

## Carbonic anhydrase inhibitors. Part 43. Schiff bases derived from aromatic sulfonamides: towards more specific inhibitors for membrane-bound versus cytosolic isoforms

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**Summary** — Schiff bases were prepared by reaction of sulfanilamide, homosulfanilamide and *p*-aminoethyl-benzenesulfonamide with substituted benzene- and heterocyclic aldehydes. The compounds were characterized by standard procedures and were assayed as inhibitors of three isoforms of carbonic anhydrase (CA). Several of these new compounds showed a modest two-fold selectivity for the membrane-bound (bovine) isozyme, CA IV (bCA IV) as compared to the cytosolic human isoforms hCA I and II, in contrast to classical inhibitors which are 17–33 times less effective against bCA IV. This greater selectivity toward bCA IV is due mainly to a decreased potency against hCA II relative to classical inhibitors. This type of compound might lead to the development of low molecular weight isozyme specific CA IV inhibitors.

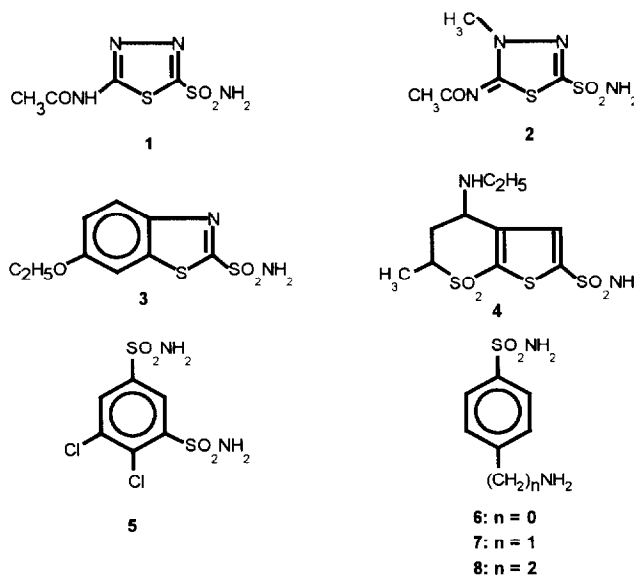
carbonic anhydrase / isoforms I, II, IV / aromatic sulfonamides / Schiff base

### Introduction

Sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors are firmly established in clinical medicine [2–4], being used in the prevention and/or treatment of a variety of disorders such as glaucoma [5, 6], epilepsy and other neurologically abnormal states [7], gastro-duodenal ulcers [8], osteoporosis [9] and acute mountain sickness [10].

Heterocyclic sulfonamides such acetazolamide **1** [2], methazolamide **2** [11], ethoxzolamide **3** [12] or dorzolamide **4** [6, 13] have been used clinically for more than 40 years [2] (the first inhibitor, acetazolamide, was introduced in clinical medicine in 1956, whereas dorzolamide was introduced in 1995 for the topical treatment of glaucoma [6]). Aromatic compounds such as dichlorophenamide **5** or sulfanilamide **6** besides possessing clinical applications have also constituted a lead for the development of other important classes of pharmacological agents: the thiazide

and high-ceiling diuretics [14, 15] and the antimicrobial sulfonamides [16].

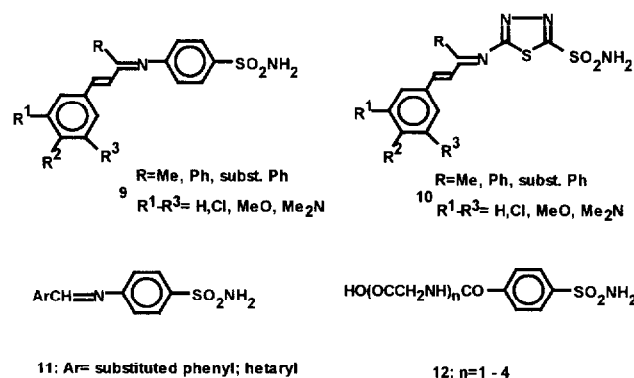


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Although aromatic sulfonamides are generally 10–100 times weaker CA inhibitors than the heterocyclic derivatives [2–4], their interest as CA inhibitors has recently been revived due to a major drawback regarding the classical type 1–6 inhibitors, ie, their lack of selectivity towards the numerous CA isozymes isolated in vertebrates [2–4, 17]. Nine CA isozymes have been described in this phylum [17]. They possess different capacities for catalyzing CO<sub>2</sub> hydration to bicarbonate (the physiological reaction in which these enzymes participate), but generally also have a high affinity for all the clinically used sulfonamide type 1–6 inhibitors mentioned above. This presents a challenge since, for many of the newly discovered isozymes, the real physiological role is largely unknown, and selective inhibition studies would presumably allow their role to be assessed [3]. In addition, isozyme-selective inhibitors may constitute improved drugs, without side-effects induced by inhibition of the enzyme in other tissues/organs [3, 7]. Sulfanilamide 6 is the first compound for which CA inhibitory properties have been reported [18], but its structurally related type 7 and 8 congeners have remained largely unstudied [3, 4]. Only recently have a large number of derivatives of heterocyclic as well as aromatic sulfonamides been prepared and assayed for the selective inhibition of different CA isozymes by ourselves and other groups [3, 4, 19–22].

Thus, Schiff bases of type 9–11, derivatives of aromatic as well as heterocyclic sulfonamides, have recently been reported by ourselves [19, 20], with the unexpected discovery that some of these derivatives are slightly more efficient in inhibiting the membrane-bound isozyme CA IV as compared to the cytosolic major isozymes CA I and II. Jain et al's group [21], on the other hand, reported type 12 inhibitors, derivatives of *p*-carboxybenzenesulfonamide, which showed competitive behavior with CO<sub>2</sub> as substrate (all other sulfonamides act as non-competitive inhibitors with the physiological substrate of the enzyme [2–4]) and relatively high affinities for CA I, an enzyme not as prone to inhibition by sulfonamides as compared to CA II or CA IV. Mention should be made that in secretory tissues and red blood cells, CA II and CA IV are considered to be responsible for physiological functions [3, 6], whereas the role of CA I, a very abundant protein in erythrocytes, is largely unknown [2, 3].

Among the recently prepared inhibitors [19, 20], the Schiff bases obtained from sulfanilamide 6 and type 11 aromatic/heterocyclic aldehydes showed the best ratio of increased activity towards CA IV versus CA II (in contrast to the classical type 1–6 inhibitors which are generally 17–33 times more potent inhibitors of CA II than CA IV [19, 23]). This exciting finding prompted us to extend the series of this type of



inhibitors. Here we report the synthesis and characterization of novel Schiff bases prepared from sulfanilamide 6, homosulfanilamide 7 or *p*-aminoethylbenzenesulfonamide 8 and aromatic/heterocyclic aldehydes. The prepared derivatives were tested for inhibition against three CA isozymes, namely CA I, II and IV. Some of the new derivatives showed a higher affinity for CA IV than for CA II as compared to classical inhibitors as well as the previously reported compounds 11, confirming that this research line might lead to low molecular weight isozyme-specific CA inhibitors.

## Results

Reaction of aromatic sulfonamides 6–8 with aromatic or heterocyclic aldehydes afforded the 21 Schiff bases 13 and 14 shown in table I, together with their inhibition data against isozymes CA I, II and IV. All the derivatives reported here are new compounds. They were characterized by standard procedures (IR, <sup>1</sup>H-NMR spectroscopy, elemental analysis), which confirmed the proposed structures.

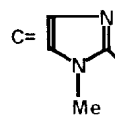
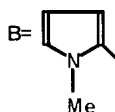
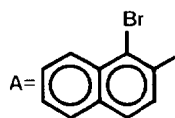
For comparison, inhibition data on the above-mentioned three isozymes with the standard inhibitors 1–5 as well as sulfonamides 6–8 are shown in table II.

The new inhibitors were assayed against the above-mentioned CAs due to the fact that CA I, II and IV are the most abundant of the nine isozymes so far isolated in vertebrates [3, 6, 17] on the one hand; and on the other, the high activity forms CA II and CA IV are considered to play a key physiological role in many tissues in which CA activity is present [2, 3, 6]. Although CA I is the second most abundant protein in blood (after serum albumin), its physiological role remains unknown [2]. However, this isozyme may be largely inhibited (around 85%) by the inorganic anions (such as chloride or phosphate) present in the plasma [2, 3]. Although CA II, one of the most power-

**Table I.** Schiff bases **13**, **14** prepared by reaction of aromatic aldehydes with sulfonamides **6–8** and their inhibition data against isozymes CA I, II and IV.

Compound	<i>n</i>	<i>R</i> <sup>1</sup>	<i>R</i> <sup>2</sup>	<i>R</i> <sup>3</sup>	<i>R</i> <sup>4</sup>	<i>R</i>	CA I <sup>b</sup> (μM)	<i>K</i> <sub>i</sub> <sup>a</sup> CA II <sup>b</sup> (× 10 <sup>8</sup> M)	CA IV <sup>c</sup> (× 10 <sup>8</sup> M)
<b>13a</b>	0	Cl	H	H	H	—	13 ± 0.5	29 ± 1.7	26 ± 2.0
<b>13b</b>	0	OMe	H	H	H	—	42 ± 2.1	17 ± 0.8	21 ± 1.4
<b>13c</b>	1	Cl	H	H	H	—	10 ± 0.5	9 ± 0.3	10 ± 0.2
<b>13d</b>	1	H	H	OMe	H	—	11 ± 0.6	12 ± 1.1	7 ± 0.4
<b>13e</b>	1	H	H	NMe <sub>2</sub>	H	—	8 ± 0.4	7 ± 0.2	9 ± 0.6
<b>13f</b>	1	H	H	AcNH	H	—	10 ± 0.4	11 ± 0.7	5 ± 0.2
<b>13g</b>	1	NO <sub>2</sub>	H	H	H	—	3 ± 0.1	30 ± 2.5	31 ± 0.9
<b>13h</b>	1	H	NO <sub>2</sub>	H	H	—	14 ± 0.8	18 ± 1.1	8 ± 0.6
<b>13i</b>	1	H	H	NO <sub>2</sub>	H	—	15 ± 0.7	14 ± 0.9	13 ± 0.2
<b>13j</b>	1	H	OMe	OMe	OMe	—	3 ± 0.1	2 ± 0.1	1 ± 0.1
<b>13k</b>	2	Cl	H	H	H	—	9 ± 0.5	21 ± 1.3	25 ± 1.0
<b>13l</b>	2	H	H	NMe <sub>2</sub>	H	—	5 ± 0.5	3 ± 0.2	5 ± 0.1
<b>13m</b>	2	H	H	AcNH	H	—	7 ± 0.1	4 ± 0.2	2 ± 0.2
<b>13n</b>	2	NO <sub>2</sub>	H	H	H	—	1 ± 0.1	25 ± 1.1	31 ± 0.9
<b>13o</b>	2	H	NO <sub>2</sub>	H	H	—	11 ± 0.5	15 ± 1.3	14 ± 0.4
<b>13p</b>	2	H	H	NO <sub>2</sub>	H	—	10 ± 0.2	2 ± 0.3	0.7 ± 0.1
<b>14a</b>	0	—	—	—	—	A	22 ± 0.9	7 ± 0.3	5 ± 0.1
<b>14b</b>	0	—	—	—	—	PhCH=CH—	24 ± 1.1	5 ± 0.2	6 ± 0.3
<b>14c</b>	0	—	—	—	—	B	2 ± 0.1	1 ± 0.1	3 ± 0.1
<b>14d</b>	1	—	—	—	—	C	2 ± 0.2	2 ± 0.1	1 ± 0.1
<b>14e</b>	2	—	—	—	—	C	2 ± 0.1	1 ± 0.1	0.8 ± 0.1

<sup>a</sup>Mean ± standard error (*n* = 2); <sup>b</sup>human (cloned) isozymes; <sup>c</sup>from bovine lung microsomes.



ful catalysts ever found in nature [24], is not as abundant as CA I; this high activity isozyme is virtually present in every cell [2, 3], where it catalyzes the hydration of CO<sub>2</sub> generated in metabolic processes. In many tissues (such as ciliary processes within the eye, gastric or pancreatic mucosa, kidneys, etc) it also participates in the processes of electrolyte secretion, generating H<sup>+</sup> or HCO<sub>3</sub><sup>-</sup> ions (the products of CO<sub>2</sub> hydration) [2, 3]. Recently [23] it has been shown that many processes in which CA II was thought to play a critical role are in fact modulated by CA IV activity.

This isozyme is membrane-bound, is very abundant in the lung, kidney, gastrointestinal tract and hepatocytes of vertebrates among others [3, 23, 25]. Like CA II, it is a high activity form. The two isozymes, unlike CA I, are quite sensitive to inhibition by sulfonamides [2, 3, 23, 25]. This former isozyme is in fact inhibited at sulfonamide concentrations 100–2500 times higher than those necessary for CA II, but it has a greater sensitivity to inorganic anions as compared to CA II or CA IV [2, 3, 26]. It is thus of considerable interest to investigate sulfonamide inhibitors that would react

**Table II.** Inhibition data of isozymes CA I, II and IV with the standard inhibitors **1–5** and the sulfonamides **6–8** used for the preparation of the new inhibitors.

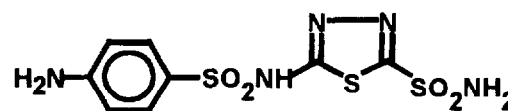
Inhibitor	CA I <sup>b</sup> ( $\mu M$ )	$K_i^a$ CA II <sup>b</sup> ( $\times 10^8 M$ )	CA IV <sup>c</sup> ( $\times 10^8 M$ )
Acetazolamide <b>1</b>	$0.2 \pm 0.01$	$0.7 \pm 0.01$	$12 \pm 0.9$
Methazolamide <b>2</b>	$0.01 \pm 0.005$	$0.8 \pm 0.04$	$14.5 \pm 1.1$
Ethoxzolamide <b>3</b>	$0.01 \pm 0.005$	$0.09 \pm 0.01$	$3.2 \pm 0.2$
Dorzolamide <b>4</b>	$> 500 \pm 10$	$0.18 \pm 0.02$	$3.0 \pm 0.1$
Dichlorophenamide <b>5</b>	$34 \pm 2.5$	$3.5 \pm 0.02$	$99 \pm 5.5$
Sulfanilamide <b>6</b>	$28 \pm 1.5$	$30 \pm 2.0$	$300 \pm 7.2$
Homosulfanilamide <b>7</b>	$25 \pm 0.9$	$17 \pm 0.9$	$280 \pm 10.5$
<i>p</i> -Aminoethylbenzenesulfonamide <b>8</b>	$21 \pm 1.4$	$18 \pm 1.15$	$245 \pm 12.6$

<sup>a</sup>Mean  $\pm$  standard error ( $n = 2$ ); <sup>b</sup>human (cloned) isozymes; <sup>c</sup>from bovine lung microsomes.

predominantly with one or the other of these physiologically important enzymes. For example, dorzolamide is highly selective for CA II relative to CA I, and only modestly selective over CA IV (table II).

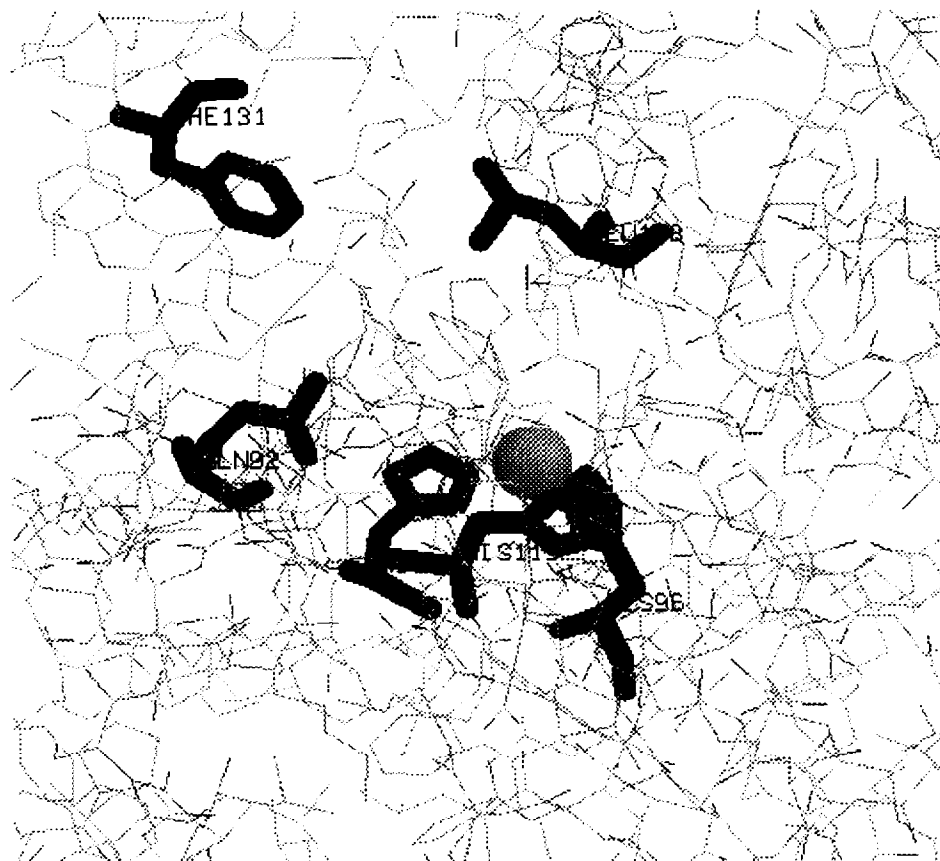
## Discussion

Except for the previously mentioned derivatives **9** and **11** recently reported by ourselves [19, 20] and four other derivatives reported by Beasley et al [27] in 1956, Schiff bases of aromatic sulfonamides have not been investigated as CA inhibitors by other researchers. All the previously investigated compounds were sulfanilamide derivatives. It was therefore of interest to synthesize some additional derivatives of this type (five such compounds are reported here, ie, **13a,b** and **14a–c**) as well as Schiff bases derived from homosulfanilamide **7** and *p*-aminoethyl-benzenesulfonamide **8**, taking into account the fact that theoretical QSAR calculations predicted that CA inhibitory properties would increase with the elongation of the inhibitor molecule in the direction of the axis intersecting the zinc ion of the enzyme and the sulfur atom of the sulfonamide [22, 28]. This was confirmed experimentally on a series of 1,3,4-thiadiazole-2-sulfonamide derivatives containing substituted pyridinium moieties in position 5 [22], as well as on a series of 20 non-congeneric sulfonamides (including among others derivatives **1–7**) in which the only structure common to all compounds was the sulfonamide moiety [28]. In addition, in the series of derivatives **12** reported by Jain et al [21], CA inhibitory properties gradually increased with *n* (compounds with  $n = 0, 1, 2, 3$  and 4 were prepared).

**15**

Examining the X-ray crystallographic structures available for several CA II-sulfonamide inhibitors [29–31], it is obvious that the increased stability of adducts, ie, higher binding energies, is associated with inhibitors possessing an elongated molecule due to supplementary favorable interactions with amino acid side chains within the active site cavity. For instance, aminobenzolamide **15**, a six-fold more potent inhibitor of human CA II compared to acetazolamide **1** [32], was shown to interact hydrophobically with the active site residues Phe 131 and Leu 198, and also to form a hydrogen bond with Gln 92 [30] (fig 1). These interactions were not seen for the acetazolamide–CA II complex [29], due to the fact that the shorter acetazolamide molecule is unable to interact with these groups of the enzyme.

Thus, in an attempt to obtain more specific CA IV inhibitors, the same approach as that for CA II was used, of elongating the molecule starting from the previously reported Schiff bases **11**, which already possessed good discriminatory properties towards the membrane-bound (CA IV) versus the cytosolic enzyme [19]. Practically, some of the **11** derivatives previously reported by this group [19] were the first examples of inhibitors having the same affinity for the two isozymes in contrast to the clinically used compounds **1–5**, which possessed 17–33 higher affinity for CA II as compared to CA IV (table II).



**Fig 1.** Detail of CA II active site with the Zn(II) ion and its three histidine ligands (His 94, His 96 and His 119) at the center. Residues important for assuring tight binding of sulfonamide inhibitors such as Gln 92, Phe 131 and Leu 198 are shown in black. The figure was generated using the program Raswin for Windows with a Texas Instruments 4000M PC. The X-ray crystallographic data are those of Hakansson et al [33], and were obtained from Brookhaven Protein Database via Internet (accession number 2CBA).

As seen from the data in table II, the 'longer' inhibitors homosulfanilamide **7** and *p*-aminoethylbenzenesulfonamide **8** are stronger inhibitors than sulfanilamide **6**, but like other sulfonamides **1–6**, they have lower affinities for CA IV as compared to CA II. It is thus clear that the molecular feature leading to equal affinity for the cytosolic and membrane-bound isozyme is connected with the presence of the Schiff base moiety in the inhibitor molecule. Since no Schiff bases for sulfonamides **7** and **8** have been described in the literature, we prepared a series of 16 such derivatives (table I).

Aldehydes used for the preparation of the new derivatives **13** and **14** were generally substituted benzaldehydes or heterocyclic derivatives, as noted in the previous work [19]. The only difference was that an aldehyde from the naphthalene series as well as

cinnamaldehyde (which is not an aromatic aldehyde) were also used for the preparation of Schiff bases in this study.

The following observations can be made regarding inhibition of isozymes CA I, II and IV with **13** and **14** (table I): i) potency in inhibiting all three isozymes generally increased with *n*, with the *p*-aminoethylbenzenesulfonamide derivatives being greater than the homosulfanilamide derivatives, which in turn were better inhibitors than the sulfanilamides bearing the same substitution pattern (compare also with data from [19], in which a larger series of sulfanilamide Schiff bases were assayed for the inhibition of these isozymes). This result is in fact in agreement with data inferred from X-ray crystallographic and QSAR studies mentioned above; ii) as shown in the previous study [19], the number of groups R<sup>1</sup>–R<sup>4</sup> substituting

the benzene nucleus of the aldehyde used to prepare the Schiff bases is not a very important parameter, their nature instead greatly influencing CA inhibitory properties. Again, derivatives of heterocyclic aldehydes **14c–e** are slightly more active than the aromatic ones [19], with no major differences between the latter and the naphthalenealdehyde or cinnamaldehyde derivatives **14a,b**. For the benzaldehyde derivatives **13**, substitution patterns leading to effective inhibitors were 3,4,5-trimethoxyphenyl (in **13j**) or nitrophenyl, with *p*- or *m*-nitrophenyl being much more active than the *o*-nitrophenyl moieties (compare **13g–i** and **13m–o**); iii) all Schiff bases are at least one order of magnitude more active CA IV inhibitors as compared to the parent sulfonamides from which they derive (**6–8**), a situation which is completely different in the case of isozymes CA I and II. Although some inhibitors **13**, **14** (such as **14a–c** or **13e,l**) are more potent than the corresponding parent sulfonamides the differences are small, and some of the Schiff bases are sometimes less active than sulfonamides **6–8** (for instance, **13b** for CA I; **13g, k, n** for CA II); iv) the most interesting feature of the new inhibitors is in some cases their discrimination in selectivity towards CA IV as compared to CA II, a fact first evidenced for the previously reported Schiff bases **11** [19]. Thus, 12 compounds of the 21 reported here, such as **13a, d, f, h–j, m, o, p, 14a, d, e** possess higher affinities for CA IV as compared to CA II, in some cases the differences of potency being relevant. For instance, **13p** is a 2.85 stronger, and **13j** and **14d** are two-fold stronger CA IV inhibitors as compared to CA II. This is up to now the highest ratio of CA IV versus CA II inhibition achieved with any sulfonamide inhibitor, and constitutes an encouraging result for the obtention of more selective, isozyme-specific CA inhibitors. It should also be mentioned that some of these compounds (**13p, 14e**) are very strong inhibitors, with potency comparable to that of acetazolamide for CA II, making them attractive candidates for further pharmacological evaluation.

## 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. <sup>1</sup>H-NMR spectra were recorded in DMSO-*d*<sub>6</sub> as solvent, with a Bruker CPX 300 or Varian EM 360L instrument. Chemical shifts are reported as  $\delta$  values, relative to Me<sub>4</sub>Si as internal standard. Elemental analysis was performed by combustion (for C, H, N) with a Carlo Erba automated analyzer (Milan, Italy). The values obtained were within  $\pm 0.4\%$  of the theoretical values calculated for the proposed formulae.

Sulfanilamide, homosulfanilamide, *p*-aminoethylbenzene-sulfonamide, acetazolamide and other sulfonamides used as standards in the enzymatic assays were from Sigma Chemical Co (St Louis, MO, USA). Aldehydes used in the synthesis were commercial reagents from Aldrich and Merck, and were used without additional purification. CA IV was isolated from bovine lung microsomes, as described by Maren et al [23].

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/HCA I and pACA/HCA II described by Forsman et al [34] (the two plasmids were a gift from S Lindskog, Umeå University, Sweden). Cell growth conditions were those described by Lindskog's group [35], and enzymes were purified by affinity chromatography according to the method of Khalifah et al [36]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM<sup>-1</sup>cm<sup>-1</sup> for CA I and 54 mM<sup>-1</sup>cm<sup>-1</sup> for CA II, respectively, based on *M<sub>r</sub>* = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively [37, 38].

**General procedure for the preparation of Schiff bases 13 and 14**  
An amount of 10 mmol sulfonamide **6–8** was dissolved in 40 mL boiling methanol and the required amount (10 mmol) of aldehyde was added to the reaction mixture. Boiling was continued for 3–8 h, then a portion of the solvent was evaporated in vacuum, and by cooling crystals of Schiff bases **13, 14** were obtained, which were recrystallized from 96% ethanol or solvents specified in other cases. Yields were generally high (see following text).

**N<sup>4</sup>-(2-Chlorobenzylidene)sulfanilamide 13a.** Pale yellow crystals (yield 68%), mp = 170–173 °C. IR (KBr), cm<sup>-1</sup>: 1140 (SO<sub>2</sub><sup>sym</sup>), 1320 (SO<sub>2</sub><sup>as</sup>), 1615 (C=N); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>),  $\delta$ , ppm: 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.13–7.35 (m, 4H, ArH from ClC<sub>6</sub>H<sub>4</sub>); 7.66 (br s, 2H, NH<sub>2</sub>); 8.17 (s, 1H, CH). Anal (C<sub>13</sub>H<sub>11</sub>N<sub>2</sub>ClO<sub>2</sub>S): C, H, N.

**N<sup>4</sup>-(2-Methoxybenzylidene)sulfanilamide 13b.** White crystals (yield 70%), mp = 191–194 °C. IR (KBr), cm<sup>-1</sup>: 1120 (OMe), 1150 (SO<sub>2</sub><sup>sym</sup>), 1330 (SO<sub>2</sub><sup>as</sup>), 1620 (C=N); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>),  $\delta$ , 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<sub>6</sub>H<sub>4</sub>); 7.62 (br s, 2H, NH<sub>2</sub>); 8.13 (s, 1H, CH). Anal (C<sub>14</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>S): C, H, N.

**4-(2-Chlorobenzylidene)homosulfanilamide 13c.** Pale yellow crystals (yield 53%), mp = 155–156 °C. IR (KBr), cm<sup>-1</sup>: 1130 (SO<sub>2</sub><sup>sym</sup>), 1310 (SO<sub>2</sub><sup>as</sup>), 1620 (C=N); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>),  $\delta$ , ppm: 4.90 (s, 2H, CH<sub>2</sub>); 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.50 (br s, 2H, NH<sub>2</sub>); 7.60–8.20 (m, 4H, ArH from ClC<sub>6</sub>H<sub>4</sub>); 8.90 (s, 1H, CH). Anal (C<sub>14</sub>H<sub>13</sub>N<sub>2</sub>ClO<sub>2</sub>S): C, H, N.

**4-(4-Methoxybenzylidene)homosulfanilamide 13d.** White crystals (yield 48%), mp = 176–177 °C. IR (KBr), cm<sup>-1</sup>: 1125 (OMe), 1150 (SO<sub>2</sub><sup>sym</sup>), 1330 (SO<sub>2</sub><sup>as</sup>), 1610 (C=N); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>),  $\delta$ , ppm: 3.80 (s, 3H, MeO); 4.90 (s, 2H, CH<sub>2</sub>); 7.05 (m, AA'BB', 4H, ArH from the phenylene of homosulfanilamide); 7.27–7.53 (m, 4H, ArH from MeOC<sub>6</sub>H<sub>4</sub>); 7.82 (br s, 2H, NH<sub>2</sub>); 8.85 (s, 1H, CH). Anal (C<sub>15</sub>H<sub>16</sub>N<sub>2</sub>O<sub>3</sub>S): C, H, N.

**4-(4-Dimethylaminobenzylidene)homosulfanilamide 13e.** Yellow crystals (yield 47%), mp = 187–189 °C. IR (KBr), cm<sup>-1</sup>: 1130 (SO<sub>2</sub><sup>sym</sup>), 1320 (SO<sub>2</sub><sup>as</sup>), 1580 (C=N); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>),  $\delta$ , ppm: 3.06 (s, 6H, 2Me); 4.80 (s, 2H, CH<sub>2</sub>); 7.05 (m, AA'BB', 4H, ArH from the phenylene of homosulfanilamide); 7.10–7.39 (m, 4H, ArH from Me<sub>2</sub>NC<sub>6</sub>H<sub>4</sub>); 7.65 (br s, 2H, NH<sub>2</sub>); 8.82 (s, 1H, CH). Anal (C<sub>16</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub>S): C, H, N.

**4-(4-Acetylaminobenzylidene)homosulfanilamide 13f.** Pale yellow crystals (yield 73%), mp = 245–246 °C. IR (KBr),  $\text{cm}^{-1}$ : 1140 ( $\text{SO}_2^{\text{sym}}$ ), 1320 ( $\text{SO}_2^{\text{as}}$ ), 1590 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 2.10 (s, 3H, Me); 4.80 (s, 2H,  $\text{CH}_2$ ); 7.05 (m, AA'BB', 4H, ArH from the phenylene of homosulfanilamide); 7.40 (br s, 2H,  $\text{NH}_2$ ); 7.50–7.95 (m, 4H, ArH from  $\text{AcNHC}_6\text{H}_4$ ); 8.52 (s, 1H, CH). Anal ( $\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}_3\text{S}$ ): C, H, N.

**4-(2-Nitrobenzylidene)homosulfanilamide 13g.** Yellow crystals (yield 53%), mp = 169–170 °C. IR (KBr),  $\text{cm}^{-1}$ : 1130 ( $\text{SO}_2^{\text{sym}}$ ), 1310 ( $\text{SO}_2^{\text{as}}$ ), 1330 ( $\text{NO}_2$ ); 1510 ( $\text{NO}_2$ ), 1590 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 4.86 (s, 2H,  $\text{CH}_2$ ); 7.15 (m, AA'BB', 4H, ArH from phenylene); 7.30 (br s, 2H,  $\text{NH}_2$ ); 7.58–8.16 (m, 4H, ArH from  $\text{O}_2\text{NC}_6\text{H}_4$ ); 8.79 (s, 1H, CH). Anal ( $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_4\text{S}$ ): C, H, N.

**4-(3-Nitrobenzylidene)homosulfanilamide 13h.** Yellow crystals (yield 60%), mp = 165–166 °C. IR (KBr),  $\text{cm}^{-1}$ : 1140 ( $\text{SO}_2^{\text{sym}}$ ), 1310 ( $\text{SO}_2^{\text{as}}$ ), 1340 ( $\text{NO}_2$ ), 1510 ( $\text{NO}_2$ ), 1640 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 4.97 (s, 2H,  $\text{CH}_2$ ); 7.15 (m, AA'BB', 4H, ArH from phenylene); 7.40 (br s, 2H,  $\text{NH}_2$ ); 7.52–8.48 (m, 4H, ArH from  $\text{O}_2\text{NC}_6\text{H}_4$ ); 8.60 (s, 1H, CH). Anal ( $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_4\text{S}$ ): C, H, N.

**4-(4-Nitrobenzylidene)homosulfanilamide 13i.** Yellow crystals (yield 53%), mp = 160–161 °C. IR (KBr),  $\text{cm}^{-1}$ : 1140 ( $\text{SO}_2^{\text{sym}}$ ), 1310 ( $\text{SO}_2^{\text{as}}$ ), 1340 ( $\text{NO}_2$ ); 1510 ( $\text{NO}_2$ ), 1595 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 4.97 (s, 2H,  $\text{CH}_2$ ); 7.15 (m, AA'BB', 4H, ArH from phenylene); 7.50 (br s, 2H,  $\text{NH}_2$ ); 7.60–8.48 (m, 4H, ArH from  $\text{O}_2\text{NC}_6\text{H}_4$ ); 8.70 (s, 1H, CH). Anal ( $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_4\text{S}$ ): C, H, N.

**4-(3,4,5-Trimethoxybenzylidene)homosulfanilamide 13j.** Pale yellow crystals, from MeOH (yield 54%), mp = 174 °C. IR (KBr),  $\text{cm}^{-1}$ : 1120 (C-OMe), 1150 ( $\text{SO}_2^{\text{sym}}$ ), 1320 ( $\text{SO}_2^{\text{as}}$ ), 1635 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 3.70 (s, 3H, 4-MeO), 3.80 (s, 6H, 3,5-MeO), 4.80 (s, 2H,  $\text{CH}_2$ ); 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.49–8.00 (s, 2H, ArH from  $\text{C}_6\text{H}_3$ ); 7.61 (br s, 2H,  $\text{NH}_2$ ); 8.50 (s, 1H, CH). Anal ( $\text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_5\text{S}$ ): C, H, N.

**4-(2-Chlorobenzylidene) aminoethylbenzenesulfonamide 13k.** Pale yellow crystals, from MeOH (yield 64%), mp = 173–174 °C. IR (KBr),  $\text{cm}^{-1}$ : 1140 ( $\text{SO}_2^{\text{sym}}$ ), 1320 ( $\text{SO}_2^{\text{as}}$ ), 1560 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 3.10 (t, 2H,  $\alpha\text{CH}_2$ ); 3.90 (t, 2H,  $\beta\text{CH}_2$ ); 7.10 (m, AA'BB', 4H, ArH from phenylene); 7.50 (br s, 2H,  $\text{NH}_2$ ); 7.60–8.20 (m, 4H, ArH from  $\text{ClC}_6\text{H}_4$ ); 8.60 (s, 1H, CH). Anal ( $\text{C}_{15}\text{H}_{15}\text{N}_2\text{ClO}_2\text{S}$ ): C, H, N.

**4-(4-Dimethylaminobenzylidene)aminoethylbenzenesulfonamide 13l.** Yellow crystals, from MeOH/DMF 1:1, v/v (yield 59%), mp = 225–226 °C. IR (KBr),  $\text{cm}^{-1}$ : 1155 ( $\text{SO}_2^{\text{sym}}$ ), 1320 ( $\text{SO}_2^{\text{as}}$ ), 1580 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 3.00 (s, 6H, 2Me); 3.10 (t, 2H,  $\alpha\text{CH}_2$ ); 3.70 (t, 2H,  $\beta\text{CH}_2$ ); 6.75 (br s, 2H,  $\text{NH}_2$ ); 7.10 (m, AA'BB', 4H, ArH from the phenylene of benzenesulfonamide); 7.30–7.90 (m, 4H, ArH from  $\text{Me}_2\text{NC}_6\text{H}_4$ ); 8.10 (s, 1H, CH). Anal ( $\text{C}_{17}\text{H}_{21}\text{N}_3\text{O}_2\text{S}$ ): C, H, N.

**4-(4-Acetylaminobenzylidene)aminoethylbenzenesulfonamide 13m.** Pale yellow crystals (yield 85%), mp = 255–256 °C. IR (KBr),  $\text{cm}^{-1}$ : 1140 ( $\text{SO}_2^{\text{sym}}$ ), 1310 ( $\text{SO}_2^{\text{as}}$ ), 1570 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 2.10 (s, 3H, Me); 3.00 (t, 2H,  $\alpha\text{CH}_2$ ); 3.80 (t, 2H,  $\beta\text{CH}_2$ ); 7.08 (m, AA'BB', 4H, ArH from the phenylene of benzenesulfonamide); 7.30 (br s, 2H,  $\text{NH}_2$ ); 7.50–8.10 (m, 4H, ArH from  $\text{AcNHC}_6\text{H}_4$ ); 8.20 (s, 1H, CH). Anal ( $\text{C}_{17}\text{H}_{19}\text{N}_3\text{O}_3\text{S}$ ): C, H, N.

**4-(2-Nitrobenzylidene)aminoethylbenzenesulfonamide 13n.** Yellow crystals, from *i*-PrOH (yield 88%), mp = 174–175 °C. IR (KBr),  $\text{cm}^{-1}$ : 1140 ( $\text{SO}_2^{\text{sym}}$ ), 1320 ( $\text{SO}_2^{\text{as}}$ ), 1340 ( $\text{NO}_2$ ); 1520 ( $\text{NO}_2$ ), 1570 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 3.00 (t, 2H,  $\alpha\text{CH}_2$ ); 3.90 (t, 2H,  $\beta\text{CH}_2$ ); 7.10 (m, AA'BB', 4H, ArH from phenylene); 7.30 (br s, 2H,  $\text{NH}_2$ ); 7.50–8.28 (m, 4H, ArH from  $\text{O}_2\text{NC}_6\text{H}_4$ ); 8.50 (s, 1H, CH). Anal ( $\text{C}_{15}\text{H}_{15}\text{N}_3\text{O}_4\text{S}$ ): C, H, N.

**4-(3-Nitrobenzylidene)aminoethylbenzenesulfonamide 13o.** Yellow crystals, from *i*-PrOH (yield 64%), mp = 151–152 °C. IR (KBr),  $\text{cm}^{-1}$ : 1130 ( $\text{SO}_2^{\text{sym}}$ ), 1310 ( $\text{SO}_2^{\text{as}}$ ), 1330 ( $\text{NO}_2$ ), 1520 ( $\text{NO}_2$ ), 1560 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 3.10 (t, 2H,  $\alpha\text{CH}_2$ ); 3.80 (t, 2H,  $\beta\text{CH}_2$ ); 7.20 (m, AA'BB', 4H, ArH from phenylene); 7.40 (br s, 2H,  $\text{NH}_2$ ); 7.42–8.30 (m, 4H, ArH from  $\text{O}_2\text{NC}_6\text{H}_4$ ); 8.50 (s, 1H, CH). Anal ( $\text{C}_{15}\text{H}_{15}\text{N}_3\text{O}_4\text{S}$ ): C, H, N.

**4-(4-Nitrobenzylidene)aminoethylbenzenesulfonamide 13p.** Yellow crystals, from MeOH (yield 71%), mp = 193–195 °C. IR (KBr),  $\text{cm}^{-1}$ : 1130 ( $\text{SO}_2^{\text{sym}}$ ), 1310 ( $\text{SO}_2^{\text{as}}$ ), 1330 ( $\text{NO}_2$ ); 1510 ( $\text{NO}_2$ ), 1590 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 3.10 (t, 2H,  $\alpha\text{CH}_2$ ); 3.95 (t, 2H,  $\beta\text{CH}_2$ ); 7.15 (m, AA'BB', 4H, ArH from phenylene); 7.40 (br s, 2H,  $\text{NH}_2$ ); 7.60–8.40 (m, 4H, ArH from  $\text{O}_2\text{NC}_6\text{H}_4$ ); 8.50 (s, 1H, CH). Anal ( $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_4\text{S}$ ): C, H, N.

**$\text{N}^4$ -(1-Bromo-2-naphthylidene)sulfanilamide 14a.** Pale yellow crystals (yield 70%), mp = 221–225 °C; IR (KBr),  $\text{cm}^{-1}$ : 1140 ( $\text{SO}_2^{\text{sym}}$ ), 1320 ( $\text{SO}_2^{\text{as}}$ ), 1610 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 7.40 (d, 2H, 8.5 part AA'BB' from phenylene); 7.66–7.74 (m, 2H,  $\text{H}^6$ ,  $\text{H}^7$  from naphthalene); 7.92 (d, 2H, 8.5 part AA'BB' from phenylene); 7.94 (m, 1H,  $\text{H}^5$  or  $\text{H}^8$  from the substituted naphthyl); 7.96 (d, 1H,  $\text{H}^3$  or  $\text{H}^4$  from the substituted naphthyl); 8.20 (d, 1H,  $\text{H}^4$  or  $\text{H}^5$  from the substituted naphthyl); 8.35 (dd, 9.7, 2.3, 1H,  $\text{H}^8$  or  $\text{H}^5$  from the substituted naphthyl); 9.06 (s, 1H, CH);  $^{13}\text{C-NMR}$ : 121.02 (CH); 124.45 (CH); 127.09 (CH); 127.20 (CH); 127.92 (CH); 128.20 (CH); 128.41 (CH); 128.61 (CH); 131.55 ( $\text{C}_q$ ); 131.69 ( $\text{C}_q$ ); 135.60 ( $\text{C}_q$ ); 141.63 ( $\text{C}_q$ ); 154.00 ( $\text{C}_q$ ); 161.35 (CH from the Schiff base moiety). Anal ( $\text{C}_{17}\text{H}_{13}\text{N}_2\text{BrO}_2\text{S}$ ): C, H, N.

**$\text{N}^4$ -(Cinnamylidene)sulfanilamide 14b.** Pale yellow crystals (yield 50%), mp = 202–204 °C; IR (KBr),  $\text{cm}^{-1}$ : 1130 ( $\text{SO}_2^{\text{sym}}$ ), 1330 ( $\text{SO}_2^{\text{as}}$ ), 1610 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 7.11 (dd, 15.9, 8.9, 1H, CH=CH-CH=N); 7.20 (d, 8.5, 2H, part AA'BB' from phenylene); 7.37 (d, 15.9, 1H, CH=CH-CH=N); 7.35–7.46 (m, 3H, ArH from Ph); 7.63 (dd, 8.1, 1.7, 2H, ArH from Ph); 7.84 (d, 8.5, 2H, part AA'BB' from phenylene); 8.35 (d, 8.9, 1H, CH=N);  $^{13}\text{C-NMR}$ : 120.82 (CH from phenylene); 126.92 (CH); 127.36 (CH); 127.53 (CH); 128.78 (CH); 129.67 (CH); 135.12 ( $\text{C}_q$ ); 140.94 ( $\text{C}_q$ ); 145.40 (CH); 154.35 ( $\text{C}_q$ ); 163.59 (CH from the Schiff base moiety). Anal ( $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_2\text{S}$ ): C, H, N.

**$\text{N}^4$ -(*N*-Methyl-2-pyrolylidene)sulfanilamide 14c.** Pale yellow crystals (yield 60%), mp = 201–204 °C. IR (KBr),  $\text{cm}^{-1}$ : 1150 ( $\text{SO}_2^{\text{sym}}$ ), 1330 ( $\text{SO}_2^{\text{as}}$ ), 1630 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 2.50 (s, 3H, Me); 7.05 (m, AA'BB', 4H, ArH from phenylene); 7.23–7.49 (m, 3H, ArH from pyrolyl); 6.28 (dd, 3.8, 2.5, 1H, from pyrolyl); 6.88 (dd, 3.8, 1.8, 1H, from pyrolyl); 7.16 (dd, 2.5, 1.8, 1H, from pyrolyl); 7.37 (d, 8.5, 2H, part AA'BB' from phenylene); 7.86 (d, 8.5, 2H, part AA'BB' from phenylene); 8.47 (s, 1H, CH from the Schiff base moiety);  $^{13}\text{C-NMR}$ : 36.18 (Me); 109.26 (CH from pyrolyl); 118.90 (CH); 121.35 (CH from pyrolyl); 127.18 (CH from phenylene); 129.94 ( $\text{C}_q$ ); 130.51 (CH); 140.16 ( $\text{C}_q$ ); 152.71 (CH from the Schiff base moiety); 155.50 ( $\text{C}_q$ ). Anal ( $\text{C}_{12}\text{H}_{13}\text{N}_3\text{O}_2\text{S}$ ): C, H, N.

**4-(N-1-Methyl-imidazol-2-ylidene)homosulfanilamide 14d.** White crystals (yield 73%), mp = 167–168 °C. IR (KBr),  $\text{cm}^{-1}$ : 1150 ( $\text{SO}_2^{\text{sym}}$ ), 1325 ( $\text{SO}_2^{\text{as}}$ ), 1645 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 4.00 (s, 3H, Me); 4.90 (s, 2H,  $\text{CH}_2$ ); 7.05 (m, AA'BB', 4H, ArH from the phenylene of homosulfanilamide); 7.50–7.95 (m, 2H, ArH from imidazolyl); 7.80 (br s, 2H,  $\text{NH}_2$ ); 8.50 (s, 1H, CH). Anal ( $\text{C}_{12}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$ ): C, H, N.

**4-(N-1-Methyl-imidazol-2-ylidene)aminoethylbenzenesulfonamide 14e.** White crystals (yield 72%), mp = 151–152 °C. IR (KBr),  $\text{cm}^{-1}$ : 1145 ( $\text{SO}_2^{\text{sym}}$ ), 1320 ( $\text{SO}_2^{\text{as}}$ ), 1645 (C=N);  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ),  $\delta$ , ppm: 3.10 (t, 2H,  $\alpha\text{CH}_2$ ); 3.95 (t, 2H,  $\beta\text{CH}_2$ ); 4.00 (s, 3H, Me); 7.00 (m, AA'BB', 4H, ArH from the phenylene of benzenesulfonamide); 7.50–7.95 (m, 2H, ArH from imidazolyl); 7.80 (br s, 2H,  $\text{NH}_2$ ); 8.20 (s, 1H, CH). Anal ( $\text{C}_{13}\text{H}_{16}\text{N}_4\text{O}_2\text{S}$ ): C, H, N.

### Pharmacology

#### Assay of CA inhibition

Inhibitors were assayed by Maren's micromethod [39] 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 carried out 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 for the formation of the E–I complex [2]. In a special  $\text{CO}_2$  bubbler cell, 0.3 mL of distilled water was added followed by 0.4 mL of phenol red indicator solution (1%) and (0.1 mL of inhibitor + 0.1 mL of CA solution, preincubated as mentioned above). The CA concentrations were 1.5 nM for CA II, 210 nM for CA I and 3.5 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 (the mean of two determinations) were determined as described by Maren. The standard error of this measurement was around 5–10% [15, 39].

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