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Synthesis and cytotoxic activity of non-naturally substituted 4-oxycoumarin derivatives

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ABSTRACT

Coumarins are a large family of natural and synthetic compounds exerting different pharmacological effects, including cytotoxic, anti-inflammatory or antimicrobial. In the present communication we report the synthesis of a series of 12 diversely substituted 4-oxycoumarin derivatives including methoxy substituted 4-hydroxycoumarins, methyl, methoxy or unsubstituted 3-aryl-4-hydroxycoumarins and 4-benzyloxycoumarins and their anti-proliferative effects on breast adenocarcinoma cells (MCF-7), human promyelocytic leukemia cells (HL-60), human histiocytic lymphoma cells (U937) and mouse neuroblastoma cells (Neuro2a). The most potent bioactive molecule was the 4-hydroxy-5,7-dimethoxycoumarin (compound **1**) which showed similar potency (IC_{50} 0.2–2 μM) in all cancer cell lines tested. This non-natural product reveals a simple bioactive scaffold which may be exploited in further studies.

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Natural products, with their inherent ability to interact with biological targets, represent a significant source of inspiration for drug discovery and development.[1] A primary aim in medicinal chemistry is the design of structural analogues of bioactive natural products with an improved pharmacological profile. Such examples have been provided with anticancer agents derived from natural products, such as microtubule targeting drugs.[2]

In the current report, we present the synthesis and biological activity of a series of 4-oxycoumarin derivatives, which show substitution patterns that are not found in nature. Coumarins are a large class of natural and/or synthetic products that have attracted interest because of their remarkable array of biological activities, usually associated to a low toxicity.[3–9] The pharmacological and biochemical properties of coumarins depend upon the pattern of substitution of the naturally occurring scaffold. Potential anti-tumour effects of coumarin derivatives have been previously examined.[10–12]

The naturally occurring 7-hydroxycoumarin derivatives have been investigated as potential lead structures for cancer drug development and the related compounds scopoletin (7-hydroxy-6-methoxycoumarin) and esculetin (6,7-dihydroxycoumarin) (Fig. 1) show anti-proliferative effects in several tumor cell lines and thus have been proposed as potential anticancer agents.[12–16]

4-Hydroxycoumarins constitute another important class of coumarin derivatives. Many of them display pharmacological activities including anticoagulant,[14] antibacterial[17] or anti-inflammatory.[18] It has been shown that 4-hydroxycoumarins such as compound (A) (Fig. 1) bearing an aryl group in the 3 position, inhibit cell proliferation.[19] Furthermore, a recent study shows that the

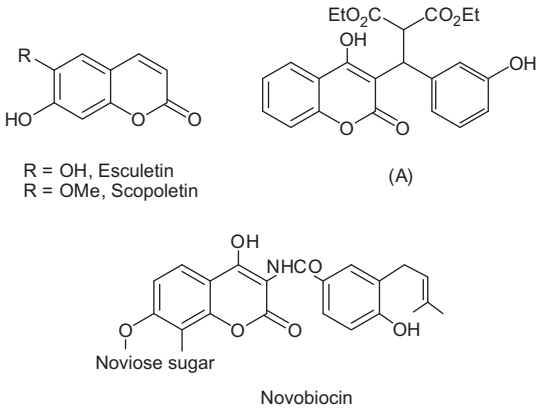

Figure 1. Chemical structures of esculetin, scopoletin, compound (A) and novobiocin.

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most simple 4-hydroxycoumarin leads to a selective cytoskeleton disorganization in melanoma cells without affecting a non-tumoral fibroblastic cell line.[20] This effect is correlated with the reduction in cell adhesion and inhibition of random motility. Because adhesion of tumor cells to extracellular matrix is required during the metastatic process, the 4-hydroxycoumarin has been suggested as useful treatment in the adjuvant therapy for melanoma.[20]

Novobiocin (Fig. 1), a member of the amino-4-hydroxycoumarin family, has been shown to possess anticancer activity, and several derivatives were prepared based on structural modifications in the amide side chain, the coumarin ring, and/or the sugar moiety.[21]

However, the most simple 4-oxycoumarin scaffold comprising a bicyclic ring system has not been explored in sufficient detail. Therefore, taking into account previous data, in the present paper we report the synthesis of a series of 12 different 4-oxycoumarin derivatives, structurally related to the mentioned compounds, but exhibiting a new pattern of substitution that does not occur in nature and is not present in previously tested compounds: two simple 4-hydroxycoumarins dimethoxy substituted in 5 and 7 or in 7 and 8 positions (compounds **1–2**); 4-hydroxycoumarins in which the group present in 3 position in compound (A) or in novobiocin is substituted by a phenyl ring (compounds **3–8**) and finally different substituted 4-(benzyloxy)coumarins (compounds **9–12**).

Different methods have been reported for the synthesis of the simple 4-hydroxycoumarin moiety: condensation of *o*-hydroxyacetophenones with diethyl carbonate in the presence of an alkali metal[22] or cyclocondensation of malonates with phenols in presence of anhydrous aluminum chloride at about 180 °C.[23]

For the sake of an easily reproducible methodology, we have synthesized in good yields the compounds **1–2** using appropriately disubstituted phenols, malonic acid, $ZnCl_2$ as Lewis agent and phosphorus oxychloride ($POCl_3$), as condensing agent (Scheme 1).[23]

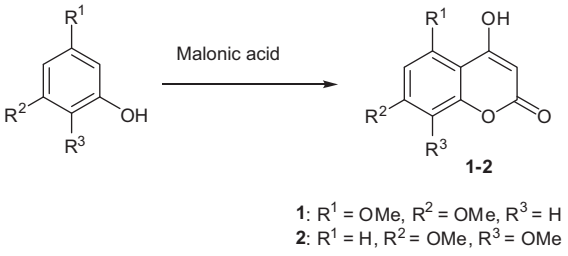
First, the synthesis of the compounds **3–8**[24–29] was achieved by the preparation of different phenyliodonium coumarinate species (**I–II**) starting from the corresponding 3-unsubstituted 4-hydroxycoumarin. Then we carried out the palladium-catalyzed Suzuki coupling reaction between phenyliodonium zwitterions and the conveniently substituted phenyl boronic acids to afford the final compounds (Scheme 2).

The preparation of compounds **9–12** is outlined in Scheme 3. For compounds **9** and **10**, the synthesis has been executed by reaction of commercially available 4-hydroxycoumarin and 4-hydroxy-6-methylcoumarin with benzyl chloride in EtOH in the presence of anhydrous potassium carbonate.[30,31] Compounds **11** and **12** were obtained by reaction of the previously synthesized compounds **3** and **4** with benzyl bromide in acetone.[32,33]

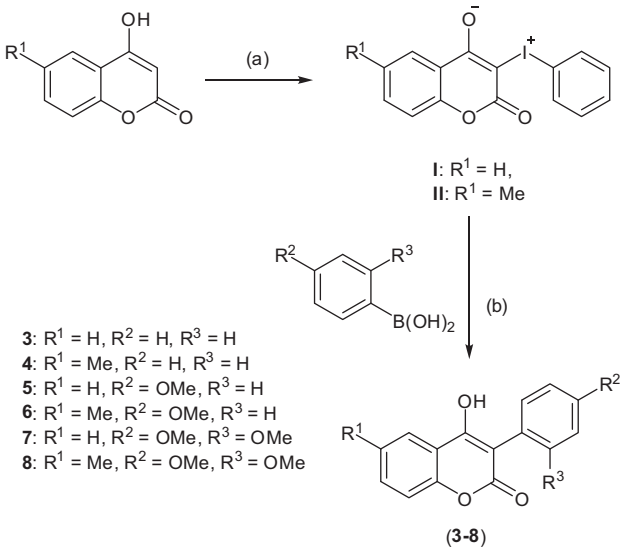
The inhibition of cell proliferation by these diversely substituted coumarins **1**–**12** was then evaluated in vitro using the human breast MCF-7 cells and the human promyelocytic leukemia HL-60 cells.

Cell proliferation assay was carried out by using the Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate) (Roche, Mannheim, Germany) based on the mitochondrial enzymatic cleavage of the WST-1 to formazan salt, whose formation has been monitored by measuring the absorbance at 450 nm, as previously described.[34,35]

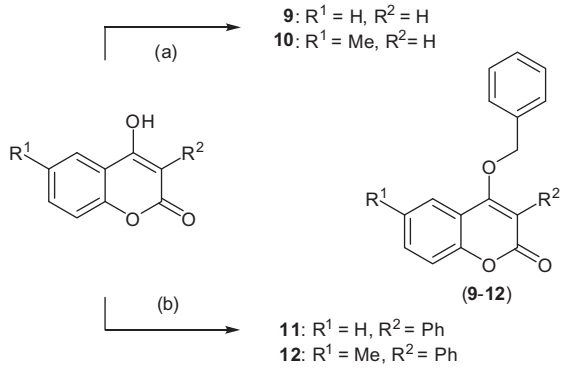
Inhibition of cell proliferation was expressed as percentage of viable cells in treated samples as compared to vehicle-treated cells and the 50% inhibitory concentration of cell proliferation (IC_{50}) was calculated by nonlinear least squares curve fitting (GraphPad Software, San Diego, CA, USA). Each value was obtained from two/three



Scheme 1. Reagents and conditions: $ZnCl_2$, $POCl_3$, reflux, 48 h.



Scheme 2. Reagents and conditions: (a) $PhI(OAc)_2$, Na_2CO_3, H_2O, r.t., 14 h; (b) $Pd(OAc)_2$, P(*t*-Bu)$_3$, LiOH, DME/H_20, r.t., 24–48 h.



Scheme 3. Reagents and conditions: (a) $PhCH_2Cl$, K_2CO_3, EtOH, reflux, 6 h; (b) $PhCH_2Br$, K_2CO_3, acetone, reflux, 6 h.

Table 1
Percentage of cell viability ± SEM for the synthesized compounds **1**–**12** at 10 μM

Compounds	Percentage cell viability ± SEM	
	MCF-7	HL-60
1	17.1 ± 0.9	23.2 ± 0.1
2	114.2 ± 3.9	28.9 ± 0.6
3	96.7 ± 5.6	73.4 ± 4.6
4	72.6 ± 0.6	73.9 ± 1.4
5	114.9 ± 8.4	86.6 ± 3.1
6	106.2 ± 3.7	83.9 ± 2.0
7	91.9 ± 5.3	100.4 ± 3.1
8	69.0 ± 6.6	74.4 ± 11.4
9	95.0 ± 2.1	72.7 ± 5.5
10	104.3 ± 2.2	76.0 ± 1.3
11	113.0 ± 10.2	94.7 ± 2.0
12	53.5 ± 0.1	92.3 ± 3.7

Table 2
IC_{50} ± SEM values of anti-proliferative activity of compounds **1**, **2** and reference compounds

Compounds	IC_{50} (μM) ± SEM			
	MCF-7	HL-60	U937	Neuro2a
1	2.1 ± 0.3	2.0 ± 0.1	0.21 ± 0.06	0.55 ± 0.12
2	62.3 ± 1.2	9.1 ± 0.2	4.3 ± 0.9	1.4 ± 0.7
Tamoxifen	8.6 ± 1.6	14.3 ± 2.6	n.d.	n.d.
Coumarin	>50	>50	>50	>50

Data are means from three independent experiments each run in triplicate.

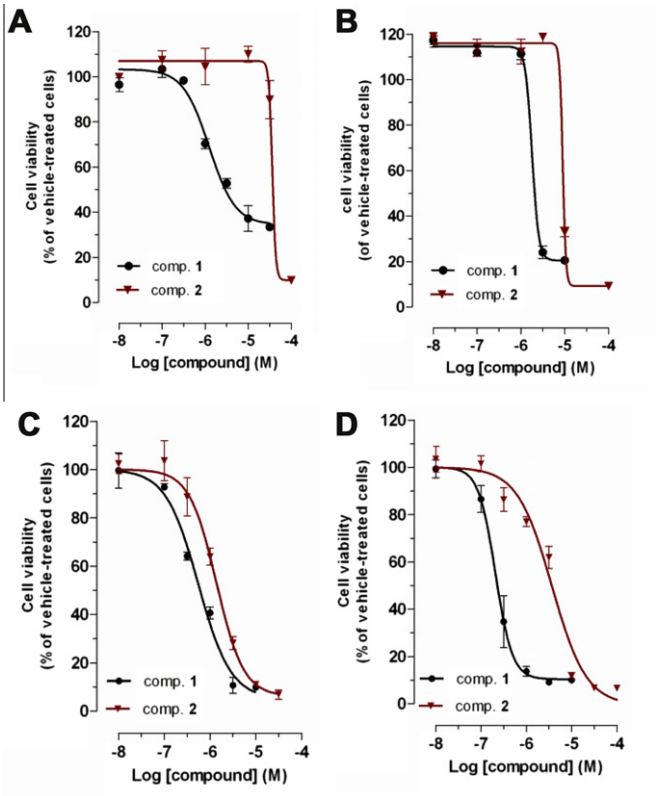


Figure 2. Curves showing inhibition of cell proliferation of MCF-7 (A) HL-60 (B) U937 (C) and Neuro2a (D) cells by compounds **1** and **2**. Data are mean values ± SEM, *N* = 6.

independent experiments carried out in triplicate. The anti-proliferative effects obtained at 10 μM with compounds **1–12** are shown in Table 1. The results show that the two structural isomers, compound **1** and **2** are the most potent molecules in the series. Therefore, we then characterized the anti-proliferative activity of compound **1** and **2** in a wide range of concentrations (0.01–100 μM) and a total of four cancer cell lines, MCF-7, HL-60, the human histiocytic lymphoma U937 cells and the mouse neuroblastoma Neuro2a cells. Interestingly, compound **1** showed the most potent anti-proliferative effect in all the tested cell lines with low μM and even nM IC_{50} values. Its structural isomer **2**, showed anti-proliferative effects in Neuro2a, U937 and HL-60 cells while being ineffective in MCF-7 cells up to 30 μM. In all cell lines, compound **2** resulted less potent than compound **1** to inhibit cell viability as reported in Table 2. In Figure 2, we report the concentration-dependent anti-proliferative effect of compound **1** (black) and **2** (red).

In MCF-7 cells, the inhibition curve of compound **1** showed a typical sigmoidal shape with a Hill coefficient close to 1 suggesting a single target, while compound **2** likely exerted membrane toxic effects only at 30 μM and 100 μM. In HL-60 cells, the proliferation inhibition showed a very steep curve for both compounds, suggesting an involvement of multiple targets. Although the cell death mechanisms were not determined experimentally, compound **1** revealed cell debris typically of apoptotic cells (data not shown). The IC_{50} values for compound **1** and **2** calculated from the curves as well as the values for the positive controls, tamoxifen and coumarin are shown in Table 2.

Within our new diversified non-natural coumarin library only the 4-oxycoumarin **1** exhibited significant cytotoxic effect (that is anti-proliferative activity with an IC_{50} value below 2 μM) and it showed a similar potency in all the tested cancer cell lines.

Intriguingly, compound **2**, a structural isomer of compound **1** in which only one methoxy group is changed from 5 to 8 position, is significantly active in Neuro2a cells, but ineffective in MCF-7 cells, and only weakly active in U937 and HL-60 cells. This clearly indicates a structural basis for biological activity (that is anti-proliferative effects) for the 5,7-dimethoxy compound (**1**) compared to the 7,8-dimethoxy isomer (**2**). Compound **1** is thirty times more active than compound **2** in the cytotoxic activity on MCF-7 cells. In agreement, compound **1** showed a stronger anti-proliferative effect than compound **2** also in U937 and Neuro2a cells (three to twenty times higher). In general U937 and Neuro2a cells were more sensitive to these dimethoxy substituted 4-hydroxycoumarins than MCF-7 and HL-60 cells. All the cell lines were cultured with 10% FBS in the medium apart from HL-60 which needed 20% of FBS. In order to exclude an effect related to the different % of FBS, we performed the cell viability experiments either in presence of 10% or 20% FBS. The results did not show any variation in the concentration-dependent anti-proliferative effects (data not shown). The solubility of compounds **1** and **2** in the assay medium was tested by microscopic inspection and compound **1** was soluble up to 20 μM whereas compound **2** was soluble up to 100 μM. From all the compounds with bulky groups on the coumarin scaffold only compound **12** shows a weak activity in the border-line range of the 53% of cell viability only on MCF-7 cells.

In conclusion, compound **1** with its simple structure (4-hydroxy-5,7-dimethoxycoumarin) represents a new simple chemical scaffold with significant anti-proliferative effects in cancer cells.

Intriguingly, in the present series we show that the introduction of aryl rings in the 3 position, or the substitution of the 4-hydroxy with a benzyloxy group causes a meaningful loss of activity.

The fact that compound **2**, a structural isomer of **1** which differs only in the two methoxy groups in 7 and 8 positions, was inactive on MCF-7 cells and significantly less active than **1** in all the other cell lines tested (IC_{50} values three to twenty times higher), reveals that the substitution in these positions of the 4-oxycoumarin scaffold significantly influences the biological activity.

Our data support the investigation of new derivatives of compounds **1** and **2** expanding on the chemical features by introducing different functional groups.

Acknowledgments

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28. *General procedure for the preparation of 3-phenyliodonium coumarinates* **I** *and* **II**: iodobenzene diacetate (10 mmol) was suspended in a solution of Na_2CO_3 (10 mmol) in water (100 mL) and was stirred for 30 min at room temperature. To this solution was added a mixture of the corresponding 4-hydroxycoumarin (10 mmol) and Na_2CO_3 (10 mmol) in water (100 mL). After the mixture was stirred at room temperature for 14 h, the precipitate was collected by filtration, washed with water (5 × 20 mL) and dried under vacuum. The resulting white solid was used without further purification.
29. *General procedure for the preparation of 3-aryl-4-hydroxycoumarins* **3**–**8**: a degassed solution of appropriated phenyl boronic acid (1.21 mmol) and P(*t*-But)$_3$ (0.109 mmol) in DME and H_2O (4:1, 12.5 mL) was added to a mixture of iodonium ylide (0.55 mmol), LiOH/H_2O (1.65 mmol) and $Pd(OAc)_2$ (0.027 mmol) under argon at room temperature. After being stirred at the same temperature for 24–48 h. The resulting mixture was purified by FC (hexane/ethyl acetate, 7:3) to give the desired compound.
4-Hydroxy-3-(2′,4′-dimethoxyphenyl)-6-methylcoumarin (**8**). It was obtained with yield 57%. Mp: 274–277 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 2.30–2.44 (s, 3H, CH_3), 3.63–3.75 (s, 3H, OCH_3), 3.75–3.86 (s, 3H, OCH_3), 6.49–6.68 (m, 2H, H3′, H5′), 7.02–7.13 (m, 1H, H6′), 7.21–7.32 (m, 1H, H8), 7.37–7.51 (m, 1H, H7), 7.66–7.76 (s, 1H, H5). ^{13}C NMR (DMSO-d_6) δ (ppm): 21.1, 55.9, 56.1, 99.2, 102.9, 105.6, 113.2, 116.5, 116.7, 123.9, 133.5, 133.7, 133.8, 151.2, 159.5, 160.9, 161.4, 162.5. MS *m*/*z* (%): 312 (M^+, 59), 137 (100), 97 (36), 83 (37), 71 (51), 59 (79). Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.24; H, 5.19.
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31. *General procedure for the preparation of 4-benzyloxycoumarins* **9**–**10**: to a solution of appropriate 4-hydroxycoumarin (1.5 mmol) in EtOH (15 mL), K_2CO_3 (4.5 mmol) and benzyl chloride (4.5 mmol) were added. The reaction was stirred at reflux for 6 h under nitrogen atmosphere. After the solution was cooled, K_2CO_3 was filtered off and the solution was evaporated to dryness. The oil residue was treated with ether obtaining a precipitate that was filtered. It was purified by FC (hexane/ethyl acetate, 7:3) or by crystallization to give the desired compounds.
4-Benzyloxy-6-methylcoumarin (**10**). It was obtained with yield 45%. Mp: 220–223 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.40 (s, 3H, CH_3), 3.45–3.74 (s, 1H, H3), 4.25–4.70 (m, 2H, CH_2), 6.79–7.03 (m, 2H, H7, H8), 7.04–7.33 (m, 4H, H2′, H3′, H4′, H5′), 7.39–7.74 (m, 2H, H5, H6′). ^{13}C NMR ($CDCl_3$) δ (ppm): 21.9, 69.0, 87.5, 116.9, 117.5, 127.0, 127.1, 127.2, 127.6, 128.8, 128.9, 132.1, 135.4, 136.3, 149.8, 162.4, 169.9. MS *m*/*z* (%): 266 (M^+, 100), 135 (57), 134 (65), 91 (52), 77 (27). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.66; H, 5.28.
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33. *General procedure for the preparation of 4-benzyloxy-3-phenylcoumarins* **11**–**12**: to a solution of appropriate 4-hydroxycoumarin (0.7 mmol) in acetone (16 mL), K_2CO_3 (1.4 mmol) and benzyl bromide (1.4 mmol) were added. The reaction was stirred at reflux for 12 h under nitrogen atmosphere. After the solution was cooled, K_2CO_3 was filtered off and the solution was evaporated to dryness. The residue was treated with ether and the precipitate was filtered. It was purified by FC (dichloromethane/petroleum ether, 9.5:0.5) or by crystallization to give the desired compounds.
4-(Benzyloxy)-3-phenylcoumarin (**11**). It was obtained with yield 46%. Mp: 204–206 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 4.65 (s, 2H, CH_2), 7.11–7.18 (m, 2H, H2′, H6′), 7.34–7.24 (m, 5H, H4′, H6, H3″, H4″, H5″), 7.37 (d, 1H, H8, *J* = 8.3), 7.45–7.41 (m, 1H, H2″), 7.48 (t, 2H, H3′, H5′, *J* = 7.4), 7.55 (d, 2H, H6″, H7, *J* = 7.0), 7.84 (d, 1H, H5, *J* = 8.0). ^{13}C NMR ($CDCl_3$) δ (ppm): 69.0, 100.3, 115.9, 117.6, 123.4, 125.3, 127.1, 127.3, 127.6, 127.8, 128.0, 128.4, 128.5, 128.6, 128.7, 128.8, 128.9, 132.6, 136.3, 152.4, 161.8, 166.9. MS *m*/*z* (%): 328 (M^+, 100), 238 (45), 208 (44), 152 (32), 92 (65), 91 (61). Anal. Calcd for $C_{22}H_{16}O_3$: C, 80.47; H, 4.91. Found: C, 80.48; H, 4.89.
4-Benzyloxy-6-methyl-3-phenylcoumarin (**12**). It was obtained with yield 50%. Mp: 173–175 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.40 (s, 3H, CH_3), 4.64 (s, 2H, CH_2), 7.13–7.16 (m, 2H, H7, H2′), 7.26 (s, 3H, H4′, H6′, H8), 7.34–7.30 (m, 3H, H3″, H4″, H5″), 7.47 (t, 2H, H3′, H5′, *J* = 7.5), 7.53 (m, 2H, H2″, H6″), 7.57 (s, 1H, H5). ^{13}C NMR ($CDCl_3$) δ (ppm): 21.9, 69.1, 100.3, 116.8, 117.5, 127.0, 127.1, 127.2, 127.6, 127.8, 128.4, 128.6, 128.7, 128.8, 128.8, 128.9, 132.1, 132.6, 135.4, 136.3, 149.8, 161.7, 166.9. MS *m*/*z* (%): 342 (M^+, 73), 208 (24), 92 (30), 91 (100). Anal. Calcd for $C_{23}H_{18}O_3$: C, 80.68; H, 5.30. Found: C, 80.66; H, 5.28.
34. The HL60 and the MCF-7 cells line were purchased from the American Type Culture Collection. HL60 cells were grown in Iscove's modified Dulbecco's medium with 2 mM L-glutamine supplemented with 20% fetal bovine serum, 1 μg/ml amphotericin B, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies Invitrogen, Basel, Switzerland). MCF-7 cells were cultured in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1 μg/ml), 2 mM L-glutamine and 10% fetal bovine serum (all from Life Technologies Invitrogen, Basel, Switzerland). All cells were grown in a humidified incubator at 37 °C and 5% CO_2. Cells were seeded into 96-well plates at a density of 2 × 10^6 per well and incubated at 37 °C with 5% CO_2. After 24 h incubation to allow cell attachment, cells were treated for 72 h with the acetylenic compounds in the range 0.1–100 μM. For the HL-60 cells it is not necessary to wait the time of incubation to allow cell attachment because the cells are in suspension. At the end of the exposure time, WST-1 was added at 1/10 of the total volume and after 60 min of incubation at 37 °C, the absorbance was measured at 450 nm with a microplate reader (Wallac; Perkin Elmer,Wellesley, MA, USA).
35. Chicca, A.; Pellati, F.; Adinolfi, B.; Matthias, A.; Massarelli, I.; Benvenuti, S.; Martinotti, E.; Bianucci, A. M.; Bone, K.; Lehmann, R.; Nieri, P. *Br. J. Pharmacol.* **2008**, *153*, 879.