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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

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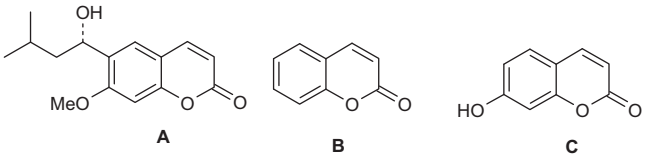
ABSTRACT

Two series of disubstituted coumarins incorporating ether and acetyl/propionyl moieties in positions 6,7- and 7,8- of the heterocyclic ring were synthesized investigated for the inhibition of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). All these coumarins were very weak or ineffective as inhibitors of the housekeeping, offtarget isoforms CA I and II. The 6,7-disubstituted series showed ineffective inhibition also for the transmembrane tumor-associated isoforms CA IX and XII, whereas the corresponding isomeric 7,8-disubstituted coumarins showed nanomolar/subnanomolar inhibition of CA IX/XII. The nature and position of the groups substituting the coumarin ring in the 7,8-positions greatly influenced CA inhibitory properties, with C1–C4 alkyl ethers being the most effective inhibitors.

Coumarins were only recently discovered as a novel class of inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).[1,2] Their mechanism of action has also been elucidated, this class of CA inhibitors (CAIs) being prodrugs and differing of all other known inhibitors of this enzyme. Coumarins in fact do not directly interact with the metal ion from the enzyme active site, which is critical both for catalysis and inhibition of CAs with other classes of compounds, such as sulfonamides, metal-complexing anions, phenols or polyamines.[1–7] Indeed, as shown by kinetic and X-ray crystallographic studies, coumarins are mechanism based inhibitors, which undergo hydrolysis under the influence of the zinc-hydroxide, nucleophilically active species of the enzyme, with generation of substituted-2-hydroxycinnamic acids.[1,2] For example, the natural product coumarin **A** or the simple non-substituted derivative **B** (but also many of its congeners possessing various substitution patterns at the coumarin ring)[2] not only act as effective CAIs against many of the mammalian isoforms CA I–CA XV, but the real enzyme inhibitor was detected to be the hydrolyzed coumarins, **A1** and **B1** (Scheme 1), formed from the original derivatives **A** and **B**.[1] These adducts have been thoroughly characterized by X-ray crystallography of enzyme-inhibitor adducts.[1,2] The 2-hydroxy-cinnamic acids **A1** and **B1** were shown to bind in an unprecedented way to the enzyme, at the entrance of the active site cavity, plugging the entire entrance to it, and blocking thus the catalytic activity of the enzyme.[1,2] Only very recently, some fullerene derivatives were shown to bind in a similar way to the CAs.[8] Occlusion of the CA active site entrance, by hydrolyzed coumarins (i.e., *cis*- or *trans*-2-hydroxycinnamic acids)[1,2] or fullerenes[8] thus constitutes a totally novel mechanism of CA inhibition, which started to be exploited to design compounds with various applications.[2]



Indeed, more recently we shown that umbelliferone (7-hydroxycoumarin) **C** and some of its derivatives are selective, although not very active (submicromolar) inhibitors of the tumor-associated isoforms CA IX and XII, whereas they basically do not inhibit CA I and II, offtarget, highly abundant CA isoforms.[9] In fact the CAIs of the sulfonamide type are clinically used for decades, for various classes of diuretics and systemically acting antiglaucoma agents, but their main drawback is just their rather potent inhibition of CA I and II.[3,4,10] However critical barriers to the use of CAIs as therapeutic agents are related to the high number of isoforms in humans (i.e., 16 CAs, of which 13 have catalytic activity), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available

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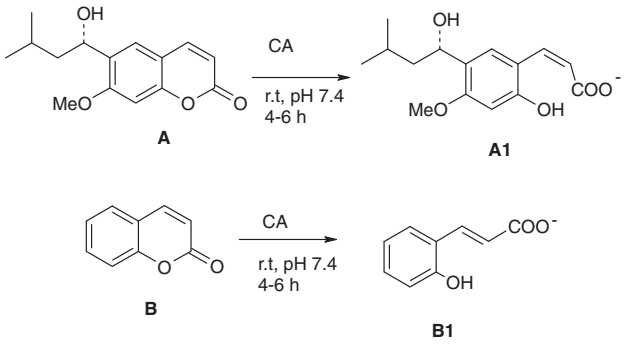
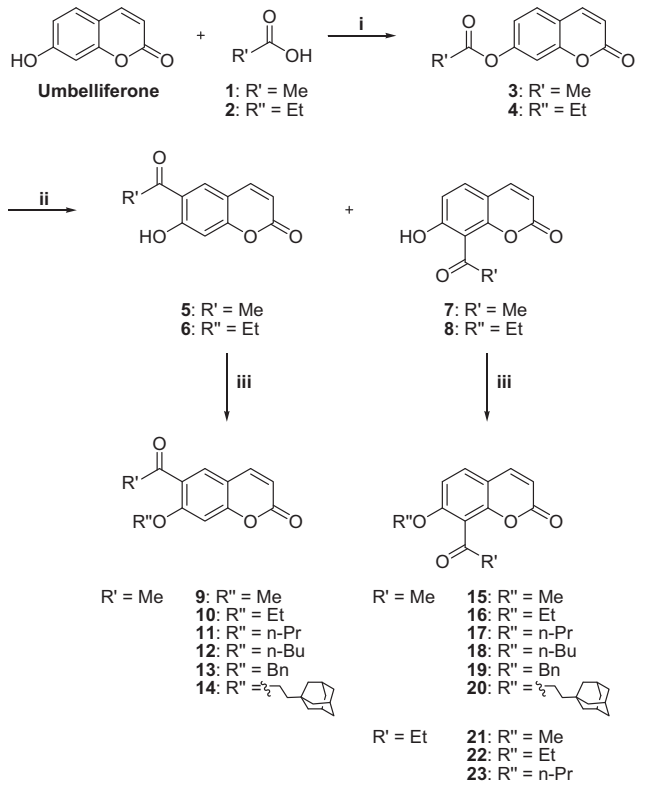


Scheme 1. Formation of 2-hydroxy-cinnamic acids **A1** and **B1** by the CA-mediated hydrolysis of coumarins **A** and **B**.

inhibitors of the sulfonamide/sulfamate type.[3,4,10–13] Thus, there is a stringent need of CAIs with a more selective inhibition profile compared to the sulfonamides and their isosteres, and the coumarins represent an interesting class due to the fact that already a certain number of isoform-selective CAIs targeting CA IX, XII, XIII among others, have been detected, even if the number of investigated coumarins up to date is rather limited (less than 50 compounds have been so far investigated for their interactions with the mammalian CAs).[1,2,9]

Considering **C** as lead molecule, and continuing our interest in investigating coumarins as CAIs, we report here the synthesis and inhibition studies of two series of coumarin derivatives prepared from umbelliferone **C** as lead, and their interaction with four physiologically relevant isoforms, CA I, II, IX and XII.[12,15]

Umbelliferone **C** was shown earlier to constitute an interesting lead for designing novel CAIs based on the coumarin scaffold.[9] Indeed, this compound is an extremely weak inhibitor of isoform hCA



i: dry DMF, DMAP, DCC, r.t, 16h; **ii**: $AlCl_3$, 180°; **iii**: dry THF, PPh_3, DIAD, r.t, 16h

Scheme 2. Preparation of the two series of coumarins **3**–**23**, investigated in this study, starting from umbelliferone (7-hydroxycoumarin) **C**.

I (K_I of 58.4 μM), it does not inhibit significantly hCA II (K_I >100 μM), but it is a submicromolar inhibitor of the tumor-associated isoforms hCA IX and XII, against which it has inhibition constants in the range of 482–754 nM.[9] We used thus this compound to prepare novel coumarin derivatives, also because its phenol moiety can be easily derivatized, leading to novel chemotypes which have not been investigated earlier as CAIs.[1,2] Indeed, disubstituted coumarins in the 6,7- and 7,8-positions have not been included in the series of such compounds tested for their interaction with various CAs in the previous work reported so far.[1,2,9]

Umbelliferone **C** has been converted to the corresponding acetyl and propionyl esters **3** and **4**, by reaction with carboxylic acids **1** and **2** in the presence of carbodimides (DCC, dicyclohexyl carbodiimide and DMAP, dimethylaminopyridine), as shown in Scheme 2. Acetyl-/propionyl esters **3** and **4** were subjected to the Fries rearrangement,[14] in the presence of $AlCl_3$ at 180°, to give the mixture key intermediates, isomeric acyl-umbelliferones **5**–**8**. These mixtures have been separated by flash-chromatography leading to the corresponding pure pairs of derivatives **5**/**7** and **6**/**8**, respectively. The ratio of 6,7-disubstituted versus 7,8-disubstituted coumarins obtained after the rearrangement was of 1:9. Compounds **9**–**23** were the obtained in Mitsunobu conditions,[15] using the appropriate aliphatic alcohol and the purified key intermediates **5–8** mentioned above, in presence of DIAD and triphenylphosphine.[16]

Inhibition data with compounds **5–23** reported here and **A–C** as standards against four CA isozymes, that is, hCA I, II, IX and XII,[17] are shown in Table 1. The following structure–activity relationship (SAR) observations can be drawn from data of Table 1 for these coumarins with 6,7- and 7,8-disubstitution pattern:

(i) The coumarins possessing 6,7-disubstituted moieties, of types **5, 6**, and **9–14**, showed very weak or total lack of CA inhibitory activity against all investigated isoforms. Thus, only **5** and **6** were micromolar hCA IX inhibitors; **9** was a micromolar inhibitor

Table 1
hCA I, II, IX and XII inhibition data with coumarins **5**–**23** and **A**–**C** (as standard inhibitors), by a stopped-flow, CO_2 hydration assay method (6 h incubation time between enzyme and coumarin)[17]

Compound	K_I*			
	hCA I[a] (μM)	hCA II[a] (μM)	hCA IX[b] (nM)	hCA XII[b] (nM)
A	0.078	0.059	54.5	48.6
B	3.1	9.2	>100	>100
C	58.4	>100	482	754
5	>100	>100	8030	>100,000
6	>100	>100	8015	>100,000
7	>100	>100	73.0	61.9
8	>100	>100	58.2	61.7
9	>100	>100	7800	6540
10	>100	>100	7400	>100,000
11	>100	>100	7580	>100,000
12	>100	>100	>100,000	>100,000
13	>100	>100	>100,000	>100,000
14	>100	>100	>100,000	77,700
15	>100	>100	78.3	60.9
16	>100	>100	70.8	1.0
17	>100	>100	56.7	0.98
18	>100	>100	61.2	8.8
19	>100	>100	72.3	22.4
20	>100	>100	63.9	31.5
21	>100	>100	37.8	26.3
22	>100	>100	46.7	33.2
23	>100	>100	50.2	38.4

[a] Full length, cytosolic isoform.
[b] Catalytic domain, recombinant enzyme.
* Errors in the range of ±5–10% of the reported value, from three different determinations.

of hCA IX and XII, whereas all other compounds generally showed inhibition constants >100 μM against all investigated isoforms. Definitely, this substitution pattern leads to a total loss of CA inhibitory properties to the compounds incorporating it.

(ii) Compounds **7, 8** as well as **15–23**, isomeric to the previously discussed ones, but possessing the substituents in the 7,8-positions of the coumarin ring, showed a totally different inhibition profile. Thus, all these compounds were ineffective as hCA I and II inhibitors, with K_I's >100 μM, similar to the lead molecule **C**. This is a desirable feature for CAI, in order to target isoforms involved in pathological processes, and not hCA I and especially hCA II (which is the physiologically dominant isoform), whose inhibition may be deleterious and lead to side effects of such a drug.[3,4]

(iii) The tumor-associated hCA IX was inhibited by umbelliferone **C**[9] in the submicromolar range (K_I of 482 nM), as discussed above, but most of its derivatives **7, 8** and **15–23** were much better inhibitors, with K_I's in the range of 37.8–78.3 nM (Table 1). Thus, for the compounds obtained after the Fries rearrangement, the activity increased from the acetyl **7** to the propionyl **8** derivatives. In the case of the ethers **15–20**, activity increased from the C1 to the C3 derivative (the *n*-propyl derivative **17** was the best hCA IX inhibitor for the acetyl subseries), to decrease then again for the benzyl and adamantylethyl derivatives **19** and **20**. However the ethyl and adamantylethyl derivatives had quite similar derivatives, proving that SAR (which generally for this class of CAIs is very much sensitive to small modifications in the scaffold of the inhibitor) is less sharp for these two derivatives differing quite a lot by the presence of such a bulky group in **20** (compared to **16**). However, for the other derivatives investigated here the reverse is true, with small modifications leading to a sharp increase or decrease of activity (compare **7** and **8**, **16** and **17**, **17** and **18**, respectively). For the propionyl derivatives **21–23**, activity was even more increased compared to the corresponding acetyl derivatives **15–17**, with inhibition constants in the range of 37.8–50.2 nM. The best hCA IX inhibitor was **21**, which with a K_I of 37.8 nM is the best coumarin hCA IX inhibitor reported so far (the previous best inhibitor had a K_I of 48 nM).[2]

(iv) The same behavior as that observed for hCA IX was also detected for the inhibition of the second tumor-associated isoform, hCA XII with coumarins **7, 8** and **15–23** investigated here. Thus, the lead **C** was a moderate hCA XII inhibitor, with a K_I of 754 nM, whereas the new compounds reported here possessing the 7,8-disubstitution pattern, behaved as much more effective inhibitors, with K_I's in the range of 0.98–61.9 nM. It is the first time that we detect subnanomolar inhibition with a coumarin derivative and we think this is a very significant finding (many sulfonamides with subnanomolar inhibition of various CA isozymes are known).[3,4,10] The following SAR was evidenced for these new hCA XII inhibitors. The two key intermediates **7** and **8** had the same potency as hCA XII inhibitors (K_I of 61.7–61.9 nM), irrespective whether an acetyl or propionyl moiety was present in the 8-position of the coumarin ring. This behavior is different from that observed with these two compounds against hCA IX, as discussed above. The ethers **15–23** showed on the other hand enhanced inhibitory properties compared to the parent phenols from which they were prepared. Thus, the methoxy derivatives **15** and **21** were better inhibitors than the parent phenols **7** and **8**, but the increase in activity was not significant (K_I's of 60.9 nM for **15** and of 26.3 nM for **21** have been measured, Table 1). However for the acetyl series, the increase of the aliphatic chain in the ether moiety from one (in compound **15**) to two and three carbon atoms led to an impressive increase in the hCA XII inhibitory activity, the compounds **16** and **17** having inhibition constants of 1 nM and of 0.98 nM, respectively (Table 1). Further increasing the length of the aliphatic chain, as in **18**, or introduction of the benzyl or adamantylethyl moieties, as in **19** and **20**, led to a decrease in activity, but these compounds were still among the best hCA XII coumarin CAIs reported so far, with K_I's of 8.8–341.5 nM. For the propionyl series, the activity remained good enough but the compounds **22** and **23** were less active compared to the corresponding acetyl derivatives **16** and **17**.

In conclusion, we report here that the 6,7-disubstituted coumarins prepared by Fries rearrangement from umbelliferone are devoid of significant CA inhibitory properties against isoforms hCA I, II, IX and XII. However, their isomers, incorporating 7,8-disubstituted moieties act as nanomolar or subnanomolar hCA IX and XII inhibitors, whereas not inhibiting significantly the offtarget isoforms hCA I and II. SAR for the inhibition of hCA IX and XII was complex but the main features associated with low nanomolar inhibitors have been delineated. This is the first report in which subnanomolar inhibition of a CA isoform with a coumarin derivative is ever observed.

Acknowledgment

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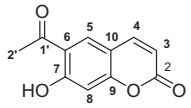
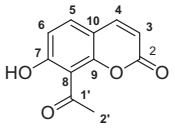
16. 6-Acetyl-7-hydroxy-2*H*-chromen-2-one (**5**):



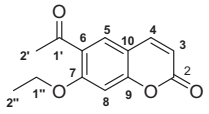
Obtained in 35% yield; mp 173–175 °C; silica gel TLC R_f 0.13 (AcOEt/*n*-Hex 20%, v/v); ν_{max} (KBr) cm^{-1}, 3071 (O–H), 2927 (C–H), 1736 (C=O), 1638 (C=C),1521 (aromatic), 1192 (O–CO); δ_H (400 MHz, DMSO-d_6) 2.71 (3H, s, 2′-H_3), 6.38 (1H, d J = 9.4 Hz, 3-H), 6.93 (1H, s, 8-H), 8.07 (1H, d J = 9.4 Hz, 4-H), 8.37 (1H, s, 5-H); δ_c (100 MHz, DMSO-d_6) 203.7 (C-1′), 164.3 (C-7), 160.5 (C-2), 159.2 (C-9), 145.2 (C-4), 138.9 (C-5), 119.7 (C-6), 114.2 (C-3), 112.6 (C-10), 104.7 (C-8), 29.1 (C-2′).

8-Acetyl-7-hydroxy-2*H*-chromen-2-one (**7**):



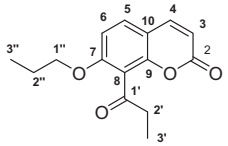
Obtained in 65% yield; mp 169–171 °C; silica gel TLC R_f 0.27 (AcOEt/*n*-Hex 20%, v/v); ν_{max} (KBr) cm^{-1}, 3061 (O–H), 2927 (C–H), 1735 (C=O), 1615 (C=C),1526 (aromatic), 1178 (*O*–C=O); δ_H (400 MHz, DMSO-d_6) 2.65 (3H, s, 2′-H_3), 6.33 (1H, d J = 9.4 Hz,
3-H), 6.95 (1H, d J = 8.6 Hz, 6-H), 7.70 (1H, d J = 8.6 Hz, 5-H), 8.03 (1H, d J = 9.4 Hz, 4-H); δ_c (100 MHz, DMSO-d_6) 201.6 (C-1′), 160.8 (C-2), 160.4 (C-7), 153.4 (C-9), 145.6 (C-4), 132.8 (C-5), 115.7 (C-8), 114.4 (C-6), 112.7 (C-10), 112.1 (C-3), 33.4 (C-2′).

6-Acetyl-7-ethoxy-2*H*-chromen-2-one (**10**):



Obtained in 60% yield; mp 164–166 °C; silica gel TLC R_f 0.16 (AcOEt/*n*-Hex 14%, v/v); ν_{max} (KBr) cm^{-1}, 2935 (C–H), 1732 (C=O), 1622 (C=C), 1533 (aromatic), 1240 (C–O), 1107 (*O*–C=O); δ_H (400 MHz, DMSO-d_6) 1.47 (3H, t J = 6.8 Hz, 2″-H_3), 2.60 (3H, s, 2′H_3), 4.29 (2H, q J = 6.8, 1′-H_2), 6.39 (1H, d J = 9.6 Hz, 3-H), 7.20 (1H, s, 8-H), 8.04 (1H, s, 5-H), 8.11 (1H, d J = 9.6 Hz, 4-H); δ_c (100 MHz, DMSO-d_6) 198.0 (C-1′), 161.6 (C-7), 160.6 (C-2), 158.4 (C-9), 145.3 (C-4), 131.4 (C-5), 125.9 (C-6), 114.4 (C-3), 112.8 (C-10), 101.7 (C-8), 66.1 (C-1″), 32.6 (C-2′), 15.4 (C-2″).

8-Propionyl-7-propoxy-2*H*-chromen-2-one (**23**):



Obtained in 80% yield; mp 82–84 °C; silica gel TLC R_f 0.12 (AcOEt/*n*-Hex 20%, v/v); ν_{max} (KBr) cm^{-1}, 2932 (C–H), 1747 (C=O), 1635 (C=C), 1534 (aromatic), 1287 (C–O), 1170 (*O*–C=O); δ_H (400 MHz, DMSO-d_6) 0.98 (3H, t J = 7.2 Hz, 3″-H_3), 1.13 (3H, t J = 7.4 Hz, 3′-H_3), 1.74 (2H, six J = 7.2 Hz, 2″-H_2), 2.83 (2H, q J = 7.4 Hz, 2′-H_2), 4.13 (2H, t J = 7.2 Hz, 1″-H_2), 6.36 (1H, d J = 9.6 Hz, 3-H), 7.19 (1H, d J = 8.8 Hz, 6-H), 7.77 (1H, d J = 8.8 Hz, 5-H), 8.06 (1H, d J = 9.6 Hz, 4-H); δ_c (100 MHz, DMSO-d_6) 202.9 (C-1′), 160.3 (C-2), 158.5 (C-7), 151.3 (C-9), 145.2 (C-4), 131.2 (C-5), 119.4 (C-8), 113. (C-10), 113.5 (C-3), 110.2 (C-6), 71.24 (C-1″), 38.3 (C-2′), 22.75 (C-2″), 11.13(C3″), 8.42 (C-3′).

17. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed 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 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 shows the inhibition after 6 h incubation, which led to the completion of the in situ hydrolysis of the coumarin and formation of the 2-hydroxy-cinnamic acids.[1,2] The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[1,2] and represent the mean from at least three different determinations. CA isofoms were recombinant ones obtained in house as reported earlier.[1,2].