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ORIGINAL ARTICLE

Salts of 5-amino-2-sulfonamide-1,3,4-thiadiazole, a structural and analog of acetazolamide, show interesting carbonic anhydrase inhibitory properties, diuretic, and anticonvulsant action

Jorge R. A. Diaz[1], Gerardo Enrique Camí[1], Malva Liu-González[2], Daniel R. Vega[3], Daniela Vullo[4], Américo Juárez[5], José C. Pedregosa[1], and Claudiu T. Supuran[4]

[1]Área De Química General E Inorgánica, Facultad De Química, Bioquímica Y Farmacia, CONICET PIP-6246, Universidad Nacional De San Luis, San Luis, Argentina, [2]S.C.S.I.E. (Unidad De Rx) – Facultad De Física, Universidad De Valencia, Valencia, Spain, [3]Grupo Materia Condensada, GlyA, GAlyANN, Comisión Nacional De Energía Atómica, San Martín, Buenos Aires, Argentina, [4]Department of Neurofarba, University of Florence, Sesto Fiorentino (Firenze), Italy, and [5]Laboratorio De Farmacología, Departamento De Farmacia, Facultad De Química, Bioquímica Y Farmacia, Universidad Nacional De San Luis, San Luis, Argentina

Abstract

Three salts of 5-amino-2-sulfonamide-1,3,4-thiadiazole (Hats) were prepared and characterized by physico-chemical methods. The *p*-toluensulfonate, the methylsulfonate, and the chlorhydrate monohydrate salts of Hats were evaluated as carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) and as anticonvulsants and diuretics, since many CAIs are clinically used as pharmacological agents. The three Hats salts exhibited diuretic and anticonvulsant activities with little neurotoxicity. The human (h) isoforms hCA I, II, IV, VII, IX, and XII were inhibited in their micromolar range by these salts, whereas pathogenic beta and gamma CAs showed similar, weak inhibitory profiles.

Keywords

Anticonvulsant, carbonic anhydrase, diuretic, sulfonamides, sulfonamide salts, thermal stability

History

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Introduction

Sulfonamide inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), such as acetazolamide, have been used clinically as diuretics, antiglaucoma, or anticonvulsant agents for a long period[1–9], whereas more recent drug design studies have evidenced other CA inhibitors (CAIs) belonging to the sulfonamide/sulfamate/sulfamide classes as molecules of interest for developing novel therapies for obesity[10,11] and cancer[12–15], based on the selective inhibition of CA isozymes involved in such pathologies, among which 15 such isoforms described so far in humans[1,16].

Epilepsy is one of the most common serious neurologicaldisorders characterized by recurrent seizures. Since several decades, acetazolamide is used as an anticonvulsant agent in the treatment ofepilepsy[1,10,11]. Despite the development of a rapid tolerance consisting in diminished therapeutic efficacy after the initial response of the patients, acetazolamide is still used in combination therapy with other antiepileptic drugs or inrefractory epilepsies[1].

We have obtained and determined the crystal structures of sulfonamides incorporating the 1,3,4-thiadiazole ring[17–23]. The results obtained by our group with these sulfonamides indicated a notable increase in the CA inhibitory properties of metal complexes of sulfonamides such as acetazolamide or its deacetylated precursor, 5-amino-1,3,4-thiadiazole-2-sulfonamide (Hats)[23]. Furthermore, continuing our studies on unsubstituted heterocyclic sulfonamides and their metal complexes[17–23], we started to study the role of new salts of Hats with regard to their biological activity. In this work, we report new salts of Hats with *p*-toluenesulfonic acid, methylsulfonic acid, and the hydrochloride monohydrate salts. We evaluated the CA inhibition and anticonvulsant/diuretic activities of these three sulfonamide salts.

Address for correspondence: Gerardo Enrique Camí, Área De Química General E Inorgánica, Facultad De Química, Bioquímica Y Farmacia, CONICET PIP-6246, Universidad Nacional De San Luis, Chacabuco 917, San Luis 5700, Argentina. E-mail: gecami88@gmail.com

Experimental

Salt formation

The synthesis of Hats was performed in two steps (Figure 1). In the first step, the deacetylation of acetazolamide (AZA) to give Hats was performed following the procedure reported in the literature[17]. This product was characterized by its FTIR spectrum resulting identical to that reported by Pedregosa et al.[17,18]. In the second step, 100 mg of Hats (0.55 mmol) were suspended at room temperature in 50 mL of absolute ethanol (99.5%, Merck, Darmstadt, Germany) with permanent stirring. Then, 1.2 g of *p*-toluenesulfonic acid (Aldrich, Seelze, Germany; 6.31 mmol); 0.45 mL of methylsulfonic acid (Aldrich, Seelze, Germany; 6.7 mmol); 0.45 mL of HCl (37%, Merck, Darmstadt, Germany; 5.5 mmol) were added maintaining the stirring during 2 h at room temperature. White powders were obtained for each

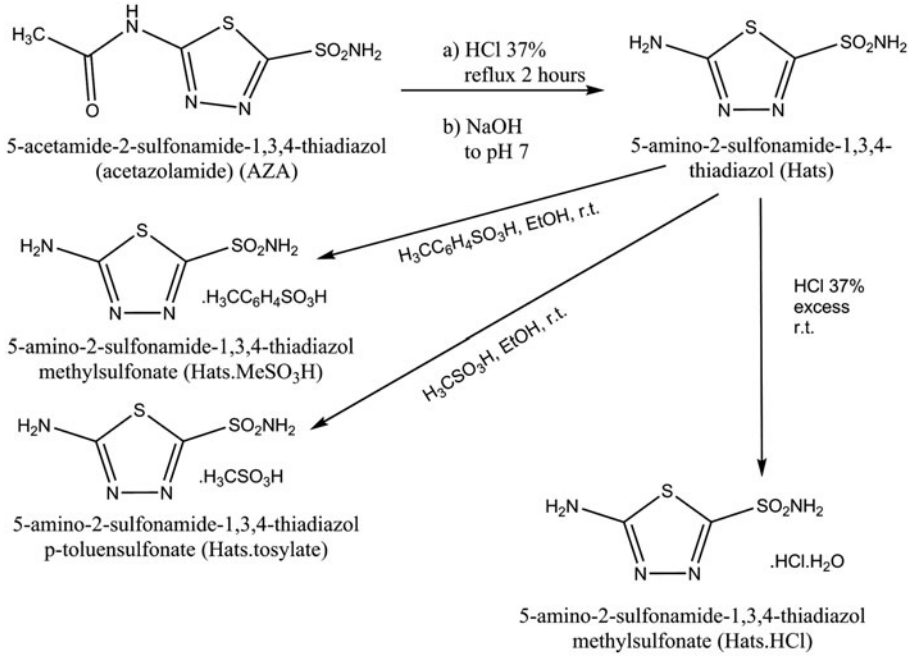


Figure 1. Preparation of 5-amino-2-sulfonamide-1,3,4-thiadiazole salts.

salt, 5-amino-1,3,4-thiadiazole-2-sulfonamide *p*-toluenesulfonate (Hats.tosylate), 5-amino-1,3,4-thiadiazole-2-sulfonamide methylsulfonate (Hats.$MeSO_3H$), and 5-amino-1,3,4-thiadiazole-2-sulfonamide hydrochloride monohydrate (Hats.HCl). Colorless prismatic single crystals of Hats.tosylate, suitable for DRX studies, were obtained after 2 weeks at 15 °C avoiding light exposure.

Methods

X-ray data for Hats.tosylate were collected with an Enraf–Nonius FR590 CCD area detector (Labx, Schwerzenbach, Switzerland) using Collect[24] and HKL Denzo–Scalepack[25] software with graphite monochromated Mo-Kα ($k = 0.71073$ Å) at 293[2] K. The structure was solved by direct methods using SHELXS-97[26] and all the non-hydrogen atoms were refined anisotropically by full-matrix least-squares on F^2 using SHELXL-97[26]. The H atoms attached to N were found in a difference Fourier map, further idealized (N–H:0.86 Å), and finally allowed to ride. All calculations, including Figures 1–3, were performed using WinGX[27] and Ortep-3 for windows[28]. The crystal structure of Hats tosylate has been deposited in the CSD with reference number CCDC 730598. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif. The compound crystallizes forming colorless triclinic prisms with the space group P1.

Elemental analysis (C, H, N, and S) was carried out with a Carlo Erba EA 1108 microanalyser (Triad Scientific, Manasquan, NJ) belonging to the Instituto de QuímicaFísica de Materiales, MedioAmbiente y Energía (INQUIMAE), Universidad de Buenos Aires, Argentine; sulfanilamide was used as the standard.

Fourier transformed infrared (FT-IR) spectra were recorded on a Nicolet Protégé 460 spectrometer (Conquer Scientific, San Diego, CA) provided with a CsI beam splitter in the 4000–250 cm^{-1} range with 32 scans and spectral resolution of 4 cm^{-1}, using the KBr pellet technique.

Thermogravimetric (TGA) and differential thermal analysis (DTA) curves were obtained with a Shimadzu TGA-51 Thermal Analyzer (Shimadzu Corporation, Kyoto, Japan) and DTA-50 Thermal Analyzer, using platinum pans, flowing air at 50 ml min^{-1} and at a heating rate of 10 °C min^{-1} from room temperature to 1000 °C.

To confirm these, melts or crystalline phase changes were used a Leitz Westlar heating microscope at this stage. The equipment used for mass spectra was a VG AUTOSPEC. The spectral data were obtained through of Electronic Impact (EI) at 70 eV and Fast Atom Bombardment (FAB). The values listed for each of the compounds are expressed in unit of *m/z*.

The reaction for obtaining Hats.tosylate occurred with a yield of 95%. Elemental analysis for $C_2H_5N_4O_2S_2^+.CH_3C_6H_4O_3S^-$: Found (calculated): C: 31.21% (30.95%); H: 2.29% (2.58%); N: 15.78% (16.05%); S: 28.53% (27.50%).

All animals used in experiments were handled in accordance with international standard for the use and care of laboratory animals. The corresponding protocols were approved by the Universidad Nacional de San Luis, following the provisions of the ANMAT[29] 6344/96.

The diuretic effect was evaluated using male and female adult Wistar rats weighing 220 g. The administration of solutions was performed by oral gavage in a volume of 5 mL/100 g body weight following the method proposed by Lipschitz et al.[30]. All animals were deprived of water 12 h before the experiment having free access to food. In addition to the urine volume, sodium excretion and urinary potassium were recorded[31] and the concentrations of these ions were quantified in a flame spectrometer Metrolab model 315 (Shimadzu Corporation, Kyoto, Japan).

The anticonvulsant activity of drugs was tested in mice Rockland (weighing 25–30 g, administering the sulfonamides intraperitoneally), against seizures induced by intraperitoneal administration of nikethamide or picrotoxin as control drugs. Through the ''rota-rod test''[32], the neurological deficit was evaluated, which is evidenced by the ability of the mouse to stay in balance (on a cylinder rotating at 15 rpm) in three trials of 1 min each. Animals were distributed into three lots: negative control (saline), positive control (phenobarbital), and experimental compounds (Hats.tosylate, Hats.$MeSO_3H$, and Hats.HCl). The compounds were administered intraperitoneally. The mice are placed on the rota-rod bar and put into operation the apparatus. Animals that do not fall off the bar in 5 min will be chosen for the test. Thirty minutes after intraperitoneal administration, the mice are placed on the moving bar and the number of falls in 5 min was registered. Then the analysis of the recorded data was achieved.

The ''chimney'' test[33] is another *in vivo* test used to evaluate the mouse *motor* coordination impairments. It is based on the time that the rodent needs to traverse a tube of glass or clear acrylic 20 cm long in a span of 10 s. In this assay, the degree of muscular relaxation in mice is evaluated. Animals were distributed into three lots: negative control (saline), positive control (phenobarbital), and experimental (sulfonamides). The rota-rod test was used to evaluate the activity of drugs that interfere with motor coordination (Test of Dunham, 1957), while the ''chimney'' test was used to estimate locomotor activity[33]. Neurotoxicity experiments were carried out with adults Rockland albino mice weighing 25–30 g. The doses of sulfonamides studied were 20, 50, and 90 mg/kg of mouse.

An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity[34]. Phenol red (at 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm with 10 mM Hepes (pH 7.4) or TRIS (pH 8.3) as buffers and 0.1 M $NaClO_4$ (for maintaining constant ionic strength), at 20 °C, following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s (the uncatalyzed reaction needs around 60–100 s in the assay conditions, whereas the catalyzed ones are of around 6–10 s), as described earlier[35–43]. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Enzyme concentrations in the assay system were in the range of 10 nM for all the enzymes considered in the present study, which have been obtained as recombinant proteins, in-house, as reported earlier[43–48].

Table 1. Crystal data of Hats.tosylate.

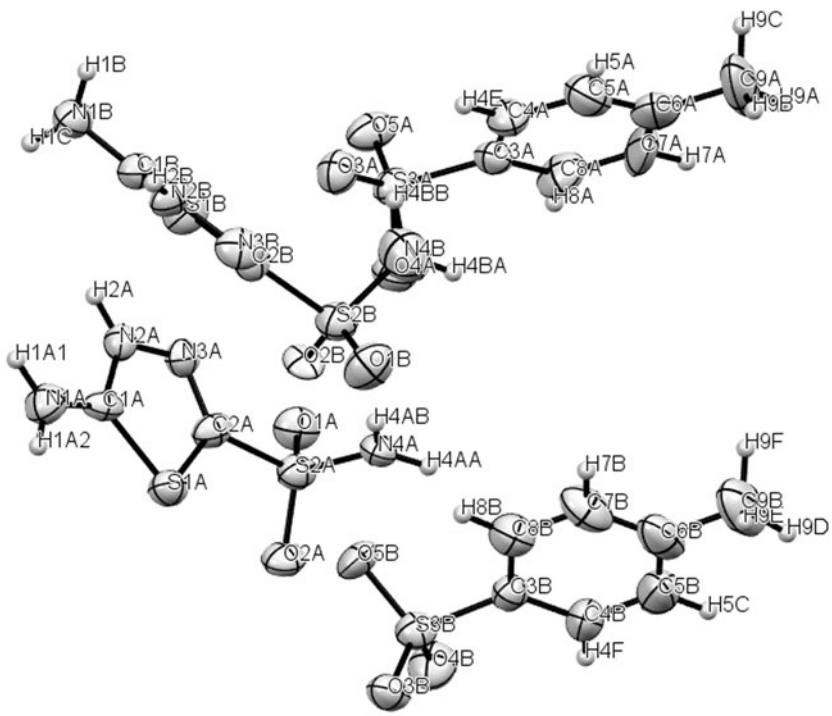
Empirical formula	$C_{18}H_{18}N_8O_{10}S_6$
Formula weigh	698.76
Temperature	293 (2) K
λ	0.71073 Å
Crystal system	Triclinic
Space group	P-1
Parameters	$a = 9.3220$ (3) Å, $\alpha = 92.7390$ (13)°
	$b = 10.8990$ (4) Å, $\beta = 101.5110$ (16)°
	$c = 14.7830$ (6) Å, $\gamma = 95.6290$ (13)°
Volume	1461.15 (9) Å^3
Z	2
Density (calc.)	1.588 g/cm^3
Absorption coefficient	0.533 mm^{-1}
F (000)	716
Crystal dimension	0.2 × 0.16 × 0.12 (mm)
θ range	1.41–27.43°
Range all indices	$-12 \le h \le 12$, $-13 \le k \le 13$,
	$-18 \le I \le 18$
Collected reflections	9081
Independent reflections	5629 [R (int) = 0,1021]
Integrity θ (max.)	$\theta = 27.43$: 84.4%
Refinement method	Full-matrix least-squares on F^2
Data/restrictions/parameters	5629/0/401
Adjusted on F^2	0.921
Indices R (final) [$I > 2\sigma(I)$]	$R_1 = 0.0760$, w$R_2 = 0.1705$
Indices R (all data)	$R_1 = 0.2487$, w$R_2 = 0.2756$
Max. difference peak and hole	0.617y–0.559 e.Å^{-3}

Results and discussion

Characterization

In a previous work, we have reported the crystal structures and spectroscopic characterization of Hats.$MeSO_3H$ and Hats.HCl[22,23]. Crystal and experimental data for Hats.tosylate are listed in Table 1. Figure 2 shows ORTEP diagram of the studied compound, and Figure 3 shows the molecular packing. Hats.tosylate consists of two H_2ats^+ and two *p*-toluenesulfonate ions in the asymmetric unit. The anion *p*-toluensulfonate and cation H_2ats^+ are distributed in alternative parallel layers along with the *b*-axis and stabilized by hydrogen bonds between the 2-sulfonamido group and sulfonate ion, 5-amino and protonated N2 (azole) group and sulfonate group (Table 2). The π-stacking between phenyl groups are reinforced by interactions between these groups and terminal methyl moieties, which stabilize the crystal lattice.

Figure 2. ORTEP diagram of Hats.tosylate.



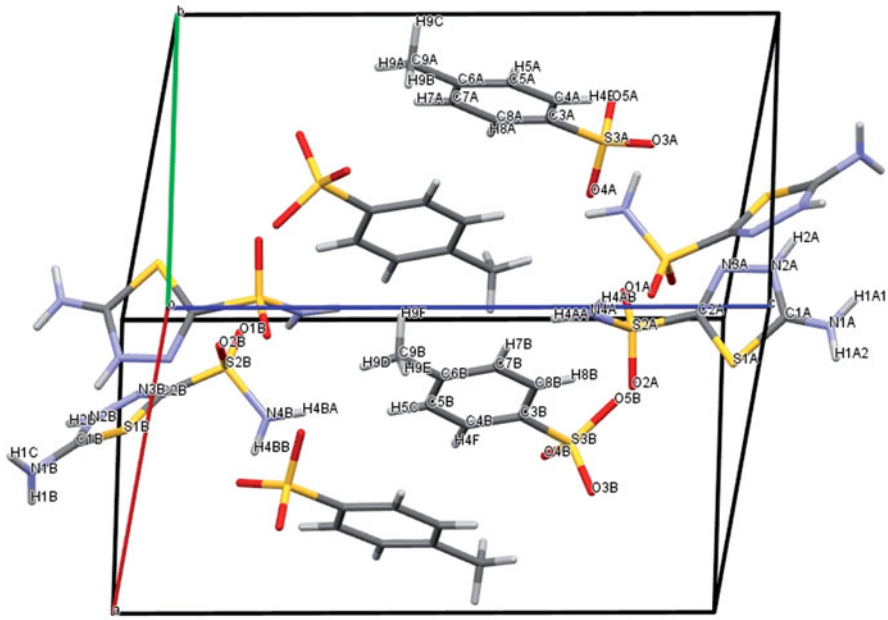


Figure 3. Unit cell with hydrogen atoms of Hats.tosylate.

Table 2. H-bonds interactions in Hats.tosylate crystal structure.

Donor–H . . . Acceptor	Distance (Å) Donor–H	Distance (Å) H . . . Acceptor	Distance (Å) Donor . . . Acceptor	Angle (deg.) Donor–H . . . Acceptor
N(1A)–H(1A1) . . . O(5B)[a]	0.860	2.033	2.833	154.35
N(1A)–H(1A2) . . . O(2B)[a]	0.860	2.522	2.948	111.50
N(2A)–H(2A) . . . O(4B)[a]	0.860	2.002	2.808	155.79
N(4A)–H(4A1) . . . O(3B)[b]	0.861	1.997	2.858	177.45
N(4A)–H(4A2) . . . O(5A)[c]	0.860	2.125	2.984	177.30
N(1B)–H(1B1) . . . O(3A)[d]	0.860	1.907	2.745	164.64
N(1B)–H(1B2) . . . O(5B)[e]	0.860	2.078	2.807	142.12
N(2B)–H(2B) . . . O(5A)[d]	0.860	1.989	2.824	163.55
N(4B)–H(4B1) . . . O(4B)[f]	0862	2.108	2.946	164.03
N(4B)–H(4B2) . . . O(4A)[c]	0.859	2.161	2.930	148.91
CG . . . CG	Centroid–centroid (Å)	Centroid-Plane (Å)	Slippage angle (deg.)	
CGB . . . CGB[h]	4.410	3.513	37.2	
CGA . . . CGB[h]	4.291	3.508	25.7	

CGA: centroid of cyclic group C3A, C4A, C5A, C6A, C7A and C8A.
CGB: centroid of cyclic group C3B, C4B, C5B, C6B, C7B and C8B.
a: $-x+1, -y+1, -z$; *b*: $x+1, y+1, z$; *c*: $-x+1, -y+1, -z+1$; *d*: $x, y, z-1$; *e*: $-x, -y+1, -z$; *f*: $x, y+1, z$; *h*: $-x+1, -y+1, -z+1$; *i*: $-x+2, -y, -z+1$.

The complete assignment of the vibrational modes of Hats has been studied by our group[23]. Based on these studies and using appropriate literature[35], it has been possible to assign the vibrational modes of Hats salts. In this work, the assignment of vibrational modes of Hats.tosylate has been done by comparison with the other two above mentioned salts and Hats. Figure 4 shows the FTIR spectra of Hats.tosylate and Table 3 proposes assignation of vibrational modes.

Analyzing the vibrational modes mentioned above, it can be seen that NH_2 stretching appears at lower frequencies and NH_2 deformation at higher frequencies in the three salts with respect to Hats. These facts are consistent with the respective crystal structures, where these groups are interacting with the hydrogen bridge formation, stabilizing the crystal structure, weakening the NH bond. Four modes corresponding to NH stretching are observed. The values observed at 3319 and 3246 cm^{-1} correspond to the asymmetric and symmetric stretching of the $–NH_2$ group attached to the carbon of the thiadiazole ring, at higher frequencies in Hats.tosylate with respect to Hats.$MeSO_3H$ and Hats.HCl, and these three frequencies are less than Hats (Table 3). The other two values observed at 3177 and 3067 cm^{-1} are very similar to those observed in Hats, Hats.$MeSO_3H$ and Hats.HCl. The NH deformations are observed at higher frequencies in the salts regarding Hats. These facts confirm the implication of the amino groups in the formation of hydrogen bonds to stabilize the crystal structure. It is also interesting to note that the corresponding modes ν_{as} SO_2 groups and CN appear at higher frequencies than Hats, suggesting that these links are strengthened with respect to Hats (Table 3). This fact indicates that the S–O and C–N bonds are strengthened. The stretching N–N appears at similar frequencies in all cases.

The result of mass spectra shows that the molecular ions are not visible due to its relative abundance which is lower than 5% and mainly due to the ionic nature of the salts. In the three salts, the fragment of *m/z* 180 (compounds Hats.tosylate and Hats.$MeSO_3H$) and *m/z* 181 (V) has a 100% relative abundance. This fragment corresponds to Hats and protonated Hats. In addition, compound Hats.tosylate appears to be a fragment of *m/z* 172 (55% relative abundance) due to *p*-toluenesulfonic acid. The Hats.$MeSO_3H$ shows a fragment at *m/z* 96 (40% relative abundance) corresponding to methylsulfonic acid. The mass spectra of three salts are presented in the Supplementary material.

Figure 4. FTIR spectrum of Hats.tosylate.

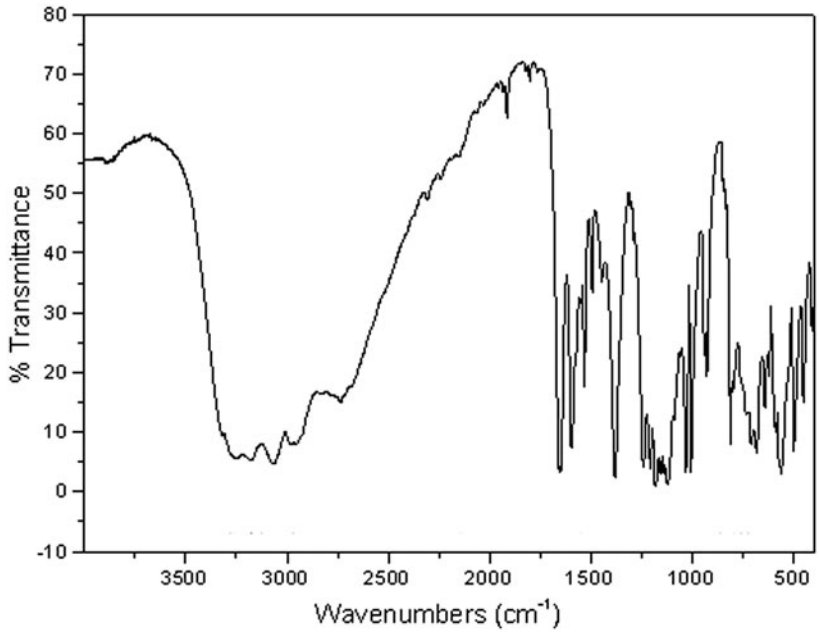


Table 3. Proposed assignment of the FTIR spectrum and a comparison of selected.

Infrared	Assignment
(a) Proposed assignment of Hats.tosylate vibrational modes.	
3319 (s)	ν_{as} (C)NH_2
3246 (s)	ν_s (C)NH_2
3177 (m)	ν_{as} (S)NH_2
3067 (m)	ν_s (S)NH_2 + ν CH phenyl + ν NH (ring)
2984 (w)	ν CH phenyl + ν CH methyl
2952 (w)	ν CH methyl
1930–1912–1820–1802 (w)	Phenyl group disustituted
1651 (s)	δ(C)NH_2
1596 (s)	δ(S)NH_2
1551 y 1532 (m)	ν C–N
1495 (w), 1448 (w)	ν C–C (ring) + ν N–C
1383(s)	ν_{as} SO_2 + ν_{as} SO_3H (IR); δ_{ip} CH
1241(s), 1208 (s)	δ CH methyl
1182(s)	ν N–N (ring)
1158(s)	δ C–H
1141(s)	ν_s SO_2 + ν_s SO_3H
1122(s)	δ CH methyl
1060 (m)	Rocking (S)NH_2
1032 (s)	δ ring
1006 (s)	δ CH methyl
940 (m)	N S–N $_{sulfonamide}$ + wagging (S)NH_2
928 (m) y 813 (s)	Δ CH methyl
798 (m)	ν C–S (ring) + δ phenyl ring
710 (s)	δ CH methyl + δ_{oop} arC-H
685 (s)	ν C–S + δ phenyl ring
643(m)	Ring torsion
622 (m) y 590 (s)	ν C–S
562 (s)	Red modes
500 (s)	Wagging (C)NH_2 + δ SO_2
452 (m)	Red modes
411(w)	Rocking SO_2
401(w)	δ SO_2 + δ_{ip} N-C (ring)

Assignment	Hats (cm^{-1})	Hats.tosylate (cm^{-1})	Hats.$MeSO_3H$ (cm^{-1})	Hats.HCl (cm^{-1})
(b) Comparison of selected vibrational modes for compounds Hats and its salts.				
ν_{as} (C)NH_2	3430	3320	3255	3280
ν_s (C)NH_2	3325	3245	3240	3190
ν_{as} SO_2	1340	1385	1375	1375
ν_s SO_2	1140	1140	1145	1140
ν NH_2	1605	1650	1645	1640
ν CN	1580–1500	1595–1530	1595–1525	1585–1530
ν NN	1175	1180	1200	1185

ν, stretching; δ, deformation; ip., in plane; oop., out of plane; s, strong; m, medium; w, weak. All values are approximate and rounded for ease in comparing them.

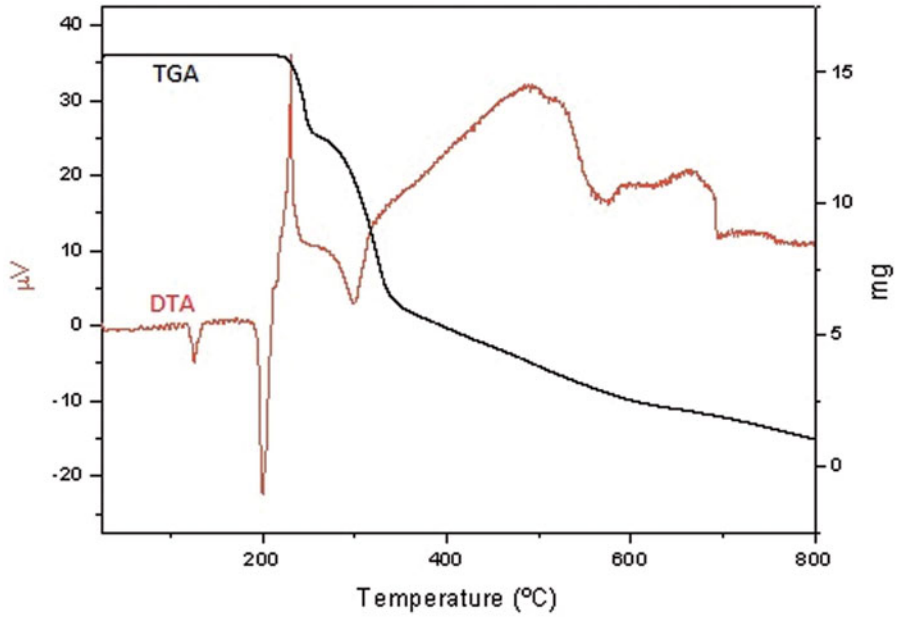


Figure 5. ATG-ATD diagram of Hats.tosylate.

A very rich thermal behavior was observed in the studied compounds. Hats.tosylate is stable up to 120 °C. Starting at 120 °C associated to an endothermic process, a change of the crystalline phase occurred. This fact was observed in a heating microscope stage. Compound Hats.$MeSO_3H$ too changed its crystalline phase below 100 °C. Compound Hats.HCl decomposed at 120 °C (onset 118 °C) with loss of water of crystallization.

Immediately before decomposition, Hats.tosylate and Hats.$MeSO_3H$ showed a melting point near 200 °C [Hats.tosylate: 212 °C (onset 203 °C); Hats.$MeSO_3H$ 200 °C (onset 196 °C)]. Hats.HCl anhydrous melted at 194 °C (on set 187 °C). Subsequently the three salts decomposed in many endothermic and exothermic steps until complete calcinations occur at 700 °C (Hats.tosylate), 925 °C (Hats.$MeSO_3H$), and 600 °C (Hats.HCl). Figure 5 shows thermal diagrams (TGA-DTA) of Hats.tosylate. Thermal diagrams of compounds Hats.$MeSO_3H$ and Hats.HCl are presented in the Supplementary material.

Biological assays

Diuretic effect

The diuretic effect is attributed to the ability of these salts to inhibit the renal CA[1,4]. This effect is demonstrated by the alkalinity of the urine excreted. However, it has been suggested that the inhibition of sodium reabsorption in different parts of the nephron also contribute to this mechanism of action. To verify this effect, six batches were prepared with eight animals each. Batch 1 (negative control) received saline physiological solution; batch 2 (positive control) received acetazolamide (20 mg/kg); batch 3 (reference) received furosemide (Sigma Ch. Co., St. Louis, MO) (10 mg/kg); batch 4 received compound III (70 mg/kg); batch 5 received compound IV (70 mg/kg), and batch 6 received compound V (70 mg/kg). Urine fractions were collected each hour for a period of 5 h after the administration of each drug. The urinary excretion volume was calculated using the following relationship:

$$\text{UVE} = \frac{\text{collected volume}}{\text{volume administered}} \times 10$$

where UVE is the urinary volume excreted.

The recorded values were expressed as means ± SEM. It applies statistical analysis one-way ANOVA and subsequent comparisons used the Turkey test. Differences below a probability level of 5% ($p < 0.05$) were considered statistically significant. To carry out this study, we used the program GraphPad Prim (GraphPad Software Inc., La Jolla, CA)[40].

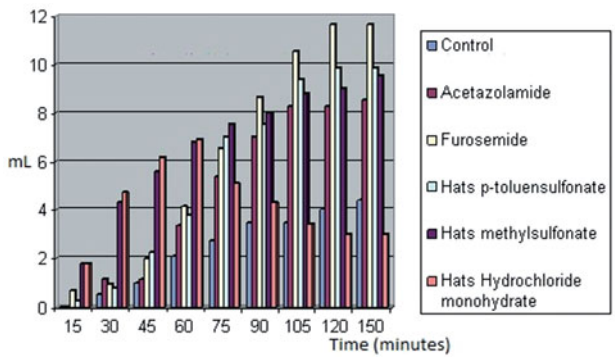


Figure 6. Diuresis of Hats salts (mL of urine versus time). From left to right (each set of columns): control, Aza, furosemide, p-toluensulfonate Hats, Hats methylsulfonate, Hats hydrochloride monohydrate.

Diuresis has two components: an increment of the urine volume (water excretion) and a net loss of solutes (electrolytes)[31]. Figure 6 shows comparative diuretic effect of Hats salts with respect to acetazolamide and furosemide. Hats.$MeSO_3H$ possesses a similar effect which Hats.HCl as to the excretion of electrolytes in the urine. However, the removal of liquid is important in the first 60 min of the test, subsequently removing liquid is similar to the batch control. Hats.$MeSO_3H$ exhibited a significant diuretic effect during the test. Diuresis was significant for the first 75 min of the test with respect to the control. Hats.tosylate also presented a diuretic effect, which was maintained throughout the test. Spectrophotometric data of urinary ions concentration yielded the results as exhibited in Table 4.

Anticonvulsant activity

In a first assay, physiological saline solution (negative control) was administered intraperitoneally to eight mice per group, whereas compounds Hats.tosylate, Hats.$MeSO_3H$, Hats.HCl, and AZA at doses of 20, 50, and 90 mg/kg of live animal, in volumes

Table 4. Biological assays.

(a) Concentration of urinary ions observed

Control (saline solution):	Na^+: 0.93 mg/L	K^+: 3.18 mg/L
Reference (furosemide):	Na^+: 2.85 mg/L	K^+: 5.4 mg/L
Hats.tosylate:	Na^+: 4.9 mg/L	K^+: 6.8 mg/L
Hats.$MeSO_3H$:	Na^+: 5.8 mg/L	K^+: 8.6 mg/L
Hats.HCl:	Na^+: 6.4 mg/L	K^+: 8.3 mg/L

(b) Effect of Hats salts and acetazolamide on nikethamide and picrotoxine-induced convulsions in mice

		% Protection	
Treatments	Dose (mg/kg)	Picrotoxine	Nikethamide
Saline	–	0	0
Hats	90	72*	79*
Hats.tosylate	90	38	58
Hats.$MeSO_3H$	90	53	75
Hats.HCl	90	44	56
AZA	90	82**	96**

(c) Effect of Hats salts on locomotor activity of mice (rota-rod test)

Treatment	Dose (mg/mL)	No. of falls in 15 min
Saline	—	1.2 ± 0.8
Phenobarbital	10	5.5 ± 0.4
Hats.tosylate	90	2.0 ± 0.5***
Hats.$MeSO_3H$	90	2.2 ± 0.9***
Hats.HCl	90	2.6 ± 0.5***

(d) Effect of Hats salts on locomotor activity of mice (Chimney assay)

Treatment	Dose (μM/kg)	Time delayed on climbing (min.)
Saline	–	6 ± 6
Hats.tosylate	90	8 ± 5***
Hats.$MeSO_3H$	90	6 ± 7***
Hats.HCl	90	7 ± 4***
Phenobarbital	10	–

*$p < 0.05$; **$p < 0.01$ in comparison with saline control. ***$p < 0.05$ in comparison with phenobarbital lot.

of 0.1 mL per 30 g mouse, were administered to other animals. After 30 min of administration of the test compounds, nikethamide was injected by the same route at 0.1 mL (dose 20 mg/kg). It was observed that a dose of 20 mg/kg of test compound is not fully effective for convulsions protection; convulsions were observed in one or two mice per group. However, at higher doses (50 and 90 mg/kg of test compound), the convulsions protection was effective, since none of the mice suffered convulsions.

In a second assay, a dose of compound of 90 mg/100 g of animal weigh was given in a volume of 0.1 mL per 10 g mouse, intraperitoneally (using eight mice per group). After 30 min of administration, test compounds were injected by the same route of picrotoxine 0.1 mL per 30 g mouse (20 μg/100 g body weight).

In the control batch, we observed that in animals treated with picrotoxine the convulsions appeared after 4 min post-administration. The animals injected with compounds Hats.tosylate and Hats.$MeSO_3H$ were protected from convulsions during 20–25 min post-administration, whereas after longer periods of time, clonic convulsions appeared.

The animals injected with compound Hats.HCl were protected from convulsions during the 20 min post-administration. Tonic convulsions appear thereafter, but the animals survived. Acetazolamide showed a weaker protective effect than Hats salts since convulsions occurred within 15 min post-administration (Table 4).

Neurotoxicity (rota-rod test)

The *rota-rod assay* has been used to examine *in vivo neurotoxicity of the sulfonamide salts.* Five groups of eight animals each were used. For the negative control group (saline solution), one fall (mean) had been recorded during the first 15 min. In the positive control group, injected with phenobarbital (10 mg/kg), five falls were observed. In the third group previously injected with compound Hats.tosylate (90 mg/kg), two falls were observed during the first 15 min. In the fourth group, injected with compound Hats.$MeSO_3H$ (90 mg/kg), two falls were observed during the first 15 min. Finally, in the fifth group, injected with compound Hats.HCl (90 mg/kg), two falls during the first 15 min were observed. The results showed no adverse effects on motor coordination with the tested drugs compared with control animals ($p > 0.05$, one-way ANOVA and subsequent Turkey test) at the doses here assayed. Table 4 shows these results.

Table 5. Inhibition of human (h) isoforms hCA I – XII with hats salts and AZA as standard drug.

	K_I(nM)					
Compound	hCA I	hCA II	hCA IV	hCA VII	hCA IX	hCA XII
Hats.tosylate	3400	320	2740	1980	255	570
Hats.$MeSO_3H$	8300	327	3630	4225	570	570
Hats.HCl	932	365	2270	623	307	421
AZA	250	12	74	2.5	25	5.70

Effect of Hats salts on locomotor activity of mice. Chimney test

Five groups of eight animals each were employed. Animals used in the first group (saline solution, negative control) showed a

Table 6. Inhibition of PgiCA from *porphyromona sgingivalis*; and Can2, from *cryptococcus neoformans* with hats salts investigated in this paper.

	K_I (μM)		
Compound	Can2	PgiCAbeta	PgiCAgamma
Hats.tosylate	7.82	3.60	0.915
Hats.$MeSO_3H$	8.62	5.87	2.01
Hats.HCl	5.24	4.16	1.95

delay of 6 s to pass through the glass tube. The group injected with phenobarbital (positive control) did not perform the test within the considered time. The group intraperitoneally injected with compound Hats.tosylate (0.1 mL/15 g mouse) led to a delay of 8 s. The group intraperitoneally injected with compound Hats.$MeSO_3H$ (0.1 mL/15 g mouse) led to a delay of 6 s. The group intraperitoneally injected the compound Hats.HCl (0.1 mL/15 g mouse) led to a delay of 7 s. The results show that there is no sedation or ataxia drug tested, compared with those animals of the control batch ($p > 0.05$, one-way ANOVA and subsequent Turkey test).

CA inhibition

Inhibition data with Hats salts against various human (h) CAs, hCA I, II, IV, VII, IX, and XII, and pathogenic beta and gamma CAs have been obtained. AZA, a well-known CAI, was used as a standard drug for comparison (Table 5). As in other CAs inhibition assays with sulfonamides and their metal complexes, phenols and thiophenols, the enzymes were incubated for 10–15 min with test compounds for allowing the formation of the enzyme-inhibitor adduct[41–45,49,50]. Working under similar conditions, Hats salts inhibited both hCAs and micro-organisms CAs in the micromolar concentration range, although they exhibited a weaker inhibitory power compared with AZA (Table 5). These results show that although the solubility in water is improved for Hats by salt formation, the interaction of the sulfonamide with the active site of the enzyme is weaker (than for acetazolamide) probably due to the fact that the acetamido moiety present in the drug allows the stabilization of the adduct through additional interactions in which Hats cannot participate. This is consistent with the X-ray crystal structures, in which the deprotonated sulfonamido group appears bound to the Zn(II) ion from the enzyme active site[7,44,45].

In previous studies, it was found that by inhibiting CAs from bacterial or other pathogens, a growth inhibition of the studied micro-organisms occurred[37,45,46]. Table 5 shows the values of enzyme inhibition for CAs in *Porphyromonas gingivalis* (responsible of periodontal pathogenicity) and *Cryptococcus neoformans* (responsible of serious, systemic fungal diseases)[1,7,8]. According to these values, the best results were obtained against PgiCAgamma (Table 6). Thus, the Hats salts can be considered as interesting candidates for possible application in designing anti-infective agents with a novel mechanism of action.

Conclusions

In this work, we obtained new salts of Hats: 5-amino-1,3,4-thiadiazole-2-sulfonamide. The crystal structure was determined for one of them, whereas spectroscopic, spectrometric, and thermal analysis showed that the new salts differentiate with respect to similar salts previously obtained in our group. The results of preclinical assays suggest that the effect of the studied sulfonamides on electrolytes excretion is similar with the thiadiazide diuretics due to the significant removal of Na^+ and K^+ ions. Hats and AZA showed the same anticonvulsant protection effects against intraperitoneal administration of niketamide and picrotoxin. The sulfonamide salts were most effective as anticonvulsants in the niketamide than in the picrotoxin-induced convulsions assay. These facts suggest that they are effective to protect against mild convulsions. The tested compounds showed no effects of neurotoxicity in the full range of effective doses in the study by the rota-rod test and chimney test. Hats salts inhibited human, bacterial, and fungal CAs. These facts led us to study more deeply these compounds as potential antiinfective agents. This work stimulates further study for their possible pharmacological applications.

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Declaration of interest

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Supplementary material available online

Supplementary Figure S1–S7