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# Synthesis, structure, and estrogenic activity of 4-amino-3-(2-methylbenzyl)coumarins on human breast carcinoma cells

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**Abstract**—A number of coumarins exhibit interesting pharmacological activities and are therefore of therapeutic use. We report here the synthesis and the structural analysis of new N-substituted 4-amino-3-(2-methylbenzyl)coumarins (compounds **8a**–**8e**) that present structural analogies with estrothiazine and 11- or 7-substituted 17β-estradiol. These derivatives were tested with respect to estrogenic activity on the estrogen receptor positive (ER+) human MCF-7 breast cancer cell line. Two of the reported compounds (**8a** and **8b**) stimulated specifically the proliferation of MCF-7 cells, but not that of estrogen receptor negative (ER−) human MDA-MB-231 breast cancer cells, suggesting that their mitogenic activity is mediated by ER. Accordingly, the stimulating effect of **8a** and **8b** was suppressed by the pure antiestrogen fulvestrant. Besides, **8a** and **8b** induced ER down-regulation similar to that produced by classical ER agonists or pure antagonists. The effects of the compounds under study on ER-mediated transcription were assessed on (ER+) MVLN cells, that is, MCF-7 cells stably transfected with a pVit-tk-Luc reporter plasmid. Derivatives **8a** and **8b**, and surprisingly compound **8c**, enhanced ER-mediated gene transactivation in that model. Finally, no coumarin was able to compete with tritiated 17β-estradiol ($[^3H]E_2$) for ER binding, suggesting unconventional interactions with the receptor, such as interactions with the second binding pocket or with the coactivator-binding region. To conclude, observations performed in this study on compound **8c** reveal that estrogenic activity can be dissociated from enhancement of cell proliferation. Furthermore, ERE-driven transactivation of transcription seems to be a condition necessary, but not sufficient, for estrogen-induced stimulation of cell growth.
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*Abbreviations*: ER, estrogen receptor; $E_2$, 17β-estradiol; ERE, estrogen response element; UV, ultra-violet; FTIR, Fourier-transformed infrared; NMR, nuclear magnetic resonance; CI-MS, chemical ionization mass spectrometry; DMSO, dimethylsulfoxide; TLC, thin-layer chromatography; mp, melting point; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DPBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; EFM, estrogen-free medium; DCC, dextran-coated charcoal; RLU, relative luciferase unit.

*Keywords*: 4-Aminocoumarins; Breast cancer cells; Estrogenic activity.

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## 1. Introduction

Coumarins are natural or synthetic benzopyranic derivatives that form a family of active compounds with a wide range of pharmacological properties. Actually, coumarins can display anticoagulant activity,[1] antipsoriasis activity,[2] inhibitory activity on viral proteases,[3] antibacterial/antitumoral activity,[4] antioxidant activity,[5] antiproliferative activity,[6] estrogen-like effects[7–9] or central nervous system modulating activities.[10]

The discovery of coumarins with weak estrogenic activity is of potential medical interest since such derivatives could be used as therapeutic agents to prevent the emergence of adverse effects associated with menopause, such as osteoporosis, cardiovascular risk (atherosclerosis), or cognitive deficiency.[11]

We recently reported promising biological properties in two new families of synthetic coumarins. The first family, represented by 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones (Chart 1), exhibits strong antiproliferative activities in MCF-7 breast carcinoma cells by a mechanism that remains to be determined.[12] The second, represented by 1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones (Chart 1), displays interesting antioxidant and estrogenic-like effects in HepG2 and MCF-7 cells, respectively.[5,13,14]

In the present paper, we report the efficient synthesis of new N-substituted-4-amino-3-(2-methylbenzyl)coumarins (**8a**–**8e**) that present some structural similarities with 1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones. UV, FTIR, $^1$H NMR spectroscopic experiments as well as CI-MS and X-ray diffraction were used to determine their structure.

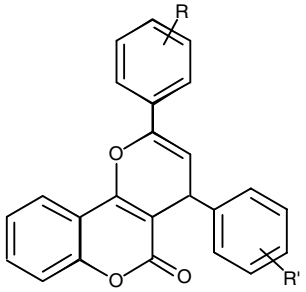
Next, compounds **8a**–**8e** were tested for their activity on ER-dependent cell signaling. For this purpose, we used the (ER+) breast carcinoma cell line MCF-7 which expresses ERα, and the MVLN cell line which corresponds to MCF-7 stably transfected with an ERE-driven luciferase reporter gene. As a negative control, these compounds were tested on (ER−) MDA-MB-231 breast carcinoma cells. Observations described herein reveal that, among the tested derivatives, compounds **8a**–**8c** enhance ER-mediated gene transactivation (enhancement of luciferase gene expression). Interestingly, compounds **8a** and **8b**, but not compound **8c**, display mitogenic activity toward MCF-7 cells. Finally, despite their unequivocal effect on ER, none of the tested compounds was found to behave as a conventional ligand for this receptor.

## 2. Chemistry

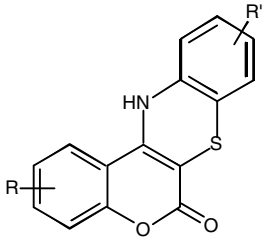
The condensation of the primary amines **4a**–**4d** on 4-hydroxycoumarin **1a** and the primary amine **4e** on 4-hydroxy-7-methoxycoumarin **1b** was efficiently carried out in refluxing ethoxyethanol (Fig. 1)[13–17] to afford the intermediary Schiff bases **5a**–**5e**, isolated as enamines (compounds **6a**–**6e**), as shown by the FTIR and $^1$H NMR data. Indeed, according to previous works, the involvement of the tautomeric forms of **1**, that is, 2,4-chromanedione **2** or 2-hydroxychromen-4-one **3** (Fig. 1), seems likely.[13,14,18,19]

The direct monoalkylation of **6a**–**6e** at the acidic position 3 of the coumarinic core structure by 2-methylbenzylbromide **7** was carried out without solvent in the molten state (135–140 °C) (Fig. 2).[20] The 4-amino-3-benzylcoumarins **8a**–**8e** were precipitated from isopropanol under vigorous stirring while the reaction mixture was slowly cooled down from 50 °C to room temperature. In such conditions, the mixture was still under a liquid state, allowing precipitation of the reaction products. Actually, at room temperature, the mixture solidified into an amorphous 'glassy' state, preventing therefore the purification of the reaction products.

The structure of the enamines **6a**–**6e** and **8a**–**8e** was assigned from UV, FTIR, $^1$H NMR, CI-MS, and X-ray data. UV spectra recorded in DMSO revealed the presence of three absorption maxima at ≈λ 260, λ 300, and λ 320 nm, corresponding to the coumarinic and benzenic chromophores.[13] FTIR and $^1$H NMR data recorded for **6a**–**6e** and **8a**–**8e** revealed that the latter were isolated under the enamine tautomeric form. Actually, FTIR analysis disclosed a strong absorption band between $\nu$ 3325 and 3265 cm$^{-1}$ as well as two strong bands between $\nu$ 1601 and 1610 cm$^{-1}$ (NH and C(3)H=C(4) protons, respectively, of the intermediates **6a**–**6e**). These results correlated with $^1$H NMR experi-

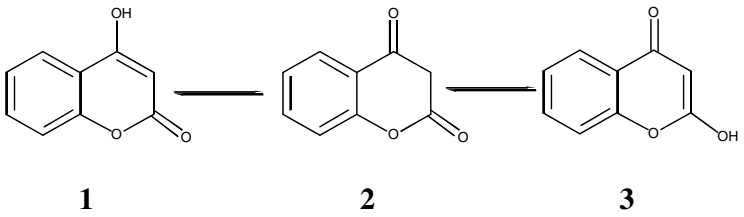



2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones

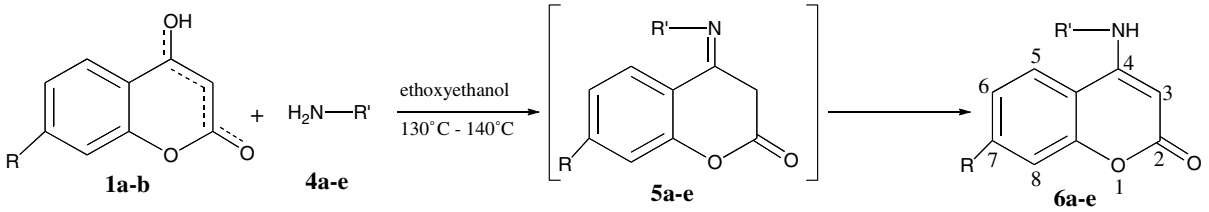



1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones

**Chart 1.** Structures of diarylpyranocoumarins and of benzopyranobenzothiazinones.



**1** **2** **3**




**1a-b** **4a-e** **5a-e** **6a-e**

**1a:** R=H
**1b:** R=$H_3CO$

**4a:** R'=$C_5H_9$
**4b:** R'=$C_6H_{11}$
**4c:** R'=$C_{12}H_{25}$
**4d:** R'=$C_8H_9$
**4e:** R'=$C_5H_{11}$

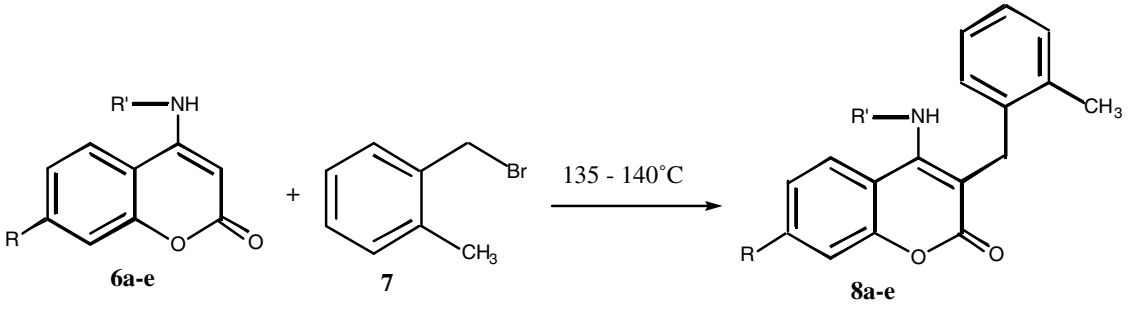
**5a:** R=H, R'=$C_5H_9$
**5b:** R=H, R'=$C_6H_{11}$
**5c:** R=H, R'=$C_{12}H_{25}$
**5d:** R=H, R'=$C_8H_9$
**5e:** R=$H_3CO$, R'=$C_5H_{11}$

**6a:** R=H, R'=$C_5H_9$
**6b:** R=H, R'=$C_6H_{11}$
**6c:** R=H, R'=$C_{12}H_{25}$
**6d:** R=H, R'=$C_8H_9$
**6e:** R=$H_3CO$, R'=$C_5H_{11}$

**Figure 1.** Synthesis of 4-aminocoumarins **6a**–**6e**.

ments, that is, a broad signal associated with the secondary amine function between $\delta$ 5.23 and 7.70 ppm, and a singlet between $\delta$ 5.20 and 5.33 ppm corresponding to the C(3)H=C(4) proton. Likewise, a strong band between $\nu$ 3325 and 3364 $cm^{-1}$ as well as a broad signal between $\delta$ 4.30 and 4.39 ppm were relevant to the NH proton of the **8a**–**8e** secondary amine at the position 4 of the coumarin core structure.

Crystallographic data of **8a** (Fig. 3a) lead us to identify a cyclopentyl ring-puckering pseudorotation (Fig. 3b). In this context, the belonging of **8a** to the triclinic space group P-1 with two molecules in the asymmetric unit was relevant to the cyclopentyl conformation. Indeed, the most important differences between these two molecules are the dihedral angles between the mean planes of the cyclopentyl ring and the coumarin moiety (70.4(2)° and 86.1(2)°) as well as between the mean planes of the cyclopentyl ring and the benzyl ring (52.2(2)° and 43.9(2)°) as shown in Figure 3b. It is noteworthy that the dihedral angle between the mean planes of the benzyl ring and the coumarin moiety is similar between the two structures (83.9(1)° and 82.3(1)°). One can also observe a twisted conformation on the C(10)–C(11) bond with puckering parameters Q(2) = 0.3533(43) Å, Phi(2) = 26.0(8)° for the first molecule (Fig. 3b) and an envelope conformation on the C(14) atom with puckering parameters Q(2) = 0.3497(50) Å, Phi(2) = 323.9(9)° for the second molecule (data not shown).[21] The analysis of ring substituents leads to an equatorial position for the N(1) atom of both structures, the angles of C(10)–N(1) with the mean plane normal of the cyclopentane ring having the same value (60.2(2)°). Moreover, it is of note that both substituents, that is, the amino group as well as the benzyl core structure, are oriented in the same direction (Fig. 4). Finally, X-ray experiment definitively confirmed the enamine tautomeric form of **8a**–**8e**.

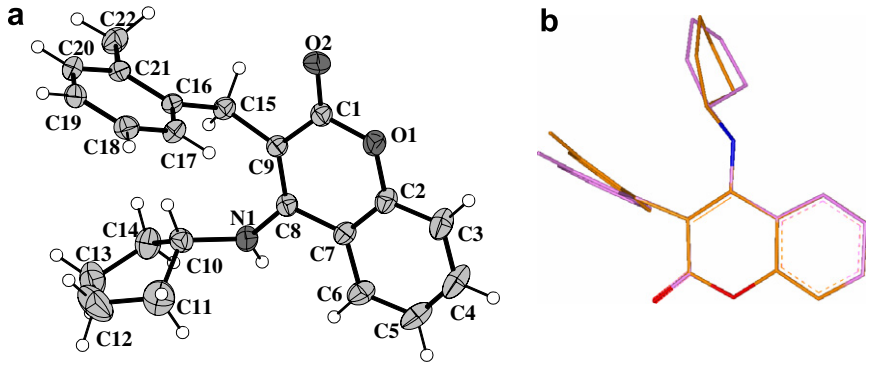



**6a-e** **7** **8a-e**

**6a:** R=H, R'=$C_5H_9$
**6b:** R=H, R'=$C_6H_{11}$
**6c:** R=H, R'=$C_{12}H_{25}$
**6d:** R=H, R'=$C_8H_9$
**6e:** R=$H_3CO$, R'=$C_5H_{11}$

**8a:** R=H, R'=$C_5H_9$
**8b:** R=H, R'=$C_6H_{11}$
**8c:** R=H, R'=$C_{12}H_{25}$
**8d:** R=H, R'=$C_8H_9$
**8e:** R=$H_3CO$, R'=$C_5H_{11}$

**Figure 2.** Synthesis of 4-amino-3-benzylcoumarins **8a**–**8e**.




**Crystal Data and Details of the Structure Determination for 8a**

| | |
|---|---|
| **Crystal Data** | |
| Formula | $C_{22}H_{23}NO_2$ |
| Formula Weight | 333.41 |
| Crystal System | Triclinic |
| Space group | P-1 (No. 2) |
| a, b, c [Å] | 11.978(2); 12.935(2); 13.321(2) |
| alpha, beta, gamma [°] | 95.69(1); 111.64(1); 105.15(1) |
| V [Å$^3$] | 1807.2(5) |
| Z | 4 |
| D(calc) [g.cm$^{-1}$] | 1.225 |
| Mu(MoK ) [mm$^{-1}$ ] | 0.078 |
| F(000) | 712 |
| Crystal Size [mm] | 0.48 x 0.18 x 0.17 |
| **Data Collection** | |
| Temperature (K) | 293(2) |
| Radiation [Å] | MoK 0.71073 |
| Theta Min-Max [°] | 2.4, 30.0 |
| Dataset | +13/-16 ; +18/-18 ; +18/-13 |
| Tot., Uniq. Data, $R_{int}$ | 21225,10421, 0.041 |
| Observed data [I > 2.0 (I)] | 6528 |
| **Refinement** | |
| $N_{ref}$, $N_{par}$ | 10421, 453 |
| R, wR2, S | 0.0416, 0.1242, 1.01 |
| Max. and Av. Shift/Error | 0.00, 0.00 |
| Min. and Max. Resd. Dens. [e/Å$^3$] | -0.32, 0.35 |

**Figure 3.** (a) ORTEP drawing of 4-aminocyclopentyl-3-(2-methylbenzyl)coumarin **8a**. Bond lengths (Å) and angles (°). O1–C2 1.366(3), O1–C1 1.372(3), O2–C1 1.211(3), N1–C8 1.355(3), N1–C10 1.450(3), N1–H1 0.860, C1–C9 1.429(3), C2–C7 1.381(4), C2–C3 1.382(4), C3–C4 1.358(4), C3–H3 0.930, C4–C5 1.371(5), C4–H4 0.930, C5–C6 1.376(4), C5–H5 0.930, C6–C7 1.400(4), C6–H6 0.930, C7–C8 1.462(3), C8–C9 1.377(3), C9–C15 1.492(3), C10–C14 1.512(4), C10–C11 1.532(4), C10–H10 0.980, C11–C12 1.465(6), C11–H11A 0.970, C11–H11B 0.970, C12–C13 1.443(6), C12–H12A 0.970, C12–H12B 0.970, C13–C14 1.480(5), C13–H13A 0.970, C13–H13B 0.970, C14–H14A 0.970, C14–H14B 0.970, C15–C16 1.515(3), C15–H15A 0.970, C15–H15B 0.970, C16–C17 1.383(4), C16–C21 1.403(3), C17–C18 1.377(4), C17–H17 0.930, C18–C19 1.369(4), C18–H18 0.930, C19–C20 1.372(4), C19–H19 0.930, C20–C21 1.376(4), C20–H20 0.930, C21–C22 1.501(4), C22–H22A 0.960, C22–H22B 0.960, C22–H22C 0.960. (b) Comparison of both crystal structures (orange and purple) after superimposition of the coumarin core structure (10 atoms, RMS ≈ 0.01 Å, InsightII Software, Accelrys, Inc., San Diego, USA).

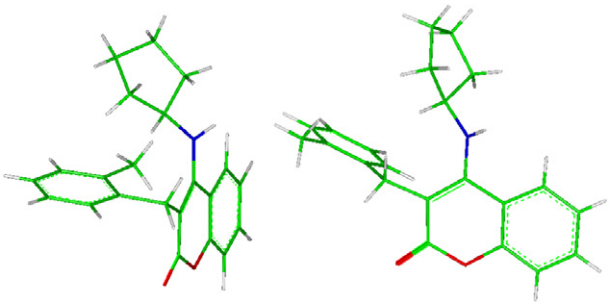


**Figure 4.** Orientation of the amino substituent and the benzyl group with regard to the coumarin core structure (InsightII Software).

## 3. Biological results and discussion

X-ray data reported above reveal that the benzyl motif of **8a**–**8e** can be oriented in such a way that it positions itself over the coumarin core structure, forming thereby a potential tetracyclic motif which presents analogies not only with 3-methoxy-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-one (estrothiazine), an estrogen agonist we recently discovered,[13,14] but also with 17β-estradiol (Fig. 5).[22] To reach such a structural analogy, substituent R of the coumarins (H or $OCH_3$) must be superimposed with the hydrogen acceptor groups of 17β-estradiol or estrothiazine. In this way, the 4-amino group R′ of the compounds is found to match the 11- or the 7-position of 17β-estradiol, depending on

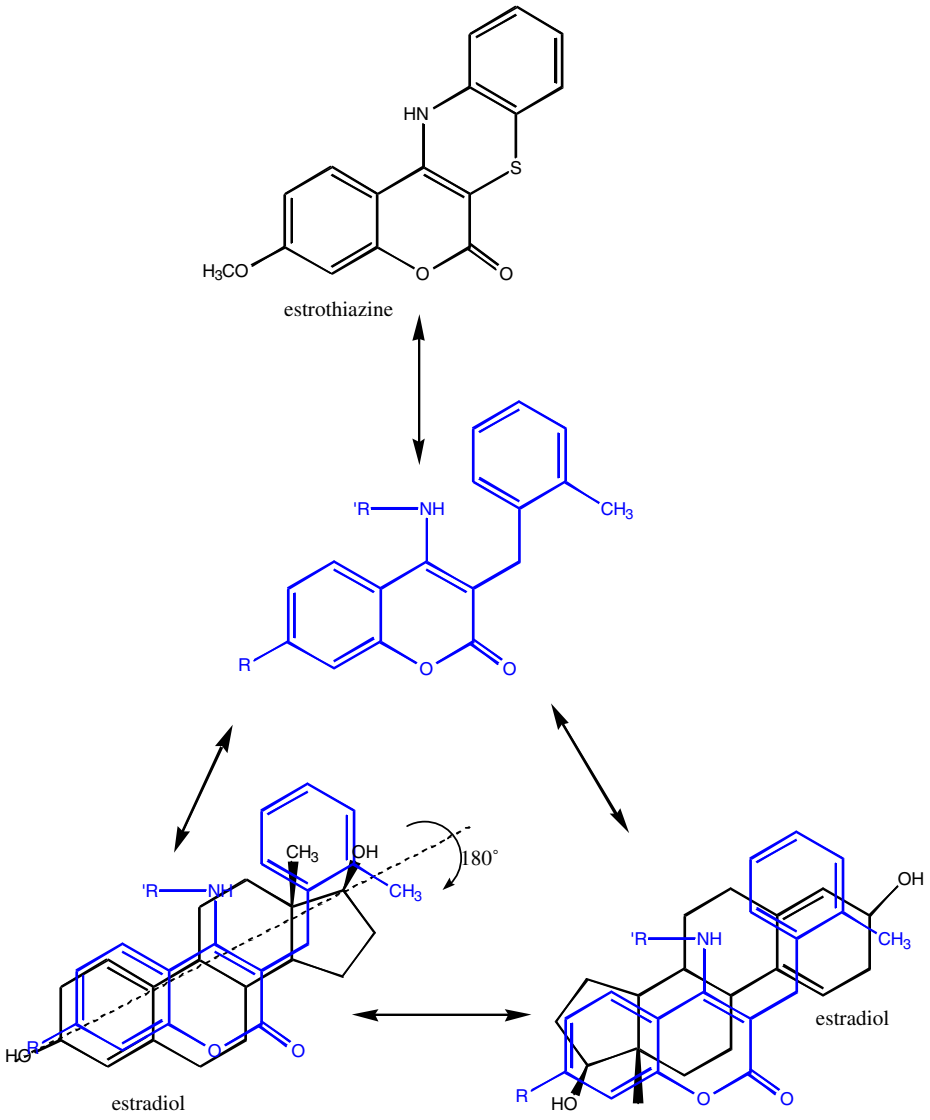



**Figure 5.** Analogies between the reported coumarins (blue) and estrothiazine or 17β-estradiol.

the orientation of the latter.[22] It is noteworthy that these two positions have been extensively explored to generate agonists, partial agonists or pure antagonists (Fig. 5).[22–24] In this regard, long functionalized hydrophobic chains have been reported to confer pure antiestrogen activity, whereas shorter chains confer agonist or partial antagonist properties. Hence, we examined in the current study the bioactivity of **8a**–**8e**, with special emphasis bestowed on their effect on cell proliferation, ER regulation, and ERE-dependent gene transcription in breast carcinoma cells.

### 3.1. Mitogenic effect on breast carcinoma cells

Substances acting as ER agonists generally exert a stimulating effect on the proliferation of estrogen-sensitive breast carcinoma cells. Thus, we examined derivatives **8a**–**8e** with respect to their action on the growth of the ER+ breast carcinoma cell line MCF-7. As illustrated in Figure 6 (upper panel), compounds **8a** and **8b** induced a significant increase in cell proliferation, while compounds **8c**–**8e** had no effect in this respect. Dose–response analysis of the mitogenic effect of compounds **8a** and **8b** on MCF-7 cells (Fig. 7) revealed that these coumarins are ≈4 orders of magnitude less potent than a bona fide estrogen such as $E_2$. Yet, their stimulating effect specifically involves ER-mediated signaling since it is suppressed by the pure antiestrogen fulvestrant (Fig. 8). Furthermore, compounds **8a** and **8b**, as well as compounds **8c**–**8e**, did not affect the proliferation of the ER− breast carcinoma cell line MDA-MB-231 (Fig. 6, lower panel).

### 3.2. Regulation of ER

The proliferative response of breast carcinoma cells and other estrogen target cells to ER agonists is almost invariably accompanied by receptor down-regulation resulting from ER breakdown via the ubiquitin-proteasome system.[25,26] This phenomenon can be documented by ER immunofluorescence staining.[27] As shown in Figure 9, ER demonstration by immunofluorescence microscopy reveals the presence of the receptor in nuclei of MCF-7 cells. In agreement with data reported previously,[28]

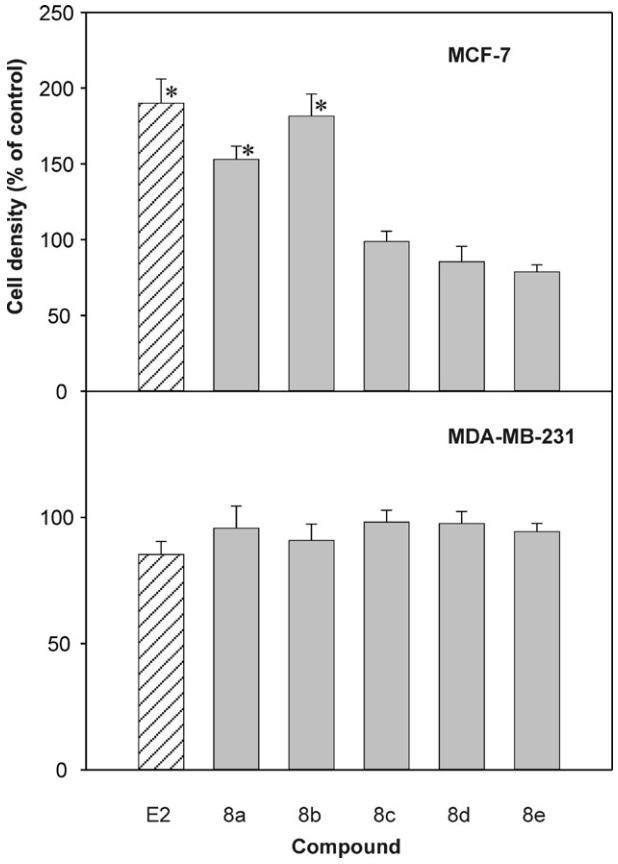



**Figure 6.** Effect of coumarin derivatives on the growth of breast carcinoma cell lines MCF-7 and MDA-MB-231. Cells were exposed for 3 days to compounds **8a**–**8e** at $10^{-6}$ M, or to $10^{-9}$ M $E_2$. Cell densities in cultures were determined by electronic counting as described in Section 5. Data are expressed relative to control (i.e., no drug addition, 100%). Each column is mean of four determinations (±SD). *Significantly higher than control, Dunnett's post hoc test.

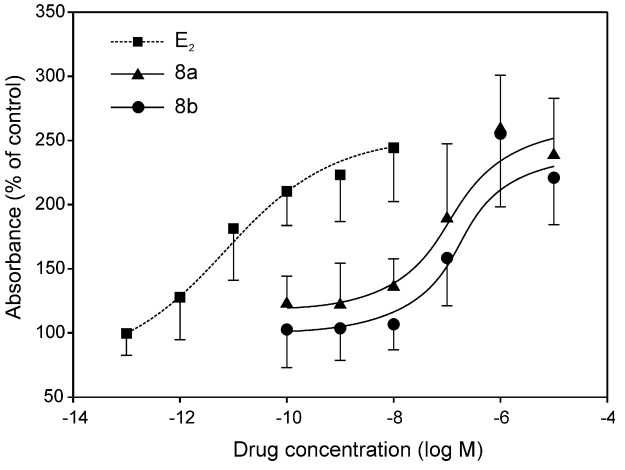



**Figure 7.** Dose–response relationships of the mitogenic effect of compounds **8a** and **8b** on MCF-7 cells, as compared to $E_2$. After 3 days of drug exposure, cells were fixed and stained with crystal violet. Cell densities were evaluated by colorimetry, as described in Section 5. Data are expressed relative to control (i.e., no drug addition, 100%). Each symbol is mean of eight determinations (±SD).

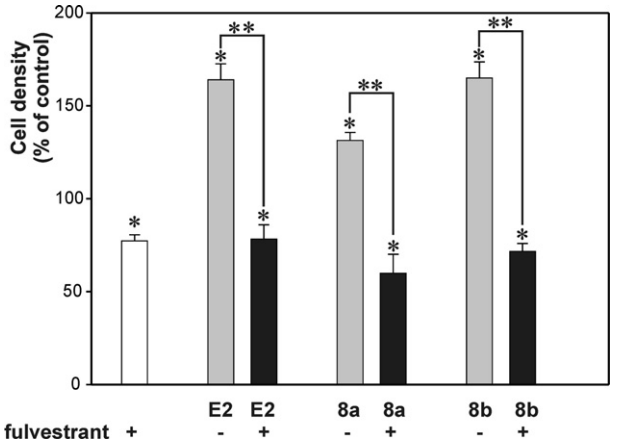



**Figure 8.** Fulvestrant-induced inhibition of the proliferative response of MCF-7 cells to coumarin derivatives (compounds **8a** and **8b** at $10^{-6}$ M) or $E_2$ ($10^{-9}$ M). Cells were exposed for 3 days to **8a**, **8b** or $E_2$ in absence or presence of fulvestrant ($10^{-7}$ M). Cell densities in cultures were determined by electronic counting as described in Section 5. Data are expressed relative to control (i.e., no drug addition, 100%). Each column is mean of four determinations (±SD). *Significantly different from control, Dunnett's post hoc test; **Significantly lower than cultures without fulvestrant, Tukey's post hoc test.

exposure to the physiological agonist $E_2$ results in a drastic decrease of immunofluorescence signal, indicative of ER down-regulation (Fig. 9). Similar receptor down-regulation occurring in cells exposed to steroidal antagonists such as fulvestrant has been reported[29] but is not relevant to the present situation since no investigated compounds displayed antagonist properties. Examination of data in Figure 9 shows that derivatives **8a** and **8b**, which carry saturated cyclic substituents, induce ER down-regulation like $E_2$, whereas derivatives bearing aliphatic or aromatic substituents, that is, compounds **8c**–**8e**, have no effect (Figs. 9 and 10). Thus, compounds **8a** and **8b**, which cause ER down-regulation, are also those which induce a marked proliferative response in MCF-7 cells (Fig. 6). Hence, saturated cyclic substituents on the nitrogen in position 4 of the coumarin confer both mitogenicity and the ability to induce ER down-regulation in breast carcinoma cells.

Figure 11 illustrates the effect of the proteasome inhibitor MG-132 on ER down-regulation induced by compounds **8a** and **8b**, as compared to $E_2$. As can be expected, MG-132 abrogates the decrease of ER immunofluorescence signal caused by $E_2$ (Fig. 8c and d). A similar effect of proteasome inhibition is seen with compound **8a** (Fig. 8e and f) and compound **8b** (Fig. 8g and h), indicating that, like $E_2$, they induce ER breakdown via the ubiquitin–proteasome pathway.

### 3.3. ER-mediated gene transactivation

ER-mediated intracellular signaling (at least the classical genomic pathway) notably involves the recruitment of the receptor to estrogen response elements (ERE) present within the promoter regions of target genes. Thus, the transactivation activity of ER can be assessed by evaluating the expression of ERE-containing reporter genes. In this study, we used MVLN cells (i.e., MCF-7 cells stably

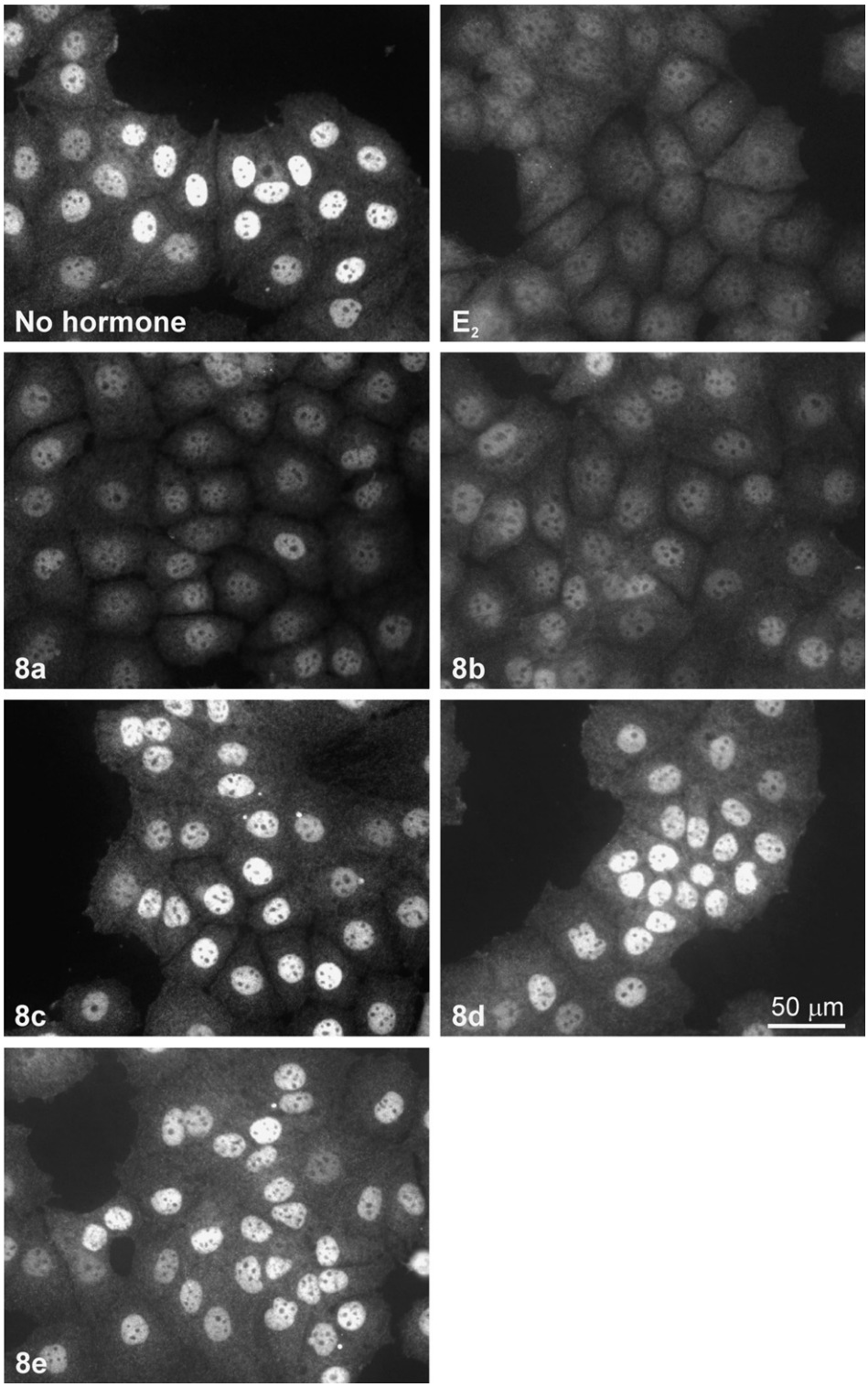



**Figure 9.** Effect of coumarins **8a**–**8e** ($10^{-6}$ M), and of $10^{-9}$ M $E_2$ on ER expression in MCF-7 cells, as demonstrated by immunofluorescence staining. Cells were treated for 24 h, fixed and processed for immunostaining with HC-20 antiserum as described in Section 5. Texas Red labeling.

transfected with a pVit-tk-Luc reporter plasmid) in order to examine the effect of derivatives **8a**–**8e** on ERE-driven gene transactivation (Fig. 12). In accordance with their mitogenic activity on MCF-7 cells, compounds **8a** and **8b** enhanced ER-mediated gene transactivation, producing 185% and 127% increases of luciferase expression, respectively. By contrast, compounds **8d** and **8e** only induced 36% and 53% increases of luciferase gene expression. Strikingly, compound **8c** induced a marked stimulation of ER-mediated gene transactivation, since it produced a 260% increase in luciferase gene expression. Thus, the effect of **8c** at $10^{-6}$ M was equivalent to that of $E_2$ at $10^{-9}$ M. Dose–effect relationship of **8c** on ERE-driven transactivation, illustrated in Figure 13, indicates

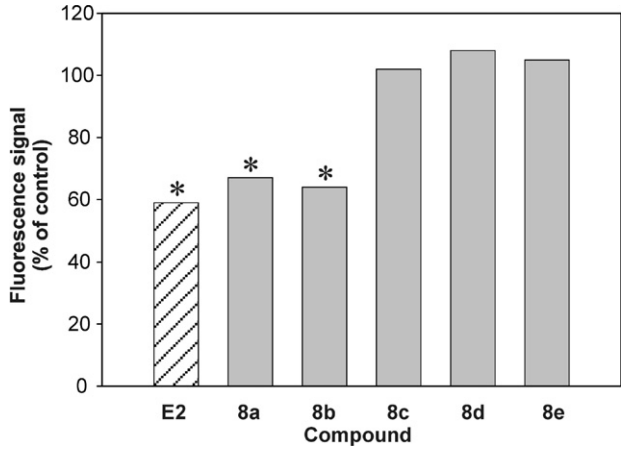



**Figure 10.** Quantitative analysis of immunofluorescence signals in nuclei of MCF-7 cells (see Fig. 9). Median signal intensities were evaluated as described in Section 5 and are expressed relative to control (i.e., no drug addition, 100%). Drug concentrations as specified in legend to Figure 9. *Significantly lower than control (untreated cells), Dunn's multiple comparison test.

an $EC_{50}$ of $\approx 10^{-7}$ M (this value is only approximative in absence of a plateau at high concentrations). In similar conditions, the $EC_{50}$ value for $E_2$ would be $10^{-11}$ M.[30] Even though **8c** appears noticeably less potent than $E_2$, its stimulating action on ER-mediated gene transactivation is specific, since it is suppressed by the pure antiestrogen fulvestrant (Fig. 13).

### 3.4. Receptor–ligand binding studies

In absence of isotopically labeled forms of our synthetic coumarin derivatives, it was not possible to evaluate their binding to ER in a direct fashion. Thus, putative interactions of compounds **8a**–**8e** with ER were explored by examining their ability to compete with [$^3$H]$E_2$ for binding to recombinant ER. With the exception of **8a**, no investigated compound acted as an effective competitor, even when tested in 1000-fold excess. In these conditions, **8a** produced a detectable inhibition (RBA $\leqslant 0.1\%$ of $E_2$) which was also recorded for ERβ (data not shown). This very weak interference with [$^3$H]$E_2$ binding must probably be artfactual since biological efficiency of **8a** is comparable to that of **8b** and **8c**, in terms of ER-mediated transcription. Thus, despite structural analogies between 17β-estradiol and coumarins **8a**–**8e**, there is no evidence for direct interaction of the latter compounds with the 17β-estradiol binding pocket. So far, it remains uncertain whether the inability of compounds **8a**–**8e** to compete with [$^3$H]$E_2$ results from the fact that they form unstable complexes with ER or from the fact that they interact with ER sites distinct from the 17β-estradiol binding pocket (see below). In this context, it is of note that other bioactive xenoestrogens devoid of [$^3$H]$E_2$ displacement ability have already been reported.[31]

## 4. Conclusion

The present paper reports the synthesis and the characterization of a new family of benzopyrans, that is, 4-amino-3-(2-methylbenzyl)coumarins **8a**–**8e**. UV, FTIR, and $^1$H NMR spectroscopic data as well as X-ray crystallography analysis led us to conclude that these compounds exist exclusively under the enamine form. In a second part of this work, we evaluated the biological activity of these compounds on the (ER+) breast carcinoma cell line MCF-7. Among the five reported coumarins, the compounds **8a** and **8b**, that is, the cyclopentyl and cyclohexyl N-substituted derivatives, stimulated cell proliferation in an ER-dependent manner. In addition to their mitogenic activity, **8a** and **8b** induced ER down-regulation and enhanced ERE-driven gene transcription, all properties typical of ER agonists. On the other hand, another compound—the *n*-dodecyl N-substituted derivative **8c**—exhibited quite unusual properties since this compound enhanced ER-mediated gene transactivation without affecting cell proliferation or causing ER down-regulation. This observation demonstrated that, in this series of compounds, a long aliphatic chain suppresses mitogenicity without loss of transactivation capacity. From the biological profile of **8c**, we can infer that ERE-driven gene transactivation is not invariably accompanied by ER down-regulation. This is a significant finding since it contradicts previous claim that ER degradation is mandatory for ER-mediated gene transactivation.[32] Yet, our observations on **8c** are in accordance with more recent work showing that ER proteolysis is not essential for transactivation activity.[33,34] Another interesting conclusion which can be drawn from the properties of compound **8c** is that—as far as estrogen-like substances are concerned—ERE-driven gene transactivation is probably necessary, but not sufficient, for mitogenic activity. In this regard, it might be worth exploring whether a cell proliferative response mediated by ER activation is always associated with a down-regulation of the receptor.

The most unexpected finding in our study is the fact that the coumarin derivatives fail to compete with $E_2$ for ER binding. In this context, the absence of ER anchoring points in the reported coumarins remains uncommon. Actually, it is well known that ligand binding to ER depends principally on the presence of polar anchoring chemical groups such as methoxy groups, alcohol functions or acidic phenolic hydroxyls, required to establish electrostatic stabilizing interactions with selected ER residues. The intracellular oxidation of the aromatic ring of the coumarinic motif into phenol by specific enzymes and the stabilization of the complex through aromatic—aromatic interactions are not excluded. However, as already shown with 1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones, the absence of activity with compound **8e** suggests that the presence of a phenolic hydrogen acceptor does not contribute to estrogenicity.[13] Alternatively, the interaction of the active coumarins **8a**–**8c** with the second binding pocket of the receptor[35,36] or with the coactivator binding site at the surface of ER should be explored.[37] Last but not least, we cannot exclude the possibility that ER activation induced by **8a**–**8c** involves indirect mechanism(s) such as nuclear receptor crosstalk or crosstalk with other signaling pathways.

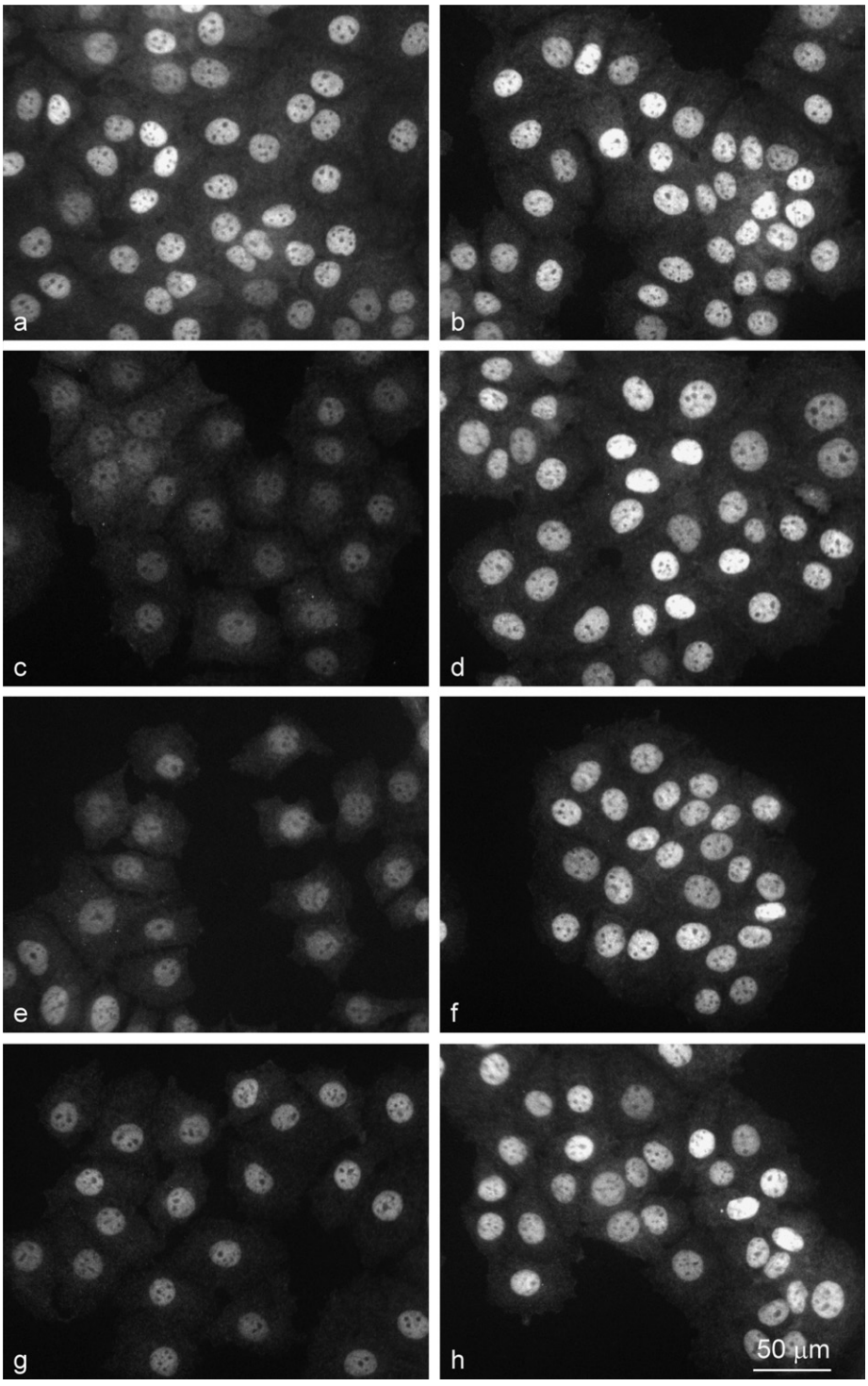



**Figure 11.** Effect of MG-132 on ER down-regulation induced by $E_2$, compound **8a** or compound **8b**. (a) Untreated cells; (b) 7 h-exposure to $10^{-5}$ M MG-132; (c) 6 h-exposure to $E_2$ ($10^{-9}$ M); (d) 6 h-exposure to $E_2$ in presence of MG-132; (e) 6 h-exposure to **8a** ($10^{-6}$ M); (f) 6 h-exposure to **8a** in presence of MG-132; (g) 6 h-exposure to **8b** ($10^{-6}$ M); (h) 6 h-exposure to **8b** in presence of MG-132. Treatment with MG-132 was initiated 1 h before addition of $E_2$, **8a** or **8b**. Cells were processed for immunofluorescence staining with HC-20 antiserum as described in Section 5. Texas Red labeling.

From a pharmacological point of view, the peculiar properties of the *n*-dodecyl N-substituted derivative **8c** might prove of value in a context of estrogen replacement therapy and therefore the mechanism of action of this particular compound deserves further investigations.

## 5. Experimental

### 5.1. Chemistry

4-Hydroxycoumarin, primary amines, and 2-methylbenzylbromide were purchased from Aldrich (Sigma–

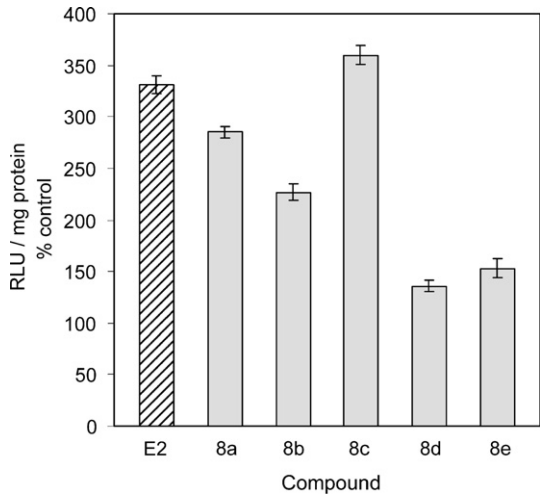



**Figure 12.** Effect of coumarins **8a**–**8e** ($10^{-6}$ M) and of $E_2$ ($10^{-8}$ M) on ERE-driven gene transcription in MVLN cells. After 24 h of drug exposure, luciferase reporter gene expression was assayed as specified in Section 5. Data are expressed as percentage of control (untreated cells, 100 ± 1%). Each column represents mean of three determinations (±SD). All values are significantly higher than control value (Dunnett's post hoc test).

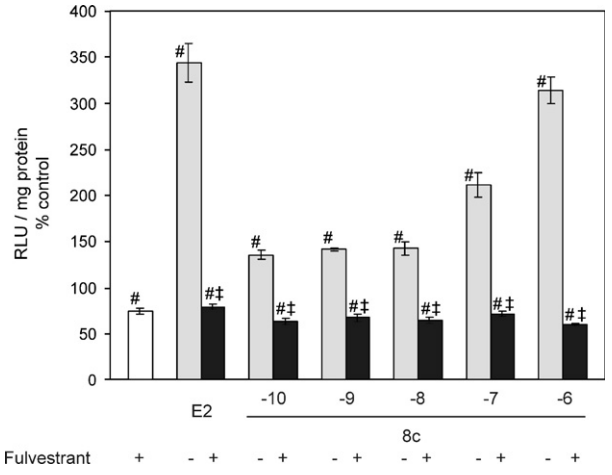



**Figure 13.** Dose–response relationship of the stimulating effect of compound **8c** on ERE-driven gene transcription. Cells were exposed for 24 h to increasing concentrations of **8c** ($10^{-10}$–$10^{-6}$ M), in the absence or the presence of fulvestrant ($10^{-7}$ M). For comparison, cells were treated in the same conditions with $E_2$ at $10^{-8}$ M. Luciferase reporter gene expression was assayed as specified in Section 5. Data are expressed as percentage of control (untreated cells, 100 ± 2%). Each column represents mean of three determinations (±SD). #Significantly different from control, Dunnett's post hoc test; ‡significantly lower than cultures without fulvestrant, Tukey's post hoc test.

Aldrich, Saint-Quentin Fallavier, France). 4-Hydroxy-7-methoxycoumarin was purchased from Acros Organics (Noisy-le-Grand, France). All melting points were determined on a Kofler Heizbank Reichert 18.43.21 without correction. The electronic absorption spectra (250–600 nm) were recorded at 20 °C in DMSO on a UVIKON 930 spectrophotometer. Infrared (IR) spectra were measured with a FTIR-8201PC spectrophotometer in potassium bromide pellets ($\nu$ in $cm^{-1}$) over a range of 4400–550 $cm^{-1}$. Absorption bands are designed as S, strong; br S, broad strong; M, medium; W, weak; VS, very strong; and br VS, broad very; strong. $^1$H NMR spectra were obtained at 293 K on a Bruker AC300 (300 MHz) spectrometer in $CDCl_3$ or DMSO-$d_6$ solutions. Chemical shifts ($\delta$) are reported in parts per million (ppm) and downfield from tetramethylsilane $Me_4Si$ (internal reference). Spin–spin coupling $J$ was exposed in Hz. Splitting patterns are designed as s, singlet; br s, broad singlet; d, doublet; br d, broad doublet; t, triplet; q, quadruplet; m, multiplet; br m, broad multiplet; dd, double doublet; br, broad and dd, double doublet. Mass spectra were recorded on a triple quadrupole tandem mass spectrometer Nermag R30-10H under positive chemical ionization (CI) using ammonia as reagent gas under a pressure of $10^{-4}$ Torr, an electron energy of 70 eV and an emission current of 100 μA. Mass spectra corresponded to averages of 30 full spectra were recorded on an EZSCAN acquisition system (Mass Evolution, Houston, TX, USA). Elemental analysis was performed by the Service de Microanalyses of the Université Pierre et Marie Curie in Paris. Thin-layer chromatographies were carried out on Alugram Sil G/UV254 plates with appropriate solvents and spots were visualized under UV light at 254 nm.

**5.1.1. Condensation of primary amines on 4-hydroxycoumarin.** After the dissolution of 10 mmol of 4-hydroxycoumarin **1a** in 50 mL of ethoxyethanol, 1.5 equiv of the primary amine freshly distilled on potassium hydroxide are added. The mixture is stirred under reflux during 6 h. The solvent is then evaporated under vacuum and the crude powder filtered and rinsed with diethyl ether.

**5.1.1.1. 4-Cyclopentylaminocoumarin (6a).** Prepared by coupling cyclopentylamine **4a** on 4-hydroxycoumarin **1a**. White powder (78%). Mp 170 °C. FTIR ($\nu$, $cm^{-1}$): 3308 (S, amine), 3076 (W, CH aromatic), 2945 (M, $CH_2$ cyclopentyl), 2850 (M, $CH_2$ cyclopentyl), 1655 (VS, C=O), 1610 (VS, C(3)=C(4) coumarin), 1549 (VS, C(3)=C(4) coumarin), 1483–1447 (M, C=C aromatic). $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 1.63–1.76 (6H, m, cyclopentyl), 2.06–2.11 (2H, m, cyclopentyl), 3.86 (1H, br dd, $^3J$ = 5.7 Hz, cyclopentyl), 5.30 (1H, s, CH=C pyranone), 6.14 (1H, d, $^3J$ = 5.7 Hz, NH), 7.22–7.27 (2H, dd, $^3J$ = 7.7 Hz, CH aromatic), 7.49 (1H, dd, $^3J$ = 7.3 Hz, CH aromatic), 7.83 (1H, d, $^3J$ = 7.5 Hz, CH aromatic). MS $m/z$: 230 (M+1) ($C_{14}H_{15}NO_2$).

**5.1.1.2. 4-Cyclohexylaminocoumarin (6b).** Prepared by coupling cyclohexylamine **4b** on 4-hydroxycoumarin **1a**. White powder (80%). Mp 184 °C. FTIR ($\nu$, $cm^{-1}$): 3325 (S, amine), 3078 (W, $CH_2$ aromatic), 2928 (S, $CH_2$ cyclohexyl), 2855 (M, $CH_2$ cyclohexyl), 1663 (VS, C=O), 1610 (VS, C(3)=C(4) coumarin), 1551 (VS, C(3)=C(4) coumarin), 1485–1447 (M, C=C aromatic). $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 1.21–1.45 (5H, m, cyclohexyl), 1.69–1.79 (3H, m, cyclohexyl), 2.00–2.15 (2H, m, cyclohexyl), 3.40 (1H, br s, CH cyclohexyl), 5.23 (1H, br s, NH), 5.33 (1H, s, CH=C pyranone), 7.20–7.32 (2H, m, CH aromatic), 7.47–7.54 (2H, m, CH aromatic). MS $m/z$: 244 (M+1) ($C_{15}H_{17}NO_2$).

**5.1.1.3. 4-*n*-Dodecylaminocoumarin (6c).** Prepared by coupling *n*-dodecylamine **4c** on 4-hydroxycoumarin **1a**. White powder (30%). Mp 108 °C. FTIR ($\nu$, $cm^{-1}$): 3321 (VS, amine), 3050 (W, CH aromatic), 2958 (S, $CH_3$ *n*-dodecyl), 2923 (VS, $CH_2$ *n*-dodecyl), 2853 (VS, $CH_2$ *n*-dodecyl), 1666 (VS, C=O), 1606 (VS, C(3)=C(4) coumarin), 1553 (VS, C(3)=C(4) coumarin), 1483–1434 (M, C=C aromatic). $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 0.89 (3H, t, $^3J = 6.9$ Hz, $CH_3$ *n*-dodecyl), 1.27–1.43 (18H, m, $CH_2$ *n*-dodecyl), 1.75 (2H, m, $CH_2$ *n*-dodecyl), 3.27 (2H, dd, $^3J = 5.3$ Hz, $CH_2$–NH *n*-dodecyl), 5.33 (1H, s, CH=C pyranone), 5.41 (1H, br s, NH), 7.24–7.34 (2H, m, CH aromatic), 7.51–7.56 (2H, m, CH aromatic). MS *m*/*z*: 330 (M+1) ($C_{21}H_{31}NO_2$).

**5.1.1.4. 4-Phenylethylaminocoumarin (6d).** Prepared by coupling phenylethylamine **4d** on 4-hydroxycoumarin **1a**. White powder (74%). Mp 178 °C. FTIR ($\nu$, $cm^{-1}$): 3265 (S, amine), 3022 (W, CH aromatic), 2940 (W, $CH_2$), 2856 (W, $CH_2$), 1659 (VS, C=O), 1609 (VS, C(3)=C(4) coumarin), 1562 (VS, C(3)=C(4) coumarin), 1497–1450 (M, W, C=C aromatic). $^1H$ NMR (DMSO-$d_6$) $\delta$ (ppm): 2.93 (2H, t, $^3J = 5.5$ Hz, $CH_2$), 3.49 (2H, dt, $^3J = 5.5$ Hz, $CH_2$–NH), 5.20 (1H, s, CH=C pyranone), 7.16–7.32 (7H, m, CH aromatic), 7.56 (1H, dd, $^3J = 8.8$ Hz, CH aromatic), 7.70 (1H, br s, $^3J = 5.9$ Hz, NH), 8.00 (1H, d, $^3J = 8.7$ Hz, CH aromatic). MS *m*/*z*: 266 (M+1) ($C_{17}H_{15}NO_2$).

**5.1.1.5. 7-Methoxy-4-*n*-pentylaminocoumarin (6e).** Prepared by coupling *n*-pentylamine **4e** on 7-methoxy-4-hydroxycoumarin **1b**. White powder (73%). Mp 105 °C. FTIR ($\nu$, $cm^{-1}$): 3306 (M, amine), 3028 (W, CH aromatic), 2920 (W, $CH_2$ *n*-pentyl), 2851 (W, $CH_2$ *n*-pentyl), 1693 (VS, C=O), 1601 (S, C(3)=C(4) coumarin), 1543 (S, C(3)=C(4) coumarin), 1497–1431 (M, C=C aromatic). $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 0.90 (3H, t, $^3J = 7.1$ Hz, $CH_3$ *n*-pentyl), 1.38 (4H, m, $CH_2$ *n*-pentyl), 1.74 (2H, m, $CH_2$ *n*-pentyl), 3.27 (2H, dd, $^3J = 7.1$ Hz, $CH_2$–NH *n*-pentyl), 3.85 (3H, s, $OCH_3$), 5.30 (1H, s, CH=C pyranone), 5.94 (1H, br s, NH), 7.23–7.30 (1H, m, CH aromatic), 7.55 (1H, dd, $^3J = 7.1$ Hz, CH aromatic), 7.70 (1H, dd, $^3J = 7.1$ Hz, CH aromatic). MS *m*/*z*: 262 (M+1) ($C_{15}H_{19}NO_3$).

**5.1.2. Condensation of methylbenzylbromide on the substituted 4-aminocoumarins (6a–6e).** For the preparation of 4-amino-3-benzylcoumarins, 10 mmol of substituted 4-aminocoumarin and 1.5 equiv of mehylbenzylbromide **7** were stirred at 135–140 °C during 8 h. On cooling at 50 °C, 15 mL of isopropanol was added to the mixture. The bromhydrate ammonium salt of the awaited product was precipitated and filtered under vacuum. The precipitate was dissolved in 15 mL of chloroform and washed three times with 10 mL of an aqueous solution of sodium hydroxide 0.1 N. After, the organic layer was washed with twice with 10 mL of water and dried on dry $MgSO_4$. The mixture was filtered and the solvent evaporated under vacuum to afford to the awaited 4-amino-3-benzylamincoumarins **8a**–**8e**.

**5.1.2.1. 4-Cyclopentylamino-3-(2-methylbenzyl)coumarin (8a).** Prepared by coupling methylbenzylbromide **7** on the 4-aminocoumarin **6a**. White powder (54%). Mp 174 °C. FTIR ($\nu$, $cm^{-1}$): 3348 (S, amine), 3050 (W, CH aromatic), 2943 (M, $CH_2$ cyclopentyl), 2866 (M, $CH_2$ cyclopentyl), 1655 (VS, C=O), 1605 (S, C(3)=C(4) coumarin), 1531 (br S, C(3)=C(4) coumarin), 1485–1458 (M, C=C aromatic). $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 1.32–1.36 (2H, m, cyclopentyl), 1.51–1.55 (4H, m, cyclopentyl), 1.84–2.00 (2H, m, cyclopentyl), 2.38 (3H, s, $CH_3$), 3.97 (2H, s, $CH_2$), 4.13 (1H, br s, CH cyclopentyl), 4.36 (1H, br s, NH), 7.01–7.28 (5H, m, CH aromatic), 7.35–7.38 (1H, d, $^3J = 8.3$ Hz, CH aromatic), 7.50 (1H, m, CH aromatic), 7.73 (1H, d, $^3J = 8.1$ Hz, CH aromatic). MS *m*/*z*: 334 (M+1) ($C_{22}H_{23}NO_2$). Anal. Calcd for $C_{22}H_{23}NO_2$: C, 79.25; H, 6.95; N, 4.20. Found: C, 79.01; H, 6.93; N, 4.16.

**5.1.2.2. 4-Cyclohexylamino-3-(2-methylbenzyl)coumarin (8b).** Prepared by coupling methylbenzylbromide **7** on the 4-aminocoumarin **6b**. White powder (51%). Mp 210 °C. FTIR ($\nu$, $cm^{-1}$): 3364 (VS, amine), 3067 (W, CH aromatic), 2928 (S, $CH_2$ cyclohexyl), 2851 (S, $CH_2$ cyclohexyl), 1651 (VS, C=O), 1605 (VS, C(3)=C(4) coumarin), 1531 (br VS, C(3)=C(4) coumarin), 1481–1454 (M, C=C aromatic). $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 1.00–1.07 (5H, m, cyclohexyl), 1.53–1.69 (3H, br m, cyclohexyl), 1.83–1.88 (2H, br m, cyclohexyl), 2.40 (3H, s, $CH_3$), 3.65 (1H, m br, CH cyclohexyl), 3.95 (2H, s, $CH_2$), 4.36 (1H, br s, NH), 6.99–7.29 (5H, m, CH aromatic), 7.40 (1H, dd, $^3J = 8.3$ Hz, $^4J = 1.1$ Hz, CH aromatic), 7.46–7.55 (1H, m, CH aromatic), 7.60 (1H, dd, $^3J = 8.1$ Hz, $^4J = 1.3$ Hz, CH aromatic). MS *m*/*z*: 348 (M+1) ($C_{23}H_{25}NO_2$). Anal. Calcd for $C_{23}H_{25}NO_2$: C, 79.51; H, 7.25; N, 4.03. Found: C, 79.48; H, 7.28; N, 4.06.

**5.1.2.3. 4-*n*-Dodecylamino-3-(2-methylbenzyl)coumarin (8c).** Prepared by coupling methylbenzylbromide **7** on the 4-aminocoumarin **6c**. White powder (42%). Mp 112 °C. FTIR ($\nu$, $cm^{-1}$): 3333 (VS, amine), 3020 (W, CH aromatic), 2926 (VS, $CH_2$ *n*-dodecyl), 2853 (VS, $CH_2$ *n*-dodecyl), 1655 (VS, C=O), 1609 (VS, C(3)=C(4) coumarin), 1541 (br VS, C(3)=C(4) coumarin), 1487–1452 (M, C=C aromatic). $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 0.88 (3H, t, $^3J = 6.9$ Hz, CH3 *n*-dodecyl), 1.16–1.35 (18H, br m, *n*-dodecyl), 1.41 (2H, br m, *n*-dodecyl), 2.38 (3H, s, $CH_3$), 3.37 (2H, m br, $CH_2$–NH *n*-dodecyl), 3.98 (2H, s, $CH_2$), 4.39 (1H, br s, NH), 7.01–7.26 (5H, m, CH aromatic), 7.35–7.38 (1H, dd, $^3J = 8.3$ Hz, $^4J = 1.0$ Hz, CH aromatic), 7.47–7.53 (1H, m, CH aromatic), 7.67 (1H, dd, $^3J = 8.1$ Hz, $^4J = 1.2$ Hz, CH aromatic). MS *m*/*z*: 434 (M+1) ($C_{29}H_{39}NO_2$). Anal. Calcd for $C_{29}H_{39}NO_2$: C, 80.33; H, 9.07; N, 3.23. Found: C, 80.58; H, 9.14; N, 3.29.

**5.1.2.4. 4-Phenylethylamino-3-(2-methylbenzyl)coumarin (8d).** Prepared by coupling methylbenzylbromide **7** on the 4-aminocoumarin **6d**. White powder (41%). Mp 152 °C. FTIR ($\nu$, $cm^{-1}$): 3329 (S, amine), 3032 (M, CH aromatic), 2939 (M, $CH_2$), 1659 (VS, C=O), 1605 (VS, C(3)=C(4) coumarin), 1539 (br VS, C(3)=C(4) coumarin), 1485–1454 (VS, M, C=C aromatic). $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.29 (3H, s, $CH_3$), 2.71 (2H, t, $^3J = 8.22$ Hz, $CH_2$, 3.65 (2H, m br, $CH_2$–NH), 3.84 (2H, s, $CH_2$), 4.35 (1H, br s, NH),

6.93–7.01 (3H, m, CH aromatic), 7.08–7.29 (7H, m, CH aromatic), 7.34–7.39 (1H, m, CH aromatic), 7.46 (1H, m, CH aromatic), 7.50–7.56 (1H, m, CH aromatic). MS *m/z*: 370 (M+1) ($C_{25}H_{23}NO_2$). Anal. Calcd for $C_{25}H_{23}NO_2$: C, 81.27; H, 6.27; N, 3.79. Found: C, 81.36; H, 6.24; N, 3.87.

**5.1.2.5. 7-Methoxy-4-n-pentylamino-3-(2-methylbenzyl)coumarin (8e).** Prepared by coupling methylbenzylbromide **7** on the 4-aminocoumarin **6e**. White powder (45%). Mp 172 °C. FTIR ($\nu$, $cm^{-1}$): 3325 (S, amine), 3052 (W, CH aromatic), 2953 (M, $CH_3$), 2926 (M, $CH_2$), 2870 (W, $CH_2$), 1655 (S, C=O), 1607 (S, C (3)=C(4) coumarin), 1541 (S, C (3)=C(4) coumarin), 1490–1420 (W, C=C aromatic). $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 0.82 (3H, t, $^3J = 7.1$ Hz, $CH_3$ *n*-pentyl), 1.12–1.21 (4H, m, *n*-pentyl), 1.37–1.44 (2H, m, *n*-pentyl), 2.37 (3H, s, $CH_3$), 3.36 (2H, m br, $CH_2$–NH *n*-pentyl), 3.87 (3H, s, $OCH_3$), 3.93 (2H, s, $CH_2$), 4.30 (1H, s, NH), 6.79–6.83 (2H, m, CH aromatic), 7.02–7.20 (4H, m, CH aromatic), 7.60 (1H, d, $^3J = 8.7$ Hz, CH aromatic). MS *m/z*: 366 (M+1) ($C_{23}H_{27}NO_3$). Exact Anal. Calcd for $C_{23}H_{27}NO_3$: C, 75.62; H, 7.40; N, 3.84. Found: C, 75.71; H, 7.20; N, 3.82.

### 5.2. X-ray crystal structure determination of 4-cyclopentylamino-3-(2-methylbenzyl)coumarin (8a)

A selected single crystal (dimensions 0.48 mm × 0.18 mm × 0.17 mm) of **8a** was mounted onto a glass fiber and set up on a Nonius Kappa-CCD diffractometer. Diffraction data were collected at room-temperature Mo Kα radiation ($\lambda = 0.71073$ Å). Unit cell parameter determination, data collection strategy, and integration were carried out with the Nonius EVAL-14 suite of programs. The data were corrected from absorption by a multi-scan method.[38] The structure was solved by direct methods with SHELXS-86,[39] refined by full least-squares on $F^2$, and completed with SHELXL-97.[40] Graphics were carried out with DIAMOND.[41] All non-H atoms were refined with anisotropic displacement parameters and H atoms were simply introduced at calculated positions (riding model). Crystallographic data: $C_{22}H_{23}NO_2$, MW, 333.41; triclinic, *P*–1 (No. 2); *a*, 11.978(2) Å; *b*, 12.935(2) Å; *c*, 13.321(2) Å; *V*, 1807.2(5) $Å^3$; *Z*, 4; $D_{calc}$, 1.225 g $cm^{-1}$; *F*(000), 712; $\mu$, 0.078 $mm^{-1}$; 6528 observed data, with $I > 2\sigma(I)$.

### 5.3. Biology

**5.3.1. Cell lines and culture.** The ER+ cell line MCF-7 (ATCC No. HTB22) was originally obtained in 1977 from the Michigan Cancer Foundation (Detroit, MI). The ER− cell line MDA-MB-231 (ATCC No. HTB26) came from the American Type Culture Collection. Routine cell propagation and experimental studies were carried out at 37 °C in a cell incubator with humid atmosphere at 5% $CO_2$. Unless specified otherwise, cells were cultured in T-flasks containing DMEM (BioWhittaker Europe, Verviers, Belgium) supplemented with Phenol Red, 10% FBS (HyClone, Logan, Utah), 25 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (DMEM-FBS) (supplements from BioWhittaker). Cells were passed once a week, with a renewal of the medium every two days. For subculture and measurement of growth, the cell monolayers were rinsed with DPBS and cells were dislodged from the vessel bottom by treatment with trypsin–EDTA solution. After vigorous pipetting, concentrations of cells in suspension were determined in an electronic cell counter (model Z1 Coulter counter, Beckman Coulter, Fullerton, CA). For routine cell maintenance, MCF-7 and MDA-MB-231 cells were plated in 75 or 25 $cm^2$ T-flasks at a density of $10^4$ cells/$cm^2$. Before measurement of cell growth, ER immunofluorescence staining or assessment of ER-mediated reporter gene transactivation, phenol red-free DMEM supplemented with 10% charcoal-stripped FBS (HyClone, Logan, Utah), 25 mM Hepes, and 2 mM L-glutamine (EFM) was substituted for DMEM-FBS and cells were grown in this medium for a minimum of 2 days.

**5.3.2. Drugs and cell treatments.** $E_2$ was obtained from Calbiochem-Novabiochem (La Jolla, CA). Fulvestrant (ICI 182,780) came from Tocris Cookson, Bristol, UK. Stock solutions of tested compounds were prepared at least 10,000-fold more concentrated in ethanol ($E_2$ and fulvestrant) or DMSO (coumarin derivatives) and stored at −20 °C. MG-132 was purchased from BIOMOL Int. (Plymouth Meeting, PA) and prepared as a 1000-fold concentrated stock in ethanol. Working solutions were made extemporaneously in culture medium.

**5.3.3. Cell proliferation.** The effects of $E_2$, fulvestrant and coumarin derivatives on cell growth were assessed either by direct cell counting[26] or by colorimetry after crystal violet staining,[42] as described previously. For measurement of cell culture growth by direct counting, cells in EFM were plated in 12-well dishes at a density of $10^4$ cells/$cm^2$. At day 1, the seeding medium was replaced by EFM containing $E_2$ ($10^{-9}$ M), fulvestrant ($10^{-7}$ M) or one of the coumarin derivatives ($10^{-6}$ M). Cells were trypsinized at day 4 and counted as described above. For the measurement of growth by crystal violet staining, MCF-7 cells in EFM were seeded in 96-well plates (2000 cells/well) and treated as indicated above for a period of 3 days with coumarin **8a** or **8b** ($10^{-10}$–$10^{-5}$ M), or with $E_2$ ($10^{-13}$–$10^{-8}$ M). At the end of drug exposure, the culture medium was removed and cells were fixed with 1% glutaraldehyde. After fixation, cells were stained with 0.1% crystal violet. Destaining was achieved under gently running tap water and cells were lysed with 0.2% Triton X-100. The absorbance of stained preparations was measured at 570 nm using a Labsystems Multiskan MS microplate reader. In preliminary experiments, we checked that there was a linear relationship between absorbance and cell density.

**5.3.4. Immunofluorescence staining.** MCF-7 cells in EFM were plated at a density of $10^4$ cells/$cm^2$ on sterile round glass coverslips in 12-well dishes. Two days after seeding, cells were fed fresh EFM containing $E_2$ ($10^{-9}$ M) or coumarin derivatives ($10^{-6}$ M), with or without MG-132 ($10^{-5}$ M) (treatment durations specified in figure legends). At the end of treatment, cell monolayers

were rinsed with Dulbecco's PBS and fixed with 4% paraformaldehyde in the same buffer. Following fixation, paraformaldehyde was changed for DPBS where cell cultures were stored at 4 °C until immunostaining which was usually performed within the next 20 h. Demonstration of ER by immunofluorescence was achieved as detailed in a previous publication.[27] In brief, cell monolayers were rinsed several times with PBS (PBS, 0.04 M $Na_2HPO_4$, 0.01 M $KH_2PO_4$, and 0.12 M NaCl, pH 7.2) containing 0.2% Triton X-100. For all subsequent incubation and rinsing steps, Triton X-100 was included in buffer to ensure cell permeabilization. Before exposure to the primary antibody, cells were preincubated for 20 min in PBS containing 0.05% casein and 0.05 M $NH_4Cl$ to prevent non-specific adsorption of immunoglobulins. Cells were exposed for 60 min to the primary antibody (rabbit polyclonal antibody HC-20 raised against residues 576–595 at the carboxy terminus of human ERα, Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:40 in PBS containing 0.05% casein. Thereafter, the cell preparations were incubated for 30 min in presence of a dextran polymer conjugated with both peroxidase and antibodies raised against rabbit immunoglobulins (EnVision™, Dakopatts, Glostrup, Denmark). The next step consisted in a 30 min incubation with rabbit anti-peroxidase antiserum (Laboratory of Hormonology, Marloie, Belgium), followed by biotinylated swine anti-rabbit immunoglobulin antibody (Dakopatts) for a further 30 min. Texas Red labeling was completed by exposing cells for 30 min to Texas Red-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After final rinses in PBS, the coverslips were mounted on glass slides using commercial anti-fading medium (Vectashield®, Vector Laboratories). Negative controls were produced by omitting the primary antibody. This modification resulted in a near complete disappearance of the signal. The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi-illumination. Excitation wavelength of 596 nm and emission wavelength of 615 nm were used for the observation of Texas Red fluorescence. The appearance of immunostained cell preparations was documented by using a PC-driven digital camera (Leica DC 300 F, Leica Microsystems AG, Heerbrugg, Switzerland). Microscopic fields were digitalized thanks to a software specifically designed for image acquisition and storage (Leica IM 50). Image adjustment and printing were achieved with appropriate software (Corel PHOTO-PAINT™ and CorelDRAW™, Corel Corporation, Ottawa, ON, Canada). Quantitative analysis of nuclear signals was performed on digitalized images using Image J™ (a public domain image software developed by W. Rasband at the Research Services Branch of the National Institute of Mental Health, NIH). Images were analyzed in the red channel after RGB split. Gray level (on a scale of 0–255, corresponding to fluorescence intensity) was determined in each nucleus. Median fluorescence intensities were computed from the analysis of approximately 80–120 nuclei in each control or treated culture.

**5.3.5. ERE-dependent luciferase expression.** In order to evaluate ER-mediated gene transactivation, assays were run on MVLN cells (MCF-7 cells stably transfected with a pVit-tk-Luc reporter plasmid).[43] ER-induced expression of the reporter gene was evaluated by measuring luciferase activity[44] using the Luciferase Assay System from Promega (Madison, WI). Cells were plated in 6-well plates at a density of $10^4$ cells/cm$^2$ in EFM, cultured for 3 days, and then incubated with $10^{-8}$ M $E_2$, $10^{-7}$ M fulvestrant, or one of the coumarin derivatives ($10^{-6}$ M). Compound **8c** was also tested at increasing concentrations ($10^{-6}$–$10^{-10}$ M), alone or in combination with $10^{-7}$ M fulvestrant. At the end of treatment, the medium was removed and cell monolayers were rinsed twice with PBS. Diluted lysis solution (250 µl, Promega E153A) was added to the cultures, which were submitted to mild agitation for 20 min in order to extract luciferase. Detergent-lysed cells were scraped and suspensions were clarified by centrifugation (5 min, 10,000*g*). Finally, 20 µl of extracts was mixed at room temperature with 100 µl of luciferase reagent mixture (Promega E151A/E152A), prepared according to the manufacturer's protocol. Luminescence was measured in a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Luciferase induction was expressed in arbitrary units (relative luciferase units, RLU) calculated per milligram of protein, and data are given as percentages of the mean value obtained from untreated cells. Protein concentrations in total cell lysates obtained by detergent extraction were determined by the BCA Protein Assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) as standard.

**5.3.6. Ligand-binding assay.** Receptor–ligand binding assay was performed in cell-free conditions using highly purified recombinant hERα (Calbiochem Novabiochem, San Diego, CA) diluted in a bovine serum albumin solution (1 mg/mL). Beforehand, recombinant ER was adsorbed onto hydroxyapatite (HAP). After removal of unbound material by centrifugation, HAP-bound ER was incubated overnight at 0–4 °C with 1 nM [$^3$H]$E_2$ (Amersham Biosciences, Roosendaal, The Netherlands) in the presence of increasing amounts of either cold $E_2$ or one of the coumarin derivatives. Radioactivity adsorbed onto HAP was then extracted with ethanol and measured by liquid scintillation counting. The relative binding affinity RBA was expressed as the concentration required to reduce the binding of [$^3$H]$E_2$ by 50%, thus RBA = ([$I_{50}$] compound/[$I_{50}$] $E_2$) × 100.

**5.3.7. Statistics.** The statistical significance of differences in cell growth or luciferase activity was assessed by ANOVA, followed by Dunnett's post hoc or Tukey's post hoc test. Statistical analysis of quantitative immunofluorescence data was performed by Kruskal–Wallis test (non-parametric ANOVA), followed by Dunn's multiple comparisons test. Level of significance was arbitrarily set at $p = 0.05$.

**5.3.8. Modeling.** Spatial coordinates of both crystal structures determined from X-ray experiments on **8a** were uploaded to Silicon Graphics O2 workstations. Drawings were performed using the Builder module of the InsightII software package, version 98 (Accelrys, Inc., San Diego, USA).

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