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Carbonic anhydrase inhibitors. Part 35*. Synthesis of Schiff bases derived from sulfanilamide and aromatic aldehydes: the first inhibitors with equally high affinity towards cytosolic and membrane-bound isozymes

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Summary — A series of Schiff bases was prepared by reaction of sulfanilamide with substituted benzene- and heterocyclic aldehydes. The compounds were characterized by standard procedures, and were assayed as inhibitors of the zinc enzyme carbonic anhydrase (CA). The new compounds act as inhibitors towards isozymes CA I and II (cytosolic) and CA IV (membrane-bound), and possess an equally high affinity for the last two, in contrast to classical inhibitors which are 17–33 times less effective against CA IV. This is the first evidence of high-affinity CA IV inhibitors, and might lead to the development of low molecular weight CA IV isozyme-specific derivatives.

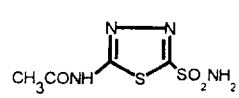
carbonic anhydrase / isozyme / sulfanilamide / Schiff base

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc enzyme widely spread in the plant and animal kingdoms, acting as a highly efficient catalyst for the reversible hydration of carbon dioxide to bicarbonate (reaction 1), but also catalyzing non-physiological reactions such as aldehyde hydration, and ester and sultone hydrolysis [2, 3].

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+ \qquad (1)$$

Sulfonamides are specific inhibitors of CA [2–4], binding in ionized form to the Zn(II) ion within the active site and displacing the water molecule bound to the metal [5, 6] which is responsible for the catalytic power of the enzyme [7]. Moreover, heterocyclic and aromatic derivatives, such as acetazolamide **1** [2], methazolamide **2** [2], ethoxzolamide **3** [2], the recently developed thienothiopyransulfonamides of type **4** [5] and dichlorophenamide **5** [2] are clinically-used pharmacological agents in the treatment of a variety of disorders, such as glaucoma [8, 9], diverse neurological diseases [2, 12] and acid-base disequilibria [2, 11], as well as in many physiological studies [11, 13].



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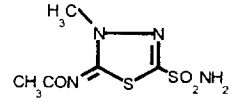

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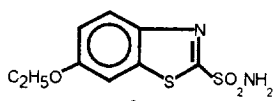

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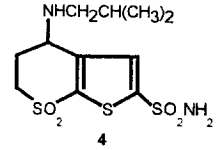

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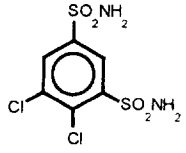

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*For part 34, see reference [1].

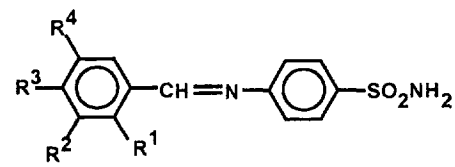
Since the report in 1940 of sulfanilamide as a strong inhibitor of red cell CA, by Mann and Keilin [14], a large number of aromatic and heterocyclic sulfonamides have been investigated [3, 15–17] for their interaction with different CA isozymes, mainly due to the above-mentioned clinical applications of such agents. Presently much effort is devoted to the design of isozyme-specific [3, 17] or organ-selective [3, 9, 10] CA inhibitors, taking into account the fact that in vertebrates at least eight distinct CA isozymes are known [3], which differ greatly in their ability to catalyze reaction 1, and also possess different susceptibilities to inhibition by unsubstituted sulfonamides [18]. The precise physiological function of many of them is also unknown [3]. On the other hand, topically-active CA inhibitors, such as derivatives **3**, are successful antiglaucoma agents, recently introduced into clinical use [9].

Taking into account our interest in the design and pharmacological actions of diverse agents from this class [3], we report here the synthesis, characterization and biological activity of a large series of Schiff bases derived from sulfanilamide and aromatic/heterocyclic aldehydes. The prepared derivatives were characterized by means of analytic and spectroscopic methods and were assayed in vitro for inhibition against isozymes CA I, II and IV. Interesting activity was detected for some members of the series against these three isozymes, but the most unexpected finding was their equally high affinity towards CA II and CA IV. This makes them a good starting point for the design of strong CA IV-specific inhibitors.

Results

The compounds prepared by condensation of sulfanilamide with aromatic/heterocyclic aldehydes, of types **6** and **7**, are shown in tables I, II, together with their inhibition data against isozymes CA I, II and IV. The pK_a values for the SO_2NH_2 moiety are also shown, as this parameter was considered important for the inhibition potency of sulfonamides [2–4].

Table I. Schiff bases **6** prepared by reaction of substituted benzaldehydes with sulfanilamide: the pK_a values of the sulfonamide moiety and inhibition data against isozymes CA I, II and IV.



6	R^1	R^2	R^3	R^4	Yield (%)	pK_a*	K_i		
							CA I (μM)	CA II ($\times 10^8$ M)	CA IV ($\times 10^8$ M)
a	H	H	H	H	95	10.2	18	27	31
b	OH	H	H	H	98	10.1	35	41	42
c	NO_2	H	H	H	75	9.6	9	21	20
d	H	H	Cl	H	90	10.1	25	28	27
e	H	H	OH	H	94	10.2	14	19	22
f	H	H	OMe	H	92	10.0	13	19	20
g	H	H	NMe_2	H	82	10.1	10	8	10
h	H	H	NO_2	H	87	9.8	13	5	8
i	H	H	CN	H	99	9.9	4	11	13
j	H	OMe	OH	H	61	9.8	5	8	9
k	H	OMe	OMe	H	96	10.1	7	3	5
l	H	OMe	OAc	H	62	9.9	3	10	15
m	OH	OH	H	CHO	97	9.8	4	2	6
n	OH	OMe	H	CHO	99	9.9	5	3	11
o	H	OMe	OMe	OMe	91	10.1	5	3	4
p	H	OMe	OH	Br	87	9.6	12	4	7

*Only the values for the ionization of sulfonamido moieties are given; in the case of derivatives containing phenolic OH groups which ionize at similar pK_a values, it is difficult to attribute the group responsible for the first ionization step.

Table II. Schiff bases of type **7**, prepared from sulfanilamide and heterocyclic aldehydes: the pK_a values of the sulfonamido moiety and inhibition data against isozymes CA I, II and IV.

$$Het\text{-}CH{=}NC_6H_4SO_2NH_2$$

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7	*Het*	*Yield (%)*	*pK_a**	*K_i*		
				CA I (μM)	*CA II ($\times 10^8$ M)*	*CA IV ($\times 10^8$ M)*
a	2-Furyl	93	9.7	3	5	8
b	5-Methyl-2-furyl	94	9.7	3	4	11
c	Pyrol-2-yl	63	9.6	5	2	4
d	Imidazol-4(5)-yl	58	9.8	1	12	13
e	2-Pyridyl	54	9.8	2	9	10
f	3-Pyridyl	76	9.9	4	8	14
g	4-Pyridyl	69	9.9	4	5	9

*See table I.

The prepared compounds were tested for inhibition against three CA isozymes, namely CA I (from human red cells), CA II (from bovine red cells) and bovine CA IV, a membrane-bound isozyme recently purified and cloned by Sly's group [19]. The first two are the major erythrocyte isozymes [2], which differ in their ability to catalyze reaction 1 (CA II has a maximum turnover number of 1.4×10^6 s^{-1}, whereas isozyme CA I is somehow slower, with a turnover of 2×10^5 s^{-1} [20]), and in their susceptibility to inhibition by sulfonamides [18] (CA II is more sensitive to these inhibitors (with K_i values in the nanomolar range [2, 3, 18], while CA I is much more resistant, with K_i values in the micromolar range [18]). Mention should be made that bovine and human CA II have very similar kinetic and inhibition characteristics, as well as a high degree of homology [2, 3, 10]. CA IV on the other hand, was recently shown to be involved in major secretory processes [18, 19], previously attributed to the cytosolic high-activity form CA II. However, in contrast to CA II, this membrane-bound isozyme is generally 17–33 times less sensitive to classical sulfonamide inhibitors of type **1–5**, as proved by Maren's group [18]. As it seems that CA IV is the major biologically-relevant isoform among the eight isozymes presently known in vertebrates [3, 18], detection of sulfonamides with high affinity towards it would allow specific inhibition studies with much greater accuracy, without interference from the other isozymes.

The inhibition data against isozymes of standard sulfonamide inhibitors, such as acetazolamide **1**, sulfanilamide (4-aminobenzenesulfonamide) and other compounds discussed here, are also shown in table III.

Discussion

Although Schiff bases derived from sulfanilamide and aromatic aldehydes were synthesized by earlier researchers interested in antibacterial derivatives [21], and subsequently the field was reinvestigated by several other groups [22–25], with very few exceptions such compounds were never tested as CA inhibitors, probably due to the fact that aromatic sulfonamides were considered weaker inhibitors than the heterocyclic derivatives [2, 3, 15]. Only recently have such sulfonamides started to be reinvestigated, mainly due

Table III. Inhibition data of isozymes CA I, II and IV with the standard inhibitors sulfanilamide and **1–5**, as well as compounds **8**, **10** and **12**, recently developed as inhibitors.

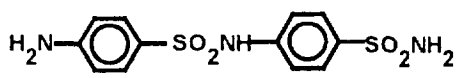
Inhibitor	*K_i*		
	CA I (μM)	*CA II ($\times 10^8$ M)*	*CA IV ($\times 10^8$ M)*
Sulfanilamide	28	30	300
Acetazolamide **1**	0.2	0.7	12
Methazolamide **2**	0.01	0.8	6.5
Ethoxzolamide **3**	0.01	0.1	3.2
MK-417 4	> 500	0.2	3.0
5	34	3.4	99
8	12	43	358
10 (R_1-R_3=Me)	31	29	485
12	15	1.5	40

to the need to prepare isozyme-specific or organ-selective inhibitors [3, 9, 10]. Until now very few compounds are known to act in this way. Thus sulfanilylsulfanilamide **8** binds very strongly to CA I [26], and has a much lower affinity for CA II, the isozyme predominantly inhibited by sulfonamide inhibitors [10]; trifluorometanesulfonamide **9** is the strongest CA III inhibitor [27] (it also appreciably inhibits the other isozymes, but CA III is a sulfonamide-resistant isozyme [3]); and positively-charged heterocyclic sulfonamides of type **10** reported by us inhibit the membrane-bound CA isozymes CA IV, and presumably the mitochondrial form CA V, but act as much stronger CA II inhibitors at the same time [17, 28]. Being membrane-impermeant, they could still show some selectivity in vivo for the membrane-bound isozymes [17].

The only Schiff bases derived from aromatic/heterocyclic sulfonamides previously tested as CA inhibitors are the four sulfanilamide derivatives **11**, reported by Beasley et al [16], prepared from sulfanilamide and the following aldehydes: 2-hydroxy-, 4-chloro-, 2,4-dichloro- and 3-methoxy-4-hydroxy-5-iodobenzaldehyde, as well as the heterocyclic derivative **12** prepared from 5-amino-1,3,4-thiadiazole-2-sulfonamide and 2-ketoglutaric acid, reported by this group [29]. On the other hand, Prugh et al [30] reported the preparation of thieno[2,3-*b*]- and thieno[3,2-*b*]-thiophene-2-sulfonamide derivatives, in which Schiff bases were intermediates but were not isolated, being further reduced to primary/secondary amines leading to compounds which acted as topically-active inhibitors in glaucoma [30, 31].

The prepared derivatives, of type **6**, obtained from substituted benzaldehydes and sulfanilamide, shown in table I, were designed taking into account the following aspects. i) The one, two, three or four groups arising from the aldehyde, substituting the benzene nucleus in different positions, may afford supplementary stabilization or destabilization of the enzyme–inhibitor adduct, thus leading respectively to stronger or weaker inhibitors. As a structure–activity study was not performed for inhibitors of this type [32], it appeared of interest to analyze a large series of diversely substituted compounds, possessing both electron-withdrawing and electron-donating groups, in order to detect effective substitution patterns. (ii) These groups substituting the second benzene ring also affect the pK_a of the sulfonamido moiety, which is an important parameter for the binding of inhibitors to enzyme [2, 3, 33], as well as for the pharmacological properties of this type of CA inhibitors [9]. The corresponding pK_a values for the SO_2NH_2 moieties of the synthesized inhibitors were therefore also determined. It is noteworthy that for compounds containing OH (phenolic) moieties, it is not always clear whether the determined pK_a is that of the sulfonamido or the phenolic moiety, since both these groups should ionize around 10 pK_a units (which is the value actually determined experimentally by us; see the *Experimental protocols* for details).

The derivatives **7**, obtained from sulfanilamide and heterocyclic aldehydes (table II), were prepared using approaches similar to those from substituted benzaldehydes. But in the case of these heterocyclic derivatives even stronger binding to the enzyme would be expected, due to the putative participation of heteroatoms belonging to the inhibitor molecule in the hydrogen bond network involving the amino acid side chain of the active site, as in the case of the



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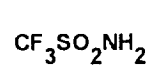

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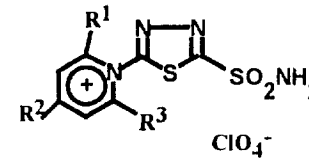
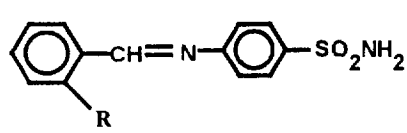
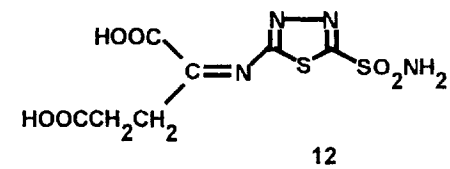


10: R^1-R^3 = Me; Ph; i-Pr



11 : R = H; Cl; OH



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enzyme–inhibitor adducts of heterocyclic sulfonamides, for which this behavior was documented by X-ray crystallographic studies [7].

As seen from tables I–III, CA inhibitory properties of derivatives **6** and **7** against the three investigated isozymes are good. In the case of CA I, it is noteworthy that all Schiff bases prepared by us are more active than the parent compound, sulfanilamide, whereas several compounds, such as **6i–o** and all derivatives **7**, have activities comparable (1/10 potency) to those of acetazolamide, the standard (and very potent) CA inhibitor. All compounds derived from heterocyclic aldehydes (**7**) are generally more active than those obtained from substituted benzaldehydes, against the three isozymes. Groups such as methoxy, hydroxy, cyano and acetoxy seem to favor CA I inhibition. Against CA II, again all Schiff bases are more active than sulfanilamide. It seems that the number of groups substituting the arylidene moiety is not a very important parameter for CA inhibition with this class of derivatives, since mono- and polysubstituted compounds had similar inhibitory effects (compare **6g**, **6j** and **6n** on CA II). The pK_a of the sulfonamido moiety seems not to be critical for the potency of these inhibitors, although generally the stronger acids **7** showed a slightly improved activity as compared to sulfonamides **6**. Of course, pK_a is an important parameter for the in vivo fate of such drugs [2, 9]. One of the exciting findings in this research was that, in contrast to classical inhibitors such as sulfanilamide and derivatives **1–4** (see table III), compounds reported by us here, as a whole class, possess a much stronger affinity towards isozyme CA IV. Up to now, no other inhibitors possessing this behavior have been detected (see also K_i values for compounds **8–12** in table III). Thus, in the case of the sulfonamides mentioned above, of types **1–4**, affinities for CA IV were 17–33-fold weaker than the corresponding affinities towards CA II [18]. From such data, it seems that Schiff bases of types **6** and **7** might lead to the development of CA IV-specific and potent inhibitors, a desirable goal to achieve, since this isozyme possesses a critical role in vivo [3, 18, 19]. The only inhibitors possessing some specificity towards CA IV reported previously were polymers [3] or positively-charged compounds [3, 17, 28] which, being membrane-impermeant, inhibited only the membrane-bound enzyme; but such compounds appreciably inhibited CA I and CA II, similarly to acetazolamide **1** or other inhibitors **2–4** and **8–12** discussed here. As seen from the data in tables I and II; derivatives **6c** and **d** have a higher affinity towards CA IV than towards CA II, and nine other derivatives (including **6b**,**f**,**j**,**o** and **7c–e**) have affinities for these two isozymes differing only slightly in favor of CA II.

In conclusion, we report here the first evidence of sulfonamides with comparable affinities for cytosolic (CA II) and membrane-bound (CA IV) enzymes, which are Schiff bases derived from sulfanilamide and substituted - benzene or heterocyclic aldehydes. Further exploring this class of inhibitors might lead to further improvement of the inhibition ratios in favor of CA IV, and the obtainment of highly selective such compounds. Work is in progress in our laboratory to attain this goal.

Experimental protocols

Chemistry

Melting points were recorded with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with a Carl Zeiss IR-80 instrument. Electronic spectra were obtained in methanolic solution, with a Cary 3 spectrophotometer, interfaced with an IBM-compatible PC. NMR spectra were recorded in DMSO-d_6 as solvent, with a General Electrics Omega instrument, working at 200 MHz for the ^{1}H-NMR spectra, and 75.57 MHz for the ^{13}C-NMR spectra. Chemical shifts are reported as δ values, relative to Me_4Si as internal standard. Thin layer chromatography (tlc) was performed on silica-gel G-60 from Merck (Darmstadt, Germany), in chloroform/methanol 10:1 (v/v). Elemental analysis was performed by combustion (for C, H, N) with a Carlo Erba Automated analyzer (Milan, Italy). The values obtained were within ±0.4% of the theoretical values calculated for the proposed formulae.

Sulfanilamide, acetazolamide, methazolamide and aldehydes used in the syntheses were commercial reagents from Sigma, Aldrich and Merck, and were used without additional purification. Bovine CA II and human CA I were from Sigma Chemical Co (Saint Louis, MO, USA). CA IV was isolated from bovine lung microsomes as described by Maren et al [18]. Other sulfonamides used as standards in the enzymatic determinations were prepared as described in the literature [3, 4, 6, 15–17, 28, 29]

*General procedure for the preparation of Schiff bases **6** and **7***
Sulfanilamide (4.3 g, 25 mmol) was dissolved in 40 mL boiling ethanol and the required aldehyde (25 mmol) was added to the reaction mixture. Boiling was continued for 3 h, then a portion of the solvent was evaporated in vacuum, and crystals of derivatives **6**, **7** were obtained by cooling; these were recrystallized from 96% ethanol. Yields were generally high (see tables I, II).

*N^4-Benzylidene sulfanilamide **6a**.* White crystals, mp 179–180 °C, lit mp: 176 °C [21], 182–185 °C [22], 185–189 °C [23]. IR (KBr), cm^{-1}: 1150, 1330, 1630; UV (MeOH) λ_{max}, nm, (log ε): 264 (3.34); with NaOH: 265 (3.40). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.12–7.38 (m, 5H, ArH from Ph); 7.61 (br s, 2H, NH_2); 8.18 (s, 1H, CH). ^{13}C-NMR δ, ppm: 118.1, 118.9, 129.8, 130.1 (all signals of Ph moiety); 114.9, 131.7, 135.4, 153.8 (all from -C_6H_4-); 178.6 (CH). Tlc, R_f = 0.71. Anal $C_{13}H_{12}N_2O_2S$ (C, H, N).

*N^4-(2-Hydroxybenzylidene) sulfanilamide **6b**.* White crystals, mp 209–210 °C, lit mp [16, 23, 24] 209–210 °C and 208–209 °C. IR (KBr), cm^{-1}: 1150, 1310, 1615; UV (MeOH) λ_{max},

nm, (log ε): 232 (4.33), 272 (4.26), 341 (4.15); with NaOH: 233 (4.43), 276 (4.32), 342 (4.16). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.21–7.43 (m, 4H, ArH from HOC_6H_4); 7.61 (br s, 2H, NH_2); 8.18 (s, 1H, CH); 8.83 (br s, 1H, OH). ^{13}C-NMR δ, ppm: 118.2, 118.9, 129.5, 136.4 (all signals of HOC_6H_4 moiety); 114.9, 131.7, 135.4, 153.8 (all from -C_6H_4-); 178.9 (CH). Tlc, R_f = 0.68. Anal $C_{13}H_{12}N_2O_3S$ (C, H, N).

N⁴-(2-Nitrobenzylidene) sulfanilamide ***6c***. Yellow crystals, mp 189–190 °C, lit mp 245–246 °C [24] and 183 °C [23]. IR (KBr), cm^{-1}: 1150, 1340 (both the sulfonamido stretching vibrations), 1350, 1530 (νNO$_2$), 1620; UV (MeOH) λ_{max}, nm, (log ε): 238 (4.43), 259 (4.40), 310 (4.02) with NaOH: 262 (4.37), 310 (3.64). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.18–7.38 (m, 4H, ArH from $O_2NC_6H_4$); 7.65 (br s, 2H, NH_2); 8.19 (s, 1H, CH). ^{13}C-NMR δ, ppm: 118.5, 118.8, 127.9, 136.9 (all signals of $O_2NC_6H_4$ moiety); 114.8, 131.7, 135.4, 153.8 (all from -C_6H_4-); 178.9 (CH). Tlc, R_f = 0.69. Anal $C_{13}H_{11}N_3O_4S$ (C, H. N).

N⁴-(4-Chlorobenzylidene) sulfanilamide ***6d***. Pale yellow crystals, mp 187–188 °C, lit mp 191–192 °C [24] and 193–194 °C [16]. IR (KBr), cm^{-1}: 1140, 1320, 1615; UV(MeOH) λ_{max}, nm, (log ε): 224 (4.06), 312 (4.12); with NaOH: 224 (4.09), 316 (4.18). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.13–7.35 (m, 4H, ArH from ClC_6H_4); 7.66 (br s, 2H, NH_2); 8.17 (s, 1H, CH). ^{13}C-NMR δ, ppm: 118.5, 118.8, 127.9, 130.9 (all signals of ClC_6H_4 moiety); 114.7, 131.7, 135.4, 153.8 (all from -C_6H_4-); 178.8 (CH). Tlc, R_f = 0.69. Anal $C_{13}H_{11}N_2ClO_2S$ (C, H, N).

N⁴-(4-Hydroxybenzylidene) sulfanilamide ***6e***. White crystals, mp 203–204 °C, lit mp 209–210 °C [24]. IR (KBr), cm^{-1}: 1145, 1320, 1620; UV (MeOH) λ_{max}, nm, (log ε): 233 (4.13), 272 (4.15), 344 (4.05); with NaOH: 234 (4.40), 276 (4.35), 345 (4.16). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.21–7.43 (m, 4H, ArH from HOC_6H_4); 7.63 (br s, 2H, NH_2); 8.15 (s, 1H, CH); 8.82 (br s, 1H, OH). ^{13}C-NMR δ, ppm: 118.2, 118.8, 129.3, 137.2 (all signals of HOC_6H_4 moiety); 114.9, 131.7, 135.5, 153.8 (all from -C_6H_4-); 178.8 (CH). Tlc, R_f = 0.40. Anal $C_{13}H_{12}N_2O_3S$ (C, H, N).

N⁴-(4-Methoxybenzylidene) sulfanilamide ***6f***. White crystals, mp 181–182 °C, lit mp 181–182 °C [23]; 192–193 °C [21]. IR (KBr), cm^{-1}: 1120 (OMe), 1150, 1330, 1620; UV (MeOH) λ_{max}, nm, (log ε): 233 (4.10), 319 (4.15); with NaOH: 232 (4.14), 319 (4.16). ^{1}H-NMR (DMSO-d_6) δ, ppm: 3.98 (s, 3H, MeO); 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.17–7.43 (m, 4H, ArH from $MeOC_6H_4$); 7.62 (br s, 2H, NH_2); 8.13 (s, 1H, CH); ^{13}C-NMR δ, ppm: 28.5 (Me); 118.2, 118.6, 129.1, 138.5 (all aromatic signals of $MeOC_6H_4$ moiety); 114.8, 131.5, 135.6, 153.8 (all from -C_6H_4-); 178.8 (CH). Tlc, R_f = 0.55. Anal $C_{14}H_{14}N_2O_3S$ (C, H, N).

N⁴-(4-Dimethylaminobenzylidene) sulfanilamide ***6g***. Yellow crystals, mp 202–203 °C, lit mp 202–203 °C [23], 226–227 °C [21], 214 °C [33]. IR (KBr), cm^{-1}: 1150, 1330, 1615, 2880-2960 (NMe_2); UV (MeOH) λ_{max}, nm, (log ε): 240 (4.22), 342 (4.71); with NaOH: 241 (4.22), 344 (4.72). ^{1}H-NMR (DMSO-d_6) δ, ppm: 4.06 (s, 6H, 2Me); 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.10–7.39 (m, 4H, ArH from $Me_2NC_6H_4$); 7.65 (br s, 2H, NH_2); 8.12 (s, 1H, CH); ^{13}C-NMR δ, ppm: 38.9 (Me), 118.1, 118.7, 129.5, 138.1 (all aromatic signals of $Me_2NC_6H_4$ moiety); 114.8, 131.5, 135.6, 153.8 (all from -C_6H_4-); 178.9 (CH). Tlc, R_f = 0.39. Anal $C_{15}H_{17}N_3O_2S$ (C, H, N).

N⁴-(4-Nitrobenzylidene) sulfanilamide ***6h***. Yellow crystals, mp 182–183 °C, lit mp 175 °C [23]. IR (KBr), cm^{-1}: 850 (νC–N); 1170, 1330 (both the sulfonamido vibrations), 1350, 1530 (νNO$_2$) 1630; UV (MeOH) λ_{max}, nm, (log ε): 222 (4.15), 285 (4.38), 350 sh (4.10); with NaOH: 264 (4.86), 350 (3.85). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.19 (m, AA'BB' 4H, ArH from $O_2NC_6H_4$); 7.60 (br s, 2H, NH_2); 8.21 (s, 1H, CH). ^{13}C-NMR δ, ppm: 118.5, 118.8, 127.6, 135.6 (all signals of $O_2NC_6H_4$ moiety); 114.9, 131.5, 135.4, 153.8 (all from -C_6H_4- of sulfanilamide moiety); 178.8 (CH). Tlc, R_f = 0.49. Anal $C_{13}H_{11}N_3O_4S$ (C, H, N).

N⁴-(4-Cyanobenzylidene) sulfanilamide ***6i***. Pale yellow crystals, mp 179–180 °C; this is a new compound. IR (KBr), cm^{-1}: 1150, 1330, 1620; 2240 (CN); UV (MeOH) λ_{max}, nm, (log ε): 229 (4.12), 270 (4.55), 320 (4.11); with NaOH: 227 (4.26), 264 (4.51). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.17 (m, AA'BB' 4H, ArH from NCC_6H_4); 7.61 (br s, 2H, NH_2); 8.18 (s, 1H, CH). ^{13}C-NMR δ, ppm: 118.5, 118.6, 127.8, 135.6, 185.3 (all signals of NCC_6H_4 moiety); 114.9, 131.5, 135.5, 153.8 (all from -C_6H_4- of sulfanilamide moiety); 178.9 (CH). Tlc, R_f = 0.48. Anal $C_{14}H_{11}N_3O_2$) (C, H. N).

N⁴-(3-Methoxy-4-hydroxybenzylidene) sulfanilamide ***6j***. Pale yellow crystals, mp 191–192 °C, lit mp 198–199 °C [34]. IR (KBr), cm^{-1}: 1150, 1320, 1620; UV (MeOH) λ_{max}, nm, (log ε): 234 (4.10), 262 (4.16), 331 (4.02); with NaOH: 230 (4.23), 260 (4.25), 381 (4.56). ^{1}H-NMR (DMSO-d_6) δ, ppm: 3.91 (s, 3H, Me); 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.15–7.47 (m, 3H, ArH from C_6H_3); 7.63 (br s, 2H, NH_2); 8.16 (s, 1H, CH); 8.80 (br s, 1H, OH). ^{13}C-NMR δ, ppm: 29.3 (Me); 118.1, 118.8, 129.5, 137.9 (all signals of C_6H_3); 114.8, 131.7, 135.9, 153.8 (all from -C_6H_4-); 178.3 (CH). Tlc, R_f = 0.40. Anal $C_{14}H_{12}N_2O_4S$ (C, H, N).

N⁴-(3,4-Dimethoxybenzylidene) sulfanilamide ***6k***. Pale yellow crystals, mp 190–193 °C, lit mp 193 °C [23]. IR (KBr), cm^{-1}: 1150 (C–OMe); 1160, 1340 (sulfonamido vibrations); 1620; UV (MeOH) λ_{max}, nm, (log ε): 233 (4.12), 264 (4.23), 332 (4.18); with NaOH: 230 (4.23), 260 (4.29); ^{1}H-NMR (DMSO-d_6) δ, ppm: 3.91 (s, 6H, 2Me); 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.21–7.48 (m, 3H, ArH from C_6H_3); 7.62 (br s, 2H, NH_2); 8.15 (s, 1H, CH). ^{13}C-NMR δ, ppm: 29.5 (Me), 118.7, 118.8, 128.9, 137.1 (all signals of C_6H_3); 114.9, 131.7, 135.9, 153.7 (all from -C_6H_4-); 178.1 (CH). R_f = 0.81. Anal $C_{15}H_{14}N_2O_4S$ (C, H, N).

N⁴-(4-Acetoxy-3-methoxybenzylidene) sulfanilamide ***6l***. White crystals, mp 178–179 °C; this is a new compound. IR (KBr), cm^{-1}: 1120 (C–O vibration) 1150, 1340, 1630, 1750 (acetate band); UV (MeOH) λ_{max}, nm, (log ε): 225 (4.45), 270 (4.43), 316 (4.36); with NaOH: 230 (4.23), 380 (4.31). ^{1}H-NMR (DMSO-d_6) δ, ppm: 1.79 (s, 3H, Me from acetyl); 3.91 (s, 3H, MeO); 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.15–7.41 (m, 3H, ArH from C_6H_3); 7.63 (br s, 2H, NH_2); 8.15 (s, 1H, CH). ^{13}C-NMR δ, ppm: 12.3 (Me from acetyl); 29.4 (MeO), 118.1, 118.5, 129.6, 137.9 (all signals of C_6H_3), 114.9, 131.7, 135.9, 153.9 (all from -C_6H_4-); 178.2 (CH). Tlc, R_f = 0.40. Anal $C_{16}H_{14}N_2O_5S$ (C, H, N).

N^4-(2,3-Dihydroxy-5-formylbenzylidene) sulfanilamide ***6m.*** Orange crystals, mp 230 °C (dec); this is a new compound. IR (KBr), cm^{-1}: 1130, 1350, 1640, 1670 (CH=O); UV (MeOH) λ_{max}, nm, (log ε): 269 (4.16), 311 (4.07), 477 (2.82); with NaOH: 289 (4.15), 337 (4.24), 393 (3.80). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.23–7.37 (m, 2H, ArH from C_6H_2); 7.60 (br s, 2H, NH_2); 8.16 (s, 1H, CH from azomethine); 8.21 (s, 1H, CH from aldehyde). ^{13}C-NMR δ, ppm: 118.1, 118.9, 119.5, 129.5, 138.4 (all signals of C_6H_2); 114.8, 131.7, 135.9, 153.8 (all from -C_6H_4-); 178.3 (CH from azomethine); 182.5 (CH from aldehyde). Tlc, R_f = 0.50. Anal $C_{14}H_{12}N_2O_5S$ (C, H, N).

N^4-(2-Hydroxy-3-methoxy-5-formylbenzylidene) sulfanilamide ***6n.*** Red crystals, mp >242 °C; this is a new compound. IR (KBr), cm^{-1}: 1140 (OMe); 1150, 1330, 1620, 1680 (CH=O); UV (MeOH) λ_{max}, nm, (log ε): 267 (4.18), 310 (4.05), 482 (2.96); with NaOH: 289 (4.25), 339 (4.32), 395 (3.74). ^{1}H-NMR (DMSO-d_6) δ, ppm: 3.90 (s, 3H, MeO); 7.05 (m, AA'BB', 4H, ArH from the phenylene); 7.23–7.36 (m, 2H, ArH from C_6H_2); 7.60 (br s, 2H, NH_2); 8.15 (s, 1H, CH from azomethine); 8.21 (s, 1H, CH from aldehyde). ^{13}C-NMR δ, ppm: 30.5 (MeO); 118.1, 118.8, 119.5, 129.5, 138.4 (all signals of C_6H_2); 114.8, 131.7, 135.9, 153.8 (all from -C_6H_4-); 178.2 (CH from azomethine); 182.5 (CH from aldehyde). Tlc, R_f = 0.57. Anal $C_{15}H_{14}N_2O_5S$ (C, H, N).

N^4-(3,4,5-Trimethoxybenzylidene) sulfanilamide ***6o.*** Pale yellow crystals, mp 221–222 °C; this is a new compound. IR (KBr), cm^{-1}: 1120 (C–OMe); 1150, 1340 (sulfonamido vibrations); 1620; UV (MeOH) λ_{max}, nm, (log ε): 233 (4.34), 319 (4.31); with NaOH: 232 (4.38), 319 (4.34); ^{1}H-NMR (DMSO-d_6) δ, ppm: 3.91 (s, 3H, 4-MeO); 3.99 (s,6H, 3,5-MeO); 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.29 (s, 2H, ArH from C_6H_2); 7.61 (br s, 2H, NH_2); 8.15 (s, 1H, CH). ^{13}C-NMR δ, ppm: 29.5, 29.9 (Me); 118.1, 119.0, 128.9, 135.4 (all signals of C_6H_2); 114.8, 131.6, 135.9, 153.7 (all from -C_6H_4-); 178.4 (CH). Tlc, R_f = 0.38. Anal $C_{16}H_{14}N_2O_5S$ (C, H, N).

N^4-(5-Bromo-4-hydroxy-3-methoxybenzylidene) sulfanilamide ***6p.*** Pale yellow crystals, mp 202–203 °C (dec); this is a new compound. IR (KBr), cm^{-1}: 1150, 1320, 1620; UV (MeOH) λ_{max}, nm, (log ε): 236 (4.30), 328 (4.28), 460 (2.73); with NaOH: 233 sh (4.28), 260 sh (4.10), 383 (4.49). ^{1}H-NMR (DMSO-d_6) δ, ppm: 3.90 (s, 3H, Me); 7.05 (m, AA'BB', 4H, ArH from the phenylene of sulfanilamide); 7.28 (s, 2H, ArH from C_6H_2); 7.64 (br s, 2H, NH_2); 8.15 (s, 1H, CH); 8.80 (br s, 1H, OH). ^{13}C-NMR δ, ppm: 29.5 (Me); 118.7, 118.8, 120.9, 129.8, 137.9 (all signals of C_6H_2); 114.9; 131.7, 135.8, 153.8 (all from -C_6H_4-); 178.5 (CH). Tlc, R_f = 0.53. Anal $C_{14}H_{11}N_2O_4SBr$ (C, H, N).

N^4-(2-Furfurylidene) sulfanilamide ***7a.*** White crystals, mp 183–184 °C; lit mp 196 °C [23]. IR (KBr), cm^{-1}: 1150, 1320, 1630; UV (MeOH) λ_{max}, nm, (log ε): 229 (3.83); 327 (4.21); with NaOH: 229 (4.01), 327 (4.39). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.19–7.46 (m, 3H, ArH from furyl); 7.66 (br s, 2H, NH_2); 8.20 (s, 1H, CH). ^{13}C-NMR δ, ppm: 134.1, 138.9, 139.8,140.2 (all signals of furyl moiety); 114.9, 131.7, 135.4, 153.7 (all from -C_6H_4-); 178.9 (CH). Tlc, R_f = 0.42. Anal $C_{11}H_{10}N_2O_3S$ (C, H, N).

N^4-(5-Methyl-2-furfurylidene) sulfanilamide ***7b.*** White crystals, mp 208–209 °C (dec). IR (KBr), cm^{-1}: 1120 (COC); 1150, 1330, 1630; UV (MeOH) λ_{max}, nm, (log ε): 229 (3.87), 327 (4.26); with NaOH: 229 (4.02), 327 (4.42). ^{1}H-NMR (DMSO-d_6) δ, ppm: 2.50 (s, 3H, Me); 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.34 (s, 2H, ArH from furyl); 7.65 (br s, 2H, NH_2); 8.20 (s, 1H, CH). ^{13}C-NMR δ, ppm: 25.7 (Me); 134.3, 138.6, 139.8, 140.2 (all signals of furyl moiety); 114.8, 131.7, 135.4, 153.7 (all from -C_6H_4-); 178.9 (CH). Tlc, R_f = 0.48. Anal $C_{12}H_{12}N_2O_3S$ (C, H, N).

N^4-(2-Pyrolylidene) sulfanilamide ***7c.*** Pale yellow crystals, mp 193–194 °C. IR (KBr), cm^{-1}: 1150, 1330, 1630; UV (MeOH) λ_{max}, nm, (log ε): 229 (3.87), 329 (4.25); with NaOH: 229 (4.06), 327 (4.44). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.23–7.49 (m, 3H, ArH from pyrolyl); 7.66 (br s, 2H, NH_2); 8.20 (s, 1H, CH); 8.35 (br s, 1H, NH). ^{13}C-NMR δ, ppm: 134.7, 136.5, 138.8, 138.9 (all signals of pyrolyl moiety); 114.9, 131.6, 135.4, 153.9 (all from -C_6H_4-); 180.5 (CH). Tlc, R_f = 0.32. Anal $C_{11}H_{11}N_3O_2S$ (C, H, N).

N^4-(4(5)-Imidazolylidene) sulfanilamide ***7d.*** Yellow crystals, mp 208–210 °C. IR (KBr), cm^{-1}: 1140, 1330, 1630; UV (MeOH) λ_{max}, nm, (log ε): 230 (3.91), 329 (4.34); with NaOH: 229 (4.11), 327 (4.28). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.33–7.56 (m, 2H, ArH from imidazolyl); 7.65 (br s, 2H, NH_2); 8.20 (s, 1H, CH); 8.39 (br s, 1H, NH). ^{13}C-NMR δ, ppm: 137.8, 138.5, 142.9 (all signals of imidazolyl moiety); 114.9, 131.7, 135.4, 153.8 (all from -C_6H_4-); 180.1 (CH). Tlc, R_f = 0.29. Anal $C_{10}H_{10}N_4O_2S$ (C, H, N).

N^4-(2-Pyridylidene) sulfanilamide ***7e.*** Pale yellow crystals, mp 189–190 °C. IR (KBr), cm^{-1}: 1130, 1330, 1630; UV (MeOH) λ_{max}, nm, (log ε): 229 (4.05), 329 (4.29); with NaOH: 229 (4.06), 327 (4.44). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.26–7.44 (m, 4H, ArH from pyridyl); 7.65 (br s, 2H, NH_2); 8.22 (s, 1H, CH). ^{13}C-NMR δ, ppm: 121.7, 130.1, 136.2, 138.5, 138.9 (all signals of pyridyl moiety); 114.8, 131.6, 135.3, 153.9 (all from -C_6H_4-); 180.2 (CH). Tlc, R_f = 0.39. Anal $C_{12}H_{11}N_3O_2S$ (C, H, N).

N^4-(3-Pyridylidene) sulfanilamide ***7f.*** Pale yellow crystals, mp 190–193 °C. IR (KBr), cm^{-1}: 1130, 1320, 1630; UV (MeOH) λ_{max}, nm, (log ε): 229 (4.00), 329 (4.28); with NaOH: 229 (4.07), 327 (4.39). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.26–7.40 (m, 4H, ArH from pyridyl); 7.65 (br s, 2H, NH_2); 8.20 (s, 1H, CH). ^{13}C-NMR δ, ppm: 123.8, 129.5, 137.1, 138.0, 138.9 (all signals of pyridyl moiety); 114.7, 131.7, 135.3, 153.9 (all from -C_6H_4-); 179.8 (CH). Tlc, R_f = 0.38. Anal $C_{12}H_{11}N_3O_2S$ (C,H, N).

N^4-(4-Pyridylidene) sulfanilamide ***7g.*** Pale yellow crystals, mp 198–201 °C. IR (KBr), cm^{-1}: 1130, 1320, 1630; UV (MeOH) λ_{max}, nm, (log ε): 229 (4.10), 329 (4.33); with NaOH: 229 (4.07), 327 (4.41). ^{1}H-NMR (DMSO-d_6) δ, ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.34 (m, AA'BB', 4H, ArH from pyridyl); 7.65 (br s, 2H, NH_2); 8.21 (s, 1H, CH). ^{13}C-NMR δ, ppm: 121.5, 129.9, 138.8 (signals of pyridyl moiety); 114.5, 131.7, 135.3, 153.9 (from -C_6H_4-); 179.9 (CH). Tlc, R_f = 0.42. Anal $C_{12}H_{11}N_3O_2S$ (C,H,N).

Determination of pK_a values

The acidity constants were determined spectrophotometrically by the method of Robinson and Pekrul [35], in aqueous ethanol (30%, v/v), at 25 °C. Standardized 0.1 N solutions of NaOH or HCl were used for preparing solutions of sulfonamides at different pH values, and the electronic spectra were registered

thereafter with a Cary 3 instrument. pH was measured with a glass microelectrode.

Pharmacology

Assay of CA inhibition

Inhibitors were assayed by Maren's micromethod [36], at 0 °C, in the conditions of the E–I (enzyme–inhibitor) technique. Stock solutions of inhibitor (1 mM) were prepared in distilled deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations [2, 4]) and dilutions up to 10 nM were prepared thereafter with distilled deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow the formation of the E–I complex [26]. In a special CO_2 bubbler cell, 0.3 mL distilled water was added, followed by 0.4 mL phenol red indicator solution (1%) and 0.1 mL inhibitor plus 0.1 mL CA solution, preincubated as mentioned above. The CA concentrations were 1.5 nM for CA II, 235 nM for CA I and 3 nM for CA IV. The hydration reaction was initiated by addition of 0.1 mL barbital buffer (pH 7.5), and the time to obtain a color change was recorded with a stopwatch. Enzyme-specific activity in the presence and in the absence of inhibitors, as well as K_i values, were determined as described by Maren [2, 40].

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