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Click-tailed coumarins with potent and selective inhibitory action against the tumor-associated carbonic anhydrases IX and XII

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ABSTRACT

Coumarins behave as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) with a mechanism of inhibition distinct from other classes of inhibitors. A series of 7-substituted coumarins incorporating aryl-triazole moieties were prepared by click chemistry procedures starting from 7-hydroxycoumarin or 4-methyl-7-aminocoumarin. The panel of new compounds was assayed for the inhibition of the cytosolic, widespread human (h) isoforms hCA I and II, and the transmembrane, tumor-associated ones hCA IX and XII. Most of the coumarins were weak inhibitors or did not inhibit significantly hCA I and II, but showed low nanomolar inhibitory action against the transmembrane isoforms (K_I of 14.3–34.4 nM against hCA IX and of 4.7–37.8 nM against hCA XII). Since many hypoxic tumors overexpress hCA IX/XII, and as these targets were recently validated for obtaining antitumor/antimetastatic agents, with one inhibitor in Phase I clinical trials, the present findings constitute an interesting extension to the knowledge of non-sulfonamide, selective inhibitors of CA isoforms involved in serious pathologies.

1. Introduction

The coumarins are a relatively new class[1] of inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).[2–4] Discovered initially in a library of natural products (the first coumarin CA inhibitor (CAI) was isolated from the Australian plant *Leionema elipticum*),[1] a large number of synthetic and natural product coumarins were investigated as inhibitors[5–9] of a large number of isoforms of the 15 presently described in humans, hCA I–hCA XIV (there are two V-type isoforms, hCA VA and hCA VB).[2,10,11]

Coumarins are an important class of CAIs for several reasons: (i) they were the first CAIs showing a high selectivity for inhibiting CA isoforms of interest for pharmacologic applications, such as the tumor-associated ones (hCA IX and XII, which are targets for anti-tumor/antimetastatic drugs)[12,13] or the mitochondrial ones (CA VA and VB, which are targets for anti-obesity agents).[14,15] Furthermore, their selectivity for the target isoforms (e.g., CA IX, XII, VA, VB, etc.) over the ubiquitous ones (such as CA I and II) was explained from the structural viewpoint, after the report of several X-ray crystal structures of adducts of some coumarins bound to hCA II.[1b,2] In fact the coumarins act as prodrug inhibitors, being hydrolysed by the esterase activity of the enzyme to 2-hydroxycinnamic acid derivatives, which bind at the entrance of the active site cavity, thus obstructing it.[1b,2] As this is the most variable part of the active site among the various hCAs,[2–4] this particular binding mode explains the very high selectivity of many coumarin derivatives for inhibiting isoforms of interest, as those mentioned above, whereas their inhibitory action against the house-keeping enzymes hCA I and II is generally low or insignificant.[1,5–11](ii) The chemical simplicity and possibility to explore various facile synthetic approaches for this ring system, allows for the possibility to generate a large number of structurally diverse coumarin derivatives easily, which may lead thereafter to interesting applications. For example some 6-glycosyl-substituted coumarins reported earlier by our group[7a] showed selective inhibitory action against hCA IX and XII (over hCA I and II), and excellent in vivo antitumor/antimetastatic activity, in an animal model of the disease.[7a,12a] Actually a rather large number of substitution patterns at the coumarin ring and a variety of diverse substituents have been investigated in order to obtain CAIs with desired physico-chemical and pharmacologic properties.[1,5–11](iii) Considering the coumarins as lead compounds, a large number of structurally diverse classes of CAIs were obtained, among which the sulfocoumarins, the thio-coumarins, thioxo-coumarins, the coumarin oximes, as well as mono-cyclic 5- and 6-membered lactones/thiolactones.[8–11]Similar to the parent compounds, most of these new classes of CAIs act as prodrug inhibitors, being hydrolysed within the enzyme active site to derivative which thereafter inhibit the enzyme.[8–11]

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0968-0896/

Although a large number of synthetic approaches were reported to date for derivatizing coumarins in order to obtain CAIs, the click chemistry has been not investigated in detail for this class of compounds (although many sulfocoumarins incorporating a variety of substitution patterns were reported by using this cycloaddition reaction).[10,11] Here we report a series of 7-substituted coumarins obtained by using click chemistry, which showed excellent CA IX/XII inhibitory activity as well as selectivity for the inhibition of the tumor-associated isoforms over the cytosolic ones CA I and II.

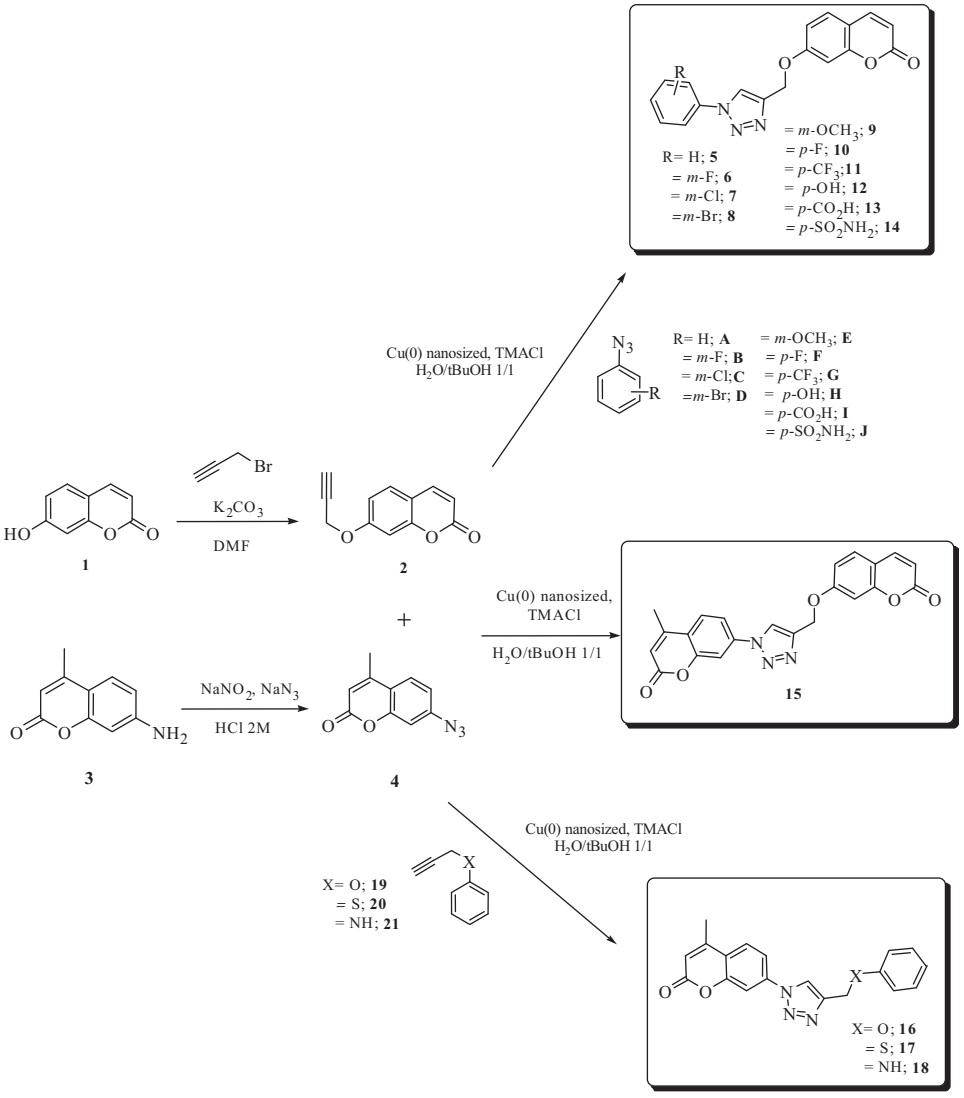
2. Results and discussion

2.1. Chemistry

The rationale of this work was the following one: click chemistry is a versatile and facile methodology for generating chemical space, which was successfully used for obtaining interesting sulfonamide and sulfamate CAIs.[16,17] Here we apply this methodology for obtaining 7-substituted coumarins, since this reaction was not used up until now for obtaining CAIs belonging to the coumarin class.

Starting from 7-hydroxy-coumarin **1**, the propargyl ether key intermediate **2** was obtained as an alkyne for the click chemistry. Reaction of **2** with aromatic azides **A–J** afforded a first series of triazoles, **5–14**, incorporating the coumarin ring and diverse aryl groups at the triazole moiety (Scheme 1).4-Methyl-7-amino-coumarin **3** was transformed into the corresponding azide **4** by diazotization followed by reaction with sodium azide, and was subsequently used for cycloaddition reactions with alkyne **2** (leading to a bis-coumarin derivative, **15**) or propargyl derivatives **19–21** (obtained from phenol, thiophenol or aniline, and propargyl bromide), leading to derivatives **16–18** (Scheme 1).

The rationale for derivatizing the 7-hydroxy-/amino coumarins was furnished by our earlier work in which we showed that coumarins incorporating various functionalities in this position lead to effective and isoform-selective CAIs.[1,5–7] Thus, we decided to incorporate substituted-aromatic moieties either directly attached to the triazole ring (as in **5–14**) or via a CH_2X linker (as in **16–18**, in which X = O, S or NH). For the first sub-group of derivatives (**5–14**), the aryl azides used incorporated various 3- or 4-substituted groups (halogens, OH, OMe, carboxyl, sulfamoyl, etc.) in order to delineate the structure–activity relationship (SAR) for the inhibition of various CA isoforms with the derivatives reported here.



Scheme 1. General synthetic scheme of compounds **5–18**.

2.2. Carbonic anhydrase inhibition

Coumarins **5**–**18** were screened for the inhibition of four human (h) CA isoforms involved in important physiologic/pathologic processes, that is, the cytosolic, hCA I and II (offtargets in this case) and the transmembrane, tumor-associated hCA IX and XII (anticancer drug targets).[2,3,12,13] Table 1 shows inhibition data of coumarins **5**–**18** and the sulfonamide acetazolamide **AAZ** (as standard inhibitor) against hCA I, II, IX and XII, after a period of 6 h of incubation of the enzyme and inhibitor solutions.[18–21] It should be mentioned that assaying the inhibition with the usual 15 min incubation period (as for the sulfonamides)[1,5] leads to the measurement of a very weak inhibition (data not shown). For this reason, a 6 h incubation time has been used for assaying all coumarins as CAIs.[1,5–11]The following SAR should be noted regarding the inhibition data of Table 1.(i) Isoform hCA I was moderately or poorly inhibited by coumarins **5**–**18** investigated here. Six derivatives (**9**, **11**–**14** and **18**) showed K_Is in the range of 172.8–246.7 nM (the same range as the sulfonamide acetazolamide, **AAZ**) whereas the remaining ones were weaker, micromolar inhibitors (**6**, **10**, **16** and **17**) or did not inhibit significantly the enzyme up until 10 μM (Table 1). The compounds showing some inhibitory activity, had the following substitution patterns at the aryl moiety linked to the triazole ring: 3-methoxyphenyl, 4-trifluoromethylphenyl, 4-hydroxy-/carboxy-and sulfamoyl-phenyl.(ii) Isoform II, the physiologically dominant one (as it is present in virtually all cells, being particularly abundant in blood red cells, gastro-intestinal tract, lungs and kidneys among others) 2–4 was poorly inhibited by the new coumarins reported here except the carboxylate derivative 13 which showed K_I of 99.6 nM. The other derivatives did not inhibit significantly the enzyme up until 10 μM (Table 1). Carboxylates may have a multitude of inhibition mechanisms towards CAs, as the COO^- moiety may be a zinc-binding group, it may anchor to the zinc-coordinated water molecule or even promotes binding outside the active site.[19–22](iii) The transmembrane isoform hCA IX was effectively inhibited by all coumarins reported here, with K_Is in the range of 14.3–34.4 nM. It may be observed that the range of the inhibitory power is very small, meaning that all the substitution patterns explored in the paper lead to highly effective CAIs (in fact **AAZ** shows an inhibition of 25 nM against this isoform and it has been shown by Williams' group to show potent antitumor activity in vivo).[12d]

(iv) A similar situation to what mentioned above for hCA IX has also been observed for the inhibition of hCA XII with the coumarins investigated here, which showed highly effective inhibitory action, with K_Is in the range of 4.7–37.8 nM. Only the bis-coumarin 15 was slightly less inhibitory (K_I of 37.8 nM) whereas the remaining derivatives showed K_Is <10 nM (except one compound, **8**, which has a K_I of 27.1 nM). Overall, the coumarins reported here show excellent inhibitory action against both tumor-associated isoforms hCA IX and XII.(v) As many coumarins and sulfocoumarins reported earlier,[1,5–11] the derivatives investigated here are hCA IX/XII selective inhibitors over hCA I and II, which constitutes a very important feature for this class of compounds. Indeed, one of the main problems with CAIs is constituted by their wide range of side effects, mainly due to inhibion of the offtarget isoforms such as hCA I and II.[2–4,23]

Table 1
Inhibition data of human CA isoforms hCA I, II, IX and XII with coumarins **5**–**18** reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO_2 hydrase assay[18]

Compound	R	X	K_I (nM)*			
			hCA I	hCA II	hCA IX	hCA XII
5	H	—	>10000	>10000	24.5	4.8
6	3-F	—	921	>10000	24.9	5.1
7	3-Cl	—	>10000	>10000	31.2	5.5
8	3-Br	—	>10000	>10000	27.5	27.1
9	3-OCH_3	—	210.7	>10000	23.6	5.2
10	4-F	—	1568	>10000	24.0	4.7
11	4-CF_3	—	239.5	>10000	23.0	5.5
12	4-OH	—	242.5	>10000	26.9	5.3
13	4-COOH	—	240.9	99.6	28.8	4.9
14	4-SO_2NH_2	—	172.8	>10000	14.3	9.9
15	—	—	>10000	>10000	22.8	37.8
16	—	O	1243.2	>10000	33.1	5.0
17	—	S	2137.9	>10000	29.6	5.1
18	—	NH	246.7	9743	34.4	4.9
AAZ			250	12	25	5.7

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ±5–10% of the reported values).

3. Conclusions

A series of 7-substituted coumarins incorporating aryl-triazole moieties were prepared by click chemistry procedures starting from 7-hydroxycoumarin or 4-methyl-7-aminocoumarin. The panel of new compounds was assayed for the inhibition of the cytosolic, widespread human hCA I and II isoforms, and the transmembrane, tumor-associated ones hCA IX and XII. Most of the coumarins were weak inhibitors or did not inhibit significantly hCA I and II, but showed low nanomolar inhibitory action against the transmembrane isoforms (K_I of 14.3–34.4 nM against hCA IX and of 4.7–37.8 nM against hCA XII). Since many hypoxic tumors overexpress hCA IX/XII, and as these targets were recently validated for obtaining antitumor/antimetastatic agents, with one inhibitor in Phase I clinical trials, the present findings constitute an interesting extension to the knowledge of non-sulfonamide, selective inhibitors of CA isoforms involved in serious pathologies.

4. Experimental protocols

4.1. Chemistry

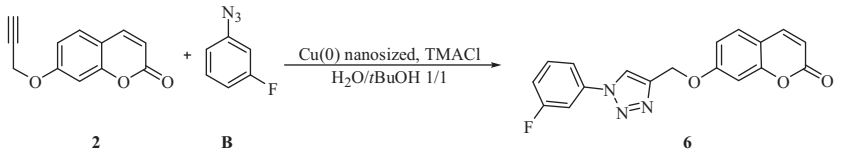
Anhydrous solvents and all reagents were purchased from Sigma–Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, DEPT-135, DEPT-90, HSQC, HMBC) spectra were recorded using a Bruker Advance III 400 MHz spectrometer in DMSO-d6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q, quartet; m, multiplet; brs, broad singlet; dd, doublet of doublets, appt, apparent triplet, appq, apparent quartet. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica gel 60 (230–400 mesh ASTM) as the stationary phase and ethyl acetate/*n*-hexane were used as eluents. Melting points (mp) were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected.

The solvents used in MS measures were acetone, acetonitrile (Chromasolv grade), purchased from Sigma–Aldrich (Milan–Italy), and mQ water 18 MΩ, obtained from Millipore's Simplicity system (Milan–Italy). The mass spectra were obtained using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA) equipped by Electrospray Source (ESI) operating in both positive and negative ions. Stock solutions of analytes were prepared in acetone at 1.0 mg mL^{-1} and stored at 4 °C. Working solutions of each analyte were freshly prepared by diluting stock solutions in a mixture of

mQ H_2O/ACN 1:1 (v/v) up to a concentration of 1.0 μg mL^{-1} The mass spectra of each analyte were acquired by introducing, via syringe pump at 10 μL min^{-1}, of the its working solution. Raw-data were collected and processed by Varian Workstation Vers. 6.8 software.

4.2. Procedure 1

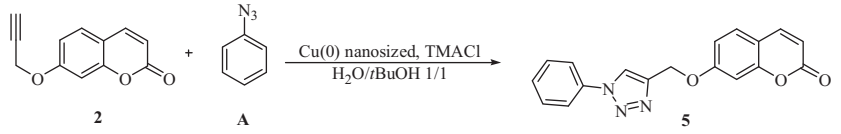
4.2.1. General synthetic procedure of compounds 5–18[24]



2 B 6

The appropriate alkyne **2**, **19**–**21** (1.0 equiv) was added to a suspension of aryl azide **A**–**J**, **4** (1.1 equiv) in H_2O/*t*BuOH 1:1 (3.5 ml) at rt, followed by copper (0) nanosized (0.1 equiv) and TMACl (1.0 equiv). The suspension was stirred at 60 °C until starting materials were consumed (TLC monitoring), then quenched with H_2O (20 ml) and the formed precipitate was filtered-off and washed with H_2O. The solid was dissolved in a minimal amount of acetone, the obtained solution was filtered through Celite 521® and then concentrated under vacuo to give a residue that was triturated with Et_2O or DCM to afford the titled compounds **5**–**18**.

4.2.1.2. Synthesis of 7-(1-phenyl-1*H*-[1,2,3]triazol-4-ylmethoxy)-chromen-2-one 5.



2 A 5

7-(1-Phenyl-1*H*-[1,2,3]triazol-4-ylmethoxy)-chromen-2-one **5** was obtained according the general procedure 1 earlier reported using phenylazide **A** (1.1 equiv), 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) in *t*BuOH/H_2O (1:1, 3.5 ml), tetramethylamonium chloride (1.0 equiv) and copper nanosize (0.1 equiv). The reaction mixture was stirred for 5 h to give the titled compound **5** as a white solid.

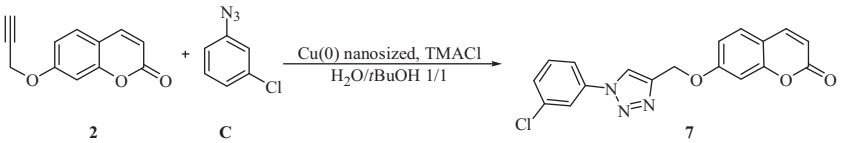
7-(1-Phenyl-1H-[1,2,3]triazol-4-ylmethoxy)-chromen-2-one ***5****:* 74% yield; mp 172–175 °C; silica gel TLC Rf 0.52 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 5.41 (s, 2H, CH_2), 6.35 (d, *J* = 9.6, 1H), 7.11 (dd, *J* = 2.4, 8.6, 1H), 7.25 (d, *J* = 2.4, 1H), 7.56 (t, *J* = 7.6, 1H), 7.66 (t, *J* = 7.6, 2H), 7.70 (d, *J* = 8.6, 1H), 7.96 (d, *J* = 7.6, 2H), 8.05 (d, *J* = 9.6, 1H), 9.05 (s, 1H); δC (100 MHz, DMSO-d6): 63.0, 102.6, 113.6, 113.7, 113.8, 121.2, 124.1, 129.8, 130.5, 130.9, 138.0, 144.1, 145.2, 156.2, 161.2, 162.0; *m*/*z* (ESI positive) 320.0 $[M+H]^+$.

4.2.1.2. Synthesis of 7-[1-(3-fluoro-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one 6.

7-[1-(3-Fluoro-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one **6** was obtained according the general procedure 1 earlier reported using 3-fluorophenylazide **B** (1.1 equiv), 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) in *t*BuOH/H_2O (1:1, 3.5 ml), tetramethylamonium chloride (1.0 equiv) and copper nanosize (0.1 equiv). The reaction mixture was stirred for 1.5 h to give the titled compound **6** as a white solid.

7-[1-(3-Fluoro-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one ***6****:* 77% yield; mp 158–160 °C; silica gel TLC R_f 0.45 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6): 5.42 (s, 2H, CH_2), 6.36 (d, *J* = 9.6, 1H), 7.11 (dd, *J* = 2.4, 8.6, 1H), 7.25 (d, *J* = 2.4, 1H), 7.42 (m, 1H), 7.71 (m, 2H), 7.86 (m, 1H), 7.91 (m, 1H), 8.05 (d, *J* = 9.6, 1H), 9.10 (s, 1H); δ_F (376 MHz, DMSO-d6): −110.52 (s, 1F); δ_C (100 MHz, DMSO-d6): 62.5, 102.5, 108.1 (d, J^2_{CF} = 26.3), 113.6, 113.7, 113.8, 116.4 (d, J^2_{CF} = 21.0), 117.0, 124.2, 130.5, 132.8 (d, J^3_{CF} = 9.0), 138.6 (d, J^3_{CF} = 10.4), 144.3, 145.1, 156.2, 161.2, 161.9, 162.8 (d, J^1_{CF} = 262.7); *m*/*z* (ESI positive) 338.0 $[M+H]^+$.

4.2.1.3. Synthesis of 7-[1-(3-chloro-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one 7.



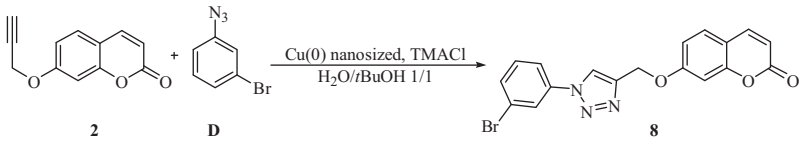
2 C 7

1-Azido-3-chlorobenzene **C** (1.1 equiv) and 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The

mixture was stirred at 60 °C for 3.5 h andthen treated as described in general procedure 1 to afford **7** as a white solid.

7-[1-(3-Chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one ***7****:* 77% yield; mp 182–183 °C; silica gel TLC R_f 0.41 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6): 5.41 (s, 2H, CH_2), 6.36 (d, J = 9.6, 1H), 7.10 (dd, J = 2.4, 8.6, 1H), 7.24 (d, J = 2.4, 1H), 7.66 (m, 3H), 7.97 (d, J = 8.1, 1H), 8.05 (d, J = 9.6, 1H), 8.11 (d, J = 2.0, 1H), 9.12 (s, 1H); δ_C (100 MHz, DMSO-d6): 62.5, 102.3, 113.6, 113.7, 113.8, 119.7, 120.9, 124.3, 129.6, 130.5, 132.6, 135.1, 138.5, 144.3, 145.2, 156.2, 161.1, 161.9; *m*/*z* (ESI positive) 354.0 $[M+H]^+$.

4.2.1.4. Synthesis of 7-[1-(3-bromo-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one 8.

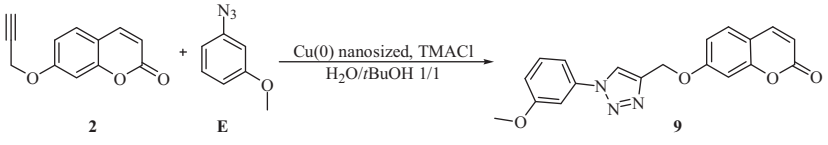


2 D 8

1-Azido-3-bromobenzene **D** (1.1 equiv) and 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 6 h andthen treated as described in general procedure 1to afford **8** as a white solid.

7-[1-(3-Bromo-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one ***8****:* 78% yield; mp 170–172 °C; silica gel TLC R_f 0.41 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6): 5.41 (s, 2H, CH_2), 6.36 (d, J = 9.6, 1H), 7.10 (dd, J = 2.4, 8.6, 1H), 7.24 (d, J = 2.4, 1H), 7.61 (t, J = 8.2, 1H), 7.71 (d, J = 8.6, 1H), 7.76 (d, J = 8.2, 1H), 8.02 (d, J = 8.2, 1H), 8.05 (d, J = 9.6, 1H), 8.23 (s, 1H), 9.12 (s, 1H); δ_C (100 MHz, DMSO-d6): 62.5, 102.6, 113.6, 113.7, 113.8, 120.1, 123.4, 123.7, 124.3, 130.5, 132.5, 132.8, 138.5, 144.3, 145.2, 156.0, 161.1, 161.9; *m*/*z* (ESI positive) 398.0 $[M+H]^+$.

4.2.1.5. Synthesis of 7-[1-(3-methoxy-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one 9.

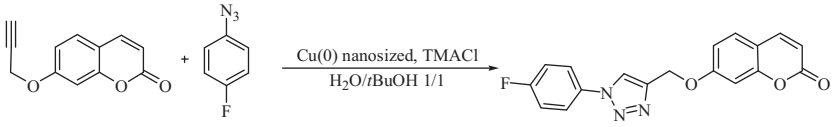


2 E 9

1-Azido-3-methoxybenzene **E** (1.1 equiv) and 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 2.5 h and then treated as described in general procedure 1 to afford **9** as a white solid.

7-[1-(3-Methoxy-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one ***9****:* 81% yield; mp 131–133 °C; silica gel TLC R_f 0.36 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6): 3.89 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 6.36 (d, J = 9.6, 1H), 7.11 (m, 2H), 7.24 (d, J = 2.4, 1H), 7.54 (m, 3H), 7.70 (d, J = 8.6, 1H), 8.05 (d, J = 9.6, 1H), 9.07 (s, 1H); δ_C (100 MHz, DMSO-d6): 56.6, 62.7, 102.6, 106.6, 113.1, 113.6, 113.7, 113.8, 115.5, 124.2, 130.5, 131.8, 138.5, 144.0, 145.2, 156.2, 131.1, 161.2, 162.0; *m*/*z* (ESI positive) 350.0 $[M+H]^+$.

4.2.1.6. Synthesis of 7-[1-(4-fluoro-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one 10.

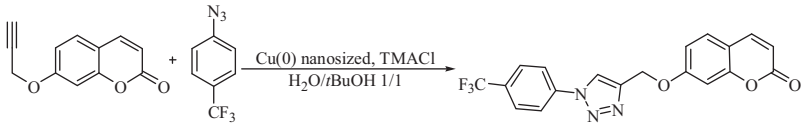


2 F 10

7-[1-(4-fluoro-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one **10** was obtained according the general procedure 1 earlier reported using 1-azido-4-fluorobenzene **F** (1.1 equiv), 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) in *t*BuOH/H_2O 1:1 (3.5 ml), tetramethylamonium chloride (1.0 equiv) and copper nanosize (0.1 equiv). The reaction mixture was stirredfor 7.5 h to give the titled compound **10** as a whitesolid.

7-[1-(4-Fluoro-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one ***10****:* 68% yield; mp 201–203 °C; silica gel TLC R_f 0.34 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6): 5.40 (s, 2H, CH_2), 6.36 (d, J = 9.2, 1H), 7.10 (dd, J = 2.4, 8.6, 1H), 7.24 (d, J = 2.4, 1H), 7.51 (m, 2H), 7.70 (d, J = 8.6, 1H), 8.00 (m, 2H), 8.05 (d, J = 9.2, 1H), 9.02 (s, 1H); δ_F (376 MHz, DMSO-d6): −112.94 (s, 1F); δ_C (100 MHz, DMSO-d6): 62.4, 102.4, 113.5, 113.6, 113.7, 117.6 (d, J^2_{CF} = 23.1), 123.4 (d, J^3_{CF} = 8.8), 124.3, 130.7, 133.9 (d, J^4_{CF} = 2.9), 144.0, 145.1, 156.1, 161.0, 161.8, 162.5 (d, J^1_{CF} = 244.4); *m*/*z* (ESI positive) 338.0 $[M+H]^+$.

4.2.1.7. Synthesis of 7-[1-(4-trifluoromethyl-phenyl)-*1H*-[1,2,3]-triazol-4-ylmethoxy]-chromen-2-one 11.

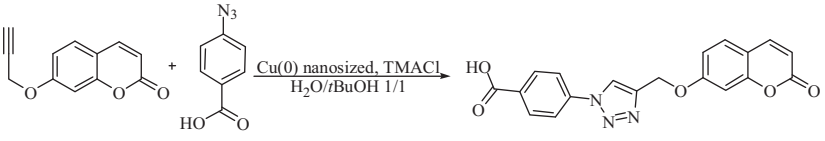


2 G 11

7-[1-(4-trifluoromethyl-phenyl)-*1H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one **11** was obtained according the general procedure 1 earlier reported using 1-azido-4-trifluoromethylbenzene **G** (1.1 equiv), 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) in *t*BuOH/H_2O 1:1 (3.5 ml), tetramethylamonium chloride (1.0 equiv) and copper nanosize (0.1 equiv). The reaction mixture was stirred for 2.5 h to give the titled compound **11** as a white powder.

*7-[1-(4-Trifluoromethyl-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one **11**:* 70% yield; mp 185–187 °C; silica gel TLC R_f 0.50 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6): 5.43 (s, 2H, CH_2), 6.35 (d, J = 9.6, 1H), 7.10 (dd, J = 2.4, 8.6, 1H), 7.24 (d, J = 2.4, 1H), 7.70 (d, J = 8.6, 1H), 8.05 (m, 3H), 8.22 (d, J = 8.4, 2H), 9.19 (s, 1H); δ_C (100 MHz, DMSO-d6): 62.5, 102.6, 113.7, 113.7, 113.8, 121.6, 123.9 (d, J^1_{CF} = 270.6), 124.4, 128.2 (q, J^3_{CF} = 3.4), 129.8 (d, J^2_{CF} = 32.7), 130.5, 140.2, 144.5, 145.2, 156.2, 161.2, 161.9; *m*/*z* (ESI positive) 388.0 $[M+H]^+$.

4.2.1.8. Synthesis of 7-[1-(4-hydroxy-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one 12.

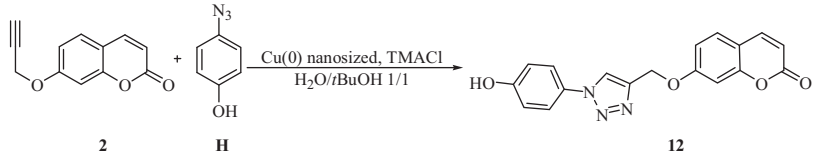


2 I 13

1-Azido-4-hydroxybenzene **H** (1.1 equiv) and 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 1.5 h and then treated as described in general procedure 1 to afford **12** as a white solid.

*7-[1-(4-Hydroxy-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one **12**:* 77% yield; mp 241–242 °C; silica gel TLC R_f 0.21 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6): 5.37 (s, 2H, CH_2), 6.35 (d, J = 9.6, 1H), 6.98 (d, J = 8.6, 2H), 7.10 (dd, J = 2.4, 8.6, 1H), 7.24 (d, J = 2.4, 1H), 7.70 (m, 3H), 8.05 (d, J = 9.6, 1H), 8.85 (s, 1H), 10.00 (s, 1H,, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d6): 62.6, 102.6, 113.6, 113.7, 113.8, 117.0, 123.0, 124.0, 129.6, 130.5, 143.7, 145.2, 156.2, 158.8, 161.2, 162.0; *m*/*z* (ESI positive) 336.0 $[M+H]^+$.

4.2.1.9. Synthesis of 7-[1-(4-carboxy-phenyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one 13.

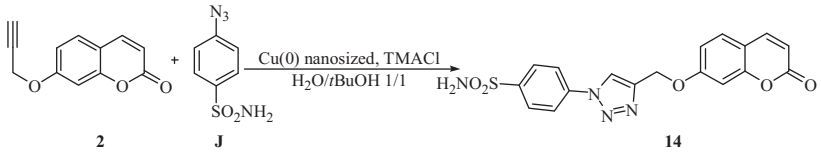


2 H 12

1-Azido-4-carboxybenzene **I** (1.1 equiv) and 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 12 h and treated as described in general procedure 1 to afford **13** as a white solid.

*7-[1-(4-Carboxy-phenyl)-1H-[1,2,3]triazol-4-ylmethoxy]-chromen-2-one **13**:* 55% yield; mp 290–292 °C d; silica gel TLC R_f 0.34 (MeOH/CH_2Cl_2 10% v/v); δ_H (400 MHz, DMSO-d6): 5.42 (s, 2H, CH_2), 6.36 (d, J = 9.2, 1H), 7.10 (dd, J = 2.4, 8.6, 1H), 7.25 (d, J = 2.4, 1H), 7.71 (d, J = 8.6, 1H), 8.05 (d, J = 9.2, 1H), 8.15 (bq, 4H), 9.16 (s, 1H), 13.34 (br s, 1H, exchange with D_2O, COO*H*); *m*/*z* (ESI negative) 362.17 $[M-H]^-$.

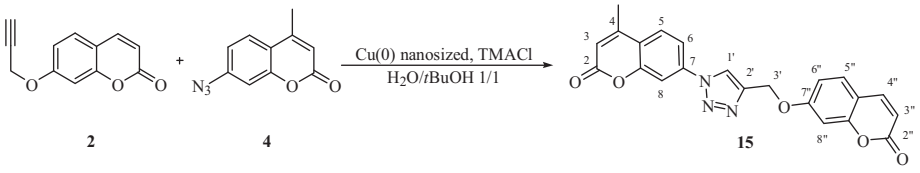
4.2.1.10. Synthesis of 4-[4-(2-oxo-2*H*-chromen-7-yloxymethyl)-[1,2,3]triazol-1-yl]-benzenesulfonamide 14.



4-Azidobenzenesulfonamide **J** (1.1 equiv) and 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 2.5 h and then treated as described in general procedure 1 to afford **14** as a light yellow solid.

4-[4-(2-Oxo-2H-chromen-7-yloxymethyl)-[1,2,3]triazol-1-yl]-benzenesulfonamide ***14****:* 80% yield; mp 279–281 °C; silica gel TLC R_f 0.43 (MeOH/CH_2Cl_2 10% v/v); δ_H (400 MHz, DMSO-d6): 5.43 (s, 2H, CH_2), 6.36 (d, J = 9.2, 1H), 7.10 (dd, J = 2.4, 8.6, 1H), 7.25 (d, J = 2.4, 1H), 7.59 (s, 2H, exchange with D_2O, SO_2NH_2), 7.71 (d, J = 8.6, 1H), 8.06 (m, 3H), 8.19 (d, J = 8.8, 2H), 9.15 (s, 1H); δ_C (100 MHz, DMSO-d6): 62.5, 102.6, 113.7, 113.8, 113.9, 121.4, 124.4, 128.5, 130.5, 139.4, 144.5, 144.9, 145.2, 156.3, 161.2, 161.9; m/z (ESI negative) 397.0 $[M-H]^-$.

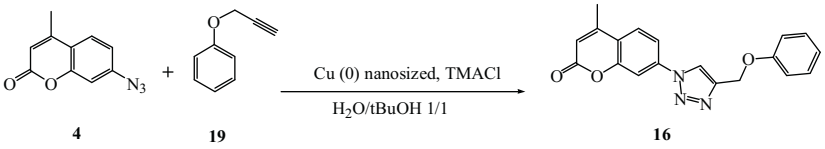
4.2.1.1. Synthesis of 4-methyl-7-[4-(2-oxo-2*H*-chromen-7-yloxymethyl)-[1,2,3]triazol-1-yl]-chromen-2-one 15.



4-Methyl-7-[4-(2-oxo-2*H*-chromen-7-yloxymethyl)-[1,2,3]triazol-1-yl]-chromen-2-one **15** was obtained according the general procedure **1** earlier[24] reported using 7-azido-4-methyl-2*H*-chromen-2-one **4** (1.1 equiv) and 7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2** (0.05 g, 1.0 equiv) in *t*BuOH/H_2O 1:1 (3.5 ml), tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol). The reaction mixture was stirred for 6 h to give a solid that was purified by silica gel column chromatography eluting with ethyl acetate in *n*-hexane from 20% to 50% to afford the title compound **15** as a yellow solid.

4-Methyl-7-(4-((2-oxo-2H-chromen-7-yloxy)methyl)-1H-1,2,3-triazol-1-yl)-2H-chromen-2-one ***15****:* 88% yield; silica gel TLC R_f 0.32 (Ethyl acetate/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d6) 2.11 (3H, s, CH_3), 5.44 (2H, s, 3′-H_2), 6.35 (1H, d, J 9.6, 3″-H), 6.53 (1H, d, J 1.2, 3-H), 7.11 (1H, dd, J 8.4, 2.4, 6″-H), 7.25 (1H, d, J 2.4, 8″-H), 7.71 (1H, d, J 8.4, 5″-H), 8.06 (4H, m, 5, 6, 8, 4″-H), 9.22 (1H, s, 1′-H); δ_C (100 MHz, DMSO-d6) 161.9, 161.0, 160.3, 156.2, 154.3, 153.7, 145.2, 144.5, 139.3, 130.5, 128.2, 124.4, 120.6, 116.6, 115.8, 113.9, 113.7, 108.6, 108.0, 102.6, 62.5, 32.2; m/z (ESI positive) 402.0 $[M+H]^+$.

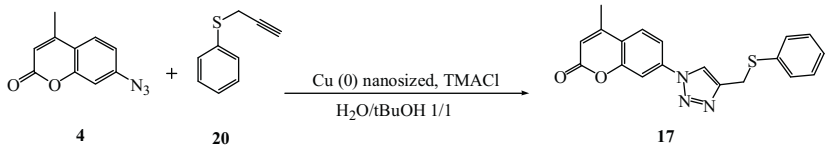
4.2.1.12. Synthesis of 4-methyl-7-(4-phenoxymethyl-[1,2,3]triazol-1-yl)-chromen-2-one 16.



7-Azido-4-methyl-2*H*-chromen-2-one **4** (1.1 equiv) and prop-2-ynyloxy-benzene **19** (0.05 g, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 2 h and then treated as described in general procedure 1 to afford **16** as a yellow solid.

4-Methyl-7-(4-phenoxymethyl-[1,2,3]triazol-1-yl)-chromen-2-one ***16****:* 70% yield; mp 208–209 °C; silica gel TLC R_f 0.40 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 2.51 (s, 3H, CH_3), 5.30 (s, 2H, CH_2), 6.53 (d, J = 1.2, 1H), 7.02 (t, J = 7.6, 1H), 7.13 (d, J = 7.6, 2H), 7.37 (t, J = 7.6, 2H), 8.06 (m, 3H), 9.18 (s, 1H); δ_C (100 MHz, DMSO-d6): 19.0, 61.8, 108.5, 115.6, 115.7, 116.5, 120.5, 121.9, 124.0, 128.2, 130.5, 139.4, 145.3, 153.7, 154.5, 158.9, 160.3; m/z (ESI positive) 334.0 $[M+H]^+$.

4.2.1.13. Synthesis of 4-methyl-7-(4-phenylsulfanylmethyl-[1,2,3]triazol-1-yl)-chromen-2-one 17.

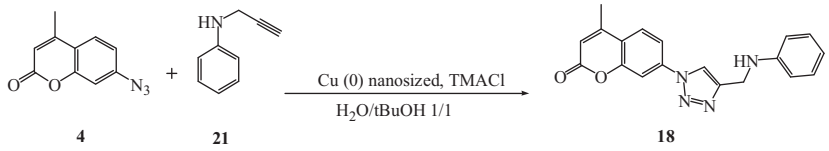


4 20 17

7-azido-4-methyl-2*H*-chromen-2-one **4** (1.1 equiv) and prop-2-ynylsulfanyl-benzene **20** (0.05 g, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 2 h and then treated as described in general procedure 1 to afford **17** as a yellow solid.

4-Methyl-7-(4-phenylsulfanylmethyl-[1,2,3]triazol-1-yl)-chromen-2-one ***17****:* 68% yield; mp 178–179 °C; silica gel TLC R_f 0.44 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 2.51 (s, 3H, CH_3), 4.44 (s, 2H, CH_2), 6.52 (d, J = 1.2, 1H), 7.24 (t, J = 7.6, 1H), 7.37 (t, J = 7.6, 2H), 7.45 (d, J = 7.6, 2H), 8.00 (m, 3H), 8.94 (s, 1H); δ_C (100 MHz, DMSO-d6): 19.0, 28.1, 108.3, 115.6, 116.3, 120.3, 122.6, 127.0, 128.2, 129.3, 130.2, 136.4. 139.3, 146.2, 153.7, 154.6, 160.4; *m*/*z* (ESI positive) 350.0 $[M+H]^+$.

4.2.1.14. Synthesis of 4-Methyl-7-(4-phenylaminomethyl-[1,2,3] triazol-1-yl)-chromen-2-one 18.



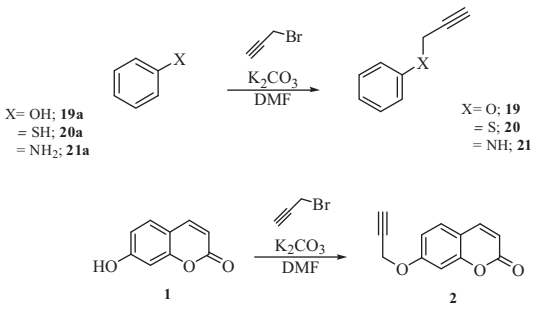
4 21 18

7-azido-4-methyl-2*H*-chromen-2-one **4** (1.1 equiv) and phenyl-prop-2-ynyl-amine **21** (0.05 g, 1.0 equiv) were dissolved in *t*BuOH/H_2O 1:1 (3.5 ml) and then tetramethylamonium chloride (1.0 equiv) and copper nanosize (10% mol) were added. The mixture was stirred at 60 °C for 2.5 h and then treated as described in general procedure 1 to afford **18** as a yellow solid.

4-Methyl-7-(4-phenylaminomethyl-[1,2,3]triazol-1-yl)-chromen-2-one ***18****:* 85% yield; mp 197–200 °C; silica gel TLC R_f 0.30 (EtOAc/*n*-hexane 50% v/v); δ_H (400 MHz, DMSO-d_6): 2.51 (s, 3H, CH_3), 4.43 (d, J = 6.0, 2H, CH_2), 6.20 (t, J = 6.0, 1H, exchange with D_2O, NH), 6.52 (d, J = 1.2, 1H), 6.60 (t, J = 7.8, 1H), 6.71 (d, J = 7.8, 2H), 7.12 (t, J = 7.8, 2H), 8.03 (m, 3H), 8.94 (s, 1H); δ_C (100 MHz, DMSO-d_6): 19.0, 39.5, 108.2, 113.3, 115.5, 116.2, 117.1, 120.2, 122.3, 128.2, 129.8, 139.5, 148.3, 149.2, 153.7, 154.6, 160.4; *m*/*z* (ESI positive) 333.0 $[M+H]^+$.

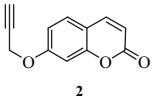
4.3. Procedure 2

4.3.1. General synthetic procedure of alkynes 2, 19–21[25]



Propargyl bromide (1.2 equiv) was added to a suspension of compound **1**, **19a**–**21a** (0.5 g, 1.0 equiv) and K_2CO_3 (2.0 equiv) in dry DMF (4 ml) under a nitrogen atmosphere and that was stirred at rt until starting material was consumed (TLC monitoring). The reaction mixture was quenched with H_2O (20 ml) and extracted with Et_2O or EtOAc (25 ml). The organic layer was washed with brine (4 × 15 ml), dried over Na_2SO_4, filtered-off and concentrated under vacuo to give the titled compounds **2**, **19**–**21**.

4.3.1.1. Synthesis 7-prop-2-ynyloxy-chromen-2-one 2.



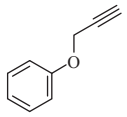
2

7-Prop-2-ynyloxy-chromen-2-one **2** was obtained according the general procedure 2 reported earlier.[25] The reaction mixture was

stirred at rt for 2 h, quenched with H_2O and extracted with EtOAc to give the titled compound **2** as a white powder.

*7-Prop-2-ynyloxy-chromen-2-one **2**:* 73% yield; mp 118–119 °C; silica gel TLC R_f 0.56 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 3.69 (t, *J* = 2.4, 1H), 4.97 (d, *J* = 2.4, 2H), 6.36 (d, *J* = 9.6, 1H), 7.03 (dd, *J* = 2.4, 8.6, 1H), 7.09 (d, *J* = 2.4, 1H), 7.70 (d, *J* = 8.6, 1H), 8.04 (d, *J* = 9.6, 1H); δ_C (100 MHz, DMSO-d6): 57.0, 79.4, 79.8, 102.7, 113.7, 113.8, 113.9, 130.4, 145.1, 156.0, 161.0, 161.1. Experimental in agreement with reported data.[26]

4.3.1.2. Synthesis of prop-2-ynyloxy-benzene 19.

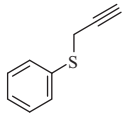


19

Prop-2-ynyloxy-benzene **19** was obtained according the general procedure **2** earlier reported. The reaction mixture was stirred at rt for 2 h to give the titled compound **19**.

*Prop-2-ynyloxy-benzene **19**:* 74%yield; silica gel TLC R_f 0.68 (EtOAc/*n*-hexane 20% v/v); δH (400 MHz, $CDCl_3$): 2.58 (t, *J* = 2.4, 1H), 4.76 (d, *J* = 2.4, 2H), 7.06 (m, 3H), 7.37 (t, *J* = 8.8, 2H); δ_C (100 MHz, $CDCl_3$): 56.1, 75.8, 79.0, 115.3, 121.9, 129.8, 157.9. Experimental in agreement with reported data.[25]

4.3.1.3. Synthesis of prop-2-ynylsulfanyl-benzene 20.

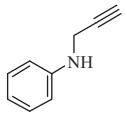


20

Prop-2-ynylsulfanyl-benzene **20**was obtained according the general procedure **2** earlier reported.[25]The reaction mixture was stirred at rt for 2 h to give the titled compound **20**.

*Prop-2-ynylsulfanyl-benzene **20**:* 96% yield; silica gel TLC R_f 0.61 (EtOAc/*n*-hexane 10% v/v); δH (400 MHz, $CDCl_3$): 2.30 (t, *J* = 2.4, 1H), 3.67 (d, *J* = 2.4, 2H), 7.32 (m, 1H), 7.39 (t, *J* = 7.6, 2H), 7.52 (m, 2H); δ_C (100 MHz, $CDCl_3$): 22.9, 71.9, 80.2, 127.3, 129.3, 129.4, 130.4. Experimental in agreement with reported data.[27]

4.3.1.4. Synthesis of phenyl-prop-2-ynyl-amine 21.



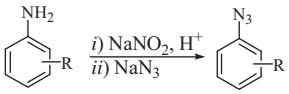
21

Phenyl-prop-2-ynyl-amine **21**was obtained according the general procedure **2** earlier reported.[25] The reaction mixture was stirred at rt for 4 h and the obtained oil was purified by silica gel column chromatography eluting with EtOAc/petroleum ether 1:20 to afford the title compounds 21.

*Phenyl-prop-2-ynyl-amine **21**:* 35% yield; silica gel TLC R_f 0.76 (EtOAc/*n*-hexane 20% v/v); δH (400 MHz, DMSO-d6): 2.24 (t, *J* = 2.4, 1H), 3.96 (d, *J* = 2.4, 2H), 6.78 (d, *J* = 7.4, 2H), 6.85 (t, *J* = 7.4, 1H), 7.31 (t, *J* = 7.4, 2H); δ_C (100 MHz, $CDCl_3$): 34.4, 72.1, 80.8, 114.5, 119.7, 129.6, 146.3. Experimental in agreement with reported data.[28]

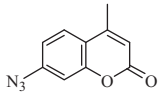
4.4. Procedure 3

4.4.1. Synthesis of phenylazides 4, A–J[6]



The proper aniline (0.5 g, 1.0 equiv) was dissolved in a 4 M HCl aqueous solution (5 ml) at 0 °C. $NaNO_2$ (1.2 equiv) was slowly added and the resulting solution was stirred at the same temperature for 0.5 h. Then NaN_3 (1.5 equiv) was added portion-wise and the mixture was stirred at rt for 0.5 h. Reaction mixture was filtered-off or extracted with Et_2O (2 × 15 ml) and the combined organic layers were dried over Na_2SO_4, filtered-off and the solvent evaporated in vacuo to afford the corresponding phenylazide which was used without further purification.

4.4.2. Synthesis of 7-azido-4-methyl-chromen-2-one 4

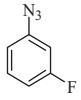


4

7-Azido-4-methyl-chromen-2-one **4** was obtained according the general procedure 3reported earlier. 7-Amino-4-methyl-coumarin**3**was treated with $NaNO_2$ and NaN_3 in a 2 M HCl aqueous solution and the formed precipitate was filtered-off to afford the title compound **4** as a yellow solid.

*7-Azido-4-methyl-chromen-2-one **4**:* 88% yield; mp 122–124 °C; silica gel TLC R_f 0.57 (EtOAc/*n*-hexane 20% v/v); δH (400 MHz, DMSO-d6): 2.46 (d, *J* = 1.2, 3H, CH_3), 6.38 (d, *J* = 1.2, 1H), 7.18 (m, 2H), 7.83 (d, *J* = 8.4, 1H); δ_C (100 MHz, DMSO-d6): 19.0, 107.7, 114.1, 116.5, 117.7, 127.9, 144.2, 153.8, 155.0, 160.4. Experimental in agreement with reported data.[29]

4.4.3. Synthesis of phenylazide A

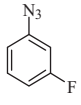


B

Phenyl azide **A** was obtained according the general procedure 3 reported earlier.

*Phenyl azide **A**:* 60% yield; silica gel TLC R_f 0.76 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6):7.12 (d, *J* = 7.6, 2H), 7.20 (t, *J* = 7.6, 1H), 7.42 (t, *J* = 7.6, 2H); δ_C (100 MHz, DMSO-d6): 120.0, 126.1, 131.0, 140.3. Experimental in agreement with reported data.[29]

4.4.4. Synthesis of 3-fluorophenylazide B



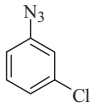
B

3-Fluorophenylazide **B** was obtained according to the general procedure3reported earlier.[30]

*3-Fluorophenyl azide **B**:* 62% yield; silica gel TLC R_f 0.81 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 7.06 (m, 3H), 7.46

(q, J = 7.2, 1H); δF (376 MHz, DMSO-d6): −110.52 (s, 1F); δ_C (100 MHz, DMSO-d6): 107.6 (d, J^2_{CF} = 25.0), 112.8 (d, J^2_{CF} = 21.0), 116.2, 132.5 (d, J^3_{CF} = 9.0), 142.5 (d, J^3_{CF} = 9.0), 163.7 (d, J^1_{CF} = 245.0). Experimental in agreement with reported data.[30]

4.4.5. Synthesis of 3-chlorophenylazide C

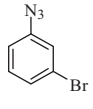


C

3-Chlorophenylazide **C** was obtained according to the general procedure 3 reported earlier.[30]

*3-Chlorophenyl azide **C**:* 86% yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 7.14 (dd, J = 2.2, 8.0, 1H), 7.24 (t, J = 2.2, 1H), 7.30 (d, J = 2.2, 1H), 7.46 (t, J = 8.0, 1H); δ_C (100 MHz, DMSO-d6): 118.9, 120.1, 126.0, 132.4, 135.1, 142.2. Experimental in agreement with reported data.[30]

4.4.6. Synthesis of 3-bromophenylazide D

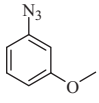


D

3-Bromophenylazide **D** was obtained according to the general procedure 3reported earlier.

*3-Bromophenylazide **D**:* 88% yield; silica gel TLC R_f 0.83 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6):7.18 (dt, J = 1.6, 7,2, 1H), 7.38 (m, 3H); δ_C (100 MHz, DMSO-d6): 119.3, 122.8, 123.5, 128.9, 132.7, 142.3. Experimental in agreement with reported data.[30]

4.4.7. Synthesis of 3-methoxyphenylazide E

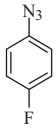


E

3-Methoxyphenyl azide **E** was obtained according to the general procedure 3 reported earlier.

*3-Methoxyphenyl azide **E**:* 74% yield; silica gel TLC R_f 0.78 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 3.80 (s, 3H, CH_3), 6.66 (t, J = 2.4, 1H), 6.74 (ddd, J = 0.8, 2.4, 8.2, 1H), 6.81 (ddd, J = 0.8, 2.4, 8.2, 1H), 7.36 (t, J = 8.2, 1H); δ_C (100 MHz, DMSO-d6): 56.3, 105.7, 112.0, 112.1, 131.7, 141.5, 161.5. Experimental in agreement with reported data.[29]

4.4.8. Synthesis of 4-fluorophenylazide F

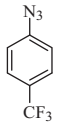


F

4-Fluorophenylazide **F** was obtained according the general procedure 3 reported earlier.[29]

*4-Fluorophenylazide **F**:* 89% yield; silica gel TLC R_f 0.79 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 67.19 (m, 2H), 7.29 (t, J = 8.8, 2H); δF (376 MHz, DMSO-d6): −117.77 (s, 1F); δ_C (100 MHz, DMSO-d6): 117.7 (d, J^2_{CF} = 23), 121.8 (d, J^3_{CF} = 9.0), 136.4, 160.3 (d, J^1_{CF} = 241.0). Experimental in agreement with reported data.[30]

4.4.9. Synthesis of 4-trifluoromethyl-phenyl azide G

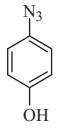


G

4-Trifluoromethylphenylazide **G** was obtained according to the general procedure 3 reported earlier.[30]

*4-Trifluoromethylphenylazide **G**:* 67% yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 7.35 (d, J = 8.8, 2H), 7.78 (d, J = 8.8, 2H); δF (376 MHz, DMSO-d6): −56.18 (s, 3F); δ_C (100 MHz, DMSO-d6): 120.8, 125.0 (d, J^1_{CF} = 269.6), 126.2 (q, J^2_{CF} = 32.0), 130.0, 144.7. Experimental in agreement with reported data.[33]

4.4.10. Synthesis of 4-hydroxy-phenyl azide H

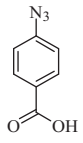


H

4-Hydroxyphenylazide **H** was obtained according the general procedure 3 reported earlier.[31]

*4-Hydroxyphenylazide **H**:* 46% yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6):6.84 (d, J = 8.8, 2H), 6.97 (d, J = 8.8, 2H), 9.56 (br s, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d6): 117.5, 121.1, 130.6, 156.0. Experimental in agreement with reported data.[31]

4.4.11. Synthesis of 4-azidobenzoic acid I

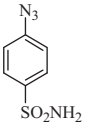


I

4-Azidobenzoic acid **I** was obtained according the general procedure 3 reported earlier. 4-Aminobenzoic acid was treated with $NaNO_2$ and NaN_3 in a 4 M HCl aqueous solution and the formed precipitate was filtered-off to afford the title compound **I** as a yellow solid.

*4-Azidobenzoic acid **I**:* 73% yield; mp 188–190 °C d; silica gel TLC R_f 0.71 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 7.25 (d, J = 8.4, 2H), 7.99 (d, J = 8.4, 2H), 13.02 (br s, 1H, exchange with D_2O, COOH); δ_C (100 MHz, DMSO-d6): 120.2, 128.3, 132.2, 144.9, 167.6. Experimental in agreement with reported data.[32]

4.4.12. Synthesis of 4-azido-benzenesulfonamide J



J

4-Azidobenzenesulfonamide **J** was obtained according the general procedure **3** reported earlier.[34] Sulfanilamide was treated with $NaNO_2$ and NaN_3 in a HCl 2 M aqueous solution and the formed precipitate was filtered-off to afford the title compound **J** as a yellow solid.

*4-Azidobenzenesulfonamide **J**:* 60% yield; mp 120–121 °C; silica gel TLC R_f 0.47 (EtOAc/*n*-hexane 50% v/v); δH (400 MHz, DMSO-d6): 7.33 (d, J = 8.8, 2H), 7.41 (s, 2H, exchange with D_2O, SO_2NH_2), 7.87 (d, J = 8.8, 2H); δ_C (100 MHz, DMSO-d6): 120.5, 128.6, 141.5, 143.9. Experimental in agreement with reported data.[34]

4.5. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[18] 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-catalysed 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 uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min–6 h at room temperature (15 min) or 4 °C (6 h) prior to assay, in order to allow for the formation of the E–I complex. Data from Table 1 were obtained after 6 h incubation of enzyme and inhibitor, as for the sulfocoumarins and coumarins reported earlier.[1,5–11] The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier,[19–21]and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[19–21]

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