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Fluorescent sulfonamide carbonic anhydrase inhibitors incorporating 1,2,3-triazole moieties: Kinetic and X-ray crystallographic studies

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ABSTRACT

Fluorescent sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) were essential for demonstrating the role played by the tumor-associated isoform CA IX in acidification of tumors, cancer progression towards metastasis and for the development of imaging and therapeutic strategies for the management of hypoxic tumors which overexpress CA IX. However, the presently available such compounds are poorly water soluble which limits their use. Here we report new fluorescent sulfonamides **7**, **8** and **10** with increased water solubility. The new derivatives showed poor hCA I inhibitory properties, but were effective inhibitors against the hCA II (K_Is of 366–127 nM), CA IX (K_Is of 8.1–36.9 nM), CA XII (K_Is of 4.1–20.5 nM) and CA XIV (K_Is of 12.8–53.6 nM). A high resolution X-ray crystal structure of one of these compounds bound to hCA II revealed the factors associated with the good inhibitory properties. Furthermore, this compound showed a three-fold increase of water solubility compared to a similar derivative devoid of the triazole moiety, making it an interesting candidate for ex vivo/in vivo studies.

1. Introduction

Fluorescent sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs)[1–3] such as compounds **A** and **B** reported earlier by our group,[4] were essential for demonstrating the role played by the tumor-associated isoform CA IX in acidification of tumors,[5] cancer progression towards metastasis,[6] and for the development of imaging[7] and therapeutic strategies[8] for the management of hypoxic tumors which overexpress high amounts of CA IX.[9] Indeed, starting from such proof-of-concept experiments,[5,7,8] many interesting, CA IX-selective inhibitors were developed,[10,11] with one such compound, **SLC-0111** currently in advanced Phase I clinical trials for the treatment of hypoxic, metastatic solid tumors (Fig. 1).[12]

However one of the main drawbacks associated with the previously fluorescent sulfonamide CAIs, that is, compounds **A** and **B**, is related to their relatively low water solubility at pH values in the range of 6.5–7.4.[4] This limits their extensive use in many biological situations in which working with higher concentrations of inhibitor is required. As a consequence, there is urgent need to develop fluorescent sulfonamides with increased water solubility, which in principle can be achieved in several ways: either by changing the linker between the benzenesulfonamide head and the fluorescent tail present in **A** and **B**, or by changing the nature of the fluorescent moiety to incorporate hydrophilic moieties, not present in the fluorescein fragment. Herein we report new compounds **7**, **8** and **10** obtained using both synthetic strategies mentioned above, their inhibition potencies and selectivity profiles against the physiologically relevant human (h) CA isoforms such as hCA I, II, IX, XII and XIV, as well an X-ray crystallographic structure at 1.3 Å resolution of one selected compound in adduct with the physiologically dominant isoform hCA II.

2. Results and discussion

2.1. Drug design and chemistry

The first synthetic approach used here consisted of replacing the alkyl spacers between the benzenesulfonamide head and the thioureido-fluorescent moiety (methylene or ethylene for **A** and **B**, respectively) by a 1,2,3-triazolyl spacer, which due to the presence of the heterocyclic ring system rich in heteroatoms should increase water solubility. Since the click chemistry methodology[13] has been successfully applied for obtaining large series of sulfonamide, sulfocoumarin or coumarin CAIs,[14–16] also in this case was the preferred choice.

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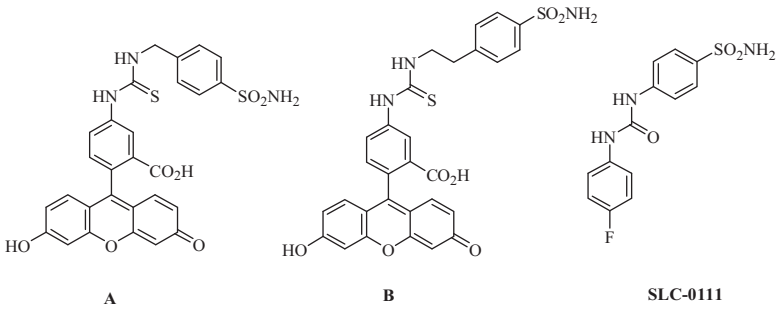
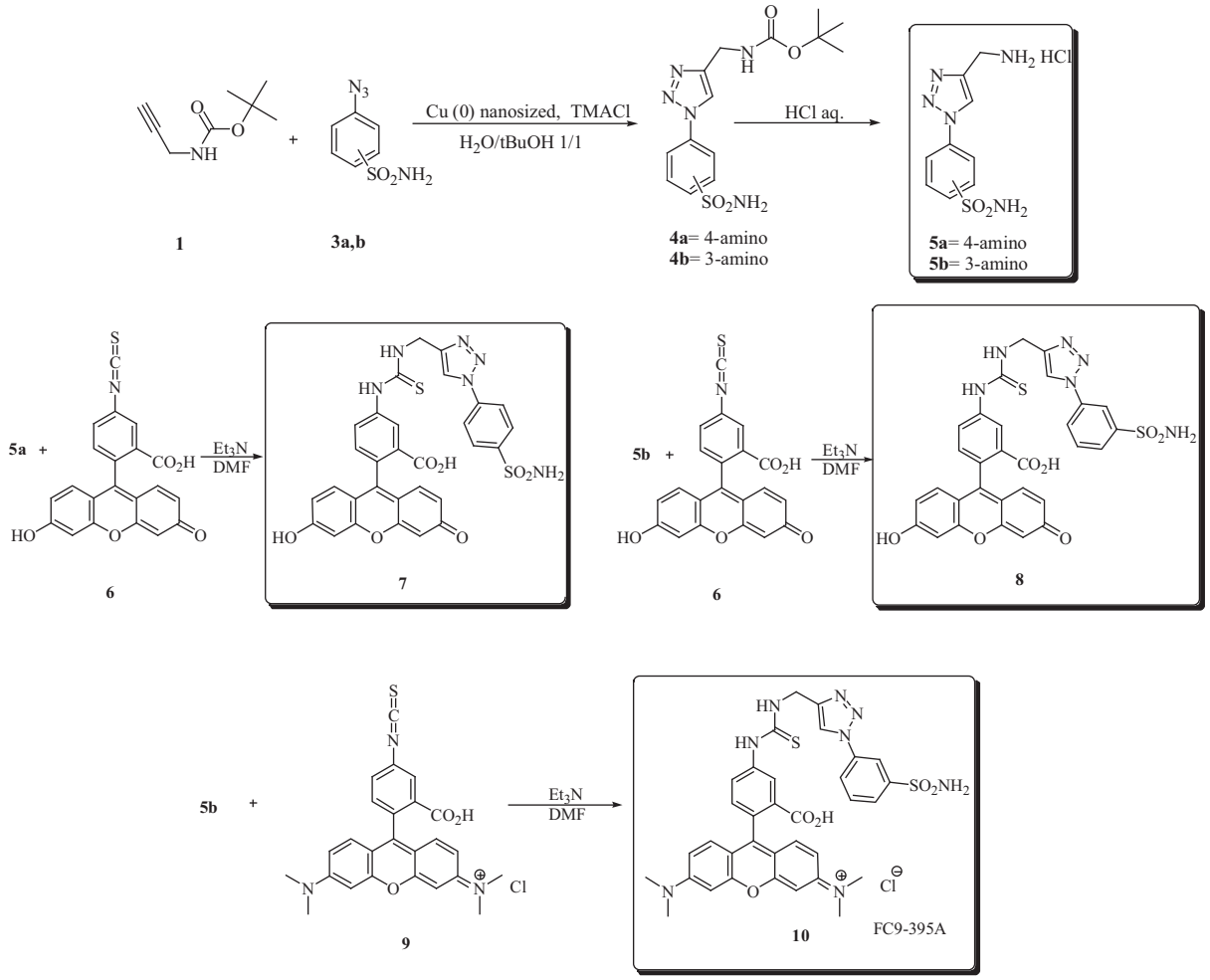


Figure 1. Structures of fluorescent sulfonamides **A**, **B** and the advanced Phase I **SLC-0111**.



Scheme 1. Synthesis of fluorescent-tagged compounds **7**, **8** and **10**.

The 1,2,3-triazolyl-benzenesulfonamide key intermediates **5a** and **5b** were prepared by routine procedures as outlined in Scheme 1. The propargylamine *Boc*-protected **1** was reacted in the presence of copper(0) nanosized catalysts with freshly prepared azidobenzenesulfonamides **3a** and **3b** to afford the desired intermediates **5a** and **5b** upon deprotection of the amino moiety with aqueous 12 M hydrochloric acid. These amines were then coupled with fluorescein isothiocyanate (FITC) leading to the thioureas **7** and **8**, which are analogs of the leads **A** and **B**, but incorporate the water-solubilizing triazole moieties in their molecule (Scheme 1).

The alternative procedure was to replace the fluorescein moiety present in **A** and **B** by a fluorescent tag which promotes a better water solubility itself. Indeed, the replacement of the 6-hydroxy-xanthen-3-one moiety from fluorescein by the (6-dimethylamino-xanthen-3-ylidene)-dimethyl-ammonium present in **10**, is supposed to increase the water solubility as well as the membrane impermeability of compound **10** compared to the structurally-related derivative **8** (Scheme 1). The synthesis of **10** was achieved again by using the amino sulfonamide **5b** and the fluorescent isothiocyanate **9** (see Section 4 for details).

We have compared the phosphate buffer (pH 7.4) solubility of **A**, one of the most investigated fluorescent CAI for a variety of in vitro and in vivo studies,[4–9] with compound **7** designed here. **A** reported a solubility of 1.2 mg/mL whereas **7** had a more than 3-fold increased solubility of 3.8 mg/mL in the same conditions (25 °C, pH 7.4 phosphate buffer).

2.2. Carbonic anhydrase inhibition

Inhibition data against five physiologically relevant isoforms, that is, hCA I, II (cytosolic), IX, XII and XIV (*trans*-membrane) with the fluorescent sulfonamides **A** and **B** (lead molecules) and the newly prepared **7**, **8** and **10**, are shown in Table 1.

The structure–activity relationship (SAR) for the inhibition of these isoforms is rather well delineated:

(i) The cytosolic isoform, hCA I, was poorly inhibited by these sulfonamides with K_Is ranging between 1300 and 7630 nM. Introduction of the triazole moiety in compounds **7**–**10** led to a decrease of the inhibitory power compared to the lead molecules **A** and **B**, which is favorable feature of the new compounds as hCA I is an off-target, highly abundant in the red blood cells and gastrointestinal tract enzyme.[1]

(ii) In the case of hCA II, one of the physiologically dominant isoforms, the nature of the benzenesulfonamide head strongly influenced the inhibitory activity. Thus, the sulfanilamide derivative **7**, similar to the related benzenesulfonamide **A** and **B** (possessing a *p*-sulfamoyl functional group) were moderate-effective inhibitors (K_Is of 36–45 nM) whereas the two metanilamide derivatives **8** and **10** showed a decreased inhibitory power (K_Is of 88–127 nM). The hydrophilic fluorescent moiety present in **10**, led to a more efficient CAI (1.44 fold) when compared to the fluorescein-containing, structurally similar derivative **8**.

(iii) hCA IX, the target isoform for anticancer CAIs,[4–8] was well inhibited by the compounds reported here, which showed a similar activity with the leads **A**, **B**. The inhibition constants ranged between 8.1 and 36.9 nM, with the best inhibitor being the sulfanilamide derivative **7** (Table 1). A similar behavior was observed for the inhibition of the second tumor-associated, transmembrane isoform, hCA XII, but the inhibitory power was even stronger (K_Is in the range of 4.1–20.5 nM), with the SAR similar to what mentioned above for the inhibition of hCA IX. Noteworthy the selectivity profile (hCA IX/hCA II) of compound **10** was slightly improved when compared to **8** (3.44 and 3.62, respectively) but resulted decreased for the second tumor associated CA isoform (hCA XII/hCA II 12.0 and 8.22 for **8** and **10**, respectively).

(iv) The other transmembrane isoform (not connected with tumors), hCA XIV,[18] was also effectively inhibited by sulfonamides investigated here, with K_Is in the range of 12.8–53.6 nM. Again the best inhibitor was the fluorescein-triazolyl derivative of sulfanilamide **7**, whereas the worst one was the corresponding metanilamide derivative **8**.

Table 1
Inhibition data of isoforms hCA I, II, IX, XII and XIV with fluorescent sulfonamides, by a stopped-flow, CO_2 hydrase assay[17]

Entry	K_I^* (nM)				
	hCA I	hCA II	hCA IX	hCA XII	hCA XIV
A[a]	1450	44	26	7.0	41
B[b]	1300	45	24	5.0	33
7	2560	36	8.1	4.1	12.8
8	7630	127	36.9	20.5	53.6
10	6425	88	24.3	10.7	31.4
AAZ	0.25	0.012	0.06	25	5.7

[*] Mean from three different assays. Errors were in the range of ±10% of the reported values, data not shown.
[a] From Ref. 4a, except CA XII and XIV data which are reported here for the first time.
[b] From Refs. 1,4a.

2.3. X-ray crystallography

Considering the interesting CA inhibitory and solubility features of **7**, we co-crystallized this compound with hCA II and solved its crystal structure by means of X-ray crystallography. It should be mentioned that the hCA II–**B** adduct has also been characterized by X-ray crystallography earlier, by Alterio et al.[4b]

The electron density of **7** bound within the hCA II active site (Fig. 2) shows density well defined for all atoms of the inhibitor, which was found coordinated to the Zn(II) ion from the active site cavity by means of the deprotonated sulfonamide moiety, as reported for all CA–sulfonamide adducts characterized by this technique to date (see Table 2 in Section 4 for details).[4a,19–22] The NH coordinated to zinc also makes a strong H-bond with the OH of Thr199, an interactions also observed in all CA–sulfonamide adducts investigated so far.[4a,19–22] The scaffold of the inhibitor occupies all the enzyme active site, with the benzenesulfonamide moiety in van der Waals contacts with Gln92, Asn67, Thr199 and Thr200 residues, whereas the 1,2,3-triazole one in van der Waals contacts with Phe131, Thr200 and Leu204 residues. No polar interactions between the inhibitor heteroatoms and the active site residues were observed, as also reported for the hCA II–**B** adduct mentioned above.[4a]

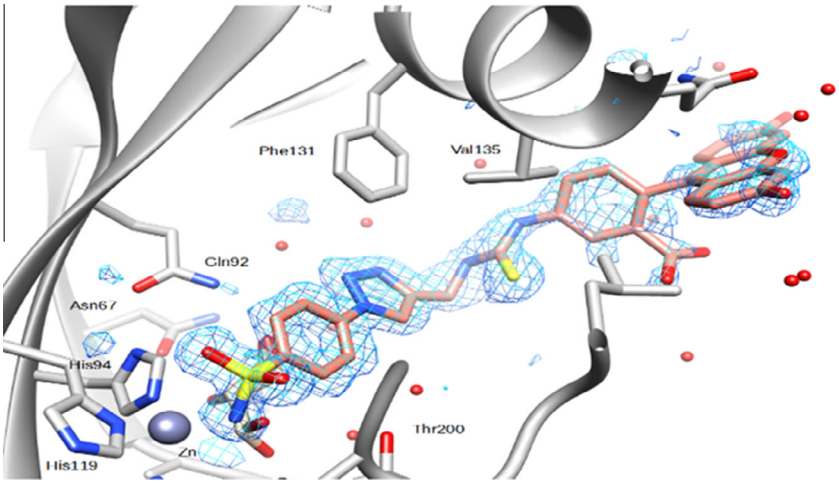


Figure 2. Crystallographic adduct of **7** with hCA II (PDB accession code: 4RH2). An $F_o - F_c$ omit electron density map is also shown contoured at 2 σ.

Table 2
Summary of data collection and atomic model refinement statistics*

	hCAII + **7**
PDB ID	4RH2
Wavelength (Å)	0.980
Space group	*P*21
Unit cell (*a*, *b*, *c*, β) (Å, °)	42.362, 41.282, 72.138, 104.30
Limiting resolution (Å)	29.1–1.3 (1.38–1.30)
Unique reflections	50,556 (4078)
R_{sym} (%)	0.022 (0.063)
Redundancy	2.07 (1.58)
Completeness overall (%)	84.7 (44.8)
<*I*/(*I*)>	23.8 (8.7)
Refinement statistics	
Resolution range (Å)	29.1–1.3
Unique reflections, working/free	50,556/2526
R_{factor} (%)	0.156
R_{free} (%)	0.171
No. of protein atoms	2177
No. of water molecules	385
No. of heterogen atoms	45
r.m.s.d. bonds (Å)	0.006
r.m.s.d. angles (°)	1.331
Ramachandran statistics (%)[2]	
Most favored	96.1
Additionally allowed	3.9
Generously outlier regions	0
Average B factor (Å^2)	
All atoms	13.12
Compound	25.74
Solvent	26.59

* Values in parentheses are for the highest resolution shell.

A superimposition of the two structures is in fact shown in Figure 3. From this figure it may be observed that only the benzenesulfonamide moieties of the two inhibitors **B** and **7** are superimposable. The ethylene spacer of **B** and the 1,2,3-triazole moiety of **7** have comparable lengths in the two inhibitors, but they do not completely superimpose, and this also leads to different orientations of the two thioureido fragments and the corresponding fluorescein tails present in **B** (Fig. 3). In **B** the fluorescein tail is closer to Phe131, an amino acid residue which delineates two sub-pockets within the hydrophobic half of the CA II active site.[19] On the contrary, in **7** the tail is in a totally different orientation, being far from Phe131 and closer to Leu204, Val135 hydrophobic pocket. This is probably also the reason why **7** is a three times better hCA II inhibitor compared to **B** (Table 1).

3. Conclusions

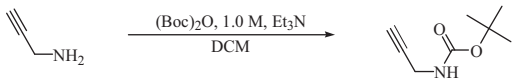
New fluorescent CAIs are reported which possess enhanced water solubility and high affinity for the transmembrane isoforms hCA IX, XII and XIV compared to previously investigated such derivatives. The synthesis has been achieved by click chemistry, which allowed the introduction of 1,2,3-triazole moieties which enhanced hydrosolubility. X-ray crystallography of hCA II complexed with one of these sulfonamides evidenced interesting interactions between the inhibitor scaffold and residues involved in the binding of inhibitors. Furthermore, the tail of the new fluorescent inhibitor had a very different orientation compared to a structurally similar compound devoid of the triazole moiety.

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. Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra were recorded using a Bruker Advance III 400 MHz spectrometer 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 ethyl acetate/*n*-hexane were used as eluents. Melting points (mp) were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected. All compounds reported here were >95% HPLC pure.

4.1.1. Synthesis of prop-2-ynyl-carbamic acid *tert*-butyl ester 1[23]



1

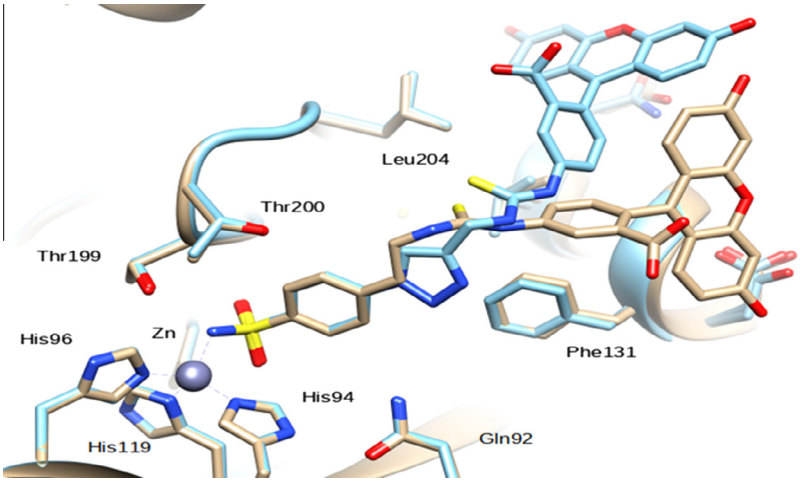


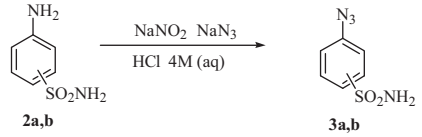
Figure 3. Superimposition of adducts **7** and **B** with hCA II (PDB accession code: 2F14).

Propargylamine (1.0 g, 1.0 equiv) and triethylamine (1.1 equiv) were dissolved in DCM (80 ml). The solution was cooled to 0 °C and *tert*-butyloxycarbonylcarbonate (1.1 equiv) dissolved in 20 ml of DCM was added drop-wise. The solution was stirred at rt for 5 h then was quenched with aqueous HCl 1.0 M (100 ml) and the organic layer was washed with H_2O (3 × 50 ml), brine (3 × 20 ml) and dried over Na_2SO_4, filtered off and concentrated under vacuo to give a brown oil that was purified by silica gel column chromatography eluting with 10% v/v Ethyl acetate/*n*-hexane to give **1** as a colorless oil.

Prop-2-ynyl-carbamic acid *tert*-butyl ester **1**: silica gel TLC R_f 0.20 (Ethyl Acetate/*n*-hexane 10% v/v); ν_{max} (KBr) cm^{-1} 3350 (C≡C—H), 2170 (C≡CH), 1760 (C═O); δ_H (400 MHz, DMSO-d_6) 1.42 (9H, s, 3 × CH_3), 3.08 (1H, t, *J* 4.0, 4-H), 3.73 (2H, m, 2-H_2), 7.29 (1H, br s, 1-H); δ_C (100 MHz, DMSO-d_6) 156.6 (C═O), 82.6, 79.1, 73.6, 30.3 (C-2) and 29.9 (3 × CH_3).

Experimental in agreement with reported data.[23]

4.2. General synthetic procedure of compounds 3a,b[24]



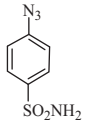
2a,b **3a,b**

2a= 4-amino
2b= 3-amino

3a= 4-amino
3b= 3-amino

The appropriate aminobenzenesulfonamide **2a**,**b** (0.5 g, 1.0 equiv) was dissolved in a 4 M HCl aqueous solution (5 ml) at 0 °C. Then $NaNO_2$ 5 M aqueous solution (1.2 equiv) was added drop-wise to the solution followed by a NaN_3 5 M aqueous solution (1.5 equiv). The reaction mixture was stirred for 0.5 h at rt and the solid formed was filtered-off, dried under vacuo and used as it is.

4.2.1. Synthesis of 4-azido-benzenesulfonamide 3a



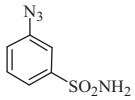
3a

4-Azido-benzenesulfonamide **3a** was obtained according the general procedure earlier reported as a yellow solid.

4-Azido-benzenesulfonamide **3a**: 65% yield; mp 120–121 °C; silica gel TLC R_f 0.50 (MeOH/DCM 10% v/v); δ_H (400 MHz, DMSO-d_6): 7.33 (d, *J* = 8.8, 2H), 7.41 (s, 2H, exchange with D_2O, SO_2NH_2), 7.87 (d, *J* = 8.8, 2H); δ_C (100 MHz, DMSO-d_6): 120.5, 128.6, 141.5, 143.9.

Experimental in agreement with reported data.[24]

4.2.2. Synthesis of 3-azido-benzenesulfonamide 3b

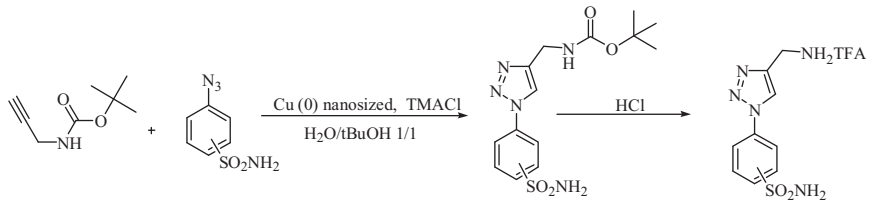


3b

3-Azido-benzenesulfonamide **3b** was obtained according the general procedure earlier reported as a pale yellow solid.

3-Azido-benzenesulfonamide **3b**: 60% yield; mp 110 °C dec.; silica gel TLC R_f 0.47 (MeOH/DCM 10% v/v); δ_H (400 MHz, DMSO-d_6): 7.37 (d, *J* = 8.8, 2H), 7.48 (s, 2H, exchange with D_2O, SO_2NH_2), 7.52 (s, 1H), 7.63 (m, 1H); δ_C (100 MHz, DMSO-d_6): 117.2, 123.1, 123.5, 131.8, 141.4, 146.9.

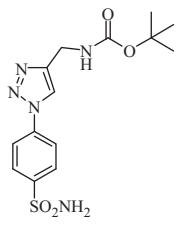
4.3. General synthetic procedure of compounds 5a,b[25]



1 **3a,b** **4a**= 4-amino **4b**= 3-amino **5a**= 4-amino **5b**= 3-amino

Freshly prepared azidobenzenesulfonamide **3a**,**b** (0.12 g, 1.1 equiv) was added to a suspension of alkyne **3a** (1.0 equiv) in H_2O/^{t}BuOH 1:1 (3 ml) at rt. Then copper(0) nanosized (0.1 equiv) and TMACl (1.0 equiv) were added to the suspension and the reaction mixture was stirred at rt until starting materials were consumed (TLC monitoring). The reaction was quenched with H_2O (20 ml) and the mixture was extracted with ethyl acetate (3 × 15 ml). The combined organic layers were washed with H_2O (3 × 15 ml), dried over Na_2SO_4, filtered-off and concentrated under vacuo to give a pale yellow residue that was purified by silica gel column chromatography eluting 50% EtOAc/*n*-hexane to afford **4a** and **b** which were treated with concentrated aqueous hydrochloric acid for 4 h at rt. The reaction was treated with Na_2CO_3 to pH 5 and then extracted with ethyl acetate (3 × 15 ml). The combined organic layers were washed with H_2O (3 × 15 ml) dried over Na_2SO_4, filtered and concentrated under vacuo to give a residue that was purified by silica gel column chromatography eluting with MeOH/DCM 10% to afford the titled compounds.

4.3.1. Synthesis of [1-(4-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-carbamic acid *tert*-butyl ester 4a[25c]

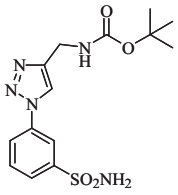


4a

1-(4-Sulfamoyl-phenyl)-1*H*-[1,2,3]triazol-4-ylmethyl]-carbamic acid *tert*-butyl ester **4** was obtained according the general procedure earlier reported as a pale yellow solid.

[1-(4-Sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-carbamic acid *tert*-butyl ester **4a**: 79% yield; silica gel TLC R_f 0.24 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d_6) 1.44 (9H, s), 4.31 (2H, d, J = 6.0, CH_2), 7.42 (1H, t, J 6.0, exchange with D_2O, –N*H*), 7.55 (2H, s, exchange with D_2O, SO_2NH_2), 8.04 (2H, d, J = 8.8, Ar-*H*), 8.16 (2H, d, J = 8.8, Ar-*H*), 8.73 (1H, s, Ar-*H* triazole); δ_C (100 MHz, DMSO-d_6): 29.1, 36.8, 79.5, 121.1, 121.9, 128.4, 139.6, 144.6, 148.0, 156.8.

4.3.2. Synthesis of [1-(3-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-carbamic acid *tert*-butyl ester 4b

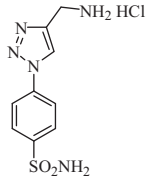


4b

[1-(3-Sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-carbamic acid *tert*-butyl ester **4b** was obtained according the general procedure earlier reported as a pale yellow solid.

[1-(3-Sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-carbamic acid *tert*-butyl ester **4b**: 79% yield; silica gel TLC R_f 0.24 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d_6) 1.42 (9H, s), 4.28 (2H, d, J = 6.2, CH_2), 7.40 (1H, t, J 6.2; exchange with D_2O, –N*H*), 7.54 (2H, s, exchange with D_2O, SO_2NH_2), 7.80 (1H, appt, J = 8.2, Ar-*H*), 7.90 (1H, d, J = 8.2, Ar-*H*), 8.13 (1H, dd, J = 8.2 3.7, Ar-*H*), 8.33 (1H, t, J 3.7), 8.69 (1H, s, Ar-*H* triazole); δ_C (100 MHz, DMSO-d_6): 29.3, 36.6, 79.8, 118.2, 123.8, 124.1, 126.7, 130.4, 132.0, 137.3, 142.5, 146.8.

4.3.3. Synthesis of 4-(4-aminomethyl-[1,2,3]-triazol-1-yl)-benzenesulfonamide hydrochloride 5a[25c]

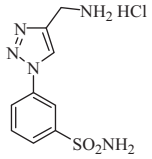


5a

4-(4-Aminomethyl-[1,2,3]-triazol-1-yl)-benzenesulfonamide hydrochloride **5a** was obtained according the general procedure earlier reported as a pale yellow solid.

(1H, s, Ar-*H* triazole); δ_C (100 MHz, DMSO-d_6): 34.8, 121.4, 123.8, 128.6, 139.4, 142.9, 145.1.

4.3.4. Synthesis of 3-(4-aminomethyl-[1,2,3]-triazol-1-yl)-benzenesulfonamide hydrochloride 5b



5b

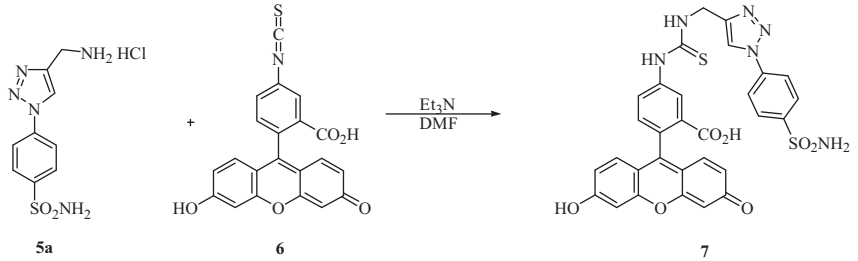
3-(4-Aminomethyl-[1,2,3]-triazol-1-yl)-benzenesulfonamide **5b** was obtained according the general procedure earlier reported as a white solid.

3-(4-Aminomethyl-[1,2,3]-triazol-1-yl)-benzenesulfonamide hydrochloride **5b**: 63% yield; silica gel TLC R_f 0.10 (MeOH/DCM 10% v/v); δ_H (400 MHz, DMSO-d_6) 4.29 (2H, s, CH_2), 7.66 (2H, s, exchange with D_2O, SO_2NH_2), 7.87 (1H, m, Ar-*H*), 7.91 (1H, m, Ar-*H*), 8.15 (1H, m, Ar-*H*), 8.36 (1H, d, J = 2.8, Ar-H), 8.46 (3H, br s, exchange with D_2O, –NH_3), 8.96 (1H, s, Ar-*H* triazole); δ_C (100 MHz, DMSO-d_6): 35.3, 118.2, 123.8, 124.1, 126.7, 132.0, 137.3, 142.5, 146.8.

4.4. General procedure for synthesis of fluorescent sulfonamides 7, 8 and 10[26]

4-(4-Aminomethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide hydrochloride salt **5a** (1.0 equiv) or 3-(4-aminomethyl-[1,2,3]-triazol-1-yl)-benzenesulfonamide hydrochloride salt **5b** (1.0 equiv) and the appropriate 5-isothiocyanato-benzoic acid fluorescent tag **6** or **9** (1.0 equiv) were poured in a two neck flask and dry DMA (1.0 ml) was added, followed by addition of TEA (1.5 equiv). The reaction was stirred at rt until starting materials were consumed (TLC monitoring), and then it was quenched with slush, treated with a 6.0 M aqueous hydrochloric acid solution and extracted with ethyl acetate (3 × 15 ml). The combined organic layers were washed with H_2O (3 × 20 ml), dried over Na_2SO_4, filtered and concentrated in vacuo to give a solid residue that was purified by silica gel column chromatography eluting with ethyl acetate afford the titled compounds.

4.4.1. Synthesis of 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-{3-[1-(4-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-thioureido}-benzoic acid 7



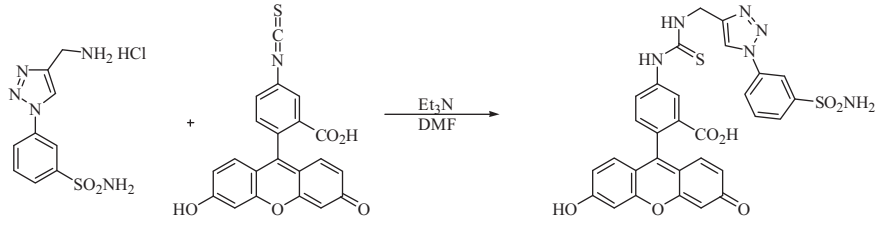
5a 6 7

4-(4-Aminomethyl-[1,2,3]-triazol-1-yl)-benzenesulfonamide hydrochloride **5a**: 72% yield; silica gel TLC R_f 0.02 (MeOH/DCM 10% v/v); δ_H (400 MHz, DMSO-d_6) 4.29 (2H, s, CH_2), 7.60 (2H, s, exchange with D_2O, SO_2NH_2), 8.10 (2H, d, J = 8.8, Ar-*H*), 8.15 (2H, d, J = 8.8, Ar-*H*), 8.42 (3H, br s, exchange with D_2O, –NH_3), 9.0

3-(4-Aminomethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide hydrochloride salt **5a** (1.0 equiv) and 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-isothiocyanato-benzoic acid **6** (1.0 equiv) were treated according to the general procedure previously reported afford the titled compound **7** as an orange powder.

2-(6-Hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-{3-[1-(4-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-thioureido}-benzoic acid **7**: 38% yield; silica gel TLC R_f 0.24 (Ethyl acetate); mp 194–198 °C; δ_H (400 MHz, DMSO-d_6) 4.96 (2H, d, *J* 5.2, CH_2), 6.61 (5H, m), 6.71 (2H, s), 7.24 (1H, d, *J* 8.8, Ar-*H*), 7.56 (2H, s, exchange with D_2O, SO_2NH_2), 7.80 (1H, dd, *J* 8.8 2.3, Ar-*H*), 8.06 (2H, d, *J* 8.8, Ar-*H*), 8.19 (2H, d, *J* 8.8, Ar-*H*), 8.31 (1H, d, *J* 2.0, Ar-*H*), 8.62 (1H, br s, exchange with D_2O, N*H*), 8.91 (1H, s, Ar-*H* triazole), 10.14 (4H, br s, exchange with D_2O, 2 × O*H*, COO*H*, N*H*); δ_C (100 MHz, DMSO-d_6) 45.4, 103.2, 110.0, 113.7, 115.4, 117.1, 126.0, 128.1, 128.7, 129.8, 130.0, 137.1, 141.9, 148.9, 153.1, 160.8, 161.9, 168.6, 168.4 (*C*=O), 182.6 (*C*=S); *m*/*z* (ESI positive) 642.2 $[M+H]^+$.

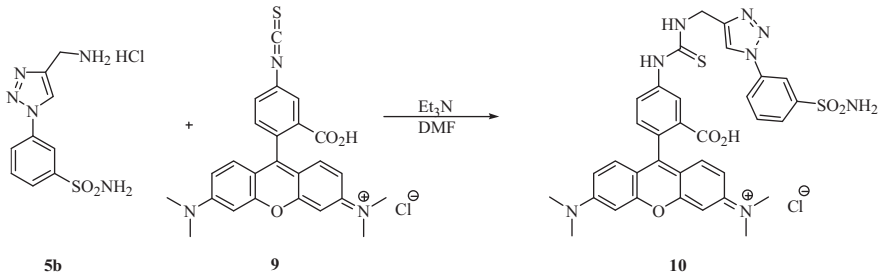
4.4.2. Synthesis of 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-{3-[1-(3-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-thioureido}-benzoic acid 8



3-(4-Aminomethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide hydrochloride salt **5b** (1.0 equiv) and 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-isothiocyanato-benzoic acid **6** (1.0 equiv) were treated according to the general procedure previously reported afford the titled compound **8b** as an orange powder.

2-(6-Hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-{3-[1-(3-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-thioureido}-benzoic acid **8**: 26% yield; silica gel TLC R_f 0.33 (Ethyl acetate); mp 180 °C; δ_H (400 MHz, DMSO-d_6) 4.94 (2H, d, *J* 5.4, CH_2), 6.62 (5H, m), 6.68 (2H, s), 7.24 (1H, d, *J* 8.8, Ar-*H*), 7.54 (2H, s, exchange with D_2O, SO_2NH_2), 7.80 (1H, dd, *J* 8.8 2.3, Ar-*H*), 7.84 (1H, m, Ar-*H*), 7.92 (1H, m, Ar-*H*), 8.13 (1H, m, Ar-*H*), 8.32 (1H, d, *J* 2.0, Ar-*H*), 8.65 (1H, br s, exchange with D_2O, N*H*), 8.89 (1H, s, Ar-*H* triazole), 10.12 (4H, br s, exchange with D_2O, 2 × O*H*, COO*H*, N*H*); δ_C (100 MHz, DMSO-d_6) 45.4, 103.2, 110.0, 113.7, 115.4, 117.1, 125.8, 126.0, 126.1, 128.1, 128.7, 129.8, 130.0, 131.4, 132.0, 137.1, 141.9, 148.9, 153.1, 160.8, 161.9, 168.6, 169.7 (*C*=O), 182.2 (*C*=S); *m*/*z* (ESI positive) 643.1 $[M+H]^+$.

4.4.3. Synthesis of [9-(2-carboxy-4-{3-[1-(3-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-thioureido}-phenyl)-6-dimethylamino-xanthen-3-ylidene]-dimethyl-ammonium chloride 10



3-(4-Aminomethyl-[1,2,3]triazol-1-yl)-benzenesulfonamide hydrochloride salt **5b** (1.0 equiv) and 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-isothiocyanato-benzoic acid **9** (1.0 equiv) were treated according to the general procedure previously reported afford the titled compound **10** as light pink powder.

[9-(2-Carboxy-4-{3-[1-(3-sulfamoyl-phenyl)-1*H*-[1,2,3]-triazol-4-ylmethyl]-thioureido}-phenyl)-6-dimethylamino-xanthen-3-ylidene]-dimethyl-ammonium chloride **10**: 18% yield; silica gel TLC R_f 0.05 (Ethyl acetate); mp >200 °C; δ_H (400 MHz, DMSO-d_6) 3.30 (12H, s), 4. 32 (2H, d, *J* 5.4, CH_2), 7.00 (4H, m), 7.14 (3H, m), 7.24 (2H, s, exchange with D_2O, SO_2NH_2), 7.41 (1H, m), 7.72 (1H, d, *J* 8.8, Ar-*H*), 7.82 (1H, m, Ar-*H*), 7.89 (1H, m, Ar-*H*), 8.13 (1H, m, Ar-*H*), 8.32 (1H, d, *J* 2.0, Ar-*H*), 8.42 (1H, br s, exchange with D_2O, N*H*), 8.90 (1H, s, Ar-*H* triazole), 10.38 (2H, br s, exchange with D_2O, COO*H*, N*H*); δ_C (100 MHz, DMSO-d_6) 35.1, 45.4, 103.4, 111.0, 113.8, 115.2, 117.0, 125.9, 126.0, 126.2, 128.0, 128.6, 129.9, 130.1, 131.4, 132.0, 137.0, 142.0, 148.8, 153.0, 160.9, 162.0, 168.2, 170.0 (*C*=O), 182.1 (*C*=S); *m*/*z* (ESI positive) 732.27 $[M+H]^+$.

4.5. Kinetic assay inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature (15 min) or 4 °C (6 h) prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear leastsquares methods using PRISM 3, as reported earlier,[20f] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier.[15,20]

4.6. Co-crystallization and X-ray data collection

Crystals of hCA II complexed with compound **7** were obtained using the sitting drop vapor diffusion method. An equal volume of 0.8 mM solution of hCA II in Tris pH = 8.0 and 1.6 mM of **7** in Hepes 20 mM pH = 7.4 was mixed and incubated for 15 minutes. 2 μl of the complex solution were mixed with 2 μl of a solution of 1.6 M sodium citrate, 50 mM Tris pH 8.0 and were equilibrated against the same solution at 296 K. Crystals of the complex grew in a few days. The crystals were flash-frozen at 100 K using a solution obtained by adding 25% (v/v) glycerol to the mother liquor solution as cryoprotectant. A data set on a crystal of the complex hCAII–compound **7** was collected using synchrotron radiation at the Xaloc beamline at ALBA (Barcelona, Spain) with a wavelength of 0.980 Å and a DECTRIS Pilatus 6 M detector. Data were integrated and scaled using the program XDS.[27] Data processing statistics are showed in Table 2.

4.7. Structure determination

The crystal structure of hCA II (PDB accession code: 4FIK) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5.[28] 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of R_{free} calculations. The initial $|F_o - F_c|$ difference electron density maps unambiguously showed the inhibitors. Atomic models for inhibitors were calculated and energy minimized using the program JLigand 1.0.39. Refinements proceeded using normal protocols of positional, isotropic atomic displacement parameters alternating with manual building of the models using COOT.[29] Solvent molecules were introduced automatically using the program ARP.[30] Final rounds of refinement for all the models included hydrogen at calculated positions and refined using a riding model. The quality of the final models was assessed with PROCHECK.[31] Crystal parameters and refinement data are summarized in Table 2. Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 4RH2). Graphical representations were generated with Chimera.[32]

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