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Mono- and three-tailed sugar and iminosugar decorated benzenesulfonamide carbonic anhydrase inhibitors†

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A collection of novel mono- and three-tailed derivatives based on a sugar (glucose) or an iminosugar (trihydroxy piperidine) featuring a terminal benzenesulfonamide were synthesized to investigate the so-called “sugar” and “azasugar” approach with the aim of exploring the activity and selectivity towards the inhibition of human carbonic anhydrases (hCAs). The synthetic approach relies on a general copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction followed by an amine-isothiocyanate coupling. Biological assays were used to collect subtle information on the role of these single or multiple hydrophilic chains. Among the sugar-based inhibitors, the single-tailed compound **10** was identified as a better inhibitor than the reference compound (**AAZ**) towards three different hCAs, while, among the three sugar tailed derivatives, potent and selective inhibition was found for compounds **25** and **26**. A promising and selective inhibitory activity was discovered for the iminosugar single-tailed compound **31** towards hCA VII (K_i = 9.7 nM).

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Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide (CO₂) to give bicarbonate (HCO₃[−]) and a proton (H⁺),¹ a fundamental reaction related to many important physiological processes such as respiration and transport of CO₂/HCO₃[−], pH regulation and CO₂ homeostasis, electrolyte secretion, bone resorption, calcification, biosynthetic reactions and tumorigenesis.²

At physiological pH, this reaction is too slow and requires to be catalyzed. Eight different genetic families, named α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-CA classes, are known to be present in living organisms (higher vertebrates, green plants, algae, bacteria, and archaea) and they differ for metal ions in the active site (*e.g.* zinc, iron, cadmium, and cobalt). Human carbonic anhydrases (hCAs) all belong to the α-family. Fifteen different α-CA isoforms have been identified and characterized and exhibit different enzyme kinetics, expression levels and

locations within the cell and tissues.³ Five hCA isozymes are cytosolic (hCAs I–III, hCA VII, and hCA XIII), two are mitochondrial (hCAs VA and VB), four are membrane-bound or transmembrane proteins (hCAs IV, IX, XII and XIV) and one is secreted into the saliva and milk (hCA VI).⁴ CA isozymes have become drug targets for biomedical applications. For example, hCAs I, II, and IV isoforms have been used to develop antiglaucoma agents, hCAs VA and VB isoforms are targets for obtaining anti-obesity drugs, while hCA VII is implicated in neuropathic pain and in the development of anticonvulsant drugs. Moreover, CAs IX and XII show close association with hypoxic tumours such as those of the lung, breast, colon, esophagus and cervix in which they are overexpressed in tissues and are absent under physiological conditions.⁵ Therefore, the inhibition (or activation) of hCA activity is employed to treat a wide range of acquired and inherited diseases.² Nevertheless, the large number of hCA isoforms requires new increasingly selective inhibitors to avoid side effects due to the indiscriminate inhibition of isoforms that are not involved in a certain pathology.⁶ The so-called “sugar-tail approach”, suggested by Winum and co-workers,^{5a} is emerging as a promising strategy to differently interact with the transmembrane protein (*i.e.*, hCA IX) and the physiologically dominant cytosolic isozymes hCAs I and II. The introduction of a sugar moiety permitted the development of more selective inhibitors with polar or charged tails, thus impairing their ability to diffuse through lipid membranes. The best candidates for this function are carbohydrates, which are widespread in biologically active compounds, influencing their pharmacokinetics, drug targeting,

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and mechanism of action. Moreover, carbohydrates have good solubility in water, a high degree of polyfunctionality and hydrophilicity and their stereochemical arrangement can potentially allow the differentiation of subtle differences in CA active site topology.⁷ Several inhibitors with a sulfonamide moiety, the most used and effective functional group for enabling CA inhibition, directly connected to the anomeric carbon (compounds **1**, Fig. 1) or to the C-6 hydroxyl group of the sugar (compounds **2**, Fig. 1) or *N*- β -glycosyl sulfamides were reported.^{5a} Wilkinson and co-workers synthesized 1,4-disubstituted-1,2,3-triazole sulfonamide glycoconjugates *via* the CuAAC (“copper(i)-catalyzed azide–alkyne cycloaddition”) reaction,⁸ exploiting the versatility of the click chemistry methodology (compounds **3**, Fig. 1).⁹ A promising related strategy developed by our group for targeting more selective carbonic anhydrase inhibitors (CAIs) is the so-called “iminosugar or aza-sugar approach”, where carbohydrates are replaced by glycomimetics in which a nitrogen atom replaces the ring oxygen or the anomeric carbon of carbohydrates, respectively.¹⁰ These compounds have a basic nitrogen atom which can be protonated at physiological pH, establishing different interactions with the enzyme active site or proximal residues with respect to the corresponding carbohydrate.¹¹

We have recently reported the synthesis of chimeric compounds obtained by conjugating a piperidine iminosugar, alkylated at the endocyclic nitrogen with a six carbon atom chain ending with a triazole, to several benzenesulfonamides through various linking moieties (ureido, amido or thiour-

eido), obtaining encouraging results for strong inhibition in the nanomolar range, especially towards hCAs II and VII isoforms.¹²

The crystallographic structures of CA II and CA IX showed that the active site cavity of these enzymes is composed of two different conserved sub-pockets containing hydrophobic and hydrophilic amino acids, respectively.¹³ Based on these results, Tanpure *et al.* first introduced the dual-tail approach as a strategy to further improve the selectivity and specificity of CA inhibitors by addressing both the hydrophobic and hydrophilic parts of the CA active site and conserving the zinc binding group.¹⁴ They synthesized dual-tailed compounds combining phenyl moieties (hydrophobic tails) and glucosyl ones (hydrophilic tails) and their corresponding single-tailed compounds as reference compounds. The compounds designed by the dual-tail approach showed less inhibitory activity with respect to the reference compound acetazolamide (AAZ), probably because the relative positions of the two tails were not optimal for synergistic effects. In contrast, the corresponding single-tailed compounds resulted in more potent inhibitors than AAZ. However, the dual-tailed compounds were tested *in vitro* only on hCAs I and II, suggesting that the information from this preliminary study is limited. Afterwards, Hou *et al.* revisited the dual-tail approach by designing novel CAIs with an amino glucosamine as a hydrophilic moiety and a cinnamamide fragment as a hydrophobic portion, differently connected to each other. Nine target compounds **4** were synthesized by this approach which revealed very good inhibition values towards CA II and IX isozymes with respect to their corresponding single-tailed compounds, thus proving the validity of the dual-tail approach.¹⁵ Nevertheless, recent X-ray crystallographic studies¹⁶ led to a more satisfactory knowledge of the active site composition for each isoform (except for CAs VA and VB) revealing that various CA isoforms do not present an exact distinction between hydrophobic and hydrophilic moieties because of many accessory pockets existing in each hCA isoform. Therefore, the simple hydrophobic/hydrophilic division of the isoform binding pocket may be inadequate. Following these data, Bonardi *et al.* reported the synthesis of 32 benzenesulfonamide derivatives incorporating three tails (5 types, Fig. 1) to increase the matching of the target–ligand interaction within the different hCA active sites.¹⁷ They found that the introduction of a third tail changed the inhibition profiles in terms of potency and selectivity of action. In particular, the presence of three lipophilic tails in the 5 structures did not lead to significant advantages, while a great variability of potency and selectivity was observed by increasing the polarity of at least one tail. In the general structure of **5**, T1 is an aromatic moiety (*e.g.* phenyl, furyl, or naphthyl), T2 can be an alkyl or an aryl group bearing two to eight carbon atoms, while T3 is an alkyl or aryl moiety that may contain a more polar group (*e.g.* cyano, amino or carboxylic acid). As a result, they demonstrated through X-ray crystallography studies and *in silico* tools that bulky **5** derivatives occupied the binding cavities with a great variability among the isoforms, thus contributing to the development of improved selectivity of action.¹⁷

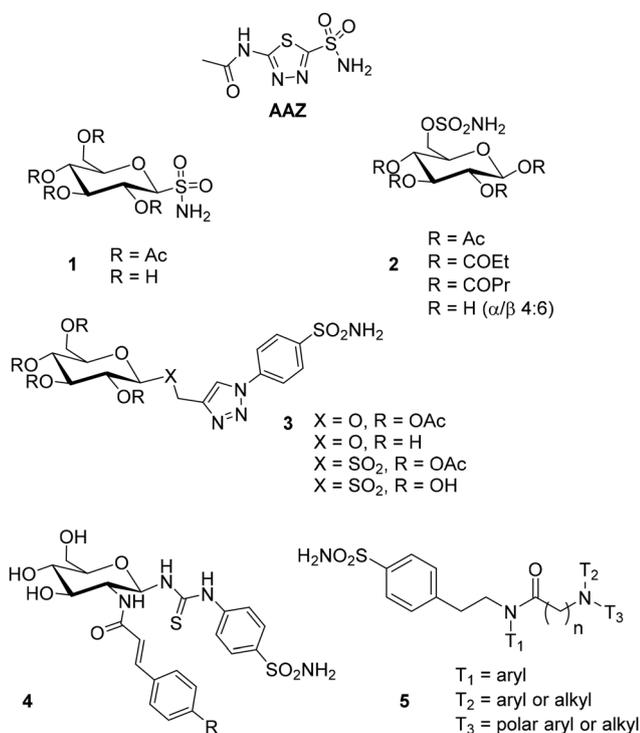


Fig. 1 Examples of hCA inhibitors containing a sugar moiety and dual- and three-tailed compounds, and the reference compound AAZ.



With these premises, we proposed to synthesize a series of benzenesulfonamides bearing multiple hydrophilic tails and screen them as CA inhibitors. Given our experience, we focused on the synthesis of three-tailed compounds containing the sulfonamide group as a zinc binding function and sugars or iminosugars as polar tails (Scheme 1). The synthesized compounds were screened against many different hCAs and compared with the corresponding single-tailed compounds. The results, as well as considerations on the differences in the behaviour of iminosugar vs. sugar moieties, are reported and discussed in this work.

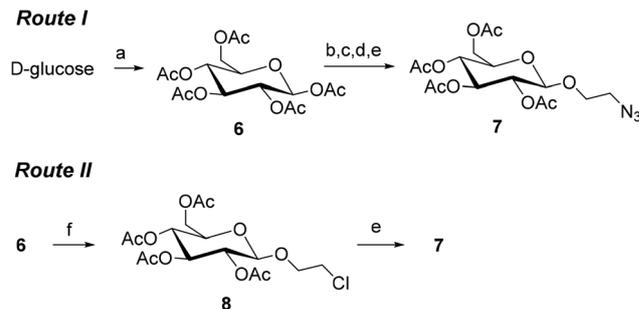
Results and discussion

The synthesis of sugar- and azasugar-linked azides or amines for coupling with benzenesulfonamides functionalized with alkyne or isothiocyanate moieties, respectively, was planned.

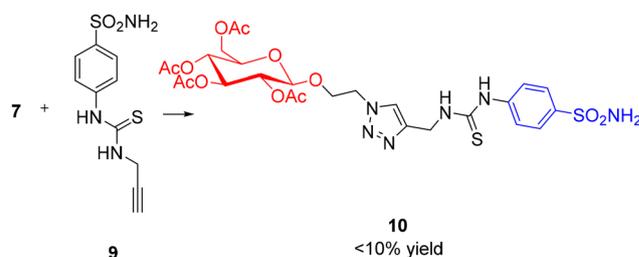
We started with the synthesis of derivative **7** with a relatively short linker between the sulfonamide and the sugar moiety, since in our previous studies¹² we did not find favourable interaction of a long linker within the active site. Thus, in this work, we wanted to bring the sulfonamide closer to the sugar portion. Azide **7** was first obtained from *D*-glucose in 16% overall yield by following a five step procedure reported in the literature (route I, Scheme 2).¹⁸ Alternatively, the same azide **7** was obtained by performing the glycosylation reaction directly on β -*D*-glucose pentaacetate **6** which reacted with 2-chloroethanol in the presence of $\text{BF}_3 \cdot \text{OEt}_2$ and 3 Å molecular sieves in dry CH_2Cl_2 to give the corresponding **8** in 60% yield (route II, Scheme 2). The treatment of **8** with NaN_3 in DMF at 50 °C for three days furnished **7** in 87% yield. With this synthetic strategy (route II), azide **7** was obtained with fewer steps and a higher overall yield (38%, Scheme 2).

Initial attempts to obtain benzenesulfonamide thiourea **10** by the copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction between sugar derived azide **7** and alkyne **9**¹⁹ were unsuccessful (Scheme 3).

Despite several attempts, varying the reaction time, temperature, reductant/catalyst ratio and solvent mixture (from



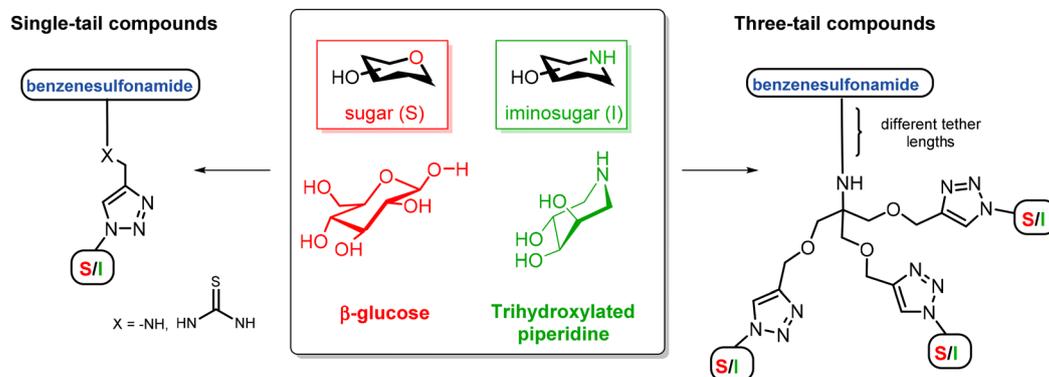
Scheme 2 (a) Ac_2O , NaOAc , reflux, 74%; (b) 1,2-diaminoethane, AcOH , dry THF, rt, 7 h, 86%; (c) Cl_3CCN , DBU, dry CH_2Cl_2 , 0 °C to rt, 5 h, 65%; (d) 2-chloroethanol, $\text{BF}_3 \cdot \text{OEt}_2$, 3 Å MS, dry CH_2Cl_2 , -76 °C, 2 h, 51%; (e) NaN_3 , dry DMF, 50 °C, 3 d, 87%; (f) 2-chloroethanol, $\text{BF}_3 \cdot \text{OEt}_2$, 3 Å MS, dry CH_2Cl_2 , 0 °C to rt, 4 h, 60%.



Scheme 3 Initial attempts to obtain **10**.

THF/water to water/*t*-BuOH), only trace amounts (<10%) of **10** were collected, with the recovery of azide **7**. We wondered whether the presence of a preformed thiourea bond might be responsible for the failure of the CuAAC reaction. However, CuAAC reactions are reported in the literature,²⁰ where a substituted thiourea moiety acts both as a reductant and a ligand. We then modified our synthetic strategy by performing the CuAAC first and successively the amine-isothiocyanate coupling.

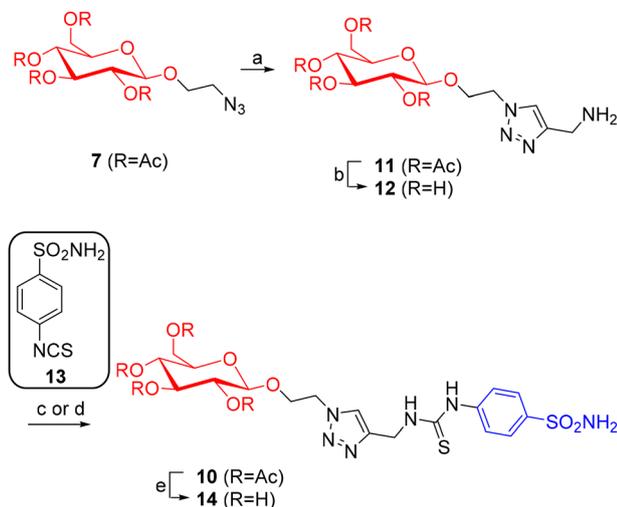
The CuAAC reaction⁸ of **7** with propargylamine in the presence of TBTA (tris((1-benzyl-4-triazolyl)methyl)amine), CuSO_4



Scheme 1 Aim of the work.



and sodium ascorbate yielded amine **11** in 38% yield (Scheme 4), after treatment with the copper scavenger resin Quadrasil MP® and purification by flash column chromatography (FCC). The TBTA ligand is necessary because it stabilizes the copper(I)-oxidation state ensuring that it does not complex the propargylamine during the reaction, while the Quadrasil MP® resin allows the removal of traces of copper residues from the reaction crude product. Subsequently, **11** was reacted with benzenesulfonamide isothiocyanate **13** and NEt_3 in CH_3CN and EtOH to afford the single-tailed thiourea **10** in 53% yield (Scheme 4). Deacetylation of **10** with MeONa in MeOH followed by treatment with the acid resin Amberlyst 15 gave **14** in 57% yield (Scheme 4).

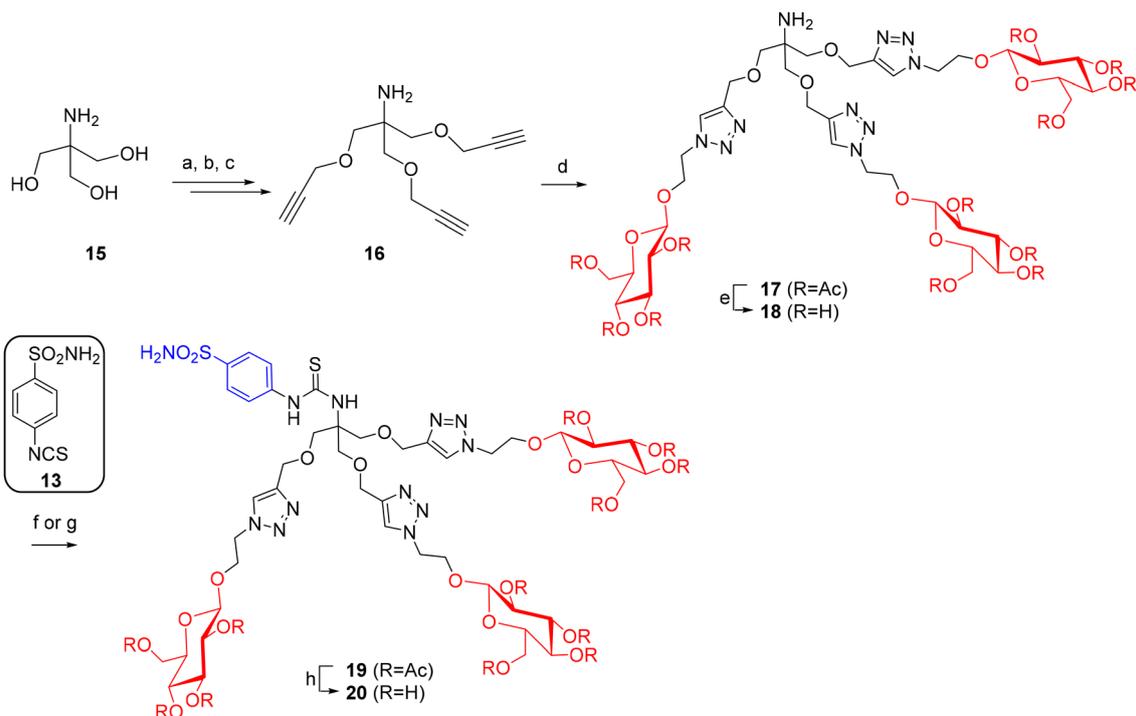


Scheme 4 (a) Propargylamine, CuSO_4 , TBTA, sodium ascorbate, $\text{THF} : \text{H}_2\text{O}$ 2 : 1, rt, 15 h, 38%; (b) Na_2CO_3 , MeOH, rt, 18 h, quantitative; (c) starting from **11**, reaction with sulfonamide **13**, NEt_3 , $\text{CH}_3\text{CN} : \text{EtOH}$ 1 : 1, 80 °C, 18 h, 53%; (d) starting from **12**, reaction with sulfonamide **13**, EtOH, rt, 20 h, 14%; (e) MeONa, MeOH, rt, 2 h, 57%.

izes the copper(I)-oxidation state ensuring that it does not complex the propargylamine during the reaction, while the Quadrasil MP® resin allows the removal of traces of copper residues from the reaction crude product. Subsequently, **11** was reacted with benzenesulfonamide isothiocyanate **13** and NEt_3 in CH_3CN and EtOH to afford the single-tailed thiourea **10** in 53% yield (Scheme 4). Deacetylation of **10** with MeONa in MeOH followed by treatment with the acid resin Amberlyst 15 gave **14** in 57% yield (Scheme 4).

Performing the two steps for obtaining **14** from **11** the other way around, that is, first deprotection to **12** and then coupling with the sulfonamide **13**, was less efficient (14% overall yield, Scheme 4). While deacetylation of **11** to **12** occurred quantitatively, the following coupling was unsatisfactory, likely due to a sluggish reaction under the conditions required by hydrophilic **12** (EtOH at room temperature).

With the single-tailed **14** in hand, the synthesis of the corresponding triple-tailed benzenesulfonamide **20** was attempted by employing the same synthetic strategy. The trivalent amine **16** was obtained from tris(hydroxymethyl)amino-methane **15** in three steps with a 25% overall yield.²¹ The CuAAC reaction of **16** with the sugar-derived azide **7** in $\text{THF} / \text{H}_2\text{O}$ with CuSO_4 and sodium ascorbate under MW irradiation at 80 °C yielded the functionalized amine **17** (89%, Scheme 5), after treatment with the copper scavenger resin Quadrasil MP® and purification by FCC. The reaction of **17** with isothiocyanate **13** in the presence of NEt_3 in $\text{CH}_3\text{CN}/\text{EtOH}$ provided



Scheme 5 (a) Boc_2O , $t\text{-BuOH}/\text{MeOH}$, rt, 18 h, 74%; (b) propargyl bromide, KOH, DMF, 0 °C to 35 °C, 24 h, 35%; (c) TFA, dry CH_2Cl_2 , rt, 2 h, 95%; (d) CuSO_4 , sodium ascorbate, $\text{THF} : \text{H}_2\text{O}$ 2 : 1, MW 80 °C, 1 h 45 min, 89%; (e) Ambersep 900-OH, MeOH, rt, 16 h, 96%; (f) from **17**, reaction with sulfonamide **13**, $\text{CH}_3\text{CN} : \text{EtOH}$ 1 : 1, 80 °C, 5 d, 36%; (g) from **18**, reaction with sulfonamide **13**, EtOH, MeOH, 50 °C, 20 h, 17%; (h) Na_2CO_3 , MeOH, rt, 18 h, 68%.



the triple-tailed benzenesulfonamide **19** in a moderate 36% yield, which can be ascribed to the bulkiness of amine **17**. Deprotection of **19** with Na_2CO_3 in MeOH led to the final triple-tailed compound **20** in 68% yield (Scheme 5). As previously mentioned, the reaction of unprotected **18** with sulfonamide **13** yielded compound **20** in a much lower yield (17%) due to poor solubility in the solvent mixture.

Since the triple-tailed compounds **19** and **20** resulted in much poorer inhibitors than **10** and **14** (see biological results), the synthesis of congeners **25** and **26** (Scheme 6) was planned, speculating that spacing the sulfonamide moiety from the polar tails would allow it to enter the active site of hCAs more easily. The trivalent alkyne **22** was obtained in 61% yield by coupling **16** with the protected amino acid **21**, which was carried out in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIPEA) in CH_2Cl_2 at room temperature. A CuAAC reaction of **22** with the sugar-derived azide **7** under MW irradiation at 80 °C gave the protected amine **23** in 79% yield (Scheme 6), after treatment with the copper scavenger resin Quadrasil MP® and purification by FCC. Deprotection of **23** in trifluoroacetic acid (TFA) furnished the free amine **24** (94%), which was added to benzenesulfonamide **13** in the presence of NET_3 in $\text{CH}_3\text{CN}/\text{EtOH}$ to afford the three-tailed compound **25** in 89% yield, after treatment over Sephadex LH-20 resin and purification by FCC. The final hydrolysis of acetates, performed with Na_2CO_3 in MeOH

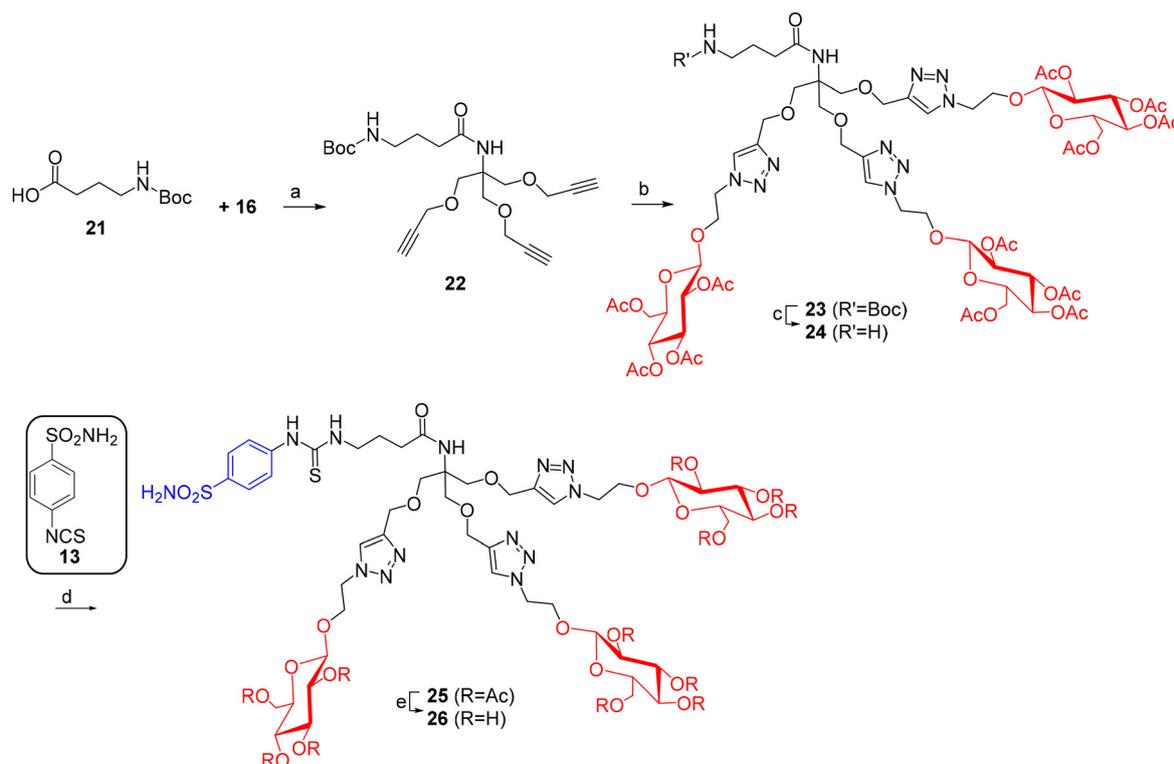
in order to prevent hydrolysis of amide which might occur under more basic conditions, gave the desired benzenesulfonamide **26** quantitatively (Scheme 6).

With the sugar derivatives in hand, our attention was focused on the synthesis of related iminosugar-linked benzenesulfonamides, in order to investigate the role of a sugar mimetic in the potency and selectivity of the inhibitors towards the different isoforms of hCAs.

3,4,5-Trihydroxypiperidine iminosugars recently showed interesting biological properties.²²

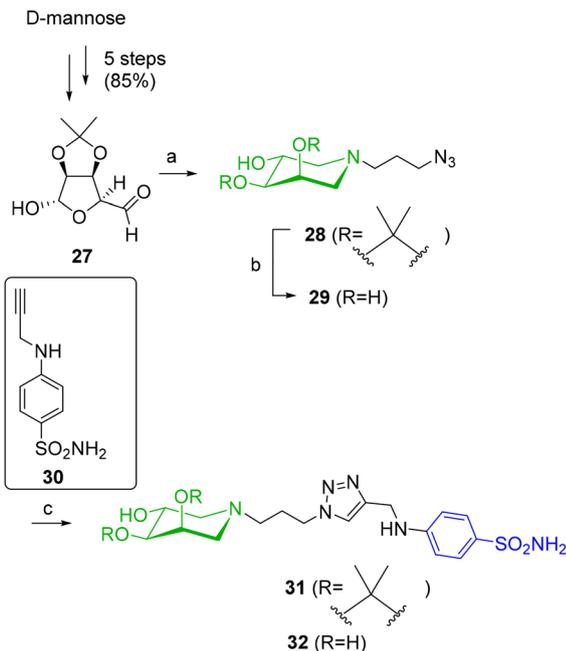
Azide **28**,²³ the precursor of all the new compounds, was synthesized from aldehyde **27**, derived in turn from inexpensive D-mannose in five steps (85% overall yield), through a double reductive amination procedure (DRA) as reported (Scheme 7).²⁴ Compound **28** was selected for having a linker between the azide and the iminosugar moiety roughly of the same length as in the sugar derivative **7**. A CuAAC reaction of **28** with benzenesulfonamide **30** bearing an alkyne moiety in the presence of CuSO_4 and sodium ascorbate yielded the protected compound **31** in 91% yield (Scheme 7), after treatment with the copper scavenger resin Quadrasil MP® and purification by FCC. Concurrently, deprotection of azide **28** to trihydroxypiperidine **29**,²³ followed by CuAAC with benzenesulfonamide **30** afforded the corresponding deprotected **32** in 70% yield after treatment with the copper scavenger resin Quadrasil MP® and purification by FCC.

Triazole-iminosugar **34**, prepared from azide **28** through the protected intermediate **33** according to the literature²⁵



Scheme 6 (a) EDC-HCl, HOBt, DIPEA, CH_2Cl_2 , rt, 3 d, 61%; (b) **7**, CuSO_4 , sodium ascorbate, $\text{THF} : \text{H}_2\text{O} 2 : 1$, MW 80 °C, 1 h 15 min, 79%; (c) TFA, rt, 2 h, 94%; (d) NET_3 , $\text{CH}_3\text{CN} : \text{EtOH} 1 : 1$, 80 °C 4 d, 89%; (e) Na_2CO_3 , MeOH, rt, 18 h, quantitative.

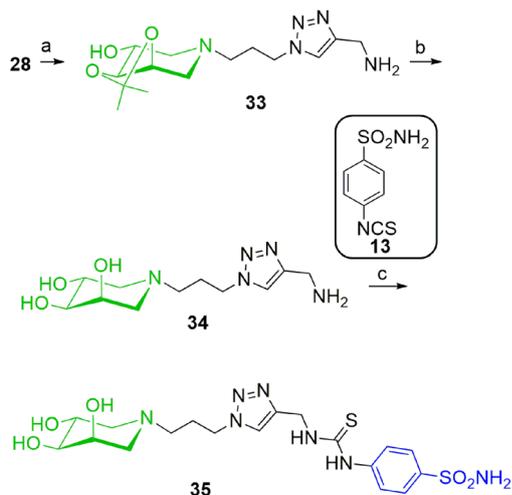




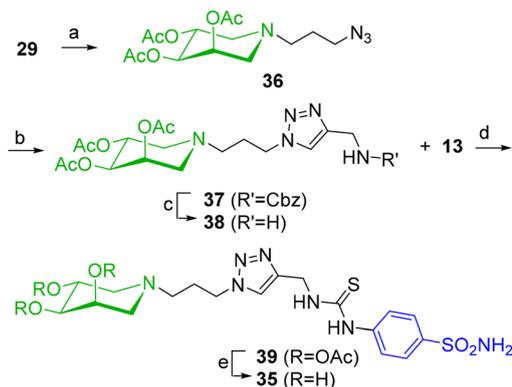
Scheme 7 (a) 3-Azido-1-propanamine, NaBH_3CN , CH_3COOH , dry MeOH, rt, 7 d, 64%; (b) HCl 12 M, MeOH, rt, 18 h, 93%; (c) CuSO_4 , sodium ascorbate, THF/ H_2O 2 : 1, rt, 18 h, 91% from **28**, 70% from **29**.

(Scheme 8), was reacted with benzenesulfonamide **13** in MeOH/EtOH at 50 °C to give thiourea **35** in 15% yield (Scheme 8). Again, the high hydrophilicity of **34** which is not soluble in the typical solvents used for the coupling reaction may be responsible for the low yield of the product.

A more satisfactory synthetic route to **35** was developed starting from azide **36**, obtained by acetylation of **29** in 90% yield (Scheme 9). The direct CuAAC reaction of **36** with propargylamine in the presence of TBTA gave only traces of the



Scheme 8 (a) Propargylamine, CuSO_4 , sodium ascorbate, THF/ H_2O 2 : 1, rt, 16 h, 96%; (b) HCl 1 M, MeOH, rt, 16 h, DOWEX 50WX8-200, 77%; (c) EtOH, MeOH, 50 °C, 20 h, 15%.



Scheme 9 (a) Ac_2O , dry pyridine, rt, 18 h, 90%; (b) *N*-Cbz-propargylamine, CuSO_4 , sodium ascorbate, THF/ H_2O 2 : 1, rt, 18 h, 54%; (c) H_2 , Pd(OH) $_2$ /C, MeOH, rt, 18 h, 93%; (d) NEt_3 , CH_3CN : EtOH, 80 °C, 18 h, 59%; (e) Na_2CO_3 , MeOH, rt, 18 h, 38%.

desired triazole **38**. Thus, a two-step procedure was employed, reacting **36** with *N*-Cbz-propargylamine in the presence of CuSO_4 and sodium ascorbate in THF/ H_2O (Scheme 9). The treatment of the resulting protected amine **37** (54%) under an H_2 atmosphere in the presence of Pd(OH) $_2$ /C led to the free amine **38** in 93% yield. A reaction with benzenesulfonamide **13** in the presence of NEt_3 in CH_3CN /EtOH at 80 °C afforded the protected compound **39** (59%), which was deacetylated with Na_2CO_3 in MeOH to the final benzenesulfonamide **35** in 38% yield. This strategy, besides increasing the yield of **35**, also allowed access to the corresponding triacetylated **39** to be compared in the biological assays.

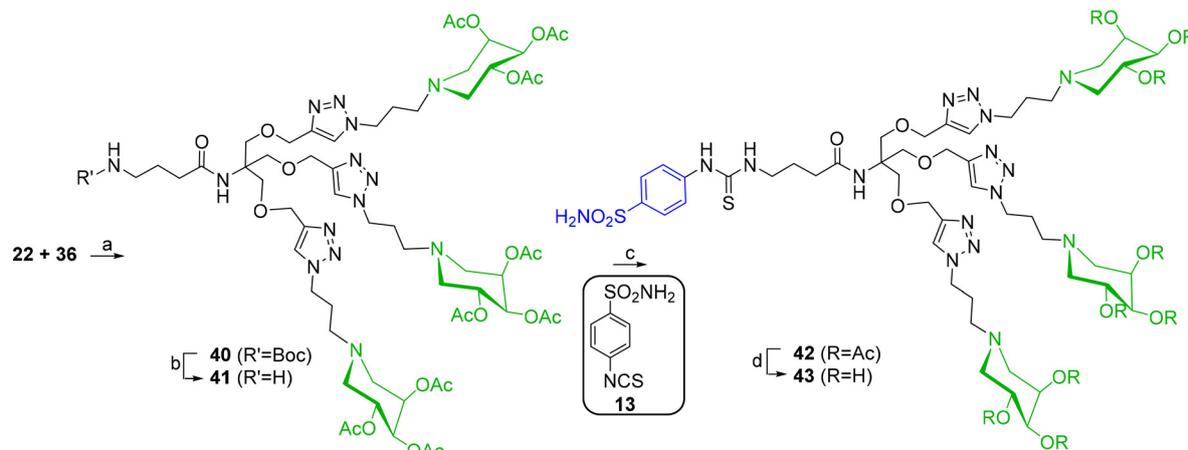
The synthesis of three-tailed iminosugar benzenesulfonamide analogues was then performed. Compound **43**, an analogue of **26** with the iminosugar moieties replacing the sugar ones, was chosen as the target, taking into account the better biological profile of **26** (the three-tailed compound with a longer linker between the sulfonamide moiety and the sugar tails) with respect to **20**.

Starting from the acetonide-protected trihydroxypiperidine **28**, the target three-tailed benzenesulfonamide **43** was recovered only in traces.

Much better results were obtained by starting with the azido-armed fully acetylated iminosugar **36**. Its CuAAC reaction with the trialkyne scaffold **22** in the presence of CuSO_4 and sodium ascorbate under MW irradiation at 80 °C yielded the protected compound **40** (96%, after treatment with the copper scavenger resin Quadrasil MP® and purification by FCC, Scheme 10). Deprotection of carbamate with TFA in dry CH_2Cl_2 furnished the free amine **41** (92%), which was reacted with benzenesulfonamide **13** in the presence of NEt_3 in CH_3CN /EtOH to give the protected compound **42** in 68% yield. Final deacetylation with Na_2CO_3 in MeOH and passage over Bio-Beads $_SX8$ resin gave the triple-tailed **43** in 55% yield (Scheme 10).

The newly synthesized single-tailed sugar (**10** and **14**) and iminosugar (**31**, **32**, **35**, and **39**), and triple-tailed sugar (**19**, **20**,





Scheme 10 (a) CuSO_4 , sodium ascorbate, THF/ H_2O 2 : 1, MW 80 °C, 1 h, 96%; (b) TFA, dry CH_2Cl_2 , rt, 2 h, 92%; (c) NEt_3 , CH_3CN , EtOH, 80 °C, 3 d, 68%; (d) Na_2CO_3 , MeOH, rt, 18 h, 55%.

25, and 26) and iminosugar (42 and 43) benzenesulfonamides were tested against different isoforms of hCAs (I, II, III, IV, VA, VB, VI, VII, IX, XII and XIII) using a Stopped Flow CO_2 Hydrase assay²⁶ in order to evaluate the role of multivalent presentation of the sugar/iminosugar compared to the corresponding monovalent compounds and also to explore the role of a sugar mimetic (iminosugar) in the selectivity of the inhibitor towards the different isoforms of hCAs. Acetazolamide (AAZ) was used as a reference.²⁷ The inhibition data are reported in Table 1.

The investigated compounds showed weak inhibitory properties against the cytosolic isoforms hCAs I and II ($K_i > 200$ nM), with the exception of 10, which strongly inhibited hCA I with $K_i = 65.2$ nM (3.83 times lower than AAZ) and hCA II with $K_i = 5.7$ nM (2.12 times lower than AAZ). This single-tailed protected sugar 10 also showed powerful inhibition of hCA IX ($K_i = 5.1$ nM), *i.e.*, 5.06 times lower than that of AAZ. In general, the introduction of an iminosugar moiety did not

increase the inhibition with respect to the reference compound AAZ. However, the protected three-tailed sugar compound 25 and the single-tailed iminosugar 31 showed very good selectivity towards hCA VII with low inhibition constant values ($K_i = 9.7$ nM for both compounds). The deprotected compound 26 featuring three sugar tails is a 1.58 times better inhibitor than AAZ towards hCA VB ($K_i = 34.2$ nM). Concerning the differences among protected and deprotected compounds in the inhibition of hCAs, 10 was found to be a better inhibitor than 14 mainly on hCAs I, II and IX ($K_i = 65.2, 5.7$ and 5.1 nM vs. $312.8, 205.2$ and 45.8 nM). The three-tailed compounds 19 and 20 with a shorter linker between the sulfonamide group and the sugar moieties are weak to moderate inhibitors against all the hCAs ($K_i = 70.3$ – 9595 nM). Probably, the more hindered compounds 19 and 20 experience adverse steric effects when the sulfonamide moiety approaches the hCA active site. However, the protected 19 showed good selectivity for hCA VII ($K_i = 70.3$ nM), the isoform of interest for the treat-

Table 1 Inhibition data of compounds 10, 14, 19, 20, 25, 26, 31, 32, 39, 35, 42, and 43 towards hCAs I, II, III, IV, VA, VB, VI, VII, IX, XII and XIII by a Stopped Flow CO_2 Hydrase assay. Acetazolamide (AAZ) was used as the reference inhibitor. Type of inhibitor: (S): sugar and (I): iminosugar

Compound	Type	K_i^a (nM)										
		hCA I	hCA II	hCA III	hCA IV	hCA VA	hCA VB	hCA VI	hCA VII	hCA IX	hCA XII	hCA XIII
10	(S)-single tail	65.2	5.7	—	6604	271.9	—	406.5	76.5	5.1	69.4	233.0
14	(S)-single tail	312.8	205.2	—	7979	711.7	—	458.5	82.2	45.8	36.5	696.7
31	(I)-single tail	547.4	6384	>10 000	8410	330.0	550.0	86.0	9.7	242.3	29.0	424.3
32	(I)-single tail	684.6	7117	>10 000	7485	339.0	237.9	73.3	34.8	85.6	26.7	397.8
39	(I)-single tail	249.6	326.6	—	3801	2709	—	93.6	75.6	47.6	62.1	638.2
35	(I)-single tail	322.1	427.3	>10 000	285.9	363.0	184.2	503.6	83.9	135.6	21.7	50.7
19	(S)-three tails	916.8	304.6	—	9595	376.0	—	766.8	70.3	1901	700.8	260.5
20	(S)-three tails	1760	304.8	—	8974	97.6	—	445.0	75.3	3871	369.1	95.3
25	(S)-three tails	8641	9073	>10 000	5884	88.0	96.3	96.4	9.7	71.4	31.0	90.7
26	(S)-three tails	668.5	477.6	>10 000	3297	188.0	34.2	149.8	30.0	121.6	85.5	206.3
42	(I)-three tails	474.4	605.8	—	9558	906.1	—	80.4	76.8	88.9	77.2	363.3
43	(I)-three tails	552.9	525.3	—	8703	96.5	—	211.9	72.4	84.2	61.2	67.6
AAZ		250.0	12.1	>10 000	74.0	63.0	54.0	11.0	2.5	25.8	5.7	17.0

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



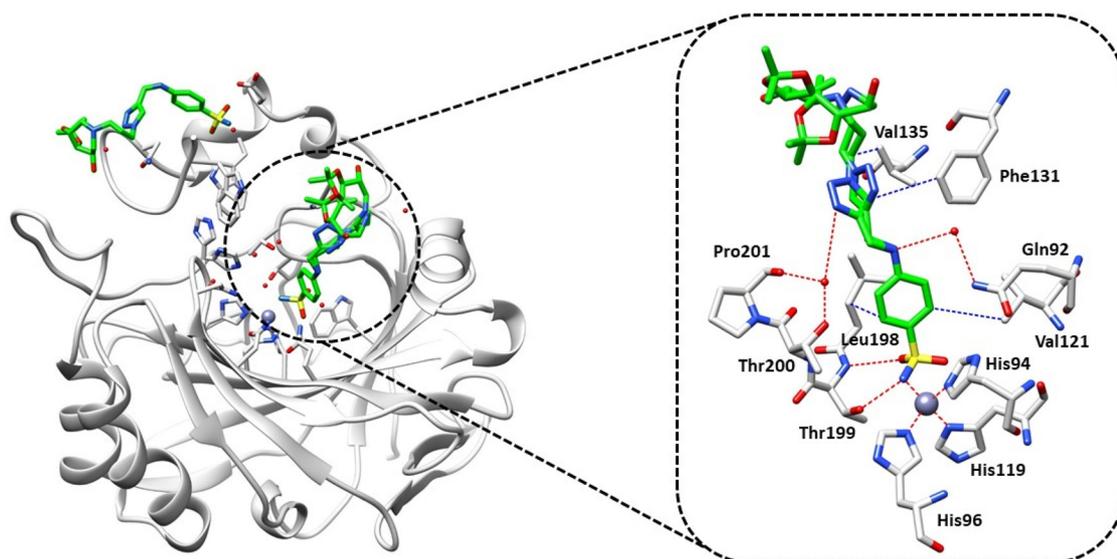


Fig. 2 X-ray crystal structure of hCA II bound with compound **31** (PDB: 8OGF). Residues involved in the binding of inhibitors are also shown; the grey sphere represents the zinc ion in the active site of the proteins.

ment of neuropathic pain. As a matter of fact, the sugar three-tailed compounds **25** and **26** bearing a longer linker between the sulfonamide and the tails resulted in better inhibitors of hCA VII ($K_i = 9.7$ and 30.0 nM, respectively) with respect to **19** and **20**. While acetylated **25** also inhibited the tumour associated hCA XII isoform ($K_i = 31.0$ nM), deprotected **26** was a 1.58 times stronger inhibitor of hCA VB ($K_i = 34.2$ nM) as compared to **AAZ**. Concerning the single-tailed iminosugar compounds **31**, **32**, **39** and **35**, they all inhibited the tumour associated hCA XII with $K_i = 21.7$ – 62.1 nM. The protected **31** showed strong inhibition and selectivity ($K_i = 9.7$ nM) towards hCA VII, in contrast to deprotected **32**. Peracetylated **39** showed good inhibition ($K_i = 47.6$ nM) of hCA IX, while the corresponding deacetylated **35** showed great selectivity towards hCA XII ($K_i = 21.7$ nM). The three-tailed iminosugar derivatives neither showed impressive levels of inhibition nor particular selectivity. It is worth noting the inverse behaviour of deprotected **43** and its corresponding peracetylated **42** towards hCA VA (**43**, $K_i = 96.5$ nM; **42**, $K_i = 906.1$ nM) and hCA VI (**43**, $K_i = 211.9$ nM; **42**, $K_i = 80.4$ nM).

According to the data presented in Table 1, compound **31** exhibited weak inhibition against hCA II. To investigate the molecular basis of CA inhibition using this derivative, we used X-ray crystallography to determine its complex with hCA II at a resolution of 1.3 Å (Fig. 2).

Although **31** is not a very potent hCA II inhibitor, we chose this derivative for crystallographic experiments because in many cases this isoform may be not a drug target but an off-target. There are in fact hundreds of highly potent hCA II inhibitors for which the crystal structure of adducts with this isoform has been reported,^{3,13} but very few weak inhibitors were characterized by this technique. Understanding the factors that are connected with less efficient hCA II inhibition can in fact be useful for drug design purposes.

Complex hCAII/**31** revealed two molecules bound to the protein, with one located inside the active site responsible for the activity, and the other bound in a cleft (N-terminal) and not involved in the mechanism of inhibition, as shown in Fig. 2; this finding is in agreement with previous reports from some of us.^{28,29}

Analysis of the electron density maps (Fig. S1 in the ESI†) revealed a clear density for the portion of the inhibitor which includes the benzenesulfonamide group and the triazole ring, confirming its binding to the enzyme. However, the triazole ring showed double conformation leading probably to the disorder of the tail that resulted in a poor electron density map, so that zero occupancy was attributed to the atoms forming the inhibitor tail. Compound **31** showed the classical binding mode of sulfonamide inhibitors by directly interacting with the zinc ion and forming a hydrogen bond with Thr199. The benzenesulfonamide moiety established hydrophobic interactions with the side chains of Val121 and Leu198, further stabilizing the complex within the active site. Additionally, a water bridge was observed between Gln92 and the secondary amine of the aminobenzenesulfonamide portion. One of the conformations of the triazole ring formed a water bridge with Thr200 and Pro201, while hydrophobic interactions were observed with Phe131. However, the second conformation lacked these interactions, providing a possible explanation for the weak potency of this inhibitor against hCA II.

Experimental

General methods

Commercial reagents were used as received. All reactions were carried out under magnetic stirring and monitored by TLC on



0.25 mm silica gel plates (Merck F254). Column chromatography was carried out on Silica Gel 60 (32–63 μm) or on silica gel (230–400 mesh, Merck). Yields refer to spectroscopically and analytically pure compounds unless otherwise stated. $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra were recorded on a Varian Gemini 200 MHz, a Varian Mercury 400 MHz or on a Varian INOVA 400 MHz instrument at 25 $^\circ\text{C}$. Chemical shifts are reported relative to CDCl_3 (^{13}C : $\delta = 77.0$ ppm, ^1H : 7.26 ppm), or CD_3OD (^{13}C : $\delta = 49.0$ ppm, ^1H : 3.31 ppm). Integrals are in accordance with assignments, coupling constants are given in Hz. For detailed peak assignments 2D spectra were measured (COSY, HSQC). Small scale microwave assisted syntheses were carried out in a microwave apparatus for synthesis (CEM Discover) with an open reaction vessel and external surface sensor. IR spectra were recorded with an IRAffinity-1S SHIMADZU system spectrophotometer. Optical rotation measurements were performed on a JASCO DIP-370 polarimeter. ESI-MS spectra were recorded with a Thermo Scientific™ LCQ fleet ion trap mass spectrometer. Elemental analyses were performed with a Thermo Scientific FlashSmart Elemental Analyzer CHNS/O.

Only the synthesis and characterization of the most relevant compounds are described in this section. For the other compounds described in this work, see the ESI.† For practical reasons, the assignment of H and C atoms in NMR characterization studies reflects the numbering of chemical structures in the ESI.†

Synthesis of compound 10

Benzenesulfonamide **13** (20 mg, 0.09 mmol) and NEt_3 (6 μl , 0.04 mmol) were added to a solution of **11** (40 mg, 0.09 mmol) in CH_3CN (1 ml) and EtOH (1 mL) and the mixture was stirred at 80 $^\circ\text{C}$ for 15 hours until a TLC control attested the disappearance of the starting material **11** ($R_f = 0.23$, CH_2Cl_2 : MeOH : NH_4OH (6%) 10:1:0.1). Then, the crude product was evaporated under vacuum and purified by flash column chromatography on silica gel (CH_2Cl_2 : MeOH : NH_4OH (6%) 25:1:0.1) to give 31 mg of **10** ($R_f = 0.39$, CH_2Cl_2 : MeOH : NH_4OH (6%) 10:1:0.1, 0.05 mmol, 53%) as pale yellow oil.

10: $[\alpha]_{\text{D}}^{20} = -12.50$ ($c = 0.75$, MeOH). $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ ppm: 7.97 (s, 1H, triazole), 7.84 (d, $J = 8.8$ Hz, AB system, 2H, Ar), 7.69 (d, $J = 8.7$ Hz, AB system, 2H, Ar), 5.23 (t, $J = 9.5$ Hz, 1H, H-3), 5.00 (t, $J = 9.8$ Hz, 1H, H-4), 4.89 (br s, 2H, H-9), 4.87–4.84 (m, 1H, H-2), 4.68 (d, $J = 7.8$ Hz, 1H, H-1), 4.60 (t, $J = 5.2$ Hz, 2H, H-8), 4.26 (dd, $J = 4.7$, 12.4 Hz, 1H, Ha-6), 4.22–4.16 (m, 1H, Ha-7), 4.15–4.10 (m, 1H, Hb-6), 4.02 (quint, $J = 5.3$ Hz, 1H, Hb-7), 3.89–3.84 (m, 1H, H-5), 2.05 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.96 (s, 3H, OAc), 1.95 (s, 3H, OAc). $^{13}\text{C-NMR}$ (50 MHz, CD_3OD) δ ppm: 182.8 (s, 1C, C=S), 172.3 (s, 1C, C=O), 171.6 (s, 1C, C=O), 171.3 (s, 1C, C=O), 171.2 (s, 1C, C=O), 145.7 (s, 1C, triazole), 144.1 (s, 1C, Ar), 140.2 (s, 1C, Ar), 128.0 (d, 2C, Ar), 125.4 (d, 1C, triazole), 123.7 (d, 2C, Ar), 101.6 (d, 1C, C-1), 74.1 (d, 1C, C-3), 72.9 (d, 1C, C-5), 72.6 (d, 1C, C-2), 69.8 (d, 1C, C-4), 69.0 (t, 1C, C-7), 63.0 (t, 1C, C-6), 51.4 (t, 1C, C-8), 40.5 (t, 1C, C-9), 20.7 (q, 2C, CH_3) 20.6 (q, 2C, CH_3).

IR (CDCl_3): $\tilde{\nu} = 3343, 3034, 2959, 1755, 1531, 1365, 1333, 1203, 1163, 1056, 1042$ cm^{-1} . MS-ESI (m/z , %) = 709.08 (100) [$\text{M} + \text{Na}$] $^+$. $\text{C}_{26}\text{H}_{34}\text{N}_6\text{O}_{12}\text{S}_2$ (686.71): calcd C, 45.47; H, 4.99; N, 12.24; S, 9.18. found C, 45.10; H, 5.28; N, 12.30; S, 9.25.

Synthesis of compound 25

Benzenesulfonamide **13** (18 mg, 0.08 mmol) was added to a solution of **24** (64 mg, 0.04 mmol) and NEt_3 (6 μl , 0.04 mmol) in EtOH (1 mL) and CH_3CN (1 mL) and the mixture was stirred at 80 $^\circ\text{C}$ for 4 days until a TLC control attested the disappearance of the starting material **24** ($R_f = 0.27$, CH_2Cl_2 : MeOH 10:1). Then, the reaction mixture was evaporated under vacuum. The crude product was purified by size exclusion chromatography by employing Sephadex LH-20® resin and eluting with MeOH and then by flash column chromatography on silica gel (CH_2Cl_2 : MeOH 8:1) to give 65 mg of **25** ($R_f = 0.46$, CH_2Cl_2 : MeOH 8:1, 0.04 mmol, 89%) as a white waxy solid.

25: $[\alpha]_{\text{D}}^{22} = -10.90$ ($c = 0.8$, CHCl_3). $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ ppm: 7.95–7.81 (m, 5H, triazole, Ar), 7.70–7.61 (m, 2H, Ar), 5.24 (t, $J = 9.5$ Hz, 3H, H-3), 5.01 (t, $J = 9.6$ Hz, 3H, H-4), 4.88–4.84 (m, 3H, H-2), 4.71 (d, $J = 8$ Hz, 3H, H-1), 4.65–4.51 (m, 12H, H-8, H-9), 4.28 (dd, $J = 4.4, 12.4$ Hz, 3H, Ha-6), 4.25–4.17 (m, 3H, Ha-7), 4.14 (d, $J = 12.0$ Hz, 3H, Hb-6), 4.05–3.95 (m, 3H, Hb-7), 3.93–3.85 (m, 3H, H-5), 3.85–3.74 (m, 6H, H-10), 3.66–3.54 (m, 2H, H-13), 2.32–2.23 (m, 2H, H-11), 2.05 (s, 9H, OAc), 2.00 (s, 9H, OAc), 1.98–1.84 (m, 20H, OAc, H-12). $^{13}\text{C-NMR}$ (50 MHz, CD_3OD) δ ppm: 182.1 (s, 1C, C=S), 175.6 (s, 1C, HN-C=O), 172.3 (s, 3C, O=CCH₃), 171.6 (s, 3C, O=CCH₃), 171.3 (s, 3C, O=CCH₃), 171.1 (s, 3C, O=CCH₃), 145.8 (s, 3C, triazole), 143.9 (s, 1C, Ar), 140.1 (s, 1C, Ar), 128.1 (d, 2C, Ar), 125.7 (d, 3C, triazole), 123.8 (d, 2C, Ar), 101.6 (d, 3C, C-1), 74.1 (d, 1C, C-3), 73.0 (d, 3C, C-5), 72.5 (d, 3C, C-2), 69.8 (t, 3C, C-10), 69.6 (d, 3C, C-4), 69.0 (t, 3C, C-7), 65.4 (t, 3C, C-8), 63.1 (t, 3C, C-6), 61.4 (s, 1C, HNC(CH₂O)₃), 51.3 (t, 3C, C-9), 45.0 (t, 1C, C-13), 34.9 (t, 1C, C-11), 26.2 (t, 1C, C-12), 20.8 (q, 6C, OAc), 20.6 (q, 6C, OAc). IR (CDCl_3) $\tilde{\nu} = 3405, 3010, 2960, 2912, 1750, 1532, 1460, 1368, 1230, 1160, 1038$ cm^{-1} . MS-ESI (m/z , %) = 915.84 (100) [$\text{M} + 2\text{Na}$] $^{2+}$, 1807.87 (48) [$\text{M} + \text{Na}$] $^+$. $\text{C}_{72}\text{H}_{99}\text{N}_{13}\text{O}_{36}\text{S}_2$ (1785.58): calcd C, 48.40; H, 5.58; N, 10.19; S, 3.59; found C, 48.28; H, 5.75; N, 10.10; S, 3.65.

Synthesis of compound 26

Sodium carbonate (4 mg, 0.04 mmol) was added to a solution of **25** (15 mg, 0.01 mmol) in MeOH (1 mL) and the mixture was stirred at room temperature for 18 hours. The mixture was filtered and the solvent was removed under vacuum to give 10 mg of **26** ($R_f = 0.06$, CH_2Cl_2 : MeOH : NH_4OH (6%) 10:1:0.1, 0.01 mmol, quantitative) as a white waxy solid.

26: $[\alpha]_{\text{D}}^{24} = -3.25$ ($c = 0.4$, MeOH). $^1\text{H-NMR}$ (400 MHz, D_2O) δ ppm: 7.97 (s, 3H, triazole), 7.79 (d, $J = 8.2$ Hz, AB system, 2H, Ar), 7.35 (d, $J = 8.4$ Hz, AB system, 2H, Ar), 4.61–4.53 (m, 6H, H-8), 4.50 (s, 6H, H-9), 4.35 (d, $J = 7.8$ Hz, 3H, H-1), 4.25–4.16 (m, 3H, Ha-7), 4.05–3.96 (m, 3H, Hb-7), 4.30 (d, $J = 12.0$ Hz, 3H, Ha-6), 3.68–3.57 (m, 9H, Hb-6, H-10), 3.49–3.31 (m, 8H, H-3, H-4, H-13), 3.30–3.23 (m, 3H, H-5), 3.16 (t, $J = 8.8$ Hz, 3H,



H-2), 2.19 (br s, 2H, H-11), 1.75 (br s, 2H, H-12). ^{13}C -NMR (50 MHz, D_2O) δ ppm: 175.6 (s, 1C, C=S), 163.5 (s, 1C, HN-C=O), 143.8 (s, 3C, triazole), 141.0 (s, 2C, Ar), 126.7 (d, 2C, Ar), 125.6 (d, 3C, triazole), 125.0 (d, 2C, Ar), 102.4 (d, 3C, C-1), 75.9 (d, 3C, C-3), 75.6 (d, 3C, C-4), 72.9 (d, 3C, C-2), 69.5 (d, 3C, C-5), 68.0 (t, 3C, C-7), 67.5 (t, 3C, C-10), 63.5 (t, 3C, C-9), 60.7 (t, 3C, C-6), 59.7 (s, 1C, HNC(CH₂O)₃), 50.3 (t, 3C, C-8), 43.8 (t, 1C, C-13), 33.1 (t, 1C, C-11), 24.1 (t, 1C, C-12). MS-ESI (m/z , %) = 639.58 (100) $[\text{M} - 2\text{H}]^{2-}$. $\text{C}_{48}\text{H}_{75}\text{N}_{13}\text{O}_{24}\text{S}_2$ (1282.31): calcd C, 44.96; H, 5.90; N, 14.20; S, 5.00; found C, 44.70; H, 6.16; N, 14.30; S, 5.26.

Synthesis of compound 31

Sulfonamide **30** (46 mg, 0.22 mmol), CuSO_4 (10 mg, 0.06 mmol) and sodium ascorbate (24 mg, 0.12 mmol) were added to a solution of azide **28** (51 mg, 0.20 mmol) in THF (2 mL) and milliQ water (1 mL). The reaction mixture was stirred at room temperature for 18 hours, until a TLC control attested the disappearance of the starting material **28** (R_f = 0.39, CH_2Cl_2 :MeOH:NH₄OH (6%) 10:1:0.1). The mixture was filtered through Celite®, the solvent was removed under vacuum and subsequently the crude product was treated with the "Quadrasil MP®" resin keeping the mixture under stirring at room temperature in the minimum amount of MeOH for 1 hour (1 g of resin for each mmol of copper). The crude product was purified by flash column chromatography on silica gel (gradient eluent from CH_2Cl_2 :MeOH:NH₄OH (6%) 15:1:0.1 to 10:1:0.1) to give 85 mg of **31** (R_f = 0.13, CH_2Cl_2 :MeOH:NH₄OH (6%) 15:1:0.1, 0.18 mmol, 91%) as a white waxy solid.

31: $[a]_D^{22} = -6.10$ (c = 1.00, MeOH). ^1H -NMR (400 MHz, CD_3OD) δ ppm: 7.89 (s, 1H, triazole), 7.61 (d, J = 7.2 Hz, AB system, 2H, Ar), 6.68 (d, J = 7.4 Hz, AB system, 2H, Ar), 4.45 (s, 2H, 10), 4.42 (t, J = 6.4 Hz, 2H, H-9), 4.25–4.19 (m, 1H, H-3), 3.86–3.81 (m, 1H, H-4), 3.81–3.74 (m, 1H, H-5), 2.76 (d, J = 12.6 Hz, 1H, Ha-2), 2.68–2.60 (m, 1H, Ha-6), 2.36 (dd, J = 3.2, 12.8 Hz, 1H, Hb-2), 2.33–2.16 (m, 2H, H-7), 2.10–1.92 (m, 3H, H-8, Hb-6), 1.46 (s, 3H, Me), 1.33 (s, 3H, Me). ^{13}C -NMR (100 MHz, CD_3OD) δ ppm: 152.6 (s, 1C, Ar), 146.9 (s, 1C, triazole), 131.4 (s, 1C, Ar), 128.4 (d, 2C, Ar), 124.6 (d, 1C, triazole), 112.8 (d, 2C, Ar), 110.1 (s, 1C, OC(CH₃)₂), 80.0 (d, 1C, C-4), 74.4 (d, 1C, C-3), 70.5 (d, 1C, C-5), 57.1 (t, 1C, C-6), 55.0 (t, 1C, C-2), 54.6 (t, 1C, C-7), 49.8 (t, 1C, C-9), 39.4 (t, 1C, C-10), 28.5 (q, 1C, OOC(CH₃)₂), 28.1 (t, 1C, C-8), 26.6 (q, 1C, OOC(CH₃)₂). IR (CDCl_3): $\tilde{\nu}$ = 3345, 2990, 2941, 2830, 2641, 2363, 2344, 2328, 2297, 2189, 1998, 1743, 1601, 1510, 1429, 1327, 1327, 1244, 1221, 1196, 1163, 1146 cm^{-1} . MS-ESI (m/z , %) = 465.20 (93) $[\text{M} - \text{H}]^-$, 930.72 (100) $[2\text{M} - \text{H}]^-$. $\text{C}_{20}\text{H}_{30}\text{N}_6\text{O}_5\text{S}$ (466.55): calcd C, 51.49; H, 6.48; N, 18.01; S, 6.87; found C, 51.28; H, 6.69; N, 17.92; S, 6.80.

Carbonic anhydrase inhibition

An applied photophysics stopped-flow instrument has been used for assaying CA-catalyzed CO_2 hydration activity.²⁶ Phenol red (at a concentration of 0.2 mM) was used as an 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 the ionic strength constant), 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 were 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 the inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares method using PRISM 3 and the Cheng-Prusoff equation, as reported earlier³⁰ and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier.³⁰

Crystallization and X-ray data collection

The crystals of hCA II were obtained using the hanging drop vapor diffusion method using a 24 well Linbro plate. 2 μl of 10 mg ml^{-1} solution of hCA II in Tris-HCl 20 mM pH 8.0 were mixed with 2 μl of a solution of 1.5 M sodium citrate and 0.1 M Tris pH 8.0 and were equilibrated against the same solution at 296 K. The complexes were prepared by soaking the hCA II native crystals in the mother liquor solution containing the inhibitors at a concentration of 10 mM for two days. All crystals were flash-frozen at 100 K using a solution obtained by adding 15% (v/v) glycerol to the mother liquor solution as a cryoprotectant. Data of the crystals of the complexes were collected using synchrotron radiation at the XRD2 beamline at Elettra Synchrotron (Trieste, Italy) with a wavelength of 1.000 Å and a DECTRIS Pilatus 6M detector. Data were integrated and scaled using the XDS program.³¹ Data processing statistics are shown in the ESI.†

Structure determination

The crystal structure of hCA II (PDB accession code: 4FIK) without solvent molecules and other heteroatoms was used to obtain initial phases using Refmac5.³² 5% of the unique reflections were selected randomly and excluded from the refinement data set for Rfree calculations. The initial $|\text{Fo}-\text{Fc}|$ difference electron density maps unambiguously showed the inhibitor molecules. The inhibitor was introduced in the model with 1.0 occupancy. Refinements were performed using normal protocols of positional and anisotropic atomic displacement parameters alternating with the manual building of the models using COOT.³³ The quality of the final models was assessed with COOT and RAMPAGE.³⁴ Crystal parameters and refinement data are summarized in the ESI.† Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 8OGF). Graphical representations were generated with Chimera.³⁵



Conclusions

Twelve mono- and three-tailed derivatives based on a sugar (glucose) or a iminosugar (trihydroxy piperidine) containing a terminal benzenesulfonamide as the zinc binding function have been synthesized with the aim of developing potent and selective carbonic anhydrase inhibitors and collecting information on the behaviour of compounds bearing multiple hydrophilic chains. Among the investigated synthetic strategies, the preferred ones involved, in general, a CuAAC reaction followed by an amine–isothiocyanate coupling and final deprotection of the hydroxy groups. The novel compounds (two sugar single tailed, four iminosugar single tailed, four sugar triple tailed and two iminosugar triple tailed) were screened against eleven hCA isoforms. Among the isoforms screened in the biological assays, the following are particularly relevant for addressing biomedical problems: hCAs I, II and IV involved in some forms of glaucoma, hCAs VA and VB that are targets for antiobesity drugs, and hCA VII is implicated in neuropathic pain and in the development of anticonvulsant drugs. Moreover, hCAs IX and XII show close association with hypoxic tumours such as those of the lung, breast, colon, esophagus and cervix. The collected inhibition data do not indicate clear and general trends for the structure/activity relationship and suggest that information must be drawn case by case. The protected mono-tailed sugar derivative **10** was found to be a more powerful hCA inhibitor than the reference compound **AAZ**, showing lower inhibition constants towards hCAs I, II and IX ($K_i = 65.2, 5.7$ and 5.1 nM, respectively). In general, the introduction of an iminosugar moiety did not furnish better inhibitors than the reference **AAZ**; however, increased selectivity may be achieved, as shown by the promising protected iminosugar derivative **31** which is a good and selective inhibitor of hCA VII ($K_i = 9.7$ nM). X-Ray analysis of hCA II in complex with the iminosugar compound **31** revealed two molecules bound to the active site, one located within the active site and responsible for the activity, and the other bound outside the active site and not involved in inhibition. A typical binding mode of sulphonamide inhibitors was found for compound **31**, with the benzenesulfonamidate moiety directly interacting with the zinc ion within the active site. A double conformation was found for the triazole and the iminosugar parts of the inhibitor, suggesting a certain disorder of the tail which is consistent with the relatively weak potency of this inhibitor against hCA II. The introduction of three hydrophilic tails is generally detrimental for the inhibition of hCAs. However, high selectivity towards a specific isoform coupled to powerful inhibition has been occasionally observed. In particular, the three-tailed peracetylated sugar **25** was a potent and selective inhibitor of hCA VII ($K_i = 9.7$ nM) and its deprotected analogue **26** was a better inhibitor of hCA VB ($K_i = 34.2$ nM) than **AAZ**.

Author contributions

Project design by F. Cardona and C. Supuran. Synthesis by M. G. Davighi and characterization by M. G. Davighi and

C. Matassini. Biological assays and crystallographic experiments and analyses by A. Angeli. The manuscript was written by M. G. Davighi, F. Cardona and A. Goti, with edits from all authors.

Conflicts of interest

There are no conflicts to declare.

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