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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, structure, and estrogenic activity of 2- and 3-substituted 2,3-dihydro-4H-1-benzopyran-4-ones

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Abstract Molecules with potent estrogenic activity are ~270 Å^3 hydrophobic structures that encompass two hydroxyls among which is at least one phenol. However, compounds with only one phenol or devoid of such a ring have been shown to enhance ERα-mediated transcription at concentrations much larger than those measured with E_2. In this context, we show here that benzopyrans sharing one hydroxyl/methoxyl and containing an additional benzylidenyl or a spirocyclohexyl motif are able to induce ERE-dependent transcription in breast carcinoma cells.

Keywords Benzylidenylbenzopyranones · Spirocyclohexylbenzopyranones · Estrogenic activity · ERα binding affinity

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Introduction

Most non-steroidal compounds with potent estrogenic activity are ~270 Å^3 hydrophobic molecules flanked by two hydroxyls (of which at least one is phenol) playing the role of corresponding functions in 17β-estradiol (E_2). These hydroxyls are commonly considered as a part of the pharmacophore for the onset of an estrogenic activity (Wurtz *et al.*, 1998). However, recent studies have revealed that they are not absolutely required: chlorinated pesticides (such as DDT, chlordecone, and methoxychlor), organophosphorus compounds (such as parathion), or pyrethroids (Lóránd *et al.*, 2010) also give estrogenic responses.

In an attempt to produce new heterocyclic estrogens (Jacquot *et al.*, 2001; Gust *et al.*, 2001; Gust *et al.*, 2002; Jacquot *et al.*, 2002; von Rauch *et al.*, 2005) and to improve reported structure/activity relationships (SAR), we synthesized two series of benzopyrans sharing structural and physicochemical similarities with apigenin (flavonoid) and genistein (isoflavonoid), two phytoestrogens (Fig. 1), i.e., 3-benzylidenyl-2,3-dihydro-4H-1-benzopyran-4-ones (homoisoflavonoid derivatives) (Farkas *et al.*, 1971; Lévai and Schág, 1979; Lévai *et al.*, 1981; Al Nakib *et al.*, 1990; Fisera *et al.*, 1997; Hoshino *et al.*, 1998; Subramaniyan *et al.*, 2002; Koorbanally *et al.*, 2006; Biju *et al.*, 2010) and 2-spirocyclohexyl-2,3-dihydro-4H-1-benzopyran-4-ones (Dike *et al.*, 1991). The ability of some of these compounds to enhance ERα-mediated transcription confirmed the importance not only of one hydroxy- but also of methoxy-containing phenyl groups. Molecular structures established by Fourier-transformed infrared (FTIR) and homo/heteronuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, and high-resolution mass spectrometry (HRMS), as well as theoretical calculations, suggest that these active compounds may localize within the ligand-binding pocket of

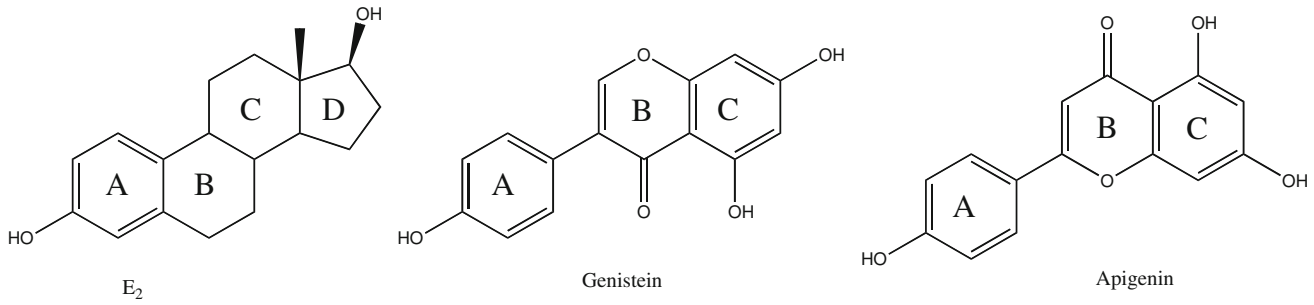


Fig. 1 Chemical structure of 17β-estradiol (E_2), genistein (isoflavone), and apigenin (flavone)

the receptor even though no interaction could be detected by classical [^{3}H]E_2 competitive binding assay.

Chemistry

3-benzylidenyl-2,3-dihydro-4H-1-benzopyran-4-ones

A series of planar 3-benzylidenyl-2,3-dihydro-4H-1-benzopyran-4-ones (molecules **3a–f**) mimicking non-steroidal estrogens (such as genistein and apigenin) were synthesized following the reaction of Knoevenagel by condensing the substituted benzaldehydes **2a–f** on the chroman-4-one **1** in solvent-free medium and in the presence of a few drops of piperidine (Scheme 1a) (Lévai and Schág, 1979). The pure expected products were obtained from direct crystallization on cooling at room temperature. During the course of the reaction, the temperature was carefully kept at 110 °C to avoid exocyclic (*cisoid* forms I or/and II) to endocyclic (*transoid* form III) isomerization (Scheme 1b), which occurs at ~150 °C and which can be accompanied by a rearrangement into 3-methylflavone (Mulvagh *et al.*, 1979; Lévai *et al.*, 1981). In this regard, it is noteworthy that the absence of electron acceptor substituents on the benzaldehyde motif would favor the formation of the *exo* (*cisoid*) forms I or II (Subramaniyan *et al.*, 2002). The *cisoid* isomer II resulting principally from a photoisomerization process of the former (Hassner and Mead, 1964; Bennett *et al.* 1972; Mulvagh *et al.*, 1979; Lévai *et al.*, 1981; Lévai *et al.*, 1999), the expected form I, would be exclusively observed.

To confirm the presence of the *cisoid* form I, we carried out a complete structural study on **3e**. The ROESY NMR spectrum revealed a correlation between the CH_2 motif in position 2 and the aromatic proton in the *ortho* position of the benzylidenyl phenyl ring (inset of Scheme 1a). Likewise, no coupling was recorded between this CH_2 and the alkenic CH in position 9, excluding therefore the *cisoid* form II. FTIR data were also in accordance with the *cisoid* form I, the band corresponding to the C=C double bond (frequency between $\overline{\nu} = 1590$ cm^{-1} and $\overline{\nu} = 1610$ cm^{-1}) being for all compounds of the same intensity as that corresponding to the C=O (frequency between $\overline{\nu} = 1651$ cm^{-1} and $\overline{\nu} = 1668$ cm^{-1}) (inset of Scheme 1a) (Hassner and Mead, 1964). Even if NMR and FTIR data suggest strongly that the synthesized 3-benzylidenyl-2,3-dihydro-4H-1-benzopyran-4-ones were isolated under the *cisoid* form I, some doubts may subsist between the latter and the thermodynamic-driven *transoid* form III. To discriminate between these two conformers, the structure of **3e** has been resolved by X-ray diffraction. Unambiguously, the X-ray structure reveals that the *cisoid* form I was the only isolated form (Fig. 2), confirming definitively the NMR and FTIR results as expected.

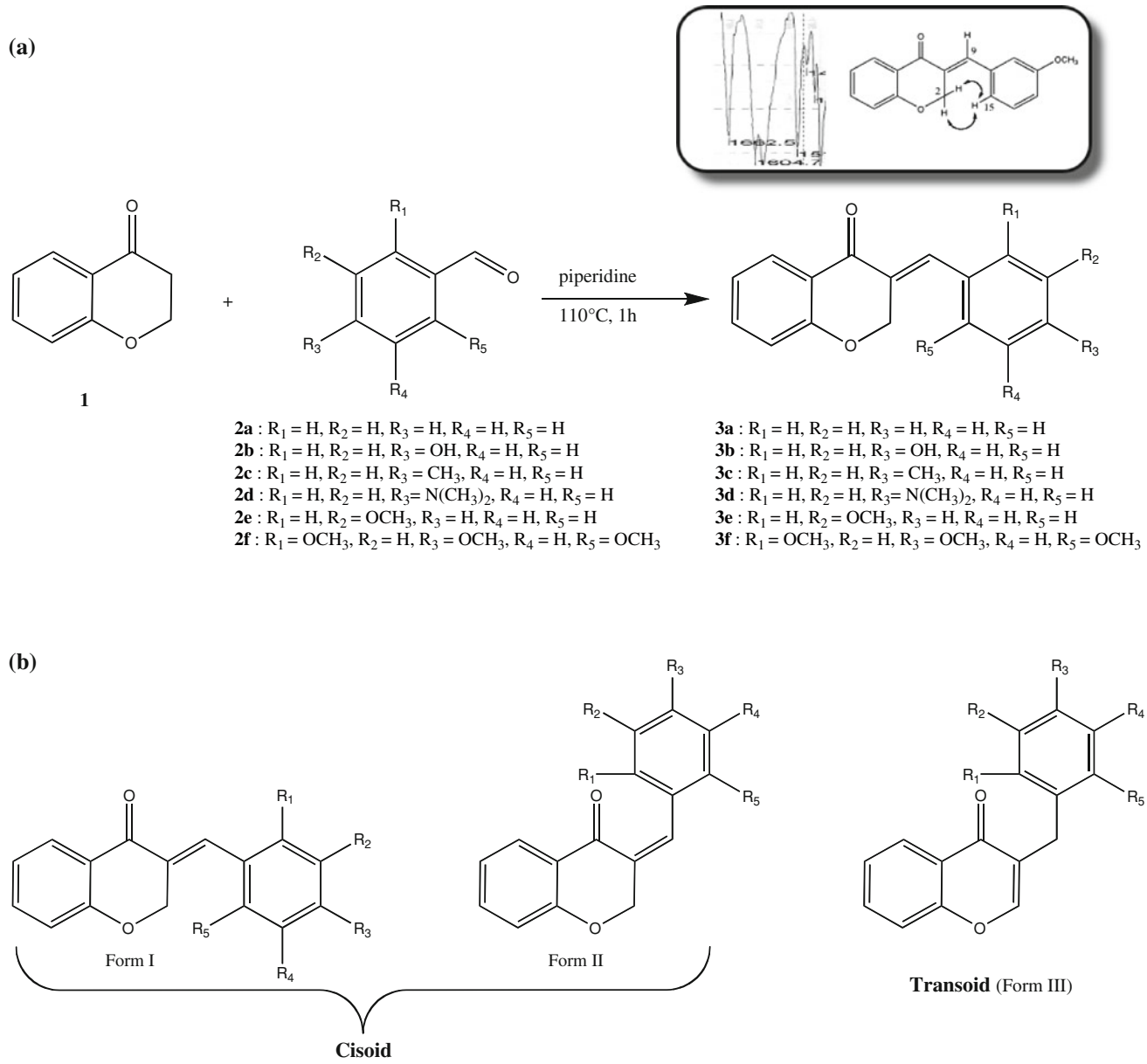
Spiro[cylohexyl-2,4′-(2,3-dihydro-4H-1-benzopyran-4-ones)]

Substituted spiro[cylohexyl-2,4′-(2,3-dihydro-4H-1-benzopyran-4-ones)] **6a–d** were obtained in one-step following the Kabbe's method (Kabbe, 1978; Bergmann and Gericke, 1990). Briefly, the cyclohexanones **5a–c** were condensed on the substituted acetophenones **4a** or **4b** (Bergmann and Gericke, 1990; Panteleon *et al.*, 2008) in refluxing toluene and in the presence of pyrrolidine (Scheme 2). The assembly was equipped with a Dean–Stark apparatus to eliminate the molecules of water resulting from the condensation reaction. The products were purified by flash column chromatography with the appropriate eluant. It should be noted that the cyclohexyl motif was structurally defined under the chair conformer, allowing therefore the substituent group in the position 13 of the crystal structure to orientate equatorially, as shown by the X-ray of compound **6c** (Fig. 3).

Results and discussion

Transcription assays

The effect of 3-benzylidenylchroman-4-ones **3a–f** on ERα-mediated transcription was assayed on MVLN breast



Scheme 1 **a** Synthesis of 3-benzylidenyl-2,3-dihydro-4H-1-benzopyran-4-ones **3a–f**. *Inset* infrared spectrum (*left*) and NMR coupling observed by using the sequence ROESY (*right*) of the compound **3e**. *Inset* a coupling between the protons in position 2 and the proton in the *ortho* position of the benzylidene ring is relevant to the *cisoid* form I (or eventually the *transoid* form III). The intensity of the two bands at $\bar{\nu} = 1{,}662\ \text{cm}^{-1}$ and $\bar{\nu} = 1{,}664\ \text{cm}^{-1}$ recorded using infrared being identical, the *cisoid* form I is likely (Hassner and Mead, 1964). **b** Structure of the two *cisoid* (isomers I and II) and the *transoid* forms

cancer cells (MCF-7 cells stably transfected with a plasmid containing an estrogen-response element (ERE) triggering the luciferase gene (Pons *et al.*, 1990)). At 10^{-6} M, a concentration usually tested for endocrine disrupting chemicals (see for example (Miksicek, 1994)), **3b** and **3e** increased luciferase activity with an almost equivalent potency (236 and 243 %, respectively; control: 100 %, Fig. 4a) while **3a** was totally ineffective. The acceptor character of the OCH_3 and OH oxygen carried by the benzylidenyl motif of these molecules seemed important for transcription suggesting some SAR connection with the role of the 17β and 3 OH action of E_2 (or corresponding position in non-steroidal estrogens) in terms of interaction with ERα (Jacquot *et al.*, 2003) even the efficiency of the hormone is at least 1,000 times higher (E_2 operates at 10^{-10} M). Thus, the presence of three methoxy groups on the benzylidenyl motif (compound **3f**: 134 %) appears deleterious probably due to steric and electronic reasons. In the same context, a low activity was observed with the *N*,*N*-dimethylamino group (**3d**: 145 %).

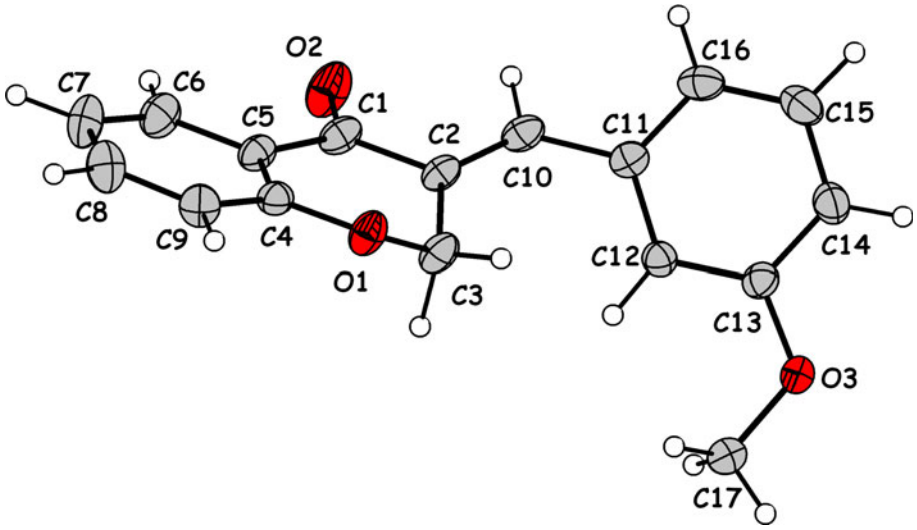
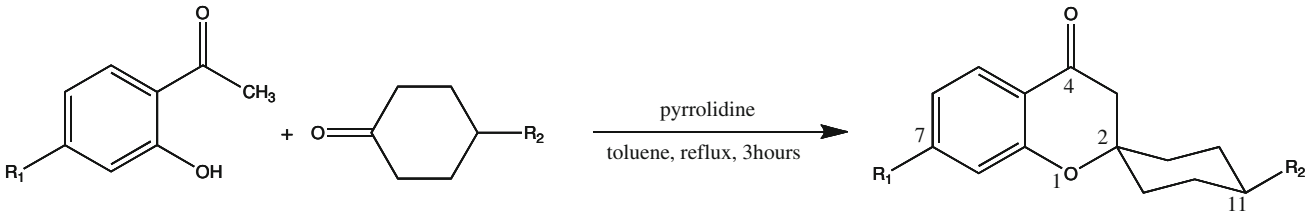


Fig. 2 ORTEP drawing of the 3-(3′-methoxybenzylidenyl-2,3-dihydro-4H-1-benzopyran-4-ones **3e**



4a : $R_1 = OCH_3$
4b : $R_1 = OH$

5a $R_2 = H$
5b $R_2 = -C(CH_3)_3$
5c $R_2 = C_6H_5$

6a $R1=OCH_3$, $R_2 = H$
6b $R1=OH$, $R_2 = H$
6c $R1=OCH_3$, $R_2 = -C(CH_3)_3$
6d $R1=OCH_3$, $R_2 = C_6H_5$

Scheme 2 Synthesis of the 2-spirocyclohexylbenzopyran-4-ones **6a–d**

With regard to the 2-spirocyclohexylchromanones **6a–d**, **6b** at 10^{-6} M solely increased luciferase activity (279 %, Fig. 4b). Hence, the donor character of the hydroxyl in position 7 of the benzopyran core structure seemed essential as the corresponding methoxylated analogue **6a** was devoid of activity.

Transcriptional activities of the most active compounds **3b**, **3e**, and **6b** were assayed at a tenfold higher concentration (10^{-5} M) to optimize their efficiency (Fig. 5). Under such conditions, luciferase expression reached values slightly lower than those measured with E_2 at 10^{-10} M. Interestingly, the antiestrogen fulvestrant (ICI) totally abrogated the capacity of these three compounds to enhance transcription, confirming unambiguously that they contribute to the acquisition by ERα of an active conformation. This property does not necessarily imply an insertion procedure within the ligand-binding pocket of ERα since a similar antiestrogen-induced suppression of transcription enhancement occurs with activating molecules operating at other receptor sites or associated proteins (coregulators), such as cyclic nucleotides (Aronica and Katzenellenbogen, 1991), bisphosphonates (Journe *et al.*, 2004), or synthetic peptides corresponding to a regulatory motif of ERα (Gallo *et al.*, 2007).

Binding assays

All investigated compounds failed to compete with [^{3}H]E_2 for ERα binding (assays carried out with a highly purified recombinant human ERα (Maaroufi and Leclercq, 1994)) raising the question regarding the mechanism by which **3b**, **3e**, and **6b** may enhance transcription. The implication of ERβ may be excluded because MVLN cells, as MCF7 cells from which they derive do not express significant amounts of this receptor (Kuiper *et al.*, 1998). Although an indirect activation of ERα through an interaction with a target contributing to a cross-talk mechanism could not be ruled out, a participation in living cells of factors that may enhance the potency of these compounds to associate with the receptor (coregulators) (Gee *et al.*, 1999) could be advocated, as proposed for other non-steroidal estrogens similarly unable to displace [^{3}H]E_2 in binding assays (Gust *et al.*, 2001; Gust *et al.*, 2002; Jacquot *et al.*, 2002; von Rauch *et al.*, 2004; Jacquot *et al.*, 2007; Laïos *et al.*, 2007).

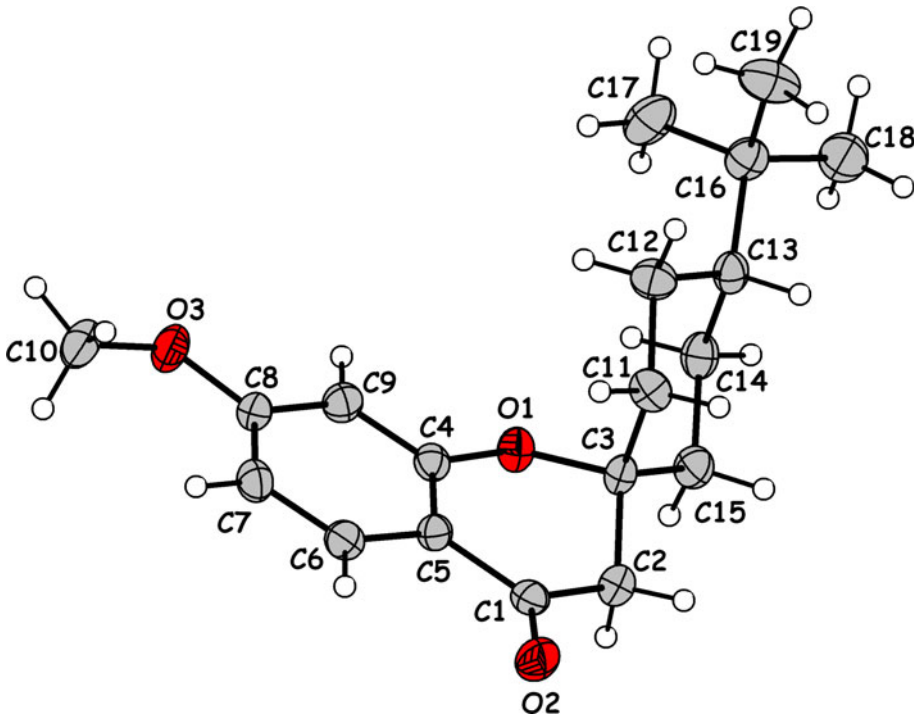


Fig. 3 ORTEP drawing of spiro[*tert*-butylcyclohexyl-2,4′-(2,3-dihydro-7-methoxy-4H-1-benzopyran-4-one)] (**6c**)

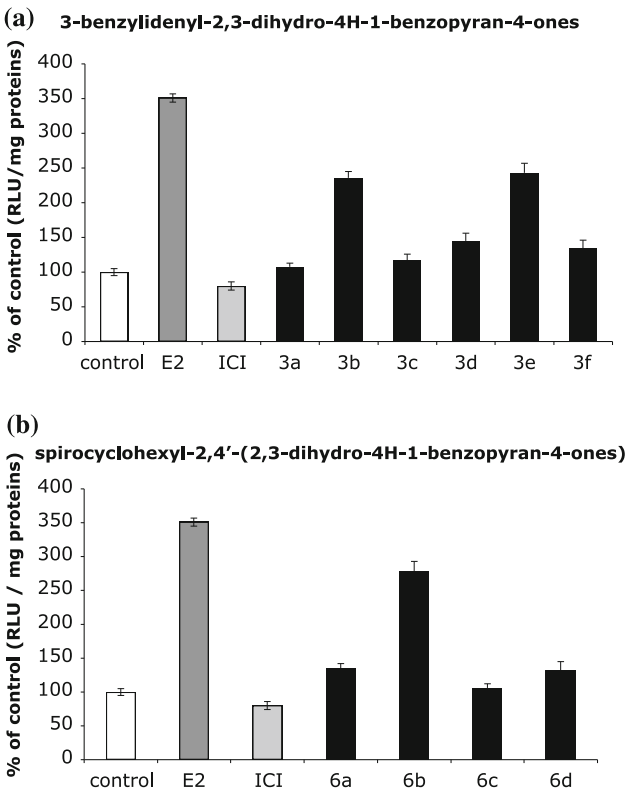


Fig. 4 Effect of compounds on ERα-mediated transcription. Assays were carried out in MVLN breast cancer cells in the presence of E_2 at 10^{-10} M, ICI (fulvestrant) at 10^{-7} M or investigated compounds at 10^{-6} M (24 h incubation). Values refer to the increase of basal luciferase expression (reporter gene) ($n = 4$, ±SD)

In fact, transcription induced by a compound usually occurs at lower concentrations than expected from its efficiency to compete with [$^3HE_2$] (RBA value), indicating that biological tests performed on living cells surpass conventional ligand binding assays in terms of sensitivity.

Potential insertion within the E_2-binding pocket

Interactions between residues of the ligand-binding pocket of ERα and hydroxyls/methoxyls of weak non-steroidal estrogens without significant binding affinity for the receptor have been reported (Gust *et al.*, 2001; Jacquot *et al.*, 2001; Jacquot *et al.*, 2002; Gust *et al.*, 2002; von Rauch *et al.*, 2005). Hence, the question of whether the ability of **3b**, **3e**, and **6b** to enhance transcription is or not related to such a capacity is of interest.

Similarities between our compounds and genistein as well as apigenin guided our analysis. Indeed, we previously reported that these two phytoestrogens enhance ERα-mediated transcription with an efficiency closely related to their binding affinity for ERα (RBA: genistein = 0.5–1.0, apigenin = 0.1–0.2; E_2 = 100) (Seo *et al.*, 2006). According to X-Ray crystallographic data, this property results from an ability of these molecules to dock within the E_2-stabilized conformation of the ligand-binding pocket. Our active compounds being unable to compete with E_2 for ERα binding, we failed to use such a docking approach because we felt that results might be subjected to controversy. We preferred to restrict our analysis to a comparison of the structural and physicochemical properties of our compounds

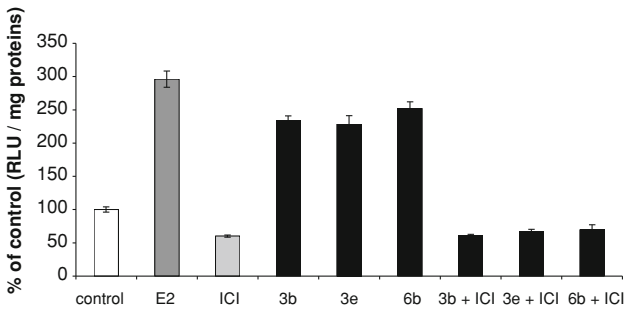


Fig. 5 Effect of active compounds on ERα-mediated transcription in the presence of ICI (fulvestrant). Assays were carried out in MVLN breast cancer cells in the presence of E_2 at 10^{-10} M, ICI (fulvestrant) at 10^{-7} M or investigated compounds at 10^{-5} M in the presence or absence of ICI (fulvestrant) at 10^{-7} M (24 h incubation). Values refer to the increase of basal luciferase expression (reporter gene) ($n = 3$, ±SD)

Table 1 Physicochemical parameter values (Molecular volumes, log*P*, and polar surface area) of the synthesized derivatives **3a–f** and **6a–d** (references: E_2, genistein, and apigenin)

Compounds	Volume (Å^3)	Log*P*	Polar surface area (Å^2)
E_2	268.74	3.43	40.46
Genistein	270.24	2.27	90.90
Apigenin	270.24	2.46	90.90
3a	216.80	3.62	26.31
3b	224.84	3.14	46.53
3c	233.36	4.07	26.30
3d	262.70	3.72	29.54
3e	242.34	3.65	35.54
3f	293.43	3.47	54.01
6a	233.19	3.61	35.54
6b	215.16	3.07	46.53
6c	299.40	4.71	35.54
6d	304.62	4.70	35.54

Molecular volumes and Polar surface area (PSA) and are expressed in Å^3 and Å^2, respectively

with those of genistein, apigenin, and E_2, taken as reference ligands.

Remarkably, the calculated physicochemical properties of compounds able to enhance transcription (**3b**, **3e**, and **6b**) were found to be similar to those observed for our reference ligands (Table 1). Thus, their possible insertion within the E_2-binding pocket appears conceivable.

Indeed, all tested compounds are characterized by reasonable smaller molecular volumes than E_2, genistein, and apigenin, suggesting that potency to enhance transcription may result, in terms of steric hindrance, from possible interactions within the ligand-binding pocket, except for **3f**, **6c**, and **6d**, which were inactive, and for which a higher value was calculated (Table 1). In this regard, the volume of active compounds is especially low, *i.e.,* 224.84 Å^3 for **3b**, 242.34 Å^3 for **3e,** and 215.16 Å^3 for **6b** (references: E_2: 268.74 Å^3, genistein, and apigenin: 270.24 Å^3). The planarity of **3b** and **3e** conjugated with some structural analogies with genistein and apigenin suggests similar interactions with residues of the binding pocket (Manas *et al.*, 2004). Indeed, the exocyclic localization of the double bond may restrain the rotation of the benzylidenyl aromatic ring in the *cisoid* form I (Scheme 1b), maintaining its orientation in an appropriate direction for such interactions.

Log*P* values (3.14 for **3b**, 3.65 for **3e**, and 3.07 for **6b**), which reflect lipophilicity, are in the same range as E_2 (3.43). Thus, not only molecular volumes but also lipophilicity of active compounds are relevant to a possible insertion within the ligand-binding pocket.

Despite the absence of polar groups at both extremities, **3e** shares a polar surface within the range calculated for our reference molecules. Important polar surface area was also recorded for **6b**. Hence, the presence of polar groups, and more specifically of donor/acceptor OCH_3 or OH groups might be crucial for an interaction with either Arg-353/Glu-394 or His-524, known to associate with the 3 and 17β hydroxyls of E_2, respectively. Hence, even if purely hypothetical, the methoxylated/hydroxylated benzylidenyl in position 3 of the 3-benzylidenylchroman-4ones (**3b** and **3e**) or in the position 7 of the benzopyran moiety of the 2-spirocyclohexylbenzopyran-4-ones (**6b**) may mimic, at least partially, the phenolic hydroxyls of genistein and apigenin to interact with these important anchoring determinants of the E_2-binding pocket.

Although our structural analysis provides pertinent data to explain the biological activity of **3b**, **3e**, and **6b**, the lack of competition between these compounds and [^{3}H]E_2 for recombinant ERα binding still raises questions about their mechanism of action. Even though an activating metabolism giving rise to a hydroxylation/O-demethylation of **3b**, **3e**, and **6b** suitable for significant ERα binding may occur in cell culture, association with another receptor site such as its coactivator-binding groove (Moore *et al.*, 2010; Leclercq *et al.*, 2011) could not be excluded. Likewise, the specific conformational modifications induced by specific ERα partners, the activity of which could be modulated by these compounds, is another alternative hypothesis.

Conclusion

In summary, we have synthesized phenol- or methoxyphenyl-containing benzopyrans which activate ERα-mediated transcription. While interactions of these compounds with residues of the ligand-binding pocket of the receptor directly implicated in the onset of estrogenic responses

seems likely, their lack of competition with [^{3}H]E_2 for ERα binding raises questions upon the mechanism by which they operate. Thus, and as already shown for other non-steroidal estrogens with similar ERα-binding properties, the capacity to interact with the receptor and to increase its ability to enhance transcription do not seem to be strictly relevant to identical chemical features. While the presence of one phenol or one methoxyphenyl may fail to confer a significant binding affinity for the receptor, such a function may be largely sufficient to induce a weak ERE-dependent transcription giving rise to an estrogenic response.

Hence, investigations are obviously required to decrypt the mechanism of action of non-steroidal estrogens unable to compete with [^{3}H]E_2 for ERα binding, such as the ones described here. In this regard, extensive structural (NMR and X-ray crystallography) or FRET studies may be fruitful in opening new avenues for pharmacomodulation since the estrogen pharmacophore appears more complex than usually thought.

Experimental protocols

Chemistry

All chemical products were purchased from Aldrich Inc. (Saint-Quentin Fallavier, France). The synthesized compounds were characterized by elemental analyses, FTIR spectroscopy, ^{1}H- and ^{13}C-NMR spectroscopy, and HRMS. Infrared spectra were recorded from potassium bromide pellets on a Shimadzu FTIR-8201 PC spectrometer ($\overline{\nu}$ in cm^{-1}). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC 300 spectrometer. Samples were dissolved in DMSO-d_6 or $CDCl_3$. All measurements were performed at 293 K, and chemical shift values were referenced to tetramethylsilane (CEA Saclay, France) as internal standard. 2D NMR experiments heteronuclear multiple bond correlation, HMQC (Heteronuclear Multiple Quantum Coherence), HSQC (Heteronuclear Single Quantum Coherence), and ROESY (Rotating frame Overhause Effect SpectroscopY) were recorded from a Bruker AC 500 apparatus equipped with a Cryo Platform (Bruker). High-resolution mass spectra were acquired using a hybrid linear ion trap LTQ-Orbitrap (Thermo Fisher Scientific, Les Ulis, France). Melting points were determined on a Büchi N°510 apparatus. Microanalyses were carried out by the Service de Microanalyses of the University Pierre et Marie Curie in Paris.

*Substituted 3-benzylidenyl-2,3-dihydro-4H-1-benzopyran-4-ones (**3a–f**)*

2,3-dihydro-4H-1-benzopyran-4-one (chroman-4-one **1**, 5 mmol) and substituted benzaldehyde **2a–f** (5 mmol, 1 eq.) were heated to 110 °C for 1 h in solvent-free medium in the presence of 10 drops of piperidine. On cooling to room temperature, the product crystallized and was filtered under vacuum, and rinsed with diethylether.

*3-Benzylidenyl-2,3-dihydro-4H-1-benzopyran-4-one (**3a**)* Prepared by condensing the benzaldehyde **2a** on the chroman-4-one **1**. White powder. 53 % yield. mp = 111 °C. FTIR (KBr) ($\overline{\nu}$ cm^{-1}): 3057, 2856, 1668, 1601, 1493, 1475, 1450, 1016; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 5.36 (2H, d, ^4J = 2.2 Hz, CH_2); 6.94 (1H, d, ^3J = 10.0 Hz, Ar–H); 7.08 (1H, m, ^3J = 8.9 Hz, Ar–H); 7.32 (2H, m, ^3J = 9.2 Hz, Ar–H); 7.41–7.53 (4H, m, Ar–H); 7.89 (1H, s, CH); 8.01–8.05 (1H, dd, ^3J = 9.5 Hz, ^4J = 2.2 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 67.33, 117.91, 121.99, 127.25, 128.79, 129.69, 130.28, 130.77, 133.76, 136.25, 136.54, 160.63, 181.18. MS (m/z): 259 [MNa^+] (calc: [MH^+] = 236.08); Anal calcd for $C_{16}H_{12}O_2$: C, 81.36, H, 5.08. Found: C, 80.87, H, 5.15.

*3-(4′-Hydroxybenzylidenyl)-2,3-dihydro-4H-1-benzopyran-4-one (**3b**)* Prepared by condensing the 4-hydroxybenzaldehyde **2b** on the chroman-4-one **1**. Gray powder. 83 % yield. mp = 213 °C. FTIR (KBr) ($\overline{\nu}$ cm^{-1}): 3163, 3063, 2947, 2866, 1651, 1593, 1508, 1450, 1018; ^{1}H-NMR (DMSO-d_6) δ (ppm) : 5.41 (2H, s, CH_2); 6.83–6.89 (3H, m, Ar–H); 7.04-7.16 (2H, m, ^3J = 10.0 Hz, Ar–H); 7.27–7.33 (1H, t, ^3J = 9.2 Hz, Ar–H); 7.56–7.67 (2H, m, CH, Ar–H); 7.87–7.91 (1H, dd, ^3J = 9.6 Hz, ^4J = 2.2 Hz, Ar–H); 9.72 (1H, s, OH). ^{13}C-NMR (DMSO-d_6) δ (ppm): 67.36, 116.70, 117.88, 121.08, 121.47, 121.92, 127.24, 129.83, 130.58, 134.96, 136.15, 157.51, 160.61, 181.16. MS (m/z): 275 [MNa^+] (calc: [MH^+] = 252.08); Anal calcd for $C_{16}H_{12}O_3$: C, 76.19, H, 4.76. Found: C, 76.34, H, 4.69.

*3-(4′-Methylbenzylidenyl)-2,3-dihydro-4H-1-benzopyran-4-one (**3c**)* Prepared by condensing the 4-methylbenzaldehyde **2b** on the chroman-4-one **1**. White powder. 40 % yield. mp = 115 °C. FTIR (KBr) ($\overline{\nu}$ cm^{-1}): 3067, 2934, 2843, 1665, 1607, 1512, 1462, 1011; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 2.42 (3H, s, CH_3); 5.38 (2H, d, ^4J = 1.7 Hz, CH_2); 6.98 (1H, m, ^3J = 8.3 Hz, Ar–H); 7.08–7.11 (1H, m, ^3J = 7.29 Hz, Ar–H); 7.24 (4H, m, Ar–H); 7.50 (1H, m, Ar–H); 7.88 (1H, s, CH); 8.03–8.06 (1H, dd, ^3J = 7.7 Hz, ^4J = 1.6 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 20.99, 67.41, 117.87, 121.49, 121.94, 127.22, 129.42, 129.94, 130.42, 130.99, 136.15, 136.59, 139.76, 160.58, 181.13. MS (m/z): 273 [MNa^+] (calc: [MH^+] = 250.10); Anal calcd for $C_{17}H_{14}O_2$: C, 81.60, H, 5.60. Found: C, 81.35, H, 5.72.

*3-(4′-N,N-Dimethylaminobenzylidenyl)-2,3-dihydro-4H-1-benzopyran-4-one (**3d**)* Prepared by condensing the 4-*N*,*N*-dimethylaminobenzaldehyde **2d** on the chroman-4-

one **1**. White powder. 26 % yield. mp = 154 °C. FTIR (KBr) ($\bar{\nu}$ cm^{-1}): 3000, 2914, 1659, 1607, 1477, 1066; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 3.08 (6H, s, CH_3); 5.45 (2H, s, CH_2); 6.74 (2H, d, ^3J = 8.8 Hz, Ar–H); 6.97 (1H, d, ^3J = 8.3 Hz, Ar–H); 7.07 (1H, t, ^3J = 7.2 Hz, Ar–H); 7.27 (2H, m, Ar–H); 7.47 (1H, m, Ar–H), 7.85 (1H, s, CH); 8.02–8.05 (1H, dd, ^3J = 7.9 Hz, ^4J = 1.7 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 39.54, 67.82, 111.71, 117.63, 121.08, 121.69, 121.87, 125.30, 127.06, 132.69, 132.94, 135.52, 137.41, 151.16, 160.27, 180.62. MS (m/z): 302.11 [MNa^+] (calc: [MH^+] = 279.13); Anal calcd for $C_{18}H_{17}NO_2$: C, 77.42, H, 6.09, N, 5.02. Found: C, 76.84, H, 6.23, N, 4.99.

*3-(3′Methoxybenzylidenyl)-2,3-dihydro-4H-1-benzopyran-4-one (**3e**)* Prepared by condensing the 3-methoxybenzaldehyde **2e** on the chroman-4-one **1**. White powder. 25 % yield. mp = 83 °C. FTIR (KBr) ($\bar{\nu}$ cm^{-1}): 3026, 2966, 2837, 1663, 1605, 1512, 1462, 1215, 1024; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 3.86 (3H, s, OCH_3); 5.37 (2H, s, CH_2); 6.85–6.91 (2H, m, ^3J = 7.8 Hz, Ar–H); 6.95–7.06 (2H, m, Ar–H), 7.06–7.11 (1H, m, ^3J = 8.0 Hz, Ar–H), 7.35–7.40 (1H, t, ^3J = 8.0 Hz, Ar–H); 7.48–7.50 (1H, t, ^3J = 6.9 Hz, ^4J = 1.3 Hz, Ar–H), 7.86 (1H, s, CH); 8.00–8,04 (1H, dd, ^3J = 9.2 Hz, ^4J = 1.9 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 55.68, 79.30, 101.39, 108.89, 114.30, 127.52, 160.89, 165.73, 190.27. MS (m/z): 289.08 [MNa^+] (calc: [MH^+] = 266.09); Anal calcd for $C_{17}H_{14}O_3$: C, 76.69, H, 5.26. Found: C, 75.59, H, 5.42.

*3-(2′,4′,6′-Trimethoxybenzylidenyl)-2,3-dihydro-4H-1-benzopyran-4-one (**3f**)* Prepared by condensing the 2,4,6-trimethoxybenzaldehyde **2f** on the chroman-4-one **1**. Yellow powder. 42 % yield. mp = 130 °C. FTIR (KBr) ($\bar{\nu}$ cm^{-1}): 3100, 2897, 1663, 1605, 1466, 1416, 1207, 1057; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 3.82 (6H, s, OCH_3); 3.88 (3H, s, OCH_3); 4.87 (2H, s, CH_2); 6.17 (2H, s, Ar–H); 6.94 (1H, d, ^3J = 8.3 Hz, Ar–H); 7.04 (1H, t, ^3J = 7.7 Hz, Ar–H); 7.46 (1H, t, ^3J = 7.8 Hz, Ar–H); 7.76 (1H, s, CH); 8.05 (1H, d, ^3J = 7.6 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 55.28, 55.53, 55.74, 69.10, 72.95, 90.71, 90.88, 104.12, 117.77, 121.21, 121.75, 127.18, 129.49, 129.99, 135.77, 159.12, 161.13, 163.09, 181.35. MS (m/z): 349.00 [MNa^+] (calc: [MH^+] = 326.12); Anal calcd for $C_{19}H_{18}O_5$: C, 69.94, H, 5.52. Found: C, 70.16, H, 5.79.

*Spiro[cylohexyl-2,4′-(2,3-dihydro-4H-1-benzopyran-4-one)] (**6a–d**)*

The synthesis of the spiro[cylohexyl-2,4′-(2,3-dihydro-4H-1-benzopyran-4-ones)] **6a–6d** was carried out by condensing the appropriate cyclohexanones **5a–5c** (1.1 eq.) with the 2′-hydroxyacetophenones **4a** or **4b** (3 mmol) in the presence of 5 mL pyrrolidine and in refluxing toluene during 3 h. The assembly was equipped with a Dean–Stark trap to remove water resulting from the reaction. Toluene was evaporated under vacuum. Then, the crude product was purified by column chromatography (ethyl acetate/cyclohexane: 3/7) to afford the final product which crystallized in <48 h.

*Spiro[cylohexyl-2,4′-(2,3-dihydro-7-methoxy-4H-1-benzopyran-4-one)] (**6a**)* Prepared by condensing the cyclohexanone **5a** on the 4-methoxy-6-hydroxyacetophenone **4a**. Yellow crystals. 45 % yield. mp = 68 °C. FTIR (KBr) ($\bar{\nu}$ cm^{-1}): 3080, 2928, 2861, 1672, 1574, 1437, 1443, 1198, 1059; ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.31–1.68 (8H, m, cyclohexyl); 1.72–1.80 (2H, m, cyclohexyl); 2.65 (2H, s, CH_2); 3.84 (3H, s, OCH_3); 6.42 (1H, d, ^4J = 3.0 Hz, Ar–H); 6.53 (1H, dd, ^3J = 10.6 Hz, ^4J = 2.9 Hz, Ar–H); 7.78 (1H, d, ^3J = 10.7 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 20.98, 24.62, 33.93, 47.04, 55.66, 80.13, 101.28, 109.05, 114.17, 127.45, 160.99, 165.75, 190.17. MS (m/z): 269.03 [MNa^+] (calc: [MH^+] = 246.13); Anal calcd for $C_{15}H_{18}O_3$: C, 73.17, H, 7.32. Found: C, 73.15, H, 7.48.

*Spiro[cylohexyl-2,4′-(2,3-dihydro-7-hydroxy-4H-1-benzopyran-4-one)] (**6b**)* Prepared by condensing the cyclohexanone **5a** on the 4,6-dihydroxyacetophenone **4b**. Yellow crystals. 68 % yield. mp = 175 °C. FTIR (KBr) ($\bar{\nu}$ cm^{-1}): 3105, 3044, 2941, 2847, 1651, 1495, 1447; 1184, 1119; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 1.24–1.56 (8H, m, cyclohexyl); 1.81–1.85 (2H, m, cyclohexyl); 2.62 (2H, s, CH_2); 6.28 (1H, s, Ar–H); 6.43 (1H, dd, ^3J = 10.6 Hz, ^4J = 3.0 Hz, Ar–H); 7.57 (1H, d, ^3J = 10.4 Hz, Ar–H); 10.49 (1H, s, OH). ^{13}C-NMR (DMSO-d_6) δ (ppm): 21.03, 24.66, 33.99, 47.10, 79.77, 102.93, 109.79, 113.30, 127.81, 160.93, 164.90, 189.88. MS (m/z): 255.09 [MNa^+] (calc: [MH^+] = 232.11); Anal calcd for $C_{14}H_{16}O_3$: C, 72.41, H, 6.90. Found: C, 72.61, H, 6.91.

*Spiro[ter-butylcyclohexyl-2,4′-(2,3-dihydro-7-methoxy-4H-1-benzopyran-4-one)] (**6c**)* Prepared by condensing the *tert*-butylcyclohexanone **5b** on the 4-methoxy-6-hydroxyacetophenone **4a**. Yellow crystals. 71 % yield. mp = 146 °C. FTIR (KBr) ($\bar{\nu}$ cm^{-1}): 3026, 2937, 2862, 1680, 1580, 1445, 1205, 1070; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 0.91 (9H, s, CH_3); 1.10 (1H, m, cyclohexyl); 1.31–1.60 (6H, m, cyclohexyl); 2.20 (2H, m, ^3J = 11.9 Hz, cyclohexyl); 2.62 (2H, s, CH_2); 3.87 (3H, s, OCH_3); 6.44 (1H, d, ^4J = 2.2 Hz, Ar–H); 6.56 (1H, dd, ^3J = 8.8 Hz, ^4J = 2.4 Hz, Ar–H); 7.82 (1H, d, ^3J = 8.8 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 21.57, 23.03, 27.36, 32.08, 34.02, 35.18, 46.69, 47.90, 55.69, 79.34, 81.63, 101.40, 109.03, 114.30, 127.50, 160.90, 165.74, 189,82. MS (m/z):

325.18 [MNa^+] (calc: [MH^+] = 302.19); Anal calcd for $C_{19}H_{26}O_3$: C, 75.49, H, 8.61. Found: C, 75.78, H, 8.36.

Spiro[4-phenylcyclohexyl-2,4′-(2,3-dihydro-7-methoxy-4H-1-benzopyran-4-one)] ***(6d)*** Prepared by condensing 4-phenylcyclohexanone **5c** on 4-methoxy-6-hydroxyacetophenone **4a**. Yellow crystals. 65 % yield. mp = 153 °C. FTIR (KBr) ($\overline{\nu}$ cm^{-1}): 3028, 2939, 2907, 2866, 1709, 1497, 1447, 1250, 1167; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 1.59–1.97 (6H, m, cyclohexyl); 2.17–2.22 (2H, m, cyclohexyl); 2.63 (1H, m, cyclohexyl); 2.92 (2H, s, CH_2); 3.84 (3H, s, OCH_3); 6.41 (1H, d, ^4J = 2.9 Hz, Ar–H); 6.54 (1H, m, ^3J = 10.7 Hz, ^4J = 2.9 Hz, Ar–H); 7.21–7.34 (5H, m, Ar–H); 7.79 (1H, d, ^3J = 10.7 Hz, Ar–H). ^{13}C-NMR (DMSO-d_6) δ (ppm): 33.33, 40.74, 41.64, 126.19, 126.67, 128.36, 145.32, 210.12. MS (m/z): 345.15 [MNa^+] (calc: [MH^+] = 322.16); Anal calcd for $C_{21}H_{22}O_3$: C, 78.26, H, 6.83. Found: C, 77.63, H, 6.47.

X-ray crystallography

Compounds **3e** and **6c** were recrystallized from a mixture of n-pentane/methanol (80/20) and n-pentane alone, respectively. Selected single crystals were mounted onto a glass fiber and set-up on a diffractometer Nonius Kappa-CCD. Data were recorded at room temperature under Mo Kα radiation (λ = 0.71073 Å). Data collection, integration, and unit cell parameters were carried out using the Nonius EVAL-14 program. The data were corrected from absorption by means of SHELXS-86 (Sheldrick, 1986) and refined by full least-squares on F^2, and completed with SHELXL-97 (Sheldrick, 1997). Graphics were carried out with DIAMOND (Brandenburg and Berndt 1999). All non-H atoms were refined with anisotropic displacement parameters, and H atoms were included in calculated positions. Crystallographic data: **3e**: $C_{17}H_{14}O_3$, monoclinic, $P2_1/c$, a = 12.545(9) Å, b = 6.789(8) Å, c = 16.290(11) Å, V = 1371.2(2) Å^3, Z = 4, 4742 observed data, with $I > 2\sigma$ (I). Crystallographic data: **6c**: $C_{19}H_{26}O_3$, monoclinic, $P2_1/a$, a = 7.572(5) Å, b = 23.507(38) Å, c = 9.624(11) Å, V = 1712.4(3) Å^3, Z = 4, 2739 observed data, with $I > 2\sigma$ (I).

Biological tests

ERα-mediated transcription

ERα-mediated transcription was assayed in MCF-7 cells stably transfected with a pVit-tk-Luc reporter plasmid (MVLN cells (Pons *et al.*, 1990)). Expression of this luciferase reporter gene was measured according to a procedure already described (Demirpence *et al.*, 1993) using the Luciferase Assay System from Promega (Madison, WI). Cells were plated in six-well plates at a density of 10^4 cells/cm^2 in 10 % DCC-treated fetal calf serum, cultured for 3 days, and then maintained in the absence of any compound (control) or exposed to one of the investigated benzopyran derivatives (10^{-6} M). 10^{-10} M E_2 or 10^{-7} M fulvestrant (ICI) were used as complementary positive and negative luciferase-inducing agents. At the end of treatment, the medium was removed, and the cell monolayers were rinsed twice with PBS. A fivefold diluted lysis solution (250 µl, Promega E153A) was then added to the plates, which were subsequently maintained under mild agitation for 20 min to extract luciferase. Lysed cells were detached with a scraper, and the resulting suspensions clarified by centrifugation (5 s, 10,000×g). Finally, 20 µl of such clarified extracts were 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, expressed in arbitrary units with regard to a blank (relative luciferase units) was normalized according to the protein content of the extract (coomassie assay) and the data given as percentages of the mean value (±SD) of control untreated cells.

ERα binding assay

"Hydroxylapatite (HAP) assay" (Maaroufi and Leclercq, 1994) was performed using highly purified recombinant hERα (Calbiochem Novabiochem, San Diego, CA) diluted in a bovine serum albumin solution (1 mg/ml). Beforehand, recombinant ERα was adsorbed onto HAP. After removal of unbound material by centrifugation, HAP-bound ERα was incubated overnight at 0–4 °C with 10^{-9} M [^{3}H]E_2 (Amersham Biosciences, Roosendaal, The Netherlands) in the presence of increasing amounts of either unlabeled E_2 (10^{-10} to 10^{-6} M) or one of the investigated compounds. Radioactivity adsorbed onto HAP was then extracted with ethanol and measured by liquid scintillation counting. Relative concentration of E_2 and compound required to reduce [^{3}H]E_2 binding by 50 % gave the relative binding affinity (RBA), i.e., RBA = ($IC_{50compound}$/IC_{50E2}) × 100.

Theoretical physicochemical properties calculations

The physicochemical properties of each compound were theoretically calculated by using the free online service Molinspiration (http://www.molinspiration.com/cgi-bin/properties). The hydrophobicity (Log$P$), the polar surface area, and the volume of the different molecules were calculated and compared with the references E_2, genistein, and apigenin.

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