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Carbonic anhydrase inhibitors. Inhibition of the cytosolic and tumor-associated carbonic anhydrase isozymes I, II and IX with some 1,3,4-oxadiazole- and 1,2,4-triazole-thiols

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Abstract

Novel mercapto-1,3,4-oxadiazole and -1,2,4-triazole derivatives were synthesized by various pathways starting from 4-(4-halogeno-phenylsulfonyl)benzoic acid hydrazides which were reacted with carbon disulfide or isothiocyanates. The heterocyclic mercaptans prepared in this way were assayed as inhibitors of three physiologically relevant isoforms of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), i.e., the cytosolic CA I and II, and the tumor-associated, transmembrane isozyme CA IX. Interesting biological activity was detected for some of the new mercaptans, with inhibition constants in the low micromolar range.

Keywords: *Carbonic anhydrase, CAI, CAII, CAIX, inhibition tumour associated, oxadiazolethiols, triazolethiols*

Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metallo-enzymes, present in prokaryotes and eukaryotes, being encoded by four distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, *Bacteria*, algae and cytoplasm of green plants), the β-CAs (predominantly in *Bacteria*, algae and chloroplasts of both mono- as well as dicotyledons) the γ-CAs (mainly in *Archaea* and some *Bacteria*), and the δ-CAs, present in some marine diatoms, respectively [1–8]. In mammals, 16 different α-CA isozymes or CA-related proteins (CARP) have been described, with very different subcellular localization and tissue distribution [1–8]. Basically, there are several cytosolic forms (I–III, and VII), five membrane-bound isozymes (IV, IX, XII, XIV and XV), two mitochondrial forms (VA and VB), as well as a secreted isozyme in saliva and milk, CA VI. Among the membrane-bound CAs, isoforms CA IV and XV are anchored to membranes by means of GPI (glycosylphosphatidylinositol) tails, whereas isozymes IX, XII and XIV are transmembrane proteins possessing just one transmembrane domain [1–8]. However, all these five isozymes have their active site outside the cell, being commonly termed as extracellular CAs [1–8]. These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiological or pathological

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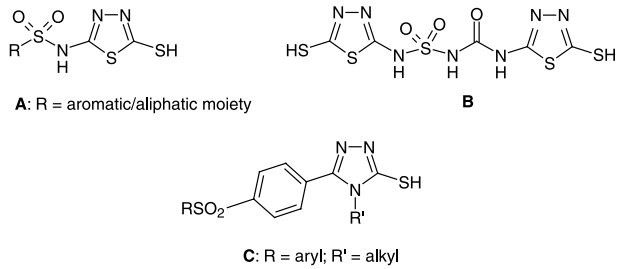
processes [1–8]. Many of these isozymes are important targets for the design of inhibitors or activators with clinical applications [1–8].

A wide variety of heterocycles, including 1,3,4-thiadiazole, 1,2,4-triazole and 1,3,4-oxadiazole derivatives have been described for their possible applications as antimicrobials, inhibitors of glycosidase, urease or carbonic anhydrase (CA) enzymes [9–13]. Among such compounds we have also investigated the interaction of a series of heterocyclic mercaptans incorporating 1,3,4-thiadiazole- 1,2,4-triazole moieties, of types **A–C**, with CA isozymes I, II, IV and IX, finding several potent inhitors, potentially useful as lead compounds for obtaining isozyme-selective derivatives [11,12].



Based on the interesting CA inhibitory activity of some heterocyclic mercaptans **A–C** investigated earlier by us, and continuing our research in this field, we report here the synthesis of some new mercapto-1,3,4-oxadiazoles/-1,2,4-triazole Mannich bases and investigated their CA inhibition activities against three physiologically relevant isoforms, the cytosolic CA I and II, and the tumor-associated, transmembrane isozyme CA IX. Our main interest was as mentioned above, the detection of enzyme inhibitors with an inhibition profile leading to isozyme-selectivity in view of the fact that the presently used CA inhibitors indiscriminately inhibit most of the 15 isoforms widely distributed in mammals [1–8]. As a consequence, clinically used CA inhibitors showed many undesired side effects [1–8].

Materials and methods

Chemistry

Melting points were determined with a Boetius apparatus and are uncorrected. The IR spectra were recorded on a FTS-135 BIO-RAD instrument in KBr pellets. The UV spectra were recorded on a SPECORD 40 Analytik Jena instrument using 2×10^{-5} M methanolic solutions. The NMR spectra were recorded on a VARIAN GEMINI 300 BB instrument at 300 MHz for ^{1}H and at 75 MHz for ^{13}C and using TMS as internal standard.

General procedure for preparation of mannich bases ***4(a–c)***, ***5(a–c)***. A mixture of 1,3,4-oxadiazole **2(a–c)** (0.01 mole) and secondary cyclic amine (0.01 mole) was refluxed in ethanol (50 mL) with 37% formaldehyde (0,02 mole) for 3 h. The resulting solid was filtered, dried and recrystallised from absolute ethanol.

*5-(4-phenylsulfonyl)phenyl-3-(piperidin-1-yl-methyl)-1,3,4-oxadiazole-2(3H)-thione (**4a**)*. m.p. 134–135°C; 92% yield. Found: C: 57.92; H: 4.95; N: 10.18. Calcd. for $C_{20}H_{21}N_3O_3S_2$ (415.54 g/mol): C: 57.81; H: 5.09; N: 10.11%; UV spectrum (methanol; λ_{max} nm; ϵ_{max}): 247 (44054); 347 (26913); IR-; ^{1}H-NMR and ^{13}C-NMR spectra–See Tables I and II

*5-[4-(4-chloro-phenylsulfonyl)phenyl]-3-(piperidin-1-yl-methyl)-1,3,4-oxadiazole-2(3H)-thione (**4b**)*. m.p. 239–241°C; 91% yield. Found: C: 53,43; H: 4.42; N: 9.41. Calcd. for $C_{20}H_{20}ClN_3O_3S_2$ (449.98 g/mol): C: 53.39; H: 4.48; N: 9.34%; UV spectrum (methanol; λ_{max} nm; ϵ_{max}): 251 (43804); 350 (26528); IR-; ^{1}H-NMR and ^{13}C-NMR spectra–See Tables I and II

*5-[4-(4-bromo-phenylsulfonyl)phenyl]-3-(piperidin-1-yl-methyl)-1,3,4-oxadiazole-2(3H)-thione (**4c**)*. m.p. 241–243°C; 94% yield. Found: C: 48.59; H: 4.02; N: 8.57. Calcd. for $C_{20}H_{20}BrN_3O_3S_2$ (494.43 g/mol): C: 48.64; H: 4.08; N: 8.50%; UV spectrum (methanol; λ_{max} nm; ϵ_{max}): 252 (44910); 350 (26162); IR-; ^{1}H-NMR and ^{13}C-NMR spectra–See Tables I and II

*5-(4-phenylsulfonyl)phenyl-3-(morpholin-4-yl-methyl)-1,3,4-oxadiazole-2(3H)-thione (**5a**)*. m.p. 148–150°C; 94% yield. Found: C: 54.71; H: 4.50; N: 10.12. Calcd. for $C_{19}H_{19}N_3O_4S_2$ (417.51 g/mol): C: 54.66; H: 4.59; N: 10.06%; UV spectrum (methanol; λ_{max} nm; ϵ_{max}): 246 (40067); 349 (26265); IR-; ^{1}H-NMR and ^{13}C-NMR spectra–See Tables I and II

*5-[4-(4-chloro-phenylsulfonyl)phenyl]-3-(morpholin-4-yl-methyl)-1,3,4-oxadiazole-2(3H)-thione (**5b**)*. m.p. 250–252°C; 92% yield. Found: C: 50.55; H: 3.97; N: 9.38. Calcd. for $C_{19}H_{18}ClN_3O_4S_2$ (451.95 g/mol): C: 50.49; H: 4.01; N: 9.30%; UV spectrum (methanol; λ_{max} nm; ϵ_{max}): 250 (42521); 350 (25607); IR-; ^{1}H-NMR and ^{13}C-NMR spectra–See Tables I and II

*5-[4-(4-bromo-phenylsulfonyl)phenyl]-3-(morpholin-4-yl-methyl)-1,3,4-oxadiazole-2(3H)-thione (**5c**)*. m.p. 251–252°C; 95% yield. Found: C: 46.02; H: 3.59; N: 8.52. Calcd. for $C_{19}H_{18}BrN_3O_4S_2$ (496.41 g/mol): C: 45.97; H: 3.65; N: 8.46%; UV spectrum (methanol; λ_{max} nm; ϵ_{max}): 253 (47884); 350 (28321); IR-; ^{1}H-NMR and ^{13}C-NMR spectra–See Tables I and II

Table I. Selected IR data for some of the new compounds **4(a–c)** and **5(a–c)**.

			υ (cm^{-1})									
							SO_2					
No	**X**	**R**	**CH aryl**	**CH_2 asym**	**CH_2 sym**	**C=C+ C=N**	**asym**	**sym**	**C=S**	**CH_2N**	**C–O–C**	**N–N**
4a	H		3060	2936	2853	1593 1441	1291 1321	1157	1248	1223	1067	1009
4b	Cl		3087	2936	2852	1581 1472	1290 1324	1159	1249	1221	1087	1010
5b	Cl		3067	2935	2853	1590 1439	1291 1321	1157	1248	1223	1068	1009
5c	Br		3087	2945	2854	1617 1572	1294 1324	1159	1252	1186	1068	1009

CA inhibition assay

An Applied Photophysics stopped-flow instrument was used for assaying the CA-catalysed CO_2 hydration activity [20]. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), and following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7–17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction was used for determining the initial velocity. The uncatalyzed rates were determined in a same manner and subtracted from the total observed rates. 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) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver-Burk plots, as reported earlier, and represent the mean from at least three different determinations [12].

Results and discussion

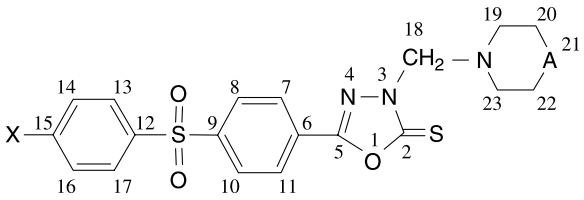
Chemistry

In the present work 4-(4-X-phenylsulfonyl)benzoic acid hydrazides **1(a–c)**, (X=H, Cl, Br) were used as key intermediates for the synthesis of compounds investigated here as enzyme inhibitors. Nucleophilic addition of 4-(4-X-phenylsulfonyl)benzoic acid hydrazides **1(a–c)**, (X = H, Cl, Br,) to different isothiocyanate led to N^4-R-N^1-[4-(4-X-phenylsulfonyl)benzoyl-thiosemicarbazides **6(a–c)**, **7(a–c)** (X=H, Cl, Br), R=–$CH(CH_3)_2$, -2-methoxyphenyl. By ring closure of these compounds in alkaline medium the 5-[4-(4-X-phenylsulfonyl)-phenyl]-4--R-*4H*-1,2,4-triazole-3-thiols [14, 15] **8(a–c)**, **9(a–c)** (X=H, Cl, Br), R=–$CH(CH_3)_2$, -2--methoxyphenyl were produced (Scheme 1).

When compounds **1(a–c)** were treated with carbon disulphide and potassium hydroxide in ethanolic medium, 5-[4-(4-X-phenylsulfonyl)phenyl]-1,3,4-oxadiazole-2-thiols **2(a–c)**, (X=H, Cl, Br) were obtained, which have been reported earlier [16] (Scheme 1). Diphenylsulfones incorporated into the triazole moiety were then synthesized by the reaction of compounds **2(a–c)** with hydrazine hydrate 99% in absolute ethanol when 4-amino-5-[4-(4-X-phenylsulfonyl)phenyl]-*4H*-1,2,4-triazole-3-thiols **3(a–c)** were obtained (X=H, Cl, Br) [16] (Scheme 1). Compounds **2(a–c)** were allowed to undergo the Mannich reaction with various secondary amines, such as morpholine or piperidine, in the presence of 37% formaldehyde (in absolute ethanol) for the synthesis of compounds **4(a–c)** and **5(a–c),** respectively (Scheme 1).

Analytical and physical data of the new compounds **4(a–c)** and **5(a–c)** are provided in the Experimental Section and Tables I and II. The structure of the Mannich bases **4(a–c)** and **5(a–c)** is supported by the IR spectral data (Table I). Thus in these spectra a new band characteristic

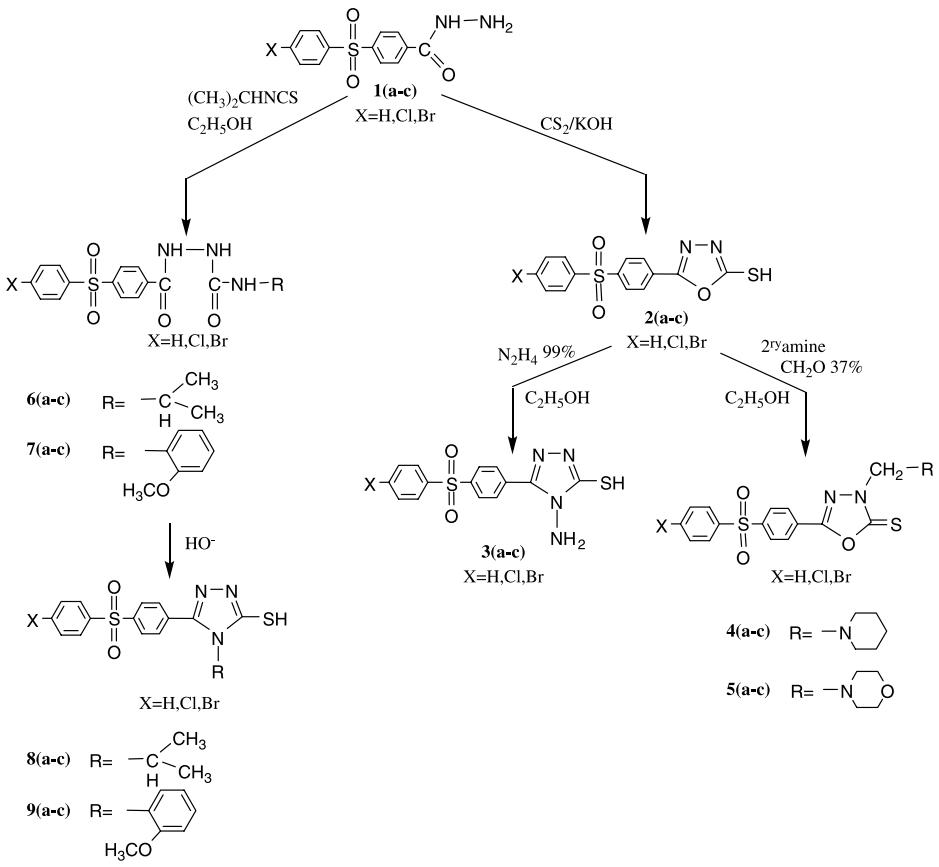
Table II. NMR data for compounds **4(a–c)**; **5(a–c)**



No	X	A	^{1}H-NMR ($CDCl_3$) δ (ppm), J (Hz)	^{13}C-NMR δ (ppm)
4a	H	CH_2	8.07s, 2H, H-7,11; 8.07s, 2H, H-8,10; 7.97dd (7.5, 1.8), 2H, H-13,17; 7.56t (7.5), 2H, H-14,16; 7.61tt (7.5, 1.8), 1H, H-15; 5.06s, 2H, H-18; 2.77bt (5.2), 2H, H-19,23; (1.35-1.62)m, 3H, H-20,21,22	178.31, C-2; 157.52, C-5; 126.72, C-6; 128.43, C-7, 11; 127.84, C-8, 10; 144.81, C-9; 140.67, C-12; 127.18, C-13, 17; 129.52, C-14,16; 133.72, C-15; 71.82, C-18; 51.58, C-19,23; 25.84,C-20,22; 23.58, C-21
4b	Cl	CH_2	8.06d, (8.9), 2H, H-7,11; 8.10d (8.9), 2H, H-8,10; 7.91d (8.7) 2H, H-13,17; 7.52d (8.7), 2H, H-14,16; 5.06s, 2H, H-18; 2.77t (5.5), 2H, H-19,23; 1.58qv (5.5), 2H, H-20, 22; 1,39m, 1H, H-21	178.10, C-2; 157.14, C-5; 126.75, C-6; 128.24, C-7, 11; 127.10, C-8, 10; 144.12, C-9; 140.37, C-12; 129.11, C-13,17; 129.71, C-14,16; 138.95, C-15; 71.63, C-18; 51.37, C-19,23; 25.63, C-20,22; 23.37, C-21
4c	Br	CH_2	7.91d, (8.8), 2H, H-7,11; 7.97d, (8.8), 2H, H-8,10; 7.82d (8.7), 2H, H-13,17; 7.66d (8.7), 2H, H-14,16; 5.06s, 2H, H-18; 2.77t (5.5), 2H, H-19,23; (1.35-1.64)m, 3H, H-20,21,22	178.37, C-2; 157.15, C-5; 126.75, C-6; 128.48, C-7,11; 127.32, C-8,10; 144.55, C-9; 139.70, C-12; 129.36, C-13,17; 132.88, C-14,16; 129.21, C-15; 71.77, C-18; 51.52,C-19,23; 25.46, C-20,22; 23.24, C-21
5a	H	–O–	8.07s, 2H, H-7,11; 8.07s, 2H, H-8,10; 7.97dd, (7.3, 1.5), 2H, H-13,17; 7.54t, (7.3), 2H, H-14,16; 7.62tt, (7.3, 1.5), 1H, H-15; 5.06s, 2H, H-18; 2.83t(4.8), 2H, H-19,23; 3.69t, (4.8), 2H, H-20,22	178.34, C-2; 157.25, C-5; 126.48, C-6; 128.46, C-7,11; 127.85, C-8,10; 145.14, C-9; 140.63, C-12; 127.21, C-13,17; 129.51, C-14,16; 133.73, C-15; 70.72, C-18; 50.49, C-19,23; 66.70, C-20,22
5b	Cl	–O–	8.06d, (8.1), 2H, H-7,11; 8.08d, (8.1), 2H, H-8,10; 7.91d, (8.7), 2H, H-13,17; 7.52d, (8.7), 2H, H-14,16; 5.06s, 2H, H-18; 2.83t(4.7), 2H, H-19,23; 3.70t, (4.7), 2H, H-20,22	178.35, C-2; 157.13, C-5; 126.73, C-6; 128.46, C-7,11; 127.31, C-8,10; 144.60, C-9; 140.62, C-12; 129.30, C-13,17; 129.87, C-14,16; 139.14 C-15; 70.75, C-18; 50.50, C-19,23; 66.70, C-20,22
5c	Br	–O–	8.06s, 2H, H-7,11; 8.07s, 2H, H-8,10; 7.83d, (8.1), 2H, H-13,17; 7.68d, (8.1), 2H, H-14,16; 5.06s, 2H, H-18; 2.83bt(4.5), 2H, H-19,23; 3.69bt, (4.5), 2H, H-20,22	178.37, C-2; 157.15, C-5; 126.75, C-6; 128.48, C-7,11; 127.32, C-8,10; 144.55, C-9; 139.69, C-12; 129.36, C-13,17; 132.89, C-14,16; 129.21, C-15; 70.77, C-18; 50.52, C-19,23; 66.71, C-20,22

s = singlet; d = doublet; t = triplet; dd = doublet of doublets; tt = triplet of triplets; qv = quartet; m = multiplet; b = broad

of the $-CH_2-N-CH_2-$ group around 1186–1223 cm^{-1} appears simultaneously with the disappearance of the band around 3100–3300 cm^{-1} characteristic for the –NH– group of 1,3,4-oxadiazole-2-thione. In the NMR spectra (Table II) a characteristic signal due to the $-N-CH_2-N-$ protons appeared at 5.06–5.07 ppm as a singlet, whereas the signal due to $-N-CH_2-N-$ carbon appeared at 70.72–71.82 ppm. On the other hand, the ^{1}H-NMR spectra exhibited features and characteristics for the diarylsulfone moiety and for the remaining functional side-chains, and presented significant similarities with



Scheme 1. Synthesis of the heterocycles **2–9**

the related heterocyclic thiols possessing a different ring system and a diphenylsulfone moiety previously reported in the literature [17–19].

Carbonic anhydrase inhibition

Heterocyclic mercaptans have been investigated earlier as CA inhibitors against isoforms I, II, IV and IX [11–13], and some interesting data were obtained. Thus, we decided to investigate other classes of such derivatives for their interaction with some isoforms. Inhibition data against three physiologically relevant CA isoforms, i.e., the cytosolic human hCA I and hCA II, and the tumor-associated, transmembrane isozyme hCA IX, were obtained by means of a stopped-flow assay [20] and the results are provided in Table III.

The following SAR can be drawn from the data of Table III: (i) against the slow [1,2] cytosolic isozyme hCA I, the heterocycles **2–5** showed good inhibitory activity, with inhibition constants in the range 5.1–9.3 μM, being on the other hand weaker inhibitors as compared to the classical sulfonamide inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), which had a K_I of 0.25 μM. Thus, all types of substitution patterns in derivatives **2–5** lead to approximately the same inhibitory pattern, whether

Table III. Inhibition data for derivatives **2-9** investigated here and the standard sulfonamide CAI (acetazolamide **AAZ**), against isozymes hCA I, II and IX.

Compound	hCA I[a]	$K_I^{\dagger}$ (μM) hCA II[a]	hCA IX[b]
AAZ	0.250	0.012	0.025
2a	7.4	3.7	13.5
2b	9.3	3.1	32.8
2c	9.2	2.7	14.2
3a	7.5	1.6	57.1
3b	6.4	2.0	59.0
3c	7.2	2.1	54.5
4a	7.5	5.9	32.4
4b	5.1	1.4	52.8
4c	6.2	1.6	31.9
5a	6.4	0.63	1.25
5b	8.0	3.0	9.20
5c	7.6	4.3	6.07
8a	340	11	129
8b	460	12	133
8c	400	31	348
9a	422	67	476
9b	443	78	452
9c	449	85	433

†Errors in the range of 5-10% of the reported value (from 3 different assays); [a] Human cloned isozyme, by the CO_2 hydration method; [b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method [20].

in the 1,3,4-oxadiazole or 1,2,4-triazole series of derivatives. Thus, substitution of the nitrogen atom close to the thiol (thione) moiety (in derivatives **4** and **5**) with the rather bulky piperidine-methyl or morpholine-methyl groups was not detrimental to the hCA I inhibitory activity of these compounds as compared to the corresponding parent, unsubstituted compounds **2**. The 4-X-phenyl moiety in all these derivatives was also not very important for their inhibitory power, since both compounds with X=H or X= halogens, showed similar activity. For the triazoles **3**, the presence of the amino moiety in the 4-position also did not negatively influence the inhibitory power. However, for the compounds incorporating the much bulkier *iso*-propyl and 2-methoxy-phenyl moieties in this position, i.e., derivatives **8a–8c**, and **9a–c**, the hCA I inhibitory activity was very much diminished as compared to the corresponding derivatives **3a–c** (possessing the more compact amino group in this position), with K_Is in the range 340–460 μM. Probably this is due to the steric hindrance produced by these bulky moieties, which interferes with the binding of the mercaptide to the zinc ion within the enzyme active site, as observed earlier for other heterocyclic mercaptans possessing bulky groups in the neighborhood of the zinc-binding group [11–13]; (ii) against the physiologically most important cytosolic isoform, hCA II, the new compounds investigated here showed good inhibitory activity, with K_Is in the range 0.63–31 μM, being again less efficient inhibitors as compared to the sulfonamide acetazolamide (K_I of 12 nM). The oxadiazoles **2**, the aminotriazoles **3** and the Mannich bases **4** and **5** showed a similar level of activity. So, similarly to what mentioned above for isozyme I, the substitution pattern of these compounds does not greatly influence their CA II inhibitory activity. The only much less active derivatives were again the *iso*-propyl/2-methoxy-phenyl substituted triazoles **8** and **9** which were less inhibitory probably for the same reason mentioned above, i.e., steric impairment due to the bulky moiety substituting the N-4 atom. Unexpectedly however, the best inhibitor was the Mannich base incorporating the thiadiazole ring **5a**, which has a rather bulky group at the nitrogen atom close to the zinc-binding function; (iii) against the tumor-associated isoform hCA IX, compounds **2–5** showed a moderate - weak inhibitory activity, with K_Is in the range of 1.25–59 μM, whereas the bulky triazoles **8** and **9** were quite weak inhibitors, with K_Is in the range 129–476 μM. The SAR is again quite similar to that mentioned above for isozymes I and II.

In conclusion, we synthesized and assayed as CA inhibitors a series of heterocyclic mercaptans incorporating oxadiazole and triazole rings, and various other substituted-diphenylsulfone side chains. Some of the new compounds proved to be moderately active inhibitors of isozymes hCA I, II and IX, with inhibition constants in the low micromolar range.

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