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Letter to the editor

Carbonic anhydrase inhibitors: synthesis and inhibitory properties of 1,3,4-thiadiazole-2,5-bissulfonamide

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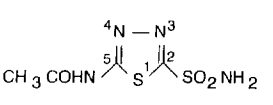
Summary — 1,3,4-Thiadiazole-2,5-bissulfonamide was prepared from 2,5-dimercapto-1,3,4-thiadiazole, by a modification of the reported literature procedure, ie, via the sulfenamide. This compound is the lead molecule for designing important classes of pharmacological agents, such as benzothiadiazine diuretics, and it seems to behave as a strong inhibitor of the zinc enzyme carbonic anhydrase (CA). Here we prove that the title compound is actually a weak CA inhibitor and try to explain why previous investigators obtained erroneous data.

carbonic anhydrase / disulfonamide / inhibitor

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc enzyme commonly found in the plant and animal kingdoms, acting as a highly efficient catalyst for the reversible hydration of carbon dioxide to bicarbonate. It also catalyzes other non-physiological reactions, such as aldehyde hydration and ester or sultone hydrolysis [1, 2].

Sulfonamides are specific inhibitors of CA [1–3], binding in an ionized form to the Zn(II) ion within the active site and displacing the water molecule bound to the metal ion [4, 5], which is responsible for the catalytic power of the enzyme [6]. Moreover, heterocyclic derivatives such as acetazolamide **1** [1], methazolamide **2** [1], or the recently developed thienothiopyran sulfonamides of type **3** [5] are widely used pharmacological agents in the treatment of glaucoma [7, 8], diverse neurological disorders [1, 9] and acid-base disequilibria [1, 10] as well as in many physiological studies [1, 11]. 1,3,4-Thiadiazole-2,5-bissulfonamide **4**, a compound prepared in the classical study of Roblin and Clapp [12] was reported by Miller et al [13] to be a very strong CA inhibitor, but it seems that subsequent attempts to prepare this compound were unsuccessful.



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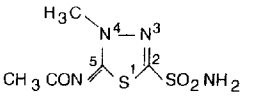

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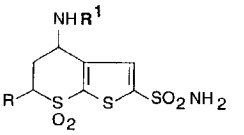
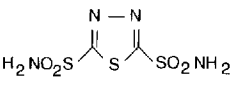


3 a R=H; R^1=Me_2CHCH_2-
b R=Me; R^1=Et



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In the present study we report synthesis of **4** from 2,5-dimercapto-1,3,4-thiadiazole via the sulfenamide and measure its inhibitory activity.

Chemistry and pharmacology

Historically, **4** is an important molecule because it constitutes a lead for designing derivatives with biological activities based on the disulfonamide structure. Thus, the simplest compound in the aromatic series from this class of CA inhibitors, benzene-1,3-disulfonamide **5**, is a strong inhibitor towards bovine CA II [14]. Starting from this structure, dichlorophenamide **6** was developed [15], which is both a strong CA

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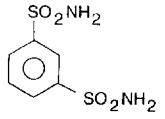

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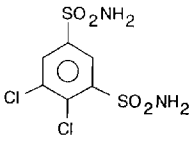

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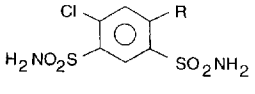
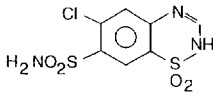


7 : R=CH_3
8 : R=NH_2



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inhibitor, as well as a diuretic drug (IC_{50} = 3 x 10^{-8} M, against human CA) [1]. This drug possesses renal chloruretic properties of the thiazide type [16], which are independent of the CA inhibitory effects [15, 16].

Initially, the pharmacological effects of the saluretic thiazides were accounted for by their CA inhibitory activity [15], and a great number of unsubstituted mono- and disulfonamides were synthesized and tested [17, 18]. Only later, Maren [1, 16] showed that the two effects are independent, although the majority of thiazides are moderately inhibitory.

The pathway towards the discovery of the benzothiazides was continued with two other molecules: disulfamide (4-chloro-6-methyl-1,3-benzenedisulfonamide) **7**, an orally active diuretic agent [19], and salamide (4-amino-6-chloro-1,3-benzenedisulfonamide) **8** [15, 20], which is both the chemical precursor and metabolite of chlorothiazide **9** [1, 15]. Except for dichlorophenamide **6**, still in clinical use as diuretic [21], other sulfonamides of type **5–8** have no clinical importance but they led to the discovery of thiazide diuretics. Cyclization of **8** with orthoesters or aldehydes gives benzothiazides of type **9** [15], which, although being moderately inhibitory, as mentioned above, produce important saluretic effects and in consequence are widely employed clinically for mobilization of body fluids in diverse pathological states [1, 21].

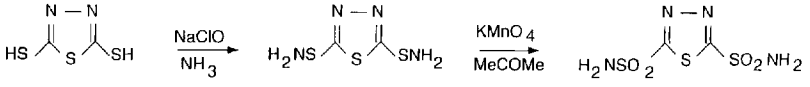
It is critical to note that the most important structural feature of all these compounds is the presence of two sulfamoyl groups in the *meta* position (possibly within a cyclic moiety, as for the benzothiadiazines of type **9**), which are responsible for the diuretic activity of these drugs. An important variant is represented by the high ceiling diuretic furosemide, in which the second sulfamoyl group is replaced by a carboxyl group or its equivalent. As will be shown, the original molecule which served as lead for developing all these derivatives, ie, 1,3,4-thiadiazole-2,5-bissulfonamide **4**, is a weak CA inhibitor (K_I = 10^{-5} M), but it was (inexplicably) originally reported as very potent ($K_I \cong 10^{-8}$ M).

Results and discussion

The title compound was prepared from 2,5-dimercapto-1,3,4-thiadiazole **10**, which is commercially available (scheme 1). This was oxidized with sodium hypochlorite in the presence of ammonia, at pH 13 (in the presence of NaOH), with the formation of 1,3,4-thiadiazole-2,5-disulfenamide **11**, which was not isolated. This was oxidized with potassium permanganate to the corresponding disulfonamide **4**. This type of transformation of mercaptans to sulfonamides, via the sulfenamide, was originally reported by Korman, for derivatives of benzothiazole-2-sulfonamide [22]. Our synthesis is different from that of Roblin and Clapp [12], who prepared **4** by the oxidative chlorination of **10** in acetic acid, and amidation of the resultant bissulfonyl chloride (only melting point and elemental analysis were reported for **4** in ref [12]).

Although the previous investigators [13] reported **4** to be a 2.4 times stronger CA inhibitor (IC_{50} = 8 x 10^{-9} M) than acetazolamide **1**, we failed to reproduce these data. Instead, we show that **4** is a very weak inhibitor (K_I = 10 μM) (table I). The assay used was essentially the same as in the literature [13].

Two questions remain unanswered about the bissulfonamide **4**, which, as we have shown, possesses historical importance for the development of diverse pharmacological agents. Why is **4** such a weak CA inhibitor, especially in light of the acidity of one of the two sulfonamide ionizations? A structurally related compound, acetazolamide **1**, is a very strong inhibitor. The second question is: why did Miller et al [13] obtain erroneous results in their assay?



10 → 11 → 4

Scheme 1.

Only hypotheses can be made about the answers of these questions. First, the weakly inhibitory properties of **4** may be accounted for by a sterically demanding interaction within the enzyme active site, mainly due to the bulky second sulfonamide group, and the lack of any additional hydrophobic moieties on the molecule, which would tend to lower the I_{50}. Second, it is possible that the compound reported by Roblin and Clapp [12] in some way was not **4** but an impurity formed in the synthesis and not correctly identified. Assuming stability is not a problem, a candidate might be the thio-analogue of acetazolamide, 5-acetylthio-1,3,4-thiadiazole-2-sulfonamide **12**, which probably possesses strong CA inhibitory properties and might be obtained in a reaction mixture containing **10**, acetic acid, chlorine and water, with impurities such as acetaldehyde or acetyl chloride. In order to test our hypothesis, we tried to synthesize **12** by the classical route, envisaging the mercapto derivative **13** as intermediate, which by oxidative chlorination should afford the corresponding sulfonyl chloride. This could be aminated thereafter to obtain **12**. Alternatively, the sulfenamide variant of this synthesis was also considered.

Thus, **10** was treated with one equivalent of an acetylating agent (acetic anhydride, acetyl chloride, acetic acid, etc) using a diversity of experimental procedures in order to obtain the key intermediate, the monoacetyl derivative **13**. Unfortunately, in all cases only a mixture of the bisacetylated derivative **14** and unreacted **10** could be isolated from such reaction mixtures. Thus, we are presently unable to answer whether 'thioacetazolamide' **12** is or is not a strong CA inhibitor and whether this is the compound that induced the error in the assay of Miller et al [13].

Experimental protocols

Melting points were recorded on a Fisher-Johns apparatus and are not corrected. IR spectra were obtained with a Perkin Elmer 1600 FTIR instrument. ^{1}H NMR spectra were recorded in deuteriochloroform with a Bruker 200XL instrument, working at 200 MHz, and ^{13}C NMR spectra with a Bruker CXP 300 apparatus, in D_2O as solvent. Chemical shifts are reported as δ values (ppm), relative to tetramethylsilane as internal standard. Elemental analyses were obtained with an automatic Carlo Erba (Milan, Italy) combustion instrument.

2,5-Dimercapto-1,3,4-thiadiazole was from Aldrich, other inorganic, organic reagent and solvents were analytical grade and were used without further purification. Hemolyzed dog red cell CA was the source of enzyme in this study [14].

Table I. Inhibitory potency (I_{50}) of selected sulfonamides against CA II at 0° C.

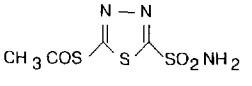
Compound	I_{50} (μM)[a]
Acetazolamide **1**	0.004
Methazolamide **2**	0.007
MK-417 **3a**	0.002
Thiadiazole-disulfonamide **4**	10
Benzene-1,3-disulfonamide **5**	0.008
Dichlorophenamide **6**	0.030
Disulfamide **7**	0.070
Salamide **8**	0.075
Chlorothiazide **9**	0.060
Benzenesulfonamide	200

[a]Maren and Wiley [14] and further work in this laboratory.

Synthesis of 1,3,4-thiadiazole-2,5-disulfonamide 4

Twenty millimoles of 2,5-dimercapto-1,3,4-thiadiazole **10** were dissolved in a solution containing 1.8 g (45 mmol) NaOH and 20 mL water. The yellow solution which formed was cooled at 0° C with ice and salt and, by means of two dropping funnels, 15 mL of concentrated ammonia solution and 25 mL of bleach solution (5.5% NaClO) respectively, were added dropwise, for 30 min, with intense magnetic stirring. The sulfenamide formed by oxidation was collected by filtration, washed with cold water until neutral pH, suspended in 15 mL acetone and treated with a small excess of saturated $KMnO_4$ solution in acetone. The precipitated MnO_2 obtained by oxidation of the sulfenamide was filtered and discarded, the excess $KMnO_4$ was removed with a small amount of oxalic acid, the acetone was evaporated in vacuum and the residue was recrystallized from 200 mL water. Three grams of **4** were obtained (62% yield, based on the dimercaptothiadiazole **10**), mp 189 °C, lit [12] mp 187–189 °C; anal: found: C, 9.9, H, 1.7, N, 22.9%; $C_2H_4N_4O_4S_3$ requires: C, 9.8, H, 1.7, N, 22.9%.

In the FTIR spectrum of **4**, the strong SO_2 vibrations were evidenced at 1107 cm^{-1} ($\nu^s(SO_2)$) and 1378 cm^{-1} ($\nu^{as}(SO_2)$), respectively. A broad NH_2 band was observed at 3080 cm^{-1} and no S-H or C=S vibrations (the latter due to possible tautomeric forms of the raw material **10**) were present in the IR spectrum of the synthesized compound. The ^{1}H NMR (recorded in $CDCl_3$) of **4** afforded only a broad singlet at 7.20 ppm, which readily disappeared when D_2O was added in the NMR tube (due to the fact that the sulfonamido protons are in fast exchange with the solvent). In the ^{13}C NMR spectrum (in D_2O) again only one signal was detected, at 162.40 ppm, due to the equivalence of the two carbon atoms in this symmetrical molecule.



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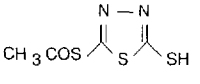

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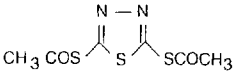

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In vitro assay of CA inhibition

The inhibitory potency of **4** was assayed at 0 °C using 100% CO_2 in a total cell volume of 7 mL with hemolyzed dog red cell CA [14]. Enzyme and inhibitor were preincubated at 0 °C for 5 min in the absence of substrate, in order to allow the formation of the enzyme–inhibitor adduct. The reaction was started by addition of 2 mL barbital buffer (50 mM, pH 7.9), and the time recorded to obtain an endpoint at pH 6.8 with bromothymol blue as indicator. The number of enzyme units in the system is given by:

$$EU = (T_{unc} - T_c)/T_c$$

where T_c is the enzyme catalyzed rate of CO_2 hydration (in seconds), and T_{unc} the corresponding uncatalyzed rate. The I_{50} is therefore the inhibitor concentration which halves the enzyme units in the system [1, 15].

Acknowledgments

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