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**Synthesis of bulky-tailed sulfonamides incorporating
pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-1(5H)-yl) moieties and evaluation
of their carbonic anhydrases I, II, IV and IX inhibitory effects**

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Abstract

Using celecoxib as lead, two novel series of sulfonamides incorporating the pyridotriazolopyrimidine scaffold have been synthesized and evaluated in vitro as inhibitors against four relevant human (h) carbonic anhydrases (CAs, EC 4.2.1.1), the cytosolic and ubiquitous hCA I and II as well as the transmembrane hCA IV and hCA IX. Most of the reported sulfonamides acted as efficient, low micromolar inhibitors of hCA I, II and IV, whereas they displayed higher efficacy in inhibiting the tumor-associated isoform hCA IX. Many derivatives herein reported showed better hCA IX versus hCA II selectivity ratios compared to celecoxib or acetazolamide. Considering isoform IX is a validated target for the diagnosis and treatment of hypoxic tumors, discovery of selective CA IX inhibitors represents a promising step to unveil more effective anticancer therapies.

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hCA IX/hCA II = 1.1
hCA IX/hCA IV = 14.5

hCA IX/hCA II = 3.4
hCA IX/hCA IV = 16.9

Synthesis of bulky-tailed sulfonamides incorporating pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5*H*)-yl) moieties and evaluation of their carbonic anhydrases I, II, IV and IX inhibitory effects

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Abstract. Using celecoxib as lead, two novel series of sulfonamides incorporating the pyridotriazolopyrimidine scaffold have been synthesized and evaluated *in vitro* as inhibitors against four relevant human (h) carbonic anhydrases (CAs, EC 4.2.1.1), the cytosolic and ubiquitous hCA I and II as well as the transmembrane hCA IV and hCA IX. Most of the reported sulfonamides acted as efficient, low micromolar inhibitors of hCA I, II and IV, whereas they displayed higher efficacy in inhibiting the tumor-associated isoform hCA IX. Many derivatives herein reported showed better hCA IX versus hCA II selectivity ratios compared to celecoxib or acetazolamide. Considering isoform IX is a validated target for the diagnosis and treatment of hypoxic tumors, discovery of selective CA IX inhibitors represents a promising step to unveil a more effective cancer therapies.

Keywords: Carbonic anhydrase, Tumour-associated isoforms, Pyridotriazolopyrimidine, Sulfonamide, Celecoxib.

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1. Introduction:

Celecoxib **I**, a sulfonamide acting as dual cyclooxygenase – carbonic anhydrase (CA, EC 4.2.1.1) inhibitor,¹ was considered to be an attractive target for designing CA inhibitors (CAIs) which may possess a dual action on these two families of enzymes involved in various pathologies which cross-react in some cases (e.g., glaucoma, cancer, arthritis).²⁻⁶ Celecoxib shows a nanomolar affinity for the cytosolic human (h) isoform hCA II ($K_i = 21$ nM), inhibiting even better the tumour associated enzymes hCA XI ($K_i = 16$ nM) and hCA XII ($K_i = 18$ nM).¹ These enzymes were recently validated as antitumor/antimetastatic targets with one compounds (SLC-0111) in Phase Ib/II clinical trials.^{2,3} The X-ray crystal structure of celecoxib bound CA II (PDB code: 6cox) reported by one of our groups,¹ afforded the deep understanding of the groups important for interaction with the CA II active site. The sulfonamide group of **I** was observed to be coordinated to Zn^{+2} in a tetrahedral coordination geometry, whereas the trifluoromethyl group occupied the hydrophobic pocket, located in one half of the active site.^{1a} Unexpectedly, the *p*-toyl moiety of the drug was observed to point towards the hydrophilic half of the active site, making favourable contacts with residues Asn67, Glu69 and Gln92.

Figure 1. Structure of some reported sulphonamide CAIs of types **I-III**.

The presence of the 2-phenyl substituent in the triazolobenzenesulfonamide derivative **II** (Figure 1) elicited moderate to strong inhibition of four CAs, namely, CA I, CA II, CA IX and CA XII, with K_i ranging between 0.041 to 0.542 μ M.⁷ Modification of the phenyl group to a ferrocene based moiety, as in compound **III** led to a decrease of the activity towards CA I and CA XII, while retaining a good inhibitory effect against CA II ($K_i = 0.036$ μ M) and CA IX ($K_i = 0.065$ μ M).⁷

Figure 2: Schematic representation of possible interactions with CA IX active site (PDB: 4q06)^{1a} of A. Celecoxib and B. Designed scaffold with possible extra interaction points C.Target PTP derivatives

An analysis of a modelling study, of celecoxib and CA IX protein, showed a similar mode of interaction between the sulfonamide and the active site, as in the hCA II – celecoxib complex analysed by X-ray crystallography (Figure 2A). Our current model of designing selective CAIs is based on designing a scaffold that can be substituted with less polar groups (Ar_1 and Ar_2 in Figure 2B), in order to fill in the unoccupied hydrophobic pocket (Figure 2B). In view of the aforementioned facts and in continuation of our earlier work on the design of inhibitors of these enzymes,¹⁻³ it was envisaged to incorporate the bulky pyridotriazolopyrimidine (**PTP**) scaffold as tail to the benzene-sulfonamide motif. We aimed to exploit the unoccupied hydrophobic region shown in Figure 2A, within the active site of CA IX, hypothesizing that the aryls at position 6 and 8 of the **PTP** scaffold may interact in a favorable manner with the enzyme active site, as shown schematically in Figure 2C. We report herein the synthesis and the carbonic anhydrases inhibition evaluation of the new such derivatives, incorporating the bulky **PTP** scaffold.

2. Results and discussion:

2.1. Chemistry:

Our proposed synthetic methodology to the desired pyridotriazolopyrimidine scaffold containing sulfonamide moiety is depicted in Scheme 1, Scheme 2 and Scheme 3. The synthesis of the 6-amino-2-thiouracil (**3**) was accomplished as reported by refluxing ethyl cyanoacetate (**1**) with thiourea (**2**) in sodium ethoxide.⁸ The condensation of the 6-amino-2-thiouracil (**3**) with different aromatic α - β unsaturated ketones, β -enaminones, diketones and aldehydes was reported as convenient and efficient strategy to synthesize substituted pyrido[2,3-

d]pyrimidines.⁹⁻¹³ Quiroga *et al.*¹² reported that the reaction of the 6-amino-2-thiouracil (**3**) with different chalcones in refluxing DMF resulted in the formation of the pyrido[2,3-*d*]pyrimidines **5** and/or **6**. When the reaction performed under argon atmosphere for short period of time, it yielded mainly the 2-thioxo-2,3,5,8-tetrahydropyrido[2,3-*d*]pyrimidin-4(1*H*)-one derivative **6**. However, under exposure to air for long time in refluxing DMF, the oxidized isomer **5** was formed. In addition, the substitutions of phenyl groups of the chalcone affect the product selectivity where the electron withdrawing group preferentially favours the formation of the oxidized form **5**.¹²

Scheme 1: Reagents and conditions: (i) NaOEt/Ethanol (ii) dry DMF /reflux 15h

The synthesis of the key pyrido[2,3-*d*]pyrimidines **5a-j** was accomplished *via* reacting 6-amino-2-thiouracil **3** and different α - β unsaturated aromatic ketones **4a-j**¹⁴ in refluxing dry DMF under air in 40-65% yields (Scheme 1). The structures assigned to compounds **5a-j** were confirmed by ¹H and ¹³C NMR. ¹H NMR spectra of the synthesized pyrido[2,3-*d*]pyrimidines showed the presence of a singlet at δ 7.56-8.07 *ppm* integrated to the pyridine ring proton and two NH singlet signals at δ 12.23-12.41 and 12.90-13.10 *ppm* corresponding to two NHs of the pyrimidine ring. Furthermore, ¹³C NMR spectra of **5a-c** revealed the signals of the pyridine H at δ 108.20-108.27 *ppm* and the signal of thioxo carbon at δ 175.41-175.43 *ppm*.

The hydrazoneyl chlorides **11a** and **11b** synthesis were started with the chlorination of the active methylene group of acetylacetone **7a** and ethyl acetoacetate **7b** using sulphuryl chloride to afford the α -chloroacetyl derivatives **8a** and **8b**, as described by Alihn in 1878.¹⁵ Then by applying Japp-Klingemann coupling of the aforementioned α -chloroacetyl derivatives (**8a** and **8b**) with the diazonium salts of sulfanilamide **10**, we have got the acetyl derivative **11a** and the ester derivative **11b** in reasonable yields (65 and 72%, respectively) (Scheme 2).¹⁶

Scheme 2: Reagents and conditions: (i) SO₂Cl₂/ 0 °C (ii) NaNO₂/HCl (iii) CH₃COONa/ 0 °C

The reaction of the pyrido[2,3-*d*]pyrimidine thiones and hydrazonoyl halides have been extensively studied.¹⁶⁻¹⁹ This reaction proceeds *via* 1,3-dipolar cycloaddition of hydrazonoyl chlorides with dipolarophile thiones.¹⁷

A literature review of this reaction almost exclusively prefers isomer **16** over **15** (Scheme 3). This suggest that the reaction process through the cyclo-addition of carbon 2 and nitrogen 3 of the pyrido[2,3-*d*]pyrimidine thiones with the hydrazonoyl chloride derivative in presence of base (Scheme 3).¹⁷ The reaction mechanism was postulated to start with S-alkylation of the thiouracil derivative to afford the thiohydrazontes **12**. In the presence of base, the nucleophilicity of the terminal hydrazone *N* increases and the compound undergoes Chapman-like rearrangement to give the corresponding thiohydrazide **13**. The latter thiohydrazide was cyclized *in situ* to afford **15** or **16**. ¹H NMR spectra of compounds **16a-t** showed disappearance of the two NH singlet signals. The acetyl derivatives displayed singlet signals resonating at δ 2.63-2.68 ppm representing the acetyl CH₃ protons. Meanwhile, ¹H NMR of the ester analogues showed a typical triplet-quartet pattern of the ethyl protons at δ 1.24-1.31 and 4.42-4.43 ppm.

Scheme 3: Reagents and conditions: (i) Dioxane, TEA, reflux, 6-14h.

Analysis of the ¹³C NMR spectra of the novel synthesized derivatives indicated that we preferentially synthesized isomer **16** rather than isomer **15**. It was reported that the chemical shift of the carbonyl carbon in pyrimidin-4-one derivatives is relying on the adjacent environment.²⁰ For instance, if the adjacent nitrogen is pyrrole type (sp³-hybridized) (as in **16**) the carbonyl carbon signals of the pyrimidinone ranged from 160-165. Nevertheless, if the neighbouring atom is a pyridine type (as in **15**) chemical shift of the carbonyl carbon usually appears at 170-175 ppm. ¹³C NMR spectral data of compounds **16a-t** revealed carbonyl carbon signals of the pyrimidinone at 159.90 and 163.21 ppm. This result is in line with previous reports of the reactions of hydrazonoyl halides with similar condensed 2-thioxopyridopyrimidines.¹⁶⁻²¹

2.2. Carbonic anhydrase inhibition:

All the synthesized derivatives **16a-16t** were evaluated for their efficacy in inhibiting four relevant CA isoforms, i.e., hCA I, II, IV and IX, by using the stopped flow carbon dioxide hydrase assay²², in comparison to acetazolamide (**AAZ**) as a standard CAI.

In general, all the assayed compounds displayed good inhibitory action against the reported hCA isoforms, with K_i spanning from the low micromolar to the low-medium nanomolar range. In detail, the following structure–activity-relationship (SAR) can be gathered from the data reported in Table 1, for each tested isoform:

Table 1: Inhibition data of human CA isoforms hCA I, II, IV and IX with sulfonamides **16a-16t**, celecoxib and the standard sulfonamide inhibitor acetazolamide (**AZ**) by a stopped flow CO_2 hydrase assay.²²

Compound	Structure	R_1	R_2	K_i (μM)			
				hCA I	hCA II	hCA IV	hCA IX
16a	I	-COCH ₃	-H	3.79	0.59	3.00	0.18
16b	I	-COOC ₂ H ₅	-H	NA	3.54	4.62	3.26
16c	I	-COCH ₃	-F	6.64	1.19	3.58	0.25
16d	I	-COOC ₂ H ₅	-F	9.35	4.66	4.90	3.24
16e	I	-COCH ₃	-Cl	7.42	2.03	3.56	0.35
16f	I	-COOC ₂ H ₅	-Cl	9.16	3.65	4.70	1.46
16g	I	-COCH ₃	-OCH ₃	5.50	0.74	3.01	0.33
16h	I	-COOC ₂ H ₅	-OCH ₃	7.37	1.58	NA	1.45
16i	I	-COCH ₃	-NO ₂	5.52	0.86	2.92	0.33
16j	I	-COOC ₂ H ₅	-NO ₂	7.29	3.05	3.56	2.46
16k	II	-COCH ₃	-H	6.78	0.73	4.79	1.12
16l	II	-COOC ₂ H ₅	-H	6.82	1.41	NA	1.90
16m	II	-COCH ₃	-F	2.65	0.35	2.45	0.19
16n	II	-COOC ₂ H ₅	-F	NA	1.48	3.96	1.62
16o	II	-COCH ₃	-Cl	NA	3.43	2.52	0.99

16p	II	-COOC ₂ H ₅	-Cl	2.64	1.71	4.33	0.39
16q	II	-COCH ₃	-OCH ₃	9.04	2.98	2.23	0.42
16r	II	-COOC ₂ H ₅	-OCH ₃	0.85	0.72	4.25	0.38
16s	II	-COCH ₃	-NO ₂	3.02	0.50	0.20	0.16
16t	II	-COOC ₂ H ₅	-NO ₂	8.71	1.75	2.98	1.10
Celecoxib				>10	0.021	0.29	0.02
AAZ				0.25	0.01	0.07	0.03

*Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5 –10% of the reported values).

(i) The cytosolic isoform hCA I was inhibited by most sulfonamides herein reported in the low micromolar range (K_{Is} between 2.64 and 9.35 μM), with the exception of compounds **16b**, **16n** and **16o** which did not inhibit hCA I up to 100 μM . Only derivative **16r** showed a submicromolar hCA I inhibitory efficacy (K_{Is} 0.85 μM). Such results ascribed to the prepared compounds better inhibitory efficacy than those of celecoxib, although worsened compared to the clinically used sulfonamide **AAZ**.

(ii) The physiologically dominant isoform hCA II was efficiently inhibited by all sulfonamides **16a–16t**, with K_{I} values ranging between 0.35 and 4.66 μM (Table 1). Despite the impossibility to drawing a clear SAR, probably elicited by the rather flat inhibitory tendency revealed against hCA II, it is worth noting the submicromolar inhibitors **16a**, **16g**, **16i**, **16k**, **16m**, **16r** and **16s** (K_{Is} between 0.35 and 86 μM). All of them bear the acetyl group in position R_1 (except **16r**), thus highlighting the better impact of such a group on the binding efficacy, compared to the carboxymethyl moiety.

(iii) Likewise hCA I, most of the reported sulfonamides showed comparable effectiveness in inhibiting the membrane-associated isoform hCA IV, with K_{I} values spanning in the low micromolar range (2.23– 4.90 μM), except **16h** and **16l** which did not inhibit hCA I up to 100 μM . Only the nitro derivative **16s**, which appended the acetyl and the thienyl groups at the **PTP** scaffold, acted as a potent hCA IV inhibitor with a K_{I} of 0.20 μM .

(iv) Finally, the tumor-associated isoform hCA IX was potently inhibited by the most of the reported sulfonamides with K_{I} reaching the low-medium nanomolar range (0.16 – 3.26 μM). In particular, the acetyl bearing derivatives **16a**, **16c**, **16e**, **16g** and **16i** from series I and **16m** and **16s** from series II, displayed a nanomolar inhibitory profile (K_{Is} of 0.18, 0.25, 0.35, 0.33, 0.33, 0.19 and 0.16 μM , respectively) likened to the corresponding carboxyethyl analogues (K_{Is}

of 3.26, 3.24, 1.46, 1.45, 2.46, 1.62 and 1.10 μM , respectively). Conversely, only the carboxyethyl bearing compounds **16p** and **16r** demonstrated to be better hCA IX inhibitors (K_{Is} of 0.39 and 0.38 μM) in comparison to the acetyl analogues **16o** and **16q** (K_{Is} of 0.99 and 0.42 μM). Moreover, the data in Table 1 undeniably highlighted the positive effect on the inhibitory efficacy against hCA IX obtained by swapping the 4-Cl-phenyl group with the thienyl one.

(v) Despite the not enviable efficacy that sulfonamides **16a-16t** showed against the tumor-associated isoform (celecoxib and **AAZ** displayed K_{Is} of 0.02 and 0.03 μM), it is reasonable to focus the attention on the interesting selective profile they possess against hCA IX versus hCA II (Table 2). Indeed, it is satisfying to note that most of the reported compounds displayed a selectivity ratio hCA IX/hCA II from two-fold to fourteen-fold higher than celecoxib or **AAZ**. Only **16k**, **16l** and **16n** were non-selective hCA IX over hCA II inhibitors. On the other hand, all the acetyl **PTP** sulfonamides from the series I and **16k** and **16m** from series II were shown to possess much better selectivity for the tumor-associated isoform hCA IX over hCA IV, compared to the corresponding carboxyethyl analogues and **AAZ**, while exhibited comparable selectivity ratio with celecoxib. A different behaviour may be highlighted for the remaining derivatives of series II. In fact, the carboxyethyl bearing sulfonamides **16p**, **16r** and **16t** were more selective inhibitors of hCA IX over hCA IV in comparison to their acetyl analogues. In addition, **16p** and **16r** displayed selectivity ratio hCA IX/IV comparable to celecoxib, being four-fold better than **AAZ**.

(vi) As more than once highlighted here, the data reported in Table 1 clearly demonstrate that the replacement of the acetyl group in position R_1 by the carboxyethyl moiety generally worsened the inhibition profiles of derivatives **16a-16t** depending on the substituents Ar_1 , R_2 and the considered isoform. It is reasonable to hypothesize that such a diminished inhibitory efficacy of the carboxyethyl derivatives in comparison to the acetyl ones might derive from the steric hindrance aroused by the **PTP**. Indeed, such a bulky core may lead to a rather rigid and fixed positioning of the tricyclic scaffold within the active site pocket, obviously depending on the substituents Ar_1 and R_2 . Hence, in most cases the replacement of the acetyl group with the bigger carboxyethyl moiety may cause clashes with amino acid residues from the hydrophobic pocket, worsening thus the inhibitory activity.

Table 2: Selectivity ratios for the inhibition of hCA IX over hCA II and hCA IX over hCA IV for the compounds **16a-16t** reported in the paper:

Compound	Selectivity ratio
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	hCA IX/hCA II	hCA IX/hCA IV
16a	3.35	16.90
16b	1.08	1.42
16c	4.68	14.12
16d	1.44	1.51
16e	5.78	10.14
16f	2.49	3.21
16g	2.23	9.12
16h	1.09	NA
16i	2.62	8.86
16j	1.24	1.45
16k	0.65	4.26
16l	0.75	NA
16m	1.84	12.83
16n	0.91	2.44
16o	3.45	2.53
16p	4.37	11.08
16q	7.06	5.29
16r	1.86	11.00
16s	3.13	1.23
16t	1.58	2.70
Celecoxib	1.05	14.5
AAZ	0.48	2.96

3. Conclusions

Two novel series of sulfonamides, **16a-16j** and **16k-16t**, containing the pyridotriazolopyrimidine scaffold have been synthesized and evaluated *in vitro* as inhibitors against four relevant hCAs, comprising the cytosolic and ubiquitous isozymes hCA I and II as well as the transmembrane hCA IV and hCA IX, the last one a validated antitumor drug target. Most of the reported sulfonamides acted as good, low micromolar inhibitors of hCA I, II and IV, whereas they displayed a better efficacy in inhibiting the tumor-associated isoform hCA IX. Many derivatives herein reported highlighted better selectivity ratios for inhibiting hCA IX over hCA II than celecoxib and **AAZ**. Conversely, some sulfonamides among the two series demonstrated a comparable selectivity for hCA IX over hCA IV, similar to celecoxib, whereas being 3 to 6-fold better than **AAZ**. Considering that isoform hCA IX is a validated target for the diagnosis and treatment of cancers, discovery of selective inhibitors represents a promising step to unveil a more effective cancer therapy, and moreover the obtained SAR might further help in the design of novel, isoform-selective inhibitors.

4. Experimental

4.1 Chemistry:

4.1.1. General:

Melting points were measured with a Stuart apparatus and were uncorrected. The NMR spectra were recorded by Varian Gemini-300BB 300, 400 and 500 MHz FT-NMR spectrometers (Varian Inc., Palo Alto, CA). ^1H spectra were run at 300, 400 and 500 MHz and ^{13}C spectra were run at 75, 100 and 125 MHz, respectively, in deuterated dimethylsulphoxide ($\text{DMSO}-d_6$). Chemical shifts (δ_{H}) are reported relative to TMS as internal standard. All coupling constant (J) values are given in hertz. The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Microanalyses were carried out using Perkin Elmer PE 2400 CHN Elemental Analyzer and the results were within $\pm 0.4\%$. Analytical thin layer chromatography (TLC) on silica gel plates containing UV indicator was employed routinely to follow the course of reactions and to check the purity of products. All reagents and solvents were purified and dried by standard techniques. 6-Amino-2-thiouracil (**3**),²³ hydrazoneyl chlorides **11a**, **11b**,¹⁸ pyrido[2,3-*d*]pyrimidines **5d**, **5e**, **5g** were prepared according to the literature procedure.¹²

4.1.2. General procedure for preparation of 5-aryl-7-(thiophen-2-yl / 4-chlorophenyl)-2-thioxo-2,3-dihydropyrido[2,3-*d*]pyrimidin-4(1*H*)-one (**5a-j**) and spectral data is depicted in the supplementary file.

4.1.3. General procedure for preparation of target compounds (**16a-t**). To a mixture of 5-aryl-7-(thiophen-2-yl / 4-chlorophenyl)-2-thioxo-2,3-dihydropyrido[2,3-*d*]pyrimidin-4(1*H*)-one (**5a-j**) (1 mmol) and the appropriate hydrazoneyl chloride **11a**, **b** (1 mmol) in dioxane (30 mL), triethylamine (0.14 mL, 1 mmol) was added. The reaction mixture was refluxed until the starting materials were fully consumed or until conversion was observed to stall (monitored by TLC). The solvent was removed under vacuum and the residue was triturated with methanol. The formed solid was washed thoroughly with water, filtered then crystallized from DMF:EtOH [v:v, 1:1] or subjected to flash chromatography (methanol/dichloromethane, 1:10) to give **16a-t**. Compounds **16k** and **16l** were previously synthesized in our laboratory.¹⁸

4.1.3.1. 4-(3-Acetyl-8-(4-chlorophenyl)-5-oxo-6-phenylpyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5*H*)-yl)benzenesulfonamide (**16a**): Yield: 46%, m.p. $>300\text{ }^{\circ}\text{C}$; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ ppm: 2.66 (s, 3H, $-\text{CH}_3$), 7.46-7.48 (m, 5H, Ar-H), 7.54 (br. s, 2H, $-\text{SO}_2\text{NH}_2$, D_2O exchangeable), 7.60 (d, $J = 8.5\text{ Hz}$ 2H, Ar-H), 7.78 (s, 1H, pyridine H), 8.10 (d,

$J = 9.0$ Hz 2H, Ar-H), 8.35 (d, $J = 8.5$ Hz 2H, Ar-H), 8.48 (d, $J = 9.0$ Hz 2H, Ar-H); ^{13}C NMR (125 MHz, DMSO- d_6) δ ppm: 30.11, 108.04, 119.31, 120.69, 127.71, 128.19, 128.61, 128.93, 129.43, 130.14, 136.20, 136.44, 139.26, 139.58, 142.23, 142.70, 147.67, 155.12, 155.52, 160.27, 160.31, 188.03; Anal. Calcd for $\text{C}_{28}\text{H}_{19}\text{ClN}_6\text{O}_4\text{S}$: C, 58.90; H, 3.35; N, 14.72. Found: C, 59.20; H, 3.56; N, 14.52.

4.1.3.2. Ethyl 8-(4-chlorophenyl)-5-oxo-6-phenyl-1-(4-sulfamoylphenyl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16b**): Yield: 57%, m.p. >300 °C; ^1H NMR (500 MHz, DMSO- d_6) δ ppm: 1.29 (t, $J = 7$ Hz, 3H, $-\text{COOCH}_2\text{CH}_3$), 4.43 (q, $J = 7$ and 7, 2 Hz, $-\text{COOCH}_2\text{CH}_3$), 7.45-7.47 (m, 5H, Ar-H), 7.53 (br. s, 2H, $-\text{SO}_2\text{NH}_2$, D_2O exchangeable), 7.59 (d, $J = 8.5$ Hz, 2H, Ar-H), 7.77 (s, 1H, pyridine H), 8.09 (d, $J = 8.5$ Hz, 2H, Ar-H), 8.34 (d, $J = 8.5$, 2H, Ar-H), 8.44 (d, $J = 8.5$, 2H, Ar-H); ^{13}C NMR (125 MHz, DMSO- d_6) δ ppm: 13.72, 63.63, 107.39, 118.77, 120.26, 127.21, 127.72, 128.18, 128.47, 128.94, 129.65, 135.73, 135.87, 135.98, 138.76, 139.03, 142.24, 146.63, 154.57, 154.76, 156.12, 159.79, 159.90; Anal. Calcd for $\text{C}_{29}\text{H}_{21}\text{ClN}_6\text{O}_5\text{S}$: C, 57.95; H, 3.52; N, 13.98. Found: C, 57.76; H, 3.87; N, 13.82.

4.1.3.3. 4-(3-Acetyl-8-(4-chlorophenyl)-6-(4-fluorophenyl)-5-oxopyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5H)-yl)benzenesulfonamide (**16c**): Yield: 51%, m.p. >300 °C; ^1H NMR (500 MHz, DMSO- d_6) δ ppm: 2.67 (s, 3H, $-\text{CH}_3$), 7.28 (t, $J = 8.5$, 2H, Ar-H), 7.52-7.55 (m, 4H, 2Ar-H + $-\text{SO}_2\text{NH}_2$, D_2O exchangeable), 7.59 (d, $J = 8.5$, 2H, Ar-H), 7.79 (s, 1H, pyridine H), 8.09 (d, $J = 8.5$, 2H, Ar-H), 8.34 (d, $J = 8.5$, 2H, Ar-H), 8.47 (d, $J = 8.5$, 2H, Ar-H); ^{13}C NMR (125 MHz, DMSO- d_6) δ ppm: 29.63, 107.56, 114.51 ($^2J_{\text{F-C}} = 21.38$ Hz), 118.88, 120.20, 127.21, 128.93, 129.64, 130.66 ($^3J_{\text{F-C}} = 8.38$ Hz), 135.26, 135.29, 135.76, 135.88, 138.74, 141.73, 142.24, 146.81, 153.50 ($^1J_{\text{F-C}} = 198.00$ Hz), 159.79, 161.24, 163.19, 187.51; Anal. Calcd for $\text{C}_{28}\text{H}_{18}\text{ClFN}_6\text{O}_4\text{S}$: C, 57.10; H, 3.08; N, 14.27. Found: C, 57.46; H, 3.36; N, 14.56.

4.1.3.4. Ethyl 8-(4-chlorophenyl)-6-(4-fluorophenyl)-5-oxo-1-(4-sulfamoylphenyl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16d**): Yield: 62%, m.p. >300 °C; ^1H NMR (500 MHz, DMSO- d_6) δ ppm: 1.30 (t, $J = 7$ Hz, $-\text{COOCH}_2\text{CH}_3$), 4.43 (q, $J = 7$ and 7.5 Hz, 2H, $-\text{COOCH}_2\text{CH}_3$), 7.29 (t, $J = 9$ Hz, 2H, Ar-H), 7.52-7.55 (m, 4H, 2Ar-H + $-\text{SO}_2\text{NH}_2$, D_2O exchangeable), 7.61 (d, $J = 8.5$, 2H, Ar-H), 7.81 (s, 1H, pyridine H), 8.09 (d, $J = 8.5$, 2H, Ar-H), 8.35 (d, $J = 9$, 2H, Ar-H), 8.44 (d, $J = 9$, 2H, Ar-H); ^{13}C NMR (125 MHz,

DMSO-*d*₆) δ ppm: 13.74, 63.65, 107.46, 114.53 ($^2J_{\text{F-C}} = 21.88$ Hz), 118.86, 120.28, 127.22, 128.97, 129.68, 130.69, 130.76 ($^3J_{\text{F-C}} = 8.63$ Hz), 135.28, 135.77, 135.87, 135.96, 138.74, 142.27, 146.62, 153.46, 154.85 ($^1J_{\text{F-C}} = 159.18$ Hz), 159.86, 159.90, 161.27, 163.21; Anal. Calcd for C₂₉H₂₀ClFN₆O₅S: C, 56.27; H, 3.26; N, 13.58. Found: C, 56.56; H, 3.59; N, 13.62.

4.4.3.5. 4-(3-Acetyl-6,8-bis(4-chlorophenyl)-5-oxopyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5*H*)-yl)benzenesulfonamide (**16e**): Yield: 40%, m.p. >300 °C; ^1H NMR (500 MHz, DMSO-*d*₆) δ ppm: 2.66 (s, 3H, -CH₃), 7.50-7.55 (m, 6H, 4Ar-H + -SO₂NH₂, D₂O exchangeable), 7.62 (d, $J = 8.5$ Hz 2H, Ar-H), 7.82 (s, 1H, pyridine H), 8.10 (d, $J = 9.0$ Hz 2H, Ar-H), 8.36 (d, $J = 8.5$ Hz 2H, Ar-H), 8.48 (d, $J = 9.0$ Hz 2H, Ar-H); ^{13}C NMR (125 MHz, DMSO-*d*₆) δ ppm: 30.36, 108.18, 118.00, 120.96, 127.91, 128.42, 129.67, 130.37, 131.05, 133.76, 136.49, 136.59, 138.57, 139.43, 142.42, 142.95, 147.57, 153.93, 155.80, 160.48, 160.64, 188.26; Anal. Calcd for C₂₈H₁₈Cl₂N₆O₄S: C, 55.55; H, 3.00; N, 13.88. Found: C, 55.79; H, 3.16; N, 13.59.

4.4.3.6. Ethyl 6,8-bis(4-chlorophenyl)-5-oxo-1-(4-sulfamoylphenyl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16f**): Yield: 52%, m.p. >300 °C; ^1H NMR (500 MHz, DMSO-*d*₆) δ ppm: 1.30 (t, $J = 7$, 3H, -COOCH₂CH₃), 4.45 (q, $J = 7$ and 7.5, 2H, -COOCH₂CH₃), 7.49-7.54 (m, 5H, Ar-H), 7.61 (d, $J = 8$ Hz, 2H, Ar-H), 7.80 (s, 1H, pyridine H), 8.09 (d, $J = 8.0$ Hz, 2H, Ar-H), 8.35 (d, $J = 8.5$ Hz, 2H, Ar-H), 8.44 (d, $J = 8.5$ Hz, 2H, Ar-H); ^{13}C NMR (125 MHz, DMSO-*d*₆) δ ppm: 14.43, 64.35, 108.00, 119.40, 120.98, 127.91, 128.42, 129.66, 130.36, 131.07, 133.78, 136.51, 136.54, 136.59, 138.53, 139.40, 142.98, 147.32, 153.85, 155.51, 156.78, 160.55, 160.64; Calcd for C₂₉H₂₀Cl₂N₆O₅S: C, 54.81; H, 3.17; N, 13.23. Found: C, 55.09; H, 3.30; N, 13.52.

4.4.3.7. 4-(3-Acetyl-8-(4-chlorophenyl)-6-(4-methoxyphenyl)-5-oxopyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5*H*)-yl)benzenesulfonamide (**16g**): Yield: 35%, m.p. >300 °C; ^1H NMR (300 MHz, DMSO-*d*₆) δ ppm: 2.68 (s, 3H, -COCH₃), 3.85 (s, 3H, -OCH₃), 7.02 (d, $J = 8.7$ Hz, 2H, Ar-H), 7.45-7.48 (m, 4H, 2Ar-H + -SO₂NH₂, D₂O exchangeable), 7.60 (d, $J = 8.7$ Hz, 2H, Ar-H), 7.76 (s, 1H, pyridine H), 8.09 (d, $J = 9.0$ Hz, 2H, Ar-H), 8.34 (d, $J = 7.8$ Hz, 2H, Ar-H), 8.48 (d, $J = 8.4$ Hz, 2H, Ar-H); Anal. Calcd for C₂₉H₂₁ClN₆O₅S: C, 57.95; H, 3.52; N, 13.98. Found: C, 58.00; H, 3.76; N, 14.24.

4.4.3.8. Ethyl 8-(4-chlorophenyl)-6-(4-methoxyphenyl)-5-oxo-1-(4-sulfamoylphenyl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16h**): Yield: 47%, m.p. >300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 1.31 (t, *J* = 7.2 Hz, 3H, -COOCH₂CH₃), 3.85 (s, 3H, -OCH₃), 4.43 (q, *J* = 6.9 and 7.2 Hz, 2H, -COOCH₂CH₃), 7.01 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.31-7.50 (m, 4H, 2Ar-H + -SO₂NH₂, D₂O exchangeable), 7.60 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.75 (s, 1H, pyridine H), 8.08 (d, *J* = 8.4 Hz, 2H, Ar-H), 8.33 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.44 (d, *J* = 8.7 Hz, 2H, Ar-H); Anal. Calcd for C₃₀H₂₃ClN₆O₆S: C, 57.10; H, 3.67; N, 13.32; Found: C, 57.32; H, 3.86; N, 13.59.

4.4.3.9. 4-(3-Acetyl-8-(4-chlorophenyl)-6-(4-nitrophenyl)-5-oxopyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5H)-yl)benzenesulfonamide (**16i**): Yield: 52%, m.p. >300 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm: 2.67 (s, 3H, -COCH₃), 7.52 (s, 2H, -SO₂NH₂, D₂O exchangeable), 7.61 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.72 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.87 (s, 1H, pyridine H), 8.11 (d, *J* = 8.5 Hz, 2H, Ar-H), 8.32 (d, *J* = 8.5 Hz, 2H, Ar-H), 8.36 (d, *J* = 8.5 Hz, 2H, Ar-H), 8.49 (d, *J* = 8.5 Hz, 2H, Ar-H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ ppm: 30.08, 107.70, 118.89, 120.79, 123.29, 127.71, 129.46, 130.15, 130.34, 136.17, 136.44, 139.14, 142.13, 142.84, 146.42, 147.40, 147.68, 152.61, 155.51, 160.08, 160.63, 187.79; Anal. Calcd for C₂₈H₁₈ClN₇O₆S: C, 54.60; H, 2.95; N, 15.92; Found: C, 54.86; H, 3.18; N, 15.83.

4.4.3.10. Ethyl 8-(4-chlorophenyl)-6-(4-nitrophenyl)-5-oxo-1-(4-sulfamoylphenyl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16j**): Yield: 63%, m.p. >300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 1.24 (t, *J* = 6.6 Hz, 3H, -COOCH₂CH₃), 4.35 (q, *J* = 6.9 and 7.6 Hz, 2H, -COOCH₂CH₃), 7.54 (s, 2H, -SO₂NH₂, D₂O exchangeable), 7.62 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.74 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.89 (s, 1H, pyridine H), 8.09 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.32 (d, *J* = 7.5 Hz, 2H, Ar-H), 8.37 (d, *J* = 8.1 Hz, 2H, Ar-H), 8.44 (d, *J* = 8.7 Hz, 2H, Ar-H); Anal. Calcd for C₂₉H₂₀ClN₇O₇S: C, 53.92; H, 3.12; N, 15.18. Found: C, 54.27; H, 3.36; N, 15.40.

4.4.3.11. 4-(3-Acetyl-6-(4-fluorophenyl)-5-oxo-8-(thiophen-2-yl)pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5H)-yl)benzenesulfonamide (**16m**): Yield: 53%, m.p. >300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 2.66 (s, 3H, COCH₃), 7.23-7.33 (m, 3H, 2Ar-H + H⁴ thiophene), 7.49 (br. s, 2H, D₂O exchangeable SO₂NH₂), 7.51 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.76 (s, 1H, pyridine H), 7.86 (m, 1H, H⁵ thiophene), 8.11 (d, *J* = 8.9 Hz, 2H, Ar-H), 8.15-

8.2 (m, 1H, H³ thiophene), 8.43 (d, J = 8.9 Hz, 2H, Ar-H); Anal. Calcd for C₂₆H₁₇FN₆O₄S₂: C, 55.71; H, 3.06; N, 14.99. Found: C, 55.86; H, 3.34; N, 15.28.

4.4.3.12. Ethyl 6-(4-fluorophenyl)-5-oxo-1-(4-sulfamoylphenyl)-8-(thiophen-2-yl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16n**): Yield: 48%, m.p. >300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 1.29 (t, J = 7.2 Hz, 3H, -COOCH₂CH₃), 4.41 (q, J = 7.2 and 7.5 Hz, 2H, -COOCH₂CH₃), 7.22-7.33 (m, 3H, 2Ar-H + H⁴ thiophene), 7.45-7.53 (m, 4H, 2Ar-H + 2H of SO₂NH₂, D₂O exchangeable), 7.77 (s, 1H, pyridine H), 7.82-7.88 (m, 1H, H⁵ thiophene), 8.10 (d, J = 8.4 Hz, 2H, Ar-H), 8.15-8.2 (m, 1H, H³ thiophene), 8.40 (d, J = 8.4 Hz, 2H, Ar-H); Anal. Calcd for C₂₇H₁₉FN₆O₅S₂: C, 54.91; H, 3.24; N, 14.23. Found: C, 55.26; H, 3.53; N, 14.56.

4.4.3.13. 4-(3-Acetyl-6-(4-chlorophenyl)-5-oxo-8-(thiophen-2-yl)pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5H)-yl)benzenesulfonamide (**16o**): Yield: 73%, m.p. >300 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm: 2.67 (s, 3H, COCH₃), 7.24 (m, 1H, H⁴ thiophene), 7.48 (d, J = 8.4 Hz, 2H, Ar-H), 7.54-7.56 (m, 4H, 2Ar-H + 2H of SO₂NH₂, D₂O exchangeable), 7.79 (s, 1H, pyridine H), 7.88 (d, J = 4.5 Hz, 1H, H⁵ thiophene), 8.12 (d, J = 8.8 Hz, 2H, Ar-H), 8.17 (d, J = 3.3 Hz, 1H, H³ thiophene), 8.45 (d, J = 8.8 Hz, 2H, Ar-H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ ppm: 30.18, 107.24, 117.80, 120.95, 127.73, 128.22, 129.47, 129.94, 130.71, 132.49, 133.47, 138.39, 139.16, 142.23, 142.83, 143.63, 147.37, 153.29, 155.32, 157.27, 160.39, 188.08; Anal. Calcd for C₂₆H₁₇ClN₆O₄S₂: C, 54.12; H, 2.97; N, 14.56. Found: C, 54.36; H, 3.26; N, 14.79.

4.4.3.14. Ethyl 6-(4-chlorophenyl)-5-oxo-1-(4-sulfamoylphenyl)-8-(thiophen-2-yl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16p**): Yield: 63%, m.p. 286-289 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 1.29 (t, J = 7.2 Hz, 3H, -COOCH₂CH₃), 4.42 (q, J = 7.2 and 6.9 Hz, 2H, -COOCH₂CH₃), 7.24 (m, 1H, H⁴ thiophene), 7.46-7.51 (m, 4H, 2Ar-H + 2H of SO₂NH₂, D₂O exchangeable), 7.53 (d, J = 8.4 Hz, 2H, Ar-H), 7.77 (s, 1H, pyridine H), 7.87 (d, J = 4.8 Hz, 1H, H⁵ thiophene), 8.10 (d, J = 8.7 Hz, 2H, Ar-H), 8.16 (m, 1H, H³ thiophene), 8.40 (d, J = 8.7 Hz, 2H, Ar-H); Calcd for C₂₇H₁₉ClN₆O₅S₂: C, 53.42; H, 3.15; N, 13.84. Found: C, 53.66; H, 3.47; N, 14.06.

4.4.3.15. 4-(3-Acetyl-6-(4-methoxyphenyl)-5-oxo-8-(thiophen-2-yl)pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5H)-yl)benzenesulfonamide (**16q**): Yield: 64%, m.p.

>300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 2.63 (s, 3H, COCH₃), 3.80 (s, 3H, -OCH₃), 6.97 (d, *J* = 9 Hz, 2H, Ar-H), 7.20 (m, 1H, H⁴ thiophene), 7.37-7.45 (m, 4H, 2Ar-H + 2H of SO₂NH₂, D₂O exchangeable), 7.56 (s, 1H, pyridine H), 7.74-8.06 (m, 3H, 2Ar-H + H⁵ thiophene), 8.27 (m, 1H, H³ thiophene), 8.39 (d, *J* = 8.1 Hz, 2H, Ar-H); Calcd for C₂₇H₂₀N₆O₅S₂: C, 56.63; H, 3.52; N, 14.68. Found: C, 56.93; H, 3.86; N, 14.37.

4.4.3.16. Ethyl 6-(4-methoxyphenyl)-5-oxo-1-(4-sulfamoylphenyl)-8-(thiophen-2-yl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16r**): Yield: 60%, m.p. >300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 1.31 (t, *J* = 7.2 Hz, 3H, -COOCH₂CH₃), 3.85 (s, 3H, -OCH₃), 4.42 (q, *J* = 7.2 and 6.9 Hz, 2H, -COOCH₂CH₃), 7.01 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.23 (m, 1H, H⁴ thiophene), 7.41 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.48 (br. s, 2H, SO₂NH₂, D₂O exchangeable), 7.72 (s, 1H, pyridine H), 7.85 (d, *J* = 4.8 Hz, 1H, H⁵ thiophene), 8.09 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.13-8.20 (m, 1H, H³ thiophene), 8.40 (d, *J* = 8.7 Hz, 2H, Ar-H); ¹³C NMR (75 MHz, DMSO) δ 14.22, 55.70, 64.08, 107.21, 113.64, 117.84, 118.75, 120.92, 127.50, 129.65, 130.46, 130.77, 131.55, 132.22, 136.37, 139.19, 142.73, 143.80, 147.07, 154.48, 155.09, 156.68, 159.92, 160.64; Calcd for C₂₈H₂₂N₆O₆S₂: C, 55.81; H, 3.68; N, 13.95. Found: C, 55.54; H, 3.37; N, 14.09.

4.4.3.17. 4-(3-Acetyl-6-(4-nitrophenyl)-5-oxo-8-(thiophen-2-yl)pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-1(5H)-yl)benzenesulfonamide (**16s**): Yield: 69%, m.p. >300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 2.67 (s, 3H, COCH₃), 7.25 (m, 1H, H⁴ thiophene), 7.52-7.70 (m, 4H, 2Ar-H + 2H of SO₂NH₂, D₂O exchangeable), 7.86 (s, 1H, pyridine H), 8.00-8.33 (m, 5H, 4Ar-H + H⁵ thiophene), 8.45-8.55 (m, 3H, 2Ar-H + H³ thiophene); Calcd for C₂₆H₁₇N₇O₆S₂: C, 53.15; H, 2.92; N, 16.69. Found: C, 53.46; H, 3.24 N, 17.02.

4.4.3.18. Ethyl 6-(4-nitrophenyl)-5-oxo-1-(4-sulfamoylphenyl)-8-(thiophen-2-yl)-1,5-dihydropyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (**16t**): Yield: 72%, m.p. >300 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm: 1.28 (t, *J* = 7.2 Hz, 3H, -COOCH₂CH₃), 4.40 (q, *J* = 7.2 and 6.9 Hz, 2H, -COOCH₂CH₃), 7.24 (t, *J* = 4.2 Hz, 1H, H⁴ thiophene), 7.50 (br. s, 2H, SO₂NH₂, D₂O exchangeable), 7.72 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.83 (s, 1H, pyridine H), 7.88 (d, *J* = 4.8 Hz, 1H, H⁵ thiophene), 8.10 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.16-8.20 (m, 1H, H³ thiophene), 8.32 (d, *J* = 8.4 Hz, 2H, Ar-H), 8.40 (d, *J* = 8.4 Hz, 2H, Ar-H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ ppm: 13.74, 63.60, 106.34, 117.00, 120.60, 122.82, 127.20, 128.89, 129.65, 130.00, 132.31, 135.78, 138.54, 142.42, 143.02, 146.00, 146.72, 147.17, 151.64, 154.54,

156.00, 157.00, 159.85; Calcd for $C_{27}H_{19}N_7O_7S_2$: C, 52.51; H, 3.10; N, 15.88. Found: C, 52.63; H, 3.31; N, 15.64.

4.1. CA activity and inhibition measurements:

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.²² Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of 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 (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier,²⁴ and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.²⁵

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