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CARBONIC ANHYDRASE INHIBITORS: SYNTHESIS OF SULFONAMIDES INCORPORATING 2,4,6-TRISUBSTITUTED-PYRIDINIUM-ETHYLCARBOXAMIDO MOIETIES POSSESSING MEMBRANE-IMPERMEABILITY AND *IN VIVO* SELECTIVITY FOR THE MEMBRANE-BOUND (CA IV) VERSUS THE CYTOSOLIC (CA I AND CA II) ISOZYMES*,†

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A new approach is proposed for the selective *in vivo* inhibition of membrane-bound versus cytosolic carbonic anhydrase (CA, EC 4.2.1.1) isozymes with a class of positively-charged, membrane-impermeant sulfonamides. Aromatic/heterocyclic sulfonamides acting as strong (but unselective) inhibitors of this zinc enzyme were derivatized by the attachment of trisubstituted-pyridinium-ethylcarboxy moieties (obtained from 2,4,6-trisubstituted-pyrylium salts and β-alanine) to the amino, imino, hydrazino or hydroxyl groups present in their molecules. Efficient *in vitro* inhibition (in the nanomolar range) was observed with some of the new derivatives against three investigated CA isozymes, i.e., hCA I, hCA II (cytosolic forms) and bCA IV (membrane-bound isozyme; h = human; b = bovine isozyme). Due to their salt-like character, the new type of inhibitors reported here, unlike the classical, clinically used compounds (such as acetazolamide, methazolamide, ethoxzolamide), are unable to penetrate biological membranes,

* See Ref. [1].

† This paper is dedicated to the memory of Tom Maren (1917–1999), a major contributor to the chemistry and physiology of carbonic anhydrase inhibitors.

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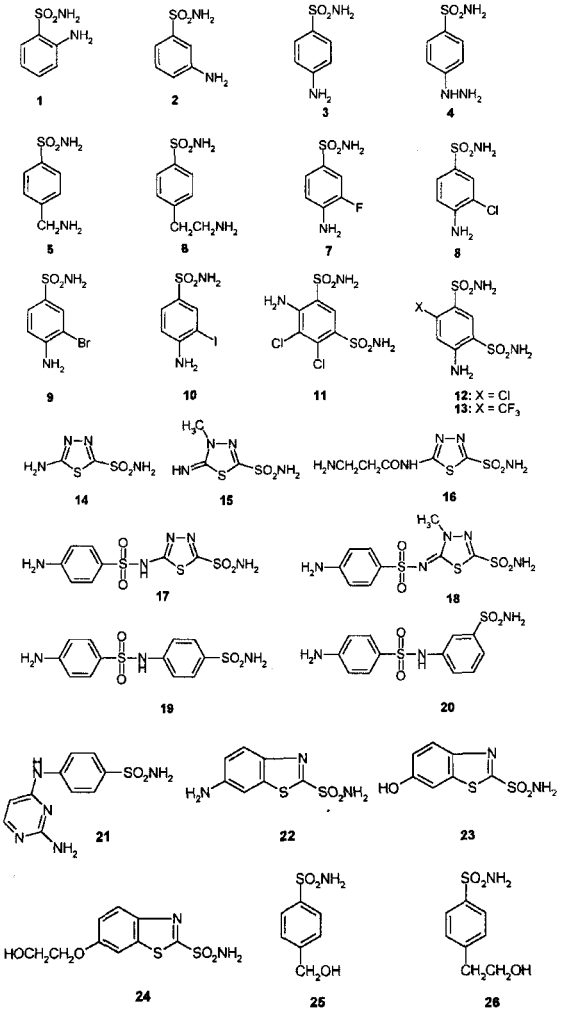
as shown by *ex vivo* and *in vivo* perfusion experiments in rats. The level of bicarbonate excreted into the urine of the experimental animals perfused with solutions of the new and classical inhibitors suggest that: (i) when using the new type of positively-charged sulfonamides, only the membrane-bound enzyme (CA IV) was inhibited, whereas the cytosolic isozymes (CA I and II) were not affected, (ii) in the experiments in which the classical compounds (acetazolamide, benzolamide, etc.) were used, unselective inhibition of all CA isozymes (I, II and IV) occurred.

Keywords: Carbonic anhydrase; Isozymes I, II, IV; Sulfonamide; Pyrylium salt; Pyridinium salt; Bicarbonate excretion

INTRODUCTION

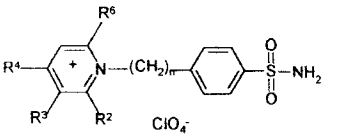
The 14 different carbonic anhydrase (CA, EC 4.2.1.1) isozymes or CA-related proteins (CA-RP) described up to now in higher vertebrates, including humans,[2] are involved in critical physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH homeostasis, electrolyte secretion in a variety of tissues/organs, as well as biosynthetic reactions, such as gluconeogenesis and ureagenesis among others.[3-5] Inhibition of some of these enzymes by aromatic/heterocyclic sulfonamides has been exploited clinically for more than 45 years in the treatment of a variety of diseases such as glaucoma, epilepsy, congestive heart failure, mountain sickness, gastric and duodenal ulcers, etc.[3,4,6] It should be also noted that a sulfonamide CA inhibitor was the first non-mercurial diuretic in clinical use in the early 1950s, and that it subsequently led to the development of the thiazide and high-ceiling diuretics, two classes of widely-used pharmacological agents.[4,7] The main draw-back of the presently available sulfonamide CA inhibitors is their total lack of specificity for the different CA isozymes. Thus, except for CA III, a muscle isozyme relatively resistant to inhibition by sulfonamides,[8] other CA isozymes, such as the cytosolic CA I, II, and VII, the membrane-bound forms CA IV, IX, XII and XIV, or the mitochondrial CA V, show very high and similar affinity (in the micro – nanomolar range) for this class of inhibitors. [2,9 14] Development of isozyme-specific or at least organ-selective inhibitors would be highly beneficial both for obtaining novel types of drugs, devoid of major side effects, as well as for many physiological studies in which specific/selective inhibitors would be valuable tools for understanding the physiology of these enzymes. Among the latest important developments in this field, one should note the recent isolation of three novel membrane-bound CA isozymes, CA IX,[10] XII[13] and XIV,[14] in addition to the "classical" one, CA IV, purified several years before by Sly's group.[15] Some of these isozymes were identified only in tumor cells, and little is known at the moment regarding the physiological consequences of their inhibition/activation.[13-16]

In previous contributions from this laboratory[17–22] it was shown that by attaching different "tails" to the molecules of aromatic/heterocyclic sulfonamides of types **1–26**, possessing free amino, hydrazino, imino or hydroxy groups, it is possible to obtain water-soluble, efficient inhibitors of several CA isozymes (such as CA I, II and IV), some of which showed promising anti-glaucoma activity via the topical route in experimental animals. It appeared thus of interest to explore a similar strategy with the intention of obtaining membrane-impermeant sulfonamide inhibitors, which should inhibit only the membrane-bound but not the cytosolic isozymes.



In some preliminary communications[21,23] we reported that the reaction of sulfanilamide **3**, and its homologues **5** and **6**, or that of the heterocyclic

derivative **14**, with substituted pyrylium salts affords new classes of tight-binding CA I, II and IV inhibitors, of the types shown below (**27–30**), which showed some selectivity *in vivo* for the inhibition of the membrane-bound isozyme bCA IV.



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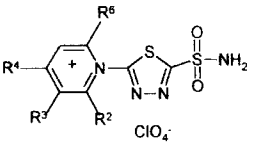
28: n = 1
29: n = 2



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In this paper we extend the above-mentioned studies,[21,23] and report the reaction of 26 aromatic/heterocyclic sulfonamides containing a free amino, imino, hydrazino or hydroxyl group, with 2,4,6-trisubstituted pyridinium-ethylcarboxylic acids of types **A–D** (obtained from β-alanine and pyrylium salts). The new compounds reported here were tested for the inhibition of three CA isozymes, hCA I, hCA II and bCA IV (h = human, b = bovine isozymes). Affinities in the nanomolar range were detected for many compounds against isozymes II and IV. In order to show that the new inhibitors are membrane-impermeant, some *ex vivo* and *in vivo* perfusion experiments in rats have also been performed. Thus, incubation of red blood cells with positively-charged, as well as classical CA inhibitors, showed that only with the latter did the erythrocytes became saturated with inhibitor, due to facilitated penetration of such compounds through the red cell membranes. The level of bicarbonate excreted into the urine of rats perfused with solutions of the new and classical inhibitors. The results showed that when using the positively-charged sulfonamides, only the membrane-bound enzyme was inhibited, whereas the cytosolic isozymes were not affected, contrary to the experiments in which the classical compounds were used (such as acetazolamide, benzolamide or methazolamide), which led to unselective inhibition of all CA isozymes (cytosolic and membrane-associated).

MATERIALS AND METHODS

Melting points were determined with a heating plate microscope (not corrected), IR spectra as KBr pellets, 400–4000 cm^{-1} on a Perkin-Elmer 16PC FTIR spectrometer and ^{1}H-NMR spectra on a Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard).

Elemental analysis was done on a Carlo Erba Instrument CHNS Elemental Analyzer, Model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Analytical and preparative HPLC was performed on a reversed-phase C_{18} Bondapack column, with a Beckman EM-1760 instrument. The pyridinium-ethylcarboxylic acids **A–D** were prepared as described in the literature[24,25] from β-alanine (Sigma) and the corresponding trisubstituted pyrylium salt. EDCI, diisopropyl carbodiimide, and triethylamine were from Sigma Chemical Co. Acetonitrile, acetone (E. Merck) or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions. Sulfonamides **1–26** used in synthesis were either commercially available compounds (from Sigma, Acros or Aldrich) or were prepared as described previously: 4-hydrazino-benzene-sulfonamide **4** by diazotization of sulfanilamide followed by reduction of the diazonium salt with tin(II) chloride;[26] halogenosulfanilamides **7–10** by halogenation of sulfanilamide as reported in the literature;[27] compound **16** from 5-amino-1,3,4-thiadiazole-2-sulfonamide (obtained from acetazolamide)[28] by acylation with the phthalimido-derivative of β-alanine, followed by hydrazinolysis,[29] and the imine **15** by deprotection of methazolamide with concentrated hydrochloric acid.[30] Aminobenzolamide **17** and the corresponding thiadiazoline **18** were obtained as reported in Ref. [31], the sulfanilyl-sulfanilamide **19** and its *meta*-derivative **20** as reported in Ref. [32] the benzothiazole-2-sulfonamide derivatives **22–24** as described in Ref. [33], and the alcohols **24** and **25** from the corresponding amines by diazotization followed by hydrolysis of the diazonium salts.

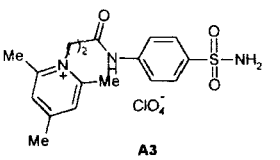
General Procedure for the Preparation of Compounds A–D

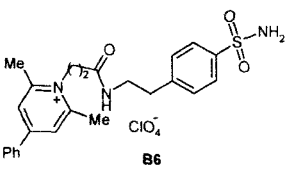
An amount of 20 mM of pyrylium salt **35(A–D)** and 1.80 g (20 mM) of β-alanine were suspended in 100 mL of anhydrous acetonitrile and heated at reflux for 15 min. Triethylamine (300 μL, 20 mM) was then added to the reaction mixture and refluxing continued for 2–3 h (TLC control). After cooling, 2 mL of glacial acetic acid was added and the pyridinium salt was precipitated with 200–300 mL of diethyl ether. Recrystallization from 5% perchloric acid afforded the pure title compounds.[24,25]

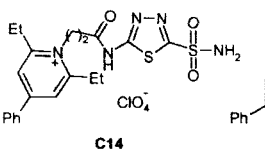
General Procedure for the Preparation of Compounds (A–D)1–26

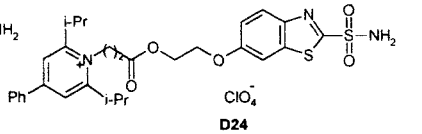
An amount of 3 mM sulfonamide **1–26** was dissolved/suspended in 50 mL of anhydrous acetonitrile and then treated with the stoichiometric amount (3 mM) of positively-charged carboxylic acid **A–D**. An amount of 570 mg

(3 mM) of (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) (EDCI)·HCl was added and the reaction mixture was magnetically stirred at room temperature for 15 min, then 90 μL (6 mM) of triethylamine was added and stirring was continued for 8–10 h at 4°C (TLC control). The solvent was evaporated *in vacuo* and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate, filtered and the solvent removed *in vacuo*. The pure compounds **(A–D)1–26** were obtained either by means of preparative HPLC (C_{18} reversed-phase μ-Bondapack or Dynamax-60A (25 × 250 mm) columns; 80% acetonitrile/8% methanol/12% water, 30 mL/min), or by recrystallization from 5% aqueous perchloric acid.









4-(2,4,6-Trimethylpyridinium-N-ethylcarboxamido)-benzenesulfonamide perchlorate **A3**, as white crystals, m.p. > 320°C. IR (KBr), cm^{-1}: 625 (ClO_4^-), 1100 (ClO_4^-), 1140 (SO_2^{sym}), 1295 (amide III), 1366 (SO_2^{as}), 1564 (amide II), 1710 (amide I), 3365 (NH, NH_2). UV (MeOH), λ_{max}, nm (lg ε): 224 sh (4.51), 275 (3.99). ^{1}H-NMR (TFA), δ, ppm: 2.62 (s, 6H, 2,6-$(Me)_2$), 2.74 (s, 3H, 4-Me), 2.85 (t, 2H, $C\underline{H}_2CH_2CO$), 3.19 (t, 2H, $CH_2C\underline{H}_2CO$), δ_A 7.18, δ_B 7.75 (AA′BB′system, 4H, $J_{AB} = 7.9$, ArH from 4-sulfamoylphenyl), 7.50 (br s, 2H, SO_2NH_2) (the CONH protons are in fast exchange with the solvent and are not seen in TFA), 8.10 (s, 2H, ArH, 3,5-H from pyridinium). ^{13}C-NMR (TFA), δ, ppm: 15.5 (s, Me), 17.9 (s, Me), 37.1 (s, $\underline{C}H_2CH_2CO$), 40.8 (s, $CH_2\underline{C}H_2CO$), 128.6, 129.4, 132.3, 133.5, 135.7, 138.4, 142.0, 172.9 ($\underline{C}ONH$). Anal. $C_{13}H_{21}N_2O_3S^+ClO_4^-$ (C, H, N, S).

4-(2,6-Dimethyl-4-phenylpyridinium-ethylcarboxamidoethyl)-benzenesulfonamide perchlorate **B6**, as white crystals, m.p. 298–299°C (dec.). IR (KBr), cm^{-1}: 625 (ClO_4^-), 1100 (ClO_4^-), 1157 (SO_2^{sym}), 1295 (amide III), 1354

(SO_2^{as}), 1570 (amide II), 1713 (amide I), 3365 (NH, NH_2). UV (MeOH), λ_{max}, nm (lg ε): 223.8 sh (4.13), 296 (4.21). ^{1}H-NMR (TFA), δ, ppm: 2.85 (t, 2H, C$\underline{H}_2$CH$_2$CO of β-Ala), 3.02 (s, 6H, 2,6-(Me)$_2$), 3.15 (t, 2H, $^3J_{HH} = 6.7$, α-CH$_2$), 3.21 (t, 2H, CH$_2$C$\underline{H}_2$CO of β-Ala), 3.80 (t, 2H, $^3J_{HH} = 6.7$, β-CH$_2$), 7.11–7.82 (m, 9H, ArH from 4-sulfamoylphenyl + 4-Ph), 7.55 (br s, 2H, SO_2NH_2) (the CONH protons are in fast exchange with the solvent and are not seen in TFA), 8.04 (s, 2H, 3,5-H from pyridinium). ^{13}C-NMR (TFA), δ, ppm: 17.4 (s, Me), 30.3 (s, $\underline{C}$H$_2$ of aminoethyl), 34.9 (s, $\underline{C}$H$_2$ of aminoethyl), 37.2 (s, $\underline{C}$H$_2$CH$_2$CO), 40.8 (s, CH$_2\underline{C}$H$_2$CO), 128.4, 130.7, 132.8, 133.6, 134.6, 145.5, 170.6 ($\underline{C}$ONH). Anal. $C_{23}H_{25}N_2O_3S^+ClO_4^-$ (C, H, N, S).

5-(2,6-Diethyl-4-phenylpyridinium-ethylcarboxamido)-1,3,4-thiadiazol-2-sulfonamide perchlorate **C14**, as white crystals, m.p. > 320°C. IR (KBr), cm^{-1}: 625 (ClO_4^-), 1100 (ClO_4^-), 1179 (SO_2^{sym}), 1290 (amide III), 1380 (SO_2^{as}), 1573 (amide II), 1714 (amide I), 3060 (NH), 3365 (NH_2). UV (MeOH), λ_{max}, nm (lg ε): 223.5 sh (4.25), 296 (4.10). ^{1}H-NMR (TFA), δ, ppm: 1.54 (t, 6H, 2 Me from Et), 2.88 (t, 2H, C$\underline{H}_2$CH$_2$CO), 3.22 (t, 2H, CH$_2$C$\underline{H}_2$CO), 3.34 (q, 4H, 2 CH$_2$ from Et), 7.09–8.41 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium), 7.45 (br s, 2H, SO_2NH_2). ^{13}C-NMR (TFA), δ, ppm: 13.8 (s, Me), 36.9 (CH$_2$ of Et), 37.7 (s, $\underline{C}$H$_2$CH$_2$CO), 40.8 (s, CH$_2\underline{C}$H$_2$CO), 128.1, 130.5, 132.9, 133.4, 134.6, 145.8, 159.3 (C-2 of thiadiazole), 170.0 (C-5 of thiadiazole), 171.7 (CONH). Anal. $C_{18}H_{21}N_5O_3S_2^+ClO_4^-$ (C, H, N, S).

6-(2,6-Di-isopropyl-4-phenylpyridinium-ethylcarboxy-ethoxy)-benzothiazole-2-sulfonamide perchlorate **D24**, as tan crystals, m.p. > 320°C. IR (KBr), cm^{-1}: 625 (ClO_4^-), 1100 (ClO_4^-), 1160 (SO_2^{sym}), 1290 (amide III), 1354 (SO_2^{as}), 1579 (amide II), 1713 (amide I), 3060 (NH), 3365 (NH_2). UV (MeOH), λ_{max}, nm (lg ε): 226 (4.01), 298 (4.17). ^{1}H-NMR (TFA), δ, ppm: 1.55 (d, 12H, 4 Me from *i*-Pr), 2.87 (t, 2H, C$\underline{H}_2$CH$_2$CO), 3.18 (t, 2H, CH$_2$C$\underline{H}_2$CO), 3.53 (heptet, 2H, 2 CH from *i*-Pr), 7.28–8.65 (m, 14H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium + 3H of benzothiazole), 8.25 (br s, 2H, SO_2NH_2). ^{13}C-NMR (TFA), δ, ppm: 10.5, 11.7, 30.3, 37.0 (s, $\underline{C}$H$_2$CH$_2$CO), 40.5 (s, CH$_2\underline{C}$H$_2$CO), 40.8, 49.3, 128.6, 130.7, 132.0, 133.9, 137.5, 141.9, 149.8, 174.1 (CONH). $C_{25}H_{27}N_3O_5S_2^+ClO_4^-$ (C, H, N, S).

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 as described by Lindskog *et al.*[34] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[35] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[36] Enzyme

concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1} cm^{-1}$ 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.[37,38] CA IV was isolated from bovine lung microsomes as described by Maren *et al.* and its concentration has been determined by titration with ethoxzolamide.[39]

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC.[40] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25°C. A molar absorption coefficient (ε) of 18,400 $M^{-1} cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis under the conditions of the experiments (pH 7.40), as reported in the literature.[40] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration and the values reported throughout the paper are the mean of such results. 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 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constant K_I was determined as described by Pocker and Stone.[40] Enzyme concentrations were 3.5 nM for hCA II, 9.6 nM for hCA I and 30 nM for bCA IV (this isozyme has a decreased esterase activity[15] and higher concentrations had to be used for the measurements).

Penetrability Through Red Cell Membranes

An amount of 10 mL of freshly isolated human red cells thoroughly washed several times with Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min were treated with 25 mL of a 2 mM solution of sulfonamide inhibitor. Incubation was done at 37°C with gentle stirring, and after incubation periods of 30, 60 and 120 min, respectively, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 10 mL of the above mentioned buffer in order to eliminate all unbound inhibitor.[41] The cells were then lysed in 25 mL of distilled water and centrifuged to remove membranes and other insoluble impurities. The obtained solution was then heated at 100°C for 5 min (in order to denature CAs) and sulfonamides possibly present were assayed in each sample

by three methods: by a HPLC method,[42,43] spectrophotometrically[44] and enzymatically.[40]

HPLC

A variant of the methods of Gomaa[42] and Iyer and Taft[43] has been developed by us, as follows. A commercially available 5 μm Bondapack C-18 column was used for the separation, with a mobile phase consisting of acetonitrile–methanol–phosphate buffer (pH 7.4), 10 : 2 : 88 (v/v), at a flow rate of 3 mL/min, with 0.3 mg/mL sulphadiazine (Sigma) as internal standard. The retention times were: 12.69 min for acetazolamide, 4.55 min for sulphadiazine, 10.54 min for benzolamide, 4.12 min for sulfanilamide, 2.15 min for **A17**, 2.45 min for **B7**, 2.78 min for **A23**, 2.96 min for **B14**, 3.06 min for **C13**, 2.87 min for **C18** and 3.24 min for **D15**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide, and wavelength in the range of 270–310 nm for the other sulfonamides).

Spectrophotometrically

A variant of the pH-induced spectrophotometric assay of Abdine *et al.*[44] has been used, working for example at 260 and 292 nm, respectively, for acetazolamide; at 225 and 265 nm, respectively, for sulfanilamide, etc. Standardized solutions of each inhibitor were prepared in the same buffer as that used for the membrane-penetrability experiments.

Enzymatically

The amount of sulfonamide present in the lysate was evaluated based on hCA II inhibition measured by the esterase method, as described above.[40] Standard inhibition curves have been obtained previously for each sulfonamide, using the pure compound, which were used thereafter for determining the amount of inhibitor present in the lysate. The three methods presented above led to results in good agreement, within the limits of the experimental errors (Table II).

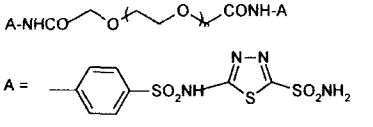
Intravenous Perfusion Experiments in Rats

Adult male Wistar rats were perfused intravenously (i.v.) with solutions of sulfonamide CA inhibitors in the concentration ranges of 3–15 mg/kg (equal volumes of inhibitor solutions were used in all experiments).

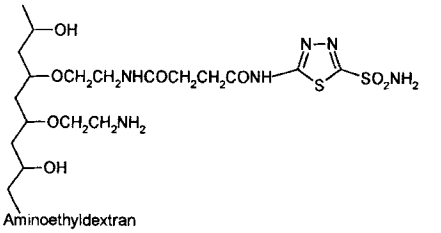
Urine was collected for the next 12 h and the amount of bicarbonate present was determined enzymatically using a phosphoenol pyruvate carboxylase–malate dehydrogenase assay, kit from Gilford Systems (Oberlin, OH, USA).[45] Three animals were used for each inhibitor, and the data reported in Table III are the mean of such assays. Small amounts (0.5 mL) of blood were also taken from these animals, at 1 and 2 h after starting the perfusion experiment in order to determine the level of sulfonamides present in the erythrocytes. Red cells were treated as above (except that they were not incubated with any inhibitor) and sulfonamides present in the cell lysate were measured by the HPLC method mentioned above.

RESULTS AND DISCUSSION

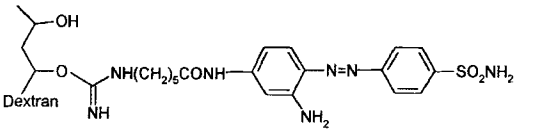
Although several thousand different aromatic/heterocyclic sulfonamide CA inhibitors have been synthesized in the last 45 years in the search for diverse pharmacological agents,[3,4,17–23,30,46–48] the number of membrane-impermeant inhibitors is very low indeed. Thus, a first approach for introducing membrane-impermeability into such compounds was that of attaching aromatic/heterocyclic sulfonamides to polymers, such as polyethyleneglycol;[31,49] aminoethyldextran;[50] or dextran.[51] Compounds such as **31–33**, possessing molecular weights in the range of 3.5–99 kDa, showed membrane-impermeability due to their high molecular weights, and were shown to selectively inhibit (*in vivo*) only CA IV and not the cytosolic isozymes (primarily CA II) being used in several valuable renal and pulmonary physiologic studies.[50–52] Due to their macromolecular nature, such inhibitors could not be developed as drugs/diagnostic tools, since *in vivo* they could induce allergic reactions. A second approach for achieving membrane-impermeability is that of using highly polar, salt-like compounds. Only one such sulfonamide has been used in physiological studies, QAS (quaternary ammonium sulfanilamide) **34**, which was reported by Henry's group[52] to inhibit only extracellular CAs in a variety of arthropods (such as the crab *Callinectes sapidus*) and fish.[52] The main draw-back of QAS is its relatively high toxicity in higher vertebrates.[4]



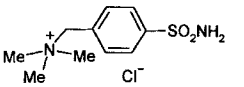
31: M_r = 3.5 kDa



32: M_r = 6.7 - 99 kDa



33: Mr = 5, 100 and 1000 kDa



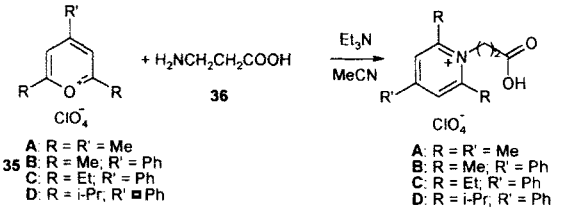
34: QAS

A large number of positively-charged sulfonamides, prepared by reaction of amino-sulfonamides with pyrylium salts, of types **27–30**, were recently reported by this group.[21,23] Based on QSAR studies on several series of CA inhibitors, including some positively-charged derivatives of type **30**,[53] it emerged that the enhancement of CA inhibitory activity is correlated with increased positive charges on the heterocyclic/aromatic ring incorporated in such molecules, as well as with "long" inhibitor molecules *per se* (i.e., molecules extending in the direction passing through the Zn(II) ion of the enzyme, the sulfonamide nitrogen atom and the long axis of the inhibitor).[53] It appeared thus of interest to try to explore this result by designing positively-charged, "long" sulfonamide inhibitors. Thus, we attached substituted-pyridinium-ethylcarboxy moieties to the amino-, hydrazino-, imino- or hydroxy groups of the sulfonamides **1–26**. The substitution pattern of the pyridinium ring had been previously shown[21,23,53] to be critical for the biological activity of this type of sulfonamide CA inhibitor. The best CA inhibitors were those incorporating 2,4,6-trimethylpyridinium or 4-phenyl-2,6-dialkyl-pyridinium moieties in their molecule.[21,23,53] These were just the types of moieties selected for the preparation of the new sulfonamides reported here (2,4,6-trimethylpyridinium-, 4-phenyl-2,6-dimethyl-pyridinium-, 4-phenyl-2,6-diethyl-pyridinium- and 4-phenyl-2,6-di-isopropylpyridinium-).

Reaction of the substituted pyrylium salts **35A–35D** with β-alanine **36** afforded the pyridinium-carboxylic acid derivatives **A–D**. Coupling of sulfonamides **1–26** with these pyridinium derivatives in the presence of EDCI or diisopropylcarbodiimide, afforded the new derivatives **(A–D) 1–26**, by a procedure relatively similar to that reported by Whitesides'[46,47] and our[17–20] groups for the preparation of amino acyl-carboxamido sulfonamides. As a large number of derivatives is reported here, each compound is designated by a letter identifying the positively-charged carboxylic acid from which it is derived (**A–D**), and a figure identifying the sulfonamide of type **1–26** at which the substituted-pyridinium-methylcarboxy moiety has been attached. For instance, **A3** is *p*-(2,4,6-trimethylpyridinium-ethylcarboxamido)-benzenesulfonamide perchlorate, **B6** is *p*-(2,6-dimethyl-4-phenylpyridinium-ethyl-carboxamidoamidoethyl)-benzenesulfonamide perchlorate, **C14** is 5-(2,6-diethyl-4-phenylpyridinium-ethylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide perchlorate and **D24** is 6-(2,6-di-isopropyl-4-phenylpyridinium-ethylcarboxyethoxy)-benzothiazole-2-sulfonamide, etc. (Scheme 1).

The new compounds reported here were characterized by standard chemical and physical methods (elemental analysis, within ±0.4% of the theoretical values; IR; ^{1}H- and ^{13}C-NMR spectroscopy) that confirmed their structure (see Experimental Section for details) and were assayed for the inhibition of isozymes hCA I, hCA II and bCA IV (Table I).

Inhibition data against three CA isozymes, hCA I, hCA II and bCA IV with the new derivatives (Table I) show that the substituted-pyridinium-ethylcarboxy moieties attached to these sulfonamides generally led to an increase in the CA inhibitory properties compared to the corresponding parent sulfonamide **1–26**. Particularly strong inhibitors were those derived from heterocyclic derivatives (1,3,4-thiadiazoles, 1,3,4-thiadiazolines, benzothiazoles). Slightly less active were the 1,3-benzene-disulfonamides **11–13** and the 3-fluoro/chloro-sulfanilamide derivatives **7**, **8**, together with the pyrimidine-substituted sulfanilamides of type **21**, and the



SCHEME 1 Prepartion of compounds A–D.

TABLE I CA inhibition data for sulfonamides **1–26** and the positively-charged derivatives **(A–D)1–26** reported in the present study, against isozymes I, II and IV

Inhibitor	K_I* (nM)		
	hCA I[a]	*hCA* II[a]	*bCA* IV[b]
1	45400	295	1310
2	25000	240	2200
3	28000	300	3000
4	78500	320	3215
5	25000	170	2800
6	21000	160	2450
7	8300	60	180
8	9800	110	320
9	6500	40	66
10	6000	70	125
11	6100	28	175
12	8400	75	160
13	7500	62	140
14	8600	60	540
15	9300	19	355
16	455	3	125
17	6	2	5
18	1	0.6	0.8
19	42	6	50
20	44	9	53
21	690	12	154
22	70	9	19
23	55	8	17
24	50	7	15
25	24000	125	560
26	18000	110	450
A1	31600	274	420
A2	23500	250	400
A3	15000	139	195
A4	33000	300	420
A5	1480	41	76
A6	750	33	70
A7	720	19	42
A8	640	20	54
A9	760	43	75
A10	800	44	69
A11	540	16	62
A12	360	17	61
A13	310	14	53
A14	300	10	46
A15	320	8	55
A16	42	6	14
A17	16	6	11
A18	15	4	9
A19	50	15	63
A20	60	13	65
A21	55	16	64
A22	14	9	48
A23	13	7	21
A24	12	6	30

TABLE I (*Continued*)

Inhibitor	K_I* (nM)		
	hCA I[a]	*hCA* II[a]	*bCA* IV[b]
A25	1800	71	125
A26	1700	70	115
B1	25000	205	275
B2	19500	200	260
B3	12600	104	151
B4	25600	215	280
B5	1230	30	46
B6	700	24	43
B7	660	11	33
B8	610	10	34
B9	600	12	30
B10	580	18	53
B11	370	11	27
B12	360	10	25
B13	300	9	23
B14	200	5	15
B15	185	5	13
B16	32	4	11
B17	10	2	9
B18	10	2	8
B19	36	6	45
B20	35	7	50
B21	32	9	50
B22	10	4	16
B23	11	5	15
B24	9	3	11
B25	1300	30	64
B26	1100	28	60
C1	27000	230	330
C2	22000	195	310
C3	15000	121	165
C4	27600	255	310
C5	1380	37	65
C6	740	26	57
C7	720	15	34
C8	600	14	41
C9	610	18	52
C10	620	22	60
C11	450	14	39
C12	350	13	35
C13	300	12	33
C14	275	6	24
C15	305	7	25
C16	36	4	15
C17	11	5	9
C18	10	3	8
C19	44	10	57
C20	43	11	53
C21	41	12	50
C22	12	6	27
C23	11	6	25
C24	10	4	22

TABLE I (*Continued*)

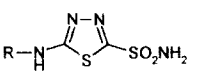
Inhibitor	K_I* (nM)		
	hCA I[a]	*hCA* II[a]	*bCA* IV[b]
C25	1700	50	78
C26	1550	43	80
D1	30000	270	320
D2	23500	225	340
D3	15000	120	170
D4	32500	300	370
D5	1420	40	75
D6	770	36	72
D7	750	21	43
D8	600	19	51
D9	650	38	72
D10	640	39	78
D11	490	14	45
D12	350	16	42
D13	300	15	36
D14	300	9	33
D15	275	8	30
D16	39	6	14
D17	13	5	11
D18	12	4	10
D19	52	15	61
D20	54	16	62
D21	40	18	66
D22	13	7	34
D23	11	8	36
D24	10	6	31
D25	1770	68	112
D26	1600	63	96

*Standard error for the determination of K_I values was of 10–20% (from 2 different assays); [a]Human (cloned) isozyme; [b]Isolated from bovine lung microsomes.

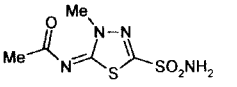
sulfanilyl-sulfanilamides **19** and sulfanilyl-metanilamides **20**. The simple aromatic derivatives were (as expected) less active than the previously mentioned heterocyclic sulfonamides. The potency of the obtained inhibitor generally varied in the following way, based on the parent sulfonamide from which it was prepared: the derivatives of *p*-hydrazino-benzenesulfonamide **4** < the orthanilamides **1** ≅ the metanilamides **2** < the sulfanilamides **3** < the homosulfanilamides **5** < the *p*-aminoethyl-benzenesulfonamides **6** ≅ the halogeno-substituted sulfanilamides **7–10** ≅ the 1,3-benzene-disulfonamides **11–13** ≅ the sulfanilyl-sulfanilamides **19** and the sulfanilyl-metanilamides **20** < the 1,3,4-thiadiazole-2-sulfonamides **14, 16** and **17** ≅ 4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamide **15** and **18** ≅ the benzothiazole-2-sulfonamides **22–24**. It is in fact well established that such heterocyclic derivatives (1,3,4-thiadiazoles; 1,3,4-thiadiazolines) are among the strongest CA inhibitors

yet reported.[3,4] Based on the carboxylic acid from which they were obtained the trimethylpyridinium (**A** type) derivatives were less active than the 2,6-diisopropyl-4-phenylpyridinium (**D** type) derivatives, which in turn were less active than the 2,6-diethyl-4-phenyl-pyridinium derivatives (**C** type compounds). The best inhibitors in the whole series were those derived from 2,6-dimethyl-4-phenylpyridinium-ethylcarboxylic acid (**B** type derivatives).

All three CA isozymes investigated here were susceptible to inhibition with this type of sulfonamides, with hCA II and bCA IV being the most sensitive, whereas by comparison hCA I was generally less susceptible to inhibition.



37: R = CH_3CO
38: R = $C_6H_5SO_2$



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In order to better understand the interaction of the sulfonamide inhibitors with CA isozymes, *ex vivo* experiments were performed with freshly isolated human blood. Thus, incubation of human red cells (which contain high concentrations of isozymes I and II, i.e., 150 μM hCA I and 20 μM hCA II, but not the membrane-bound CA IV)[54] with millimolar concentrations of different sulfonamide inhibitors, such as sulfanilamide **3**, acetazolamide **37** or methazolamide **39**, led to saturation of the two isozymes present in erythrocytes with inhibitor within a short period of incubation (30 min), whereas for benzolamide **38** a similar effect was achieved after a somewhat longer period (60 min) (Table II). This is obviously due to the high diffusibility through membranes of the first three inhibitors whereas benzolamide, with a pK_a of 3.2 for the second sulfonamido group,[55] exists mainly as an (di)anion at the pH at which the experiment was done (7.4) and is thus less diffusible and penetrates membranes more slowly. Different cationic sulfonamides synthesized by us here, such as **A17**, **A23**, **B7**, **B14**, **C13**, **C18**, **D15**, etc., under the same conditions were detected only in very small amounts within the blood red cells, proving that they were unable to penetrate through the membranes, obviously due to their cationic nature. Even after incubation times as long as 1 h (and longer, data not shown), only traces of such cationic sulfonamides were present inside the blood red cells, as proved by the three assay methods used for their identification in the cell lysate, which were in good agreement with each other (Table II). This demonstrates that the approach taken here for achieving

TABLE II Levels of sulfonamide CA inhibitors (μM) in red blood cells at 30 and 60 min, after exposure of 10 mL of blood to solutions of sulfonamide (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4). The concentrations of sulfonamide was determined by three methods: HPLC, electronic spectroscopy (ES) and the enzymatic method (EI) – see Experimental for details

Inhibitor	[*Sulfonamide*], μM*					
	$t = 30$ min			$t = 60$ min		
	HPLC[a]	*ES*[b]	*EI*[c]	*HPLC*[a]	*ES*[b]	*EI*[c]
Sulfanilamide **3**	148 ± 4	151 ± 5	143 ± 3	159 ± 3	164 ± 5	159 ± 5
Acetazolamide **37**	136 ± 7	139 ± 5	140 ± 4	160 ± 8	167 ± 5	163 ± 5
Benzolamide **38**	110 ± 5	108 ± 3	112 ± 2	148 ± 4	146 ± 6	149 ± 2
Methazolamide **39**	170 ± 9	169 ± 8	165 ± 5	168 ± 5	168 ± 4	167 ± 5
A17	0.6 ± 0.02	0.7 ± 0.04	0.5 ± 0.05	0.9 ± 0.06	0.8 ± 0.03	0.8 ± 0.02
A23	1.1 ± 0.05	1.1 ± 0.01	1.0 ± 0.04	1.2 ± 0.05	1.3 ± 0.04	1.2 ± 0.03
B7	0.9 ± 0.06	1.2 ± 0.04	1.1 ± 0.02	1.0 ± 0.03	1.3 ± 0.011	1.2 ± 0.05
B14	0.7 ± 0.02	0.6 ± 0.03	0.8 ± 0.01	0.9 ± 0.03	0.9 ± 0.04	1.1 ± 0.05
C13	1.4 ± 0.05	1.5 ± 0.03	1.3 ± 0.04	1.5 ± 0.03	1.6 ± 0.07	1.4 ± 0.06
C18	0.6 ± 0.05	0.5 ± 0.02	0.7 ± 0.04	0.8 ± 0.02	0.6 ± 0.04	0.8 ± 0.05
D15	0.7 ± 0.03	0.8 ± 0.05	0.6 ± 0.03	0.9 ± 0.03	0.9 ± 0.05	1.0 ± 0.03

*Mean ± standard error (from 2 determinations) by: [a]the HPLC method[55]; [b]the electronic spectroscopic method[57]; [c]the enzymatic method.[53]

TABLE III Renal excretion of bicarbonate in rats, after administration of the classical sulfonamide CA inhibitors acetazolamide **37** and benzolamide **38**, as well as the positively-charged sulfonamides **B14**, **C18** and **D15**, and the level of sulfonamide present in red cells 2 h after administration of the drug

Inhibitor	*Drug concentration* (mg/kg)	*Excreted* HCO_3^- (mM)*	[*Sulfonamide*][a] (μM)*
Acetazolamide	10	110 ± 3	164 ± 9
Benzolamide	5	115 ± 6	155 ± 8
B14	5	37 ± 4	0.013 ± 0.003
C18	4	39 ± 5	0.015 ± 0.006
D15	5	41 ± 6	0.011 ± 0.007

*Mean ± standard error (from 2 determinations).[a]By the HPLC method.

membrane-impermeability works well for the designed positively-charged sulfonamide CA inhibitors.

In order to show that *in vivo* membrane-bound CA but not cytosolic isozymes are inhibited by the cationic sulfonamides reported here, additional experiments of i.v. perfusion in rats were performed and the amount of bicarbonate excreted into the urine of the experimental animals analyzed, together with the level of sulfonamides present in their blood 2 h after administration of the drugs (Table III).[31,50–52] It is well established[31,50–52] that at least two CA isozymes, CA II and IV, are involved in bicarbonate reabsorption by the proximal, and to a less extent the distal, tubules in the

kidneys of vertebrates (except those of *Reptilia*, in which other mechanisms have been shown to be present[4b]). Administration of CA inhibitors (such as acetazolamide, benzolamide, ethoxzolamide, etc) to vertebrates produces an alkaline urine, due to the renal excretion of bicarbonate, Na^+, K^+ and osmotically obligated water, as a consequence of CA inhibition.[4,31] Up to now, studies of selective inhibition of different CA isozymes present in the diverse compartments of renal tubular cells have been performed only with high molecular weight CA inhibitors of types **31–33**,[4,31,56] with molecular weights in the range of 3.5–99 kDa. These being membrane-impermeant due to their high molecular weight, were shown to selectively inhibit only CA IV and not the cytosolic isozymes (primarily CA II). The particular nature of the compounds reported by us here allows for the first time to perform this type of study with low molecular weight inhibitors.

From the data of Table III it can be seen that administration of classical low molecular weight inhibitors, such as acetazolamide **37** or benzolamide **38** (which inhibit both cytosolic as well as membrane-bound isozymes) leads to a peak of 105–110 mM of bicarbonate excreted into urine in 12 h, as shown in the studies of Maren's group,[4,31] and reconfirmed in the present work. Administration of positively-charged sulfonamides such as **B14**, **C18** or **D15**, under the same conditions as for the classical inhibitors mentioned above, produced bicarbonate elimination but in a lower quantity, due to the fact that only CA IV was inhibited by these membrane-impermeant compounds. On the other hand, after such experiments, only traces of positively-charged sulfonamides were detected in the red cells of the experimental animals, in contrast to the situation after the administration of acetazolamide or benzolamide, which produced a rapid saturation of the cytosolic isozymes present in the erythrocytes (Table III). Our data compare well with the recent data from Maren's laboratory[31] who reported an excretion in rats of about 40 mM bicarbonate into urine after administration of 100 mg/kg of the 3.5 kDa inhibitor **31**. In their work it was concluded that both CA II and CA IV participate in the normal full renal reabsorption of bicarbonate, these data being in agreement with data from Sly's laboratory[56] as well as that reported by us here. Thus, correlating the results of Tables II and III, it is clear that *ex vivo* and *in vivo*, positively-charged sulfonamides such as those described here are able to discriminate between membrane-bound and cytosolic isozymes. This is not surprising after all, since many positively-charged compounds, such as some reversible anticholinesterase agents (neostigmine, edrophonium, pyridostigmine, demecarium; etc),[57] the quaternary ammonium antimuscarinic agents (such as methantheline),[58] or the neuromuscular blocking agents (tubocurarine, alcuronium, gallamine,

atracurium, decamethonium; etc),[59] all possessing quaternary ammonium or pyridinium moieties in their molecules, are known to be membrane-impermeant due to their cationic nature.

In conclusion, we report here a general approach for the preparation of positively-charged, membrane-impermeant sulfonamide CA inhibitors with high affinity for the cytosolic isozymes CA I and CA II, as well as for the membrane-bound one CA IV. They were obtained by attaching pyridinium-ethylcarboxylic acid moieties to the molecules of aromatic/heterocyclic sulfonamides incorporating free amino, imino, hydrazino or hydroxyl groups in their molecule. Ring systems which have been derivatized by the above mentioned procedures included: 2-, 3- or 4-aminobenzenesulfonamides, 4-(ω-aminoalkyl)-benzenesulfonamides, 3-halogeno-substituted-sulfanilamides, 1,3-benzene-disulfonamides, 1,3,4-thiadiazole-2-sulfonamides and benzothiazole-2-sulfonamides among others, and were chosen in such a way as to show that the proposed approach is a general one. *Ex vivo* and *in vivo* studies, in two model systems (human red cells, and perfusion experiments in rats, respectively), showed the new class of inhibitors reported here to discriminate for the membrane-bound versus the cytosolic isozymes, selectively inhibiting only CA IV.

Acknowledgments

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[1] This paper is part 84 of the series. Preceding part: A. Scozzafava, F. Briganti, M.A. Ilies and C.T. Supuran (2000) *J. Med. Chem.* (in press).
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