 and 199.5 confirmed the presence of ketone and unsaturated ketone in the metabolite **1e**. The downfield shift of signal for C-18 methyl group and appearance of signal at δ 85.5 confirmed that one of the hydroxyl groups was introduced at C-14 carbon. The presence of signals at δ 35.5, 21.5 and 20.0 for C-1, C-11 and C-16 carbons eliminated the possibility of another hydroxylation at ring A, C and D. By comparing the spectral data with earlier reports the position of another hydroxyl group was confirmed at C-7. Further, the coupling constants observed for multiplet at δ 4.31 were not more than 5 Hz which confirmed that the corresponding hydroxyl group at C-7 was in axial position with α-orientation. The melting point and optical rotation of the compound obtained was 249-251 ºC and +179 (*c* = 0.53 in chloroform) respectively, was matched well with earlier reports,[30,31] and the compound was identified as 7α,14α-dihydroxyprogesterone (**1e**).

The HRMS of the compound with R_f 0.32 (**1f**) displayed the molecular ion at *m/z* 369.2038 $[M+Na]^+$ corresponding to molecular formula $C_{21}H_{30}O_4$ (calcd. 369.2042 for $[M+Na]^+$) indicating increase in mass by 32 units and introduction of two hydroxyl groups to the parent steroid **1**. In the UV spectrum of compound **1f**, absorbance was observed at 239 nm revealed the presence of α,β-unsaturated carbonyl group. The IR spectrum showed three absorption band at 3435, 1701, 1655 cm^{-1} corresponding to the presence of –OH, -C=O, -C=C-C=O functional groups in the isolated metabolite. In ^{13}C NMR spectrum signals were observed for three methyl, seven methylene, five methine and six quaternary carbons indicating introduction of hydroxyl groups at methylene and methine carbons. The presence of signals at δ 34.2, 21.3 and 20.0 characteristic for C-2, C-11 and C-16 carbons, respectively eliminated the possibility of hydroxylation at methylene carbon in ring A, C and D. The shift of signals for C-18 carbon from δ 13.7 to 17.2, revealed introduction of one hydroxyl groups at C-14 carbon. The downfield shift of signal for C-19 methyl carbon from δ 17.7 to 19.5 showed the presence of another hydroxyl group at C-6. It was further confirmed by downfield shift of C-19 methyl protons from δ 1.21 to 1.40 in the ^1H NMR spectrum. Thus, the new downfield signal for the oxygen bearing methine protons at δ 4.31 (1H, m) was assigned to C-6 proton. The coupling constant for the signal was less than 5 Hz indicated that the proton

is in equatorial position and the corresponding hydroxyl group was in axial position with β-orientation. By comparing the spectral data, melting point and optical rotation of compound **1f** with earlier reports[30,31] it was identified as 6β,14α-dihydroxyprogesterone (**1f**).

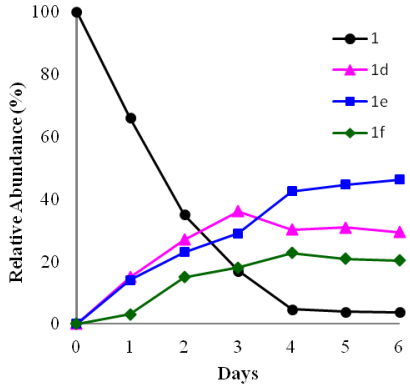
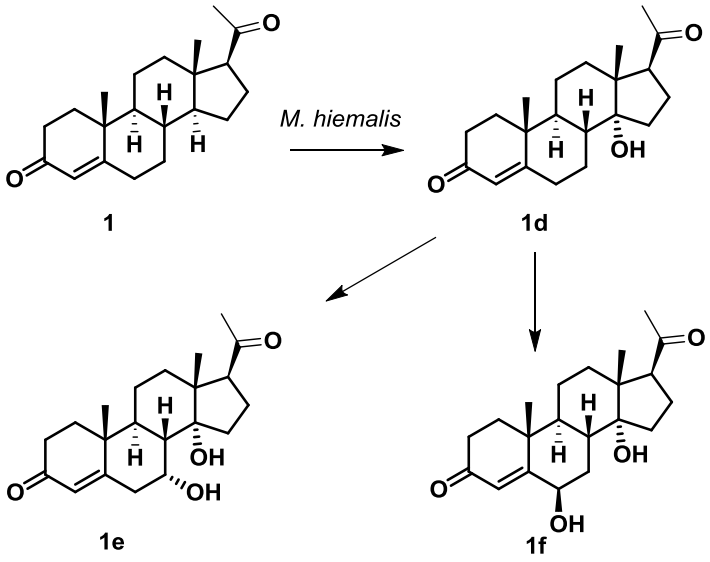


Figure 4.2: Time course study of Progesterone (**1**) transformation with *M. hiemalis*: (●) progesterone (**1**), (▲) 14α-hydroxyprogesterone (**1d**), (■) 7α,14α-dihydroxyprogesterone (**1e**), (♦) 6β,14α-dihydroxyprogesterone (**1f**).

Time-course experiment (Fig. 4.2) indicated that *M. hiemalis* was able to transform almost 83% of progesterone (**1**) into 14α-hydroxyprogesterone (**1d**), 7α,14α-dihydroxyprogesterone (**1e**), 6β,14α-dihydroxyprogesterone (**1f**) at the end of three days of incubation. The relative percentage of metabolites did not alter even after prolonged incubation beyond four days. Incubation of 14α-hydroxyprogesterone (**1d**) with *M. hiemalis* for four days provided all other metabolites. Based on these results, the biotransformation pathway of progesterone (**1**) by *M. hiemalis* was deduced (Scheme



Scheme 4.1: Transformation of progesterone (**2**) by *Mucor hiemalis*

4.1). It was observed that, initially hydroxylation was occurred at 14α-position to form **1d** as the major metabolite which then dihydroxylated at different positions.

4.1.2: Biotransformation of testosterone (2)

A batch of 20 flasks was inoculated with *M hiemalis* and the substrate **2** (0.7 g/L) was added to each flask. At the end of five days of incubation period, contents of all the flasks were pooled and processed as described in materials and methods (Chapter 2). The crude extract (1.92 g) upon TLC, HPLC and LC-MS analyses indicated the presence of one major and three minor metabolites (Fig.4.3). The crude extract was subjected to column chromatography on silica gel (230-400 mesh, 50 g) and elution of metabolites carried out with methanol/dichloromethane (CH_2Cl_2) gradient mixture. The gradual increase in % of methanol in dichloromethane up to 3.5% yielded different fractions containing four pure metabolites (93 mg, 169 mg, 503 mg, 195 mg, R_f: 0.46, 0.40, 0.35, 0.32 system: I; R_t: 20.5 min, 17 min, 13.5 min and 10 min, respectively). From the spectral data analyses and by comparing the data with earlier reports [32,33] the metabolites were identified.

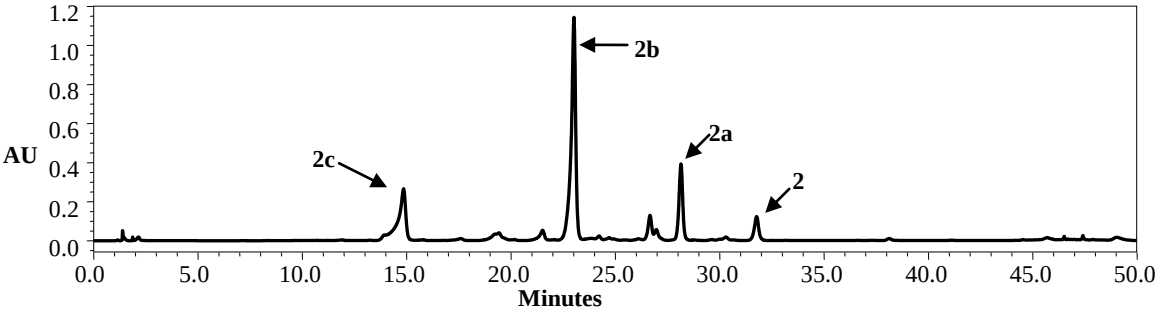


Figure 4.3: The HPLC analysis of testosterone (**2**) transfromation with *M. hiemalis* after 5 days

The IR spectrum of the compound with R_f 0.40 (**2d**) exhibited an absorption at 3417, 1651 cm^{-1} corrosponding to hydroxyl and α, β unsaturated ketone, respectively. The HRMS spectrum of metabolite showed molecular ion peak at *m/z* 305.2107 $[M+H]^+$ corresponding to molecular formula $C_{19}H_{28}O_3$ (calcd. 305.2116 for $[M+H]^+$), indicating introduction of a hydroxyl group in the parent compound **2**. In ^{13}C NMR spectrum of metabolite **2d** the number of signals for quaternary carbon was increased by one with loss of one tertiary carbon signal, indicated that hydroxylation was ouccerd at tertiary carbon. The appearance of one new downfield signal at δ 83.3 and shift of signal for C-18 methyl carbon from δ 11.6 to 14.6 indicated introduction of a hydroxyl group at C-14 position. In the ^1H NMR spectrum (CD_3OD, 500 MHz) of **2d** no new signal was observed in the range from δ

3.3 to 4.5. The downfield shift of signal for C-17 proton further confirmed the introduction of hydroxyl group at C-14, with α-orientation. The melting point and optical rotation of the compound obtained was 173-174 ºC and +116 (*c* = 2.03) in methanol, respectively. The compound was identified as 14α-hydroxytestosterone (**2d**) and the spectral data was matched well with the earlier reports.[32,33]

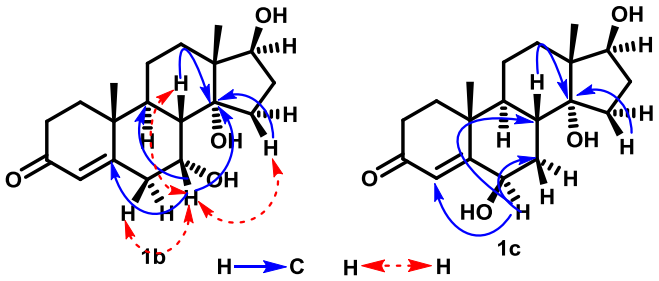


Figure 4.4: Key HMBC (H→C) and NOESY (H ↔ H) correlations of compound **2e** and **2f**

The HRMS spectrum of metabolite **2e** (Rf 0.35) showed molecular ion peak at *m/z* 321.2056 $[M+H]^+$ corresponding to the molecular formula $C_{19}H_{28}O_4$ [calcd. 321.2066 for $(M+H)^+$], indicating introduction of two oxygen atoms to the parent compound **2**. The IR spectrum exhibited absorptions at 3444 (broad) and 1663 cm^{-1} directing the presence of hydroxyl and conjugated carbonyl groups, respectively. In the ^{13}C NMR spectrum, the number of signals for quaternary carbon was increased by one with loss of signal for one methylene carbon indicating the introduction of a hydroxyl group at methylene carbon and another at methine carbon which was further confirmed by the appearance of only one new multiplet at δ 4.26 (1H, m) in the ^1H NMR spectrum of **2e**. Downfield shift of signal for C-17Hα from δ 3.61 to 4.17 indicated the hydroxylation at C-14 tertiary carbon with α-orientation is consistent with a 1,3-diaxial type interaction between 17Hα and the 14α-hydroxyl group. Downfield shift of C-18 methyl and appearance of a new signal at δ 85.5 (quaternary carbon) in the ^{13}C NMR spectrum confirmed the position of hydroxyl group at C-14 which was further supported by the correlations between C-14 with H-8 and H-15 in HMBC experiment (Fig. 4.4). The signal for hydroxylated carbon at δ 69.9 correlated with the proton at δ 4.26 in HSQC spectrum and the same proton (δ 4.26) showed correlations with C-9, C-14 and C-5 in HMBC spectrum indicating the hydroxylation at C-7 position. From the NOESY experiment, the orientation of H-7 was confirmed to be β on the basis of correlations with H-8β, H-16β and H-6β. Thus from the analyses of spectral data the compound was identified as 7α,14α-dihydroxytestosterone (**2e**).

Similarly, from the HRMS analysis, molecular formula for compound **2f** (Rf 0.32) was deduced as $C_{19}H_{28}O_4$ which corresponded to the ion peak at *m/z* 321.2057, (calcd. 321.2066 for $[M+H]^+$) indicating dihydroxylation of the substrate **2**. The IR spectrum of metabolite **2f** showed absorption bands at 3423 and 1658 cm^{-1} predicting the presence of hydroxyl and conjugated carbonyl groups. In the ^{13}C NMR spectrum, two new downfield signals at δ 84.2 and 74.1 for quaternary and tertiary carbons, respectively was appeared with loss of one methylene carbon and increase in the number of quaternary carbons indicating the hydroxylations on methine and methylene carbons. In HMBC, the quaternary carbon at δ 84.2 showed correlations with H-8 and H-15 revealing the presence of hydroxyl group at C-14 position (Fig. 4.4). In the ^1H NMR spectrum of **2f**, the downfield shift of signals for C-18 methyl proton from δ 0.82 to 0.93 and that of 17Hα from δ 3.61 to 4.25 further confirmed the hydroxylation at C-14 tertiary carbon with α-orientation. The appearance of new signal at δ 4.34 (1H, t, J=2.9 Hz) and the downfield shift of signal for C-19 methyl protons indicated introduction of another hydroxyl group at C-6 position. The low coupling constant (< 5 Hz) for the signal at δ 4.34 for H-6 with C-7 protons revealed the presence of proton in equatorial position and the corresponding hydroxyl group in β-orientation. The HMBC spectrum further supported the position of hydroxyl group through correlations between H-6 and C-4, C-7 and C-8 and the compound was identified as 6β,14α-dihydroxytestosterone (**2f**).

The molecular formula of compound **2g** with R_f 0.46 was $C_{19}H_{26}O_3$, as evidenced from HRMS which showed $[M+H]^+$ ion peak at 303.1952 (calcd-303.1960) and suggested incorporation of one oxygen into parent compound. The IR analysis of compound **2g** showed absorption bands at 3368, 1713 and 1651 cm^{-1} explaining the presence of hydroxyl, carbonyl and conjugated carbonyl groups. In the ^{13}C NMR spectrum (CD_3OD, 125 MHz) of compound the presence of signals for two methyl, eight methylene, three methine and six quaternary carbons indicated that the new hydroxylation was occurred at methine carbon. The appearance of signal at high downfield region δ 222.1 indicated the presence of carbonyl group in the five member ring. Further the signal for C-17 carbon was disappeared which indicated the oxidation of C-17 hydroxyl group. It was further confirmed by the disappearance of the signal for C-17Hα in the ^1H NMR spectrum. The signal at δ 81.2 for quaternary carbon and downfield shift of signal for C-8 from δ 36.9 to 39.3 and C-15 carbon from δ 24.2 to 30.4 confirmed the introduction of hydroxyl group at C-14 position. The

stereochemistry of newly introduced hydroxyl group was confirmed by comparing the melting point (263-265 ºC), optical rotation (+133, *c* = 0.5 in methanol) and spectral data with earlier reports[30,3130] and the metabolite **2g** was identified as 14α-hydroxy-4-androstene-3,17-dione (**2g**).

From the substrate concentration studies, the optimum concentration for the transformation of testosterone (**2**) was found to be 0.7 g/L. Further increase in the substrate concentration decreased the level of metabolites formation. Time course experiments (Fig. 4.5) indicated that during early hours of incubation (24 h), **2d** was the major metabolite (63-65%) in the fermentation medium. With prolonged incubation,

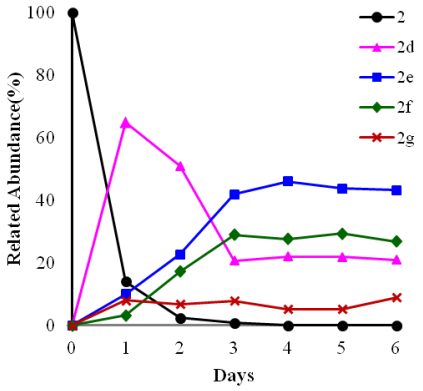
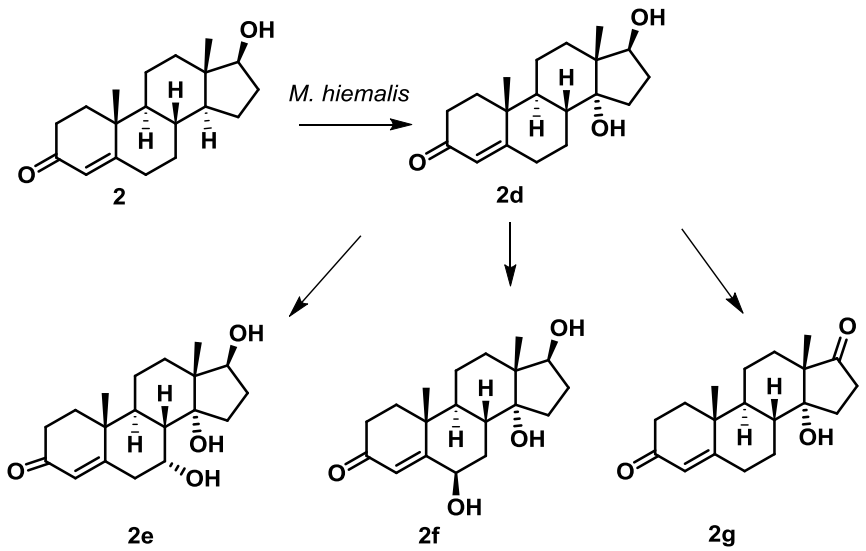


Figure 4.5: Time course study of testosterone (**2**) transformation with *M. hiemalis*: (●) testosterone (**2**), (▲) 14α-hydroxytestosteone **(2d)**, (■) 7α,14α-dihydroxytestosterone **(2e)**, (♦) 6β,14α-dihydroxytestosterone **(2f)**, (**x**)14α-hydroxy-4-androstene-3,17-dione (**2g**)

accumulation of other metabolites was observed (**2e**, **2f** and **2g**) with decrease in the level of **2d**. After four days of incubation, no significant change in the level of metabolites was observed. Upon incubation of **2d** with *M. hiemalis* for 4 days,



Scheme 4.2: Transformation of testosterone (**2**) by *Mucor hiemalis*

subsequent hydroxylation occurred at 6β- or 7β-position to form other metabolites **2e** and **2f**. Furthermore formation of **2g** was observed which might be formed by oxidation of 17β-hydroxyl group of **2d**. Similar results were observed when **2d** was incubated with resting cells, which indicated that the *M. hiemalis* initiates the biotransformation of **2** by hydroxylation at 14α- position to form **2d** as the major metabolite. When prolonging the incubation, the fungal system performed another hydroxylation, either at 6β- or 7β-position to form dihydroxy metabolites **2e** and **2f.** Based on these observations, the biotransformation pathway of testosterone (**2**) by *M. hiemalis* was deduced (Scheme 4.2).

4.2: Cell free preparation and investigation of *in vitro* 14α-hydroxylase activity in *M .hiemalis*

Usually steroid hydroxylase systems in fungi are inducible by steroid substrates. To study *in vitro* hydroxylase activity in *M. hiemalis*, either of **1** or **2** was used as an inducer. Active crude cell lysate was prepared by crushing the testosterone induced well grown mycelia (36 h) in the extraction buffer with glass beads in bead beater as discussed in the materials and methods (Chapter 2). Hydroxylase assay was carried out in total volume of 1 mL buffer containing protein (5 mg), 0.3 mM of progesterone and NADPH by incubating at 30 ºC for 6 h. After this incubation period, assay mixture was extracted with ethyl acetate (3 mL × 3), concentrated and analyzed by using HPLC for the formation of metabolites.

Different parameters controlling the 14α-hydroxylase activity in the cell free extract such as substrate for induction, pH of extraction buffer and incubation temperature were further standardized to obtain optimum *in vitro* activity. The hydroxylase activities were observed in presence of NADPH or NADPH generating system. However, no hydroxylase activity observed when the assay was carried out using NADH. Thus, the 14α-hydroxylase in *M. hiemalis* is NADPH dependent. Loss in the hydroxylase activity was observed in the absence of protease inhibitor in the extraction buffer. The optimum concentration of phenylmethanesulfonylfluoride (PMSF; a common protease inhibitor) in the extraction buffer to obtain maximum relative activity was found to be 2.5 mM.

4.2.1: Localization of 14α-hydroxylase system in *M. hiemalis*

The hydroxylase activity observed for samples obtained after centrifugation of crude lysate at various centrifugal forces revealed that all the components of 14α-hydroxylase

system were membrane bound proteins (Fig. 4.6) as complete hydroxylase activities observed were from microsomal pellet obtained at 1,20,000 × g.

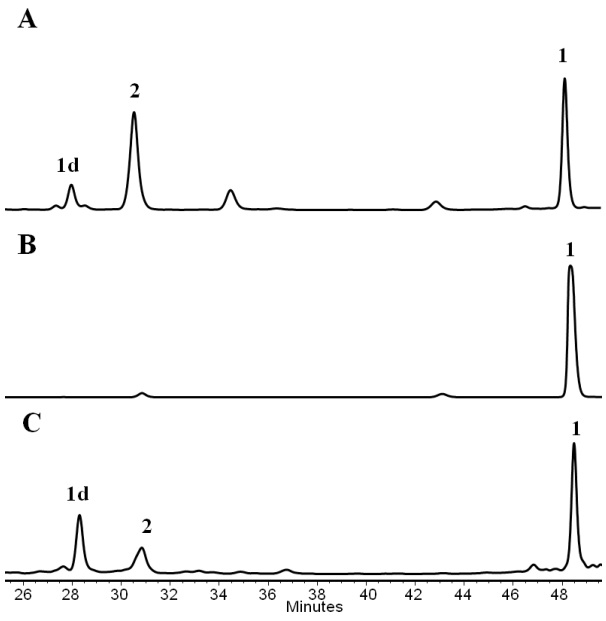


Figure 4.6:. LC-MS analysis of hydroxylase assays with (A) 10250 × g suspension, (B) 120000 × g supernatant, (C) microsomal suspension. – Testosterone (**2**) used for induction, Progesterone (**1**) substrate for hydroxylase assay, 14α-hydroxyprogesterone (**1d**) product formed in the assay.

4.2.2: Induction with different substrates

On enzyme induction with different inducers and progesterone as substrate in the hydroxylase assay (Table 4.1), maximum activity in microsomal pellet was obtained when progesterone was used as inducer with 12 h induction period. Induction with testosterone and 17α-methyltestosterone resulted in microsomal suspension with 14α-hydroxylase activities. However, there was no hydoxylase activity observed with microsomal pellets obtained from the mycelia induced with androsterone and cholesterol. Further no 14α-hydroxylase activities were observed with un-induced

Table 4.1: Effect of different inducers on hydroxylase activity in microsomal suspension

No.	Inducer (15 mg/100 mL for 12 h)	% relative activity
1	Progesterone	100%
2	Testosterone	65%
3	17α-methyltestosterone	59%
4	Estradiol	44%
5	Androsterone	No activity
6	Cholesterol	No activity
7	Phenabarbitol	No activity
8	Uninduced	No activity

mycelial microsomal preparations. These results clearly indicated that the 14α-hydroxylase system in *M. hiemalis* is inducible and the level of induction depends on the inducer being used.

4.2.3: Optimization of pH and incubation temperature

In this study, Tris-HCl buffers of different pH were used for lysate and microsomal suspension preparations (Fig. 4.7A). Screening the extraction buffers with range of pH from 7.2 to 8.7 revealed that, the extraction buffer of pH 8.2 yielded a microsomal suspension with maximum hydroxylase activity. Moderate activity (57%) was observed at pH 7.5 which decreased to 17-20% with the extraction buffer of pH 7.0. Considerable loss of the activity was observed when pH of extraction buffer was increased up to 8.7.

Hydroxylase assay carried out at various incubation temperatures in the range of 10 ºC to 50 ºC indicated that 30 ºC is the optimum incubation temperature for 14α-hydroxylase activity (Fig. 4.7B).

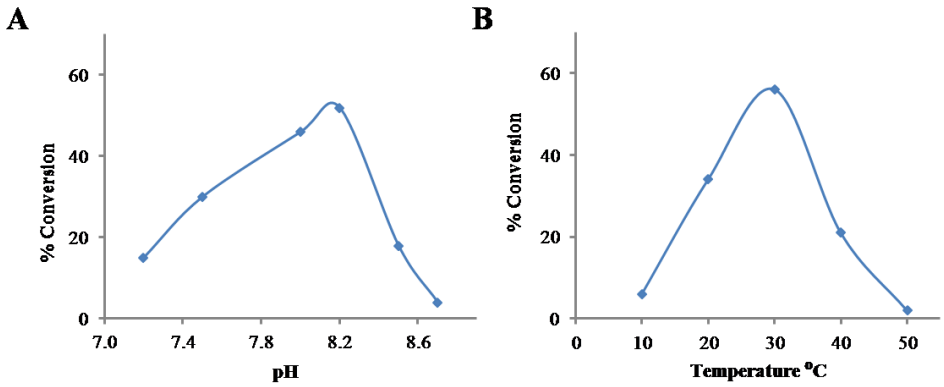


Figure 4.7: Effect of (**A**) pH and (**B**) temperature on 14α-hydroxylase activity

Thus the extraction buffer composed of glucose (0.25 M), EDTA (10 mM), glycerol (20% v/v), KCl (10 mM), PMSF (2.5 mM), DTT (5 mM) in Tris-HCl (0.1 M, pH 8.2) and the hydroxylase assay with NADPH generating system as source of co-factor with incubation at 30 °C were ideal experimental parameters to obtain active microsomal suspension from *M. hiemalis*.

4.2.4: Effect of inhibitors on 14α-hydroxylase activity

When the hydroxylase assay carried out in the presence of different known cytochrome P450 inhibitors (Fig. 4.8) such as SKF-525A, carbon monoxide, cytochrome c, N-methylmaleimide and ketocanazol, significant inhibition in the 14α-hydroxylase activity was observed. Loss of activity up to 70-75% was observed when the assay was carried out in presence of SKF-525A (1 mM) and cytochrome c (50 µM). Similarly, drastic reduction in the hydroxylase activity was observed when the assay mixture was bubbled

with carbon monoxide for 30 sec (1-2 bubble per sec). Inhibition of 14α- hydroxylase activity from *M. hiemalis* by known cytochrome P450 inhibitors indicated that the hydroxylase system belongs to cytochrome P450 family.

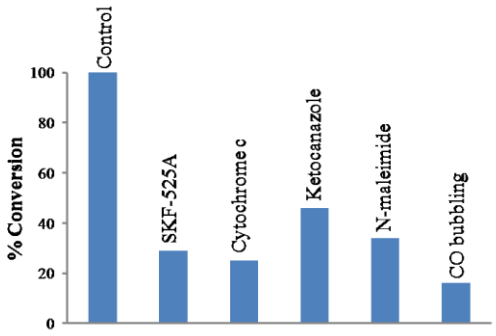


Figure 4.8: Effect of different inhibitors on 14α-hydroxylase activity

4.2.5: Spectral data analysis of 14α-hydroxylase system

In the spectral characterization, microsomal suspension exhibited spectra characteristic of cytochrome P450 (Fig. 4.9). The dithionite-reduced protein sample showed maximum absorption at 428 nm and bubbling with CO resulted in a change of the absorption peak to 420 nm. The characteristic spectra for cytochrome family of enzyme further confirmed that these proteins belong to cytochrome P450 family. In general, cytochrome P450s bound to carbon monoxide have an absorbance peak at 450 nm, in this case with microsomal suspension from *M. hiemalis* it was observed at 420 nm which indicated the P450 system is very unstable or rapidly changes to the P420 form.

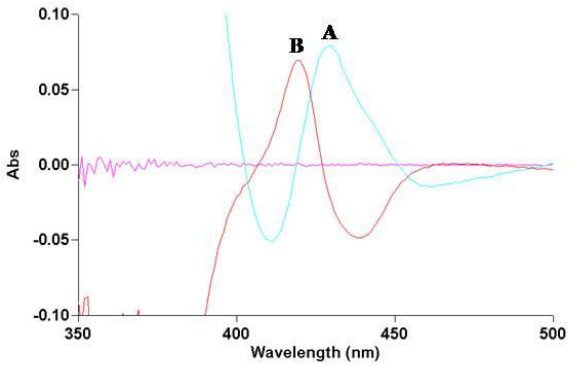


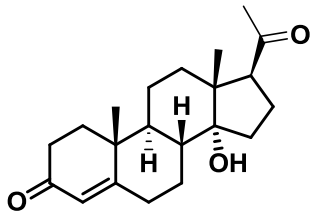
Fig. 4.9: Spectral analysis of microsomal suspension from *M. hiemalis* Graph **A**: spectrum of reduced form of cytochrome P450 (428 nm), Graph **B**: Spectrum obtained on bubbling of CO gas into microsomal preparation after reduction with sodium dithionate (420 nm).

4.3: Summary and Conclusion

In conclusion, the fungal system *M. hiemalis* was able to carry out the efficient hydroxylation at 14α-position on two hormonal steroids, progesterone (**1**) and testosterone (**2**) to form corresponding 14α-hydroxy metabolites which further transformed into 6β/7α, 14α-dihydroxy derivatives on extended incubation. The biotransformation pathway of **1** and **2** by *M. hiemalis* was deduced based on the time course and resting cell experiments using substrates and isolated metabolites. The hydroxylase assays with microsomal suspension prepared from *M. hiemalis* with varying experimental parameters such as inducer, pH, temperature etc. outlined the optimum conditions required to obtain maximum 14α-hydroxylase activity *in vitro*.

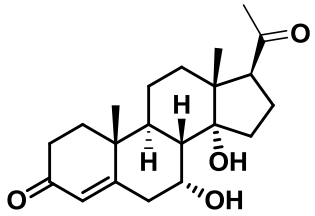
4.4: Spectral data

14α-hydroxyprogesterone (1d)



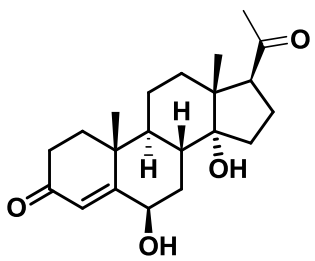
Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 195-196 ºC, $[\alpha]_D^{22}$: +195 (*c* = 2.16 in $CHCl_3$), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3442 (OH), 1713 (CO), 1651 (conj. CO);^{1}H NMR (500 MHz, $CDCl_3$, Me_4Si): 0.79 (3H, s, H-18), 1.20 (3H, s, H-19), 5.74 (1H, s, H-4),2.13 (3H, s, H-21) 3.22 (1H, t, J= 8.8 Hz, H-17), ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): 210.4 (C-20), 199.5 (C-3), 170.6 (C-5), 123.9 (C-4), 85 (C-14), 59.4 (C-17), 47.9 (C-13), 46.2 (C-9), 38.6 (C-10), 38.2 (C-8), 35.7 (C-1), 33.9 (C-2), 33.3 (C-12), 32.5 (C-6), 31.4 (C-21), 30.9 (C-15), 27.1 (C-7), 21.3 (C-11), 20.1 (C-16), 17.2 (C-18), 17.2 (C-19); HRMS: *m/z* 353.2087 $[M+Na]^+$ [calcd- *m/z* 353.2093].

7α ,14α-dihydroxyprogesterone (1e)



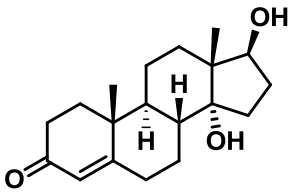
Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 249-251 ºC, $[\alpha]_D^{22}$: +179 (*c* = 0.53 in $CHCl_3$), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}):): 3447 (OH), 1705 (CO), 1651 (conj. CO); ^{1}H NMR (500 MHz, $CDCl_3$, Me_4Si): 76 (3H, s, H-18), 1.21 (3H, s, H-19), 5.79 (1H, s, H-4), 2.12 (3H, s, H-21), 4.31 (1H, m, H-7β); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): 210.6 (C-20), 198.9 (C-3), 166.7 (C-5), 127.0 (C-4), 85.5 (C-14), 69.6 (C-7), 59.2 (C-17), 48.0 (C-13), 41.2 (C-6), 40.4 (C-9), 39.6 (C-8), 38.6 (C-10), 35.5 (C-1), 33.9 (C-2), 33.0 (C-12), 31.4 (C-21), 30.8 (C-15), 21.5 (C-11), 20.0 (C-16), 17.1 (C-18), 17.1 (C-19); HRMS: *m/z* 369.2033 $[M+Na]^+$ [calcd- *m/z* 369.2042].

6β,14α-dihydroxyprogesterone (1f)



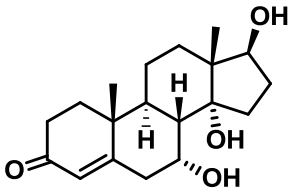
Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 242-244 ºC, $[\alpha]_D^{22}$: +117 (*c* = 1.59 in $CHCl_3$), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3435 (OH), 1701 (CO), 16552 (conj. CO); ^1H NMR (500 MHz, $CDCl_3$, Me_4Si): 0.82 (3H, s, H-18), 1.40 (3H, s, H-19), 5.82 (1H, s, H-4), 2.13 (3H, s, H-21) 4.43 (1H, m, H-6α); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): 210.1 (C-20), 200.1 (C-3), 167.5 (C-5), 126.4 (C-4), 85.3 (C-14), 73.3 (C-6), 59.4 (C-17), 48.1 (C-13), 46.3 (C-9), 38.0 (C-10), 37.2 (C-1), 34.2 (C-2), 33.8 (C-7), 33.3 (C-12), 32.5 (C-8), 31.4 (C-21), 30.9 (C-15), 21.3 (C-11), 20.0 (C-16), 19.5 (C-19), 17.2 (C-18); HRMS: *m/z* 369.2038 $[M+Na]^+$ [calcd- *m/z* 369.2042].

14α-hydroxytestosterone (2d)



Colorless crystals (crystallized from $CHCl_3$/methanol), mp: 173-174 ºC, $[\alpha]_D^{22}$: 116 (*c* = 2.03 in $CHCl_3$), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3422 (OH), 1652 (conj. CO); ^1H NMR (500 MHz, CD_3OD, Me_4Si): 0.92 (3H, s, H-18), 1.21(3H, s, H-19), 5.73 (1H, s, H-4), 4.31 (1H, m, H-17α); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): 199.7 (C-3), 170.8 (C-5), 123.8 (C-4), 83.3 (C-14), 78.5 (C-17), 46.9 (C-13), 46.7 (C-9), 38.7 (C-8), 38.7 (C-10), 35.7 (C-1), 33.9 (C-2), 32.7 (C-12), 32.5 (C-6), 29.6 (C-16), 28.5 (C-15), 26.1 (C-7), 19.7 (C-11), 17.2 (C-19), 14.6 (C-18); HRMS: *m/z* 305.2107 $[M+H]^+$ [calcd- *m/z* 304.2038].

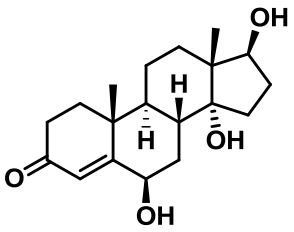
7α,14α-dihydroxytestosterone (2e)



Colorless crystals (crystallized from $CHCl_3$/methanol), mp: 236-238 ºC, $[\alpha]_D^{22}$: +109 (*c* = 2.63 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3425 (OH), 1669 (conj. CO); ^1H NMR (500 MHz, CD_3OD, Me_4Si): 0.88 (3H, s, H-18), 1.26 (3H, s, H-19), 5.72 (1H, s, H-4), 4.17 (1H, m, H-17α); 4.26 (1H, m, H-7β); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): 201.8 (C-3), 172 (C-5), 126.9 (C-4), 85.6 (C-14), 79.2 (C-17), 69.9 (C-7), 48.4 (C-13), 42.1 (C-6), 41.8 (C-9), 41.4 (C-8), 40.0 (C-10), 36.7 (C-1), 34.8 (C-2), 33.4 (C-

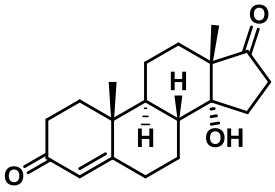
12), 30.0 (C-16), 29.8 (C-15), 20.8 (C-11), 17.3 (C-19), 15.5 (C-18); HRMS: *m/z* 321.2056 $[M+H]^+$ [calcd- *m/z* 321.2066]

6β,14α-dihydroxytestosterone (2f)



Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 193-195 ºC, $[\alpha]_D^{22}$: +38 (*c* = 1.85 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3418 (OH), 1649 (conj. CO); ^1H NMR (500 MHz, CD_3OD, Me_4Si): 0.93 (3H, s, H-18), 1.42 (3H, s, H-19), 5.79 (1H, s, H-4), 4.25 (1H, m, H-17α); 4.34 (1H,t, *J*= 2.9 Hz H-6α); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): 201.8 (C-3), 172 (C-5), 126.9 (C-4), 85.6 (C-14), 79.2 (C-17), 69.9 (C-7), 48.4 (C-13), 42.1 (C-6), 41.8 (C-9), 41.4 (C-8), 40.0 (C-10), 36.7 (C-1), 34.8 (C-2), 33.4 (C-12), 30.0 (C-16), 29.8 (C-15), 20.8 (C-11), 17.3 (C-19), 15.5 (C-18); HRMS: *m/z* 321.2057 $[M+H]^+$ [calcd- *m/z* 321.2066].

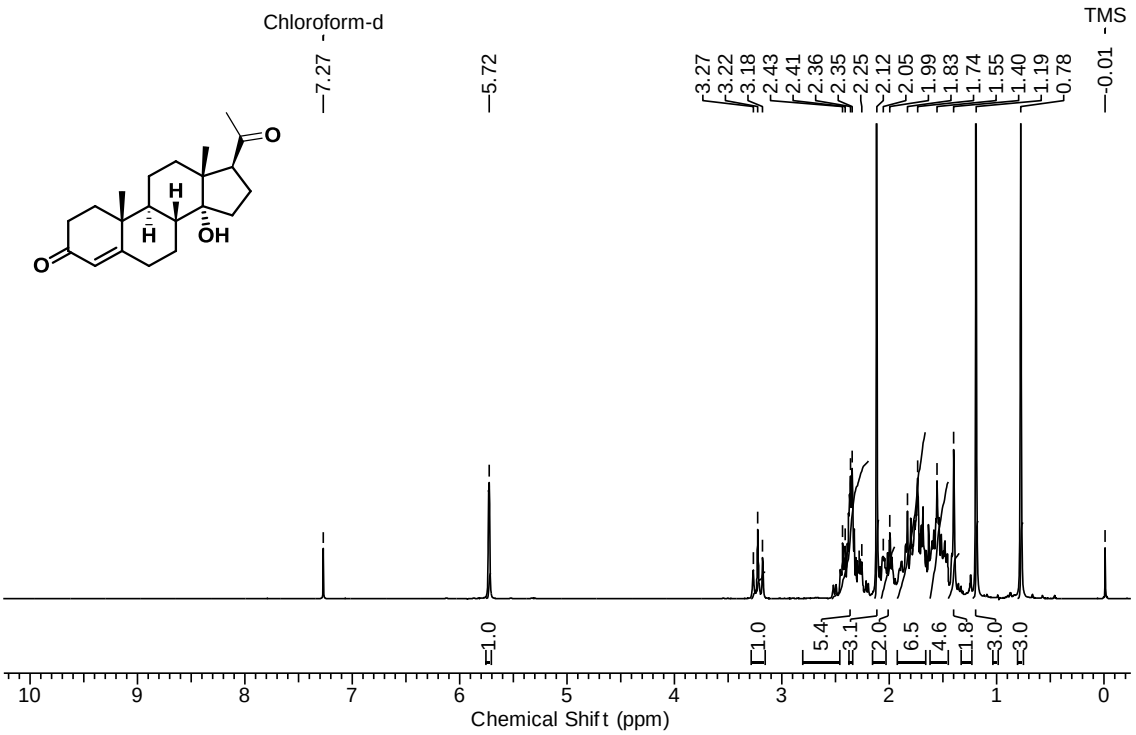
14α-hydroxy-4-androstene-3,17-dione (2g)



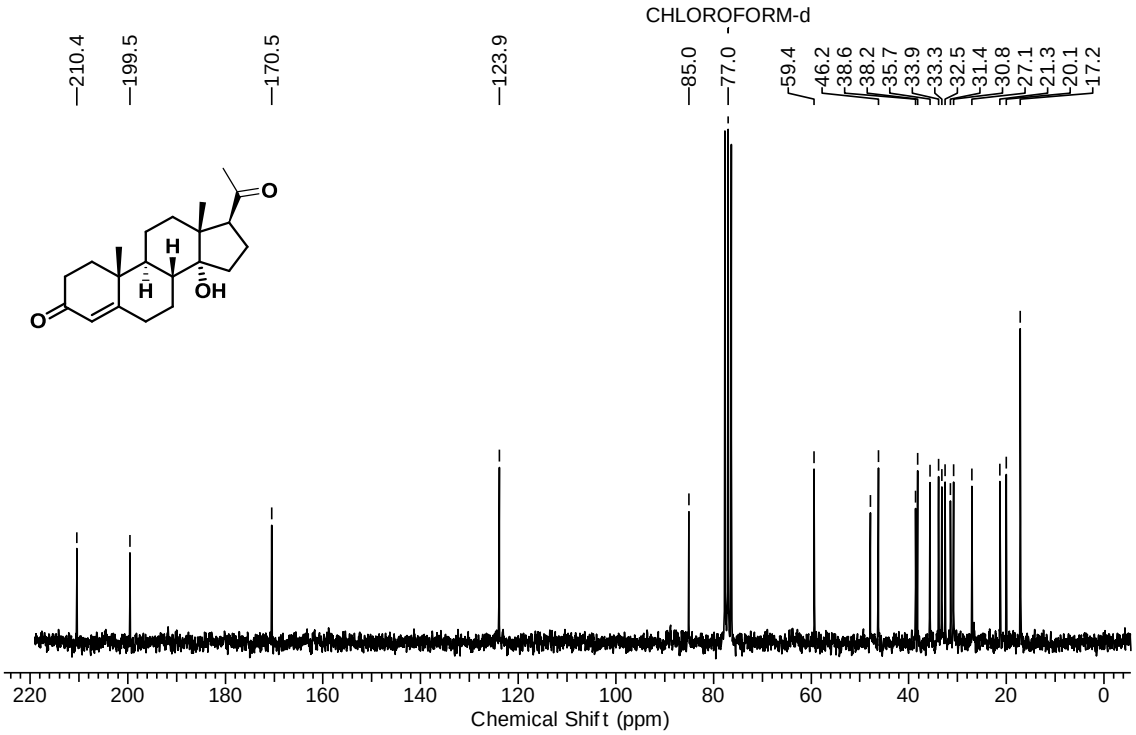
Colorless crystals (crystallized from $CHCl_3$/methanol); mp: 263-265 ºC, $[\alpha]_D^{22}$: +133 (*c* = 0.6 in MeOH), λ_{max} (MeOH): 239 nm; IR ν_{max} (cm^{-1}): 3381 (OH), 1744 (CO), 1668 (conj. CO); ^1H NMR (500 MHz, CD_3OD, Me_4Si): 0.81 (3H, s, H-18), 1.36 (3H, s, H-19), 5.71 (1H, s, H-4); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): 222.1 (C-17), 202.3 (C-3), 174.7 (C-5), 124.2 (C-4), 81.2 (C-14), 54.1 (C-13), 48.4 (C-9), 40.1 (C-10), 39.3 (C-8), 36.9 (C-1), 34.7 (C-12), 34.1 (C-2), 33.6 (C-6), 30.4 (C-15), 25.8 (C-16), 25.1 (C-7), 20.3 (C-11), 18.15 (C-19), 17.6 (C-18); HRMS: *m/z* 303.1952 $[M+H]^+$ [calcd- *m/z* 303.1955].

4.5: Selected spectra

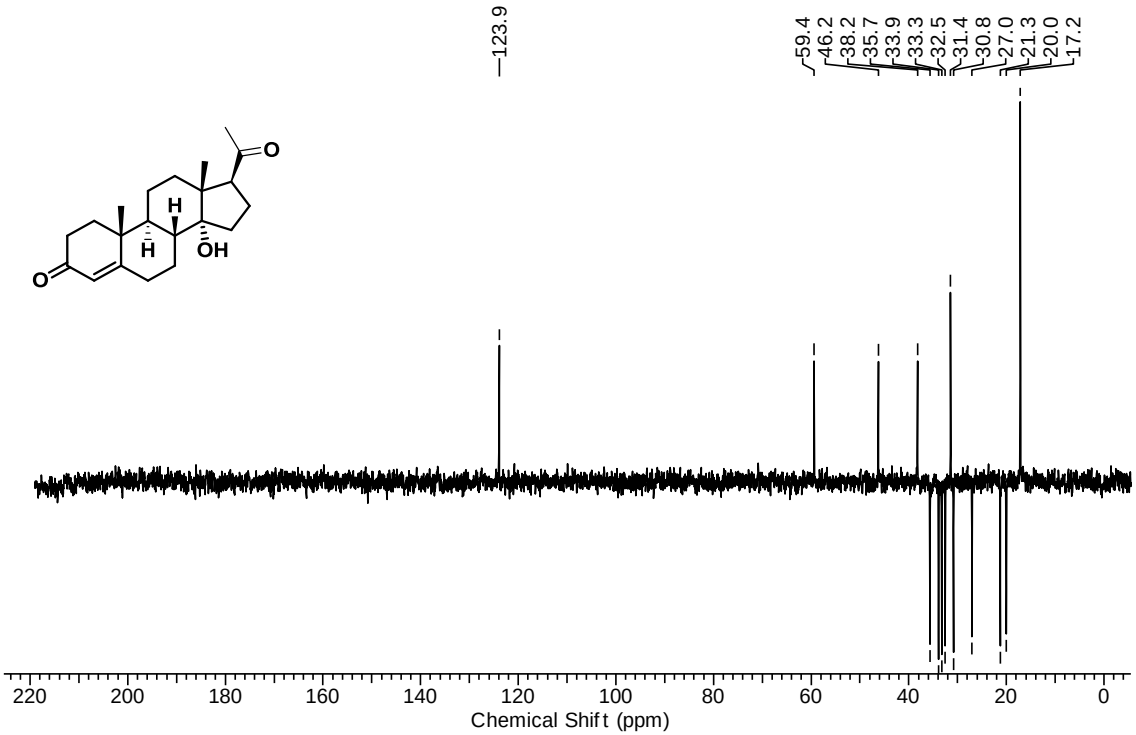
14α-hydroxyprogesterone (1d)



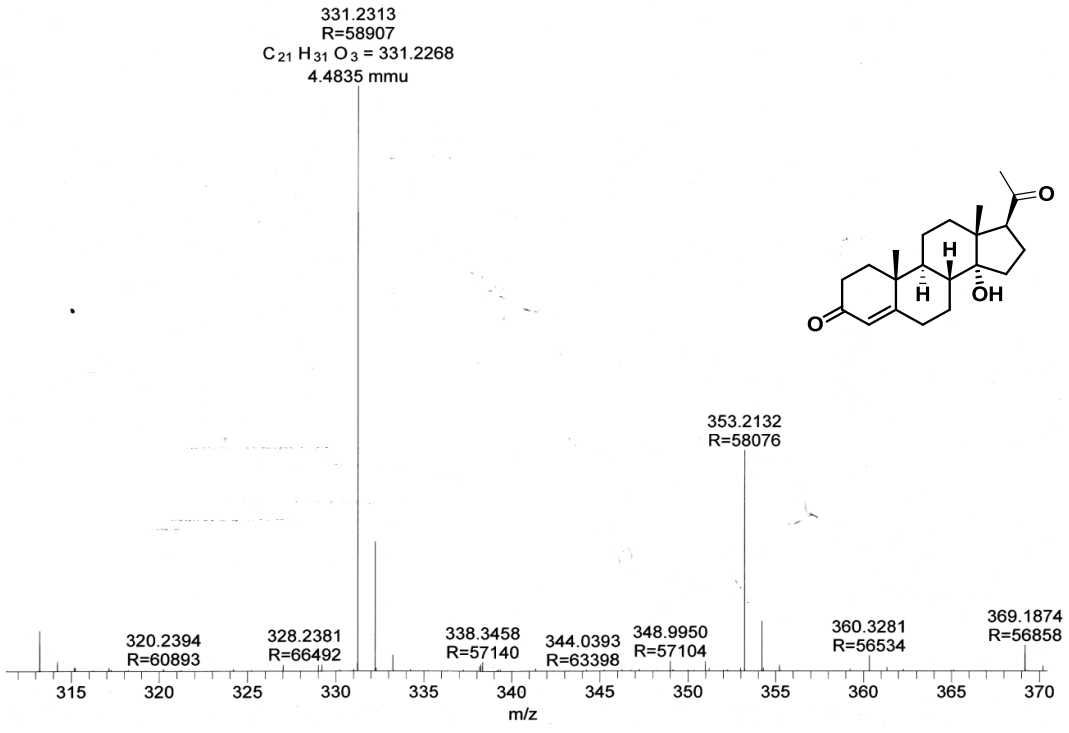
^{1}H NMR spectrum of **1d** in $CDCl_3$ at 500 MHz



^{13}C NMR spectrum of **1d** in $CDCl_3$ at 500 MHz

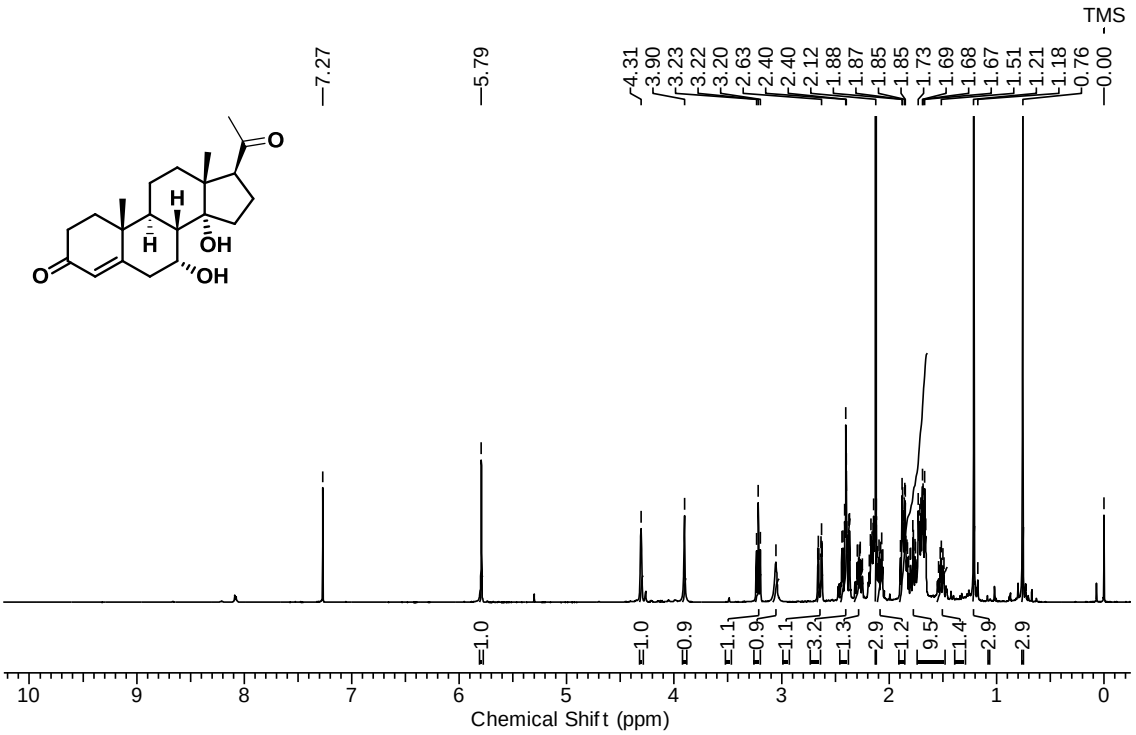


DEPT NMR spectrum of **1d** in $CDCl_3$ at 500 MHz

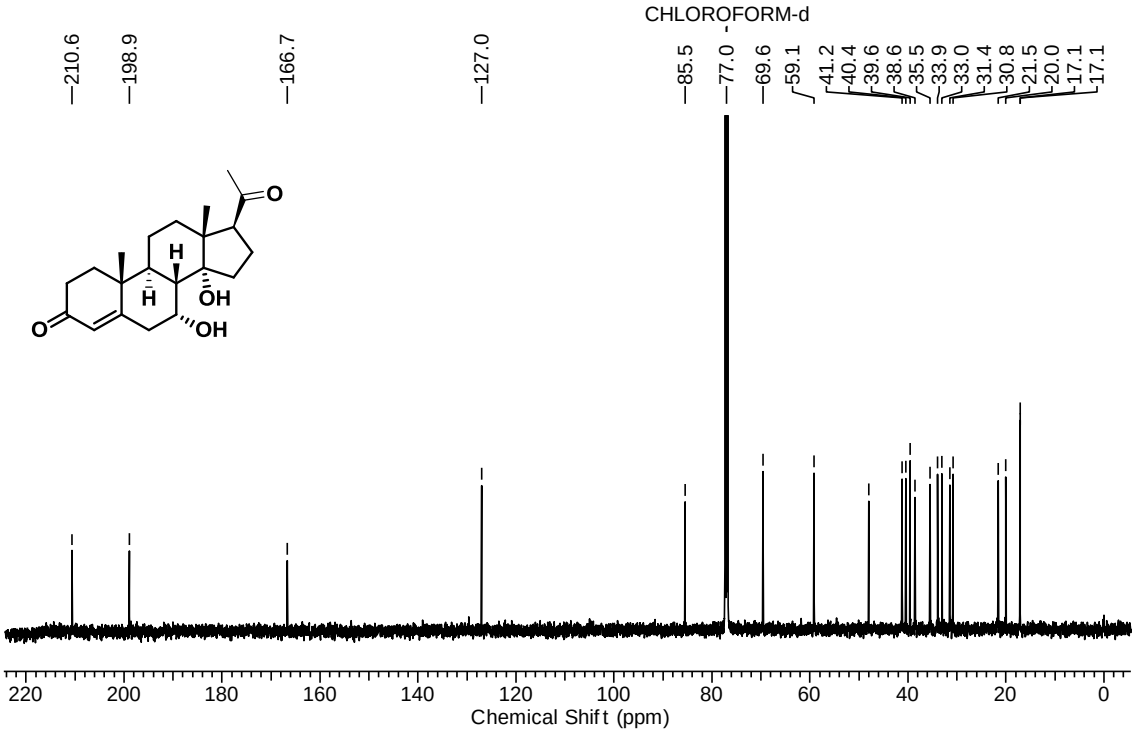


Mass spectrum of **1d** (ESI+ mode)

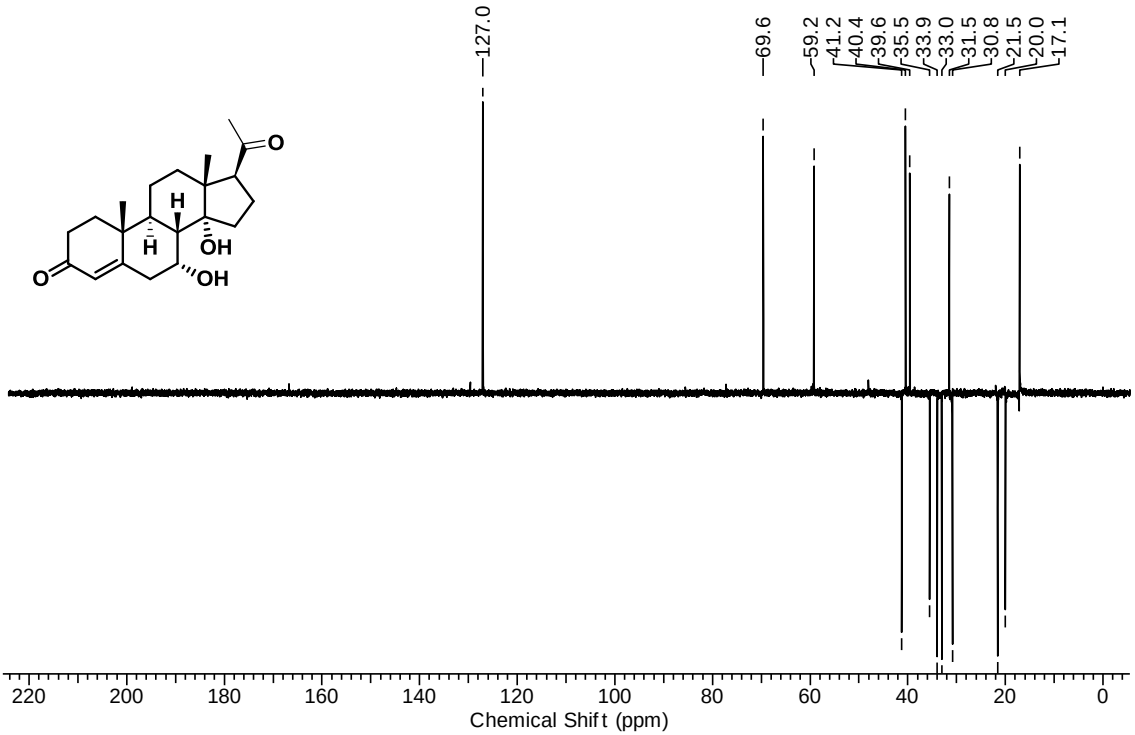
7α ,14α-dihydroxyprogesterone (1e)



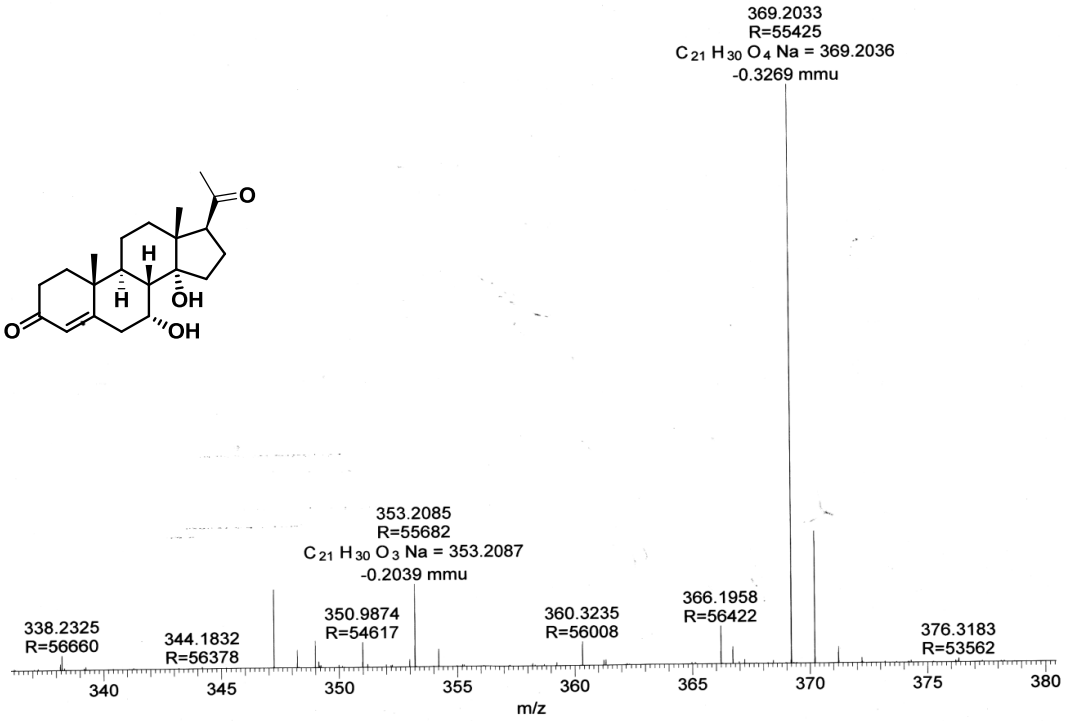
^{1}H NMR spectrum of **1e** in $CDCl_3$ at 500 MHz



^{13}C NMR spectrum of **1e** in $CDCl_3$ at 500 MHz

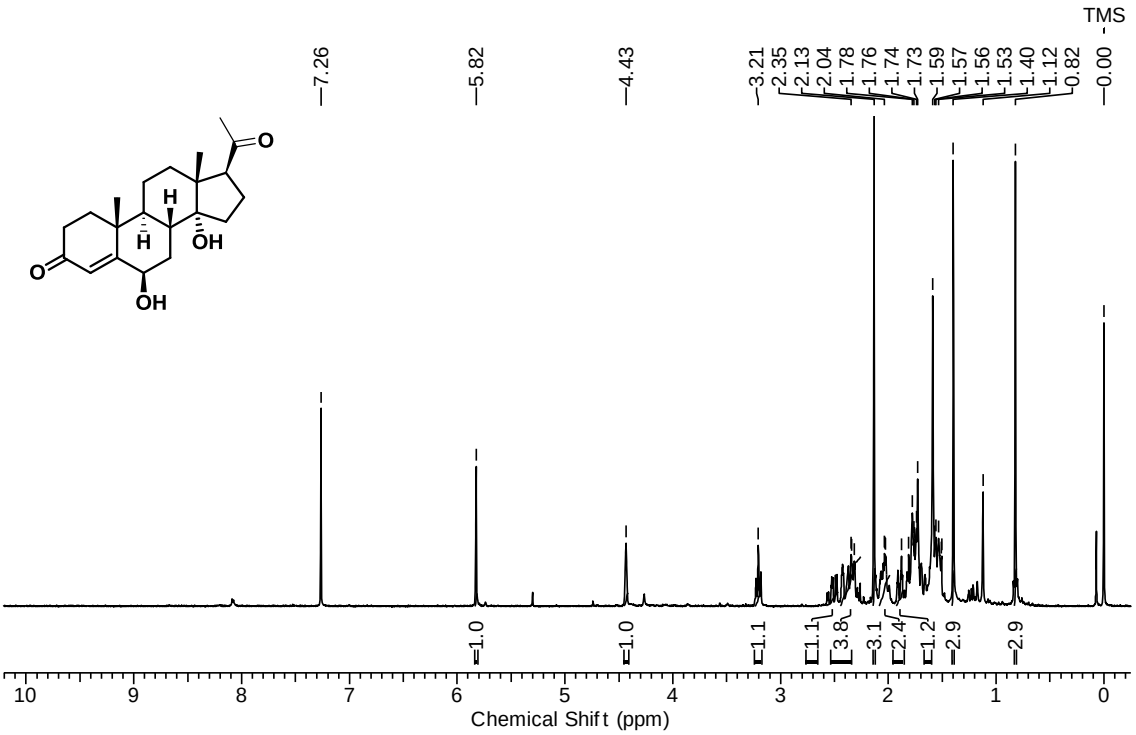


DEPT NMR spectrum of **1e** in $CDCl_3$ at 500 MHz

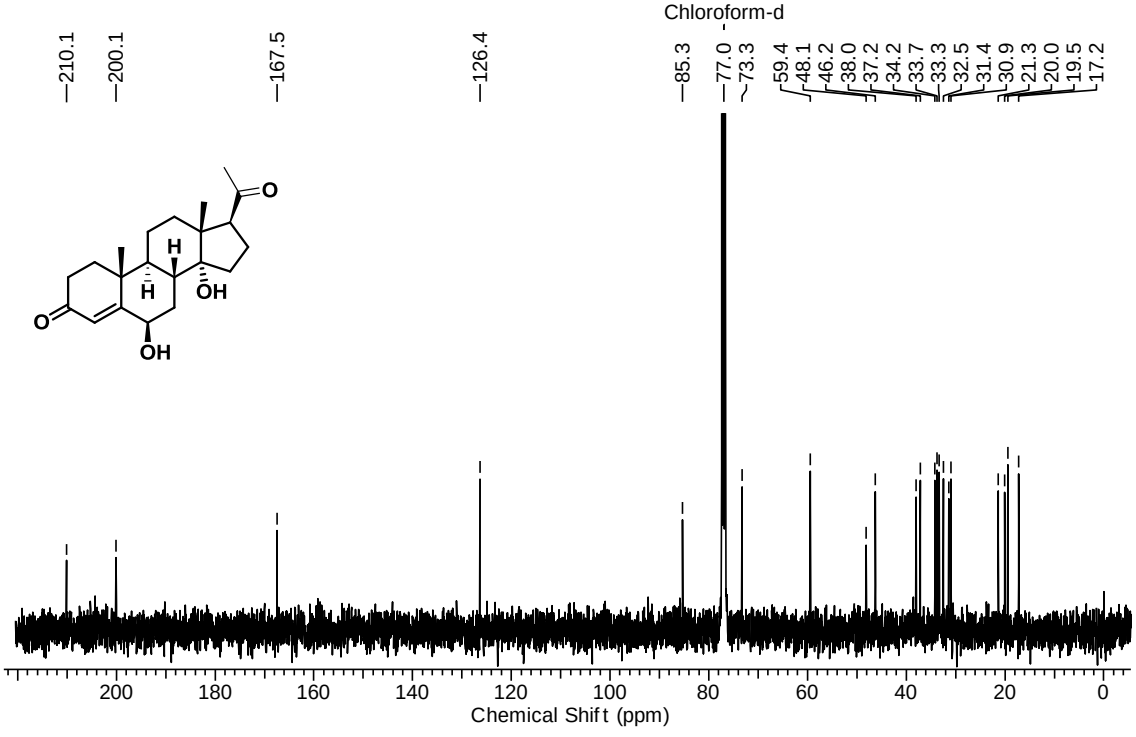


Mass spectrum of **1e** (ESI+ mode)

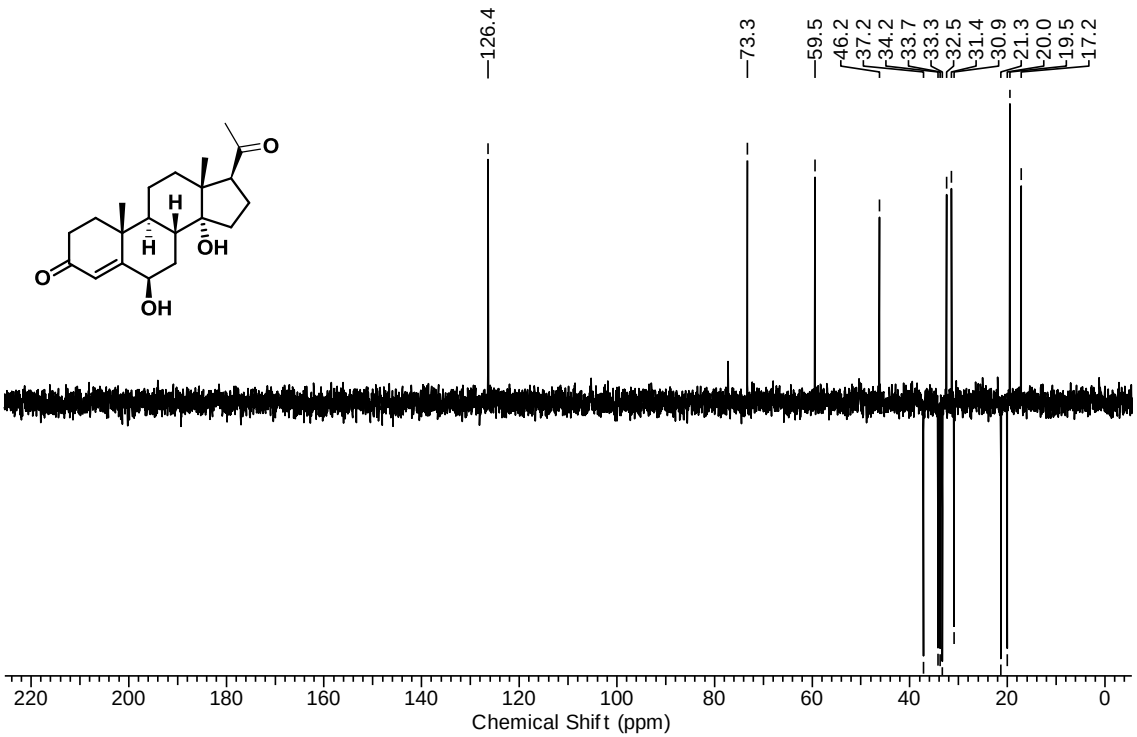
6β ,14*α*-dihydroxyprogesterone (1f)



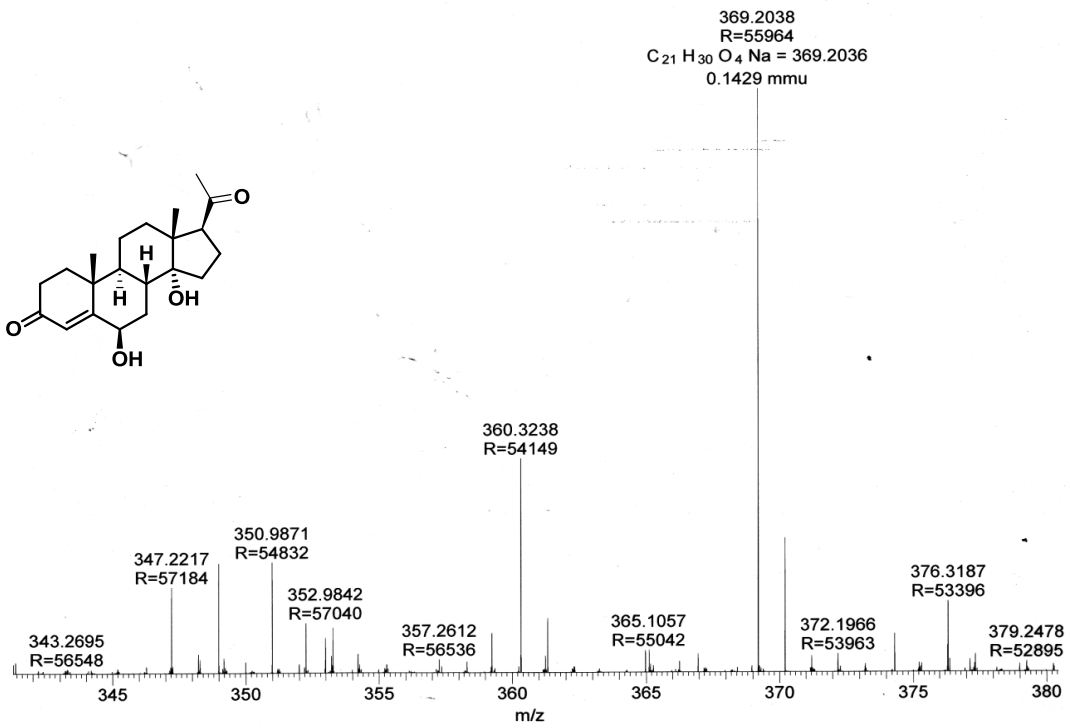
^{1}H NMR spectrum of **1f** in $CDCl_3$ at 500 MHz



^{13}C NMR spectrum of **1f** in $CDCl_3$ at 500 MHz

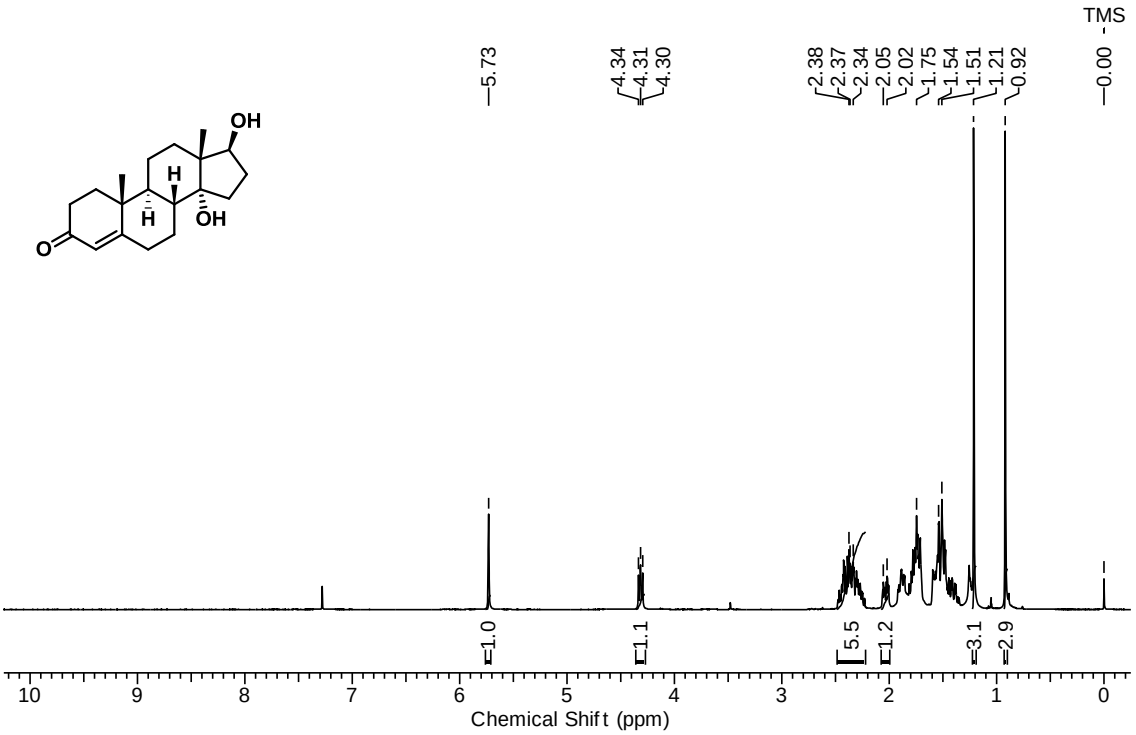


DEPT NMR spectrum of **1f** in $CDCl_3$ at 500 MHz

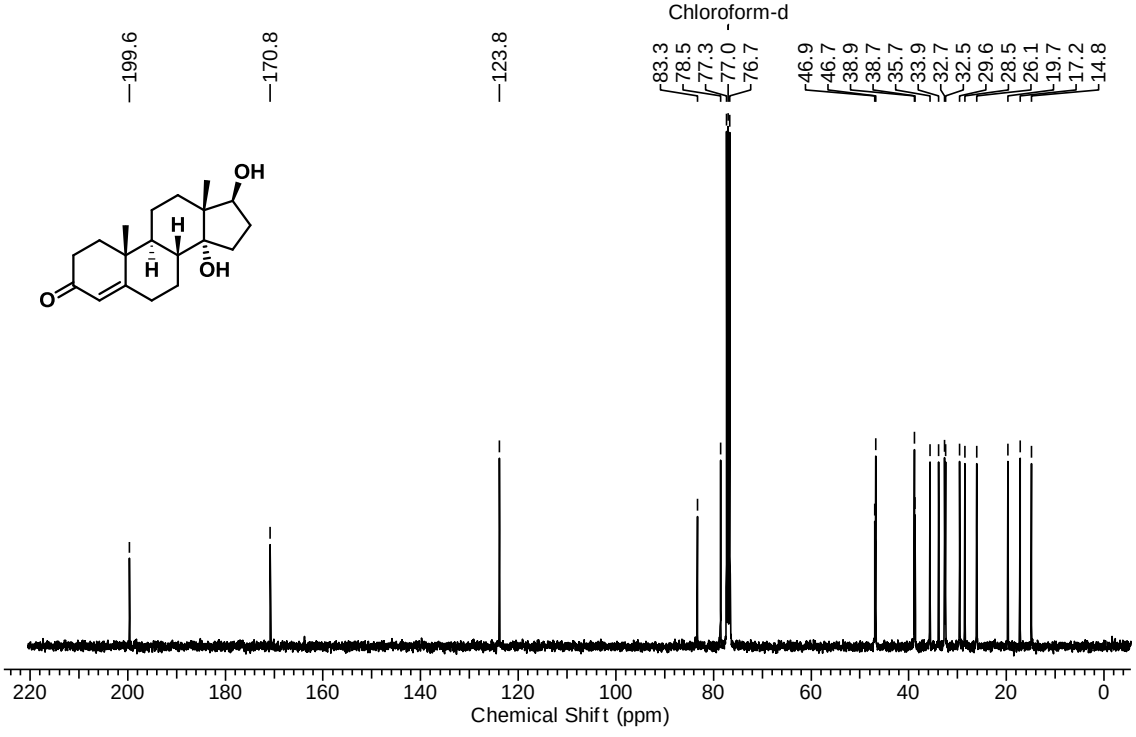


Mass spectrum of **1f** (ESI+ mode)

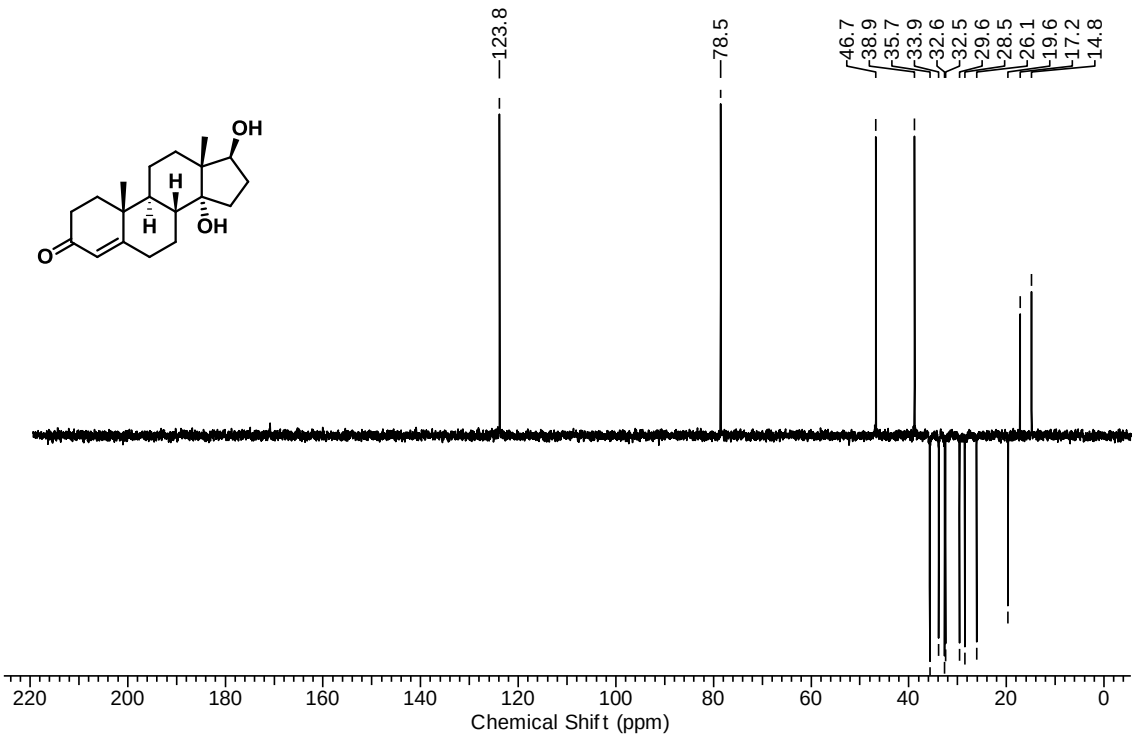
14*α*-hydroxytestosterone (2d)



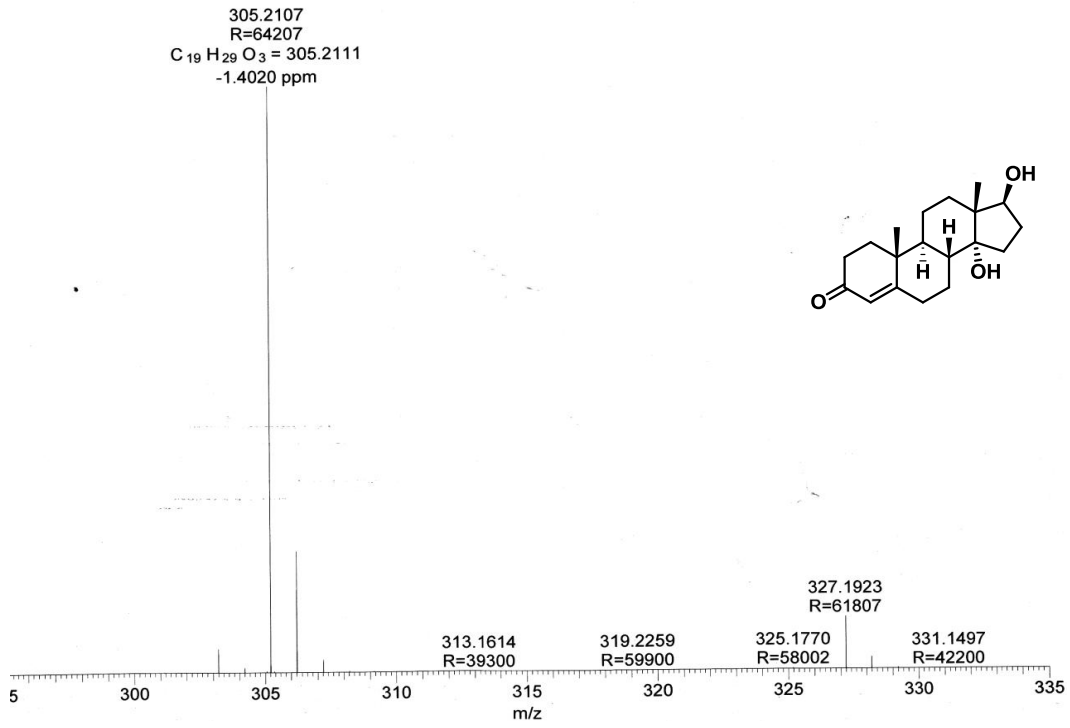
^{1}H NMR spectrum of **2d** in $CDCl_3$ at 400 MHz



^{13}C NMR spectrum of **2d** in $CDCl_3$ at 400 MHz

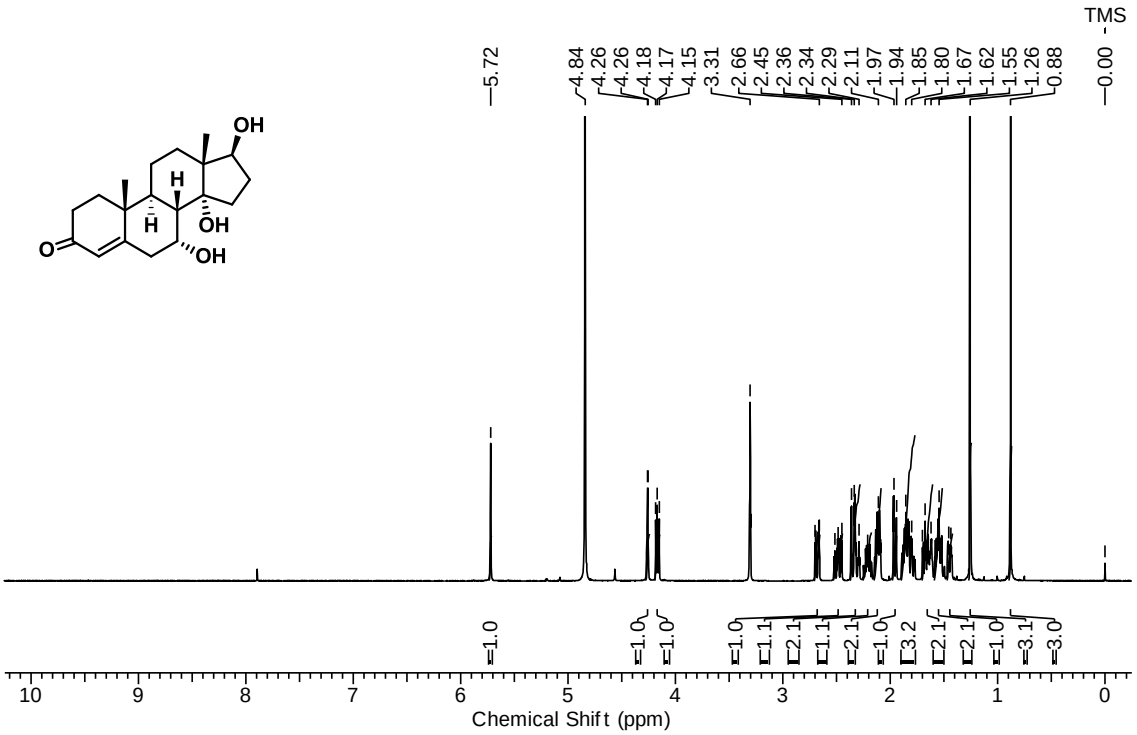


DEPT NMR spectrum of **2d** in $CDCl_3$ at 400 MHz

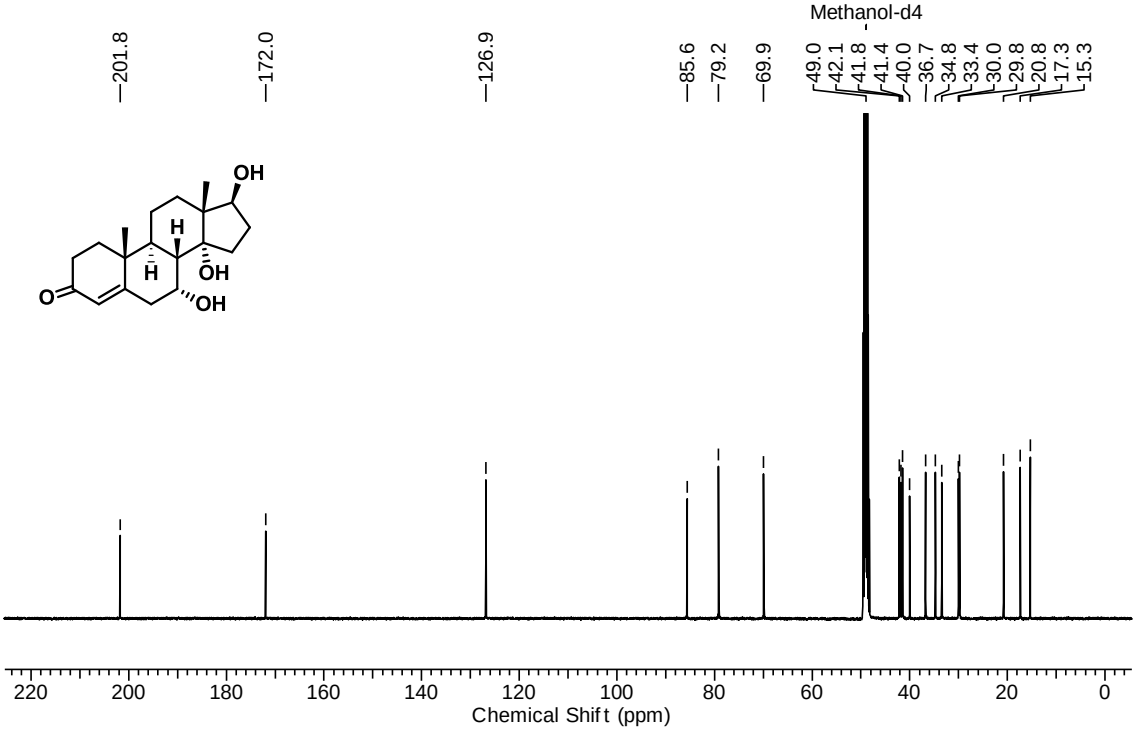


Mass spectrum of **2d** (ESI+ mode)

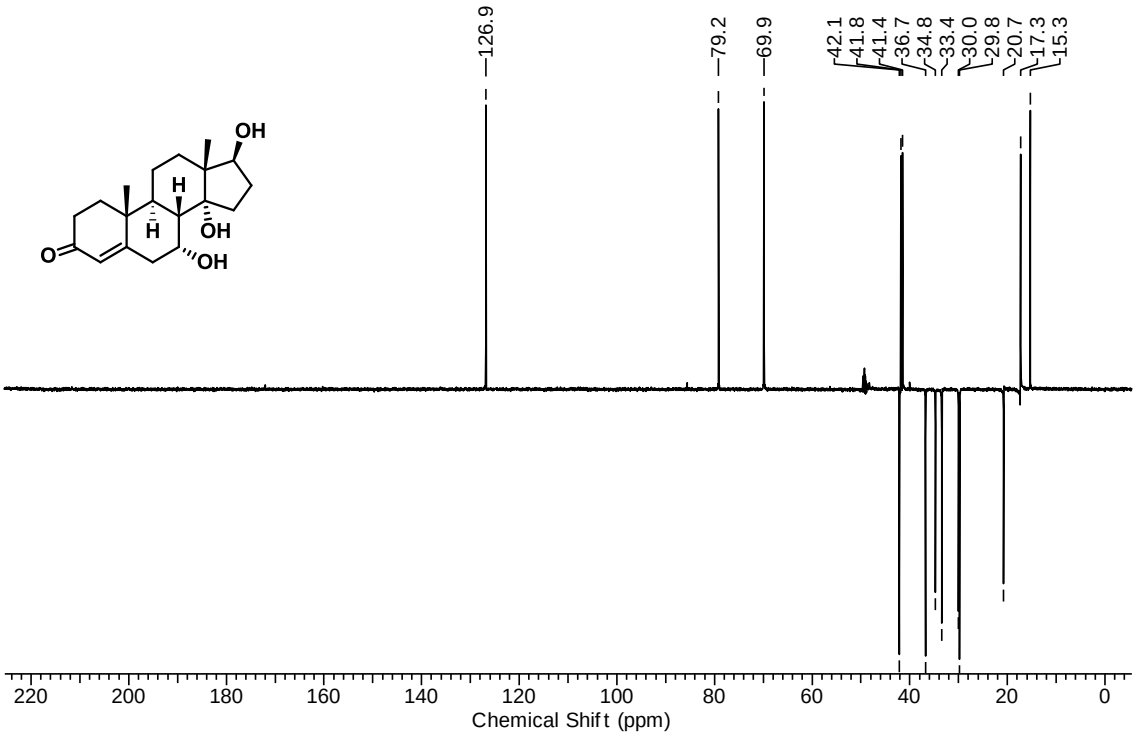
7α,14α-dihydroxytestosterone (2e)



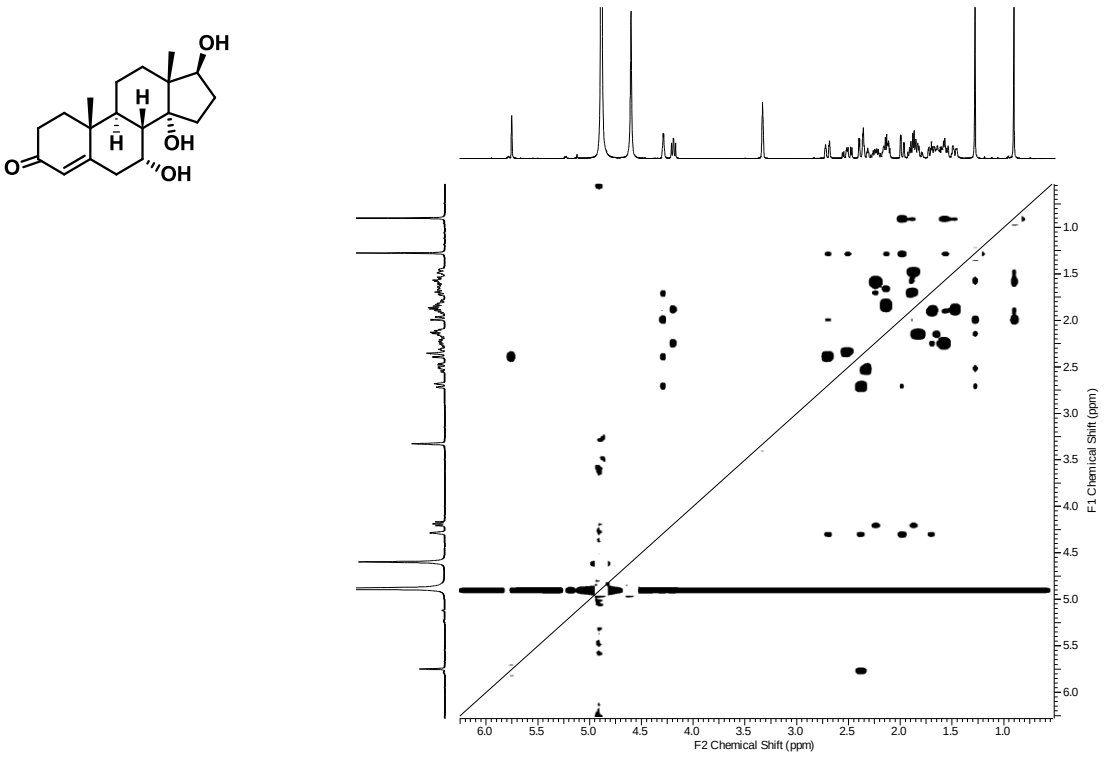
^{1}H NMR spectrum of **2e** in CD_3OD at 500 MHz



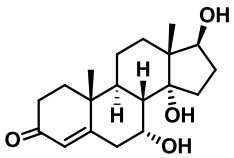
^{13}C NMR spectrum of **2e** in CD_3OD at 500 MHz

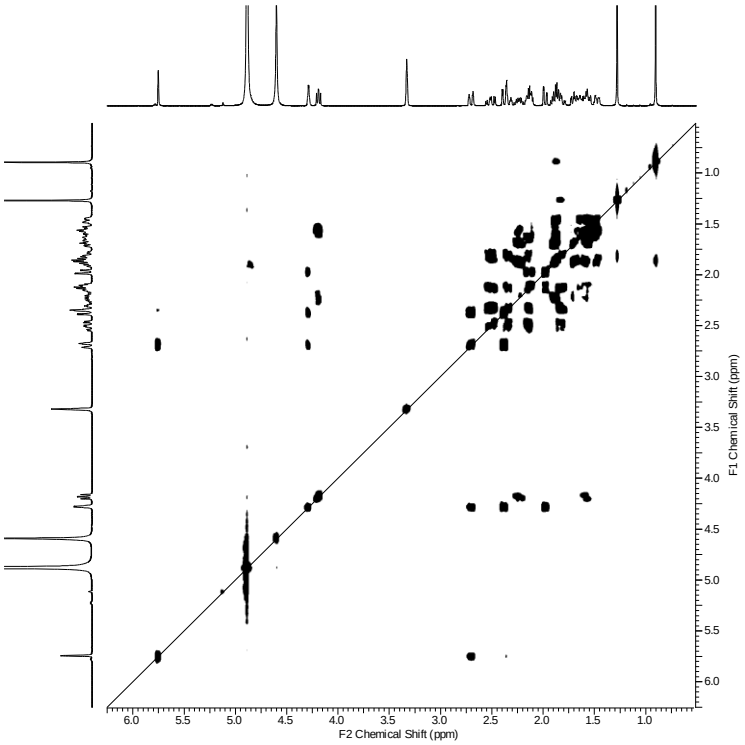


DEPT NMR spectrum of **2e** in CD_3OD at 500 MHz

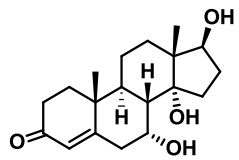


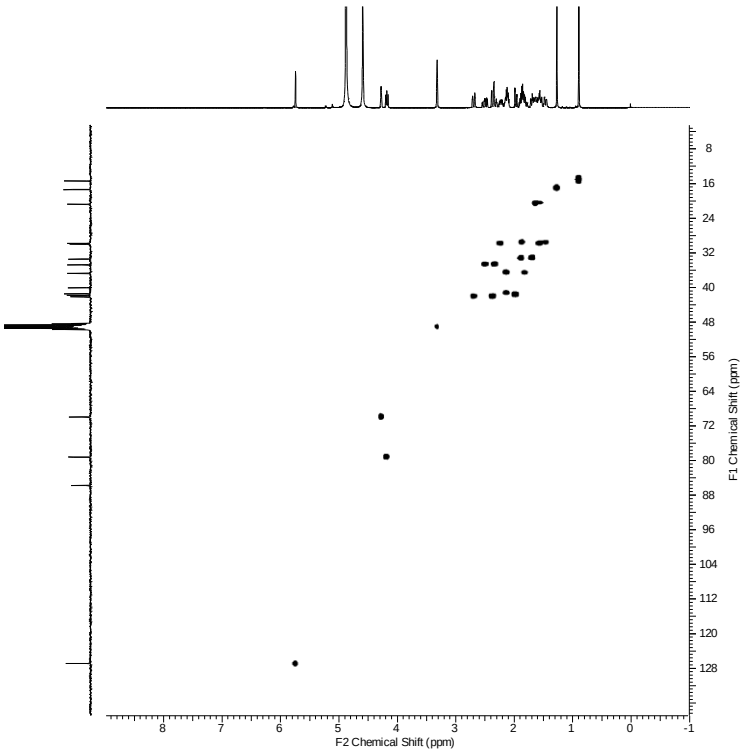
NOESY spectrum of **2e** in CD_3OD



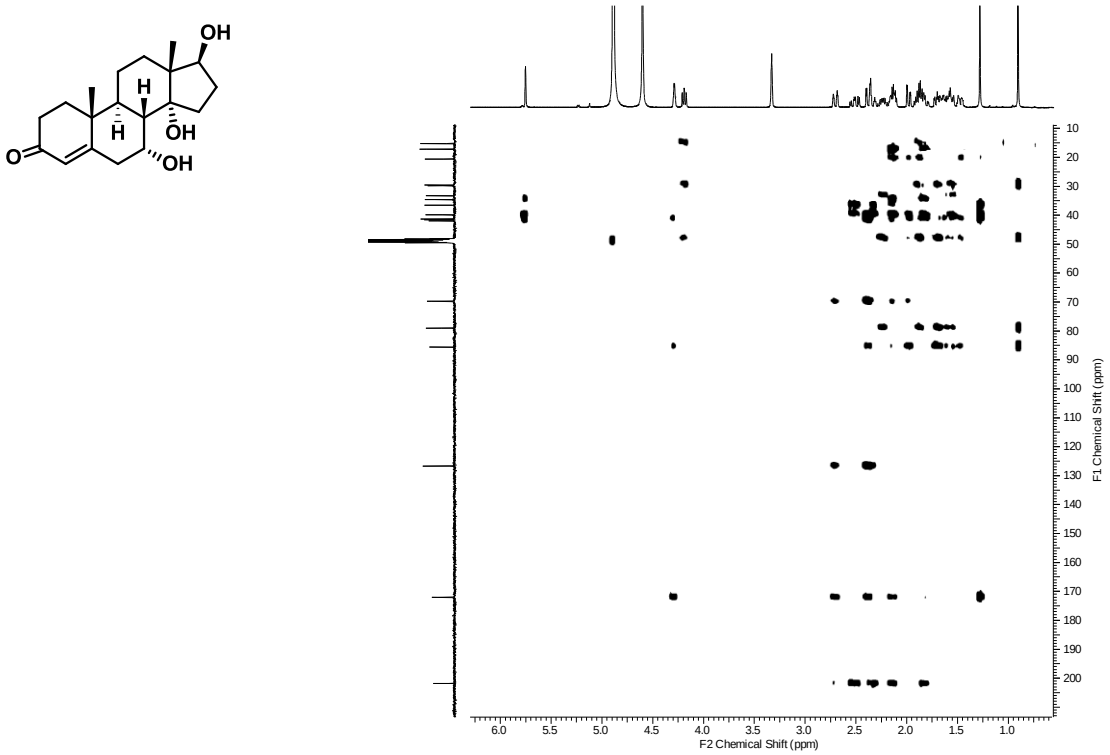


COSY spectrum of **2e** in CD_3OD

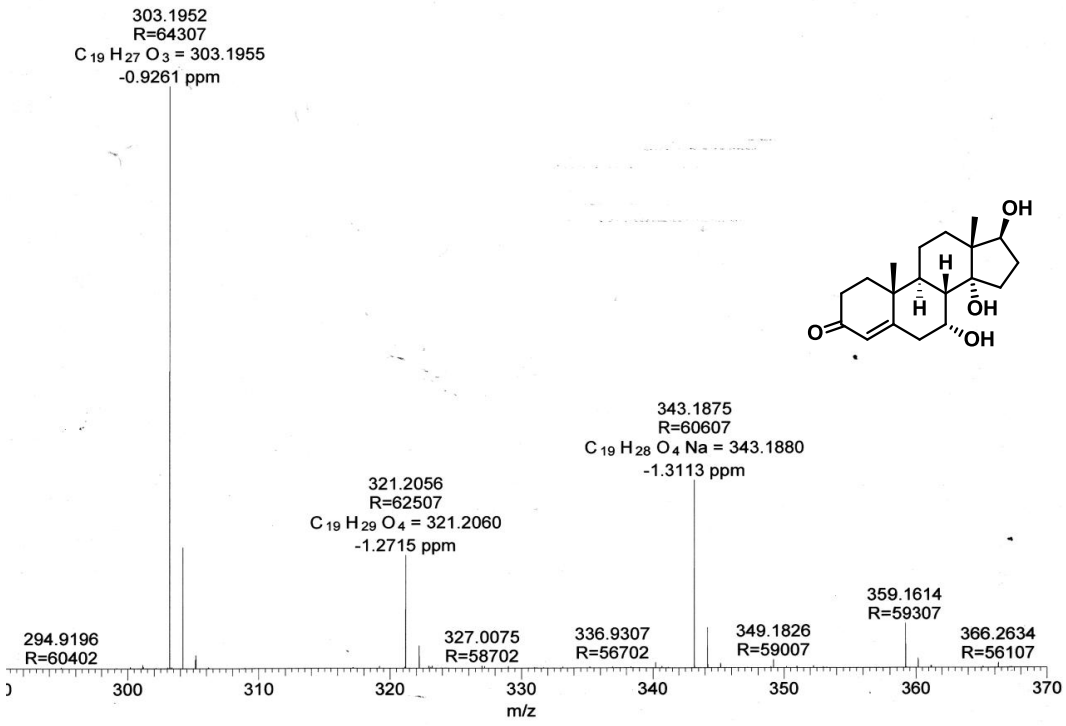




HSQC spectrum of **2e** in CD_3OD

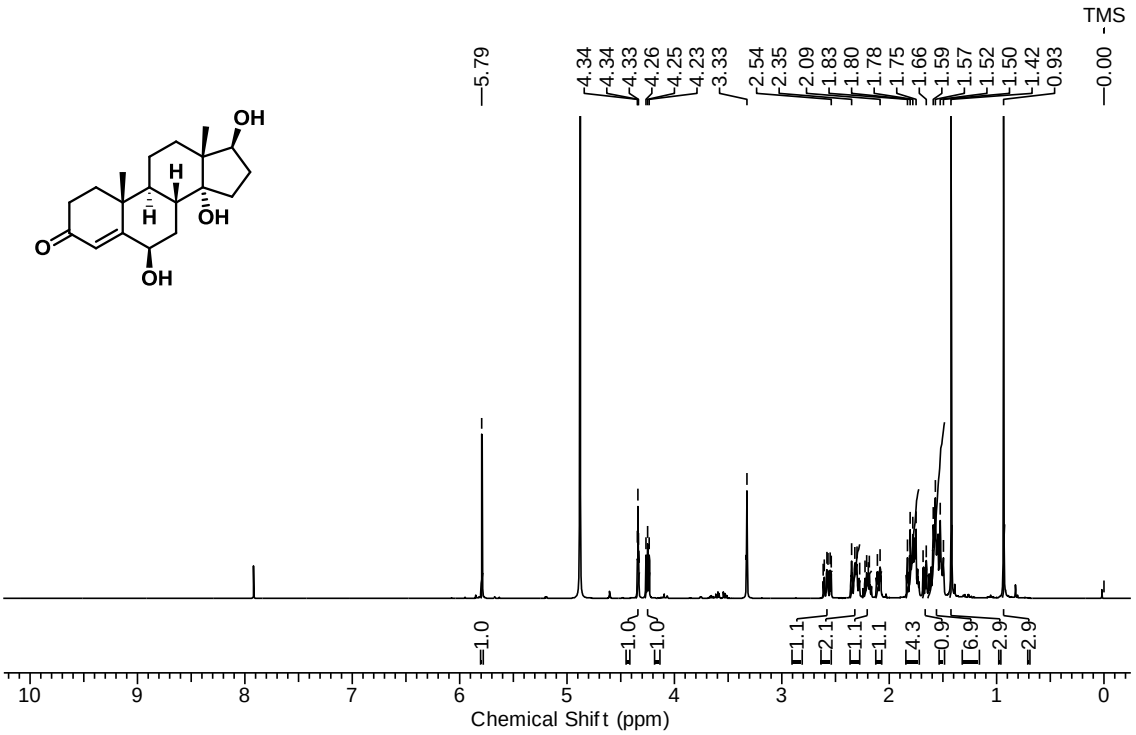


HMBC spectrum of **2e** in CD_3OD

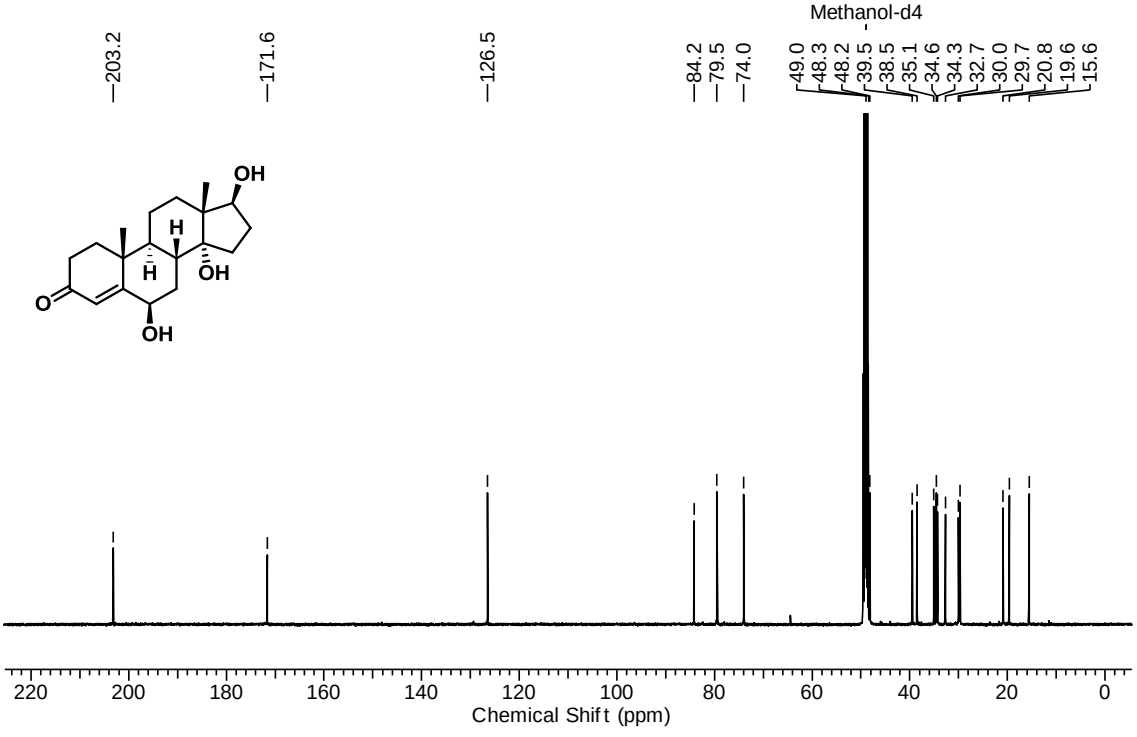


Mass spectrum of **2e** (ESI+ mode)

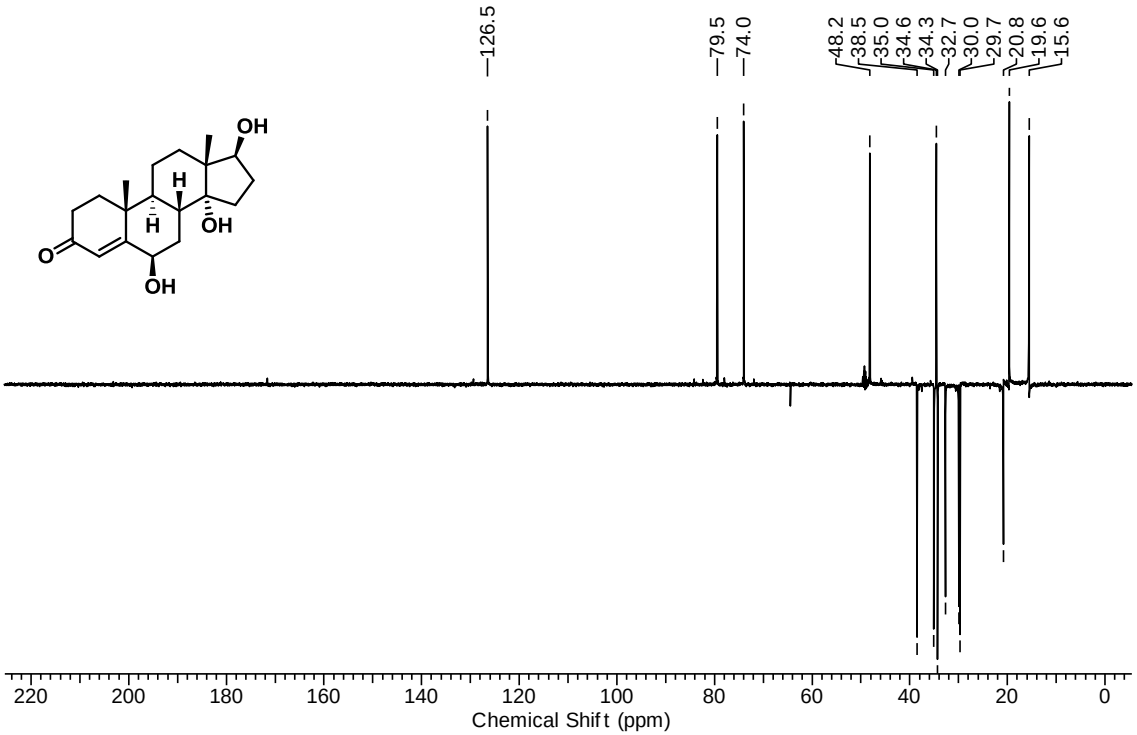
6β,14α-dihydroxytestosterone (2f)



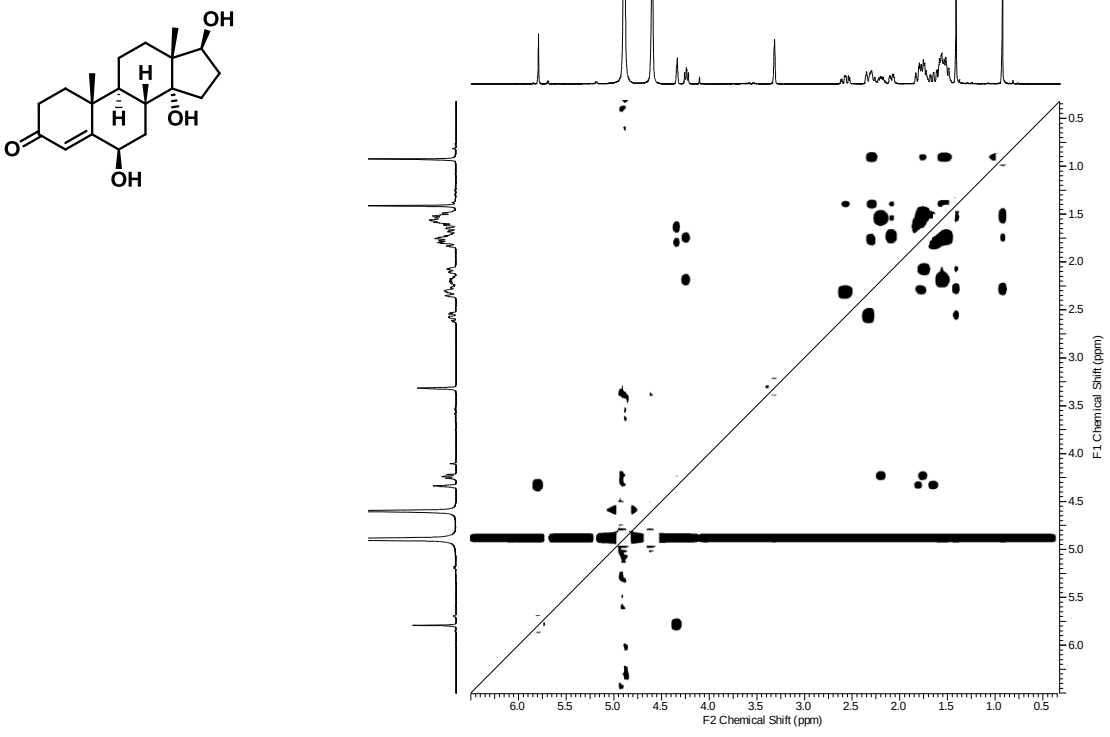
^{1}H NMR spectrum of **2f** in CD_3OD at 500 MHz



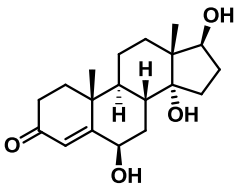
^{13}C NMR spectrum of **2f** in CD_3OD at 500 MHz

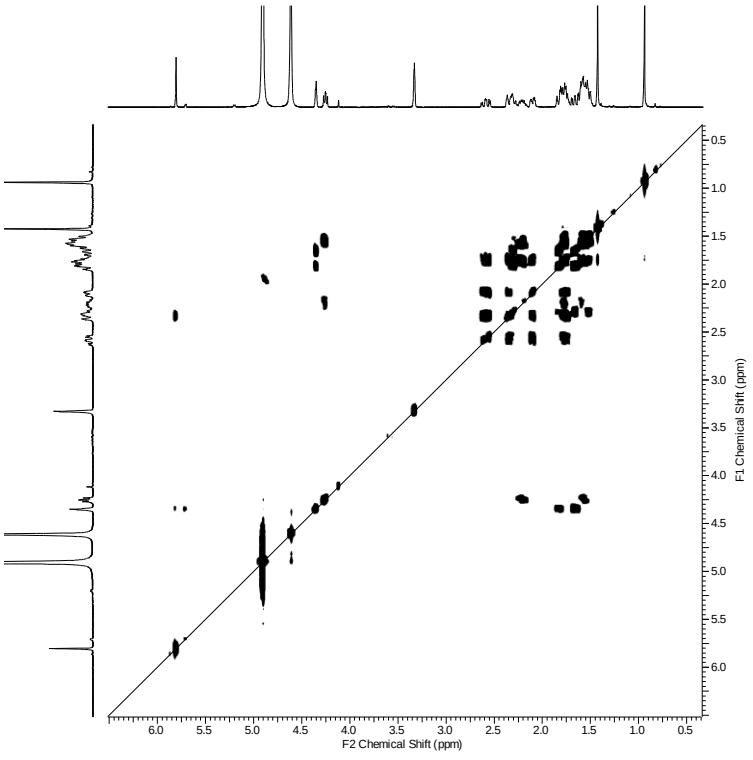


DEPT NMR spectrum of **2f** in CD_3OD at 500 MHz

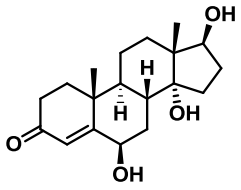


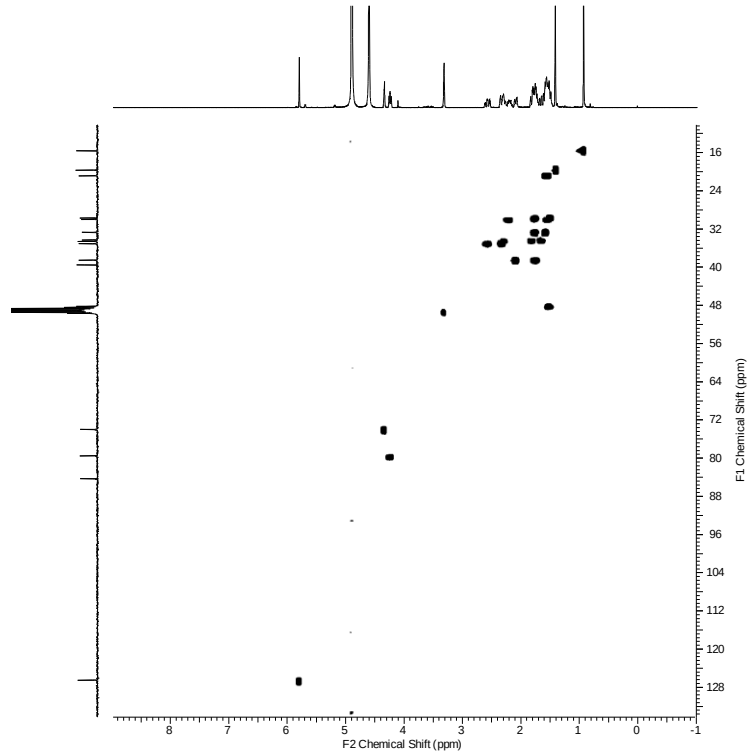
NOESY spectrum of **2f** in CD_3OD



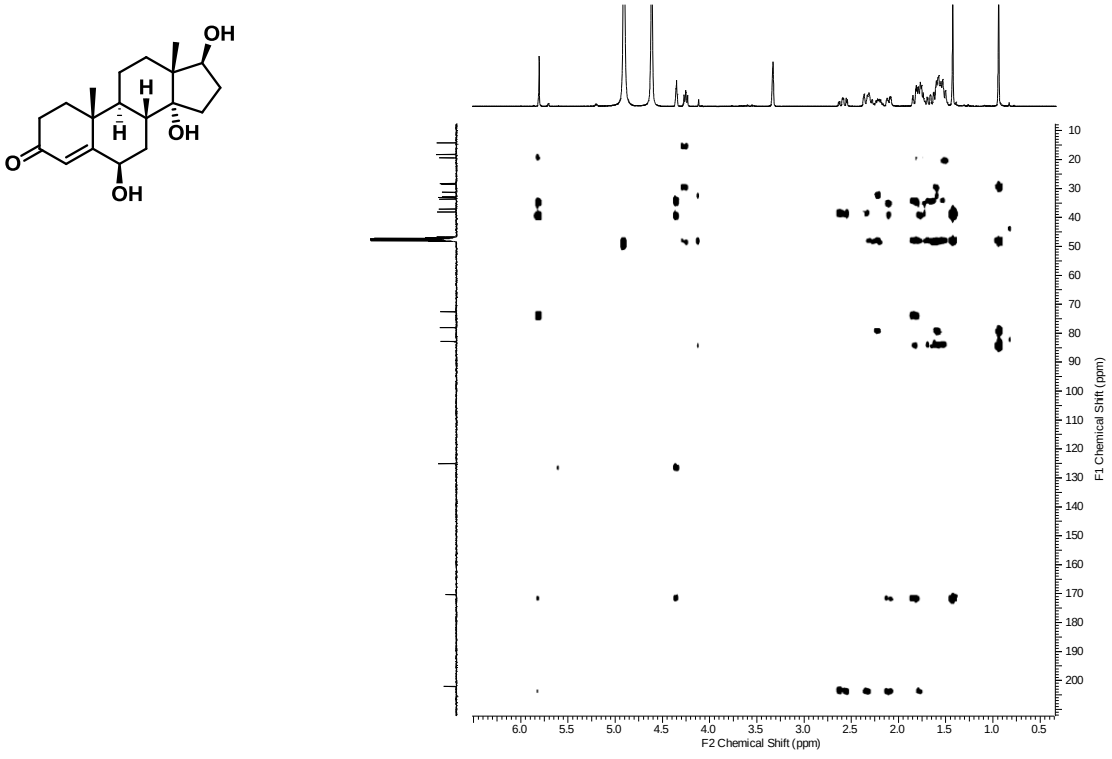


COSY NMR spectrum of **2f** in CD_3OD

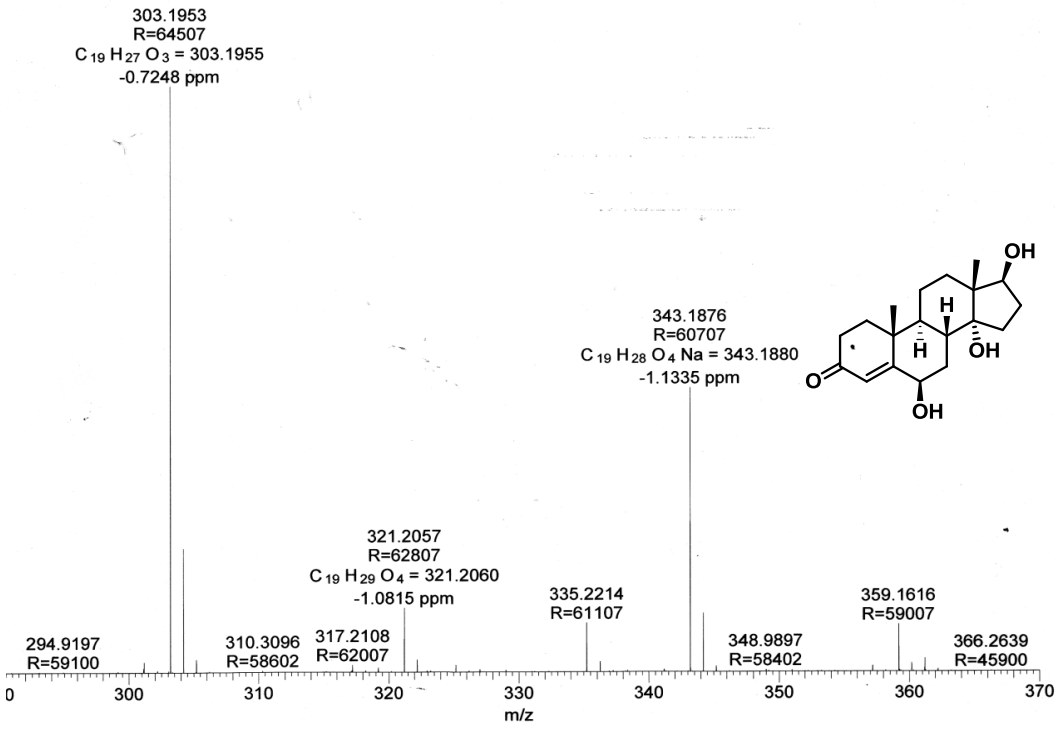




HSQC NMR spectrum of **2f** in CD_3OD

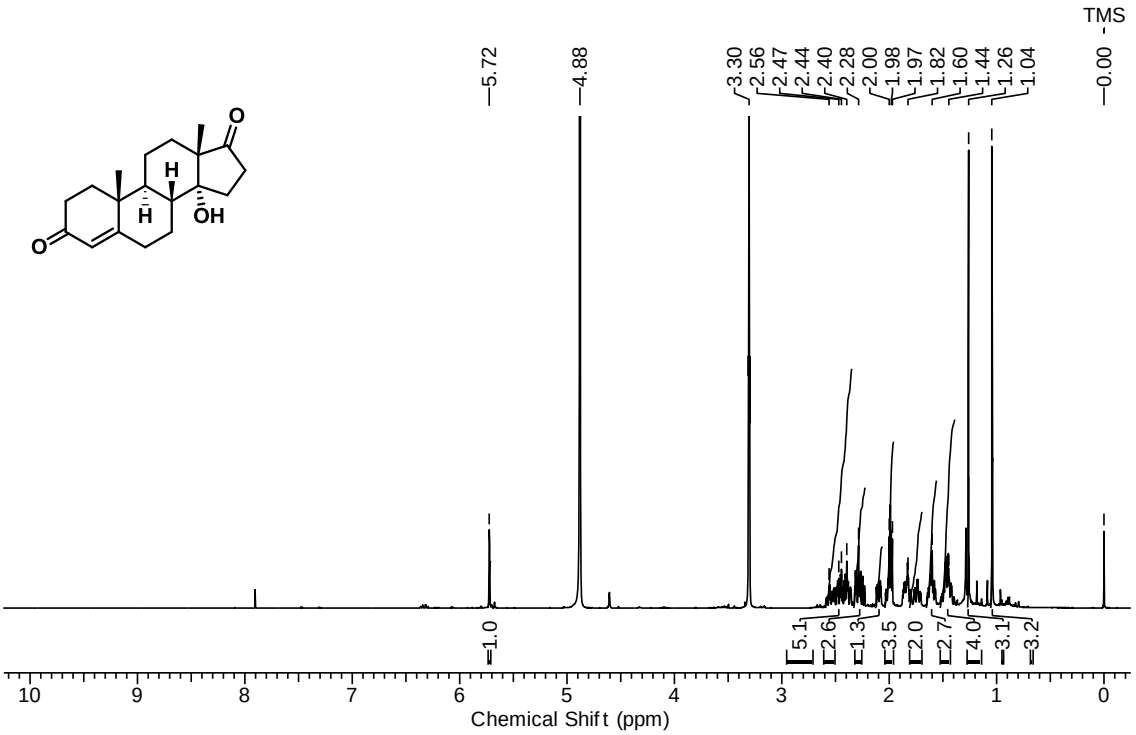


HMBC NMR spectrum of **2f** in CD_3OD

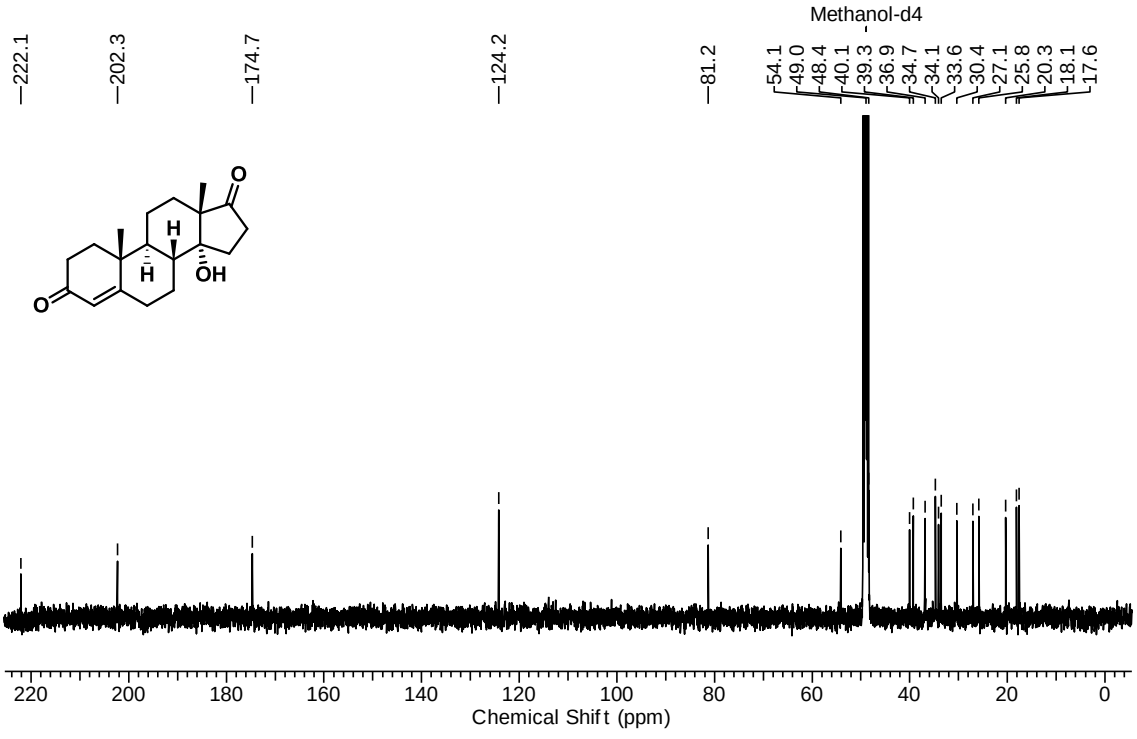


Mass spectrum of **2f** (ESI+ mode)

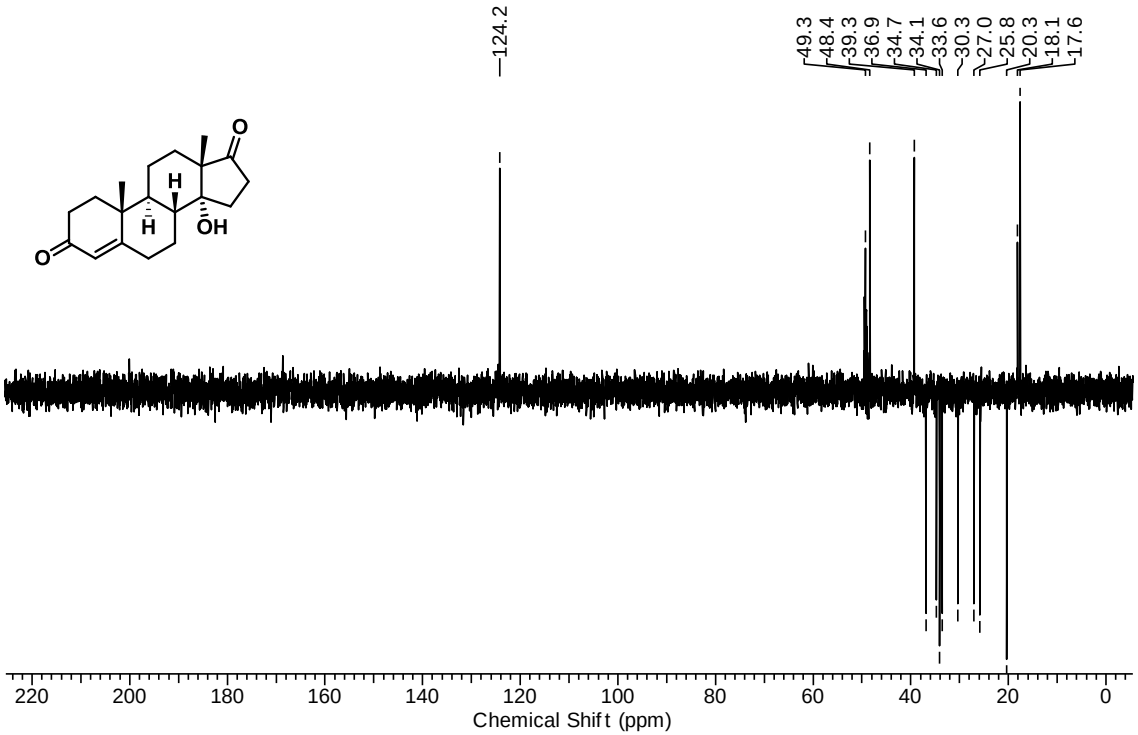
14α-hydroxy-4-androstene-3,17-dione (2g)



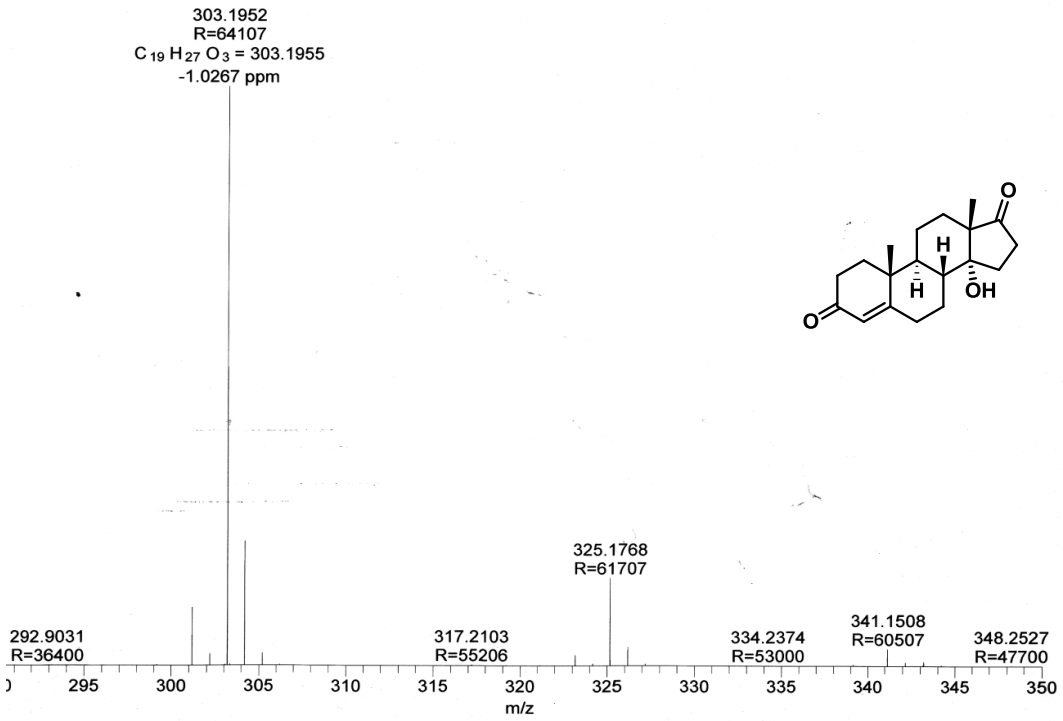
^{1}H NMR spectrum of **2g** in CD_3OD at 500 MHz



^{13}C NMR spectrum of **2g** in CD_3OD at 500 MHz



DEPT NMR spectrum of **2g** in CD_3OD at 500 MHz



Mass spectrum of **2g** (ESI+ mode)

4.6: References

(1) Barnes, P. J.; Adcock, I. *Trends Pharmacol. Sci.* **1993**, *14*, 436-441.
(2) Chlebowski, R. T.; Herrold, J.; Ali, I.; Oktay, E.; Chlebowski, J. S.; Ponce, A. T.; Heber, D.; Block, J. B. *Cancer* **1986**, *58*, 183-186.
(3) Tan, K.; Roda, R.; Ostrow, L.; McArthur, J.; Nath, A. *Neurology* **2009**, *72*, 1458-1464.
(4) Templeton, J. F.; Kumar, V. P. S.; Bose, D.; Smyth, D. D.; Kim, R. S.; Labella, F. S. *Can. J. Physiol. Pharmacol.* **1988**, *66*, 1420-1424.
(5) Yaderets, V. V.; Andryushina, V. A.; Voishvillo, N. E.; Stytsenko, T. S.; Zeinalov, O. A. *Pharm. Chem. J.* **2009**, *43*, 55-58.
(6) Kitawaki, J.; Kim, T.; Kanno, H.; Noguchi, T.; Yamamoto, T.; Okada, H. *J. Steroid Biochem. Mol. Biol.* **1993**, *44*, 667-670.
(7) Andre, A. F. S.; Macphillamy, H. B.; Nelson, J. A.; Shabica, A. C.; Scholz, C. R. *J. Am. Chem. Soc.* **1952**, *74*, 5506-5511.
(8) Wife, R. L.; Kyle, D.; Mulheirn, L. J.; Volger, H. C. *J. Chem. Soc. Chem. Comm.* **1982**, 306-307.
(9) Madyashtha, K. M.; Joseph, T. *J. Steroid Biochem. Mol. Biol.* **1993**, *45*, 563-569.
(10) Singh, K.; Sehgal, S.; Vèzina, C. *Can. J. Microbiol.* **1967**, *13*, 1271.
(11) Eppstein, S. H.; Meister, P. D.; Peterson, D. H.; Murray, H. C.; Osborn, H. M. L.; Weintraub, A.; Reineke, L. M.; Meeks, R. C. *J. Am. Chem. Soc.* **1958**, *80*, 3382-3389.
(12) Hu, S. H.; Genain, G.; Azerad, R. *Steroids* **1995**, *60*, 337-352.
(13) Smith, K. E.; Latif, S. A.; Kirk, D. N. *J. Steroid Biochem. Mol. Biol.* **1991**, *38*, 249-256.
(14) Madyashtha, K. M.; Joseph, T. *J. Steroid Biochem. Mol. Biol.* **1993**, *45*, 563-569.
(15) Nega, E.; Grunwaldt, G. *J. Basic. Microb.* **1997**, *37*, 139-145.
(16) Petric, S.; Hakki, T.; Bernhardt, R.; Zigon, D.; Cresnar, B. *J. Biotechnol.*, *150*, 428-437.
(17) Mutafov, S.; Angelova, B.; Avramova, T.; Boyadjieva, L.; Dimova, I. *Process Biochem.* **1997**, *32*, 585-589.
(18) Vitas, M.; Rozman, D.; Komel, R.; Kelly, S. L. *J. Biotechnol.* **1995**, *42*, 145-150.
(19) Vitas, M.; Pajic, T.; Kelly, S. L.; Komel, R. *J. Steroid Biochem. Mol. Biol.* **1997**, *63*, 345-350.
(20) Ahmed, F.; Williams, R. A. D.; Smith, K. E. *J. Steroid Biochem. Mol. Biol.* **1996**, *58*, 337-349.
(21) Samanta, T. B.; Ghosh, D. K. *J. Steroid Biochem. Mol. Biol.* **1987**, *28*, 327-332.
(22) Cotillon, A. C.; Doostzadeh, J.; Morfin, R. *J. Steroid Biochem. Mol. Biol.* **1997**, *62*, 467-475.
(23) Irrgang, S.; Schlosser, D.; Fritsche, W. *J. Steroid Biochem. Mol. Biol.* **1997**, *60*, 339-346.
(24) Makovec, T.; Breskvar, K. *Eur. J. Physiol.* **2000**, *439*, R111-R112.
(25) Suzuki, K.; Sanga, K.; Chikaoka, Y.; Itagaki, E. *Biochim. Biophys. Acta.* **1993**, *1203*, 215-223.
(26) Strijewski, A. *Eur. J. Biochem.* **1982**, *128*, 125-135.
(27) El-Kadi, I. A.; Mostafa, M. E. *Folia Microbiol.* **2004**, *49*, 285-290.
(28) Kristan, K.; Rizner, T. L. *J. Steroid Biochem. Mol. Biol.* **2012**, *129*, 79-91.
(29) Antoniou, T. N. H.; Curran, B. P. G.; Smith, K. E. *Microbiology* **1994**, *140*, 1633-1640.
(30) Templeton, J. F.; Kumar, V. P. S.; Marat, K.; Kim, R. S.; Labella, F. S.; Cote, D. *J. Nat. Prod.* **1987**, *50*, 463-467.
(31) Habibi, Z.; Yousefi, M.; Ghanian, S.; Mohammadi, M.; Ghasemi, S. *Steroids* **2012**, *77*, 1446-1449.
(32) Hunter, A. C.; Watts, K. R.; Dedi, C.; Dodd, H. T. *J. Steroid Biochem. Mol. Biol.* **2009**, *116*, 171-177.
(33) Brzezowska, E.; DmochowskaGladysz, J.; Kolek, T. *J. Steroid Biochem. Mol. Biol.* **1996**, *57*, 357-362.

List of Research Publications

- "Biocatalyst Mediated Production of 6β,11α-dihydroxy derivatives of 4-ene-3-one Steroids" **Swati P. Kolet**, Siddiqui Niloferjahan, Saikat Haldar, Rajesh Gonnade and Hirekodathakallu V. Thulasiram*****, *Steroids*, **2013**, *78*, 1152-1158.
- "Biocatalysis: fungi mediated novel and selective 12- or 17-hydroxylation on the basic limonoid skeleton" Saikat Haldar, **Swati P. Kolet** and Hirekodathakallu V. Thulasiram*, *Green Chem.*, **2013**, *15*, 1311-1317.
- "Expedient preparative isolation, quantification and rapid characterization of basic limonoids" Saikat Haldar, Prasad B. Phapale, **Swati P. Kolet** and Hirekodathakallu V. Thulasiram*****, *Anal. Methods*, **2013**, *5*, 5386-5391.
- "One-Pot Fluorescent Labeling Protocol for the Complex Hydroxylated Bioactive Natural Product" Saikat Haldar, Santosh Kumar, **Swati P. Kolet**, Harshal S. Patil, Dhiraj Kumar, Gopal C. Kundu, and Hirekodathakallu V. Thulasiram*****, *J. org. chem.*, **2013**, *78*, 10192-10202.
- "Transformation of testosterone and progesterone by *Mucor hiemalis*: Investigation of Kinetics, metabolism and 14α-hydroxylase activity" **Swati P. Kolet,** Saikat Haldar, Siddiqui Niloferjahan and Hirekodathakallu V. Thulasiram* (Communicated).

Symposium Attended/ Poster Presentations

- Attended the National level Symposium: "CRSI", 2009 at National Chemical Laboratory, Pune and presented poster on "Microbial transformation of steroids". **Swati P. Kolet**, Manish Ranjan and Hirekodathakallu V. Thulasiram*.
- Attended the International level Symposium: "INSA", 2009 at National Chemical Laboratory, Pune and presented poster on "Microbial transformation of steroids" **Swati P. Kolet**, Siddiqui Niloferjahan and Hirekodathakallu V. Thulasiram*.
- Attended the Biodesign India symposium 2010, University of Kerala, Thiruvananthapuram and presented poster on "Microbial transformation of steroids: Isolation of steroids hydroxylase" **Swati P. Kolet**, Siddiqui Niloferjahan and Hirekodathakallu V. Thulasiram*.
- Attended the international conference Biotrans 2013, Manchester, London and presented poster on "Biocatalysis: Fungi Mediated Novel and Selective Hydroxylations on the Steroids and Basic Limonoid Skeletons" **Swati P. Kolet**, Saikat Haldar and Hirekodathakallu V. Thulasiram*.

Erratum

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Erratum