CARLA LOPES VARELA

DESIGN, SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIPS STUDIES ON STEROIDAL AROMATASE AND 5α-REDUCTASE INHIBITORS AS ANTI-TUMORS

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DESIGN, SÍNTESE E ESTUDOS DE RELAÇÃO ESTRUTURA-ATIVIDADE DE ESTERÓIDES INIBIDORES DA AROMATASE E DA 5α-REDUCTASE COMO ANTI-TUMORAIS

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Aos meus pais,

Guiomar e Carlos

À minha avó Trindade,

in memoriam

Trabalho realizado sob a orientação do(a) Professor(a)

Doutor Elisiário José Tavares da Silva, Faculdade de Farmácia da Universidade de Coimbra

Doutora Fernanda Maria Fernandes Roleira, Faculdade de Farmácia da Universidade de Coimbra

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ABSTRACT

Cancer is a major public health problem in all countries. Among the several forms of cancer, breast and prostate cancers are the ones most frequently diagnosed among Women and Men, and a large number of them are hormone-dependent.

Breast cancer is the most common malignancy in women being the leading cause of cancer death worldwide. The natural history of this illness suggests that many of these tumors are dependent on estrogens for their development and growth. Among the several therapeutic options available, endocrine therapy controlling estrogen production has been the guiding principle for more than a century. In the biosynthesis of estrogens it is involved the enzyme aromatase, and its inhibition assumes a rational way to treat this disease. Nowadays, it has been observed a meaningful evolution in the development of aromatase inhibitors (AIs). The recent successful elucidation of the crystallized 3D structure of the enzyme, which provided the structural basis for the specificity to the interaction with its substrate, was an important milestone. This has clarified the establishment of hydrogen bonds between the oxygen atoms of C-3 and C-17 keto groups and specific amino acid residues of the active site of the enzyme. Besides this, it was also observed that C-6 linear side chains of some inhibitors protrude into an access channel cavity immobilizing catalytic residues. This new data is very useful to understand the action of the inhibitors and for the development of promising molecules able to overcome some of the major drawbacks of AIs, such as the side effects, which are bone loss, joint pain and heart problems and the acquired resistance that they can led to after some years of usage.

In this work, we were interested in the design, synthesis, biochemical and biological evaluation of the anti-aromatase activity of steroidal compounds obtained by structural modifications within the A-, B- and D-rings of androstenedione, the natural substrate of the enzyme. Supported on the results obtained, the features of the steroidal compounds determinant for achieving inhibitory activity were elucidated.

It is known that aromatase establishes two main hydrogen bonds with the carbonyl functions at C-3 and C-17 of androstenedione. Also, former studies have revealed that, in spite of this, the presence of the carbonyl group at C-3 is not completely mandatory to bind steroid molecules to the enzyme. On the other side, the C-17 carbonyl group seems to have a major role on the activity of steroidal AIs. Based on this rationale, we have transformed the C-3 carbonyl group into a hydroxyl group. From the studied molecules, we observed that C-3 hydroxyl derivatives of androstenedione are very active inhibitors specially when the hydroxyl group assumes a 3β-stereochemistry. Changing both carbonyl groups, at C-3 and C-17, led to a significant decrease in activity. These results allowed establishing that the existence of a carbonyl group at C-3 is not mandatory for a steroid to be a potent inhibitor, as long as there is one at C-17.

According to previous results reported on the literature, it was inferred that some planarity in the A-ring and in the A,B-ring junction would be required for steroids to inhibit the enzyme. Based on this, we have synthesized new AIs and compared the influence of double bonds and epoxide functions in several positions along the A-ring. It was observed that when introducing the double bond in 4,5-position, the compound obtained revealed the highest activity, pointing out the importance of the planarity in the position closer to the A,B-ring junction. However, among the epoxide series, the 3,4-

epoxysteroid revealed to be the most active inhibitor, evidencing the possibility of the oxiran oxygen atom to establish hydrogen bonds within the active site of the enzyme.

According to former information, it is known that the enzyme may have a hydrophobic binding pocket with a limited accessible volume, but that can accommodate non-bulky substituents located in the C-6 and C-7 position of the steroidal framework. Also, most recently, it was observed that the enzyme has an access channel, which allows the accommodation of C-6 side chains present in some inhibitors. Further, previous works have revealed the evidence that the aromatase inhibitory activity is similar when the same kind of substituent is in the referred C-6 or C-7 position. With this knowledge in mind, we have synthesized new 6α-methyl and 7α-allyl androstenedione derivatives that revealed to be strong AIs.

Previous work pointed out the importance of the C-17 carbonyl group in the steroidal D-ring as a structural feature required to reach maximum aromatase inhibitory activity. Based on this, and in order to achieve new structure-activity relationships (SAR), we have substituted the C-17 carbonyl group by a hydroxyl, acetyl and also by a thionyl isoster group, to compare inhibitory activities. In almost all synthesized compounds the C-17 carbonyl derivatives revealed to be stronger AIs.

We were also interested in preparing and studying some derivatives of the two steroidal AIs that were/are in clinical use for the treatment of breast cancer, formestane and exemestane. The C-4 acetoxy and acetylsalicyloxy derivatives from formestane were synthesized and evaluated showing that bulky substituents in C-4 position diminish the inhibitory activity. With respect to epoxy derivatives of exemestane, which are potential metabolites, they revealed to be potent AIs.

The activity of the most potent AIs evaluated in placental microsomes was also tested in MCF-7aro cells being some of them able to inhibit cell viability and cell

proliferation even more efficiently than exemestane. Some of the compounds were also studied in SK-BR-3 and LTEDaro cells.

Concerning prostate cancer, this type of tumor depends on androgens for its development and progression. Another illness with this association, and which is also a recurrent disease in men, is benign prostate hyperplasia (BPH). In both these situations, it is always observed an increase in the activity of the enzyme 5α-reductase, the one responsible for converting testosterone into dihydrotestosterone, the main androgen implicated in the differentiation and growth of prostate. Hence, the inhibition of 5α-reductase is a rational way for treating these hyperandrogenic disorders. Despite the potency and success of the molecules used nowadays, they still present some disadvantages, such as increase in both bone and muscle loss, and impotency. Further, when these molecules are used in a profilatic way, they can cause high-grade prostate cancers.

In this thesis we designed and synthesized steroids with the 3-keto-Δ^4 moiety in the steroidal A-ring combined with carboxamide, carboxyester or carboxylic acid functions at the C-17β position of the D-ring. This rationale was mainly focused on analogs of finasteride and dutasteride, two potent irreversible 5α-reductase inhibitors (RIs) used in the clinic, sharing in the same molecule the 3-keto-Δ^4 moiety present in testosterone, the natural substrate of the enzyme, with the C-17β carboxamide group present in the referred RIs. The results obtained suggest that lipophilic amides favour the enzyme inhibition, being the synthesized steroid with a C-17β *N-tert*-butylcarboxamide group the best inhibitor.

Coumarins constitute another group of compounds that can structurally resemble steroids in inhibiting aromatase. Theregore, in this thesis we have also synthesized new 3-thiophenyl coumarin derivatives in order to open a new pathway for the synthesis and evaluation of this class of compounds as AIs. Some preliminary results concerning their synthesis will be presented.

Keywords: steroids, breast cancer, aromatase, aromatase inhibitors, prostate cancer, benign prostate hyperplasia, 5α-reductase, 5α-reductase inhibitors, design and synthesis, structure-activity relationships, coumarins.

RESUMO

O cancro é dos problemas de saúde pública com maior incidência a nível mundial. Dos diferentes tipos de cancro, o cancro de mama e da próstata são dos mais frequentemente diagnosticadas na Mulher e no Homem, sendo que um número significativo deles são hormono-dependentes.

O cancro de mama é das doenças mais comuns nas mulheres e constitui a principal causa de morte por cancro na população feminina, em todo o mundo. Desde há muito que se sabe que a maioria deste tipo de cancro depende de estrogénios, para o seu desenvolvimento e crescimento. Entre as diversas opções terapêuticas disponíveis, a terapia endócrina de controlo da produção de estrogénios tem sido o princípio orientador seguido durante mais de um século. Na biossíntese de estrogénios existe a enzima aromatase e a sua inibição constitui uma forma muito eficiente de tratar esta doença. Atualmente, tem-se observado uma evolução significativa no desenvolvimento dos inibidores da aromatase. A recente elucidação da estrutura cristalina 3D da enzima constitui um importante ponto de viragem, já que esclareceu as bases estruturais para a especificidade da interação da enzima com o seu substrato. Este esclarecimento mostrou que se estabelecem ligações de hidrogénio entre os átomos de oxigénio dos grupos carbonilo em C-3 e C-17 e resíduos específicos de aminoácidos, no local ativo da enzima. Para além disso, também foi observado que cadeias lineares posicionadas em C-6 de alguns inibidores esteróides se projetam para o interior de um canal de acesso à enzima, imobilizando os resíduos catalíticos. Esta nova informação revelou-se muito útil na tentativa de se compreender o mecanismo de inibição da aromatase e também no desenvolvimento de moléculas promissoras capazes de ultrapassarem algumas das

principais desvantagens dos inibidores da aromatase em uso, como sejam, os efeitos secundários relacionados com a perda de massa óssea, dores articulares e problemas cardíacos, bem como a resistência adquirida, que estes tendem a desenvolver após alguns anos de utilização.

Neste trabalho estivemos interessados no design, na síntese e na avaliação biológica da atividade inibidora da aromatase de compostos esteróides obtidos por modificações estruturais nos anéis A, B e D da androstenodiona, o substrato natural da enzima. As características determinantes para que estes compostos sejam potentes inibidores foram elucidadas com base nos resultados obtidos.

Sabe-se que a aromatase estabelece duas ligações de hidrogénio principais com os átomos de oxigénio das funções carbonilo em C-3 e C-17 da androstenodiona. Estudos anteriores revelaram que, apesar disso, a presença do grupo carbonilo em C-3 não é absolutamente essencial para que a molécula se ligue à enzima. Por outro lado, o grupo em C-17 parece ter um papel predominante na atividade inibitória da aromatase. Desta forma, decidimos transformar o grupo carbonilo de C-3 num grupo hidroxilo. Das moléculas estudadas, observámos que os derivados C-3 hidroxilados são inibidores bastante potentes, especialmente se o grupo hidroxilo assumir a estereoquímica 3β. A alteração de ambos os grupos carbonilo em C-3 e C-17 levou, por seu turno, a uma diminuição muito significativa da atividade. Estes resultados permitiram estabelecer que, de facto, não é essencial a existência de um grupo carbonilo em C-3 num esteróide para que este seja um potente inibidor, desde que exista um outro destes grupos em C-17.

De acordo com resultados apresentados na literatura, revelou-se necessário a existência de alguma planaridade no anel A e na junção dos anéis A,B, para que os compostos esteróides inibam a enzima. Assim, sintetizámos novos inibidores da

aromatase e comparámos a influência que as funções, dupla ligação e epóxido, tinham quando ocupavam diferentes posições do anel A. Perante os resultados obtidos, verificou-se que, quando a dupla ligação estava na posição 4,5, o composto apresentava maior atividade, reforçando a importância da planaridade na posição mais próxima da junção dos anéis A,B. Todavia, para a série dos epóxidos, o derivado 3,4 mostrou ser o inibidor mais ativo, o que levantou a hipótese do oxigénio do anel oxirano conseguir estabelecer ligações de hidrogénio com algum aminoácido do local ativo da enzima.

De acordo com dados anteriores, sabe-se que a enzima poderá ter uma bolsa hidrofóbica, com um volume limitado, mas que poderá acomodar substituintes pouco volumosos localizados nas posições C-6 e C-7 dos esteróides. Recentemente, também se observou que a enzima tem um canal de acesso que permite a entrada dos esteróides e que poderá acomodar cadeias laterais na posição C-6 destes compostos. Para além disso, outros trabalhos revelaram que a atividade inibitória é semelhante quando o mesmo tipo de substituinte está na posição C-6 ou na C-7. Desta forma, sintetizámos novos derivados 6α-metilo e 7α-alilo da androstenodiona que revelaram ser potentes inibidores da aromatase.

Estudos anteriores mostraram a importância do grupo carbonilo em C-17 no anel D esteróide como característica estrutural requerida para atingir atividade inibitória máxima. Deste modo, e de forma a estabelecer novas relações estrutura-atividade, substituímos o grupo carbonilo em C-17 pelo grupo hidroxilo, acetilo e também pelo isóstero tionilo. Em quase todos os compostos preparados, os que contêm o grupo carbonilo revelaram ser inibidores mais potentes.

Estivemos também interessados em preparar e estudar alguns derivados dos dois fármacos usados/em uso na clínica para o tratamento do cancro de mama, o formestano e o exemestano. Os derivados C-4 acetoxilo e acetisaliciloxilo do formestano foram

sintetizados e avaliados. Ficou evidente que substituintes nesta posição diminuem a atividade inibitória. Relativamente aos epoxi-derivados do exemestano, que são potenciais metabolitos, estes mostraram ser potentes inibidores.

A actividade dos inibidores da aromatase mais potentes avaliados nos microssomas da placenta foi também testada em células MCF-7aro sendo que alguns deles revelaram inibir a viabilidade e proliferação celulares de uma forma ainda mais eficiente que o exemestano. Alguns destes compostos foram ainda estudados nas linhas celulares SK-BR-3 e LTEDaro.

Relativamente ao cancro de próstata, este depende de androgénios para o seu desenvolvimento e progressão. Outra doença que também partilha destas características é a hiperplasia benigna da próstata. Em ambas as situações é sempre observada uma atividade anormalmente elevada da enzima 5α-reductase, enzima responsável pela conversão da testosterona na di-hidrotestosterona, o principal androgénio implicado na diferenciação e crescimento da próstata. Assim, a inibição da 5α-reductase surge como uma forma lógica de promover o tratamento destas situações. Apesar das moléculas que existem atualmente em uso clínico serem potentes, elas apresentam algumas desvantagens, como a perda de massa óssea e de massa muscular e impotência. Além disso, quando eles são usados de uma forma profilática podem causar cancro de próstata de elevado grau.

Nesta tese fizemos o design e sintetizámos esteróides com a função 3-ceto-Δ^4 do anel A do esteróide combinada com funções carboxamida, carboxiéster e ácido carboxílico na posição C-17β do anel D. Na base deste design estão os compostos finasteride e dutasteride, dois inibidores potentes e irreversíveis da 5α-reductase usados na terapêutica. Assim, procurou-se reunir na mesma molécula, o anel A que existe na

testosterona, o substrato natural da enzima, com os substituintes presentes em C-17β dos referidos fármacos. Os resultados obtidos sugerem que as amidas lipofílicas favorecem a inibição da enzima, sendo que o composto com o grupo *N*-*terc*-butilcarboxamida em C-17β constitui o melhor inibidor.

As cumarinas constituem outro grupo de compostos que podem assemelhar-se quimicamente aos esteróides, na inibição da aromatase. Assim, também sintetizámos novos derivados 3-tiofenil da cumarina, de forma a abrir um novo caminho para a síntese e a avaliação desta classe de compostos como inibidores da aromatase. Alguns resultados preliminares relacionados com a sua síntese são apresentados nesta tese.

Palavras-chave: esteróides, cancro de mama, aromatase, inibidores da aromatase, cancro de próstata, hiperplasia benigna da próstata, 5α-reductase, inibidores da 5α-reductase, design e síntese, relações estrutura-atividade, cumarinas.

LIST OF ABBREVIATIONS

AIs	Aromatase inhibitors
ATR	Attenuated total reflectance
BOP	(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
BPH	Benign prostate hyperplasia
BRCA1	Breast cancer gene type 1
BRCA2	Breast cancer gene type 2
$CDCl_3$	Deuterated chloroform
^{13}C NMR	Carbon nuclear magnetic resonance
COSY	Correlation spectroscopy
DCC	Dicyclohexylcarbodiimide
DDQ	5,6-Dicyanobenzoquinone
DHT	Dihydrotestosterone
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
E1	Estrone
E1 mechanism	Unimolecular elimination mechanism
E2	17β-Estradiol
ER	Estrogen receptor
ESI-MS	Electrospray ionization-Mass spectrometry
FDA	Food and drug administration
HER 2	Human epidermal growth factor receptor 2

^{1}H NMR	Proton nuclear magnetic resonance
HPLC	High-pressure liquid chromatography
HRMS	High-resolution mass spectrometry
IC_{50}	Inhibitory concentration for half of maximum activity
IR	Infra-red spectroscopy
J	Coupling constant
K_i	Inhibition constant
LC-MS	Liquid chromatography-Mass spectrometry
LNCaP cells	Human androgen-responsive prostate cancer cell line
LTEDaro cells	Long-term estrogen deprivation human breast cancer cell line
MCF-7aro cells	ER^+ aromatase-overexpressing human breast cancers cell line
Mp	Melting point
NADPH	Nicotinamide adenine dinucleotide phosphate
NOESY	Nuclear Overhauser effect spectroscopy
NSAIDs	Nonsteroidal anti-inflammatory drugs
***p*-TSA**	*p*-Toluenesulfonic acid
Rf	Retention factor
RIs	5α-Reductase inhibitors
rt	Room temperature
SAR	Structure-activity relationships
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SK-BR-3	ER^- human breast cancer cell line
S_N2	Bimolecular nucleophilic substitution
T	Testosterone

TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultra-violet spectroscopy

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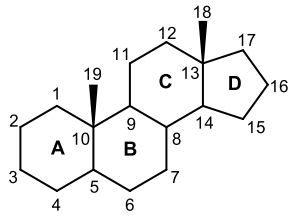
OUTLINE OF THE THESIS

The present thesis focuses on:

i) achieving new compounds for breast and prostate cancer treatment;

ii) establishing structure-activity relationships (SAR) of steroidal compounds as aromatase and 5α-reductase inhibitors.

For that, several compounds where designed, synthesized and screened for their aromatase and 5α-reductase inhibitory activity, in order to evaluate their anti-tumor potency towards breast and prostate cancer and for BPH treatment, respectively.



Ring-lettering and atom-numbering of the steroidal framework

This work is divided in three main sections:

CHAPTER I – STEROIDAL AROMATASE INHIBITORS AS ANTI-TUMORS

In this part, it is made an introduction where the scope of the disease, the molecular target, and the compounds used in breast cancer treatment are revised.

This chapter is subdivided into five subjects where there are presented: the rationale behind the prepared compounds; the synthetic strategies to obtain them and the discussion of the encountered SAR. The conclusion about the studies performed on cell lines are also made. Molecular modelling studies were performed for some compounds

and its conclusions are presented. The experimental part of the prepared compounds in chapter I is also disclosed.

CHAPTER II - STEROIDAL 5α-REDUCTASE INHIBITORS AS ANTI-TUMORS AND AS DRUGS FOR THE TREATMENT OF BENINGN PROSTATE HYPERPLASIA (BPH)

In this part, it is made an introduction presenting the disease and the molecular target involved as well as the molecules used in the treatment.

This chapter presents the rationale behind the synthesis of the desired compounds as well as the synthetic strategies behind it. It is also made a study of the SAR and the conclusion about the studies performed in cell lines. The experimental part of this section is enclosed in the end of the chapter.

CHAPTER III - FUTURE WORK: DEVELOPMENT OF COUMARINS AS AROMATASE INHIBITORS

This part of the thesis arose from our interest in extending the study of aromatase inhibition to other type of compounds, namely coumarins. A complementary study of this kind of compounds was made at the Department of Organic Chemistry of the Faculty of Pharmacy of the University of Santiago de Compostela, Spain. The presentation of the compounds prepared is made in this chapter.

The biochemical assays and biological studies of the compounds prepared in this thesis were performed in the Laboratory of Biochemistry, Department of Biological

Sciences, Faculty of Pharmacy, University of Porto, and the Institute for Molecular and Cell Biology, University of Porto.

The molecular modeling studies were performed in collaboration with the CCLab, Department of Health Science, University “Magna Græcia” of Catanzaro, Italy.

The X-ray analysis was made in collaboration with the Group of Electronic and Magnetic Structure of Materials from the Center of Materials Study through X-ray Diffraction at the Physics Department of the Faculty of Sciences and Technology, University of Coimbra.

CHAPTER I

STEROIDAL AROMATASE INHIBITORS AS ANTI-TUMORS

INTRODUCTION

A.1. Breast Cancer: Some Facts

A.2. Estrogens

A.2.1. Estrogen Receptors

A.2.2. Estrogen Biosynthesis

A.3. Aromatase

A.4. Breast cancer treatment

A.4.1. Surgery

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A.4.5.2. Aromatase Inhibitors (AIs)

AIM OF THE WORK

1.1. DERIVATIVES OF EXEMESTANE

1.1.1. Design and synthesis

1.1.2. Chemistry

1.1.3. Biochemistry and biology

1.1.4. Structure-activity relationships discussion and conclusions

1.2. DERIVATIVES OF FORMESTANE

1.2.1. Design and synthesis

1.2.2. Chemistry

1.2.3. Biochemistry

1.2.4. Structure-activity relationships discussion and conclusions

1.3. A- AND D-RING MODIFIED DERIVATIVES OF ANDROSTENEDIONE

1.3.1. ***C-3 Hydroxyl Derivatives of Androstenedione and Testosterone***

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INTRODUCTION

A.1. Breast Cancer: Some Facts

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in women worldwide, with an estimated 1.4 million of new cases and 458,000 deaths in 2008.[1] In Figure 1.1 are indicated the most common cancers in women in 1984, 2007 and predicted to occur in 2030, for the United Kingdom.[2]

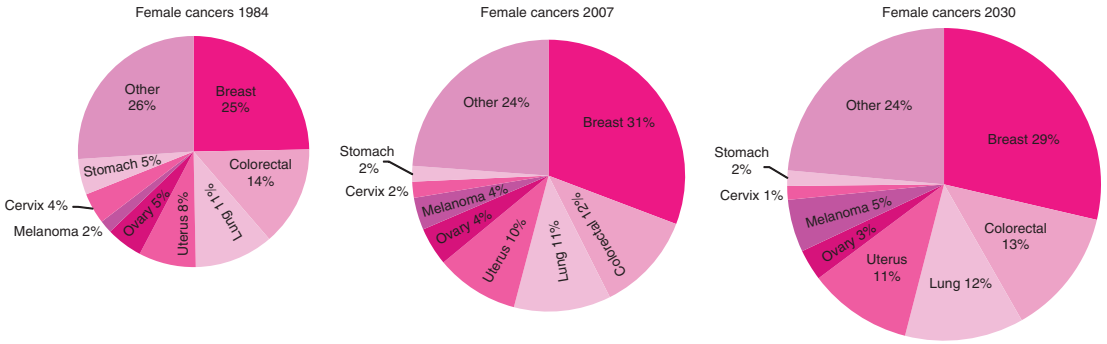


Figure 1.1 - Pie charts of the most common cancers in women in 1984, 2007 and predicted for 2030, for the United Kingdom. The areas of the pies are proportional to the numbers of cases[2]

The three most commonly types of cancer among women expected to be diagnosed in 2030 are breast, lung and colorectal, accounting for more than half of the estimated cases of cancer in women. Breast cancer alone is expected to account for 29% of all the estimated cases. These same predictions were also made for 2012, for the United States.[3]

In spite of the rising in breast cancer incidence, the mortality rate is falling in developed countries (approximately 6-19 per 100,000 cases) because of the more favorable life style, the earlier diagnosis of the disease and the discovery of new specific

drugs.[4] Due to the combination between the high incidence and the favourable prognostic, breast cancer is the one with higher survival rate. It is estimated that approximately 4,4 millions of women, worldwide, that were formerly diagnosed with breast cancer within the last five years, are still alive.[5]

In Portugal, in the beginning of the 90's, it was observed an inversion in the increased tendency of mortality due to breast cancer. The decrease rate was of 2% per year (Figure 1.2). This can be explained by the early detection and the easy access to effective treatments.[5] The incidence rates in the United Sates, as well as in other western countries including United Kingdom, have decreased since the early 2000s largely due to reduction in the use of hormone replacement-therapy, in post-menopausal women.[6,7]

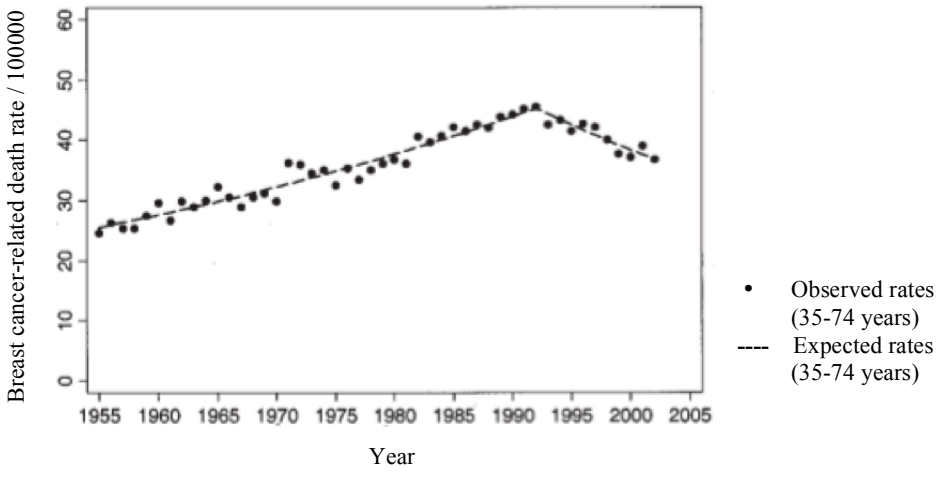


Figure 1.2 - Breast cancer-related mortality in Portuguese women (35-74 years), between 1955-2002[5]

There are a number of factors that are known to increase the risk of developing breast cancer, being some of them modifiable, accounting to control the development of the disease. Besides the gender, age is the most important risk factor for breast cancer. Hence, for example, a 20-year old woman has a probability of 0.06% to develop breast

cancer in the next 10 years, while a 60-year old woman has a risk of 3.45%.[8] However, in the last years, it has been observed a more frequent diagnosis of breast cancer among young women. Women with family history of breast cancer, especially in a first-degree relative (mother, sister, daughter, father, or brother), are at increased risk of developing the disease. Compared to women without a family history, the risk of breast cancer is 1.8 times higher for women with one first-degree female relative. This risk also increases when the first diagnosis occurs at a younger age.[8] Genetic factors are also decisive. It is estimated that 5-10% of breast cancer cases arise from inherited mutations, including in the breast cancer susceptibility genes BRCA1 and BRCA2. Mutations in these two genes account for about 15-20% of familiar breast cancers.[8,9] Reproductive hormones are thought to influence breast cancer risk. Early menarche (<12 years) and older age at menopause (>55 years) may increase the risk for breast cancer.[8,10] Postmenopausal women with high levels of endogenous hormones (estrogen or testosterone) have about twice the risk for developing breast cancer.[8,11] Younger age of child bearing and a greater number of pregnancies decrease the risk of breast cancer.[1,8] Recently, it has been attributed to the use of oral contraceptives the slightly increase in the risk of breast cancer. Also, the use of hormone replacement therapy, with combined estrogen and progestin, appears to increase the risk of developing and dying from breast cancer.[8] There are many other risk factors for breast cancer, such as, high breast tissue, high bone density, a fat-rich diet and environmental agents, frequently referred to as "endocrine disruptors", like cigarette smoke, alcohol and exposure to harmful radiations.[8,10]

In conclusion, it is accepted that sex-steroid hormones are involved in the development of breast tumors. However, some aspects are still to be fully understood.

A.2. Estrogens

Estrogens are essential for life. They are a group of steroid hormones that are involved in numerous physiological processes including the development and maintenance of the female sexual organs, reproductive cycle, reproduction and several neuroendocrine functions. However, these hormones also play crucial roles in certain disease states, especially in mammary and endometrial carcinomas.[12,13]

A.2.1. Estrogen Receptors

Estrogens induce cellular transformations by different mechanisms being the estrogen receptor (ER) (Figure 1.3) the central piece of them.[14,15] Estrogens diffuse through the cell membrane into the cell and, after the involvement of other non-ER plasma membrane-associated estrogen binding proteins,[16] they bind to the ER that is located in the nucleus belonging to the nuclear hormone receptor superfamily.[17,18] It was in 1986 that Green, P. *et al.*[19] and Greene, G. L. *et al.*[20] have cloned the first ER, and later in 1996 Kuipier, G. G. *et al.*[14] have reported a second ER. These are nowadays known as ERα and ERβ, respectively.

Figure 1.3 - Crystal structure of ERα ligand binding domain complexed with estradiol[15]

The two ER forms present a very high degree of similarity when compared at the aminoacid level and they have a similar tertiary structure.[21] They share common regions, which participate in the formation of independent but interacting functional domains: the N-terminal transactivation domain, the DNA-binding domain and the ligand-binding domain. They are encoded by separate genes, *ESR1* and *ESR2*, which are found in different chromosomal locations.[17,18,22]

ERα and ERβ can be detected in different tissues, being possible to exist both receptors in some cells, whereas in others there is only one subtype predominant. ERα is mainly expressed in the uterus, prostate stroma, theca cells of the ovary, Leydig cells of testes, epididymis, bone, breast, some regions of the brain and white adipose tissue. ERβ is in turn expressed in the colon, bone marrow, salivary gland, vascular endothelium and in some parts of the brain.[18]

ERs play therefore functional roles both in the physiology and disease. Concerning breast, it is known that normal and cancer tissues present different ERα and ERβ distribution, which might influence breast cancer risk, hormone responsiveness and survival.[18] Although, it has been shown that also ERβ is expressed in breast tumors, ERα is indeed the predominant estrogen receptor in the female reproductive tract and mammary glands, being also the receptor most required for the known estrogenic responses.[13,23] ERα is the only form clinically measured for medical decision-making and treatment.[18] Approximately three quarter of breast tumors express hormone receptors like ER (ER-positive tumors).

A.2.2. Estrogen Biosynthesis

The biosynthesis of steroidal hormones occurs from the precursor cholesterol and it is a multi-step process, which involves a series of transformations with different enzymes (Figure 1.4). These enzymes promote the synthesis of different families of steroidal hormones, not only in specific secretory organs but also in peripheral tissues.[24]

The most predominant estrogen in circulation is 17β-estradiol (E2), which is the most biologically active in breast tissue.[24] In premenopausal women the synthesis of estrogens occurs in the ovaries, and also in the placenta, in pregnant ones. However, in postmenopausal women the ovary function and the production of estrogens cease. Hence, in this case, peripheral tissues such as liver, skin, muscle as well as adipose tissue provide the major source of estrogen synthesis.[13,25]

The last step of biosynthesis of the most potent endogenous estrogen E2 is made from testosterone by aromatase, which is generally considered rate-limiting in estrogen production. The crucial role played by aromatase in estrogen production has led to enormous interest both in the enzyme and its inhibitors.[13,26]

A.3. Aromatase

The human aromatase enzyme is a member of the cytochrome P450 group and is the product of CYP19A1 gene. Aromatase is bound in the endoplasmic reticulum[27,28] of the cell and works in a heterodimer system composed by two major proteins: cytochrome $P450_{arom}$, which contains a heme group and a steroid binding site, and an ubiquitous flavoprotein NADPH-cytochrome P450 reductase, which transfers reducing NADPH equivalents to cytochrome $P450_{arom}$.

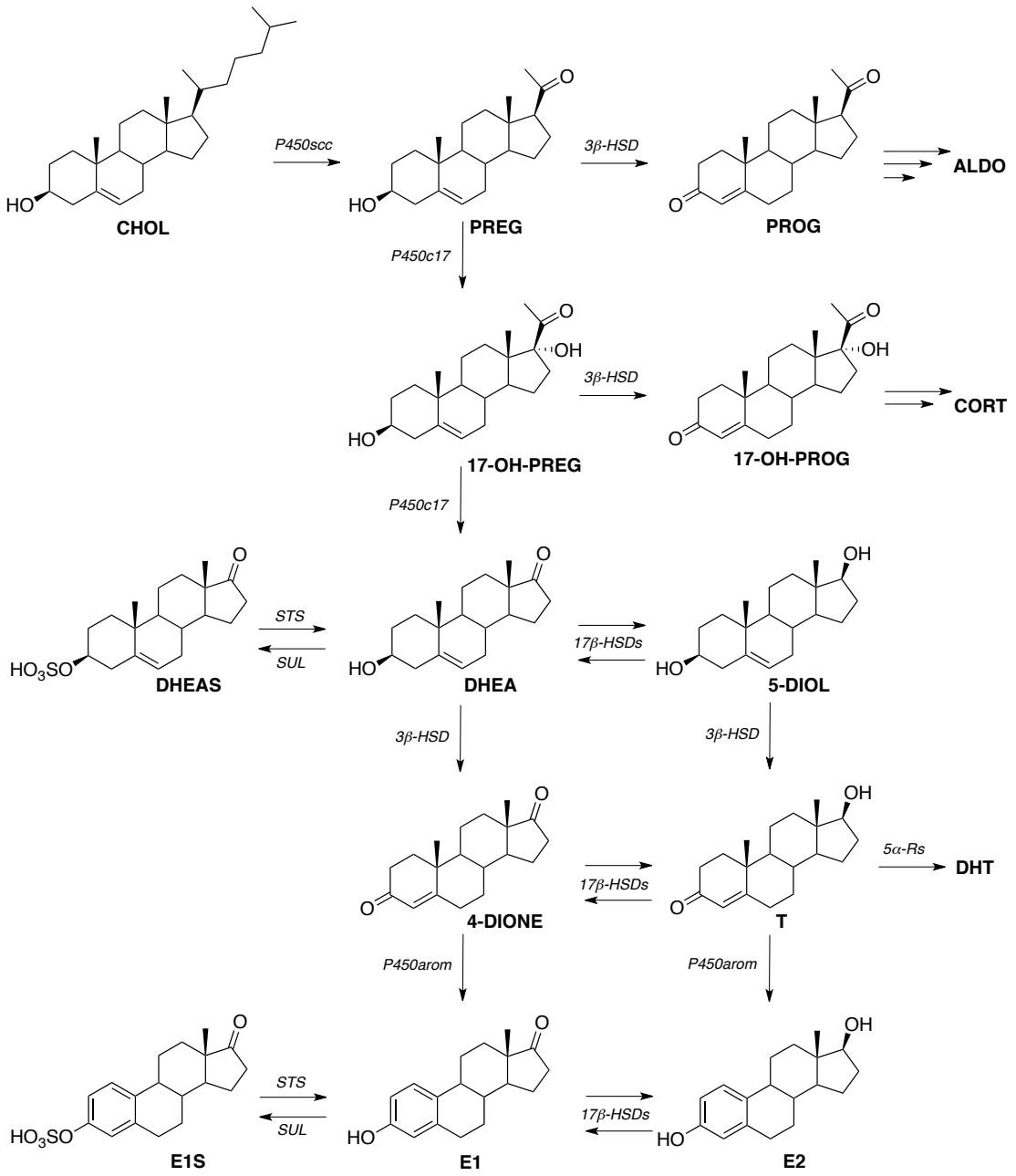


Figure 1.4 - The biosynthesis of steroidal hormones. The enzymes are: cholesterol side-chain cleavage (**P450scc**), 3β-hydroxysteroid dehydrogenase (**3β-HSD**), 17α-hydroxylase/17,20-lyase (**P450c17**), steroid sulfatase (**STS**), steroid sulfotransferase (**SUL**), 17β–hydroxysteroid dehydrogenases (**17β-HSDs**), aromatase (**P450arom**), 5α-reductases (**5α-Rs**). The steroids are: cholesterol (**CHOL**), pregnenolone (**PREG**), 17α–hydroxypregnenolone (**17-OH-PREG**), progesterone (**PROG**), 17α–hydroxyprogesterone (**17-OH-PROG**), aldosterone (**ALDO**), cortisol (**CORT**), dehydroepiandrosterone sulphate (**DHEAS**), dehydroepiandrosterone (**DHEA**), 5-androstene-3β,17β-diol (**5-DIOL**), 4-androstene-3,17-dione (**4-DIONE**), testosterone (**T**), dihydrotestosterone (**DHT**), estrone sulphate (**E1S**), estrone (**E1**), and estradiol (**E2**) (adapted from Poirier, D. **2008**[24])

Aromatase promotes the aromatization of androgens being the only enzyme in vertebrates known to aromatize a six membered ring.[12,28,29] Its substrates are androstenedione (Figure 1.5) and testosterone.

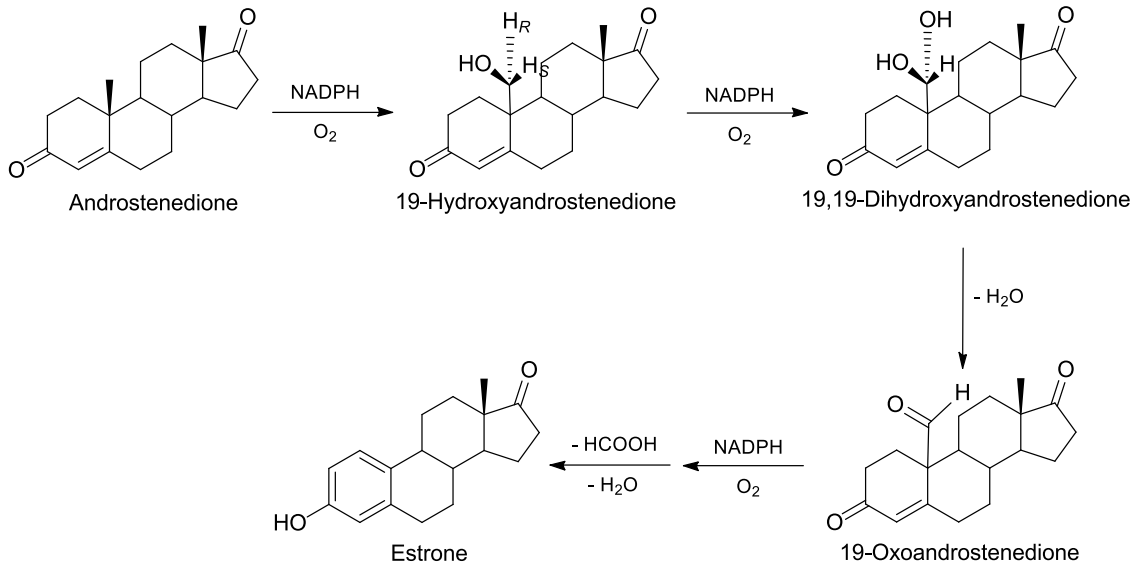


Figure 1.5 - Proposed mechanism for the aromatization of androgens[29]

Aromatization of androstenedione, the preferred substrate, proceeds through three successive oxidation steps, each using one mole of NADPH as an electron donor and one mole of oxygen, in order to convert one mole of substrate into one mole of estrone (Figure 1.5). The first oxidative step is the hydroxylation at the C-19 methyl group to generate a 19-hydroxymethyl function. This is then subjected to a stereospecific second hydroxylation at the C-19 *pro*-R position to provide the C-19 geminal diol, which is the hydrated form of the C-19 aldehyde. In the last step, the C-19 aldehyde derivative undergoes the third final hydroxylation, probably at the 2β position, and aromatizes spontaneously.[30] This last step of the aromatization mechanism is not thoroughly understood.[12,13,26]

Aromatase is present in high levels in a variety of places including ovary, placenta, adipose tissue, testis and skin. Breast cancer tissues have been shown to express aromatase and to produce higher levels of estrogens than non-cancerous cells. This is one of the main reasons why aromatase has generated such a huge interest as a target in the treatment of breast cancer.[12,13]

During the last years, much of the research that was being performed worldwide in order to elucidate the mechanism of aromatase action has relied on site directed mutagenesis experiments with specific aromatase inhibitors. Very recently, in 2009, there was a significant breakthrough in this field since Gosh *et al*.[28] successfully solved the 3D structure of human aromatase, after crystallizing it along with its substrate, androstenedione, hence providing a structural basis for its binding to the natural substrate (Figure 1.6).

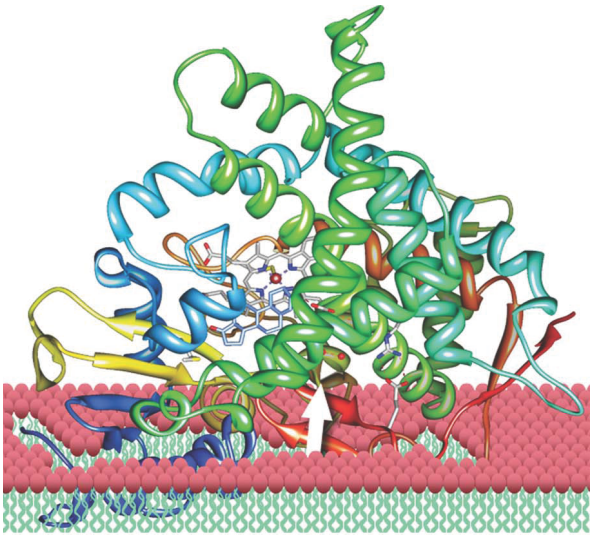


Figure 1.6 - The overall structure of aromatase[28]

From the 3D crystal structure of the enzyme aromatase, it was found that the active site is a distal cavity of the heme-binding pocket and the heme iron atom is the

reaction center of the enzyme. The catalytic site of aromatase is located at the juncture of the I and F helices (Figure 1.7).[31]

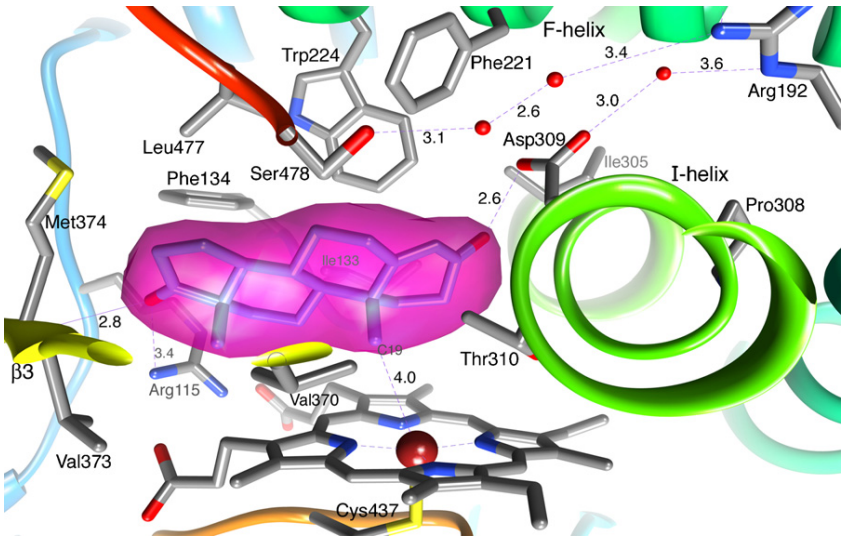


Figure 1.7 - Close-up view of aromatase active site showing the bound androstenedione and some of the amino acids involved[31]

The amino acid residues within the catalytic cleft are Ile305, Ala306, Asp309 and Thr310 from the I-helix, Phe221 and Trp224 from the F-helix, Ile133 and Phe134 from the B-C loop, Val370, Leu372 and Val373 from the K-helix-β3 loop, Met374 from β3, and Leu477 and Ser478 from β8-β9 loop. Concerning the substrate, the 17-keto oxygen of androstenedione makes a hydrogen bond (2.8Å) with the backbone amide of Met374 and a weak contact (3.4Å) with NH1 of Arg115. The 3-keto is 2.6Å from the carboxylate moiety of Asp309 and may be protonated. Androstenedione binds with its β-face oriented towards the heme group and the C-19 of the methyl group positioned at a distance of 4.0Å from the heme Fe atom with which is coordinated.[28,31,32]

The steroid-binding pocket that fits an androstenedione molecule is delineated by a confluence of tight packing hydrophobic residues. The hydrophobic residues and porphyrin rings of heme pack tightly against the steroid backbone. The side chains of residues Arg115, Ile133, Phe134, Phe221, Trp224, Ala306, Thr310, Val370, Val373, Met374 and Leu477 make direct van der Waals contacts with the bound substrate. Ile133, Phe134, Phe221, Trp224 and Leu477 approach the substrate from the α-face and follow the contour and puckering of the steroid backbone, while the side chains of Arg115, Ala306 and Met374 make contacts at its edge, and Thr310, Val370 and Val373 on the β-face. The combined surface creates a pocket that encloses androstenedione very tightly (Figure 1.8).[33] The volume of the binding pocket is estimated to be no more than 400Å^3, which is considerably smaller than in other xenobiotic-metabolizing human P450s.[28,31]

In summary, the crystal structure of aromatase revealed to be a highly tuned molecular machine of estrogen production. This important and recent discovery will be useful in the structure-based design development of new compounds.[28,33]

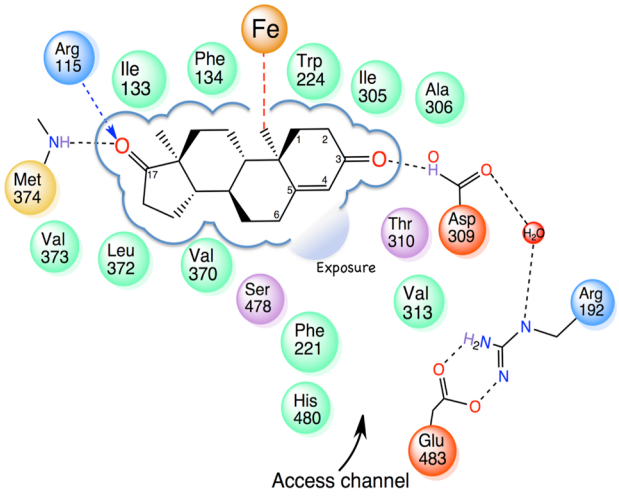


Figure 1.8 - Binding interactions and exposure of ligands to the enzyme interaction spaces with androstenedione[33]

A.4. Breast cancer treatment

Although there have been recent declines in breast cancer mortality rates in some European Union countries, breast cancer still remains of key importance to public health not only in Europe but also worldwide.

The major decision about the treatment to follow is determined by the patient and by the physician, after being considered the biological characteristics of the tumor, the age of the patient and the risks and benefits from each treatment. Therefore, the therapeutic strategies available are: surgery, chemotherapy, radiation therapy, targeted therapy and hormonal therapy.

A.4.1. Surgery

The main objective of breast cancer surgery is the removal of the tumor itself and to assess the stage of the disease. The techniques usually performed are lumpectomy and simple or total mastectomy.

A.4.2. Chemotherapy

There are many factors that influence the outcome of chemotherapy such as the size of the tumor and the number of lymph nodes affected. Drugs like doxorubicin (Adriamycin®) are used to weaken and destroy cancer cells within the body, being also used to shrink cancer that has metastasized. In most cases, combinations of drugs are more effective than one drug alone for breast cancer treatment.[8]

A.4.3. Radiation therapy

This procedure is used to destroy cancer cells that eventually have remained in the breast, chest wall or underarm area after surgery. It might also be needed after mastectomy when cancer is larger than 5 cm in size or when it is dissiminated in the lymph nodes. Radiotherapy might be applied externally or internally, depending on the type, stage and location of the tumor. In the last decades, this therapy has become an accurate targeted process, which allowed reducing side effects and treatment time.[8]

A.4.4. Targeted therapy

In about 15-30% of breast tumors there is an over-production of protein HER2 (Human epidermal growth factor receptor 2), which allows the tumor to grow faster. Monoclonal antibodies that target this protein, like trastuzumab (Herceptin®), offer a survival benefit for women who have breast cancer that overexpress HER2. Hence, all invasive breast cancers should be tested for the gene that encodes the HER2 protein to identify women that would benefit from this therapy.[8]

A.4.5. Hormonal therapy

Many studies have shown that endocrine therapy plays an important role in the treatment of hormone-dependent breast cancer, being used in most of the cases in complement with the above-mentioned approaches. There are two major hormonal treatment modalities to prevent the effects of estrogens in the growth of tumors:[24] (1) the use of estrogen antagonists to block the action of the estrogens on its receptor, and (2) the use of inhibitors of the biosynthesis of estrogens, to reduce their circulating levels (Figure 1.9).

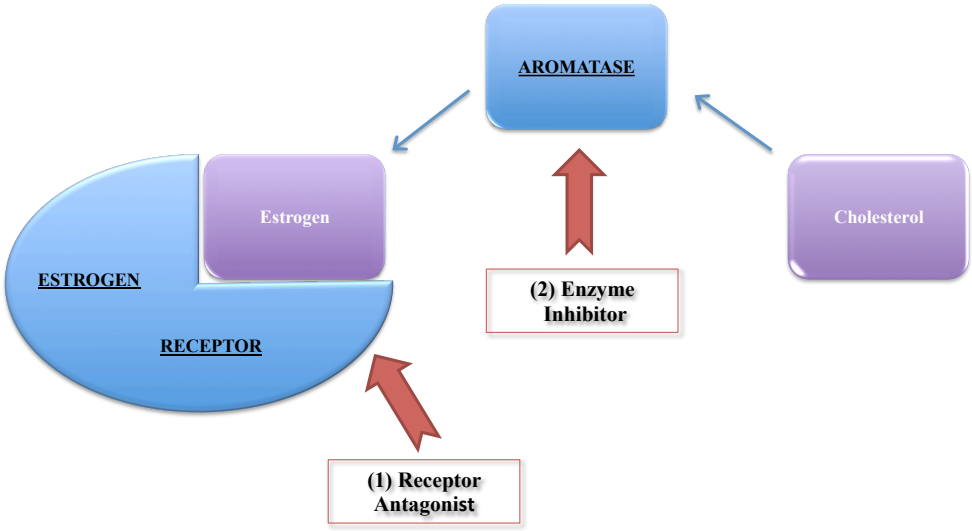


Figure 1.9 - Strategies for hormonal therapy

A.4.5.1. Estrogen Receptor Antagonists

One of the breakthroughs in breast cancer treatment was the discovery of drugs that targeted the ER, also called antiestrogens. These compounds compete for binding to the ER and reduce the number of receptors available for binding to endogenous estrogens. This approach has proven to be very effective in estrogen-dependent breast cancers. There are two types of antiestrogens: SERMs (Selective Estrogen Receptor Modulators) and SERDs (Selective Estrogen Receptor Downregulators). SERMs are chemically diverse compounds but with a conformational similarity that allow them to bind to ER, presenting both selective agonist or antagonist action on several target tissues depending on its content on ER.[34] SERDs block estradiol action and induce receptor downregulation by causing its degradation.

Tamoxifen (Figure 1.10) was the first antiestrogen drug for breast cancer treatment approved in the 1970s and is the SERM most widely used.[13,35] Like other SERMs, Tamoxifen exerts an agonist effect on the ER of some tissues, while acting as

antagonist on others. For this reason, it is quite effective as ER antagonist in the treatment of hormone receptor-positive breast cancer, both in pre and postmenopausal women. However, the agonist effects in the ER of the uterus and vascular system, after prolonged usage, causes an increased risk of endometrial cancer and thromboembolism.[12,36] On the other side, the agonist effect in the bone ER, prevents bone loss and osteoporosis. Other examples of recent SERMs are raloxifene and toremifene, which are structurally related to tamoxifen.[37]

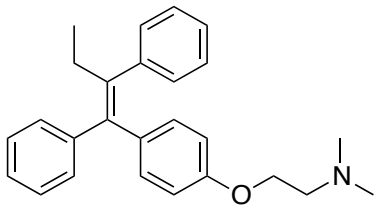


Figure 1.10 - Tamoxifen

Fulvestrant (Figure 1.11) is a SERD compound that has an affinity for the ER approximately 100 times higher than tamoxifen. Besides, it has no estrogen-like activity on the uterus, and has the capacity to block completely the stimulatory activities of both agonists and antagonists, like tamoxifen. The clinical efficacy of fulvestrant has been compared with that of tamoxifen in postmenopausal women with breast cancer, and its efficacy in tamoxifen-resistant breast cancer has also been demonstrated.[37]

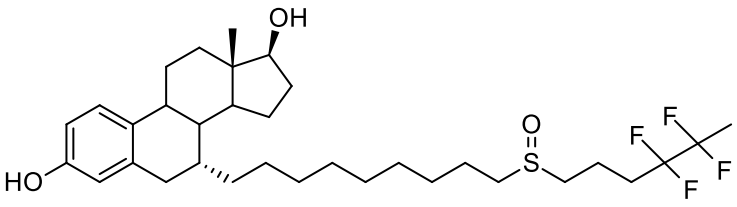


Figure 1.11 - Fulvestrant

A.4.5.2. Aromatase Inhibitors (AIs)

Hormonal treatment has also been focused into the reduction of estrogen levels, which has revealed to be a very successful approach. This is achieved by using aromatase inhibitors (AIs), by blocking the production of estrogens. Investigations on the development of aromatase inhibitors began in the 1970s and have expanded greatly in the past three decades.[13]

AIs do not interact directly with the ER but block the conversion of adrenal androgens into estrogens in peripheral tissues, including the breast tissue.[36]

The evolution of AIs has seen a transition from prototype first generation agents, which were not particularly potent or selective, to the actual third-generation drugs, which are much more effective. This last generation of compounds represent a significant advance in ER-positive breast cancer therapy in postmenopausal women, being approved both for early- and late-stage cases.[38]

AIs are traditionally subdivided into two main classes, according to their structure. Type I inhibitors are associated to the substrate-binding site of the enzyme and invariably have an androgen structure, and are usually referred to as steroidal inhibitors. On the other side, type II inhibitors are "nonsteroidal", and are essentially azole type compounds.[39] All AIs are similar in the way that they inhibit the estrogen synthesis which is by blocking aromatase activity. However, they have distinct mechanisms of action.

The steroidal or type I AIs can be classified as competitive or mechanism-based inhibitors according to the way they promote the inhibition of the enzyme, binding reversibly or irreversibly to its active site. Nonsteroidal or type II inhibitors are mostly competitive inhibitors. Thus, competitive inhibitors are molecules that compete with the substrate for noncovalent binding to the active site of the enzyme, decreasing the

amount of product formed. A mechanism-based inhibitor mimics the substrate, being then converted by the enzyme to a reactive intermediate, which irreversibly binds to the enzyme promoting its inactivation. The term "mechanism based" is used because the inhibitor uses the mechanism of the enzyme to be activated.[13] Other denominations are also used for these inhibitors such as "enzyme-activated irreversible inhibitors", "suicide substrates" and "suicide inactivators", since aromatase is inactivated because of its own mechanism of action.[38]

On the other side, nonsteroidal AIs interact noncovalently with the heme moiety of the enzyme and occupy its substrate-binding site, thereby preventing binding of androgens to the catalytic site. This antagonism is reversible, being these AIs competitively displaced from the active site.[38] The nonsteroidal inhibitors contain a nitrogen heteroatom with a free electron pair that coordinates with the heme iron (Fe^{3+}) mimicking the C-10 methyl group of steroids. Some of these inhibitors are likely to be less enzyme specific and can inhibit other cytochrome P_{450}-mediated hydroxylations, which results in significant toxicity.[35]

The evolution of AIs has seen the development of agents of both type I and type II classes that have progressively increased in both specificity and potency, within each new generation.

First-Generation Drugs

First-generation AIs were used without the knowledge at that time that they had anti-aromatase properties, as was the case of testololactone and aminoglutethimide (Figure 1.12).

Testololactone (Teslac®) was initially used as androgen, based on the conviction that existed at that time, that androgens might counteract the action of estrogens. It is structurally related to testosterone and became available for studies in the early 1960s.[40] Segaloff *et al.*[40] performed the first clinical study and reported on the effectiveness of the drug in advanced breast cancer. It demonstrated a very low rate of toxic effects and did not present virilism or other hormonal activity present in testosterone. It was only in 1975 that Siiteri and Thompson[41] discovered that this compound had aromatase inhibitory activity. It was used for over two decades as a treatment for breast cancer since it was found to inhibit aromatase.[25]

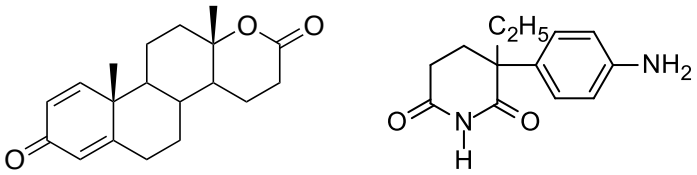


Figure 1.12 - Testololactone (left) and aminoglutethimide (right)

Aminoglutethimide (Cytadren®) was originally an antiepileptic agent that was removed from the market due to serious side effects, namely renal failure.[13,40] It first entered in preliminary clinical trials in advanced breast cancer as a result of the observation that it inhibited adrenal steroidogenesis, being introduced as a form of chemical adrenalectomy.[39]

Aminoglutethimide was the prototype for the nonsteroidal aromatase inhibitors,[40] being referred to as the first-generation AI. Although it was an important AI, it was clear that it was far from being an ideal agent. The drug was only partially effective in supressing plasma estrogen levels, and it lacked specificity since it also

inhibited a number of other steroidogenic CYP-450 enzymes, requiring a concomitant administration of corticosteroids. This resulted in significant toxicity.[13,39]

Second-Generation Drugs

Second-generation inhibitors were developed with greater selectivity and potency than their first-generation counterparts. Among the second-generation AIs that reached the clinic, the most notables were the steroidal, formestane, and the nonsteroidal, fadrozole (Figure 1.13).

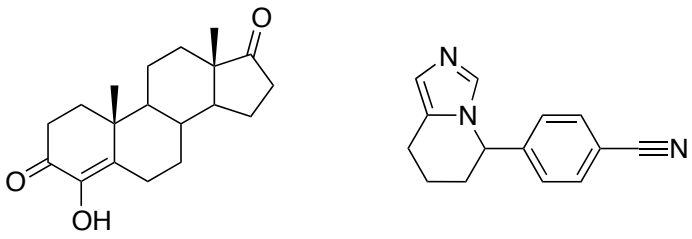


Figure 1.13 - Formestane (left) and fadrozole (right)

Formestane (Lentaron®) was one of about 200 compounds that were specifically designed and assayed as AIs by Harry and Angela Brodie in the 1970s, which revealed to be a potent inhibitor of aromatase.[39,42,43] It is structurally related to androstenedione, the natural substrate of the enzyme, having a hydroxyl group at C-4. Due to this inhibitory activity it was indeed used for breast cancer treatment in postmenopausal women.[44]

Formestane competes rapidly with androstenedione for the active site of the enzyme initiating a time-dependent reactive process which results in either covalent or

very tight binding to the enzyme, causing its inactivation.[42] It appeared to be converted by the enzyme to a reactive intermediate that bound irreversibly to aromatase.[39] This kind of inhibitor presents lasting effects, because new enzyme synthesis is required to overcome this mode of inhibition. Hence, it is not necessary the continued presence of the drug to maintain inhibition and the probability of toxic side effects are reduced.[35,42] Formestane was administrated as a suspension by intramuscular injection because, when given orally, it had poor biological activity due to glucuronidation of the critical 4-hydroxy group through the first-pass liver metabolism, which results in its elimination from the body.[39]

Formestane is about 60-fold more potent than aminoglutethimide.[39] It is also more effective than tamoxifen, and it does not have estrogenic activity on other tissues as tamoxifen.[25]

Fadrozole (Afema®) is the representative compound of second-generation type II inhibitor. This compound is an imidazole analog of aminoglutethimide, but shows higher potency, inhibiting aromatase *in vitro* 200 to 400 times higher.[45] Another interesting difference when compared to the first generation drugs is that, at concentrations that maximally inhibit the enzyme, it had relatively small effects on other cytochrome P_{450}-related enzymes. This means that with fadrazole there is no need for corticoid replacement.[39]

Although fadrozole is a highly potent compound, it has a relatively short half-life, which is responsible for its lower *in vivo* activity when compared with other triazole derivatives, that are cleared more slowly.[46]

Third-Generation Drugs

Among the recent third-generation of AIs, the most significant ones include type I, exemestane, and type II, anastrazole and letrozole (Figure 1.14).

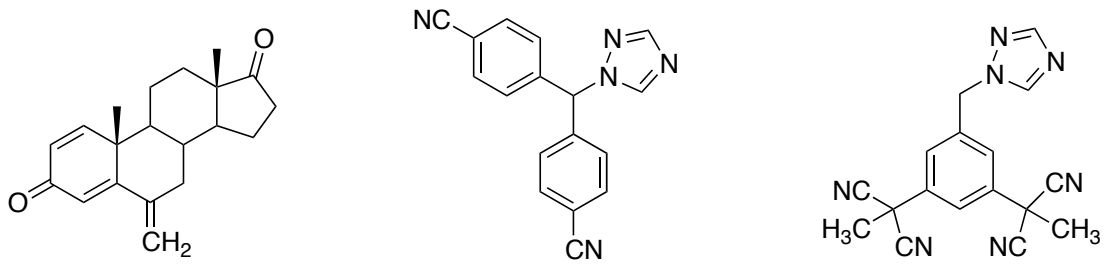


Figure 1.14 - Exemestane (left), letrozole (middle) and anastrazole (right)

These molecules are substantially more potent than aminoglutethimide in inhibiting aromatase activity *in vitro* (Table 1.1). For the first-generation drug it is necessary a micromolar concentration while for the second- and third-generation drugs the active concentration ranges the nanomolar scale. Their superior pharmacokinetic profiles also mean that they are even more effective *in vivo*.[39]

Exemestane (Aromasin®) is an orally active, irreversible steroidal inhibitor developed by Di Salle and Lombardi to overcome the unfavourable metabolism and poor oral availability of formestane.[47,48] Exemestane entered in preclinical development in 1986, and successfully performed clinical trials during the 1990s led to FDA approval in 1999 for the treatment of advanced breast cancer in postmenopausal women.[48] It differs from nonsteroidal inhibitors in that it leads to irreversible inhibition of aromatase, probably by covalent binding to the enzyme. This derivative of

androstenedione is recognized as a false substrate by the enzyme and processed through the normal catalytic mechanism to a transformed product, which covalently binds to and irreversibly inactivates the substrate-binding site of the enzyme, an effect also known as "suicide inhibition".[49] The irreversible nature of its inhibitory activity is responsible for the long-lasting effect (3 to 5 days according to the dose) on estrogen biosynthesis.[48] Treatment with this steroidal compound has been shown to be well tolerated and chronic treatment was found to suppress plasma estrogen levels by 85 to 95% when the drug was administered at a dose ≥10 mg per day.[50] It also shows a great potential to be more effective as first-line treatment than other substances, including antiestrogens and nonsteroidal AIs.[48]

Exemestane undergoes a complex metabolism, being the first step the reduction of the 17-keto group to give the 17β-hydroxy derivative (the primary metabolite to be identified in plasma), followed by the P_{450}-catalyzed oxidation of the 6-methylidene group with formation of many secondary metabolites (Figure 1.15).[53,54] These metabolites were found to be either inactive or less potent than exemestane.[48,53,55-57] However, the 17β-hydroxy derivative was further studied by Goss and collaborators[58] which found that it produced similar effects to exemestane, such as, bone sparing and favorable changes in circulating lipid levels. Buzzetti *et al.* have synthesized some potential metabolites through oxidation of the 6-methylidene group.[47] Recently, some new exemestane metabolites were detected in urine by gas chromatography coupled to mass spectrometry.[53]

Table 1.1 - Comparison of aromatase inhibitory activity of first, second and third generation drugs[39,51,52]

	Placental microsomes *IC_{50} (nM)*	***Breast cancer homogenates*** *IC_{50} (nM)*	***Inhibition***[a)] *(%)*
Aminoglutethimide	3000	4500	90.6
Anastrazole	12	10	96.7
Letrozole	12	2.5	98.9
Formestane	50	30	91.9
Exemestane	50	15	97.9

[a)] Activity determined *in vivo*; drugs were given orally with exception for formestane that was administered intramuscularly.

Nonsteroidal third-generation, anastrazole (Arimidex®) and letrozole (Femara®) are reversible, imidazole-based potent AIs and with high specificity to aromatase. These new agents are 100-3000 times more active than aminoglutethimide, and inhibit whole-body aromatization by greater than 96%.[13] Both agents have shown to cause profound suppression of plasma estrogen levels in postmenopausal women.[59] Since they do not interact significantly with other P_{450} enzymes, no substantial suppression of cortisol, progesterone and aldosterone levels is evident.[13] Besides, they have few side effects and low toxicity when compared with other generation nonsteroidal inhibitors.[35]

Anastrazole, which was the first AI from the third-generation drugs to enter into clinical trials, in an *in vitro* system, revealed to be a potent inhibitor of human placental aromatase (200 times more potent than aminoglutethimide and twice more potent than formestane). Treatment with anastrazole does not change lipid profiles, and studies have

confirmed that it has fewer thromboembolism and ischemic cerebrovascular events, and it does not demonstrate androgenic, progestogenic or estrogenic effects.[60]

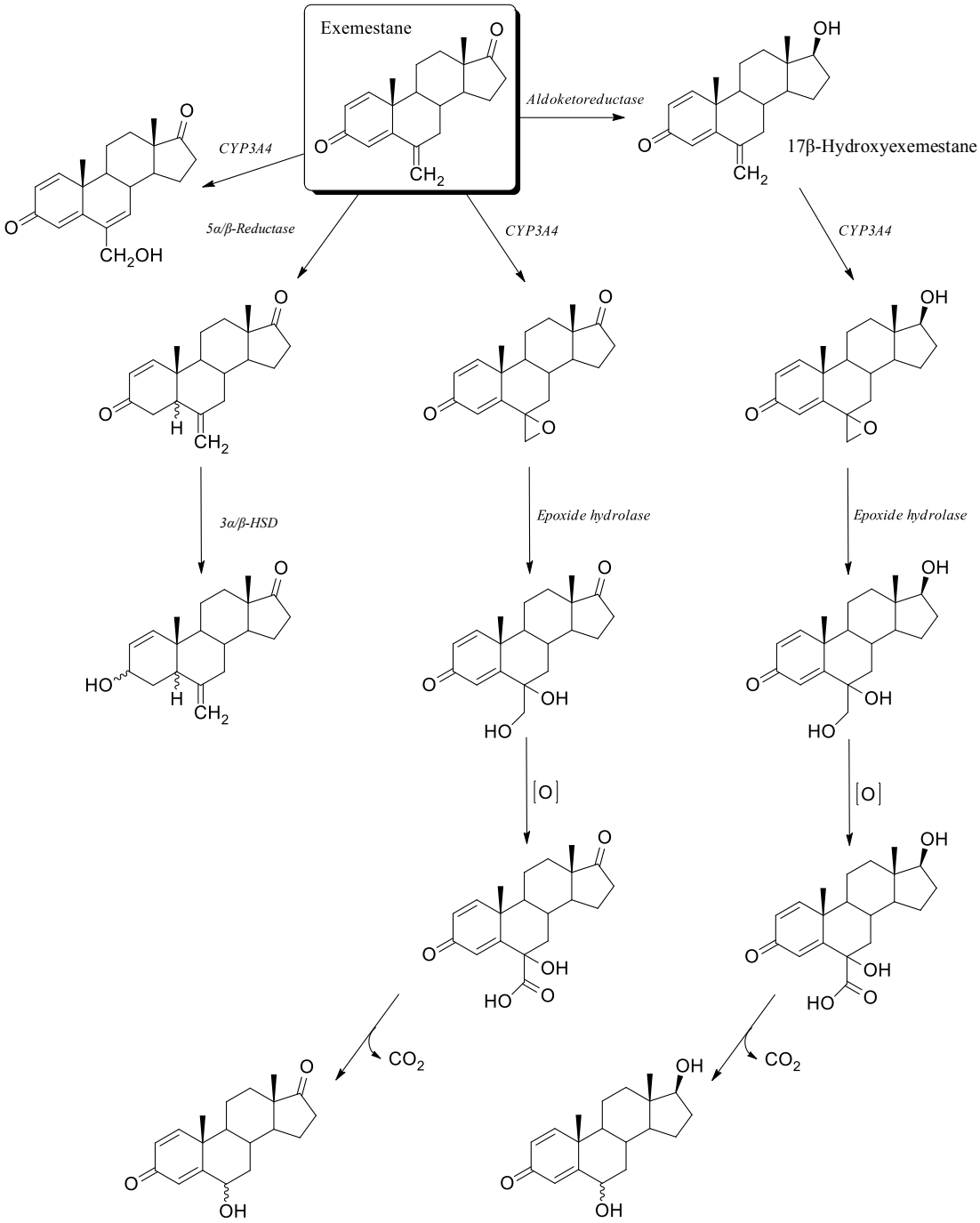


Figure 1.15 - Proposed metabolic pathways for exemestane (3α/β-HSD = 3α/β-hydroxysteroid-dehydrogenase)

Letrozole is more potent and effective, suppressing plasma estrogen concentration to undetectable levels. It has proven to be superior in several aspects to the other drugs having survival benefits and better tolerability.[61] A cross-over study of letrozole and anastrazole revealed that treatment with the first one supressed levels of *in vivo* aromatization below the detection limit of the assays in all patients, while this degree of inhibition with anastrazole was only achieved in one case. Essentially, letrozole achieves a greater aromatase inhibition at its lower dose (0.5 mg, daily) when compared with anastrazole at its higher dose (10 mg, daily).[59,61] This suggests that letrozole might translate into more effective therapy than anastrozole for postmenopausal women.[62]

Clinically, letrozole produces tumor remission in postmenopausal women with breast cancer resistant to other endocrine treatments and chemotherapy.[39]

Third-generation AIs have their efficacy in breast cancer demonstrated, being the treatment of choice for the advanced disease, as well as for the adjuvant setting, the preoperative setting and even with potential in chemoprevention.[63]

Resistance to Aromatase Inhibitors

Whilst substantial number of patients with breast cancer benefit from treatment with AIs, others do not and, even in responding patients, remissions are not usually permanent and disease returns in a resistant form.[64] Overall, the long-term use of antihormonal therapy can result in the development of resistance.[35]

Once AIs play their role by causing estrogen deprivation, many of the mechanisms by which resistance occurs are likely to be shared by other forms of

endocrine deprivation. These mechanisms include the loss of ERs with the treatment,[65] the outgrowth of hormone-intensive cells, the ineffective estrogen suppression and/or endocrine compensation, and a switch to dependence on other mitogens.[39,66]

Irrespective of the cause of the phenotype of aromatase, certain tumors may be more sensitive/resistant to individual AIs. Since steroidal and nonsteroidal AIs have a different mechanism of action, it is not observed cross-resistance. Potential explanations for this lack of cross-resistance include: the nature of the interaction with the enzyme active site, differential sensitivities of aromatase variants to specific compounds, androgen-agonistic effects, and inherent differences in potencies among AIs.[38,67] Furthermore, this lack of cross-resistance between distinct AIs offers the possibility of its sequential use in advanced disease.[36]

Advantages/Disadvantages of Aromatase Inhibitors

Aromatase inhibitors have revealed several advantages over other endocrine therapies, such as:[39]

i. The actions of AIs are not totally irreversible and estrogen levels return to normal on discontinuation of treatment;

ii. AIs specifically decrease the estrogen production, while surgical ablation of endocrine organs will affect the normal production of other steroid hormones. For this reason, AIs have fewer side effects and lower morbidity;

iii. AIs have the potential to totally block estrogen production since biosynthesis is not restricted to endocrine glands but occurs in many other tissues, such as breast cancer.

On the other side, specific AIs also present some disadvantages in the treatment of estrogen-dependent breast cancers:[39]

i. They do not affect estrogens exogenously derived or levels of other types of steroids with estrogenic activity;

ii. They are unproven to be effective in premenopausal women once they cannot stop the ovaries from producing estrogen due to the hypophyseal feedback mechanism;

iii. They increase significantly musculoskeletal symptoms, arthralgia, osteopenia, osteoporosis and fracture rate, since estrogens have a major role in the maintenance of bone mass;[12,25]

iv. Although no significant changes in cardiovascular events have been described, a trend toward its increase is of concern;[25]

v. They may induce cellular resistance, by long-term estrogen deprivation.[25,35]

AIM OF THE WORK

Recent data has shown significant success in improving the survival rates among breast cancer patients by using aromatase inhibition therapy. This can be explained by the more insightful choice of the appropriate drugs for each patient, based on the clinical information available and by using more potent drugs, such as exemestane, anastrazole and letrozole.

Third-generation AIs appear to be extremely potent and highly specific inhibitors of the enzyme, showing strong anti-tumor effects in postmenopausal women, and being well tolerated. However, they still present some side effects, such as increase of bone loss, joint pain and cardiac events.[68] In addition, after some years of usage they can develop cellular resistance.[51] For these reasons it is necessary to discover new drugs that allow to surpass the referred limitations. These new molecules can also allow studying the mechanism of enzyme inhibition and can help to clarify the aromatization mechanism of aromatase, which still remains unknown.

The recent elucidation of the 3D-structure of aromatase and the establishment of the molecular basis of the enzyme-substrate interaction[36] allowed us a more rational design of AIs. The design, synthesis and biological study of the new substrate- and structure-based inhibitors in this thesis would greatly contribute to understand the interactions of the enzyme with the inhibitors and to establish new SAR.

Based on this, the aim of the work was to design and synthesize new steroidal AIs for subsequent biochemical and biological evaluation, and in some cases to do molecular modelling studies, in order to establish new and solid SAR.

1.1. DERIVATIVES OF EXEMESTANE

1.1.1. Design and synthesis

Exemestane (**3**) (Scheme 1.1), chemically described as 6-methylenandrosta-1,4-diene-3,17-dione, is the only steroidal AI in the market being orally active, long-lasting and safe for the treatment of hormone-responsive breast cancer in postmenopausal women.[48]

As described before, exemestane is extensively metabolized (Figure 1.15). Frequently, these metabolites are still active compounds and there is a huge interest in studying them biochemically and biologically. Hence, we were interested in synthesizing some selected exemestane derivatives. By this reason, our first challenge began with the synthesis of exemestane itself.

Synthesis of Exemestane

Buzzetti *et al.*, in the US patent 4,808,616, disclosed the synthesis of exemestane for the first time in 1989.[69] They considered its preparation by dehydrogenation of 6-methylenadrosta-4-ene-3,17-dione using 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) and anhydrous dioxane as solvent. The starting material 6-methylenadrosta-4-ene-3,17-dione was prepared by reaction of androstenedione with formaldehyde acetal and phopshoryl chloride, according to the general method of Annen *et al.*[70]

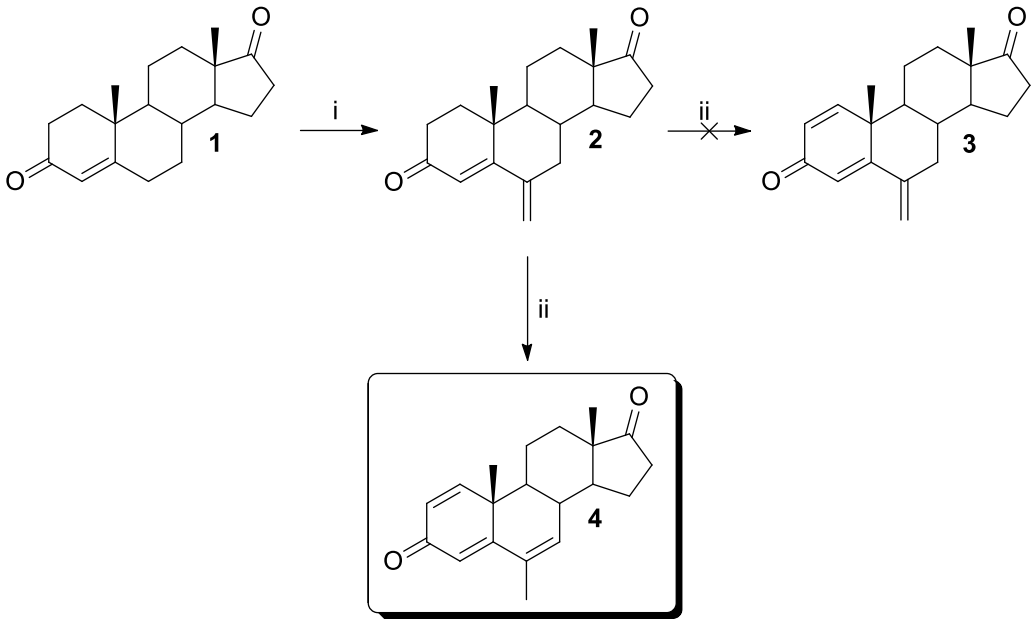
Other synthetic strategies were disclosed in order to overcome the disadvantages and drawbacks of this method, specially the low yields and high price of DDQ as well as purification processes, which were not affordable in a scaled up synthesis. Therefore,

a practical approach, using the commercially available dehydrotestosterone, disclosed a curious *Mannich* reaction on a cross-conjugated dienone moiety by directly introducing the methylidene group at the C-6 position using, for this matter, paraformaldehyde and dimethylamine in isoamylic alcohol. Exemestane was then obtained after the *Jones* oxidation of the 17β-hydroxyl group of its precursor.[48,71] Another strategy developed also made use of androstenedione as starting material and it also involved the intermediate 6-methylenadrosta-4-ene-3,17-dione, but using other reagents to prepare it, and being followed by three other reactions until the production of exemestane.[48] Other groups explored other approaches studying the influence of an acid catalyst with the dehydrogenation agent,[72] and others even studied this approach using also at least one co-oxidant in the dehydrogenation reaction.[73,74]

In this thesis, we decided to attempt the synthesis of exemestane (**3**) using a two-step strategy (Scheme 1.1).[69] Hence, we used androstenedione (**1**) as the starting material to which the 6-methylidene group was introduced. We followed the method developed by Annen *et al.*,[70] who succeeded in performing this transformation in an one-step reaction overcoming the former synthesis that had many steps. Once prepared the 6-methylidene derivative (**2**), a subsequent dehydrogenation using DDQ[47] was made in order to achieve exemestane (**3**). However, in our hands we did not succeed in this step ending up by obtaining compound **4** (Scheme 1.1). Marcos-Escribano *et al.*[74] have also reported the same problem we encountered. In general, the 1,2-dehydrogenation of steroidal 4-en-3-ones with DDQ predominates in the presence of weak acids, or in uncatalyzed reactions, while the 6,7-dehydrogenation occurs in the presence of strong acids. In fact, the reaction of androstenedione (**1**) with DDQ in benzene or dioxane, under reflux, leads to the 1,2-dehydrogenated derivative as the major one.[74] However, when these conditions were applied to compound **2** the reaction proceeded with the

desired 1,2-dehydration but, besides that, it was also observed an isomerization of the exocyclic C-6 double bond forming the most stable conjugated system leadind to derivative **4**.

Scheme 1.1 - Attempt to synthesize exemestane (**3**) from androstenedione (**1**)



Reagents and Conditions: (i) anhydrous sodium acetate, formaldehyde dimethyl acetal, phosphoryl chloride, anhydrous chloroform, reflux, 10 h; (ii) DDQ, anhydrous dioxane, reflux, 11 h 10 min.

The structure of compound **2**, which is a key synthetic precursor of exemestane, was characterized by X-ray analysis and its ORTEP view is shown in Figure 1.16.[75] Results from the single-crystal diffraction revealed that it adopts a 1α-sofa conformation, slightly distorted towards a 1α,2β-halfchair. The pseudo-torsion angle C19-C10$^{\cdots}$C13-C18 indicates that the structure of **2** is slightly twisted. It was observed that the 6-methylidene group is in a beta equatorial position with an angle of 63.8(2)º relative to the plane of the molecule.

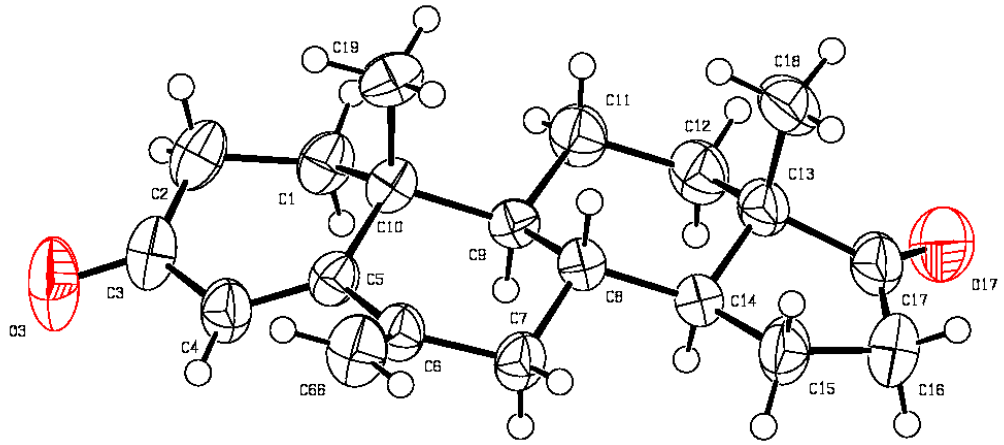


Figure 1.16 – ORTEP view of the molecular structure of compound **2** obtained by X-ray analysis[75]

Synthesis of Derivatives of Exemestane

Due to the huge clinical interest of exemestane and since it is extensively metabolized being some of the metabolites active, we decided to synthesize some epoxide derivatives that are proposed as potential metabolites in the metabolic pathways for exemestane.[48,53-55] Besides, we have already observed for other potent AIs that the substitution of double bonds by epoxide functions, which have similar bond geometries, allowed the molecule to maintain planarity and to exhibit strong aromatase inhibitory activity.[76,77] Because of this, we have synthesized the epoxide derivatives **5** and **6** of exemestane.

Two different reaction conditions for the epoxidation reaction were explored in order to obtain distinct epoxide derivatives of exemestane (Scheme 1.2).

For the epoxidation of the 6-exomethylidene group, we subjected exemestane to treatment with performic acid generated *in situ*.[78] Buzzetti *et al.*[47] also synthesized compound **5** using different reaction conditions (*m*-chloroperbenzoic acid). For the

oxidation of the C-1/C-2 double bond we used alkaline hydrogen peroxide,[79] which allowed obtaining the respective epoxide **6**.

We have also been interested in preparing a 3-deoxy-derivative based on the fact that formerly our team has concluded that the presence of the carbonyl group at C-3 was not mandatory to bind the inhibitors to the enzyme.[80] With this idea in mind, we subjected exemestane to the reaction conditions of Hanson *et al.*[81] using sodium borohydride in a mixture of trifluoroacetic acid, acetonitrile, acetic acid and dichloromethane to promote the deoxygenation of the C-3 carbonyl group. This reaction allowed the preparation of compound **8** along with compound **7**, which is the main *in vivo* metabolite of exemestane (Scheme 1.2).

1.1.2. Chemistry

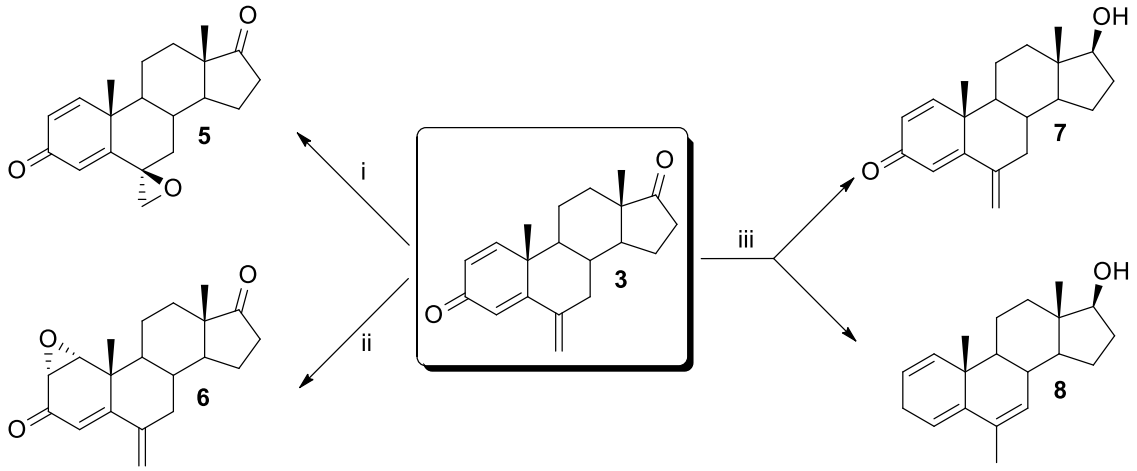
Androstenedione (**1**) (Scheme 1.1) was prepared through the oxidation of testosterone with a solution of chromium trioxide in aqueous sulphuric acid (*Jones* reagent), as reported by Rasmusson and Arth,[82] in 98% yield.

The direct γ-alkylenation of enone **1** was achieved by reaction with phosphoryl chloride and formaldeyde dimethyl acetal.[70] After 10 h at reflux, a mixture of several products was formed, being compound **2** isolated by silica gel column chromatography, in 17% yield.

Compound **2** (Scheme 1.1) was then subjected to a reaction with DDQ in order to promote the dehydrogenation at C-1/C-2,[47] and therefore produce exemestane (**3**). The reaction conditions allowed indeed the dehydrogenation at the mentioned position of the A-ring. However, they also promoted the isomerization of the 6-methylidene

double bond with formation of compound **4**, which was isolated in the pure form by silica gel column chromatography, in 98% yield, as a white solid.

Scheme 1.2 - Synthesis of derivatives of exemestane (**3**)

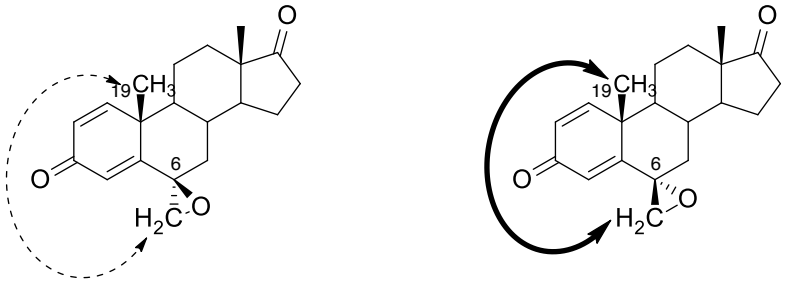


Reagents and Conditions: (i) H_2O_2, HCOOH, dichloromethane, rt, 96 h; (ii) H_2O_2, NaOH, methanol, rt, 24 h; (iii) CF_3COOH, CH_3COOH, CH_3CN, $NaBH_4$, anhydrous dichloromethane, rt, 11 h.

The synthesis of compound **5** (Scheme 1.2) was made by treating exemestane (**3**) with performic acid in dichloromethane, at room temperature, for 96 h.[78,80] The crude mixture was then purified by column chromatography affording the desired epoxide **5**, in 21% yield. In other fraction, a mixture of **5** with its 6α-isomer was obtained in a 65:35 proportion, respectively.

Although the C-6 epoxide derivative of exemestane is already described, its stereochemistry had not been established. Therefore, two-dimensional NOESY experiments were used to assign the C-6 stereochemistry of compound **5**. The most significant correlation to be studied was between the H-atoms of groups C-6 -O-CH_2 and 19-CH_3 (Figure 1.17). For the 6α-isomer it was observed a strong correlation

between the H-atoms of the C-6 -O-CH_2 and 19-CH_3 groups, which implies that these H-atoms are spatially close enough to correlate. Hence the C-6 -O-CH_2- will be pointing towards the beta face of the molecule being the epoxide functional group with alpha stereochemistry. For the 6β-isomer **5**, it was not observed any correlation between the C-6 -O-CH_2- and 19-CH_3 H-atoms. This observation together with the study of the chemical shifts for the 19-CH_3 H-atoms, which are shifted downfield when compared with that of the 6α-isomer, due to the higher influence of the electronegativity of the oxygen atom, allows one to conclude that the more abundant and isolated compound is the 6β-isomer **5**.



6β-Epoxide isomer (compound **5**) **6α**-Epoxide isomer

Figure 1.17 - Compound **5** (6β-isomer) and its 6α-isomer showing NOESY correlations (dashed line – absence of correlation; full line – strong correlation)

The synthesis of epoxide **6** (Scheme 1.2) was performed by treatment of a methanol solution of exemestane (**3**) with an alkaline oxidizing solution of 35% hydrogen peroxide in a 4 N sodium hydroxide solution.[79] The reaction was carried out at room temperature during 24 h and the crude product was purified by column chromatography affording the desired epoxide **6** in 11% yield.

Despite the reaction has not been complete, only compound **6** was detected.

NOESY experiments were also used to unequivocally identify compound **6** (Figure 1.18). The most significant signal analyzed was the correspondent to the resonance of 1-H atom at 3.58 ppm. It was observed a strong correlation between this H-atom and the H-atoms of group 19-CH_3, which reveals that the 1-H must be assuming the beta configuration allowing an enhanced spatial proximity towards the C-19 angular methyl group (Figure 1.18). Hence, the isolated isomer will be the 1α,2α-epoxide compound **6**.

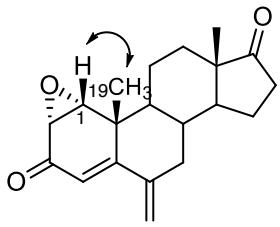


Figure 1.18 - Compound **6** showing NOESY correlations (full line – existence of correlation)

The reaction of exemestane (**3**) (Scheme 1.2) with a mixture of sodium borohydride in trifluoroacetic acid, glacial acetic acid and acetonitrile was performed in a nitrogen atmosphere.[81] It led to a mixture of several compounds, being **7** and **8** the two main products, which were isolated through column chromatography. Compound **7** was isolated in higher yield (39%) as a pure white solid residue and compound **8** was isolated in 12% yield.

As the ^{1}H NMR studies did not allow to unequivocally elucidate the position of the double bonds within the A-ring in compound **8**, two-dimentional COSY experiments were performed. The most relevant signals analyzed in COSY spectrum were: the signal of the H-atoms from -CH_2 group in C-3 at 2.77 ppm and the signals of the H-atoms of the double bonds at 5.66 ppm, 5.70 ppm and 5.92 ppm (Figure 1.19).

The analysis of the spectrum showed a significant correlation between the two H-atoms of the C-3-methylidenegroup and the H-atoms resonating at 5.66 and 5.70 ppm, and a weaker correlation with the H-atom resonating at 5.92 ppm. This correlation pattern is consistent with the structure of compound **8** (Figure 1.19).

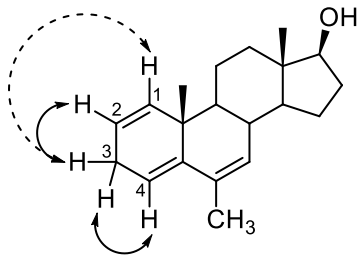


Figure 1.19 - Compound **8** showing COSY correlations (dashed line – weak correlation; full line – strong correlation)

1.1.3. Biochemistry and biology

Inhibition of aromatase activity by the synthesized steroids (**5**, **6** and **7**) was evaluated in human placental microsomes by a radiometric assay in which tritiated water, released from [1β-^{3}H] androstenedione into the incubation medium, was used as an index of estrogen formation.[83] A screening assay was performed in human placental microsomes and the results obtained are shown in Table 1.2 as a percentage of inhibition (%). The assay was performed for all compounds at 2 μM, against an assay carried out in the absence of the inhibitor. The aromatase inhibitor formestane at 0.5 μM concentration (99.65 ± 0.06%) was used as reference.[84,85]

The IC_{50} of these compounds was also evaluated in MCF-7aro cells, an ER^+ aromatase-overexpressing human breast cancers cell line, using the same radiometric assay, as reported in previous works of our group.[84,85]

In addition, cell proliferation and cell viability were evaluated, also in MCF-7aro cells, according previous works of our group.[84,85] All derivatives revealed to promote a decrease in both cell proliferation and cell viability. However, compound **6** promotes this effect in a more efficient way, even surpassing exemestane itself (unpublished results).

Table 1.2 - Percentage of aromatase inhibition and IC_{50} values of tested compounds in human placental microsomes *a)* and in MCF-7aro cells *b)*

Compounds	Aromatase Inhibition[a)] (%) ± SEM	IC_{50}[a)] (μM)	IC_{50}[b)] (μM)
5	93.64 ± 0.89	0.67	0.70
6	86.78 ± 0.99	1.04	0.75
7	-	-	4.20
Formestane (9)	99.65 ± 0.06	0.042	-
Exemestane (3)*	-	0.027	0.90

*Results for the IC_{50} of exemestane (**3**) described in the literature.[47]

1.1.4. Structure-activity relationships discussion and conclusions

As pointed out before, exemestane is metabolized *in vivo* into active compounds. In this work, it were synthesized some potential metabolites of exemestane by substituing the double bonds of the A- and B-rings by epoxide groups (compounds **5** and **6**) and by reducing the C-17 carbonyl group to a hydroxyl group (compound **7**). Looking at compounds **5** and **6** (Figures 1.17 and 1.18 and Table 1.2), we observed that the substitution of the double bond, both in the C-6 exocyclic and in C-1/C-2 position,

by the epoxide group, led to derivatives less potent in microsomes but even more potent in MCF-7aro cells than exemestane (**3**), being the exocyclic substitution (compound **5**) slightly more potent, with an IC_{50} of 0.67 μM, in microsomes, and 0.70 μM in MCF-7aro cells, than the substitution at C-1/C-2 (compound **6**), with an IC_{50} of 1.04 μM and 0.75 μM, in microsomes and MCF-7aro cells, respectively. In these two molecules it was maintained the integrity of the C-17 carbonyl group, which is known to be important to reach maximum aromatase inhibitory activity. In compound **7**, this group was transformed into a hydroxyl group and a significant decrease in activity was observed (IC_{50} of 4.20 μM) in MCF-7aro cells. This decreasing of activity was already observed in other aromatase inhibitors for the same transformation. We believe that, contrary to what happens with the carbonyl group, the hydroxyl group does not allow the establishment of the hydrogen bond with Met374 residue of the active site of the enzyme (Figure 1.7), which is essential for a strong inhibitory activity.

When it was studied the cell viability and cell proliferative effects of the synthesized molecules (**5**, **6** and **7**), we observed that they induced a decline in cell viability and proliferation in a more efficient way than exemestane. When the two epoxide inhibitors were compared, we observed that compound **6**, although slightly less potent in inhibiting aromatase than **5**, was the most effective derivative in reducing the cell viability and proliferation. This reveals that other mechanisms of cell death than aromatase inhibition may be involved, requiring further studies to elucidate the biological effects observed (unpublished results).

1.2. DERIVATIVES OF FORMESTANE

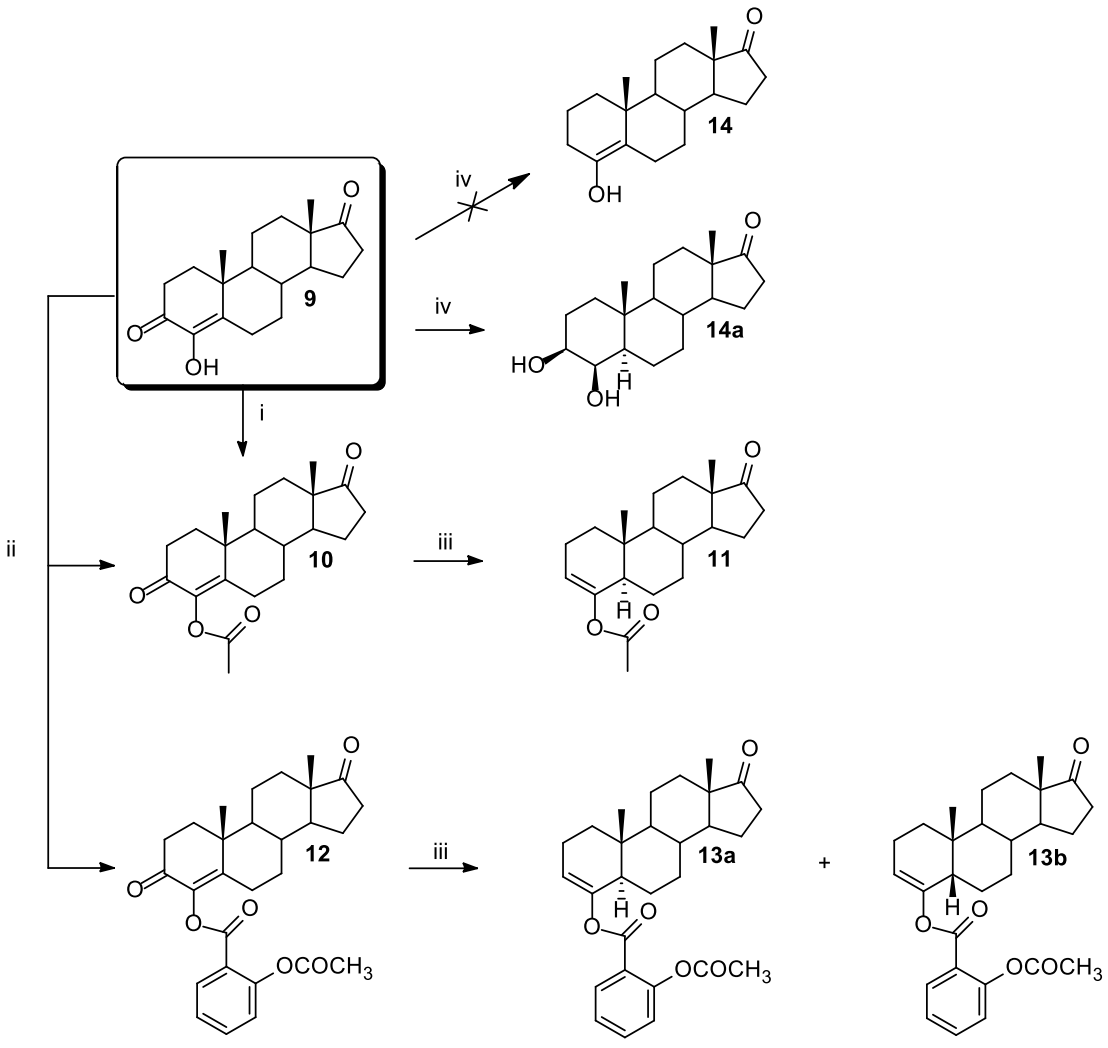
1.2.1. Design and synthesis

Formestane (**9**) (Scheme 1.3), chemically described as 4-hydroxyandrost-4-ene-3,17-dione, was the first selective aromatase inhibitor to become available in the clinic. Due to the high activity of this molecule and to the fact that its C-4 acetoxy derivative have also revealed to be a strong aromatase inhibitor,[43] we decided to prepare several other C-4 ester derivatives related to formestane in order to study their activity against aromatase (Scheme 1.3).[87] Moreover, an interesting feature related to breast cancer concerns the existence of an elevated expression of the enzyme cyclooxygenase 2 (COX-2) in the tumor tissues. Based on this, it was hypothesized that the inhibition of COX-2 might decrease carcinogenesis by decreasing cell proliferation, angiogenesis and metastasis and increasing apoptosis.[86-88] Further, epidemiologic studies suggest that the use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid, protects against breast cancer development. Hence, we felt that it would be interesting to develop C-4 acetoxy and acetylsalicyloxy derivatives of formestane (**9**) (Scheme 1.3).[76] In the case of the C-4 acetylsalicyloxy derivative, we intended to combine in the same steroidal structure, the dual capacity of aromatase and COX-2 inhibition. Hence, the acetyl derivatives **10** and **12** were prepared through classical acetylation conditions from the corresponding alcohols using the respective acyl chloride.

Based on the rationale that the presence of the carbonyl group at C-3 is not mandatory to bind the molecule to the enzyme,[80] we were also interested in preparing the corresponding C-3 deoxyderivatives **11**, **13a** and **14**. For this, compounds **10** and **12** were submitted to a *Clemmenson*-type reduction with zinc dust in acetic acid solution. This allowed to obtain, respectively, compounds **11** and **13a** in mixture with **13b**. In

order to achieve compound **14**, we subjected formestane to the reaction conditions of Hanson *et al.*[81] where it was used sodium borohydride in a mixture of trifluoroacetic acid, acetonitrile, acetic acid and dichloromethane to promote the deoxygenation of the C-3 carbonyl group. However, this only allowed to isolate **14a**.

Scheme 1.3 - Synthesis of derivatives of formestane (**9**)



Reagents and Conditions: (i) acetyl chloride, anhydrous pyridine, rt, 21 h 50 min; (ii) *o*-acetylsalicyloyl chloride, anhydrous pyridine, rt, 24 h 40 min; (iii) Zn dust, acetic acid, rt; (iv) CF_3COOH, CH_3COOH, CH_3CN, $NaBH_4$, anhydrous dichloromethane, rt, 45 min.

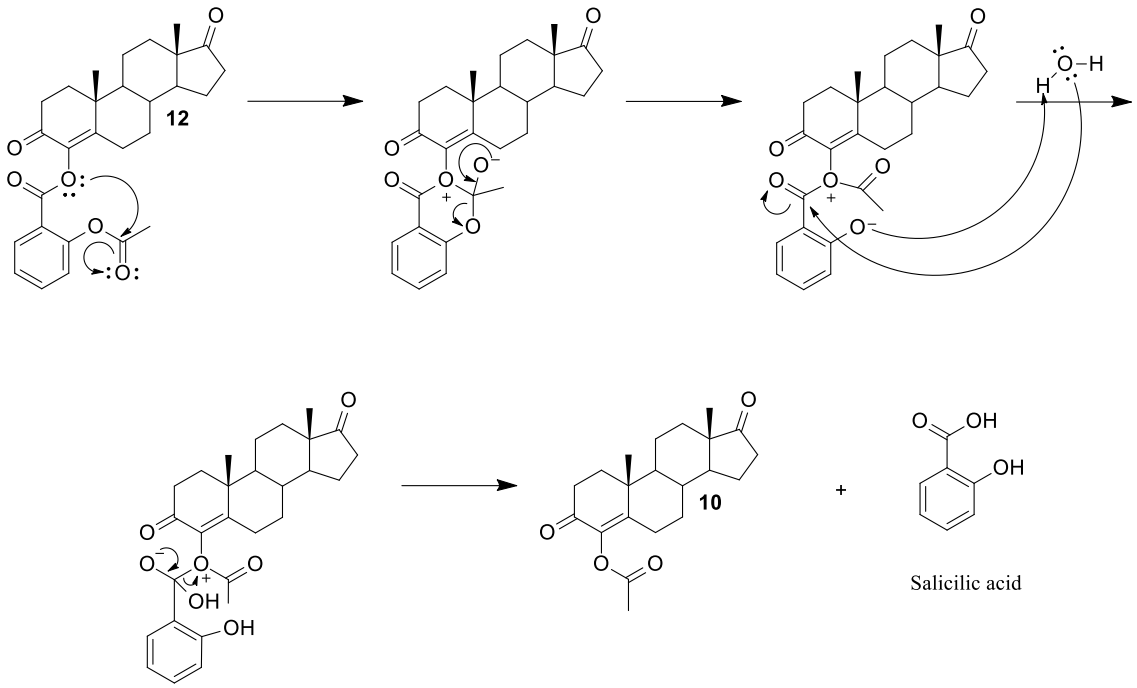
1.2.2. Chemistry

Compound **9** was treated with acetyl chloride in anhydrous pyridine leading, after crystallization, to the pure compound **10**, in 72% yield. Subsequently, compound **10** was treated with zinc dust in acetic acid solution leading to a mixture of 5α- and 5β-epimers, which after crystallization with petroleum ether allowed the isolation of the desired pure 5α-epimer, compound **11**.

Treatment of **9** with *o*-acetylsalicyloyl chloride in anhydrous pyridine led, after purification by column chromatography, to a main fraction (one single TLC spot), which after NMR analysis revealed to be composed by a mixture of compounds **10** and **12**, in a 40:60 proportion, respectively. The formation of compound **10** probably resulted from an interestingly intramolecular transesterification occurred in **12**, according to the proposed mechanism (Scheme 1.4). This may involve the formation of a cyclic intermediate, which then opens through alkaline catalysis leading to formation of **10** and salicilic acid. Further purification of this mixture by another column chromatography, using a different mixture of solvents, allowed the isolation of the pure compound **12**. Treating **12** with zinc dust in acetic acid solution led to an inseparable epimeric mixture of **13a**, and its 5β-epimer (**13b**) in a 70:30 proportion, respectively (NMR analysis).

The reaction of formestane (**9**) with the reductive mixture of sodium borohydride in trifluoroacetic acid, glacial acetic acid and acetonitrile was performed under a nitrogen atmosphere, in order to attempt the preparation of the 3-deoxy derivative **14**.[81] However, instead of **14**, a mixture of compounds was obtained from which **14a** was isolated through a column chromatography as a white solid, in 65% yield.

Scheme 1.4 - Proposed mechanism for the intramolecular transesterification of **12** to obtain derivative **10**



1.2.3. Biochemistry

Inhibition of aromatase activity by the synthesized steroids **11** and **12** was evaluated in human placental microsomes as mentioned formerly (Section 1.1.3). A screening assay was performed and the results obtained are shown in Table 1.3 as a percentage of inhibition (%).[76]

1.2.4. Structure-activity relationships discussion and conclusions

As pointed out previously, some recent studies suggest that the use of NSAIDs, such as acetylsalicylic acid, protects against breast cancer development.[86-88]

Additionally, the C-4 acetoxy derivative **10** of formestane (**9**), the first steroidal AI clinically used also revealed to be a strong inhibitor.[89] In this manner, a new AI (compound **12**) based on **9** was designed and synthesized, by esterification of its C-4 hydroxyl group with the acetylsalicylic acid moiety. However, the C-4 acetylsalicyloxy derivative **12** showed a pronounced decrease in aromatase inhibition when compared with its precursor **9**.

Table 1.3 - Percentage of aromatase inhibition of tested compounds in human placental microsomes[76]

Compounds	Aromatase Inhibition (%) ± SEM
10*	81
11	33.90 ± 1.68
12	68.73 ± 3.53
Formestane (9)	99.65 ± 0.06

*Data for compound **10** described in the literature.[89]

The results obtained suggest that the presence of bulky substituents in C-4 diminishes the aromatase inhibitory activity, which is consistent with the short volume of the binding pocket of aromatase. The C-4 acetoxy derivative **11** also showed a dramatic reduction in the aromatase inhibitory activity (Table 1.3), when compared with that of **10** showing that the C-3 carbonyl group would be potentially important, in this case. This decrease in activity can be also due to the displacement of the 4,5 double bond to the 3,4 position, reinforcing the importance of having planarity in the A,B-ring

junction at C-5 as previously observed by our group.[76,80,90] This hypothesis might be corroborated when we compare **11** with the C-4 acetoxy derivative of Δ^4-olefin (Figure 1.20), which is a strong competitive inhibitor (IC_{50} = 0.66 μM).[77,91] It is also interesting to compare the activity of **11** with that of Δ^3-olefin **27a** (Scheme 1.6 – Section 1.3.2). We observed a decrease in the activity when we go from Δ^3-olefin **27a** to its C-4 acetoxy derivative **11**. In this case, the introduction of a C-4 acetoxy group did not maintain the aromatase inhibitory activity, in contrary to that observed for **10** relatively to **9**.

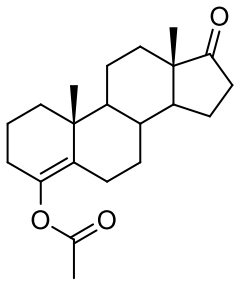


Figure 1.20 - Structure of 4-acetoxyandrost-4-en-17-one

Further studies in COX-2 inhibition were not performed due to the weak aromatase inhibitory activity of compound **12**.

1.3. A- AND D-RING MODIFIED DERIVATIVES OF ANDROSTENEDIONE

1.3.1. *C-3 Hydroxyl Derivatives of Androstenedione and Testosterone*

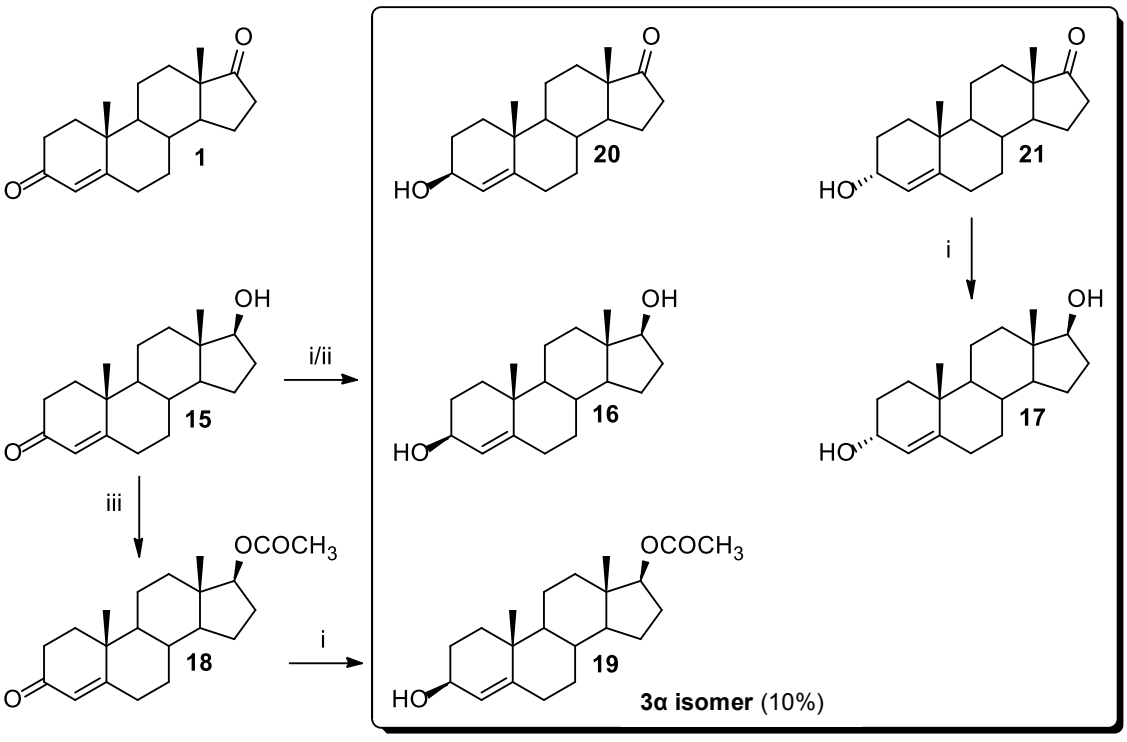
1.3.1.1. Design and synthesis

After the elucidation of the active site of the enzyme aromatase and the establishment of the structural bases of the enzyme-substrate interaction,[28] a more rational design of new AIs was allowed. Among other interactions, aromatase establishes two main hydrogen bonds with the oxygen atoms of the carbonyl groups at C-3 and C-17 of androstenedione.[28] From a previous study, it was concluded that the presence of the carbonyl group at C-3 is not absolutely necessary to allow the binding of steroid molecules to the enzyme.[80] Other authors described strong aromatase inhibitors without the C-17 carbonyl group.[92] Recently, our group also confirmed those facts and postulated that it is necessary, at least, one carbonyl group (C-3 or C-17) in order to allow the binding of steroid molecules to aromatase.[90]

In this work, we were interested in studying steroid molecules as aromatase inhibitors in which the carbonyl group at C-3 (a hydrogen bond acceptor) of androstenedione, was replaced by a hydroxyl group (a hydrogen bond donor) and also in which the two carbonyl groups (C-3 and C-17) were replaced by two hydroxyl groups. The design of these compounds was based in the structure of the substrates of the enzyme, androstenedione and testosterone (substrate-based design), and in the interactions of these substrates with the enzyme. The effect of the stereochemistry of the hydroxyl group at C-3 was also explored (Scheme 1.5). For this purpose, we studied reduction conditions of the carbonyl group by using two different hydride reagents, sodium borohydride and lithium tri-*t*-butoxy aluminum hydride, in order to obtain

compound **16** from compound **15**. Compound **17** was in turn prepared from the commercially available compound **21**. Reduction of testosterone acetate derivative **18**, with sodium borohydride gave an isomeric mixture of 3α (10%) and 3β (compound **19**). The effect of different functional groups at C-17, such as carbonyl (compound **20**), hydroxyl (compound **16**) and acetyl (compound **19**), was also studied for the series of 3β-OH derivatives.

Scheme 1.5 - Synthesis of C-3 hydroxyl derivatives of androstenedione (**1**) and testosterone (**15**)



Reagents and conditions: (i) $NaBH_4$, methanol, rt; (ii) (*t*-butoxi)$_3$AlLiH, tetrahydrofuran, reflux, 8 h 20 min; (iii) $(CH_3CO)_2O$, pyridine, rt, 21 h 25 min.

1.3.1.2. Chemistry

The synthesis of derivative **16** was performed from **15** through two different methods (Scheme 1.5), in an attempt to achieve stereoselectivity in the reduction of the C-3 carbonyl group. In the first approach, testosterone (**15**) reacted with sodium borohydride, in methanol, at room temperature yielding a mixture of the 3β- and 3α-isomers, which after crystallization afforded 21% of compound **16** (Method A). In the second method, testosterone (**15**) was refluxed in tetrahydrofuran with lithium tri-*t*-butoxyaluminum hydride.[93] However, this reduction also led to a mixture of the 3β- and 3α-isomers, which after crystallization gave a similar amount of compound **16** (Method B). Testosterone (**15**) was also converted to its acetate derivative **18**, by acetylation with acetic anhydride, in 84% yield (Scheme 1.5).[94] Compound **18** was then reduced with sodium borohydride, leading to a mixture of the 3β- (compound **19**) and 3α-epimers (90:10 respectively, NMR and HPLC). Attempts to isolate 3β-isomer by crystallization led however to enrichment of the obtained mixture in the 3α-isomer. Compound **17** was obtained quantitatively from the commercially available compound **21**, by reduction with sodium borohydride in methanol, at room temperature (Scheme 1.5).

1.3.1.3. Biochemistry and biology

Inhibition of aromatase activity by the synthesized steroids (**16**, **17**, **19**, **20** and **21**) was evaluated in human placental microsomes as mentioned formerly (Section 1.1.3). The screening assay was performed in human placental microsomes and the results obtained are shown in Table 1.4 as a percentage of inhibition (%).[76]

For the most potent inhibitor of this series, steroid **20**, the IC_{50} was determined in human placental microsomes and also in MCF-7aro cells as described formerly

(Section 1.1.3), and the kinetic studies to characterize the type of binding to the active site of the enzyme and the apparent inhibition constant, were also performed (Table 1.5). This steroid revealed to be a powerful competitive inhibitor.[76]

The effect of derivative **20** in cell viability and cell proliferation was assessed in MCF-7aro cells. Compound **20** inhibits aromatase in MCF-7aro cells.[95] It was also observed that **20** induced a significant decrease in cell viability and proliferation in a dose- and time-dependent maner.[95,96] To evaluate if the biological effect of **20** in MCF-7aro cells was depend on ER, it was also studied the effect in an ER^- human breast cancer cell line SK-BR-3. It was also observed a decrease in their viability, which means that **20** induces its effect in cells in a ER-independent way.[95]

Table 1.4 – Aromatase inhibition of tested compounds in human placental microsomes[76]

Compounds	Aromatase Inhibition (%) ± SEM
16	56.82 ± 5.59
17	6.59 ± 2.00
19*	4.60 ± 0.40
20	93.14 ± 2.36
21	60.84 ± 2.61
Formestane (9)	99.65 ± 0.06

* 90% was the best purity achieved

Table 1.5 – IC_{50} determined in human placental microsomes *a)* and in MCF-7aro cells *b)* and kinetic studies for the most potent inhibitor[76,95]

Compounds	IC_{50} (μM)[a)]	IC_{50} (μM)[b)]	Type of Inhibition	*V*m (mol/min./μg prot)	K_i (μM)	Real Affinity (*K*m/K_i) (nM)
20	0.183	0.600	competitive	0.225 ± 0.025	0.100	1.026 ± 0.026
Formestane (9)	0.042	-	-	-	-	-

1.3.1.4. Structure-activity relationships discussion and conclusions

It is known that aromatase establishes two main hydrogen bonds with the carbonyl functions at C-3 and C-17 of its natural substrate **1**.[28] Nevertheless, it was observed that the presence of the carbonyl group at C-3 is not mandatory to bind steroid molecules to the enzyme aromatase and to get aromatase inhibition.[80] The C-17 carbonyl group, however, seems to have a more important role in steroidal AIs. In any case, at least one of the referred carbonyl groups must exist in order to allow the binding of steroid molecules to the enzyme aromatase.[90]

In this chapter, several steroid molecules were studied as AIs, in which the carbonyl group at C-3 (a hydrogen bond acceptor) of the substrate of the enzyme (**1**), was replaced by a hydroxyl group (a hydrogen bond donor). In another group of compounds, the two carbonyl groups (C-3 and C-17) were replaced by two hydroxyl groups. Looking at compounds **20** and **21** (Scheme 1.5 and Tables 1.4 and 1.5), we observed that the substitution of the C-3 carbonyl group of **1** by a C-3 hydroxyl group, maintaining the C-17 carbonyl group, led to a potent AI when the C-3-OH group assumes the 3β stereochemistry. Compound **20** revealed to be a very strong aromatase inhibitor, with an IC_{50} of 0.180 μM and 0.600 μM in microsomes and in MCF-7aro

cells, respectively, and having also a high affinity to the enzyme (K_i of 0.1 μM) (Table 1.5). The C-3α-OH analog **21** was not as good AI as **20**. Changing the two C-3 and C-17 carbonyl groups of **1** by hydroxyl groups, a dramatic decrease in the activity was observed (compounds **16** and **17**), particularly if the C-3-OH assumes the 3α stereochemistry (compound **17**) (Table 1.4). This decrease was also observed for other compounds submitted to the same type of transformation in C-17.[90] The lack of both C-3 and C-17 carbonyl groups in **16** and **17** can explain the inability of these steroids to bind conveniently to the enzyme with the consequent loss of activity. As the C-3-OH stereochemistry seems to play an important role in the aromatase inhibitory capacity of this kind of compound, a molecular docking study in the aromatase active site was performed for compounds **20** and **21**.

Molecular modelling studies revealed the ability of both compounds to establish one hydrogen bond (1.8Å) between the C-17 carbonyl group and the Met374 residue. Accordingly, the different aromatase recognition of **20** and **21** can be addressed to the hydrogen bond network of C-3-OH. In the former case, **20** donates and accepts two hydrogen bonds with Thr310 (2.1Å) and Ile305 (2.4Å), respectively, (Figure 1.21), and in the latter case, **21** establishes only one hydrogen bond with Asp309 residue (2.1Å) (data not shown).

Concerning compound **19** (Scheme 1.5), the substitution of the C-17 hydroxyl group by the C-17 acetoxy group dramatically reduces the aromatase inhibitory activity (Table 1.4). The acetoxy moiety, that is a bulky group, may cause steric hindrance at the enzyme active site. This is in accordance with previous studies[90] and is consistent with the short volume (400Å^3) of the aromatase binding pocket, as recently described, which only accommodates molecules with the appropriate dimensions and with small substituents.[28]

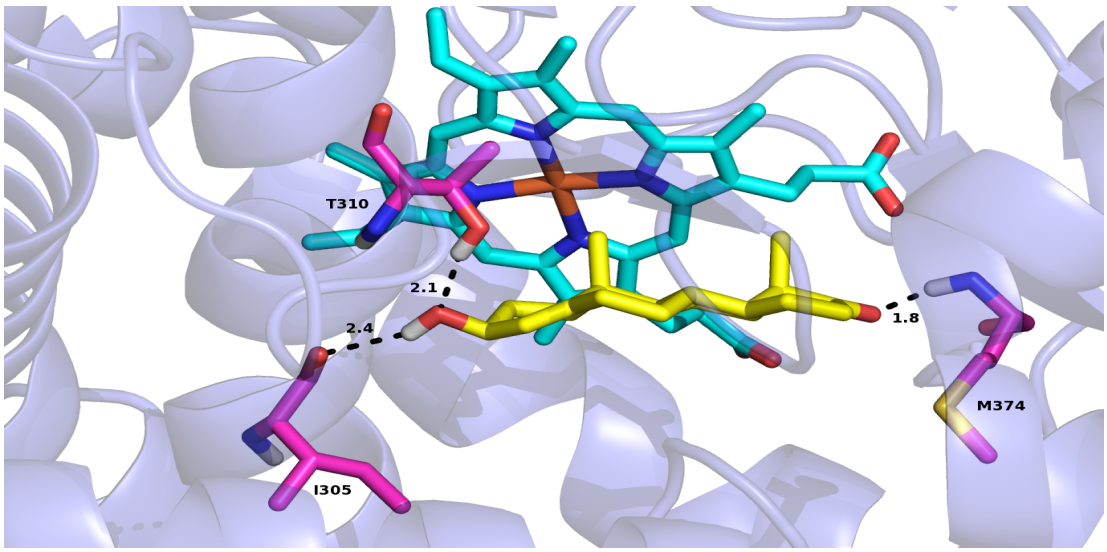


Figure 1.21 - Best pose of inhibitor **20** within the aromatase binding pocket shown as a transparent cartoon. The ligand is represented as a yellow carbon polytube model. The heme and the labelled residues interacting via hydrogen bonds are, respectively, displayed by cyan and magenta carbon poytubes models. Hydrogen bonds are depicted as dashed black lines, and their distances are measured in Å.

1.3.2. *A-Ring Olefins and Epoxides Derivatives of Androstenedione*

1.3.2.1. Design and synthesis

An important role in the manifestation of biological activity towards aromatase is played by the A-ring. Hence, there are some important features to attend when designing and synthesizing new compounds as AIs, like the pattern of substitution among the several positions and the stereochemistry at C-5 in the A,B-ring fusion. These will in turn be of huge influence in the planarity of A-ring, one of the most important features to allow a better fitting of steroids in the active site of the enzyme.[77,91,97]

In our recent studies we also noticed that some planarity in the A-ring and in the A,B-ring junction is important for the inhibitory activity of steroids against aromatase.[80,90] This planarity can be conferred by a double bond or by an epoxide function both containing similar bond geometries. Recently, we were interested in studying the influence of the position of the double bond or the epoxide function along the A-ring, in aromatase inhibitory activity. For this, we prepared two series of steroid compounds: Δ^4, Δ^3, Δ^2 and Δ^1-olefins (compounds **25**, **27a**, **29** and **34**, respectively) and 4,5-, 3,4-, 2,3- and 1,2-epoxides (compounds **26a**, **28**, **30** and **35**, respectively) (Scheme 1.6) and studied their inhibitory activity against aromatase.

For the synthesis of the Δ^4-olefin **25** (Scheme 1.6), we followed a strategy already used by Numazawa *et al.*[98] where androstenedione (**1**) was subjected to a protection reaction of its C-3-carbonyl group followed by a reduction using sodium metal in liquid ammonia.

In the meanwhile, we have developed another synthetic strategy based on the C-3 deoxygenation of testosterone acetate **36**,[81] followed by the acetate group hydrolysis, ending with the hydroxyl group oxidation (Scheme 1.6). Although this new strategy involves another step when compared with the former one, its overall yield is much better (62% *vs* 38%) and it does not form by-products.

For the synthesis of the Δ^3-olefin **27a** we followed the method of Mckenna *et al.*[99] formerly optimized by our group, where androstenedione (**1**) was reduced with zinc dust in glacial acetic acid at reflux temperature (Scheme 1.6).[100]

For the preparation of the Δ^1-olefin **34** we used a four step approach developed by Bowers *et al.*[101] which involved an initial reduction of the enone **31** with lithium tri-*t*-butoxy aluminium hydride, followed by reaction of the formed allylic alcohol **32** with thionyl chloride and then, lithium aluminium hydride giving compound **33a** in mixture with **33b**, which were oxidized with *Jones* reagent to compound **34** and **29** (Scheme 1.6). We have also intended another alternative strategy to obtain the Δ^1-olefin **34**. Hence, we adapted the one used by Numazawa *et al.* for the synthesis of Δ^4-olefin **25**, which involved the C-3 carbonyl group protection followed by reduction with sodium metal in liquid ammonia.[98] However, in this case, right in the first step of the strategy, the reaction of protection of the C-3 carbonyl group with ethane-1,2-dithiol, it was observed the production of several compounds being the desired protected one obtained only in very small yield which was an obstacle to proceed with this strategy.

The 4,5-, 3,4-, 2,3- and 1,2-epoxides were prepared using Δ^4, Δ^3, Δ^2 and Δ^1-olefins, respectively, as starting materials (Scheme 1.6). For these syntheses, the referred olefins were treated with performic acid generated *in situ*, which allowed the formation of the desired epoxides.[100] For 2,3-epoxide **30** it was also used the technique

formerly used by Campbell *et al.*[102] where the oxidation reagent was peracetic acid instead of the performic acid, in order to try to increase the yield.

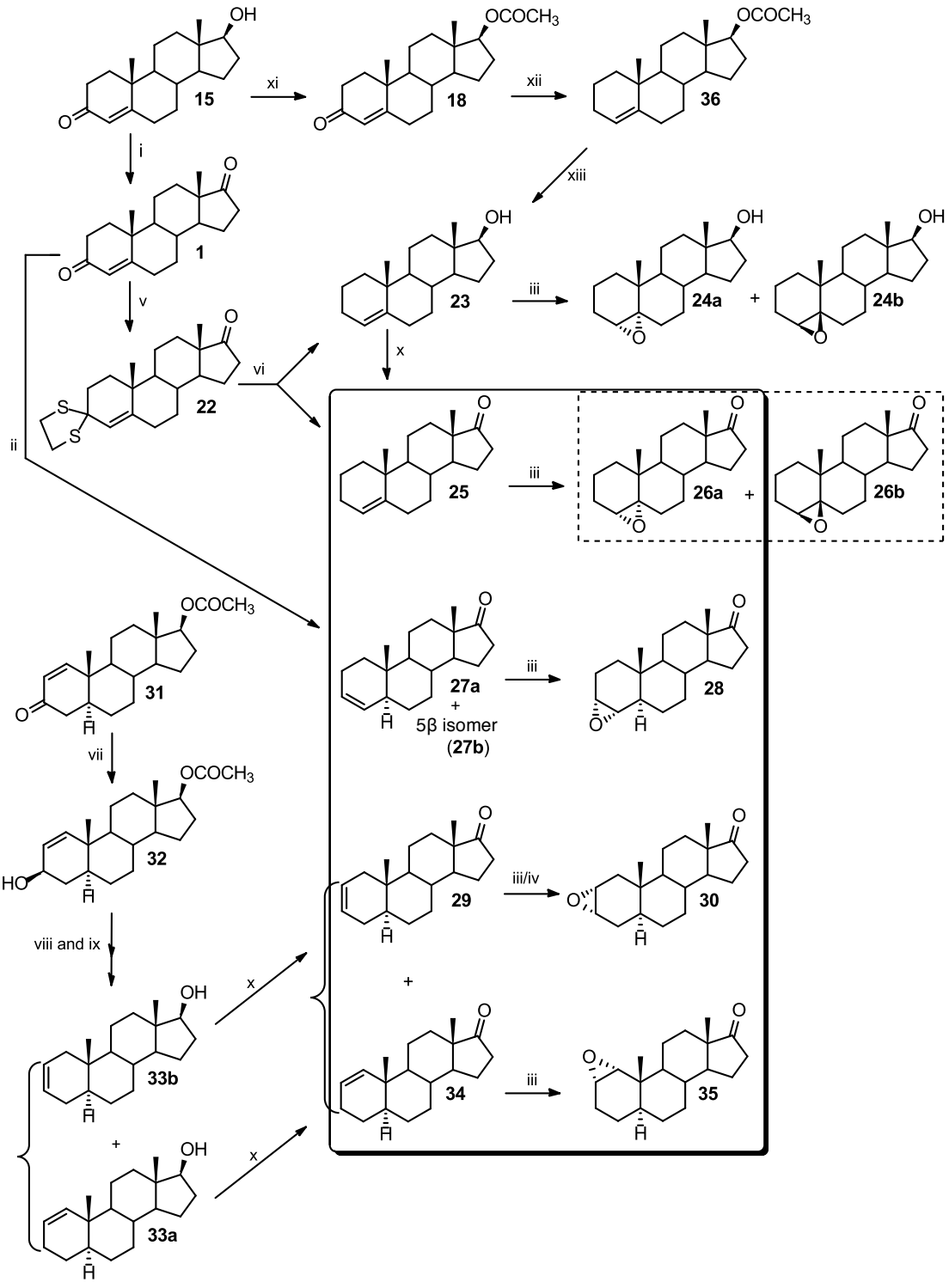
We were also interested in synthesizing the Δ^4-olefin **25** (Scheme 1.6) using the four step strategy from Bowers *et al.*,[101] previously used in the synthesis of the Δ^1-olefin **34**. Surprisingly, in this case, instead of compound **25**, it was obtained the $\Delta^{3,5}$-diene **40** (Scheme 1.7).[94]

Preparation of compound **40**, which is also an AI, was previously described by different synthetic strategies.[103-108] However, this is the first time it is prepared by this way. Hence, it was performed the C-3 carbonyl reduction of testosterone acetate derivative (**18**) using lithium tri-*t*-butoxy aluminium hydride, followed by reaction of the obtained **37** with thionyl chloride, giving **38**, which by hydrolysis with lithium aluminium hydride afforded **39**. *Jones* oxidation of **39** led to **40**.

1.3.2.2. Chemistry

Androstenedione (**1**) was prepared through Jones oxidation of testosterone (**15**) as described before (Section 1.1.2). Protection of the C-3 carbonyl group of androstenedione (**1**) was undertaken by treatment of **1** with ethane-1,2-dithiol in anhydrous tetrahydrofuran and in the presence of anhydrous *p*-toluenesulfonic acid (Scheme 1.6).[109] The crude product obtained was purified by column chromatography affording the protected compound **22** in 84% yield. Desulfurization of compound **22** with sodium-ammonia in anhydrous tetrahydrofuran,[98] afforded compound **25** in 46% yield and compound **23** in 26% yield.

Scheme 1.6 - Synthesis of A-ring olefin and epoxide derivatives of androstenedione (**1**)



Reagents and conditions: (i) CrO_3, H_2SO_4, acetone, rt; (ii) Zn dust, acetic acid reflux, 15 min; (iii) H_2O_2, HCOOH, dichloromethane, rt; (iv) CH_3COOOH, $CH_3COONa.3H_2O$, chloroform, rt, 7 h 30 min; (v) $HSCH_2CH_2SH$, *p*TSA, anhydrous tetrahydrofuran, rt, 4 h; (vi) Na, NH_3, anhydrous tetrahydrofuran, -65 ºC, 25 min; (vii) (*t*-butoxi)$_3$AlLiH, anhydrous tetrahydrofuran,

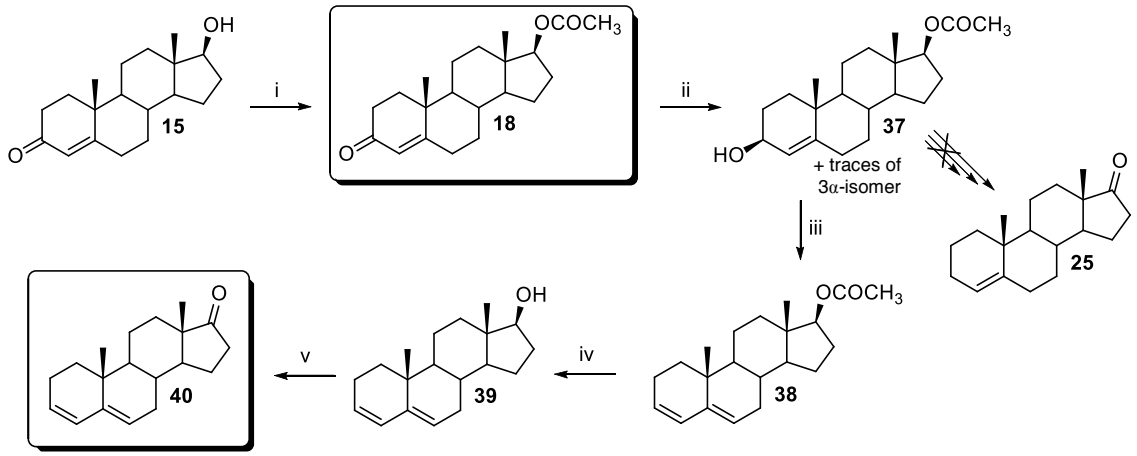
reflux, 3 h 15 min; (viii) $SOCl_2$, benzene, 5-8 ºC, 3 h 15 min; (ix) $AlLiH_4$, diethyl ether, reflux, 10 h 30 min; (x) CrO_3, H_2SO_4, acetone, 0 ºC; (xi) $(CH_3CO)_2O$, pyridine, rt, 21 h 25 min; (xii) $NaBH_4$, CF_3COOH, CH_3COOH, CH_3CN, dichloromethane, rt, 3 h 30 min; (xiii) 2% NaOH, dioxane/water, rt, 52 h.

The crude material resulting from the treatment of compound **25**, in dichloromethane, with performic acid was purified by column chromatography allowing the isolation of one main fraction (one TLC spot). NMR analysis of this fraction revealed to be a mixture of the isomers **26a** and **26b**, in 66:34 proportion. Further purification by column chromatography allowed the isolation of the pure compound **26a** in 38% yield and **26b** in 63%. Compound **23** was treated in the same way as compound **25**, allowing to obtain a fraction which after NMR analysis revealed to be an inseperable mixture of the two 4,5-epoxide isomers **24a** and **24b** (Scheme 1.6).

In agreement with a previous description of our group,[80] a *Clemmenson*-type reduction of **1** with zinc dust in acetic acid solution gave a mixture of 5α- (**27a**) and 5β-epimers (**27b**) from which the **27a** was isolated by *n*-hexane crystallization, in 60% yield. Treatment of **27a** with performic acid in dichloromethane led to the epoxide derivative **28**, in 96% yield (Scheme 1.6).[80]

Compound **30** was synthesized from the commercially available **29** using two different protocols: with performic acid, Procedure A,[90] and with peracetic acid, Procedure B (Scheme 1.6).[102] As the starting material **29** was only available in 92% purity (it is supplied in a 92:8 inseparable mixture with its isomer **27a**), the resulting 2,3-epoxide **30** was also obtained in the same purity (92%) (NMR and HPLC analysis), by both procedures.

Scheme 1.7 - Synthesis of aromatase inhibitor **40** from **15**



Reagents and conditions: (i) $(CH_3CO)_2O$, anhydrous pyridine, rt, 21 h 25 min; (ii) (*t*-butoxi)$_3$AlLiH, anhydrous tetrahydrofuran, reflux, 3 h 30 min; (iii) $SOCl_2$, benzene, 5-8 ºC, 5 h 30 min; (iv) $AlLiH_4$, ethyl ether, reflux, 8 h; (v) CrO_3/pyridine, pyridine, rt, 19 h.

Compound **34** was prepared following a described strategy.[101] Treatment of enone **31** with lithium tri-*t*-butoxy aluminum hydride led to the desired allylic alcohol **32**, in 94% yield (Scheme 1.6). The allylic alcohol **32** was then treated with thionyl chloride in benzene. From this reaction, an untractable crude (TLC, NMR and LC-MS control) was obtained, which was used as starting material in the next reaction. Treatment of this crude with lithium aluminium hydride led, after conventional workup, followed by column chromatography, to an isomeric mixture (one TLC spot) of compounds **33a** and its Δ^2-isomer **33b** (90:10, NMR analysis) (Scheme 1.6).

An attempt to separate compounds **33a** and **33b** was made using a neutral alumina column chromatography and *n*-hexane/dichloromethane. However, the isolated fractions, subjected to ^{1}H NMR analysis, always revealed to be isomeric mixtures with variable compositions (from 25:75 to 90:10 of **33a** and **33b**, respectively). The oxidation of the isomeric mixture (90:10) of these compounds was then performed

using *Jones* reagent, giving a mixture of **29** and **34**. This mixture was then subjected to a laborious purification process by column chromatography and consecutive recrystallizations, allowing the isolation of Δ^1-olefin **34** in the required purity for further biological studies (96% by LC-MS control).

Although the formation of the Δ^2-isomer **33b** has not been previously referred in the followed synthetic strategy,[101] our results revealed that isomerisation of the double bound from the Δ^1- to the more stable Δ^2-position occurred in a considerable extension. In fact, the Δ^1- and Δ^2-isomers (**34** and **29**) revealed similar physico-chemical properties, presenting the same Rf values over several chromatography solvents, and similar crystallization conditions. Moreover, as they have the same exact mass, it was difficult to distinguish them by mass spectrometry and elemental analysis techniques. Facing these difficulties, the complete diagnosis of the double bond position in these compounds could only be achieved by NMR spectroscopy. The Δ^1-isomer **34** presents two proton signals at 5.52 ppm (2-H) and 5.83 ppm (1-H) for the olefinic H-atoms, whereas the Δ^2-isomer **29** presents only one common typical multiplet around 5.9 ppm, for the two olefinic 2-H and 3-H atoms, allowing the accurate identification of both **34** and **29**. Therefore, the NMR analysis appears to be the most adequate technique to follow the formation of these compounds.

Treatment of **34** with a solution of performic acid in dichloromethane allowed to prepare and isolate compound **35** in 15% yield.

In order to also obtain the Δ^4-olefin **25** through a different strategy, to overcome the low yield of the first strategy described, it was applied the same approach of the four step pathway used for the Δ^1-olefin. However, in this case, instead of Δ^4-olefin we obtained the $\Delta^{3,5}$-diene **40** (Scheme 1.7). For this, testosterone (**15**) was converted into

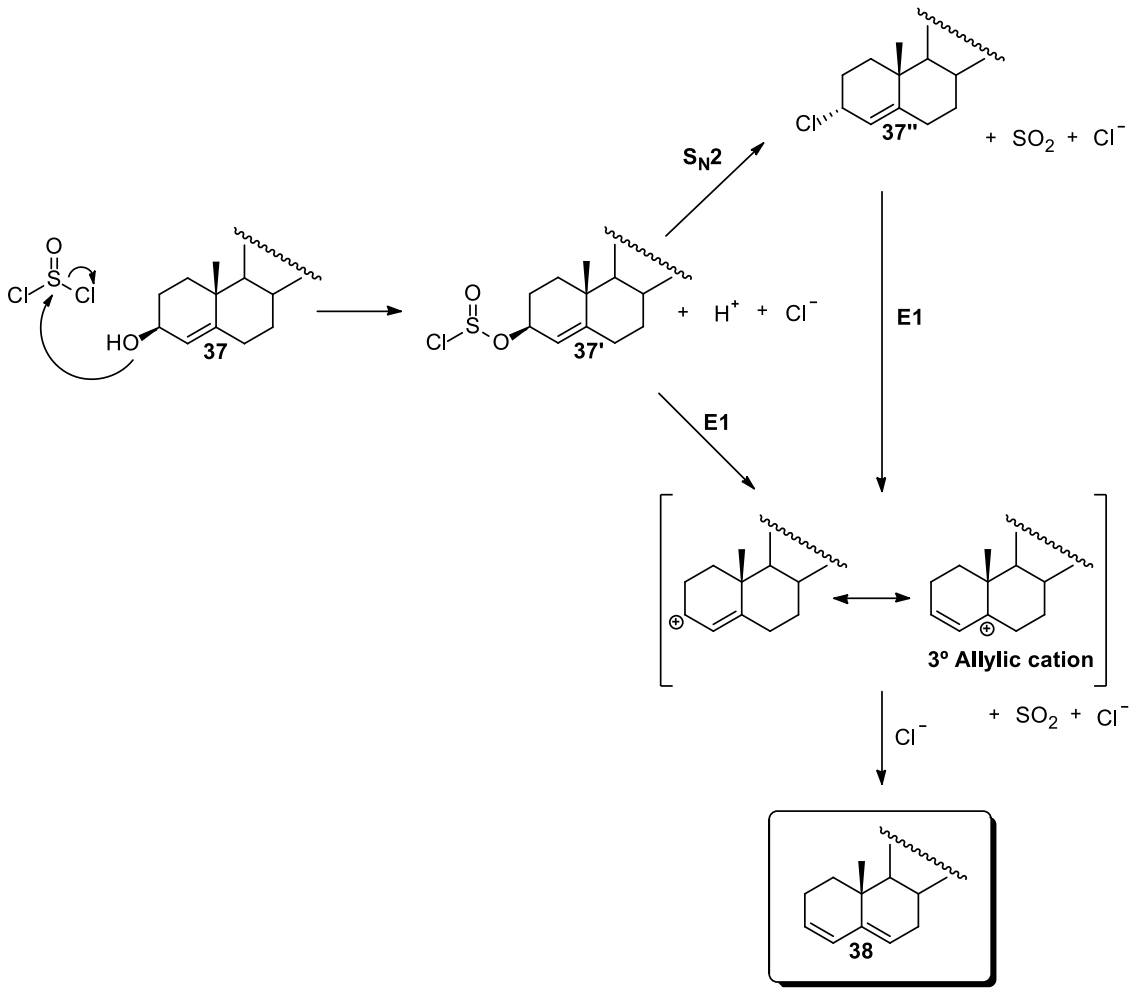
its acetate derivative **18**, in 84% yield. Treatment of **18** with lithium tri-*t*-butoxyaluminum hydride, in the conditions previously described, led to the Δ^4-3β-allylic alcohol **37** with traces of its 3α-isomer. Treatment of this mixture with thionyl chloride in benzene, contrarily to what was expected, gave the $\Delta^{3,5}$-diene **38**, according to the proposed mechanism (Scheme 1.8). Therefore, considering an E1 elimination mechanism, an allylic cation would be formed from the chlorosulfinate ester intermediate **37'**. This is a particularly stable tertiary allylic cation that favours the elimination reaction to form **38** (Scheme 1.8). On the other side, a S_N2 nucleophilic substitution displacement would provide a C-3 allylic chloride **37''** with inversion of configuration, which could also afford compound **38** through the same allylic cation formed by the E1 mechanism (Scheme 1.8). As observed, both mechanisms concur for the formation of the stabilized tertiary allylic cation, explaining diene **38** as the only product formed.

To complete the synthetic strategy, the diene **38** was treated with lithium aluminium hydride giving the $\Delta^{3,5}$-diene derivative **39**, which after oxidation with chromium trioxide in pyridine[110] led to $\Delta^{3,5}$-diene **40**. The classical *Jones* reaction did not allow the oxidation of compound **39**. This approach allowed to find a new way to prepare compound **40**, which is also an AI.[108]

As the ^{1}H NMR did not allow to unequivocally elucidate the position of the diene C=C bonds in the A,B-ring system, two-dimensional COSY experiments of compound **38** were performed. The most relevant signals analysed in the COSY spectrum were for the three olefinic H-atoms at 5.9, 5.6, and 5.4 ppm; for the 17α-H atom at 4.5 ppm; and for the 18-CH_3 and 19-CH_3 H-atoms at 0.82 and 0.95 ppm, respectively (Figure 1.22). The 17α-H atom correlates with 18-CH_3 H-atoms (^3J), which

resonates at 0.82 ppm, therefore the resonance at 0.95 ppm is unequivocally due to 19-CH_3 H-atoms.

Scheme 1.8 - Proposed mechanism for the formation of **38**



Focusing on the three olefinic H-atoms and concerning the $\Delta^{3,5}$-diene isomer **38** (Figure 1.22), the C-4 H-atom will correlate strongly with the C-3 H-atom and weakly with the C-6 H-atom. In fact, we observed that an olefinic H-atom at 5.9 ppm (the 4-H) strongly correlates with another olefinic H-atom at 5.6 ppm (the 3-H) (^3J); the same

olefinic H-atom at 5.9 ppm has a very weak correlation with an olefinic H-atom at 5.4 ppm (the 6-H) (^4J) and there is no observable correlation between the olefinic H-atoms at 5.6 ppm (the 3-H) and 5.4 ppm (the 6-H) (^5J), respectively (Figure 1.22). Therefore, the signal at 5.6 ppm unequivocally belongs to C-3 H-atom, the signal at 5.9 ppm to C-4 H-atom, and the signal at 5.4 ppm to C-6 H-atom, which is in agreement with the structure of compound **38**.

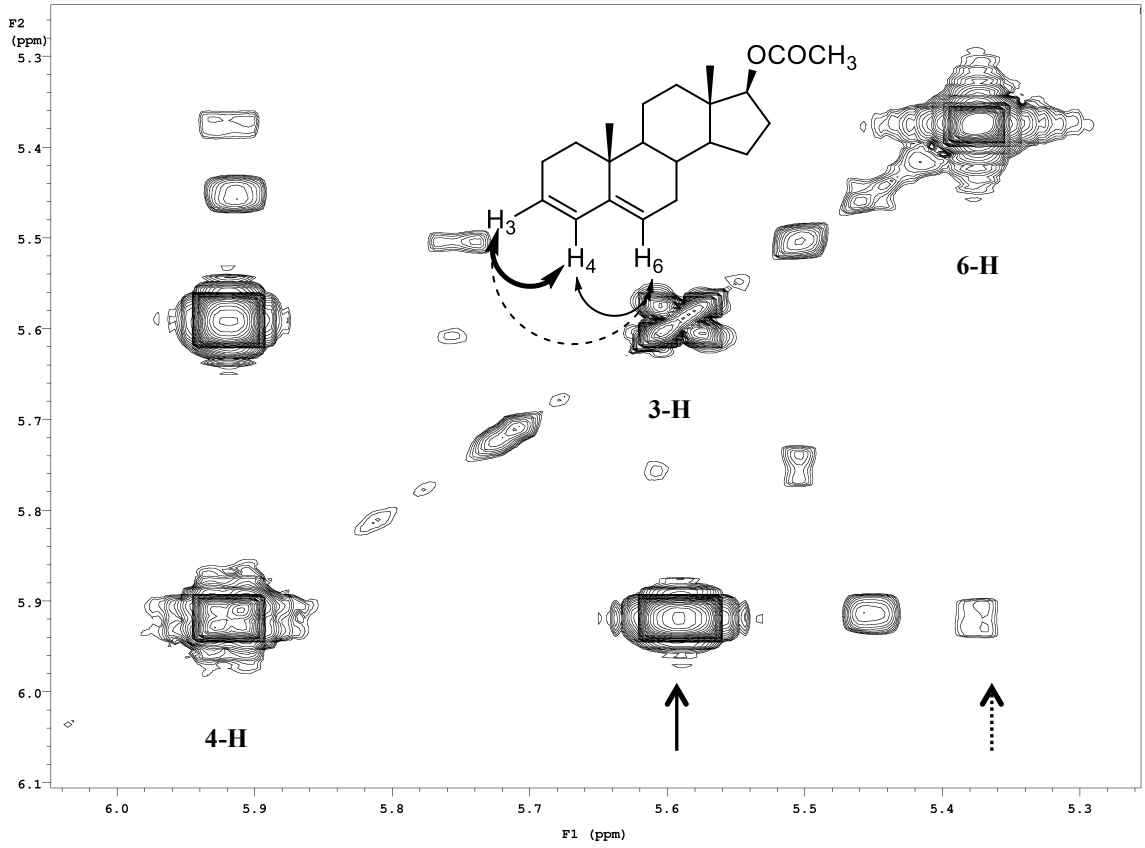


Figure 1.22 - Expansion of the COSY spectrum of compound **38** in the region of the olefinic H-atoms (bold line – strong correlation; plain line – weak correlation; dashed line – absence of correlation). Each olefinic H-atom is identified in the figure. It is also highlighted the strong correlation (**full arrow**), between 4-H and 3-H, and the very weak one (**dashed arrow**), between 4-H and 6-H

Since the former strategy did not turn out as we expected, Δ^4-olefin **25** was prepared by a new alternative synthetic strategy, which is depicted in Scheme 1.6. In

this case, testosterone (**15**) was converted to its acetate derivative **18** with acetic anhydride, in 84% yield. Then compound **18** was treated with a mixture of sodium borohydride in trifluoracetic acid, glacial acetic acid and acetonitrile in nitrogen atmosphere,[81] giving compound **36** in 99% yield. This compound was then submitted to a base-catalyzed hydrolysis giving quantitatively compound **23**, which was then subjected to *Jones* oxidation leading to the desired Δ^4-olefin **25**, in 75% yield. This new strategy has the advantage of allowing a better overall yield without the formation of isomers, hence with easier purification procedures.

1.3.2.3. Biochemistry and biology

Inhibition of aromatase activity by the synthesized steroids (**25**, **26a**, **26b**, **27a**, **28**, **29**, **30**, **34** and **35**) was evaluated in human placental microsomes as mentioned formerly (Section 1.1.3). The results obtained are shown in Table 1.6 as a percentage of inhibition (%).[76]

For the most powerful AIs **25**, **26a**, **26b**, **27a**, **28**, **29** and **30** the IC_{50} in human placental microsomes was determined, and kinetic studies, to characterize the type of binding to the active site of the enzyme and the apparent inhibition constant, were also performed (Table 1.7). These steroids revealed to be competitive inhibitors of aromatase.[76]

Previously described values for the IC_{50} and K_i for compound **40** were also presented in Table 1.7.[108]

For compounds **25**, **26a** and **29** the IC_{50} was also determined in MCF-7aro cells as described formerly (Section 1.1.3) (Table 1.7).[95]

Additionally, the effect of derivatives **25**, **26a** and **29** in cell viability and cell proliferation was assessed in MCF-7aro cells. These compounds inhibit aromatase in MCF-7aro cells causing a significant decrease in their viability and proliferation in a dose- and time-dependent manner, being compound **25** the most efficient while **29** is the less potent in decreasing cell viability.[95,96]

To evaluate if the biological effects of **25**, **26a** and **29** in MCF-7aro cells were ER-dependent or not, they were also studied in the ER^- human breast cancer cell line SK-BR-3. All compounds induced a decrease on the viability of SK-BR-3 cells in a dose-dependent manner, suggesting that these AIs can induce their effects in cells in an ER-independent manner. Results indicate that compounds **25** and **26a** induced a decrease in MCF-7aro cell viability in an aromatase-dependent way but ER-independent manner, whereas for compound **29** the reduction was both aromatase- and ER-independent.[95]

The biological effects of compounds **25** and **26a** were also investigated in LTEDaro cells (long-term estrogen deprivation human breast cancer cell line) that represent a good model to study AI acquired resistance. It was also observed a decrease in these cells viability being compound **25** the most efficient. An interesting observation was that exemestane, the only steroidal AI in clinical use, which was used as a reference, has no effect in this cell line,[95] revealing compound **25** to be very promising as a drug for resistant breast cancer.

1.3.2.4. Structure-activity relationships discussion and conclusions

As reported before, some planarity in the A-ring and in the A,B-ring junction reveals to be important for steroids to have anti-aromatase activity.[80,90] This planarity

could be achieved by introducing double bonds or epoxide functions into the A-ring, both possessing similar bond geometries. In this part of the work, we were interested in studying the effect of the double bonds and the epoxide groups in several positions along the A-ring, in aromatase inhibitory activity.

Table 1.6 – Aromatase inhibition of tested compounds in human placental microsomes[76]

Compounds	Aromatase Inhibition (%) ± SEM
25	97.84 ± 0.19
26a	84.59 ± 0.51
26b	84.54±0.62
27a	95.90 ± 0.60
28	96.40 ± 0.10
29	72.05 ± 2.60
30	70.70 ± 4.25
34	55.99 ± 1.86
35	40.01 ± 2.05
Formestane (9)	99.65 ± 0.06

Considering the A-ring olefins (**25**, **27a**, **29** and **34**) and the corresponding epoxides (**26a**, **28**, **30** e **35**) (Scheme 1.6), it was observed that 4,5-olefin **25** and 1,2-olefin **34** are better AIs than the corresponding epoxides **26a** and **35**, respectively (Tables 1.6 and 1.7). Interestingly, and on the contrary, the 3,4-epoxide **28** and the 2,3-epoxide **30** are better AIs than the corresponding olefins **27a** and **29** (Tables 1.6 and 1.7). We believe that this is probably due to the possibility of the oxiran oxygen atom of

the epoxide **28** and **30**, which is near the C-3 atom, may resemble the C-3 carbonyl oxygen of the aromatase substrate **1**, allowing it to establish hydrogen bonds with aromatase active site residues and contributing for a strong binding of the inhibitor with aromatase. In fact, this hypothesis was already explored in a recently published work of our group, for the 3α,4α-epoxy-5α-androstan-17β-ol.[90] Computer-assisted molecular modelling studies also indicate epoxide **28** as being able to accept two hydrogen bonds with the aromatase. Notably, they are both established with the Thr310 residue (Figure 1.23), in particular with the NH (2.4Å) backbone and OH side chain (2.3Å). Interestingly, in the lowest energy pose, inhibitor **28** fits the receptor core adopting an inverted positioning when compared with aromatase substrate **1**, keeping the C-19 methyl group in the opposite side of the heme group.[76]

When we compare 4,5-epoxides **26a** and **26b**, we see that, contrary to what usually happens, in this particular case the beta stereochemistry still allows a derivative with enhanced inhibitory activity (IC_{50} = 0.530 *vs* 0.970 μM) (Tables 1.6 and 1.7). This must be a consequence of the similar conformations that the steroidal A-ring adopts in both epoxides.

Another observation related with SAR of A-ring olefins and epoxides is that when the double bond is closer to the A,B-ring junction in C-5, the highest aromatase inhibition is reached (Tables 1.6 and 1.7), confirming that the planarity in the C-5 A,B-ring junction is very important to aromatase inhibition. In fact, among the studied olefin and epoxide compounds, 4,5-olefin **25** showed the best aromatase inhibition. Numazawa *et al*. had already described this compound as a very strong aromatase inhibitor.[98] Among the epoxides, compound **28** showed the best aromatase inhibitory activity (IC_{50}=0.145 μM).

Table 1.7 – IC_{50} determined in human placental microsomes *a)* and in MCF-7aro cells *b)* and kinetic studies for the most potent inhibitors of this series[76,95]

Compounds	IC_{50} *a)* (μM)	IC_{50} *b)* (μM)	Type of Inhibition	*V*m (mol/min./μg prot)	K_i (μM)	Real Affinity (*K*m/K_i) (nM)
25	0.135	5.0	-	-	-	-
26a	0.970	8.5	competitive	0.015 ± 0.001	0.086	0.636 ± 0.058
26b	0.530	-	-	-	-	-
27a	0.225	-	competitive	-	0.050	-
28	0.145	-	competitive	-	0.038	-
29	1.733	12.5	competitive	0.200 ± 0.010	9.501	0.012 ± 0.002
30	1.150	-	-	-	-	-
40*	0.340	-	competitive	-	0.058	-
Formestane (9)	0.042	-	-	-	-	-

* Results described in the literature.[108]

It is known that the introduction of additional double bonds into androstenedione based compounds gives derivatives with enhanced inhibitory activity.[111] However, when we compare the biochemical results of compound **27a** with that of **40** (Table 1.7), which has an additional double bound in C-5/C-6, we observed a decrease in potency (IC_{50} = 0.225 *vs* 0.340 μM).

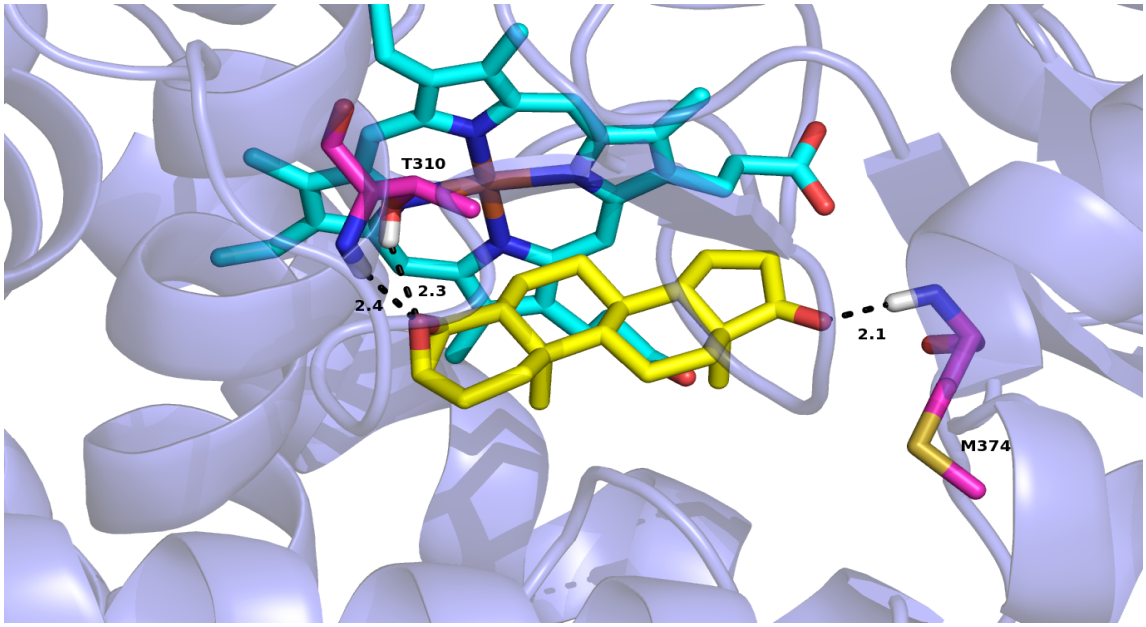


Figure 1.23 - Best pose of inhibitor **28** within the aromatase binding pocket shown as transparent cartoon. The ligand is represented as a yellow carbon polytube model. The heme and the labelled residues interacting via hydrogen bonds with **28** are, respectively, displayed by cyan and magenta carbon polytube models

1.3.3. *Derivatives of 5α-Androst-3-en-17-one*

1.3.3.1. Design and synthesis

From previous studies of our group and in order to justify the strong activity of the 3,4-epoxide **28** (Scheme 1.6) when compared with the other A-ring epoxides, it was postulated that its oxiran oxygen atom could resemble the C-3 carbonyl group of androstenedione[76,90,112] in establishing a hydrogen bond with residues of the active site of aromatase.[28,113] To further explore this hypothesis we decided to synthesize the cyclopropane derivative **41** (Scheme 1.9). For this we used the *Rawson and Harrison* method[114] where the Δ^3-olefin was set to react with a mixture of zinc dust and cuprous chloride in anhydrous diethyl ether with methylidenediiodide.

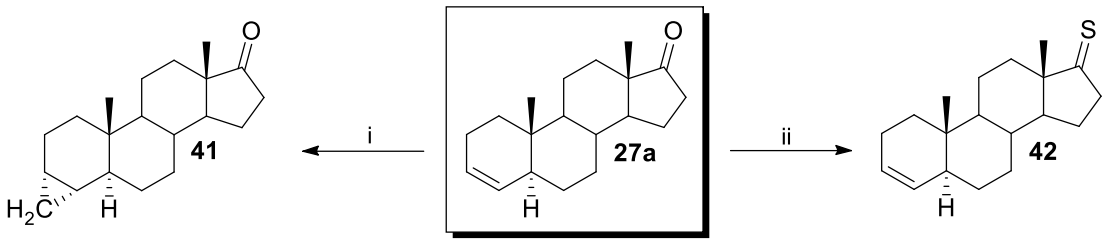
On the other side, based on the importance of the C-17 carbonyl group in steroidal AIs,[80,90] it was also performed a chemical modification by substituting the C-17 carbonyl oxygen atom of compound **27a** by its sulfur isoster **42** (Scheme 1.9). This was achieved by using *Lawesson's* reagent, which allows the chemical conversion of carbonyl to thiocarbonyl compounds.[115]

1.3.3.2. Chemistry

The method of *Simmons and Smith*[116] allows the stereospecific addition of an unsubstituted methylidenegroup to an olefin to obtain the corresponding cyclopropane derivative. In this method, the methylidenediiodide and a zinc-copper couple in anhydrous diethyl ether is usually used. The method of preparation of this couple is an important fact in determining its reactivity towards methylidenediiodide. Although this

is a convenient and very used method to promote this entry, in fact the experimental procedure requires the preparation of a rather irreproducible zinc-cooper reagent. Therefore, *Rawson and Harrison*[114] developed a method where it was not required a separate preparation of the zinc-copper couple, making use of the fact that a mixture of zinc dust with a cuprous halide is even more effective. By this reason, we selected this later method to prepare compound **41**.

Scheme 1.9 - Synthesis of aromatase inhibitors **41** and **42** from **27a**



Reagents and conditions: (i) Zn, CuCl, CH_2I_2, I_2, anhydrous diethyl ether, reflux, 90 h; (ii) *Lawesson's* reagent, anhydrous toluene, reflux, 7 h.

For this, olefin **27a** was subjected to a reaction with a mixture of zinc dust and cuprous chloride in anhydrous diethyl ether with methylidenediiodide, under a nitrogen atmosphere, in ultra-sound at reflux temperature (Scheme 1.9). After 10 h of reaction, the TLC control revealed the presence of a product with slightly higher Rf when compared to the starting material. The reaction proceeded in the referred conditions for a total of 90 h, with further addition of reagents for several times. Even so, the reaction was never complete. The residue obtained after work up was purified through silica gel

column chromatography, which allowed isolating 10.7 mg (20% yield) of the pure compound **41**.

The elucidation of the stereochemistry of the cyclopropane ring of derivative **41** was achieved by NOESY experiments. The α-H atom of the -CH_2- cyclopropyl group presents the strongest correlation with the geminated β-H atom and also correlates with 5α-H and 1α-H atoms. The α-H atom with the signal at -0.12 ppm showed the following correlations: a very strong one with the H-atom resonating at 0.65 ppm, which corresponds to the β-H atom of the -CH_2- cyclopropane ring; a moderate correlation with the H-atom resonating at 0.55 ppm, which corresponds to the 5α-H atom, and a weak correlation with the 1α-H atom resonating at 0.88 ppm (Figure 1.24, in green). On the other hand, the β-H atom from the -CH_2- cyclopropane ring presents a strong correlation with the geminal α-H atom and strong correlations with 3β-H and 4β-H atoms (Figure 1.24, in red). These two H-atoms correlate strongly with each other, and 3β-H atom also correlates with 2α-H and 2β-H atoms and in a smaller intensity with 1β-H atom (Figure 1.24, in blue). In the spectrum, it were observed strong correlations between the H-atoms resonating at 0.35 and 0.80 ppm, which corresponds to 3β-H and 4β-H atoms. The H-atom resonating at 0.35 ppm has other correlations: a strong one with H-atom resonating at 1.66 ppm, a moderate one with H-atom at 1.37 ppm, and a weak one with H-atom at 0.59 ppm. By this reason, the signal at 0.35 ppm was unequivocally attributed to the 3β-H atom and that at 0.80 ppm to the 4β-H atom. On the other side, signals at 1.66, 1.37 and 0.59 ppm are due to the resonance of 2β-H, 2α-H and 1β-H atoms, respectively. On the other hand, 1α-H atom correlates with 2α-H and 2β-H atoms, although in a stronger way with 2α-H atom (Figure 1.24, in grey). In summary, the main resonance signals for the most important H-atoms of the cyclopropryl derivative **41** are at: -0.12 ppm for the α-H atom of the -CH_2- cyclopropyl

group; 0.35 ppm for 3β-H atom; 0.55 ppm for 5α-H atom, 0.65 ppm for β-H atom of the -CH_2- cyclopropyl group, and 0.80 ppm for 4β-H atom.

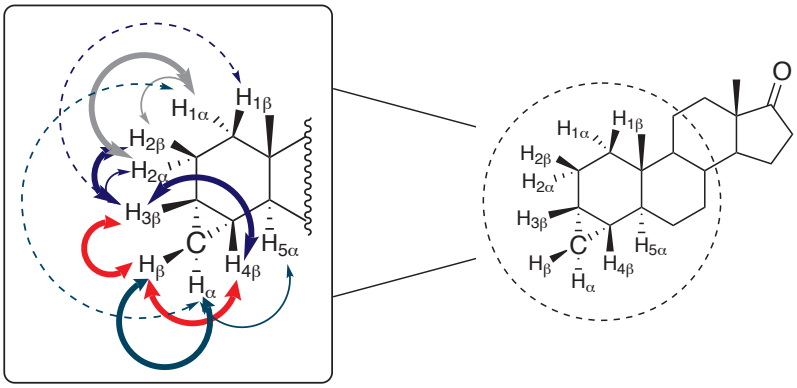


Figure 1.24 - Compound **41** showing NOESY correlations (bold line – strong correlation; plain line – moderate correlation; dashed line – weak correlation)

The synthesis of compound **42** was performed under anhydrous conditions with *Lawesson's* reagent through thionation of the C-17 carbonyl group of **27a** (Scheme 1.9). The reaction occurred during 7 h, as reported for other compounds,[115] however in our case it was never complete. After this period of time, the reaction mixture was worked up with a prior elimination of *Lawesson's* reagent by an aluminium oxide neutral column chromatography. This chromatographic procedure constitutes an additional step to the conventional work up and it was implemented after observing that there was an undesirable reaction of *Lawesson's* reagent with the silica gel, leading to a complete decomposition of the product, when the crude was directly chromatographed through a silica gel column. After the reagent elimination, the crude product was then finally purified by silica gel column chromatography affording thione **42**, in 54% yield.

1.3.3.3. Biochemistry and biology

Inhibition of aromatase activity by the modified steroids was evaluated as mentioned formerly (Section 1.1.3.). A screening assay was performed and the results are shown as a percentage of inhibition (%) for compounds **41** and **42**[76] (Table 1.8). The IC_{50} was also determined for compound **41** in human placental microsomes and in MCF-7aro cells (unpublished results) (Table 1.8).

Table 1.8 – Aromatase inhibition for compounds **41** and **42** in human placental microsomes and IC_{50} determined in human placental microsomes *a)* and in MCF-7aro cells *b)* for compound **41**

Compounds	Aromatase Inhibition (%) ± SEM	IC_{50} *a)* (μM)	IC_{50} *b)* (μM)
41	95.35 ± 0.57	0.110	1.880
42	41.45 ± 2.05	-	-
Formestane (9)	99.65 ± 0.06	0.042	-

1.3.3.4. Structure-activity relationships discussion and conclusions

In Scheme 1.9, we developed the synthesis of the cyclopropane derivative **41** from the olefin **27a**. Our expectation was that this cyclopropane derivative **41** would be less potent than 3,4-epoxide **28**, in order to justify the strong activity of this epoxide through the establishment of a hydrogen bond in the active site of aromatase. However, and interestingly, derivative **41** revealed to have higher anti-aromatase activity than epoxide **28** (IC_{50}=0.110 *vs* 0.145 μM) (Tables 1.7 and 1.8). We think that in this case,

some direct van der Waals contacts between the cyclopropane ring and the side chains of some aminoacids, must be more determinant for the interaction of the inhibitor with the active site of aromatase than the hydrogen bond that can be established between the oxiran oxygen atom of epoxide **28** and residues of the active site, hence justifying the higher inhibitory activity of **41**. Compound **41** is effectively the most potent AI synthesized and evaluated that is described in this thesis.

Compound **42** was designed based on the isosterism concept by performing the chemical substitution of the C-17 carbonyl oxygen atom by the corresponding sulfur atom. It was observed that this transformation resulted in a loss of the aromatase inhibitory activity, which prompted us to conclude that the sulfur atom was not as able as the oxygen atom to establish a hydrogen bond with the Met374 residue, in the aromatase active site.

1.4. C-6 METHYL DERIVATIVES OF ANDROSTENEDIONE

1.4.1. Design and synthesis

From SAR studies based on steroidal androstanes with alkyl and phenyl groups in the C-6 position, it was accepted that the referred substituents were beneficial for achieving aromatase inhibitory activity. It was believed that a hydrophobic binding pocket of the enzyme would interact with these groups, favoring aromatase inhibition.[111,117-121] However, when the 3D structure of aromatase, in a complex with its substrate androstenedione, was elucidated, no peculiar binding pocket near the C-6 region was found. Nevertheless, a shallow hydrophobic crevice was observed to accommodate the extra C-6-methylidene group of exemestane (Figure 1.14), being this group surrounded by the side-chain atoms of Thr310, Val370 and Ser478 (Figure 1.25), when its molecule was built into the active site of aromatase.[28,33]

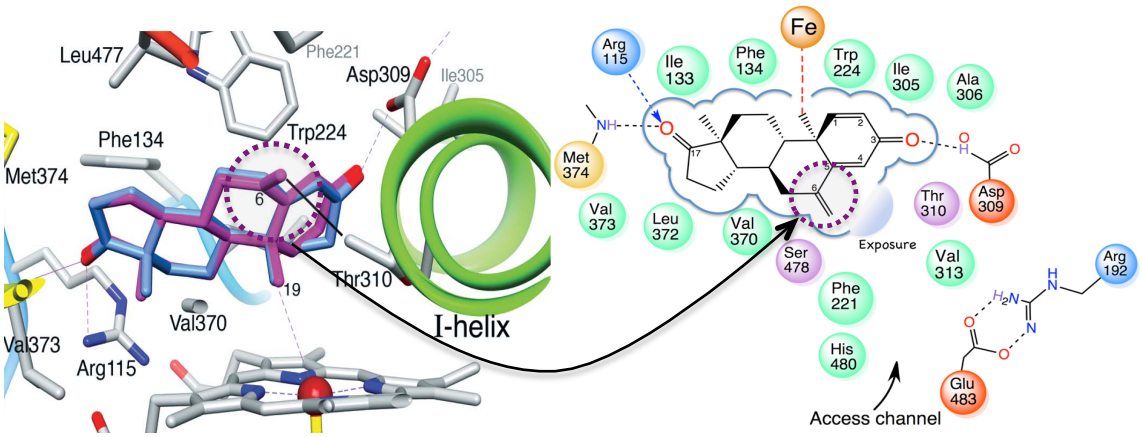


Figure 1.25 - Modelling of exemestane (C atoms in magenta) after being built in the active site of aromatase, showing that it superimposes very well with androstenedione (C atoms in blue) (left image), with detail of the access channel (right image)[28,33]

In a recent work, Ghosh *et al.*[33] have determined the crystal structures of inhibited complexes of aromatase with steroidal C-6-substituted 2-alkynyloxy compounds. It was found that, the linear C-6-alkynyloxy side chains protrude into an access channel cavity immobilizing the catalytic residues, which resulted in very potent inhibitors of aromatase. Numazawa *et al.*[118-121] have synthesized C-6 alkyl derivatives and one of its most potent inhibitors was the 3-deoxy derivative of androstenedione with a C-6α methyl group. With all this information in mind, we decided to develop new molecules based in two of our better AIs, the Δ^3-olefin **27a** and the 3,4-epoxide **28**, but with an additional methyl group in its C-6α position, in order to try to increase its inhibitory activity.

Therefore, we embarked on the preparation of these compounds through the synthesis presented in Scheme 1.10.

1.4.2. Chemistry

The direct γ-alkylenation of enone **1** was achieved by subjecting this to reaction with phosphoryl chloride and formaldehyde dimethyl acetal.[70] After 9 h of reflux a mixture of several products was formed, being compound **2** isolated after silica gel column chromatography, in 17% yield.

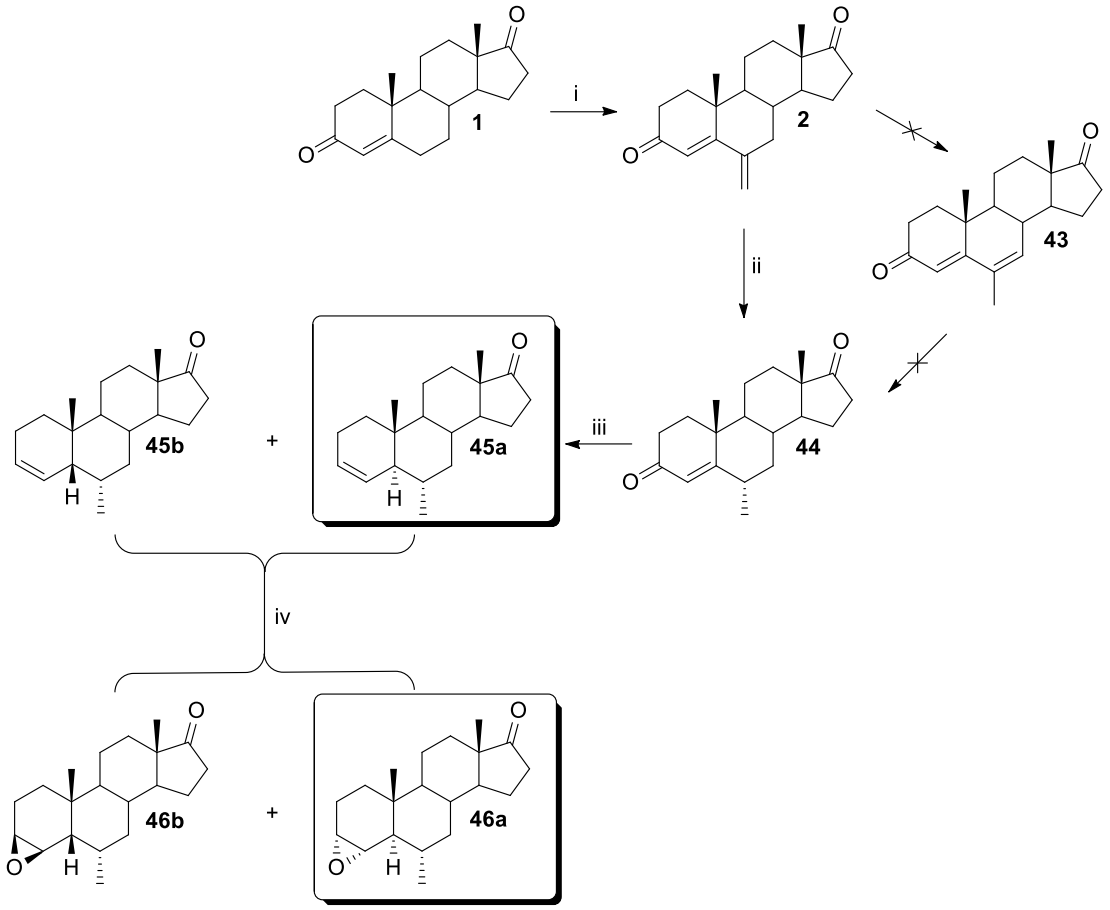
In order to try to prepare **44** and as a first approach, we followed the most recent strategy from Buzzetti *et al.*[47] where compound **2** isomerizes to **43** and then **43** is catalytically reduced to compound **44**. However, in our hands, when it was attempted the isomerization of the 6-methylene-4-en-3-one system of **2** into the 6-methyl-4,6-dien-3-one of **43** with 5% palladium charcoal (Pd-C), in refluxing ethanol, under benzyl alcohol addition,[122] there was no successful reaction. For this reason, compound **44** was

then prepared by another catalytic reduction of **2**, in order to selectively reduce its C-6 methylidene group.[123] Therefore, compound **2** was subjected to reflux in a solution of ethanol containing cyclohexene, as hydrogen donor, in the presence of 5% Pd-C catalyst. The reaction was followed by UV, since λ_{max} of the starting material **2** is 260 nm while the λ_{max} of the desired product **44** is 240 nm. After 3 h, the reaction was complete and the residue obtained after filtration of the catalyst was purified by silica gel column chromatography, which allowed isolating derivative **44** in 71% yield. A *Clemmenson*-type reduction of **44**, with zinc dust in a glacial acetic acid solution, at reflux temperature afforded an isomeric mixture of 5α- and 5β-olefins **45a** and **45b** (1.7:1, by NMR). Attempt to separate these isomers was made by silica gel column chromatography allowing to obtain the pure compound **45b**. In the case of **45a**, the best purity achieved was 90%, after two subsequent chromatographic columns. Subsequently, the whole mixture of olefins **45a** and **45b** was treated with performic acid generated *in situ*, giving a mixture of epoxides **46a** and **46b**. This mixture was then purified by silica gel column chromatography, which allowed isolating the pure 3α,4α-epoxide **46a**, in 44% yield, and the pure 3β,4β-epoxide **46b**, in 29% yield.

1.4.3. Biochemistry

Inhibition of aromatase activity by the synthesized steroids (**45a** and **46a**) was evaluated in human placental microsomes as formerly mentioned (Section 1.1.3). The results obtained are shown as a percentage of inhibition (%), in Table 1.9. The IC_{50} for both steroids was also determined (Table 1.9) (unpublished results).

Scheme 1.10 - Synthesis of aromatase inhibitors **45a** and **46a** from androstenedione (**1**)



Reagents and conditions: (i) anhydrous sodium acetate, formaldehyde dimethyl acetal, phosphoryl chloride, anhydrous chloroform, reflux, 9 h; (ii) cyclohexene, 5% Pd-C, ethanol, reflux, 3 h 15 min; (iii) zinc dust, glacial acetic acid, reflux, 5 h; (iv) H_2O_2, HCOOH, dichloromethane, rt, 9 h 30 min.

1.4.4. Structure-activity relationships discussion and conclusions

Compounds **45a** and **46a** revealed to be very active (IC_{50} = 0.560 and 0.175 μM, respectively) (Table 1.9), although slightly less potent than the corresponding derivatives without the C-6 alkyl substituent, **27a** and **28** (IC_{50} = 0.225 and 0.145 μM) (Table 1.7). Contrarily to that observed by Numazawa *et al.*[118-121] for the Δ^4-olefin, our C-6α methyl derivative **45a** revealed to be a weaker AI when compared with the

corresponding olefin **27a**. As it has been observed for compounds **27a** and **28**, where the epoxide derivative **28** was more potent than the respective olefin **27a**, also for these C-6α methyl derivatives, the epoxide **46a** is more potent than the corresponding olefin **45a**. This reinforces the hypothesis that the oxiran oxygen atom in the 3,4 position could resemble the C-3 carbonyl oxygen atom of the aromatase substrate androstenedione (**1**), hence establishing hydrogen bonds with aromatase active site residues which contributes for a stronger inhibition of the enzyme.

Table 1.9 – Aromatase inhibition and IC_{50} of tested compounds in human placental microsomes

Compounds	Aromatase Inhibition (%) ± SEM	IC_{50} (μM)
45a	93.47 ± 1.06	0.560
46a	96.72 ± 0.37	0.175
Formestane (9)	99.65 ± 0.06	0.042

1.5. C-7 ALLYL DERIVATIVES OF ANDROSTENEDIONE

1.5.1. Design and synthesis

Many studies suggest that the presence of alkyl or phenyl groups in the C-6 or C-7 positions of the steroidal androstane scaffold is beneficial for achieving aromatase inhibitory activity.[111,124-128] It was believed that this activity was due to the existence of a hydrophobic binding pocket in this region of the enzyme and that it would interact with those groups leading to a more effective ligand-enzyme interaction. Surprisingly, when recently the 3D structure of aromatase was elucidated,[28] no particular binding pocket near the C-6 and C-7 region of androstenedione was found. Although, it was identified a narrow hydrophobic cleft that accommodated the extra C-6-methylidene group of exemestane. The recent work of Ghosh and collaborators[33] has revealed the development of very potent C-6-substituted steroidal AIs whose linear side chains protruded into the access channel cavity, resulting in a very efficient inhibition of the enzyme. In addition, Brueggemeier and co-workers[124-127] have also reported the synthesis of many effective C-7α-substituted androstanes as AIs, having the most part of the substituents a phenyl moiety. Labrie *et al.*[128] have also described a rather active AI with an allyl functional group in the C-7α position. Moreover, evidence from previous reported works[127,129] show that the aromatase inhibitory activity is similar when the same kind of substituent is in position C-6 or C-7 of a given steroidal framework.

Keeping this in mind, we have been interested in performing the synthesis of derivatives of 7α-allylandrostenedione (**50**) (Scheme 1.11) where two main structural features were considered: the introduction of a double bond in C-1 position and the reduction of the carbonyl group at C-3 position. The introduction of a double bond in

C-1 position was based on the rationale that the A-ring planarity in steroidal AIs is important for aromatase inhibition, as reported in previous works,[76,80,90] and also because it has been established that the introduction of a double bond at C-1/C-2 of androstenedione affords AIs that causes mechanism-based inactivation of aromatase.[29,74,130,131] In fact, the aromatization process of androstenedione encompasses the C-1 and C-2 hydrogen atoms elimination,[132] as observed in Figure 1.5. There are also several studies revealing that, in some AIs, the C-3 carbonyl group, present in the natural substrate of the enzyme, is not essential for aromatase inhibition.[76,77,80,91] For this reason, we have designed and synthesized a series of 7α-allyl derivatives according to the strategy depicted in Scheme 1.11. Further, for each set of derivatives it was studied the influence of the functional group (carbonyl, hydroxyl or acetyl) at C-17 position in aromatase inhibitory activity.

1.5.2. Chemistry

Testosterone (**15**) was converted into its acetate derivative **18**, in 84% yield. Dehydrogenation of **18** with chloranil was undertaken in an anhydrous environment, leading to a brown-greenish solid residue.[133] This residue was then purified by silica gel column chromatography affording the dienone **47**, in 58% yield. Subsequently, a *Sakurai* reaction was performed in **47** with allyltrimethylsilane and titanium tetrachloride, at controlled atmosphere and temperature, in order to introduce the allyl group at the C-7α position.[128,134] After work-up, an oily crude was obtained which was purified over silica gel column chromatography allowing the isolation of the 7α-allyl derivative **48**, in 56% yield. Compound **48** was next submitted to alkaline hydrolysis

giving the alcohol **49**, in 96% yield. This was subsequently oxidized using *Jones* reagent, leading to ketone **50** in 73% yield after insolubilization in diisopropylether.

In order to obtain the 3-deoxo derivative **51**, the reaction of **48** with a mixture of sodium borohydride in a mixture of trifluoracetic acid, glacial acetic acid, acetonitrile and dichloromethane was performed under a nitrogen atmosphere[81] giving, after column chromatography, the almost pure compound **51**. An analytical sample of this compound was obtained by ethanol/water crystallization. Compound **51** was then submitted to base-catalyzed hydrolysis, at room temperature, giving a crude product which, after column chromatography allowed obtaining a fraction containing alcohol **52**, with traces of inseparable impurities. Treatment of this fraction with *Jones* reagent gave rise, after column chromatography, to the pure ketone **53**, in 79% yield. Compound **52** was then further reobtained, in its pure form, as a white solid, in 29 % yield, by reduction of ketone **53** with sodium borohydride in ethanol.[135]

Aiming to prepare the Δ^1-derivative **54**, compound **49** was treated with DDQ and benzoic acid in refluxing toluene.[136] After several filtrations and purification by silica gel column chromatography, the alcohol **54** was isolated in 50% yield. Compound **54** was then treated with acetic anhydride in pyridine giving a crude product that after ethyl acetate/*n*-hexane crystallization led to acetate **55**. *Jones* oxidation of **54** led, after column chromatography purification, to the pure ketone **56**, in 88% yield.

1.5.3. Biochemistry

Inhibition of aromatase activity by the modified steroids was evaluated as previously mentioned (Section 1.1.3.). A screening assay was performed and the results are shown as a percentage of inhibition (%) for all compounds (Table 1.10).[137]

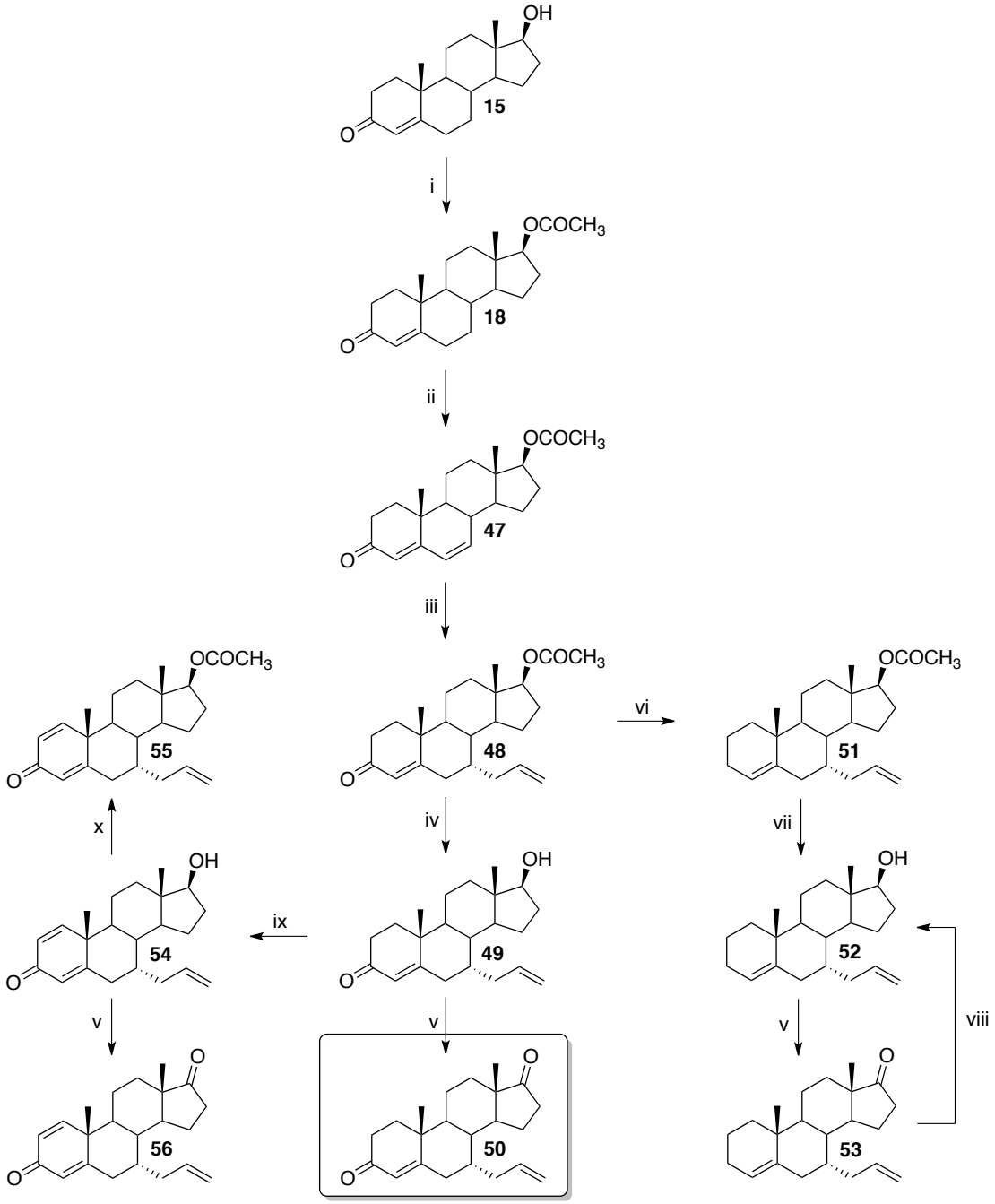
For steroids **50**, **53**, **54** and **56** the IC_{50} was determined, and kinetic studies, to characterize the type of binding to the active site of the enzyme, and the apparent inhibition constant were also performed (Table 1.11). All these steroids revealed to be competitive inhibitors of aromatase.[137]

1.5.4. Structure-activity relationships discussion and conclusions

Regarding the 7α-allyl-3-deoxo-4-androstenes **51**, **52** and **53**, it was observed that the 3-deoxo analog **53** is slightly less potent than **50** (IC_{50} = 0.75 *vs* 0.59 μM) (Table 1.11) showing that, in this case, the C-3 carbonyl group appears to be important for aromatase inhibitory activity. In addition, when one substitutes the C-17 carbonyl group of **53** by a hydroxyl group, as in **52**, the activity also decreased, as expected (Table 1.10). In this case, no C-3 or C-17 carbonyl group is present, being therefore difficult the binding of the inhibitor to the enzyme active site. Substituting the C-17 carbonyl group of **53** by an acetate group, as in **51**, the aromatase inhibitory activity decreased even more (Table 1.10). This allows us to conclude that the short volume of the aromatase active site (400$Å^3$),[28] does not allow bulky substituents to be present in this position of the androstane framework if one aims to obtain strong aromatase inhibitors.

Concerning the 7α-allyl-3-oxo-1,4-androstadienes **54**, **55** and **56**, it was observed that the Δ^1 analogue **56** is a very strong AI (IC_{50} = 0.47 μM) with great affinity for the enzyme aromatase (Tables 1.10 and 1.11).

Scheme 1.11 - Synthesis of steroidal 7α-allyl derivatives as aromatase inhibitors



Reagents and conditions: (i) $(CH_3CO)_2O$, anhydrous pyridine, rt, 21 h 30 min; (ii) chloranil, acetic acid, toluene, reflux, 3 h; (iii) $TiCl_4$, allyltrimethylsilane, anhydrous dichloromethane, -78 ºC, 40 min; (iv) 2% NaOH, dioxane/water, rt, overnight; (v) *Jones* reagent, acetone/dioxane, 0 ºC; (vi) CF_3COOH, CH_3COOH, CH_3CN, $NaBH_4$, anhydrous dichloromethane, rt, 1 h 30 min; (vii) 2% NaOH, dioxane/water, rt, 48 h; (viii) $NaBH_4$, ethanol, -10 to 5 ºC, overnight; (ix) DDQ, benzoic acid, toluene, reflux, 34 h; (x) $(CH_3CO_2)_2O$, anhydrous pyridine, rt, 33 h 15 min.

Compound **56** is even more potent than **50** and also more potent than the corresponding 3-deoxo compound **53** (Tables 1.10 and 1.11), proving that the introduction of a double bond in C-1, which increases the planarity of the A-ring, is more beneficial for aromatase inhibition than the C-3 carbonyl group reduction, in the studied compounds. Compound **56** is structurally similar to exemestane (**3**) differing by the longer alkenyl group (allyl) at C-7 position, instead of the shorter one (methylidene group) at C-6 position of exemestane (**3**).

Table 10 – Aromatase inhibition of tested compounds in human placental microsomes[137]

Compounds	Aromatase Inhibition (%) ± SEM
48	10.12 ± 0.74
49	24.74 ± 1.56
50	83.14 ± 1.95
51	31.33 ± 2.24
52	52.44 ± 3.85
53	84.29 ± 3.23
54	87.94 ± 3.04
55	13.01 ± 0.35
56	94.49 ± 1.04
Formestane (9)	99.65 ± 0.06

Surprisingly, when one substitutes the C-17 carbonyl group of **56** by a hydroxyl group, as in **54**, the aromatase inhibitory activity is almost maintained (IC_{50} = 0.45 μM), showing **54** lower affinity to aromatase (Tables 1.10 and 1.11). In this case, the decrease

is much lower than that observed in the 3-deoxo derivatives series (**53** and **52**), probably because one carbonyl group, at C-3 position, remains in the molecule, allowing the establishment of hydrogen bonding with aromatase active site residues, and also because the A-ring planarity is increased, by the presence of the Δ^1 double bond. By this reason, the A-ring planarity seems to assume an important role, since the same type of substitution in **50** to give **49**, produces a dramatic decrease in the aromatase inhibition (Table 1.10). The substitution of the C-17 carbonyl group of **56** by an acetate group, as in **55,** decreases the aromatase inhibitory activity (Table 1.10), even more than that observed in the 3-deoxo derivatives series. This dramatic decrease was also observed when it was made the substitution of the C-17 carbonyl group of **50** by an acetate group, as in **48**. This effect can be due to stereochemical conflicts of these bulky groups with aromatase active site, because of the larger size of inhibitors **48** and **55,** when compared with **51**.

Table 1.11 – IC_{50} and kinetic studies for compounds **50**, **53**, **54** and **56**[137]

Compounds	IC_{50} (µM)	Type of Inhibition	*V*m (mol/min./µg prot)	K_i (µM)	Real Affinity (*K*m/K_i) (nM)
50	0.59	competitive	0.009 ± 0.001	80.00	0.458 ± 0.042
53	0.75	competitive	0.017 ± 0.003	60.00	0.014 ± 0.003
54	0.45	competitive	0.004 ± 0.002	65.00	0.835 ± 0.041
56	0.47	competitive	0.006 ± 0.001	45.00	0.926 ± 0.055
Formestane (9)	0.042	-	-	-	-

In summary, several 7α-allyl derivatives of androstenedione were prepared and found to be strong aromatase inhibitors. Concerning its SAR, the presence of a double bond in C-1, as in **54** and **56**, which increases the planarity of the steroidal A-ring, seems to be beneficial for aromatase inhibition. The reduction of the C-3 carbonyl group did not particularly favour the aromatase inhibitory activity, in some of these compounds, proving the importance of this group in the establishment of a hydrogen bond with the enzyme. The introduction of a double bond in C-1 seems to be more beneficial for aromatase inhibition since it increases the planarity in the A-ring. The substitution of the C-17 carbonyl group by a hydroxyl group usually decreases the aromatase inhibition, except if a double bond in C-1 is present, as in **54**. The substitution of the C-17 carbonyl group by an acetate group dramatically decreases the aromatase inhibitory activity. This effect seems to be lower when the volume of the steroid molecule is also smaller as in **51**.

1.6. CONCLUDING STRUCTURE-ACTIVITY RELATIONSHIPS

As main conclusions emerging from the observed results, it can be established that if one aims to design and synthesize an ideal steroidal AI we have to consider some important aspects, such as:

- The existence of at least a carbonyl group at C-3 or C-17, but it is better if considering both of them. If we have to loose one of these groups, we should keep the C-17 carbonyl group.
- Put enough planarity in A-ring and A,B-ring junction, through double bonds or other functions, like epoxides or cyclopropanes, since the planarity in this region is very important to inhibit aromatase.
- Try to conjugate and extend the number of double bonds in convenient positions of the A and B-rings of the steroid, specially in positions C-1, C-4 and C-6, like in exemestane.
- Avoid bulky groups in specific positions of the steroidal framework since the active site of the enzyme is small.
- Some substituents in C-6 and C-7 positions can be beneficial to achieve strong aromatase inhibition.

1.7. EXPERIMENTAL SECTION

Melting points (mps) were determined on a Reichert Thermopan hot block apparatus and were not corrected.

IR spectra were recorded on a Jasco 420FT/IR spectrometer using a NaCl disk with chloroform solution and, when needed, KBr disk.

The ^{1}H NMR spectra were recorded at 600 MHz, on a Varian Unity 600. The ^{13}C NMR spectra were recorded at 150 MHz on a Varian Unity 600. The characterization of CH_3, CH_2, CH and $C_{quaternary}$ carbons was made by the APT technique. Chemical shifts were recorded in δ values in parts per million (ppm) downfield from tetramethylsilane as an internal standard. All *J*-values are given in Hz.

ESI-MS (electrospray ionization-mass spectrometry) and LC-MS (liquid chromatography-mass spectrometry) sepctra were obtained with a mass spectrometer QIT-MS Thermo Finningan, model LCQ Advantage MAX coupled to a Liquid Chromatograph of High Performance Thermo Finningan.

Reactions were monitored by thin layer chromatography (TLC) in silica gel 60 F_{254} aluminium sheets. The chromatographic separation of products was made using silica gel 60 (0.063-0.200 mm) columns. The TLC plates were revealed using ultra-violet lamp (254 nm) and/or revealed by pulverization with a mixture of sulphuric acid/ethanol 95%, followed by heating at 100 ºC.

Testosterone (**15**) was purchased from STEROID, (Cologno, Monzese, MI). Exemestane (**3**) was purchased from Sequoia Research Products (Pangbourne, United Kingdom). Formestane (**9**) was purchased from Sigma-Aldrich, Inc. (St. Louis, USA). 3β-Hydroxyandrost-4-en-17-one (**20**), 3α-hydroxyandrost-4-en-17-one (**21**), 5α-androst-2-en-17-one (**29**) and 3-oxo-5α-androst-1-en-17β-yl acetate (**31**) were purchased from

Steraloids, Inc. (Newport RI, USA). Reagents and solvents were used as obtained from the suppliers without further purification, except dioxane, which was dried through reflux and distilled from sodium;[138] chloroform, dichloromethane[138] and pyridine,[139] which were dried from calcium hydride, and diethyl ether from sodium.[138]

Yields have not been optimized.

All compounds that were evaluated biochemically and biologically possess a purity superior to 95%, except compounds **19** and **45a** (90% purity) and compounds **29** and **30** (92% purity). The purity was checked by HPLC (high-pressure liquid chromatography) with a C18-reversed phase column and water/acetonitrile 40:60 as solvent. The purity of individual compounds was determined from the peak areas in the chromatogram of the sample solution.

Scheme 1.1 - *Attempt to synthesize exemestane (**3**) from androstenedione (**1**)*

6-Methylenandrost-4-ene-3,17-dione (2).

A suspension of anhydrous sodium acetate (1.0 g, 12.19 mmol) in anhydrous chloroform (30.0 mL) containing formaldehyde dimethyl acetal (30 mL, 340.0 mmol) and phosphoryl chloride (1.9 mL, 20.0 mmol) was stirred at reflux temperature, for 1 h. After that, androstenedione (**1**) (773.5 mg, 2.70 mmol) was added and the mixture was treated dropwise with phosphoryl chloride (1.9 mL, 20.0 mmol), over a period of 3 h 30 min, and let to stir, at reflux temperature, under a stream of dry nitrogen, for a total of 10 h. Subsequently, the reaction mixture was allowed to cool to room temperature and, under vigorous stirring, a saturated aqueous solution of sodium carbonate was added until the aqueous layer became alkaline. This mixture was afterwards diluted with chloroform (200 mL) and the organic layer was washed with water (4 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness. The obtained residue was purified by silica gel 60 column chromatography (hexane/diethyl ether) affording the pure compound **2** (134.8 mg, 17%). Further crystallization from acetone/*n*-hexane afforded an analytical sample of **2**. $Mp_{(actenone/n\text{-hexane})}$ 162-164 ºC [lit.,[70] 165 ºC]. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3084 (=C-H), 1738 (C_{17}=O), 1671 (C_3=O), 1599 (C=C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.78 (3H, s, 18-H_3), 1.00 (3H, s, 19-H_3), 4.87 (1H, t, =CH_2), 4.97 (1H, t, =CH_2), 5.79 (1H, s, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.5 (C-18), 14.9 (C-19), 18.2, 19.5, 29.0, 31.6, 32.9, 33.0, 33.6, 36.6, 36.9, 45.3, 48.9, 50.3, 112.4 (=CH_2), 119.6 (C-4), 143.2 (C-6), 166.3 (C-5), 197.4 (C-3), 217.8 (C-17).

6-Methylandrosta-1,4,6-trien-17-one (4).

A mixture of compound **2** (253.2 mg, 0.85 mmol) and DDQ (288.9 mg, 1.27 mmol) in anhydrous dioxane (20 mL) was refluxed under a stream of dry nitrogen for 11 h 10 min, time after which nearest all starting material had been transformed. The reaction mixture was then filtered through alumina in order to remove the oxidizing agent. Afterwards, the solvent was removed under vacuum and the crude product was dissolved in ethyl acetate (150 mL); the yellow organic layer was washed with water (3 x 100 mL), dried over anhydrous $MgSO_4$, and concentrated to dryness. The resulting dark yellow oily residue was purified by silica gel column chromatography (petroleum ether 40-60 ºC/diethyl ether) allowing the isolation of the pure compound **4** (247.0 mg, 98%) as a white solid. $Mp_{(\text{petroleum ether 40-60 ºC/diethyl ether})}$ 216-219 ºC [lit.,[47] 222 ºC]. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 1737 (C_{17}=O), 1763 (C_3=O), 1611 (C=C). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.98 (3H, s, 18-H_3), 1.18 (3H, s, 19-H_3), 1.93 (3H, m, 20-H_3), 5.89 (1H, bs, 7-H), 6.20 (1H, d, $J_{4,2}$=1.6, 4-H), 6.27 (1H, dd, $J_{2,1}$=10.1, $J_{2,4}$=1.7, 2-H), 7.06 (1H, d, $J_{1,2}$=10.1, 1-H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 13.8 (C-18), 19.3 (C-19), 20.6 (C-20), 21.2, 21.3, 31.2, 35.5, 37.2, 47.7, 48.5, 48.9, 121.8 (C-7), 127.8 (C-4), 132.4 (C-6), 132.7 (C-2), 153.2 (C-1), 162.9 (C-5), 186.4 (C-3), 219.2 (C-17).

Scheme 1.2 - *Synthesis of derivatives of exemestane (**3**)*

6β-Spirooxiranandrosta-1,4-diene-3,17-dione (5).

To a solution of exemestane (**3**) (250.4 mg, 0.84 mmol) in dichloromethane (7 mL), a solution of performic acid generated *in situ* by addition of HCOOH 98-100% (0.12 mL) to H_2O_2 35% (0.31 mL), was added and the reaction was stirred at room temperature for 29 h, time after which an equivalent amount of performic acid solution was added and let to react for more 67 h. The reaction was worked up by addition of dichloromethane (200 mL) and the organic layer was washed with 10% aqueous $NaHCO_3$ (2 x 100 mL) followed by water (4 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness, giving a white solid residue. Purification of this residue by column chromatography (petroleum ether 40-60ºC/ethyl acetate) afforded the pure compound **5** (55.7 mg, 21%) as a white crystalline solid and a mixture of compound **5** and its 6α-isomer (76.1 mg of a 65:35 mixture of 6β:6α, respectively, by NMR).

6β-Spirooxiranandrosta-1,4-diene-3,17-dione (**5**): Mp (petroleum ether 40-60 ºC/ethyl acetate) 222-224 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3045 (=CH_2), 1722 (C=O), 1662 (C=C), 1053 (C-O). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.96 (3H, s, 18-H_3), 1.37 (3H, s, 19-H_3), 2.79 (1H, d, $J_{a\text{-}b}$=4.2, -CHa-O-), 3.13 (1H, d, $J_{b\text{-}a}$=4.2, -CHb-O-), 6.15 (1H, d, $J_{4\text{-}2}$=1.8, 4-H), 6.26 (1H, dd, $J_{2\text{-}1}$=10.2, $J_{2\text{-}4}$=1.8, 2-H), 7.08 (1H, d, $J_{1\text{-}2}$=10.2, 1-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.8 (C-18), 18.5 (C-19), 21.7, 21.9, 31.1, 32.9, 35.5, 38.9, 44.8, 47.6, 50.3 (-CH_2-O-), 50.4, 51.6, 59.6 (C-6), 124.9 (C-2), 127.4 (C-1), 155.7 (C-4), 161.4 (C-5), 185.9 (C-3), 219.4 (C-17). ESI: 313.2 ($[M+H]^+$, 100%).

6α-Spirooxiranandrosta-1,4-diene-3,17-dione (from the 6β:6α mixture): ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.95 (3H, s, 18-H_3), 1.27 (3H, s, 19-H_3), 2.69 (1H, d, J_{a-b}=5.7, -CHa-O-), 3.01 (1H, dd, J_{b-a}=5.7, -CHb-O-), 6.26 (1H, dd, J_{2-1}=10.1, J_{2-4}=1.8, 2-H), 6.32 (1H, d, J_{4-2}=1.8, 4-H), 7.04 (1H, d, J_{1-2}=10.1, 1-H).

Note: **a** and **b** are the $–CH_2$ protons of the exocyclic oxiran ring.

1α,2α-Epoxy-6-methylenandrost-4-ene-3,17-dione (6).

To a solution of exemestane (250.4 mg, 0.84 mmol) in methanol (10 mL), in an ice bath, it was added dropwise a cooled solution of 35% H_2O_2 (0.81 mL, 34.83 mmol) followed by a cooled solution of 4 N aqueous NaOH (0.61 mL, 2.44 mmol). The reaction was stirred at 0 ºC over 30 min, and then at room temperature for 24 h. The alkaline solution was neutralized by addition of 0.5 N aqueous HCl. After that, water (150 mL) was added and the product extracted with dichloromethane (3 x 100 mL). The organic layer was washed successively with 10% aqueous $NaHCO_3$ (2 x 100 mL) and water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue. This residue was purified by silica gel 60 column chromatography (petroleum ether 40-60 ºC/ethyl acetate) affording the pure compound **6** (28.7 mg, 11%) as a white solid. Mp $_{\text{(petroleum ether 40-60 ºC/ethyl acetate)}}$ 201-203 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3029 (=CH_2), 1722 (C=O), 1675 (C=C), 1052 (C-O). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.93 (3H, s, 18-H_3), 1.18 (3H, s, 19-H_3), 3.46 (1H, dd, $J_{2β-1β}$=3.9, $J_{2β-4}$=1.9, 2β-H), 3.58 (1H, d, $J_{1β-2β}$=3.9, 1β-H), 4.98 (1H, dd, J_{a-b}=1.75, $J_{a-7β}$=1.75, C_6=CHa), 5.08 (1H, dd, J_{b-a}=1.75, $J_{b-7β}$=1.75, C_6=CHb), 5.88 (1H, d, $J_{4-2β}$=1.9, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.7 (C-18), 18.9 (C-19), 21.3, 21.7, 31.0, 34.9, 35.6, 38.6, 41.3, 47.3, 47.5, 50.7, 55.1 (C-2), 60.6 (C-1), 114.6 (C_6=CH_2), 118.6 (C-4), 145.1 (C-6), 163.9 (C-5), 194.3 (C-3), 219.7 (C-17). ESI: 313.4 ($[M+H]^+$, 100%).

3-Oxo-6-methylenandrosta-1,4-dien-17β-ol (7) and 6-methylandrosta-1,4,6-trien-17β-ol (8).

Sodium borohydride (63.2 mg, 1.66 mmol) was added in small proportions with stirring and cooling to a previously cooled mixture of trifluoracetic acid (0.4 mL), glacial acetic acid (0.4 mL) and acetonitrile (0.4 mL). A solution of **3** (100.0 mg, 0.337 mmol) in dry dichloromethane (8 mL) was added to the former mixture. After this, the reaction mixture was let to react at room temperature, with stirring and under a stream of nitrogen, until all the starting material had been consumed (11 h, TLC control). The reaction mixture was then neutralized with a solution of 10% aqueous $NaHCO_3$ and extracted with dichloromethane (3 x 100 mL). The organic layer was washed with water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue (102.5 mg). This residue was purified by silica gel column chromatography (petroleum ether 40-60 ºC/ethyl acetate) affording 39.1 mg of compound **7** (39%) and 11.3 mg of compound **8** (12%).

3-Oxo-6-methylenandrosta-1,4-dien-17β-ol (**7**): $Mp_{(\text{petroleum ether 40-60 ºC/ethyl acetate})}$ 87-90 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3421 (O-H), 3037 (H-C=), 1735 (C=C), 1656 (C=C), 1057 (C-O) . ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.81 (3H, s, 18-H_3), 1.14 (3H, s, 19-H_3), 3.66 (1H, dd, $J_{17\alpha,16\alpha}$= $J_{17\alpha,16\beta}$=8.6, 17α-H), 4.94 (1H, t, C=C$\underline{H}_2$), 5.01 (1H, t, C=C$\underline{H}_2$), 6.14 (1H, t, $J_{4,2}$= 1.8, 4-H), 6.23 (1H, dd, $J_{2,1}$=10.2, $J_{2,4}$=1.8, 2-H), 7.08 (1H, d, $J_{1\text{-}2}$=10.2, 1-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.1 (C-18), 19.7 (C-19), 22.4, 23.4, 30.3, 35.8, 36.2, 40.0, 43.0, 43.8, 50.0, 50.5, 81.4 (C-17), 111.9 (C=$\underline{C}H_2$), 122.5 (C-4), 127.6 (C-2), 145.8 (C-6), 154.6 (C-1), 167.9 (C-5), 186.6 (C-3). ESI: 299.3 ($[M+H]^+$, 100%).

6-Methylandrosta-1,4,6-trien-17β-ol (**8**): ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.81 (3H, s, 18-H_3), 0.99 (3H, s, 19-H_3), 1.81 (3H, s, 6-H_3), 2.77 (2H, m, 3-H), 3.65 (1H, dd, $J_{17\alpha,16\alpha}$= $J_{17\alpha,16\beta}$=8.4, 17α-H), 5.36 (1H, s, 7-H), 5.66 (1H, t, $J_{4,2\alpha}$= $J_{4,2\beta}$=4.3, 4-H), 5.70 (1H, m, 2-H), 5.92 (1H, dt, $J_{1\text{-}2}$=10.0, $J_{1\text{-}3\alpha}$=$J_{1\text{-}3\beta}$=2.0, 1-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.1 (C-18), 20.0 (C-19), 20.6, 21.2 (C-20), 23.2, 27.1, 30.5, 36.5, 36.7, 37.1, 43.6, 49.1, 49.5, 81.6 (C-17), 117.8 (C-4), 122.6 (C-2), 126.1 (C-1), 131.4 (C-5), 133.3 (C-7), 142.4 (C-6).

Scheme 1.3 - *Synthesis of derivatives of formestane (**9**)*

4-Acetoxyandrost-4-ene-3,17-dione (10).

To a solution of **9** (750.6 mg, 2.48 mmol) in anhydrous pyridine (12.5 mL), at 0 ºC, acetyl chloride (0.27 mL, 3.80 mmol) was added dropwise. The reaction was stirred for 15 min at 0 ºC and then the temperature was raised to the ambient. Three subsequent additions of acetyl chloride (3 x 0.1 mL) were made allowing the reaction to be complete (total reaction time: 21 h 50 min, TLC). The solvent was then evaporated under vacuum and the obtained residue was crystallized with ethyl acetate/*n*-hexane after activated charcoal decolouration giving the pure compound **10** as white crystals (616.1 mg, 72%). $Mp_{(chloroform)}$ 169-171 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3018 (=CH), 1739 (C=O), 1680 (C=C), 1059 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.91 (3H, s, 18-H_3), 1.26 (3H, s, 19-H_3), 2.23 (3H, s, CH_3COO). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.7 (C-18), 17.6 (C-19), 20.2, 20.3, 21.7, 23.9, 29.7, 31.2, 33.3, 34.6, 34.7, 35.7, 39.1, 47.4, 50.7, 53.8, 139.2, 154.9, 168.6, 190.4 (C-3), 220.2 (C-17).

4-Acetoxy-5α-androst-3-en-17-one (11).

To a solution of **10** (90.3 mg, 0.26 mmol) in glacial acetic acid (7.5 mL), zinc dust (500.0 mg, 7.65 mmol) was added. The reaction was sonicated in an ultrasound bath at room temperature for 25 min, time after which an excess of zinc dust (500.0 mg, 7.65 mmol) was added and the reaction proceeded until all the starting material had been consumed (2 h, TLC). Zinc was then filtered, washed with diethyl ether (50 mL) and the filtrate was concentrated under vacuum. To the oily residue obtained, water (100 mL) was added and the product was extracted with dichloromethane (3 x 100 mL). The organic phase was sequentially washed with 10% aqueous $NaHCO_3$ (2 x 100 mL)

and water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving an oily residue (105.9 mg) composed by a mixture of **11** and its 5β-epimer. Further crystallization with petroleum ether afforded the pure compound **11**, as white crystals. $Mp_{(petroleum\ ether)}$ 116-119 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3018 (=CH), 1737 (C=O), 1681 (C=C), 1158 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.86 (3H, s, 18-H_3), 0.88 (3H, s, 19-H_3), 2.11 (3H, s, CH_3COO), 5.24 (1H, dd, $J_{3\text{-}2\beta}$=6.6, $J_{3\text{-}2\alpha}$=3.3, 3-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 12.4 (C-18), 13.9 (C-19), 20.5, 20.6, 20.7, 21.4, 21.7, 30.1, 31.5, 33.4, 34.7, 35.8, 36.5, 47.0, 47.8, 51.3, 53.0, 112.5 (C-3), 148.8 (C-4), 169.7, 221.0 (C-17). HRMS: *m/z* $[M + Na]^+$ calcd for $C_{21}H_{30}O_3$: 353.2087; found: 353.2080.

4-(*o*-Acetylsalicyloxy)androst-4-ene-3,17-dione (12).

To a solution of **9** (500.2 mg, 1.65 mmol) in anhydrous pyridine (6.5 mL) at 0 ºC, *o*-acetylsalicyloyl chloride (492.0 mg, 2.48 mmol) was added. The reaction mixture was stirred at room temperature for 22 h 30 min and after that an excess of *o*-acetylsalicyloyl chloride (247.4 mg, 1.25 mmol) was added. The reaction proceeded until complete transformation of the starting material (24 h 40 min, TLC). After evaporation of the solvent under vacuum, the residue was dissolved in dichloromethane (100 mL) and the organic layer was sequentially washed with 0.25 N aqueous HCl (4 x 100 mL), 10% aqueous $NaHCO_3$ (2 x 100 mL) and water (2 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a yellow oily residue (865.8 mg). This residue was then purified by silica gel 60 column chromatography (petroleum ether 40-60 ºC/ethyl acetate) allowing to separate 610.6 mg of compound **12** in mixture with compound **10** (60:40 respectively, NMR). A portion of this mixture (127.7 mg) was further purified by another silica gel 60 column chromatography (*n*-

hexane/diethyl ether) allowing to isolate the pure compound **12** (51.2 mg) as a white crystalline residue. $Mp_{(diethyl\ ether/n\text{-}hexane)}$ 183-185 ºC. IR (KBr disk) υ_{max} cm^{-1}: 3453 (CH_{Ar}), 1769 (C=O ester), 1739 (C=O), 1687 (C=C), 1606 ($C{=}C_{Ar}$), 1195 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.92 (3H, s, 18-H_3), 1.32 (3H, s, 19-H_3), 2.28 (3H, s, CH_3COO), 7.12 (1H, d, $J_{4\text{-}3}$=7.8, 4-H_{Ar}), 7.32 (1H, dd, $J_{3\text{-}4}$=7.8, $J_{3\text{-}2}$=7.8, 3-H_{Ar}), 7.57 (1H, dd, $J_{2\text{-}3}$=7.8, $J_{2\text{-}1}$=9.2, 2-H_{Ar}), 8.13 (1H, d, $J_{1\text{-}2}$=9.2, 1-H_{Ar}). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.7 (C-18), 17.8 (C-19), 20.4, 20.9, 21.7, 24.0, 29.9, 31.5, 33.4, 34.8, 34.9, 35.7, 39.4, 47.4, 50.9, 54.0, 122.8, 123.8 (C_{Ar}-4), 125.9 (C_{Ar}-3), 132.2 (C_{Ar}-2), 134.0 (C_{Ar}-1), 139.2, 151.1, 155.3, 162.1, 169.3, 189.8 (C-3), 219.5 (C-17). ESI: 463.7 ($[M\text{-}H]^+$, 100%).

4-(*o*-Acetylsalicyloxy)-5α-androst-3-en-17-one (13a).

A solution of a crude containing **12** as the main product (272.9 mg) in glacial acetic acid (25 mL), was sonicated with an ultrasound probe in the presence of excess of zinc dust (< 10 μm) (4.73 g, 17.43 mmol) until the transformation of all the starting material (20 min, TLC). Zinc was filtered and washed with glacial acetic acid and then the filtrate was concentrated under vacuum. To the oily residue obtained, water (200 mL) was added and the product was extracted with dichloromethane (3 x 100 mL). The organic phase was sequentially washed with 10% aqueous $NaHCO_3$ (2 x 150 mL) and water (3 x 150 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness giving an oily residue (242.9 mg). This residue was purified by silica gel 60 column chromatography (petroleum ether 40-60 ºC/ethyl acetate) allowing to isolate 101.1 mg of **13a** in an inseparable mixture with its 5β-epimer (**13b**) (70:30 respectively, by NMR).

4-(*o*-Acetylsalicyloxy)-5α-androst-3-en-17-one (**13a**): ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.87 (3H, s, 18-H_3), 0.93 (3H, s, 19-H_3), 2.33 (3H, s, CH_3COO), 5.36 (1H, dd, $J_{3\text{-}2\beta}$=6.6, $J_{3\text{-}2\alpha}$=3.3, 3-H), 7.12 (1H, d, $J_{4\text{-}3}$=8.7, 4-H_{Ar}), 7.33 (1H, dd, $J_{3\text{-}4}$=8.7, $J_{3\text{-}2}$=8.7, 3-H_{Ar}), 7.58 (1H, dd, $J_{2\text{-}3}$=8.7, $J_{2\text{-}1}$=9.6, 2-H_{Ar}), 8.07 (1H, d, $J_{1\text{-}2}$=9.6, 1-H_{Ar}).

4-(*o*-Acetylsalicyloxy)-5β-androst-3-en-17-one (**13b**): ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.87 (3H, s, 18-H_3), 1.05 (3H, s, 19-H_3), 2.34 (3H, s, CH_3COO), 5.51 (1H, dd, $J_{3\text{-}2\beta}$=6.9, $J_{3\text{-}2\alpha}$=3.6, 3-H), 7.12 (1H, d, $J_{4\text{-}3}$=8.7, 4-H_{Ar}), 7.33 (1H, dd, $J_{3\text{-}4}$=8.7, $J_{3\text{-}2}$=8.7, 3-H_{Ar}), 7.58 (1H, dd, $J_{2\text{-}3}$=8.7, $J_{2\text{-}1}$=9.6, 2-H_{Ar}), 8.05 (1H, d, $J_{1\text{-}2}$=9.6, 1-H_{Ar}).

3β,4β-Dihydroxy-5α-androstan-17-one (14a).

Sodium borohydride (62.2 mg, 1.64 mmol) was added in small portions with stirring and cooling to a previously cooled mixture of trifluoracetic acid (0.4 mL), glacial acetic acid (0.4 mL) and acetonitrile (0.4 mL). A solution of **9** (100.1 mg, 0.331 mmol) in anhydrous dichloromethane (8 mL) was added to the former mixture. After this, the reaction mixture was let to react at room temperature, with stirring and under a stream of nitrogen, until all the starting material had been consumed (45 min, TLC control). The reaction mixture was then neutralized with a solution of 10% aqueous $NaHCO_3$ and extracted with dichloromethane (3 x 100 mL). The organic layer was washed with water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue (95.7 mg). This residue was purified by silica gel column chromatography (petroleum ether 40-60ºC/ethyl acetate) affording 66.3 mg of compound **14a** (65%). $Mp_{(\text{petroleum ether 40-60ºC/ethyl acetate})}$ 213-216 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3340 (O-H), 3265 (O-H), 1747 (C=O), 1066 (C-O), 1033 (C-O). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.86 (3H, s, 18-H3), 1.05 (3H, s, 19-H3),

2.06 (1H, ddd, $J_{16\alpha,16\beta}$=18.5, $J_{16\alpha,15\beta}$=9.2, $J_{16\alpha,15\alpha}$=9.2, 16α-H), 2.44 (1H, ddd, $J_{16\beta,16\alpha}$=18.5, $J_{16\beta,15\beta}$=9.2, $J_{16\beta,15\alpha}$=9.2, 16β-H), 3.57 (1H, ddd, $J_{3\alpha,2\beta}$=7.2, $J_{3\alpha,2\beta}$=4.1, $J_{3\alpha,4\alpha}$=3-4, 3α-H), 3.76 (1H, dd, $J_{4\alpha,3\alpha}$=3-4, $J_{4\alpha,5\alpha}$=3.1, 4α-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ:13.8 (C-18), 14.7 (C-19), 19.9, 21.8, 25.6, 25.9, 31.2, 31.5, 35.0, 35.6, 35.8, 36.8, 47.8, 48.8, 51.5, 55.3, 72.2 (C-3), 74.6 (C-4), 221.3 (C-17).

***Scheme 1.5** – Synthesis of C-3 hydroxyl analogs of androstenedione (**1**) and testosterone (**15**)*

Androst-4-ene-3β,17β-diol (16).

Method A. To a solution of testosterone (**15**) (1.0 g, 3.47 mmol) in anhydrous methanol (30.0 mL), sodium borohydride (350.1 mg, 9.25 mmol) was added and the reaction was stirred at room temperature for 1 h 20 min, after which a supplement of 100 mg of $NaBH_4$ was added to completely transform the starting material (2 h 40 min, TLC). After methanol removal under vacuum, water (100 mL) was added and the product extracted with ethyl acetate (3 x 200 mL). The organic layer was then washed with water (200 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue (1.04 g) composed by a mixture of **16** and its 3α-epimer **17**. Crystallization from methanol gave the pure compound **16** (214.7 mg, 21%) as white crystalline plates. $Mp_{(methanol)}$ 149-151 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3361 (O-H), 1656 (C=C), 1051 (C-O). ^1H (600 MHz, DMSO-d_6) δ: 0.64 (3H, s, 18-H_3), 0.98 (3H, s, 19-H_3), 3.41 (1H, ddd, $J_{17\alpha\text{-}16\alpha}$=8.5, $J_{17\alpha\text{-}16\beta}$=8.5, $J_{17\alpha\text{-}17\beta OH}$=4.5, 17α-H), 3.90 (1H, m, 3α-H), 4.44 (1H, d, $J_{17\beta OH\text{-}17\alpha}$=4.5, 17β-OH), 4.54 (1H, d, $J_{3\beta OH\text{-}3\alpha}$=5.4, 3β-OH), 5.17 (1H, bs, 4-H). ^{13}C (150 MHz, DMSO-d_6) δ: 11.2 (C-18), 18.5 (C-19), 20.2, 23.0, 29.0, 29.7, 31.5, 32.3, 35.3, 35.5, 36.4, 36.8, 42.4, 50.2, 54.2, 65.9 (C-3), 79.9 (C-17), 125.3 (C-4), 144.2 (C-5). ESI: 289.2 ($[M-H]^+$, 84%).

Method B. To a solution of testosterone (**15**) (1.0 g, 3.47 mmol) in anhydrous tetrahydrofuran (40.0 mL), lithium tri-*t*-butoxyaluminium hydride (1.15 g, 4.51 mmol) was added and the reaction was heated under reflux for 2 h 30 min. To completely transform the starting material (TLC), 874 mg of tri-*t*-butoxyaluminum hydride were added in several portions and the reaction stirred at room temperature for more 8 h 20

min. After methanol removal under vacuum, water (100 mL) was added and the product extracted with ethyl acetate (3 x 200 mL). The organic layer was then washed with water (200 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue (1.04 g) composed by a mixture of **16** and **17**. Crystallization from methanol gave the pure compound **16** (222.1 mg, 22%) as white crystalline plates.

Androst-4-ene-3α,17β-diol (17).

To a solution of **21** (10 mg, 0.035 mmol) in methanol (3 mL), sodium borohydride (4.64 mg, 0.123 mmol) was added and the reaction mixture was stirred at room temperature until all the starting material has been consumed (10 min, TLC). After methanol evaporation and dissolution of the residue obtained with ethyl acetate (50 mL), the organic layer was washed with water (3 x 40 mL), dried over anhydrous $MgSO_4$, filtered and evaporated affording the pure compound **17**, in quantitative yield as a white residue. $Mp_{(dichloromethane/methanol)}$ 202-205 ºC. IR (KBr) ν_{max} cm^{-1}: 3328 (O-H), 1655 (C=C), 1054 (C-O). ^1H NMR (600 MHz, DMSO-d_6) δ: 0.66 (3H, s, 18-H_3), 0.92 (3H, s, 19-H_3), 3.41-3.45 (1H, m, 17-H), 3.85 (1H, bs, 3-H), 4.29 (1H, d, *J*=4.8, 3α-OH or 17β-OH), 4.38 (1H,d, *J*=4.8, 3α-OH or 17β-OH), 5.30 (1H, s, *J*=4.2, 4-H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 11.1 (C-18), 18.0 (C-19), 20.7, 23.0, 27.7, 29.7, 31.3, 31.6, 32.1, 35.4, 36.5, 36.9, 40.0, 42.4, 50.3, 53.2, 62.3 (C-3), 79.9 (C-17), 122.2 (C-4). ESI: 289.5 ($[M-H]^+$, 100%).

3-Oxoandrost-4-en-17β-yl acetate (18).

To a solution of testosterone (**15**) (2.0 g, 6.93 mmol) in anhydrous pyridine (48.0 mL), acetic anhydride (7.9 mL, 83.9 mmol) was added and the reaction was

stirred for 21 h 25 min, at room temperature, until all the starting material was consumed (TLC control). Dichloromethane (250 mL) was added and the organic layer was washed with 10% aqueous $NaHCO_3$ (3 x 150 mL), 10% aqueous HCl (3 x 150 mL) and water (3 x 150 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness. Crystallization of the obtained residue from ethyl acetate gave the pure compound **18** (1.92g, 84%). $Mp_{(ethyl\ acetate)}$ 141-142 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3018 (=CH), 1736 (C=O), 1675 (C=C), 1248 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.82 (3H, s, 18-H_3), 1.18 (3H, s, 19-H_3), 2.03 (3H, s, CH_3COO), 4.58 (1H, dd, $J_{17\alpha\text{-}16\alpha}$=9.0, $J_{17\alpha\text{-}16\beta}$=8.0, 17α-H), 5.71 (1H, s, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 12.0 (C-18), 17.4 (C-19), 20.5, 21.1, 23.4, 27.4, 31.4, 32.7, 33.9, 35.4, 35.7, 36.6, 38.6, 42.4, 50.2, 53.7, 82.4 (C-17), 123.9 (C-4), 170.9 (C-5), 171.1 (OC=O), 199.4 (C3=O).

3β-Hydroxyandrost-4-en-17β-yl acetate (19).

To a solution of **18** (676.5 mg, 2.04 mmol) in anhydrous methanol (20 mL), sodium borohydride (127.5 mg, 3.37 mmol) was added and the reaction was stirred at room temperature until complete transformation of the starting material (45 min, TLC). After removal of methanol under vacuum, water (200 mL) was added and the product extracted with ethyl acetate (3 x 200 mL). The organic layer was washed with water (200 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue (706.4 mg). This residue was purified by silica gel 60 column chromatography (petroleum ether 60-80ºC/ethyl acetate) affording 430.0 mg of a 90:10 3β/3α-epimeric mixture of 3-hydroxyandrost-4-en-17β-yl acetate (NMR and HPLC control). An attempt to isolate by crystallization the 3β-epimer **19**, just enriched the mixture in the 3α-epimer.

3β-Hydroxyandrost-4-en-17β-yl acetate (**19**): ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.76 (3H, s, 18-H_3), 0.99 (3H, s, 19-H_3), 1.98 (3H, s, CH_3COO), 3.90 (1H, m, 3α-H), 4.49 (1H, dd, $J_{17\alpha\text{-}16\alpha}$=9.0, $J_{17\alpha\text{-}16\beta}$=8.0, 17α-H), 4.54 (1H, d, $J_{3bOH\text{-}3\alpha}$=5.5, 3β-OH), 5.19 (1H, bs, 4-H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 11.8 (C-18), 18.4 (C-19), 20.0, 20.8, 22.9, 27.0, 28.9, 31.4, 32.2, 35.1, 35.2, 36.2, 36.7, 41.9, 49.7, 53.8, 65.8 (C-3), 81.8 (C-17), 125.5 (C-4), 143.9 (C5), 170.2 (OC=O). ESI: 331.0 ($[M-H]^+$, 100%).

3α-Hydroxyandrost-4-en-17β-yl acetate (from the mixture with **19**): ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.77 (1H, s, 18-H_3), 0.92 (1H, s, 19-H_3), 1.98 (3H, s, CH_3COO), 3.85 (1H, m, 3β-H), 4.36 (1H, d, $J_{3\alpha OH\text{-}3\beta}$=4.4, 3α-OH), 4.50 (1H, dd, $J_{17\alpha\text{-}16\alpha}$=9.0, $J_{17\alpha\text{-}16\beta}$=8.0, 17α-H), 5.31 (1H, d, $J_{4\text{-}3\beta}$=4.5, 4-H).

Scheme 1.6 *– Synthesis of A-ring olefin and epoxide derivatives of androstenedione* ***(1)***

Androst-4-ene-3,17-dione (1).

To a solution of testosterone (**15**) (11.6 g, 40.2 mmol) in acetone (500 mL) it was slowly added a solution of aqueous chromium trioxide in sulphuric acid – *Jones* reagent (15 mL), until the reaction mixture acquired a persistent red coloration. After stirring at room temperature for 15 min, the excess of the oxidant agent was destroyed with methanol, until it reacquired the green coloration. Then, it was diluted with water (250 mL), the acetone evaporated under vacuum and it was poured into a mixture of water with ice (900 mL). The white precipitate formed was filtered and dried affording the pure compound **1** (10.4 g, 91%). IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3012 (H-C=), 1736 (C_{17}=O), 1662 (C3=O), 1617 (C=C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.92 (3H, s, 18-H_3), 1.22 (3H, s, 19-H_3), 5.76 (1H, s, 4-H).

3,3-(Ethylenedithio)androst-4-en-17-one (22).

A solution of androstenedione (**1**) (501.0 mg, 1.75 mmol) in anhydrous THF (7 mL) was treated with anhydrous *p*-toluenesulfonic acid (904.2 mg, 5.25 mmol) and ethane-1,2-dithiol (176 μL, 2.10 mmol). A clear slightly yellowed solution was obtained. The reaction mixture was then stirred at room temperature for 4 h and after that it was diluted with water (50 mL) and brine (20 mL) and extracted with ethyl acetate (3 x 100 mL). The organic layer was washed with brine (4 x 70 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a slightly yellowed residue was obtained. Crystallization from ethyl acetate gave the pure compound **22** (253.6 mg) as white crystals. Purification of the crystallization mother liquor by silica gel 60 column chromatography (*n*-hexane/ethyl acetate) afforded an additional portion

of **22**, as a white crystalline solid (total yield 84%). A minor product (37.1 mg, 5%) was also isolated revealing to be 3,3,17,17-bis-(ethylenedithio)androst-4-ene. Compound **22**: $Mp_{(n\text{-hexane/ethyl acetate})}$ 169-170 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3016 (=CH), 1737 (C=O), 1647 (C=C), 663 (C-S). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.87 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 3.22-3.36 (4H, m, -SCH_2CH_2S-), 5.50 (1H, bs, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.7 (C-18), 18.5 (C-19), 20.5, 21.7, 31.3, 31.4, 31.8, 35.3, 35.8, 36.7, 37.2, 37.9, 39.5, 40.0, 47.6, 50.9, 54.2, 65.6, 124.6 (C-4), 145.7 (C-5), 220.9 (C-17).

Androst-4-en-17-one (25) and Androst-4-en-17β-ol (23).

Method A. To liquid ammonia (70 mL) at -65 ºC sodium metal (1.16 g, 50.5 mmol) was added. To this stirred solution, compound **22** (500.2 mg, 1.38 mmol) in anhydrous tetrahydrofuran (10mL) was added over a period of 10 min. Ammonia was removed at room temperature with a stream of argon and methanol (27 mL), followed by chilled water (18 mL), were cautiously added affording a yellow residue. The residue was then acidified with a solution of 5% aqueous HCl, diluted with water (75 mL) was extracted with ethyl acetate (3 x 100mL). The organic layer was washed with a solution of 5% aqueous $NaHCO_3$ (5 x 100 mL) and water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a slightly yellowed residue. This crude was then purified by silica gel 60 column chromatography (*n*-hexane/ethyl acetate) yielding compound **25** (174.7 mg, 46%) as a white crystalline solid and compound **23** (99.4 mg, 26%) as a white solid.

Androst-4-en-17-one (**25**): $Mp_{(n\text{-hexane/ethyl acetate})}$ 74-76 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3018 (=CH), 1738 (C=O), 1657 (C=C). ^1H NMR (600 MHz, $CDCl_3$)

δ: 0.88 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 5.32 (1H, m, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.7 (C-18), 19.2 (C-19), 19.4, 20.6, 21.8, 25.7, 31.5, 32.1, 32.3, 35.5, 35.8, 37.1, 37.8, 47.7, 51.2, 54.5, 119.6 (C-4), 144.3 (C-5), 221.3 (C-17). ESI: 271.0 ($[M-H]^+$, 50%).

Androst-4-en-17β-ol (**23**): Mp (n-hexane/ethyl acetate) 143-146 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3278 (OH), 2959 (=CH), 1651 (C=C). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.65 (3H, s, 18-H_3), 0.97 (3H, s, 19-H_3), 3.43 (1H, ddd, $J_{17\alpha\text{-}16\alpha}$=9, $J_{17\alpha\text{-}16\beta}$=9, $J_{17\alpha\text{-}17\beta OH}$=5, 17α-H), 4.43 (1H, d, $J_{17\beta OH\text{-}17\alpha}$=5, 17β-OH), 5.24 (1H, m, 4-H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 11.2 (C-18), 18.8 (C-19), 19.1, 20.6, 23.0, 25.1, 29.8, 31.9, 32.5, 35.5, 36.4, 36.5, 37.2, 42.4, 50.3, 54.1, 79.9 (C-17), 118.7 (C-4), 144.2 (C-5).

3-Oxoandrost-4-en-17β-yl acetate (18). As described in ***Scheme 1.5***.

Androst-4-en-17β-yl acetate (36).

Sodium borohydride (566.2 mg, 14.97 mmol) was added in small proportions with stirring and cooling to a previously cooled mixture of trifluoracetic acid (3.5 mL), glacial acetic acid (3.5 mL) and acetonitrile (3.5 mL). A solution of **18** (1.0 g, 3.03 mmol) in anhydrous dichloromethane (18 mL) was added to the former mixture. After this, the reaction mixture was let to react at room temperature, with magnetic stirring and under a stream of nitrogen, until all the starting material had been consumed (3 h 30min, TLC control). The reaction mixture was then neutralized with a solution of 10% aqueous $NaHCO_3$ and extracted with dichloromethane (4 x 100 mL). The organic layer was washed with water (4 x 100mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving 945.0 mg (99%) of compound **36** as a white solid

residue. $Mp_{(methylidenechloride/n\text{-}hexane/ethanol)}$ 95-99 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3024 (=CH), 1737 (C=O), 1663 (C=C), 1043 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.80 (3H, s, 18-H_3), 1.01 (3H, s, 19-H_3), 2.03 (3H, s, CH_3COO), 4.58 (1H, dd, $J_{17\alpha H\text{-}16\alpha H}$=9, $J_{17\alpha H\text{-}16\beta H}$=8, 17α-H), 5.29 (1H, m, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 12.0 (C-18), 19.2 (C-19), 19.4, 20.9, 21.2, 23.5, 25.7, 32.4, 32.8, 35.8, 36.9, 37.1, 37.8, 42.5, 50.5, 54.4, 82.8 (C-17), 119.3 (C-4), 144.7 (C-5), 171.2 (C=O).

Androst-4-en-17β-ol (23).

Method B. Compound **36** (945.0 mg, 2.99 mmol) was added to a mixture of dioxane/water (85:15) (90 mL) with 2% aqueous NaOH (18 mL), at room temperature. The reaction mixture was let to react until total transformation of the starting material (52 h, TLC control) being after this time neutralized with a solution of 5% aqueous HCl. The dioxane was evaporated under vacuum leading to a white solid residue that was diluted with water (200 mL) and extracted with ethyl acetate (4 x 100 mL). The organic layer was then washed with water (4 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving 819.0 mg (99%) of compound **23** as a white solid.

Androst-4-en-17-one (25).

Method B. Jones reagent (2.7 mL) was added dropwise to a solution of **23** (839.8 mg, 3.06 mmol) in acetone/dioxane (60:10) (190 mL), at 0 ºC, with stirring, until the orange colour of the reagent remains. The excess of oxidant agent was destroyed with the addition of 2-propanol. The dioxane and acetone were evaporated under vacuum. To the remaining residue water (200 mL) was added and the resulting solution extracted with ethyl acetate (4 x 100 mL). The organic layer was then washed with 10% aqueous $NaHCO_3$ (3 x 100 mL) and water (3 x 100 mL), dried over anhydrous Na_2SO_4,

filtered and concentrated to dryness giving a white solid residue (129.3 mg) after the addition of some drops of diethyl ether. This solid was then purified by silica gel column chromatography (hexane/ethyl acetate) affording the pure compound **25** (624.2 mg, 75%) as a white crystalline solid.

5α-Androst-3-en-17-one (27a).

To a boiling solution of androstenedione (**1**) (1.0 g, 2.50 mmol) in glacial acetic acid (60 mL), zinc dust (12.0 g, 183.5 mmol) was added in two portions and after 10 min the reaction was complete. The zinc suspension was filtered, the zinc washed with glacial acetic acid and the filtrate evaporated to dryness. The resulting residue was diluted with water (100 mL) and extracted with diethyl ether (3 x 120 mL). The organic layers were washed with 10% aqueous $NaHCO_3$ (3 x 100 mL) and water (3 x 100 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness to give a white crystalline solid composed by a mixture of 5α-olefin **27a** and 5β-olefin **27b**. Crystallization of the mixture from *n*-hexane allowed obtaining the pure **27a** (570.0 mg, 60%). ^{1}H NMR (600, $CDCl_3$) δ: 0.79 (3H, s, 18-H_3), 0.87 (3H, s, 19-H_3), 5.29 (1H, ddd, $J_{4,3}$=9.6, $J_{4,5\alpha}$=3.8, $J_{4,2\alpha}$=1.9, 4-H), 5.66 (1H, ddd, $J_{3,4}$=9.8, $J_{3,2\beta}$=6.4, $J_{3,2\alpha}$=3.2, 3-H).

3β-Hydroxy-5α-androst-1-en-17β-yl acetate (32).

To a solution of compound **31** (500.4 mg, 1.51 mmol) in anhydrous tehrahydrofuran (20 mL) under nitrogen, lithium tri-*t*-butoxyaluminum hydride (499.7 mg, 1.96 mmol) was added and the reaction was heated under reflux for 2 h, time after which an excess of lithium tri-*t*-butoxyaluminum hydride (99.9 mg, 0.39 mmol) was added. The reaction proceeded until complete transformation of the starting material (3 h 15 min, total). After removal of the solvent under vacuum, water (200 mL) was added

and the aqueous layer extracted with dichloromethane (3 x 200 mL). The organic layer was then washed with water (200 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. Crystallization from methanol/water gave the pure compound **32** (471.5 mg, 94%) as a white solid. $Mp_{(methanol/water)}$ 142-144 ºC (lit.,[101] 142-144 ºC). IR υ_{max} (NaCl plates, $CHCl_3$) cm^{-1}: 3388 (OH), 3024 (=CH), 1735 (C=O), 1248 (C-O). ^1H NMR (600 MHz, DMSO-d_6) δ: 0.74 (3H, s, 18-H_3), 0.85 (3H, s, 19-H_3), 1.98 (3H, s, CH_3COO), 4.06 (1H, m, 4α-H), 4.50 (1H, dd, $J_{17\alpha\text{-}16\alpha}$=8.0, $J_{17\alpha\text{-}16\beta}$=8.0, 17α-H), 4.63 (1H, d, $J_{3\beta OH\text{-}3\alpha}$=5.8, 3-OH), 5.39 (1H, d, $J_{1\text{-}2}$=10.0, 1-H), 5.75 (1H, dd, $J_{2\text{-}1}$=10.0, $J_{2\text{-}3\alpha}$=2.0, 2-H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 12.0 (C-18), 15.4 (C-19), 20.2, 20.8, 22.9, 27.0, 27.6, 30.9, 34.8, 35.3, 36.4, 37.5, 42.2, 43.0, 50.0, 50.8, 66.8 (C-3), 81.8 (C-17), 130.6 (C-1), 135.3 (C-2), 170.2 (C=O). ESI: 333.4 ($[M+H]^+$, 14 %).

5α-Androst-1-en-17β-ol (33a).

A solution of **32** (418.0 mg, 1.25 mmol) in benzene (10 mL) was kept at 5-8 ºC, under nitrogen, treated with thionyl chloride (0.42 mL, 5.59 mmol) and stirred for 3 h 15 min, never allowing complete transformation of the starting material. Benzene was evaporated under vacuum at room temperature and an oily residue was obtained. To this residue, solid $NaHCO_3$ was added followed by 10% aqueous $NaHCO_3$ (100 mL). The aqueous layer was extracted with dichloromethane (3 x 100 mL) and the resulting organic layer was washed with water (3 x 100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness giving 422.0 mg of a transparent oily residue. To a solution of this residue in ethyl ether (63 mL), lithium aluminium hydride (292.4 mg, 7.66 mmol) was added cautiously, under nitrogen, and the reaction heated under reflux for 10 h 30 min. A saturated sodium potassium tartrate solution (150 mL) was added and the mixture extracted with diethyl ether (4 x 100 mL). The organic layer was then

washed with water (3 x 100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness giving a white solid residue (one TLC spot). ^{1}H NMR analysis of this residue revealed to be composed by a mixture of **33a** and its Δ^2 isomer (**33b**) in similar proportions. This residue was further purified by column chromatography over neutral alumina and *n*-hexane/dichloromethane as solvent giving 239.0 mg of a white solid (one TLC spot). ^{1}H NMR and LC-MS analysis of this product revealed to be composed by **33a** in 90% purity.

5α-Androst-1-en-17β-ol (**33a**): ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.64 (3H, s, 18-H_3), 0.80 (3H, s, 19-H_3), 3.42 (1H, ddd, $J_{17\alpha\text{-}16\alpha}$=9.0, $J_{17\alpha\text{-}16\beta}$=9.0, $J_{17\alpha\text{-}17\beta OH}$ =5.0, 17α-H), 4.42 (1H, d, $J_{17\beta OH\text{-}17\alpha}$=5.0, 17β-OH), 5.45 (1H, m, 2-H), 5.82 (1H, bd, $J_{1\text{-}2}$=10.0, 1-H).

5α-Androst-2-en-17β-ol (**33b**) (from the mixture with **33a**): ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.63 (3H, s, 18-H_3), 0.71 (3H, s, 19-H_3), 3.42 (1H, ddd, $J_{17\alpha\text{-}16\alpha}$=9.0, $J_{17\alpha\text{-}16\beta}$=9.0, $J_{17\alpha\text{-}17\beta OH}$ =5.0, 17α-H), 4.42 (1H, d, $J_{17\beta OH\text{-}17\alpha}$=5.0, 17β-OH), 5.56 (2H, m, 2-H and 3-H).

5α-Androst-1-en-17-one (34).

Jones reagent (0,3 mL) was added dropwise to a solution of a mixture **33a** and **33b** (90:10) (100.1 mg, 0.36 mmol) in acetone (5 mL), at 0 ºC, with stirring until the orange colour of the reagent remains. Then, the mixture was stirred for more 3 min, after which the reaction was poured into water (100 mL) and the product extracted with dichloromethane (3 x 100 mL). The organic layer was then washed with 10% aqueous $NaHCO_3$ (2 x 100 mL) and water (2 x 100 mL), dried over anhydrous Na_2SO_4, filtered

and concentrated to dryness giving a white solid residue. This residue (one TLC spot) was composed by a mixture of **29** and **34**. Column chromatography purification (*n*-hexane/diethyl ether) followed by consecutive recrystallizations from methanol gave compound **34** in 96% purity (LC-MS analysis).

5α-Androst-1-en-17-one (**34**): ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.86 (3H, s, 19-H_3), 0.87 (3H, s, 18-H_3), 5.52 (1H, m, 2-H), 5.83 (1H, bd, $J_{1\text{-}2}$=10.0, 1-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.9 (C-19), 15.7 (C-18), 20.4, 21.8, 25.3, 25.9, 28.2, 30.9, 31.6, 35.3, 35.8, 37.6, 44.6, 47.9, 51.5, 51.8, 125.4 (C-2), 135.4 (C-1), 221.3 (C-17). ESI: 271.3 ($[M\text{-}H]^+$, 100%).

5α-Androst-2-en-17-one (**29**) (from the mixture with **34**): ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.78 (3H, s, 19-H_3), 0.87 (3H, s, 18-H_3), 5.59 (2H, m, 2-H and 3-H).

General procedure to obtain 4α,5α-epoxyandrostan-17β-ol (24a) and 4β,5β-epoxyandrostan-17β-ol (24b), 4α,5α-epoxyandrostan-17-one (26a) and 4β,5β-epoxyandrostan-17-one (26b), 3α,4α-epoxy-5α-androstan-17-one (28), 2α,3α-epoxy-5α-androstan-17-one (30), and 1α,2α-epoxy-5α-androstan-17-one (35).

To a solution of the olefin (**23**, **25**, **27a, 29** or **34**) in dichloromethane, a solution of performic acid, generated *in situ* by addition of HCOOH 98-100% to H_2O_2 35%, was added. The reaction mixture was stirred at room temperature until complete transformation of the starting material. Dichloromethane (100 mL) was added and the organic layer was washed successively with 10% aqueous $NaHCO_3$ (2 x 100 mL) and water (4 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness.

The obtained residue was purified by silica gel 60 column chromatography (*n*-hexane/ethyl acetate).

4α,5α-Epoxyandrostan-17β-ol (24a) and 4β,5β-epoxyandrostan-17β-ol (24b).

Olefin **23** (82.7 mg, 0.30 mmol); dichloromethane (4.0 mL); HCOOH 98-100% (0.04 mL); H_2O_2 35% (0.11 mL); total reaction time: 7 h (TLC). Before column chromatography, 83.6 mg of a white solid residue was obtained. Purification by column chromatography afforded 39.7 mg of an inseparable epimeric mixture (60:40, by NMR) of **4α,5α-** (**24a**) and **4β,5β-** (**24b**) **epimers**, respectively.

4α,5α-Epoxyandrostan-17β-ol (**24a**) (from the mixture with **24b**): ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.65 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 3.42-3.46 (1H, m, 17α-H), 2.86 (1H, d, $J_{4\beta\text{-}3\alpha}$=4.3, 4β-H), 4.42 (1H, d, $J_{17\beta OH\text{-}17\alpha}$=4.8, 17β-OH).

4β,5β-Epoxyandrostan-17β-ol (**24b**) (from the mixture with **24a**): ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.64 (3H, s, 18-H_3), 0.93 (3H, s, 19-H_3), 3.42-3.46 (1H, m, 17α-H), 2.86 (1H, d, $J_{4\alpha\text{-}3\alpha}$=4.6, 4α-H), 4.44 (1H, d, $J_{17\beta OH\text{-}17\alpha}$=4.9, 17β-OH).

4α,5α-Epoxyandrostan-17-one (26a) and 4β,5β-epoxyandrostan-17-one (26b).

Olefin **25** (300.0 mg, 1.01 mmol); dichloromethane (15.0 mL); HCOOH 98-100% (0.14 mL); H_2O_2 35% (0.44 mL); total reaction time: 10 h 45 min (TLC). Before column chromatography it was obtained 309.7 mg of a white solid residue. Purification by column chromatography afforded 111.3 mg (35%) and 183.4 mg (58%) of 4α,5α- and 4β,5β-epimers **26a** and **26b**, respectively.

4α,5α-Epoxyandrostan-17-one (**26a**): $Mp_{(chloroform)}$ 142-145 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 1734 (C=O), 1216 (C-O-C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.88 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 2.46 (1H, ddd, $J_{16\beta\text{-}16\alpha}$=19.0, $J_{16\beta\text{-}15\beta}$=9.0, $J_{16\beta\text{-}15\beta}$=1.0, 16β-H), 2.90 (1H, d, $J_{4\beta\text{-}3\alpha}$=4.6, 4β-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.7 (C-18), 15.3 (C-19), 19.2, 20.5, 21.8, 23.6, 29.3, 29.6, 31.2, 31.4, 34.7, 35.8, 36.4, 46.7, 47.7, 51.2, 61.3, 65.3 (C-5), 220.9 (C-17). ESI: 287.1 ($[M-H]^+$, 100%).

4β,5β-Epoxyandrostan-17-one (**26b**): $Mp_{(chloroform)}$ 148-150 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 1738 (C=O), 1016 (C-O-C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.88 (3H, s, 18-H_3), 1.08 (3H, s, 19-H_3), 2.43 (1H, ddd, $J_{16\beta\text{-}16\alpha}$=19.0, $J_{16\beta\text{-}15\beta}$=9.0, $J_{16\beta\text{-}15\beta}$=1.0, 16β-H), 2.94 (1H, d, $J_{4\alpha\text{-}3\beta}$ =4.1, 4α-H). ESI: 289.3 ($[M+H]^+$, 100%).

3α,4α-Epoxy-5α-androstan-17-one (28).

Olefin **27a** (67.5 mg, 0.25 mmol); dichloromethane (1.0 mL); HCOOH 98-100% (0.05 mL); H_2O_2 35% (0.05 mL); total reaction time: 6 h (TLC). It was afforded 75 mg of **28** (96%). ^1H NMR (500 MHz, $CDCl_3$) δ: 0.79 (3H, s, 19-H_3), 0.86 (3H, s, 18-H_3), 2.08 (1H, ddd, $J_{16\alpha\text{-}16\beta}$=19.0, $J_{16\alpha\text{-}15\alpha/15\beta}$=9.5, 16α-H), 2.44 (1H, ddd, $J_{16\beta\text{-}16\alpha}$=19.0, $J_{16\beta\text{-}15\beta}$=5.5, $J_{16\beta\text{-}15\alpha}$=0.0-1.0, 16β-H), 2.71 (1H, d, $J_{4\beta\text{-}5\alpha}$=4.0, 4β-H), 3.17 (1H, dd, $J_{3\beta\text{-}2\alpha}$=3.0, $J_{3\beta\text{-}2\beta}$=3.0, 3β-H).

2α,3α-Epoxy-5α-androstan-17-one (30).

Method A. Olefin **29** (100.0 mg, 0.37 mmol); dichloromethane (2.0 mL); HCOOH 98-100% (0.1 mL); H_2O_2 35% (0.3 mL); total reaction time: 6 h (TLC). Before column chromatography it was obtained 103.5 mg of a white solid residue. Purification by column chromatography (chloroform) afforded compound **30** (6.7 mg,

6%) in 92% purity (NMR and HPLC analysis). IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 1738(C=O), 1013 (C-O-C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.78 (3H, s, 19-H_3), 0.84 (3H, s, 18-H_3), 2.05 (1H, ddd, $J_{16\alpha\text{-}16\beta}$=19.0, $J_{16\alpha\text{-}15\beta}$=9.0, $J_{16\alpha\text{-}15\alpha}$=9.0, 16α-H), 2.42 (1H, ddd, $J_{16\beta\text{-}16\alpha}$=19.0, $J_{16\beta\text{-}15\beta}$=9.0, $J_{16\beta\text{-}15\alpha}$=1.0, 16β-H), 3.11 (1H, m, $J_{2\beta\text{-}1\alpha}$=6.0, $J_{2\beta\text{-}3\beta}$=3.95, 2β-H) and 3.16 (1H, m, $J_{3\beta\text{-}4\alpha}$ =6.0, $J_{3\beta\text{-}2\beta}$=3.95, $J_{3\beta\text{-}4\beta}$=1.69, 3β-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 15.6 (C-19), 16.3 (C-18), 22.8, 24.4, 30.7, 31.6, 33.1, 34.1, 36.4, 37.8, 38.5, 38.9, 40.9, 50.2, 53.5, 53.9, 55.0 (C-2), 56.4 (C-3) and 223.9 (C-17). ESI: 287.0 ([M-H]$^+$, 100%).

Method B. To a stirred 9% aqueous peracetic acid solution (1.0 mL) at 10 ºC, trihydrated sodium acetate (79.6 mg) and olefin **29** (200.2 mg, 0.73 mmol) in chloroform (2 mL) were added. The reaction was then stirred at room temperature until complete transformation of the starting material (7 h 30 min, TLC). Dichloromethane (150 mL) was added and the organic layer was washed with 10% aqueous $NaHCO_3$ (100 mL), water (4 x 100 mL), dried over anhydrous $MgSO_4$ and concentrated to dryness giving 162.9 mg of a white residue. Purification by column chromatography (*n*-hexane/ethyl acetate) followed by crystallization with methanol/water gave compound **30**, also in 92% purity.

1α,2α-Epoxyandrostan-17-one (35).

Olefin **34** (46.4 mg, 0.17 mmol); dichloromethane (3 mL); HCOOH 98-100% (0.03 mL); H_2O_2 35% (0.07 mL); total reaction time: 9 h 30 min (TLC). Before column chromatography, 38.6 mg of an oily residue was obtained. Purification by column chromatography afforded 7.4 mg (15%) of the pure compound **35**. $Mp_{(chloroform)}$ 119-122 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 1738 (C=O), 1049 (C-O). ^1H NMR (600 MHz,

$CDCl_3$) δ: 0.87 (3H, s, 18-H_3), 0,92 (3H, s, 19-H_3), 2.07 (1H,ddd, $J_{16\alpha\text{-}16\beta}$=19.0, $J_{16\alpha\text{-}15\alpha}$=9.0, $J_{16\alpha\text{-}15\beta}$=9.0, 16α-H), 2.42 (1H, ddd, $J_{16\beta\text{-}16\alpha}$=19.0, $J_{16\beta\text{-}15\beta}$=9.0, $J_{16\beta\text{-}15\alpha}$=1.0, 16β-H), 2.99 (1H, d, $J_{1\beta\text{-}2\beta}$=4.0, 1β-H), 3.12 (1H, dd, $J_{2\beta\text{-}1\beta}$=4.0, $J_{2\beta\text{-}3\alpha}$=3.0, 2β-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.4 (C-18), 13.8 (C-19), 20.4, 21.7, 22.7, 23.4, 27.6, 30.4, 31.3, 34.9, 35.8, 36.6, 37.3, 47.7, 49.0, 51.3, 52.9 (C-1), 59.1 (C-2), 221.1 (C=O). ESI: 286.9 ($[M\text{-}H]^+$, 100%).

Scheme 1.7 - *Synthesis of aromatase inhibitor* ***40*** *from* ***15***

3-Oxoandrost-4-en-17β-yl acetate (18). As described in ***Scheme 1.5***

3β-Hydroxyandrost-4-en-17β-yl acetate (37).

To a solution of **18** (2.0 g, 6.05 mmol) in anhydrous tetrahydrofuran (75 mL) under nitrogen, lithium tri-*t*-butoxyaluminum hydride (2.0 g, 7.86 mmol) was added and the reaction was heated under reflux for 2 h, time after which an excess of lithium tri-*t*-butoxyaluminum hydride (500.1 mg, 1.97 mmol) was added. The reaction proceeded until complete transformation of the starting material (3 h 30 min, total). After removal of the solvent under vacuum, water (200 mL) was added and the aqueous layer extracted with dichloromethane (3 x 200 mL). The resulting organic layer was washed with water (200 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness giving 1.97 g of a crude mainly composed by compound **37**. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.76 (3H, s, 18-H_3), 0.99 (3H, s, 19-H_3), 1.98 (3H, s, CH_3COO), 3.90 (1H, m, 3α-H), 4.49 (1H, dd, $J_{17\alpha H\text{-}16\alpha H}$=9.0, $J_{17\alpha H\text{-}16\beta H}$=8.0, 17α-H), 4.54 (1H, d, $J_{3\beta OH\text{-}3\alpha H}$=5.5, 3β-OH), 5.19 (1H, bs, 4-H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 11.8 (C-18), 18.4 (C-19), 20.0, 20.8, 22.9, 27.0, 28.9, 31.4, 32.2, 35.1, 35.2, 36.2, 36.7, 41.9, 49.7, 53.8, 65.8 (C-3), 81.8 (C-17), 125.5 (C-4), 143.9 (C5), 170.2 (OC=O).

Androsta-3,5-dien-17β-yl acetate (38).

A solution of the previously obtained crude (500.4 mg) in benzene (10 mL) was kept at 5-8 ºC, under nitrogen, treated with thionyl chloride (0.5 mL, 6.72 mmol) and stirred for 2 h 25 min, time after which an excess of thionyl chloride (0.1 mL, 1.38 mmol) was added. The reaction proceeded never allowing the complete transformation

of the starting material (5 h 30 min, total). Benzene was evaporated under vacuum, at room temperature giving an oily residue to which solid $NaHCO_3$ (500 mg) was added followed by 10% aqueous $NaHCO_3$ (100 mL). The aqueous layer was extracted with dichloromethane (3 x 100 mL) and the resulting organic layer was washed with water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue. This residue was purified by silica gel 60 column chromatography (petroleum ether 60-80ºC/ethyl acetate) to afford 263.4 mg of the title compound **38** as a white crystalline residue in an overall yield of 56% from **18**. $Mp_{(\text{petroleum ether 60-80 ºC/ethyl acetate})}$ 116-119 ºC. IR υ_{max} (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3018 (=CH), 1736 (C=O ester), 1648 (C=C), 1244 (C-O). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.83 (3H, s, 18-H_3), 0.96 (3H, s, 19-H_3), 2.04 (3H, s, CH_3COO), 4.61 (1H, dd, $J_{17\alpha\text{-}16\alpha}$=9.0, $J_{17\alpha\text{-}16\beta}$=8.0, 17α-H), 5.38 (1H, m, 6-H), 5.59 (1H, m, 3-H), 5.92 (1H, m, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 12.0 (C-18), 18.8 (C-19), 20.4, 21.2, 22.9, 23.5, 27.5, 31.3, 31.6, 33.7, 35.2, 36.8, 42.5, 48.3, 51.2, 82.8 (C-17), 122.6 (C-6), 125.1 (C-3), 128.8 (C-4), 141.5 (C-5), 171.2 (OC=O). ESI: 315.1 ($[M+H]^+$, 76%).

Androsta-3,5-dien-17β-ol (39).

To a solution of **38** (100.8 mg, 0.32 mmol) in ethyl ether (15 mL), lithium aluminium hydride (76.2 mg, 2.01 mmol) was added cautiously under nitrogen and the reaction heated under reflux for 8 h. A saturated sodium potassium tartrate solution (150 mL) was added and the mixture extracted with ethyl ether (4 x 100 mL). The organic layer was washed with water (4 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving 79.4 mg (91%) of the pure compound **39** as a white solid. $Mp_{(\text{ethyl acetate/}n\text{-hexane})}$ 140-142 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3302 (OH), 3021 (=CH), 1646 (C=C), 1054 (C-O). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.67 (3H, s,

18-H_3), 0.89 (3H, s, 19-H_3), 3.45 (1H, ddd, $J_{17\alpha\text{-}17\beta OH}$=5.0, $J_{17\alpha\text{-}16\alpha}$=9.0, $J_{17\alpha\text{-}16\beta}$=9.0, 17α-H), 4.45 (1H, d, $J_{17\beta OH\text{-}17\alpha}$=5.0, 17β-OH), 5.34 (1H, m, 6-H), 5.57 (1H, m, 3-H), 5.88 (1H, m, 4-H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 11.2 (C-18), 18.5 (C-19), 20.1, 22.4, 22.9, 29.8, 30.8, 31.4, 33.2, 34.6, 36.3, 42.3, 48.0, 50.9. 79.9 (C-17), 122.6 (C-6), 124.5 (C-3), 128.8 (C-4), 140.8 (C-5). ESI: 271.2 ([M-H]$^+$, 100%).

Androsta-3,5-dien-17-one (40).

To a solution of **39** (62.0 mg, 0.23 mmol) in pyridine (3 mL), a pyridine solution (2.3 mL) of chromium trioxide (98 mg, 0.98 mmol) was added at 0 ºC. The reaction was stirred at room temperature for 19 h, until total transformation of the starting material (TLC control). The mixture was then diluted with ethyl ether (150 mL) and poured into water (50 mL). The organic phase was brined (6 x 150 mL), washed with water (3 x 200 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a yellow residue which was purified by neutral column chromatography (petroleum ether 40-60 ºC) giving 9.0 mg (38%) of pure compound **40**. $Mp_{(chloroform)}$ 81-83 ºC [lit.,[140] 80-82 ºC]. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3018 (=CH), 1739 (C=O), 1652 (C=C). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.91 (3H, s, 19-H_3), 0.97 (3H, s, 18-H_3), 5.40 (1H, m, 6-H), 5.61 (1H, m, 3-H), 5.93 (1H, m, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.7 (C-19), 18.8 (C-18), 20.2, 21.8, 22.9, 30.6, 31.3, 31.4, 33.7, 35.3, 35.8, 47.7, 48.5, 51.9, 122.1 (C-6), 125.3 (C-3), 128.7 (C-4), 141.6 (C-5), 221.0 (C=O). ESI: 269.1 ([M-H]$^+$, 99%).

Scheme 1.9 - *Synthesis of aromatase inhibitors **41** and **42** from **27a***

3α,4α-Methylen-5α-androstan-17-one (41).

A mixture of zinc dust <10 μm (409.7 mg, 6.26 mmol) and cuprous chloride (618.9 mg, 13.07 mmol) in anhydrous diethyl ether (15 mL) was stirred and heated to reflux, using a ultra-sound bath, under an atmosphere of anhydrous nitrogen, for 30 min. A solution of **27a** (50.0 mg, 0.18 mmol) in anhydrous diethyl ether (2 mL) was added to the former suspension followed by the addition of methylidenediiodide (0.3 mL, 3.35 mmol) and iodine (14.6 mg). The reaction mixture was maintained at reflux for 37 h, time after which a new addition of the mixture of dust zinc (204.6 mg, 3.13 mmol), cuprous chloride (309.3 mg, 6.54 mmol) in anhydrous diethyl ether (5 mL) with methylidenediiodide (0.15 mL, 1.68 mmol) and iodine (7 mg) (this mixture was kept at reflux temperature with stirring for 1 h prior to the addition to the reaction mixture) was made. The reaction proceeded in the same conditions for more 53 h. After this, the reaction mixture was filtered through an oxide aluminium filtration plate and washed with ethyl acetate (50 mL) and diethyl ether (100 mL). The filtrates were concentrated and the residue was diluted with diethyl ether (150 mL) and washed with 5% aqueous HCl (3 x 50 mL), 5% aqueous $NaHCO_3$ (3 x 50 mL) and water (3 x 50 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to dryness giving a white solid residue. This residue was then purified by silica gel column chromatography (hexane/ethyl acetate) that allowed obtaining the pure compound **41** in 10.7 mg (21%). $Mp_{(hexane/ethyl\ acetate)}$ 120-123 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 1708 (C=O). ^1H NMR (600 MHz, $CDCl_3$) δ: -0.12 (1H, m, α cyclopropyl-H), 0.35 (1H, m, 3β–H), 0.55 (1H, m, 5α-H), 0.65 (1H, m, β cyclopropyl-H), 0.80 (1H, m, 4β–H), 0.83 (3H, s, 19-H_3), 0.85 (3H, s, 18-H_3), 2.05 (1H, ddd, $J_{16\alpha,16\beta}$=19.2, $J_{16\alpha,15\beta}$=$J_{16\alpha,15\alpha}$=9.1,

16α-H), 2.42 (1H, ddd, $J_{16\beta,16\alpha}$=19.2, $J_{16\beta,15\beta}$=8.9, $J_{16\beta,15\alpha}$=1.0, 16β-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 8.9 (C-3), 12.4 (CH_2 cyclopropyl), 12.7 (C-19), 13.9 (C-18), 14.2 (C-4), 19.3, 20.6, 21.8, 29.3, 30.9, 31.65, 32.3, 34.7, 35.1, 35.9, 47.8, 48.7, 51.5, 52.9, 221.4 (C-17). ESI: 285.5 ($[M-H]^+$, 100%).

5α-Androst-3-ene-17-thione (42).

To a solution of olefin **27a** (420.7 mg, 1.46 mmol) in anhydrous toluene (30 mL), *Lawesson's* reagent (624.9 mg, 1.54 mmol) was added and the reaction mixture was heated under reflux for 7 h, in an inert atmosphere. The remaining *Lawesson's* reagent was removed through a neutral alumina column leading to an orange residue, which was further purified by silica gel 60 column chromatography (petroleum ether 40-60 ºC) affording the pure compound **42** (238.5 mg, 54%), as a light orange solid. $Mp_{(petroleum\ ether\ 40\text{-}60ºC)}$ 95 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3015 (=CH), 1650 (C=C), absence of C=O (peak around 1715 in compound **8**). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.79 (3H, s, 19-H_3), 0.88 (3H, s, 18-H_3), 2.60 (1H, ddd, $J_{16\alpha\text{-}16\beta}$=22.0, $J_{16\alpha\text{-}15\beta}$=9.0, $J_{16\alpha\text{-}15\alpha}$=9.0, 16α-H), 2.93 (1H, ddd, $J_{16\beta\text{-}16\alpha}$=22.0, $J_{16\beta\text{-}15\beta}$=9.0, $J_{16\beta\text{-}15\alpha}$=1.0, 16β-H), 5.27 (1 H, ddd, $J_{4\text{-}3}$=9.5, $J_{4\text{-}5\alpha}$=4.0, $J_{4\text{-}2\alpha}$=2.0, 4-H), 5.54 (1 H, ddd, $J_{3\text{-}4}$=9.5, $J_{3\text{-}2\beta}$=6.0, $J_{3\text{-}2\alpha}$=3.0, 3-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.5 (C-19), 20.5 (C-18), 23.7, 26.1, 27.1, 29.9, 34.3, 36.7, 37.7, 38.3, 38.6, 48.5, 51.8, 55.7 (2 carbons), 62.1, 128.2 (C-4), 133.7 (C-3), 274.1 (C=S). ESI: 287.2 ($[M-H]^+$, 100%).

Scheme 1.10 - *Synthesis of aromatase inhibitors* ***45a*** *and* ***46a*** *from androstenedione (**1**)*

6-Methylenandrosta-4-ene-3,17-dione (2). As described in ***Scheme 1.1***

6α-Methylandrost-4-ene-3,17-dione (44).

To a solution of **2** (402.9 mg, 1.35 mmol) and cyclohexene (0.81 mL, 7.96 mmol) in absolute ethanol (26 mL), 5% Pd-C (80.6 mg, 0.76 mmol) was added. The suspension was set to reflux temperature with stirring. The UV spectra of aliquots were periodically determined at appropriate times and after 3 h 15 min it was observed a complete change from λ_{max} 260 nm to λ_{max} 240 nm, meaning that the starting material had been completely transformed. The catalyst was afterwards removed by filtration and the filtrate was concentrated under reduced pressure giving an oily residue. This residue was purified by column chromatography (hexane/ethyl acetate) allowing to isolate the pure compound **44** (276.4 mg, 71%) as a white solid. $Mp_{(hexane/ethyl\ acetate)}$ 164-166 ºC [lit.,[118,123] 166-168 ºC, 167-169 ºC]. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3050 (H-C=), 1739 (C=O), 1671 (C=C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.92 (3H, s, 18-H_3), 1.09 (3H, d, *J*=6.5, 6α-H_3), 1.21 (3H, s, 19-H_3), 5.80 (1H, bs, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) *δ*: 13.7 (C-18), 18.3 (C-20), 18.4 (C-19), 20.5, 21.7, 31.3, 33.6, 33.7, 35.0, 35.8, 35.9, 38.9, 39.7, 47.5, 50.7, 54.1, 121.6 (C-4), 173.5 (C-5), 199.6 (C-3), 220.3 (C-17).

6α-Methyl-5α-androst-3-en-17-one (45a) and 6α-methyl-5β-androst-3-en-17-one (45b).

To a boiling solution of **44** (259.3 mg, 0.863 mmol) in gacial acetic acid (40 mL), zinc dust <10 μm (1.48 g, 22.66 mmol) was added in several portions during 1 h.

After 3 h another amount of zinc dust (1.48 g, 22.66 mmol) was added, within 1 h. The reaction proceeded with a total of 5 h 15 min, although, without total transformation of the starting material. The zinc suspension was filtered, the zinc was washed with glacial acetic acid and the filtrate evaporated to dryness. The resulting residue was then diluted with water (100 mL) and extracted with diethyl ether (3 x 100 mL) and ethyl acetate (100 mL). The organic layer was washed with 10% aqueous $NaHCO_3$ (3 x 100 mL) and water (3 x 100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. The NMR analysis of this crude revealed to be composed by both isomers **45a** and **45b** in a relation of 1.7:1, respectively. This crude was then purified by silica gel column chromatography (hexane/ethyl acetate), which allowed isolating compound **45b** in 13% yield as a white solid residue. A mixture of isomers **45a** and **45b** (193.7 mg, 78% yield) was also obtained. A new silica gel column chromatography of this mixture allowed obtaining compound **45a** in 90% purity (NMR and HPLC control).

6α-Methyl-5α-androst-3-en-17-one (**45a**): IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3030 (H-C=), 1740 (C=O), 1651 (C=C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.80 (3H, s, 19-H_3), 0.87 (3H, s, 18-H_3), 0.93 (3H, d, J=6.1, 6α-H_3), 5.61 (1H, ddd, $J_{3,4}$=10.0, $J_{3,2\beta}$=6.5, $J_{3,2\alpha}$=3.3, 3-H), 5.67 (1H, ddd, $J_{4,3}$= 10.2, $J_{4,5\alpha}$=3.9, $J_{4,2\alpha}$=2.0, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 12.6 (C-19), 13.9 (C-18), 19.9 (C-20), 20.3, 21.7, 23.2, 29.5, 31.6, 34.2, 34.7, 35.3, 35.8, 35.9, 47.8, 51.4, 52.3, 53.3, 126.2 (C-3), 127.2 (C-4), 221.4 (C-17). ESI: 287.3 ($[M+H]^+$, 100%).

6α-Methyl-5β-androst-3-en-17-one (**45b**): $Mp_{(hexane/ethyl\ acetate)}$ 84-87 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3025 (H-C=), 1740 (C=O), 1647 (C=C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.86 (3H, s, 18-H_3), 0.96 (3H, d, J=6.9, 6-H_3), 0.97 (3H, s, 19-H_3), 2.03 (1H,

ddd, $J_{16\alpha,16\beta}$=18.9, $J_{16\alpha,15\beta}$=9.3, $J_{16\alpha,15\alpha}$=9.0, 16α-H), 2.42 (1H, dd, $J_{16\beta,16\alpha}$=19.2, $J_{16\beta,15\beta}$=8.8, 16β-H), 5.56 (1H, dd, $J_{4,3}$=10.3, $J_{4,5\beta}$=1.7, 4-H), 5.70 ($J_{3,4}$=9.8, $J_{3,2\alpha}$=3.5, 3-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.8 (C-18), 14.1 (C-20), 19.3 (C-19), 20.8, 21.8, 22.3, 22.7, 22.9, 29.3, 31.8, 34.3, 34.5, 35.6, 35.9, 40.8, 49.1, 51.1, 125.7 (C-4), 127.9 (C-3), 221.5 (C-17).

3α,4α-Epoxy-6α-methyl-5α-androstan-17-one (46a) and 3β,4β-epoxy-6α-methyl-5β-androstan-17-one (46b).

A stirred solution of a crude mixture of compounds **45a** and **45b** (112.4 mg, 0.39 mmol) in dichloromethane (30 mL) was treated with a solution of performic acid, generated *in situ* by the addition of 98-100% formic acid (0.10 mL) and 30% hydrogen peroxide (0.21 mL), at room temperature. The reaction mixture was stirred at room temperature for 9 h 30 min. Dichloromethane was then added (150 mL), and the organic layer was washed successively with 10% aqueous $NaHCO_3$ (100 mL) and water (4 x 100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness giving a slightly yellow oily residue. This residue was purified by silica gel column chromatography (hexane/ethyl acetate) allowing to isolate 52.0 mg (44%) of the pure 3α,4α-epimer **46a** as a white solid, and 34.6 mg (29%) of the pure 3β,4β-epimer **46b**.

3α,4α-Epoxy-6α-methyl-5α-androstan-17-one (**46a**): $Mp_{(hexane/ethyl\ acetate)}$ 153-156 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 1740 (C=O), 1013 (C-O). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.79 (3H, s, 19-H_3), 0.85 (3H, s, 18-H_3), 1.07 (3H, d, J=6.4, 6α-H_3), 2.43 (1H, dd, $J_{16\beta,16\alpha}$=19.2, $J_{16\beta,15\beta}$=8.7, 16β-H), 2.92 (1H, dd, $J_{4\beta,5\alpha}$=4.1, $J_{4\beta,6\beta}$=1.3, 4β-H), 3.15 (1H, dd, $J_{3\beta,2\beta}$=$J_{3\beta,2\alpha}$=3.4, 3β-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.8 (C-19), 13.9

(C-18), 19.8, 20.6, 20.9, 21.7 (C-20), 29.5, 30.8, 31.5, 34.4, 34.7, 35.9, 40.2, 47.7, 51.2, 51.9, 52.3, 53.3 (C-4), 53.4 (C-3), 221.0 (C-17). ESI: 303.1 ($[M+H]^+$, 100%).

3β,4β-Epoxy-6α-methyl-5β-androstan-17-one (**46b**): $Mp_{(hexane/ethyl\ acetate)}$ 83-87 ºC. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 1738 (C=O), 1050 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.84 (3H, s, 18-H_3), 0.87 (3H, s, 19-H_3), 1.06 (3H, d, J=6.9, 6α-H_3), 2.44 (1H, dd, $J_{16\beta,16\alpha}$=18.3, $J_{16\beta,15\beta}$=8.7, $J_{16\beta,15\alpha}$=0.8, 16β-H), 2.91 (1H, d, $J_{4\alpha,5\beta}$=3.9, 4α-H), 3.17 (1H, dd, $J_{3\alpha,2\alpha}$=$J_{3\alpha,2\beta}$=2.5, 3α-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.6 (C-18), 13.7 (C-19), 18.9, 20.2 (C-20), 22.1, 28.9, 29.5, 31.6, 33.6, 35.1, 35.5, 35.80, 35.81, 35.82, 42.6, 48.9, 51.1, 52.9 (C-4), 53.0 (C-3), 220.9 (C-17).

Scheme 1.11 *– Synthesis of 7α-allyl derivatives as aromatase inhibitors*

3-Oxoandrost-4-en-17β-yl acetate (18). As described in ***Scheme 1.5***

3-Oxoandrosta-4,6-dien-17β-yl acetate (47).

To a solution of **18** (3.8 g, 11.50 mmol) in acetic acid (50 mL) and toluene (10 mL), under a stream of nitrogen, chloranil (2.5 g, 10.17 mmol) was added and the reaction mixture was refluxed for 3 h (UV spectrophotometric control). After cooling, the mixture was extracted with dichloromethane (3 x 200 mL) and the organic layer was sequentially washed with 2.5 N aqueous NaOH, 10% aqueous $NaHCO_3$ (3 x 150 mL) and water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness yielding a crude product, which was purified by silica gel chromatography (petroleum ether 40-60 ºC/diethyl ether) gave the pure compound **47** (2.2 g, 58%), as a yellow crystalline solid. $Mp_{(ethyl\ acetate/n\text{-}hexane)}$ 141-143 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3024 (=CH), 1734 (C=O), 1658 (C=C), 1617 (C=C), 1030 (C-O). ^1H NMR ($CDCl_3$, 600 MHz) δ: 0.88 (3H, s, 18-H_3), 1.11 (3H, s, 19-H_3), 2.05 (3H, s, CH_3COO), 4.63 (1H, dd, $J_{17\alpha,16\alpha}$=$J_{17\alpha,16\beta}$=9.0, 17α-H), 5.67 (1H, s, 4-H), 6.09 (1H, d, $J_{6,7}$=10.4, 6-H), 6.12 (1H, dd, $J_{7,6}$ =10.4, $J_{7,8}$= 2.4, 7-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.9 (C-18), 16.3 (C-19), 20.2, 21.1, 23.1, 27.4, 33.8, 33.9, 36.0, 36.5, 37.3, 43.4, 48.0, 50.6, 82.1 (C-17), 123.8 (C-7), 128.2 (C-6), 139.9 (C-4), 163.4 (C-5), 171.1 (OC=O), 199.4 (C-3).

7α-Allyl-3-oxoandrost-4-en-17β-yl acetate (48).

To a solution of **47** (0.55 g, 1.67 mmol) in anhydrous dichloromethane (50 mL), under stirring and in an inert atmosphere, cooled at -78 °C, titanium tetrachloride (3.0

mL, 27.29 mmol) was added. After 5 min, a solution of allyltrimethylsilane (3.0 mL, 18.6 mmol) in anhydrous dichloromethane (5.0 mL) was carefully added to the previous solution. The resulting mixture was stirred for 40 min and then allowed to warm to 30 °C. After that, water was added, the mixture was extracted with dichloromethane (3 x 100 mL) and the organic layer was washed with 10% aqueous $NaHCO_3$ (2 x 100 mL) and water (3 x 100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness yielding an oily product (0.674 g). Purification of this product by silica gel flash column chromatography (petroleum ether 40-60 ºC/diethyl ether) afforded the pure compound **48** (0.35 g, 56%). $Mp_{(petroleum\ ether\ 40\text{-}60\ ºC/diethyl\ ether)}$ 150-152 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3012 (=CH), 1745 (C=O ester), 1722 (C=O), 1663 (C=C), 1041 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.84 (3H, s, 18-H_3), 1.20 (3H, s, 19-H_3), 2.04 (3H, s, CH_3COO), 4.61 (1H, dd, $J_{17\alpha,16\alpha}$=8.9, $J_{17\alpha,16\beta}$=8.2, 17α-H), 4.98 (1H, ddd, $CH=CH_2$), 5.02 (1H, ddd, $CH=CH_2$), 5.64 (1H, m, $CH=CH_2$), 5.71 (1H, s, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.9 (C-18), 17.9 (C-19), 20.7, 21.1, 22.8, 27.3, 30.2, 33.9, 35.9, 36.0, 36.1, 36.4, 38.3, 38.6, 42.5, 45.9, 46.9, 82.4 (C-17), 116.8 (C-24), 126.2 (C-4), 136.8 (C-23), 169.2 (C-5), 171.1 (C=O), 199.1 (C-3). ESI: 369.53 ($[M-H]^+$, 100%).

7α-Allyl-3-oxoandrost-4-en-17β-ol (49).

To a mixture of dioxane/water (85:15) (50 mL) with 2% aqueous NaOH (10 mL), compound **48** (0.62 g, 1.67 mmol) was added and the resulting solution was stirred overnight, at room temperature, after which chilled water was added leading to the formation of a precipitate. This precipitate was separate by filtration, dissolved in ethyl acetate/dichloromethane (4:1), and the resulting solution was dried over anhydrous $MgSO_4$ and concentrated to dryness yielding the pure compound **49** (0.57 g, 96%) as a white solid. $Mp_{(ethyl\ acetate/dichloromethane)}$ 216-218 ºC [lit.,[128] 208-210 ºC]. IR (NaCl plates,

$CHCl_3$) υ_{max} cm^{-1}: 3421 (OH), 1722 (C=O), 1645 (C=C), 1061 (C-O). ^1H NMR (600 MHz, DMSO-d_6) δ: 0.69 (3H, s, 18-H_3), 1.16 (3H, s, 19-H_3), 3.46 (1H, ddd, $J_{17\alpha,16\alpha}$=8.5, $J_{17\alpha,16\beta}$=8.5, $J_{17\alpha,17\beta\text{-OH}}$=4.8, 17α-H), 4.47 (1H, d, $J_{17\beta\text{-OH},17\alpha}$=4.8, 17β-OH), 4.94 (1H, ddd, CH=CH_2), 5.00 (1H, m, CH=CH_2), 5.56 (1H, s, 4-H), 5.72 (1H, m, CH=CH_2). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 11.0 (C-18), 17.4 (C-19), 20.4, 22.3, 29.6, 29.8, 33.6, 35.2, 35.5, 35.7, 36.0, 37.8, 38.2, 42.4, 45.5, 46.4, 79.8 (C-17), 116.1 (C-22), 125.2 (C-4), 137.4 (C-21), 169.2 (C-5), 197.5 (C-3). ESI: 327.31 ($[M-H]^+$, 100%).

General procedure to obtain 7α-allylandrost-4-ene-3,17-dione (50), 7α-allylandrost-4-en-17-one (53) and 7α-allylandrosta-1,4-diene-3,17-dione (56).

Jones reagent was added dropwise to solutions of **49**, **52** or **54** in acetone/dioxane (60:10), at 0 ºC, with stirring until the orange colour of the reagent remains. The excess of oxidant agent was destroyed by addition of 2-propanol. The reaction mixture was added to water (150 mL), extracted with ethyl acetate (3 x 100 mL) and the organic layer washed with 10% aqueous $NaHCO_3$ (2 x 100 mL) and water (2 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness.

7α-Allylandrost-4-ene-3,17-dione (50).

Jones reagent (2.6 mL); compound **49** (0.53 g, 1.61 mmol); acetone/dioxane (60:10) (70 mL); the residue obtained was insolubilized in diisopropylether giving the pure compound **50** (0.386 g, 73%) as a white crystalline compound. $Mp_{(n\text{-hexane/ethyl acetate})}$ 218-221 ºC [lit.,[128] 219-221 ºC]. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3076 (=CH), 1734 (C=O), 1669 (C=C). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.92 (3H, s, 18-H_3), 1.22 (3H, s, 19-H_3), 5.01 (1H, ddd, CH=C$\underline{H}_2$), 5.05 (1H, ddd, CH=C$\underline{H}_2$), 5.67 (1H, m, C$\underline{H}$=CH_2),

5.73 (1H, s, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.5 (C-18), 17.9 (C-19), 20.5, 21.3, 30.2, 31.1, 33.9, 35.4, 35.6, 35.9, 36.0, 38.1, 38.7, 46.7, 47.1, 47.5, 117.1 (C-22), 126.4 (C-4), 136.4 (C-21), 168.5 (C-5), 198.9 (C-3), 220.0 (C-17). ESI: 325.13 ($[M-H]^+$, 100%).

7α-Allylandrost-4-en-17-one (53).

Jones reagent (1.1 mL); compound **52** (134.7 mg, 0.43 mmol); acetone/dioxane (60:10) (23 mL); the solid residue obtained (129.3 mg) was purified by silica gel column chromatography (*n*-hexane/ethyl acetate) affording the pure compound **53** (105.6 mg, 79%) as a white crystalline solid. $Mp_{(n\text{-hexane/ethyl acetate})}$ 139-142 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3082 (=CH), 1734 (C=O), 1640 (C=C). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.89 (3H, s, 18-H_3), 1.05 (3H, s, 19-H_3), 2.44 (1H, ddd, $J_{16\beta,16\alpha}$=18.9, $J_{16\beta,15\beta}$=8.5, $J_{16\beta,15\alpha}$=1.3, 16β-H), 4.99 (2H, m, CH=C$\underline{H}_2$), 5.28 (1H, dd, $J_{4,3\alpha}$=2.2, $J_{4,3\beta}$=2.2, 4-H), 5.72 (1H, m, C$\underline{H}$=CH_2). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.5 (C-18), 19.2, 19.9 (C-19), 20.7, 21.4, 25.7, 29.5, 31.4, 35.2, 35.7, 35.7, 37.2, 37.9, 38.3, 46.9, 47.3, 47.7, 115.8 (C-22), 122.2 (C-4), 138.0 (C-21), 140.5 (C-5), 221.1 (C=O). ESI: 311.39 ($[M-H]^+$, 100%).

7α-Allylandrosta-1,4-diene-3,17-dione (56).

Jones reagent (0.3 mL); compound **54** (25.1 mg, 0.077 mmol); acetone/dioxane (60:10) (10 mL); the solid residue obtained (27.5 mg) was purified by silica gel column chromatography (*n*-hexane/ethyl acetate) affording the pure compound **56** (22.1 mg, 88%) as a white crystalline solid. $Mp_{(n\text{-hexane/ethyl acetate})}$ 205-207 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3076 (=CH), 1737 (C=O), 1661 (C=C), 1617 (C=C). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.95 (3H, s, 18-H_3), 1.26 (3H, s, 19-H_3), 5.01 (1H, ddd, CH=C$\underline{H}_2$),

5.08 (1H, ddd, CH=C<u>H</u>$_2$), 5.67 (1H, m, C<u>H</u>=CH$_2$), 6.06 (1H, dd, $J_{4,2}$=1.8, $J_{4,6\alpha}$=1.7, 4-H), 6.24 (1H, dd, $J_{2,1}$=10.1, $J_{2,4}$=1.8, 2-H), 7.05 (1H, d, $J_{1,2}$=10.1, 1-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.5 (C-18), 19.0 (C-19), 21.4, 21.7, 29.4, 30.9, 35.2, 35.3, 36.7, 37.7, 43.4, 44.9, 46.3, 47.6, 117.2 (C-22), 126.5 (C-4), 127.6 (C-2), 136.1 (C-21), 154.9 (C-1), 165.2 (C-5), 185.6 (C-3), 219.4 (C-17). ESI: 323.39 ($[M-H]^+$, 100%).

7α-Allylandrost-4-en-17β-yl acetate (51).

Sodium borohydride (202.0 mg, 5.33 mmol) was added in small portions to a stirred and cooled mixture of trifluoracetic acid (1.24 mL), glacial acetic acid (1.24 mL) and acetonitrile (1.24 mL) followed by a solution of **48** (400.2 mg, 1.08 mmol) in anhydrous dichloromethane (20 mL). The temperature was raised up at room temperature and the reaction was stirred under nitrogen, until all the starting material had been consumed (1 h 30 min, TLC control). The reaction mixture was then neutralized with 10% aqueous $NaHCO_3$, extracted with dichloromethane (4 x 100 mL) and the organic layer was washed with water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue (380.8 mg). This residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate) affording 293.8 mg of a fraction containing compound **51**, which was further purified by crystallization from ethanol/water giving the pure **51** (26.9 mg) as clear and bright needle-like crystals. $Mp_{(ethanol/water)}$ 129-130 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3076 (=CH), 1728 (C=O), 1041 (C-O). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.81 (3H, s, 18-H$_3$), 1.03 (3H, s, 19-H$_3$), 2.04 (3H, s, CH_3COO), 4.59 (1H, dd, $J_{17\alpha,16\alpha}$=8.5, $J_{17\alpha,16\beta}$=8.5, 17α-H), 4.96 (2H, m, CH=C<u>H</u>$_2$), 5.25 (1H, dd, $J_{4,3\alpha}$=2.3, $J_{4,3\beta}$=2.3, 4-H), 5.69 (1H, m, C<u>H</u>=CH$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.8 (C-18), 19.2, 19.9 (C-19), 20.9, 21.2, 22.9, 25.8, 27.4, 29.5, 35.3, 36.2, 36.7, 37.1, 37.9, 38.5, 42.6, 46.1, 47.2, 82.8 (C-17),

115.9 (C-24), 121.9 (C-4), 138.4 (C-23), 140.9 (C-5), 171.2 (C=O). ESI: 355.49 ([M-H]$^+$, 100%).

7α-Allylandrost-4-en-17β-ol (52).

From 51: Compound **51** (223.2 mg, 0.63 mmol) was added to a mixture of dioxane/water (85:15) (18 mL) with 2% aqueous NaOH (3.6 mL), at room temperature, and the reaction mixture was stirred until total transformation of the starting material (48 h, TLC control) being after this time neutralized with a solution of 5% aqueous HCl. The dioxane was evaporated under vacuum leading to a white solid residue that was then dissolved with ethyl acetate (50 mL), diluted with water (100 mL) and extracted with ethyl acetate (3 x 100 mL). The organic layer was washed with water (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness giving a white solid residue (209.3 mg). A portion of this residue was separated by silica gel column chromatography (*n*-hexane/diethyl ether) affording compound **52** in mixture with some impurities.

A pure sample of **52** was obtained from **53** by the following procedure:

From 53: To a stirring solution of **53** (15.3 mg, 0.049 mmol) in ethanol (3.0 mL), under nitrogen, at -10 ºC, sodium borohydride (0.6 mg, 0.016 mmol) was added. The mixture was stirred for 1 h and after two subsequent additions of sodium borohydride (0.6 mg, 0.016 mmol; 0.7 mg, 0.018 mmol), the reaction was stirred, at room temperature, overnight. The reaction mixture was then cooled at -10 ºC and 3 N aqueous HCl was added until pH 5 to 7. Ethanol was evaporated under vacuum and the oily residue obtained was poured in water (50 mL) and extracted with ethyl acetate (3 x 50 mL). The organic layer was then washed with water (3 x 100 mL), dried over

anhydrous $MgSO_4$, filtered and concentrated to dryness giving a solid residue (18.2 mg). This residue was washed with chilled *n*-hexane leading to the pure compound **52** (4.4 mg, 29%) as a white solid. $Mp_{(ethyl\ acetate)}$ 140-143 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3297 (OH), 3076 (=CH), 1640 (C=C), 1059 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.77 (3H, s, 18-H_3), 1.04 (3H, s, 19-H_3), 3.64 (1H, dd, $J_{17\alpha,16\alpha}$=8.6, $J_{17\alpha,16\beta}$=8.6, 17α-H), 4.96 (2H, m, CH=C$\underline{H}_2$), 5.25 (1H, dd, $J_{4,3\alpha}$=2.3, $J_{4,3\beta}$=2.3, 4-H), 5.70 (1H, m, C$\underline{H}$=CH_2). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 10.9 (C-18), 19.3, 19.9 (C-19), 21.1, 22.8, 25.8, 29.5, 30.4, 35.3, 36.2, 36.6, 37.2, 37.9, 38.8, 42.9, 46.3, 47.3, 82.0 (C-17), 115.5 (C-22), 121.8 (C-4), 138.5 (C-21), 141.1 (C-5). ESI: 313.49 ($[M-H]^+$, 100%).

7α-Allyl-3-oxoandrosta-1,4-dien-17β-ol (54).

To a stirred solution of **49** (538.7 mg, 1.64 mmol) in toluene (55 mL), DDQ (645.0 mg, 2.84 mmol) and benzoic acid (215.0 mg, 1.76 mmol) were added. After 18 h at reflux, an additional amount of DDQ (250 mg, 1.10 mmol) and benzoic acid (150 mg, 1.23 mmol) was added and the reaction proceeded for more 16 h. The reaction mixture was cooled at room temperature, filtered and the resulting filtrate was evaporated giving an oily residue, which was dissolved with dichloromethane and then mixed with silica gel. This mixture was filtered through basic alumina and washed with petroleum ether 40-60 ºC/ethyl acetate (8:2) (250 mL). Evaporation of the filtrate afforded a dark residue that was purified by silica gel column chromatography (toluene/diethyl ether) affording compound **54** (268 mg, 50%) as a pure solid. $Mp_{(petroleum\ ether\ 40\text{-}60\ ºC/toluene)}$ 175-178 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3378 (OH), 3018 (=CH), 1652 (C=C), 1053 (C-O). ^1H NMR (600 MHz, $CDCl_3$) δ: 0.83 (3H, s, 18-H_3), 1.24 (3H, s, 19-H_3), 3.65 (1H, dd, $J_{17\alpha,16\alpha}$=8.6, $J_{17\alpha,16\beta}$=8.6, 17α-H), 5.00 (1H, ddd, CH=C$\underline{H}_2$), 5.05 (1H, ddd, CH=C$\underline{H}_2$), 5.64 (1H, m, C$\underline{H}$=CH_2), 6.06 (1H, dd, $J_{4,2}$=1.8,

$J_{4,6\alpha}$=1.7, 4-H), 6.23 (1H, dd, $J_{2,1}$=10.1, $J_{2,4}$=1.8, 2-H), 7.06 (1H, d, $J_{1,2}$=10.1, 1-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 10.9, 19.1, 22.2, 22.9, 29.4, 30.2, 35.4, 36.1, 37.5, 38.2, 43.1, 43.6, 45.2, 45.9, 81.4 (C-17), 116.9 (C-22), 126.3 (C-4), 127.5 (C-2), 136.7 (C-21), 155.6 (C-1), 166.1 (C-5), 185.9 (C-3). ESI: 325.39 ($[M-H]^+$, 100%).

7α-Allyl-3-oxoandrosta-1,4-dien-17β-yl acetate (55).

To a solution of **54** (50.0 mg, 0.15 mmol) in anhydrous pyridine (4.0 mL), acetic anhydride (0.2 mL, 1.79 mmol) was added and the reaction was stirred for 33 h 15 min at room temperature, until all the starting material had been consumed (TLC control). Dichloromethane (100 mL) was added and the organic layer was washed with 10% aqueous $NaHCO_3$ (2 x 100 mL), 10% aqueous HCl (2 x 100 mL) and water (2 x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness. The resulting residue was crystalized from ethyl acetate/*n*-hexane giving the pure compound **55** (29.6 mg, 54%) as white needles. $Mp_{(\text{ethyl acetate/}n\text{-hexane})}$ 156-159 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3076 (=CH), 1731 (C=O), 1661 (C=C), 1617 (C=C), 1042 (C-O). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.87 (3H, s, 18-H_3), 1.24 (3H, s, 19-H_3), 2.04 (3H, s, CH_3COO), 4.59 (1H, dd, $J_{17\alpha,16\alpha}$=8.5, $J_{17\alpha,16\beta}$=8.5, 17α-H), 5.01 (1H, ddd, CH=C$\underline{H}_2$), 5.05 (1H, ddd, CH=C$\underline{H}_2$), 5.64 (1H, m, C$\underline{H}$=CH_2), 6.04 (1H, dd, $J_{4,2}$=1.6, $J_{4,6\alpha}$=1.5, 4-H), 6.23 (1H, dd, $J_{2,1}$=10.1, $J_{2,4}$=1.6, 2-H), 7.05 (1H, d, $J_{1,2}$=10.1, 1-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.9 (C-18), 19.2 (C-19), 21.1, 22.1, 23.1, 27.3, 29.4, 35.4, 36.3, 37.5, 38.0, 42.7, 43.5, 45.0, 45.7, 82.2 (C-17), 117.1 (C-24), 126.4 (C-4), 127.6 (C-2), 136.5 (C-23), 155.4 (C-1), 165.9 (C-5), 171.0 (C=O), 185.8 (C-3). ESI: 367.49 ($[M-H]^+$, 100%).

CHAPTER II

STEROIDAL 5α-REDUCTASE INHIBITORS AS ANTI-TUMORS AND AS DRUGS FOR THE TREATMENT OF BENIGN PROSTATE HYPERPLASIA (BPH)

INTRODUCTION

B.1. Prostate cancer and Benign Prostate Hyperplasia (BPH): Some Facts

B.2. Androgens and 5α-Reductase Enzyme

B.3. 5α-Reductase Inhibitors

B.3.1. Steroidal 5α-Reductase Inhibitors
B.3.2. Nonsteroidal 5α-Reductase Inhibitors

AIM OF THE WORK

2.1. STEROIDAL C-17 CARBOXY DERIVATIVES

2.1.1. Design and synthesis
2.1.2. Chemistry
2.1.3. Biochemistry and biology
2.1.4. Structure-activity relationships discussion and conclusions

2.2. EXPERIMENTAL SECTION

INTRODUCTION

B.1. Prostate Cancer and Benign Prostate Hyperplasia (BPH): Some Facts

Androgens are responsible for many physiological functions in both males and females. Their action is mediated by specific intracellular hormone receptors expressed in androgen responsive cells,[141] being essential for sex-determination and mature sexual development in men.[142] Among the several organs responsible for androgen production, testes produce these hormones in the greatest amount.[143] Testosterone (T), the major circulating androgen, is secreted by Leydig cells of the testes under the stimulation of pituitary-derived luteinizing hormone (LH).[141] The effects of these hormones in male accessory sex organs are mediated primarily through the reduction of T to dihydrotestosterone (DHT).[144] DHT is more potent than T in stimulating most of the androgen actions in the prostate, binding with greater affinity with androgen receptor.[145]

Despite their physiological role, androgens also play an important part in the genesis and progression of prostate cancer and benign prostate hyperplasia (BPH).[146-149] Besides these actions, an excessive accumulation of DHT may likewise cause male pattern baldness, acne, alopecia in men and hirsutism in women.[150]

Prostate cancer is typically a slow growth tumor that affects older men, being the average age at time of diagnosis of 70 years old. It is the most frequently diagnosed cancer and the leading cause of cancer death in the United States and United Kingdom (Figure 2.1).[2] There has been a significant increase in the clinical detection of prostate cancer due to the progressive nature of the disease and to the increase in life expectancy of the male population.[151]

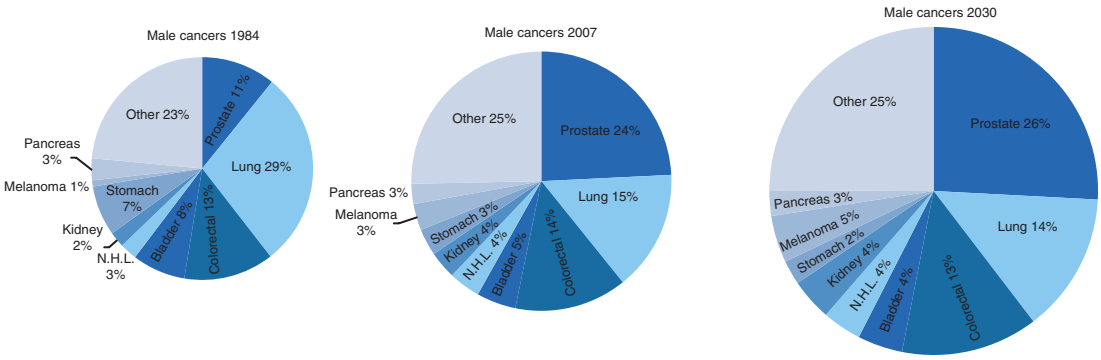


Figure 2.1 - Pie charts of the most common cancers in men in 1984, 2007 and predicted for 2030, in the United Kingdom[2]

BPH is a leading disorder of the elderly male population where there is an enlargement of the prostate gland due to an over-proliferation of the stromal and glandular elements of the prostate. The incidence of BPH is about 50% of males at the age of 50, 70% at the age of 70 and 90% of males by the age of 80, being the major responsible for men morbidity.[145,153]

B.2. Androgens and 5α-Reductase Enzyme

The conversion of T to DHT (Figure 2.2) is made by a microssomal steroid 5α-reductase enzyme (3-oxo-steroid-4-ene dehydrogenase), which works in a two membrane enzyme system together with NADPH, at the level of prostatic stromal and basal cells.[145,149,152]

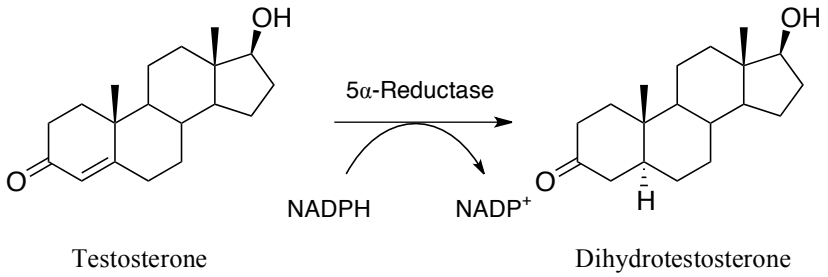


Figure 2.2 - Reduction of testosterone to dihydrotestosterone by 5α-reductase

In the proposed chemical mechanism for the above mentioned conversion (Figure 2.3),[145,149,150,152] it is considered the formation of a binary complex between the enzyme and NADPH, followed by the formation of a ternary complex with the substrate T. A delocalized carbocation is formed due to the activation of the enone system by a strong interaction with an electrophilic residue (Enz^+) present in the active site. Enolate of DHT is formed by the direct hydride ion transfer from NADPH to the α face of the delocalized carbocation leading to a selective reduction at C-5. This enolate, which is coordinated with the formed $NADP^+$ on the α face, is then attacked by a proton on the β-face at C-4 giving the ternary complex Enz-$NADP^+$-DHT. Binary $NADP^+$-enzyme complex is formed after departure of DHT and finally the release of $NADP^+$ leaves the enzyme free for further catalytic cycles.

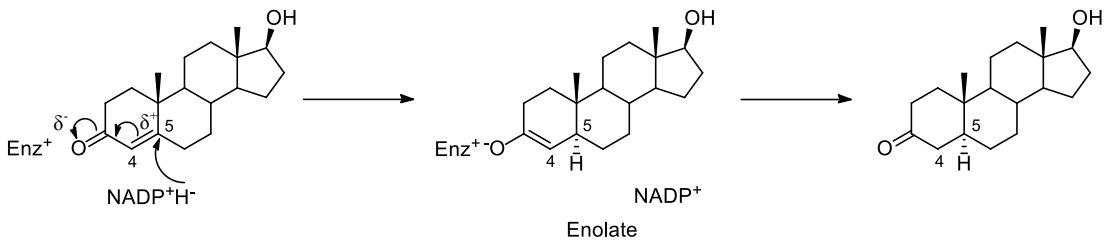


Figure 2.3 - Mechanism for 5α-reduction of testosterone

The crystal structure of 5α-reductase is not yet known due to the unstable nature of the enzyme not allowing its isolation and purification. Nevertheless, the modern molecular biology techniques allowed the identification of two different types of 5α-reductase enzyme: type I and type II.[145,152] They differ in several aspects, such as, size, molecular weight, localization, etc. The comparison of the properties of the two isoenzymes is summarized in Table 2.1.

Recently, a third type of 5α-reductase enzyme (type III) has been identified in hormone-refractory prostate cancer cells, and it is known that it also converts T to DHT, being active at pH 6.9. It is indeed a ubiquitous enzyme in mammals.[145]

Table 2.1 – Properties of human type I and type II 5α-reductase[145,152,154]

Properties	***Type I 5α-reductase***	***Type II 5α-reductase***
Amino acids	259	245
Molecular weight (Da)	29 462	27 000
Optimal pH	6-8.5	5-5.5
Biochemical properties	Hydrophobic	Hydrophobic
In vitro inhibition by Finasteride	IC_{50} = 410 nM	IC_{50} = 9.4 nM
In vitro inhibition by Dutasteride	IC_{50} = 2.4 nM	IC_{50} = 0.5 nM
Location	Peripheral skin, hair follicles	Prostate, male external genitalia
Selectivity to the inhibitors	Inhibitors with 4-methyl-4-aza functionalities are very potent	4-Aza, 6-aza and charged 3-substituents derivatives are very selective

B.3. 5α-Reductase Inhibitors

Based on the fact that the problems of the prostate are frequently associated to high DHT levels, the inhibition of 5α-reductase enzyme becomes a logical approach for their treatment. Hence, a large number of molecules have been synthesized as potential 5α-reductase inhibitors over the past years. In addition, several compounds may act as androgen receptor antagonists by preventing the natural ligands of the androgen receptor, such as T and DHT, from binding to the receptor. Combination of these two therapeutical approaches provides effective androgen receptor blockage.[145]

5α-Reductase inhibitors (RIs) are classified as steroidal and nonsteroidal, based on their structure.

B.3.1. Steroidal 5α-Reductase Inhibitors

The first inhibitors synthesized were designed upon modifications of the natural substrate T. One of the main modifications was the substitution of one carbon atom of the A-, B-, C- and D-rings of the steroid framework by a nitrogen heteroatom leading to the discovery of potent inhibitors of human 5α-reductase, such as 4-azasteroids, 6-azasteroids and 10-azasteroids. Therefore, there are several categories among these azasteroids, depending on the position of the nitrogen atom.[145,152,155,156]

Other derivatives, with a carboxylic acid group at the C-3 position were studied. They were designed to mimic the enzyme-bound enolate intermediate (Figure 2.3) by incorporating sp^2-hybridized centers at C-3 and C-4. Nitro, sulphonic acid, phosphonic acid and phosphinic acid derivatives also proved to be efficient inhibitors of 5α-reductase.[145]

Other categories of steroidal inhibitors developed were: 4-substituted, with cyano and trifluoromethyl groups, 16-methyl substituted derivatives, 6-methylidene derivatives, as well as other oximes and tetrahydrooxazin-2-ones derivatives.[145]

Among the azasteroid derivatives developed, two of them, finasteride and dutasteride, are nowadays clinically used.

Finasteride (Proscar®) was the first 5α-reductase inhibitor to be approved by the US Food and Drug Administration (FDA) (Figure 2.4). This inhibitor blocks the reduction of T through the inhibition of type II 5α-reductase isoenzyme in a very potent fashion, when compared to the inhibition of the type I (9.4 nM *versus* 410 nM of IC_{50}, respectively) (Table 1). At clinical dose, 5 mg/day, it causes a decrease of 65-80% of DHT levels in the plasma and studies have demonstrated an improvement in the symptoms of BPH.[145] The decrease in the DHT production results in an average reduction in the prostate volume of 20-25% over one year.[157] Finasteride and closed analogs are mechanism-based inactivators of type II 5α-reductase.[145] It is usually used in combination with an α-blocker* since it is expected to have additive benefit in the treatment of the symptomatic BPH.[157] Although, the Prostate Cancer Prevention Trial (PCPT) has demonstrated a reduced prevalence of prostate cancer with finasteride,[151,158,159] it has not been approved by the FDA to be used for prevention of prostate cancer (www.drugs.com/pro/finasteride), since it leads to the development of high grade prostate cancers.[160]

* **α-Blockers** – one of the classes of drugs used in BPH treatment; compounds such as doxazosin, alfuzosin and tansulosin are selective α_1-andrenergic receptor blockers that have lower side effects; their effect occurs through reducing smooth muscle tone in the prostate.[157]

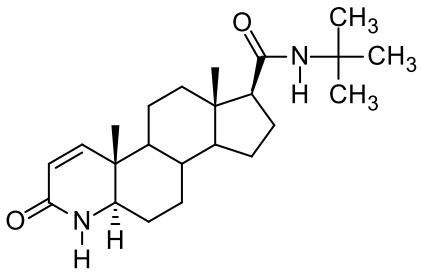


Figure 2.4 - Finasteride

Lately, in 2002, the FDA approved dutasteride (Avodart®) (Figure 2.5), which is currently available as a drug for the treatment of prostate diseases such as BPH and prostate cancer, and also for acne, male pattern baldness and hirsutism.[161]

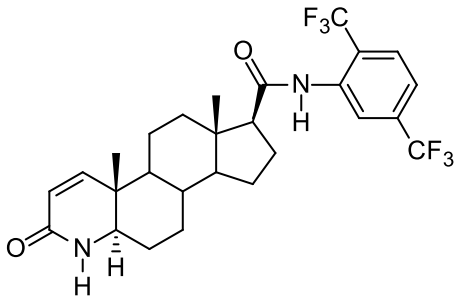


Figure 2.5 - Dutasteride

Dutasteride has emerged as the most potent dual inhibitor being highly selective towards type I and II 5α-reductase isoenzymes (Table 2.1), not binding to the androgen receptor. It reduces DHT levels over 90% following one year of oral administration,[162] and in spite of being a time-dependent inhibitor, it is 60 more potent than finasteride with a surprisingly long half-life.[141,145] Being a dual inhibitor, dutasteride is more beneficial than selective type II inhibitors since it does not allow the escape of DHT

formed through the type I isoenzymes. This provides a greater efficacy and consistency in the suppression of DHT production. Dutasteride is usually well tolerated with exception to what is reported in some literature where it is described that it alters libido and erectile dysfunction.[161]

Although the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial has presented a decrease in the absolute prostate cancer risk, dutasteride has not been approved by the FDA to be used for prevention of prostate cancer, since it also leads to the development of high grade prostate cancer.[151,159,163]

B.3.2. Nonsteroidal 5α-Reductase Inhibitors

A number of classes of nonsteroidal RIs have emerged over the last years. Due to the potential interaction with other enzymes or receptors with hormonal action exhibited by steroidal compounds, the research towards the discovery of nonsteroidal inhibitors has gained some importance in the last years.[145,163]

Nonsteroidal RIs can be classified according to their structure. Their design is based on (aza)steroidal inhibitors, generally by removing one or more rings from the (aza)steroidal structure. These derivatives are generally thought to act by mimicking T, being competitive inhibitors of 5α-reductase. They include benzo[f]quinolinones, pyridones and quinolinones, benzo[c]quinolinones and benzo[c]quinolizinones which mimic 4-azasteroid, 6-azasteroid and 10-azasteroid inhibitors, respectively, and also indol derivatives.[145,156] Among these latest derivatives (the indol group), compound FK-143 (Figure 2.6) was disclosed as a potent dual inhibitor[145,164-166] being expected to be available for clinical use in the treatment of BPH.

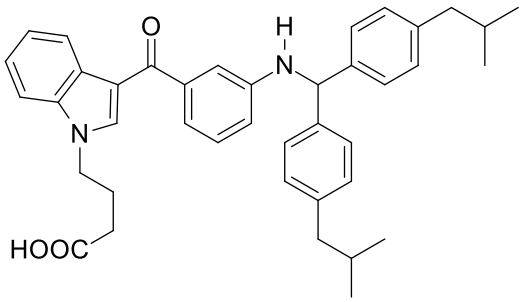


Figure 2.6 - FK-143

AIM OF THE WORK

Although finasteride and dutasteride are the only two 5α-reductase inhibitors clinically used nowadays, the research in this field has been very active over the last 40 years and many molecules have been prepared, some of them with very promising results. Though the most recent research of these inhibitors has been focused on azasteroidal derivatives, in fact the first inhibitors designed were based on modifications of the natural substrate of the enzyme, T.

Since the diseases of the prostate have a huge incidence in the older men, and once the population is aging, it seems really important to continue searching for new 5α-reductase inhibitors, with higher potency but with less side effects such as decrease in bone and muscle mass, and impotency. Therefore, we focused on the design, synthesis and on the establishment of new SAR of new RIs. With these molecules, we intend to investigate the biological effects of the most potent screened compounds. It is our hope to contribute for the development of new potent RIs.

2.1. STEROIDAL C-17 CARBOXY DERIVATIVES

2.1.1. Design and synthesis

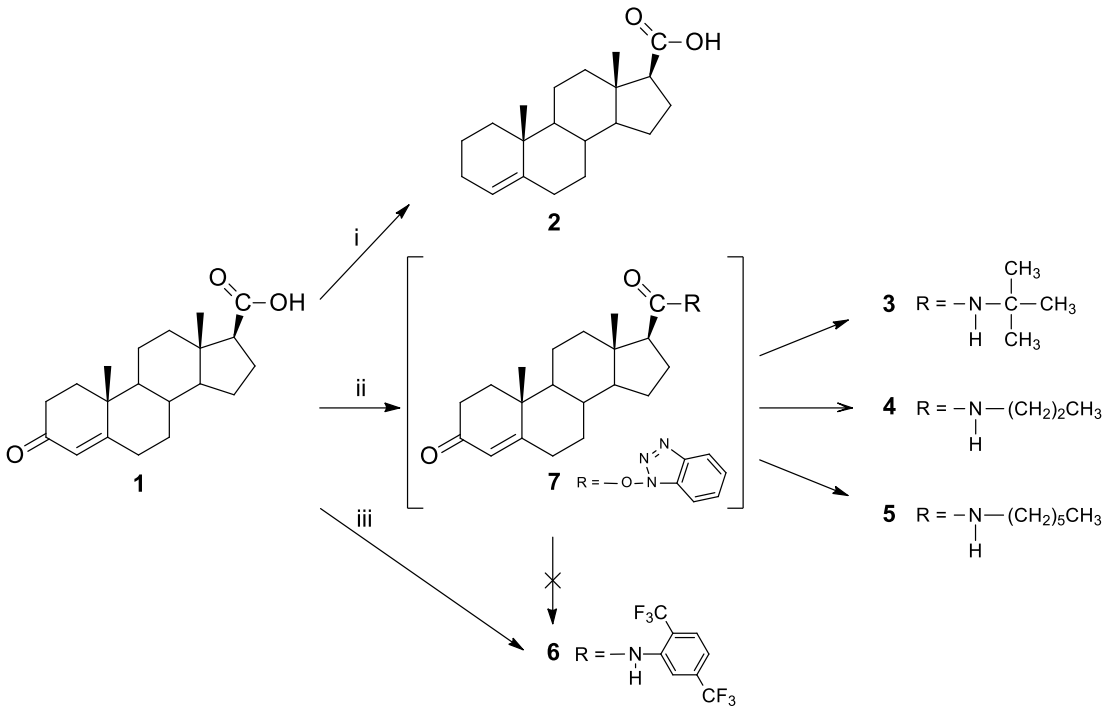
In the A-ring of T, the C-3 carbonyl group works presumably as an H-bond acceptor from a residue in the enzyme, which would donate a hydrogen to stabilize the intermediate enolate. In addition, it has been described the importance of a sp^2-hybridized center at C-3 and C-4 for the 5α-reductase inhibitory activity.[145] Further, it was also observed that the enzyme tolerated a wide variety of side chains at the 17β-position,[167] and the presence at C-17β of lipophilic side chains, containing amide or ester groups, enhances potency by binding to a lipophilic pocket on the enzyme.[145] Besides this, previous studies have highlighted the importance of the 3-keto-Δ^4-androstan-17β-carboxamide steroids as RIs. [149,168,169]

Based on this, we were interested in designing and synthesizing steroids keeping the 3-keto-Δ^4 moiety in the A-ring, as in T, and having a carboxamide, carboxyester or carboxylic acid function at the C-17β position (Scheme 2.1).

The rationale particularly focuses on the synthesis of derivatives of finasteride (Figure 2.4) and dutasteride (Figure 2.5) obtained by combining in the same molecule, the A-ring of the substrate T with the C-17β carboxamide group of the referred RIs (Scheme 2.1).

Compound **1** (Scheme 2.1) was used as starting material in the synthesis of these derivatives and itself was previously described as a potent inhibitor of 5α-reductase, for the microsomal enzyme of human skin.[145,152,170,171]

Scheme 2.1 - Synthesis of 5α-reductase inhibitors from 4-androsten-3-one-17β-carboxylic acid (**1**)



Reagents and conditions: (i) CF_3COOH, CH_3COOH, CH_3CN, $NaBH_4$, anhydrous dichloromethane, rt, 3 h 30 min; (ii) dimethylformamide, triethylamine, *tert*-butylamine, or *n*-propylamine, or *n*-hexylamine, or 2,5-bis-(trifluoromethyl)aniline, BOP, dichloromethane, rt; (iii) 1st: $SOCl_2$, anhydrous tetrahydrofuran, anhydrous pyridine, rt, 40 min; 2nd: 2,5-bis-(trifluoromethyl)aniline, anhydrous tetrahydrofuran, anhydrous pyridine, 66 ºC, 5 h.

The rationale behind the design of compound **2** was to study the effect of the absence of the C-3 carbonyl group in the inhibition of the enzyme, maintaining the carboxylic acid function at the C-17β. This compound was prepared by reaction conditions of Hanson *et al.*[81] where it was used sodium borohydride in a mixture of trifluoroacetic acid, acetonitrile, acetic acid and dichloromethane.

Concerning the amides **3**, **4**, **5** and **6** (Scheme 2.1), they combine in the same molecule, the A-ring of the substrate T and different C-17β carboxamide groups,

namely the C-17β carboxamide group of finasteride (compound **3**) and the C-17β carboxamide group of dutasteride (compound **6**). The other amides, with different *N*-alkyl chain lengths (**4** and **5**), were synthesized in order to evaluate the effect of the chain length in the enzyme inhibition. Amide derivatives **3** to **5** were prepared by reacting **1** with *tert*-butylamine, *n*-propylamine or *n*-hexylamine, in dimethylformamide (DMF) and dichloromethane, using triethylamine as catalyst and the coupling agent (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP). The attempt to prepare amide **6** by this procedure, led to carboxyester **7**. Amide **6** was then prepared by reaction of **1** with thionyl chloride followed by reaction of the resulting acyl chloride with 2,5-bis-(trifluoromethyl)aniline.

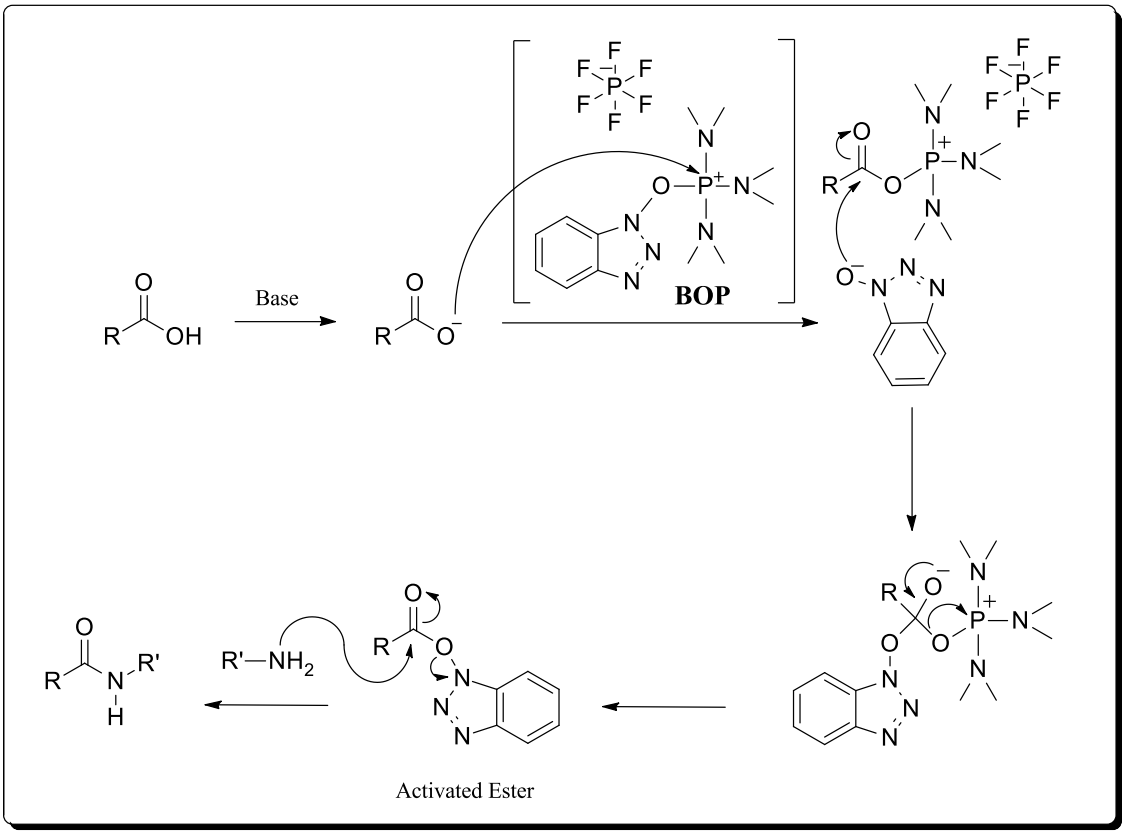
2.1.2. Chemistry

The synthesis of compound **2** (Scheme 2.1), which is the C-3 deoxygenated derivative of **1**, was performed by submitting **1** to a reduction with sodium borohydride in a mixture of trifluoroacetic acid, glacial acetic acid, and acetonitrile giving **2**, in 71% yield.[81]

The amides **3**, **4** and **5** (Scheme 2.1) were obtained in 69%, 54% and 35% yield, respectively, by reacting carboxylic acid **1** with *tert*-butylamine, *n*-propylamine or *n*-hexylamine, in DMF and dichloromethane, using triethylamine as catalyst and the coupling agent BOP, which promotes the activation of the carboxylic acid **1** for further nucleophilic acyl substitution, through the mechanism presented in Scheme 2.2.[172,173]

Although amide **3** has already been prepared in 89%[174] and in 95%[175] using thionyl chloride followed by *tert*-butylamine, in this work we present a one-step reaction using milder conditions.

Scheme 2.2 - Mechanism for amide synthesis using BOP as activating agent



An attempt to prepare amide **6** from **1** (Scheme 2.1) using the above mentioned conditions and the amine 2,5-bis-(trifluoromethyl)aniline, afforded compound **7** in 73% yield. In this case, the activated intermediate ester **7** was unable to react with the amine 2,5-bis-(trifluoromethyl)aniline to give **6**, probably due to the weak nucleophilicity of the deactivated aromatic amine 2,5-bis-(trifluoromethyl)aniline. In order to overcome this limitation, amide **6** was obtained by a two-step reaction strategy.[141] In the first step, the carboxylic acid **1** was activated to the respective acyl chloride by treatment with thionyl chloride, in anhydrous tetrahydrofuran and in the presence of anhydrous pyridine. This step allowed to transform the initial carboxylic acid into the acyl halide. In the second step, the acyl chloride was subjected to reaction with 2,5-bis-

(trifluoromethyl)aniline in anhydrous tetrahydrofuran and in the presence of anhydrous pyridine providing compound **6**, in 35% yield.

2.1.3. Biochemistry and biology

The 5α-reductase inhibitory activity of each steroidal compound was evaluated in human prostatic microsomes by a DLLME-GC-MS method previously developed by our group, that identifies and quantifies T and DHT, using the internal standards T-d_3 and DHT-$^{13}C_3$.[176] The ratio between T/T-d_3 and DHT/DHT-$^{13}C_3$ allowed the quantification of T and DHT produced after 5α-reductase reaction estimating the anti-5α-reductase activity of each compound.[177]

The percentage of 5α-reductase inhibition (%) of the synthesized RIs was made using the RI finasteride as reference compound (Table 2.2).[178] For compounds **3**, **4** and **5**, with percentage of 5α-reductase inhibition higher than 60%, the IC_{50} was also determined (Table 2.2).[177]

The effects of the most potent RIs **3**, **4**, **5** and finasteride were investigated in the viability of LNCaP cells, which is a human androgen-responsive prostate cancer cell line. It was observed that the tested compounds induced a significant decrease in cell viability, being compound **3** the most potent, even more than finasteride.[177]

2.1.4. Structure-activity relationships discussion and conclusions

In this study, it was investigated the inhibitory activity of 5α-reductase of a set of synthesized steroids. It is known that one of the key structural requirements for 5α-reductase inhibition is the presence of a 4-en-3-one system in the A-ring and a lipophilic

17β-side chain in the D-ring of steroids.[145] Furthermore, it has been found that the presence at C-17β position of lipophilic amides or esters enhances potency by binding to a lipophilic pocket on the enzyme.[145] We have investigated these features by studying different C-17β chemical modifications (amide, ester or carboxylic acid functions) in the potential steroidal RIs.

Table 2.2 – Percentage of 5α-reductase inhibition and IC_{50} values of tested compounds in human prostatic microsomes[177]

Compounds	5α-Reductase Inhibition (%) ± SEM	IC_{50} (μM)
1	29.55 ± 3.09	-
2	11.04 ± 3.42	-
3	73.08 ± 3.05	0.37
4	69.33 ± 1.03	0.46
5	63.56 ± 1.08	0.61
6	22.44 ± 2.03	-
7	49.20 ± 2.51	-
Finasteride	84.62 ± 1.21	0.096[167]

Based on the results obtained (Table 2.2), it is possible to infer that C-17β carboxylic acid analogs (**1** and **2**) (Scheme 2.1) of T are weak RIs, particularly the 3-deoxo analog **2**. In fact, several descriptions referred the importance of a sp^2-hybridized center at C-3 and C-4 positions of steroids for the 5α-reductase inhibitory activity.[145] Therefore, the lack of that center at C-3 as in **2**, results in a decrease in the activity. Concerning analog **1**, it was already reported as a competitive RI, with 87.7 %

inhibition for the microsomal 5α-reductase enzyme of human skin.[145,179] However, in our experimental conditions, using the prostate microsomal enzyme, analog **1** showed only 29.55 % of inhibition.

Among the amides synthesized and evaluated, the *N*-*tert*-butylcarboxamide **3** (Scheme 2.1) showed the best inhibitory activity. In this case, we combined in the same molecule, the A-ring of the substrate T and the C-17β carboxamide group of finasteride (Figure 2.4), which resulted in a potent inhibitor.

Regarding the *N*-propylcarboxamide **4** and the *N*-hexylcarboxamide **5** (Scheme 2.1) it is possible to infer that these steroids are also strong inhibitors (IC_{50} of 0.46 and 0.61 μM, respectively) (Table 2.2). Further, the *N*-propylcarboxamide **4** is slightly more active than the *N*-hexylcarboxamide **5**, however both of them are less active than *N*-*tert*-butylcarboxamide **3**, suggesting that a hindered *N*-alkyl group, as in **3**, or a shorter *N*-alkyl linear chain, as in **4**, in the C-17β carboxamide function favors the 5α-reductase inhibitory activity.

Concerning compound **6**, in spite of having in the C-17β position the same group as the potent dutasteride, the *N*-[2,5-bis(trifluoromethyl)phenyl]carboxamide, surprisingly it only exhibits minimal inhibitory activity (22.44 %). This result reveals that along with the C-17β carboxamide group, the steroidal A-ring type also determines the potency of inhibitors. Regarding the ester derivative **7**, our results demonstrate that it is a moderate inhibitor (49.20 % inhibition).

In this study, it was also evaluated the effect of the most potent RIs, steroids **3**, **4**, **5** and finasteride in LNCaP cells viability. This cell line is a human androgen-responsive prostate cancer cell line, that is a good model to study hormonal therapies for this disease.[180,181] This cell line responds positively to growth stimulation induced by androgens *in vitro* and *in vivo*.[182,183]

The results obtained with T-treated LNCaP cells with RIs **3**, **4**, **5** and finasteride indicate that compounds induced a significant decrease in cell viability, being steroid **3** the most potent, even more than finasteride.

Further, our results suggested that these steroids induced the reduction in cell viability in a 5α-reductase-dependent manner, by inhibiting 5α-reductase and the conversion of T into DHT.

In summary, taking into account the studied compounds, it is possible to conclude that the C-17β lipophilic carboxamide chemical groups along with the 3-keto-Δ^4 moiety in the A-ring of the steroidal framework, seem to be favourable key features for achieving 5α-reductase inhibitory activity, being the steroid with the C-17 *N-tert*-butlycarboxamide group (**3**), the best RI. Furthermore, steroids **3**, **4** and **5** as well as finasteride induced a decrease in viability of stimulated LNCaP cells in a 5α-reductase dependent-manner, being the synthesized steroids **3**, **4**, and **5** even more effective than finasteride. This study will help in the future design of new steroidal RIs contributing to the discovery of new drugs with fewer side effects.

2.2. EXPERIMENTAL SECTION

Melting points (mps) and IR spectra were collected as mentioned before (section **1.6.**), except for compound **3** whose IR was recorded using the ATR (attenuated total reflectance) device. ^{1}H, ^{13}C NMR, ESI-MS and LC-MS mass spectra were recorded as mentioned in section **1.7**.

Reactions were monitored by thin layer chromatography (TLC) in silica gel 60 F_{254} aluminium sheets, as described in section **1.7**.

4-Androsten-3-one-17β-carboxylic acid (**1**) was purchased from Fountain Limited (Malta). Reagents and solvents were used as obtained from suppliers without further purification, with exception to dichloromethane,[138] and pyridine,[139] which were dried through reflux and distillation from CaH_2, being stored away from light in a brown bottle with type 4Å molecular sieves, under an atmosphere of dry N_2.

Yields have not been optimized.

All compounds possess a purity superior to 98%. The purity was checked by HPLC with a C18-reversed phase column and water/acetonitrile 30:70 as solvent. The purity of individual compounds was determined from the peak areas in the chromatogram of the sample solution.

Scheme 2.1 - *Synthesis of 5α-reductase inhibitors from 4-androsten-3-one-17β-carboxylic acid (**1**)*

4-Androstene-17β-carboxylic acid (2).

Sodium borohydride (590.3 mg, 15.6 mmol) was added in small portions to a cooled and stirred mixture of trifluoroacetic acid (3.6 mL), glacial acetic acid (3.6 mL) and acetonitrile (3.6 mL). After this, a solution of compound **1** (1.0 g, 3.16 mmol) in dry dichloromethane (53 mL) was added and the reaction proceeded at room temperature, under a stream of dry nitrogen, until all the starting material had been consumed (3 h 20 min by TLC). The reaction mixture was then neutralized with 10% aqueous $NaHCO_3$, extracted with chloroform (4 x 100 mL) and the organic layer washed with water (4 x 100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. Crystallization from ethyl acetate/hexane afforded the pure compound **2** (680.5 mg, 71%) as white crystals. $Mp_{(ethyl\ acetate/hexane)}$ 205-208 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3399 (O-H carboxylic acid), 3020 (H-C=), 1701 (C=O carboxylic acid), 1676 (C=C), 1216 (C-O carboxylic acid). ^1H NMR ($CDCl_3$, 600 MHz) δ: 0.75 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 2.38 (1H, dd, $J_{17\alpha,16\alpha}$=9.4, $J_{17\alpha,16\beta}$=9.4, 17α-H), 5.30 (1H, bs, 4-H), 10.46 (1H, bs, COOH). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.3 (C-18), 19.2 (C-19), 19.4, 21.2, 23.4, 24.5, 25.7, 32.5, 33.2, 36.1, 37.1, 37.8, 38.2, 44.3, 54.4, 55.1, 55.9, 119.3 (C-4), 144.7 (C-5), 179.9 (C_{20}=O). ESI: 303.4 ($[M+H]^+$, 100%).

General method for obtaining N-tert-butyl-3-oxoandrost-4-ene-17β-carboxamide (3), N-propyl-3-oxoandrost-4-ene-17β-carboxamide (4), N-hexyl-3-oxoandrost-4-ene-17β-carboxamide (5) and 1H-benzo[d][1,2,3]triazol-1-yl 3-oxoandrost-4-ene-17β-carboxylate (7).

Compound **1** was dissolved in dimethylformamide and triethylamine. The solution was cooled in an ice-water bath and the amine was added followed by a solution of BOP in dichloromethane. The reaction mixture was stirred at 0 ºC for 30 min and then at room temperature for 1 h 20 min to 3 h. Dichloromethane was then removed under reduced pressure and the resulting solution diluted with water (25 mL) and extracted with ethyl acetate (2 x 25 mL). The organic layer was washed successively with 1 N aqueous HCl (3 x 25 mL), water (25 mL), 1 M aqueous $NaHCO_3$ (3 x 25 mL) and water (2 x 25 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to dryness. The residue obtained was purified by silica gel column chromatography (petroleum ether 40-60 ºC/ethyl acetate) giving the pure compounds.

***N-tert*-Butyl-3-oxoandrost-4-ene-17β-carboxamide (3).**

Compound **1** (200.0 mg, 0.632 mmol); dimethylformamide (1.3 mL); triethylamine (0.1 mL, 0.72 mmol); *tert*-butylamine (0.1 mL, 0.95 mmol); BOP (279.1 mg, 0.63 mmol); dichloromethane (1.6 mL); reaction time: 3 h; yield (162.0 mg, 69%) as white solid. $Mp_{(\text{petroleum ether 40-60 ºC/ethyl acetate})}$ 219-221 ºC [Lit.[174] 218-219 ºC from acetone]. IR (ATR) υ_{max} cm^{-1}: 3366 (N-H), 1661 (C=O amide and C=O ketone), 1615 (C=C). ^1H NMR ($CDCl_3$, 600 MHz) δ: 0.72 (3H, s, 18-H_3), 1.18 (3H, s, 19-H_3), 1.35 (9H, s, 3x -CH_3), 5.08 (1H, s, -NH), 5.73 (1H, s, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.1 (C-18), 17.4 (C-19), 21.0, 23.2, 24.4, 29.0 (3x CH_3), 31.9, 32.8, 34.0, 35.6, 35.7,

38.5, 38.6, 43.5, 51.1, 53.8, 55.6, 57.5, 123.9 (C-4), 171.1 (C-5), 171.6 (C_{20}=O), 199.5 (C_3=O). ESI: 372.6 ($[M+H]^+$, 100%).

***N*-Propyl-3-oxoandrost-4-ene-17β-carboxamide (4).**

Compound **1** (200.0 mg, 0.632 mmol); dimethylformamide (1.3 mL); triethylamine (0.1 mL, 0.72 mmol); *n*-propylamine (0.1 mL, 0.95 mmol); BOP (279.1 mg, 0.63 mmol); dichloromethane (1.6 mL); reaction time: 1 h 20 min; yield (122.6 mg, 54%). $Mp_{(petroleum\ ether\ 40\text{-}60\ ºC/ethyl\ acetate)}$ 147-149 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3368 (N-H), 1656 (C=O amide and C=O ketone), 1617 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$, 600 MHz) δ: 0.72 (3H, s, 18-H3), 0.92 (3H, t, *J*=7.4Hz, -CH_3), 1.18 (3H, s, 19-H3), 2.40 (1H, dd, $J_{17\alpha,16\alpha}$=14.6, $J_{17\alpha,16\beta}$=4.9, 17α-H), 3.12-3.33 (2H, m, -CH_2-), 5.38 (1H, bs, -NH), 5.72 (1H, s, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 11.4 (C-18), 13.2 (-CH_3), 17.3 (C-19), 20.9, 23.1, 23.6, 24.4, 31.9, 32.8, 33.9, 35.6, 35.7, 38.6, 41.2, 43.6, 53.8, 55.5, 57.0, 123.8 (C-4), 171.1 (C-5), 172.4 (C_{20}=O), 199.5 (C_3=O). ESI: 358.8 ($[M+H]^+$, 100%).

***N*-Hexyl-3-oxoandrost-4-ene-17β-carboxamide (5).**

Compound **1** (177.8 mg, 0.562 mmol); dimethylformamide (1.3 mL); triethylamine (0.09 mL, 0.64 mmol); *n*-hexylamine (0.11 mL, 0.84 mmol); BOP (167.2 mg, 0.38 mmol); dichloromethane (1.6 mL); reaction time 3 h; yield (78.4 mg, 35%). $Mp_{(petroleum\ ether\ 40\text{-}60\ ºC/ethyl\ acetate)}$ 56-61 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3389 (N-H), 1656 (C=O amide and C=O ketone), 1621 (C=C). ^1H NMR ($CDCl_3$, 600 MHz) δ: 0.72 (3H, s, 18-H3), 0.87 (3H, t, *J*=7.0 Hz, -CH_3), 1.18 (3H, s, 19-H3), 2.40 (1H, dd, $J_{17\alpha,16\alpha}$=14.5, $J_{17\alpha,16\beta}$=5.0, 17α-H), 3.15-3.35 (2H, m, -CH_2-), 5.34 (1H, bs, -NH), 5.72 (1H, s, 4-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.2 (C-18), 13.9 (-CH3), 17.4 (C-19),

20.9, 22.5, 23.6, 24.4, 26.6, 29.8, 31.4, 31.9, 32.8, 33.9, 35.6, 35.7, 38.3, 38.6, 39.5, 43.6, 53.8, 55.5, 57.0, 123.9 (C-4), 171.1 (C-5), 172.3 (C_{20}=O), 199.4 (C_3=O). ESI: 400.6 ($[M+H]^+$, 100%).

1H-Benzo[d][1,2,3]triazol-1-yl 3-oxoandrost-4-ene-17β-carboxylate (7).

Compound **1** (200.1 mg, 0.632 mmol); dimethylformamide (1.3 mL); triethylamine (0.1 mL, 0.72 mmol); 2,5-bis(trifluoromethyl)aniline (0.15 mL, 0.95 mmol); BOP (279.8 mg, 0.63 mmol); dichloromethane (3 mL); reaction time: 5 h, without completion. Crystallization from ethyl acetate gave the pure compound **7** (200.5 mg, 73%) as white shiny needles. $Mp_{(ethyl\ acetate)}$ 210-213 ºC. IR (NaCl plates, $CDCl_3$) υ_{max} cm^{-1}: 3311 (H-C= aromatic), 3021 (H-C=), 1809 (C=O ester), 1669 (C=C), 1616 and 1447 (C=C arom), 1070 (C-O ester). ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 0.98 (3H, s, 18-H3), 1.22 (3H, s, 19-H3), 2.86 (1H, dd, $J_{17\alpha,16\alpha}$=9.4 Hz, $J_{17\alpha,16\beta}$=9.4 Hz, 17α-H), 5.75 (1H, s, 4-H), 7.41 (1H, m, *J*=7.4, *J*=0.9, *J*=0.9, 3'-H or 6'-H), 7.42 (1H, m, *J*=7.9, *J*=7.1, *J*=0.8, 4'-H or 5'-H), 7.54 (1H, m, J=7.9, J=7.1, J=0.8, 4'-H or 5'-H), 8.06 (1H, m, *J*=7.4, *J*=0.9, *J*=0.9, 3'-H or 6'-H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.8 (C-18), 17.4 (C-19), 20.9, 24.0, 24.5, 31.8, 32.7, 33.9, 35.70, 35.74, 37.9, 38.6, 45.2, 52.7, 53.5, 55.5, 108.2 (C-4), 120.5, 124.0, 124.7, 128.5, 128.6, 143.5, 170.1 (C-5), 170.4 (C_{20}=O), 199.3 (C_3=O). ESI: 434.5 ($[M+H]^+$, 100%).

17β-*N*-[2,5-bis(Trifluoromethyl)phenyl]3-oxoandrost-4-ene-17β- carboxamide (6).

A solution compound **1** (400.0 mg, 1.26 mmol) in anhydrous tetrahydrofuran (15 mL) and anhydrous pyridine (0.1 mL), under a stream of dry nitrogen, was set at 2 ºC. To this solution, thionyl chloride (0.12 mL, 1,64 mmol) was added and the reaction mixture was stirred at 2 ºC for 20 min and then at room temperature for 40 min. The

reaction mixture was then filtered and the obtained residue washed with anhydrous toluene. The filtered was concentrated under vacuum giving a yellow oil which was diluted with anhydrous tetrahydrofuran (20 mL) and anhydrous pyridine (0.1 mL). To the resulting solution it was added 2,5-bis-(trifluoromethyl)aniline (0.22 mL, 1.39 mmol) and the reaction mixture was refluxed for 5 h. After that, it was diluted with chloroform (300 mL), and the resulting organic layer was then washed with 1 N aqueous HCl (4 x 100 mL), brine (4 x 100 mL) and water (100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue obtained was purified by silica gel column chromatography (hexane/ethyl acetate) giving the pure compound **6** (230.9 mg, 35%) as a white solid. $Mp_{(hexane/\ ethyl\ acetate)}$ 204-207 ºC. IR (NaCl plates, $CHCl_3$) υ_{max} cm^{-1}: 3315 (H-C= aromatic), 3017 (H-C=), 1706 (C=O ketone), 1664 (C=O amide), 1645 (C=C), 1616 and 1473 (C=C aromatic), 1137 (C-F), 1591 (N-H). ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 0.82 (3H, s, 18-H3), 1.19 (3H, s, 19-H3), 5.74 (1H, s, 4-H), 7.44 (1H, d, $J_{4',3'}$=8.2, 4'-H), 7.50 (1H, s, 6'-H), 7.72 (1H, d, $J_{3',4'}$=8.2, 3'-H), 8.78 (1H, s, -NH). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.2 (C-18), 17.4 (C-19), 20.9, 23.6, 24.3, 31.9, 32.7, 33.9, 35.6, 35.7, 37.9, 38.6, 44.5, 53.6, 55.7, 58.4, 120.2 (C-4'), 120.4 (C-6'), 124.0 (C-4), 126.7 (C-3'), 170.7 (C-5), 171.4 (C_{20}=O), 199.4 (C_3=O). ESI: 528.5 ($[M+H]^+$, 100%).

CHAPTER III

FUTURE WORK:

DEVELOPMENT OF COUMARINS AS

AROMATASE INHIBITORS

INTRODUCTION

AIM OF THE WORK

3.1. 3-THIOPHENE COUMARIN DERIVATIVES

3.1.1. Design and Synthesis

3.1.2. Chemistry

3.2. EXPERIMENTAL SECTION

INTRODUCTION

Phytochemicals are powerful food factors, found in fruits, vegetables, and other whole foods, that promote profound effects on the human health. It has been found that some of these compounds interact with enzymes and nuclear receptors, which leads to the modulation of selective physiological mechanisms.[184]

For instance, genistein (Figure 3.1) in soybeans is known to be an agonist of ER,[184] thus competing with estrogen for binding to the receptor. Chen *et al.* have also shown that isoflavones and flavones (Figure 3.1) are agonists of estrogen-related receptors (ERRs)[185] and have also isolated and identified procyanidin B dimers (Figure 3.1) that act as competitive inhibitors of aromatase, supressing the growth of aromatase-mediated tumors in mice.[186]

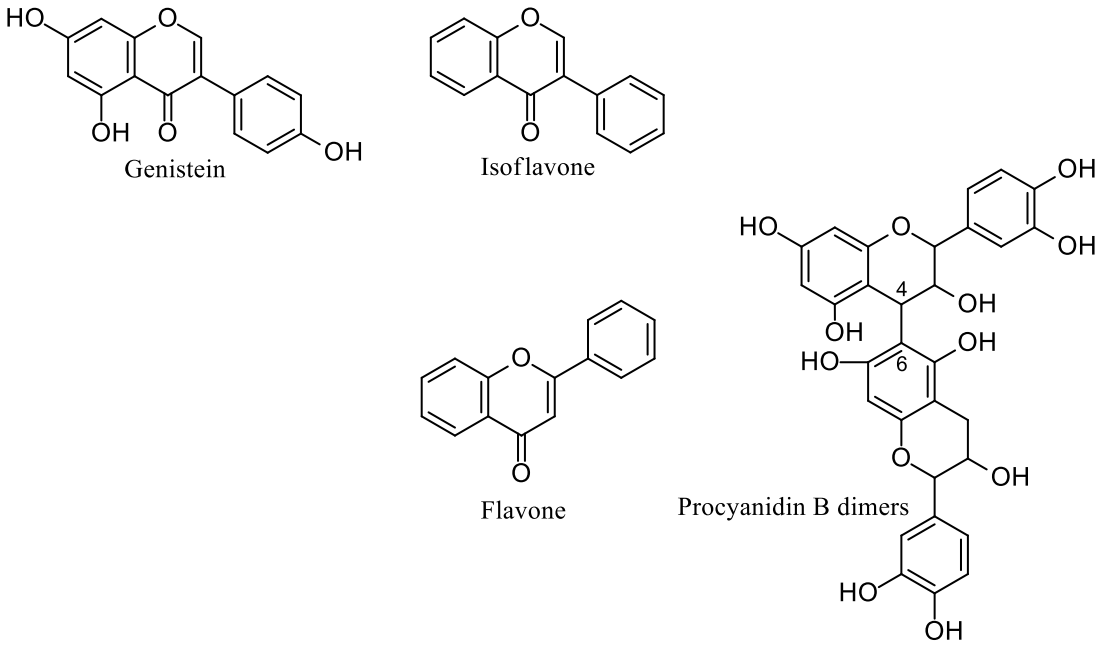


Figure 3.1 - Structures of some phytochemicals

Among phytochemicals, coumarin also called 1,2-benzopyrone (Figure 3.2) and its derivatives (coumarins) are widely distributed in nature and many of them exhibit useful and diverse biological activities.[187-189]

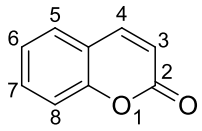


Figure 3.2 - Structure of coumarin

Coumarin derivatives have been found to have several potential therapeutic activities[190] including photochemotherapy, anti-tumor and antiviral therapy,[191,192] stimulation of central nervous system, antibacterial,[193-195] anti-inflamatory and anti-coagulant activities.[196,197]

The pattern of substitutions on the coumarin basic chemical structure is believed to influence both the pharmacological and biochemical properties of coumarins, determining therapeutic applications and toxicity profiles.[190,198] For example, coumarin and its active metabolite, 7-hydroxycoumarin (umbelliferone) (Figure 3.3), have demonstrated growth-inhibitory activity in human breast cancer cell lines such as MCF-7 and prostate cancer cell lines such as LNCaP.[199,200]

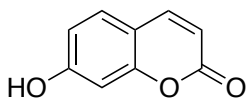
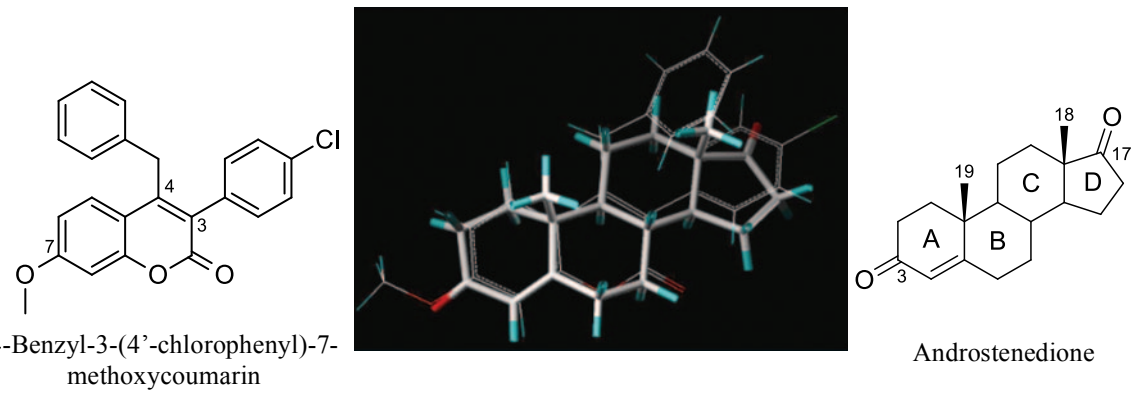


Figure 3.3 - Structure of 7-hydroxycoumarin

Among the diverse biological activities of coumarins, the most intriguing one is the notable effect of some derivatives against breast cancer.[201] By this reason, some coumarin derivatives have been evaluated in the last years for breast cancer therapy.[202,203] Chen *et al.*[184] found *4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin* (Figure 3.4) to be a potent competitive AI (IC_{50}=0.08 μM), suppressing aromatase activity and the proliferation of ER-positive MCF-7 breast cancer cells.[204]

The molecular superimposition of the chemical structure of this coumarin derivative with the chemical structure of the substrate of aromatase, androstenedione (Figure 3.4), allowed establishing the following similarities:[184]

- the coumarin rings mimic the A- and B-rings of androstenedione;
- the 3-(4'-chlorophenyl) group of the coumarin mimics the D-ring and aligns near the steroidal C-17 keto oxygen;
- the 4-benzyl group of the coumarin aligns very closely to the C-19 methyl group of the steroidal substrate;
- the coumarin 7-methoxyl group also aligns very closely with the C-3 keto oxygen of the steroidal substrate.



4-Benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin

Androstenedione

Figure 3.4 - Alignment of androstenedione with 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin (adopted from Chen *et al.* [184])

The described alignment strongly suggests that the three functional groups – *3-(4'-chlorophenyl)*, *4-benzyl* and *7-methoxyl* – share the same enzymatic interactions with the mentioned groups within androstenedione being superimposable with them. By these reasons, the referred functional groups are believed to be responsible for the inhibition of aromatase by the mentioned coumarin derivatives. Also, a favourable interaction at the 7-position of the coumarin ring is due to the formation of a hydrogen bond by the 7-oxygen atom.[204]

AIM OF THE WORK[‡]

The main goal of this work was to synthesize a set of coumarin compounds in order to be further biologically evaluated against aromatase. For this aim, we used the *4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin* (Figure 3.4) as a hit compound since it has formerly proved to be a potent competitive aromatase inhibitor. Subsequently we modified the chemical groups that are believed to be important for the inhibitory activity against the enzyme aromatase. With this rationale we intended to explore the interactions that aromatase establishes with these groups, in order to understand the SAR of this kind of compounds, which will allow the future design and synthesis of valuable AIs. It is important to mention that this is a very recent ongoing work and that the biological assays are just starting. In this way, here we present our first approaches to the design and synthesis of coumarin compounds as potential aromatase inhibitors.

[‡] This work was developed in the Department of Organic Chemistry of the Faculty of Pharmacy of the University of Santiago de Compostela, Spain.

3.1 3-THIOPHENE COUMARIN DERIVATIVES

3.1.1. Design and synthesis

Although there have been elucidated some features in the coumarin framework that enhance the inhibitory activity towards aromatase, as mentioned above, in our work we have also been interested in exploring the following modifications:

i. To substitute the 3-(4'-chlorophenyl) group of the hit coumarin 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin (Figure 3.4) by a smaller aromatic ring with an electronegative hetero atom, as in thiophene ring in order to explore: a) the different positioning of the sulphur atom in the thiophene ring, and b) the effect of an additional electronegative atom in that ring, such as a bromine atom.

ii. To remove the 4-benzyl group in order to study how it would affect the interaction with the enzyme aromatase.

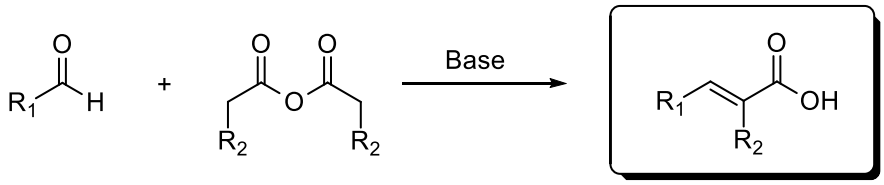
iii. To evaluate the influence of different functional groups such as (methyl, methoxyl, acetyl and hydroxyl) between C-5 and C-8 positions.

There are several methods for preparing coumarin derivatives. These include, just to mention a few, the classic *Pechmann*, *Claisen*, *Perkin* and *Wittig* reactions.[204-206]

In the present work, it was followed the *Perkin* method, which is indeed the most versatile process and the one that can use a higher number of starting materials at lower cost. The *Perkin* reaction involves the condensation of a carboxylic acid anhydride with an aldehyde in the presence of a weak base to give unsaturated carboxylic acids (Scheme 3.1).[207,208] The first example of this reaction was described by *Perkin* in 1868 and curiously it involved the synthesis of a coumarin compound by heating the sodium salt of salicylaldehyde with acetic anhydride.[207,209] The *Perkin*

approach is a very important method for the synthesis of substituted coumarins and the reaction presumably proceeds through cinnamic acids as intermediates.[207]

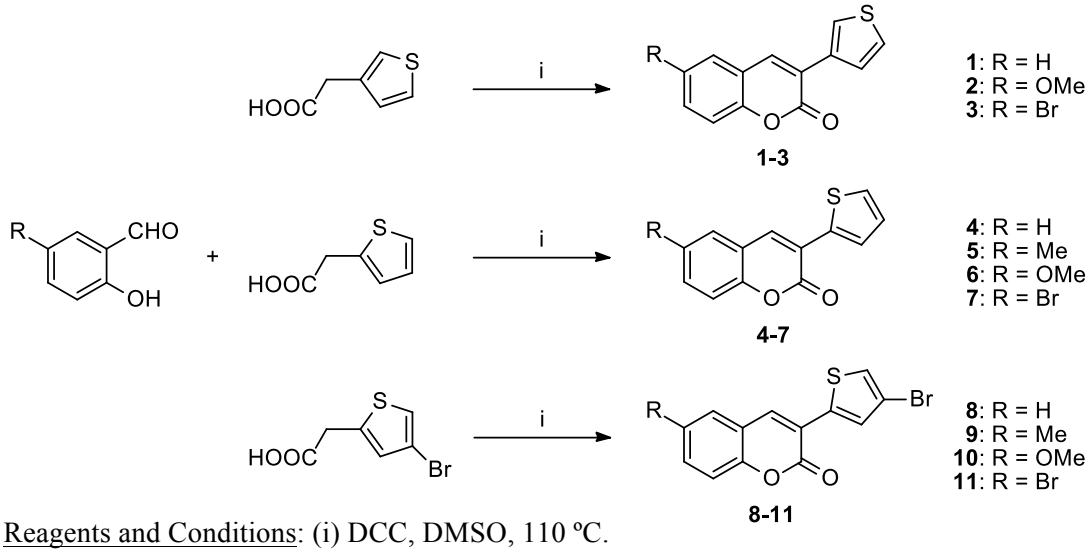
Scheme 3.1 - General *Perkin* reaction



3.1.2. Chemistry

Coumarin derivatives **1-11** were efficiently synthesized according to the protocol outlined in Scheme 3.2. This protocol consists in a direct synthetic route involving the classical *Perkin* procedure followed by an intramolecular esterification reaction forming the cyclic ester, in this case, the desired coumarin.[210-212]

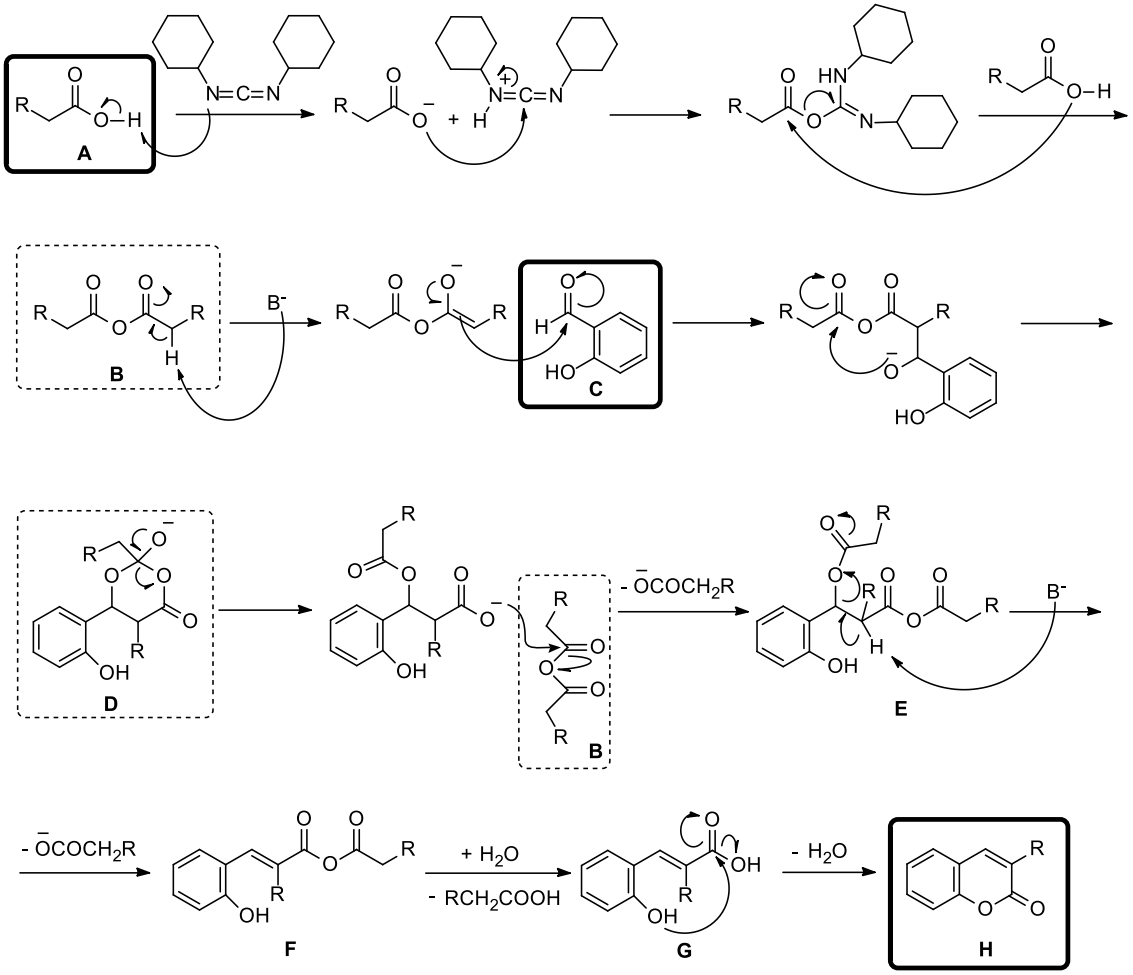
Scheme 3.2 - Synthesis of coumarin derivatives using *Perkin* conditions



Reagents and Conditions: (i) DCC, DMSO, 110 ºC.

The reactions were accomplished by condensation of the appropriate substituted salicylaldehydes with the adequate substituted α-aryl acetic acids, using *N,N'*-dicyclohexylcarbodiimide (DCC), as dehydrating agent. This is a very versatile reaction affording different families of substituted coumarins. The mechanism for the formation of the desired coumarins is depicted bellow (Scheme 3.3).

Scheme 3.3 – Proposed mechanism for the *Perkin* reaction using DCC

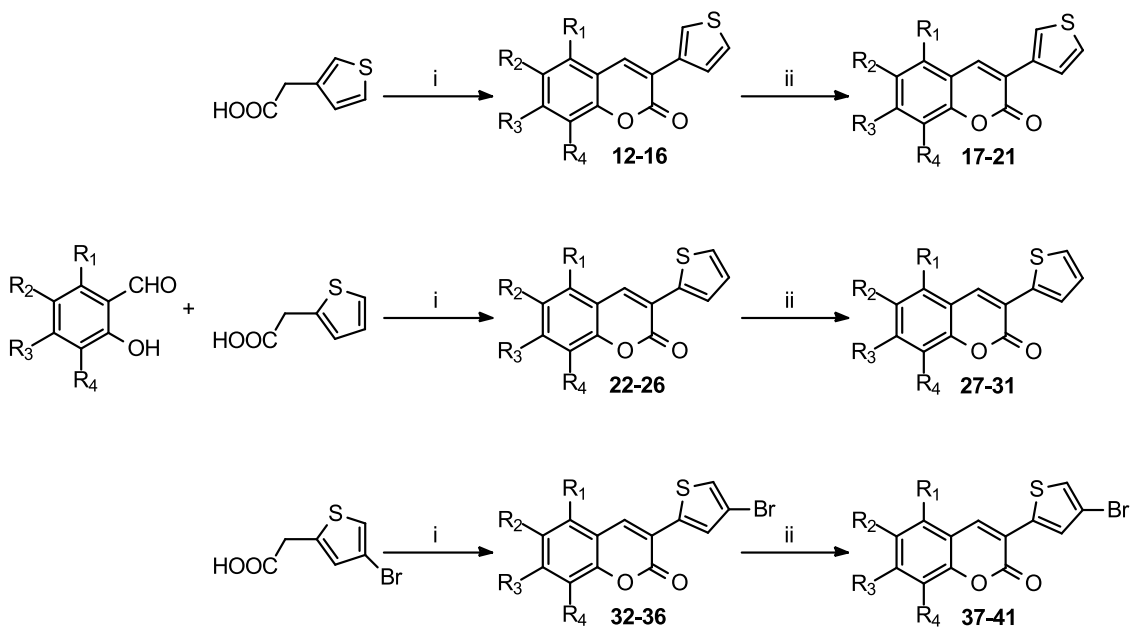


The referred mechanism involves DCC to promote the condensation between two molecules of the α-aryl acetic acid (**A**) in order to form the anhydride (**B**). This anhydride will then react with salicylaldehyde (**C**) leading afterwards to the cyclic intermediate (**D**), which will then open and react with another molecule of the anhydride (**B**). The reaction proceeds with the formation of derivative (**G**) through species (**E**) and (**F**), which will then cyclize affording the desired coumarin (**H**).

The acetoxylated coumarin derivatives (**12-16**, **22-26**, **32-36**) were synthesized by the *Perkin-Oglialoro* reaction using the convenient α-arylacetic acids and salicylaldehyde, in acetic anhydride and potassium acetate (Scheme 3.4).[213,214] The corresponding hydroxylated derivatives (**17-21**, **27-31**, **37-41**) were obtained by hydrolysis of the previously obtained esters in the presence of aqueous HCl solution and methanol (Scheme 3.4).[213,214]

The *Perkin-Oglialoro* reaction is indeed the most important modification of the *Perkin* reaction, where it occurs the condensation of aromatic aldehydes with α-arylacetic acids in acetic anhydride and in the presence of a weak base to obtain α-arylcinnamic acids, being this adaptation an improvement in the classical synthesis of coumarins.[206,208]

Scheme 3.4 - Synthesis of coumarin derivatives using *Perkin-Oglialoro* conditions



12, **22** and **32:** R_1 = H, R_2 = OAc, R_3, R_4 = H
13, **23** and **33:** R_1, R_2 = H, R_3 = OAc, R_4 = H
14, **24** and **34:** R_1, R_2, R_3 = H, R_4 = OAc
15, **25** and **35:** R_1, R_2 = H, R_3, R_4 = OAc
16, **26** and **36**: R_1 = OAc, R_2 = H, R_3 = OAc, R_4 = H

17, **27** and **37:** R_1 = H, R_2 = OH, R_3, R_4 = H
18, **28** and **38:** R_1, R_2 = H, R_3 = OH, R_4 = H
19, **29** and **39:** R_1, R_2, R_3 = H, R_4 = OH
20, **30** and **40:** R_1, R_2 = H, R_3, R_4 = OH
21, **31** and **41:** R_1 = OH, R_2 = H, R_3 = OH, R_4 = H

Reagents and Conditions: (i) CH_3COOK, Ac_2O, reflux; (ii) HCl 2 N, CH_3OH, reflux.

3.2. EXPERIMENTAL SECTION

The ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively, on a Bruker AXM spectrometer. Chemical shifts were recorded in δ values in parts per million (ppm) downfield from tetramethylsilane as an internal standard. All *J*-values are given in Hz.

Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer.

Reactions were monitored by thin layer chromatography (TLC) in silica gel 60 F_{254} aluminium sheets. The chromatographic separation of products was made using silica gel 60 (230-00 mesh) flash column chromatography. The TLC plates were revealed using ultra-violet lamp (254 nm).

Yields have not been optimized.

Scheme 3.2 - *Synthesis of coumarin derivatives using Perkin conditions*

General procedure for the preparation of 3-thiophenylcoumarins (1-11).

A solution of 2-hydroxybenzaldehyde/2-hydroxy-5-methylbenzaldehyde/2-hydroxy-5-methoxybenzaldehyde and the corresponding thiophene acetic acid, 3-thiophene acetic acid/2-thiophene acetic acid/4-bromo-2-thiophene acetic acid, in dimethyl sulfoxide was prepared. DCC was added and the mixture was heated at 110 ºC for the adequate period of time, until total transformation of the starting material. Ice and acetic acid were added to the reaction mixture. After keeping it at room temperature for 2 hours, the mixture was extracted with diethyl ether (3 x 50 mL). The organic layer was extracted with 5% aqueous $NaHCO_3$ (3 x 50 mL) and then water (3 x 50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. The residue was purified by flash column chromatography (hexane/ethyl acetate).

3-(3-Thiophenyl)coumarin (1).

2-Hydroxybenzaldehyde (506.6 mg; 4.09 mmol); 3-thiophene acetic acid (728.9 mg; 5.13 mmol); DCC (1.31 g; 6.35 mmol); DMSO (4 mL). Yield 44 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.26-7.39 (m, 3H, H-5', H-8'), 7.47-7.55 (m, 3H, H-2', H-4', H-7'), 7.92 (s, 1H, H-4), 8.18 (m, 1H, H-5). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 116.4, 119.4, 122.7, 122.9, 124.5, 125.7, 126.0, 126.2, 127.7, 131.1, 134.4, 137.2, 152.6. MS: 228.9 ($[M+H]^+$, 28 %).

6-Methoxy-3-(3-thiophenyl)coumarin (2).

2-Hydroxy-5-methoxybenzaldehyde (500.8 mg; 3.29 mmol); 3-thiophene acetic acid (728.9 mg; 4.11 mmol); DCC (1.06 g; 5.14 mmol); DMSO (5 mL). Yield 41 %. ^{1}H

NMR (250 MHz, $CDCl_3$) δ: 3.86 (s, 3H, -OCH_3), 6.97 (d, 1H, $J_{5,7}$=2.9, H-5), 7.09 (dd, 1H, $J_{7,8}$=9.0, $J_{7,5}$=2.9, H-7), 7.29 (d, 1H, $J_{8,7}$=9.0, H-8), 7.39 (dd, 1H, $J_{4',5'}$=5.1, $J_{4',2'}$=3.0, H-4'), 7.52 (dd, 1H, $J_{5',4'}$=5.1, $J_{5',2'}$=1.3, H-5'), 7.89 (s, 1H, H-4), 8.20 (dd, 1H, $J_{2',5'}$=3.0, $J_{2',4'}$=1.3, H-2'). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 55.7, 109.5, 110.5, 117.3, 118.9, 119.7, 121.5, 125.6, 126.0, 126.1, 134.3, 135.4, 136.9, 156.1. MS: 258.8 ($[M+H]^+$, 33 %).

6-Bromo-3-(3-thiophenyl)coumarin (3).

2-Hydroxy-5-bromobenzaldehyde (500.2 mg; 2.49 mmol); 3-thiophene acetic acid (442.7 mg; 3.11 mmol); DCC (801.9 mg; 3.89 mmol); DMSO (4 mL). Yield 58 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.24 (d, 1H, $J_{8,7}$=9.7, H-8), 7.41 (m, 1H, H-4'), 7.50 (d, 1H, $J_{5',4'}$=5.1, H-5'), 7.59 (m, 1H, H-7), 7.68 (bs, 1H, H-2'), 7.84 (s, 1H, H-4), 8.21 (m, 1H, H-5). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 116.3, 118.3, 121.5, 122.6, 126.4, 126.7, 126.9, 130.4, 133.9, 134.3, 137.0, 151.5, 158.9. MS: 307.8 ($[M+H]^+$, 100 %).

3-(2-Thiophenyl)coumarin (4).

2-Hydroxybenzaldehyde (504.3 mg; 4.13 mmol); 2-thiophene acetic acid (728.3 mg; 5.12 mmol); DCC (1.33 mg; 6.44 mmol); DMSO (4 mL). Yield 44 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.13 (m, 1H, H-4'), 7.33 (m, 2H, H6, H8), 7.42 (dt, 1H, H-5'), 7.52 (m, 2H, H-5, H-7), 7.80 (dt, 1H, H-3'), 7.99 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 110.2, 116.3, 119.3, 124.6, 126.9, 127.4, 127.6, 129.3, 131.1, 132.6, 135.4, 152.6, 163.8. MS: 229.0 ($[M+H]^+$, 26%).

6-Methyl-3-(2-Thiophenyl)coumarin (5).

2-Hydroxy-5-methylbenzaldehyde (500.9 mg; 3.67 mmol); 2-thiophene acetic acid (651.9 mg; 4.59 mmol); DCC (1.18 g; 5.73 mmol); DMSO (4 mL). Yield 53 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 2.42 (s, 3H, -CH_3), 7.12 (dd, 1H, $J_{4',5'}$=5.1, $J_{4',3'}$=3.8, H-4'), 7.29 (m, 3H, H-5, H-7, H-8), 7.42 (dd, 1H, $J_{5',4'}$=5.1, $J_{5',3'}$=0.9, H-5'), 7.78 (dd, 1H, $J_{3',4'}$=3.8, $J_{3',5'}$=0.9, H-3'), 7.95 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.7, 116.0, 119.1, 121.7, 124.6, 126.8, 127.4, 132.2, 134.1, 135.5, 150.7, 159.6. MS: 243.0 ($[M+H]^+$, 31 %).

6-Methoxy-3-(2-thiophenyl)coumarin (6).

2-Hydroxy-5-methoxybenzaldehyde (500.6 mg; 3.29 mmol); 2-thiophene acetic acid (585.3 mg; 4.12 mmol); DCC (1.06 g; 5.14 mmol); DMSO (5 mL). Yield 47 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 3.86 (s, 3H, -OCH_3), 6.97 (d, 1H, $J_{5,7}$=2.9, H-5), 7.09 (dd, 1H, $J_{7,8}$=9.1, $J_{7,5}$=2.9, H-7), 7.13 (dd, 1H, $J_{4',5'}$=5.2, $J_{4',3'}$=3.9, H-4'), 7.28 (d, 1H, $J_{8,7}$=9.0, H-8), 7.43 (dd, 1H, $J_{5',4'}$=5.2, $J_{5',3'}$=0.7, H-5'), 7.80 (dd, 1H, $J_{3',4'}$=3.9, $J_{3',5'}$=0.9, H-3'), 7.96 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 55.7, 100.0, 106.5, 109.1, 111.7, 117.3, 118.3, 119.0, 126.9, 127.4, 127.7, 135.2, 142.8, 156.1. MS: 258.8 ($[M+H]^+$, 27 %).

6-Bromo-3-(2-thiophenyl)coumarin (7).

2-Hydroxy-5-bromobenzaldehyde (502.7 mg; 2.50 mmol); 2-thiophene acetic acid (443.8 mg; 3.12 mmol); DCC (801.3 mg; 3.89 mmol); DMSO (4 mL). Yield 51 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.20 (m, 1H, H-4'), 7.42 (d, 1H, $J_{8,7}$=8.8, H-8), 7.71 (m, 2H, H-7, H-5'), 7.84 (d, 1H, $J_{3',4'}$=3.5, H-3'), 8.00 (d, 1H, $J_{5,7}$=2.2, H-5), 8.49 (s, 1H, H-

4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 95.5, 116.5, 118.3, 119.2, 121.4, 127.2, 127.6, 129.5, 130.4, 133.8, 134.8, 147.9, 151.3. MS: 307.8 ($[M+H]^+$, 100 %).

3-(4-Bromo-2-thiophenyl)coumarin (8).

2-Hydroxybenzaldehyde (250.4 mg; 2.05 mmol); 4-bromo-2-thiophene acetic acid (568.4 mg; 2.57 mmol); DCC (658.5 mg; 3.19 mmol); DMSO (5 mL). Yield 38 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 7.33 (d, 1H, $J_{5',3'}$=1.4, H-5'), 7.38 (dd, 2H, *J*=8.0, *J*=1.6, H-5, H-8), 7.54 (t, 1H, *J*=7.6, H-6), 7.55 (t, 1H, *J*=7.4, H-7), 7.68 (d, 1H, $J_{3',5'}$=1.4, H-3'), 8.01 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, DMSO) δ: 110.7, 115.9, 116.4, 119.2, 124.5, 126.6, 127.1, 130.8, 138.5, 139.7, 156.9, 157.8, 168.9. MS: 307.8 ($[M+H]^+$, 100 %).

6-Methyl-3-(4-bromo-2-thiophenyl)coumarin (9).

2-Hydroxy-5-methylbenzaldehyde (252.4 mg; 1.85 mmol); 4-bromo-2-thiophene acetic acid (508.2 mg; 2.29 mmol); DCC (595.9 mg; 2.89 mmol); DMSO (5 mL). Yield 34 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 2.42 (s, 3H, -CH_3), 7.26 (m, 3H, H-5, H-7, H-8), 7.31 (m, 1H, H-5'), 7.66 (d, 1H, *J*=1.4, H-3'), 7.95 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.6, 100.0, 108.4, 116.2, 121.8, 124.9, 127.6, 128.9, 132.8, 134.5, 134.6, 135.9, 153.7, 155.4. MS: 321.7 ($[M+H]^+$, 100 %).

6-Methoxy-3-(4-bromo-2-thiophenyl)coumarin (10).

2-Hydroxy-5-methoxybenzaldehyde (250.5 mg; 1.65 mmol); 4-bromo-2-thiophene acetic acid (457.1 mg; 2.07 mmol); DCC (532.5 mg; 2.58 mmol); DMSO (5 mL). Yield 36 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 3.89 (s, 3H, -OCH_3), 6.98 (d, 1H, $J_{5,7}$=2.9, H-5), 7.12 (dd, 1H, $J_{7,8}$=9.1 , $J_{7,5}$=2.9, H-7), 7.27 (d, 1H, $J_{8,7}$=6.0, H-8), 7.32

(d, 1H, $J_{5',3'}$=1.5, H-5'), 7.68 (d, 1H, $J_{3',5'}$=1.4, H-3'), 7.97 (s, 1H, H-4). MS: 338.0 ($[M+H]^+$, 100 %).

6-Bromo-3-(4-bromo-2-thiophenyl)coumarin (11).

2-Hydroxy-5-bromobenzaldehyde (251.8 mg; 1.25 mmol); 4-bromo-2-thiophene acetic acid (344.4 mg; 1.56 mmol); DCC (401.1 mg; 1.94 mmol); DMSO (5 mL). Yield 59 %. ^{1}H NMR (250 MHz, DMSO) δ: 7.45 (d, 1H, $J_{8,7}$=8.9, H-8), 7.79 (dd, 1H, $J_{7,8}$=8.7, $J_{7,5}$=2.3, H-7), 7.84 (bs, 2H, H-3', H-5'), 7.96 (d, 1H, $J_{5,8}$=2.4, H-5), 8.63 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, DMSO) δ: 99.8, 100.8, 102.9, 115.1, 125.9, 134.4, 141.8, 147.5, 160.5, 164.2, 171.5, 173.4, 175.4. MS: 387.1 ($[M+H]^+$, 23 %).

Scheme 3.4 - *Synthesis of coumarin derivatives using Perkin-Oglialoro conditions*

General procedure for the preparation of 3-thiophenylcoumarins (12-16, 22-26 and 32-36).

The solution containing CH_3COOK, the appropriate thiophene acetic acid, and hydroxylated benzaldehyde in Ac_2O was refluxed (138 °C) with stirring during different periods of time. The reaction mixture was then cooled, neutralized with 10% aqueous $NaHCO_3$, and extracted with ethyl acetate. The organic layers were washed with water, dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness. The products were purified by recrystallization in ethyl acetate or ethyl acetate/ethanol.

6-Acetoxy-3-(3-thiophenyl)coumarin (12).

2,5-Dihydroxybenzaldehyde (501.1 mg; 3.63 mmol); 3-thiophene acetic acid (515.1 mg; 3.62 mmol); CH_3COOK (640.4 mg; 6.52 mmol); Ac_2O (4 mL). Yield 54 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 2.34 (s, 3H, -OAc), 7.23 (dd, 1H, $J_{7,8}$=8.9, $J_{7,5}$=2.7, H-7), 7.35 (d, 1H, $J_{8,7}$=8.9, H-8), 7.30 (d, 1H, $J_{5,7}$=2.7, H-5), 7.40 (dd, 1H, $J_{5',4'}$=5.1, $J_{5',2'}$=3.2, H-5'), 7.49 (dd, 1H, $J_{4',5'}$=5.1, $J_{4',2'}$=1.2, H-4'), 7.88 (s, 1H, H-4), 8.20 (m, 1H, H-2'). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.9, 117.3, 119.7, 119.8, 124.5, 125.8, 126.0, 126.1, 126.4, 126.5, 133.9, 136.3, 146.6, 169.2. MS: 287.0 ($[M+H]^+$, 10 %).

7-Acetoxy-3-(3-thiophenyl)coumarin (13).

2,4-Dihydroxybenzaldehyde (502.9 mg; 3.64 mmol); 3-thiophene acetic acid (515.8 mg; 3.63 mmol); CH_3COOK (639.4 mg; 6.52 mmol); Ac_2O (4 mL). Yield 38 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 2.30 (s, 3H, -OAc), 7.04-7.09 (m, 2H, H-6, H-8), 7.35-7.49 (m, 3H, H-4', H-5', H-5), 7.87 (s, 1H, H-2'), 8.14 (s, 1H, H-4). ^{13}C NMR (75.5

MHz, $CDCl_3$) δ: 21.0, 99.9, 109.6, 109.7, 118.4, 125.6, 125.9, 126.1, 128.3, 130.7, 133.9, 136.5, 139.8, 152.3, 168.4. MS: 286.9 ($[M+H]^+$, 5 %).

8-Acetoxy-3-(3-thiophenyl)coumarin (14).

2,3-Dihydroxybenzaldehyde (500.4 mg; 3.62 mmol); 3-thiophene acetic acid (515.3 mg; 3.62 mmol); CH_3COOK (639.0 mg; 6.51 mmol); Ac_2O (4 mL). Yield 45 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.42 (s, 3H, -OAc), 7.23-7.26 (m, 2H, H-6, H-7), 7.35-7.39 (m, 2H, H-5', H-5), 7.48 (dd, 1H, $J_{4',2'}$=1.3, $J_{4',5'}$=5.1, H-4'), 7.89 (s, 1H, H-4), 8.15 (dd, 1H, *J*=1.3, *J*=2.9, H-2'). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.6, 120.4, 120.7, 124.1, 124.2, 124.4, 125.1, 125.7, 126.1, 126.3, 133.9, 136.6, 137.3, 158.7, 168.6. MS: 287.0 ($[M+H]^+$, 5 %).

7,8-Diacetoxy-3-(3-thiophenyl)coumarin (15).

2,3,4-Trihydroxybenzaldehyde (500.5 mg; 3.25 mmol); 3-thiophene acetic acid (461.1 mg; 3.24 mmol); CH_3COOK (573.5 mg; 5.84 mmol); Ac_2O (4 mL). Yield 49 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.35 (s, 3H, 7-OAc), 2.44 (s, 3H, 8-OAc), 7.13 (d, 1H, *J*=8.6, H-6), 7.39-7.51 (m, 3H, H-4', H-5', H-5), 7.90 (s, 1H, H-2'), 8.17 (m, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.2, 20.6, 118.3, 119.0, 122.3, 124.7, 125.8, 126.0, 126.3, 129.8, 133.8, 136.3, 144.6, 145.6, 158.6, 167.3, 167.8. MS: 344.9 ($[M+H]^+$, 14 %).

5,7-Diacetoxy-3-(3-thiophenyl)coumarin (16).

2,4,6-Trihydroxybenzaldehyde (501.7 mg; 3.26 mmol); 3-thiophene acetic acid (461.7 mg; 3.25 mmol); CH_3COOK (573.0 mg; 5.84 mmol); Ac_2O (4 mL). Yield 35 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.33 (s, 3H, -OAc), 2.43 (s, 3H, -OAc), 6.99 (d, 1H,

$J_{8,6}$=2.1, H-8), 7.04 (d, 1H, $J_{6,8}$=2.1, H-6), 7.39 (dd, 1H, $J_{5',4'}$=5.1, $J_{5',2'}$=3.0, H-5'), 7.48 (dd, 1H, $J_{4',5'}$=5.1, $J_{4',2'}$=1.2, H-4'), 7.87 (s, 1H, H-4), 8.13 (dd, 1H, $J_{2',4'}$=2.9, $J_{2',5'}$=1.2, H-2'). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.9, 21.0, 107.3, 110.9, 112.2, 122.1, 125.8, 126.2, 126.4, 130.4, 134.0, 146.9, 152.0, 153.4, 168.2, 168.3. MS: 345.0 ($[M+H]^+$, 6 %).

6-Acetoxy-3-(2-thiophenyl)coumarin (22).

2,5-Dihydroxybenzaldehyde (500.5 mg; 3.62 mmol); 2-thiophene acetic acid (515.5 mg; 3.63 mmol); CH_3COOK (640.1 mg; 6.53 mmol); Ac_2O (4 mL). Yield 53 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 2.34 (s, 3H, -OAc), 7.13 (dd, 1H, $J_{4',3'}$=5.1, $J_{4',5'}$=3.8, H-4'), 7.23 (dd, 1H, $J_{7,8}$=8.9, $J_{7,5}$=2.5, H-7), 7.31 (d, 1H, $J_{5,7}$=2.5, H-5), 7.36 (d, 1H, $J_{8,7}$=8.9, H-8), 7.45 (dd, 1H, $J_{5',4'}$=5.1, $J_{5',3'}$=1.0, H-5'), 7.81 (dd, 1H, $J_{3',4'}$=3.8, $J_{3',5'}$=1.0, H-3'), 7.94 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.8, 117.3, 117.4, 119.7, 122.2, 124.5, 127.4, 127.5, 128.1, 134.5, 135.5, 146.8, 150.0, 169.1, 169.3. MS: 287.0 ($[M+H]^+$, 3 %).

7-Acetoxy-3-(2-thiophenyl)coumarin (23).

2,4-Dihydroxybenzaldehyde (501.9 mg; 3.63 mmol); 2-thiophene acetic acid (515.7 mg; 3.63 mmol); CH_3COOK (639.9 mg; 6.52 mmol); Ac_2O (4 mL). Yield 52 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 2.35 (s, 3H, -OAc), 7.11 (m, 3H, H-8, H-6, H-4'), 7.42 (dd, 1H, $J_{5',4'}$=5.1, $J_{5',3'}$=1.0, H-5'), 7.54 (d, 1H, $J_{5,6}$=8.5, H-5), 7.79 (dd, 1H, $J_{3',4'}$=3.7, $J_{3',5'}$=1.0, H-3'), 7.98 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 21.2, 100.0, 109.9, 117.1, 118.6, 123.6, 127.1, 127.5, 127.7, 128.3, 132.9, 134.8, 147.8, 152.7, 169.1. MS: 287.0 ($[M+H]^+$, 4 %).

8-Acetoxy-3-(2-thiophenyl)coumarin (24).

2,3-Dihydroxybenzaldehyde (500.8 mg; 3.62 mmol); 2-thiophene acetic acid (515.3 mg; 3.62 mmol); CH_3COOK (640.8 mg; 6.52 mmol); Ac_2O (4 mL). Yield 42 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.44 (s, 3H, -OAc), 7.13 (dd, 1H, $J_{4',5'}$=5.1, $J_{4',3'}$=3.8, H-4'), 7.28 (m, 2H, H-6, H-7), 7.42 (m, 2H, H-5, H-5'), 7.79 (dd, 1H, $J_{3',4'}$=3.8, $J_{3',5'}$=1.1, H-3'), 7.99 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.6, 120.6, 122.2, 124.4, 124.5, 125.0, 127.4, 127.6, 127.9, 134.9, 135.6, 137.4, 143.1, 158.2, 168.5. MS: 287.0 ($[M+H]^+$, 7 %).

7,8-Diacetoxy-3-(2-thiophenyl)coumarin (25).

2,3,4-Trihydroxybenzaldehyde (500.3 mg; 3.24 mmol); 2-thiophene acetic acid (462.3 mg; 3.24 mmol); CH_3COOK (575.6 mg; 5.84 mmol); Ac_2O (4 mL). Yield 41 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.34 (s, 3H, -OAc), 2.43 (s, 3H, -OAc), 7.13 (m, 2H, H-6, H-4'), 7.42 (m, 2H, H-5, H-5'), 7.78 (d, 1H, $J_{3',4'}$=3.8, H-3'), 7.96 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.2, 20.6, 99.9, 117.9, 119.2, 121.7, 123.5, 124.6, 127.4, 127.6, 127.9, 129.9, 134.6, 144.5, 167.1, 167.7, 168.2. MS: 345.0 ($[M+H]^+$, 3 %).

5,7-Diacetoxy-3-(2-thiophenyl)coumarin (26).

2,4,6-Trihydroxybenzaldehyde (500.4 mg; 3.24 mmol); 2-thiophene acetic acid (461.2 mg; 3.24 mmol); CH_3COOK (575.8 mg; 5.84 mmol); Ac_2O (4 mL). Yield 54 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.33 (s, 3H, -OAc), 2.45 (s, 3H, -OAc), 7.01 (d, 1H, $J_{8,6}$=2.1, H-8), 7.07 (d, 1H, $J_{6,8}$=2.0, H-6), 7.13 (dd, 1H, $J_{4',5'}$=5.0, $J_{4',3'}$=3.9, H-4'), 7.44 (bd, 1H, $J_{5',4'}$=5.1, H-5'), 7.77 (bd, 1H, $J_{3',4'}$=3.7, H-3'), 7.94 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.9, 21.0, 97.6, 107.4, 109.9, 112.3, 127.4, 127.5, 128.1, 128.6, 130.9, 136.7, 146.8, 151.8, 153.1, 168.1, 168.2. MS: 345.0 ($[M+H]^+$, 13 %).

6-Acetoxy-3-(4-bromo-2-thiophenyl)coumarin (32).

2,5-Dihydroxybenzaldehyde (300.0 mg; 2.17 mmol); 4-bromo-2-thiophene acetic acid (479.9 mg; 2.17 mmol); CH_3COOK (387.3 mg; 3.95 mmol); Ac_2O (4 mL). Yield 52 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.35 (s, 3H, -OAc), 7.32 (m, 4H, H-5', H-5, H-7, H-8), 7.69 (s, 1H, H-3'), 7.94 (s, 1H, H-4). MS: 365.9 ($[M+H]^+$, 27 %).

7-Acetoxy-3-(4-bromo-2-thiophenyl)coumarin (33).

2,4-Dihydroxybenzaldehyde (312.5 mg; 2.26 mmol); 4-bromo-2-thiophene acetic acid (499.5 mg; 2.26 mmol); CH_3COOK (402.8 mg; 4.10 mmol); Ac_2O (4 mL). Yield 55 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.35 (s, 3H, -OAc), 7.10 (m, 1H, H-5), 7.16 (bs, 1H, H-8), 7.32 (bd, 1H, $J_{5',3'}$=1.2, H-5'), 7.55 (dd, 1H, $J_{7,5}$=8.4, $J_{7,8}$=0.7, H-7), 7.67 (bd, $J_{3',5'}$=1.2, H-3'), 7.97 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 21.1, 109.5, 110.1, 117.2, 119.5, 126.2, 126.6, 128.2, 129.6, 136.9, 153.1, 158.9, 168.9, 175.5, 183.7. MS: 365.8 ($[M+H]^+$, 20 %).

8-Acetoxy-3-(4-bromo-2-thiophenyl)coumarin (34).

2,3-Dihydroxybenzaldehyde (299.9 mg; 2.17 mmol); 4-bromo-2-thiophene acetic acid (481.4 mg; 2.18 mmol); CH_3COOK (384.4 mg; 3.92 mmol); Ac_2O (4 mL). Yield 64 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.44 (s, 3H, -OAc), 7.30 (m, 3H, H-5', H-6, H-7), 7.43 (m, 1H, H-5), 7.67 (d, 1H, $J_{3',5'}$=1.3, H-3'), 7.98 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 25.5, 114.5, 124.6, 125.4, 130.1, 130.6, 131.2, 131.9, 133.6, 141.6, 141.9, 142.2, 149.0, 163.0, 173.5. MS: 365.8 ($[M+H]^+$, 25 %).

7,8-Diacetoxy-3-(4-bromo-2-thiophenyl)coumarin (35).

2,3,4-Trihydroxybenzaldehyde (348.8 mg; 2.26 mmol); 4-bromo-2-thiophene acetic acid (500.6 mg; 2.26 mmol); CH_3COOK (401.2 mg; 4.09 mmol); Ac_2O (4 mL). Yield 58 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.34 (s, 3H, -OAc), 2.42 (s, 3H, -OAc), 7.18 (bd, 1H, $J_{6,5}$=8.6, H-6), 7.33 (bs, 1H, H-5'), 7.42 (bd, 1H, $J_{5,6}$=8.6, H-5), 7.66 (bs, 1H, H-3'), 7.96 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.1, 20.5, 101.5, 109.6, 118.2, 119.1, 120.1, 126.1, 126.9, 128.6, 136.7, 137.0, 144.9, 157.9, 158.0, 167.6, 168.2. MS: 423.8 ($[M+H]^+$, 7 %).

5,7-Diacetoxy-3-(4-bromo-2-thiophenyl)coumarin (36).

2,4,6-Trihydroxybenzaldehyde (349.6 mg; 2.27 mmol); 4-bromo-2-thiophene acetic acid (502.2 mg; 2.27 mmol); CH_3COOK (404.0 mg; 4.09 mmol); Ac_2O (4 mL). Yield 31 %. ^1H NMR (250 MHz, $CDCl_3$) δ: 2.34 (s, 3H, -OAc), 2.47 (s, 3H, -OAc), 7.03 (d, 1H, $J_{8,6}$=2.1, H-6), 7.35 (d, 1H, $J_{5',3'}$=1.2, H-5'), 7.63 (d, 1H, $J_{3',5'}$=1.3, H-3'), 7.94 (s, 1H, H-4). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 20.9, 21.0, 98.7, 107.4, 110.4, 112.5, 125.6, 129.0, 129.2, 136.4, 139.4, 147.1, 151.6, 152.5, 153.3, 168.0, 168.1. MS: 424.0 ($[M+H]^+$, 25 %).

General procedure for the preparation of hydroxylated thiophenylcoumarins (17-21, 27-31 and 37-41).

Hydroxylated 3-thiophenylcoumarins were obtained by hydrolysis of their acetoxylated counterparts. Hence, the appropriate acetoxylated coumarin was mixed with 2 N aqueous HCl and MeOH and refluxed (100 °C) with stirring during different periods of time. The resulting reaction mixture was cooled in an ice-bath and the

reaction products, obtained as solids, were filtered, washed with cold distilled water, and dried under vacuum.

6-Hydroxy-3-(3-thiophenyl)coumarin (17).

Compound **12** (251.4 mg; 0.88 mmol); 2 N HCl (20.6 mL); MeOH (8.8 mL). Yield 92 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 7.01 (d, 1H, $J_{5,7}$=2.8, H-5), 7.06 (dd, 1H, $J_{7,8}$=9.0, $J_{7,5}$=2.8, H-7), 7.27 (d, 1H, $J_{8,7}$=8.8, H-8), 7.65 (dd, 1H, $J_{5',4'}$=5.0, $J_{5',2'}$=2.9, H-5'), 7.71 (dd, 1H, $J_{4',5'}$=5.2, $J_{4',2'}$=1.3, H-4'), 8.23 (dd, 1H, $J_{2',5'}$=2.9, $J_{2',4'}$=1.2, H-2'), 8.42 (s, 1H, H-4), 9.78 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 112.4, 116.9, 119.8, 120.0, 121.5, 125.7, 126.4, 127.1, 134.9, 137.4, 138.5, 145.9, 154.0. MS: 245.0 ($[M+H]^+$, 29 %).

7-Hydroxy-3-(3-thiophenyl)coumarin (18).

Compound **13** (227.4 mg; 0.79 mmol); 2 N (20.6 mL); MeOH (8.8 mL). Yield 91 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.76 (bs, 1H, J=1.6, H-2'), 6.83 (dd, 1H, $J_{6,5}$=8.4, $J_{6,8}$=2.1, H-6), 7.58 (d, 1H, $J_{5,6}$=8.5, H-5), 7.63 (m, 2H, H-4', H-5'), 8.14 (bs, 1H, J=2.6, H-8), 8.39 (s, 1H, H-4), 10.62 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 101.8, 111.9, 113.6, 113.7, 117.0, 124.3, 126.2, 126.8, 129.9, 139.2, 154.5, 161.3. MS: 344.9 ($[M+H]^+$, 14 %).

8-Hydroxy-3-(3-thiophenyl)coumarin (19).

Compound **14** (250.7 mg; 0.88 mmol); 2 N HCl (20.6 mL); MeOH (8.8 mL). Yield 91 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 7.09-7.18 (m, 3H, H-5, H-6, H-7), 7.66 (d, 1H, J=2.8, H-5'), 7.72 (bd, 1H, J=5.1, H-4'), 8.26 (bs, 1H, J=2.8, H-2'), 8.45 (s, 1H, H-4), 10.27 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 117.9, 118.0, 118.5,

118.6, 124.6, 124.8, 125.7, 126.4, 127.0, 138.9, 142.9, 144.5, 153.1. MS: 245.0 ($[M+H]^+$, 25 %).

7,8-Dihydroxy-3-(3-thiophenyl)coumarin (20).

Compound **15** (250.2 mg; 0.73 mmol); 2 N HCl (20.6 mL); MeOH (8.8 mL). Yield 91 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.85 (d, 1H, $J_{6,5}$=8.4, H-6), 7.09 (d, 1H, $J_{5,6}$=8.4, H-5), 7.63 (dd, 1H, $J_{5',4'}$=4.9, $J_{5',2'}$=2.0, H-5'), 7.67 (dd, 1H, $J_{4',5'}$=4.9, $J_{4',2'}$=1.1, H-4'), 8.15 (d, 1H, $J_{2',5'}$=2.9, H-2'), 8.36 (s, 1H, H-4), 9.44 (s, 1H, -OH), 10.17 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 112.7, 113.1, 116.9, 119.2, 124.3, 126.2, 126.9, 131.9, 135.2, 139.8, 149.6, 159.8, 176.4. MS: 261.0 ($[M+H]^+$, 24 %).

5,7-Dihydroxy-3-(3-thiophenyl)coumarin (21).

Compound **16** (249.8 mg; 0.72 mmol); 2 N HCl (20.6 mL); MeOH (8.8 mL). Yield 92 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.23 (d, 1H, $J_{8,6}$=2.9, H-8), 6.29 (d, 1H, $J_{6,8}$=2.0, H-8), 7.60 (m, 2H, H-4, H-5), 8.09 (dd, 1H, H-2'), 8.27 (s, 1H, H-4), 10.44 (s, 1H, -OH), 10.77 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 93.8, 98.5, 102.2, 105.4, 123.7, 126.2, 126.7, 134.0, 135.4, 140.7, 155.4, 156.3, 162.1. MS: 260.8 ($[M+H]^+$, 20 %).

6-Hydroxy-3-(2-thiophenyl)coumarin (27).

Compound **22** (257.3 mg; 0.90 mmol); 2 N HCl (20.6 mL); MeOH (8.8 mL). Yield 91 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 7.03 (dd, 1H, $J_{7,8}$=8.9, $J_{7,5}$=1.9, H-7), 7.10 (d, 1H, $J_{5,7}$=2.0, H-5), 7.19 (m, 1H, H-4'), 7.30 (d, 1H, $J_{8,7}$=8.7, H-8), 7.68 (d, 1H, $J_{5',4'}$=5.0, H-5'), 7.87 (d, 1H, $J_{3',4'}$=3.7, H-3'), 8.52 (s, 1H, H-4), 9.82 (s, 1H, -OH). ^{13}C

NMR (75.5 MHz, DMSO-d_6) δ: 112.3, 116.9, 119.8, 119.9, 120.0, 126.7, 127.4, 129.0, 129.0, 135.5, 136.3, 154.2, 154.2. MS: 245.0 ($[M+H]^+$, 11 %).

7-Hydroxy-3-(2-thiophenyl)coumarin (28).

Compound **23** (300.0 mg; 1.05 mmol); 2 N HCl (24.7 mL); MeOH (10.6 mL). Yield 86 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.78 (bs, 1H, H-8), 6.85 (m, 1H, H-6), 7.16 (m, 1H, H-4'), 7.62 (m, 2H, H-5', H-5), 7.76 (m, 1H, H-3'), 8.45 (s, 1H, H-4), 10.68 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 101.9, 111.9, 113.9, 116.3, 125.5, 127.4, 127.7, 136.0, 137.3, 154.3, 159.5, 161.4. MS: 244.8 ($[M+H]^+$, 23 %).

8-Hydroxy-3-(2-thiophenyl)coumarin (29).

Compound **24** (301.2 mg; 1.05 mmol); 2 N HCl (25.0 mL); MeOH (10.6 mL). Yield 43 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 7.10 (dd, 1H, H-4'), 7.19 (m, 3H, H-5, H-6, H-7), 7.69 (d, 1H, H-5'), 7.88 (d, 1H, H-3'), 8.54 (s, 1H, H-4), 10.33 (bs, 1H, H-4). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 118.0, 118.6, 120.4, 120.5, 124.9, 126.6, 126.7, 127.5, 128.9, 135.5, 136.8, 144.5, 150.7. MS: 244.9 ($[M+H]^+$, 6 %).

7,8-Dihydroxy-3-(2-thiophenyl)coumarin (30).

Compound **25** (155.6 mg; 0.45 mmol); 2 N HCl (13.0 mL); MeOH (5.5 mL). Yield 51 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.86 (d, 1H, $J_{6,5}$=8.4, H-6), 7.13 (d, 1H, $J_{5,6}$=8.5, H-5), 7.15 (dd, 1H, $J_{4',5'}$=5.1, $J_{4',3'}$=3.7, H-4'), 7.61 (dd, 1H, $J_{5',3'}$=1.0, $J_{5',4'}$=5.1, H-5'), 7.77 (dd, 1H, $J_{3',4'}$=3.7, $J_{3',5'}$=1.0, H-3'), 8.45 (s, 1H, H-4), 9.84 (bs, 2H, -OH, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 112.7, 113.3, 119.3, 125.5, 127.4, 127.7, 129.1, 132.1, 136.2, 137.9, 139.8, 142.6, 149.9. MS: 260.8 ($[M+H]^+$, 16 %).

5,7-Dihydroxy-3-(2-thiophenyl)coumarin (31).

Compound **26** (250.0 mg; 0.73 mmol); 2 N HCl (25.0 mL); MeOH (8.8 mL). Yield 98 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.25 (d, 1H, $J_{8,6}$=2.1, H-8), 6.31 (d, 1H, $J_{6,8}$=2.1, H-6), 7.12 (dd, 1H, $J_{4',3'}$=5.1, $J_{4',5'}$=3.8, H-4'), 7.56 (dd, 1H, $J_{3',4'}$=5.1, $J_{3',5'}$=1.0, H-3'), 7.72 (dd, 1H, $J_{5',3'}$=1.0, $J_{5',4'}$=3.8, H-5'), 8.33 (s, 1H, H-4), 10.52 (s, 1H, -OH), 10.88 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 94.0, 102.4, 107.1, 113.9, 125.2, 127.1, 127.6, 132.3, 136.6, 149.5, 155.2, 156.4, 162.4. MS: 260.9 ($[M+H]^+$, 29 %).

6-Hydroxy-3-(4-bromo-2-thiophenyl)coumarin (37).

Compound **32** (250.0 mg; 0.68 mmol); 2 N HCl (20.6 mL); MeOH (8.8 mL). Yield 60 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.42 (d, 1H, $J_{5,7}$=8.4, H-5), 6.82-7.02 (m, 3H, H-3', H-5', H-4), 7.24 (d, 1H, $J_{7,8}$=9.5, H-7), 7.97 (d, 1H, $J_{8,7}$=9.7, H-8), 10.18 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 112.6, 116.4, 117.3, 118.4, 118.9, 119.9, 122.4, 124.7, 127.2, 144.3, 150.1, 153.9, 159.8. MS: 323.7 ($[M+H]^+$, 18 %).

7-Hydroxy-3-(4-bromo-2-thiophenyl)coumarin (38).

Compound **33** (100.3 mg; 0.27 mmol); 2 N HCl (8.2 mL); MeOH (8.2 mL). Yield 45 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.79 (d, 1H, $J_{8,5}$=2.1, H-8), 6.87 (dd, 1H, $J_{6,5}$=8.5, $J_{6,8}$=2.1, H-6), 7.59 (d, 1H, $J_{5,6}$=8.6, H-5), 7.72 (d, 1H, $J_{5',3'}$=1.2, H-5'), 7.77 (d, 1H, $J_{3',5'}$=1.2, H-3'), 8.62 (s, 1H, H-4), 10.78 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 101.9, 109.1, 111.5, 113.9, 114.6, 125.1, 126.7, 130.1, 137.5, 138.0, 154.3, 157.5, 161.7. MS: 323.9 ($[M+H]^+$, 100 %).

8-Hydroxy-3-(4-bromo-2-thiophenyl)coumarin (39).

Compound **34** (100.3 mg; 0.27 mmol); 2 N HCl (8 mL); MeOH (5 mL). Yield 90 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 7.18 (m, 3H, H-5, H-6, H-7), 7.79 (s, 1H, H-5'), 7.88 (s, 1H, H-3'), 8.66 (s, 1H, H-4), 10.37 (bs, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 103.3, 118.5, 118.7, 118.9, 120.0, 125.0, 126.4, 127.9, 136.9, 137.7, 139.1, 140.9, 144.4. MS: 323.8 ($[M+H]^+$, 100 %).

7,8-Dihydroxy-3-(4-bromo-2-thiophenyl)coumarin (40).

Compound **35** (130.0 mg; 0.31 mmol); 2 N HCl (16.2 mL); MeOH (4.6 mL). Yield 80 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.88 (d, 1H, $J_{6,5}$=8.4, H-6), 7.11 (d, 1H, $J_{5,6}$=8.4, H-5), 7.72 (s, 1H, H-5'), 7.78 (s, 1H, H-3'), 8.59 (s, 1H, H-4), 9.54 (s, 1H, -OH), 10.37 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 109.3, 112.5, 113.5, 114.5, 119.6, 125.3, 126.9, 132.1, 132.2, 137.8, 138.9, 150.4, 159.4. MS: 339.9 ($[M+H]^+$, 100 %).

5,7-Dihydroxy-3-(4-bromo-2-thiophenyl)coumarin (41).

Compound **36** (50.0 mg; 0.12 mmol); 2 N HCl (8 mL); MeOH (2 mL). Yield 75 %. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.26 (d, 1H, $J_{8,6}$=2.0, H-8), 6.31 (d, 1H, $J_{5,8}$=2.0, H-5), 7.67 (d, 1H, $J_{5',3'}$=1.3, H-5'), 7.76 (d, 1H, $J_{3',5'}$=1.3, H-3'), 8.44 (s, 1H, H-4), 10.61 (s, 1H, -OH), 10.94 (s, 1H, -OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 111.1, 114.4, 124.2, 131.2, 136.3, 143.9, 147.9, 159.8, 165.9, 166.5, 173.5, 174.1, 184.6. MS: 339.7 ($[M+H]^+$, 100 %).

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