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Original article

Carbonic anhydrase inhibitors. Synthesis, molecular structures, and inhibition of the human cytosolic isozymes I and II and transmembrane isozymes IX, XII (cancer-associated) and XIV with novel 3-pyridinesulfonamide derivatives

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ABSTRACT

A series of novel 3-pyridinesulfonamide derivatives (**2**–**5**, **9**–**11** and **13**–**15**) have been synthesized and investigated as inhibitors of five isoforms of zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), that is, the cytosolic ubiquitous CA I and II, and isozymes CA IX and XII (cancer-associated), and XIV. Against the human isozyme hCA I the new compounds showed K_Is in the range of 0.089–251 μM, whereas toward hCA II, K_Is = 50.5–487 nM. Isozyme hCA IX was inhibited with K_Is in the range of 5.2–18.3 nM, while hCA XII with K_Is = 6.0–16.4 nM, and hCA XIV with K_Is = 76.4–152.0 nM. All of the new compounds **2**–**5**, **9**–**11** and **13**–**15** showed excellent hCA IX inhibitory efficacy, with K_Is = 5.2–18.3 nM, being much more effective as compared to the clinically used **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (K_Is = 24–50 nM).

1. Introduction

The connections between carbonic anhydrase (CA, EC 4.2.1.1) and cancer is known for approximately fifteen years since two tumor-associated membrane carbonic anhydrase isozymes (CA IX and CA XII) have been identified cloned, and sequenced [1–3]. Many reports have been published on the role of CA IX in tumor physiology [4–6]. In addition its role in the control of tumor pH, there are evidences that CA IX can also influence other processes in the cell microenvironment that promote cell proliferation, invasion, and metastasis [7]. Upregulated expression of CA IX was reported in carcinoma cells derived from various organs including breast, cervix, bladder, esophagus, lung, kidney, colon and rectum, head and neck. This protein constitutes an endogenous marker of cellular hypoxia, a natural phenotype of solid tumors [8] and its predicative and prognostic potential has been demonstrated in various clinical studies [9]. Considering the abnormally high expression of CA IX in may hypoxic tumors and its demonstrated role in the tumor acidification processes and oncogenesis, CA IX constitutes a candidate target for anticancer therapy. Thus, agents that can selectively inhibit CA IX activity may have therapeutic value and offer opportunities for the treatment or prevention of a variety of cancers. The potential use of CA inhibitors as antitumor agents opens thus a new important research direction [10–12].

For example, it has recently been demonstrated that sulfonamide or coumarin potent CA IX inhibitors have a potent effect in inhibiting the growth of both primary tumors and metastases in an animal model of breast cancer [13,14].

Recently, we have reported on the strong inhibition of human cytosolic isozymes CA I and II and tumor-associated isozymes IX and XII with some 4-chloro-5-methyl-2-(R-thio)benzenesulfonamides of type **I** [15–17] (Fig. 1). We also described the syntheses a number of 3-pyridinesulfonamides of type **II** and **III** (Fig. 1), and their inhibitory activities against human isozymes hCA I, II, IX, XII and XIV [18,19]. Numerous of these compounds exhibit excellent hCA IX inhibitory efficacy with inhibition constants of 4.6–12.0 nM, being much more effective as compared to the clinically used sulfonamides **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (K_Is = 24–50 nM). These findings prompted us to synthesize and investigate of the related 3-pyridinesulfonamides of types **II** and **IV**–**VI** (Fig. 1).

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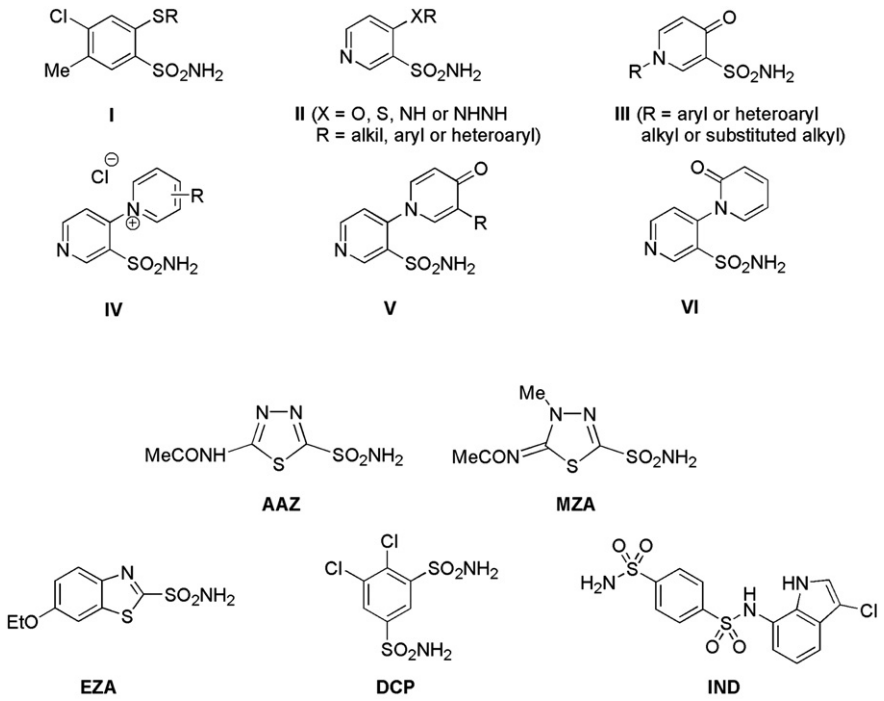
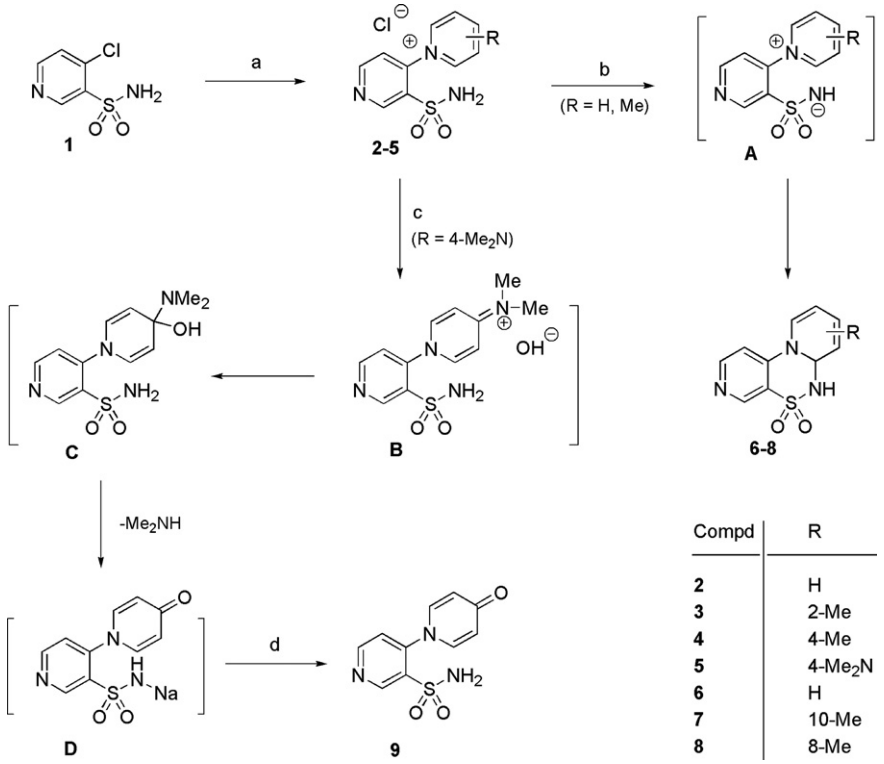


Fig. 1. General structures of known **I**–**III** and novel **IV**–**VI** arylsulfonamides, and clinically used sulfonamides **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (standard CA inhibitors).

2. Results and discussion

2.1. Chemistry

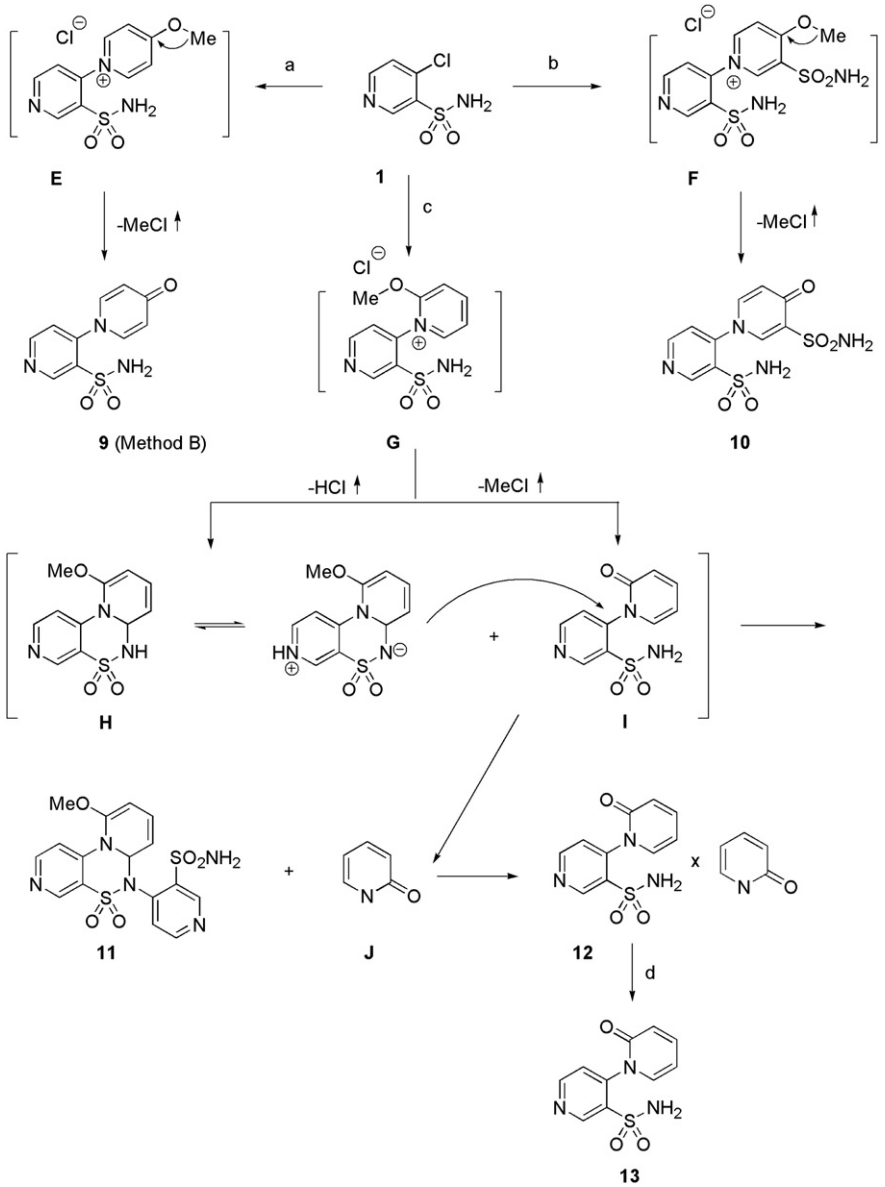
As shown in Scheme 1, the syntheses of the target 3-pyridinesulfonamide derivatives **2**–**9** were achieved by convenient procedures starting from commercially available 4-chloro-3-pyridinesulfonamide **1**. First, the reactions of **1** with pyridine, 2-methylpyridine, 4-methylpyridine or 4-dimethylpyridine carried out in boiling acetonitrile led to the formation of 1-(3-sulfamoylpyrid-4-yl)pyridinium chlorides **2**–**5** in a very good yields (87–95%). Then, upon treatment of compd. **2**–**4** with NaOH (1.2 M equiv.) in water at



Compd	R
2	H
3	2-Me
4	4-Me
5	4-Me_2N
6	H
7	10-Me
8	8-Me

Scheme 1. Synthesis and proposed mechanism of the formation of 3-pyridinesulfonamide derivatives **2**–**9**. Reagents, conditions and yields: (a) pyridine, 2-methylpyridine, 4-methylpyridine or 4-dimethylaminopyridine (2 M equiv.), dry acetonitrile, reflux 18 h, 87–95%; (b) NaOH (1.2 M equiv.), water room temperature, 3 h, 60–85%; (c) NaOH (2.0 M equiv.) water, 0–10 °C, 1 h, room temperature, 36 h; (d) hydrochloric acid to pH 2.5, room temperature, 24 h, 70%.

room temperature, the desired dipyrido[2,1-*c*: 4′,3′-*e*][1,2,4]thiadiazine derivatives **6**–**8** were obtained in 60–85% yield. The attempted transformation of **5** into analogous pyridodithiazine by this route failed. However, upon treatment of **5** with an excess of NaOH (2 M equiv.) in water at temperatures 0–20 °C corresponding 4-(1,4-dihydro-4-oxopyrid-1-yl)-3-pyridinesulfonamide **9** was obtained. In this conditions the pyridinium chloride **5** was converted successively to the unstable ammonium hydroxide **B** and the addition product of type **C**, which undergoes a spontaneous elimination of Me_2NH leading to the formation of the salt **D**, which upon acidification afforded free sulfonamide **9** in 70% yield. Compound **9** was also obtained in 68% yield by a convenient procedure (method B, Scheme 2) starting from 4-chloro-3-pyridinesulfonamide **1** and 4-methoxypyridine in dry acetonitrile. The initial step is believed to be formation of intermediate pyridinium salt **E** which undergoes S_N2 substitution on the methyl by chloride to form the target sulfonamide **9**. Analogously, by reaction with 4-methoxy-3-pyridinesulfonamide was prepared the target sulfonamide **10** as shown in Scheme 2. However, we found that analogue reaction of 4-chloro-3-pyridinesulfonamide **1** with 2-methoxypyridine led to the formation a mixture of 4-substituted 3-pyridinesulfonamide **11** and the adduct of 4-(1,2-dihydro-2-oxopyrid-1-yl)-3-pyridinesulfonamide with 2-pyridone **12**, which were separated in 12.6 and 38.5% yields. We propose a reaction sequence for the transformation as shown in Scheme 2. The initial step is believed to be the formation of intermediate 1-(3-sulfomoypyrid-4-yl)-2-methoxpyridinium chloride **G** which undergoes elimination of hydrochloride or methyl chloride to form the mixture of intermediate dipyrido[2,1-*c*:4′, 3′-*e*][1,2,4]thiadiazine **H** and 4-(1,2-dihydro-2-oxopyrid-1-yl)-3-pyridinesulfoamide **I**, respectively. Then, the reaction of intermediate **H** with **I** led to the final product **11** and 2-pyridone **J** which undergoes addition to the intermediate **I** to form the adduct **12**. Then upon treatment of adduct **12**



Scheme 2. Synthesis and proposed mechanism of the formation of 3-pyridinesulfonamide derivatives **9**–**13**. Reagents, conditions and yields: (a) 4-methoxypyridine (1.2 M equiv.) dry acetonitrile, room temperature 24 h, reflux 45 h, 68%; (b) 4-methoxy-3-pyridinesulfonamide (1.0 M equiv.) dry acetonitrile, reflux 26 h 82%; (c) 2-methoxypyridine (1.3 M equiv.) dry acetonitrile, room temperature 24 h, reflux 45 h, 12.6% for **11** and 38.5% for **12**; (d) methanol, reflux 0.5 h water, room temperature 16 h, 91%.

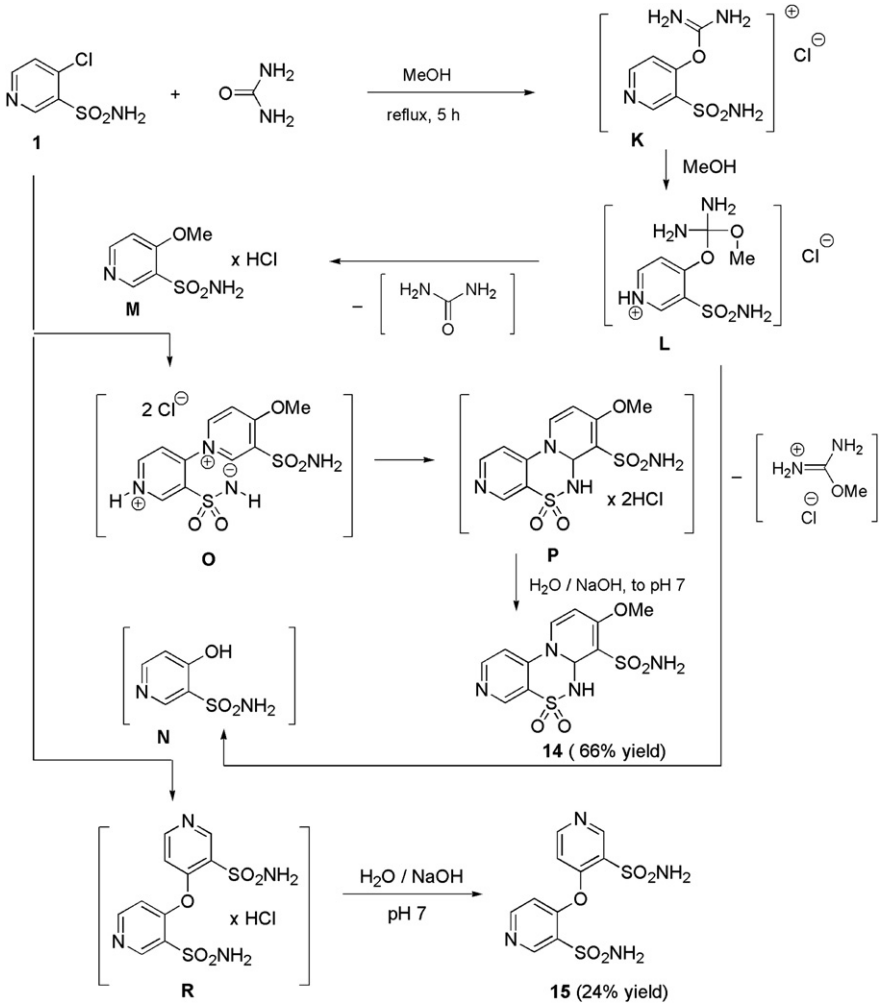
with methanol and water at reflux, the free sulfonamide **13** was obtained in 91% yield.

We have found in our studies that the reaction of 4-chloro-3-pyridinesulfonamide **1** with urea led to the formation of the expected uronim chloride **K**, which in the presence of methanol undergoes a further transformation. The proposed mechanism leading to the formation of the products **14** and **15** is outlined in Scheme 3. The initial step is believed to be formation of unstable $1H^{+}$-pyridinium chloride **L**, which after spontaneous elimination of urea or 2-methyluronium chloride gives two intermediate products: 4-methoxy-3-pyridinesulfonamide hydrochloride **M** and 4-hydroxy-3-pyridinesulfonamide **N**, respectively. In the next stages of reaction the intermediate **M** and **N** reacts with starting compound **1** led to formation of pyridinium chloride **O**, which undergoes cyclocondensation to afford the hydrochloride **P** of final product **14**, and hydrochloride **R** of final product **15**, respectively. Then, upon neutralization of the salts **P** and **R** with NaOH in water the desired free sulfonamides **14** and **15** were obtained in 66% and 24% yield, respectively.

All final products were characterized by IR and NMR spectroscopy as shown in experimental protocols. Elemental analyses (C, H, N) were in accordance with the proposed structure. X-ray crystallography was undertaken in order to investigate in detail molecular structures of the adduct **12** and its free sulfonamide **13** (Figs. 2 and 3).

2.2. CA inhibition studies

The compounds **2**–**5**, **9**–**11** and **13**–**15** as well as standard, clinical used CAIs, such as acatazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND** (Fig. 1) have been tested for the inhibition of two cytosolic ubiquitous isozymes of human origin, that is, hCA I and hCA II, and two transmembrane isoforms (cancer-associated) hCA IX, XII, and hCA XIV isozyme (Table 1). The following should be noted regarding CA inhibitory data of Table 1: (a) against the slow cytosolic izoform hCA I, the sulfonamides investigated here showed high to weak inhibitory properties. Thus, derivatives **2**, **3**, **9**, **10** and **15** exhibited weak inhibition of this izoform, with K_Is in the range of 5.35–251.0 μM, being thus much weaker inhibitors as compared to the clinically used sulfonamides **AAZ**–**IND** (Table 1). The compounds **4**, **5** and **11** were slightly more effective inhibitors against hCA I, with K_Is in the range of 2.46–4.56 μM. However, only the one compound **14** acted as a strong hCA I inhibitor (K_Is = 0.089 μM), with a comparable potency as the references compounds (*i.e.*, **EZA** or **IND**); (b) against the ubiquitous and dominant rapid cytosolic isozyme hCA II, all compounds **2**–**5**, **9**–**11** and **13**–**15** acting as weaker inhibitors (K_Is in the range 50.5–487.0 μM)(Table 1); (c) all of the new compounds **2**–**5**, **9**–**11** and **13**–**15** showed excellent hCA IX inhibitory efficacy, with inhibition constants of 5.2–18.3 nM, being much more effective as



Scheme 3. Proposed mechanism of the formation of 3-pyridinesulfonamide derivatives **14** and **15** in the reaction of 4-chloro-3-pyridinesulfonamide with urea in boiling methanol.

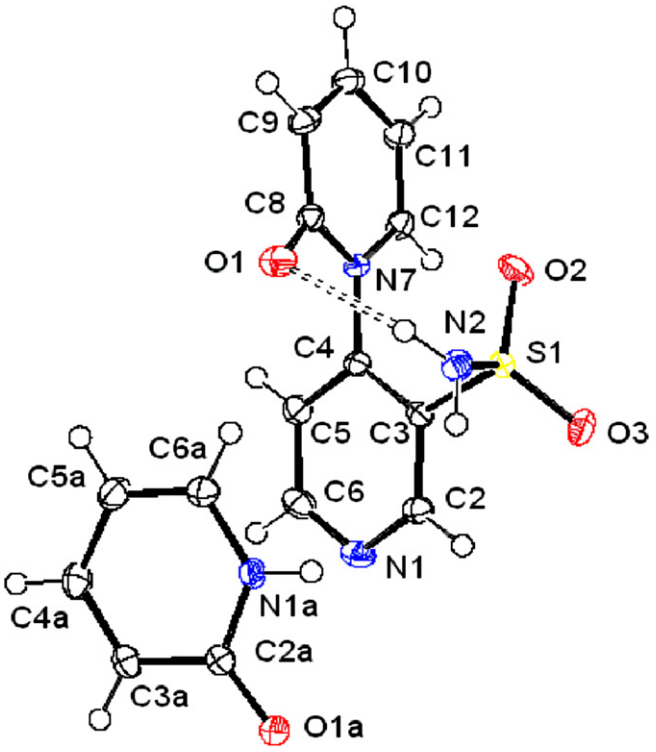


Fig. 2. ORTEP [23] representation of molecular structure of **12**. Displacement ellipsoids are shown at the 50% probability level.

compared to the clinical used **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (K_Is = 24–50 nM); (d) a quite good inhibition profile of the second tumor-associated isoform, that is, hCA XII has also been observed for all investigated sulfonamides of types: **2**–**5** (K_Is = 8.2–16.4 nM); **9**, **10** and **13** (K_Is = 6.1–9.1 nM); **11** and **14** (K_Is = 7.5–7.8 nM), and **15** (K_Is = 6.0 nM) (Table 1); (e) all of the investigated sulfonamides **2**–**5**, **9**–**11** and **13**–**15** showed moderate inhibition properties toward hCA XIV isozyme with K_Is in the range of 76.4–152.0 nM (Table 1).

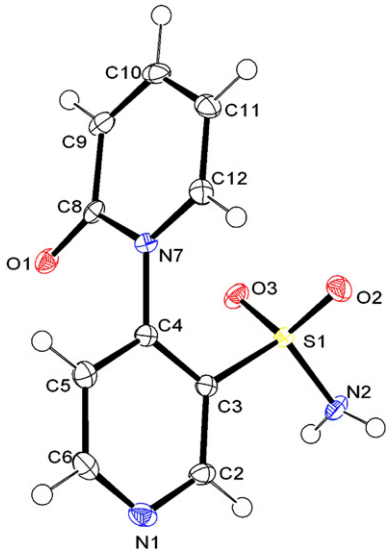


Fig. 3. ORTEP [23] representation of molecular structure of **13**. Displacement ellipsoids are shown at the 50% probability level.

3. Conclusions

We have developed methods for the preparation of the novel series of 3-pyridinesulfonamide derivatives. The several new sulfonamides have been assayed for the inhibition of the physiologically relevant CA isozymes, such as CA I and II, the tumor-associated isozymes CA IX and XII, as well as membrane-associated CA XIV. Toward the human isozyme CA I the new compounds showed inhibition constants in the range of 0.089–251 μM, while against hCA II in the range of 50.5–487 nM. Moreover, hCA IX was inhibited with K_Is in the range of 5.2–18.3 nM, while second membrane-associated isozyme hCA XII was inhibited with K_Is in the range of 6.0–16.4 nM. Compound **14** has a distinctive activity against hCA I (K_Is = 0.089 μM) comparable with the clinical used sulfonamides **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (K_Is = 0.025–1.20 μM). Is noteworthy, that all of the new compounds **2**–**5**, **9**–**11** and **13**–**15** showed excellent hCA IX inhibitory efficacy, with inhibition constants of 5.2–18.3 nM, being much more effective as compared to the clinically used **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (K_Is = 24–50 nM).

4. Experimental protocols

4.1. Synthesis

The following instruments and parameters were used: melting points Buchi 535 apparatus, IR spectra: KBr pellets, 400–4000 cm^{-1} Perkin–Elmer FT IR spectrophotometer; ^1H and ^{13}C NMR: Varian Gemini 200 apparatus at 200 and 50 MHz, respectively; chemical shifts are expressed in parts per million (ppm) relative to TMS as internal standard. The results of elemental analyses for C, H and N were within ±0.3% of theoretical values.

4.1.1. Procedures for the preparation of 1-(3-sulfamoylpyrid-4-yl) pyridinium chlorides (*2*–*5*)

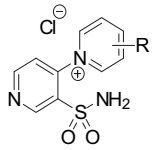
A mixture of the appropriate pyridine derivative (24 mmol) and 4-chloro-3-pyridienesulfonamide **1** (2.31 g, 12 mmol) in acetonitrile (15 ml) was refluxed with stirring for 18 h and left to stand at room temperature overnight. The precipitate was filtered off, washed with acetonitrile (5 × 2 ml) and dried. In this manner the following aminium chlorides were obtained.

*4.1.1.1. 1-(3-Sulfamoylpyrid-4-yl)pyridinium chloride (**2**).* Starting from pyridine (1.9 g), the title compound **2** was obtained (3.1 g, 95%): m.p. 201–202 °C dec.; IR (KBr) 3190, 3125 (SO_2NH_2), 1630, 1565 (pyridine rings) 1335, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.08 (d, J = 5.2 Hz, 1H, H-5, pyridine), 8.35 (t, J = 7.3 Hz, 2H, H-3 and H-5, pyridinium), 8.47 (s, 2H, SO_2NH_2), 8.88 (t, J = 7.3 Hz, 1H, H-4 pyridinium), 9.17 (d, J = 5.2 Hz, 1H, H-6, pyridine), 9.36 (s, 1H, H-2 pyridine), 9.39 (dd, J_{ortho} = 7.3 Hz, J_{meta} = 1.3 Hz, 2H, H-2 and H-6, pyridinium) ppm; ^{13}C NMR (DMSO-d_6) δ 122.99, 127.78, 134.23, 145.27, 146.58, 149.55 149.99, 155.14 ppm. Anal. ($C_{10}H_{10}ClN_3O_2S$) C, H, N.

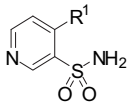
*4.1.1.2. 2-Methyl-1-(3-sulfamoylpyrid-4-yl)pyridinium chloride (**3**).* Starting from 2-methylpyridine (2.2 g) the title compound **3** was obtained (3.1 g, 90%): m.p. 207–208 °C dec.; IR (KBr) 3190, 3140 (SO_2NH_2), 1635, 1565 (pyridine rings), 1345, 1185 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.59 (s, 3H, CH_3), 8.07 (d, J = 5.2 Hz, 1H, H-5, pyridine), 8.24–8.27 (m, 1H, H-4, pyridinium), 8.44 (s, 2H, SO_2NH_2), 8.74 (d, J = 8.0 Hz, 1H, H-3 pyridinium), 9.18 (d, J = 5.2 Hz, 1H, H-6, pyridine), 9.22 (d, J = 8.0 Hz, 1H, H-5 pyridinium), 9.32–9.34 (m, 1H, H-6, pyridinium), 9.37 (s, 1H, H-2, pyridine) ppm. Anal. ($C_{11}H_{12}ClN_3O_2S$) C, H, N.

*4.1.1.3. 4-Methyl-1-(3-sulfamoylpyrid-4-yl)pyridinium chloride (**4**).* -Starting from 4-methylpyridine (2.2 g), the title compound **4** was

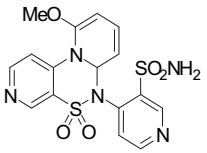
Table 1
Carbonic anhydrase inhibition data for compounds **2**–**5**, **9**–**11** and **13**–**15** and standard inhibitors against human isozymes hCA I, II, IX, XII and XIV by a stopped-flow, CO_2 hydration assay [23].



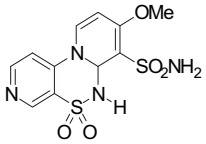
2-5



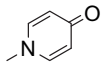
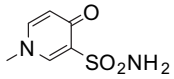
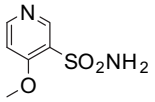
9,10,13,15



11



14

Compd	R	R^1	K_I[a]				
			hCA I[b] (μM)	hCA II[b] (nM)	hCA IX[c] (nM)	hCA XII[c] (nM)	hCA XIV[d] (nM)
AAZ			0.31	12	25	5.7	41
MZA			0.78	14	27	3.4	43
EZA			0.025	8	34	22	25
DCP			1.20	38	50	50	345
IND			0.031	15	24	3.4	104
2	H	–	7.60	102	7.6	16.4	152
3	2-Me	–	5.45	336	7.4	8.2	113
4	4-Me	–	4.56	391	10.1	9.0	104
5	4-Me_2N	–	2.91	149	9.0	9.9	87.5
9	–		6.12	98.3	18.3	6.1	98.5
10	–		7.13	50.5	5.2	8.7	93.8
11	–	–	2.46	220.0	8.7	7.5	76.4
13	–		5.38	487.0	10.2	9.1	80.6
14	–	–	0.089	90.0	13.5	7.8	86.1
15	–		251.0	54.6	9.0	6.0	127.0

[a] Errors in the range of ±5–10% of the reported value (from 3 different assays).
[b] Human (cloned) isozymes, by CO_2 hydration method.
[c] Catalytic domain of human, cloned isozymes [24,25], by the CO_2 hydration method.
[d] Full-length human (cloned) isozyme [26].

obtained (3.2 g, 93%): m.p. 226–227 °C dec.; IR (KBr) 3180, 3115 (SO_2NH_2), 1640, 1575 (pyridine rings), 1360, 1175 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.76 (s, 3H, CH_3), 8.05 (d, J = 4.9 Hz, 1H, H-5, pyridine), 8.20 (d, J = 6.5 Hz, 2H, H-3 and H-5, pyridinium), 8.46 (s, 2H, SO_2NH_2), 9.17 (d, J = 4.9 Hz, 1H, H-2, pyridine), 9.29 (d, J = 6.5 Hz, 2H, H-2 and H-6 pyridinium), 9.37 (s, 1H, H-2, pyridine) ppm. Anal. ($C_{11}H_{12}ClN_3O_2S$) C, H, N.

*4.1.1.4. 4-Dimethylamino-1-(3-sulfamoylpyrid-4-yl)pyridinium chloride (**5**).* Starting from 4-dimethylaminopyridine (3.4 g) the title compound 5 was obtained (3.3 g, 87%): m.p. 242–243 °C dec.; IR (KBr) 3400, 3150 (SO_2NH_2), 1655, 1575 (pyridine rings), 1355, 1170 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.28 (s, 6H, CH_3–N–CH_3), 7.18 (d, J = 7.5 Hz, 2H, H-3 and H-5 pyridinium), 7.86 (d, J = 5.1 Hz, 1H, H-5, pyridine), 8.34 (s, 2H, SO_2NH_2), 8.43 (d, J = 7.5 Hz, 2H, H-2 and H-6 pyridinium), 9.07 (d, J = 5.1 Hz, 1H, H-6, pyridine), 9.31 (s, 1H, H-2, pyridine) ppm; ^{13}C NMR (DMSO-d_6) δ 107.19, 123.98, 135.26, 142.84, 145.45, 149.83, 155.10, 156.69 ppm. Anal. ($C_{12}H_{15}ClN_4O_2S$) C, H, N.

*4.1.2. Procedures for the transformations of pyridinium chlorides **2**–**4** into 6,6a-dihydrodipyridio[2,1-c:4′,3′-e][1,2,4]thiadiazine 5,5-dioxide derivatives (**6**–**8**)*

To a solution of the appropriate pyridinium chloride **2**, **3** or **4** (4 mmol) in water (5 ml), solution of NaOH (0.19 g, 4.8 mmol) in water (5 ml) was added. The reaction mixture was stirred at room temperature for 3 h, and the resulting precipitate was collected by filtration, washed successively with water (3 × 2 ml) and acetonitrile (2 × 1 ml), and dried. In this manner the following pyridothiadiazines were obtained.

*4.1.2.1. 6,6a-Dihydrodipyridio[2,1-c:4′,3′-e][1,2,4]thiadiazine 5,5-dioxide (**6**).* Starting from pyridinium chloride **2** (1.09 g), the title compound **6** was obtained (0.8 g, 85%): m.p. 228–230 °C dec.; IR (KBr) 3120 (NH), 1660, 1585 (C=N and C=N), 1330, 1170 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 5.28–5.46 and 6.10–6.25 (2m, 2 × 2H, H-6a,7,8 and H-9), 7.04 (d, J = 7.7 Hz, 1H, H-10), 7.42 (d, J = 6.1 Hz, 1H, H-1), 8.48 (d, J = 6.1 Hz, 1H, H-2), 8.74 (s, 1H, H-4), 8.99 (s, 1H, H-6) ppm. Anal. ($C_{10}H_9ClN_3O_2S$) C, H, N.

*4.1.2.2. 10-Methyl-6,6a-dihydrodipyridio[2,1-c:4′,3′-e][1,2,4]thiadiazine 5,5-dioxide (**7**).* Starting from pyridinium chloride **3** (1.14 g), the title compound **7** was obtained (0.7 g, 70%): m.p. 238–240 °C dec.; IR (KBr) 3115 (NH), 1670, 1590 (C=N and C=N), 1330, 1175 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.80 (s, 3H, CH_3), 5.27 (s, 1H, H-6a), 5.95 (s, 1H, H-8), 6.08 (s, 1H, H-7), 6.94 (d, J = 6.9 Hz, 1H, H-9), 7.44 (d, J = 5.8 Hz, 1H, H-1), 8.49 (d, J = 5.8 Hz, 1H, H-2), 8.78 (s, 1H, H-4), 8.84 (s, 1H, H-6) ppm. Anal. ($C_{11}H_{11}N_3O_2S$) C, H, N.

*4.1.2.3. 8-Methyl-6,6a-dihydrodipyridio[2,1-c:4′,3′-e][1,2,4]thiadiazine 5,5-dioxide (**8**).* Starting from pyridinium chloride **4** (1.14 g), the title compound **8** was obtained (0.6 g, 60%): m.p. 210–212 °C dec.; IR (KBr) 31150 (NH), 1675, 1585 (C=N and C=N), 1325, 1170 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.80 (s, 3H, CH_3), 5.21 (s, 2H, H-6a and H-7), 6.14 (d, J = 5.9 Hz, 1H, H-9), 7.05 (d, J = 5.9 Hz, 1H, H-10), 7.43 (d, J = 5.8 Hz, 1H, H-1), 8.48 (d, J = 5.8 Hz, 1H, H-2), 8.75 (s, 1H, H-4), 8.88 (s, 1H, H-6) ppm. Anal. ($C_{11}H_{11}N_3O_2S$) C, H, N.

*4.1.3. Synthesis of 4-(1,4-dihydro-4-oxopyrid-1-yl)-3-pyridinesulfonamide **9** from pyridinium chloride **5** (method A), and from 4-chloro-3-pyridinesulfonamide **1** (method B)*

Method A (Scheme 1). To an ice-cold solution of NaOH (0.32 g, 8 mmol) in water (15 ml), pyridinium chloride **5** (1.26 g, 4 mmol) was added. The reaction solution was stirred at 0–10 °C for 1 h, followed at room temperature for 36 h. A small amount of insoluble side product was filtered out together with charcoal added. The filtrate (pH ~11) was acidified with 1% hydrochloric acid to pH 2.5. After 24 h of stirring the title product **9** was collected by filtration, washed with water (3 × 2 ml) and dried at temperatures gradually increasing to 100 °C. Yield 0.7 g (70%), m.p. 235–236 °C; IR (KBr) 3310, 3190 (SO_2NH_2), 1640 (C=O), 1340, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.19 (d, J = 7.6 Hz, 2H, H-3 and H-5 pyridone), 7.70 (d, J = 5.1 Hz, 1H, H-5, Py–SO_2), 7.75 (d, J = 7.6 Hz, 2H, H-2 and H-6, pyridone), 8.01 (s, 2H, SO_2NH_2), 8.96 (d, J = 5.1 Hz, 1H, H-6, Py–SO_2), 9.20 (s, 1H, H-2, Py–SO_2) ppm; ^{13}C NMR (DMSO-d_6) δ 117.37, 123.90, 135.18, 141.28, 146.88, 149.77, 155.02, 177.85 ppm. Anal. ($C_{10}H_9N_3O_3S$) C, H, N.

Method B (Scheme 2). A mixture of **1** (1.93 g, 10 mmol) and 4-methoxypyridine (1.3 g, 12 mmol) in dry acetonitrile (35 ml) was stirred a room temperature for 24 h, followed at reflux for 45 h. After cooling to room temperature the suspension was left overnight. The precipitate was collected by filtration, washed successively with acetonitrile (3 × 1.5 ml), water (5 × 3 ml) and acetonitrile (3 × 2 ml), the dried at temperatures gradually increasing to 100 °C. Yield 1.7 g (68%), m.p. 234–236 °C; IR and ^1H NMR were identical with authentic sample **9** described in method A.

*4.1.4. Synthesis of 4-(1,4-dihydro-4-oxo-3-sulfomoylpyridin-1-yl)-3-pyridinesulfonamide (**10**)*

A solution of **1** (1.35 g, 7 mmol) and 4-methoxy-3-pyridinesulfonamide (1.32 g, 7 mmol) in dry acetonitrile was refluxed with stirring for 26 h. After cooling to room temperature the suspension was left overnight. The precipitate was collected by filtration, washed with acetonitrile (4 × 1.5 ml) and dried at temperatures gradually increasing to 100 °C. Yield 1.9 g (82%), m.p. 247–249 °C; IR (KBr) 3325, 3220, 3150 (SO_2NH_2), 1645 (C=O), 1575 (C=N), 1340, 1160 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.45 (d, J = 7.8 Hz, 1H, H-5 of 4-pyridone ring), 6.94 (s, 2H, SO_2NH_2 of pyridone ring), 7.80 (d, J = 5.2 Hz, 1H, H-5 of pyridine ring), 7.89 (dd, J_{ortho} = 7.8 Hz, J_{meta} = 2.1 Hz, 1H, H-6 of pyridone ring), 8.09 (s, 2H, SO_2NH_2 of pyridine ring), 8.28 (d, J = 2.1 Hz, 1H, H-2 of pyridone ring), 8.99 (d, J = 5.2 Hz, 1H, H-6 of pyridine ring), 9.20 (s, 1H, H-2 of pyridine ring) ppm; ^{13}C NMR (DMSO-d_6) δ 119.41, 124.25, 130.19, 135.19, 141.68, 142.66, 146.08, 149.56, 155.26, 172.92 ppm. Anal. ($C_{10}H_{10}N_4O_5S_2$) C, H, N.

*4.1.5. One-pot synthesis of 2-[10-methoxy-5,5-dioxo-6,6a-dihydrodipyrido[2,1-c:4′,3′-e][1,2,4]thiadiazin-6-yl]benzenesulfonamide (**11**) and the adduct of 4-(1,2-dihydro-2-oxopyrid-1-yl)-3-pyridinesulfonamide with 2-pyridone (**12**)*

A mixture of 4-chloro-3-pyridinesulfonamide **1** (5. 78 g, 30 mmol) and 2-methoxypyridine (4.26 g, 39 mmol) in dry acetonitrile (60 ml) was stirred at room temperature for 24 h, followed at reflux for 45 h. After cooling to room temperature a pitchy side products was filtered out with charcoal. The solvent was partially evaporated under normal pressure and the suspension (~15 g) was left overnight. The precipitate of adduct **12** was collected by filtration, washed with acetonitrile (3 × 1.5 ml) (Filtrate **A** was left for further work-up) and dried. Yield 2.0 g (38.5%), m.p. 165–166 °C; IR (KBr), 3310, 3290, 3240, 3125 (NH and SO_2NH_2), 1700, 1660 (C=O), 1620 (C=N), 1350, 1175 cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.14 (t, J = 6.2 Hz, 1H, arom.), 6.28–6.40 (m, 2H, arom.), 6.50 (d, J = 9.8 Hz, 1H, arom.), 7.33–7.44 (m, 2H, arom.), 7.53–7.66 (m, 5H, SO_2NH_2 and 3H arom.), 8.95 (d, J = 5.0 Hz, 1H, H-6, Py–SO_2), 9.17 (s, 1H, H-2, Py–SO_2), 11.55 (br.s, 1H, HN–C=O ↔ N=C–OH of 2-pyridone moiety) ppm; ^{13}C NMR (DMSO-d_6) δ 105.11, 106.17, 120.30, 120.48, 125.07, 135.76, 136.13, 138.72, 141.22, 141.92, 145.30, 149.72, 154.73, 161.65, 162.65 ppm. Anal. ($C_{15}H_{14}N_4O_4S$) C, H, N.

The filtrate **A** was evaporated under reduced pressure. To the resulting oily residue 2-propanol (60 ml) and charcoal (0.3 g) was added and the resulting mixture was heated under reflux for 1 h. The charcoal and side products were separated by suction and the hot filtrate was left at room temperature for 24 h. The precipitate of **11** was collected by filtration, washed with 2-propanol (3 × 2 ml) and dried, Yield 0.8 g (12.6%), m.p. 201–203 °C dec.; IR (KBr), 3360, 3295, 3190 (SO_2NH_2), 1645, 1565 (C=N and C=C), 1345, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.75 (s, 3H, CH_3O), 6.46 (d, J = 7.7 Hz, 1H, H-1), 6.85–7.1 (m, 2H, H-6a and H-7), 7.40–7.68 (m, 2H, H-8 and H-9), 7.80 (d, J = 5.2 Hz, H-5 of Py–SO_2NH_2), 7.91 (dd, J_{ortho} = 7.7 Hz, J_{meta} = 2.4 Hz, 1H, H-2), 8.12 (s, 2H, SO_2NH_2), 8.29 (d, J_{meta} = 2.4 Hz, 1H, H-4), 9.00 (d, J = 5.2 Hz, 1H, H-6 of Py–SO_2NH_2), 9.22 (s, 1H, H-2 of Py–SO_2NH_2) ppm. Anal. ($C_{16}H_{15}N_5O_5S_2$) C, H, N.

*4.1.6. Transformation of adduct **12** to free 4-(1,2-dihydro-2-oxopyrid-1-yl)-3-pyridinesulfonamide (**13**)*

A solution of adduct **11** (1.38 g, 40 mmol) in methanol (35 ml) was refluxed with stirring for 0.5 h, and then water (7 ml) was added. After cooling to room temperature the suspension was left overnight. The precipitate was collected by filtration washed with methanol (2 × 1 ml) and dried at temperatures gradually increasing to 90 °C. Yield 0.92 g (91%), m.p. 206–207 °C dec.; IR (KBr) 3240, 3180 (SO_2NH_2), 1655 (C=O) 1580, 1560, 1530 (pyridine rings) 1335, 1160 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.30–6.42 (m, 1H, arom.), 6.45–6.58 (m, 1H, arom.), 7.58 (br.s, 5H, SO_2NH_2 and 3H arom.), 8.95 (t, J = 3.4 Hz, 1H, H-6 of Py–SO_2NH_2), 9.17 (d, J = 2.0 Hz, H-2 of Py–SO_2NH_2) ppm; ^{13}C NMR (DMSO-d_6) δ 106.18, 120.48, 125.08, 136.13, 138.72, 141.93, 145.31, 149.72, 154.74, 161.66 ppm. Anal. ($C_{10}H_9N_3O_3S$) C, H, N.

*4.1.7. One-pot synthesis of 2-[8-methoxy-5,5-dioxo-6,6a-dihydrodipyrido[2,1-c:4′,3′-e][1,2,4]thiadiazine]sulfonamide (**14**) and 4,4′-oxydipyridine-3-sulfonamide (**15**) by the reaction of 4-chloro-3-pyridinesulfonamide (**1**) with urea in boiling methanol*

A solution of 4-chloropyridine-3-sulfonamide **1** (1.93 g, 10 mmol) and urea (0.63 g, 10.5 mmol) in methanol (10 ml) was stirred at reflux for 5 h, then methanol was evaporated under reduced pressure. The solid residue was dissolved in water (5 ml, 20 °C) then neutralized pH 7 with 1% aqueous NaOH solution. The precipitate of compound **14** thus obtained was filtered off, washed with water (5 × 2 ml) and methanol (6 × 2 ml) (filtrate **B** was left for further work-up), and dried. Yield 1.14 g (66%), m.p. 206–208 °C

dec.; IR (KBr) 3380, 3265, 3115 (SO_2NH_2), 1660, 1475 (C=C and C=N) 1350, 1335, 1160 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.64 (s, 3H, CH_3O), 4.69 (d, $J = 3.3$ Hz, 1H, H-6a), 6.40 (s, 1H, H-9), 6.97 (s, 2H, SO_2NH_2), 7.48 (d, $J = 5.7$ Hz, 1H, H-10), 7.53 (d, $J = 5.7$ Hz, 1H, H-1), 8.62 (d, $J = 5.7$ Hz, 1H, H-2), 8.87 (s, 1H, H-4), 9.20 (br.s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6) δ 55.63, 69.07, 86.77, 112.22, 115.31, 126.12, 131.93, 145.01, 146.54, 149.83, 153.43 ppm. Anal. ($C_{11}H_{12}N_4O_5S_2$) C, H, N.

The water–methanol filtrate mixture **B** was evaporated under reduced pressure. To the residue obtained a solution of acetic acid (1.5 ml) in water (8 ml) was added and stirred at room temperature for 2 h. The title product **15**, which precipitated, was collected by filtration, washed successively with water (2 × 1 ml) and methanol (1 ml), and dried at temperatures gradually increasing to 105 °C. Yield 0.4 g (24%), m.p. 248–249 °C; IR (KBr), 3380, 3330, 3150 (SO_2NH_2), 1645, 1580, 1570 (C=N and C=C), 1340, 1325, 1160 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.45 (d, $J = 7.7$ Hz, 1H, H-5), 6.94 (s, 2H, SO_2NH_2), 7.89 (dd, $J_{ortho} = 7.7$ Hz, $J_{meta} = 2.4$ Hz, 1H, H-6), 8.27 (d, $J_{meta} = 2.4$ Hz, 1H, H-2), and 7.80 (d, $J = 5.2$ Hz, 1H, H-5′), 8.09 (s, 2H, SO_2NH_2′), 8.89 (d, $J = 5.2$ Hz, 1H, H-6′), 9.20 (s, 1H, H-2′) ppm. Anal. ($C_{10}H_{10}N_4O_5S_2$) C, H, N.

4.2. X-ray crystal structure analysis

The diffraction data for single crystals of **12** and **13** were collected at 100K with Oxford Diffraction XCaliburE diffractometer using Mo Kα radiation. The intensity data were collected and processed using CrysAlisPro Software. The structures were solved by direct methods with the program SHELXS-97 [20] and refined by full-matrix least-squares method on F^2 with SHELXL-97 [21,22]. Hydrogen atoms from N–H groups were freely refined and those bonded to C atoms refined as riding on their carriers.

Crystal data for **12**: $C_{10}H_9N_3O_3S \cdot C_5H_5NO$, triclinic, space group $P-1$, $a = 8.8829$ (4), $b = 10.0844(6)$, $c = 10.5080$ (7) Å, $\alpha = 112.601$ (6), $\beta = 112.306$ (5), $\gamma = 95.617$ (4)°, $V = 770.55$ (10) Å^3, $Z = 2$, $T = 100$ K, $d_x = 1.493$ g cm^{-3}, $\mu(Mo\ K\alpha) = 0.239$ mm^{-1}, 8998 data were collected up to $\theta_{max} = 28.97°$ ($R_{int} = 0.0200$, $R\sigma = 0.0242$). Final R indices for 3091 reflections with $I > 2\sigma(I)$ and 163 refined parameters are: $R_1 = 0.0311$, $wR_2 = 0.0829$ ($R_1 = 0.0362$, $wR_2 = 0.0842$ for all 3559 data).

Crystal data for **13**: $C_{10}H_9N_3O_3S$, monoclinic, space group $P2_1/c$, $a = 9.6132$ (8), $b = 7.7939(8)$, $c = 14.4521$ (12) Å, $\beta = 98.822$ (9)°, $V = 1070.00$ (17) Å^3, $Z = 4$, $T = 100$ K, $d_x = 1.560$ g cm^{-3}, $\mu(Mo\ K\alpha) = 0.302$ mm^{-1}, 11855 data were collected up to $\theta_{max} = 29.03°$ ($R_{int} = 0.0203$, $R\sigma = 0.0162$). Final R indices for 2336 reflections with $I > 2\sigma(I)$ and 163 refined parameters are: $R_1 = 0.0292$, $wR_2 = 0.0808$ ($R_1 = 0.0327$, $wR_2 = 0.0818$ for all 2590 data).

Crystallographic data for compounds **12** and **13** have been deposited with the Cambridge Crystallographic Data Centre, with the deposition numbers CCDC 821393 and 821394.

4.3. CA inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity [23]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of E–I complex. The inhibition constants were obtained by non-linear last-squares methods using PRISM 3, as reported earlier [24–26], and represent the mean from at least three different determinations.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.07.011.

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