The development of 19F NMR as a tool for analysing steroid degradation in active urine samples

MARIA MAGDALENA CZYZEWSKA

The development of ^{19}F NMR as a tool for analysing steroid degradation in active urine samples

Maria Magdalena Czyzewska

A thesis submitted for the degree of Doctor of Philosophy

Department of Chemistry

University of Durham

2017

Abstract

The detection of prohibited performance-enhancing drugs in sports is often carried out using urine samples. The reason for this is that urine samples can be collected under non-sterile conditions and do not require the presence of a sanctioned medical officer. Improper storage of the urine samples from athletes can lead to microbial contamination, which can cause changes in the steroids profile, leading to false positive or false negative results for a particular athlete. To address this problem, a new analytical method was proposed that employs a fluorinated steroid as an internal standard and fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F-NMR) spectroscopy to identify both microbial and thermally-induced changes in the urine samples.

In **Chapter 2**, synthesis of fluorinated steroids was carried out using method that involve the reaction of Selectfluor® with enolates/enols of steroids. A range of fluorinated steroids was prepared (2 novel F-steroids) in moderate yields and varying diastereoselectivities. Several synthesised steroids were recrystallized and crystals suitable for X-ray were obtained.

In **Chapter 3**, selected fluorinated steroids were incubated with microorganisms such as *Escherichia coli, Bacillus subtilis, Bacillus megaterium* and *Streptomyces griseus*. Fluorinated steroids were transformed to various oxidised metabolites upon incubation with *Streptomyces griseus*. *Escherichia coli* did not produce any metabolites due to lack of cytochrome P450 enzymes. Incubation of *Bacillus subtilis* and *Bacillus megaterium* was not successful and therefore metabolites were not detected.

In **Chapter 4**, hydroxy steroids were reacted with pentafluoropyridine (PFP) to form perfluoropyridine ethers in good yield. Several novel steroids were synthesised and the structures of 4 perfluoropyridine ethers were confirmed for the first time by X-ray structure. It was found that the hydroxy steroid PFP adducts have very similar ^{19}F NMR spectra however they can be distinguished using this technique. This novel derivatisation technique could be potentially used for identification of hydroxy steroids in biological material by ^{19}F NMR.

Declaration

This work was conducted in the Department of Chemistry at Durham University between October 2012 and September 2016. The work has not been submitted for a degree in this, or any other university. It is my own work, unless otherwise indicated.

Copyright

Acknowledgment

Dla mojego Michałka, który jest dla mnie prawdziwie Bożym darem.

Abbreviations

Ac	Acetyl
Boc	*tert*-Butoxycarbonyl
b.p.	Boiling point
Bu	Butyl
cm^{-1}	Wavenumbers
COSY	Correlation spectroscopy
d	Doublet (spectral)
DAST	Diethylaminosulfurtrifluoride
DCM	Dichloromethane
dd	Doublet of doublets (spectral)
ddd	Doublet of doublet of doublets (spectral)
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
ds	Diastereoselectivity
eq	Equivalent
Et	Ethyl
g	Gramme
GC	Gas Chromatography
GC-MS	Gas Chromatography / Mass Spectrometry
h	Hour(s)
Hz	Hertz
IAAF	International Amateur Athletics Federation
IOC	International Olympic Committee
IR	Infrared

J	Coupling constant
LDA	Lithium diisopropylamide
lit.	Literature
m	Multiplet (spectral)
m/z	Mass-to-charge ratio
Me	Methyl
min	Minutes
mmol	Millimole(s)
m.p.	Melting point
MS	Mass spectrometry
MSTFA	*N*-Methyl-*N*-(trimethlysilyl)trifluoroacetamide
NFOBS	*o*-Benzenedisulfonamide
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
NSFI	Fluorobenzenesulfonamide
Nu	Nucleophile
Ph	Phenyl
PPHF	Olah's reagent
ppm	Part(s) per million
q	Quartet (spectral)
R	Alkyl
RT	Room temperature
s	Singlet (spectral)
TBME	*tert*-Butyl methyl ether
t-Bu	*tert*-Butyl
temp	Temperature

THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
WADA	World Anti-Doping Agency

Contents

1 Introduction

1.1 Doping in Sports

The first recognition of performance-enhancing drugs in sport occurred in the early 1920s, when the International Amateur Athletics Federation (IAAF) prohibited the use of stimulants.[1] However, with a lack of any effective means of testing for banned substances available the IAAF often had to rely solely on the word of the athlete in question. In 1966 the Union Cycliste Internationale and the F´ed´eration Internationale de Football Association joined the IAAF in their fight against performance enhancing drugs when they introduced tests for the detection of doping substances into their World Championships.[2] The International Olympic Committee (IOC) formed a medical commission in 1967 to deal with doping in sports and in 1968 they introduced compulsory anti-doping testing for athletes at both the Summer and Winter Olympics. In the 1980s, the IOC recruited a number of laboratories worldwide to perform banned substance testing on a routine basis and at the 1988 Olympic Games Ben Johnson became the first Olympic competitor to be sanctioned for doping.[3] In 1999 an independent agency The World Anti-Doping Agency (WADA) was created to promote, coordinate, and monitor the fight against doping. In the subsequent years since formation, WADA has established detailed anti-doping guidelines together with a comprehensive and ever changing list of prohibited substances.[4] Selected examples of prohibited substances, as determined by WADA, are given in **Table 1.1**.

Table 1.1 Overview of the 2016 Prohibited Drugs List compiled by WADA (1th January 2016).

Entry	Class	Class name	Selected examples
1	S1.1a	Exogenous steroids	1-androstenedio, 1-androstenedione, bolandiol, bolasterone, boldenone, boldione, calusterone; clostebol, danazol.
2	S1.1.b	Endogenous steroids	androstenediol, androstenedione, dihydrotestosterone, dehydroepiandrosterone, testosterone.
3	S1.2	Other Anabolic Agents	Clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol.
4	S2.1	Peptide hormones, growth factors and related substances	Erythropoiesis-Stimulating Agents [e.g. erythropoietin (EPO), darbepoetin (dEPO), hypoxia-inducible factor (HIF) stabilizers, methoxy polyethylene glycol-epoetin beta (CERA), peginesatide (Hematide)].
5	S3	Beta-2 agonists	Salbutamol, formoterol
6	S4.1	Hormone and metabolic modulators	aminoglutethimide, anastrozole, androstatrienedione, 4-androstene-3,6,17 trione (6-oxo), exemestane, formestane, letrozole, testolactone
7	S5	Diuretics and other masking agents	Acetazolamide, amiloride, bumetanide, canrenone, chlorthalidone, etacrynic acid, furosemide, indapamide, metolazone, spironolactone, thiazides

Class description:

- **S0. Non-approved substances**

Any pharmacological substance which is not included in the subsequent sections of the List and with no current approval by any governmental regulatory health authority for human therapeutic use is prohibited at all times (e.g. drugs under pre-clinical or clinical development or discontinued, designer drugs, substances approved only for veterinary use).

- **S1. Anabolic agents**

Anabolic Androgenic Steroids (AAS): “exogenous” (refers to a substance which is not ordinarily produced by the body naturally) and “endogenous” (refers to a substance which is ordinarily produced by the body naturally).

- **S2. Peptide hormones, growth factors, related substances and mimetics**

The following substances, and other substances with similar chemical structure or similar biological effect(s), are prohibited: Erythropoiesis-Stimulating Agents (e.g. erythropoietin (EPO), darbepoetin (dEPO), hypoxia-inducible factor (HIF) stabilizers); Chorionic Gonadotrophin (CG) and Luteinizing Hormone (LH) and their releasing factors;

Corticotrophins and their releasing factors (e.g. corticorelin acetate); Growth Hormone (GH) and its releasing factors (e.g. hexarelin, alexamorelin); Insulin-like Growth Factor-1 (IGF-1); Fibroblast Growth Factors (FGFs), Hepatocyte Growth Factor (HGF), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Vascular-Endothelial Growth Factor (VEGF)

- **S3. Beta-2 agonists**
  - All beta-2 agonists, including all optical isomers where relevant, are prohibited. Except:
  - Inhaled salbutamol (maximum 1600 μg over 24 hours);
  - Inhaled formoterol (maximum delivered dose 54 μg over 24 hours);
  - Inhaled salmeterol in accordance with the manufacturers' recommended therapeutic regimen.

- **S4. Hormone and metabolic modulators**

Hormones - substances targeting wide range of organs to regulate physiology and behaviour. Metabolic modulators - modify the effects of hormones, accelerate or slow down specific enzyme reactions.

- **S5. Diuretics and masking agents**

Diuretics- any substance that increases production of urine. Masking agent- any substance that is used to hide or prevent detection of a banned substance.

Figure 1.1 Anti-doping headlines in sports.

Athletes' use of illicit substances continues to hit the headlines since the 1970s sometimes even with rumours of state-sponsored doping **(Figure 1.1)**. In 2004 the British sprinter, Dwain Chambers was banned from competition for two years after being found guilty of taking the anabolic steroid THG.[5] In January 2013, the retired American cyclist Lance Armstrong admitted to doping and accepted the punishment by the U.S. Anti-Doping Agency (USADA). He was stripped of his seven Tour de France wins and banned from sport for life. USADA's allegations were based on testimony the agency took from eyewitnesses, members of Armstrongs' USPS and Discovery Channel cycling teams who say they saw Armstrong taking the drugs or in possession of them. Some of the performance enhancers allegedly taken by Armstrong included:

- **Erythropoietin (EPO)**: is used by athletes to increase the number of red blood cells in their circulatory system, which are available to carry oxygen. Athletes implemented a number of means to avoid detection of EPO use, including: micro-dosing (using smaller amounts of EPO to reduce the clearance time of the drug), intravenous injections (injecting the drug directly into the vein rather than subcutaneously to reduce clearance time), saline, plasma or glycerol infusions.
- **Blood transfusions (blood doping):** this generally involve the extraction of an athlete's own blood pre-competition and re-infusion of that blood shortly before or during competition (e.g. in the evening or on a rest day in a multistage race) to increase the athlete's oxygen carrying red blood cells.
- **Testosterone:** it is an anabolic agent and can increase muscle mass and strength. In smaller doses anabolic agents such as testosterone can promote muscle recovery from strenuous exercise and increase endurance.
- **Human Growth Hormone (hGH)**: it is used to increase strength and lean muscle mass, to assist in weight loss and promote recovery.
- **Corticosteroids (e.g. cortisone):** reduce inflammation, assist in recovery and can provide a burst of energy and create a temporary feeling of increased energy and well-being.

More recently in December 2014, a German TV documentary alleged as many as 99% of Russian athletes were guilty of doping, although the Russian Athletics Federation described the allegations as "lies". The World Anti-Doping Agency had recommended a blanket ban for all Russian athletes from the Olympic Games in Rio 2016. After numbers of appeals by

Russian athletes who live and train outside Russia. The court of Arbitration for Sport (CAS) has cleared a total of 271 from 389 Russian athletes to take part in the Rio Olympic Games. Since then, there have been numerous further allegations of doping in athletics.[6]

1.2 Methods of Detection in Anti-Doping Screening

Currently, anti-doping laboratories tend to rely mainly on mass spectrometric (MS) based analytical techniques in combination with gas and liquid chromatography to detect for example steroids that are on WADA's list of prohibited substances. A typical MS procedure for the detection of anabolic steroids in an athlete's urine sample is outlined in **Figure 1.2**.

The initial step of the sample preparation involves enzymatic hydrolysis of any water soluble steroids to remove any sulphate or glucuronate modifications. The resulting non-polar steroids are then extracted with *t*-butyl methyl ether (TBME) or *n*-pentane. Subsequently, recovered steroids are derivatized using a mixture of *N*-methyl-*N*-(trimethlysilyl), trifluoroacetamide (MSTFA), ammonium iodide, and ethanethiol. The trimethyliodosilane (TMS) formed in this mixture traps enols of ketosteroids and forms trimethylsilyl derivatives of the steroids. Once the TMS steroid derivatives are prepared, the sample is analysed by capillary GC-MS. The results of each experiment are reviewed manually comparing retention times and mass fragments to those of a standard urine sample containing each metabolite.

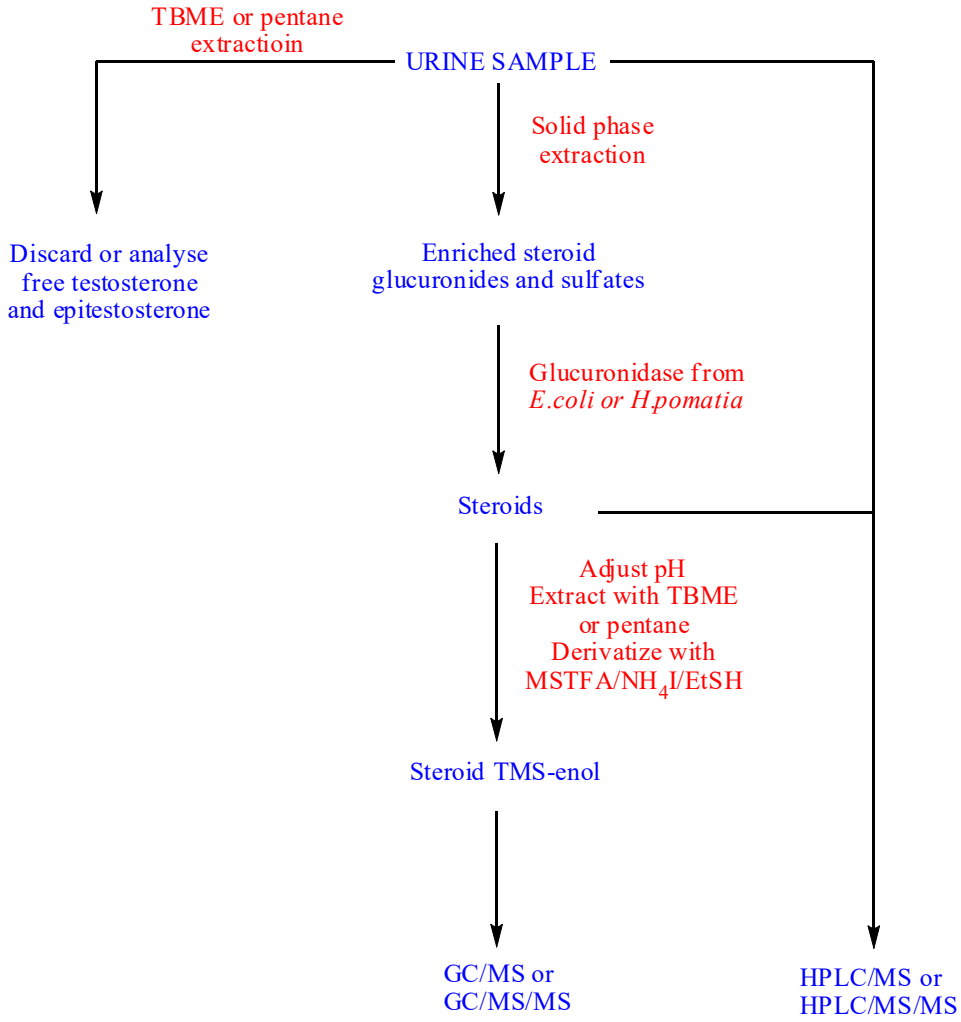


Figure 1.2 Procedure for steroid extraction and detection in athlete urine samples.

At the Unitéd' Analyse du Dopage, an IOC-accredited laboratory in Lausanne (Switzerland), GC-MS is used in six out of the seven standard tests for dopant detection.[7] Identifying and confirming an illegal substance is sufficient in most GC-MS procedures. Quantification is only required for naturally occurring substances (e.g. testosterone) and other drugs with concentration limits such as caffeine. Interfering peaks and background noise can complicate GC-MS data reading because screening methods are designed to detect an entire classes of compounds and they are not optimized for individual compounds within the mixture. Furthermore, a single ion fragment may not be unique to a given compound (e.g. regio-isomers). These issues limit the use of automated software programs for GC-MS data reading and mean that even today the tests require that an experienced analysts evaluate all of the data collected. In contrast, LC-MS/MS and GC-MS/MS measure the relative abundance of precursor/product ion pairs (transitions). The likelihood of a

target compound and an interfering substance having the same precursor/product ion pairs is relatively small. The data usually are easier to interpret compared to GC-MS data and can be more easily evaluated by computer software. GC-MS is not suitable for every banned substance. Substances in low concentrations, volatiles or those which undergo thermal decomposition can be detected with alternative tests, such as immunoassays.

HPLC is the most versatile of all chromatography methods but also the most complex. It is routinely used for quantitative analysis in biological samples such as blood, urine and other body fluids. HPLC can be coupled to various detectors such as UV, fluorescence or mass spectrometry (LC/MS and LC/MS/MS). For instance, accredited laboratories detect corticosteroids (a specific class of steroid hormones, which are known to increase motor activity) by using high-performance liquid chromatography (HPLC) in conjunction with MS or tandem mass spectrometry (MS/MS). HPLC-MS detection is effective here as it minimises the chance of corticosteroids being denaturated and hence an inaccurate test result (e.g. false negative) being recorded. Corticosteroids, cannot be reliably detected by GC-MS. This is because this class of compound is not stable when heated and they are slightly volatile.

To perform testing for banned substances at concentrations that are too low for standard GC-MS/HPLC-MS detection, immunoassays are often employed.[7] The hormone human chorionic gonadotropin (hCG) and human growth hormone (hGH) are examples of banned substances that must be detected using an immunoassay. To distinguish pituitary and human growth hormone (hGH) a method like the immune luminometric assay is used. This employs two antigen-specific monoclonal antibodies that recognize the antigen (hGH) at different domains. These antibodies are immobilized on the inner surface of a column together with a secondary antibody that is luminescence-labelled. Luminescence is detected by using an automatic luminometer.

Blood and urine testing are capable of detecting many prohibited substances, but not blood transfusions. One method introduced to aid the detection of such transfusions is the biological passport. Brought in by WADA in 2009, an athlete's biological passport aims to reveal the effects of doping rather than detect the substance or method itself.[8] The passport is an electronic document that contains data about certain markers from throughout their career (e.g. testosterone level). If these change dramatically, it alerts officials that the athlete might be doping. Some scientists have questioned the passport's efficiency -

especially when complicating factors such as training at altitude are factored in - but also its sensitivity to micro-dosing, a little-but-often approach to doping.[8]

Another important factor in antidoping testing is microbial contamination. Improper storage of the urine samples from athletes can cause changes in the steroids concentrations, leading to false positive or false negative results for a particular athlete. At the present time WADA does not provide a detailed standard protocol regarding storage or transportation of the urine samples collected.[9,3] The occurrence of degraded urine samples vary depending of the season of the year, with a peak during hot months, as well as the duration of transportation and the storage time. Up to the current time, no preservative is added systematically to sport urine samples because it is reasoned that the introduction of a chemical substance into athletes' samples after the collection procedure may lead to legal challenges.[10] If it is not possible to analyse refrigerated samples in a short time, an effective method of preserving urine specimen is desirable. Up to the current time, both physical and chemical methods have been proposed to protect urine samples from degradation that can arise due to improper storage conditions.[11]

The physical methods include: heating, ultrasonication, ultraviolet radiation, and membrane filtration. Heating is very efficient as a method but can be used only for thermostable products. Sterilization by UV radiation (~260 nm) is being used increasingly for heat-sensitive materials. It is quite lethal but does not penetrate glass, water, or other substances very effectively. Because of this, UV radiation is used as a sterilizing method in only a few particular situations. Pathogens and other microorganisms are destroyed when a thin layer of liquid is passed under the UV lamp.[12] Membrane filtration is also used for the sterilization of thermally unstable material. Within the context of the WADA regulations, the athlete maintains control of the sample at all times until it is sealed. Therefore, the athlete would be the one responsible for performing the filtration. Membrane filtration of large-volume urine samples using a syringe-mounted filter was found to be impractical because the pores of the membrane were saturated after a while. The possibility of deactivating enzymes or destroying microorganisms by ultrasonic waves (UWs) has been widely explored for laboratory applications in microbiology, immunology, and enzymology. However, at ambient temperature and pressure, ultrasonication has little lethal effect on microorganisms. Different species of microorganisms may be more susceptible to ultrasound treatment than others. Gram-positive cells such as Staphylococcus aureus and

coccus-shaped cells are more resistant to ultrasound than gram-negative cells and rod-shaped bacteria.

Chemical methods employ various additives to preserve the urine samples (enzyme and protein synthesis inhibitors, inorganic salts and antimicrobial agents).[13,14] A wide variety of chemicals currently used are bacteriostatic (inhibit growth) at low concentrations and bacteriocidal at high concentrations (kill the microorganisms but not necessarily their spores). Most common preservatives include:

- **NaN_3** - It has been used in concentrations ranging from 0.1% to 1% (w/v) in doping control laboratories for preservation of the analytes. Its major disadvantage is the high toxicity, even though the exact mechanism of intoxication remains unknown. The use of azide as a urine preservative was rejected by the UK.
- **Boric acid** - At concentrations between 10 and 20 g/L, boric acid is bacteriostatic or fungistatic for nearly all of the common urinary pathogens. If the concentration of borate exceeds 40 g/L and the exposure times are longer than 6 h, borate has bactericidal effects on various gram-negative species of urinary pathogens.
- **Penicillin–streptomycin–amphotericin** - A commercial antibiotic and antimycotic liquid mixture has been used in the literature to maintain sterility. Penicillin acts by inhibiting bacterial cell wall synthesis. Streptomycin inhibits prokaryote protein synthesis by preventing the transition from initiation complex to chain-elongating ribosome and causes miscoding. Amphotericin B is used as an antifungal agent.
- **Chloramphenicol** - Inhibits protein synthesis in prokaryotic cells and is widely used for suppressing bacterial growth in fungal media. It is primarily bacteriostatic, although it may be bacteriocidal to certain species.
- **Pepstatin** - Is a low-molecular-weight, highly specific inhibitor of acid proteases. It has been shown to inhibit virtually all acid proteases and proteases of microbial origin. It is being routinely used in the EPO stability test.
- **Phenylmethylsulfonyl fluoride (PMSF)** - Is an irreversible serine protease inhibitor that acts by sulfonating serine residues at the active site. It also inhibits cysteine proteases and mammalian acetylcholinesterase.
- **Pefabloc (AEBSF)** [4-(2-aminoethyl) benzenesulfonyl fluoride] - Is an irreversible serine inhibitor. It reacts covalently with the serine residue at the catalytic centre.

- **Protease inhibitor cocktails and EDTA** - Various commercial protease inhibitor cocktails with broad specificity for the inhibition of serine, cysteine, and aspartic proteases as well as metalloproteases are available. Usually, they contain Pefabloc, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA.

Both physical and chemical methods are either impractical or very costly to implement. For instance, one approach proposes the addition of complex cocktail of antibiotics to the urine samples to inhibit any microbial growth. This approach is prohibitively expensive and in a wider context it could also contribute to the growing problem of antibacterial resistance. To address this problem from a fresh perspective we undertake a study to develop a ^{19}F NMR protocol, a simple and robust analytical protocol that could be used to identify samples in which microbial growth has occurred in anti-doping samples. In order to do this a range of fluorinated steroids are required (**Chapter 2**). The metabolism of each fluorinated steroid prepared will be assessed by range of microorganisms.

1.2 Selected Aspects of Organofluorine Chemistry

1.2.1 Introduction

Fluorine is a p-block element in group XVII and is the lightest halogen. It was isolated by Henri Moissan, a French chemist, in 1886 by electrolysis of a solution of hydrofluoric acid in potassium hydrogen fluoride. At standard conditions, fluorine forms diatomic molecules, which exist as a pale yellow gas. Fluorine is the 13th most abundant element in the earth's crusts, although never occurs as a free element in nature. The most important fluorine minerals are fluorspar, fluorapatite, and cryolite. Only 13 naturally occurring organic fluorin-containing compounds are known reflecting the significant challenge in forming C-F bond under natural aqueous conditions.[15] Chapter 3 will provide a more in depth discussion regarding fluorinated natural products and the role of fluorine in nature. Synthetic fluorine containing compounds have found widespread use in a range of chemical products including; coolants, aerosol propellants, surfactants, polymers and drugs (**Figure 1.3**).[16,17]

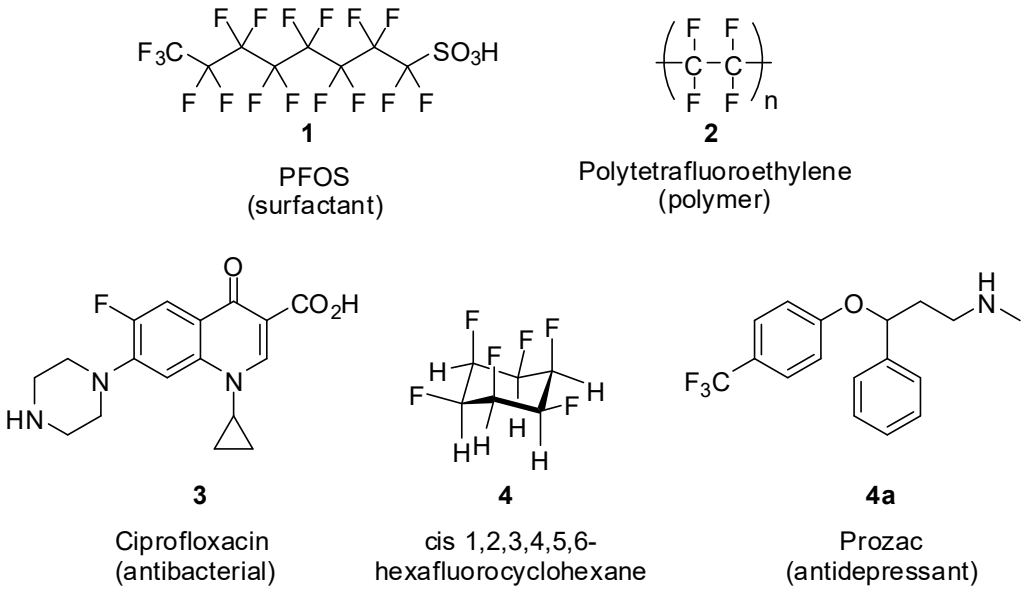


Figure 1.3 Selected examples of important organofluorine compounds.

1.2.2 Fluorine bond length and strength

Fluorine is the most electronegative element, therefore formation of F^- or a covalent bond is highly favourable. In comparison to all of the other halogens, fluorine forms strong bonds with many atoms and in particular, the silicon-fluorine bond is one of the strongest single bonds known (**Table 1.2**).[18,19]

Table 1.2 Dissociation bond energies of halogen containing compounds.

X	X-X	H-X	B-X	Al-X	C-X	Si-X
F	159	574	645	582	459	808
Cl	243	428	444	427	327	471
Br	193	363	368	360	272	372
I	151	294	272	285	239	293

Due to the low F-F bond energy (159 kJ/mol) fluorine gas (F_2) reacts readily with other elements or compounds often in an extremely exothermic manner making it very difficult to handle. Many of the reactions involving fluorine are driven by its tendency to gain an electron and form the neon core [$(He)2s^2 2p^6$]. Fluorine has a relatively small covalent

radius (64 pm)[19]; therefore, the bonds between fluorine and other atoms are, in general shorter that the equivalent bonds between other halogens and the corresponding atoms (**Table 1.3**).[20]

Table 1.3 Common bond lengths for halogen atoms.

X	X-X	H-X	B-X	C-X	Si-X
F	1.417	0.917	1.36	1.39	1.60
Cl	2.009	1.274	1.74	1.78	2.05
Br	2.283	1.408	2.01	0.27	0.31
I	0.267	1.608	2.22	0.22	0.21

1.2.3 ^{19}F NMR applications in biological systems

Naturally occurring fluorine is monoisotopic, consisting solely of ^{19}F and it has a sensitivity to NMR detection that is 83% of the sensitivity of ^{1}H nucleus. Nevertheless, there are 17 stable radioisotopes with half-lives ranging from 109.771 minutes for the longest-lived radioisotope ^{18}F to 4.1 x 10^{-22} seconds the least stable isotope ^{15}F.

Compounds containing the ^{18}F radioisotope have found applications in positron emission tomography (PET). ^{18}F containing compounds are routinely used as tracers to probe metabolic functions. One such species is ^{18}F-fluorodeoxyglucose, commonly abbreviated ^{18}F-FDG, which can be used to assess glucose metabolism in brain tumours. The major challenge for application of PET as a cancer detection tool is the rapid and clean synthesis of bioactive, ^{18}F-radiolabeled compounds.[21]

In comparison to hydrogen, the fluorine nucleus is on average surrounded by nine electrons and therefore the range of fluorine chemical shifts is much wider extending typically from 200 ppm to -200 ppm (**Figure 1.4**). A valuable aspect of ^{19}F NMR is that the area under each peak is proportional to the number of fluorine atoms responsible for such signals. Due to the wide chemical shift range, there is no single fluorine-containing compound convenient for use as a universal standard in ^{19}F NMR analysis and usually a reference similar to the compound under examination is used. The most commonly used internal standards for ^{19}F NMR are trifluoroacetic acid (TFA), trichlorofluoromethane and hexafluorobenzene (**Table 1.4**).[22]

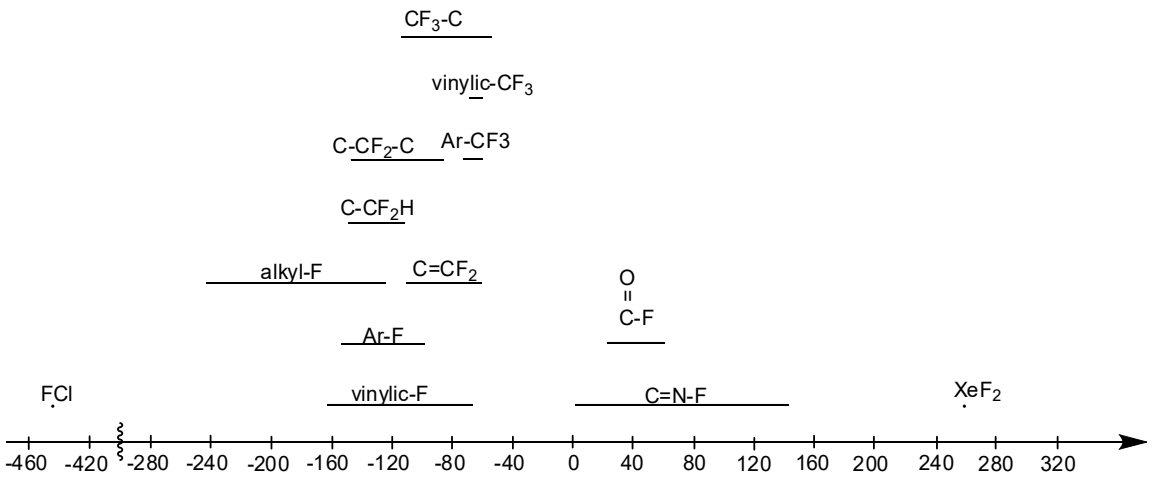


Figure 1.4 Examples of fluorine (^{19}F) NMR chemical shifts.

Table 1.4 Chemical shifts of common internal standards for ^{19}F NMR

^{19}F NMR Reference Standards vs. $CFCl_3$ δ(ppm)

Trichlorofluoromethane	0.00
Trifluoroacetic acid	-76.55
Hexafluorobenzene	-164.9
Fluorobenzene	-113.15
Trifluorochloromethane	-28.6
Elemental fluorine	+422.92
Fluoroacetonitrile	-251
Difluoro, tetrachloroethane	-67.80
Trifluorotoluene	-63.72

It is worth noting that fluorine chemical shifts are significantly more sensitive to their local environment within a molecule compared to proton chemical shifts.[23] In particular in biological systems, the fluorine shifts changes that are observed upon protein binding or folding/unfolding can reach 8 ppm.[22] This is in contrast to ^{1}H NMR spectroscopy where shifts observed in similar processes are usually < 0.3 ppm. For example, M.A. Danielson and J.J. Falke have found that addition of D-galactose or D-glucose to a sample of the 5-fluoro-tryptophan labelled galactose-binding protein produces an adduct which could be observed by ^{19}F NMR.[23] In this particular case the chemical shift of the Trp183 position changed upon addition of D-galactose or D-glucose by ~3 ppm (**Figure 1.5**).[22]

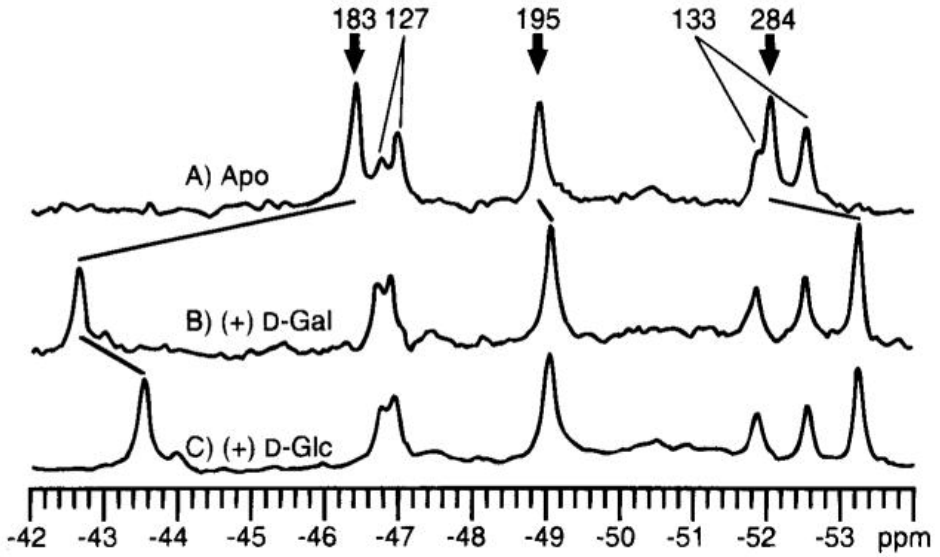


Figure 1.5 **^{19}F NMR** shift changes of 5-fluoro-tryptofan induced by addition of D-galactose and D-glucose.[10]

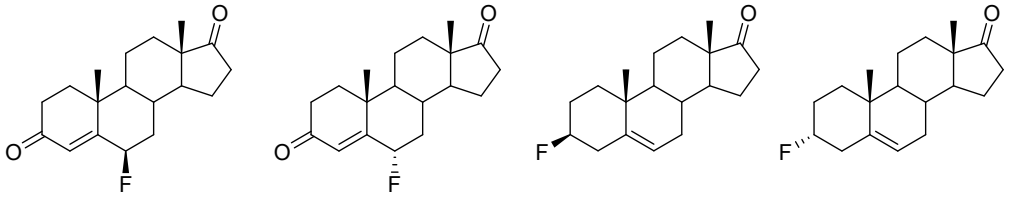
Interestingly small shift changes could be also induced by a solvent isotope effect. For instance, Hansen, Detman and Sykes have investigated the binding of fluorinated aromatic compounds to cyclodextrins.[24] In this study they have found that the fluorine signals from the water soluble aromatic compounds were de-shielded when the amount of H_2O increased in the sample (**Table 1.5**). Variations of chemical shifts with pH were also observed for the compounds bearing ionisable group (e.g. *m*-fluorophenol -36.941 ppm at pH = 6.5; *m*-fluorophenolate -38.315 ppm at pH = 10.9).

Table 1.5 Shift changes with D_2O/H_2O ratio.

%D_2O	***m*-Fluorobenzoic acid (ppm)***	***m*-Fluorophenol (ppm)***
100	-0.945	-0.373
50	-0.883	-0.296
25	-0.870	-0.251

*$\delta=(\delta_{\text{fully bound}}-\delta_{\text{free}})$

Similarly to ^{1}H NMR, the ^{19}F NMR spectra of fluorinated compounds show signal splitting as a result of heteronuclear spin-spin coupling (**Table 1.6**).[25,26]



5b **5a** **6b** **6a**

Table 1.6 Coupling values of selected fluoro-steroids.

Steroid	J(X*-^{19}F) Hz										
	4-H	6-H	3-H	2-C	3-C	4-C	5-C	6-C	7-C	8-C	10-C
5β	5	50	-	-	<1	9.2	12	167	23	<1	2
5α	<1	49	-	-	<1	14.7	11	185	18.4	11	2.7
6β			49.5	17.7	174.3	19.5	12.7	1.2	1.2	-	-
6α			48.4	20.4	166.3	21.6	<1	-	-	-	-

***X**-proton (4-H, 6-H, 3-H) or carbon (2-C, 4-C, 5-C, 6-C, 7-C, 8-C, 10-C).

The germinal and vicinal coupling constants observed in ^{19}F NMR spectra H -^{19}F are relatively large compared to H-H (e.g. $^{2}J_{HF}$ = 40-80Hz, $^{3}J_{H,F}$ = 1-45Hz). In ^{13}C NMR spectra the typical coupling constants are of the order: $^{1}J_{C,F}$ = 160-175 Hz and $^{2}J_{C,F}$ = 13-30 Hz. ^{13}C NMR experiments also show long-rang C-F couplings which can be significant. Homonuclear coupling between fluorine atoms are relatively large compared with those between hydrogen atoms. Coupling between geminal fluorine atoms ($^{2}J_{F-F}$) give a large value of 250 to 300 Hz. Three bond coupling ($^{3}J_{F-F}$) in saturated aromatic hydrocarbons usually range from 0 Hz to 16 Hz.

More recently, ^{19}F NMR has been applied as a tool in medical diagnosis. Due to the 100% isotopic abundance of ^{19}F this technique possesses excellent potential in detecting metabolic changes of therapeutic compounds. For example, it was shown that 4-fluoro-2-nitrophenyl-beta-D-galactopyranoside is rapidly cleaved by the enzyme beta-galactosidase[27] an enzyme that is commonly used in molecular biology as a reporter marker to monitor gene expression. Enzymatic cleavage of galactopyranoside produces a new ^{19}F NMR signal (-46.49 ppm) that is well separated from the chemical shift of the original parent compound (-42.75 ppm) and thus enzyme action can be easily and rapidly monitored.

1.3 Methods for preparing fluorinated steroids

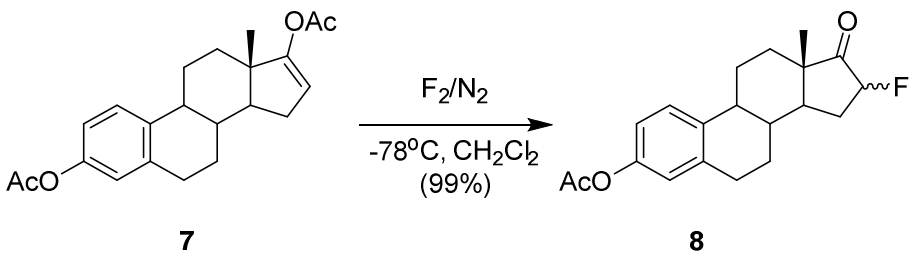
Many of the earliest examples of fluorination involved the use of elemental fluorine (F_2) or electrolysis of an organic compound in a solution of hydrogen fluoride.[28] However, given the unique properties of the F atom there are now several different alternative methods and reagents for the formation of C-F bonds, which avoid these original harsh conditions. The following sections briefly outline some of the most commonly encountered methods and reagents utilised in the preparation of fluorinated steroids.

1.3.1 Electrophilic fluorination

Electrophilic fluorination involves transfer of F^+ to an electron rich centre. Elemental fluorine (F_2) and reagents containing the F-O bond (e.g. CF_3OF) can be used for this purpose, however there are now alternative reagents containing nitrogen-fluorine bonds which have proven to be safer and more stable to handle.

1.3.2 Elemental Fluorine

High reactivity and poor regio-selectivity often make it difficult to work with elemental fluorine (F_2), therefore this reagent is usually diluted with an inert gas such as nitrogen (N_2) or argon (Ar). Patrick *et al*. showed that fluorination of estronediacetate (**7**) with a 5-10% mixture of F_2 in nitrogen leads to the formation of 16-fluoroestrone (**8**) in a good yield and with moderate regio-selectivity (56% yield, α:β = 10:1), **Scheme 1.1**.[29] It is worth noting that a very high yield and good selectivity was observed in the fluorination of **7** with XeF_2 (99% yield, α:β=9:1).



7 **8**

Scheme 1.1 Synthesis of 16-fluoroestrone (**8**) using elemental fluorine.

The preparation of **8** was performed by bubbling F_2/N_2 through a solution of the 3,17-diacetylestrone in DCM but a more convenient and practical method for using F_2 was developed by Chambers and Sandford at Durham University (**Figure 1.6**).[30] They constructed a micro-channel reactor which enables efficient mixing of a gas and liquid phase in controlled and safe manner. Yields obtained using this flow reactor set-up have been shown to be in most cases as high as for fluorination reactions carried out using standard equipment and elemental fluorine.

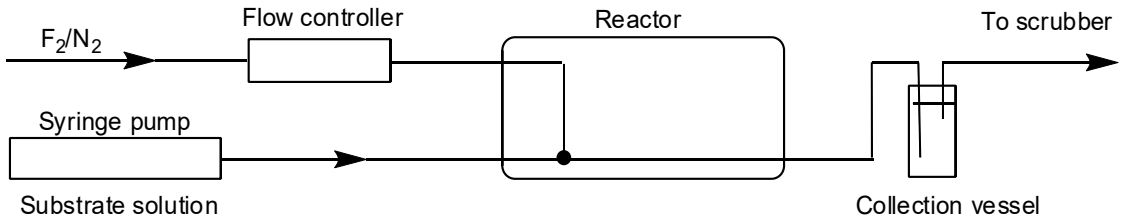
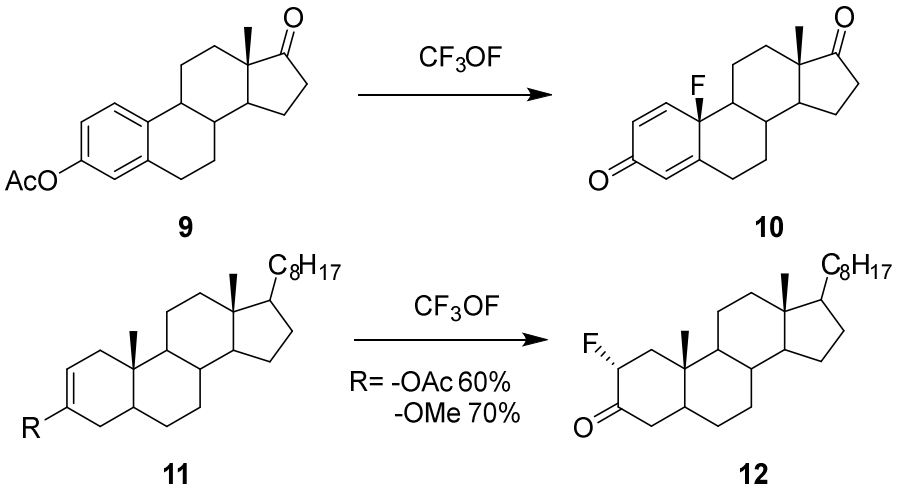


Figure 1.6 Micro-channel reactor developed for fluorination reactions with F_2.

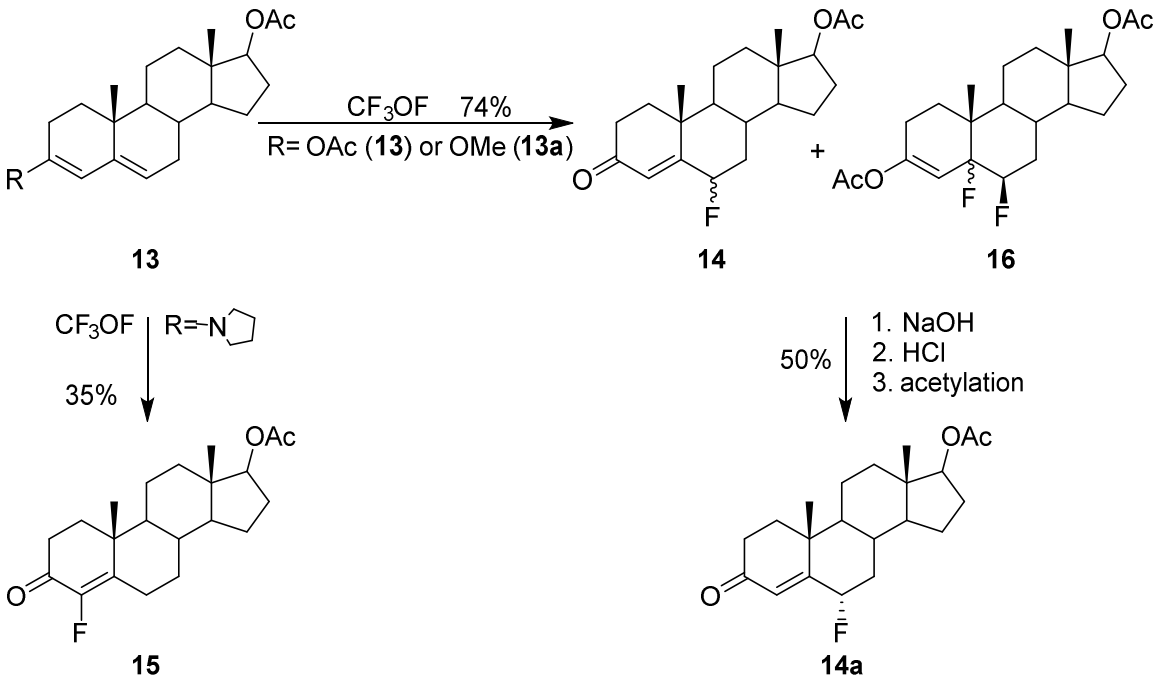
1.3.3 Organofluoroxy Reagents

The first electrophilic fluorinating reagent, fluoroxytrifluoromethane (CF_3OF), was discovered in 1968 by Barton and co-workers.[31] This reagent effectively and rapidly reacts with activated olefins and can be used with complex substrates in the presence of keto-, alkoxy, and acyloxy-groups.[31,32] For example, treatment of oestrone methyl ether or oestrone acetate (**9**) with a small excess of CF_3OF selectively produces 10β-fluoro-19-norandrosta-1,4-dien-3,17-dione (**10**) (**Scheme 1.2**). The utility of CF_3OF in the fluorination of steroid enol-esters was also demonstrated in the reaction of cholestanone (**11**). The fluorination of **11** gave exclusively 2α-fluorocholestanone (**12**) in a good yield (**Scheme 1.2**).[32] The fluorination reactions with trifluoromethyl hypofluorite (CF_3OF) are usually carried out in trichlorofluoromethane ($CFCl_3$) however tetrachloromethane (CCl_4), chloroform ($CHCl_3$) or dichloromethane (CH_2Cl_2) can be used to improve the solubility of the substrate.



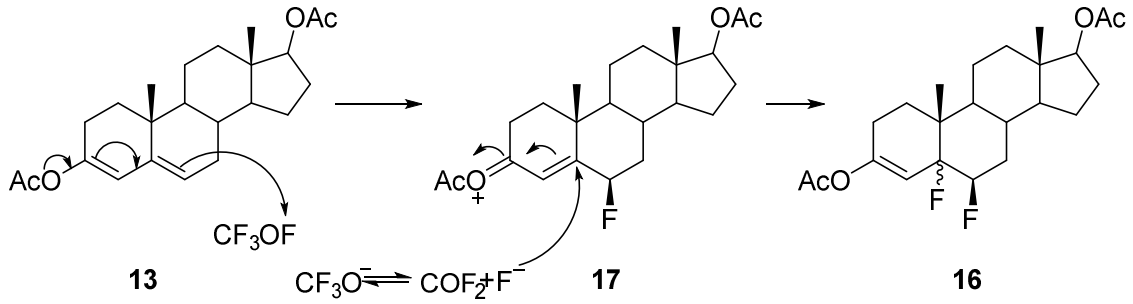
Scheme 1.2 Examples of fluorination with fluoroxytrifluoromethane (CF_3OF).

Although, fluoroxytrifluoromethane, tends to be more selective for mono-fluorination than *N*-F reagents[32] Barton has showed that small quantities of bis-fluorinated products can be sometimes observed (**Scheme 1.3**).[33] For instance, fluorination of bicycle testosterone (**13**) (R = OAc) gave a mixture of 6α- and 6β-testosterone (**14**) with a small amount of the bis-fluorinated product **16** also being obtained. The mixture of α/β testosterone (**14**) can be equilibrated to give predominately the 6α-fluorotestosterone (**16**) isomer under acidic conditions.[33] Interestingly, the regio-selectivity of CF_3OF can be changed from position 6 to 4 on the steroid by using the imine of testosterone which reacts gives 4-fluorotestosterone (**15**) in a 35% yield.[33]



Scheme 1.3 Synthesis of fluorinated testosterone derivatives using fluoroxytrifluoromethane (CF_3OF).

The formation of a bis-fluorinated product (**16**) can be explained by the fluoride ion attacking intermediate **17** (**Scheme 1.4**). Formation of the carbonyl difluoride means that the hydroxyl and N-H groups present in a substrate may be acylated to a small extent. This can be avoided through the addition of methanol to the reaction mixture or by subsequent hydrolysis of the acetate during the reaction work-up.



Scheme 1.4 Potential mechanism for the formation bis-fluorinated product (**16**).

1.3.4 *N*-F Reagents

Due to the reactivity and toxicity associated with elemental fluorine (F_2) and hydrogen fluoride (HF), the introduction of fluorine into organic molecules still presents a difficult challenge to synthetic organic chemists. Major progress in the field of electrophilic fluorinating agents came with the discovery of reagents containing nitrogen-fluorine (N-F) bond. These N-F reagents have largely replaced the organofluoroxy reagents due to their enhanced stability and ease of handling. A range of *N*-fluorinated amines, quaternary salts, amides and sulphonamides were synthesised for selective electrophilic fluorination under mild conditions (**Figure 1.7**).[34] In comparison to the previous reagents discussed, the N-F reagents are less expensive to produce and conventional glass equipment is often suitable for their use.

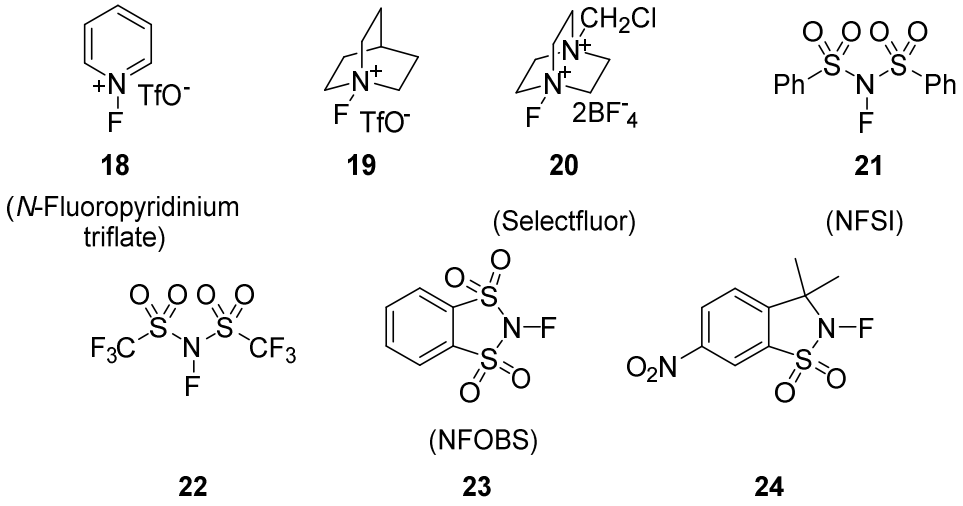


Figure 1.7 Selected examples of N-F fluorinating reagents.

1.3.5 *N*-Fluoropyridiniumtriflates

In 1986 Umemoto and co-workers reported the first stable *N*-fluoropyridinium salts, which had good activities and were suitable for commercial production.[35] They observed that the non-nucleophilic counter anions were essential to their stability, reactivity and selectivity (e.g. OTf, BF_4, SbF_6, ClO_4). The fluorinating power of these N-F salts can be controlled by varying the ring substituents and increasing electron density on the N-F site leads to the formation of a less reactive reagent. The structures and relative reactivity of common N-F salts that have been developed are presented in **Figure 1.8**.

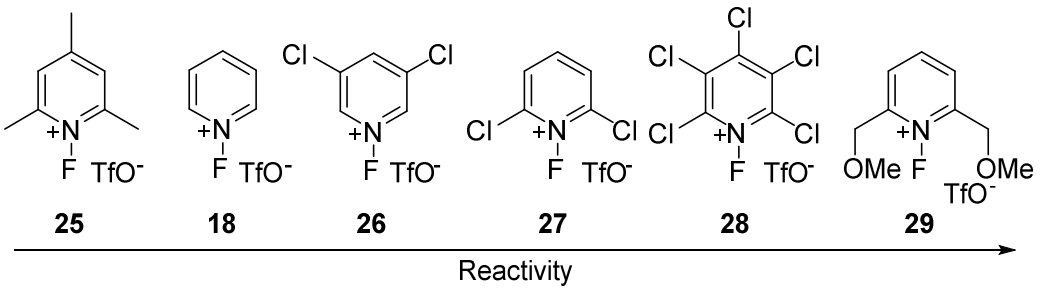
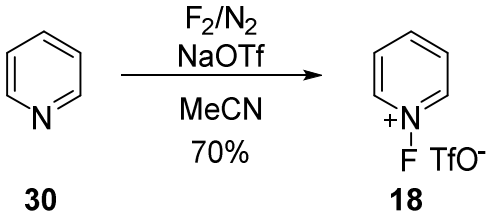


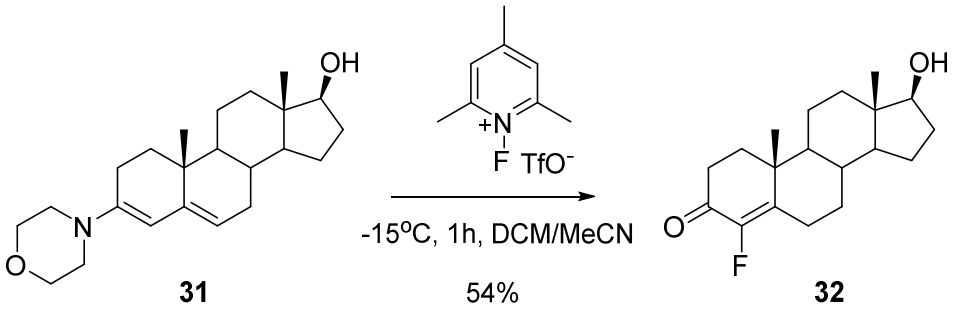
Figure 1.8 Reactivity of selected *N*-fluoropyridinium triflate reagents.

The less reactive reagent **25** is ideal for fluorinating reactive or easily oxidized compounds, such as enamines, carbanions and sulphides.[35] The most potent reagents (e.g. **28** and **29**) are suitable for fluorinating alkenes and aromatic rings. Compounds like the salt **18** which have moderate reactivity are suitable for fluorinating electron-rich substrates, such as enol alkyl ethers, enolsilyl ethers, and activated vinyl acetates. Compound **26** reacts with activated aromatic compounds at elevated temperature.[35] All of the *N*-F salts in **Figure 1.8** can be prepared by bubbling F_2/N_2 (1:9) mixture through a solution of the relevant pyridine/pyridine derivative and sodium triflate in acetonitrile (**Scheme 1.5**).



Scheme 1.5 Synthesis of *N*-fluoropyridiniumtriflates.

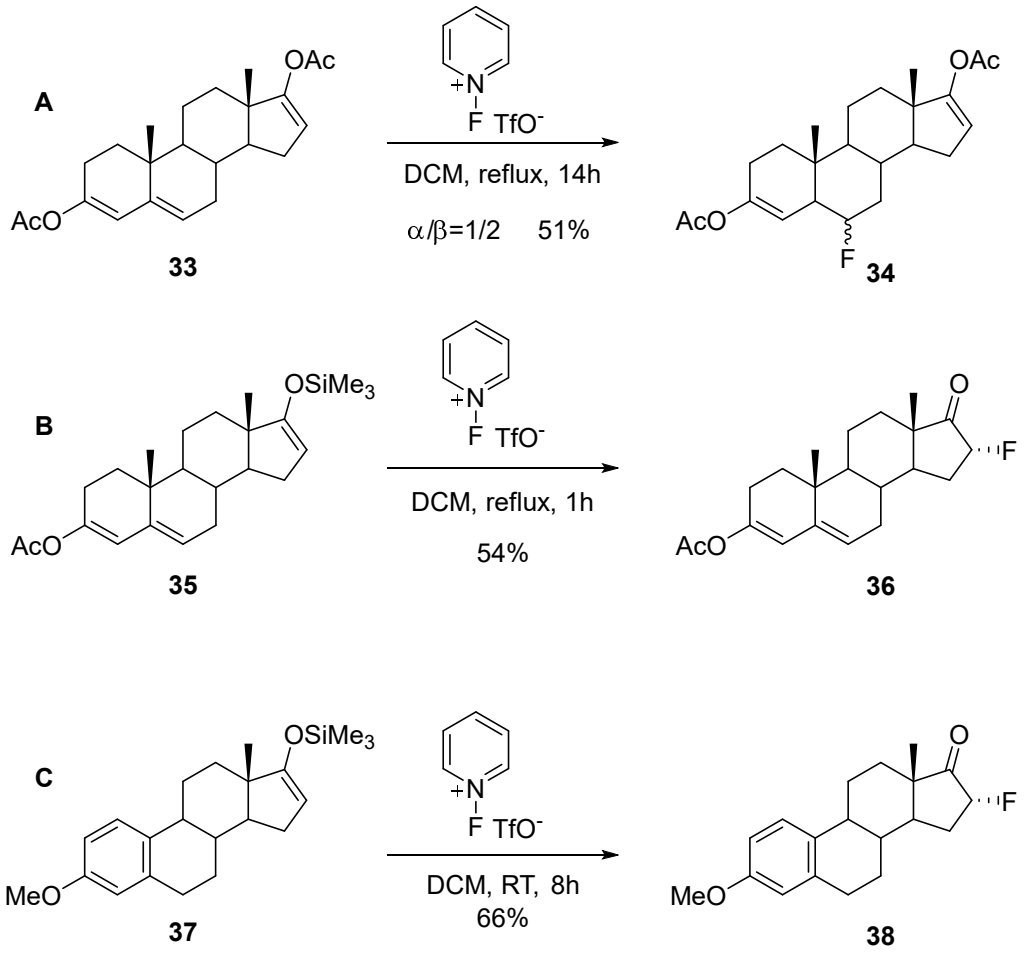
Umemoto and others have also demonstrated that *N*-fluoropyridinium salts can be used in a synthesis of fluoro-steroids.[36] For example, fluorination of the steroid enamine **31** using N-F salt **25** in DCM/MeCN gave 4-fluorotestosterone (**32**) in 54% yield (**Scheme 1.6**).



Scheme 1.6 Use of *N*-fluoropyridinium salts to synthesise fluoro-steroids.

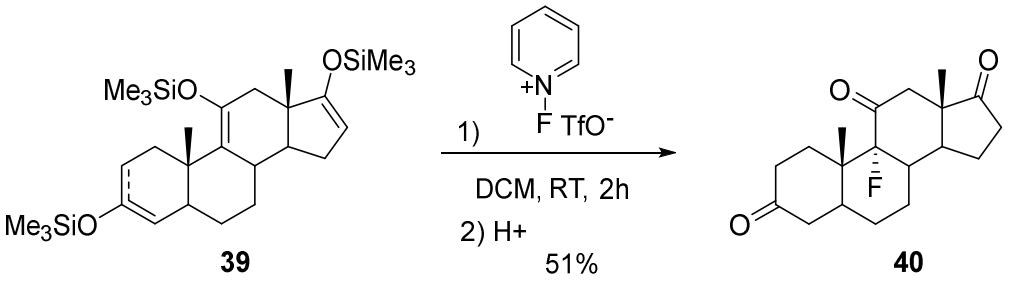
N-Fluoropyridinium triflate (**25**) also shows high regioselectivity with steroids having two reactive sites. During fluorination of **33**, *N*-fluoropyridinium triflate reacted selectively with the conjugated vinyl acetate in the presence of an enol acetate, whereas during the fluorination of steroid **35** it reacts preferentially with the silylenol ether moiety in the presence of a conjugated vinyl acetate. The reaction of *N*-fluoropyridinium triflate with estrone **37** gives selectively 16α-fluoro-estrone (**38**) in the presence of an activated aromatic ring (**Scheme 1.7,** Reaction A).[36]

It has also been reported that the steric bulk of the substituents on the aromatic ring of the pyridinium salt can play a very important role in directing the stereoselectivity of the fluorination.[18]For example, the fluorination of 17β-diacetoxy-3,5-androstadiene (**33**), with *N*-fluoropyridinium triflate gave a 1 : 2 mixture of the 6α/6β-fluoro steroid **34** (**Scheme 1.7**, Reaction B), while the bulkier salt *N*-fluoro-2,4,6-trimethylpyridinium triflate affords a 1:8.5 mixture of 6α/6β-fluoro-steroid (**35**) in 55% yield (**Scheme 1.7**, Reaction C).



Scheme 1.7 Selectivity of fluorination with *N*-Fluoropyridinium triflate.

Remarkable regio-selectivity was obtained during the fluorination of the silyl enol ether steroid **39** by Umemoto *et al*. (**Scheme 1.8**).[37] Here they used 1.0 equivalent of *N*-fluoropyridinium triflate and 9-α-fluoro steroid (**40**) was produced in a 51% yield. The outcome of this reaction suggested that the pyridinium salt reacts almost exclusively with the tri-substituted enol ether moiety.



Scheme 1.8 Synthesis of 9α-fluoro-steroid (**40**).

1.3.6 Selectfluor® and derivatives

Selectfluor® is an exceptionally stable fluorinating reagent developed by Banks and co-workers.[38] It is soluble in polar solvents such as acetonitrile (MeCN), dimethylformamide (DMF) and water. Recently, ionic liquids have also been successfully used as solvents for fluorination reactions involving Selectfluor®.[39] The fluorination power of this reagent can be increased or decreased by replacing the CH_2Cl group on the *N*-fluoroquinuclidine ring (**Figure 1.8**). Derivatives synthesised vary from simple methyl derivative **41** (R = Me) to the highly reactive trifluoroethyl reagent **41** (R = CF_3CH_2).[40]

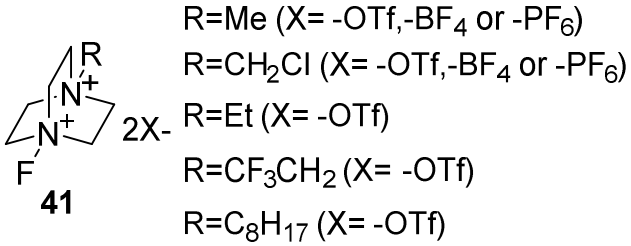
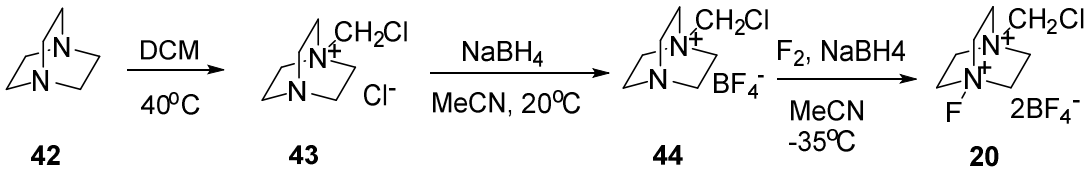


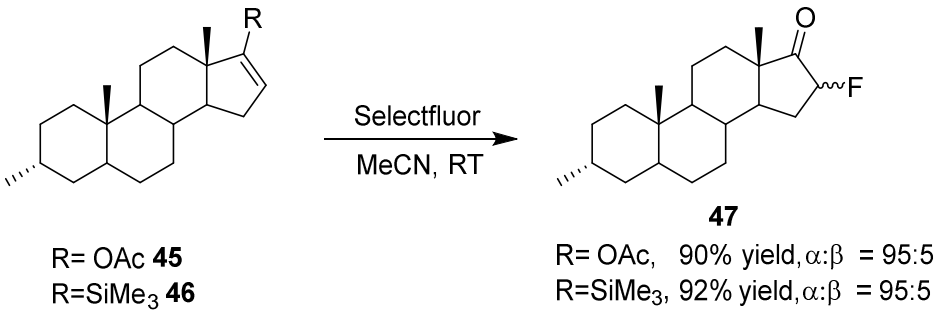
Figure 1.8 Examples of fluorinating agents derived from DABCO (**42**).

The synthesis of Selectfluor® is very simple which has allowed it to be produced on a multiple ton per year scale (**Scheme 1.9**).[41,42] The procedure involves alkylation of 1,4-diazabicyclo[2.2.2]octane (DABCO) **42** with DCM, counter ion exchange with sodium tetrafluoroborate and subsequent fluorination with elemental fluorine in acetonitrile.



Scheme 1.9 The synthesis of Selectfluor® (**20**).

It was shown by Lal *et al.* that Selectfluor® can be used in a synthesis of fluorinated steroids (**Scheme 1.10**).[43] The fluorination reaction of the enol ester **45** and the silylenol ether **46** gave the 16α-fluoro steroid **48** in very good yield and stereoselectivity.



Scheme 1.10 Synthesis of fluorinated steroids with Selectfluor®.

Herrinton and co-workers reported the reaction of Selectfluor® with a conjugated enol ester **13** (**Table 1.7**).[44] The fluorination reaction with Selectfluor® and *N*-fluoropyridinium salts gave the target product with similar yield and stereo-selectivity. The reaction performed with N-Fluorodibenzenesulfonimide (NFSI) gave the product with lower yield but produced mainly β isomer of **14**.

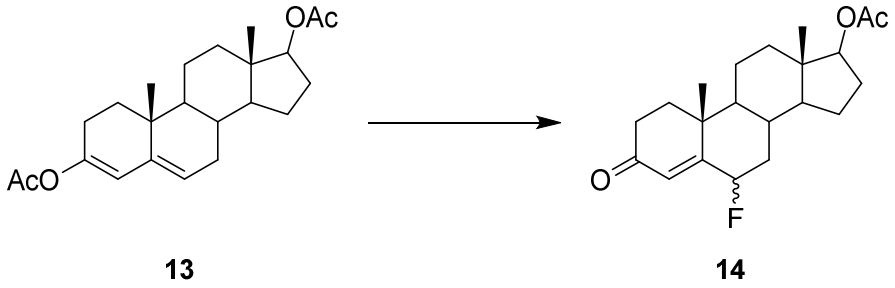
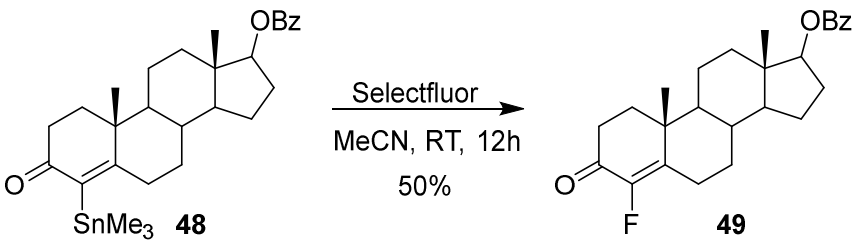


Table 1.7 Stereo-selectivity of selected fluorinating agents in the formation of **14**.

Entry	Reagent	Solvent	Conditions	Yield [%]	14 (α:β)
1[45]	Selectfluor®	MeCN	3 h, 0°C	95	1:1.4
2[46]	NFSI	THF	24 h, 40°C	60	5:95
3[47]	NFPy	MeCN	2 days, 40°C	96	50:50

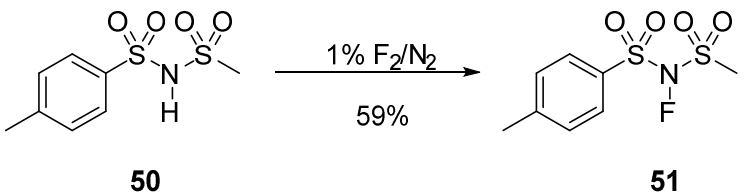
Widdowson and co-workers showed that fluorination with Selectfluor® can be carried out with steroid derivatives containing a vinyl stannane group.[48] For example, when testosterone derivative (**48**) was treated with Selectfluor® 4-fluoro testosterone (**49**) was obtained in a 50% yield (**Scheme 1.11**).



Scheme 1.11 Fluorination of steroid **48**.

1.3.7 Sulfonyl derivatives

The first application of *N*-alkyl-*N*-fluorosulfonamides in electrophilic fluorinations was described by Barnette and co-workers in 1984.[49] *N*-alkyl-*N*-fluorosulfonamides can be easily prepared by treatment of the appropriate amide with a mixture of elemental fluorine (F_2) and nitrogen. For example, fluorination of sulphonamide **50** in $CFCl_3$/ $CHCl_3$ gave the fluorosulphonamide (**51**) in 59% after purification by flash column chromatography (**Scheme 1.12**).[49]

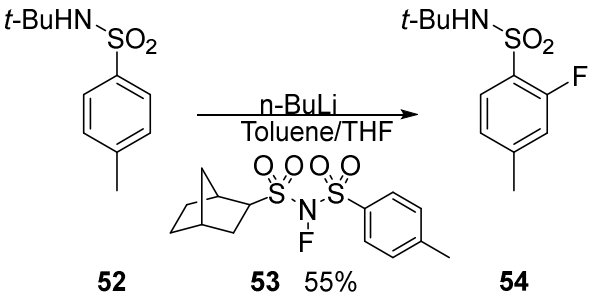


50 **51**

Scheme 1.12 Formation of fluorosulphonamide (**52**).

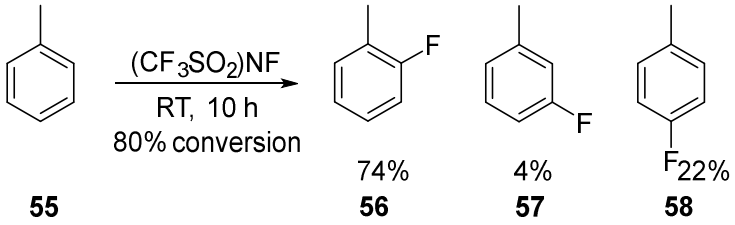
In comparison to the *N*-fluoropyridinium salts the *N*-alkyl-*N*-fluorosulfonamides reagents are neutral and are therefore less electrophilic. It was shown by Barnette, that they react

with various carbanions such as malonates, ketones, acids, amide enolates, alkyl and aryl organometallics.[49] A typical fluorination procedure involves anion generation with strong base followed by addition of *N*-fluorosulfonamide (**Scheme 1.13**).



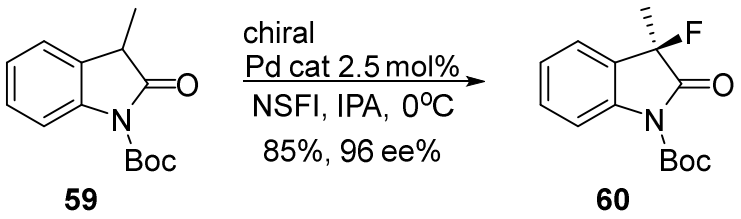
Scheme 1.13 Ortho lithiation followed by fluorination with *N*-fluorosulfonamide.

After Barnette's discovery, Des-Marteau reported the synthesis of perfluoroalkylsulfonamides.[50] These compounds are among the most powerful electrophilic fluorinating reagents known. For instance, fluorination of toluene with $(CF_3SO_2)_2NF$ occurred at room temperature to give predominantly 2-fluorotoluene (**56)** (**Scheme 1.14**).



Scheme 1.14 Fluorination of toluene (**55**) using $(CF_3SO_2)_2NF$.

Although, the perfluoroalkylsulfonamides are very powerful fluorinating reagents their preparation requires the use of neat elemental fluorine (F_2). Consequently, less reactive sulphonamides such as *N*-fluorobenzenesulfonamide (NSFI (**21**)) and *o*-benzenedisulfonamide (NFOBS (**23**)) have become more popular. These reagents are more reactive than *N*-alkyl-*N*-fluorosulfonamides and they are commercially available. Recently NSFI (**21**), was successfully used by Hamashima in an enantioselective fluorination of oxindoles (**Scheme 1.15**).[51] The high enantioselectivity obtained in this reaction can be explained by the formation of an intermediate chiral palladium enolate which undergoes subsequent fluorination.



59 **60**

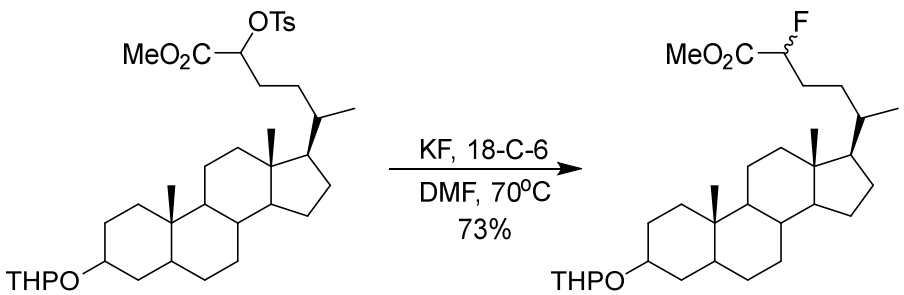
Scheme 1.15 Enantioselective fluorination of oxindole (**59**).

1.3.8 Nucleophilic fluorination

Another approach to the preparation of fluoro-organic compounds relies on employment of nucleophilic fluorination (F^-) reagents. Some of the most commonly used methods of nucleophilic fluorination are briefly discussed in the following sections, and, where possible relevant examples of their application in the preparation of fluorinated steroids have been included.

1.3.9 Fluoride Ion

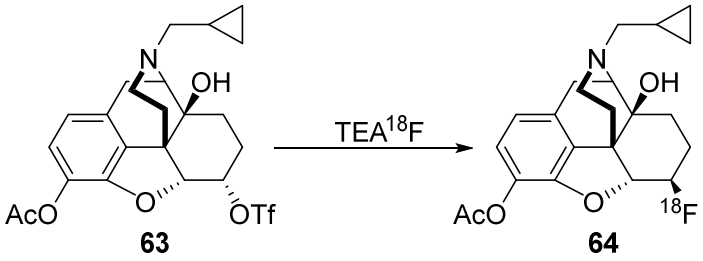
Fluoride ion (F^-) is a very poor nucleophile in protic solvents as it is heavily solvated and it forms tight ion pairs in aprotic media. Given this dipolar aprotic solvents, such as DMF or MeCN, tend to give the best results in combination with soluble tetraalkylammonium fluorides (e.g. tetra-*n*-butylammonium fluoride (TBAF)). Alternatively, metal fluorides can be used in conjugation with a crown ether to increase solubility. For example, cholenic methyl ester **61** was fluorinated at the α-position using potassium fluoride (KF) and 18-crown-6 in DMF (**Scheme 1.16**).[52]



61 **62**

Scheme 1.16 Nucleophilic fluorination with potassium fluoride (KF).

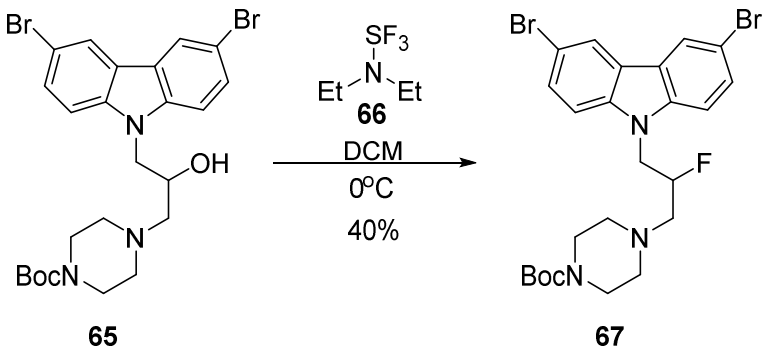
Nucleophilic fluorination is often use for the labelling of compounds with the ^{18}F isotope. Changing synthesised ^{18}F-acetylcyclopfoxy **64** to study opiate receptors employing PET-scanning technique.[53] The synthesis involved displacement of the triflate group with fluoride using tetraethyl ammonium fluoride (**Scheme 1.17**).



Scheme 1.17 ^{18}F isotope labelling.

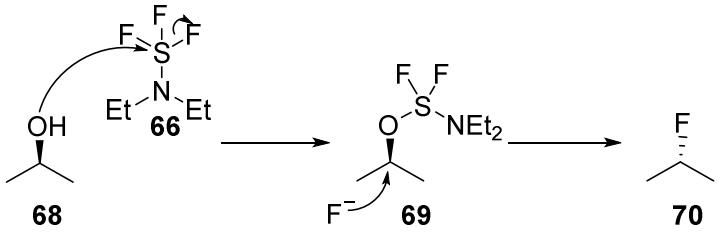
1.3.10 DAST and its derivatives

Diethylaminosulfurtrifluoride (DAST (**66**)), a reagent derived from SF_4, was first prepared by Middleton in 1975 and is now one of the most widely used and commercially available fluorinating agents.[54] DAST (**66**) can be used to transform alcohols directly to the corresponding alkyl fluorides as well as aldehydes and ketones to the corresponding geminal difluorides. Upon heating, DAST decomposes rapidly to produce the highly explosive $(Et_2N)_2SF_2$, therefore fluorination reactions that use this reagent are usually carried out below 50^{o}C, typically rt. In 1990 Lal *et al* addressed the issue of the thermal instability by preparing the DAST related bismethoxyethyl derivative (Deoxofluor (**71**)). Deoxofluor is more stable due to coordination of the methoxy group to sulphur centre. For example, Bombrun *et al* recently used DAST (**66**) to obtain the fluorinated analogue of 3,6-dibromocarbazole piperazine derivative **65** (**Scheme 1.18**).[55] It was found that this compound is a potent modulator of the Cytochrome c release via Bax Channel Modulation.



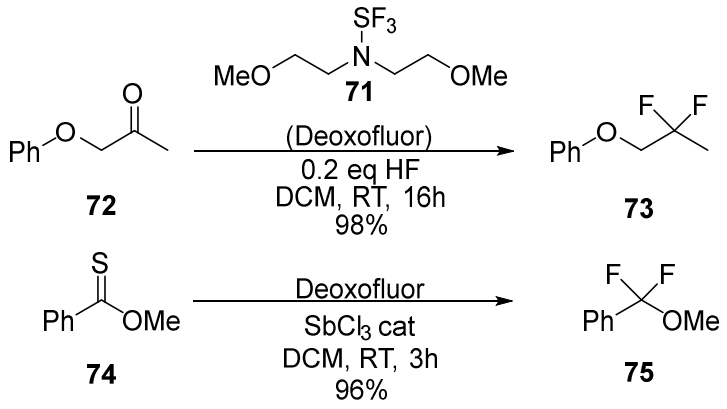
Scheme 1.18 DAST (**66**) fluorination of 3,6-dibromocarbazole piperazine derivative (**65**).

DAST and related fluorinating agents proceed via a fluorodeoxygenation mechanism. Fluorodeoxygenation relies on the transformation of the hydroxyl group into a good leaving group (e.g. intermediate **69**, **Scheme 1.19**)[56] and this is subsequently displaced by fluoride ion (**Scheme 1.19**). The fact that the reaction proceeds with inversion of stereochemistry allows introduction of the fluorine with stereochemical control.



Scheme 1.19 The mechanism of DAST (**66**) fluorodeoxygenation.

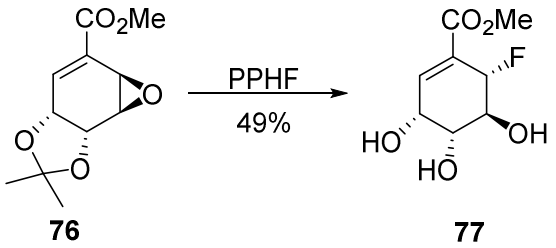
DAST type fluorinating reagents can also be used to convert carbonyl and thiocarbonyl compounds to appropriate germinal difluoro derivatives. This and related nucleophilic reactions were used by Lal and co-workers to prepare various organofluoro compounds.[57,58] For example, α-phenoxy carbonyl compound **72** was fluorinated in a very good yield to provide difluoro derivative **73** (**Scheme 1.20**). Similarly, thioester **74** was converted to difluoro compound **75** in an excellent yield. More recently, Rozen achieved this type of transformation using BrF_3.[59]



Scheme 1.20 Bis-fluorination of carbonyl compounds with Deoxofluor (**71**).

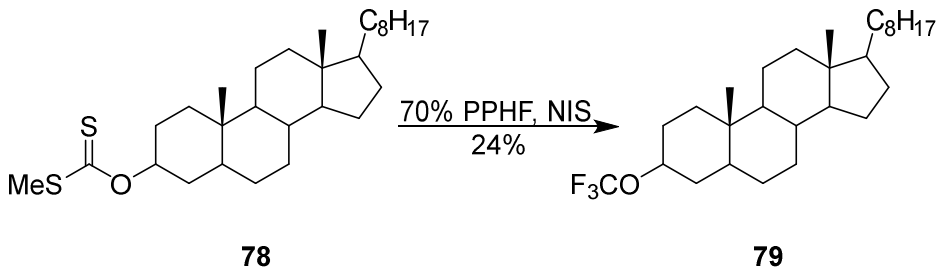
1.3.11 HF Reagents

The wide spread use of HF as a fluorinating reagent is limited for various reasons. Firstly, HF is very volatile, highly toxic and corrosive to glass equipment. In addition, HF is a relatively weak acid (pKa = 3.17) providing only low concentrations of fluoride ions in solutions. Consequently, other HF equivalent reagents have been developed over the years. In these alternative reagents the corrosive and reactive nature of HF was modulated by using it in combination with various amines. Reagents of this class tend to be more nucleophilic than HF on its own, making them valuable reagents for nucleophilic fluorination reactions. The most common reagent in this class is Olah's reagent, (full name, PPHF) which consists of a mixture of 70% hydrogen fluoride and 30% pyridine. Applications of Olah's reagent include reactions with alcohols and alkenes to give alkyl fluorides, acyl chlorides or anhydrides to give acyl fluorides.[60] Olah's reagent, can also be used to ring open epoxides, a strategy that was utilised in the preparation of the fluorinated derivative of shikimic acid (**76**) (**Scheme 1.21**).[61]



Scheme 1.21 Epoxide opening with Olah's reagent (PPHF).

Kanie employed PPHF to introduce three fluorine atoms into a steroid core (**78**).[62] This was achieved by activating the substrate with *N*-Iodosuccinamide (NSI) to facilitate the attack by fluoride (**Scheme 1.22**).



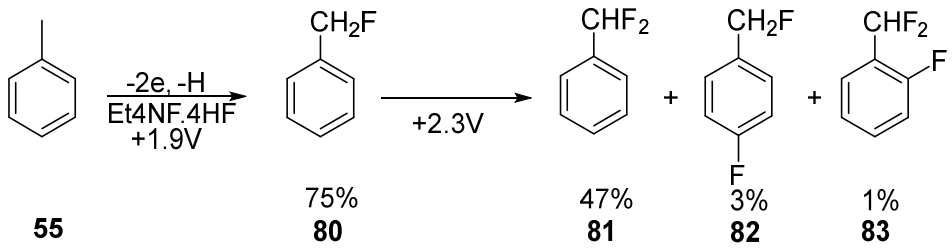
78 **79**

Scheme 1.22 Introduction of a trifluoromethylether group using Olah's reagent (PPHF).

1.3.12 Electrochemical fluorination

Electrochemical fluorination was introduced by J. H. Simons in the 1940's and today it is called the Simons Process.[63] This technique employs anhydrous hydrogen fluoride (HF) as a solvent and some process variations inorganic fluorides.[64] By this route, it is possible to produce a range of perfluorinated amines, ethers, carboxylic acids, and sulfonic acids.

A major breakthrough in the field of electrochemical fluorination came in 1970 when triethylamine-3HF dissolved in acetonitrile gave mono fluorinated naphthalene.[65] Since then, selective fluorinations of a variety of aliphatic and aromatic substrates have been reported in the literature.[66] For example, Morita and co-workers have showed that electrolytic fluorination of toluene in the presence of $Et_4NF{\cdot}4HF$ gave exclusively benzylfluoride (**80**) in 75% yield. Further fluorination afforded mainly difluoromethylbenzene (**81**), along with small amounts of ring-fluorinated side products **82** and **83** (**Scheme 1.23**).[66]



55 **80** **81** **82** **83**

Scheme 1.23 Electrochemical fluorination.

1.4 Aims of the project

The detection of prohibited performance-enhancing drugs in sports is often carried out using urine samples. The reason for this is that urine sample can be collected under non-sterile conditions and do not require the presence of a WADA sanctioned medical officer. Once the urine sample has been collected the sample is divided into two and preserved within sealed containers. If first sample has revealed the presence of a prohibited substance second sample will be analysed. Although these measurements help to eliminate errors during analysis at the present time WADA does not provide the national anti-doping organizations with a detailed standard protocol regarding storage or transportation of the urine samples collected. Consequently, improper storage of the urine samples from athletes can lead to microbial contamination, which can cause changes in the testosterone concentrations, leading to false positive results for a particular athlete. This is highlighted in the case of the British athlete Diana Modhal. Modhal was banned from competing on to have the ban overturned when it was found that microbial contamination was responsible for the positive results obtained. To overcome any potential problems with microbial contamination both physical and chemical methods have been developed to protect urine samples from degradation that can arise due to improper storage conditions.[67,68] However, many of these methods are either impractical or very costly to implement. For instance, one approach proposes the addition of complex cocktail of antibiotics to the urine samples that will function to inhibit any microbial growth. This approach is prohibitively expensive and in a wider context it could also contribute to the growing problem of antibacterial resistance.

To address the problem from a fresh perspective we undertake a study to develop a simple and robust analytical protocol that could be used to identify samples in which microbial growth has occurred in anti-doping samples. The key technique that will be employed to do this is ^{19}F NMR. To enable this critical aspect of the early work on the project will involve the preparation of fluorine containing steroids (examples-**Figure 1.10**). As discussed earlier, there are several different synthetic procedures available in literature that have been successfully utilised to introduce a fluorine atom into steroid structures. These methods will be employed when appropriate and new synthetic strategies will be investigated when required. Of particular interest is to try and expand the work that has

been carried out to create fluorinated steroids using both Selectfluor® and elemental fluorination.

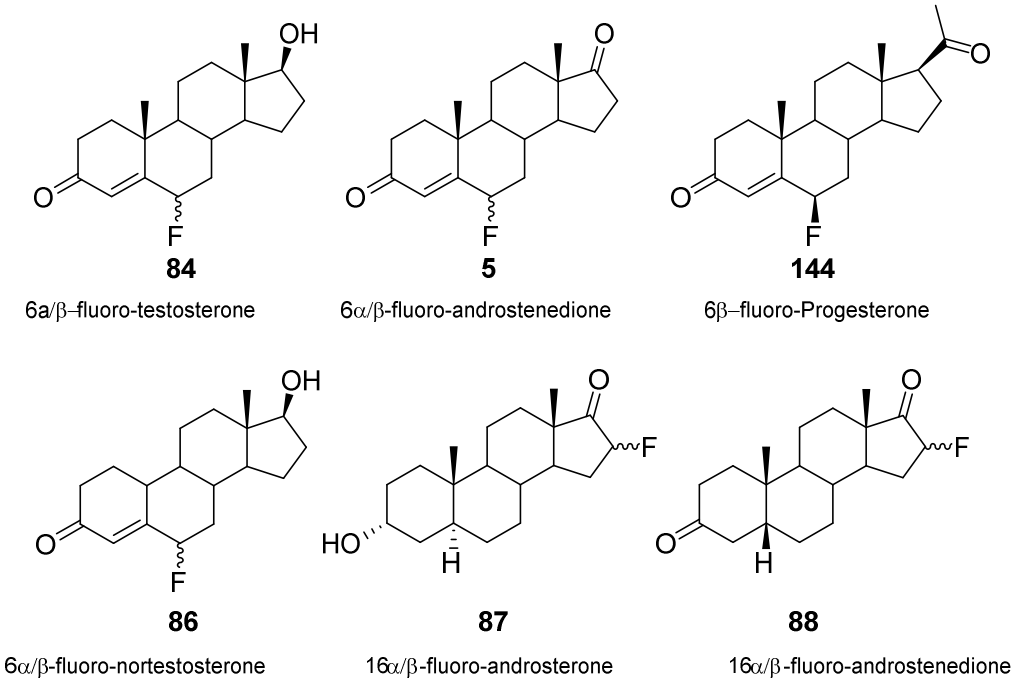


Figure 1.10 Examples of fluorine containing steroids.

The metabolism of each fluorinated steroid prepared will be assessed by a range of microorganisms such as *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *E. coli*, *Enterococcus faecalis* and *Candida albicans*. These microorganisms are representative of species commonly encounter in human microbial flora, urinary tract infections and indoor air. The biotransformations will be monitored by ^{19}F NMR and the metabolites produced analysed by HPLC, ESI-MS and GC-MS. This work will be carried out in collaboration with the group of Dr Cormac Murphy at University College Dublin.

Each fluorinated steroid prepared will also be incubated under a range of conditions (temperature, exposure to air, etc.) and the chemical changes or degradation arising will be investigated.

1.5 Reference

[1] J. L. Fourcroy,'*Pharmacology, Doping and Sports'*, Routledge, chapter, **2009,** *2*, 9.
[2] L. D. Bowers, *Annu. Rev. Anal. Chem*., **2009**, *2*, 485.
[3] M. Tsivou, H. A. Dimopoulou, I. P. Leontiou, D. G. Georgakopoulos, M. A. Koupparis, J. Atta-Politou, C. G. Georgakopoulos, *Clin. Chim. Acta*, **2010**, *411*, 448.
[4] http://list.wada-ama.org/by-substance/
[5] http://news.bbc.co.uk/sport1/hi/athletics/3492427.stm
[6] R. W. Pound, Richard H. McLaren, G. Younger, '*The Inndependent commission Report'*, **2015**.
[7] J. S. MacNeil, '*Today's Chemist at Work'*, **2001**, *5*.
[8] O. van Maren, *'In blood we trust? The Kreuziger Biological Passport Case. By Thalia Diathesopoulou*', **2015**, *1*.
[9] R. de la Torre, X. de la Torre, C. Alia, J. Segura, T. Baro, J.M. Torres-Rodriguez, *Anal*. *Biochem*., **2001**, *289*, 116.
[10] M. Rowbottom, *'Diane Modahl wins her appeal against drugs ban'. The Independent*. **27/07/1995**.
[11] A.T. Kicman, J.K. Fallon, D.A. Cowan, C. Walker, S. Easmon, D. Mackintosh, *Clin. Chem*., **2002**, *48*, 1799.
[12] M. Tsivou, D. Livadara, D. G. Georgakopoulos, M. Koupparis, J. Atta-Politou, C. G. Georgakopoulos, Preservation of urine doping control samples: preliminary results, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (Eds.), *'Recent Advances in Doping Analysis'*, **2008**, *16*, 41.
[13]P. Elliott, T. C. Peakman, *Int. J. Epidemiol*., **2008**, *37*, 234.
[14] M. Tsivou, D. Livadara, DG. Georgakopoulos, MA. Koupparis, J. Atta-Politou, CG. Georgakopoulos, *Anal. Biochem.*, **2009**; *388*, 179.
[15] C. C. H. Chan, S. O. C. Mundle, T. Eckert, X. Liang, S Tang, G. Lacrampe-Couloume, E. A. Edwards, B. Sherwood Lollar, *Environ. Sci. Technol*., **2012**, *46*, 10154.
[16] D. E. C. Adams, R. U. Halden, '*Fluorinated Chemicals and the Impacts of Anthropogenic Use'*, **2010**, *27*, 539.
[17] N. S. Keddie, A. M. Z. Slawin, T. Lebl, D. Philp. D. O'Hagan, Nat. Chem., **2015**, *7*, 483.
[18] S. Patia, Z. Rappoport, '*The Chemistry of Organic Silicon Compounds'*, **1989**, *3*.
[19] F.H. Allen, O.Kennard, *J.Chem.Soc. Perkin Trans,* **1987**, *2*.
[20] L. E. Sutton, '*Tables of Interatomic Distances and Configuration in Molecules and Ions'*, *The Chemical Society*, **1958**.
[21] M. M. Alauddin, *Am J Nucl Med Mol Imaging*. **2012**, *1*, 55.
[22] W. R. Dolbier, 'G*uide to Fluorine NMR for Organic Chemists*', Wiley, **2009**.
[23] M. A. Danielson, J. J. Falke, *Annu. Rev. Biophys. Biomol. Struct*, **1996**, *25*, 163.
[24] P. E. Hansen, H. D. Dettman, B. D. Sykes, *J. Magn. Reson*., **1985**, *62*, 487.
[25] A. Kasal, M. Budesinsky, W. J. Griffiths, '*Spectroscopic Methods of Steroids Analysis*', **2010**,84.
[26] H. L. Holland, *Tet. Lett*., **1978**, *10*, 881.
[27] W. Cui, P. Otten, Y. Li, K. S. Koeneman, J. Yu and R. P. Mason, *Magn. Reson. Med.*, **2004,** *51*, 616.
[28] J. H. Simons, H. T. Francis, J. A. Hogg, *J. Electrochem. Soc*., **1949**, *95*, 53.
[29] T. B. Patrick, R. Mortezania, *J. Org. Chem*., **1988**, *53*, 5153.

[30] R. D. Chambers, D. Holling, R. C. H. Spink, G. Sandford, *Lab on a Chip*, **2001**, *1*, 132.
[31] D. H. R. Barton, A. K. Ganguly, R. H. Hesse, S. N. Loo, M. M. Pechet, *Chem. Commun*., **1968**, 806.
[32] S. Rozen, *Chem. Rev*., **1996**, *96*, 1717.
[33] D. H. R. Barton, A. K. Ganguly, R. H. Hesse, S. N. Loo, M. M. Pechet, *Chem. Commun*., **1968**, 804.
[34] J. Liu, J. Hu, *Future Med. Chem*., **2009**, *1*(*6*), 1189.
[35] T. Umemoto, K. Kawada, K. Tomita, *Tet.Lett*., **1986**, *27*, 4465.
[36] T. Umemoto, S. Fukami, G. Tomizawa, K. Harasawa, K. Kawada, K. Tomita, *J. Am. Chem. Soc*., **1990**, *112*, 8563,
[37] G. Tomizawa,T. J. Umemoto, *Fluorine Chem*., **1991**, *54*, 205.
[38] R. E. Banks, *J. Fluorine Chem*., **1998**, *87*, 1.
[39] K. K. Laali, G. I. Borodkin, *J. Chem. Soc. Perkin Trans.*, **2002**, *2*, 953.
[40] R. E. Banks,M. K. Besheesh, S. N. Mohialdin-Khaffaf, I. Sharif, *J. Chem. Soc. Perkin Trans.*, **1996**, *1*, 2069.
[41] R. E. Banks, '*Air Products and Chemicals*', **1992**, *178*, 9.
[42] J. J. Hart, R. G. Syvret, *J. Fluorine Chem*., **1999**, *100*, 157.
[43] G. S. Lal, *J. Org. Chem*., **1993**, *58*, 2791.
[44] V. Reydellet-Casey, D. J. Knoechel, P. M. Herrinton, *Org. Process Res. Dev*., **1997**, *1*, 217.
[45] R. E. Banks, S. N. Mohialdin-Khaffaf, G. S Lal, I Sharif, R. G. Syvret, *J. Chem. Soc., Chem. Commun*., **1992**, 595.
[46] V. Reydellet-Casey, D. J. Knoechel, P. M. Herrinton, *Org. Process Res. Dev.*,**1997**, *1*, 217.
[47] A. J. Poss, D. Van Der Puy, D. Nalewajek, G. A. Shia, W. J. Wagner, R. L. Frenette, *J. Org. Chem*., **1991**, *56*, 5962.
[48] H. F. Hodson, D. J. Madge, D. A. Widdowson, *J. Chem. Soc. Perkin Trans.*,**1995**, *1*, 2965.
[49] W. E. Barnette, *J. Am. Chem. Soc*., **1984**, *106*, 452.
[50] S. Singh, D. DesMarteu, S. Zuberi, M. Witz, H. Huang, *J. Am. Chem. Soc*., **1987**, *109*, 7194.
[51] Y. Hamashima, T. Suzuki, H. Takano, Y. Shimura, M. Sodeoka, *J. Am. Chem. Soc*., **2005**, *127*, 10164.
[52] Y. Kobayashi, T. Taguchi, T. Terada, J. Oshida, M. Morisaki, N. Ikekawa, *Tet. Lett.*, **1979**, *20*, 2023.
[53] M. Channing, W. Eckelman, J. Bennett, T. Burke, K. Rice, *Int. J. Radiat. Isot.*, **1985**, *36*, 429.
[54] W. Middleton, *J. Org. Chem*., 1975, *40*, 574.
[55] A. Bombrun, P. Gerber, G. Casi , O. Terradillos , B. Antonsson , S. Halazy, *J. Med. Chem*., **2003**, 46 (*21*), 4365.
[56] R. P. Singh, J. M. Shreeve, *Synthesis*, **2002**, *17,* 2561.
[57] G. S. Lal, G. P. Pez, R. J. Pesaresi, F. M. Prozonic, *J. Chem. Soc. Chem. Commun.*, **1999**, *2*, 215.
[58] G. S. Lal, G. P. Pez, R. J. Pesaresi, F. M. Prozonic, H. Cheng, *J. Org. Chem.*, **1999**, *64*, 7048.
[59] S. Rozen, *Acc. Chem. Res*., **2005**, *38*, 803.
[60] G. A. Olah, J. G. Shih, G. K. S. Prakash, *J. Fluorine Chem*., **1986**, *33*, 377.
[61] S. Bowles, M. M. Campbell, M. Sainsbury, G. M. Davies, *Tetrahedron Lett*.,**1989**, *30*, 3711.
[62] K. Kanie, Y. Tanaka, K. Suzuki, M. Kuroboshi, T. Hiyama, *Bull. Chem. Soc. Jpn*., **2000**, *73*, 471.
[63] T. Okazoe, *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci*., **2009**, 85(*8*), 276.
[64] J. H. Simons, *J. Electrochem. Soc*.,**1949**, *95*, 47.
[65] V. Dinoiu, *Revue Roumaine de Chimie*, **2007**, *52*, 453.
[66] K. Momota, K. Mukai, K. Kato, M. Morita, *Electrochim. Acta.,* **1998**, *43*, 2503.
[67]P. Elliott, T. C. Peakman, *Int. J. Epidemiol*., **2008**, *37*, 234.

[68] M. Tsivou, D. Livadara, DG. Georgakopoulos, MA. Koupparis, J. Atta-Politou, CG. Georgakopoulos, *Anal. Biochem.*, **2009**; *388*, 179.

2 The Synthesis of Fluorinated Steroids

2.1 Introduction

In order to evaluate ^{19}F NMR as a tool for detecting bacterial contamination in antidoping samples we want to look at the metabolism of a range of fluorinated steroids in several different micro-organisms. To carry out this work a range of fluorine containing steroids presented in **Figure 2.1** were synthesised. In order to access the fluorinated steroids a range of different synthetic methods have been employed where appropriate and new synthetic strategies have also been investigated. Initially the reactivity of a series of protected steroids towards the fluorinating agents Selectfluor® and NSFI were examined. The fluorinated steroids produced were then incubated with a range of microorganisms; *Streptomyces griseus, Escherichia coli, Bacillus subtilis* and *Bacillus megaterium* and the resulting metabolites were examined by ^{19}F NMR, LC-MS and GC-MS (**Chapter 3**). In addition to the studies carried out in bacteria the chemical stabilities of the fluorinated steroids were also examined (**Chapter 3**).

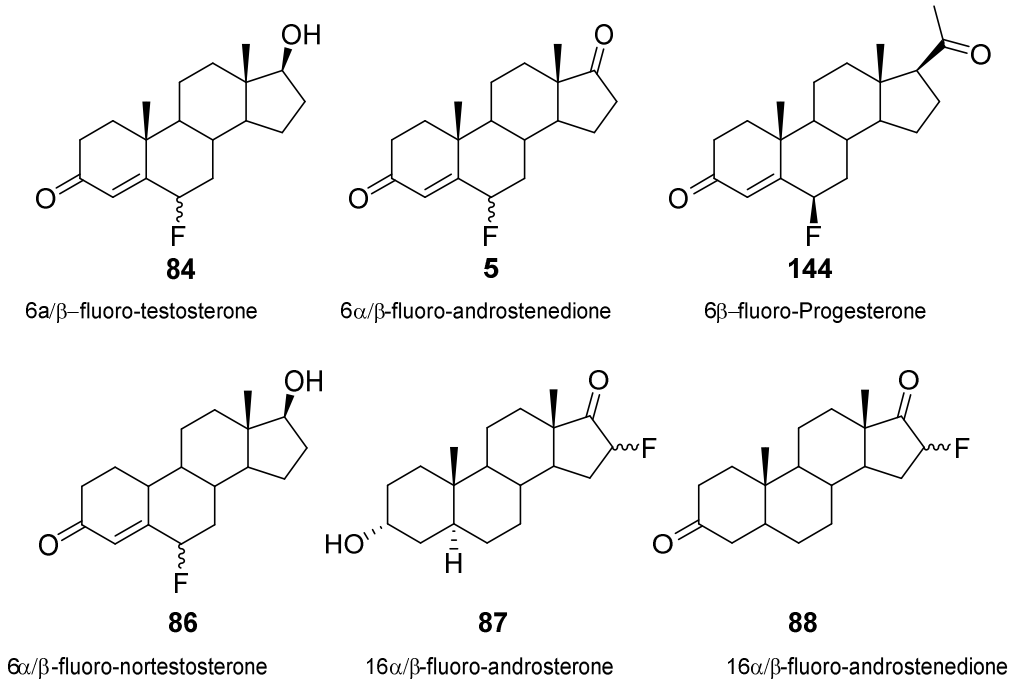
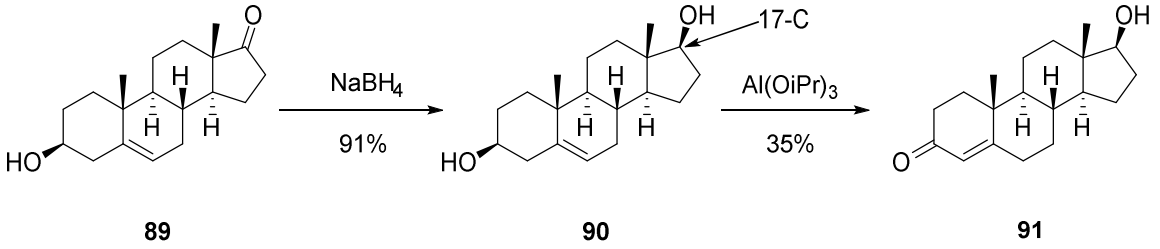


Figure 2.1 Structures of the fluorinated steroids prepared in this study.

2.2 Synthesis of Fluorinated Steroids

2.2.1 Synthesis of 6α/β-fluoro-testosterone

The synthesis of 6α/β-fluoro-testosterone (**84**) could be carried out from commercially available testosterone (**91**). However, as dehydroepiandrosterone (DHEA) (**89**) was already available in the Cobb group an alternative approach was initially investigated, in which testosterone (**91**) was prepared prior to fluorination. The synthesis of testosterone (**91**) was attempted using the sequence of reactions shown in **Scheme 2.1**.



89 **90** **91**

Scheme 2.1 Synthesis of testosterone (**91**) from DHEA (**89**).

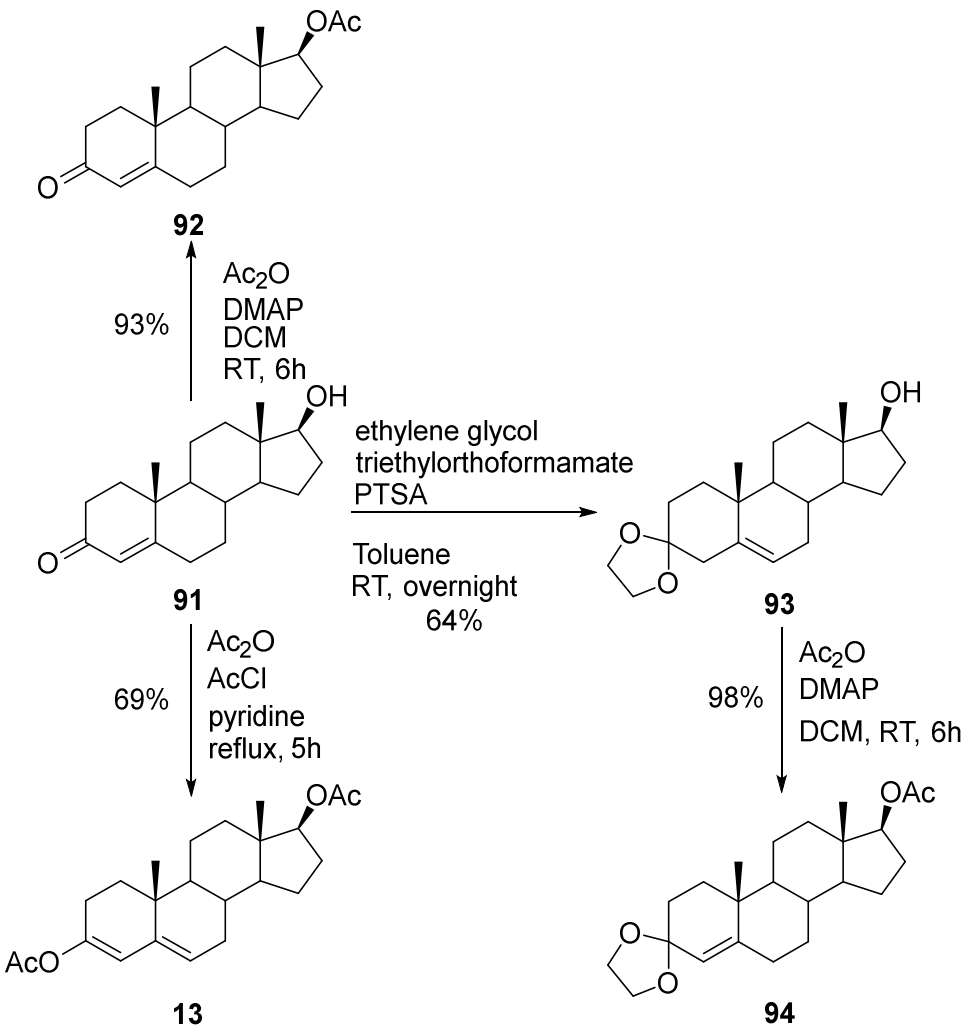
The known steroid alcohol (**90**) was prepared according to a literature procedure which involved the reduction of DHEA (**89**) in ethanol with sodium borohydride (**Scheme 2.1**).[1] This reaction was successful and gave the desired alcohol (**90**) in an excellent yield (91%). The product structure was confirmed by analysis of the IR spectrum, which showed a broad signal at 3441 cm^{-1} corresponding to the hydroxyl group, and the ^1H NMR spectrum which contained a characteristic peak at $\delta_H = 3.64$ ppm corresponding to the proton on 17-C.

With the alcohol (**90**) in hand, attention turned to the subsequent oxidation reaction. A search of literature revealed that the particular reaction required had previously been described by Kuwada *et al*.[2] In this early work, the 3β,17β-dihydroxyandrost-5-ene (**90**) was oxidised and isomerised to the α/β unsaturated compound using aluminium isopropoxide and acetone as a very mild oxidising agent. The subtle difference between 3-C and 17-C hydroxyl group reactivity allowed regioselective oxidation to produce thermodynamic product in 40% yield.

Following the protocol developed by Kuwada, the alcohol (**90**) in toluene was treated with aluminium isopropoxide and acetone. The resulting crude reaction mixture was then concentrated under vacuum, and purified by flash column chromatography (silica gel) to give by ^1H NMR low purity testosterone (**91**) in 35% crude yield. Disappointingly, the

synthesised testosterone was not sufficiently pure for the further investigation therefore the commercial material was used for the synthesis of fluorinated analogues.

In order to prepare fluorinated testosterone derivatives, the synthesis of a range of protected steroids needed to be carried out first. The synthesis of the di-protected testosterone (**94**) began with the formation of ethylene glycol acetal (**93**) followed by acetylation with acetic anhydride (**Scheme 2.2**). The reaction proceeded as expected and gave the desired product (**93**) in an excellent yield. Acetylation of testosterone was carried out in a similar manner to produce **92** in a 93% yield. Some minor problems were encountered during the formation of the bis-acetylated steroid **13**. Initially the reaction was carried out according to a literature procedure.[3] This involved treatment of testosterone (**94**) with acetyl chloride and acetic anhydride in pyridine. The reaction was heated at reflux and reaction progress was monitored by ^{1}H NMR. It was found that the product showed some degree of instability under these reaction conditions. In addition, the conjugated enol ester **13** reacts with water at temperature > 25ºC and decomposes slowly on silica. To circumvent these problems, the original literature procedure was modified. Specifically, it was found that the reaction could be more effectively carried out using DMF instead of pyridine as a solvent. This change in solvent improved the handling of the reaction mixture (in pyridine the reaction initially solidifies) and it simplifies the work-up procedure (multiple aqueous washes were required to remove the large excess of pyridine). Furthermore, it was found that when the reaction was quenched at 0ºC it gives a much cleaner product, which could be simply purified by trituration with ethanol.



Scheme 2.2 Protection of testosterone (**91**).

Products **92** and **93** were obtained as crystalline solids and their structures were confirmed by X-ray diffraction (**Figure 2.2**). Key evidence to confirm the formation of **94** was also obtained from the ^{1}H NMR spectra. The ^{1}H NMR spectrum showed the expected characteristic signal at δ_H = 3.80-3.92 ppm corresponding to the glycol group. The formation of steroid **13** could easily be confirmed from the presence of a pair of singlets in the ^{1}H NMR at δ_H = 2.05 ppm and δ_H = 2.13 ppm, corresponding to the acetyl group protons.

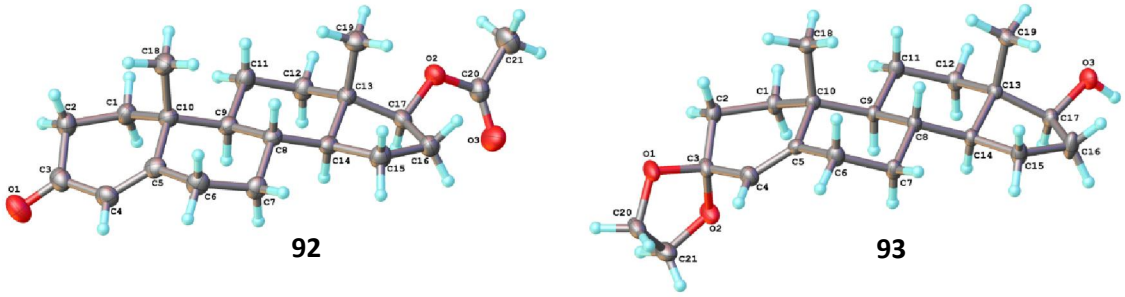
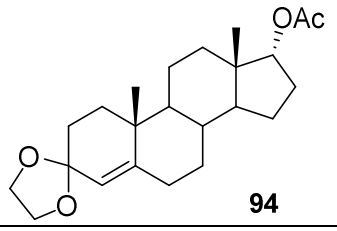
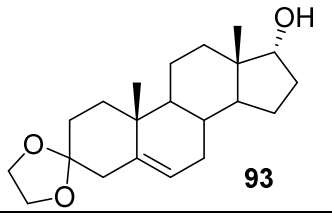
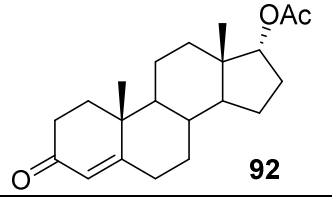


Figure 2.2 X-ray structures of protected testosterones **92** and **93**.

The resulting protected steroids (**13**, **92**, **93**, **94**) were subsequently used to generate their fluorinated analogues. Initially fluorination with F_2 gas was carried out with steroids **92**, **93** and **94**. A solution of the appropriate steroid in MeCN was sparged with a mixture of F_2/N_2 at 0-5°C for 1h. The reaction mixture was concentrated and analysed by ^{1}H and ^{19}F NMR. It was found that the reactions gave a mixture of fluorinated steroids with the main component being unreacted starting material (> 90%). Unfortunately, any attempts to obtain pure single species from the crude reaction mixtures by flash column chromatography were unsuccessful and the experiments could not be repeated due to limited access to the specialized F_2, fluorination equipment.

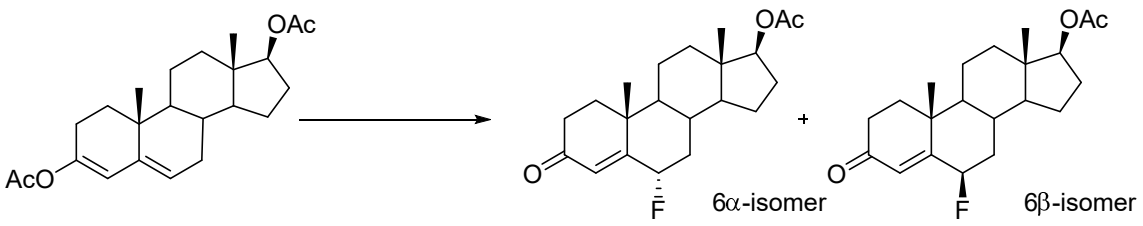
Attention then turned to the reaction of the protected steroids with the known electrophilic fluorinating agent, Selectfluor®. To a solution of the steroid (100 mg) in MeCN was added the fluorinating reagent (1.2 eq) under argon at RT. The reaction mixtures were stirred at RT for 8h. After that time, TLC analysis showed only the presence of starting material for all of the reactions carried out. Given this the temperature of the reaction was increased to reflux and the mixtures were allowed to stir overnight. The crude reaction mixtures were then analysed by ^{19}F NMR and GC-MS. The results obtained are presented in **Table 2.1**.

Table 2.1. Fluorination of protected steroids using Selectfluor®.

Entry	Starting material	Products observed
1	 **94**	Fluorinated products present by ^{19}F NMR Main product: de-protected steroid (MW 288) and fluorinated products (MW 392)
2	 **93**	Fluorinated products present by ^{19}F NMR Main product: de-protected steroid (MW 288) and fluorinated products (MW 350) Minor products: de-protected/fluorinated product (MW 306)
3	 **92**	Fluorinated products present by ^{19}F NMR Mainly starting material Trace of fluorinated product (MW 348) and de-protected starting material

When steroid **92** and **93** were treated with Selectfluor® fluorinated products were detected by GC-MS (**Table 2.1**, Entry 1-3). The reaction of Selectfluor® with **93** gave the most fluorinated products by ^{19}F NMR, therefore an attempt was made to purify the crude reaction mixture by flash column chromatography (on silica gel). The fractions containing the fluorinated products were combined based on ^{19}F NMR. Unfortunately, the identification of a major product was not possible. Nevertheless, it is speculated that the most nucleophilic position 6-C would have been preferentially fluorinated under the electrophilic fluorination conditions used.

The reactivity of **13** towards Selectfluor® and then *N*-fluorobenzenesulfonimide (NFSI) was investigated. The testosterone derivative **13** is more nucleophilic than steroids **92**, **93** and **94** previously investigated and, therefore, it was anticipated that fluorination will proceed selectively at 6-C. **13** was stirred under argon for up to 48 h in the presence of 1.1 eq of the aforementioned fluorinating agents and **Table 2.2** summarises the products obtained.



13 **14a** **14b**

Table 2.2 Attempted fluorination of protected testosterone (**13**).

Entry	Reagent	Solvent	Temp. (°C)	Time (h)	Isolated yield (%)	α/β
1	Selectfluor®	DMF	RT	48	98	40/60
2	Selectfluor®	MeCN	RT	24	Starting material only	-
3	Selectfluor®	DMF	0°C	24	47	40/60
4	Selectfluor®	DMA	RT	24	not isolated	50/50
5	NFSI	DMF	RT	48	not isolated	25/75

With the exception of the reaction in acetonitrile (**Table 2.2**, Entry 2), which gave only unreacted starting material (**13**), all of the reaction conditions investigated led to the formation of 6α/β-fluoro-testosterone (**14**) (as determined by ^{19}F NMR). The reaction in DMF at RT produced a 40:60 mixture of α/β-diastereoisomers (**14a** and **14b**) in very good yield (**Table 2.2**, Entry 1). Speculating that diastereoselectivity of the fluorination could be improved; additional experiments were performed and reaction temperature, solvent and fluorinating agent were varied. From these experiments it was found that both solvent and temperature did not affect the selectivity of the reaction to any great extent. In addition, it was found that the reaction performed with NFSI gives initially the β-isomer, although this appears to be thermodynamically unstable and gradually isomerise to the α-isomer finally giving a 25:75 (α:β) mixture after 48h (**Table 2.2**, Entry 5).

Formation of the fluorinated mixture of α/β steroids (**14a** and **14b**) was confirmed by examination of the ^{19}F NMR spectrum, which revealed new signals at $\delta_{F\beta}$ = -165.53 ppm and $\delta_{F\alpha}$ = -183.23 ppm corresponding to the CH_2F. The configuration of the diastereoisomers was assigned on the basis of 2D correlation NMR experiments (**Figure 2.3**). In the α isomer 10-**C**-CH_3 group showed correlation between proton 6C-**H** and 8C-**H**. The ^1H NOESY spectrum of the isomer **14b** showed correlation between 4C-**H**, 6C-**H** and 7C-**H** indicating that the fluorine is placed in the axial position.

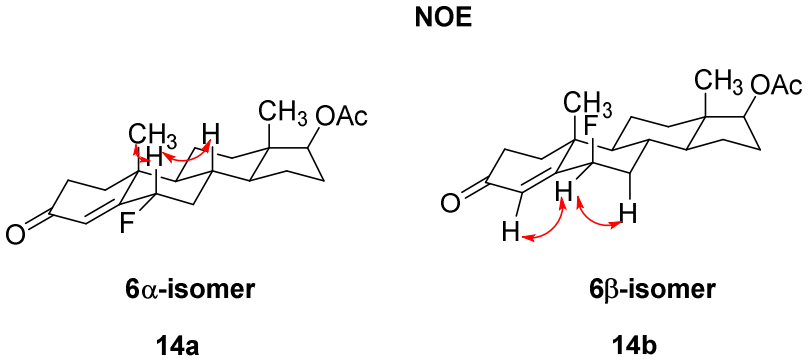


Figure 2.3 Nuclear Overhauser Effect (NOE) interactions identified in compound **14**.

With the acyl protected 6α/β-fluoro-testosterone acetate (**14**) in hand, the next stage was to attempt the deprotection reaction. In theory, the removal of an acyl protecting group can be achieved by either treatment with acid or base, and a range of reaction conditions was examined (**Table 2.3**). The progress of the reactions was monitored by ^{1}H NMR and TLC.

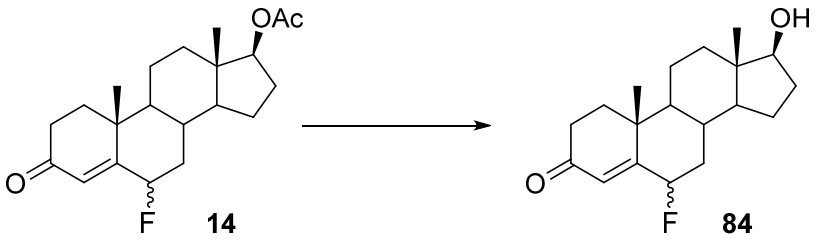
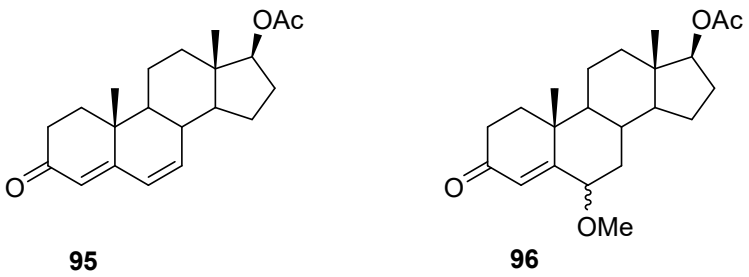


Table 2.3 Deprotection of **14** under various conditions.

Entry	Solvent	Time (h)	Temp.	Reagent	Results
1	THF	24	RT	0.5M NaOH	trace of product
2	MeOH	36	RT	0.2M NaOH	Decomposition
3	MeOH/H_2O	36	RT	K_2CO_3	Decomposition
4	MeOH/H_2O	1.5	Reflux	K_2CO_3	trace of product
5	MeOH/H_2O	24	30°C	K_2CO_3	trace of product
6	MeOH/H_2O	36	RT	Cs_2CO_3	Decomposition
7	THF	48	40°C	2M HCl	Product

From the data presented in **Table 2.3** it can be seen that the product is not stable under basic conditions (**Table 2.3**, Entry 1-6). It was observed by ^{1}H NMR that the starting material could easily undergo an elimination reaction to form de-fluorinated product (**95**) which showed new signals at δ_H = 6.12 ppm, 6.05 ppm, 5.75 ppm (no fluorine was observed by ^{19}F NMR). The starting material was also converted to methanol adduct (**96**) based on ^{1}H NMR which showed characteristic signal at δ_H = 3.42 ppm (3H) corresponding to OC***H***$_3$ protons



95 **96**

Figure 2.4 Side products generated during the de-protection of the acyl protected 6α/β-fluoro-testosterone acetate (**14**).

The best conversion achieved under basic reaction conditions was when the reaction was carried out at reflux for 1.5 h (**Table 2.3**, Entry 4). However, after flash column chromatography (silica gel) the product was isolated in a low yield (35%) and the purity as determined by ^{1}H NMR was found also found to be low (e.g. contained methanol adducts). These unsuccessful attempts led us to investigate acetyl hydrolysis under acidic conditions. A solution of the starting material **14** in THF was treated with 2M HCl. Subsequent purification of the crude reaction mixture afforded the desired compound **84** (58% yield) as confirmed by analysis of the ^{1}H NMR spectrum, which showed disappearance of a signal at δ_H = 2.05 ppm arising from loss of the OAc protons.

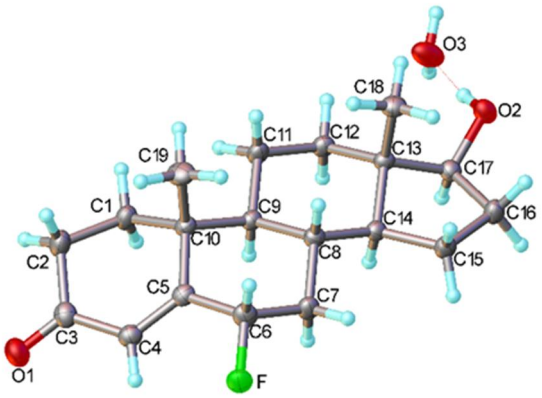
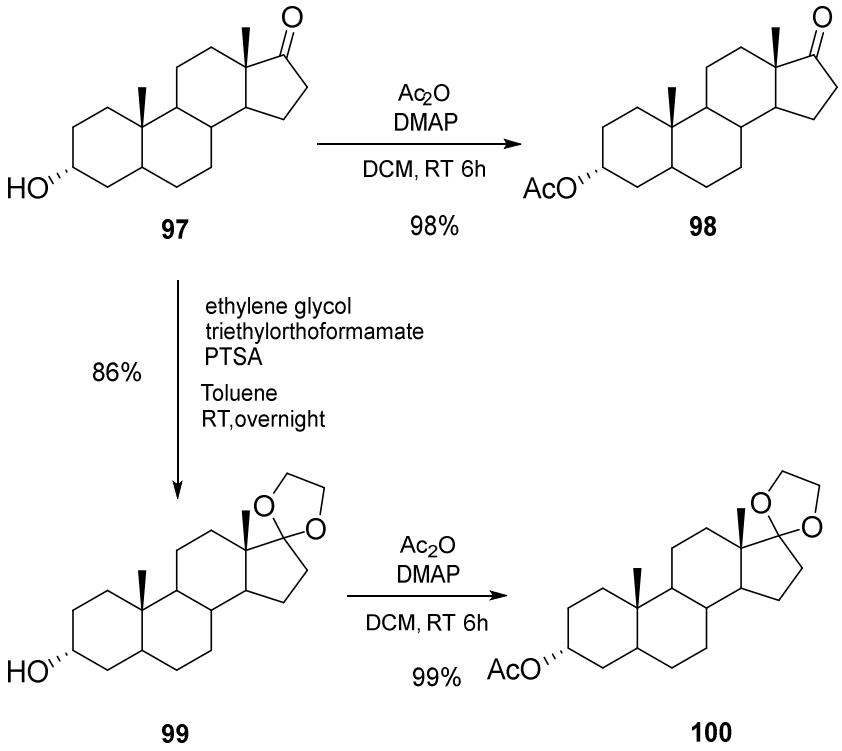


Figure 2.5 X-ray structure of 6α-fluoro-testosterone (**84**).

2.2.2 Synthesis of Fluorinated Androsterone

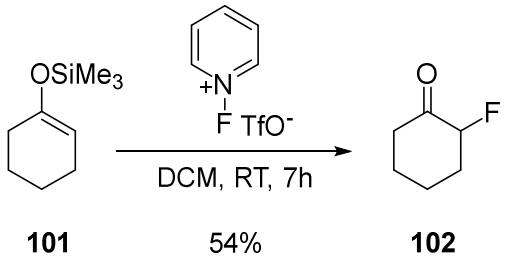
The first step in the preparation of fluorinated androsterone requires protection of the androsterone hydroxyl group at 3-C. This was achieved using acetic anhydride under basic conditions in DCM (**Scheme 2.3.**). The reaction gave the desired product **98** in very good yield (98%) and purity after purification on a silica pad. The carbonyl group was then protected with ethylene glycol and a catalytic amount of *p*-toluenesulfonic acid (PTSA). Triethyl-orthoformate was used to remove water from the reaction mixture. An excellent yield was obtained for both the non-acylated (**99**) (86%) and acylated androsterone (**100**) (99%).



Scheme 2.3 Protection of androsterone (**97**).

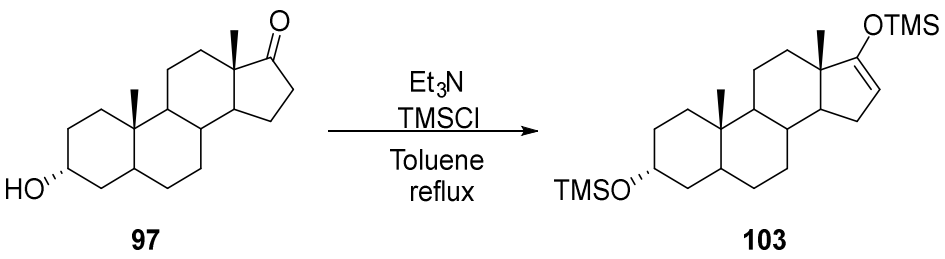
The formation of **98** was supported by mass spectrometry showing a molecular ion of *m/z* = 332 $[M+H]^+$. In addition, the ^1H NMR spectrum showed a signal attributed to the acetyl group at δ_H = 0.85 ppm (3H). Key evidence for the formation of glycol protected compounds was found in the ^1H NMR spectrum where signal corresponding to the glycol group were observed at around δ_H = 3.80-3.97 ppm for **99** and at around δ_H = 3.80 - 3.96 ppm for **100**.

It was speculated that the presence of a TMS group would make the molecule more reactive towards electrophilic fluorination. This was based on the fact that this type of protecting group had been employed in the preparation of α-fluoro-carbonyls (e.g. **102**) by Umemoto.[4] For example, Umemoto found that *N*-fluoropyridinium triflate can be used in the fluorination of ether **101**, to give 2-fluorocyclohexanone **102** in an excellent yield (87%).



Scheme 2.4 Fluorination of a silylenolether (**101**)[4].

The synthesis of the TMS protected androsterone (**103**) was carried out under thermodynamic conditions using triethylamine to generate the enol and trimethylsilyl chloride (TMS-Cl) as a trapping agent. The reaction mixture was stirred at reflux and monitored by ^{1}H NMR. After 24h at reflux, a mixture of the starting material and the product was detected. No further changes were observed after an additional 24h, therefore the reaction mixture was worked-up and the product was purified by flash column chromatography. Unfortunately, the purification was unsuccessful due to instability of the product on silica gel. It was later found that the desired fluoro-steroid (**87**) could be synthesised by generating the androsterone enol under acidic conditions (catalytic amount of H_2SO_4), so no further attempts to synthesise the di-TMS protected steroid were carried out.

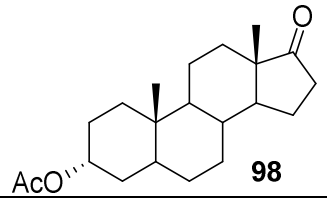
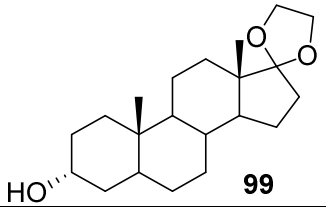
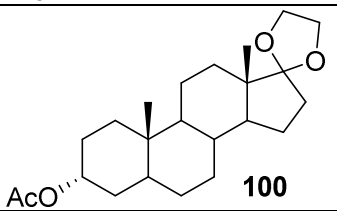


Scheme 2.5 Attempted synthesis of TMS protected androsterone (**103**).

The resulting protected steroids (**98**, **99** and **100**) were then subsequently reacted with F_2 gas. A solution of the appropriate steroid in MeCN was sparged with a mixture of F_2/N_2 at 0-5ºC for 1h. The reaction mixture was concentrated and analysed by ^{1}H and ^{19}F NMR. It was found that the reaction gave a mixture of fluorinated steroids with the main component being the unreacted starting material. Unfortunately, any attempts to purify out a single product from the crude reaction mixture was unsuccessful.

Attention then turned to the reactions of the protected steroids with Selectfluor®. The reactions were carried out on 100 mg scale and used similar conditions using (e.g. Selectfluor® (1.1eq) in MeCN) to those used previously **Table 2.4**. The reaction mixtures were stirred at RT for 8h then refluxed overnight. The reaction mixtures were analysed by NMR and GC-MS. The results obtained are summarised in **Table 2.4**.

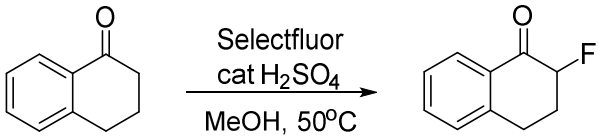
Table 2.4 Fluorination of protected steroids using Selectfluor®.

Entry	Starting material	Products
1	 **98**	No fluorinated product Mainly starting material Trace of de-protected starting material (MW 290)
2	 **99**	No fluorinated product Mainly starting material and de-protection/oxidation product (MW 288) Minor products: de-protected steroid and oxidation product (MW332 possibly 3-keto steroid)
3	 **100**	No fluorinated product Mainly Starting material Trace of de-protected/oxidised product (MW332)

Compounds **98**, **99** and **100** (**Table 2.4**, Entry 1-3) did not appear to be sufficiently nucleophilic to be fluorinated with Selectfluor® given that the major product recovered was unreacted starting material. Trace amounts of the corresponding de-protected and oxidised products were also detected by GC-MS but no fluorinated product was formed (as indicated by ^{19}F NMR).

The α-fluorination of carbonyl compounds could be achieved by electrophilic fluorination of enol ethers derivatives. Methods for direct α-fluorination of carbonyl compounds have

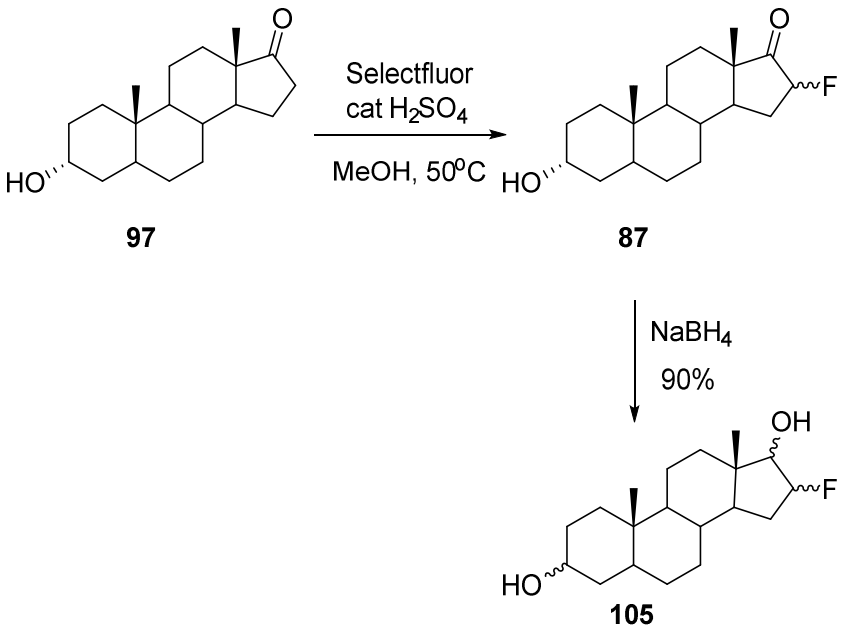
been extensively studied by the Batey group.[5] They found that the presence of a catalytic amount of sulphuric acid in the reaction mixture is sufficient to generate enol which reacts with Selectfluor® to give α-fluorinated product. For example, fluorination of tetralone (**104**) gave the product in 93% yield after flash column chromatography (**Scheme 2.6**).



104 93%

Scheme 2.6 Fluorination of tetralone (**104**).

Following the protocol developed in Batey's group, androsterone was treated with Selectfluor® and sulphuric acid in methanol. The reaction was successful however extended stirring times were necessary to achieve good conversion (**Scheme 2.7**). The formation of product **87** was supported by ^{19}F NMR spectroscopy indicated the presence of a fluorine atom at δ_F = -183.45 ppm, δ_F = -192.58 ppm (α/β mixture).



97 **87** **105**

Scheme 2.7 Fluorination of androsterone (**97**) using Selectfluor® under acidic conditions.

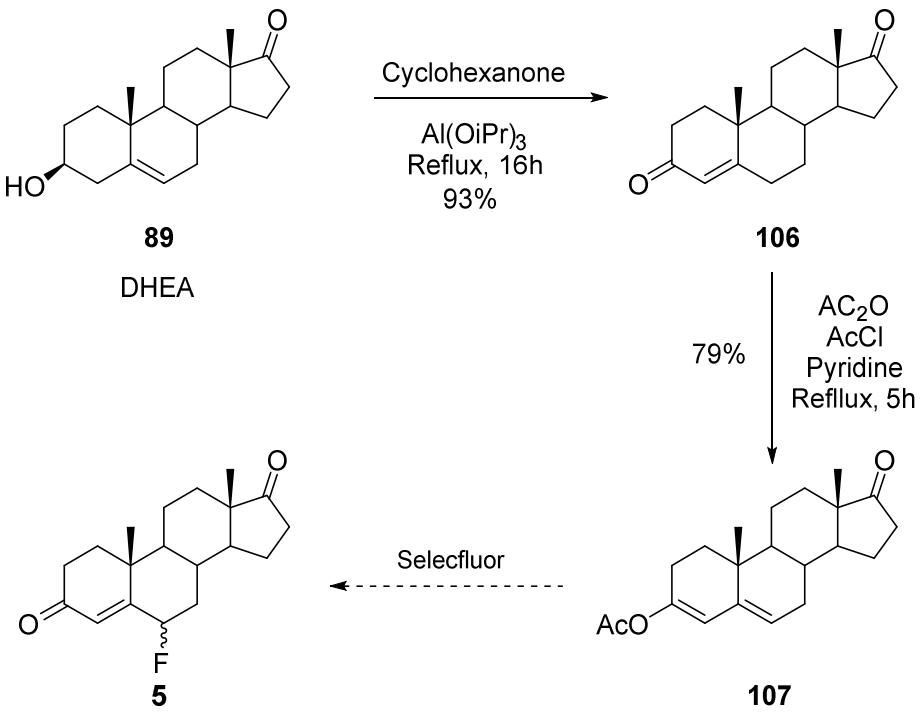
The synthesis of 16α/β-Fluoro-3β,17β-dihydroxyandrostane (**105**) was carried out by reducing 16α/β-fluoro-androsterone (**87**) with sodium borohydride in a mixture of DCM and MeOH overnight at RT. This gave the desired product in 90% yield as a mixture of

four diastereoisomers. The formation of **105** was confirmed by the ^{19}F NMR spectrum, which showed peaks at δ_F = - 179.82 ppm, - 185.28 ppm, - 191.93 ppm, - 196.35 ppm corresponding to the four 16-fluoro-isomers. ^{1}H NMR revealed formation of the corresponding alcohol δ_H = 350-3.62 ppm (0.5H, m), and δ_H = 3.76-3.77 ppm (0.4H, m).

2.2.3 Synthesis of Fluorinated Androstenedione

Androstenedione (**106**) (called andro) is banned by the WADA but it is currently available without a prescription and marketed primarily to athletes and bodybuilders. The number of people regularly using androstenedione is not known. It was found by Leder that when given in dosages of 300 mg/day, this increases serum testosterone and estradiol concentrations in some healthy men.[6] There was marked variability in individual responses for all of the measured sex steroids.

It was envisaged that the target 6α/β-fluoro-androstenedione (**5**) could be potentially generated in three steps following the procedure presented below in **Scheme 2.8**. The first step in the synthesis involves an Oppenauer Oxidation reaction of commercially available dehydroepiandrosterone (DHEA (**89**)).



Scheme 2.8 Attempted synthesis of 6α/β-fluoro-androstenedione (**5**).

Following the protocol outlined by Almeida *et all.*[7] the synthesis of androstenedione (**106**) was carried out in toluene with aluminium isopropoxide (5.65 eq) and cyclohexaneone (21 eq). After 16h at reflux, the formation of the product was confirmed by ^{1}H NMR analysis. The reaction was worked-up and the product was purified by re-crystallisation from hexane (31% yield). A substantial amount of the product was detected in the mother liquors; however, attempts to purify further product from the mother liquor by flash column chromatography were unsuccessful. In addition, it was also found that low purity androstenedione product did not give the desired product (**107**) at the next stage.

In order to improve the reaction yield and product purity, various modifications to the original procedure were carried out. It was found that the reaction yield could be improved by using freshly distilled toluene and by distilling off the excess cyclohexanone before carrying out the work-up (yield was increased to 60%). It was also found, that a very good reaction profile could be achieved for the reaction performed according to the procedure published by Pavlovic *et all*.[8] The reaction was carried out in a similar fashion however the charge of aluminium isopropoxide was significantly reduced (0.6 eq) and the reagent was added very slowly. The work-up procedure was modified based on the previous observations and the product was isolated by re-crystallisation from hexane in a very good yield (93%) and purity. Evidence for the formation of diketone **106** was ascertained from the ^{13}C NMR spectrum which contained two signals corresponding to the carbonyl groups at δ_C = 200 ppm (conjugated) and at δ_C = 220 ppm.

The synthesis of acetyl androstenedione (**107**) was performed following the procedure developed for the synthesis of 6α/β-fluoro-testosterone (**84**). A solution of androstenedione (**106**) in DMF was treated with pyridine, acetylchloride and acetic anhydride and the resultant solution was heated at reflux for 5h. This gave the acetyl androstenedione as expected, however minor problems were encountered during the isolation of the product. It was found that the product decomposes on silica during purification if the reaction is quenched with water at RT. The first problem was addressed by quenching the reaction slowly at 0ºC. This gave the acetyl androstenedione (**107**) with a good crude yield. Purification on deactivated silica (pre-washed with Et_3N) and alumina were not successful with the product obtained of lower purity that the input material. A pure product was obtained by trituration of the crude material with Et_2O and subsequent slurry in methanol. As previously, evidence confirming the formation of **107** was obtained from the ^{1}H NMR spectrum which showed the appearance of the double bond protons (δ_H = 5.70 ppm and δ_H

= 5.41 ppm) and the acyl signal (δ_H = 2.13 ppm) respectively. The product appears to be unstable and slowly decompose upon storage.

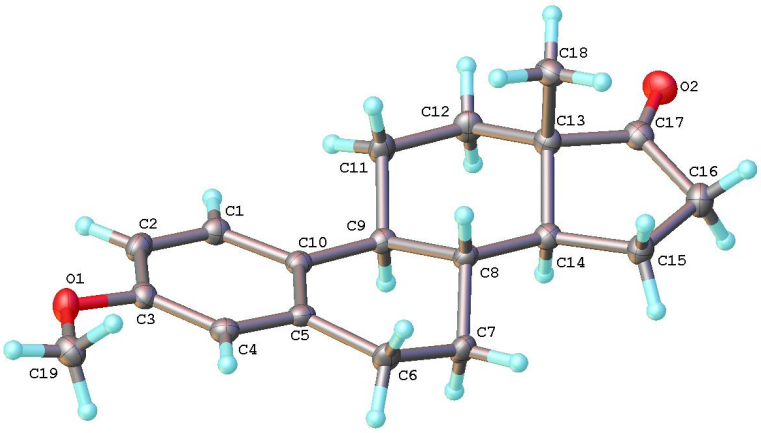
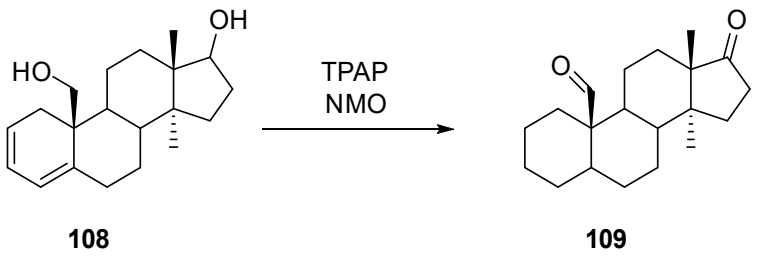


Figure 2.6 X-ray structure of 3-acetoxyandrost-3,5-diene-17-one (**107**).

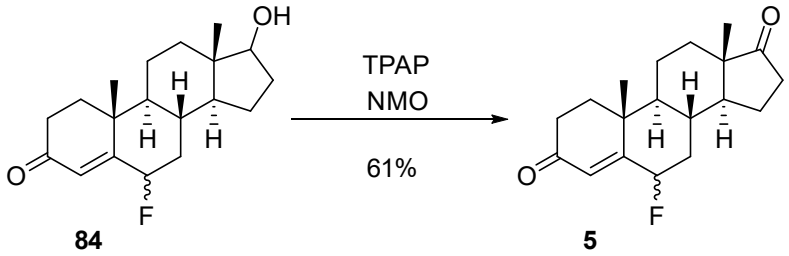
The final stage of the synthesis was performed according to the previously developed protocol (Section 2.2.1, 6α/β-fluoro-testosterone (**84**)). To a solution of the acetyl androstenedione (**107**) in DMF was added Selectfluor® and the reaction mixture was stirred at RT. After 24h, NMR indicated complete consumption of the starting material and the formation of a complex mixture of fluorinated products (16 signals were observed by ^{19}F NMR). Purification by flash column chromatography (silica gel) was not successful; therefore, in order to improve the yield and selectivity of the fluorination reaction, several experiments were carried out in which the reaction time, solvents and temperature were all varied. Unfortunately, all attempts to efficiently generate 6α/β-fluoro-androstenedione (**5**) were unsuccessful. Therefore, attention turned to the use of the previously prepared 6α/β-fluoro-testosterone (**84**) as an alternative precursor in the preparation of 6α/β-fluoro-androstenedione (**5**).

It was suggested that under mild conditions oxidation of 6α/β-fluoro-testosterone (**84**) could be achieved without defluorination of the starting material/product occurring. There are many examples in the literature reporting the oxidation of steroids. Of these, a reaction carried out Langer and co-workers appeared to be the most relevant.[9] Here, a mixture of **108**, tetrapropylammonium perruthenate (TPAP) and 4-methylmorpholine *N*-oxide (NMO) was stirred at RT for 24 h, to afford steroid **109** in 55% yield (**Scheme 2.9**).



Scheme 2.9 Oxidation of steroids with TPAP and NMO.[9]

Following the procedure developed by Langer, to a solution of 6α/β-fluoro-testosterone (**84**) in dry MeCN, TPAP (0.05 eq) and NMO (1.5 eq) were added (**Scheme 2.9**). The reaction mixture was stirred at RT and, following work-up, product was obtained in a very good yield (61%).



Scheme 2.10 Synthesis of 6α/β-fluoro-androstenedione (**5**) via oxidation.

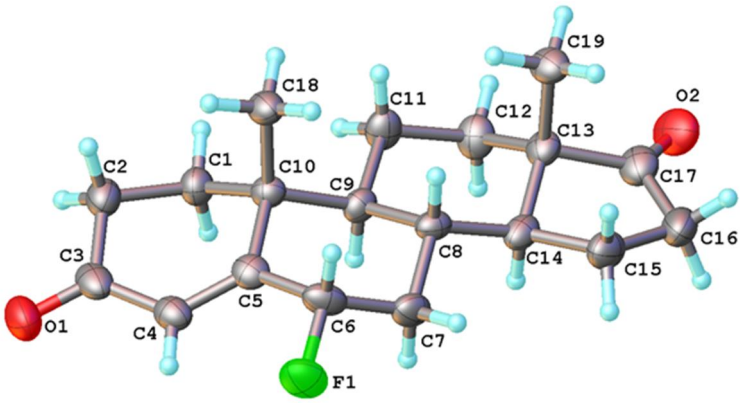


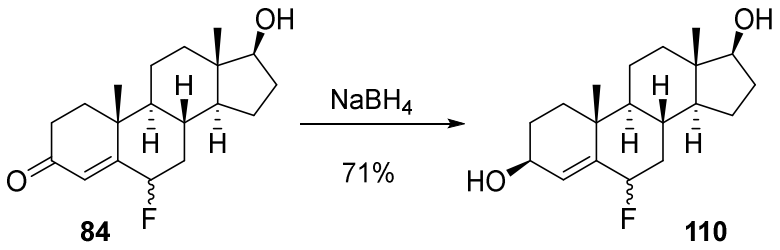
Figure 2.7 X-ray structures of 6α-fluoro-androstenedione (**5**).

The spectroscopic data were as expected similar to those reported for 6α/β-fluoro-testosterone (**84**) providing indirect evidence for the formation of the desired fluorinated

product **5**. Compound **5** also showed the characteristic signals corresponding to the two carbonyl groups at δ_C = 219.4 ppm and δ_C = 198.3 ppm and also signals attributed to the fluorine at δ_F = - 183.61 ppm (α isomer) and δ_F = - 165.66 ppm (β isomer).

2.2.4 Synthesis of 6α/β-Fluoro-3β,17β-dihydroxyandrost-4-ene (110)

We envisaged that the synthesis of 6α/β-Fluoro-3β,17β-dihydroxyandrost-4-ene (**110**) could be carried out by reducing the already available 6α/β-fluoro-testosterone (**84**). It was found that this could indeed be achieved using sodium borohydride in a mixture of DCM and MeOH. After 3h at RT, the reaction was quenched with HCl_{aq}, the product was filtered off and dried in a vacuum oven. This gave the product desired product in a 71% yield and as a mixture of two diastereoisomers.



Scheme 2.11 Synthesis of 6α/β-fluoro-3β,17β-dihydroxyandrost-4-ene (**110**).

The formation of **110** was confirmed by analysis of the ^{19}F NMR spectrum, which showed peaks at δ_F = - 161.41 ppm, δ_F = - 184.19 ppm, corresponding to the two 6-fluoro-isomers. The configuration of the two main products could be deduced from 2D correlation NMR experiments (**Figure 2.8**). The stereochemistry of the α isomer was elucidated through ^1H NOESY experiments, and provided evidence that the proton 3-**H** was located axial to the proton 8-**H**. The ^1H NOESY spectrum of the β isomer showed correlation between the proton 6-**H** and 7-**H** indicating a close proximity. Unfortunately, it was not possible to fully separate the isomers by flash column chromatography.

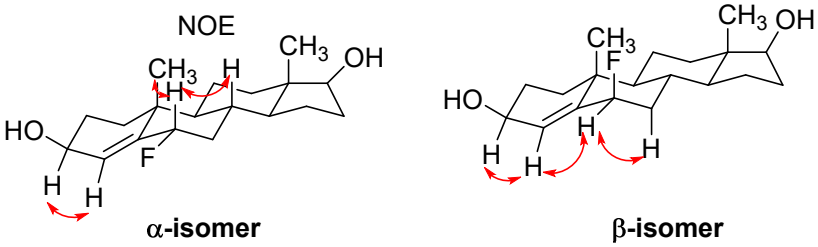
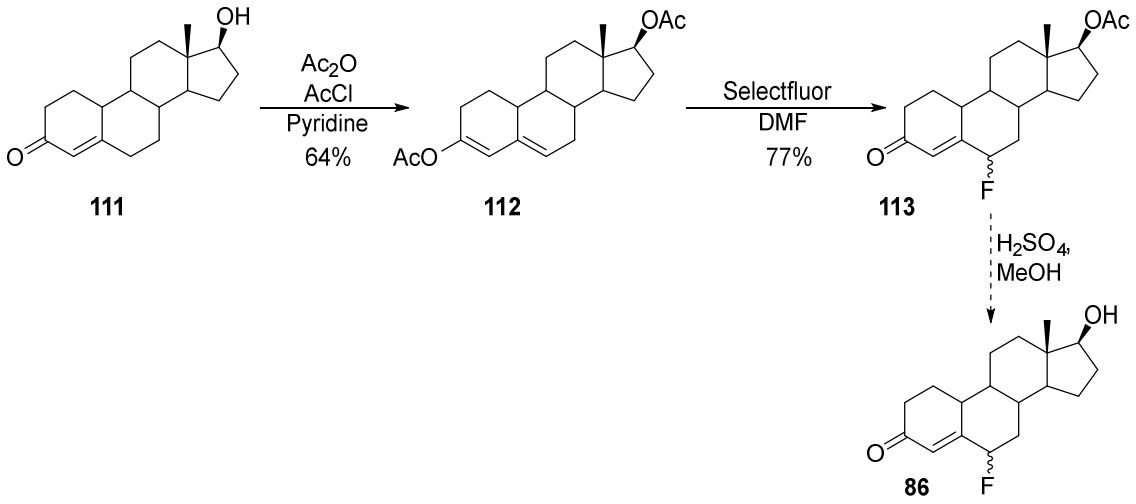


Figure 2.8 6α/β-Fluoro-3,17-dihydroxyandrost-4-ene (**110**) isomers.

2.2.5 Synthesis of 6α/β-Fluoro-Nortestosterone

Following the procedure developed for the synthesis of 6α/β-fluoro-testosterone (**84**), a solution of nortestosterone (**111**) in pyridine was treated with acetyl chloride and acetyl anhydride to give the protected intermediate (**112**) (**Scheme 2.12**).



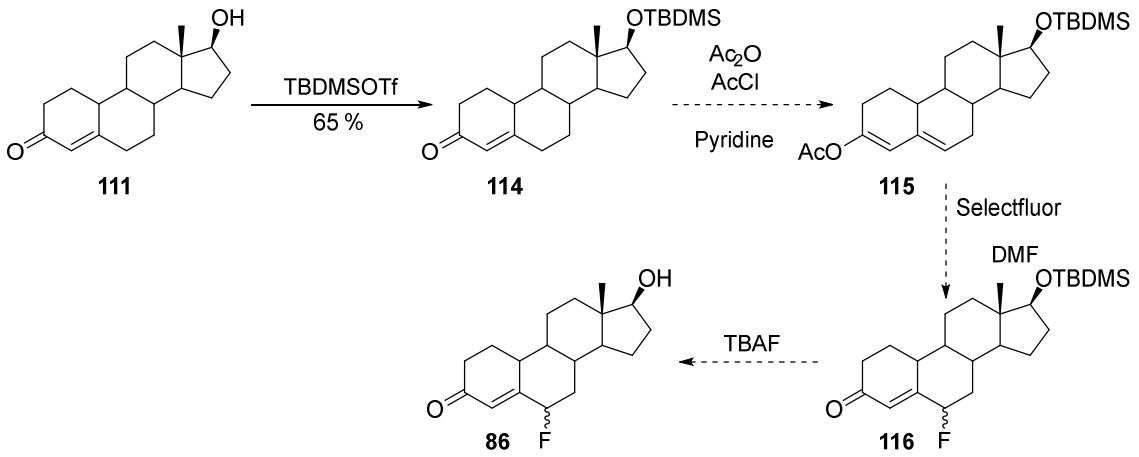
Scheme 2.12 Attempted synthesis of 6α/β-fluoro-nortestosterone (**86**).

Evidence confirming the formation of **112** was obtained from the ^{1}H NMR spectrum which showed the appearance of the double bond protons (δ_H = 5.47 ppm and δ_H = 5.76 ppm) and the acetyl signals (δ_H = 2.07 ppm and δ_H = 2.15 ppm) respectively. The next stage was to attempt the formation of the corresponding acetyl protected 6α/β-fluoro-nortestosterone (**113**). A solution of **112** in DMF was treated with Selectfluor®. The reaction mixture was heated to 40°C overnight to give the product in 77% yield after purification by flash column chromatography. Fluorination of the acetyl protected nortestosterone (**113**) was confirmed by analysis of the ^{19}F NMR spectrum, which showed signal at δ_F = -181 ppm for the α isomer and signal at δ_F = - 170 ppm for the β isomer. Finally, hydrolysis of the acyl group was attempted. As for the previous synthesis of 6α/β-fluoro-testosterone (**84**) this was carried out under both basic and acidic conditions and the results are summarised in **Table 2.5**. Disappointingly, all of the reaction conditions investigated led to the formation of an intractable mixture of products.

Table 2.5 Attempted hydrolyses of the acetyl group in **113.**

Entry	Solvent	Reagent	Temp (°C)	Time	Outcome (^{1}H NMR)
1	MeOH	2M HCl	40	3 days	starting material only
2	MeOH/H_2O	K_2CO_3	40	3 days	trace of product
3	MeOH/H_2O	0.5M NaOH	40	3 days	trace of product/ decomposition
4	THF	2M HCl	Reflux	3 days	trace of product/ decomposition
5	THF	2M HCl	40	3 days	starting material only

The failure of the above synthesis prompted investigations into an alternative method for the preparation of the 6α/β-fluoro-nortestosterone (**86**). This involved the synthesis of TBDMS protected alcohol, acylation, fluorination and TBDMS deprotection (**Scheme 2.13**). Unlike the acyl protected alcohol, this could be deprotected under milder conditions with a fluoride source (e.g. TBAF). It is worth noting that this strategy precedes via Selectfluor® fluorination, which could potentially cleave the TBDMS group (as seen by Rogers *et al*.).[10]



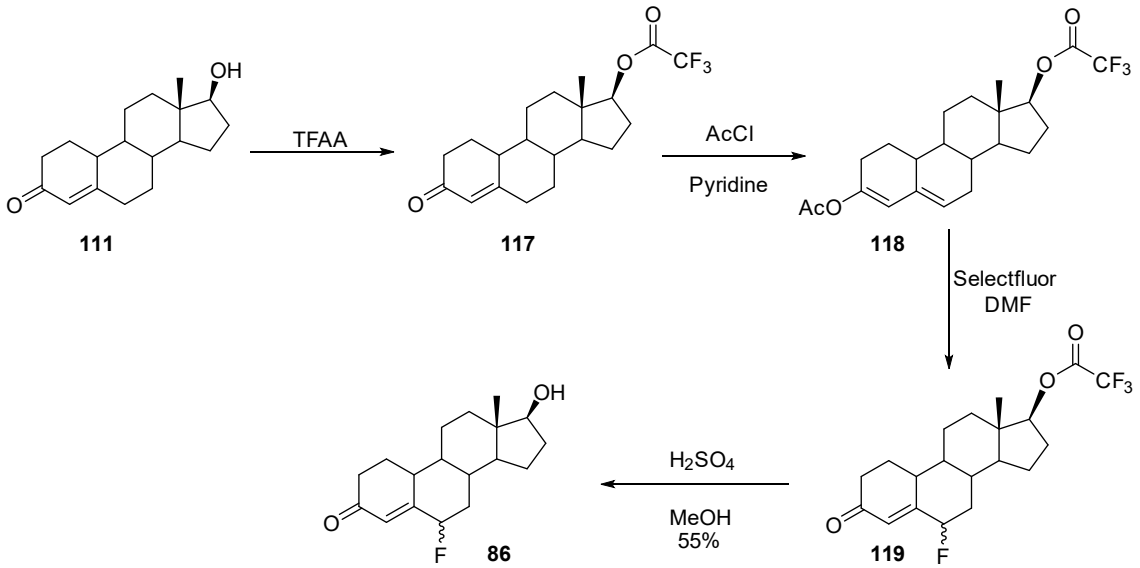
Scheme 2.13 Attempted synthesis of 6α/β-fluoro-nortestosterone (**86**).

With this in mind, nortestosterone (**111**) was reacted with TBDMSOTf to give silyl ether (**114**) in a 65% yield (**Scheme 2.13**). It was found that the reaction could be carried out in

the presence of either TBDMSOTf or TBDMSCl, however significantly better conversion was achieved for the reaction with triflate (TBDMSOTf 65%, TBDMSCl 50%). Evidence for the formation of the silyl ether (**114**) was confirmed by analysis of the ^{1}H NMR and the MS data. The ^{1}H NMR spectrum showed peaks at $\delta_H = 0.88$ ppm and $\delta_H = 0.00$ ppm, which can be attributed to the *t*-butyl and methyl groups respectively.

The next step involved the reaction of the silyl protected nortestosterone (**115**) with acyl chloride and acetic anhydride in pyridine. This reaction was performed at 80ºC overnight. Unfortunately, the reaction was not successful and it was found that the TBDMS group was not stable under the reaction conditions used.

Given the lack of success in the employment of TBDMS group in the synthesis of 6- fluoro-nortestosterone (**86**), attention turned to the use of a trifluoroacetate protecting group. It was speculated that the presence of an electron-withdrawing substituent on the acetate would encourage the final ester hydrolysis. Consequently, nortestosterone (**111**) was reacted with trifluoroacetic anhydride (TFAA) in DMF to give the trifluoroacetate (**117**) (**Scheme 2.14**). The presence of the desired compound in the crude product was established by NMR spectroscopy, where comparison of the ^{1}H NMR spectrum of the starting material (**111**) and the product (**117**) showed the disappearance of the OH signal. Additional evidence for the formation of product **117** was found in the ^{19}F NMR spectrum where a signal corresponding to the CF_3 group was observed at $\delta_F = -75$ ppm. Subsequently, it was found that this reaction could be carried out in one pot with the acylation reaction. The synthesis began with the reaction of nortestosterone (**111**) with TFAA to afford intermediate trifluoroacetate (**117**) which after 15 min at RT was treated with acetyl chloride to give the acylated nortestosterone (**118**). Evidence for the formation of the acylated nortestosterone was provided by the ^{1}H NMR spectrum, in which a characteristic signals corresponding to the conjugated double bonds at $\delta_H = 5.84$ ppm and $\delta_H = 5.77$ ppm were observed. Unfortunately, all attempts to purify and fully characterise this compound were unsuccessful and led to decomposition. Unsaturated enol ester (**118**) was then treated with Selectfluor® to give the corresponding fluoro steroid (**119**). The formation of this product was confirmed by analysis of the ^{19}F NMR spectrum, which showed peaks at $\delta_F = -170$ ppm and $\delta_F = -181$ ppm for the α and β isomers respectively. Following the procedure used previously to hydrolyse the acyltestosterone, the trifluoroacylnortestosterone was treated with dilute sulphuric acid. The hydrolysis proceeded as expected and gave the desired alcohol (**86**) in 55% yield.



Scheme 2.14 Synthesis of 6α/β-fluoro-nortestosterone (**86**).

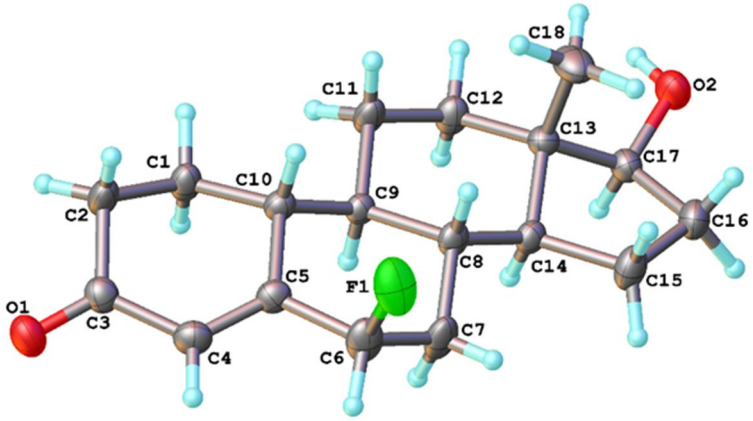
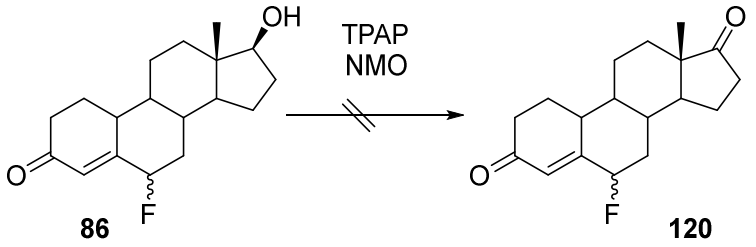


Figure 2.9 X-ray structure of 6-fluoro-β-nortestosterone (**86**).

2.2.6 Attempted Synthesis of Fluorinated Norandrostenedione

It was expected that the synthesis of 6-fluoro-norandrostenedione (**120**) could be carried out by oxidation of 6α/β-fluoro-nortestosterone (**86**) using condition outlined previously for the synthesis of 6-fluoro-androstenedione. The oxidation of 6α/β-fluoro-nortestosterone (**86**) was carried out with TPAP (0.05 eq) and NMO (1.5 eq) in MeCN (**Scheme 2.15**). The reaction mixture was stirred at RT overnight. After that time, no product was detected and therefore another portion of TPAP (0.05 eq) and NMO (1.5 eq) were added. 6α/β-fluoro-nortestosterone (**86**) was not oxidized under these conditions and

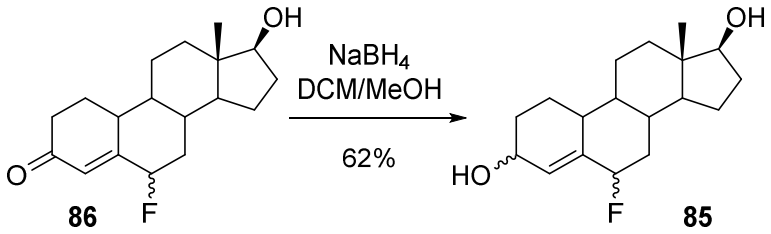
only the starting material was recovered. Subsequently, a Swern oxidation 6α/β-fluoro-nortestosterone (**86**) was attempted. To a solution of DMSO and oxalyl chloride in DCM was added 6α/β-fluoro-nortestosterone (**86**) at -78ºC. After 30 min at -78ºC, Et_3N was added and the reaction mixture was allowed to warm-up to RT. Analysis of the reaction mixture by ^{19}F NMR showed a complex mixture of products. No product was detected by GC-MS. It is speculated that the different conformation of the 6α/β-fluoro-nortestosterone (**86**) in comparison to 6α/β-fluoro-testosterone (**84**) was the reason for the unsuccessful oxidation.



Scheme 2.15 Attempted synthesis of 6-fluoro-norandrostenedione (**120**).

2.2.7 Synthesis of Fluorinated Norandrostenediol

The synthesis of 6-fluoro-norandrostenediol (**85**) was carried out by reducing 6α/β-fluoro-nortestosterone (**86**). It was found that this could be achieved with sodium borohydride in a mixture of DCM and MeOH. After 3h at RT, the reaction was complete; the product was filtered off and dried in a vacuum oven. This gave the product **85** in a 62% yield and as a mixture of two isomers.



Scheme 2.16. Synthesis of 6α/β-fluoro-norandrostenediole (**85**).

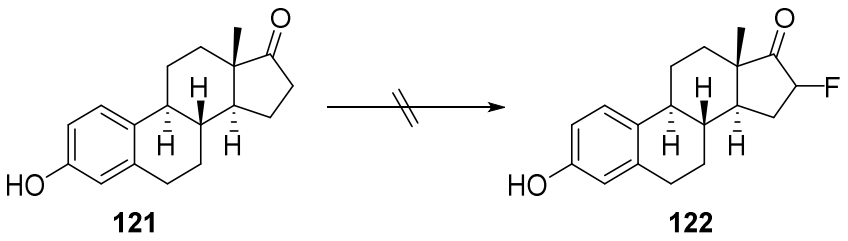
The formation of **85** was confirmed by analysis of the ^{19}F NMR spectrum, which showed peaks at $\delta_{F\beta}$ = - 167.12 ppm, $\delta_{F\alpha}$ = - 181.84 ppm, for the 6-fluoro isomers. Analysis of ^{1}H

NMR revealed formation of the corresponding allylic alcohol δ_H = 4.10-4.27 ppm. Based on NOE experiments and available literature for similar steroids the reduction gave 3β alcohol.[11] Unfortunately, it was not possible to separate the isomers by flash column chromatography on silica.

2.2.8 Synthesis of Fluorinated Estrone

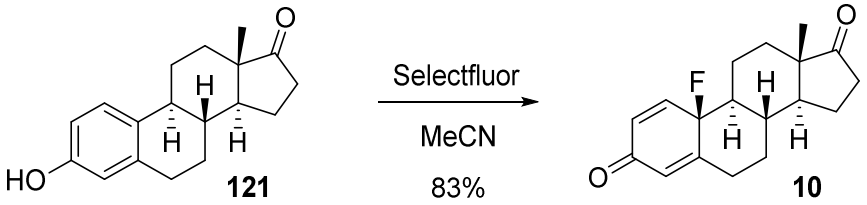
Initially, an attempt was made to introduce a fluorine into the 16-C position of the steroid framework under acidic conditions. Following the procedure previously developed for the preparation of 16α/β-fluoro-androsterone (**88**), a solution of estrone (**121**) in MeOH was treated with H_2SO_4 and Selectfluor® as shown on **Scheme 2.17**. The reaction mixture was then stirred at RT for 1 day. After this time, ^{19}F NMR analysis suggested the formation of a mixture of fluorinated products. Unfortunately, all attempts to purify the fluorinated products by flash column chromatography (silica gel) were unsuccessful.

Literature precedent suggested that the 16-C position could be fluorinated via the initial formation of enol ester or displacement of a good leaving group from the α position.[12] These strategies, however, were not explored.



Scheme 2.17 Attempted synthesis of 16-fluoroestrone (**122**).

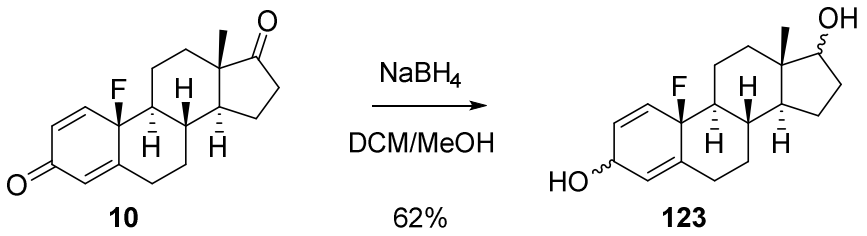
Based on a literature search estrone could also be fluorinated at the 10-C position (**Scheme 2.18**). Following the protocol outlined by Stavber, [13] the synthesis of 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**) was carried out in acetonitrile with Selectfluor® (1.2 eq). After 4 h at 50°C, the fluorination of estrone was confirmed by ^{19}F NMR analysis, which showed peak at δ_F= -165.32 ppm. Analysis of ^1H NMR and MS data of the isolated product showed formation of the ketone product (**10**) (10β-fluoro-3,17-dihydroxy-1,4-androstene). The fluorination gave the ketone product in 83% yield.



121 10

Scheme 2.18 Synthesis of 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**).

The synthesis of 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**) was then carried out by reducing the corresponding diketone (**Scheme 2.19**). It was found that this could be achieved with sodium borohydride in a mixture of DCM and MeOH. After 3h at RT, the reaction was worked-up to give the product in 62% yield as a mixture of four isomers based on ^{19}F NMR.



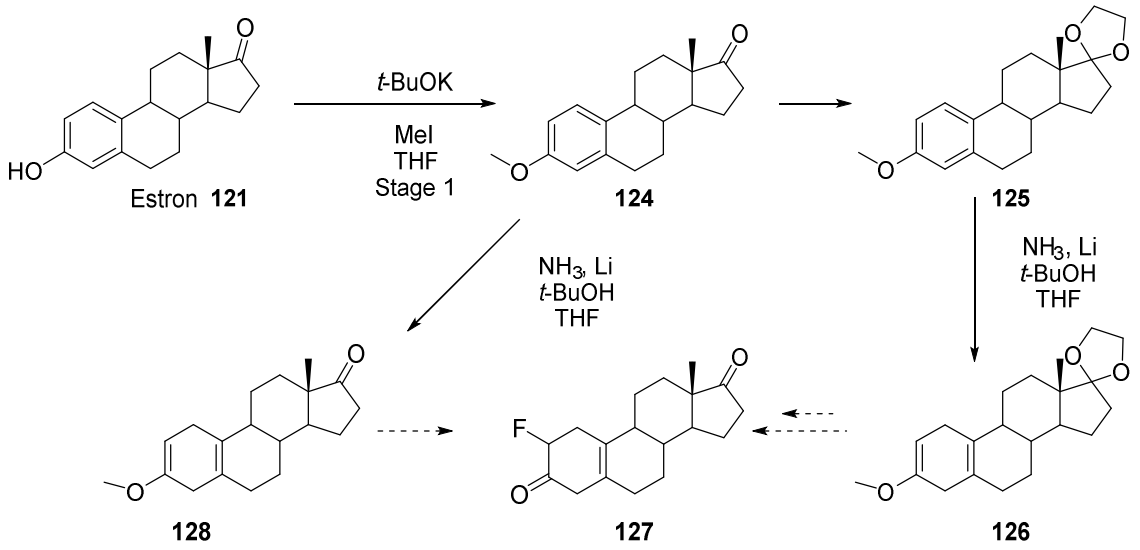
10 123

Scheme 2.19 Synthesis of 10α/β-fluoro-3,17-dihydroxy-1,4-androstene (**123**).

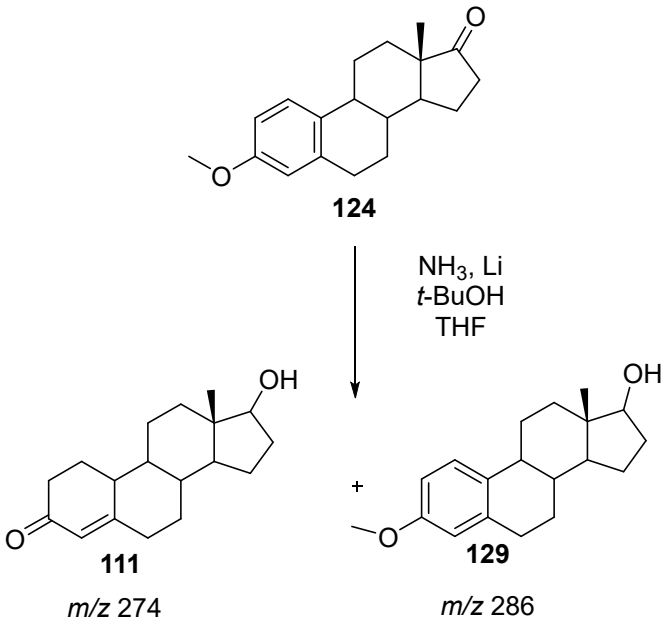
The formation of the alcohol product was confirmed by the ^{1}H NMR spectrum, which showed new signals at δ_H = 2.79-2.83 ppm and δ_H = 3.73 ppm corresponding to C***H***OH protons, and MS data, which showed the appearance of ions at *m/z* 292. The ^{19}F NMR spectrum, showed peaks at δ_F = -144.58 ppm, δ_F = -145.75, δ_F = -157.25 and δ_F = -157.89 ppm, for the fluoro diastereoisomers. Unfortunately, it was not possible to separate the isomers by flash column chromatography.

We also speculated that estrone (**121**) could be converted to the 3-fluoro-androstenedione derivative (**127**). In an effort to achieve this, methylation of estrone was carried out using *t*-BuOK/MeI in THF. The reaction proceeded as expected and gave the desired product (**124**) in a good yield. Evidence for the formation of the **124** was obtained from the ^{1}H NMR spectrum. Compound **124** shows characteristic signals corresponding to the OMe protons at δ_H = 3.78 ppm. The methylated steroid was reduced under Birch conditions using lithium in liquid ammonia. After 20 h, the ^{1}H NMR completion check indicated almost complete consumption of the starting material and a mixture of two major products

(**Scheme 2.21**). A comparison of the ^{1}H NMR spectrum of the starting material and the product showed the appearance of the triplet at δ_H = 3.73 ppm suggesting reduction of the carbonyl group. The presence of the methyl estradiol was also confirmed by GC-MS which showed mass at *m/z* 286. The methyl estradiol was probably further reduced to form compound at *m/z* 274. It was not possible to obtain a pure sample of this material by flash column chromatography, but based on ^{1}H NMR analysis of one of the cleanest column fraction the structure of this product was presented in **Scheme 2.21**.

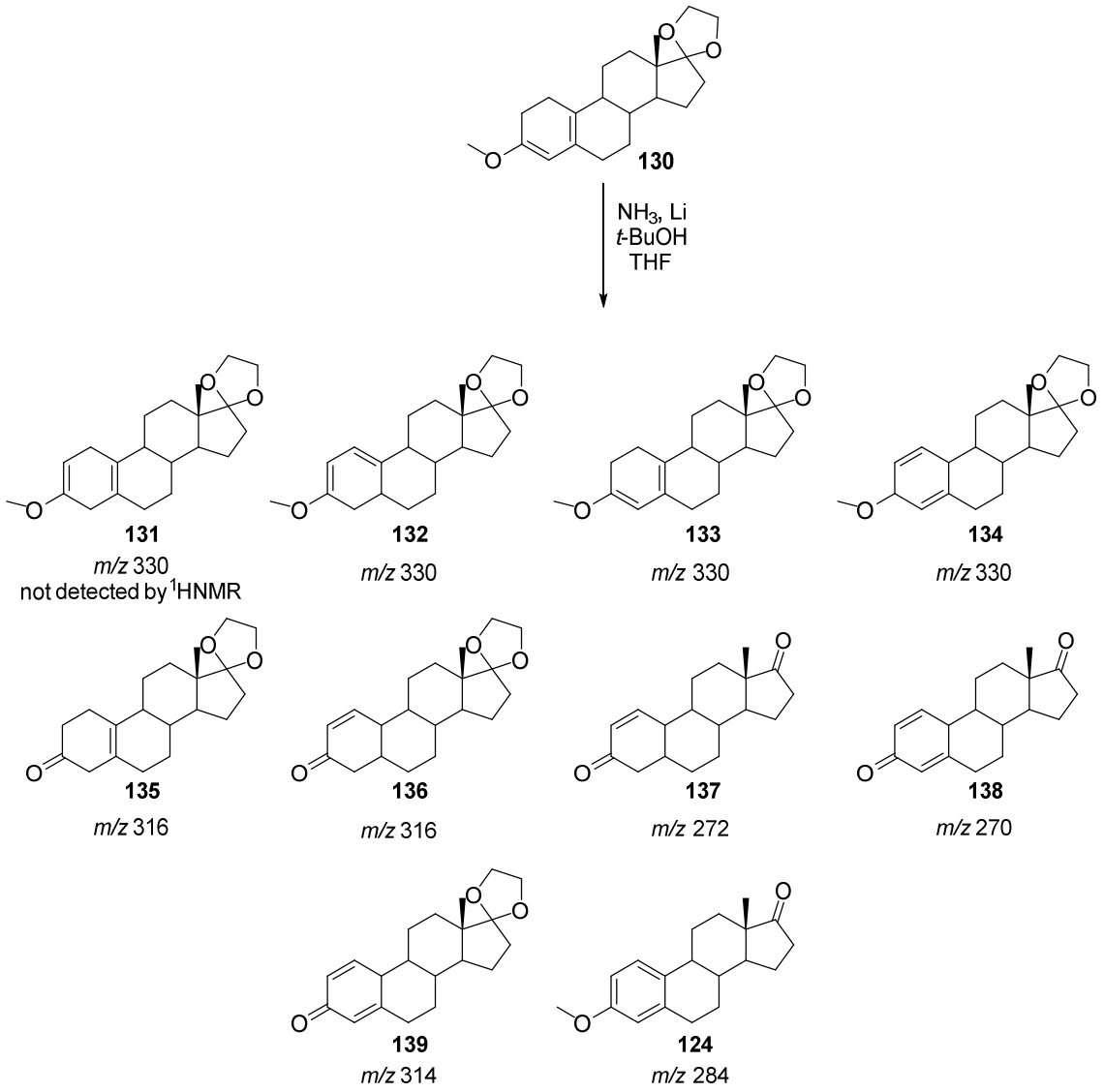


Scheme 2.20 Attempted synthesis of 3-fluoro-androstenedione (**127**).



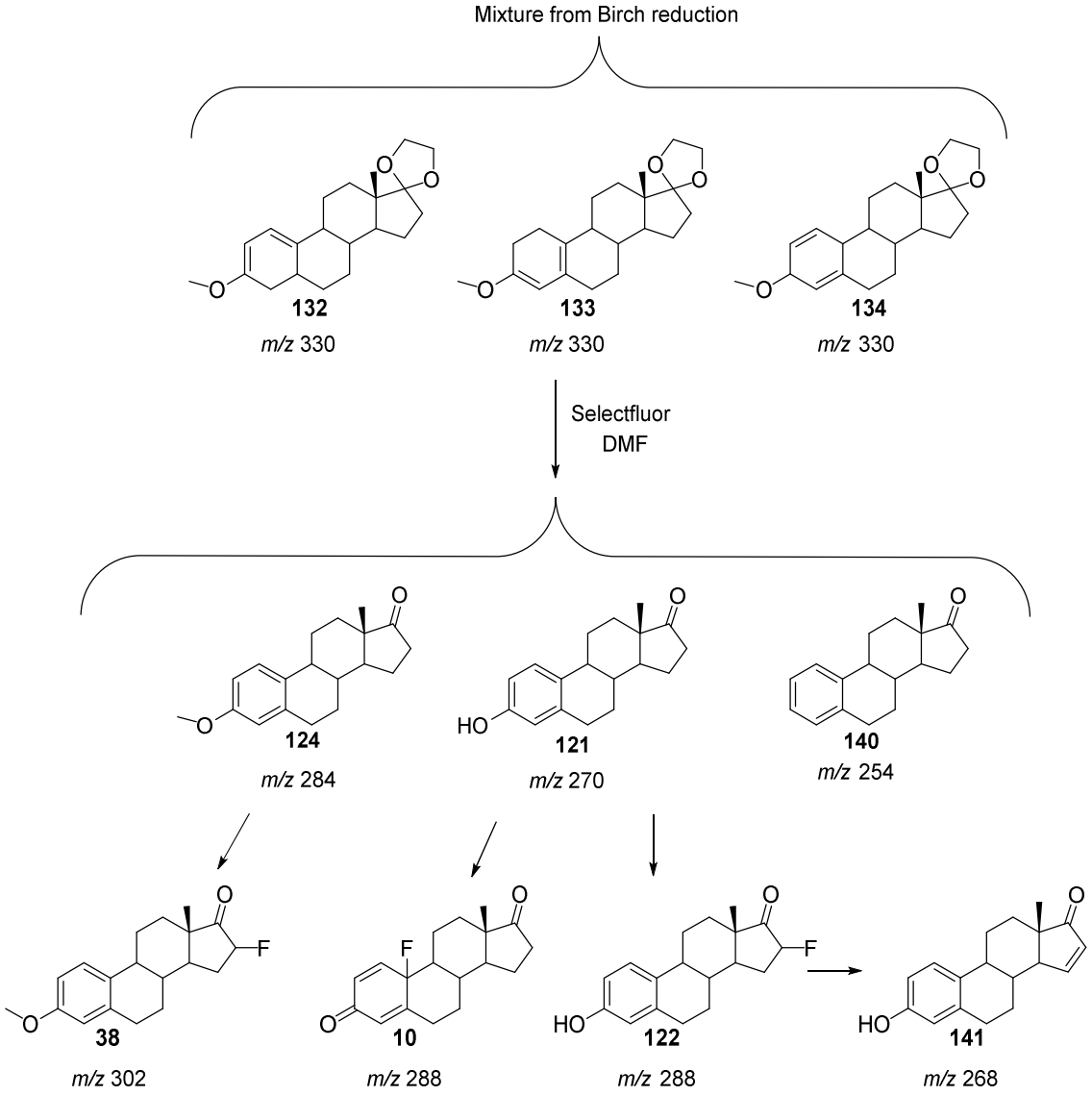
Scheme 2.21 Reduction of methylestrone (**124**).

At this stage it was hypothesised that the reduction profile could be improved by protecting the carbonyl group. This was carried out in a similar fashion to the previously synthesised protected androsterone using ethylene glycol and PTSA. The reaction was successful and gave the product **125** in a 95% yield. The formation of the protected product was confirmed by analysis of the ^{1}H NMR spectrum, which showed peak at δ_H = 3.88-3.98 (4H) for the protecting group. The protected steroid was then reduced under Birch conditions using lithium in liquid ammonia. Based on the ^{1}H NMR and GC-MS completion check after 20h and a mixture of products was obtained (**Scheme 2.22**). The expected product **131** (*m/z* 330), based on the literature procedure, was not detected by ^{1}H NMR. This molecule probably isomerised to the more stable conjugated enol ethers (e.g. **132** and **133**). The crude product was triturated with hexane, acetone and purified on silica this gave a mixture of two products based on GC-MS analysis *m/z* 330 and *m/z* 316. Analysis of ^{1}H NMR suggested a mixture of three major components **132**, **133** and **134** (which has a mass of *m/z* 330). Compound **132** appears to be the major component of this mixture and gives three signals for the enol ether system δ_H = 3.57 ppm (OC**H**$_3$), δ_H = 5.21 ppm (2C-**H**) and δ_H = 5.32 ppm (1C-**H**). Compound **133** gives two signals for the enol ether system δ_H = 3.48 ppm (OC**H**$_3$) and δ_H = 5.83 ppm (4C-**H**). Compound **134** appears to be a minor component of this mixture and gives four signals for the steroidal A ring δ_H = 3.24 ppm (OC**H**$_3$), δ_H = 6.17 ppm (4C-**H**), δ_H = 6.63 ppm (2C-**H**) and δ_H = 7.14 ppm (1C-**H**). Compound **135** could be another component of this mixture detected by GC-MS (*m/z* 316). This molecule does not give any signal in the aromatic/double bond region.



Scheme 2.22 Suggested structures of the products arising from the Birch reduction based on GC-MS and ^{1}H NMR data.

The mixture of products obtained from the Birch reduction was taken through the next stage of the synthesis. It was hoped that after the fluorination reaction the main product could be separated from the mixture. The reaction was carried out in DMF and Selectfluor® at RT for 48 h (**Scheme 2.23**). After that time ^{1}H NMR and GC-MS indicated formation of a mixture of products with two major components **140** (*m/z* 254) and **124** (*m/z* 284). In addition, ^{1}H NMR suggested that all products lost the glycol protecting group and that the majority of products contain an aromatic ring. The aromatisation reaction probably occurred in a stepwise manner via fluorination of the starting material and elimination of HF. Small quantities of fluorinated products were also detected. These were probably formed through the fluorination of methylestrone **124** and estrone **121**.

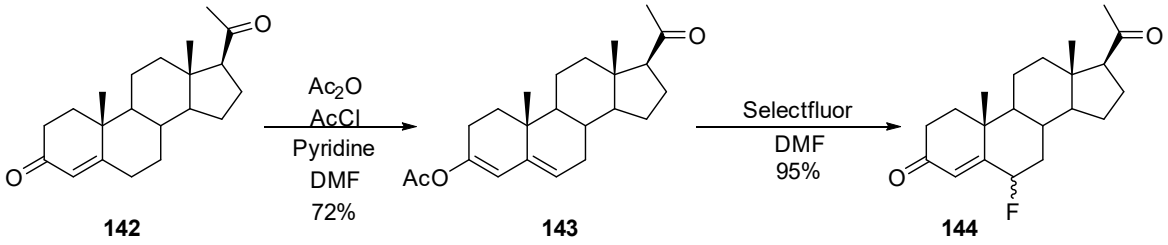


Scheme 2.23 Potential products (**38**, **10**, **116**, **134**) obtained after fluorination of a mixture obtained from Birch reduction (**132**, **133**, **134**).

2.2.9 Synthesis of Fluorinated Progesterone

Following the protocol developed previously for the fluorination of testosterone the synthesis of 6α/β-fluoro-progesterone (**144**) was carried out in two steps (**Scheme 2.24**). During the first stage progesterone (**142**) was treated with AcCl and pyridine in DMF. The reaction proceeded as expected and the conjugated enol ester was obtained in 72% yield. Evidence for the formation of the acylated progeserone (**143**) was provided by the ^{1}H NMR spectrum, in which a characteristic signals corresponding to the conjugated double bonds at δ_H = 5.39 ppm and δ_H = 5.69 ppm were observed. Additional evidence for the formation of **143** was found in the GC-MS spectrum where the signal corresponding to the correct

mass was detected (*m/z* = 356). Acyl protected progesterone (**143**) was then treated with Selectfluor® to give the corresponding 6-fluoro-steroid **144** in a 95% yield. The formation of this product was confirmed by analysis of the ^{19}F NMR spectrum, which showed peaks at δ_F = -161.41 ppm and δ_F -184.19 ppm for the α and β isomer respectively. Structures of **143** and **144** were confirmed by X-ray crystallography (**Figure 2.10** and **Figure 2.11**)



142 **143** **144**

Scheme 2.24 Synthesis of 6α/β-fluoro-progesterone (**144**).

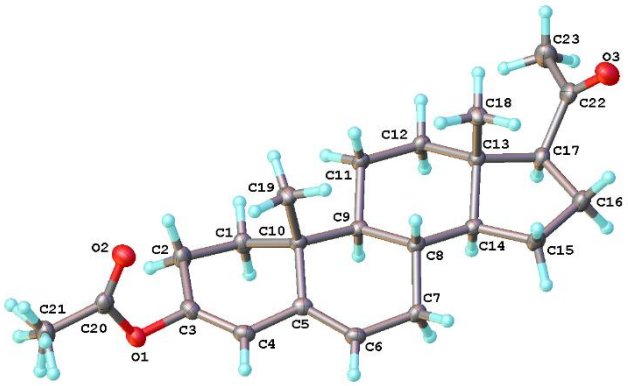
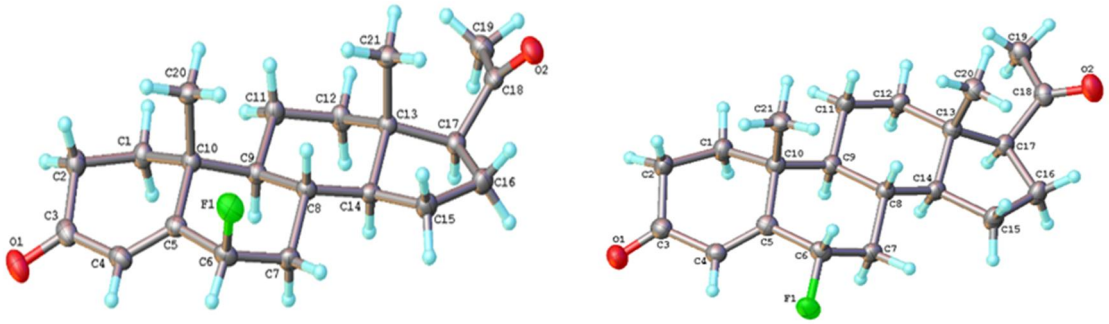


Figure 2.10 X-ray structure of acyl protected progesterone (**143**).



6β-fluoro-progesterone 6α-fluoro-progesterone

Figure 2.11 X-ray structures of isomers 6β-fluoro-progesterone and 6α-fluoro-progesterone (**144**).

2.3 Conclusions

The work described in this chapter was focused on the synthesis of fluorinated steroids using methods that involve electrophilic fluorination. Selectfluor® was chosen as the optimum reagent, and a process was developed using that reagent. This was based on the experience within the group and the literature precedents. It was reported that *N*-

fluoropyridinium salts requires high temperatures or long reaction times for complete reaction and usually low yields are observed for steroids 3,5-dienol acetates.[14] On the other hand, the neutral *N*-fluorosulfonimides gives usually high β-fluoro selectivity and moderate yields for these steroids.14 This could be explained by stereoselective elimination of HF from the α-fluoro steroid isomer. The elimination process is much slower for Selectfluor®. In comparison to gaseous F_2, the key benefit of this electrophilic fluorination agent is the ability to provide facile access to fluorinated steroids without the need to use specialised equipment for F_2 manipulation. The optimised, standard protocol for the synthesis of fluorinated steroids involved the preparation of the reaction of steroid with Selectfluor® in DMF or MeCN at RT. Fluorinted-testosterone (**84**), fluorinated-androstenedione (**5**), fluorinated-androstenediol (**110**), fluorinated-notestosterone (**86**), fluorinated-norandrostenediol (**85**), fluorinated-progesterone (**144**) and, fluorinated-estrone (**10**) derivative were synthesised using this method in good yield (83%) and moderated diastereoselestivity. Fluorinated-androsterone (**87**) was synthesised using Selectfluor® under acidic conditions in methanol at 50ºC (93%).

In addition, several novel X-ray structures of fluorinated steroids were obtained (e.g: 6α-fluoro-testosterone **Figure 2.12**). Experiments with gaseous fluorine were also carried out, however, due to limited access to the fluorination equipment fluorination of steroids with F_2 was not fully explored.

With a range of fluorinated steroids in hand, attention then turned to their metabolism by microorganisms (**Chapter 3**).

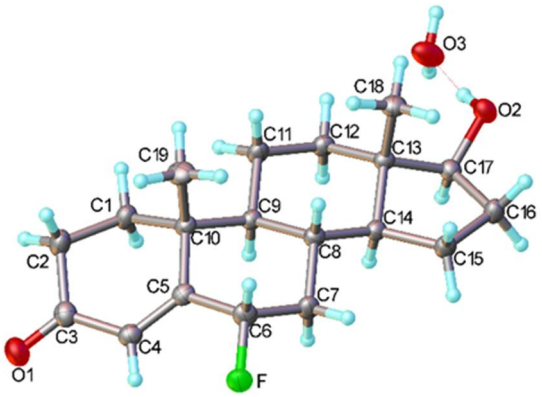


Figure 2.12 X-ray structures of 6α-fluoro-testosterone (**84**).

2.4 Reference

[1] G. E. Turfitt, *Biochem. J.*, **1948**, 42 (*3*), 376.
[2] S. Kuwada, T. Joyama, *J. Pharm. Soc. Jpn*., **1937**, *57*, 247.
[3] Y. S. Choe, J. A. Katzenellenbogen, *Steroids*., **1995**, *60*, 414.
[4] T. Umemoto, S. Fukami, G. Tomizawa, K. Harasawa, K. Kawada, K.Tomita, *J. Am. Chem. Soc.* **1990**, *112*, 8563.
[5] J. Liu, J. Chan, C. M. Bryant, P. A. Duspara, E. E. Lee, D. Powell, H. Yang, Z. Liu, C. Walpole, E. Roberts, R.A. Batey, *Tetrahedron Lett*., **2012**, *53*, 2971.
[6] B. Z. Leder, C. Long Cope, D. H. Catlin, B. Ahrens, D. A. Schoenfeld, J. S. Finkelstein, *JAMA;* **2000**, *283*, 779.
[7] M. L. S. Almeida, P. Kocovsky, J. Backvall, *J. Org. Chem.*, **1996**, *61*, 6587.
[8] V. D. Pavlović, M. M Dabović, S. Martinović, L. B Lorenc, J. Kalvoda, *J. Serb. Chem. Soc.*, **2004**, 69(*11*), 861.
[9] P. Langer, *J. Prakt.Chem.*, **2000***, 342,* 7.
[10] C. Rogers, *Synth. Commun.*, **2002**, 32(*19*), 2991.
[11] Fukushima, Dobriner, *J. Org. Chem*., **1961**, *26*, 3022.
[12] Liu, Carlson, Katzenellenbogen, *J. Med. Chem*., **1992**, *35*, 2113.
[13] S. Stavber, M. Jereb, M. Zupan, *Synlett*, **1999**, *9*, 1375.
[14] V. Reydellet-Casey, D.J. Knoechel, P.M. Herrinton, *Org. Process Res. Dev.*, **1997**, *1*, 217.

3 Biotransformations of Steroids

3.1 Introduction

Organofluorine compounds are virtually absent in nature and to date, only a handful of naturally occurring fluorinated metabolites have been identified in plants or microorganisms (**Figure 3.1**).[1,2] In addition, there are currently no reports of the biosynthesis of fluorine containing molecules in animals. The first organofluorine compound reported in the literature was fluoroacetate **145**, which was isolated by Marais in 1943, from the shrub *Dichapetalum cymosum*.[1,2] It was found that this plant is toxic to animals due to the high level of **145** that accumulates in its leaves.

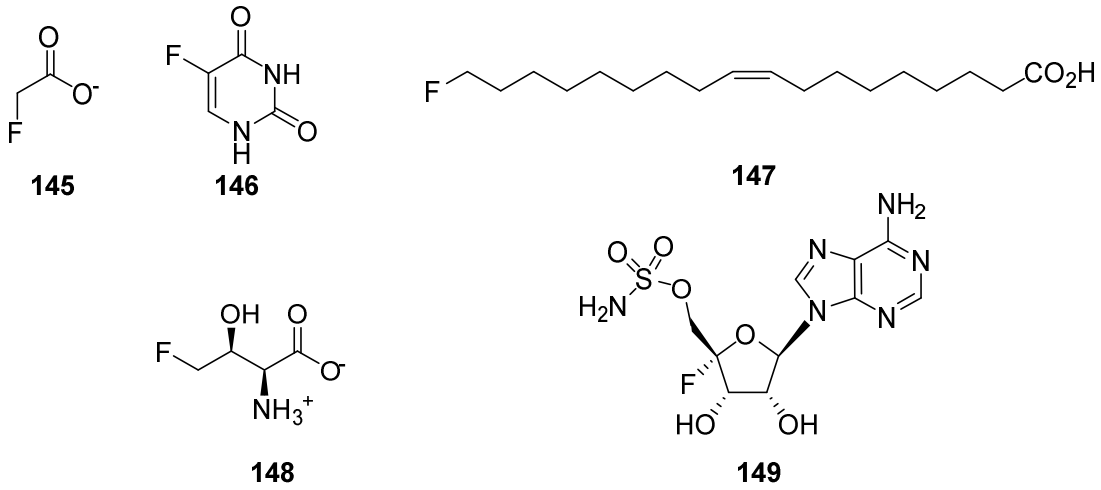


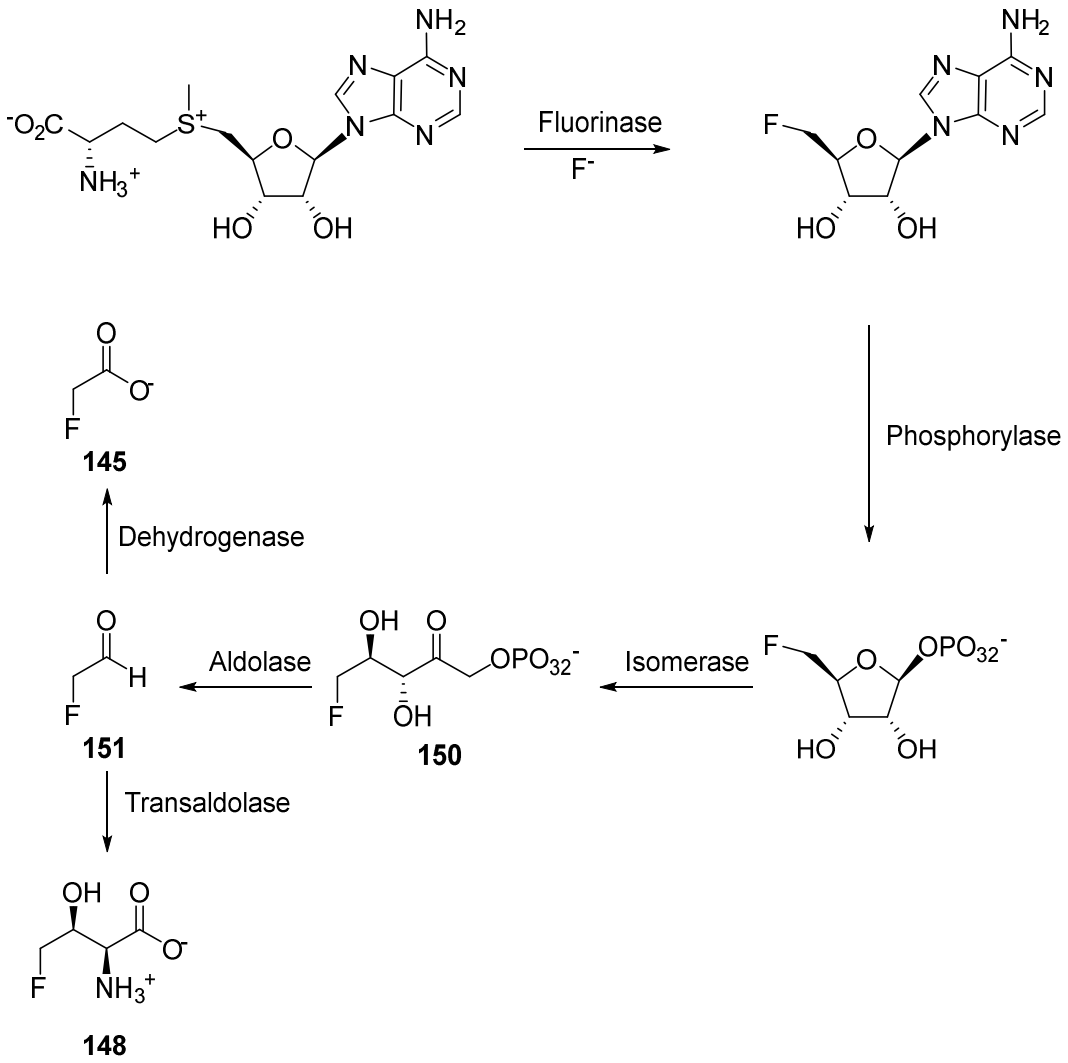
Figure 3.1 Fluorinated metabolites isolated from natural sources.

It is believed that fluoroacetate **145** is a building block of ω-fluorooleic acid **147** isolated by Peters *et al*. from the shrub *Dichapetalum toxicarium*.[3] More recent studies on this plant, by Hamilton *et al.* have resulted in the discovery of other ω-substituted fluoro acids.[4]

The identification of fluorine containing metabolites from microorganisms is equally rare. The bacterium *Streptomyces calvus* was the first reported microorganism found to be capable of biosynthesising a fluoroorganic natural product. The research on this bacterium carried out by Thomas *et al*. resulted in the isolation of nucleocidin **149** in 1957.[5] Interestingly the structure of nucleocidin (**149**) was not fully elucidated until 1969 when the first total synthesis was completed.[6] For over 20 years attempts to repeat biosynthesis of nucleocidin **149** have been unsuccessful. However, recently it has been shown by

O'Hagan group that publicly deposited strain has lost the ability to produce the **149**. It has been reported that a mutation of the *bldA* gene is responsible for the loss of ability to form C-F bond by *S.calvus*.[7] O'Hagan group used an in-house strain of *S.calvus* T-3018 held by Pfizer. The strain T-3018 does not have the mutation and is able to produce nucleocidin **149**.

Currently, *Streptomyces catteleya*, *Streptomyces sp.* MA37, *Streptomyces xinghaiensis* are the only reported microorganisms known to produce secondary metabolites that contain a C-F bond.[8,9] The initial research on this *Streptomyces* spp. was carried out by Sanada and co-workers in 1986.[10] They discovered that in a fluoride containing environment (KF) *S. cattleya* produces the metabolites fluoroacetate **145** and 4-fluorothreonine **146**. Subsequent detailed studies performed by the O'Hagan group have identified not only the first ever fluorination enzyme, the fluorinase, but also all of the intermediates on the biosynthetic pathway from inorganic fluoride (KF) to the secondary metabolites fluoroacetate **145** and 4-fluorothreonine **148** (**Scheme 3.1**).[11,12]



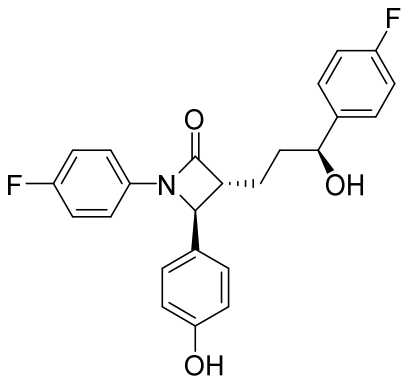
Scheme 3.1 The biosynthetic pathway to fluoroacetate **145** and 4-fluorothreonine **148** from inorganic fluoride (KF).

A wide range of fluorinated amino acids can be prepared using micro-organisms. This approach was used to synthesise a fluorinated lipase by Markel *et al.*[13] ME5355 strain of *E. coli* was incubated in a medium containing three different fluorinated amino acids. This led to incorporation of these amino acids at 24 positions of the lipase enzyme in a single experiment. Interestingly this modification did not abolish the lipase enzyme activity.

One could also achieve incorporation of fluorinated amino acids into proteins by having the amino acid present in the diet of an organism. For example, Gerig carried out ^{19}F NMR study on cyanomethemoglobin prepared from haemoglobin isolated from rabbits fed with D,L-4-fluorophenylalanine.[14] In this experiment, the amino acid was inserted randomly in all phenylalanine positions of α- and β-globin chains.

Unfortunately, the above methods for the preparation of fluorinated proteins have a disadvantage. During the biosynthesis, fluorinated amino acids are inserted at all locations in the sequence occupied by residues of a given kind of amino acid. Therefore, if the synthesis of a protein with a single position in the sequence occupied by a fluorinated amino acid is required, chemical synthesis has to be carried out.

Despite the fact that only a few naturally occurring fluorinated metabolites have been identified in plants or microorganisms, over 30% of currently available and commercially valuable pharmaceuticals contain a fluorine atom. Indeed, a common strategy for modifying drug stability, lipophilicity and increasing the half-life of a drug *in vivo* is to introduce a fluorine atom.[15,16,17,18] An example of how dramatic this approach can be seen in the development of the cholesterol uptake inhibitor drug Ezetimibe (**153**) (**Figure 3.2**). Fluorination of the phenyl ring, prevents oxidation to the corresponding phenol derivative and substitution of a methoxy group with fluorine prevents demethylation. These changes led to significantly reduced metabolic degradation and a 400-fold increase in activity.[19,20]



Ezetimibe (**153**)

Figure 3.2 An example of commercial pharmaceutical Ezetimibe (**153**) containing fluorine.

3.2 Drug metabolism

Before a drug can be approved for use in humans, extensive screening has to be performed to establish its safety. One of the important factors in this evaluation process is the knowledge of a drug's metabolism. Drug metabolism studies are generally based on the use of tissue culture (*in vitro*) or small animal models (*in vivo*). Traditionally, *in vivo* studies have involved administration of the drug to laboratory animals and subsequent examination of its fluids and tissues for the presence of the parent drug and its metabolites. In many cases this is very challenging as metabolites are usually present in only very small

quantities making them difficult to isolate and identify. Once the metabolite is isolated, identification is usually accomplished using sensitive spectroscopic techniques such as GC-MS, LC-MS, ^{1}H NMR. It was shown by Smith and Rosazza[45] that these studies are expensive and in many cases could be replaced by the use of microbial systems. Information gained from drug metabolism studies is usually used to increase efficiency of the drug, selectivity, duration of action or decreasing its toxic side effect. However, information gathered via studies of this kind information could be also used to identify banned substances taken by an athlete.

3.2.1 Metabolism studies in mammalian system

Most drugs entering the mammalian system are altered biochemically. Metabolic transformations are usually classified as either Phase I or Phase II. Phase I includes oxidation, reduction and hydrolysis reactions, while Phase II involves conjugation reactions (e.g. glucuronidation, acetylation, glutathione conjugation, glycine conjugation). These reactions generally convert drugs into more polar substances which can be readily excreted from the organism. Metabolites are usually less active than the parent drug; however enhancement of the biological activity is also possible (**Figure 3.3**).[45,21] Some complex molecules usually have tens of metabolites which were formed by sequential or parallel biotransformations. These significantly complicate metabolic studies.[22]

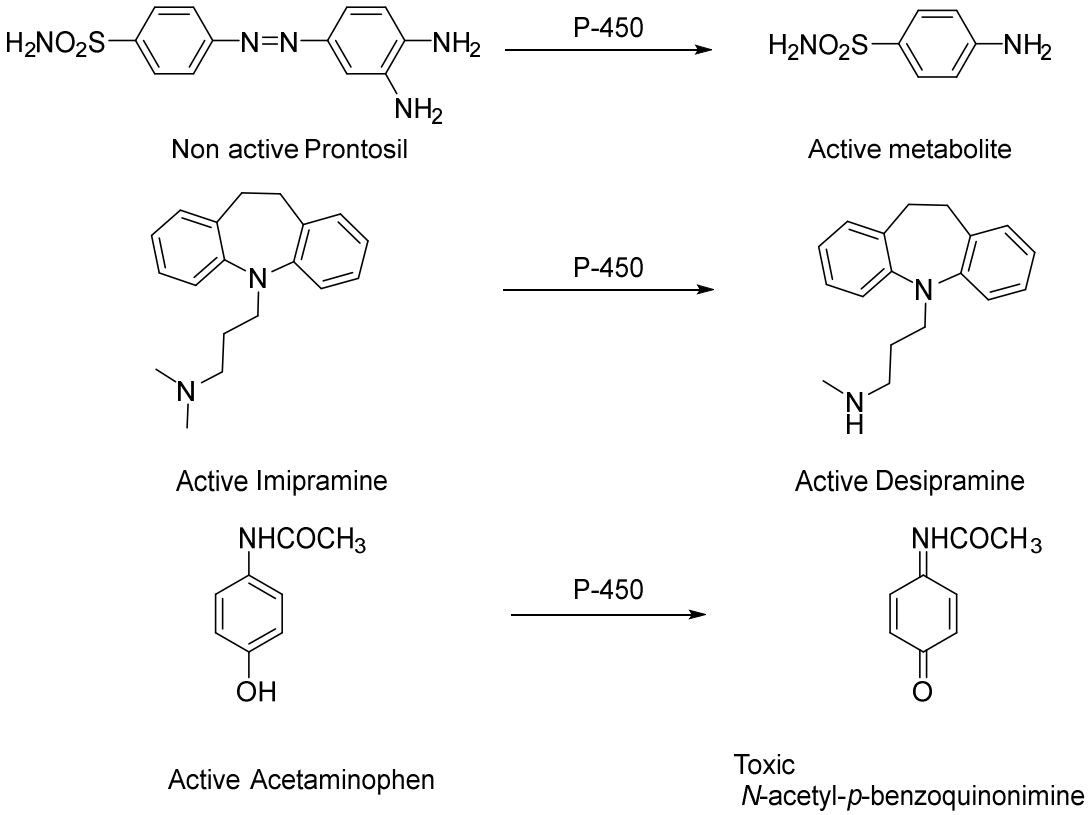


Figure 3.3 Example of metabolites more active than the parent drug - enhancement of the biological activity.45

An important reaction in the biotransformation process is the oxidation reaction. To understand the basic mechanism of this reaction numerous studies have been conducted since 1962. New classes of enzymes participating in the oxidation process have been discovered, including cytochrome P-450 enzyme systems, flavoprotein oxygenases, and α-ketoglutarate-requiring oxygenases. Majority of oxidation reaction are catalysed by cytochrome P-450, which is located mainly in the liver of mammals but also in the kidneys, lungs, small intestine, spleen, testes, ovary and placenta (**Table 3.1**).[23] It is believed that cytochrome P-450 catalyses hydroxylation, oxidation, dealkylation and deamination reactions. In most cases, this results in the formation of unstable intermediates that spontaneously decompose to the stable metabolites. All of the oxidative reactions, except oxidative deamination, that occur in mammalian tissue can be duplicated *in vitro* with microsomal fractions.[24] Microsomal fractions are form as microglobular bodies during disruption of the endoplasmic reticulum of cells. Enzymes associated with oxidation of substances appear to show a low degree of substrate specificity. However, within certain classes of compounds oxidation could be selective. This could be difficult to interpret

because a compound could be oxidised by different enzymes or different amounts of enzyme.

Table 3.1 Examples of reactions catalysed by cytochrome P-450.

No.	Reaction name	Substrate	Intermediate	Product
1	Hydroxylation	Alkanes	-	Alcohols
		Aromatic compounds	Epoxide	Phenols
2	*N*-Dealkylation	Secondary and tertiary amines	α-Hydroxyamine	Aldehyde and primary or secondary amine
3	*O*-Dealkylation	Ethers	α-Hydroxyether	Aldehyde and alcohol
4	*S*-Dealkylation	Sulfide	α-Hydroxysulfide	Aldehyde and thiol
5	*S*-Oxidation	Sulfide	-	Sulfoxide
		Sulfoxide	-	Sulfone
6	*N*-Oxidation	Primary and secondary Amines	-	Hydroxyamine
		Primary and secondary Amide	-	Hydroxyamide
		Tertiary amine	-	N-oxide
7	Deamination	Primary and secondary Amines	α-Hydroxyamine	Aldehyde and ammonia or primary amine
8	Dehydrogenation	Primary alcohol	-	Aldehyde
		Secondary alcohol	-	Ketone
		Aldehyde	-	Carboxylic acid
		trans-Dihydrodiol	-	Catechol

Studies on *Pseudomonas putida* showed that the active site of cytochrome P-450 contains an iron hem with two axial ligands (**Figure 3.4**). The first axial ligand is a thiolate group that is attached to the nearby protein, while the second axial ligand varies depending on the enzymatic cycle.[25] The second ligand is displaced by oxygen during the catalytic cycle.

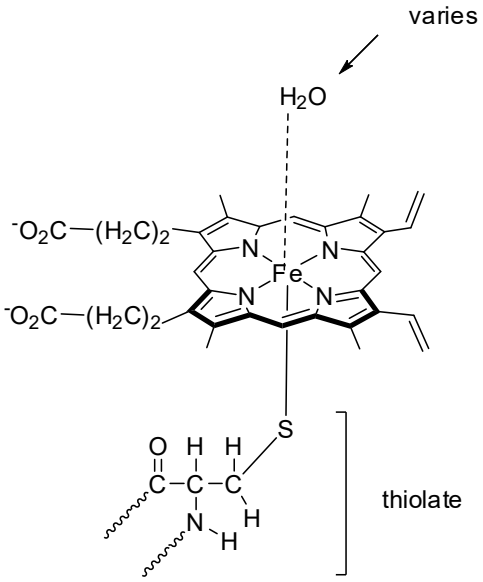
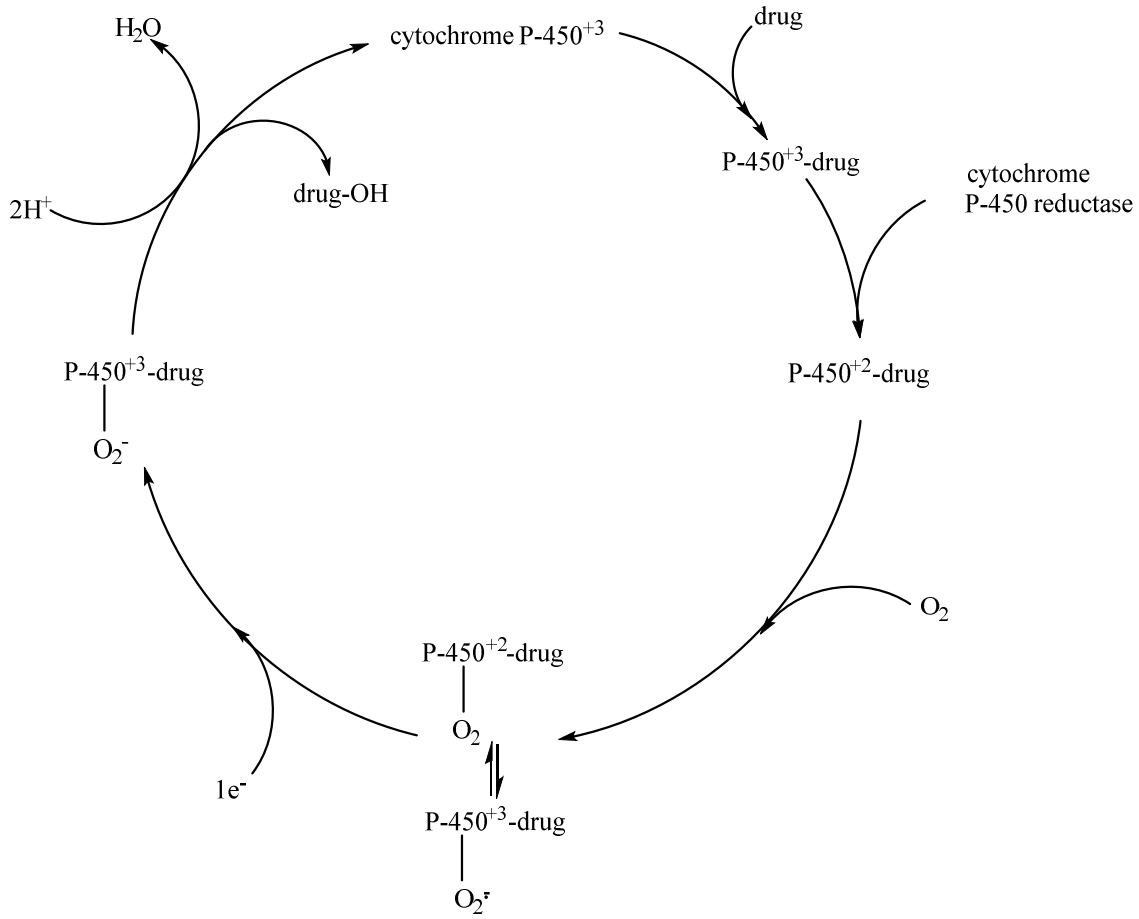


Figure 3.4 Active site of cytochrome P-450. [24]

Based on the mammalian and microbial studies, the catalytic cycle of cytochrome P-450 was deduced (**Scheme 3.2**).[26] In the first step, substrate binds to the apoprotein, which is in close proximity to the cofactor. This induces a change in the conformation of the active site, often displacing a water molecule and changing the state of the heme iron (Fe^{+3}) from low-spin to high-spin. Depending on the source of cytochrome P-450, a flavin nucleotide, iron/sulphur proteins and/or cytochrome b_5 are providing two equivalents of the reducing agent to the cofactor from NADPH. The resulting complex undergoes rapid reduction *via* cytochrome P-450 reductase followed by formation of a reduced cytochrome-substrate-oxygen complex. Subsequently the oxygen is reduced by cytochrome iron, and the resulting complex undergoes further reduction and disproportionation with the transfer of one oxygen to bound substrate and protonation of the second oxygen. Finally, the complex breaks down to water, oxidised drug and oxidised cytochrome P-450.



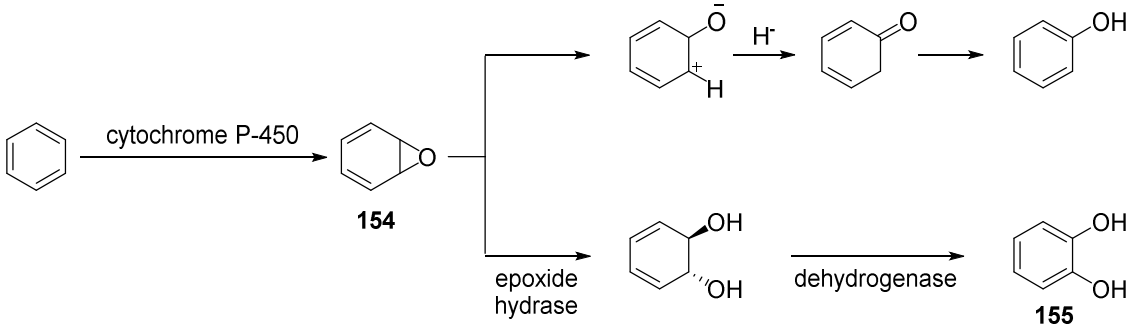
Scheme 3.2 The catalytic cycle of cytochrome P-450**.**

Given the huge diversity of P-450 systems generated from mammalian and microbial origin, a nomenclature system has been developed to categorize individual CYPs. The system was developed by Dr. David Nelson and it is based upon sequence identity where P-450s are grouped into families (1, 2, 3...), subfamilies (A, B, C...) and a number for each individual P-450 (1, 2, 3 ...), e.g. CYP105A1.[27] Currently there are > 10 thousand CYP gene sequences in genomic data bases, and of these sequences > 30% are of microbial origin. P-450s can be also divided into four classes depending on how electrons from NAD(P)H are delivered to the catalytic cycle:

- Class I proteins needs FAD containing reductase and an iron sulphur redoxin
- Class II proteins require FAD/FMN containing P-450 reductase for transfer of electrons
- Class III enzymes do not require an electron donor
- Class IV electrons are obtained directly from NAD(P)H

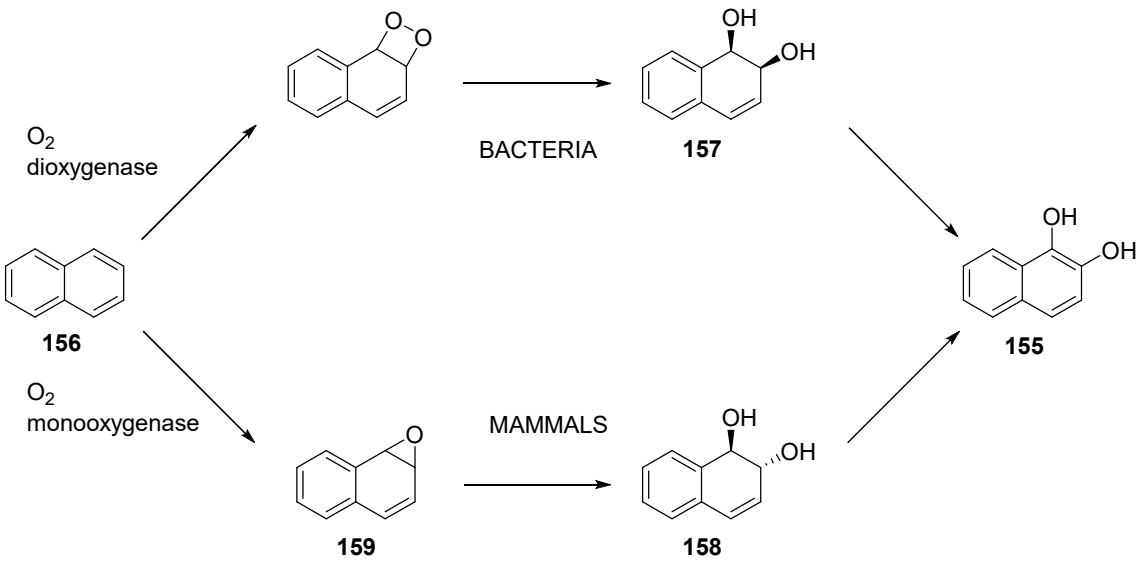
Humans have 57 genes and more than 59 pseudogenes divided among 18 families of cytochrome P-450 genes and 43 subfamilies. However, only 7 known isoforms are responsible for more than 90% of the metabolism of all pharmaceuticals currently in use.[28] For example CYP1 CYP2 and CYP3 are responsible for drug and steroid metabolism. CYP11, CYP17, CYP19, CYP21 are involved in the biosynthesis of steroids.

Aromatic compounds are often oxidated to phenols in mammalian systems. This reaction is similar to electrophilic aromatic substitution. Activated electron rich aromatic rings are easily hydroxylated; whereas rings bearing electron withdrawing groups are hydroxylated slowly or not at all. Furthermore, it was observed that the hydroxyl group is introduced in *ortho* and *para* positions to the activating group, while deactivated rings are hydroxylated in the *meta* position.[29,30,31] Aromatic hydroxylation involves formation of an intermediate epoxide (**154**) which then can decompose to the corresponding phenol via non enzymatic rearrangement. Alternatively the epoxide can be transformed to the catechol (**155**) through epoxide hydrase and dehydrogenase (**Scheme 3.3**).[32,33,34] More stable epoxides such as naphthalene-1,2-oxide may participate in an alkylation reaction with cellular components. This process is responsible for toxicity of certain aromatic compounds.[35,36]



Scheme 3.3 Biotransformation of aromatic compounds.[37]

In bacteria, the oxidation reaction is catalysed mainly by dioxygenase (**Scheme 3.4**).[38] In contrast to mammalian systems the oxidation process proceeds through *cis*-dihydrodiol intermediate (**157**). This was isolated by Jerina and characterised by NMR spectroscopy.[39] The catechol (**155**) is further deprotonated to carbon dioxide and water in bacteria. Some microorganisms such as *Pseudomonas* species,[40] *Claviceps purpurea*[41] and yeasts[42] oxidise aromatic compounds via an epoxide intermediate (**159**).



Scheme 3.4 Oxidation of naphthalene (**156**) in bacterial and mammalian systems.

The microbial systems demonstrated substantial capacity to hydroxylate simple aromatic compounds. It was shown by Smith and Rosanzza that in general, a good correlation could be obtained between the mammalian and microbial systems (**Table 3.2**).[43,44,45] Nitrobenzene was the only substrate which did not yield a hydroxylated product in microbial systems.[45] This compound gave 2-, 3-, and 4-hydroxylated product in mammalian systems. The relative reactivities of simple aromatic compounds toward microbial hydroxylases follow the order NH_2>OMe, CH_3>H, Cl, $NHCOCH_3$>NO_2.[46] Interestingly, it was found that comparison between *in vivo* and *in vitro* metabolic systems is not always valid for simple aromatic compounds.

Table 3.2 The hydroxylation of simple aromatic compounds by microbial and mammal an metabolites systems.

No	Substrate	Microbial metabolite[1]	Mammalian metabolites	
			In vitro	***In vivo***
1	Acetanilide	2-Hydroxyacetanilide, Aniline (*a, c, d, h*)	2- and 4-Hydroxyacetanilide, Aniline	4-Hydroxyacetanilide, Aniline
2	Acronycine	9-, 11-, 9- and 11-hydroxyacronycines (*b, h, i*)	N/A	9-, 11-, 9-and 11-hydroxyacronycines
3	Aniline	Acetanilide; 2-Hydroxyacetanilide, 4-Hydroxyaniline (*b, c, i*)	4-Hydroxyaniline	Acetanilide; 2-, 3- and 4-Hydroxyanilines
4	Anisole	2- and 4- Hydroxyanisoles, Phenol (*a, b, c, d, e, f, i*)	2- and 4- Hydroxyanisoles, Phenol	2- and 4- Hydroxyanisoles,
5	Benzene	Phenol (*a, b, d, e, i*)	Phenol	Phenol
6	Benzoic acid	2- and 4-Hydroxybenzoic acids, 3,4-dihydroxybenzoic acid (*c, g, i*)	3-Hydroxybenzoic acid	2-, 3- and 4-Hydroxybenzoic acids
7	Biphenyl	2- and 4-Hydroxybiphenyls, 4,4-dihydroxybiphenyl (*b, e, g, i*)	2- and 4-Hydroxybiphenyls	4-Hydroxy-, 3,4-dihydroxy-, 4,4'-dihydroxybiphenyls
8	Chlorobenzene	2- and 4-Hydroxychlorobenzenes (*c, d, e, f, i*)	2-, 3- and 4-Hydroxychlorobenzenes	2-, 3- and 4-Hydroxychlorobenzenes
9	Coumarin	7-Hydroxycoumarin (*d, e, i*)	7-Hydroxycoumarin	3-, 4-, 5-, 6-, 7- and 8-Hydroxycoumarins
10	Naphthalene	1- and 2-Hydroxynaphthalenes (*b, c, e, f, i*)	1- and 2-Hydroxynaphthalenes	1- and 2-Hydroxynaphthalenes
11	Nitrobenzene	-	4-Hydroxynitrobenzene	2-, 3- and 4-Hydroxynitrobenzenes; 2-, 3- and 4-Hydroxyanilines
12	*trans*-Stilbene	*trans*-4-Hydroxy- and *trans*-4,4'-dihydroxystilbenes (*b, c, e, f, i*)	*trans*-4-Hydroxy- and *trans*-4,4'-dihydroxystilbenes	*trans*-4-Hydroxy-, *trans*-3,4-dihydroxy- and *trans*-4,4'-dihydroxystilbenes
13	Toluene	2- and 4-Hydroxytoluenes (*a, c, e, f, i, h*)	2- and 4-Hydroxytoluenes; benzyl alcohol	Benzoic acid and its conjugates

[1]Microorganisms used: a) *Penicillium chrysogenum, b) Cunninghamella blakesleeana, c) Aspergillus ochraceous, d) Gliocladium deliquescens, e) Streptomyces* species*, f) Rhizopus stolonifer, g) Curvularia lunata, h) Streptomyces rimosus, i) Cunninghamella bainieri*.

Hydroxylation of sp^3 carbons next to nitrogen, oxygen or sulphur generally leads to dealkylation due to the instability of the hydroxyl intermediate. Tertiary amines substituted with two methyl groups are often quickly dealkylated to the corresponding secondary amines, which subsequently are dealkylated to the primary amines much more slowly.[47] *N*-demethylation rate is proportional to lipid solubility and inversely proportional to pka value.[48] An *n-tert*-butyl group is cleaved by a sequence of oxidations reactions beginning with hydroxylation of methyl group.[49] This type of the oxidation becomes probably more important as the size of the *N*-alkyl function increases. In a similar fashion *O*-alkyl groups can be removed during drug metabolism. It was shown by Axelrod that aromatic *para*-substituted ethers are more easily cleaved than their corresponding *ortho*- and *meta*-analogs.[50] Some substituted ethers such as the dimethylaminoethyl ether appears to be more resistant to oxidation and are often incorporated into drug molecules (e.g. diphenhydramine).[51] *S*-Dealkylation has not been extensively investigated, because thioethers are not widely incorporated into drug molecules. However it has been suggested that this process proceeds via similar mechanism to *N*- and *O*-dealkylation.[52] It was also found that sulfides in heterocyclic systems are more prone to *S*-oxidation than *S*-dealkylation.

3.2.2 Metabolism studies in microbial systems

During metabolism studies, several practical problems are usually encountered: development of a suitable analytical method to detect drug/metabolites in a biological matrix; comparison of results from metabolic studies between different species; isolation of sufficient amounts of metabolites for characterisation. The last problem could be addressed through preparative synthesis, although this is usually expensive and time consuming. An alternative solution was proposed by Smith and Rosazza who suggested that it is often possible to find microbial systems which mimic biotransformations observed in humans.[45] These systems are called "microbial models of mammalian metabolism". It is important to realise that it is very unlikely that a single microorganism can perform the same transformations as the mammalian system. However, if common metabolites are detected in mammalian and microbial system, the microbial system could be used to obtain gram quantities of metabolites via routine fermentation scale-up techniques.

Many bacterial species have been identified with a few species now having their complete genomes sequenced. Most actinobacteria, particularly streptomyces and mycobacteria, encode usually large numbers of CYP genes (e.g. *Streptomyces avermitilis* 33, *Streptomyces scabies* 25, *Mycobacteriumvanbaalenii* 51).[53] A substantial number of actinobacterial CYPs are associated with pathways encoding compounds which have been applied in human medicine. For instance, *Streptomyces* cytochrome P-450 enzyme systems catalyse many transformations of xenobiotics. These oxidative transformations have been studied with alkaloids,[54] coumarins[55] and other molecules. The most significant of these biotransformations are aliphatic and aromatic hydroxylations, *N*-oxidation and *O*- and *N*-dealkylations (*N*-oxidation, *O*- and *N*-dealkylations are usually an effect of α hydroxylation). These have been exploited by industry in the synthesis of pravastatin,[56] 16-hydroxylation of steroids[57] and in the preparation of metabolites for toxicological studies. In spite of the relatively widespread application of microbial hydroxylation, it is only in recent years that the mechanism of this reaction has been understood to some extent. The majority of work on the hydroxylation reactions has been performed using growing or resting cultures of fungi. This is attributed to the fact that isolation of enzymes might be difficult due to their instability. From the available data, it was suggested that all hydroxylating enzymes contains cytochrome P-450 species. These enzymes are classified as monooxogenases because there are capable of incorporation of one molecule of molecular oxygen. In addition to that, they are widely distributed among almost all forms of life and based on available data they function by a similar mechanism. Hydroxylating enzymes which are able to oxidise different substrates in regio- and stereospecific manners are dependent on cofactors. The cofactors are responsible for the binding of oxygen, its activation and delivery to the substrate which is bound (if necessary activated) to the apo-enzyme. The apo-enzyme therefore is responsible for the regio- and stereospecificity of the reaction. It was observed that the overall catalytic cycle of cytochrome P-450 is independent of the origin of the enzyme.

It has been suggested that there is a specific relationship between substitution of the substrate and the site of hydroxylation.[58,59] This has been explored by using steroids that were substituted with oxygens at different positions.[60] Varying these locations in a systematic manner, a relationship was established for several microorganisms. For instance, the fungus *Calonectria decora* transformed androstenone substrates into dihydroxy analogs in a specific manner (**Figure 3.5**). This relationship is valid for several

steroids.[61] No specific activation of the C-H bond is provided by the enzyme, but rather the position of hydroxylation is controlled by the shape of the active site. However, not all C-H bonds are equally reactive and therefore this could also play a role in determining the position of hydroxylation.

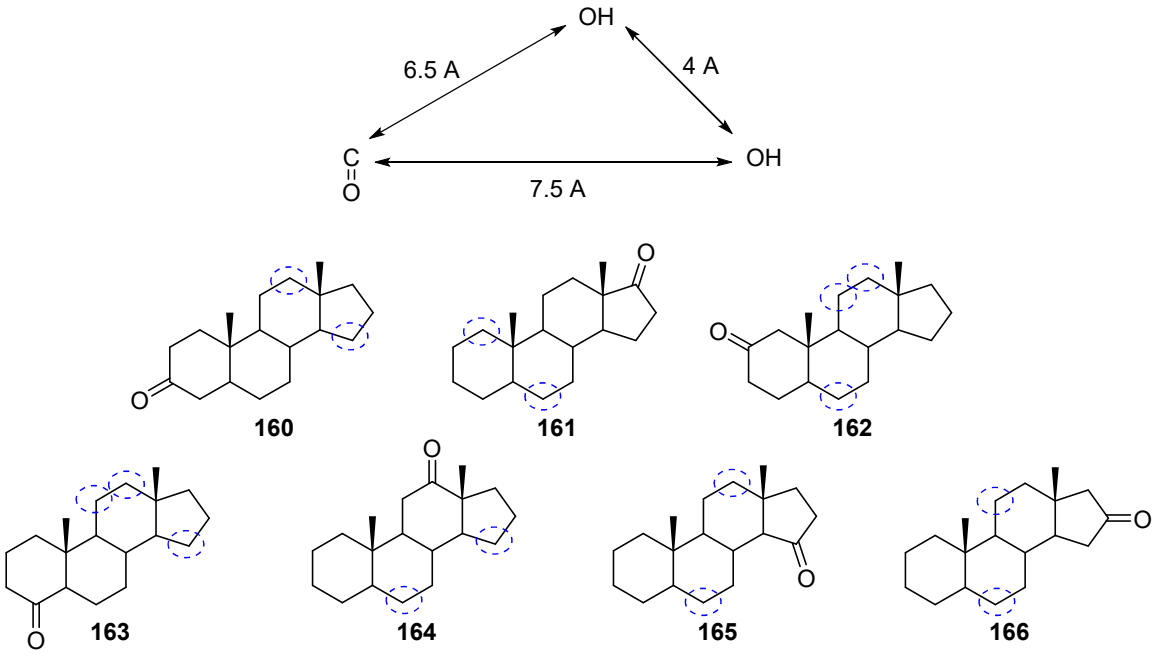


Figure 3.5 That relationship between substitution of the substrate and the site of hydroxylation.

From the work with bis-substituted steroids and *Calonectria decora* a few additional relationships were observed (**Figure 3.6**).[61] It appears that the presence of the carbonyl or hydroxyl group in the ring A or D have the major directing effect on hydroxylation and substituents in the B or C ring do not show a strong directing influence. In addition both carbonyl and hydroxyl group exhibit similar directing effects at a given location.

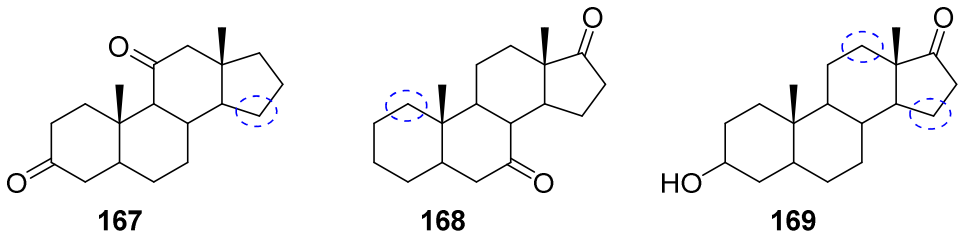
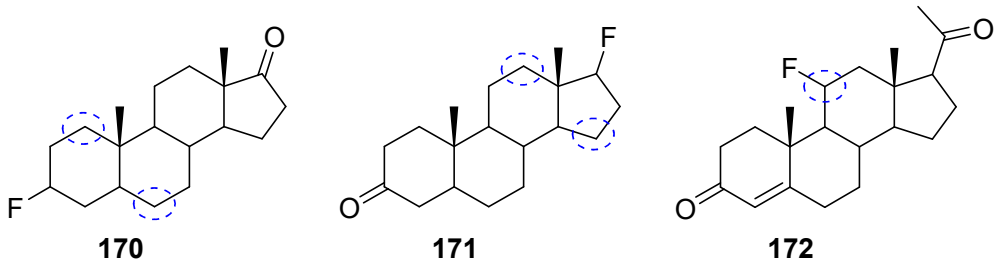


Figure 3.6 The presence of the carbonyl or hydroxyl group in the ring A or D have the major directing effect on hydroxylation.

Based on the studies performed on *Calonectria decora,* substituents such as enol ethers and acetals could also have directing effects but, usually with reduced specificity and yield.

Hydroxylation of monoketohalosteroids is controlled by the carbonyl group.[62] If a halogen is present at a preferred site of hydroxylation the reaction in general occur at a different place (with *R. nigricans* (*stolonifer*), however, sometimes hydroxylation may also occur at the preferred site irrespective of the presence of halogen).[63] It is worth noting that many microorganisms do not show triangular tendency of hydroxylation (e.g. *Rhizopus circinnans, Absidia regnieri, Syncephalastrum racemosum*).[64] On the other hand, *Aspergillus ochraceus* hydroxylate a wide range of substances (androstanes, pregnanes) at C-11α irrespective of the location of substituents.[65,66]

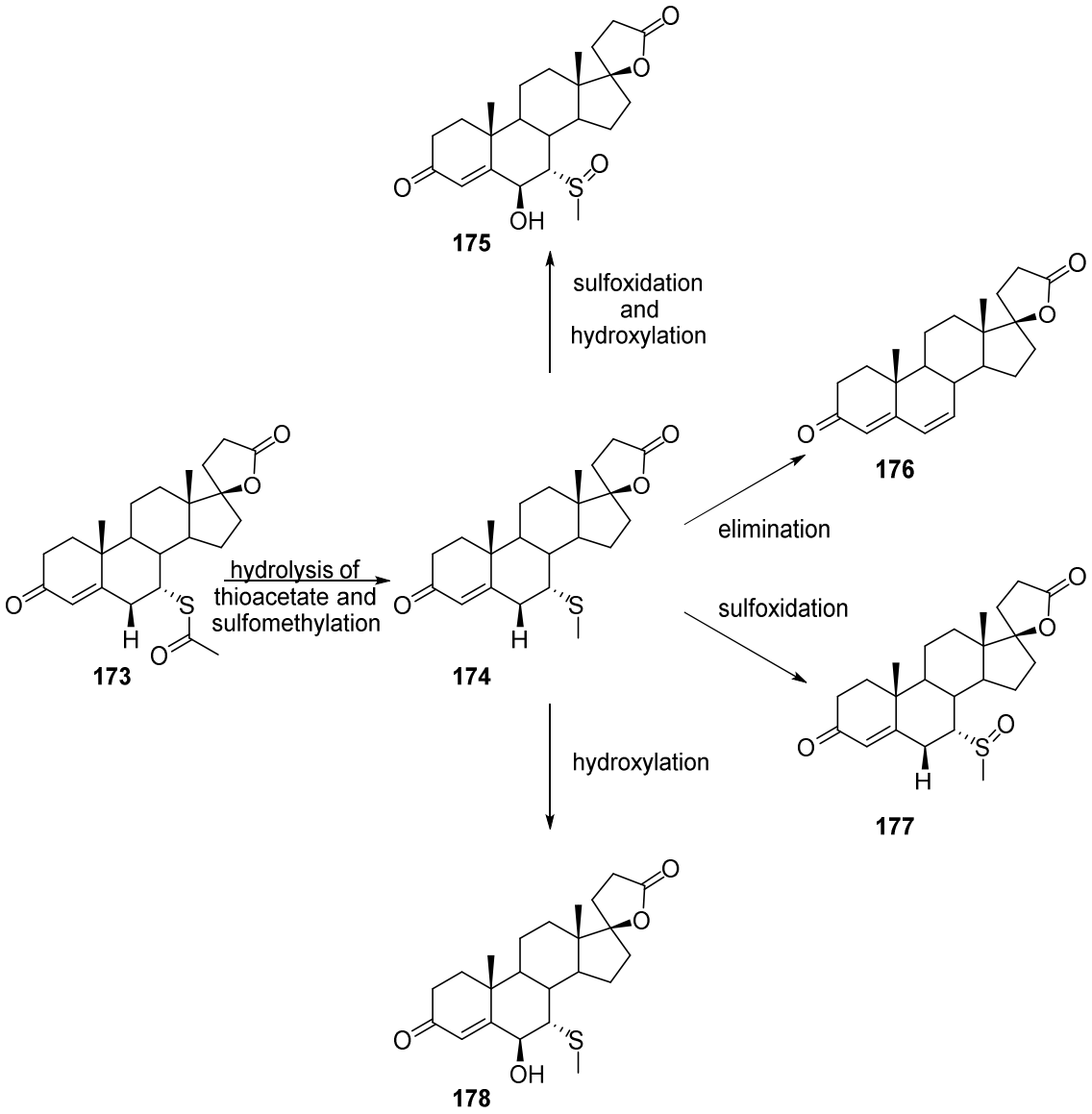


170 **171** **172**

Figure 3.7 Hydroxylation of monoketohalosteroids.

For microbial studies a large number of microorganisms are selected based on a number of factors including literature and researcher experience. A typical procedure usually involves a two-stage fermentation. The first stage involves submerging selected organisms in an Erlenmeyer flask containing a medium that supports growth of the cultures (e.g. a soybean meal-glucose medium, a peptone-glucose medium).[67,68,69] These cultures are incubated with shaking for 1-2 days depending on growth rate of the organisms. After this period, the cultures are transferred to fresh medium containing the drug substrate usually dissolved in solvents such as water, DMF, EtOH or DMSO (Stage 2 fermentation). Drug substrates are usually added at a concentration of 500 µg/ml although any charge can be used which is non-toxic to the organism. Subsequently, the cultures are incubated for a certain period of time which greatly depends on used microorganisms (1-14 days). During the incubation period the culture broth is usually sampled and analysed for the presence of the drug and its metabolites by LC or GC. Because of the simplicity of the fermentation procedure a large number of the microorganisms can be screened simultaneously for their ability to metabolise the drug. In addition, the process is usually straightforward and can be performed by technicians and students. The majority of work in the microbial metabolism

field has been dedicated to steroids, drugs and antibiotics. For example, initial studies on spironolactone (**173**) indicated that a key intermediate in its mammalian metabolism was the methylsulfide (**174**) (**Scheme 3.5**). The work on humans resulted in the identification of its three analogs hydroxysulfoxide **175**, dienone **176** and sulfoxide **177**. To assess action of these compounds on the human body larger quantities of these metabolites were required. Marsheck and Karim found that the fungus *Chaetomium cochloides* converted the intermediate methylsulfide **174** to the sulfoxide metabolites **175** and **177**.[70,71] In addition, *C. cochloides* converted the methylsulfide **174** to the hydroxyl metabolite **178** which was postulated as an intermediate in the human metabolites of spironolactone to **175** and **177**.

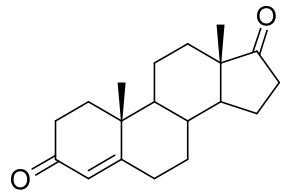
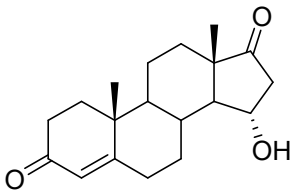
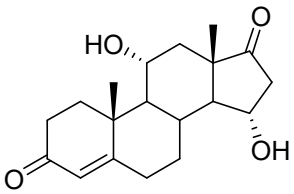
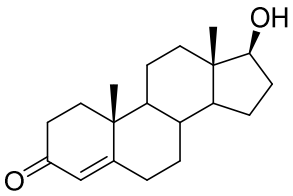
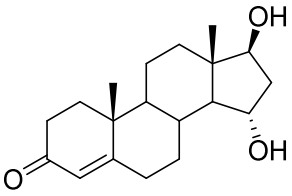
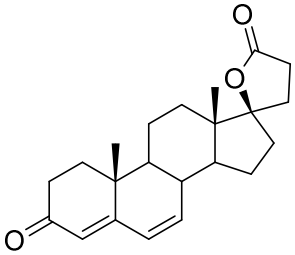
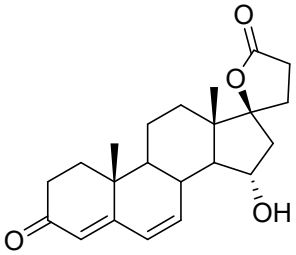
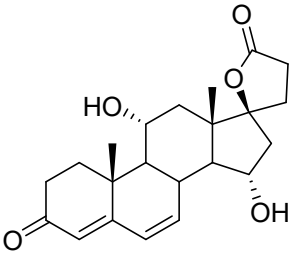
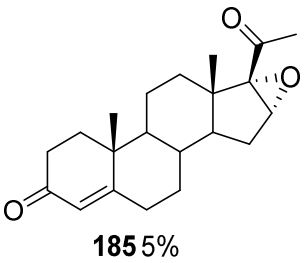
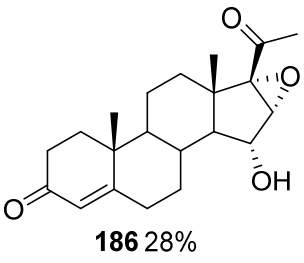
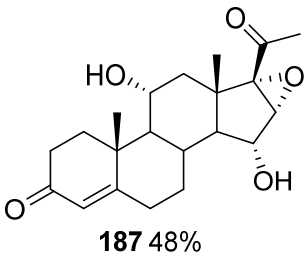
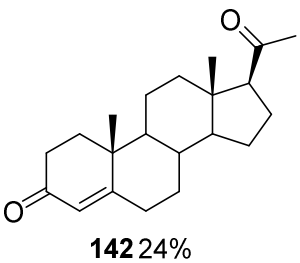
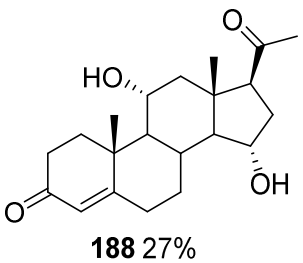


Scheme 3.5 Example of initial metabolism studies by *Chaetomium cochloides* on spironolactone (**173**).71

Steroids are widely used as anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic and anticancer agents.[72] Given the complex structure of the steroid molecule the total chemical synthesis might be complicated. Because of that numerous bioconversion of steroids have been reported and some of them could be applied for the production of pharmaceutical ingredients and their precursors.[73,74,75] The first commercialised microbial process was in the production of 11α-hydrohyprogesterone, a compound with antiandrogenic and blood pressure regulating activity. This fermentation was performed with *Mucor racemosus*.[76]

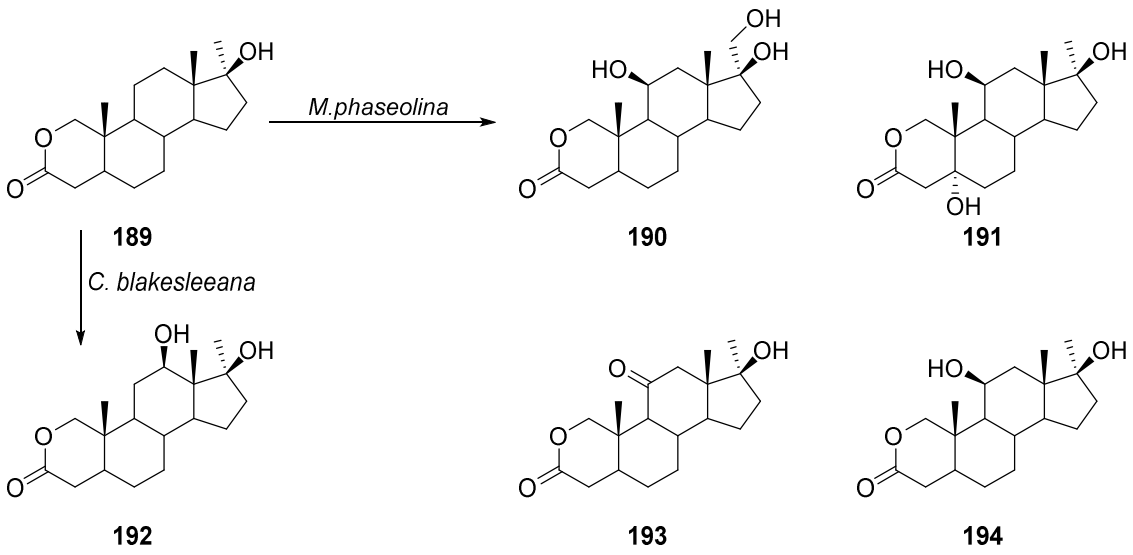
Most of the publications in the field of microbial metabolism are focused on hydroxylation and dehydrogenation. It was observed that in the hydroxylation process the stereochemistry of the carbon atom which is hydroxylated is always preserved. The newly formed hydroxyl group has the same configuration as had the hydrogen atom which occupied the same site prior to reaction. Theoretically, it is possible to hydroxylate steroid in different position by using different microorganism and/or directing groups (hydroxylation at following positions has been reported: 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16 and 17).[77] Products of fermentation are usually obtained in moderate yields and often as a mixture of products. For example, *Colletotrichum lini* ST-1 hydroxylated androst-4-ene-3,17-dione (**106**), testosterone (**91**), canrenone (**182**), 16α,17α-epoxyprogesterone (**185**) and progesterone (**142**) in 15α/β and 11α position in ≤ 64% yield (**Table 3.3**).[78]

Table 3.3 Example of initial metabolism studies by *Colletotrichum lini* ST-1 on steroids.

No	Substrate recovered (%)	Product yield (%)	
1	 **106** 4%	 **179** 5%	 **180** 64%
2	 **91** 6%	 **181** 60%	
3	 **182** 5%	 **183** 22%	 **184** 47%
4	 **185** 5%	 **186** 28%	 **187** 48%
5	 **142** 24%	 **188** 27%	

Another important fungus was studied by Ferris, because of its metabolic similarities to mammalian systems. Ferris *et al* found that *Cunninghamella bainieri* fungi, found in soil and plant material, metabolise a wide range of drugs in a similar manner to mammalian

systems.[79] It was shown that naphthalene (**156**) was converted into a *trans*-dihydrodiol (**158**), demonstrating the presence of an epoxide hydrase. Anisole was hydroxylated at the 2 and 4 position as well as *O*-dealkylated. These metabolites were also detected in mammalian systems, although in different ratios. More recently, it was found that *Cunninghamella* species possess cytochrome P-450 monooxygenase systems analogous to those in mammals and phase II drug metabolism enzymes.[80] Given this, members of this genus are often used to investigate the metabolism of drugs. For example, Smith investigated metabolism of oxandrolone (**189**) which is used by athletes because it does not aromatise and nor affect the production of testosterone (**91**) in the body when used in low dose (**Scheme 3.6**).[81] Fermentation of this anabolic steroid with *Cunninghamella blakesleeana* and *Macrophomina phaseolina* afforded six new metabolites which might be useful in the investigation of the mammalian drug metabolism.



Scheme 3.6 Metabolism of oxandrolone (**189**) by *Cunninghamella blakesleeana* and *Macrophomina phaseolina*.

3.3 Biological studies objectives

3.3.1 Introduction

In order to use ^{19}F NMR as an analytical tool to study the microbial metabolism of steroids a range of fluorine containing steroids were prepared as described in **Chapter 2**. A fluorine atom is comparable in size to a hydrogen atom and does little to alter the overall structure of a molecule. Therefore, if the fluorinated analogue of a naturally occurring metabolite is prepared then it will act as a substrate for the same enzymes as the natural product and both molecules will be metabolised via the same pathways **(Figure 3.8)**.[5,6] Initially, it was necessary to identify microorganisms that would allow the biotransformation of fluorinated steroids. Selection of fluorinated steroids were incubated under a range of conditions (temperature, exposure to air, etc.) and the resulting chemical changes were investigated.

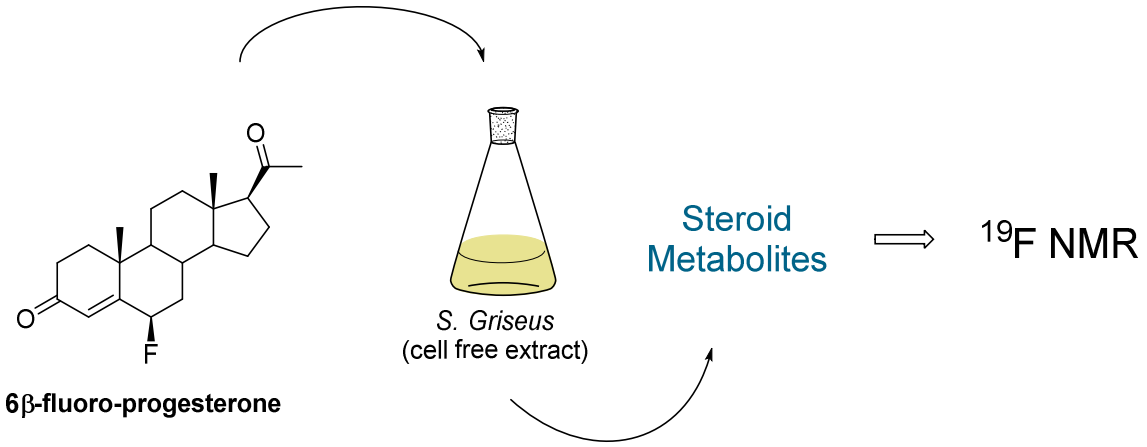


Figure3.8 Proposed method of detecting metabolic changes by ^{19}F NMR spectroscopy.

3.3.2 Metabolic changes of fluorinated steroids in pure bacterial cultures

Biological studies to identify biotransformation products of fluorinated steroids in pure bacterial cultures using ^{19}F-NMR were carried out in DurhamUniversity departments of Chemistry, and Pharmacy and at University College Dublin (UCD). At Durham University all experiments were carried out with the bacteria *Streptomyces griseus*. It was found that this bacterium has the ability to transform 6-fluoro-progesterone (**144**) to various metabolites. The experiments performed in Stockton were performed with *Escherichia coli, Bacillus subtilis* and *Bacillus megaterium*. Finally, the studies carried out at University College Dublin were focused on the metabolism of fluoro-steroid by *Streptomyces griseus*.

3.3.3 General culture conditions and metabolite extraction procedures used in Durham and Dublin

Bacteria were inoculated from a plate into Erlenmeyer flasks containing soya bean meal media and the pH was adjusted to 7. The medium consists of: soya bean meal (5 g/L), Glycerol (20 g/L), Yeast extract (5 g/L), K_2HPO_4 (5g/L). Cultures were incubated with rotary agitation at 27ºC for 72 h. After that time, following solutions in Erlenmeyer flasks were prepared:

- medium + starting culture bacteria
- medium + starting culture bacteria + with addition of fluorinated steroid after 72h
- medium + after 72 h fluorinated steroid was added (negative probe)

The Erlenmeyer flasks were incubated with rotary agitation at 27ºC for 6 days. The cultures were sonicated and centrifuged. The supernatant and pellet fractionns were then extracted (**Figure 3.3**).

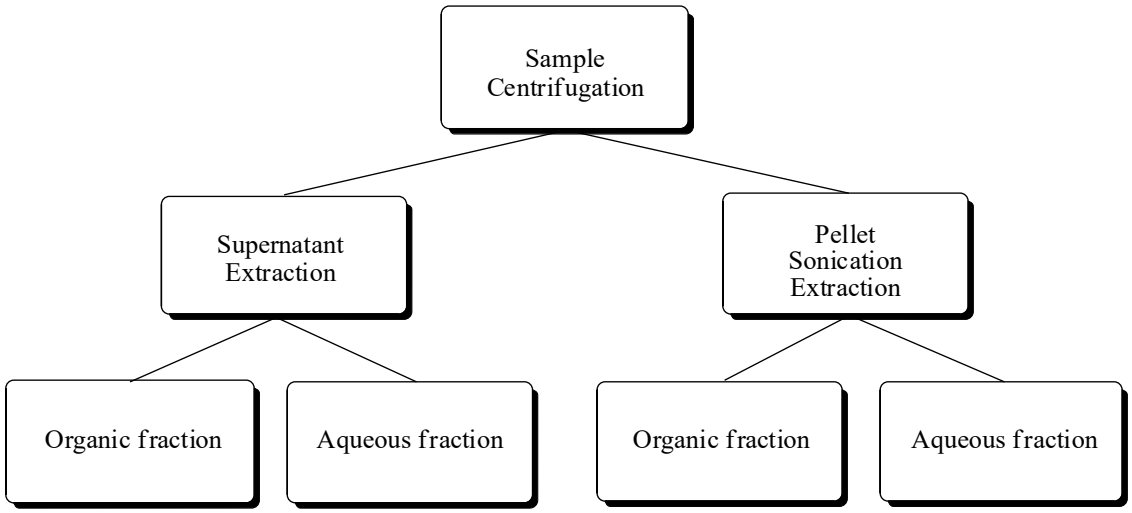


Figure 3.9 Extraction procedure for metabolic identification.

3.3.4 Feeding experiments with fluorinated steroids and *S. griseus*

S. griseus is a Gram positive, aerobic, filamentous bacteria commonly found in soil. It helps to naturally decompose organic matter contributing in part to the earthy odour of soil and decaying leaves, and to the fertility of soil. It forms a threadlike net called a mycelium that bears chains of spores at maturity. Their branching strands are 0.5 to 1.0 micrometre in diameter and the optimal temperature for *S. griseus* growth is at 25-35ºC. *S. griseus* contains cytochrome P-450 that mimic oxidation processes. It is very commonly used in the production of antibiotics along with most other Streptomycetes. Antibiotics specifically made from *S. griseus* include: Streptomycin, Cycloheximide, Candicidin B and Grisein. *S. griseusis* is well known for its ability to transform xenobiotics and it has also previously been shown to transform fluorinated compounds such as Flurbiprofen.[82] *S. griseus* has 27 putative P-450 genes in its genome.[83] It was shown by Makino that one of the three CYP154 enzymes (CYP154C3) catalyzed regio and stereospecific hydroxylation of various steroids at the 16 position of the D ring.[84] Thus it was expected that fluorinated steroids will be transformed to various hydroxylated metabolites upon incubation with *S. griseus*. The studies presented in this Chapter have utilised the fluorinated steroids synthesised in **Chapter 2** (**Figure 3.10**).

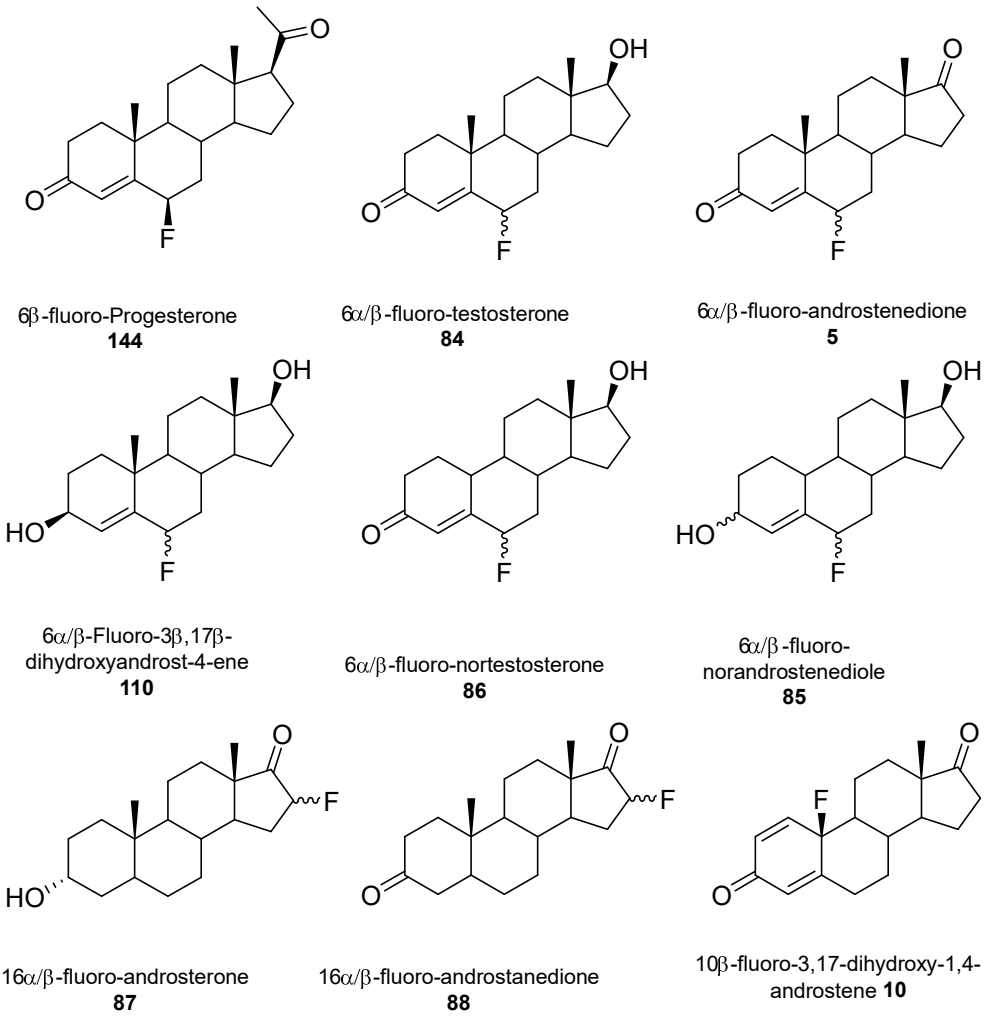


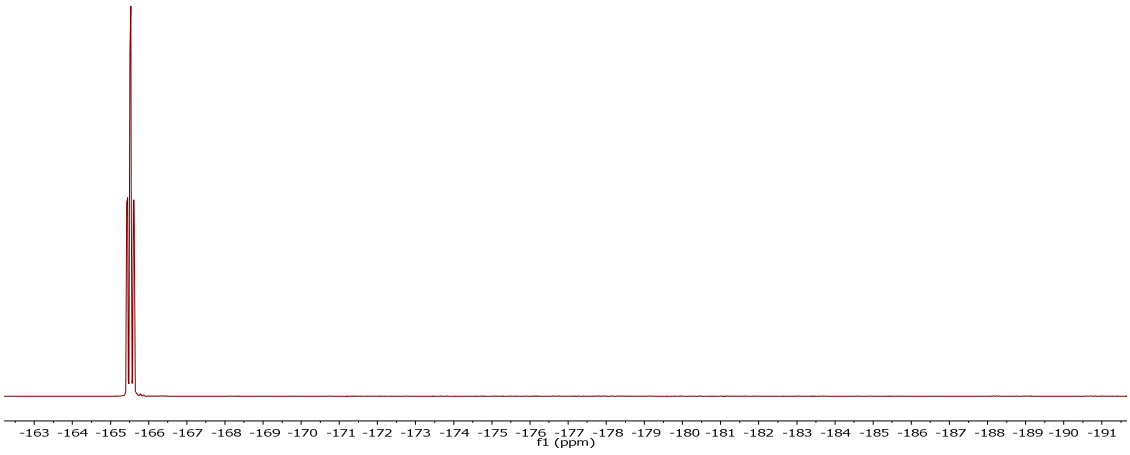
Figure 3.10 Fluorinated steroids used in the microbial feeding experiments.

3.3.5 Feeding experiments with 6β-fluoro-progesterone

The first experiment involved incubation of *S. griseus* in soya bean meal with 6β-fluoro-progesterone (**144**). Upon completion of the incubation period the cultures were sonicated and centrifuged. The supernatants and pellets were then extracted with ethyl acetate (as **Figure 3.9**). ^{19}F NMR and GC-MS analysis of the extracts indicated formation of various metabolites (**Figures 3.11** and **3.12**). Analysis revealed that all the steroid metabolites were located in the supernatant organic extracts. The aqueous phase of the supernatant did not contain any fluorinated compounds. The extraction of the pellet yielded an organic phase which contained mainly 6β-fluoro-progesterone (**144**) with a small amount of fluorinated metabolites detected previously in the supernatant organic extract. Analysis of the aqueous extract of the pellet using ^{19}F NMR revealed that it did not contain any fluorinated steroids (the signal observed at -122.11 ppm is characteristic of inorganic fluoride). Observed strong

peaks in pallet fractions are coming from large amount of starting material used in the experiments.

A – ^{19}F NMR spectral analysis of the Pellet (organic fraction)



B - ^{19}F NMR spectral analysis of the Supernatant (organic fraction)

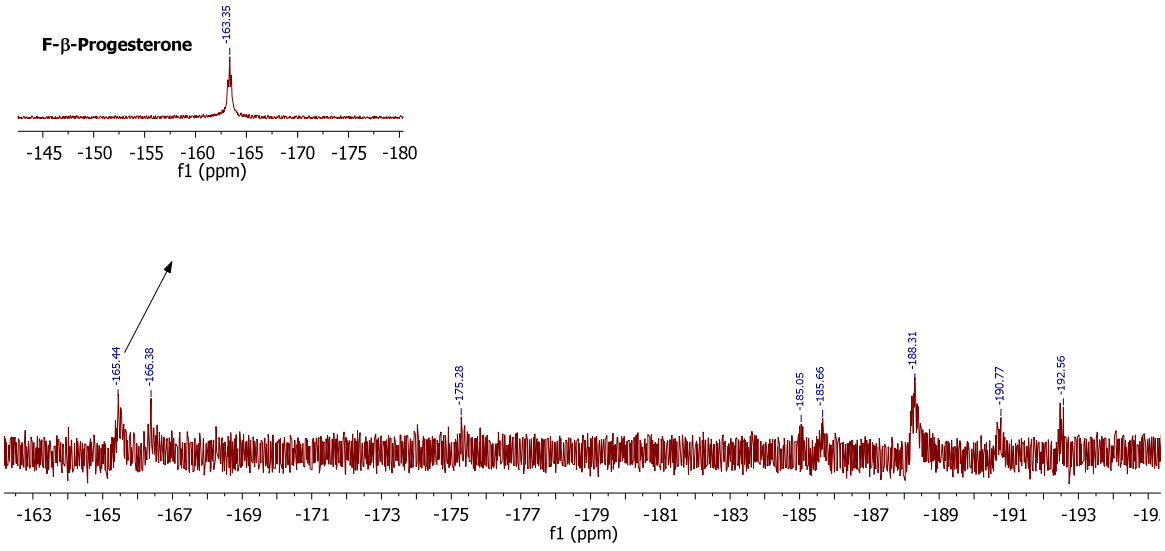


Figure 3.11 ^{19}F NMR analysis of 6β-fluoro-progesterone (**144**) incubated with *S. griseus*. **A**- ^{19}F NMR of pellet organic fraction in d-chloroform. **B**- ^{19}F NMR of supernatant organic fraction in d-chloroform. The other fractions (pellet and supernatant aqueous fraction in D_2O) showed no peaks during ^{19}F NMR analysis.

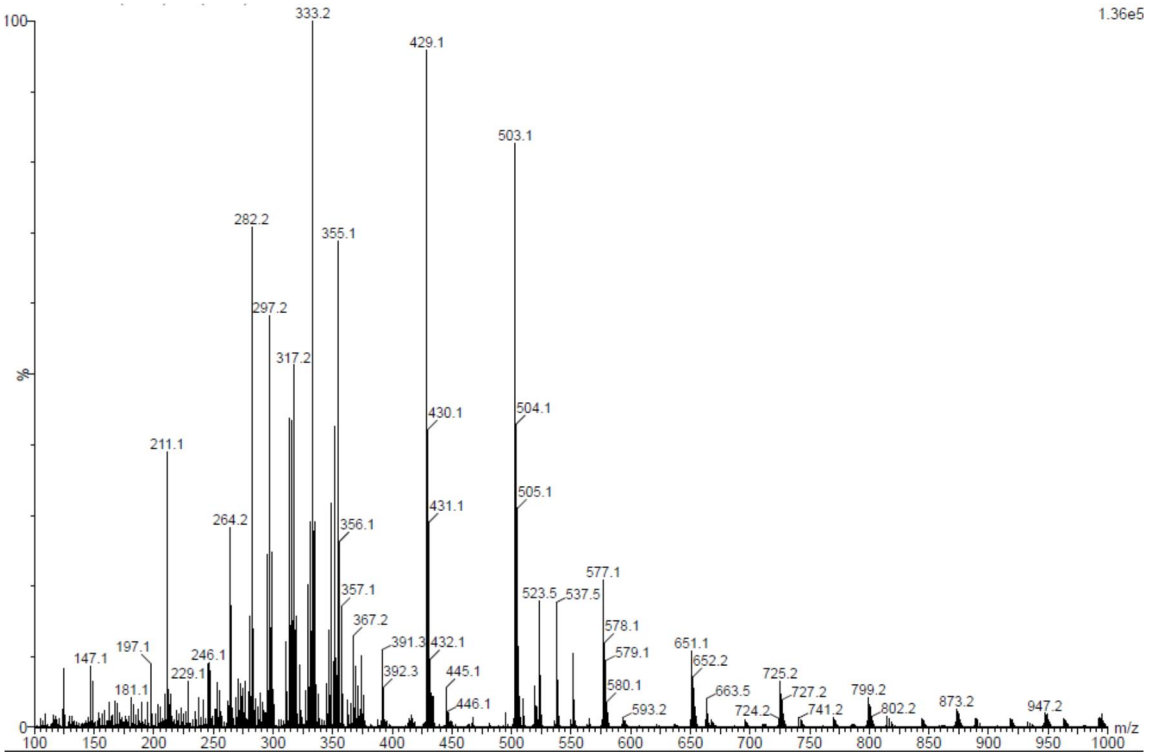


Figure 3.12 Transformation of 6β-fluoro-progesterone (**144**) by *S.griseus* observed by GC-MS (organic extract of the pellet).

Interestingly a small variation in the metabolic profile was observed when the experiment was repeated in a baffled flask. This was most likely related to better air-medium mixing (**Figure 3.13**).

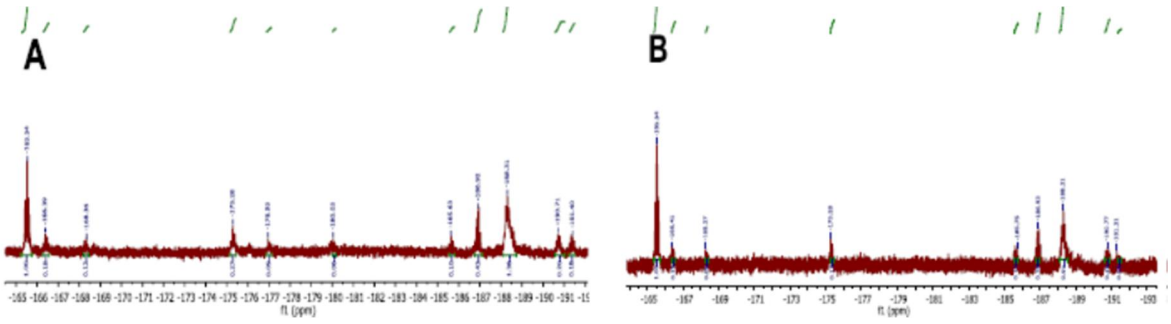


Figure 3.13 Spectra **A** incubation in normal erlenmeyer flask. Spectrum **B** incubation in baffled flask.

Identification of the metabolites base on crude ^{19}F NMR and GC-MS could be challenging; therefore, the compounds present in the supernatant organic extract were partially separated using preparative TLC. The plate was run using an 80% DCM/EtOAc solvent system. As

shown in the figure below, nine fractions were identified (**Figure 3.14**). The silica from the TLC plate was removed and extracted and these extracts were analysed using ^{19}F NMR / GC-MS and presented in **Figure 3.12** and **Figure 3.15**.

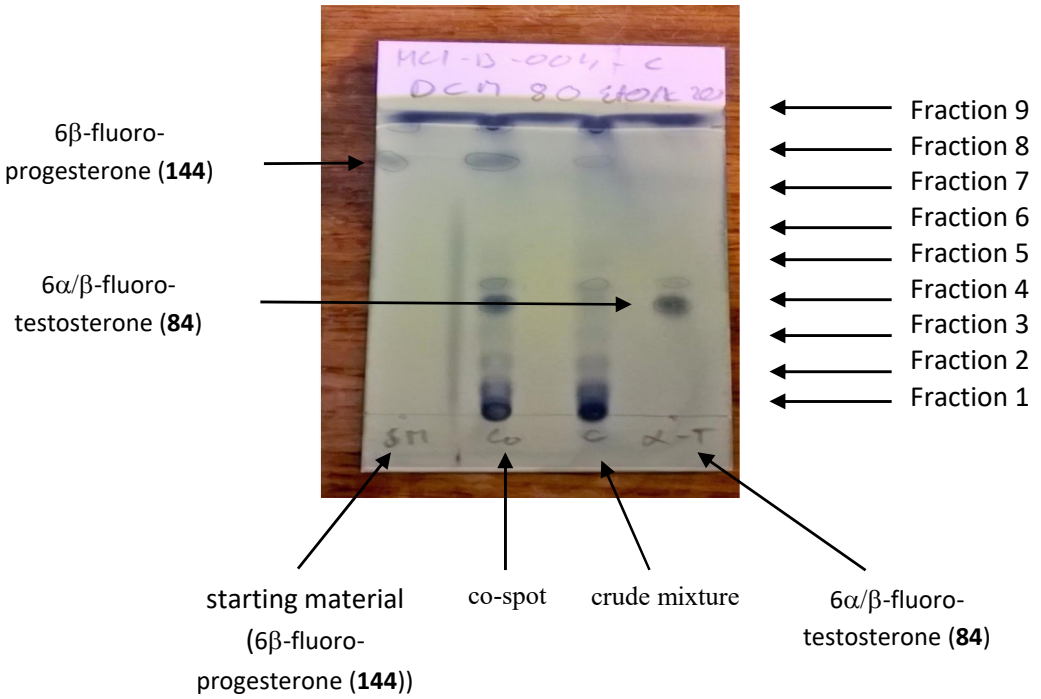


Figure 3.14 TLC plate presented with crude mixture. Transformation of 6β-fluoro- progesterone (**144**) by *S. griseus*.

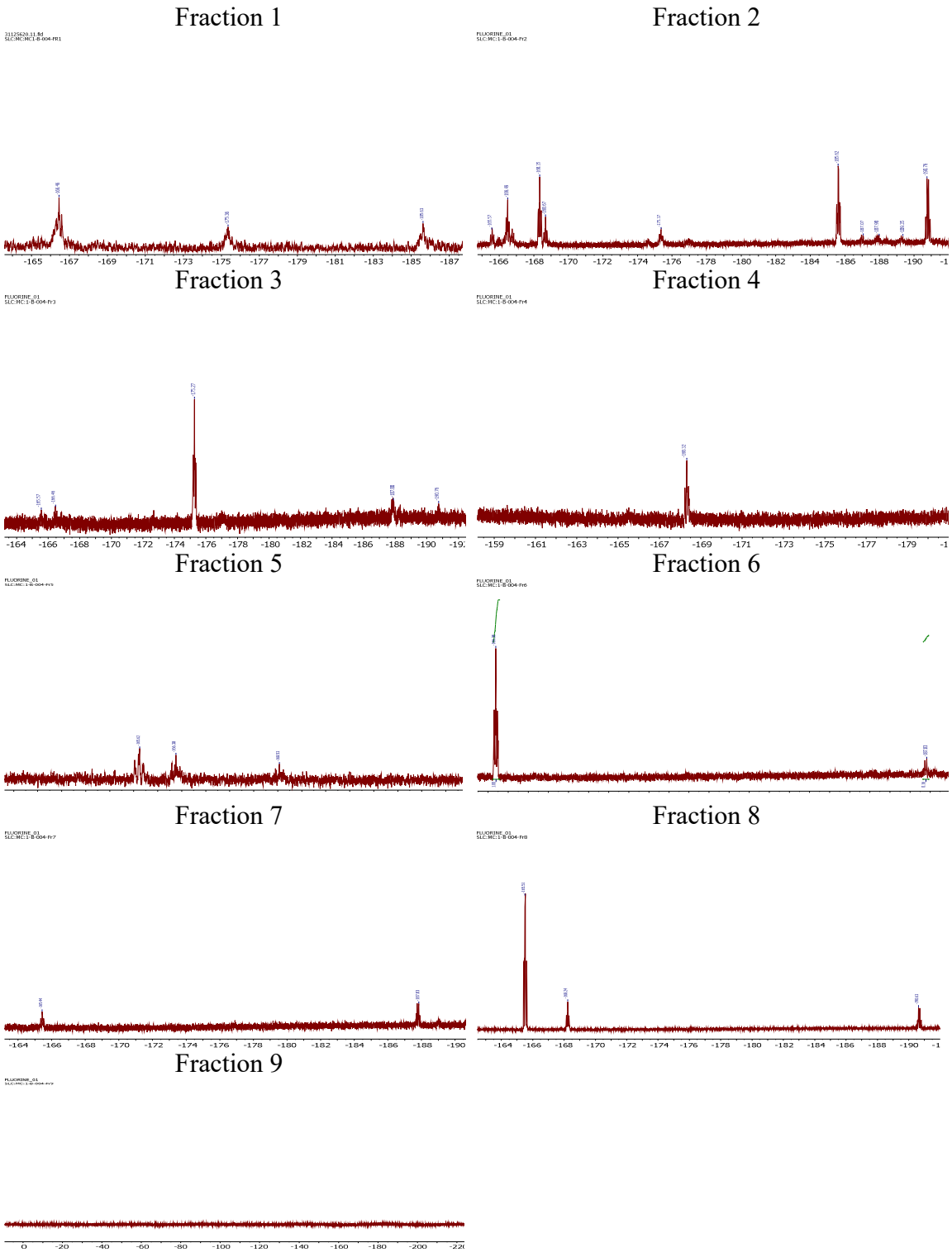


Figure 3.15 ^{19}F NMR of the fractions collected after separation of the crude mixture by preparative TLC technique in 80% DCM/EtOAc solvent system.

The ^{19}F NMR data presented in **Figure 3.15** is summarised in **Table 3.4**. It appears from the data obtained that some of the metabolites were not stable on silica gel and decomposed or formed new products. It is possible that some of the metabolites (e.g. ^{19}F NMR signals at -185.05 ppm, -188.31 ppm, -192.56 ppm) were conjugates with sugars or unstable hydroxy compounds that underwent hydrolysis or elimination under the acidic conditions during the TLC separation. In addition, the data suggests that two major products were formed on the TLC plate which gave ^{19}F NMR signal at -168 ppm (fraction 1: -168.35 ppm; fraction 4: -168.32 ppm; fraction 8: -168.24 ppm) and -187 ppm (fraction 2: -187.98 ppm; fraction 3: -187.88 ppm; fraction 6: -187.83 ppm; fraction 7: -187.83 ppm). It is also possible that these peaks correspond to new products with similar ^{19}F NMR signals.

Table 3.4 Summarised data of the ^{19}F NMR of each fraction collected.

Experiment	**^{19}F NMR signals (ppm)**	
	Original metabolites	**New signals after TLC**
Crude	-165.44, -166.38, -175.28, -185.05, -185.66, -188.31, -190.77, -192.56	-
Fraction 1	-166.46, -175.36, -185.63	-
Fraction 2	-165.57, -166.49, -175.37, -185.62, -190.76	-168.35, -168.67-187.07, -187.98, -189.35
Fraction 3	-165.57, -166.46, -175.27, -190.76	-187.88,
Fraction 4	-	-168.32
Fraction 5	-165.62, -166.38	-168.53
Fraction 6	-166.38	-187.83
Fraction 7	-165.44	-187.83
Fraction 8	-165.51, -190.63	-168.24
Fraction 9	-	-

Comparison of GC-MS analysis for fraction 1 and 6β-fluoro-progesterone (**144**) indicated similarities in the fragmentation pattern (**Figure 3.12** and **3.16**). Characteristic fragmentation behaviour of 6β-fluoro-progesterone (**144**) was the elimination of HF (*m/z* 312 (72), (**195**)). A concomitant loss of carbonyl group (C-20, (**196**)) gave rise to an

abundant fragment ion at *m/z* 43 (70) (**198**). Fragmentation of D ring generated the product ion at *m/z* 227 (100) (**197**).

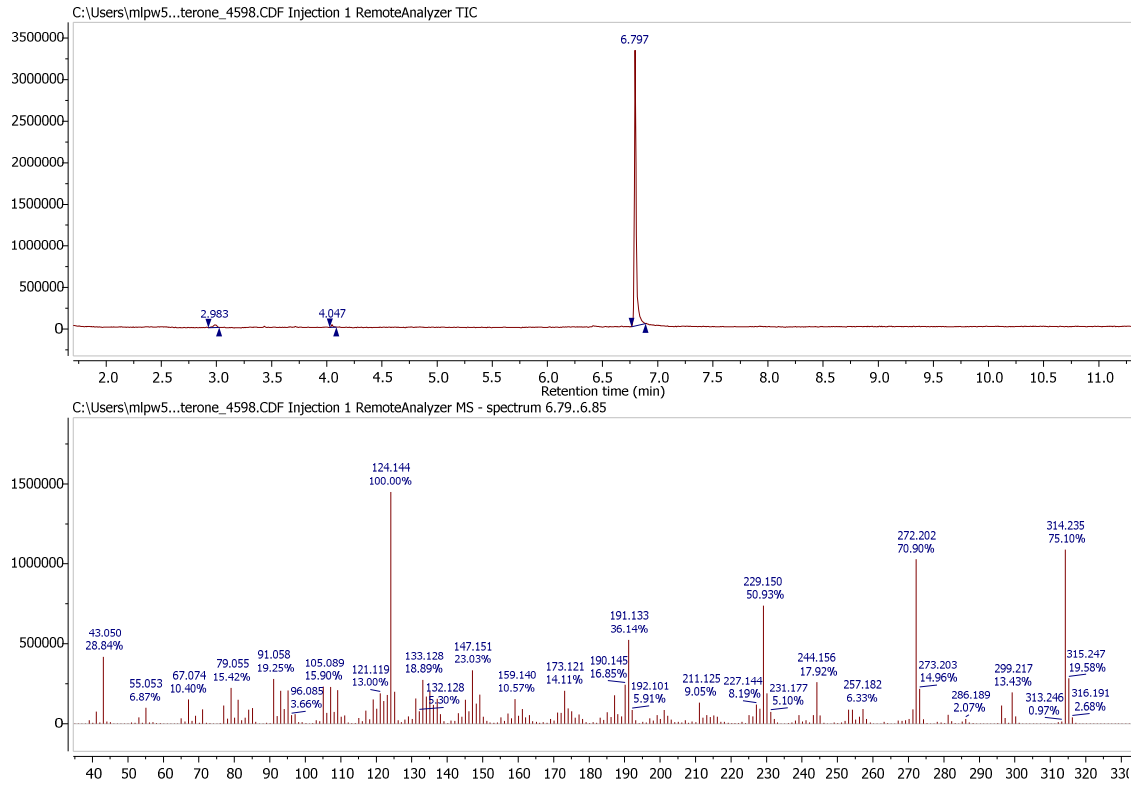
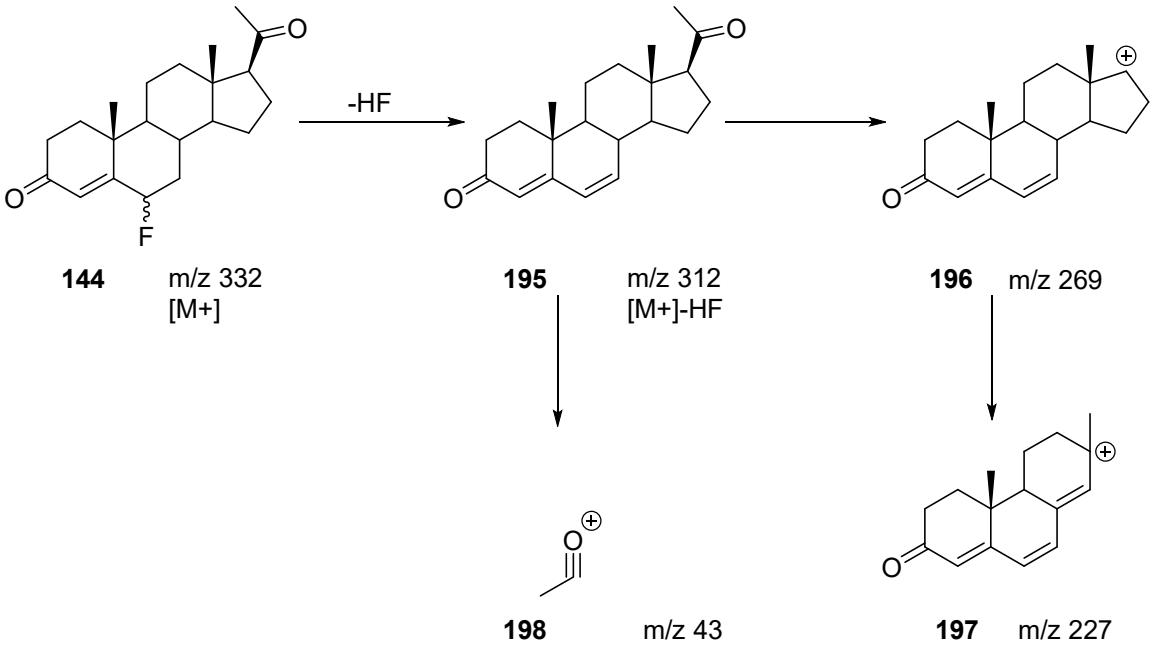


Figure 3.16 GC-MS spectrum of 6β-fluoro-progesterone (**144**).



Scheme 3.7 Proposed fragmentation pathway of 6β-fluoro-progesterone (**144**).

Abundant fragment ions at *m/z* 333 ([M+1]) and *m/z* 313 ([M+1]-HF) could indicate the presence of 6β-fluoro-progesterone (**144**) in fraction 1 but other characteristic fragments of 6β-fluoro-progesterone (**144**) such as *m/z* 269 and *m/z* 227 were not detected. The ion at *m/z* 313 could also be the molecular ion of de-fluorinated 6β-fluoro-progesterone (**144**), which could form during the feeding experiment and/or on silica. Given that fraction 1 does not contain 6β-fluoro-progesterone (**144**) based on TLC, the ion at *m/z* 333 was most likely generated during a fragmentation process in MS.

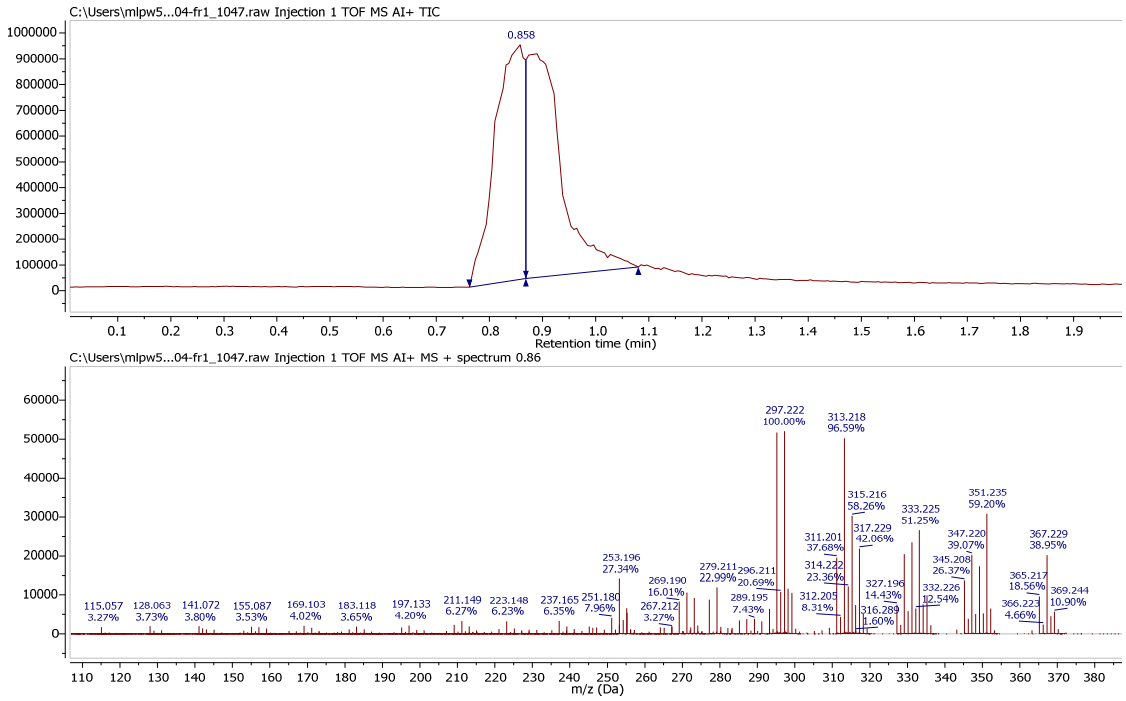
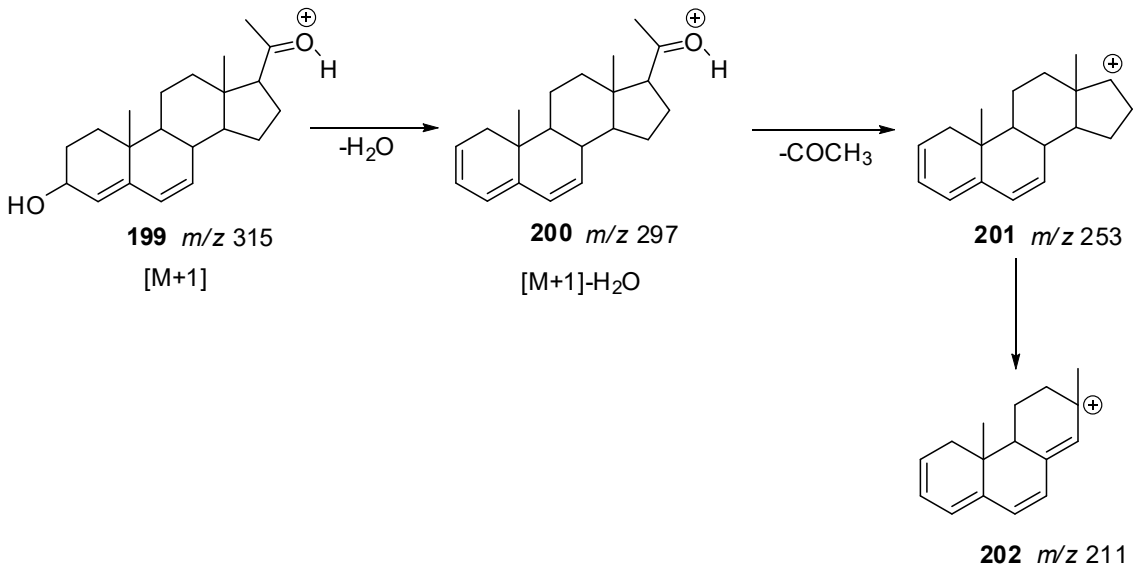


Figure 3.17 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 1.

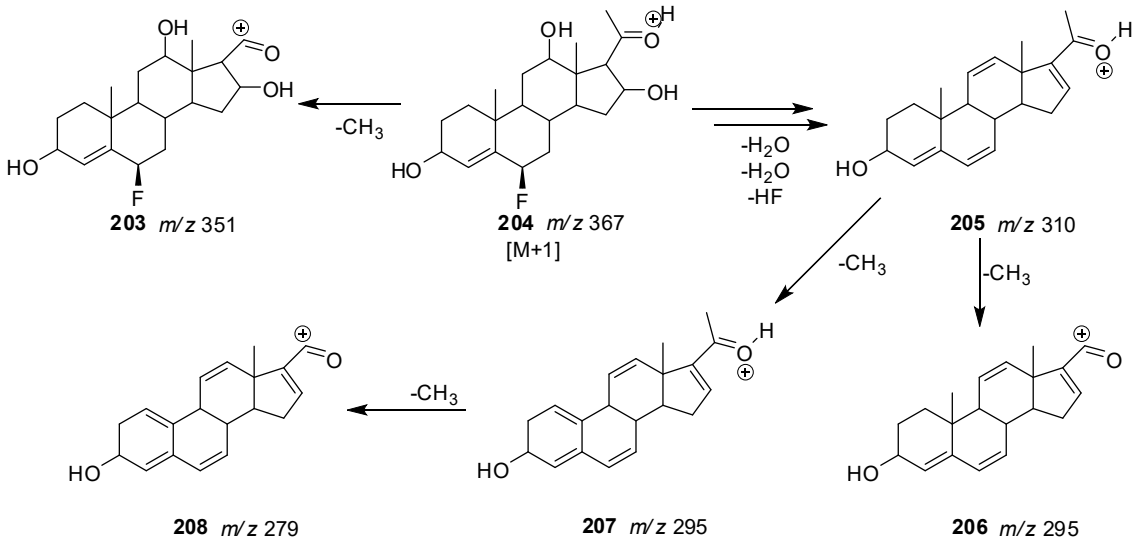
The presence of ion at *m/z* 315 in fraction 1 could be attributed to 3-hydroxy-4,6-pregnen-20-one ([M+1], (**199**)). The ion mass spectrum of fraction 1 includes the most abundant ion at *m/z* 297 (**200**). Its generation is initiated from protonated molecule **199** by loss of water shown in **Scheme 3.8**. The dissociation of the carbonyl group from 17-C gave rise to the product ion at *m/z* 253 (**201**). Fragmentation of the D-ring yielded a charged fragment ion at *m/z* 211 (**202**).



Scheme 3.8 Proposed GC-MS fragmentation pathway of 3-hydroxy-4,6-pregnen-20-one potentially detected in fraction 1.

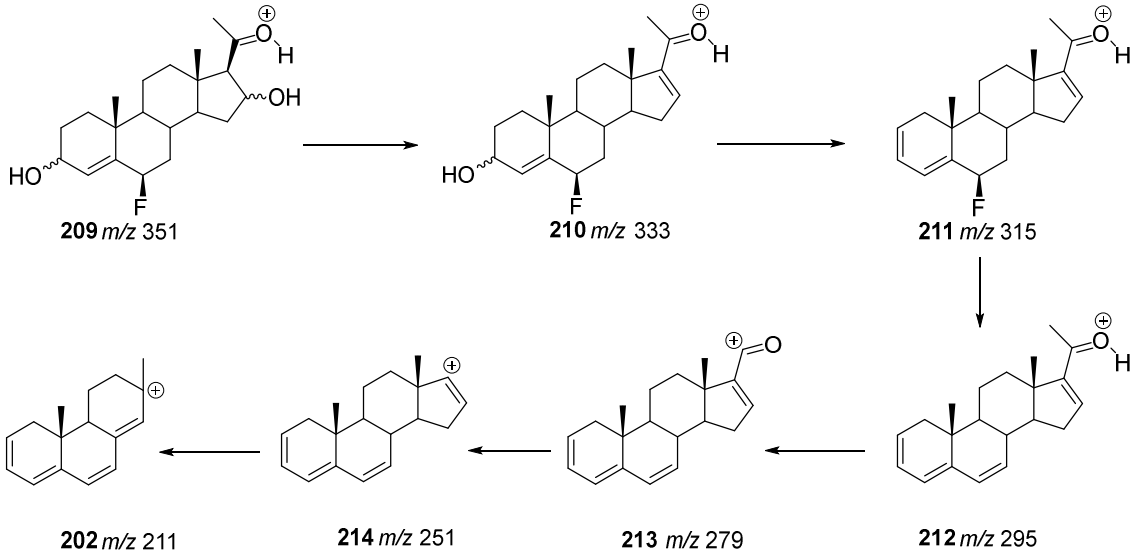
The formation of 3-hydroxy-4,6-pregnen-20-one (**199**) could be attributed to the microbial reduction of the carbonyl group. The reduction of α,β-unsaturated carbonyl group is usually highly stereospecific and has been described for numerous substances.[85] The elimination of HF from 6β-fluoro-progesterone (**144**) could potentially occur via microbial transformation and/or due to instability of the fluorinated metabolite during isolation step or purification on silica.

The ion detected at *m/z* 367 detection in fraction 1, is proposed to originate from 6-fluoro-3,12,16-trihydroxy-4-pregnen-20-one [M+1] (**204**) (**Scheme 3.9**). Postulated fragmentation routes of this molecule giving rise to ion at *m/z* 351 (**203**), which was formed through dissociation of a CH_3 group from molecule (most likely 21-C). The product ion mass spectrum of fraction 1 includes an abundant product ion at *m/z* 295 (**207**). Its generation is initiated from the dihydroxylated 4-pregnen-20-one through the elimination of water (presumably from 16-C and 12-C), HF and a methyl group. Elimination of another methyl group leads to the formation of ion at *m/z* 279 (**208**).



Scheme 3.9 Proposed GC-MS fragmentation pathway of 6-fluoro-3,12,16-trihydroxy-4-pregnen-20-one potentially detected in fraction 1.

Alternatively, it is also plausible that the ion at *m/z* 351 (**209**) originates from the monohydroxylated-4-pregnen-20-one derivative (**209**) (**Scheme 3.10**). The proposed fragmentation pathway of this molecule involves elimination of water to produce ions at *m/z* 333 (**210**) and *m/z* 315 (**211**), elimination of HF to produce ion at *m/z* 295, fragmentation of the carbonyl group (*m/z* 279 (**213**) and *m/z* 251 (**214**)) and fragmentation of the metabolite D ring (*m/z* 211 (**202**)).



Scheme 3.10 Proposed GC-MS fragmentation pathway of monohydroxylated-4-pregnen-20-one potentially detected in fraction 1.

Given the aforementioned analysis a summary of the proposed metabolites contained in fraction 1 are tabulated below (**Table 3.5**).

Table 3.5 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus.* (Detected GC-MS ions in fraction 1).

Entry	*m/z*	Proposed Metabolite
1	315 [M+1], 297 [M+1]-H_2O, 253, 211	3-Hydroxy-4,6-pregnen-20-one (**199**)
2	367 [M+1], 351 [M^+]-CH_3, 295, 279	6-Fluoro-3,11,16-trihydroxy-4-pregnen-20-one (**204**)
3	351 [M+1], 333 [M+1]-H_2O, 315, 295, 279, 211	6-Fluoro-11-hydroxy-4-pregnen-20-one (**209**)
4	313 [M+1]	4,6-pregnen-3,20-dione (**195**)

The presence of dihydroxylated 4-pregnen-20-one (**209**) as a metabolite is supported by existing literature. Hydroxylation of various steroids at the 16α position by *S. griseus* cytochrome P-450 CYP154C3 was studied by Makino.[86] Here they reported stereospecific hydroxylation of testosterone (**91**), progesterone (**142**), androstene-3,17-dione (**106**), androsterone (**97**), 1,4-androstadiene-3,17-dione, dehydroepiandrosterone, 4-pregnane-3,11,20-trione and deoxycorticosterone. There are no reports in the literature of *S. griseus* 11-C hydroxylation. The typical microorganisms that are used for 11-C α hydroxylation are *Rhizopusnigricans* and *Aspergillus niger*, whereas strains of *Cunninghamella blackesleena* and *Curvularia lunata* are used for 11C-β hydroxylation.

The GC-MS spectrum of fraction 2 is presented below (**Figure 3.18**). This fraction showed many similarities in the fragmentation pattern to fraction 1; however, a few additional ions were detected at *m/z* 362, 282, 264 and 246. The origin of these ions is currently unknown. Assuming that ion at *m/z* 362 or 282 is the molecular ion [M+1], the molecule would have an odd molecular weight. This indicates that an odd number of nitrogen atoms are present in the molecule (nitrogen rule). Ions at *m/z* 264 and 246 are most likely to be the fragments generated from ion at *m/z* 282 through the loss of water.

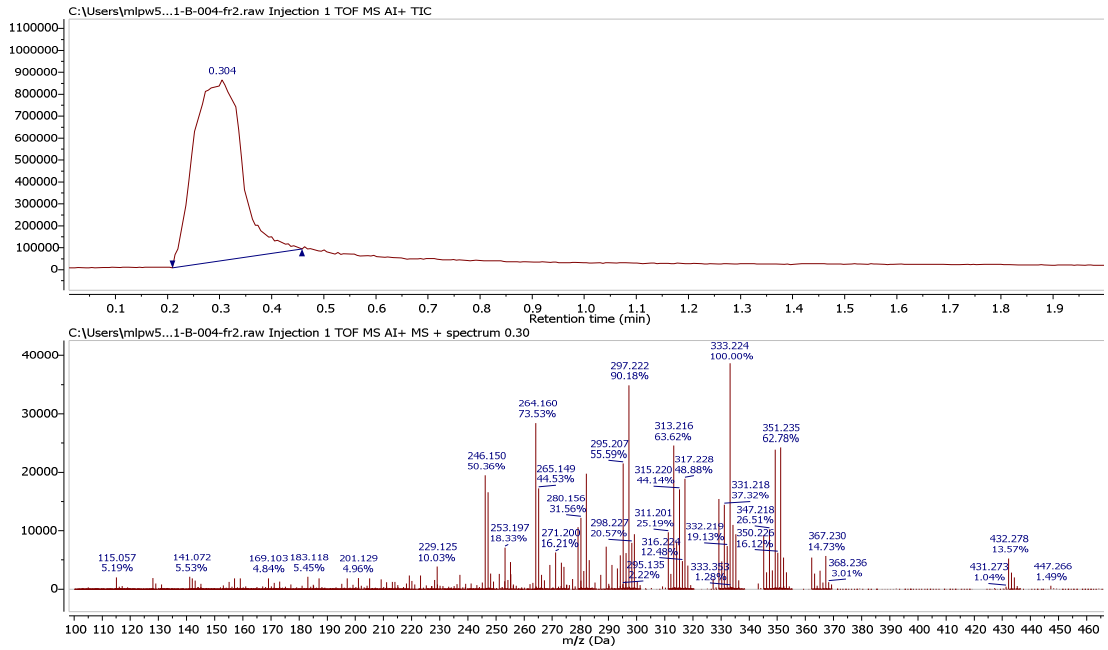


Figure 3.18 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 2.

Table 3.6 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 2.)

Entry	*m/z*	Proposed Metabolite
1	297 [M+1]-H_2O, 253, 211	2,4,6-pregnen-20-one (**200**) or 3-Hydroxy-4,6-pregnen-20-one (if water was eliminated during analysis) (**199**)
2	367 [M+1], 351 [M^+]-CH_3	6-Fluoro-3,11,16-trihydroxy-4-pregnen-20-one (**204**)
3	351 [M+1], 333, 313 [M+1]-H_2O, 211	6-Fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**)
4	313 [M+1]	4,6-pregnen-3,20-dione (**195**)

When comparing fraction 3 to fraction 2, there were two additional ions at *m/z* 335 and *m/z* 315. The ion at *m/z* 335 is proposed to originate from 6-fluoro-3-hydroxy-4-pregnen-20-one (**215**) [M+1] (**Scheme 3.11**). The fragment ion of 6-fluoro-3-hydroxy-4-pregnen-20-one (**199**) at *m/z* 315 is proposed to originate from loss of HF. Elimination of water from *m/z* 315 produced ion at *m/z* 297 (**200**) and subsequent fragmentation of the ketone led to the formation of ion at *m/z* 253 (**201**). Alternatively, ions at *m/z* 315 and 297 could be the molecular ions if elimination of water and HF occurred during feeding experiment, isolation or purification of metabolites.

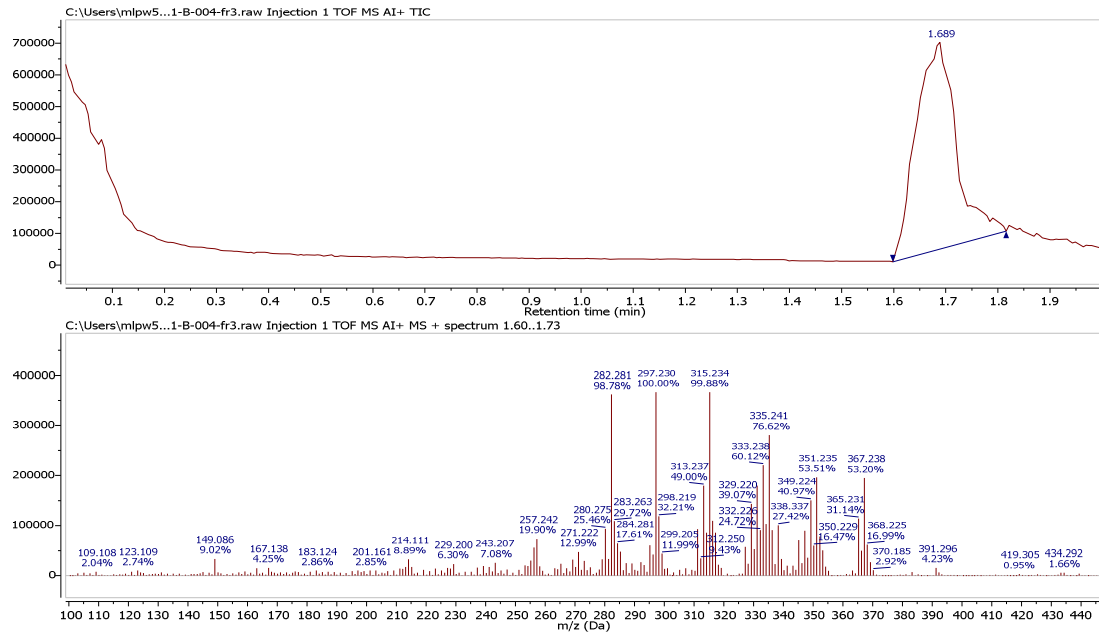
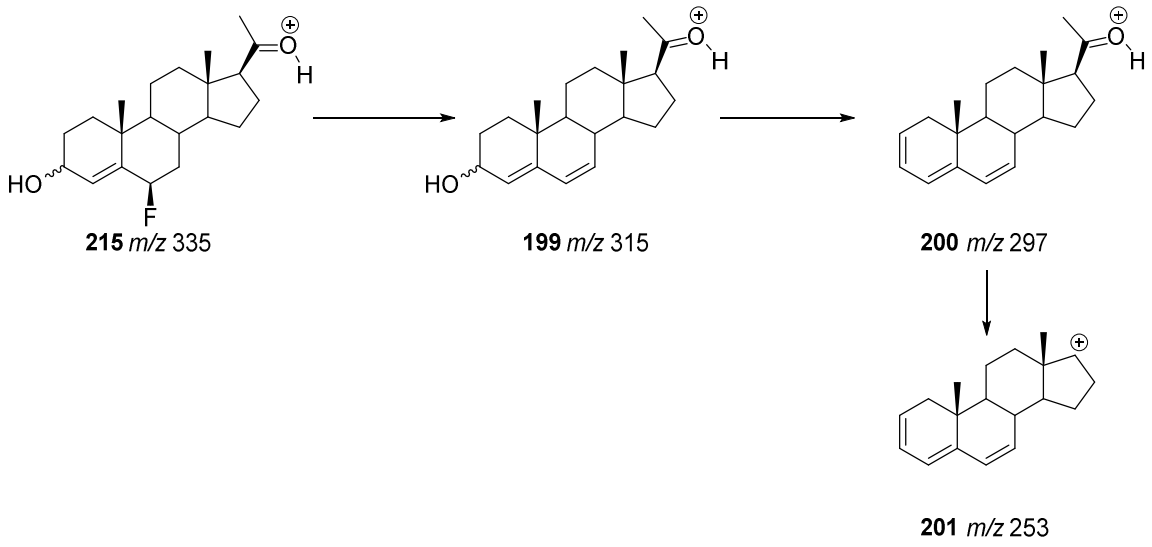


Figure 3.19 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 3.

Table 3.7 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 3.)

Entry	*m/z*	Proposed Metabolite
1	315 [M+1], 297 [M+1]-H_2O, 253	3-Hydroxy-4,6-pregnen-20-one (**199**)
2	367 [M+1], 351 [M^+]-CH_3	6-Fluoro-3,11,16-trihydroxy-4-pregnen-20-one (**204**)
3	351 [M+1]	6-Fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**)
4	335 [M+1], 297, 253	6-Fluoro-3-hydroxy-4-pregnen-20-one **(215)**



Scheme 3.11 Proposed GC-MS fragmentation pathway of 6-fluoro-3-hydroxy-4-pregnen-20-one potentially detected in fraction 3.

Fraction 4 contained 6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**) (*m/z* 351 [M+1], *m/z* 333 [M+1]-H_2O, 313 [M+1]-H_2O-HF) and 4,6-pregnen-3,20-dione (**195**) (*m/z* 313 [M+1]). These compounds were also detected in the previous fractions. The ion at *m/z* 333 could indicate presence of 6β-fluoro-progesterone (**144**) [M+1], however, ^{19}F NMR did not confirm that this compound was present. A new ion at *m/z* 349 could be potentially attributed to 6-fluoro-16-hydroxy-4-pregnen-3,20-one (**216**). A proposed mechanism of fragmentation of *m/z* 349 ([M+1]) is depicted below in **Scheme 3.12**. This mechanism involves elimination of a methyl group, water, and fragmentation of the A ring as this could explain the formation of ions at *m/z* 273 (**220**), 257 (**222**) and 237 (**223**). Ions at *m/z* 331 [M+1]-H_2O (**217**), 299 (**219**) and 257 (**222**) included in **Scheme 3.12** were detected in the next 5th fraction were *m/z* 349 (**216**) was the major component. It is worth noting that a similar fragmentation pattern of the A ring was observed by Thevis for androst-4,9-diene-17β-ol-3-one.[87] Fraction 4 also contained the abundant ion at *m/z* 292. The nitrogen rule suggested that this molecular ion [M+1]/fragment contained an odd number of nitrogen atoms. The origin of this ion is currently unknown.

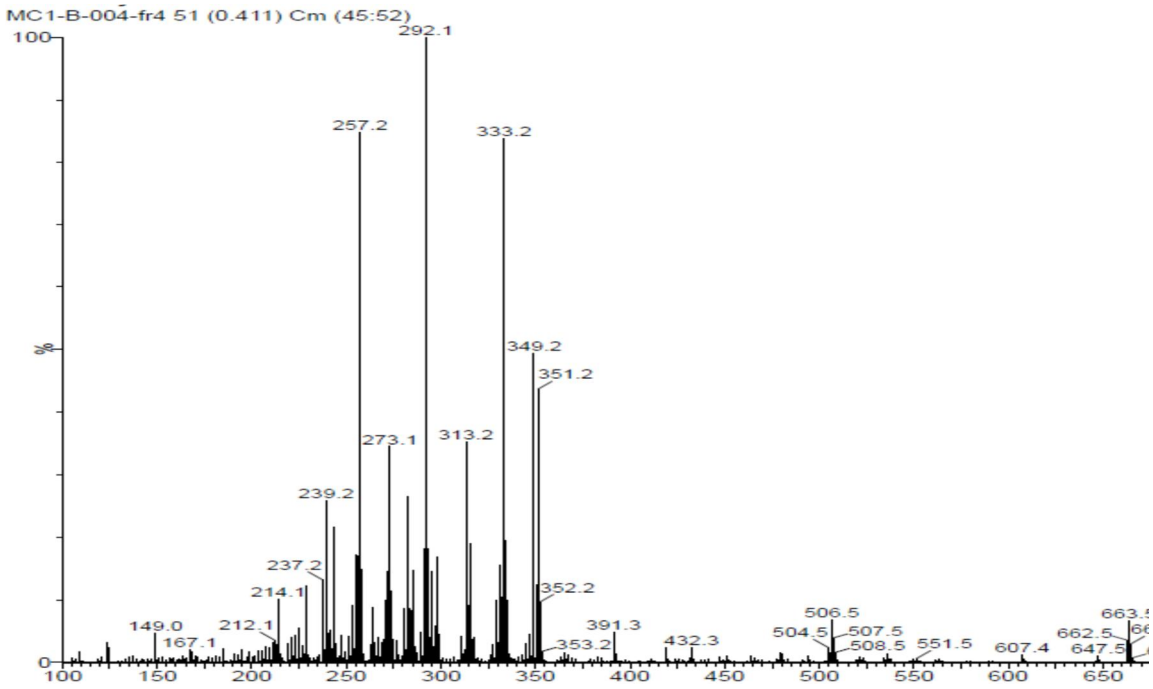
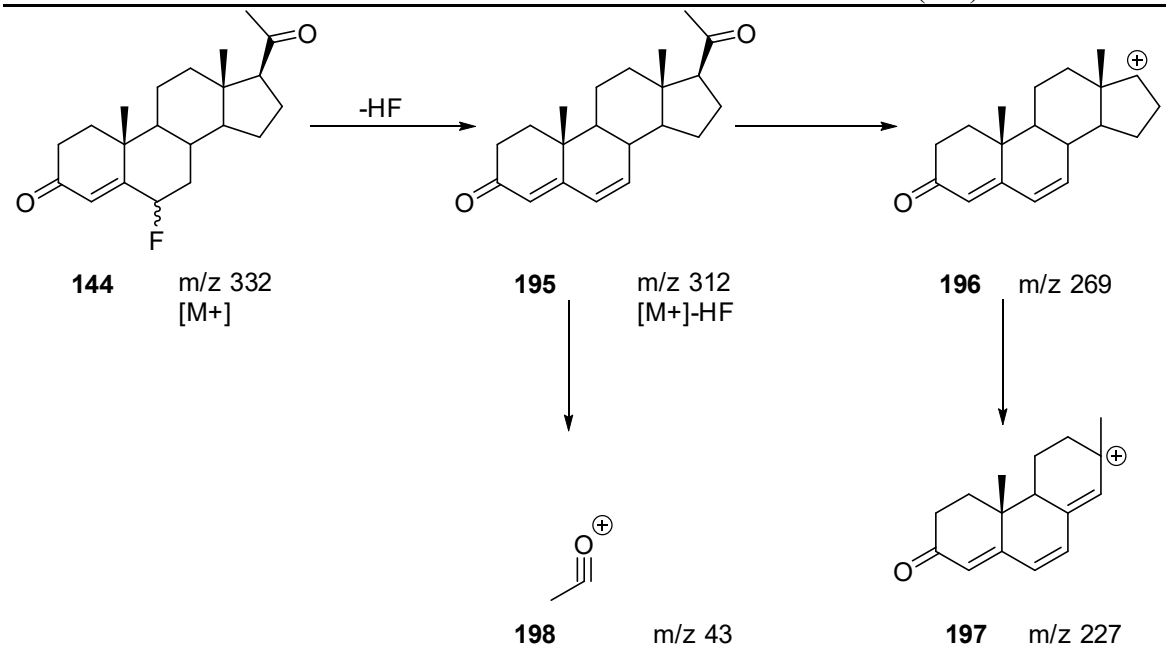


Figure 3.20 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 4.

Table 3.8 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 4.)

Entry	*m/z*	Proposed Metabolite
1	351 [M+1], 333 [M+1]-H_2O, 313 [M+1]-H_2O-HF	6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**)
2	349 [M+1], 273, 257, 237	6-Fluoro-16-hydroxy-4-pregnen-3,20-one (**216**)



Scheme 3.12 Proposed GC-MS fragmentation pattern for 6-fluoro-16-hydroxy-4-pregnen-3,20-one in fraction 4.

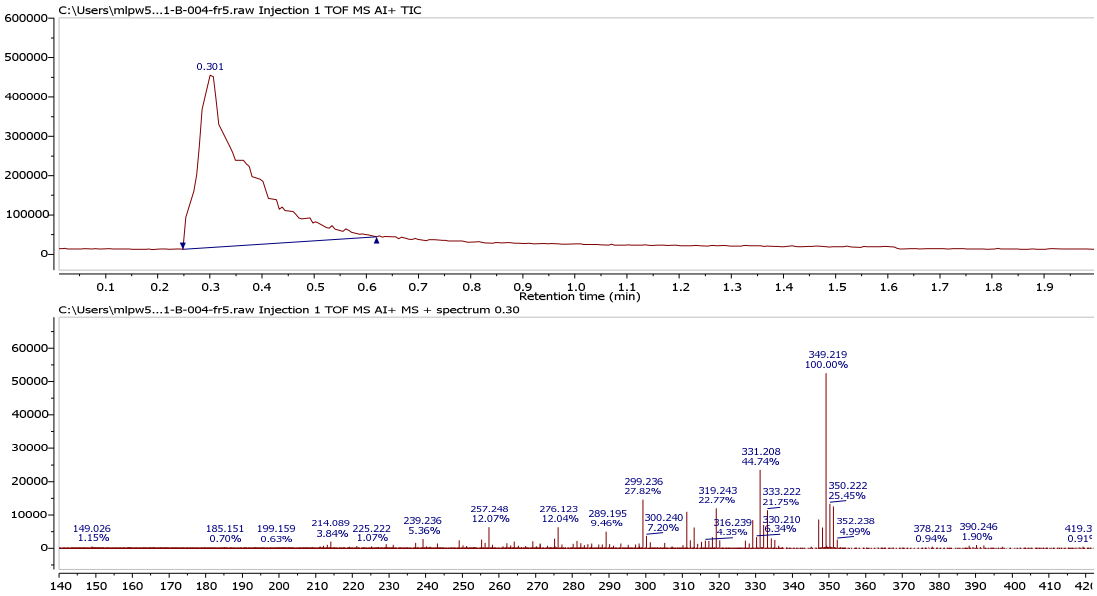


Figure 3.21 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 5.

Table 3.9 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 5.)

Entry	*m/z*	Proposed Metabolite
1	349 [M+1], 331, 299, 257	6-Fluoro-16-hydroxy-4-pregnen-3,20-one (**216**)

As mentioned earlier, fraction 5 showed an abundant ion at *m/z* 349 which could be attributed to 6-fluoro-16-hydroxy-4-pregnen-3,20-one (**216**). Formation of this compound was further supported by the presence of ions at *m/z* 331 [M+1]-H_2O (**217**), 299 (**219**) and 257 (**222**) (**Scheme 3.12**).

Fraction 6 contained two fluorinated compounds based on ^{19}F NMR analysis. GC-MS suggested presence of 6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**) (*m/z* 351 [M+1], minor component) and 6-fluoro-3-hydroxy-4-pregnen-20-one (**215**) (*m/z* 335 [M+1]). Fragmentation of 6-fluoro-3-hydroxy-4-pregnen-20-one (**215**) was described earlier in **Scheme 3.11** (fraction 3), showing fragments at *m/z* 315 [M+1]-HF (**199**), 297 [M+1]-HF–H_2O (**200**) and *m/z* 253 (**201**). However, a different fragmentation pattern was observed for fraction 6 suggesting that both compounds present in fraction 6 and 3 are diastereoisomers (possibly at 3-C) or structural isomers (e.g. reduction of 3-C vs 20-C by bacteria). Fragmentation of the compound observed in fraction 6 is shown in **Scheme 3.13**. The protonated molecule is the starting point of the proposed dissociation pathway that leads to

cleavages of the bonds between 20-C and 21-C as well as 17-C and 20-C accompanied by the elimination of HF.

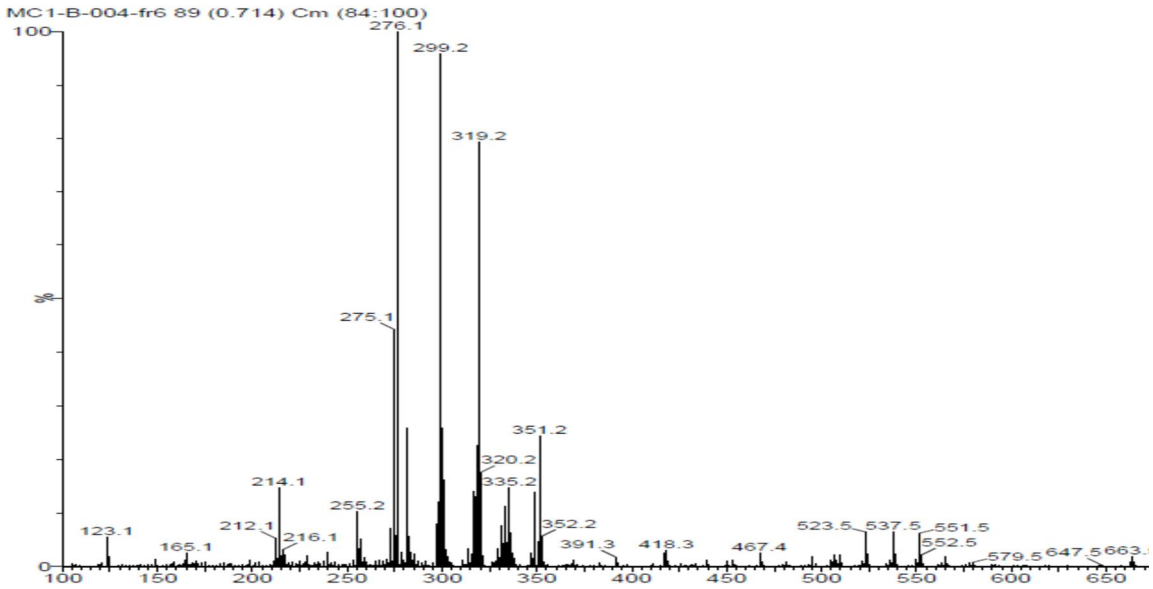
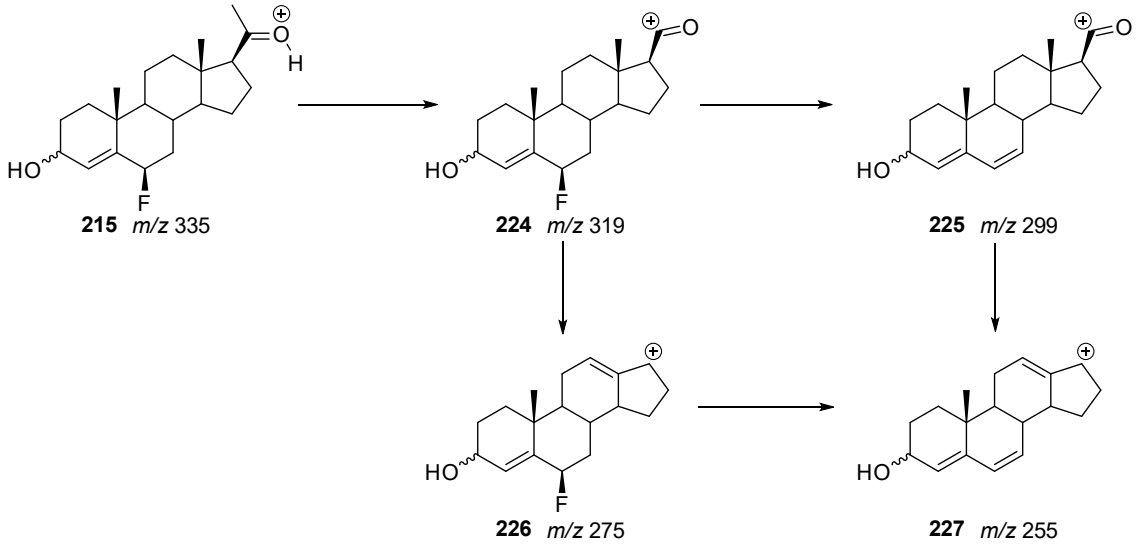


Figure 3.22 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 6.

Table 3.10 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 6.)

Entry	*m/z*	Proposed Metabolite
1	351 [M+1]	6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**)
2	335 [M+1], 319, 299, 275, 255	6-fluoro-3-hydroxy-4-pregnen-20-one (**215**)



Scheme 3.13 The ion mass of most abundant ion and proposal of mechanism for fraction 6.

The most abundant ion of fraction 7 appeared at *m/z* 351. This ion was described previously and was assigned to 6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**) ([M+1]). The fragmentation pathway of ion at *m/z* 351 in fraction 7 differs to that described earlier in fraction (351 [M+1], 333 [M+1]-H_2O, 315, 295, 279, 211; **Scheme 3.10**). The ions at *m/z* 315, 295, 279 and 211 are not observed, instead the ion at *m/z* 333 [M+1]-H_2O (**210**) loses water and the methyl group 21-C to produce ion at *m/z* 299 (**228**). It is highly possible that both fractions contained the diastereoisomer of 6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**) (3-C and/or 16-C). Alternatively, 6β-fluoro-progesterone (**144**) may have been hydroxylated at a position other than 16-C- (most likely alternatives are 11-C or 12-C).

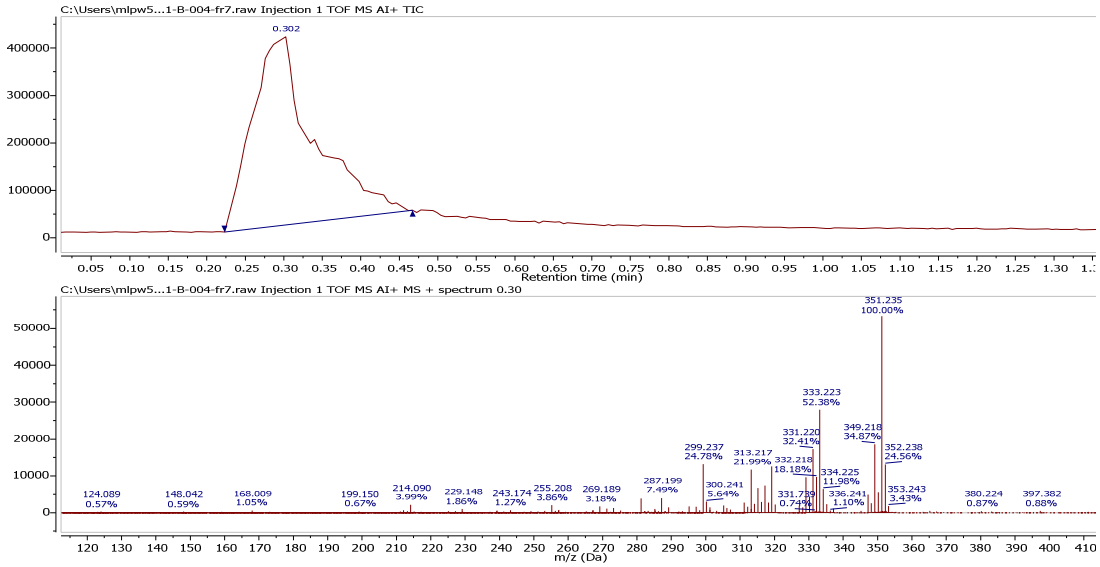
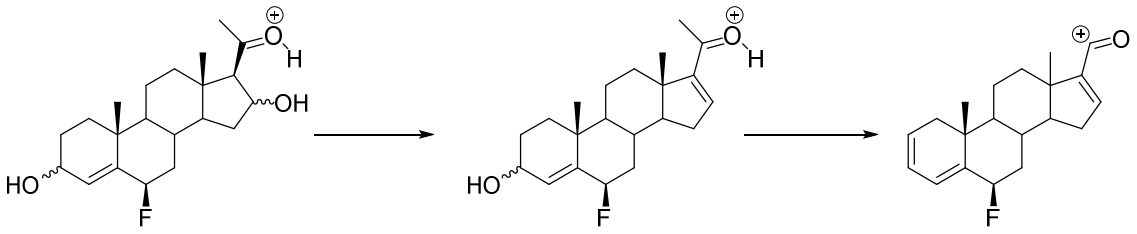


Figure 2.23 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 7.

Table 3.11 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 7.)

Entry	*m/z*	Proposed Metabolite
1	351 [M+1], 333, 299	6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**)



209 *m/z* 351 **210** *m/z* 333 **228** *m/z* 299

Scheme 3.14 The ion mass of most abundant ion and proposal of mechanism for fraction 7.

Fraction 8 mainly contained 6β-fluoro-progesterone (**144**) based on the evidence from the GC-MS analysis and TLC. The molecular ion [M+1] at *m/z* 333 (**144**) fragments by losing HF to produce ion at *m/z* 313 (**312**). The minor component of fraction 8 is the streptomycin (*m/z* 581, [M^+] (**229**); **Scheme 3.15**) antibiotic produce by this bacteria. This molecule fragments by losing the methyl group and the aldehyde group to produce ions at *m/z* 566 (**230**) and 552 (**231**) respectively.

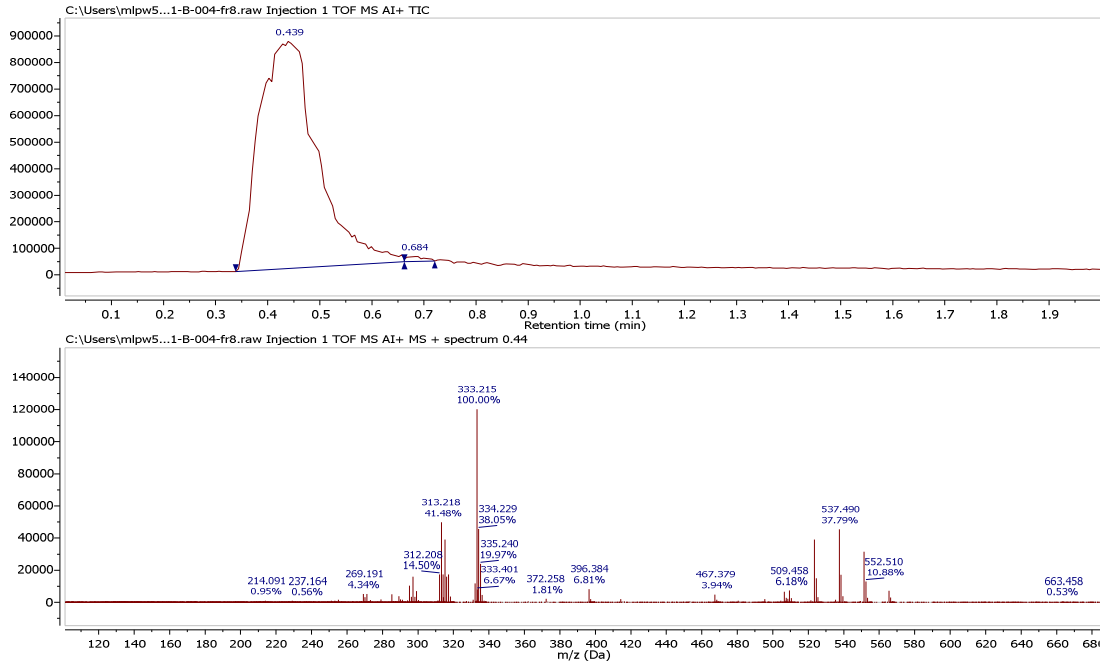
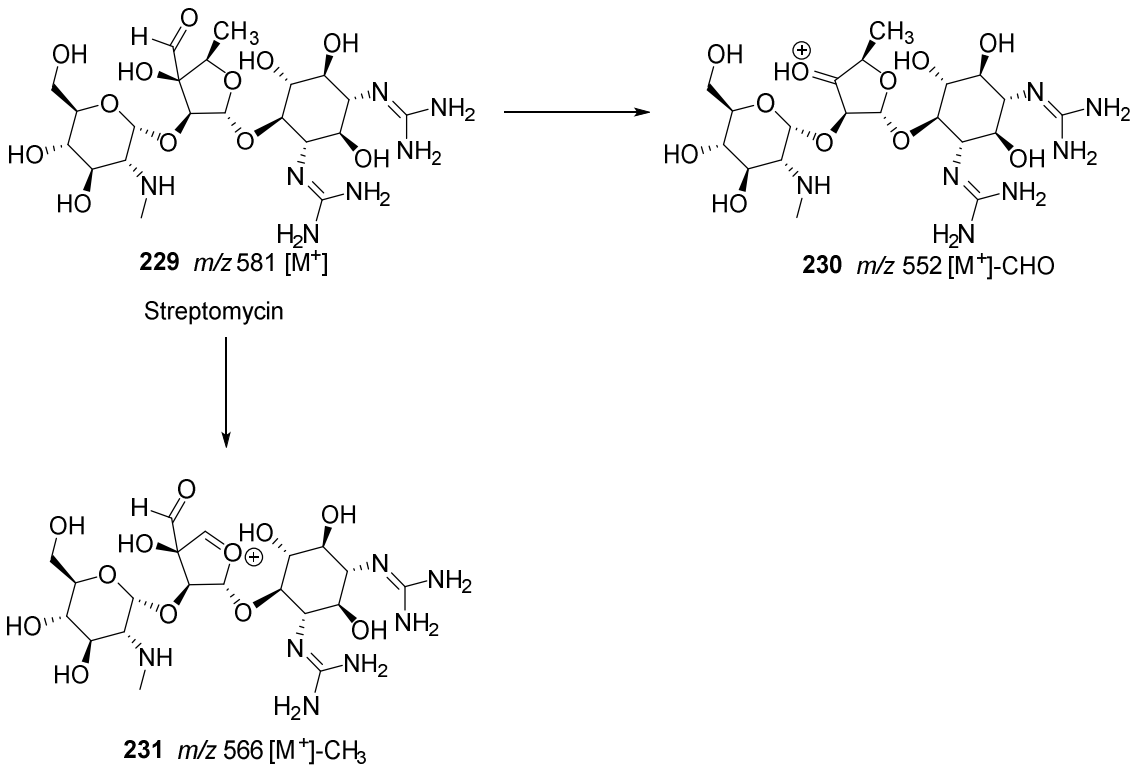


Figure 3.24 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 8.

Table 3.12 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 8.)

Entry	*m/z*	Proposed Metabolite
1	333 [M+1], 313 [M+1]-HF	6-fluoro--progesterone (**144**)
2	581 [M+1], 566, 552	Streptomycin (**229**)



Scheme 3.15 Proposed minor metabolites for 6β-fluoro-progesterone (**144**) for fraction 8 and 9 is streptomycin antibiotic produce by *S. griseus*.

Fraction 9 does not contain steroids or fluorinated compounds and the major compound of this fraction is Streptomycin (**229**) (*m/z* 581, $[M]^{+\cdot}$) produced by the bacteria.

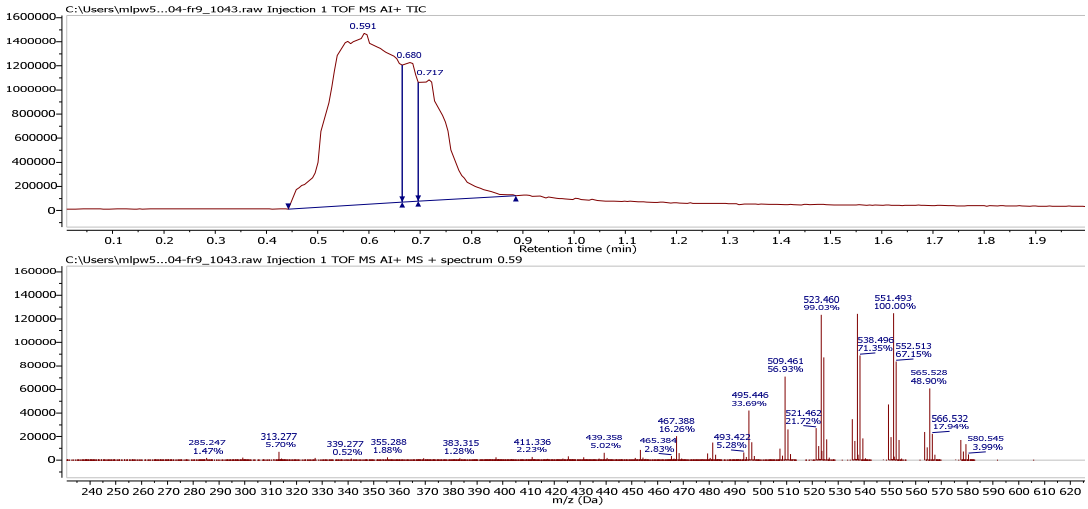
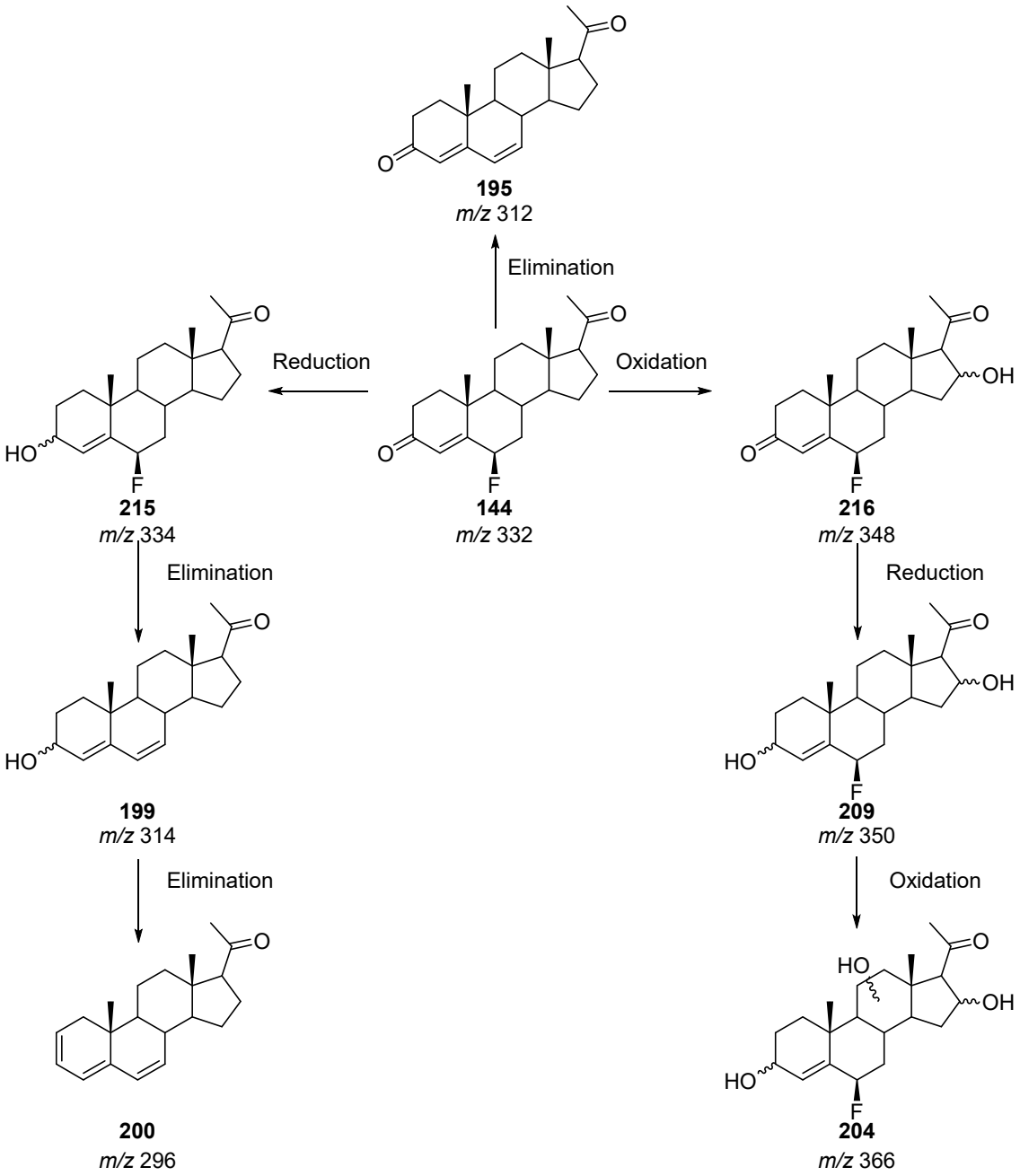


Figure 3.25 GC-MS spectrum for the transformation of 6β-fluoro-progesterone (**144**) by *S. griseus* for fraction 9.

Table 3.13 Summary of metabolites produced via the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus*. (Detected GC-MS ions in fraction 9.)

Entry	m/z	Proposed Metabolite
1	581 $[M]^{+\cdot}$, 566, 552	Streptomycin (**229**)

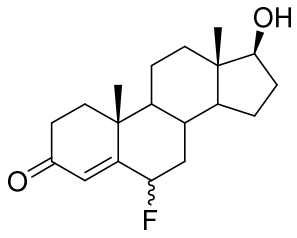
In summary, seven different metabolites are proposed (**Scheme 3.11**). It is highly probable that some of the steroids such as 3-hydroxy-4,6-pregnen-20-one (**199**), 2,4,6-pregnen-20-one (**200**) or 4,6-pregnen-3,20-dione (**195**) were formed during the isolation or purification stages via elimination of water or/and HF. Two metabolites were formed by reduction of 3-C carbonyl group (**215**) and hydroxylation at 16-C carbon (**216**) (16α-hydroxylation). These reactions are known and were reported in the literature for different steroids. As the ion at *m/z* 351 [M+1] was detected in 6 different fractions, it is highly probable that different diastereoisomers of 6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**) were formed (alternatively fluoro-progesterone was hydroxylated at a different position).



Steroid	144	215	199	200	216	209	204	195
Mass	332	334	314	296	348	350	366	312
Identified by GC-MS analysis of Fraction	8	3,6	1,2,3	2	4,5	1,2,3,4 ,6,7	1,2,3	2

Scheme 3.16 Summary of the compounds detected by GC-MS after the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus.*

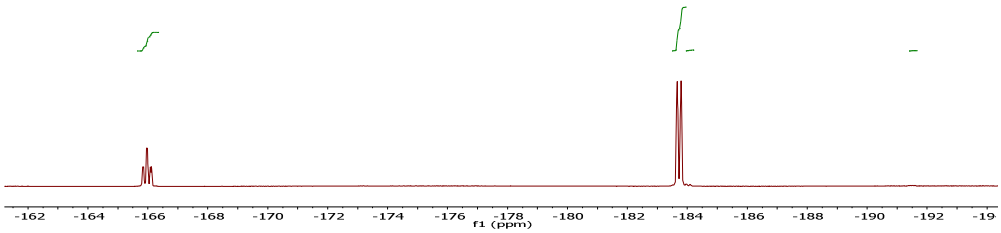
3.3.6 Feeding experiments with 6α/β-fluoro-testosterone (84)



84

The biological experiments with *S. griseus* and 6α/β-fluoro-testosterone (**84**) ware carried out according to the general procedure described in paragraph **3.3.1**. The organic and aqueous extracts were analysed using GC-MS/LC-MS and ^{19}F NMR. From ^{19}F NMR analysis it could be seen that only a few metabolites were generated by the bacteria (**Table 3.14**). Unfortunately, the concentration of these metabolites was very low and therefore only one metabolite was identified by GC-MS. This metabolite gave an ion at *m/z* 304 and it is most likely the product of the oxidation of 6α/β-fluoro-testosterone (**84**) (**Scheme 3.17**).

A- ^{19}F NMR spectral analysis of control (organic fraction)



B - ^{19}F NMR spectral analysis of Pellet (organic fraction)

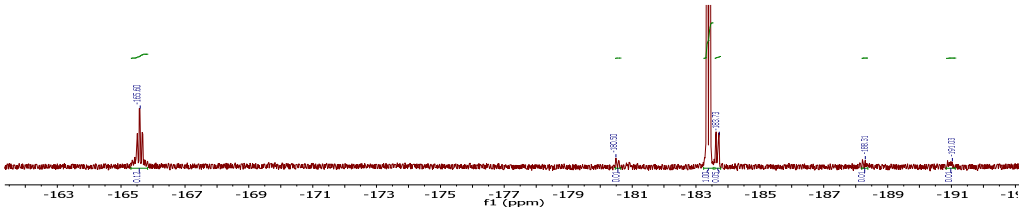
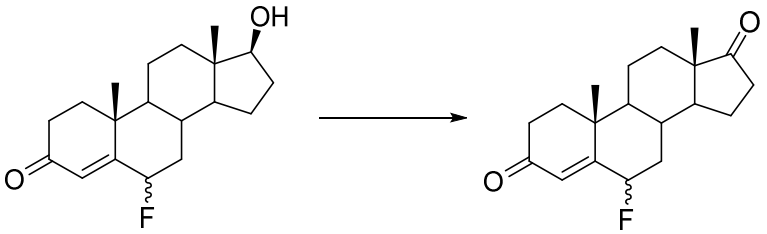


Figure 3.26 ^{19}F NMR analysis of 6α/β-fluoro-testosterone (**84**) incubated with *S. griseus*. **A-** ^{19}F NMR of control organic fraction in d-chloroform. **B-** ^{19}F NMR of the pellet organic fraction in d-chloroform. The other fractions (pellet and supernatant aqueous fraction in D_2O) show no peaks during ^{19}F NMR analysis.

Table 3.14 New metabolites shown by ^{19}F NMR analysis of 6α/β-fluoro-testosterone (**84**) incubated with *S. griseus*.

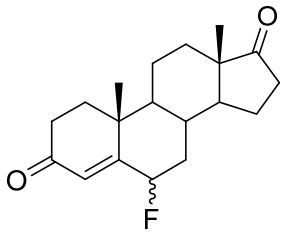
Experiment	^{19}F NMR analysis
Supernatant (aqueous)	No fluorinated metabolites detected.
Pellet (organic)	-95.74, -108.70, -123.4, -130.10, -148.50, -149.93, -151.74, -165.75, -180.50, -180.82, -184.35, -188.22
Pellet (aqueous)	No metabolites detected.



92 *m/z* 306 **5** *m/z* 304

Sheme 3.17 Detected GC-MS ions in transformation of 6α/β-fluoro-testosterone (**84**) by *S. griseus* and structure of proposed metabolites.

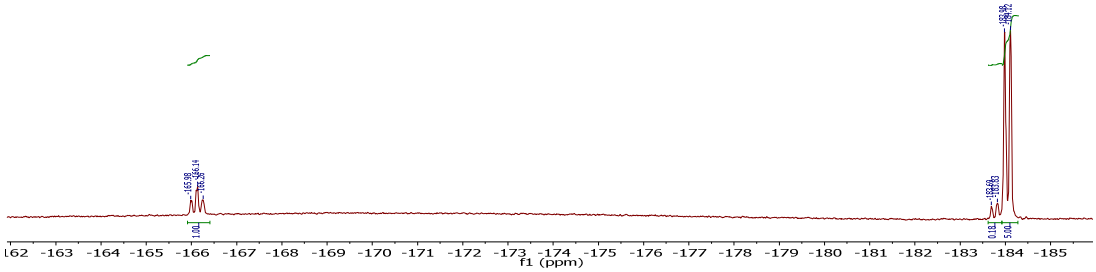
3.3.7 Feeding experiments with 6α/β-fluoro-androstenedione (5)



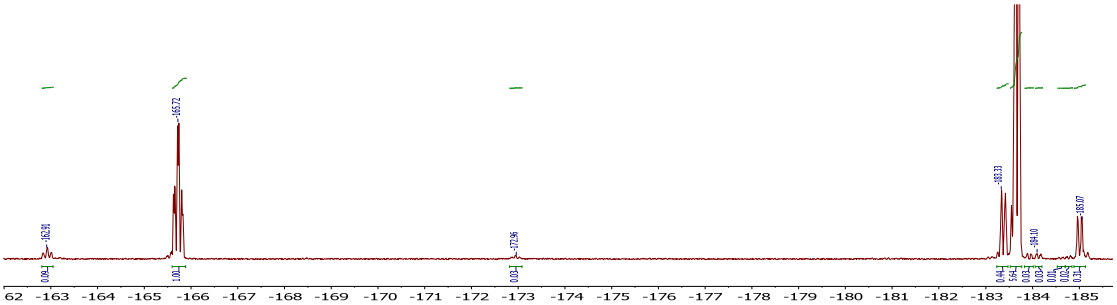
5

In a similar fashion *S. griseus* was fed with a mixture of 6α/β-fluoro-androstenedione (**5**) (sample contained a trace amount of 6α/β-fluoro-testosterone (**84**) which was used to make this starting material, signal at ^{19}F NMR signal at -183.45 ppm). The supernatants and pellets were extracted and analysed by ^{19}F NMR and GC-MS (**Figure 3.27**). All steroid metabolites were located in the pellet organic extracts. The aqueous phase of the supernatant and pellet did not contain any fluorinated compounds. The extraction of the supernatant gave only a trace amount of the starting material 6α/β-fluoro-androstenedione (**5**).

A – ^{19}F NMR spectral analysis of the Control (organic fraction)



B – ^{19}F NMR spectral analysis of the Pellet (organic fraction)



C - ^{19}F NMR spectral analysis of the Supernatant (organic fraction)

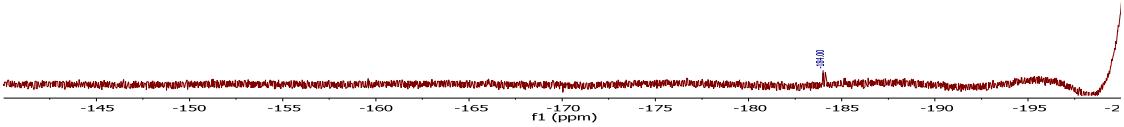


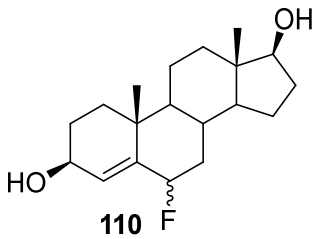
Figure 3.27 ^{19}F NMR analysis of 6α/β-fluoro-androstenedione (**5**) incubated with *S. griseus*. **A**- ^{19}F NMR of control organic fraction in d-chloroform. **B**- ^{19}F NMR of the pellet organic fraction in d-chloroform. **C**- ^{19}F NMR of supernatant organic fraction in d-chloroform. The other fractions (pellet and supernatant aqueous fraction in D_2O) showed no peaks in the ^{19}F NMR analysis.

Based on ^{19}F NMR analysis two metabolites could be detected (**Table 3.15**). Unfortunately, the concentration of these two compounds was very low and therefore identification by GC-MS was not possible (GC-MS showed the starting material and de-fluorinated compound only).

Table 3.15 New metabolites detected by ^{19}F NMR analysis of 6α/β-fluoro-androstenedione (**5**) incubated with *S. griseus*.

Experiment	^{19}F NMR Metabolites
Pellet (organic)	-122.56, -156.18, -172.96 (detected in control sample), -190.27 (detected in control sample)

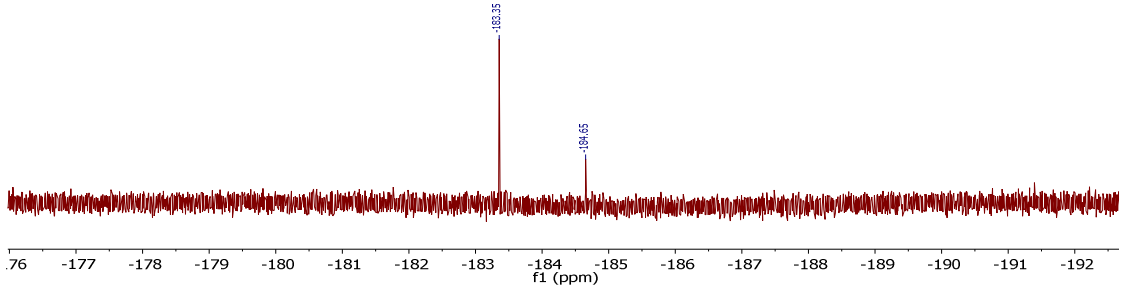
3.3.8 Feeding experiments with labelled 6α/β-fluoro-3β,17β–dihydroxyandrost-4-ene (110)



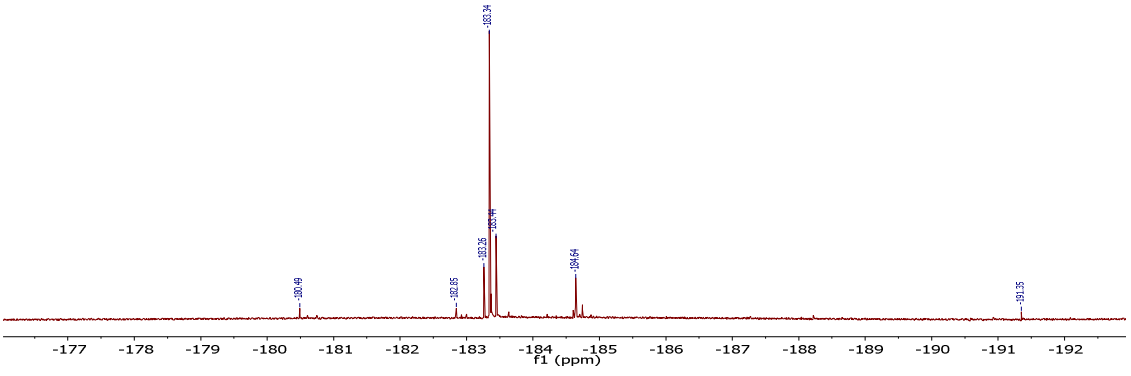
110

S. griseus was fed with a mixture of 6α/β-fluoro-3β,17β–dihydroxyandrost-4-ene (**110**). The aqueous extracts contained only the starting material and the organic phases were free from any fluorinated steroids. The ^{19}F NMR and GC-MS results are presented below **(Figure 3.28).**

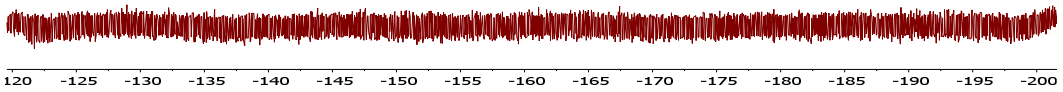
A – ^{19}F NMR (decoupled) spectral analysis of the Control (organic fraction)



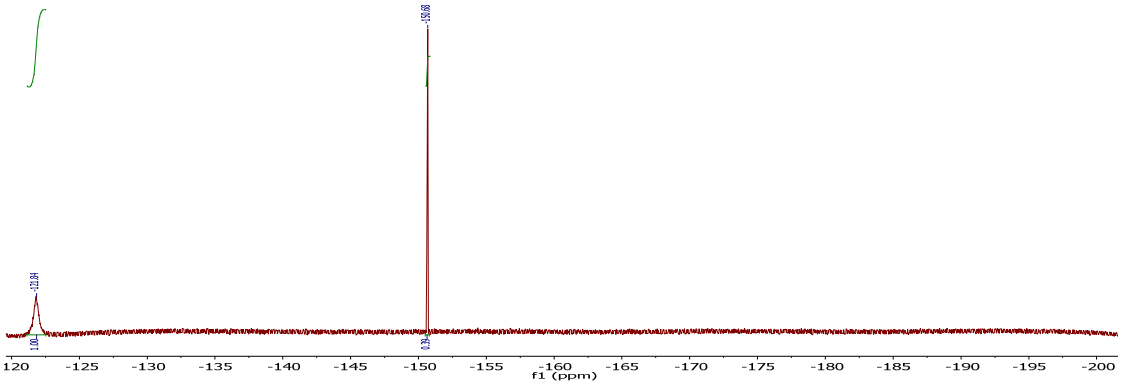
B – ^{19}F NMR (decoupled) spectral analysis of the Pellet (organic fraction)



C – ^{19}F NMR (decoupled) spectral analysis of the Supernatant (organic fraction)



D – ^{19}F NMR (decoupled) spectral analysis of the Supernatant (aqueous fraction)



E – ^{19}F NMR (decoupled) spectral analysis of the Pellet (aqueous fraction)

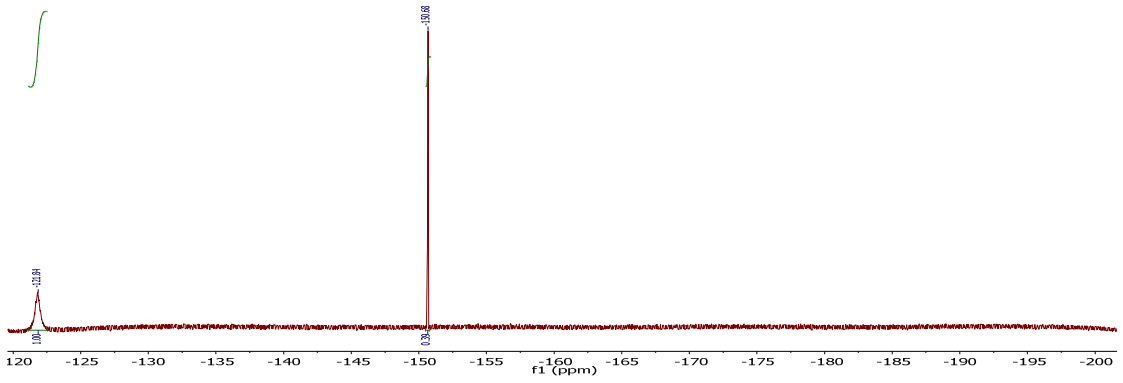
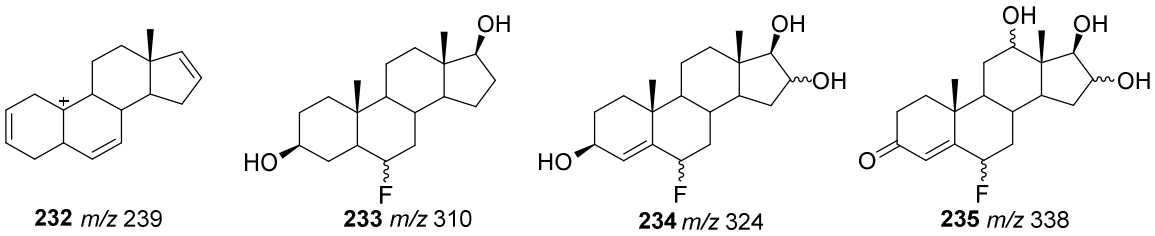


Figure 3.28 ^{19}F NMR (decoupled) analysis of 6α/β-fluoro-3β,17β–dihydroxyandrost-4-ene (**110**) incubated with *S. griseus*. **A**- ^{19}F NMR of control organic fraction in d-chloroform. **B**- ^{19}F NMR of pellet organic fraction in d-chloroform. **C**- ^{19}F NMR of supernatant organic fraction in d-chloroform. **D**- ^{19}F NMR of supernatant aqueous fraction in D_2O. **E**- ^{19}F NMR of pellet aqueous fraction in D_2O. The other fractions (pellet and supernatant aqueous fraction in D_2O) show no peaks during ^{19}F NMR analysis.

Table 3.16 New metabolites detected by ^{19}F NMR analysis of 6α/β-fluoro-3β,17β–dihydroxyandrost-4-ene (**110**) incubated with *S. griseus*.

Experiment	^{19}F NMR Signals (ppm)
Supernatant (organic)	No fluorinated metabolites detected.
Supernatant (aqueous)	- 150.68, -121.84
Pellet (organic)	-180.61, -180.75, -183.00, -184.21, -188.22
Pellet (aqueous)	- 150.68, -121.84

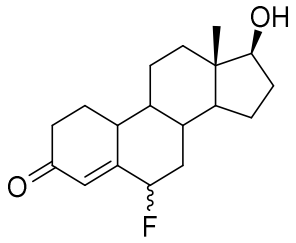
The organic phase of the supernatant contained ions in the GC-MS analysis that suggested the presence of steroids: *m/z* 239, *m/z* 310, *m/z* 324, *m/z* 338. The proposed structures for these ions are presented below (**Figure 3.29**). From the analysis of the GC-MS data, it appears that *S. griseus* hydroxylated the starting material (as discussed previously hydroxylation is most likely to occur at C-16, dihydroxylation at C-11/C-12) and reduced the double bond. Formation of the ion at *m/z* 239 (**232**) was generated through the elimination/fragmentation reaction which probably occurred during analysis. The organic extracts of the pellet contained one major compound by GC-MS. This compound gave ions at *m/z* 301 (M^+) and *m/z* 286 (M^+-CH_3). The structure of this potential metabolite is not easy to predict because an odd molecular weight suggests presence of an odd numbers of nitrogen atoms. Based on the ^{19}F NMR data, the organic extracts of the pellet contained mainly the starting material (peaks at -161.41 ppm and -184.19 ppm) what suggest that both compounds give similar fluorine spectrum. In addition to that, ^{19}F NMR analysis suggests formation of a few metabolites in small quantities.



232 *m/z* 239 **233** *m/z* 310 **234** *m/z* 324 **235** *m/z* 338

Figure 3.29 Proposed metabolites arising from 6α/β-fluoro-3β,17β–dihydroxyandrost-4-ene (**110**) incubation with *S. griseus*.

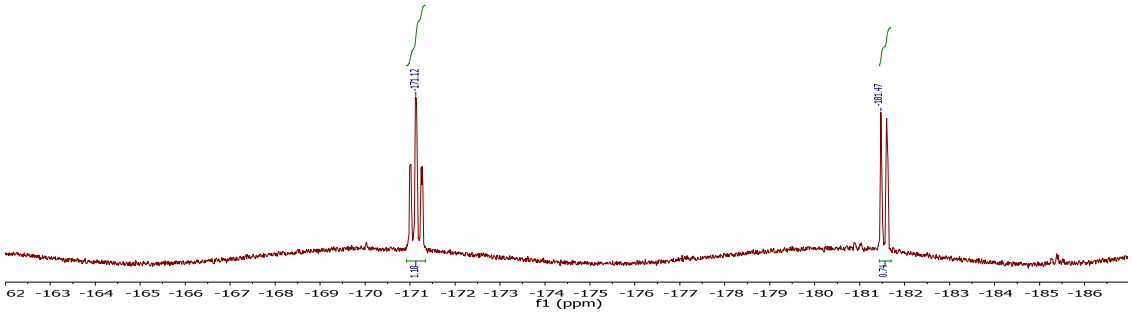
3.3.9 Feeding experiment with labelled 6-fluoro-nortestosterone (86)



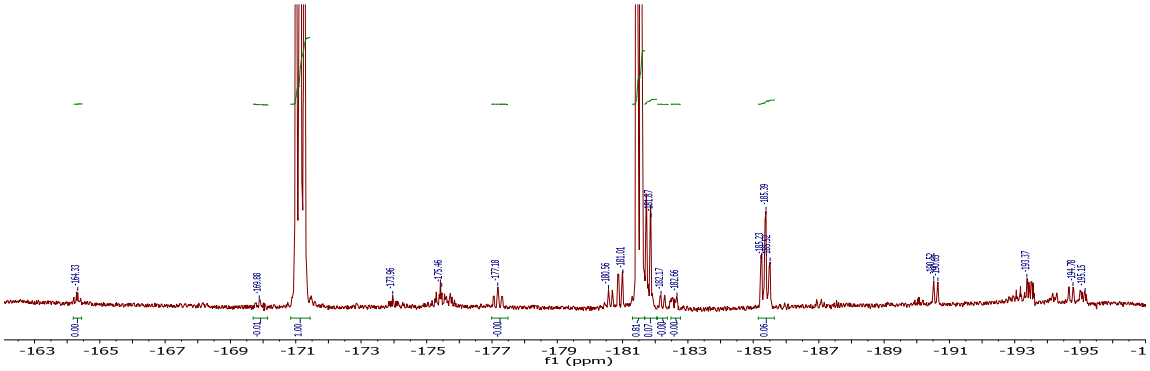
86

When *S. griseus* was fed with a mixture of 6α/β-fluoro-nortestosterone (**86**) few metabolites were generated. All of the steroids metabolites were found to be located in the pellet and supernatant organic extracts. The aqueous phase of the pellet contained F^- ions (-122.2 ppm). The results are summarised in the table below.

A – ^{19}F NMR spectral analysis of the Control (organic fraction)



B – ^{19}F NMR spectral analysis of the Pellet (organic fraction)



C – ^{19}F NMR spectral analysis of the Supernatant (organic fraction)

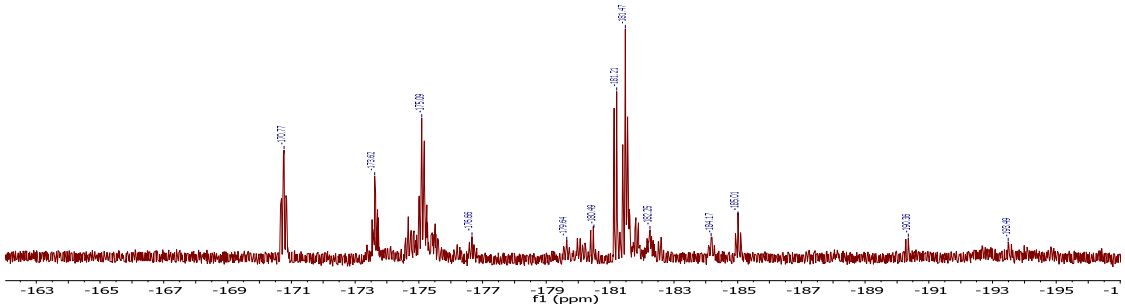


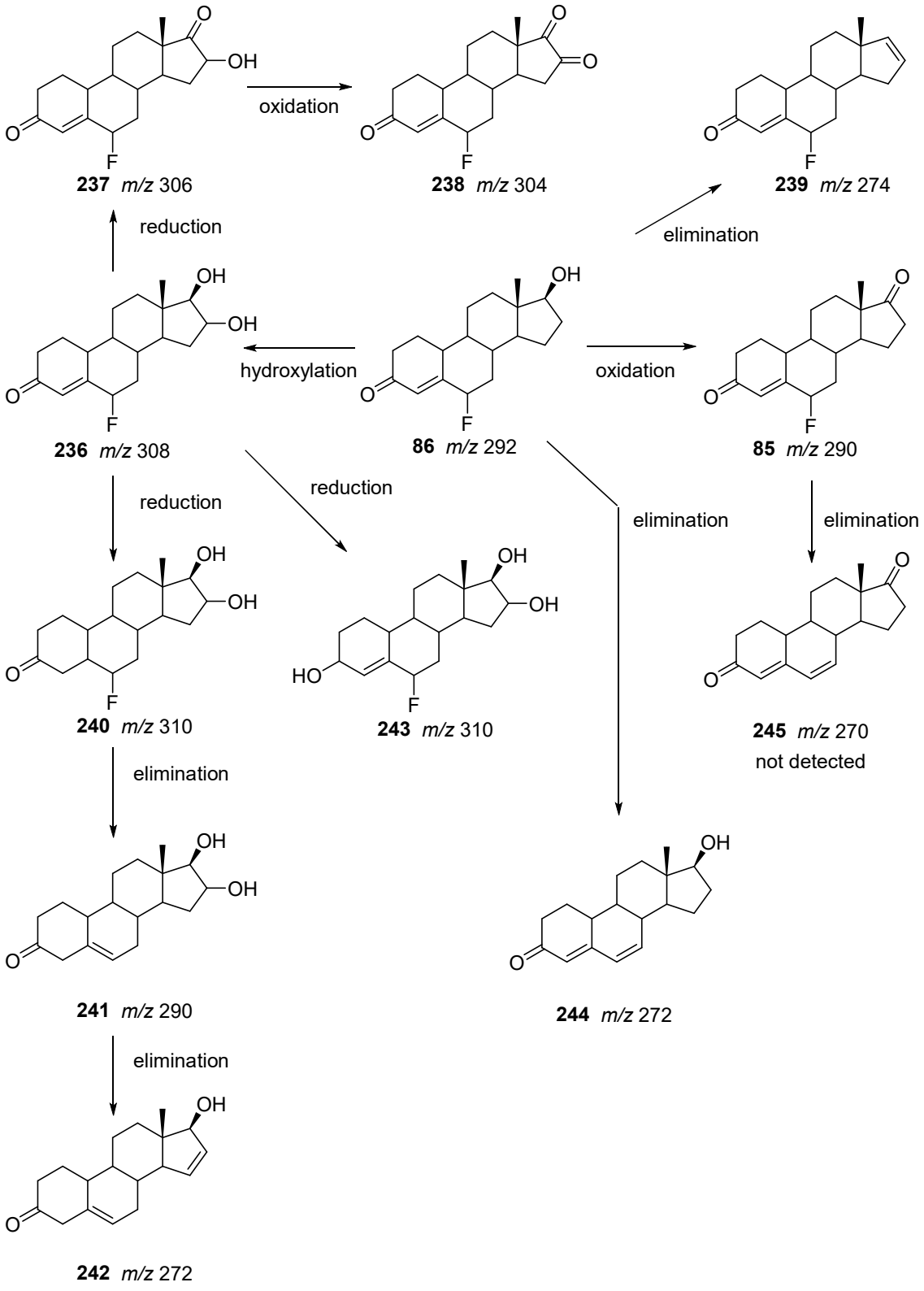
Figure 3.30 ^{19}F NMR analysis of 6α/β-fluoro-nortestosterone (**86**) incubated with *S. griseus*. **A**- ^{19}F NMR of the control organic fraction in d-chloroform. **B**- ^{19}F NMR of the pellet organic fraction in d-chloroform. **C**- ^{19}F NMR of the supernatant organic fraction in d-chloroform. The other fractions (pellet and supernatant aqueous fraction in D_2O) showed no peaks during ^{19}F NMR analysis.

Table 3.17 New matabolites detected by ^{19}F NMR analysis of 6α/β-fluoro-19-nortestosterone (**106**) incubated with *S. griseus*.

Experiment	^{19}F NMR Signal (ppm)
Supernatant (organic)	-173.62, -175.65, -176.66, -179.64, -180.49, -181.12, -181.81, -182.25, -182.61, -184.17, -185.01
Supernatant (aqueous)	No fluorinated metabolites detected.
Pellet (organic)	-164.33, -169.88, -173.96, -175.46, -177.18, -180.56, -181.01, -182.17, -182.66, -185.39, -190.52, -193.37, -194.78, -195.15
Pellet (aqueous)	No metabolites detected.

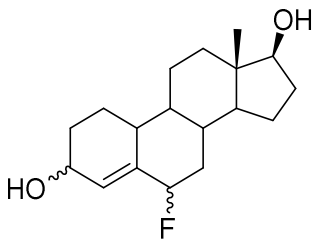
In comparison to 6α/β-fluoro-testosterone (**84**), 6α/β-fluoro-nortestosterone (**86**) underwent more extensive metabolism in *S. griseus*. The organic phase of the supernatant contained ions suggesting presence of 5 different metabolites: *m/z* 308, *m/z* 306, *m/z* 304, *m/z* 290 and *m/z* 272. The proposed structures for these ions are presented in **Scheme 3.18**. One of the major metabolites appears to be the hydroxylated starting material (probably hydroxylation at 16-C). This compound could be then transformed into three different metabolites. Ions at *m/z* 306 (**237**) and *m/z* 304 (**238**) suggest oxidation of the hydroxylated starting material to di- and tri-carbonyl compound respectively. The hydroxylated starting material could be also reduced. This was supported by the presence of ions at *m/z* 310 in the pellet organic fraction (**240**, **243**). Presence of ion at *m/z* 290 (**85**) was an effect of

oxidation of the starting material. Elimination of HF from the starting material led to the formation of ion at *m/z* 272 (**244**). The pellet (organic fraction) contained products most likely generated through reduction (*m/z* 310) and elimination reactions (*m/z* 290, *m/z* 272, *m/z* 274). The ion in the GC-MS at *m/z* 310 could be generated through the reduction of the mono hydroxylated metabolite (*m/z* 308). Elimination of HF and water from the reduced metabolite led to the formation of ions at *m/z* 290 and *m/z* 272 respectively. Ions at *m/z* 274 suggested elimination of water from 6α/β-fluoro-nortestosterone (**86**).



Scheme 3.18 Proposed metabolites arising from the incubation of 6α/β-fluoro-nortestosterone (**86**) in *S. griseus*.

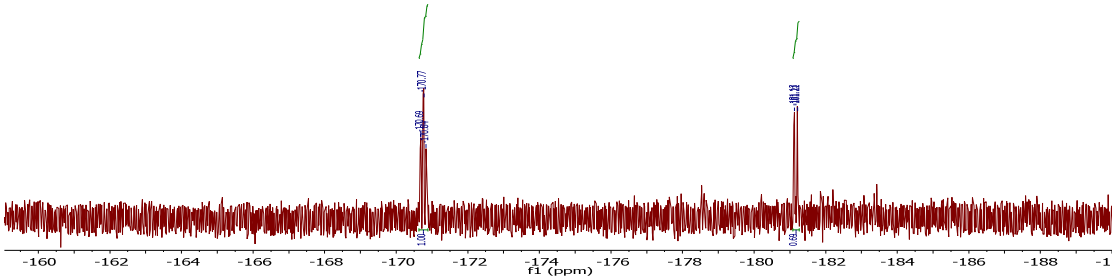
3.3.10 Feeding experiments with 6α/β-fluoro-norandrostenediole (85)



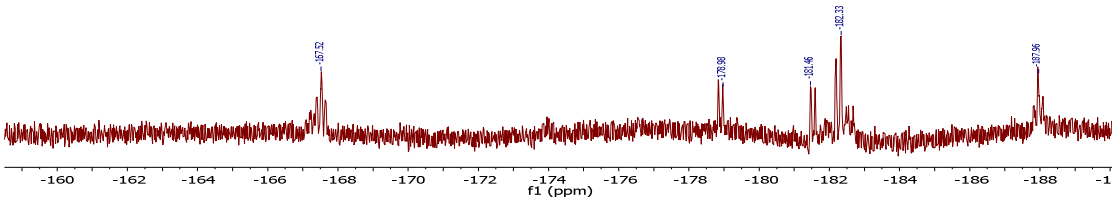
85

When *S. griseus* was fed with a mixture of 6α/β-fluoro-norandrostenediole (**85**) it was found to generate several metabolites. All of fluorinated steroid metabolites were located in the pellet and supernatant organic extracts. The aqueous phase of the pellet contained F^- ions (^{19}F NMR peak at -122.2 ppm) and possibly a non-steroidal compound (-150.5 ppm). The results obtained are summarised in **Table 3.18**.

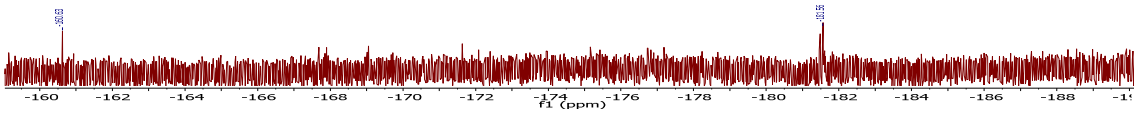
A – ^{19}F NMR spectral analysis of the Control (organic fraction)



B – ^{19}F NMR spectral analysis of the Pellet (organic fraction)



C – ^{19}F NMR spectral analysis of the Supernatant (organic fraction)



D – ^{19}F NMR (decoupled) spectral analysis of the Pellet (aqueous fraction)

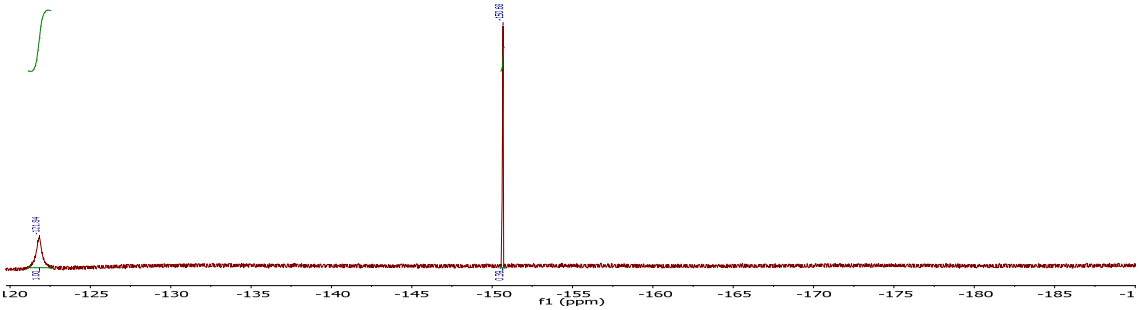


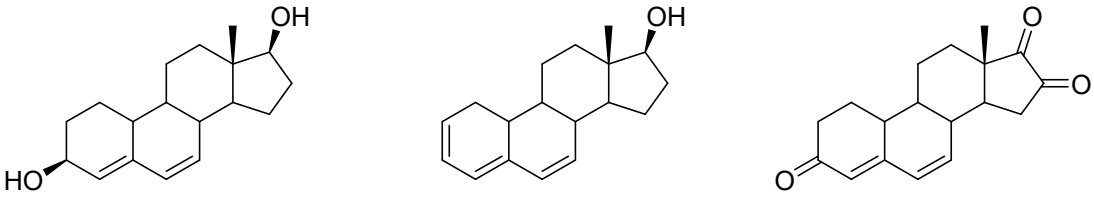
Figure 3.31 ^{19}F NMR analysis of 6α/β-fluoro-norandrostenediole (**85**) incubated with *S. griseus*. **A-** ^{19}F NMR of control organic fraction in d-chloroform. **B-** ^{19}F NMR of pellet organic fraction in d-chloroform. **C-** ^{19}F NMR of supernatant organic fraction in d-chloroform. **D-** ^{19}F NMR (decoupled) of pellet aqueous fraction in D_2O. The other fractions (pellet and supernatant aqueous fraction in D_2O) show no peaks during ^{19}F NMR analysis.

Table 3.18 New metabolites detected by^{19}F NMR analysis of 6α/β-fluoro-norandrostenediole (**85**) incubated with *S. griseus*.

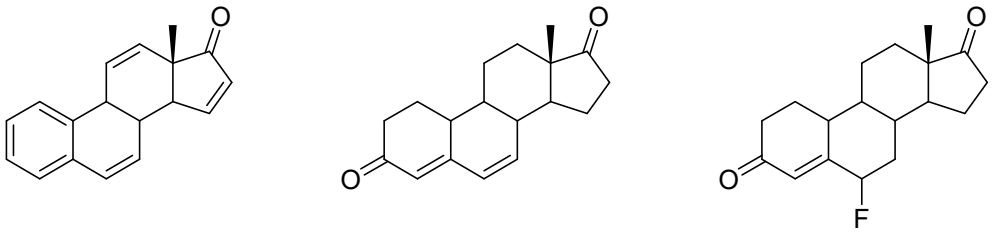
Experiment	^{19}F NMR signals (ppm)
Supernatant (organic)	-170.43
Supernatant (aqueous)	No fluorinated metabolites detected
Pellet (organic)	-167.18, -178.86, -181.59, -187.91
Pellet (aqueous)	No fluorinated steroid metabolites (-122.24 fluorine-ions, -150.52 non steroid molecule)

Analysis of the GC-MS data suggested formation of three compounds presented in **Figure 3.32**. Interestingly the starting material was not detected during this analysis. This suggests that elimination of HF could occur during incubation/isolation or on the GC

column and therefore ion at *m/z* 274 was detected. Ion at *m/z* 256 suggests elimination of water and HF from the starting material. This ion could also be formed during GC-MS analysis. Ion at *m/z* 284 corresponds to the oxidised starting material. It is possible that *S. griseus* first hydroxylated the starting material at position C-16 and then oxidised the hydroxyl groups (HF elimination could occurred during GC-MS analysis). Unfortunately, no corresponding intermediates to support this theory were detected. The main component of the supernatant organic fraction is a compound with a molecular ion *m/z* 248 [M^+] (**249**) and fragmentation ion at *m/z* 233 [M^+-CH3]. This steroid probably originated from complex metabolic transformations (oxidation/reduction/elimination) and therefore its structure is difficult to predict. The figure below presents just an example of compound with *m/z* 248 (**249**), which could potentially be formed via oxidation of 17-C, hydroxylation at 12-C and 16-C, elimination of hydroxyl groups and aromatization. Ions at *m/z* 290 [M^+] (**89**) and *m/z* 270 [M^+-HF] (**245**) were also detected suggesting oxidation of two hydroxyl groups.



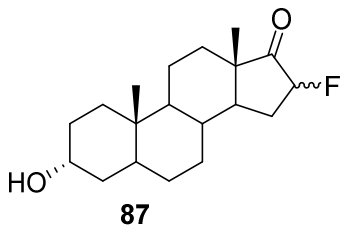
246 *m/z* 274 **247** *m/z* 256 **248** *m/z* 284



249 *m/z* 248 **245** *m/z* 270 **89** *m/z* 290

Figure 3.32 Proposed metabolites for 6α/β-fluoro-norandrostenediole (**85**)

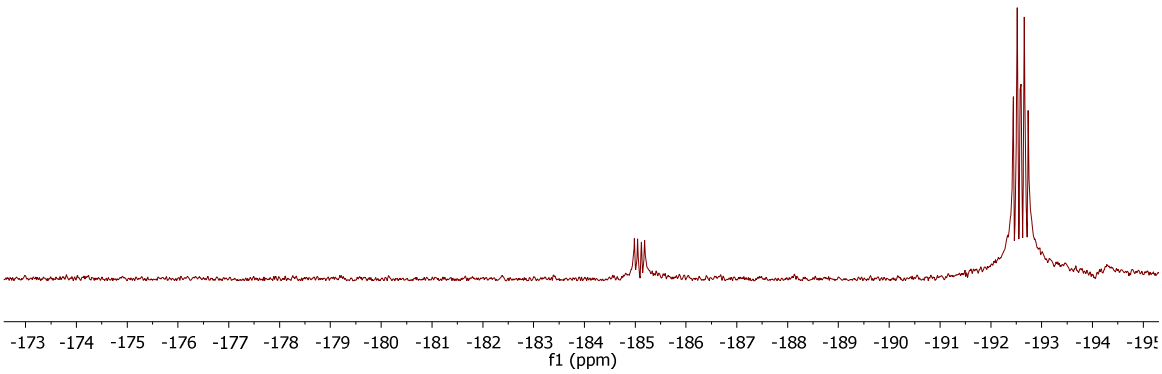
3.3.11 Feeding experiments with 16α/β-fluoro-androsterone (87)



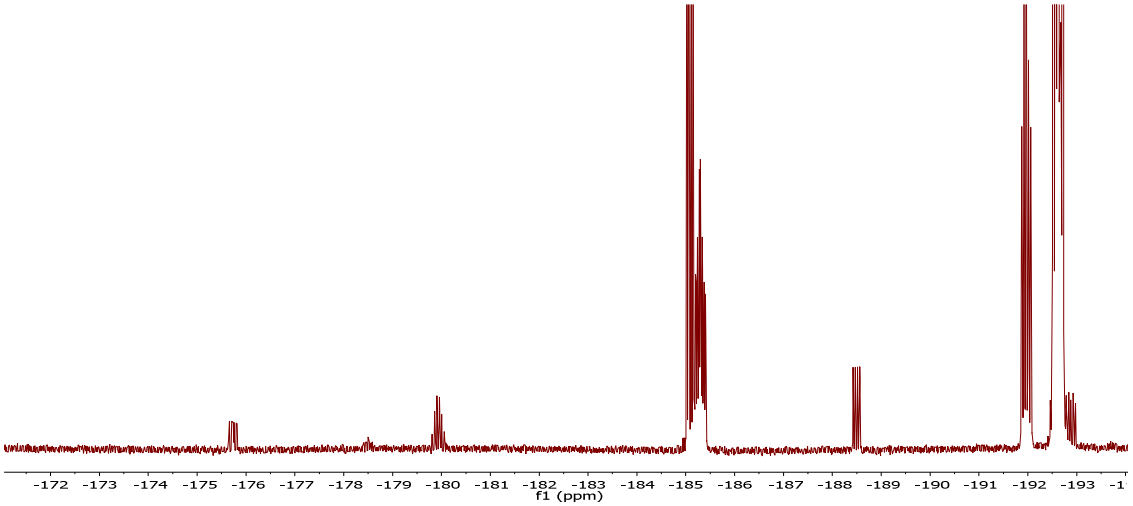
87

The following feeding experiment was carried out with 16α/β-fluoro-androsterone (**87**) and *S. griseus*. Upon completion of the experiment the mixture was worked-up and the extracts were analysed using ^{19}F NMR and GC-MS (**Figure 3.33**).

A – ^{19}F NMR spectral analysis of the Control (organic fraction)



B – ^{19}F NMR spectral analysis of the Pellet (organic fraction)



C – ^{19}F NMR spectral analysis of the Supernatant (organic fraction)

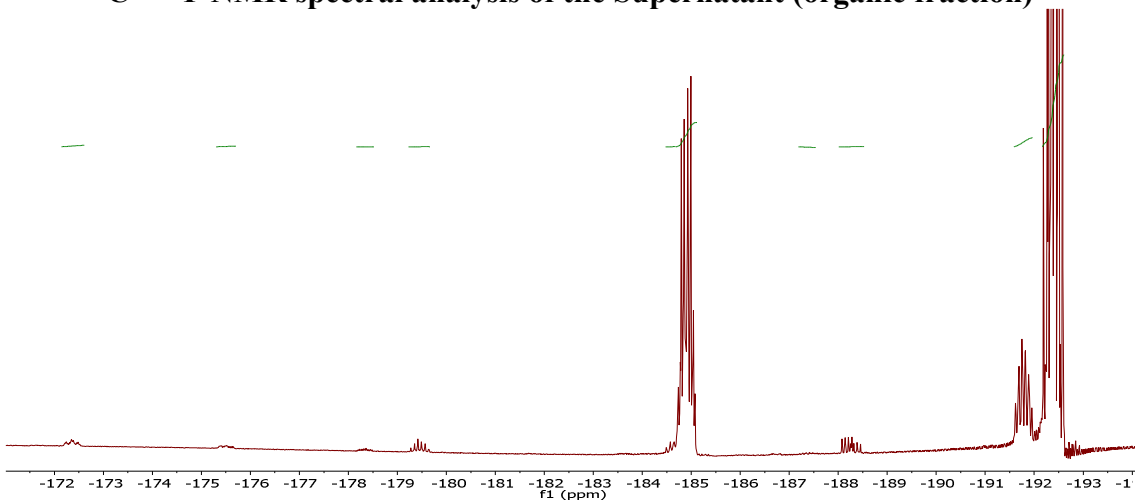


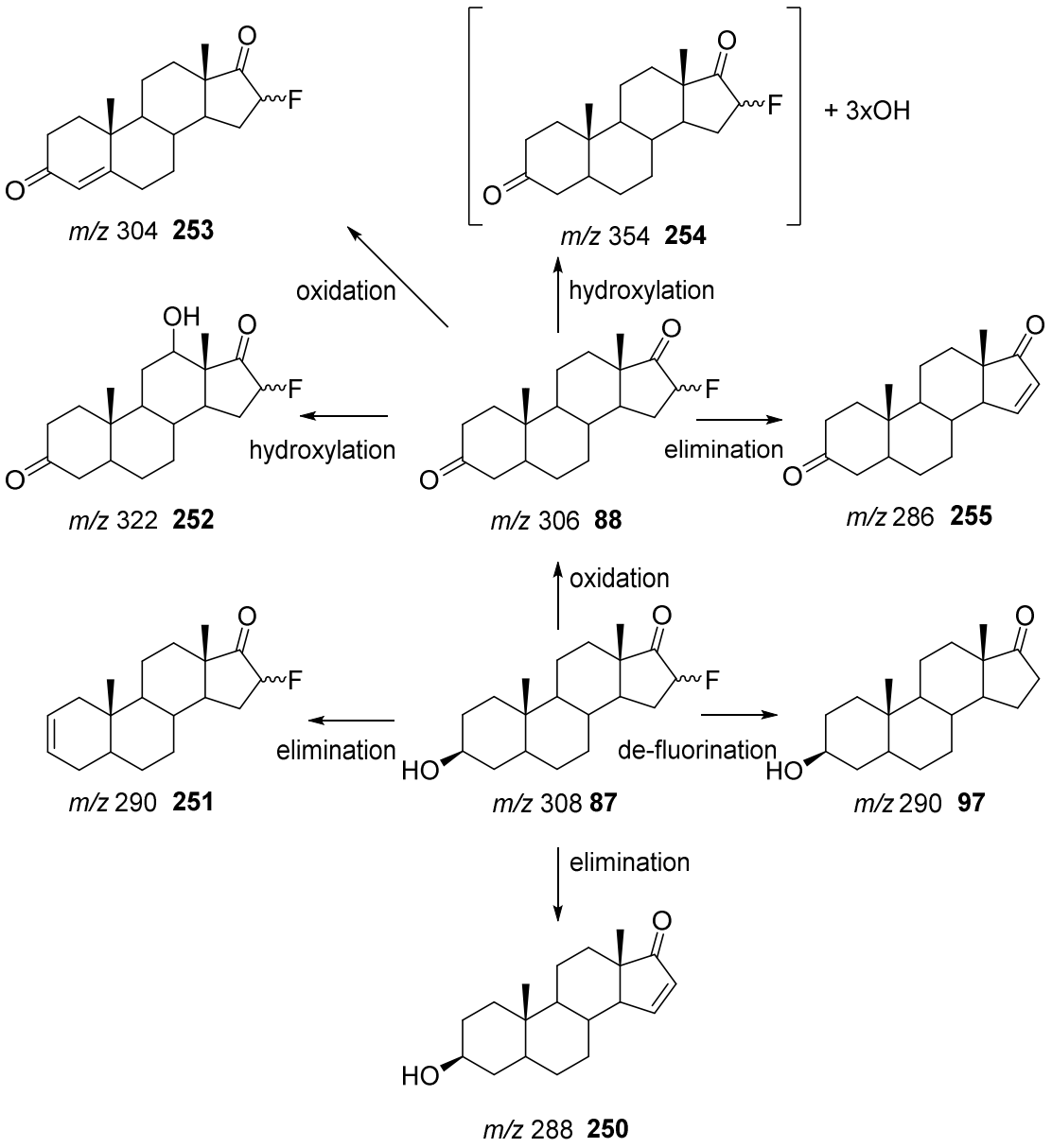
Figure 3.33 ^{19}F NMR analysis of 16α/β-fluoro-androsterone (**87**) incubated with *S. griseus*. **A**- ^{19}F NMR of control organic fraction in d-chloroform. **B**- ^{19}F NMR of pellet organic fraction in d-chloroform. **C**- ^{19}F NMR of supernatant organic fraction in d-chloroform. The other fractions (pellet and supernatant aqueous fraction in D_2O) showed no peaks during ^{19}F NMR analysis.

Table 3.19 New metabolites detected by ^{19}F NMR analysis of 16α/β-Fluoro-androsterone (**87**) incubated with *S. griseus*.

Experiment	^{19}F NMR signals (ppm)
Supernatant (organic)	-172.35, -175.49, -178.29, -179.45
Supernatant (aqueous)	No fluorinated metabolites detected.
Pellet (organic)	-175.79, -178.48, -179.96, -185.29, -192.89
Pellet (aqueous)	No fluorinated metabolites detected.

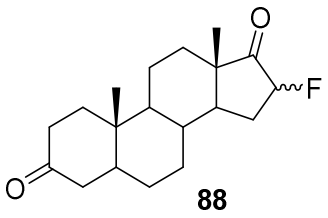
Based on ^{19}F NMR analysis 5 potential metabolites were detected in the pellet organic fraction and 4 potential metabolites in the supernatant. GC-MS analysis of the pellet organic fraction detected the starting material (**87**) (*m/z* 308, *m/z* 290 [M^+-H_2O]) and one potential metabolite at *m/z* 354 (**254**) presented in **Scheme 3.19**. In theory this metabolite could be formed by oxidation of hydroxyl group at 3-C and triple hydroxylation. However, intermediate metabolites were not detected (e.g. dihydroxylated molecule), therefore formation of a triple hydroxylated molecule would be unlikely. Ion at *m/z* 306 (**88**) was not present in the GC-MS spectra of the starting material and the control sample and corresponds to the major metabolite 16α/β-fluoro-5α-androstanedione (**88**). This molecule is then oxidised to the hydroxylated derivative (**252**) (*m/z* 322), reduced to unsaturated

ketone (**253**) (*m/z* 304) or transformed into non fluorinated steroid (**255**) (*m/z* 286). Ion at *m/z* 288 (**250**) and *m/z* 290 (**251**) was formed by elimination of HF and water respectively from the starting material. A less probable explanation for the formation of ion at *m/z* 290 (**97**) is de-fluorination of the starting material.



Scheme 3.19 Proposed metabolites for 16α/β-Fluoro-androsterone (**87**).

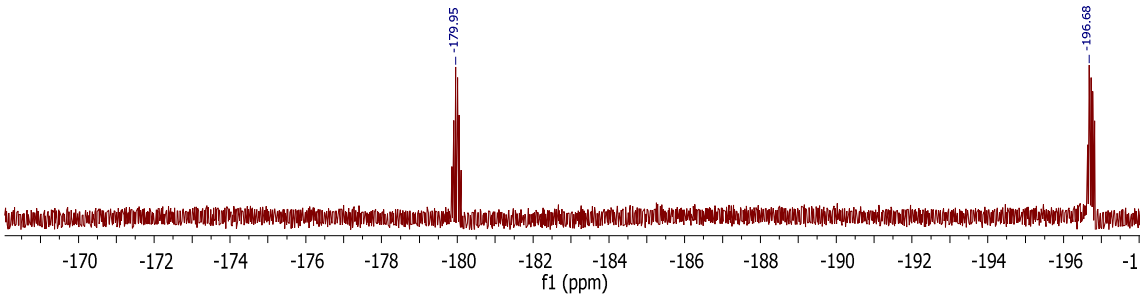
3.3.12 Feeding experiments with 16α/β-fluoro-5α-androstanedione (88)



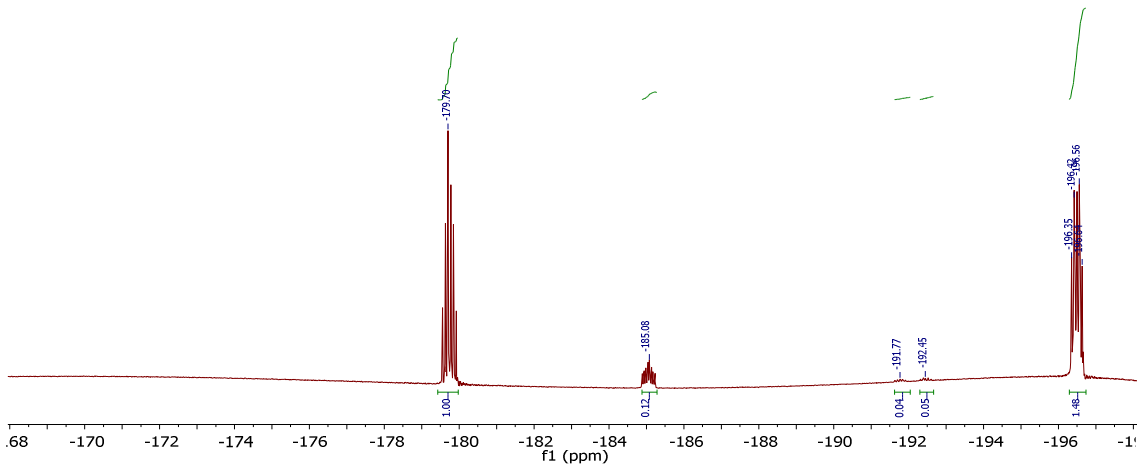
88

The experiment below was carried out with 16α/β-fluoro-androstenedione (**88**). As before *S. griseus* was fed with the steroid and upon completion of the experiment the bacteria were centrifuged and extraction was carried out. Analysis of the ^{19}F NMR and GC-MS data revealed formation of a few metabolites (**Figure 3.33**).

A – ^{19}F NMR spectral analysis of the Control (organic fraction)



B – ^{19}F NMR spectral analysis of the Pellet (organic fraction)



C – ^{19}F NMR spectral analysis of the Supernatant (organic fraction)

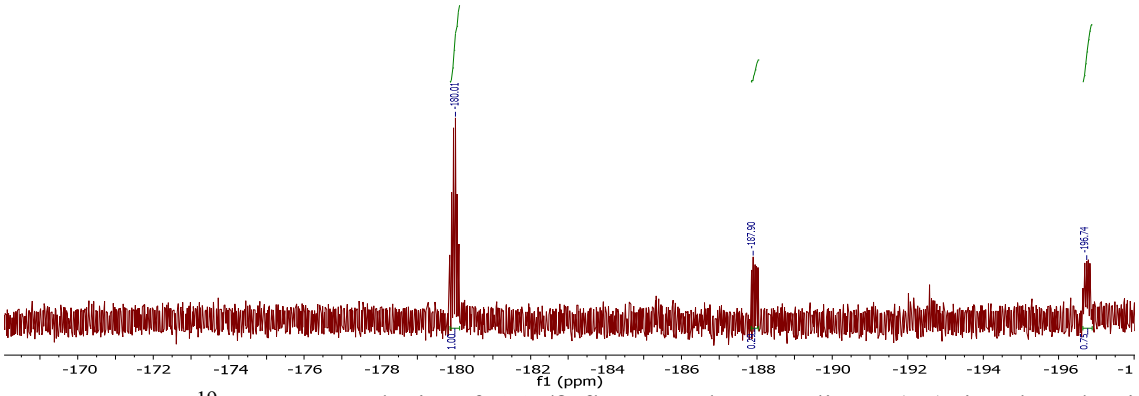


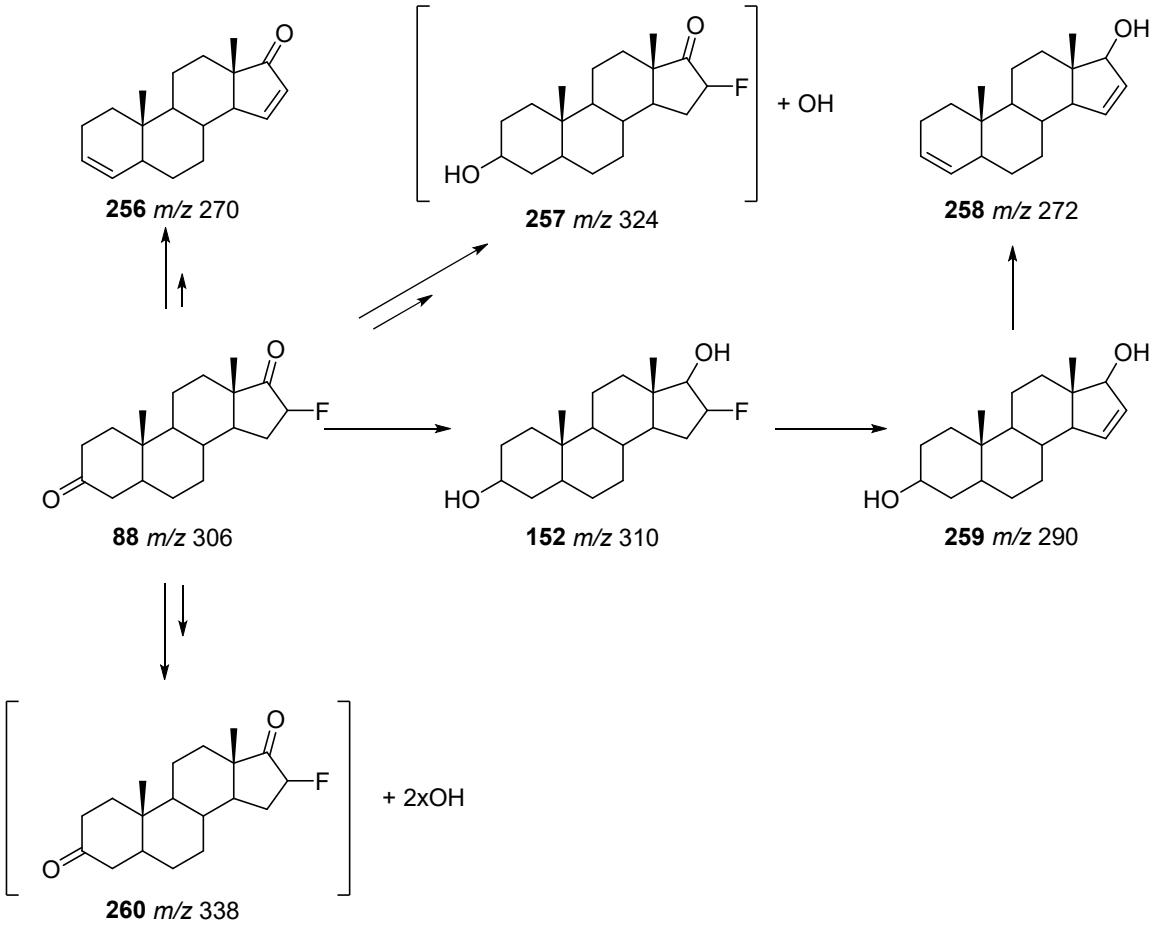
Figure 3.33 ^{19}F NMR analysis of 16α/β-fluoro-androstenedione (**88**) incubated with *S. griseus*.**A**- ^{19}F NMR of control organic fraction in d-chloroform. **B**- ^{19}F NMR of pellet organic fraction in d-chloroform. **C**- ^{19}F NMR of supernatant organic fraction in d-chloroform. The other fractions (pellet and supernatant aqueous fraction in D_2O) show no peaks during ^{19}F NMR analysis.

Table 3.20 New metabolites shown by^{19}F NMR analysis of 16α/β-fluoro-androstenedione (**88**) incubated with *S. griseus*.

Experiment	^{19}F NMR Metabolites
Supernatant (organic)	-187.90, -192.06, -192.61
Supernatant (aqueous)	No fluorinated metabolites detected.
Pellet (organic)	-185.08, -191.77, -192.45
Pellet (aqueous)	No fluorinated metabolites detected.

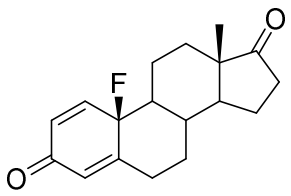
Based on ^{19}F NMR analysis 3 potential metabolites were detected in the pellet organic fraction and 3 potential metabolites in the supernatant organic. Analysis of the GC-MS data suggested the formation of the compounds presented in **Scheme 3.20**. Interestingly different metabolic profiles were detected for samples obtained from extraction of the supernatant and pellet (organic fractions). This suggests that some of the metabolites were formed exclusively inside the cells (pellet organic) and the others were formed on the surface of the cells or were excreted to supernatant. Pellet organic extracts contained three metabolites (ions at *m/z* 310, 270 and 272). Ion at *m/z* 310 (**171**) suggested reduction of the starting material to 16α/β-fluoro-5α-androstenedione (**88**). This was supported by observation of ion at *m/z* 290 (**259**) which was generated from *m/z* 310 through the elimination of HF. Elimination of water from ion at *m/z* 290 led to the formation of ion at

m/z 272 (**258**). Ion at *m/z* 270 (**256**) could be generated from the metabolite formed through the reduction and elimination of HF/water from the starting material. Ions at *m/z* 310 and 270 were also detected in the supernatant along with other metabolites (*m/z* 338, 324 and 290). Ions at m/z 338 (**260**) and 324 (**257**) suggest formation of mono and bis hydroxylated metabolites.



Sheme 3.20 Proposed metabolites for 6α/β-fluoro-5α-androstan-3,17-one (**88**).

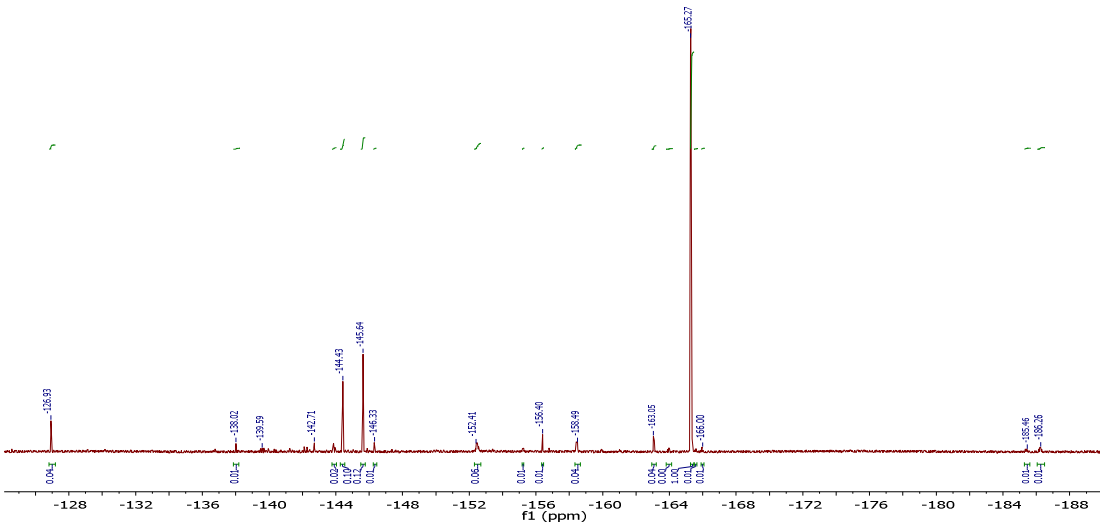
3.3.13 Feeding experiments with 10β-fluoro-3,17-dihydroxy-1,4-androstene (10)



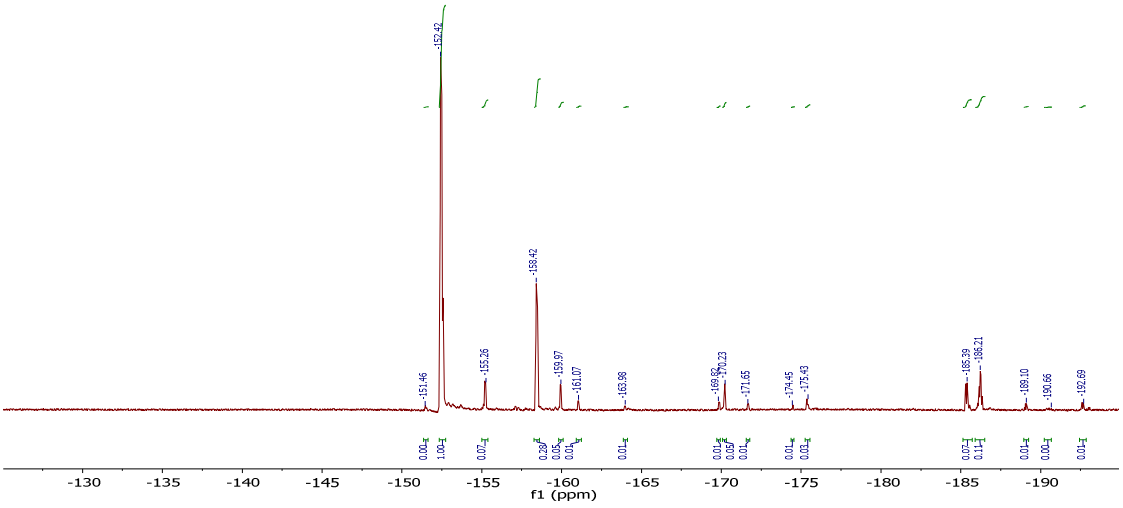
10

The experiment below was carried out with 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**). As before *S. griseus* was fed with the steroid and upon completion of the experiment the bacteria were centrifuged and extraction was carried out. Analysis of the ^{19}F NMR and GC-MS data revealed formation of a few metabolites (**Figure 3.34**).

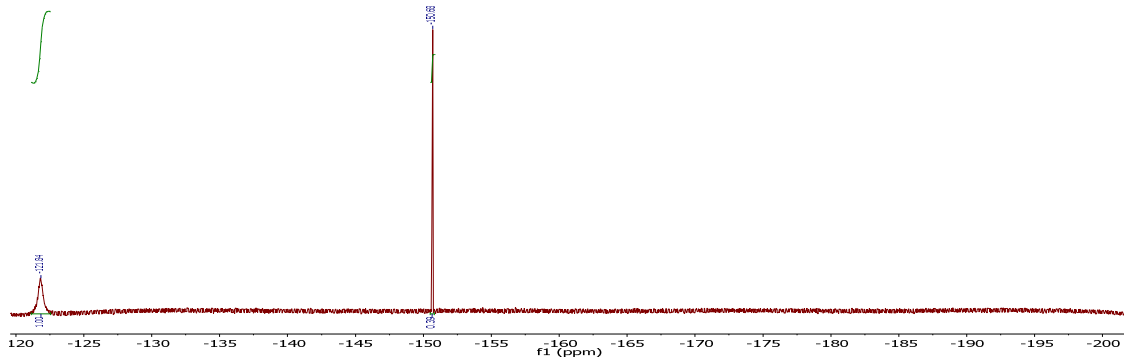
B – ^{19}F NMR (decoupled) spectral analysis of the Pellet (organic fraction)



C – ^{19}F NMR (decoupled) spectral analysis of the Supernatant (organic fraction)



D – ^{19}F NMR (decoupled) spectral analysis of the Supernatant (aqueous fraction)



E – ^{19}F NMR (decoupled) spectral analysis of the Pellet (aqueous fraction)

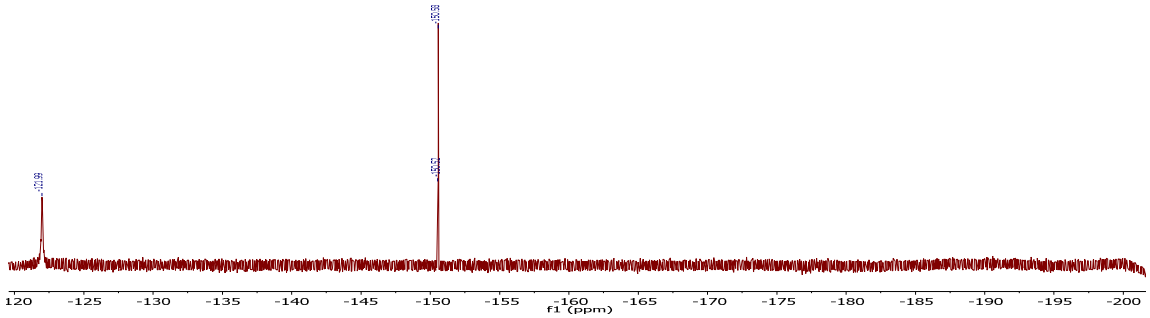
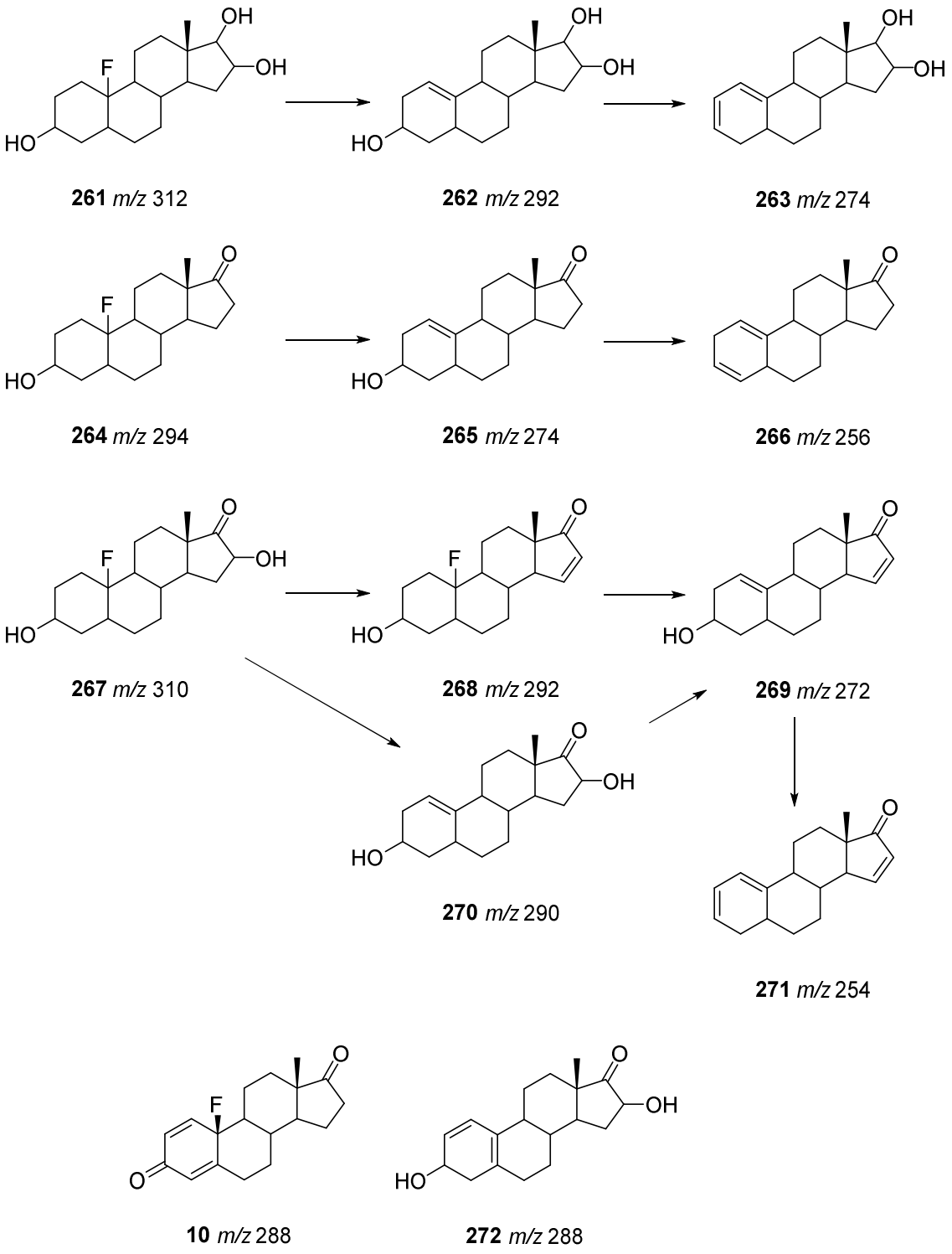


Figure 3.34 ^{19}F NMR (decoupled) analysis of 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**) incubated with *S. griseus*. **A-** ^{19}F NMR of control organic fraction in d-chloroform. **B-** ^{19}F NMR of the pellet organic fraction in d-chloroform. **C-** ^{19}F NMR of supernatant organic fraction in d-chloroform. **D-** ^{19}F NMR of supernatant aqueous fraction in D_2O. **E-** ^{19}F NMR of pellet aqueous fraction in D_2O. The other fractions (pellet and supernatant aqueous fraction in D_2O) show no peaks during ^{19}F NMR analysis.

Table 3.21 New metabolites detected by^{19}F NMR analysis of 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**) incubated with *S. griseus*.

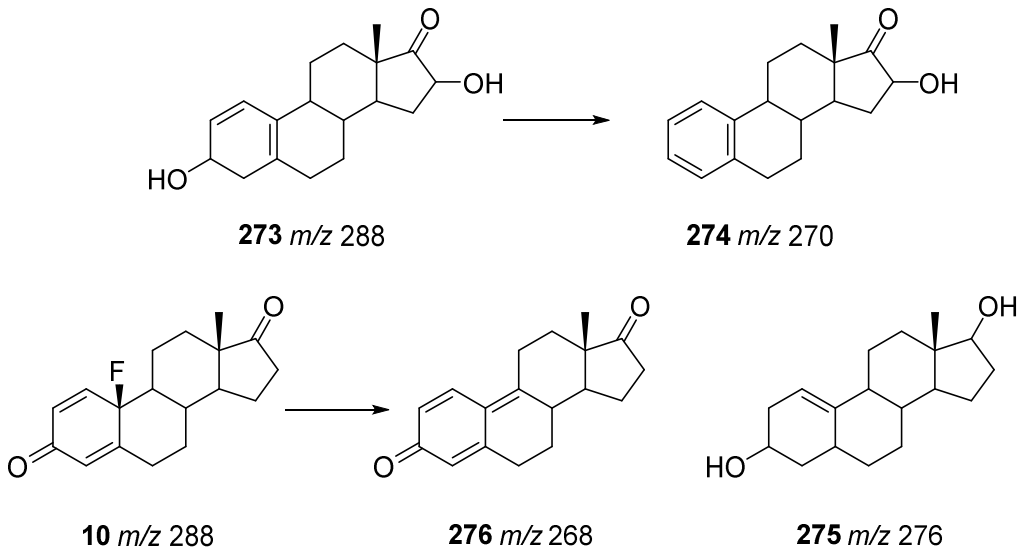
Experiment	^{19}F NMR Metabolites
Supernatant (organic)	-152.42, -155.22, -158.50, -159 90, -161.07, -163.58, -169.82, -170.23, -171.65, -174.45, -175.43, -185.48, -186.22, -189.10, -190.66
Supernatant (aqueous)	-150.62
Pellet (organic)	-126.93, -138.02, -139.59, -142.71, -144.43, -145.64, -146.33, -152.41, -156.40, -158.49, -163.65, -166.00, -185.46, -186.26
Pellet (aqueous)	-150.58, -150.52



Scheme 3.21 Proposed metabolites for 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**).

The organic phase of the supernatant contained ions suggesting presence of 4 metabolites: **261** *m/z* 312, **264** *m/z* 294, **267** *m/z* 310, **10**/**272** *m/z* 288 as shown at **Scheme 3.21**. The proposed structures for these ions are presented in **Scheme 3.21**. From the analysis of the GC-MS data, it appears that *S. griseus* reduced the starting material to form compound with

264 *m/z* 294. This compound was also hydroxylated most likely at 16-C to form the metabolite at *m/z* 310 (**267**). Formation ion at *m/z* 312 (**261**) was an effect of reduction and hydroxylation reactions. In addition, ion at m/z 288 (**272**/ **10**) was detected suggesting formation of the oxidised starting material or hydroxylated product with no fluorine. This ion (*m/z* 288) was also detected in the organic pellet phase alongside three other ions (**Scheme 3.22**, *m/z* 270, *m/z* 268 and *m/z* 276). Ion at *m/z* 270 is a fragmentation ion formed from *m/z* 288 by elimination of water. Ion at *m/z* 268 could be formed from a different ion at *m/z* 288 by elimination of HF. Reduction of the double bonds, carbonyl group and elimination of HF would lead to the formation of ion at *m/z* 276. The proposed metabolites arising from this data are presented below in **Scheme 3.22**.



273 *m/z* 288 **274** *m/z* 270

10 *m/z* 288 **276** *m/z* 268 **275** *m/z* 276

Scheme 3.22 Proposed metabolites from 10β-fluoro-3,17-dihydroxy-1,4-androstene (**10**).

^{19}F NMR analysis of the pellet aqueous phase revealed 2 metabolites at -150.58 ppm and -150.52 ppm. Unfortunately, these metabolites decomposed during preparative LC based on ^{19}F NMR. The LC-MS trace of crude sample showed ion with 100% abundance at *m/z* 703, suggesting presence of cell debris. The aqueous phase of the supernatant did not contain any fluorinated metabolites.

3.3.14 Stockton experiments using *Escherichia coli* MG1655, *Bacillus subtilis* and *Bacillus megaterium* 14581

The experiments performed in Stockton were performed with *Escherichia coli, Bacillus subtilis* and *Bacillus megaterium*. *Escherichia coli* consist of a diverse group of bacteria that normally live in the intestines of people and animals. They are harmless and actually play important roles in healthy animal intestinal tracts. *Bacillus subtilis* is an extremely common bacterium. It is found in soil, water, air, decomposing plant matter and the gastrointestinal tract of ruminants and humans. *Bacillus megaterium* is ubiquitous in the human environment. It is found in soil, various foods and on a variety of surfaces. The studies presented below have focused on the selected fluorinated steroids synthesised in **Chapter 2** (**Figure 3.35**).

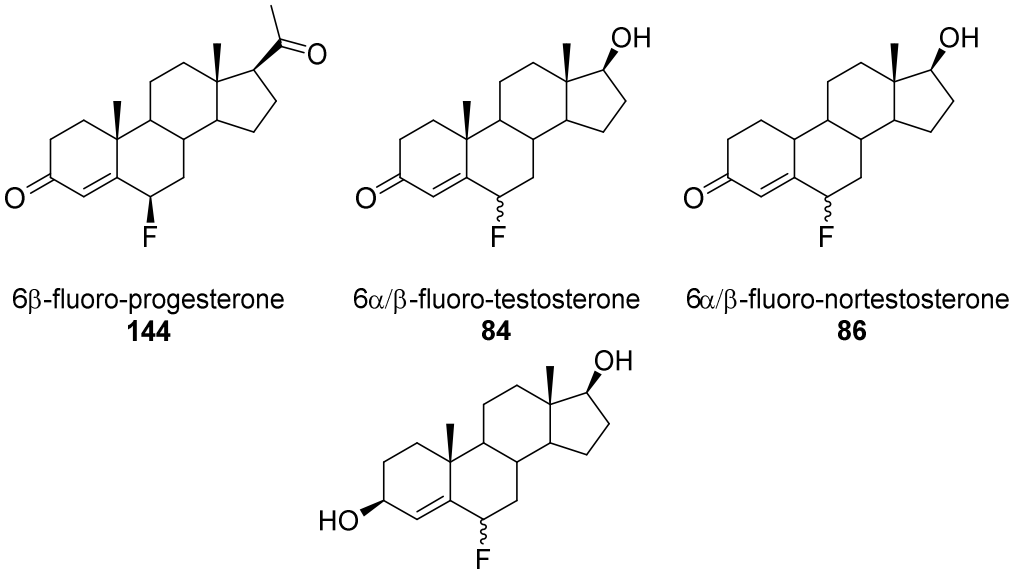


Figure 3.35 Steroids used in Stockton biological feeding experiments.

3.3.15 Incubation experiments

The first experiment involved incubation of 6β-fluoro-progesterone (**144**), 6α/β-fluoro-testosterone (**84**), 6α/β-fluoro-nortestosterone (**86**) and 6α/β-fluoro-3,17-dihydroxyandrost-4-ene (**110**) with *Escherichia coli* MG1655, *Bacillus subtilis* and

Bacillus megaterium 14581 (bacteria available in-house). Bacteria were inoculated from plate into Erlenmeyer flasks containing soya bean meal media and the pH was adjusted to 7. Cultures were incubated with rotary agitation at 27ºC for 72 h. After that time, the following solutions in Erlenmeyer flasks were prepared:

- medium + starting culture bacteria
- medium + starting culture bacteria + after 72 h fluorinated steroid was added
- medium + after 72 h fluorinated steroid was added (negative probe)

The Erlenmeyer flasks were incubated with rotary agitation at 27ºC for 6 days. After that time, minimal microbial growth was observed. The cultures were sonicated and centrifuged. The supernatants and pellets were then extracted with ethyl acetate. ^{19}F NMR and GC-MS analysis of the extracts indicated that bacteria did not transform 6β-fluoro-progesterone (**144**), 6α/β-fluoro-testosterone (**84**), 6α/β-fluoro-nortestosterone (**86**) and 6α/β-fluoro-3,17-dihydroxyandrost-4-ene (**110**).

It was suggested that the experiment was not successful due to the incubation protocol. Consequently, different conditions were explored to promote bacterial growth:

- Media:
- LB (Lysogeny broth) medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl
- NB (Nutrient broth) medium: 10 g, peptone, 10 g beef extract, 5 g NaCl
- 868 medium [88]: 10 g pepton, 10 g yeast extract, 20 g glucose (This medium was described for the growth of *B. subtilisin* the literature: bacterial cells were grown for 48 h at 30°C with shaking (130 rpm) in 868 medium)
- Temperature: (30, 37ºC)
- Agitation: (150, 200 rpm)
- Volumes (5, 10, 25, 50 ml)
- Incubation time with steroid (24, 48 h)

As before, the incubation experiments involved 6β-fluoro-progesterone (**144**), 6α/β-fluoro-testosterone (**84**), 6α/β-fluoro-nortestosterone (**86**) and 6α/β-fluoro-3,17-dihydroxyandrost-4-ene (**110**). *Escherichia coli* MG1655*, Bacillus subtilis* and *Bacillus*

megaterium 14581 were grown in 50 ml falcon tubes/Erlenmeyer flasks in various medium (LB, NB, 868). Volumes of medium were varied from 5 to 50 ml, agitation was set to 200 rpm and the temperature was maintained at 30ºC or 37ºC. The conditions are summarised in the table below (**Table 3.22**).

Table 3.22 Conditions used for the incubation of selected fluorinated-steroids with *Escherichia coli* MG1655*, Bacillus subtilis* and *Bacillus megaterium* 14581.

Bacteria	Steroid	Media	Volume of media (ml)	Volume of bacteria	Compound (g)	TºC
B. subtilis	6β-fluoro-progesterone (**144**) 6-fluoro-testosterone (**84**) 6α/β-fluoro-nortestosterone (**86**)	LB, NB, 868	5	100 µl	0.00548 (LB) 0.00528 (NB)	30, 37
			10	200 µl	0.00548 (LB) 0.00510 (NB)	30, 37
			25	0.5 ml	0.00576 (LB) 0.00516 (NB)	30, 37
			50	1 ml	0.01140 (LB) 0.01088 (NB)	30, 37
***B. megaterium* 14581**	6α/β-fluoro-3,17-dihydroxyandrost-4-ene (**110**)	LB, NB, 868	10	200 µl	0.00543 (LB) 0.00548 (NB)	30, 37
***E. coli* MG1655**		LB, 868	10	200 µl	0.00529	30, 37

Unfortunately, it was not possible to achieve good bacterial growth by varying the conditions listed above. Based on in house expertise, modified buffered conditions were proposed for *Bacillus subtilis*. This involved adding a buffer to 100 ml of NB medium:

- 0.5 ml Metal Mix (14 ml 1 M Ca-chloride; 10 ml 0.1 M Mn chloride; 20 ml 1 M Mg chloride; 56 ml water), sterile-filtered or made from pre-sterilized stock solutions
- 0.05 ml Fe-solution (32.4 mg Fe(III)-chloride; 1 ml 1 M HCl in 100 ml water), sterile-filtered
- 5 ml Phosphate buffer (95 g K_2HPO_4 x 3 H_2O, 124 g KH_2PO_4, ad 1l water), autoclaved

Disappointingly, all attempts to promote bacterial growth were unsuccessful. It was therefore suggested that problems encountered with the bacterial growth are associated with

the laboratory environment. Due to lack of the success with the feeding experiments it was decided to try subcloning technique. This was done for positive-testing cells, if there was more than one clone in the original plate, it diluted the population to one or a few cells and regrow. This technique was used to achieve a purely monoclonal culture. Subcloning was repeated several times to ensure a completely monoclonal colony.

After successful subcloning, the feeding experiments were carried out with selected fluorinated-steroids and bacteria (*Escherichia coli* MG1655, *Bacillus subtilis* and *Bacillus megaterium* 14581) as presented in **Table 3.22**. Although the growth of all bacteria was achieved the transformation of the fluorinated steroids was not detected. Therefore, no further feeding experiments were carried out in Stockton.

3.4 Stability experiments

3.4.1 Introduction

Stability tests are widely carried out to identify the likely degradation of chemicals and biological samples over time. The nature of the stress testing will depend on the individual drug substance and the type of drug products involved. The experiments are usually carried out on a single batch of tested substance. It usually includes the effect of temperature (in 10^{o}C increments), humidity, oxidation, photostability.[89] Where appropriate, the testing should also evaluate the susceptibility of the substance to hydrolysis across a wide range of pH values. Studying the products of degradation is useful in establishing degradation pathways, altering substance structure to improve stability, modifying storage conditions and developing suitable analytical procedures. In addition to long term stability studies, accelerated testing is performed. These studies use exaggerated storage conditions to increase the rate of chemical degradation or physical change of a substance. However, results from accelerated testing studies are not always predictive of physical changes. All these studies help to establish the date of a substance designating the time prior to which a batch of the product is expected to remain within the approved shelf life specification if stored under defined conditions.

3.4.2 Biological fluids

Storing and transporting biological fluids for doping analysis is carried out by the organisations associated with the World Anti-Doping Agency (WADA). Currently, there

are no specific protocols for the transport of biological samples to anti-doping laboratories. As a result, microbial and thermal degradation of chemical substances in the samples may occur, which could lead to false negative or false positive results. Given the social consequences of positive tests, the storage and transport of the biological samples should be strictly regulated. This is supported by numerous publications dealing with biological fluids. For example, Konings has stressed in his research that cooling of a urine sample during transport is essential (≤ 5ºC).[90] In principle, urine in the bladder is sterile, however, can be contaminated when it leaves the urinary tract and is collected in non-sterile containers. As urine is a rich source of nutrient, it can support bacterial growth. At temperatures between 5ºC - 40ºC and pH 4.5 - 8.5 microorganisms can multiply at very high rates with generation times as 30 min. As a result, a single bacterium can produce 10^9 cells in 15h and this could completely change the chemical profile of a urine sample.

The importance of urine storage conditions was also highlighted by Zaitsu.[91] In their research the long term stability of various drugs in urine was examined along with preventive measurements against their decomposition. The urine samples were collected under sterile and non-sterile conditions and spiked (500 ng/ml) with methampletamine, amphetamine, nitrazepam, estazolam, 7-aminoflunitrazepam, cocaine and 6-acetylmorphine. The samples were then stored for 150 days at 25ºC, 4ºC and -20ºC and the results obtained by Zaitsu are summarised in **Table 3.23.**

Table 3.23 Stability of selected drugs in urine.[91]

Drug	Filtration 25 ºC	NaN_3 25ºC	Non sterile 25ºC	Filtration 4ºC	NaN_3 4ºC	Non sterile 4ºC
Methamophetamine	no change	no change	loss of ~150ng/ml	no change	no change	no change
Amphetamine	no change	no change	loss of ~200ng/ml	no change	no change	no change
Nitrazepam	no change	no change	full decomp.	no change	no change	no change
Estazolam	no change	no change	no change	no change	no change	no change
7-Aminoflunitrazepam	loss of ~450ng/ml	loss of ~200ng/ml	loss of ~310ng/ml	loss of ~450ng/ml	loss of ~100ng/ml	loss of ~450ng/ml
Cocaine	loss of ~100ng/ml	full decomp.	full decomp.	no change	no change	no change
6-Acethylmorphine	no change	loss of ~200ng/ml	full decomp.	no change	no change	no change

In all cases samples were stored at -20ºC were preserved. It was found that samples contaminated with microorganisms contained significantly reduced level of 7-

aminoflunitrazepam, nitrazepam, cocaine and 6-acetylmorphine after 150 days of storage. Degradation of the drugs investigated was significantly inhibited in samples filtered using aseptic urine collection kit. In addition, it was also observed that common food preservative NaN_3 can in some cases accelerate the hydrolysis of a drug (cocaine, 6-acetylmorphine).

The work of Zaitsu correlates well with studies performed by Jimenez.[92] Jimenez with his co-workers investigated the stability of ephedrine derivatives (ephedrine, norephedrine, methylephedrine, pseudoephedrine, norpseudoephedrine) and amphetamine derivatives (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine). Sterile and non-sterile urine samples were spiked with the appropriate drug and then stored at 4ºC or -20ºC for 24 months (non-sterile 6 months). No significant loss of the drugs under study was observed at any of the investigated conditions.

3.4.3 Results from stability tests

Once the metabolism of fluorinated steroids under *in vitro* microbial growth conditions had been confirmed we looked to determine the ability of fluorinated steroids to act as an internal standard to assay for chemical or thermal degradation. This work was carried out as the chemical stability is the key to the application of a fluorinated steroid as in internal standard in anti-doping controls. The purpose of this stability testing was to investigate how the quality of fluoro-steroids varies with time under the influence of variety of environmental factors such as temperature and medium. The choice of test conditions defined in these studies was based on an analysis of the effects of storage conditions on the chemical profile of urine samples.

To perform the stability tests, three steroids were selected (6α/β-fluoro-testosterone (**84**), 6β-fluoro-progesterone (**144**), 6-fluoro-androsterone (**87**)). A sample of an appropriate steroid was dissolved in DMSO and then the solution was diluted with water. Some samples were treated with additives such as sodium chloride or buffer and then the samples were stored at 5ºC, room temperature and 36ºC for 10 months. The samples were prepared in duplicates to eliminate any potential errors. The results from these experiments are presented below (**Table 3.24-3.32**). All tested steroids were found to be stable under the investigated conditions for at least 1 year.

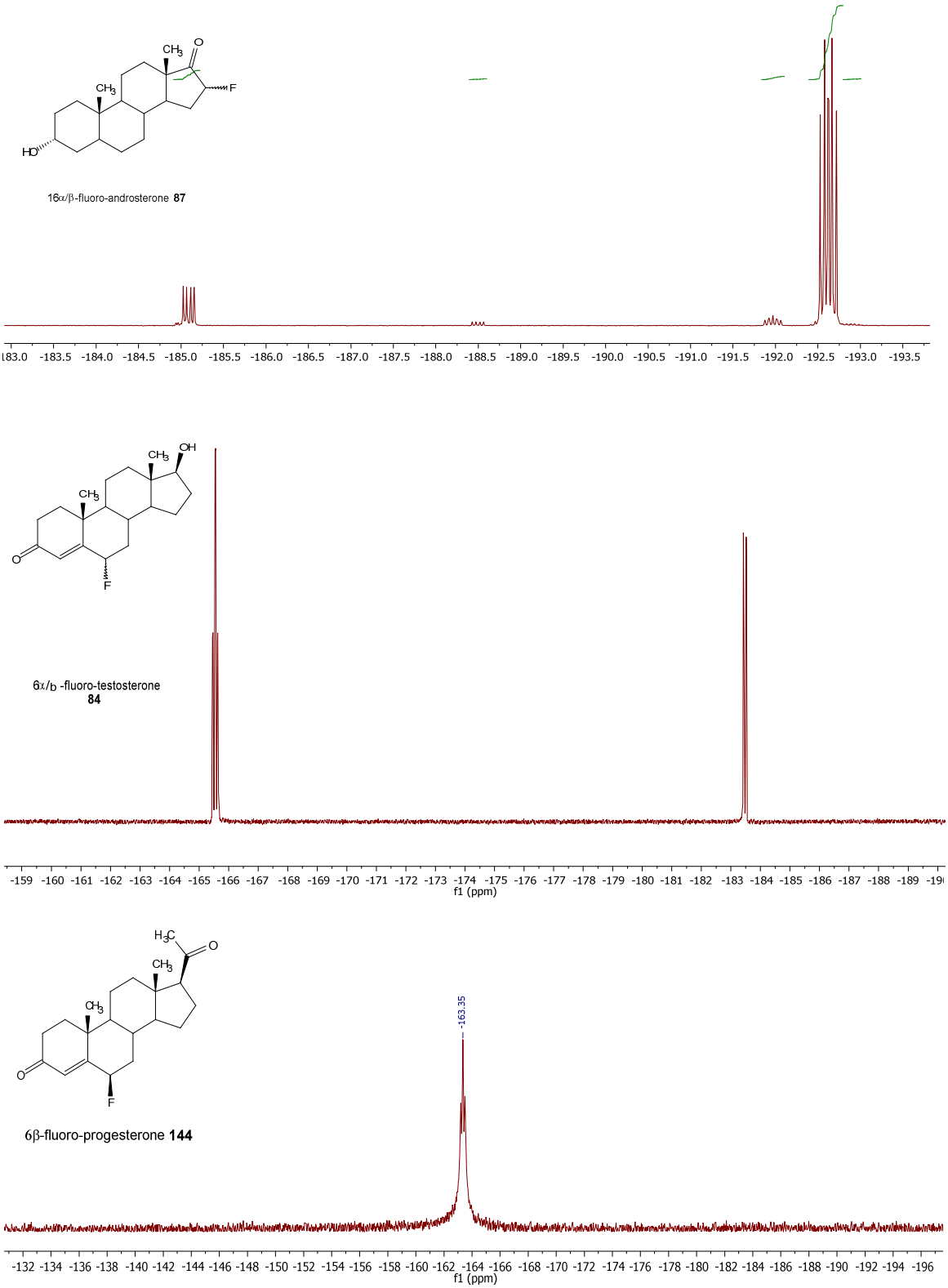


Figure 3.36 Reference ^{19}F NMR spectra of the fluorinated steroids used for stability experiments in d-chloroform.

Table 3.24 Stability of 6α/β-fluoro-testosterone (**84**) at 22-25ºC as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O (ml)	NaCl (mg)	Temp. (ºC)	1 month	1 year
1	2	200	-	500	-	22-25	✓	✓
1 control	2	200	-	500	-	22-25	✓	✓
2	2	200	-	500	2	22-25	✓	✓
2 control	2	200	-	500	2	22-25	✓	✓
3	2	200	LB	500	-	22-25	✓	✓
3 control	2	200	LB	500	-	22-25	✓	✓
4	2	100	-	600	-	22-25	✓	✓
4 control	2	100	-	600	-	22-25	✓	✓
control	2	700	-	0	-	22-25	✓	✓

Table 3.25 Stability of 6β-fluoro-progesterone (**144**) at 22-25ºC as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O (ml)	NaCl (mg)	Temp. (ºC)	1 month	1 year
5	2	200	-	500	-	22-25	✓	✓
5 control	2	200	-	500	-	22-25	✓	✓
6	2	200	-	500	2	22-25	✓	✓
6 control	2	200	-	500	2	22-25	✓	✓
7	2	200	LB	500	-	22-25	✓	✓
7 control	2	200	LB	500	-	22-25	✓	✓
8	2	100	-	600	-	22-25	✓	✓
8 control	2	100	-	600	-	22-25	✓	✓
control	2	700	-	0	-	22-25	✓	✓

Table 3.26 Stability of 6-fluoro-androsterone (**87**) at 22-25ºC as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O (ml)	NaCl (mg)	Temp. (ºC)	1 month	1 year
9	2	200	-	500	-	22-25	✓	✓
9 control	2	200	-	500	-	22-25	✓	✓
10	2	200	-	500	2	22-25	✓	✓
10 control	2	200	-	500	2	22-25	✓	✓
11	2	200	LB	500	-	22-25	✓	✓
11 control	2	200	LB	500	-	22-25	✓	✓
12	2	100	-	600	-	22-25	✓	✓
12 control	2	100	-	600	-	22-25	✓	✓
control	2	700	-	0	-	22-25	✓	✓

Table 3.27 Stability of 6α/β-fluoro-testosterone (**84**) at 5ºC as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O(ml)	NaCl (mg)	Temp. (ºC)	1 month	1 year
13	2	200	-	500	-	5	✓	✓
13 control	2	200	-	500	-	5	✓	✓
14	2	200	-	500	2	5	✓	✓
14 control	2	200	-	500	2	5	✓	✓
15	2	200	LB	500	-	5	✓	✓
15 control	2	200	LB	500	-	5	✓	✓
16	2	100	-	600	-	5	✓	✓
16 control	2	100	-	600	-	5	✓	✓
control	2	700	-	0	-	5	✓	✓

Table 3.28 Stability of 6β-fluoro-progesterone (**144**) at 5ºC as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O (ml)	NaCl (mg)	Temp. (ºC)	1 month	1 year
17	2	200	-	500	-	5	✓	✓
17 control	2	200	-	500	-	5	✓	✓
18	2	200	-	500	2	5	✓	✓
18 control	2	200	-	500	2	5	✓	✓
19	2	200	LB	500	-	5	✓	✓
19 control	2	200	LB	500	-	5	✓	✓
20	2	100	-	600	-	5	✓	✓
20 control	2	100	-	600	-	5	✓	✓
control	2	700	-	0	-	5	✓	✓

Table 3.29 Stability of 6-fluoro-androsterone (**87**) at 5ºC as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O(ml)	NaCl (mg)	Temp. (ºC)	1 month	1 year
21	2	200	-	500	-	5	✓	✓
21 control	2	200	-	500	-	5	✓	✓
22	2	200	-	500	2	5	✓	✓
22 control	2	200	-	500	2	5	✓	✓
23	2	200	LB	500	-	5	✓	✓
23 control	2	200	LB	500	-	5	✓	✓
24	2	100	-	600	-	5	✓	✓
24 control	2	100	-	600	-	5	✓	✓
control	2	700	-	0	-	5	✓	✓

Table 3.30 Stability of 6α/β-fluoro-testosterone (**84**) at 37°C as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O(ml)	NaCl (mg)	Temp. (°C)	1 month	1 year
25	2	200	-	500	-	37	✓	✓
25 control	2	200	-	500	-	37	✓	✓
26	2	200	-	500	2	37	✓	✓
26 control	2	200	-	500	2	37	✓	✓
27	2	200	LB	500	-	37	✓	✓
27 control	2	200	LB	500	-	37	✓	✓
28	2	100	-	600	-	37	✓	✓
28 control	2	100	-	600	-	37	✓	✓
control	2	700	-	0	-	37	✓	✓

Table 3.31 Stability of 6β-fluoro-progesterone (**144**) at 37°C as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O (ml)	NaCl (mg)	Temp. (°C)	1 month	1 year
29	2	200	-	500	-	37	✓	✓
29 control	2	200	-	500	-	37	✓	✓
30	2	200	-	500	2	37	✓	✓
30 control	2	200	-	500	2	37	✓	✓
31	2	200	LB	500	-	37	✓	✓
31 control	2	200	LB	500	-	37	✓	✓
32	2	100	-	600	-	37	✓	✓
32 control	2	100	-	600	-	37	✓	✓
control	2	700	-	0	-	37	✓	✓

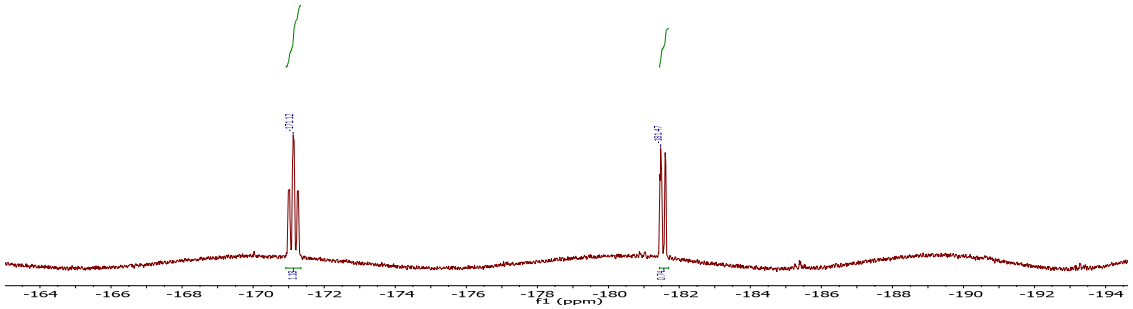
Table 3.32 Stability of 6-fluoro-androsterone (**87**) at 37°C as determinated by ^{19}F NMR.

No.	Mass (mg)	d_6-DMSO (ml)	Buffer (D_2O)	D_2O (ml)	NaCl (mg)	Temp. (°C)	1 month	1 year
33	2	200	-	500	-	37	✓	✓
33 control	2	200	-	500	-	37	✓	✓
34	2	200	-	500	2	37	✓	✓
34 control	2	200	-	500	2	37	✓	✓
35	2	200	LB	500	-	37	✓	✓
35 control	2	200	LB	500	-	37	✓	✓
36	2	100	-	600	-	37	✓	✓
36 control	2	100	-	600	-	37	✓	✓
control	2	700	-	0	-	37	✓	✓

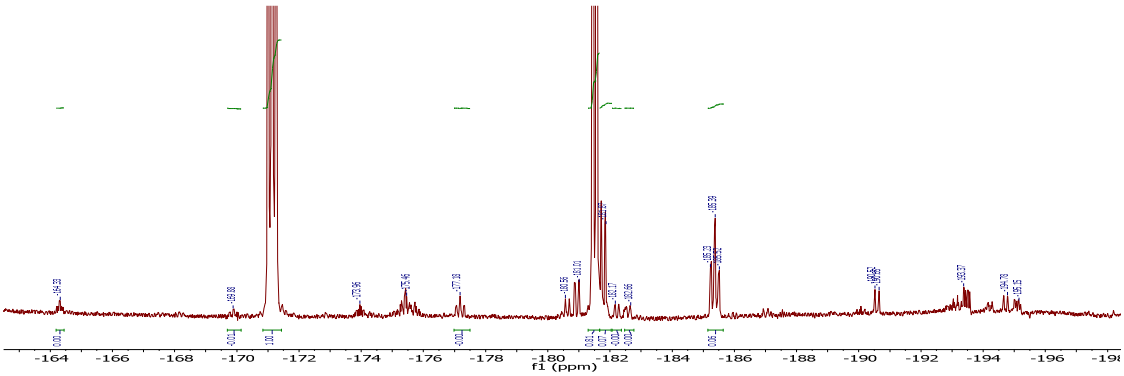
3.5 Conclusions

Recent literature reports have shown that under certain conditions microorganisms cytochrome P-450 enzyme systems can facilitate the transformation of endogenous steroids such as androsterone into banned substances such as the 19-norsteroids in urine samples.[93] It is believed that cytochrome P-450 catalyses various reactions such as hydroxylation, oxidation, dealkylation and deamination reactions. In most cases, this results in the formation of unstable intermediates that spontaneously decompose to the stable metabolites. This is potentially problematic in that the concentrations of banned steroids that were detected could approach threshold levels giving rise to a false positive. At the present time the role of microbial growth cannot be completely ruled out and this is an issue that we are addressing in **Chapter 3**. The synthetic fluorinated steroids were assessed for their biotransformation by a range of microorganisms (pure cultures). The studies were carried out at Durham University in Chemistry Department, Stockton department of Pharmacy and at University College Dublin (UCD). At Durham University and at University College Dublin experiments were carried out with the bacteria *Streptomyces griseus.* The experiments in Stockton were employed *Escherichia coli, Bacillus subtilis* and *Bacillus megaterium*. The strains were cultured in standard media (e.g. tryptone soya broth, LB, NB, 868) for 24 h and the fluorinated steroids added to the cultures. After further incubation (24-48h) the biotransformation products were extracted and analysed by ^{19}F NMR. The degradation of several fluorinated steroids could be easily detected using ^{19}F NMR. A new metabolite formed by bacteria was represented by a new signal in ^{19}F NMR. For example, 6-fluoro-nortestosterone (**86**) was transformed by *Streptomyces griseus* into several different fluorinated molecules as could be observed by ^{19}F NMR (**Figure 3.37**).

A – ^{19}F NMR analysis of the Control (organic fraction) for 6-fluoro-nortestosterone (86)



B – ^{19}F NMR analysis of the Pellet (organic fraction) 6-fluoro-nortestosterone (86)



In an attempt to characterise these metabolites and establish the metabolic pathways the purification of the crude extracts by preparative TLC and HPLC was carried when possible. Subsequently identification via GC-MS and LC-MS was attempted. As reported in section **3.2.3**, metabolism of steroids by microorganisms involves enzymes P-450 which is responsible for oxidation. This reaction is usually highly regio selective and involves a few positions (C-1, C-6, C-11, C-12, C-15, C-16) depends on steroid substitution pattern and microorganism involved (**Figure 3.38**). The oxidation of fluorinated steroids was confirmed by MS, however due to time constrains the structures of metabolites were not confirmed. Based on LC-MS and GC-MS de-fluorinated products were also detected. These could be potentially formed in microorganisms, during isolation/purification of biological samples and/or during analysis. No reports on the investigated fluoro-steroids/microorganisms combinations were found in the literature.

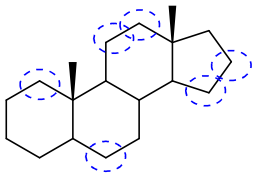


Figure 3.38 Positions presented for regio-selective oxidation reaction.

The key milestone of the project was accomplished by showing that the fluorinated steroids can be bio-transformed by common bacteria found in human environment. In addition, it was confirmed that ^{19}F NMR can be used as a tool to monitor steroid metabolism/degradation by bacteria. This work supports the hypothesis that bacterial contamination of urine samples could lead to false positive or false negative results. It has to be emphasised that work in this area remains incomplete due to time constrain and further

studies are needed to fully/characterise the fluorinated metabolites generated in the feeding experiments.

Once the metabolism of the fluorinated steroids under *in vitro* microbial growth conditions had been confirmed we investigated the ability of the fluorinated steroids to act as internal standards to assay for steroid chemical and thermal degradation. To perform the stability tests, three steroids were selected: 6α/β-fluoro-testosterone (**84**), 6-fluoro-androsterone (**87**) and 6β-fluoro-progesterone (**144**). A solution of an appropriate steroid in DMSO was diluted with water and treated with additives such as sodium chloride or buffer. The samples were prepared in duplicates and then stored at 5ºC, room temperature and 36ºC. Short-term (>30 days) and long-term (>12 months) stability of selected fluoro-steroids was investigated. All tested steroids appear to be stable to chemical breakdown under the investigated conditions for at least 1 year. Further experiments are required to investigate a wider range of conditions (e.g. different pH, different buffers, temperatures: 4, 25ºC and 37ºC) to build a more complete picture of fluoro-steroid's stability. In addition, long term stability studies need to be carried out with fake urine samples (imitate real human urine and it is used in drug testing labs to calibrate their urine screening equipment) and samples from healthy volunteers for selected fluorinated steroids.

3.6 Reference

[1] J. S. C. Marias, J. Onderstepoort, *Vet. Sci. Anim. Ind*., **1943**, *18*, 203.
[2] J. S. C. Marias, J. Onderstepoort, *Vet. Sci. Anim. Ind*., **1944**, *20*, 67.
[3] R. A. Peters, P. J. Hall, P. F. V. Ward, N. Sheppard, *J. Biochem*., **1960**, *77*, 17.
[4] J. T. G. Hamilton, D. B. Harper, *Phytochemistry*, **1997**, *44*, 1179.
[5] S. O. Thomas, V. L. Singleton, J. A. Lowery, R. W. Sharpe, L. M. Pruess, J. N. Porter, J. H. Mowat, N. Bohonos, *Antibiotics Ann*., **1957**, *716*, 1956.
[6] D. A. Schuman, R. K. Robins, M. J. Robins, *J. Am. Chem. Soc.*, **1969**, *91*, 3391.
[7] A. Bartholome, J. E. Janso, U. Reillyb, D. O'Hagan, *Org. Biomol. Chem.*, **2017**,*15*, 61.
[8] D. O'Hagan, H. Deng, *Chem. Rev*., **2015**, *115*, 634.
[9] L. Ma, Y. Li, L. Meng, H. Deng, Y. Li, Q. Zhange A. Diaoab, *RSC Adv*., **2016**, *6*, 27047.
[10] M. Sanada, T. Miyano, S. Iwadare, J. M. Williamson, B. H. Arison, J. L. Smith, A. W. Douglas, J. M. Leisch, E. Inamine, *J. Antibiotics*, **1986**, *39*, 259.
[11] C. D. Murphy, D. O'Hagan, C. Schaffrath, *Angew. Chem. Int. Ed*., **2001**, *40*, 4479.
[12] H. Deng, S. M. Cross, R. P. McGlinchey, J. T. G. Hamilton, D. O'Hagan, *Chem. Biol*., **2008**, *15*, 1268.
[13] L. Merkel, M. Schauer, G. Antranikian, N. Budisa, *Chem. Bio. Chem*, **2010**, *11*, 1505.
[14] J. T. Gerig, J. C. Klinkenborg, R. A. Nieman, *Biochemistry*, **1983**, 22 (*9*), 2076.
[15] C. D. Murphy, B. R. Clark, J. Amadio, *App. Microbiol. Biotechnol.*, **2009**, *84*, 617.
[16] C. Isanbor, D. O'Hagan, *J. Fluorine Chem.*, **2006**, *127*, 303.
[17] K. L. Kirk, *J. Fluorine Chem.*, **2006**, *127*, 1013.
[18] K. Muller, C. Faeh, F. Diederich, *Science*, **2007**, *317*, 1881.
[19] S. B. Rosenblum, T. Huynh, A. Afonso, H. R. Davis, N. Yumibe, J. W. Clader, D. A. Burnett, *J. Med. Chem.*, **1998**, *41*, 973.
[20] M. VanHeek, C. F. France, D. S. Compton, R. L. McLeod, N. P. Yumibe, K. B. Alton, E. J. Sybertz, H. R. Davis, *J.Pharmacol. Exp. Ther.*, **1997**, *283*, 157.
[21] A. M. Clark, J. D. McChesney, C. D. Hufford, *Med. Res. Rev.*, **1985**, *5*, 231.
[22] H. Goldenberg, V. Fishman, *Proc. Soc. Exp. Biol. Med*., **1961**, *109*, 178.
[23] S. Orrenius, L. Ernster, "*Molecular Mechanisms of Oxygen Activation*", New York, **1974**, 216.
[24] H.O. Klingele, P.K. Knoefel, "Absorption, Distribution, Transformation and Excretion of Drugs", Ed. C.C. Thomas, *Springfield*, **1972**, *77*.
[25] S. Shaik, P. de Visser, '*Cytochrome P-450: Structure, Mechanism, and Biochemistry*', **2005**.
[26] J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, G. J. Mannering, "Microsomes and Drug Oxidations", **1969**.
[27] D. R. Nelson, L. Koymans, T. Kamataki, J. J. Stegman, R. Feyereisen, D. J. Waxman, M. R. Waterman, O. Gotoh, M. J. Coon, R. W. Estrabrook, *Pharmacogenetics*, **1996**, *6*, 1.
[28] M. J. Groot, *Drug Discov. Today*, **2006**, *11*, 601.
[29] J. Daly, *Biochem. Pharmacol*., **1970**, *19*, 2979.
[30] J. Daly, D. Jerina, B. Witkop, *Arch. Biochem. Biophys*., **1968**, *128*, 517.
[31] J. Daly, G. Guroff, S. Udenfriend, B. Witkop, *Biochem Pharmacol*., **1968**, *17*, 31.
[32] J. Daly, "*Handbook of Experimental Pharmacology*", **1971**, *28*, 285.
[33] D. Jerina, J. Daly, B. Witkop, P. Zaltzman-Nirenberg, S. Udenfriend, *Biochemistry*, **1970**, *9*, 147.
[34] G. J. Kasperek, T. C. Bruice, *J. Amer. Chem. Soc*., **1972**, *94*, 198.
[35] J. W. Daly, D. M. Jerina, B. Witkop, *Experientia*, **1972**, *28*, 1129.
[36] D. M. Jerina, J. W. Daly, *Science*, **1974**,*185,* 573.
[37] D. T. Gibson, *Science*, **1968**, *161*, 1093.

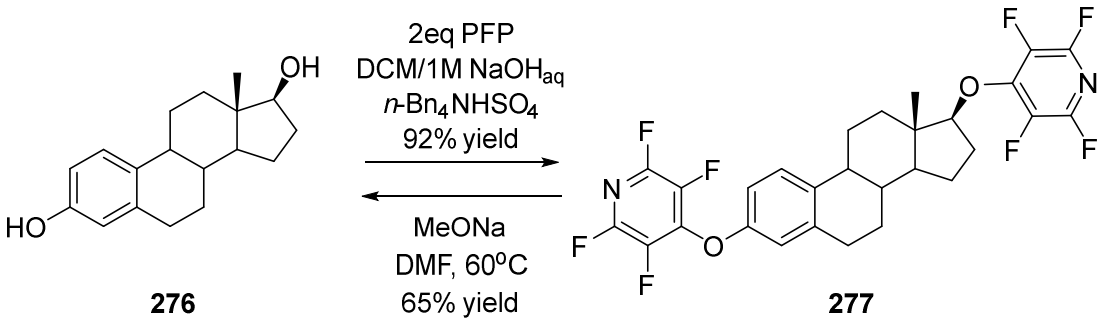
[38] O. Hayaishi, *Bacteriol. Rev*., **1966**, *30*,720.
[39] D. M. Jerina, J. W. Daly, A. M. Jeffrey, D. T. Gibson, *Arch. Biochem. Biophys*., **1971**, *142*, 394.
[40] I. C. Gunsalus, J. R. Meeks, J. D. Lipscomb, P. DeBrunner, E. Munck, "*Molecular Mechanisms of Oxygen Activation*", **1974**, 559.
[41] S. H. Ambike, R. M. Baxter, *Phytochemistry*, **1970**, *9*, 1995.
[42] W. Duppel, J. M. LeBeult, M. J. Coon, *Eur. J. Biochem*., **1973**, *36*, 583.
[43] R. E. Betts, D. E. Walters, J. P. Rosazza, *J. Med. Chem*., **1974**, *17*, 599.
[44] R. V. Smith, J. P. Rosazza, R. A. Nelson, *J. Chromatogr*., **1974**, *95*, 247.
[45] R. V. Smith, J. P. Rosazza, *J. Pharm. Scien*., **1975**, *64*, 1737.
[46] R. V. Smith, J. P. Rosazza, *Arch. Biochem. Biophys*., **1974**, *161*, 551.
[47] E. Gram, B. B. Brodie, J. R. Gillette,"*Handbook of Experimental Pharmacology*", **1971**, *28*, 334.
[48] R. E. McMahon, N. R. Easton, *J. Med. Pharm. Chem*., **1961**, *4*, 437.
[49] J. J. Kamm, A. Szuna, R. Kuntzman, *J. Pharmacol. Exp. Ther*., **1972**, *182*, 507.
[50] J. Axelrod, *Biochem. J*., **1956**, *63*, 634.
[51] R. E. McMahon, H. W. Culp, J. Mills, F. J. Marshall, *J. Med. Chem*., **1963**, *6*, 343.
[52] E. Gram, B. B. Brodie, J. R. Gillette," Handbook *of Experimental Pharmacology*", *Springer-Verlag,* **1971**, *28*, 334.
[53] D.C. Lamb, B. Zhao, F.P. Guengerich, S.L. Kelly, M.R. Waterman, *Streptomyces: molecular biology and biotechnology*, **2011**, 450.
[54] F. S. Sariaslani, J. P. Rosazza, *Appl, Environ. Microbiol.*, **1983**, *46,* 467.
[55] F. S. Sariaslani, F. M. Eckenrode, J. M. Beale, J. P. Rosazza, *J. Med. Chem*., **1984**, *27*, 749.
[56] N. Serizawa, T. Matsuoka, *Biochim. Biophys. Acta*, **1991**, *1084*, 35.
[57] J. R. Berrie, R. A. D. Williams, K. E. Smith, *J. Steroid. Biochem*, **1999**, *71*, 153.
[58] G. S. Fonken, M. E. Herr, H. C. Murray, L. M. Reineke, *J. Am. Chem, Soc*., **1967**, *89*, 672.
[59] G. S. Fonken, M. E. Herr, H. C. Murray, R. A. Johnson, *J. Org. Chem*, **1968**, *33,* 3182.
[60] E. R. H. Jones, *Pure Appl. Chem*., **1973**, *33*, 39.
[61] A. M. Bell, P. C. Cherry, I. M. Clark, W. A. Denny, E. R. H. Jones, G. D. Meakins, D. Woodgate, *J. Chem. Soc., Perkin Trans. 1*, **1972**, 2081.
[62] E. R. H. Jones, G.vD. Meakins, J. O. Miners, A. L. Wilkins, *J. Chem. Soc, Perkin Trans 1*, **1975**, 2308.
[63] H. L. Holland, E. M. Thomas, *Can. J. Chem*., **1982**, *60*, 160.
[64] A. M. Bell, I. M. Clark, W. A. Denny, E. R. H. Jones, G. D. Meakins, W. E. Muller, E. E. Richards, *J. Chem. Soc., Perkin Trans. 1*, **1973**, 2131.
[65] A. M. Bell, I. W. Browne, W. A. Denny, E. R. H. Jones, G. D. Meakins, A. Kasal, *J. Chem. Soc., Perkin Trans 1.*, **1972**, 2930.
[66] A. S. Clegg, W. A. Denny, E. R. H. Jones, G. D. Meakins, *J. Chem. Soc., Perkin Trans 1*, **1973**, 2137.
[67] C. D. Hufford, G. A. Capiton, A. M. Clark, J. K. Baker, *J. Pharm. Sci.*, **1981**, *70*, 151.
[68] C. D. Hufford, A. M. Clark, J. K. Baker, *J. Pharm. Sci.*, **1981**, *70*, 155.
[69] C. D. Hufford, A. M. Clark, J. D. McChesney, *Antimicrob. Ag. Chemother.*, **1981**, *19*, 337.
[70] W. J. Marsheck, A. Karim, *Appl. Microbiol*., **1973**, *25*, 647.
[71] A. Karim, E. A. Brown, *Steroids*, **1972**, *20*, 41.
[72] S. B. Mahato, S. Garai, *Steroids*, **1997**, *62*, 332.
[73] P. C. Peart, K. P. McCook, F. A. Russell, F. R. William, P. B. Reese, *Steroids*, **2011,** *76*, 1317.
[74] H. Li, H. M. Liu, W. Ge, L. Huang, L. Shan, *Steroids*, **2005**, *70*, 970.
[75] A. S. Lamm, A. R. M. Chen, W. F. Reynolds, P. B. Reese, *Steroids*, **2007**, *72*, 713.
[76] E. Minofy, H. Hamid, A. Elsalam, *Egypt, J. Microbiol*., **2000**, *34*, 153.
[77] W. Charney, H. L. Herzog, "*Microbial Transformations of Steroids*", **1967**, *2*, 14.

[78] Y. Wu, H. Li, X. M. Zhang, J. S. Gong, Z. M. Rao, J. S. Shi, X. J. Zhang, Z. H. Xu, *J. Mol. Catalysis:B Enzymatic*, **2015**, *120*, 111.
[79] J. P. Ferris, M. J. Fasco, F. L. Stylianopoulou, D. M. Jerina, J. W. Daly, A. M. Jeffrey, *Arch. Biochem. Biophys*., **1973**, *156*, 97.
[80] L. Sun, H. H. Huang, L. Liu, D. F. Zhong, *Appl. Environ. Microbiol*., **2004**, *70*, 2722.
[81] C. Smith, A. Wahab, M. S. A. Khan, M. S. Ahmad, D. Farran, M. I. Choudhary, E. Baydoun, *Steroids*, **2015**, *102*, 39.
[82] T. V. Bright, F. Dalton, V. L. Elder, C. D. Murphy, N. K. O'Connor, G. Sandford, *Org. Biomol. Chem.*, **2013**, *11*, 1135.
[83] N. Funa, M. Funabashi, Y. Ohnishi, S. Horinouchi, *J. Bacteriol*., **2005**, *187*, 8149.
[84] T. Makino, Y. Katsuyama, T. Otomatsu, N. Misawa, Y.Ohnishia, *Appl. Environ. Microbiol*., **2014**, *80*, 1371.
[85] N. Desrut, A. Kergomard, M. F. Renard, H. Veschambre, *Biochem. Biophys. Res. Commun.*, **1983**, *110*, 908.
[86] T. Makino, Y. Katsuyama, T. Otomatsu, N. Misawa, Y. Ohnishi, *App. Environ. Microbiol.*, **2014**, *80*, 1371.
[87] M. Thevis, W. Schanzer, *J. Am. Soc. Mass Spectrom.*, **2005**, *16*, 1660.
[88] N. K. O'Connor, A. S. Hudson, S. L. Cobb, D. O'Neil, J. Robertson, V. Duncan, C. D. Murphy, *Amino Acids*, **2014**, *46*, 2745.
[89] Stability Testing of New Drug Substances and Products, ICH Harmonised Tripartite Guideline, **2003**.
[90] J. G. Kuenen, W. N. Konings, *Accred. Qual. Assur*., **2010**, *15*, 133.
[91] K. Zaitsu, A. Miki, M. Katagi, H. Tsuchihashi, *Forensic Sci. Int.*, **2008**, *174*, 189.
[92] C. Jimenez, R. De la Torre, M. Ventura, J. Segura, R. Ventura, *J. Chrom. B*, **2006**, *843*, 84.
[93] M. Tsivou, D. Livadara, D. G. Georgakopoulos, M. A. Koupparis, J. Atta-Politou, C. G. Georgakopoulos, *Anal. Biochem*., **2009**, *388*, 146.

4 A New Method for Steroid Derivatisation

4.1 Steroids derivatisation with pentafluoropyridine (285) as a method of analysis in anti-doping

Jarman studied synthesis and cleavage of various perfluoroaryl ethers for the selective protection of alcohols.[1] He found that estradiol treated with pentafluoropyridine under Schotten-Baumann conditions gave bis-PFP estradiol adduct in a 92% yield. This adduct was subsequently deprotected with MeONa and estradiol was recovered in 65% yield.



276 **277**

Scheme 4.1 Steroid derivatisation with pentafluoropyridine (**283**).

As a part of the studies concerning the detection of anabolics in an athlete's urine sample, the reactivity of pentafluoropyridine (PFP (**285**)) with steroids was explored. It was expected that PFP could react cleanly with a range of steroids and give adducts that would be easily detected by ^{19}F NMR. Our hypothesis proposed that PFP could be used as a novel derivatisation technique which allows identification of hydroxy steroids in biological material by ^{19}F NMR (**Figure 4.1**).

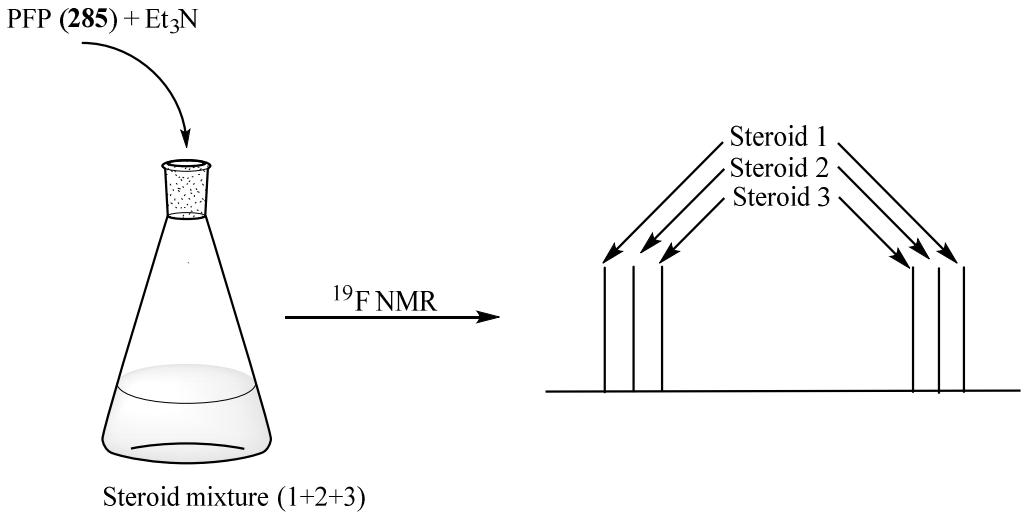


Figure 4.1 ^{19}F NMR as a novel method of detecting steroid in a mixture. This could be potentially used as an easy tool in detecting and identifying various steroids.

Initial studies focused on the synthesis of derivatized steroids presented below (**Figure 4.2**).

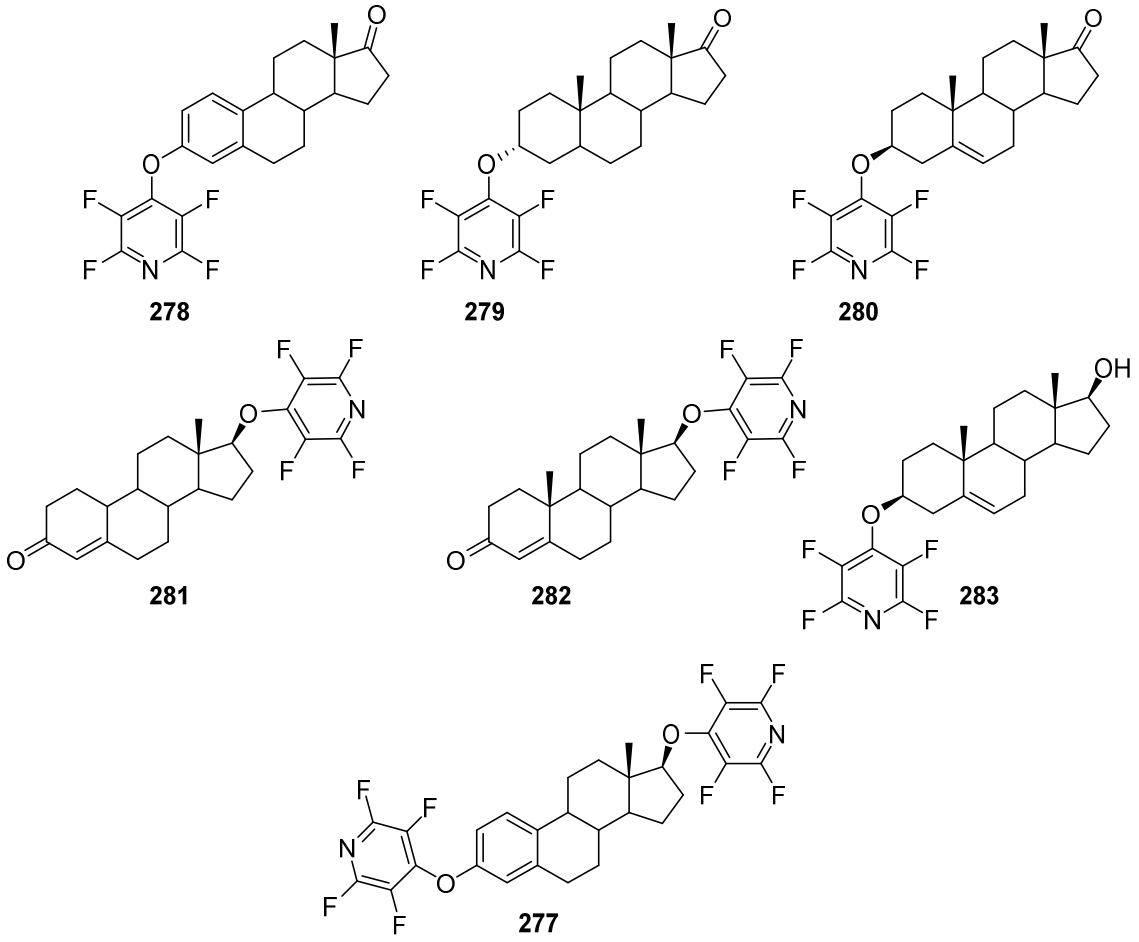
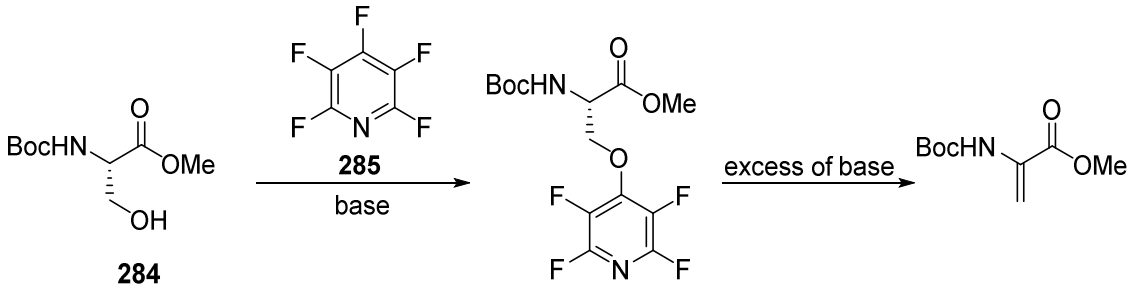


Figure 4.2 Target structures of pentafluoropyridine steroid adducts.

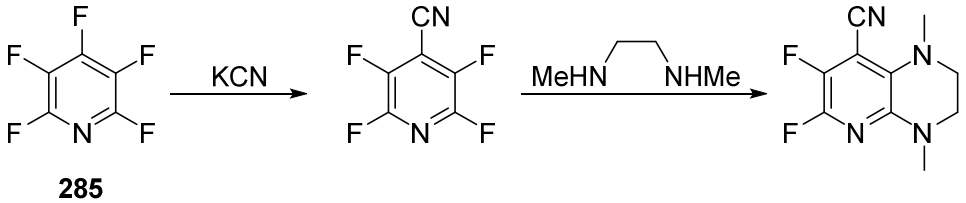
4.2 Current application of pentafluoropyridine

Previous studies in the Cobb group have focused on exploring the chemistry of PFP (**285**) to provide a novel strategy for tagging and protecting amino acids. For example, Webster *et al.* showed that **285** reacts with protected amino acids to generate novel peptide building blocks, which could be elaborated into dehydroalanine derivatives (**Scheme 4.2**).[2] A range of novel dehydrobutyrines, potentially useful in peptide chemistry, were generated and showed that pentafluoropyridine can successfully react with a variety of nucleophiles to afford 4-substituted tetrafluoropyridine. The regioselectivity of nucleophilic substitution in this process may be explained by high nucleophilicity of oxygen and the activating influence of pyridine ring nitrogen that significantly activates the para position.



Scheme 4.2 Synthesis of dehydroalanine derivatives using PFP (**285**).

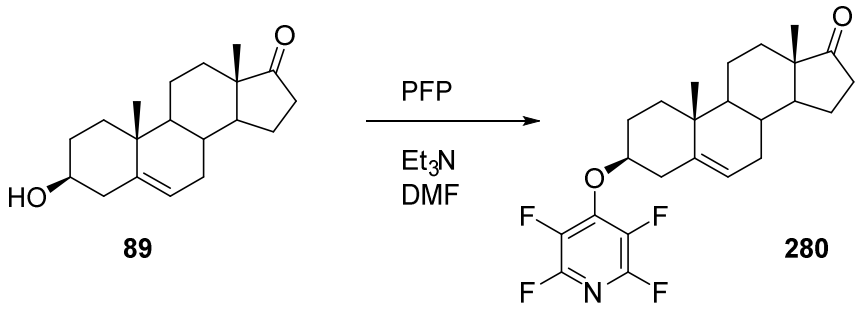
The chemistry of PFP (**285**) has been also explored by Prof. G. Sandford (Durham University) who has shown that PFP can be used to generate novel pyridine derivatives bearing five functional groups.[2] For example, polyfunctional tetrahydropyrido[3,4-b]pyrazine scaffolds have been synthesized easily by a one-pot annulation reaction of PFP (**285**) with various diamines (**Scheme 4.3**).[3] This general approach has special relevance to the development of new fluorinated drug small molecules, which are playing an increasingly important role in the pharmaceutical industry.



Scheme 4.3 Functionalization of PFP (**285**).

4.2.1 Synthesis of PFP tagged steroids

In order to find appropriate conditions for the synthesis of pentafluoropyridine steroid adducts, several different conditions were explored using dehydroepiandrosterone (DHEA (**89**)) (**Table 4.1**). To a solution of the steroid (100 mg) in an appropriate solvent, base (1.2 eq. compared to starting steroid) and pentafluoropyridine (2 eq. compared to starting steroid) were added under argon at room temperature. The reaction mixtures were stirred at room temperature or at 50°C and monitored by $^{19}F/^{1}H$ NMR. The results are summarised in the table below (**Table 4.1**).



Sheme 4.4 Synthesis of a DHEA-PFP adduct.

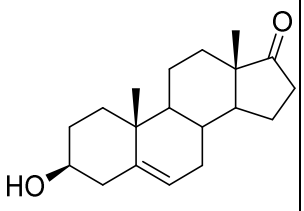
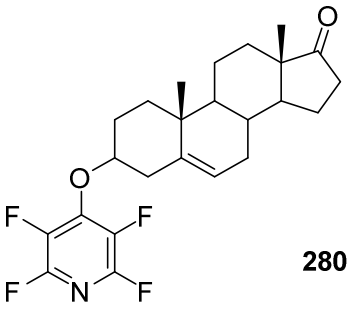
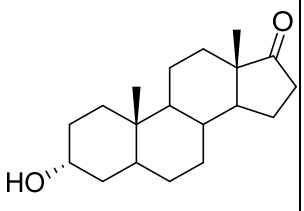
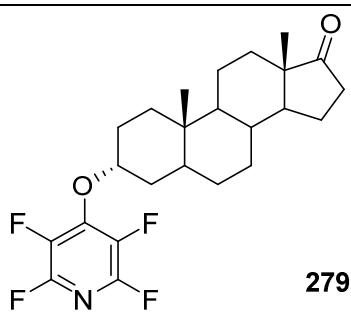
Table 4.1 Attempted synthesis of DHEA pentafluoropyridine adducts under various reaction conditions.

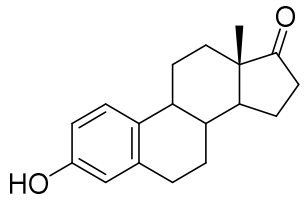
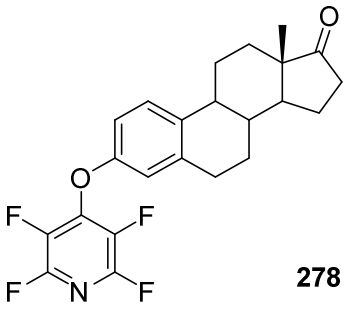
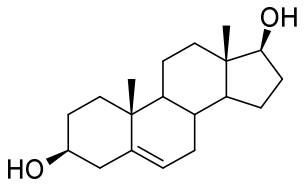
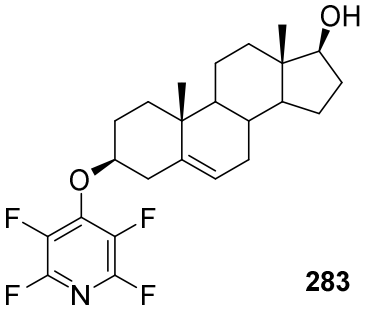
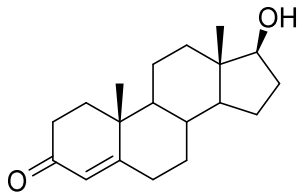
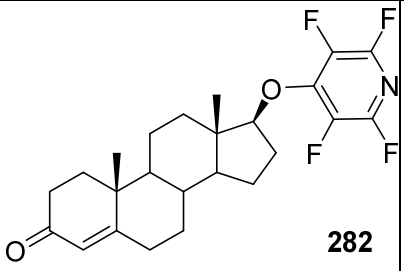
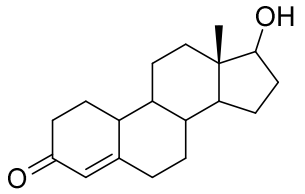
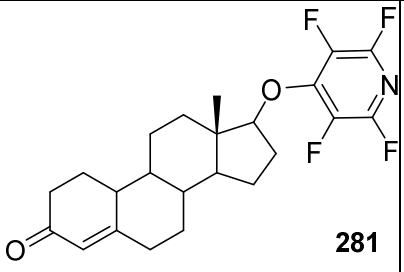
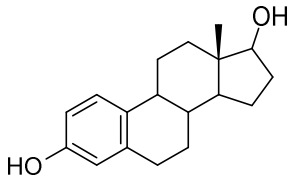
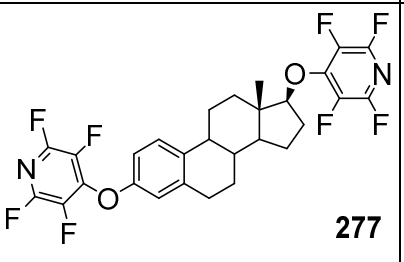
Entry	Base	Solvent	Temp. (°C)	Time (h)	Conversion (%) (base on ^{1}H NMR)
1	K_2CO_3	DCM	RT	72	No product/ side products
2	K_2CO_3	DMF	RT	48	14
3	K_2CO_3	DMF	50	4	55
4	K_2CO_3	MeCN	RT	48	No product
5	Et_3N	DMF	RT	72	16
6	Et_3N	DMF	50	4	86
7	Et_3N	DCM	RT	72	No product/ side products
8	Et_3N	MeCN	RT	72	No product/ side products
9	1M NaOH	DCM	RT	24	No product/ side products

From the initial experiments highlighted above, it appeared that the nucleophilic substitution does not progress at room temperature (DCM, MeCN) or it progress as very slowly (DMF). At higher temperature the reaction was accelerated with the fastest rate achieved for the reaction carried out in DMF with Et_3N (**Table 4.1**, Entry 6). This reaction was worked-up after 4h at 50ºC and gave the product **280** in a 86% yield. Formation of the fluorinated product was confirmed by examination of the ^{19}F NMR spectrum, which revealed new signals at δ_{Fo} = - 90.80 ppm and δ_{Fm} = - 158.14 ppm corresponding to the fluorine atoms in ortho and meta positions.

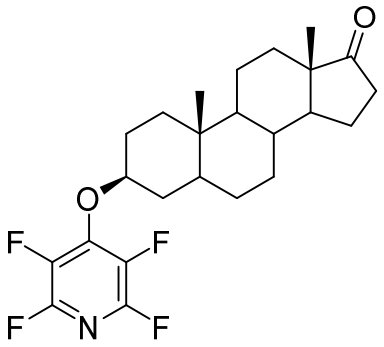
From the initial results, it appeared that the PFP-steroid adduct could be easily synthesised using Et_3N in DMF. Following this protocol, Et_3N (1.2 eq. compared to the starting steroid) and PFP (2.2 eq) were added to a solution of the appropriate steroid (0.1 g) in DMF (1 ml). The reaction mixture was then stirred for 3 days at 50ºC. After that time, ^1H NMR analysis indicated reaction completion with some starting material remained unreacted. The results are summarized in the table below (**Table 4.2**).

Table 4.2 Synthesis of PFP – steroid abducts.

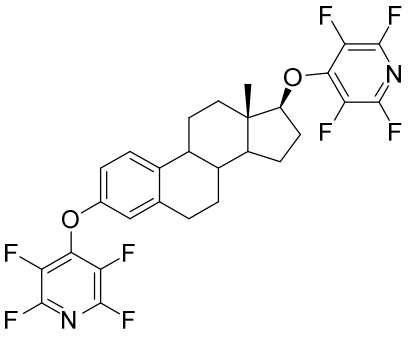
Entry	Starting material	Product	^{19}F NMR	Isolated Yield (%)
1	 **89**	 **280**	-90.80; -158.14	86
2	 **97**	 **279**	-91.00; -158.11	76

3	 **121**	 **278**	-88.89; -154.41	60
4	 **90**	 **283**	-90.87; -158.14	67
5	 **91**	 **282**	-90.74; -158.12	52
6	 **111**	 **281**	-90.68; -158.12	68
7	 **290**	 **277**	-88.85; -90.77; -154.46; -158.11	70

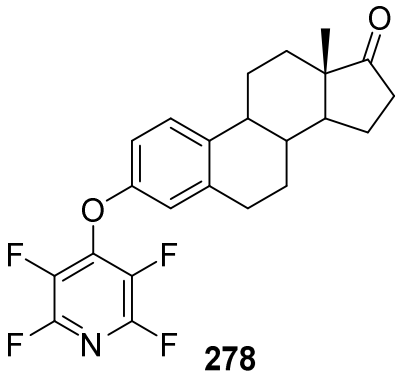
The formation of the products in **Table 4.2** were confirmed by ^{19}F NMR, GC-MS/ASAP experiments. The ^{19}F NMR spectrum showed two characteristic signals corresponding to the *ortho* and *meta* fluorine atoms. In addition, re-crystallisation of the products from $CHCl_3$ gave crystals suitable for X-ray diffraction (**Figure 4.3**).



279



277



278

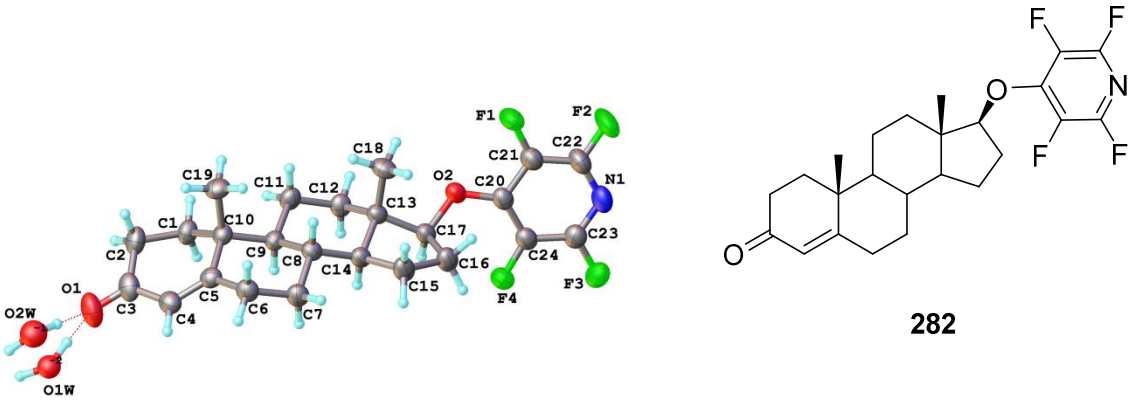
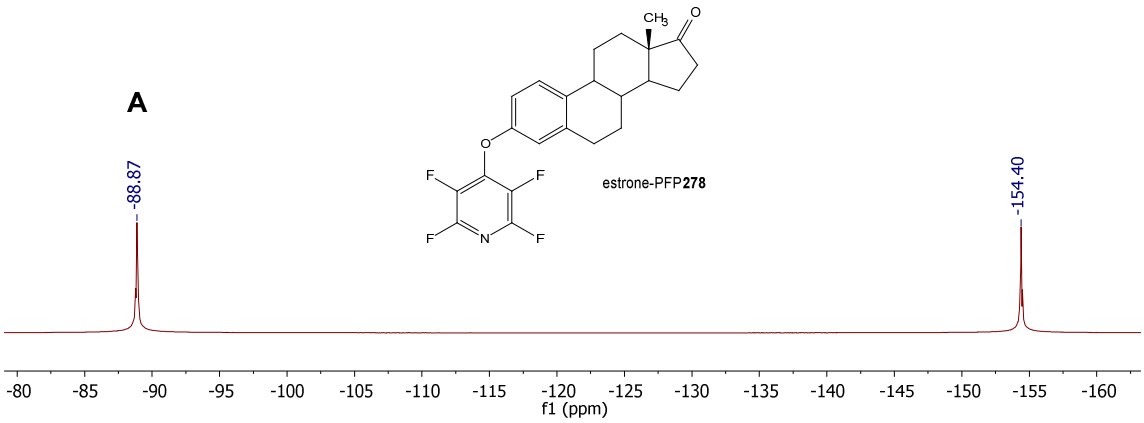
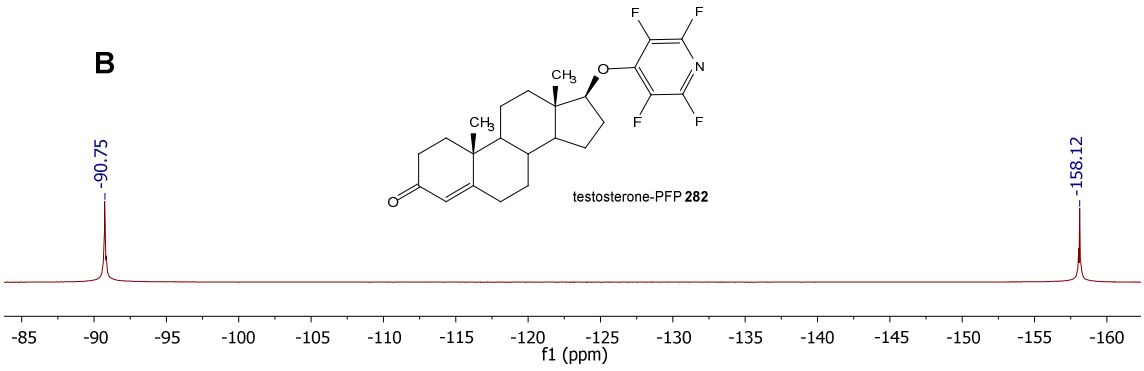


Figure 4.3 X-ray crystal structures for selected PFP derivatives for steroids.

4.2.2 ^{19}F NMR studies of PFP-tagged steroids

It was hypothesised that various steroids could be derivatised with PFP (**185**) to give adducts easily detectable by ^{19}F NMR. This could be potentially used as an easy tool in detecting and identifying various steroids in complex mixtures. In order to prove this, a simple experiment was designed employing PFP. A mixture of several steroids in equal portions (DHEA (**89**), testosterone (**91**), estrone (**121**), androsterone (**97**)) were dissolved in DMF and treated with PFP (**185**). Upon reaction completion the reaction mixture was worked-up and the crude reaction mixture was analysed by ^{19}F NMR. Based on these preliminary results it appears that this technique could be used to identify a range of steroids. However, due to a different reactivity of the hydroxylated steroids, quantitative measurements may not be possible. To circumvent this problem, the reaction conditions should be modified to provide steroid derivative in quantitative yield.





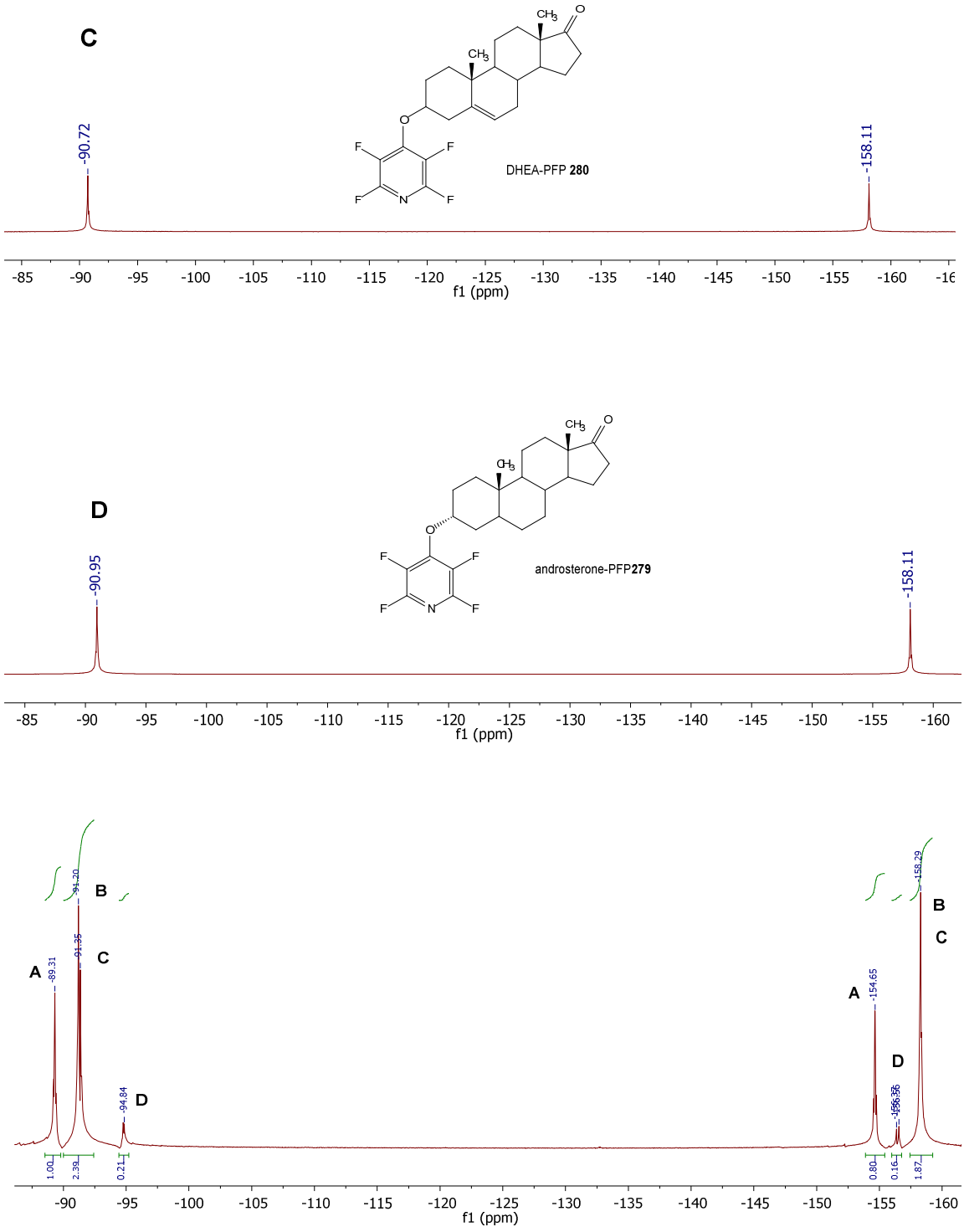
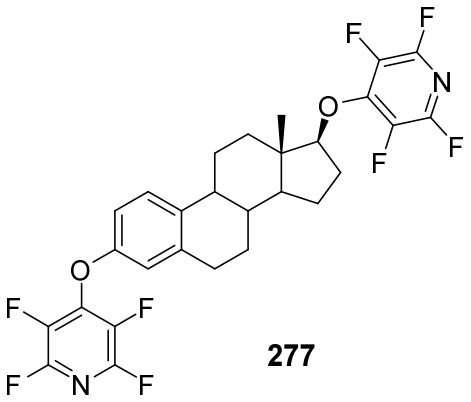


Figure 4.4 ^{19}F NMR of steroids mixture: **A** – estrone-PFP, **B** – testosterone-PFP, **C** - DHEA-PFP, **D** – androsterone-PFP.

4.3 Metabolism experiments

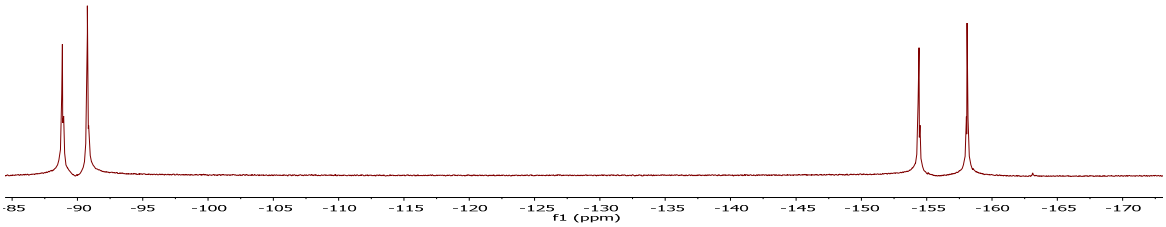
4.3.1 3,17-Bistetrafluoropyridine-estradiol (277)



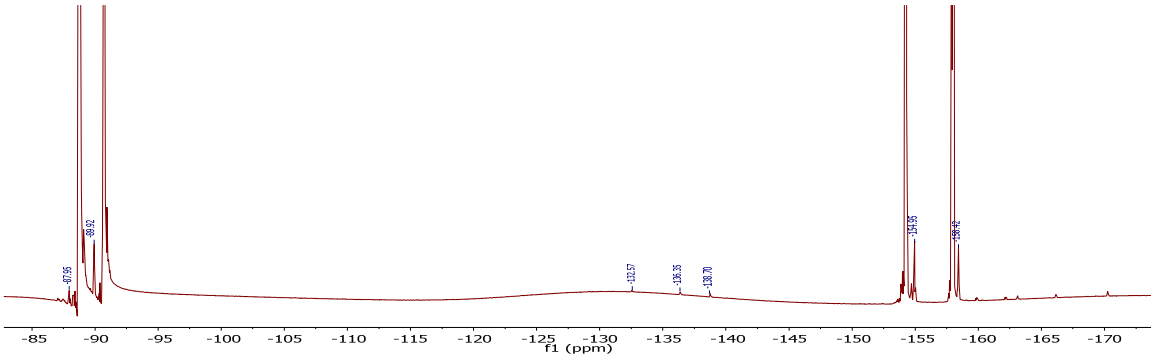
277

S. griseus was fed with 3,17-bistetrafluoropyridine-estradiol (**277**) following the general protocol described earlier (**Chapter 3, Section 3.3.1**). The bacteria were centrifuged and extraction of the pallet and supernatant was carried out. The extracts were analysed by ^{19}F NMR and GC-MS (**Figure 4.4**).

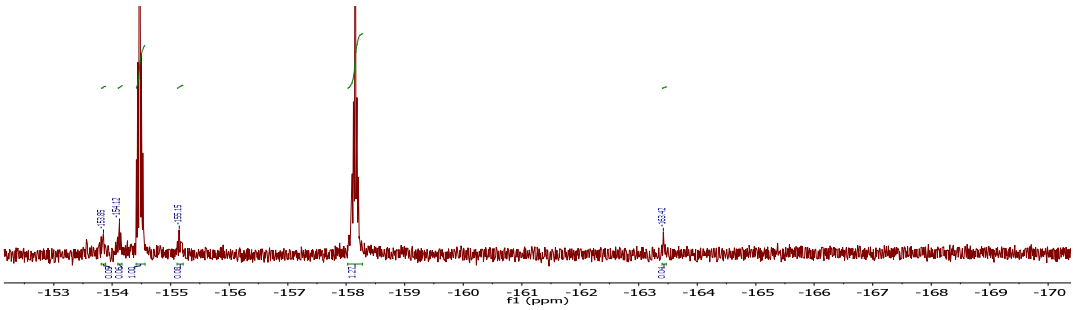
A – ^{19}F NMR spectral analysis of the Control (organic fraction)



B – ^{19}F NMR spectral analysis of the Pellet (organic fraction)



C – ^{19}F NMR spectral analysis of the Supernatant (organic fraction)



E – ^{19}F NMR (decoupled) spectral analysis of the Pellet (aqueous fraction)

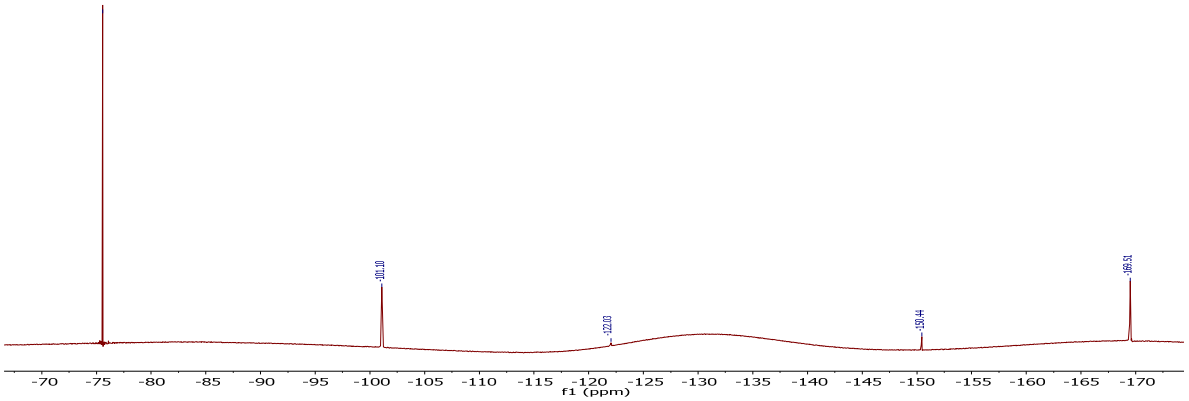
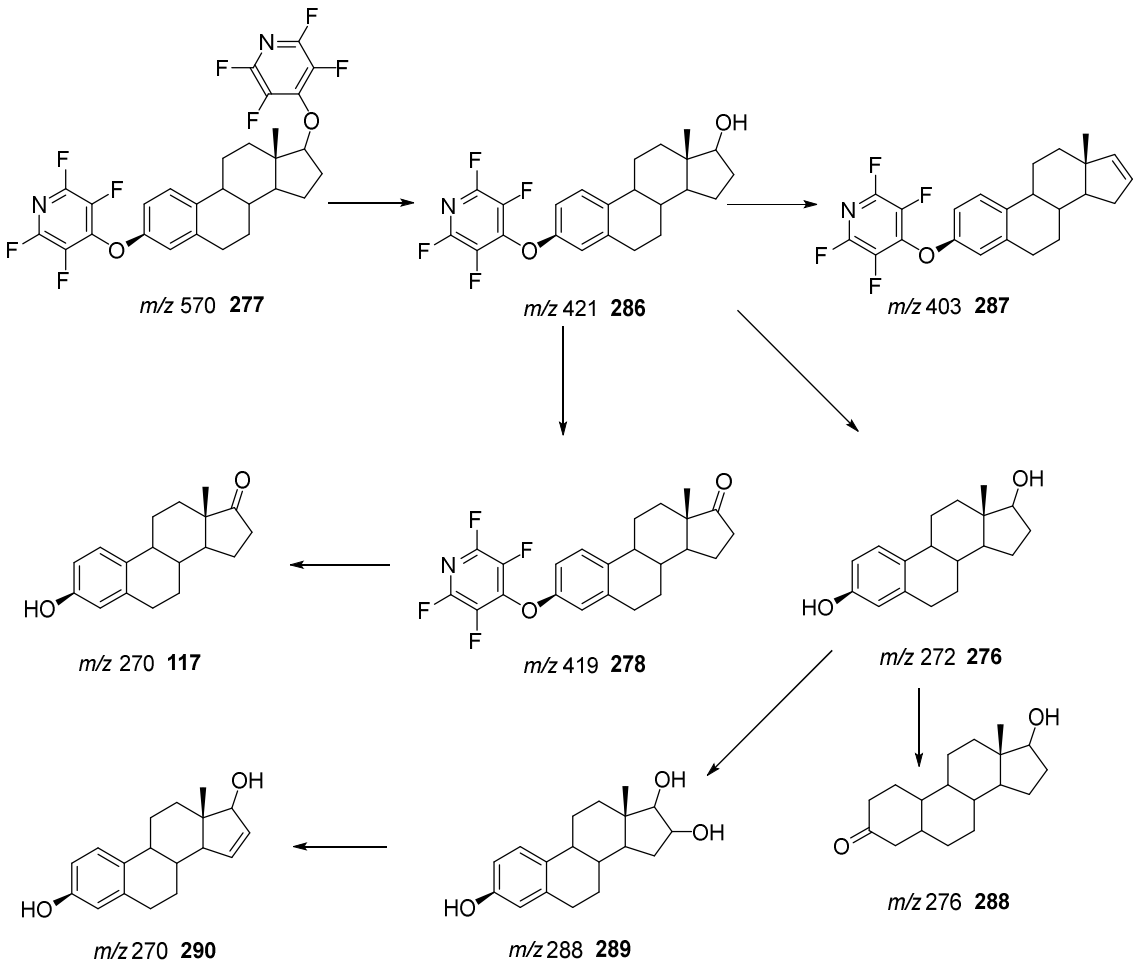


Figure 4.4 ^{19}F NMR analysis of 3,17-bistetrafluoropyridine-estradiol (**277**) incubated with *S. griseus*. **A**- ^{19}F NMR of control organic fraction in d-chloroform. **B**- ^{19}F NMR of pellet organic fraction in d-chloroform. **C**- ^{19}F NMR of supernatant organic fraction in d-chloroform. **E**- ^{19}F NMR (decoupled) of pellet aqueous fraction in D_2O. The other fractions (pellet and supernatant aqueous fraction in D_2O) showed no peaks during ^{19}F NMR analysis.

Table 4.3 New metabolites detected by ^{19}F NMR analysis of 3,17-bistetrafluoropyridine-estradiol (**277**) incubated with *S. griseus*.

Experiment	^{19}F NMR signal (ppm)
Supernatant (organic)	- 153.85, -154.12, -155.15, -163.13
Supernatant (aqueous)	No fluorinated metabolites
Pellet (organic)	- 132.78, - 136.60, - 138.98, -135.84, - 154.10, -154.81, - 155.17, - 158.60, -160.06, - 162.44, - 166.34, - 170.46
Pellet aqueous	No fluorinated metabolites

GC-MS analysis of the pellet organic fraction and the supernatant organic fraction detected a few potential metabolites presented in **Scheme 4.5**. An ion at *m/z* 421 was not present in the GC-MS spectra of the starting material and the control sample and corresponds to the estradiol with one fluoropyridine group (**286**). This molecule is then oxidised to the estrone derivative (**278**, *m/z* 419) or cleaved to estradiol (**276**, *m/z* 272). The product at *m/z* 270 (**117**, estrone) could be created by cleavage of the pyridine units and oxidation of 17-C hydroxyl group (alternatively by oxidation of estradiol). The ion at *m/z* 270 could also correspond to the unsaturated estradiol which could be formed by cleavage of the pyridine units (**290**), oxidation at 16-C and subsequent elimination of water (**289**, the intermediate triol at *m/z* 288 was not detected). The ion at *m/z* 276 corresponds to the reduced estradiol (**288**).



Scheme 4.5 Proposed metabolites produced after 3,17-bistetrafluoropyridine-estradiol (**277**) incubation with *S. griseus*.

4.4 Conclusions and Future Work

Pentafluoropyridine has special relevance to the development of new fluorinated drug small molecules.[1] In addition, it could be also used for the selective protection of alcohols.[4] As a part of the studies concerning the detection of anabolics in an athlete's urine sample, the reactivity of pentafluoropyridine with hydroxy steroids was explored. An easy and fast method of preparing fluorine derivatised hydroxyl steroids has been developed. This involved reaction of steroid with pentafluoropyridine, triethylamine in DMF. A range of PFP steroid adducts were synthesised in moderate to excellent yields (52-86%). Several novel hydroxy steroid-PFP adducts were synthesised and the structures of 4 steroid PFP derivatives were confirmed by X-ray analisis.

In the second phase of the work the analytical potential of the hydroxy steroid-PFP adducts was examined. It was found that each of the steroid-PFP adducts have characteristic ^{19}F NMR spectra which could be used for their detection or confirmation in mixed samples. For example, a mixture of four steroids (DHEA (**89**), testosterone (**91**), estrone (**121**), androsterone (**97**)) when treated with pentafluoropyridine formed a mixture of the corresponding adducts easily detectable by ^{19}F NMR.

The future work should investigate the scope of this new derivatisation method. This will involve a synthesis of various hydroxy steroid-PFP adducts to generate a library of the reference compounds. Subsequently biological samples could be spiked with steroid mixtures and, after derivatisation, ^{19}F NMR analysis could be carried out to investigate the detection limits of this technique.

4.5 Reference

[1] M. Jarman, R. McCague, *J. Chem. Research* (S), **1985**, 114.
[2] A.M. Webster, C.R. Coxon, A.M. Kenwright, G. Sandford, S.L. Cobb, *Tetrahedron*, **2014**, *70*, 4661.
[3] G. Sandford , R. Slater, D.S. Yufit, J.A. Howard, A. Vong, *J. Org .Chem.,* **2005**, *70*, 7208.

5 Conclusions and Future work

5.1 Conclusion

The use of performance-enhancing drugs in sport was prohibited in the early 1920s, however, no effective testing methods for banned substances were available at that time. To address this problem, in 1999 an independent agency The World Anti-Doping Agency (WADA) was created to promote, coordinate, and monitor the fight against doping in sports. This led to formation of detailed anti-doping guidelines together with a comprehensive and ever changing list of banned substances. WADA's approved anti-doping laboratories tend to rely mainly on GC-MS and LC-MS techniques to detect prohibited substances. In sport the detection of the use of banned substances is routinely carried out using urine samples as, unlike blood samples, these do not require medical sampling officers for collection. The collected urine sample is divided into two and preserved within sealed containers. A second sample will be only analysed if a positive result was obtained for the first sample. Unfortunately, anti-doping organizations do not have a detailed standard protocol regarding storage or transportation of the urine samples. The endogenous steroid profile could be altered due to microbial growth in the urine as a result of factors such as variability in temperature during transport.

This thesis deals with the development of a novel method that utilizes ^{19}F NMR spectroscopy which could be used either as a standalone procedure or as an additional protocol alongside existing methods to identify urine samples that could generate false positives or false negatives as a result of microbial growth. It was hypothesised that a urine sample could be spiked with a reference fluorinated steroid and its chemical degradation or metabolism caused by microorganisms could be observed by ^{19}F NMR. The work described in **Chapter 2** was focused on the synthesis of fluorinated steroids using a method that involve the reaction of Selectfluor® (a convenient source of electrophilic fluorine) with enolates/enols of varius steroids. In comparison to gaseous F_2, the key benefit of this electrophilic fluorination agent is the ability to provide facile access to fluorinated steroids without the need to use specialised equipment for F_2 manipulation. Modification of the existing fluorination protocol with Selectfluor® (a novel 'one pot' approach) provided access to 11 fluorinated steroids and 3 novel fluoro-steroids, as presented in **Figure 5.1**. Those molecules were synthesised in moderate yields and varying diastereoselectivity.

Several synthesised steroids were re-crystallised and crystals suitable for X-ray were obtained. The first ever X-ray crystal structures for several fluorinated steroids have been obtained: e.g. 6α-fluoro-testosterone (**84**), (**Figure 5.2**).

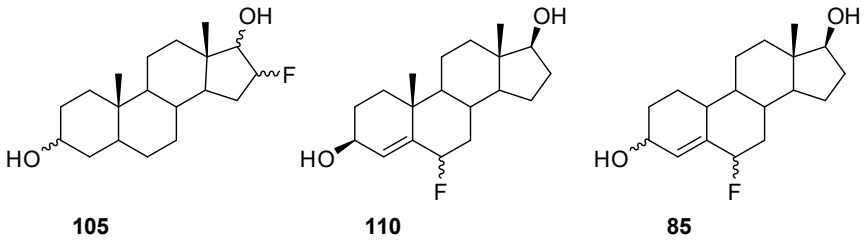


Figure 5.1 Synthesised novel fluoro-steroids.

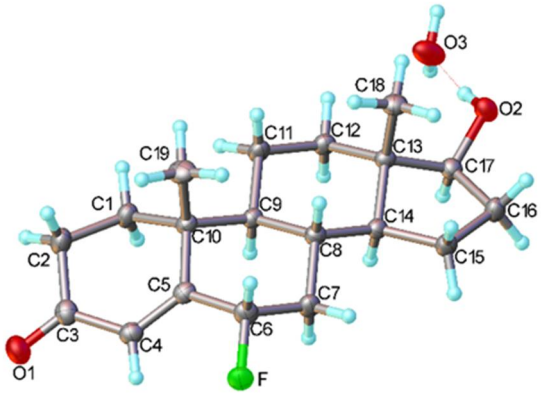


Figure 5.2 X-ray structures of 6α-fluoro-testosterone (**84**).

It is known that under certain conditions microorganisms' cytochrome P-450 enzyme systems can facilitate various reactions such as hydroxylation, oxidation, dealkylation and deamination reactions. In most cases, this result in the formation of unstable intermediates that spontaneously decompose to the stable metabolites. One of the key aims of this project was to assess fluorinated steroids for their biotransformation by a range of microorganisms (pure cultures). The biological experiments were carried out in the laboratories of University of Durham, Stockton department of Pharmacy and University College Dublin (UCD). The work performed at Durham University and UCD focused on the bacteria *Streptomyces griseus.* The experiments in Stockton employed *Escherichia coli, Bacillus subtilis* and *Bacillus megaterium*. The strains were cultured in standard media (e.g. tryptone soy broth,) for 24 h and the fluorinated steroids added to the cultures. After further incubation (24-48h), the cultured strains were sonicated, the biotransformation products were extracted and analysed by ^{19}F NMR. The degradation of fluorinated steroids by

microorganisms could easily detected using ^{19}F NMR. The most significant transformation was observed with 6β-fluoro-progesterone (**144**) (**Figure 5.3**) and 6-fluoro-nortestosterone (**86**). Other steroids were metabolised to the lesser extent. In an attempt to characterise the products, purification was carried out by preparative TLC and HPLC. Each fraction was analysed (GC-MS/LC-MS and ^{19}F NMR) and metabolic pathways were proposed. As reported in the literature, metabolism of steroids by microorganisms involves enzymes P-450 which is responsible for oxidation and this was shown by MS, however due to time constrains the structures of metabolites were not confirmed. This showed that steroids can be bio-transformed by common bacteria in human environment. A summary of the transformations achieved for 6β-fluoro- progesterone (**144**) with *S. griseus* is presented on **Scheme 5.1**.

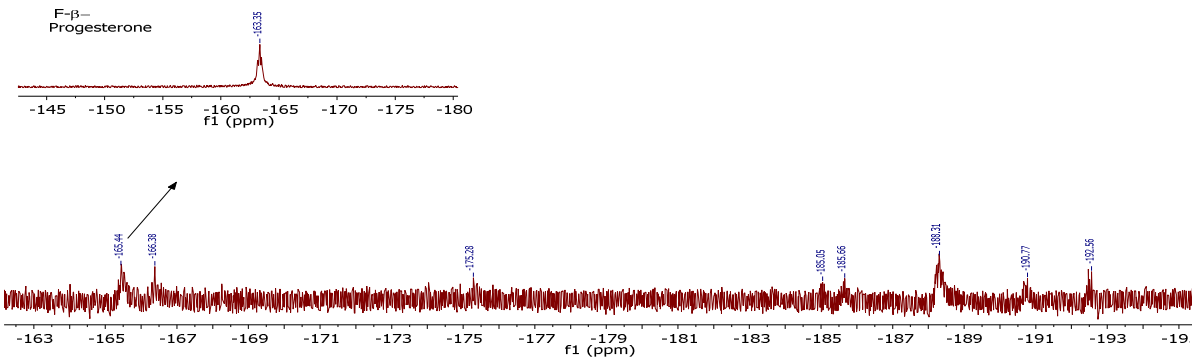
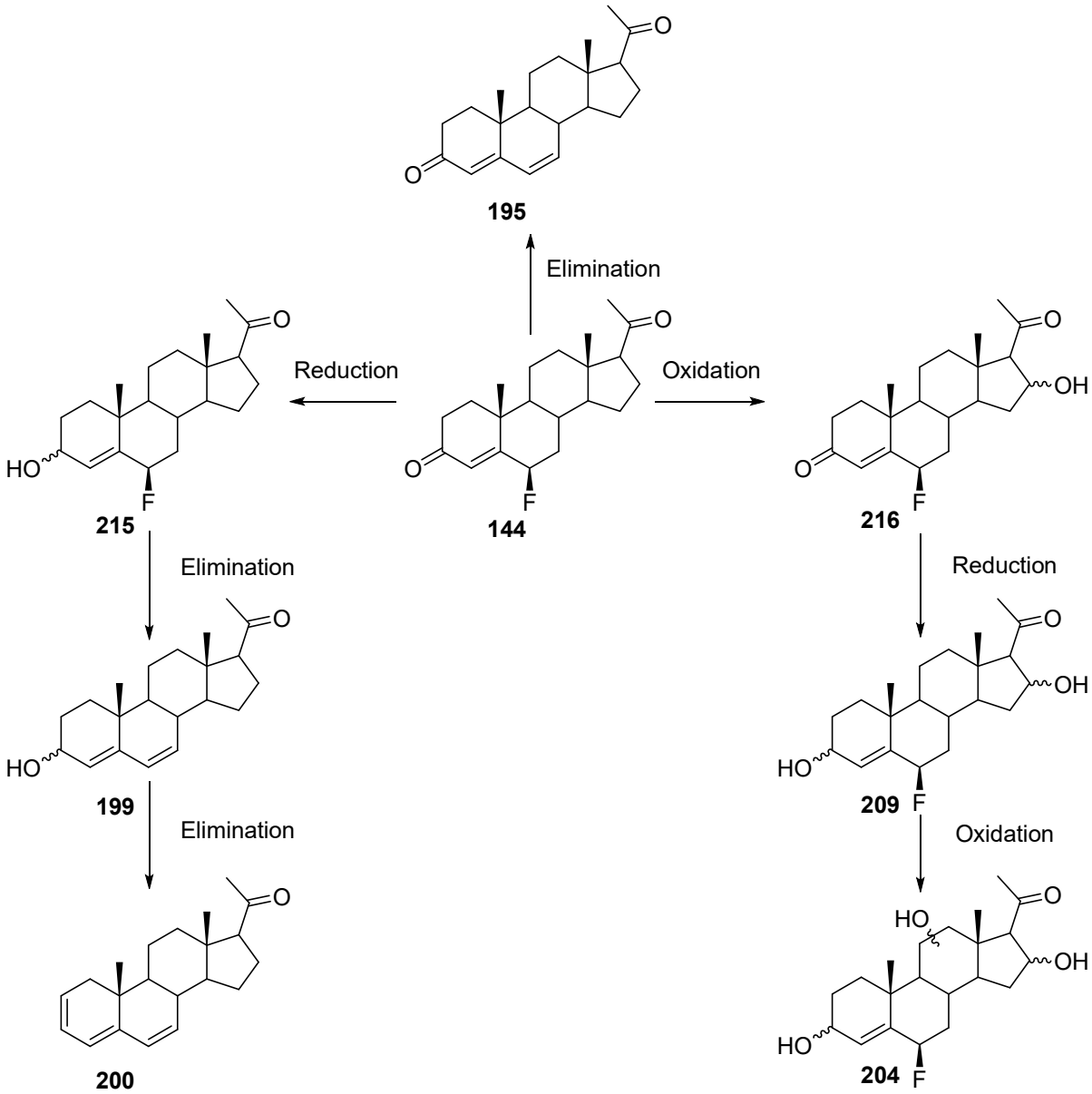


Figure 5.3 ^{19}F NMR analysis of 6β-fluoro-progesterone (**144**) incubated with *S. griseus*.

For 6β-fluoro-progesterone (**144**), seven different metabolites were proposed (**Scheme 5.1**). Certain steroids such as 3-hydroxy-4,6-pregnen-20-one (**199**), 2,4,6-pregnen-20-one or 3-Hydroxy-4,6-pregnen-20-one (**200**) and (**195**) could be formed during the isolation or purification stages via elimination of water or/and HF. Two metabolites were formed by reduction of C-3 carbonyl group and hydroxylation at C-16 carbon (16α-hydroxylation). These reactions are known and were reported in the literature for different steroids. Because ion at *m/z* 351 [M+1] was detected in 6 different fractions, it is highly probable that different diastereoisomers of 6-fluoro-3,16-dihydroxy-4-pregnen-20-one (**209**) were formed.

Through this work we confirmed the hypothesis that ^{19}F NMR can be used to detect microbial contamination that could lead to false positive or false negative results during anti-doping tests.



Scheme 5.1 Summary of the compounds potentially detected after the biotransformation of 6β-fluoro-progesterone (**144**) by *S. griseus.*

Escherichia coli do not contain cytochrome P450 enzymes that mimic oxidation processes *in vivo* and therefore no metabolites were detected. The experiments carried out with *Bacillus subtilis* and *Bacillus megaterium* did not produce any metabolites it could be due

to problems with the growth of the microorganisms. Finally, as a part of the studies concerning the detection of anabolics in a urine sample, reactivity of pentafluoropyridine with hydroxyl steroids was explored. It was hypothesised that pentafluoropyridine would react cleanly with a range of hydroxyl steroids and give corresponding ethers easily detectable by ^{19}F NMR. It was shown in **Chapter 4** that hydroxy steroids treated with Et_3N and pentafluoropyridine in DMF form perfluoropyridine ethers in good yield. Several novel hydroxy steroid-PFP adducts (**Figure 5.3**) were synthesised and the structures of 4 steroid PFP derivatives were confirmed for the first time by X-ray structure (**Figure 5.4**). An easy and fast method of preparing fluorine tagged steroids was developed.

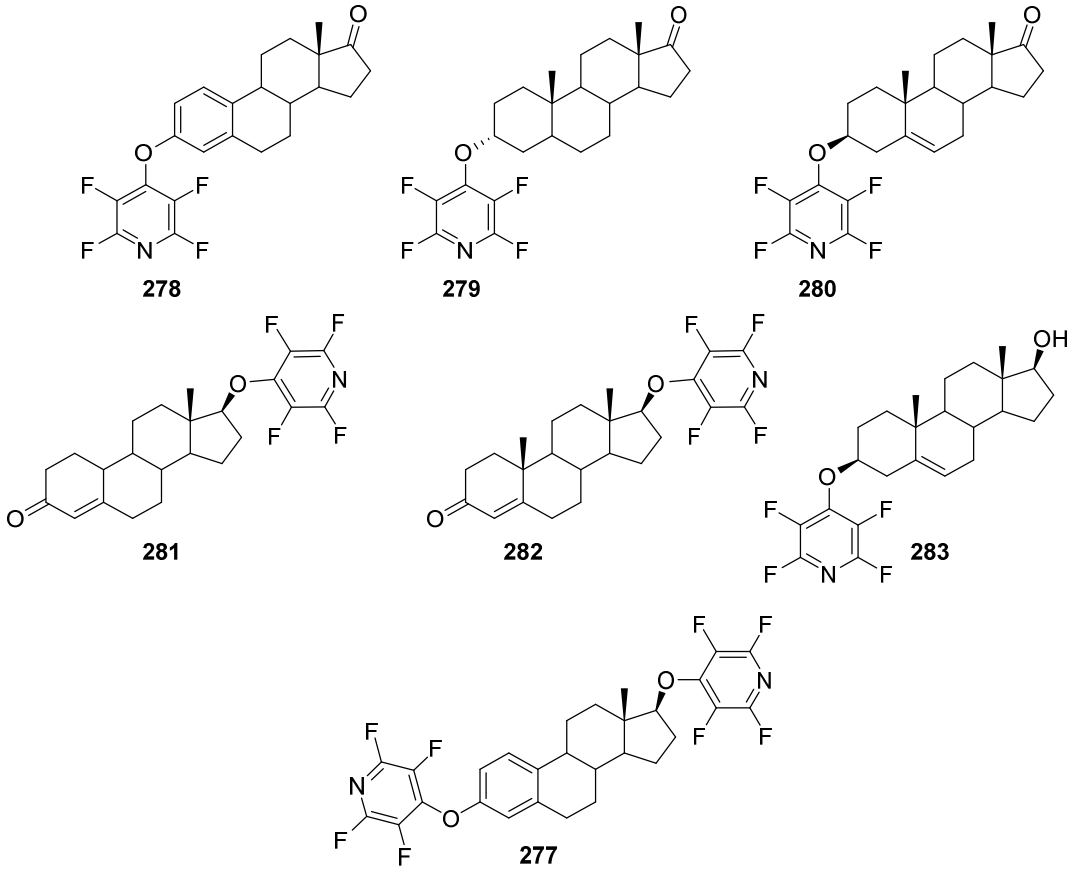
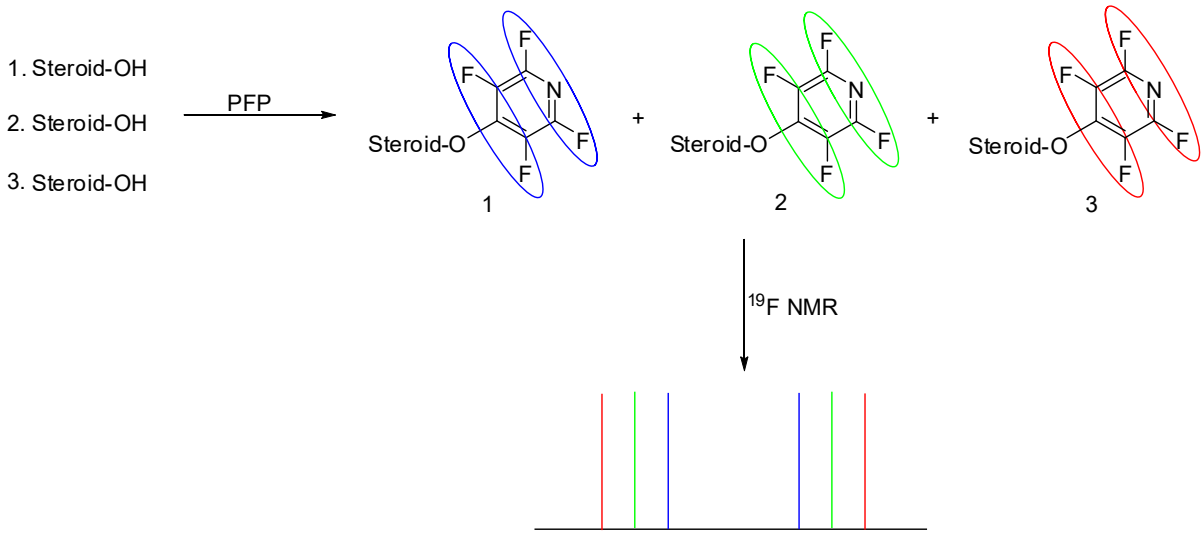


Figure 5.4 Structures of pentafluoropyridine steroid adducts prepared.

It was proposed that this reaction could be used as a novel derivatisation technique which allows identification of hydroxy steroids in biological material by ^{19}F NMR (**Scheme 5.2**). It was found that the investigated hydroxy steroid-PFP adducts have very similar ^{19}F NMR spectra however they can be distinguished using this technique.



Scheme 5.2 ^{19}F NMR as a novel method of detecting steroid in a mixture

5.2 Future work

To address the problem of microbial contamination of urine samples in sports we initiated a study to develop a new protocol that will utilize fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F NMR). This protocol employs fluorinated steroid as an internal standard which should act as a substrate for the same enzymes as the natural steroid and both molecules should be metabolised via the same pathways. The protocol allows detection of microbial growth by ^{19}F NMR, which could lead to false positive or false negative results in anti-doping samples. Future work in this area should focus on exploring the metabolism of fluorinated steroids by common bacteria and fungi found in human environment. This will involve the synthesis of new fluorinated steroids for feeding experiments to produce more detailed information about microbial degradation of fluorinated-steroids. In addition, the biological experiments should be scaled-up to isolate sufficient amount of the metabolites for their characterisations. If necessary, the synthesis of the isolated metabolites should be carried out to confirm structure. Future work should also focus on the investigation of the stability of fluorine labelled steroids in fake urine and subsequently in urine samples from healthy volunteers. The microorganisms in the urine responsible for steroids transformation could be isolated by initially streaking loopfuls of the urine onto agar plates containing some fluorine labelled steroid. The colonies that are most apparent should be further examined by re-culturing in liquid media containing the fluorinated steroid, and the supernatants should be examined for the presence of fluoro-metabolites by ^{19}F-NMR. Additionally, to determine if the microbial community is altered by the presence of the fluorinated steroid, restriction fragment length polymorphism analysis could be conducted on spiked and non-spiked samples.

In the second phase of the work the analytical potential of the hydroxysteroid-PFP adducts was examined. It was found that these adducts are easily synthesised by reacting pentafluoropyridine with steroids under basic conditions in DMF. The steroid-PFP adducts have characteristic ^{19}F NMR spectra which could be used for their detection or confirmation in biological samples. The future work should investigate the scope of this new derivatisation method. This will involve a synthesis of various hydroxy steroid-PFP adducts to generate a range of the reference compounds. Subsequently biological samples could be

spiked with steroid mixtures and after derivatisation ^{19}F NMR analysis could be carried out to investigate detection limits of this technique.

6. Experimental

6.1 General Experimental

All air- and/or moisture-sensitive reactions were carried out under an argon atmosphere in oven-dried glassware.

Chromatography

Thin layer chromatography (TLC) was performed using commercially available aluminium-backed plates coated with silica gel 60 F_{254} (UV_{254}) or neutral aluminium oxide 60 F_{254} (UV_{254}), and visualised under ultra-violet light (at 254 nm), or through staining with ethanolic phosphomolybdic acid followed by heating. Flash column chromatography was carried out using 200-400 mesh silica gel 40-63 μm or neutral alumina

Gas chromatography

Gas Chromatography was carried out on a Hewlett-Packard 5890 series II gas chromatograph fitted with a 25 cm column and connected to a flame ionisation detector.

Infrared spectroscopy

Infrared spectra were recorded using a Diamond ATR (attenuated total reflection) accessory (Golden Gate) or as a solution in chloroform via transmission IR cells on a Perkin-Elmer FT-IR 1600 spectrometer.

NMR spectroscopy

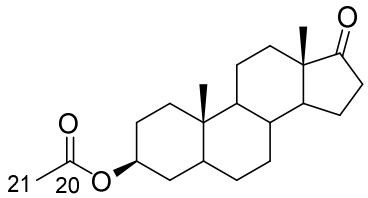
^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded in $CDCl_3$ (unless otherwise stated) on, Varian Mercury-400 (^{1}H, ^{13}C, ^{19}F), Bruker Avance-400 (^{1}H, ^{13}C), Varian Inova-500 (^{1}H, ^{13}C) or Varian VNMRS-700 (^{1}H, ^{13}C) spectrometers and reported as follows: chemical shift δ (ppm) (number of protons, multiplicity, coupling constant *J* (Hz), assignment). The chemical shifts are reported using the residual signal of $CHCl_3$ as the internal reference (δ_H = 7.26 ppm; δ_C = 77.0 ppm). All chemical shifts are quoted in parts per million relative to tetramethylsilane (δ_H= 0.00 ppm) and coupling constants are given in Hertz to the nearest 0.5 Hz. Assignment of spectra was carried out using COSY, NOESY, HSQC, and HMBC experiments.

Mass spectrometry

Gas-chromatography mass spectra (EI) were taken using a Thermo-Finnigan Trace with a 25 cm column connected to a VG Mass Lab Trio 1000. Electrospray mass spectra (ES) were obtained on a Micromass LCT Mass Spectrometer. High resolution mass spectra were obtained using a Thermo-Finnigan LTQFT mass spectrometer or XevoQToF mass spectrometer (Waters UK, Ltd) by Durham University Mass Spectrometry service.

6.2 Synthesis of fluorinated steroids

3β-Acetoxyandrostan-17-one (98) [1]

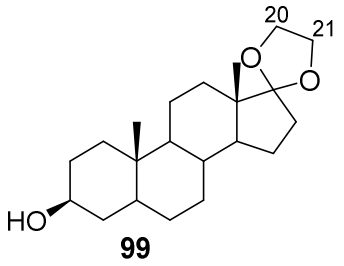


98

To a solution of androsterone (**97**) (0.60 g, 2.1 mmol) in dry DCM (50 ml) was added acetic anhydride (0.42 ml, 4.4 mmol) and DMAP (0.84 g, 6.9 mmol) under argon. The reaction mixture was stirred at RT for 4 h. After that time, the reaction mixture was poured into water and neutralized with saturated aqueous solution of $NaHCO_3$ (50 ml) The layers were separated and the aqueous layers extracted with DCM (3×20 ml). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by filtration through a plug of silica (eluting with hexane / ethyl acetate 5:1) to give **98** as a white solid (0.67 g, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.68-0.73 (1H, m, 9-C**H**), 0.85 (3H, s, 18-C**H**$_3$), 0.86 (3H, s, 19-C**H**$_3$), 0.94-1.06 (3H, m, 7-C**H**), 1.16-1.40 (7H, m, 4-C**H**, 5-C**H**, 6-C**H**, 11-C**H**, 12-C**H**, 14-C**H**), 1.46-1.67 (5H, m, 2-C**H**, 8-C**H**, 15-C**H**), 1.71-1.84 (2H, m, 4-C**H**, 11-C**H**), 1.89-1.95 (2H, m, 1-C**H**, 2-C**H**, 7-C**H**, 12-C**H**), 2.02 (3H, s, 3-OCOC**H**$_3$), 2.03 (1H, m, 16-C**H**), 2.43 (1H, dd, J = 19.5 Hz , J = 9.0 Hz, 16-C**H**), 4.68 (1H, m, 3-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 12.3 (19-**C**), 13.7 (18-**C**), 20.3 (11-**C**), 21.4 (21- **C**H_3C=O), 21.7 (15-**C**), 27.3 (2-**C**), 28.1 (6-**C**), 30.6 (7-**C**), 31.4 (12-**C**), 33.8 (4-**C**), 34.9 (8-**C**), 35.5 (10-**C**), 35.8 (16-**C**), 36.7 (1-**C**), 44.7 (5-**C**), 47.6 (13-**C**), 51.2 (14-**C**), 54.2 (9-**C**), 73.4 (3-**C**), 170.6 (20-CH_3**C**=O), 221.1 (17-**C**); IR (neat) 2921, 1730, 1233, 1019, 606 cm^{-1}; MS *m/z* (relative intensity, %); 332 ([M]$^{+\cdot}$, 19), 272 (100), 257 (46), 218 (39), 201 (58), 107 (96), 105 (55), 92 (67), 90 (53), 80 (40), 78 (57), 66 (56), 54 (47), 42 (73), 40 (33). Spectra and physical data matched that previously published.[2]

3β-Hydroxy-5α-androstan-17-one (99)



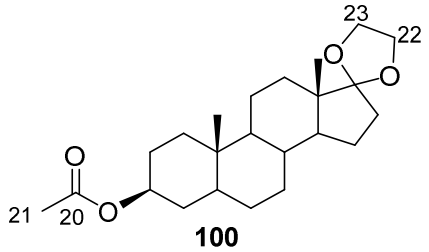
99

To a solution of androsterone (**97**) (1.00 g, 3.4 mmol) in dry toluene (40 ml), ethylene glycol (7.70 ml, 137.7 mmol) was added followed by triethyl orthoformate (1.72 ml, 10.3 mmol) and PTSA (0.06 g, 0.3 mmol) under argon at RT. The reaction mixture was stirred aqueous at RT overnight. The reaction mixture was quenched with saturated solution of $NaHCO_3$ (20 ml) and diluted with ethyl acetate. The layer was separated and the aqueous layer was extracted with ethyl acetate (3×20 ml). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification of the product by flash column chromatography on silica gel (hexane / ethyl acetate 8:2) to afforded **99** as a white solid (0.98 g, 86%).

H^1 NMR (400 MHz, $CDCl_3$) δ; 0.66-0.73 (1H, m, 9-C**H**), 0.83 (5H, s, 18-C**H**, 19-C**H**), 0.85-0.97 (2H, m), 0.98-1.07 (1H, m), 1.13-1.32 (6H, m), 1.33-1.46 (4H, m), 1.47-1.72 (6H, m), 1.73-1.87 (3H, m), 1.91-2.00 (1H, m), 3.81-3.94 (4H, m, 20/21-C$\mathbf{H}_2$C$\mathbf{H}_2$), 4.77-4.85 (1H, m, O**H**);^{13}C NMR (100 MHz, $CDCl_3$) δ; 12.2 (19-**C**), 14.4 (18-**C**), 20.6 (11-**C**), 22.6 (14-**C**), 27.4, 28.4, 30.6, 31.2, 33.9, 34.7, 35.5, 35.7, 36.7, 44.6, 45.9, 50.2 (14-**C**), 54.0 (9-**C**), 64.5 (21-**C**), 65.1 (22-**C**), 71.6 (3-**C**), 119.4 (17-**C**).

Spectra and physical data matched that previously published. [3]

3β-Acetoxy-5α-androstan-17-ethyleneketal (100)



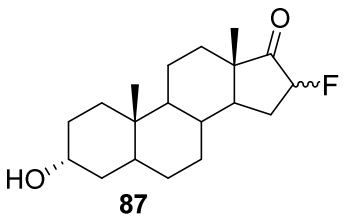
100

To a solution of steroid **99** (1.14 g, 3.4 mmol) in dry DCM (100 ml) was added acetic anhydride (0.80 ml, 8.5 mmol) and DMAP (1.60 g, 13.1 mmol) under argon at RT. The reaction mixture was stirred at RT for 4 h. After that time, the reaction mixture was poured into water and neutralized with saturated aqueous solution of $NaHCO_3$ (50 ml). The layers were separated and the aqueous layer was extracted with DCM (3×20 ml). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by filtration through a plug of silica (eluting with hexane / ethyl acetate 6:1) to give the title compound as a white solid (1.26 g, 99%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.65-0.73 (1H, m, 9-C**H**), 0.82 (3H, s, 18-C**H**$_3$), 0.83 (3H, s, 19-C**H**$_3$), 0.84-1.06 (2H, m, 7-C**H**), 1.15-1.41 (7H, m, 4-C**H**, 5-C**H**, 6-C**H**), 1.42-1.84 (5H, m), 1.92-1.98 (1H, m), 2.01 (3H, s, 21-C**H**), 3.80-396 (4H, m, 22/23-C**H**$_2$C**H**$_2$), 4.62-4.72 (1H, m, 3-C**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ; 12.3 (19-**C**), 17.7 (18-**C**), 20.4 (11-**C**), 21.7 (21-**C**) 22.6 (15-**C**), 27.4 (2-**C**), 28.4 (6-**C**), 31.2 (12-**C**), 31.5 (4-**C**), 34.0, 35.0, 35.6, 35.8, 36.7, 36.8, 45.9, 51.3 (14-**C**), 54.3 (9-**C**), 63.7 (3-**C**), 64.5 (22-**C**), 65.1 (23-**C**), 119.4 (17-**C**), 170.6 (20-**C**); MS *m/z* (relative intensity, %); 376 ($[M]^{+\cdot}$, 71), 99 (100), 86 (41), 78 (22), 54 (27), 42 (39), 40 (15).

Spectra and physical data matched that previously published.[4]

16α/β-Fluoro-androsterone (87)



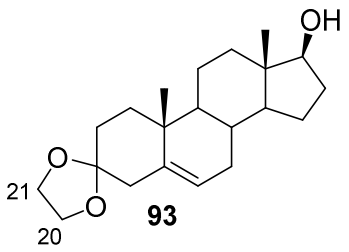
87

To a solution of androsterone (**97**) (2.00 g, 6.9 mmol) and Selectfluor® (3.00 g, 8.5 mmol) in MeOH (34 ml) was added H_2SO_4 (18M, 40µl, 0.7 mmol). The reaction mixture was stirred under N_2 at 50 °C overnight. After that time, water (200 ml) was added and the solids ware filtered off. The product was dried at 50ºC overnight. This gave the product **87** as a white solid (2.08 g, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.68-0.73 (1H, m, 9-C**H**), 0.85(3H, s, 18-C**H**$_3$), 0.86 (3H, s, 19-C**H**$_3$), 0.94-1.06 (2H, m, 7-C**H**), 1.16-1.40 (7H, m, 4-C**H**, 5-CH, 6-C**H**, 11-C**H**, 12-C**H**, 14-C**H**), 1.46-1.67 (3H, m, 2-C**H**, 8-C**H**, 15-C**H**), 1.71-1.84 (2H, m, 4-C**H**, 11-C**H**), 1.89-1.95 (4H, m, 1-C**H**, 2-C**H**, 7-C**H**, 12-C**H**), 2.02-2.09 (3H, m), 3.51-3.64 (1H, m, 3-C**H**), 4.58-4.77 (0.30H, m, α, β, isomers, 16-C**H**), 5.49-5.15 (1H, m, α, β, isomers, 16-C**H**); ^{13}C NMR (150 MHz, $CDCl_3$) δ; 12.2 (18-**C**), 14.3 (19-**C**), 20.0 (11-**C**), 28.2, 29.6 (15-**C**), 29.8, 29.8, 30.5, 31.2, 34.8, 35.6, 37.9, 44.7, 47.7, 48.3, 54.2 (9-**C**), 71.0 (3-**C**), 81.7 (3-**C**) 89.5 (16α-**C**), 90.7, (16β-**C**), 213.1 (17α-**C**), 213.2 (17β-**C**); ^{19}F NMR (564 MHz, $CDCl_3$) δ; -183.45 (dt, J = 47.9, J = 26.5 Hz); -192.58 (dt, J = 51.9 Hz, J = 28.2Hz), IR (neat) 3498, 2930, 1748, 1444, 1046 cm$^{-1;}$ GC-MS m/z (relative intensity, %); 309 ([M]$^{+\cdot}$, 2), 288 (11), 234 (34), 216 (27), 108 (100), 107 (74), 90 (68).

Spectra and physical data matched that previously published.[5]

17β-Hydroxy-androst-5-ene 3-ethylene ketal (93)



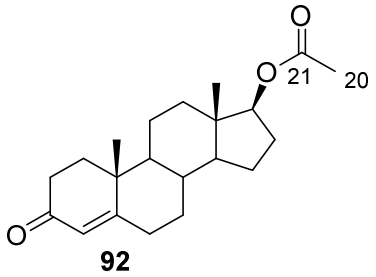
93

To a solution of testosterone (**91**) (1.00 g, 3.5 mmol) in dry toluene (40 ml) at RT ethylene glycol (7.70 ml, 13.8 mmol) was added followed by triethyl orthoformate (1.72 ml, 10.3 mmol) and PTSA (0.07 g, 0.34 mmol) under argon. The reaction mixture was stirred at RT overnight. The reaction mixture was quenched with saturated aqueous solution of $NaHCO_3$ (40 ml) and diluted with ethyl acetate (20 ml). The layers were separated and the aqueous layer was extracted with ethyl acetate (3×20 ml). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification of the product by flash column chromatography on silica (ethyl acetate / hexane 6:4) afforded the product as a white solid (0.78 g, 64%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.76 (3H, s, 18-C**H**$_3$), 0.90-1.00 (1H, m, 9-C**H**), 1.03 (3H, s, 19-C**H**$_3$), 1.04-1.14 (1H, m), 1.18-1.37 (3H, m), 1.38-1.53 (3H, m), 1.54-1.70 (3H, m), 1.72-1.86 (3H, m), 1.93-2.15 (3H, m), 2.52-2.60 (3H, m, 17-C**H**), 3.58-3.68 (1H, m, O**H**), 3.87-4.02 (4H, m, 20/21-C**H**$_2$C**H**$_2$), 5.32-5.37 (1H, m, 6-C**H**).

Spectra and physical data matched that previously published.[6]

Testosterone acetate (92)



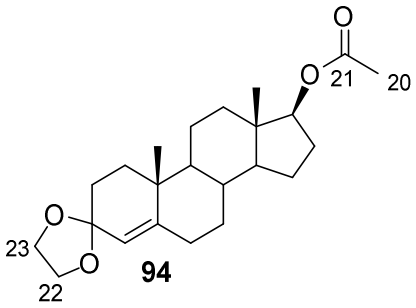
92

To a solution of testosterone (**91**) (0.50 g, 1.7 mmol) in dry DCM (30 ml) was added acetic anhydride (0.40 ml, 4.4 mmol) and DMAP (0.70 g, 5.7 mmol) under argon. The reaction mixture was stirred at RT for 4 h. After that time the reaction mixture was poured into water and neutralized with saturated aqueous solution of $NaHCO_3$ (30 ml). The layers were separated and the aqueous layer was extracted with DCM (3×20 ml). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by filtration through a plug of silica (eluting with hexane / ethyl acetate 4:2) to give **92** as a white solid (0.52 g, 93%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.83 (3H, s, 18-C**H**$_3$), 0.86-1.10 (3H, m) 1.18 (3H, s, 19-C**H**$_3$), 1.23-1.43 (4H, m), 1.44-1.88 (8H, m), 2.04 (3H, s, 20-C**H**), 2.12-2.33 (2H, m), 2.33-2.47 (2H, m, 2-C**H**), 4.59 (1H, t, *J* = 8.4 Hz, 17-C**H**), 5.73 (1H, s, 4-C**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ; 11.0 (18-**C**), 16.3 (19-**C**), 19.5 (11-**C**), 20.1 (20-**C**), 22.4 (15-**C**), 26.4 (16-**C**), 30.4 (7-**C**), 31.7 (6-**C**), 32.9 (2-**C**), 34.4, 34.6 (1-**C**), 35.6 (12-**C**), 37.6 (10-**C**), 41.44 (13-**C**), 49.2 (14-**C**), 52.6 (9-**C**), 81.4 (17-**C**), 122.9 (4-**C**),168.1 (5-**C**) 170.1 (21-**C**), 198.3 (3-**C**), MS *m/z* (relative intensity, %); 330 ($[M]^{+\cdot}$, 12), 288 (13), 228 (15), 185 (18), 147 (41), 146 (38), 131 (17), 124 (68), 104 (35), 90 (37), 78 (40), 66 (25), 54 (33), 43 (100).

Spectra and physical data matched that previously published. [7]

17β-Acetoxyandrost-5-ene-3-ethylene ketal (94)



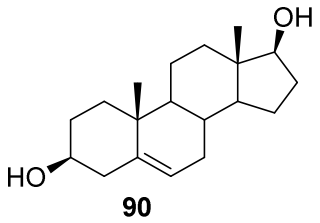
94

To a solution of 17β-Hydroxy-androst-5-ene 3-ethylene ketal (**93**) (0.12 g, 0.4 mmol) in dry DCM (10 ml) was added acetic anhydride (0.10 ml, 1.0 mmol) and DMAP (0.17 g, 1.4 mmol) under argon. The reaction mixture was stirred at RT for 4 h. After that time, the reaction mixture was poured into water and neutralized with saturated aqueous solution of $NaHCO_3$, (10 ml). The layers were separated and the aqueous layer was extracted with DCM (3×20 ml). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by filtration through a plug of silica (eluting with hexane / ethyl acetate 5:1) to give **94** as a white solid (0.67 g, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.73 (3H, s, 18-C**H**$_3$), 0.95 (3H, s, 19-C**H**$_3$), 0.97-1.63 (12H, m), 1.63-1.77 (3H, m), 1.96 (3H, s, 17COCOC**H**$_3$), 1.99-2.14 (2H, m), 2.44-2.52 (1H, m), 3.80-3.92 (4H, m, 3-COC**H**$_2$C**H**$_2$O), 4.52 (1H, t, *J* = 8.4 Hz, 17-C**H**), 5.24-5.29 (1H, m, 4-C**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ; 11.9 (18-**C**), 18.9 (19-**C**), 20.54 (11–**C**), 21.19 (20-**C**), 23.5 (15-**C**), 27.5 (16-**C**), 31.0, 31.3, 36.3, 36.6, 36.7, 51.0 (9-**C**), 64.4 (23-**C**), 64.2 (22-**C**), 82.7 (17-**C**), 106.5, 109.5 (3-**C**), 121.7 (4-**C**), 140.2 (5-**C**), 149.3 (5-**C**), 171.3 (21-**C**); GC-MS *m/z* (relative intensity, %); 374 ([M]$^{+\cdot}$, 10), 99 (100), 91 (19), 54 (35), 41 (10).

Spectra and physical data matched that previously published.[4]

3β,17β-Dihydroxyandrost-5-ene (90)



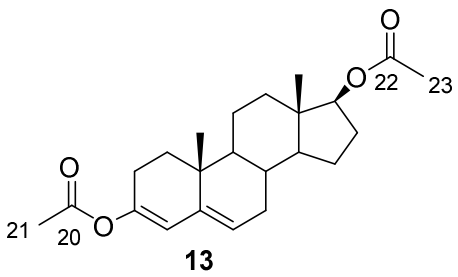
90

To a solution of DHEA (**89**) (10.00 g, 34.7 mmol) in ethanol (200 ml) was added $NaBH_4$ (0.66 g, 17.4 mmol). The reaction mixture was stirred at RT in open flask for 3 h. After that time the mixture was neutralised with 1M HCl_{aq} (3 ml) and diluted with water (150 ml). The reaction mixture was filtered and the filter cake was washed with water (50 ml). The filter cake was dried at 50°C to give the product as a white solid (9.18 g, 91%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.76 (3H, s, 18-C**H**$_3$), 0.91-1.00 (1H, m, 9-C**H**), 1.02 (3H, s, 19-C**H**$_3$), 1.03-1.14 (2H, m), 1.15-1.34 (3H, m), 1.15-1.34 (2H, m), 1.36-1.67 (6H, m), 1.73-1.90 (3H, m), 1.93-2.12 (2H, m, 7-C**H**), 2.18-2.33 (2H, m, 4-C**H**), 3.48-3.56 (1H, m, 17-C**H**), 3.61-3.67 (1H, m, 3-C**H**), 5.33-5.36 (1H, m, 6-C**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ; 10.9 (18-**C**), 19.4 (19-**C**), 20.6 (11–**C**), 23.4 (15-**C**), 30.5 (16-**C**), 31.5 (2-**C**), 31.6 (8-**C**), 31.9 (7-**C**), 36.6 (12-**C**), 37.3 (1-**C**), 42.3 (4-**C**), 42.7 (13-**C**), 50.2 (9-**C**), 51.3 (14-**C, C**), 71.7 (3-**C**), 81.9 (17-**C**), 121.4 (6-**C**), 140.8 (5-**C**); GC-MS *m/z* (relative intensity, %); 290 ($[M]^{+\cdot}$, 30), 257 (58), 118 (100), 116 (56), 66 (93). Structure was also confirmed by X-ray diffraction analysis.

Spectra and physical data matched that previously published.[8]

3,17β-Diacetoxyandrost-4,5-diene (13)



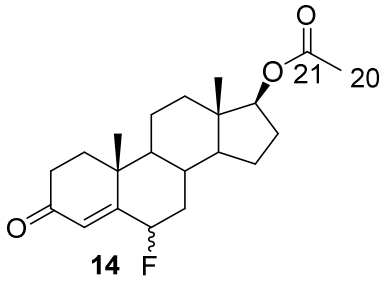
13

To testosterone (**91**) (0.50 g, 1.7 mmol) was added acetic anhydride (0.43 ml, 4.5 mmol), pyridine (0.11 ml, 1.4 mmol) and acethyl chloride (0.95 ml, 13.3 mmol) dropwise under argon at 25ºC. The reaction mixture was refluxed for 5 h and then was stirred overnight at RT. The mixture was concentrated under reduced pressure and the residue was triturated with ethanol. The resulting material was dried at 50ºC to give **13** as a white solid (0.44 g, 69%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.83 (3H, s, 18-C**H**$_3$), 1.01 (3H, s, 19-C**H**$_3$), 1.14-1.26 (2H, m), 1.27-1.73 (8H, m), 1.73-1.81 (2H, m), 2.05 (3H, s, 17-OCOC**H**$_3$), 2.07-2.12 (1H, m), 2.13 (3H, s, 3-OCOC**H**$_3$), 2.14-2.24 (3H, m), 2.38-2.51 (1H, m), 4.61 (1H, dd, *J* = 8.0 Hz, *J* = 7.6 Hz, 17-C**H**), 5.38-5.40 (1H, m, 6-C**H**), 5.69 (1H, d, *J* = 2.4 Hz, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 11.9 (18-**C**), 18.8 (19-**C**), 21.0 (23-**C**), 21.1 (21-**C**), 23.2, 26.2 (2-**C**), 27.4, 27.21, 27.9 (16-**C**), 30.8 (1-**C**), 36.5 (10-**C**), 36.6 (12-**C**), 40.56, 42.6 (13-**C**), 43.5 (9-**C**), 51.1 (14-**C**), 82.7 (17-**C**), 117.5 (4-**C**), 123.6 (6-**C**), 134.5 (5-**C**), 148.6 (3-**C**) 171.1 (22-**C**), 169.2 (20-**C**); GC-MS *m/z* (relative intensity, %); 372 ($[M]^{+\cdot}$, 5), 330 (100), 133 (12), 91 (12), 43 (62).

Spectra and physical data matched that previously published. [9]

6α/β-Fluorotestosterone acetate (14)

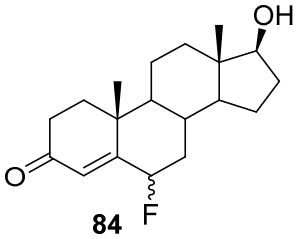


To a solution of 3,17β-diacetoxyandrost-4,5-diene (**13**) (0.97 g, 2.6 mmol) in DMF (10 ml) was added Selectfluor® (1.02 g, 2.9 mmol) under argon at RT. The reaction mixture was stirred for 2 days at RT. After that time, water (10 ml) was added and the resulting precipitate was filtered off. The filter cake was dried at 50ºC overnight to give **14** as a white solid (0.90 g, 99%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.85 (3H, s, 18-C**H**$_3$), 0.93-1.01 (1H, m, 9-C**H**), 1.05-1.13 (1H, m, 14-C**H**), 1.14-1.18 (1H, m), 1.12-1.29 (3H, m), 1.31 (3H, s, 19- C**H**$_3$), 1.34-1.87 (5H, m), 1.91-1.99 (1H, m), 2.05 (3H, 20-C**H**), 2.11-2.30 (2H, m), 2.34-2.47 (1H, m), 2.50-2.59 (1H, m), 4.61 (1H, t, *J*= 8.3, 17-C**H**), 4.92 (0.41H, t, α-isomers, *J* = 2.4 Hz, 6-C**H**), 5.00-5.06 (0.49H, m, α/β-isomers, 6-C**H**), 5.12-5.18 (0.12H, m, α-isomer, 6-C**H**), 5.87 (0.78H, d, β-isomer, *J* = 4.8 Hz, 4-C**H**), 6.08 (0.19H, s, α-isomer, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 12.2 (18-**C**), 20.4 (19-**C**), 21.3 (11–**C**), 23.3 (20-C), 27.4 (16-**C**), 29.7 (8-**C**), 34.3 (2-**C**), 36.2 (1-**C**), 36.3 (12-**C**), 36.4 (7-**C**), 36.7 (10-**C**), 42.5 (13-**C**), 50.2 (14-**C**), 53.2 (9-**C**), 82.3 (17-**C**), 87.5 (α6-**C**), 92.8 (β6-**C**), 119.6 (α4-**C**), 128.3 (β4-**C**), 163.4 (5-**C**), 170.3 (21-**C**), 199.7 (3-**C**); ^{19}F NMR (379 MHz, $CDCl_3$) δ; -165.53 (β, dt, *J* = 47.9 Hz, *J* = 10.1Hz), -183.45 (α, d, *J* = 47.9 Hz); MS *m/z* (relative intensity, %); 348 ($[M]^{+\cdot}$, 29), 306 (46), 288 (59), 273 (16), 246 (38), 231 (19), 203 (24), 148 (17), 145 (29), 133 (41), 97 (35), 93 (50), 91 (61), 55 (50), 43 (100), 41 (33).

Spectra and physical data matched that previously published.[10]

6α/β-Fluoro-testosterone (84)



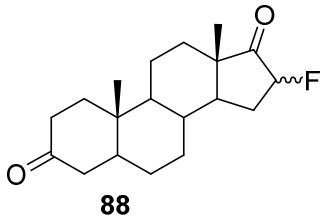
84

To a solution of 6α/β-fluorotestosterone acetate (**14**) (0.10 g, 0.3 mmol) in THF (2 ml) was added 2M HCl (2 ml). The reaction mixture was stirred at RT for 48 h. After that time, the reaction mixture was neutralized with saturated aqueous solution of $NaHCO_3$ and concentrated under reduced pressure. The residue was dissolved in DCM (10 ml), washed with water (2×20 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography on silica gel (ethyl acetate / DCM 1:5) gave the product (**84**) as white solid (0.05 g, 58%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.81 (3H, s, 18-C**H**$_3$), 0.89-1.14 (3H, m), 1.15-1.27 (1H, m), 1.31 (3H, s, 19- C**H**$_3$), 1.34-1.56 (4H, m), 1.57-1.80 (3H, m), 1.80-2.01 (2H, m), 2.02-2.33 (3H, m), 2.34-2.61 (2H, m), 3.62-3.70 (1H, m, 17-C**H**), 4.92 (0.41H, t, α-isomers, *J* = 2.4 Hz, 6-C**H**), 5.00-5.06 (0.49H, m, α/β-isomers, 6-C**H**), 5.12-5.18 (0.12H, m, α-isomer, 6-C**H**), 5.87 (0.78H, d, 4-C**H,** β-isomer, *J* = 4.8 Hz), 6.08 (0.19H, s, β-isomer, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 12.2 (18-**C**), 20.4 (19-**C**), 21.3 (11–**C**), 23.3, 27.4 (16-**C**), 29.7 (8-**C**), 34.3 (2-**C**), 36.2 (1-**C**), 36.3 (12-**C**), 36.4 (7-**C**), 36.7 (10-**C**), 42.5 (13-**C**), 50.2 (14-**C**), 53.2 (9-**C**), 82.3 (17-**C**), 87.5 (α6-**C**), 92.8 (β6-**C**), 119.6 (α4-**C**), 128.3 (β4-**C**), 163.4 (5-**C**), 171.0 (20-**C**), 199.7 (3-**C**); ^{19}F NMR (564 MHz, $CDCl_3$) δ; -165.53 (β, dt, *J* = 47.9 Hz, *J* = 10.1Hz), -183.45 (α, d, *J* = 47.9 Hz); MS *m/z* (relative intensity, %); 306 ($[M]^{+\cdot}$, 12), 287 (17), 184 (23), 150 (24), 146 (38), 141 (36), 121 (24), 108 (46), 104 (53), 97 (50), 90 (100), 78 (97), 66 (78), 54 (94), 40 (67).

Spectra and physical data matched that previously published. [11]

16α/β-Fluoro-5α-androstanedione (88)



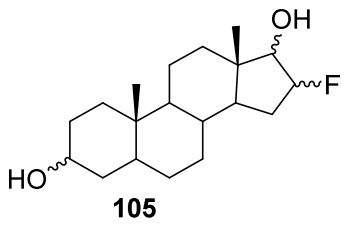
88

To a solution of 16α/β-fluoro-androsterone (**87**) (1.00 g, 3.2 mmol) in dry MeCN (10 ml) was added TPAP (0.06 g, 0.16 mmol), NMO (0.66 g, 4.9 mmol) and Aº4 molecular sieves (~0.50 g). The reaction mixture was stirred at RT overnight then the mixture was filtered and the solvent was evaporated. The residue was diluted with water (10 ml) and 1 M HCl was added to achieve pH 6-7. The resulting suspension was stirred at RT for 10 min. The solid were filtered off, washed with H_2O (10 ml) and dried at 40ºC overnight. This gave the product **88** as an off white solid (0.85 g, 88%).

^{1}H NMR (600MHz, $CDCl_3$) δ; 0.79-0.87 (3H, m), 0.92 (3H, s, 18-C**H**$_3$), 0.95-1.05 (11H, m), 1.15-1.20 (1H, m), 1.21-1.23 (3H, m), 1.27-1.47 (10H, m), 1.50-1.67 (6H, m),1.68-1.73 (3H, m), 1.75-1.85 (3H, m),1.87-1.98 (2H, m), 1.99-2.03 (2H, m), 2.05-2.13(3H, m), 2.21-2.32 (4H, m), 2.33-2.40 (2H, m), 2.44-2.49 (1H, m), 4.64 (0.4H, t, β/α16-C**H**, *J* = 8.3 Hz), 4.72 (0.4H, t, β/α16-C**H**, *J* = 8.3 Hz), 5.03 (0.5H, d, β/α16-C**H**, *J* = 7.1 Hz), 5.11 (0.5H, d, β/α16-C**H**, *J* = 7.1 Hz); ^{13}C NMR (150MHz, $CDCl_3$) δ; 11.4 (18-**C**), 14.0, 14.5, 20.2, 28.4, 30.1, 31.1, 34.7, 35.7, 37.9, 38.2, 44.4, 46.3, 48.1, 53.6,89.4, 90.6, 92.1, 211.2, 212.7; ^{19}F NMR (379 MHz, $CDCl_3$) δ; 179.9 , -196.7; IR (neat) 2937, 1752, 1709, 1443, 1039, 730 cm^{-1}; GC-MS *m/z* (relative intensity, %); 306 ([M]$^{+\cdot}$, 37), 232 (91), 217 (100);

Spectra and physical data matched that previously published.[12]

16α/β-Fluoro-3β,17β-dihydroxyandrostane (105)

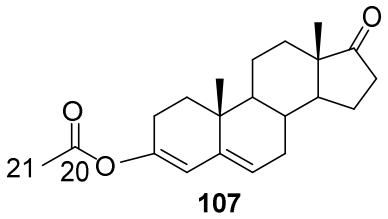


105

To a solution of 16α/β-fluoro-androsterone (**87**) (2.00 g, 6.4 mmol) in DCM / MeOH (1:1, 40 ml) in 0°C was added $NaBH_4$ (0.77 g, 20.0 mmol). The reaction mixture was stirred at RT in open flask overnight. The solvent was evaporated, water (40 ml) was added and pH was adjusted to 6-7 with 1M HCl. The solid was filtered off washed with H_2O, dried at 40°C overnight. This gave the product as a white solid (5.40 g, 90%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 0.66 (3H, s, 18-C**H**$_3$), 0.69-0.75 (1H, m), 0.80 (3H, s, 19-C**H**$_3$), 0.93-1.01 (2H, m), 1.08-1.15 (2H, m), 1.21-1.35 (4H, m), 1.36-1.43 (2H, m), 1.44-1.49 (1H, m), 1.53-1.66 (4H, m), 1.68-1.75 (2H, m), 1.76-1.88 (4H, m), 2.14-2.19 (0.4H, m), 3.50-3.62 (0.5H, m), 3.76-3.77 (0.4, m), 5.15-5.19 (0.2H, m), 5.25-5.5.28 (0.2H, m); ^{13}C NMR (150MHz, $CDCl_3$) δ; 12.4 (18-**C**), 17.2 (19-**C**), 20.1, 28.6, 31.0, 32.3, 32.7, 35.3, 37.0, 38.2, 44.9, 45.4, 47.2, 54.0, 71.3 (3-**C**), 78.1, 94.1 (17-**C**), 95.3 (16-**C**); IR (neat) 3359, 2930, 1371, 1033, 606 cm^{-1}; ^{19}F NMR (189 MHz, $CDCl_3$) δ; -179.82, -185.28, -191,93 and -196.35 (mixture of 4 isomers); MS *m/z* (relative intensity, %); 310 ([M]$^{+\cdot}$, 31), 290 (81), 272 (22), 248 (39), 233 (55), 215 (100).

3-Acetoxyandrost-3,5-diene-17-one (107)



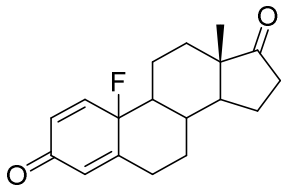
107

To androst-4-ene-3,17-dione (**106**) (2.00 g, 6.9 mmol) was added acetic anhydride (0.88 ml, 18.1 mmol), pyridine (0.55 ml, 6.9 mmol) and acethyl chloride (1.92 ml, 54.4 mmol) dropwise under argon. The reaction mixture was refluxed for 7 h and then stirred overnight at RT. The mixture was concentrated under reduced pressure and the residue was triturated with ethanol. The mother liquor was concentrated and purified by column chromatography on silica gel (hexane / EtOAc 95/5%) This gave the product **107** as white solid (1.78 g, 79%)

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.91 (3H, s, 18-C**H**$_3$), 1.02 (3H, s, 19-C**H**$_3$), 1.04-1.12 (1H, m), 1.28-1.38 (3H, m), 1.40-1.50 (1H, m), 1.51-1.60 (1H, m), 1.67-1.77 (2H, m), 1.79-1.89 (3H, m), 1.91-1.99 (1H, m), 2.04-2.12 (1H, m), 2.13 (3H, s, 21-C**H**), 2.24-2.33 (1H, m), 2.40-2.51 (2H, m), 5.40-5.42 (1H, m, 6-C**H**), 5.70 (1H, d, 4-C**H**, J = 2.4 Hz); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 13.6 (18-**C**), 18.8 (19-**C**), 20.5 (11–**C**), 21.1 (21-**C**), 21.8 (15-**C**), 24.7 (2-**C**), 30.75 (16-**C**), 31.4 (8-**C**), 33.7 (1-**C**), 35.0 (10-**C**), 35.8 (12-**C**), 47.7 (13-**C**), 48.1 (9-**C**), 51.8 (14-**C**), 116.8 (6-**C**), 123.4 (4-**C**), 139.5 (5-**C**), 147.1 (3-**C**), 169.3 (20-C), 218.7 (17-**C**); IR (neat) 2944, 1733, 1670, 1641, 888 cm^{-1}; MS *m/z* (relative intensity, %); 328 ([M]$^{+\cdot}$, 8), 287 (21), 286 (100), 271 (7), 137 (6).

Spectra and physical data matched that previously published. 9

10β-Fluoro-3,17-dihydroxy-1,4-androstene (10)



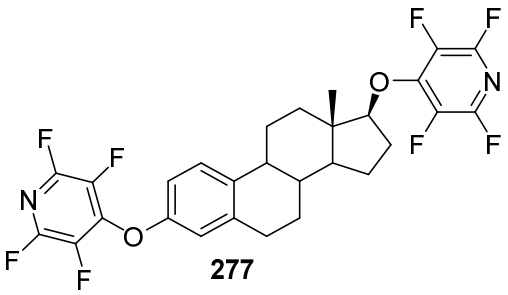
10

To a solution of estrone (**121**) (10.00 g, 37.0 mmol) and Selectfluor® (15.80 g, 44.6 mmol) in MeCN (70 ml). The reaction mixture was stirred under N_2 at 50°C overnight. The solvent was evaporated. The reaction mixture was diluted with DCM (70 ml) and the layers were separated. The organic layer was dried over $MgSO_4$ filtered and concentrated to give the crude product as a brown oil. The crude product was purified by flash column chromatography on silica (DCM / EtOAc 85/15%). This gave the product **10** as a white solid (7.25 g, 83%).

^{1}H NMR (700 MHz, $CDCl_3$) δ; 0.69 (3H, s, 18-C**H**$_3$), 1.15-1.21 (1H, m), 1.24-1.33 (3H, m), 1.58-1.65 (1H, m), 1.84-1.91 (2H, m), 1.93-1.99 (2H, m), 2.03-2.12 (3H, m), 2.46-2.50 (2H, m), 6.04 (1H, s, 4-C**H**), 6.24 (1H, d, 1-C**H,** *J* = 6.9 Hz), 7.06 (1H, d, 2-C**H**, *J* =6.9 Hz); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 13.6 (18-**C**), 21.9, 22.1, 30.9, 31.5, 31.7, 35.3, 35.5, 47.6, 49.9, 54.0, 54.1, 123.8 (4-**C**), 129.6 (2-**C**), 144.9 (1-**C**), 159.6 (5-**C**), 184.8 (3-**C**), 219.6 (17-**C**); ^{19}F NMR (379 MHz, $CDCl_3$) δ; -165.32 (d, *J* = 37.9 Hz).; IR (neat) 2944, 1733, 1670, 1641, 888 cm^{-1}; GC-MS *m/z* (relative intensity, %); 288 ([M]$^{+\cdot}$, 47), 247 (19), 163 (26), 150 (75), 149 (46), 145 (29), 126 (100), 107 (34), 93 (34), 79 (25).

Spectra and physical data matched that previously published.[13]

3,17-Bistetrafluoropyridine-estradiol (277)

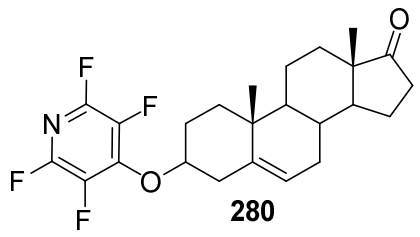


277

To a suspension of estradiol (**276**) (1.22 g, 4.5 mmol) in dry DMF (3 ml) was added triethylamine (0.75 ml, 5.4 mmol) and PFP (1.1 ml, 10 mmol). The reaction mixture was stirred for 2 days at 50ºC. After that time, water (10 ml) was added and the mixture was stirred for 20 min. The solids were filtered off. The crude product was dried at 40ºC overnight then purified by flash column chromatography on silica (DCM / EtOAc 85/15%). This gave the product **277** as a white solid (1.79 g, 70%).

^{1}H NMR (700 MHz, $CDCl_3$) δ; 1.00 (3H,s, 18-C**H**$_3$), 1.25-1.43 (4H, m),1.49-1.58 (3H, m), 1.79-1.93 (3H, m), 1.96-2.00 (1H, m),2.21-2.33 (3H, m), 2.82-2.91 (2H, m), 4.66-4.68 (1H, t, 17-C**H**, *J* = 9.0 Hz), 6.77-6.76 (1H, m, 4-C**H**), 6.82-6.84 (1H, m, 2-C**H**), 7.26-7.27 (1H, m, 1-C**H**);^{13}C NMR (175 MHz, $CDCl_3$) δ; 11.4 (18-**C**), 23.1 (15-**C**), 25.9 (14–**C**), 26.81, 28.0 (9-**C**), 29.4 (6-**C**), 36.6, 38.1, 43.7, 44.2, 49.3 (15-**C**), 93.0 (17-**C**), 113.8 (2-**C**), 116.5 (4-**C**),126.6 (**C**), 134.5-134.7 (**C**, m), 135.3-135.5 (**C**, m), 136.0-136.2 (**C**, m) 136.8 (**C**), 126.8-137.0 (**C**, m), 138.8 (**C**), 143.3-143.5 (**C**, m), 144.5-144.7 (**C**, m), 144.7-144.9 (**C**, m), 147.5-147.6 (**C**, m), 153.8 (**C**, m); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -88.85 (ortho positions), -90.77 (ortho positions), -154.46 (meta positions), -158.11 (meta positions); IR (neat) 2939, 1642, 1474, 1019, 975 cm^{-1}; ASAP-MS *m/z* (relative intensity, %); 571($[M+1]^{+\cdot}$, 94), 503 (11), 429 (13), 405 (41), 404 (100), 355 (12), 308 (16).

Androst-5ene-17-one 3-(2,3,5,6-tetrafluoropyridine)ether (DHEA-PFP) (280)

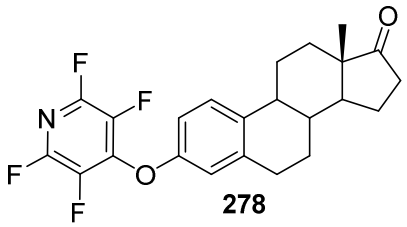


280

To a suspension of DHEA (**89**) (0.10 g, 6.9 mmol) in dry DMF (1 ml) was added triethylamine (0.11 ml, 8.3 mmol) and PFP (1.70 ml, 1.5 mmol). The reaction mixture was stirred for 2 days at 50ºC. After that time, water (10 ml) was added and the mixture was stirred for 20 min. The solids were filtered off. The crude product was dried at 40ºC overnight then purified by flash column chromatography on silica (DCM / EtOAc 85/15%). This gave the product **280** as a white solid (0.25 g, 86%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 0.99 (3H, s, 18-C**H**$_3$), 1.01-1.06 (1H, m), 1.09 (3H, s, 19-C**H**$_3$), 1.14-1.19 (1H, m), 1.25-1.32 (1H, m), 1.47-1.59 (3H, m), 1.64-1.72 (3H, m), 1.78-1.87 (2H, m), 1.94-1.98 (2H, m), 2.04-2.17 (3H, m), 2.45-2.59 (3H, m), 4.50-4.57 (1H, m, 3-C**H**), 5.46 (1H, s, 6-C**H**); ^{13}C NMR (150 MHz, $CDCl_3$) δ; 13.7 (18-**C**), 19.5 (19-**C**), 20.5, 22.0, 28.8 29.4,30.9, 31.5, 35.9, 36.8, 39.2, 47.6, 50.2, 51.8, 84.2 (3-**C**), 122.9 (6-**C**), 134.0-134.4 (**C**, m), 136.6-136.9 (**C**), 139.2 (**C**), 142.9 (**C**, t, J = 17 Hz), 145.4 (C, t, J = 15 Hz), 146.2-146.6 (**C**, m); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -90.80 (ortho positions), -158.14 (meta positions); IR (neat) 2994, 1702, 1492, 1119, 975 cm^{-1}; ASAP-MS *m/z* (relative intensity, %); 438 ($[M+1]^{+\cdot}$ (100), 429 (29), 355 (28), 271 (19).

Estron 3-(2,3,5,6-tetrafluoropyridine)ether (estrone-PFP) (278)

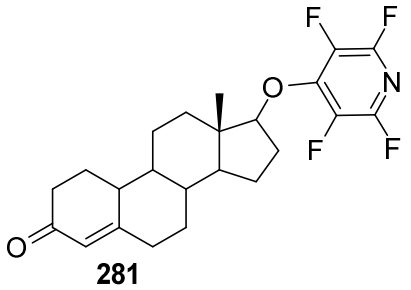


278

To a suspension of estrone (**121**) (0.10 g, 3.7 mmol) in dry DMF (1 ml) was added triethylamine (0.06 ml, 4.4 mmol) and PFP (0.09 ml, 8.1 mmol). The reaction mixture was stirred for 2 days at 50ºC. After that time, water (10 ml) was added and the mixture was stirred for 20 min. The solids were filtered off. The crude product was dried at 40ºC overnight then purified by flash column chromatography on silica (DCM / EtOAc 85/15%). This gave the product **278** as a white solid (0.093 g, 60%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 0.95 (3H, s, 18-C**H**$_3$), 1.43-1.67 (7H, m), 1.96-199 (1H, m), 2.00-2.09 (2H, m), 2.12-2.18 (1H, m), 2.26-2.32 (1H, m), 2.38-2.43 (1H, m), 2.49-2.55 (1H, m), 2.89-2.92 (2H, m), 6.77 (1H, s, 4-C**H**), 6.83 (1H, d, 1-C**H,** *J* = 5.9 Hz), 7.28 (1H, d, 2-C**H**, *J*=5.9 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ; 13.7 (18-**C**), 21.5, 25.7, 26.21, 29.4, 31.4, 35.7, 37.9, 43.9, 47.8, 50.3, 113.8, 116.4, 126.8,134.7-1351 (**C**, m), 136.7, 137.3-137.7 (**C**, m), 138.7, 142.9 (**C**, t, *J* = 17 Hz), 144.4-144.7 (**C**, m), 145.3 (**C**, t, *J* = 14 Hz), 153.8 (3-**C**), 220.5 (17-**C**); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -88.89 (ortho positions), -154.41 (meta positions); IR (neat) 2927, 1702, 1464, 1043, 965 cm^{-1}; ASAP-MS *m/z* (relative intensity, %); 420 ($[M+1]^{+\cdot}$ 100), 402 (95), 355 (41), 299 (12).

Nortestosterone 17-(2,3,5,6-tetrafluoropyridine)ether (nortestosterone-PFP) (281)

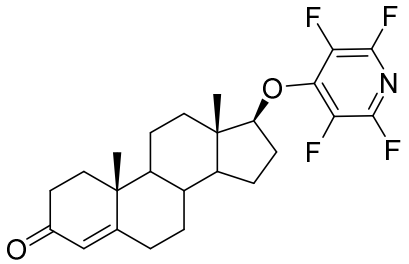


281

To a suspension of nortestosterone (**111**) (0.10 g, 3.6 mmol) in dry DMF (1 ml) was added triethylamine (0.04 ml, 4.4 mmol) and PFP (0.09 ml, 8.1 mmol). The reaction mixture was stirred for 2 days at 50°C. After that time, water (10 ml) was added and the mixture was stirred for 20 min. The solids were filtered off. The crude product was dried at 40°C overnight then purified by flash column chromatography on silica (DCM / EtOAc 85/15%). This gave the product **281** as a white solid (0.105 g, 68%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 1.00 (3H, s, 18-C**H**$_3$), 1.25-1.43 (4H, m), 1.49-1.58 (3H, m), 1.79-1.93 (3H, m), 1.96-2.00 (1H, m), 2.21-2.33 (3H, m), 2.82-2.91 (2H, m), 4.66-4.68 (1H, t, 17-C**H**, *J* = 6.0 Hz), 6.77-6.76 (1H, m, 4-C**H**), 6.82-6.84 (1H, m, 2-C**H**), 7.26-7.27 (1H, m, 1-C**H**);^{13}C NMR (150 MHz, $CDCl_3$) δ; 11.6 (18-**C**), 23.3, 26.0, 26.7, 28.1, 30.6, 35.4, 36.5, 40.2, 42.5, 44.1, 49.2, 49.4 (9-**C**), 93.1 (17-**C**), 124.9 (4-**C**), 166.2 (5-**C**), 200.0 (3-**C**), PFP carbons not detected; ^{19}F NMR (376 MHz, $CDCl_3$) δ; –90.68 (ortho positions), -158.12 (meta positions); IR (neat) 2933, 1474, 1093, 726 cm^{-1}; ASAP-MS *m/z* (relative intensity, %); 424 ([M+1]$^{+\cdot}$, 100), 356 (18), 355 (40), 257 (19).

Testosterone 17-(2,3,5,6-tetrafluoropyridine)ether (testosterone-PFP) (282)

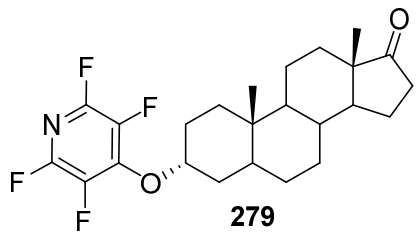


282

To a suspension of testosterone (**91**) (0.10 g, 3.5 mmol) in dry DMF (1 ml) was added triethylamine (0.06 ml, 4.1 mmol) and PFP (0.09 ml, 8.1 mmol). The reaction mixture was stirred for 2 days at 50ºC. After that time, water (10 ml) was added and the mixture was stirred for 20 min. The solids were filtered off. The crude product was dried at 40ºC overnight then purified by flash column chromatography on silica (DCM / EtOAc 85/15%). This gave the product **282** as a white solid (0.079 g, 52%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 0.94 (1H, m), 0.99 (3H, s, 19-C**H**$_3$), 1.02-1.10 (2H, m), 1.27 (3H, s, 18-C**H**$_3$), 1.42-1.49 (2H, m), 1.57-1.62 (3H, m), 1.69-1.75 (3H, m), 1.80-1.85 (2H, m), 1.86-1.89 (1H, m), 2.01-2.04 (1H, m), 2.20-2.34 (3H, m), 2.35-2.46 (3H, m), 4.57 (1H, t, 17-C**H**, *J* = 5.9 Hz), 5.73 (1H, s, 4-C**H**);^{13}C NMR (150 MHz, $CDCl_3$) δ; 11.8 (19-**C**), 17.7 (18-**C**), 20.8, 23.7, 28.4, 31.7, 33.0, 34.2, 35.7, 36.1, 36.8, 38.9, 44.2, 50.2, 54.0, 92.9, 93.3 (17-**C**), 124.4 (4-**C**), 170.8 (5-**C**), 199.7 (3-**C**), PFP carbons not detected; ^{19}F NMR (376 MHz, $CDCl_3$) δ; -90,74 (ortho positions), -158.12 (meta positions); IR (neat) 2942, 1660, 1505, 1471, 1091, 980 cm^{-1}; ASAP-MS *m/z* (relative intensity, %); 438 ($[M+1]^{+\cdot}$ (100), 429 (29), 355 (28), 271 (19).

Androstan-17-one 3-(2,3,5,6-tetrafluoropyridine)ether (androsterone-PFP) (279)

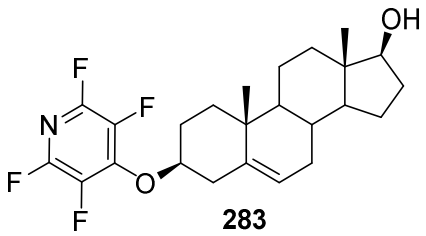


279

To a suspension of androsterone (**97**) (0.20 g, 0.5 mmol) in dry DMF (1 ml) was added triethylamine (0.06 ml, 4.1 mmol) and PFP (0.18 ml, 0.6 mmol). The reaction mixture was stirred for 2 days at 50ºC. After that time, water (10 ml) was added and the mixture was stirred for 20 min. The solids were filtered off. The crude product was dried at 40ºC overnight then purified by flash column chromatography on silica (DCM / EtOAc 85/15%). This gave the product **279** as a white solid (0.23 g, 76%).

^{1}H NMR (700 MHz, $CDCl_3$) δ; 0.68-0.72 (1H, m), 0.84 (3H, s, 18-C**H**$_3$), 0.87 (3H, s, 19-C**H**$_3$), 0.93-1.00 (2H, m), 1.02-1.06 (1H, m), 1.15-1.29 (3H, m), 1.30-1.35 (3H, m), 1.44-1.50 (1H, m), 1.52-156 (1H, m), 1.57-1.59 (1H, m), 1.61-1.67 (1H, m), 1.68-1.77 (2H, m), 1.78-1.82 (2H, m), 1.88-1.93 (1H, m), 1.97-2.06 (2H, m), 2.41 (1H, dd, *J* = 10.4 Hz, *J* = 10.4 Hz) 4.57-4.62 (1H, m, 3-C**H**); ^{13}C NMR (175MHz, $CDCl_3$) δ; 12.2 (18-**C**), 13.7 (19-**C**), 20.4, 21.6, 28.2, 28.3, 30.7, 31.4, 34.7, 34.9, 35.6, 35.7, 36.5, 44.5, 47.6, 51.2, 54.1, 84.0, 134.5-134.6 (**C**, m), 134.7-134.8 (**C**, m), 136.2-136.5 (**C**, m), 143.2-143.4 (**C**, m), 144.8-145.0 (**C**, m), 220.8 (17-**C**); IR (neat) 2937, 1738, 1642, 1502, 1473, 1109, 985 cm^{-1}; ^{19}F NMR (376 MHz, $CDCl_3$) δ; -91.00 (ortho positions), -158.11 (meta positions); ASAP-MS *m/z* (relative intensity, %); 440 ($[M+1]^{+\cdot}$ (13), 423 (42), 422 (100), 273 (24), 255 (46).

17β-Hydroxyandrost-5-ene 3-(2,3,5,6-tetrafluoropyridine)ether (283)

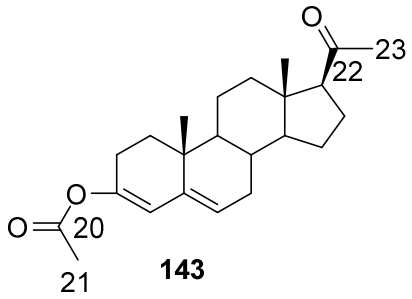


283

To a suspension of **90** (2.00 g, 6.9 mmol) in dry DMF (15 ml) was added triethyloamine (0.84 ml, 8.3 mmol) and PFP (5.13 ml, 30.3 mmol). The reaction mixture was stirred for 2 days at 50°C. After 48 h, water (40 ml) was added and the mixture was stirred for 20 min. The solids were filtered off. The crude product was dried at 40°C overnight. To give the product **283** as a white solid (2.02 g, 67%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0. 0.96 (3H, s, 18-C**H**$_3$), 0.98-1.01 (2H, m), 1.08 (3H, s, 19-C**H**$_3$), 1.09-1.15 (1H, m), 1.16-1.30 (2H, m), 1.33-1.61 (8H, m), 1.66-1.73 (1H, m), 1.77-1.87 (4H, m), 1.91-1.97 (1H, m), 1.99-2.08 (3H, m), 2.17-2.30 (2H, m), 2.44-2.57 (2H, m), 1.78-1.82 (2H, m), 1.88-1.93 (1H, m), 4.46-4.46 (2H, m), 5.34 (0.34H, m), 5.41 (1H, s, -C**H**), 5.41 (1H, m, 6-C**H**); ^{13}C NMR (150 MHz, $CDCl_3$) δ; 11.52 (18-**C**), 19.4 (19-**C**), 20.6, 23.6, 28.2, 31.4, 31.8, 36.6, 36.7, 36.9, 39.2, 43.9, 49.6, 50.7, 71.8, 82.0, 84.3, 93.3, 121.2, 123.1, 134.0-134.5 (**C**, m), 136.6-137.1 (**C**, m), 139.1 (**C**), 142.9-143.2 (**C**, m), 145.3-145.6 (**C**, m), 146.5-146.8 (C, m), 147.7-147.9 (C, m); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -90.87 (ortho positions), -158.14 (meta positions); IR (neat) 3273, 2944, 1641, 1471, 1093, 753 cm^{-1}; MS *m/z* (relative intensity, %); 290 (M – PFP, 100), 272 (56), 257 (38), 218 (48), 201 (26), 107 (30),

3-Acetoxypregh-3,5-diene-20one 143



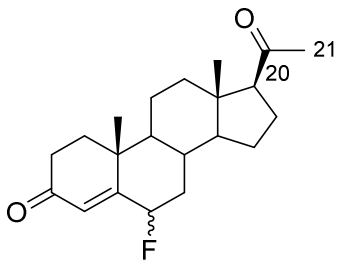
143

To progesterone (**142**) (0.50 g, 1.6 mmol) was added acetic anhydride (0.40 ml, 4.2 mmol), pyridine (0.12 ml, 1.4 mmol) and acetyl chloride (1.00 ml, 14.1 mmol) under argon. The reaction mixture was refluxed for 3 h and then was stirred overnight at RT. The mixture was concentrated under reduced pressure and the residue was triturated with acetonitrile. The resulting material was dried at 50°C to give **143** as a white solid (0.41 g, 72%).

^{1}H NMR (700 MHz, $CDCl_3$) δ; 0.65 (3H, s, 18-C**H**$_3$), 0.99 (3H, s, 19-C**H**$_3$), 1.05-1.10 (1H, m), 1.16-1.29 (3H, m), 1.31-1.37 (1H, m), 1.42-1.50 (2H, m, 12-C**H**), 1.62-1.73 (6H, m), 1.84-1.87 (1H, m, 8-C**H**), 2.03-2.09 (2H, m, 11-C**H**), 2.12 (6H, s, 21/23-C**H**$_3$) 2.14-2.23 (3H, m), 2.37-2.41 (1H, m, 7-C**H**), 2.50-2.57 (1H, m, 17-C**H**), 5.38 (1H, s, 6-C**H**), 5.68 (1H, s, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 13.4 (18-**C**), 18.9 (19-**C**), 21.2 (11–**C**), 22.9, 24.3, 24.9 (23-CO**C**H_3), 31.8 (21-CO**C**H_3), 33.9 (6-**C**), 35.0 (9-**C**), 38.9, 44.2 (12-**C**), 48.0, 57.13, 63.8 (17-**C**), 98.45 (6-**C**) 117.0 (4-**C**), 123.7 (4-**C**), 139.4 (5-**C**), 147.1 (3-**C**), 169.4. (20-**C**), 209.0 (22-**C**); IR (neat) 2939, 1748, 1703, 1365, 1218, 1119 cm^{-1}; GC-MS *m/z* (relative intensity, %); 356 ([M]$^{+\cdot}$, 8), 315 (23) 314 (100), 43 (12).

Spectra and physical data matched that previously published.[14]

6α/β-Fluoro-progesterone (144)



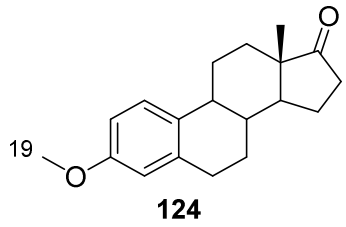
144

To 3-acetoxypregh-3,5-diene-20one (**143**) (7.70 g, 2.2 mmol) in dry DMF (80 ml) was added Selectfluor® (9.60 g, 2.7 mmol), at 0°C under argon. The reaction mixture was stirred at RT overnight. After that time, water (150 ml) was added and the solids were filtered off. The product was purified by flash column chromatography on silica. This gave the product **144** as a white solid (6.87 g, 92%).

^{1}H NMR (700 MHz, $CDCl_3$) δ; 0.70 (3H, s, 19-C**H**$_3$), 1.00 (1H, m, 9-C**H**), 1.16-1.28 (2H,m), 1.30 (3H, s, 18-C**H**$_3$), 1.32-1.34 (1H, m), 1.42-1.46 (1H, m, 11-C**H**), 1.49-1.55 (1H, m, 8-C**H**), 1.63-1.77 (4H,m), 1.90-1.95 (1H, m), 2.06-2.11 (2H, m) 2.13 (3H, s, 21-C**H**), 2.18-2.24 (2H, m, 16-C**H**), 2.39-2.43 (1H, m, 17-C**H**), 2.52-2.58 (2H, m, 2-C**H**), 4.96 (0.51H, t, β-isomers, *J* = 2.4 Hz, 6-C**H**), 5.03 (0.49H, t, β-isomers, *J* = 2.4 Hz, 6-C**H**), 5.88 (1H, d, β-isomer, *J* = 2.4 Hz, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 13.2 (19-**C**), 18.3 (18-**C**), 20.8 (11–**C**), 22.8 (16-**C**), 24.2 (15-**C**), 29.9, 31.4 (21 -CO**C**H_3), 34.1 (2-**C**, 17-**C**), 36.8, 37.26 (1-**C**, 8-**C**, 7-**C**), 37.8 (10-**C**), 38.4 (12-**C**), 43.9 (13-**C**), 53.0 (9-**C**), 55.8 (14-**C**), 63.3(2-C, 17-**C**), 92.7 (6-**C**), 93.6. (6-**C**), 128.4 (4-**C**), 161.5 (5-**C**), 199.7 (20-**C**), 209.0 (3-**C**); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -161.412 (β, t, *J*= 56.4 Hz), -184.19 (α, d, *J*= 48.5 Hz); IR (neat) 1680, 1696, 2932, cm$^{-1;}$ GC-MS *m/z* (relative intensity, %); 332 ([M]$^{+\cdot}$, 92), 312 (72), 270 (84), 227 (100), 142 (73).

Spectra and physical data matched that previously published.14

Esterone 3-methyl ether (124)



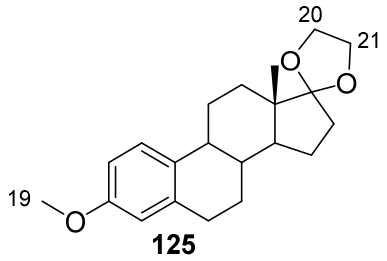
124

To a suspension of potassium *tert*-butoxide (10.00 g, 8.8 mmol) in dry THF (200 ml) was added estrone (**121**) (20.00 g, 7.4 mmol) at 5°C. The reaction mixture was stirred for 1h. After that time, MeI (5.07 ml, 8.1 mmol) was added, reaction mixture was warmed up to RT and stirred for 3h. The solvent was evaporated and DCM (200 ml) water (100 ml) was added. The layers were separated and the aqueous layer was extracted with DCM (3 x 50 ml). The organic layers ware combined, dried over $MgSO_4$, filtered and concentrated to give the product as a white solid (20.30 g, 97%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 0.91 (3H, s, 18-C**H**$_3$), 1.41-1.66 (3H, m), 1.67-1.76 (3H, m), 1.93-1.98 (2H, m, 6-C**H**), 1.99-2.08 (1H, m), 2.11-2.18 (1H, m), 2.23-2.29 (1H, m), 2.36-2.43 (1H, m, 8-C**H**), 2.48-2.53 (1H, m) 2.88-2.93 (2H, m), 3.78 (3H, s, 19-C**H**), 6.65 (1H, s, 4-C**H**), 6.73 (1H, d, *J* = 5.99 Hz), 7.21 (1H, d, *J* = 5.99 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ; 14.0 (18-**C**), 21.7, 26.0 (8–**C**), 26.7, 29.8 (16-**C**), 31.7 (6-**C**), 36.0, 38.5, 44.1 (9-**C**), 48.1, 50.5, 55.3 (19-**C**), 111.7 (2-**C**), 114.0 (4-**C**), 126.4 (1-**C**), 132.1 (10-**C**), 137.8 (5-**C**), 157.7 (3-**C**), 221.0 (17-**C**); IR (neat) 2915, 1736, 1504, 1237, 1036 cm^{-1}; GC - MS *m/z* (relative intensity, %); 284 ([M], 100), 285 ([M]$^{+\cdot}$, 23), 199 (39), 160 (27).

Spectra and physical data matched that previously published [15]

3-Methoxy-1,3,5(10)-estratrien 17-ethylene ketal (125)



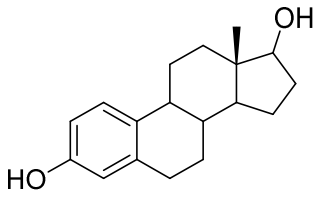
125

To a solution of steroid **124** (20.30 g, 7.1 mmol) in toluene (100 ml) was added ethylene glycol (353.00 ml, 285.5 mmol), triethyl orthoformate (2.48 ml, 21.4 mmol), and PTSA (1.23 g, 0.7 mmol) under argon. The reaction mixture was stirred overnight at RT. After that time, the mixture was quenched with saturated aqueous solution Na_2CO_3 and diluted with ethylacetate (100 ml). The organic phase was wash with water (3 x 30 ml), brine (30 ml), dried over $MgSO_4$, and filtered. The solvent was removed under reduced pressure to give the product as an off white solid **125** (15.35 g, 95%).

^{1}H NMR (600 MHz, MeOD) δ; 0.88 (3H, s, 18-C**H**$_3$), 1.31-1.50 (4H, m), 1.52-1.55 (1H, m), 1.61-1.66 (1H, m), 1.74-1.80 (2H, m), 1.82-1.91(2H, m), 2.00-2.05 (1H, m), 2.20-2.27 (1H, m), 2.29-2.34 (1H, m), 2.81-2.89 (2H, m), 3.77 (3H, s,19-C**H**), 3.87-3.98 (4H, m, 20/21-C**H**$_2$C**H**$_2$), 6.62 (1H, s, 4-C**H**), 6.71 (1H, d, = 10.4 Hz, 1-C**H**), 7.21 (1H, d, J = 9.7 Hz, 2-C**H**); ^{13}C NMR (150 MHz, MeOD) δ; 14.4 (18-**C**), 22.5, 26.0, 26.3, 27.1, 29.9, 30.8, 34.3, 39.2, 43.7, 46.3, 49.5, 55.3, 64.7 (20-**C**), 65.3 (21-**C**), 111.6, 113.9, 119.5, 126.4 (1-**C**), 132.8 (10-**C**), 138.1 (5-**C**), 157.5 (3-**C**), 221.0 (17-**C**); GC-MS *m/z* (relative intensity, %); 329 ($[M+1]^{+\cdot}$, 10), 328 ($[M]^{+\cdot}$, 42), 283 (12), 267 (30), 266 (100), 227 (14), 99 (96); IR (neat) 2932, 1737, 1498, 1037, 729 cm^{-1}.

Spectra and physical data matched that previously published.[16]

Estradiol (276)



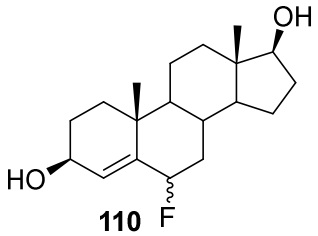
276

To a solution of estrone (**121**) (5.35 g, 2.0 mmol) in DCM / MeOH (50% / 50%, 80 ml) was added $NaBH_4$ (37.83 g, 1.0 mmol). The reaction mixture was stirred at RT overnight. The solvent was evaporated, water was added (100 ml), and pH was adjusted to 6-7 with 1 M HCl. The solid was filtered off, washed with H_2O, and dried at 40ºC overnight. This gave the product **276** as a white solid (5.30 g, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.78 (3H, s, 18-C**H**$_3$), 1.15-1.22 (2H, m), 1.27-1.50 (6H, m),1.66-1.75 (1H, m), 1.83-1.90 (1H, m), 1.92-1.97 (1H, m), 2.08-2.16 (1H, m), 2.16-2.20 (1H, m), 2.27-2.33 (1H, m), 2.80-2.85 (2H, m), 3.73 (1H, t, 17-C**H,** *J* = 8.4 Hz), 6.56 (1H, s, 4-C**H**), 6.62 (1H, d, 1-C**H**, *J* = 8.4 Hz), 7.15 (1H, d, 2-C**H,** *J* = 8.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ; 11.0 (18-**C**), 23.1, 26.3, 27.1, 29.6, 30.6, 36.7, 38.8, 43.2, 43.9, 50.0, 81.9 (17-**C**), 112.6. (2-**C**), 115.2 (4-**C**), 126.5 (1-**C**), 132.7 (10-**C**), 138.3 (5-**C**), 153.2 (3-**C**); IR (neat) 2915, 1452, 1232, 1054 cm^{-1}; GC-MS *m/z* (relative intensity, %); 273 ([M+1]$^{+\cdot}$, 19), 272 ([M]$^{+\cdot}$, 100), 213 (18), 172 (13), 160 (13), 146 (13).

Spectra and physical data matched that previously published.[17]

6α/β-Fluoro-3β,17β–dihydroxyandrost-4-ene (110)

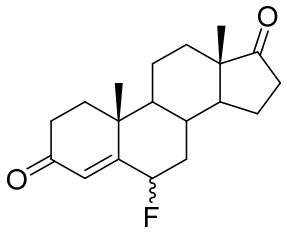


110

To a solution of 6α/β-fluoro-testosterone (**84**) (1.00 g, 0.3 mmol) in DCM / MeOH (50 / 50% 12 ml) in 0°C was added $NaBH_4$ (0.30 g, 0.8 mmol). The reaction mixture was stirred at RT in open flask overnight. The solvent was evaporated and water was added (10 ml) and pH was adjusted to 6-7 with 1 M HCl. The solid was filtered off washed with H_2O, and dried at 40°C overnight. To give the product **110** as a white solid (0.70 g, 71%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.74-0.76 (1H, m, 9-C**H**), 0.78 (3H, s, 18-C**H**$_3$), 0.89-0.99 (1H, m), 1.00-1.15 (3H, m), 1.18 (1H, s, 19-C**H**$_3$), 1.25-1.37 (3H, m), 1.39-1.50 (3H, m), 1.51-1.64 (3H, m), 1.66-1.70 (1H, m), 1.71-1.76 (1H, m), 1.80-1.91 (2H, m), 1.97-2.16 (3H, m), 3.63 (1H, t, 17-C**H**, *J* = 8.4Hz), 4.09-4.23 (1H, m, 3-C**H**), 4.80 (0.45H, t, β, isomers, *J* = 2.8Hz), 4.87-4.91 (0.09H, m, α, isomers, 4-C**H**), 4.92 (0.46H, t, β, isomers, *J* = 2.8Hz, 4-C**H**), 5.00-5.04 (0.12H, m, α, isomers, 4-C**H**), 5.66 (1H, s, 6-C**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ; 11.0 (18-**C**), 20.4 (19-**C**), 23.2 (11–**C**), 29.1, 30.4 (8-**C**), 30.5 , 36.4, 36.6, 37.4, 37.6, 42.9, 50.7 (14-**C**), 53.9. (9-**C**), 67.8, 81.7 (17-**C**), 93.7 (6α-**C**), 95.3(6α-**C**), 131.9 (4-**C**), 142.7 (3-**C**); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -161.41 (β, t, *J* = 56.4 Hz), -184.19 (α, d, *J* = 48.5 Hz).

6α/β-Fluoroandrost-4-ene-3,17-dione 5



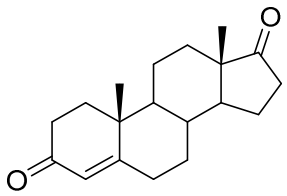
5

To a solution of 6α/β-fluorotestosterone (**84**) (1.00 g, 3.3 mmol) in dry MeCN (10 ml) was added TPAP (0.06 g, 0.2 mmol), NMO (0.66g, 4.9 mmol) and Aº4 molecular sieves (~0.50 g). The reaction mixture was stirred at RT overnight. The solvent was evaporated, water was added (10 ml) and pH was adjusted to 6-7 with 1 M HCl. The solids were filtered off, washed with H_2O and dried at 40ºC overnight. This gave the product **5** as an off white solid (0.56 g, 56%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 0.90 (3H, s, 18-C**H**$_3$), 0.99-1.03 (1H, m), 1.20 (3H, s, 19-C**H**$_3$), 1.23-1.45 (7H, m), 1.57-1.63 (2H, m), 1.66-1.71 (2H, m), 1.73-1.82 (3H, m), 1.86-1.89 (2H, m), 1.96-2.04 (2H, m), 2.05-2.07 (2H, m), 2.08-2.15 (2H, m) 2.34-2.51 (5H, m), 5.05-5.10 (0.63H, m, α, isomers), 5.17-5.22 (0.55H, m, α, isomers), 6.10 (1H, s, 4-C**H**);^{13}C NMR (150 MHz, $CDCl_3$) δ; 13.6 (18-**C**), 18.0 (19-**C**), 20.1 (11–**C**), 21.6 (15-**C**), 31.0 (12-**C**), 33.0, 33.59, 35.5 (16-**C**), 36.2, 37.2, 39.0 (10-**C**), 47.4 (13-**C**), 50.4. (14-**C**), 53.5 (9-**C**), 87.3 (6α-**C**), 88.4 (6α-**C**), 119.8 (4-**C**), 165.1 (5-C), 198.3 (3-**C**), 219.4 (17-**C**); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -165,66 (β, t, J = 50.3 Hz), and -183.61 (α, d, J = 54.8 Hz); IR (neat) 2938, 1733, 1662,1056 cm^{-1}; GC-MS *m/z* (relative intensity, %); 304 ([M]$^{+\cdot}$, 100), 260 (31), 142 (33).

Spectra and physical data matched that previously published.[18]

Androstenedio (106)



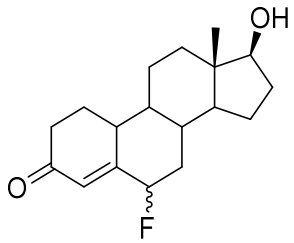
106

To a solution of DHEA (**89**) (5.00 g, 1.7 mmol) in dry freshly distillated toluene (60 ml) was added cyclohexanone (42 ml, 39.9 mmol) under argon. The reaction mixture was heated at reflux employing Dean-Stark (10 ml of toluene was removed). $Al(OiPr)_3$ (2.3 g, 1.1 mmol) in toluene (80 ml) was added at reflux dropwise $Al(OiPr)_3$ at the rate which corresponded to the rate of solvent distillation. The mixture was then refluxed for 2h. The reaction mixture was diluted with toluene (100 ml) and saturated solution of sodium potassium tartarete (100 ml). The layers were separated, the organic was layer dried over $MgSO_4$, filtered and concentrated. This gave the crude product as an yellow oil. The crude product was treated with hexane (60 ml) and stirred at RT for 30 min. The solids were filtered off and dried at 40^{o}C overnight. This gave the product as a white solid (4.61 g, 93%).

^{1}H NMR (600 MHz, $CDCl_3$) δ; 0.92 (3H, s, 18-C**H**$_3$), 0.97-1.01 (1H, m), 1.08-1.15 (1H, m), 1.21 (3H, s, 19C**H**$_3$), 1.25-1.32 (2H, m), 1.42-1.49 (2H, m), 1.53-1.60 (2H, m), 1.67-1.76 (4H, m), 1.85-1.88 (1H, m), 1.95-2.00 (2H, m), 2.02-2.06 (1H, m), 2.07-2.14 (1H, m), 2.31-2.37 (2H, m), 2.39-2.44 (1H, m), 2.44-2.50 (2H, m) 5.75 (1H, s, 4-C**H**); ^{13}C NMR (150 MHz, $CDCl_3$) δ; 13.6 (18-**C**), 17.3 (19-**C**), 20.2 (11–**C**), 21.6 (15-**C**), 30.7 (7-**C**), 31.2 (1-**C**), 32.5 (12-**C**), 33.8 (2-**C**), 35.1 (8-**C**), 35.6 (16-**C**), 35.7, 38.5 (10-**C**), 47.4 (13-**C**), 50.8 (14-**C**), 53.8 (9-**C**), 124.1 (4-**C**), 170.2 (5-**C**), 199.2 (3-**C**), 220.3 (17-**C**); IR (neat) 2919, 1730, 1659 cm^{-1}; GC-MS *m/z* (relative intensity, %); 286 ([M]$^{+\cdot}$, 100), 244 (48), 148 (44), 124 (66), 79 (36).

Spectra and physical data matched that previously published.[19]

6α/β-Fluoro-nortestosterone (86)



86

To a solution of nortestosterone (**111**) (2.42 g, 8.8 mmol) in dry DMF (36 ml) was added pyridine (4.26 ml, 52.9 mmol) and TFAA (1.64 ml, 9.3 mmol). The mixture was heated at 40ºC and stirred for 15 min. After that time AcCl (2.50 ml, 35.3 mmol) was added and the reaction was heated at 70ºC. The mixture was stirred overnight. The reaction mixture was cooled down to -10ºC, diluted with EtOAc (30 ml) and water (30 ml). The layers were separated, the aqueous layer was extracted with EtOAc (3 x 30 ml). The combined organic layers were washed with brine (30 ml) dried over $MgSO_4$, filtered and concentrated. The crude product (**117**) was used without purification at the next stage.

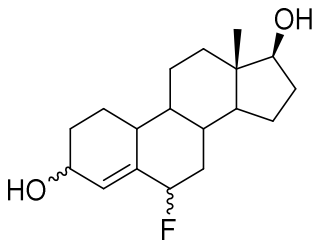
To the crude product in dry DMF (25 ml) was added Selectfluor® (4.06 g, 1.1 mmol). The reaction mixture was stirred at RT for 48 h. After that time $^1H/^{19}F$ NMR show complete consumption of the starting material. The reaction was diluted with 2M HCl (25 ml) and THF (25 ml). The reaction mixture was stirred at RT for 24 h. The reaction mixture was neutralized with saturated solution of $NaHCO_3$ and concentrated under reduced pressure. The residue was dissolved in DCM (10 ml), washed with water (2×20 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography on silica gel (ethyl acetate/DCM 1:5) gave the product **86** as an off white solid (1.42 g, 55%).

^1H NMR (700 MHz, $CDCl_3$) δ; 0.82 (3H, s, 18-C**H**$_3$), 0.86-0.91 (1H, m), 0.99-1.03 (1H, m, 14-C**H**), 1.09-1.13 (1H, m), 1.20-1.29 (2H, m), 1.30-1.37 (2H, m), 1.45-1.50 (2H, m, 16-

C**H**), 1.58-1.67 (2H, m, 15-C**H**), 1.79-1.88 (3H, m), 2.06-2.11 (1H, m), 2.18-2.22 (1H, m), 2.29-2.33 (1H, m), 2.35-2.37 (1H, m), 2.41-2.50 (2H, m), 3.66 (1H, t, 17-C**H**, J = 15.3 Hz), 5.00 (0.54H, s, β-isomers, 6-C**H**), 5.07 (0.51H, s, β-isomers, 6-C**H**), 4.93-4.96 (0.14H, m, β-isomer, 6-C**H**), 5.03-5.04 (0.07H, s, α-isomer, 6-C**H**), 5.94 (1H, s, 6-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 11.2 (18-**C**), 23.1 (15-**C**), 26.0, 26.3, 30.5 (16–**C**), 26.4, 38.6, 43.6, 49.7 (14-**C**), 81.7 (17-**C**), 91.6 (β6-**C**), 92.6 (β6-**C**), 127.7 (β4-**C**), 200.0 (3-**C**); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -171.10 (β, t, J = 49.5 Hz), -181,49 (α, d, J = 54.5 Hz); IR (neat) 3431, 2948, 1666 cm^{-1}; GC-MS *m/z* (relative intensity, %); 292 ($[M]^{+\cdot}$, (49), 274 [M^+- F] (22.3), 272 (88), 254, 213 (100), 128 (57).

Spectra and physical data matched that previously published.14

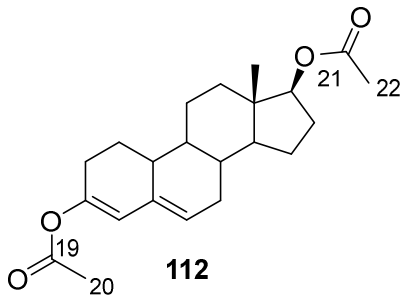
6α/β-Fluoro-norandrostenediole (85)



85

To a solution of 6α/β-fluoro-nortestosterone (**86**) (1.00 g, 0.3 mmol) in DCM / MeOH (50% / 50% 12 ml) in 0°C was added $NaBH_4$ (0.30 g, 7.9 mmol). The reaction mixture was stirred at RT in open flask overnight. The solvent was evaporated, water (10 ml) was added (ml) and pH was adjusted to 6-7 with 1 M HCl. The solids were filtered off, washed with H_2O and dried at 40°C overnight. This gave the product **85** as a white solid (0.71 g, 71%).^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.56-0.68 (1H, m), 0.70-0.80 (3H, m, 18-C**H**$_3$), 0.82-1.00 (2H, m), 1.02-1.21 (6H, m), 1.22-1.34 (4H, m),1.34-1.50 (6H, m), 1.53-1.64 (1H, m), 1.65-1.85 (6H, m), 2.02-2.18 (7H, m), 3.60-3.69 (1H, m, 3- C**H**), 4.11-4.27 (1H, m, 17 - C**H**), 4.73-4.80 (0.32H, m, α-isomers, 6-C**H**), 4.82-4.92 (0.83H, m, α/β-isomers, 6-C**H**), 4.96-4.99 (0.45H, m, α-isomer, 6-C**H**), 5.75 (1H, s, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$); δ 12.2 (18-**C**), 21.4, 21.2, 23.6, 26.6, 27.5, 27.8, 28.3, 31.2, 36.9, 40.9, 42.9, 43.8, 50.6, 83.1 (17-**C**), 117.9 (4-**C**),124.0 (6-**C**), 134.9 (21-**C**), 149.0 (23-**C**), 169.5 (22-**C**), 171.5 (20-**C**); ^{19}F NMR (376 MHz, $CDCl_3$); δ= -167.12 (β, t, *J* = 48.8 Hz), -181.84 (α, d, *J* = 56.4 Hz);

3β,17β-Diacetoxynorandrost-3,5-diene (112)



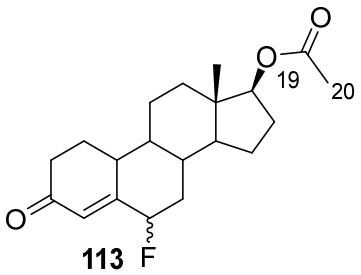
112

To nortestosterone (**111**) (0.50 g, 1.8 mmol) were added acetic anhydride (0.45 ml, 4.7 mmol), pyridine (0.33 ml, 1.8 mmol) and acetyl chloride (1.12 ml, 14.0 mmol) under argon. The reaction mixture was refluxed for 6 h and was then stirred overnight at RT. The mixture was concentrated under reduced pressure and the residue was triturated with ethanol. The solids were filtered off, dried at 40°C to give pure product. The mother liquors ware concentrated and purified by flash column chromatography on silica gel (hexane 95 / EtOAc 5%) This gave the product as off white solid (0.42 g, 64%).

^{1}H NMR (700 MHz, CDdCl$_3$) δ; 0.82 (3H, s, 18-CH$_3$), 0.91-0.97 (1H, m), 1.08-1.13 (1H, m), 1.16-1.28 (2H, m), 1.30-1.36 (1H, m), 1.42-147 (1H, m), 1.48-1.53 (2H, m), 1.61-1.72 (2H, m), 1.75-1.79 (1H, m), 1.86-1.93 (2H, m), 2.04 (3H, s, 21-C**H**), 2.09-2.12 (1H, m), 2.13 (3H, s, 23-C**H**), 2.14-2.20 (3H, m), 2.41 (1H, m), 4.62 (1H, t, 17-C**H**, *J* = 8.38 Hz), 5.47 (1H, s, 6-C**H**), 5.76 (1H, s, 4-C**H**); ^{13}C NMR (175 MHz, CDCl$_3$) δ; 12.1 (18-**C**), 21.2 (20-**C**), 21.3 (22-**C**), 23.4, 26.4, 27.4, 27.6, 28.1, 31.0, 36.7, 36.8, 40.7, 42.9, 43.8, 50.6 (14-**C**), 83.1 (17-**C**), 117.9 (4-**C**), 124.0 (6-**C**), 134.9 (3-**C**), 149.0 (5-**C**), 169.5 (21-**C**), 171.5 (19-**C**); IR (neat) 2984, 1757, 1684, 1267 cm^{-1}; MS *m/z* (relative intensity, %); 358 ([M]$^{+\cdot}$, 10), 316 (100),

Spectra and physical data matched that previously published.[20]

17β-Acetoxy-6α/β fluoronortestosterone (113)



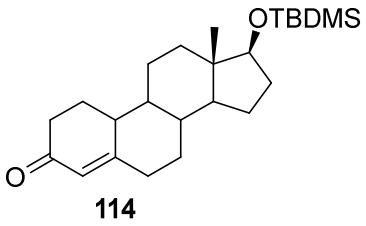
113

To a solution of steroid **112** (0.30 g, 8.4 mmol) in DMF (3 ml) was added Selectfluor® (0.32 g, 0.9 mmol) under argon at RT. The reaction mixture was stirred for 2 days at RT. After that time, water (10 ml) was added and the resulting precipitate was filtered off. The filter cake was dried at 40ºC overnight to give the product **113** as a white solid (0.28 g, 77%).

^{1}H NMR (400 MHz, $CDCl_3$) δ; 0.87 (3H, s, 18-C**H**$_3$), 1.05-1.13 (1H, m, 9-C**H**), 1.17-1.26 (2H, m), 1.29-1.43 (3H, m), 1.49-1.59 (1H, m), 1.60-1.72 (2H, m), 1.77-1.88 (3H, m), 2.04 (3H, 20-C**H**), 2.14-2.23 (1H, m), 2.26-2.37 (3H, m), 2.38-2.40 (1H, m), 2. 41-2.50 (1H, m), 4.59-4.65 (1H, m, 17-C**H**), 4.89-4.95 (0.29H, m, α-isomers, 6-C**H**), 4.98 (0.50H, s, β-isomers, 6-C**H**), 5.01-5.07 (0.28H, m, α-isomer, 6-C**H**), 5.11 (0.52H, s, β-isomers, 6-C**H**), 5.87 (0.73H, s, β-isomer, 4-C**H**), 6.07 (0.32H, s, α-isomer, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; 12.2 (18-**C**), 20.4 (20-**C**), 21.3, 23.3, 27.4, 29.7, 34.3, 36.2, 36.7, 42.5, 50.2, 53.2 (9-**C**), 82.3 (17-**C**), 87.5 (α6-**C**), 92.8 (β6-**C**), 119.6 (α4-**C**), 127.6 (β4-**C**), 158.6 (5-**C**), 171.0 (19-**C**), 199.8 (3-**C**); ^{19}F NMR (376 MHz, $CDCl_3$) δ; -170.67 (β, t, J = 52.6 Hz), -181.18 (α, d, J = 56.4 Hz); GC-MS *m/z* (relative intensity, %); 334 ($[M]^{+\cdot}$, 26), 314 (32), 292 (45), 277 (34), 274 (80), 256 (25), 207 (40), 147 (43), 128 (41), 105 (27), 91 (25), 77 (28), 55 (23), 44 (45), 43 (100), 41 (34).

Spectra and physical data matched that previously published.14

TBDMS-nortestosterone (114)



114

To a solution of nortestosterone (**111**) (0.50 g, 0.2 mmol) in dry DMF (5 ml) was added TBDMSCl (0.66 g, 0.4 mmol) and imidazole (0.15 g, 0.2 mmol) under argon at 0ºC. The reaction was stirred in 45-50ºC overnight. TBDMS (0.66 g, 0.4 mmol) imidazole (0.15 g) were added the mixture was stirred at 45-50ºC for 6h. The reaction mixture was diluted with DCM (30 ml) and water (20 ml). The organic layer was separated and washed with water (6 x 20 ml). The organic layer was dried over $MgSO_4$ filtered and concentrated to give the crude product. The crude product was purified by flash column chromatography on silica (hexane 80% / DCM 20%). This gave the product **114** as an off white solid (0.34 g, 47%).

^{1}H NMR (700 MHz, $CDCl_3$) δ; 0.00 (6H, d, Si-C**H**$_3$), 0.75 (3H, s, 18-CH_3), 0.76-0.82 (1H, m), 0.86 (9H, s, Si-C**H**$_3$), 0.89-0.96 (2H, m), 0.97-1.03 (2H, m), 1.20-1.34 (2H, m), 1.40-1.47 (1H, m, 16-C**H**), 1.49-1.58 (2H, m), 1.74-1.78 (1H, m), 1.79-1.84 (2H, m), 1.85-1.90 (1H, m, 16-C**H**), 2.04-2.09 (1H, m) 2.21-2.28 (3H, m, 10/6-C**H**), 2.36-2.40 (1H, m, 2-C**H**), 2.42-2.47 (1H, m, 2-C**H**), 3.55 (1H, t, 17-C**H**, *J* = 8.3 Hz), 5.80 (1H, s, 4-C**H**); ^{13}C NMR (175 MHz, $CDCl_3$) δ; -4.8 (Si-**C**H_3), -4.5 (Si-**C**H_3), 11.3 (18–**C**), 18.0 (Si-(**C**H_3)$_3$), 23.3, 25.6, 25.8, 26.2, 26.5, 30.7, 30.8, 35.5, 36.5, 36.8, 40.5, 42.6, 43.3, 49.3, 49.7, 81.5 (17-**C**), 124.4 (4-**C**), 166.8 (5-**C**), 199.8 (3-**C**); MS *m/z* (relative intensity, %); 373 (1), 332 (28), 331 (100), 255 (11), 75 (31); IR (cm^{-1}); 2928, 1664, 830.

Spectra and physical data matched that previously published.[21]

6.3 Experimental for biological experiments

Synthesised fluorinated steroids were used in the biological experiments. Soya meal medium was send from our collaborator Dr Cormac Murphy from UCD Dublin. Medium LB (Lysogeny broth) was purchased from Sigma Aldrich. *Streptomyces griseus* ATCC13273 was obtained from LGC Standards UK, *Escherichia coli* MG1655*, Bacillus subtilis* and *Bacillus megaterium* 14581 bacteria were available in house.

General culture condition

S. griseus was cultured in 250-ml Erlenmeyer flasks containing 50 ml soya bean meal media as described previously (Murphy 2010). Cultures were incubated with rotary agitation 200 rpm at 27ºC for 6 days. After first 72 h, labelled steroids were added (3-5 mg). At the end of the incubation period, cells were harvested by centrifugation, separated from supernatant and washed with water. The cells in water were disrupted by sonication (5 min in total, bursts of 1 s with 1-s interval) using an ice bath to prevent overheating. Supernatant and cells in water were extracted with ethyl acetate.

Control experiments were conducted in which the microorganism was incubated in the absence of fluorinated-steroids compound and the compounds were incubated in the absence of microorganism.

Media

The following media were used:

- LB (Lysogeny broth) medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl
- NB (Nutrient broth) medium: 10 g, peptone, 10 g beef extract, 5 g NaCl
- 868 medium[22]: 10 g pepton, 10 g yeast extract, 20 g glucose.
- Soya bean meal (5 g/L), Glycerol (20 g/L), Yeast extract (5 g/L), K_2HPO_4 (5g/L).

Final pH for all media were adjusted to 7.

6.4 Reference

[1] T. Tohru, Monde, Kenji, *J. Am. Chem. Soc.*, **2012**, 134(*8*), 3695.

[2] I. M. Merlani, L. S. Amiranashvili, I. N. Men'shova, *Chem. Nat. Compd.*, **2007**, *43*, 97.

[3] P. Gartnera, C. Novaka, C. Einzingera, W. Felzmanna, M. Knollmüllera, G. Gmeinerb, W. Schanzerc, *Steroids*, **2003**, *68*, 85.

[4] D. Chambers, T. Nakano, M. Parsons, G. Sandford, A. S. Badsonov, J. A. K. Howard, *J. Fluorine Chem.*, **2008**, *129*, 811.

[5] J. Liu, J. Chan, C. M. Bryant, P. A. Duspara, E. E. Lee, D. Powell, H. Yang, Z. Liu, C. Walpole, E. Roberts, R. A. Batey*, Tetrahedron Lett.*, **2012,** 53(*24*), 2971.

[6] M. Berube, Y. Laplante, D Poirier, *Med. Chem.*, **2006**, 2(*4*), 329.

[7] J. A.Morales-Serna, A. Vera, E. Paleo, E. Garcia-Rios, R. Gavino, G. Garcia de la Mora, J. Cardenas, *Synthesis*, **2010**, *24*, 4261.

[8] X. K. Liu, B. J. Ye, Y. Wu, J. X. Nan, Z. H. Lin, H. R. Piao, *Chem. Biol. Drug Des*., **2012**, 79 (*4*), 523.

[9] P. Marwah, A. Marwah, H. A. Lardy, *Tetrahedron*, **2003**, 59 (*13*), 2273.

[10] D. Trafalisb, E. Geromichaloua, P. Dalezisb, N. Nikoleousakosb, V. Sarlia, *Steroids*, **2016**, *115*, 1.

[11] Y. S. Choe, J. A. Katzenellenbogen, *Steroids*, **1995**, 60 (*5*), 414.

[12] A. Liu, K. E. Carlson, J. A. Katzenellenbogen, *J. Med. Chem*., **1992**, 35 (*11*), 2113.

[13] W. T. Pennington, G. Resnati, D. D. DesMarteau, *J. Org. Chem*., **1992**, 57 (*5*), 1536.

[14] Y. S. Choe, J. A. Katzenellenbogen, *Elsevier Science Inc*., **1995**, *60*.

[15] M. R. Tremblay, D. Poirier, *J. Chem. Soc*., **1996**, *1*, 2765.

[16] D. J. Collins, J. Sjovall, *Aust. J. Chem*., **1983**, *36*, 339.

[17] B. Wang, L. Qin, K. D. Neumann, S. Uppaluri, R. L. Cerny, S. G. DiMagno, *Org. Lett.*, **2010**, 12 (*15*), 3352.

[18] H. Kunzer, M. Stahnke, G. Sauer, R. Wiechert, *Tetrahedron Lett*., **1990**, 31*(27*), 3859.

[19] V. D. Pavlović, M. M Dabović, S. Martinović, L. B Lorenc, J. Kalvoda, *J. Serb. Chem. Soc.*,**2004**, 69(*11*), 861.

[20] L. Velluz, B. Goffinet, G. Amiard, *Tetrahedron*, **1958**, 4(*3–4*), 241.

[21] D. Menberu, K. D. Onan, P. W. L, Q, S. Choe, *J. Org. Chem*., **1992**, 57 (*7*), 2100.

[22] N.K. O'Connor, A.S. Hudson, S.L. Cobb, D. O'Neil, J. Robertson, V. Duncan, C.D. Murphy, *Amino Acids*, **2014**, *46*, 2745.