Bioorganic & Medicinal Chemistry 21 (2013) 21–27

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Facile synthesis and characterization of novel pyrazole-sulfonamides and their inhibition effects on human carbonic anhydrase isoenzymes

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ARTICLE INFO

Article history:
Received 11 September 2012
Revised 1 November 2012
Accepted 4 November 2012
Available online 27 November 2012

Keywords:
Pyrazole
Sulfonamide
Diazonium
Carbonic anhydrase
Inhibition

ABSTRACT

In the current study, a series of pyrazole-sulfonamide derivatives (**2–14**) were synthesized, characterized, and the inhibition effects of the derivatives on human carbonic anhydrases (hCA I and hCA II) were investigated as in vitro. Structures of these sulfonamides were confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR and LC–MS analysis. ^{1}H NMR and ^{13}C NMR revealed the tautomeric structures. hCA I and hCA II isozymes were purified from human erythrocytes and inhibitory effects of newly synthesized sulfonamides on esterase activities of these isoenzymes have been studied. The K_i values of compounds were 0.062–1.278 μM for hCA I and 0.012–0.379 μM for hCA II. The inhibition effects of **7** for hCA I and **4** for hCA II isozymes were almost in nanomolar concentration range.

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1. Introduction

Carbonic anhydrase (CA), a metalloenzyme (EC 4.2.1.1) containing Zn^{+2} ion in its active region catalyses the hydration of CO_2 and dehydration of HCO_3^{-}.[1,2] Beside the hydration reaction of CO_2, CA catalyses the reaction of cyanamide to carbamic acid or urea to cyanamide, hydration of aldehydes to geminal diol. It also hydrolyses carboxylic, sulfonic, and phosphoric acid esters.[3]

Sixteen CA isoenzymes isoenzymes have been identified till now. It was shown that the vital functions of these isoenzymes differ according to tissues and organs. Lung, kidney, gastric mucosa, eye lenses, salivary glands, muscles, nerve myelin sheath, pancreas, prostate and endometrial tissues come first among these tissues. CA enzyme is characterized from most of these tissues and functions of it are being tried to be determined.[4]

Carbonic anhydrase inhibitors (CAIs) block the function of carbonic anhydrase enzyme. Primary sulfonamides, having the molecular formula of RSO_2NH_2 are the most well-known CA inhibitors. Sulfonamides, like acetazolamide, are useful antiglaucoma agents. However, the usages of sulfonamides are limited because of numerous side effects. Sulfonamides, as well as the usage as antiglaucoma agent, are used to treat cancer and some neurological diseases.[5] For this reason, there are several attempts to illuminate the inhibition mechanism of CA and to synthesize new compounds with higher inhibition potential. Novel CA inhibitors have been designed for pharmacological and medicinal approaches, and many inhibitors have been synthesized recently.[6]

Pyrazole derivatives have attracted great attention due to widespread applications in pharmaceutical and agrochemical industries.[7–9] The presence of pyrazole as the central core in the biologically active molecules has grown rapidly in the past decade.[7,10,11] Among various properties of pyrazole derivatives, for example, anti-bacterial, anti-inflammatory, anti-obesity, antidepressant, anticoagulant, leishmanicidal and cytotoxic activities have been reported.[12–18]

The present study was carried out in order to synthesize, characterize and evaluate biological activity of a new series of pyrazole-sulfonamide derivatives. In this current study, our main aim was to check whether the new derivatives may show higher inhibitory effect on CA enzymes with respect to the compounds previously investigated. The synthesized molecules have been characterized by various techniques including NMR, FT-IR and LC–MS. For in vitro inhibition studies human erythrocyte carbonic anhydrase (CA I and CA II) isoenzymes were purified using Sepharose-4B L-tyrosine-sulfanilamide affinity chromatography. The inhibitory effects of novel compounds on the activities of purified human erythrocyte CA I and CA II isozymes were investigated.

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0968-0896/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmc.2012.11.012

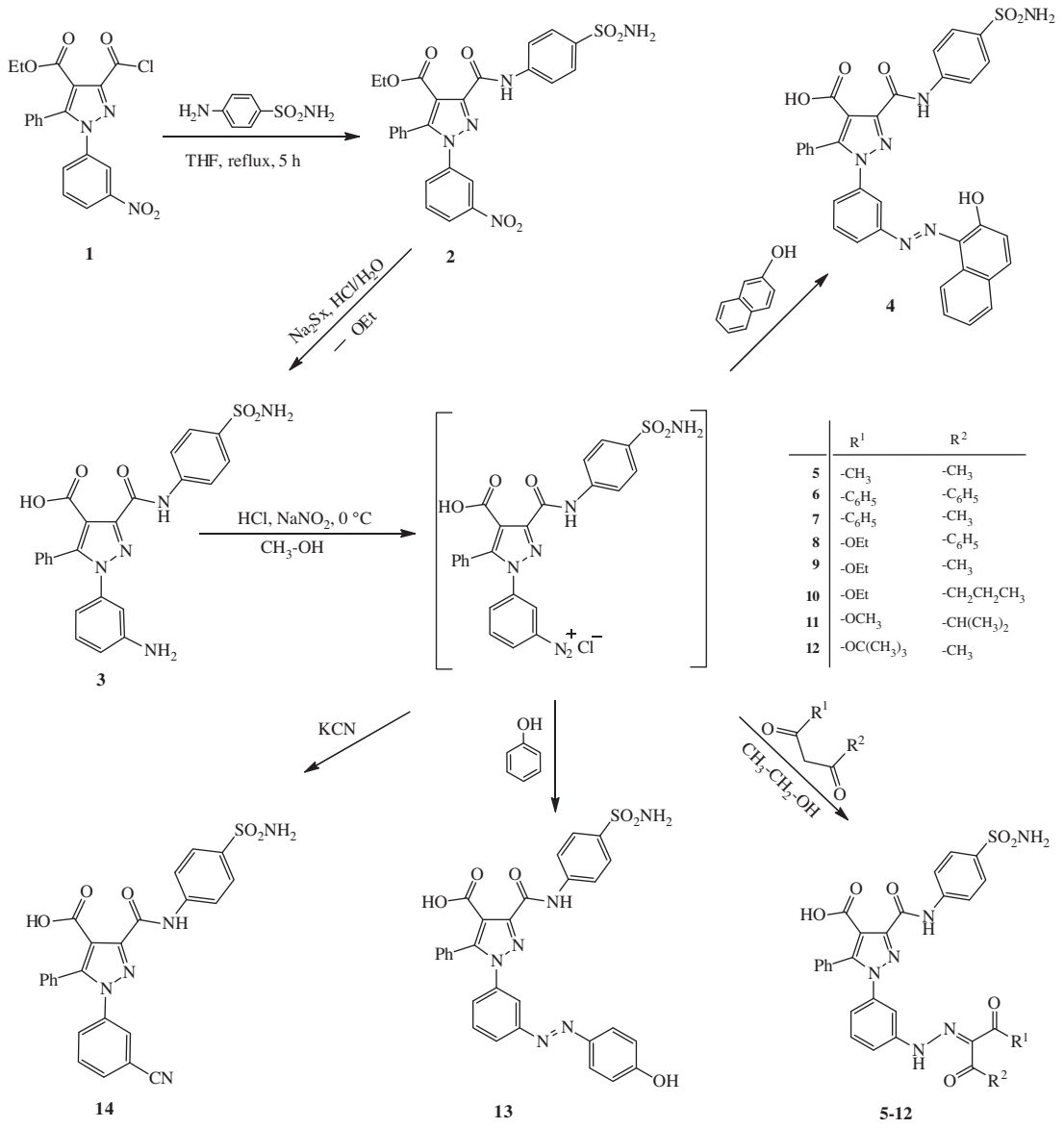
2. Results and discussion

2.1. Chemistry

In this study first, ethyl 1-(3-nitrophenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylate (**2**) was synthesized by the reaction of ethyl 3-(chlorocarbonyl)-1-(3-nitrophenyl)-5-phenyl-1*H*-pyrazole-4-carboxylate (**1**) with 4-amino benzenesulfonamide. Subsequent reduction of the nitro group of **2** by sodium polysulphur hydrogenation ($Na_2S/S/H_2O$) afforded **3**. The spectral analysis indicated the simultaneous hydrolysis of the ester group of **2** with the reduction reaction. The hydrolysis of the reactant (**2**) is explained by heating the mixture with HCl solution. Compound **3** then underwent diazoniation and final reactions with phenol, 2-naphthol and 1,3-dicarbonyl compounds to yield novel pyrazole-sulfonamides (**4**–**13**). A nitrile derivative (**14**) was also synthesized by interference of diazonium compound with KCN. The synthesis of compounds is shown in Scheme 1.

Isotropic chemical shifts of Ar–OH and N**H** appear as downfield shifts in ^{1}H NMR spectra. The peaks of Ar–OH and N**H** are used to distinguish between possible tautomeric structures. The ^{1}H NMR and ^{13}C NMR results showed that **5**, **7** and **9**–**12** show tautomeric structures while **6** and **8** prefer keto-hydrazo form (See Fig. 1). The relative integral of the enol and keto peaks of compounds **5**, **7**, **9**, **10**, **11**, **12** are 1:0.95, 1:4.55, 1:6.69, 1:7.44, 1:11.90, 1:5.44, respectively. Compound **5** has almost equally probable tautomeric structures. Compounds **7** and **9**–**12** favor keto-hydrazo form with respect to the enol form on the NMR time scale. As the –R moiety is varied from the phenyl group to the alkyl groups, the novel molecules start showing tautomeric forms. In other words, the phenyl group contributes to the stability of the molecule keeping only one form rather than showing resonance structures.

On the other hand, the thermal decomposition of the compounds upon heating did not allow the determination of melting temperatures (see Fig. 2 and Table 1 showing the TGA results). Compound **12** exhibited the lowest degradation temperature while compound **2** had the highest value. Compounds **4**, **13** and **14** started degradation right above 280 °C. Interestingly, **7**, **8** and **9** began decomposition in the same temperature range of 215–225 °C. Such similar thermal degradation temperatures are attributed to similar chemical structures. We think that the release of the –R moiety occurred at relatively elevated temperatures. It was even observed that the TGA



	R1	R2
5	-CH3	-CH3
6	-C6H5	-C6H5
7	-C6H5	-CH3
8	-OEt	-C6H5
9	-OEt	-CH3
10	-OEt	-CH2CH2CH3
11	-OCH3	-CH(CH3)2
12	-OC(CH3)3	-CH3

Scheme 1. Synthesis of target inhibitors **2**–**14**.

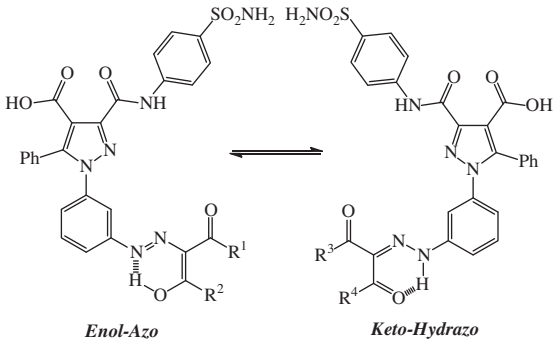


Figure 1. Only keto-hydrazo for (**6**,**8**) and tautomeric structures of keto-hydrazo and enol-azo for (**5**,**7**, **9**–**12**).

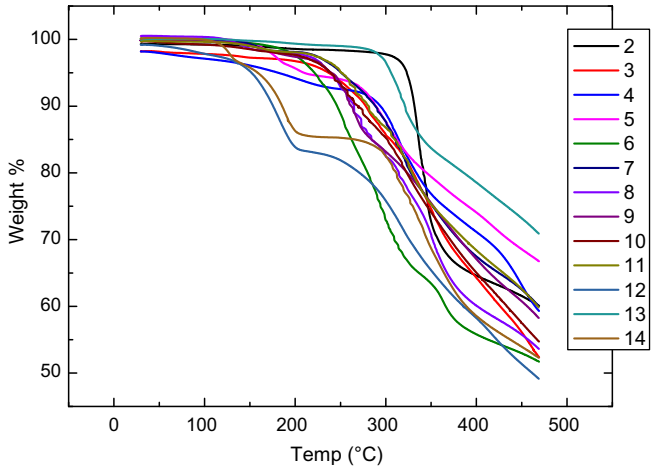


Figure 2. TGA results of compounds **2**–**14**.

Table 1
Thermal decomposition temperatures of the compounds

Compound	Thermal degradation temperature (°C)
2	300
3	210
4	280
5	265
6	187
7	210
8	218
9	225
10	195
11	183
12	120
13	280
14	280

pans were enlarged at the end of the experiments, which is explained by the release of the –R moiety to the gas phase. The –R moiety release did not allow to performing DSC experiments for determining the melting temperatures of the molecules.

2.2. Biological activity studies

CA inhibitors (CAIs) have been used in many pharmacological applications including treatment of glaucoma, diuretics, anticonvulsants, antiobesity and/or antitumor agent/diagnostic tools.[19] Since CA II plays an important role in the production of aqueous humor in humans,[20] CA II inhibitors are particularly used in the treatment of glaucoma, epilepsy, and used as diuretics.

In the current study, isoenzymes of CA I and CA II were purified from human erythrocytes, intensive resource of CAs. For the purification procedure Sepharose-4B L-tyrosine affinity chromatography method was employed. Following the purification step, the inhibitory effects of compounds from **2** to **14** on the activities of purified human erythrocyte CA I and CA II isozymes were studied. It was not possible to investigate the inhibitory effect of **3**. K_i values of the compounds are given in Table 2. Almost all of the compounds inhibited the isoenzymes in a noncompetitive manner, except compounds **4** and **7**. Compound **7** inhibited CA I and similarly compound **4** inhibited CA II uncompetitively. Interestingly the compounds showing the uncompetitive inhibitory effects on CA I and CA II had the smallest K_i values. The strongest inhibitor on CA I was the compound **7** with a K_i value of 0.0623 μM. The compound **4** showed the strongest inhibition on CA II with a K_i value of 0.0124 μM. K_i values of these two compounds are almost in nanomolar concentration range. According to decreasing inhibition power on CA I, compounds can be ranked as **7**, **2**, **8**, **6**, **4**, **12**, **5**, **10**, **11**, **14**, **13** and **9**. The inhibition decreasing list of CA II is in the order of **4**, **12**, **10**, **8**, **6**, **11**, **2**, **7**, **9**, **5**, **13** and **14** (See Table 2). The compounds have smaller K_i values for CA II in general. The smaller the K_i values mean higher inhibitory effect.

The structure–activity relationship (SAR) mainly depends on the tautomeric structures and the –R moiety. Compounds **8**, **6** and **4** being stable only in one form depict higher inhibition effect on both of the enzymes CA I and II. However, compounds **7**, **10**, **11** and **12** having resonance structures do not show consistent inhibition effect. For instance, compound **10** has relatively lower inhibition effect on CA I than CA II. Both of the compounds **13** and **14** exhibit the lowest inhibition on the enzymes. These results indicate that varying the –R moiety has significant effect on the biological activity of the sulfonamide derivatives. The compound **4** including 2-naphtol group is the most effective over CA II isoenzyme. We proposed that the OH group in this compound increase the inhibition effect by interacting with other amino acids in CA II enzyme. The values obtained for this compound are consistent with one of our previous works.[21] Also the coupling reactions of **3** with 1,3-dicarbonyl compounds gave eight novel sulfonamide derivatives. Although inhibition effects of these compounds are relatively weak compare with **4**, the values are still quite good (0.0623–1.278 μM for CA I and 0.0954–0.259 μM for CA II). Among these compounds, compound **10** includes propyl and ethoxy groups as R substituent. Also **12** includes methyl and *t*-butoxide. It is estimated that these compounds have relatively less inhibition effects on CA II because of the steric effect of whole or some part of these R substituents. K_i values of **10** and **12** (0.09 and 0.111 μM for CA II, respectively) are consistent with the K_i values of the compounds, which have similar R substituents, in our previous studies.[22,23] Although the nitrile substituent in **14** is less bulky according to others, this compound exhibited less inhibition effect on CA II. K_i value of this compound (0.379 μM for CA II) is consistent with the K_i value (0.410 μM for CA II) of nitrile derivative in our previous study.[21]

Our inhibition type results (Table 2) in this study are consistent with those of our previous ones.[24] Almost all of the chemicals inhibited the enzyme noncompetitively. However, Lineweaver–Burk graphs of the compounds **7** and **4** depict uncompetitive inhibition on CA I and CA II, respectively (Fig. 3). In an uncompetitive inhibition, inhibitors bind to enzyme-substrate complex not to the enzyme itself. The inhibitor prevents formation of the products by binding to this complex. Small K_i values indicate the stability of enzyme-substrate-inhibitor complex and production of fewer amount of the products. Consequently, **4** and **7** are better inhibitors than the other compounds on activity of CA isozymes.

Table 2
K_i values and inhibition types of chemicals for CA I and CA II

İnhibitor	hCA I		hCA II	
	K_i (μM)	Inhibition type	K_i (μM)	Inhibition type
2	0.102 ± 0.0503	Noncompetitive	0.144 ± 0.0227	Noncompetitive
3	—	—	—	—
4	0.318 ± 0.159	Noncompetitive	0.0124 ± 0.0037	Uncompetitive
5	0.448 ± 0.202	Noncompetitive	0.259 ± 0.0713	Noncompetitive
6	0.219 ± 0.115	Noncompetitive	0.128 ± 0.0424	Noncompetitive
7	0.0623 ± 0.0227	Uncompetitive	0.159 ± 0.0298	Noncompetitive
8	0.115 ± 0.0266	Noncompetitive	0.126 ± 0.0482	Noncompetitive
9	1.278 ± 0.046	Noncompetitive	0.197 ± 0.0503	Noncompetitive
10	0.485 ± 0.0887	Noncompetitive	0.111 ± 0.0123	Noncompetitive
11	0.490 ± 0.191	Noncompetitive	0.136 ± 0.0743	Noncompetitive
12	0.384 ± 0.174	Noncompetitive	0.0954 ± 0.0398	Noncompetitive
13	1.079 ± 0.437	Noncompetitive	0.349 ± 0.0144	Noncompetitive
14	0.806 ± 0.0202	Noncompetitive	0.379 ± 0.0764	Noncompetitive

Changes in CA activity can be related with diabetes and related metabolic diseases such as hypertension.[25] The inhibition studies on CAs help in understanding mechanisms of enzyme catalysis. Studies about the enzyme's tissue distribution and understanding the vital functions of the enzymes in the tissues gained significance. Due to such important factors, syntheses of enzyme inhibitors and activators attract more attention.[3,23,26] Up to now, inhibitory effects of various different anions, metal ions, drugs, phenols and sulfonamides, functioning as specific inhibitors, have been investigated on the activity of CA isozymes.[22,27–30] In the current contribution, the compounds showed strong inhibitory effects on CA isozymes, especially on CA II. Among these compounds, **4** and **7** one are powerful inhibitors for CA and may be used in the generation of potent CAIs.

3. Experimental protocols

3.1. Chemicals

Deuterated dimethyl sulfoxide (d-DMSO) with 99.98% purity, ethyl benzoyl acetate, benzoyl acetone, CNBr-activated Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma-Aldrich. Acetyl acetone, 2-naphtol, sodium azide, sodium nitrite, L-tyrosine, sodium acetate, and solvents such as propanol, ethanol and tetrahydrofuran were purchased from Merck. Dibenzoyl methane and 4-aminobenzene sulfonamide were obtained from Fluka. Tetrahydrofuran was freshly distilled before use. All reactions were monitored by analytical thin-layer chromatography (TLC) on 0.25 mm pre-coated Kieselgel 60F 254 plates (Merck); compounds were visualized by Camag TLC devices UV (254 and 366 nm).

3.2. Characterization

NMR: All the compounds were dissolved in DMSO-*d* for NMR measurements. NMR experiments were performed on a JEOL 500 Lambda instrument (operating at 500 MHz for ^{1}H, 125 MHz for ^{13}C) with a multi-nuclei 5 mm solution probe (50TH5/FG2). The ^{1}H NMR spectra were acquired with recycle delay time of 10 s, and with 32 repetitions. The proton spectra were internally referenced to the signal of the solvent at 2.50 ppm. The ^{13}C NMR spectra were acquired by cross-polarization pulse sequence including proton decoupling with 2 s recycle delay time and with 1024 repetitions. The ^{13}C NMR spectra were again internally referenced to the solvent signal at 39.5 ppm.

FT-IR: The FT-IR transmission spectra of each compound were recorded on Bruker Optics, Vertex 70 FT-IR spectrometer. For each sample, 100 scans were taken at a resolution of 4 cm^{-1} with N_2 exposure.

LC–MS: The LC–MS data were acquired by positive ionization on a Bruker Esquire HCT connected with an electrospray ionization unit.

TGA: The thermal decomposition temperatures were measured with a Thermogravimetric analyzer. TGA measurements were per-

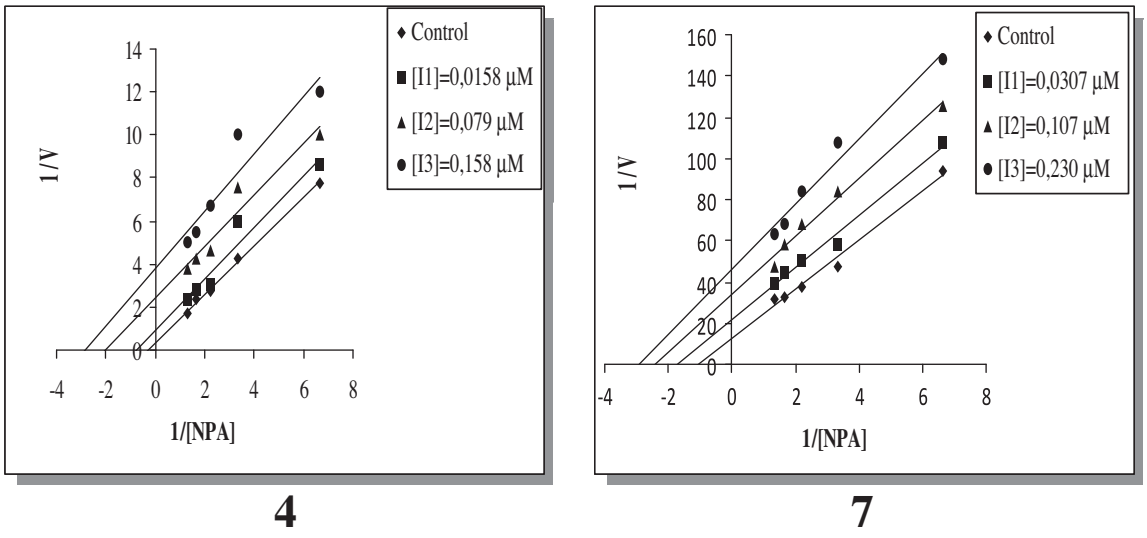


Figure 3. Lineweaver–Burk graphs of the compound **4** on CA II and compound **7** on CA I.

formed on Netzch STA 449 C (Selb, Germany) with the component of DSC (/TG) HIGH RG 4 unit, at a heating rate of 10 °C min^{-1} under a helium purge of 65 ml min^{-1}.

3.3. Synthesis

Compound **1** was prepared as the starting material by following the previous literature reported procedures.[23]

3.3.1. Ethyl 1-(3-nitrophenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylate (2)

Compound **1** (0.399 g, 1 mmol) was dissolved in 20 mL dry THF. To this solution 4-aminobenzenesulfonamide (0.344 g, 2 mmol) was added. Mixture was refluxed for 5 h and solvent was evaporated. The crude product was washed with water and crystallized from ethanol. Yield: 96%; IR (ν, cm^{-1}): 3353 and 3221 (NH), 3059 (ArCH), 2980 (Aliphatic CH), 1690 (C=O, ester), 1670 (C=O, amide), 1594–1427 (C=N and C=C), 1322 and 1131 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.02 (s, 1H, CONH), 8.19–7.32 (m, 13H, ArH), 7.30 (s, 2H, SO_2NH_2), 4.04 (q, J = 7.1 Hz, 2H, OCH_2), 0.95 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 162.3 (C=O, ester), 160.9 (C=O, amide), 148.6 (=C−NO_2), 148.2, 146.2 and 120.1 (pyrazole C-3, C-5 and C-4), 61.2 (OCH_2), 14.1 (CH_3), 141.9, 139.4, 132.2, 131.2, 130.7, 130.5, 129.0, 127.8, 127.3, 123.9, 120.9, 113.9; MS (ESI): *m/z* 536.1 [M+1] (calcd for $C_{25}H_{21}N_5O_7S$: 535.12).

3.3.2. 1-(3-Aminophenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (3)

Compound **3** containing aromatic amine group was prepared as described previously[21] and crystallized from methanol. Yield: 75%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3308 (NH), 3176 and 3053 (ArCH), 1706 (C=O, acid), 1630 (C=O, amide), 1587–1421 (C=N and C=C), 1324 and 1151 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.03 (s, 1H, CONH), 7.83–6.26 (m, 13H, ArH), 7.29 (s, 2H, SO_2NH_2), 6.54 (m, 2H, Ar–NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 164.0 (C=O, acid), 161.7 (C=O, amide), 149.7 (=C−NH_2), 146.3, 146.0, and 120.4 (pyrazole C-3, C-5 and C-4), 141.9, 139.6, 139.4, 130.5, 129.7, 128.8, 128.6, 127.2115.2, 114.5, 113.8, 111.7; MS (ESI): *m/z* 478.0 [M+1] (calcd for $C_{23}H_{19}N_5O_5S$: 477.11).

3.3.3. General procedure for the syntheses of compounds 4–13

1-(3-aminophenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (**3**) (0.477 g, 1 mmol) was dissolved in a mixture of methanol (30 ml) and concentrated hydrochloric acid (2 ml). The solution was then cooled to 0–5 °C. Sodium nitrite (0.104 g, 1.5 mmol) in water (10 ml) was then added to this solution dropwise with vigorous stirring while keeping at 0−5 °C. After dissolving an aromatic or β-dicarbonyl compound (1 mmol) in a sufficient amount of ethanol, the solution was cooled and added dropwise into the already prepared diazonium salt solution. The pH of the coupling mixture, in each case, was maintained at 7−8 through the coupling process by adding aqueous sodium acetate. Stirring was continued for 1 h at 0−5 °C and 2 h at room temperature. The precipitated products were filtered off, washed with water several times, dried, and recrystallized from an appropriate solvent.

3.3.4. 1-(3-[(2-Hydroxynaphthalen-1-yl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenyl carbamoyl)-1*H*-pyrazole-4-carboxylic acid (4)

Synthesized from **3** (0.477 g, 1 mmol) and β-naphtol (0.144 g, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 88%; IR (ν, cm^{-1}): 3590 (OH), 3500–2500 (COOH), 3250 (NH), 3060 (ArCH), 1732 (C=O, acid), 1683 (C=O, amide), 1591–1447 (C=N and C=C), 1318 and 1151 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 15.47 (s, 1H, Ar–OH), 14.61 (br, s, 1H, CONH), 8.20–6.76 (m, 19 H, ArH), 7.30 (s, 2H, SO_2NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 165.9 (C=O, acid), 160.9 (C=O, amide), 146.1, 145.8, and 119.9 (pyrazole C-3, C-5 and C-4), 145.2 (=C−OH), 142.9 (=C–N=N), 141.6, 140.7, 138.6, 132.9, 131.0, 130.8, 130.3, 129.9, 129.8, 129.5, 129.2, 128.3, 128.3, 127.2, 126.9, 124.7, 1221.0, 119.4, 114.7, 108.7; MS (ESI): *m/z* 633.1 [M+1] (calcd for $C_{33}H_{24}N_6O_6S$: 632.15).

3.3.5. 1-(3-[(2-Hydroxy-4-oxopent-2-en-3-yl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenyl carbamoyl)-1*H*-pyrazole-4-carboxylic acid (5)

Synthesized from **3** (0.477 g, 1 mmol) and acetylacetone (0.103 ml, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 95%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3257 (NH), 3064 (ArCH), 2986 (Aliphatic CH), 1720 and 1671 (C=O), 1589–1430 (C=N and C=C), 1313 and 1159 (S=O, assym. and sym.); ^{1}H NMR (125 MHz, DMSO-d_6) δ (ppm): 13.69 (s, 1H, C=C−*OH*), 11.59 (br, s, 1H, CONH), 7.86–7.08 (m, 13H, ArH), 7.29 (s, 2H, SO_2NH_2), 2.39 and 2.26 (s, 6H, $2CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 197.8 and 197.3 (C=O, keton), 164.2 (C=O, acid), 161.4 (C=O, amide), 147.1 (=C–N=N), 146.1, 145.9 and 120.3 (pyrazole C-3, C-5 and C-4) 31.6 and 26.8 (CH_3), 142.8, 141.9, 139.9, 139.3, 135.1, 134.6, 129.9, 128.2, 122.6, 121.8, 116.9, 114.9, 113.5; MS (ESI*): m/z* 611.1 [M+Na] (calcd for $C_{28}H_{24}N_6O_7S$: 588.14).

3.3.6. 1-(3-[2-(1,3-Dioxo-1,3-diphenylpropan-2-ylidene)hydrazinyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (6)

Synthesized from **3** (0.477 g, 1 mmol) and dibenzoylmethane (0.224 g, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 79%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3256 (NH), 3059 (ArCH), 1722 and 1625 (C=O), 1591–1447 (C=N and C=C), 1327 and 1156 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.66 (s, 1H, Ar–*NH*–N=), 11.05 (s, 1H, CONH), 8.09–6.79 (m, 23H, ArH), 7.30 (s, 2H, SO_2NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 194.2 and 191.1 (C=O, benzoyl), 163.8 (C=O, acid), 161.7 (C=O, amide), 145.9, 144.0 and 120.3 (pyrazole C-3, C-5 and C-4), 141.9, 139.7, 139.4, 138.2, 137.1, 136.6, 136.3, 134.9, 133.6, 130.5, 130.4, 129.6, 129.4, 129.0, 128.9, 128.7, 128.6, 127.7, 127.3, 115.6, 114.4, 93.7; MS (ESI): *m/z* 735.2 [M+Na] (calcd for $C_{38}H_{28}N_6O_7S$: 712.17).

3.3.7. 1-(3-[(3-Hydroxy-1-oxo-1-phenylbut-2-en-2-yl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (7)

Synthesized from **3** (0.477 g, 1 mmol) and benzoylacetone (0.162 g, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 86%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3258 (NH), 3060 (ArCH), 2970 (aliphatic CH), 1721 (C=O), 1591–1421 (C=N and C=C), 1323 and 1156 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 13.74 (s, 1H, C=C−*OH* enol tautomer), 11.15 (s, 1H, Ar–*NH*–N= keto tautomer), 11.04 (s, 1H, CONH), 7.86–6.89 (m, 18H, ArH), 7.29 (s, 2H, SO_2NH_2), 2.38 (s, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 196.9 (C=O, acetyl), 195.4 (C=O, benzoyl), 163.9 (C=O, acid), 161.6 (C=O, amide), 147.4, 146.1, 120.3 (pyrazole C-3, C-5, C-4), 25.3 (CH_3), 144.1, 141.8, 140.0, 139.7, 139.4, 135.8, 135.1, 130.6, 129.6, 129.2, 128.8, 128.7, 127.2, 126.4, 120.4, 115.4, 114.4, 112.3, 97.4; MS (ESI): *m/z* 651.2 [M+1] $C_{33}H_{26}N_6O_7S$: 650,16).

3.3.8. 1-(3-[2-(1-Ethoxy-1,3-dioxo-3-phenylpropan-2-ylidene)hydrazinyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (8)

Synthesized from **3** (0.477 g, 1 mmol) and ethylbenzoylacetate (0.172 ml, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 82%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3253 (NH), 3055 (ArCH), 2971 (aliphatic CH), 1726 (C=O), 1590–1422 (C=N and C=C), 1326 and 1157 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.89 (s, 1H, Ar–*NH* –N=), 11.02 (s, 1H, CONH), 7.86–6.83 (m, 18H, ArH); 7.29 (s, 2H, SO_2NH_2), 4.26 (q, J = 7.1 Hz, 2H, OCH_2), 1.20 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 189.6 (C=O, benzoyl), 163.8 (C=O, acid), 162.7 (C=O, ester) 161.5 (C=O, amide), 147.4, 146.1, 120.3 (pyrazole C-3, C-5, C-4), 62.0 (OCH_2), 14.3 (CH_3), 143.5, 141.9, 139.8, 139.4, 137.3, 133.3, 130.6, 130.3, 130.1, 129.9, 128.9, 128.8, 128.7, 128.4, 127.2, 120.9, 115.9, 114.4, 112.7; MS (ESI): *m/z* 681.2 [M+1] (calcd for $C_{34}H_{28}N_6O_8S$: 680.17).

3.3.9. 1-(3-[(1-Ethoxy-3-hydroxy-1-oxobut-2-en-2-yl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (9)

Synthesized from **3** (0.477 g, 1 mmol) and ethyl acetoacetate (0.126 ml, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 91%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3260 (NH), 3060 (ArCH), 2970 (Aliphatic CH), 1722 and 1683 (C=O), 1590–1402 (C=N and C=C), 1313 and 1158 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 13.89 (s, 1H, C=C–*OH* enol tautomer), 11.52 (s, 1H, Ar–*NH*–N= keto tautomer), 11.29 (s, 1H, CONH), 7.88–6.97 (m, 13H, ArH), 7.29 (s, 2H, SO_2NH_2), 4.24 (q, J = 7.1 Hz, 2H, OCH_2), 2.42 (s, 3H, $COCH_3$) 1.21 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 194.8 (C=O, acetyl), 164.0 (C=O, acid), 162.8 (C=O, ester), 161.5 (C=O, amide), 147.2, 146.1, 120.3 (pyrazole C-3, C-5, C-4), 62.0 (OCH_2), 25.9 and 14.3 (CH_3), 143.4 141.9, 139.9, 139.3, 132.4, 130.7, 130.6, 129.9, 128.8, 128.7, 127.2, 121.1, 115.8, 114.8, 112.6; MS (ESI): *m/z* 641.2 [M+Na] (calcd for $C_{29}H_{26}N_6O_8S$: 618.15).

3.3.10. 1-(3-[(1-Ethoxy-3-hydroxy-1-oxohex-2-en-2-yl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (10)

Synthesized from **3** (0.477 g, 1 mmol) and ethyl butyrylacetate (0.160 ml, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 86%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3259 (NH), 3064 (ArCH), 2970 (Aliphatic CH), 1735 and 1684 (C=O), 1592–1458 (C=N and C=C), 1335 and 1157 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm):13.81 (s, 1H, C=C–*OH* enol tautomer), 11.46 (s, 1H, Ar–*NH*–N= keto tautomer), 11.23 (s, 1H, CONH), 7.88–6.96 (m, 13H, ArH), 7.29 (s, 2H, SO_2NH_2), 4.24 (q, J = 7.1 Hz, 2H, OCH_2), 2.66 (t, J = 7.2 Hz, 2H, $COCH_2CH_2$) 1.51 (hextet, J = 7.3 Hz, 3H, $CH_2CH_2CH_3$), 1.21 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 0.85 (t, J = 7.3 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 197.0 (C=O, keton), 164.0 (C=O, acid), 162.9 (C=O, ester), 161.5 (C=O, amide), 147.3, 146.0, 120.3 (pyrazole C-3, C-5, C-4), 61.4 (OCH_2), 18.0 and 14.5 (CH_2), 14.3 and 14.1 (CH_3), 143.5, 141.9, 139.9, 139.3, 132.3, 130.7, 130.6, 129.9, 128.7, 128.6, 127.2, 120.9, 115.7, 114.9, 112.4; MS (ESI): *m/z* 647.2 [M+1] (calcd for $C_{31}H_{30}N_6O_8S$: 646.18).

3.3.11. 1-(3-[(3-Hydroxy-1-methoxy-4-methyl-1-oxopent-2-en-2-yl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (11)

Synthesized from **3** (0.477 g, 1 mmol) and methyl isobutyrylacetate (0.158 ml, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 88%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3259 (NH), 3059 (ArCH), 2971 (Aliphatic CH), 1735 and 1683 (C=O), 1593–1436 (C=N and C=C), 1312 and 1158 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm):13.65 (s, 1H, C=C–*OH* enol tautomer), 11.47 (s, 1H, Ar–*NH*–N= keto tautomer), 11.04 (s, 1H, CONH), 7.88–7.02 (m, 13H, ArH), 7.30 (s, 2H, SO_2NH_2), 3.76 (s, 3H, OCH_3), 3.38 (m, 1H, $CH(CH_3)_2$) 0.98 (d, J = 6.8 Hz, 6H, $CH(CH_3)_2$); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 200.6 (C=O, keton), 163.9 (C=O, acid), 163.4 (C=O, ester), 161.5 (C=O, amide), 147.4, 145.9, 120.3 (pyrazole C-3, C-5, C-4), 52.8 (OCH_3), 34.3 (CH), 19.3 ($2CH_3$), 143.4, 141.8, 139.8, 139.4, 131.0, 130.8, 129.9, 128.8, 128.5, 127.2, 121.1, 120.4, 115.8, 114.5, 112.3; MS (ESI): *m/z* 633.2 [M+1] (calcd for $C_{30}H_{28}N_6O_8S$: 632.17).

3.3.12. 1-(3-[(1-*tert*-Butoxy-3-hydroxy-1-oxobut-2-en-2-yl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (12)

Synthesized from **3** (0.477 g, 1 mmol) and *t*-butyl acetoacetate (0.163 ml, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 86%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3227 (NH), 3060 (ArCH), 2971 (Aliphatic CH), 1738 and 1674 (C=O), 1592–1435 (C=N and C=C), 1327 and 1151 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm):13.69 (s, 1H, C=C–*OH* enol tautomer), 11.87 (s, 1H, Ar–*NH*–N= keto tautomer), 11.51 (s, 1H, CONH), 7.88–6.96 (m, 13H, ArH), 7.30 (s, 2H, SO_2NH_2), 2.24 (s, 3H, $COCH_3$), 1.46 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 194.9 (C=O, acetyl), 164.4 (C=O, acid), 161.9 (C=O, ester), 161.4 (C=O, amide), 146.9, 146.1, 120.2 (pyrazole C-3, C-5, C-4), 84.0 ($OC(CH_3)_3$), 26.4 and 25.8 (CH_3), 143.3, 142.1, 139.9, 139.2, 132.2, 130.7, 130.6, 129.8, 128.9, 128.7, 127.2, 121.2, 115.9, 115.7, 112.8; MS (ESI): *m/z* 669.2 [M+Na] (calcd for $C_{31}H_{30}N_6O_8S$: 646.18).

3.3.13. 1-(3-[(4-Hydroxyphenyl)diazenyl]phenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (13)

Synthesized from **3** (0.477 g, 1 mmol) and phenol (0.094 g, 1 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 75%; IR (ν, cm^{-1}): 3385 (OH), 3500–2500 (COOH), 3260 (NH), 3064 (ArCH), 1736 (C=O, acid), 1624 (C=O, amide), 1590–1457 (C=N and C=C), 1312 and 1156 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.07 (s, 1H, CONH), 7.89–6.90 (m, 18H, ArH and ArOH), 7.30 (s, 2H, SO_2NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 163.8 (C=O, acid), 161.9 (C=O, amide), 152.8 (=C–OH), 147.4, 146.4, and 120.4 (pyrazole C-3, C-5 and C-4), 145.5, 141.8, 139.7, 139.4, 130.7, 130.7, 130.6, 130.6, 130.5, 130.1, 128.8, 128.5, 127.2, 125.7, 123.8, 122.6, 118.5, 116.6, 114.6; MS (ESI): *m/z* 583.1 [M+1] (calcd for $C_{29}H_{22}N_6O_6S$: 582.13).

3.3.14. 1-(3-Cyanophenyl)-5-phenyl-3-(4-sulfamoylphenylcarbamoyl)-1*H*-pyrazole-4-carboxylic acid (14)

The diazonium salt solution of **3** (0.477 g, 1 mmol) was prepared according to the general procedure. After dissolving potassium cyanide (2 mmol) in 2 ml water, the solution was cooled and added dropwise into the already prepared diazonium salt solution. Following the addition, the cold mixture is allowed to warm up to room temperature. When the temperature reached about 15 °C, the formation of nitrogen gas began. Then the solution was placed on a steam bath and heated at 60 °C for 45 min to complete the decomposition. Finally the resulting precipitate was filtered under vacuum and dried. The residue was purified by crystallization from ethanol. Yield: 87%; IR (ν, cm^{-1}): 3500–2500 (COOH), 3263 (NH), 3032 (ArCH), 2110 (CN), 1719 and 1624

(C=O), 1589–1424 (C=N and C=C), 1329 and 1155 (S=O, assym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 11.06 (CONH), 7.88–6.99 (m, 13H, ArH), 7.30 (s, 2H, SO_2NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 163.8 (C=O, acid), 161.7 (C=O, amide), 147.5, 146.3 and 120.4 (pyrazole C-3, C-5, C-4), 116.9 (CN), 141.8, 140.8, 139.9, 139.4, 131.1, 130.7, 128.8, 128.5, 127.2, 126.4, 125.6, 122.6, 121.7, 116.1; MS (ESI): *m/z* 510.1 [M+Na] (calcd for $C_{24}H_{17}N_5O_5S$: 487.10).

3.4. Biological activity evaluation

3.4.1. Hemolysate preparation

Fresh human blood obtained from the University Hospital Blood center and erythrocytes were purified. After low-speed centrifugation (1.500 rpm for 15 min) and removal of plasma and buffy coat, the red blood cells were isolated, washed twice with 0.9% NaCl, and hemolyzed with 1.5 volumes of ice-cold water. Then, ghost and intact cells were removed by high-speed centrifugation (20.000 rpm for 30 min) at 4 °C and the pH of the hemolysate adjusted to 8.7 with solid Tris.

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

Sepharose-4B L-tyrosine affinity chromatography column was prepared according to our previous studies.[21–24,31] During the purification procedures of hCA I and hCA II, the absorbance was measured at 280 nm to monitor protein elution by affinity chromatog raphy. CO_2-hydratase activity was determined in eluted fractions and the active fractions were collected.[32,33]

3.4.3. Hydratase activity assay

Carbonic anhydrase hydratase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[32]

3.4.4. Esterase activity assay

Esterase activity of human erythrocyte carbonic anhydrase was assayed by following the change in absorbance at 348 nm of 4-nitrophenyl acetate to 4-nitrophenolate ion over a period of 3 min at 25 °C using a spectrophotometer according to the method described by Verpoorte et al.[34]

3.4.5. Quantitative protein determination

Quantitative protein determination was done by measuring the absorbance at 595 nm according to Bradford, using bovine serum albumin as a standard.[35]

3.4.6. SDS–polyacrylamide gel electrophoresis

The control of enzyme purity was carried out by using Laemmli's procedure in two different acrylamide concentrations, 3% and 8% for running and stacking gel, respectively.[24,36]

3.4.7. In vitro inhibition studies

The inhibitory effects of some synthesized sulfonamide derivatives on carbonic anhydrase enzyme activity purified from human erythrocyte were tested in triplicate at each concentration by using esterase activity assay. CA activities were measured in the presence of different substrate concentrations as previously described. Lineweaver–Burk curves were used for determination of K_i and inhibitor type.[24,37]

Acknowledgements

The authors are very grateful to The Scientific and Research Council of Turkey (TUBITAK) for providing financial support for this project (Grant No. 106T180). The facility of King Fahd University of Petroleum and Minerals (KFUPM) is used, and KFUPM is gratefully acknowledged for the support of this project. The authors are thankful Stefan Walter and Kerstin Ruecker at Chemistry/Biology Branch of Osnabrück University for LC/MS and TGA measurements, respectively. S.O. is also grateful to German Science Foundation (DFG) for the support of summer research stay at Osnabrück University/Germany with Grant number STE 1127/15-1.

References and notes

1. Wilkinson, B. L.; Bomaghi, L. F.; Houston, T. A.; Innocenti, A.; Vullo, D.; Supuran, C. T. *J. Med. Chem.* **2007**, *50*, 1651.
2. Krungkrai, S. R.; Suraveratum, N.; Rochanakij, S.; Krungkrai, J. *Int. J. Parasitol.* **2001**, *31*, 661.
3. Supuran, C. T.; Scozzafava, A. *Curr. Med. Chem.* **2001**, *1*, 61.
4. Hewett-Emmett, D. *In the Carbonic Anhydrase–New Horizons*; Birkhäuser: Basel, 2000. pp 29–78.
5. Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3467.
6. Balaydın, H. T.; Soyut, H.; Ekinci, D.; Göksu, S.; Beydemir, S.; Menzek, A.; Sahin, E. *J. Enzyme Inhib. Med. Chem.* **2012**, *27*, 43.
7. Machado, P.; Lima, G. R.; Rotta, M.; Bonacorso, H. G.; Zanatta, N.; Martins, M. A. P. *Ultrason. Sonochem.* **2011**, *18*, 293.
8. Matsumoto, H. Mode of Action of Pyrazole Herbicides Pyrazolate and Pyrazoxyfen: HPPD Inhibition by the Common Metabolite, New Discoveries in Agrochemicals, 2004, pp 161–171.
9. Sherman, T. D.; Duke, M. V.; Clark, R. D.; Sanders, E. F.; Matsumoto, H.; Duke, S. O. *Pestic. Biochem. Physiol.* **1991**, *40*, 236.
10. Wei, F.; Zhao, B. X.; Huang, B.; Zhang, L.; Sun, C. H.; Dong, W. L.; Shin, D. S.; Miao, J. Y. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6342.
11. Dang, T. T.; Dang, T. T.; Langer, P. *Tetrahedron Lett.* **2007**, *48*, 3591.
12. Prakash, O.; Kumar, R.; Sehrawat, R. *Eur. J. Med. Chem.* **2009**, *44*, 1763.
13. Maggio, B.; Daidone, G.; Raffa, D.; Plescia, S.; Mantione, L.; Cutuli, V. M. C.; Mangano, N. G.; Caruso, A. *Eur. J. Med. Chem.* **2001**, *36*, 737.
14. Lange, J. H. M.; Kruse, C. G. *Drug Discovery Today* **2005**, *10*, 693.
15. Bailey, D. M.; Hansen, P. E.; Hlavac, A. G.; Baizman, E. R.; Pearl, J.; Defelice, A. F.; Feigenson, M. E. *J. Med. Chem.* **1985**, *28*, 256.
16. Jia, Z. Z.; Wu, Y. H.; Huang, W. R.; Zhang, P. L.; Song, Y. H.; Woolfrey, J.; Sinha, U.; Arfsten, A. E.; Edwards, S. T.; Hutchaleelaha, A.; Hollennbach, S. J.; Lambing, J. L.; Scarborough, R. M.; Zhu, B. Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1229.
17. Bernardino, A. M. R.; Gomez, A. O.; Charret, K. S.; Freitas, A. C. C.; Machado, G. M. C.; Canto-Cavalheiro, M. M.; Leon, L. L.; Amaral, V. F. *Eur. J. Med. Chem.* **2006**, *41*, 80.
18. Choi, W.-K.; El-Gamal, M. I.; Choi, H. S.; Baek, D.; Oh, C.-H. *Eur. J. Med. Chem.* **2011**, *46*, 5754.
19. Supuran, C. T. *Curr. Top. Med. Chem.* **2007**, *7*, 825.
20. Sugrue, M. F. *Prog. Retin. Eye Res.* **2000**, *19*, 87.
21. Kasımoğulları, R.; Bülbül, M.; Günhan, H.; Güleryüz, H. *Bioorg. Med. Chem.* **2009**, *17*, 3295.
22. Kasımoğulları, R.; Bülbül, M.; Mert, S.; Güleryüz, H. *J. Enzyme Inhib. Med. Chem.* **2011**, *26*, 231.
23. Kasımoğulları, R.; Bülbül, M.; Arslan, B. S.; Gökçe, B. *Eur. J. Med. Chem.* **2010**, *45*, 4769.
24. Şen, E.; Alım, Z.; Duran, H.; İşgör, M. M.; Beydemir, Ş.; Kasımoğulları, R.; Ok, S. *J. Enzyme Inhib. Med Chem* **2011**, 651456. http://dx.doi.org/10.3109/14756366.
25. Gülcin, I.; Beydemir, S.; Büyükokuroğlu, M. E. *Biol. Pharm. Bull.* **2004**, *27*, 613.
26. Beydemir, S.; Gülcin, I. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 193.
27. Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1583.
28. Coban, T. A.; Beydemir, S.; Gulcin, I.; Ekinci, D.; Innocenti, A.; Vullo, D.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 5791.
29. Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 641.
30. Ekinci, D.; Beydemir, S.; Kufrevioglu, O. I. *J. Enzyme Inhib. Med. Chem.* **2007**, *22*, 745.
31. Beydemir, S.; Ciftci, M.; Ozmen, I.; Buyukokuroglu, M. E.; Ozdemir, H.; Küfrevioglu, O. I. *Pharmacol. Res.* **2000**, *42*, 187.
32. Wilbur, K. M.; Anderson, N. G. *J. Biol. Chem.* **1948**, *176*, 147.
33. Rickli, E. E.; Ghazanfar, S. A. S.; Gibbons, B. H.; Edsall, J. T. *J. Biol. Chem.* **1964**, *239*, 1065.
34. Verpoorte, J. A.; Mehta, S.; Edsall, J. T. *J. Biol. Chem.* **1967**, *242*, 4221.
35. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
36. Laemmli, U. K. *Nature* **1970**, *227*, 680.
37. Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *57*, 658.