Synthesis and Antiproliferative Activity of 3 and 7-Styrylcoumarins

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Abstract

A series of styrylcoumarins were obtained via Mizoroki-Heck reactions between 3-bromo-4-methyl-7-(octyloxy)-2*H*-chromen-2-one or 2-oxo-2*H*-chromen-7-yl trifluoromethanesulfonate and functionalized styrenes. The structures of the products were elucidated by spectroscopic analysis. All compounds were evaluated against SW480 and CHO-K1 cell lines. A number of hybrids showed good antiproliferative activity. Among the tested compounds, hybrids **6e**, **10c** and **10d**, exhibited the highest activity ($IC_{50\text{- }SW480/48h}$ = 6,92; 1,01 and 5,33 µM, respectively) and selectivity (IS_{48h} = >400; 67,8 and 7,2, respectively). In addition, these compounds were able to preserve their activities over time. The results achieved by these hybrids were even better than the lead compounds (coumarin and resveratrol) and the standard drug (5-FU). As regards structure-activity relationship it seems that the location of the styryl group on the coumarin structure and the presence of the hydroxyl group on the phenyl ring were determinant for the activity.

Keywords: coumarin, styrylcoumarin, antiproliferative activity, colorectal cancer

Abbreviations: 5-FU: 5-Fluorouracil; CRC: Colorectal Cancer; DMSO: dimethylsulfoxide; NF-κB: Nuclear factor-κB.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death worldwide (Arnold *et al*., 2016). This pathology presents a great geographical distribution and the patterns are very similar among men and women, being the third most common cancer in men and the second one in women (Globocan, 2012).

Current treatments for CRC include combinations of chemotherapeuthic agents such as FOLFOX (5-FU/leucovorin/oxaliplatin) and FOLFIRI (folic acid/5-FU/irinotecan) which are composed of 5-fluorouracil as the backbone of treatment, that, although effective, cause undesirable gastrointestinal and neurological side-effects which many times result in dose limitations or cessation of the anti-cancer therapy (McQuade *et al*., 2014; Pointet & Taieb, 2017). Thus, it becomes necessary to discover new, more potent, and selective agents for treating this disease.

In this regard, many pharmacological studies dealing with coumarins as a key structural motif have been reported. The pharmacological actions with which coumarins are endowed include anti-HIV (Kashman 1992), anti-inflammatory (Resch *et al.,* 1998), anticoagulant (Madari *et al.,* 2003), antimicrobial (Ankleka *et al.,* 2003), antiplatelet (Vilar *et al.,* 2006), antiprotozoal (Arango *et al.,* 2010; Pierson *et al.,* 2010) and antioxidant (Gacche *et al.,* 2012). Besides, some coumarins have exhibited antimycobacterial activity which is potentiated when they bear a fatty acid residue in their structure (Yee *et al.,* 2005). Furthermore, coumarins have also shown inhibition of cell proliferation in different types of cancer cell lines (Egan *et al.,* 1997; Kawaii *et al.,* 2001; Budzisz *et al*., 2003; Yang *et al*., 2006; Riveiro *et al*., 2008). According to this, some coumarin antibiotics can bind to the carboxyl terminus of Hsp90 molecular chaperone, which modulate multiple oncogenic pathways and is overexpressed in human malignancies, causing its inhibition (Neckers *et al*., 2002; Zhao *et al*., 2011). On the other hand, natural product resveratrol, which belongs to the family of compounds known as stilbenes, has great significance because of its wide variety of biological properties (Baur *et al.,* 2006; Kedzierski *et al.,* 2007; Udenigwe *et al.,* 2008; Gülçin *et al.,* 2010), including among them anticancer activity (Borriello *et al.,* 2014). Resveratrol inhibits CRC in all phases acting on different targets such as NF-κB, AP-1, cyclooxygenase and kinases (Athar *et al*., 2009). This compound enhances the activation of tumor suppressor p53 which ultimately induces apoptosis of human colon cancer cells. It also activates caspases 3 and 8 and increases the BAX/Bcl-2 ratio (Temraz *et al.,* 2013).

The combination of two pharmacological agents into a single molecule, which leads to what is known as a hybrid molecule, has resulted in an emerging strategy in medicinal chemistry and drug discovery research (Meunier, 2008). These hybrid molecules may display dual activity, but

do not necessarily act on the same biological target (Tsogoeva, 2010, atención este review sobre moléculas híbridas es más reciente que el de 2010, quizá sea interesant incluirlo también: Shaveta, Mishra, S, Singh. P (2016) Hybrid molecules: The privileged scaffolds for various pharmaceuticals. Eur J Med Chem 124: 500-536). Combining chemotherapy with agents having different mechanisms of action is one of the methods adopted for treating cancer (Mayur *et al.,* 2009; Solomon *et al.,* 2009). Several stilbene-coumarin hybrids were identified as very promising candidates by their excellent antiproliferative potency or by their remarkable apoptosis-inducing activity (see fig. 1). Compound **1a** and **1b** showed high activity against lung carcinoma H460 with IC_{50} values of 0.45 and 0.29 µM, respectively (Belluti *et al.,* 2010). Styrylcoumarin **1c** was evaluated for its antitumor activity against MCF-7 and HCT-28 tumor cell lines. This compound showed varying degrees of growth inhibition on the above mentioned cell lines with IC_{50} values of 7.32 and 3.78 µM, respectively (Shen *et al.,* 2013). 3-Phenylcoumarin **1d** exhibited potent antiproliferative activity with IC_{50} values of 5.2 and 7.5 µM against HL-60 and A549 cell lines, respectively (Yang *et al.,* 2011). Benzimidazole-coumarin hybrid **1e** was screened for *in vitro* antitumor activity on different cell lines. This compound, tested at a concentration of 10 µM, induced more than 50% inhibition of most of the cell lines, with higher selectivity against leukemic cancer cell lines (Paul *et al.*, 2013). Coumarin-chalcone hybrid **1f** showed around 30 fold more selectivity towards C33A (cervical carcinoma) cells over normal fibroblast NIH3T3 cells with an IC_{50} value of 3.59 µM (Sashidhara *et al.,* 2010). The resveratrol-imidazole hybrid **1g** showed cytotoxic activity against leukemia and renal cancer cell lines (4.1 and 8.3 µM, respectively) (Bellina *et al.*, 2015). Resveratrol-chalcone conjugate **1h** showed high selectivity towards certain ovarian cancer, non-small cell lung cancer and breast cancer cell lines, with GI_{50} values in the range of 1.28-34.1 µM (Kumar *et al.*, 2014).
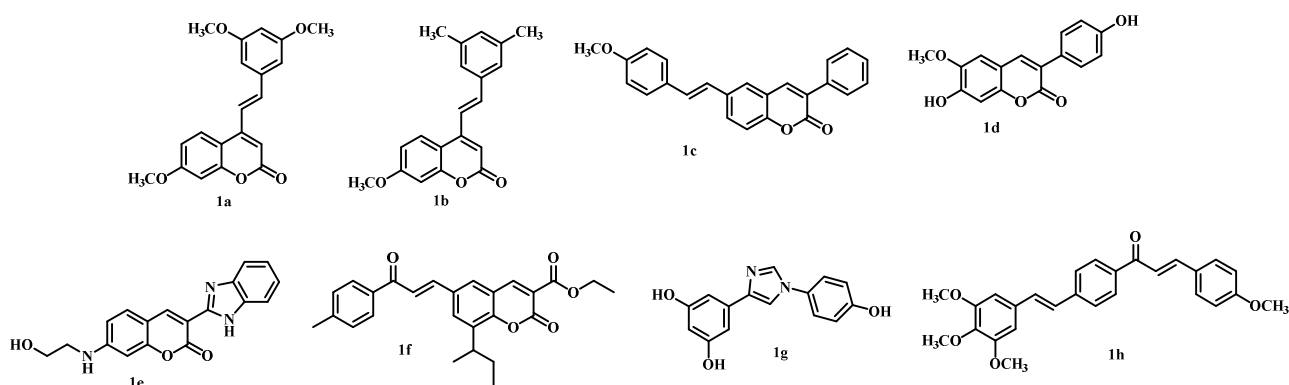

Fig. 1. Hybrid compounds endowed with antitumor activity

Hybridizing the coumarin nucleus with other moieties has afforded new molecules with improved anticancer activity profile (Agarwal, 2000). In the search for new therapeutic alternatives to treat colorectal cancer, we have designed and synthesized a series of stilbene-coumarin hybrids (styrylcoumarins) (fig. 2). In addition, the anti-cancer activity of our hybrid molecules have been evaluated *in vitro*.
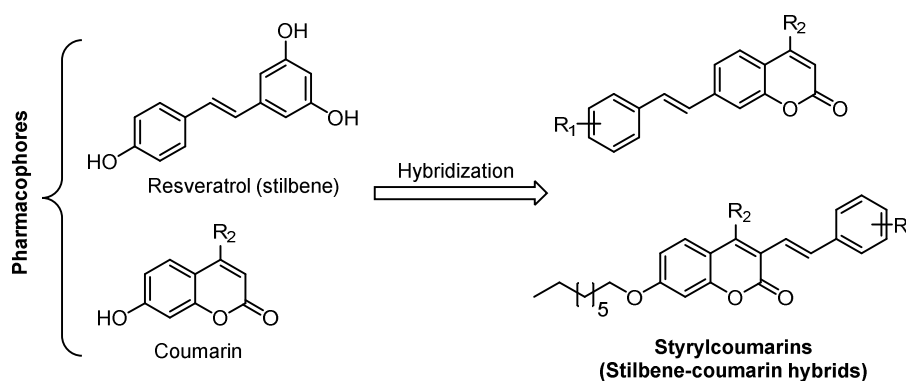

**Fig. 2**. Design of stilbene-coumarin hybrids as anti-cancer agents.

Materials and Methods

Chemistry

Microwave reactions were carried out in a CEM Discover microwave reactor in sealed vessels (monowave, maximum power 300 W, temperature control by IR sensor, fixed temperature). ^{1}H and ^{13}C NMR spectra were recorded on a Varian instrument operating at 500 (300) and 125 (75) MHz, respectively. The signals of the deuterated solvent (CDCl$_3$) were used as reference (CDCl$_3$: $\delta = 7.27$ ppm for ^{1}H NMR and $\delta = 77.00$ ppm for ^{13}C NMR). Carbon atom types (C, CH, CH$_2$, CH$_3$) were determined by using the DEPT or APT pulse sequence. Signals were assigned using two-dimensional heteronuclear correlations (COSY and HSQC). High resolution mass spectra were recorded using electrospray ionization mass spectrometry (ESI-MS). A QTOF Premier instrument with an orthogonal Z-spray-electrospray interface (Waters, Manchester, UK) was used operating in the W-mode. The drying and cone gas was nitrogen set to flow rates of 300 and 30 L/h, respectively. Methanol sample solutions (ca. 1×10^{-5} M) were directly introduced into the ESI spectrometer at a flow rate of 10 µL/min. A capillary voltage of 3.5 kV was used in the positive scan mode, and the cone voltage set to $Uc = 10$ V. For accurate mass measurements, a 2 mg/L standard solution of leucine enkephalin was introduced via the lock spray needle at a cone voltage set to 85 V and a flow rate of 30 µL/min. IR spectra were recorded on a Spectrum RX I FT-IR

system (Perkin-Elmer, Waltham, MA, USA) in KBr disks. Silica gel 60 (0.063–0.200 mesh, Merck, Whitehouse Station, NJ, USA) was used for column chromatography, and precoated silica gel plates (Merck 60 F254 0.2 mm) were used for thin layer chromatography (TLC). Monitoring of the reaction progress and product purification was carried out by TLC.

Synthesis of 4-methyl-7-(octyloxy)-2H-chromen-2-one (**3**): Coumarin (1g, 5.7 mmol), potassium hydroxide (955 mg, 17 mmol) and acetonitrile (20 mL) were placed in a 50 mL flat-bottomed flask equipped with a magnetic stirring bar. The mixture was stirred and heated to reflux for a period of 5 min. Then, 1-bromooctane (1.1 mL, 1.22 g, 6.3 mmol) was added to the reaction mixture which was then refluxed for 6h. The crude reaction mixture was concentrated on a rotatory evaporator and the residue was purified by column chromatography over silica gel. Elution with hexane and a mixture of hexane-ethyl acetate (9:1 ratio) gave rise to compound **3** (1.4 g, 85% yield).
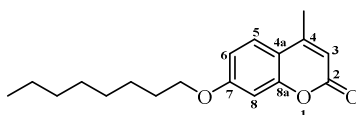

M.p. 48-50°C; IR (KBr, cm^{-1}): ν_{max} 2924 (C-H), 1718 (C=O), 1620 (C=C), 1512 (C=C$_{Ar}$), 1294 (C-O-C), 1199 ((C=O)-O), 839 (C-H$_{Ar}$). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.93 (CH$_3$, t, J = 6.5 Hz), 1.35 (3CH$_2$, m), 1.51 (CH$_2$, m), 1.64 (CH$_2$, m), 1.86 (CH$_2$, m), 2.44 (CH$_3$, s), 4.05 (-CH$_2$O-, t, J = 6.5 Hz), 6.17 (H$_3$, s), 6.85 (H$_8$, d, J = 2.3 Hz), 6.90 (H$_6$, dd, J = 8.7, 2.3 Hz) 7.53 (H$_5$, d, J = 8.7 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.11 (CH$_3$), 18.70 (CH$_3$) 22.68 (CH$_2$), 26.01 (CH$_2$), 28.99 (CH$_2$), 29.24 (CH$_2$), 29.34 (CH$_2$), 31.80 (CH$_2$), 68.65 (-OCH$_2$-), 101.34 (C$_8$), 111.82 (C$_6$), 112.74 (C$_3$), 113.40 (C$_{4a}$), 125.46 (C$_5$), 152.63 (C$_{8a}$), 155.32 (C$_4$), 161.47 (C=O), 162.29 (C$_7$). EIMS: m/z 289.1800 [M + H]$^+$, (calculated for [C$_{18}$H$_{25}$O$_3$]$^+$: 289.1804).

Synthesis of 3-bromo-4-methyl-7-(octyloxy)-2H-chromen-2-one (**4**): A solution of alkyloxycoumarin **3** (1g, 3.5 mmol) and NBS (623 mg, 3.5 mmol) in methanol (20 mL) was stirred in an ice bath. Then, *p*-TsOH (170 mg, 1.0 mmol) in methanol (10 mL) was added to the reaction mixture for 10 min. Then, the reaction mixture was stirred at room temperature for 1h. The crude reaction mixture was concentrated on a rotatory evaporator and the residue was purified by column chromatography over silica gel. Elution with hexane and a mixture of hexane-ethyl acetate (95:5 ratio) afforded compound **4** (0.96 g, 75% yield).
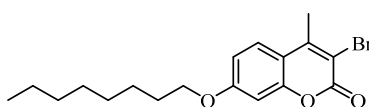
Yellow oil; IR (KBr, cm^{-1}): ν_{max} 2924 (C-H), 1737 (C=O), 1614 (C=C), 1468 (C=C$_{Ar}$), 1285 (C-O-C), 1201 ((C=O)-O), 811 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.93 (CH$_3$, t, J = 6.5 Hz), 1.35 (3CH$_2$, m), 1.51 (CH$_2$, m), 1.74 (CH$_2$, m), 1.86 (CH$_2$, m), 2.63 (CH$_3$, s), 4.05 (-CH$_2$O-, t, J = 6.5 Hz), 6.84 (H$_8$, d, J = 2.5 Hz), 6.92 (H$_6$, dd, J = 8.9, 2.5 Hz), 7.53 (H$_5$, d, J = 8.9 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.13 (CH$_3$), 19.50 (CH$_3$) 22.66 (CH$_2$), 25.98 (CH$_2$), 28.97 (CH$_2$), 29.23 (CH$_2$), 29.33 (CH$_2$), 31.81 (CH$_2$), 68.79 (-OCH$_2$-), 101.17 (C$_8$), 109.50 (C$_3$), 113.20 (C$_6$), 113.42 (C$_{4a}$), 126.01 (C$_5$), 151.23 (C$_4$), 153.63 (C$_{8a}$), 157.48 (C$_7$), 162.37 (C=O). EIMS: m/z 367.0908 [M + H]$^+$, (calculated for [C$_{18}$H$_{24}$BrO$_3$]$^+$: 367.0909).

Synthesis of 2-oxo-2H-chromen-7-yl trifluoromethanesulfonate (**9**): A solution of 7-hydroxycoumarin (**8**) (1.0 g, 6.17 mmol) in pyridine (5mL) was cooled to 0°C and treated with triflic anhydride (2.1 mL, 12.34 mmol, 20 min addition). The reaction mixture was allowed to warm to room temperature and stirred for an additional 2.5 h. The crude mixture was partitioned between saturated aqueous CuSO$_4$ and ethyl acetate. After separation of the fractions, the organic fraction was dried (Na$_2$SO$_4$) and concentrated under reduced pressure. The crude product was purified by column chromatography over silica gel. Elution with a mixture of hexane-ethyl acetate (8:2 ratio) afforded compound **9** in 85% yield (1.54 mg, 85% yield).
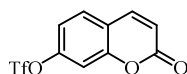
M.p. 78-80 °C; IR (KBr, cm^{-1}): ν_{max} 1720 (C=O), 1421 y 1220 S(=O)$_2$, 1130 (CF$_3$), 1294 (C-O-C), 1110 ((C=O)-O), 748-979 (C-O-S). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 6.53 (H$_3$, d, J = 9.7 Hz), 7.25 (H$_6$, dd, J = 2.3, 8.6 Hz), 7.31 (H$_8$, d, J = 2.3 Hz), 7.62 (H$_5$, d, J = 8.6 Hz), 7.76 (H$_4$, d, J = 9.7 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 110.60 (C$_8$), 117.81 (C$_3$, C$_6$), 118.66 (–SO$_3$CF$_3$, q, $J_{C–F}$ = 321 Hz), 118.80 (C$_{4a}$), 129.48 (C$_5$), 142.18 (C$_4$), 150.84 (C$_7$), 154.57 (C$_{8a}$), 159.35 (C = O). EIMS: m/z 294.9880 [M + H]$^+$, (calculated for [C$_{10}$H$_6$F$_3$O$_5$S]$^+$: 294.9884).

Styrene synthesis:

Method 1: Wittig Reaction under microwave conditions.

Potassium carbonate (0.10 mol) and (ethyl)triphenylphosphonium iodide (0.016 mol) were mixed and crushed in a mortar to a fine powder and transferred into a 250 mL Erlenmeyer flask. Aldehyde (0.016 mol) was added to the powdered mixture and thoroughly mixed. The Erlenmeyer flask was covered with a watch glass and placed into a microwave oven along with a 1L beaker of ice (to ensure that the reaction mixture did not overheat). The microwave was run at full power for 12 minutes, making sure to pause the heating and replace the ice provided it melted. A tiny sample of the crude reaction product was removed, diluted in a small amount of hexanes and analyzed by TLC

to check the reaction progress. Upon completion, the crude solids were extracted with hexanes (3 x 20 mL). The combined hexane extracts were placed in a round-bottomed flask and concentrated by rotary evaporation to obtain a yellow oil which was purified by flash chromatography on silica gel using hexanes or a mixture of hexanes/ethyl acetate as eluent.

Method 2: Knoevenagel condensation reactions:

To a solution of aldehyde (5 mmol) and malonic acid (20 mmol) in pyridine (21 mL) was added piperidine (0.75 mL, 7.6 mmol). The mixture was heated at reflux for a period of 30 min. under microwave irradiation. Then toluene (40 mL) was added to the cooled reaction mixture and the solvent volume reduced in vacuo at 30-40°C. Additional toluene (20 mL) was then added and the solvent again removed in vacuo in order to remove all traces of pyridine. This procedure afforded a residue which was purified by column chromatography over silica gel. Elution with 9:1 hexane/ethyl acetate afforded the corresponding vinylphenol.

General procedure for the Mizoroki-Heck reactions: Styrene (3mmol), 3-bromo-7-alkyloxycoumarin (**4**) or 2-oxo-2*H*-chromen-7-yl trifluoromethanesulfonate (**9**) (3 mmol), triethanolamine (3 mL) and Pd(OAc)$_2$ (0.03 g) were placed in a 25 mL flat-bottomed flask equipped with a magnetic stirring bar. The mixture was stirred and heated to reflux for a period of 30 min, under microwave irradiation. The mixture was allowed to cool and then passed through a pad of silica using ethyl acetate as the solvent. Then the filtrate was evaporated and the crude residue was purified by preparative chromatography (silica gel, hexane/methylene chloride (1:1) mixture) affording the coupling products in 35-58% yield.

3-[(*E*)-2-(2,4-dimethoxyphenyl)ethenyl]-4-methyl-7-(octyloxy)-2*H*-chromen-2-one
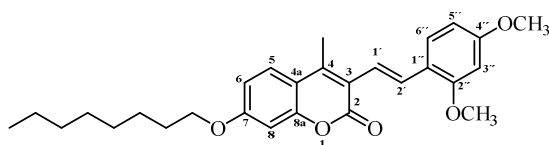
Yield: 0.39 mmol, 175mg, 39%; M.p. 106-108 °C; IR (KBr, cm^{-1}): ν $_{max}$ 2922 (C-H), 1714 (C=O), 1610 (C=C), 1504 (C=C$_{Ar}$), 1251 (C-O-C), 1153 ((C=O)-O), 834 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.94 (CH$_3$, t, *J* = 6.8 Hz), 1.35 (3CH$_2$, m), 1.52 (CH$_2$, m), 1.62 (CH$_2$, m), 1.86 (CH$_2$, m), 2.60 (CH$_3$, s), 3.89 (OCH$_3$, s), 3.91 (OCH$_3$, s) 4.06 (-CH$_2$O-, t, *J* = 6.5 Hz), 6.51 (H$_{3''}$, d , *J* = 2.5 Hz), 6.57 (H$_{5''}$, dd, J = 8.8, 2.5 Hz), 6.85 (H$_8$, d, *J* = 2.5 Hz), 6.90 (H$_6$, dd, *J* = 8.8, 2.5 Hz), 7.12 (H$_{1'}$, d, *J* = 16.6 Hz), 7.56 (H$_{6''}$, d, *J* = 8.8 Hz), 7.60 (H$_5$, d, J = 8.8 Hz), 7.74 (H$_{2'}$, d, *J* = 16.6 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.14 (CH$_3$), 15.63 (CH$_3$) 22.68 (CH$_2$), 26.0 (CH$_2$), 29.05 (CH$_2$),

29.25 (CH_2), 29.35 (CH_2), 31.82 (CH_2) 53.43 (OCH_3) 55.59 (OCH_3) 68.61 (-OCH_2-), 98.46 ($C_{3''}$) 100.90 (C_8), 104.93 ($C_{5''}$), 112.81 (C_6), 114.45 (C_{4a}), 119.76 ($C_{1''}$) 119.92 ($C_{1'}$), 120.24 (C_3) 125.82 (C_5) 127.90 ($C_{6''}$) 130.80 ($C_{2'}$) 145.38 (C_{8a}) 153.55 (C_4), 158.39 ($C_{2''}$), 160.76 (C_7), 160.87 ($C_{4''}$) 161.44 (C=O). EIMS: m/z 451.2482 $[M + H]^+$, (calculated for $[C_{28}H_{35}O_5]^+$: 451.2484).

3-[(E)-2-(4-methoxyphenyl)ethenyl]-4-methyl-7-(octyloxy)-2H-chromen-2-one
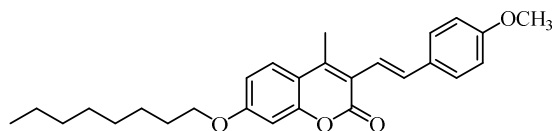
Yield: 0.37mmol, 155mg, 37%; M.p. 114-116 °C; IR (KBr, cm^{-1}): ν $_{max}$ 2926 (C-H), 1710 (C=O), 1610 (C=C), 1512 (C=C_{Ar}), 1242 (C-O-C), 1178 ((C=O)-O), 834 (C-H_{Ar}); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.94 (CH_3, t, J = 6.7 Hz), 1.35 (3CH_2, m), 1.52 (CH_2, m), 1.63 (CH_2, m), 1.86 (CH_2, m), 2.60 (CH_3, s), 3.88 (OCH_3, s), 4.06 (-CH_2O-, t, J = 6.5 Hz), 6.85 (H_8, d, J = 2.5 Hz), 6.91 (H_6, dd, J = 9.0, 2.5 Hz), 6.95 ($H_{3''}$, $H_{5''}$, d, J = 9.0 Hz), 7.07 ($H_{1'}$, d, J = 16.2 Hz), 7.53 ($H_{2''}$, $H_{6''}$, d, J = 9.0 Hz), 7.61 ($H_{2'}$, d, J = 16.2 Hz), 7.62 (H_5, d, J = 9.0 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.13 (CH_3), 15.47 (CH_3) 22.68 (CH_2), 25.98 (CH_2), 29.03 (CH_2), 29.23 (CH_2), 29.34 (CH_2), 31.81 (CH_2), 66.73 (OCH_3), 68.59 (-OCH_2-), 100.93 (C_8), 112.90 (C_6), 114.11 ($C_{3''}$, $C_{5''}$), 114.34 (C_{4a}), 118.89 ($C_{1'}$), 119.34 (C_3), 125.86 (C_5), 128.02 ($C_{2'}$, $C_{6'}$), 130.53 ($C_{1''}$), 135.08 ($C_{2'}$), 147.75 (C_{8a}), 153.57 (C_4), 159.66 ($C_{4''}$), 160.57 (C_7), 161.62 (C=O). EIMS: m/z 421.2377 $[M + H]^+$, (calculated for $[C_{27}H_{33}O_4]^+$: 421.2379).

3-[(E)-2-(3,4-dimethoxyphenyl)ethenyl]-4-methyl-7-(octyloxy)-2H-chromen-2-one
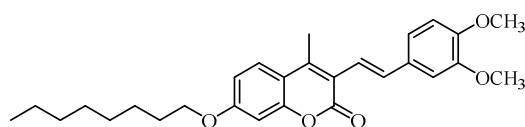
Yield: 0.38mmol, 171mg, 38%; M.p. 94-96 °C; IR (KBr, cm^{-1}): ν $_{max}$ 2920 (C-H), 1712 (C=O), 1618 (C=C), 1514 (C=C_{Ar}), 1251 (C-O-C), 1161 ((C=O)-O), 813 (C-H_{Ar}); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.94 (CH_3, t, J = 6.8 Hz), 1.35 (3CH_2, m), 1.52 (CH_2, m), 1.62 (CH_2, m), 1.87 (CH_2, m), 2.61 (CH_3, s), 3.96 (OCH_3, s), 3.99 (OCH_3, s), 4.07 (-CH_2O-, t, J = 6.5 Hz), 6.86 (H_8, d, J = 2.5 Hz), 6.92 (H_6, $H_{5''}$, d, J = 8.8, 2.5 Hz), 7.07 ($H_{1'}$, d, J = 16.6 Hz), 7.13 ($H_{2''}$, d, J = 1.8 Hz), 7.15 ($H_{6''}$, dd, J = 8.8, 1.8 Hz), 7.57 ($H_{2'}$, d, J = 16.6 Hz), 7.62 (H_5, d, J = 8.8 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.12 (CH_3), 15.54 (CH_3), 22.68 (CH_2), 26.02 (CH_2), 29.03 (CH_2), 29.24 (CH_2), 29.35 (CH_2), 31.81 (CH_2), 53.98 (2OCH_3), 68.71 (-OCH_2-), 100.90 (C_8), 109.37 ($C_{2''}$), 111.21 (C_6), 112.98 ($C_{5''}$), 114.31 (C_{4a}), 119.17 ($C_{6''}$), 119.30 (C_3), 119.90 ($C_{1'}$), 125.87 (C_5), 130.86 ($C_{1''}$),

135.37 ($C_{2'}$), 145.90 ($C_{3''}$), 149.11 ($C_{4''}$), 149.27 (C_{8a}) 153.59 (C_4), 160.61 (C_7), 161.65 (C=O).

EIMS: m/z 451.2483 [M + H]$^+$, (calculated for $[C_{28}H_{35}O_5]^+$: 451.2484).

3-[(*E*)-2-(4-hydroxy-3-methoxyphenyl)ethenyl]-4-methyl-7-(octyloxy)-2*H*-chromen-2-one
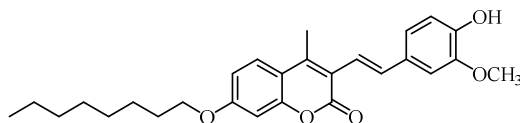

Yield: 0.36mmol, 157mg, 36%; M.p. 114-116 °C; IR (KBr, cm^{-1}): ν $_{max}$ 2924 (C-H), 1716 (C=O), 1610 (C=C), 1512 (C=C$_{Ar}$), 1257 (C-O-C), 1161 ((C=O)-O), 813 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.94 (CH$_3$, t, *J* = 6.8 Hz), 1.34 (3CH$_2$, m), 1.52 (CH$_2$, m), 1.69 (CH$_2$, m), 1.86 (CH$_2$, m), 2.60 (CH$_3$, s), 3.99 (OCH$_3$, s), 4.06 (-CH$_2$O-, t, *J* = 6.5 Hz), 6.85 (H$_8$, d, *J* = 2.5 Hz), 6.91 (H$_6$, dd, *J* = 8.8, 2.5 Hz), 6.96 (H$_{5''}$, d, *J* = 8.8 Hz), 7.04 (H$_{1'}$, d, *J* = 16.3 Hz), 7.10 (H$_{2''}$, d, *J* = 2.5 Hz), 7.12 (H$_{6''}$, dd, *J* = 8.8, 2.5 Hz), 7.55 (H$_{2'}$, d, *J* = 16.3 Hz), 7.62 (H$_5$, d, J = 8.8 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.12 (CH$_3$), 15.52 (CH$_3$), 22.66 (CH$_2$), 25.99 (CH$_2$), 29.03 (CH$_2$), 29.24 (CH$_2$), 29.36 (CH$_2$), 31.83 (CH$_2$), 56.0 (OCH$_3$), 68.67 (-OCH$_2$-), 100.90 (C_8), 109.03 ($C_{2''}$), 112.96 (C_6), 114.31 (C_{4a}), 114.58 ($C_{5''}$),118.85 ($C_{6''}$), 119.33 (C_3), 120.29 ($C_{1'}$), 125.89 (C_5), 130.38 ($C_{1''}$), 135.56 ($C_{2'}$), 145.83 ($C_{4''}$), 145.95 ($C_{3''}$), 146.69 (C_4), 153.56 (C_{8a}) 160.64 (C_7), 161.62 (C=O).EIMS: m/z 437.2331 [M + H]$^+$, (calculated for $[C_{27}H_{33}O_5]^+$: 437.2328).

3-[(*E*)-2-(4-hydroxy-3,5-dimethoxyphenyl)ethenyl]-4-methyl-7-(octyloxy)-2*H*-chromen-2-one
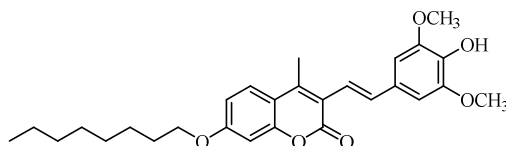

Yield: 0.41mmol, 191mg, 41%; M.p. 55-57 °C; IR (KBr, cm^{-1}): ν $_{max}$ 2927 (C-H), 1708 (C=O), 1616 (C=C), 1514 (C=C$_{Ar}$), 1253 (C-O-C), 1153 ((C=O)-O), 858 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.94 (CH$_3$, t, *J* = 6.8 Hz), 1.34 (3CH$_2$, m), 1.52 (CH$_2$, m), 1.66 (CH$_2$, m), 1.86 (CH$_2$, m), 2.61 (CH$_3$, s), 3.99 (2OCH$_3$, s), 4.02 (-CH$_2$O-, t, *J* = 6.5 Hz), 6.82 (H$_{2''}$, H$_{6''}$, s), 6.85 (H$_8$, d, *J* = 2.0 Hz), 6.91 (H$_6$, dd, *J* = 8.9, 2.0 Hz), 7.04 (H$_{1'}$, d, *J* = 16.1 Hz), 7.54 (H$_{2'}$, d, *J* = 16.1 Hz), 7.62 (H$_5$, d, J = 8.9 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.12 (CH$_3$), 15.58 (CH$_3$), 22.67 (CH$_2$), 26.0 (CH$_2$), 29.03 (CH$_2$), 29.24 (CH$_2$), 29.34 (CH$_2$), 31.83 (CH$_2$), 56.38 (2OCH$_3$), 68.67 (-OCH$_2$-), 100.90 (C_8), 103.66 ($C_{2''}$,$C_{6''}$), 112.99 (C_6), 114.26 (C_{4a}), 119.10 ($C_{1'}$),119.18 (C_3), 125.91 (C_5), 129.31($C_{2'}$),135.21 ($C_{1''}$),135.78 ($C_{4''}$),145.96 (C_4), 147.22 ($C_{3''}$, $C_{5''}$), 160.61 (C_{4a}), 161.70 (C_7), 162.47 (C=O). EIMS: m/z 467.2426 [M + H]$^+$, (calculated for $[C_{28}H_{35}O_6]^+$: 467.2434).

3-[(*E*)-2-(1,3-benzodioxol-5-yl)ethenyl]-4-methyl-7-(octyloxy)-2*H*-chromen-2-one
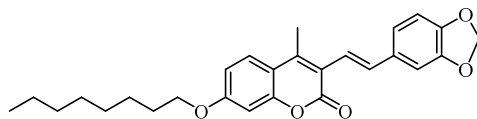

Yield: 0.49mmol, 214mg, 49%; M.p. 122-125 °C; IR (KBr, cm^{-1}): ν $_{max}$ 2926 (C-H), 1716 (C=O), 1614 (C=C), 1500 (C=C$_{Ar}$), 1234 (C-O-C), 1178 ((C=O)-O), 808 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.94 (CH$_3$, t, *J* = 6.8 Hz), 1.35 (3CH$_2$, m), 1.51 (CH$_2$, m), 1.67 (CH$_2$, m), 1.86 (CH$_2$, m), 2.58 (CH$_3$, s), 4.05 (-CH$_2$O-, t, *J* = 6.5 Hz), 6.02 (O-CH$_2$-O) 6.83 (H$_8$, d, *J* = 2.5 Hz), 6.84 (H$_{5''}$, d, *J* = 8.8, 2.5 Hz), 6.90 (H$_6$, dd, *J* = 8.8, 2.5 Hz), 7.01 (H$_{1'}$, H$_{6''}$, m), 7.12 (H$_{2''}$, d, *J* = 2.5 Hz), 7.59 (H$_{2'}$, d, *J* = 16.1 Hz), 7.60 (H$_5$, d, J = 9.1 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.13 (CH$_3$), 15.37 (CH$_3$), 22.66 (CH$_2$), 25.99 (CH$_2$), 29.03 (CH$_2$), 29.24 (CH$_2$), 29.34 (CH$_2$), 31.81 (CH$_2$), 68.60 (-OCH$_2$-), 100.83 (C$_8$), 101.18 (O-CH$_2$-O), 105.53 (C$_{2''}$), 108.45 (C$_{5''}$), 112.94 (C$_6$), 114.20 (C$_{4a}$), 118.99 (C$_{6''}$), 119.24 (C$_{1'}$), 122.07 (C$_3$), 125.87 (C$_5$), 132.33 (C$_{1''}$), 135.07 (C$_{2'}$), 146.02 (C$_{4''}$), 147.64 (C$_{3''}$), 148.17 (C$_{8a}$) 153.57 (C$_4$), 160.45 (C$_7$), 161.66 (C=O). EIMS: m/z 435.2179 [M + H]$^+$, (calculated for [C$_{27}$H$_{31}$O$_5$]$^+$: 435.2171).

3-[(*E*)-2-(2,5-dimethoxyphenyl)ethenyl]-4-methyl-7-(octyloxy)-2*H*-chromen-2-one
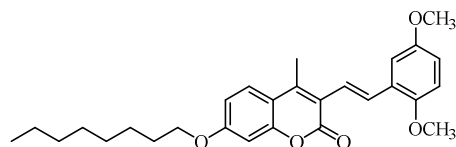
Yield: 0.35mmol, 158mg, 35%; M.p. 74-77 °C; IR (KBr, cm^{-1}): ν $_{max}$ 2922 (C-H), 1705 (C=O), 1614 (C=C), 1489 (C=C$_{Ar}$), 1253 (C-O-C), 1184 ((C=O)-O), 790 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.94 (CH$_3$, t, *J* = 6.9 Hz), 1.35 (3CH$_2$, m), 1.52 (CH$_2$, m), 1.63 (CH$_2$, m), 1.86 (CH$_2$, m), 2.61 (CH$_3$, s), 3.87 (OCH$_3$, s), 3.89 (OCH$_3$, s) 4.06 (-CH$_2$O-, t, *J* = 6.5 Hz), 6.86 (H$_8$, H$_{3''}$, H$_{4''}$, m), 6.91 (H$_6$, dd, *J* = 8.9, 2.5 Hz), 7.19 (H$_{6''}$, d, *J* = 2.7 Hz), 7.22 (H$_{1'}$, d, *J* = 16.1 Hz), 7.61 (H$_5$, d, J = 8.9 Hz), 7.81 (H$_{2'}$, d, *J* = 16.1 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.13 (CH$_3$), 15.64 (CH$_3$), 22.68 (CH$_2$), 26.0 (CH$_2$), 29.05 (CH$_2$), 29.25 (CH$_2$), 29.35 (CH$_2$), 31.81 (CH$_2$), 55.84 (2OCH$_3$), 68.61 (-OCH$_2$-), 100.91 (C$_8$),112.24 (C$_6$, C$_{3''}$),112.84 (C$_{4a}$), 114.12 (C$_{4''}$), 114.32 (C$_{6''}$),119.80 (C$_{1'}$),122.17 (C$_{1''}$), 126.0 (C$_3$), 127.65 (C$_5$), 130.80 (C$_{2'}$), 146.36 (C$_{2''}$), 151.76 (C$_{5''}$), 153.71 (C$_{8a}$), 153.73 (C$_4$), 160.60 (C$_7$), 161.66 (C=O). EIMS: m/z 451.2484 [M + H]$^+$, (calculated for [C$_{28}$H$_{35}$O$_5$]$^+$: 451.2484).

7-[(*E*)-2-(2,5-dimethoxyphenyl)ethenyl]-2*H*-chromen-2-one
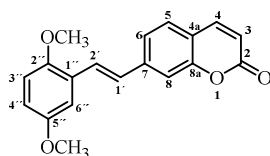
Yield: 0.51mmol, 158mg, 51%; yellow solid, M.p. 125-128 °C; IR (KBr, cm^{-1}): ν $_{max}$ 1724 (C=O), 1606 (C=C), 1494 (C=C$_{Ar}$), 1220 (C-O-C), 1041 ((C=O)-O), 833 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 500 MHz): δ 3.84 (OCH$_3$, s), 3.89 (OCH$_3$, s) 6.39 (H$_3$, d, *J* = 9.6 Hz), 6.84-6.88 (H$_{3''}$, H$_{4''}$, m), 7.12 (H$_{1'}$, d, *J* = 16.5 Hz), 7.16 (H$_{6''}$, s$_{apparent}$), 7.43-7.48 (H$_5$, H$_6$, H$_8$, m), 7.59 (H$_{2'}$, d, *J* = 16.5 Hz), 7.68 (H$_4$, d, *J* = 9.6 Hz). ^{13}C NMR (CDCl$_3$, 125 MHz): δ 55.8 (OCH$_3$) 56.2 (OCH$_3$) 111.9 (C$_{3''}$), 112.4 (C$_{6''}$), 114.3 (C$_8$), 114.8 (C$_{4''}$), 115.8 (C$_3$) 117.9 (C$_{4a}$), 122.6 (C$_6$) 126.2 (C$_{1''}$) 126.8 (C$_{1'}$) 127.4 (C$_{2'}$) 127.9 (C$_5$) 142.0 (C$_7$), 143 (C$_4$), 151.8 (C$_{2''}$), 153.8 (C$_{5''}$), 154.6 (C$_9$), 160.9 (C=O).
EIMS: m/z 309.1127 [M + H]$^+$, (calculated for [C$_{19}$H$_{17}$O$_4$]$^+$: 309.1134).

7-[(*E*)-2-(2,3-dimethoxyphenyl)ethenyl]-2*H*-chromen-2-one
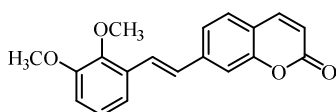
Yield: 0.58mmol, 179mg, 58%; orange solid, M.p. 154-157 °C; IR (KBr, cm^{-1}): ν $_{max}$ 1720 (C=O), 1604 (C=C), 1475 (C=C$_{Ar}$), 1276 (C-O-C), 1143 ((C=O)-O), 837 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 500 MHz): δ 3.91 (OCH$_3$, s), 3.92 (OCH$_3$, s) 6.41 (H$_3$, d, *J* = 9.4 Hz), 6.91 (H$_{4''}$, dd, *J* = 8.1, 1.3 Hz), 7.11 (H$_{5''}$, dd, *J* = 8.0, 8.0 Hz), 7.17 (H$_{1'}$, d, *J* = 16.5 Hz), 7.28-7.30 (H$_{6''}$, m), 7.46-7.51 (H$_5$, H$_6$, H$_8$, m), 7.61 (H$_{2'}$, d, *J* = 16.4 Hz), 7.71 (H$_4$, d, *J* = 9.5 Hz). ^{13}C NMR (CDCl$_3$, 125 MHz): δ 55.9 (OCH$_3$), 61.1 (OCH$_3$) 112.3 (C$_{4''}$), 114.4 (C$_8$), 115.9 (C$_3$), 118.0 (C$_{4a}$), 122.6 (C$_6$) 124.2 (C$_{5''}$) 126.4 (C$_{1'}$) 127.9 (C$_{2'}$) 128.0 (C$_5$) 130.6 (C$_{1''}$), 142.0 (C$_7$), 143.0 (C$_4$), 147.4 (C$_{2''}$), 153.1 (C$_{3''}$), 154.6 (C$_{8a}$), 160.8 (C=O). EIMS: m/z 309.1127 [M + H]$^+$, (calculated for [C$_{19}$H$_{17}$O$_4$]$^+$: 309.1131).

7-[(*E*)-2-(4-hydroxy-3,5-dimethoxyphenyl)ethenyl]-2*H*-chromen-2-one
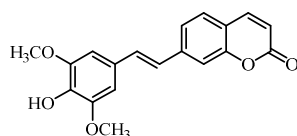
Yield: 0.51 mmol, 165mg, 51%; yellow solid, M.p. 159-161 °C; IR (KBr, cm^{-1}): ν $_{max}$ 3433 (OH), 1724 (C=O), 1612 (C=C), 1512 (C=C$_{Ar}$), 1211 (C-O-C), 1112 ((C=O)-O), 833 (C-H$_{Ar}$); ^{1}H NMR

(CDCl$_3$, 500 MHz): δ 3.97 (2-OCH$_3$, s), 6.38 (H$_3$, d, J = 9.5 Hz), 6.80 (H$_{2''}$, H$_{6''}$, s), 6.97 (H$_{1'}$, d, J = 16.1 Hz), 7.15 (H$_{2'}$, d, J = 16.1 Hz), 7.40-7.46 (H$_5$, H$_6$, H$_8$, m), 7.68 (H$_4$, d, J = 9.5 Hz). ^{13}C NMR (CDCl$_3$, 125 MHz): δ 56.4 (OCH$_3$), 103.8 (C$_{2''}$, C$_{6''}$), 103.9 (C$_8$), 115.2 (C$_3$), 117.8 (C$_{4a}$), 122.3 (C$_6$) 125.0 (C$_{1'}$) 127.9 (C$_5$) 128.0 (C$_{1''}$) 132.1 (C$_{2'}$), 135.7 (C$_{4''}$), 141.5 (C$_7$), 143.0 (C$_4$), 147.3 (C$_{3''}$, C$_{5''}$), 154.6 (C$_9$), 160.9 (C=O). EIMS: m/z 325.1076 [M + H]$^+$, (calculated for [C$_{19}$H$_{17}$O$_5$]$^+$: 325.1069).

7-[(E)-2-(4-hydroxy-3-methoxyphenyl)ethenyl]-2H-chromen-2-one
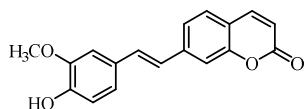
Yield: 0.50 mmol, 147mg, 52%; yellow solid, M.p. 204-206 °C; IR (KBr, cm^{-1}): ν $_{max}$ 3402 (OH), 1705 (C=O), 1606 (C=C), 1512 (C=C$_{Ar}$), 1267 (C-O-C), 1124 ((C=O)-O), 835 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 500 MHz): δ 3.83 (-OCH$_3$, s), 6.41 (H$_3$, d, J = 9.5 Hz), 6.80 (H$_{5''}$, d, J = 8.1 Hz), 7.05 (H$_{6''}$, d, J = 8.3 Hz), 7.16 (H$_{1'}$, d, J = 16.3 Hz), 7.25 (H$_{2''}$, s$_{apparent}$), 7.38 (H$_{2'}$, d, J = 16.3 Hz), 7.54 (H$_8$, s$_{apparent}$), 7.55 (H$_6$, d, J = 8.9 Hz), 7.67 (H$_5$, d, J = 8.0 Hz), 8.02 (H$_4$, d, J = 9.5 Hz). ^{13}C NMR (CDCl$_3$, 125 MHz): δ 56.1 (OCH$_3$), 110.7 (C$_{2''}$), 113.4 (C$_8$), 115.4 (C$_{4a}$), 116.5 (C$_3$) 117.9 (C$_{5'}$) 121.5 (C$_{6''}$) 122.9 (C$_6$) 124.2 (C$_{1'}$), 128.4 (C$_{1''}$), 129.0 (C$_5$), 132.6 (C$_{2'}$), 142.3 (C$_7$), 144.4 (C$_4$), 148.2 (C$_{4''}$), 148.4 (C$_{3''}$), 154.6 (C$_9$), 160.6 (C=O). EIMS: m/z 295.0970 [M + H]$^+$, (calculated for [C$_{18}$H$_{15}$O$_4$]$^+$: 295.0964).

7-[(E)-2-(1,3-benzodioxol-5-yl)ethenyl]-2H-chromen-2-one
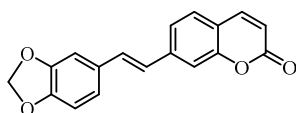
Yield: 0.55mmol, 161mg, 55%; yellow solid, M.p. 193-196 °C; IR (KBr, cm^{-1}): ν $_{max}$ 1720 (C=O), 1606 (C=C), 1494 (C=C$_{Ar}$), 1249 (C-O-C), 1041 ((C=O)-O), 846 (C-H$_{Ar}$); ^{1}H NMR (CDCl$_3$, 500 MHz): δ 6.01 (H$_{6''}$, s), 6.38 (H$_3$, d, J = 9.5 Hz), 6.83 (H$_{5''}$, d, J = 8.0 Hz), 6.95 (H$_{1'}$, d, J = 16.3 Hz), 6.99 (H$_{6''}$, dd, J = 8.0, 1.5 Hz), 7.10 (H$_{2''}$, d, J = 1.5 Hz), 7.15 (H$_{2'}$, d, J = 16.3 Hz), 7.39 (H$_8$, d, J = 1.5 Hz), 7.40 (H$_6$, d, J = 6.0 Hz), 7.44 (H$_5$, d, J = 8.5 Hz), 7.68 (H$_4$, d, J = 9.6 Hz). ^{13}C NMR (CDCl$_3$, 125 MHz): δ 101.3 (OCH$_2$O), 105.7 (C$_{2''}$), 108.5 (C$_{5''}$), 113.9 (C$_8$), 115.8 (C$_4$), 117.9 (C$_{4a}$) 122.3 (C$_{6''}$) 122.4 (C$_6$) 125.1 (C$_5$), 127.9 (C$_{1'}$), 131.0 (C$_{1''}$), 131.7 (C$_{2'}$), 141.6 (C$_7$), 143.0 (C$_4$), 148.1 (C$_{4''}$), 148.3 (C$_{3''}$), 154.6 (C$_9$), 160.8 (C=O). EIMS: m/z 293.0814 [M + H]$^+$, (calculated for [C$_{18}$H$_{13}$O$_4$]$^+$: 293.0808).

Biological activity assays

Cell lines and culture medium

An adenocarcinoma colon cancer cell line (SW480) and non-malignant cells (CHO-K1) were used for biological assays. These were obtained from The European Collection of Authenticated Cell Cultures (ECACC, England) and maintained in Dulbecco's Modified Eagle Medium, supplemented with 10% heat-inactivated (56°C) horse serum, 1% penicillin/streptomycin and 1% non-essential amino acids (Gibco Invitrogen, Carlsbad, USA). For all experiments, horse serum was reduced to 3%, and the medium was supplemented with 5 mg/ml transferrin, 5 ng/ml selenium and 10 mg/ml insulin (ITS-defined medium; Gibco, Invitrogen, Carlsbad, USA) (Maldonado *et al.*, 2009).

Cell Viability by Trypan Blue Dye Exclusion Assay

To assess the effect of styrylcoumarins on the viability of both cell lines, a trypan blue dye exclusion test was performed, which allows to differentiate live cells since these can exclude the dye (Lamy *et al.*, 2007). Cells were seeded to a final density of $1x10^5$ cells/well in 12-well tissue culture plates and incubated at 37 °C in a 5% CO_2 atmosphere. The cultures were allowed to grow for 24 h and then were treated for 24 and 48 h with either 0,5% DMSO (vehicle control) or various concentrations (10 - 100 µM) of different styrylcoumarins, as well as coumarin, resveratrol (lead compounds) and 5-fluorouracil (5-FU; the standard drug). After treatment, cells were washed with buffer versene and they were collected by trypsinization (0.5% trypsin/2.6 mM ethylenediaminetetraacetic acid) followed by centrifugation. Then, trypan blue (1/1, vol/vol) was added to the pellet. The number of unstained cells (live cells) was determined by optical microscope, using hemocytometer. The inhibition percentage was calculated with regard to untreated cells (vehicle control) and IC_{50} values were obtained from dose response curves for each compound. Selectivity index (SI) was calculated by the ratio of IC_{50} values in non-malignant CHO-K1 cells to IC_{50} of SW480 cells.

Antiproliferative activity of styrylcoumarins

The antiproliferative effect of styrylcoumarins was evaluated by using *Sulforhodamine B (SRB) dye*, a colorimetric assay based on staining of total cellular protein of adherent cells. Cells were seeded to a final density of 2500 cells/well in 96-well tissue culture plates and incubated at 37°C in a 5% CO_2 atmosphere. The cultures were allowed to grow for 24 h and then were treated either with increasing concentration of styrylcoumarins (0.02 – 50 µM, the ranges depended on the result of the

IC$_{50}$ value) or 0.5% DMSO (control), for 0, 2, 4, 6 and 8 days. Culture media was replaced every 48 hours. After each incubation time, cells were fixed with trichloroacetic acid (50% v/v) (MERCK) for an hour. Cell proteins were determined by staining with 0.4% (w/v) SRB (Sigma-Aldrich, United States). Cells were then washed with 1% acetic acid for the removal of unbound SRB, and left for air-drying. Protein bound SRB was solubilized in 10 mM Tris-base and the absorbance was measured at 492 nm in a microplate reader (Mindray MR-96A) (Pérez *et al.*, 2014). All experiments were performed in triplicate.

Statistical analysis

All experiments were performed at least three times. Data are reported as mean ± SE (standard error). Statistical differences between control group (non-treated) and treated cells were evaluated by one-way ANOVA followed by the Dunnett's test. Values with $p \leq 0.05$ were considered significant. Data were analyzed with GraphPad Prism version 7.03 for Windows (Graph Pad Software, San Diego, California, USA).

Results and Discussion

Chemistry

Synthesis of 3-styrylcoumarins

Synthesis of 3-styrylcoumarins was performed following the scheme 1, which began with formation of the coumarin (**2**) by microwave assisted Pechmann reaction between resorcinol (**1**) and ethyl acetoacetate (Manhas *et al.,* 2006). Compound (**3**), obtained by microwave –assisted Williamson etherification reaction of coumarin (**2**) (Otero *et al.,* 2014) in 85% yield, was brominated with NBS (Adhikari *et al.,* 2002) to give the alkyloxybromocoumarin (**4**) in 75% yield. This compound was subjected to cross-coupling with various styrenes (**5**) under palladium catalysis (Heck reaction) (Sabrina *et al.,* 2011), leading to the formation of seven 3-styrylcoumarins (**6a–6g**) in 35%–49% yields. In order to improve these results other methodologies were tested such as (Pd(Ph$_3$)$_4$, AgOAc, DMF) (Martins *et al.,* 2012) or Pd(AcO)$_2$, PPh$_3$, CH$_3$CO$_2$Na (Gordo *et al.,* 2011) but the results were not as expected. Styrenes (**5**) were synthesized via microwave assisted Wittig reaction between substituted aldehydes and methyltriphenylphosphonium bromide (Martin *et al.,* 2007) and Knoevenagel condensation reactions between 4-hydroxybenzaldehydes with malonic acid (Simpson *et al.,* 2005).
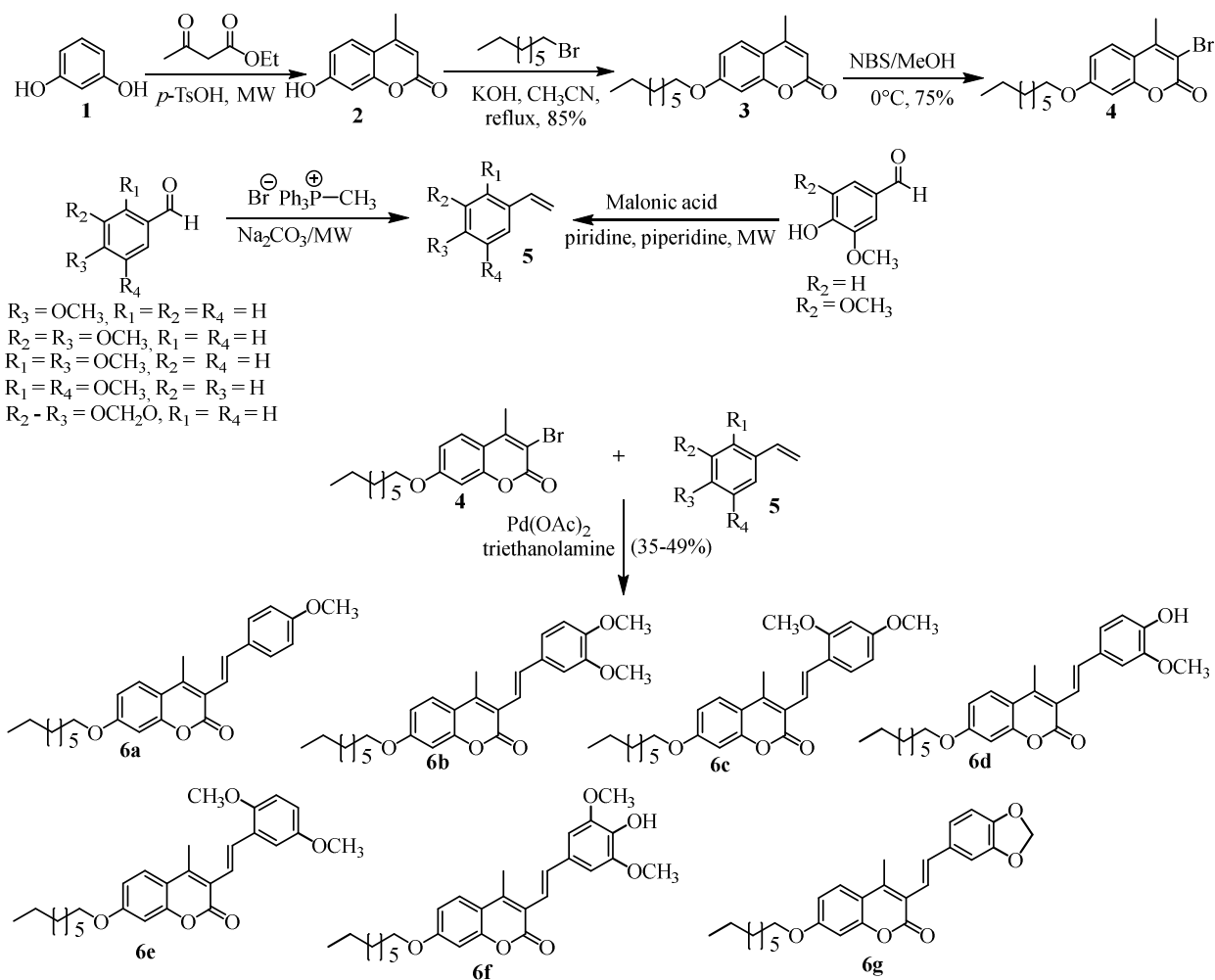
Scheme 1. Synthetic pathway to 3-styrylcoumarins

Synthesis of 7-styrylcoumarins

Scheme 2 shows the synthesis of 7-styrylcoumarins, which began with the formation of the 7-hydroxycoumarin (**8**) by microwave assisted condensation between resorcinol (**1**) and malic acid (**7**) (Symeonidis *et al.,* 2009). Compound (**8**) was treated with trifluoromethanesulfonic anhydride to obtain triflate **9** (Cano *et al.,* 2013) in 85% yield, that in turn was subjected to cross-coupling with various styrenes (**5**) under palladium catalysis (Sabrina *et al.,* 2011), to afford the styrylcoumarins under study (**10a-10e**) in 50%–58% yields. The product of the reaction of coumarin **8** with styrenes **5f** and **5g** showed in the NMR duplication of several signals, which could indicate a mixture of isomers. For this reason, these compounds were not taken into account in this study.
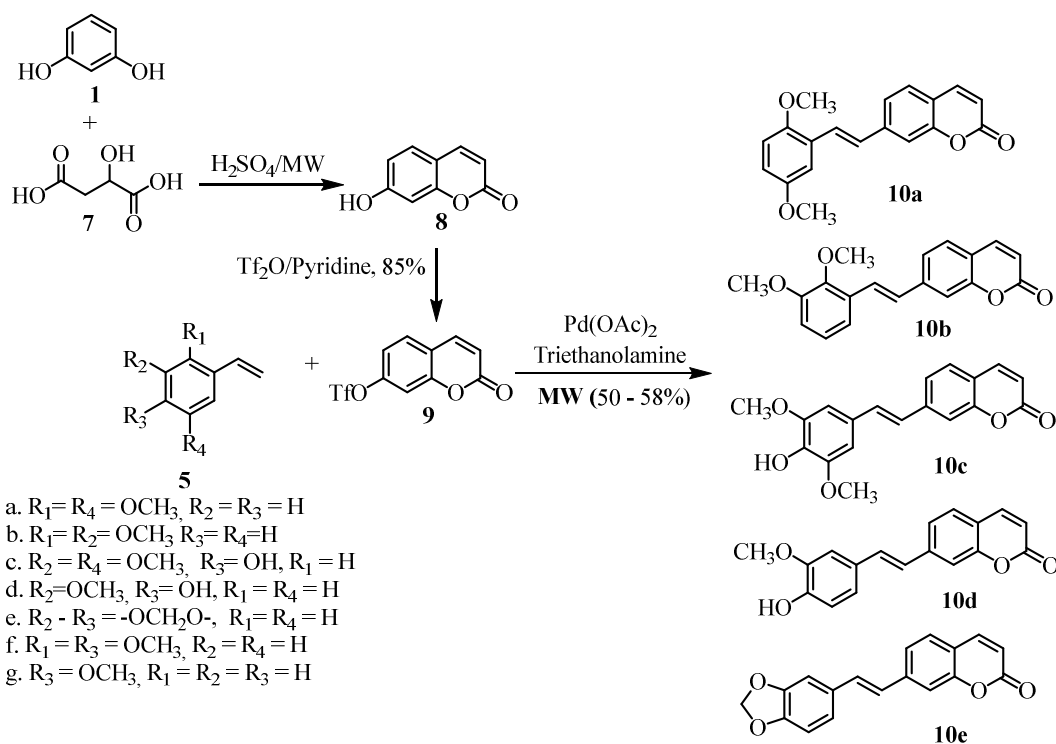
Scheme 2. Synthetic pathway to 7-styrylcoumarins

The structures of all compounds have been established by a combined study of IR, ESI-MS, ^{1}H-NMR, ^{13}C-NMR and COSY spectra. IR spectra exhibited characteristic absorption peaks corresponding to C=O, C=C, C=C$_{Ar}$, C-O-C, and C-H$_{Ar}$. ESI-MS spectra showed characteristic [M+H]$^{+}$ peaks corresponding to their molecular weights. The assignments of all the signals to individual H or C-atoms have been performed on the basis of typical δ-values and *J*-constants. The ^{1}H-NMR spectra of 3-styrylcoumarins dissolved in CDCl$_3$ showed signals of -CH$_3$ (~2.60 ppm), -OCH$_2$- (~4.00 ppm), H-C=C-H$_{trans}$ (~7.00 and 7.60 ppm) and 7-styrylcoumarins showed signals of H-C=C-H$_{cis}$ (~6.50 and 8.00 ppm) and H-C=C-H$_{trans}$ (~7.15 and 7.40 ppm). ^{13}C-NMR spectra 3-of styrylcoumarins showed at around 14, 68, 118, 135 and 161 ppm, corresponding to -CH$_3$, –CH$_2$O-, H-C=C-H$_{trans}$, and C=O, respectively. 7-Styrylcoumarins showed signals around 116, 145, 124, 132 and 160 ppm, corresponding to H-C=C-H$_{cis}$, H-C=C-H$_{trans}$, and C=O, respectively.

Effect of styrylcoumarins on SW480 and CHO K1 cell viability

A series of 3- and 7-styrylcoumarin hybrids were previously synthesized, and evaluated against SW480 and CHO-K1 cell lines, in order to asses their effect on the viability, through the trypan blue dye exclusion method. As shown in Figure 3, the activity was time- and concentration-dependent, with a higher cytotoxic effect on SW480 cells in comparison with CHO-K1 cells. Cytotoxicity was reported as 50% inhibitory concentration (IC$_{50}$) values.
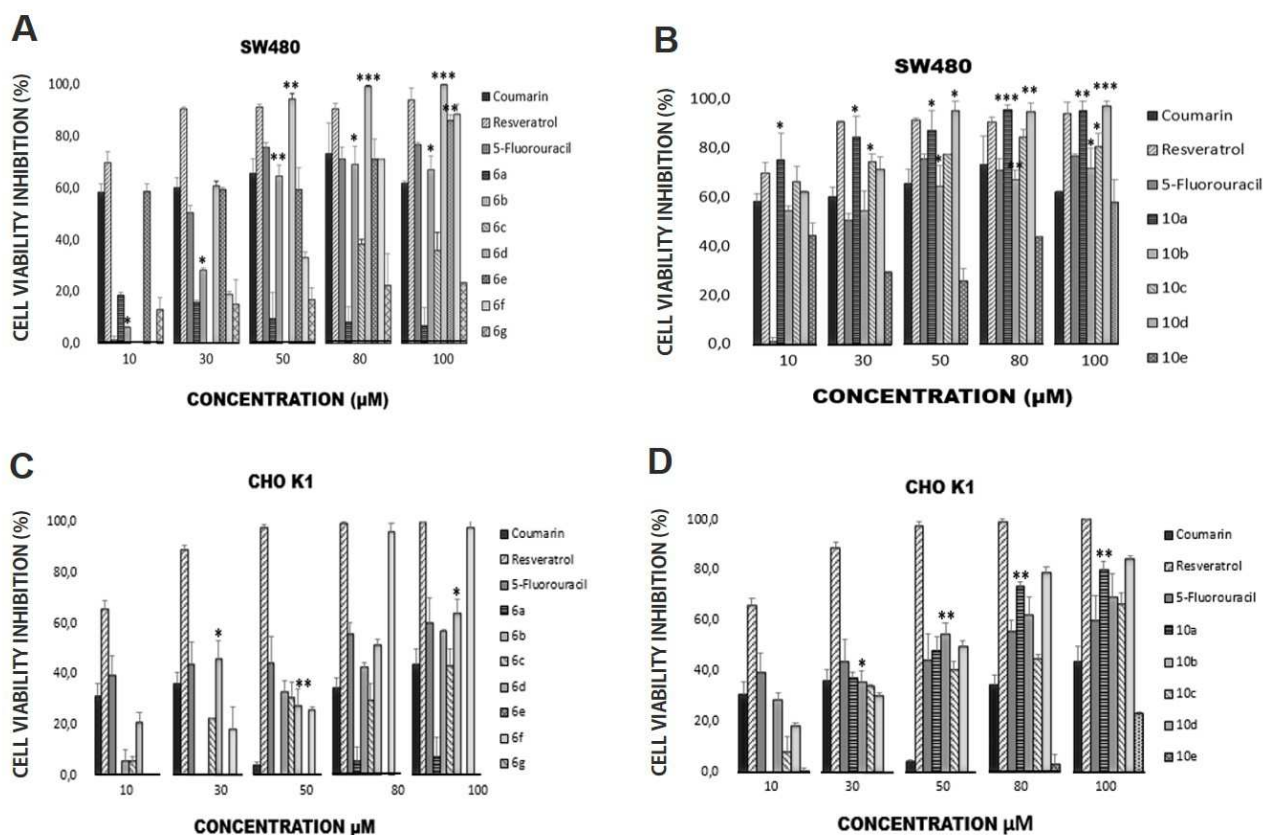
Fig. 3. Effect of 3- and 7-styrylcoumarins on cell viability of SW480 (A and B) and CHO K1 cells (C and D), respectively, 48 hours post-treatment with diferent concentrations of styrylcoumarins (10-100µM). Viable cells were counted and growth inhibition was calculated using 100% viability of control. Zero values indicate that there was not inhibition at that concentration. Data are presented as the mean ± SE of three independent experiments (*P< 0.05; **P < 0.01; ***P < 0.001).

All results regarding to the cytotoxic effect are summarized in Table 1. Among the compounds tested, hybrids **6a**, **6c**, **6g** and **10e** exhibited the lowest activity at both 24 and 48 hours after treatment. Besides, excluding compound **10e**, the increase of IC_{50} concentration at 48 hours suggests a loss in the cytotoxic activity of these hybrids. On the other hand, although compound **6f** exhibited good activity against SW480 cancer cell line after 24 hours of treatment (IC_{50}=23,77 µM), this effect decreased after 48 hours (IC_{50}=50,64 µM) as evidenced by the rise in the IC_{50}. In addition, compound **6f** showed a low selectivity index (24h: 1,51; 48h: 0,9). Moreover, compounds **6b** and **6d** exhibited moderate activity against SW480 cell line after 24 hours of treatment (**6b**: IC_{50}=40,53 µM; **6d**: IC_{50}=49,75 µM), and even though the activity of **6d** improved after 48 hours (**6d**: IC_{50}=26,07 µM), the selectivity index decreased due to increased toxicity in the non-malignant cell line (IS = 24h: 5,27; 48h: 2,7). The opposite occurred with compounds **10a** and **10b** which

displayed the highest activity after 24 hours of treatment (IC_{50}= 0,10 and 0,031 µM, respectively), but decreased after 48 hours (IC_{50}= 0,68 and 7,92 µM, respectively). Despite the loss in the activity and selectivity of these two compounds over time, it was worthwhile to include them in further studies since they were more potent than the lead and standard compounds at lowest concentrations and time. When compound **10c** was tested at 24 hours, it exhibited higher toxicity against the non-malignant cell ($IC_{50-CHOK1}$= 4,66 µM; $IC_{50-SW480}$= 9,40 µM) than the malignant one. However, after 48 hours of treatment the cytotoxicity varied in a significant way for both cell lines ($IC_{50-CHOK1}$= 68,63 µM; $IC_{50-SW480}$= 1,01 µM), being these values even better than coumarin and 5-FU ($IC_{50-SW480}$= 3.91 µM; $IC_{50-SW480}$= 35,43 µM, respectively). This fact was also reflected in the increase of the selectivity index for compound **10c**. Finally, compounds **6e** and **10d** showed better activity against SW480 cells (**6e**: IC_{50-24h}=11,15 µM, IC_{50-48h}= 6,92µM; **10d**: IC_{50-24h}= 5,47µM, IC_{50-48h}=5,33 µM) in comparison with the non-malignant cell line (**6e**: $IC_{50-24h/48h}$= >3000 µM; **10d**: IC_{50-24h}=9,48 µM, IC_{50-48h}=38,14 µM) and the activity was kept over time. Once again, these two compounds exhibited higher activity than the lead and standard compounds, which shows that the improvement in the activity and selectivity could be due to a synergistic action of the lead compounds when they are linked to form a single structure in the hybrid. This study is consistent with the findings of Belluti *et al* (2010) which found similar results in the activity of some stilbene-coumarin hybrids when they were tested in other cancer cell lines (A431 epidermoid carcinoma, JR8 melanoma cell and H460 non-small cell lung cancer).

Table 1 Cytotoxic effect (µM) of styrylcoumarins on SW480 and CHO K1 cell lines

Compounds	24 hours			48 hours		
	IC$_{50}$ (µM) CHO cells	IC$_{50}$ (µM) SW480 cells	SI	IC$_{50}$ (µM) CHO cells	IC$_{50}$ (µM) SW480 cells	SI
6a	>3000	61,81	>40	>3000	>3000	>1
6b	66,26	40,53	1,63	110,07	45,50	2,4
6c	419,69	109,49	3,83	213,10	576,91	0,4
6d	262,41	49,75	5,27	69,81	26,07	2,7
6e	>3000	11,15	>200	**>3000**	**6,92**	**>400**
6f	35,88	23,77	1,51	43,61	50,64	0,9
6g	>3000	295,09	>10	>3000	>3000	>1
10a	254,18	0,10	2534,80	43,90	0,68	64,8
10b	4,55	0,031	147,26	40,86	7,92	5,2
10c	4,66	9,40	0,50	**68,63**	**1,01**	**67,8**
10d	9,48	5,47	1,73	**38,14**	**5,33**	**7,2**
10e	1546,22	2020,28	0,16	>3000	92,88	>30
Coumarin	2541,85	32,12	79,13	1251,66	3,91	319,7
Resveratrol	9,00	8,90	1,01	2,87	0,89	3,2
5-Fluorouracil	90,99	61,68	1,48	47.63	35,43	1.34

The IC$_{50}$ values were obtained from dose response curves for each compound. . Selectivity index (SI) was calculated by the ratio of IC$_{50}$ values in non-malignant CHO-K1 cells to IC$_{50}$ of SW480 cells.

All compounds that exhibited good activity and selectivity with the trypan blue method (**6b**, **6d**, **6e**, **10a**, **10b**, **10c** and **10d**) were tested in order to determine their antiproliferative effect with sulforhodamine B assay, which is used for cell density determination based on the measurement of cellular protein content. After comparing each treatment with the control, results indicated that the activity was time- and concentration-dependent. Among the results obtained, hybrids **6e**, **10c** and **10d** displayed significant antiproliferative activity even at low concentrations (IC$_{50}$ values lower than 12 µM) from day 4 of the treatment, while hybrid **6d** required a higher concentration (>20 µM)

to exert a significant antiproliferative effect in the same time. Furthermore, molecule **6b** required less time (2 days) but a higher concentration (50 µM) to display a significant activity (p≤ 0.05). On the other hand, none of the concentrations evaluated for hybrids **10a** and **10b** caused cell growth inhibition over time (Fig. 4).
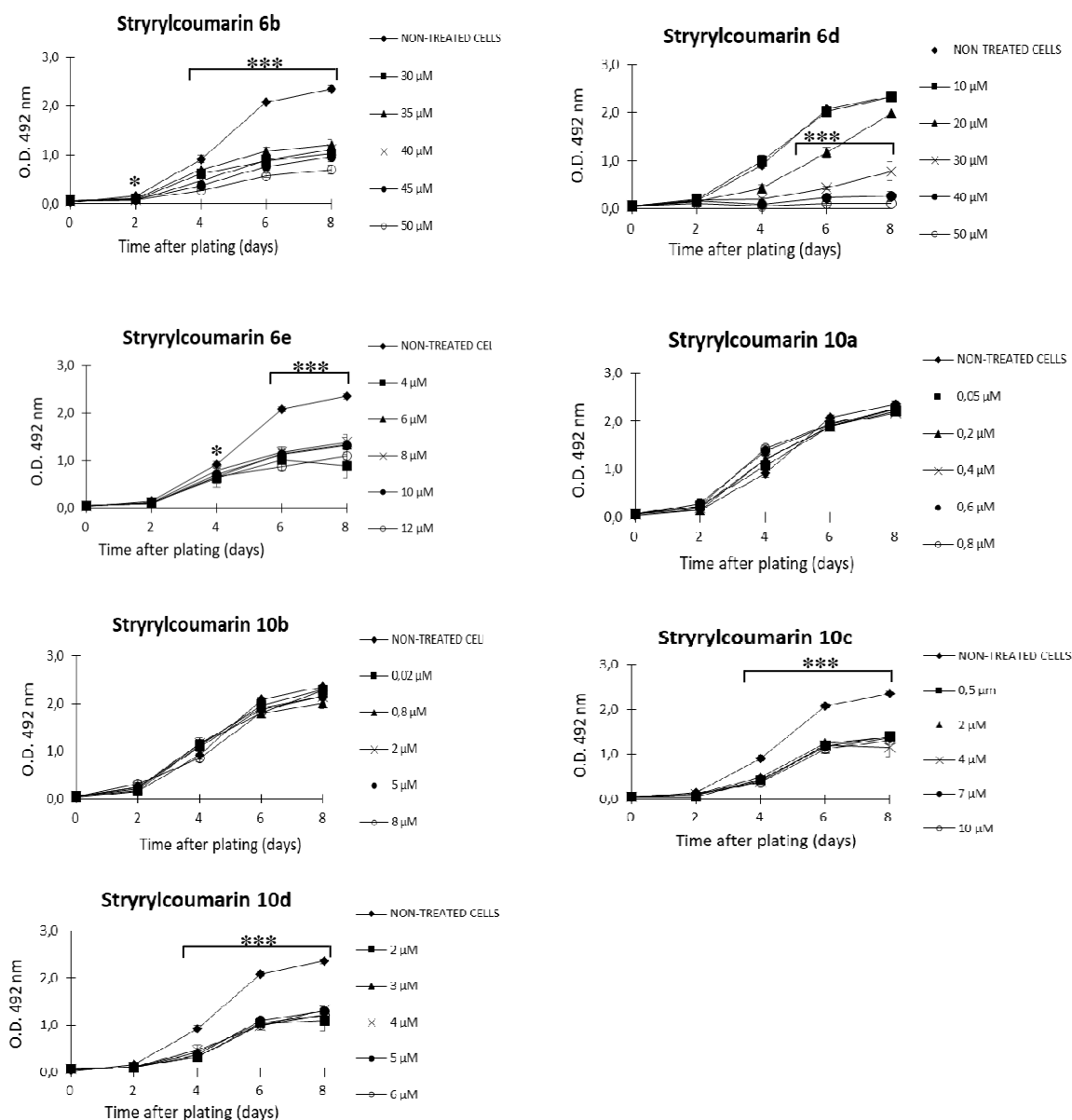

Fig. 4. Antiproliferative effect of styrylcoumarins on SW480 colon cancer cell growth. Data are presented as the mean ± SE of at least three independent experiments (*P<0.05; **P < 0.01; ***P < 0.001). Optical Density (O.D.) is directly proportional to cell mass of adherent cells.

On a structure-activity relationship, it is interesting to note that in general, the position of styryl group is important for the activity. Thus, when series **6** and **10** are compared a higher prevalence in the toxicity against SW480 cells, with better selectivity indexes for **10** series, is observed (see compounds **10a-10d**). For instance, when compounds **6f** and **10c**, bearing the 4-hydroxy-3,5-

dimethoxystyryl motif, are compared a divergence of activities is observed, being the hybrid **10c** the one which is able to preserve the best activity over time. On the other hand, we found that oxygenated positions in 3,4 as well as 2,5 positions were determinant for the activity. These results are similar to the ones obtained by Belluti *et al.* (2010) on H460 cells when they were treated with 7-methoxycoumarin having 3,5-disubstitution pattern in trans-vinylbenzene moiety. However, we noticed that the presence of a hydroxyl group in 4-position was necessary to preserve the activity through time (**6d**, **10c** and **10d** vs **10a**) (See Figure 4). The effect of the hydroxyl groups may be due to a better molecular recognition ability towards the target bioreceptors upon hydrogen bond formation (Patrick, 2013). Thus, the presence of specific substituents in certain positions proved to be crucial in endowing the compounds with inhibitory effect and antiproliferative. The 4-methoxy and methylendioxy group were detrimental, since the presence of these groups in compounds **6a**, **6g**, and **10e** resulted in a decrease of activity.

Conclusions

Twelve hybrid molecules combining styryl and coumarin motifs have been designed and synthesized and their antiproliferative activity has been measured against SW480 and CHO-K1 cell lines. Among the tested compounds, hybrids **6e**, **10c** and **10d** exhibited the highest activity which make them promising candidates for further studies. The SAR analysis showed that styryl group in the position 7 and the presence of hydroxyl groups in position 4 or methoxy groups in 2,5 and 3,4-positions were determinant for their biological activity.

Conflict of interest

The authors declare that they have no conflict of interest.

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