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Synthesis of 1,2,4-triazole-5-on derivatives and determination of carbonic anhydrase II isoenzyme inhibition effects

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ABSTRACT

Carbonic anhydrase (CA) II plays major roles in pH regulation of body, protection of electrolyte balance, transportation of water and some metabolic pathways. Therefore, CA II inhibitors are very important molecules for drug design and have many pharmacological applications. CA II as a target molecule is also important for eliminating some pathological conditions such as glaucoma, cancer, epilepsy, ulcer and obesity. In this study, some 1,2,4-triazole derivatives were synthesized and CA II inhibition potentials of these molecules were examined. It has been found that molecule **7c** was the most potent inhibitor with the lowest IC_{50} value at micromolar level among the examined molecules. The inhibition in the range of 18.41–64.97% was seen in the presence of newly synthesized molecules at their reachable maximum concentration in the reaction mixtures. Kinetic studies showed that the inhibition mechanism of compound **7c** on carbonic anhydrase activity was reversible and uncompetitive. Molecular docking studies also indicated that compound **7c** could bind to the active site of the enzyme by weakly interacting with especially Gln102, Leu240, Ala241 and Trp243. ADME properties of these newly synthesized (**3a-e**, **6**, **7a-e**) were also studied and showed good oral drug candidate like properties.

1. Introduction

Carbonic anhydrases (CAs) catalyze the hydration of carbon dioxide to bicarbonate and protons. They form a family of metal-containing enzymes and can be found in bacteria, archaea and eukaryotes [1,2]. Human carbonic anhydrase (hCA) is included in the class of α-carbonic anhydrase (α-CA). There are several isoenzymes in this subclass. CA I – III, CA VII and CA XIII are cytosolic enzymes, CA IV, CA IX, CA XII, CA XIV and CA XV are membrane-bound enzymes, CA VA and CA VB are mitochondrial enzymes, and CA VI is a secreted isoenzyme [2–8].

The reaction catalyzed by carbonic anhydrases is involved in many important processes: gas exchange, transport of CO_2 and bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, secretion of electrolytes in different tissue and organs, biosynthetic reactions in gluconeogenesis, lipogenesis and ureagenesis, bone resorption, calcification and tumorigenicity [9–18].

Many of the CA isozymes involved in these processes are important therapeutic targets. They are inhibited to treat a range of disorders such as obesity, epilepsy, glaucoma, oedema, infectious diseases and osteoporosis [19]. When the tumor-associated CAs are inhibited, growth of the primary tumors and metastases are blocked. Also, population of cancer stem cells reduces. Thus this class of enzyme inhibitors has beneficial anticancer action [20–23].

CA II, which is a physiologically predominant isoform in CA enzymes, is found in red blood cells and many other tissues and its inhibitors have found a wide range of medical applications as diuretics, antiglaucoma and antiepileptic agents [24]. It was also found that excess amounts of CA II are present in various types of cancer cells alone or in combination with tumor-associated enzymes CA IX and CA XII [25].

However, CA inhibitors that have been used up to now have not been sufficiently safe compounds for reasons such as the large number of enzyme isoforms, the widespread presence in many organs and tissues, and the inability of existing drugs to be selective to isoenzymes [26,27]. For example, commercially available CA II inhibitors such as Acetazolamide (AZA), methazolamide (MZA), and ethoxzolamide (EZA) are strong inhibitors but not specific. These compounds not only inhibit CA II enzymes but also other isoenzymes and as a result of this, various side effects such as drowsiness, tingling, depression, fatigue, weight loss, gastrointestinal disturbances, metabolic acidosis, myopia may be

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observed. Topiramate as another inhibitor of CA II has also a very weak efficiency. All these situations lead and encourage investigators to study on the discovery of new CA inhibitors [28].

Many heterocyclic compounds containing 1,2,4-triazole ring is known to have important pharmacological properties such as antimicrobial, antiviral, antioxidant, analgesic, anti-inflammatory, antitumor, anticonvulsant, enzyme inhibitory, antihypertensive and anti-HIV [29–32]. Furthermore, some of the modern daily drugs such as Ribavirin (antiviral agent), Anastrozole, Letrozole (nonsteroidal aromatase inhibitors), Fluconazole, Itraconazole, Terconazole, Posaconazole, Voriconazole (antifungal), Rizatriptan (antimigrane) and Nefazodone, Etoperidone (antidepressant) are example to medicines bearing 1,2,4-triazole ring [29,33–36]. In addition, it is known that certain Schiff bases bearing 1,2,4-triazole ring have also significant biological activities [37,38]. On the other hand, hydrazine-hydrazone derivatives having −CONHN=CH− functional structure are used as both pharmacological active compounds such as anticancer, antibacterial, anticonvulsant, analgesic, anti-inflammatory, antiviral, antimalarial, antidepressant and antituberculosis [39,40] and starting material in the synthesis of compounds such as imidazoles, pyrazoles, triazoles, oxadiazoles, thiazolidinones, and 2-Azetidinones [39,41].

We emphasized above that triazole derivative compounds have some kind of biological activities such as antifungal, antimicrobial etc and it has been reported by many researchers [29–32]. It is clearly understood from the literature that sulfonamides are the top inhibitor molecules for CA activity [42]. In addition to this, it is recently reported that some compounds containing phenolic groups have good potentials in terms of CA inhibition [43]. Therefore, these encouraged us to synthesize new hybrid molecules having both triazole ring and phenolic group as being alternative molecules for sulfonamides and examine them in terms of CA inhibitor efficiencies. In this study, novel 1,2,4-triazole compounds were synthesized and their inhibitory effects on the activity of bovine erythrocyte carbonic anhydrase was examined by performing biochemical, kinetic and molecular modelling studies.

2. Results and discussion

2.1. Chemistry

In the current study, synthesis, carbonic anhydrase II inhibition and molecular docking studies of 1,2,4-triazole-5-one derivatives were revealed. Synthesis of the intermediate and target compounds (**1–7**) was showed in Scheme 1. All new compounds were characterized by the IR, ^{1}H NMR, ^{13}C NMR, mass spectra and elemental analyses and the spectral data were confirmed with the suggested structures.

In all IR spectra, 1,2,4-triazole C=O absorption bands were observed in the 1667–1684 cm^{-1} range. The C=O absorption peaks of hydrazide (**5**) and hydrazide-hydrazones (**7a-e**) were also observed at 1715–1720 cm^{-1}. Compounds **1**, **2** and **4** were synthesized according to the literature we published earlier [44]. 1,2,4-Triazole-Schiff bases (**3a-e**) were synthesized by the condensation of compound **2** with suitable 2-hydroxy-5-substitue benzaldehydes in an oil bath maintained at 160–165 °C. In the ^{1}H NMR spectrum, the NH_2 group belonging to compound **2** disappeared, and N=CH and OH singlet signals were observed at about 9.90 and 10.60 ppm, respectively. Also, in the ^{13}C NMR spectrum, the carbon peaks of the N=CH group were observed at about 148 ppm. The reaction of compound **4** with ethyl bromoacetate in the presence of sodium ethoxide resulted in the formation of ethyl acetate derivative (**5**). This compound was transformed into acetohydrazide derivative (**6**). In the ^{1}H NMR spectra of compound **6**, the singlet signal NH_2 and NH protons were appeared at 4.55 and 9.55 ppm, respectively. The hydrazide C=O observed at 165.90 ppm in the ^{13}C NMR spectrum. In addition, the signals of the ester OCH_2CH_3 group disappeared.

The reaction of compound **6** with various substitute benzaldehydes in ethanol gave hydrazide-hydrazone derivatives (**7a-e**). In the IR spectra, the OH, NH, hydrazide C=O and C=N stretching bands belonging to arylidene hydrazide structure were observed at about 3210, 3077, 1680 and 1588 cm^{-1}, respectively. In the ^{1}H and ^{13}C NMR spectra of these compounds, benzyl CH_2, NCH_2, N=CH and OH proton signals and triazole C-5, C=O and CONH carbon signals were observed as double singlets. According to the literature, hydrazine-hydrazone derivatives may exist as *E/Z* geometric isomers around –C=N− double bond and as *cis/trans* amide conformers about the amide CO−NH bond (Scheme 2) [45–48]. In general, when hydrazine-hydrazones are dissolved in polar solvents like DMSO-d_6, the *E* geometrical isomers of these compounds undergo a rapid *trans/cis* amide equilibrium, in which the *trans* conformer predominates [45–48].

In the ^{1}H NMR spectrum of selected compound (**7b**), the proton signals belonging to CH_3, N-CH_2, Ar-H, −N=CH, OH and NH protons were observed as double singlets at 2.12 (*trans*)/2.16 (*cis*), 4.81 (*trans*)/4.43 (*cis*), 7.76 (*trans*)/7.65 (*cis*), 8.29 (*trans*)/8.41(*cis*), 10.37 (*trans*)/10.98 (*cis*) and 11.75 (*trans*)/12.08 (*cis*) ppm, respectively (Fig. 1). Furthermore, using ^{1}H NMR data, the percentage ratios of *E* isomer and *trans/cis* conformer of the compound can be calculated. This compound showed the signals belonging to *cis* and *trans* amide conformers in 29% and 71%, respectively, (Fig. 1). The N-CH_2 and aromatic proton signals of the *trans* conformers are found downfield compare to the *cis* conformers, because of steric hindrance [45–48].

In the ^{13}C NMR spectra of this compound, CH_3, N-CH_2, some aromatic carbons, triazole C-5 and N=CH carbon signals were also observed as double singlets. In addition, the signals belonging to triazole C=O and hydrazide C=O appeared at about 163 and 167 ppm, respectively. As results, compounds **7a-e** showed that had the *E* isomers and *trans* conformer structures (**I**) to be dominant forms among the four possible structures [45–48].

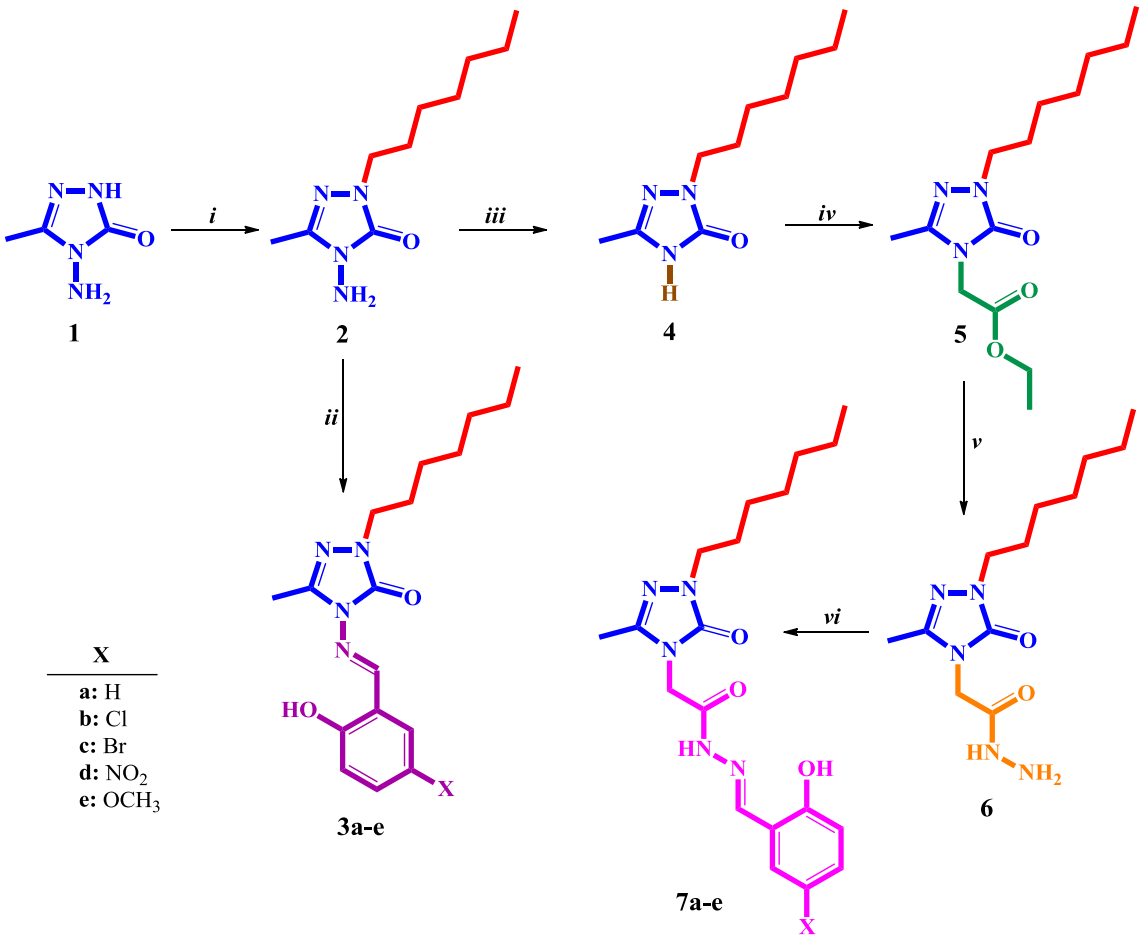
2.2. Biological activity

2.2.1. Inhibition studies for carbonic anhydrase activity

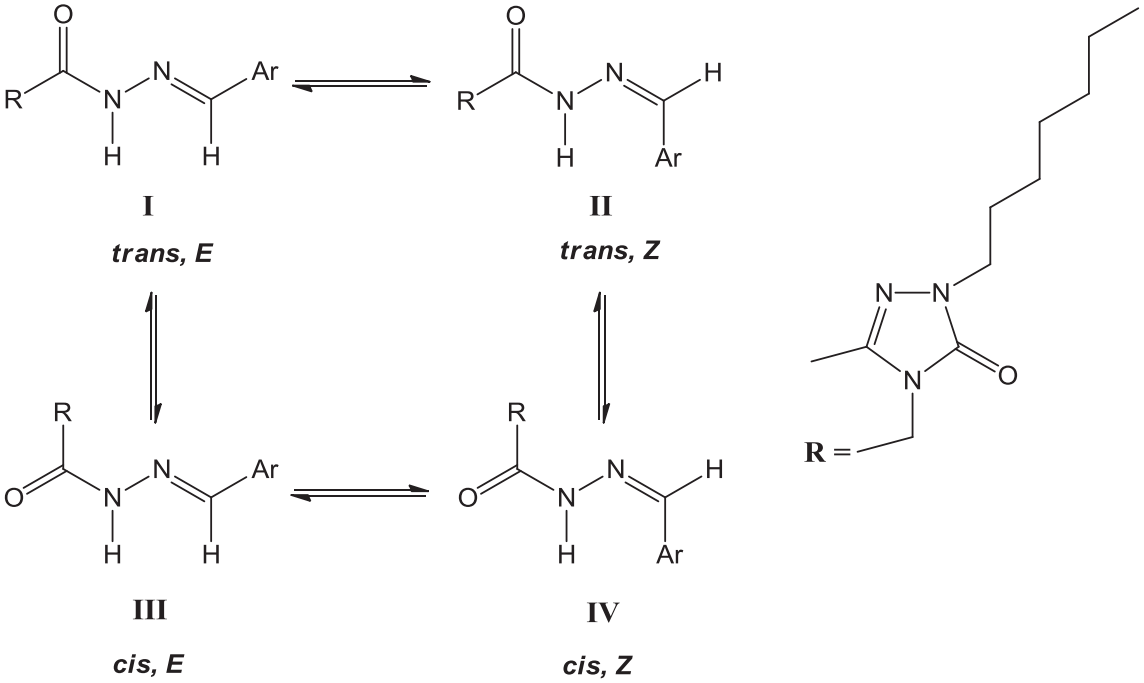
Prior to initiation of inhibition studies, carbonic anhydrase activity was optimized in accordance with the literature (Table 2). It was determined that carbonic anhydrase from bovine erythrocytes (Sigma, C3934) had optimum temperature and optimum pH at 25 °C and pH 7.4, respectively. Optimum values for reaction time and enzyme concentration were determined as 9 min and 5 μg/mL, respectively. Although K_m value was calculated as 2.07 mM PNPA, the studies were done by using 1.5 mM PNPA because of solubility problems.

In this study, some of the newly synthesized 1,2,4-triazole compound derivatives have been examined in terms of carbonic anhydrase inhibition potentials. Because these molecules have largely hydrophobic groups, suitable for membrane penetration if they will be drug candidate, they were solved in ethanol and DMSO. Because of organic solvents inhibit enzymes in the presence of their higher concentration than 3% [49], organic solvent in the reaction mixture was adjusted to not exceed 3%. For this reason, suitable blank mixtures were prepared considering the inhibitory effect of the solvent and relative activities are calculated according to this fact. Besides that even if the organic compounds are prepared at a given concentration in organic solvent mixture, it has been observed that there is occasional collapse in the reaction medium. It can be attributed to the fact that the inhibitor organic molecules can precipitate in reaction mixtures containing largely buffer solution. Because of each organic molecule used in the study has a different behavioral profile, the concentration range of inhibitor molecules used in the carbonic anhydrase inhibition studies was also different from the molecule to molecule (Table 1).

Enzyme inhibition studies were carried out in the presence of PNPA substrate at 1.5 mM. Percentage relative activities versus inhibitor concentrations were separately plotted for each organic molecule and IC_{50} values were determined. As can be seen in Table 2, molecule **7c** having the lowest IC_{50} value (60.80 μM) was found the most effective inhibitor at micromolar level for carbonic anyhdrase activity among the



Scheme 1. Reagents and conditions: *i*: absolute ethanol, NaOEt/1-bromoheptane, reflux; *ii*. 160–165 °C, oil bath heating; *iii*. H_3PO_2 solution, $NaNO_2$, room temperature; *iv*. absolute ethanol, NaOEt/ethyl bromoacetate, reflux; *v*. butanol, $NH_2NH_2{\cdot}H_2O$, reflux; *vi*. ethanol, ArCHO, reflux,



Scheme 2. *E/Z* Geometrical isomers and *cis/trans* conformers of compounds **7a-e.**

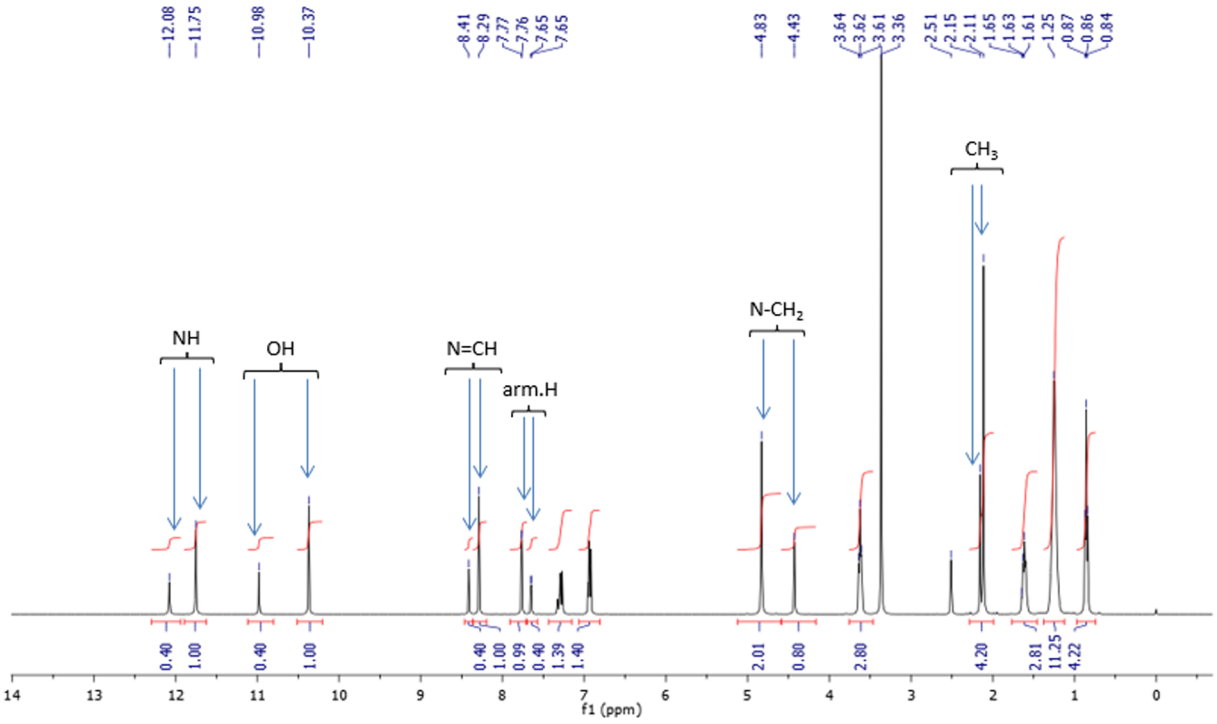


Fig. 1. ^{1}H NMR of compound **7b.**

examined molecules. In the presence of other inhibitor molecules, inhibition was observed in different ratios (18.41–64.97%).

In one study some derivatives of 2-substituted quinazolin-4(3H)-ones was synthesized and evaluated against carbonic anhydrase-II. IC_{50} values of the compound 1, compounds 21 and 22 were determined as 108.30 ± 0.93 µM, 191.93 ± 2.72 µM and 61.33 ± 2.38, respectively [50]. A series of Schiff bases of thiazole was synthesized and screened for their possible carbonic anhydrase inhibitory potential. IC_{50} values for these molecules ranged from 69.49 ± 4.07 µM to 435.46 ± 9.9 µM [51]. After the 2,4-dichloro-1,3,5-triazine derivatives of some sulfa drugs were synthesized, CA inhibition was determined by using bovine cytosolic carbonic anhydrase isozyme II. IC50 values for these molecules were between 1.49 ± 0.006 and 105.3 ± 0.16 µM [52]. In another study, 1,4-dihydropyrimidinone substituted diaryl urea and thiourea derivatives were evaluated against the activity of human carbonic anhydrase II. hCA II was inhibited by all the compounds, with IC_{50} values ranging from 63.09 to 169.71 µM. The best hCA II inhibitor among the compounds investigated was 4-(triflouro substituted) phenyl derivative [53].

To determine inhibition type and K_i value, Lineweaver-Burk graphs were plotted in the absence of molecule 87 and at its two different concentrations for monitoring the changes in K_m and V_{max} values (Table 2, Fig. 2).

Table 2
Type of carbonic anyhdrase inhibition in the presence of molecule 7c and some kinetic parameters.

[Inhibitor] (µM)	K_m (µM)	V_{max} (µmol/min)	Type of inhibition	K_i (µM)
0	0.20	2.86	Uncompetitive	60.67 ± 0.54
15	0.11	1.69		
200	0.07	0.95		

As can be seen in Table 2 that K_m and V_{max} values were determined to be 0.07 µM and 2.86 µmol/min, respectively, in the absence of inhibitor. When the concentration of the molecule **7c** was increased 15–200 µM, both K_m and V_{max} values decreased. From this point of view it can be said that type of the carbonic anhydrase inhibition by molecule **7c** is uncompetitive. Molecule **7c** causes an inhibition for carbonic anhydrase activity by binding enzyme-substrate complex reversibly

Table 1
Optimization of carbonic anhydrase activity and inhibition potentials of the organic compounds.

Reaction Conditions		IC_{50}, µM		Maximum Inhibition		Binding Affinity (ΔG, kcal/mol)
				%	[I] µM	
		3a	> 5	18.41 ± 0.01	5	−5.6
		3b	> 50	37.16 ± 0.80	50	−6.1
		3c	> 50	41.09 ± 1.00	50	−6.6
		3d	> 50	39.05 ± 0.58	50	−6.9
		3e	> 15	22.97 ± 0.20	15	−6.2
		6	> 50	22.53 ± 0.19	50	−6.1
		7a	2050	64.97 ± 0.05	5000	−6.5
pH	7.4	**7b**	> 100	28.66 ± 0.11	100	−6.2
Temperature	25 °C	**7c**	60.80	67.07 ± 0.69	200	−6.5
Final carbonic anhydrase concentration	5.0 µg/mL	**7d**	> 50	20.26 ± 0.74	50	−7.0
Final PNPA concentration	1.5 mM	**7e**	> 1500	49.38 ± 0.07	1500	−6.3
Reaction time	9 min	**Sulfanilamide**	3	93.00 ± 0.24	10	

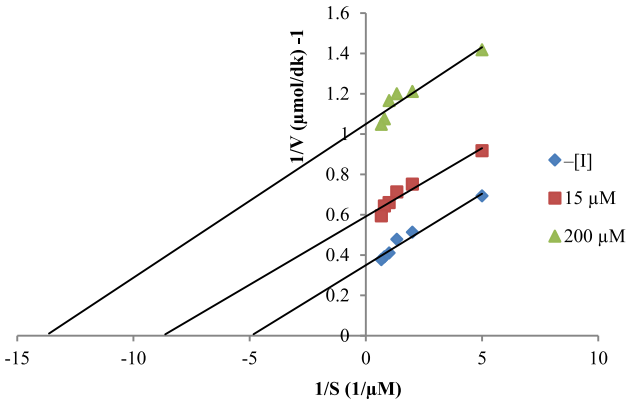


Fig. 2. Plot of 1/[S] vs. 1/*v*. Determination of inhibition type, and changes in K_m and V_{max} values in the presence of **7c** (15 µM and 200 µM) or its absence.

with weak interactions at a site other than the active site. In addition, K_i value was determined as 60.67 ± 0.54 µM as a result of this study.

It was reported that new fluorescent sulfonamide molecules having increased water solubility was tested for carbonic anhydrase inhibition. The new derivatives were found to be effective inhibitors against the hCA II. The K_i values of them were determined in the range of 366–127 nM [54]. Some newly synthesized mercapto-1,3,4-oxadiazole and mercapto-1,2,4-triazole derivatives were assayed as CA II inhibitor. The compounds showed good inhibitory activity and their K_i values are in the range 0.63–31 µM [55]. In another study, a series of some sulfonamides having curcumin scaffold were synthesized and investigated for inhibitors of carbonic anhydrase I (human) and II (bovine). Enzyme kinetic studies of compound 14, 16 and 30 against bCAII enzyme showed that K_i values of these molecules were 0.71, 0.67 and 0.71 µM, respectively [56]. Also, some novel glycine and phenylalanine sulfonamide derivatives were studied as carbonic anhydrase inhibitors against human cytosolic carbonic anhydrase I and II. G1-4 and P1-4 molecules showed good inhibition effect. Their K_i values are 14.66–315 µM for hCA I and 18.31–143.8 µM for hCA II, respectively [57].

2.3. Computational analysis

2.3.1. Molecular modeling studies

The compounds were studied by docking them into the crystal structure of bovine carbonic anhydrase to observe the common behavior of interaction of these compounds with the enzyme. The top 9 predicted docked conformations generated by AutoDock Vina were retained for analyzing the binding affinity of the compounds.

The results of the docking studies show that molecule **7d** has the highest binding affinity and binds to the enzyme efficiently as a strong inhibitor among the examined molecules. But IC_{50} value of this molecule could not be determined because of the solubility problem. For this reason molecule **7c** having the lowest IC_{50} was used to determine the type of the carbonic anhydrase inhibition. In addition, the results of the docking studies also show that compound **7c** is one of the four molecule with highest binding affinity and binds to the enzyme efficiently as a strong inhibitor among the examined molecules (Table 2). As known, the results of docking and biochemical kinetic studies may not coincide completely. In addition, inhibitor molecules were interacted with target proteins in its active site when performing docking studies. But biochemical kinetic studies showed that molecule **7c** inhibited carbonic anhydrase as uncompetetively. It means that molecule **7c** interacted with carbonic anhydrase in a binding site other than active site as explained above.

Interactions of different groups of the molecule **7c** with the amino acid side chains in the appropriate position in the three-dimensional structure of the enzyme are important in the formation of the enzyme-inhibitor complex (Fig. 3). Some of these highlights are listed as follows; (1) π-alkyl and π-π stacked interactions of triazole ring of molecule **7c** with Ala241 and Trp243, respectively, (2) conventional interaction of hydroxyl group of bromo phenol ring with Ala241, (3) π-sigma interaction of bromo phenol ring with Leu240 and, (4) conventional interaction of carbonyl group with Gln102.

2.3.2. ADME studies

The absorption, distribution, metabolism and excretion (ADME) properties were studied for **3a-e**, **6** and **7a-e** compounds by using Molinspiration online property calculation toolkit (Molinspiration, 2015) and the results were given in Table 3. It is clearly known that the prediction of the ADME parameters helps to the researchers to develop new drug molecule. It is seen Table 3 that all investigated molecules have good ADME properties (except for *n*-ON acceptors of **7d**) in terms of their TPSA, MW, miLogP, *n*-ON acceptors and *n*-OHNH donors properties. Because there is no violation more than one of this rule, new molecules synthesized in the present study can be considered likely to be a good orally active drug candidate (referans). It was also observed that the compounds exhibited a good % absorption (%ABS) ranging from 58.17% to 84.02% (Table 3). Compound **7c** as a most potent inhibitor among the tested molecules showed 73.98% absorption. Furthermore, ADME does not proves whether a compound is biologically active or not. So, biological activity studies should perform for the new drug candidate molecules. As seen in Table 1, we examined CA II inhibitory potentials of these molecules as drug candidates in case of the treatment of some diseases related to high CA II activity. It was declared above that **7c** was found the most potential in terms of CA inhibitory efficiency. In addition to the result of in vitro biochemical inhibition studies, ADME results strengthen the speculations of being a drug candidate molecule of **7c**.

3. Conclusions

Carbonic anyhdrase inhibition has a pharmacological importance. So, some triazole derivative molecules were newly synthesized and evaluated in terms of their inhibition efficiencies and ADME properties. It was determined that the molecule **7c** among eleven molecules was found the most potential, its IC_{50} value at micromolar level being determined, for carbonic anyhdrase inhibition. It can be speculated that further chemical and pharmacological studies on carbonic anyhdrase inhibition may perform for this molecule.

4. Experimental section

4.1. General

Chemicals and solvents were purchased from Sigma-Aldrich, Merck, Alfa Aesar and Acros, and they were used without further purification. Reactions were followed by thin-layer chromatography (TLC) on silica gel HSGF254 plates (0.2 ± 0.03 mm thickness). The mobile phase was ethyl acetate:diethyl ether (1:1), and detection was made using UV light. The melting points were determined on Thermo Scientific digital 9200 series melting point apaparatus. The IR spectrum (cm^{-1}) was recorded on a Perkin-Elmer FT-IR spectrometer Frontier. ^1H and ^{13}C NMR spectra were taken on a Bruker 400 MHz NMR instrument in DMSO-d_6 solvent. All chemical shifts were reported in δ (ppm) using TMS as an internal standard. The mass spectra were recorded at *m/z* values by using LC-MS. The elemental analysis was performed on a Costech Elemental Combustion System CHNS-O elemental analyzer. All the compounds gave C, H and N analysis within ± 0.4% of the theoretical values. Enzyme activity measurements and incubation processes were performed by using Perkin Elmer UV–vis. spectrofotmeter and a thermoblock (Boeco), respectively.

Compounds 1, 2 and 4 were synthesized according to the literature we published earlier [44].

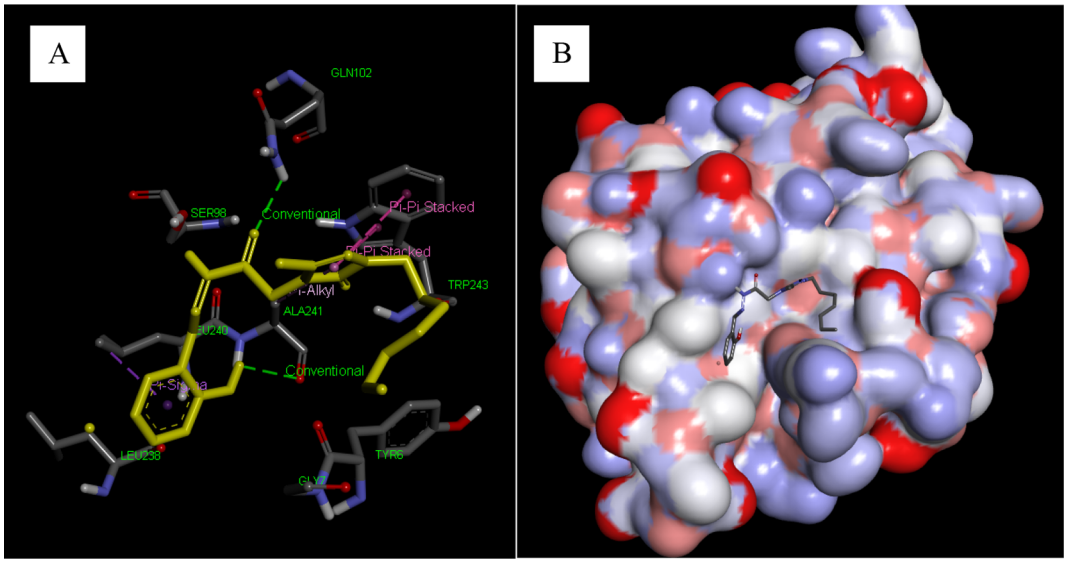


Fig. 3. Predicted conformation of the molecule **7c** inside the binding pocket of carbonic anhydrase (A) micro environment which shows various types of interactions of the compounds atoms with the amino acid residues and (B) general projection.

Table 3
Pharmacokinetic parameters important for good oral bioavailability of synthesized compound **3a-e**, **6** and **7a-e.**

Entry	% ABS	TPSA (A^2)	*n*-ROTB	MV	MW	miLog *P*	*n*-ON acceptors	*n*-OHNH donors	Nviolations
Rule	**–**	**–**	**–**	**–**	**≤500**	**≤5**	**< 10**	**< 5**	**≤1**
3a	84.02	72.42	6	272.62	288.35	2.58	6	1	0
3b	84.02	72.42	6	286.16	322.80	3.23	6	1	0
3c	84.02	72.42	6	290.51	367.25	3.36	6	1	0
3d	68.20	118.25	7	295.95	333.35	2.51	9	1	0
3e	80.83	81.66	7	298.17	318.38	2.61	7	1	0
6	76.24	94.95	6	229.41	241.29	−1.14	7	3	0
7a	73.98	101.52	10	354.41	373.46	3.23	8	2	0
7b	73.98	101.52	10	367.95	407.90	3.88	8	2	0
7c	73.98	101.52	8	338.69	424.30	3.00	8	2	0
7d	58.17	147.34	9	344.14	390.40	2.15	11	2	1
7e	77.70	110.75	9	346.35	375.43	2.25	9	2	0
Sulfanilamide	79.26	86.19	1	138.05	172.21	−0.29	4	4	0

% ABS: percentage absorption, TPSA: topological polar surface area, *n*-ROTB: number of rotatable bonds, MV: molecular volume, MW: molecular weight, miLog P: logarithm of partition coefficient of compound between *n*-octanol and water, *n*-ON acceptors: number of hydrogen bond acceptors, *n*-OHNH donors: number of hydrogen bonds donors.

*4.2. General procedure for the synthesis of 1,2,4-triazole-Schiff bases (**3a-e**)*

4-Amino-1,2,4-triazole-3-one (10 mmol, 2.12 g) was heated at 160–165 °C for 1,5h with suitable 2-hydroxy-5-substitue benzaldehydes (10 mmol) and then cooled. The solid product was recrystallized from ethanol to afford compound **3a**, **3b**, **3c**, **3d** or **3e**, respectively.

*4.2.1. 1-Heptyl-4-(2-hydroxybenzylidenamino)-3-methyl-1,5-dihydro-1H-1,2,4-triazole-5-one (**3a**)*

White solid (2.63 g, 83% yield); m.p. 103–104 °C; IR (FTIR-ATR, cm^{-1}): 1710 (C=O), 1594 (C=N); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.85 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.25 (bs, 8H, N−$(CH_2)_2$−$(CH_2)_4$−CH_3), 1.63–1.66 (m, 2H, N−CH_2−CH_2−$(CH_4)_4$−CH_3), 2.29 (s, 3H, CH_3), 3.65 (t, 2H, N−CH_2−$(CH_2)_5$−CH_3, J = 8.0 Hz), Ar−H: [6.89–6.97 (m, 2H), 7.33–7.37 (m, 1H), 7.79–7.82 (m, 1H)], 9.96 (s, 1H, −N=CH), 10.29 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, δ, ppm): 10.96 (CH_3), 13.84 (CH_3, N−$(CH_2)_6$−CH_3), 21.98 (CH_2, N−$(CH_2)_5$−CH_2−CH_3), 25.84 (CH_2, N−$(CH_2)_2$−CH_2−$(CH_2)_3$−CH_3), 27.88 (CH_2, N−CH_2−CH_2−$(CH_2)_4$−CH_3), 28.13 (CH_2, N−$(CH_2)_3$−CH_2−$(CH_2)_2$−CH_3), 31.08 (CH_2, N−$(CH_2)_4$−CH_2−CH_2−CH_3), 44.27 (CH_2, N−CH_2−$(CH_2)_5$−CH_3) Ar−C: [116.42 (CH), 119.42 (CH), 119.46, 126.40 (CH), 132.86 (CH), 142.75], 149.35 (triazol C-5), 151.32 (−N=CH), 157.60 (C=O); MS (ES, 70 eV) *m/z* (%): 339.37 ($[M + Na]^+$, 100), 317.36 ($[M + 1]^+$, 13), 171.19 (12), 100.13 (6); Anal. Cald. for $C_{17}H_{24}N_4O_2$: C, 64.53; H, 7.65; N, 17.71; found: C, 64.67; H, 7.54; N, 17.93.

*4.2.2. 4-(5-Chloro-2-hydroxybenzylidenamino)-1-Heptyl-3-methyl-1,5-dihydro-1H-1,2,4-triazole-5-one (**3b**)*

White solid (3.12 g, 89% yield); m.p. 109–110 °C; IR (FTIR-ATR, cm^{-1}): 1711 (C=O), 1592 (C=N); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.85 (t, 3H,N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.26 (bs, 8H, N−$(CH_2)_2$−$(CH_2)_4$−CH_3), 1.63–1.66 (m, 2H, N−CH_2−CH_2−$(CH_4)_4$−CH_3), 2.30 (s, 3H, CH_3), 3.65 (t, 2H, N−CH_2−$(CH_2)_5$−CH_3, J = 8.0 Hz), Ar−H: [6.98 (d, 1H, J = 8.0 Hz), 7.36–7.39 (m, 1H), 7.78 (bs, 1H)], 9.95 (s, 1H, −N=CH), 10.61 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, δ, ppm): 10.95 (CH_3), 13.85 (CH_3, N−$(CH_2)_6$−CH_3), 21.97 (CH_2, N−$(CH_2)_5$−CH_2−CH_3), 25.83 (CH_2, N−$(CH_2)_2$−CH_2−$(CH_2)_3$−CH_3), 27.84 (CH_2, N−CH_2−CH_2−$(CH_2)_4$−CH_3), 28.12 (CH_2, N−$(CH_2)_3$−CH_2−$(CH_2)_2$−CH_3), 31.08 (CH_2, N−$(CH_2)_4$−CH_2−CH_2−CH_3), 44.27 (CH_2, N−CH_2−$(CH_2)_5$−CH_3), Ar−C: [118.30 (CH), 121.30, 123.32, 124.81 (CH), 132.24 (CH), 142.88], 148.82 (−N=CH), 149.29 (triazol C-5), 156.35 (C=O); MS (ES, 70 eV) *m/z* (%): 373.33 ($[M + Na]^+$, 100), 351.32 ($[M]^+$, 28), 339.37 (25), 171.19 (9); Anal. Cald. for $C_{17}H_{23}ClN_4O_2$: C, 58.20; H, 6.61; N, 15.97; found: C, 58.58; H, 6.63; N, 16.09.

4.2.3. 4-(5-Bromo-2-hydroxybenzylidenamino)-1-heptyl-3-methyl-1,5-dihydro-1H-1,2,4-triazole-5-one (3c)

White solid (3.15 g, 80% yield); m.p. 126–127 °C; IR (FTIR-ATR, cm^{-1}): 1714 (C=O), 1591 (C=N); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.85 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1,26 (bs, 8H, N−$(CH_2)_2$−$(CH_2)_4$−CH_3), 1.63–1.66 (m, 2H, N−CH_2−CH_2−$(CH_4)_4$−CH_3), 2.30 (s, 3H, CH_3), 3.65 (t, 2H, N−CH_2−$(CH_2)_5$−CH_3, J = 8.0 Hz), Ar−H: [6.93 (d, 1H, J = 8.0 Hz), 7.46–7.49 (m, 1H), 7.90 (bs, 1H)], 9.94 (s, 1H, −N=CH), 10.63 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, δ, ppm): 10.95 (CH_3), 13.84 (CH_3, N−$(CH_2)_6$−CH_3), 21.98 (CH_2, N−$(CH_2)_5$−CH_2−CH_3), 25.84 (CH_2, N−$(CH_2)_2$−CH_2−$(CH_2)_3$−CH_3), 27.85 (CH_2, N−CH_2−CH_2−$(CH_2)_4$−CH_3), 28.14 (CH_2, N−$(CH_2)_3$−CH_2−$(CH_2)_2$−CH_3), 31.09 (CH_2, N−$(CH_2)_4$−CH_2−CH_2−CH_3), 44.26 (CH_2, N−CH_2−$(CH_2)_5$−CH_3), Ar−C: [110.80, 118.71 (CH), 121.86, 127.75 (CH), 135.01 (CH), 142.84], 148.72 (−N=CH), 149.27 (triazol C-5), 156.76 (C=O). MS (ES, 70 eV) m/z (%): 419.29 ([M + Na]$^+$, 100), 395.26 ([M]$^+$, 28), 259.17 (52), 171.14 (73); Anal. Cald. for $C_{17}H_{23}BrN_4O_2$: C, 51.65; H, 5.86; N, 14.17; found: C, 51.84; H, 5.93; N, 13.93.

4.2.4. 1-Heptyl-4-(2-hydroxy-5-nitrobenzylidenamino)-3-methyl-1,5-dihydro-1H-1,2,4-triazole-5-one (3d)

White solid (2.79 g, 77% yield); m.p. 137–138 °C; IR (FTIR-ATR, cm^{-1}): 1677 (C=O), 1593 (C=N), 1339, 1298 (NO_2); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.86 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.27 (bs, 8H, N−$(CH_2)_2$−$(CH_2)_4$−CH_3), 1.63–1.67 (m, 2H, N−CH_2−CH_2−$(CH_4)_4$−CH_3), 2.33 (s, 3H, CH_3), 3.65 (t, 2H, N−CH_2−$(CH_2)_5$−CH_3, J = 8.0 Hz), Ar−H: [7.13 (d, 1H, J = 8.0 Hz), 8.24 (bs, 1H), 8.60 (bs, 1H)], 10.02 (s, 1H, −N=CH), 11.95 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, δ, ppm): 10.96 (CH_3), 13.86 (CH_3, N−$(CH_2)_6$−CH_3), 21.97 (CH_2, N−$(CH_2)_5$−CH_2−CH_3), 25.82 (CH_2, N−$(CH_2)_2$−CH_2−$(CH_2)_3$−CH_3), 27.83 (CH_2, N−CH_2−CH_2−$(CH_2)_4$−CH_3), 28.12 (CH_2, N−$(CH_2)_3$−CH_2−$(CH_2)_2$−CH_3), 31.08 (CH_2, N−$(CH_2)_4$−CH_2−CH_2−CH_3), 44.29 (CH_2, N−CH_2−$(CH_2)_5$−CH_3), Ar−C: [117.08 (CH), 120.36, 121.49 (CH), 127.85 (CH), 140.00, 142.87], 147.81 (−N=CH), 149.25 (triazol C-5), 162.88 (C=O); MS (ES, 70 eV) m/z (%): 384.31 ([M + Na]$^+$, 30), 362.29 ([M]$^+$, 5), 259.17 (1 0 0), 237.15 (48), 171.14 (7); Anal. Cald. for $C_{17}H_{23}N_5O_4$: C, 56.50; H, 6.41; N, 19.38; found: C, 56.85; H, 6.19; N, 19.67.

4.2.5. 1-Heptyl-4-(2-hydroxy-5-methoxybenzylidenamino)-3-methyl-1,5-dihydro-1H-1,2,4-triazole-5-one (3e)

White solid (3.12 g, 90% yield); m.p. 97–98 °C; IR (FTIR-ATR, cm^{-1}): 1714 (C=O), 1594 (C=N), 1281 (C−O); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.85 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.26 (bs, 8H, N−$(CH_2)_2$−$(CH_2)_4$−CH_3), 1.63–1.66 (m, 2H, N−CH_2−CH_2−$(CH_4)_4$−CH_3), 2.30 (s, 3H, CH_3), 3.65 (t, 2H, N−CH_2−$(CH_2)_5$−CH_3, J = 8.0 Hz), 3.73 (s, 3H, OCH_3), Ar−H: [6.89 (d, 1H, J = 8.0 Hz), 6.97–7.00 (m, 1H), 7.31 (bs, 1H)], 9.86 (s, 1H, −N=CH), 9.91 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, δ, ppm): 10.95 (CH_3), 13.86 (CH_3, N−$(CH_2)_6$−CH_3), 21.97 (CH_2, N−$(CH_2)_5$−CH_2−CH_3), 25.82 (CH_2, N−$(CH_2)_2$−CH_2−$(CH_2)_3$−CH_3), 27.87 (CH_2, N−CH_2−CH_2−$(CH_2)_4$−CH_3), 28.12 (CH_2, N−$(CH_2)_3$−CH_2−$(CH_2)_2$−CH_3), 31.08 (CH_2, N−$(CH_2)_4$−CH_2−CH_2−CH_3), 44.27 (CH_2, N−CH_2−$(CH_2)_5$−CH_3), 55.44 (CH_3, OCH_3), Ar−C: [109.29 (CH), 117.51 (CH), 119.59, 119.95 (CH), 142.80, 149.34], 151.03 (−N=CH), 151.90 (triazol C-5), 152.26 (C=O); MS (ES, 70 eV) m/z (%): 369.36 ([M + Na]$^+$, 19), 259.17 (1 0 0), 237.15 (64), 171.08 (45); Anal. Cald. for $C_{18}H_{26}N_4O_3$: C, 62.41; H, 7.56; N, 16.17; found: C, 62.49; H, 7.55; N, 16.01.

4.3. 2-(1-Heptyl-5-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazole-4-yl] acetohydrazide (6)

Compound **3** (1,97 g, 10 mmol) was refluxed with equivalent amount of sodium (0.23 g, 10 mmol) in absolute ethanol (100 mL) for 2 h while being protected from moisture. Then, ethyl bromoacetate (1,13 mL, 10 mmol) was added, and this mixture was refluxed for additional 12 h (monitored by TLC, ethyl acetate:hexane 4:1). The solvent was evaporated under reduced pressure, and the oily product (ethyl-2-(1-heptyl-5-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazole-4-yl]acetate, **5**) was form. The oily portion was extracted with dichloromethane, and then the extraction solvent was dried with sodium sulphate. The dichloromethane was removed from rotary evaporator. The remaining viskos part (**5**) was used in the next stage without further purification.

Compound **5** (2,8 g, 10 mmol) and hydrazine hydrate (1,52 mL, 25 mmol) in *n*-butanol (50 mL) were refluxed for 6 h (monitored by TLC, ethyl acetate:hexane 4:1). After cooling to room temperature, a colorless solid appeared. The solid mass that separated was filtered, dried and recrystallized from ethanol to get the desired product as a solid.

White solid (2.12 g, 79% yield); m.p. 128–129 °C; IR (FTIR-ATR, cm^{-1}): 3300 (NH_2), 1686, 1675 (C=O), 1578 (C=N), 1281 (C−O); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.85 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.24–1.27 (m, 8H, N−$(CH_2)_2$−$(CH_2)_4$−CH_3), 1.58–1.61 (m, 2H, N−CH_2−CH_2−$(CH_4)_4$−CH_3), 2.11 (s, 3H, CH_3), 3.58 (t, 2H, N−CH_2−$(CH_2)_5$−CH_3, J = 8.0 Hz), 4.20 (s, 2H, CH_2) 4.55 (s, 2H, NH_2), 9.35 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, δ, ppm): 11.30 (CH_3, N−$(CH_2)_6$−CH_3), 13.85 (CH_3), 21.97 (CH_2, N−$(CH_2)_5$−CH_2−CH_3), 25.84 (CH_2, N−$(CH_2)_2$−CH_2−$(CH_2)_3$−CH_3), 28.17 (CH_2, N−CH_2−CH_2−$(CH_2)_4$−CH_3), 28.25 (CH_2, N−$(CH_2)_3$−CH_2−$(CH_2)_2$−CH_3), 31.11 (CH_2, N−$(CH_2)_4$−CH_2−CH_2−CH_3), 41.54 (CH_2, N−CH_2), 44.15 (CH_2, N−CH_2−$(CH_2)_5$−CH_3), 143.74 (triazol C=O), 153.02 (triazol C-5), 165.90 (hydrazide C=O); MS (ES, 70 eV) m/z (%): 292.33 ([M + Na]$^+$, 100), 270.25 ([M + 1]$^+$, 18), 170.94 (16); Anal. Cald. for $C_{12}H_{23}N_5O_2$: C, 53.51; H, 8.61; N, 26.00; found: C, 53.87; H, 8.60; N, 25.87.

4.4. General procedure for the synthesis of hydrazide-hydrazone derivatives (7a-e)

10 mmol 2-(1-Heptyl-5-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazole-4-yl]acetohydrazide (5) and various aldehydes (10 mmol) were mixed in ethanol (50 mL), then this mixed was refluxed for 5–6 h (TLC, ethyl acetate:hexane 4:1). At the end of the reaction, the solvent was evaporated under reduced pressure, and the solid residue was recrystallized from ethyl acetate:petroleum ether (1:3).

4.4.1. 2-(1-Heptyl-3-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazol-4-yl)-N′-(2-hydroxybenzylidene)acetohydrazide (7a)

White solid (3.09 g, 83% yield); m.p. 182–183 °C; IR (FTIR-ATR, cm^{-1}): 3215 (NH), 1687, 1667 (C=O), 1588 (C=N), 1284 (C−O); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.86 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.23–1.26 (m, 8H, N−$(CH_2)_2$−$(CH_2)_4$−CH_3), 1.60–1.62 (m, 2H, N−CH_2−CH_2−$(CH_4)_4$−CH_3), 2.12 and 2.16 (s, 3H, CH_3, *trans and cis conformers*), 3.62 (t, 2H, N−CH_2−$(CH_2)_5$−CH_3, J = 8.0 Hz), 4.81 and 4.43 (s, 2H, N−CH_2, *trans and cis conformers*), Ar−H: [6.85–6.93 (m, 2H), 7.23–7.29 (m, 1H), 7.74–7.76 ve 7.55–7.57 (m, 1H, *trans and cis conformers*)], 8.36 and 8.44 (s, 1H, −N=CH, *trans and cis conformers*), 10.06 and 10.92 (s, 1H, OH, *trans and cis conformers*), 11.69 and 12.01 (s, 1H, NH, *trans and cis conformers*); ^{13}C NMR (DMSO-d_6, δ, ppm): 11.26 and 11.33 (CH_3, *trans and cis conformers*), 13.86 (CH_3, N−$(CH_2)_6$−CH_3), 21.98 (CH_2, N−$(CH_2)_5$−CH_2−CH_3), 25.83 (CH_2, N−$(CH_2)_2$−CH_2−$(CH_2)_3$−CH_3), 28.17 (CH_2, N−CH_2−CH_2−$(CH_2)_4$−CH_3) 28.23 (CH_2, N−$(CH_2)_3$−CH_2−$(CH_2)_2$−CH_3), 31.13 (CH_2, N−$(CH_2)_4$−CH_2−CH_2−CH_3), 41.81 and 42.09 (CH_2, N−CH_2, *trans and cis conformers*), 44.16 (CH_2, N−CH_2−$(CH_2)_5$−CH_3), Ar−C: [116.16 and 116.34 (CH, *trans and cis conformers*), 119.33 (CH), 118.56 and 119.37 (*trans and cis conformers*), 126.27 and 129.16 (CH, *trans and cis conformers*), 131.30 and 131.52 (CH, *trans and cis conformers*), 156.46 and 157.31 (*trans and cis conformers*)], 141.85 and 147.67 (−N=CH, *trans and cis conformers*), 143.78 and 153.23 (triazol C-5, *trans and cis conformers*), 163.03 (triazol C=O), 167.62 (hydrazide C=O). The ratio of *trans/cis conformers*: 64.00/36.00; MS (ES, 70 eV) m/z (%): 396.39

([M + Na]$^+$, 92), 374.36 ([M + 1]$^+$, 41), 273.21 (100), 170.95 (7); Anal. Cald. for $C_{19}H_{27}N_5O_3$: C, 61.11; H, 7.29; N, 18.75; found: C, 61.23; H, 7.27; N, 18.57.

4.4.2. 2-(1-Heptyl-3-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazol-4-yl)-N′-(5-chloro-2-hydroxybenzylidene)acetohydrazide (7b)

White solid (3.34 g, 82% yield); m.p. 202–203 °C; IR (FTIR-ATR, cm^{-1}): 3210 (NH), 1710, 1683 (C=O), 1586 (C=N); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.86 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.25 (bs, 8H, N−$(CH_2)_2$−$(\underline{CH_2})_4$−CH_3), 1.60–1.65 (m, 2H, N−CH_2−$\underline{CH_2}$−$(CH_4)_4$−CH_3), 2.11 and 2.15 (s, 3H, CH_3, *trans and cis conformers*), 3.62 (t, 2H, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3, J = 8.0 Hz), 4.83 and 4.43 (s, 2H, N−CH_2, *trans and cis conformers*), Ar−H: [6.92–6.95 (m, 1H), 7.26–7.33 (m, 1H), 7.76 and 7.65 (s, 1H, *trans and cis conformers*)], 8.29 and 8.41 (s, 1H, −N=CH, *trans and cis conformers*), 10.37 and 10.98 (s, 1H, OH, *trans and cis conformers*), 11.75 ve 12.08 (s, 1H, NH, *trans and cis conformers*); ^{13}C NMR (DMSO-d_6, δ, ppm): 11.24 and 11.32 (CH_3, *trans and cis conformers*), 13.87 (CH_3, N−$(CH_2)_6$−CH_3), 21.98 (CH_2, N−$(CH_2)_5$−$\underline{CH_2}$−CH_3), 25.82 (CH_2, N−$(CH_2)_2$−$\underline{CH_2}$−$(CH_2)_3$−CH_3), 28.17 (CH_2, N−CH_2−$\underline{CH_2}$−$(CH_2)_4$−CH_3), 28.23 (CH_2, N−$(CH_2)_3$−$\underline{CH_2}$−$(CH_2)_2$−CH_3), 31.13 (CH_2, N−$(CH_2)_4$−$\underline{CH_2}$−CH_2−CH_3), 41.94 and 42.11 (CH_2, N−CH_2, *trans and cis conformers*), 44.15 (CH_2, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3), Ar−C: [117.96 (CH), 120.54 and 121.90 (*trans and cis conformers)*, 123.02 and 123.30 (*trans and cis conformers)*, 124.85 and 127.34 (CH, *trans and cis conformers),* 130.68 (CH), 155.19 ve 155.90 (*trans and cis conformers*)], 139.76 and 145.33 (−N=CH, *trans and cis conformers*), 143.95 and 153.23 (triazol C-5, *trans and cis conformers*), 163.23 (triazol C=O), 167.91 (hydrazide C=O). The ratio of *trans/cis conformers*: 71.00/29.00; MS (ES, 70 eV) *m/z* (%): 430.37 ([M + Na]$^+$, 100), 408.35 ([M + 1]$^+$, 57), 238.22 (63), 134.98 (48); Anal. Cald. for $C_{19}H_{26}ClN_5O_3$: C, 55.95; H, 6.42; N, 17.17; found: C, 56.21; H, 6.45; N, 17.27.

4.4.3. 2-(1-Heptyl-3-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazol-4-yl)-N′-(5-bromo-2-hdroxybenzylidene)acetohydrazide (7c)

White solid (3.39 g, 86% yield); m.p. 208–209 °C; IR (FTIR-ATR, cm^{-1}): 3336 (NH), 1687, 1669 (C=O), 1589 (C=N); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.86 (t, 3H, N−$(CH_2)_6$-CH_3, J = 8.0 Hz), 1.23–1.29 (m, 8H, N−$(CH_2)_2$−$(\underline{CH_2})_4$−CH_3), 1.59–1.61 (m, 2H, N−CH_2−$\underline{CH_2}$−$(CH_4)_4$−CH_3), 2.11 and 2.15 (s, 3H, CH_3, *trans and cis conformers*), 3.62 (t, 2H, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3, J = 8.0 Hz), 4.82 and 4.42 (s, 2H, N−CH_2, *trans and cis conformers*), Ar−H: [6.87–6.90 (m, 1H), 7.38–7.41 (m, 1H), 7.89 and 7.77 (s, 1H, *trans and cis conformers*)], 8.27 ve 8.40 (s, 1H, −N=CH, *trans and cis conformers*), 10.40 ve 10.99 (s, 1H, OH, *trans and cis conformers*), 11.74 ve 12.07 (s, 1H, NH, *trans and cis conformers*); ^{13}C NMR (DMSO-d_6, δ, ppm): 11.24 and 11.32 (CH_3, *trans and cis conformers*), 13.88 (CH_3, N−$(CH_2)_6$−CH_3), 21.97 (CH_2, N−$(CH_2)_5$−$\underline{CH_2}$−CH_3), 25.81 (CH_2, N−$(CH_2)_2$−$\underline{CH_2}$−$(CH_2)_3$−CH_3), 28.16 (CH_2, N−CH_2−$\underline{CH_2}$−$(CH_2)_4$−CH_3), 28.22 (CH_2, N−$(CH_2)_3$−$\underline{CH_2}$−$(CH_2)_2$−CH_3), 31.12 (CH_2, N−$(CH_2)_4$−$\underline{CH_2}$−CH_2−CH_3), 41.94 and 42.11 (CH_2, N−CH_2, *trans and cis conformers*), 44.15 (CH_2, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3), Ar−C: [110.46 and 110.85 (*trans and cis conformers*), 118.43 and 118.63 (CH, *trans and cis conformers*), 121.15 and 122.45 (*trans and cis conformers)*, 127.75 and 130.21 (CH, *trans and cis conformers*), 133.52 and 133.73 (CH, *trans and cis conformers*), 155.61 and 156.30 (*trans and cis conformers*)], 139.67 ve 145.16 (−N=CH, *trans and cis conformers*), 143.96 and 153.23 (triazol C-5, *trans and cis conformers*), 163.23 (triazol C=O), 167.90 (hydrazide C=O); The ratio of *trans/cis conformers*: 71.00/29.00; MS (ES, 70 eV) *m/z* (%): 476.29 ([M + Na]$^+$, 38), 259.242 (29), 237.21 (1 0 0), 171.20 (24); Anal. Cald. for $C_{19}H_{26}BrN_5O_3$: C, 50.45; H, 5.79; N, 15.48; found: C, 50.63; H, 5.70; N, 15.61.

4.4.4. 2-(1-Heptyl-3-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazol-4-yl)-N′-(2-hydroxy-5-nitrobenzylidene)acetohydrazide (7d)

White solid (3.05 g, 73% yield); m.p. 191–192 °C; IR (FTIR-ATR, cm^{-1}): 3255 (NH), 1693, 1660 (C=O), 1580 (C=N), 1338, 1291 (NO_2); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.86 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.24 (bs, 8H, N−$(CH_2)_2$−$(\underline{CH_2})_4$−CH_3), 1.60–1.63 (m, 2H, N−CH_2−$\underline{CH_2}$−$(CH_4)_4$−CH_3), 2.13 and 2.16 (s, 3H, CH_3, *trans and cis conformers*), 3.63 (t, 2H, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3, J = 8.0 Hz), 4.87 and 4.44 (s, 2H, N−CH_2, *trans and cis conformers*), Ar−H: [7.10 (d, 1H, J = 8.0 Hz), 8.15–8.19 (m, 1H), 8.56 and 8.59 (s, 1H, *trans and cis conformers*)], 8.34 and 8.52 (s, 1H, −N=CH, *trans and cis conformers*), 11.73 and 11.88 (s, 1H, OH, *trans and cis conformers*), 11.88 and 12.17 (s, 1H, NH, *trans and cis conformers*); ^{13}C NMR (DMSO-d_6, δ, ppm): 11.25 and 11.32 (CH_3, , *trans and cis conformers*), 13.87 (CH_3, N−$(CH_2)_6$−CH_3), 21.97 (CH_2, N−$(CH_2)_5$−$\underline{CH_2}$−CH_3), 25.81 (CH_2, N−$(CH_2)_2$−$\underline{CH_2}$−$(CH_2)_3$−CH_3), 28.16 (CH_2, N−CH_2−$\underline{CH_2}$−$(CH_2)_4$−CH_3), 28.21 (CH_2, N−$(CH_2)_3$−$\underline{CH_2}$−$(CH_2)_2$−CH_3), 31.12 (CH_2, N−$(CH_2)_4$−$\underline{CH_2}$−CH_2−CH_3), 41.88 and 42.14 (CH_2, N−CH_2, *trans and cis conformers*), 44.16 (CH_2, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3), Ar−C: [116.74 and 117.07 (CH, *trans and cis conformers*), 119.80 and 120.93 (*trans and cis conformers*), 121.41 and 123.62 (CH, *trans and cis conformers*), 126.56 (CH), 140.07, 162.44 and 163.42 (*trans ve cis konformer*)], 138.95 and 143.98 (−N=CH, *trans and cis conformers*), 144.01 and 153.22 (triazole C-5, *trans and cis conformers*),161.90 (triazole C=O), 167.91 (hydrazide C=O); The ratio of *trans/cis conformers*: 71.00/29.00; MS (ES, 70 eV) *m/z* (%): 457.34 ([M + K]$^+$, 32), 441.38 ([M + Na]$^+$, 19), 419.42 ([M + 1]$^+$, 34), 134.98 (1 0 0); Anal. Cald. for $C_{19}H_{26}N_6O_5$: C, 54.54; H, 6.26; N, 20.08; found: C, 54.71; H, 5.98; N, 20.32.

4.4.5. 2-(1-Heptyl-3-methyl-5-oxo-1,5-dihydro-4H-1,2,4-triazol-4-yl)-N′-(2-hydroxy-5-methoxybenzylidene)acetohydrazide (7e)

White solid (3.08 g, 83% yield); m.p. 182–183 °C; IR (FTIR-ATR, cm^{-1}): 3215 (NH), 1687, 1667 (C=O), 1588 (C=N), 1284 (C−O); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.85 (t, 3H, N−$(CH_2)_6$−CH_3, J = 8.0 Hz), 1.25 (bs, 8H, N−$(CH_2)_2$−$(\underline{CH_2})_4$−CH_3), 1.59–1.63 (m, 2H, N−CH_2−$\underline{CH_2}$−$(CH_4)_4$−CH_3), 2.11 ve 2.15 (s, 3H, CH_3, *trans and cis conformers*), 3.62 (t, 2H, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3 J = 8.0 Hz), 3.72 (s, 3H, OCH_3), 4.81 ve 4.41 (s, 2H, N−CH_2, *trans and cis conformers*), Ar−H: [6.82–6.90 (m, 2H), 7.28 ve 7.14 (s, 1H, *trans and cis conformers*)], 8.31 ve 8.41 (s, 1H, −N=CH, *trans and cis conformers*), 9.62 and 10.35 (s, 1H, OH, *trans and cis conformers*), 11.68 and 11.98 (s, 1H, NH, *trans and cis conformers*); ^{13}C NMR (DMSO-d_6, δ, ppm): 11.27 (CH_3), 13.88 (CH_3, N−$(CH_2)_6$−CH_3), 21.97 (CH_2, N−$(CH_2)_5$−$\underline{CH_2}$−CH_3), 25.81 (CH_2, N−$(CH_2)_2$−$\underline{CH_2}$−$(CH_2)_3$−CH_3), 28.16 (CH_2, N−CH_2−$\underline{CH_2}$−$(CH_2)_4$−CH_3), 28.22 (CH_2, N−$(CH_2)_3$−$\underline{CH_2}$−$(CH_2)_2$−CH_3), 31.12 (CH_2, N−$(CH_2)_4$−$\underline{CH_2}$−CH_2−CH_3), 41.88 and 42.10 (CH_2, N−CH_2, *trans and cis conformers*), 44.15 (CH_2, N−$\underline{CH_2}$−$(CH_2)_5$−CH_3), 55.45 (CH_3, OCH_3), Ar−C: [109.39 and 111.88 (CH, *trans and cis conformers*), 117.20 (CH), 118.21 ve 118.48 (CH, *trans and cis conformers),* 118.84 and 120.24 (*trans and cis conformers*), 150.67 and 151.35 (*trans and cis conformers*), 153.23]. 141.50 and 146.93 (−N=CH, *trans and cis conformers*), 143.98 and 153.20 (triazol C-5, *trans and cis conformers*), 163.04 (triazol C=O), 167.90 (hydrazide C=O); The ratio of *trans/cis conformers*: 75.00/25.00; MS (ES, 70 eV) *m/z* (%): 426.43 ([M + Na]$^+$, 100), 404.40 ([M + 1]$^+$, 57), 332.32 (62), 238.22 (27); Anal. Cald. for $C_{20}H_{29}N_5O_4$: C, 59.54; H, 7.24; N, 17.36; found: C, 59.68; H, 7.21; N, 17.43.

4.5. Carbonic anhydrase assay, activity optimization

The esterase activity of carbonic anhydrase (CA) was determined by the method based on the principle of hydrolysis *p*-nitrophenylacetate (PNPA) to *p*-nitrophenol and acetaldehyde. The assay mixture consisted of 150 μL of enzyme solution (prepared in Tris-SO_4 buffer, 50 mM, pH 7.4), 500 μL Tris-SO_4 buffer (50 mM, pH 7,4), 100 μL purified water and 750 μL *p*-nitrophenyl acetate (PNPA, 3 mM stock solution) in a 1 cm spectrophotometric cell. The mixture was allowed to stand at room temperature for 3 min for lag phase. The change in absorbance at 348 nm was measured over 9 min against the blank (obtained by preparing the same mixture without enzyme). One unit of enzyme activity

was expressed as 1 μmol p-nitrophenol released per minute at room temperature [58,59].

Optimum pH, temperature, reaction time and optimum protein concentration, and K_m and V_{max} values were determined separately before inhibition studies for avoiding errors.

4.6. Inhibition studies

Stock solutions of **3a-e**, **6** and **7a-e** organic molecules in DMSO:ethanol (1:1) were prepared and their inhibitory potencies were examined. 50 μL of each inhibitor solutions having different concentration were added into CA solution and preincubated at 25 °C for 10 min. Substrate solution (PNPA) was then added to each reaction mixture at the indicated volume and the subsequent treatments were carried out as described above. The control mixture was prepared by using organic solvent instead of the inhibitor solution. The inhibitor concentrations for each organic molecule were plotted against the relative enzyme activity and IC_{50} values for each molecule was calculated. The inhibitory activities of examined organic molecules were expressed as the concentration, which inhibits 50% of the enzyme activity (IC_{50}).

4.7. Determination of K_m, V_{max}, K_i and inhibition type

To determine K_m, V_{max} and K_i values, and inhibition type, carbonic anhydrase activities were assayed in the absence and presence of two different inhibitor concentrations of the most potent inhibitor molecule (**7c**) (15 μM and 200 μM) in the presence of PNPA. After then Linewevar-Burk graphics were plotted.

4.8. Enzyme and compounds preparation for docking

Initially, in order to prepare for docking work, the evaluated organic molecules given in Table 1 were optimized by using the Spartan16 program. Conformer distribution with molecular mechanics/MMFF and, equilibrium geometry with semi empirical/PM6 and density functional/M06-2X/6-31G** were used as the optimization method and the most appropriate conformations of the compounds were identified [60]. Crystalline form of the bovine carbonic anhydrase II selected as the target receptor for which the docking of the compounds is to be performed was obtained in the form of the PDB format (1V9E) from the Protein Data Bank (www.rcsb.org) web site after crystalline form was found from literature [61]. Enzyme was purified by Autodock Tools-1.5.6 and then ligand-protein interactions of the organic compounds in the binding pocket were investigated using AutoDock Vina 1.1.2 [62]. The binding energies of organic molecules were calculated and the inhibition potentials were theoretically monitored. Interactions between receptor and ligands were investigated and monitored by using Discovery Studio 4.5 Client program.

4.9. Determination of ADME properties

ADME properties of newly synthesized compounds (**3a-e**, **6**, **7a-e**) were investigated as a second computational study in order to estimate drug likeness of these compounds. In the present study, we have calculated % ABS (percentage absorption), TPSA (topological polar surface area), *n*-ROTB (number of rotatable bonds), MV (molecular volume), MW (molecular weight), miLog P (logarithm of partition coefficient of compound between *n*-octanol and water), *n*-ON acceptors (number of hydrogen bond acceptors), *n*-OHNH donors (number of hydrogen bonds donors) by using Molinspiration online property calculation toolkit (Molinspiration, 2015) [63,64]. Absorption (% ABS) was calculated by using the equation of $\%\ ABS = 109 - (0.345 \times TPSA)$ [65].

4.10. Statistical analysis

Statistical analyses were performed using SPSS software (version 23), and the data were compared by ONE WAY ANOVA test. The differences between groups were analyzed by Post-Hoc test. Tukey test P values less than 0.05 were considered as significant.

Conflict of interest

All authors declare no conflict of interest.

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Supporting information summary

The ^1H and ^{13}C NMR datas of the synthesized compounds are included as a support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.10.042.

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