António Sérgio Vieira Pereira

NEW CHIRAL STEROID DERIVATIVES THROUGH [8Π+2Π] CYCLOADDITION REACTIONS OF 1,2-DIAZAFULVENIUM METHIDES

Thesis prepared for the degree of Master of Science under supervision of Professor Jorge António Ribeiro Salvador and Professor Teresa Margarida Dias de Pinho e Melo and submitted to the Faculty of Pharmacy of University of Coimbra

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Universidade de Coimbra

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by

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Thesis submitted in partial fulfillment of the requirements for the degree of

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in

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Supervisor: Professor Jorge António Ribeiro Salvador

Co-supervisor: Professor Teresa Margarida de Vasconcelos Dias de Pinho e Melo

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“Science seeks the truth. And it does not discriminate. For better or worse it finds things out. Science is humble. It knows what it knows and it knows what it doesn’t know. It bases its conclusions and beliefs on hard evidence - evidence that is constantly updated and upgraded. It doesn’t get offended when new facts come along. It embraces the body of knowledge. It doesn’t hold on to medieval practices because they are tradition.”

- Ricky Gervais

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Abstract

This master thesis, in continuity with previous work, aims primarily at the synthesis of hexacyclic steroidal derivates containing heteroatoms in the E and F rings, and also at the reduction of their functional groups in order to achieve greater antineoplastic activity.

Steroids are abundant in the natural world and display a wide array of biological acivities, exhibiting contraceptive, anti-inflammatory and antineoplastic properties. Introducing heteroatoms in the steroidal scaffold usually results in changes in their pharmacological properties, and is a strategy currently used in design and development of CYP17 inhibitors that show potential for treatment of benign prostatic hyperplasia and prostate cancer.

In this frame of reference, the reactivity of diazafulvenium methides was explored towards 16-dehydropregnenolone, a steroidal substrate bearing an unsaturated bond in the D ring. This steroid, acting as a dipolarophile, intercepted the 1,7- dipoles, participating in a [8π+2π] cycloaddition, originating hexacyclic chiral steroids containing a D-ring fused bicyclic system. Afterwards, the ester and ketone groups of the synthesized compounds were reduced to hydroxymethyl and hydroxyl groups, aiming at increasing their pharmacological potency.

This thesis' work began with the synthesis of the 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-carboxylates acting as precursors of diazafulvenium methides, through thermal extrusion of sulfur dioxide.

By forming these dipoles in presence of the steroidal substrate, three hexacyclic steroidal compounds derived from 16-dehydropregnenolone were obtained. Finally, the reduction of the ester groups on the pyrazole ring and the ketone group on the steroidal scaffold was carried out.

Resumo

Esta dissertação de mestrado, dando continuidade a um projecto prévio, tem como principal objectivo a síntese de compostos esteróides hexacíclicos contendo heteroátomos nos anéis E e F e, posteriormente, a redução dos grupos funcionais dos compostos para potenciar a sua actividade anticancerígena.

Compostos esteróides são abundantes na natureza e exibem uma variedade de actividades biológicas, apresentando efeitos contraceptivos, anti-inflamatórios ou anticancerígenos. A introdução de heteroátomos ou a fusão de anéis heterocíclicos ao esqueleto esteróide resulta normalmente em alterações nas suas propriedades farmacológicas, e é uma estratégia actualmente usada no design e desenvolvimento de inibidores da CYP17 que demonstram potencial no combate à hiperplasia benigna da próstata e cancro da próstata.

Neste contexto, explorou-se a reactividade de aniões metil-diazafulvénio para com a 16-desidropregnenolona (16-DHP), um substrato esteróide com uma ligação dupla no anel D. Este esteróide, actuando como dipolarófilo, intercepta os 1,7-dipolos, participando em reacções de cicloadição [8π+2π], originando compostos esteróides hexacíclicos quirais com um sistema bicíclico fundido ao anel D. Posteriormente, os grupos funcionais cetona e éster dos compostos sintetizados são reduzidos a grupos hidroxilo, com o intuito de aumentar a sua potência farmacológica.

O trabalho laboratorial iniciou com a síntese dos 2,2-dioxo-1*H*,3*H*-pirazolo[1,5-*c*][1,3]tiazole-carboxilatos que funcionam, mediante extrusão térmica de dióxido de enxofre, como precursores dos aniões metil-diazafulvénio.

Por formação destes compostos na presença do substrato esteróide foi possível obter três compostos hexacíclicos derivados da 16-desidropregnenolona. Por fim foi efectuada a redução dos grupos éster do anel pirazole e do grupo cetona do esqueleto esteróide.

Contents

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Nomenclature

16-DHP	16-dehydropregnenolone
16-DPA	16-dehydropregnenolone acetate
AI	aromatase inhibitor
BPH	benign prostatic hyperplasia
BTMSA	bis(trimethylsilyl)acetylene
cDNAs	complementary deoxyribonucleic acid
CRPC	castration resistant prostate cancer
CYP17	cytochrome P450 17α hydroxylase/17,20 lyase
d	doublet
dd	double doublet
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DMAD	dimethyl acetylenedicarboxylate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EI	electron ionization
EMATE	estrone 3-O-sulfamate
EPAC	ethyl phenylacetilenecarboxilate
ESI	electrospray ionization
FDA	Food and Drug Administration
FVP	fast vacuum pyrolysis
HOMO	highest occupied molecular orbital
HSD	hydroxysteroid dehydrogenase
HSDI	hydroxysteroid dehydrogenase inhibitor
IC50	half maximal inhbitory concentration
IR	infrared spectroscopy
IUPAC	International Union of Pure and Applied Chemistry

LUMO	lowest unoccupied molecular orbital
m	multiplet
m.p.	melting point
MCPBA	meta-chloroperoxybenzoic acid
MW	microwave
NMR	nuclear magnetic resonance
NPM	N-phenylmaleimide
r.t.	room temperature
s	singlet
SAR	structure–activity relationship
STS	steroid sulfatase
STSI	steroid sulfatase inhibitor
t	triplet
TCB	1,2,4-trichlorobenzene
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran
TMS	tetramethylsilane
TMSA	trimethylsilylacetylene

Part I

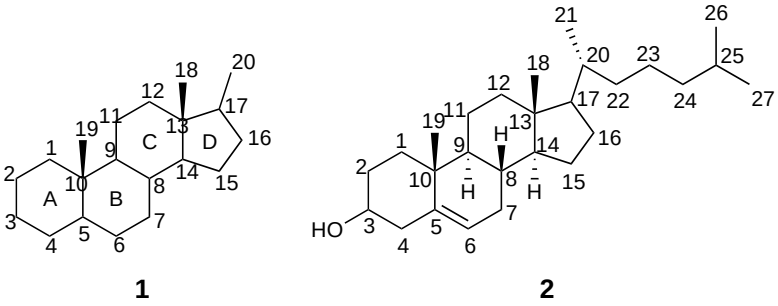
INTRODUCTION

In this part, steroid structure and steroidogenesis are initially focused, followed by pharmacological properties and examples. Antineoplastic activity is analysed in greater detail, with a general view on enzymatic targets. In the end, reactions leading to penta- and hexacyclic steroids are analyzed, with a greater focus on molecules containing heteroatoms.

1 STEROIDS: STRUCTURE, BIOSYNTHESIS AND PHARMACOLOGICAL PROPERTIES

1.1 STRUCTURE AND BIOSYNTHESIS

Steroids are a specific group of lipids including a wide variety of naturally-available structures in both animals and plants. Performing important *in vivo* roles, acting as signalling molecules (e.g. hormones) and as components of cell membranes, they regulate a wide variety of development and physiological processes from embryo stage to adulthood. Steroidal hormones are similar to the classic 4-ringed structure (**1**) and all derive from cholesterol (**2**).



1 **2**

Figure 1.1: Basic numbered structure for classic 4-ringed steroid (**1**) and cholesterol (**2**).

The biological process by which steroids are generated from cholesterol and changed into other active steroidal hormones is called steroidogenesis.[1] In humans, it occurs in a number of locations:

- All human tissues which produce steroids must first convert cholesterol to pregnenolone. This conversion is the rate-limiting step of steroid synthesis, which occurs inside the mitochondria of the respective tissue.
- Corticosteroids are produced in the adrenal cortex.
- Oestrogen and progesterone are made primarily in the ovary and the placenta during pregnancy, and testosterone in the testes.
- Testosterone is also converted to oestrogen to regulate the supply of each, both in females and males.

Steroidogenesis consists of a series of enzymatic transformations of cholesterol's D-ring, and several types of steroids are synthesized by the human organism by conversion of

cholesterol into progestogens (21C), androgens (19C) and oestrogens (18C) through various enzymatic pathways.[2]

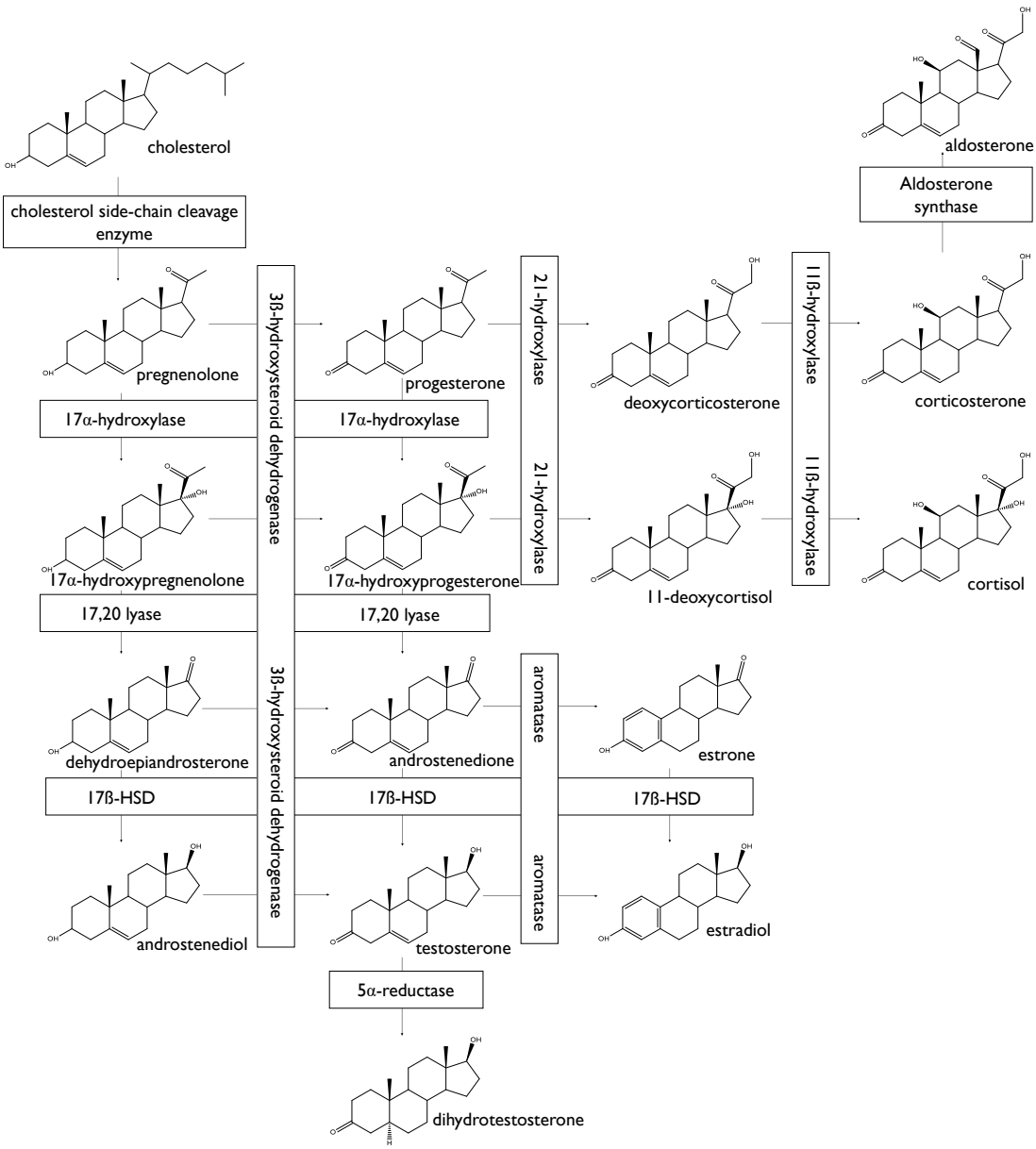


Figure 1.2: Schematic representation of steroidogenesis.

Isolation of some steroidogenesis key enzymes and a better understanding of their cDNAs and genes showed that there are fewer enzymes than reactions, given that usually, a certain enzyme catalyses the same reaction in different tissues. This means steroidogenesis can be seen as a simple process that has small variations, depending on the tissue it occurs in.

1.2 PHARMACOLOGICAL PROPERTIES

Since their discovery in 1935, steroids have been used for treatment of diseases: initially, they were thought to only be helpful against Addison's Disease,[3] but over the course of the following decade, scientists found the range of steroid medical uses broadening from areas so far apart as contraceptives to anti-inflammatory drugs.[4]

Nowadays, steroid use in medicine is mainly related to anti-inflammatory and immune system modulating properties.[5] Due to their wide array of pharmacological properties, steroids are still a common starting point for new drug research, particularly when a small structural change can trigger a completely different biological response.[2] Most steroidal drugs derive from steroids themselves, although some can be obtained through *de novo* synthesis.

Usually, the process by which drug molecules affect physiological functions starts with a bond to the transmembrane receptors on the cellular surface, triggering the release of secondary messengers into the cytosol and/or nucleus. Steroids, on the other hand, act directly on the cellular nucleus: they permeate the cell wall rapidly, due to their lipophilicity. Once inside the cell membrane, steroids may undergo chemical reactions in order to bind specifically to the nuclear receptors, present in the cytosol. Several steroids have pharmacological use due to their anti-inflammatory, anti-tumoral and anti-viral properties.

1.2.1 Steroids as anti-neoplastic drugs

Most of the steroidal anti-cancer drugs have been developed as cytotoxic (through non-hormonal targets) or anti-hormonal/anti-proliferative drugs. In the second group, enzyme inhibitors include steroid sulfatase inhibitors (STSI), aromatase inhibitors (AI) and hydroxysteroid dehydrogenase inhibitors (17βHSDI); receptor inhibitors are comprised mainly of anti-oestrogens and anti-progestins.

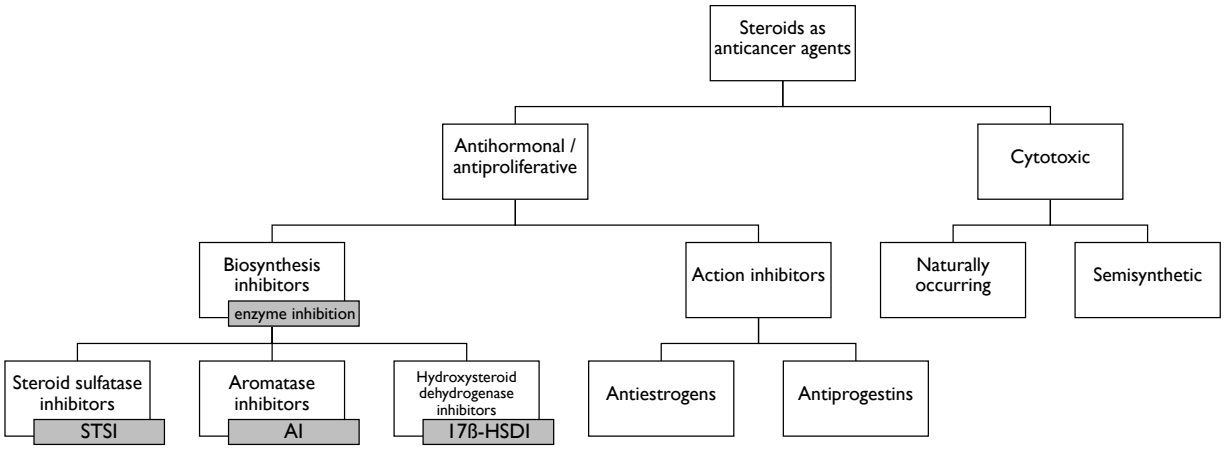


Figure 1.3: Classification of steroidal anti-cancer agents.

1.2.2 Glucocorticoids

Another important class of steroidal drugs, glucocorticoids, are used to suppress various allergic, inflammatory, autoimmune disorders and are also administered as post-transplantory immunosuppressants. Glucocorticoids are the second-line treatment for asthma, because of their potent anti-inflammatory properties: the primary mechanism is the synthesis of lipocortin-1, which indirectly inhibits several leukocyte inflammatory events (epithelial adhesion, emigration, chemotaxis, phagocytosis, respiratory burst, etc.). Typical examples of commercially available corticosteroids are dexamethasone (**3**), used for treatment of rheumatic problems, skin diseases and severe allergies; prednisolone (**4**), used for inflammatory and autoimmune conditions;[6] and fludrocortisone (**5**), used in cases of Addison's disease and chronic low blood pressure.

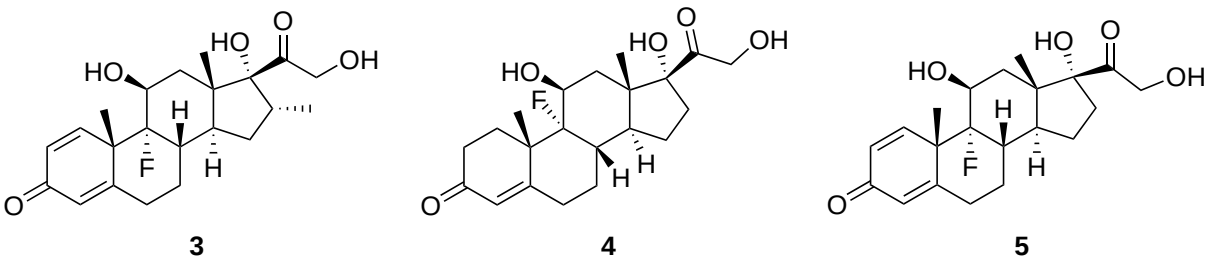


Figure 1.4: Structures of dexamethasone (**3**), prednisolone (**4**), and fludrocortisone (**5**).

1.2.3 Neuroactive steroids

Neuroactive steroids, also known as neurosteroids, are endogenous or exogenous steroids that rapidly alter neuronal excitability through interaction with ligand-gated ion channels and other cell surface receptors.[7] In addition to their actions on neuronal membrane receptors, some of these steroids may also exert effects on gene expression via nuclear steroid hormone receptors. Neurosteroids have a wide range of potential clinical applications, from sedation to treatment of epilepsy and traumatic brain injury.[8,9]

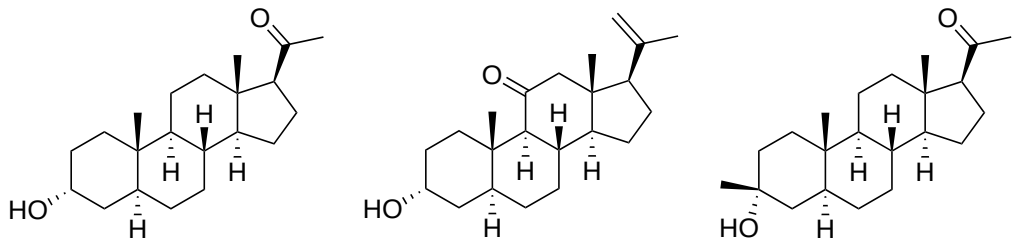
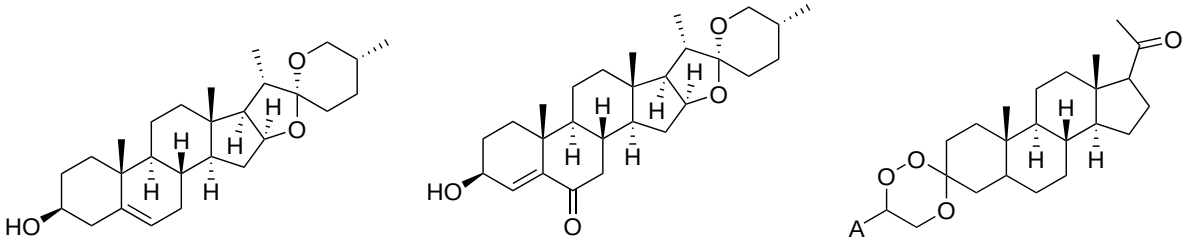


Figure 1.5: Examples of neuroactive steroids: allopregnanolone (**6**), alfaxalone (**7**), and ganaxolone (**8**).

1.2.4 Anti-malaric drugs

Some steroid derived compounds may also present anti-malaric activity, with many diosgenin (**9**) and diosgenone derivatives being studied for this effect, like compound **10**, which presents an IC_{50} of 11.3 ± 1.6 μM and 9.8 ± 1.0 μM against the FcB2 (chloroquine-resistant) and NF-54 *P. falciparum* strains, respectively.[10]

Steroid-based 1,2,4-trioxanes have been synthesized and evaluated for their anti-malaric activity in Swiss mice models of *Plasmodium yoelii*. According to the authors, further Structure-Activity Relationship data is needed, although the biological activity shows a strong dependence on the size and the nature of the steroidal side chain: trioxanes which have a shorter side chain appear to be significantly more active, such as **11**, for example.[11]

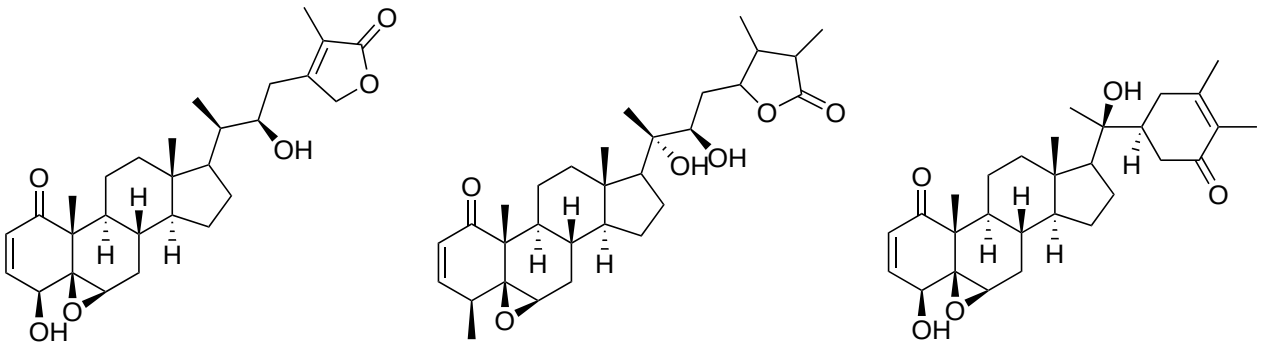


9 **10** **11**

Figure 1.6: Example of diosgenin (**9**), diosgenone derivative (**10**) and a 1,2,4-trioxane (**11**) with anti-malaric activity.

1.2.5 Withanolides

Withanolides are a group of naturally occurring compounds exhibiting a broad range of biological properties: anti-microbial, anti-inflammatory, hepatoprotective, immunomodulatory, insect anti-feedant and anti-tumour. Structurally, they consist of an ergostane skeleton, oxidized at C1 and bearing a C22,26 δ-lactone group. Structure-Activity Relationship data has determined that anti-tumour activity requires some structural specificity: an α,β-unsaturated ketone on ring A, a 5β,6β-epoxide on ring B, and a C9 side-chain with an α,β-unsaturated δ-lactone group. Withaferin A (**12**) has all these structural requisites and is the most known anti-tumoural withanolide, causing suppression of tumour cell proliferation both *in vitro* and *in vivo*. Ixocarpalacton A (**13**), isolated from *Physalis philadelphica*, shows promising results as an anti-tumour agent,[12] and withanolide D (**14**) was reported to have significant anti-tumour activity against carcinoma of the nasopharynx, both *in vitro* and *in vivo*.[13]



12 **13** **14**

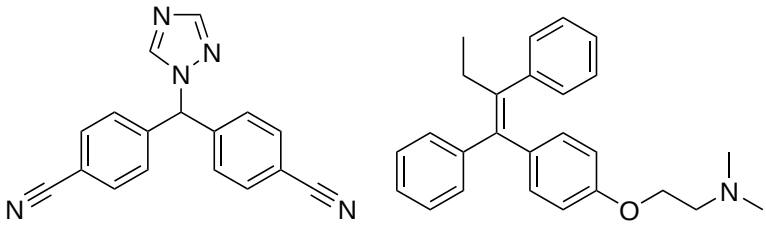
Figure 1.7: Structures of withaferin-A (**12**), ixocarpalacton-A (**13**) and withanolide-D (**14**).

2 STEROIDS AS ANTI-NEOPLASTIC DRUGS

2.1 BREAST CANCER

Breast cancer is a neoplastic growth of breast tissue. In Europe, breast cancer is the deadliest among women, responsible for 1 out of every 6 cancer-related casualties.[14] Oestrogens are necessary for growth of regular tissue, but are also responsible for the development of some tumours: benign prostatic hyperplasia, prostate and endometrial cancer, and also breast cancer. Oestrogen is the main hormone involved in the development and growth of breast tumours and oestrogen deprivation remains a key therapeutic approach.[15] Oestrogens are known to be important in the growth of breast cancers in both pre- and postmenopausal women. Although oestrogens are no longer made in the ovaries after menopause, peripheral tissues - such as adipose and liver tissue - produce sufficient concentrations to stimulate tumour growth.[16] Oestradiol concentrations in breast cancer tissues are 5 and 23 times higher than in plasma of pre- and postmenopausal women, respectively.[17] This data suggests there are biosynthetic pathways in tumours that are responsible for surprisingly high concentrations of some steroids, and local synthesis of oestradiol is one of the culprits: breast cancer tissue contains all enzymes needed to locally synthesize oestradiol from circulating inactive steroid precursors present in plasma. There are two biological pathways in play: through aromatase conversion of androgens into oestrogens, and through sulfatase conversion of estrone sulfate into estrone, further converted into oestradiol by the reductive activity of 17β-hydroxysteroid dehydrogenase.[17]

Molecules that act as inhibitors of the enzymes involved in oestradiol synthesis have been used as anti-neoplastic drugs, with great results in breast cancer, particularly for aromatase inhibitors such as letrozole (**15**) and tamoxifen (**16**).



15 **16**

Figure 2.1: Structures of letrozole (**15**) and tamoxifen (**16**).

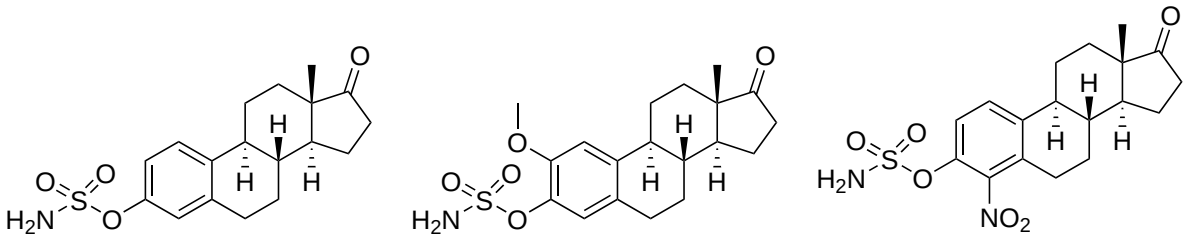
2.1.1 Steroid sulfatase

Steroid sulfatase (STS) is a microsomal enzyme, found in the endoplasmic reticulum, in several tissues including placenta and skin tissue. It is responsible for regulating the form-

ation of biologically active steroids from inactive steroid sulfates (e.g. dehydroepiandrosterone sulfate and estrone sulfate),[18] catalysing the hydrolysis of the sulfate esters of 3-hydroxy steroids, which are inactive transport or precursor forms of the active 3-hydroxy steroids.[19] The reverse reaction is catalysed by a different enzyme - sulfotransferase - and is, in normal conditions, in equilibrium with the reaction carried by sulfatase.[20]

Oestrone sulfate is the main source of active steroids in receptor positive breast cancer tissue, responsible for oestrone concentrations ten times higher than aromatase, and also with a significantly higher lifespan.[21] Steroid sulfates are highly polar molecules and therefore have no affinity towards oestrogen receptors: they function as oestrogen provisions, in order to protect neighbouring tissues from excessive stimulation. Intratumoral STS mRNA levels are significantly higher when compared to adjacent non-malignant tissue[18] and have been associated to the progression of tumours, not only in breast tissue, but also in endometrial, ovarian and prostatic tissue.[22]

Over the last decade, STS has received increasing attention as a drug target due to its role in various diseases, in particular of estrogen-dependent tumours. Current STS inhibitors have been designed based on natural substrates, or discovered through screening of compound libraries: rational design was not as efficient, since only recently did the structure of STS become available.[23] STS inhibitors can be classified as reversible and irreversible. Most reversible inhibitors are substrate-based, have a steroid skeleton and are, usually, less potent than irreversible inhibitors. All irreversible inhibitors feature the arylsulfamate moiety.[19]



17 **18** **19**

Figure 2.2: Structure of EMATE (**17**) and its derivates.

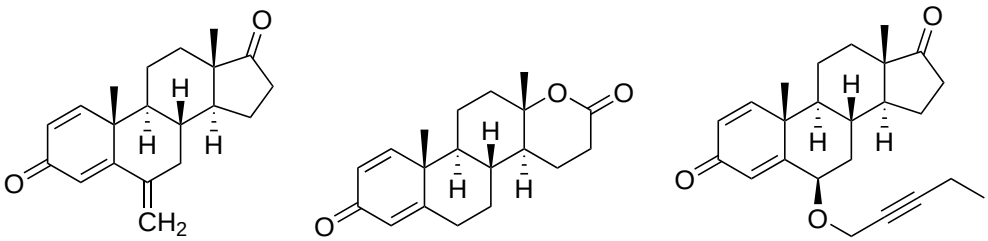
The main goal of STS inhibitors is to inhibit growth of oestrogen receptor-positive tumour tissue and so, any oestrogenic activity of the inhibitors is higly prohibitive. Surprisingly though, EMATE (**17**), a prototype STS inhibitor, displayed high oestrogenicity levels, which limited its potential use in breast cancer therapy.[24,25] It triggered a search for irreversible inhibitors based on EMATE's structure, that maintained its potency but lacked its oestrogenicity. Several molecules were synthesized: the compounds **18** and **19** are the most potent, with IC_{50} values of 0.004 μM and 0.0008 μM, respectively, in placental microsomes

assay.[19]

2.1.2 Aromatase

Aromatase is the enzyme responsible for the crucial and rate-limiting step of the synthesis of oestrogens. It is a member of the cytochrome P450 family, responsible for the aromatization of androgens (C19) into oestrogens (C18) through a series of hydroxylations, and elimination of C19 as formic acid.[26] The expression of this enzyme occurs mainly in the ovaries and placenta, but it is also present, even if in smaller concentrations, in other tissues including gonads, brain, adipose tissue, placenta, blood vessels, skin, and bone. Although it has beneficial effects on sexual development and even in neuroprotection,[27] it is also over-expressed and aiding the development of tissue of endometriosis, uterine fibroids, breast cancer, and endometrial cancer. Aromatase inhibitors (AIs) have become useful in the management of patients with breast cancer whose lesion was found to be oestrogen receptor positive.[28] In fact, studies have demonstrated that the third generation of AIs can be used as a first weapon against advanced breast cancer receptor positive tumours, in postmenopausal women. This class of compounds can also be used in chemoprevention.

AIs can be divided into type I and type II inhibitors. Type I inhibitors are androstenedione analogues, competitively binding to the active site of the enzyme, like exemestane (**20**). Testolactone (**21**) is used as treatment for advanced breast cancer, and a derivate of exemestane (**22**), with a pent-2-yn-1-yloxy group on C6, has an IC_{50} close to that of letrazole's (11.9 nM vs 9.9 nM).[29] Type II inhibitors are non-steroidal and bind reversibly to the enzyme's heme group.



20 **21** **22**

Figure 2.3: Structures of aromatase inhibitors.

2.1.3 17β-hydroxysteroid dehydrogenase

17β-hydroxysteroid dehydrogenases are a group of alcohol oxidoreductases responsible for catalysing the dehydrogenations of 17-hydroxysteroids. This includes interconversion

of estrone and estradiol. Estrone sulfate is the main source of active steroids in breast cancer tissue: after being converted to estrone by steroid sulfatase, it is converted into estradiol by 17βHSD. For this role, 17β-hydroxysteroid dehydrogenase is also considered a pharmaceutical target for inhibitor development.[30] It has been shown that this enzyme is present in malignant epithelial cells in breast cancer and that its reductive action is greater than the inverse oxidative action.[17] Dehydroepiandrosterone can also be reduced by 17βHSD into androestenediol, which is also a good candidate for binding to oestrogen receptors, stimulating the growth of breast cancer tissue.

2.2 PROSTATE CANCER AND BENIGN PROSTATIC HYPERPLASIA

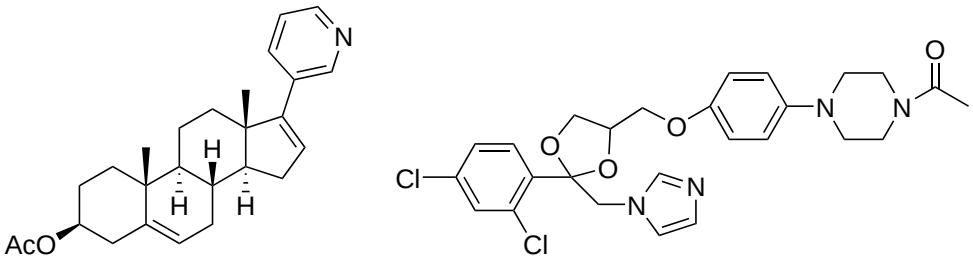
The prostate is an exocrine gland of the male reproductive system. Its function is to secrete a slightly alkaline fluid that usually constitutes 30% of the volume of the semen.[31] Its normal development and maintenance is dependent on androgens and the androgen receptors are important in the development and progression of prostate cancer.[32] Prostate cancer and benign prostatic hyperplasia (BPH) are the two main pathologies of the prostate. In later stages, prostate cancer can lead to difficulty urinating, blood in the urine, or pain in the pelvis and back. Treatment of aggressive prostate cancers may involve hormonal (steroidal) therapy.[33] Benign prostatic hyperplasia is a noncancerous increase in size of the prostate. BPH involves hyperplasia of prostatic stromal and epithelial cells resulting in large nodules. When sufficiently large, the nodules impinge on the urethra and increase resistance to the flow of urine from the bladder and may produce similar symptoms to prostate cancer: need to urinate frequently, involuntary urination, and urinary hesitancy and intermittency.[34]

Androgens (testosterone and related hormones) are considered to play a permissive role in the development of BPH: androgens have to be present for BPH to occur but do not necessarily directly cause the condition. On the other hand, studies suggest that administering exogenous testosterone is not associated with a significant increase in the risk of BPH symptoms, so the role of testosterone in prostate cancer and BPH is still unclear.[35]

Up to 95% of circulating androgens come from the testes, and the early therapy to both prostate cancer and benign prostatic hyperplasia was chemical or surgical castration.[36,37] Although this approach yielded good results, some cases displayed resistance to treatment after androgen-levels lowering therapy, due to increased androgen receptor sensitivity. The proposed resistance mechanism involves an increase in both the local production of androgens and in the expression of the androgen receptor, in order to compensate for lower circulatory levels of testosterone.[38] Studies indicate that castration resistant prostate cancer (CRPC) is related to androgen receptors' overexpression. The phase I clinical tri-

als of abiratherone acetate (**23**) - CYP17 inhibitor - strengthened this idea, since it led to a lowering of the prostate's specific antigen, leading to its approval by the FDA in 2011.[39]

The evolution of prostate cancer is related to the balance between cell growth and death. Androgens are the main regulators of this balance, stimulating cell multiplication and inhibiting apoptosis. Androgenic hormones - testosterone and DHT in particular - exert their effect on the prostate through binding to the androgen receptors, resulting in the synthesis of specific proteins and cellular proliferation. For this reason, one of the approaches for treatment of prostate cancer and BPH targets the enzymes involved in the androgen synthesis, such as 5α-reductase and CYP17.[40] Cetoconazole (**24**), initially used as an antimicotic, has been successful in treatment for prostate cancer, due to its CYP17 inhibitor traits.[41]



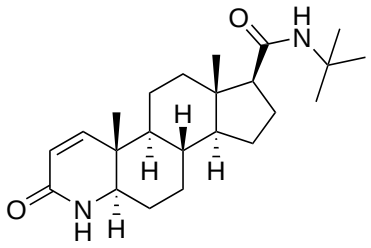
23 **24**

Figure 2.4: Structures of abiratherone acetate (**23**) and cetoconazole (**24**).

2.2.1 5α-reductase

5α-reductase is an enzyme that catalyses an irreversible conversion of the C4-C5 double bond of testosterone into the corresponding 3-oxo-5α-testosterone, commonly known as dihydrotestosterone. It is a critical mediator of prostatic growth, ten times more potent than testosterone, since it dissociates from the androgen receptor more slowly. Although testosterone is the main substrate for this enzyme, other endogenous steroids are known to bind to it as well.

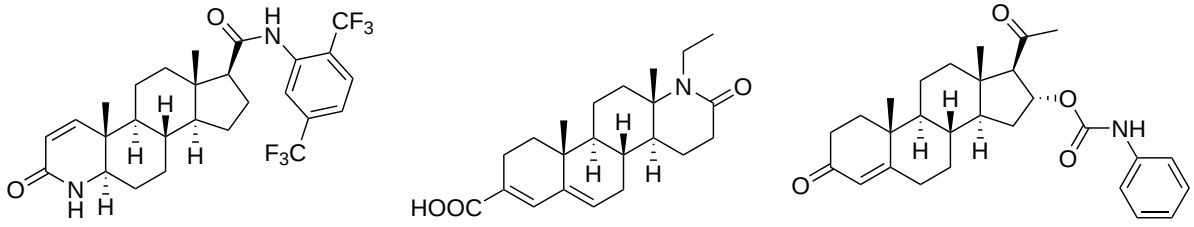
The 5α-reductase enzyme family consists of 3 isoenzymes, of which the isoforms I and II are the most well-known. Isoform I is the most common, present in skin, liver, kidneys, brain and lung tissues. It has an increased activity in prostate cancer, where it plays a more significant role than in BPH. Isoform II, towards which testosterone has a higher affinity, is predominantly located in prostatic and genital tissues in general and has a fundamental role in BPH.[42] Due to its unstable nature, it has not yet been possible to isolate this enzyme and its crystalline structure remains unknown. Therefore, the design of 5α-reductase inhibitors has been mostly based on the substrate structures, the enzyme mechanism, and previous data on SAR.[43]



25

Figure 2.5: Structure of finasteride.

Finasteride (**25**) was the first 5α-reductase steroidal inhibitor approved by the FDA for therapy against BPH, and displays a greater selectivity for the isoform II. This molecule served as starting grounds for the development of other azasteroids that showed affinity towards both isoforms I and II, such as dutasteride (**26**), which is approximately 60 times more potent. Dutasteride is used for treatment of BPH, but although it is successful in reducing symptoms in the urinary tract, it is also responsible for decreasing muscle and bone levels, and causing impotence. Finasteride derivates with a carboxylic acid on C3 have also been studied as 5α-reductase inhibitors, and molecule **27** has shown to be a potent inhibitor of type II 5α-reductase, while also reducing the size of the prostate.[44,45] Progesterone and desoxycorticosterone have also been used as a starting point for new 5α-reductase inhibitors, since they are testosterone competitors for the enzyme's binding site, resulting in drugs like **28**, designed taking into account the common aspects of both molecules.[46]



26 **27** **28**

Figure 2.6: Structures of 5α-reductase inhibitors.

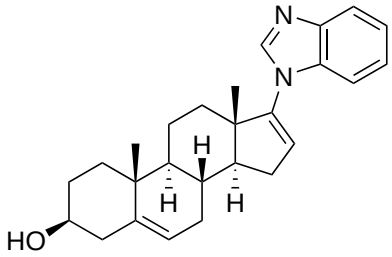
2.2.2 CYP17

CYP17, also known as 17α-hydroxylase, is a cytochrome P450 enzyme that performs a vital role in the biological synthesis of androgens. It catalyses the hydroxylation of progesterone into 17α-hydroxyprogesterone on C17, and breaks the C17-C20 bond, creating androestenedione and dehydroepiandrosterone (DHEA). It contains a heme group in its active site, responsible for this catalytic activity. The steroids originated by CYP17 are weak

androgens but function as substrates for other enzymes - 5α-reductase and 17βHSD- which will originate far more potent androgens - testosterone and DHT, discussed in section 2.2.1. One of the catalytic effects of this enzyme - hydroxilation of pregnenolone - originates 17α-hydroxypregnenolone, which is a starting material for the biosynthesis of corticosteroids. Therefore, ideal CYP17 inhibitors must focus solely on the C17-C20 lyase and leave the hydroxilation of pregnenolone untouched.

Most CYP17 inhibitors consist of heterocycles containing nitrogen atoms in place of C17, capable of coordinating to the iron present in the enzyme's heme group.[47] Galecterone (**29**), currently in phase III clinical trials,[48] contains a benzimidazole group at C17, and possesses a unique dual mechanism of action, acting as both an androgen receptor antagonist and as a CYP17 inhibitor.[49]

Recently, the X-Ray crystalline structure of CYP17 in presence of potent steroidal inhibitors was discovered,[50] which will most likely contribute to the design and discovery of new CYP17 inhibitors.



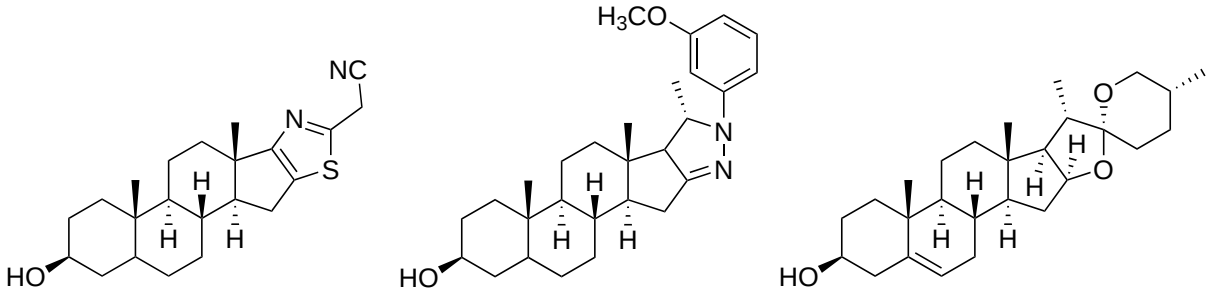
29

Figure 2.7: Structure of galecterone.

3 BUILDING UPON THE STEROID SKELETON: PENTA- AND HEXACYCLIC STEROIDS

3.1 PENTACYCLIC STEROIDS

Pentacyclic steroids have been the focus of research for more than half a century, becoming molecules of interest, both in chemical and biological terms. Pentacyclic steroids are known to exhibit interesting and diverse biological properties and there are many examples of pharmacological and biological importance, especially those containing a heteroatom. This has led to an increased effort in finding synthetic processes which originate new steroidal compounds.[51] There are several examples of anti-neoplastic steroids with uses in breast cancer (**30**), acute promyelocytic leukemia (**31**), and cell apoptosis induction in cancerous tissue (**32**).[52,53,54]



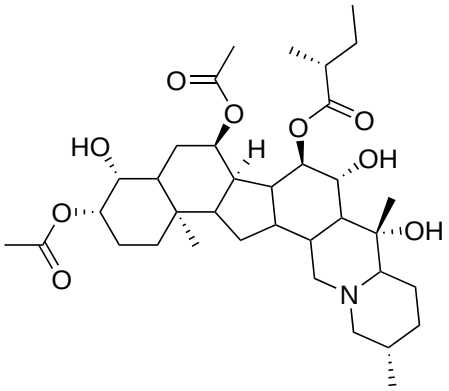
30 **31** **32**

Figure 3.1: Structures of pentacyclic steroids with pharmaceutical properties.

A change in a steroid's biological properties is often an expected result of introducing a heteroatom in its skeleton.[55] Steroids with heterocycles display a wide array of biological properties in addition to their typical hormonal activity: these include anti-tubercular, anti-oxidant, anti-neoplastic properties and promote immune reactions.[56,57] Introducing heterocycles in steroidal structures is, actually, a frequently used strategy in drug development and enzyme-inhibitor design, and the incorporation of short carbon bridges linking certain positions of the steroid skeleton is a popular approach to making new biologically active steroid hormone analogues. Most of the synthetic effort so far has been aimed at annulation of ring A, D, and bridging rings A and B. Most of the published work focuses on the synthesis of these steroid derivates, and little has yet been published concerning their biological activities.[51]

3.2 HEXACYCLIC STEROIDS

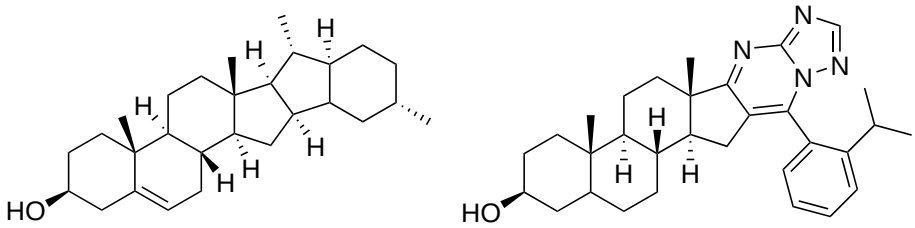
Hexacyclic steroids are a much less explored group of molecules: most published work consists in the isolation and evaluation of biological effects. The two largest groups of hexacyclic steroid derivatives are saponines and steroid-alkaloids, both occurring in the plant kingdom. The first scientific reference of a medical use of a hexacyclic steroid dates back to 1952, when after being isolated from *pure veratrum*, neogermitrine (**33**) was associated with hypotensive activity.[58]



33

Figure 3.2: Structure of neogermitrine.

Recently published work has solanidine (**34**) showing promising preliminary results in cell proliferation rate, cell cycle distribution and apoptosis in human 1547 osteosarcoma cell line tests.[53] A triazolopyrimidine derivative (**35**) has also shown cytotoxic activity *in vitro* against human prostatic carcinoma, human breast carcinoma and human esophageal carcinoma cell lines.[59]



34 **35**

Figure 3.3: Structures of hexacyclic steroids with pharmaceutical properties.

Part II

RESULTS AND DISCUSSION

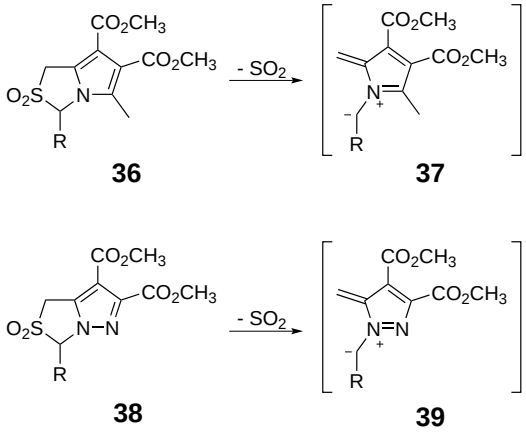
The research work focused the synthesis of new hexacyclic steroid derivates through [8π+2π] cycloaddition reactions of 1,2-diazafulvenium methides. In this part, after introducing previous work on this particular reactivity, the results obtained throughout the research work are presented and discussed.

4 [8π+2π] CYCLOADDITION

Pericyclic reactions of extended dipoles (those with more than 4π electrons) are of considerable value in organic synthesis,[60] and [8π+2π] cycloaddition reactions are of special importance within pericyclic reactions, especially in the synthesis of compounds containing 6-membered heterocycles.

Considering the first reports of the reactions involving diazafulvenium methides and 16-DPA resulted in chiral steroid-fused compounds,[61] it is expected that [8π+2π] cycloadditions will prove being of great synthetic use in the near future.

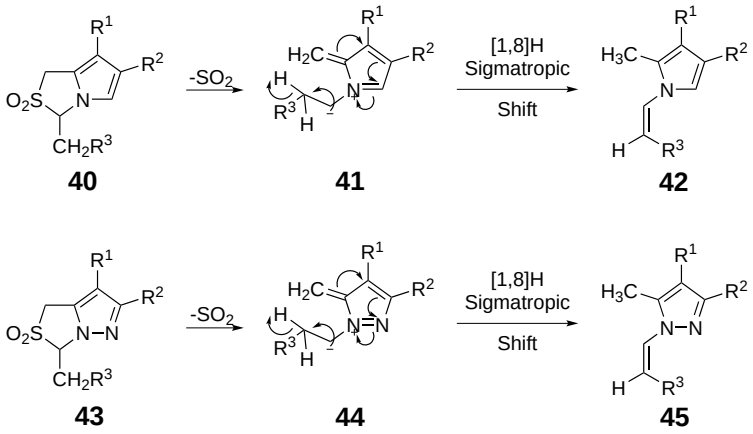
1-Azafulvenium methides (**37**) and 1,2-diazafulvenium methides (**39**) are dipoles with a conjugated system of 8π electrons which can act as 4π 1,3-dipoles or as 8π 1,7-dipoles.[62] Reactivity of pyrrolo[1,2-*c*]thiazole-2,2-dioxides (**36**) and pyrazolo[1,5c][1,3]thiazole-2,2-dioxides (**38**) was reported by Sutcliffe *et al*, proving that these are 'masked' aza- and diazafulvenium methides,[60] respectively, which are obtained by thermal extrusion of sulphur dioxide (Scheme 1).[61]



Scheme 1: SO_2 extrusion of **36** and **38** leading to aza and diazafulvenium methides.

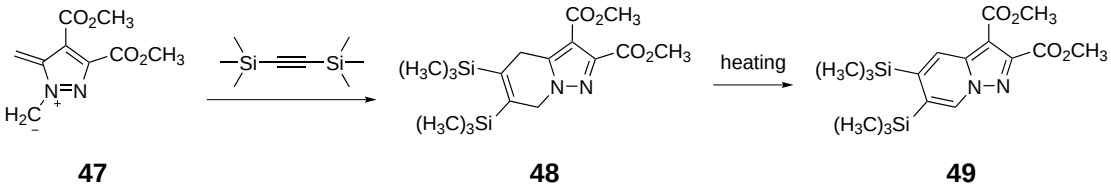
Padwa *et al* reported unsuccessful attempts at the extrusion of SO_2 through thermal and photochemical methods in 1989, but Sutcliffe and co-workers achieved this goal under flash vacuum pyrolysis (FVP) a decade later.[60,63] Sutcliffe *et al* also reported that the SO_2 extrusion from the pyrazolo sulfone occurs more easily than from the pyrrolo sulfone,[62] hinting that the extra nitrogen atom works as an electron-withdrawing group, facilitating the process.

It was also reported that aza- and diazafulvenium methides bearing methyl or benzyl groups at C1 or C7 undergo sigmatropic [1,8]H shifts giving vinyl-pyrroles (**42**) and vinyl-pyrazoles(**45**), respectively (Scheme 2).[61,62]



Scheme 2: Sigmatropic [1,8]H shift of diazafulvenium methides **41** and **44**.

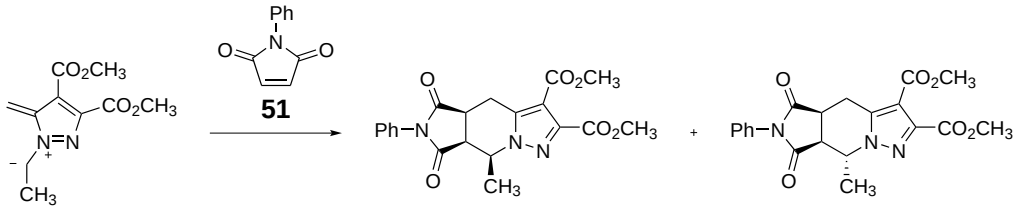
Cycloaddition of diazafulvenium methides was first explored by Sutcliffe *et al*, who reported that no adducts were formed by generating **47** *in situ* in presence of *N*-phenylmaleimide or dimethyl acetylenedicarboxylate. However, in presence of bis(trimethylsilyl)-acetylene (an electron-rich dienophile), the cycloadduct **48** was obtained in 34% yield and further heating resulted in the aromatised adduct **49** in 24% yield: this was the first evidence for the formation of these dipolar systems by intermolecular trapping (Scheme 3).[60]



Scheme 3: Reaction of 1,2-diazafulvenium methide with bis(trimethylsilyl)-acetylene.

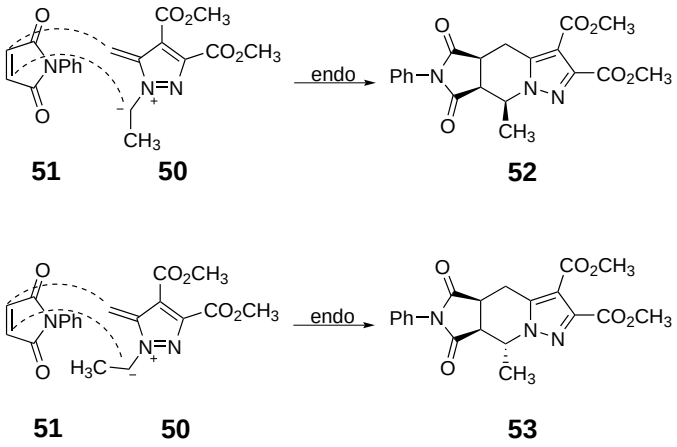
Under the same conditions, the extrusion of SO_2 in presence of the asymmetrical dienophile trimethylsilylacetylene yielded a mixture of regioisomers. According to Sutcliffe et al, the lack of regioselectivity is related to the small difference in the HOMO/LUMO coefficients between the dipole and the dipolarophile. It was also noted that the dipole underwent cycloaddition across the 1,7 but not the 1,3 positions.[60]

Contrary to Storr's results, work by Soares *et al* reported that the 1,7-dipolar cycloaddition of 1,2-diazafulvenium methide **50** with *N*-phenylmaleimide **51** (an electron-deficient dipolarophile) resulted in two diastereoisomeric products, cycloadducts **52** (67%) and **53** (24%) (Scheme 4).[64]



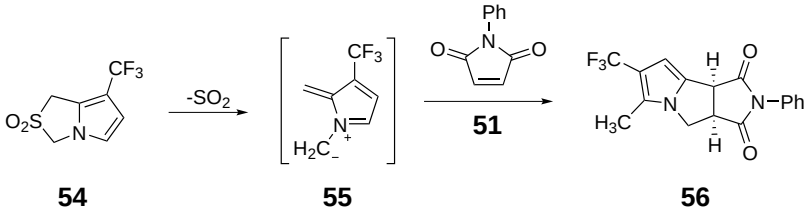
Scheme 4: Reaction of 1,2-diazafulvenium methide with *N*-phenylmaleimide.

Scheme 5 reflects the two different configurations of the diazafulvenium methide being involved in the formation of the *endo* cycloadducts resulting in diastereoisomers. Although the configuration in which the methyl group is facing the interior of the dipolar system is less stable, it is plausible that it is involved on the cycloaddition, since this very same configuration is the one responsible for the sigmatropic [1,8]H shift that leads to the formation of the vinylpyrazole, illustrated in Scheme 2.[64]



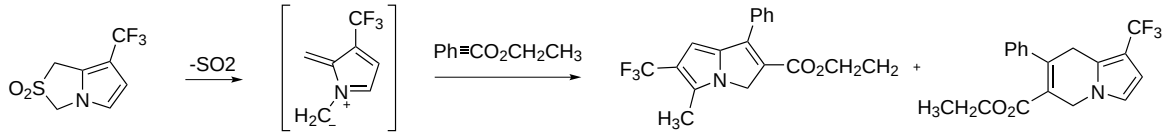
Scheme 5: Possible configurations of the diazafulvenium methide **50** in the *endo* approach.

The first evidence of azafulvenium methides reacting as 1,3-dipoles was reported by Nunes *et al*, after observing the formation of **56** when the azafulvenium methide **55** was formed in presence of *N*-phenylmaleimide. The 1,3-cycloaddition occurred exclusively, and no 1,7-cycloadducts were detected (Scheme 6).[65]



Scheme 6: Azafulvenium methide **55** reacting as a 1,3-dipole.

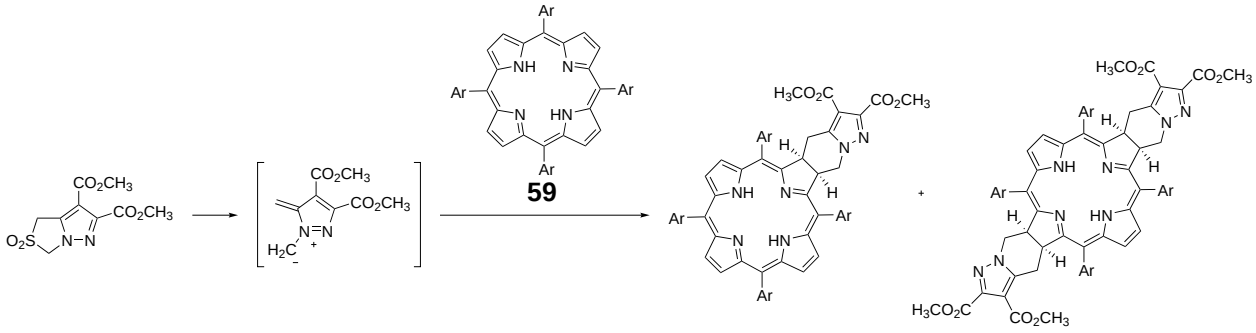
The same azafulvenium methide **55**, however, in presence of ethyl phenylpropiolate, reacted as both a 1,3- and 1,7-dipole, originating a mixture of cycloadducts **57** and **58** (82:18) (Scheme 7).[65]



54 **55** **57** **58**

Scheme 7: Azafulvenium methide **55** reacting as both a 1,3-dipole and 1,7-dipole.

The reactivity of aza- and diazafulvenium methides is also applied to synthesizing molecules with biological activity. Pereira *et al* have described the use of [8π+2π] cycloadditions in order to obtain chlorins **60** and bacteriochlorins **61** from microwave-generated diazafulvenium methides and porphyrins (**59**) (Scheme 8).[66,67] These compounds' absorption and fluorescence spectra and quantum yield measurements show their potential in various bio-medical applications. In particular, the bacteriochlorins are excellent candidates for NIR imaging.[67]

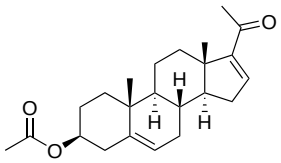


46 **47** **60** **61**

Scheme 8: Synthesis of chlorins **60** and bacteriochlorins **61** through diazafulvenium methide **47**.

5 RESULTS AND DISCUSSION

In previous works,[68,61] diazafulvenium methides were both thermally and microwave-generated in presence of 16-dehydropregnenolone acetate **62** (16-DPA) (Figure 5.1), leading to chiral compounds. The dipole then reacted with the steroid's C16-C17 double bond, originating a hexacyclic steroid, with a double ring fused to the original steroid's D-ring. These were obtained exclusively or selectively with the approach of the 1,7-dipole by the less hindered α-face of 16-DPA. Quantum chemical calculations at the DFT level were carried out in order to rationalize the stereochemistry outcome, and indicated that *endo* cycloadditions of the more stable dipole conformation, having the 1-substituent pointing outward, are significantly more favorable than the alternative *exo* cycloaddition.



63

Figure 5.1: Structure of 16-dehydropregnenolone acetate (16-DPA).

Through this approach, several hexacyclic steroids were synthesized, using 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazoles as precursors to the *in situ* generated diazafulvenium methides.

This research work aimed to synthesize new hexacyclic steroids exploring the reactivity of diazafulvenium methides: the available sulfones **46**, **64** and **65** were used and the synthesis of new 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazoles was attempted (Figure 5.2). The interest in synthesizing the new sulfones **66**, **67**, **68** and **69** originated with cytotoxicity values that display a correlation of higher IC_{50} values with the presence of a benzyl group.[69]

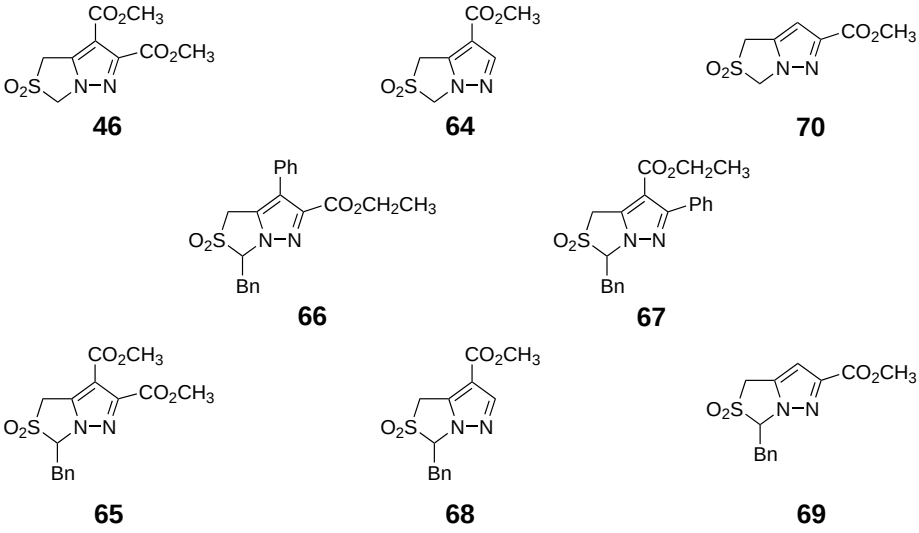


Figure 5.2: 2,2-Dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazoles planned for this work.

In this work, 16-dehydropregnenolone (16-DHP) (Figure 5.3) was used as steroidal substrate and captured the diazafulvenium methides generated *in situ*, originating hexacyclic compounds. Subsequently, some of the hexacyclic steroids' ketone and ester groups were reduced in order to potenciate their anticancer activity.

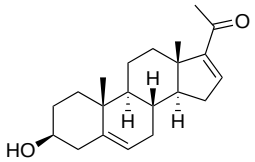
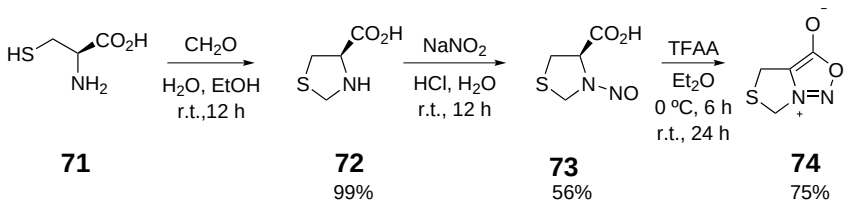


Figure 5.3: Structure of 16-dehydropregnenolone (16-DHP).

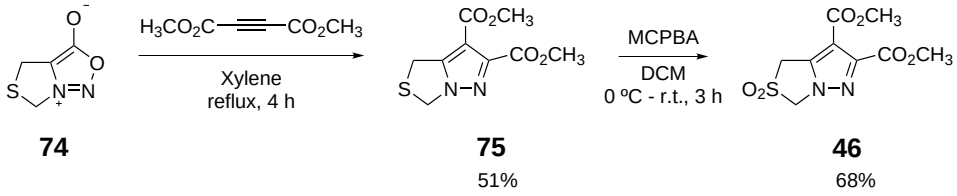
5.1 SYNTHESIS OF 2,2-DIOXO-1*H*,3*H*-PYRAZOLO[1,5-*C*][1,3] THIAZOLES

Scheme 9 shows the initial synthetic pathway towards **74**, to be used as precursor to the 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazoles. L-cysteine **71** reacted with formaldehyde, resulting in thiazolidine **72** in high yield (99%). Its reaction with sodium nitrite in acidic conditions resulted in compound **73** (56%). The mesoionic compound **74** was obtained in good yield (75%) after treating **73** with trifluoroacetic anhydride.



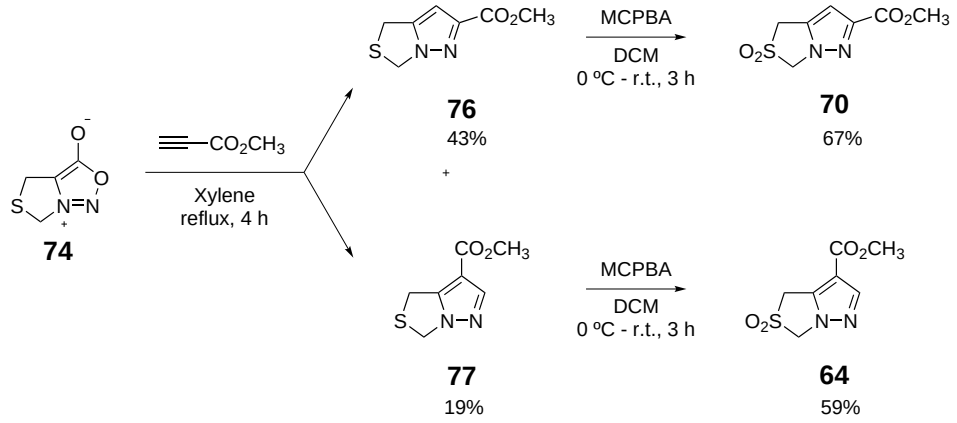
Scheme 9: Synthetic pathway towards compound **74**.

Sydnone **74** is a highly reactive species, able to participate in 1,3-dipole cycloadditions, and adding it to a dipolarophile such as dimethyl acetylenedicarboxylate (DMAD) results in a bicyclic product: the reaction yielded compound **75** as only product in 51% yield. Oxidation of the pyrazolo[1,5-*c*][1,3]thiazole **75** with chloroperoxybenzoic acid (MCPBA) resulted in the target sulfone **46** in 68% yield (Scheme 10).



Scheme 10: Synthesis of compound **46**.

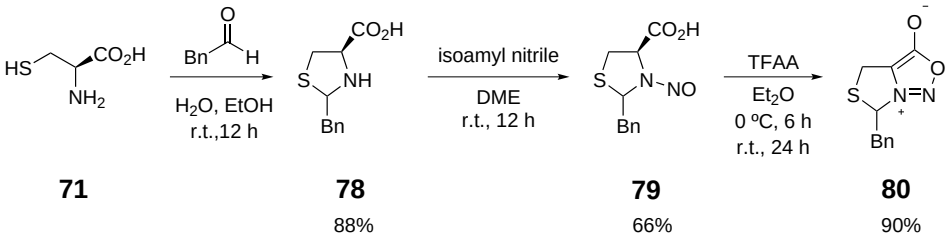
Reaction of **74** with methyl propiolate yielded two regioisomers **76** and **77** (70:30) in 62% yield. Oxidation of the pyrazolo[1,5-*c*][1,3]thiazoles **76** and **77** with MCPBA resulted in the target sulfones **70** and **64** (Scheme 11). In this work, reactivity of compound **70** was not explored further, due to the formation of an unseparable mixture of regioisomers after originating the corresponding diazafulvenium methides in presence of 16-DHP.



Scheme 11: Synthesis of compounds **70** and **64**.

A similar synthetic sequence was carried out, with the objective of obtaining 3-benzyl-2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazoles. The initial sequence towards the mesoionic

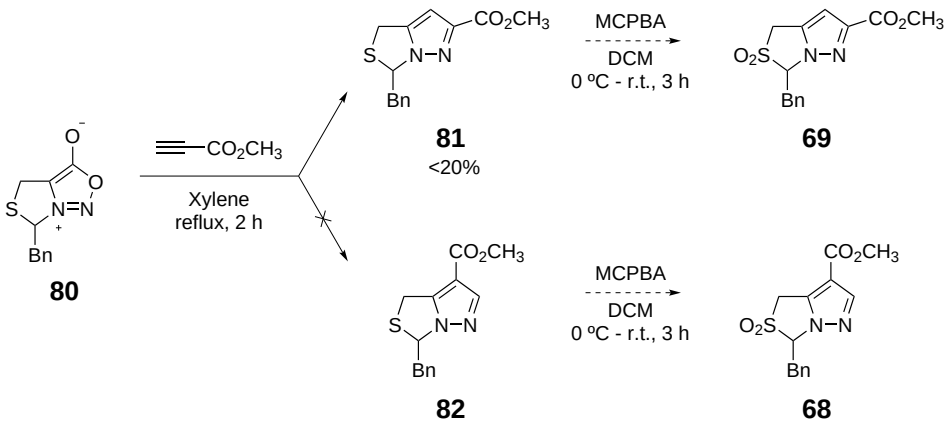
species **80** is shown in Scheme 12. L-cysteine **71** reacted with phenylacetaldehyde, resulting in thiazolidine **78** in high yield (88%). The reaction of **78** with isoamyl nitrile in dimetoxyethane (DME) resulted in compound **79** (66%). The mesoionic compound **80** was obtained in good yield (90%) after treating **79** with trifluoroacetic anhydride.



71 **78** 88% **79** 66% **80** 90%

Scheme 12: Synthetic pathway towards **80**.

The expected outcome from adding sydnone **80** to dipolarophile methyl propiolate was to obtain a mixture of regioisomers **81** and **82** that would originate the respective sulfones after treatment with MCPBA, as illustrated in Scheme 13. Only the regioisomer **81** was identified and isolated in low yields (<20%), and proved unstable, degrading at room temperature. Alternatively, direct treatment of the reaction media with MCPBA without prior isolation was done, in an attempt to obtain the more stable sulfones **69** and **68**. NMR analysis showed that no product was obtained, and so, further reactivity of these compounds was not pursued.



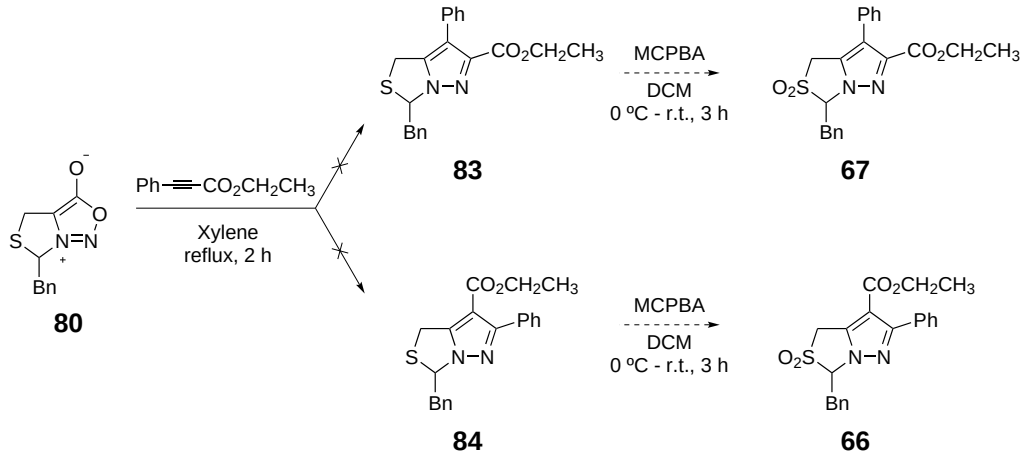
80 **81** <20% **69** **82** **68**

Scheme 13: Synthesis of compounds **69** and **68**.

Another synthetic sequence was carried out, aimed at obtaining compounds **67** and **66**. The mesoionic species **80** was refluxed in xylene with ethyl phenylpropiolate. The expected outcome was to obtain regioisomers **83** and **84**, which, through oxidation with MCPBA would ideally result in the targeted products **67** and **66** (Scheme 14).

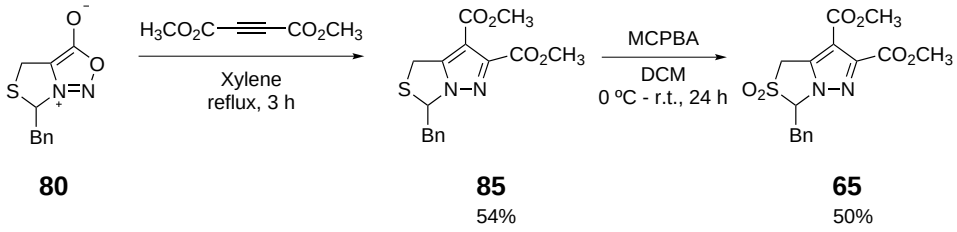
Unfortunately, NMR analysis showed that none of the regioisomers was obtained, possibly

due to poor stability at room temperature, as was observed previously with compounds **69** and **68**.



Scheme 14: Synthesis of compounds **67** and **66**.

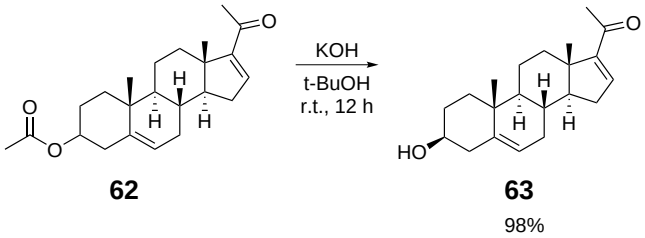
Finally, sydnone **80** reacted with dimethyl acetylenedicarboxylate (DMAD) resulting in compound **85** as only product in 54% yield. Oxidation of the pyrazolo[1,5-*c*][1,3]thiazole **85** with MCPBA resulted in the target sulfone **65** in 50% yield (Scheme 15).



Scheme 15: Synthesis of compound **65**.

5.2 SYNTHESIS OF 16-DHP

16-DHP was synthesized by treating 16-DPA (**62**) with potassium hydroxide in tert-butanol (Scheme 16). The NMR ^{1}H spectrum confirmed that 16-DHP was obtained as the single product in 98% yield (Figure 5.4).



Scheme 16: Synthesis of 16-DHP.

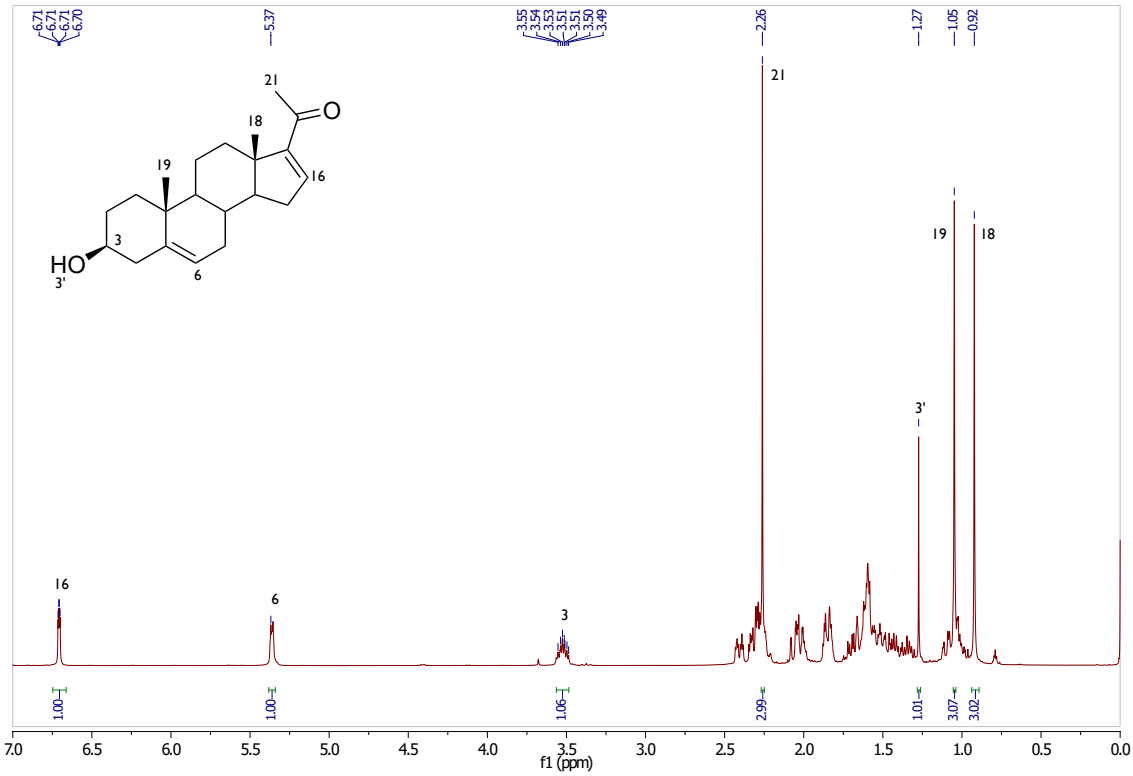


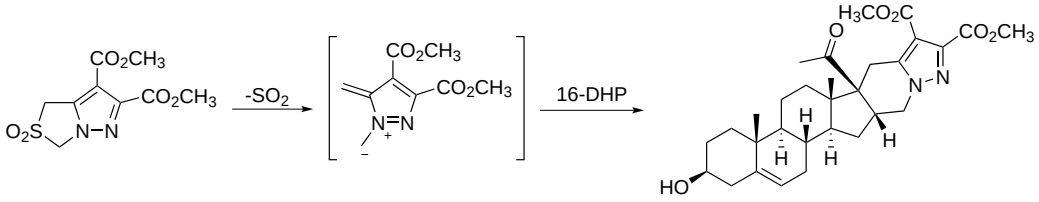
Figure 5.4: ^{1}H NMR spectrum of 16-DHP.

5.3 REACTIVITY OF 16-DHP AND 1,2-DIAZAFULVENIUM METHIDES THROUGH [8π+2π] CYCLOADDITION REACTIONS

5.3.1 Dimethyl 4,5,6,7- tetrahydropyrazolo[1,5-*a*]pyridine- 2,3-dicarboxylate fused with 16-DHP

The reaction of 16-DHP with diazafulvenium methide **86** described in previous work was optimized (Table 1).[68] Using 2.5 equivalents of sulfone **46** under microwave irradiation at 250 ºC for 10 min in presence of 16-DHP gave the expected compound **87** in a regio- and stereoselective manner in 41% yield (Table 1, Entry 1). Using 3 or 4 equivalents of sulfone gave the same compound but in lower yields: 37% and 38%, respectively (Table 1, Entries 2 and 3). The conventional heating of 2.5 equivalents of sulfone in presence of 16-DHP resulted in 24% yield (Table 1, Entry 4).

Table 1: Reaction of diazafulvenium methide **86** with 16-DHP.



46 **86** **87**

Entry	**46**	Reaction Conditions			Yield
1	2.5 sulfone equiv.	MW	250 °C	10 min	41%
2	3 sulfone equiv.	MW	250 °C	10 min	37%
3	4 sulfone equiv.	MW	250 °C	10 min	38%
4	2.5 sulfone equiv.	TCB, reflux		4 h	24%

Comparison of the ^{1}H NMR spectrum with the one from 16-DHP (Figure 5.4) shows that the H-16 proton goes from a triplet at 6.72 ppm to a multiplet at 3.70 ppm, as expected from the saturation of the C16-C17 bond. The protons on C31 and C33 appear as singlets at 3.87 ppm and 3.93 ppm, respectively. In the new six-membered ring, the two protons on C29 appear as two doublets at 3.25 and 3.36 ppm, and the two protons on C24 appear as a double doublet at 4.29 ppm and a multiplet at 3.83 ppm (Figure 5.5).

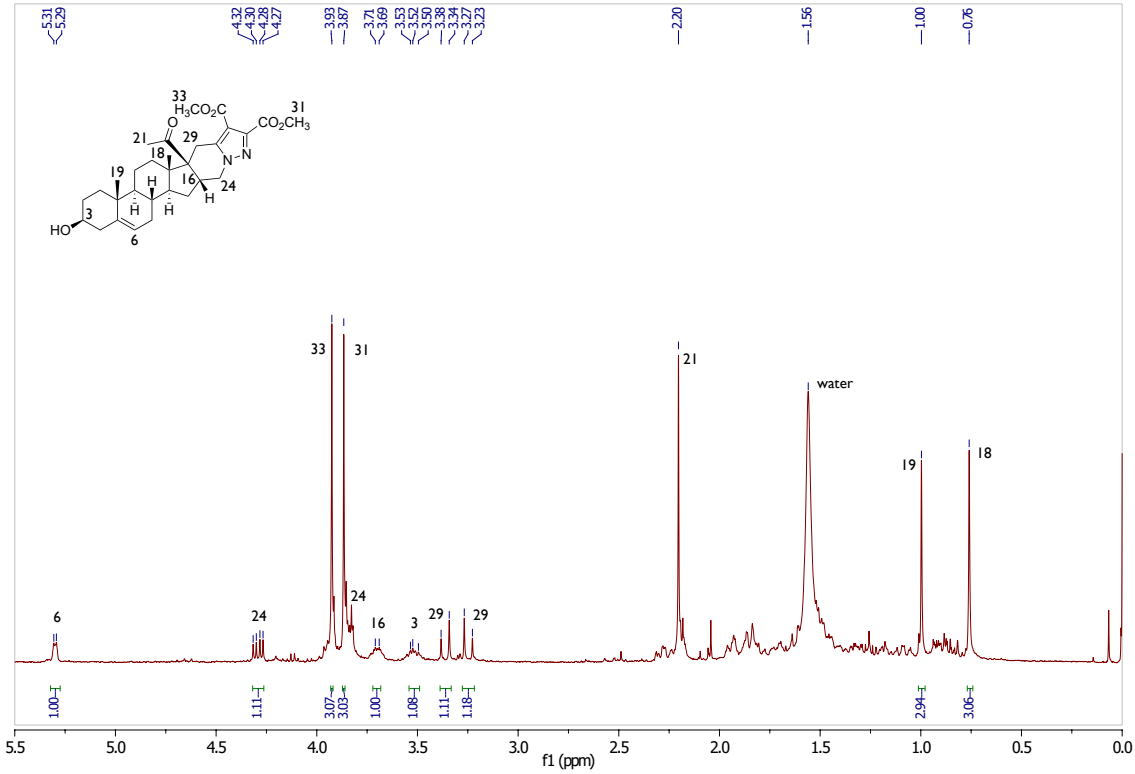
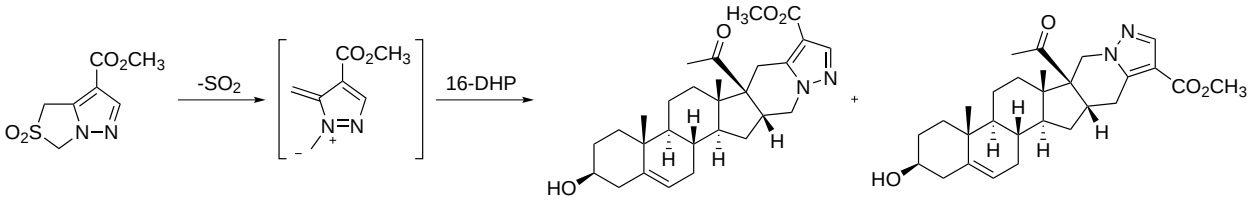


Figure 5.5: ^{1}H NMR spectrum of the hexacyclic steroid **87**.

5.3.2 Methyl 4,5,6,7-tetrahydropyrazolo[1,5- *a*]pyridine-3-carboxylate fused with 16-DHP

Reactivity of 16-DHP towards the diazafulvenium methide **88** was also explored (Table 2). The 1,7-dipole was generated *in situ* from of the sulfone **64** in presence of 16-DHP, resulting in a mixture of regioisomers **89** and **90**, with the latter always being the minor product and sometimes only present in trace amounts or not at all. Using 2.5 equivalents of sulfone under microwave irradiation at 250 ℃ for 10 min ga ve compound **89** in 24% yield (Table 2, Entry 1). The use of 3 equivalents of sulfone resulted in lower yield (16%) (Table 2, Entry 2). In the conventional heating assay, 2.5 equivalents of sulfone **64** were refluxed in 1,2,4-trichlorobenzene for 4 hours in presence of 16-DHP, resulting in compound **89** in 47% yield and compound **90** in 2% yield (Table 2, Entry 3).

Table 2: Reaction of diazafulvenium methide **88** with 16-DHP.



64 **88** **89** **90**

Entry	**64**	Reaction Conditions			Yield **89**	Yield **90**
1	2.5 sulfone equiv.	MW	250 °C	10 min	24%	-
2	3 sulfone equiv.	MW	250 °C	10 min	16%	-
3	2.5 sulfone equiv.	TCB, reflux		4 h	47%	2%

The NMR spectra for compounds **89** and **90** are mostly similar (Figures 5.6 and 5.7). Attributing the structure to each spectrum was done with analysis of the signals of protons at C24 and C29: from molecule **89** to **90**, as expected due to a greater distance to the electronegative nitrogen atoms, the pair of double doublets from C24 protons show an upfield shift from 4.26 and 3.82 ppm for molecule **89** to 3.63 and 2.51 ppm for molecule **90**. Inversely, the two doublets from the protons on C29 shift downfield, from 3.29 and 3.39 ppm for molecule **89** to 4.17 and 4.58 for molecule **90**, due to closer proximity to the electronegative nitrogen atoms.

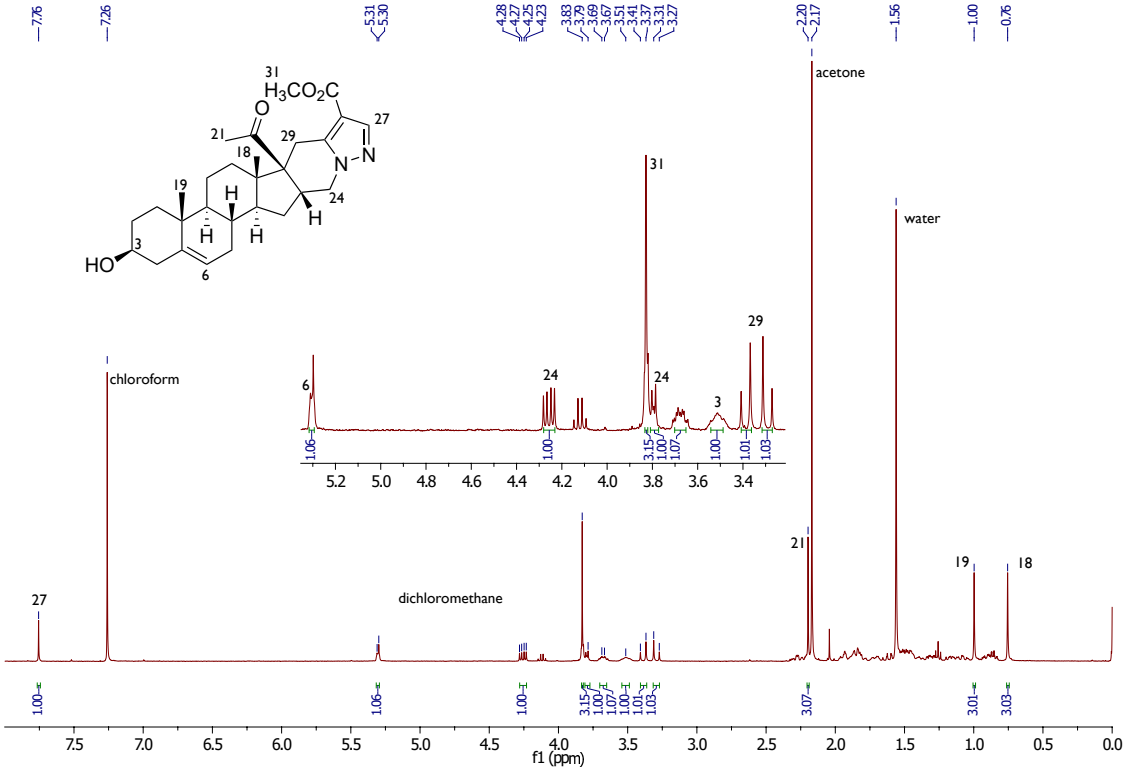


Figure 5.6: ^{1}H NMR spectrum of the hexacyclic steroid **89**.

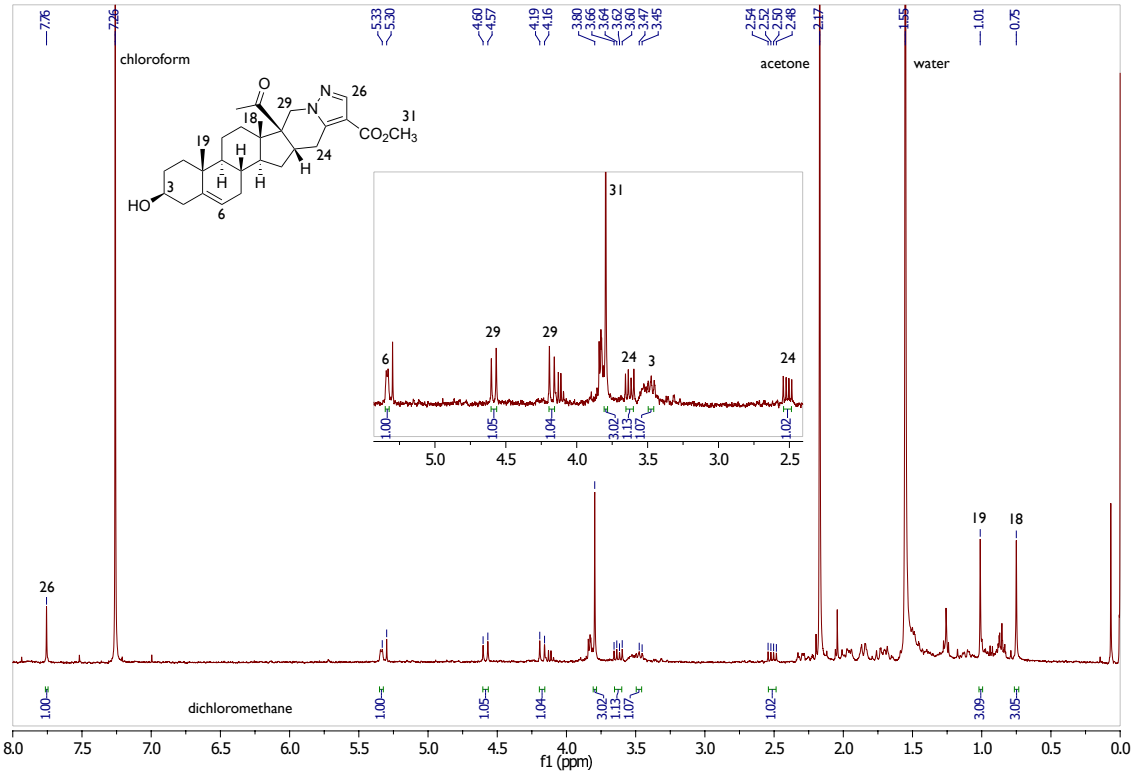


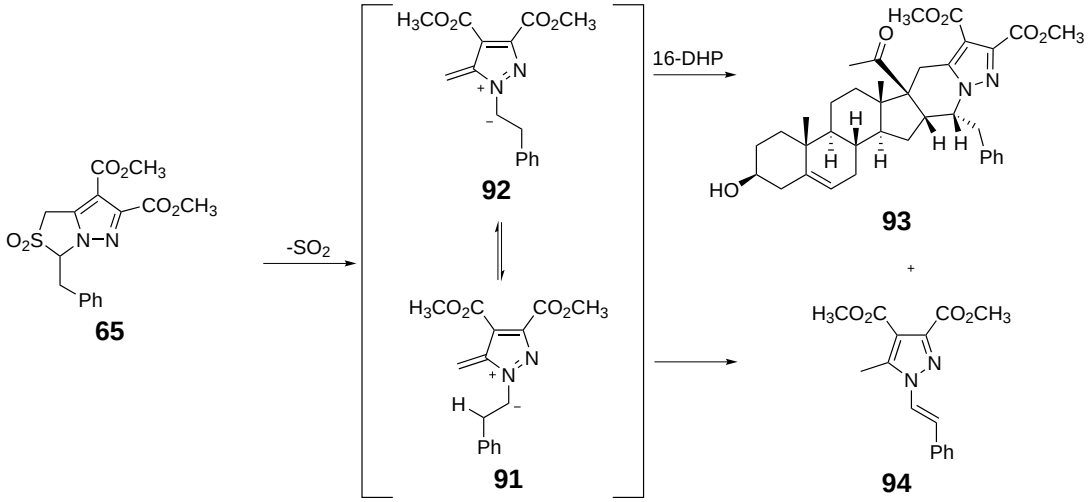
Figure 5.7: ^{1}H NMR spectrum of the hexacyclic steroid **90**.

5.3.3 Dimethyl (*S*)-7-benzyl-4,5,6,7- tetrahydropyrazolo[1,5-*a*]pyridine-2,3- dicarboxylate fused with 16-DHP

The reactivity of sulfone **65** in presence of 16-DHP was explored (Table 3). In addition to obtaining the target compound **93** in a regio- and stereoselective manner, the expected competitive synthesis of **94** was observed, resulting from a sigmatropic [1,8]H shift mentioned in Scheme 2.

The hexacyclic steroid **93** resulted from the interception of **92** by 16-DHP using 1.5 equivalents of sulfone in microwave conditions in 29% yield for **93** and 45% yield for **94** (Table 3, Entry 1). Conventional heating of 1.5 equivalents of sulfone **65** in presence of 16-DHP resulted in 39% and 23% yields for compounds **93** and **94**, respectively (Table 3, Entry 2).

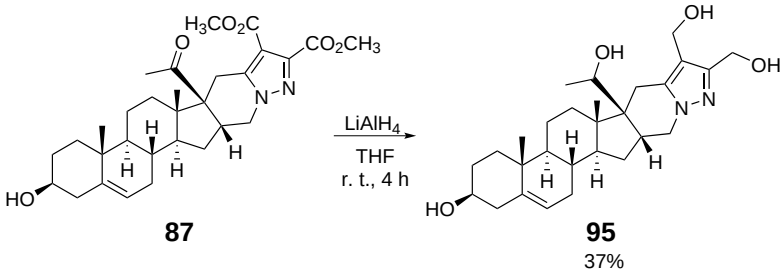
Table 3: Reaction of diazafulvenium methide **92** with 16-DHP.



Entry	**65**	Reaction Conditions			Yield **93**	Yield **94**
1	1.5 sulfone equiv.	MW	250 ℃	10 min	29%	45%
2	1.5 sulfone equiv.	Δ	250 ℃	4 h	39%	23%

5.4 REDUCTION OF SYNTHESIZED HEXACYCLIC STEROIDAL COMPOUNDS

Previous work originated an interest in reducing the ester groups of hexacyclic steroids due to a correlation with higher values of biological activity. The strategy to obtain hexacyclic steroidal molecules containing hydroxyl groups as substituents in the ring system fused to the steroidal substrate was to make the reduction with lithium aluminium hydride.



Scheme 17: Reduction of compound **87**.

The initial procedure was to add the hexacyclic steroid in a dichloromethane solution to a suspension of lithium aluminium hydride in diethyl ether. Thin-layer chromatography showed the presence of less polar reaction products, hinting that reduction of the ester groups was not occurring in a complete fashion, leading to low yields. One possible reason to this partial reduction of the hexacyclic steroid is that as its ketone and one ester group are reduced, the molecule's polarity shifts, lowering its solubility in diethyl ether and making it no longer accessible to the reducing agent.

A new approach to the reduction of the ester groups was adopted: lithium aluminium hydride was added directly to an ice-cold solution of the hexacyclic steroid in tetrahydrofuran (Scheme 17). This approach was successful in reducing the ester groups and resulted in the reduction of the ketone group in C20 as well. Unfortunately, isolation of the products through flash chromatography proved challenging: due to its high polarity, traditional ethyl acetate/hexane mixtures proved uncapable of separating the reaction products. Separation was achieved using a chloroform/methanol/ammonium hydroxide (50:5:0.25) eluent, and the final product was isolated in 37% yield.

With the contributions from the HSQC, COSY and NOESY NMR spectra, it was possible to identify most peaks in the ^{1}H NMR spectrum of compound **95** (Figure 5.8):

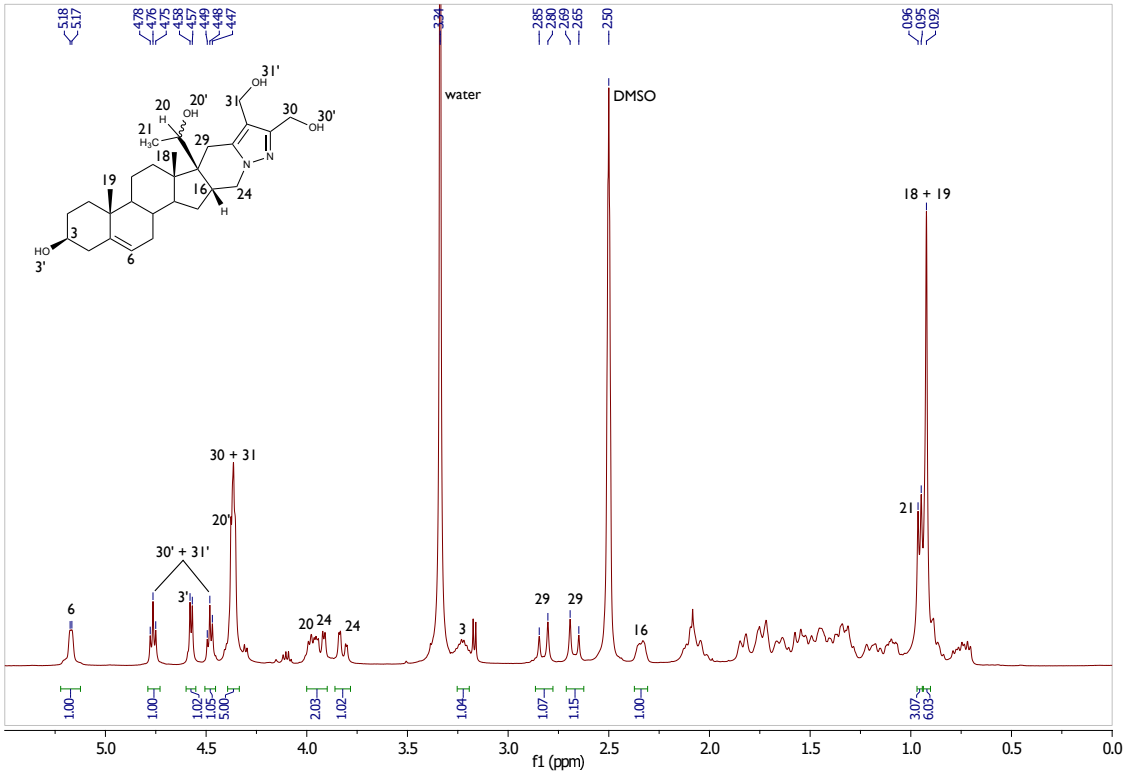


Figure 5.8: ^{1}H NMR spectrum of the hexacyclic steroid **95**.

The HSQC NMR spectrum (Figure 5.9) showed that the doublet at 4.57 ppm and the two triplets at 4.48 and 4.76 ppm were due to protons attached to oxygen atoms, since they did not show any proton-carbon single bond correlations. Also, the multiplet from 4.34 to 4.39 was due to the protons on C30 and C31: their shift was coherent with what would be expected from CH_2OH groups, and they showed proton-carbon single bond correlations with two carbon atoms at 53.5 and 56.1 ppm. Yet, the integration value from said peak in the ^{1}H NMR spectrum hinted that one extra proton was overlapped with the four CH_2 protons: since it displayed no extra signal on the HSQC spectrum, it was identified as being the proton from the expected fourth hydroxyl group.

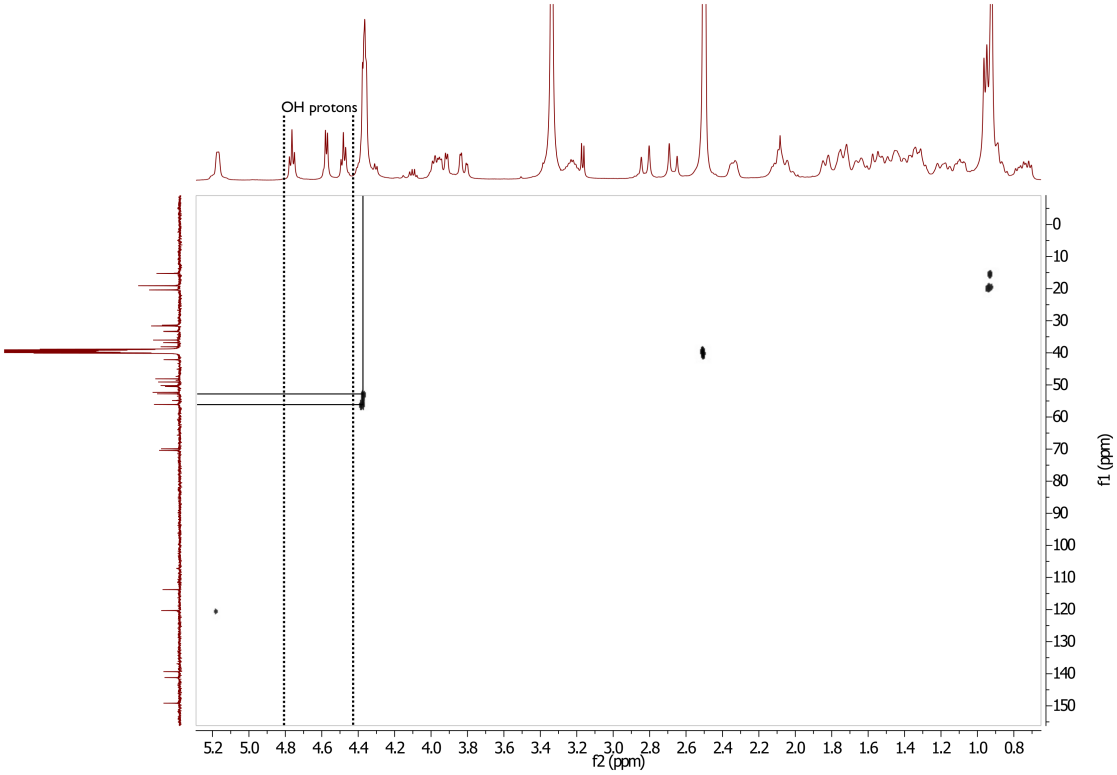


Figure 5.9: HSQC NMR spectrum of the hexacyclic steroid **95**.

The analysis of the COSY spectrum of compound **95** allowed the identification of several proton peaks: the peaks corresponding to hydroxyl groups attached to C3 and C20 were identified, as for the peaks for the protons on C16, C20, C21, C24, C29, C30 and C31.

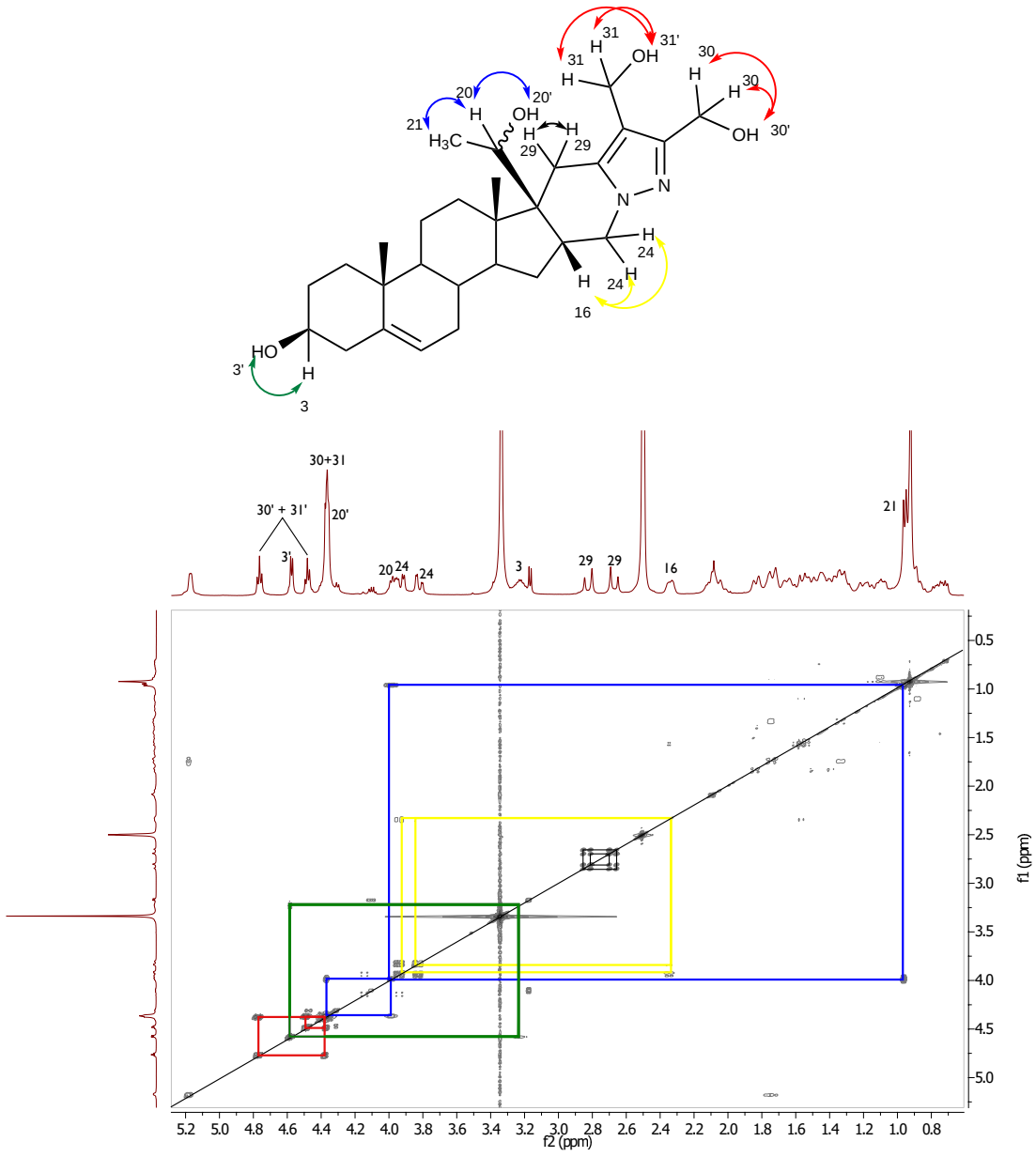


Figure 5.10: COSY NMR spectrum of the hexacyclic steroid **95** and couplings found.

The NOESY spectrum (Figure 5.11) shows a correlation between the proton at C16 and the one at C20, but due to rotation on the C17-C20 bond it is insufficient to draw conclusions on the configuration of C20 and X-ray crystallography would be necessary to obtain an absolute configuration.

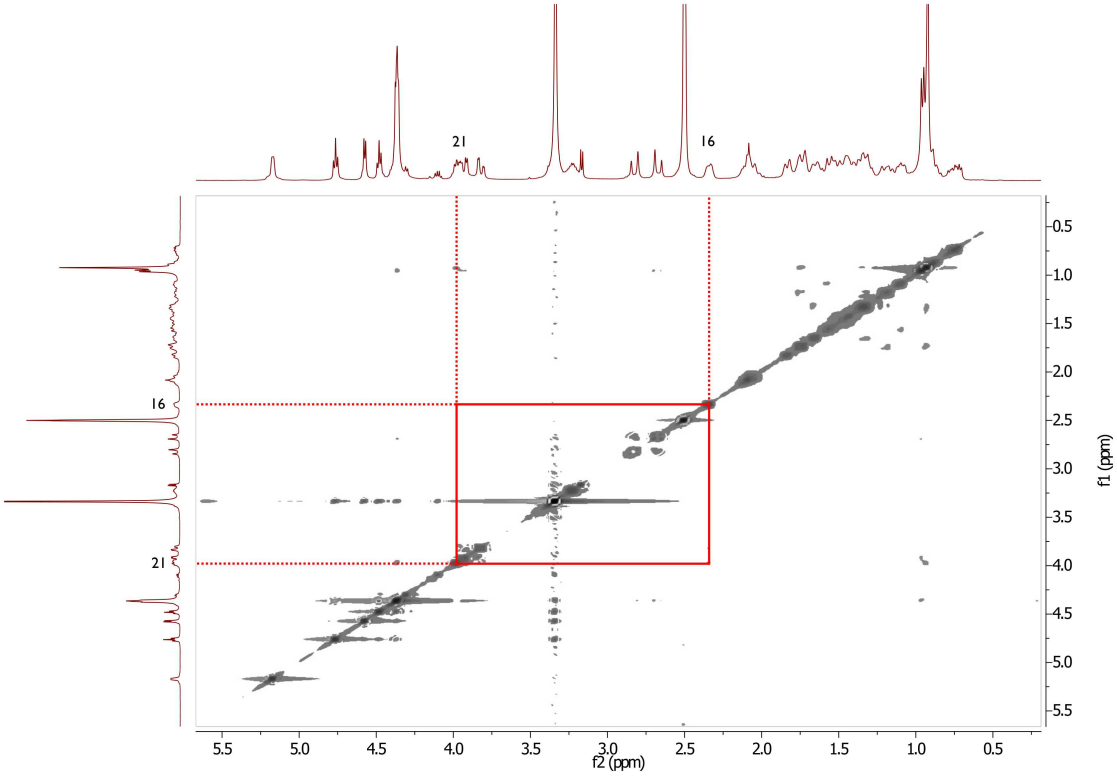
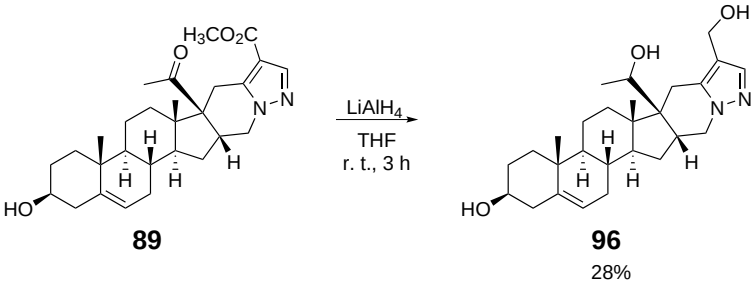


Figure 5.11: NOESY NMR spectrum of the hexacyclic steroid **95**.

In similar fashion, compound **89** was reduced to compound **96** (Scheme 18), and obtained as an ambar oil due to presence of a contaminant visible in ^{1}H NMR spectra. All attempts at purifying the product through multi-solvent recrystallization were unsuccessful, and in the end, the purified product was obtained in a 28% yield after precipitating in distilled water. The definition of the NMR spectra is lacking, but it is still possible to identify ^{1}H NMR peaks (Figure 5.12): the singlet of the vinyllic proton on C6 appears at 5.16 ppm, the two doublets from the protons on C29 appear at 2.86 ppm and 2.65 ppm, the doublet from the protons on C21 appears at 0.97 ppm, and the overlapped singlets of the protons on C18 and C19 appear at 0.92 ppm. Through analysis of COSY and HSQC NMR spectra it is also possible to identify the presence of the triplet from the C30 hydroxyl group at 4.63 ppm and the doublet from the C20 hydroxyl group at 4.56 ppm.



Scheme 18: Reduction of compound **89**.

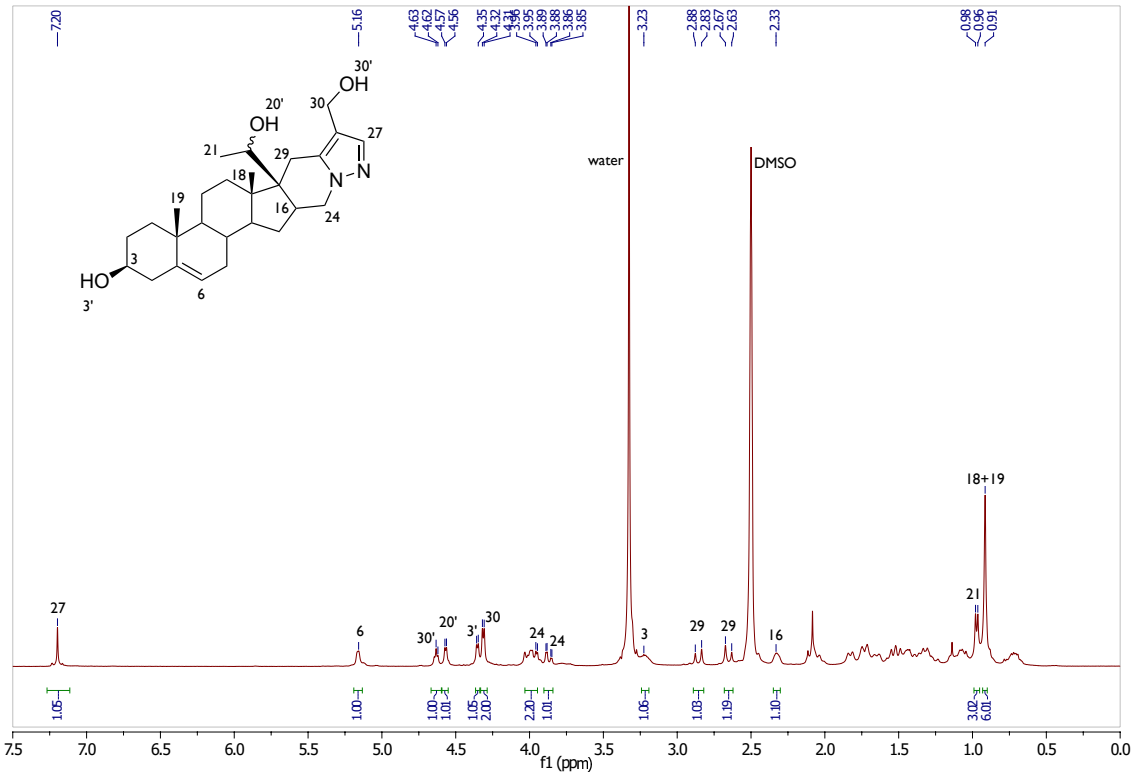
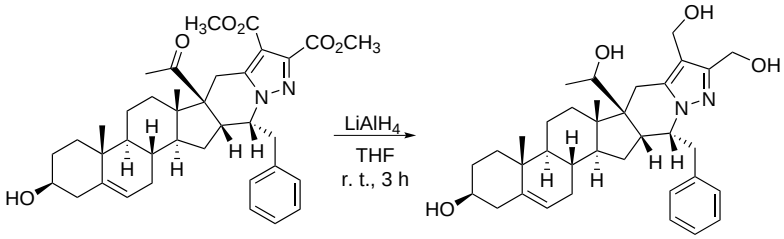


Figure 5.12: ^{1}H NMR spectrum of the hexacyclic steroid **96**.

Ultimately, compound **93** was reduced to compound **97** and obtained in 24% yield after precipitating in distilled water (Scheme 19). As before, it is possible to identify important peaks in the ^{1}H NMR spectrum (Figure 5.13): the aromatic protons from C34-C38 appear from 7.23 to 7.32 ppm as a multiplet. The protons from hydroxyl groups attached to C30 and C31 appear as triplets at 4.78 ppm and 4.51 ppm and protons from the hydroxyl groups attached to C3 and C20 appear as doublets at 4.56 ppm and 4.30 ppm. The peaks from the protons on C30 and C31 appear at 4.41 ppm as a multiplet with an integration value of 4. Only on of the two doublets from the C29 protons is visible at 3.05 ppm with the other possibly overlapping with the water signal. Lastly, the doublet from the C21 protons appears at 0.93 ppm, and the singlets from C18 and C19 protons appear at 0.89 and 0.76 ppm.



93 **97**
24%

Scheme 19: Reduction of compound **93**.

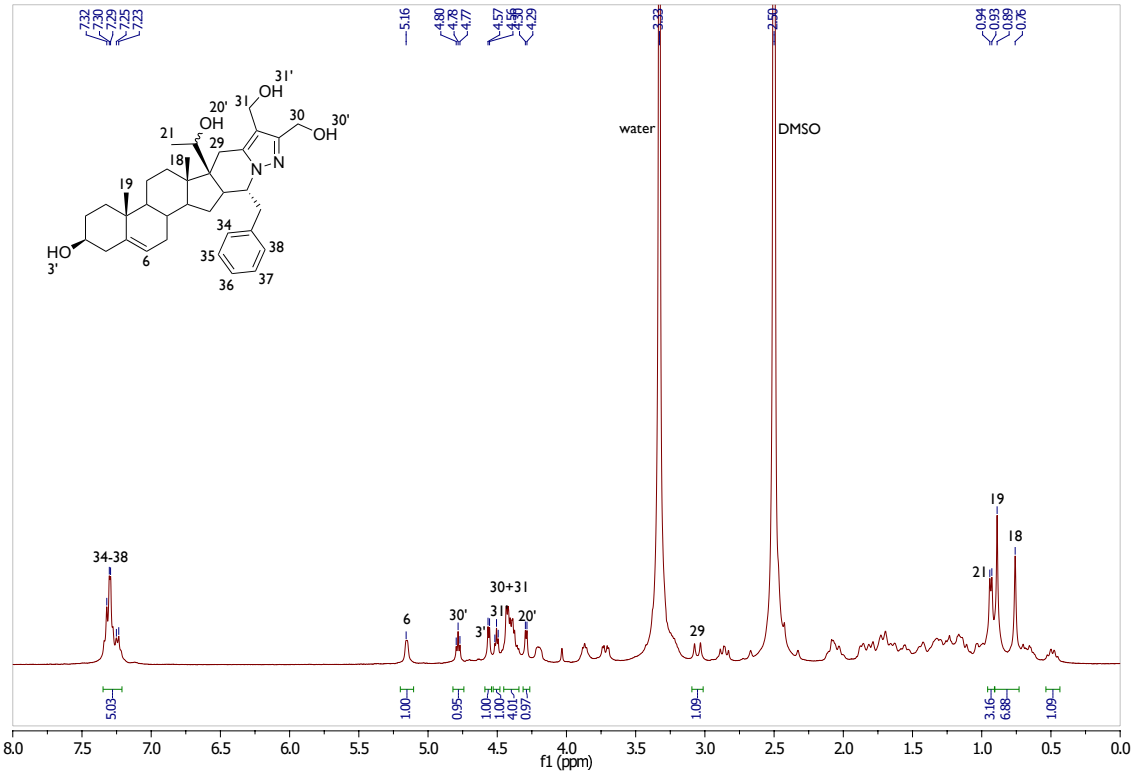


Figure 5.13: ^{1}H NMR spectrum of the hexacyclic steroid **97**.

Part III

CONCLUSION

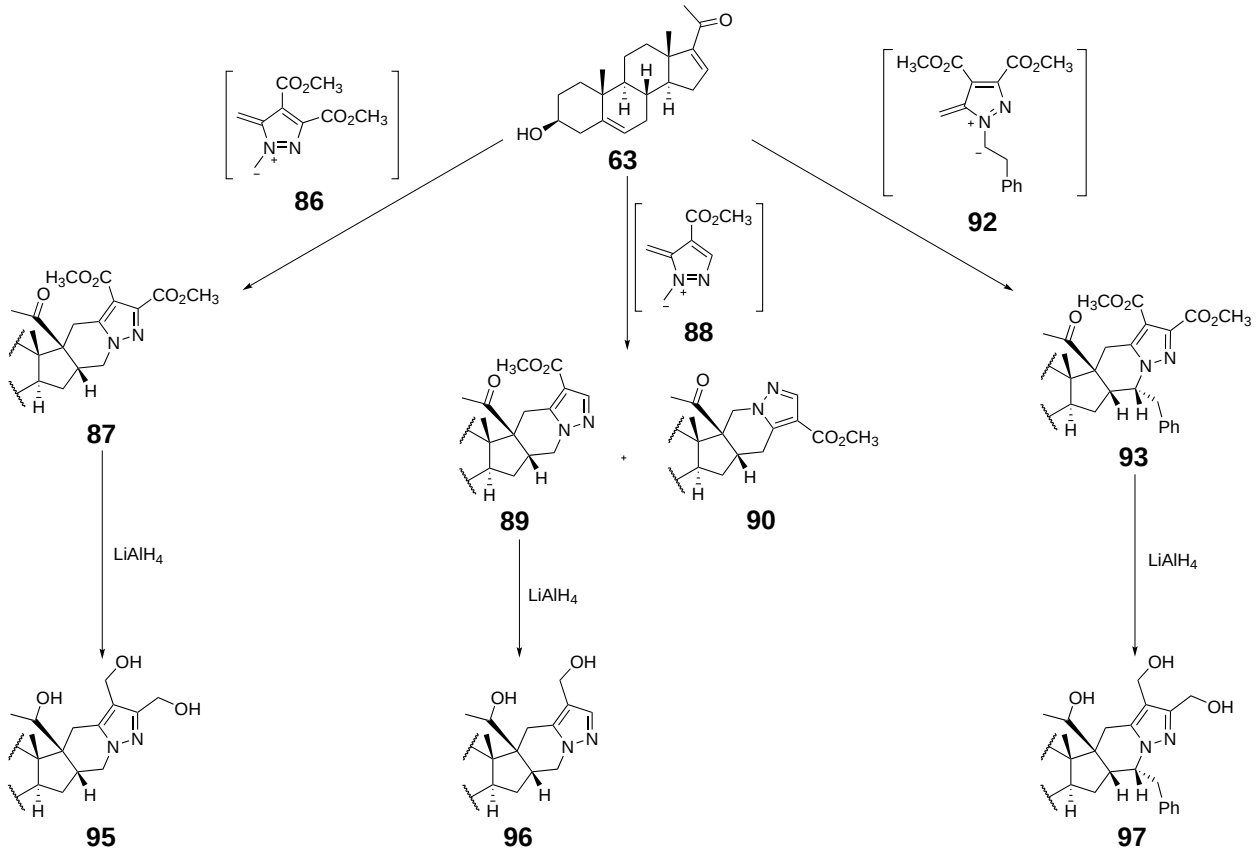
This project's aim consisted in exploring the reactivity of 16-DHP towards several 1,2-diazafulvenium methides through [8π+2π] cycloaddition reactions, in order to synthesize new chiral hexacyclic steroidal molecules.

The planned synthesis of the 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-carboxylates **46**, **64**, **70** and **65** was successful.

Unsuccessful attempts were made at the synthesis of compounds **66**, **67**, **68** and **69**.

It was shown that thermal elimination of sulphur dioxide from 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-carboxylates originated the corresponding 1,2-diazafulvenium methides that participate in the cyclization reactions with the steroidal substrate.

The hexacyclic steroids **87**, **89**, **90** and **93** were stereoselectively synthesized through [8π+2π] cycloaddition reactions of the chiral steroidal scaffold 16-DHP with the 1,2-diazafulvenium methides originated by 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-carboxylates **46**, **64** and **65** (Scheme 20).



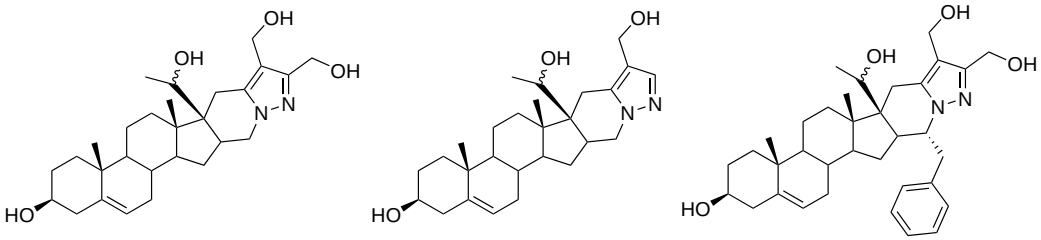
Scheme 20: [8π+2π] cycloaddition reactions of 16-DHP with 1,2-diazafulvenium methides and ensuing reduction reactions.

The cycloaddition of 16-DHP with the 1,2-diazafulvenium methide **86** gave the expected product **87** in a regio- and stereoselective manner with better yields when the reaction was done with microwave irradiation.

The cycloaddition of 16-DHP with the 1,2-diazafulvenium methide **88** resulted in a mixture of regioisomers. Product **89** was always obtained in major yield, and product **90** was only observable in trace amounts and only when the reaction occurred in thermal conditions. Yield of the major product **89** was higher in conventional thermolysis conditions.

Cycloaddition of 16-DHP with the 1,2-diazafulvenium methide **92** also gave the expected product **93** in a regio- and stereoselective manner with better yields when the reaction was done in conventional thermolysis conditions.

The synthesized hexacyclic steroids **87**, **89** and **93** had their functional groups reduced, leading to three new hexacyclic steroids: compounds **95**, **96** and **97**, all derived from successful reductions of the ketone and ester groups (Figure 5.14).



95 **96** **97**

Figure 5.14: Reduced hexacyclic steroid derivatives.

Part IV

EXPERIMENTAL

6 EXPERIMENTAL

6.1 LABORATORIAL EQUIPMENT

Melting Points Melting points were determined with a Falc Melting Point heated plate microscope, with the use of open capillaries. Raw results.

Infra-Red Spectroscopy Infrared spectra were obtained with a Agilent Technologies Cary 630 FTIR spectrometer through the Attenuated Total Reflectance method (ATR).

Nuclear Magnetic Resonance Spectroscopy Nuclear magnetic resonance spectra were obtained with Brucker Avance III spectrometers, operating at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR). Deuterated chloroform was the solvent used (except when stated otherwise). Chemical shift values are presented in parts per million (ppm), relative to tetramethylsilane (TMS), and the coupling constants (*J*) are expressed in Hz.

Microwave Microwave-assisted reactions were performed using a Discover S-Class device, from CEM Focused Synthesis System, using 10 mL microwave tubes.

Chromatography When the chemical reactions' evolution was analysed with thin layer chromatography, Merck's 60 F254 silica plaques with aluminium support were used. Most compounds were purified using column chromatography, using Merck's, Macherey-Nagel's or Fluka's silica gel (0.040-0.063 mm).

6.2 SOLVENTS AND REAGENTS

All the solvents and reagents not mentioned below were obtained from Aldrich, Merck or Fluka, and were used directly, without additional purification.

Ethyl Acetate Distilled, after being refluxed for 3 hours with potassium carbonate.

Acetone Distilled and stored on 3 Å molecular sieves, after being refluxed for 3 hours with potassium carbonate.

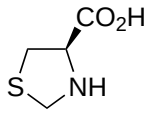
Dichloromethane and Chloroform Distilled and stored on 4 Å molecular sieves, after being refluxed for 3 hours with calcium chloride.

Ethanol and Methanol Distilled from sodium alcoxide and stored on 3 Å molecular sieves, after being refluxed for 2 hours with magnesium (5 g/L) with iodine scraps (0.5 g/L).

Diethyl Ether, Hexane and Toluene Distilled and stored on 4 Å molecular sieves, after being refluxed with sodium scraps, using benzophenone as an indicator.

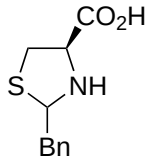
6.3 EXPERIMENTAL PROCEDURES

6.3.1 Synthesis of 1,3-thiazolidine-4-carboxylic acids



72

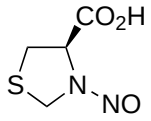
1,3-thiazolidine-4-carboxylic acid: the synthesis of this compound was based on literature procedure.[70] A solution of formaldehyde (40 mmol) in ethanol (30 mL) was added to a solution of L-cysteine (40 mmol) in water (40 mL). The reaction mixture was stirred at room temperature overnight. The product was filtrated and washed with diethyl ether, and obtained as a white solid (>99%). The product was identified by TLC comparison with a sample previously available.



78

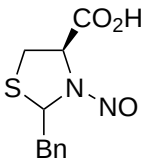
(4*R*)-2-benzylthiazolidine-4-carboxylic acid: the synthesis of this compound was based on literature procedure.[70] A solution of phenylacetaldehyde (40 mmol) in ethanol (30 mL) was added to a solution of L-cysteine (40 mmol) in water (40 mL). The reaction mixture was stirred at room temperature overnight. The product was filtrated, washed with diethyl ether, and obtained as a white solid (88%). The product was identified by TLC comparison with a sample previously available.

6.3.2 Synthesis of *N*-nitrosothiazolidine-4-carboxylic acids



73

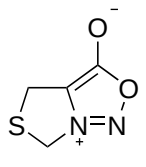
***N*-nitrosothiazolidine-4-carboxylic acid:** the synthesis of this compound was based on literature procedure.[60] Concentrated hydrochloric acid was added to a stirred suspension of 1,3-thiazolidine-4-carboxylic acid (123.7 mmol) in water (126 mL) until total dissolution. A sodium nitrite (247.4 mmol,) aqueous solution (63 mL) was slowly added. The reaction mixture was stirred at room temperature for 12 h. Upon completion, the mixture was extracted with ethyl acetate. The organic phase was washed with water and Brine, dried over anhydrous sodium sulphate and the solvent was evaporated. After crystallizing with petroleum ether, the final product was obtained as a yellow solid (56%). The product was identified by TLC comparison with a sample previously available.



79

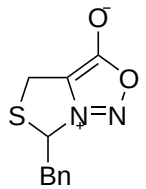
2-Benzyl-*N*-nitrosothiazolidine-4-carboxylic acid: the synthesis of this compound was based on literature procedure.[71] Isopentyl nitrile (67 mmol) was added to a solution of (4*R*)-2-benzylthiazolidine-4-carboxylic acid (45 mmol) in dimethoxyethane (400 mL). The reaction mixture was stirred at room temperature for 16 h. After this time the solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate and washed with a 1 M hydrochloric acid solution. The organic phase was dried over anhydrous sodium sulphate and the solvent was evaporated. Purification of the crude product by flash chromatography [ethyl acetate/hexane (1:4), (1:3) and (1:2)] gave the product as an orange oil (66%). The product was identified by TLC comparison with a sample previously available.

6.3.3 Synthesis of 4*H*,6*H*-thiazolo[3,4-*c*][1,2,3]oxodiazole-7-ium-3-oxides



74

4*H*,6*H*-thiazolo[3,4-*c*][1,2,3]oxadiazol-7-ium-3-oxide: the synthesis of this compound was based on literature procedure.[60] Trifluoroacetic anhydride (21.65 mmol,) was slowly added to a suspension of *N*-nitrosothiazolidine-4-carboxylic acid (21.65 mmol) in anhydrous diethyl ether (230 mL) at 0 ℃. The reaction mixture was stirred at 0 ℃ for 6 h and then stirred at room temperature for 24 h. The resulting mixture was neutralized with saturated sodium bicarbonate solution until pH=7, and then extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulphate and the solvent was evaporated, and the final product was obtained as a yellow solid (75%). The product was identified by TLC comparison with a sample previously available.

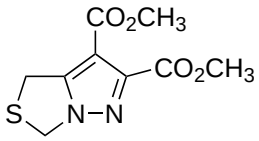


80

6-Benzyl-4*H*,6*H*-thiazolo[3,4-*c*][1,2,3]oxadiazol-7-ium-3-oxide: the synthesis of this compound was based on literature procedure.[72] Trifluoroacetic anhydride (21.65 mmol,) was slowly added to a suspension of 2-benzyl-*N*-nitrosothiazolidine-4-carboxylic acid (21.65 mmol) in anhydrous diethyl ether (230 mL) at 0 ℃. The reaction mixture was stirred at 0 ℃ for 6 h and then left at room temperature. After stirring at room temperature for 24 h the solution was neutralized with a saturated sodium chloride solution. The organic phase was dried over anhydrous sodium sulphate and the solvent was evaporated. Purification of the crude product by

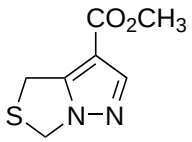
flash chromatography [ethyl acetate/hexane (1 :2)] gave the product as a yellow solid (90%). The product was identified by TLC comparison with a sample previously available.

6.3.4 Synthesis of 1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-carboxylates

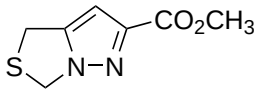


75

Dimethyl 1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6,7-dicarboxylate: the synthesis of this compound was based on literature procedure.[60] A solution of 4*H*,6*H*-thiazolo[3,4-*c*][1,2,3]oxadiazol-7-ium-3-oxide (7 mmol) and dimethyl acetylenedicarboxylate (11.25 mmol) in xylene (10 mL) was refluxed under a N_2 atmosphere for 3h. The reaction was then cooled to room temperature and the solvent was evaporated. Purification of the product by flash chromatography [ethyl acetate/hexane (1:2) and (1:1)] gave the product as a white solid (51%). The product was identified by TLC comparison with a sample previously available.

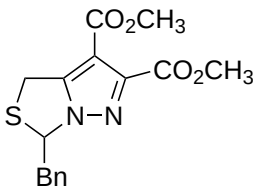


76



77

Methyl 1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6-carboxylate and methyl 1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-7-carboxylate: the synthesis of this compound was based on literature procedure.[60] A solution of 4*H*,6*H*-thiazolo[3,4-*c*][1,2,3]oxadiazol-7-ium-3-oxide (7 mmol) and methyl propiolate (11.1 mmol) in xylene (10 mL) was refluxed under a N_2 atmosphere for 4 h. The reaction was then cooled to room temperature and the solvent was evaporated. Purification of the product by flash chromatography [ethyl acetate/hexane (1:2)] gave, in order of elution, compound **77** as a white solid (19%), and compound **76** as a white solid (53%). The products were identified by TLC comparison with samples previously available.

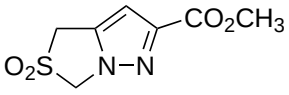


98

Dimethyl 6-Benzyl-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6,7-dicarboxylate: the synthesis of this compound was based on literature procedure.[60] A solution of 6-Benzyl-4*H*,6*H*-thiazolo[3,4-*c*][1,2,3]oxadiazol-7-ium-3-oxide (7 mmol) and dimethyl acetylenedicarboxylate (11.25 mmol) in xylene (10 mL) was refluxed under N_2 atmosphere for 3h. Purification of the product by flash chromatography

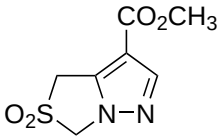
[ethyl acetate/hexane (1:2)] gave the product as a white solid (54%). The product was identified by TLC comparison with a sample previously available.

6.3.5 Synthesis of 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-carboxylates



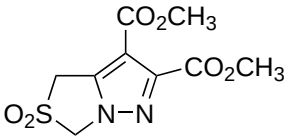
70

Methyl 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6-carboxylate: the synthesis of this compound was based on literature procedure.[61] 3-Chloroperoxybenzoic acid (11.8 mmol) was added to a stirred ice-cold solution of 1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6-carboxylate (3.94 mmol) in dry dichloromethane (30 mL) under a N_2 atmosphere. The cooling bath was removed and the mixture was stirred at room temperature for 4 h. The reaction mixture was washed with 10% (w/v) aqueous sodium bisulfite solution (2 × 80 mL) and with 10% (w/v) aqueous sodium bicarbonate solution (2 × 80 mL). The organic phase was dried over anhydrous sodium sulphate and the solvent was evaporated. The product was obtained as a white solid (56%). The product was identified by TLC comparison with a sample previously available.



64

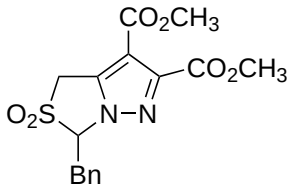
Methyl 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-7-carboxylate: the synthesis of this compound was based on literature procedure.[61] 3-Chloroperoxybenzoic acid (11.8 mmol) was added to a stirred ice-cold solution of 1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-7-carboxylate (3.94 mmol) in dry dichloromethane (30 mL) under a N_2 atmosphere. The cooling bath was removed and the mixture was stirred at room temperature for 4 h. The reaction mixture was washed with 10% (w/v) aqueous sodium bisulfite solution (2 × 80 mL) and with 10% (w/v) aqueous sodium bicarbonate solution (2 × 80 mL). The organic phase was dried over anhydrous sodium sulphate and the solvent was evaporated. The product was obtained as a white solid (67%). The product was identified by TLC comparison with a sample previously available.



46

Dimethyl 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*]-[1,3]thiazole-6,7-dicarboxylate: the synthesis of this compound was based on literature procedure.[60] 3-Chloroperoxybenzoic acid (11.8 mmol) was added to a stirred ice-cold solution of dimethyl 1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6,7-dicarboxylate (3.94 mmol) under a

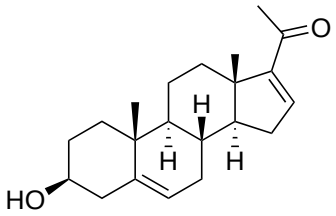
N_2 atmosphere. The cooling bath was removed and the mixture was stirred at room temperature for 3 h. The reaction mixture was washed with 10% (w/v) aqueous sodium bisulfite solution (2 × 80 mL) and with 10% (w/v) aqueous sodium bicarbonate solution (2 × 80 mL). The organic phase was dried over anhydrous sodium sulphate and the solvent was evaporated. The product was obtained as a white solid (68%). The product was identified by TLC comparison with a sample previously available.



99

Dimethyl 3-benzyl-2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6,7-dicarboxylate: the synthesis of this compound was based on literature procedure.[61] 3-Chloroperoxybenzoic acid (11.8 mmol) was added to a stirred ice-cold solution of dimethyl 6-Benzyl-1*H*,3*H*-pyrazolo[1,5-*c*][1,3]thiazole-6,7-dicarboxylate (3.94 mmol) under a N_2 atmosphere. The cooling bath was removed and the mixture was stirred at room temperature for 24 h. The reaction mixture was washed with 10% (w/v) aqueous sodium bisulfite solution (2 × 80 mL) and with 10% (w/v) aqueous sodium bicarbonate solution (2 × 80 mL). After evaporation, the product was crystallized in cold diethyl ether and obtained as a white solid (50%). The product was identified by TLC comparison with a sample previously available.

6.3.6 Synthesis of steroidal substrate

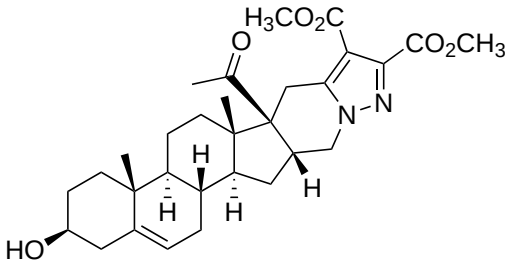


63

16-dehydropregnenolone (16-DHP): the synthesis of this compound was based on literature procedure.[73] Potassium hydroxide was added to a solution of 16-DPA in tert-butanol and the mixture was stirred at room temperature overnight. The reaction mixture was poured into distilled water, and extracted with ethyl acetate. The organic phase was then washed with distilled water and aqueous sodium chloride, followed by phase separation.The organic phase was dried over anhydrous sodium sulphate and the solvent was evaporated. The product was crystallized in hexane and obtained as a white solid (98%).

^{1}H NMR: δ 6.71 (dd, *J* = 3.3, 1.8 Hz, 1H), 5.37 (s, 1H), 3.59 – 3.41 (m, 1H), 2.12 – 1.97 (m, 3H), 1.27 (s, 1H), 1.05 (s, 5H), 0.92 (s, 3H).

6.3.7 Synthesis of steroid derivatives through [8π+2π] cycloaddition reactions of 1,2-diazafulvenium methides



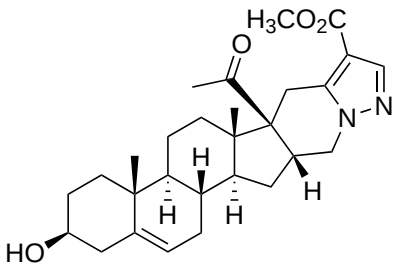
87

Dimethyl 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-2,3-dicarboxylate fused with 16-DHP: the synthesis of this compound was based on literature procedure.[69]

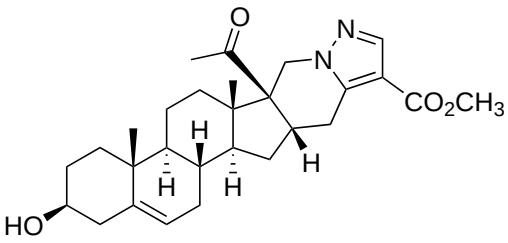
Microwave: a mixture of 16-DHP (0.3 mmol) and sulfone **46** (2.5 eq.) in 1 mL 1,2,4-trichlorobenzene was irradiated in a microwave oven at 250 ℃, for 10 min. After cooling to room temperature, purification of the reaction mixture by flash chromatography [hexane, ethyl acetate/hexane (1:1) and (2:1)] gave compound **87** as a white solid (41%).

Conventional heating: a mixture of 16-DHP (0.3 mmol) and sulfone **46** (2.5 eq.) in 2 mL 1,2,4-trichlorobenzene was refluxed under N_2 atmosphere for 3 h. After cooling to room temperature, purification of the reaction mixture by flash chromatography [hexane, ethyl acetate/hexane (1:1) and (2:1)] gave compound **87** as a white solid (24%).

^{1}H NMR: δ 5.30 (d, *J* = 5.1 Hz, 1H), 4.29 (dd, *J* = 13.4, 6.3 Hz, 1H), 3.93 (s, 4H), 3.87 (s, 4H), 3.70 (d, *J* = 7.7 Hz, 2H), 3.58 – 3.45 (m, 2H), 3.30 (dd, *J* = 46.1, 16.2 Hz, 3H), 1.00 (s, 3H), 0.76 (s, 3H).



89



90

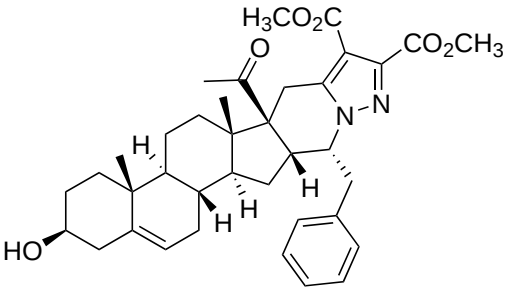
Methyl 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-3-carboxylate fused with 16-DHP: the synthesis of this compound was based on literature procedure.[69]

Microwave: a mixture of 16-DHP (0.3 mmol) and sulfone **64** (2.5-3 eq.) in 1 mL 1,2,4-trichlorobenzene was irradiated in a microwave oven at 250 ℃, for 10 min. After cooling to room temperature, purification of the reaction mixture by flash chromatography [hexane, ethyl acetate/hexane (1:1) and (2:1)] gave compound **89** as a white solid (24%).

Conventional heating: a mixture of 16-DHP (0.3 mmol) and sulfone **64** (2.5-3 eq.) in 2 mL 1,2,4-trichlorobenzene was refluxed under N_2 atmosphere for 4 h. After cooling to room temperature, purification of the reaction mixture by flash chromatography [hexane, ethyl acetate/hexane (1:1) and (2:1)] gave compounds **89** and **90** as white solids in 47% and 2% yields, respectively.

^{1}H NMR (compound **89**): δ 7.76 (s, 1H), 5.30 (d, *J* = 5.1 Hz, 1H), 4.26 (dd, *J* = 13.3, 6.2 Hz, 1H), 3.86 – 3.75 (m, 5H), 3.68 (d, *J* = 7.7 Hz, 1H), 3.57 – 3.47 (m, 1H), 3.34 (dd, *J* = 37.9, 16.1 Hz, 2H), 2.20 (s, 3H), 1.00 (s, 3H), 0.76 (s, 2H).

^{1}H NMR (compound **90**):δ 7.76 (s, 1H), 5.33 (s, 1H), 4.59 (d, *J* = 13.8 Hz, 1H), 4.18 (d, *J* = 13.8 Hz, 1H), 3.80 (s, 2H), 3.63 (dd, *J* = 15.8, 7.9 Hz, 2H), 3.46 (d, *J* = 8.4 Hz, 2H), 2.51 (dd, *J* = 15.9, 8.1 Hz, 2H), 1.01 (s, 3H), 0.75 (s, 3H).



93

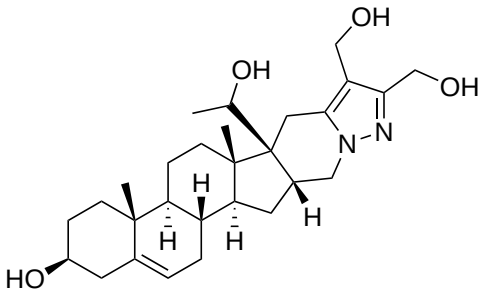
Dimethyl (*S*)-7-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-2,3-dicarboxylate fused with 16-DHP: the synthesis of this compound was based on literature procedure.[69]

Microwave: a mixture of 16-DHP (0.3 mmol) and sulfone **65** (1.5-2.5 eq.) in 1 mL 1,2,4-trichlorobenzene was irradiated in a microwave oven at 250 ℃, for 10 min. After cooling to room temperature, purification of the reaction mixture by flash chromatography [hexane, ethyl acetate/hexane (1:1) and (2:1)] gave compound **93** as a white solid (29%).

Conventional heating: a mixture of 16-DHP (0.3 mmol) and sulfone **65** (1.5-2.5 eq.) in 2 mL 1,2,4-trichlorobenzene was refluxed under N_2 atmosphere for 3.5 h. After cooling to room temperature, purification of the reaction mixture by flash chromatography [hexane, ethyl acetate/hexane (1:1) and (2:1)] gave compound **93** as a white solid (39%).

^{1}H NMR: δ 7.42 – 7.27 (m, 1H), 5.25 (s, 1H), 4.22 (d, *J* = 8.0 Hz, 1H), 4.13 (dd, *J* = 12.0, 4.8 Hz, 1H), 3.96 (s, 1H), 3.89 (s, 1H), 3.48 (d, *J* = 7.0 Hz, 1H), 3.04 (dd, *J* = 14.1, 10.3 Hz, 1H), 2.42 – 2.31 (m, 1H), 2.14 (s, 1H), 2.04 (s, 1H), 0.95 (s, 1H), 0.65 (s, 1H).

6.3.8 Synthesis of reduced steroid derivatives



95

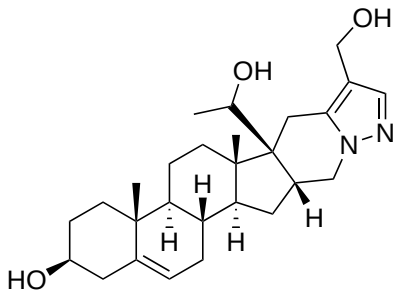
2,3-dihydroxymethyl-(4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-2,3-diyl) fused with reduced 16-DHP: lithium aluminium hydride (2.40 mmol) was added to a solution of compound **87** (0.20 mmol) in dry tetrahydrofuran at 0 ºC. The reaction mixture was stirred at 0 ºC for 15 min and then left at room temperature. After stirring for 4 h, drops of ethyl acetate were added followed by 1 mL distilled water. After stirring for 30 min, drops of hydrochloric acid were added until pH=7, the mixture was filtered over celite, dried over anhydrous sodium sulphate and the solvent was evaporated. After purification of the reaction mixture by flash chromatography [chloroform/methanol/ammonium hydroxide (50:5:0.25)] the product was crystallized in distilled water and obtained as a white powder (37%).

m.p. > 275 ℃ (brown colouring at 210 ℃).

IR 1010, 1060, 1458, 1521, 1552, 2888, 2926 and 3213 cm^{-1}.

^{1}H NMR (400 MHz, DMSO-*d6*): δ 5.17 (d, *J* = 3.5 Hz, 1H), 4.76 (t, *J* = 5.5 Hz, 1H), 4.57 (d, *J* = 4.5 Hz, 1H), 4.48 (t, *J* = 5.1 Hz, 1H), 4.37 (d, *J* = 4.8 Hz, 6H), 4.00 – 3.89 (m, 2H), 3.82 (dd, *J* = 13.2, 2.8 Hz, 1H), 3.23 (d, *J* = 4.7 Hz, 1H), 2.75 (dd, *J* = 61.4, 17.2 Hz, 3H), 2.34 (m, 1H), 0.96 (d, *J* = 6.3 Hz, 4H), 0.92 (s, 6H).

^{13}C NMR (101 MHz, DMSO-*d6*): δ 149.2, 141.2, 139.3, 120.3, 113.8, 70.4, 69.9, 56.1, 54.9, 52.8, 52.3, 50.5, 50.2, 49.1, 48.1, 42.1, 38.1, 36.8, 36.0, 33.4, 33.3, 31.6, 31.4, 20.4, 19.1, 15.3.



96

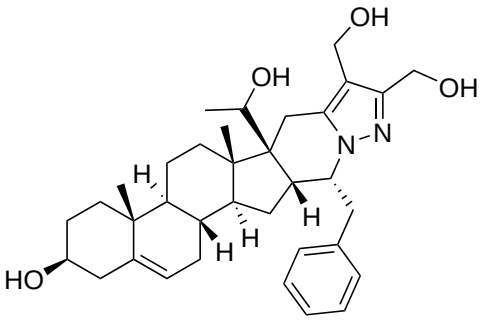
3-hydroxymethyl-(4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridin-3-yl) fused with reduced 16-DHP: lithium aluminium hydride (1.60 mmol) was added to a solution of compound **89** (0.20 mmol) in dry tetrahydrofuran at 0 ºC. The reaction mixture was stirred at 0 ºC for 15 min and then left at room temperature. After stirring for 3 h, drops of ethyl acetate were added followed by 1 mL distilled water. After stirring for 30 min, drops of hydrochloric acid were added until pH=7, the mixture was filtered over celite, dried over anhydrous sodium sulphate and the solvent was evaporated. After purification of the reaction mixture by flash chromatography [chloroform/methanol/ammonium hydroxide (50:5:0.25)] the product was crystallized in distilled water and obtained as a white powder (28%).

m.p. = 177.6-178.0 ℃.

IR 809, 1005, 1046, 1375, 1420, 1458, 2865, 2929 and 3311 cm^{-1}.

^{1}H NMR (400 MHz, DMSO-*d6*): δ 7.20 (s, 1H), 5.16 (s, 3H), 4.63 (s, 2H), 4.57 (d, *J* = 3.8 Hz, 2H), 4.35 (d, *J* = 4.1 Hz, 3H), 2.75 (dd, *J* = 81.5, 17.0 Hz, 8H), 0.97 (d, *J* = 6.0 Hz, 9H), 0.92 (s, 18H).

^{13}C NMR (101 MHz, DMSO-*d6*): δ 141.2, 138.4, 137.1, 120.2, 116.1, 70.4, 70.0, 53.6, 52.9, 51.0, 50.5, 49.2, 48.0, 42.2, 36.8, 36.0, 33.6, 33.2, 31.6, 31.5, 31.4, 30.7, 30.5, 29.6, 29.2, 29.1, 28.4, 28.3, 28.00, 27.6, 27.3, 26.7, 26.3, 20.4, 20.3, 19.1, 15.4.



97

2,3-dihydroxymethyl-(*S*)-(7-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-2,3-diyl) fused with reduced 16-DHP: lithium aluminium hydride (2.40 mmol) was added to a solution of compound **93** fused with 16-DHP (0.20 mmol) in dry tetrahydrofuran at 0 ºC. The reaction mixture was stirred at 0 ºC for 15 min and then left at room temperature. After stirring for 4h, drops of ethyl acetate were added followed by 1 mL distilled water. After stirring for 30 min, drops of hydrochloric acid were added until pH=7, the mixture was filtered over celite, dried over anhydrous sodium sulphate and the solvent was evaporated. After purification of the reaction mixture by flash chromatography [chloroform/methanol/ammonium hydroxide (50:5:0.25)] the product was crystallized in distilled water and obtained as a white powder (24%).

m.p. > 275 ℃ (yellow colouring at 245 ℃).

IR 700, 1009, 1044, 1385, 1454, 2869, 2932 and 3355 cm^{-1}.

^{1}H NMR (400 MHz, DMSO-*d6*): δ 5.16 (s, 1H), 4.78 (t, *J* = 5.4 Hz, 1H), 4.56 (d, *J* = 4.4 Hz, 1H), 4.51 (t, *J* = 4.8 Hz, 1H), 4.45 – 4.36 (m, 5H), 4.29 (d, *J* = 4.6 Hz, 1H), 0.93 (d, *J* = 5.8 Hz, 4H), 0.89 (s, 3H), 0.76 (s, 3H).

^{13}C NMR (101 MHz, DMSO-*d6*): δ 148.7, 141.1, 140.1, 138.3, 129.1, 128.4, 126.3, 120.4, 114.0, 70.1, 69.9, 59.0, 56.2, 53.9, 53.0, 50.4, 48.9, 47.7, 42.4, 42.1, 36.8, 36.0, 35.4, 33.8, 31.8, 31.7, 31.4, 26.6, 20.5, 20.5, 19.0, 18.5, 15.9.

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