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Sulfonamides incorporating 1,3,5-triazine moieties selectively and potently inhibit carbonic anhydrase transmembrane isoforms IX, XII and XIV over cytosolic isoforms I and II: Solution and X-ray crystallographic studies ☆

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ABSTRACT

Reaction of cyanuryl chloride with D,L-amino acids and amino alcohols afforded a new series of triazinyl-substituted benzenesulfonamides incorporating amino acyl/hydroxyalkyl-amino moieties. Inhibition studies of physiologically relevant human carbonic anhydrase (CA, EC 4.2.1.1) isoforms, such as CA I, II, IX, XII and XIV with these compounds are reported. They showed moderate-weak inhibition of the cytosolic, offtarget isozymes CA I and II, but many of them were low nanomolar inhibitors of the transmembrane, tumor-associated CA IX and XII (and also of CA XIV). The X-ray crystal structure of two of these compounds in adduct with CA II allowed us to understand the features associated with this strong inhibitory properties and possibly also their selectivity. Two of these compounds were also investigated for the inhibition of other human isoforms, that is, hCA IV, VA, VB, VI, VII and XIII, as well as inhibitors of the fungal pathogenic CAs Nce103 (*Candida albicans*) and Can2 (*Cryptococcus neoformans*), showing interesting activity. The 1,3,5-triazinyl-substituted benzenesulfonamides constitute thus a class of compounds with great potential for obtaining inhibitors targeting both α-class mammalian, tumor-associated, and β-class from pathogenic organisms CAs.

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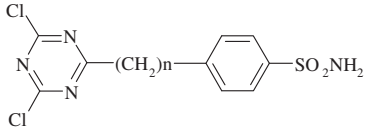
1. Introduction

In previous studies from this group[1] the synthesis and carbonic anhydrase (CA, EC 4.2.1.1) inhibition with benzenesulfonamides incorporating 1,3,5-triazine moieties of types **1–3** have been investigated.[1] Such compounds have been prepared from cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) and aromatic sulfonamides, by a procedure already reported in 1959 by D'Alelio and White.[2] Although this chemistry is very simple, several interesting facts emerged regarding compounds **1–3**: (i) these derivatives showed effective inhibition of several CA isoforms, of the 16 presently known in mammals,[3] such as the cytosolic CA I and II, and the transmembrane, tumor-associated CA IX.[3–5] For example, **1** inhibited CA I and II with inhibition constants in the range of 106–120 nM, whereas CA IX was inhibited in the subnanomolar range (K_I of 0.15 nM), with a selectivity ratio for inhibiting the tumor-associated isoform over the physiologically dominant one CA II of greater than 700, the highest ever observed for a sulfonamide inhibitor.[1a] However its close congeners, **2** and **3**, differing by only one or two extra CH_2 moieties, respectively, compared to **1**, were much weaker CA IX inhibitors (K_Is of 124–138 nM) but their efficacy for CA I and II was enhanced compared to **1** (K_Is of 13–21 nM against CA II and of 75–136 nM against CA I);[1a] (ii) the different reactivity and possibility to sequentially replace one or two chlorine atoms from the scaffolds of **1–3**, by reaction with various nucleophiles, affords a methodology to prepare large and chemically diverse classes of CA inhibitors (CAIs), by means of a facile and straightforward chemistry.[1,2]



1-3
n = 0, 1, 2

☆ Coordinates and structure factors have been deposited in the Protein Data Bank as entries; compound **7**, 3MMF, and compound **11**, 3MNA.

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Previous studies have examined the reactivity of sulfonamides **1–3** towards alcohols,[1a] phenols,[1a] primary and secondary aliphatic amines,[1a,b] ammonia,[1b] hydrazine,[1b] as well as a small number of amino acids and their derivatives (i.e., the amino acid esters of glycine and β-alanine).[1b] Similar to the leads **1–3**, the compounds obtained by the above-mentioned reactions showed very interesting properties as CAIs, against the tested isoforms, that is, hCA I and II (h = human isoform) and hCA IX.[1] Indeed, many such compounds were low nanomolar hCA IX inhibitors whereas their affinity for the cytosolic offtarget isozymes I and II was low, making them CA IX-selective inhibitors, with selectivity ratios in the range of between 10 and 700.[1] As CA IX was recently shown to be a promising antitumor target for the development of therapeutic and imaging anticancer agents, by us and other groups,[3–10] it appeared of interest to prepare more members belonging to this family of compounds and investigate their binding to all the transmembrane CA isoforms, in addition to CA IX. Indeed, similar to CA IX, CA XII[10–13] and XIV[10,14] are transmembrane isoforms with an extracellular active site, and they share a certain degree of homology with CA IX.[10–14] CA XII is also present in many tumors, like CA IX, whereas CA XIV is not associated with cancers but is widespread in many tissues such as the kidneys, liver and brain among others.[10] The precise function of these isozymes in all the tissues in which they are present is poorly understood,[10–14] except for tumors,[5,6] in which it has been demonstrated that they participate in the process of pH regulation during tumorigenesis. In fact all hypoxic tumors overexpress CA IX[5–8] (and many of them also CA XII)[5] as a consequence of the hypoxia inducible factor-1 (HIF-1) regulatory pathway.[7] The overall consequence of the strong CA IX/XII over-expression is the pH imbalance of the tumor tissue, with most hypoxic tumors having acidic pHe values around 6.5, in contrast to the normal tissue which has characteristic pHe values around 7.4.[5–9] Expression of CA IX is strongly increased in many types of solid tumors, such as gliomas/ependymomas, mesotheliomas, papillary/follicular carcinomas, as well as carcinomas of the bladder, uterine cervix, kidneys, esophagus, lungs, head and neck, breast, brain, vulva, and squamous/basal cell carcinomas, among others.[5–9,3b] CA IX/XII inhibition with sulfonamides was recently shown to reverse the effect of tumor acidification,[5] leading to inhibition of the primary tumor and metastases growth, and CA IX/XII have been proposed as novel therapeutic antitumor targets.[3,5,7,8]

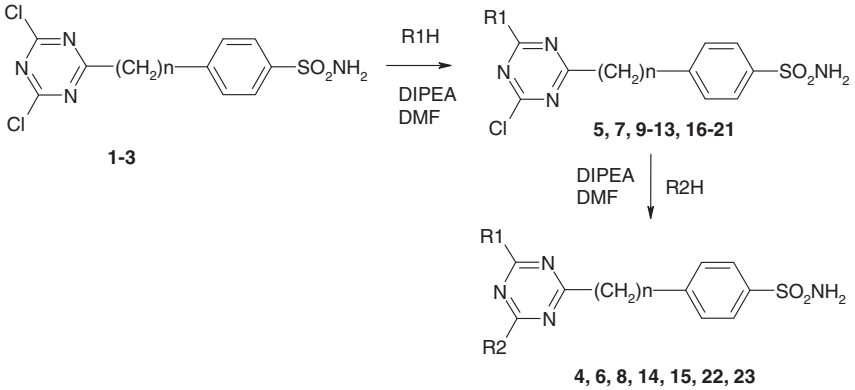
Reported here are novel sulfonamide CAIs obtained considering compounds **1–3** as leads, which incorporate the 1,3,5-triazine moiety, connected to potent and selective inhibition of the transmembrane, tumor-associated isoforms CA IX over the cytosolic CA I and II. The synthesis of derivatives which contain amino acyl and amino alcohol moieties in their molecules, due to the enhanced hydrophilicity of such derivatives, has been explored. Furthermore, the inhibition studies have been expanded to account for two transmembrane isoforms, CA XII and XIV, and report the X-ray crystal structures of two adduct of these new sulfonamides with hCA II, which afford new insights in the drug design of CAIs targeting various isoforms.

2. Results and discussion

2.1. Chemistry

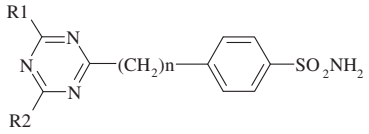
The rationale for obtaining novel sulfonamide CAIs based on the 1,3,5-triazine scaffold reported in this paper, was that of incorporating moieties which may induce an enhanced hydrophilic character, due to the fact that **1–3** and some of the compounds reported earlier,[1] showed poor water solubility.[1] Some α- and β-amino acids, aminoalcohols or polyols seemed to be a good option, as they generally induce favorable such properties, and in the previous communication[1b] we showed that three such compounds (the glycine, glycine methyl ester and β-alanine derivatives of **3**) possess excellent enzyme inhibitory properties against the tumor-associated isoform CA IX. Thus, these findings extend previous studies, and report here the synthesis of a larger series of such compounds, obtained by reaction of the dichloro-substituted triazines **1–3**[1] with one or two equivalents of nitrogen or oxygen nucleophiles, such as α- and β-amino acids and their esters, aminoalcohols as well as a monosilylated derivative of ethyleneglycol (Scheme 1). These nucleophiles were chosen in such a way as to contain moieties which may enhance water solubility and affinity to the enzyme for the new CAIs, and amino acids as well as amino alcohols seem to be an interesting choice.

Reaction of the key intermediate sulfonamides **1–3,** possessing two reactive chlorine atoms at the 1,3,5-triazine ring with these nucleophiles (R1H, R2H), in the molar ratio of 1:1, afforded by the replacement of one chlorine atom, the formation of a rather large series of mono-substituted derivatives (**5, 7, 9–13** and **16–21**) (Scheme 1). When working in molar ratios between the dichlorotriazines **1–3** and the nucleophile of 1:2, both chlorine atoms from **1–3** were replaced, leading to the disubstituted compounds **4, 6, 8, 14, 15, 22** and **23** (Scheme 1). The stepwise replacement of one, two or three chlorine atoms from cyanuryl chloride by means of aromatic nucleophilic substitution reactions was already documentated in the 50s.[2] The nucleophiles R1H, R2H employed in our syntheses included amino acids such as Gly, β-Ala, Ser, DOPA; amino acid esters, such as the methyl esters of Gly and Ala; aminoalcohols such as 2-aminoethanol and 4-amino-1-butanol, as well as the mono-*tert*-butyl-dimethylsilyl derivative of ethyleneglycol (Table 1). All chiral amino acids were racemic mixtures. They were



Scheme 1. Preparation of sulfonamides **4–23** by reaction of the dichloro-triazines **1–3** with nucleophiles (R1H, R2H) in the presence of diisopropylethylamine (DIPEA), in DMF.

Table 1
Inhibition data of human CA isoforms hCA I, II, IX, XII and XIV with sulfonamides **1–24** and standard sulfonamide inhibitors by a stopped flow CO2 hydrase assay[15]

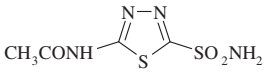


1-23

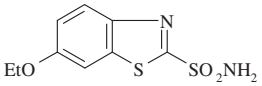
No.	*n*	R1	R2	K_I^* (nM)				
				hCA I	hCA II	hCA IX	hCA XII	hCA XIV
1	0	Cl	Cl	120[a]	106[a]	0.15[a]	0.35	34.3
2	1	Cl	Cl	136[a]	13[a]	124[a]	56.2	24.1
3	2	Cl	Cl	75[a]	21[a]	138[a]	43.8	15.4
4	0	$NHCH_2COOH$	$NHCH_2COOH$	4550	376	8.4	6.0	7.2
5	0	$NHCH_2COOH$	Cl	3032	351	7.9	10.2	9.3
6	0	$NHCH_2COOMe$	$NHCH_2COOMe$	502	435	9.4	8.7	7.0
7	0	$NHCH_2COOMe$	Cl	3075	41	8.2	6.7	6.5
8	0	$NH(CH_2)_2COOH$	$NH(CH_2)_2COOH$	673	368	8.9	0.85	0.92
9	0	NHCHMeCOOH	Cl	1324	561	0.96	5.8	10.1
10	0	$NHCH(CH_2OH)$–COOH	Cl	109	412	8.9	8.5	7.4
11	0	$NH(CH_2)_2OH$	Cl	1098	37	0.75	1.6	3.8
12	0	$NH(CH_2)_4OH$	Cl	1245	119	34.8	43.9	27.7
13	0	$O(CH_2)_2OSi(Me_2)$-*t*-Bu	Cl	3472	356	113	248	583
14	1	$NHCH_2COOMe$	$NHCH_2COOMe$	607	453	9.2	9.3	9.1
15	1	NHCHMeCOOH	NHCHMeCOOH	1659	435	8.5	9.2	8.9
16	1	$NH(CH_2)_2COOH$	Cl	2360	258	34.1	20.7	16.9
17	1	$NHCH[CH_2C_6H_3(OH)_2]$–COOH	Cl	3021	475	95	80	72
18	2	$NHCH_2COOH$	Cl	33[a]	32[a]	1.0[a]	2.6	10.4
19	2	$NHCH_2COOMe$	Cl	39[a]	33[a]	1.4[a]	2.9	23.5
20	2	$NH(CH_2)_2COOH$	Cl	35[a]	29[a]	1.7[a]	3.5	17.9
21	2	$NHCH[CH_2C_6H_3(OH)_2]$–COOH	Cl	1549	371	91	82	77
22	2	NHCHMeCOOH	NHCHMeCOOH	2040	409	8.4	8.6	8.8
23	2	$NHCH(CH_2OH)COOH$	$NHCH(CH_2OH)COOH$	3704	517	9.0	7.1	7.6
24	–	–	–	7[c]	12[c]	1.3	1.5	4.1
AAZ[b]	–	–	–	250	12	25	5.7	41
EZA[b]	–	–	–	25	8	34	22	2.5
DCP[b]	–	–	–	1200	38	50	50	345

[a] From Ref. 1.
[b] From Ref. 3a.
[c] From Ref. 20.
[*] Mean from three different assay, by a stopped flow technique (errors were in the range of ±5–10% of the reported values).

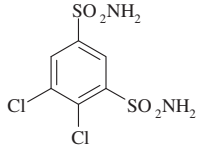
chosen in such a way as to afford supplementary insights regarding the structure–activity relationship (SAR) features of this novel family of CAIs.



AAZ



EZA



DCP

2.2. CA inhibition

The inhibition of five CA isoforms has been investigated with derivatives **1–23** and clinically used, standard CAIs such as acetazolamide (**AAZ**), ethoxzolamide (**EZA**) and dichlorophenamide (**DCP**).[15] The isoforms included in the study were the cytosolic hCA I and II as well as the transmembrane hCA IX, XII and XIV, all of which are involved in a host of important physiological and pathological functions in vertebrates.[3–10]

The following structure–activity relationship (SAR) can be observed from data of Table 1, for the inhibition of the CA isoforms investigated here with the new group of sulfonamides **1–23** reported here:

(i) The slow cytosolic isoform hCA I was inhibited by compounds **1–23** with K_Is in the range of 33–4550 nM. Most of these compounds were ineffective as hCA I inhibitors, except for the previously reported derivatives **18–20** which incorporate the aminoethylbenzenesulfonamide moiety ($n = 2$) and one Gly, GlyOMe or β-Ala moieties, which had K_Is in the range of 33–39 nM, comparable to that of **EZA** (K_I of 25 nM). Medium efficacy as hCA I inhibitors has been observed for the leads **1–3** and for compound **10**, which showed K_Is in the range of 75–136 nM. The remaining compounds had inhibition constants >500 nM, being thus rather inefficient hCA I inhibitors (Table 1).

(ii) The investigated sulfonamides inhibited hCA II, the physiologically dominant and highly active cytosolic isoform,[3] with K_Is in the range of 13–517 nM. Several compounds, among which **2, 3, 7**, **11** and **18–20** showed effective hCA II inhibition. Similar to the clinically used derivatives **AAZ**, **EZA** and **DCP**, these compounds inhibited this isoforms with inhibition constants of 13–41 nM. In

addition to the leads 2 and 3, which contain two chlorine atoms on the 1,3,5-triazine ring, the other effective hCA II inhibitors possess one chlorine atom and one Gly, GlyOMe or β-Ala moieties in their molecule. The compounds with a longer spacer ($n = 2$) were more effective as hCA II inhibitors compared to the ones incorporating shorter such linkers ($n = 0$ or 1). It may be also observed that the presence of two amino acyl moieties on the triazine ring leads to less effective inhibitors compared to the compounds with only one such moiety (compare 6 and 7 for example). The SAR is in fact not very regular, although generally, for the same/similar substitution pattern on the triazine ring, the longer spacer compound ($n = 2$) was more effective an inhibitor than the compound with the intermediate spacer ($n = 1$) which in turn was more effective than the short spacer derivative ($n = 0$). Rather bulky amino acid residues (e.g., DOPA, Ser, etc), or two such groups in the molecule, also led to less effective hCA II inhibitors compared to compounds possessing more compact, one such moiety. The short aminoalcohol derivative **11** was also more effective than the bulkier compounds **12** (with a longer spacer between the amino and OH moieties compared to **11**) and **13** (incorporating the bulky *tert*-butyl-dimethylsilyloxyethyloxy moiety).

(iii) The tumor-associated isoform hCA IX was inhibited by compounds **1–23** with K_Is in the range of 0.15–138 nM (Table 1). Several subnanomolar and low nanomolar inhibitors of this important drug target have been evidenced, in addition to **1**, reported earlier.[1] They are: **9, 11** (K_Is <1 nM) and **4–8, 10, 14, 15, 18–20, 22** and **23** (K_Is in the range of 1.0–9.4 nM). The least effective inhibitors were **2, 3, 13, 17** and **21**, with K_Is in the range of 91–138 nM. These observations therefore indicate that the sulfonamides incorporating the 1,3,5-triazinyl moiety lead to highly effective hCA IX inhibitors, for a rather large number of substitution patterns. In the series of reported compounds, the best inhibitors were those incorporating sulfanilamide ($n = 0$), one chlorine as substituent of the triazine moiety and either one Ala or aminoethanol moieties as the second substituent of the triazine ring (compounds **9** and **11**). Highly effective were also the Gly, GlyOMe or β-Ala monoderivatives (**18–20**) with the longer spacer ($n = 2$) in their molecules, whereas the loss of activity was correlated with the presence of bulkier moieties substituting the 1,3,5-triazine ring (as in **13, 17** and **21**). It is interesting to note (but rather difficult to explain) that even if leads 2 and 3 are rather ineffective as hCA IX inhibitors, all substitutions done in these compounds by replacing one or both chlorine atoms with amino acyl or amino acyl ester moieties, led to compounds with enhanced hCA IX inhibitory properties. On the other hand, not the same can be said on the lead **1**. All substitution done on this compound led to less effective hCA IX inhibitors, but the loss of activity was (with few exceptions, **12** and **13**) not significant and an important feature of the new derivatives reported here is their enhanced hydrophilicity compared to **1**, which is poorly soluble in most organic solvents and water.[1] It may be also observed that most of the new triazinyl sulfonamides reported here show much better hCA IX inhibitory effects compared to the standard inhibitors **AAZ, EZA** and **DCP**.

(iv) The inhibition of the second isoform associated to tumors, hCA XII, has not been investigated earlier with this family of derivatives. It may be observed from data of Table 1, that similar to hCA IX, the lead **1** was a highly effective hCA XII inhibitor (K_I of 0.35 nM) whereas its close congeners with longer spacers **2** and **3** ($n = 1$ and 2) were much less effective, with K_Is in the range of 43.8–56.2 nM. The substitution of the chlorine atom(s) from **1**, in compounds such as **4–12** ($n = 0$) led to a modest loss of hCA XII inhibitory activity for derivatives **4–11** (K_Is of 0.85–10.2 nM) and to a more important loss for the bulkier derivatives **12** and **13** (K_Is of 43.9–248 nM). As for hCA IX, in the case of the leads **2** and **3**, all substitution patterns explored here led to more effective hCA XII inhibitors (K_Is in the range of 2.6–9.3 nM for compounds **14–16, 18–20, 22** and **23**), except compounds **17** and **21**, both incorporating the bulky DOPA moieties, which were weaker inhibitors (K_Is of 80–82 nM) compared to the parent sulfonamides from which they were obtained. Thus, as for hCA IX discussed above, many of the new triazinyl sulfonamides reported here showed highly effective hCA XII inhibitory properties.

Table 2
Inhibition profile of isoforms human (h) CA IV, VA, VB, VI, VII and XIII, as well as the fungal enzyme Nce103 (*Candida albicans*) and Can2 (*Cryptococcus neoformans*) with two of the triazinyl sulfonamides reported in the paper, compounds **5** and **18**. AAZ (standard inhibitor) inhibition data are also shown

Enzyme	K_I* (nM)		
	5	**18**	**AAZ**
hCA IV	89	97	74
hCA VA	48	65	63
hCA VB	24	23	54
hCA VI	59	76	11
hCA VII	9.4	8.3	2.5
hCA XIII	62	68	5.7
Nce103	9.0	9.7	132
Can2	5.5	8.2	10.5

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

(v) The inhibition of the third transmembrane isoform, hCA XIV, was also investigated here for the first time with triazinyl sulfonamides. The leads **1–3** showed significant inhibitory activity, with K_Is in the range of 34.3–15.4 nM. There is a rather regular SAR for these derivatives, as the inhibition of hCA XIV increases with the increase of the spacer from $n = 0$ in the sulfanilamide derivative **1**, to $n = 2$ in the aminoethylbenzenesulfonamide derivative **3**, which was more than two times a better hCA XIV inhibitor compared to **1**. Most of the derivatives obtained from **1–3** as leads, were also highly effective hCA XIV inhibitors except for **12, 13, 17** and **21**, all incorporating rarher bulky groups, which were the least effective inhibitors (K_Is in the range of 27.7–583 nM). Thus, the di-β-Ala derivative **8** was a subnanomolar hCA XIV inhibitor (and the most potent such inhibitor reported to date).[3] Effective inhibition (K_Is <10 nM) was observed for many other such derivatives, among which **4–7, 10, 11, 14, 15, 22** and **23**. However, the SAR for the inhibition of this isoform is rather different compared to that for the inhibition of hCA IX and XII (which, as outlined above, were in fact quite similar).

(vi) In order to explore the complete inhibition profile of this class of sulfonamide CAIs, two of the most active compounds (against the isoforms discussed above), **5** and **18**, have been assayed for the inhibition of the remaining six catalytically active human isoforms (hCA IV–XIII)[3] as well as two β-CAs from the pathogenic fungi *Candida albicans* (Nce103) and *Cryptococcus neoformans* (Can2).[16] These two enzymes play an important role in the life cycle of these organisms and their inhibition has recently been proposed as a new approach to design antifunglas.[16] Data of Table 2 show that hCA IV, hCA VA, hCA VB, hCA VI and hCA XIII are significantly inhibited by these two sulfonamides, with K_Is in the range of 23–97 nM, but one human isoform (hCA VII) as well as the two fungal β-CAs are even more sensitive to inhibition, with K_Is in the range of 5.5–9.7 nM. Thus, this family of sulfonamide CAIs may have potential to also design inhibitors of β-CAs, enzymes quite widespread in many pathogenic fungi and bacteria, such as among others *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, *Brucella suis, Streptococcus pneumoniae*.[3,16,17]

2.3. X-ray crystallography

In order to rationalize inhibition of CAs with this series of compounds, X-ray crystallographic studies were performed on adducts

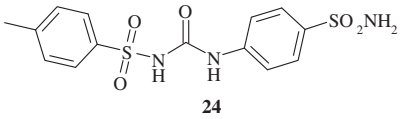
Table 3
Data and final model statistics for the hCA II adducts with compounds **11** and **7**

Data-collection statistics		
PDB Accession number	3MMF	3MNA
Compound	**11**	**7**
Temperature (K)	100	100
Wavelength (Å)	1.5418	1.5418
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å,°)	a = 42.34	42.36
	b = 41.24	41.23
	c = 72.05	72.02
	β = 104.31,	β = 104.21,
	$\alpha = \gamma$ = 90	$\alpha = \gamma$ = 90
Total number of possible reflections	38930	38929
Total number of unique reflections	38628	38812
Resolution (Å)	23.8–1.5 (1.55–1.5)*	25.3–1.5 (1.55–1.5)
R_{sym}	6.4% (34.1%)	7.1 (31.6)
$I/\sigma(I)$	15.8 (3.4)	15.0 (4.5)
Completeness	99.2 (97.1)	99.7 (99.0)
Redundancy	3.6 (3.3)	4.0 (3.7)
Final model statistics		
[a]R_{cryst} (%)	15.9	14.5
[b]R_{free} (%)	18.2	17.0
Residue Nos.	4–261	4–261
No. of protein atoms (including alternate conformations)	2086	2078
No. of drug atoms	22	28
No. of H_2O molecules	312	340
R.m.s.d. for bond lengths (Å), angles (°)	0.010, 1.4	0.009, 1.3
Ramachandran statistics (%)	88.9	88.0
Most favored, additionally allowed and generously allowed regions	10.6 0.5	11.6 0.5
B factors (Å^2)		
Average, main-, side-chain, compound, solvent	16.6, 21.2, 21.2, 31.8	13.2, 17.9, 19.9,29.5

[c] R_{free} is calculated in same manner as [b]R_{cryst}, except that it uses 5% of the reflection data omitted from refinement.
[a] $R_{sym} = (\Sigma|I - \langle I\rangle|/\Sigma\langle I\rangle) \times 100$.
[b] $R_{cryst} = (\Sigma||F_o| - |F_c||/\Sigma|F_{obs}|) \times 100$.
* Values in parenthesis represent highest resolution bin.

of hCA II with two such compounds which showed good affinity for this isoform, that is, compounds **7** and **11** (K_Is of 41 and 37 nM, respectively, Table 1). The final model statistics for the hCA II adducts with compounds **7** and **11** are shown in Table 3.

The electron density of all moieties from inhibitors **7** and **11** was clearly seen in the two adducts with the enzyme (Fig. 1). In the refined models sulfonamides **7** and **11** bind to the zinc ion in the active site through the N1 atom of the sulfonamide moiety at distances of ~2.0 Å. As in other published hCA II-sulfonamide complexes,[17–20] additional hydrogen bonds from Thr199 N and OG to the sulfonamide oxygen atoms are present. The inhibitors are both rigid molecules, and their C10 extensions make no hydrophobic contacts with the enzyme (Figs. 1 and 2). The chlorine atom of compounds **7** and **11** contact the side-chain atoms of Ile91 and Gln92, in addition to making hydrogen bonds with water molecules (Fig. 2). The triazine ring of compounds **7** and **11** stacks upon the aromatic carbon atoms of the Phe131 phenyl ring. Both compounds are significantly non-planar, possibly due to the steric contact between the benzene and triazine rings across the C4–N7–C8 valence angle. The twist can be described by two torsion angles: C(5)–C(4)–N(7)–C(8), 161.3° (compound **11**) and 156.2° (compound **7**), and C(4)–N(7)–C(8)–N(9), −178.9° and 175.8°, respectively. Neither of the torsion angle C9–C10–N11–C15 deviates by more than 13° from the triazine ring. Both terminal C15 substituents are approximately normal to the planes of the triazine ring. The electron density of the C15 terminal substituent of compounds **7** or **11** are weak and neither interact directly with protein atoms lining the active site, but rather an array of ordered water molecules. The terminal alcohol oxygen atom in compound **11** is located



24

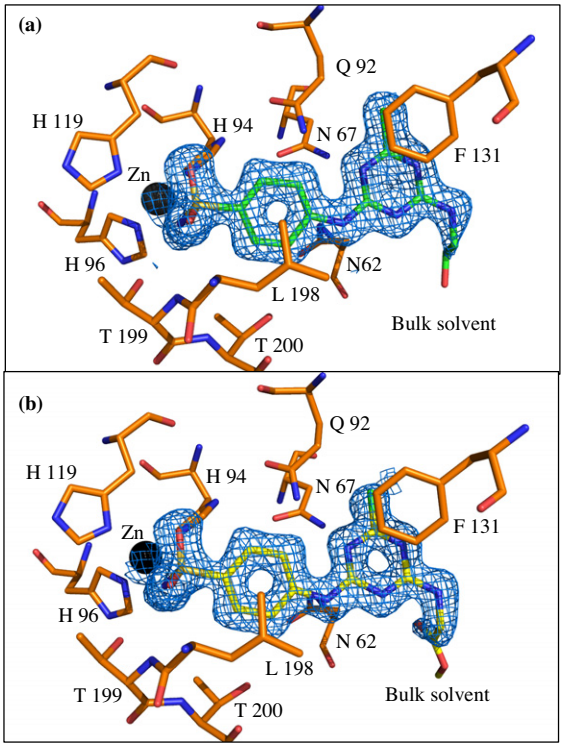


Figure 1. Stick representation of compound (a) **11** (green) and (b) **7** (yellow) bound in the active site of hCA II. The electron density is represented by a 2σ-weighted $2F_o - F_c$ Fourier map (blue mesh). Amino acids are as labeled. Figure made using PyMOL (DeLano Scientific).

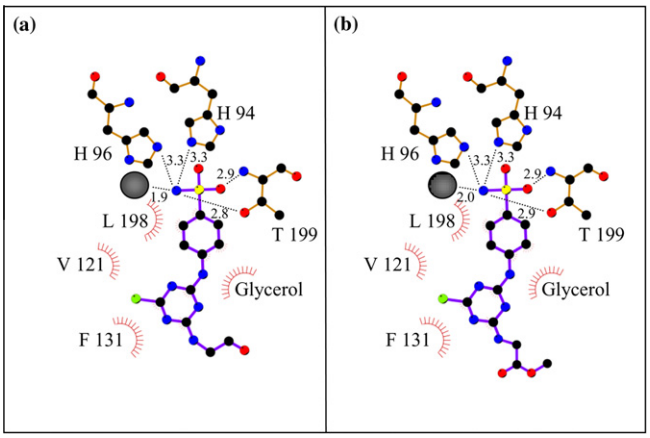


Figure 2. Schematic 2D-representation of hCA II—compound (a) **11** and (b) **7** interactions when bound to the enzyme. Hydrophobic contacts are indicated by red hash marks. H-bond lengths and the zinc coordination (Å) are indicated by black dashed lines. Atoms are represented as spheres: zinc, grey; carbon, black; oxygen, red; nitrogen, blue; sulfur, yellow; chlorine, green). Figure made using Ligplot.

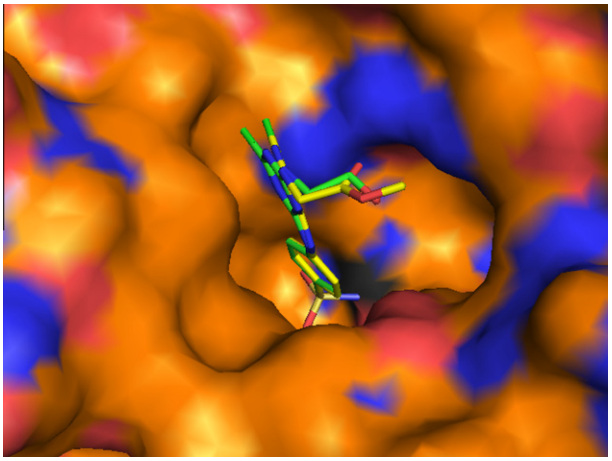


Figure 3. View of compound **7** and **11** superposed in the active site of hCA II. hCA II is depicted as a surface representation (oxygen, red; nitrogen, blue; carbon, yellow). Compounds **11** (green) and **7** (yellow) are represented as sticks. Atoms of inhibitor molecules are colored as in Figure 1. Figure made using PyMOL (DeLano Scientific).

approximately in the position of the methyl ester of compound **7** (Fig. 3).

In order to rationalize why the triazinyl-substituted benzenesulfonamides as an entire class show such strong CA inhibitory properties against the physiologically dominant isoforms (e.g., CA II, IX and XII) and in some cases also selectivity for inhibiting transmembrane over cytosolic isoforms, we include in our analysis sulfonamide **24**,[20] the tosylureido derivative of sulfanilamide. The synthesis and hCA I, II and IV inhibition data with this compound have been reported earlier,[20] and alsoits X-ray crystal structure in adduct with CA II is available (PDB file 1ZFK). Similar to the triazinyl derivatives investigated here, such as among others **7** and **11**, compound **24** possesses the 4-amino-benzenesulfonamide scaffold to which a diverse tail is attached compared to the triazinyl moieties. Furthermore, **24** is a very strong inhibitor of all CA isoforms investigated here, hCA I, II, IX, XII and XIV, with inhibition constants in the range of 1.3–12 nM. Thus, the main difference between the triazinyl substituted compounds **7** and **11** compared to **24**, is that the latter demonstrated no significant selectivity for inhibiting the transmembrane over the cytosolic isoforms. Comparing the X-ray crystal structures of the adducts of hCA II with the triazinyl derivative **11** and the tosylureido sulfanilamide **24** (Fig. 4), one may observed that the sulfanilamide fragment of the two inhibitors are totally superposable when bound to the enzyme active site. However, the triazinyl-substituted tail from **11** and the tosylureido one from **24**, are orientated towards opposite parts of the active site. Thus, we hypothesize that these two very different binding modes of the tails fragments of the two inhibitors are responsible for the important difference in selectivity for the inhibition of the cytosolic over transmembrane isoforms with these types of CAIs. The triazinyl portion of the molecule, through the variable twisting evidenced above for the two adducts described here, allows for an orientation of the inhibitor molecule in regions of the active site where no other classes of sulfonamides have been observed earlier.[1,12,13]

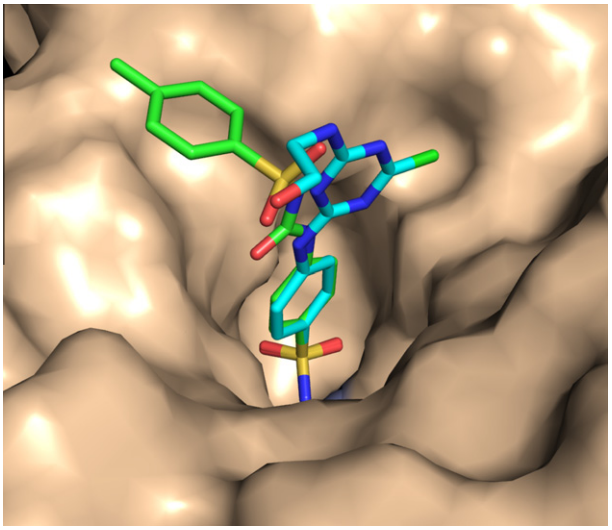


Figure 4. View of compound **11** and **24** (PDB file 1zfk) superposed within the active site of hCA II. Figure made using PyMOL (DeLano Scientific).

3. Conclusions

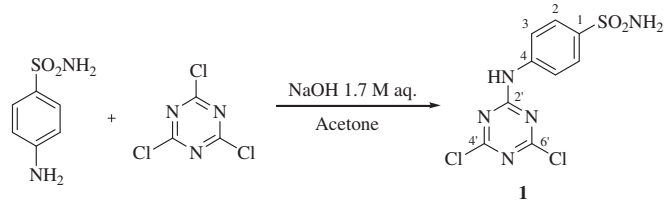
A new series of triazinyl-substituted benzenesulfonamides incorporating amino acyl moieties are reported, together with inhibition studies of physiologically relevant CA isoforms, such as CA I, II, IX, XII and XIV. These compounds showed moderate-weak inhibition of the cytosolic, offtarget isozymes CA I and II, but many of them were low nanomolar inhibitors of the transmembrane, tumor-associated CA IX and XII (and also of CA XIV). The X-ray crystal structure of two of these compounds in adduct with hCA II allowed for observing structural features associated with this strong inhibitory properties and possibly also their selectivity. Two of these compounds were also investigated for the inhibition of other human isoforms, that is, hCA IV, VA, VB, VI, VII and XIII, as well as inhibitors of the fungal pathogenic CAs Nce103 (*Candida albicans*) and Can2 (*Cryptococcus neoformans*), showing interesting activity. The 1,3,5-triazinyl-substituted benzenesulfonamides constitute thus a class of compounds with great potential for obtaining inhibitors targeting both mammalian, tumor-associated and pathogenic organisms β-class CAs.

4. Experimental protocols

4.1. Chemistry

Anhydrous solvents and all reagents were purchased from Sigma–Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. NaH 60% in oil dispersion was washed with *n*-hexane until a homogeneous white solid was obtained, dried and stored under a nitrogen atmosphere prior to use. Infrared (IR) spectra were recorded as KBr plates and are expressed in ν (cm^{-1}). Nuclear magnetic resonance (^1H NMR, ^{13}C NMR, DEPT-135, DEPT-90, HSQC, HMBC) spectra were recorded using a Bruker Advance III 400 MHz spectrometer in $CDCl_3$, MeOH-d_4 or in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q, quadruplet; m, multiplet; br s, broad singlet; dd, double of doubles, appt, apparent triplet, appq, apparent quartet. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica Gel 60 (230–400 mesh ASTM) as the stationary phase and ethylacetate/*n*-hexane or MeOH/DCM were used as eluents. Melting points (mp) were carried out in open capillary tubes and are uncorrected.

4.1.1. Synthesis of 4-(4′,6′-dichloro-1′,3′,5′-triazin-2′-ylamino)-benzenesulfonamide 1[1,2]

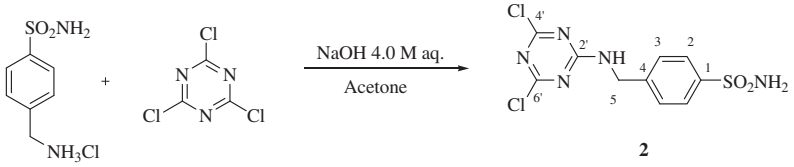


1

A 1.0 M solution of 4-aminobenzenesulfanilamide (17.2 g, 0.1 mol, 1.0 equiv) in acetone (100 ml) was added dropwise to a vigorously stirred suspension of cyanuric chloride (18.4 g, 1.0 equiv) in the same solvent (100 ml) at 0 °C. The white slurry was stirred at the same temperature for 30 min. and then a 1.7 M aqueous solution of NaOH (4.0 g, 1.0 equiv) was added over a period of 20 min. Stirring was continued for 1 h, the reaction was quenched by addition of slush (100 ml) and the solid filtered off. Crystallization from acetone afforded the title compound **1** as a white solid.

4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1**: 77% yield; mp 190–193 °C; δ_H (400 MHz, DMSO-d_6) 7.34 (2H, s, SO_2NH_2, exchange with D_2O), 7.80 (2H, d, J 8.8, 2 × Ar-H), 7.87 (2H, d, J 8.8, 2 × Ar-H); δ_C (100 MHz, DMSO-d_6) 170.3, (C-4′). 164.9 (C-2′), 140.9 (*ipso*), 140.8 (*ipso*), 127.6, 122.1.

4.1.2. Synthesis of 4-[(4′,6′-dichloro-1′,3′,5′-triazin-2′-ylamino)-methyl]benzenesulfonamide 2[1a]

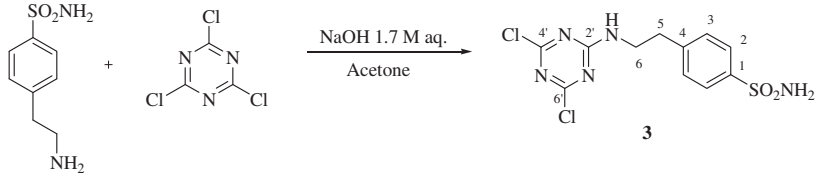


2

The reaction was carried out according to the previous procedure using 4-aminomethylbenzenesulfonamide hydrochloride (26.9 g, 1.0 equiv), cyanuric chloride (22.3 g, 1.0 equiv) and NaOH (9.7 g, 2.0 equiv).

4-[(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)methyl]benzenesulfonamide **2**: mp 210–221 °C; δ_H (400 MHz, DMSO-d_6) 4.63 (2H, d, J 6.2, 5-H_2), 7.38 (2H, s, SO_2NH_2, exchange with D_2O), 7.51 (2H, d, J 8.4, 2 × Ar-H), 7.82 (2H, d, J 8.4, 2 × Ar-H), 9.69 (1H, t, J 6.2, N*H*, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 170.6. 169.7, 166.7 (C-2′), 144.0 (*ipso*), 142.5 (*ipso*), 128.6, 126.8, 44.6 (C-5).

4.1.3. Synthesis of 4-[6-(4′,6′-dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide 3[1]

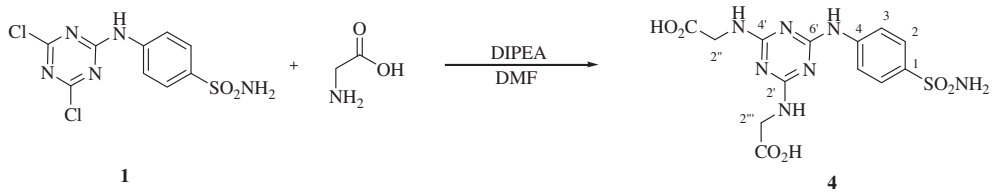


3

The reaction was carried out according to the previous procedure using 4-(2-aminoethyl)benzenesulfonamide (20.0 g, 1.0 equiv), cyanuric chloride (18.4 g, 1.0 equiv) and NaOH (4.0 g, 1.0 equiv). Crystallization from acetone afforded the title compound **3** as a white solid.

4-[6-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide **3**: 63% yield; mp 298–300 °C; δ_H (400 MHz, DMSO-d_6) 2.95 (2H, t, J 6.2, 5-H_2), 3.59 (2H, q, J 6.2, 6-H_2), 7.34 (2H, s, SO_2NH_2, exchange with D_2O), 7.46 (2H, d, J 8.4, 2 × 3-H), 7.78 (2H, d, J 8.4, 2 × 2-H), 9.26 (1H, t, J 6.2, N*H*, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 170.0, 166.2 (C-2′), 143.8 (*ipso*), 143.2 (*ipso*), 130.2 (C-3), 126.6 (C-2), 42.7 (C-6), 34.6 (C-5).

4.1.4. Synthesis of 2″,2‴-[6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazine-2′,4′-diyl]-bis(azanediyl)diacetic acid 4



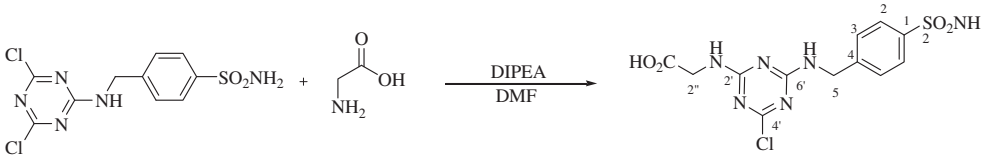
1 4

4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.1 g, 1.0 equiv) and glycine (2.4 equiv) were dissolved in dry

DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred at 60 °C under a nitrogen atmosphere until starting material was consumed (TLC monitoring). Then the reaction was quenched with slush and the precipitate formed was collected by filtration, washed whit H_2O and dried under high vacuo to give the title compound as a white solid.

2″,2‴-[6′-(4-Sulfamoylphenylamino)-1′,3′,5′-triazine-2′,4′-diyl]-bis(azanediyl)diacetic acid **4**: 32% yield; mp 304 °C with decomposition; ν_{max} (KBr) cm^{-1}, 3370, 2838, 1730 (C=O, acid), 1598 (aromatic); δ_H (400 MHz, DMSO-d_6, 80 °C) 3.95 (2H, d, *J* 5.0, 2″/2‴-H_2), 4.10 (2H, d, *J* 5.0, 2″/2‴-H_2), 7.20 (2H, s, SO_2NH_2, exchange with D_2O), 7.30 (2H, d, *J* 8.2, 2 × 3-H), 7.80 (2H, d, *J* 8.2, 2 × 2-H), 7.90 (2H, m, 2 × CH_2NH, exchange with D_2O), 9.82 (1H, br s, N*H*, exchange with D_2O); δ_C (100 MHz, DMSO-d_6, 80 °C) 171.0 (C=O), 164.8, 157.0, 143.6, 138.2, 127.2, 120.4, 43.3 (C-2″).

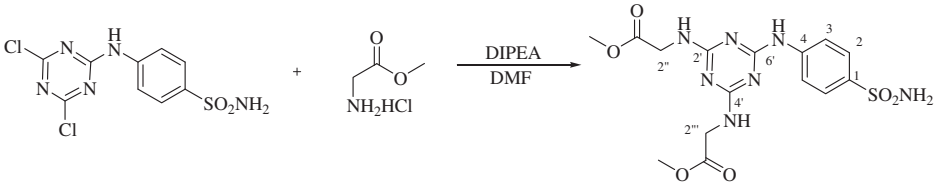
4.1.5. Synthesis of 2″-[4′-chloro-6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazin-2′-ylamino]acetic acid 5



4-[(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)methyl]benzenesulfonamide **2** (0.1 g, 1.0 equiv) and glycine (2.4 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring). Then the reaction was quenched with slush and the precipitate formed was collected by filtration, washed whit H_2O and dried under high vacuo to give the title compound as a white solid.

2″-[4′-Chloro-6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazin-2′-ylamino]acetic acid **5**: 28% yield; mp 226–231 °C; ν_{max} (KBr) cm^{-1}, 3365, 2838, 1732 (C=O, acid), 1560 (aromatic); δ_H (400 MHz, DMSO-d_6, 80 °C) 3.69 (2H, d, *J* 5.0, 2″-H_2), 4.50 (2H, d, *J* 5.0, 5-H_2), 6.30 (1H, br s, exchange with D_2O), 7.34 (2H, s, exchange with D2O, SO_2NH_2), 7.49 (2H, m, , 2 × 3-H), 7.80 (2H, m, 2 × 2-H); δ_C (100 MHz, DMSO-d_6, 80 °C) 173.4 (C=O), 172.3 (C-4′), 168.7 (C-6′), 165.8 (C-2′), 144.3 (*ipso*), 143.5 (*ipso*), 128.3 (C-3), 126.5 (C-2), 44.4 (C-5), 42.2 (C-2″).

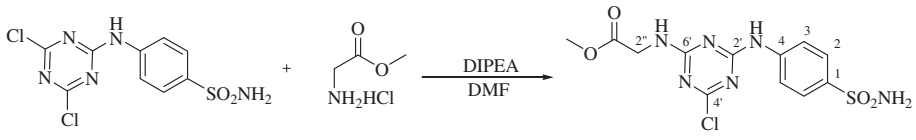
4.1.6. Synthesis of dimethyl 2′,2‴-[6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)diacetate 6



4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.1 g, 1.0 equiv) and methyl 2-aminoacetate hydrochloride (4.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was treated according to the procedure reported above to afford the title compound as a white solid.

Dimethyl 2′,2‴-[6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)diacetate **6**: 36% yield; mp 254 °C with decomposition; ν_{max} (KBr) cm^{-1}, 3330, 2820, 1760 (C=O, ester), 1566 (aromatic); δ_H (400 MHz, DMSO-d_6) 3.67 (6H, s, 2 × CH_3), 4.10 (2H, d, *J* 5.0, 2 × 2″-H_2), 7.20 (2H, s, SO_2NH_2, exchange with D_2O), 7.30 (2H, d, *J* 8.2, 2 × 3-H), 7.80 (2H, d, *J* 8.2, 2 × 2-H), 8.90 (2H, m, 2 × CH_2–N*H*, exchange with D_2O), 10.40 (1H, br s, –N*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 172.0, 167.3, 149.9, 139.2, 138.0, 127.6, 121.1, 54.0, 44.2.

4.1.7. Synthesis of methyl 2″-(4′-chloro-6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazin-2′-ylamino)acetate 7

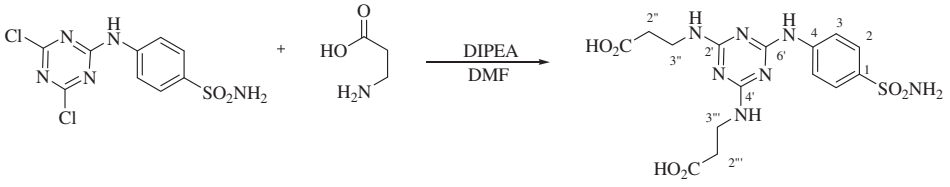


4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.4 g, 1.0 equiv) and methyl 2-aminoacetate hydrochloride (0.17 g, 2.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (0.36 g, 4.4 equiv) was added. The reddish solution was stirred

O.N. at 90 °C then quenched with a 1.0 M aqueous solution of hydrochloric acid (40 ml). The precipitate formed was collected by filtration, washed with H_2O and dried under vacuo to afford the title product as a white solid.

Methyl 2″-(4′-chloro-6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazin-2′-ylamino)acetate **7**: 35% yield; mp 271–273 °C; ν_{max} (KBr) cm^{-1}, 3360, 2820, 1762 (C═O, acid), 1559 (aromatic); δ_H (400 MHz, DMSO-d_6) 3.70 (3H, s, CH_3), 4.12 (2H, d, *J* 5.0, 2″-H_2), 7.30 (2H, s, SO_2NH_2, exchange with D_2O), 7.79 (4H, m, Ar-H), 8.70 (1H, br s, CH2–N*H*–, exchange with D_2O), 10.56 (1H, br s, –N*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 171.0, 169.3, 166.7, 164.3, 142.6, 139.0, 127.3, 120.5, 52.8 (*C*H_3), 43.2 (C-2″).

4.1.8. Synthesis of 3″,3‴-[6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)dipropanoic acid 8



1 β-Ala **8**

4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.1 g, 1.0 equiv) and 3-aminopropanoic acid (4.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was treated according to the procedure reported above to afford the title compound as a white solid.

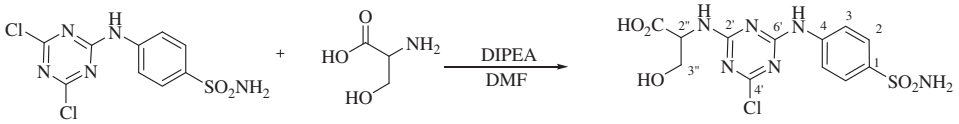
3″,3‴-[6′-(4-Sulfamoylphenylamino)-1′,3′,5′-triazine-2′,4′-diyl] bis(azanediyl)dipropanoic acid **8**: 55% yield; mp 308–310 °C; ν_{max} (KBr) cm^{-1}, 3348, 2820, 1715 (C═O, acid), 1560 (aromatic); δ_H (400 MHz, DMSO-d_6) 2.60 (4H, br m, 2 × 3″-H_2), 3.63 (4H, br m, 2 × 2″-H_2), 7.27 (2H, d, *J* 8.2, 2 × 3-H), 7.76 (2H, d, *J* 8.2, 2 × 2-H), 7.89 (2H, s, SO_2NH_2, exchange with D_2O), 9.80 (2H, m, 2 × CH_2–N*H*, exchange with D_2O), 10.40 (1H, br s, N*H*, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 174.0, 167.2, 158.2, 143.6, 138.4, 128.5, 120.0, 37.2, 34.7.

4.1.9. Synthesis of D,L-2″-[4′-chloro-6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazin-2′-ylamino]propanoic acid 9

4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.1 g, 1.0 equiv) and D,L-alanine (1.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (2.5 equiv) was added. The reaction was treated according to the procedure reported above to afford the title compound as a white solid.

D,L-2″-[4′-Chloro-6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazin-2′-ylamino]propanoic acid **9**: 27% yield; mp 322–325 °C; ν_{max} (KBr) cm^{-1}, 3332, 2850, 1722 (C═O, acid), 1555 (aromatic); δ_H (400 MHz, DMSO-d_6) 1.40 (3H, d, *J* 5.4, CH_3), 4.40 (1H, br m, 2″-H), 7.20 (2H, d, *J* 8.2, 2 × 3-H), 7.80 (2H, d, *J* 8.2, 2 × 2-H), 7.90 (2H, s, SO_2NH_2, exchange with D_2O), 9.80 (2H, br s, CH_2N*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 175.2, 170.0, 165.3, 157.8, 143.7, 138.0, 128.2, 120.0, 50.1, 19.8.

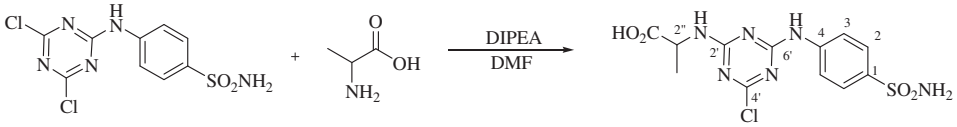
4.1.10. Synthesis of D,L-2″-[4′-chloro-6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazin-2′-ylamino]-3″-hydroxypropanoic acid 10



1 D,L-Ser **10**

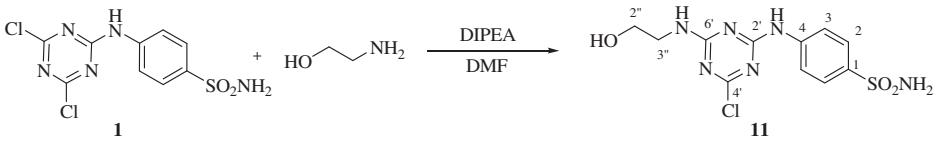
4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.1 g, 1.0 equiv) and D,L-serine (1.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (2.5 equiv) was added. The reaction was treated according to the procedure reported above to afford the title compound as a white solid.

D,L-2″-[4′-Chloro-6′-(4-sulfamoylphenylamino)-1′,3′,5′-triazin-2′-ylamino]-3″-hydroxypropanoic acid **10**: 32% yield; mp 255–257 °C; ν_{max} (KBr) cm^{-1}, 3350, 2827, 1712 (C═O, acid), 1560 (aromatic); δ_H (400 MHz, DMSO-d_6) 3.30 (1H, m, 3″-H*H*), 3.72 (1H, br m, 2″-H), 4.20 (1H, m, 3″-*H*H), 7.28 (2H, s, SO_2NH_2, exchange with D_2O), 7.42 (2H, d, *J* 8.2, 2 × 3-H), 7.77 (2H, d, *J* 8.2, 2 × 2-H), 8.10 (1H, br s, CHN*H*-, exchange with D_2O), 10.34 (1H, br s, N*H*); δ_C (100 MHz, DMSO-d_6) 175.3, 167.0, 166.4, 165.0, 145.2, 141.6, 130.2, 128.5, 68.2, 56.0.



1 D,L-Ala **9**

4.1.11. Synthesis of 4-[4′-chloro-6′-(2″-hydroxyethylamino)-1′,3′,5′-triazin-2′-ylamino] benzenesulfonamide 11

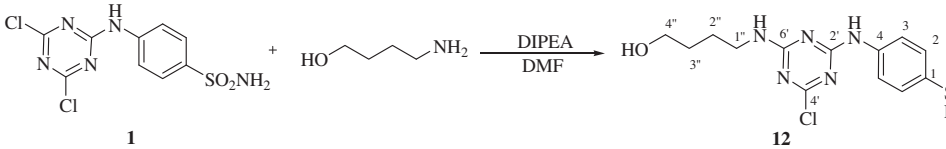


1 11

4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (1.0 g, 1.0 equiv) was dissolved in dry DMF (20 ml) followed by addition of ethanolamine (0.19 g, 1.0 equiv) and DIPEA (0.48 g, 1.2 equiv). The reaction was treated according to the procedure reported above to afford the title compound as a white solid.

4-[4′-Chloro-6′-(2″-hydroxyethylamino)-1′,3′,5′-triazin-2′-ylamino] benzenesulfonamide **11**: 65% yield; mp 290–293 °C; ν_{max} (KBr) cm^{-1}, 3260, 2830, 1559 (aromatic); δ_H (400 MHz, DMSO-d_6, 2 atropoisomers were detected in 1/0.5 ratio. Only the major one is reported herein) 3.42 (2H, m, 3″-H_2), 3.59 (2H, m, 2″-H_2), 4.81 (1H, t, *J* 5.2, O-*H*, exchange with D_2O), 7.30 (2H, s, SO_2NH_2, exchange with D_2O), 7.78 (2H, m, Ar-H), 7.92 (2H, m, Ar-H), 8.27 (1H, br m, CH_2NH–, exchange with D_2O), 10.35 (1H, br s, –N*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 169.2, 166.5, 164.3, 143.0, 138.7, 127.3, 120.3, 60.1, 44.2.

4.1.12. Synthesis of 4-[4′-chloro-6′-(4″-hydroxybutylamino)-1′,3′,5′-triazin-2′-ylamino]benzenesulfonamide 12

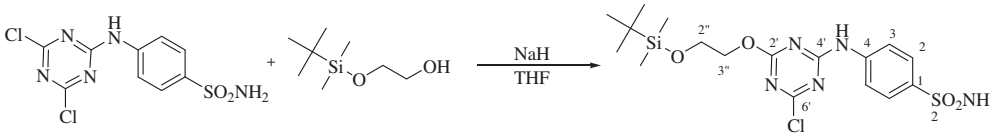


1 12

4-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.1 g, 1.0 equiv) and 4-amonobutan-1-ol (1.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (2.5 equiv) was added. The reaction was treated according to the procedure reported above to afford the title compound as a white solid.

4-[4′-Chloro-6′-(4″-hydroxybutylamino)-1′,3′,5′-triazin-2′-ylamino]benzenesulfonamide **12**: mp 270–271 °C; silica gel TLC R_f 0.10 (MeOH/DCM 10%); ν_{max} (KBr) cm^{-1}, 3295, 2813, 1560 (aromatic); δ_H (400 MHz, DMSO-d_6, 2 atropoisomers were detected in 1/0.4 ratio. Only the major one is reported herein) 1.54 (4H, m, 2″-H_2, 3″-H_2), 3.11 (2H, m, 1″-H_2), 3.47 (2H, m, 4″-H_2), 4.20 (1H, t, *J* 5.4, O-*H*, exchange with D_2O), 7.28 (2H, s, SO_2NH_2, exchange with D_2O), 7.88 (4H, m, Ar-H), 8.39 (1H, br m, CH_2NH-, exchange with D_2O), 10.37 (1H, br s, –N*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 173.2, 172.4, 165.1, 145.0, 131.1, 130.5, 118.6, 66.0, 45.1, 32.0, 26.0.

4.1.13. Synthesis of 4-(4′-(2″-(*tert*-butyldimethylsilyloxy)-ethoxy)-6′-chloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide 13

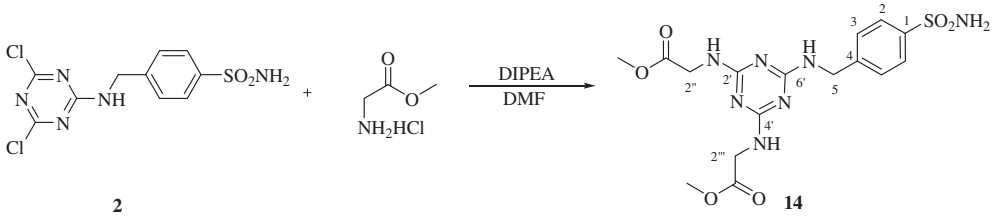


1 13

2-(*tert*-Butyldimethylsilyloxy)ethanamine (0.22 g, 1.0 equiv) was added to a suspension of neat NaH (0.036 g, 1.2 equiv) in dry THF (3.0 ml) and the mixture was stirred under a nitrogen atmosphere at rt for 30 min. The above mixture was slowly added via cannula to a freshly prepared suspension of 4-(4′,6′-dichloro-1′,3′,5′-triazin-2′-ylamino)benzenesulfonamide **1** (0.40 g, 1.0 equiv) in dry THF (17 ml) and the resulting orange mixture was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring). The reaction was quenched with H_2O (20 ml), extracted with ethyl acetate (3 × 15 ml) and the combined organic layers were washed with brine (2 × 20 ml), dried over Na_2SO_4, filtered off and the solvent was removed in vacuo to give a residue that was purified by silica gel column chromatography eluting with 5% MeOH/DCM to afford the title compound as a pale yellow solid.

4-(4′-(2″-(*tert*-Butyldimethylsilyloxy)ethoxy)-6′-chloro-1′,3′,5′-triazin-2′-ylamino) benzenesulfonamide **13**: 18% yield; mp 120–124 °C; silica gel TLC R_f 0.02 (MeOH/DCM 5% v/v); ν_{max} (KBr) cm^{-1}, 3280, 2814, 1530 (aromatic); δ_H (400 MHz, DMSO-d_6, 2 atropoisomers were detected in 1/0.9 ratio. Only the major one is reported herein) 0.098 (6H, s, –Si(CH_3)$_2$), 0.89 (9H, s, –SiC(CH_3)$_3$), 3.92 (2H, br m, 2″-H_2), 4.33 (2H, br m, 3″-H_2), 7.27 (2H, s, SO_2NH_2, exchange with D_2O), 7.86 (4H, m, Ar-H), 10.37 (1H, br s, –N*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 173.1, 172.6, 165.4, 145.1, 131.0, 130.5, 118.2, 74.2, 65.0, 26.0, 19.1, −4.2.

4.1.14. Synthesis of dimethyl 2″,2‴-[6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)diacetate 14

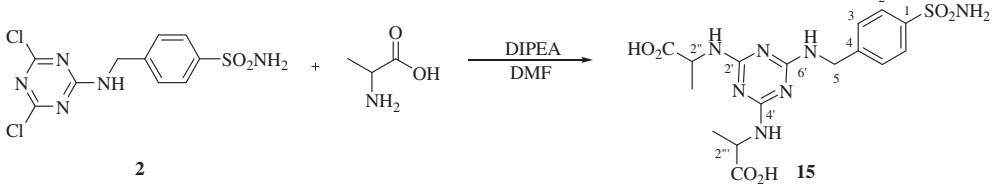


2 14

4-[(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)methyl]benzenesulfonamide **2** (0.1 g, 1.0 equiv) and methyl 2-aminoacetate hydrochloride (4.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.

Dimethyl 2″,2‴-[6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)diacetate **14**: 34% yield; mp 245–246 °C; ν_{max} (KBr) cm^{-1}, 3332, 2835, 1762 (C=O, ester), 1560 (aromatic); δ_H (400 MHz, DMSO-d_6, 3 atropoisomers were detected in 1/0.3/0.29 ratio. Only the major one is reported herein) 3.52 (6H, s, 2 × CH_3), 3.65 and 3.92 (4H, d, J 5.6, 2 × 2′-H_2), 4.50 (2H, d, J 5.6, 5-H_2), 7.30 (2H, s, SO_2NH_2, exchange with D_2O), 7.42 (2H, d, J 8.2, 2 × 3-H), 7.78 (2H, d, J 8.2, 2 × 2-H), 8.20 and 8.60 (1H, t, J 5.6, 2 × CH_2–N*H*, exchange with D_2O), 10.52 (1H, br s, –N*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 171.9, 169.0, 165.2, 144.5. 129.0, 126.8, 53.0, 44.3, 43.0, 42.4.

4.1.15. Synthesis of D,L-2′,2‴-[6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazine-2′,4′-diyl]-bis(azanediyl)dipropanoic acid 15

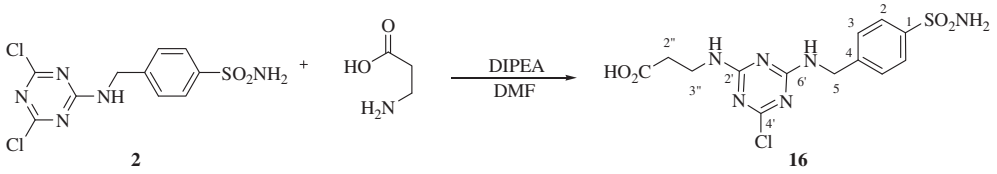


2 15

4-[(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)methyl]benzenesulfonamide **2** (0.1 g, 1.0 equiv) and D,L-alanine (4.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.

D,L-2′,2‴-[6′-(4-Sulfamoylbenzylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)dipropanoic acid **15**: 34% yield; mp 260–261 °C; ν_{max} (KBr) cm^{-1}, 3350, 2827, 1718 (C=O, acid), 1551 (aromatic); δ_H (400 MHz, DMSO-d_6) 1.32 (6H, d, J 5.6, 2 × CH_3), 4.50 (4H, m, 5-H_2, 2 × 2″-H), 7.28 (2H, s, SO_2NH_2, exchange with D_2O), 7.50 (2H, d, J 8.2, 2 × 3-H), 7.80 (2H, d, J 8.2, 2 × 2-H); δ_C (100 MHz, DMSO-d_6) 174.3, 169.6, 165.1, 158.0, 144.1, 138.0, 128.6, 50.1, 44.2, 19.3.

4.1.16. Synthesis of 3″-[4′-chloro-6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazin-2′-ylamino]propanoic acid 16

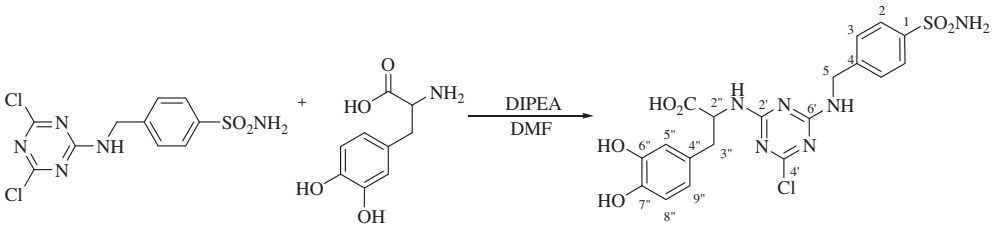


2 16

4-[(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)methyl]benzenesulfonamide **2** (0.1 g, 1.0 equiv) and 3-aminopropanoic acid (2.4 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a light brown solid.

3″-[4′-Chloro-6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazin-2′-ylamino]propanoic acid **16**: 35% yield; mp 165–167 °C; ν_{max} (KBr) cm^{-1}, 3322, 2833, 1716 (C=O, acid), 1555 (aromatic); δ_H (400 MHz, DMSO-d_6) 2.58 (2H, br m, 3″-H_2), 3.65 (2H, br m, 2″-H_2), 4.53 (2H, br s, 5-H_2), 7.28 (2H, s, SO_2NH_2, exchange with D_2O), 7.40 (2H, d, J 8.2, 2 × 3-H), 7.80 (2H, d, J 8.2, 2 × 2-H), 9.78 (1H, br s, -N*H*-, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 175.0, 170.1, 167.2, 162.3, 145.2, 139.6, 128.2, 119.7, 45.3, 37.2, 34.7.

4.1.17. Synthesis of D,L-2″-[4′-chloro-6′-(4-sulfamoylbenzylamino)-1′,3',5′-triazin-2′-ylamino]-3″-(6″,7″-dihydroxyphenyl)propanoic acid 17

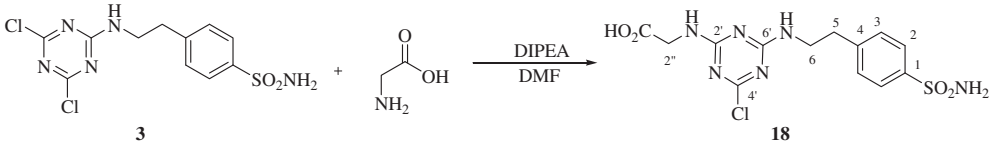


2 17

4-[(4′,6′-Dichloro-1′,3',5′-triazin-2′-ylamino)methyl]benzenesulfonamide **2** (0.1 g, 1.0 equiv) and D,L-3,4-dihydroxyphenylalanine (2.4 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a light brown solid.

D,L-2″-[4′-Chloro-6′-(4-sulfamoylbenzylamino)-1′,3′,5′-triazin-2′-ylamino]-3″-(6″,7″-dihydroxyphenyl)propanoic acid **17**: 37% yield; mp 299–301 °C; ν_{max} (KBr) cm^{-1}, 3360, 2812, 1700 (C=O, acid), 1567 (aromatic); δ_H (400 MHz, DMSO-d_6) 3.57 (2H, m, 2″-H, 3″-H*H*), 4.55 (3H, m, 3″-*H*H, 5-H_2), 6.53 (3H, m, 5″-H, 8″-H, 9″-H), 7.29 (2H, s, SO_2NH_2, exchange with D_2O), 7.40 (2H, d, *J* 8.2, 2 × 3-H), 7.78 (2H, d, *J* 8.2, 2 × 2-H), 8.30 (2H, br s, CHN*H*–, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 174.3, 166.8, 167.1, 165.2, 148.1, 145.0, 144.8, 143.0, 136.0, 129.8, 129.0, 121.6, 119.5, 114.0, 67.8, 45.6, 38.0.

4.1.18. Synthesis of 2″-[4′-chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2'-ylamino]acetic acid 18[1b]

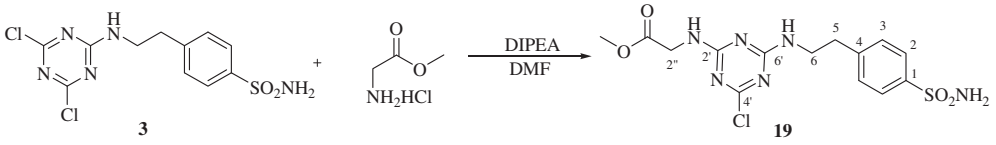


3 18

4-[6-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide **3** (0.1 g, 1.0 equiv) and glycine (2.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.

2″-[4′-Chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2′-ylamino]acetic acid **18**: 30% yield; mp 199–201 °C; ν_{max} (KBr) cm^{-1}, 3350, 2827, 1730 (C=O, acid), 1565 (aromatic); δ_H (400 MHz, DMSO-d_6, 80 °C) 2.90 (2H, m, 5-H_2), 3.48 (2H, m, CH_2 glycine), 3.92 (2H, m, 6-H_2), 6.80 (1H, br s, exchange with D_2O), 7.32 (2H, s, SO_2NH_2, exchange with D_2O), 7.44 (2H, d, *J* 8.2, 2 × 3-H), 7.78 (2H, d, *J* 8.2, 2 × 2-H); δ_C (100 MHz, DMSO-d_6, 80 °C) 172.2, 169.0, 166.9 (C-6), 166.0 (C-2′), 144.4 (*ipso*), 143 (*ipso*), 130.1 (C-3), 126.7 (C-2), 43.4, 42.2, 35.1 (C-6).

4.1.19. Synthesis of methyl 2″-[4′-chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2′-ylamino]acetate 19[1b]

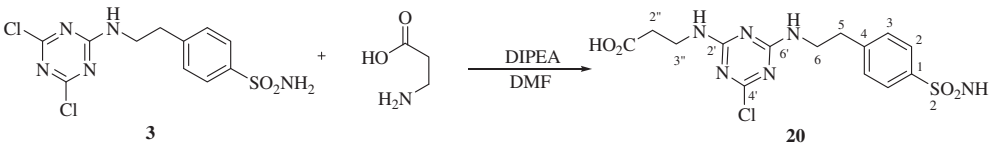


3 19

4-[6-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide **3** (0.1 g, 1.0 equiv) and methyl 2-aminoacetate hydrochloride (2.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.

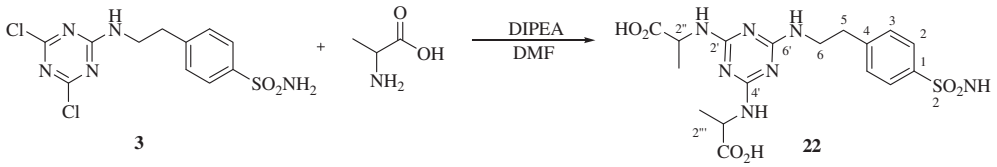
Methyl 2″-[4′-chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2′-ylamino]acetate **19**: 42% yield; mp 234–236 °C; ν_{max} (KBr) cm^{-1}, 3340, 2829, 1766 (C=O, ester), 1561 (aromatic); δ_H (400 MHz, DMSO-d_6, 80 °C) 2.90 (2H, m, 5-H_2), 3.44 (2H, m, CH_2 glycine), 3.62 (3H, s, CH_3), 4.04 (2H, m, 6-H_2), 7.32 (2H, s, SO_2NH_2, exchange with D_2O), 7.43 (2H, d, *J* 8.2, 2 × 3-H), 7.79 (2H, d, *J* 8.2, 2 × 2-H), 8.11 (2H, m, exchange with D_2O); δ_C (100 MHz, DMSO-d_6, 80 °C) 171.3 (C=O), 168.7 (C-4′), 166.6 (C-6′), 166.0 (C-2′), 144.3 (*ipso*), 143.0 (*ipso*), 130.0, 126,6, 52.7, 43.3, 42.4, 35.0 (CH_3).

4.1.20. Synthesis of 3″-[4′-chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2′-ylamino]propanoic acid 20[1b]



3 20

4-[6-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide **3** (0.1 g, 1.0 equiv) and 3-aminopropanoic acid (2.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.

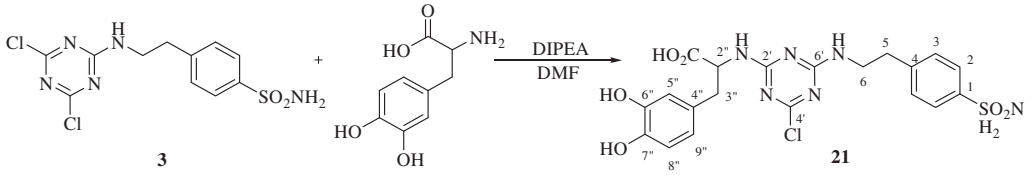


3 **22**

3″-[4′-Chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2′-ylamino]propanoic acid **20**: 28% yield; mp 265–269 °C; ν_{max} (KBr) cm^{-1}, 3350, 2827, 1712 (C=O, acid), 1560 (aromatic); δ_H (400 MHz, DMSO-d_6) 2.60 (2H, br m, 3″-H_2), 2.90 (2H, m, 5-H_2), 3.65 (2H, br m, 2″-H_2), 3.99 (2H, m, 6-H_2), 7.35 (2H, s, SO_2NH_2, exchange with D_2O), 7.38 (2H, d, J 8.2, 2 × 3-H), 7.72 (2H, d, J 8.2, 2 × 2-H), 9.65 (1H, br s, CH_2NH-, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 175.3, 169.0, 167.1, 162.2, 145.0, 140.0, 128.1, 120.2, 44.6, 38.1, 37.1, 36.5.

4.1.21. Synthesis of D,L-2″-[4′-chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2′-ylamino]-3″-(6″,7″-dihydroxyphenyl)-propanoic acid 21

4-[6-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide **3** (0.1 g, 1.0 equiv) and DL-3,4-dihydroxy-phenylalanine (2.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.



3 **21**

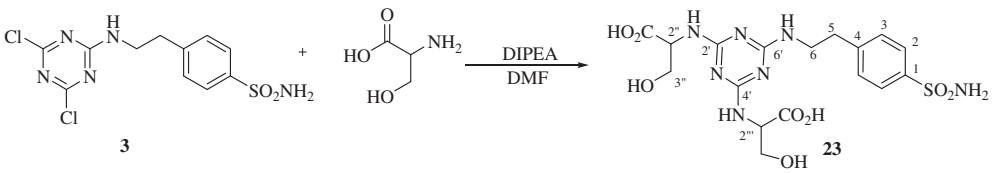
D,L-2″-[4′-Chloro-6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazin-2′-ylamino]-3″-(6″,7″-dihydroxyphenyl)propanoic acid **21**: 28% yield; mp 319–321 °C with decomposition; ν_{max} (KBr) cm^{-1}, 3366, 2818, 1703 (C=O, acid), 1563 (aromatic); δ_H (400 MHz, DMSO-d_6) 2.90 (2H, br m, 5-H_2), 3.62 (4H, m, 6-H_2, 2″-H, 3″-HH), 4.50 (1H, m, 3″-HH), 6.50 (3H, m, 5″-H, 8″-H, 9″-H), 7.33 (2H, s, SO_2NH_2, exchange with D_2O), 7.42 (2H, d, J 8.2, 2 × 3-H), 7.81 (2H, d, J 8.2, 2 × 2-H), 8.27 (2H, br s, CHNH-, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 175.2, 167.0, 166.8, 165.3, 148.0, 145.2, 145.1, 142.2, 135.4, 130.1, 128.7, 122.0, 118.0, 115.3, 68.6, 44.1, 38.4, 36.2.

4.1.22. Synthesis of D,L-2″,2‴-[6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazine-2′,4′-diyl] bis(azanediyl)dipropanoic acid 22

4-[6-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide **3** (0.1 g, 1.0 equiv) and D,L-alanine (4.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until starting material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.

D,L-2″,2‴-[6′-(4-Sulfamoylphenethylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)dipropanoic acid **22**: 48% yield; mp 274–276 °C; ν_{max} (KBr) cm^{-1}, 3362, 2818, 1717 (C=O, acid), 1558 (aromatic); δ_H (400 MHz, DMSO-d_6) 1.35 (6H, d, J 5.6, 2 × CH_3), 2.88 (2H, m, 5-H_2), 4.00 (2H, m, 6-H_2), 4.35 (2H, m, 2 × 2″-H), 7.32 (2H, s, SO_2NH_2, exchange with D_2O), 7.41 (2H, d, J 8.2, 2 × 3-H), 7.80 (2H, d, J 8.2, 2 × 2-H); δ_C (100 MHz, DMSO-d_6) 174.1, 168.0, 165.0, 160.2, 144.0, 135.7, 129.0, 55.2, 44.2, 36.4, 19.3.

4.1.23. Synthesis of D,L-2″,2‴-[6′-(4-sulfamoylphenethylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)bis(3″-hydroxypropanoic acid) 23



3 **23**

4-[6-(4′,6′-Dichloro-1′,3′,5′-triazin-2′-ylamino)ethyl]benzenesulfonamide **3** (0.1 g, 1.0 equiv) and D,L-serine (4.2 equiv) were dissolved in dry DMF (15 ml) and DIPEA (5.0 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt until start-

ing material was consumed (TLC monitoring) and treated according to the procedure reported above to afford the title product as a white solid.

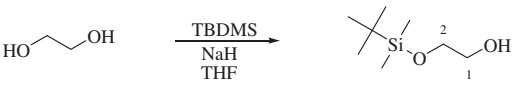
D,L-2″,2‴-[6′-(4-Sulfamoylphenethylamino)-1′,3′,5′-triazine-2′,4′-diyl]bis(azanediyl)bis(3″-hydroxypropanoic acid) **23**: 42% yield; mp 278–280 °C with decomposition; ν_{max} (KBr) cm^{-1}, 3342, 2830, 1710 (C=O, acid), 1562 (aromatic); δ_H (400 MHz, DMSO-d_6) 2.88 (2H, br m, 5-H_2), 3.34 (4H, m, 6-H_2, 2 × 3″-H*H*), 3.77 (2H, br m, 2 × 2″-H), 4.40 (2H, m, 2 × 3″-*H*H), 7.32 (2H, s, SO_2NH_2, exchange with D_2O), 7.45 (2H, d, *J* 8.2, 2 × 3-H), 7.76 (2H, d, *J* 8.2, 2 × 2-H), 8.00 (2H, br s, CHN*H*-, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 174.9, 165.3, 164.0, 145.0, 141.4, 129.0, 127.5, 69.4, 58.0, 44.2, 36.0.

The preparation of some of the intermediates used in the syntheses are also shown below.

4.1.24. Synthesis of methyl 2-aminoacetate hydrochloride[21]

Thionyl chloride (1.58 g, 1.0 equiv) was added drop-wise to a solution of glycine (1.0 g, 1.0 equiv) in MeOH (50 ml) at 0 °C. The solution was stirred under reflux until starting material was consumed (TLC monitoring). Then solvent was evaporated in vacuo to afford the title product as a white solid and does not need further purification.

4.1.25. Synthesis of 2-(*tert*-butyldimethylsilyloxy)ethanol[22]



Dry ethane-1,2-diol (1.0 g, 1.0 equiv) was added to a suspension of neat NaH (0.65 g, 1.0 equiv) in dry THF (20 ml). The suspension was stirred at rt for 30 min followed by addition over 20 min of a solution of *tert*-butyldimethylsilylchloride (2.43 g, 1.0 equiv) in the same solvent (20 ml) and the resulting mixture was stirred under a nitrogen atmosphere at rt for 50 min. Then the reaction was treated with diethyl ether (70 ml), washed with a 10% aqueous solution of K_2CO_3 (3 × 30 ml), brine (3 × 20 ml) and the organic layers were dried over Na_2SO_4, filtered off and the solvent evaporated under vacuo to give a pale yellow oil that was purified by silica gel column chromatography eluting with 10% ethyl acetate in *n*-hexane to afford the title compound as a thin colourless oil. 2-(*tert*-Butyldimethylsilyloxy)ethanol: 89% yield; silica gel TLC R_f 0.22 (ethyl acetate/petroleum ether 10% v/v); δ_H (400 MHz, DMSO-d_6) -0.98 (6H, s, –Si(CH_3)$_2$), 0.92 (9H, s, –SiC(CH_3)$_3$), 3.46 (2H, q, *J* 5.6, 1-H_2), 3.63 (2H, t, *J* 5.6, 2-H_2), 4.59 (1H, t, *J* 5.6, O-*H*, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 65.5 (C-1), 63.4 (C-2), 26.8 (–SiC(CH_3)$_3$), 19.0 (–SiC(CH$_3$)$_3$), −4.3 (–Si(CH_3)$_2$).

4.2. Expression and Purification of hCA II

The plasmid encoding hCA II was transformed into *E. coli* BL21 cells through standard procedures and the transformed cells were expressed at 37 °C in LB medium containing 100 μg/ml ampicillin.[23] hCA II production was induced by the addition of isopropyl thiogalactoside to a final concentration of 1 mM at an O.D$_{600}$ of 0.6 AU. The cells were harvested after 4hrs of post induction. The cell pellets were lysed and hCA II was purified through affinity chromatography using pAMBS resin as has been described elsewhere.[24]

4.3. Co-crystallization and X-ray data collection

Co-crystals of hCA II complexed with compounds **7** and **11** were obtained using the hanging drop vapor diffusion method.[25] 10 μl drops (0.2 mM hCA II; 0.4 mM compound either **1** or **11**; 0.8 M sodium citrate; 50 mM Tris-Cl; pH 8.0) were equilibrated against precipitant solution (1.6 M sodium citrate; 50 mM Tris-Cl; pH 8.0) at room temperature (~20 °C).[26] Useful crystals were observed 4 days after the crystallization setup. A crystal was cryoprotected by quick immersion into 25% glycerol precipitant solution and flash-cooled by exposure to a gaseous stream of nitrogen at 100 K. X-ray diffraction data were obtained using an R-AXIS IV^{++} image plate system with Osmic Varimax HR optics and a Rigaku RU-H3R Cu rotating anode operating at 50 kV and 22 mA. The detector-crystal distance was set to 80 mm. The oscillation steps were 1° with a 6 min exposure per image. Indexing, integration, and scaling were performed using HKL2000.[27]

4.4. Structure determination

The crystal structure of hCA II (PDB accession code: 2ILI)[26] was used to obtain initial phases of the structures using *PHENIX*.[28] The solvent molecules were removed to avoid model bias and 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of R_{free} calculations. For both compounds, the initial $|F_o - F_c|$ difference electron density maps unambiguously showed the triazine, 4-aminobnzene, and sulfonamide positions. Atomic models for compounds **7** and **11** were calculated and energy minimized using the PRODRG2 server.[29] Refinements proceeded using normal protocols of positional, isotropic atomic displacement parameters, TLS refinement, and automated solvent additions, alternating with manual refitting of the models using *COOT*.[30] Final rounds of refinement for both models included riding hydrogen positions, which decreased R_{work} and R_{free} values by about 1% each. The quality of the final models were assessed with *PROCHECK*.[31] Crystal and refinement data are summarized in Table 2.

4.5. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[15] 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 7.4) and 20 mM $NaBF_4$ (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 (10 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[1,3] and represent the mean from at least three different determinations. All CAs were recombinant proteins obtained as reported earlier by these groups.[1,12,13,16]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.005.

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