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Laboratory note

Carbonic anhydrase inhibitors – Part 29[1]: Interaction of isozymes I, II and IV with benzolamide-like derivatives

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Abstract – Reaction of 5-amino-1,3,4-thiadiazole-2-sulfonamide and 5-imino-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline with sulfonyl halides/sulfonic acid anhydrides afforded benzolamide-like derivatives possessing very strong inhibitory effects towards three isozymes of carbonic anhydrase (CA), CA I, II and IV. Some of the compounds were designed in such a way to possess good leaving groups (such as nitro-; 2,4,6-triphenyl-pyridinium, etc.) for aromatic nucleophilic substitution reactions with fluoride, in order to introduce positron-emitting isotopes in their molecule, such as ^{18}F. Reactions done initially with the stable isotope of fluorine were not very effective, as the yields in the desired fluoro-derivatives were low, and a complex reaction mixture was obtained. By using this type of approach, and optimizing the synthetic procedure, CA inhibitors for positron emission tomography (PET) applications might be obtained (in the case of utilizing a carrier, which is the non-radioactive derivative itself, since the affinities of such derivatives for the receptor are in the nanomolar range). Further improving of such synthetic procedures might lead to better yields and the respective compounds should be used as selective ligands (also in carrier-free systems) in assessing the role of membrane bound CA isozymes or for new diagnostic tools based on PET. © Elsevier, Paris

carbonic anhydrase / inhibitors / isozymes I, II, IV / positron emitting isotopes / positron emission tomography (PET) / heterocyclic sulfonamides / aromatic nucleophilic substitution

1. Introduction

The eight isozymes of carbonic anhydrase (CA, EC 4.2.1.1), CA I–VIII, and two CA-like proteins (CA IX, XI) presently described in vertebrates [2] play critical functions in important physiological processes such as respiration and transport of CO_2/HCO_3^- between metabolizing tissues and lungs [3–5]; secretion of electrolytes in different epithelia, such as aqueous humor formation [6, 7], cerebrospinal fluid secretion [3], pancreatic [3] and gastric juice formation [3, 8], urinary production of acid within the cells of proximal and distal tubule of kidneys [3, 9] and many others [3].

CA inhibitors of the sulfonamide type [3, 10, 11] such as acetazolamide **1**, methazolamide **2** or ethoxzolamide **3** were extensively used in clinical medicine for the last 40 years in the management of diverse diseases such as edema [3, 9]; glaucoma [3, 7, 12]; epilepsy [13]; mountain sickness [14]; gastric ulcers [15] and some neurological disorders [16] among others. On the other hand, recently, thienothiopyran sulfonamides of type **4** were developed [17] and introduced in clinical use as topical antiglaucoma agents, devoid of the side effects seen with systemic administration of such drugs [3, 7, 12] (see *figure 1*).

Another compound from this class of pharmacological agents, benzolamide **5**, an orphan drug [18], occupies a special place among the known CA inhibitors, possessing nanomolar affinity for the human isozyme, HCA II, and also having a highly acidic proton in its molecule (pK_a 3.2 of the SO_2NH moiety) [3]. These characteristics confer to the drug special properties, such as selective inhibition of

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[1]For the preceding part, see [1].

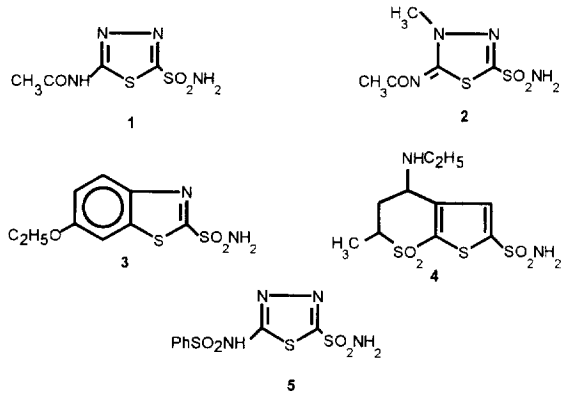

Figure 1.

renal CA (at doses of 1 mg/kg, in animals and humans [3]) due to its lack of access to intracellular isozymes [3, 16, 18], when the drug is ionized at physiological pH.

Although benzolamide has been a valuable drug in many physiological studies [19], few structurally-related inhibitors of this type were synthesized, since the initial report of Vaughan et al. [20] of **5** and five other substituted congeners (*p*-amino-; *p*-acetylamino-; *p*-methyl-; *p*-chloro- and *p*-bromo-derivatives), presumably due to the fact that benzolamide could not be developed for wide-spread clinical use [18]. Thorough characterization as well as spectral data for these inhibitors have never been published on the other hand.

Our interest in developing isozyme-specific and/or organ-selective CA inhibitors [7–12, 21–26] prompted us to investigate their possible applications in the design of compounds suitable to be used as NMR contrast agents or for positron emission tomography (PET) imaging studies. Therefore, we reinvestigated the benzolamide analogs, based on the following two aspects: (i) in order to find compounds with specificity towards the membrane-bound CA isozyme (CA IV) or eventually other known isozymes, such as CA I or CA II. This fact is primarily based on the relatively high acidity (pK_a values for the SO_2NH moieties) of such compounds, and as a consequence, in ionized state (as anions) in solutions at the physiological pH, they would probably have an impaired access through biological membranes; (ii) the eventual possibility to introduce positron-emitting isotopes (such as ^{18}F; ^{11}C, etc.) into their molecules, by means of rapid chemical reactions. Up to now, only one such CA inhibitor has been reported, [^{11}C-carbonyl]-acetazolamide [27] which allowed the first in vivo localization of CA in the heart and lung [28]. However, as acetazolamide readily penetrates biological membranes, and inhibits all CA isozymes, a membrane-impermeant (ie, CA IV specific) and effective CA inhibitor labeled with a positron-emitting isotope could allow quantitative imaging of membrane-bound CA in a variety of tissues, where this isozyme is thought to provide critical catalysis of CO_2 reactions. In this paper we report the preparation o a large series of 5-sulfonylamino-1,3,4-thiadiazole-2-sulfonamide and 5-sulfonylimino-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline derivatives, their inhibition properties against three CA isozymes, CA I, II and IV, and propose two approaches for introducing fluorine into some of these molecule. Such investigations exploited the aromatic nucleophilic substitution reactions of nitro- or triphenylpyridinium-containing derivatives, which by reaction with fluoride would allow the introduction of the fluoro moiety in their molecule. Our study may be considered a model in order to introduce positron-emitting isotopes, such as ^{18}F, in the molecules of enzyme inhibitors. The reactions reported here, done with the stable isotope of fluorine have initially not been very effective, as the yield in the desired fluoro-derivatives were low, but by improving this type of approach, CA inhibitors for positron emission tomography studies might be obtained in higher yields, which might subsequently be used as selective ligands in assessing the role of membrane bound CAs as well as other imaging applications.

2. Experimental protocols

2.1. Chemistry

Melting points were recorded with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with a Carl Zeiss IR-80 instrument. NMR spectra were recorded in DMSO-d_6, trifluoroacetic acid (TFA) or D_2O as solvents, with a General Electrics Omega or Bruker CPX200 instrument, working at 300 and 200 MHz, respectively (for the ^{1}H-NMR spectra), and 75 57 MHz, respectively, for the ^{13}C-NMR spectra. Chemical shifts are reported as δ values, relative to Me_4Si as internal standard. Thin layer chromatography (TLC) was done on precoated silicagel 60 plates, from Merck, and spots were visualized in UV light. HPLC was performed with a Beckman instrument, using a Rheodyne pump and column (reverse phase Bondapack μ-C18). Elemental analysis was done by combustion (for C, H, N) with a Carlo Erba automated analyzer (Milan, Italy). The values obtained were within ± 0.4% of the theoretical values, calculated for the proposed formulae.

Sulfonyl chlorides, benzyl chloromethyl ether, solvents as well as inorganic reagents were from Aldrich, Merck and Carlo Erba. 2-Sulfobenzoic acid cyclic anhydride was from Acros. Amine **6** and imine **7** were prepared by literature procedures [20, 29] from acetazolamide and methazolamide (Sigma), respectively, by desacetylation with concentrated hydrochloric acid, followed by neutralization with sodium bicarbonate of the corresponding hydrochlorides. Kryptofix [2.2.2] used as catalyst was from Merck. Pyrylium salts were prepared by literature procedures [30].

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 29.3 kDa for CA II, respectively [34, 35]. CA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide [36].

*2.1.1. General procedure for the preparation of compounds **8–49***

5 mMoles (0.90 g) of 5-amino-1,3,4-thiadiazole-2-sulfonamide **6**, or 5 mMoles (0.97 g) of 5-imino-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline **7** were dissolved in 15 mL solution 2.5 M NaOH and cooled to 2–5 °C in a salt-ice bath. 5 mMoles of finely powdered sulfonyl chloride were added in small portions, concomitantly with 10 mL of a 2 M NaOH solution, maintaining the temperature under 10 °C. The reaction mixture was then stirred at room temperature for 20–25 h, then the pH was adjusted to 2 with 5 N HCl, and the precipitated sulfonamides were filtered and recrystallized from aqueous ethanol. Compounds **8–16, 18–22, 26–28** and **29–37, 39–43, 47–49** were prepared in this way. Derivatives **23** and **44** were prepared by reaction of **6** or **7** with 2-sulfobenzoic acid cyclic anhydride in equimolar amount, in anhydrous acetonitrile at reflux for 2 h.

*2.1.2. Preparation of aminoderivatives **17** and **38***

The two compounds were prepared by desacetylation of the corresponding acetamido derivatives **16**, **37**, with concentrated hydrochloric acid, at reflux for 1 h. Mention should be made that **16** and **17** were reported previously by Young et al. [20], whereas **37** and **38** are new compounds.

*2.1.3. Preparation of pyridinium salts **24, 25** and **45, 46***

5 mMoles of aminoderivatives **17** or **37** and 5.2 mMoles of 2,4,6-trisubstituted pyrylium salts **54**, **55** were suspended in 100 mL of absolute ethanol, and the reaction mixture was refluxed for 3 h. The solvent was evaporated in vacuum, 3 mL of concentrated ammonia solution were added (for converting the unreacted pyrylium salt into the corresponding pyridine), then the obtained pyridine was extracted with 30 mL of diethyl ether, and the residue, after acidification to pH 4 (with $HClO_4$ and HBF_4, respectively) was recrystallized from ethanol–water (4:1, v/v). The pyridinium derivatives were obtained with yields of 56–73%.

*2.1.4. Reaction of 4-nitrobenzolamide **19** with benzyl-chloromethyl ether*

0.4 g of **19** were dissolved in 15 mL acetonitrile and 0.3 mL of diisopropylethylamine and 70 μL of benzyl-chloromethyl ether were added. The reaction mixture was stirred at room temperature for 20 min, the solvent was evaporated in vacuum and the glue retaken in ethanol. In this solvent, the monoderivatized compound **50** is soluble, whereas the tris-derivatized one **51** is poorly soluble. It is interesting to note that no bis-derivatized compound (at the two different sulfonamido moieties) is formed in this reaction, and different attempts to prepare it always failed. In the ^{1}H-NMR spectrum of **50** prepared in this way, were evidenced the following signals of the protecting groups, absent from the spectrum of **19**: 2.45 (s, 2H, NCH_2); 4.52 (s, 2H, OCH_2); 7.20 (m, 5H, Ph); 7.75–8.25 (m, AA'BB', 4H, C_6H_4); 8.70 (s, 2H, SO_2NH_2).

*2.1.5. Reaction of nitroderivatives **19, 50, 51** with potassium fluoride and cryptand [2.2.2]*

2 mMoles of nitroderivative, 2 mMoles of anhydrous KF and 2 mMoles of cryptand [2.2.2] were dissolved in 15 mL of DMSO and heated to 125 °C for 15 min. The reaction mixture was followed by TLC or by HPLC in order to evidence the possible formation of the corresponding fluoroderivatives **11**, **52** and **53**, respectively. The HPLC elution system contained sodium dihydrogen phosphate 0.07 M, at 3 mL/min.

*2.1.6. Reaction of triphenylpyridinium salts **25, 46** with potassium fluoride and cryptand [2.2.2]*

2 mMoles of triphenylpyridinium derivative **25, 46**, 2 mMoles of KF and 2 mMoles of cryptand [2.2.2] were dissolved in 25 mL DMSO and heated at 125 °C for 2 h. The formation of fluorosulfonamides **11, 32** was evidenced by means of TLC on silicagel plates or HPLC, in the same eluting system as described above.

*2.1.7. 5-Methylsulfonamido-1,3,4-thiadiazole-2-sulfonamide **8***

White crystals, m.p. 215–217 °C (from ethanol–water 1:1, v/v); IR (KBr), cm^{-1}: 710, 876, 1131, 1174, 1362, 1615, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.71 (s, 3H, Me); 8.12 (br s, 2H, SO_2NH_2); 8.19 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 24.6 (Me); 160.3 (C-2); 173.2 (C-5). Anal. $C_3H_6N_4O_4S_3$ (C, H N).

*2.1.8. 5-Phenylmethylsulfonamido-1,3,4-thiadiazole-2-sulfonamide **9***

White crystals, m.p. 201–203 °C (from ethanol–water 1:1, v/v); IR (KBr), cm^{-1}: 715, 862, 1130, 1177, 1360, 1490, 1615, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.35 (s, 2H, CH_2); 7.10–7.49 (m, 5H, ArH); 8.12 (br s,2H, SO_2NH2); 8.19 (s,1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 36.0 (Me); 118.0; 119.9; 131.5; 136.2; 160.9 (C-2); 173.5 (C-5). Anal. $C_9H_{11}N_4O_4S_3$ (C, H N).

*2.1.9. 5-(4-Methylbenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **10***

White crystals, m.p. 269 °C, lit. [20] m.p. 267–268 °C; IR (KBr), cm^{-1}: 693, 712, 889, 1130, 1170, 1291, 1357, 1425, 1581, 1611, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.51 (s, 3H, Me); 7.21–7.57 (m, AA'BB',4H, ArH); 8.06 (br s, 2H, SO_2NH_2); 8.18 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.3 (Me); 118.1; 118.9; 129.6; 130.4; 159.3 (C-2 from thiadiazole); 171.6 (C-5 from thiadiazole). Anal. $C_9H_{10}N_4O_4S_3$ (C, H, N).

*2.1.10. 5-(4-Fluorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide **11***

White crystals, m.p. 192–194 °C (from ethanol–water 1:1, v/v); IR (KBr), cm^{-1}: 684, 715, 883, 1132, 1170, 1284, 1357, 1425, 1572, 1604, 3065; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.10–7.58 (m, AA'BB',4H, ArH); 8.03 (br s, 2H, SO_2NH_2); 8.22 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 118.1; 118.7; 130.0; 130.9; 160.1 (C-2 from thiadiazole); 173.1 (C-5 from thiadiazole). Anal. $C_8H_7N_4O_4S_3F$ (C, H, N).

2.1.11. 5-(4-Chlorobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***12***

White crystals, m.p. 272–274 °C (from ethanol–water 1:1, v/v), lit. [20] m.p. 270–271 °C; IR (KBr), cm^{-1}: 681, 702, 765, 837, 1132, 1176, 1284, 1361, 1420, 1575, 1609, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.10–7.55 (m, AA'BB',4H, ArH); 8.12 (br s, 2H, SO_2NH_2); 8.27 (s,1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 118.3; 118.9; 130.9; 133.2; 160.7 (C-2 from thiadiazole); 173.8 (C-5 from thiadiazole). Anal. $C_8H_7N_4O_4S_3Cl$ (C, H, N).

2.1.12. 5-(4-Bromobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***13***

White crystals, m.p. 272–273 °C, lit. [20] m.p. 271–272 °C; IR (KBr), cm^{-1}: 665, 774, 890, 1135, 1170, 1279, 1360, 1421, 1490, 1584, 1600, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.50–7.90 (m, AA'BB',4H, ArH); 8.05 (br s, 2H, SO_2NH_2); 8.20 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 127.1; 130.1; 134.5; 141.5; 160.1 (C-2 from thiadiazole); 173.4 (C-5 from thiadiazole). Anal. $C_8H_7N_4O_4S_3Br$ (C, H, N).

2.1.13. 5-(4-Iodobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***14***

White crystals, m.p. 286–288 °C, IR (KBr), cm^{-1}: 669, 712, 765, 890, 953, 1136, 1175, 1284, 1369, 1420, 1482, 1585, 1609, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.51–7.96 (m, AA'BB',4H, ArH); 8.08 (br s, 2H, SO_2NH_2); 8.20 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 128.9; 130.0; 134.2; 139.7; 161.0 (C-2 from thiadiazole); 172.8 (C-5 from thiadiazole). Anal. $C_8H_7N_4O_4S_3I$ (C, H, N).

2.1.14. 5-(4-Methoxybenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***15***

White crystals, m.p. 249–251 °C; IR (KBr), cm^{-1}: 690, 711, 772, 838, 925, 1130, 1164, 1285, 1362, 1422, 1595, 1610, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.50 (s, 3H, MeO); 7.29–7.72 (m, AA'BB',4H, ArH); 8.03 (br s,2H, SO_2NH_2); 8.21 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 41.8 (Me); 118.4; 119.9; 128.0; 130.5; 159.7 (C-2 from thiadiazole); 172.0 (C-5 from thiadiazole). Anal. $C_9H_{10}N_4O_5S_3$ (C, H, N).

2.1.15. 5-(4-Acetylaminobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***16***

White crystals, m.p. 297–299 °C (dec), lit. [20] m.p. 285–290 °C; IR (KBr), cm^{-1}: 610, 687, 820, 1128, 1255, 1370, 1408, 1647, 3030; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.36 (s, 3H, Me); 7.04–7.52 (m, AA'BB',4H, ArH); 7.82 (br s, 4H, SO_2NH_2 + SO_2NH + CONH). Anal. $C_{10}H_{11}N_5O_5S_3$ (C, H, N).

2.1.16. 5-(4-Aminobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***17***

White crystals, m.p. 260–261 °C, lit. [20] m.p. 259–261 °C; IR (KBr), cm^{-1}: 618, 675, 778, 850, 1073, 1137, 1264, 1369, 1450, 1500, 3050; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.00–7.48 (m, AA'BB',4H, ArH); 8.04 (br s, 3H, SO_2NH_2 + SO_2NH), 8.47 (s, 2H, NH_2). Anal. $C_8H_9N_5O_4S_3$ (C, H, N).

2.1.17. 5-(3-Aminobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***18***

White crystals, m.p. 242–244 °C, IR (KBr), cm^{-1}: 601, 653, 787, 850, 915, 1065, 11417, 1260, 1369, 1450, 1520, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.00–7.39(m, 4H, ArH); 8.10 (br s, 3H, SO_2NH_2 + SO_2NH), 8.35 (s, 2H, NH_2); Anal. $C_8H_9N_5O_4S_3$ (C, H, N).

2.1.18. 5-(4-Nitrobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***19***

Pale yellow crystals, m.p. 247 °C (dec.); IR (KBr), cm^{-1}: 658, 760, 789, 825, 1131, 1174, 1284, 1362, 1418, 1581, 1603, 3059; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.75–8.10 (m, AA'BB',4H, ArH); 8.20 (br s,3H, SO_2NH_2 + SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 127.5; 128.0; 129.8; 130.1; 159.8 (C-2 from thiadiazole); 173.3 (C-5 from thiadiazole). Anal. $C_8H_7N_5O_6S_3$ (C, H, N).

2.1.19. 5-(3-Nitrobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***20***

Yellow crystals, m.p. 270–271 °C (dec.); IR (KBr), cm^{-1}: 671, 732, 795, 822, 1130, 1178, 1280, 1366, 1419, 1580, 1610, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.42–8.00 (m, 4H, ArH); 8.15 (br s, 3H, SO_2NH_2 + SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 126.8; 128.9; 129.9; 130.5; 159.8 (C-2 from thiadiazole); 173.5 (C-5 from thiadiazole). Anal. $C_8H_7N_5O_6S_3$ (C, H, N).

2.1.20. 5-(2-Nitrobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***21***

Yellow crystals, m.p. 242–244 °C (dec.); IR(KBr), cm^{-1}: 638, 712, 755, 847, 1133, 1182, 1280, 1365, 1413, 1580, 1610, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.49–8.08 (m, 4H, ArH); 8.17 (br s, 3H, SO_2NH_2 + SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 125.3; 129.4; 129.9; 131.5; 159.6 (C-2 from thiadiazole); 173.5 (C-5 from thiadiazole). Anal. $C_8H_7N_5O_6S_3$ (C, H, N).

2.1.21. 5-(N,N-Dimethylaminosulfamoyl)-1,3,4-thiadiazole-2-sulfonamide ***22***

Colorless crystals, m.p. 210–212 °C. IR (KBr) cm^{-1}: 610, 684, 739, 880, 1142, 1185, 1334, 1482, 1610, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 4.80 (s, 6H, Me_2N); 8.10 (br s, 2H, SO_2NH_2); 8.37 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 54.8 (Me); 160.3 (C-2); 173.2 (C-5). Anal. $C_4H_9N_5O_4S_3$ (C, H, N).

2.1.22. 5-(2-Carboxybenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***23***

White crystals, m.p. 275–276 °C (dec.); IR(KBr), cm^{-1}: 685, 729, 755, 879, 1138, 1182, 1280, 1365, 1415, 1586, 1610, 1720, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.34–7.80 (m, 4H, ArH); 8.19 (br s, 3H, SO_2NH_2 + SO_2NH), 10.43 (s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 125.9; 129.0; 129.9; 130.2; 159.6 (C-2 from thiadiazole); 173.5 (C-5 from thiadiazole); 195.4 (COOH). Anal. $C_9H_7N_4O_6S_3$ (C, H, N).

2.1.23. 1-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-trimethyl-pyridinium perchlorate ***24***

White crystals, m.p. > 300 °C; IR (KBr), cm^{-1} (bands in italics are due to the anion): 595, *625*, 664, 787, 803, 884, 915, *1100*, 1150, 1190, 1200, 1285, 1360, 1495, 1604, 3065; ^{1}H-NMR (D_2O), δ, ppm: 3.08 (s, 6H, 2,6-Me_2); 3.11 (s, 3H, 4-Me), 7.30–8.06 (m, AA'BB',4H, ArH from phenylene); 9.05 (s,2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal. $C_{16}H_{18}N_5O_4S_3^+ ClO_4^-$ (C, H, N).

2.1.24. 1-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-triphenhyl-pyridinium tetrafluoroborate ***25***

Pale yellow crystals, m.p. > 300 °C; IR (KBr), cm^{-1} (bands in italics are due to the anion): *610*, 635, 703, 785, 896, *1090*, 1150, 1204, 1355, 1410, 1520, 1600, 3065; ^{1}H-NMR (D_2O), δ, ppm: 7.50–8.60 (m, 19H, ArH, 3Ph + C_6H4); 9.27 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal. $C_{31}H_{24}N_5O_4S_3^+$ BF_4^- (C, H, N).

2.1.25. 5-(2,4-Dinitrobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***26***

Yellow crystals, m.p. 226–228 °C (dec.); IR (KBr), cm^{-1}: 645, 693, 754, 790, 807, 898, 1137, 1170, 1290, 1361, 1426, 1605, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.90–8.15 (m, 3H, ArH); 8.25 (br s, 3H, SO_2NH_2 + SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 132.4; 139.7; 142.0; 144.8; 161.5 (C-2 from thiadiazole); 174.0 (C-5 from thiadiazole). Anal. $C_8H_6N_6O_8S_3$ (C, H, N).

2.1.26. 5-(4-Chloro-3-nitrobenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***27***

Yellow crystals, m.p. 222–223 °C (dec.); IR (KBr), cm^{-1}: 660, 687, 769, 794, 810, 903, 1141, 1175, 1358, 1432, 1598, 3058; ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.65–7.95 (m, 3H, ArH); 8.20 (br s, 3H, SO_2NH_2 + SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 122.4; 127.6; 137.1; 143.5; 163.7 (C-2 from thiadiazole); 172.8 (C-5 from thiadiazole). Anal. $C_8H_6N_5O_6S_3Cl$ (C, H, N).

2.1.27. 5-(2,4,6-Trimethylbenzenesulfonamido)-1,3,4-thiadiazole-2-sulfonamide ***28***

White crystals, m.p. 233–235 °C, IR (KBr), cm^{-1}: 690, 726, 895, 1130, 1175, 1296, 1360, 1420, 1588, 1607, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.51 (s, 3H, 4-Me); 2.68 (s, 6H, 2,6-Me_2); 7.35 (s, 2H, ArH); 8.01 (br s,2H, SO_2NH_2); 8.19 (s, 1H, SO_2NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.3 (Me); 25.4 (Me); 118.3; 118.9; 129.0; 130.8; 159.9 (C-2 from thiadiazole); 171.2 (C-5 from thiadiazole). Anal. $C_{11}H_{14}N_4O_4S_3$ (C, H, N).

2.1.28. 5-Methylsulfonylimido-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***29***

White crystals, m.p. 210–212 °C (from ethanol–water 1:1, v/v); IR (KBr), cm^{-1}: 712, 870, 1136, 1174, 1363, 1428, 1615, 3063; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.70 (s, 3H, $MeSO_2$); 3.91 (s, 3H, N-Me); 8.02 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 24.7 ($MeSO_2$); 38.0 (Me-N); 160.8 (C-2); 166.2 (C-5). Assignements of thiadiazoline signals in the ^{13}C-NMR spectra for derivatives of this type was previously reported by Katritzky et al. [37, 38] and Supuran et al. [39]. Anal. $C_4H_8N_4O_4S_3$ (C, H, N).

2.1.29. 5-Phenylmethylsulfonylimido-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***30***

White crystals, m.p. 238–239 °C (from ethanol–water 1:1, v/v); IR (KBr), cm^{-1}: 705, 851, 924, 1035, 1132, 1175, 1360, 1615, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.35 (s, 2H, CH_2); 3.90 (s, 3H, N-Me); 7.10–7.54 (m, 5H, ArH); 8.12 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 35.7 (CH_2); 38.0 (Me-N); 118.4; 119.9; 133.4; 136.8; 160.9 (C-2); 166.5 (C-5). Anal. $C_{10}H_{13}N_4O_4S_3$ (C, H N).

2.1.30. 5-(4-Methylbenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***31***

White crystals, m.p. 201–205 °C (dec.); IR(KBr), cm^{-1}: 652, 673, 834, 900, 1134, 1170, 1357, 1418, 1530, 1604, 3067; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.52 (s, 3H, Me); 3.90 (s, 3H, N-Me); 7.05–7.47 (m, AA'BB',4H, ArH); 8.02 (br s,2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 21.7 (Me from tosyl); 38.6 (N-Me); 118.1; 118.8; 128.5; 129.4 (all from C_6H_4); 158.9 (C-2 from thiadiazoline); 165.5 (C-5 from thiadiazoline). Anal. $C_{10}H_{12}N_4O_4S_3$ (C, H, N).

2.1.31. 5-(4-Fluorobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***32***

White crystals, m.p. 189 °C (dec); IR (KBr), cm^{-1}: 647, 693, 875, 1135, 1174, 1362, 1421, 1584, 1615, 3064; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.88 (s, 3H, Me); 7.10–7.55 (m, AA'BB',4H, ArH); 8.05 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.8 (Me); 118.5; 118.9; 130.5; 131.2 (all from C_6H_4); 159.8 (C-2 from thiadiazoline); 165.7 (C-5 from thiadiazoline). Anal. $C_9H_9N_4O_4S_3F$ (C, H, N).

2.1.32. 5-(4-Chlorobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***33***

White crystals, m.p. 194–196 °C (dec); IR (KBr), cm^{-1}: 623, 680, 739, 852, 1130, 1175, 1362, 1420, 1585, 1619, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.90 (s, 3H, Me); 7.13–7.61 (m, AA'BB',4H, ArH); 8.09 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.9 (Me); 118.1; 118.9; 130.7; 131.3 (all from C_6H_4); 159.8 (C-2 from thiadiazoline); 165.6 (C-5 from thiadiazoline). Anal. $C_9H_9N_4O_4S_3Cl$ (C, H, N).

2.1.33. 5-(4-Bromobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***34***

White crystals, m.p. 196–198 °C (dec); IR (KBr), cm^{-1}: 618, 693, 755, 859, 926, 1135, 1177, 1365, 1429, 1595, 1610, 3063; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.89 (s, 3H, Me); 7.11–7.62 (m, AA'BB',4H, ArH); 8.08 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.9 (Me); 118.0; 118.9; 130.9; 131.7 (all from C_6H_4); 159.9 (C-2 from thiadiazoline); 165.5 (C-5 from thiadiazoline). Anal. $C_9H_9N_4O_4S_3Br$ (C, H, N).

2.1.34. 5-(4-Iodobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***35***

White crystals, m.p. 225–256 °C (dec); IR (KBr), cm^{-1}: 650, 699, 721, 875, 938, 1135, 1179, 1361, 1421, 1584, 1620, 3064; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.91 (s, 3H, Me); 7.06–7.63 (m, AA'BB',4H, ArH); 8.09 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.8 (Me); 118.2; 119.4; 130.7; 131.9 (all from C_6H_4); 160.5 (C-2 from thiadiazoline); 165.9 (C-5 from thiadiazoline). Anal. $C_9H_9N_4O_4S_3I$ (C, H, N).

2.1.35. 5-(4-Methoxybenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***36***

White crystals, m.p. 218–220 °C (dec); IR(KBr), cm^{-1}: 658, 734, 880, 927, 1134, 1170, 1360, 1428, 1534, 1610, 3065; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.50 (s, 3H, MeO); 3.90 (s, 3H, N-Me); 7.05–7.51 (m, AA'BB',4H, ArH); 8.08 (br s,2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 35.9 (Me from MeO); 38.6 (N-Me); 118.5; 118.8; 128.3; 129.7 (all from C_6H_4); 159.0 (C-2

from thiadiazoline); 165.2 (C-5 from thiadiazoline). Anal. $C_{10}H_{12}N_4O_5S_3$ (C, H, N).

2.1.36. 5-(4-Acetylaminobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***37***

White crystals, m.p. 227 °C (dec); IR (KBr), cm^{-1}: 675, 702, 774, 814, 886, 1132, 1171, 1357, 1392, 1410, 1443, 1545 (acetamide bands); 1650, 3071; ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.81 (s, 3H, Me from acetyl); 3.87 (s, 3H, N-Me); 6.45 (s, 1H, CONH); 7.03–7.41 (m, AA'BB',4H, ArH); 8.00 (br s,3H, SO_2NH_2). ^{13}C-NMR (DMSO-d_6), δ, ppm: 18.2 (Me from acetyl); 38.7 (N-Me); 117.6; 118.0; 127.9; 128.3 (all from C_6H_4); 159.0 (C-2 from thiadiazoline); 165.3 (C-5 from thiadiazoline); 178.4 (CO). Anal. $C_{11}H_{13}N_5O_5S_3$ (C, H, N).

2.1.37. 5-(4-Aminobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***38***

White crystals, m.p. 280–284 °C (dec); IR (KBr), cm^{-1}: 675, 715, 758, 825, 894, 1130, 1167, 1362, 1442, 1596, 3068. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.90 (s, 3H, Me); 5.21 (s, 2H, NH_2); 7.05–7.45 (m, AA'BB',4H, ArH); 7.97 (br s, 2H, SO_2NH_2). ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.5 (N-Me); 117.6; 118.4; 126.5; 128.0 (all from C_6H_4); 158.8 (C-2 from thiadiazoline); 163.1 (C-5 from thiadiazoline); Anal. $C_9H_{11}N_5O_4S_3$ (C, H, N).

2.1.38. 5-(3-Aminobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***39***

White crystals, m.p. 278–281 °C (dec); IR (KBr), cm^{-1}: 637, 690, 788, 854, 896, 1131, 1166, 1365, 1450, 1596, 3065. ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.90 (s, 3H, Me); 5.24 (s, 2H, NH_2); 7.17–7.59 (m, 4H, ArH); 8.01 (br s, 2H, SO_2NH_2). ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.9 (N-Me); 117.3; 118.6; 126.9; 129.5 (all from C_6H_4); 158.4 (C-2 from thiadiazoline); 163.7 (C-5 from thiadiazoline); Anal. $C_9H_{11}N_5O_4S_3$ (C, H, N).

2.1.39. 5-(4-Nitrobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***40***

Pale yellow crystals, m.p. 236–238 °C (dec); IR (KBr), cm^{-1}: 634, 697, 750, 815, 893, 1132, 1175, 1298, 1361, 1426, 1595, 3067; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.90 (s, 3H, N-Me); 7.50–8.02 (m, AA'BB',4H, ArH); 8.18 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.7 (N-Me); 127.6; 128.0; 129.1; 130.0 (all from C_6H_4); 159.9 (C-2 from thiadiazoline); 174.1 (C-5 from thiadiazoline). Anal. $C_9H_9N_5O_6S_3$ (C, H, N).

2.1.40. 5-(3-Nitrobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***41***

Pale yellow crystals, m.p. 234–235 °C (dec); IR (KBr), cm^{-1}: 649, 714, 752, 810, 943, 1134, 1175, 1296, 1360, 1421, 1595, 3063; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.90 (s, 3H, N-Me); 7.34–7.82 (m, 4H, ArH); 8.11 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.8 (N-Me); 126.4; 128.9; 129.5; 130.7 (all from C_6H_4); 160.1 (C-2 from thiadiazoline); 174.5 (C-5 from thiadiazoline). Anal. $C_9H_9N_5O_6S_3$ (C, H, N).

2.1.41. 5-(2-Nitrobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***42***

Pale yellow crystals, m.p. 231–233 °C; IR (KBr), cm^{-1}: 640, 745, 871, 933, 1133, 1170, 1286, 1364, 1416, 1590, 3063; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.89 (s, 3H, N-Me); 7.20–7.61 (m, 4H, ArH); 8.20 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.7 (N-Me); 127.1; 128.4; 129.2; 130.8 (all from C_6H_4); 159.8 (C-2 from thiadiazoline); 174.1 (C-5 from thiadiazoline). Anal. $C_9H_9N_5O_6S_3$ (C, H, N).

2.1.42. 5-(N,N-Dimethylaminosulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***43***

Colorless crystals, m.p. 227–229 °C. IR (KBr) cm^{-1}: 618, 644, 757, 809, 935, 1140, 1171, 1338, 1480, 1600, 3066; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.89 (s, 3H, N-Me); 4.80 (s, 6H, Me_2N); 8.10 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.7 (4-N-Me); 54.9 (Me from Me_2NSO_2); 160.3 (C-2); 172.9 (C-5). Anal. $C_5H_{11}N_5O_4S_3$ (C, H, N).

2.1.43. 5-(2-Carboxybenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***44***

White crystals, m.p. 259–262 °C (dec.); IR(KBr), cm^{-1}: 635, 690, 736, 758, 924, 1130, 1182, 1280, 1361, 1410, 1588, 1610, 1722, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.89 (s, 3H, N-Me); 7.34–7.80 (m, 4H, ArH); 8.19 (br s, 2H, SO_2NH_2), 10.40 (s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.7 (4-N-Me); 125.6; 128.9; 129.5; 130.8; 159.9 (C-2 from thiadiazole); 171.3 (C-5 from thiadiazole); 195.8 (COOH). Anal. $C_{10}H_9N_4O_6S_3$ (C, H, N).

2.1.44. 1-[2-Sulfamoyl-4-methyl-δ^2-1,3,4-thiadiazolidin-5-yl-(iminosulfonyl-4-phenyl)]-2,4,6-trimethyl-pyridinium perchlorate ***45***

White crystals, m.p. > 300 °C; IR (KBr), cm^{-1} (bands in italics are due to the anion): *625*, 671, 785, 804, 923, *1100*, 1153, 1187, 1215, 1362, 1487, 1612, 3070; ^{1}H-NMR (TFA: trifluoroacetic acid), δ, ppm: 2.76 (s, 6H, 2,6-Me_2); 2.85 (s, 3H, 4-Me), 3.88 (s, 3H, N-Me); 7.09–7.47 (m, AA'BB',4H, ArH from phenylene); 7.62 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal. $C_{17}H_{20}N_5O_4S_3^+$ ClO_4^- (C, H, N).

2.1.45. 1-[2-Sulfamoyl-4-methyl-δ^2-1,3,4-thiadiazolidin-5-yl-(iminosulfonyl-4-phenyl)]-2,4,6-triphenhylpyridinium tetrafluoroborate ***46***

Pale yellow crystals, m.p. > 300 °C; IR(KBr), cm^{-1} (bands in italics are due to the anion): *609*, 632, 707, 794, *1090*, 1151, 1210, 1357, 1515, 1590, 1612; ^{1}H-NMR (TFA), δ, ppm: 3.93 (s, 3H, N-Me); 7.25–7.90 (m, 19H, ArH, 3Ph + C_6H_4); 8.30 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal. $C_{32}H_{26}N_5O_4S_3^+$ BF_4^- (C, H, N).

2.1.46. 5-(2,4-Dinitrobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***47***

Yellow crystals, m.p. 229–232 °C (dec.); IR (KBr), cm^{-1}: 650, 699, 733, 779, 820, 890, 1139, 1170, 1364, 1420, 1605, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.92 (s, 3H, N-Me); 7.90–8.18 (m, 3H, ArH); 8.20 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.7 (4-N-Me); 132.7; 139.5; 142.0; 144.8; 161.7 (C-2 from thiadiazole); 170.0 (C-5 from thiadiazole). Anal. $C_9H_8N_6O_8S_3$ (C, H, N).

2.1.47. 5-(4-Chloro-3-nitrobenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***48***

Yellow crystals, m.p. 227–228 °C; IR (KBr), cm^{-1}: 668, 712, 754, 799, 840, 935, 1141, 1172, 1360, 1421, 1599, 3058; ^{1}H-NMR

(DMSO-d_6), δ, ppm: 3.92 (s, 3H, N-Me); 7.65–8.01 (m, 3H, ArH); 8.20 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 38.9 (4-N-Me); 122.2; 127.5; 137.1; 143.5; 163.9 (C-2 from thiadiazole); 170.3 (C-5 from thiadiazole). Anal. $C_9H_8N_5O_6S_3Cl$ (C, H, N).

2.1.48. 5-(2,4,6-Trimethylbenzenesulfonylimido)-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline ***49***

White crystals, m.p. 244–245 °C, IR (KBr), cm^{-1}: 650, 714, 798, 857, 1133, 1168, 1299, 1360, 1418, 1593, 1607, 3060; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.51 (s, 3H, 4-Me); 2.70 (s, 6H, 2,6-Me_2); 3.92 (s, 3H, 4-N-Me); 7.39 (s, 2H, ArH); 8.00 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.3 (Me); 25.5 (Me); 38.9 (4-N-Me); 118.5; 119.6; 129.5; 130.8; 159.9 (C-2 from thiadiazole); 169.5 (C-5 from thiadiazole). Anal. $C_{12}H_{16}N_4O_4S_3$ (C, H, N).

2.2. Pharmacology

2.2.1. Determination of water (buffer) solubility

A standard solution was prepared by dissolving a precisely weighted amount (generally 1 mg) of inhibitor in 10 mL of methanol. The UV absorption maximum of each compound has been determined (with a Cary 3 spectrophotometer) eventually diluting the solution (with MeOH) as necessary. A saturated solution of each compound was then prepared by stirring magnetically a small volume of 0.039 M phosphate buffer (pH 7.4) in the presence of excess inhibitor for 3 h. The obtained saturated solution was filtered in order to remove solid compound through a Millipore 0.45 μm filter and scanned by UV at the wavelength of the absorption maximum previously determined. Total solubility was determined by the relationship: $C' = A'C/A$, where C = concentration of standard solution (mg/mL); A = absorbance of standard solution; A' = absorbance of the saturated solution; C' = concentration of the saturated solution (mg/mL).

2.2.2. Partition coefficient determinations

Chloroform–buffer partition coefficients were obtained by equilibrating the test compound between chloroform and 0.1-ionic strength pH 7.4 phosphate buffer. The concentration in each phase was determined by UV spectrophotometry [40].

2.2.3. Carbonic anhydrase inhibition

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 [41]. 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, in the conditions of the experiments (pH 7.40), as reported in the literature [41]. Non-enzymic 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 [21, 22]) 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 [41]. Enzyme concentrations were 3.5 nM for CA II, 10 nM for CA I and 35 nM for CA IV (this isozyme has a decreased esterase activity [42] and higher concentrations had to be used for the measurements).

3. Results and discussion

Reaction of sulfonyl chlorides/anhydrides with 5-amino-1,3,4-thiadiazole-2-sulfonamide **6** or 5-imino-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline **7**, in Schotten–Baumann conditions, afforded derivatives **8–49**, by the method of Vaughan et al. [20] (*figure 2* and *tables I* and *II*). The new compounds were characterized by standard chemical and spectroscopic procedures that confirmed their structure (see the experimental protocols for details).

CA inhibition data against three isozymes, CA I, II and IV as well as buffer solubility and chloroform–buffer partition coefficients for the prepared compounds **8–49** and the lead molecule, benzolamide **5**, are shown in *tables I* and *II*. Inhibition data with the standard inhibitors **1–4** are shown in *table III*.

The interest in 5-alkyl/aryl-sulfonylamido-1,3,4-thiadiazole-2-sulfonamides and the corresponding thiadiazolines as putative tight-binding and/or isozyme specific CA inhibitors might be rationalized on the following aspects emerged after the recent report of the X-ray crystallographic structure of the adduct of HCA II with aminobenzolamide **17** by Liljas' group [43] (*figure 3*).

As many other sulfonamide inhibitors [17, 44], aminobenzolamide is coordinated as the fourth ligand to the Zn(II) ion within the CA active site, and the NH^- moiety of the primary sulfonamido group forms a hydrogen bond with the OH moiety of Thr 199 [43]. But in contrast to other sulfonamide inhibitors for which the X-ray crystallographic structure has been reported, such as acetazolamide [44] or the thienothiopyran sulfonamides [17], benzolamides participates in supplementary interactions with the active site, and this probably accounts for the higher affinity of this inhibitor for HCA II (as compared to acetazolamide for instance). Thus, an oxygen atom of

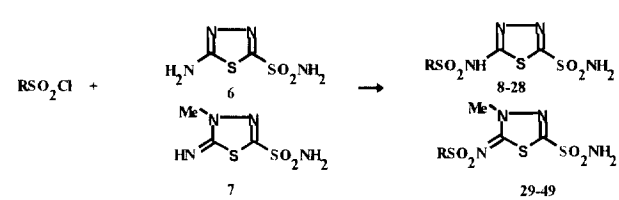

Figure 2.

Table I. Compounds of type **8–28** prepared, their inhibition data (K_I) towards HCA I, HCA II, and BCA IV, chloroform–buffer partition coefficients, and solubility data.

No.	R	K_I (nM)			Log P[c]	S[d] (mg/100 mL)
		HCA I[a]	HCA II[a]	BCA IV[b]		
5	C_6H_5 (benzolamide)	15	9	12	0.0001	40
8	Me	10	6	5	0.024	542
9	$PhCH_2$	7	5	6	0.104	67
10	4-Me-C_6H_4	5	4	3	0.025	2030
11	4-F-C_6H_4	4	4	7	0.019	1232
12	4-Cl-C_6H_4	4	3	5	0.027	347
13	4-Br-C_6H_4	3	2	4	0.140	295
14	4-I-C_6H_4	2	1	2	0.165	280
15	4-$MeOC_6H_4$	5	3	4	0.030	195
16	4-$AcNHC_6H_4$	10	3	8	0.024	900
17	4-H_2N-C_6H_4	6	2	5	0.015	1050
18	3-H_2N-C_6H_4	9	1	7	0.016	975
19	4-O_2N-C_6H_4	3	1	2	0.013	1700
20	3-O_2N-C_6H_4	2	0.9	1	0.014	1278
21	2-O_2N-C_6H_4	5	3	4	0.026	549
22	Me_2N	19	8	13	0.382	60
23	2-$HO_2CC_6H_4$	1	0.5	0.6	0.001	176
24 [e]	4-$Me_3Py^+C_6H_4$	18	4	10	< 0.0001	475
25 [f]	4-$Ph_3Py^+C_6H_4$	360	110	320	0.430	320
26	2,4-$(O_2N)_2C_6H_3$	12	5	28	0.740	50
27	4-Cl-3-O_2N-C_6H_3	9	3	7	0.085	407
28	2,4,6-$Me_3C_6H_2$	15	9	12	0.540	72

[a] Human (cloned) isozymes.
[b] From bovine lung microsomes.
[c] Chloroform–buffer partition coefficient.
[d] Solubility in pH 7.40 buffer, at 25 °C.
[e] As perchlorate salt.
[f] As tetrafluoroborate salt.

the secondary sulfonamido moiety forms a 3.2 Å hydrogen bond with the ε-2 nitrogen atom of Gln 92, whereas the phenyl ring of the inhibitor has edge-to-face van der Waals interactions with Phe 131, which strongly favour the stability of the enzyme-inhibitor adduct [43, 45]. These data should imply that compounds of type **8–49** reported by us here should be good candidates for tight-binding at least for HCA II, since they possess the two structural elements mentioned above: the secondary sulfonamido group, and (in most cases) a substituted aromatic ring originating from the sulfonyl chloride used in the synthesis. The nature of the moieties substituting the secondary sulfonamido group (R in formulas **8–49**) is thus of critical importance for the efficiency as CA inhibitor of the compounds containing them. For this reason we included a large variety of such groups, which obviously, also influence the solubility and pharmacological properties of these enzyme inhibitors. Mention should be made that some very bulky groups were introduced in some cases in the 4-position of the phenyl ring of benzolamide – our lead molecule – in order to exploit the above-mentioned factors, evidenced in the structure of the HCA II – aminobenzolamide adduct, together with some other possible interactions, involving hydrophobic 'patches' situated in the lip of HCA II active site. This type of approach has been previously used by Whitesides group [46] for designing strong CA II inhibitors, prepared from 4-carboxy-benzenesulfonamide and phenylglycylglycylglycine benzyl ester, or by Christianson's group [47] for obtaining a sulfonamide probe with nanomolar afinity to HCA II, used for fluorescence anisotropy detection of zinc with a CA-based biosensor. These approaches used the attachment of bulky/long moieties to benzenesulfonamide derivatives, in order to exploit these cooperative interactions which assure increased stability to the E–I adduct. Finally, QSAR studies of this group rationalized theoretically why such a 'long' molecule would bind tighter to CA [48, 49].

Table II. Compounds **29–49** synthesized, their inhibition towards HCA I, HCA II and BCA IV, partition coefficient and solubility data.

No.	R	K_I (nM)			Log P^c	S^d (mg/100 mL)
		HCA I[a]	HCA II[a]	BCA IV[b]		
29	Me	17	4	8	0.104	240
30	$PhCH_2$	6	8	9	0.097	115
31	4-Me-C_6H_4	5	3	3	0.009	50
32	4-F-C_6H_4	8	4	7	0.144	330
33	4-Cl-C_6H_4	8	3	5	0.217	134
34	4-Br-C_6H_4	5	2	6	0.286	127
35	4-I-C_6H_4	1	0.6	1	0.310	78
36	4-$MeOC_6H_4$	6	3	5	0.209	64
37	4-$AcNHC_6H_4$	2	0.7	2	0.066	800
38	4-H_2N-C_6H_4	1	0.6	0.8	0.395	110
39	3-H_2N-C_6H_4	1	0.5	0.8	0.338	125
40	4-O_2N-C_6H_4	8	4	6	0.402	75
41	3-O_2N-C_6H_4	7	2	5	0.396	81
42	2-O_2N-C_6H_4	5	1	3	0.542	62
43	Me_2N	9	5	8	0.441	60
44	2-$HO_2CC_6H_4$	1	0.2	0.5	0.002	117
45[e]	4-$Me_3Py^+C_6H_4$	17	4	12	< 0.0001	75
46[f]	4-$Ph_3Py^+C_6H_4$	455	110	180	0.345	64
47	2,4-$(O_2N)_2C_6H_3$	10	4	8	0.852	77
48	4-Cl-3-O_2N-C_6H_3	7	2	5	0.098	140
49	2,4,6-$Me_3C_6H_2$	13	7	9	0.659	38

[a] Human (cloned) isozymes.
[b] From bovine lung microsomes.
[c] Chloroform–buffer partition coefficient.
[d] Solubility in pH 7.40 buffer, at 25 °C.
[e] As perchlorate salt.
[f] As tetrafluoroborate salt.

Table III. CA inhibition data with standard sulfonamide inhibitors **1–4**.

Inhibitor	K_I (nM)		
	HCA I[a]	HCA II[a]	BCA IV[b]
Acetazolamide **1**	900	12	220
Methazolamide **2**	780	14	240
Ethoxzolamide **3**	25	8	13
Dorzolamide **4**	> 50 000	9	43

[a] Human (cloned) isozymes.
[b] From bovine lung microsomes.

Derivatives **8–49** prepared by considering the above-mentioned rationale contained alkylsulfonyl-, dimethylaminosulfamoyl- or halogeno-, alkyl-, methoxy-, amino-, and nitro-substituted-phenylsulfonyl moieties, and were chosen in order to obtain derivatives with different physico-chemical properties (i.e., enhanced lipophilicity or conversely, enhanced hydrosolubility, etc.) but also groups that would allow an easy derivatization, which might be exploited to prepare isotopically labeled compounds, envisaging a possible application of such inhibitors in PET imaging.

Two approaches were considered for this latter scope: (i) a nucleophilic displacement of the 4-substituent of the benzene nucleus, as for instance the replacement of nitro groups in compounds **19–21**, **40–42** by diverse nucleophiles, since they are among the best leaving-groups in aromatic nucleophilic substitution reactions (being also activated by the SO_2N – hetaryl moieties, as well as the other substituents of the benzene nucleus), and (ii) reaction of amino groups in compounds of type **17, 18, 38, 39** with pyrylium salts, when pyridinium derivatives of type **24, 25, 45, 46** can be obtained, which might be labeled with ^{11}C by using labeled pyrylium salts. Alternatively, they can be used in nucleophilic substitution reactions of the type mentioned above, since it was

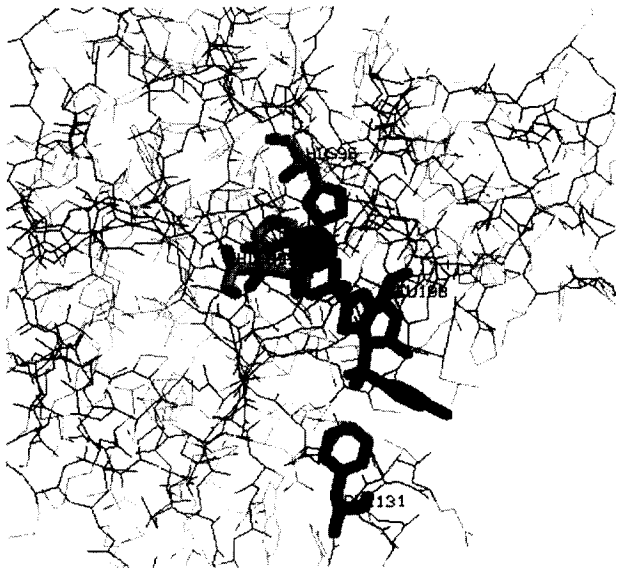

Figure 3. HCA II – aminobenzolamide (**17**) complex. The active site is shown with the Zn(II) ion (central sphere), its three histidine ligands (His 94, 96 and 119) and the coordinated aminobenzolamide molecule. The amino-phenyl ring is pointing outward of the active site and is interacting with the phenyl ring of Phe 131, whereas an oxygen of the secondary sulfonamido moiety forms a hydrogen bond with Gln 92. The figure has been generated from the X-ray crystallographic coordinates of Vidgren et al. [43] by using the program RasMol for Windows 2.6, with a Texas Instruments 4000 M Notebook. This structure is not deposited in Brookhaven Protein Database.

proved by Katritzky [50] that triarylpyridinium moieties are good leaving groups in a variety of nucleophilic substitutions.

By the first approach it was thought that the good leaving-group potential of the nitro group in compound **19** could be exploited in order to replace it by fluorine, for introducing the relatively long-lived positron-emitting isotope ^{18}F in the molecule of an inhibitor of this type (*figure 4*). The model reaction, reported by us here, has been done with the stable isotope of fluorine.

The main complication in a reaction of this type consists in the high acidity (pK_a around 3) of the SO_2NH moiety in compound **19**, which could impair the desired reaction, i.e., the nucleophilic displacement of NO_2 by F. As the nitrobenzolamide **19** gave a low yield of fluorobenzolamide **11** (first reaction in *figure 4*), protection of the sulfonamido moieties was considered, by means of benzyl-chloromethyl ether. The monoprotected derivative **50** as well as the triprotected one **51** were prepared in this way (*figure 4*) (no diprotective derivative could be prepared). But their reactions with KF in the presence of cryptand [2.2.2] gave again low (0.5–2%) yields of fluorinated sulfonamides **52**, **53**, according to reactions shown in *figure 4*. Thus, this type of aromatic nucleophilic displacement, using DMSO as solvent and working at high temperatures is ineffective for introducing fluorine in the molecule of a CA inhibitor, although it was successfully applied for the production of ^{18}F-16-α-

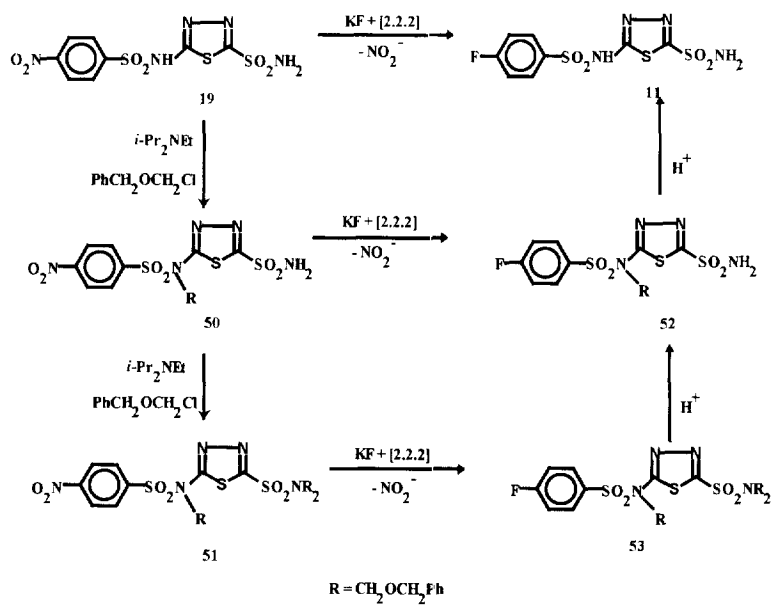

Figure 4.

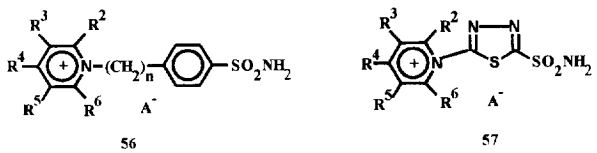

Figure 5.

fluoroestradiol [51] or ^{18}F-fluorodeoxyglucose [52, 53], which are extensively used ligands in a variety of PET medical imaging [54, 55] or visualization of biochemical processes in vivo [56].

By the second approach mentioned above, 4-aminobenzolamide **17** and its analogue **38**, were reacted with 2,4,6-trisubstituted pyrylium salts, in order to obtain the pyridinium derivatives **24, 25** and **45, 46**, respectively. Mention should be made that the preparation of positively-charged CA inhibitors by using this approach was extensively explored by Supuran et al. [57, 58] both for aromatic as well as heterocyclic derivatives, of type **56**, **57**. Some compounds from these series have shown very good CA inhibitory properties against bovine CA (a HCA II type isozyme) and HCA I [57, 58] (see *figure 5*).

By using 2,4,6-trimethyl- and 2,4,6-triphenylpyrylium salts **54, 55** and the above-mentioned aminoderivatives **17**, **38**, the trisubstituted pyridinium derivatives **24, 25, 45** and **46** were prepared (*figure 6*).

^{11}C-Isotopically labeled derivatives of this type might be also obtained easily, since the syntheses of ^{13}C-labeled 2,4,6-trimethylpyrylium salts were reported by Balaban's group [59], and they could be readily adapted for preparing ^{11}C-labeled **54**. As the ^{11}C is a relatively short-lived isotope, here we have explored another approach for a possible introduction of the longer-lived positron emitting isotope ^{18}F into the molecule of a CA inhibitor, ie, the nucleophilic displacement reaction of the triphenylpyridinium moiety from **25** or **46** by fluoride (*figure 7*). Certainly, only the model reaction is reported, involving the stable isotope of fluorine.

Thus, the reaction of the triphenyl-pyridinium derivatives **25** and **46** with KF and cryptand [2.2.2] in DMSO has been done, in order to obtain the fluoroderivatives **11** and **32**, respectively. Unfortunately, this approach also proved to be inefficient, as the fluoroderivatives could be isolated with an overal yield of 0.5–1%. A large number (around 7) of unidentified reaction products were evidenced too.

Although the procedures envisaged above did not lead for the moment to encouraging results regarding the nucleophilic substitution of nitro or triarylpyridinium moieties by fluoride in benzolamide-like derivatives, and whence their applications in PET imaging, this type of approach might be optimized by designing other leaving groups or reactions that would proceed with less complications. In fact, in both types of experiments reported here, the nucleophilic substitutions led to complex reaction mixtures (around 5–7 compounds were evidenced but the structure could not be fully assigned for all of them) in which the desired fluorosulfonamide amounted

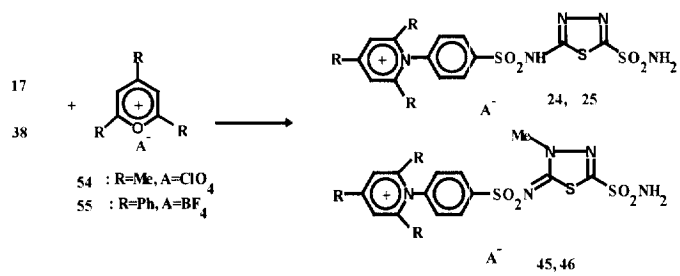

Figure 6.

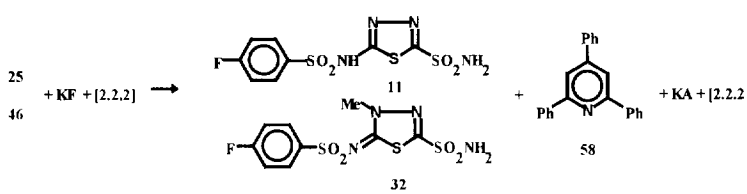

Figure 7.

for less than 3%. This is probably also due to the high temperatures at which the reactions were performed. But one of the positive aspects of this research was that many of the prepared compounds showed excellent inhibitory properties against all three CA isozymes investigated.

As seen from data of *tables I* and *II*, and also by comparison with the data of *table III*, where standard CA inhibitors were assayed in the same conditions, it is obvious that the whole class of 5-alkyl/arylsulfonylamido-1,3,4-thiadizole-2-sulfonamides and the corresponding thiadiazolines behave as very potent inhibitors against all three CA isozymes investigated. The most susceptible isozyme to inhibition was CA II, followed by CA IV and then CA I. Still, in contrast to classical inhibitors **1–4**, the compounds reported by us have a much greater affinity for CA I. Thus the ratio of the K_I's CA I/CA II ranged between 50–5500 for the classical inhibitors **1**, **2** and **4**, whereas for ethoxzolamide is 3 (but ethoxzolamide has a somewhat atypical behaviour). Typically, heterocyclic sulfonamides have a 50–100 times lesser affinity for HCA I as compared to HCA II [3, 10, 11]. The above ratio for derivatives **8–49** is on the other hand always in the range 2–5 (typically around 3), proving that a small but important improvement for designing a CA I-specific inhibitor has been done. Good inhibition profiles were observed for CA IV too, but without reaching any selectivity as reported previously for some other classes of inhibitors by us [22, 23]. Still in the case of CA IV, some kind of specificity might be expected in vivo, since at physiological pH values, these inhibitors should be highly dissociated and thus membrane-impermeant, case in which they would interfere principally with the membrane-bound isozyme, CA IV. Substitution patterns leading to very powerful inhibitors included such moieties as nitro, iodo, fluoro, amino and especially carboxy attached to the phenyl group of the lead molecule, benzolamide. Obviously, these groups participate in the supplementary interactions with amino acid residues at the entrance of the enzyme active site mentioned earlier. Generally, the thiadiazolines were slightly more active than the corresponding thiadiazole-sulfonamides. It should be also stressed the large difference of solubility and lipophilicity between pairs of such compounds (compare for instance **19** and **40**) that might be very important for eventual applications in physiological studies, with the thiadiazolines much more liposoluble and whence diffusible than the thiadiazoles.

In conclusion, in the present study we report two series of benzolamide-like compounds which behave as strong inhibitors against three isozymes, HCA I, HCA II and BCA IV. Some of these derivatives were tried to be derivatized in order to introduce fluorine into their molecule as model reactions for putative PET applications. Such compounds might already be used in the presence of a carrier (such as the non-radioactive corresponding inhibitor) for studying ligand–receptor interactions in PET, since the affinity of the inhibitors for the receptor is very high (in the nanomolar range, for HCA IV).

Note added in proof: Transformation of nitrobenzolamide **19** into ^{18}F-fluorobenzolamide **11** has been achieved with high yields (> 50%) by the first reaction of *figure 4* (without the need of protecting the sulfonamide moieties) working at 80 °C and in acetonitrile as solvent. In these much milder conditions no secondary reaction products were formed and the radio-labeled fluorobenzolamide could be easily separated by HPLC from the reaction mixture. Thus, the original approach described in this paper for obtaining CA inhibitors for application as PET diagnostic agents is indeed of help, now that the chemical synthesis process has been optimized. The results of these new experiments will be published shortly [60].

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