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CARBONIC ANHYDRASE INHIBITORS: INHIBITION OF ISOZYMES I, II AND IV WITH *N*-HYDROXYSULFONAMIDES – A NOVEL CLASS OF INTRAOCULAR PRESSURE LOWERING AGENTS *

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A series of *N*-hydroxy sulfonamides has been prepared by reaction of alkyl-, arylalkyl- and arylsulfonyl halides or sulfonic acid anhydrides with hydroxylamine. Structurally related inhibitors were also obtained from acyl chlorides and hydroxylamine, as well as by reaction of tosyl isocyanate with hydroxylamine, sulfamic acid and sulfamide. 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. Good inhibitors, as well as compounds with moderate activity against these isozymes were detected, depending on the R group to which the SO_2NHOH or CONHOH moieties were attached. Susceptibility to inhibition was generally: hCA II > bCA IV ≫ hCA I. Some of the new inhibitors showed very good antiglaucoma action when administered directly into the eye in experimental animals, acting 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.

Keywords: *N*-hydroxysulfonamides; Hydroxylamines; Hydroxamic acids; Carbonic anhydrase; Isozyme I, II, IV; Antiglaucoma drugs

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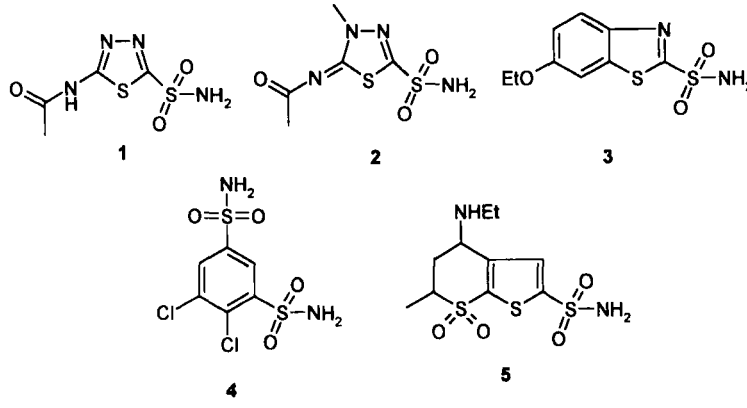
INTRODUCTION

Carbonic anhydrase (CA, EC 4.2.1.1), an enzyme playing a central role to both transport and metabolic processes involving CO_2 and bicarbonate, is present in a variety of tissues of higher vertebrates in the form of eight isozymes.[2–4] By catalyzing the reversible interconversion between the two chemical species mentioned above, in metabolically active tissues (such as the muscle), cytoplasmic (CA I–III) and sarcolemmal (CA III) isozymes facilitate CO_2 transport out of the cell.[3] The only membrane-bound isozyme known (CA IV), which is highly abundant in the kidneys and lungs, has been shown to possess an extracellular orientation of the active site, and to be critical in acidifying the outer boundary layer through the protons formed by CO_2 hydration according to Eq. (1).[5,6] This process further facilitates cellular ammonia transport by providing the H^+ ion for the protonation of NH_3 (Eq. 2), thus maintaining the trans-membrane ammonia gradient.[3,5]

$$O{=}C{=}O + H_2O \Leftrightarrow HCO_3^- + H^+, \quad (1)$$

$$NH_3 + H^+ \rightarrow NH_4^+. \quad (2)$$

The mitochondrial isozyme (CA V) is known to supply bicarbonate/CO_2 for the initial reaction of gluconeogenesis and ureagenesis in many mammalian tissues,[7,8] as well as for the pyruvate carboxylation in the *de novo* lipogenesis in adipocytes.[9]



STRUCTURES 1–5

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Sulfonamides possessing CA inhibitory properties[4,10–12] such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3** and dichlorophenamide **4** have been used for more than 40 years as pressure lowering systemic drugs in the treatment of open-angle glaucoma.[13] Their effect is due to inhibition of at least two CA isozymes present within cilliary processes of the eye, i.e., CA II and CA IV, which is followed by lowered bicarbonate formation and reduction of aqueous humor secretion.[14–17] Their main drawback is constituted by side effects such as fatigue, augmented diuresis, paresthesias, etc., due to CA inhibition in other tissues/organs than the target one, i.e., the eye.[13]

The above-mentioned side effects are absent in the case in which the inhibitor has topical activity, and is applied directly into the eye. This route was demonstrated only as recently as 1983 by Maren's group[14] and was followed by the development of the first clinical agent of this type, dorzolamide **5**.[16,17] Dorzolamide (Trusopt®) has been introduced into clinical use in 1995 in USA and Europe and it constituted the beginning of a radically new treatment of glaucoma, devoid of the severe side effects observed with the systemic inhibitors.[12–17] The success of topical anti-glaucoma CA inhibitors fostered much research in the synthesis and clinical evaluation of other types of such compounds.[12–15]

It was recently reported by this group[11a] that in addition to the unsubstituted sulfonamides of the type RSO_2NH_2, certain derivatives possessing the general formula RSO_2NHX (where X may be a group such as OH, OCH_3, NH_2, NHMe, Cl, etc.) act as very powerful inhibitors of isozymes I and II, sometimes their potency being greater than that of the corresponding unsubstituted sulfonamide. Since few isozyme-specific CA inhibitors are presently[4,10–12,18] known for the many CA isozymes isolated in vertebrates,[2] it appeared of interest to test whether some inhibitors from the classes recently described[11a] might be developed as anti-glaucoma drugs. In this paper we report the synthesis of a series of *N*-hydroxysulfonamides, obtained by reaction of alkyl-, arylalkyl- and arylsulfonyl halides or sulfonic acid anhydrides with hydroxylamine. Some other derivatives structurally related to the *N*-hydroxysulfonamides mentioned above have also been prepared, such as two hydroxamic acids of the type ArCONHOH, as well as compounds which were obtained by reaction of tosyl isocyanate with hydroxylamine, sulfamic acid and sulfamide. The new derivatives have been tested for inhibition of CA isozymes I, II and IV, and several effective inhibitors were evidenced for all of them. Some compounds were then assayed *in vivo*, for their intraocular pressure (IOP) lowering properties, in rabbits, in an experimental animal model of glaucoma. Some

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compounds reported by us here proved very effective IOP lowering agents, with potencies much greater than those of dorzolamide **5**, the clinically used antiglaucoma agent from this class of derivatives.

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 Varian 300CXP in DMSO-d_6 as solvent. Chemical shifts are expressed as δ values relative to Me_4Si as standard. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer, and were $\pm 0.4\%$ of the theoretical values.

Sulfonyl halides, sulfonic acid anhydrides, triethylamine, sulfamic acid, sulfamide and hydroxylamine hydrochloride used in synthesis were commercially available (from Sigma, Acros or Aldrich). Tosyl isocyanate was from Acros. Acetonitrile (Merck) and other solvents used in synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions. Sulfonamides **1–5** used as standards in the enzymatic assay were commercially available from Sigma, Aldrich or Merck, Sharp and Dohme.

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.*[19] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group,[20] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[21] 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.[22,23] bCA IV was isolated from bovine lung microsomes, and its concentration has been determined by titration with ethoxzolamide.[24,25]

Inhibitors were assayed by Maren's micromethod,[26] at 0°C, in 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.*[26] Stock solutions of inhibitor (1 mM) were prepared in distilled–deionized water, eventually also containing 10% (v/v) DMSO (which is not inhibitory at these concentrations)[4] for poorly soluble

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compounds, 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.[26] 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 measurement is around 5%–10%.[4,13,26]

General Procedure for the Preparation of Compounds 6–25

The *N*-hydroxysulfonamides **6–25** were prepared as previously described by this[11a] and Blackburn's groups,[27] by reaction of arylsulfonyl halides or sulfonic acid anhydrides, with hydroxylamine in alcoholic–aqueous medium.

Methods A and B An amount of 10 mM sulfonyl halide (chloride for method A, and fluoride for method B) was dissolved/suspended in 50 ml of methanol and the stoichiometric amount (10 mM, 0.69 g) of hydroxylamine hydrochloride dissolved in 10 ml of water was added. The mixture was magnetically stirred at 4°C for 10 min, then the calculated amount of solid $NaHCO_3$ was added and stirring was continued for 4–6 h (slightly longer times were needed for method B, i.e., 8–10 h) till all the sulfonyl halide was consumed in the reaction with the nucleophile. The solvent was evaporated under reduced pressure and the precipitated *N*-hydroxysulfonamides were recrystallized from ethanol.

Method C Seventy mg (10 mmol) of hydroxylamine hydrochloride and 0.84 ml (5 mmol) of triflic anhydride were suspended in 10 ml of acetone and 0.70 ml (10 mmol) of triethylamine were added dropwise. The mixture was magnetically stirred at 4°C for 15 h. The solvent was then evaporated in vacuo, and the tan residue treated with 5 ml of cold water. The triflate salts being water soluble were thus separated from CF_3SO_2NHOH (much less water soluble) by a simple filtration. The latter compound was recrystallized from *iso*-propanol.

Method D Seventy mg (10 mmol) of hydroxylamine hydrochloride, 0.70 ml (10 mmol) of triethylamine and 10 mmol of sulfobenzoic cyclic anhydride or tetrabromo-*O*-sulfobenzoic cyclic anhydride were heated at

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refluxation in 50 ml of anhydrous acetonitrile for 2 h, with a small amount of *p*-toluenesulfonic acid added as catalyst. After evaporation of the solvent, the products were treated with 10 ml of water, the precipitated filtered and recrystallized from ethanol.

Synthesis of Hydroxamic Acids 26 and 27

An amount of 10 mmol of acyl chloride in 20 ml of anhydrous terahydrofuran was treated with the stoichiometric amount of hydroxylamine hydrochloride suspended in 10 ml of anhydrous methanol. The calculated amount of triethylamine was added in order to neutralize the HCl, and the mixture magnetically stirred at 4°C for 2–4 h. After evaporation of the solvent, a small amount of water was added to the oil obtained and the hydroxamic acids **26** and **27** which precipitated after several hours at 4°C were recrystallized from methanol.

Synthesis of Derivatives 28–30

An amount of 0.76 ml (5 mmol) tosyl isocyanate dissolved in 10 ml of anhydrous acetonitrile was treated with 5 mmol of hydroxylamine hydrochloride (and the stoichiometric amount of Et_3N necessary to neutralize the HCl), sulfamic acid or sulfamide, dissolved in 40 ml of anhydrous acetonitrile. The mixture was stirred at room temperature for 1 h, then the solvent was evaporated and the precipitated derivatives were washed with 10 ml of water, filtered off and recrystallized from ethanol–water (1 : 2, v/v). Yields were almost quantitative.

N,N-Dimethyl-N'-hydroxy-sulfamide, **6** Colorless crystals, mp 130–2°C. IR (KBr), cm^{-1}: 1140 (SO_2^{sym}), 1334 (SO_2^{as}), 3060 (NH), 3370 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 4.80 (s, 6H, Me_2N); 8.66 (s, 1H, SO_2NH); 9.89 (s, 1H, OH). Found, C, 17.09; H, 5.61; N, 19.72. $C_2H_8N_2O_3S$ requires C, 17.14; H, 5.75; N, 19.99%.

N-Hydroxy-phenylmethylsulfonamide, **7** Colorless crystals, mp 128–9°C. IR (KBr), cm^{-1}: 1176 (SO_2^{sym}), 1365 (SO_2^{as}), 3060 (NH), 3380 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.17 (s, 2H, $PhCH_2$); 7.15–7.49 (m, 5H, ArH from Ph); 9.08 (s, 1H, SO_2NH); 10.03 (s, 1H, OH). Found, C, 44.54; H, 4.69; N, 7.20. $C_7H_9NO_3S$ requires C, 44.91; H, 4.85; N, 7.48%.

N-Hydroxy-trifluoromethylsulfonamide, **8** Colorless crystals, mp 204–5°C (dec). IR (KBr), cm^{-1}: 1169 (SO_2^{sym}), 1345 (SO_2^{as}), 3060 (NH), 3390 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 0.90 (t, 3H, Me from ethyl); 9.85 (s, 1H, SO_2NH); 10.80 (s, 1H, OH). Found, C, 6.95; H, 1.19; N, 8.37. $CH_2F_3NO_3S$ requires C, 7.28; H, 1.22; N, 8.48%.

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N-Hydroxy-4-fluorophenylsulfonamide, **9** Colorless crystals, mp 213–5°C. IR (KBr), cm^{-1}: 1171 (SO_2^{sym}), 1360 (SO_2^{as}), 3060 (NH), 3380 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.11–7.49 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-F-phenylene); 9.30 (s, 1H, SO_2NH); 10.09 (s, 1H, OH). Found, C, 37.62; H, 3.19; N, 7.27. $C_6H_6FNO_3S$ requires: C, 37.70; H, 3.16; N, 7.33%.

N-Hydroxy-4-chlorophenylsulfonamide, **10** Colorless crystals, mp 220–1°C. IR (KBr), cm^{-1}: 1175 (SO_2^{sym}), 1366 (SO_2^{as}), 3060 (NH), 3385 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.10–7.49 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-Cl-phenylene); 9.30 (s, 1H, $S0_2NH$); 10.12 (s, 1H, OH). Found, C, 37.89; H, 2.60; N, 6.38. $C_6H_6ClNO_3S$ requires: C, 34.71; H, 2.91; N, 6.75%.

N-Hydroxy-4-bromophenylsulfonamide, **11** Colorless crystals, mp 211–2°C. IR (KBr), cm^{-1}: 1179 (SO_2^{sym}), 1370 (SO_2^{as}), 3060 (NH), 3365 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.15–7.47 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-Br-phenylene); 9.25 (s, 1H, SO_2NH); 10.12 (s, 1H, OH). Found, C, 28.25; H, 2.11; N. 5.30. $C_6H_6BrNO_3S$ requires: C, 28.59; H, 2.40; N, 5.56%.

N-Hydroxy-4-iodophenylsulfonamide, **12** Colorless crystals, mp 223–5°C. IR (KBr), cm^{-1}: 1185 (SO_2^{sym}), 1377 (SO_2^{as}), 3060 (NH), 3390 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.10–7.54 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-I-phenylene); 9.19 (s, 1H, SO_2NH); 10.11 (s, 1H, OH). Found, C, 23.80; H, 1.95; N, 4.39. $C_6H_6INO_3S$ requires: C, 24.10; H, 2.02; N, 4.69%.

N-Hydroxy-p-toluenesulfonamide, **13** Colorless crystals, mp 149°C, lit.[28a,b] mp 148°C. IR (KBr), cm^{-1}: 1170 (SO_2^{sym}), 1315 (SO_2^{as}), 3060 (NH), 3375 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, Me); 7.10–7.60 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-Me-phenylene); 9.21 (s, 1H, SO_2NH); 10.12 (s, 1H, OH). ·Found, C, 44.95; H, 4.66; N, 7.19. $C_7H_9NO_3S$ requires: C, 44.91; H, 4.85; N, 7.48%.

N-Hydroxy-4-nitrophenylsulfonamide, **14** Yellow crystals, mp 118–21°C. IR (KBr), cm^{-1}: 1152 (SO_2^{sym}), 1368 (SO_2^{as}), 3060 (NH), 3380 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.08–7.66 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-O_2N-phenylene); 9.17 (s, 1H, SO_2NH); 10.19 (s, 1H, OH). Found, C, 32.85; H, 2.54; N, 12.70. $C_6H_6N_2O_5S$ requires: C, 33.03; H, 2.77; N, 12.84%.

N-Hydroxy-3-nitrophenylsulfonamide, **15** Yellow crystals, mp 110–2°C. IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1342 (SO_2^{as}), 3065 (NH), 3380 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.08–7.50 (m, 4H, ArH, *m*-O_2N-phenylene); 9.15 (s, 1H, SO_2NH); 10.21 (s, 1H, OH). Found, C, 32.73; H, 2.49; N, 12.61. $C_6H_6N_2O_5S$ requires: C, 33.03; H, 2.77; N, 12.84%.

N-Hydroxy-2-nitrophenylsulfonamide, **16** Yellow crystals, mp 101–2°C. IR (KBr), cm^{-1}: 1173 (SO_2^{sym}), 1380 (SO_2^{as}), 3060 (NH), 3370 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.12–7.58 (m, 4H, ArH, *o*-O_2N-phenylene);

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9.14 (s, 1H, SO_2NH); 10.08 (s, 1H, OH). Found, C, 33.12; H, 2 71; N, 12.75. $C_6H_6N_2O_5S$ requires: C, 33.03; H, 2.77; N, 12.84%.

N-Hydroxy-3-nitro-4-chlorophenylsulfonamide, **17** Yellow crystals, mp 119–22°C. IR (KBr), cm^{-1}: 1155 (SO_2^{sym}), 1340 (SO_2^{as}), 3060 (NH), 3380 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.09–7.60 (m, 3H, ArH); 9.25 (s, 1H, SO_2NH); 10.20 (s, 1H, OH). Found, C, 28.65; H, 1.72; N, 10.78. $C_6H_5ClN_2O_5S$ requires: C, 28.53; H, 1.99; N, 11.09%.

N-Hydroxy-4-acetylaminophenylsulfonamide, **18** Colorless crystals, mp 147–9°C. IR (KBr), cm^{-1}: 1152 (SO_2^{sym}), 1350 (SO_2^{as}), 1533 (amide II), 1680 (amide I), 3060 (NH), 3390 (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.80 (s, 3H, Me from Ac); 6.18 (s, 1H, Ac*NH*); 7.07–7.60 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-AcNH-phenylene); 9.18 (s, 1H, SO_2NH); 10.20 (s, 1H, OH). Found, C, 41.82; H, 4.41; N, 12.10. $C_8H_{10}N_2O_4S$ requires: C, 41.73; H, 4.38; N, 12.17%.

N-Hydroxy-4-aminophenylsulfonamide, **19** Colorless crystals, mp 204–5°C. IR (KBr), cm^{-1}: 1155 (SO_2^{sym}), 1348 (SO_2^{as}), 3060 (NH, NH_2), 3385 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 5.46 (s, 2H, H_2N-phenylene) 7.05–7.65 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-H_2N-phenylene); 9.19 (s, 1H, SO_2NH); 10.23 (s, 1H, OH). Found, C, 38.15; H, 4.45; N, 14.72. $C_6H_8N_2O_3S$ requires: C, 38.29; H, 4.28; N, 14.88%.

N-Hydroxy-3-aminophenylsulfonamide, **20** Tan crystals, mp 203–6°C. IR (KBr), cm^{-1}: 1172 (SO_2^{sym}), 1360 (SO_2^{as}), 3060 (NH, NH_2), 3385 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 5.18 (s, 2H, H_2N-phenylene) 7.21–7.45 (m, 4H, ArH, *m*-H_2N-phenylene); 9.15 (s, 1H, SO_2NH); 10.18 (s, 1H, OH). Found, C, 38.52; H, 4.02; N, 14.79. $C_6H_8N_2O_3S$ requires: C, 38.29; H, 4.28; N, 14.88%.

N-Hydroxy-pentafluorolphenylsulfonamide, **21** Colorless crystals, mp 105–8°C (dec.). IR (KBr), cm^{-1}: 1148 (SO_2^{sym}), 1332 (SO_2^{as}), 3060 (NH), 3385 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 9.78 (s, 1H, SO_2NH); 10.44 (s, 1H, OH). Found, C, 27.22; H, 0.62; N, 5.14. $C_6H_2F_5N0_3S$ requires: C, 27.39; H, 0.77; N, 5.32%.

N-Hydroxy-2-carhoxyphenylsulfonamide, **22** Colorless crystals, mp 91–3°C. IR (KBr), cm^{-1}: 1153 (SO_2^{sym}), 1358 (SO_2^{as}), 1720 (COOH), 3060 (NH), 3390 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.15–7.43 (m, 4H, ArH, *o*-HOOC-phenylene); 9.14 (s, 1H, SO_2NH); 10.15 (br s, 1H, COOH); 10.26 (s, 1H, OH). Found, C, 38.54; H, 3.09; N, 6.28. $C_7H_7NO_5S$ requires: C, 38.71; H, 3.25; N, 6.45%.

N-Hydroxy-2-carhoxytetrabromophenylsulfonamide, **23** Colorless crystals, mp 111–2°C. IR (KBr), cm^{-1}: 1158 (SO_2^{sym}), 1377 (SO_2^{as}), 1720 (COOH),

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3060 (NH), 3400 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 9.29 (s, 1H, SO_2NH); 10.32 (br s, 1H, COOH); 10.45 (s, 1H, OH). Found, C, 15.50; H, 0.43; N, 2.61. $C_7H_3Br_4NO_5S$ requires: C, 15.78; H, 0.57; N, 2.63%.

N-Hydroxy-p-methoxyphenylsulfonamide, **24** Colorless crystals, mp 140–1°C. IR (KBr), cm^{-1}: 1170 (SO_2^{sym}), 1319 (SO_2^{as}), 3060 (NH), 3385 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.50 (s, 3H, MeO); 7.10–7.66 (m, AA′BB′, $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-MeO-phenylene); 9.20 (s, 1H, SO_2NH); 10.12 (s, 1H, OH). Found, C, 41.56; H, 4.13; N, 6.79. $C_7H_9NO_4S$ requires: C, 41.37; H, 4.46; N, 6.89%.

N-Hydroxy-2,4,6-trimethylphenylsulfonamide, **25** Colorless crystals, mp 119–22°C. IR (KBr), cm^{-1}: 1170 (SO_2^{sym}), 1312 (SO_2^{as}), 3060 (NH), 3370 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, 4-Me); 2.75 (s, 6H, 2,6-Me_2); 7.10–7.63 (m, 2H, ArH); 9.20 (s, 1H, SO_2NH); 10.17 (s, 1H, OH). Found, C, 50.12; H, 6.01; N, 6.28. $C_9H_{13}NO_3S$ requires: C, 50.22; H, 6.09; N, 6.51%.

Isonicotinoylhydroxamic acid, **26** Tan crystals, mp 145–7°C. IR (KBr), cm^{-1}: 1280 (amide III), 1565 (amide II), 1620 (C=N), 1680 (amide I), 3060 and 3190 (NH), 3390 (OH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.15–7.72 (m, AA′BB′, $J_{AB} = 7.9$ Hz, 4H, ArH); 7.95 (s, 1H, CONH); 8.65 (s, 1H, OH); Found, C, 52.25; H, 4.09; N, 20.11. $C_6H_6N_2O_2$ requires: C, 52.17; H, 4.38; N, 20.28%.

3,4-Dichlorophenylhydroxamic acid, **27** White crystals, mp 152–4°C. IR (KBr), cm^{-1}: 1285 (amide III), 1550 (amide II), 16845 (amide I), 3060 and 3180 (NH), 3390 (OH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.05–7.64 (m, 3H, ArH); 7.90 (s, 1H, CONH); 8.53 (s, 1H, OH). Found, C, 45.10; H, 3.09; N, 12.01. $C_7H_5Cl_2NO_2$ requires: C, 45.37; H, 3.22, N, 12.21%.

O-(4-Tosylamidocarbonyl)-hydroxylamine, **28** Colorless crystals, mp 198–201°C. IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1295 (amide III), 1360 (SO_2^{as}), 1528 (amide II), 1670 (amide I), 3060 and 3190 (NH), 3360 br (OH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, Me from tosyl); 7.05–756 (m, AA′BB′, $J_{AB} = 7.1$ Hz, 4H, ArH, phenylene from tosyl); 8.82 (s, 2H, ONH_2); 8.91 (s, 1H, SO_2NH). Found, C, 41.86; H, 4.23; N, 11.95. $C_8H_{10}N_2O_4S$ requires: C, 41.73; H, 4.38; N, 12.17%.

O-(4-Tosylamidocarbonyl)-sulfamic acid, **29** Colorless crystals, mp 234–7°C (dec.). IR (KBr), cm^{-1}: 1120 (SO_2^{sym}), 1290 (amide III), 1340 (SO_2^{as}), 1535 (amide II), 1680 (amide I), 3060 and 3190 (NH); ^{1}H-NMR (DMSO-d_6). δ, ppm: 2.50 (s, 3H, Me from tosyl); 7.05–7.55 (m, AA′BB′, $J_{AB} = 7.1$ Hz, 4H, ArH, phenylene from tosyl); 7.91 (s, 1H, SO_2NH); 8.50 (s, 2H, SO_2NH_2). Found, C, 32.76; H, 3.30; N, 9.29. $C_8H_{10}N_2O_6S_2$ requires: C, 32.65; H, 3.42; N, 9.52%.

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N-(4-Tosylamidocarbonyl)-sulfamide, **30** Colorless crystals, mp 265–6°C (dec.). IR (KBr), cm^{-1}: 1125 (SO_2^{sym}), 1290 (amide III), 1350 (SO_2^{as}), 1550 (amide II), 1680 (amide I), 3060 and 3190 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, Me from tosyl); 7.05–7.55 (m, AA′BB′, $J_{AB} = 7.1$ Hz, 4H, ArH, phenylene from tosyl); 7.91 (s, 1H, SO_2NH); 8.70 (s, 2H, SO_2NH_2); 9.12 (s, 1H, $CONHSO_2$). Found, C, 32.55; H, 3.70; N, 14.25. $C_8H_{11}N_3O_5S_2$ requires: C, 32.76; H, 3.78; N, 14.33%.

Measurement of Tonometric IOP

Adult male New Zealand albino rabbits weighing 3–3.5 kg were used in the experiments (three animals were used for each inhibitor studied). The experimental procedures conform to those of the Association for Research in Vision and Ophthalmology Resolution on the use of animals. 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 water or DMSO–water (1 : 4, v/v) (in the case of compounds **9** and **29**, **30**) 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/increasing effects.

IOP was measured using a Digilab 30R pneumatonometer (BioRad, Cambridge, MA, USA) as described by Maren's group.[29,30] 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.[29,30] All data are expressed as mean ± SE, using a one-tailed *t* test.

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RESULTS AND DISCUSSION

Compounds **6–25** have been synthesized by standard procedures, starting from sulfonyl halides or sulfonic acid anhydrides and hydroxylamine (Table I). The related derivatives **26** and **27** were obtained by reaction of acyl chlorides with the same nucleophile, whereas derivatives **28–30**, as shown in Scheme 1, were obtained from tosyl isocyanates and hydroxylamine, sulfamic acid or sulfamide, respectively.

The compounds **6–30** were characterized by standard physico-chemical methods (elemental analysis, spectroscopy, etc.) which confirmed their structures. A remark should be made regarding the reaction of tosyl isocyanate with nucleophiles (Scheme 1). It is well-known[31] that this very reactive isocyanate converts alcohols (ROH), amines (RNH_2, but secondary amines also react), as well as other active hydrogen containing compounds, to the corresponding carbamates or ureas possessing the general formulas TsNHCOOR and TsNHCONHR, respectively. Since both hydroxylamine (H_2N-OH), as well as sulfamic acid ($HO-SO_2-NH_2$) contain an OH as well as an NH_2 group that might be derivatized by this reagent, it appeared of interest to investigate which group reacts

TABLE I *N*-Hydroxysulfonamides (RSO_2NHOH) **6–25** prepared in the present study and their method of synthesis

Compound	*R*	*Yield*	*Method of synthesis*
6	Me_2N-	54	A
7	$PhCH_2$-	51	B
8	CF_3-	60	C
9	*p*-F-C_6H_4-	62	A
10	*p*-Cl-C_6H_4-	76	A
11	*p*-Br-C_6H_4-	73	A
12	*p*-I-C_6H_4-	85	A
13	*p*-CH_3-C_6H_4-	69	A
14	*p*-O_2N-C_6H_4-	55	A
15	*m*-O_2N-C_6H_4-	55	A
16	*o*-O_2N-C_6H_4-	42	A
17	3-Cl-4-O_2N-C_6H_3-	55	A
18	*p*-AcNH-C_6H_4-	89	A
19	*p*-H_2N-C_6H_4-	50	B
20	*m*-H_2N-C_6H_4-	39	B
21	C_6F_5-	82	A
22	*o*-HOOC-C_6H_4-	93	D
23	*o*-HOOC-C_6Br_4-	88	D
24	*p*-CH_3O-C_6H_4-	69	A
25	2,4,6-$(CH_3)_3$-C_6H_2-	69	A

A – hydroxylamine + RSO_2Cl; B – hydroxylamine + RSO_2F; C – hydroxylamine + triflic anhydride; D – hydroxylamine + sulfobenzoic cyclic anhydride.

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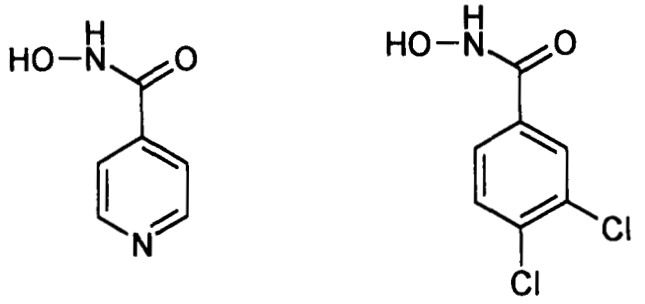
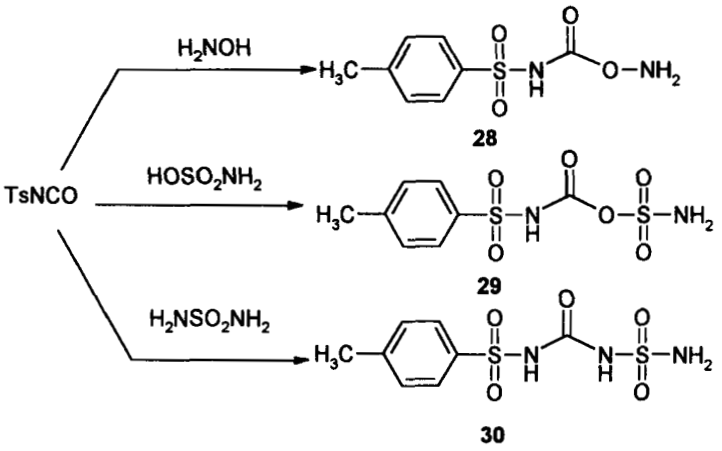


26 **27**

STRUCTURES 26 and 27



SCHEME 1

first. Spectroscopic data of the prepared compounds (**28–30**) indicated (see Experimental part for details) that it is the hydroxy group which reacted when one mole of TsNCO and one mole of the above-mentioned nucleophiles were mixed, although bis-derivatized compounds (at the OH and NH_2 groups) could also be prepared and purified (data not shown, since these derivatives did not show interesting CA inhibitory properties).

Inhibition data with compounds **6–30** as well as standard CA inhibitors **1–5**, against three isozymes, CA I, II and IV, are shown in Table II.

The following remarks can be made with regard to the inhibition data of Table II. *N*-hydroxy-sulfonamides **6–25**, hydroxamic acids **26** and **27**, as well as tosylisocyanate derived compounds **28–30** genarally act as efficient

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TABLE II CA inhibition data for compounds **6**–**30** and standard CA inhibitors **1**–**5** (IC_{50} – the mean of two different assays – represents the molarity of inhibitor producing a 50% decrease of enzyme specific activity for the CO_2 hydration reaction, by Maren's micro-method[26])

Compound	IC_{50} (nM)*		
	hCA I[a]	*hCA II*[a]	*bCA IV*[b]
1 (acetazolamide)	200 ± 14	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
4 (dichlorophenamide)	900 ± 50	12 ± 0.6	139 ± 3
5 (dorzolamide)	30,000 ± 500	2 ± 0.1	3 ± 0.1
6	12,000 ± 600	4 ± 0.5	29 ± 1
7	19,300 ± 200	190 ± 4	460 ± 10
8	20,400 ± 300	3 ± 0.3	16 ± 0.2
9	15,600 ± 450	19 ± 1	90 ± 5
10	27,000 ± 200	21 ± 0.8	120 ± 7
11	38,000 ± 500	18 ± 2	39 ± 5
12	15,000 ± 300	13 ± 2	45 ± 3
13	51,000 ± 600	70 ± 4	127 ± 8
14	57,000 ± 500	9 ± 0.3	70 ± 5
15	17,000 ± 100	5 ± 0.5	24 ± 1
16	15,000 ± 200	5 ± 0.4	13 ± 0.7
17	17,000 ± 600	4 ± 0.3	12 ± 0.9
18	40,000 ± 500	29 ± 3	40 ± 2
19	62,000 ± 800	37 ± 1	55 ± 3
20	39,000 ± 300	45 ± 3	124 ± 10
21	30,000 ± 200	0.8 ± 0.1	13 ± 1
22	25,000 ± 100	09 ± 0.1	12 ± 2
23	18,000 ± 100	08 ± 0.1	9 ± 1
24	68,000 ± 300	51 ± 6	110 ± 8
25	72,000 ± 1000	62 ± 4	156 ± 13
26	200 ± 11	10 ± 0.7	30 ± 2
27	510 ± 20	18 ± 2	39 ± 4
28	6,900 ± 300	5 ± 0.3	19 ± 1
29	12,000 ± 100	2 ± 0.08	25 ± 3
30	20,000 ± 200	1 ± 0.1	30 ± 2

*Mean ± average spread (from two determinations).
[a]Human (cloned) isozyme; [b]Isolated from bovine lung microsomes.

inhibitors of CA II and CA IV, being less effective against CA I. The affinity of these new classes of inhibitors towards the different isozymes is quite diverse. Thus, in the case of the *N*-hydroxysulfonamides, hCA II is quite susceptible to inhibition with these derivatives (IC_{50} values for the most active compounds in the series in the nanomolar range), whereas bCA IV is 2–14 times less susceptible. Still, it should be mentioned that all the new inhibitors reported here have higher affinity for bCA IV as compared to the classical inhibitors acetazolamide **1**, methazolamide **2** or dichlorophenamide **4**. hCA I on the other hand may be inhibited at only much

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larger (micromolar) concentrations of derivatives **6**–**25**, in contrast to the classical sulfonamide inhibitors of the type **1**–**4**, which possess an increased affinity for this slow isozyme (Table II). This feature is anyhow considered a positive one from the point of view of the putative ophthalmologic applications of these CA inhibitors (see later in the text), since the isozymes involved in aqueous humor secretion are CA II and IV, but not CA I.[13–17]

In the series of *N*-hydroxysulfonamides reported here, the nature of the groups to which the SO_2NHOH moiety is linked, is very important for the biological activity of the obtained compounds. Thus, the most potent hCA II inhibitors were those containing the trifluoromethyl- (**8**), pentafluorophenyl- (**21**) and 2-carboxyphenyl- (**22** and **23**) moieties. Very good inhibition was also observed for the trisubstituted sulfamide **6**, whereas the only such aralkyl compound in the series, (**7**) was one of the least active inhibitors against both hCA II and bCA IV. In the series of *N*-hydroxy-substituted-benzenesulfonamides (**9**–**20**), substitution patterns leading to good biological activity were essentially those involving nitro groups (such as in **14**–**17**). Still, the most potent CA II and CA IV inhibitors were compounds **21**–**23** mentioned above.

The two hydroxamic acids **26** and **27** were much more efficient hCA I inhibitors, as compared to the above-mentioned *N*-hydroxysulfonamides. These two compounds also acted as good hCA II and bCA IV inhibitors. In fact only recently Christianson's group[32] reported the X-ray crystallographic structure for the adducts of hCA II with $CH_3CONHOH$ and $CF_3CONHOH$, the first CA inhibitors of this type. These last two compounds possess a lower affinity for the enzyme (IC_{50} in the micromolar range, i.e., 47 μM for the former compound, and 3.8 μM for the latter both against hCA II),[32] but their binding mode is quite interesting as they interact with the Zn(II) ion of the CA active site by mean of the ionized nitrogen atom of the hydroxamate moiety, and in the case of the trifluoro-derivative, also by means of a fluorine atom. In addition, hydrogen bonds between the hydroxamate OH and active site residue Thr 199 were also seen,[32] which presumably further stabilize the E–I adduct (Figure 1).

As seen from the data of Table II, the aromatic/heterocyclic hydroxamic acids described here are much more effective CA inhibitors than the aliphatic derivatives reported by Christianson *et al.*[32] This is probably due to the presence of the bulky aromatic/heterocyclic ring which contributes to a further stabilization of the E–I adduct. The binding mode of these aromatic/heterocyclic hydroxamates described by us is probably similar to that of $CF_3CONHOH$, mentioned above, involving coordination to the

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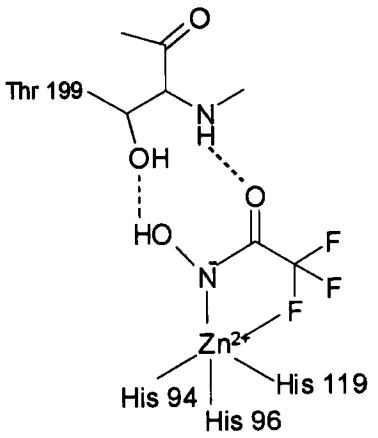

FIGURE 1 Schematic binding of trifluoromethyl hydroxamate to hCA II (adapted from Ref. 32).

Zn(II) ion within the CA active site, by means of the ionized nitrogen atom of the hydroxamic acid moiety.

Finally, derivatives **28–30** constitute completely new structural types of CA inhibitors, since they either do not possess at all a primary sulfonamido group (derivative **28**), or this group is not directly linked to an aromatic/heterocyclic ring (compounds **29**, **30**). The only CA inhibitors previously reported with which these compounds might be somehow related are the sulfamates of the type $Ar-OCH_2CH_2O-SO_2NH_2$ prepared and investigated by Maren's group.[33] Still, there are considerable differences between our and Maren's group's compounds, primarily due to the presence of the carbonyl moieties in the first derivatives. These groups not only probably greatly influence physico-chemical properties of the inhibitors (such as the pK_a value of the sulfonamido moiety), but might contribute to supplementary interactions between the inhibitor and the amino acid residues within the CA active site, in the E–I adduct. Indeed, these compounds act as very strong hCA II inhibitors, with potencies comparable to acetazolamide ethoxzolamide or dorzolamide (it should be mentioned that except for $CF_3SO_2NH_2$ and related perhaloalkylsulfonamides, aliphatic sulfonamides are extremely weak CA inhibitors, with affinity constants for hCA II in the millimolar range).[4,13a]

The promising *in vitro* CA inhibitory activity of some of the newly prepared compounds prompted us to investigate them *in vivo*, for their effects on the intraocular pressure (IOP), after topical application directly into the

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eye, in normotensive rabbits, frequently used as an animal model of glaucoma.[14,15,29,30,34] Some of these results are shown in Table III and Figure 2.

The inhibitors selected for *in vivo* studies were among the most active against hCA II and IV, in the prepared series, i.e., **6, 9, 14, 18, 21, 22, 26, 29** and **30**. The following facts should be noted regarding the data of Table III: (i) a first group of compounds, such as **14, 18, 21** and **29**, showed much more effective IOP lowering effects as compared to dorzolamide **5**, both at 30 min after administration of the inhibitor within the rabbit eye, as well as the other times for which measurements were recorded (1, 1.5 and 2 h, respectively); (ii) a second group of inhibitors (**6, 9, 26** and **30**), showed no IOP lowering effects at half-an-hour after administration, but exhibited an important such effect (comparable or better than those of dorzolamide) at 1 h or later periods after administration. This is probably due to different kinetics of penetration through the eye tissues of these compounds, which generally belong to classes of CA inhibitors which were not investigated previously as IOP lowering agents; (iii) although highly water soluble, and behaving as a very efficient CA inhibitor (Table II), compound **22** did not show any IOP lowering effect. Probably its physico-chemical properties do not allow an easy penetration through biological membranes, similarly to the classical CA inhibitors, such as acetazolamide, methazolamide, or ethoxzolamide, which do not possess topical IOP lowering effects.

In conclusion, in the present paper we present novel classes of effective CA inhibitors, such as the *N*-hydroxysulfonamides, some aromatic/heterocyclic hydroxamates as well as compounds obtained by reaction of tosyl isocyanate with hydroxylamine, sulfamic acid and sulfamide. These

TABLE III IOP after treatment with one drop (50 μL) solution 2% of CA inhibitor directly into the rabbit eye, at 30, 60 and 90 min after administration

Inhibitor	ΔIOP (mmHg)*			
	$t = 0$	$t = 30$ min	$t = 60$ min	$t = 90$ min
5 (dorzolamide)	0	2.2 ± 0.10	4.1 ± 0.15	2.7 ± 0.08
6	0	0	2.5 ± 0.12	7.5 ± 0.18
9	0	0	2.2 ± 0.10	3.1 ± 0.14
14	0	5.1 ± 0.10	5.1 ± 0.08	4.0 ± 0.07
18	0	6.5 ± 0.15	9.5 ± 0.10	6.5 ± 0.09
21	0	3.1 ± 0.05	7.5 ± 0.13	7.5 ± 0.10
22	0	0	0	0
26	0	0.5 ± 0.06	4.0 ± 0.09	6.2 ± 0.11
29	0	4.0 ± 0.12	10.1 ± 0.10	10.0 ± 0.15
30	0	0	10.3 ± 0.20	11.2 ± 0.09

*$\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$; Mean ± average spread ($n = 3$).

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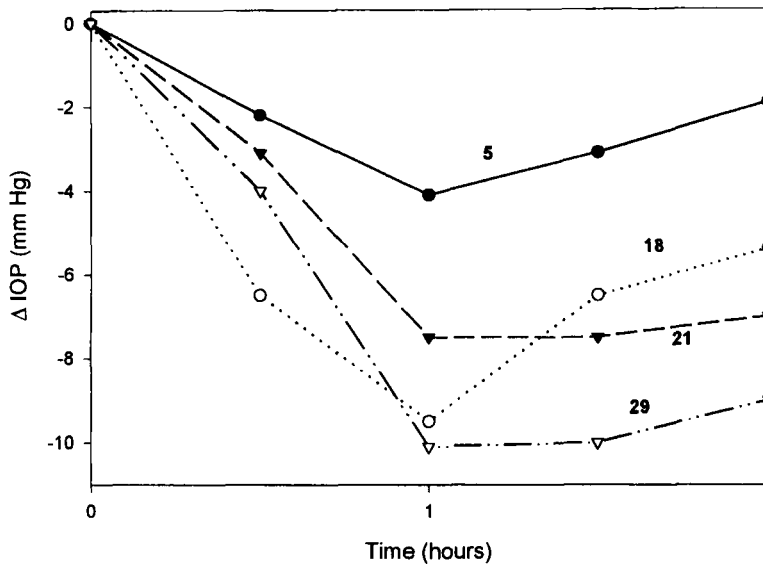

FIGURE 2 Lowering of IOP in the rabbit eyes after topical administration of one drop (50 μL) of 2% solution of the following CA inhibitors: dorzolamide **5** (as standard), and compounds **18**, **21** and **29**.

compounds showed good *in vitro* CA II and IV inhibitory properties, and some of them were more effective *in vivo* as IOP lowering agents as compared to the clinical drug dorzolamide, *via* the topical route.

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