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Novel coumarins and 2-thioxo-coumarins as inhibitors of the tumor-associated carbonic anhydrases IX and XII

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ABSTRACT

A series of coumarins incorporating *tert*-butyl-dimethylsilyloxy- or allyoxy- moieties in positions 4-, 6 or 7 of the heterocyclic ring have been synthesized and then converted to the corresponding 2-thioxo-coumarins. Other derivatives incorporating hydroxyethyloxy-, tosylethoxy- and 2-fluroethyloxy- moieties in position 7 of the coumarin ring were synthesized together with derivatives of 4-methyl-7-amino coumarin incorporating acetamido, 3,5-dimethylphenylureido- and *tert*-butyloxycarbonylamido functionalities. All these compounds were assayed as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). The human (h) cytosolic isoforms hCA I and II were weakly inhibited (hCA I) or not inhibited at all (hCA II) by these (thioxo)coumarins whereas the tumor-associated transmembrane isoforms hCA IX and XII were inhibited with efficiencies from the submicromolar to the low micromolar range by many of these derivatives. The structure–activity relationship for these classes of less investigated CA inhibitors are delineated, with the potential of using them as leads to obtain isoform-selective inhibitors with excellent affinity for CA IX and XII (validated antitumor targets) which do not significantly inhibit the cytosolic offtarget isoforms hCA I and II.

1. Introduction

Coumarins are a class of widely spread natural compounds possessing a variety of biological activities, which was only recently discovered to inhibit the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1).[1–5] CAs are ubiquitous enzymes in organisms throughout the tree of life, with several genetically distinct families encoding them in prokaryotes and eukaryotes.[4] They are inhibited by metal complexing anions, sulfonamides and their isosteres (sulfamates, sulfamides, etc.), phenols and polyamines,[4] which bind either to the metal ion within the enzyme active site or are anchored to the water molecule coordinated to it.[1–7] The coumarin α-CA inhibition mechanism is different from that of the other classes of inhibitors, and was only recently deciphered.[1–3] A natural product coumarin, 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one **A**, isolated from the Australian plant *Leionema ellipticum*,[1a] was shown to possess significant CA inhibitory activity.[1] By means of X-ray crystallography of its adduct with the human (h) isoform hCA II, it was evidenced the presence of a substituted 2-hydroxy-cinnamic acid in the enzyme active site, which is the hydrolysis product (mediated by the CA) of the original coumarin **A**.[1b] The same situation has been thereafter observed for the simple, unsubstituted coumarin **B**.[2] The 2-hydroxy-cinnamic acids formed from the original coumarins were found to occlude the entrance to the enzyme active site, a mechanism never evidenced before for CA inhibition.[1,2] Thiocoumarins (possessing the endocyclic sulfur atom in position 1 of the heterocyclic ring) were also shown to behave in a similar manner to the coumarins, but this class was scarcely investigated until now, with only one such compound reported to possess CA inhibitory properties,[2] whereas derivatives incorporating the sulfur atom in position 2 (exocyclic sulfur) were not investigated at all up to now as CA inhibitors (CAIs). Several observations emerged during these studies of coumarins/thiocoumarins as CAIs: (i) they bind in hydrolyzed form and do not interact with the catalytically crucial metal ion from the active site cavity, constituting a new category of mechanism-based inhibitors (they act as 'prodrug inhibitors');[1–3] (ii) the substituted-2-hydroxy-cinnamic acids formed from the original coumarin by hydrolysis were observed bound within the active site either as the *cis* isomer,[1b] as well as *trans* isomers[2] (by means of X-ray crystallography of enzyme-inhibitor adducts), depending on the substitution pattern at the original coumarin prodrug; (iii) these inhibitors were observed bound at the entrance of the enzyme active site, plugging its entrance, and blocking thus the catalytic activity of the enzyme.[1,2] An important observations was also that this region of the CA active site is the most variable one among the 16 CA isoforms present in mammals,[3,8,9] and this may explain why many coumarin derivatives show a high selectivity ratio for inhibiting various CA isoforms (e.g., CA VA, VII, IX, XII, XIII and XIV).[1–3] Many of these isoforms have pharmacologic applications for obtaining antiglaucoma, antiobesity, anticonvulsant or antitumor drugs/diagnostic agents.[10]

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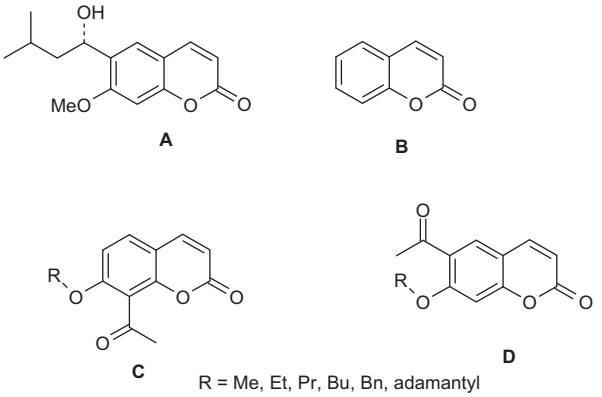


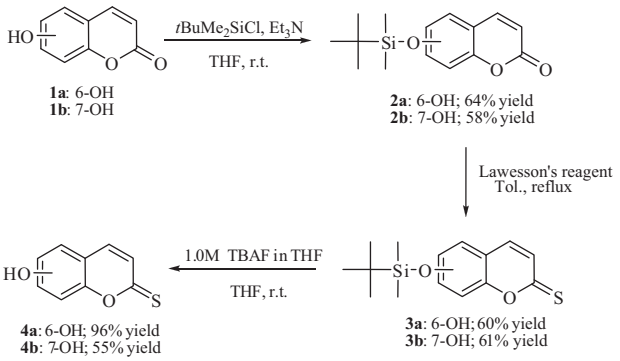
More recently we demonstrated[3] that coumarins incorporating hydroxy and acetylated moieties may lead to interesting CAIs, some of which show high selectivity for inhibiting the tumor-associated isoforms hCA IX and XII.[3,4] For example, for the isomeric 7,8-disubstituted- and 6-7-disubstituted coumarins **C** and **D**, only the first substitution pattern led to highly effective CA IX/XII inhibitors (in the subnanomolar–nanomolar range)[3b] whereas the corresponding isomers **D** were ineffective as CA I, II and XII inhibitors and much less effective (compared to **C**) as hCA IX inhibitors.[3b] Considering that the substitution pattern and the nature (and number) of moieties present in the various positions of the coumarin ring are the main factors influencing CA inhibitory properties as well as selectivity profile in this class of inhibitors, we report here the synthesis and enzyme inhibitory activity of a series of variously substituted coumarins and 2-thioxo-coumarins incorporating silylated, allyl, ether, arylureido and carbamate moieties. The rationale of this study is based on the scarcity of data regarding this type of substitution patterns in various positions of the coumarin ring system. Furthermore, we obtained a rather large number of 2-thioxo-coumarins (not investigated up until now as CAIs) which complete our insights into the structure–activity relationship (SAR) for this class of inhibitors.

2. Results and discussion

2.1. Chemistry

In order to generate chemical diversity for obtaining coumarins/2-thioxo-coumarins with CA inhibitory activity, the 6-/7-hydroxy-substituted coumarins **1a** and **1b** were used as starting materials. These compounds were silylated with *tert*-butyt-dimethylsilyl chloride leading to the key intermediates **2a,b** which were thereafter thionylated at the C2 position by treatment with Lawesson's reagent (Scheme 1).[11] The 2-thioxo-coumarins **3a,b** thus obtained afforded 6-/7-hydroxy-2-thioxo-coumarins **4a,b** by treatment with tetrabutyl ammonium fluoride (TBFA), as shown in Scheme 1.

Alternatively, 6-, 7- or 4-hydroxycoumarins **1a–1c** were transformed to the corresponding allyl ethers **5a–5c** by treatment with allyl bromide in the presence of cesium salts. Coumarins **5a–5c** were then converted to the corresponding 2-thioxo-coumarins incorporating allylether functionalities, **6a–6c**, by reaction with Lawesson's reagent (Scheme 2). Compounds **7–9** were then obtained in order to devise a facile and rapid reaction for incorporating fluorine in the molecule of a coumarin CAI, with the scope of eventually extending this reaction to ^{18}F-containing compounds, which may be useful as PET imaging agents. It has been in fact reported by our group that fluorescent compounds incorporating



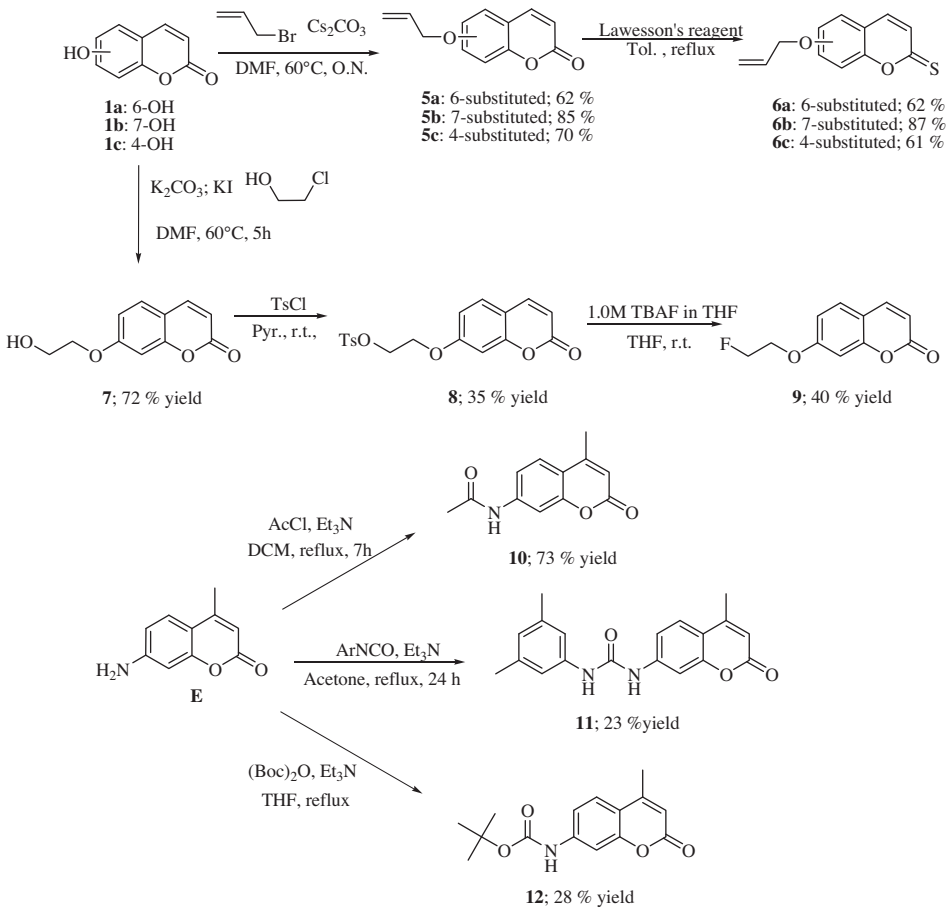
Scheme 1. Preparation of silylated coumarins/2-thioxo-coumarins incorporating 6- and 7-hydroxy moieties.

fluorescein- or near infrared (NIR) tails are highly effective in visualizing hypoxic tumor regions in experimental animals possessing different xenografted tumors.[12,13] Thus, 7-hydroxycoumarin **1b** was treated with chloroethanol in the presence of potassium carbonate and potassium iodide, leading to 7-hydroxyethyloxycoumarin **7**. This compound was tosylated by reaction with tosyl chloride in pyridine leading to the tosylate **8**, which has been converted to 7-(2-fluoroethyloxy)-coumarin **9** by a nucleophilic substitution reaction with TBAF in THF (Scheme 2). Another group of derivatives, compounds **10–12**, were obtained from 4-methyl-7-amino-coumarin **E** (commercially available compound) which was reacted with acetyl chloride, an arylisocyanate (i.e., 3,5-dimethylphenylisocyanate) or Boc anhydride, in order to explore the reactivity of the 7-amino group from coumarin **E** and its propensity to be derivatized for generating chemical diversity for this type of CAIs. Indeed, both the acetamide **10**, the urea derivative **11** and the carbamate **12** were obtained in a one step reaction with an excellent yield (see Section 4). All compounds investigated here were characterized by physico-chemical procedures (^{1}H, ^{13}C and ^{19}F NMR, MS) and elemental analysis which confirmed their structures (see Section 4).

2.2. Carbonic anhydrase inhibition studies

Inhibition data with compounds **2–12** reported here and the simple coumarin **B** as standard, against four CA isozymes, that is, hCA I, II, IX and XII, are shown in Table 1.[14] The following structure–activity relationship (SAR) observations can be drawn from data of Table 1 for these coumarin/2-thioxo-coumarin derivatives:

(i) The cytosolic isoform hCA I (h = human enzyme) was weakly inhibited by the simple, hydroxyl-substituted coumarins **1a–1c** investigated earlier,[3a] as well as by the variously substituted (thioxo)coumarin derivatives **2–12**, with inhibition constants in the range of 7.17–91.7 μM. Aminocoumarins **E** and the parent unsubstituted coumarin **B** were slightly more effective as hCA I inhibitors (K_Is of 3.10–5.56 μM). The most effective hCA I inhibitors (but they are all rather weak inhibitors) were the simple coumarins/2-thioxo-coumarins **2a–4a** incorporating the *tert*-butyl-dimethylsilyl moiety (or without such a substitution) and it may be seen that there is no important difference of activity between the coumarins/2-thioxo-coumarins, and also between the compounds with or without the silyl moiety (compare **3a/3b** with **4a/4b**, for example). Another group of compounds showing a modest hCA I activity was **6a–6c** (K_Is of 7.60–9.24 μM), which are 2-thioxo-coumarins incorporating allyl ether substitutions in various positions of the ring. These compounds were much more effective



Scheme 2. Preparation of coumarin/2-thioxo-coumarin derivatives **5**–**12**.

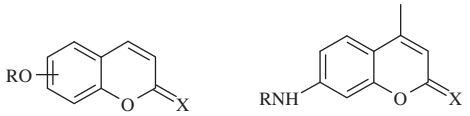
compared to the corresponding coumarins **5a–5c** (K_Is of 30.3–72.9 μM). The 7-substituted derivatives were generally better inhibitors compared to the 6- or 4-substituted compounds bearing the same substituent (Table 1). The remaining substitution patterns, present in derivatives **7–12** led to much less effective hCA I inhibitors (K_Is of 24.4–91.7 μM). In fact, a modest inhibition of this isoform is a desired feature of such CAIs which should normally target selectively the tumor-associated isoforms hCA IX and XII, and should not possess potent hCA I and II inhibitory effects.

(ii) The ubiquitous, offtarget isoforms hCA II was not inhibited by all of the coumarins investigated here (**1–12** and **E**), which showed K_Is in the range of >200 μM. The unsubstituted coumarin **B** was a weak hCA II inhibitor, with a K_I of 9.2 μM, as reported earlier.[1]

(iii) The tumor associated isoform hCA IX was weakly or not at all inhibited by coumarin **B** (K_Is of >200 μM), whereas the compounds investigated here were effective or medium potency inhibitors, with K_Is in the range of 0.19–3.23 μM (Table 1). Thus, the silylated derivatives **2** and **3** as well as the hydroxy-substituted 2-thioxo-coumarins **4a,b** were submicromolar inhibitors with K_Is in the range of 0.78–0.96 μM, but were slightly less effective compared to the parent hydroxylated coumarins from which they were prepared (Table 1) investigated earlier.[3a] The sylilation however did not lead to a strong loss of CA IX inhibitory activity. The transformation of the coumarin to the corresponding 2-thioxo-coumarin, as well as the introduction of the *tert*-butyldimethylsilyl moiety did not markedly influence the CA inhibitory effects of these compounds which all showed a very similar inhibitory behavior towards this isoform. The allyl-substituted coumarins **5a–5c** were also effective hCA IX inhibitors with K_Is in the range of 0.21–0.93 μM. For this substitution pattern, the position of the allylether functionality strongly influenced the inhibitory power, with the 4-substituted compound **5c** being 4.4-times a better inhibitor compared to the 6-substituted derivative **5a**. The 7-substituted compound **5b** showed an intermediate behavior between the two isomers mentioned above (Table 1). Conversion of the allyl-substituted coumarins **5a–5c** to the corresponding 2-thioxo-coumarins **6a–6c** led to an important loss of hCA IX inhibitory effects, the thiocoumarins being an order of magnitude weaker inhibitors compared to the parent coumarins **5a–5c** (K_Is in the range of 3.04–3.26 μM). Compounds **7–9** showed interesting, submicromolar hCA IX inhibitory effects. The best inhibitor was the tosylate **8** (K_I of 0.53 μM), followed by the fluoroderivative **9** (K_I of 0.68 μM), whereas the hydroxyethyl derivative **7** was a slightly weaker inhibitor (K_I of 0.92 μM). All three amino-coumarin derivatives **10–12**, incorporating acetamido, ureido or carbamate functionalities were also effective, submicromolar hCA IX inhibitors (K_I in the range of 0.39-0.97 μM), being much better hCA IX inhibitors compared to the parent, unsubstituted aminocoumarin **E** (K_I of 7.64 μM).

(iv) hCA XII is also present in many tumor types, being like hCA IX a transmembrane isoform, with an extracellular active site, and involved in many physiologic and pathologic processes.[4] It may be observed that again coumarin **B** was ineffective as hCA XII inhibitor (K_I >200 μM), whereas the

Table 1
Inhibition data against hCA I, II, IX and XII with (thio)coumarins **2–12** by a stopped-flow CO_2 hydrase assay (incubation time of enzyme with inhibitor for 6 h)[14]



1-9 **E, 10-12**

No.	X	R	hCA I	K_I[a] (μM)		hCA XII
				hCA II	hCA IX	
1[ab]	O	H	>100	>100	0.19	0.68
1b[b]	O	H	58.4	>100	0.48	0.75
1c[b]	O	H	95.0	>100	0.41	6.30
2a	O	*t*-Bu(Me_2)Si	8.78	>200	0.80	0.28
3a	S	*t*-Bu(Me_2)Si	7.57	>200	0.86	0.31
4a	S	H	7.17	>200	0.80	0.34
2b	O	*t*-Bu(Me_2)Si	8.32	>200	0.85	0.83
3b	S	*t*-Bu(Me_2)Si	8.18	>200	0.96	0.35
4b	S	H	8.02	>200	0.78	0.32
5a	O	CH_2=CH–CH_2	30.3	>200	0.93	0.80
5b	O	CH_2=CH–CH_2	72.9	>200	0.73	0.64
5c	O	CH_2=CH–CH_2	43.2	>200	0.21	0.88
6a	S	CH_2=CH–CH_2	8.51	>200	3.26	1.25
6b	S	CH_2=CH–CH_2	7.60	>200	3.23	2.83
6c	S	CH_2=CH–CH_2	9.24	>200	3.04	1.27
7	O	$HOCH_2CH_2$	24.4	>200	0.92	0.63
8	O	Ts–OCH_2CH_2	66.3	>200	0.53	0.90
9	O	FCH_2CH_2	42.9	>200	0.68	0.59
10	O	Ac	47.7	>200	0.39	0.91
11	O	3,5-$Me_2C_6H_3NHCO$	38.8	>200	0.61	0.64
12	O	*t*-Bu–OCO	91.7	>200	0.97	0.58
E	O	H	5.56	>200	7.64	9.13
Coumarin			3.1	9.2	>200	>200

[a] Mean from 3 assays. Errors were in the range of 5–10% of the reported value (data not sown).
[b] From Ref. 3a.

derivatives **1–12** investigated here were low micromolar or submicromolar inhibitors (K_Is in the range of 0.28–2.83 μM). Again the silylated derivatives **2** and **3** as well as the hydroxy-substituted 2-thioxo-coumarins **4a,b** were submicromolar inhibitors with similar K_Is, in the range of 0.28–0.83 μM, but in this case the sylilation improved the inhibitory power compared to the parent hydroxyl-substituted coumarins investigated earlier[3a] (Table 1). As for the case of hCA IX inhibition discussed above, the transformation of the coumarin to the corresponding 2-thioxo-coumarin, as well as the introduction of the *tert*-butyl-dimethylsilyl moiety did not influence significantly the CA XII inhibitory effects of these compounds. For the allyl-substituted coumarins/2-thioxo-coumarins **5** and **6**, the first group of derivatives led to effective submicromolar inhibitors (K_Is in the range of 0.64–0.88 μM) whereas the 2-thioxo-coumarins were less effective (K_Is in the range of 1.25–2.83 μM) (Table 1). The remaining compounds **7–12**, incorporating functionalities derived from the hydroxyethyloxy derivative **7** or the 7-aminocoumarin **E**, were again quite effective as hCA XII inhibitors, with K_Is in the range of 0.58–0.91 μM. As for hCA IX, aminocoumarin **E** was a much weaker hCA XII inhibitor compared to its derivatives **10–12**.

3. Conclusion

We extend our earlier investigations on coumarins as CAIs.[1–3] We report here a series of coumarins incorporating *tert*-butyl-dimethylsilyloxy- or allyoxy- moieties in positions 4-, 6 or 7 of the heterocyclic ring, which have been obtained by a simple reaction step and then converted to the corresponding 2-thioxo-coumarins, a class of not investigated CAIs up until now. Other coumarin derivatives incorporating hydroxyethyloxy-, tosylethoxy- and 2-fluroethyloxy- moieties in position 7 of the coumarin ring were synthesized in order to obtain compounds which may be labeled with radioactive fluorine, for PET applications. Furthermore, derivatives of 4-methyl-7-amino coumarin incorporating acetamido, 3,5-dimethylphenylureido- and *tert*-butyloxycarbonylamido functionalities have also bee prepared from 7-aminocoumarin. All these compounds have been assayed as inhibitors of several CA isoforms with medicinal chemistry applications. The cytosolic isoforms hCA I and II were weakly inhibited (hCA I) or not inhibited at all (hCA II) by these (thio)coumarins whereas the tumor-associated transmembrane isoforms hCA IX and XII were inhibited with efficiencies from the submicromolar to the low micromolar range by many of these derivatives. The structure–activity relationship of these classes of less investigated CA inhibitors are delineated, with the potential of using them as leads to obtain isoform selective inhibitors with excellent affinity for CA IX and XII (validated antitumor targets) which do not significantly inhibit the cytosolic offtarget isoforms hCA I and II.

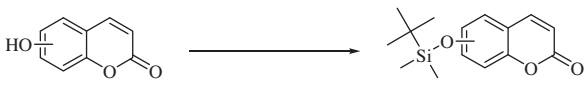
4. Experimental

4.1. Chemistry

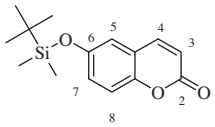
^1H, ^{13}C, DEPT, COSY, HMQC and HMBC spectra were recorded using a Bruker Advance III 400 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). For all new compounds DEPT, COSY, HMQC and HMBC were routinely used to definitely assign the signals of ^1H and ^{13}C. Infrared spectra were recorded on a Perkin Elmer Spectrum R XI spectrometer as solids on KBr plates. 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} aluminium backed plates. Elution of the plates was carried out using ethyl acetate/*n*-hexane or MeOH/DCM systems. Visualization was achieved with UV light at 254 nm, by dipping into a 0.5% aqueous potassium permanganate solution, by Hanessian's Stain solution and heating with a hot air gun or by exposure to iodine.

Coumarins **1a–1c** and **B**, **E**, solvents and other chemicals were used as supplied from Aldrich Chemical Co., Acros, Fisher, Alfa Aesar or Lancaster Synthesis.

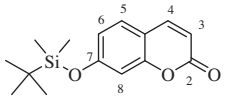
4.1.1. Synthesis of 6-(*tert*-butyldimethylsilyloxy)-2*H*-chromen-2-one (2a) and 7-(*tert*-butyldimethylsilyloxy)-2*H*-chromen-2-one (2b)



A solution of 6-hydroxy-2*H*-chromen-2-one or 7-hydroxy-2*H*-chromen-2-one (0.5 g, 1.0 equiv) was treated at rt with *tert*-butyldimethylsilyl chloride (1.1 equiv) and Et_3N (1.0 equiv) in THF. The reaction was stirred at rt until starting material was consumed (TLC monitoring) then quenched with H_2O (40 ml) and extracted with ethyl acetate (3 × 15 ml). The combined organic layers were washed with H_2O (2 × 20 ml), dried over Na_2SO_4, filtered-off and concentrated under vacuo to give a residue that was purified by silica gel column chromatography eluting with 20% ethyl acetate/*n*-hexane *v/v*.

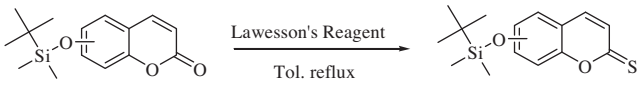


4.1.1.1. 6-(*tert*-Butyldimethylsilyloxy)-2*H*-chromen-2-one (2a). Yield 64% yield; δ_H (400 MHz, DMSO-d_6) 0.25 (6H, s, –Si-(CH_3)$_2$), 1.00 (9H, s, –Si-C(CH_3)$_3$), 6.51 (1H, d *J* 9.6, 3-H), 7.13 (1H, dd, *J* 9.4, 2.4, 7-H), 7.25 (1H, d, *J* 2.4, 5-H), 7.33 (1H, d, *J* 9.4, 8-H), 8.00 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 161.0 (*C*=O), 152.2, 149.2, 144.8, 124.9, 120.3, 118.6, 118.3, 117.4, 26.4, 18.8, −3.8.

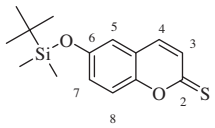


4.1.1.2. 7-(*tert*-Butyldimethylsilyloxy)-2*H*-chromen-2-one (2b). Yield 58% yield; δ_H (400 MHz, DMSO-d_6) 0.29 (6H, s, –Si-(CH_3)$_2$), 1.00 (9H, s, –Si-C(CH_3)$_3$), 6.34 (1H, d *J* 9.6, 3-H), 6.88 (1H, dd, *J* 9.4, 2.4, 6-H), 6.92 (1H, d, *J* 2.4, 8-H), 7.65 (1H, d, *J* 9.4, 5-H), 8.04 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 162.2 (*C*=O), 156.4, 145.4, 130.6, 118.1, 114.2, 112.3, 107.9, 103.1, 26.7, 18.7, −2.3.

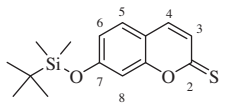
4.1.2. Synthesis of 6-(*tert*-butyldimethylsilyloxy)-2*H*-chromene-2-thione (3a) and 7-(*tert*-butyldimethylsilyloxy)-2*H*-chromene-2-thione (3b)



6-(*tert*-Butyldimethylsilyloxy)-2*H*-chromen-2-one (**2a**) or 7-(*tert*-butyldimethylsilyloxy)-2*H*-chromen-2-one (**2b**) (0.5 g, 1.0 equiv) was dissolved in dry toluene (20 ml) and treated with Lawesson's reagents (1.5 equiv) at reflux for 3 h. The mixture was cooled down to rt, solvent was removed under vacuo and the residue was partitioned between H_2O and ethyl acetate. The organic layer was washed with H_20 (3 × 15 ml), dried over Na_2SO_4, filtered and concentrated in vacuo o give a residue that was purified by silica gel column chromatography eluting with 20% ethyl acetate/*n*-hexane *v*/*v*.



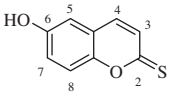
4.1.2.1. 6-(*tert*-Butyldimethylsilyloxy)-2*H*-chromene-2-thione (3a). Yield 60% yield; silica gel TLC R_f 0.40 (ethyl acetate/*n*-hexane 20% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 0.27 (6H, s, –Si-(CH_3)$_2$), 1.01 (9H, s, –Si-C(CH_3)$_3$), 7.25 (1H, dd *J* 9.2, 2.8, 7-H), 7.29 (1H, d, *J* 9.6, 3-H), 7.32 (1H, d, *J* 2.8, 5-H), 7.55 (1H, d, *J* 9.2, 8-H), 7.90 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 198.0 (*C*=S), 153.3, 152.2, 136.8, 130.1, 126.0, 122.2, 118.4, 118.3, 26.4, 18.8, −3.8.



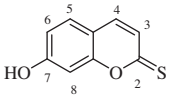
4.1.2.2. 7-(*tert*-Butyldimethylsilyloxy)-2*H*-chromene-2-thione (3b). Yield 61% yield; silica gel TLC R_f 0.38 (ethyl acetate/*n*-hexane 20% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 0.31 (6H, s, –Si-(CH_3)$_2$), 1.00 (9H, s, –Si-C(CH_3)$_3$), 7.01 (1H, dd *J* 9.2, 2.8, 6-H), 7.03 (1H, d, *J* 2.8, 8-H), 7.17 (1H, d, *J* 9.6, 3-H), 7.76 (1H, d, *J* 9.2, 5-H), 7.90 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 198.2 (*C*=S), 159.0, 158.2, 137.2, 130.9, 127.5, 126.1, 119.8, 115.9, 26.3, 18.9, −3.8.

4.1.3. Synthesis of 6-hydroxy-2*H*-chromene-2-thione (4a) and synthesis of 7-hydroxy-2*H*-chromene-2-thione (4b)

6-(*tert*-Butyldimethylsilyloxy)-2*H*-chromene-2-thione (**3a**) or 7-(*tert*-butyldimethylsilyloxy)-2*H*-chromene-2-thione (**3b**) (0.3 g, 1.0 equiv) was dissolved in THF (2.0 ml) and treated at rt with TBAF 1.0 M in THF (1.1 equiv). The reaction was stirred at rt until starting material was consumed (TLC monitoring) and then was quenched with 3.0 M aqueous hydrochloric acid, extracted with ethyl acetate (3 × 15 ml). The combined organic layers were washed with H_2O (3 × 20 ml), brine (3 × 20 ml) dried over Na_2SO_4, filtered, concentrated under vacuo to give a residue that was purified by silica gel column chromatography eluting with 50% ethyl acetate/*n*-hexane *v*/*v*.



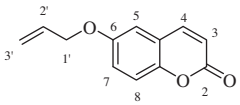
4.1.3.1. 6-Hydroxy-2*H*-chromene-2-thione (4a). Yield 96% yield; silica gel TLC R_f 0.35 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 7.12 (1H, d *J* 2.8, 5-H), 7.18 (1H, dd, *J* 9.2, 2.8, 7-H), 7.25 (1H, d, *J* 9.6, 3-H), 7.50 (1H, d, *J* 9.2, 8-H), 7.87 (1H, d, *J* 9.6, 4-H), 10.05 (1H, br s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 197.8 (*C*=S), 155.8, 151.1, 137.0, 129.9, 122.1, 121.9, 118.2, 112.9.



4.1.3.2. 6-Hydroxy-2*H*-chromene-2-thione (4b). Yield 55% yield; silica gel TLC R_f 0.40 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 6.93 (2H, m, 6-H, 8-H), 7.09 (1H, d, *J* 9.6, 3-H), 7.68 (1H, d, *J* 9.2, 5-H), 7.85 (1H, d, *J* 9.6, 4-H), 10.96 (1H, br s, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6) 198.1 (*C*=S), 163.3, 159.0, 137.8, 130.9, 126.0, 115.9, 114.1, 102.8.

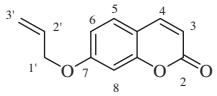
4.1.4. Synthesis of allyloxycoumarines 5a–c

Hydroxycoumarin (1.0 g, 1.0 equiv), Cs_2CO_3 (3.0 equiv) and allylbromide (3.0 equiv) were dissolved in dry DMF (30 ml) and the mixture was stirred at 60 °C O.N. The reaction was quenched with slush and extracted with DCM (3 × 20 ml). The combined organic layers were washed with brine (3 × 20 ml), H_2O (5 × 20 ml), dried over Na_2SO_4, filtered and concentrated under vacuo to give a residue that was crystallized from MeOH/H_2O.

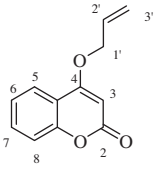


4.1.4.1. 6-(Allyloxy)-2*H*-chromen-2-one yield (5a). 62% yield; δ_H (400 MHz, DMSO-d_6) 4.64 (2H, d *J* 8.0, 1′-H_2), 5.32 (1H, dd, *J* 13.2, 4.8, 3′-H*H*), 5.48 (1H, dd, *J* 15.6, 4.8, 3′-*H*H), 6.10 (1H, m, 2′-H), 6.16 (1H, d, *J* 9.6, 3-H), 7.25 (1H, dd, *J* 9.2, 2.4, 7-H), 7.41 (1H, d, *J*

2.4, 5-H), 7.36 (1H, d, *J* 9.2, 8-H), 8.03 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 161.0 (C=O), 155.4, 148.8, 144.9, 134.3, 120.8, 120.1, 118.7, 118.3, 117.5, 112.7, 69.7.



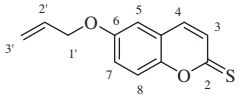
4.1.4.2. 7-(Allyloxy)-2*H*-chromen-2-one yield (5b). 85% yield; δ_H (400 MHz, DMSO-d_6) 4.73 (2H, d *J* 8.0, 1′-H_2), 5.32 (1H, dd, *J* 13.2, 4.8, 3′-H*H*), 5.45 (1H, dd, *J* 15.6, 4.8, 3′-*H*H), 6.09 (1H, m, 2′-H), 6.33 (1H, d, *J* 9.6, 3-H), 7.00 (1H, dd, *J* 9.2, 2.4, 6-H), 7.05 (1H, d, *J* 2.4, 8-H), 7.67 (1H, d, *J* 9.2, 5-H), 8.03 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 162.0 (C=O), 155.0, 148.3, 144.2, 135.1, 119.2, 118.7, 118.5, 118.0, 117.2, 112.6, 69.5.



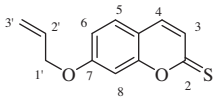
4.1.4.3. 4-(Allyloxy)-2*H*-chromen-2-one yield (5c). 70% yield; δ_H (400 MHz, DMSO-d_6) 4.87 (2H, d *J* 8.0, 1′-H_2), 5.40 (1H, dd, *J* 13.2, 4.8, 3′-H*H*), 5.59 (1H, dd, *J* 15.6, 4.8, 3′-*H*H), 5.96 (1H, s, 3-H), 6.15 (1H, m, 2′-H), 7.41 (1H, m, 7-H, 8-H), 7.22 (1H, dd, *J* 8.8, 8.4, 6-H), 7.89 (1H, d, *J* 8.8, 5-H); δ_C (100 MHz, DMSO-d_6) 165.4 (C=O), 162.5, 153.7, 133.7, 132.6, 125.2, 123.8, 119.7, 117.4, 116.1, 92.0, 70.7.

4.1.5. Synthesis of allyloxy-2*H*-chromene-2-thiones 6a–c

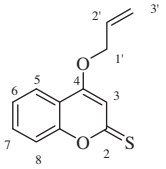
The proper allyloxycoumarine **5**a-c (1.0 equiv) was dissolved in dry toluene and treated with Lawesson's reagent (2.0 equiv). The reaction mixture was refluxed until consumption of the starting material (TLC monitoring). Then solvent was removed in vacuo and the residue obtained was purified by silica gel column chromatography eluting with ethyl acetate in *n*-hexane to afford the corresponding thione.



4.1.5.1. 6-(Allyloxy)-2*H*-chromene-2-thione (6a). 62% yield; δ_H (400 MHz, DMSO-d_6) 4.68 (2H, d *J* 8.0, 1′-H_2), 5.32 (1H, dd, *J* 13.2, 4.8, 3′-H*H*), 5.49 (1H, dd, *J* 15.6, 4.8, 3′-*H*H), 6.09 (1H, m, 2′-H), 7.30 (1H, d, *J* 9.6, 3-H), 7.36 (1H, dd, *J* 9.2, 2.4, 7-H), 7.38 (1H, d, *J* 2.4, 5-H), 7.59 (1H, d, *J* 9.2, 8-H), 7.89 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 197.9 (C=S), 156.3, 151.9, 145.7, 136.8, 134.0, 130.2, 121.9, 118.8, 118.4, 112.1, 69.8.



4.1.5.2. 7-(Allyloxy)-2*H*-chromene-2-thione 6b. 87% yield; silica gel TLC R_f 0.32 (ethyl Acetate/*n*-hexane 20% *v*/*v*); ν_{max} (KBr) cm^{-1} 1760, 1519, 1215; δ_H (400 MHz, DMSO-d_6) 4.77 (2H, dt, *J* 5.6, 1.6, 1′-H_2), 5.35 (1H, dq, *J* 12.0, 1.6, 3′-H*H*), 5.47 (1H, dq, *J* 17.2, 1.6, 3′-*H*H), 6.11 (1H, m, 2′-H), 7.11 (1H, dd, *J* 8.8, 2.4, 6-H), 7.12 (1H, d, *J* 9.2, 3-H), 7.27 (1H, d, *J* 2.4, 8-H), 7.77 (1H, d, *J* 8.8, 5-H), 7.88 (1H, d, *J* 9.2, 4-H); δc (100 MHz, DMSO-d_6), 198.2 (C=S), 162.9, 158.9, 137.5, 133.7, 130.6, 127.1, 119.2, 115.8, 115.2, 102.0, 70.0; Anal. Calcd C, 66.03; H, 4.62; S, 14.69. Found: C, 66.56; H, 4.32; S, 14.68.



4.1.5.3. 4-(Allyloxy)-2*H*-chromene-2-thione (6c). 61% yield; δ_H (400 MHz, DMSO-d_6) 4.95 (2H, d *J* 6.0, 1′-H_2), 5.42 (1H, dd, *J* 13.2, 4.8, 3′-H*H*), 5.59 (1H, dd, *J* 15.6, 4.8, 3′-*H*H), 6.15 (1H, m, 2′-H), 6.97 (1H, s, 3-H), 7.51 (1H, t, *J* 8.8, 7-H), 7.63 (1H, d, *J* 8.8, 8-H), 7.80 (1H, t, *J* 8.8, 6-H), 7.96 (1H, d, *J* 8.8, 5-H); δ_C (100 MHz, DMSO-d_6) 198.5 (C=S), 160.9, 157.2, 134.4, 132.5, 126.6, 123.8, 119.9, 117.5, 117.2, 107.6, 71.2.

4.1.6. Synthesis of 7-(2′-hydroxyethoxy)-2*H*-chromen-2-one (7)

A mixture of 7-hydroxy-2*H*-chromen-2-one (0.5 g, 1.0 equiv), K_2CO_3 (5.0 equiv), K_I (1.0 equiv) and chloroethanol (1.0 equiv) in DMF dry (10 ml) was stirred at 60 °C for 5 h. The reaction mixture was cooled down to 0 °C, quenched with 6 M aqueous hydrochloric acid (50 ml) and extracted with ethyl acetate (3 × 20 ml). The combined organic layers were washed several timed with H_2O, dried over Na_2SO_4, filtered-off and concentrated under vacuo to afford a residue that was purified by silica gel column chromatography eluting with 50% ethyl acetate/ *n*-hexane *v*/*v*.

4.1.6.1. 7-(2′-Hydroxyethoxy)-2*H*-chromen-2-one (7). 72% yield; silica gel TLC R_f 0.10 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 3.78 (2H, m, 2′-H_2), 4.13 (2H, m, 1′-H_2), 4.97 (1H, t, *J* 5.6, exchange with D_2O, O-*H*), 6.30 (1H, d, *J* 9.6, 3-H), 6.97 (1H, dd, *J* 9.2, 2.4, 6-H), 7.02 (1H, d, *J* 2.4, 8-H), 7.65 (1H, d, *J* 9.2, 5-H), 8.01 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 162.8, 161.3, 156.3, 145.3, 130.4, 113.7, 113.4, 102.1, 71.3, 65.9, 60.3.

4.1.7. Synthesis of 2′-(2-oxo-2*H*-chromen-7-yloxy)ethyl 4″-methylbenzenesulfonate (8)

7-(2′-Hydroxyethoxy)-2*H*-chromen-2-one (**7**) (0.2 g, 1.0 equiv) was dissolved in dry pyridine (5 ml) and treated at 0 °C with TsCl (1.1 equiv). The yellow solution was stirred at rt until starting material was consumed (TLC monitoring) and then quenched with a 1.0 M aqueous hydrochloric acid at 0 °C. The mixture was extracted with ethyl acetate (3 × 15 ml) and the combined organic layers were washed with brine (3 × 20 ml), H_2O (3 × 20 ml) dried over Na_2SO_4, filtered-off and concentrated under vacuo to afford a residue that was purified by silica gel column chromatography eluting with 50% ethyl acetate/ *n*-hexane *v*/*v*.

4.1.7.1. 2′-(2-Oxo-2*H*-chromen-7-yloxy)ethyl 4″-methylbenzenesulfonate (8). 35% yield; silica gel TLC R_f 0.36 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 2.44 (3H, s, CH_3). 4.32 (2H, m, 1′-H_2), 4.41 (2H, m, 2′-H_2), 6.34 (1H, d, *J* 9.6, 3-H), 6.87 (1H, dd, *J* 9.2, 2.4, 6-H), 6.92 (1H, d, *J* 2.4, 8-H), 7.49 (2H, d *J* 8.4, 2 × 2″-H/3″-H), 7.64 (1H, d, *J* 9.2, 5-H), 7.83 (2H, d *J* 8.4, 2 × 2″-H/3″-H), 8.02 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 161.7, 161.1, 156.1, 145.9, 145.1, 133.1, 131.0, 130.4, 128.6, 113.7, 113.6, 113.5, 102.3, 69.7, 66.9, 21.9.

4.1.8. Synthesis of 7-(2′-fluoroethoxy)-2*H*-chromen-2-one (9)

2′-(2-Oxo-2*H*-chromen-7-yloxy)ethyl 4″-methylbenzenesulfonate (**8**) (0.1 g, 1.0 equiv) was dissolved in THF(1.0 ml) and treated

with TBAF 1.0 M in THF (1.05 equiv). The yellow solution was stirred at rt for 15 min. Then solvents were removed in vacuo and the residue was purified by silica gel column chromatography eluting with 50% ethyl acetate/ *n*-hexane *v*/*v*.

4.1.8.1. 7-(2′-Fluoroethoxy)-2*H*-chromen-2-one (9). 40% yield; silica gel TLC R_f 0.40 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 4.36 (1H, m, 1′-H*H*), 4.44 (1H, m, 1′-*H*H), 4.74 (1H, m, 1′-*H*H), 4.87 (1H, m, 1′-H*H*), 6.34 (1H, d, *J* 9.6, 3-H), 7.02 (1H, dd, *J* 9.2, 2.4, 6-H), 7.09 (1H, d, *J* 2.4, 8-H), 7.68 (1H, d, *J* 9.2, 5-H), 8.03 (1H, d, *J* 9.6, 4-H); δ_C (100 MHz, DMSO-d_6) 162.2, 161.1, 145.2, 130.5, 113.63, 113.60, 113.5, 102.3, 82.0 (d, $^1J_{C\text{-}F}$ 166, C-2′), 68.6 (d, $^2J_{C\text{-}F}$ 18, C-1′), δ_F (376 MHz, DMSO-d_6) –222.23 (1F, s).

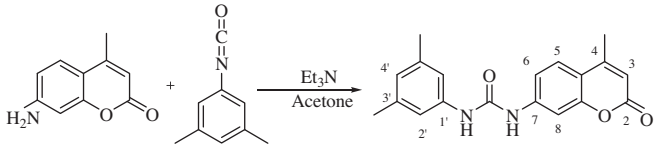
4.1.9. Synthesis of *N*-(4-methyl-2-oxo-2*H*-chromen-7-yl)acetamide (10)

10

A suspension of 7-amino-4-methyl-2*H*-chromen-2-one (0.1 g, 1.0 equiv) in DCM dry (5.0 ml) was treated with acetyl chloride (1.0 equiv) and Et_3N (1.0 equiv) under reflux for 7 h. Solvents were removed under vacuo and the residue was purified by silica gel column chromatography eluting with 50% ethyl acetate/ *n*-hexane *v*/*v*.

4.1.9.1. *N*-(4-Methyl-2-oxo-2*H*-chromen-7-yl)acetamide (10). 73% yield; silica gel TLC R_f 0.11 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 2.14 (3H, s, 1′-C*H*$_3$), 2.43 (3H, s, 4-C*H*$_3$), 6.29 (1H, s, 3-H), 7.50 (1H, dd, *J* 9.2, 2.4, 6-H), 7.74 (1H, d, *J* 9.2, 5-H), 7.79 (1H, d, *J* 2.4, 8-H), 10.40 (1H, br s, exchange with D_2O, N-*H*); δ_C (100 MHz, DMSO-d_6) 170.0, 161.0, 154.6, 154.0, 143.5, 126.8, 115.9, 115.7, 113.0, 106.3, 25.1, 18.9.

4.1.10. Synthesis of 1-(3′,5′-dimethylphenyl)-3-(4-methyl-2-oxo-2*H*-chromen-7-yl)urea (11)

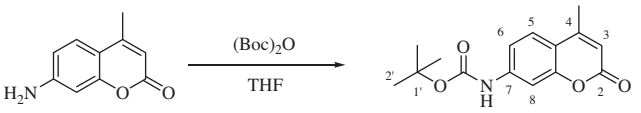


E 11

7-amino-4-methyl-2*H*-chromen-2-one (0.1 g, 1.0 equiv) in acetone (10 ml) was treated at reflux with 3,5-dimethyyl isocyanate (1.0 equiv) and Et_3N (1.1 equiv) for 24 h. Then the solvents were removed in vacuo and the residue was purified by silica gel column chromatography eluting with 50% ethyl acetate/ *n*-hexane *v*/*v*.

4.1.10.1. 1-(3′,5′-Dimethylphenyl)-3-(4-methyl-2-oxo-2*H*-chromen-7-yl)urea (11). 23% yield; silica gel TLC R_f 0.22 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 2.28 (6H, s, 2 × 3′-C*H*$_3$), 2.43 (3H, s, 4-C*H*$_3$), 6.25 (1H, s, 3-H), 6.69 (1H, s, 4′-H), 7.13 (2H, s, 2 × 2′-H), 7.39 (1H, dd, *J* 9.2, 2.4, 6-H), 7.65 (1H, d, *J* 2.4, 8-H), 7.71 (1H, d, *J* 9.2, 5-H), 8.72 (1H, s, exchange with D_2O, N-*H*), 9.20 (1H, s, exchange with D_2O, N-*H*); δ_C (100 MHz, DMSO-d_6) 161.1, 254.2, 153.4, 144.5, 140.6, 140.0, 138.9, 138.6, 126.9, 124.9, 124.3, 117.3, 116.8, 115.3, 22.1, 19.0.

4.1.11. Synthesis of *tert*-butyl 4-methyl-2-oxo-2*H*-chromen-7-ylcarbamate (12)



E 12

A suspension of 7-amino-4-methyl-2*H*-chromen-2-one (0.1 g, 1.0 equiv) in THF dry (2.0 ml) was treated at reflux with di-*tert*-butyl dicarbonate (1.0 equiv) and Et_3N (1.1 equiv) for 24 h. Then the solvents were removed in vacuo and the residue was purified by silica gel column chromatography eluting with 50% ethyl acetate/ *n*-hexane *v*/*v*.

4.1.11.1. *tert*-Butyl 4-methyl-2-oxo-2*H*-chromen-7-ylcarbamate (12). 28% yield; silica gel TLC R_f 0.42 (ethyl acetate/*n*-hexane 50% *v*/*v*); δ_H (400 MHz, DMSO-d_6) 1.54 (9H, s, 3 × 2′-C*H*$_3$), 2.42 (3H, s, 4-C*H*$_3$), 6.26 (1H, s, 3-H), 7.44 (1H, dd, *J* 9.2, 2.4, 6-H), 7.57 (1H, d, *J* 2.4, 8-H), 7.70 (1H, d, *J* 9.2, 5-H), 9.92 (1H, s, exchange with D_2O, N-*H*); δ_C (100 MHz, DMSO-d_6) 161.0, 154.8, 154.2, 153.4, 144.1, 126.8, 115.1. 113.0, 105.2. 80.9, 28.9, 27.8, 18.9.

4.2. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[14] 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 assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min–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 coumarin and formation of the 2-hydroxy-cinnamic acids. 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 isoforms were recombinant ones obtained in house as reported earlier.[2,3]

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