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6-Substituted 1,2-benzoxathiine-2,2-dioxides are Isoform-Selective Inhibitors Towards Human Carbonic Anhydrases IX, XII and VA

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A series of 6-substituted 2-benzoxathiine-2,2-dioxides were synthesized starting from 2,5-dihydroxybenzaldehyde, and then screened in vitro for their inhibition properties against five human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms. All the compounds showed excellent selectivity against the mitochondrial (hCA VA) and the tumor associated (hCA IX and XII) enzymes.

The 1,2-benzoxathiine 2,2-dioxides, also called sulfocoumarines, are bioisosters of the coumarines and were recently reported as potent and isoform selective carbonic anhydrase inhibitors (CAIs).[1] In analogy to the coumarine class, their inhibition mechanism starts with the CA-mediated hydrolysis of the intramolecular sulfonic acid ester to give, upon geometrical isomerisation, the *trans* vinyl sulfonic acid, which in turn binds to the zinc-coordinated water molecule.[2]

CAIs of the sulfonamide type are in clinical use for the treatment of glaucoma, obesity, epilepsy and as diuretics for almost 60 years.[3-5] The use of CAIs for pharmaceutical applications relies on the diverse distribution of the CAs in physio/pathological conditions within the tissues. For instance the antiglaucoma drugs mainly target CA II, IV and XII; diuretics CA II, IV, XII and XIV; antiepileptics CA VII and XIV, whereas the isoforms IX and XII are strictly correlated to the tumors.[6-10] The main drawback associated to their use is the lack of selectivity in inhibiting the various enzymatic isoforms, which results in a plethora of side effects.[3-10]

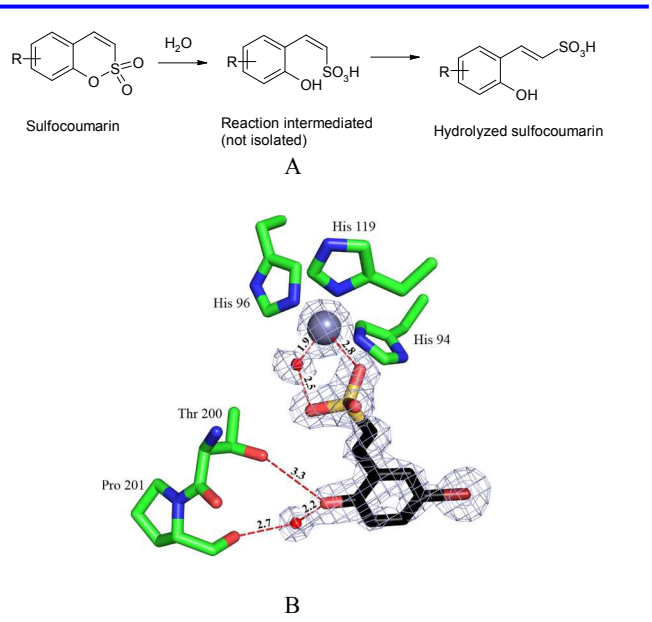

Fig. 1: CA inhibition mechanism of sulfocoumarines. A) The sulfocoumarin undergoes an enzyme-mediated hydrolysis with formation of the *trans*-2-hydroxy-phenyl-ω-ethenylsulfonic acid. B. The sulfonic acid binds to the CA II active site, by anchoring of the sulfonic acid group to the zinc-coordinated water molecule. The Zn(II) ion (central larger sphere), its three His ligands (His94, 96 and 119), water molecule coordinated to the zinc (small sphere) as well as active site residues Thr200 and Pro201 involved in the binding of the hydrolyzed sulfocoumarin are shown, as determined by X-ray crystallography (PDB file 4BCW).[1]

Since the identification of the sulfonamides and their bioisosters, the sulfamates and the sulfonates, as CAIs many efforts have been made for the development of specific inhibitors. In this contest the "tail

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† Electronic supplementary information (ESI) available: Analytical data and spectra (1H, 13C NMR) for all products.

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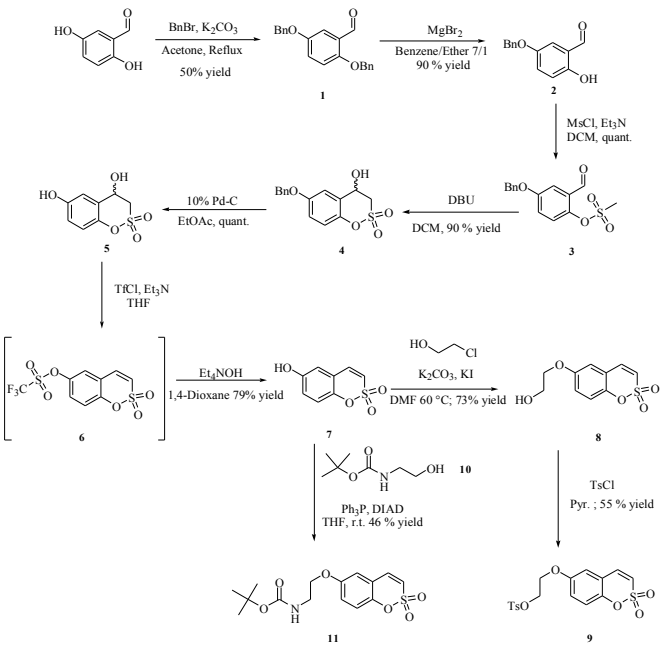
approach" was the preferred one. It consisted of the chemical manipulation of a compound at its tail-end, which resulted in a modification of its physical/chemical properties.[3,4] In addition several and novel CAI scaffolds have been identified such as the polyamines,[11] phenols,[12] dithiocarbamates,[13] xanthates,[14] the coumarines[2] and their structural derivatives such as the thiocoumarines, 2-thioxo-coumarines and coumarine oximes.[6a,15] The sulfocoumarines represent the latest class of CAIs identified and show high selectivity profiles.[1,7] The inhibition mechanisms of many of these compounds were determined by X-ray crystallography of the enzyme-inhibitor adducts.[2, 11-14]

In our previous reports we explored the CA kinetic profiles of a small series of 6-tetrazolyl and 6-triazolyl-substituted sulfocoumarines as well as the effects derived by the introduction of different substituents at the 7 position.[1,7] As extension herein we report the synthesis, characterization and *in vitro* CA inhibition of a small series of 6-substituted sulfocoumarines.



Scheme 1:Synthesis of the 6-substituted sulfocoumarines **7-9** and **11**.

All the compounds were prepared according to the general strategy of Zalubovskis's group[16] by reacting the commercially available 2,5-dihydroxybenzaldehyde with benzyl bromide to afford **1** which was treated with magnesium bromide for selective de-benzylation.[17] Then mesylation of the phenol **2** was followed by intramolecular cyclisation in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to afford the racemic 4-hydroxy-6-benzyloxy-3,4-dihydrosulfocoumarin **4,** which was deprotected under Pd-C catalyzed hydrogenation and then dehydrated by means of its triflate derivative **6**. As expected such a compound proved to be unstable and was directly subjected to treatment with tetraethylammonium hydroxide to afford the 6-hydroxy-substituted sulfocoumarine **7**. At this point the hydroxyl group in 7 was alkylated with 2-chloroethanol through an *in situ* Filkenstein reaction in the presence of potassium carbonate as a base to afford **8**, which in turn was treated with tosylchloride in pyridine to give **9**. Finally compound **11** was obtained via the Mitsunobu coupling reaction of 7 with the freshly prepared Boc-protected 2-aminoethanol **10** (Scheme 1).

Table 1. hCA I, II, VA, IX and XII inhibition data with sulfocoumarins **4–11**, by a stopped-flow CO2 hydrase assay. [19]

Entry	Ki *				
	hCAI(μM)	hCA II(μM)	hCAVA(μM)	hCAIX(nM)	hCAXII(nM)
4	>100	>100	89	83.7	36.2
5	>100	>100	42	36.2	16.5
7	91**	>100	96	300**	204**
8	>100	>100	0.06	26.8	10.4
9	>100	>100	42	50.3	684
11	>100	>100	87	11.5	9.8
AAZ	0.25	0.012	0.06	25	5.7

* Errors in the range of ±5 % of the reported values, from three different assays.

** Data retrieved from reference 1.

The obtained compounds **4, 5, 7-9** and **11** were screened *in vitro* as inhibitors against the most abundant and cytosolic CAs (hCAI and II), the mitochondrial (hCA VA) and the transmembrane tumor-associated hCA IX and XII in comparison with acetazolamide (AAZ) as a standard. In consideration of the previous reports[1,7] we can summarize that the main influencing factor for isozyme selectivity is the substitution at the phenyl ring. In particular all the compounds showed to be ineffective inhibitors of the cytosolic hCAs I and II. (Table 1) Herein and for the first time we report the kinetic profiles of the 6-substituted sulfocoumarines **7-9**, **11** and their precursors **4** and **5** against the mitochondrial hCA VA, with KIs in the range of 0.06-96 μM (Table 1). Such an isoform is raising much attention as it was proved to be a suitable target for the development of new and effective antiobesity agents.[5b,18] As preliminary Structure-Activity-Relationship (SAR) here we report that the introduction at 7-position in the sulfocoumarine scaffold of the ethanolic moiety (as for the compound **8**) resulted in a significant reduction of the Ki for the hCA VA (0.06 μM). Further manipulations at the alcoholic terminal such as in compounds **9** and **11,** or elimination of the alkyl chain as for **7**, suppressed any isoform selectivity (Table 1). Currently an exhaustive SAR investigation on this kind of compounds is ongoing. All the compounds of the series are potent inhibitors of the transmembrane and tumor-associated isoform hCAs IX and XII with KIs spanning between 9.8-684 nM (Table 1). As for the hCA VA isoform a defined SAR is not feasible at the moment. However some key elements are evident: i) the introduction of an alkyl substituent, as for the compound **8**, drastically reduced the Ki values (26.8 and 10.4 nM for the hCA IX and XII respectively). Such a behaviour is analogous to the previously reported for the mitochondrial isoform. ii) The introduction of the lipophilic and bulky substituent Boc-moiety at the end of the chain, as for the compound **11**, accounted for selectivity against the tumor associated CAs. Such an effect was not observed when the tosyl group was introduced instead (compound **9**). Probably hydrophobic interactions occurring between the Boc-group of **11** with specific aminoacids present at the rim of the enzymatic cavities particularly contribute to the stabilization of the inhibitor-enzyme complex.

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Conclusions

Herein we reported a series of 6-substituted sulfocoumarines obtained by intramolecular cyclization of the corresponding methanesulfonic acid esters. We investigated compounds **4**, **5**, **7-9** and **11** for their inhibition activities against five hCA isoforms.

As expected for the 6-substituted sulfocoumarines, the compounds showed to be ineffective in inhibiting the cytosolic hCAs I and II, whereas showed interesting profiles against the tumor associated hCA IX and XII with Kis in a range of 9.8-684 nM.

For the first time we report the 6-substituted sulfocoumarines as effective inhibitors of the mitochondrial hCA VA isoform, with compound **8** as the strongest in the series (Ki 0.06 μM).

These findings are of particular importance for the future development of new and effective inhibitors against the mitochondrial CA isoforms having antiobesity pharmacological applications. In particular the lack of safe and effective drugs for the treatment of obesity and/or obesity-related pathologies makes these compounds particularly interesting for developing new therapeutics.

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19. An Applied Photophysics stoppedflow instrument has been used for assaying the CA catalysed CO2 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 Na2SO4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO2 hydration reaction for a period of 10–100 s. The CO2 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—6 h 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. Data from Table 1 were obtained after 6 h incubation of enzyme and inhibitor, as for the sulfocoumarins and coumarins reported earlier.[1,7] The inhibition constants were obtained by non-linear leastsquares methods using PRISM 3, as reported earlier,[8a] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[1,7]