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A novel and one-pot synthesis of new 1-tosyl pyrrol-2-one derivatives and analysis of carbonic anhydrase inhibitory potencies

Cemalettin Alp [a,b], Deniz Ekinci [c], Mehmet Serdar Gültekin [a,*], Murat Şentürk [a,d,*], Ertan Şahin [a], Ömer İrfan Küfrevioğlu [a]

[a] Atatürk University, Science Faculty, Department of Chemistry, Erzurum, Turkey
[b] Erzincan University, Çayırlı Vocational School, Erzincan, Turkey
[c] Ondokuz Mayıs University, Faculty of Agriculture, Department of Agricultural Biotechnology, Enzyme and Microbial Biotechnology Division, Samsun, Turkey
[d] Ağrı İbrahim Çeçen University, Department of Chemistry, Ağrı, Turkey

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ABSTRACT

Here we propose a novel one-pot synthesis of new tosyl-pyrrole derivatives. By means of the new developed method, pyrrole derivatives were synthesized at room temperature in a single step, and a useful method is proposed for the synthesis of similar compounds. Moreover, inhibitions of two human cytosolic carbonic anhydrase (hCA, EC 4.2.1.1) isozymes I and II by 1-tosyl-pyrrole and 1-tosyl-pyrrol-2-on derivatives were investigated. 1-Tosyl-pyrrole, 1-tosyl-1*H*-pyrrol-2(5*H*)-one, 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one and 5-oxo-1-tosyl-2,5-dihydro-1*H*-pyrrol-2-yl acetate showed inhibitory action with K_i values in the range of 14.6–42.4 μM for hCA I and 0.53–37.5 μM for hCA II, respectively. All pyrrole derivatives were competitive inhibitors with 4-nitrophenylacetate as substrate. Some new synthesized pyrrole derivatives showed very effective hCA II inhibitory effects, in the same range as the clinically used sulfonamide acetazolamide, and might be used as leads for generating enzyme inhibitors targeting other CA isoforms.

1. Introduction

Carbonic anhydrase (EC 4.2.1.1., CA) is a pH regulatory/metabolic enzyme in all life kingdoms, being found in organisms all over the phylogenetic tree. It catalyzes the hydration of carbon dioxide to bicarbonate and the corresponding dehydration of bicarbonate in acidic medium with regeneration of CO_2.[1] At least 16 CA isozymes have been described up to now in mammals, the most active ones as catalysts for carbon dioxide hydration being CA II and CA IX.[1–3] The mammalian carbonic anhydrases have generally monomeric structures.[4] Sixteen isozymes of the zinc-binding enzyme have been described that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB) and one is secreted in saliva (CA VI). Hilvo et al.[5] has reported that CA XV isoform is not expressed in humans or in other primates, but it is abundant in rodents and other higher vertebrates.[2] These CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as oedema, glaucoma, obesity, cancer, epilepsy and osteoporosis.[1,3] Thus, carbonic anhydrase inhibitors are valuable molecules for drug design and pharmacological applications. Our group recently investigated the interaction of hCA I and hCA II cytosolic isozymes with several types of phenols, such as the simple phenol and several of its substituted derivatives, for example, diphenols, salicylates and some of their derivatives.[6] In this study, towards the discovery of new class of inhibitors, we synthesized novel pyrrole derivatives and determined their inhibition effects on human CA-I and II isozymes, because it is critically important to explore further classes of potent CAIs in order to detect compounds with different inhibition profiles.

We investigated a novel series of 1-tosyl pyrrole derivatives, some of which are widely used as prodrugs. Especially, 5-lactams such as pyrrol-2-one and its derivatives have shown anti-tumour activity. In the recent years, N-substituted maleimides and 5-ylidene pyrrol-2(5*H*)-ones have received growing attention,[7] due to their biological activity features and potencies for organic synthesis as key compounds for synthesis of bio-active molecules, which are *Oteromycin, Talaroconvolutin A, Azaspirene, Fusarin, Clausenamide*..., etc.[8] Pyrrol-2-one and its derivatives are also active and important reagents as dienophiles in Diels–Alder reactions, as a 1,3-dipolar reagent for Michael addition[9] and a material for organic complexes.

* Corresponding authors. Address: Ağrı İbrahim Çeçen University, Faculty of Art and Sciences, Department of Chemistry, 04100 Ağrı, Turkey. Tel.: +90 442 2314428; fax: +90 442 2360948 (M.S.G.); tel.: +90 442 2314414; fax: +90 442 2360948 (M.S.).
E-mail addresses: gultekin@atauni.edu.tr (M.S. Gültekin), senturkm36@gmail.com (M. Şentürk).

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Many methods have been developed to synthesize these useful functional heterocycles. There are some studies for the synthesis of 5-hydroxy-2(5*H*)-pyrrol-2-one skeleton in the literature.[10] Most notably, cyclo addition of phenylisocyanate with dialkyl acetylene has been extensively studied and pyrrol-2-one has been obtained with several steps in good yields.[10] One of the methods developed for the synthesis of 1,5-dihydro-pyrrol-2(5*H*)-one is based on a formal (2+3) cycloaddition reaction of diphenyl-cyclopropenone with imines or diimines. In general, pyrrol-2-one structure has been synthesized from acetylene, ketone or aldehyde and 1,3-dipolar compounds. A number of synthetic routes are available to build into lactams core structure in pyrrol-2-one skeleton.

Carbonic anhydrase inhibitors are a class of pharmaceuticals that suppress the activity of carbonic anhydrase. Their clinical use has been established as antiglaucoma agents, diuretics, antiepileptics, in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders or osteoporosis. Thus, discovery of novel effective carbonic anhydrase inhibitors is a great afford for pharmacological and medical applications. The aim of the current study is to develop a novel synthesis method for new pyrrole-2-one derivatives and to discover novel powerful carbonic anhydrase inhibitors.

We selected pyrroles as CA inhibitors (CAIs) because the simple aromatic/heterocyclic atoms have been shown to be inhibitors with CO_2 as substrate for the main isoform of CA, that is, human CA II (hCA II). In an excellent study, Supuran's group reported the X-ray crystal structure for the adduct of hCA II with aromatic/heterocyclic atoms,[12] showing this compound to bind to CA by anchoring its electronegative moiety to the zinc-bound water/hydroxide ion of the enzyme active site. All of the inhibitors used in this study were determined to inhibit the enzyme in competitive manner.

The toluene moiety of tosyl was found to lay in the hydrophobic part of the hCA II active site, where CO_2, the physiologic substrate of the CAs, binds in the precatalytic complex explaining thus the behaviour of tosyl as a unique CO_2 competitive inhibitor. Only recently, Supuran's group investigated the interactions of phenol and some of its substituted derivatives (as well as bicyclic phenols) with all mammalian isozymes, CA I–XV,[12] evidencing some low micromolar/submicromolar inhibitors as well as the possibility to design isozyme selective CAIs. Indeed, the inhibition profile of various isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the submicromolar range for many simple phenols.[6,11,12] It appeared thus of interest to extend the previous studies.[12]

2. Results and discussion

2.1. Chemistry

We previously investigated the addition reactions of phenyliodine bis (trifluoroacetate) (PIFA) to monocyclic and bicyclic alkenes and synthesized corresponding addition products.[10a] In this study, we investigated the reaction between 1-tosyl-pyrrol and PIFA and synthesized compounds **2** and **3** in high yields. We have developed a novel method for one-pot synthesis of 1-tosyl-pyrrol-2-one skeleton using 1-tosyl-pyrrole as starting material and PIFA (phenyliodine bis(trifluoroacetate)) (Scheme 1).

For this purpose, 1 equiv of 1-tosyl-pyrrole molecule was reacted with 1.15 and 2.2 equiv of PIFA. Using 1 equiv of 1-tosyl-pyrrole and 1.15 equiv of PIFA, 1-tosyl-1*H*-pyrrol-2(5*H*)-one **2** was obtained with 81% yield and 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one **3** with 19% yield. When 1-tosyl-pyrrole was reacted with 2.2 equiv of PIFA, 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one **3** was obtained in 97% yield. Both reactions were initiated at 5 °C and stirred for 10 h at room temperature.

Scheme 2 shows the mechanism proposed for the formation of **2** and **3**. In this mechanism, PIFA is added to the olephinic bond of heterocyclic dien **1** initially and trifluoroacetate (CF_3COO^-) of PIFA group is removed from iodine, attacks to iodoform ring from opposite side and the addition comes true. Trifluoroacetate is 1,2 added to the ring, 2-trifloroacetoxy-1-tosyl-pyrrole **6** is formed after elimination of iodobenzene and trifluoroacetic acid from **5**. 1-Tosyl-1*H*-pyrrol-2(5*H*)-one **2** is obtained via PIFA oxidation of this product and 1,3 shift of the proton on the ring. When 2.2 equiv of PIFA is used, it is added to **6,** and **9** is formed via attacking of free trifloroacetate to iodophor ring. Via elimination of this molecule and hydrolysis of the product, **3** is formed (Scheme 2). Figure 1 shows the X-ray spectrum for 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one **3**.

5-Acetoxy-1-tosyl-1*H*-pyrrole-2(5*H*)-one **12** was synthesized in 72% yield via acetylation of hydroxyl group of **3** to pyridine/acetic anhydride (Scheme 3).

2.2. Crystal structure

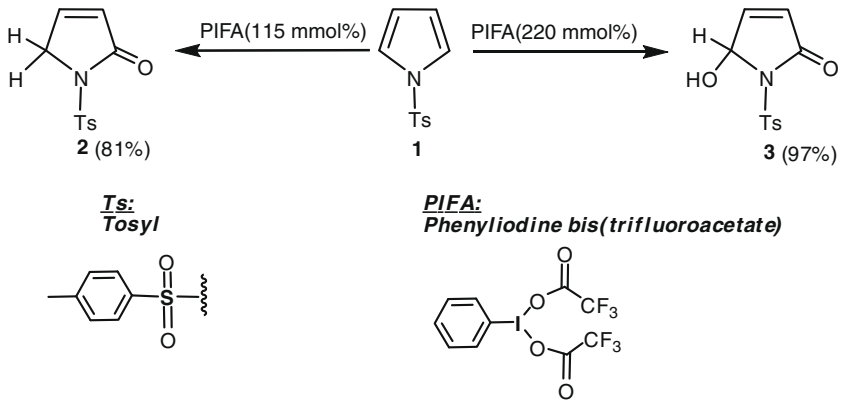
An X-ray diffraction analysis of *tosylated pyrrole compound* (**3**) was undertaken. Molecular structure and packing diagram of the compound are shown in Figure 1a and b, respectively. The compound crystallizes in the monoclinic space group *P21/c*, with four molecules in the unit cell. It contains the 1-tosyl pyrrole scaffold with the substituted hydroxyl and ketone moieties to pyrrole part and has the configuration shown in Figure 1a, which is the (*R*)-configuration at the chiral centre (related to the asymmetric C atom at C11). The bond lengths and angles are within normal ranges. Phenyl and pyrrole rings are planar and they are oriented at a dihedral angle of 88.10(8)°. C–O_{ketone} bond length is 1.210(3) Å. The structures of related tosyl pyrroles, that is, 2-bromo-1-(*p*-toluenesulfonyl)pyrrole,[13] bis(1-tosyl-2-pyrroleyl)ethyne[14] and 2-chloromethyl-1-(4-methylphenylsulfonyl)pyrrole[15] have been reported.

The geometry around S atom is significantly deviated from that of regular tetrahedral. The maximum and minimum angles around S are 119.9(2) and 103.9(2), respectively. S–O bond lengths differ by only 0.006 Å which implies that there is no delocalization of N1 lone pair into the sulfonyl group. S–O distances are similar to those found in analogous structures.[13–15] These distances do not vary significantly even in the sulfonamide structures, despite the different interaction patterns observed.[4] The S–N bond distance lies [1.652(2) Å] within the expected range of 1.63–1.69 Å. In all essential details, the geometry of the molecule regarding bond lengths and angles of the compound is in good agreement with the values observed in similar structures.[13–16]

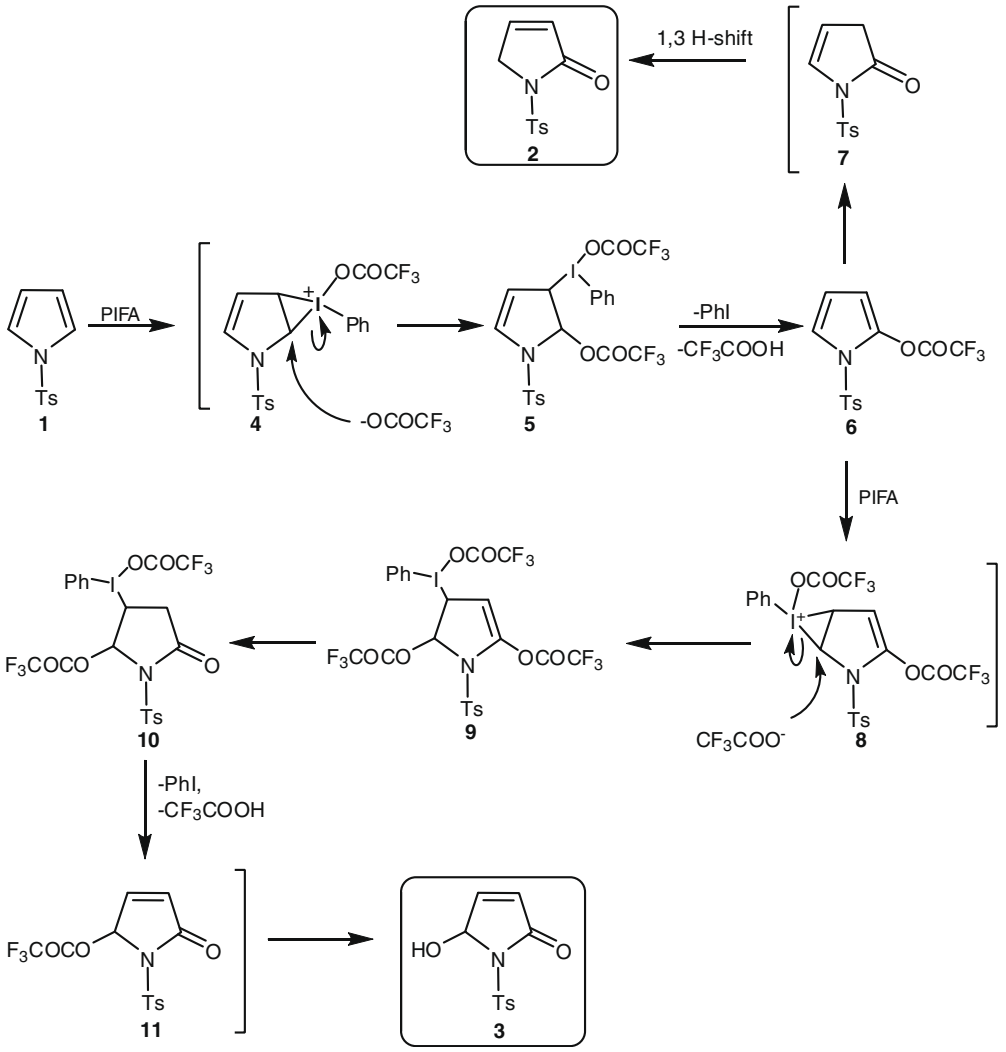
The crystal structure consists of 1D polymeric chain along the *c*-axis, where the hydroxyl and ketone atoms are involved in intramolecular C11–H11···O4 ($-x$, $-1/2+y$, $1/2-z$) hydrogen bonds, with H···O = 2.38 Å and C–H···N angle of 148°. In addition, the hydroxyl group is involved in one intermolecular O4–H4···O1 hydrogen bond, with sulfonyl O atom, (H···N = 2.49 Å and C–H···N = 122°) (Fig. 1).

2.3. CA purification, assay and inhibition by 1-tosyl pyrrole (1) and 1-tosyl pyrrol-2-one derivatives (2, 3, 12)

The purification of the two CA isozymes was performed in a simple single-step method by means of Sepharose-4B-aniline-sulfanilamide affinity column chromatoghrapy.[17] hCA I was purified 105.5-fold with a specific activity of 975.92 EU mg^{-1} and overall yield of 51.7%, and hCA II was purified 646.5-fold with a specific activity of 5980 EU mg^{-1} and overall yield of 55% (Table 1).[18–21] Inhibitory effects of tosylated compounds on enzyme activities were tested under in vitro conditions; K_i values were calculated from Lineweaver–Burk graphs and are given in Table 2.[22]



Scheme 1. Synthesis of compounds **2** and **3** with PIFA (phenyliodine bis(trifluoroacetate)).



Scheme 2. The mechanism for formation of compounds **2** and **3**.

We report here the first study on the inhibitory effects of tosyl pyrroles **1, 2, 3 and 12** on the esterase activity of hCAs I and II. The acetazolamide (**13**) was used as reference compound in our experiments for comparison reasons. Data of Table 2 and 3 show the inhibition of hCAs I and II with tosyl pyrroles **1, 2, 3 and 12**, by an esterase assay,[19] with 4-nitrophenylacetate (4-NPA) as substrate:

(i) Against the slow cytosolic isozyme hCA I, compounds **1–2** behave as moderate inhibitors, with K_i values in the range of 37.3–42.4 μM. A second group of derivatives, including **3** and **12**, showed better inhibitory activity as compared to the previously mentioned tosyl pyrroles, with K_i values of 14.6–25.7 μM, (Table 2). Thus, the nature of the groups strongly influences hCA I inhibitory activity. Acetazolamide **5** is also a medium-weak CAI with

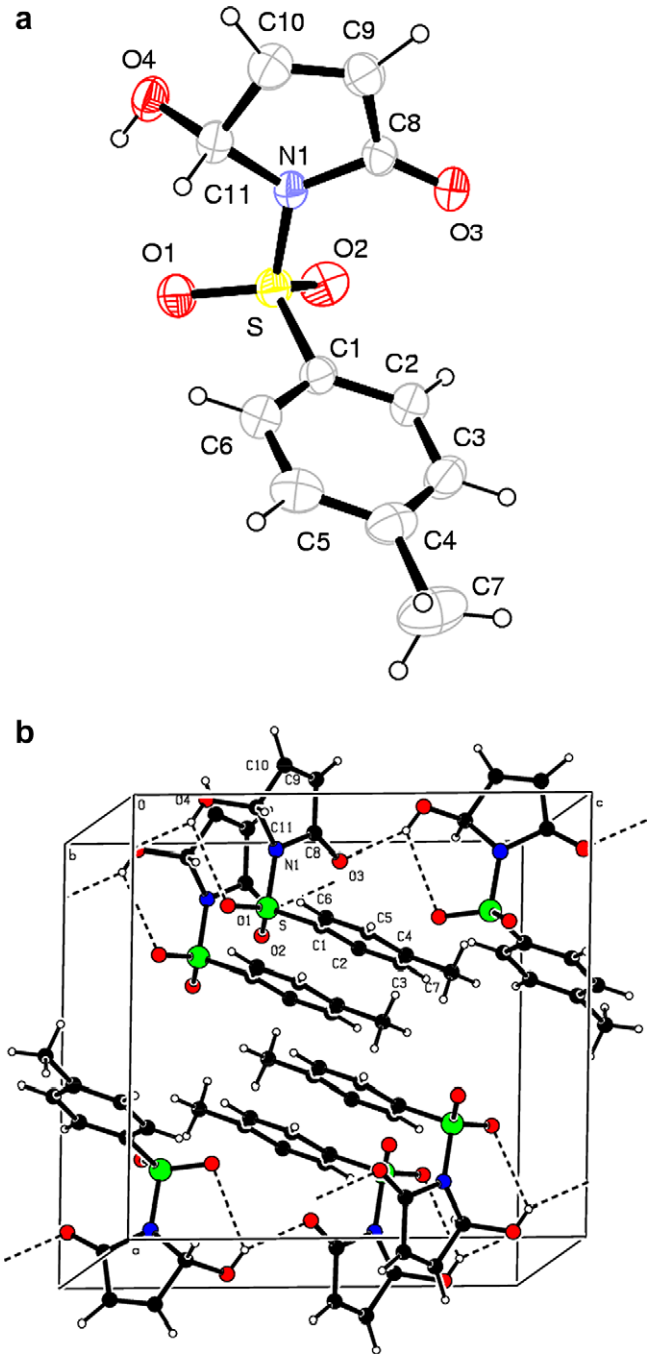
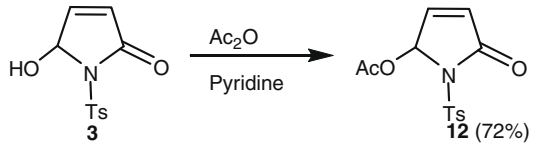


Figure 1. (a) The molecular structure of **3**, with the atom-numbering scheme. Displacement ellipsoids are drawn at the 50% probability level. (b) The crystal packing of (**3**), viewed down the *b*-axis. H-bonds are shown as dashed lines.



Scheme 3. Acetylation of compound **3** with pyridine/acetic anhydrate.

this assay and substrate against hCA I (K_i of 36.2 μM).[8b] Kinetic investigations (Lineweaver–Burk plots, data not shown) indicate that similar to sulfonamides and inorganic anions,[4–6,23–25] all the investigated compounds act as competitive inhibitors with 4-NPA as substrate, that is, they bind in the same regions of the active site cavity as compared to the substrate. However, the binding site of 4-NPA itself is unknown, but it is presumed to be in the same region as that of CO_2, the physiological substrate of this enzyme.[26]

(ii) A better inhibitory activity has been observed with compounds **3–4** investigated here for the inhibition of the rapid cytosolic isozyme hCA II (Table 2). Thus, two derivatives, that is, **1** and **2**, showed weak hCA II inhibitory activity with K_i-s in the range of 23.1–37.5 μM, Table 2), whereas the remaining two derivatives were quite effective hCA II inhibitors, with K_i-s in the range of 0.51–0.73 μM, (Table 2). Methoxy **4** is also effective hCA II inhibitor. The best hCA II inhibitor in this series of derivatives was the bulky, 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one **3**, which with a K_i of 0.51 μM is a better inhibitor than acetazolamide, a clinically used sulfonamide. It must be stressed that K_is measured with the esterase method is always in the micromolar range because hCAs I and II are weak esterases.[27] It should be noted that esterase method usually gives K_i-s an order of magnitude higher as compared to the CO_2 hydrase assay.

In a recent study, thioxolone, a simple compound lacking the sulfonamide, sulfamate or related functional groups that are typically found in all known CA inhibitors, was reported to act as a CAI inhibitor and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies (thioxolone acts as a prodrug, being hydrolyzed in situ with the formation of a mercapto phenol derivative which is the real enzyme inhibitor).[28] However, Innocenti et al.[29] showed that compared to sulfonamides, thioxolone was inefficient for generating isozyme-selective inhibitors, since except for hCA I which was inhibited in the nanomolar range (K_i of 91 nM), the remaining 12 mammalian CA isoforms (CA II–CA XV) were inhibited with a very flat profile by this compound (K_i-s in the range of only 4.93–9.04 μM). Thus, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

3. Conclusions

We have developed a novel and one-pot synthesis for 1-tosyl-1*H*-pyrrol-2(5*H*)-one, 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one. By means of the new developed method, **2** and **3** were synthesized at room temperature using only PIFA reactive in a single step, and also an available method is proposed for the synthesis of molecules which have similar structures. Tosylated pyrrole compounds **1, 2, 3 and 12** affect the activity of CA isozymes due to the presence of the different functional groups. Our findings here indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols/biphenyl diphenols. Indeed, some tosylpyrroles investigated here showed effective hCAs I and II inhibitory activity, in the low micromolar range, by the esterase method which usually gives K_i-s an order of magnitude higher as compared to the CO_2 hydrase assay.[30] These findings point out that these compounds may lead to generate potent CAIs against all CA isoforms and might be used for the treatment of CA-related disorders.

4. Experimental

4.1. General

All chemicals used were of reagent grade. Solvents were dried over appropriate drying agents and freshly distilled before use.

Table 1
Summary of purification procedure for human carbonic anhydrase isozymes (hCA I and hCA II) by a Sepharose-4B-aniline-sulfanilamide affinity column chromatography

Purification steps		Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate		163	43	18.88	811.84	7009	8.63	100	1
Sepharose-4B-aniline-sulfanilamide affinity column chromatography	hCA I	486	8	0.52	4.16	3888	934.62	55.47	108.30
	hCA II	794	5	0.13	0.65	3970	6107.70	56.64	707.73

The progress of synthetic reactions was monitored by thin layer chromatography (TLC) in a suitable solvent system. Column chromatography was performed on Fluka Silica Gel 60 (70–230 mesh, 254–366 mesh). Melting points were determined by a Gallen Kamp melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 400 spectrometer in deuterated chloroform ($CDCl_3$), depending on the solubility of the product. Chemical shifts are reported in δ ppm downfield with respect to tetramethylsilane as an internal standard in ^{1}H and ^{13}C NMR spectra. Elementary analyzed spectra were recorded using a Leco CHNS-932 spectrometer. Infrared spectra were recorded using Perkin–Elmer 337 Greating Spectrophotometer.

4.2. Chemicals

Tosylchloride, Sepharose 4B, protein assay reagents and 4-nitrophenylacetate were obtained from Sigma–Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

4.3. Synthesis of 1-tosyl-pyrrol-2-one derivatives

4.3.1. Synthesis of 1-tosyl-1*H*-pyrrol-2(5*H*)-one (2)

1 g (4.51 mmol) of 1-tosyl pyrrole (**1**) was dissolved in 30 ml of dichloromethane and stirred magnetically at −15 °C. 2.23 g (5.20 mmol, 1.15 equiv) of PIFA were added slowly to the reaction solution at −15 °C. Then the reaction mixture was warmed to room temperature and stirred at room temperature for 16 h. The reaction mixture was washed with $NaHCO_3$ solution and dried over sodium sulfate, filtered and evaporated to afford a residue. The crude residue was purified on column chromatography (silica gel) using ethyl acetate-hexane to yield 81% (0.87 g, 3.66 mmol) of 1-tosyl-1*H*-pyrrol-2(5*H*)-one (**2**) and 19% (0.22 g, 0.86 mmol) of 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one (**3**). 1-Tosyl-1*H*-pyrrol-2(5*H*)-one (**2**) was recrystallized from ether–hexane solution (mp: 127–129 °C).

^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ = 2.41 (s, 6H), 4.47 (t, 4H, *J* = 2.0 Hz), 6.04 (dt, 2H, *J* = 6.1, 2.0 Hz), 7.25 (dt, 2H, *J* = 6.1, 2.0 Hz), 7.32 (d, 4H, *J* = 8.42 Hz), 7.93 (d, 4H, *J* = 8.42 Hz). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): 23.5, 54.3, 129.0, 129.9, 131.7, 137.7, 147.0, 148.4, 170.1. IR (KBr, cm^{-1}): 3101, 2925, 1726, 1596, 1444, 1400, 1359, 1169, 1092. Anal. Calcd for $C_{11}H_{11}NO_3S$ (237.27): C, 55.68; H, 4.67; N, 5.90; S, 13.51. Found: C, 55.92; H, 4.52; N, 5.81; S, 13.26.

4.3.2. Synthesis of 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one (3)

1 g (4.51 mmol) of 1-tosyl pyrrole (**1**) was dissolved in 50 ml of dichloromethane and stirred magnetically at room temperature. 4.27 g (9.92 mmol, 2.2 equiv) of PIFA were added slowly to the reaction solution at room temperature and stirred at room temperature for 18 h. The reaction mixture was washed with $NaHCO_3$ solution and dried over sodium sulfate, filtered and evaporated to afford a residue. The crude residue was purified on column chromatography on silica gel ethyl acetate–hexane to yield 97% (1.11 g, 4.38 mmol) of 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one (**3**). 5-Hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one (**3**) was recrystallized from ether–hexane solution (mp: 140–142 °C).

^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 2.42 (s, 3H), 6.06 (dd, 1H, *J* = 6.1, 0.9 Hz), 7.05 (dd, 1H, *J* = 6.1, 1.83 Hz) 6.24 (m, 1H) 7.33 (d, 2H, *J* = 8.23 Hz), 7.95 (d, 2H, *J* = 8.23 Hz). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): 23.6, 78.4 129.4, 130.1, 131.7, 137.5, 147.3, 149.9, 168.5. IR (KBr, cm^{-1}): 3456, 3103, 2918, 1731, 1597, 1490, 13-401, 1354, 1168, 1112, 1055. Anal. Calcd for $C_{11}H_{11}NO_4S$ (253.27): C, 52.16; H, 4.38; N, 5.53; S, 12.66. Found: C, 51.88; H, 4.33; N, 5.47; S, 12.47.

4.3.3. Synthesis of 5-oxo-1-tosyl-2,5-dihydro-1*H*-pyrrole-2-yl acetate (12)

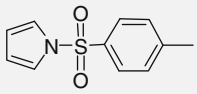
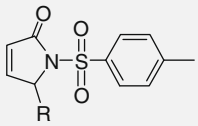
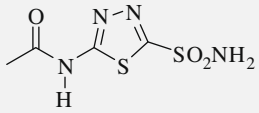
To a mixture of 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one **(3)** (1 g, 3.95 mmol) and pyridine (5 ml, 60 mmol) in dichloromethane (20 ml) was added slowly acetic anhydride (0.81 g, 7.90 mmol, 2 equiv). The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was washed with 5% HCl. Then the organic layer was washed with $NaHCO_3$ solution and dried over sodium sulfate, filtered and evaporated to afford a residue. Purification of the residue was by silica gel column chromatography using ethyl acetate–hexane to yield 72% (0.84 g, 2.84 mmol) of 5-oxo-1-tosyl-2,5-dihydro-1*H*-pyrrole-2-yl acetate (**12**). Compound **12** was recrystallized from ether–hexane solution (mp: 136–138 °C).

^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 2.16 (s, 3H), 2.43(s, 3H), 6.12 (dd, 1H, *J* = 6.8, 1.4 Hz), 7.10 (m, 2H), 7.34 (d, 2H, *J* = 8.24 Hz) 7.94 (d, 2H, *J* = 8.24 Hz). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): 22.5, 23.5, 83.0, 130.0, 130.1, 131.5, 137.8, 147.1, 147. 168.2, 171.0. IR (KBr, cm^{-1}): 3105, 2920, 1749, 1596, 1367, 1229, 1170, 1097. Anal. Calcd for $C_{13}H_{13}NO_5S$ (295.31): C, 52.87; H, 4.44; N, 4.74; S, 10.86. Found: C, 52.88; H, 4.50; N, 5.65; S, 10.77.

4.4. Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Centre of the Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% of NaCl and hemolyzed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20,000 rpm for 30 min at 4 °C. The pH of the hemolysate was adjusted to 8.7 with solid Tris. Firstly, Sepharose-4B was oxidized by $KMnO_4$ and subsequently activated by $SOCl_2$. After that aniline was attached to the activated gel as a spacer arm and finally diazotized sulfanilamide was clamped to the *para* position of aniline molecule as ligand. The hemolysate was applied to the prepared Sepharose 4B-aniline-sulfanylamide affinity column which had been equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4 °C.[13]

Table 2
hCAs I and II inhibition data with pyrrole derivatives **1–3, 12** and acetazolamide[6b] **13**, by an esterase assay with 4-nitrophenylacetate as substrate

Structure	Compounds	R	K_i[a] (μM)	
			hCA I	hCA II
	**1**	–	42.4 ± 3.1	37.5 ± 3.3
	**2**	H	37.3 ± 2.7	23.1 ± 2.9
	3	OH	14.6 ± 1.6	0.51 ± 0.04
	12	OAc	25.7 ± 3.7	0.73 ± 0.02
	**13**	–	36.2	0.37

[a] Mean from at least three determinations. Errors in the range of 5–10% of the reported value (data not shown).

Table 3
I_{50} values from in vitro study of hCA-I and hCA-II in the presence of different tosylpyrrole derivatives

Inhibitor	I_{50} values for hCA-I (μM)	I_{50} values for hCA-II (μM)
N-*p*-tosyl-pyrrole (**1**)	23.14	14.29
1-Tosyl-1*H*-pyrrole-2(5*H*)-one (**2**)	18.18	11.24
5-Hydroxy-1-tosyl-1*H*-pyrrole-2(5*H*)-one (**3**)	6.03	0.21
5-Oxo-1-tosyl-2,5-dihydro-1*H*-pyrrole-2-yl acetate (**12**)	7.97	0.37

4.5. Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[18] CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_o - t_c/t_c)$ where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

4.6. Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer (CHEBIOS UV–VIS) according to the method described by Verpoorte et al.[19] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M Tris–SO_4 buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL of H_2O and 0.1 mL of enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of 1-tosyl-pyrrole (**1**), 1-tosyl-1*H*-pyrrol-2(5*H*)-one (**2**), 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one (**3**) and 5-oxo-1-tosyl-2,5-dihydro-1*H*-pyrrol-2-yl acetate (**12**) were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. HCA-I enzyme activities were measured for *N*-*p*-tosyl-pyrrole (**1**) (10.50–39.5 μM), 1-tosyl-1*H*-pyrrole-2(5*H*)-one (**2**) (12.75–25.6 μM), 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one (**3**) (2.5–10.2 μM) and 5-oxo-1-tosyl-2,5-dihydro-1*H*-pyrrol-2-yl acetate (**12**) (5.0–10.7) at cuvette concentrations and HCA-II enzyme activities were measured for 1-*p*-tosyl-pyrrol (**1**) (10.15–21.5 μM), 1-tosyl-1*H*-pyrrol-2(5*H*)-one (**2**) (7.65–15.8 μM), 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one (**3**) (0.05–0.55 μM) and 5-oxo-1-tosyl-2,5-dihydro-1*H*-pyrrol-2-yl acetate (**12**) (0.10–0.75) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor, an Activity (%)–[Inhibitor] graph was drawn. To determine K_i values, three different inhibitor concentrations were tested. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver–Burk curves were drawn.[22]

4.7. Protein determination

Protein quantity was determined spectrophotometrically at 595 nm according to the Bradford method during the purification steps, using bovine serum albumin as the standard.[20]

4.8. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure. A 20-mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye.[21]

4.9. Crystal structure determination

For the crystal structure determination, the single-crystal of 5-hydroxy-1-tosyl-1*H*-pyrrol-2(5*H*)-one **3** was used for data collection on a four-circle Rigaku R-AXIS RAPID-S diffractometer (equipped with a two-dimensional area IP detector). The graphite-monochromatized Mo Kα radiation ($\lambda = 0.71073$ Å) and oscillation scans technique with $\Delta\omega = 5°$ for one image were used for data collection. The lattice parameters were determined by the least-squares methods on the basis of all reflections with $F^2 > 2\sigma(F^2)$. Integration of the intensities, correction for Lorentz and polarization effects and cell refinement was performed using CrystalClear (Rigaku/MSC Inc., 2005) software.[31] The structures

Table 4
Crystal data and structure refinement

Empirical formula	$C_{11}H_{11}NO_4S$
Formula weight	253.3
Temperature (K)	293(2)
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	$P21/c$
Unit cell dimensions	
a (Å)	12.2054(2)
b (Å)	7.3819(2)
c (Å)	12.8944(4)
α (°)	90
β (°)	93.93(3)
γ (°)	90
Volume (Å^3)	1159.0(1)
Z	4
Density (calcd) (Mg/m^3)	1.45
Absorption coefficient (mm^{-1})	0.281
F(0 0 0)	528
Crystal	Plate; colourless
Crystal size (mm$^{3)}$	0.20 × 0.02 × 0.02
θ range for data collection (°)	3.2–30.6
Index ranges	$-17 \leqslant h \leqslant 17$, $-9 \leqslant k \leqslant 10$, $-18 \leqslant l \leqslant 18$
Reflections collected	33639
Independent reflections	3558 [R_{int} = 0.079]
Completeness to θ = 30.2°	99.90%
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	2561/0/156
Goodness-of-fit on F^2	1.054
Final R indices [$F^2 > 2\sigma(F^2)$]	R_1 = 0.058, wR_2 = 0.159
R indices (all data)	R_1 = 0.084, wR_2 = 0.179
Largest diff. peak and hole	0.216 and −0.414 e Å^{-3}

were solved by direct methods using SHELXS-97[32] and refined by a full-matrix least-squares procedure using the program SHELXL-97.[32] Hydrogen atom involved in hydrogen bonding was located in the difference Fourier map and allowed to refine freely. All other hydrogen atoms were added at calculated positions and refined using a riding model. Their isotropic temperature factors were fixed to 1.2 times (1.5 times for methyl groups) the equivalent isotropic displacement parameters of the parent carbon atom. Anisotropic thermal displacement parameters were used for all non-hydrogen atoms. The final difference Fourier maps showed no peaks of chemical significance. Crystal data and structure refinement parameters of compound (**3**) are given in Table 4.

Supplementary data

Crystallographic data (excluding structure factors) for the structures reported in this article have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 765673. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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