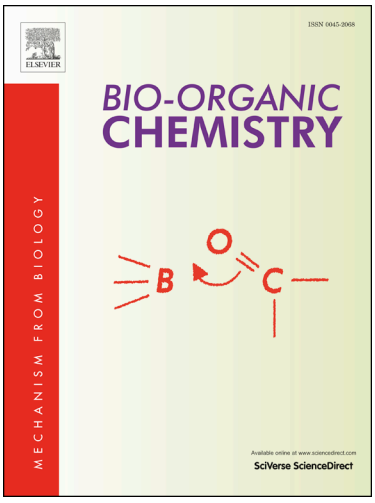

Novel sulfonamides incorporating 1,3,5-triazine and amino acid structural motifs as inhibitors of the physiological carbonic anhydrase isozymes I, II and IV and tumor-associated isozyme IX

Peter Mikuš, Dominika Krajčiová, Mária Mikulová, Branislav Horváth, Daniel Pecher, Vladimír Garaj, Silvia Bua, Andrea Angeli, Claudiu T. Supuran

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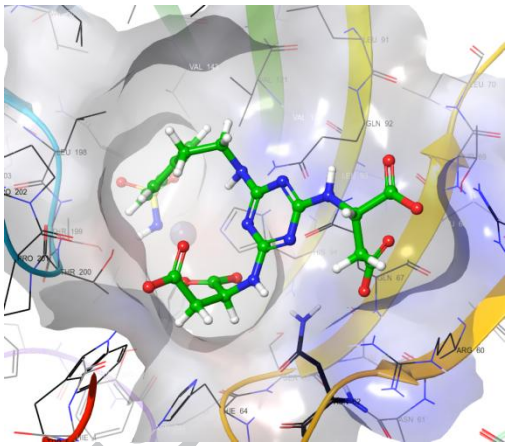

Glide docking model of 2,2'-((6-((4-sulfamoylphenethyl)amino)-1,3,5-triazine-2,4-diyl)bis(imino))disuccinic acid associate with carbonic anhydrase IX

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ABSTRACT

A new series of thirty s-triazinyl-substituted aminoalkylbenzenesulfonamides, incorporating a symmetric pair of amino acid moieties, is reported, together with inhibition studies of physiologically relevant human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms. Specifically, against the cytosolic hCA I, II, transmembrane hCA IV and the tumor-associated, membrane-bound hCA IX.

The compounds were prepared by nucleophilic substitution of chlorine atoms from cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) using environmentally friendly water-based synthetic conditions. The products yields ranged in the interval of 43-97%. Purity of the products was verified by the HPLC-DAD-ESI-Q-TOF MS method. Identity of the products was confirmed by the same method plus NMR and IR.

The products showed weak inhibition of the cytosolic, off-target isozyme hCA II, but some of them were low nanomolar (i.e. strong) inhibitors of the tumor-associated hCA IX. The series offered

representatives selective towards isozymes hCA I, IV and IX. 2,2'-((6-((4-sulfamoylphenethyl)amino)-1,3,5-triazine-2,4-diyl)bis(imino))disuccinic acid demonstrated highest selectivity to the tumor-associated isoform hCA IX over off-target isozymes, with impressive K_I ratio (hCA II / hCA IX) 213.9 and inhibition constant equal to acetazolamide (K_I = 25.8 nM). Although the selectivities of some other products, e.g. those conjugating Leu and Glu, were a bit lower (188.7 and 84.3, respectively) their inhibition constants were similar to acetazolamide too (24.0 and 27.1, respectively).

The selected most impressive results from the inhibition study were interpreted via molecular modeling experiment (docking in Glide) revealing different inter-molecular enzyme-substrate interaction of 2,2'-((6-((4-sulfamoylphenethyl)amino)-1,3,5-triazine-2,4-diyl)bis(imino))disuccinic acid within specific hCA IX and hCA II microregions. Therefore, several selected compounds from this study can be considered as highly effective and selective inhibitors of hCA IX, worthy to further (preclinical) investigation.

Keywords:
1,3,5-Triazine
Benzene sulfonamides
Amino acids
Carbonic anhydrase
Enzyme inhibition
Isoform selectivity

1. Introduction

Carbonic anhydrases (CAs) are a family of zinc containing metalloenzymes catalyzing the reversible hydration of carbon dioxide to bicarbonate ion and hydrogen proton. Sixteen CA isoforms have been well defined in humans, from which transmembrane hCA IX have shown increased expression in hypoxia-induced tumor cells [1, 2]. The upregulation of hypoxia inducible factor 1 (HIF-1) directly correlates with hCA IX overexpression and activation in many tumor cells [3], including head and neck squamous cell carcinoma [4], non-small lung [5] and breast cancer [6,7], astrocytic [8] and gastrointestinal tumors [9], colorectal [10], renal [11] and cervical carcinoma [12,13]. Human CA IX plays a major role in tumor cell proliferation, pH regulation, migration and adhesion, thus the suppression of hCA IX activity results in a reduction of these processes and metastatic cascade [14]. Since hCA IX is not demonstrated in majority of normal physiological tissues, but is associated with a wide variety of tumors as mentioned above, hCA IX and the inhibition of its activity have become a promising and broadly studied target for therapy or immunotherapy with monoclonal antibodies and for tumor hypoxia imaging.

In comparison with hCA IX, the hCA II isozyme is not regulated by HIF-1. Human CA II is physiologically dominant and highly active cytosolic isoform [15]. It is present under physiological conditions in almost every human tissue and organ [16]. Other physiologically relevant human carbonic anhydrase isoforms include e.g. cytosolic hCA I, that is found at the highest level in erythrocytes and is also expressed in normal colorectal mucosa [17, 18], or membrane-bound hCA IV, that is present in the eye [19]. In order to eliminate the hypoxia-induced tumor cells with a minimum impact on the healthy cells, a big effort is put on the selectivity enhancement of newly developed potential hCA inhibitors.

In recent years, many sulfonamides, phenols, inorganic anions, and coumarin derivatives have been shaped as main classes of hCA inhibitors, including hCA IX and XII [20-24]. Predominantly benzene sulfonamides, in which the incorporation of 1,3,5-triazine moiety with 4-aminobenzene sulfonamide has offered improved specificity and efficacy targeting hCA IX and hCA XII, have become more frequently explored by working group of Supuran. The conjugates created by an implementation of various moieties into the 1,3,5-triazinyl benzene sulfonamide intermediates have been investigated in several consecutive studies [25-30]. In 2005 [26] hydrazino, ethylamino, isopropylamino, N,N-diethylamino- or N-methyl-N-propylamino- moieties were incorporated into the 1,3,5-triazinyl benzene sulfonamide intermediates inhibiting hCA I with K_Is 31-8500 nM, hCA II with K_Is 14-765 nM and hCA IX with K_Is 1-640 nM. In

2014 [27] ammonia, anilines, morpholine, tert-butylamine, N-methyl-2-aminoethanol and 1, 3-diaminopropane were tested as nucleophiles for the substitution of the triazinyl moiety. Therein, hCA II was moderately inhibited by most of the new compounds (K_Is in the range of 12.5–130 nM), and hCA IX and hCA XII were potently inhibited with K_Is in the range of 1.2–34.1 and of 2.1–33.9 nM, respectively.

In 2011 Supuran et al. [26] employed in their synthetic procedures among various nucleophiles (DOPA, 2-aminoethanol and 4-amino-1-butanol, mono-tert-butyl-dimethylsilyl derivative of ethyleneglycol) also amino acids such as Gly, β-Ala, L-Ala, Ser as well as methyl esters of Gly and L-Ala. This series contained a majority of monosubstituted derivatives and some disubstituted derivatives, showing a moderate-weak inhibition of the cytosolic hCA I and II but a low nanomolar (i.e. strong) inhibition activity of hCA IX, XII and XIV. The K_I values for these isozymes were 4550, 376, 8.4, 6.0, 7.2 nM, respectively, for the 1,3,5-triazinyl aminobenzene sulfonamide derivatives disubstituted with Gly; 673, 368, 8.9, 0.85, 0.92 nM respectively, with β-Ala; and 502, 435, 9.4, 8.7, 7.0 nM, respectively, with methylester of Gly. The K_Is were 1659, 435, 8.5, 9.2, 8.9 nM, respectively, for the 1,3,5-triazinyl aminomethylbenzene sulfonamide derivatives disubstituted with L-Ala; and 607, 453, 9.2, 9.3, 9.1 nM, respectively, with methylester of Gly. They were 2040, 409, 8.4, 8.6, 8.8 nM, respectively, for the 1,3,5-triazinyl aminoethylbenzene sulfonamide derivatives disubstituted with L-Ala; and 3704, 517, 9.0, 7.1, 7.6 nM, respectively, with Ser. In 2013 [29] the fluorine-substituted 1,3,5-triazine moiety was studied in the reaction with amino acid such as glycine and its methyl ester providing mono-fluoro-substituted products. Their inhibition studies revealed rather weak inhibition of hCA III, IV, V and XIII with K_I in the range of 602-9015 nM, a moderate inhibition of hCA I, VI, and IX with K_I in the range of 81.5-250 nM, and strong inhibition of the physiologically relevant hCA II, VII and XII with K_I 4.5-8.3 nM. It is apparent from the above mentioned results that some conjugates created by an implementation of amino acids into the 1,3,5-triazinyl aminoalkyl benzene sulfonamide intermediates could be potent inhibitors of the hCA isoforms related to hypoxia-induced tumor cells. However, no systematic study on the amino acids has been carried out in this context so far. Therefore, we decided to evaluate such amino acids conjugates in detail and systematically, covering essential as well as non-essential amino acids and their derivatives. Incorporation of amino acid moiety enhances water-solubility and was used in design of hundreds of CAIs [31]. General structures of triazinyl derivatives incorporating amino acid moieties, that have been published so far, are summarized in Fig. 1.

The aim of this work was to synthesize and characterize novel 1,3,5-triazine (amino-/aminomethyl-/aminoethyl-)benzene sulfonamide conjugates disubstituted with 10 essential amino acids including (i) less polar neutral amino acids as well as (ii) acidic amino acids. All amino acids used contained one amino group as the only nucleophile in the molecule enabling selective chlorine substitution. The optimized synthetic procedure in a water environment reported in our very recent paper [32] for 1,3,5-triazine benzene sulfonamide conjugates disubstituted with Gly and β-Ala was adapted/optimized in this work for the preparation of new amino acid conjugates. The inhibition data of the synthesized products with approved chemical structures, i.e. the minimum inhibition concentrations towards hCA I, hCA II, hCA IV, hCA IX and the selectivity expressed as a ratio of hCA IX : hCA II (or other relevant ratios), were evaluated and discussed.

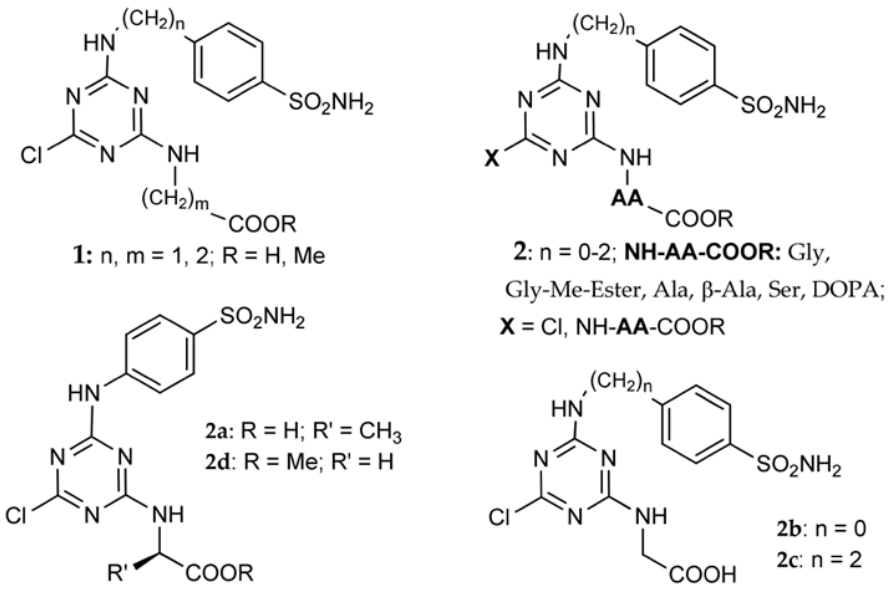
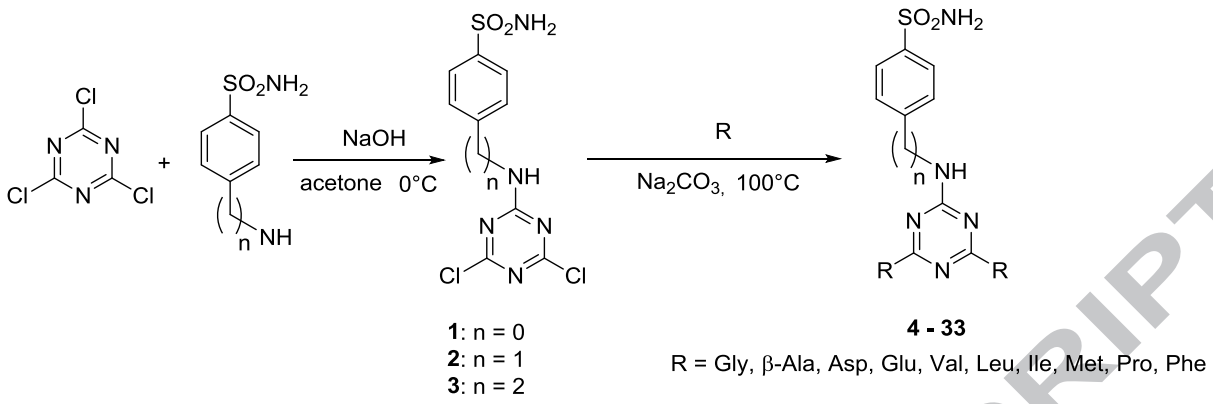


Figure 1. Published general structures of triazinyl derivatives incorporating amino acid moieties [31].

2. Results and Discussion

2.1 Chemistry

Final compounds (i.e. amino acids conjugates) were prepared in two steps by nucleophile substitution of chlorine atoms in cyanuric chloride (2,4,6-trichloro-1,3,5-triazine). For the preparation of the key monosubstituted *s*-triazine intermediates (**1-3**) the previously reported protocol was used [25, 33] Trisubstituted s-triazines (**4-33**) were prepared by the reaction of a corresponding intermediate (**1-3**) with amino acid (present in its L-enantiomeric form, if applicable) and anhydrous sodium carbonate according to Scheme 1. This water-based environmentally friendly synthetic procedure, accompanied with a simple purification, provided very high yields for majority of the products.



Scheme 1. General scheme for the synthesis of target *s*-triazine derivates.

A series of the synthesized *s*-triazinyl-amino-/aminomethyl-/aminoethyl-benzenesulfonamide conjugates substituted with amino acids, including (i) the group of less polar neutral amino acids as well as (ii) acidic amino acids, was proved for the purity and chemical structure by the HPLC-DAD/MS, NMR, and IR methods (for the analytical methods see section 4.1). The products were prepared as free acids with exception of Pro that was present as sodium salt. The reaction yields, ranging in the interval of 42.8-97.2%, depended on both (i) the length of the linker between sulfonamide and triazine (i.e. amino-, aminomethyl-, aminoethyl-) and (ii) amino acid structure (for the particular data see section 4.2). Generally, the prolongation of the linker resulted in the decreasing of the yield (average values for the amino-, aminomethyl- and aminoethyl-derivatives were 89.2%, 77.3%, and 71.3%, respectively). Although there was found a correlation between the length of the linker and the yield, the effect of amino acid type seemed to be random. For example, the highest yields (calculated as an average of the all amino-/aminoalkyl-benzenesulfonamide derivatives of given amino acid) were found for Gly (90.8%), Phe (92.2%) and Leu (88.7%) conjugates while the lowest ones for Val (56.9%), Pro (72.3%) and Glu (70.2%) conjugates. The HPLC-UV measurements did not indicate any significant samples impurities. The purity of each conjugate was higher than 90% with the average value of the all samples 94.52%. The identity of the all conjugates was unambiguously confirmed via exact molecular weights and additional structural information documented by the data in section 4.2 and by Figs. S1-S30 in Supplementary material. Such approved conjugates were then suitable for testing on their biological activities.

2.2 CA inhibition

Inhibition data against cytosolic hCA isoforms I and II as well as transmembrane hCA IV and IX with the thirty original benzenesulfonamides with s-triazine moiety disubstituted with amino acids (**4 – 33**), as well as their intermediates (**1 – 3**) and the standard sulfonamide CAI acetazolamide (**AAZ**) are shown in Table 1. They were obtained by a stopped-flow, CO_2 hydrase assay [34]. The following structure–activity relationship (SAR) data were observed, when considering inhibition data in Table 1 and comparing them with previously reported inhibition data of intermediates **1–3** monosubstituted with amino acids [26,27] summarized in Table 2:

(i) Most of the tested compounds **4-33** were ineffective as hCA I inhibitors. Compounds **12, 15** and **22**, possessing Asp, Glu and Ile amino acid substituents, are inactive against hCA I. The other derivatives with these amino acids are weak micromolar inhibitors as well as all Gly derivatives **4-6** and compounds **27** and **33** substituted with Met and Phe respectively and both with 4-aminoethylbenzenesulfonamide (n = 2) on s-triazine core. Substitution with Leu causes weak micromolar inhibition at compounds **19** (n = 0) and **21** (n = 2), while the compound **20** with intermediate spacer (n = 1) is moderately effective inhibitor with K_I = 96.9 nM. Other effective hCA I inhibitors are compound **32** substituted with Phe and

homosulfanilamide (n = 1) with lowest K_I = 67.1 nM, and compound **28** substituted with Pro and sulfanilamide (n = 0) with K_I = 87.0 nM. Compound **28** exhibits also high isozyme selectivity with selectivity ratio hCA II / hCA I = 35.8. Much more selective hCA I inhibitor already exists [35], although it happens rarely to find one for this isozyme. For derivatives substituted with Pro, prolongation of linker between benzenesulfonamide and s-triazine core decreases the activity. Except the three aforementioned active inhibitors of hCA I **20, 28, 32**, disubstitution of Cl atoms with amino acids decreases the inhibition activity. Monosubstitution of Cl on intermediates **2, 3** with amino acid can increase the activity (Tab. 2; compounds X2, X3, X5 and X8) [27].

(ii) The investigated sulfonamides showed to be weak inhibitors of hCA II, the physiologically dominant and highly active cytosolic isoform [15], with K_Is in the range of 235.7–7125 nM. Disubstitution of intermediate **1** with Gly decreases inhibition activity 3.5 times (**4**) and approximately as much if any intermediate (**1-3**) is monosubstituted with amino acid (Tab. 2). A very steep decline of inhibition activity is observed for the rest of compounds **5-33**. Among derivatives substituted with hydrophobic amino acids Val, Leu, Ile or with Pro, the weakest inhibitors of hCA II are those with sulfanilamide substituent (n = 0). On the contrary, among derivatives substituted with aromatic amino acid Phe, the least active inhibitor is compound **33** with long spacer (n = 2). Disubstitution with acidic amino acids Asp and Glu decreases effectiveness towards hCA II to micromolar K_Is except compound **14** with K_I = 0.7 μM.

(iii) The membrane-associated isoform hCA IV was inhibited by the tested sulfonamides **4–33**, with inhibition constants in a wide range. The activity entirely diminished with disubstitution by acidic amino acid Asp. If disubstituted with Glu, only compound **14** with intermediate linker (n = 1) retained weak activity (K_I = 4.1 μM). For hydrophobic amino acids Val and Leu, increasing the length of linker increases also the inhibition activity against hCA IV. For derivatives with all other amino acid substituents including Ile is applicable the same SAR as for Glu derivatives, meaning that compounds with homosulfanilamide (n = 1) have the best inhibition activity. The best hCA IV inhibitor is compound **26** with Met (K_I = 45.6 nM) which is also selective hCA IV inhibitor, as the selectivity ratios hCA I / hCA IV = 11.7, hCA II / hCA IV = 69.5 and hCA IX / hCA IV = 28.0. Compounds **23** and **32** have inhibition activity towards hCA IV comparable with AAZ (with K_Is 76.3 and 61.9 resp.).

(iv) The tumor-associated hCA IX was the most inhibited isoform among the four investigated isozymes, with K_Is ranging between 8.4 and 2592.4 nM (Tab. 1). The most active hCA IX inhibitor among the intermediate products **1-3**, with chlorine atoms at positions 3 and 5 on s-triazine ring, is compound **1** with sulfanilamide substituent (n = 0) [32]. The same applies to derivatives where both chlorine atoms are substituted with Gly, β-Ala, Leu or Ile. On the contrary, disubstitution with Val, Pro and acidic amino acids Asp and Glu demands long linker (n = 2) to retain high activity. In the case of the acidic amino acids Asp and Glu, prolonging in the linker provides significant effect on the inhibition of hCA IX while there is almost no effect on the inhibition of hCA II. This makes the compounds **12** and **15** also very selective towards hCA IX.. Compounds **4** and **7** with Gly and β-Ala substituents and short linker (n = 0) are strong hCA IX inhibitors with K_Is 8.4 and 8.9 nM respectively and selectivity ratio against hCA II >40. Monosubstituted intermediate **1** with Gly seems to have the same properties towards all tested CAs (Tab. 2; compound X1) [27] as disubstituted compound **4** (Tab. 1). While monosubstitution on intermediates **2** and **3** with Gly strongly increases inhibition activity and selectivity towards hCA IX (Tab. 2; compounds X1, X2), subsequent substitution of last chlorine atom with Gly cancels this effect (**5, 6**). Good inhibitors are also compounds **12, 15, 19** and **30** with K_Is in the range 24 – 27 nM, comparable with AAZ. All four compounds are selective towards isozyme hCA IX and the selectivity of compound **12** is the highest having selectivity ratio hCA II / hCA IX = 214 (hCA II / hCA IX selectivity was confirmed also by molecular docking in Glide, see chapter 2.3) and being inactive against isozymes hCA I and IV.

Table 1. Inhibition data for synthesized *s*-triazine derivatives **4**-**33** and the standard sulfonamide inhibitor acetazolamide (AAZ) against hCA I, hCA II, hCA IV and hCA IX; their selectivity ratios for inhibition of isozyme hCA IX over hCA II. (Methodology based on stopped flow assay was employed for the evaluation [34]; see section 4.3 Carbonic anhydrase inhibition assay)

Compound	n	-R	K_I* (nM)				selectivity
			hCA I	hCA II	hCA IV	hCA IX	hCA II / hCA IX
AAZ			250.0	12.0	74.0	25.8	0.5
1	0	Cl	120.0[a]	106.0[a]	NT	0.15[a]	706.7
2	1	Cl	136.0[a]	13.0[a]	NT	124.0[a]	0.1
3	2	Cl	75.0[a]	21.0[a]	NT	138.0[a]	0.2
4	0	Gly	4550.0	376.0	>10000	8.4	44.8
5	1	Gly	9362.4	478.4	867.3	145.8	3.3
6	2	Gly	4023.8	428.1	3415.2	330.4	1.3
7	0	β-Ala	673.0	368.0	9596.0	8.9	41.3
8	1	β-Ala	655.4	661.6	4476.0	1818.5	0.4
9	2	β-Ala	960.7	892.1	>10000	134.2	6.6
10	0	Asp	8259.8	6219.1	>10000	211.9	29.3
11	1	Asp	3700.0	5953.7	>10000	2364.1	2.5
12	2	Asp	>10000	5519.6	>10000	25.8	213.9
13	0	Glu	9260.8	7125.0	>10000	202.4	35.2
14	1	Glu	6214.1	695.2	4125.8	193.2	3.6
15	2	Glu	>10000	2284.3	>10000	27.1	84.3
16	0	Val	398.7	5335.4	>10000	2111.1	2.5
17	1	Val	377.0	839.3	656.8	1371.8	0.6
18	2	Val	932.2	804.1	476.3	130.9	6.1
19	0	Leu	4191.9	4528.8	2380.9	24.0	188.7
20	1	Leu	96.9	396.0	516.8	167.3	2.4
21	2	Leu	4854.5	912.5	367.1	123.8	7.4
22	0	Ile	>10000	2948.9	4021.2	92.6	31.8
23	1	Ile	664.5	628.5	76.3	164.3	3.8
24	2	Ile	7337.2	1556.2	1421.3	189.3	8.2
25	0	Met	346.7	803.7	3294.0	2222.2	0.4
26	1	Met	531.3	3170.6	45.6	1274.9	2.5
27	2	Met	4527.8	5017.9	2336.1	2592.4	1.9
28	0	Pro	87.0	3112.8	9315.0	295.0	10.6
29	1	Pro	256.4	773.1	350.4	265.5	2.9
30	2	Pro	958.4	1070.8	2206.9	25.7	41.7
31	0	Phe	305.4	866.1	9387.0	191.5	4.5
32	1	Phe	67.1	235.7	61.9	119.6	2.0
33	2	Phe	4893.8	6161.8	374.5	223.1	27.6

NT = not tested

[a] From ref. 25

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

Table 2. Inhibition data for *s*-triazine derivatives monosubstituted with amino acids against hCA I, hCA II, and hCA IX reported in previous works [26,27]; their selectivity ratios for inhibition of isozyme hCA IX over hCA II.

Compound	n	$-R_1$	$-R_2$	K_I* (nM)			selectivity
				hCA I	hCA II	hCA IX	hCA II / hCA IX
X1	0	Gly	Cl	3032[a]	351[a]	7.9[a]	44
X2	1	Gly	Cl	35[b]	29[b]	1.7[b]	17
X3	2	Gly	Cl	33[b]	32[b]	1.0[b]	32
X4	0	Ala	Cl	1324[a]	561[a]	0.96[a]	584
X5	2	Ala	Cl	33[a]	32[a]	1.0[a]	32
X6	0	Ser	Cl	109[a]	412[a]	8.9[a]	12
X7	1	β-Ala	Cl	2360[a]	258[a]	34.1[a]	7.6
X8	2	β-Ala	Cl	31[b]	28[b]	1.2[b]	23

[a] From ref. 27

[b] From ref. 26

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10% of the reported values) [26,27].

2.3 Molecular modeling

The docking of compound **12** into the human isozymes II and IX showed interaction with residues in the same region as similar compounds docked by Saluja et al. [28]. When compared with crystal structure of methyl N-{4-chloro-6-[(4-sulfamoylphenyl)amino]-1,3,5-triazin-2-yl}glycinate complexed to hCA II (Fig. 2) [27], it is obvious that substitution of chlorine atom on s-triazine with bulkier amino acid prevents the compound **12** from π-π interaction of s-triazine with Phe-131 and moves it closer to Trp-5 and His-64 (marked as HIE 64 in the Fig. 2). The weak H-bond between Cl and Gln-92 residue is substituted with stronger H-bond provided by carboxylic group of compound **12**.

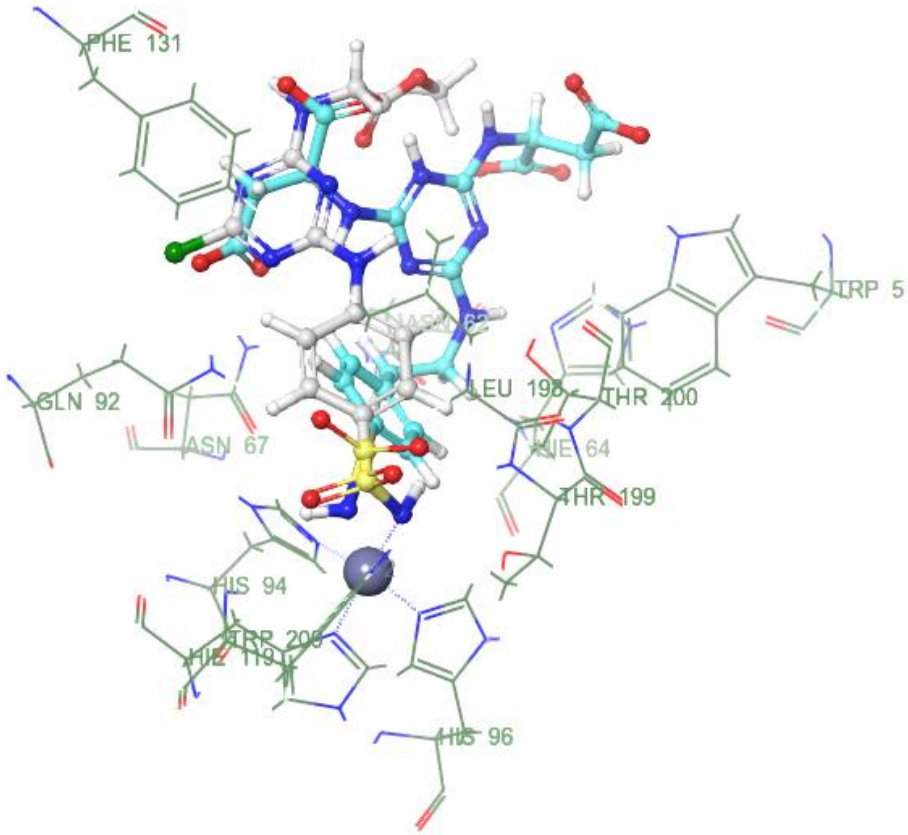


Figure 2. Superposition of molecule **12** (light blue carbons) docked in the active site of hCA II and crystal structure of N-{4-chloro-6-[(4-sulfamoylphenyl)amino]-1,3,5-triazin-2-yl}glycinate (white carbons) complexed with hCA II (PDB ID: 3MNA) [27].

The pose of molecule **12** in hCA IX is slightly different from the pose in hCA II (Fig. 3, 4). While Arg-60 residue in hCA IX creates H-bond with carboxylic group of **12** (1.86 Å), Leu-60 residue in hCA II is repulsive due to steric hindrance and lipophilic character. This repulsion prevents the other carboxylic groups to create H-bonds with Arg-58 and Asn-62 residues. This explains the higher affinity of the compound towards isozyme IX.

The computed scores were in agreement with the experimentally found selectivity. The docking score of the molecule **12** in hCA IX was -8.33 while in hCA II only -4.35.

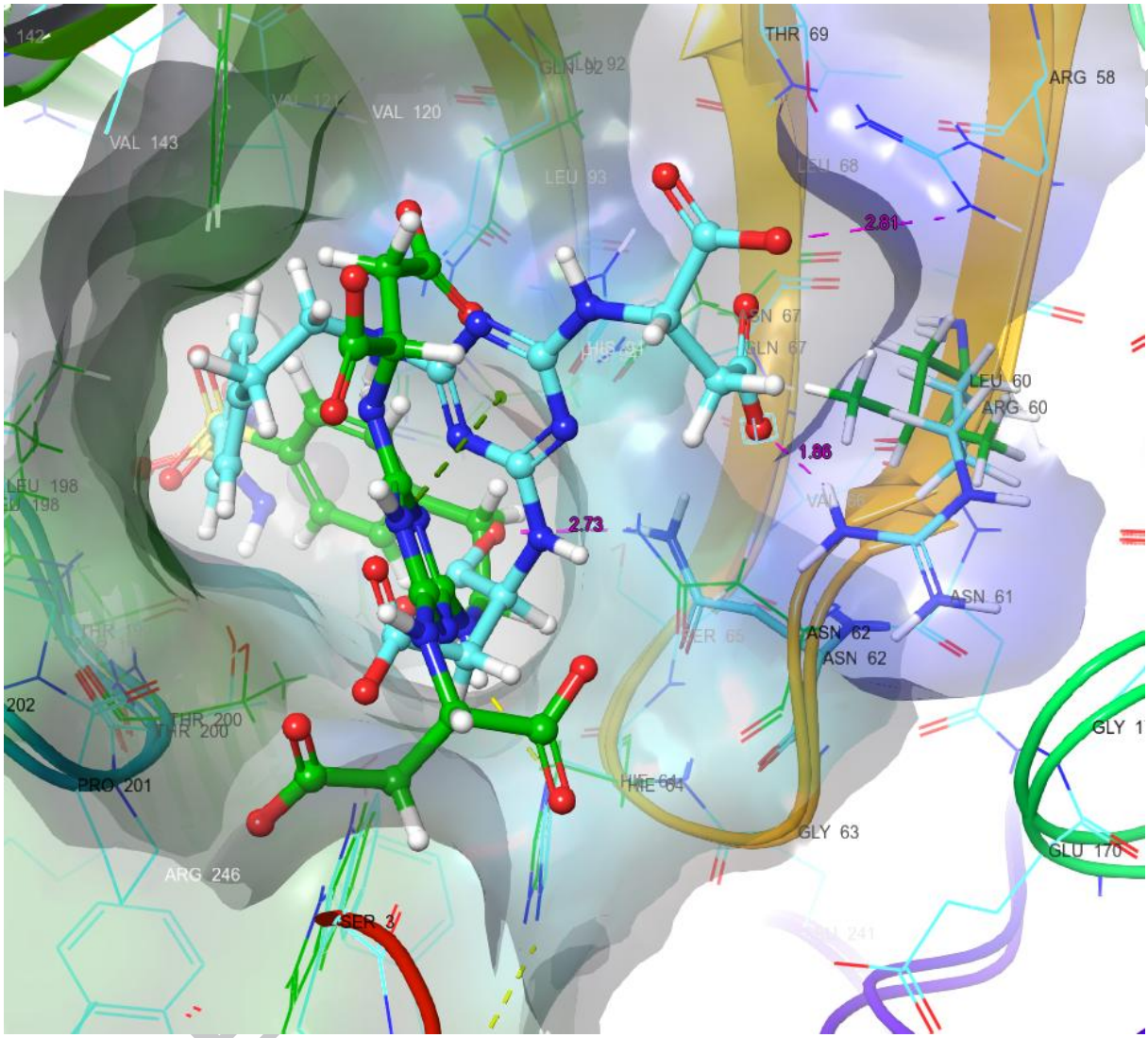
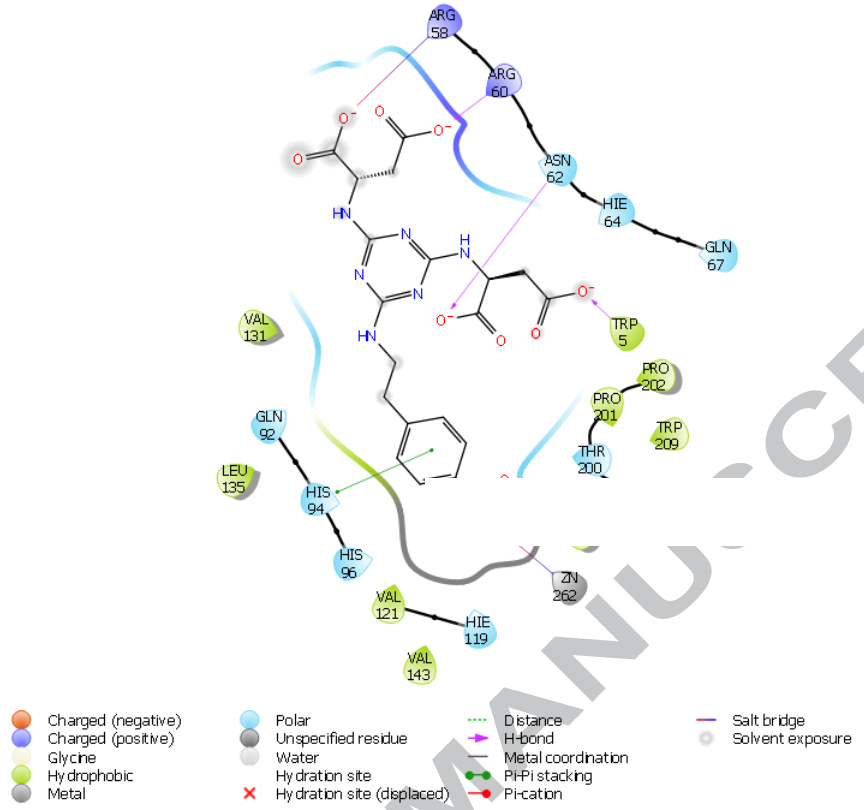


Figure 3. Superposition of molecule **12** docked in the active site of hCA IX (light blue carbons of all associating groups) creating H-bond with Arg-60 and the same molecule docked in the active site of hCA II (green carbons of all associating groups) repulsed by Leu-60.



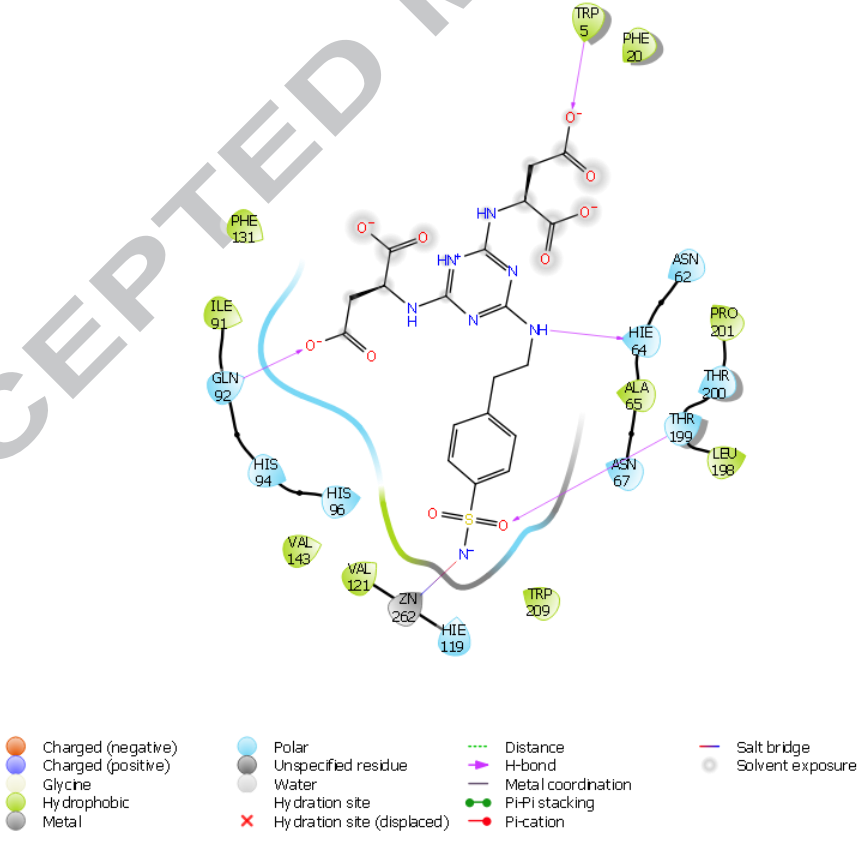


Figure 4. 2D interaction diagram of compound **12** dockes into the active site of hCA IX (upper panel) and hCA II (lower panel).

3. Conclusion

A new set of thirty triazinyl-substituted benzenesulfonamides, incorporating a symmetric pair of amino acid moieties was prepared by easy, environment friendly procedure with high yields. The inhibition studies of physiologically relevant hCA isoforms, such as hCA I, II, IV and tumor-associated hCA IX with the set of sulfonamide inhibitors are reported. The results demonstrate that the tested compounds showed only modest activity as inhibitors of hCA I (K_Is ≥ 67.1 nM) and hCA IV (K_Is ≥ 45.6 nM) and their activity towards hCA II was weak (K_Is in the range 235.7 – 7125 nM). However, this set of compounds showed high diversity in isozyme selectivity, offering selective hCA I inhibitor **28** with two Pro residues, selective hCA IV inhibitor **26** with two Met residues and selective hCA IX inhibitors **15**, **19** and **12** with two Glu, Leu and Asp residues, respectively (selectivity ratios hCA II / hCA IX were 84, 189 and 214, respectively). Best inhibitors of hCA IX, considering K_I values, are compounds **4** and **7** (K_Is with values 8.4 and 8.9 nM, respectively, and selectivity against physiologically most relevant isozyme hCA II over 40). Undoubtedly, the presented findings will stimulate a further more detail investigation of some of these new highly potent hCA inhibitors. In a continuation of the structure – activity study, following valuable modifications are proposed for this group of conjugates: (i) the substitution of triazinyl-aminoalkyl-benzenesulfonamides with other amino acids (and their derivatives) and (ii) peptides, and, especially, (iii) complexing such conjugates (ligands) with metal cations [36]. With the selectivity and capability to chelate radioactive metal isotopes by free carboxylate groups, also some derivatives from this study could constitute excellent leads for the development of new, unconventional group of anticancer drugs or diagnostics targeting hypoxia-induced CA isoforms such as CA IX. For example, compound **12** has good inhibition activity (K_I = 25.8 nM) and high selectivity towards hCA IX (selectivity ratio hCA II / hCA IX = 214) and possesses four carboxylic groups that can serve as chelating groups for radioactive metal isotopes. Such metal complexes could constitute new class for treatment/diagnostics of hypoxic tumors and will be the subject of our further investigations.

4. Experimental section

4.1 Experimental materials and methods

All reagents and solvents were purchased from AppliChem GmbH (Darmstadt, Germany) and Sigma Aldrich (St.Louis, MO, USA) and used without further purification. All the reactions were monitored by the thin-layer chromatography (TLC) with the UV visualization (245 nm) using Merck Silica gel plates 60 F_{254} as a stationary phase and hexane/ethyl acetate or dichloromethane/methanol as mobile phases. The nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) spectra were recorded on a Varian MERCURY plus 300 MHz spectrometer at 90 °C using DMSO-d_6 as a solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm). Splitting patterns are designated as follows: s- singlet, d- doublet, t- triplet, q- quartet, m- multiplet, br-broad and dd-doublet of doublet. Infrared (IR) spectra (in KBr plates) were recorded on Perkin-Elmer UATR Two (PerkinElmer Ltd., Beaconsfield, UK). The measured data are expressed in wavenumbers (cm^{-1}) and the signal strength is given as weak (w), medium (m) or strong (s). All of the HPLC-UV/MS measurements were performed on an LC Agilent Infinity System (Agilent Technologies, Santa Clara, CA, USA) equipped with a gradient pump (1290 Bin Pump VL), an automatic injector (1260 HiPals), and a column thermostat (1290 TCC). Zorbax Extend-C18 column, 2.1x50 mm, 1.7 µm (Agilent Technologies, Santa Clara, CA, USA) and SeQuant® ZIC®-HILIC column, 2.1x100 mm, 3.5 µm (Merck KGaA, Darmstadt, Germany), were employed for the HPLC analyses. The LC system was coupled with a photodiode array detector (Infinity 1290 DAD) and a quadrupole time-of-flight mass spectrometer (6520 Accurate Mass Q-TOF LC/MS). Q-

TOF was equipped with an electrospray ionization source operated in positive ionization mode. All the samples were dissolved in 20% acetonitrile and filtered through 0.22 µm nylon syringe filter before the HPLC-HRMS analyses. The HPLC-HRMS methods were optimized with respect to particular groups of the analyzed compounds, see Table 3. The flow rate was set at 0.5 mL/min and 1.0 µL of the sample was injected into the column. Gradient elution was used with the column oven temperature 35 °C. All measurements were performed with following MS parameters: drying gas temperature 350 °C, drying gas flow 10 $L.min^{-1}$, nebulizing gas pressure 60 psi, ESI source voltage 3500 V, fragmentor voltage 140 V, collision gas N2.

Table 3. The HPLC-HRMS methods used for the analyses of the amino acid conjugates **4-33**.

Analyzed compound	Column type	Mobile phase A	Mobile phase B
Majority of compounds	Zorbax Extend-C18	0.1% formic acid in demineralized water	acetonitrile
Compounds 9 and 25	Zorbax Extend-C18	10 mM ammonium acetate in demineralized water with pH 6.8	acetonitrile
Dicarboxylic compounds 10-15	SeQuant® ZIC®-HILIC	10 mM ammonium acetate in demineralized water with pH 6.8	acetonitrile

4.2 Synthetic chemistry and products characterization

*4.2.1 General procedure for the synthesis of 4-(4´,6´-dichloro-1´,3´,5´-triazine-2´-ylamino/aminomethyl/aminoethyl)benzenesulfonamide intermediates **1-3*** [27]

The 1.0 M solution of 4-amino-/aminomethyl-/aminoethyl-benzenesulfonamide (1 equiv) in acetone (100 ml) was added dropwise into a vigorously stirred suspension of cyanuric chloride (1 equiv) in acetone (100 ml) at 0°C. The white reaction mixture was stirred at the same temperature for 30 min. After then the 1.7 M aqueous solution of NaOH (1 equiv) was added during a period of 20 min. Stirring was continued for 1 h, and the reaction was quenched by the addition of slush (100 mL). After reaction was completed, the resulted solid was filtered off, washed and dried under high vacuum. The product was crystalized from acetone affording the compounds **1-3** as a white solid.

*4.2.2 General procedure for the synthesis of 2,2´-((6-(4-Sulfamoylphenyl-/benzyl-/phenethyl-amino)-1,3,5-triazine-2,4-diyl)bis(imino)) conjugates with monocarboxylic acids **4-9, 16-33***

Reaction mixture containing 4-(4´,6´-dichloro-1´,3´,5´-triazine-2´-ylamino/aminomethyl/aminoethyl)benzenesulfonamide (0.2 g, 1 equiv) and amino acid (3 equiv) was stirred in H_2O (2 mL) at room temperature for 10 min. The aqueous solution of Na_2CO_3 (4 equiv) was added dropwise into the reaction mixture. Then the mixture was refluxed for 24 h and evaluated by TLC during the reaction. The synthesis was terminated by 1M HCl addition (up to pH=2.0-4.5, depending on amino acid) until a maximum amount of precipitate was produced. The product was isolated by filtration and dried under high vacuum. After then the product purity and structural characterization were established by a combination of IR spectra, HPLC-UV/MS and NMR (for the recorded spectra see Figures S1-S24 in the Supplementary material).

4.2.2.1 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))diacetic acid **4** [32]: White solid; yield 96.4%. IR (KBr, cm^{-1}): 2968 (w), 1683 (m), 1598 (m), 1507 (s), 1407 (s), 1159 (s). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 12.10 (br s, 2H, COOH), 9.17 (s, 1H, Ar-NH), 7.89 (d, 2H, *J* = 8.9 Hz, Ar-H(a)), 7.67 (d, 2H, *J* = 8.9 Hz, Ar-H(b)), 6.94 (br s, 4H, SO_2NH_2, 2 x CH_2-**NH**), 3.95 (d, 4H, *J* = 5.2 Hz, 2 x **CH_2**-NH); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 172.02, 166.23, 164.39, 144.03, 136.91, 126.65, 119.32, 42.69. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{13}H_{15}N_7SO_6H]^+$ 398.0877; Found: 398.0878.

4.2.2.2 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))diacetic acid **5**:
White solid; yield 97.1%. IR (KBr, cm^{-1}): 3288 (w, NH), 1709 (w, C=O), 1621 (s), 1557 (s), 1399 (m), 1331 (m, SO_2NH_2), 1152 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.77 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.68 (br s, 1H, Ar-**NH**), 7.47 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.13 (br s, 2H, **NH**-CH), 7.02 (s, 2H, SO_2NH_2), 4.50 (d, 2H, *J* = 3.6 Hz, NH-$\mathbf{CH_2}$), 3.92 (s, 4H, 2 x H-C(2)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 171.51, 164.62, 163.74, 144.22, 143.24, 128.22, 126.15, 43.79, 42.70. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{14}H_{17}N_7SO_6H]^+$ 412.1034; Found: 412.1036.

4.2.2.3 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))diacetic acid **6**:
White solid; yield 78.9%. IR (KBr, cm^{-1}): 3288 (m, NH), 1716 (w, C=O), 1620 (s), 1557 (s), 1514 (s), 1407 (m), 1308 (m, SO_2NH_2), 1150 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.74 (d, 2H, *J* = 8.2 Hz, 2 x Ar-H(a)), 7.39 (d, 2H, *J* = 8.2 Hz, 2 x Ar-H(b)), 7.01 (s, 2H, SO_2NH_2), 6.61 (br s, 3H, 3 x Ar-**NH**), 3.90 (d, 4H, *J* = 3.1 Hz, 2 x H-C(2)), 3.46 (m, 2H, NH-$\mathbf{CH_2}$), 2.89 (m, 2H, $\mathbf{CH_2}$-Ar); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 172.05, 165.52, 150.49, 144.42, 142.56, 129.40, 126.18, 42.92, 42.70, 35.57. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{15}H_{19}N_7SO_6H]^+$ 426.1190; Found: 426.1191.

4.2.2.4 3,3´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))dipropanoic acid **7** [32]:
White solid; yield 94%. IR (KBr, cm^{-1}): 3290 (w, NH), 2967 (m), 1717 (w, C=O), 1630 (m), 1592 (s), 1509 (s), 1399 (m), 1163 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.00 (br s, 1H, Ar-**NH**), 7.92 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(a)), 7.66 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(b)), 6.88 (s, 2H, SO_2NH_2), 6.57 (br s, 2H, 2 x **NH**-CH), 3.51 (dt, 4H, *J* = 7.1 Hz, *J* = 5.8 Hz, 2 x H-C(3)), 2.52 (t, 4H, *J* = 7.1 Hz, 2 x H-C(2)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.24, 166.30, 164.62, 144.33, 136.72, 126.71, 119.10, 36.95, 34.72. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{15}H_{19}N_7SO_6H]^+$ 426.1190; Found: 426.1190.

4.2.2.5 3,3´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))dipropanoic acid **8**:
White solid; yield 73.7%. IR (KBr, cm^{-1}): 3430 (m, NH), 3266 (w, NH), 1667 (m), 1634 (s), 1551 (s), 1521 (m), 1408 (m), 1329 (m, SO_2NH_2), 1149 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.74 (d, 2H, *J* = 8.1 Hz, 2 x Ar-H(a)), 7.45 (d, 2H, *J* = 8.1 Hz, 2 x Ar-H(b)), 6.99 (s, 2H, SO_2NH_2), 6.92 (br s, 1H, Ar-**NH**), 6.21 (br s, 2H, 2 x **NH**-CH), 4.47 (d, 2H, *J* = 6.2 Hz, NH-$\mathbf{CH_2}$), 3.42 (dt, 4H, *J* = 7.1 Hz, *J* = 5.5 Hz, 2 x H-C(3)), 2.45 (t, 4H, *J* = 7.1 Hz, 2 x H-C(2)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.32, 166.34, 166.18, 145.37, 142.94, 127.98, 126.00, 43.70, 36.80, 34.81. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{16}H_{21}N_7SO_6H]^+$ 440.1347; Found: 440.1349.

4.2.2.6 3,3´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))dipropanoic acid **9**:
White solid; yield 50.6%. IR (KBr, cm^{-1}): 3301 (m, NH), 2939 (m), 1723 (w, C=O), 1622 (m), 1548 (s), 1408 (m), 1331 (m), 1307 (m, SO_2NH_2), 1153 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.39 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.00 (s, 2H, SO_2NH_2), 6.42 (br s, 1H, Ar-**NH**), 6.26 (br s, 2H, 2 x **NH**-CH), 3.46 (m, 6H, NH-$\mathbf{CH_2}$, 2 x H-C(3)), 2.90 (m, 2H, $\mathbf{CH_2}$-Ar), 2.48 (t, 4H, *J* = 7.1 Hz, 2 x H-C(2)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.32, 165.88, 165.81, 144.52, 142.55, 129.39, 126.19, 41.78, 36.81, 35.72, 34.81. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{17}H_{23}N_7SO_6H]^+$ 454.1503; Found: 454.1506.

4.2.2.7 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-methylbutanoic acid) **16**:
White solid; yield 80.6%. IR (KBr, cm^{-1}): 3262 (w, NH), 2967 (w), 1717 (w, C=O), 1622 (m), 1590 (m), 1558 (s), 1497 (s), 1404 (m), 1327 (m, SO_2NH_2), 1154 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.22 (br s, 1H, Ar-**NH**), 7.90 (d, 2H, *J* = 8.9 Hz, 2 x Ar-H(a)), 7.70 (d, 2H, *J* = 8.9 Hz, 2 x Ar-H(b)), 6.93 (br s, 2H, SO_2NH_2), 6.68 (br s, 2H, 2 x **NH**-CH), 4.42 (dd, 2H, *J* = 8.1 Hz, *J* = 6.0 Hz, 2 x H-

C(2)), 2.17 (m, 2H, 2 x H-C(3)), 0.98 (d, 6H, *J* = 6.8 Hz, 2 x CH_3), 0.97 (d, 6H, *J* = 6.8 Hz, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.44, 165.42, 163.81, 143.57, 137.37, 126.77, 119.65, 59.32, 30.44, 19.47, 18.85. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{19}H_{27}N_7SO_6H]^+$ 482.1820; Found: 482.1821.

4.2.2.8 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-methylbutanoic acid) **17**:

White solid; yield 42.8%. IR (KBr, cm^{-1}): 3283 (w, NH), 2967 (w), 1717 (w, C=O), 1622 (s), 1558 (s), 1471 (w), 1394 (w), 1328 (m, SO_2NH_2), 1156 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.77 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.61 (br s, 1H, Ar-**NH**), 7.48 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.02 (s, 2H, SO_2NH_2), 6.78 (br s, 2H, 2 x **NH**-CH), 4.52 (br s, 2H, NH-**CH_2**), 4.35 (m, 2H, 2 x H-C(2)), 2.12 (m, 2H, *J* = 6.6 Hz, 2 x H-C(3)), 0.93 (d, 6H, *J* = 6.8 Hz, 2 x CH_3), 0.92 (d, 6H, *J* = 6.8 Hz, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.25, 164.20, 163.29, 144.29, 143.26, 128.15, 126.09, 59.25, 43.81, 30.36, 19.39, 18.71. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{20}H_{29}N_7SO_6H]^+$ 496.1973; Found: 496.1975.

4.2.2.9 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-methylbutanoic acid) **18**:

White solid; yield 47.4%. IR (KBr, cm^{-1}): 3284 (w, NH), 2966 (w), 1717 (w, C=O), 1622 (s), 1558 (s), 1471 (w), 1395 (w), 1331 (m, SO_2NH_2), 1157 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.41 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.02 (s, 3H, SO_2NH_2, Ar-**NH**), 6.73 (br s, 2H, 2 x **NH**-CH), 4.37 (dd, 2H, *J* = 7.7 Hz, *J* = 5.9 Hz, 2 x H-C(2)), 3.51 (dt, 2H, *J* = 7.4 Hz, *J* = 7.2 Hz, NH-**CH_2**), 2.91 (t, 2H, *J* = 7.4 Hz, **CH_2**-Ar), 2.13 (m, 2H, 2 x H-C(3)), 0.95 (d, 12H, *J* = 6.9 Hz, 4 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.32, 163.79, 163.17, 144.14, 142.69, 129.43, 126.18, 59.29, 46.25, 35.37, 30.42, 19.40, 18.77. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{21}H_{31}N_7SO_6H]^+$ 510.2129; Found: 510.2131.

4.2.2.10 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(4-methylpentanoic acid) **19**:

White solid; yield 92.3%. IR (KBr, cm^{-1}): 3263 (br, w, NH), 2959 (w), 1717 (w, C=O), 1623 (m), 1590 (m), 1558 (s), 1496 (s), 1404 (m), 1318 (m, SO_2NH_2), 1154 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.07 (br s, 1H, Ar-**NH**), 7.91 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(a)), 7.68 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(b)), 6.91 (br s, 2H, SO_2NH_2), 6.76 (d, 2H, *J* = 8.2 Hz, 2 x **NH**-CH), 4.53 (dt, 2H, *J* = 8.2 Hz, *J* = 6.6 Hz, 2 x H-C(2)), 1.81-1.56 (m, 6H, 2 x H-C(4), 2 x H-C(3)), 0.93 (d, 6H, *J* = 6.4 Hz, 2 x CH_3), 0.90 (d, 6H, *J* = 6.4 Hz, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 174.77, 165.78, 164.19, 143.91, 137.09, 126.72, 119.41, 52.28, 25.06, 23.20, 22.09. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{21}H_{31}N_7SO_6H]^+$ 510.2129; Found: 510.2137.

4.2.2.11 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(4-methylpentanoic acid) **20**:

White solid; yield 87.9%. IR (KBr, cm^{-1}): 3263 (br, w, NH), 2957 (w), 1723 (w, C=O), 1622 (s), 1539 (s), 1456 (w), 1403 (w), 1333 (m, SO_2NH_2), 1157 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.46 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.32 (br s, 1H, Ar-**NH**), 7.01 (br s, 2H, SO_2NH_2), 6.71 (br s, 2H, 2 x **NH**-CH), 4.56-4.42 (m, 4H, 2 x H-C(2), NH-**CH_2**), 1.76-1.51 (m, 6H, 2 x H-C(3), 2 x H-C(4)), 0.89 (d, 6H, *J* = 6.4 Hz, 2 x CH_3), 0.86 (d, 6H, *J* = 6.4 Hz, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 174.66, 164.74, 164.52, 144.66, 143.15, 128.11, 126.08, 52.24, 43.70, 24.98, 23.15, 22.10. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{22}H_{33}N_7SO_6H]^+$ 524.2286; Found: 524.2294.

4.2.2.12 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(4-methylpentanoic acid) **21**:

White solid; yield 85.8%. IR (KBr, cm^{-1}): 3286 (w, NH), 2958 (m), 1723 (w, C=O), 1622 (s), 1557 (s), 1470 (w), 1441 (w), 1334 (m, SO_2NH_2), 1157 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.2 Hz, 2 x Ar-H(a)), 7.41 (d, 2H, *J* = 8.2 Hz, 2 x Ar-H(b)), 7.02 (br s, 5H, SO_2NH_2, 2 x Ar-**NH, NH**-CH_2), 4.48 (m, 2H, NH-**CH_2**), 3.50 (br s, 2H, 2 x H-C(2)), 2.91 (t, 2H, *J* = 7.4 Hz, **CH_2**-Ar), 1.78-1.54 (m, 6H, 2 x H-C(3), 2 x H-C(4)), 0.92 (d, 6H, *J* = 6.4 Hz, 2 x CH_3), 0.89 (d, 6H, *J* = 6.4 Hz, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 174.44, 164.08, 163.36, 144.12, 142.70, 129.42, 126.21, 52.35, 35.36, 25.00, 23.15, 22.09. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{23}H_{35}N_7SO_6H]^+$ 538.2442; Found: 538.2450.

4.2.2.13 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-methylpentanoic acid) **22**:

White solid; yield 79.9%. IR (KBr, cm^{-1}): 3266 (w, NH), 2966 (w), 1717 (w, C=O), 1623 (m), 1590 (m), 1558 (s), 1497 (s), 1406 (m), 1328 (m, SO_2NH_2), 1154 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.21 (br s, 1H, Ar-**NH**), 7.91 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(a)), 7.70 (d, 2H, *J* = 8.9 Hz, 2 x Ar-H(b)), 6.93 (br s, 2H, SO_2NH_2), 6.68 (br s, 2H, 2 x **NH**-CH), 4.48 (m, 2H, 2 x H-C(2)), 1.92 (m, 2H, 2 x H-C(3)), 1.52 (m, 2H, H-C(4)), 1.28 (m, 2H, H-C(4)), 0.95 (d, 6H, *J* = 6.8 Hz, 2 x C(3)-**CH_3**), 0.90 (t, 6H, *J* = 7.4 Hz, 2 x H-C(5)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.44, 165.33, 163.83, 143.57, 137.41, 126.74, 119.68, 58.26, 37.04, 25.59, 16.01, 11.64. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{21}H_{31}N_7SO_6H]^+$ 510.2129; Found: 510.2133.

4.2.2.14 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-methylpentanoic acid) **23**:

White solid; yield 89.3%. IR (KBr, cm^{-1}): 3284 (w, NH), 2966 (w), 1716 (w, C=O), 1622 (s), 1557 (s), 1463 (w), 1408 (w), 1334 (m, SO_2NH_2), 1157 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.76 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.66 (br s, 1H, **NH**-CH_2), 7.48 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.02 (s, 2H, SO_2NH_2), 6.84 (br s, 2H, 2 x **NH**-CH), 4.52 (br s, 2H, NH-**CH_2**), 4.43 (m, 2H, 2 x H-C(2)), 1.87 (m, 2H, 2 x H-C(3)), 1.49 (m, 2H, H-C(4)), 1.47 (m, 2H, H-C(4)), 0.90 (d, 6H, *J* = 7.0 Hz, 2 x C(3)-**CH_3**), 0.86 (t, 6H, *J* = 7.5 Hz, 2 x H-C(5)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.22, 163.68, 162.47, 144.22, 143.26, 128.16, 126.12, 58.19, 43.77, 37.03, 25.50, 15.96, 11.61. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{22}H_{33}N_7SO_6H]^+$ 524.2286; Found: 524.2290.

4.2.2.15 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-methylpentanoic acid) **24**:

White solid; yield 84.6%. IR (KBr, cm^{-1}): 3265 (w, NH), 2965 (w), 1719 (w, C=O), 1621 (s), 1551 (s), 1459 (w), 1406 (w), 1332 (m, SO_2NH_2), 1157 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.41 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.02 (br s, 3H, SO_2NH_2, Ar-**NH**), 6.72 (br s, 2H, 2 x **NH**-CH), 4.43 (m, 2H, 2 x H-C(2)), 3.51 (m, 2H, NH-**CH_2**), 2.91 (m, 2H, **CH_2**-Ar), 1.89 (m, 2H, 2 x H-C(3)), 1.49 (m, 2H, H-C(4)), 1.25 (m, 2H, H-C(4)), 0.92 (d, 6H, *J* = 6.9 Hz, 2 x C(3)-**CH_3**)), 0.88 (t, 6H, *J* = 7.5 Hz, 2 x H-C(5)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.31, 166.35, 163.87, 144.13, 142.68, 129.43, 126.17, 58.19, 47.18, 37.06, 35.36, 25.53, 15.99, 11.61. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{23}H_{35}N_7SO_6H]^+$ 538.2442; Found: 538.2449.

4.2.2.16 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(4-methylthiobutanoic acid) **25**:

White solid; yield 80.4%. IR (KBr, cm^{-1}): 3243 (w, NH), 1717 (w, C=O), 1623 (m), 1589 (m), 1558 (s), 1496 (s), 1404 (m), 1320 (m, SO_2NH_2), 1154 (s, SO_2NH_2). ^{1}H NMR (300MHz, DMSO-d_6, 90 °C) δ: 9.26 (br s, 1H, Ar-**NH**), 7.89 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(a)), 7.69 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(b)), 7.11 (br s, 4H, SO_2NH_2, 2 x **NH**-CH), 4.60 (br s, 2H, 2 x H-C(2)), 2.57 (m, 4H, 2 x H-C(4)), 2.15-1.97 (m, 4H, 2 x

H-C(3)), 2.05 (s, 6H, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.82, 165.09, 163.68, 143.56, 137.41, 126.75, 119.67, 53.09, 31.60, 30.61, 15.15. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{19}H_{27}N_7S_3O_6H]^+$ 546.1258; Found: 546.1259.

4.2.2.17 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(4-methylthiobutanoic acid) **26**:
White solid; yield 88.4%. IR (KBr, cm^{-1}): 3282 (w, NH), 2969 (w), 1717 (w, C=O), 1623 (m), 1558 (s), 1507 (m), 1398 (m), 1330 (m, SO_2NH_2), 1156 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.76 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.47 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.25 (br s, 1H, Ar-**NH**), 7.01 (s, 2H, SO_2NH_2), 6.75 (br s, 2H, 2 x **NH**-CH), 4.51 (m, 4H, 2 x H-C(2), NH-**CH_2**), 2.52 (m, 4H, 2 x H-C(4)), 2.09-1.90 (m, 4H, 2 x H-C(3)), 2.03 (s, 6H, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.92, 165.19, 165.00, 144.74, 143.12, 128.14, 126.08, 53.02, 43.70, 31.72, 30.55, 15.14. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{20}H_{29}N_7S_3O_6H]^+$ 560.1419; Found: 560.1423.

4.2.2.18 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(4-methylthiobutanoic acid) **27**:
White solid; yield 83.2%. IR (KBr, cm^{-1}): 3281 (w, NH), 2919 (w), 1717 (w, C=O), 1617 (s), 1558 (m), 1540 (m), 1507 (m), 1398 (w), 1328 (m, SO_2NH_2), 1154 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(a)), 7.41 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(b)), 7.12 (br s, 3H, 2 x **NH**-CH, Ar-**NH**), 7.02 (s, 2H, SO_2NH_2), 4.56 (m, 2H, 2 x H-C(2)), 3.50 (m, 2H, NH-**CH_2**), 2.91 (m, 2H, **CH_2**-Ar), 2.55 (m, 4H, 2 x H-C(4)), 2.08-1.91 (m, 4H, 2 x H-C(3), 2.05 (s, 6H, 2 x CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.64, 163.01, 162.79 , 144.11, 142.68, 129.44, 126.16, 53.05, 41.81, 35.36, 31.60, 30.51, 15.14. HRMS (ESI/QTOF) *m/z*: $[M + H]^+$ Calcd. for $[C_{21}H_{31}N_7S_3O_6H]^+$ 574.1571; Found: 574.1577.

4.2.2.19 N,N´-(6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(pyrrolidine-2-carboxylic acid) **28**:
White solid; yield 97.2%. IR (KBr, cm^{-1}): 2978 (w), 2882 (w), 1728 (w, C=O), 1624 (m), 1582 (m), 1539 (s), 1475 (s), 1343 (s, SO_2NH_2), 1156 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.05 (br s, 1H, Ar-**NH**), 7.90 (d, 2H, *J* = 8.7 Hz, 2 x Ar-H(a)), 7.67 (d, 2H, *J* = 8.7 Hz, 2 x Ar-H(b)), 6.88 (s, 2H, SO_2NH_2), 4.46 (br s, 2H, 2 x H-C(2)), 3.60 (br s, 4H, 2 x H-C(4)), 2.25 (m, 2H, H-C(6)), 2.04-1.90 (m, 6H, H-C(6), 2 x H-C(5)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 174.30, 164.10, 163.60, 144.14, 136.77, 126.73, 119.16, 59.04, 46.71, 30.25, 23.67. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{19}H_{23}N_7SO_6H]^+$ 478.1508; Found: 478.1511.

4.2.2.20 N,N´-(6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(pyrrolidine-2-carboxylic acid) **29**:
Beige solid; yield 62.3%. IR (KBr, cm^{-1}): 2969 (w), 1733 (w), 1717 (w, C=O), 1653 (m), 1601 (s), 1539 (s), 1457 (m), 1396 (w), 1331 (m, SO_2NH_2), 1156 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 8.28 (br s, 1H, **NH**-CH_2), 7.79 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.50 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.03 (br s, 2H, SO_2NH_2), 4.55 (br s, 2H, 2 x H-C(2)), 4.43 (br s, 2H, NH-**CH_2**), 3.59 (br s, 4H, 2 x H-C(4)), 2.23 (dq, 2H, *J* = 8.2 Hz, H-C(6)), 2.02-1.92 (m, 6H, H-C(6), 2 x H-C(5)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.25, 167.19, 164.54, 143.45, 134.15, 128.38, 126.25, 59.72, 47.22, 43.78, 29.88, 23.71. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{20}H_{25}N_7SO_6H]^+$ 492.1660; Found: 492.1668.

4.2.2.21 N,N´-(6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(pyrrolidine-2-carboxylic acid) **30**:
Beige solid; yield 57.5%. IR (KBr, cm^{-1}): 2968 (w), 2880 (w), 1733 (w), 1717 (w, C=O), 1653 (m), 1598 (s), 1539 (s), 1457 (m), 1396 (w), 1332 (m, SO_2NH_2), 1156 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.74 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.41 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.02 (br s, 2H, SO_2NH_2), 4.44 (br s, 2H, 2 x H-C(2)), 3.57 (m, 6H, 2 x H-C(4), NH-C**H_2**), 2.92 (t, 2H, *J* = 7.1 Hz, **CH_2**-

Ar), 2.22 (m, 2H, H-C(6)), 2.03-1.89 (m, 6H, H-C(6), 2 x H-C(5)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.60, 164.60, 160.96, 143.92, 142.73, 129.49, 126.21, 59.56, 47.07, 41.80, 35.26, 29.98, 23.69. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{21}H_{27}N_7SO_6H]^+$ 506.1816; Found: 506.1825.

4.2.2.22 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-phenylpropanoic acid) **31**:
White solid; yield 93.8%. IR (KBr, cm^{-1}): 3281 (br, w, NH), 1724 (w, C=O), 1623 (m), 1588 (m), 1557 (s), 1495 (s), 1404 (m), 1327 (m, SO_2NH_2), 1154 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.09 (br s, 1H, Ar-**NH**), 7.83 (d, 2H, *J* = 8.9 Hz, 2 x Ar-H(a)), 7.67 (d, 2H, *J* = 8.9 Hz, 2 x Ar-H(b)), 7.25 – 7.14 (m, 10H, Ar-H), 6.91 (br s, 2H, SO_2NH_2), 6.70 (br s, 2H, 2 x **NH**-CH) 4.72 (ddd, 2H, *J* = 8.6 Hz, *J* = 7.8 Hz, *J* = 5.2 Hz, 2 x H-C(2)), 3.17 (dd, 2H, *J* = 14.0 Hz, *J* = 5.2 Hz, 2 x H-C(3)), 3.05 (dd, 2H, *J* = 14.0 Hz, *J* = 8.6 Hz, 2 x H-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.59, 165.57, 164.11, 143.76, 138.31, 137.16, 129.54, 128.61, 126.66, 119.56, 119.28, 55.15, 37.45. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{27}H_{27}N_7SO_6H]^+$ 578.1821; Found: 578.1824.

4.2.2.23 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-phenylpropanoic acid) **32**:
White solid; yield 92.5%. IR (KBr, cm^{-1}): 3287 (w, NH), 1717 (w, C=O), 1625 (s), 1551 (s), 1397 (m), 1333 (m, SO_2NH_2), 1158 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.74 (d, 2H, *J* = 8.6 Hz, 2 x Ar-H(a)), 7.41 (d, 2H, *J* = 8.6 Hz, 2 x Ar-H(b)), 7.28-7.13 (m, 10H, Ar-H), 7.00 (br s, 3H, SO_2NH_2, Ar-**NH**), 6.27 (br s, 2H, 2 x **NH**-CH) 4.64 (ddd, 2H, *J* = 8.4 Hz, *J* = 7.8 Hz, *J* = 5.3 Hz, 2 x H-C(2)), 4.44 (d, 2H, *J* = 6.21 Hz, NH-**CH$_2$**), 3.09 (dd, 2H, *J* = 14.0 Hz, *J* = 5.4 Hz, 2 x H-C(3)), 2.99 (dd, 2H, *J* = 14.0 Hz, *J* = 8.4 Hz, 2 x H-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.77, 165.95, 165.67, 145.00, 143.02, 138.34, 129.48, 128.51, 126.68, 126.17, 125.94, 55.00, 43.65, 37.52. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{28}H_{29}N_7SO_6H]^+$ 592.1973; Found: 592.1979.

4.2.2.24 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))di(3-phenylpropanoic acid) **33**:
White solid; yield 90.4%. IR (KBr, cm^{-1}): 3285 (w, NH), 1717 (w, C=O), 1623 (s), 1551 (s), 1455 (w), 1398 (w), 1332 (m, SO_2NH_2), 1157 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.74 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(a)), 7.38 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(b)), 7.28-7.14 (m, 10H, Ar-H), 7.01 (br s, 2H, SO_2NH_2), 6.74 (br s, 1H, Ar-**NH**), 6.55 (br s, 2H, 2 x **NH**-CH), 4.68 (ddd, 2H, *J* = 8.4 Hz, *J* = 7.9 Hz, *J* = 5.3 Hz, 2 x H-C(2)), 3.44 (dt, 2H, *J* = 7.4 Hz, *J* = 4.8 Hz, NH-**CH$_2$**), 3.13 (dd, 2H, *J* = 14.0 Hz, *J* = 5.3 Hz, 2 x H-C(3)), 3.02 (dd, 2H, *J* = 14.0 Hz, *J* = 8.4 Hz, 2 x H-C(3)), 2.86 (t, 2H, *J* = 7.4 Hz, **CH$_2$**-Ar); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.59, 164.82, 164.61, 144.28, 142.62, 138.26, 129.50, 128.60, 126.78, 126.32, 126.02, 55.16, 41.75, 37.50, 35.45. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{29}H_{31}N_7SO_6H]^+$ 606.2129; Found: 606.2135.

*4.2.3 General procedure for the synthesis of 2,2´-((6-(4-Sulfamoylphenyl-/benzyl-/phenethyl-amino)-1,3,5-triazine-2,4-diyl)bis(imino)) conjugates with dicarboxylic acids **10-15***
Reaction mixture containing 4-(4´,6´-dichloro-1´,3´,5´-triazine-2´-ylamino/aminomethyl/aminoethyl)benzenesulfonamide (0.2 g, 1 equiv) and glutamic or aspartic acid (3 equiv) was stirred in H_2O (3 mL) at room temperature for 10 min. The aqueous solution of Na_2CO_3 (5 equiv) was added dropwise into the reaction mixture. Then the mixture was refluxed for 24 h and evaluated by TLC during the reaction. The synthesis was terminated by 1M HCl addition (up to pH=3.0-4.0) until a maximum amount of precipitate was produced. The product was isolated by filtration and dried under high vacuum. Preliminary analysis indicated some impurities in the samples so that further purification (recrystallization from water) was needed to achieve the purity higher than 90%. After then the product purity and characterization were established by a combination of IR spectra, HPLC-UV/MS and NMR (for the recorded spectra see Figures S25-S30 in the Supplementary material).

4.2.3.1 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))disuccinic acid **10**:
White solid; yield 90.2%. IR (KBr, cm^{-1}): 3286 (w, NH), 2967 (w), 1715 (m, C=O), 1589 (s), 1554 (s), 1496 (s), 1380 (m), 1154 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.12 (s, 1H, Ar-**NH**), 7.89 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(a)), 7.68 (d, 2H, *J* = 8.8 Hz, 2 x Ar-H(b)), 6.89 (s, 2H, SO_2NH_2), 6.60 (br s, 2H, 2 x **NH**-CH), 4.68 (t, 2H, *J* = 6.4 Hz, 2 x H-C(2)), 2.72 (d, 4H, *J* = 6.4 Hz, 2 x H-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.44, 172.41, 165.77, 164.55, 144.00, 136.95, 126.80, 119.29, 50.59, 38.24. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{17}H_{19}N_7SO_{10}H]^+$ 514.0992; Found: 514.0996.

4.2.3.2 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))disuccinic acid **11**:
White solid; yield 78.9%. IR (KBr, cm^{-1}): 3290 (m, NH), 1717 (m, C=O), 1623 (s), 1559 (s), 1541 (s), 1396 (w), 1328 (m, SO_2NH_2), 1151 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.47 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.09 (br s, 1H, Ar-**NH**), 6.99 (s, 2H, SO_2NH_2), 6.38 (br s, 2H, 2 x **NH**-CH), 4.73 (dd, 2H, *J* = 6.4 Hz, *J* = 5.9 Hz, 2 x H-C(2)), 4.48 (d, 2H, *J* = 6.3 Hz, **CH_2**-Ar), 2.71 (dd, 2H, *J* = 16.2 Hz, *J* = 5.9 Hz, 2 x H_a-C(3)), (dd, 2H, *J* = 16.2 Hz, *J* = 6.4 Hz, 2 x H_b-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.25, 172.20, 166.22, 165.80, 145.09, 143.00, 128.15, 126.11, 50.45, 43.66, 37.06. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{18}H_{21}N_7SO_{10}H]^+$ 528.1143; Found: 528.1149.

4.2.3.3 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))disuccinic acid **12**:
Beige solid; yield 68.2%. IR (KBr, cm^{-1}): 3281 (w, NH), 1717 (m, C=O), 1617 (s), 1559 (s), 1541 (s), 1396 (m), 1328 (m, SO_2NH_2), 1152 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(a)), 7.41 (d, 2H, *J* = 8.3 Hz, 2 x Ar-H(b)), 7.00 (br s, 2H, SO_2NH_2), 6.60 (br s, 1H, Ar-**NH**), 6.41 (br s, 2H, 2 x **NH**-CH), 4.76 (dd, 2H, *J* = 6.4 Hz, *J* = 5.9 Hz, 2 x H-C(2)), 3.46 (m, 2H, NH-**CH_2**), 2.89 (m, 2H, **CH_2**-Ar) 2.75 (dd, 2H, *J* = 16.2 Hz, *J* = 5.9 Hz, 2 x H_a-C(3)), 2.72 (dd, 2H, *J* = 16.2 Hz, *J* = 6.4 Hz, 2 x H_b-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 173.24, 172.18, 165.86, 165.57, 144.44, 142.55, 129.43, 126.19, 50.49, 41.79, 37.19, 37.01, 35.63. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{19}H_{23}N_7SO_{10}H]^+$ 542.1300; Found: 542.1309.

4.2.3.4 2,2´-((6-(4-Sulfamoylphenylamino)-1,3,5-triazine-2,4-diyl)bis(imino))diglutaric acid **13**:
White solid; yield 87.6%. IR (KBr, cm^{-1}): 3242 (m, NH), 2967 (br, w), 1713 (m, C=O), 1622 (m), 1589 (s), 1557 (s), 1495 (s), 1404 (m), 1318 (m, SO_2NH_2), 1151 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 9.02 (br s, 1H, Ar-**NH**), 7.89 (d, 2H, *J* = 8.9 Hz, 2 x Ar-H(a)), 7.68 (d, 2H, *J* = 8.9 Hz, 2 x Ar-H(b)), 6.89 (br s, 2H, SO_2NH_2), 6.66 (br s, 2H, 2 x **NH**-CH), 4.45 (t, 2H, *J* = 6.8 Hz, 2 x H-C(2)), 2.34 (t, 4H, *J* = 7.6 Hz, 2 x H-C(4)), 2.12-1.91 (m, 4H, 2 x H-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 174.21, 174.06, 166.11, 164.49, 144.04, 136.88, 126.75, 119.23, 53.51, 31.28, 27.48. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{19}H_{23}N_7SO_{10}H]^+$ 542.1305; Found: 542.1308.

4.2.3.5 2,2´-((6-(4-Sulfamoylbenzylamino)-1,3,5-triazine-2,4-diyl)bis(imino))diglutaric acid **14**:
Beige solid; yield 59.8%. IR (KBr, cm^{-1}): 3242 (m, NH), 1716 (m, C=O), 1622 (s), 1558 (s), 1404 (m), 1324 (m, SO_2NH_2), 1152 (s, SO_2NH_2). ^1H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(a)), 7.46 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(b)), 6.99 (br s, 3H, SO_2NH_2, Ar-**NH**), 6.36 (br s, 2H, 2 x **NH**-CH), 4.46 (m, 4H, 2 x H-C(2), **CH_2**-Ar), 2.30 (m, 4H, 2 x H-C(4)), 2.16-1.83 (m, 4H, 2 x H-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 174.17, 174.11, 166.23, 166.14, 145.17, 143.00, 128.16, 126.07, 53.22, 43.64, 30.97, 27.28. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{20}H_{25}N_7SO_{10}H]^+$ 556.1456; Found: 556.1462.

4.2.3.6 2,2´-((6-(4-Sulfamoylphenethylamino)-1,3,5-triazine-2,4-diyl)bis(imino))diglutaric acid **15**:

Beige solid; yield 66.3%. IR (KBr, cm^{-1}): 3242 (m, NH), 2968 (w), 1717 (m, C=O), 1622 (s), 1558 (s), 1540 (s), 1405 (m), 1312 (m, SO_2NH_2), 1152 (s, SO_2NH_2). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C) δ: 7.75 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(a)), 7.40 (d, 2H, *J* = 8.4 Hz, 2 x Ar-H(b)), 7.00 (s, 2H, SO_2NH_2), 6.57 (br s, 1H, Ar-**NH**), 6.51 (br s, 2H, 2 x **NH**-CH), 4.43 (br s, 2H, 2 x H-C(2)), 3.46 (m, 2H, NH-**CH_2**), 2.89 (t, 2H, *J* = 7.4 Hz, **CH_2**-Ar), 2.33 (m, 4H, 2 x H-C(4)), 2.12-1.86 (m, 4H, 2 x H-C(3)); ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C) δ: 174.05, 165.46, 153.82, 144.42, 142.56, 129.44, 126.15, 53.25, 41.77, 35.52, 30.91, 27.21. HRMS (ESI/QTOF, *m/z*): $[M + H]^+$ Calcd. for $[C_{21}H_{27}N_7SO_{10}H]^+$ 570.1613; Found: 570.1621.

4.3 Carbonic anhydrase inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity [34]. 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 Tris (pH 8.3) 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 0.005 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 ChengPrusoff equation, as reported earlier, and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house. [37-40]

4.4. Protein preparation

The hCA IX isoenzyme structure was prepared for modeling using the 2.2 Å resolution hCA IX-acetazolamide crystal complex (PDB ID: 3IAI) [41]. The hCA II isoenzyme structure was prepared for modeling using the 1.5 Å resolution hCA II- 4-({4-chloro-6-[(2-hydroxyethyl)amino]-1,3,5-triazin-2-yl}amino)benzenesulfonamide crystal complex (PDB ID: 3MMA) [27]. Schrödinger's 'Protein Preparation Wizard' [42] was used for the preparation with water molecules within 5 Å of the ligand initially retained, but deleted for subsequent docking. Bond orders were assigned and hydrogen atoms added, with protonation states for basic and acidic residues based on residue pKa's at normal pH (7.0). However, subsequent optimization of hydroxyl groups, histidine C/N atom "flips" and protonation states, and side chain O/N atom "flips" of Asn and Gln residues was based on optimizing hydrogen bonding patterns, so that the final assignments were checked on visual inspection of the protein.

4.5 Molecular docking

LigPrep [43] was used to prepare ligand **12** (geometry optimization by OPLS 3, generation of all optical isomers, low-energy ring conformations) and Epik [44–46] for the generation of the probable ionized and tautomerized structures within a pH range of 7 ±2. Sulfonamide group was N-deprotonated as it is well known that in this form it serves as zinc binding site [47].

Docking to hCA IX and hCA II was performed in program Glide with XP precision [48,49]. Penalization for low probable ionization state and constraint for bond between zinc and zinc binding group were used.

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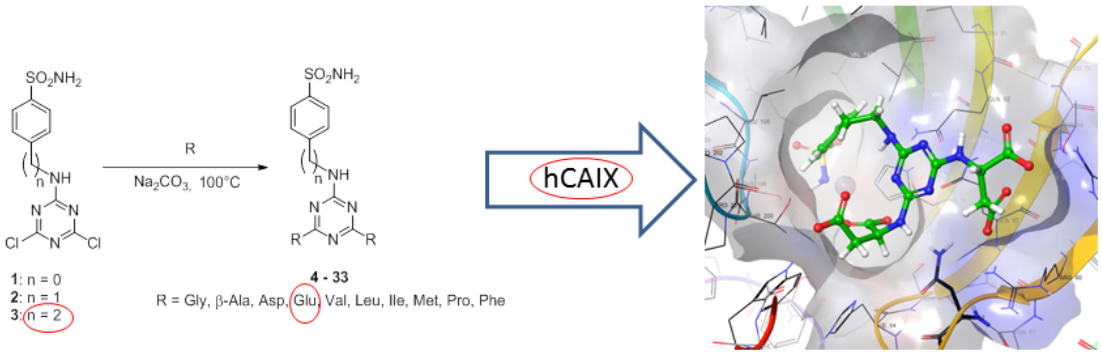
Graphical abstract



Highlights

- Novel sulfonamides incorporating 1,3,5-triazine and amino acid structural motifs
- Green synthesis of disubstituted amino acid products with high selectivity and yield
- Study on inhibition activity of products toward carbonic anhydrases I, II, IV, IX
- Found highly effective inhibitors toward tumor-associated hCA IX
- Docking study on the most promising enzyme-substrate complex