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CARBONIC ANHYDRASE INHIBITORS: INHIBITION OF ISOZYMES I, II AND IV WITH HETEROCYCLIC MERCAPTANS, SULFENAMIDES, SULFONAMIDES AND THEIR METAL COMPLEXES[#]

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A series of sulfenamides, sulfonamides and sulfonamide metal complexes have been prepared starting from 4,5-disubstituted-3-mercapto-1,2,4-triazole derivatives. The heterocyclic mercaptans were oxidized to the corresponding sulfenamides by hypochlorite in the presence of ammonia. The sulfonamides were obtained by oxidation of sulfenamides with potassium permanganate. The Zn(II) and Cu(II) complexes of the new heterocyclic sulfonamides have been prepared via the sodium salt of the ligand. Inhibition of three carbonic anhydrase (CA) isozymes, hCA I, hCA II and bCA IV (h = human, b = bovine) with the prepared compounds has been investigated. Mercaptans were generally less inhibitory than sulfenamides, which in turn behaved as weaker inhibitors than the sulfonamides. The strongest inhibitors were the Zn(II) and Cu(II) complexes of the heterocyclic sulfonamides. Susceptibility to inhibition was generally: hCA II > bCA IV > hCA I. Although none of the obtained simple inhibitors (mercaptans, sulfenamides, sulfonamides) possessed antiglaucoma action when administered directly into the eye in experimental animals, the Zn(II) and Cu(II) complexes of some sulfonamides acted as more efficient intraocular pressure lowering agents as compared to the clinical drug dorzolamide. This constitutes an encouraging result for obtaining novel antiglaucoma drugs from this class of CA inhibitors.

[#] See Ref. [1].

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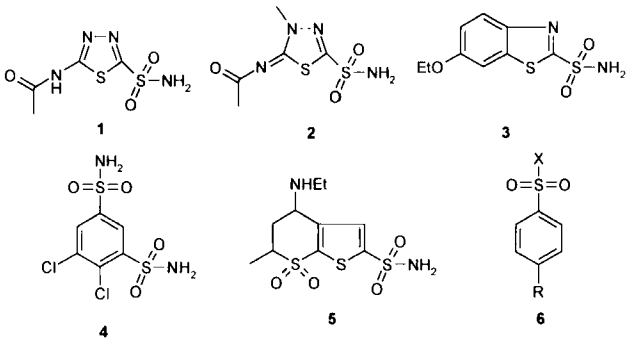
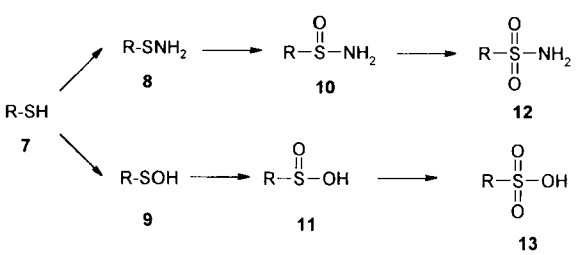
INTRODUCTION

Although catalyzing one of the simplest physiological reactions, the reversible hydration of CO_2 to bicarbonate, carbonic anhydrases (CA's, EC 4.2.1.1) are widely spread enzymes in bacteria, archaea, plants and animals, where they are involved in a variety of physiologic, metabolic or regulatory processes.[2–4] In higher vertebrates at least eight isozymes[2] and two CA-like proteins have been isolated so far.[5] The precise physiological function for many of these proteins is presently unknown,[6] but the major isozymes such as CA II (cytosolic)[6] and CA IV (membrane-bound)[7] were shown to play a critical role in the transport of CO_2/bicarbonate from metabolizing tissues to the lungs,[8] secretion of electrolytes,[8 10] and pH homeostasis[11] among others.

Like many metallo-enzymes, CA's are inhibited by metal complexing anions such as cyanide, cyanate, sulfide, azide, etc.,[12,13] which directly coordinate to the Zn(II) ion within the active site cavity.[14,15] Unlike other zinc enzymes, they possess another class of very potent inhibitors, the unsubstituted sulfonamides possessing the general formula $R\text{-}SO_2NH_2$ (R = perfluoroalkyl, aryl or hetaryl).[6,16] Similarly to some inorganic anions, sulfonamide inhibitors also bind as anions to the Zn(II) ion, in some cases with very high affinity, by substituting a catalytically important zinc-bound water molecule present in the uninhibited enzyme.[12 15] Sulfonamides possessing CA inhibitory properties have clinical applications in the prevention or treatment of a variety of diseases correlated with disfunction of secretory processes in which CA isozymes are involved, such as ocular and cerebrovascular fluids production,[8,9,17,18] hydrochloric acid secretion in the stomach,[8,19] or acidification of urine in the kidneys[20] among others. Several pharmacological agents from this class of compounds, such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, dichlorophenamide **4** and dorzolamide **5** are used clinically as diuretics (**4**),[8,21] antiglaucoma agents (**1**–**3** for systemic administration (now obsolete), whereas **5** is a topical inhibitor recently introduced in clinics in USA and Europe),[22,23] anti-epileptic drugs,[24] but also as diagnostic tools in positron emission tomography (PET) or phase-contrast nuclear magnetic resonance imaging (NMRI) of cerebrovascular disease.[25,26]



Recently this group has proved[27] that the structure-activity relationship of sulfonamide CA inhibitors are more intricate than generally considered, since appreciable inhibitory properties have been detected for compounds of type **6**, which do not possess free SO_2NH_2 moieties in their molecules, in addition to the unsubstituted aromatic sulfonamides that have been used as lead molecules for designing these new classes of inhibitors. Other types of organic compounds possessing high affinity for CA I and II investigated up to now were the heterocyclic mercaptans **7** (R = mono- or bicyclic heterocyclic moiety, such as 1,3,4-thiadiazolyl; benzimidazolyl; benzothiazolyl, etc)[28] and the aromatic sulfenamides **8** (R = 2- and 4-nitrophenyl).[27] Since some compounds of type **6**–**8** were shown to possess CA inhibitory properties similar or even stronger than the aromatic sulfonamides containing the same moieties in their molecules,[27,28] and by taking into account the well-known sulfur chemistry illustrated in Scheme 1, it appeared of interest to investigate in further detail some of these derivatives for their interaction with different CA isozymes.



SCHEME 1

Of the compounds of Scheme 1, excepting for sulfonamides of type **12**, sulfenamides **8** and mercaptans **7**, mentioned above, we have recently shown[27] that aromatic sulfinates **11** behave as stronger CA inhibitors when compared with the corresponding sulfonates **13** (the free acids are shown in Scheme 1, but the species interacting with the enzyme are obviously the corresponding anions). As no systematic study has been published up to now regarding the comparative CA inhibition of a series of derivatives **7**–**13**, here we report such an investigation for heterocyclic mercaptans, sulfenamides and sulfonamides, derivatives of 1,2,4-triazole. Unfortunately, sulfenic acids of type **9**, as well as sulfinamides **10** could not be prepared sufficiently pure in order to be included in the present study. The Zn(II) and Cu(II) complexes of the new sulfonamides reported here have also been prepared and included in the CA inhibition studies since it has been recently demonstrated that some inhibitors from this class act as very powerful intraocular pressure (IOP) lowering agents, *in vivo*, and might be developed as anti-glaucoma drugs.[29] Three CA isozymes were used in the enzymatic assay, i.e., human (h) hCA I, hCA II and bovine (b) bCA IV, and interesting differences have emerged regarding their behavior towards these novel classes of inhibitors. This work might constitute a good starting point for the design of isozyme-specific CA inhibitors.

MATERIALS AND METHODS

Melting points were determined with a heating plate microscope and are not corrected; IR spectra were obtained in KBr pellets with a Perkin-Elmer 16PC FTIR spectrometer and ^{1}H-NMR spectra with a Bruker 200CXP apparatus in solvents specified in each case. Chemical shifts are expressed as δ values relative to Me_4Si as standard. EPR spectra of crystalline powders were recorded on a Varian E-9 spectrometer at room temperature. The field was calibrated using crystalline diphenylpicrylhydrazyl ($g = 2.0036$). Magnetic susceptibility measurements of the metal complexes were carried out at room temperature with a fully automated AZTEC DSM8 pendulum-type susceptometer. Mercury(II) tetrakis-(thiocyanato)cobaltate(II) was used as a susceptibility standard. Corrections for the diamagnetism were estimated from Pascal's constants. Conductimetric measurements were done at room temperature (1 mM concentration of complex) in DMF solution with a Fisher conductimeter. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer, and gravimetrically for the

metal ions, and were $\pm 0.4\%$ of the theoretical values. Thermogravimetric measurements have been done in air, at a heating rate of 10°C/min., with a Perkin-Elmer 3600 thermobalance.

Heterocyclic mercaptans **14a–e** used in synthesis were prepared as described in the literature.[30] Other reagents (metal salts, potassium permanganate, ammonia, etc) were from Acros, Aldrich or Merck and were used without further purification. Acetone (Merck) and other solvents used in the synthesis or enzyme assays were doubly distilled and kept over molecular sieves in order to maintain them in anhydrous conditions.

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.*[31] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group,[32] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[33] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of $49\,mM^{-1}\,cm^{-1}$ for CA I and $54\,mM^{-1}\,cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.30 kDa for CA II, respectively.[34,35] bCA IV was isolated from bovine lung microsomes as described by Maren *et al.*, and its concentration was determined by titration with ethoxzolamide.[36]

Inhibitors were assayed by Maren's micromethod,[37] at 0°C, under the conditions of the E-I (enzyme-inhibitor) technique. Water saturated with 100% CO_2 (at 0°C) was used as substrate, as originally described by Maren *et al.*[37] 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)[6a] and dilutions up to 0.1 nM were done 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.[37] In a special 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 of 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 IC_{50} values (the mean of two determinations) were determined as described by Maren (the standard error of this measurements is around 5–10%).[37]

Adult male New Zealand albino rabbits weighing 2–3 kg were used in the tonometric measurements of intraocular pressure (IOP) (three animals were used for each inhibitor studied). The experimental procedures conform to the Association for Research in Vision and Ophthalmology Resolution on the use of animals and were done at the Ophthalmologic Clinic of the University of Florence. The rabbits were kept in individual cages with food and water provided *ad libitum*. The animals were maintained on a 12 h : 12 h light/dark cycle in a temperature controlled room, at 22–26°C. Solutions of inhibitors (2%, by weight) were obtained in DMSO-water (2 : 3, v/v) due to the lower water solubility of some of these derivatives. Control experiments with DMSO (at the same concentration as that used for obtaining the inhibitors solutions) showed that it does not possess IOP lowering or increasing effects. Dorzolamide used as standard in these measurements was from Merck.

IOP was measured using a Digilab 30R pneumatonometer (BioRad, Cambridge, MA, USA) as described by Maren's group.[38] The pressure readings were matched with two-point standard pressure measurements at least twice each day using a Digilab Calibration verifier. All IOP measurements were done by the same investigator with the same tonometer. One drop of 0.2% oxybuprocaine hydrochloride (novesine, Sandoz) diluted 1 : 1 with saline was instilled in each eye immediately before each set of pressure measurements. IOP was measured three times at each time interval, and the means reported. IOP was measured first immediately before drug administration, then at 30 min after the instillation of the pharmacological agent, and then each 30 min for a period of several hours. For all IOP experiments drug was administered to only one eye, leaving the contralateral eye as an untreated control. The ocular hypotensive activity is expressed as the average difference in IOP between the treated and control eye, in this way minimizing the diurnal, seasonal and interindividual variations commonly observed in the rabbit.[38] All data are expressed as mean ± SE, using a one-tailed *t*-test.

General Procedure for the Preparation of Sulfenamides 15a–d

The method previously described by us for the preparation of 1,3,4-thiadiazole-2,5-bis-sulfonamide (*via* the corresponding bis-sulfenamide)[39] has been applied for the preparation of the title derivatives. An amount of 10 mMol of mercaptan **14a–e** was dissolved in a solution obtained from 0.8 g NaOH and 10 mL water. To this solution were added dropwise and *concomitantly* 10 mL of a solution of concentrated ammonia (25%) and a

solution of sodium hypochlorite (15 mL; 5.6% NaClO), respectively, over a period of 30 min, with good magnetic stirring and maintaining the temperature at 0°C. The precipitated sulfenamide that formed was filtered and thoroughly washed with water till neutral pH. Sulfenamides **15a–d** obtained in this way were recrystallized from acetone. Yields were in the range of 35–62%. The mercaptan **14e** could not be transformed in these conditions to the corresponding sulfenamide, due to the cleavage of its 1,2,4-triazole moiety. Protection of the imidazolic NH group of **14e** (with 2-nitrosulfenyl chloride, as the corresponding sulfenamide, or with tosyl chloride, as the corresponding tosylamide) did not improve the situation, as the obtained compounds did not afford the $S\text{-}NH_2$ derivative by treatment with hypochlorite and ammonia under the conditions described above.

General Procedure for the Preparation of Sulfonamides 16a–d

Sulfenamides **15a–d** (5 mMol) were dissolved in 30 mL of anhydrous acetone and treated with a small excess of saturated $KMnO_4$ solution in the same solvent, with good magnetic stirring, at 0°C. The excess of $KMnO_4$ was destroyed by addition of a small amount of oxalic acid, the precipitated MnO_2 was filtered and discarded, and the acetone solution of sulfonamides **16a–d** evaporated in vacuo. Recrystallization from ethanol-water (1 : 1) afforded the title compounds with good yields (75–84%).

General Procedure for the Preparation of Complexes 17–24

An amount of 6 mmol of sodium salt of **16a–d** was prepared by reacting the corresponding sulfonamide with the required amount of an alcoholic 1N NaOH solution, in ethanol as solvent. To these solutions were added the metal salt (Zn(II), and Cu(II) chlorides) aqueous solutions, working in the molar ratio $RSO_2NH^- : M^{n+}$ of 2 : 1. The aqueous-alcoholic reaction mixtures were heated on a steam bath for 1 h and after being cooled at 0°C the precipitated complexes were filtered and thoroughly washed with alcohol–water 1 : 1 (v/v), and then air-dried. Yields were in the range of 85–90%. The obtained powders of compounds **17–24** (white for the Zn(II) complexes; green for the Cu(II) derivatives, respectively) melted with decomposition at temperatures higher than 300°C, and were poorly soluble in water and alcohol, but possessed good solubilities in DMSO, DMF as well as mixtures of DMSO–water, DMF–water.

4-Ethyl-5-[4-(phenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfenamide **15a**, as white crystals, m.p. 197–9°C. IR (KBr), cm^{-1}: 550, 635, 771, 848, 995, 1010, 1095, 1165 (SO_2^{sym}), 1313 (SO_2^{as}), 1480, 1600, 3070 (NH_2); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.30 (t, 3H, Me from ethyl; J = 7.5); 4.21 (q, 2H, CH_2 from ethyl; J = 7.5); 5.17 (br s, 2H, SNH_2); 7.50–8.31 (m, 9H, ArH); Found: C: 55.47; H, 4.13; N, 11.88; S, 18.69. $C_{16}H_{15}N_3O_2S_2$ requires: C: 55.63; H, 4.38; N, 12.16; S, 18.56%.

4-Ethyl-5-[4-(4-chlorophenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfenamide **15b**, as white crystals, m.p. 226–9°C. IR (KBr), cm^{-1}: 590, 625, 748, 851, 995, 1010, 1093, 1166 (SO_2^{sym}), 1323 (SO_2^{as}), 1470, 1590, 3075 (NH_2); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.30 (t, 3H, Me from ethyl; J = 7.4); 4.24 (q, 2H, CH_2 from ethyl; J = 7.4); 5.28 (br s, 2H, SNH_2); 7.55–8.36 (m, 8H, ArH); Found: C: 50.35; H, 3.50; Cl, 9.21; N, 10.81; S, 16.97. $C_{16}H_{14}ClN_3O_2S_2$ requires: C: 50.59; H, 3.71; Cl, 9.33; N, 11.06; S, 16.88%.

4-Ethyl-5-[4-(4-bromophenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfenamide **15c**, as white crystals, m.p. 261–2°C. IR (KBr), cm^{-1}: 570, 621, 733, 765, 830, 995, 1010, 1085, 1160 (SO_2^{sym}), 1320 (SO_2^{as}), 1470, 1590, 3070 (NH_2); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.30 (t, 3H, Me from ethyl; J = 7.1); 4.25 (q, 2H, CH_2 from ethyl; J = 7.1); 5.31 (br s, 2H, SNH_2); 7.60–8.53 (m, 8H, ArH); Found: C: 45.06; H, 3.17; Br, 18.59; N, 9.61; S, 14.98. $C_{16}H_{14}BrN_3O_2S_2$ requires: C: 45.29; H, 3.33; Br, 18.83; N, 9.90; S, 15.11%.

4-Cyclohexyl-5-[4-(4-chlorophenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfenamide **15d**, white crystals, m.p. 165–7°C. IR (KBr), cm^{-1}: 597, 633, 689, 752, 819, 995, 1015, 1090, 1160 (SO_2^{sym}), 1319 (SO_2^{as}), 1478, 1600, 3075 (NH_2); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.10–2.48 (m, 11H, C_6H_{11}); 5.21 (br s, 2H, SNH_2); 7.51–8.47 (m, 8H, ArH); Found: C: 55.19; H, 4.54; Cl, 8.23, N, 9.41; S, 14.89. $C_{20}H_{20}ClN_3O_2S_2$ requires: C: 55.35; H, 4.65; Cl, 8.17; N, 9.68; S, 14.78%.

4-Ethyl-5-[4-(phenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfonamide **16a**, as white crystals, m.p. 234–6°C. IR (KBr), cm^{-1}: 558, 651, 718, 775, 852, 995, 1010, 1090, 1154 and 1165 (SO_2^{sym}), 1313 and 1325 (SO_2^{as}), 1490, 1600, 3060 (NH_2); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.32 (t, 3H, Me from ethyl; J = 7.3); 4.25 (q, 2H, CH_2 from ethyl; J = 7.3); 7.12 (br s, 2H, SO_2NH_2); 7.54–8.40 (m, 9H, ArH); Found: C: 50.73; H, 3.75; N, 11.04; S, 16.96. $C_{16}H_{15}N_3O_4S_2$ requires: C: 50.92; H, 4.01; N, 11.13; S, 16.99%.

4-Ethyl-5-[4-(4-chlorophenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfonamide **16b**, as white crystals, m.p. 268–9°C. IR (KBr), cm^{-1}: 545, 598, 635, 714, 757, 850, 995, 1010, 1090, 1150 and 1166 (SO_2^{sym}), 1323 and 1329 (SO_2^{as}), 1480, 1600, 3065 (NH_2; ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.35 (t, 3H, Me from ethyl; J = 7.1); 4.26 (q, 2H, CH_2 from ethyl; J = 7.1); 7.20 (br s, 2H,

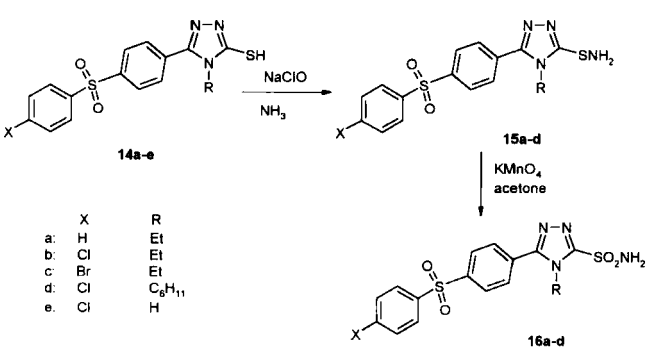
SO_2NH_2); 7.55–8.40 (m, 8H, ArH); Found: C: 46.71; H, 3.25; Cl, 8.41; N, 10.13; S, 15.70. $C_{16}H_{14}ClN_3O_4S_2$ requires: C: 46.66; H, 3.43; Cl, 8.61; N, 10.20; S, 15.57%.

4-Ethyl-5-[4-(4-bromophenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfonamide **16c**, as white crystals, m.p. 285–8°C (dec.). IR (KBr), cm^{-1}: 540, 592, 611, 731, 776, 839, 990, 1015, 1085, 1149 and 1161 (SO_2^{sym}), 1320 and 1329 (SO_2^{as}), 1475, 1600, 3065 (NH_2); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.34 (t, 3H, Me from ethyl; J = 7.2); 4.24 (q, 2H, CH_2 from ethyl; J = 7.2); 7.25 (br s, 2H, SO_2NH_2); 7.60–8.55 (m, 8H, ArH); Found: C: 41.82; H, 2.97; Br, 17.33; N, 9.15; S, 14.08. $C_{16}H_{14}BrN_3O_4S_2$ requires: C: 42.11; H, 3.09; Br, 17.51; N, 9.21; S, 14.05%.

4-Cyclohexyl-5-[4-(4-chlorophenylsulfonyl)-phenyl]-1,2,4-triazole-3-sulfonamide **16d**, as white crystals, m.p. 211–3°C. IR (KBr), cm^{-1}: 542, 590, 638, 691, 750, 824, 995, 1013, 1090, 1151 and 1160 (SO_2^{sym}), 1319 and 1330 (SO_2^{as}), 1480, 1600, 3065 (NH_2); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.10–2.51 (m, 11H, C_6H_{11}); 7.20 (br s, 2H, SO_2NH_2); 7.50–8.45 (m, 8H, ArH); Found: C: 51.24; H, 4.09; Cl, 7.38, N, 8.81; S, 14.02. $C_{20}H_{20}ClN_3O_4S_2$ requires: C: 51.55; H, 4.33; Cl, 7.61; N, 9.02; S, 13.76%.

RESULTS AND DISCUSSION

Treatment of the sodium salts of the heterocyclic mercaptans **14a–e** with sodium hypochlorite and ammonia in aqueous medium at 0°C afforded the corresponding sulfenamides **15** by the method previously described for the preparation of 1,3,4-thiadiazole-2,5-bis-sulfenamide (Scheme 2).[39] Compound **14e** could not be transformed under these conditions into the corresponding sulfenamide.



SCHEME 2

Sulfenamides **15** isolated as white powders stable enough at neutral and alkaline pH (but readily decomposed in acidic media), were oxidized thereafter to the corresponding sulfonamides of type **16** with potassium permanganate in acetone as solvent (Scheme 2).[39] Sulfonamides **16a–d** were prepared in this way in good yields. Mention should be made that no acceptable experimental procedure has been found for the transformation of sulfenamides **15** to the corresponding sulfinamides (of type **10**, see also Scheme 1), working in the presence of a variety of oxidizing agents, such as: $(NH_4)_2[Ce(NO_3)_6]$, perbenzoic acid ($PhCO_3H$), pyridinium chlorochromate, or sodium periodate.[40,41] In all these experiments only mixtures containing high amounts of sulfonamides and low amounts of sulfinamides were obtained, and the latter could not be isolated thereafter sufficiently pure in order to include them in the CA inhibition studies. Mention should be made that no compound containing the $SONH_2$ moiety has been tested up to now for its interaction with CA. Based on the well-known behavior of the sulfonamide inhibitors and of the aromatic sulfenamides investigated earlier,[27] we predict that sulfinamides of the type R-$SONH_2$ (R = aromatic or heterocyclic moiety) might also behave as strong CA inhibitors.

Since metal complexes of aromatic/heterocyclic sulfonamides were shown to possess important CA inhibitory properties,[42–44] as well as IOP lowering effects in experimental animals,[29] it appeared of interest to prepare such compounds derived from sulfonamides **16a–d**. The Zn(II) and Cu(II) complexes containing the conjugate bases of sulfonamides **16** as ligands, of type **17–24**, prepared in the present study are shown in Table I. The two metal ions mentioned above were chosen due to the excellent inhibitory properties of complexes containing such cations and sulfonamides of type **1–3** and **5**, previously reported by this group.[42,45]

Compounds **17–24** were characterized by elemental analysis (Table I) as well as physico-chemical measurements (Table II) that led to the formulation of their structure (see later in the text).

The main differences in the IR spectra of complexes **17–24** as compared to the corresponding spectra of ligands **16** from which they were obtained, involve the sulfonamido and C=N vibrations. Thus, the symmetrical SO_2 vibrations (of the sulfonamido moiety, not of the sulfone one, which are unmodified in the spectra of the ligand and of the complexes, data not shown), present at 1149–1154 cm^{-1} in the spectra of **16a–d** are shifted towards lower wavenumbers in the spectra of **17–24**, appearing at 1120–1132 cm^{-1} for the Zn(II) derivatives, and 1130–1140 cm^{-1}, for the Cu(II) complexes, respectively (Table II). The same type of shift is observed for

TABLE I Complexes **17**–**24** containing the conjugate bases (L) of sulfonamides **16a**–**d** as ligands, prepared in the present study and their elemental analysis data

No.	*Complex*	*Ligand** (*HL*)	*Analysis (calculated/found)*			
			%*M*[a]	%*C*[b]	%*H*[b]	%*N*[b]
17	$[ZnL_2]$	**16a**	8.66/8.35	50.91/50.72	3.71/3.39	5.56/5.24
18	$[ZnL_2]$	**16b**	7.94/7.65	46.64/46.48	3.15/3.01	5.10/5.05
19	$[ZnL_2]$	**16c**	7.16/7.09	42.10/42.25	2.85/2.63	4.60/4.48
20	$[ZnL_2]$	**16d**	6.56/6.19	48.22/48.31	3.81/3.72	4.22/3.93
21	$[CuL_2(OH_2)_2]$	**16a**	8.06/8.21	48.70/48.51	4.05/3.69	5.32/5.04
22	$[CuL_2(OH_2)_2]$	**16b**	7.41/7.27	44.79/44.50	3.49/3.32	4.89/4.56
23	$[CuL_2(OH_2)_2]$	**16c**	6.71/6.80	40.58/40.49	3.17/2.95	4.43/4.09
24	$[CuL_2(OH_2)_2]$	**16d**	6.17/5.84	46.62/46.75	4.07/3.83	4.08/4.05

*The sulfonamide deprotonated species of compounds **16a**–**d** (see later in the text).
[a]By gravimetry; [b]By combustion.

TABLE II Spectroscopic, thermogravimetric and conductimetric data for complexes **17**–**24**

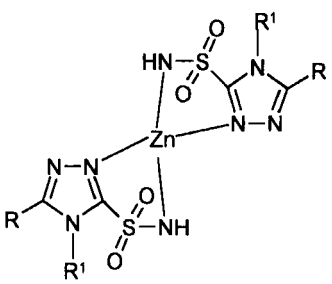
	Comp. IR spectra[a], cm^{-1}			*Electronic spectra*[b] υ (cm^{-1})	*TG analysis*[c] *calc./found*	*Conductimetry*[d] Λ_M ($\Omega^{-1} \times cm^2 \times mol^{-1}$)
	$\nu(SO_2)^s$	$\nu(SO_2)^{as}$	ν(C=N)			
17	1132	1310	1580	e	f	9
18	1130	1312	1583	e	f	7
19	1128	1315	1580	e	f	7
20	1120	1300	1590	e	f	10
21	1140	1305	1585	16,660	4.56/4.73 g	8
22	1140	1303	1585	16,750	4.19/4.25 g	11
23	1140	1306	1585	16,800	3.80/3.71 g	10
24	1130	1302	1585	16,500	3.49/3.30 g	11

[a]In KBr; [b]Reflectance diffuse spectrum in ZnO as standard; [c]Weight loss between 130–250°C; [d]10^{-3} M solution, in DMF, at 25°C; [e]No absorption seen; [f]No weight loss seen under 250°C; [g]Corresponding to two coordinated water molecules, lost at 170–180°C.

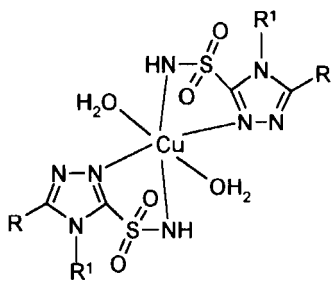
the antisymmetrical sulfonamido vibration, appearing at 1325–1330 cm^{-1} in the spectra of the ligands, and at 1300–1315 cm^{-1} in those of the complexes (Table II). The ν(C=N) vibrations from 1600 cm^{-1} in the spectra of compounds **16** are shifted to 1580–1590 cm^{-1} in the spectra of the new complexes. All these data show that the sulfonamido moieties together with the endocyclic nitrogen(s) of the triazole ring are involved in the interaction with the metal ions, a behavior similar to that of other heterocyclic sulfonamides (such as acetazolamide **1**, methazolamide **2**, or ethoxzolamide **3**) in their metal complexes, previously prepared and characterized by us and Borras' group.[42–45]

Magnetic moments of the prepared Cu(II) complexes **21**–**24** were in the range of 1.90–1.95 BM (data not shown), which correlated with the presence of a large, structureless band in the range 16,500–16,800 cm^{-1} in the reflectance diffuse spectra (Table III) and axial EPR spectra with the

parameters $g_{\perp} = 2.06-2.07$, and $g_{\parallel} = 2.35-2.37$ (data not shown), suggest an octahedral surrounding of Cu(II) in these compounds.[46] Thermogravimetric analysis showed the two water molecules present in the copper complexes to be lost in one step, between 170–180°C, proving this to be coordinated water. All the prepared complexes are non-electrolytes at room temperature in DMF as solvent (Table II). The diamagnetic Zn(II) complexes showed no absorption maxima in the region investigated here. The presence of coordinated or lattice water was not seen in their structure (Table II). The above data lead to the conclusion that Cu(II) is present in octahedral geometry whereas Zn(II) is in tetrahedral geometry in the newly prepared complexes. The donor system of the conjugate bases of sulfonamides **16** is probably constituted by the sulfonamido nitrogen and an endocyclic nitrogen atom of the 1,2,4-triazole ring, similarly to that of other heterocyclic sulfonamides previously investigated.[42–45] The most probable candidate is N-2, since in this case five-membered chelate rings free of strain would be formed. Based on the above assumption, the structures proposed for complexes **17–24** are shown below.



17-20



21-24

CA inhibition data with compounds **14–24** and standard sulfonamide inhibitors are shown in Table III, against isozymes I, II and IV.

As seen from the data of Table III, all compounds tested in the present study, i.e., heterocyclic mercaptans **14**, sulfenamides **15**, sulfonamides **16** and their metal complexes **17–24**, act as good inhibitors in the micromolar – nanomolar range, against the three investigated CA isozymes, hCA I, hCA II and bCA IV. Inhibitory power increased in the order: mercaptans < sulfenamides < sulfonamides < Zn(II) complexes of sulfonamides < Cu(II) complexes of sulfonamides. Important differences among the three CA isozymes were seen regarding their susceptibility to inhibition by the investigated derivatives. Thus, generally hCA II was the most easy to be inhibited, followed by bCA IV, whereas hCA I was the most resistant to

TABLE III Biological activity data for the new CA inhibitors **14–24** prepared in the present study and standard inhibitors **1–3** and **5**

Compound	IC_{50} (nM)*		
	hCA I[a]	*hCA II*[a]	*bCA IV*[b]
1 (acetazolamide)	200 ± 4	7 ± 0.2	120 ± 9
2 (methazolamide)	10 ± 1	9 ± 0.5	145 ± 6
3 (ethoxzolamide)	8 ± 0.9	2 ± 0.2	4 ± 0.2
5 (dorzolamide)	> 50,000 ± 50	2 ± 0.1	3 ± 0.1
14a	1200 ± 60	189 ± 5	170 ± 6
14b	1130 ± 20	175 ± 4	160 ± 7
14c	1040 ± 30	162 ± 4	160 ± 2
14d	1560 ± 45	210 ± 8	194 ± 10
14e	870 ± 12	96 ± 8	121 ± 11
15a	18 ± 2	16 ± 3	30 ± 5
15b	15 ± 3	13 ± 2	25 ± 3
15c	11 ± 2	10 ± 1	24 ± 0.8
15d	57 ± 5	49 ± 3	70 ± 4
16a	17 ± 1	5 ± 0.5	7 ± 1
16b	15 ± 2	5 ± 0.4	6 ± 0.7
16c	11 ± 0.9	4 ± 0.3	6 ± 0.9
16d	48 ± 5	27 ± 3	40 ± 2
17	6 ± 0.8	2 ± 0.1	5 ± 0.2
18	3 ± 0.3	0.9 ± 0.1	4 ± 0.1
19	3 ± 0.2	0.5 ± 0.1	3 ± 0.1
20	25 ± 1	9 ± 1	32 ± 2
21	5 ± 1	0.8 ± 0.2	3 ± 1
22	2 ± 0.3	0.5 ± 0.1	1 ± 0.1
23	2 ± 0.1	0.2 ± 0.08	1 ± 0.1
24	20 ± 1	10 ± 0.7	30 ± 3

*IC_{50} represents the molarity of inhibitor producing a 50% decrease of enzyme specific activity for the CO_2 hydration reaction, by Maren's micromethod.[37] Mean ± average spread (from two determinations). [a]Human (cloned) isozyme; [b]Isolated from bovine lung microsomes.

inhibition. Still, some differences from this general scheme were seen: thus, mercaptans **14** were the most inhibitory against bCA IV, followed by hCA II, whereas they were much less active against hCA I. As far as we know, this is the first example of an inhibitor with higher affinity for CA IV as compared to CA II or CA I. Sulfenamides **15** on the other hand had a very similar affinity for hCA I and hCA II, being less inhibitory against bCA IV (the classical inhibitors – the sulfonamides, such as acetazolamide, methazolamide, ethoxzolamide, etc., possess a completely different behavior, generally having a higher affinity for hCA II and a lower one for hCA I, whereas the inorganic anions, the second class of compounds inhibiting CAs, possessing a higher affinity for hCA I as compared to hCA II).[6,8,12,13]

The substitution pattern of the 1,2,4-triazole ring also greatly influenced the biological activity of all compounds tested as CA inhibitors in the present study. Thus, for the mercaptans **14**, the most active compound was

14e, which is the only one not bearing a substituent to the N-3 atom. For the N-3-substituted derivatives, the compounds possessing an N–ethyl moiety were more active than the derivative with the N-cyclohexyl group, obviously due to a steric hindrance associated with the presence of the bulky cyclohexyl moiety. This is also true for the other types of derivatives, such as the sulfenamides **15** and the sulfonamides **16**. In the series of compounds **14a–c** (and also **15a–c** and **16a–c**), activity increased by substituting the hydrogen atom in the *para*-position of the second aromatic ring with halogens, with the chloro-derivatives more active than the unsubstituted compounds, and in turn, the bromo-derivatives more active than the chloro ones. Thus, even if the prepared series included a relatively small number of compounds, they offered clear-cut responses regarding the structure-activity correlations for these CA inhibitors.

The most active inhibitors of all the prepared compounds were the metal complexes of heterocyclic sulfonamides, of type **17–24**. Their activity closely paralleled that of the parent ligand from which they were obtained, with the only difference that the complexes were generally 3–20 times as active as the corresponding ligand. It should also be noted that these compounds were generally much more active than the clinically used inhibitors acetazolamide and methazolamide, and possessed comparable (or slightly better activities) than ethoxzolamide or dorzolamide (Table III).

The most active inhibitors from each class, i.e., the mercaptan **14e**, the sulfenamide **15c**, the sulfonamide **16c**, and the metal complexes **19** and **23**, were tested *in vivo* as intraocular pressure (IOP) lowering agents in animal (rabbit) models of glaucoma, together with the clinical drug dorzolamide **5**. Values of IOP after the treatment with these inhibitors after 0.5 h and 1 h after instillation into the eye of a 2% solution of inhibitor are shown in Table IV.

TABLE IV IOP lowering following topical application of CA inhibitors, 0.5 and 1 h after instillation into the eye of a drop (50 μL) of 2% solution of inhibitor

Inhibitor	$\Delta IOP \pm SE$[a] (mm Hg)	
	0.5 h	1 h
Dorzolamide, **5**	2.2 ± 0.10	4.1 ± 0.15
14e	0 ± 0.10	0 ± 0.09
15c	0 ± 0.08	0 ± 0.04
16c	0 ± 0.05	0 ± 0.06
19	4.9 ± 0.14	7.5 ± 0.21
23	2.0 ± 0.09	5.3 ± 0.12

[a] Δ IOP = $IOP_{control\ eye} - IOP_{treated\ eye}$ (n = 3).

As seen from the above data, mercaptan **14e**, sulfenamide **15c** and sulfonamide **16c**, although strong CA inhibitors, have no effect on IOP after direct instillation within the eye, similarly to the classical sulfonamide inhibitors, such as acetazolamide, methazolamide, ethoxzolamide.[22,23] In contrast to the above inhibitors, dorzolamide **5** and the sulfonamide complexes **19** and **23** are powerful IOP lowering agents after topical application. In fact we have recently reported[29] the first example of metal complexes of a heterocyclic sulfonamide which is inactive *per se* as IOP lowering agents, whereas its metal (Zn(II) and Cu(II)) complexes possessed powerful such properties. This also seems to be the situation with the metal complexes of sulfonamide **16c**. Thus, its Zn(II) complex **19** and the corresponding Cu(II) derivative **23** greatly reduced IOP after topical application, with an enhanced efficiency compared to dorzolamide. More than that, the time dependence of this effect (Figure 1), showed that the reduced IOP obtained after the administration of the Zn(II) complex, persists for a longer period as compared to the decrease of IOP after dorzolamide or the Cu(II) complex **23**. Such properties, which are directly correlated to the nature of the metal ion contained in the complex CA inhibitor, might be extremely important for the design of new generations of topical anti-glaucoma agents, since complexes of heterocyclic sulfonamides containing a large variety of metal ions have been described by this group.[42–45]

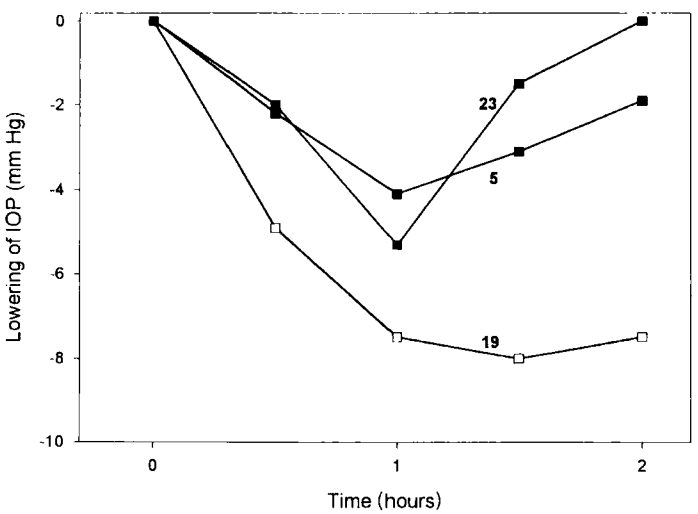


FIGURE 1 Time-dependence of IOP lowering with dorzolamide **5**; the zinc complex **19** and the copper complex **23** after topical administration of one drop (50 μL) of 2% solution of inhibitor in the rabbit.

A last remark should be made about the possible mechanism of action of this new class of IOP lowering agents. Obviously, their activity is due to inhibition of CA isozymes present in the cilliary processes within the eye, similarly to that of the topically active sulfonamides.[9,22–23] The fact that the sulfonamide *per se* is *inactive* via the topical route, whereas the metal complexes give much better results than the drug dorzolamide, indicates that the presence of metal ions in the molecules of these CA inhibitors is essential and confers on them completely new properties. Our hypothesis for explaining this fact is that the presence of the metal ion in the molecules of the complex inhibitors induces a dramatic change in their physicochemical properties as compared to those of the parent sulfonamide. This phenomenon is certainly governed by the strong polarization induced by the metal ions. In this way, it is probable that the right balance between the lipo- and hydrosolubility of these compounds is achieved, which has been considered to be the critical factor for not observing topical activity in the classical CA inhibitors, such as acetazolamide, methazolamide and ethoxzolamide (which were either too lipophilic or too hydrosoluble).[6–9] But by choosing different metal ions and diverse sulfonamides, much larger possibilities arise to finely tune the pharmacological properties and the potential value of a drug from this class of compounds.

In conclusion we described here some novel classes of CA inhibitors, derivatives of heterocyclic mercaptans, sulfenamides, sulfonamides and some of their metal complexes. The last derivatives act as IOP lowering agents when administered directly into the eye, in experimental animals. These derivatives appear to be very active and longer lasting than the drug dorzolamide, and might lead to a new generation of antiglaucoma drugs.

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