DOCKING STUDIES ON SELECTED SAPOGENINS TARGETING ANDROGEN AND ESTROGEN RECEPTORS

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ABSTRACT

DOCKING STUDIES ON SELECTED SAPOGENINS TARGETING ANDROGEN AND ESTROGEN RECEPTORS

Natural products have been used in the treatment of various diseases in history, and they are still in use today. Sapogenins are natural products derived from plant and animal sources that also have numerous biological activities. Furthermore, some sapogenins have been found to be active in common cancers such as prostate and estrogen alpha-mediated breast cancer and exert their effects via the androgen and estrogen receptors. For this reason, identifying alternative ligands for these receptors may aid in enhancing the benefits or avoiding adverse effects. Traditional or advanced molecular screening techniques are available with their respective applications. However, these applications have some limitations, such as being complicated and costly or requiring a significant amount of time due to the large number of molecules involved. With advancements in technology, in-silico methods such as molecular docking have developed into a highly accurate and cost-effective method for high throughput screening. Additionally, rapid and high-quality in-silico visualization of docked ligands and their interactions serves as a preliminary step toward determining structure-activity relationships. The molecular docking method was used in this study to identify novel androgen and estrogen receptor ligands, and to evaluate the structure-activity relationship of sapogenin molecules, which were selected from our research group's molecule library. Moreover, the Swiss Target Prediction web service was used to determine the probability of bindings prior to molecular docking. The molecular docking results demonstrated that nine of the selected sapogenins were more bindable to the androgen receptor than testosterone, whereas another nine were found to be more bindable to the estrogen receptor than estradiol. Additionally, immunoblotting was utilized to validate the activity of several molecules by examining their effects on PSA levels for androgen receptor binding.

ÖZET

ANDROJEN VE ÖSTROJEN RESEPTÖRLERİNİ HEDEFLEYEN SEÇİLMİŞ SAPOGENİNLER ÜZERİNDE DOKİNG ÇALIŞMALARI

Doğal ürünler tarih boyunca çeşitli hastalıkların tedavisinde kullanılmış ve günümüzde de kullanılmaktadır. Sapogeninler, aynı zamanda çok sayıda biyolojik aktiviteye sahip olan bitki ve hayvan kaynaklarından elde edilen doğal ürünlerdir. Ayrıca, bazı sapogeninlerin prostat ve östrojen alfa aracılı meme kanseri gibi yaygın kanserlerde aktif oldukları ve etkilerini androjen ve östrojen reseptörleri aracılığıyla uyguladıkları bulunmuştur. Bu nedenle, bu reseptörler için alternatif ligandların belirlenmesi, faydaların arttırılmasına veya olumsuz etkilerden kaçınılmasına yardımcı olabilir. Geleneksel veya gelişmiş moleküler tarama teknikleri, ilgili uygulamalarıyla birlikte mevcuttur. Bununla birlikte, bu uygulamaların karmaşık ve maliyetli olması veya çok sayıda molekül içermesi nedeniyle önemli miktarda zaman gerektirmesi gibi bazı sınırlamaları vardır. Teknolojideki gelişmelerle birlikte, moleküler yerleştirme gibi in-siliko yöntemler, yüksek verimli tarama için son derece doğru ve uygun maliyetli bir yönteme dönüşmüştür. Ek olarak, kenetlenmiş ligandların ve bunların etkileşimlerinin hızlı ve yüksek kaliteli in-siliko görselleştirilmesi, yapı-aktivite ilişkilerinin belirlenmesine yönelik bir ön adım olarak hizmet eder. Bu çalışmada, araştırma grubumuzun molekül kütüphanesinden seçilen yeni androjen ve östrojen reseptör ligandlarını belirlemek ve sapogenin moleküllerinin yapı-aktivite ilişkisini değerlendirmek için moleküler docking yöntemi kullanılmıştır. Ayrıca, moleküler yerleştirmeden önce bağlanma olasılığını belirlemek için Swiss Target Prediction web hizmeti kullanılmıştır. Moleküler yerleştirme sonuçları, seçilen sapogeninlerden dokuzunun androjen reseptörüne testosterondan daha fazla bağlanabildiğini, diğer dokuzunun ise östrojen reseptörüne estradiolden daha fazla bağlanabildiğini gösterdi. Ek olarak, androjen reseptörü bağlanması için PSA seviyeleri üzerindeki etkilerini inceleyerek birkaç molekülün aktivitesini doğrulamak için immünoblotlama kullanılmıştır.

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CHAPTER 1

INTRODUCTION

1.1. Natural Products

Natural products or secondary metabolites are organic compounds produced by living organisms or cells, which are not crucial for reproduction, growth, and development of living organisms, but provide advantages for their survival (Puglisi 2004; Williams, Foye, and Lemke 2002). It has been known that secondary metabolism has important role on sustaining the cell viability under environmental stress conditions, toxic or waste products, etc. (Monfil and Casas-Flores 2014).

Today, most of the isolated secondary metabolites are still screened for their potential as drug candidates, and their synthetic analogs are produced to improve their efficacy against various diseases (Koehn and Carter 2005).

1.1.1. Natural Products as Drug Molecules

In the history, secondary metabolites have been used for centuries in medicinal applications since they have curative and/or preventive effects on a wide range of diseases. The first records of natural products used for medicinal purposes are back to 2600 B.C., since then, oils of *Cedrus sp.*, *Papaver somniferum*, *Cupressus sempervinens*, and *Commiphora sp.* are still used for the treatment of cough and flu. In 1100 B.C. Egyptian Ebers Papyrus introduced 700 drugs to the medicinal era, including preparations obtained from plants. Chinese Medica Materia has also brought numerous medicinal plants to the literature in the same century. In 100 A.D., the Greek philosopher Dioscorides has recorded his knowledge of medicinal plants where he learned while travelling with Roman armies. Between the 5^{th} and 12^{th} centuries, the knowledge about medicinal plants was kept in the monasteries of France, Germany and England; however,

the society that preserve this Greco-Roman knowledge and that established the first pharmacies were Arabs (Cragg and Newman 2005a).

At the beginning of 19th century, morphine and aspirin, the two of very well-known examples for plant derived drugs, were obtained from the plant *Papaver somniferum* and *Salix alba*, respectively. With the groundbreaking discovery of penicillins, a group of antibiotics obtained from the fungus *Penicillium notatum*, by Alexander Fleming, the importance of natural products has been shown once more. Howard Florey and Ernst Boris, who were awarded with Nobel Prize in 1945 together with Alexander Fleming Chain, demonstrated the antibacterial effects of penicillin, and developed its production methods (Dias, Urban, and Roessner 2012).

The structural and chemical diversity and evolutionary superiority of natural products enables them to be the best drug candidates for screening novel drugs compared to the synthetic molecules. At the end of the 20th century, based on the huge demand for an effective and time saving method with lower cost instead of classical screening methods, many pharmaceutical companies have shifted their efforts to combinatorial chemistry approaches, which provide the high-intensity, high-throughput screening of secondary metabolite libraries. However, this method requires high-cost automated systems and big databases (Lahana 1999; Li and Vederas 2009).

The advancement of technology has made it easier to manage large amounts of data over time. This situation has facilitated the advancement of screening studies by lowering the costs associated with them (B. Liu, Li, and Hu 2004). The review study of Newman and Cragg (2020) reports 1881 drug molecules discovered between 1 January 1981 – 30 September 2019. From the drug molecules, 71 are natural products, 356 are the molecules derived from natural products, and 217 are synthetic molecules based on natural products. Figure 1 summarizes the findings of that study.

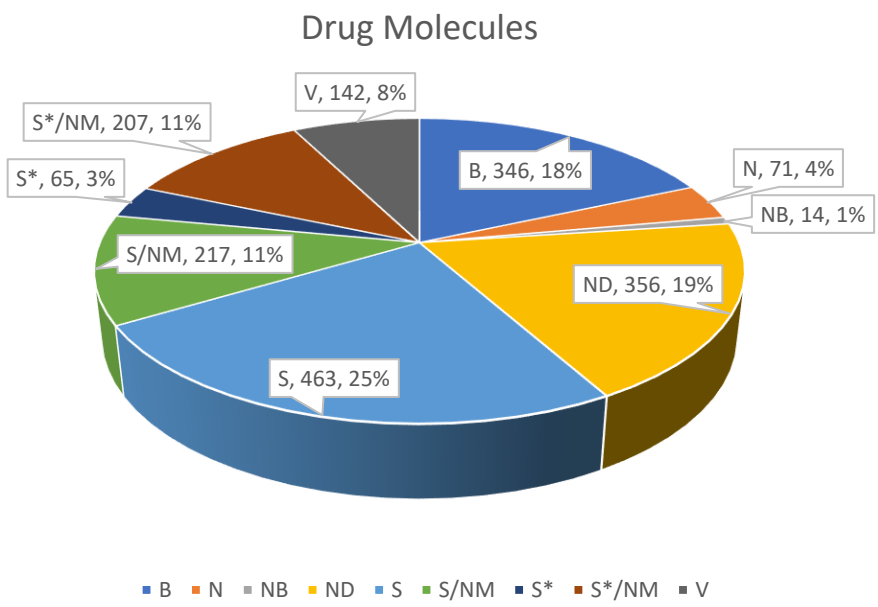


Figure 1. Drug molecules found between 01/1981 – 09/2019 – B: Biological Macromolecule, N: Unaltered Natural Products, NB: Botanical Drug (Defined Mixture), ND: Natural Product Derivative, S: Synthetic Drug, S*: Synthetic Drug (NP Derivative), /NM: Mimic of Natural Product, V: Vaccine (Newman and Cragg 2020).

As mentioned previously, natural products have played a crucial role in medicinal era over the history, and they are still being exploited for the treatment of various diseases (Cragg & Newman, 2005b; Jonathan L. Hartwell, 1982). Up to date, more than 60% of anticancer drugs originate from natural sources such as plants, fungi, marine organisms and microorganisms , which are still in use (Cragg and Newman 2005b; Newman, Cragg, and Snader 2003). Natural products, without a doubt, will continue to play a significant role in drug discovery and development.

1.2. Androgen Receptor

Development of reproductive system and secondary sexual characteristics are dependent to male sex hormones known as androgens such as dihydrotestosterone and testosterone (MacLean et al. 1993). The actions of these sex hormones are controlled via

the ligand-dependent nuclear transcription factor, androgen receptor, which is a member of the family of steroid hormone nuclear receptors (Chang et al. 1995). Rather than its roles in the development of the reproductive system and secondary sexual characteristics, androgen receptors play a role in the development of cardiovascular, immune, and musculoskeletal systems (Rana, Davey, and Zajac 2014). As well as other nuclear receptors, the androgen receptor contains three domains: the N-terminal domain, DNA-binding domain, and ligand-binding domain. N-terminal domain, which is the least conserved region, is the first exon that codes the transcriptional regulatory region of the protein. The transactivation of the androgen receptor is operated by this domain. Moreover, a critical region for gene transcription known as activation function 1 region is located on this domain. The second and third exons are known as DNA-binding domain, which is a highly conserved region and recognizes specific DNA sequences and operates binding to chromatin by two zinc-finger motifs. Lastly, the exon 4-8 belongs to the ligand-binding domain, mediating the interaction with 5-alpha-dihydrotestosterone and heat shock proteins (Bruchovsky et al. 2000; Grino, Griffin, and Wilson 1990; Davey and Grossmann 2016; He et al. 1999; Tyagi et al. 2000). The structure of androgen receptor was shown in Figure 2.

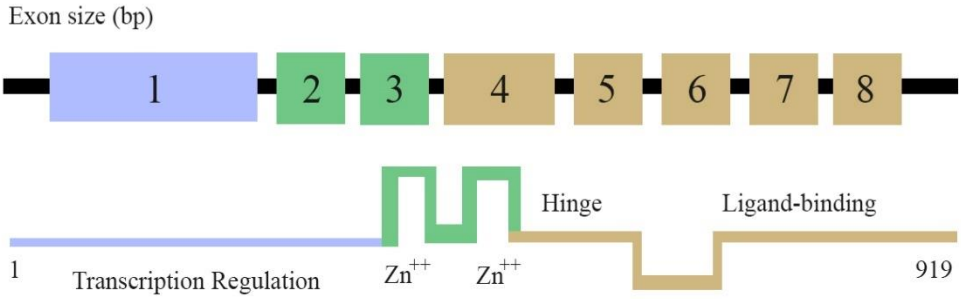


Figure 2. Structure of Androgen Receptor (Quigley et al. 1995).

Androgen receptor is known to play a role in various diseases such as cancer. The most frequently diagnosed cancer and a major cause of cancer-related death in men is prostate cancer. In 2018, approximately 29.400 deaths and more than 160.000 cases of prostate cancer were reported (“Prostate Cancer: Statistics | Cancer.Net” n.d.). A transcription program is initiated with the activation of the androgen receptor that drives

the proliferation of the cancer. Therefore inhibition of the androgen receptor is a standard cure for the patients with prostate cancer (Agoulnik and Weigel 2006). Without ligand binding, the androgen receptor (AR) is predominantly found in the cytoplasm, where it associates with heat shock proteins, which are thought to tether the AR to cytoskeletal proteins and modulate its conformation in preparation for efficient ligand binding. AR signaling is initiated when androgens cross the plasma membrane, enter the cytoplasm, and bind to the AR, resulting in the dissociation of chaperone proteins and translocation of the complex to the nucleus, where it dimerizes and binds to the androgen response element, modulating gene transcription and, ultimately, protein synthesis. As a result, prostate specific antigen is expressed (J and GA 2004).

1.2.1. Androgens

Androgens, which are steroid-based hormones, are found both in male and female reproductive systems. Furthermore, androgens are responsible for sex differentiation, development of external genital organs and secondary sex character (hair growth, voice thickening, libido, erection, skin thickening, and activity of sebaceous glands), and spermatogenesis (Pawlina and Ross 2020; Kierszenbaum 2006). Apart from this, hematopoiesis, protein production, bone formation, lipid, and carbohydrate metabolism are known metabolic activities affected by androgens (Froesch, Takayama, and Reed 1998; Palacios et al. 1983; Turner, Wakley, and Hannon 1990; Nishizawa et al. 2002). The main androgen known as testosterone that is produced in Leyding cells (95%) of testis and adrenal glands (5%), is synthesized by the metabolic pathway gamma-5 (Debes and Tindall 2002). The free testosterone molecule enters the cytoplasm by passing through the cell membrane through passive diffusion and stays in the cytoplasm of the target cell or is converted into dihydrotestosterone by the 5α-reductase enzyme. The structure of testosterone and dihydrotestosterone was given in Figure 3.

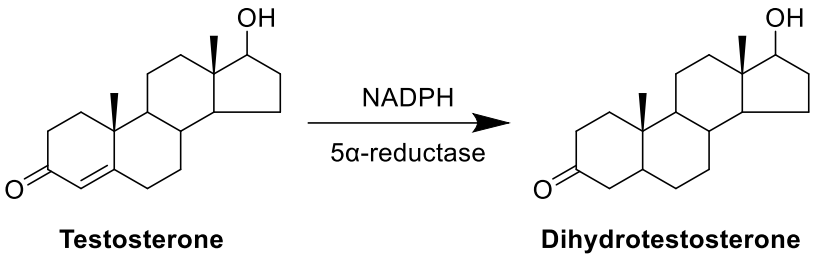


Figure 3. Structures of testosterone and dihydrotestosterone, and conversion scheme (Avendaño and Menéndez 2015).

The binding act of hormone to the receptor take place in nucleus or cytoplasm. Conformational changes on the androgen receptor occur due to this binding act. This conformational change facilitates the receptor's binding to the DNA segments located in the "promoter" and "enhancer" regions on the androgen receptor gene. With the other transcription factors, the androgen receptor regulates mRNA expression and creates a specific androgen response as a result of related protein synthesis (Wickham et al. 2000).

1.3. Estrogen Receptor

Breast cancer is highly diagnosed in women globally, with an incidence ratio of 125 to 1 in women to men (Ly et al. 2013). One of the reasons for it is hormonal factors known as steroid hormones that increase the risk of getting the disease due to its excess levels. As a result of excess levels of steroid hormones, stimulation of estrogen receptor alpha (ERα) occurs (Shyamala 1997; Chlebowski et al. 2003). Estrogen receptors (ERs) are activated by the binding of ligands known as estrogen. For this reason, estrogen receptors are also known as ligand-activated transcription factors. Estrogen receptors are classified as type 1 nuclear receptors due to generally found in the cytoplasm and move into the nucleus upon estrogen or ligand binding (Leung et al. 2006). ERα and ERβ are the two types of estrogen receptors (Jordan 2003; Greene et al. 1986; Kuiper et al. 1996; Hewitt and Korach 2002). ERα and ERβ are 90% structurally homological, whereas 53% of the amino acid identity of their ligand-binding domain is the same, which suggests that they recognize and bind to similar estrogen recognition element on DNA (Weihua et al. 2003). It has been shown that both receptors displayed similar binding affinities against

estradiol experimentally (Kuiper et al. 1998). The ERα that is expressed by tissues such as testis, mammary gland, kidney, bone and central nervous system is mainly found in breast, uterus and ovary (Cordera and Jordan 2006; Koehler et al. 2005). Furthermore, ERα has a critical role in hormone-related cancers such as breast cancer. Therefore, the discovery of the new ligands may affect the role of this receptor in such diseases (Y. Liu, Ma, and Yao 2020).

1.3.1. Estrogens

A nuclear hormone family member of steroidal compounds, estrogens, has a crucial role in a woman's body in reproductive endocrinology and also important for maintaining homeostasis (Jordan 2003). The most common estrogens found in the human body are estradiol, estrone and estriol. Figure 4 shows structures of common estrogen molecules.

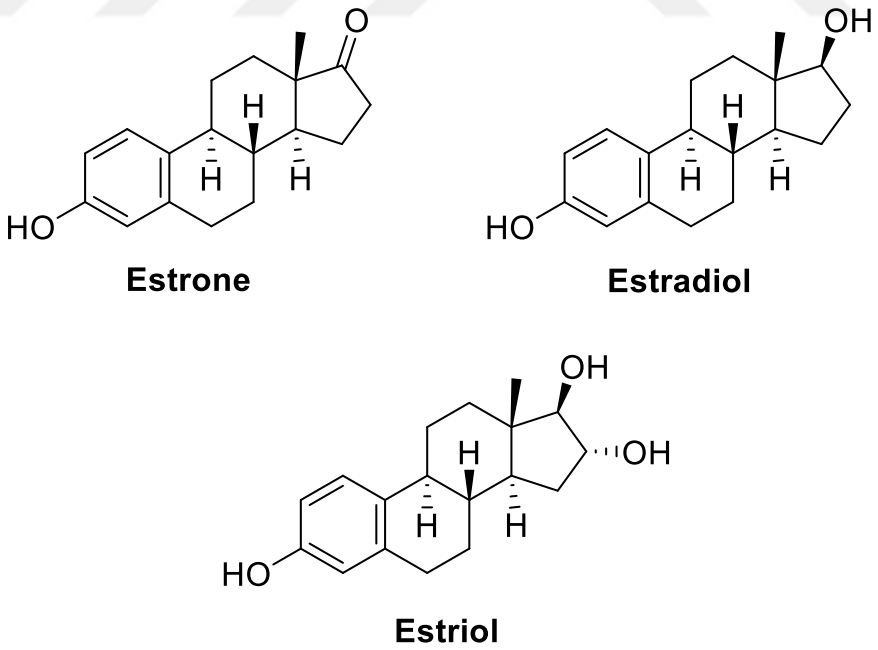


Figure 4. Structures of common estrogen molecules (Pauwels et al. 2008).

The main sources of estrogens are the ovaries and testis (Enmark and Gustafsson 1999). In contrast, other tissues such as the brain and bone can also synthesize them via the reaction of aromatase enzymes with androgens (Korach, Taki, and Sean Kimbro 1997). The effects of estrogens are mediated through estrogen receptors which are known as ligand-activated transcription factors. To treat postmenopausal symptoms by estrogen replacing therapy and understanding oral contraceptives, understanding the biological effects of estrogens are significant (Drill 1977; Mazhar Uddin et al. 2018). Throughout the menopausal years, hormone replacement therapy applied for the prevention and treatment of osteoporosis has shown to be very beneficial (Kearney and Purdie 1998). In contrast, estrogen replacement therapy has shown to increase the risk of breast cancer (Calle et al. 1997). As a result of these findings, alternative treatments for breast cancer such as antiestrogens and selective estrogen receptor modulators were developed.

1.4. Sapogenins

Sapogenins are the molecules obtained from both plant and animal sources and belong to the family of compounds known as steroids. The general structures of steroids contain 17 carbon atoms arranged in 4 rings shown in Figure 5.

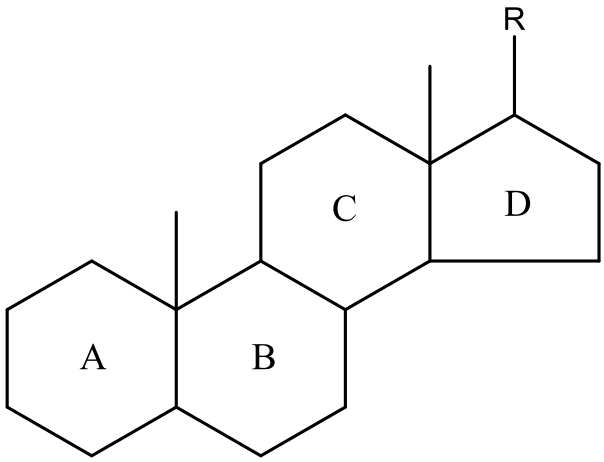


Figure 5: Structure of steroid skeleton (Mensah-Nyagan et al. 2009).

Sapogenins are mostly found in plants as glycosides, the saponins. This group of molecules foams in aqueous solutions due to the ability to lower the surface tension of water. Furthermore, saponins can form complexes with cholesterol and shows hemolytic activity, and toxicicity against cold-blooded animals (Wiechert 1971; Zerbo 2003). Based on the aglycone structure, saponins can be subdivided into two groups, steroidal and triterpene saponins, known to be derived from oxidosqualene. By the loss of 3 methyl groups, steroidal saponins contain 27 carbon atoms while triterpene saponins possesses 30 carbon framework intact (Vincken et al. 2007). Cholestane saponins, furostane saponins and spirostane saponins are the types of steroidal saponins, shown in Figure 6.

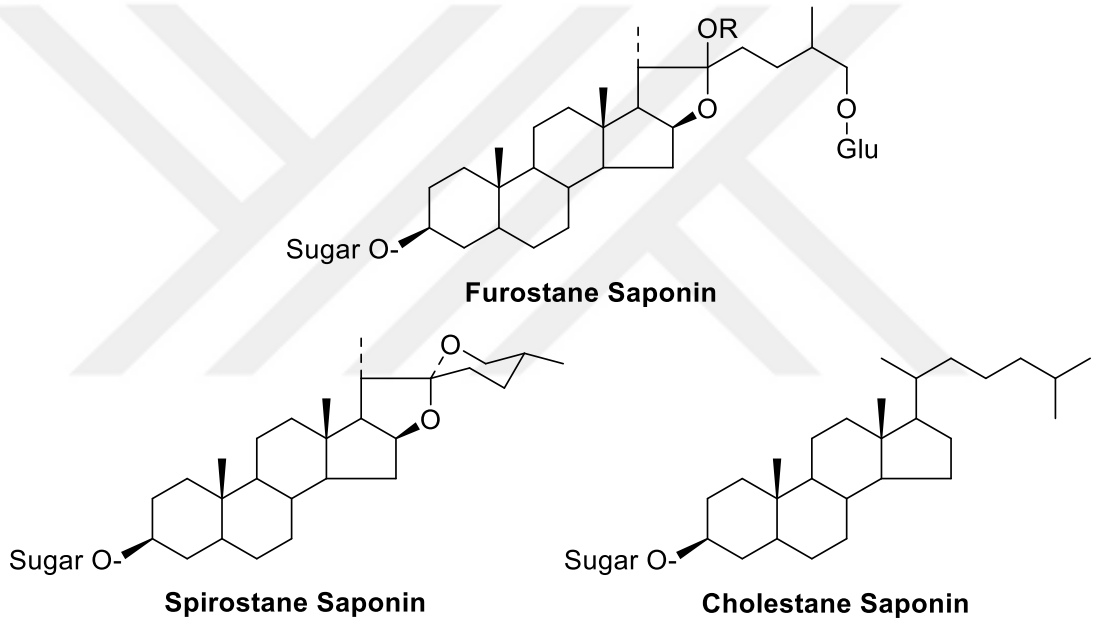


Figure 6. Structures of steroidal saponin types (Juang and Liang 2020).

Dammaranes, lupanes, hopanes, oleananes, cycloartanes, lanostanes, cucurbitanes, taraxasteranes, ursanes, tirucallanes, and steroidals are the distinguished classes of saponins. Oleananes, cycloartanes, dammaranes, lupanes and steroids are the classes which have showed strong antitumor effects on many types of cancer (Man et al. 2010). Additionally, neuroprotective, immunomodulatory, hypoglycemic, anti-inflammatory, antifungal and anticancer activities of saponins were shown in the

literature (Podolak, Galanty, and Sobolewska 2010). The semi-synthetic derivatization method can be used to enhance the anti-cancer activity of saponins and investigate the structure-activity relationship (SAR) (Man et al. 2010).

Structures of oleananes, cycloartanes, dammaranes and lupanes were illustrated in Figure 7.

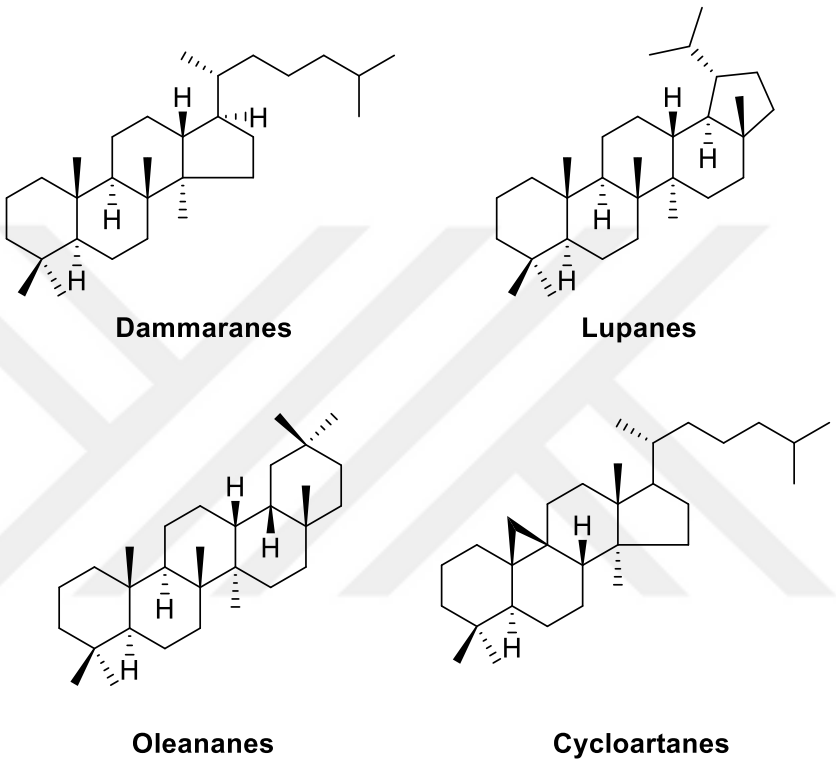


Figure 7. Structures of dammaranes, lupanes, oleananes and cycloartanes (Vincken et al. 2007).

One of the cycloartane-type saponin known as astragaloside IV has shown therapeutic effects versus prostate cancer (Tan et al. 2018). Furthermore, other cycloartanes known as astragaloside IV and cycloastragenol have been shown to inhibit ROS-associated endoplasmic reticulum stress causing the activation of cell death and known to be related to cancer (Yadav et al. 2014; Tan et al. 2018). In the preliminary studies done by our group, a sapogenin molecule, namely 20(27)-octanor cycloastragenol, has been found to show activity on androgen receptor.

1.5. Molecular Docking

Computer-aided drug design methodologies play a great role in drug discovery. These in-silico methods have benefits such as limiting the need for animal models, aiding the rational design of novel and safe drug candidates, cost-effective identification and timesaving (Brogi et al. 2020). One of the in-silico methods that has been used to evaluate the potentials of virtual drug candidates by the optimization and assessing structure is known as molecular docking. The active site of receptor is screened from the view of optimal binding mode of the ligand by the usage of algorithms in this method. The main aim of this method is to reveal the potential of drug candidate which binds to given receptor stronger than its natural ligand, and results in the alteration of the biochemical process (Thomsen and Christensen 2006).

The schematic representation of docking process has been showed in Figure 8.

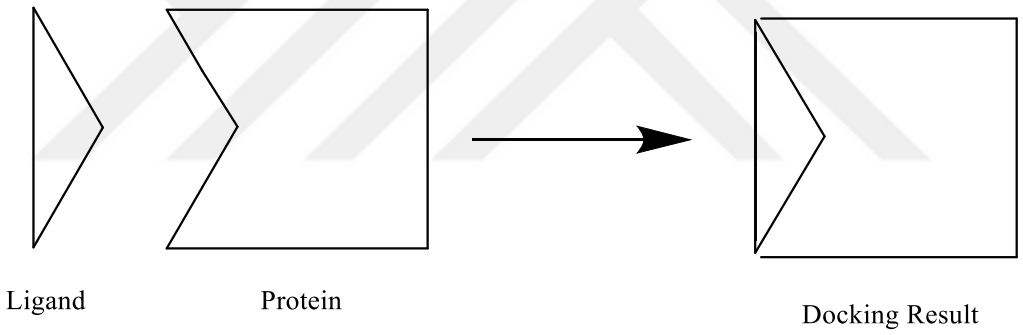


Figure 8. Schematic representation of docking process.

The usage of this method offers a faster and cheaper identification of potent drug molecules for immediate clinical trials, synthesis, and toxicological studies. Potent drug molecules are identified by the energy-based scoring function that results with the most stable receptor and ligand conformation. The lower the score, the more potent drug candidates are identified. To summarize, the molecular docking can be defined as a method that optimizes the problem during the ligand-binding process at the lowest energy score (Huang and Zou 2010). By the application of the docking algorithms which create a conformational degree of freedom by the introduction of small ligands to the receptor's

active site, the process starts. The biological activity is predicted by the scoring functions through the interrelations between the ligands and the receptors (Kitchen et al. 2004).

1.5.1. Protein - Ligand Docking

The user-defined binding site known as the grid box is the initial step of the protein-ligand docking process. By identifying the locations on the grid box, that ligand can bound to the protein, the process begins. The major problem in this process is the conformational diversity of the ligands. However, ligand docking programs like iGEMDOCK are useful for getting over these problems. There are five main modules used to construct iGEMDOCK. Mod_cav, mod_lig and mod_ga are the docking/screening module components, whereas mod_ac and mod_kc are the components of post analyzing module. When a ligand bounded PDB file is introduced to the software, mod_cav generates the binding site from the ligand bonded to the protein. Introduced ligands are processed and prepared for the docking process by the mod_lig, and the docking algorithms are used to predict the bounded poses of the protein-ligand complex by mod_ga. Each docked poses atom composition is analyzed by mod_ac, and docked poses are clustered by the interaction with protein by the mod_kc component. The main advantage of using iGEMDOCK as docking software is the graphical interface and fully automized, easy use architecture. In contrast, most of the docking software require complex coding skills and the knowledge of bash system. Furthermore, iGEMDOCK software is tested on the screening sets of estrogen receptor alpha (PDB entry: 3ERT) on ten known antagonists and 990 random selected compounds by comparing with four other accurate docking software, and the true positive rate of results was 100% (J.-M. Yang and Shen 2005). As scoring function iGEMDOCK uses GEMDOCK scoring function which is an empirical scoring function and based on evolutionary approach that is more robust than standard evolutionary approaches (Bäck 1996; Fogel 1995; Goldberg and Holland 1988; J. M. Yang and Chen 2004).

As our research group has isolated a number of triterpenic saponins and modified them via semi-synthesis/biotransformation to form a chemical library, a screening study is needed to assess the drug potential of these molecules, some of which are quite similar structurally to steroidal hormones. The aim of this thesis is to discover novel androgen

and estrogen receptor ligands and to evaluate the structure-activity relationship and binding potential of selected sapogenin molecules from the aforementioned library.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The materials used in this thesis are listed below.

2.1.1. Workstation

The workstation with AMD Ryzen 7 3750H CPU @ 2.30GHz processor, NVIDIA GeForce RTX 2060 graphics card, and 24 Gb Ram was used in molecular docking and analysing studies conducted within the scope of the thesis. The same workstation was used to screen and compile protein and molecule libraries, prepare the molecular structures for docking simulations, compile docking simulation results, and obtain data.

2.1.2. Databases Utilized Within the Scope of the Thesis

RCSB Protein Data Bank (PDB): RCSB PDB is a database that contains 3D structures of proteins and molecules (Burley et al. 2019). The structure files of proteins and the crystal structure of the ligands that are bound to the proteins used in this thesis were obtained from RCSB Protein Data Bank.

Swiss Institute of Bioinformatics - SwissTargetPrediction: SwissTargetPrediction is a web service that aims to predict the most likely protein targets for small ligands (Daina, Michielin, and Zoete 2019). All of the ligands were evaluated in SwissTargetPrediction webtool. The predictions with high scores obtained from this webtool was used, while deciding the target protein receptors.

2.1.3. Softwares Utilized Within the Scope of the Thesis

PyMol: An open-source software that enables the visualization of the 3D structures of molecules and proteins in-silico (Schrödinger, LLC 2015). The visualization of docked structures was provided by PyMol.

iGEMDOCK: A software with graphical interference that eases the preparation of ligands and proteins, virtual screening, and post-screening analysis of these from the point of pharmacological and chemical interactions (Hsu et al. 2011). The docking processes of the ligands were evaluated with iGEMDOCK. The assessments regarding to the scores obtained from this software were used as post-screening analysis.

2.1.4. Biological Studies

RPMI 1640 medium (BI), Fetal Bovine Serum (FBS) (Panbiotech), L-glutamine (Thermo Scientific), Ammonium persulfate (Amresco), SDS (Amresco), Mammalian Protease Inhibitor Cocktail (Promega), Sodium azide (Sigma-Aldrich) were purchased for bioactivity studies. Tween-20, Triton X-100, Nonidet P-40, Ponceau S Stain, glycerol, Tris, TEMED, 2-mercaptoethanol, Ethylenediaminetetraacetic acid (EDTA), Ethylenglycol Tetraacetic Acid (EGTA), sodium chloride, sodium phosphate monobasic anhydrate, sodium phosphate dibasic anhydride were purchased from Amresco.

Anti-PSA and Anti-GAPDH antibodies were purchased from Cell Signaling Technology

Buffers for immunoblotting:

a-) 10x PBS (phosphate buffered saline): 87.5 g sodium chloride, 11.5 g sodium monohydrogen phosphate and 2.3 g sodium dihydrogen phosphate was dissolved in 1 L distilled water.

b-) 2x RIPA: 40 mg SDS (sodium dodecyl sulfate) and 200 mg deoxycholic acid were dissolved in 7 ml 10xPBS containing 0.4 ml NP-40. Then the solution was completed with distilled water to 20 ml.

c-) Resolving Buffer: 1.5 M Tris HCl (90.855 g), 0.4% w/v TEMED (2 ml) and 0.4% a/h SDS (2 g) were dissolved in 500 ml distilled water, and pH of the solution was adjusted to 8.9.

d-) Stacking Buffer: 0.5 M Tris HCl (30.285 g), 0.4% w/v TEMED (2 ml), 0.4% a/h SDS (2 g) were dissolved in 500 ml distilled water, and pH of the solution was adjusted to 6.8.

e-) 10x SDS-PAGE Running Buffer: 30.2 g Tris Base, 144 g glycin and 10 g SDS were dissolved in 1 L distilled water.

f-) 10x SDS-PAGE Transfer Buffer: 30.33 g Tris base and 144 g glycin were dissolved in 1 L distilled water.

g-) Washing Buffer for SDS-PAGE: 100 ml 10x PBS was completed to 1 L with distilled water, and 1 ml Tween-20 was added.

2.2. Methods

The procedures followed in this thesis are listed below.

2.2.1. Ligand Preparation

For the preparation of ligands, all molecules were converted from 2D structure to 3D structure by the usage of ChemDraw and Chem3D software. The 2D structures were drawn manually by using the drawing tools located on the left menu in ChemDraw. Each structure was copied to the Chem3D software, and the 3D structure of each molecule were exported as .pdb file by clicking File → Export button from the menu. All of the molecules that are converted to 3D were visualized and double checked in PyMol software.

2.2.2. Swiss-Target Prediction

The SMILES of the molecules were obtained after drawing in ChemDraw software and copied into the text box found in "http://www.swisstargetprediction.ch/". Some of the molecules were redrawn by using ChemAxon's Marvin JS drawing service found on the right side of the SMILES text box. After drawing in the web service, the Predict Target button was clicked to generate the results of the search by selecting the species as *Homo sapiens* in the "Select a species" menu. Figure 9 represents the graphical interface of the Swiss-Target Prediction web service.

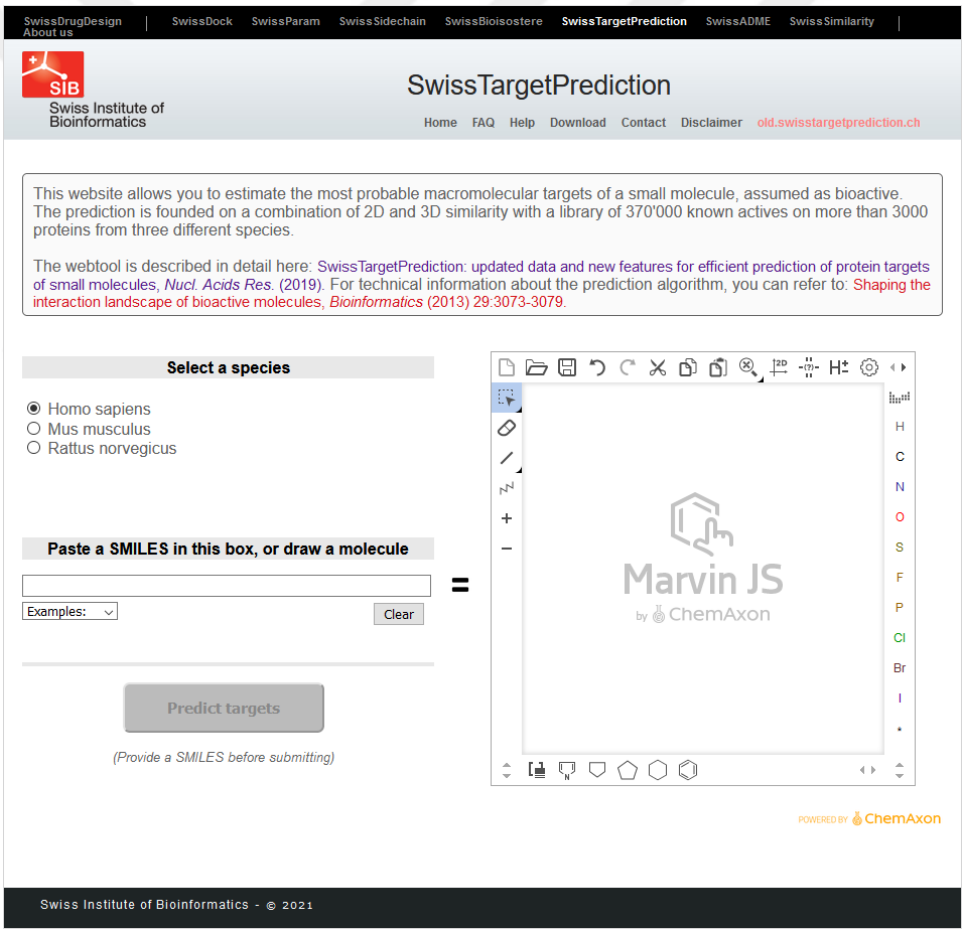


Figure 9. Graphical interface of Swiss-Target Prediction web service. ("SwissTargetPrediction" n.d.).

Results of the Swiss-Target Prediction system were saved as a screenshots file for each molecule, named the same as the related molecule.

2.2.3. Determination of Protein Structures to Be Used as Receptor

2PNU, 4HLW and 3B66 PDB files were selected as androgen receptor model from RCSB PDB. 4HLW model was previously bounded with the ligand testosterone, a steroid hormone, whereas 2PNU was bounded to ENM and 3B66 was bounded to molecule B66.

3D structures of 2PNU, 4HLW and 3B66 were given in Figures 10, 11 and 12.

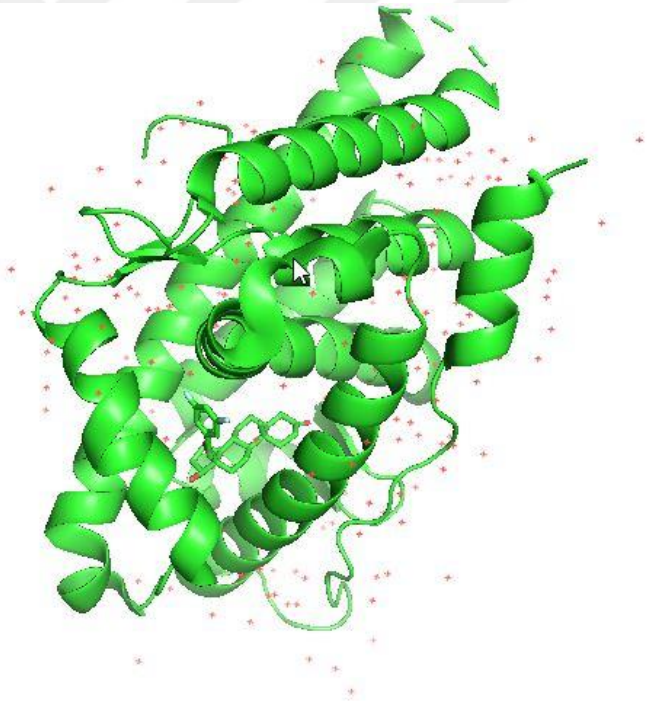


Figure 10. 3D Structure of model 2PNU complexed with small molecules and ENM.

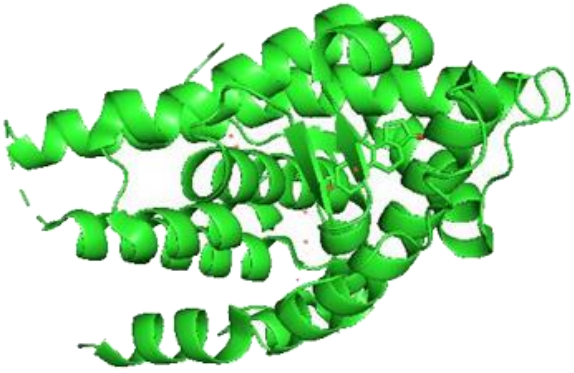


Figure 11. 3D Structure of model 4HLW complexed with small molecules and Testosterone.

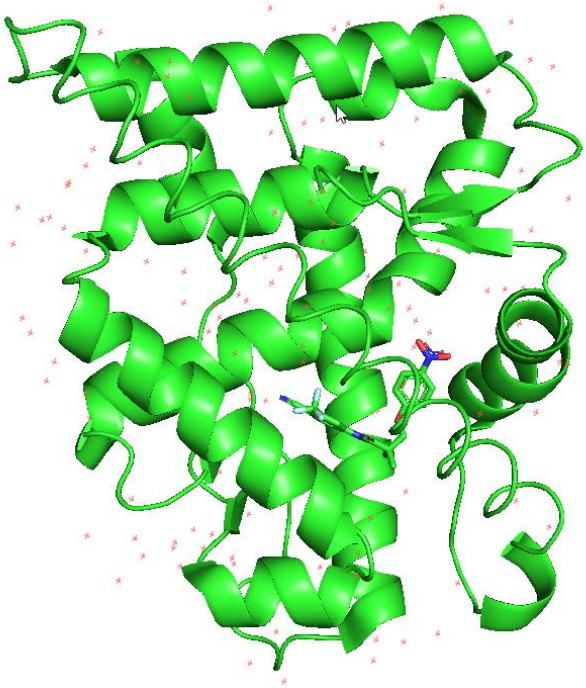


Figure 12. 3D Structure of model 3B66 complexed with B66.

The PDB file with the id 1A52 was selected as the model for estrogen receptor alpha, which was bound to estradiol (Figure 13).

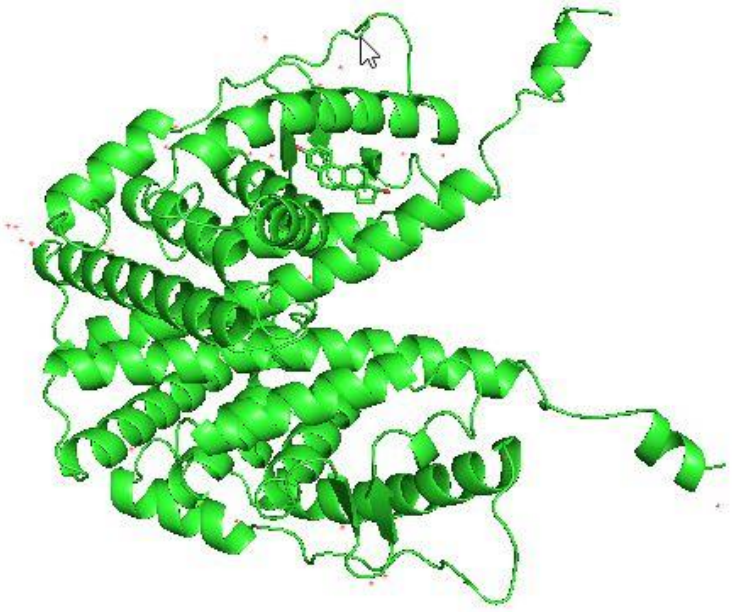


Figure 13. 3D Structure of model 1A52 complexed with Estradiol.

All PDB models and 3D models of the bounded ligands were downloaded from RCSB PDB.

2.2.4. Molecular Docking

iGEMDOCK software is used for molecular docking studies. Prepared ligands and receptors were arranged in folders. The configuration of the docking settings was done in the Protein-Ligand Docking/Screening section. After clicking the prepare binding site button in iGemdock software, the prepared protein .pdb file and binding site center is selected regarding to the bounded ligand. Prepared ligand PDB files were added by prepare compounds button, and the accurate docking option is selected as the default setting.

Screening and binding site preparation sections of the program were shown in Figure14.

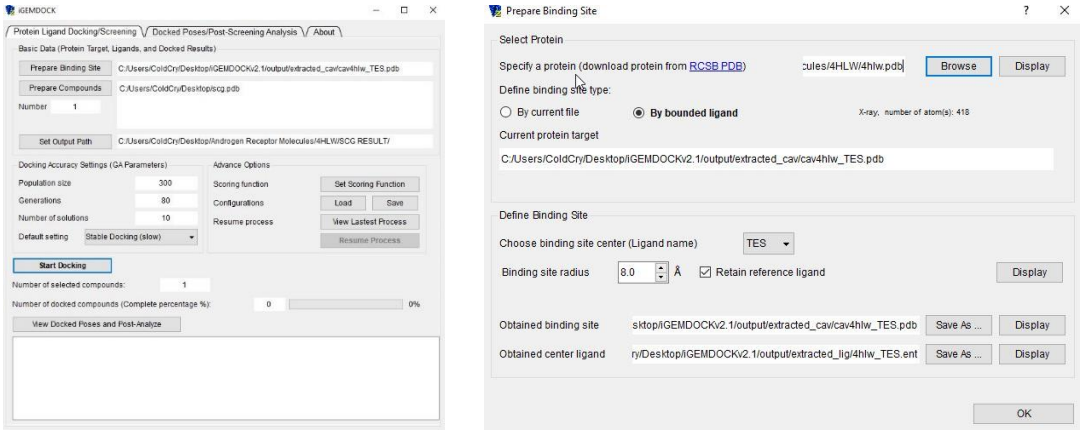


Figure 14. iGEMDOCK screening and binding site preparation section.

Docking results were exported as excel tables from the Docked Poses/Post-Screening Analysis section, shown in Figure 15.

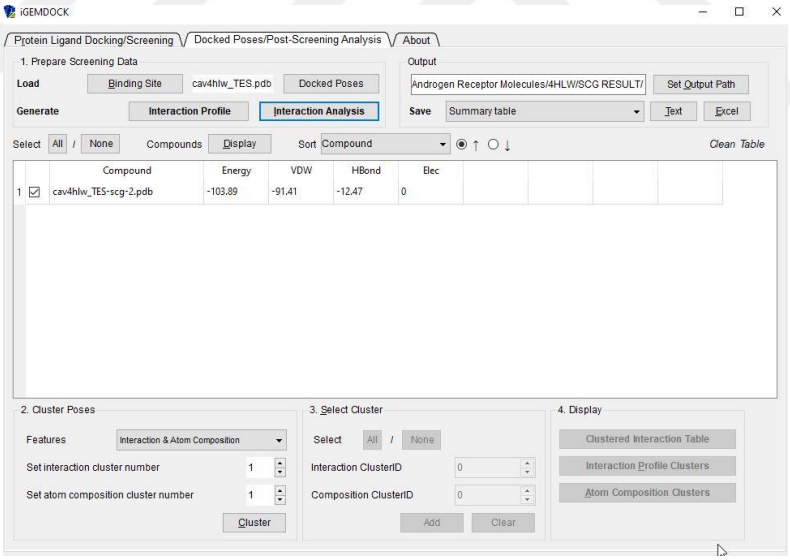


Figure 15. iGEMDOCK Post Screening Analysis section.

The energy score is calculated by the sum of the scores of Van der Waals and hydrogen bonds. The lowest score means a more stable structure.

2.2.5. Cell Culture and Immunoblotting Studies

LNCaP cells were incubated in an incubator containing 5% CO_2 at 37 °C. Cells were maintained with RPMI-1640 medium with 10% FBS. To create serum starvation condition, cells were first incubated with 2% CT-FBS for 24 h, and then CT-FBS concentration was reduced to 0.5%. After 1 h, various concentrations of selected molecules that dissolved in DMSO were added to cells.

To immunoblotting studies, cells were seeded into six or twelve well plates. After 24 h treatment with compounds, cells were trypsinized and harvested with 1xPBS in Eppendorf tubes following the indicated treatment time. Following that, the cell suspension was centrifuged at 10000 g for 3 minutes at 4 °C, and the supernatant was removed. The same procedure was repeated twice for a total of two and one minutes. As lysis buffer, 1x PBS and 2x RIPA (1:1) containing 25x PIC (protein inhibitor cocktail) were used. Every 5 minutes, samples containing an appropriate amount of lysis buffer were vortexed vigorously 5 times. After centrifuging samples at 14000 g for 10 minutes at 4 °C, the supernatant was transferred to a clean Eppendorf tube.

To determine the total protein content of samples, a BCA assay is used to equalize loading. 2 µl samples and 8 µl distilled water were added to 190 µl BCA solution (reagent A and B in a 50:1 ratio). After 30 minutes of incubation at 37 °C, photometric absorbance at 562 nm was determined. According to the BCA assay data, samples were prepared and loaded with a 4x loading buffer to a final concentration of 1x. At 95 °C for 5 minutes, samples were denatured in a dry bath.

Samples were run on 10% SDS gels prepared as described in Table 1 using a running buffer. They were initially run at 60 V for 40 minutes before switching to 120V for the remainder of the run.

Proteins on gels were transferred to a PVDF (Polyvinylidene difluoride) membrane following the running process. A sandwich composed of sponge, two filter papers, gel, PVDF membrane, two filter papers, and the sponge was assembled sequentially in a transfer cassette for transfer process at 200 mA for 90 minutes (on ice) or 20 mA for overnight.

PVDF membranes were treated with 5% milk for 1 hour before blotting with primer antibodies. Antibodies were incubated for either 1 hour or overnight, depending on the antibody type used. After 30 minutes of washing with 31 wash buffer, the

membranes were incubated with appropriate secondary antibodies for 1 h. Following the final 30-minute wash step, chemiluminescence images were captured using the Vilber Lourmat Fx-7 imaging system.

Table 1. Resolving and Stacking Gel Preparation

Resolving Gel	
Gel percentage	**10%**
Acrylamide (30%)	3.33 ml
4x Resolving Buffer	2.5 ml
Distilled water	4.1 ml
AP (10%)	75 µl
Stacking Gel	
Acrylamide (30%)	0.35 ml
4x Resolving Buffer	0.75 ml
Distilled water	1.9 ml
AP (10%)	25 µl

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Swiss Target Prediction Results

The Swiss-Target Prediction web service was used to screen selected 130 molecules to determine which ones had the potential to bind to androgen or estrogen receptors. The molecules predicted to bind these receptors are listed in Table 2.

Table 2. Swiss target prediction web service results with a high potential for the androgen and estrogen receptors.

Molecules				
C31	A-AG-01	A-SCG-01	A-SCG-03	A-SCG-06
A-SCG-07	A-SKG-01	A-SKG-03	A-SKG-06	A-SKG-09
A-SKG-11	E-SCG-01	E-SCG-02	Pr-SCA-01	Pr-SCA-02
SCG-02			CG-02	

In terms of the results, SCG-02 had the highest probability of binding to both receptors out of all these molecules, while the others had a similar low probability.

3.2. Ligand Docking Results for Androgen Receptor

The receptor models 2PNU, 4HLW and 3B66 was used in the literature to assess the androgenic potentials of the ligands previously (Wahl and Smieško 2018; Trisciuzzi et al. 2017). There were observed to be minor differences between these receptor models. Figure 16 depicts the structural alignment of these models.

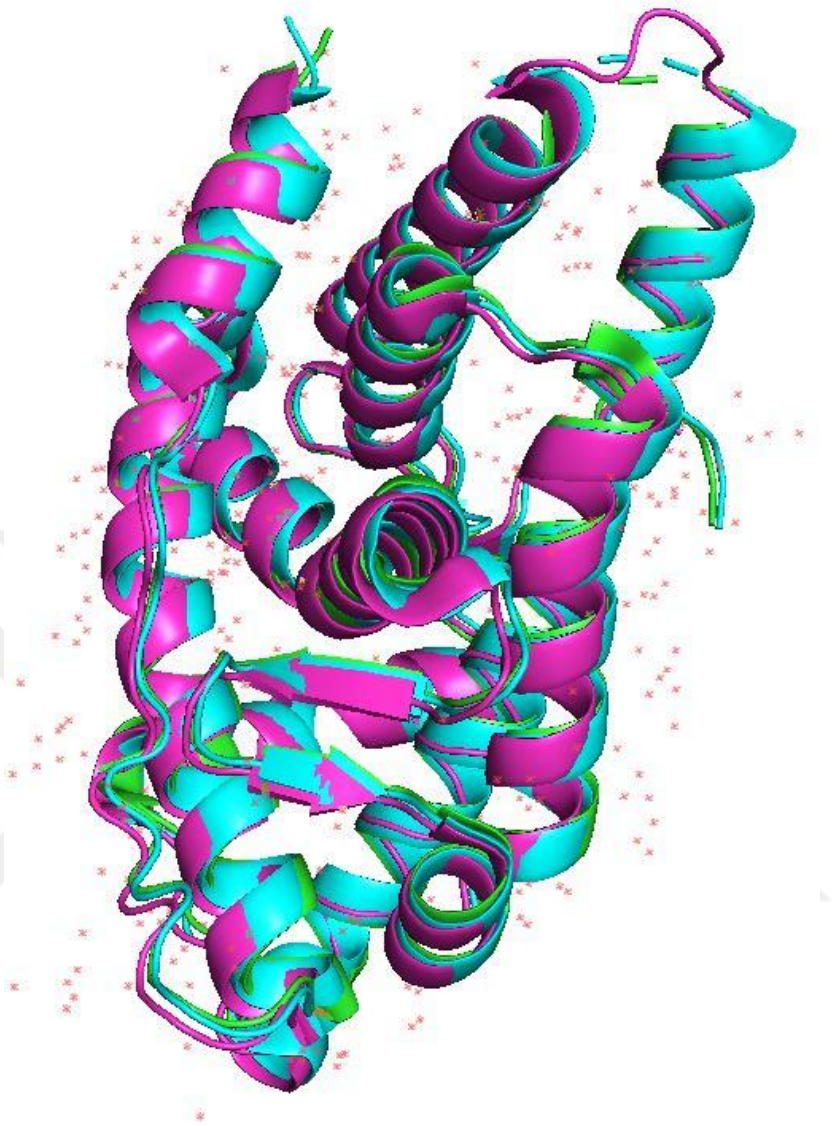


Figure 16. Alignment of structures 2PNU(Cyan), 4HLW(Green) and 3B66(Purple).

Energy scores of the selected molecules were calculated using iGEMDOCK software for each receptor. The binding site was determined based on the Testosterone molecule which was illustrated in Figure 17.

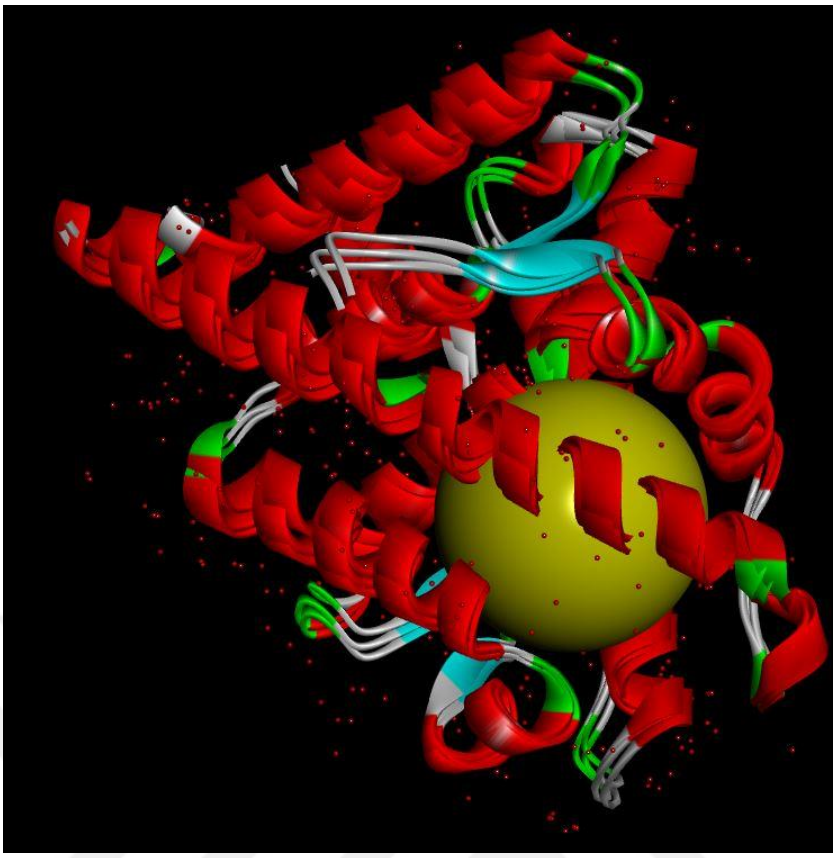


Figure 17. Binding site determined for ligands.

Structure-activity relationship of molecules that may potentially bind to androgen receptor was assessed regarding docking results. Ligand docking results of receptors 2PNU, 4HLW and 3B66 were given in Tables 3, 4 and 5, respectively.

Table 3. Docking results for receptor 2PNU.

Ligand Name	Total Energy
ENM-Model	-140.124
ENM-Ideal	-128.763
CA	-110.202
A-SCG-07	-105.477
A-SCG-06	-102.663
B25	-101.691
Pr-SCA-02	-101.441

(cont. on next page)

Table 3 (cont.)

C29	-100.934
A-CA-05	-100.025
SCG-07	-99.5377
AG	-98.4125
A-SCG-01	-97.4149
B15	-97.3416
A-SCG-03	-97.1845
E-SCG-02	-97.1393
A24	-97.0719
SCG	-96.3123
B6	-95.5882
A30	-95.4123
A-AG-01	-95.2643
A33	-94.8351
B4	-94.3938
AG	-93.3147
A27	-93.2523
B21	-92.9946
E-SCG-01	-92.8732
A29	-92.1988
A-CA-02	-92.1946
B5	-91.5795
Nh-CA-01	-91.3768
Cycloastragenol-PubChem	-91.0718
SCG-02	-91.0502
B9	-90.8911
C19	-90.7173
B2	-90.2525
Pr-SCA-01	-89.7267
C14	-89.6863
SKG	-89.6396
A-CA-07	-89.0893
B3	-89.0803
CG-04	-88.1527
C4	-87.8715
A35	-87.0011
AG-03	-86.838
AG-02	-86.8232
Nh-CA-02	-86.6884
A-SKG-03	-86.3875

(cont. on next page)

Table 3 (cont.)

C5	-86.1618
A37	-85.7943
A-AG-03	-85.5154
A-AG-06	-85.1696
A28	-85.0051
C31	-84.9496
B20	-84.7876
B22	-84.289
A-CA-04	-84.1118
A20	-83.5988
C26	-83.0725
A-SKG-06	-83.0522
CG-02	-82.4561
C17	-81.401
C18	-81.3893
A21	-81.1643
AG-08	-81.0679
AG-05	-81.055
B23	-80.7824
A19	-80.7789
C20	-80.4362
A36	-80.2339
C3	-80.1103
C10	-79.9818
A-SKG-10	-79.9754
A32	-79.7597
C13	-79.698
A-SKG-11	-79.245
C36	-79.0817
A-SKG-01	-78.9473
B1	-78.802
A-SKG-05	-78.2695
C27	-78.2065
C35	-78.0955
C21	-77.7665
A8	-77.1128
A4	-77.1079
A17	-77.1075
A10	-77.1056
A3	-77.1045

(cont. on next page)

Table 3 (cont.)

A7	-77.1034
A6	-77.1027
A5	-77.1015
A15	-77.1015
A13	-77.1011
A11	-77.1002
A18	-77.0963
A2	-77.0962
A9	-77.0952
A12	-77.093
A14	-77.0876
A16	-77.0849
A25	-77.077
A-AG-07	-76.9753
C6	-76.7857
B8	-76.7503
C34	-76.53
C2	-76.5262
C12	-76.3588
B19	-76.0823
C28	-76.0218
C8	-76.0016
A31	-75.4131
A-SKG-09	-75.3691
C16	-75.2876
C25	-75.2324
C32	-74.8526
C22	-74.703
B24	-73.3296
SCG-05	-72.8969
C15	-72.5613
C11	-72.1286
C7	-71.4213
SCG-06	-69.9728
C24	-69.8256
C33	-69.0982
C23	-68.0737
MG-01	-67.7692
A26	-36.8588
C9	-33.1975

(cont. on next page)

Table 3 (cont.)

A22	-29.519
CG-03	20.1473
SCG-03	42.6462
C30	48.5717
SCG-01	72.6865
CG-05	96.8677
SCG-04	495.633

Table 4. Docking results for receptor 4HLW.

Ligand Name	Total Energy
A-SCG-07	-112.702
A-SCG-06	-110.002
Pr-SCA-02	-108.588
A-SCG-01	-105.983
SCG	-103.883
E-SCG-02	-103.48
A-SCG-03	-101.58
Pr-SCA-01	-100.323
SCG-02	-99.6038
Tes-Model	-98.0479
Tes-Ideal	-96.8832
A30	-89.6213
A-CA-05	-89.3695
AG	-86.8674
SCG-06	-85.5674
B22	-84.908
A-AG-03	-84.7811
A-AG-06	-84.577
SCG-05	-84.2638
A-CA-02	-83.7665
C19	-83.7464
A20	-83.5784
A19	-83.4752
B23	-83.4295
E-SCG-01	-82.6481

(cont. on next page)

Table 4 (cont.)

AG	-81.9072
Nh-CA-02	-81.6432
A25	-81.5157
A24	-81.2172
A31	-81.2062
A37	-79.6807
A-AG-01	-79.6656
B21	-79.3443
A4	-79.0231
A21	-78.9427
A14	-78.8612
A17	-78.858
A8	-78.8574
A6	-78.8565
A3	-78.8564
A9	-78.8529
A13	-78.8529
A2	-78.8523
A16	-78.8522
A11	-78.8516
A18	-78.8503
A10	-78.8498
A15	-78.847
A5	-78.8462
A7	-78.8446
A12	-78.8412
C31	-78.7917
A-SKG-09	-78.5788
C6	-78.3022
A-SKG-11	-77.8744
C3	-77.734
C2	-77.3788
A-SKG-10	-77.3755
A29	-77.3382
C8	-77.3295
A-CA-04	-77.092
C4	-76.9494
B5	-76.6356
CA	-76.5467
AG-03	-76.501

(cont. on next page)

Table 4 (cont.)

AG-02	-76.4799
B9	-76.4515
C14	-76.4486
B19	-76.3024
Nh-CA-01	-76.1791
A32	-75.9286
CycloastragenolPubChem	-75.76
A-SKG-05	-75.7355
B8	-75.6693
C17	-75.6517
C34	-75.6462
C18	-75.6238
C26	-75.5582
C10	-75.3718
B4	-75.3174
A-CA-07	-75.2602
B25	-75.2132
C32	-74.9383
C22	-74.9346
AG-08	-74.8335
A33	-74.8019
A28	-74.5066
A-SKG-03	-74.4945
A-SKG-06	-74.3832
C20	-74.2498
A-SKG-01	-74.1677
A35	-74.1085
C25	-74.0834
C7	-73.9549
C11	-73.6124
B24	-73.5238
SCG-01	-73.2761
C16	-72.8995
C15	-72.6801
A27	-72.6415
C36	-72.5306
B15	-72.364
B1	-72.188
B3	-71.9519
C13	-71.8378

(cont. on next page)

Table 4 (cont.)

C23	-71.8084
C29	-71.8003
CG-02	-71.5752
B6	-71.4405
C21	-71.0604
A-AG-07	-70.9417
C12	-70.7092
SCG-07	-69.9773
CG-04	-69.4144
C35	-69.2104
C28	-68.9332
B2	-68.8005
B20	-67.22
SKG	-66.173
A36	-65.059
C33	-64.0007
C24	-61.8554
C27	-60.8996
MG-01	-58.6481
CG-03	19.6885
A26	32.549
SCG-04	37.9733
C30	42.2754
CG-05	63.8606
C9	97.0171
A22	130.893
C5	196.168
AG-05	231.93
SCG-03	298.379

Table 5. Docking results for receptor 3B66.

Ligand Name	Total Energy
B66-Model	-158.139
A-SCG-06	-101.303
A-SCG-07	-100.838

(cont. on next page)

Table 5 (cont.)

A-SCG-03	-99.6938
A-SCG-01	-98.34
Pr-SCA-02	-96.969
E-SCG-02	-95.179
B19	-93.6846
CA	-93.5484
C2	-92.25
A-SKG-03	-88.7482
SCG-02	-87.8567
A-SKG-09	-87.6617
C8	-87.5684
C20	-87.399
C28	-87.2857
C4	-86.9742
C6	-86.9667
Pr-SCA-01	-86.2701
A-SKG-10	-85.7581
C35	-85.4603
A-CA-02	-85.4092
C24	-85.3614
A31	-85.3575
C36	-85.2424
A-SKG-01	-85.0596
B66-Ideal	-84.8528
A30	-84.808
A-AG-03	-84.46
A-CA-04	-84.4534
C18	-84.0829
C17	-84.0699
A32	-83.8686
A36	-83.4266
C27	-83.4159
A25	-83.1462
C3	-83.1004
CG-04	-82.7915
A17	-82.2229
A8	-82.2196
A14	-82.2154
A11	-82.2136
A18	-82.2117

(cont. on next page)

Table 5 (cont.)

A6	-82.2021
A9	-82.2019
A15	-82.2003
A7	-82.2002
A4	-82.1998
A2	-82.1978
A5	-82.1974
A16	-82.1947
A12	-82.1933
A13	-82.19
A3	-82.1869
C34	-82.1284
A10	-82.0921
SCG-07	-82.0078
B24	-81.9513
B20	-81.7675
A-AG-07	-81.6646
A20	-81.6084
A21	-81.5627
C14	-81.5352
B22	-81.5116
SKG	-81.258
B4	-81.1615
CG-02	-80.7652
A-CA-07	-80.7494
A-SKG-05	-80.6748
B2	-80.6532
C21	-80.3308
C25	-79.907
B5	-79.6613
C31	-79.2443
C11	-79.1415
C22	-79.0667
C26	-79.0601
B23	-78.6614
A19	-78.6166
C10	-78.6125
A-CA-05	-78.5372
B6	-78.5193
C16	-78.3209

(cont. on next page)

Table 5 (cont.)

B1	-78.0247
A-AG-06	-78.0051
A-AG-01	-77.8827
C32	-77.6626
AG-08	-77.5852
B21	-77.54
A-SKG-11	-77.4741
SCG-01	-77.4514
SCG	-77.4279
B25	-77.3219
C19	-77.2795
C15	-77.2773
C7	-77.1783
C29	-77.0121
A35	-76.8189
C12	-76.8101
C13	-76.7916
B8	-76.705
B3	-76.5161
Cycloastragenol-PubChem	-76.4554
C23	-76.3999
Nh-CA-02	-76.2217
A-SKG-06	-75.9534
AG	-75.8918
A37	-74.8825
C33	-74.8268
A24	-74.7971
A27	-74.5469
AG-03	-73.8395
AG	-73.8376
AG-02	-73.8081
Nh-CA-01	-73.7937
SCG-06	-73.0961
A33	-72.8177
B15	-72.5991
B9	-72.371
SCG-05	-71.4388
A28	-69.8915
E-SCG-01	-69.4922

(cont. on next page)

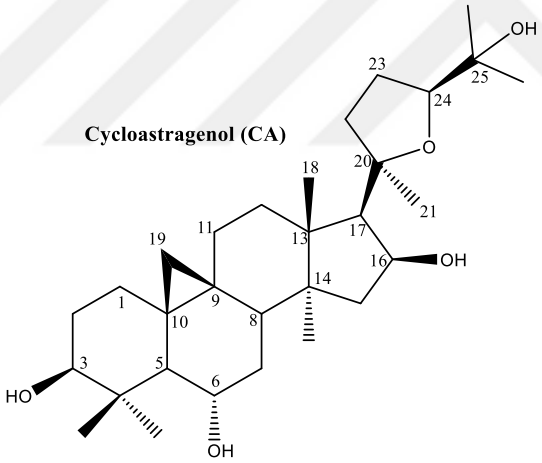
Table 5 (cont.)

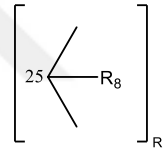
A29	-66.7756
MG-01	-66.5357
SCG-04	4.71995
A22	39.2739
C5	47.2671
CG-05	50.1218
A26	78.8617
C9	132.294
C30	138.157
AG-05	187.812
CG-03	348.313
SCG-03	372.947

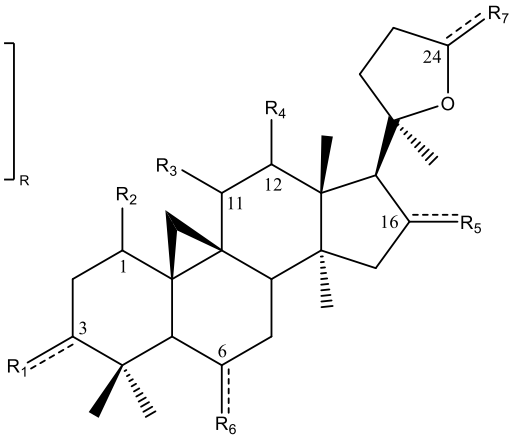
Selected ligands were the derivatives of Cycloastragenol (CA), Astragenol (AG), 20(27)-octanor cycloastragenol (SCG) and Cyclocanthogenol (SKG). For this reason, these four molecules energy score were calculated with the same method to assess structure activity relationships. None of the molecules scored lower than ENM molecule for the PDB id 2PNU receptor model. In contrast, A-SCG-07, A-SCG-06, Pr-SCA-02, A-SCG-01, SCG, E-SCG-02, A-SCG-3, Pr-SCA-01, and SCG-02 all had lower energy scores than TES-Model, which was testosterone bound to the receptor 4HLW, and TES-Ideal, which was the computationally optimized model of TES-Model. None of the molecules scored lower than B66-Model for the receptor model 3B66, but all the molecules scored lower than TES-Model scored lower than B66-Ideal. The docking data for the receptors 4HLW and 3B66 indicate that A-SCG-07, A-SCG-06, Pr-SCA-02, A-SCG-01, SCG, E-SCG-02, A-SCG-3, Pr-SCA-01 and SCG-02 may have a high potential for use as androgen receptor ligands. On the other hand, other molecules with an energy score less than zero may also be used as androgen receptor ligands due to the increased stability of the complex with a lower score. The change of grid boxes, the areas selected for the docking process can explain the difference in the energy scores for different receptors due to different bound ligands. iGEMDOCK software automatically assigns grid box regarding the selected ligand previously, ENM for the receptor 2PNU, TES for the 4HLW and B66 for the 3B66. When these ligands were compared with the selected ligands, the most structurally similar ligand was TES. This situation may indicate that the docking results of the receptor 4HLW can be more accurate. Besides that, the molecules

that scored higher than the reference molecules may have a lower affinity for binding to the receptor or it might not bind at all. Tables 6, 7, 8 and 9 show structures of the selected ligand molecules and their associated receptor energy scores.

Table 6. Structures and docking results for Cycloastragenol derivatives.







Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	2PNU-Score	4HLW-Score	3B66 -Score
Cycloastragenol (CA)	β-OH	-	-	-	β-OH	α-OH	R	OH	**-110.202**	**-76.5467**	**-93.5484**
Nh-CA-01	O	α-OH	-	-	β-OH	α-OH	R	OH	**-91.3768**	**-76.1791**	**-73.7937**
Nh-CA-02	O	-	β-OH	-	β-OH	α-OH	R	OH	**-86.6884**	**-81.6432**	**-76.2217**
A-CA-02	O	-	-	β-OH	O	α-OH	R	OH	**-92.1946**	**-83.7665**	**-85.4092**
A-CA-04	β-OH	-	-	α-OH	β-OH	α-O-SO3H	R	OH	**-84.1118**	**-77.092**	**-84.4534**
C2	β-Propionate	-	-	-	β-OH	α -Propionate	R	OH	**-76.5262**	**-77.3788**	**-92.25**

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Table 6 (cont.)

Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	2PNU-Score	4HLW-Score	3B66 - Score
C3	β-Butyrate	-	-	-	β-OH	α-Butyrate	R	OH	**-80.1103**	**-77.734**	**-83.1004**
C4	β-Isobutyrate	-	-	-	β-OH	α- Isobutyrate	R	OH	**-87.8715**	**-76.9494**	**-86.9742**
C5	β-Benzoate	-	-	-	β-OH	α-Benzoate	R	OH	**-86.1618**	**196.168**	**47.2671**
C6	β-Propionate	-	-	-	O	α -Propionate	O	-	**-76.7857**	**-78.3022**	**-86.9667**
C7	β-Butyrate	-	-	-	O	α-Butyrate	O	-	**-71.4213**	**-73.9549**	**-77.1783**
C8	β-Isobutyrate	-	-	-	O	α- Isobutyrate	O	-	**-76.0016**	**-77.3295**	**-87.5684**
C9	β-Benzoate	-	-	-	O	α-Benzoate	O	-	**-33.1975**	**97.0171**	**132.294**
C10	β-Pivalate	-	-	-	β-OH	α-Pivalate	R	OH	**-79.9818**	**-75.3718**	**-78.6125**
C11	β-OH	-	-	-	β-OH	α-Pivalate	R	OH	**-72.1286**	**-73.6124**	**-79.1415**
C12	β-Pivalate	-	-	-	β-OH	α-OH	R	OH	**-76.3588**	**-70.7092**	**-76.8101**
C13	O	-	-	-	O	O	R	OH	**-79.698**	**-71.8378**	**-76.7916**
C14	β-OH	-	-	-	β-OH	O	R	OH	**-89.6863**	**-76.4486**	**-81.5352**
C15	β-OH	-	-	-	β-OH	α-Trifluoroacetate	R	OH	**-72.5613**	**-72.6801**	**-77.2773**
C16	β-OH	-	-	-	β- Trifluoroacetate	α-OH	R	OH	**-75.2876**	**-72.8995**	**-78.3209**

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Table 6 (cont.)

Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	2PNU-Score	4HLW-Score	3B66 - Score
C17	β-Succinate	-	-	-	β-OH	α-Succinate	R	OH	**-81.401**	**-75.6517**	**-84.0699**
C18	β- Butyrate	-	-	-	β-OH	α-OH	R	OH	**-81.3893**	**-75.6238**	**-84.0829**
C19	O	-	-	-	O	O	O	-	**-90.7173**	**-83.7464**	**-77.2795**
C20	β-Acetate	-	-	-	β-OH	α-Acetate	R	OH	**-80.4362**	**-74.2498**	**-87.399**
C21	β-OH	-	-	-	β-OH	α-Acetate	R	OH	**-77.7665**	**-71.0604**	**-80.3308**
C22	β-Acetate	-	-	-	β-OH	α-OH	R	OH	**-74.703**	**-74.9346**	**-79.0667**
C23	β-OH	-	-	-	β-OH	α-Butyrate	R	OH	**-68.0737**	**-71.8084**	**-76.3999**
C24	β-Propionate	-	-	-	β-Propionate	α-Propionate	R	Propionate	**-69.8256**	**-61.8554**	**-85.3614**
C25	β-Acetate	-	-	-	β-Acetate	α-Acetate	R	Acetate	**-75.2324**	**-74.0834**	**-79.907**
C26	Hydroxyamine	-	-	-	Hydroxyamine	Hydroxyamine	R	OH	**-83.0725**	**-75.5582**	**-79.0601**
C27	β-Isobutyrate	-	-	-	β-Isobutyrate	α-Isobutyrate	R	Isobutyrate	**-78.2065**	**-60.8996**	**-83.4159**
C28	β-Butyrate	-	-	-	β-Butyrate	α-Butyrate	R	OH	**-76.0218**	**-68.9332**	**-87.2857**
C29	β-Methoxy	-	-	-	β- Methoxy	α-Methoxy	R	Methoxy	**-100.934**	**-71.8003**	**-77.0121**
C30	β-Tosylate	-	-	-	β-OH	α-OH	R	OH	**48.5717**	**42.2754**	**138.157**

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Table 6 (cont.)

Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	2PNU-Score	4HLW-Score	3B66 - Score
C31	O	-	-	-	O	α-OH	O	-	**-84.9496**	**-78.7917**	**-79.2443**
C32	O	-	-	-	O	α-OH	R	OH	**-74.8526**	**-74.9383**	**-77.6626**
C33	β-Acetate	-	-	-	β-Acetate	α-Acetate	R	Acetate	**-69.0982**	**-64.0007**	**-74.8268**
C34	O	-	-	-	O	O	R	Acetate	**-76.53**	**-75.6462**	**-82.1284**
C35	O	-	-	-	O	O	R	Propionate	**-78.0955**	**-69.2104**	**-85.4603**
C36	O	-	-	-	O	O	R	Butyrate	**-79.0817**	**-72.5306**	**-85.2424**
CG-05	β-Tosylate	-	-	-	β-OH	O	R	OH	**96.8677**	**63.8606**	**50.1218**

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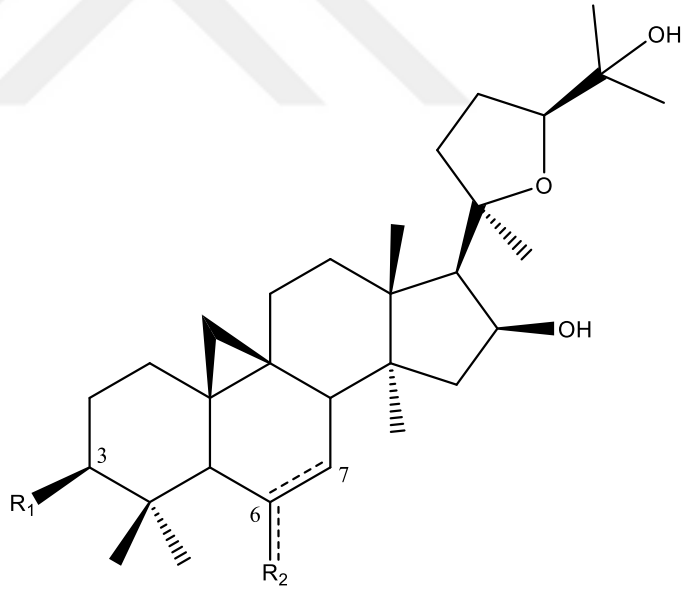
Table 6 (cont.)



Molecule	R_1	R_2	Δ^6	2PNU-Score	4LHW-Score	3B66 - Score
CG-02	β-OH	-	-	**-82.4561**	**-71.5752**	**-80.7652**
CG-03	β-Tosylate	-	-	**20.1473**	**19.6885**	**348.313**
CG-04	Methylsulfonate	-	-	**-88.1527**	**-69.4144**	**-82.7915**
CG-05	β-Tosylate	O	=	**96.8677**	**63.8606**	**50.1218**

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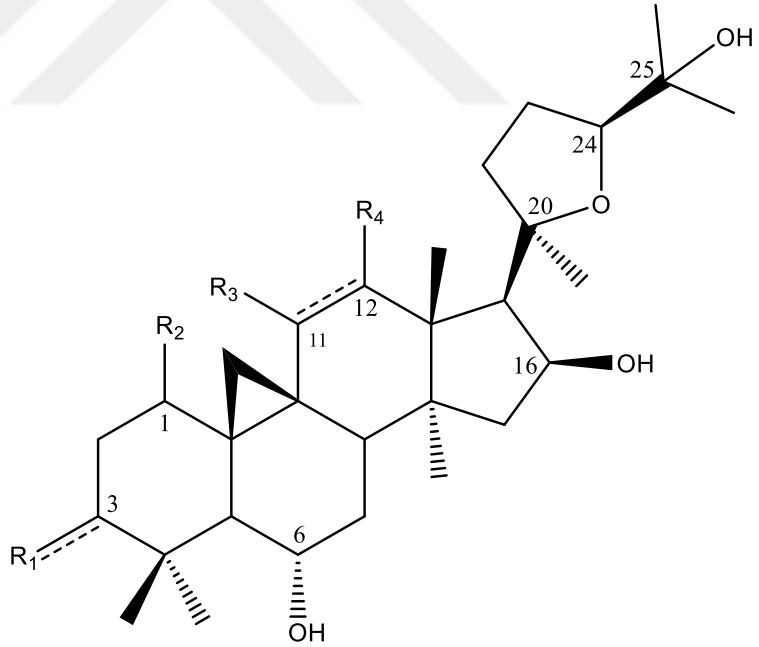
Table 6 (cont.)



Molecule	R_1	R_2	R_3	R4	Δ^{11}	2PNU-Score	4HLW-Score	3B66 - Score
B2	β-OH	α-OH	-	-	-	**-90.2525**	**-68.8005**	**-80.6532**
B3	β-OH	-	-	-	=	**-89.0803**	**-71.9519**	**-76.5161**

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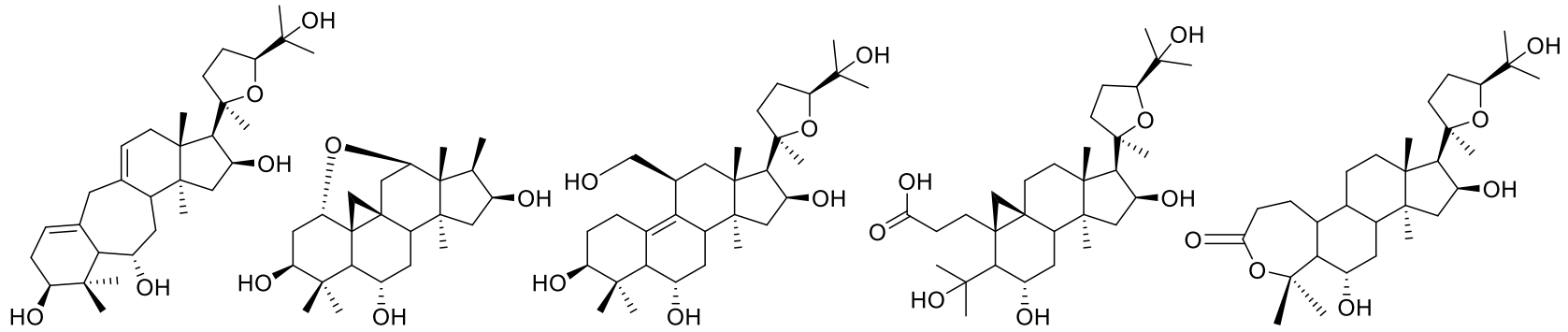
Table 6 (cont.)

B4	β-OH	-	β-OH	-	-	**-94.3938**	**-75.3174**	**-81.1615**
B5	β-OH	-	-	β-OH	-	**-91.5795**	**-76.6356**	**-79.6613**
B6	β-OH	-	-	β-OH	-	**-95.5882**	**-71.4405**	**-78.5193**
B9	O	-	-	-	-	**-90.8911**	**-76.4515**	**-72.371**



A-CA-05 **A-CA-07** **B-1** **B-8** **B-15**

Molecule	2PNU-Score	4HLW-Score	3B66-Score
A-CA-05	**-100.025**	**-89.3695**	**-78.5372**
B-1	**-78.802**	**-72.188**	**-78.0247**

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Table 6 (cont.)

Molecule	2PNU-Score	4HLW-Score	3B66-Score
B-15	**-97.3416**	**-72.364**	**-72.5991**
A-CA-07	**-89.0893**	**-75.2602**	**-80.7494**
B-8	**-76.7503**	**-75.6693**	**-76.705**

Table 6 summarizes the docking energy values for Cycloastragenol (CA) analogs. The lower score corresponds to a better binding affinity. Nh-CA-02, A-CA-02, A-CA-04, C2, C3, C4, C6, C8, C19, B5 and A-CA-05 all had lower scores than CA, revealing higher affinity for binding. Carbonyl groups in C19, C6, C8 and Nh-CA-02 may contribute significantly to the increase in binding affinity score, whereas bulky benzoate groups in C5 and C9 may do the opposite. Furthermore, the acylation of the hydroxy group at position 25 as acetate, butyrate, isobutyrate, methoxy and propionate groups negatively affected the binding affinity. When C27 is compared to C4, the only difference is the acylation at positions 16 and 25 in C27. Yet C4 had a higher binding affinity than C27 (Figure 18).

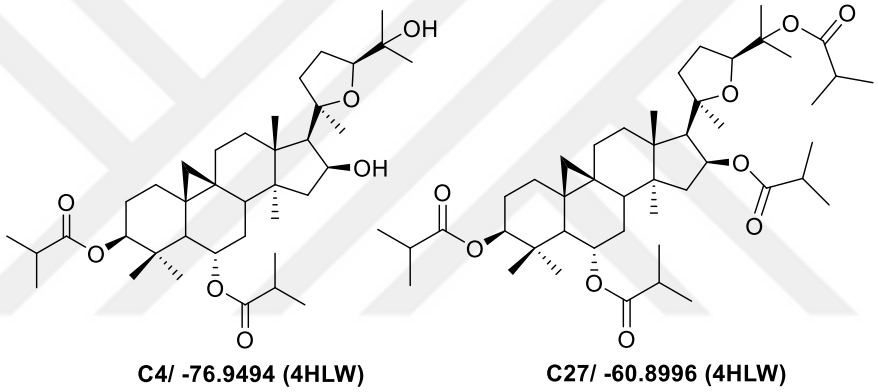


Figure 18. Structures of C4 and C27.

The same situation is effective for C3/C28 and C2/C24 couples, with the exception that the butyrate group (C3/C28) and propionate group (C2/C24) are present instead of the isobutyrate group. The scores of C6, C7 and C8 also imply that the carbonyl group might have a significant effect for the increase of binding affinity, where the only difference is the presence of carbonyl group at the 6^{th} and 24^{th} positions compared to compounds C24, C28 and C27, respectively (Figure 19).

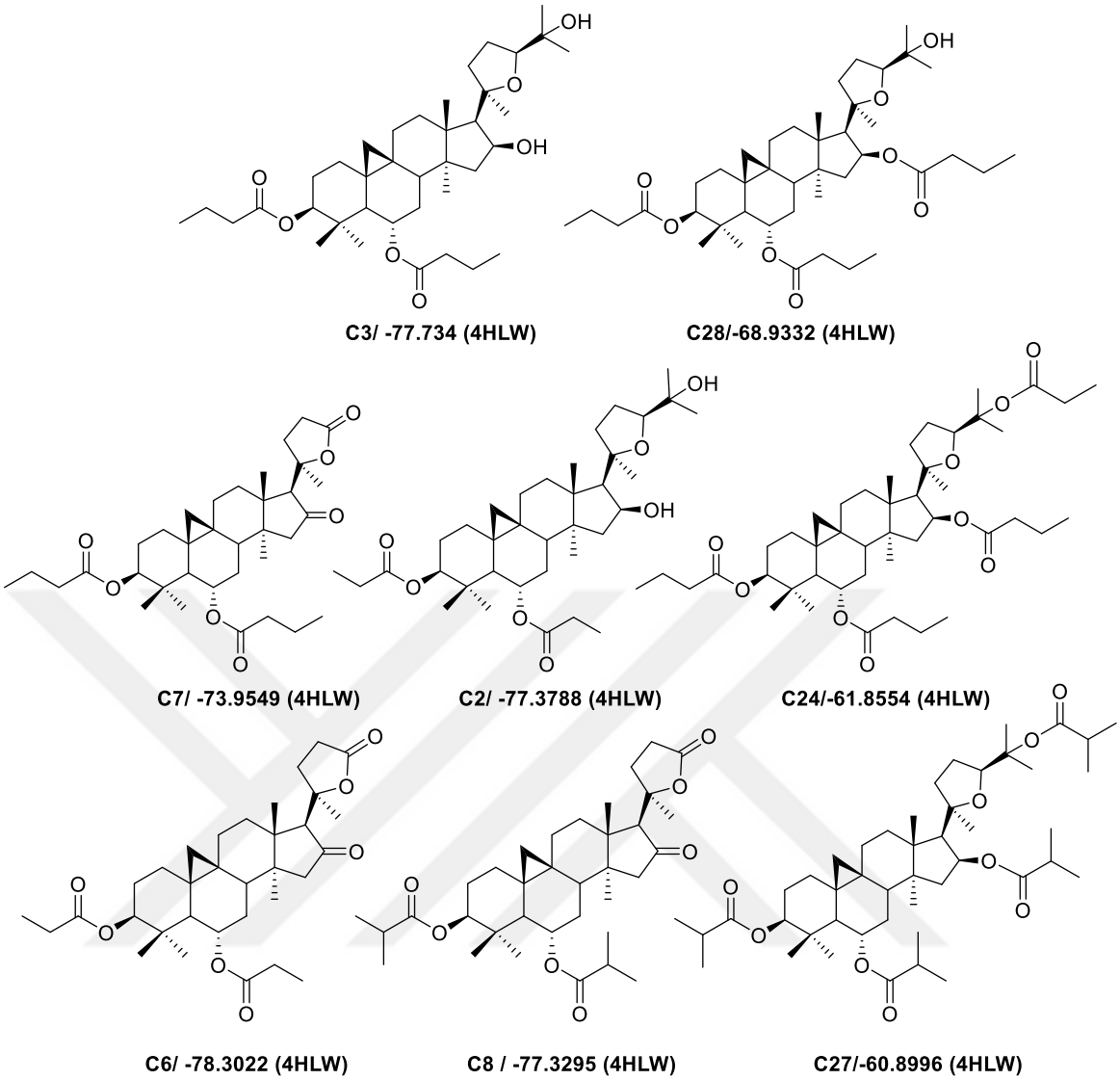


Figure 19. Structures of C3, C28, C7, C2, C24, C6, C8 and C27.

When C19 and C13 are compared, the carbonyl group at position 24 as part of a lactone ring appears to be the cause of the decrease in energy score. The unpaired electrons of the oxygen atom found in the carbonyl group acts like a hydrogen bond acceptor while the hydroxy group can make hydrogen bond as donor or acceptor. Probably, the carbonyl group in the ligand and amino acid side chains in the docking region form stronger and more stable hydrogen bonds. The ketone group at position 3 of Nh-CA-02 may also contribute to the decrease in the energy score due to same reason. Double bonds are known to be a type of covalent bonds that four electrons are shared between two atoms. The pi and sigma electron orbitals are spatially arranged differently,

causing the orbitals to overlap. As a result, the double bond is formed, providing a more rigid structure, and preventing the rotation around double bond. Due to its higher electron density, the double bonds can be more reactive in ligand receptor interactions (Alberts et al. 2008; Carey and Sundberg 2007; Fry and Page 2005). The double bond in the rings A and C, and the loss of R group in position 3 in A-CA-05 might have an effect in the increase of binding affinity (Figure 20).

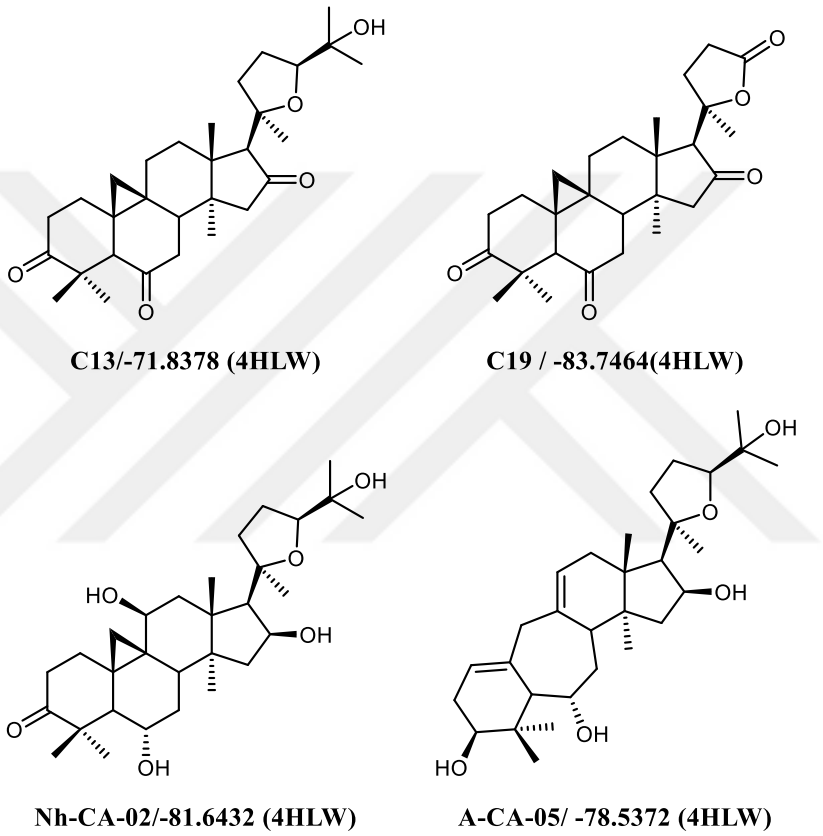
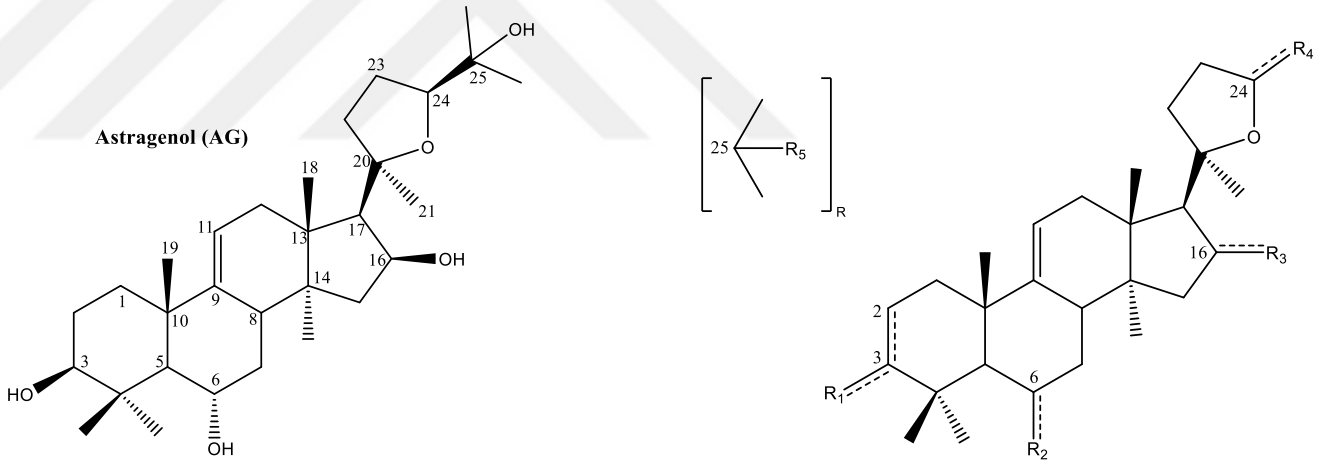


Figure 20. Structures of C13, C19, Nh-CA-02 and A-CA-05.

Table 7. Structures and docking results for Astragenol derivatives.



Molecule	R_1	Δ^3	R_3	R_2	R_4	R_5	2PNU-Score	4HLW-Score	3B66-Score
Astragenol (AG)	β-OH	-	β-OH	α-OH	R	OH	**-98.4125**	**-86.8674**	**-75.8918**
A-AG-01	O	-	β-OH	α-OH	R	OH	**-95.2643**	**-79.6656**	**-77.8827**
AG-02	-	=	β-OH	α-OH	R	OH	**-86.8232**	**-76.4799**	**-73.8081**
AG-05	β-Tosylate	-	β-OH	O	R	OH	**-81.055**	**231.93**	**187.812**
A2	β-Propionate	-	β-OH	α- Propionate	R	OH	**-77.0962**	**-78.8523**	**-82.1978**

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Table 7 (cont.)

Molecule	R_1	Δ^3	R_3	R_2	R_4	R_5	2PNU-Score	4HLW-Score	3B66-Score
A3	β-Butyrate	-	β-OH	α-Butyrate	R	OH	**-77.1045**	**-78.8564**	**-82.1869**
A4	O	-	O	O	O	OH	**-77.1079**	**-79.0231**	**-82.3998**
A5	O	-	O	O	R	OH	**-77.1015**	**-78.8462**	**-82.1974**
A6	-	=	β-OH	α-OH	R	OH	**-77.1027**	**-78.8565**	**-82.2021**
A7	$=CH_2$	-	β-OH	α-OH	R	OH	**-77.1034**	**-78.8446**	**-82.2002**
A8	β-Tosylate	-	β-OH	α-OH	R	OH	**-77.1128**	**-78.8574**	**-82.2196**
A10	β-Isobutyrate	-	β-OH	α-Isobutyrate	R	OH	**-77.1056**	**-78.8498**	**-82.0921**
A11	β-Isobutyrate	-	β-Isobutyrate	α-Isobutyrate	R	OH	**-77.1002**	**-78.8516**	**-82.2136**
A12	β-Benzoate	-	β-OH	α-Benzoate	R	OH	**-77.093**	**-78.8412**	**-82.1933**
A13	β-Benzoate	-	β-OH	α-OH	R	OH	**-77.1011**	**-78.8529**	**-82.19**
A14	β-Pivalate	-	β-OH	α-Pivalate	R	OH	**-77.0876**	**-78.8612**	**-82.2154**
A15	β-Pivalate	-	β-OH	α-OH	R	OH	**-77.1015**	**-78.847**	**-82.2003**
A16	β-Acetate	-	β-OH	α-Acetate	R	OH	**-77.0849**	**-78.8522**	**-82.1947**

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Table 7 (cont.)

Molecule	R_1	Δ^3	R_3	R_2	R_4	R_5	2PNU-Score	4HLW-Score	3B66-Score
A17	β-Acetate	-	β-Acetate	α-Acetate	R	OH	**-77.1075**	**-78.858**	**-82.2229**
A18	β-Acetate	-	β-Acetate	β-Acetate	R	Acetate	**-77.0963**	**-78.8503**	**-82.2117**
A26	Oxime	-	O	Oxime	R	OH	**-36.8588**	**32.549**	**78.8617**
A27	Oxime	-	Oxime	Oxime	R	OH	**-93.2523**	**-72.6415**	**-74.5469**
A28	O-methyloxime	-	O	O-methyloxime	O	-	**-85.0051**	**-74.5066**	**-69.8915**
A31	TSC	-	O	O	O	-	**-75.4131**	**-81.2062**	**-85.3575**
A33	O	-	O	O-methyloxime	O	-	**-94.8351**	**-74.8019**	**-72.8177**
A35	O	-	O	α-OH	R	OH	**-87.0011**	**-74.1085**	**-76.8189**
A37	β-OH	-	β-O-Me	α-OH	R	OH	**-85.7943**	**-79.6807**	**-74.8825**

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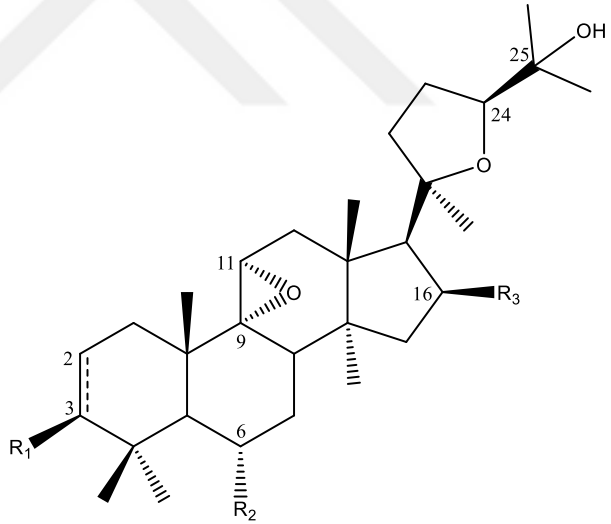
Table 7 (cont.)



Molecule	R_1	R_2	R_3	Δ^3	2PNU-Score	4HLW-Score	3B66-Score
A19	β-OH	α-OH	β-OH	-	**-80.7789**	**-83.4752**	**-78.6166**
A20	β-Acetate	α-Acetate	β-Acetate	-	**-83.5988**	**-83.5784**	**-81.6084**
A21	β-Butyrate	α-OH	β-OH	-	**-81.1643**	**-78.9427**	**-81.5627**
A22	β-Benzoate	α-Benzoate	β-OH	-	**-29.519**	**130.893**	**39.2739**

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Table 7 (cont.)

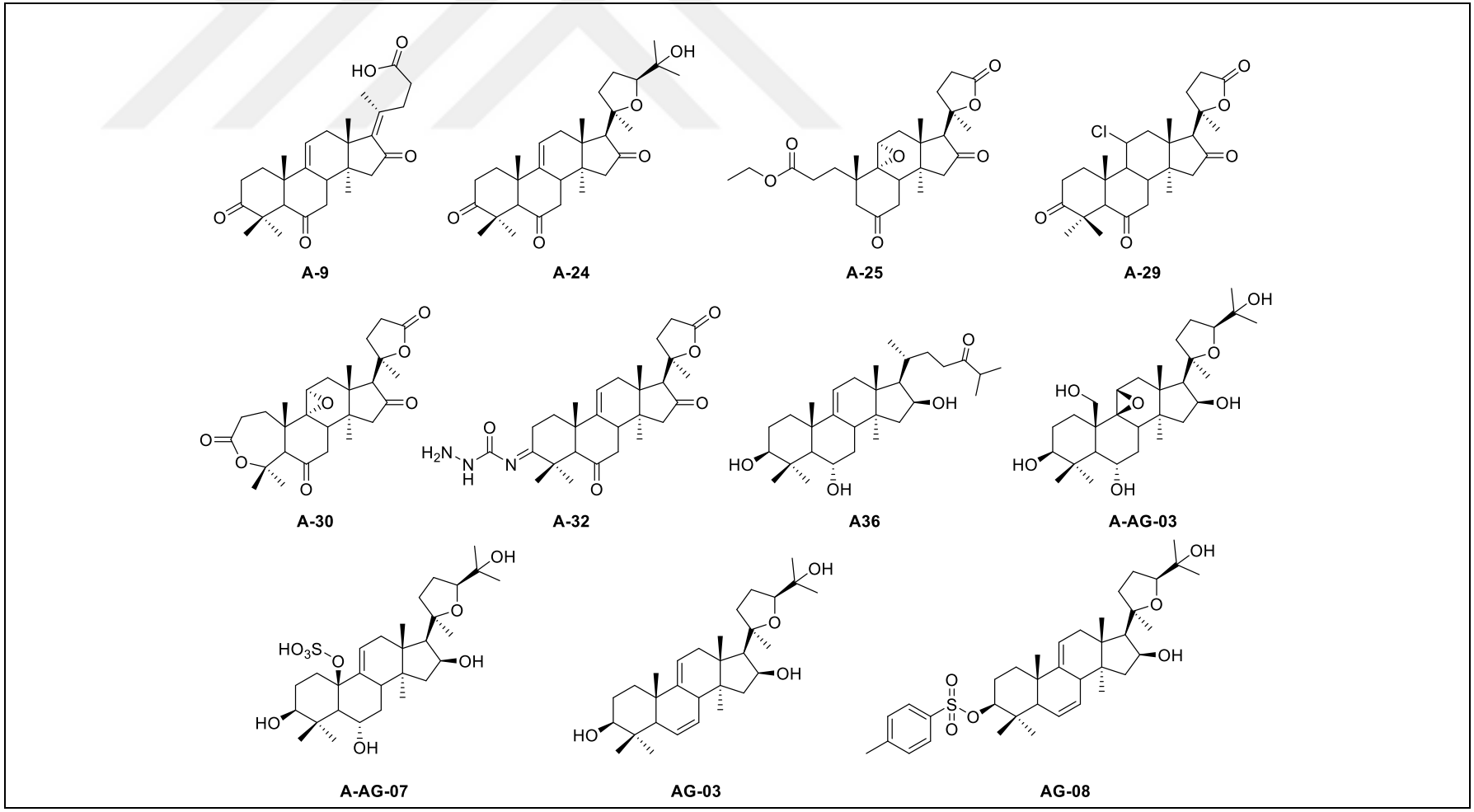

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Table 7 (cont.)

Molecule	2PNU-Score	4HLW-Score	3B66-Score
A9	**-77.0952**	**-78.8529**	**-82.2019**
A24	**-97.0719**	**-81.2172**	**-74.7971**
A25	**-77.077**	**-81.5157**	**-83.1462**
A29	**-92.1988**	**-77.3382**	**-66.7756**
A30	**-95.4123**	**-89.6213**	**-84.808**
A32	**-79.7597**	**-75.9286-**	**-83.8686**
A36	**-80.2339**	**-65.059**	**-83.4266**
A-AG-03	**-85.5154**	**-84.7811**	**-84.46**
A-AG-07	**-76.9753**	**-70.9417**	**-81.6646**
AG-03	**-86.838**	**-76.501**	**-73.8395**
AG-08	**-81.0679**	**-74.8335**	**-77.5852**

Energy scores of astragenol (AG) derivatives were summarized in Table 7. A-AG-01, A-AG-03, A-AG-07, AG-08, A2 – A21, A25, A30 – A32, A35 and A36 were all scored lower than AG, indicating that they may have a higher binding affinity. A31 (Figure 21) has the lowest energy score for 3B66 which has three carbonyl groups at carbons 6, 16 and 25 together with a thiosemicarbazide (TSC) group at position 3. Additionally, A30, which had two rare substructures including a seven membered lactone ring and an epoxy functionality, scored the lowest energy towards 4HLW (Figure 21). All the molecules that scored lower than AG had very similar results for the receptor 3B66, and the presence of carbonyl groups at different positions were also significant, viz. A4 (Figure 21).

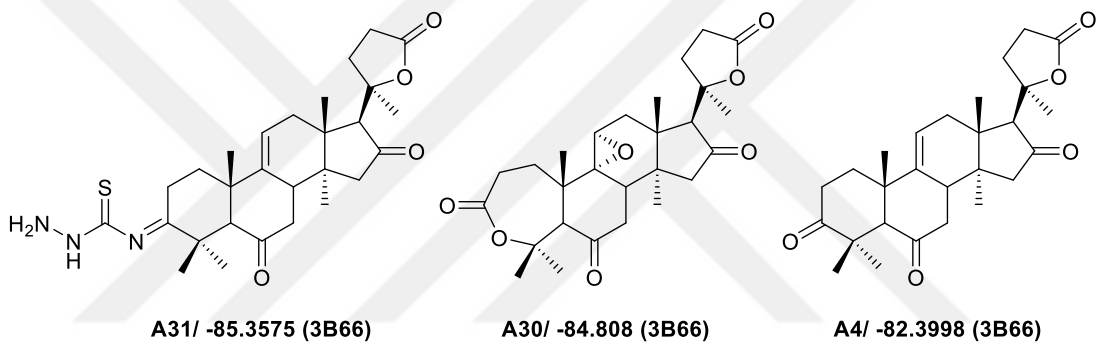


Figure 21.Structures of A31, A30 and A4.

The other molecules with good scores, viz. A8, A15 and A21 (Figure 22), contain an ester or sulfonester group at position 3. Furthermore these molecules possess higher electron density and hydrogen bond acceptors in the vicinity of carbon three. A-AG-03 (Figure 22) which has free hydroxly groups at C-3, C-6 and C16 has scored lower than the similar A-AG-07 molecule (Figure 22), signifying the presence of 9(11)-epoxy group as in A30.

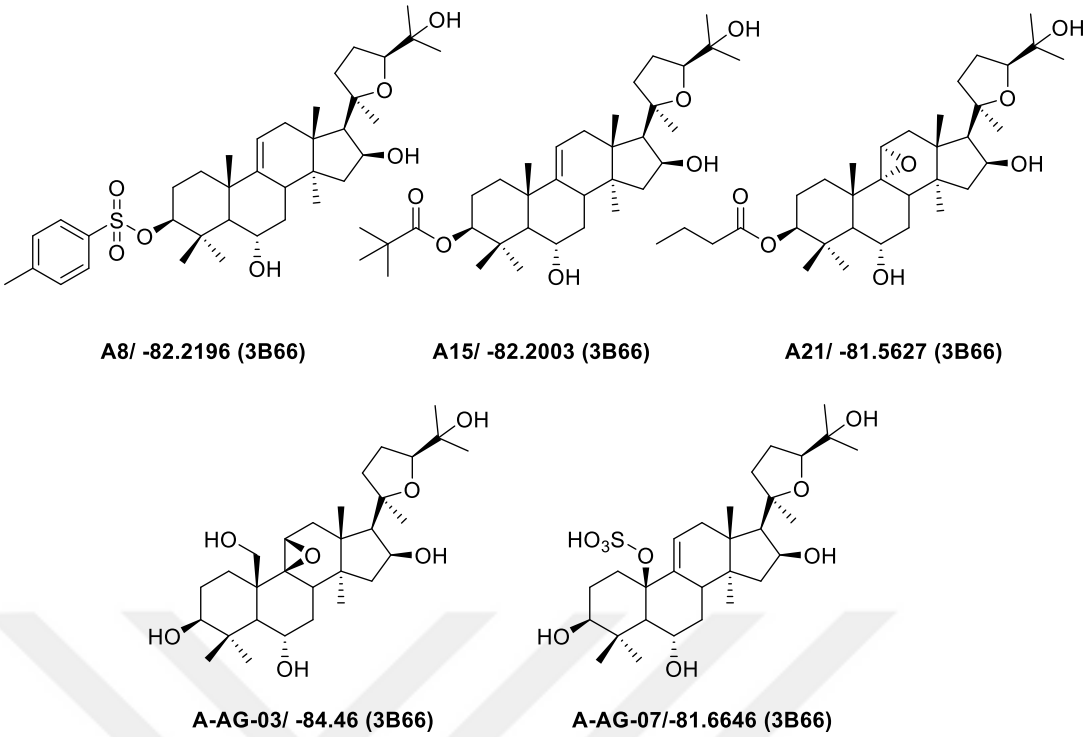


Figure 22. Structures of A8, A15, A21, A-AG-03 and A-AG-07.

Table 8 shows the docking results of 20(27)-octanor cycloastragenol (SCG). SCG-02, SCG-07, A-SCG-03, A-SCG-07, Pr-SCA-01, Pr-SCA-02 A-SCG-01, A-SCG-06 and E-SCG-02 had energy scores lower than SCG. A-SCG-07, Pr-SCA-02, A-SCG-01 and A-SCG-06 scored lower in all receptors. For the receptors 2PNU and 4HLW, A-SCG-07 (Figure 23) scored the lowest. This could be explained by the ketone group at position 16 and the hydroxy group located on the hydrophobic face of the sapogenin framework (extending from C-11 as primary alcohol). For receptor 3B66, A-SCG-06 (Figure 23) has the lowest score possessing a similar structure to A-SCG-07 in terms of a hydrophilic group extending from the upper side as C-1(OH).

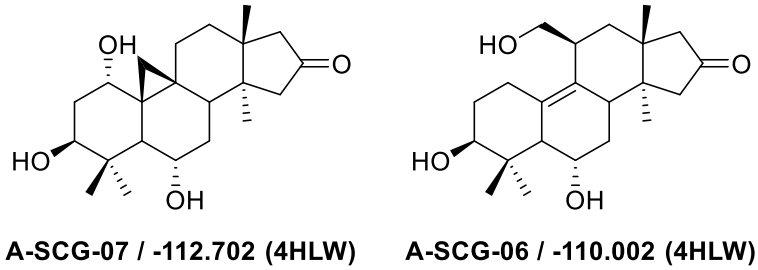


Figure 23. Structures of A-SCG-07 and A-SCG-06.

It was observed from docking pose that A-SCG-07 interacted with LEU (704), GLN (711), ARG (752) and MET (742), whereas A-SCG-06 displayed interactions with LEU (704), GLY (708), PHE (764), GLN (711), ARG (752), LEU (707) and MET (742). SCG-04 containing two tosyl groups at positions 3 and 16 afforded the most positive scores. It is highly probable that the bulky substituents prevent the entry of the ligand to the active site of the receptor.

Docking results of the Cyclocanthogenol (SKG) derivatives are shown in Table 9. B-19 (Figure 24) had the lowest energy score for receptor 3B66, whereas A-SKG-09 (Figure 24) gave the lowest score for receptor 4HLW. As mentioned in the previous part for the SCG derivatives, the primary alcohol group extending from carbon 11 seems to be important. One should also consider the presence of $\Delta^{9(10)}$ double bond as an important feature resulting in planarity right at the ring junction(s).

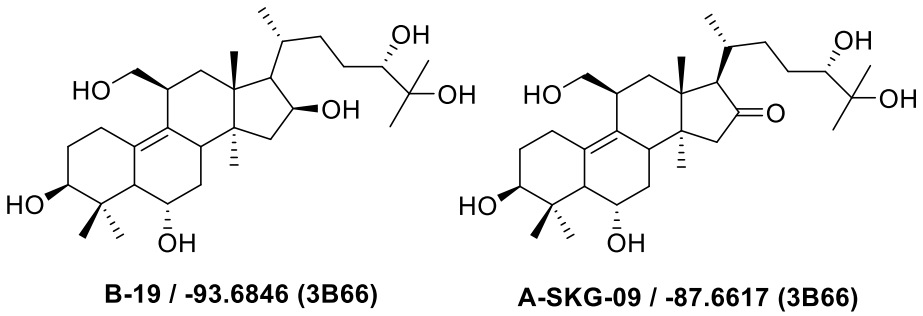
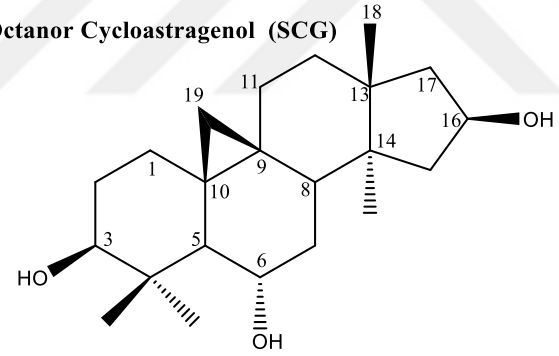


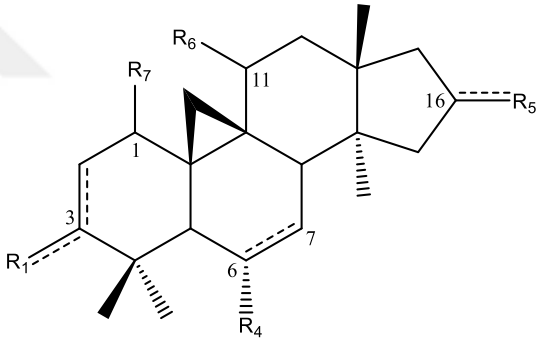
Figure 24. Structures of B-19 and A-SKG-09.

In terms of compounds B-19 and A-SKG-09, the carbonyl group at position 16 was not as significant as 3B66 for the receptor 4HLW (Table 9). For SKG derivatives, all compounds had lower energy scores for 4HLW, whereas all derivatives afforded lower energy scores for 3B66 with the exception of three analogs (A-SKG-05, A-SKG-06 and A-SKG-11). In comparison, none of the derivatives demonstrated a better binding affinity for the receptor 2PNU. This could be because of the active regions chosen based on the structure of the attached ligands, as B66 and TES have similar structures to the selected ligands, ENM has a completely different skeleton.

Table 8. Structures and docking results for SCG derivatives.

20(27)-Octanor Cycloastragenol (SCG)



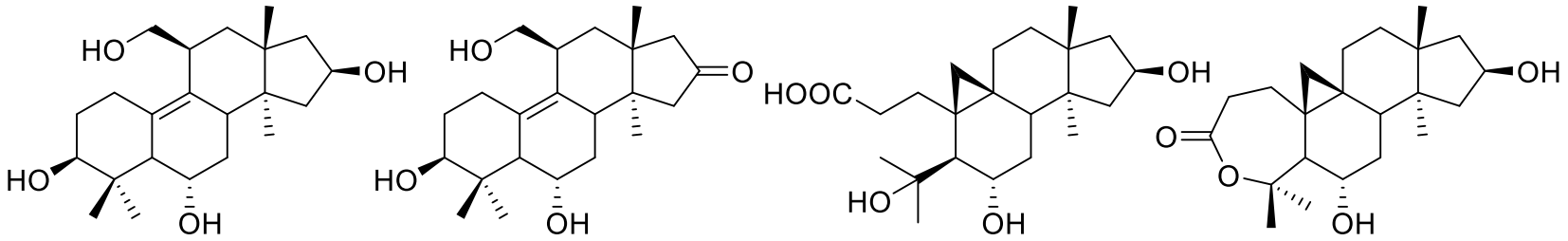


Molecule	R_1	Δ^3	R_5	Δ^6	R_4	R_6	R_7	2PNU-Score	4HLW-Score	3B66-Score
SCG	β-OH	-	β-OH	-	α-OH	-	-	**-96.3123**	**-103.883**	**-77.4279**
SCG-01	β-OH	-	β-Tosylate	-	α-OH	-	-	**72.6865**	**-73.2761**	**-77.4214**
SCG-02	β-OH	-	β-OH	=	-	-	-	**-91.0502**	**-99.6038**	**-87.8567**
SCG-03	-	=	β-OH	-	α-Tosylate	-	-	**42.6462**	**298.379**	**372.947**
SCG-04	β-Tosylate	-	β-Tosylate	=	-	-	-	**495.633**	**37.9733**	**4.71995**
SCG-05	β-OH	-	β-Tosylate	=	-	-	-	**-72.8969**	**-84.2638**	**-71.4388**

(cont. on next page)

Table 8 (cont.)

SCG-06	β-Methanesulfonic acid	-	β-Methanesulfonic acid	=	-	-	-	**-69.9728**	**-85.5674**	**-73.0961**
SCG-07	β-OH	-	β-Methanesulfonic acid	-	-	-	-	**-99.5377**	**-69.9773**	**-82.0078**
Molecule	**R_1**	**Δ^3**	**R_5**	**Δ^6**	**R_4**	**R_6**	**R_7**	**2PNU-Score**	**4HLW-Score**	**3B66-Score**
A-SCG-03	O	-	O	-	α-OH	β-OH	-	**-97.1845**	**-101.58**	**-99.6938**
A-SCG-07	β-OH	-	O	-	-	α-OH	α-OH	**-105.477**	**-112.702**	**-100.838**
Pr-SCA-01	O	-	O	-	α-OH	-	-	**-89.7267**	**-100.323**	**-86.2701**
Pr-SCA-02	β-OH	-	O	-	α-OH	-	-	**-101.441**	**-108.588**	**-96.969**



A-SCG-01 **A-SCG-06** **E-SCG-01** **E-SCG-02**

(cont. on next page)

Table 8 (cont.)

Molecule	2PNU-Score	4HLW-Score	3B66-Score
A-SCG-01	**-97.4149**	**-105.983**	**-98.34**
A-SCG-06	**-102.663**	**-110.002**	**-101.303**
E-SCG-01	**-92.8732**	**-82.6481**	**-69.4922**
E-SCG-02	**-97.1393**	**-103.48**	**-95.179**

Table 9. Structures and docking results for Cyclocanthogenol (SKG) analogs.

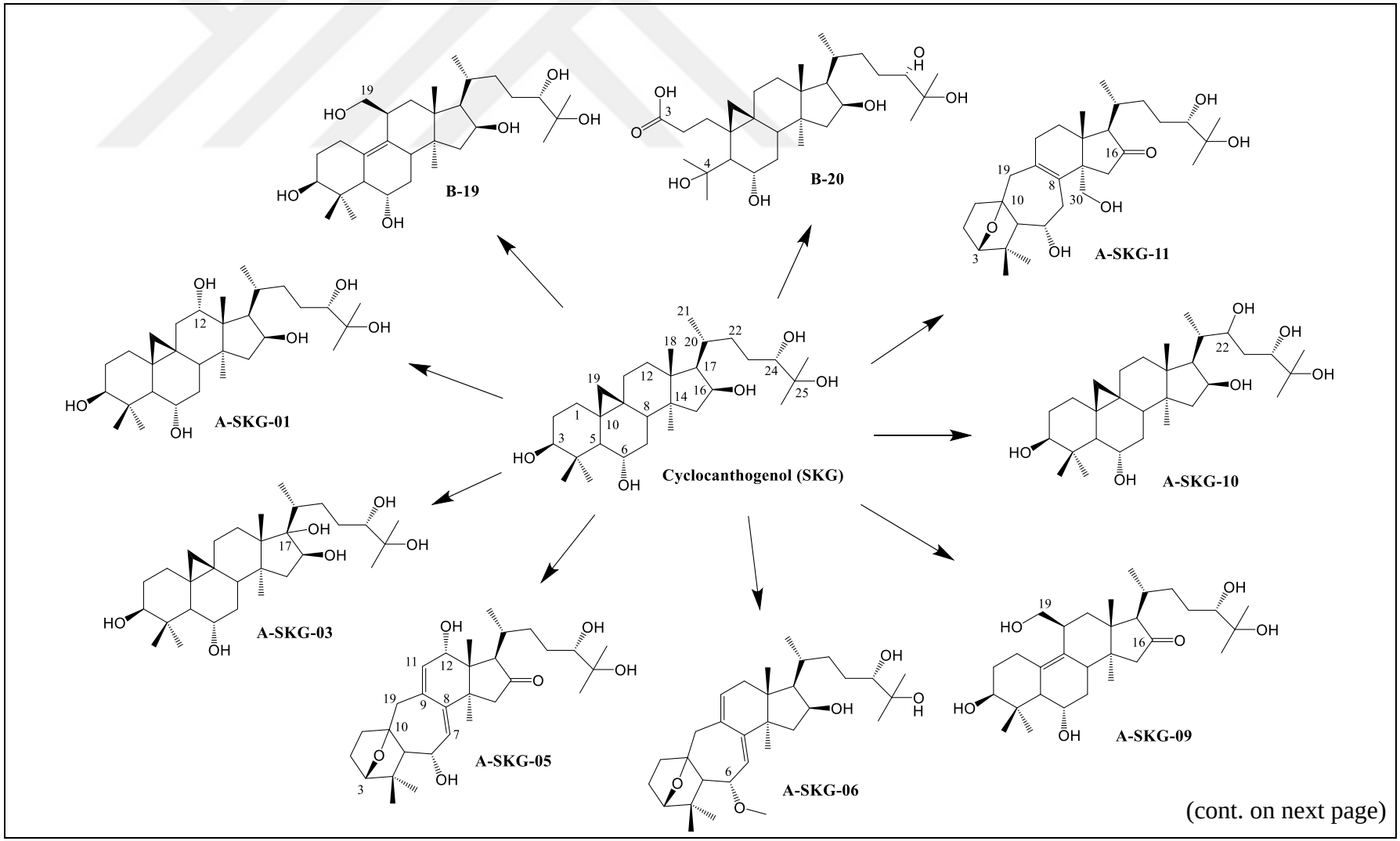


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Table 9 (cont.)

Molecule	2PNU-Score	4HLW-Score	3B66-Score
SKG	**-89.6396**	**-66.173**	**-81.258**
A-SKG-01	**-78.9473**	**-74.1677**	**-85.0596**
A-SKG-03	**-86.3875**	**-74.4945**	**-88.7482**
A-SKG-05	**-78.2695**	**-75.7355**	**-80.6748**
A-SKG-06	**-83.0522**	**-74.3832**	**-75.9534**
A-SKG-09	**-75.3691**	**-78.5788**	**-87.6617**
A-SKG-10	**-79.9754**	**-77.3755**	**-85.7581**
A-SKG-11	**-79.245**	**-77.8744**	**-77.4741**
B-19	**-76.0823**	**-76.3024**	**-93.6846**
B-20	**-84.7876**	**-67.22**	**-81.7675**

3.3. Ligand Docking Results for Estrogen Receptor

The docking results of the receptor model 1A52 was given in Table 10.

Table 10. Docking results of model 1A52

Ligand Name	Total Energy
A-SCG-01	-105.405
A-SCG-06	-103.577
A-SCG-03	-102.818
Pr-SCA-02	-98.583
A-SCG-07	-97.8115
E-SCG-02	-97.4456
Pr-SCA-01	-94.802
SCG-02	-92.6006
C16	-92.2028
Estradiol-Model	-92.1518
A29	-90.8457
A32	-90.5351
A35	-89.5583
Estradiol-Ideal	-89.1129
B19	-88.8604
C34	-88.2
AG-08	-88.0973
C19	-86.5539
E-SCG-01	-86.5183
A-AG-01	-85.9832
B21	-85.8108
B8	-85.4115
SCG	-84.9971
SCG-07	-83.8043
A-CA-07	-83.1297
A-SKG-03	-82.9744
A31	-82.0417
A-SKG-05	-82.0196
C25	-81.7059
B25	-81.6923
C31	-81.663
SCG-05	-81.4747
A-SKG-01	-81.2172
C14	-81.1511

(cont. on next page)

Table 10 (cont.)

SCG-01	-81.094
B20	-80.9276
CG-02	-80.8508
C8	-80.6484
A21	-80.319
A-CA-05	-79.721
C33	-79.4431
A-AG-03	-79.3756
C26	-79.2834
B24	-79.131
A36	-79.1308
C6	-79.0951
A-SKG-10	-79.0251
A-AG-06	-79.0083
C15	-78.9126
A20	-78.8831
A-SKG-06	-78.8556
A-AG-07	-78.6951
A-SKG-11	-78.5669
B3	-78.4621
SKG	-78.1272
A-SKG-09	-78.023
C32	-77.7703
A25	-77.6735
A37	-77.3499
AG-03	-77.0307
AG-02	-77.02
AG	-76.9661
A-CA-04	-76.9157
A19	-76.6376
B23	-76.6303
A3	-76.5964
A7	-76.5796
A5	-76.5782
A17	-76.5773
A10	-76.5681
A13	-76.5627
A2	-76.5621
A14	-76.5586
A4	-76.551
A9	-76.5463
A12	-76.5426
A11	-76.5387

(cont. on next page)

Table 10 (cont.)

A8	-76.5386
A6	-76.5382
A16	-76.5357
A18	-76.529
A15	-76.5061
AG	-76.5021
B22	-76.4102
C13	-76.3423
B1	-75.4659
C22	-75.4113
C11	-75.1916
C17	-75.1276
C18	-75.121
CG-04	-74.9927
C4	-74.8612
C35	-74.8114
B6	-74.747
C36	-74.5227
C7	-74.3354
C24	-73.6617
CA	-73.3868
Nh-CA-02	-73.0688
SCG-06	-73.0334
C3	-72.829
B15	-72.7852
B5	-72.6541
C28	-72.5855
C12	-72.5353
A33	-72.5217
A28	-72.3791
B4	-72.3686
B2	-72.3173
C21	-72.3154
C20	-72.2949
B9	-71.9189
C2	-71.3533
C23	-71.3434
Nh-CA-01	-71.3128
A-CA-02	-70.9916
C29	-70.986
A24	-70.8178
A30	-69.532

(cont. on next page)

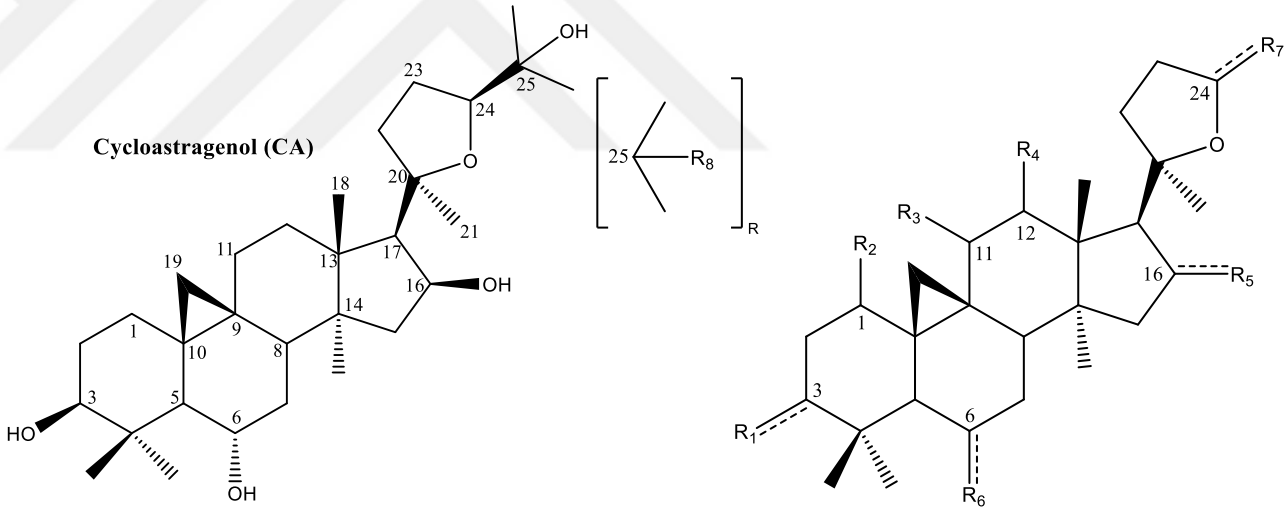
Table 10 (cont.)

C27	-69.4701
C10	-67.1851
MG-01	-62.7807
SCG-03	-31.2223
A26	-9.90001
C5	9.5789
AG-05	18.9157
A27	21.4887
CG-03	43.1912
A22	43.3032
C30	81.4726
CG-05	113.777
SCG-04	145.268
C9	186.493

As in the androgen receptor docking studies, selected ligands for the receptor 1A52 were the derivatives of Cycloastragenol (CA), Astragenol (AG), 20(27)-octanor cycloastragenol (SCG) and Cyclocanthogenol (SKG). All the scores were calculated using the same properties as in the previous section, but rather than using multiple receptor models, the estrogen receptor docking process used only 1A52 model. The main reason for this is the results of the experiments indicate that iGEMDOCK has a success rate of 78 percent (root-mean-square derivations less than 2.0 angstrom) on 305 protein-compound complexes. The virtual screening studies conducted with this software included estrogen receptor α, indicating that the docking program's success rate may be quite accurate against these receptor models, of which 1A52 is one of them. The reference molecules were EST-Model and EST-Ideal, where EST-Model is the three-dimensional structure of Estradiol bound to the receptor 1A52 and EST-Ideal is the computationally optimized version of it. A-SCG-01, A-SCG-06, A-SCG-03, Pr-SCA-02, A-SCG-07, E-SCG-02, Pr-SCA-01, SCG-02 and C16 had lower energy values than Estradiol, indicating that they may have a high probability of binding to estrogen receptor. Furthermore, A29, A32 and A35 scored lower than EST-Ideal suggesting that these molecules could also bind to estrogen receptor. C5, AG-05, A27, CG-03, A22, C30, CG-05, SCG-04 and C9, on the other hand, had scores greater than zero, indicating that these molecules would not bind to the estrogen receptor. Additionally, the molecules that scored higher than the

reference molecules may have a lower affinity for binding to the receptor or may not bind at all. Structures of the selected ligand molecules and their associated receptor energy scores are shown in Tables 11, 12, 13 and 14.

Table 11. Structures and docking results of Cycloastragenol derivatives for receptor 1A52.



Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	1A52-Score
Cycloastragenol (CA)	β-OH	-	-	-	β-OH	α-OH	R	α-OH	**-73.3868**
Nh-CA-01	O	α-OH	-	-	β-OH	α-OH	R	OH	**-71.3128**
Nh-CA-02	O	-	β-OH	-	β-OH	α-OH	R	OH	**-73.0688**
A-CA-02	O	-	-	β-OH	O	α-OH	R	OH	**-70.9916**
A-CA-04	β-OH	-	-	α-OH	β-OH	α-O-SO_3H	R	OH	**-76.9157**

(cont. on next page)

Table 11 (cont.)

Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	1A52-Score
C2	β-Propionate	-	-	-	β-OH	α -Propionate	R	OH	**-71.3533**
C3	β-Butyrate	-	-	-	β-OH	α-Butyrate	R	OH	**-72.829**
C4	β-Isobutyrate	-	-	-	β-OH	α- Isobutyrate	R	OH	**-74.8612**
C5	β-Benzoate	-	-	-	β-OH	α-Benzoate	R	OH	**9.5789**
C6	β-Propionate	-	-	-	O	α -Propionate	O	-	**-79.0951**
C7	β-Butyrate	-	-	-	O	α-Butyrate	O	-	**-74.3354**
C8	β-Isobutyrate	-	-	-	O	α- Isobutyrate	O	-	**-80.6484**
C9	β-Benzoate	-	-	-	O	α-Benzoate	O	-	**186.493**
C10	β-Pivalate	-	-	-	β-OH	α-Pivalate	R	OH	**-67.1851**
C11	β-OH	-	-	-	β-OH	α-Pivalate	R	OH	**-75.1916**
C12	β-Pivalate	-	-	-	β-OH	α-OH	R	OH	**-72.5353**
C13	O	-	-	-	O	O	R	OH	**-76.3423**
C14	β-OH	-	-	-	β-OH	O	R	OH	**-81.1511**

(cont. on next page)

Table 11 (cont.)

Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	1A52-Score
C15	β-OH	-	-	-	β-OH	α-Trifluoroacetate	R	OH	**-78.9126**
C16	β-OH	-	-	-	β- Trifluoroacetate	α-OH	R	OH	**-92.2028**
C17	β-Succinate	-	-	-	β-OH	α-Succinate	R	OH	**-75.1276**
C18	β- Butyrate	-	-	-	β-OH	α-OH	R	OH	**-75.121**
C19	O	-	-	-	O	O	O	-	**-86.5539**
C20	β-Acetate	-	-	-	β-OH	α-Acetate	R	OH	**-72.2949**
C21	β-OH	-	-	-	β-OH	α-Acetate	R	OH	**-72.3154**
C22	β-Acetate	-	-	-	β-OH	α-OH	R	OH	**-75.4113**
C23	β-OH	-	-	-	β-OH	α-Butyrate	R	OH	**-71.3434**
C24	β-Propionate	-	-	-	β-Propionate	α-Propionate	R	Propionate	**-73.6617**
C25	β-Acetate	-	-	-	β-Acetate	α-Acetate	R	Acetate	**-81.7059**
C26	Hydroxyamine	-	-	-	Hydroxyamine	Hydroxyamine	R	OH	**-79.2834**
C27	β-Isobutyrate	-	-	-	β-Isobutyrate	α-Isobutyrate	R	Isobutyrate	**-69.4701**

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Table 11 (cont.)

Molecule	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	1A52-Score
C28	β-Butyrate	-	-	-	β-Butyrate	α-Butyrate	R	OH	**-72.5855**
C29	β-Methoxy	-	-	-	β- Methoxy	α-Methoxy	R	Methoxy	**-70.986**
C30	β-Tosylate	-	-	-	β-OH	α-OH	R	OH	**81.4726**
C31	O	-	-	-	O	α-OH	O	-	**-81.663**
C32	O	-	-	-	O	α-OH	R	OH	**-77.7703**
C33	β-Acetate	-	-	-	β-Acetate	α-Acetate	R	Acetate	**-79.4431**
C34	O	-	-	-	O	O	R	Acetate	**-88.2**
C35	O	-	-	-	O	O	R	Propionate	**-74.8114**
C36	O	-	-	-	O	O	R	Butyrate	**-74.5227**
CG-05	β-Tosylate	-	-	-	β-OH	O	R	OH	**113.777**

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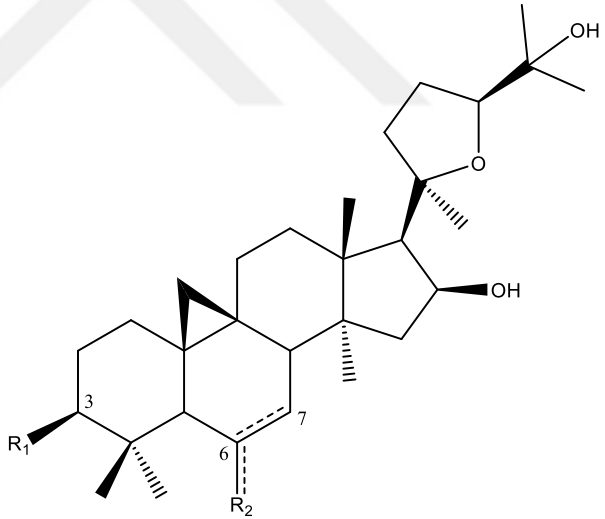
Table 11 (cont.)



Molecule	R_1	R_2	Δ^6	1A52-Score
CG-02	β-OH	-	-	**-80.8508**
CG-03	β-Tosylate	-	-	**43.1912**
CG-04	β-Methylsulfonate	-	-	**-74.9927**
CG-05	β-Tosylate	O	=	**113.777**

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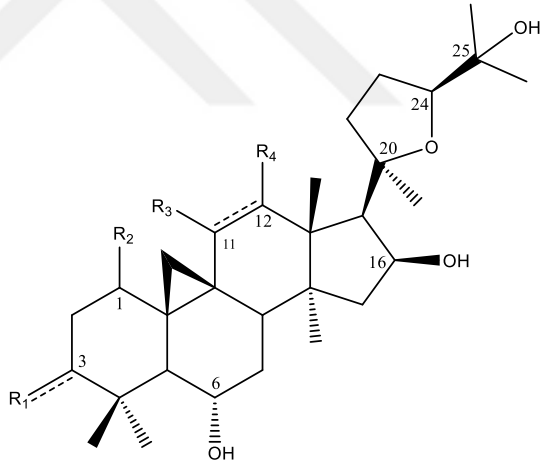
Table 11 (cont.)



Molecule	R_1	R_2	R_3	R4	Δ^6	1A52-Score
B2	β-OH	α-OH	-	-	-	**-72.3173**
B3	β-OH	-	-	-	=	**-78.4621**
B4	β-OH	-	β-OH	-	-	**-72.3686**
B5	β-OH	-	-	β-OH	-	**-72.6541**
B6	β-OH	-	-	β-OH	-	**-74.747**
B9	O	-	-	-	-	**-71.9189**

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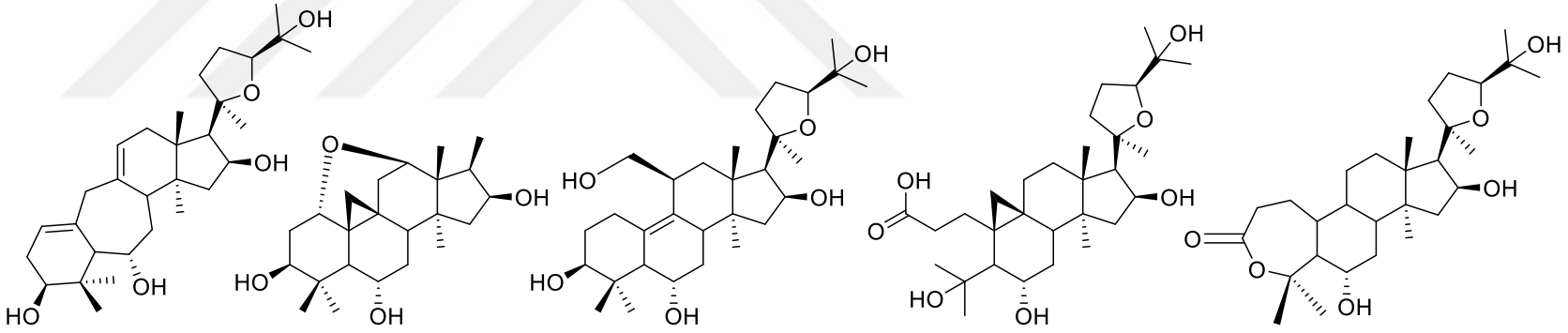
Table 11 (cont.)



A-CA-05 A-CA-07 B-1 B-8 B-15

Molecule	1A52-Score	Molecule	1A52-Score
A-CA-05	-79.721	A-CA-07	-83.1297
B-1	-75.4659	B-8	-85.4115
B-15	-72.7852	-	

Table 11 summarizes the docking results of Cycloastragenol derivatives. B-1, B-6, B-3, B-8, A-CA-04, A-CA-05, A-CA-07, CG-02, CG-04, C4, C6, C7, C8, C11, C13 – C19, C22, C24, C25, C26 and C31 – C36 scored lower than CA. C-34 and C-16 were the molecules scoring the lowest. C-34 has ketone groups at positions 3, 6 and 16 together with acetate group at 25th position. In parallel to androgen receptor scores, the carbonyl groups seem important for lower energy scores. When comparing C34 to C19, the only dissimilarity is acetate including isopropyl group extending from carbon 24 instead of a carbonyl group in C19. Both compounds showed similar scores implying that the modification of tetrahydrofuran side chain is not crucial. The trifluoroacetate group substituted from carbon 16 in C16 afforded a meaningful decrease in energy score. The ability of fluorine atoms to form hydrogen bond might be the overriding basis for such a positive result. On the other hand, C15 possessing a trifluoroacetate group at position 6 provided more positive score than C16, suggesting that the locality of acyl group was important. In CA derivatives, C9 with benzoate groups at positions 3 and 6 had the highest positive score. This positive score could be a result of the molecule's bulkiness as mentioned above for androgen receptor docking. Structures of C34, C19, C16, C15 and C9 are illustrated in Figure 25.

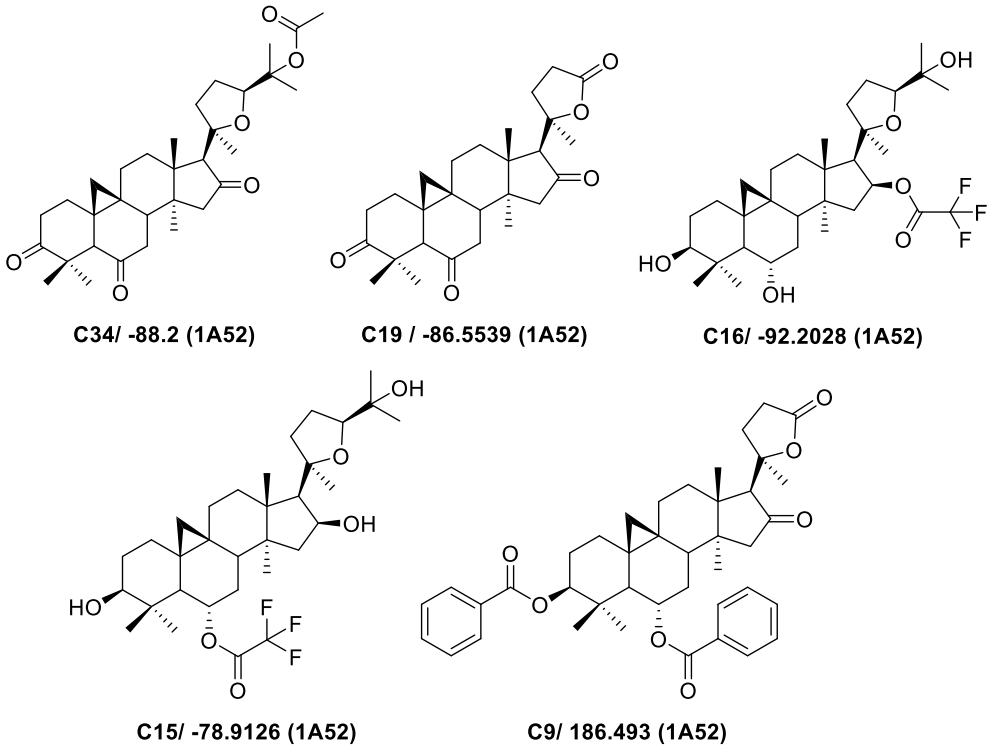


Figure 25. Structures of C34, C19, C16, C15 and C9.

Energy docking results of Astragenol derivatives were illustrated in Table 12. A-AG-01, A-AG-03, A-AG-07, AG-02, AG-03, AG-08, A9, A20, A21, A24, A25, A29, A30, A31, A32, A35, A36 and A37 had energy scores lower than AG. Among these, A29 and A32 (Figure 26) scored the lowest. Both molecules have carbonyl groups on positions of 6, 16 and 24. While A29 contains a ketone group at position 3 together with a chlorine atom extending from carbon 11, A32 possesses a semicarbazide group at 3, and these structural differences do not make a significant alteration in the score. In accordance with previous results, A22 (Figure 26), containing benzoate substitutions at positions 3 and 6, afforded positive scores. This situation lends support that the bulkiness of the benzoate groups might be the overriding basis for reduced estrogen receptor binding affinity. Furthermore, another bulky substituent in AG-05, viz. tosylate at position 3 (Figure 26), results in negative effect on the binding affinity.

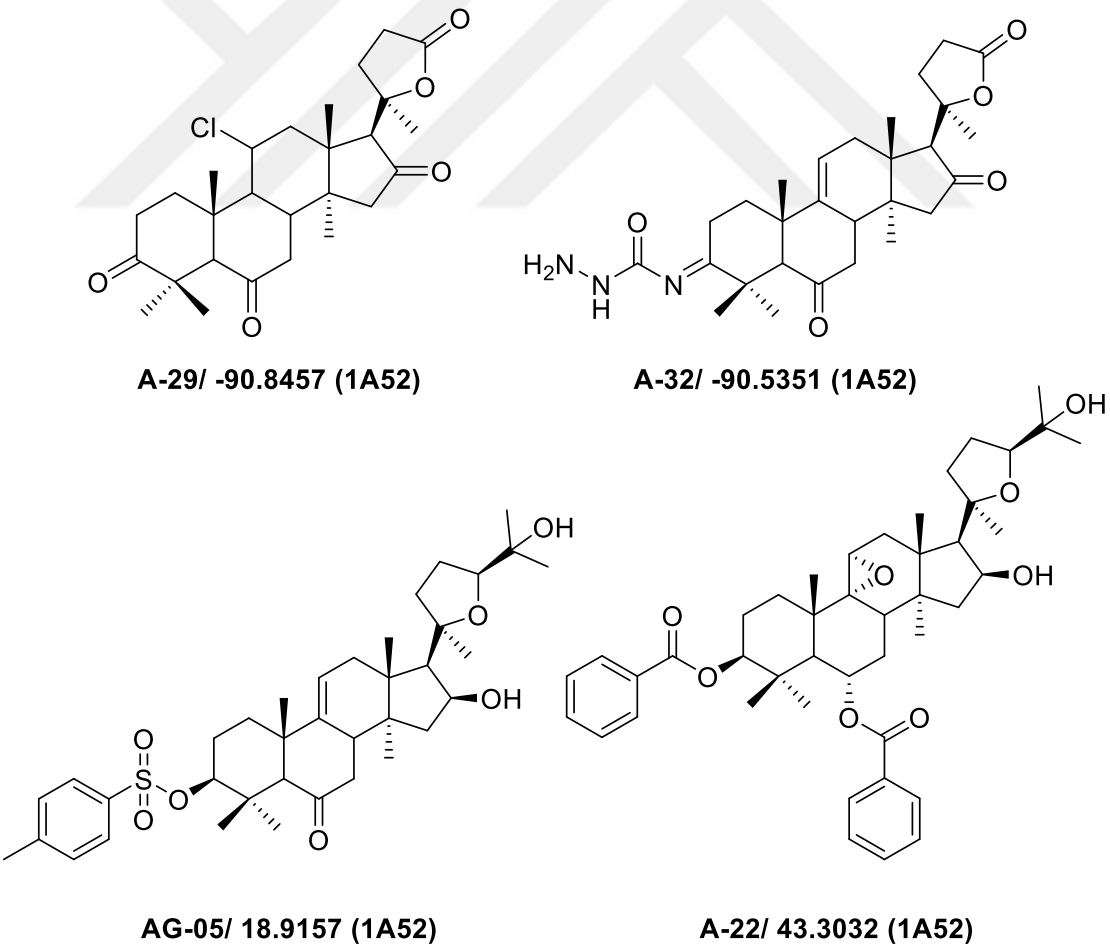
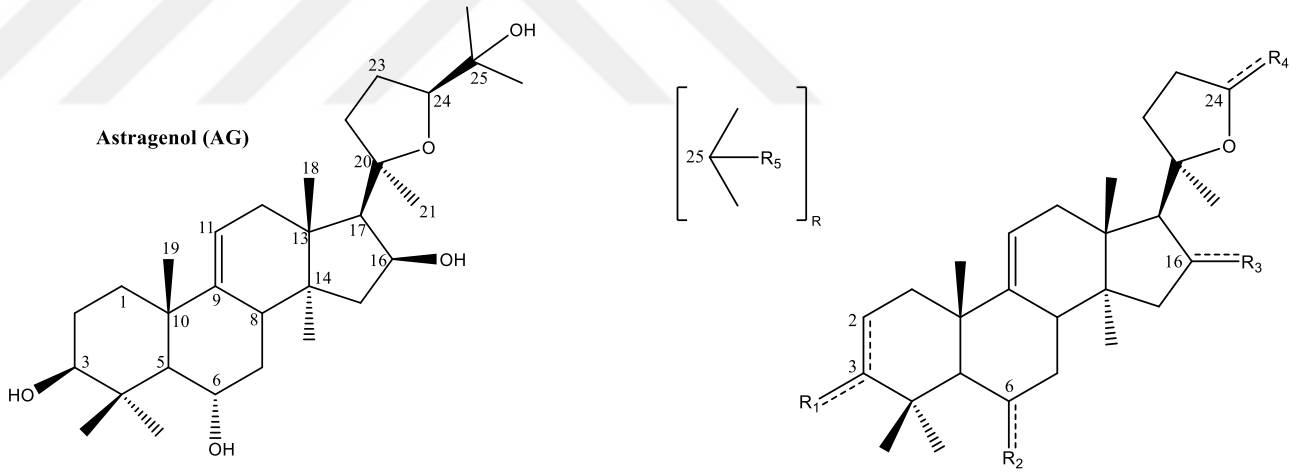


Figure 26. Structures of A29, A32, A22 and AG-05.

Table 12. Structures and docking results of Astragenol derivatives for receptor 1A52



Molecule	R_1	Δ^3	R_2	R_3	R_4	R_5	1A52-Score
Astragenol (AG)	β-OH	-	β-OH	α-OH	R	OH	**-76.9661**
A-AG-01	O	-	β-OH	α-OH	R	OH	**-85.9832**
AG-02	-	=	β-OH	α-OH	R	OH	**-77.02**

(cont. on next page)

Table 12 (cont.)

Molecule	R_1	Δ^3	R_2	R_3	R_4	R_5	1A52-Score
AG-05	β-Tosylate	-	β-OH	O	R	OH	**18.9157**
A2	β-Propionate	-	β-OH	α- Propionate	R	OH	**-76.5621**
A3	β-Butyrate	-	β-OH	α-Butyrate	R	OH	**-76.5964**
A4	O	-	O	O	O	OH	**-76.551**
A5	O	-	O	O	R	OH	**-76.5782**
A6	-	=	β-OH	α-OH	R	OH	**-76.5382**
A7	CH_3	-	β-OH	α-OH	R	OH	**-76.5796**
A8	β-Tosylate	-	β-OH	α-OH	R	OH	**-76.5386**
A10	β-Isobutyrate	-	β-OH	α-Isobutyrate	R	OH	**-76.5681**
A11	β-Isobutyrate	-	β-Isobutyrate	α-Isobutyrate	R	OH	**-76.5387**
A12	β-Benzoate	-	β-OH	α-Benzoate	R	OH	**-76.5426**
A13	β-Benzoate	-	β-OH	α-OH	R	OH	**-76.5627**
A14	β-Pivalate	-	β-OH	α-Pivalate	R	OH	**-76.5586**

(cont. on next page)

Table 12 (cont.)

A15	β-Pivalate	-	β-OH	α-OH	R	OH	**-76.5061**
A16	β-Acetate	-	β-OH	α-Acetate	R	OH	**-76.5357**
A17	β-Acetate	-	β-Acetate	α-Acetate	R	OH	**-76.5773**
A18	β-Acetate	-	β-Acetate	β-Acetate	R	Acetate	**-76.529**
A26	Oxime	-	O	Oxime	R	OH	**-9.90001**
A27	Oxime	-	Oxime	Oxime	R	OH	**21.4887**
A28	O-methyloxime	-	O	O-methyloxime	O	-	**-72.3791**
A31	TSC	-	O	O	O	-	**-82.0417**
A33	O	-	O	O-methyloxime	O	-	**-72.5217**
A35	O	-	O	α-OH	R	OH	**-89.5583**
A37	β-OH	-	β-O-Me	α-OH	R	OH	**-77.3499**

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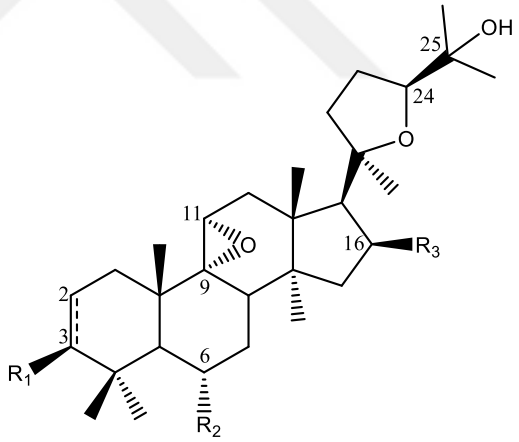
Table 12 (cont.)



Molecule	R_1	R_2	R_3	Δ^3	1A52-Score
A19	β-OH	α-OH	β-OH	-	**-76.6376**
A20	β-Acetate	α-Acetate	β-Acetate	-	**-78.8831**
A21	β-Butyrate	α-OH	β-OH	-	**-80.319**
A22	β-Benzoate	α-Benzoate	β-OH	-	**43.3032**

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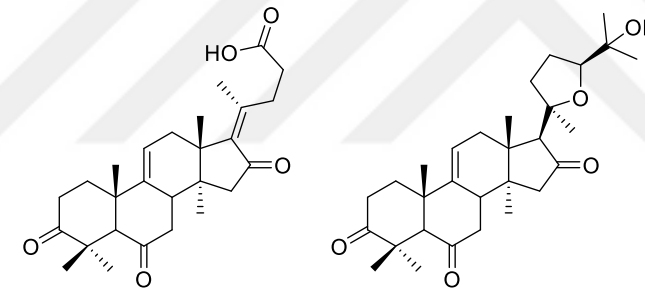
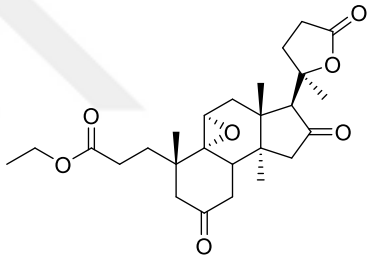
Table 12 (cont.)

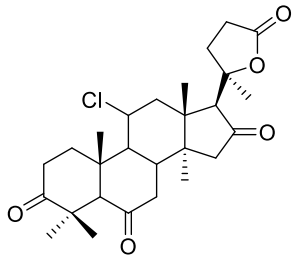


A-9

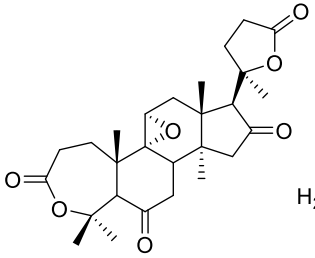
A-24



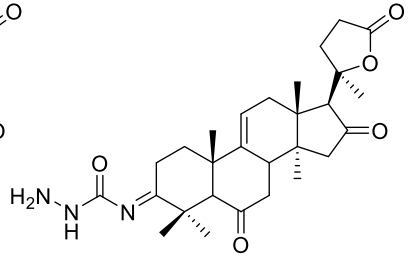
A-25



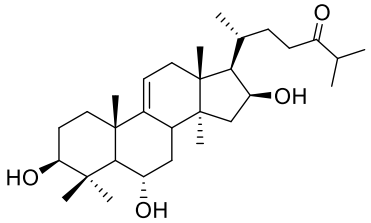
A-29



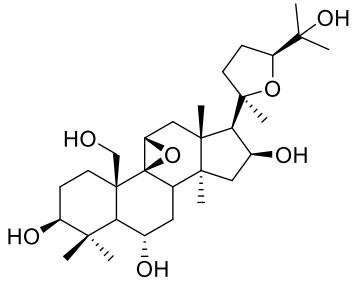
A-30



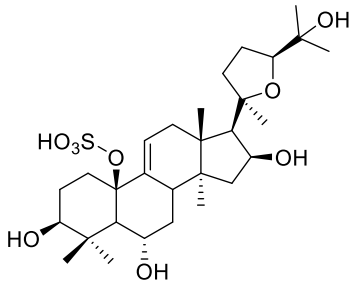
A-32



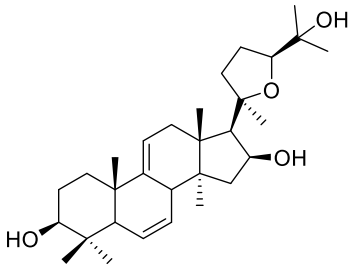
A36



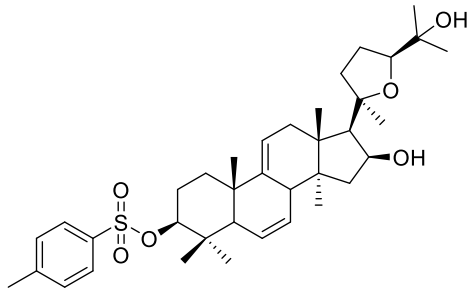
A-AG-03



A-AG-07



AG-03



AG-08

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Table 12 (cont.)

Molecule	1A52-Score	Molecule	1A52-Score
A9	**-76.5463**	**A36**	**-79.1308**
A24	**-70.8178**	**A-AG-03**	**-79.3756**
A25	**-77.6735**	**A-AG-07**	**-78.6951**
A29	**-90.8457**	**AG-03**	**-77.0307**
A30	**-69.532**	**AG-08**	**-88.0973**
A32	**-90.5351**	**-**	

The energy scores obtained as docking results for 20(27)-octanor cycloastragenol (SCG) derivatives are shown in Table 13. SCG-02, A-SCG-03, A-SCG-07, Pr-SCA-01, Pr-SCA-02, A-SCG-01, A-SCG-06, E-SCG-01 and E-SCG-02 provided lower energy scores than SCG. A-SCG-06 and A-SCG-01 were the molecules giving the lowest energy scores compared to other SCG derivatives. The only difference between these molecules is the ketone group present on position 16 in A-SCG-06, instead of hydroxy group in A-SCG-01 (Figure 27). The hydroxymethyl group at position 19 on both of these structures may also contribute to the lower energy scores. As the other low-score molecules, viz. A-SCG-03 and A-SCG-07 (Figure 27), possess hydrophilic substituents (hydroxy groups at C-3 and C-11 positions, respectively) on the upper side of the steroidal backbone, in acordance with our previous findings, such modifications are proposed to be important for interaction with the receptor. SCG-04 (Figure 27), containing tosyl group at positions 3 and 6 together with a double bond in the ring B, had the highest positive score when compared to the other derivatives, again stressing the bulky groups to be avoided for hitting active compounds.

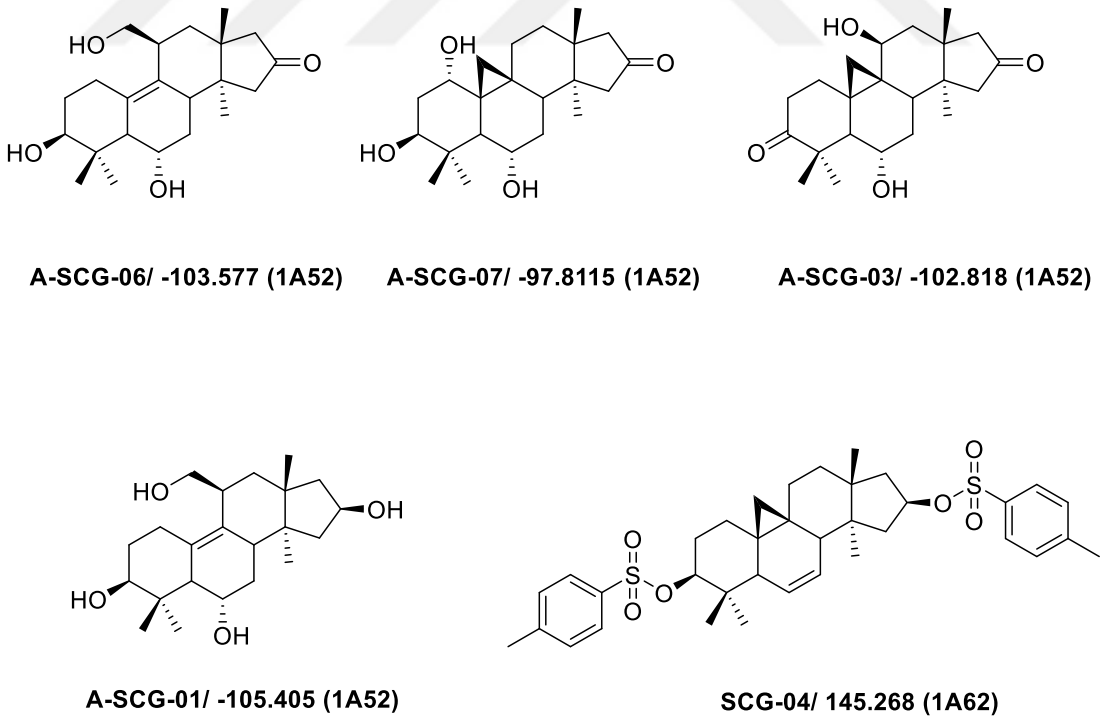
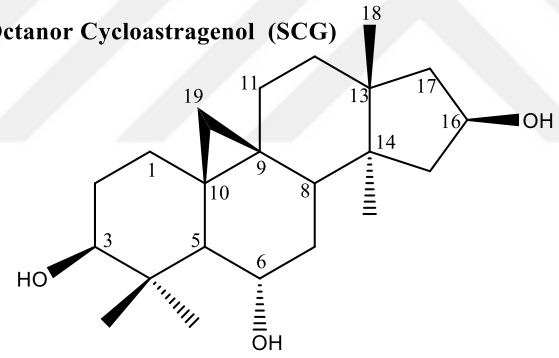


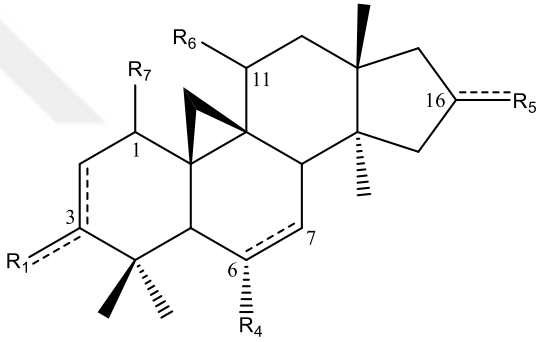
Figure 27. Structures of A-SCG-06, A-SCG-07, A-SCG-03, A-SCG-01 and SCG-04.

The energy scores for cyclocanthogenol derivatives is summarized in Table 14. Except for the A-SKG-09, all derivatives scored lower than SKG but all of the results were similar to SKG. A-SKG-09 (-78.023) and B-19 (-88.8604) shown in figure 24 are structurally similar. The ketone group on position 16 in A-SKG-09 instead of hydroxy group in B-19, provided a higher score compared to the most negative score of B-19. This result is conflicting compared to the results of other sapogenins towards androgen and estrogen receptors including CG, AG and SCG derivatives.

Table 13. Structures and docking results of SCG derivatives for receptor 1A52.

20(27)-Octanor Cycloastragenol (SCG)



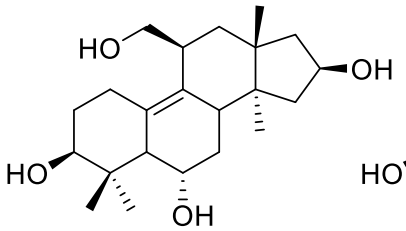


Molecule	R_1	Δ^3	R_5	Δ^6	R_4	R_6	R_7	1A52-Score
SCG	β-OH	-	β-OH	-	α-OH	-	-	**-84.9971**
SCG-01	β-OH	-	β-Tosylate	-	α-OH	-	-	**-81.094**
SCG-02	β-OH	-	β-OH	=	-	-	-	**-92.6006**
SCG-03	-	=	β-OH	-	α-Tosylate	-	-	**-31.2223**
SCG-04	β-Tosylate	-	β-Tosylate	=	-	-	-	**145.268**
SCG-05	β-OH	-	β-Tosylate	=	-	-	-	**-81.4747**
SCG-06	β-Methanesulfonic acid	-	β-Methanesulfonic acid	=	-	-	-	**-73.0334**
SCG-07	β-OH	-	β-Methanesulfonic acid	-	-	-	-	**-83.8043**

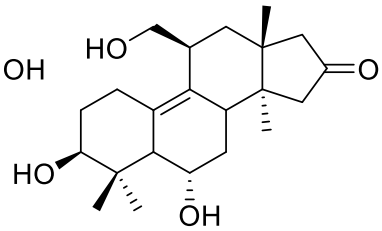
(cont. on next page)

Table 13 (cont.)

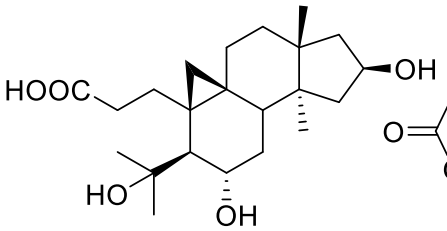
Molecule	R_1	Δ^3	R_5	Δ^6	R_4	R_6	R_7	1A52-Score
A-SCG-03	O	-	O	-	α-OH	β-OH	-	**-102.818**
A-SCG-07	β-OH	-	O	-	-	α-OH	α-OH	**-97.8115**
Pr-SCA-01	O	-	O	-	α-OH	-	-	**-94.802**
Pr-SCA-02	β-OH	-	O	-	α-OH	-	-	**-98.583**



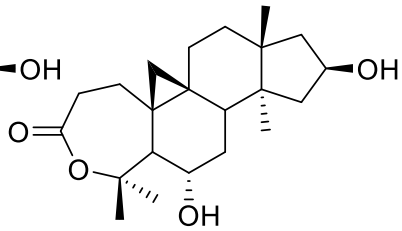
A-SCG-01



A-SCG-06



E-SCG-01



E-SCG-02

Molecule	1A52-Score	Molecule	1A52-Score
A-SCG-01	**-105.405**	**E-SCG-01**	**-86.5183**
A-SCG-06	**-103.577**	**E-SCG-02**	**-97.4456**

Table 14. Structures and docking results of Cyclocanthogenol derivatives for receptor 1A52.

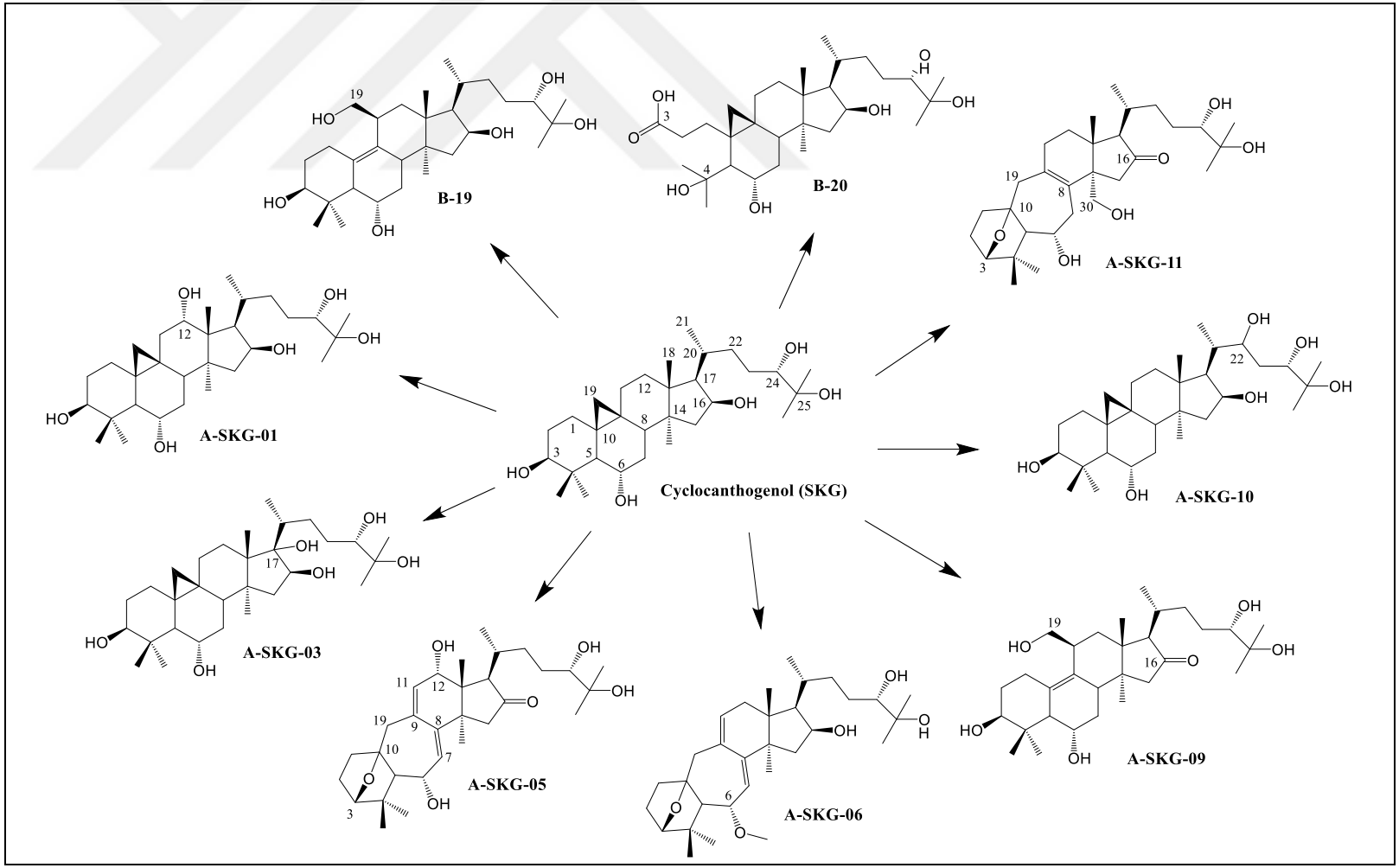


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Table 14 (cont.)

Molecule	1A52-Score	Molecule	1A52-Score
Cyclocanthogenol (SKG)	-78.1272	A-SKG-09	-78.023
A-SKG-01	-81.2172	A-SKG-10	-79.0251
A-SKG-03	-82.9744	A-SKG-11	-78.5669
A-SKG-05	-82.0196	B-19	-88.8604
A-SKG-06	-78.8556	B-20	-80.9276

3.4. Results of Immunoblotting Studies

The docking results indicated that SCG and its analogs had higher binding affinities towards androgen receptors. Thus, the derivatives of SCG with the binding affinities higher than Testosterone in docking studies were chosen for immunoblotting studies. These derivatives were A-SCG-07, A-SCG-06, Pr-SCA-02, A-SCG-01, E-SCG-02, A-SCG-03, Pr-SCA-01 and SCG-02. A-SCG-06, A-SCG-03, Pr-SCA-01 and SCG-02 could not be used in this study due to a lack of sufficient quantity. Generally, amino acid residues and ligands are established to interact via hydroxy and ketone groups present in the molecules. Figure 28 depicts docking poses of SCG, Pr-SCA-02, A-SCG-01, A-SCG-07, E-SCG-02 and Testosterone (TES).

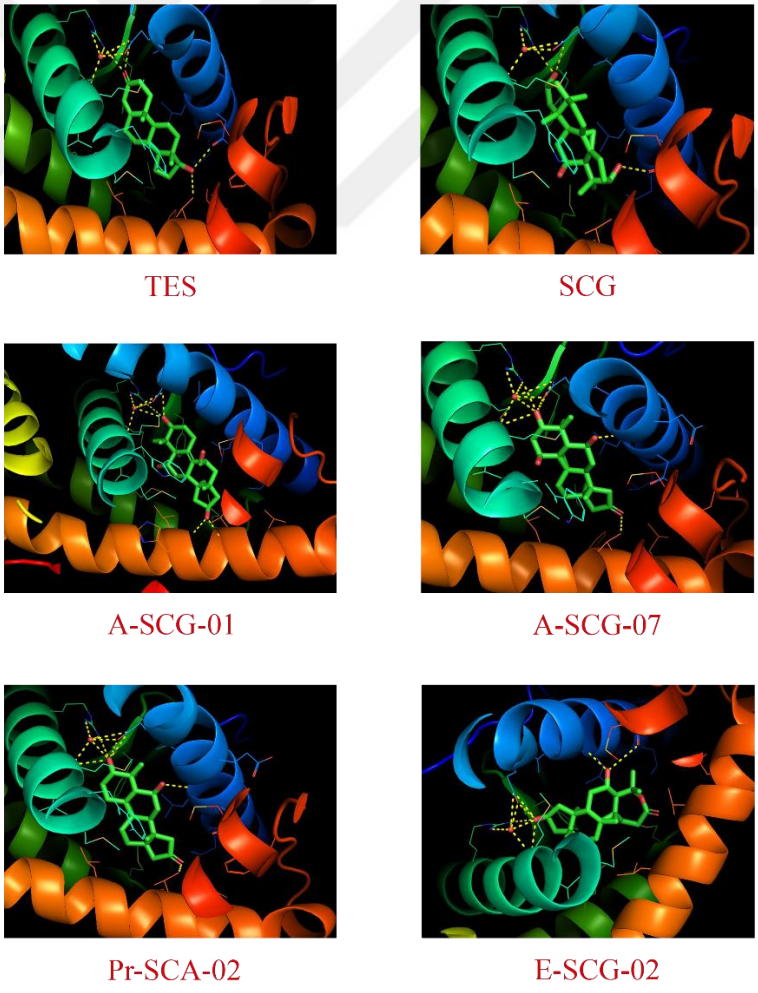

Figure 28. Docking poses of Testosterone, SCG, A-SCG-01, A-SCG-07, Pr-SCA-02 and E-SCG-02.

Based on the iGEMDOCK, interaction table was created from post-screening analysis section (Figure 29). The hydrogen bonding with side chain of GLN-711, Van der Walls interactions with LEU-704 and PHE-764 were found common in tested molecules. Furthermore, A-SCG-07, Pr-SCA-02 and E-SCG-02 exhibited similar interactions compared to Testosterone.

Compound	Energy	H-M LEU 704	H-M ASN 705	H-S ASN 705	H-M GLY 708	H-S GLN 711	H-M MET 745	H-S ARG 752	H-M LEU 873	H-M THR 877	H-S THR 877	V-M LEU 704	V-S LEU 704	V-S LEU 707	V-M GLY 708	V-S GLN 711	V-S MET 742	V-M MET 745	V-S MET 745	V-S MET 749	V-S PHE 764	V-S MET 780	V-S LEU 873	V-S THR 877
		☑	☐	☐	☐	☑	☐	☑	☐	☐	☑	☑	☑	☑	☐	☑	☑	☑	☑	☐	☑	☐	☐	☑
A-SCG-07-6.pdb	-112.7	-2.5	0	0	-3.5	-5.8	-1.7	-3.5	0	0	-2.5	-4.9	-9.6	-4.5	1.4	-4.9	-4.6	-4.5	-6.3	-3.1	-6.7	-3.1	-3.8	-4.3
Pr-SCA-02-3.pdb	-108.6	-2.5	0	0	-3.5	-5.8	-1.7	-3.5	0	0	-2.5	-4.9	-9.6	-4.5	1.4	-4.9	-3.7	-3.9	-5.6	-3.1	-6.7	-3.1	-3.6	-4.3
A-SCG-01-6.pdb	-106	0	0	0	0	-6	0	-1.7	-2.5	-3.5	0	-5.5	-9.4	-3.7	-2.7	-2.8	-5.7	-3.9	-1.7	-2.4	-11	-4	-5.6	-3.2
SCG(Cyclocephagenol)-9.pdb	-103.9	0	-1.6	-2.5	0	-3.5	0	-3.5	0	0	0	-6.5	-10.7	-4.3	-5.5	-2.4	-4.4	-4.8	-4.5	-2.9	-6.3	-2.9	-3.7	-3.4
E-SCG-02-7.pdb	-103.5	-2.5	-3.5	-0.7	0	-6	-2.5	-2.1	0	-1.8	0	-2.1	-10.6	-2.5	-2	-3.2	-4.1	-4.6	-3.5	-4.7	-5.1	-1.9	-7.1	-0.3
testmodel-7.pdb	-98	0	0	-2.5	0	-3.5	0	-3.5	0	0	-2.5	-4.7	-7.1	-3.9	-3.9	-4.6	-5.1	-3.9	-5.4	-2.7	-6.7	-2	-3.6	-4.4

Figure 29. Interaction table created by iGEMDOCK; H: Hydrogen Bond, V: Van der Waals interactions, M: Main Chain, S: Side Chain.

MG-01 was chosen as a negative control because its energy score with the most positive score below zero was significantly greater than that of SCG or Testosterone for all the androgen receptors. Figure 30 shows the effect of SCG molecule on PSA protein under non-starvation (A) and starvation (B) conditions.

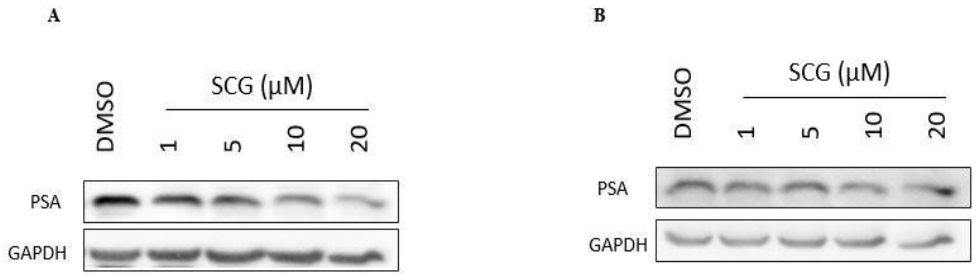

Figure 30. Effect of SCG on PSA protein under non-starvation (A) and starvation (B) conditions. GAPDH was used as the loading control.

In a dose dependent manner, SCG affected PSA protein level notably under non-starvation conditions, whereas a moderate change was observed under starvation conditions too. As shown in Figure 31, the immunoblotting studies on the selected

analogs revealed that A-SCG-07, E-SCG-02, Pr-SCA-02 and A-SCG-01 all had similar effect on PSA levels, but MG-01.

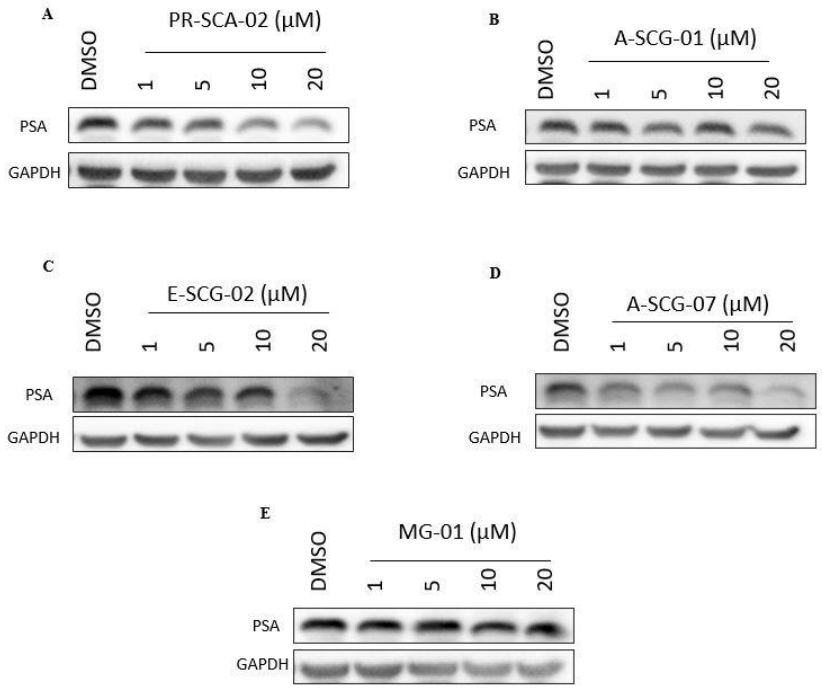

Figure 31. Effects of Pr-SCA-02 (A), A-SCG-01 (B), E-SCG-02 (C), A-SCG-07 (D) and MG-01 (E) on PSA protein levels. GAPDH was used as the loading control.

In parallel to the docking results, A-SCG-07 provided the greatest effect on PSA levels among the test molecules. The structure of MG-01, which is found to be ineffective as expected, is shown in Figure 32.

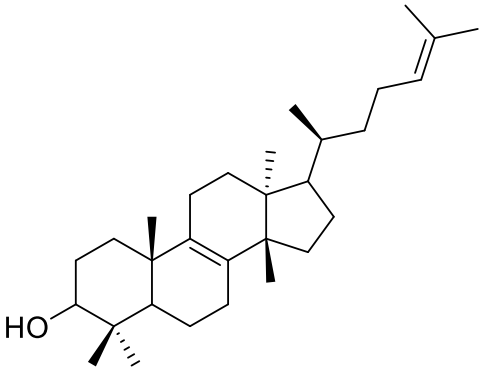

Figure 32. Structure of MG-01.

Consequently, these findings are in consistent with the results of docking studies for the androgen receptor, underscoring importance of the computational chemistry for drug screening studies. However, further in vitro and in-vivo studies are warranted to confirm the observed effects.

CHAPTER 4

CONCLUSION

In this study, sapogenins, derived from the previous projects of our group (TUBITAK 114Z958 and 109S435), and from the thesis of Göklem ÜNER (Üner 2019), were screened against androgen and estrogen receptors via in-silico methods. Initially, all molecules were drawn in two dimensions and then converted and saved as three-dimensional PDB files. Furthermore, all these molecules were screened for their potential targets to predict a probability of binding for estrogen or androgen receptors using the Swiss-Target Prediction web tool. This was followed by the selection of the receptor models and docking process of ideal and model ligands, viz. Testosterone, Estradiol, ENM and B66.

iGEMDOCK software was used for the docking process of the selected sapogenins. Compounds A-SCG-07, A-SCG-06, Pr-SCA-02, A-SCG-01, SCG, E-SCG-02, A-SCG-3, Pr-SCA-01 and SCG-02 had lower energy scores than Testosterone for the androgen receptor, suggesting that these molecules had high probabilities to interact with the androgen receptor.

On the other hand, A-SCG-01, A-SCG-06, A-SCG-03, Pr-SCA-02, A-SCG-07, E-SCG-02, Pr-SCA-01, SCG-02 and C16 had lower energy scores than Estradiol.

Additionally, from medicinal chemistry point of view, the docking results implied that the presence of carbonyl groups in the steroidal framework (i.e., at carbons 3, 16 and 24) is important on the binding affinity for both receptors together with the hydrophilic functional groups substituting from the upper side of the molecules (i.e., positions 1 and 11).

Since SCG and its derivatives had the most negative scores among all derivatives screened including Testosterone, they were selected for immunoblotting studies. The results of the docking studies for these derivatives were in accordance with in vitro findings.

For future work, the absorption, distribution, metabolism, and excretion properties of the potent molecules can be calculated. Studies on molecular dynamics simulations

may be conducted. Additionally, thorough in vitro and in vivo studies should be carried out with the active molecules to confirm our preliminary findings.

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