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DEPARTMENT OF PHARMACDYNAMIC AND BIOPHARMACY

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Estrogen like agents as anticancer drugs : Theory , synthesis and analysis

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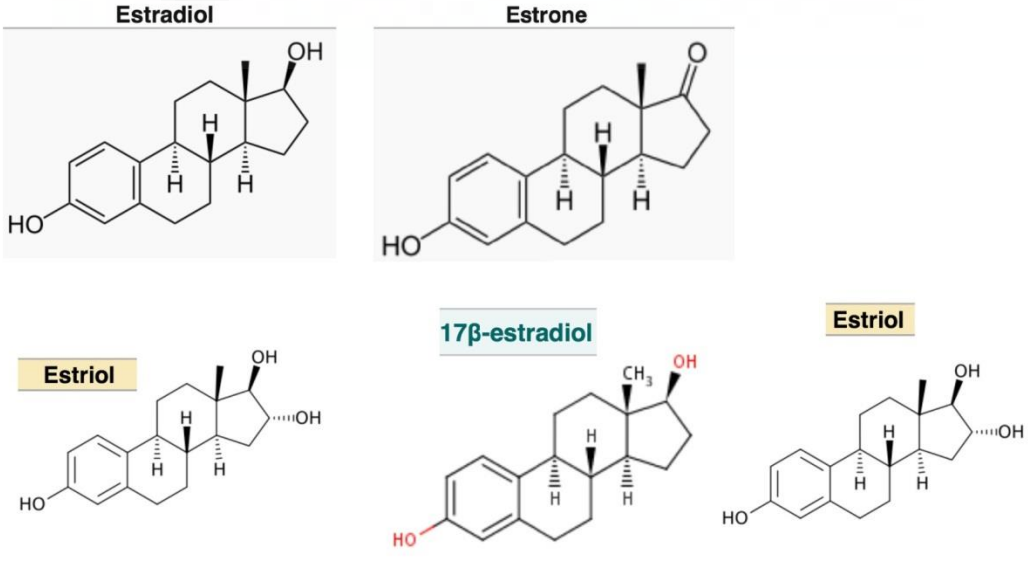
1. Estrogen receptor and its structure and ligands [77]

The human's body is formed from 10^{13}-10^{14} cells which form cellular layers and specialized tissues and organs and many biomolecules act as signals and at its core, intercellular communication involves the collection, integration and execution of a program based upon extracellular inputs. Eukaryotic organisms have developed several approaches to facilitate cellular communication at both the local/regional and distant/organism all levels. Some cell signaling molecules, such as cytokines and growth factors are hydrophilic and act on the cell surface receptors which typically initiate a cascade of protein-protein interactions to affect cellular response. Others are hydrophobic and can diffuse across the cellular membrane and their receptors are intracellular receptors. Examples for lipophilic messengers are steroid hormones, vitamin-D_3, thyroxin, retinoic acid and fatty acid derivatives such as the leukotrienes and eicosanoids. Despite the chemical diversity of the aforementioned molecules, they act via strikingly similar molecular mechanisms involving the nuclear receptor (NR) superfamily.

Estrogens (estradiol, estriol, estrone) are steroid hormones whose biological effects are mediated by the estrogen receptor alpha (ERα). The predominant endogenous estrogen is 17beta-estradiol and is what is typically implied by the term estrogen.



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1.1 several examples of Ligands for Estrogen receptor

A ligand is any molecule that binds specifically to other one. There are many known and unknow ligands for both ERα and ERβ receptors. The structure of the unknown ligands don't need to resemble the structure of estradiol for the molecule to have the same effects, and therefore it is a time-consuming task to determine the effects a given compound has on the ER ,in this part I collect a brief description of these ligands.

Estradiol and Estriol [77]

Estradiol is the agonist for the ER (natural agonist) - its structure is shown in last page. Estradiol is synthesized from cholesterol and secreted by the ovaries and cholesterol source can be food or biosynthesis. It is present in women and in men, in which case it is secreted from their testes.

Estradiol is the main female sex hormone and it is essential for growth and reproductive endocrinoligy . Whnen it has an over-production - or an exposure to estrogen-like compounds - there is a high risk of devoloping breast and endometrial cancer. When women reach menopause their ability to produce estradiol decreases and it can help to develop osteoporosis because estradiol is essential for bone growth and maintainance also Estradiol decreases the concentration of low-density lipoprotein (LDL) and increase the concentration of high density lipoprotein (HDL) and because HDL removes excess cholesterol from blood and tissue, women have a lower rate of coronary artery disease (CAD) than men before 50 years old. Estriol is a natural estrogen, which is produced during pregnancy. It is the major estrogen produced in the normal human fetus. There are indications that estriol maybe less carcinogenic than estradiol. Research has shown that estriol does not induce endometrial growth to the extent of the other estrogens, even at doses where estriol is effective for the relief of postmenopausal symptoms. The structure of estriol is also shown in last page.

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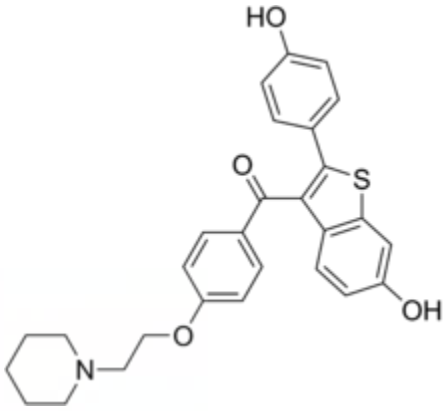
[77]

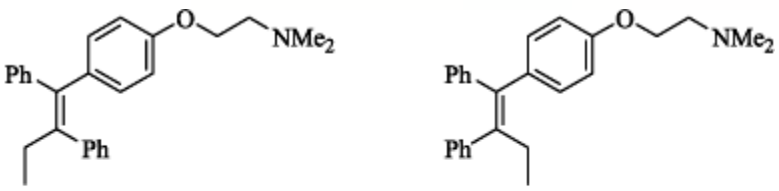
Raloxifen



Raloxifen is one of the antagonists for ERα and it was first developed to prevent breast cancer several years ago . Today, it is used to treat and prevent osteoporosis in menopause women and it is being tested for prevention and treatment of breast cancer. Raloxifen is a very efficient antagonist in the reproductive tissue, but it is a partial agonists in bone tissue and lowers cholesterol levels in the blood.

Tamoxifen E and Z [77]

Tamoxifen isn't one of the active compound in the body, but it is the prodrug for the active metabolit 4-hydroxytamoxifen (OHT). It is an agonist in some tissue and an antagonist in other tissue and it is the most important in treatment. In breast tissue, it is an antagonist, whereas in bone and uterine tissue, it is an agonist. Tamoxifen is the number one choice for the treatment of all stages of breast cancer; see [9]. Unfortunately, tamoxifen treatment has side-effects like hot flashes, vaginal bleeding, and skin aches. There are two different kinds of tamoxifen: Tamoxifen E and tamoxifen Z - structural differences between the two kinds are shown in the figure 1. Tamoxifen E is an agonist and tamoxifen Z is an antagonist in ERα;



(*Z*)-Tamoxifen (**1**)
Antiestrogenic activity

(*E*)-Tamoxifen (**2**)
Estrogenic activity

Figure 1. Structures of (*Z*)-tamoxifen (**1**) and (*E*)-tamoxifen (**2**).

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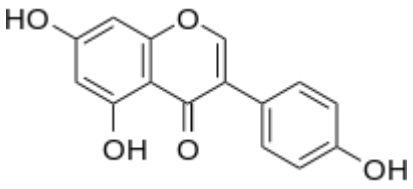
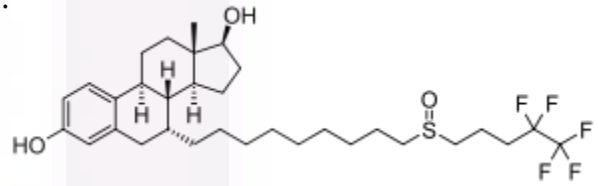
Genistein [77]



Genistein is a partial agonist in ERβ and it is found in soya beans and soy products . Genistein is a phyto-estrogen a plant-derived non-steroidal compound which has estrogen-like

Fulvestrant



Fulvestrant is an antagonist for both ERα and ERβ . It is used to treat advanced ER-positive breast cancer in post-menopausal women. It is used primarily for cancer that has relapsed either during or following treatment with standard anti-estrogen treatments, such as tamoxifen. Unfortunately, it has a negative effect on bone density;.

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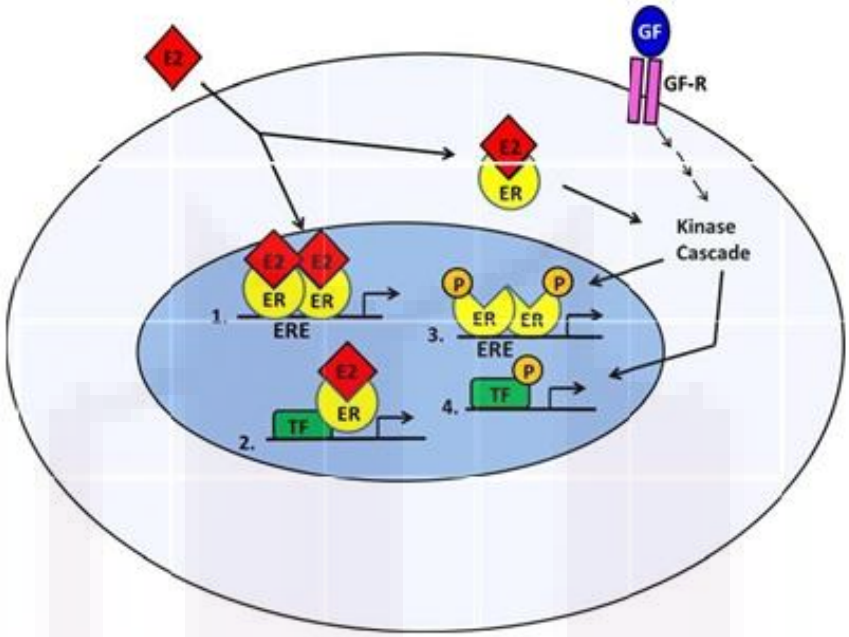

Figure 1-1: A schematic illustrating the role of ERα in cellular signaling through various mechanisms: (1) Classical mechanism of ER action. Upon its diffusion through the cell membreane, estradiol (E2) binds to ERα and induces dimerization. The resulting E2 ERα dimer in turn binds directly to EREs at target gene promoters. (2) ERE-independent genomic action. E2-complexed ERα can engage in tethering through protein-protein interactions thereby binding a transcription factor (TF) engaging its promoter. (3) Ligand-independent genomic actions. Growth factors (GF) bind to GF-receptors (GF-R) and activate protein-kinase cascades. Activated kinases can then phosphorylate (P) and activate nuclear ER at EREs. (4) Nongenomic actions. Membrane and cytosolic E2-ERα complexes can also activate protein-kinase cascades, thereby modulating protein function, e.g. activation of eNOS or regulation of gene expression through phosphorylation (P) and activation of a TF. Adapted from [2]

ERα (NR3A1) is a member of the ligand-modulated transcription factors collectively known as the NR superfamily [1-4]. Recent studies indicate ERα regulates biological processes by utilizing a variety of signaling pathways (Figure 1-1) [5, 6]. Classically, ERα is activated by binding estradiol followed by receptor dimerization and subsequent engagement of estrogen response elements (EREs) located within the promoters of target genes thereby influencing gene expression [7-9]. It should be noted binding of estradiol induces conformational change within the ligand binding (LB) domain of ERα which allows for the recruitment of transcriptional co-activators and co-repressors to ERE-containing genes [10]. The cellular context of co-activators and co-repressors in which ERα operates is one mechanism by which ligand-binding can produce differential responses in

different tissues. Additionally, ERα can influence genes not containing an ERE by act as a transcriptional co-regulator via protein-protein interactions with other transcription factor complexes in a ligand-dependent manner. Around one-third of genes which show estrogen-dependence do not harbor an ERE-like promoter motif [11] which suggests this indirect genomic function of ERα can have significant biological impact. Typical examples of transcription factors influenced by liganded-ERα include the activator protein-1 (AP1, Jun/Fos) [12] and specificity protein-1 (SP-1) [13]. Genes under AP1 control upregulated in response to estradiol include Cyclin D1 [14], insulin-like growth factor-1 (IGF-1) [15], and collagenase [16]. An example of an SP-1 dependent gene influenced by ERα is the low-density lipoprotein receptor (LDL-R) [17] [74].

Estrogens also induce rapid cellular responses which occur on the time scale of seconds to minutes after estrogen exposure. These effects are much too fast to involve transcription. Non-genomic effects often employ cellular kinases, phosphatases, ion flux and secondary messengers to elicit a timely response [6]. Such rapid cellular phenomena are commonly elicited by steroid hormones [18.19]. Several cell lines have been studied demonstrating mitogen activated protein kinase (MAPK) activation in response to estradiol [20-23]. Additional actions of ERα reported include activation of adenylyl cyclase leading to increased levels of cyclic andnoside monophosphate (cAMP) [24] as well as intracellular calcium ion release [25] [74] .

Finally, as opposed to the above estrogen-dependent mechanisms, ERα can also be activated in response to cellular kinases which phosphorylate ERα itself and/or one or more of its coregulators thereby affecting gene expression [6, 26, 27]. This adds another

layer of complexity likely supplementing classical estrogen-dependent gene expression and permits ERα to carry out its biological actions even in the absence of ligand.

It is clear that the ability of ERα to impact gene expression and cellular physiology is an integrated process consisting of both genomic and nongenomic events which often intersect. The eventual cellular response is therefore a dependent upon the cellular context in which ERα operates. The amounts of receptor, transcription factors, co-activators and co-repressors, the presence of growth factor stimuli and elicited kinase activity all play a role in determining the cellular reaction to estrogens. Given that the molecular mechanisms in response to estrogen are multifactorial it should come as no surprise the wide array of biology estrogen and ERα influence.

2 . Estrogen receptor has a important role in health and many diseases [74]

Estrogen is a hormone that is produced mainly by the ovaries but exerts its effects systemically on distant tissues. It has been long acknowledged the role estrogen plays in the development of female secondary sexual characteristics and reproductive capacity. However, the efforts of gene targeting and transgenic mouse models have drastically expanded our knowledge of ERα biology. Estrogen and its receptor are now implicated in several physiological processes including cardiovascular health, bone homeostasis, and neurological function [28]. Given its broad physiological scope, estrogen acting via ERα is central to the initiation or progression of several pathological processes including but not limited to multiple types of cancers, coronary artery disease, osteoporosis, neurodegenerative disease, autoimmune conditions, and obesity [28, 29].

2.1 Reproductive Health [74]

The results of the estrogen receptor knockout (ERKO) studies serve as an important lesson in the definition of estrogen biology [30]. ERα knockout, not surprisingly, results in female infertility as well as non-development of breast tissue [30, 31]. Murine females are born with a rudimentary epithelial ductal system embedded in stromal tissue. During puberty, rises in ovarian steroids induce maturation and elongation of the ductal epithelium leading to the development of secondary sexual characteristics. When the ERα is knocked out, proper breast tissue fails to develop and remains rudimentary through life [32, 33]. Function of ERα is also requisite for proper regulation of the menstrual cycle. In particular, ERα is critical to a negative feedback loop which down-regulates pituitary-derived luteinizing hormone leading to ovulation. Knockout of ERα leads to a disruption of the balance of the so-called hypothalamo-pituitary-gonadal axis leading to an anovulatory setting. Estrogen is also critical to growth and maintenance in preparation for implantation of an embryo and disruption of ERα leads to the inability for implantation to occur [32]. These results highlight the vital nature of functional estrogen-ERα signaling to female reproductive

In addition to the aforementioned reproductive effects observed in females, it turns out that ERα is also critical to male fertility [34]. This was a surprising observation as it was previously thought that ERα only played a role in female fertility. Testicular tissue of the knockout mice undergoes atrophy over time and sperm is lost due to fluid accumulation within the seminiferous tubules [30]. ERα is required by the somatic cells within the testes for the proper maturation of sperm and is not an inherent property of sperm lacking ERα [35, 36].

2.2 Cardiovascular Health [74]

The incidence of cardiovascular events in premenopausal women is low but rises to levels seen in men in the postmenopausal setting. This suggests a potential role of ERα in carioprotection of women. Clinical data supports this picture as reduced levels of ERα is associated with the development of coronary artery disease in female patients [37]. Furthermore, methylation of the ERα gene is observed in coronary atherosclerotic plaques compared to controls [38]. Estrogen is also associated with favorable changes in lipid profiles. Estrogen has been associated with increased high-density lipoprotein (HDL) and lower low-density lipoprotein (LDL) levels [39, 40]. Thus, cardioprotective effects of estrogen are likely to be largely a product of the circulating lipid profiles established by estrogen and ERα.

2.3 Osteoporosis [74]

Osteoporosis is the condition of a decline in bone strength or mechanical integrity marked by a decline in bone mineral density. It has long been viewed as a condition predominantly of the postmenopausal population, suggesting a plausible role of estrogen in the maintenance of bone mineral density in premenopausal women. The Women s Health Initiative demonstrated hormone replacement therapy was clinically efficacious for the prevention of osteoporosis in postmenopausal women [41]. Likely molecular mechanisms behind these observations have been established. Estrogen prevents bone resorption through stimulation of bone-forming osteoblast activity while reducing the bone-resorbing activity of osteoclasts [42-45]. This is accomplished through ERα-dependent expression of osteoprotegrin by osteoblasts which is a soluble scavenger receptor for the molecule receptor activator of nuclear factor kappa-B ligand (RANKL)

which is key to osteoclast development and activity [45]. Thus, osteoprotegrin effectively lowers the amount of the RANKL available to osteoclasts which is critical to inducing their activity. In addition to its effect on bone in females, estrogen has been demonstrated to play a direct role in bone maintenance in males with the description of an estrogen-insensitive osteoporotic male patient [29, 46]. Today the selective estrogen receptor raloxifene (which acts as an ERα agonist in bone) is used in the prevention of postmenopausal osteopenia in women [28].

2.4 Breast Cancer [74]

Breast cancer is the leading cause of cancer (excluding skin malignancies) and second leading cause of cancer associated death in females [47]. Perhaps the most familiar risk factor for development of breast cancer is prior familial history of the disease, and in particular the heritable BRCA1/2 mutations. Inheriting such a mutation is associated with upwards of an 80% lifetime risk of developing breast cancer. Despite such a high likelihood of developing cancer in patients possessing BRCA mutations, such heritable forms of breast cancer only represent five to ten percent of total breast cancer cases. Clearly risk factors other than heritable factors contribute to the development of the majority cases.

Breast cancer is one of the hormone-dependent cancers. Epidemiological evidence demonstrates a linear increase in age-related incidence with a decrease in the slop of the curve at the time of menopause. In addition, three factors which have a dramatic impact on breast cancer risk are age of menarche, nulliparity and age at menopause [48]. Obesity has also been demonstrated to increase the risk of breast cancer, likely through additional estrogen synthesis by adipose tissue [49]. Furthermore,

prolonged hormone replacement therapy in postmenopausal women has also been associated with an increased breast cancer risk [50]. Taken together, such evidence supports the notion of cumulative lifetime estrogen exposure as a factor contributing to breast carcinogenesis.

Two prevailing hypotheses exist attempting to explain the mechanism of estrogen tumorigenesis [32]. The first suggests that increased estrogen exposure leads to an increased number of cell divisions with each division carrying a certain risk of mutation. Over time, such mutations become more likely and accumulate. These genetic lesions then lead to tumor formation. The second employs genotoxic byproducts of oxidative estrogen metabolism via the catechol pathway. This pathway utilizes the cytochrome P450 family of enzymes and one such enzyme, cytochrome P450 1B1 is constitutively expressed in breast [48]. Cytochrome P4501 1B1 hydroxylates estradiol forming 4-hydroxyestradiol which can be further converted into estradiol-3,4-quinone by peroxidases [51, 52]. Estradiol-3,4-quinone can then react with adenine and guanine bases in DNA forming unstable adducts leading to depurination [48, 51]. Through both mechanisms, in addition to those yet to be discovered, estrogen proves to be a capable tumor inciting factor.

Initial clinical suggestion for a role of estrogen in breast carcinogenecis came over a century ago with a report from British surgeon Gorge Beatson. In 1896 Beatson demonstrated estrogen-withdrawal through oophorectomy in young women with advanced breast cancer resulted in regression of tumor size and improved survival [53]. The strongest evidence linking estrogen to breast cancer and its outcomes comes from decades of clinical experience with the SERM tamoxifen and improvement of patient

survival as well as breast cancer prevention. Clinical trials consistently demonstrate a survival advantage in tamoxifen treated patients irrespective of invasive status in early stage breast cancer as well as recurrence prevention in the contralateral breast [54]. One meta-analysis of multiple clinical trials found tamoxifen reduced breast cancer risk by 38 percent in high risk women [55]. This risk reduction applies only to ER-positive tumors further indicating tamoxifen clinical effects are limited to ER-dependent pathways. The success of tamoxifen has spawned the development of additional SERMs such as raloxifene as well as the estrogen synthesis blocking aromatase inhibitors (AIs) which are used in the clinic today. The clinical utility of the multiple pharmaceuticals targeting estrogen-dependent signaling via differing means (ligand competition in the case of SERMs and ligand depletion in the case of AIs) exemplifies the central role of estrogen in breast carcinogenesis.

3 .ERα shares a modular domain organization common to all nuclear receptors [74]

ERα is a member of a family of ligand-modulated transcription factors that have come to be known as nuclear receptors (NRs) [1-4]. In humans, there are currently 48 known members of the NR family and all share a core modular architecture comprised of a central DNA-binding domain flanked between an N-terminual trans-activation (TA) domain and a C-terminal ligand-binding (LB) domain (Figure 1-2) [56-58] . Intuitively, the DB domain is the region of the molecule which confers DNA-binding capacity to the nuclear receptors. The DB domains of NRs are comprised of conserved tandem zinc-fingers and bind to DNA motifs known as hormone response elements [59]. The ligand binding function of the LB domain is specific for its cognate ligand which ensures specificity of the desired physiological response [59]. In addition to ligand binding, the

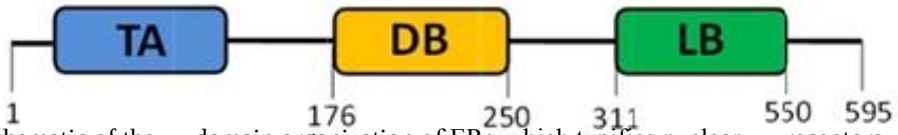

Figure 1-2: Schematic of the domain organization of ERα which typifies nuclear receptors. A central DNA-binding domain (DB) is flanked by an N-terminal Transactivation (TA) domain and a C-terminal Ligand Binding (LB) domain. The numbers represent the domain boundaries used in this work.

LB domain additionally serves as a platform for the recruitment of a multitude of cellular proteins, such as transcription factors, co-activators and co-repressors, to the site of DNA transcription and thereby allowing nuclear receptors to exert their action at genomic level in a concerted fashion [62, 63]. While the trans-activation function of the LB domain is ligand-dependent, the TA domain operates in an autonomous manner and it is believed to be responsive to growth factors acting through MAPK signaling and may further synergize the action of various co-activators and co-repressors recruited by the LB domain at the site of DNA transcription [26,62]. In this manner, nuclear receptors orchestrate a diverse array of cellular functions, and the success of coupling a DNA-binding module to a ligand-binding one has allowed nuclear receptors to become the primary players controlling organ-specific physiology [59].

14. The ERα-ERE interaction is governed by its DB domain [74]

Vital to the physiological actions of ERα is its ability to function as a transcription factor. The defining feature of transcription factors is their capacity to directly bind DNA through protein-DNA interactions. The DB domain of ER□ binds DNA as a homodimer to the AGGTCAnnnTGACCT consensus motif, termed estrogen response element (ERE), located within the promoters of target genes [63]. The crystal structure revealed that DNA-binding is accomplished through a pair of tandem C4-type Zinc fingers, with each finger containing a Zn^{2+} ion coordinated in a tetrahedral arrangement by four highly

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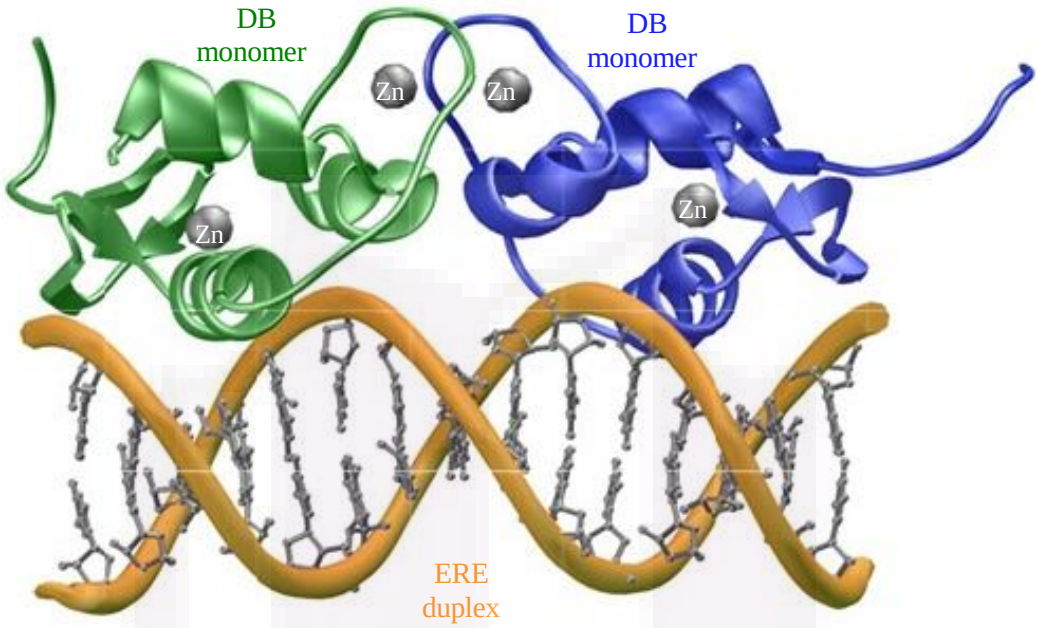

Figure 1-3: 3D crystal structure of the DB domain of ERα in complex with ERE. DB as a homodimer in complex with ERE duplex containing the AGGTCAcagTGACCT consensus sequence as determined by Rhodes and co-workers [66]. One monomer of the DB domain is shown in green and the other in blue. The Zn^{2+} divalent ions are depicted as gray spheres. The DNA backbone is shown in yellow and the bases are colored gray for clarity.

conserved cysteine residues [64, 65]. The first Zinc finger (ZF-I) within each monomer of DB domain recognizes the hexanucleotide sequence 5 -AGGTCA-3 within the major groove at each end of the ERE duplex, whilst the second Zinc finger (ZF-II) is responsible for the homodimerization of DB domain (Figure1-3). DNA sequence recognition has been demonstrated to be exclusively a property of the DB domain through construction of a protein chimera in which the DB domain of glucocorticoid receptor was swapped into ERα resulting in a receptor which responded to estradiol but bound only to a glucocorticoid response element [66]. Furthermore, it was demonstrated that this DNA sequence specificity of the DB domains is largely due to three amino acids within ZF-I of the DB domains [67].

From the DNA-side, it has remained unclear which nucleotides within the ERE are the most critical or make the strongest contact with the DB domain in large part due to differences in laboratory technique in the studies [68, 69]. Several lines of evidence suggest that genetic variations within the cognate response elements play a key role in modulating the affinity and specificity of binding of androgen, glucocorticoid and progesterone nuclear receptors [70-73]. However, determination of the impact of nucleotide variation on ERα binding affinity has remained elusive, a surprising fact given the consensus motif was determined in 1989 [63]. This is an important consideration as most estrogen-regulated genes contain imperfect ERE sequences [63].

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Experimental part

First Experiment

Synthesis of anticancer steroid compounds and determination of their effects and NMR [75]

Abstract

A simple and suitable synthetic route is reported for the formation of new 2a-triazolylcholestane derivatives. The scheme involves transformation of the starting cholestanone to the corresponding azido compound and efficient conversions of 2a-azido-5a-cholestan-3-one (3).The antiproliferative activities of the synthesized 2-triazolyl-3-ketones against three human cancer cell lines were screened. but, the result was not so acceptable in cell-growth inhibition.

Introduction

there are a number of reactions that fulfill the Sharpless criteria, the Cu-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) is perhaps the best-known example of this group.

The 1,2,3-triazole unit has a several advantageous properties, like high chemical stability, good hydrogen-bond-accepting ability, a strong dipole moment and an aromatic character.Therefore a number of compounds containing 1,2,3-triazoles have been reported to exert biological activity, including antibacterial, antiallergic and anti-HIV effects.

We tried to synthesis of steroidal heterocycles, and we have tried to develop an effective route for the production of various 2-triazolyl derivatives of cholestanone, 4c,4g, it was decided to screen these compounds in vitro for their activities against a panel of three human cancer cell lines (HeLa, MCF7 and A431). Herein, we hope we can describe the details of the synthesis of 2a-azido-5a-cholestan-3-one (3), followed by Huisgen 1,3-dipolar cycloaddition with different terminal alkynes and subsequent reduction of the resultant triazolyl ketones.

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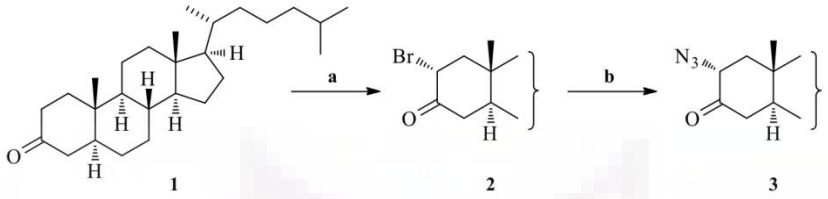
Results and Discussion

For the preparation of these steroid derivatives via copper(I)-catalysed azide-alkyne cycloaddition (CuAAC), 2a-azido-5a-cholestan-3-one (3) was chosen as starting compound. The synthetic strategy for the preparation of the starting azide is illustrated in Scheme 1.



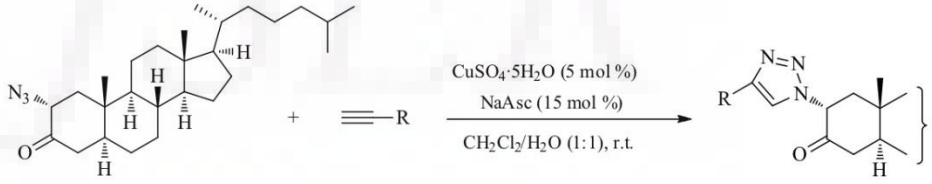
Scheme 1. Synthesis of 2a-azido-5a-cholestan-3-one. Reagents and conditions: (a) Br_2, HBr, AcOH; (b) NaN_3, DMF, 8 h. [75]

2a-Bromo-5a-cholestan-3-one (2) is synthesised via bromination from readily available cholestanone (1). After purification of the a-bromo ketone, the compound was stirred for 8 h in the presence of sodium azide to provide the desired 2a-azido ketone (3) in good yield.

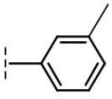
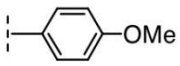
Substitutions a to carbonyl groups are known to follow an S_N2 mechanism, however in this particular case only 2a-azido ketone could be isolated. A base (NaN_3)-catalysed epimerization of the unisolated 2b-azido ketone can be assumed.

Two number of A-ring-substituted 1,2,3-triazolylcholestan-3-ones (**4c , 4g**) are synthesized in very good yields by the reactions of 3 with two kinds of terminal alkynes (Table 1).

Table 1. 1,3-Dipolar cycloaddition with terminal alkynes [75]



3 4a-m

Entry	R	Triazolyl ketone	Time (h)	Yield[a] (%)
1.		**4c**	3	91
2		**4g**	3	89

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For generation of the active catalyst, we choosed one of the most common techniques was chosen. The copper(I) species was generated in situ by the reduction of copper(II) sulfate with sodium ascorbate. Therefore, an unusual solvent system (CH_2Cl_2 as a co-solvent with water) was used to eliminate the need for ligands and to simplify the reaction protocol.

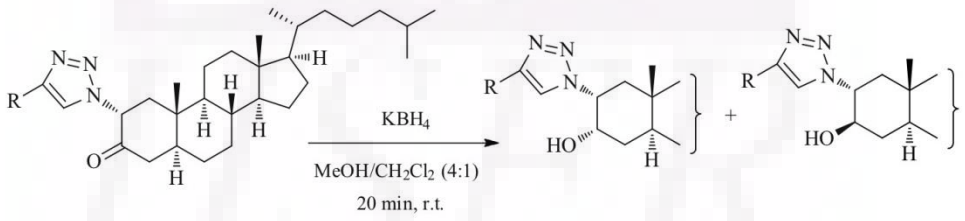
In all cases, total consumption of the starting compound was observed within 1.5-8 h at normal temperature. The reactions are very selective, and the triazolyl ketones could generally be isolated in 84-92% yields. The trace quantities of copper and reagents remaining in the reaction mixtures were removed by flash chromatography.

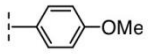
Treatment of **4c , 4g** with KBH_4 in $MeOH/CH_2Cl_2$ (4:1) resulted in two diastereomeric 3-hydroxy-2-triazolylcholestanes in an overall yield of ~ 95%.

The structures of all synthesized compounds were confirmed by ^1H and ^{13}C NMR measurements.

The ^1H NMR spectra of **4c , 4g** revealed the appearance of the new signals of the incorporated aryl groups at around 7-8 ppm as compared with the spectra of the starting material (3), while the 5'-H singlet was identified at around 7.7-8.1 ppm.

The exo-heterocyclic steroidal ketones **4c, 4g** were tested in vitro on three malignant cell lines. None of the newly prepared compounds elicited greater than 50% inhibition of cancer cell proliferation, even at the higher applied concentration. Although there is no generally accepted threshold for efficacy, when the inhibition of cell growth is less than 25% at 30 μM, such a substance may be considered ineffective. [75]



Entry	R	Triazolyl alcohols (**5** and **6**)	Yield[a] (%) of **5**	Yield[a] (%) of **6**
1		**c**	29	66
2		**g**	28	65

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[75]

Table 3. Antiproliferative effects of some selected triazolyl ketones

		Growth inhibition % (±SEM)		
Product	μM	HeLa	A431	MCF7
4g	10	25.0 (±2.6)	9.7 (±1.1)	14.2 (±2.2)
	30	32.1 (±1.6)	25.1 (±1.4)	28.6 (±2.2)
Cisplatin	10	42.6 (±2.3)	88.6 (±0.5)	53.0 (±2.3)
	30	99.9 (±0.3)	90.2 (±1.8)	86.9 (±1.3)

Conclusions

In conclusion, the efficient syntheses of two types of A-ring-substituted 1,2,3-triazolylcholestane

derivatives were been achieved by means of Huisgen 1,3-dipolar cycloaddition. The fast and reliable reactions were carried out under mild conditions that furnished the desired compounds in very good yields. An unusual, two-phase solvent system was applied to increase the solubility of the steroid and to eliminate the need for ligands. The cycloadducts were tested in vitro as concerns their antiproliferative activities, According to our observations different structural elements on the heteroring might have an impact on the cytostatic effects.The application of 'click chemistry' to further sterane skeletons is in progress and the promising results will be reported in due course.

Experimental Section [75]

General. Melting points (mp) were determined on a Kofler block and are uncorrected. Specific rotations were measured in $CHCl_3$ (c 1) at 20 °C with a POLAMAT-A (Zeiss-Jena) polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick); solvent systems (ss): (A) CH_2Cl_2/EtOAc (95:5 v/v), (B) CH_2Cl_2/EtOAc (85:15 v/v).

The spots were detected by spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid. The Rf values were determined for the spots observed by illumination at 254 and 365 nm. Flash chromatography: Merck silica gel 60, 40-63 μm. All solvents were distilled prior to use. Elementary analysis data were determined with a PerkinElmer CHN analyzer model 2400. IR spectra were recorded on a PerkinElmer FT-IR Spectrum 100. NMR spectra were recorded on a Bruker DRX 500 instrument at 500 MHz (1H NMR) or 125 MHz (13C NMR). Chemical shifts are reported in ppm (d scale), and coupling constants (J) in Hz. For the determination of multiplicities, the J-MOD pulse sequence was used.

Synthesis of 2a-azido-5a-cholestan-3-one (3). To a solution of 2a-bromo-5a-cholestan-3-one 2 (931 mg, 2 mmol) in 20 mL DMF was added sodium azide (195 mg, 3 mmol). The reaction mixture was stirred at 40 oC for 8 h and then poured into water. The precipitate that formed was filtered off and washed with water. Purification by column chromatography (CH2Cl2/hexane 1:1) afforded 3 as a white solid (720 mg, 84%), mp 146-148 oC (lit.16 mp 147-150 oC). The spectroscopic data were consistent with those reported in the literature.

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General procedure for the preparation of (4c,4g)

Compound 3 (428 mg, 1 mmol) was dissolved in CH2Cl2 (10 mL), and a solution of CuSO4•5H2O (12.5 mg, 5 mol%) and sodium ascorbate (30 mg, 15 mol%) in water (10 mL) was poured into the organic phase. The appropriate terminal alkyne (1 mmol) was added to the reaction mixture, which was then stirred for 2-6 h at normal temperature. After the consumption of the starting material (TLC monitoring), the two-phase solution was diluted with water (30 mL) and extracted with CH2Cl2 (2 x 30 mL). The combined organic layers were washed with water, dried over Na2SO4 and evaporated in vacuo. The resulting crude product was purified by flash chromatography with CH2Cl2/EtOAc (98:2) as eluent.

2a-[4-(3-Tolyl)-1H-1,2,3-triazol-1-yl]-5a-cholestan-3-one (**4c**). Alkyne: 3-tolylacetylene (0.13 mL). After purification, **4c** was obtained as a white solid (495 mg, 91%), mp 172-174 ^{o}C,

R_f = 0.62 (ss A); $[a]_{D20}$ + 54 (c 1 in $CHCl_3$), IR (KBr): 2946, 1732, 1612, 1591, 1469, 1445, 1383, 1228, 1054, 793 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$); □ [ppm] = 0.67 (s, 3H, 18-H_3), 0.85-0.9 (overlapping multiplets, 9H, 21-, 26- and 27-H_3), 1.21 (s, 3H, 19-H_3), 2.34 (dd, 1H, J = 14 Hz and J = 3.5 Hz), 2.39 (s, 3H, 3”-H_3), 5.53 (dd, 1H, J = 13.5 Hz and J = 6 Hz, 2-H), 7.13 (d, 1H, J = 7.5 Hz, 4”-H), 7.30 (t, 1H, J = 7.5 Hz, 5”-H), 7.61 (d, 1H, J = 7.5 Hz, 6”-H), 7.70 (s, 1H, 2”-H),

7.81 (s, 1H, 5’-H); ^{13}C NMR (125 MHz, $CDCl_3$); □ [ppm] = 12.0 (C-18), 12.4 (C-19), 18.6 (C-21), 21.4 (3”-CH_3), 21.5, 22.5 and 22.8 (C-26 and C-27), 23.8, 24.1, 28.0, 28.2, 28.4, 31.5,

34.9, 35.7, 36.1, 37.3, 39.5, 39.6, 42.5, 43.9, 47.0, 47.9, 53.6, 56.0, 56.1, 65.1 (C-2),

119.7 (C-5’), (122.8, 126.4, 128.6, 128.8): (4C, C-2”, C-4”, C-5”, C-6”), 130.5 (C-1”), 138.4 (C-3”), 147.7 (C-4’), 202.7 (C-3); Anal. Calcd for $C_{36}H_{53}N_3O$: C, 79.51; H, 9.82; N, 7.73. Found: C, 79.62; H,

9.95; N, 7.65. 2a-[4-(4-Methoxyphenyl)-1H-1,2,3-triazol-1-yl]-5a-cholestan-3-one**(4g).**Alkyne:

4-methoxyphenylacetylene (132 mg). After purification, 4g was obtained as a white solid (498 mg, 89%), mp 179-181 ^{o}C, R_f = 0.41 (ss A); $[a]_{D20}$ + 52 (c 1 in $CHCl_3$), IR (KBr): 2934, 1737, 1618, 1563, 1499, 1466, 1444, 1249, 1027, 834, 804 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$); □ [ppm] =

0.67 (s, 3H, 18-H_3), 0.85-0.9 (overlapping multiplets, 9H, 21-, 26- and 27-H_3), 1.21 (s, 3H, 19-H_3), 2.34 (dd, 1H, J = 14 Hz and J = 3.5 Hz), 3.83 (s, 3H, OCH_3) 5.52 (dd, 1H, J = 13.5 Hz and J = 5.5 Hz, 2-H), 6.95 (d, 2H, J = 8.5 Hz, 3”- and 5”-H), 7.73 (s, 1H, 5’-H), 7.76 (d, 2H, J = 8.5 Hz, 2”- and 6”-H); ^{13}C NMR (125 MHz, $CDCl_3$); □ [ppm] = 12.0 (C-18), 12.4 (C-19), 18.6

(C-21), 21.6, 22.5 and 22.8 (C-26 and C-27), 23.8, 24.2, 28.0, 28.2, 28.5, 31.5, 34.9, 35.7, 36.1,

37.4, 39.5, 39.7, 42.6, 43.9, 47.0, 47.9, 53.7, 55.3 (O-CH_3), 56.1, 56.2, 65.1 (C-2), 114.2 (2C, C-3” and C-5”), 118.9 (C-5’), 123.5 (C-1”), 127.0 (2C, C-2” and C-6”), 147.5 (C-4’), 159.5 (C-4”), 202.7 (C-3); Anal. Calcd for $C_{36}H_{53}N_3O_2$: C, 77.24; H, 9.54; N, 7.51. Found: C, 77.36; H, 9.68; N, 7.88.

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Determination of antiproliferative effects [75]

Human cancer cell lines were purchased from ECACC (Salisbury, UK). HeLa (cervix adenocarcinoma), A431 (skin epidermoid carcinoma) and MCF7 (breast adenocarcinoma) cells were cultivated in minimal essential medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids and an antibiotic-antimycotic mixture.

Near-confluent cancer cells were seeded onto a 96-well microplate (5000/well) and attached to the bottom of the well overnight. On the second day, 200 µL of new medium containing the tested compound (at 10 or 30 µM) was added. After incubation for 72 h at 37 ºC in humidified air with 5% CO_2, the living cells were assayed by the addition of 20 µL of 5 mg/mL MTT solution. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 h contact period. The medium was then removed and the precipitated crystals were dissolved in 100 µL DMSO during a 60 min period of shaking at 25 ºC. Finally, the reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells were utilized as controls (Mosmann, 1983).[17] All in vitro experiments were carried out on two microplates with at least five parallel wells. Cisplatin was used as positive control. Stock solutions of the tested substances (10 mM) were prepared with DMSO. The DMSO content of the medium (0.1% or 0.3%) did not have any significant effect on the cell proliferation.

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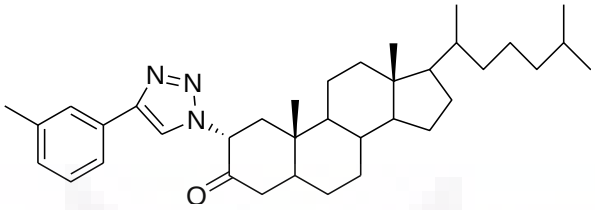
Proof of the structures of the compounds obtained with NMR

structure of the compounds, and evaluation of 1H and 13C-NMR spectra confirmed. The Figure 15-16 shows 13C-NMR spectrum 2α-[4 - (3-tolyl)-1H-1 ,2,3-triazol-1-yl]-5α-cholestane-3-one .



It can be seen that of 202 ppm in the spectrum of the starting compounds remaining carbonyl group is also present in the signal. 119.7 ppm 5'C-triazole is the incorporated atoms and the aromatic CH 4 pieces (122.8, 126.4, 128.6, 128.2 ppm). Also appears at 65.1 ppm for the ring 2-C atom that is linked to the triazole ring.

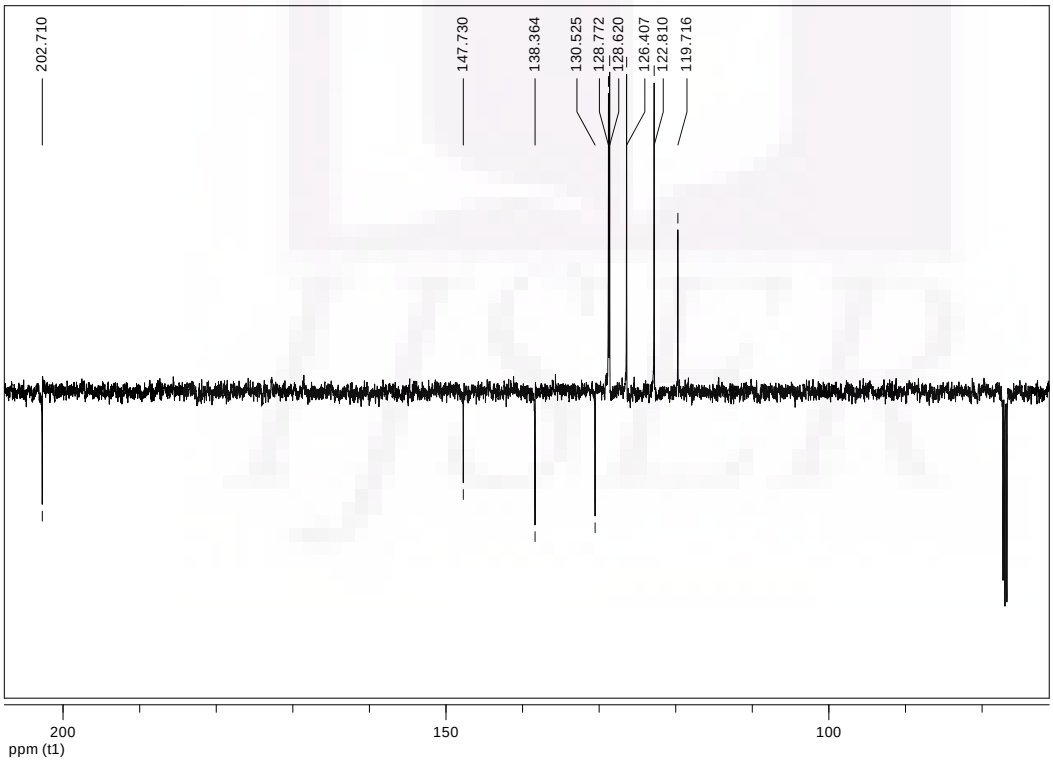


Figure. 15.

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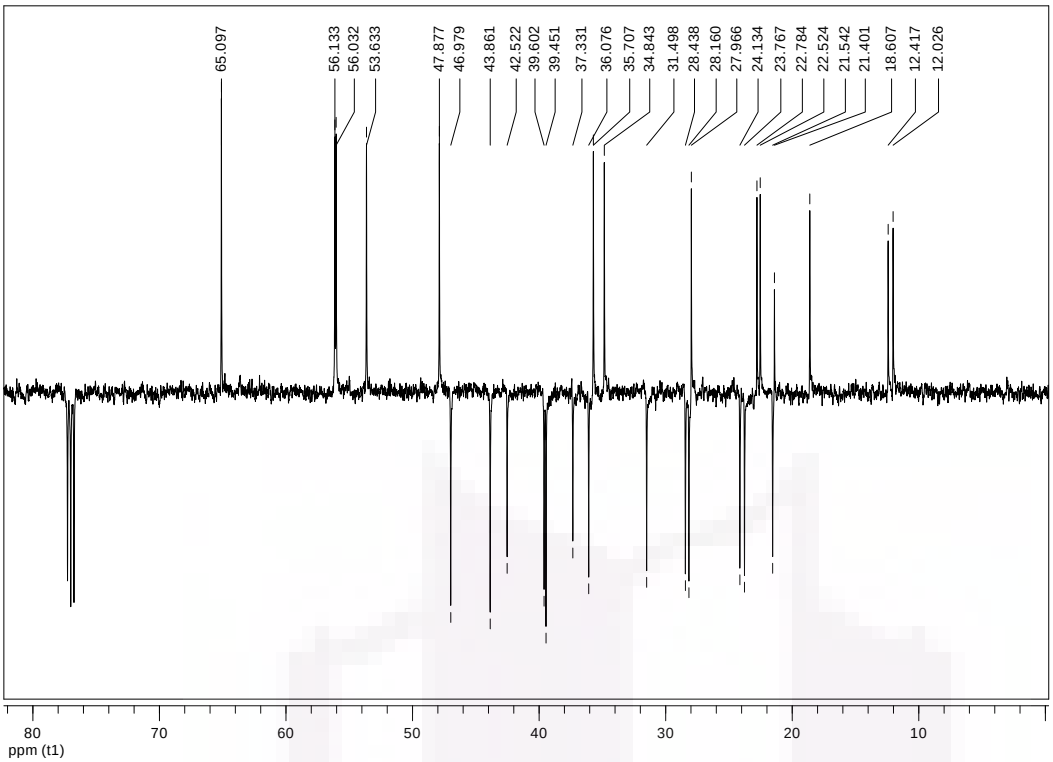

Figure. 16

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The Figure 17 2α-[4 - (3-tolyl)-1H-1 ,2,3-triazol-1-yl]-5α-cholestane-3-one 1 H-NMR spectrum. Two spectra is the angular methyl group and 1.21 0.67 ppm. 2.39 ppm methyl group linked to the aromatic ring signal, 5.5 ppm for the C-2 proton-linked 7-8 ppm 4 protons of the aromatic ring, and finally the triazole ring atom of a single-5C' proton signal is bserved.

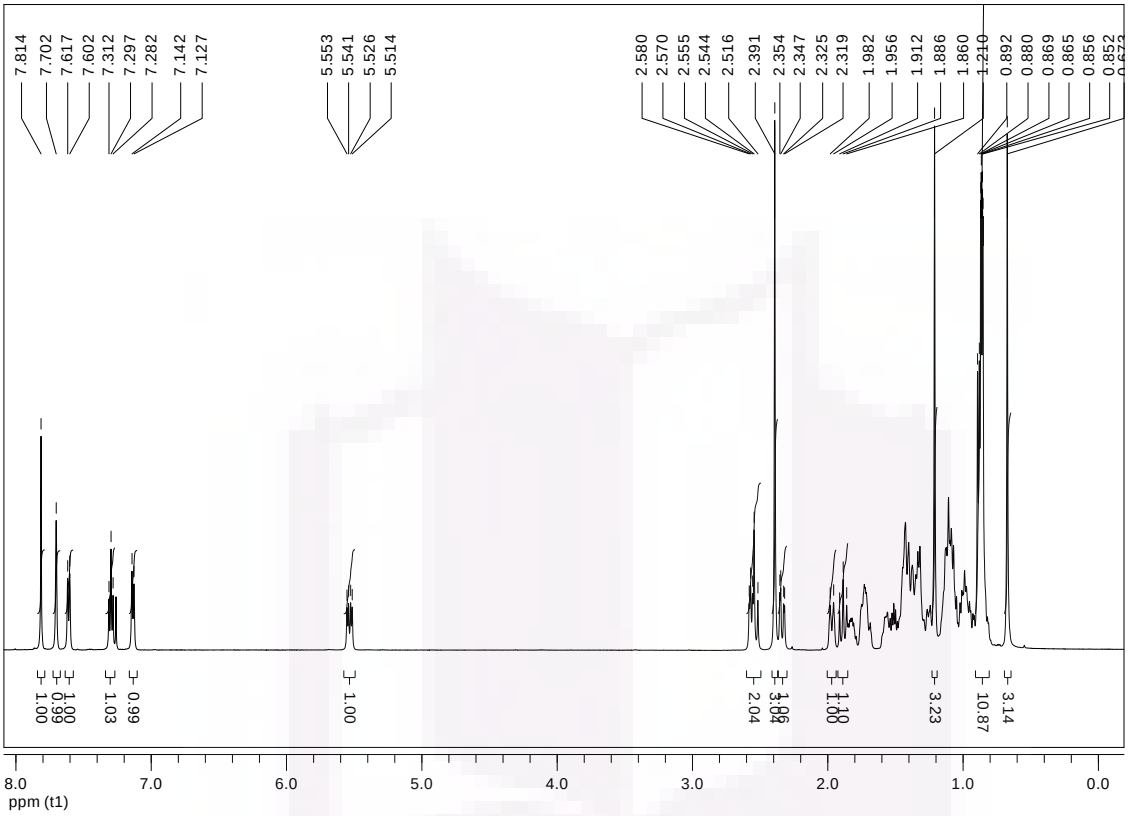
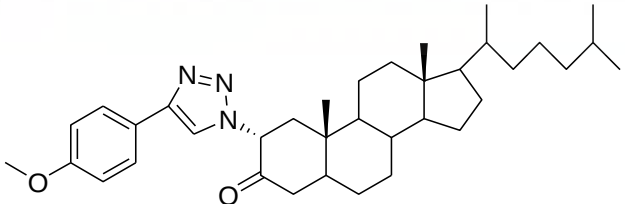


Figure.17

The Figure 18-19 shows the 2α-[4 - (4-methoxyphenyl)-1H-1 ,2,3-triazol-1-yl]-5α-cholestane-3-one 13C-NMR spectrum. 114.1 ppm, aromatic C-4 piece appears, which provides two signals, which shows that the incorporated aromatic moiety is symmetrical. 118.9 ppm of the triazole ring atom 5'C, of 65 ppm for the 2-carbon atom is displayed.



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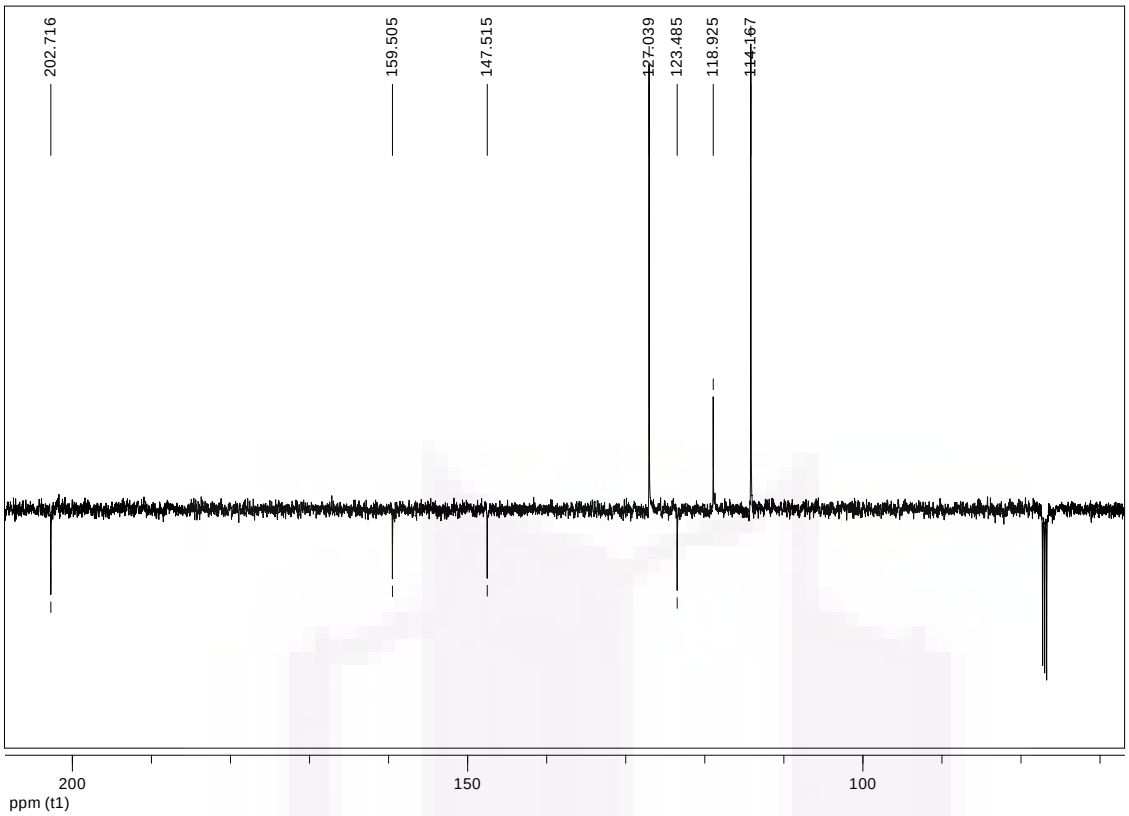

Figure. 18

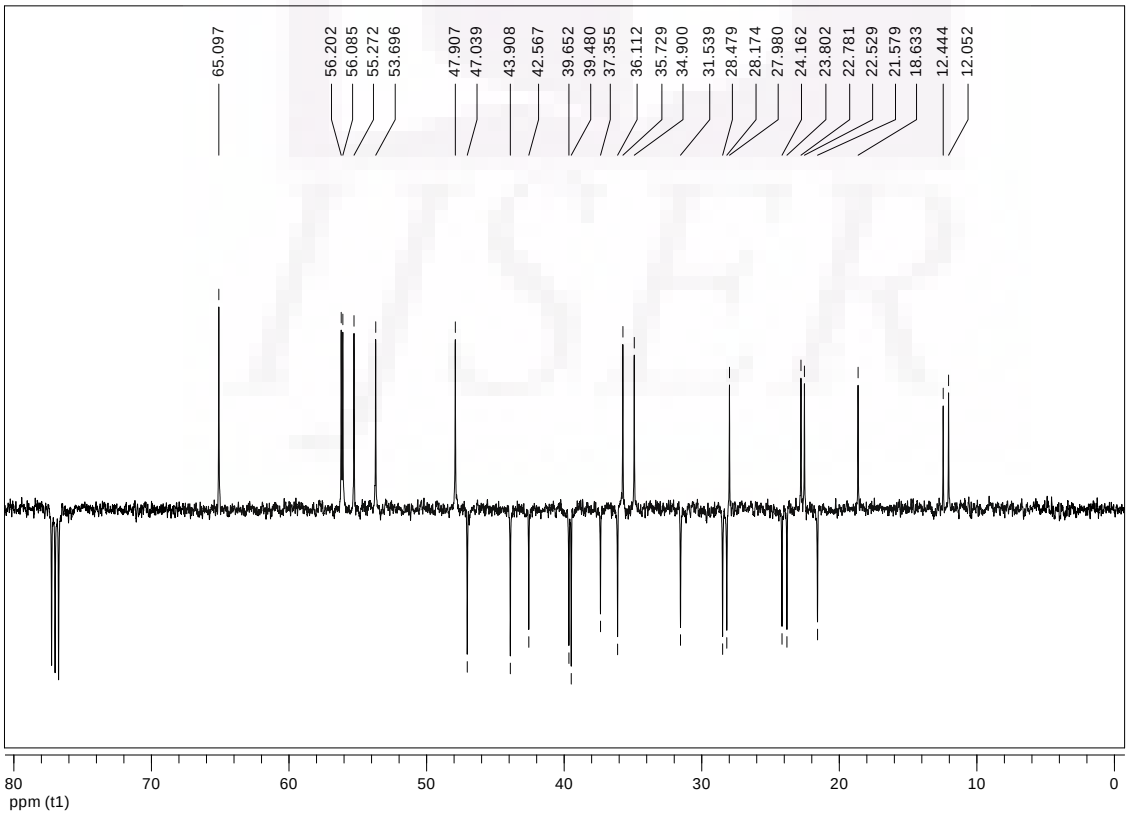

Figure. 19

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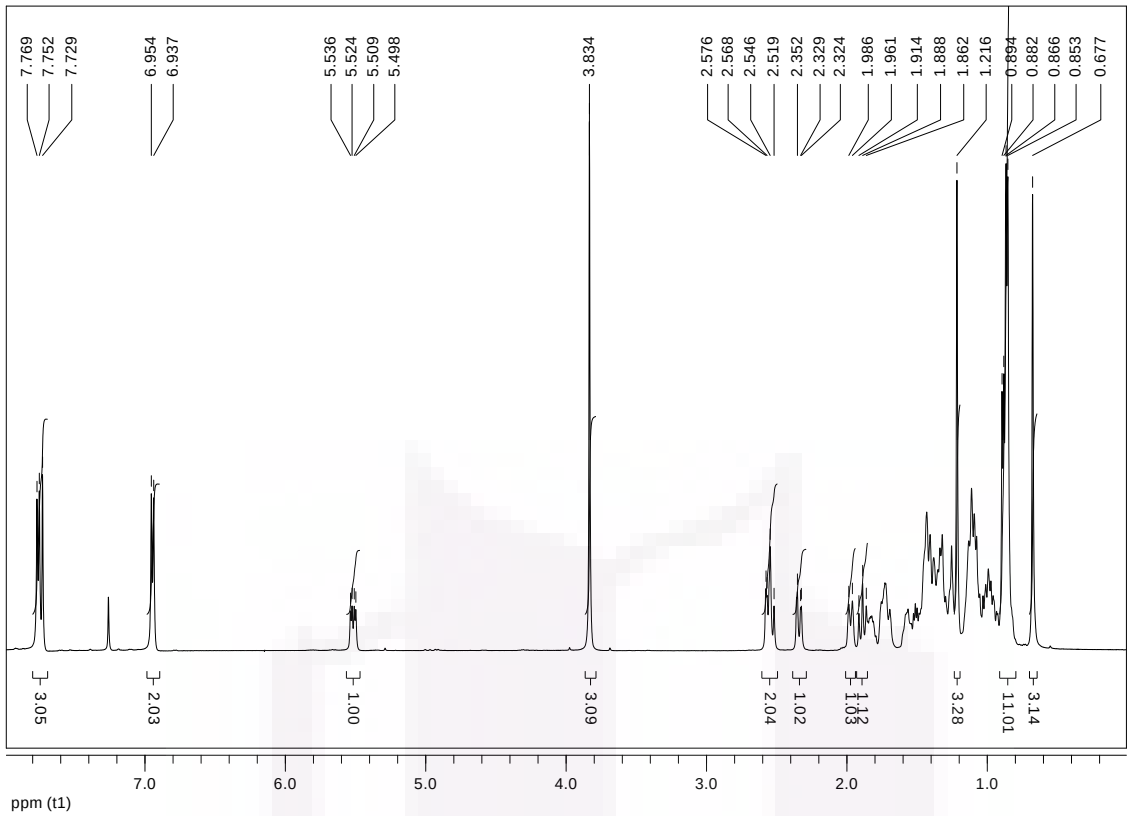

20.

Figure. 20

The Figure 20 .shows 1 H-NMR spectrum 2α-[4 - (4-methoxyphenyl)-1H-1 ,2,3-triazol-1-yl]-5α-cholestane-3-one of Fig. 3.83 ppm clearly visible on the aromatic methoxy group present, and appears at 5.5 ppm for the ring atoms of the C 2-proton, or 6.9 ppm two parts 7 and 7.7 ppm three-proton signal. This chemical shift value aa-triazole ring protons are there only appears where the incorporated aromatic ring protons of 2, hence the integral value here.

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Synthesis steroidal compounds with helping the microwave apparatus in solvent free for antimicrobial activity. [76]

2nd experiment :

We measured 5.968 gram from A1 steroidal compound and mix with half milimole of POCl3 and DMF (dimethyl formamide) and put in the microwave CEM Discovery SP and set the temperature in 140 C and for time interval 10 minute , and after synthesis we did TLC and we saw 2 different compounds (A and B) and with helping of column chromatography we sperated them, A compound was major and B compound was minor and we measured A compound and it was 3.925 g .

For TLC we used silica gel 60-F254 and result , and we used eluent dichloromethane and hexane in ratio 3 : 1

After TLC and recrystallization we reacted 0.5 mmole (0.166 g) of compound A which we synthesize it with 1 mmole (0.127 g) of p-chloroaniline in CEM microwave apparatus , Duration is 10 minute at 150 C and after that we did again TLC , but we did TLC twice with different eluents and we used column chromatography for purification and we recrystallized our compound . now our compound is ready to analysis with NMR and etc .

3rd experiment :[76]

In 3rd experiment we used A compound from last experiment and we react it with phenyl hydrazine in microwave and we measured 0.17 gram and 74 microliter of phenyl hydrazine and put in Microwave and set temperature in 150 and time in 10 minute. Then after synthesis we did TLC and we predicated our compound with column chromatography. And eluent was hexane and diisopropyl ether in ratio 9:1 , and after purification we recrystallized our compound and now it is ready to analysis with NMR and etc.

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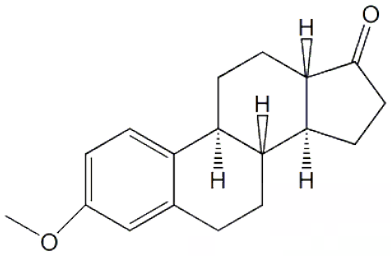
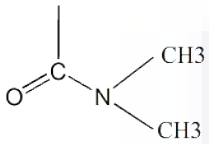
2nd experiment .[76]

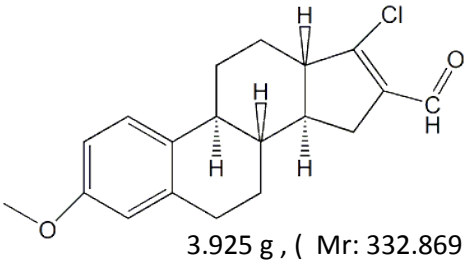
A1



5.968 g , (Mr: 284.14 g/mol)



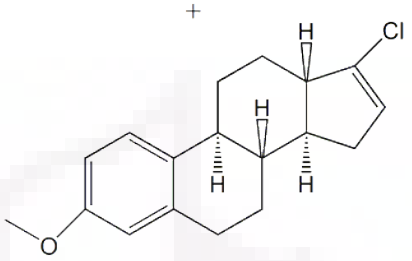
+ DMF



A

3.925 g , (Mr: 332.869 g/mol)

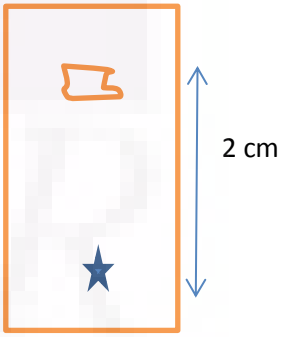
+



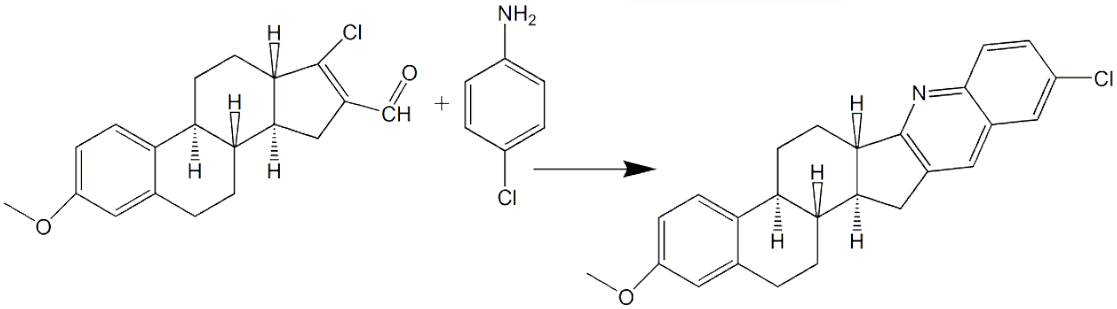
B

Mr: 302.843 g/mol

Then we did TLC with silica gel 60 – F254 and result is:



in dichloromethane + hexane (3 : 1)



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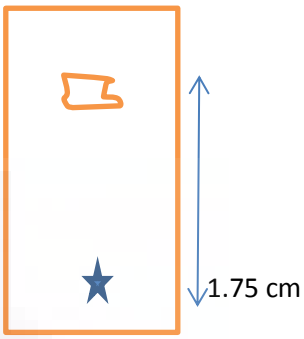
After TLC and recrystallization we reacted 0.5 mmole (0.166 g) of compound A which we synthesize it with 1 mmole (0.127 g) of p-chloroaniline in CME microwave apparatus , Duration is 10 minute at 150 C and after that we did again TLC.

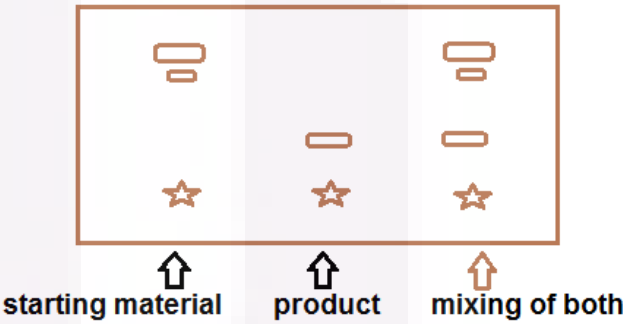
Then we did TLC with silica gel 60 – F254 and result is:

in dichloromethane + hexane (3 : 1)



We repeat TLC with starting material and product :

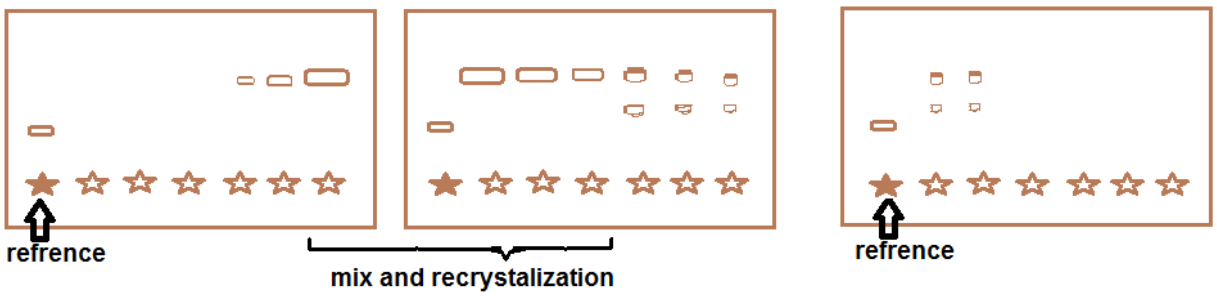
In diisopropyl ether + hexane (1:1)



After this TLC we did column chromatography , and we did TLC for every beaker and reference solution (first spot from each TLC)

The TLC result of column chromatography :

In diisopropyl ether + hexane (1:1)



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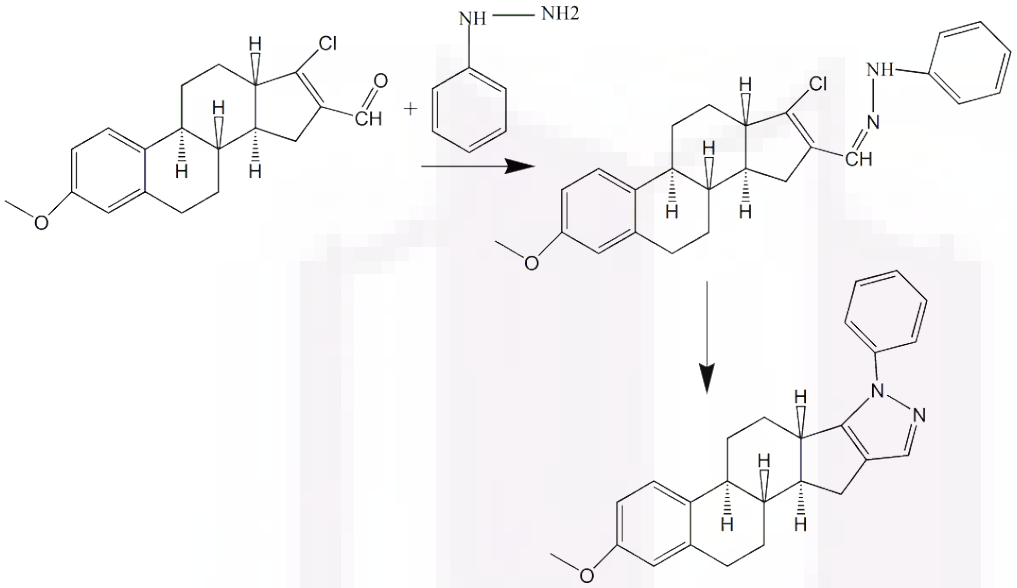
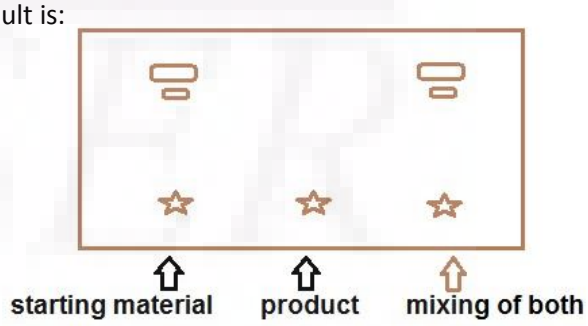
After recrystallization we have pure compound and now after these process we can analysis our compound with NMR and etc.

3rd experiment .[76]



Then we did TLC with silica gel 60 – F254 and result is:

In Diisopropyl ether + hexane (9:1)



The TLC result of column chromatography :

In Diisopropyl ether + hexane (1:1)

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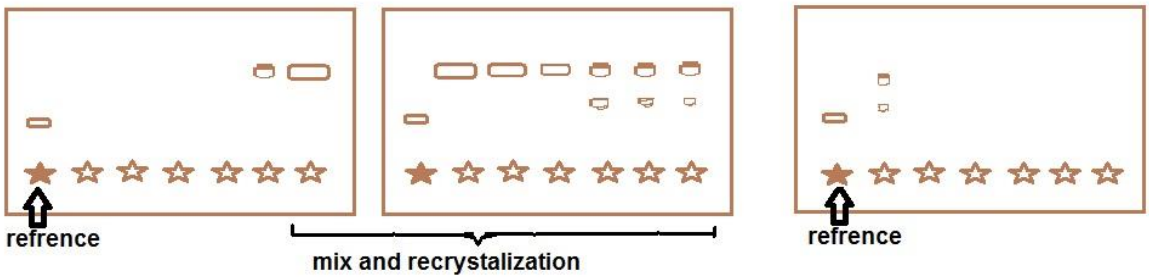

After recrystallization we have pure compound and now after these process we can analysis our compound with NMR and etc

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