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Sulfocoumarins (1,2-Benzoxathiine-2,2-dioxides): A Class of Potent and Isoform-Selective Inhibitors of Tumor-Associated Carbonic Anhydrases

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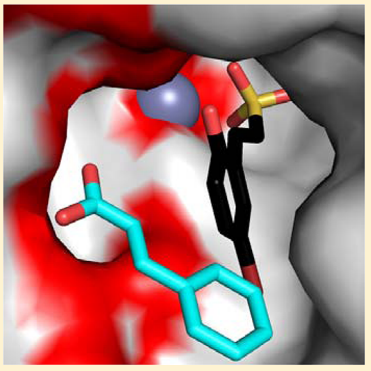
Supporting Information

ABSTRACT: Coumarins were recently shown to constitute a novel class of mechanism-based carbonic anhydrase (CA, EC 4.2.1.1) inhibitors. We demonstrate that sulfocoumarins (1,2-benzoxathiine 2,2-dioxides) possess a similar mechanism of action, acting as effective CA inhibitors. The sulfocoumarins were hydrolyzed by the esterase CA activity to 2-hydroxyphenyl-vinylsulfonic acids, which thereafter bind to the enzyme in a region rarely occupied by other classes of inhibitors. The X-ray structure of one of these compounds in adduct with a modified CA II enzyme possessing two amino acid residues from the CA IX active site, allowed us to decipher the inhibition mechanism. The sulfonic acid was observed anchored to the zinc-coordinated water molecule, making favorable interactions with Thr200 and Pro201. Some other sulfocoumarins incorporating substituted-1,2,3-triazole moieties were prepared by using click chemistry and showed low nanomolar inhibitory action against the tumor-associated isoforms CA IX and XII, being less effective against the cytosolic CA I and II.



INTRODUCTION

Coumarins such as **1**, a natural product isolated from the Australian plant *Leionema ellipticum*, P. G. Wilson (Rutaceae), or the simple unsubstituted coumarin **2**, were recently discovered to act as effective inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).[1−3] A large number of diversely substituted coumarins was subsequently screened for their inhibitory activity against all the 13 catalytically active mammalian CA isoforms,[4−6] CA I−VII, IX, XII−XV. Many of these isoforms are established drug targets for designing agents with various applications, such as diuretics, antiglaucoma drugs, anticonvulsants, antiobesity agents, or antitumor drugs/cancer diagnostic tools.[7−9]

CAs are involved in numerous physiological and pathological processes, including respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiological and pathological processes in humans,[3,4,9] as well as the growth and virulence of various fungal/bacterial/protozoan pathogens.[10−12] Many of the isoforms/enzymes involved in these processes are therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[2,4,9−11] However, a critical barrier to the design of CA inhibitors (CAIs) as therapeutic agents is related to the high number of isoforms, their diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available classical inhibitors (sulfonamides and their bioisosteres).[2−4] The recently discovered coumarins represented a completely new structural motif for the inhibition of this enzyme class and also led to the first isoform-selective inhibitors with a very high selectivity ratio for inhibiting transmembrane isoforms (such as CA IX and XII) over the cytosolic, widespread, off-target ones (CA I and II).[1−6]

Coumarins **1** and **2** were shown to act as "prodrug" inhibitors, being hydrolyzed by the esterase CA activity to the corresponding 2-hydroxy-cinnamic acids **3Z** and **4E**, which are the de facto CAIs, as shown by kinetic, X-ray crystallographic, and MS methods (Scheme 1).[1,2] Interestingly, these compounds were found bound at the entrance of the CA active site cavity[1,2] in a region where only CA activators were observed earlier in the many CA modulators of activity adducts reported in the literature.[7−9,13] In this region of the enzyme active site, the classical CAIs such as the sulfonamides,[8] sulfamates,[8,9]

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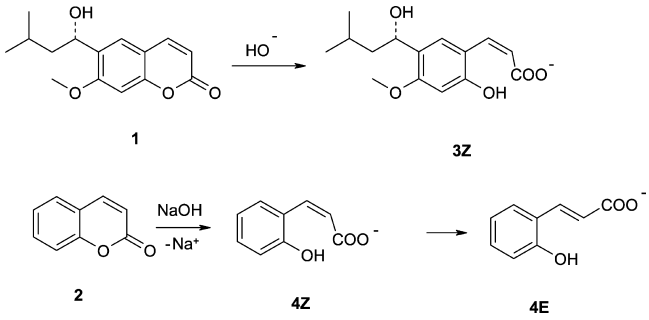
Scheme 1. Active Site Hydrolysis of Coumarins 1 and 2 When Bound to CA, Affords the Hydroxy-cinnamic Acids 3Z or 4E as Evidenced by X-ray Crystallography of Enzyme-Inhibitor Adducts[1a,2]



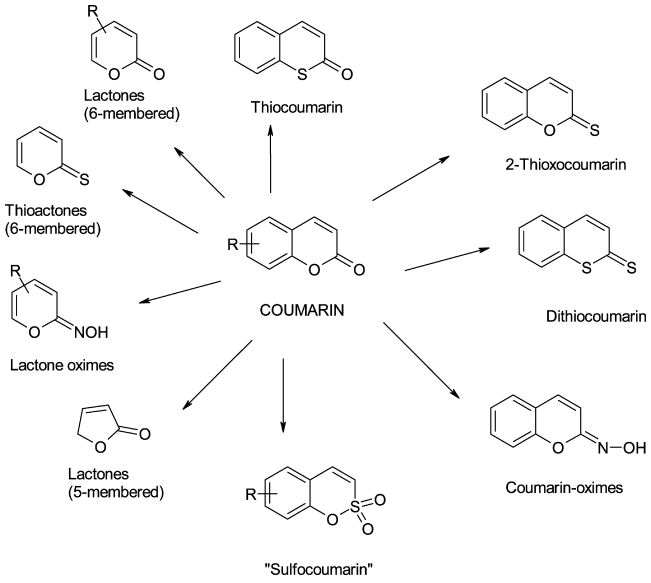
sulfamides,[8,9,14] dithiocarbamates,[15] or other (in)organic anions[16] were never observed. All these classes of CAIs in fact directly coordinate to the catalytically crucial Zn(II) ion from the active site by substituting the water molecule/hydroxide ion coordinated to it[7−9] or by adding to the metal ion coordination sphere, leading to trigonal bipyramidal geometries of the Zn(II).[8,9] Thus, coumarins are a relevant class of CAIs because: (i) they are mechanism-based, "prodrug" inhibitors, (ii) they bind in a totally different active site region compared to the classical inhibitors (sulfonamides and congeners), and (iii) they led to highly isoform-selective compounds for many mammalian CA isoforms, such as CA IX, XII, XIII, and XIV among others.[1−6]

In addition, coumarins or their derivatives are easy to synthesize, being possible to incorporate in their molecule a large variety of substitution patterns which lead to the possibility of exploring a vast chemical space, accessible with difficulty for other classes of CAIs. For example, thiocoumarins,[2,16] 2-thioxocoumarins,[16] dithiocoumarins,[16] coumarin-*N*-oximes,[16] 5-/6-membered lactones[17] and thiolactones,[17] or lactone oximes,[17] were recently investigated for their CA inhibitory properties (Chart 1). "Sulfocoumarins", i.e., 1,2-benzoxathiin 2,2-dioxides were reported recently by one of our groups[18] as coumarin bioisosteres, but their CA inhibitory properties were not yet explored. In this paper, we report that sulfocoumarins are indeed a new class of mechanism-based CAIs, we decipher their mechanism of action at the molecular level (which is similar but not identical to that of the coumarins), and report a series of new such derivatives prepared by using click chemistry. Some of these compounds showed low nanomolar inhibitory activity against two of the most interesting CA isoforms, the tumor-associated CA IX and XII (these enzymes are overexpressed in many hypoxic tumors),[9,19] being much weaker inhibitors against the off-target, widely spread cytosolic isoforms CA I and II.

RESULTS AND DISCUSSION

Chemistry and CA Inhibition. A series of mono- or disubstituted 1,2-benzoxathiin 2,2-dioxides possessing various functionalities on the benzene ring of types **5**−**12** were reported recently[18] by one of our groups. They were synthesized by an intramolecular aldol cyclization reaction of mesylsalicyl aldehydes in the presence of strong bases, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and the X-ray structure of one of them, the bromo derivative **9**, was reported too.[18] These compounds were stable at room temperature and

Chart 1. Compounds Investigated as CAIs Considering the Coumarin Scaffold As Lead[a]



[a]The "sulfocoumarins" are reported in the present work as a novel class of CAIs.

in the presence of air humidity.[18] Compounds **5**−**12** were, however, not investigated for their possible CA inhibitory properties. This may be expected, as they are coumarin bioisosteres (Chart 1), and structurally related such compounds were recently studied as CA inhibitors.[1−6,16,17]

As compounds **5**−**7** and **9**−**11** reported earlier[18] incorporated simple functionalities, such as the hydroxyl, methanesulfonyl, benzyloxy, bromo, nitro and amino (in position 6 of the 1,2-benzoxathiin 2,2-dioxide ring), or the 5,6-benzo- **8** and 6,8-disubstituted derivative **12**, it appeared of interest to incorporate a larger chemical diversity in this ring system by using click chemistry.[20] Thus, the amine **11** reported earlier was diazotized and subsequently converted to the azide **13**, which was then reacted with a series of alkynes, being transformed to the 1,2,3-triazoles **14**−**23** by the classical click chemistry cycloaddition reaction[20] (Scheme 2). Compounds **14**−**23** incorporated several groups at the triazole ring (such as substituted aryl, carboxyalkyl, substituted silyl, alkylaminomethyl, etc.), being structurally diverse when compared to compounds **5**−**12**. These structural variations are essential in order to investigate the structure−activity relationship of this potentially new class of CAIs. The new compounds **14**−**23** were fully characterized and their structure confirmed (see Experimental protocols for details).

Sulfocoumarins **5**−**23** have been investigated for their inhibitory activity against four human (h) CA isoforms, the cytosolic, widespread hCA I and II (offtargets in this case) as well as the transmembrane, tumor-associated hCA IX and XII (anticancer drug targets).[9−19] Several of these derivatives were also investigated for the inhibition of an engineerized protein, a hCA II in which two amino acid residues present in the hCA IX active site have been introduced, denominated here CA II/IX mimic and reported recently by McKenna's group.[21] This is a double mutant of hCA II with Ala65 replaced by Ser and Asn67 replaced by Gln (as these are the corresponding amino acids found in hCA IX).[22] It has been demonstrated earlier that this CA IX mimic may provide a useful model to design isozyme-

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Scheme 2. Preparation of Derivatives 14−23 by Reaction of Azide 13 with Alkynes

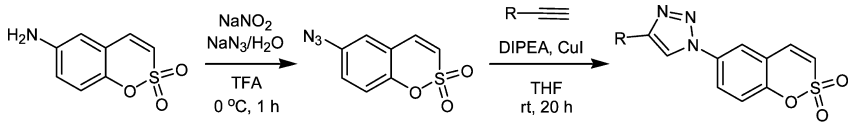
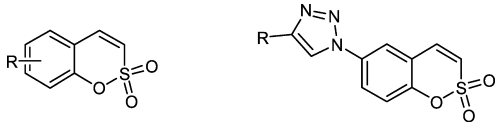


Table 1. Inhibition of Human (h) Isozymes hCA I, II, IX, XII, and a CA II/IX Active Site Mimic with Coumarins 1, 2, and Sulfocoumarins 5−23, by a Stopped-Flow, CO_2 Hydration Assay Method.[23]



		K_I (μM)[a,d]				
compd	R	hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]	CA II/IX mimic
1		0.080	0.062	54.5	48.6	
2		3.10	9.20	>100	>100	
5	6-OH	91	>100	0.300	0.234	0.80
6	6-$MeSO_3$	99	>100	0.324	0.254	2.03
7	6-BnO	93	>100	0.275	0.219	2.37
8	5,6-benzo	>100	>100	0.375	0.717	
9	6-Br	>100	>100	6.83	4.51	0.93
10	6-O_2N	92	>100	3.77	3.16	
11	6-H_2N	6.78	8.89	0.046	0.023	
12	6,8-Cl_2	>100	>100	3.26	2.93	
14	Ph	6.86	7.76	0.029	0.032	
15	COOMe	8.05	6.33	0.095	0.012	
16	COOEt	8.88	9.21	0.086	0.013	
17	Me_3Si	6.00	7.20	0.060	0.009	
18	$HOCH_2$	7.20	9.29	0.058	0.016	
19	Et_2NCH_2	8.11	9.37	0.025	0.007	
20	4-F_3CO-C_6H_4	8.43	9.64	0.074	0.014	
21	4-MeO-C_6H_4	8.93	9.35	0.018	0.039	
22	3-F_3C−C_6H_4	6.71	7.72	0.048	0.013	
23	3-MeO-C_6H_4	7.47	8.61	0.049	0.021	
acetazolamide		0.25	0.012	0.025	0.005	

[a]Preincubation of 6 h between enzyme and inhibitor. [b]Cytosolic full length, recombinant enzyme. [c]Catalytic domain, transmembrane, recombinant isoform. [d]Errors in the range of ±10% of the reported data, from three different assays.

specific CA IX inhibitors.[21] We have used this CA II/IX mimic in this work due to the fact that CA IX is difficult to crystallize, whereas this mimic easily crystallized in complex with one of the compounds investigated here (see later in the text).

Table 1 shows inhibition data of sulfocoumarins **5−23** (as well as coumarins **1** and **2** as standards) against hCA I, II, IX, and XII after a period of 6 h of incubation of the enzyme and inhibitor solutions. It should be mentioned that assaying the inhibition with the usual 15 min incubation period (as for the sulfonamides)[23] leads to the measurement of a very weak inhibition (data not shown). This was also the case with the coumarins.[1,2] For this reason, a 6 h incubation time has been used for assaying all sulfocoumarins as CAIs. Compounds **5−7** and **9** were also investigated for the inhibition of the CA II/IX mimic mentioned above. The following should be noted regarding the inhibition data of Table 1:

(i) The cytosolic isoform hCA I was poorly inhibited by the simple sulfocoumarins **5−12** (K_Is in the range of 91 to >100 μM), except the amino derivative **11**, which was a medium potency inhibitor (K_I of 6.78 μM). A similar behavior of medium potency inhibitors was also observed for the remaining sulfocoumarins, incorporating the substituted triazole scaffold, of types **14−23**, which showed K_Is in the range of 6.00−8.93 μM (Table 1). It should be noted that the natural product coumarin **1** was an effective hCA I inhibitor (K_I of 80 nM), whereas the simple coumarin **2** was a less effective inhibitor (K_I of 3.10 μM), as reported earlier by us.[1b] Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used sulfonamide,[9] showed an inhibition constant of 0.25 μM against this isoform (Table 1).

(ii) The physiologically dominant isoform hCA II was also poorly inhibited by sulfocoumarins **5−10** and **12** (K_Is > 100 μM) and moderately inhibited by coumarin **2** as well as sulfocoumarins **11** and **14−23**, with K_Is in the range of 6.33−9.64 μM. Coumarin **1** was a more effective hCA II inhibitor (K_I of 62 nM), whereas acetazolamide was a very effective one (K_I of 12 nM).

(iii) Some of the simple sulfocoumarins investigated here, e.g., compounds **5−8**, showed submicromolar hCA IX

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inhibition, with K_Is in the range of 0.275−0.375 μM, being thus much more effective CAIs compared to the coumarins **1** and **2** (K_Is in the range of 54.5 to >100 μM). Sulfocoumarins **9** and **10** were slightly less effective as hCA IX inhibitors, with inhibition constants in the range of 3.77−6.83 μM. Among the simple sulfocoumarins, only the amino derivative **11** was an effective hCA IX inhibitor with a K_I of 46 nM, whereas all triazole-containing derivatives were quite effective inhibitors of this isoform, with nanomolar inhibition constants ranging between 18 and 95 nM. Acetazolamide has a K_I of 25 nM against hCA IX. The SAR is thus quite interesting both for the simple derivatives **5**−**12** as well as for the triazole-substituted ones **14**−**23**. For the later derivatives, the 6-OH moiety and its derivatization (as sulfonic ester or ether, such as in compounds **5**−**7**) induced an effective, submicromolar inhibition of isoform hCA IX. 6-Bromo-**9** and 6-nitro-derivatives **10** were on the other hand less beneficial substitution patterns, leading to an order of magnitude loss of efficacy, whereas the 6-amino moiety restored and enhanced hCA IX inhibitory activity 82 times compared to the corresponding 6-nitro derivative **10**. For the compounds incorporating the substituted 1,2,3-triazole scaffold in position 6 of the sulfocoumarin ring, again SAR was quite interesting. The best substitution patterns at the triazole incorporated the phenyl (compound **14**), diethylaminomethyl (**19**), and 3- and 4-substituted phenyl (**20**−**23**) moieties. These compounds showed inhibition constants in the range of 18−49 nM (Table 1). The least effective triazole derivative as hCA IX inhibitor was **15**, which incorporated a methoxycarbonyl moiety (K_I of 0.95 μM), but its congener with an extra CH_2 group, compound **16**, was 11 times more effective as an inhibitor (K_I of 86 nM). It is obvious that very small structural changes in the scaffold of sulfocoumarins lead to a dramatic change in inhibitory activity against hCA IX.

(iv) Rather similar SAR with what was discussed above for hCA IX has been observed for the inhibition of the second transmembrane isoform, hCA XII. Thus, several sulfocoumarins, such as **9**, **10**, and **12**, were low micromolar hCA XII inhibitors (inhibition constants in the range of 2.93−4.51 μM). The remaining sulfocoumarins were much more effective inhibitors of this isoform, with K_Is in the range of 7 nM to 0.717 μM. The simple sulfocoumarins **5**−**8** were the least effective inhibitors in this subseries (K_Is in the range of 0.234−0.717 μM), whereas all the derivatives incorporating 1,2,3-triazole moieties, of types **14**−**23**, were low nanomolar hCA XII inhibitors (K_Is in the range of 7−39 nM). In this case, all substitution patterns at the triazole ring were highly effective in inducing excellent inhibition (Table 1). It should be also noted that coumarins **1** and **2** are ineffective hCA XII inhibitors, whereas acetazolamide is a low nanomolar one.

(v) The CA II/IX active site mimic was inhibited in the submicromolar−low micromolar range by the sulfocoumarins **5**−**7** and **9**, with inhibition constants of 0.80−2.37 μM. Considering the limited number of compounds tested against this hybrid enzyme, it can be assessed that the inhibition profile of the CA II/IX active site mimic more closely resembles that of CA IX.

X-ray Crystallography. To understand the inhibition mechanism with this new class of CAI, we resolved the X-ray crystal structure (at a resolution of 1.5 Å) of the CA II/IX mimic enzyme complexed with sulfocoumarin **9**. This is due to the fact that hCA IX is crystallizable with difficulty, whereas CA II, which easily forms crystals appropriate for X-ray investigations, has a low affinity for most of the simple sulfocoumarins investigated here. The statistics for the refinement and data collection of the CA II/IX−**9** adduct are shown in Table 2.

Table 2. Data Processing, Refinement, and Validation Statistics of the CA II/IX−9 Adduct

space group	$P2_1$
cell dimensions	
a (Å)	42.2
b (Å)	41.5
c (Å)	72.0
β (deg)	104.1
resolution (Å)	20−1.5
highest resolution shell (Å)	1.6−1.5
no. of reflections	34350
no. of reflections in test set	1805
completeness (%)	93 (80[a])
R_{merge}	0.07 (0.32)
$\langle I/\sigma I \rangle$	7.8 (2.0)
average multiplicity	2.0 (1.2)
R-factor	0.18 (0.30)
R_{free}	0.22 (0.36)
average B factor (Å^2)	13
average B factor for inhibitor (Å^2)	15
⟨B⟩ from Wilson plot (Å^2)	12
no. of protein atoms	2070
no. of inhibitor atoms	14
no. of solvent molecules	253
rms deviations from ideal values	
bond lengths (Å)	0.011
bond angles (deg)	1.48
outliers in Ramachandran plot	0.0

[a]Values in parentheses are for the high resolution bin.

Inspection of the electron density maps (Figure 1) at various stages of the refinement showed features compatible with the presence of one molecule of inhibitor bound within the active site, but compound **9** could not be fitted in the observed electron density, as in the case of the coumarins **1** and **2** investigated earlier.[1b,2] Instead, its hydrolysis product, 2-dihydroxy-5-bromophenyl-vinyl sulfonic acid **24** (Scheme 3), perfectly fitted within this electron density (Figure 1). It should be mentioned that compound **9** (as well as other derivatives investigated here of type **5**−**23**) have been incubated for 6−24 h with the buffer of the enzyme assay (see above and Experimental Protocols) in order to see whether the hydrolysis process depicted in Scheme 3 takes place. No hydrolysis products of type **24** in the case of **9** could be detected (by means of HPLC) after this incubation time, which proves that the hydrolysis of the sulfocoumarin to the vinyl sulfonic acid is mediated by the zinc hydroxide nucleophile from the CA active site, as for the coumarins which are transformed to 2-hydroxy-cinnamic acids.[1,2]

As seen from Figure 1, the electron density of **24E** is perfectly defined for all its atoms. It is probable that the

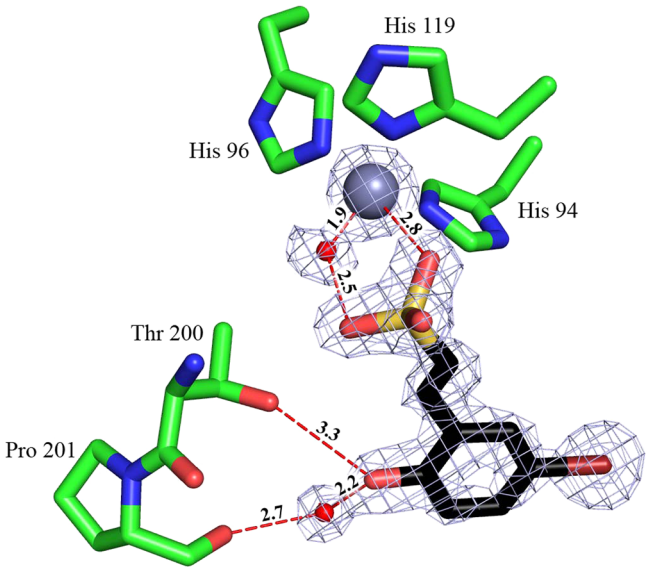
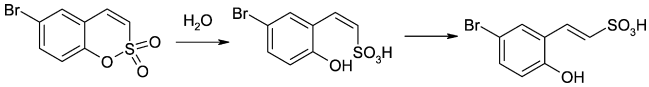


Figure 1. Binding of hydrolyzed compound **9** within the active site of the CA II/IX mimic. The $2F_o - F_c$ map was calculated in the absence of ligand and contoured at 1σ. For the sake of clarity, the map is shown only around the ligand (in black), zinc ion (gray sphere), and water molecules (red spheres). Polar interactions are shown with dashed lines, and distances are indicated in Å. The figure was generated by using PyMol.[27]

Scheme 3. Active Site, CA-Mediated Hydrolysis of 9 to 24E



sulfocoumarin **9** is initially hydrolyzed (by the zinc hydroxide, nucleophilic species of the enzyme) to the *cis* vinyl sulfonic acid **24Z**, which subsequently isomerizes spontaneously to the *trans* derivative **24E** (Scheme 3), which is observed in the electron density of the CA II/IX − **9** adduct (Figure 1). Interestingly, the sulfonic acid moiety (presumably as a sulfonate group, at the pH of 7.8, at which the crystallization was done) is not coordinated to the Zn(II) ion but it is anchored to the zinc-bound water molecule/hydroxide ion (Figure 1). This binding mode was initially observed for phenol,[12a] then for spermine,[24] a carboxylic acid methyl ester,[25] and recently for several other phenols and aromatic carboxylic acids.[26] With the present structure, it is obvious that anchoring to the zinc-coordinated water molecule may be considered as a quite general CA inhibition mechanism (we prefer to use the term "anchoring to the zinc-coordinated water" for the inhibition mechanism of these compounds, as originally proposed by Christianson,[12a] over the more recent and unhappy term "nucleophile recognition" proposed by Martin and Cohen[26]). The distance between the zinc-coordinated water/hydroxide ion and an oxygen atom of the sulfonate moiety of **24E** was of 2.5 Å. Another oxygen of this moiety was at 2.8 Å from the metal ion, being "half-coordinated". The organic scaffold of **24E** did not participate in other interactions with residues from the enzyme active site, except for the OH moiety *ortho* to the ethenylsufonate group. This moiety participates in a bifurcated hydrogen bond, with the hydroxyl of Thr200 (of 3.3 Å) and through a bridging water molecule, with the carbonyl oxygen of Pro201 (of 2.2 Å). The relatively few interactions between the inhibitor scaffold and the active site may in fact explain the not so good inhibitory power of this compound against the CA II/IX mimic (K_I of 0.93 μM, Table 1). We stress this again, that in contrast to structurally similar sulfonamides (such as acetazolamide which bind to the metal ion, Figure 2), the

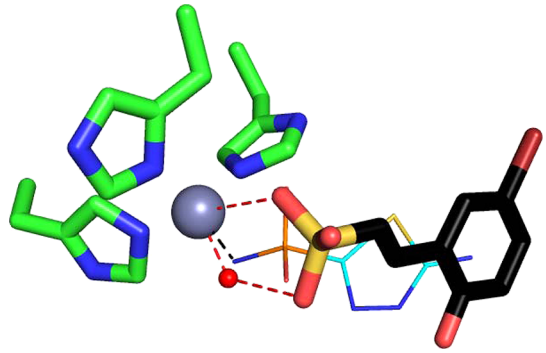


Figure 2. Comparison of binding modes of hydrolyzed sulfocoumarin (compound **9**, thick lines with black carbons), and the sulfonamide CAI acetazolamide (PDB code 2HNC, thin lines with light-blue carbons and dark-blue nitrogens). The Zn(II) ion (gray sphere) and its coordinating histidines (His94, 96, and 119, stick representation, in blue and green) are superposable in both cases. It should be noted that the sulfonamide inhibitor displaces the Zn-coordinating water molecule (red sphere), whereas the sulfocoumarin anchors to it. The interactions between the inhibitor, the zinc ion, and the water molecule coordinated to zinc are shown as dotted lines.

hydrolyzed sulfocoumarins did not replace the Zn-coordinating water molecule but merely interact with it (Figure 2). The hydrolyzed compound **9** also did not make any interactions with the mutated residues within the active site. From the superposition showed in Figure 2 between the hCA II–acetazolamide adduct and the adduct of the hydrolyzed sulfocoumarin **9** with the CA II/IX mimic, it can be observed the very different inhibition mechanism of the new class of CAIs reported here, the sulfocoumarins, compared to the widely investigated sulfonamide inhibitors.

CONCLUSION

We demonstrate in this paper that sulfocoumarins, i.e., 1,2-benzoxathiine 2,2-dioxides, possess a similar mechanism of CA inhibition as the coumarins, acting as effective inhibitors of this enzyme. The sulfocoumarins were hydrolyzed by the esterase CA activity to 2-hydroxyphenyl-vinylsulfonic acids, which thereafter bound within the enzyme active site in a region rarely occupied by other classes of inhibitors such as the sulfonamides or the dithiocarbamates, which are Zn(II) ion binders. The X-ray crystal structure of one of the investigated 6-hydroxy-1,2-benzoxathiine 2,2-dioxides in adduct with a modified CA II enzyme (possessing two amino acid residues from the CA IX active site), allowed us to decipher the inhibition mechanism. The vinylsulfonic acid formed by hydrolysis of the initial sulfocoumarin was observed anchored to the zinc-coordinated water molecule, making favorable interactions with Thr200 and Pro201. Some other sulfocoumarins incorporating substituted-1,2,3-triazole moieties were prepared by means of click chemistry. The simple sulfocoumarins were usually micromolar inhibitors of hCA IX and XII, being ineffective against hCA I and II. The compounds incorporating 1,2,3-triazole moieties showed low nanomolar inhibitory action against the tumor-associated isoforms hCA IX

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and XII, being less effective against the cytosolic hCA I and II. Sulfocoumarins, similar to coumarins, constitute a versatile, effective, and isoform-selective novel class of inhibitors targeting the tumor-associated isoforms, hCA IX and XII.

EXPERIMENTAL PROTOCOLS

Chemistry. Commercial reagents were purchased from Sigma Aldrich, Acros, or Alfa Aesar and used without further purification. Compounds **5**−**12** were prepared as reported earlier.[18] Reactions were monitored using thin-layer chromatography (TLC) on EMD Merck Silica Gel 60 F254 plates. Visualization of the developed plates was performed under UV light (254 nm). Flash chromatography was carried out using Merck silica gel (230−400 mesh). Organic solutions were concentrated under reduced pressure on a Büchi rotary evaporator. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 400 spectrometer. ^{1}H and ^{13}C NMR spectra were internally referenced to the residual solvent signal. Elemental analyses were performed on a Carlo Erba CHNSeO EA-1108 apparatus. High resolution mass spectra (HRMS) were obtained on a Q-TOF *Micro* Spectrometer (ESI). Melting points were determined on an OptiMelt automated melting point system. Purity of the new compounds was assessed by HPLC and was >98%.

6-Azido-1,2-benzoxathiine 2,2-dioxide (13). A solution of amine **11** (2.50 g, 12.7 mmol) in trifluoric acetic acid (17 mL) was cooled to 0 °C, and $NaNO_2$ (0.964 g, 13.8 mmol) was added. The reaction was stirred at 0 °C for 30 min. NaN_3 (0.826 g, 12.7 mmol) solution in water (4.5 mL) was cooled to 0 °C and added to reaction mixture and stirred for 1 h at 0 °C. Precipitate was collected by filtration and dried under vacuum to yield 2.52 g (89%) of **13** as orange crystalline solid: mp 113.5−114.5 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.31 (dd, J = 2.8, 8.8 Hz, 1H), 7.49 (d, J = 8.8 Hz, 1H), 7.55 (d, J = 2.8 Hz, 1H), 7.59 (d, J = 10.4 Hz, 1H), 7.69 (d, J = 10.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 119.9, 120.0, 120.1, 122.9, 123.6, 135.8, 137.5, 147.6. Anal. Calcd for $C_8H_5N_3O_3S$: C, 43.05; H, 2.26; N, 18.83. Found: C, 42.73; H, 2.32; N, 18.69.

General Procedure for the Synthesis of 1,2,3-Triazolyl Derivatives. To a solution of **13** (1.0 equiv) in dry THF (1 mL per mmol of **13**) DIPEA (50 equiv), the appropriate alkyne (1.1 or 2.0 or 5.0 equiv) and CuI (2 equiv) were added. The resulting mixture was stirred at room temperature under an argon atmosphere for 20 h and then poured into the mixture of ice and water, and extracted with EtOAc, and dried over Na_2SO_4, and the solvent was evaporated to obtain a crude product.

1-(2,2-Dioxido-1,2-benzoxathiin-6-yl)-4-phenyl-1*H*-1,2,3-triazole (14). Compound **14** was obtained from **13** (0.142 g, 0.64 mmol), phenylacetylene (0.08 mL, 0.70 mmol), DIPEA (5.57 mL, 32 mmol), and CuI (0.244 g, 1.28 mmol). The crude product was recrystallized from EtOH to yield 0.111 g (53%) of **14** as yellow crystalline solid: mp 239−240 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.38−7.43 (m, 1H), 7.49−7.55 (m, 2H), 7.71 (d, J = 10.4 Hz, 1H), 7.76 (d, J = 8.8 Hz, 1H), 7.85 (d, J = 10.4 Hz, 1H), 7.92−7.97 (m, 2H), 8.15 (dd, J = 8.8, 2.8 Hz, 1H), 8.41 (d, J = 2.8 Hz, 1H), 9.35 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 119.7, 119.8, 120.0, 121.2, 123.5, 123.8, 125.2, 128.2, 128.9, 129.8, 134.1, 135.7, 147.3, 149.9. HRMS-ESI (m/z) calcd for $C_{16}H_{12}N_3O_3S$ $[M + H]^+$ 326.0599, found 326.0641.

Protein Production and Purification. The CA II gene with the two CA IX mutations in active site[21] was ordered in Genscript and sequence cloned in modified pET14b vector (Novagen) without a 6xHis-tag. The plasmid was transformed in *Escherichia coli* strain BL21(DE3) and cells grown in LB medium containing 50 mg/L ampicillin at 37 °C until OD_{590} reached 0.5. IPTG was added to 1 mM concentration, cells grown for another 4 h, and harvested by centrifugation. Cells were resuspended in a buffer containing 40 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20 mM $MgSO_4$, 0.1% Triton-X100, 0.1 mg/mL DNase, and 1 mg/mL lysozyme and additionally lysed by sonification. The lysate was clarified by centrifugation and loaded on a His Trap HP affinity column (GE Healthcare). Most of the CA II mutant protein was adsorbed on the column due to two naturally occurring histidines (His3 nd 4) close to the N-terminus. The protein was eluted by using 300 mM imidazole in 40 mM tris-HCl buffer and further purified by gel filtration on Superdex 200 gel filtration and Mono-Q ion exchange columns (GE Healthcare).

Crystallization and Data Collection. The protein was concentrated into 8 mg/mL in 20 mM tris-HCl, pH 8.0, using a 10 kDa cutoff Amicon concentrator. Crystallization was done by the sitting drop technique in 96-well MRC plates (Molecular Dimensions). Then 1 μL of protein solution was mixed with 1 μL of bottom solution, (3 M $(NH_4)_2SO_4$, 100 mM tris-HCl pH 8.0), and 0.2 μL of 100 mM compound **9** dissolved in 100% DMSO. The obtained crystals were soaked in the mother liquor containing 30% glycerol and flash-frozen in liquid nitrogen. Data were collected at beamline I911−2, MAX-Lab Synchrotron, in Lund, Sweden.

Structure Determination. Images were processed by MOSFLM[28] and scaled by SCALA.[29] The structure was refined by REFMAC[30,31] using unliganded CA II mutant (PDB code 3DC9)[21] as an initial model. The parameter files for compound **9** were generated by LIBCHECK.[30,31] Ligand was fitted in electron density by using COOT,[32] followed by further REFMAC runs.[33] Data scaling, refinement, and validation statistics are listed in Table 2.

CA 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 distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min to 24 h at room temperature (15 min) or 4 °C (all other incubation times) prior to assay in order to allow for the formation of the E−I complex or for the eventual active site mediated hydrolysis of the inhibitor. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier,[1b,2] and represent the mean from at least three different determinations. Data of Table 1 represent the values after 6 h inhibition of enzyme and inhibitor. All CA isoforms were recombinant ones obtained in-house as reported earlier.[1b,2,21]

ASSOCIATED CONTENT

Supporting Information

The detailed physicochemical characterization of compounds **15**−**23** reported in the paper is provided. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The X-ray coordinates of the hCA II−**9** adduct are available in PDB with the ID code 4bcw.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CA, carbonic anhydrase; CAI, CA inhibitor; DIPEA, diisopropylethylamine; hCA, human CA; MS, mass spectrometry; THF, tetrahydrofuran

■ REFERENCES

(1) (a) Vu, H.; Pham, N. B.; Quinn, R. J. Direct screening of natural product extracts using mass spectrometry. *J. Biomol. Screening* **2008**, *13*, 265−275. (b) Maresca, A.; Temperini, C.; Vu, H.; Pham, N. B.; Poulsen, S. A.; Scozzafava, A.; Quinn, R. J.; Supuran, C. T. Non-zinc mediated inhibition of carbonic anhydrases: coumarins are a new class of suicide inhibitors. *J. Am. Chem. Soc.* **2009**, *131*, 3057−3062.

(2) Maresca, A.; Temperini, C.; Pochet, L.; Masereel, B.; Scozzafava, A.; Supuran, C. T. Deciphering the mechanism of carbonic anhydrase inhibition with coumarins and thiocoumarins. *J. Med. Chem.* **2010**, *53*, 335−344.

(3) (a) Maresca, A.; Supuran, C. T. Coumarins incorporating hydroxy- and chloro- moieties selectively inhibit the transmembrane, tumor-associated carbonic anhydrase isoforms IX and XII over the cytosolic ones I and II. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4511−4514. (b) Maresca, A.; Scozzafava, A.; Supuran, C. T. 7,8-Disubstituted- but not 6,7-disubstituted coumarins selectively inhibit the transmembrane, tumor-associated carbonic anhydrase isoforms IX and XII over the cytosolic ones I and II in the low nanomolar/subnanomolar range. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 7255−7258.

(4) (a) Lou, Y.; McDonald, P. C.; Oloumi, A.; Chia, S. K.; Ostlund, C.; Ahmadi, A.; Kyle, A.; Auf dem Keller, U.; Leung, S.; Huntsman, D. G.; Clarke, B.; Sutherland, B. W.; Waterhouse, D.; Bally, M. B.; Roskelley, C. D.; Overall, C. M.; Minchinton, A.; Pacchiano, F.; Carta, F.; Scozzafava, A.; Touisni, N.; Winum, J. Y.; Supuran, C. T.; Dedhar, S. Targeting Tumor Hypoxia: Suppression of Breast Tumor Growth and Metastasis by Novel Carbonic Anhydrase IX Inhibitors. *Cancer Res.* **2011**, *71*, 3364−3376. (b) Touisni, N.; Maresca, A.; McDonald, P. C.; Lou, Y.; Scozzafava, A.; Dedhar, S.; Winum, J.-Y.; Supuran, C. T. Glycosyl coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors. *J. Med. Chem.* **2011**, *54*, 8271−8277.

(5) Davis, R. A.; Vullo, D.; Maresca, A.; Supuran, C. T.; Poulsen, S. A. Natural product coumarins that inhibit human carbonic anhydrases. *Bioorg. Med. Chem.* **2012**, in press (DOI: 10.1016/j.bmc.2012.07.021).

(6) Balboni, G.; Congiu, C.; Onnis, V.; Maresca, A.; Scozzafava, A.; Winum, J. Y.; Supuran, C. T. Flavones and structurally related 4-chromenones inhibit carbonic anhydrases by a different mechanism of action compared to coumarins. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3063−3066.

(7) Aggarwal, M.; McKenna, R. Update on carbonic anhydrase inhibitors: a patent review (2008−2011). *Expert Opin. Ther. Pat.* **2012**, *22*, 903−915.

(8) Alterio, V.; Di Fiore, A.; D'Ambrosio, K.; Supuran, C. T.; De Simone, G. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? *Chem. Rev.* **2012**, *112*, 4421−4468.

(9) (a) Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nature Rev. Drug Discovery* **2008**, *7*, 168−181. (b) Neri, D.; Supuran, C. T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nature Rev. Drug Discovery* **2011**, *10*, 767−777.

(10) (a) Supuran, C. T.; Scozzafava, A.; Conway, J., *Carbonic Anhydrase—Its Inhibitors and Activators*; CRC Press: Boca Raton, New York, London, 2004; pp 1−363; (b) Winum, J. Y.; Rami, M.; Scozzafava, A.; Montero, J. L.; Supuran, C. Carbonic Anhydrase IX: a new druggable target for the design of antitumor agents. *Med. Res. Rev.* **2008**, *28*, 445−463. (c) Supuran, C. T.; Scozzafava, A.; Casini, A. Carbonic anhydrase inhibitors. *Med. Res. Rev.* **2003**, *23*, 146−189.

(11) (a) Fisher, S. Z.; Aggarwal, M.; Kovalevsky, A. Y.; Silverman, D. N.; McKenna, R. Neutron Diffraction of Acetazolamide-Bound Human Carbonic Anhydrase II Reveals Atomic Details of Drug Binding. *J. Am. Chem. Soc.* **2012**, *134*, 14726−14729. (b) Alterio, V.; Vitale, R. M.; Monti, S. M.; Pedone, C.; Scozzafava, A.; Cecchi, A.; De Simone, G.; Supuran, C. T. Carbonic anhydrase inhibitors: X-ray and molecular modeling study for the interaction of a fluorescent antitumor sulfonamide with isozyme II and IX. *J. Am. Chem. Soc.* **2006**, *128*, 8329−8335. (c) Köhler, K.; Hillebrecht, A.; Schulze Wischeler, J.; Innocenti, A.; Heine, A.; Supuran, C. T.; Klebe, G. Saccharin inhibits carbonic anhydrases: possible explanation for its unpleasant metallic aftertaste. *Angew. Chem., Int. Ed. Engl.* **2007**, *46*, 7697−7699.

(12) (a) Nair, S. K.; Ludwig, P. A.; Christianson, D. W. Two-site binding of phenol in the active site of human carbonic anhydrase II: structural implications for substrate association. *J. Am. Chem. Soc.* **1994**, *116*, 3659−60. (b) Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors: interactions of phenols with the 12 catalytically active mammalian isoforms (CA I−XIV). *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1583−1587. (c) Innocenti, A.; Hilvo, M.; Scozzafava, A.; Parkkila, S.; Supuran, C. T. Carbonic anhydrase inhibitors: inhibition of the new membrane-associated isoform XV with phenols. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3593−3596.

(13) (a) Ilies, M.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase activators. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C.T., Scozzafava, A., Conway, J. Eds.; CRC Press: Boca Raton, FL, 2004; pp 317−352; (b) Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine. *Biochemistry* **1997**, *36*, 10384−10392. (c) Scozzafava, A.; Supuran, C. T. Carbonic anhydrase activators: high affinity isozymes I, II and IV activators, incorporating a *β*-alanyl-histidine scaffold. *J. Med. Chem.* **2002**, *45*, 284−291.

(14) Winum, J.-Y.; Scozzafava, A.; Montero, J.-L.; Supuran, C. T. Therapeutic Potential of Sulfamides as Enzyme Inhibitors. *Med. Res. Rev.* **2006**, *26*, 767−792.

(15) (a) Carta, F.; Aggarwal, M.; Maresca, A.; Scozzafava, A.; McKenna, R.; Supuran, C. T. Dithiocarbamates: a new class of carbonic anhydrase inhibitors. Crystallographic and kinetic investigations. *Chem. Commun.* **2012**, *48*, 1868−1870. (b) Monti, S. M.; Maresca, A.; Carta, F.; De Simone, G.; Mühlschlegel, F. A.; Scozzafava, A.; Supuran, C. T. Dithiocarbamates strongly inhibit the beta-class fungal carbonic anhydrases from *Cryptococcus neoformans*, *Candida albicans*, and *Candida glabrata*. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 859−862. (c) Carta, F.; Aggarwal, M.; Maresca, A.; Scozzafava, A.; McKenna, R.; Masini, E.; Supuran, C. T. Dithiocarbamates strongly inhibit carbonic anhydrases and show antiglaucoma action in vivo. *J. Med. Chem.* **2012**, *55*, 1721−1730.

(16) Carta, F.; Maresca, A.; Scozzafava, A.; Supuran, C. T. Novel coumarins and 2-thioxo-coumarins as inhibitors of the tumor-associated carbonic anhydrases IX and XII. *Bioorg. Med. Chem.* **2012**, *20*, 2266−2273.

(17) Carta, F.; Vullo, D.; Maresca, A.; Scozzafava, A.; Supuran, C. T. New chemotypes acting as isozyme-selective carbonic anhydrase inhibitors with low affinity for the offtarget cytosolic isoform II. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 2182−2185.

(18) Grandane, A.; Belyakov, S.; Trapencieris, P.; Zalubovskis, R. Facile synthesis of coumarin bioisosteres—1,2-benzoxathiine 2,2-dioxides. *Tetrahedron* **2012**, *68*, 5541−5546.

(19) (a) Ahlskog, J. K. J.; Dumelin, C. E.; Trüssel, S.; Marlind, J.; Neri, D. In vivo targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4851−4855. (b) Buller, F.; Steiner, M.; Frey, K.; Mircsof, D.; Scheuermann, J.; Kalisch, M.; Bühlmann, P.; Supuran, C. T.; Neri, D. Selection of Carbonic Anhydrase IX Inhibitors from One Million DNA-Encoded Compounds. *ACS Chem. Biol.* **2011**, *6*, 336−344.

(20) (a) Giguère, D.; Patnam, R.; Bellefleur, M. A.; St. Pierre, C.; Sato, S.; Roy, R. Carbohydrate triazoles and isoxazoles as inhibitors of galectins-1 and -3. *Chem. Commun.* **2006**, 2379−2381. (b) Pirali, T.; Tron, G. C.; Zhu, J. One-pot synthesis of macrocycles by a tandem three-component reaction and intramolecular [3 + 2] cycloaddition. *Org. Lett.* **2006**, *8*, 4145−4148.
(21) Genis, C.; Sippel, K. H.; Case, N.; Cao, W.; Avvaru, B. S.; Tartaglia, L. J.; Govindasamy, L.; Tu, C.; Agbandje-McKenna, M.; Silverman, D. N.; Rosser, C. J.; McKenna, R. Design of a carbonic anhydrase IX active-site mimic to screen inhibitors for possible anticancer properties. *Biochemistry* **2009**, *48*, 1322−31.
(22) Alterio, V.; Hilvo, M.; Di Fiore, A.; Supuran, C. T.; Pan, P.; Parkkila, S.; Scaloni, A.; Pastorek, J.; Pastorekova, S.; Pedone, C.; Scozzafava, A.; Monti, S. M.; De Simone, G. Crystal structure of the extracellular catalytic domain of the tumor-associated human carbonic anhydrase IX. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 16233−16238.
(23) Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246*, 2561−2573.
(24) Carta, F.; Temperini, C.; Innocenti, A.; Scozzafava, A.; Kaila, K.; Supuran, C. T. Polyamines inhibit carbonic anhydrases by anchoring to the zinc-coordinated water molecule. *J. Med. Chem.* **2010**, *53*, 5511−5522.
(25) Davis, R. A.; Hofmann, A.; Osman, A.; Hall, R. A.; Mühlschlegel, F. A.; Vullo, D.; Innocenti, A.; Supuran, C. T.; Poulsen, S. A. Natural Product-Based Phenols as Novel Probes for Mycobacterial and Fungal Carbonic Anhydrases. *J. Med. Chem.* **2011**, *54*, 1682−1692.
(26) Martin, D. P.; Cohen, S. M. Nucleophile recognition as an alternative inhibition mode for benzoic acid based carbonic anhydrase inhibitors. *Chem. Commun.* **2012**, *48*, 5259−5261.
(27) DeLano, W. L. *The PyMOL Molecular Graphics System*; DeLano Scientific: San Carlos, CA, , 2002.
(28) Leslie, A. G. W. *Joint CCP4 ESF-EAMCB Newsletter on Protein Crystallography*; Daresbury Laboratory: Warrington, UK, 1992; Vol. *26*, pp 24−28.
(29) Evans, P. R. SCALA. *Joint CCP4 ESF-EAMCB Newsletter on Protein Crystallography*; Daresbury Laboratory: Warrington, UK, 1997; Vol. 33, pp 22−24
(30) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1997**, *53*, 240−255.
(31) Vagin, A. A.; Murshudov, G. N.; Strokopytov, B. V. BLANC: the program suite for protein crystallography. *J. Appl. Crystallogr.* **1998**, *31*, 98−112.
(32) Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2004**, *60*, 2126−2132.
(33) Kleywegt, G. J.; Jones, T. A. Phi/psi-chology: Ramachandran revisited. *Structure* **1996**, *4*, 1395−1400.