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RESEARCH ARTICLE

7-Aryl-triazolyl-substituted sulfocoumarins are potent, selective inhibitors of the tumor-associated carbonic anhydrase IX and XII

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Abstract

Sulfocoumarins behave as interesting inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). Here, we report a new series of 7-substituted derivatives which were obtained by the click chemistry approach from 7-propargyloxy-sulfocoumarin and aryl azides incorporating halogens, hydroxy, methoxy and carboxyl moieties in their molecules. The new compounds were screened for the inhibition on four physiologically relevant human CA (hCA) isoforms, the cytosolic hCA I and II and the transmembrane tumor-associated hCA IX and XII. The new compounds did not inhibit the cytosolic isoforms but were low nanomolar inhibitors of the tumor-associated ones hCA IX and XII.

Keywords

Carbonic anhydrase, click chemistry, human carbonic anhydrase IX, human carbonic anhydrase XII, sulfocoumarin, tumor-associated isoforms

History

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Introduction

1,2-Benzoxathiines-2,2-dioxides, also referred as sulfocoumarins, were recently validated as a novel class of inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1)[1]. The design of sulfocoumarins as CA inhibitors (CAIs) was inspired by their corresponding bioisosters coumarins[2–9], whose inhibition mechanism relies on the CA-mediated esterase activity. Indeed the sulfocoumarins, as well as coumarins, undergo a CA-mediated hydrolysis within the active site cavity with the generation of the inhibitory species. The inhibition mechanism of such hydrolysis products is different at the molecular level when compared to the classical sulfonamide-based CAIs[10,11], that coordinate in the deprotonated form to the zinc metal ion, thus substituting the fourth ligand represented by a water molecule/hydroxide ion. As demonstrated by means of X-ray crystallographic and kinetic studies, the hydrolyzed coumarine derivative (i.e. cinnamic acid) is placed at the rim of the enzymatic cavity thus occluding it. The resulting ligand-enzyme complex is stabilized by interactions with the amino acids present therein that constitute the most variable region within CA isoforms known to date (15 human CA (hCA) isoforms are reported)[2–8,10]. Conversely, 2-hydroxyphenyl-ω-ethenyl sulfonic acids which are formed from the original sulfocoumarins, tightly bind to the zinc-coordinated water molecule by means of hydrogen bonding, whereas the scaffold of the inhibitor establishes additional favorable interactions within the cavity (Figure 1)[1].

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The highly selective CA inhibition profiles shown by the coumarins and sulfocoumarins rely on this particular inhibition mechanism, which in turn depends from different binding modes of the hydrolysis products within the CAs catalytic cavities[10]. Such a feature is highly desired in the current CAIs research as the main problem associated to the sulfonamide-type clinically used CAIs is represented by their indiscriminate inhibition of the hCAs leading thus to a plethora of side effects[10,12–17]. Since many CA isoforms are involved in diverse physio/pathological conditions such as glaucoma (hCA I, II, IV and XII), edema (hCA II, IV, XIV as the most important), central nervous system (CNS)-related pathologies (hCAVII and XIV are particularly involved in the epilepsy) and tumors (hCA IX and hCA XII are strictly associated with hypoxic tumors), it is not surprising that CAIs are used in the clinic for some applications for almost 70 years[18–24]. Many synthetic efforts have been made for the development of specific CAIs: in the last 15 years, in addition to the classical ''tail approach''[10–17,25–28] which is mainly applied to classical sulfonamide inhibitors and their isosters, novel CAIs scaffolds have been also identified such as the polyamines[29], phenols[30–32], dithiocarbamates[33–36], xanthates[37], coumarins, thiocoumarins, 2-thioxocoumarins, coumarine oximes[4,38,39]. Sulfocoumarins are the latest CAI class identified and similarly to the coumarins showed the most selective inhibition profiles against the pathologically valuable CA isoforms[1,9,40–44].

In analogy to coumarins, the substitution pattern at the sulfocoumarin scaffolds strongly influences the potency and selectivity profile against different hCA isoforms[1,40–44]. Indeed, in our previous reports[40–43], we investigated large series of sulfocoumarins bearing the tetrazolyl, triazolyl or aryl/alkyl moieties at 6 position, which showed low nanomolar hCAIX/XII inhibitory potencies, without particular effects on the cytosolic hCAI/II. On the contrary, derivatives bearing small

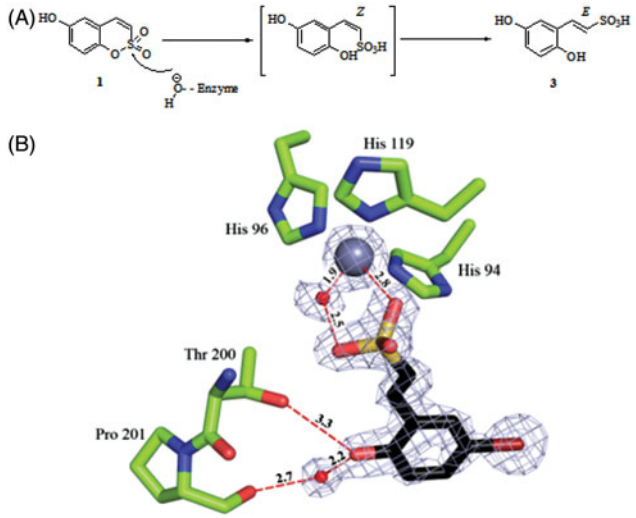


Figure 1. CA inhibition mechanism of sulfocoumarins. (A) The sulfocoumarin undergoes an enzyme-mediated hydrolysis with the formation of the trans-2-hydroxy-phenyl-ω-ethenylsulfonic acid **3**. (B) The sulfonic acid **3** binds to the hCA II active site, by anchoring to the zinc-coordinated water molecule. The Zn(II) ion (central larger sphere), its three His ligands (His94, 96 and 119), the water molecule coordinated to the zinc (small sphere) as well as active site residues Thr200 and Pro201 involved in the binding of the hydrolyzed sulfocoumarin are shown, as determined by X-ray crystallography (PDB file 4BCW)[1].

substituents as well as benzyl esters at the 7 position act as low nanomolar hCAII inhibitors[40].

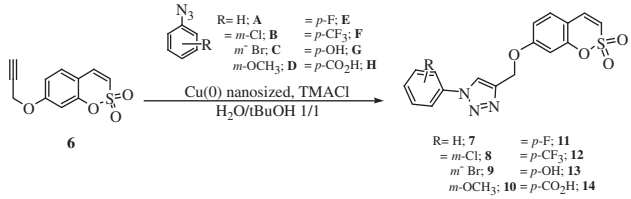
To date, sulfocoumarins incorporating bulky and flexible moieties at 7 position were not reported, thus herein we report the synthesis, characterization and *in vitro* inhibition profiles against four relevant hCA isoforms (hCAI, II, IX and XII) of a small series of 7-substituted sulfocoumarins bearing the aryl-triazolyl moieties linked through an oxymethylene group, which were synthesized by means of the click chemistry approach.

Materials and methods

Chemistry

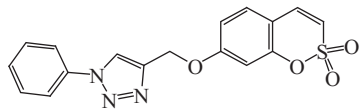
Anhydrous solvents and all reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Haverhill, MA) and TCI (Eschborn, Germany). All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringe techniques to transfer solutions. Nuclear magnetic resonance (1H-NMR, 13C-NMR) spectra were recorded using a Bruker Avance III 400 MHz spectrometer in deuterated dimethyl sulfoxide (DMSO-d6). Chemical shifts are reported in parts per million 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 (OH and NH) 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 American Society for Testing and Materials (ASTM)) as the stationary phase and ethyl acetate/n-hexane were used as eluents. Melting points (m.p.) were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected. All compounds were >95% pure by high performance liquid chromatography (HPLC).

General synthetic procedure of compounds 7–14[45]



7-Prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (1.0 eq) was added to a suspension of aryl azide **A–H** (1.1 eq) in H_2O/^{t}BuOH 1/1 (3.5 ml) at room temperature (r.t.), followed by addition of copper (0) nanosized (0.1 eq) and tetramethylammoniun chloride (TMACl) (1.0 eq). 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 dichloromethane (DCM) to afford the titled compounds **7–14.**

*Synthesis of 4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-phenyl-*1H*-[1,2,3]-triazole **7***

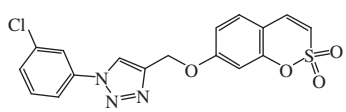


7

4-(2,2-Dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-phenyl-1*H*-[1,2,3]-triazole **7** was obtained according to the general procedure earlier reported using phenylazide **A** (1.1 eq), 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq) in ^{t}BuOH/H_2O (1/1, 3.5 ml), TMACl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 7 h to give the titled compound **7** as a white solid.

*4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-phenyl-1*H*-[1,2,3]triazole **7**.* Seventy-two percent yield; m.p. 141–143 °C; silica gel TLC R_f 0.39 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 5.41 (s, 2H, CH_2), 7.16 (dd, $J = 2.4$, 8.8, 1H), 7.30 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.55 (t, $J = 7.6$, 1H), 7.67 (m, 4H), 7.95 (d, $J = 7.6$, 2H), 9.05 (s, 1H); δ_C (100 MHz, DMSO-d_6): 62.7, 105.6, 113.2, 114.4, 120.3, 121.2, 124.2, 129.8, 130.7, 132.2, 137.4, 137.4, 143.9, 153.2, 161.9; *m/z* (ESI positive) 356.0 $[M + H]^+$.

*Synthesis of 1-(3-chloro-phenyl)-4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1*H*-[1,2,3]-triazole **8***

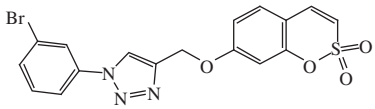


8

1-azido-3-chlorobenzene **B** (1.1 eq) and 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq) were dissolved in ^{t}BuOH/H_2O 1/1 (3.5 ml) and then TMACl (1.0 eq) and copper (0) nanosize (10% mol) were added. The mixture was stirred at 60 °C for 3.5 h, and then treated as described in general procedure earlier reported to afford **8** as a white solid.

*1-(3-Chloro-phenyl)-4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1*H*-[1,2,3]-triazole 8.* Seventy-one percent yield; m.p. 174–176 °C; silica gel TLC R_f 0.26 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 5.41 (s, 2H, CH_2), 7.16 (dd, $J = 2.4$, 8.8, 1H), 7.28 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.66 (m, 4H), 7.97 (d, $J = 8.8$, 1H), 8.10 (s, 1H), 9.11 (s, 1H); δ_C (100 MHz, DMSO-d_6): 62.6, 105.6, 113.2, 114.4, 119.7, 120.3, 121.0, 124.4, 129.6, 132.2, 132.6, 135.2, 137.4, 138.5, 144.1, 153.2, 161.8; *m/z* (ESI positive) 390.0 $[M + H]^+$.

*Synthesis of 1-(3-bromo-phenyl)-4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1*H*-[1,2,3]-triazole **9***

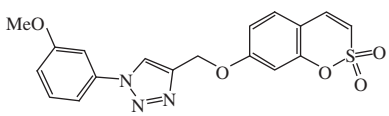


9

1-Azido-3-bromobenzene **C** (1.1 eq) and 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq) were dissolved in ^{t}BuOH/H_2O 1/1 (3.5 ml) and then TMACl (1.0 eq) and copper (0) nanosize (10% mol) were added. The mixture was stirred at 60 °C for 3.5 h and then treated as described in general procedure earlier reported to afford **9** as a white solid.

*1-(3-Bromo-phenyl)-4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1*H*-[1,2,3]-triazole **9**.* Seventy-nine percent yield; m.p. 171–173 °C; silica gel TLC R_f 0.49 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 5.41 (s, 2H, CH_2), 7.16 (dd, $J = 2.4$, 8.8, 1H), 7.30 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.61 (t, $J = 8.4$, 1H), 7.69 (m, 2H), 7.76 (d, $J = 8.4$, 1H), 8.01 (d, $J = 8.4$, 1H), 8.23 (t, $J = 2.0$, 1H), 9.11 (s, 1H); δ_C (100 MHz, DMSO-d_6): 62.7, 105.6, 113.3, 114.4, 120.2, 120.4, 123.4, 123.8, 124.4, 132.2, 132.6, 132.8, 137.4, 138.6, 144.1, 153.2, 161.9; *m/z* (ESI positive) 434.0 $[M + H]^+$.

*Synthesis of 4-(2,2-Dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(3-methoxy-phenyl)-1*H*-[1,2,3]-triazole **10***

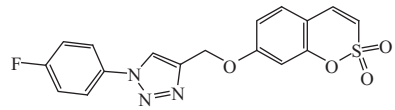


10

4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(3-methoxy-phenyl)-1*H*-[1,2,3]-triazole **10** was obtained according the general procedure earlier reported using 1-azido-3-methoxybenzene **D** (1.1eq), 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq) in ^{t}BuOH/H_2O (1/1, 3.5 ml), TMACl (1.0 eq) and copper nanosize (0.1 eq). The reaction mixture was stirred for 6.5 h to give the titled compound **10** as a white solid.

*4-(2,2-Dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(3-methoxy-phenyl)-1*H*-[1,2,3]-triazole **10**.* Sixty-eight percent yield; m.p. 129–131 °C; silica gel TLC R_f 0.40 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 3.90 (s, 3H, CH_3), 5.41 (s, 2H, CH_2), 7.11 (d, $J = 7.2$, 1H), 7.16 (dd, $J = 2.4$, 8.8, 1H), 7.30 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.54 (m, 3H), 7.69 (m, 2H), 9.06 (s, 1H); δ_C (100 MHz, DMSO-d_6): 56.6, 62.7, 105.6, 106.8, 113.1, 113.2, 114.3, 115.5, 120.3, 124.3, 131.8, 132.1, 137.4, 138.5, 143.9, 153.2, 161.1, 161.9; *m/z* (ESI positive) 386.0 $[M + H]^+$.

*Synthesis of 4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(4-fluoro-phenyl)-1*H*-[1,2,3]-triazole **11***

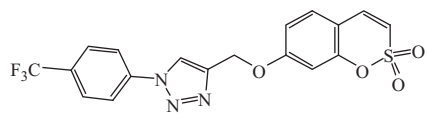


11

1-Azido-4-fluorobenzene **E** (1.1 eq) and 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq) were dissolved in ^{t}BuOH/H_2O 1/1 (3.5 ml) and then TMACl (1.0 eq) and copper (0) nanosize (10% mol) were added. The mixture was stirred at 60 °C for 2.5 h and then treated as described in general procedure earlier reported to afford **11** as a white solid.

*4-(2,2-Dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(4-fluoro-phenyl)-1*H*-[1,2,3]-triazole **11**.* Sixty-three percent yield; m.p. 168–170 °C; silica gel TLC R_f 0.33 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 5.41 (s, 2H, CH_2), 7.16 (dd, $J = 2.4$, 8.8, 1H), 7.29 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.51 (t, $J = 8.8$, 2H), 7.69 (m, 2H), 8.00 (m, 2H), 9.01 (s, 1H); δ_F(376 MHz, DMSO-d_6): −112.92 (s, 1F); δ_C (100 MHz, DMSO-d_6): 62.7, 105.6, 113.2, 114.4, 117.7 (d, $J^2CF = 23.0$), 120.3, 123.6 (d, $J^1CF = 8.9$), 124.5, 132.2, 134.0, 137.4, 144.0, 153.2, 161.9, 162.7 (d, $J^1CF = 245.2$); *m/z* (ESI positive) 374.0 $[M + H]^+$.

*Synthesis of 4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(4-trifluoromethyl-phenyl)-*1H*-[1,2,3]-triazole **12***

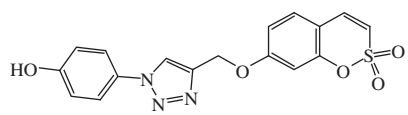


12

1-Azido-4-trifluoromethylbenzene **F** (1.1 eq) and 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq) were dissolved in ^{t}BuOH/H2O 1/1 (3.5 ml) and then TMACl (1.0 eq) and copper (0) nanosize (10% mol) were added. The mixture was stirred at 60 °C for 5 h and then treated as described in general procedure earlier reported to afford **12** as a white solid.

*4-(2,2-Dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(4-trifluoromethyl-phenyl)-1*H*-[1,2,3]-triazole **12**.* Seventy-four percent yield; m.p. 210–212 °C; silica gel TLC R_f0.40 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 5.43 (s, 2H, CH_2), 7.16 (dd, $J = 2.4$, 8.8, 1H), 7.29 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.69 (m, 2H), 8.04 (d, $J = 8.4$, 2H), 8.22 (d, $J = 8.4$, 2H), 9.19 (s, 1H); δ_C (100 MHz, DMSO-d_6): 62.7, 105.6, 113.3, 114.4, 120.4, 121.7, 124.5, 124.8 (d, $J^1CF = 270.4$), 128.2, 129.8 (d, $J^2CF = 32.0$), 132.2, 137.4, 140.3, 144.4, 153.3, 161.9; *m/z* (ESI positive) 324.0 $[M + H]^+$.

*Synthesis of 4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(4-hydroxy-phenyl)-1*H*-[1,2,3]-triazole **13***



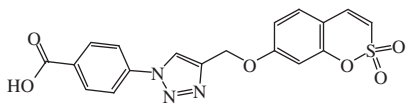
13

1-Azido-4-hydroxybenzene **G** (1.1 eq) and 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq)

were dissolved in ^{t}BuOH/H_2O 1/1 (3.5 ml) and then TMACl (1.0 eq) and copper (0) nanosize (10% mol) were added. The mixture was stirred at 60 °C for 4 h and then treated as described in general procedure earlier reported to afford **13** as a white solid.

*4-(2,2-Dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1-(4-hydroxy-phenyl)-*1H*-[1,2,3]-triazole* 13. Eighty-two percent yield; m.p. 244–246 °C; silica gel TLC R_f 0.35 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 5.38 (s, 2H, CH_2), 6.98 (d, $J = 8.8$, 2H), 7.15 (dd, $J = 2.4$, 8.8, 1H), 7.28 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.69 (m, 4H), 8.84 (s, 1H), 10.00 (s, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6): 62.7, 105.6, 113.2, 114.3, 117.0, 120.3, 123.0, 124.1, 129.6, 132.1, 137.42, 143.5, 153.2, 158.8, 161.9; *m/z* (ESI positive) 372.0 $[M + H]^+$.

*Synthesis of 1-(4-carboxy-phenyl)-4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1*H*-[1,2,3]-triazole* ***14***

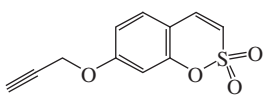


14

1-azido-carboxybenzene **H** (1.1 eq) and 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide **6** (0.05 g, 1.0 eq) were dissolved in ^{t}BuOH/H_2O 1/1 (3.5 ml) and then TMACl (1.0 eq) and copper (0) nanosize (10% mol) were added. The mixture was stirred at 60 °C for 12.5 h and then treated as described in general procedure earlier reported to afford **14** as a white solid.

*1-(4-Carboxy-phenyl)-4-(2,2-dioxo-2*H*-2λ^6-benzo-[e][1,2]-oxathiin-7-yloxymethyl)-1*H*-[1,2,3]-triazole* ***14***. Twenty-two percent yield; m.p. >300 °C; silica gel TLC R_f 0.42 (MeOH/DCM 10% *v/v*); δ_H (400 MHz, DMSO-d_6): 5.43 (s, 2H, CH_2), 7.17 (dd, $J = 2.4$, 8.8, 1H), 7.30 (d, $J = 2.4$, 1H), 7.38 (d, $J = 10.4$, 1H), 7.69 (m, 2H), 8.11 (d, $J = 8.4$, 2H), 8.19 (d, $J = 8.4$, 2H), 9.16 (s, 1H), 13.31 (bs, 1H, exchange with D_2O, COO*H*); *m/z* (ESI positive) 400.0 $[M + H]^+$.

Synthesis 7-prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide 6

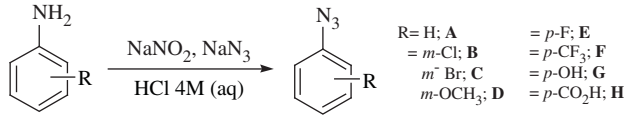


6

Propargyl bromide (1.2 eq) was added to a suspension of compound **5** (0.5 g, 1.0 eq) and K_2CO_3 (2.0 eq) in dry DMF (4 ml) under a nitrogen atmosphere and the mixture was stirred at r.t. for 2 h. The reaction mixture was quenched with H_2O (20 ml) and extracted with EtOAc (3 × 15 ml). The organic layer was washed with brine (4 × 15 ml), dried over Na_2SO_4, filtered off and concentrated under *vacuo* to give a residue that was triturated with Et_2O to afford the titled compound **6** as a white powder.

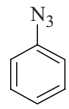
7-Prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide ***6***. Fifty-four percent yield; m.p. 150–151 °C; silica gel TLC R_f 0.83 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 3.70 (t, $J = 2.4$, 1H), 4.98 (d, $J = 2.4$, 2H), 7.07 (dd, $J = 2.4$, 8.4, 1H), 7.15 (d, $J = 2.4$, 1H), 7.37 (d, $J = 10.4$, 1H), 7.68 (m, 2H); δ_C (100 MHz, DMSO-d_6): 57.21, 79.28, 80.03, 105.70, 113.43, 114.42, 120.51, 132.11, 137.40, 153.07, 161.03.

Synthesis of phenylazides A–H[46]



The proper aniline (0.5 g, 1.0eq) was dissolved in a 4 M HCl aqueous solution (5 ml) at 0 °C. $NaNO_2$ (1.2 eq) was slowly added and the resulting solution was stirred at the same temperature for 0.5 h. Then NaN_3 (1.5 eq) was added portion-wise and the mixture was stirred at r.t. for 0.5 h. The 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.

Synthesis of phenylazide ***A***



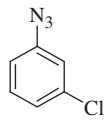
A

Phenylazide **A** was obtained according to the general procedure reported earlier.

Phenylazide A. Sixty percent yield; silica gel TLC R_f 0.76 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6):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-d_6): 120.0, 126.1, 131.0, 140.3.

Experimental in agreement with reported data[46].

Synthesis of 3-chlorophenylazide ***B***



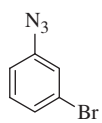
B

3-Chlorophenylazide **B** was obtained according to the general procedure reported earlier.

3-Chlorophenylazide ***B***. Eighty-six percent yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6):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-d_6): 118.9, 120.1, 126.0, 132.4, 135.1, 142.2.

Experimental in agreement with reported data[47].

Synthesis of 3-bromophenylazide ***C***



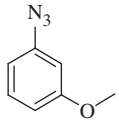
C

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

3-Bromophenylazide ***C***. Eighty-eight percent yield; silica gel TLC R_f 0.83 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6):7.18 (dt, $J = 1.6$, 7,2, 1H), 7.38 (m, 3H); δ_C (100 MHz, DMSO-d_6): 119.3, 122.8, 123.5, 128.9, 132.7, 142.3.

Experimental in agreement with reported data[47].

Synthesis of 3-methoxyphenylazide D



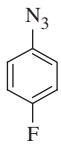
D

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

*3-Methoxyphenylazide **D**.* Seventy-four percent yield; silica gel TLC R_f 0.78 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 3.80 (s, 3H, C*H*$_3$), 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-d_6): 56.3, 105.7, 112.0, 112.1, 131.7, 141.5, 161.5.

Experimental in agreement with reported data[46].

Synthesis of 4-fluorophenylazide E

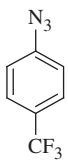


E

4-Fluorophenylazide **E** was obtained according to the general procedure reported earlier.

*4-Fluorophenylazide **E**.* Eighty-nine percent yield; silica gel TLC R_f 0.79 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 67.19 (m, 2H), 7.29 (t, $J = 8.8$, 2H); δ_F (376 MHz, DMSO-d_6): −117.77 (s, 1F); δ_C (100 MHz, DMSO-d_6): 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[47].

*Synthesis of 4-trifluoromethyl-phenylazide **F***



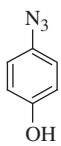
F

4-Trifluoromethylphenylazide **F** was obtained according to the general procedure reported earlier.

*4-Trifluoromethylphenylazide **F**.* Sixty-seven percent yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 7.35 (d, $J = 8.8$, 2H), 7.78 (d, $J = 8.8$, 2H); δ_F(376 MHz, DMSO-d_6): −56.18 (s, 3F); δ_C (100 MHz, DMSO-d_6): 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[48].

*Synthesis of 4-hydroxy-phenylazide **G***



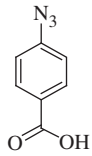
G

4-hydroxyphenylazide **G** was obtained according to the general procedure reported earlier.

4-Hydroxyphenylazide G. Forty-six percent yield; silica gel TLC R_f 0.84 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 6.84 (d, $J = 8.8$, 2H), 6.97 (d, $J = 8.8$, 2H), 9.56 (bs, 1H, exchange with D_2O, O*H*); δ_C (100 MHz, DMSO-d_6): 117.5, 121.1, 130.6, 156.0.

Experimental in agreement with reported data[49].

*Synthesis of 4-azidobenzoic acid **H***



H

4-azidobenzoic acid **H** was obtained according to the general procedure 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 **H** as a yellow solid.

4-Azidobenzoic acid H. Seventy-three percent yield; m.p. 188–190 °C dec; silica gel TLC R_f 0.71 (EtOAc/*n*-hexane 50% *v/v*); δ_H (400 MHz, DMSO-d_6): 7.25 (d, $J = 8.4$, 2H), 7.99 (d, $J = 8.4$, 2H), 13.02 (bs, 1H, exchange with D_2O, COO*H*); δ_C (100 MHz, DMSO-d_6): 120.2, 128.3, 132.2, 144.9, 167.6. Experimental in agreement with reported data[50].

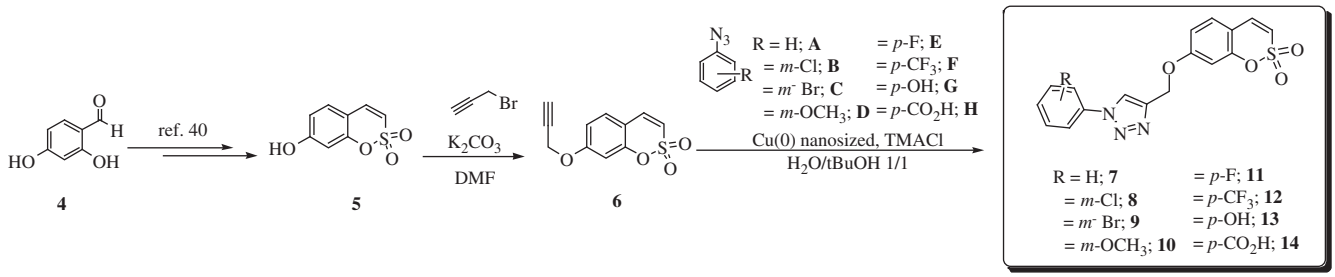
CA inhibition

An applied photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[51]. 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

Table 1. Inhibition data of human CA isoforms hCA I, II, IX and XII with sulfocoumarins 7–14 reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped-flow CO_2 hydrase assay[51].

		K_I (nM)*			
Compound	R	hCAI	hCA II	hCA IX	hCA XII
7	–	>10 000	>10 000	25.3	4.5
8	3-Cl	2301	>10 000	25.6	4.5
9	3-Br	>10 000	>10 000	36.5	5.5
10	3-OCH3	>10 000	>10 000	24.1	4.3
11	4-F	984	>10 000	26.8	5.5
12	4-CF3	>10 000	>10 000	19.0	5.2
13	4-OH	>10 000	>10 000	19.6	19.1
14	4-COOH	>10 000	>10 000	19.2	4.6
AAZ	–	250	12	25	5.7

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



Scheme 1. Synthesis of 7-substituted sulfocoumarins **7–14**.

dilutions up to 0.01 nm were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min to 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[1–4,7]. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier[2,5], and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier[52–56].

Results and discussion

CA inhibition

Sulfocoumarins **7–14** were screened *in vitro* for the inhibition against four physiologically relevant hCA isoforms, the cytosolic hCAI and II and the trans-membrane tumor-associated hCA IX and XII[10,12,24,57–60]. Table 1 shows the inhibition data obtained and compared to the standard clinically used sulfonamide acetazolamide (**AAZ**) after a period of incubation of 6 h of the enzyme and inhibitors. Noteworthy the assay inhibition performed within the usual 15 min incubation period (as for the sulfonamides)[51] led to the very weak inhibition constants (data not shown). For this reason, herein is reported a 6 h incubation time instead.

The following structure-activity relationship (SAR) should be noted:

(i) According to our previous reports[40–44], isoform hCA I was not or was poorly inhibited by sulfocoumarins **7–14**. Compounds **8** and **11** are high micromolar inhibitors, whereas the remaining ones did not significantly inhibit the enzyme below 10 μm inhibitor concentration.

(ii) The inhibition profile shown by sulfocoumarins **7–14** on the remaining three CA isoforms was quite surprising and unexpected. Indeed the 7-substituted sulfocoumarins earlier[40] reported were effective and selective inhibitors against the hCA II over the tumor-associated isoforms IX and XII. Surprisingly derivatives **7–14** herein reported did not exhibit any activity against the cytosolic isoform hCA II and were low nanomolar inhibitors against the transmembrane isoforms IX and XII (Table 1). Such a behavior might be ascribed that the previously synthesized derivatives incorporated small moieties or benzyl esters at the 7 position, whereas sulfocoumarins **7–14** bear flexible and bulky aryltriazolyl moieties via a CH_2O linker.

(iii) As mentioned, the transmembrane isoforms were strongly inhibited by the new sulfocoumarins **7–14** here reported, with K_I values spanning between 19.0 and 36.5 nM for the hCA IX and 4.3–19.1 nM for the hCA XII, respectively. These values were comparable with the clinically used sulfonamide **AAZ** (K_I: 2.5–5.7 nM). The definition of a proper SAR for hCA IX and hCA XII is not feasible, considering that all these compounds showed similar enzyme affinities. However the data we reported clearly showed that the nature of the substitution pattern -R on the phenyl moiety has a weak influence on the inhibitory properties, whereas the substitution itself within the sulfocoumarin scaffold at 7 position plays a pivotal role addressing the selectivity profiles.

Chemistry

The general strategy of Zabulovski's group[1,9] for the preparation of 6-substituted sulfocoumarins was recently validated by us for the synthesis of 7-substituted such derivatives. As reported[40], the regioselective protection of the commercially available 2,4-dihydroxybenzaldehyde **4**, followed by intramolecular cyclization and aromatization, afforded the desired 7-hydroxysulfocoumarin **5**, which finally was converted to the derivative **6** through reaction with propargyl bromide (Scheme 1). Then, **6** was reacted with various freshly prepared aryl azides **A–H** in the presence of copper (0) nanosized as catalyst[45] to afford the desired 1,2,3-triazolyl derivatives **7–14** (Scheme 1).

Conclusion

Herein, we report a series of 7-substituted sulfocoumarins bearing the 1,2,3-triazolyl moieties via a CH_2O linker obtained by the click chemistry approach. Unlike 7-substituted sulfocoumarins investigated earlier,[40] which were potent and selective hCA II inhibitors and ineffective as hCA I, IX and XII inhibitors, compounds from this new series exhibit effective and selective inhibitory properties for the tumor-associated isoforms over hCA IX and XII.

As hCA IX and hCA XII were recently validated as antitumor/antimetastatic drug targets, with one inhibitor in Phase I clinical trials[61], this prodrug, isoform-selective CAIs, reported here might be considered of interest for such biomedical applications.

Declaration of interest

This work was financed in part by a Marie Curie ITN FP7 project (Dynano). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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