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Effects of new 5-amino-1,3,4-thiadiazole-2-sulfonamide derivatives on human carbonic anhydrase isozymes

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ABSTRACT

Pyrazole carboxylic acid amides of 5-amino-1,3,4-thiadiazole-2-sulfonamide 1 (inhibitor 1) were synthesized from 4-benzoyl-1-(4-nitrophenyl)-5-phenyl-1*H*-pyrazole-3-carbonyl chloride and 4-benzoyl-1-(3-nitrophenyl)-5-phenyl-1*H*-pyrazole-3-carbonyl chloride compounds. Human carbonic anhydrase isoenzymes (hCA-I and hCA-II) were purified from erythrocyte cells by the affinity chromatography. The inhibitory effects of inhibitor **1**, acetazolamide (**AAZ**), and of 16 newly synthesized amides (**8–11, 12a–f, 13a–c, 14a–b, and 15**) on hydratase and esterase activities of these isoenzymes have been studied in vitro. The average IC_{50} values of the new compounds (**8–11,12a–f, 13a–c, 14a–b, and 15**) for hydratase activity ranged from 3.25 to 4.75 μM for hCA-I and from 0.055 to 2.6 μM for hCA-II. The mean IC_{50} values of the same inhibitors for esterase activity were in the range of 2.7–6.6 μM for hCA-I (with the exception of inhibitor 10, which did not inhibit the esterase activity of hCA-I) and of 0.013–4.2 μM for hCA-II. The K_i values for new compounds (**8–11, 12a–f, 13a–c, 14a–b, and 15**) were observed well below that of the parent compound inhibitor **1** and were also comparable to that of **AAZ** under the same experimental conditions. The comparison of newly synthesized amides to inhibitor **1** and to **AAZ** indicated that the new derivatives preferentially inhibit hCA-II and are more potent inhibitors of hCA-II than the parent inhibitor **1** and **AAZ**.

1. Introduction

Carbonic anhydrase (CA; EC 4.2.1.1) catalyzes the reversible hydration of carbon dioxide to bicarbonate and protons, a very simple but critically important physiological reaction for organisms.[1–6] In animals, the primary function of the enzyme is to transport CO_2 out of the cells and also to maintain acid–base balance in blood and other tissues by converting CO_2 to bicarbonate. CA is a zinc-containing enzyme and zinc ion prosthetic group is coordinated by histidine side chains in the active site. This enzyme has 16 different isoenzymes presently known in human. Several of these isoenzymes (CA-II and CA-IV) are expressed in human eyes.[7–10]

Glaucoma is a group of diseases characterized by gradual loss of visual field due to an elevation in intraocular pressure (IOP); the disease is the second leading cause of blindness worldwide.[10,11] Since carbonic anhydrase inhibitors have been shown to reduce intraocular pressure exclusively by lowering the aqueous humor flow, these compounds have been used for the treatment of glaucoma for years.[12,13] Sulfonamides are the best-known inhibitors of carbonic anhydrase enzyme, currently used for the treatment of glaucoma in clinical medicine.[1–14]

5-Amino-1,3,4-thiadiazole-2-sulfonamide 1 has several biological activities and antibacterial properties.[10] Several derivatives of this drug have been synthesized and used in treatment of glaucoma; one of them is acetazolamide (AAZ). AAZ is used systematically (i.e., an oral medication) and reduce intraocular pressure by lowering the fluid formation in the eye. A number of side effects of this drug are however experienced such as numbness and tingling in the fingers and toes, taste alterations, blurred vision, kidney stones, and an increase in urination. Dorzolamide (DZA) and brinzolamide (BRZ) are two other carbonic anydrase inhibitors, formulated for topical ophthalmic use (see Fig. 1 for the structures).[1,15,16] Nevertheless, people taking these two drugs also experience side effects as stinging, burning, blurred vision, upset stomach, dry eye, headache or dizziness.[1,17–19] The purpose of the present study was thus to synthesize and investigate new inhibitors of carbonic anydrase isoenzymes with potential use in the treatment of glaucoma.

2. Results and discussion

Treatment of glaucoma is primarily aimed at decreasing the intraocular pressure, which may lead to optic nerve damage and subsequently to vision loss. Several carbonic anydrase inhibitors in use lower the intraocular pressure by decreasing the aqueous

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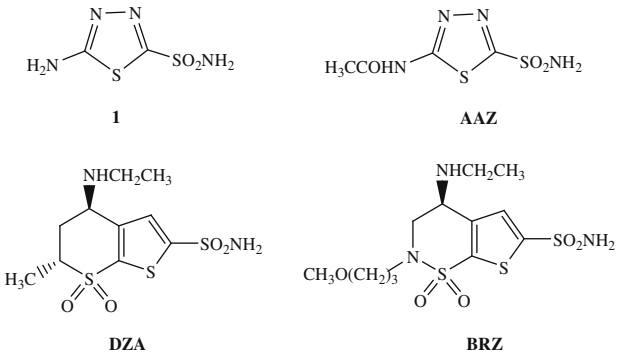


Figure 1. Structures of several CA inhibitors used in glaucoma treatment.

humor production.[1] However, as mentioned in Introduction, all medications whether topical or oral have some benefits as well as some side effects. Because of the ocular side effects after administration of the sulfonamide derivatives, the development of new carbonic anhydrase inhibitors as candidate drugs with fewer side effects will likely become valuable for the treatment of glaucoma. In this study, 16 derivatives of sulfanomides have been synthesized and their effects on purified human carbonic anydrase I and II isoenzymes have been investigated.

In order to synthesize new compounds, first activated pyrazole carboxylic acids were reacted with inhibitor **1**, and **4** new amide derivatives (named compound **4–7**) were obtained (Scheme 1). After that, using these newly synthesized amide derivatives, our target inhibitors were prepared by reducing NO_2 groups to NH_2 groups (the target compounds are named **8–11**).[20,21]

In our work, diazotization method was generally used to synthesize new derivatives of compound **8** which is an aromatic primer amine. At first, as described previously, diazonium salt of compound was prepared.[22] Then, coupling and replacement reactions were performed. During reactions, temperature was controlled (0–5 °C).

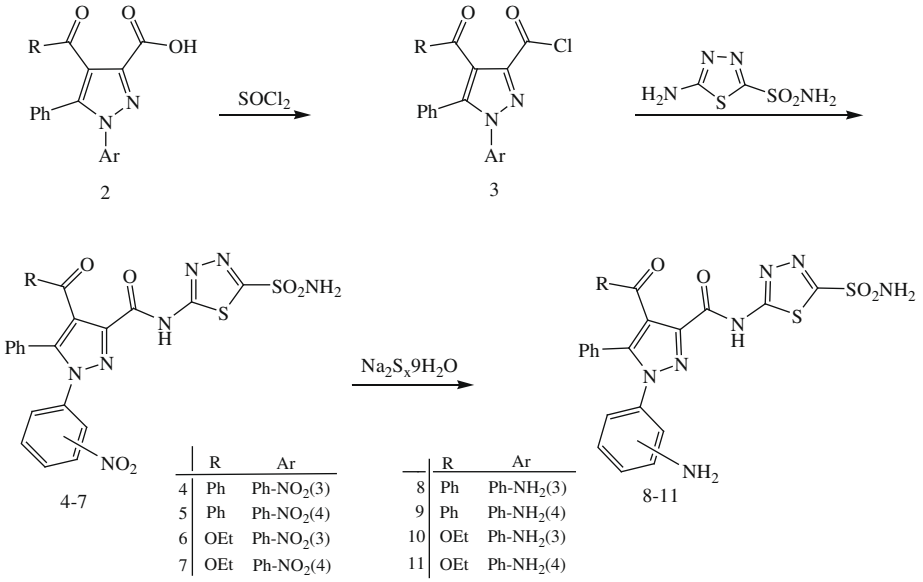
Some pH intervals were evaluated during the experiments and it is observed that the best pH value was around 3.5–4. Products are generally colorful, reaction conditions are mild, and processes are simple. The pureness of each product was checked by TLC workings which were performed for reactions.

Some hydrazo derivatives (**12a–f**) were produced in high yields by reaction of compound **8** with aliphatic 1,3-dicarbonile compounds which include active C–H protons.[23] In these compounds, because of unshared electron couples of nitrogen, proton which was transferred to base results in the resonance form; therefore the resulting products may have tautomeric structures as azo (–N=N–) or hydrazo (–NH–N=C).[24] In the literature, it has been reported that such compounds in solid phases and in chloroform have only keto-hydrazo tautomeric structures because of intermolecular hydrogen bonds.[25] In our research, when the results of ^1H NMR spectrums are examined, it is obvious that signals of **12a–f** compounds at δ = 11.88–11.27 ppm intervals are caused by hydrogen atoms in covalent linkage with nitrogens. Thus, these compounds including aliphatic active C–H groups prefer keto-hydrazo (–NH–N=C) tautomeric structures after their coupling.

Compound **13a** was synthesized in 34% yield by interference of diazonium compound with KCN, and compound **13b** was produced in 65% yield by interference of diazonium compound with KI. As source of CN, CuCN was also used along with KCN. Therein, Cu^+ ion acts as a catalyzer. For transfer of ion of I^- which is a strong nucleophile, KI was sufficient without any catalyzer. After heating of diazonium compound with H_2O to 100 °C, compound **13c** was produced in 53% yield. Structures of compounds were evaluated with spectral data. Compound **8** gave azo compounds by diazo coupling reactions with aromatic compounds like phenol or β-naphthol which are very active. Finally compound **14a** was produced in 34% yield, and derivatives of compound **14b** were produced in 43% yield. Structures of compounds were confirmed by spectral data (see Experimental), and compound **15** was produced in 57% yield by reaction of compound **8** with acetonylchloride in DMF at 60 °C for 72 h.[26] Syntheses of compounds **12a–f**, **13a–c**, **14a–b,** and **15** from compound **8** are concluded in Scheme 2.

Next, effects of these new inhibitors on human carbonic anhydrases I (hCA-I) and II (hCA-II) purified from red blood cells were investigated. The CA isozymes have been purified to homogeneity by affinity chromatography.

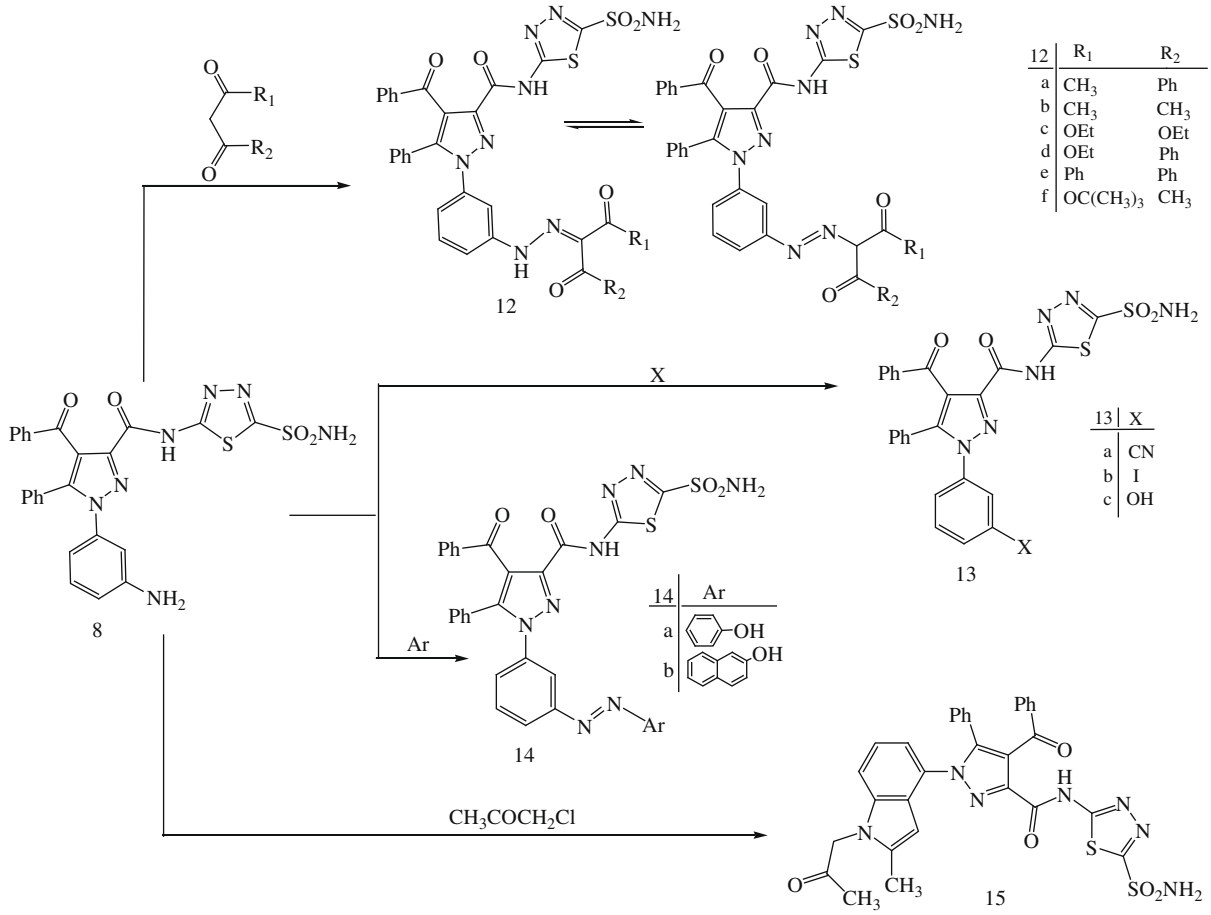
The concentration required to inhibit hCA-I and hCA-II activities of the purified proteins by 50% (IC_{50}) inhibition constant K_i was determined for each compound. The IC_{50} and K_i values were used to compare the inhibitory potential of parent inhibitor **1**, **AAZ,** and the newly synthesized derivatives (**8–11, 12a–f, 13a–c, 14a–**



	R	Ar
4	Ph	Ph-NO_2(3)
5	Ph	Ph-NO_2(4)
6	OEt	Ph-NO_2(3)
7	OEt	Ph-NO_2(4)

	R	Ar
8	Ph	Ph-NH_2(3)
9	Ph	Ph-NH_2(4)
10	OEt	Ph-NH_2(3)
11	OEt	Ph-NH_2(4)

Scheme 1. Synthesis of compounds **8–11**.



Scheme 2. Synthesis of compounds **12a–f**, **13a–c**, **14a–b,** and **15**.

b, and **15**) on hCA-I and hCA-II. As seen in the Table 1, the IC_{50} values of the new compounds (**8–11,12a–f, 13a–c, 14a–b,** and **15**) are lower than the IC_{50} values of inhibitor **1** and **AAZ** for hydratase activity of erythrocyte hCA-II. IC_{50} determination indicates that about 82% of esterase activity of hCA-II was lost in the presence of 0.065 μM of inhibitor 11.

As we examine IC_{50} value of our synthesized compounds on hydratase activity of enzyme obtained after inhibition experiments (Table 1); our synthesized compounds are more efficient than initial compounds on hCA-II enzyme. When IC_{50} values of inhibitors on hydratase activity of hCA-I were examined, compounds **12a–12f, 13a–13c, 14a, 14b,** and **15** of our synthesized compounds are more effective as inhibitors than initial compounds (**1** and **AAZ**).

As we examine IC_{50} values of synthesized compounds on esterase activity of carbonic anhydrase (Table 1); Compounds **8, 10, 11,**

Table 1
IC_{50} and K_i values of hCA-I and hCA-II isoenzyme hydratase and esterase activities obtained after inhibition experiments

İnhibitor	Hydratase IC_{50} (μM)		Esterase IC_{50} (μM)		K_i values (μM)	
	hCA-I	hCA-II	hCA-I	hCA-II	hCA-I	hCA-II
1	3.25 ± 1.77	2.9 ± 0.57	2.6 ± 1.98	3.6 ± 0.57	5.7–4.2	8.2–6.3
AAZ	3.75 ± 3.18	3.9 ± 2.26	2.9 ± 0.85	8.3 ± 0.99	6.1–3.5	2–3.2
8	4.75 ± 0.35	2.6 ± 0.57	2.7 ± 0.71	2.8 ± 0.42	3.09–2.7	0.561–0.33
9	4.2 ± 0.14	1.59 ± 0.30	4.3 ± 0.42	4.2 ± 0.28	2.42–3.5	1.56–4.6
10	3.25 ± 0.21	0.055 ± 0.01	0.44–0.21	0.013 ± 0.001	no	4.09–6.4
11	4.1 ± 0.07	0.08 ± 0.014	6.6 ± 2.69	0.015 ± 0.004	2.9–4.7	1.24–2.5
12a	0.42–0.13	0.77–0.59	0.41–0.9	0.55–0.78	0,3–0.8	0.2–079
12b	0.44–0.36	0.09–0.085	0.8–0.45	0,18–0.33	0.24–0.4	0.41–0.122
12c	0.14–0.055	0.80–0.71	0,085–0.08	0,1–0,094	1.31–0.75	0.20–0.166
12d	0.56–0.43	0.80–0.6	0,097–0.14	0,04–0.07	0.09–0.081	0.61–0.58
12e	0.21–0.39	0.22–0.87	0.25–0.08	0.02–0.05	0.43–0.2	0.04–0.06
12f	0.02–0.065	0.53–0.41	0,14–0.09	0,06–0.055	0.17–0.15	0.57–0.02
13a	0.25–0.158	0.08–0,04	0,419–0.33	0,32–0.25	0.55–0.45	0,41–0.32
13b	0,8–0.52	0,48–0.13	3,798–2.65	0,36–0.92	2,534–1.863	0,38–0.136
13c	0.6–1.3	0.05–0.01	0.39–0.8	0.26–0.31	0.53–0.4	0.22–0.83
14a	0.17–0.65	0,04–0.06	0,575–0.374	0,081–0.022	0.714–0.523	0.034–0.05
14b	0.31–0.08	0.02–0.08	0.21–0.55	0.01–0.03	0.12–0.15	0.02–0.06
15	0.4–0.12	0.32–0.96	0.2–0.35	0.1–0.23	0.39–0.22	0.4–0.21

12a–12f, 13a–13c, 14a, 14b, and 15 are more effective than initial compounds (1 and AAZ). Especially compounds **10, 11, 12d, 12e, 12f, 14a, and 14b** are the most effective compounds. When we examine hCA-I esterase IC_{50} values, compounds **10, 12a–f, 13a, 13c, 14a**, **14b,** and **15** are more effective than initial compounds (**1** and **AAZ**). Especially **12c** and **12d** compounds are the most effective compounds on this enzyme.

When the effects of synthesized compounds on K_i values (Table 1) were investigated, hCA-II K_i values of these compounds **8, 11, 12a–f, 13a–c, 14a, 14b,** and **15** are lower than initial compounds of **1** and **AAZ**, indicating that they are more effective. Especially compounds **12e, 14a,** and **14b** are the most effective compounds. Similarly, hCA-I K_i values of synthesized compounds are less than K_i values of initial compounds, therefore their potential inhibitory effects are quite higher. As seen in Table 1, **12c** and **12d** are the most effective inhibitors.

If we discuss the results in general, it can be said that our synthesized compounds show more effective inhibition property than initial compounds, so they can be seen as a candidate medicine for treatment of glaucoma.

3. Experimental protocols

3.1. Syntheses

Melting points were measured with Barnstead Electrothermal 9200 apparatus, and were uncorrected. IR spectrum data of compounds were determined by Mattson 1000 FT-IR using KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were evaluated by BRUKER DPX-400, (400 MHz), and High Performance Digital FT NMR (100 MHz) spectrometers. Mass spectra data were determined by Varian Mat III 80 eV. At the end of the each experiments TLC was performed using DC Alufolien Kiesegel 60F/254 Merck and Camag TLC devices. Elemental analyses were carried out on a Leco CHNS-932 instrument.

3.2. General procedure for synthesis of compounds 8–11

$Na_2S{\cdot}9H_2O$ (1 mmol) and sulfur (S, 2 mmol) were stirred and dissolved by boiling in 20 ml of water. This solution (sodium polysulfur) was then added dropwise to a stirred solution of warm compound **4**, **5**, **6,** or **7** (1 mmol) in methanol–water. This mixture was refluxed for 45 min and then HCl solution was added to this mixture, refluxed again for 15 min. Precipitated solid was filtrated and ammonia was added to the solution. The mixture was incubated overnight and finally the solid products were filtrated and crystallized from ethanol–water mixture. Finally, target inhibitors (**8–11**) were obtained by reducing NO_2 to NH_2 groups.

3.2.1. 1-(3-Aminophenyl)-4-benzoyl-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazole-2-yl)-1*H*-pyrazole-3-carboxamide (8)

(480 mg, 88%); Mp 216 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.20 (m, 1H, CONH), 5.42 (m, 2H, Ar-NH_2), 8.35 (m, 2H, –SO_2NH_2), 7.79–6.92 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.80 (Benzoyl C=O), 165.28 (Amide C=O), 161.43 and 160.54 (thiadiazol C-2 and C-5), 148.82, 144.34, 142.41, 137.77, 137.69, 133.96, 130.23, 130.14, 129.58, 129.06, 128.84, 128.20, 127.92, 127.43, 127.34, 122.40, 114.36, IR(KBr) (ν, cm^{-1}): 3351, 1740, 1668, 1494, 1426, 1354, 1295, 1248, 1167, 1060; MS(CI) *m/z* 546.1(M+1); Anal. Calcd for $C_{25}H_{19}N_7O_4S_2$: C, 55.04; H, 3.51; N, 17.97; S, 11.75. Found: C, 55.35; H, 3.45; N, 17.82; S, 11.55.

3.2.2. 1-(4-Aminophenyl)-4-benzoyl-5–phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazole-2-yl)-1*H*-pyrazole-3-carboxamide (9)

(465 mg, 85%); Mp 183 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 6.51 (m, 2H, Ar-NH_2), 8.37 (m, 2H, SO_2NH_2), 7.80–6.82 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.58 (benzoyl C=O), 165.36 (amide C=O), 161.44 and 160.44 (thiadiazol C-2 and C-5), 152.20, 148.80, 144.57, 143.36, 137.58, 134.10, 130.48, 130.13, 129.64, 129.06, 127.65, 127.40, 127.30, 122.91, 114.40; IR(KBr) (ν, cm^{-1}): 3312–3216, 3026, 1740, 1665, 1511, 1426, 1365, 1294, 1238, 1172, 1062; MS(CI) *m/z* 546.1(M+1); Anal. Calcd for $C_{25}H_{19}N_7O_4S_2$: C, 55.04; H, 3.51; N, 17.97; S, 11.75. Found: C, 55.32; H, 3.59; N, 17.75; S, 11.60.

3.2.3. Ethyl-1-(3-aminophenyl)-5-phenyl-3-(5-sulfamoyl-1,3,4-thiadiazole-2-ylcarbamoyl)-1*H*-pyrazole-4-carboxylate (10)

(410 mg, 76%); Mp 253 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.60 (m, 1H, CONH), 1.01 (t, 3H, –CH_3), 4.09 (q, 2H, –CH_2), 6.52 (m, 2H, Ar-NH_2), 8.42 (m, 2H, –SO_2NH_2), 7.44–6.78 (m, 9H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 164.57 (ester C=O), 164.10 (amide C=O), 163.65 and 161.36 (thiadiazol C-2 and C-5), 152.21, 144.49, 144.09, 139.70, 130.57, 130.37, 129.94, 129.71, 128.67, 128.63, 128.57, 116.19, 114.66, 62.50, 13.95; IR(KBr) (ν, cm^{-1}): 3451, 2969, 2877, 1740, 1676, 1566, 1490, 1436, 1375, 1327, 1222, 1108, 1057; MS(CI) *m/z* 514.2 (M+1); Anal. Calcd for $C_{21}H_{19}N_7O_5S_2$: C, 49.11; H, 3.73; N, 19.09; S, 12.49. Found: C, 49.10; H, 3.83; N, 19.12; S, 12.35.

3.2.4. Ethyl-1-(4-aminophenyl)-5-phenyl-3-(5-sulfamoyl-1,3,4-thiadiazole-2-ylcarbamoyl)-1*H*-pyrazole-4-carboxylate (11)

(364 mg, 71%); Mp 275 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.94 (m, 1H, CONH), 0.99 (t, 3H, CH_3), 4.11 (q, 2H, CH_2), 6.50 (m, 2H, Ar-NH_2), 8.43 (m, 2H, SO_2NH_2), 7.40–6.68 (m, 9H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 165.55 (ester C=O), 161.77 (amide C=O), 161.41 and 160.99 (thiadiazol C-2 and C-5), 147.26, 146.57, 146.11, 143.30, 130.80, 130.51, 128.95, 127.50, 126.85, 125.10, 114.80, 61.24, 13.94; IR(KBr) (ν, cm^{-1}): 3310–3214, 2967, 2740, 1738, 1659, 1524, 1495, 1440, 1365, 1327, 1251, 1174, 1105, 1065; MS(CI) *m/z* 514 (M+1); Anal. Calcd for $C_{21}H_{19}N_7O_5S_2$: C, 49.11; H, 3.73; N, 19.09; S, 12.49. Found: C, 49.23; H, 3.71; N, 18.97; S, 12.45.

3.3. General procedure for synthesis of compounds 12a–f

Sodium acetate (3 g) was dissolved in 10 ml H_2O and 2 ml HCl. Aromatic amine (1 mmol) and alcohol were added to mixture until it dissolved. Temperature was adjusted to 0–5 °C. Solution of $NaNO_2$ (83 mg, 1.2 mmol) in 10 aqua was added to mixture slowly, and process of diazotization was performed. In another place, aromatic and β-diketone (1 mmol) compounds were dissolved in ethanol and added to diazonium salt dropwise which was prepared previously. Final colored precipitate was filtered by trompe, and purified from ethyl alcohol.

3.3.1. 4-Benzoyl-1-(3-(2-(1,3-dioxo-1-phenylbutan-2-ylidene) hydrazinyl)phenyl)-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (12a)

(488 mg, 68%); Mp 174 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.82 (s, 1H, CONH), 11.27 (s, 1H, Ar-NH–N=C), 8.38 (s, 2H, SO_2NH_2), 2.50 (s, 3H, CH_3), 7.95–7.08 (m, 19H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 196.52 (acetyl C=O), 195.39 and 190.56 (benzoyl C=O), 165.35 (amide C=O), 161.39 and 160.38 (thiadiazol C-2 and C-5), 25.47 (CH_3), 144.40, 144.27, 143.28, 140.14, 139.71, 137.63, 135.88, 134.94, 134.08, 130.64, 130.48, 130.15, 130.04, 129.64, 129.55, 129.20, 129.09, 128.96, 127.89, 123.03, 120.17, 112.14; IR(KBr) (ν, cm^{-1}): 3381, 3250, 3063, 2899, 2833, 1667, 1601, 1519, 1450, 1425, 1364, 1322, 1299, 1217, 1174; Anal. Calcd for $C_{35}H_{26}N_8O_6S_2$: C, 58.49; H, 3.65; N, 15.59; S, 8.92. Found: C, 58.55; H, 3.68; N, 15.65; S, 8.89.

3.3.2. 4-Benzoyl-1-(3-((2,4-dioxopentan-3-yl)diazenyl)phenyl)-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (12b)

(328 mg, 50%); Mp 175 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.77 (s, 1H, CONH), 8.37 (s, 2H, –SO_2NH_2), 2.45, 2.29 (s, 6H, CH_3), 7.81–7.01 (m, 14H, ArH); ^{13}C NMR NMR (100 MHz, DMSO-d_6) δ (ppm): 197.56 and 196.82 (acetyl C=O), 190.52 (benzoyl C=O), 165.33 (amide C=O), 161.39 and 160.35 (thiadiazol C-2 and C-5), 31.70 and 26.76 (CH_3), 144.55, 144.48, 143.40, 142.97, 139.86, 139.58, 137.63, 137.59, 134.61, 134.06, 130.51, 130.14, 129.63, 129.07, 127.79, 127.67, 123.10, 122.93; IR(KBr) (ν, cm^{-1}): 3380, 3232, 3061, 2889, 2838, 1677, 1598, 1519, 1498, 1448, 1427, 1363, 1302, 1251, 1175; MS(CI) *m/z* 657.1 (M+1); Anal. Calcd for $C_{30}H_{24}N_8O_6S_2$: C, 54.87; H, 3.68; N, 17.06; S, 9.77.Found: C, 54.72; H, 3.85; N, 17.11; S, 9.75.

3.3.3. Diethyl-2-((3-(4-benzoyl-5-phenyl-3-(5-sulfamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)-1*H*-pyrazol-1-yl)phenyl) diazenyl)malonate (12c)

(343 mg, 48%); Mp 181 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.80 (m, 1H, CONH), 8.38 (s, 2H, SO_2NH_2), 4.32 (q, 2H, CH_2), 3.50 (s, 1H, N-CH), 1.25 (t, 3H, CH_3), 7.80–7.15 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.52, (benzoyl C=O), 170.42 (ester C=O), 165.30 (amide C=O), 161.38 and 160.40 (thiadiazol C-2 and C-5), 15.3 (CH_3), 46.03 (CH_2), 61.82 (CH), 144.40, 143.70, 139.71, 137.61, 137.30, 134.04, 133.05, 130.56, 130.30, 130.05, 129.60, 129.09, 128.90, 128.88, 128.74, 127.65, 122.94; IR(KBr) (ν, cm^{-1}): 3468, 3380, 3162, 3060, 2899, 1683, 1612, 1596, 1525, 1496, 1448, 1427, 1367, 1323, 1301, 1216, 1174; MS(CI) *m/z* 717.2 (M+1); Anal. Calcd for $C_{32}H_{28}N_8O_8S_2$: C, 53.62; H, 3.94; N, 15.63; S, 8.95. Found: C, 53.48; H, 3.99; N, 15.61; S, 8.87.

3.3.4. Ethyl-2-((3-(4-benzoyl-5-phenyl-3-(5-sulfamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)-1*H*-pyrazol-1-yl)phenyl) diazenyl)-3-oxo-3-phenylpropanoate (12d)

(381 mg, 51%); Mp 145 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.79 (m, 1H, CONH), 11.99 (s, 1H, Ar-NH–N=C), 8.37 (s, 2H, –SO_2NH_2), 4.20 (q, 2H, CH_2), 3.82 (s, 1H, N-CH), 1.26 (t, 3H, CH_3), 7.54–7.06 (m, 19H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.46 and 189.26 (benzoyl C=O), 165.34 (ester C=O), 162.70 (amide C=O), 161.38 and 160.40 (thiadiazol C-2 and C-5), 14.33 (CH_3), 46.03 (CH_2), 61.80 (CH), 144.43, 143.71, 139.71, 137.61, 137.32, 134.09, 133.07, 130.52, 130.38, 130.32, 130.04, 129.60, 129.30, 129.08, 129.01, 128.94, 128.88, 128.73, 127.64, 122.94, 120.79, 115.96, 112.91 IR(KBr) (ν, cm^{-1}): 3379, 3242, 3062, 2903, 2829, 1670, 1599, 1526, 1497, 1448, 1425, 1368, 1323, 1299, 1218, 1175; MS(CI) *m/z* 749.2 (M+1); Anal. Calcd for $C_{36}H_{28}N_8O_7S_2$: C, 57.74; H, 3.77; N, 14.96; S, 8.56. Found: C, 57.78; H, 3.97; N, 14.90; S, 8.51.

3.3.5. 4-Benzoyl-1-(3-((1,3-dioxo-1,3-diphenylpropan-2-yl) diazenyl)phenyl)-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (12e)

(686 mg, 88%); Mp 128 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.80 (m, 1H, CONH), 11.76 (s, 1H, Ar-NH–N=C) 8.40 (s, 2H, SO_2NH_2), 4.89 (s, 1H, N–CH), 8.19–7.02 (m, 24H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.53 and 185.80 (benzoyl C=O), 165.35 (amide C=O), 161.39 and 160.30 (thiadiazol C-2 and C-5), 93.73 (CH), 144.53, 143.39, 139.59, 137.60, 135.04, 134.05, 133.50, 130.60, 130.47, 130.13, 130.04, 129.63, 129.54, 129.32, 129.07, 129.04, 128.97, 128.64, 127.89, 127.69, 122.93, IR(KBr) (ν, cm^{-1}): 3468, 3380, 3231, 3061, 2885, 2831, 1680, 1598, 1523, 1495, 1427, 1367, 1301, 1224, 1174; MS(CI) *m/z* 781.2 (M+1); Anal. Calcd for $C_{40}H_{28}N_8O_6S_2$: C, 61.53; H, 3.61; N, 14.35; S, 8.21. Found: C, 61.42; H, 3.68; N, 14.27; S, 8.35.

3.3.6. *tert*-Butyl2-((3-(4-benzoyl-5-phenyl-3-(5-sulfamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)-1*H*-pyrazol-1-yl) phenyl)diazenyl)-3-oxobutanoate (12f)

(285 mg, 40%), Mp 217 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.79 (m, 1H, CONH), 11.59 (s, 1H, Ar-NH–N=C), 8.36 (s, 2H, –SO_2NH_2), 2.27 (s, 3H, CH_3), 1.52 (m, 9H, $C(CH_3)_3$), 7.79–7.13 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 194.31 (acetyl C=O), 190.55 (benzoyl C=O), 165.34 (ester C=O), 161.95 (amide C=O), 161.39 and 160.38 (thiadiazol C-2 and C-5), 28,36 and 26,48 (CH_3), 83,59 ($C(CH_3)_3$), 144.46, 143.58, 143.26, 139.74, 137.63, 134.08, 132.38, 130.56, 130.50, 130.45, 130.16, 129.62, 129.08, 127.83, 123.01, 120.97, 116.25, 112.77; IR(KBr) (ν, cm^{-1}): 3414, 3064, 2900, 2833, 1686, 1609, 1525, 1498, 1450, 1427, 1368, 1321, 1299, 1217, 1174; Anal. Calcd for $C_{33}H_{30}N_8O_7S_2$: C, 55.45; H, 4.23; N, 15.68; S, 8.97. Found: C, 55.39; H, 4.28; N, 15.56; S, 9.05.

3.3.7. 4-Benzoyl-1-(3-cyanophenyl)–5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (13a)

Diazonium salt solution of aromatic amine compound was prepared according to method in General procedure-II. A solution of CuCN and KCN which was prepared in another vessel was heated to 60 °C, and added slowly to diazonium salt solution prepared before. After complete mixing at same temperature, mixture was cooled to room temperature. Precipitated yellow product was filtered and purified from alcohol. (189 mg, 34%); Mp 173 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.80 (m, 1H, CONH), 8.39 (s, 2H, –SO_2NH_2), 7.81–7.28 (m, 15H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.52 (benzoyl C=O), 165.35 (amide C=O), 161.42 and 160.42 (thiadiazol C-2 and C-5), 152.07, 144.48, 143.25, 141.12, 139.68, 138.82, 137.63, 134.06, 130.53, 130.27, 129.62, 129.08, 128.97, 127.88, 126.26, 123.07, 122,92, 116.13; IR(KBr) (ν, cm^{-1}): 3415, 3062, 2108 (CN), 1688, 1598, 1522, 1496, 1449, 1426, 1366, 1299, 1244, 1174; Anal. Calcd for $C_{26}H_{17}N_7O_4S_2$: C, 56.21; H, 3.08; N, 17.65; S, 11.54. Found: C, 56.20; H, 3.15; N, 17.59; S, 11.58.

3.3.8. 4-Benzoyl-1-(3-iodophenyl)-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (13b)

Diazonium salt solution of aromatic amine compound was prepared according to method in General procedure-II. In another vessel, KI (656 mg, 1 mmol) was dissolved in ethanol and cooled to 0–5 °C and added dropwise to diazonium salt solution which was prepared before. Precipitated yellow precipitate was filtered with tromp and purified from alcohol. (426 mg, 65%); Mp 191 °C; ^{1}H NMR NMR (400 MHz, DMSO-d_6) δ (ppm): 13.20 (m, 1H, CONH), 8.37 (s, 2H, SO_2NH_2), 7.81–6.52 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.52 (benzoyl C=O), 165.34 (amide C=O), 161.43 and 160.38 (thiadiazol C-2 and C-5), 144.48, 143.25, 143.06, 139.70, 139.61, 137.63, 134.07, 130.57, 130.26, 129.98, 129.83, 129.63, 129.07, 128.92, 127.91, 123.09, 122.59; IR(KBr) (ν, cm^{-1}): 3377, 3061, 2904, 1687, 1609, 1523, 1497, 1449, 1425, 1367, 1298, 1235, 1174; MS(CI) *m/z* 657.0 (M+1); Anal. Calcd for $C_{25}H_{17}IN_6O_4S_2$: C, 45.74; H, 2.61; N, 12.80; S, 9.77. Found: C, 45.80; H, 2.55; N, 12.81; S, 9.91.

3.3.9. 4-Benzoyl-1-(3-hydroxyphenyl)–5–phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (13C)

Diazonium salt solution of aromatic amine compound was prepared according to method in General procedure-II. Prepared diazonium solution was heated in water bath at 100 °C until nitride gas output had stopped. After cooling of mixture to room temperature, pH adjustment (pH 3–4) was performed. After sometime, precipitated product was filtered with tromp. Produced yellow solid was purified from ethanol. (289 mg, 53%); Mp 200 °C; ^{1}H NMR

(400 MHz, DMSO-d_6) δ (ppm): 13.80 (m, 1H, CONH), 8.37 (s, 2H, -SO_2NH_2), 7.81–6.92 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.52 (benzoyl C=O), 165.30 (amide C=O), 161.43 and 160.37 (thiadiazol C-2 and C-5), 152.13 (=C–OH), 144.48, 143.23, 139.70, 137.62, 134.07, 130.56, 130.26, 129.89, 129.77, 129.63, 129.08, 128.91, 127.90, 127.41, 123.08, 114.97; IR(KBr) (ν, cm^{-1}): 3370, 3241, 3061, 1688, 1600, 1521, 1495, 1448, 1426, 1366, 1300, 1237, 1173; MS(CI) *m/z* 546.1; Anal. Calcd for $C_{25}H_{18}N_6O_5S_2$: C, 54.94; H, 3.32; N, 15.38; S, 11.73. Found: C, 54.80; H, 3.45; N, 15.36; S, 11.79.

3.3.10. 4-Benzoyl-1-(3-((4-hydroxyphenyl)diazenyl)phenyl)-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (14a)

Produced according to method in general procedure-II, (221 mg, 34%); Mp 207 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.63 (m, 1H, CONH), 8.37 (s, 2H, SO_2NH_2), 10.05 (m, 1H, Ar-OH), 8.02–7.29 (m, 18H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.51 (benzoyl C=O), 165.35 (amide C=O), 161.42 and 160.39 (thiadiazol C-2 and C-5), 160.30 (=C–OH), 145.80, 144.51, 143.23, 139.70, 137.64, 134.07, 132.05, 130.93, 130.27, 130.18, 130.06, 129.64, 129.08, 128.03, 127.90, 126.26, 125.67, 123.30, 123.09, 116.58; IR(KBr) (ν, cm^{-1}): 3384, 3064, 1689, 1597, 1522, 1500, 1449, 1426, 1365, 1301, 1245, 1214, 1174; MS(CI) *m/z* 651.2 (M+1); Anal. Calcd for $C_{31}H_{22}N_8O_5S_2$: C, 57.22; H, 3.41; N, 17.22; S, 9.86. Found: C, 57.15; H, 3.53; N, 17.15; S, 9.93.

3.3.11. 4-Benzoyl-1-(3-((2–hydroxynaphthalen–1-yl)diazenyl) phenyl)-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (14b)

Produced according to method in general procedure-II; (300 mg, 43%); Mp 195 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.80 (m, 1H, CONH), 8.39 (s, 2H, SO_2NH_2), 6.86 (d, 1H, Ar-OH), 8.26–7.14 (m, 20H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.54 (Benzoyl C=O), 165.34 (Amide C=O), 162.72 (=C–OH), 161.45 and 160.36 (Thiadiazol C-2 and C-5), 145.53, 144.46, 143.25, 141.53, 140.08, 137.69, 134.12, 132.95, 130.93, 130.32, 130.18, 130.06, 129.77, 129.68, 129.46, 129.11, 128.39, 127.89, 126.82, 124.74, 124.50, 123.29, 122.11, 120.12, 114.76, 106.27; IR(KBr) (ν, cm^{-1}): 3416, 3062, 1687, 1601, 1518, 1451, 1426, 1364, 1298, 1254, 1206, 1174; MS(CI) *m/z* 701.0 (M+1); Anal. Calcd for $C_{35}H_{24}N_8O_5S_2$: C, 59.99; H, 3.45; N, 15.99; S, 9.15. Found: C, 60.18; H, 3.43; N, 15.90; S, 9.19.

3.3.12. 4-Benzoyl-1-(2-methyl-1-(2-oxopropyl)-1*H*-indol-4-yl)-5-phenyl-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-3-carboxamide (15)

Aromatic compound of amine (1 mmol, 545 mg) was dissolved in 15 ml DMF. Chloroacetone (0,182 ml) was added into mixture. Reaction was maintained at 60 °C for three days in back cooler which has $CaCl_2$ headpiece. After evaporation of solvent of dark colored mixture in rotary evaporator, remainder dense liquid was added to water and mixed. Granulated product was filtered with trompt and purified from ethanol. (310 mg 57%); Mp 183 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.80 (m, 1H, CONH), 8.36 (s, 2H, SO_2NH_2), 4.35 (s, 2H, CH_2), 2.51 and 2.24 (s, 6H, CH_3), 7.79–7.22 (m, 13H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 202.20 (acetyl C=O), 190.56 (benzoyl C=O), 165.33 (amide C=O), 161.41 and 160.46 (thiadiazol C-2 and C-5), 44.25 (CH_2), 31.24 and 29.08 (CH_3), 143.44, 139.34, 137.61, 134.03, 131.89, 130.98, 130.41, 130.15, 129.96, 129.65, 129.07, 129.01, 127.76, 127.43, 123.45, 122.88, 121.50, 121.04, 117.03; IR(KBr) (ν, cm^{-1}): 3378, 3064, 2903, 1685, 1606, 1524, 1496, 1449, 1424, 1363, 1301, 1241, 1173; MS(CI) m/z 640.15 (M+1); Anal. Calcd for $C_{31}H_{25}N_7O_5S_2$: C, 58.20; H, 3.94; N, 15.33; S, 10.02. Found: C, 58.35; H, 3.87; N, 15.28; S, 10.12.

3.4. Purification of carbonic anhydrases I and II from human erythrocytes

Carbonic anhydrases I and II were purified as described previously.[27,28] Briefly, human blood was centrifuged at 1500 rpm for 20 min, and after removal of the plasma, the erythrocytes were washed with an isotonic solution (0.9% NaCl). After that, the erythrocytes were lysed with 1.5 volume of ice-cold water. The lysate was centrifuged at 20000 rpm for 30 min to remove cell membranes and non-lysed cells. The pH of the supernatant was adjusted to 8.7 and was then loaded onto an affinity column containing Sepharose-4B-L-tyrosine-*p*-aminobenzene sulfonamide as the binding group. After extensive washing with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7), the hCA-I and II isozymes were eluted with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6). Amount of purified protein was estimated by the Bradford method[29] and SDS–PAGE was carried out to determine whether the eluate contained the enzyme.[30]

3.5. Determination of hydratase and esterase activities

CO_2-hydratase activity of the enzyme was determined at 0 °C in a veronal buffer (pH 8.15) with pH-state method. Enzyme activity was calculated by using the equation $(t_0 - t_c/t_c)$ where t_o and t_c are the time taken for the pH change of the non-enzymatic and enzymatic reactions, respectively.[30,31] Esterase activities of carbonic anhydrase enzymes were assayed by the hydrolysis of *p*-nitrophenylacetate. The absorbance was determined at 348 nm after 3 min.[32] The % carbonic anhydrase activity values were assayed by following the hydration of CO_2. IC_{50} values for inhibitor **1, AAZ,** and the synthesized compounds (**8–11**) were determined on hCA-I and hCA-II by measuring hydratase activity in the presence of various inhibitor concentrations. Regression analysis graphs were drawn by plotting inhibitor concentrations versus enzyme activity by using Microsoft Excel Program. The data were then fitted with non-linear regression using the second degree polynomial equation. The IC_{50} value was obtained by solving the equation derived from the second degree polynomial.[31–36]

3.6. K_i Determination

To determine K_i value as well as the inhibition type, three different inhibitor concentrations giving 30%, 50%, and 70% inhibition (2.4 μM, 4.8 μM, and 7.3 μM, respectively) were selected. At each of these inhibitor concentrations, enzyme activity was measured in the presence of various substrate concentrations (0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, and 0.7 mM) and the data were linearized with Lineweaver-Burke plot for V'_{max} (apparent V_{max}) and the K_i determination.[31] Enzyme activity was also measured in the presence of the same substrate concentrations but in the absence of any inhibitor to determine the V_{max}.

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