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Carbonic anhydrase inhibitors. Part 36*. Inhibition of isozymes I and II with Schiff bases derived from chalkones and aromatic/heterocyclic sulfonamides

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Summary — A series of 27 Schiff bases was prepared by reaction of chalkones with sulfanilamide and 5-amino-1,3,4-thiadiazole-2-sulfonamide. The new compounds were characterized by analysis and standard physico-chemical methods. They possess good inhibitory properties towards isozymes **I** and **II** of carbonic anhydrase. Structure–activity effects in this series of inhibitors are also discussed.

sulfonamide / carbonic anhydrase / Schiff base / enzyme inhibition

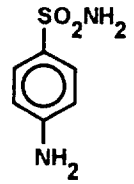
Introduction

Inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) [2, 3] are used in the clinical treatment of diverse diseases including glaucoma [4], gastroduodenal ulcers [5], acid-base disequilibria [6], and various neurological disorders [7]. A large number of CA isozymes are presently known (in vertebrates at least nine forms have been described [8]). The physiological function of many of them is not well understood [2, 3], and selective or isozyme-specific inhibitors are urgently needed, because they would allow the assessment of the physiological role of these isozymes.

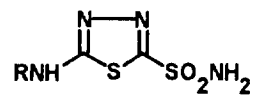
Although CA inhibition by sulfanilamide **1** has already been reported by Mann and Keilin [9], and subsequently other inhibitors have been developed for clinical use (such as acetazolamide **2** and thienothiopyransulfonamides of type **3**), little progress has been registered in the development of compounds with high specificity towards some isozymes. Thus, positively-charged sulfonamides of types **4** and **5**, with substituted pyridinium moieties, are membrane-impermeant and in this way inhibit only the membrane-bound isozyme CA IV [10–12]. In certain dosages, benzolamide **6** is a specific inhibitor for renal CA II [13], together with certain of its congeners of type **7** (X = NO_2, halogen, or a substituted-pyridinium moiety), recently developed by this group [14]. Such compounds constitute exceptions as they predominantly inhibit only a specific isozyme, whereas the great majority of sulfonamides have large affinities for all of them (although these affinities greatly differ among the different isozymes, with CA II being the most easy to inhibit, followed by CA IV, CA I, and CA III, which is the most resistant to inhibition by aromatic/heterocyclic sulfonamides [15]; data for other isozymes are not presently available [2]).

It is thus of critical importance to synthesize and assay CA inhibitors from diverse classes of substances, in order to detect compounds with specificity towards certain isozymes [16, 17]. Although a large number of aromatic/heterocyclic sulfonamides have been prepared in this regard [2, 18], Schiff bases had been relatively little studied. Thus, Beasley et al [19] prepared four derivatives of sulfanilamide and aromatic aldehydes of type **8**, and recently a much larger series of such compounds was reinvestigated by this group [1], interesting activities being detected for some inhibitors. We also reported the Schiff base **9** and some of its metal complexes, which possess very good inhibitory properties towards CA I [20]. Other approaches in which Schiff bases were intermediates but were not isolated include the synthesis of derivatives **11** by Pierce [21], by the reductive condensation

*For Part 35, see reference [1].

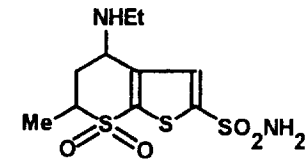


1



2 : R=Ac

10: R=H



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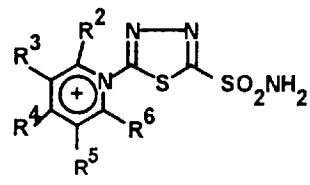

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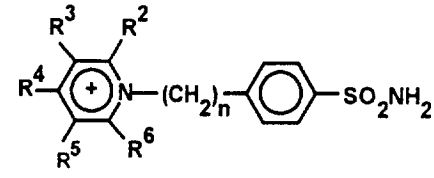
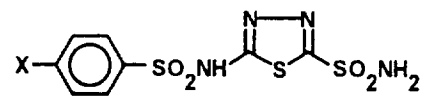


5 : n = 0, 1, 2

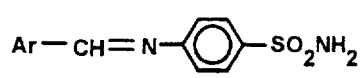


6 : X = H

7 : X = halogeno, NO_2 ; etc.

of 5-amino-1,3,4-thiadiazole-2-sulfonamide **10** with ketoacids (when Schiff bases of type **9** formed initially), and that of thieno[2,3-*b*]thiophene-2-sulfonamide derivatives **12**, reported by Prugh et al [22], in order to obtain topically-active inhibitors for use as possible antiglaucoma agents.

It therefore appeared to be of interest to prepare a large series of Schiff bases derived from aromatic



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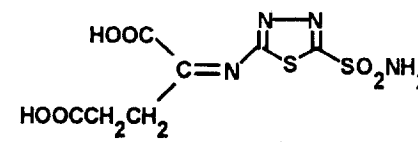

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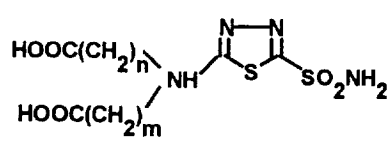
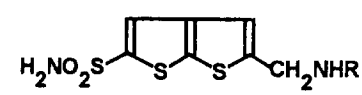


11 : n=0,1; m=1,2,3



12 : R=alkyl

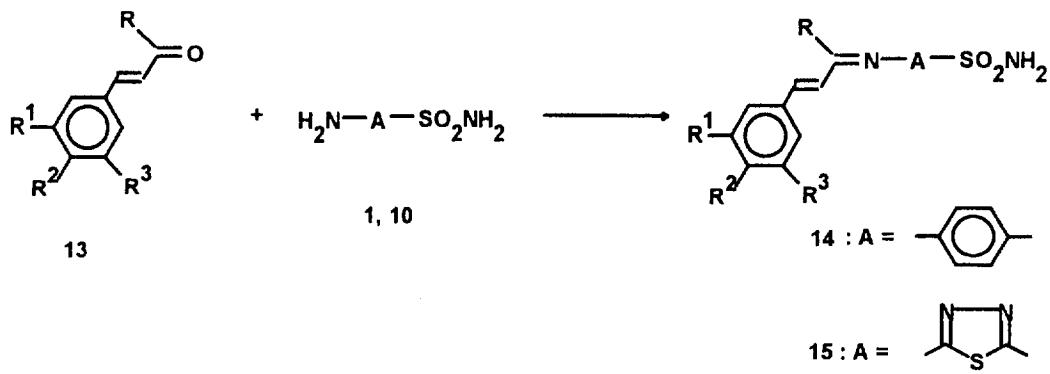
and/or heterocyclic sulfonamides, and to investigate their inhibitory properties towards the major CA isozymes. Thus far we have undertaken studies on CA I and CA II and work is in progress in this laboratory to test the prepared inhibitors against other CAs as well, such as CA III (from muscle), CA IV (from plasma membranes) and CA V (from mitochondrial matrix membrane). Our attention was focused on sulfanilamide as well as 1,3,4-thiadiazole-2-sulfonamide derivatives, as these ring systems are found in historically important CA inhibitors, such as sulfanilamide **1** and acetazolamide **2** [23]. In this paper we report the preparation, characterization and CA inhibitory properties of two series of Schiff bases, obtained from chalkones and sulfanilamide 1 or 5-amino-1,3,4-thiadiazole-2-sulfonamide **10** respectively. Structure–activity correlations for these inhibitors are also discussed.

Results

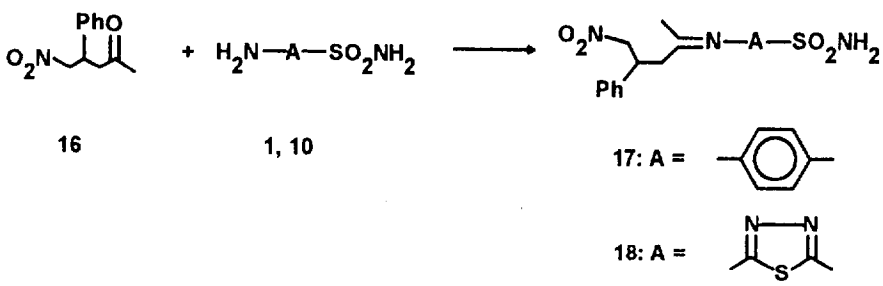
Reaction of substituted chalkones **13** [24] with sulfanilamide **1** or 5-amino-1,3,4-thiadiazole-2-sulfonamide **10** afforded Schiff bases of types **14** and **15** (scheme 1).

The prepared compounds **14** and **15** and their CA inhibitory properties are presented in tables I and II. Moieties present in the molecules of raw materials **13** included methoxy, dimethylamino and nitro, and were chosen in order to induce increased liposolubility (MeO) or higher hydrosolubility (NO_2, Me_2N), respectively, in the prepared CA inhibitors **14**, **15**. In addition, the substitution pattern also influences the pK_a of the sulfonamido group, which is an important parameter for the inhibition efficiency of CA inhibitors [2, 3].

Two more compounds **17**, **18** were prepared by a related procedure, in order to investigate this type of structure–activity correlation. 5-Nitro-4-phenyl-2-pentanone **16** was prepared [25] by Michael addition of sodium nitromethane to chalkone (benzylideneacetone). Its condensation with amines **1**, **10**, afforded the Schiff bases **17**, **18** respectively, as shown in scheme 2. In contrast to the inhibitors **14**, **15**, these last derivatives do not possess the double bond, but contain the nitro group which can further acidify the SO_2NH_2 protons, and thus contribute to increasing the CA inhibitory power.

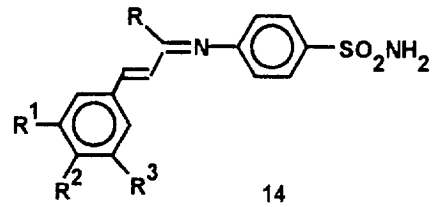


Scheme 1.



Scheme 2.

Table I. Schiff bases **14** derived from sulfanilamide and substituted chalkones prepared in this work and their inhibition data against CA I and CA II. For comparison, IC_{50} values of sulfanilamide against these two isozymes are 28 μM against CA I and 30×10^{-7} M against CA II (values from [2]).

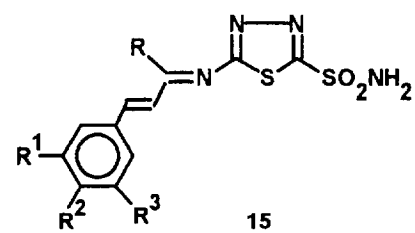


14

14	*R*	*R¹*	*R²*	*R³*	*CA I; IC_{50} ($\times 10^6$ M)*[a]	*CA II; IC_{50} ($\times 10^7$ M)*[b]
a	Me	H	H	H	14.5	5.4
b	Me	H	OMe	H	9.6	2.1
c	Me	H	NMe_2	H	0.8	1.3
d	Me	OMe	OMe	OMe	1.5	3.8
e	Ph	H	H	H	21.1	7.1
f	Ph	H	OMe	H	19.2	17.5
g	Ph	H	NMe_2	H	16.1	18.2
h	Ph	OMe	OMe	OMe	10.9	24.1
i	4-$MeOC_6H_4$	OMe	OMe	OMe	12.7	14.3
j	4-$MeOC_6H_4$	H	H	NO_2	6.5	11.4
k	4-$H_2NC_6H_4$	OMe	OMe	OMe	10.8	10.1
l	4-PhC_6H_4	OMe	OMe	OMe	25.2	27.0

[a]Human isozyme; [b]bovine isozyme; the IC_{50} values represent averages of at least two determinations which differed by less than 5%.

Table II. Schiff bases **15** prepared from 5-amino-1,3,4-thiadiazole-2-sulfonamide and substituted chalkones, and their IC_{50} data against isozymes CA I and CA II. For comparison, the corresponding values of acetazolamide **2** are given: 0.2 μM against CA I, and 0.7×10^{-8} M against CA II [2].



15

15	*R*	*R¹*	*R²*	*R³*	*CA I; IC_{50} ($\times 10^6$ M)*[a]	*CA II; IC_{50} ($\times 10^8$ M)*[b]
a	Me	H	H	H	1.7	1.5
b	Me	H	OMe	H	0.3	1.1
c	Me	H	NMe_2	H	0.1	0.1
d	Me	OMe	OMe	OMe	0.2	0.3
e	Ph	H	H	H	3.1	18.2
f	Ph	H	OMe	H	2.0	14.0
g	Ph	H	NMe_2	H	1.4	21.3
h	Ph	OMe	OMe	OMe	2.1	9.4
i	4-$MeOC_6H_4$	OMe	OMe	OMe	4.5	24.5
j	4-$MeOC_6H_4$	H	H	NO_2	0.9	8.2
k	4-$H_2NC_6H_4$	OMe	OMe	OMe	5.6	25.2
l	4-PhC_6H_4	OMe	OMe	OMe	14.1	38.6
m	4-$MeOC_6H_4$	H	NO_2	H	1.5	9.1

[a,b]See table I.

The newly synthesized compounds **14**, **15**, **17** and **18** were characterized by elemental analysis and spectroscopic methods (IR and ^{1}H-NMR), in order to confirm their structure (see the *Experimental protocols* for details). Some of the chalkones **13** are also new compounds and they have been characterized (see later in the text). These compounds were prepared by condensation of (substituted) benzaldehydes with ketones (acetone, acetophenone and substituted acetophenones), as described by Drake and Allen [24].

Discussion

The newly synthesized derivatives were characterized by standard procedures. Elemental analysis data were within ±0.4% of the theoretical values calculated for the proposed formulas (for C, H, N). In the IR spectra the following vibrations were detected: (i) the intense sulfonamido bands, at 1130–1180 cm^{-1} ($\nu^{sym}(SO_2)$) and 1300–1370 cm^{-1} ($\nu^{assym}(SO_2)$) for sulfanilamide derivatives **14**, **17**, and 1110–1180 cm^{-1} ($\nu^{sym}(SO_2)$) and 1310–1370 cm^{-1} ($\nu^{assym}(SO_2)$) respectively for thiadiazoles **15** and **18**; (ii) the Schiff base vibration, ν(C=N) at 1560–1610 cm^{-1}; and (iii) the NH_2 stretching vibration, as a broad band in the region 3100–3300 cm^{-1}. In the ^{1}H-NMR spectra, the signals of the aromatic protons of the sulfanilamide moiety were detected as an AA'BB' multiplet centered generally at 7.2 ppm (for compounds **14**), the complicated multiplet of the CH=CH protons was detected in the region 6.5–6.8 ppm, as well as the characteristic signals of other moieties present in these molecules (see the *Experimental protocols* for details).

From the data in tables I and II it can be seen that all of the prepared compounds possess good inhibitory properties towards both the CA isozymes investigated. The most important structural features which were correlated with the CA inhibitory activity of the synthesized derivatives (in addition to the moiety 'A', which was aromatic for the sulfanilamides **14** and heterocyclic for the thiadiazoles **15**) were the nature of groups R (resulting from the ketone used to prepare chalkones **13**) and the groups substituting the benzene ring of **13** (resulting from the aldehydes used in the preparation of chalkones [24]). As seen from the data in tables I and II, the inhibitors were generally much stronger in both series when R was methyl, than when R was a bulkier moiety (phenyl, substituted phenyl or biphenyl). On the other hand, R^1–R^3 moieties such as methoxy, dimethylamino or nitro led to stronger inhibitors compared to the corresponding unsubstituted derivatives (**14a** and **15a** respectively), for the two series of CA inhibitors prepared by us. Such groups are also important from a pharmacological point of view, because they can induce an increased lipophilicity (one or more methoxy groups), or higher hydrosolubility (dimethylamino or nitro) to these inhibitors, and it was proved that in order for a compound to act as a strong CA inhibitor, an appropriate balance between its hydro- and liposolubility must be attained [2–4]. This can certainly be correlated with the architecture of the CA active site [26, 27], which contains a hydrophobic half as well as a hydrophilic one. Thus, a good CA inhibitor must possess moieties which can interact both with hydrophobic and hydrophilic amino acid residues [2]. As seen from the data in tables I and II, the substitution pattern greatly modulated the CA inhibitory power of derivatives **14** and **15**. The number of groups R^1–R^3 does not seem to be a critical parameter, since mono- or trisubstituted compounds possessed similar CA inhibitory properties (compare, for instance, **14c** and **14d** against CA II, or **15c**, **15d** against CA I). Their nature is much more important; derivatives containing the dimethylamino moiety (and a methyl R group, because these were the strongest inhibitors), such as **14c** and **15c**, were the most potent CA I and CA II inhibitors in the series reported here. These were followed by the trimethoxy-substituted derivatives **14d** and **15d**, and then by the monomethoxy derivatives **14b** and **15b**. The other inhibitors prepared were all active but several general considerations may be drawn. Thus, Schiff bases **14** are all much stronger inhibitors than sulfanilamide, the parent compound from which they derive. Even the very bulky derivatives, such as the biphenyl-substituted compound **14l**, are slightly better inhibitors of CA I and CA II than sulfanilamide **1**. Probably this is also due to the fact that the active site of CA I is slightly larger than that of CA II [2, 26], and in this way the bulkier inhibitors are easier to accommodate. The same is not true for the thiadiazoles **15**, which with few exceptions are weaker inhibitors than acetazolamide **2** (but with comparable potencies), of both CA isozymes investigated here. Thus, in this series, only compounds **14b–d** have CA I and CA II inhibitory properties similar to those of acetazolamide, two of these compounds being in fact stronger CA II inhibitors than **2**. It is also important to note the fact that the heterocyclic derivatives **15** are almost an order of magnitude stronger CA inhibitors (towards both isozymes) than the substituted sulfanilamides **14**, a fact which has been known since the first years of research in the field of sulfonamide CA inhibitors [2, 3, 28]. Another point is that generally these inhibitors are 10–100 times more active against CA II than against CA I, which is a general characteristic of these types of sulfonamides [2, 29].

Thus, this series of CA inhibitors verifies a rule that we recently demonstrated [12] by means of QSAR calculations on a series of derivatives of type **4**, ie, that the linear dimensions of a CA inhibitor can be

extended only on two axes of the molecule. When the molecule becomes bigger in the third direction, the inhibitor becomes much weaker (as it is the case for those derivatives **14** and **15** which contain bulkier R moieties). On the other hand, increasing the size of groups only on one axis (elongating the molecule) leads to stronger CA inhibitors, as documented in the literature [12] and in the present study.

Derivatives **17** and **18** possessed good CA inhibitory properties. Thus, in the case of the sulfanilamide derivative **17**, IC_{50} values were 0.3 μM against CA I, and 1.5×10^{-7} M against CA II, whereas for the thiadiazole **18** the corresponding values were 0.1 μM and 0.2×10^{-8} M respectively, making the two inhibitors among the strongest in these series. This may be explained by the acidifying effect of the NO_2 groups on the sulfonamido protons, as well as by the stabilizing interaction of enzyme–inhibitor complexes, in which the nitro groups might be involved. Other compounds which contain a nitro group, ie, **14j** and **15j**, are again slightly stronger inhibitors than similarly substituted derivatives without the NO_2 element, such as **14i**, **15i**. As far as we know, a detailed study of CA inhibitors containing nitro groups has not been published, but we observed a similar effect on benzolamide-like derivatives of type **7** containing such a substitution [14].

In conclusion, in the present study we report the synthesis and characterization of 27 new sulfonamide inhibitors of the enzyme carbonic anhydrase, which we have tested for their action against two isozymes, CA I and CA II. Some of these compounds possessed interesting inhibitory properties towards both isozymes. Due to groups present in their molecule (such as the double bond, nitro and amino moieties), they can be further derivatized [30] in order to introduce other groups important for biological activity.

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 Beckman IR-4860 instrument. ^{1}H-NMR spectra were registered in DMSO-d_6 as solvent, with a Varian EM360L instrument, working at 60 MHz. Chemical shifts are reported as δ values, relative to Me_4Si 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 ±0.4% of the theoretical values calculated for the proposed formulae.

Sulfanilamide, acetazolamide, nitromethane, aldehydes and ketones (acetone, acetophenone and 4-substituted acetophenones), as well as solvents and inorganic compounds used in the syntheses, were commercial reagents from Sigma, Aldrich and Merck, and were used without additional purification. 5-Amino-1,3,4-thiadiazole-2-sulfonamide **10** was prepared from acetazolamide by acid hydrolysis in the presence of concentrated HCl, as described by Le Bar et al [30]. Bovine CA II and human CA I were from Sigma Chemical Co (Saint Louis, MO, USA).

Inhibitors were assayed by Maren's micromethod [31], at 0 °C, by the EI (enzyme–inhibitor) technique. IC_{50} values represent the molarity of inhibitor producing a 50% decrease of CA specific activity for the CO_2 hydration reaction, and are reported as the average of at least two determinations.

General procedure for preparation of chalkones 13

The appropriate ketone (22 mmol), 4 mmol of freshly distilled (substituted)benzaldehyde and 40 mL water were introduced into a three-necked reaction flask equipped with a mechanical stirrer and immersed thermometer. To this mixture, 10% NaOH solution (10 mL) was added dropwise, maintaining the temperature at 25–31 °C. The reaction mixture was then stirred at room temperature for 2–2.5 h. At the end of this time the solution was acidified with 5% HCl to pH 3. The layers which formed were separated, and the aqueous one was extracted with 20 mL benzene. The benzenic extract was added to the yellow oil originally separated, washed with 10 mL of water, dried, and the solvent evaporated in vacuo. The residue was distilled at 2–25 mmHg, when chalkones **13** were obtained with yields of 37–96%.

General procedure for preparation of Schiff bases 14, 15, 17 and 18

Sulfanilamide **1** (5 mmol) or 5-amino-1,3,4-thiadiazole-2-sulfonamide **10** (5 mmol) was suspended in 25 mL ethanol and an equimolar amount of chalkone **13** (dissolved in a small amount of the same solvent) added. The reaction mixture was refluxed for 5–8 h, then left overnight. The solvent was evaporated in vacuo, and the residue was recrystallized from methanol. Yields were of 27–97%.

Preparation of 5-nitro-4-phenyl-2-pentanone 16

This compound was prepared by the method of Kohler [25]. Benzylideneacetone **13a** (7 g) was dissolved in 15 mL methanol and the solution heated to 50 °C. Then sodium nitromethane (obtained from 3 g nitromethane and 1 g Na in the minimum amount of ethanol) was added with intense stirring. The solution, which became intensely orange, was cooled in a water ice bath and acidified with 3 g of glacial acetic acid, with magnetic stirring. The obtained precipitate was abundantly washed with water and methanol. For purification it was dissolved in chloroform, water was separated, the solvent was evaporated and the raw product was recrystallized from methanol. The yield was 48%, mp 99 °C (lit [24] mp 99–100 °C).

4-Phenyl-3-buten-2-ylidenesulfanilamide 14a. White crystals, mp 129–132 °C (yield of 64%). IR (KBr), cm^{-1}: 452, 531, 668, 715, 932, 1045, 1165, 1347, 1415, 1580, 3080, 3160. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.45 (s, 3H, Me); 5.70 (br s, 2H, SO_2NH_2); 6.20 (m, 2H, CH=CH); 6.4–8.2 (m, 9H, ArH). Analysis $C_{16}H_{16}N_2O_2S$ (C, H, N).

4-(4-Methoxyphenyl)-3-buten-2-ylidenesulfanilamide 14b. White crystals, mp 154–155 °C (yield of 79%). IR (KBr), cm^{-1}: 457, 534, 670, 714, 926, 1045, 1145, 1312, 1415, 1587, 3160. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.25 (s, 3H, Me); 3.71 (s, 3H, MeO); 5.70 (br s, 2H, SO_2NH_2); 6.60 (m, 2H, CH=CH); 6.8–8.2 (m, 8H, ArH). Analysis $C_{17}H_{18}N_2O_3S$ (C, H, N).

4-(4-Dimethylaminophenyl)-3-buten-2-ylidenesulfanilamide 14c. Yellow crystals, mp 107–110 °C (yield of 94%). IR (KBr), cm^{-1}: 465, 535, 670, 710, 932, 1010, 1140, 1310, 1423,

1580, 3260. ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.53 (s, 3H, Me); 3.00 (br s, 2H, SO_2NH_2); 3.40 (s, 6H, Me_2N); 6.30–7.50 (m, 10H, CH=CH + ArH). Analysis $C_{18}H_{21}N_3O_2S$ (C, H, N).

4-(3,4,5-Trimethoxyphenyl)-3-buten-2-ylidenesulfanilamide ***14d.*** White crystals, mp 147–148 °C (yield of 87%). IR (KBr), cm^{-1}: 460, 534, 685, 714, 924, 1050, 1140, 1310, 1415, 1590, 3200. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.20 (s, 3H, Me); 3.70 (s, 3H, 4-MeO); 3.90 (s, 6H, 3,5-$(MeO)_2$); 5.70 (br s, 2H, SO_2NH_2); 6.80–7.20 (m, 8H, CH=CH + ArH). Analysis $C_{19}H_{22}N_2O_5S$ (C, H, N).

1,3-Diphenyl-2-propen-1-ylidenesulfanilamide ***14e.*** White crystals, mp 154–156 °C (yield of 76%). IR (KBr), cm^{-1}: 542, 659, 710, 953, 1025, 1154, 1315, 1443, 1580, 3310. ^{1}H-NMR (DMSO-d_6), δ, ppm: 5.80 (br s, 2H, SO_2NH_2); 6.50 (m, 2H, CH=CH); 7.4–8.2 (m, 14H, ArH). Analysis $C_{21}H_{18}N_2O_2S$ (C, H, N).

1-Phenyl-3-(4-methoxyphenyl)-2-propen-1-ylidenesulfanilamide ***14f.*** White crystals, mp 171–173 °C (yield of 52%). IR (KBr), cm^{-1}: 462, 535, 670, 716, 935, 1050, 1147, 1310, 1420, 1580, 3165. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.70 (s, 3H, MeO); 5.75 (br s, 2H, SO_2NH_2); 6.60 (m, 2H, CH=CH); 7.3–8.2 (m, 13H, ArH). Analysis $C_{22}H_{20}N_2O_3S$ (C, H, N).

1-Phenyl-3-(4-dimethylaminoyphenyl)-2-propen-1-ylidene-sulfanilamide ***14g.*** Orange crystals, mp 111–113 °C (yield of 96%). IR (KBr), cm^{-1}: 462, 542, 671, 711, 935, 1020, 1150, 1310, 1420, 1580, 3310. 1H-NMR (DMSO-d_6), δ, ppm: 3.20 (s, 6H, Me2N); 5.80 (br s, 2H, SO_2NH_2); 6.60 (m, 2H, CH=CH); 7.0–8.0 (m, 13H, ArH); analysis $C_{23}H_{230}N_3O_2S$ (C, H, N).

1-Phenyl-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidenesulfanilamide ***14h.*** Pale yellow crystals, mp 120 °C (yield of 97%). IR (KBr), cm^{-1}: 465, 537, 692, 717, 925, 1010, 1130, 1300, 1425, 1580, 3240. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (br s, 2H, SO_2NH_2); 3.70 (s, 3H, 4-MeO); 3.90 (s, 6H, 3,5-$(MeO)_2$); 7.30–8.10 (m, 13H, CH=CH + ArH). Analysis $C_{24}H_{24}N_2O_5S$ (C, H, N).

1-(4-Methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidenesulfanilamide ***14i.*** White crystals, mp 131–132 °C (yield of 78%). IR (KBr), cm^{-1}: 462, 543, 701, 721, 918, 1020, 1140, 1310, 1425, 1570, 3230. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.30 (s, 6H, 4-MeO); 3.80 (s, 6H, 3,5-$(MeO)_2$); 5.80 (br s, 2H, SO_2NH_2); 6.40–8.00 (m, 12H, CH=CH + ArH). Analysis $C_{24}H_{24}N_2O_5S$ (C, H, N).

1-(4-Methoxyphenyl)-3-(3-nitrophenyl)-2-propen-1-ylidenesulfanilamide ***14j.*** Pale yellow crystals, mp 125–126 °C (yield of 86%). IR (KBr), cm^{-1}: 471, 554, 710, 923, 1030, 1180, 1370, 1435, 1610, 3350. 1H-NMR (DMSO-d_6), δ, ppm: 3.90 (s, 3H, 4-MeO); 5.80 (br s, 2H, SO_2NH_2); 6.50–8.50 (m, 14H, CH=CH + ArH). Analysis $C_{21}H_{19}N_3O_5S$ (C, H, N).

1-(4-Aminophenyl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidenesulfanilamide ***14k.*** Yellow crystals, mp 78–80 °C (yield of 91%). IR (KBr), cm^{-1}: 459, 554, 720, 742, 985, 1015, 1140, 1320, 1475, 1560, 3250. ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.30 (s, 2H, NH_2); 3.20 (br s, 2H, SO_2NH_2); 3.60 (s, 3H, 4-MeO); 3.80 (s, 6H, 3,5-$(MeO)_2$); 7.00–8.00 (m, 12H, CH=CH + ArH). Analysis $C_{23}H_{24}N_3O_5S$ (C, H, N).

1-(Biphen-4-yl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidenesulfanilamide ***14l.*** White crystals, mp 124–125 °C (yield of 78%). IR (KBr), cm^{-1}: 454, 551, 710, 721, 927, 1015, 1140, 1330, 1430, 1580, 3360. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (s, 6H, 4-MeO); 3.60 (s, 6H, 3,5-$(MeO)_2$); 5.80 (br s, 2H, SO_2NH_2); 6.90–8.00 (m, 17H, CH=CH + ArH). Analysis $C_{30}H_{28}N_2O_5S$ (C, H, N).

4-Phenyl-3-buten-2-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15a.*** White crystals, mp 159–162 °C (yield of 57%). IR (KBr), cm^{-1}: 505, 591, 673, 738, 939, 1033, 1122, 1202, 1380, 1580, 3160. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (s, 3H, Me); 5.70 (br s, 2H, SO_2NH_2); 7.2–8.0 (m, 7H, CH=CH + ArH). Analysis $C_{12}H_{12}N_4O_2S_2$ (C, H, N).

4-(4-Methoxyphenyl)-3-buten-2-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15b.*** White crystals, mp 164–166 °C (yield of 65%). IR (KBr), cm^{-1}: 510, 598, 677, 742, 943, 1027, 1120, 1211, 1370, 1590, 3175. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (s, 3H, Me); 3.70 (s, 3H, MeO); 5.50 (br s, 2H, SO_2NH_2); 7.2–7.9 (m, 6H, CH=CH + ArH). Analysis $C_{13}H_{14}N_4O_3S_2$ (C, H, N).

4-(4-Dimethylaminophenyl)-3-buten-2-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15c.*** Yellow crystals, mp 64–67 °C (yield of 84%). IR (KBr), cm^{-1}: 512, 605, 671, 734, 955, 1010, 1180, 1221, 1350, 1570, 3320. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.20 (s, 9H, Me + Me_2N); 3.50 (br s, 2H, SO_2NH_2); 7.0–8.0 (m, 6H, CH=CH + ArH). Analysis $C_{14}H_{18}N_5O_2S_2$ (C, H, N).

4-(3,4,5-Trimethoxyphenyl)-3-buten-2-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15d.*** White crystals, mp 87–90 °C (yield of 97%). IR (KBr), cm^{-1}: 513, 592, 665, 774, 948, 1015, 1120, 1211, 1320, 1590, 1640, 3100. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (s, 3H, Me); 3.70 (s, 3H, MeO); 3.80 (s, 6H, 3,5-$(MeO)_2$); 5.50 (br s, 2H, SO_2NH_2); 7.0–7.9 (m, 4H, CH=CH + ArH). Analysis $C_{15}H_{18}N_4O_5S_2$ (C, H, N).

1,3-Diphenyl-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15e.*** White crystals, mp 104–105 °C (yield of 66%). IR (KBr), cm^{-1}: 552, 688, 715, 975, 1025, 1161, 1372, 1598, 3305. 1H-NMR (DMSO-d_6), δ, ppm: 5.80 (br s, 2H, SO_2NH_2); 6.50 (m, 2H, CH=CH); 7.1–8.2 (m, 10H, ArH). Analysis $C_{17}H_{14}N_4O_2S_2$ (C, H, N).

1-Phenyl-3-(4-methoxyphenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15f.*** White crystals, mp 80–81 °C (yield of 65%). IR (KBr), cm^{-1}: 545, 659, 705, 977, 1020, 1170, 1370, 1605, 3300. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.80 (s, 3H, MeO); 5.90 (br s, 2H, SO_2NH_2); 6.50 (m, 2H, CH=CH); 7.0–8.3 (m, 9H, ArH). Analysis $C_{18}H_{16}N_4O_3S_2$ (C, H, N).

1-Phenyl-3-(4-dimethylaminophenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15g.*** Yellow crystals, mp 120 °C (yield of 97%). IR (KBr), cm^{-1}: 531, 650, 738, 975, 1010, 1150, 1226, 1340, 1570, 3370. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.10 (s, 6H, Me_2N); 3.40 (br s, 2H, SO_2NH_2); 7.0–8.0 (m, 11H, CH=CH + ArH). Analysis $C_{19}H_{20}N_5O_2S_2$ (C, H, N).

1-Phenyl-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15h.*** White crystals, mp 148 °C (yield of 80%). IR (KBr), cm^{-1}: 534, 652, 725, 977, 1010, 1120, 1330, 1595, 3270. 1H-NMR (DMSO-d_6), δ, ppm: 3.80 (s, 3H, MeO);3.90 (s, 6H, 3,5-$(MeO)_2$); 5.90 (br s, 2H, SO_2NH_2); 6.50 (m, 2H, CH=CH); 7.2–8.0 (m, 7H, ArH). Analysis $C_{20}H_{20}N_4O_5S_2$ (C, H, N).

1-(4-Methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15i.*** Pale yellow crystals, mp 72–73°C (yield of 90%). IR (KBr), cm^{-1}: 552, 650, 712, 970, 1010, 1170, 1360, 1600, 3300. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.80 (s, 6H, MeO); 3.90 (s, 6H, 3,5-$(MeO)_2$); 5.90 (br s, 2H, SO_2NH_2); 6.50–8.30 (m, 8H, CH=CH + ArH). Analysis $C_{21}H_{22}N_4O_6S_2$ (C, H, N).

1-(4-Methoxyphenyl)-3-(3-nitrophenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15j.*** Pale yellow crystals, mp 129–130 °C (yield of 86%). IR (KBr), cm^{-1}: 493, 575, 721, 962, 1030, 1180, 1320, 1350, 1485, 1610, 3350. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.80 (s, 3H, 4-MeO); 5.60 (br s, 2H, SO_2NH_2); 6.80–8.60 (m, 10H, CH=CH + ArH). Analysis $C_{17}H_{14}N_5O_5S_2$ (C, H, N).

1-(4-Aminophenyl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15k.*** Yellow-orange crystals, mp 145 °C (yield of 87%). IR (KBr), cm^{-1}: 495, 565, 720, 742, 998, 1015, 1110, 1310, 1475, 1580, 3270. ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.30 (s, 2H, NH_2); 3.10 (br s, 2H, SO_2NH_2); 3.60 (s, 3H, 4-MeO); 3.70 (s, 6H, 3,5-$(MeO)_2$); 7.00–8.00 (m, 8H, CH=CH + ArH). Analysis $C_{20}H_{21}N_5O_5S_2$ (C, H, N).

1-(Biphen-4-yl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15l.*** White crystals, mp 123 °C (yield of 27%). IR (KBr), cm^{-1}: 545, 716, 794, 935, 1010, 1150, 1350, 1430, 1590, 3060. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (s, 6H, 4-MeO); 3.50 (s, 6H, 3,5-$(MeO)_2$); 4.20 (br s, 2H, SO_2NH_2); 7.10–8.00 (m, 13H, CH=CH + ArH). Analysis $C_{26}H_{24}N_4O_5S_2$ (C, H, N).

1-(4-Methoxyphenyl)-3-(4-nitrophenyl)-2-propen-1-ylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***15m.*** Yellow crystals, mp 165 °C (yield of 92%). IR (KBr), cm^{-1}: 493, 575, 721, 962, 1030, 1180, 1320, 1350, 1487, 1530, 1610, 3350. 1H-NMR (DMSO-d_6), δ, ppm: 3.80 (s, 3H, 4-MeO); 5.60 (br s, 2H, SO_2NH_2); 6.80–8.60 (m, AA'BB', 10H, CH=CH + ArH). Analysis $C_{17}H_{14}N_5O_5S_2$ (C, H, N).

4-Phenyl-5-nitro-2-pentylidene-sulfanilamide ***17.*** White crystals, mp 174–177 °C (yield of 51%). IR (KBr), cm^{-1}: 451, 530, 674, 717, 918, 1033, 1173, 1338, 3070. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (s, 3H, Me); 3.65–3.95 (m, 3H, $CHCH_2$); 4.20 (br s, 2H, SO_2NH_2); 6.80–7.00 (m, 5H, ArH from phenyl); 6.95–8.00 (m, AA'BB',4H, ArH from phenylene) J = 8.1 Hz. Analysis $C_{17}H_{18}N_3O_4S$ (C, H, N).

4-Phenyl-5-nitro-2-pentylidene-5-amino-1,3,4-thiadiazole-2-sulfonamide ***18.*** White crystals, mp 197–201 °C (yield of 53%). IR (KBr), cm^{-1}: 507, 595, 664, 716, 889, 936, 1025, 1132, 1206, 1380, 1420, 3070. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.40 (s, 3H, Me); 3.65–3.90 (m, 3H, $CHCH_2$); 4.22 (br s, 2H, SO_2NH_2); 7.00–7.70 (m, 5H, ArH from phenyl). Analysis $C_{13}H_{14}N_5O_4S_2$ (C, H, N).

Chalkones **13c,d,f–m** are new compounds and were characterized by means of elemental analysis, and IR (for the sake of simplicity, only the ν(C=C), ν(C=O) and ν(C–H) bands are given below), and ^{1}H-NMR spectroscopy.

4-Dimethylaminobenzylidene acetone ***13c.*** Yellow-orange crystals, mp 120 °C (yield of 96%). IR (KBr), cm^{-1}: 1580 (C=C), 1660 (C=O); 2910 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 2.30 (s, 3H, Me); 3.00 (s, 6H, Me_2N); 6.35–7.50 (m, 6H, CH=CH + ArH). Analysis $C_{12}H_{13}NO$ (C, H, N).

3,4,5-Trimethoxybenzylidene acetone ***13d.*** Pale yellow crystals, mp 103 °C (yield of 95%). IR (KBr), cm^{-1}: 1580 (C=C), 1665 (C=O); 2930 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 2.30 (s, 3H, Me); 3.90 (s, 9H, 3MeO); 6.50–7.60 (m,4H, CH=CH + ArH). Analysis $C_{13}H_{14}O_4$ (C, H).

4-Methoxybenzylidene acetophenone ***13f.*** Pale yellow crystals, mp 69–70 °C (yield of 75%). IR (KBr), cm^{-1}: 1605 (C=C), 1665 (C=O); 2985 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 3.80 (s, 3H, MeO); 6.80–7.06 (m, 2H, CH=CH); 7.10–8.20 (m, 9H, ArH). Analysis $C_{16}H_{14}O_2$ (C, H).

4-Dimethylaminobenzylidene acetophenone ***13g.*** Orange crystals, mp 106 °C (yield of 37%). IR (KBr), cm^{-1}: 1590 (C=C), 1660 (C=O); 2900 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 3.10 (s, 6H, Me_2N); 6.80–7.80 (m, 11H, CH=CH + ArH). Analysis $C_{17}H_{17}NO$ (C, H, N).

3,4,5-Trimethoxybenzylidene acetophenone ***13h.*** Pale yellow crystals, mp 110 °C (yield of 85%). IR (KBr), cm^{-1}: 1590 (C=C), 1700 (C=O); 2920 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 3.80 (s, 9H, 3MeO); 5.40–5.60 (m, 2H, CH=CH); 7.3–7.9 (m, 7H, ArH). Analysis $C_{18}H_{18}O_4$ (C, H).

3,4,5-Trimethoxybenzylidene (4'-methoxyacetophenone) ***13i.*** Pale yellow crystals, mp 70 °C (yield of 61%). IR (KBr), cm^{-1}: 1600 (C=C), 1680 (C=O); 2930 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 2.50 (s, 3H, MeO); 3.80 (s, 9H, 3MeO); 6.60–8.10 (m, 8H, CH=CH + ArH). Analysis $C_{19}H_{20}O_5$ (C, H).

3-Nitrobenzylidene (4'-methoxyacetophenone) ***13j.*** Yellow crystals, mp 116–117 °C (yield of 79%). IR (KBr), cm^{-1}: 1600 (C=C), 1650 (C=O); 1350, 1520 (NO_2). ^{1}H-NMR ($CDCl_3$), δ, ppm: 3.90 (s, 3H, MeO); 6.80–7.05 (m, 2H, CH=CH); 7.10–8.50 (m, 8H, ArH). Analysis $C_{16}H_{13}NO_4$ (C, H, N).

3,4,5-Trimethoxybenzylidene (4'-aminoacetophenone) ***13k.*** Yellow crystals, mp 72 °C (yield of 66%). IR (KBr), cm^{-1}: 1590 (C=C), 1660 (C=O); 2900 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 2.60 (s, 2H, NH_2); 3.90 (s, 9H, 3MeO); 6.60–8.00 (m, 8H, CH=CH + ArH). Analysis $C_{18}H_{19}NO_4$ (C, H, N).

3,4,5-Trimethoxybenzylidene (4'-phenyl-acetophenone) ***13l.*** Yellow crystals, mp 108 °C (yield of 84%). IR (KBr), cm^{-1}: 1580 (C=C), 1670 (C=O); 2980 (C–H). ^{1}H-NMR ($CDCl_3$), δ, ppm: 3.90 (s, 9H, 3MeO); 7.20 (m, 2H, CH=CH); 7.25–8.10 (m, 11H, ArH). Analysis $C_{24}H_{22}O_4$ (C, H).

4-Nitrobenzylidene (4'-methoxyacetophenone) ***13m.*** Yellow crystals, mp 111–112 °C (yield of 84%). IR (KBr), cm^{-1}: 1580 (C=C), 1650 (C=O); 1330, 1510 (NO_2). ^{1}H-NMR ($CDCl_3$), δ, ppm: 3.90 (s, 3H, MeO); 6.80–7.20 (m, 2H, CH=CH); 7.20–8.40 (m, 8H, ArH). Analysis $C_{16}H_{13}NO_4$ (C, H, N).

Assay of CA inhibition

Inhibitors were assayed by Maren's micromethod [31], 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]) 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 for the formation of the E–I complex [29]. At least two assays were performed for each inhibitor at each concentration, and

the results differed by no more than 5% from each other. The reported IC_{50} values are the average such assays. 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 of inhibitor plus 0.1 mL of CA solution, preincubated as described above. The CA concentrations were 1.5 nM for CA II and 235 nM for CA I. 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 absence of inhibitors, as well as IC_{50} values were determined as described by Maren et al [15, 31].

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