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Deciphering the Mechanism of Carbonic Anhydrase Inhibition with Coumarins and Thiocoumarins

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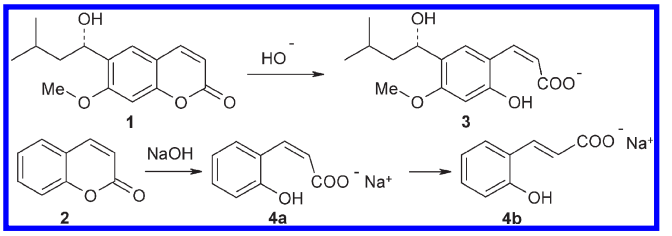
Coumarin derivatives were recently shown to constitute a totally new class of inhibitors of the zinc metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), being hydrolyzed within the CA active site to 2-hydroxycinnamic acids. We explore here a new series of variously substituted coumarins and a thiocoumarin for their interaction with 13 mammalian CA isoforms, detecting low nanomolar and isoform selective inhibitors. The mechanism of action of this class of inhibitors is delineated in detail by resolving the X-ray crystal structure of CA II in complex with *trans*-2-hydroxy-cinnamic acid, the in situ hydrolysis product of simple coumarin. Thiocoumarins also act as efficient CAIs, similarly to coumarins. The versatility of the (thio)coumarin chemistry, the *cis*−*trans* isomerization evidenced here, and easy derivatization of the (thio)coumarin rings, coupled with the nanomolar inhibition range of several isozymes, afford isoform-selective CAIs with various biomedical applications, which render these classes of compounds superior to the clinically used sulfonamides.

Introduction

In a recent paper,[1] we reported a novel class of inhibitors of the metalloenzyme carbonic anhydrase (CA,[a] EC 4.2.1.1)[2] belonging to a completely new chemotype, the coumarins.[3] The CA family of enzymes is widespread all over the phylogenetic tree (with 16 different α-CA isozymes presently known in mammals) and is inhibited by compounds which bind to the catalytically critical Zn(II) ion from the enzyme active site (or the water/hydroxide ion coordinated to it): the sulfonamides, their bioisosteres (sulfamates, sulfamides, *N*-substituted sulfonamides, etc), some metal complexing anions, and (thio)-phenols among others,[2,4] whereas the coumarins, such as the natural product **1** for which this effect was initially reported,[1,3] do not have any obvious functionality to confer them potent CA inhibitory activity.

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 (e.g., gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiological and pathological processes in humans as well as the growth and virulence of various fungal/bacterial pathogens.[2,4−11] In addition to the established role of CA inhibitors (CAIs) as diuretics and antiglaucoma drugs, it has recently emerged that they have potential as anticonvulsant, antiobesity, anticancer, and anti-infective drugs.[2,4−11] Many of the mammalian CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[2,4] However a critical barrier to the design of CAIs as therapeutic agents is related to the high number of isoforms in humans, their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors of the sulfonamide/sulfamate type.[2,4] However, in the preceding paper,[1] we showed that the natural product 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one **1** as well as the simple, unsubstituted coumarin **2** are hydrolyzed within the CA active site with formation of the 2-hydroxy-cinnamic acids **3** and **4**, respectively, which represent the de facto enzyme inhibitors. At least two other interesting facts emerged during this study:[1] (i) this new class of CAIs, the coumarins, binds (in hydrolyzed form) at the entrance of the CA active site and does not interact with the metal ion, constituting thus an entirely new category of mechanism-based inhibitors, and (ii) for the specific case of compound **1**, the formed substituted-cinnamic acid **3** was observed bound within the CA active site as the cis isomer, although these derivatives are stable in solution as trans isomers.[12] The tentative explanation for this unusual geometry of the bound inhibitor was[1] that as **3** would be too bulky as trans isomer, in the restricted space within the CA active site, in the enzyme−inhibitor adduct characterized in detail by means of X-ray crystallography,[1] the unstable in solution cis isomer is stabilized when bound

Chart 1. Hydrolysis of Coumarins **1** and **2** to the Corresponding 2-Hydroxy-cinnamic Acid Derivatives **3** and **4a**/**4b**



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[a] Abbreviations: CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; hCA, human CA; SAR, structure−activity relationship.

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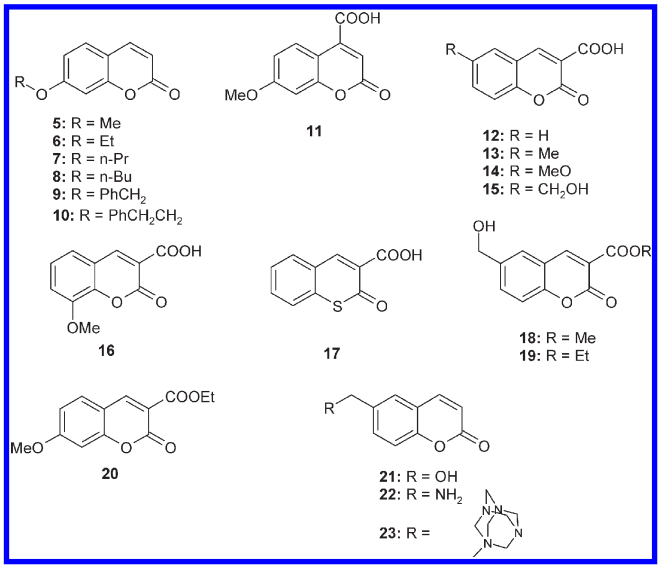
within the enzyme cavity. In the same work,[1] we prepared (by chemical hydrolysis of coumarin **2** in the presence of NaOH) the sodium salt of 2-hydroxy-cinnamic acid **4**, stable in solution as the trans isomer **4b**, and showed it to possess the same CA inhibitory properties as the parent coumarin **2**. It should be also mentioned that coumarins **1** and **2** were potent inhibitors against all investigated human CA isoforms, i.e., CA I−CA XV, which makes this entire class of derivatives of paramount interest for designing novel applications for the CAIs (Chart 1).

To understand in greater detail the CA inhibition mechanism with the coumarins, which might be useful for the design of new pharmacological applications, we investigate here both the simple lead compound **2** for its interaction with the CA active site by means of high resolution X-ray crystallography as well as a series of derivatives of **2** possessing various moieties substituting the coumarin ring in the 3-, 6-, 7-, 3,6-, 4,7-, and 3,8-positions. A thiocoumarin derivative is also investigated here for the first time for its interaction with the CAs in order to understand whether this heterocyclic ring may undergo the same transformation as the coumarin one and thus lead to a novel class of CAIs. This is the first structure−activity relationship (SAR) study for this new type of CAIs, bringing novel insights regarding the inhibition mechanism as well as the structural requirements necessary to be present in the molecules of such heterocyclic compounds for achieving high affinity for various CA isoforms.

Results and Discussion

Chemistry and CA Inhibition. In addition to the previously investigated simple coumarins **1**, **2**, and **5**,[1] a series of diversely substituted coumarins and a thiocoumarin, of type **6**−**23**, were included in this study.



Derivatives **5**−**23** incorporate various moieties by substituting the (thio)coumarin ring in the 3-, 6-, 7-, 3,6-, 4,7-, and 3,8- positions, in order to delineate the main SAR features for this class of CAI, considering the fact that the side chains present in the natural product coumarin **1**, the first CAI of this family of compounds investigated in detail, were shown to interact extensively with the enzyme active site when bound (in hydrolyzed form) within it.[1] Thus, the presence of a different numbers of side chains, as well as their chemical nature, represent important factors influencing the activity of these CAIs. In the previous work,[1] we also showed that the simple, unsubstituted coumarin **2** already possesses interesting enzyme inhibitory properties in addition to the more complicated scaffold of the natural product coumarin **1**. Thus, we considered these core structures to which various substituents were incorporated in order to delineate SAR for the whole coumarin class as CAIs. The 7-monosubstituted coumarins **5**−**10** investigated here possess various 7-alkoxy moieties, such as the aliphatic C1−C4 groups, together with the benzyl- and phenethyl- moieties. They were chosen to be investigated as the 7-methoxy group was present in the lead **1**, and X-ray crystal data for the hCA II−**1** adduct, showed the MeO group to participate in hydrogen bonds with ordered water molecules present within the CA active site, stabilizing thus the enzyme−inhibitor complex.[1] Thus, understanding how the length and nature of this moiety influence enzyme inhibitory activity may constitute an important aspect of the SAR. Compounds **6**−**10** were prepared from the commercially available 7-hydroxy-coumarin by alkylation with alkyl/aralkyl halides, as described in the literature[12] (see Supporting Information for additional details).

Disubstituted coumarins **11**−**16**,[13] possessing a carboxylic acid as well as methyl-, methoxy-, and hydroxymethyl moieties in various positions of the ring, were also included in the study. Thiocoumarin **17**[14] was the only compound incorporating this ring system in our study and was chosen for understanding whether substitution of oxygen by sulfur in the coumarin ring leads to CA inhibitory properties for this class of compounds never before investigated, but also because the corresponding carboxy-substituted coumarin **12** was present among the investigated derivatives. Disubstituted coumarins **18**−**20**[13] incorporate the ester moieties instead of the corresponding carboxylic acid one present in the parent compound **15**, whereas **20** is an ester analogue of the simple lead **5**, investigated earlier.[1] Because the lead **1** possessed a bulky moiety in position 6 of the coumarin ring, derivatives **21**−**23**[13] were included in the study due to the presence of both smaller, simpler such moieties (hydroxymethyl and aminomethyl), which can be easily derivatized, and also of the much bulkier hexamethylene-tetramine one, present in **23**. Compounds **6**−**23** were reported earlier in the search of serine protease inhibitors by Delarge's and Masereel's groups.[13,14]

Inhibition data for compounds **1**−**23** against all 13 catalytically active mammalian (h = human, m = murine) CA isoforms, CA I−IV, VA, VB, VI, VII, IX, and XII−XV, are presented in Table 1. The data have been obtained by a stopped flow technique monitoring the physiological reaction catalyzed by these enzymes, i.e., CO_2 hydration to bicarbonate and protons.[15] As for the previously studied compounds **1**, **2**, and **5**,[1] coumarins **6**−**23** investigated here were incubated with the enzymes for 6 h in order to allow for the complete hydrolysis (within the enzyme active site) of the (thio)coumarin ring and formation of the substituted 2-hydroxy-/mercapto-cinnamic acids. Thus, data of Table 1 report the inhibition constants for these (thio)coumarins **1**−**23** following 6 h incubation with all the CA isoforms. The following should be noted regarding inhibition data of Table 1:

(i) Isoform hCA I was inhibited by all coumarins/thiocoumarin **1**−**23**, with inhibition constants in the range of 78 nM−37.0 μM. The best inhibitors were

Table 1. Inhibition of Mammalian Isozymes CA I−XV (h = Human, m = Murine Isoform) with Coumarins/Thiocoumarins **1**, **2**, **5**−**23**, by a Stopped-Flow, CO_2 Hydration Assay Method (6 h Incubation Time between Enzyme and (Thio)coumarin)[15]

	K_I (μM)[c]										
isozyme[a]	**1**[d]	**2**[d]	**5**[d]	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
hCA I	0.078	3.1	5.9	31.4	23.1	37.0	26.9	31.4	3.72	9.3	6.8
hCA II	0.059	9.2	0.066	12.4	145	213	224	243	0.099	44.7	42.5
hCA III	> 500	> 500	161	29.0	38.6	50.3	40.2	49.5	> 500	9.1	13.4
hCA IV	3.8	62.2	7.8	22.7	24.6	24.9	19.2	18.5	72.3	3.8	5.6
hCA VA	96.0	> 500	> 500	7.3	8.1	9.0	7.6	6.6	> 500	9.0	8.6
hCA VB	17.7	> 500	48.6	60.9	71.7	76.4	65.1	69.4	> 500	6.8	8.3
hCA VI	35.7	> 500	61.2	5.8	37.4	26.8	9.7	8.5	> 500	31.3	31.7
hCA VII	27.9	> 500	9.1	7.2	7.7	15.0	11.8	15.9	9.4	5.1	29.6
hCA IX[b]	54.5	> 500	> 500	1.6	3.3	4.5	1.7	3.9	> 500	4.7	7.7
hCA XII[b]	48.6	> 500	167.4	4.7	8.6	8.5	8.3	5.2	> 500	9.0	8.6
mCA XIII	7.86	> 500	6.0	27.4	34.3	8.2	9.6	9.8	31.7	7.3	7.4
hCA XIV	7.80	> 500	9.7	4.7	5.0	4.3	2.0	5.5	82.3	7.4	13.0
mCA XV	93.1	> 500	> 500	76.3	54.7	65.2	43.1	44.5	> 500	5.6	6.7

	K_I (μM)[c]									
isozyme[a]	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
hCA I	6.6	9.3	1.3	0.100	0.098	3.3	2.4	7.8	7.1	4.1
hCA II	15.3	44.5	30.1	6.2	0.032	50.5	31.4	32.4	3.7	3.2
hCA III	14.5	9.7	17.1	9.0	8.9	9.6	9.1	9.8	9.6	8.1
hCA IV	4.9	4.3	5.8	4.1	6.5	0.048	5.7	5.1	5.4	6.2
hCA VA	8.7	6.2	9.4	8.4	8.4	3.0	9.5	8.9	9.7	8.2
hCA VB	7.6	8.6	6.5	7.5	7.1	2.9	8.0	6.8	6.2	5.6
hCA VI	10.5	15.6	25.6	8.8	9.5	1.5	42.0	9.9	9.2	8.9
hCA VII	4.9	6.6	6.1	7.3	7.3	0.045	20.3	6.9	8.1	3.1
hCA IX[b]	6.6	5.6	7.4	0.047	0.045	0.047	6.5	0.093	6.7	0.048
hCA XII[b]	9.0	8.9	8.6	8.7	5.6	8.4	8.8	8.2	8.6	3.2
mCA XIII	3.8	0.048	7.8	0.042	0.041	6.1	6.2	0.046	0.040	3.8
hCA XIV	39.6	8.4	1.0	7.4	7.3	4.6	9.6	9.5	8.8	7.7
mCA XV	7.2	8.6	6.4	4.8	7.4	0.046	6.6	7.4	7.1	8.2

[a] h = human; m = murine isozyme. [b] Catalytic domain. [c] Errors in the range of ±5% of the reported data from three different assays. [d] From ref 1.

the natural coumarin **1**, the thiocoumarin **17**, as well as the hydroxy-methyl-substituted ester **18** (K_Is of 78−100 nM). Substitution patterns leading to reduced hCA I inhibitory activity were those present in **6**−**10** (longer aliphatic/aralkyl chains in position 6 are thus detrimental to the inhibitory activity of these compounds compared to the unsubstituted **2** or methoxy-substituted compound **5**) and **11** (possessing a 4-carboxy moiety) because these derivatives showed K_Is > 9.3 μM. The remaining coumarins investigated here were medium potency hCA I inhibitors, with inhibition constants in the range of 1.3−9.3 μM (Table 1).

(ii) The physiologically dominant cytosolic isozyme hCA II was also inhibited by all these derivatives, with inhibition constants in the range of 32 nM−243 μM. The best inhibitor was again the methyl ester **18** (K_I of 32 nM, the best coumarin inhibitor detected so far) together with the natural product **1**, the methoxyderivative **5**, and the carboxylic acid **11** (K_Is of 59−99 nM). Again, the monosubstituted derivatives **6**−**10** incorporating bulky chains in position 6 of the coumarin ring showed reduced inhibitory activity compared to the lead **5** (K_Is of 12.4−243 μM), proving that substituents other than methoxy are not tolerated in that position for effective binding to hCA II. Not very effective hCA II inhibitors were also derivatives **12**−**16**, possessing the free COOH moiety in position 3. The thiocoumarin **17** was on the other hand a more effective inhibitor (K_I of 6.2 μM). It is, however, worth noting that there is a very large difference of activity between the carboxylic acid **15** and the corresponding esters **18** and **19**, with the methyl ester **18** being a very potent, nanomolar inhibitor (K_I of 32 nM), whereas the free acid **15** and the ethyl ester **19** were 1390−1578 times less effective hCA II inhibitors. Such a sharp SAR for minimal structural changes (i.e., a CH_2 group) was never before evidenced for other classes of CAIs, such as the sulfonamides or the sulfamates,[16] and constitutes a valuable feature for this type of inhibitor.

(iii) The muscle isoform hCA III, which is not easily inhibited by sulfonamides,[17] was weakly or not at all inhibited by coumarins **1**−**3** and **11** (K_Is of 161 → 500 μM), whereas coumarins **6**−**10** and **13**, **14** showed more efficient inhibitory activity, with K_Is in the range of 13.4−50.3 μM. Even better activity was observed for the thiocoumarin **17** and coumarins **12**, **15**, **18**−**23**, with K_Is in the range of 8.1−9.8 μM (Table 1).

(iv) The coumarin ester **19** was a nanomolar inhibitor of the extracellular isoform hCA IV (K_I of 48 nM), whereas most other such derivatives (e.g., **1**, **5**, **12**−**18** and **20**−**23**) showed effective inhibition, in the low micromolar range, with K_Is of 3.8−7.8 μM. The lead **1** was the least effective hCA IV inhibitor (K_I of 62.2 μM), whereas compounds with bulky groups in position 6 of the coumarin ring such as **6**−**10** showed also weak inhibition (K_Is of 18.5−24.9 μM) compared to the methoxy-substituted coumarin **5**.

(v) The mitochondrial isoform hCA VA was not inhibited by **2**, **5**, and **11** (K_I > 500 μM) and was weakly

inhibited by the natural product **1** (K_I of 96 μM). All other coumarins investigated here and the thiocoumarin **17** were on the other hand effective, low micromolar hCA VA inhibitors (K_Is < 10 μM), with the best inhibitor being the ester **19** (K_I of 3.0 μM). Thus, in the case of this isozyme, even the bulky derivatives **6**−**10** act as efficient CAIs.

(vi) The second mitochondrial isoform, hCA VB, showed a very different inhibition profile with compounds **1**−**23** compared to hCA VA. Thus, **2** and **11** showed no inhibitory activity (K_I > 500 μM), the natural product **1** as well as the monosubstituted derivatives **5**−**10** were weak inhibitors (K_Is in the range of 17.7−76.4 μM), whereas the remaining coumarins **12**−**16** and **18**−**23**, as well as the thiocoumarin **17**, were effective hCA VB inhibitors, with inhibition constants in the range of 2.9−8.6 μM. It is obvious that minimal structural changes in the scaffold of the coumarins influence the inhibitory activity very much.

(vii) The secreted (saliva, milk) isoform hCA VI was not inhibited by **2** and **11** (K_I > 500 μM), and was weakly inhibited by the following coumarins: **1**, **5**, **7**, **8**, **12**, **13**, **15**, **16**, and **20** (K_Is in the range of 15.6−61.2 μM). Thus, SAR is less defined here compared to other CA isoforms discussed above (e.g., for the bulky-substituted compounds **5**−**10**, there is no linear correlation between the length of the substituent present in the 6-position and the hCA VI inhibitory activity, see Table 1). Effective hCA VI inhibitory activity (K_Is in the range of 1.5−10.5 μM) was observed for derivatives **6**, **9**, **10**, **14**, **17** (thiocoumarin), **18**, **19**, and **21**−**23**. The best inhibitor was the ester **19** (K_I of 1.5 μM).

(viii) Except **2**, which was not inhibitory, the cytosolic isoform hCA VII was inhibited by all compounds **1**, **5**−**23** investigated here, with K_Is in the range of 45 nM−29.6 μM. The only nanomolar inhibitor was the ester **19** (K_I of 45 nM), whereas most of these derivatives were low micromolar inhibitors. The least effective inhibitors were **1**, **13**, and **20** (K_Is in the range 20.3−29.6 μM). Again small structural variations in the coumarin scaffold lead to important differences of activity (compare **12** and **13** which differ by a CH_2 groups but by a factor of almost 6 in their CA VII inhibitory activity).

(ix) The tumor-associated isoform hCA IX was weakly inhibited by compound **1** and not inhibited by **2**, **5**, and **11** (K_I > 500 μM). However, a large number of derivatives showed effective or very effective inhibitory activity against this isoform, which represents a new drug target for developing antitumor therapies or diagnostic agents.[7,9] Thus, thiocoumarin **17** and several coumarins (**18**, **19**, **21**, and **23**) showed low nanomolar affinity for this enzyme, with inhibition constants in the range of 45−98 nM. These coumarins incorporate the 6-hydroxymethyl- and 3-ester moieties (**18** and **19**), with no important differences of activity between the methyl and ethyl esters in this case. The monosubstituted derivatives **21** and **23** on the other hand contained either a compact (CH_2OH) or a rather bulky (hexamethylenetetramine) group in position 6 of the coumarin ring, which render our findings quite important, as it is clear that for effective CA IX inhibition, a large variations of structural motifs are allowed in the 3- and 6-positions of the (thio)coumarin ring. It is interesting to note that the isostructural (to **21**) amine **22**, was 72 times less inhibitory than the alcohol **21**. It is not improbable that the enhanced basicity of the amine **22** compared to the alcohol **21** is detrimental to binding within the enzyme active site due to a different pattern hydrogen bonds between the two moieties and amino acid residues at the entrance of the cavity, where presumably these moieties are found. However this hypothesis should be checked by X-ray crystallography, which will allow us to understand how the various substituents of the coumarin ring interact with amino acid residues within the enzyme active site. The remaining coumarins, e.g., **6**−**10**, **12**−**16**, **20**, and **21**, showed efficient, low micromolar inhibition of CA IX, with inhibition constants in the range of 1.6−7.7 μM. It may be seen that many substitution patterns on the (thio)coumarin ring lead to effective nanomolar−low micromolar CA IX inhibitors, affording for drug design campaigns for this important drug target.[2]

(x) hCA XII, another transmembrane isoform present in tumors,[18] was poorly inhibited by **2**, **5**, and **11**, whereas the natural product coumarin **1** was slightly more inhibitory (K_I of 48.6 μM). However, most of the investigated (thio)coumarins were effective, low micromolar hCA XII inhibitors, with K_Is in the range of 3.2−9.0 μM. Thus, a lot of substitution patterns present in compounds **6**−**23** investigated here lead to effective hCA XII inhibitors, although compounds with nanomolar affinity for this isozyme were not evidenced so far (Table 1).

(xi) Several low nanomolar mCA XIII inhibitors were observed among the investigated compounds, such as the thiocoumarin **17** and the coumarins **15**, **18**, **21**, and **22**, possessing inhibition constants of 40−48 nM. Together with CA IX, CA XIII is thus the isoform leading to the highest number of nanomolar inhibitors in this class of derivatives. Unlike other isoforms, CA XIII was equally inhibited by the free carboxylic acid **15** and its methyl ester **18** (K_Is of 41−48 nM), whereas the longer ethyl ester **19** was 127−148 times less inhibitory than **15**/**18**. Moderate inhibitory activity against CA XIII was observed with the coumarins **6**, **7**, and **11**, whereas compounds **5**, **8**−**10**, **12**−**14**, **16**, **19**, **20**, and **23** were medium potency, low micromolar inhibitors (K_Is of 3.8−9.6 μM).

(xii) The transmembrane isoforms hCA XIV showed an inhibition profile with the investigated derivatives rather similar to that of hCA XII, with which it shares some relevant sequence homology.[19] Thus, **2**, **11**, and **14** were ineffective inhibitors (K_Is of 39.6 → 500 M), whereas all other coumarins and the thiocoumarin investigated here showed effective, low micromolar inhibitory activity (K_Is of 1.0−13.0 μM). The best hCA XIV inhibitor was the 3,8-disubstituted coumarin **16** (K_I of 1.0 μM), which urges us to investigate also this substitution pattern represented here only by this unique compound.

(xiii) mCA XV, the latest mammalian CA isoform described so far,[20] was not inhibited by **2**, **5**, and **11** and

was weakly inhibited by coumarins **1**, and **6**−**10** (K_Is of 43.1−93.1 μM). The ester **19** was on the other hand a low nanomolar mCA XV inhibitor (K_I of 46 nM), whereas the remaining coumarins and the thiocoumarin **17** showed effective, low micromolar inhibitory activity (K_Is of 4.8−8.6 μM).

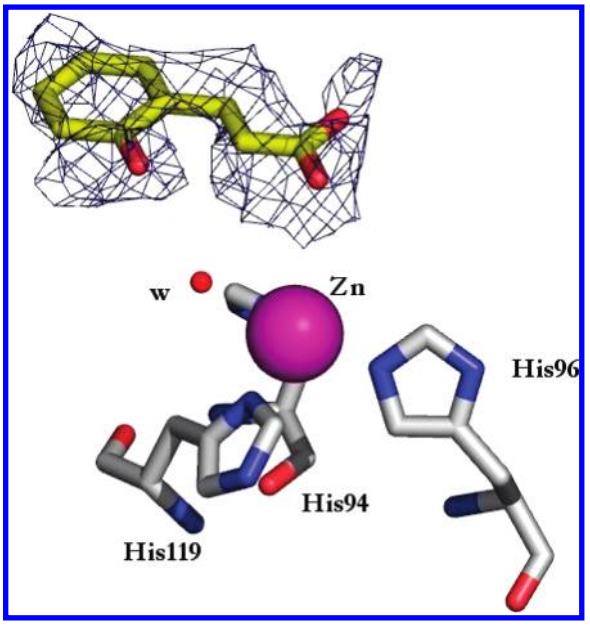


Figure 1. Electron density map at 1σ of the hydrolyzed coumarin **2** (i.e., *trans*-2-hydroxy-cinnamic acid **4b**) bound within the hCA II active site. The Zn(II) ion and its three protein ligands, (His94, His96, His119, CPK colors) and the coordinated water molecule (w, in red) are also shown.

All these data show the potential of the coumarins/thiocoumarins to selectively and potently inhibit some CA isoforms among the 13 catalytically active ones described in mammals, which are in fact undiscriminately inhibited by sulfonamides and sulfamates. The complex SAR features evidenced above afford the possibility to achieve this goal of obtaining selective inhibitors for some of these enzymes. For example, **23** is a low nanomolar inhibitor of only CA IX (K_I of 48 nM), whereas it inhibits in the micromolar range all other 12 CAs, a feature never evidenced before for a sulfonamide CAI.[2,16] The same is true for **15** and **22**, which act as nanomolar inhibitors against mCA XIII (K_Is of 40−48 nM), whereas the remaining 12 isoforms inhibited in the micromolar range by these compounds (Table 1).

Inhibition Mechanism and X-Ray Crystallography. To better understand the inhibitory mechanism with this new class of CAI, and hopefully the SAR discussed above, we resolved the X-ray crystal structure (at a resolution of 2.0 Å) of another coumarin,[1] the simple, unsubstituted derivative **2** in adduct with the physiologically dominant CA isoform, hCA II.[2,21]

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 (Figure 2) as for the previously reported structure of coumarin **1** in adduct with hCA II.[1] However, again,[1] in this

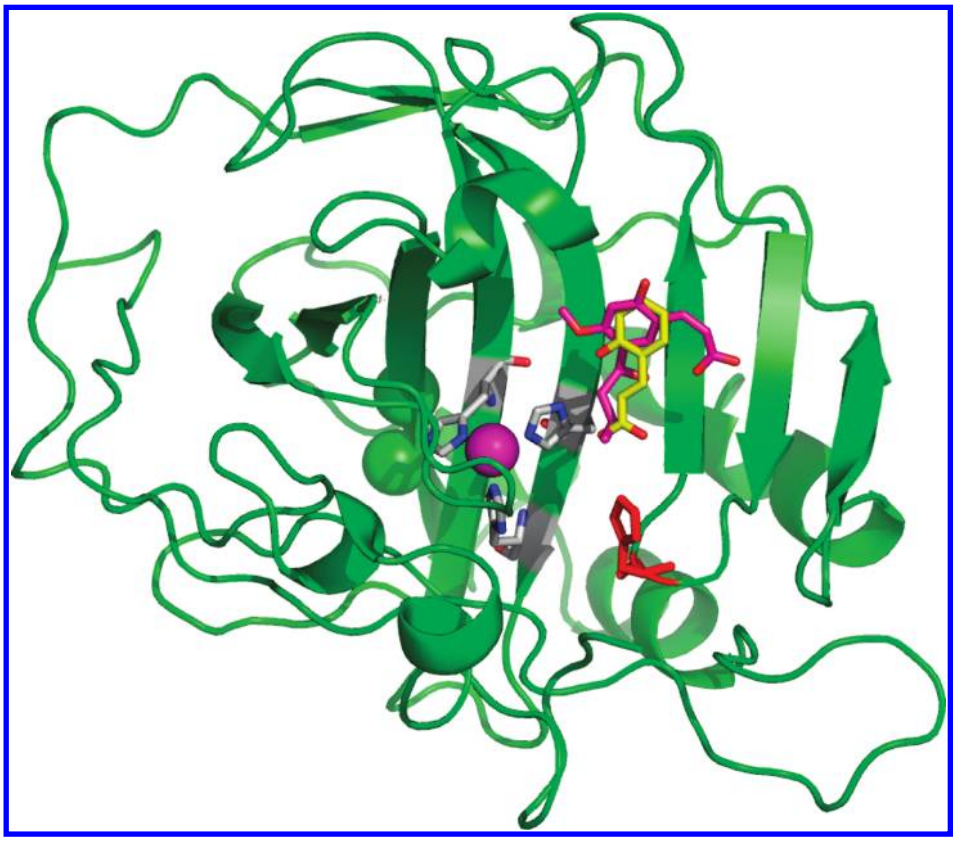


Figure 2. Binding of the coumarin **2** hydrolysis product (*trans*-2-hydroxy-cinnamic acid **4b** in yellow) and coumarin **1** hydrolysis product (*cis*-2-hydroxycinnamic acid **3**, magenta) to the hCA II active site. The protein backbone is shown as green ribbon, the catalytic Zn(II) ion as violet sphere, with its three protein ligands (His94, 96, and 119, CPK colors) also evidenced. The proton shuttle residue (His64) is shown in red.

electron density, we could not fit the structure of the coumarin **2**. Instead, its hydrolysis product, the *trans*-2-hydroxy-cinnamic acid **4b** (Chart 1) perfectly fitted within the electron density shown in Figure 1. All atoms of the hydrolyzed inhibitor had an average *B* factor of 47.0, proving that the occupancy of the inhibitor was of 70%, which represents a good occupancy level for the external part of the enzyme active site where the degree of disorder is rather high.[5] Thus, as for the similar case reported earlier,[1] the zinc hydroxide species of the enzyme, responsible for the various catalytic activities of CAs[2−4] including the esterase one,[2] appears likely to have hydrolyzed the lactone ring of **2**, leading to formation of the 2-hydroxycinnamic acid **4**.

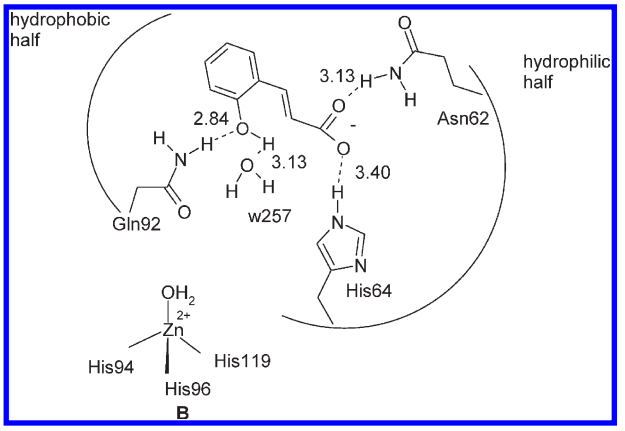


Figure 3. Hydrogen bonds in which **4b** participates when bound to the hCA II active site with residues Asn62, His64, Gln92, and a water molecule (w257). Figures represent distances (in Å).

However, unlike the bulky derivative **3** investigated earlier,[1] in this case the thermodynamically stable *trans*-2-hydroxy-cinnamic acid **4b** has been evidenced as bound within the CA active site. Interactions between the protein and Zn^{2+} ion were entirely preserved in the hCA II−**4b** adduct (data not shown).[21] The inhibitor **4b** was found bound at the entrance of the active site cavity (Figure 2), in the same region where the *cis*-hydroxycinammic acid **3** binds.[1] However, as shown in Figure 2 where the two substituted-2-hydroxycinnamic acids **3** and **4** are superposed (when bound to the enzyme active site), different orientations and conformations were adopted by the two inhibitors. Thus, the bulky 2-hydroxycinnamic acid **3** has the cis geometry, as mentioned above (probably due to steric impairment because of the second bulky, hydroxypentyl chain present in its molecule), whereas the less bulky compound **4** has the trans geometry **4b** (Figure 2). The second important difference between the two coumarin hydrolysis products is related to the orientation of the carboxyethylene moiety of the two inhibitors **3** and **4**. In fact, they are orientated toward opposite parts of the active site, the one of **3** pointing toward the exit of the cavity (and interacting with Glu238 from a symmetry-related enzyme molecule),[1] whereas the one of **4b** points toward the hydrophilic part of the CA II active site and interacting with two amino acid residues (Asn62 and His64) by means of two hydrogen bonds involving the COOH moiety of the inhibitor (Figure 3). Thus, the carboxylate moiety of inhibitor **4b** is anchored by means of two hydrogen bonds (of 3.40 and 3.13 Å, respectively) to the NH of the imidazole of His64, and the NH_2 of Asn62, amino acid residues lining the hydrophilic half of the hCA II active site (Figure 2 and 3). The phenolic moiety of inhibitor **4b** also participates in two hydrogen bonds, with

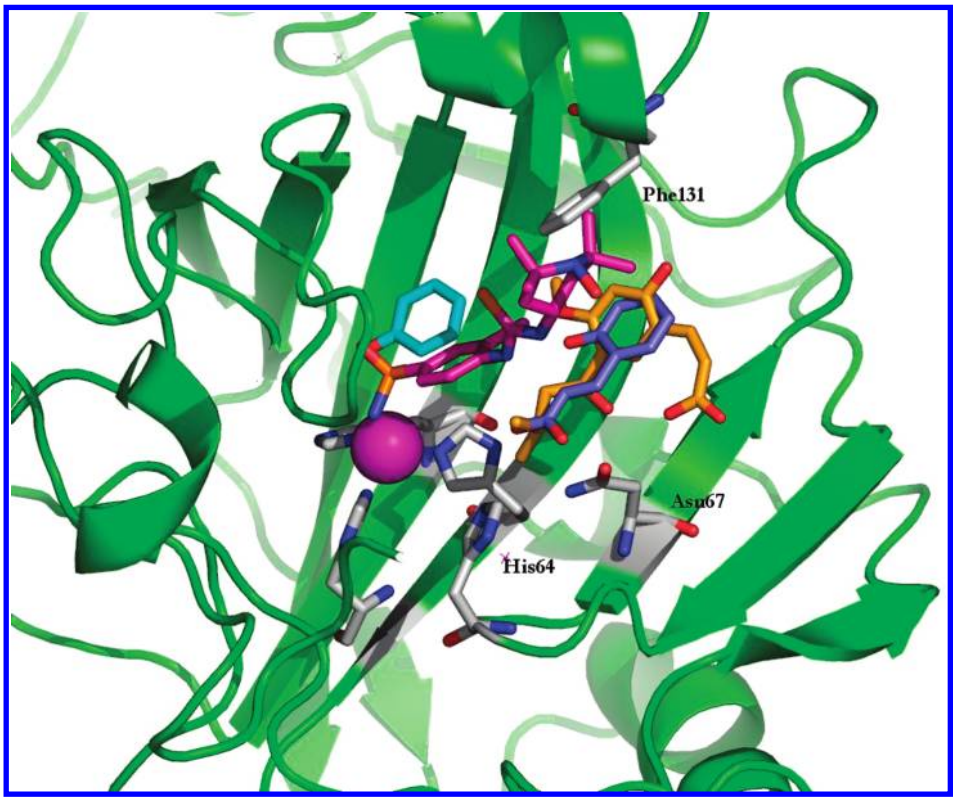
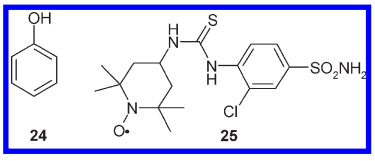


Figure 4. Superposition of the hCA II−**3** adduct (gold, PDB file 3F8E), hCA II−**4b** adduct (violet, PDB file 4F8E) with the hCA II−phenol **24** adduct[22] (file not available in PDB, sky blue) and hCA II−sulfonamide **25** adduct[24] (magenta, PDB file 3EFT). The protein backbone is shown as green ribbon, the catalytic Zn(II) ion as pink−violet sphere, with its three protein ligands (His94, 96, and 119) evidenced. Some of the amino acid residues involved in the binding of hydrolyzed coumarins (His64, Asn67, and Phe131) are also shown (CPK colors).

the amide of Gln92 (of 2.84 Å) and with a water molecule, w257 (of 3.13 Å), as shown schematically in Figure 3. Thus, this hydrolyzed coumarin interacts with different amino acid residues (i.e., Asn62, His64, and Gln92) compared to the hydrolysis product of **1**, which primarily interacted with Asn67, Phe131, and Glu238sym.[1]

To stress the novelty of this binding mode of coumarins (hydrolyzed to *cis*-/*trans*-hydroxy-cinnamic acids) to the CA active site, in Figure 4 we present a superposition of the hCA II−**3**/**4b** adducts with those of phenol **24**[22,23] and a benzene sulfonamide inhibitor recently reported by us, compound **25**.[24] Indeed, sulfonamides and phenols represent the other classes of effective CAIs, with the sulfonamides and their bioisosteres (sulfamates, sulfamides) having clinical applications.[2,16,22−24] It may be observed that sulfonamide **25** is coordinated to the Zn(II) ion within the hCA II active site, whereas its organic scaffold fills the entire enzyme cavity, making an extensive series of van der Waals and polar interactions with amino acid residues both at the bottom, middle, and entrance of the active site cavity. A water molecule/hydroxide ion, which is the fourth zinc ligand present in the noninhibited enzyme, is thus displaced by the sulfonamidate nitrogen of the inhibitor when sulfonamides bind to CAs. In contrast, phenol **24** was shown to be anchored to the zinc bound water molecule/hydroxide ion of the enzyme, and to make two hydrogen bonds with Thr199, an amino acid residue conserved in all α-CAs, by Christianson's group.[22] However, due to the limited number of interactions, phenol is a quite weak CAI[23] (**24** has a K_I of 5.5 μM against hCA II).[23] Sulfonamide **25**, which makes a host of various interactions both with the metal ion and amino acid residues within the enzyme active site, behaves as a very potent CAI (K_I of 12 nM against hCA II).[24] As seen from this superposition presented in Figure 4, the hydrolysis product of coumarins **1** and **2**, the *cis*/*trans*-2-hydroxy-cinnamic acids derivative **3** and **4b** bind very differently to the hCA II active site compared both to sulfonamide **25** or the phenol **24**. It may be seen that the scaffolds of these substituted-cinnamic acids are present in an active site region where only the terminal tail moiety of sulfonamide **25** lies, i.e., toward the entrance of the active site cavity, which is also the region with most variation in amino acid side chains among the various CA isoforms.[2,16,19] This may explain the very interesting inhibition profile of coumarins against the 13 CA isoforms investigated here.



Considering these facts exposed above, and also the observation that thiocoumarins in addition to coumarins do act as CAIs, we propose the following general inhibition mechanism with these two classes of CAIs (Chart 2).

Coumarins/thiocoumarins may possess various tautomeric forms, such as the zwitterionic benzo(thio)pyrylium phenoxides, which may bind within the CA active site similarly to phenols (Chart 2, step A),[7] i.e., by anchoring to the zinc-bound water molecule/hydroxide ion.[22] Alternatively, the chromenone tautomer may bind to the enzyme, similarly to phenols, as illustrated in Chart 2A. In fact, as we shown in the earlier work,[1] coumarins initially bind CAs with micromolar affinity (when incubated with the enzyme for 15 min) similarly to phenols. We demostrated in an earlier work[23] that a bicyclic phenol, which has some structural features in common with the above-mentioned zwitterionic form of the (thio)coumarins, inhibits hCA II in the same micromolar range as many (thio)coumarin derivatives studied in the present paper. The benzo(thio)pyrylium phenoxide shown in **A** may then undergo hydrolysis by the zinc-activated water molecule/hydroxide ion from the enzyme cavity, which acts as a very potent nucleophile.[2] It is thus probable that a *cis*-2-hydroxy-cinnamic acid intermediate is formed (Chart 2 step B), which cannot bind effectively in the restricted space near the Zn(II) ion (where simple phenols and sulfonamides bind), due to its bulky pendant arms, being thus reoriented toward the exit of the active site cavity (where it has been evidenced,[1] as stable cis derivative for the bulky coumarin **1**). A rearrangement of the enzyme−inhibitor adduct may then occur, leading to the *trans*-hydroxy/mercapto-cinnamic acids shown in Chart 2, step C, provided that the R and R′ moieties from the initial (thio)coumarin are not too bulky to interfere with the binding to the enzyme active site. Indeed, for the case investigated here (R = R′ = H), the *trans*-2-hydroxycinnamic acid **4b** was evidenced bound to the CA active site, and not the cis isomer observed for the bulky coumarin **1**.[1] The proposed mechanism is a general one (for coumarins and thiocoumarins) and may thus account for both the kinetic and X-ray crystallographic data presented here and in the earlier work.[1]

Chart 2. Proposed Inhibition Mechanism of CAs by Coumarins/Thiocoumarins, Leading to *cis*- or *trans*-2-Hydroxy/mercapto-cinnamic Acids

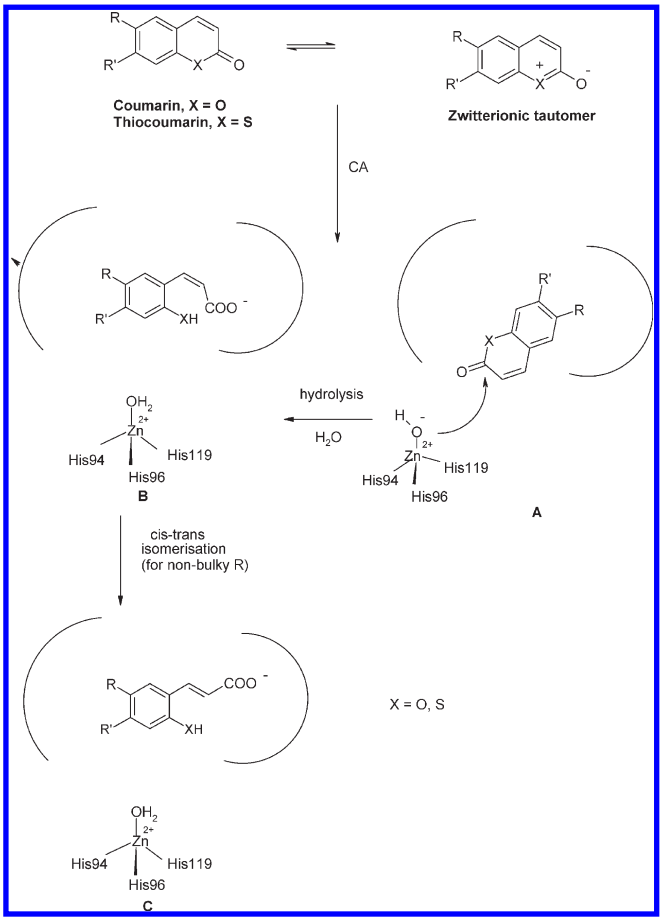


Table 2. Crystallographic Parameters and Refinement Statistics for the hCA II−**4b** Adduct

parameter	value
Crystal Parameter	
space group	$P2_1$
cell parameters	a = 42.2 Å
	b = 41.5 Å
	c = 72.4 Å
	β = 104.5°
Data Collection Statistics (20.0−2.0 Å)	
no. of total reflections	27978
no. of unique reflections	16696
completeness (%)[a]	99.4 (99.8)
$F2/\sigma(F2)$	17.2 (2.9)
R-sym (%)	14.3 (37.0)
Refinement Statistics (20.0−2.0 Å)	
R factor (%)	22.0
R-free (%)[b]	29.0
rmsd of bonds from ideality (Å)	0.012
rmsd of angles from ideality (deg)	1.53

[a] Values in parentheses relate to the highest resolution shell (2.1−2.0 Å). [b] Calculated using 5% of data.

Conclusions

We report here a detailed investigation of substituted coumarins and a thiocoumarin derivative, as inhibitors of the zinc enzyme CA. All 13 catalytically active mammalian CA isoforms, i.e., CA I−VA, VB, VI, VII, IX, XII, and XIII−XV, were inhibited by these classes of compounds with activity in the low nanomolar−micromolar range. The substitution pattern at the (thio)coumarin ring is the main player in controlling the inhibitory power. Several beneficial substitution patterns were detected, among some 3-, 6-, 7-mono-, and 3,6-, 4,7-, and 3,8-disubstituted coumarins incorporating alkyl- alkoxy-, carboxy-, ester, hydroxy/aminoethyl, or hexamethylenetetramine moieties. Importantly, various CA isoforms showed very different inhibition profiles with these compounds, leading thus to many (thio)coumarins acting as isozyme-selective inhibitor. An X-ray crystal structure for the adduct of the simple, unsubstituted coumarin with CA II showed a binding mode different of the one reported earlier for a natural product possessing a 5,6-disubstitution pattern incorporating a bulky 6-moiety. We show here that these two classes of CAIs, the coumarins and the thiocoumarins, are formed by the hydrolytic attack of the zinc hydroxide species of the enzyme to the (thio)coumarin bound in its neighborhood within the enzyme active site. The initially formed *cis*-2-hydroxy/mercapto-cinnamic acid may be stabilized and binds toward the exit of the active site cavity for (thio)coumarins substituted with bulky moieties in the 6 position. Alternatively, this intermediate isomerizes to the trans derivative (for less bulky coumarins/thiocoumarins), being bound in the same active site region, but with different orientations compared to the *cis*-2-hydroxy-cinnamic acids. The versatility of the (thio)coumarin chemistry, the *cis*−*trans* isomerization evidenced here and easy derivatization of these heterocyclic rings, coupled with the nanomolar inhibition range of several isozymes with biomedical applications, may afford isoform-selective CAIs which render these classes of compounds superior to the clinically used sulfonamides/sulfamates.

Experimental Protocols

Chemistry. Coumarins **2**, **5**, **11**, and 6-hydroxycoumarin were commercially available (Sigma-Aldrich, Milan, Italy). Coumarins **6**−**10** were prepared by alkylation of 7-hydroxycoumarin with alkyl/aralkyl halides[12] (see Supporting Information for details). Compounds **12**−**23** were reported earlier by one of our groups.[13] CA isozymes were recombinant ones prepared in our laboratory as reported earlier.[1,6−9] ^{1}H spectra were recorded using a Bruker Advance III 300 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in hertz (Hz). Melting points (mp) were measured in open capillary tubes, unless otherwise stated, using a Büchi melting point B-540 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out on Merck Silica Gel 60 F_{254} aluminum backed plates. Elution of the plates was carried out using MeOH/DCM or MeOH/$CHCl_3$ systems. Visualization was achieved with UV light at 254 nm by dipping into a 0.5% aqueous potassium permanganate solution or by exposure to iodine. Flash column chromatography was carried out using silica gel (obtained from Aldrich Chemical Co.) as the adsorbent. The crude product was introduced into the column as a solution in the same elution solvent system, alternatively as a powder obtained by mixing the crude product with the same weight of silica gel in acetone and then removing the solvent in vacuo at room temperature or dissolved into a minimum amount of DCM or carbon tetrachloride. All moisture or air sensitive reactions were carried out in oven-dried glassware under a positive pressure of nitrogen or argon using standard syringe/septa techniques. All the inert gases used (nitrogen and argon) were passed through jacket columns fitted with activated silica gel containing cobalt(II) chloride adsorbed as humidity indicator The purity of all compounds has been determined by means of analytical HPLC, performed on a reversed-phase C_{18} Bondapack column, with a Beckman EM-1760 instrument. Both conbustion and HPLC confirmed a purity of >99.5% for the compounds reported/investigated here.

7-Ethoxy-2*H*-chromen-2-one 6. δ_H (300 MHz, DMSO-d_6) 1.37 (3H, t J = 13.8, 2′-H_3), 4.15 (2H, q J = 13.8, 1′-H_2), 6.30 (1H, d J = 9.5, 3-H), 6.95 (1H, dd J = 8.4 2.4, 6-H), 6.99 (1H, d J = 2, 8-H_2), 7.63 (1H, d, J = 8.4, 5-H), 8.00 (1H, d J = 9.5, 4-H). m/z (ESI+) 191.18 ([M + H]$^+$ 12%), 213.15 ([M + Na]$^+$ 25%), 403.17 ([2M + Na]$^+$ 100%); mp: 91.8 °C.

7-Propoxy-2*H*-chromen-2-one 7. δ_H (400 MHz, DMSO-d_6) 0.99 (3H, t J = 14.6, 3′-H_3), 1.73−1.78 (2H, m J = 14.6, 2′-H_2), 4.04 (2H, t J = 12.8, 1′-H_2), 6.28 (1H, d J = 9.4, 3-H), 6.94 (1H, dd J = 8.4 2.4, 6-H),; 6.97 (1H, d J = 2, 8-H), 7.62 (1H, d J = 8.4, 5-H), 7.99 (1H, d, J = 9.4, 4-H). m/z (ESI+) 205.23 ([M + H]$^+$ 5%), 227.20 ([M + Na]$^+$ 100%), 241.19 (4); mp: 67.6 °C.

CA Inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[15] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) 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 72 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. Data reported in Table 1 show the inhibition after 6 h incubation, which led to the completion of the in situ hydrolysis of the (thio)coumarin and formation of the hydroxy/mercapto-cinnamic acid.[1]

The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier,[1,6,7] and represent the mean from at least three different determinations.

Crystallization, X-Ray Data Collection, and Refinement. Crystals of the hCA II−**4b** complex were obtained by using the hanging-drop method for cocrystallizing the protein with the ligand, as previously described.[1] A monochromatic experiment at the Cuα wavelength was performed on a crystal of hCA II grown in the presence of **2** (10 μM) by the rotation method on a PX-Ultra sealed-tube diffractometer (Oxford Diffraction) at 100 K. The crystal diffracted up to 2.0 Å resolution (resolution: 20.0−2.0 Å), with 16696 unique reflections out of 27978 reflections, and belonged to the space group $P21$ (a = 42.2 Å, b = 41.5 Å, c = 72.4 Å and $\alpha = \gamma = 90°$, $\beta = 104.5°$). Data were processed with CrysAlis RED (Oxford Diffraction 2006).[25] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model. The refinement was carried out with the program REFMAC5,[26] and model building and map inspections were performing using the COOT program.[27] The final model of the complex hCA II−**4b** adduct had an R factor of 22.0% and R-free 29.0% in the resolution range 20.0−2.0 Å, with a rms deviation from standard geometry of 0.012 Å in bond lengths and 1.53° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[28] Crystallographic parameters and refinement statistics are shown in Table 2.

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Supporting Information Available: The synthesis and characterization of compounds **6**−**10** are described in detail. This material is available free of charge via the Internet at http://pubs.acs.org.

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