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Chemometrics approach based on chromatographic behavior, *in silico* characterization and molecular docking study of steroid analogs with biomedical importance

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ABSTRACT

Physicochemical characterization of steroid analogs (triazole, tetrazole, toluenesulfonylhydrazide, nitrile, dinitrile and dione) is considered to be a very important step in further drug selection. This study applies to the determination of lipophilicity of previously synthesized steroid derivatives using reversed-phase high-performance liquid chromatography (RP HPLC). Chemometric aspect of chromatographic lipophilicity is given throughout multiple linear regression (MLR) quantitative structure-retention relationships (QSRR) approach. Minimal inhibitory concentration (MIC) is determined for two steroid derivatives possessing antimicrobial activity against *Staphylococcus aureus*. Molecular docking study was performed in order to identify the compound with the most promising potential as human cytochrome P450 CYP17A1inhibitor. Identified 3β-hydroxyandrost-5-eno[16,17-*d*]-1,2,3-triazole (**I.2.**) could be recommended for further trials for anticancer drugs and subjected to the absorption, distribution, metabolism, excretion and toxicity (ADMET) evaluation.

1. Introduction

Steroid molecules act as ligands or substrates for hormone receptors and enzymes in various biological processes, cell growth and proliferation (Gupta et al., 2013). A number of steroid derivatives have been synthesized to exert the antiproliferative activity against human cancers (Elmegeed et al., 2011; Abdelhalim et al., 2011). Numerous steroid analogs have been proved to have antiproliferative activity against human prostate cancer cell lines (Attard et al., 2005; Moreira et al., 2008; DeVore and Scott, 2012; Yu et al., 2013; Savić et al., 2013; Richmond et al., 2014). Abiraterone is a 17α-hydroxylase inhibitor, approved by U.S. FDA for the treatment of prostate cancer (Wölfling et al., 2004).

The first step in the evaluation of the possible application of synthesized steroid derivatives is the determination of their physicochemical parameters. One of the most important and most analyzed physicochemical parameter of biologically active compounds is lipophilicity, because the intermolecular interactions in biological systems can be compared with the interactions that occur in chromatographic systems (Sârbu et al., 2012; Kovačević et al., 2015). High-performance liquid chromatography (HPLC) has proved to be a method of choice for lipophilicity determination because of its high throughput ability, good accuracy and low sample consumption. Steroids in medicines and

Abbreviations: ACN, acetonitrile; ADMET, absorption, distribution, metabolism, excretion and toxicity; Caco2, *in vitro* Caco2 cell permeability (Human colorectal carcinoma); ANN, artificial neural networks; CADD, computer-aided drug design; DAD, diode array detector; ECCEN, eccentric connectivity index; EI, enzyme inhibitor; FMF, fraction of molecular framework; LR, linear regression; MBC, minimal bactericidal concentration; MDCK, *in vitro* MDCK cell permeability (Madin Darby Canine Kidney); MH agar/broth, Muller-Hinton agar/broth; MIC, minimal inhibitory concentration; MinPA, minimal projection area; MLR, multiple linear regression; MM2, molecular mechanics force field; NRL, nuclear receptor ligand; PC, principal component; PCA, principal component analysis; PC agar, plate count agar; PCR, principal component regression; PLS, partial least squares; PR, polynomial regression; QSAR, quantitative structure–activity relationship; QSPR, quantitative structure–property relationship; QSRR, quantitative structure-retention relationships; RMS, root mean square; RMSD, root mean square deviation; RMSE, root mean square error; RP HPLC, reversed-phase high-performance liquid chromatography; SD agar/broth, Sabouraud dextrose agar/broth; SP, skin permeability; SS, stepwise selection; VIF, variance inflation factor.

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drugs (Lau et al., 2003; Klinsunthorn et al., 2011; Kovačević et al., 2016a), animal feed (Gonzalo-Lumbreras et al., 2007), cosmetics (Gagliardi et al., 2002) and urine (Andersen et al., 2008; Saito et al., 2010) have been studied using HPLC. For chromatographic lipophilicity assessment, logarithm of the retention factor (log*k*) could be used and it can be determined using different software packages (Kovačević et al., 2016a; Virtual Computational Chemistry Laboratory ALOGPS 2.1 Online Program, n.d.). Lipophilicity assessment studies explain more detailed the appearance of molecular lipophilicity phenomenon (Andrić and Héberger, 2015a, 2015b; Andrić et al., 2016).

Microbiological activity of various steroid molecules against bacterial and fungal strains has been published in few papers. Androgen derivatives were tested against *Escherichia coli* and *Staphylococcus aureus* and the absence of antibacterial activity against *S. aureus* was noticed (Figueroa-Valverde et al., 2015). Antimicrobial activity of oxysterols and novel steroidal chalcones was tested against *E. coli*, *S. aureus* and *Candida albicans* and these compounds expressed antimicrobial activity against these two microorganisms (Savage et al., 2012; Shingate et al., 2013; Kakati et al., 2013). Antimicrobial activity of steroids isolated from two *Chromolaena* species and cationic steroid compounds were tested against *E. coli* and *S. aureus* (Taleb-Continiv et al., 2003; Epand et al., 2007). Tested steroid derivatives have antimicrobial activity against *E. coli* and *S. aureus*.

As one of the most commonly used techniques in the structure-based drug design, protein/ligand docking occurs. Molecular docking could be defined as a task to identify the ligand conformation with the most favorable binding energy (Tuccinardi et al., 2014). Structure-based drug design has a task to identify and optimize interactions between ligand and their host molecule, such as protein, given their three-dimensional structures (Bissantz et al., 2010). Docking a small molecule to a bioactive macromolecule in order to measure the interactions between them and visualize the optimal pose of ligand-protein complex is a widely used method in computer-aided drug design (CADD). Molecular docking is widely used for protein inhibitors binding models (Erić et al., 2012; Randjelović et al., 2013; Kozielewicz et al., 2014). Based on molecular docking results, a potential mechanism of action of a selected compound is proposed and further development of this steroid derivative as an anticancer agent is discussed.

The retention behavior of investigated steroid analogs such as triazole, tetrazole, toluenesulfonylhydrazide, nitrile, dinitrile and dione on reversed phase HPLC was investigated (Jevrić et al., 2013). As the investigated steroid analogs have biomedical importance, chromatographic behavior and *in silico* molecular modeling contribute to their characterization and better understanding of the interactions in given chromatographic system.

Therefore, the aim of this study was to assess the chromatographic lipophilicity and select the molecular characteristics that mostly contribute to the behavior of the analyzed steroid analogs in the applied chromatographic system using multiple linear regression (MLR) quantitative structure-retention relationships (QSRR) approach. As the studied steroid derivatives have biomedical importance, the evaluation of the binding affinity of the selected steroid derivatives with the best potential as human cytochrome P450 CYP17A1inhibitor was investigated.

2. Material and Methods

2.1. Investigated Steroid Analogs

The synthesis procedures of the investigated compounds and their antiproliferative activity (IC_{50}) toward androgen receptor negative prostate cancer cell line (PC-3) have been published earlier (Sakač et al., 2008; Sakač et al., 2009; Penov-Gaši et al., 2013; Nikolić et al., 2015). The IUPAC names and chemical structures of the analyzed analogs are presented in Table 1. The set of twenty-nine studied steroid analogs is divided into four groups according to their substituents. The substituents that occur within these four groups are: OH (hydroxyl), O (oxo), AcO (acetoxy) and BnO (benzyloxy) functional groups. The investigated molecules are grouped in the following way: triazole and tetrazole (**I.1 – I.7**), toluenesulfonylhydrazide (**II.8 – II.11**), nitrile and dinitrile (**III.12 – III.27**) and dione (**IV.28** and **IV.29**).

2.2. Chromatographic Instrumentation and Chemicals

Agilent Technologies 1200 Series HPLC system (Santa Clara, California, USA) with diode array detector (DAD) was used for chromatographic analysis. HPLC system contained binary pump, degasser and automatic injector connected to a computer for data processing with AgilentChemStation program. As the stationary phases, chromatographic column ZORBAX Eclipse Plus C8, Rapid Resolution HT (2.1 × 100 mm, 1.8 μm) (Santa Clara, California, USA) was used. Acetone used for dissolving and acetonitrile for mobile phase were both HPLC grade, purchased from J. T. Baker (Deventer, Netherlands). Ultrapure water was obtained using Millipore, Elix UV system and Simplicity Water Purification System (Molsheim, France).

2.3. Chromatographic Procedure

All investigated compounds were dissolved in acetone to obtain a concentration of 1 mg/mL and filtered throughout Captiva Econofilter (nylon membrane, 25 mm diameter, 0.45 μm pore size) (Santa Clara, California, USA). A binary mixture of acetonitrile and water was used as a mobile phase, with acetonitrile volume fractions in the range of 60–90 v/v. Isocratic elutions were performed at the flow rate and injection of 1.0 mL/min and 0.1 μL, respectively. The column temperature was kept at 30 °C. Detection of compounds on DAD was done at 210 nm. Retention data were expressed as the logarithm of retention factor (log*k*) values, defined by Eq. (1), and they were used for chromatographic lipophilicity modeling. All analyses were done in triplicate.

$$\log k = \log\left(\frac{t_r - t_0}{t_0}\right) \quad (1)$$

where t_r is retention time of a compound and t_0 dead time (the retention time of the first peak on the chromatogram).

2.4. Microorganisms and Growth Conditions

Dichloromethane used for dissolving the investigated compounds was purchased from Lach-Ner (Neratovice, Czech Republic). Laminar-flow Biobase Biosafety BSC-1100IIA2-X chamber, Jinan Biobase Biotech Co., Ltd., (Jinan, China) was used for manipulation with microorganisms, as well as for dichloromethane evaporation. The bacterial strains used for this study were: *Escherichia coli* ATCC 25922 (gram-negative) and *Staphylococcus aureus* ATCC 25923 (gram-positive), while *Candida albicans* ATCC 10231 was chosen as test yeast strain. All microorganisms were maintained according to ISO11133:2014 standard. Muller-Hinton broth (MHB, HiMedia, Mumbai, India) for bacterial strains and Sabouraud dextrose broth (SDB, HiMedia, Mumbai, India) was inoculated with working cell suspension made from the stocked strains that are kept in deep freezer at − 80 °C (Snijders Labs, Tiliburg, Holland) and incubated at 37 °C.

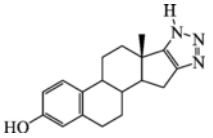
2.5. Antimicrobial Assay

A modified microdilution method (standard broth micro-dilution technique as per CLSI guidelines) in a series of glass test tubes

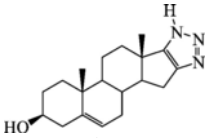
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Table 1
IUPAC names and chemical structures of investigated steroids.

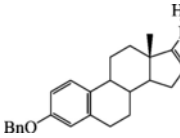
I.1. 3-Hydroxyestra-1,3,5(10)-trieno[16,17-*d*]-1,2,3-triazole



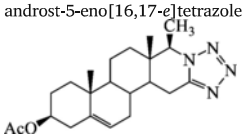
I.2. 3β-Hydroxyandrost-5-eno[16,17-*d*]-1,2,3-triazole



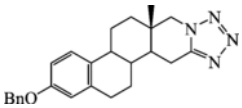
I.3. 3-Benzyloxyestra-1,



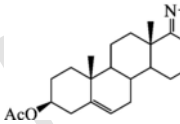
I.5. 3β-Acetoxy-17-aza-17a-homo-17aβ-methyl-androst-5-eno[16,17-*e*]tetrazole



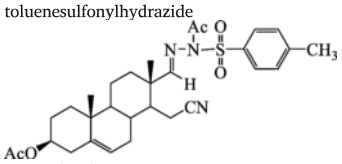
I.6. 3-Benzyloxy-17-aza-17a-homoestra-1,3,5(10)-trieno[16,17-*e*]tetrazole



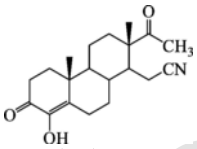
II.9. 3β-Acetoxy-17-oxo toluenesulfonylhydrazid



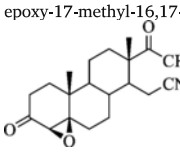
II.11. 3β-Acetoxy-*N´*-acetyl-17-oxo-16,17-secoandrost-5-ene-16-nitirile *p*-toluenesulfonylhydrazide



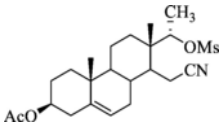
III.12. 3,17-Dioxo-4-hydroxy-17-methyl-16,17-secoandrost-4-ene-16-nitrile



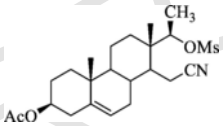
III.13. 3,17-Dioxo-4β,5β epoxy-17-methyl-16,17-



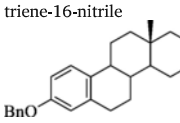
III.15. (17*S*)-3β-Acetoxy-17-(methylsulfonyloxy)-17-methyl-16,17-secoandrost-5-ene-16-nitrile



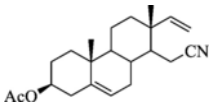
III.16. (17*R*)-3β-Acetoxy-17-(methylsulfonyloxy)-17-methyl-16,17-secoandrost-5-ene-16-nitrile



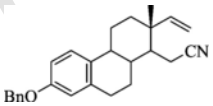
III.17. 3-Benzyloxy-17-(methyl triene-16-nitrile



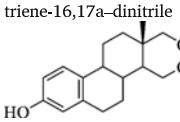
III.19. 3β-Acetoxy-17-methylene-16,17-secoandrost-5-ene-16-nitrile



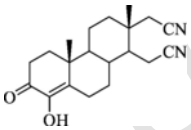
III.20. 3-Benzyloxy-17-methylene-16,17-secoestra-1,3,5(10)-triene-16-nitrile



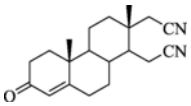
III.21. 3-Hydroxy-17a-h triene-16,17a–dinitrile



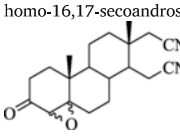
III.23. 4-Hydroxy-3-oxo-17a-homo-16,17-secoandrost-4-ene-16,17a–dinitrile



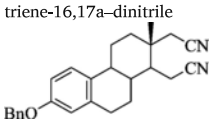
III.24. 3-oxo-17a-homo-16,17-secoandrost-4-ene-16,17a–dinitrile



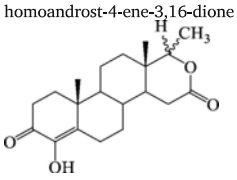
III.25. 4α,5α- and 4β,5β homo-16,17-secoandros



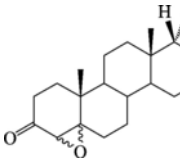
III.27. 3-Benzyloxy-17a-homo-16,17-secoestra-1,3,5(10)-triene-16,17a–dinitrile



IV.28. 17aα- and 17aβ-methyl-4-hydroxy-17-oxa-homoandrost-4-ene-3,16-dione



IV.29. 4α,5α- and 4β,5β



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(11 × 7.5 cm) was used to determine the minimum inhibitory concentration (MIC) of the investigated steroid analogs (CLSI, 2002; CLSI, 2012). Investigated compounds were dissolved in cooled dichloromethane in eppendorf tubes in the initial concentration of 10 mg/mL. From the initial solution serial dilution (1:1) was made up to the final concentration 0.078 μg/mL by using the same solvent. For one test microorganism 50 μL of substance solution was used. After transferring aliquots (50 μL) directly on the bottom of the sterile glass test tubes they are exposed to the air flow in a laminar-flow chamber until the entire volume of the solvent evaporates. For all tested microorganisms a serial dilution in MH/SD broth was made from the culture in the late exponential growth phase. In each tube 100 mL of cell suspension (approx. Conc. 3×10^3 CFU/mL) prepared in MH/SD broth was poured and incubated for 24 h at 37 °C. After the incubation and homogenization a serial dilution up to 1:1000 were prepared in BPW (BPW, HiMedia, Mumbai, India). An aliquot (100 μL) from each prepared suspension were streaked onto the MH/SD agar plates which were incubated for 24–48 h at 37 °C. After the incubation colonies were counted and the number of cell was determined and expressed as CFU/mL. The concentration of antimicrobial agent that led to the death of > 90% of cells is considered to be the minimum inhibitory concentration, while those that led to the death of > 99.99% of cells is considered to be the minimum bactericidal concentration (MBC). All analyses were done in triplicate.

2.6. Molecular Descriptors Calculation and Selection

Molecular structure was designed using MarvinSketch 15.3.26 (MarvinSketch 15.3.26, 2013) for 2D structure and ChemBio3D Ultra 12.0 (Cambridge Soft Corporation, Perkin Elmer Inc., 2012) for 3D structure. For the calculation of 380 molecular descriptors following programs were used: ChemBioDraw Ultra 12.0 (Cambridge Soft Corporation, Perkin Elmer Inc., 2012), ChemBio3D Ultra 12.0 (Cambridge Soft Corporation, Perkin Elmer Inc., 2012), PaDEL Descriptor (PaDEL Descriptors, n.d.), ALOGPS 2.1 (Virtual Computational Chemistry Laboratory ALOGPS 2.1 Online Program, n.d.), PreADMET online program (PreADMET Software, n.d.), Molinspiration online program (Molinspiration Cheminformatics, n.d.), MarvinSketch 15.3.26 (MarvinSketch 15.3.26, 2013). The stepwise selection (SS) method was used for determining the adequate molecular descriptors for MLR modeling. In the stepwise selection, after each step descriptor is added in the model and all others are being checked for their significance. The criterion for adding or removing the variables was the root mean square error (RMSE). Minimum RMSE was set at 0.05 and each descriptor that increased the RMSE was removed from the model. Descriptors selected with this method were used as input data for MLR model establishment. All molecular descriptors were calculated on the basis of 2D structures and structural optimization and energy minimization were not needed. Structural optimization and energy minimization were done in the case of molecular descriptors calculated by ChemBio3D Ultra 12.0 (Cambridge Soft Corporation, Perkin Elmer Inc., 2012). After 3D structures building, energy minimization had to be done using molecular mechanics force field method (MM2). The energy minimization was performed until the root mean square (RMS) value reached a value smaller than 0.1 kcal/Å mol.

The values of the selected calculated descriptors that contribute to the QSRR modeling are shown in Supplementary Data (Table S1). Table includes lipophilicity (ALOG*P*s, ALOGp*S* and ALogP), ADMET (absorption, distribution, metabolism, excretion and toxicity) (SP – skin permeability; Caco2 - *in vitro* Human colorectal carcinoma cell permeability; MDCK - *in vitro* Mandin Darby Canine Kidney cell permeability; EI - enzyme inhibitor; NRL - nuclear receptor ligand), geometrical (MinPA – minimal projection area), topological (ECCEN – eccentric connectivity index) and physicochemical descriptors (FMF – fraction of molecular framework).

2.7. Chemometric Techniques

Principal component analysis (PCA) is an statistical analysis technique mostly used to reduce the amount of data and/or obtain orthogonal variables, especially in the presence of collinearity (Estrada and González, 2003; González-Díaz et al., 2004; González-Díaz et al., 2007a, 2007b). It gives insight into the existence of regularities in the big data sets giving the information regarding compounds that behave in a similar way. The result of PCA analysis is shown using scores and loadings plots. Scores are the new coordinates of the projected objects and loadings that reflect the direction with respect to the original variables (Kovačević et al., 2013). The results of this kind of analysis could be used as input data for other multivariate techniques instead of original variables (Tewari and Irudayaraj, 2005; Ciosek and Wróblewski, 2006). PCA analysis was carried out using log*k* values for all five volume fractions in order to group similar compounds.

Multiple linear regression (MLR) is the most common used regression method that has found wide application in QSRR (Fragkaki et al., 2009; Đaković-Sekulić et al., 2011; Karadžić et al., 2015a, 2015b), QSAR (quantitative structure–activity relationship) (Dudek et al., 2006; Pourbasheer et al., 2010; Minovski et al., 2011; Podunavac-Kuzmanović et al., 2012) and QSPR (quantitative structure–property relationship) (Luilo and Cabaniss, 2010) modeling. MLR model includes more than one independent variable and in order to avoid over-parametrization and to properly interpret model and descriptors influence it is necessary to avoid occurrence of multicollinearity using variance inflation factor (*VIF*). *VIF* > 1 indicates the present of multicollinearity to some extent while the literature tolerates *VIF* < 10 (O'Brien, 2007; Young et al., 2008). When forming MLR model the ratio of molecular descriptors and the number of data (number of tested compounds) that participate in the formation of the model must be taken into account. This relationship is defined by Topliss-Costello rule (Topliss and Costello, 1972):

$$\frac{N}{n} \geq 5 \tag{2}$$

where *N* is the number of data points and *n* is the number of molecular descriptors. If a large number of molecular descriptors is introduced to the model that can lead to destabilization of the model and to the reduction of its predictive ability.

Established models had to be statistically validated. As the first step in the selection of the generated models standard statistical parameters were used and external validation was done in order to reflect relevance (Chiro and Gramatica, 2011; Chiro and Gramatica, 2012; Gramatica and Sangion, 2016).

2.8. Molecular Docking

Four selected steroid derivatives were drawn with ChemBioDraw Ultra 12.0, converted in 3D structure, subjected to energy minimization and saved as .mol2 files for docking simulation (Cambridge Soft Corporation, Perkin Elmer Inc., 2012). PDB structure (PDB: 3RUK) of human cytochrome P450 CYP17A1 in complex with abiraterone retrieved from Protein Data Bank database was prepared for docking procedure by removing chain A (Berman et al., 2000). Docking was done using protein monomer because only residues from individual monomers directly interact with the substrate (Kovačević et al., 2016b). Water molecules were also removed, missing hydrogens were added, correct bond types were set and Gasteiger charges were assigned. The residues within the 5 Å radius around the ligand molecule

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were selected. Visualization, modeling and molecular docking procedure were conducted using OpenEye Babel software (Babel, 2010), Chimera software (UCSF Chimera, 2004) and DOCK 6.5 software (UCSF DOCK 6.5, 1998).

3. Results and Discussion

3.1. Chromatographic Retention

Retention data expressed as the logarithm of the retention factor (log*k*) values are presented in Table 2. In Supplementary Data (Fig. S1) log*k* values are shown for all investigated mobile phase combinations.

It can be noticed that as the organic solvent volume fraction increases, the retention time decreases. Regarding that, the highest log*k* values were obtained when acetonitrile volume fraction was 60 v/v. Additionally, polarity increases going on from the first to the last molecule within each group. In accordance with polarity, all investigated compounds behave according to the polarity of the functional groups present in molecules and the following order could be established: benzyloxy < acetoxy < oxo < hydroxyl. All of the above is compatible with general chromatographic theory (Kovačević et al., 2016a).

The influence of the introduction or substitution of the functional groups on the retention could be best described comparing the compounds that differ in the presence of selected substituent. Compounds paired in this way and their Δlog*k* values are presented in Table 3. The introduction of the methyl group, as it is the case with pairs **I.4** and **I.5**, **I.6** and **I.7**, **III.14** and **III.15**, **III.14** and **III.16**, **III.17** and **III.18**, leads to the retention increase. If hydroxyl group in position 3 is replaced by benzyloxy group (pair **III.21** and **III.27**) and by acetoxy group (pair **III.22** and **III.26**) the retention significantly increases.

Linear regression parameters between logk values and acetonitrile volume fraction for both organic solvents are shown in Supplementary Data (Table S2).

3.2. Determination of Minimal Inhibitory Concentration

Solubility in water was calculated for investigated steroid analogs using computer software (MarvinSketch 15.3.26, 2013). According to MarvinSketch referent values, all compounds were organized in three groups as presented in Supplementary Data (Table S3). The first group includes compounds with high solubility in water (> 0.06 mg/mL), than with moderated solubility (0.01–0.06 mg/mL) and compounds with low solubility in water (< 0.01 mg/mL). Antimicrobial activity of

Table 2
Logarithm of the retention factor (log*k*) for investigated compounds (acetonitrile-water ratio).

Compound	log*k* (± SD)				
	60:40	70:30	75:25	80:20	90:10
I.1	[a]	[a]	[a]	[a]	[a]
I.2	− 0.787 (± 0.000)	− 0.925 (± 0.001)	− 1.289 (± 0.001)	− 1.427 (± 0.000)	− 1.532 (± 0.001)
I.3	0.429 (± 0.000)	0.033 (± 0.004)	− 0.164 (± 0.000)	− 0.359 (± 0.005)	− 0.798 (± 0.000)
I.4	0.133 (± 0.001)	− 0.202 (± 0.002)	− 0.367 (± 0.001)	− 0.522 (± 0.001)	− 0.871 (± 0.003)
I.5	0.269 (± 0.000)	− 0.071 (± 0.002)	− 0.235 (± 0.007)	− 0.394 (± 0.001)	− 0.735 (± 0.003)
I.6	0.429 (± 0.000)	0.040 (± 0.001)	− 0.144 (± 0.002)	− 0.331 (± 0.001)	− 0.698 (± 0.002)
I.7	0.555 (± 0.001)	0.143 (± 0.007)	− 0.056 (± 0.001)	− 0.246 (± 0.000)	− 0.677 (± 0.001)
II.8	− 0.265 (± 0.002)	− 0.726 (± 0.000)	− 0.952 (± 0.002)	− 1.206 (± 0.007)	− 1.637 (± 0.001)
II.9	[a]	[a]	[a]	[a]	[a]
II.10	0.738 (± 0.001)	0.241 (± 0.002)	− 0.005 (± 0.000)	− 0.253 (± 0.005)	− 0.849 (± 0.004)
II.11	0.744 (± 0.001)	0.290 (± 0.002)	0.072 (± 0.002)	− 0.141 (± 0.002)	− 0.623 (± 0.001)
III.12	[a]	[a]	[a]	[a]	[a]
III.13	− 0.266 (± 0.001)	− 0.604 (± 0.003)	− 0.787 (± 0.004)	− 0.912 (± 0.004)	− 1.474 (± 0.000)
III.14	0.188 (± 0.001)	− 0.198 (± 0.002)	− 0.401 (± 0.000)	− 0.600 (± 0.001)	− 1.054 (± 0.002)
III.15	0.236 (± 0.001)	− 0.160 (± 0.001)	− 0.359 (± 0.000)	− 0.560 (± 0.006)	− 0.996 (± 0.002)
III.16	0.304 (± 0.001)	− 0.082 (± 0.006)	− 0.271 (± 0.000)	− 0.465 (± 0.003)	− 0.865 (± 0.001)
III.17	0.494 (± 0.002)	0.047 (± 0.002)	− 0.179 (± 0.000)	− 0.408 (± 0.003)	− 0.930 (± 0.004)
III.18	0.596 (± 0.003)	0.146 (± 0.003)	− 0.076 (± 0.002)	− 0.300 (± 0.001)	− 0.854 (± 0.001)
III.19	0.740 (± 0.004)	0.357 (± 0.002)	0.176 (± 0.001)	− 0.001 (± 0.001)	− 0.384 (± 0.003)
III.20	1.028 (± 0.001)	0.568 (± 0.001)	0.348 (± 0.004)	0.140 (± 0.001)	− 0.318 (± 0.007)
III.21	− 0.557 (± 0.001)	− 0.977 (± 0.000)	− 1.307 (± 0.004)	− 1.905 (± 0.003)	− 1.980 (± 0.003)
III.22	− 0.572 (± 0.002)	− 0.929 (± 0.004)	− 1.225 (± 0.002)	− 1.507 (± 0.000)	− 2.201 (± 0.006)
III.23	− 0.419 (± 0.000)	− 0.815 (± 0.001)	− 0.962 (± 0.007)	− 1.405 (± 0.003)	− 2.377 (± 0.004)
III.24	− 0.461 (± 0.006)	− 0.864 (± 0.001)	− 0.979 (± 0.002)	− 1.271 (± 0.001)	− 1.980 (± 0.004)
III.25	− 0.139 (± 0.002)	− 0.737 (± 0.001)	− 0.881 (± 0.002)	− 1.114 (± 0.005)	− 1.980 (± 0.000)
III.26	0.194 (± 0.001)	− 0.171 (± 0.004)	− 0.353 (± 0.000)	− 0.541 (± 0.004)	− 0.929 (± 0.000)
III.27	0.511 (± 0.000)	0.075 (± 0.001)	− 0.145 (± 0.004)	− 0.361 (± 0.003)	− 0.827 (± 0.006)
IV.28	[a]	[a]	[a]	[a]	[a]
IV.29	− 0.226 (± 0.001)	− 0.539 (± 0.003)	− 0.704 (± 0.002)	− 0.871 (± 0.001)	− 1.187 (± 0.001)

[a] Compounds coeluted with acetone.

Table 3
Δlog*k* values for introduction or substitution of the functional groups.

Compounds to compare	Differences	Δlog*k* (φ = 0.60)	Δlog*k* (φ = 0.70)	Δlog*k* (φ = 0.75)	Δlog*k* (φ = 0.80)	Δlog*k* (φ = 0.90)
I.4 and **I.5**	17-CH_3	0.136	0.131	0.132	0.128	0.136
I.6 and **I.7**	17-CH_3	0.126	0.103	0.088	0.085	0.021
III.14 and **III.15**	17-CH_3	0.048	0.038	0.042	0.040	0.058
III.14 and **III.16**	17-CH_3	0.116	0.116	0.130	0.135	0.189
III.17 and **III.18**	17-CH_3	0.102	0.099	0.103	0.108	0.076
III.21 and **III.27**	3-OH → 3-BnO	1.068	1.052	1.162	1.544	1.153
III.22 and **III.26**	3-OH → 3-AcO	0.766	0.758	0.872	0.966	1.272

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twenty analyzed steroid derivatives was investigated using modified microdilution method. For microbiological tests randomly selected derivatives from three groups were used. In blank test (non-volatile residues of pure solvent) and control test (MHB/SDB broth) intensified growth and reproduction were noticed according to turbidity that was confirmed with the streaking the aliquot onto MHA/SDA following the incubation for 24–48 h at 37 °C. A number of colonies on the surface were too large and the growth was too thick. In this way, the confirmation of the proper solvent selection was confirmed and demonstrated that non-volatile residues of the solvent do not affect the inhibition of used test cultures. The results of minimal inhibitory concentration investigation with respect to *S. aureus* are shown in Fig. 1.

Only two compounds have shown the bacteriostatic effect toward *S. aureus*, compound **III.25** with moderated solubility in water and **IV.29** with high solubility in water. Compound **III.25** has shown the bacteriostatic effect at the minimal inhibitory concentration of 0.15625 mg/mL and compound **IV.29** at 5 mg/mL. These two molecules with detected antimicrobial activity have an epoxy group as one of the most reactive groups in both molecules. Molecule **III.13** also contains an epoxy group and its antimicrobial activity was tested, but it is assumed that the starting concentration of this compound was not high enough to express any activity. In further synthesis and investigations attention should be directed to steroid analogs with an epoxy group.

In addition, Savage et al. (2012) tested a multiple series of cationic steroid antibiotics against *S. aureus* and obtained MIC in the range of 0.40 to 3.13 μg/mL. Taleb-Continiv et al. (2003) used steroids isolated from two *Chromolaena* species and tested them against *S. aureus* using well diffusion method. Given MIC were in the range of 64 to 250 μg/mL. Epand et al. (2007) tested cationic steroid compounds by microdilution test. Obtained MIC was in the range of 0.4 to 9.0 μg/mL for four tested compounds.

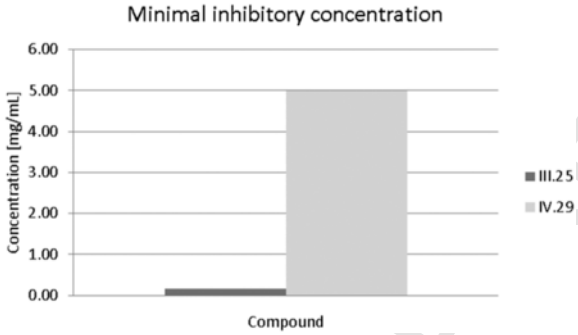


Fig. 1. The detected minimal inhibitory concentration of two steroid analogs.

All investigated derivatives have not manifested any inhibitory effect toward *E. coli* and *C. albicans* in the concentration of 10 mg/mL. The absence of the inhibitory effect could be explained regarding the cell wall that presents the barrier for the investigated derivatives.

3.3. Principal Component Analysis

The result of conducted PCA analysis is expressed through two principal components. These two PCs describe 99.20% of total variance. PC1 contributes with 96.56% and PC2 with 2.64% of total variance. The scores and loadings plots for this PCA analysis are presented in Fig. 2. On the position of the investigated steroid analogs on the scores plot all five volume fractions distributed on loadings plot have almost the same influence, except ACN 90. All five volume fractions have negative coefficients of latent variables regarding PC1 axis. Volume fractions labeled as ACN 60, ACN 70 and ACN 75 have positive and volume fractions labeled as ACN 80 and ACN 90 have a negative coefficient of latent variables regarding PC2 axis. Scores plot indicates that investigated compounds are grouped according to their polarity, regarding PC1 axis. More polar compounds that contain oxo and hydroxyl group are positioned on the positive end of PC1 axis and less polar (with acetyl and benzyl group) compounds are positioned on the negative and of PC1 axis. The analysis was done using the program Statistica v. 10 (StatSoft Inc., 2011). The outliers were not detected and significant differences between the groups in the whole series were not observed so further QSRR modeling was based on the whole data set.

3.4. QSRR Modeling

Since chromatographic behavior is considered to be a multiplex process and more than one factor influences the chromatographic retention, MLR approach was used to define QSRRs. QSRR modeling of chromatographic lipophilicity was performed in order to obtain good physicochemical profiles of investigated compounds as future drug candidates of biomedical importance. Molecular descriptors figuring in QSRR-MLR models were selected using SS procedure. In accordance with Topliss-Costello rule, a maximum number of molecular descriptors that can occur in the model is four. For building up the QSRR models presented in Table 4, NCSS 2007 program was used (Hintze, 2007).

The established MLR models contain one lipophilicity or ADMET descriptor. All four MLR models were validated by standard, *cross-* and external statistical parameters. The statistical parameters are shown in Table 4. *VIF* values were calculated for every independent variable in the model and multicollinearity is within the acceptable limits ($VIF < 10$). Very high values of R^2 (higher than 0.9435) and R^2_{adj}

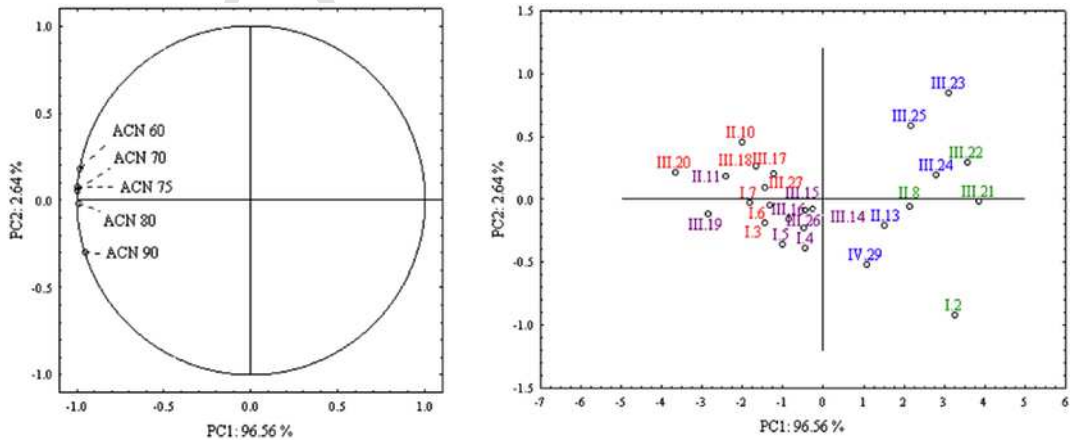


Fig. 2. PCA (scores and loadings plots) based on determined log*k* values (the compounds with OH substituents are green, with O are blue, with AcO are purple and with BnO are red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
MLR models and their statistical parameters.

Parameters	MLR1	MLR2	MLR3	MLR4
	ACN 70	ACN 75	ACN 90	ACN 90
a	0.2948 (± 0.0671)	0.2981 (± 0.6666)	0.0307 (± 0.0042)	0.5783 (± 0.0934)
x_1	**ALOGPs**	**ALOGPs**	**Caco2**	**ALogP**
b	− 0.9816 (± 0.1026)	− 1.0757 (± 0.1017)	− 0.0048 (± 0.0003)	− 0.0042 (± 0.0005)
x_2	**ALOGp*S***	**ALOGp*S***	**MDCK**	**MDCK**
c	− 0.3060 (± 0.0540)	− 0.3463 (± 0.535)	2.0902 (± 0.3849)	1.4805 (± 0.2380)
x_3	**SP**	**SP**	**EI**	**NRL**
d	− 6.5642 (± 0.8983)	− 7.4329 (± 0.8913)	0.0208 (± 0.0062)	0.0015 (± 0.0003)
x_4	**FMF**	**FMF**	**MinPA**	**ECCEN**
Intercept	− 4.6502 (± 0.3347)	− 5.1179 (± 0.3321)	− 3.2833 (± 0.5163)	− 2.7431 (± 0.3095)
R	0.9714	0.9739	0.9796	0.9793
R^2	0.9435	0.9485	0.9596	0.9590
R^2_{adj}	0.9285	0.9347	0.9488	0.9481
F	62.66	69.00	88.99	87.73
RMSE	0.1322	0.1312	0.1433	0.1442
p	0.000000	0.000000	0.000000	0.000000
VIF	3.5 $_{ALOGPs}$	3.5 $_{ALOGPs}$	3.4 $_{Caco2}$	1.3 $_{ALogP}$
	5.2 $_{ALOGpS}$	5.2 $_{ALOGpS}$	1.2 $_{MDCK}$	2.5 $_{MDCK}$
	5.1 $_{SP}$	5.1 $_{SP}$	3.1 $_{EI}$	2.4 $_{NRL}$
	6.3 $_{FMF}$	6.3 $_{FMF}$	3.0 $_{MinPA}$	4.3 $_{ECCEN}$
R^2_{cv}	0.8893	0.9095	0.8990	0.9137
TSS	4.6428	5.0065	7.6128	7.6128
PRESS	0.5140	0.4530	0.7691	0.6569
PRESS/TSS	0.1107	0.0905	0.1010	0.0863
SD_{PRESS}	0.1603	0.1505	0.1961	0.1812
R_{test}	0.9939	0.9903	0.8487	0.8418
R^2_{test}	0.9879	0.9808	0.7204	0.7086
$RMSE_{test}$	0.0444	0.0522	0.1783	0.1820

(higher than 0.9285) suggests the very good statistical validity of generated models. Low values of *cross*-validation statistical parameters as *PRESS* values and SD_{PRESS} and high values of R^2_{cv} (higher than 0.8893) describe the good statistical quality of models. The external test set gives the most reliable information about the predictive power of established models. Parameters of external validation R^2_{test} higher than 0.9879 and $RMSE_{test}$ lower than 0.1820 indicate very strong correlation.

All models include at least one lipophilicity descriptor (ALOGPs, ALOGp*S* and ALogP), except model MLR3. This indicates the dependence of the retention behavior of the studied steroid analogs from their lipophilicity. Additionally, the presence of at least one of ADMET descriptors (SP, Caco2, MDCK, EI and NRL) in formed models can be observed. The appearance of ADMET descriptors also indicates the dependence of the retention behavior of the studied steroid analogs of their lipophilicity, due to the fact that the lipophilicity of the compound dictates its movement across the biological membrane and has a crucial effect on its absorption, distribution, metabolism and elimination. Besides mentioned, models include one geometrical (MinPA), topological (ECCEN) and physicochemical descriptor (FMF).

In addition to these parameters Fig. 3 presents a graphical test of the predictive power of established models. Experimentally observed

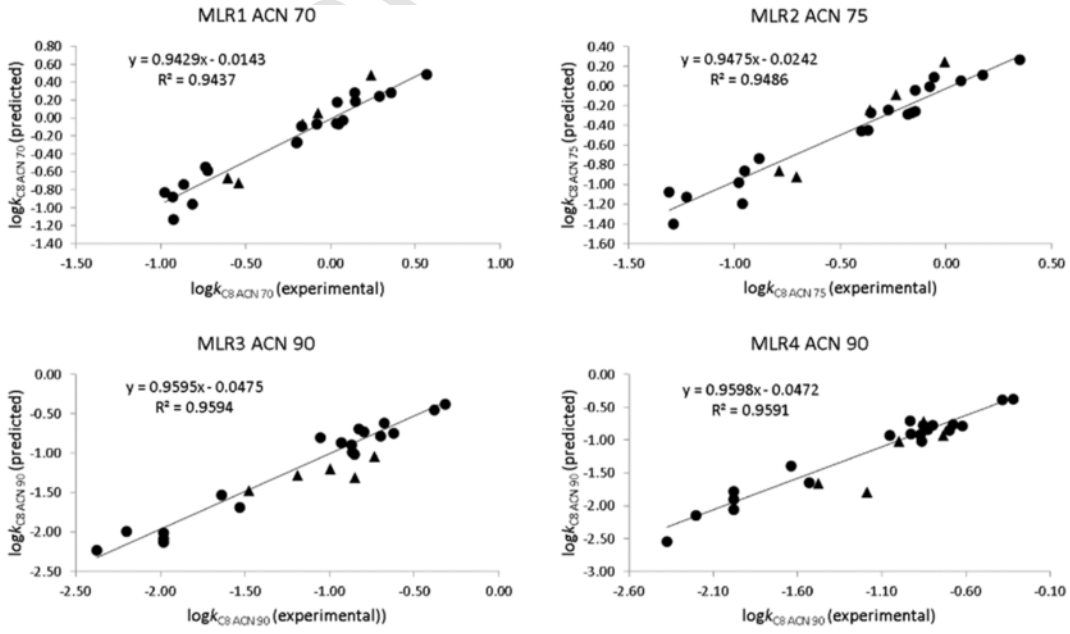


Fig. 3. Experimental *versus* predicted log*k* values for the MLR models.

log*k* *versus* predicted values are plotted for all models. Comparison of experimentally observed and predicted log*k* values indicates that there is a good fit. In Fig. 4 experimentally observed log*k* values *versus* residuals for four MLR models were shown. The residuals are randomly distributed around $y = 0$ axis that also indicates the randomness of the error. Better models were derived for systems that had the higher volume fraction of acetonitrile in the mobile phase. The established MLR models have quite a good standard, *cross*- and external validation parameters.

MLR-QSRR approach was previously used for the chromatographic lipophilicity prediction of various structurally similar steroids. The chromatographic lipophilicity of 17α-picolyl and 17(*E*)-picolinylidene androstane derivatives was predicted using linear regression (LR), polynomial regression (PR), MLR, principal component regression (PCR), partial least squares (PLS) and artificial neural networks (ANN) (Kovačević et al., 2016a). LR- and MLR-QSRR modeling was used for the chromatographic lipophilicity prediction of steroid triazole and tetrazole, toluenesulfonylhydrazide, dione, nitrile and dinitrile (Jevrić et al., 2013). Together with obtained MLR-QSRRs previously reported models can be used for chromatographic lipophilicity prediction of new steroid derivatives and in further quantitative retention-activity (QRAR) studies.

3.5. Molecular Docking Study

The following protein structure (PDB: 3RUK), taken from the Protein Data Bank (PDB), was used in this paper for docking procedure with four steroid derivatives (**I.2**, **I.4**, **I.5**, **I.6**) that were used as ligands (Berman et al., 2000). Compounds **I.2** (3β-hydroxyandrost-5-eno[16,17-*d*]-1,2,3-triazole) and **I.5** (3β-acetoxy-17-aza-17a-homo-17aβ-methyl-androst-5-eno[16,17-*e*]tetrazole) have satisfactory and compounds **I.4** (3β-acetoxy-17-aza-17a-homoandrost-5-eno[16,17-*e*]tetrazole) and **I.6** (3-benzyloxy-17-aza-17a-homoestra-1,3,5(10)-trieno[16,17-*e*]tetrazole) have moderate antiproliferative activity against PC-3 cell lines.

Molecular docking procedure was validated and abiraterone was successfully re-docked using X-ray structure of human cytochrome P450 CYP17A1 with an RMSD (root mean square deviation) of 0.494 Å. Molecular docking procedure reliability check is presented in Fig. 5 and it implies that the methods used in this study are suitable for molecular docking simulation with high precision. Lipophilic and hydrophilic surfaces of the investigated compounds are presented in Supplementary Data (Table S4). They can give insight into the lipophilic/hydrophilic areas capable to interact with lipophilic/hydrophilic parts of protein's active site.

Experimental IC_{50} values for PC-3 cell lines of synthesized steroid derivatives are presented in Table 5. Results indicate that only 3β-hydroxyandrost-5-eno[16,17-*d*]-1,2,3-triazole (**I.2**) has the ability to interact with the enzyme. Interaction distances were checked according to proposed typical interaction distances (Bissantz et al., 2010). Oxygen atom O3 of compound **I.2** interacts with iron ion (Fe^{2+}) (5351 Å). Strong interactions, achieved through hydrogen bonds, occur between hydrogen atom H21 and glycine 258.A (2536 Å), hydrogen atom H11 and aspartic acid 259.A (2709 Å), nitrogen atom N22 and glycine 262.A (3348 Å), hydrogen atom H18 and alanine 75.A (3381 Å), as well as between nitrogen atom N23 and asparagine 172.A (3508 Å). Other three compounds are not suitable for this kind of the enzyme interaction, molecule **I.5** is dislocated from binding site and regarding molecules **I.4** and **I.6** there is no suitable interaction with Fe^{2+}. In Fig. 6 molecular docking results are presented. The skeleton of 3β-hydroxyandrost-5-eno[16,17-*d*]-1,2,3-triazole could be used for further antiproliferative studies as the potential drug for prostate cancer.

Comparative analysis showed that compounds **III.22**, **III.23** and **III.24**, which docking analysis was previously reported (Nikolić et al., 2015), have in positions three and/or four hydroxyl or an oxo group, as well as compound **I.2**. Compound **I.5** has a stronger antiproliferative activity (15.32 μM) from compound **III.22** (19.12 μM), but its polarity is conditioned by the existence of the acetoxy group at position three and it dictates its interaction with the active site of the protein.

4. Conclusion

The best MLR-QSRR models were presented, statistically validated and the most important molecular features of the molecules influencing the molecular lipophilicity were highlighted. The presented results of the experimental lipophilicity determination and its prediction are very important step in the further biological characterization of selected steroid analogs. As these steroid analogs have significant anticancer ac-

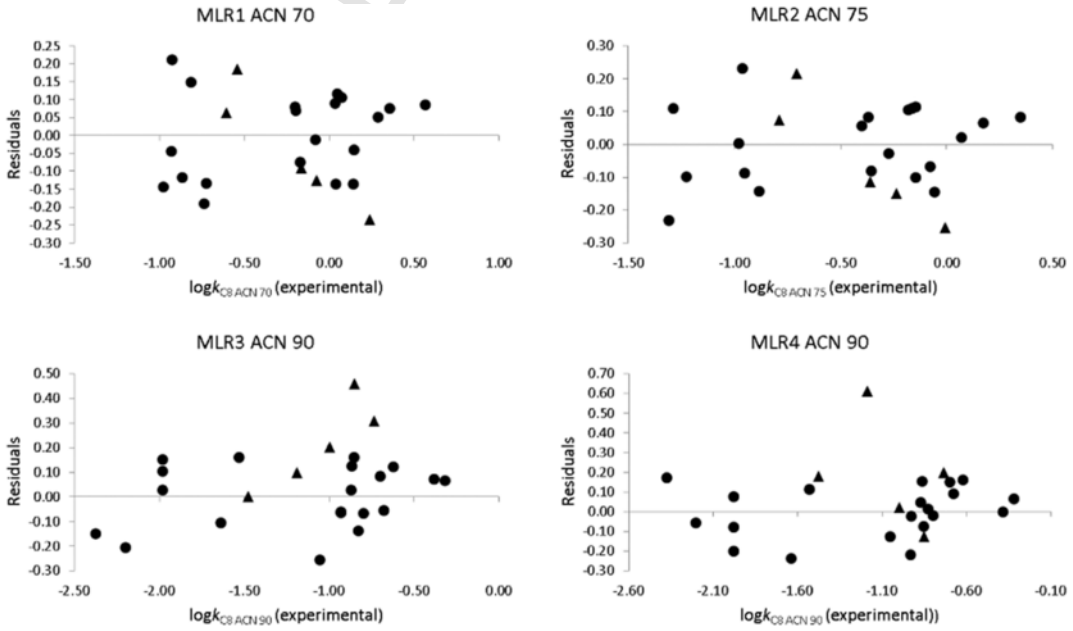


Fig. 4. The distribution of the residuals around $y = 0$ axis for the MLR models.

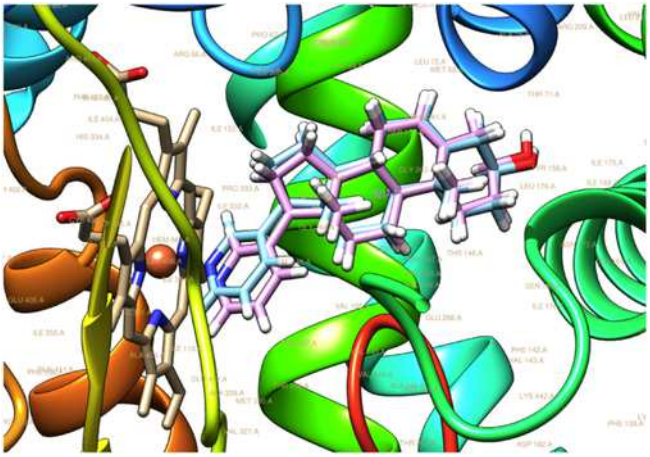


Fig. 5. Molecular docking procedure reliability check (RMSD = 0.494 Å). The structure of the initial complex was retrieved from Protein Data Bank – PDB Base (Berman et al., 2000).

Table 5
Experimental IC_{50} values for PC-3 cell lines of synthesized steroid derivatives.

Compound	IC_{50} (μM)	Reference
I.2	12.27	Sakač et al., 2009
I.4	78.96	Penov-Gaši et al., 2013
I.5	15.32	Penov-Gaši et al., 2013
I.6	65.45	Penov-Gaši et al., 2013

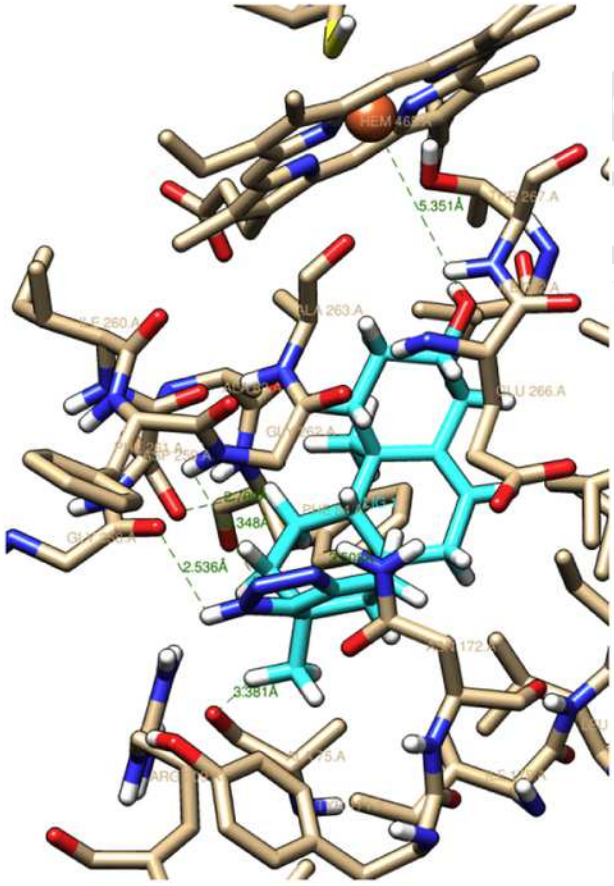


Fig. 6. Molecular docking simulation for 3β-hydroxyandrost-5-eno[16,17-*d*]-1,2,3-triazole (**I.2**) (ALA – alanine; ASN – asparagine; ASP – aspartic acid; GLY – glycine).

tivity, the obtained QSRR models can be useful for evaluation of chromatographic lipophilicity of new steroid derivatives and in further quantitative retention-activity (QRAR) studies. Microbiological activity addressed that further evaluation should be focused on steroid analogs with an epoxy group introduced in the steroid core. Modifications of the studied steroids, especially of 3β-hydroxyandrost-5-eno[16,17-*d*]-1,2,3-triazole, could lead to the development of more specific compounds with highly selective action toward human prostate cancer cells.

Uncited reference

Chem Axon, Ltd., n.d

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Appendix A. Supplementary Data

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