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Research Article

Investigation of 1,2,4-Triazole Derivatives as Potential Anti-Diabetic Agents: *In vitro* Enzyme Inhibition and *In silico* Pharmacokinetic Studies

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Abstract

Diabetes mellitus (DM) is a metabolic disease that causes an increase in blood glucose levels, leading to postprandial hyperglycemia and numerous secondary problems, such as kidney failure, blindness, cardiovascular diseases, and nerve damage. Inhibitors of α-amylase and α-glucosidase are widely used in the treatment of type-II DM because they can inhibit the digestion of starch. In this study, the inhibition potentials of previously synthesized fluorine-containing 1,2,4-triazole-5-one derivatives (**4a-d, 6a-b, 7a-b, 8a-b**) were screened against α-amylase and α-glucosidase activities. All the compounds exhibited varying degrees of α-amylase inhibitory potential, ranging from 185.2 ± 3.4 to 535.6 ± 5.5 μM. For α-glucosidase, the IC_{50} values ranged from 202.1 ± 3.8 to 803.2 ± 10.3 μM, compared to the positive control acarbose (IC_{50} = 411.3 ± 6.4 μM for α-amylase and IC_{50} = 252.0 ± 4.8 μM for α-glucosidase). **4c** exhibited excellent inhibitory potential in both cases, and we evaluated the mode of inhibition of α-amylase and α-glucosidase through kinetic studies. Furthermore, we calculated the physicochemical and pharmacokinetic properties of molecule **4c** using SwissADME software. The results of our research suggest that compound **4c** may be a promising candidate for the treatment of type-II DM.

Keywords: α-Amylase, α-glucosidase, inhibition kinetics, pharmacokinetics, triazoles

1,2,4-Triazol Türevlerinin Potansiyel Anti-Diyabetik Ajanlar Olarak Araştırılması: *In vitro* Enzim İnhibisyonu ve *In Silico* Farmakokinetik Çalışmaları

Öz

Diabetes Mellitus (DM), kan glukoz seviyesinin yükseldiği, postprandiyal hiperglisemiye neden olan, böbrek yetmezliği, körlük, kardiyovasküler hastalıklar ve sinir hasarı gibi pek çok sekonder probleme neden olan metabolik bir hastalıktır. α-Amilaz ve α-glukozidaz doğrudan tip II DM ile ilgilidir ve bu enzimlerin inhibitörleri nişasta sindirimini inhibe edebildiğinden DM tedavisinde yaygın olarak kullanılmaktadır. Bu çalışmada daha önce sentezlenen flor içeren 1,2,4-triazol-5-on türevlerinin (**4a-d, 6a-b, 7a-b, 8a-b**) α-amilaz ve α-glukozidaza karşı inhibisyon potansiyelleri araştırıldı. Tüm moleküller, pozitif kontrol akarboza kıyasla (α-amilaz için IC_{50} = 411,3 ± 6,4 uM, α-glukozidaz için IC_{50} = 252,0 ± 4,8 μM), 185,2 ± 3,4 ila 535,6 ± 5,5 μM arasında değişen farklı oranlarda α-amilaz inhibisyonu sergiledi; α-glukozidaz varlığında ise, IC_{50} değerleri 205,0 ± 3,8 ila 803,2 ± 10,3 μM arasında değişim gösterdi. 10 farklı inhibitör molekülü arasında **4c**'nin her iki durumda da mükemmel inhibe edici potansiyele sahip olduğu tespit edildi ve α-amilaz ve α-glukozidazın inhibisyon türü kinetik çalışmalarla değerlendirildi. Ayrıca SwissADME yazılımı kullanılarak **4c** molekülünün fizikokimyasal ve farmakokinetik özellikleri hesaplandı. Mevcut araştırmanın sonuçları, tip II DM'nin tedavisi için umut vaat eden bir aday olarak **4c** molekülünün potansiyelini desteklemektedir.

Anahtar Kelimeler: α-Amilaz, α-glukozidaz, inhibisyon kinetiği, farmakokinetik, triazoller

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1. Introduction

Diabetes Mellitus (DM) is a chronic disease caused by either complete or partial functional insufficiency or deficiency of insulin hormone secretion, which regulates the use of blood sugar secreted from the pancreas [1-3]. There are three types of diabetes, which are gestational DM, Type 1 DM, and Type 2 DM, depending on the etiology of the disease [4]. Type 2 DM is the most common type, characterized by hyperglycemia, hyperinsulinemia, dyslipidemia, and hypertension. It occurs due to decreased β-cell function and insulin insensitivity in peripheral tissues [5, 6].

α-Amylase (E.C.3.2.1.1) catalyzes the hydrolysis of inner α-1,4-glycosidic links in starch and produces short oligosaccharide units such glucose, maltose and dextrin [7-9]. Also α-glucosidase is the key enzyme in carbohydrates digestion [10]. It catalyzes the hydrolysis of 1,4-α bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides. The treatment of diabetes aims to reduce postprandial blood sugar. This can be achieved by slowing the absorption of glucose in the digestive tract by inhibiting the carbohydrate hydrolysis enzymes α-amylase and α-glycosidase. Inhibitors of these enzymes postpone carbohydrate assimilation and extend carbohydrate digestion time. This causes a decrease in the rate of glucose absorption. As a result, postprandial plasma glucose elevation is prevented [11, 12]. Although acarbose, metformin and miglitol are now widely used amylase inhibitors, they have side effects such as liver disorders, weight gain, bloating, stomach pain, diarrhea and flatulence [2, 3, 13]. Therefore, studies have attempted to discover new pharmacological agents which could function as α-amylase and α-glycosidase inhibitors.

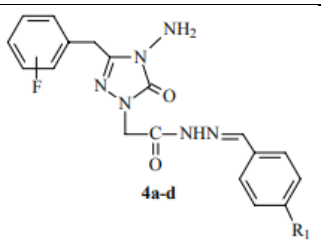
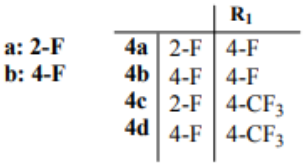
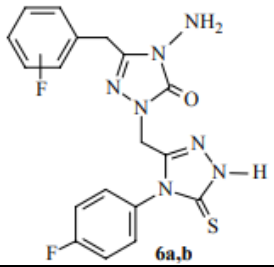
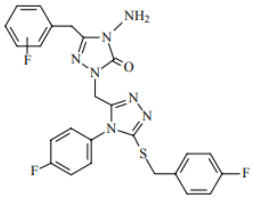
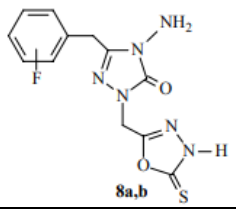
Triazoles are nitrogen-containing heterocyclic compounds and they exist in two isomeric forms i.e. 1,2,4-triazole and 1,2,3-triazole. They are both used as core molecules for the design and synthesis of many pharmacologically active molecules, due to their structural properties, like moderate dipole character, hydrogen bonding capability, ion-dipole, π-π stacking, cation-π, hydrophobic effect, van der Waal forces, rigidity, and stability [14]. Among the two isomeric forms, 1,2,4-triazoles and their derivatives have gained significant attention as potential drug candidates. They have been reported to exhibit antimicrobial, antioxidant, analgesic, anti-urease, anti-inflammatory, anticancer, and antidiabetic properties in various studies. As such, triazoles and their derivatives hold great promise as an important class of therapeutically active compounds, and further research in this area may lead to the development of new drugs for various medical conditions [15-18].

Fluorine-containing 1,2,4-triazole-5-one derivatives (**4a-d, 6a-b, 7a-b, 8a-b**) were synthesized and designed by Bekircan et al. (2016), and their inhibitory effects on urease and xanthine oxidase enzymes were investigated before [19]. The present study was conducted to investigate the inhibitory potentials of these compounds on the α-amylase and α-glycosidase enzymes. The best inhibitor molecule, **4c**, was selected and its inhibition mode was determined. In addition, the physicochemical properties, including Lipinski parameters,

pharmacokinetics, drug-likeness, and medicinal chemistry properties, were calculated using the SwissADME website to evaluate the individual ADME behavior and interpret the results. The findings of this study may provide insights into the potential of fluorine-containing 1,2,4-triazole-5-one derivatives as inhibitors of α-amylase and α-glycosidase enzymes and inform the development of new therapeutics for the treatment of diabetes.

2. Material and Methods

Table1. The 2D structure and nomenclature of the titled compounds

Compounds	Nomenclature	2D Structure
4a	2-[4-Amino-3-(2-fluorobenzyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-1-yl]-N'-[(4-fluorophenyl)methylidene]acetohydrazide	 **4a-d** 
4b	2-[4-Amino-3-(4-fluorobenzyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-1-yl]-N'-[(4-fluorophenyl)methylidene]acetohydrazide	
4c	2-[4-Amino-3-(2-fluorobenzyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-1-yl]-N'-{[4-(trifluoromethyl)phenyl]methylidene}acetohydrazide	
4d	2-[4-Amino-3-(4-fluorobenzyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-1-yl]-N'-{[4-(trifluoromethyl)phenyl]methylidene}acetohydrazide	
6a	4-Amino-5-(2-fluorobenzyl)-2-{[4-(4-fluorophenyl)-4,5-dihydro-1H-1,2,4-triazol-5-thione-3-yl]methyl}-2,4-dihydro-*3H*-1,2,4-triazol-3-one	 **6a,b**
6b	4-Amino-5-(4-fluorobenzyl)-2-{[4-(4-fluorophenyl)-4,5-dihydro-1H-1,2,4-triazol-5-thione-3-yl]methyl}-2,4-dihydro-*3H*-1,2,4-triazol-3-one	
7a	4-Amino-5-(2-fluorobenzyl)-2-{[4-(4-fluorophenyl)-5-(4-fluorobenzythio)-4H-1,2,4-triazol-3-yl]methyl}-2,4-dihydro-*3H*-1,2,4-triazol-3-one	 **7a,b**
7b	4-Amino-5-(4-fluorobenzyl)-2-{[4-(4-fluorophenyl)-5-(4-fluorobenzythio)-4H-1,2,4-triazol-3-yl]methyl}-2,4-dihydro-*3H*-1,2,4-triazol-3-one	
8a	4-Amino-5-(2-fluorobenzyl)-2-[(4,5-dihydro-1,3,4-oxadiazol-5-thione-2-yl)methyl]-2,4-dihydro-*3H*-1,2,4-triazol-3-one	 **8a,b**
8b	4-Amino-5-(4-fluorobenzyl)-2-[(4,5-dihydro-1,3,4-oxadiazol-5-thione-2-yl)methyl]-2,4-dihydro-*3H*-1,2,4-triazol-3-one	

2.1. Chemicals

In this research, analytical grade chemicals and solvents were used, which were obtained from reputable suppliers such as Merck, Sigma, and Fluka. The 2D structure and nomenclature of the compounds investigated in this study are presented in Table 1.

2.2. α-Amylase Inhibition Assay

To evaluate the anti α-amylase activity of the synthesized compounds, a modified assay from Taha et al. [20]. In this assay, 40 µL of porcine pancreatic α-amylase solution (102 µg/mL) was mixed with 3.6 µL of varying concentrations of inhibitor solutions, prepared in a sodium phosphate buffer (0.02 M, pH 7.0) containing NaCl (0.006 M). The mixture was incubated at 35 °C for 10 minutes, followed by the addition of 40 µL of 0.96 % starch solution. The reaction mixture was further incubated at 35 °C for 10 minutes, and the reaction was stopped by adding 100 µL of dinitrosalicylic acid (DNS). The tubes were boiled for 5 minutes and then cooled to room temperature. The absorbance was measured at 540 nm after diluting the reaction mixture with 1 mL of distilled water. The IC_{50} values of inhibitor molecules were determined from the inhibitor concentration- relative enzyme activity (%) graph, with acarbose being used as the standard inhibitor. By using this method, the inhibitory effects of the compounds on the α-amylase enzyme were evaluated.

2.3. α-Glucosidase Inhibition Assay

The α-glucosidase inhibition potential of the compounds was evaluated using a modified method described by Quan et al. [21]. In this method, 40 µL of 0.25 U/mL *Saccharomyces cerevisiae* α-glucosidase and 40 µL of various concentrations of the samples were incubated for 10 minutes at 37 ºC. After the incubation, 5 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) substrate was added and the reaction tubes were further incubated for 15 minutes at 37 ºC. The reaction was then stopped by adding 100 µL of 0.1 M Na_2CO_3, and the absorbance was measured at 405 nm. The IC_{50} values of the inhibitors were determined from the inhibitor concentration-relative enzyme activity (%) graph, with acarbose used as the inhibitor standard.

2.4. Kinetic characterization of α-amylase and α-glucosidase inhibition

To calculate the kinetic values and determine the inhibition type of the molecule with the best inhibitory potential among the studied compounds, a series of activity analyses were performed following the protocols described above. Different substrate concentrations were used for α-amylase and α-glucosidase assays, including 0.1 to 1.5 % starch and 1.25 to 20 mM *p*NPG, respectively. Enzyme activities were measured in the absence or presence of various concentrations of the inhibitor (100 µM and 400 µM for α-amylase; 50 µM and 300 µM for α-glucosidase). The inhibition type of the compound was determined by comparing K_m and V_{max} values and analyzing the Lineweaver-Burk plot [22].

2.5. SwissADME studies

Computational studies of the compound with the best inhibitory potential were performed to generate the ADME/pharmacokinetic profile by pasting the Simplifed Molecular Input Line Entry System (SMILES) format of the molecule on the swissADME (Swiss Institute of Bioinformatics, Switzerland) online server [23].

2.6. Statistical analysis

The experiments for this study were conducted in triplicate and the results are presented as mean ± standard deviation. Statistical analysis was performed using One-Way ANOVA.

3. Results and Discussion

3.1. *In vitro* α-amylase and α-glucosidase inhibitory potentials

1,2,4-Triazoles and their derivatives have been widely known for their synthetic and biological importance [19] and this ring system is also part of a wide variety of therapeutically important drug candidates. By this purpose, ten triazole derivatives were screened for their in vitro α-amylase and α-glucosidase inhibitory potential and the IC_{50} values are summarized in Table 2. All tested compounds showed good inhibitory potential for α-amylase showing IC_{50} values ranging from 185.2 ± 3.4 to 535.6 ± 5.5 µM under the positive control of acarbose (IC_{50} = 411.3 ± 6.4 µM); while for α-glucosidase, IC_{50} values changing from 202.1 ± 3.8 to 803.2 ± 10.3 µM when compared to standard acarbose (IC_{50} = 252.0 ± 4.8 µM). Among the studied compounds, **4c** was found to have excellent dual inhibitory potential against α-amylase and α-glucosidase when the IC_{50} values were examined.

1,2,4-triazole derivatives have been found to exhibit inhibitory effects on the enzymes α-amylase and α-glucosidase [16-18]. However, the extent of inhibition can vary depending on the specific chemical structure of the derivative. Some derivatives may bind to the active site of the enzyme, while others may bind to the substrate binding site, resulting in different inhibition mechanisms. Therefore, the inhibitory effects of 1,2,4-triazole derivatives on α-amylase and α-glucosidase enzymes can be influenced by their chemical structure. Here compound **4c** with 2-F and 4-CF_3 substituents on benzene rings has revealed excellent efficacy. Therefore, the synthetic molecule **4c** may serve as a lead candidate for drug improvement for type-II DM.

Table 2. Anti α-amylase and anti-α-glucosidase activities of the tested molecules

Compounds	Anti-α-amylase activity			Anti-α-glucosidase activity		
	IC_{50} (µM)	**Max inhibition (%)**	**Max concentration (µM)**	**IC_{50} (µM)**	**Max inhibition (%)**	**Max concentration (µM)**
4a	322.8±6.3	94.0±0.6	1500	552.3±10.2	87.1±0.5	2000
4b	330.9±5.9	95.6±0.5	1000	612.5±9.9	88.3±0.6	2500
4c	**185.2±3.4**	**92.7±0.4**	**1000**	**202.1±3.8**	**91.2±0.3**	**525**
4d	380.8±6.2	90.7±0.4	750	655.8±11.3	89.2±0.5	1500
6a	453.5±6.4	97.2±0.2	4500	388.1±6.5	90.4±0.3	750
6b	521.8±9.8	81.9±0.4	3600	384.3±5.3	85.3±0.3	750
7a	284.1±5.1	84.2±0.3	4200	456.5±8.7	87.6±0.4	1100
7b	320.8±6.5	91.6±0.3	2550	575.2±9.8	92.7±0.5	1750
8a	531.6±7.9	91.0±0.4	2500	778.9±10.1	90.5±0.5	3725
8b	535.6±5.5	90.0±0.6	5400	803.2±10.3	88.6±0.3	4500
Acarbose	411.3±6.4	97.9±0.7	650	252.0±4.8	94.3±0.6	850

*The results were presented as the mean and standard deviation (mean±SD).

3.2. Determination of inhibition kinetics

Among the ten compounds, it was stated that **4c** was found to be the most potent inhibitor. Subsequently, kinetic analyses were carried out to determine the enzyme inhibition mechanism of **4c** and Lineweaver–Burk plots were employed to derive its kinetic constants K_m, V_{max} and K_i.

It was observed from Table 3 that the K_m and the V_{max} value of **4c** decreased with increasing concentration, in the presence of both enzymes. So, the inhibition type was suggested to be uncompetitive with both α-amylase and α-glucosidase. The determined uncompetitive type of inhibition was also confirmed by a series of approximate parallel lines seen in Lineweaver-Burk graphs (Figure 1).

Table 3. Kinetic parameters and the inhibition type of **4c**

Enzyme	Inhibitor (4c), (µM)	K_m	V_{max} (µmol/min)	Type of inhibition	K_i (µM)
α-amylase	0	0.48 %	0.12	Uncompetitive	289.2
	100	0.34 %	0.08		
	400	0.25 %	0.06		
α-glucosidase	0	15.3 mM	0.04	Uncompetitive	59.2
	50	6.2 mM	0.02		
	300	3.3 mM	0.01		

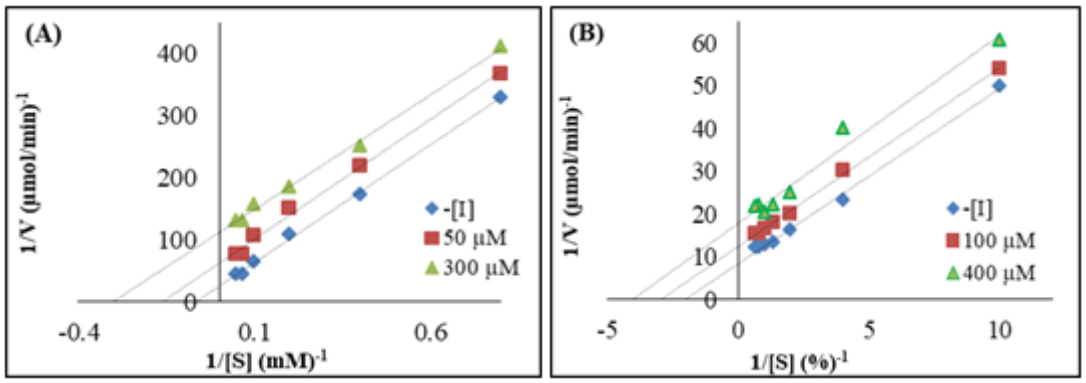


Figure 1. Lineweaver-Burk plots for inhibition type of α-glucosidase (A) and (B) α-amylase in the presence of compound **4c**

3.3. SwissADME prediction of molecule 4c

Computer-based drug design has been used to predict the ADME properties of drugs, leading to drug discovery at the budding stage [25]. Since the computational approaches are safer, easier, and faster for designing and developing new drugs, they become widely used to evaluate the compounds' pharmacokinetic profile [26]. So, we preferred the Swiss-ADME link to go around the various Swiss Drug Design tools and estimate the pharmacokinetic features of molecule **4c** as a new drug candidate. Some physicochemical parameters of **4c** were previously calculated [27]. Here we aimed to discuss molecule **4c** in terms of pharmacokinetics, lipophilicity and solubility.

Brain Or IntestinaL EstimateD permeation (BOILED Egg) method is a graphical version that operates by estimating the lipophilicity and polarity of the molecules [28]. Graphical

calculations of gastrointestinal absorption and blood-brain barrier (BBB) penetration of the **4c** molecule was shown in Figure 2. According to the BOILED Egg plot, molecule **4c** is located within the white ellipse representing the greater extent of intestinal absorption and good bioavailability. Since it can not exceed the blood-brain barrier (BBB) and does not cause a depression in the central nervous system, **4c** can be a promising drug that can very easily be absorbed by the gastrointestinal system without potential BBB permeability.

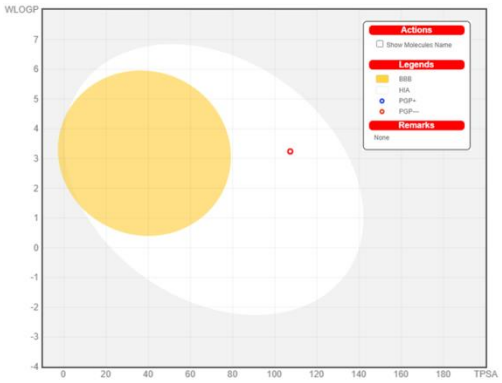


Figure 2. Graphical view of molecule **4c** according to the BOILED-EGG predictive model

The bioavailability radar shows the relation of six physicochemical parameters as solubility, flexibility, lipophilicity, size, saturation, polarity and the pink area indicates the biophysical range [29]. The radar image of molecule **4c** exhibited appropriate values for all indices except the saturation value which exceeds the limit (Figure 3). Also, the bioavailability score which is calculated as 0.55 affirms that it has good absorption since it may have more than 10 % of bioavailability in rats [30]. In addition to the Lipinski rule of five, the other four drug-likeness rules called Ghose, Egan, Veber and Muegee, have been contemporarily satisfied by molecule **4c**. Finally, the result of the pan assay interference structures (PAINS) model, designed to keep out the small molecules that probably show false positive outcomes in biological assays, post no alert for molecule **4c**.

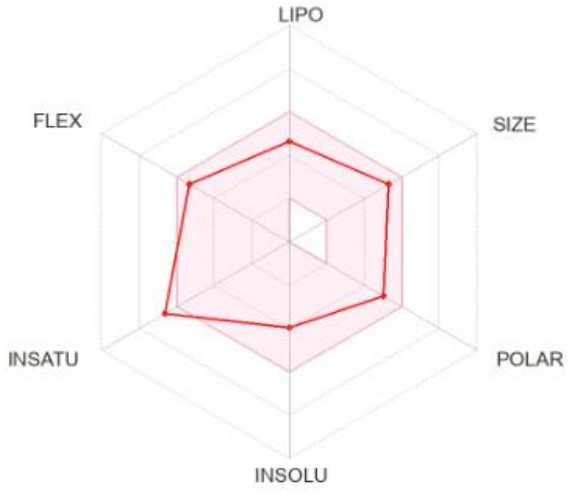


Figure 3. Bioavailability radar of the molecule **4c**

The other two important parameters are lipophilicity and solubility which are investigated for desired drug improvement. Predicted lipophilicity and solubility values of molecule **4c** by SwissADME are summarized in Table 4 and Table 5. The Log $P_{o/w}$ as the partition coefficient factor between n-octane and water solvents is the main term of the molecule lipophilicity. The estimated consensus Log $P_{o/w}$ value was 2.78 and for molecule **4c**, predicating its high miscibility in cell lipids.

Table 4. Lipophilicity of molecule **4c**

iLOGP	XLOGP3	WLOGP	MLOGP	SILICOS-IT	Consensus Log $P_{o/w}$
2.52	2.37	3.24	3.18	2.60	2.78

***** iLOGP, a physics based method lean on free energies of solvation in n-octanol and water calculated by the generalized-born and solvent accessible surface area (GB/SA) model; XLOGP3, an atomistic accost including corrective factors and knowledge based library; WLOGP, application of purely atomistic method stationed on fragmental system; MLOGP, an archetype of topological method suggested on a linear relationship with implemented 13 molecular descriptors; SILICOS-IT, an mongrel method entrust on 27 fragments and 7 topological descriptors; Consensus Log $P_{o/w}$ is an arithmetic mean of the values predicted by the five proposed methods [24].

Table 5. Water solubility of molecule **4c**

Log *S* (ESOL)	-3.92
Solubility (mol/L)	1.21e-04
Class	Soluble
Log *S* (Ali)	-4.26
Solubility (mol/L)	5.45e-05
Class	Moderately soluble
Log *S* (SILICOS-IT)	-5.75
Solubility (mol/L)	1.77e-06
Class	Moderately soluble

*****ESOL model; Solubility class: Log S Scale: Insoluble<-10 poorly<-6, moderately<-4 soluble<-2 very<0<highly, Ali model; Solubility class: Log S Scale: Insoluble<-10 poorly<-6, moderately<-4 soluble<-2very<0<highly, SILICOS-IT model; Solubility class: Log S Scale: Insoluble<-10 poorly<-6, moderately<-4 soluble<-2 very<0<highly [24].

4. Conclusion

Type II DM is considered to be the most common and life-threatening illness, affecting millions of people globally. For this reason, studies on the treatment of diabetes are becoming more important day by day. In this study, the anti α-amylase and anti α-glucosidase potential of fluorine-containing 1,2,4-triazole-5-one derivatives against acarbose, which is widely used in α-amylase inhibition, was explored. Among the **4a-d, 6a-b, 7a-b** and **8a-b** inhibitor molecules, it was determined that **4c** was the most effective inhibitor molecule. Further ADME analysis of **4c** by the SwissADME online web server reveals that **4c** has good druglikeness, lipophilicity, bioavailability and GI absorption.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Acknowledgments

We thank Prof. Dr. Olcay BEKIRCAN for synthesizing the inhibitor molecules used in this study.

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