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Isocoumarins as estrogen receptor beta selective ligands: Isomers of isoflavone phytoestrogens and their metabolites

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Abstract—In a search for new ligands selective for the estrogen receptor beta (ERβ), we prepared a series of non-steroidal compounds having an isocoumarin core structure. An interesting feature of these derivatives is that they bear the same functionalities as the well-known ERβ -selective, isoflavone phytoestrogens daidzein and genistein, but in an isomeric arrangement. These compounds could be prepared efficiently by electrophilic cyclization of acetylenic ester precursors, followed by simple manipulations to introduce additional substituents. Through a reduction of some of the isocoumarins, we also obtained isomeric analogs of the isoflavone metabolites equol and dehydroequol. The compounds we prepared were evaluated in ER binding assays, and selected compounds were studied further in cell-based gene transcription assays. Several of the isocoumarins and their analogs are high-affinity ligands that show considerable selectivity for ERβ in terms of binding affinity, and strikingly high ERβ selectivity in terms of potency in gene transcription assays. Two of the best compounds, which combine high transcriptional potency with an ERβ selectivity greater than 1000, should prove to be excellent probes of ERβ function in vivo.

1. Introduction

The estrogen receptors (ERs), members of the nuclear hormone receptor superfamily, mediate the activity of estrogens in many different organs, including the reproductive, skeletal, cardiovascular, and central nervous systems.[1–3] Two different ER subtypes, the product of different genes, are known: ERα, the first discovered, and ERβ, which was only discovered in 1996.[4,5] Both estrogen receptors bind 17β-estradiol with high affinity and bind to classical estrogen response elements in a similar way; however, there are differences in their amino acid sequence, transcriptional activity, and pattern of tissue distribution.

The overall amino acid sequence identity of the two ER subtypes is 44%; the DNA-binding domain is very well conserved, while the amino acid sequence identity of the ligand-binding domain (LBD) is 59%. Despite these differences, the two ER ligand-binding pockets (LBP) are almost identical, the principal differences being a somewhat smaller internal volume for ERβ and the substitution of just two amino acids, with Met421 in ERα corresponding to Ile373 in ERβ, and Leu384 in ERα corresponding to Met336 in ERβ.[6]

The tissue distribution patterns of ERα and ERβ are also quite different. Since ERβ is mostly expressed in the prostate, ovary, colon, urinary tract, and some brain regions, but is less expressed in certain reproductive organs,[7] a selective ERβ agonist might maintain the beneficial effects of estrogen therapy in these ERβ-rich tissues without increasing the risk of cancer in other organs that are ERα rich, such as the breast and uterus. In addition, ERβ has been shown to be antiproliferative when present along with ERα in breast cancer cells.[8] For this reason, many researchers have focused their attention on the synthesis of compounds selective for ERβ.

Among non-steroidal compounds, we have reported that the diarylpropionitrile derivative **1** (DPN)[9] and the indazole compound **2** are very ERβ selective;[10] others have reported the benzoxazole product **3** also to be highly ERβ selective (Fig. 1).[11] It is interesting that the soy isoflavones, such as daidzein (**4a**, Fig. 2) and genistein (**4b**),

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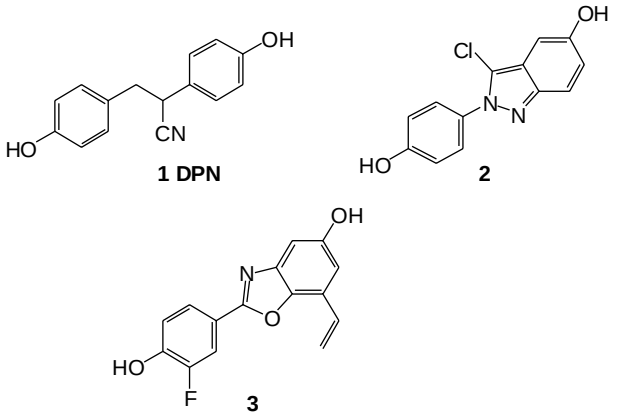


Figure 1. ER-selective ligands.

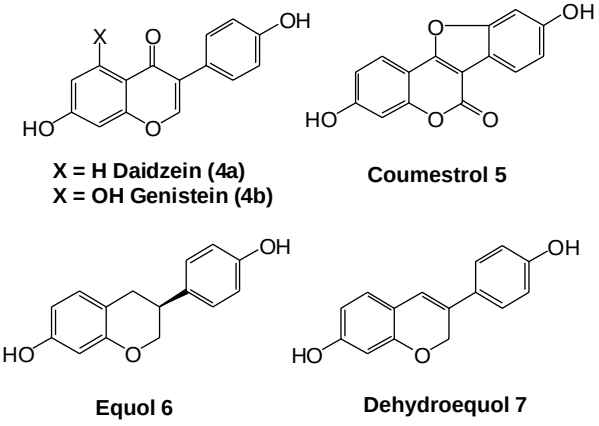


Figure 2. Phytoestrogens and analogs.

and the clover coumarin coumestrol (**5**), long known to be estrogenic (i.e., phytoestrogens), were among the first compounds noted to be ligands with selective affinity for ERβ, a property shared by the daidzein enteric metabolite, (*S*)-equol (**6**).[9,12] Dehydroequol (**7**) is also known to show estrogenic activity,[13] but its selectivity for ERβ has not been reported. Despite their considerable selectivity for ERβ in terms of binding,[9,12] however, the phytoestrogens **4**–**6** showed only modest selectivity for ERβ in terms of potency in transcription assays.[12]

Since we are interested in developing compounds that are selective for ERβ not just in terms of their binding affinity for the ERs but also in terms of their potency in cell-based assays and ultimately in vivo, we decided to investigate a series of compounds that possess a scaffold related to daidzein (**4a**), coumestrol (**5**), and (±)-equol (**6**), but have an isomeric arrangement of the central atoms of the heterocyclic core. For this reason we turned our attention to compounds having an isocoumarin core structure, a system that can also be reduced to give isomeric analogs of (±)-equol (**6**) and dehydroequol (**7**) (Fig. 3). The isocoumarin core structure is found in many natural products that exhibit a broad range of biological activities. For example, unsymmetrical 3-aryl-4-substituted isocoumarins are reported to possess cytotoxic activity against human cancer cell lines in vitro.[14] Thus, a series of C-4 substituted isocoumarins was also synthesized.

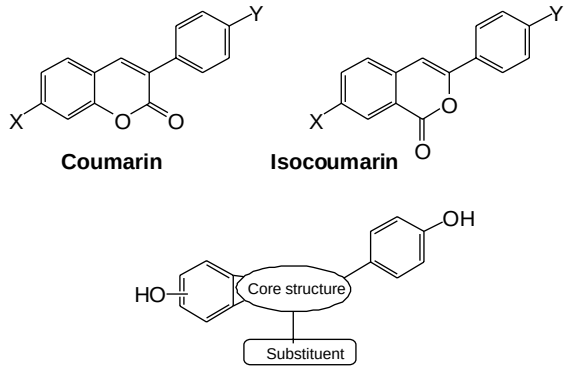


Figure 3. Coumarin and isocoumarin cores; ER pharmacophore model.

The isocoumarin scaffold conforms to a pharmacophore model for ERβ (Fig. 3) that has emerged from our work and that of others.[10] This model—in which the size of the central region of the ligand is rather small—is based on the fact that the ERβ binding pocket is smaller by about 100 Å^3 than that of ERα,[6] so that ERβ selectivity can often be engendered by reducing ligand size in this region, as well as by including some polar functions.[9,10] In this regard, it is of note that one of the two phenol rings in the isocoumarin structure is fused to form a heterocycle, which reduces size and introduces core polarity. Ideally, as well, the nature of substituents in this ERβ pharmacophore should be such that they interact favorably with the Ile373 or Met336 residues in ERβ and/or unfavorably with the Met421 and Leu384 in ERα, in this way also engendering better selectivity for ERβ.[15,16]

Herein, we present the synthesis and biological evaluation of a series of isocoumarins and derivatives, some of which show high selectivity for ERβ both in terms of binding affinity and transcriptional potency.

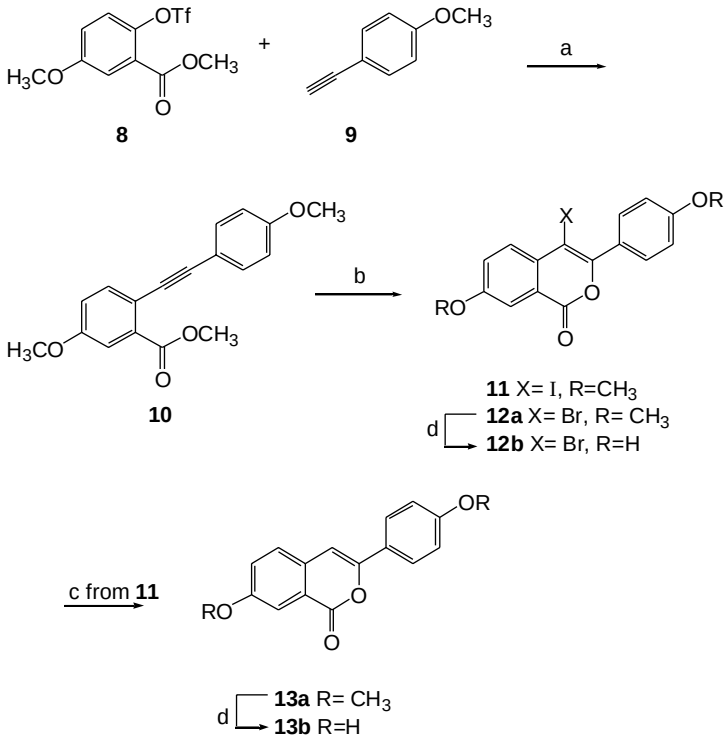
2. Results and discussion

2.1. Chemical synthesis

All the compounds synthesized by us can be divided in to two groups: isocoumarins and their reduced analogs.

2.1.1. Isocoumarin derivatives. The synthesis of the isocoumarin core structure was accomplished as reported in Scheme 1. We were able to obtain the diarylacetylene **10** via a palladium-catalyzed Sonogashira coupling reaction between derivative **8** and 4-ethynylanisole (**9**). The original literature procedure[17] worked satisfactorily on a 1 mmol scale, giving the desired compound > 80% yield, but it was unsatisfactory on a larger (10 mmol) scale. By making several changes (using 5 mol % of $Pd(PPh_3)_4$, 15 mol % of CuI, and 2 mmol of Bu_4NI), we were able to obtain a 75% yield at the larger scale.

According to a literature precedent,[14] the acetylenic ester **10** can be converted easily to the isocoumarin



Scheme 1. Reagents: (a) $Pd(PPh_3)_4$, CuI, Bu_4NI; Et_3N–CH_3CN; (b) I_2, CH_3CN (for **11**), Br_2, CH_3CN (for **12a**); (c) HCO_2H, Et_3N, PPh_3, $Pd(OAc)_2$, DMF; (d) BBr_3, CH_2Cl_2.

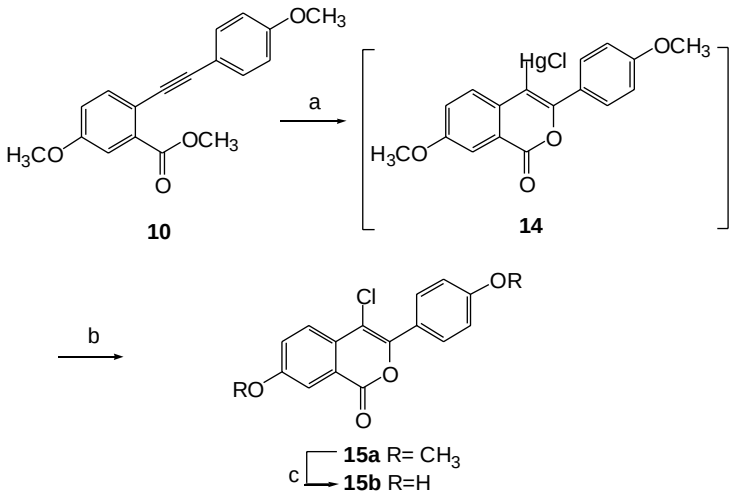
13a by an iodo-lactonization reaction, giving the iodo enol lactone **11**, from which the iodine group can be removed readily by hydrogenolysis. Although there are only few examples of bromo-lactonization reported in the literature,[18] we found that the corresponding bromo enol lactone **12a** could also be easily obtained following the reaction conditions used for the synthesis of **11**, with bromine replacing iodine.

Compounds **11** and **12a** were obtained not only in >70% yield but also regioselectively (>90%). Their structure was confirmed as the six-membered-ring enol lactone structure—as opposed to the potential alternative lactonization product, the five-membered alkylidine benzobutyrolactone—by the carbonyl stretch in the IR spectra: 1726 cm^{-1} for derivative **11**, and 1734 cm^{-1} for **12a**; the butyrolactone is expected to have a much higher stretching frequency (>1750 cm^{-1}).[19–22] Derivatives **12a** and **13a** were deprotected using BBr_3 to give the final free phenolic compounds **12b** and **13b**. Attempts to deprotect the iodo derivative **11** failed because this compound was unstable under the hydrolytic conditions employed.

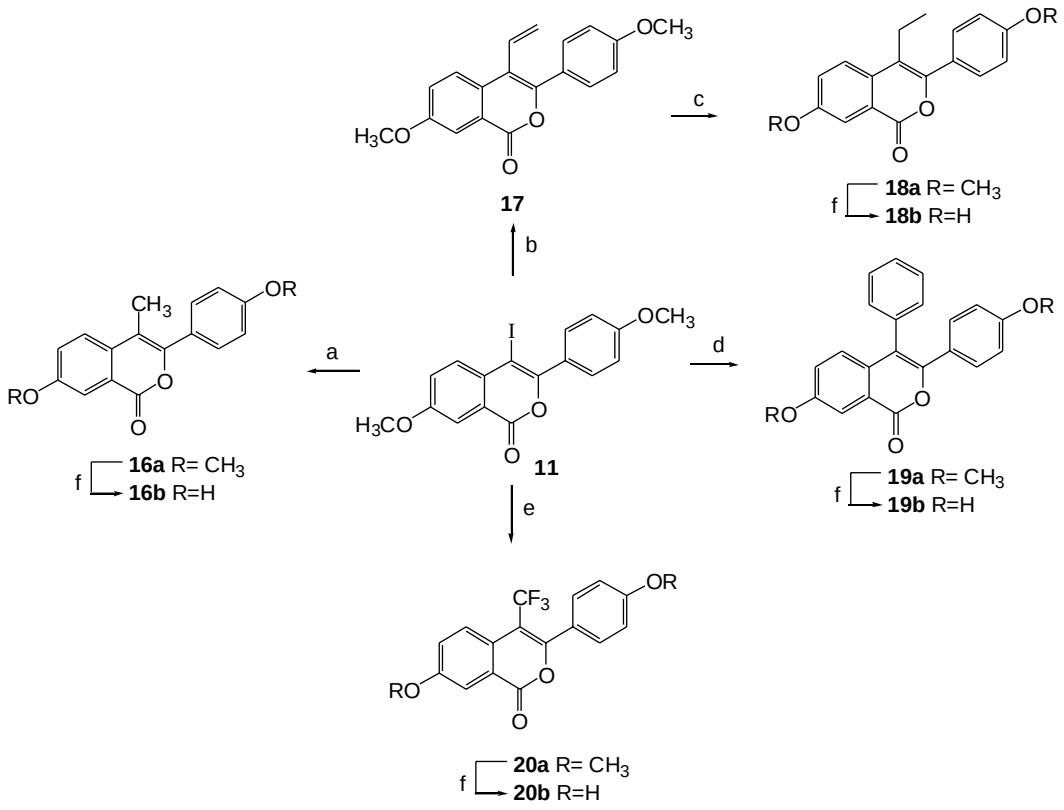
Isocoumarin **13b** is a known compound (though synthesized by different methodology).[23,24] There is, however, a large discrepancy in the melting points reported for compound **13b** in the two previous papers in which it was described: Rose et al.[23] report a mp of > 300 °C, whereas Mahto et al.[24] report a mp of 226–227 °C. Because other analytical data for the compounds prepared in these two earlier reports are not available for comparison, we cannot ascertain whether this discrepancy represents an inaccuracy in melting point determination or an error in attribution of structure. In any case the melting point that we have measured for derivative **13b** (>300 °C) is consistent with that reported by Rose et al.[23]

As shown in Scheme 2, the corresponding chloro compound **15b** could be obtained by reaction of the acetylenic acid **10** with mercuric acetate to give the corresponding isocoumarin mercurial **14**.[25] Direct replacement of the mercury group by chlorine was accomplished using copper(II) chloride, and the chloro enol lactone obtained (**15a**) was then deprotected to give **15b**.

Starting from compound **11**, several different polar substituents were introduced at the C-4 position by displacement of the iodine group (Scheme 3). Through a Stille coupling reaction,[26] the methyl (**16a**) and vinyl (**17**) compounds were synthesized, and from the reduction of vinyl compound **17**, ethyl derivative **18a** could be readily obtained. A Suzuki coupling reaction between compound **11** and phenyl boronic acid was employed for the synthesis of phenyl-substituted **19a**. For the synthesis of the CF_3 analog **20a**, we followed a literature procedure described for the replacement of an aromatic bromine group with a trifluoromethyl moiety using an unusual fluorosulfonylfluoroacetate reagent as a precursor for the in situ generation of the trifluoromethide species.[27] Although examples of this last reaction have never been reported for our particular heterocyclic system, we were able to obtain the desired compound in good yield, within a few hours. With all of these



Scheme 2. Reagents: (a) $Hg(OAc)_2$, AcOH, NaCl; (b) $CuCl_2$, THF; (c) BBr_3, CH_2Cl_2.

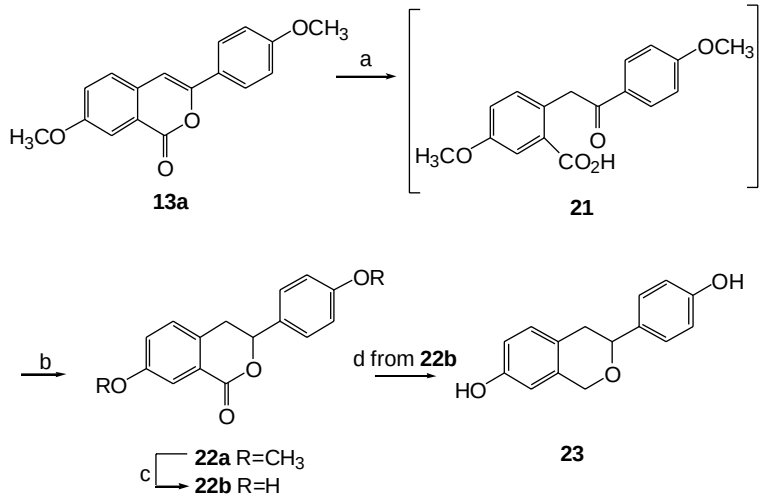


Scheme 3. Reagents: (a) $(CH_3)_4Sn$, $Pd_2(dba)_3$, $PtBu_3$, CsF, dioxane; (b) $VinylSn(Bu)_3$, $Pd_2(dba)_3$, $P'Bu_3$, CsF, dioxane; (c) Pd/C, H_2, EtOH; (d) $PhB(OH)_2$, $Pd(PPh_3)_4$, Cs_2CO_3, DMF; (e) $FSO_2CF_2CO_2CH_3$, CuI, DMF; (f) BBr_3, CH_2Cl_2.

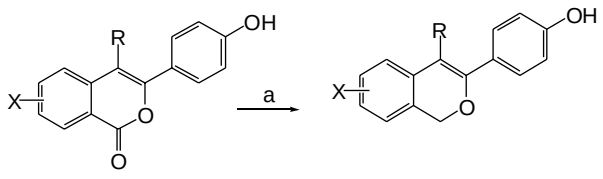
compounds, the methyl ether protecting groups were cleaved with boron tribromide.

2.1.2. Reduced analogs. Starting from the basic enol lactone **13a**, the keto-acid intermediate **21** could be obtained by an alkaline hydrolysis (Scheme 4). Reduction of this compound to the racemic hydroxyl acid and subsequent cyclodehydration using acetic anhydride gave the desired reduced lactone **22a**.[28] Derivative **22a** was then deprotected to give product **22b**, which was in turn reduced with $LiAlH_4$ to obtain isoequol **23** as a racemate.

The synthesis of dehydroisoequol **25** and its analogs **26** and **27** (Scheme 5) was accomplished by a simple reduction of the enol lactones **13b**, **16b**, and **24**[19] with $LiAlH_4$. In general, the reduction of the carboxyl group gave us reasonable yields even if different reaction conditions were employed.



Scheme 4. Reagents: (a) KOH, EtOH; (b) $NaBH_4$, EtOH, Ac_2O; (c) BBr_3, CH_2Cl_2; (d) $LiAlH_4$, THF.



Scheme 5. Reagents: (a) $LiAlH_4$, THF.

2.2. Biological results

2.2.1. Estrogen receptor binding affinity. The compounds synthesized were evaluated in competitive radiometric binding assays to determine their binding affinities for human ERα and ERβ. The results of these assays are summarized in Table 1. In this table the binding-affinity values of daidzein (**4a**), genistein (**4b**), (±) equol (**6**), and dehydroequol (**7**) are also reported.

Binding affinities are expressed relative to that of estradiol (100%), as relative binding affinity (RBA) values (Table 1, left), from which the K_i values have also been calculated (Table 1, center). We have used RBA values when discussing affinity comparisons. One should note, however, that estradiol has a 2.5-fold higher affinity for ERα than for ERβ (K_d [ERα] = 0.2 nM vs [ERβ] = 0.5 nM). Thus, the K_i values, which represent the inherent affinity of the competing ligand itself for the two ER subtypes, are the appropriate ones to use in comparing binding affinity with transcriptional potency (see next section).

Among the family of isocoumarin derivatives, the best results in terms of binding affinity selectivity are observed for compounds **13b** and **16b**, these being 82- and 40-fold ERβ selective, respectively, in terms of RBA values, with the methyl derivative **16b** having an ERβ RBA of 24%, and compound **13b** an ERβ RBA of 2.1%. It is also important to point out that these compounds show selectivity greater than that reported for the phytoestrogens, daidzein (**4a**), genistein (**4b**), and (±)-equol (**6**).[12]

In general, the other compounds of this series show ERβ binding affinities near or higher than that reported for estradiol, as in the case of derivative **20b**, **15b**, and **12b** with RBA values of 55%, 63%, and 129%, respectively, and ERβ-selectivities between 6- and 8-fold. The only exception is represented by derivative **19b**, which is slightly ERα-selective; the phenyl substituent is probably too big for the ligand binding pocket of ERβ.[6,10] In fact, if we compare the selectivity of derivatives **13b, 16b**, **18b**, and **19b**, it is evident that an increase of the dimension of the C-4 substituent leads to a dramatic reduction in selectivity. However, when the C-4 position remains unsubstituted (**13b**), the receptor binding affinity is low, especially for ERα. This result is consistent with the earlier observations of Rose et al.[23] who found **13b** and its analogs to be inactive in animal assays. In general, from this set of data we can conclude that the introduction in position C-4 of different polar groups is well tolerated by both receptors, but that small substituents seem to increase selectivity toward ERβ (**13b** vs **16b** vs **18b**).

The reduced analogs (**22b**, **23**, **25**–**27**) show lower affinities than those of the family of isocoumarin derivatives, but some of these compounds are the most ERβ selective. In fact, derivatives **25** and **27**, with a selectivity of 62- and 102-fold, respectively, and an RBA around 2.5%, are the best compounds of this series in terms of their ERβ affinity selectivity. It is particularly interesting to note that an increase in selectivity is obtained when the hydroxyl group is moved from the C-7 to C-6 position (**25** vs **27**). Curiously, the same change in the isocoumarin derivatives leads to a dramatic reduction both in activity and selectivity (**13b** vs **24**). Some other trends in the binding data should be noted: (1) When the double bond of the heterocyclic system is reduced, a decrease in ERβ binding affinity is observed (**13b** vs **22b**, **25** vs **23** and **7** vs **6**). (2) When the oxygen in the reduced isomeric analogs **23** and **25** is moved from the benzylic position to the aryl position (**6** and **7**), an

Table 1. ERα and ERβ binding affinities (relative binding affinity [RBA] and K_i values), transcriptional potencies (EC_{50} values), and affinity and potency selectivities for isocoumarin and related ligands

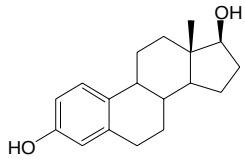
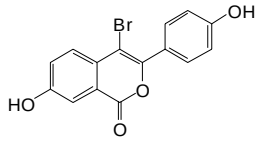
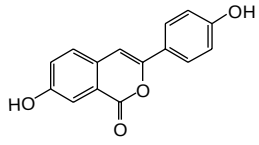
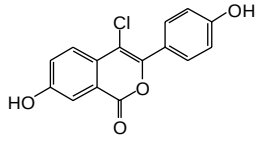
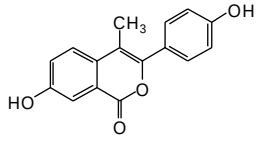
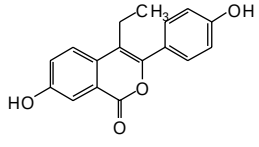
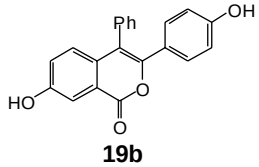
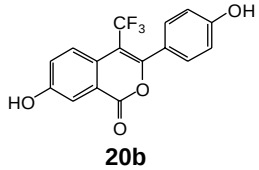
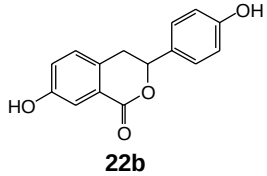
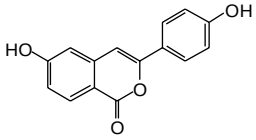
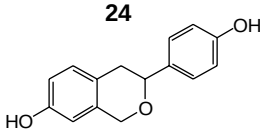
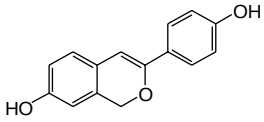
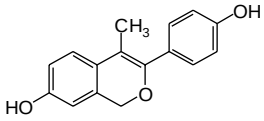
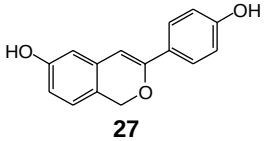
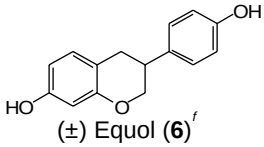
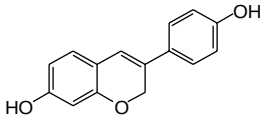
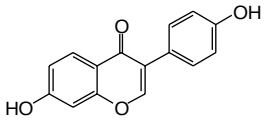
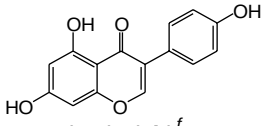
Compound	Ligand binding						Transcription potency		
	RBA[a] (%)		β/α ratio[d]	K_i[b] (nM)		β/α ratio[d]	EC_{50}[c] (nM)		β/α ratio[d]
	ERα	ERβ		ERα	ERβ		ERα	ERβ	
 Estradiol	[100]	[100]	[1]	0.2	0.5	0.4	0.021	0.11	0.19
 **12b**	16.2 ± 4.8	129 ± 24	7.9	1.23	0.38	3.2	27	0.06	450
 **13b**	0.026 ± 0.004	2.14 ± 0.08	82	770	23	33	NT[e]	NT	NT
 **15b**	9.8 ± 1.3	63 ± 13	6.4	2	0.8	2.5	110	0.06	1830
 **16b**	0.59 ± 0.01	23.9 ± 4.5	40	34	2.1	16	600	1.4	430
 **18b**	5.0 ± 1.1	16.0 ± 4.3	3	4	3.1	1.3	300	0.11	2700
 **19b**	32.2 ± 9.3	6.4 ± 1.4	0.2	0.62	7.8	0.08	NT	NT	NT
 **20b**	9.1 ± 1.4	55 ± 17	6.0	2.2	0.9	2.4	7.1	0.04	180
 **22b**	0.015 ± 0.002	0.026 ± 0.005	1.7	1300	1900	0.68	NT	NT	NT

Table 1 (*continued*)

Compound	Ligand binding						Transcription potency		
	RBA[a] (%)		β/α ratio[d]	K_i[b] (nM)		β/α ratio[d]	EC_{50}[c] (nM)		β/α ratio[d]
	ERα	ERβ		ERα	ERβ		ERα	ERβ	
 **24**	0.012 ± 0.004	0.34 ± 0.05	28	1700	150	11	NT	NT	NT
 **23**	0.035 ± 0.007	0.27 ± 0.01	7.7	570	190	3	NT	NT	NT
 **25**	0.042 ± 0.01	2.6 ± 0.7	62	480	19	25	ND[e]	12	ND
 **26**	0.23 ± 0.06	8.3 ± 1.8	36	87	6	14.5	600	1.5	400
 **27**	0.024 ± 0.001	2.45 ± 0.02	102	833	20	37	ND	170	ND
 (±) Equol (**6**)[f]	0.20 ± 0.02	1.66 ± 0.5	8	100	29	3.2	200	74	2.7
 Dehydroequol (**7**)	0.046 ± 0.01	4.3 ± 1.2	93	430	12	36	3200	7.2	440
 Daidzein (**4a**)[f]	0.010 ± 0.006	0.040 ± 0.001	4	2000	1300	1.5	250	100	2.5
 Genistein (**4b**)[f]	0.7	13	19	29	3.8	7.6	20	6.0	3.3

[a] Relative binding affinity (RBA) values are determined by competitive radiometric binding assays and are expressed as $IC_{50}^{[estradiol]}/IC_{50}^{[compound]} \times 100$ (RBA, estradiol = 100). In these assays, the K_d for estradiol is 0.2 nM on ERα and 0.5 nM on ERβ. For details, see Section 4.

[b] K_i = equilibrium binding competition constant, calculated from the K_d of estradiol on ERα (0.2 nM) or ERβ (0.5 nM): $(K_d/RBA) \times 100$.

[c] Transcriptional activity measured using a cotransfection assay in HEC-1 cells. Transcriptional potency = EC_{50}.

[d] For each index, the β/α ratio is calculated such that the ratio is > 1 for compounds having higher affinity or greater potency on ERβ than on ERα.

[e] NT = not tested; ND = not determined.

[f] Data are from Meyers et al.[9] As noted in Muthyala, et al.[12] large variations and some lower values have been reported for the ERα affinity of genistein, which would indicate a somewhat higher ERβ/ERα affinity selectivity.

increase in affinity and selectivity is noted. (3) Dehydroequol (**7**), with an ERβ RBA of 4.3% and a 93-fold ERβ binding selectivity, has ERβ selectivity greater than that of genistein, daidzein, and (*S*)-equol (32-fold beta selective) with activity comparable with that reported for (*S*)-equol (3.2% ERβ RBA).[12]

2.2.2. Activity and potency in transient transfection reporter gene assays. The transcriptional activities of some derivatives were assayed in human endometrial cancer (HEC-1) cells transfected with expression vectors for ERα and ERβ, and an estrogen-responsive reporter gene construct. The activities were normalized to that obtained with 10^{-8} M estradiol, which is set to 100. Nine derivatives were selected for testing in this transcription assay (**12b**, **15b**, **16b**, **18b**, **20b**, **25**, **26**, **27** and dehydroequol **7**), based on their ERβ affinity and selectivity. The dose–response curves for these compounds are shown in Figure 4, and the EC_{50} values (which are comparable to the K_i values for binding affinity) are summarized in Table 1 (right). In this table, the EC_{50} and K_i values of daidzein (**4a**), genistein (**4b**), and (±)-equol (**6**), determined in our previous work,[9,12] are also reported for the purpose of comparison. As noted above, it is most appropriate to compare the K_i binding affinity values (Table 1, center) with transcriptional potency EC_{50} values (Table 1, right).

In general, all compounds tested were agonists on both ER subtypes, having full (**7**, **12b**, **15b**, **16b**, **25**, and **27**) or nearly full (**18b**, **20b**, and **26**) efficacy, and they show very pronounced ERβ potency selectivity that, in all cases but one (**27**), is greater—often far greater—than their ERβ binding affinity selectivities. In particular, derivatives **12b**, **15b**, and **20b** (bromo, chloro, and CF_3 isocoumarin derivatives, respectively) show good activity on ERβ at 10^{-10} M, and thus have a potency on this ER subtype that is close to that of estradiol; by contrast, their activity on ERα is evident only at much higher concentrations. Similar behavior is also observed for compounds **16b**, **18b**, and **26** (methyl and ethyl isocoumarins, and methyl dehydroisoequol derivatives, respectively), although overall they are somewhat less potent. Of these six compounds, the greatest ERβ potency selectivity is found with compounds **15b**, **16b**, **18b**, and **26**; at 10^{-8} M, these compounds fully activate ERβ, yet have negligible activity on ERα (Fig. 4). Thus, their ERβ potency selectivity is 400 to >1000 (Table 1, right). The simple dehydroisoequol derivative **25** and its isomer **27** show interesting activity: they have low

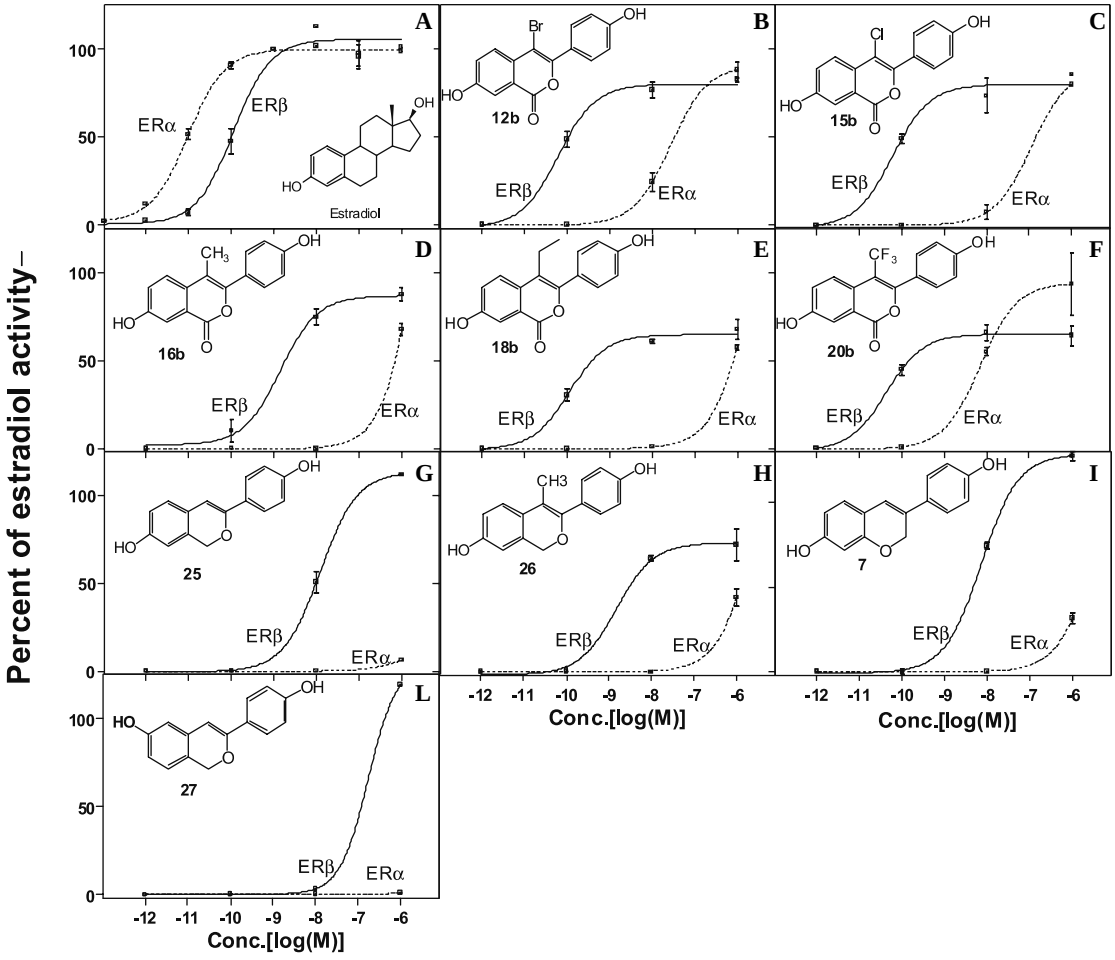


Figure 4. Transcriptional activation by ERα and ERβ in response to estradiol (E2), **12b**, **15b**, **16b**, **18b**, **20b**, **25**, **26**, **27**, and **7**. Human endometrial cancer (HEC-1) cells were transfected with expression vectors for ERα or ERβ, and the estrogen responsive gene 2EREp-pS2-Luc and were incubated with the indicated concentrations of estradiol or ligands for 24 h. Luciferase activity was measured as noted in the Section 4.

to no activity for ERα at concentrations of 10^{-8} M and below, but at 10^{-6} M, they are as active as estradiol on ERβ. Curiously, these compounds are some of the most selective ones in the binding assay (RBA values show 60-fold beta selectivity for derivative **25**, 102-fold for compound **27**), although their affinities and potencies on ERβ are rather low.

If we compare the results of the transcription assays on these isocoumarin analogs with those reported for the isoflavonoids daidzein (**4a**), genistein (**4b**), and (±) equol (**6**) (Table 1, right), we can clearly see that these new isomeric derivatives (isocoumarins) are much cleaner pharmaceutical modulators of the ERβ subtype than are the isoflavonoid soy phytoestrogens; at an appropriate dose, the isocoumarins are capable of fully activating ERβ, with no activating effect on ERα.

One aspect of our findings is of note: we find that the ERβ potency selectivity (the ratio of EC_{50} values) of several compounds in the isocoumarin series greatly exceeds their affinity selectivity (the ratio of K_i values). This is evident, especially, with **12b**, **15b**, and **18b**, where ERβ potency selectivities exceed the affinity selectivities by more than 100. We previously noted a similar discordance in ERβ potency and affinity selectivities in the indazole compounds on which we reported earlier.[10] While we do not have an explanation of this difference between affinity and potency selectivities for these compounds, it is well appreciated that whereas affinity represents binding, potency can also depend on cell and promoter-specific interactions.[29] Thus, differences in the conformation of ERβ-ligand and ERα-ligand complexes could have significant effects on their ability to interact with coactivator proteins that support their activity; these differences could then lead to the enhanced selectivity in terms of potency as opposed to affinity that we observe. However, the experimental protocol of the two experiments may account for some of the differences. The binding affinity is measured in a competitive binding assay that involves two ligands (the test compound and estradiol), and is thus dependent upon their respective interaction (association and dissociation) rates with the receptor protein. The potency experiment is assayed with the test compound alone and, in this way, might be a more direct measure of its potency.

3. Conclusion

We have synthesized isocoumarin compounds and their derivatives bearing the same core functionalities as the isoflavonoid phytoestrogens daidzein (**4a**), genistein (**4b**), coumestrol (**5**), (±) equol (**6**), and dehydroequol (**7**), but in an isomeric arrangement. Compared to the phytoestrogens, some of the isocoumarins have comparable to better ERβ affinity selectivities; those with the highest ERβ affinity selectivities are **13b**, **25**, and **27**. What is most striking, however, is the very high ERβ potency selectivity shown by some of the isocoumarins in the transcription assays: some show up to two orders of magnitude higher ERβ potency selectivities than daidzein and genistein, these ERβ potency selectivities also being greater than their binding selectivities.

Two sets of compounds stand out among those we have studied. The halogen-substituted isocoumarins, **12b** and **15b**, have transcriptional potencies on ERβ that equal or exceed that of estradiol, combined with very favorable ERβ transcriptional potency selectivities. The two alkyl-substituted isocoumarins, **16b** and **18b**, also have very high ERβ potency selectivities while retaining good ERβ potency. These two sets of compounds should facilitate investigations of the biological and physiological roles of ERβ, and they might also be useful for examining the structural conformation of ERβ agonist complexes.

4. Experimental

4.1. Materials and methods

All reagents and solvents were obtained from Aldrich or Fisher. Compound **7** (dehydroequol) was prepared as described in the literature.[30] Tetrahydrofuran, diethyl ether, toluene, and dichloromethane were dried by the solvent delivery system (SDS) (neutral alumina columns) designed by J. C. Meyer.[31] Glassware was oven dried, assembled while hot, and cooled under an inert atmosphere. Unless otherwise stated, all reactions were conducted in an inert atmosphere. Reactions using moisture- or air-sensitive reagents were performed in anhydrous solvents. Reaction progress was monitored using analytical thin-layer chromatography (TLC) on 0.25 mm Merck F-254 silica gel glass plates. Visualization was achieved by either UV light (254 nm) or potassium permanganate indicator. Flash chromatography was performed with Woelm silica gel (0.040–0.063 mm) packing.

^{1}H NMR and ^{13}C NMR spectra were obtained on a 400 or 500 MHz instrument. The chemical shifts are reported in parts per million and are referenced to either tetramethylsilane or the solvent. Mass spectra were recorded under electron impact conditions at 70 eV. Melting points were obtained on a Thomas–Hoover MelTemp apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Service Laboratory of the University of Illinois. Those final components that did not give satisfactory combustion analysis gave satisfactory exact mass determinations and were found to be at least 96% pure by HPLC analysis.

4.1.1. 5-Methoxy-2-trifluoromethansulfonyloxy benzoic acid methyl ester (8). To a solution of 2-hydroxy-5-methoxy-benzoic acid methyl ester (910 mg, 5 mmol), and trifluoromenthanesulfonic anhydride (5 mmol, 1 mL) dissolved in toluene (5 mL), Et_3N (3 mmol, 2.09 mL) was added dropwise at 0 °C. The reaction mixture was stirred at this temperature for 30 min and then allowed to warm to rt for 1 h. The reaction mixture was quenched with $NaHCO_3$ (5% soln) and extracted with EtOAc (3× 20 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum. The crude product was purified by flash chromatography (10% diethyl

ether/hexanes) to give a colorless oil (1.4 g, 90% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 (d, J = 3.2, 1H), 7.20 (d, J = 9.0, 1H), 7.09 (dd, J = 9.1, 3.3, 1H), 3.95 (s, 3H), 3.85 (s, 3H); MS (EI) m/z 314 (M^+, 18).

4.1.2. 5-Methoxy-2-(4-methoxyphenylethynyl)benzoic acid methyl ester (10). Compound **8** (1.2 g, 3.8 mmol) was dissolved in Et_3N–CH_3CN (1:5, 6 mL) and $Pd(PPh_3)_4$ (220 mg, 5 mol %), CuI (108 mg, 15 mol %), Bu_4NI (2.8 g, 4.5 mmol), and 4-ethynylanisole (**9**) (591 μL, 4.5 mmol) were added. The reaction mixture was heated at reflux for 4 h and then allowed to cool to rt and quenched with water (10 mL). The mixture was extracted with EtOAc (3× 20 mL), dried over Na_2SO_4 and the solvent removed under vacuum. The crude product was purified by flash chromatography (20%, diethyl ether/hexanes) to give a pale yellow oil (1.0 g, 75%) that crystallized on standing (mp 65–67 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52–7.45 (m, 4H), 7.00 (dd, J = 8.8, 3.0, 1H), 6.85 (dd, J = 8.7, 2H), 3.94 (s, 3H), 3.80 (s, 3H), 3.77 (s, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 166.06, 159.58, 158.77, 135.11, 132.99, 132.84, 118.28, 116.21, 115.73, 115.01, 113.97, 92.71, 86.96, 55.47, 55.23, 52.21; MS (EI) m/z 296 (M^+, 100).

4.1.3. 4-Iodo-7-methoxy-3-(4-methoxyphenyl)isocoumarin (11). Derivative **10** (800 mg, 2.7 mmol) was dissolved in CH_3CN (15 mL), and I_2 (2.0 g, 8.1 mmol) was added. The reaction mixture was stirred at rt for 2 h, then quenched with a saturated solution of $Na_2S_2O_3$ (15 mL), and extracted with EtOAc (3× 20 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum. The crude product was purified by crystallization in EtOAc to give a white solid (805 mg, 73%, mp 181–182 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, J = 8.8, 1H), 7.71 (d, J = 2.7, 1H), 7.66 (dd, J = 6.8, 2, 2H), 7.35 (dd, J = 9.2, 3.2, 1H), 6.98 (dd, J = 6.8, 2, 2H), 3.94 (s, 3H), 3.87 (s, 3H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 162.08, 160.84, 160.22, 152.70, 133.33, 132.20, 131.79, 127.57, 124.94, 121.09, 113.46, 110.17, 75.59, 56.10, 55.50; MS (EI) m/z 408 (M^+, 100).

4.1.4. 4-Bromo-7-methoxy-3-(4-methoxyphenyl)isocoumarin (12a). Derivative **10** (700 mg, 2.36 mmol) was dissolved in CH_3CN (16 mL) and Br_2 was added (181 μL, 3.54 mmol). The reaction mixture was stirred at rt for 1 h, then quenched with water (10 mL) and extracted with EtOAc (3× 20 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum. The crude product was purified by crystallization in EtOAc to give a white solid (700 mg, 82%, mp 151–153 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (d, J = 9, 1H), 7.76 (dd, J = 6.9, 2.1, 2H), 7.72 (d, J = 2.8, 1H), 7.38 (dd, J = 9, 2.8, 1H), 6.97 (dd, J = 6.9 ,2.1, 2H), 3.93 (s, 3H), 3.87 (s, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 161.63, 160.85, 160.15, 149.79, 131.37, 130.61, 128.45, 125.18, 124.81, 121.62, 113.57, 110.38, 100.55, 56.04, 55.49; MS (EI) m/z 361 (M^+, 100).

4.1.5. General method for deprotection of methoxy groups with BBr_3. The methyl ether protected compound (0.5 mmol) was dissolved in CH_2Cl_2 (1 mL), and BBr_3 (1.5 mmol) was added at 0 °C. The reaction mixture was stirred at rt for 24 h. If at the end of this time period, the starting material was not totally consumed, an additional amount of BBr_3 (1.5 mmol) was added, and the reaction mixture was stirred for other 24 h. The reaction mixture was quenched with water (5 mL) and extracted with EtOAc (3× 10 mL). The combined organic layers were dried over Na_2SO_4, and concentrated under vacuum. The crude product was purified by flash chromatography or by re-crystallization.

4.1.6. 4-Bromo-7-hydroxy-3-(4-hydroxyphenyl)isocoumarin (12b). Compound **12b** was purified by flash chromatography (50% EtOAc/hexanes) to give a pale yellow solid (60% yield) that was re-crystallized in acetone (mp > 300 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 10.56 (s, OH), 10.01 (s, OH), 7.75 (d, J = 8.7, 1H), 7.57 (dd, J = 6.9, 2.1, 2H), 7.50 (d, J = 2.5, 1H), 7.40 (dd, J = 9, 2.8, 1H), 6.86 (dd, J = 6.8, 2, 2H); ^{13}C NMR (500 MHz, acetone-d_6) δ 160.52, 158.87, 158.32, 148.90, 131.12, 128.28, 128.12, 124.58, 123.29, 121.18, 114.96, 113.13, 99.84; MS (EI) m/z 333 (M^+, 8). HRMS (EI) calcd for $C_{15}H_9BrO_4$, 331.9684; found, 332.9765. Anal. ($C_{15}H_9BrO_4$) C, H.

4.1.7. 7- Methoxy-3-(4-methoxyphenyl)isocoumarin (13a). Derivative **11** (200 mg, 0.5 mmol) was dissolved in DMF (6 mL) and formic acid (37 μL, 1 mmol), Et_3N (110 μL, 1.5 mmol), PPh_3 (10 mg, 8 mol %), and $Pd(OAc)_2$ (4 mg, 4 mol %) were added. The reaction mixture was heated at 60 °C overnight, then quenched with water and extracted with diethyl ether (3× 5 mL). The organic extracts were dried over $MgSO_4$, the solvent concentrated under vacuum, and the crude product purified by flash chromatography (50% diethyl ether/hexanes) to give a pale yellow solid (120 mg, 0.42 mmol, 86% yield) crystallized in EtOAc (mp 147–149 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (dd, J = 8.5,1.5, 2H), 7.67 (s, 1H), 7.37 (d, J = 8.8, 1H), 7.28 (dd, J = 7.5,1.7, 2H), 6.94 (d, J = 7.9, 2.0, 1H), 6.77 (s, 1H), 3.91 (s, 3H), 3.85 (s, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 162.78, 160.85, 159.32, 151.92, 131.74, 127.42, 126.56, 124.87, 124.80, 121.26, 114.29, 109.96, 100.13, 55.83, 55.48. MS (EI) m/z 282 (M^+, 100).

4.1.8. 7-Hydroxy-3-(4-hydroxyphenyl)isocoumarin (13b). Compound **13b** was purified by flash chromatography (50% EtOAc/hexanes) to give a pale yellow solid (65% yield), that was re-crystallized in ethyl acetate. Mp > 300 °C dec. (lit.[23] mp > 300 °C), ^{1}H NMR (500 MHz, methanol-d_4) δ 7.70 (dd, J = 8.8, 2.2, 2H), 7.54 (d, J = 2.6, 1H), 7.47 (d, J = 8.6, 1H), 7.25 (dd, J = 8.5, 2.5, 1H), 7.00 (s, 1H), 6.86 (dd, J = 8.8, 2.1, 2H); ^{13}C NMR (500 MHz, methanol-d_4) δ 160.25, 158.84, 152.74, 132.16, 128.98, 127.44, 125.65, 124.91, 122.05, 116.70, 113.78, 101.01. MS (EI) m/z 254 (M^+, 25). HRMS (EI) calcd for $C_{15}H_{10}O_4$, 254.0579; found, 254.0581. Anal. ($C_{15}H_{10}O_4 \cdot 0.2H_20$) C, H.

4.1.9. 4-Chloro-7-methoxy-3-(4-methoxyphenyl)isocoumarin (15a). To a suspension of $Hg(OAc)_2$ (318 mg, 1 mmol) in CH_3CO_2H (3 mL), derivative **10** (300 mg, 1 mmol) was added. The reaction mixture was stirred at rt for 1 h, and then at 0 °C a saturated solution of

NaCl (3 mL) was added. The reaction mixture was allowed reach to rt and stirred for 30 min. The separated organomercurial was removed by filtration, washed well with hexane, and dissolved in chloroform. The chloroform solution was filtered through silica gel, and the organic solvent was evaporated. The crude product (**14**) was used in the next step without any further purification. Product **14** was dissolved in THF (3 mL), and anhydrous $CuCl_2$ (270 mg, 2 mmol) was added. The reaction mixture was heated at reflux for 4 h, and then the precipitate was removed by filtration. The crude product was purified by flash chromatography (30% EtOAc/hexane) to give a white solid (90 mg, 0.28 mmol, 28% yield), re-crystallized in EtOAc/hexane (mp 155–157 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87–7.83 (m, 3H), 7.74 (d, J = 2.6, 1H), 7.42 (dd, J = 9.0, 2.8, 1H), 6.69 (dd, J = 6.8, 2.1, 2H), 3.94 (s, 3H), 3.87 (s,3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 161.44, 160.87, 160.17, 148.51, 130.96, 130.03, 125.86, 124.85, 123.97, 121.68, 113.75, 113.49, 110.51, 56.05, 55.52. MS (EI) m/z 316 (M^+, 100).

4.1.10. 4-Chloro-7-hydroxy-3-(4-hydroxyphenyl)isocoumarin (15b). Compound **15a** (20 mg, 0.06 mmol) was deprotected according to the procedure described above with BBr_3 in CH_2Cl_2. Derivative **15b** was purified by flash chromatography (50% EtOAc/hexane) to give a pale yellow solid essentially pure (11 mg, 60% yield). Mp > 300 °C dec. ^{1}H NMR (500 MHz, acetone-d_6) δ 9.5 (br s, OH), 9.0 (br s, OH), 7.85 (d, J = 8.8, 1H), 7.72 (dd, J = 6.7, 2.1, 2H), 7.66 (d, J = 2.6, 1H), 7.50 (dd, J = 9.0, 2.8, 1H), 6.68 (dd, J = 6.8, 2.1, 2H). MS (EI) m/z 288 (M^+, 100); HRMS (EI) calcd for $C_{15}H_9ClO_4$, 288.0189; found, 288.0192. Purity >95% (HPLC).

4.1.11. 7-Methoxy-3-(4-methoxypheny)-4-methylisocoumarin (16a). Derivative **11** (150 mg, 0.36 mmol) was dissolved in dioxane (2 mL), and $Pd_2(dba)_3$ (4.5 mol %), P^{t}Bu (18 mol %), CsF (120 mg, 0.79 mmol), and $Sn(CH_3)_4$ (100 μL, 0.72 mmol) were added. The reaction mixture was heated in a sealed tube at 80 °C for 12 h. After that, the precipitate was removed by filtration and the solvent removed under vacuum. The crude product was purified by flash chromatography (30% EtOAc/hexane) to give a white solid (95 mg, 0.32 mmol, 89% yield) re-crystallized in EtOAc/hexane (mp 157–159 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 2.9, 1H), 7.55–7.50 (m, 3H), 7.36 (dd, J = 8.8, 2.9, 1H), 6.95 (dd, J = 6.8, 2.0, 2H), 3.91 (s, 3H), 3.85 (s, 3H), 2.28 (s, 3H);^{13}C NMR (400 MHz, $CDCl_3$) δ 162.92, 160.19, 159.18, 149.33, 132.82, 130.96, 125.82, 125.08, 124.38, 121.86, 113.70, 110.15, 108.40, 55.88, 55.47, 13.81. MS (EI) m/z 296 (M^+, 96).

4.1.12. 7-Methoxy-3-(4-methoxyphenyl)-4-vinylisocoumarin (17). Derivative **11** (150 mg, 0.36 mmol) was dissolved in dioxane (2 mL), and $Pd_2(dba)_3$ (4.5 mol %), P^{t}Bu$_3$ (18 mol %), CsF (120 mg, 0.79 mmol), and vinylSn(Bu)$_3$ (93 μL, 0.36 mmol) were added. The reaction was performed as in the procedure given for **16a**, and the crude product was purified by flash chromatography (30% EtOAc/hexane) to give a pale yellow solid (85 mg, 0.27 mmol, 76% yield) re-crystallized in EtOAc (mp 144–145 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77–7.75 (m, 2H), 7.64 (dd, J = 6.9, 2.1, 2H), 7.31 (dd, J = 8.8, 2.8, 1H), 6.91 (dd, J = 6.8, 2.1, 2H), 6.57 (dd, J = 18, 11.4, 1H), 5.63 (dd, J = 11.4, 1.5, 1H), 5.47 (dd, J = 1.5, 18, 1H), 3.91 (s, 3H), 3.84 (s, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 162.41, 160.30, 159.26, 149.16, 131.22, 131.13, 130.41, 126.26, 125.63, 124.29, 122.63, 121.68, 113.55, 112.65, 110.21, 55.86, 55.41. MS (EI) m/z 308 (M^+, 100).

4.1.13. 4-Ethyl-7-methoxy-3-(4-methoxyphenyl)isoumarin (18a). Derivative **17** (40 mg, 0.13 mmol), was dissolved in EtOH (10 mL), and Pd/C (10%, 15 mg) was added. The reaction mixture was stirred at rt under H_2 (1 atm) for 6 h. The catalyst was filtered off and the solvent removed under vacuum to give a white solid (quant. yield) re-crystallized in EtOAc (mp 142–144 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 3.2, 1H), 7.57 (d, J = 9.2, 1H), 7.49 (dd, J = 6.8, 2.0, 2H), 7.35 (dd, J = 8.8, 3.2, 1H), 6.96 (dd, J = 6.8, 2.0, 2H), 3.91 (s, 3H), 3.86 (s, 3H), 2.70 (q, J = 7.6, 2H). 1.27 (t, J = 7.6, 3H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 162.88, 160.26, 159.11, 149.42, 131.66, 130.43, 126.01, 125.15, 124.43, 122.43, 114.71, 113.78, 110.36, 55.88, 55.46, 20.37, 14.97. MS (EI) m/z 310 (M^+, 65).

4.1.14. 7-Methoxy-3-(4-methoxyphenyl)-4-phenylisocoumarin (19a). Derivative **11** (150 mg, 0.36 mmol) was dissolved in DMF (1.5 mL), and $Pd(PPh_3)_4$ (5 mol %), Cs_2CO_3 (164 mg, 0.5 mmol) and $PhB(OH)_2$ (53.7 mg, 0.44 mmol) were added. The reaction mixture was heated at 80 °C for 4 h, then quenched with water (10 mL), and extracted with diethyl ether (3× 10 mL). The organic extracts were dried over $MgSO_4$ and the solvent removed under vacuum. The crude product was purified by flash chromatography (50% diethyl ether/hexane) to give a white solid (110 mg, 0.30 mmol, 85% yield) re-crystallized in diethyl ether/hexane (mp 135–137 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, J = 2.6, 1H), 7.43–7.40 (m, 3H), 7.27–7.19 (m, 5H), 7.10 (d, J = 8.8, 1H), 6.70 (dd, J = 6.8, 2.2, 2H), 3.93 (s, 3H), 3.75 (s, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 162.70, 159.33, 149.08, 134.96, 132.96, 131.33, 130.61, 129.25, 128.14, 127.00, 125.49, 124.38, 121.43, 115.81, 113.40, 109.90, 55.94, 55.30. MS (EI) m/z 358 (M^+, 100).

4.1.15. 7-Methoxy-3-(4-methoxyphenyl)-4-trifluoromethylisocoumarin (20a). Derivative **11** (100 mg, 0.25 mmol) was dissolved in DMF (1 mL), and $FSO_2CF_2CO_2CH_3$ (149 μL, 1.17 mmol), and CuI (46 mg, 0.25 mmol) were added. The reaction mixture was heated at 80 °C for 6 h, after which the solid obtained from the reaction was filtered off, and the solvent removed under vacuum. The crude product was purified by flash chromatography (30% EtOAc/hexane) to give a colorless oil (70 mg, 0.2 mmol, 81% yield) that solidified on standing (mp 115–118 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76–7.73 (m, 2H), 7.50 (dd, J = 6.9, 2.2, 2H), 7.38 (dd, J = 9.0, 3.0, 1H), 6.98 (dd, J = 6.9, 2.2, 2H), 3.93 (s, 3H), 3.86 (s, 3H); MS (EI) m/z 350 (M^+, 29).

4.1.16. 7-Hydroxy-3-(4-hydroxyphenyl)-4-methylisocoumarin (16b). Compound **16a** (50 mg, 0.17 mmol) was

deprotected according to the procedure described above with BBr_3 in CH_2Cl_2. After the quenching with water, the solid product obtained was removed by filtration, and the filtrate washed with EtOAc, to give a compound essentially pure (25 mg, 0.09 mmol, 55% yield). Mp > 300 °C dec. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.29 (br s, OH), 9.88 (br s, OH), 7.62 (d, J = 8.8, 1H), 7.51 (d, J = 2.7, 1H), 7.38 (dd, J = 8.5, 1.9, 2H), 7.34 (dd, J = 8.8, 2.8, 1H), 6.86 (dd, J = 8.5, 2.0, 2H), 2.19 (s, 3H);^{13}C NMR (500 MHz, DMSO-d_6) δ 161.62, 158.17, 157.24, 148.12, 130.72, 130.56, 125.79, 124.02, 123.75, 121.17, 115.10, 112.73, 107.80, 13.31. MS (EI) m/z 254 (M^+, 25). HRMS (EI) calcd for $C_{16}H_{12}O_4$, 268.0736; found, 268.0736. Anal. ($C_{16}H_{12}O_4 \cdot 0.2H_2O$) C, H.

4.1.17. 7-Hydroxy-3-(4-hydroxyphenyl)-4-ethylisocoumarin (18b). Compound **18b** (40 mg, 0.13 mmol) was deprotected according to the procedure described above with BBr_3 in CH_2Cl_2. Derivative **18a** was purified by flash chromatography (60% EtOAc/hexane) to give a pale yellow solid (34 mg, 0.12 mmol, 92% yield) re-crystallized in EtOAc (mp > 300 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 9.10 (br s, OH), 8.75 (br s, OH), 7.72–7.69 (m, 2H), 7.45 (dd, J = 6.7, 2.1, 2H), 7.41 (dd, J = 8.8, 2.8, 1H), 6.98 (dd, J = 6.7, 2.1, 2H), 2.70 (q, J = 7.5, 2H), 1.25 (t, J = 7.3, 3H);^{13}C NMR (500 MHz, acetone-d_6) δ 162.41, 159.09, 157.90, 149.92, 131.24, 131.04, 126.48, 126.01, 124.43, 123.45, 116.00, 115.04, 114.22, 20.75, 15.04. MS (EI) m/z 282 (M^+, 11). HRMS (EI) calcd for $C_{17}H_{14}O_4$, 282.0892; found, 282.0894. Anal. ($C_{17}H_{14}O_4 0.5\ H_2O$) C, H.

4.1.18. 7-Hydroxy-3-(4-hydroxyphenyl)-4-phenylisocoumarin (19b). Compound **19b** (50 mg, 0.14 mmol) was deprotected according to the procedure described above with BBr_3 in CH_2Cl_2. Derivative **19a** was purified by flash chromatography (50% EtOAc/hexane) to give a pale yellow solid (40 mg, 0.12 mmol, 85% yield) re-crystallized in EtOAc. ^{1}H NMR (500 MHz, acetone-d_6) δ 9.0 (br s, OH), 7.70 (d, J = 2.6, 1H), 7.46–7.39 (m, 3H), 7.31–7.23 (m, 5H), 7.17 (dd, J = 8.6, 2.5, 1H), 6.68 (dd, J = 6.7, 2.1, 2H); ^{13}C NMR (500 MHz, acetone-d_6) δ 162.21, 158.67, 158.09, 149.64, 136.06, 132.42, 132.12, 131.41, 129.81, 128.69, 127.84, 125.57, 124.39, 122.41, 116.20, 115.54, 113.83. MS (EI) m/z 330 (M^+, 5). HRMS (EI) calcd for $C_{21}H_{14}O_4$, 330.0892; found, 330.0888. Anal. ($C_{21}H_{14}O_4 0.5\ H_2O$) C, H.

4.1.19. 7-Hydroxy-3-(4-hydroxyphenyl)-4-trifluoromethylisocoumarin (20b). Compound **20a** (70 mg, 0.2 mmol) was deprotected according to the procedure described above with BBr_3 in CH_2Cl_2. Derivative **20b** was purified by flash chromatography (50% EtOAc/hexane) to give a white solid (60 mg, 0.18 mmol, 94% yield) re-crystallized in EtOAc (mp > 230 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 9.25 (br s, OH), 7.73–769 (m, 2H), 7.48–7.46 (m, 3H), 6.98 (dd, J = 6.7, 2.2, 2H); MS (ESI) m/z 323 (M+H, 100). HRMS (ESI) calcd for (M+H), 323.0531; found, 323.0522. Anal. ($C_{16}H_9F_3O_4$) C, H.

4.1.20. 7-Methoxy-3-(4-methoxyphenyl)-3,4-dehydroisocoumarin (22a). Compound **13a** (400 mg, 1.4 mmol) was suspended in EtOH (4 mL) and KOH (5% soln, 4 mL) was added. The reaction mixture was heated under reflux for 3 h. The reaction mixture was allowed to reach rt and EtOH removed under vacuum. The aqueous phase was washed with diethyl ether, acidified with HCl (1M), and extracted with EtOAc (3× 10 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum to give a pale yellow solid (**21**) pure enough to be used for the next step without any further purification. Derivative **21** (380 mg, 1.4 mmol) was dissolved in EtOH (4 mL), and $NaBH_4$ (310 mg, 8.4 mmol) was added. The reaction mixture was heated under reflux for 4 h. EtOH was then evaporated, the residue diluted with water (10 mL), and acidified with HCl (1 M) to give a precipitate, which was extracted with EtOAc (3× 10 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum. The racemic alcohol obtained was dissolved in acetic anhydride (1 mL) and heated under reflux for 2 h. The reaction mixture was then allowed to reach rt, quenched with water, and extracted with CH_2Cl_2 (3× 10 mL). The combined extracts were washed with NaOH (5% soln) dried over Na_2SO_4, and the solvent removed under vacuum. The crude product was purified by flash chromatography (60% diethyl ether/hexane) to give a white solid (160 mg, 0.56 mmol, 40% yield) re-crystallized in EtOAc (mp 138–139 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, J = 2.7, 1H), 7.40 (dd, J = 6.5, 1.7, 2H), 7.19 (d, J = 8.5, 1H), 7.13 (dd, J = 8.3, 2.7, 1H), 6.94 (dd, J = 6.8, 2.2, 2H), 5.47 (dd, J = 11.9, 3.2, 1H), 3.86 (s, 3H), 3.82 (s, 3H), 3.28 (dd, J = 16.1, 12, 1H), 3.03 (dd, J = 16.3, 3.1, 1H);^{13}C NMR (500 MHz, $CDCl_3$) δ 165.56, 159.76, 159.05, 131.34, 130.65, 128.47, 127.62, 125.88, 121.79, 113.93, 112.88, 80.16, 55.60, 55.29, 34.61. MS (EI) m/z 284 (M^+, 26).

4.1.21. 7-Hydroxy-3-(4-hydroxyphenyl)-3,4-dehydroisocoumarin (22b). Compound **22a** (50 mg, 0.17 mmol) was deprotected according to the procedure described above with BBr_3 in CH_2Cl_2. Derivative **22b** was purified by flash chromatography (50% EtOAc/hexane) to give a white solid (30 mg, 0.12 mmol, 70% yield) re-crystallized in EtOAc (mp > 230 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 8.75 (br s, OH), 7.48 (d, J = 2.7, 1H), 7.37 (dd, J = 6.7, 2.2, 2H), 7.23 (d, J = 8.1, 1H), 7.10 (dd, J = 8.2, 2.6, 1H), 6.87 (dd, J = 6.4, 1.9, 2H), 5.48 (dd, J = 12, 3.2, 1H), 3.25 (dd, J = 16.3, 12.2, 1H), 3.06 (dd, J = 16.3, 3.2, 1H);^{13}C NMR (500 MHz, acetone-d_6) δ 165.56, 158.46, 157.53, 131.62, 131.26, 129.74, 128.80, 127.10, 122.06, 116.10, 116.03, 80.93, 34.87. MS (EI) m/z 256 (M^+, 33). HRMS (EI) calcd for $C_{15}H_{12}O_4$, 256.0736; found, 256.0733. Anal. ($C_{15}H_{12}O_4 0.1\ H_2O$) C, H.

4.1.22. 3-(4-Hydroxyphenyl)isochroman-7-ol (isoequol, 23). Compound **22b** (30 mg, 0.12 mmol), was dissolved in THF (2 mL), and $LiAlH_4$ (6.6 mg, 0.18 mmol) was added at rt. The reaction mixture was heated under reflux for 2 h and then at 0 °C quenched with water (2 mL). After the evaporation of organic solvent, the aqueous phase was acidified with H_2SO_4 (5% soln) and extracted with EtOAc (3× 5 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum. The crude product was purified by flash chromatography (40% EtOAc/hexane) to give a white solid

(20 mg, 0.08 mmol, 71% yield) essentially pure (mp 228–230 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 8.28 (br s, OH), 8.16 (br s, OH), 7.28 (dd, J = 6.7, 2.1, 2H), 6.98 (d, J = 8.4, 1H), 6.83 (dd, J = 6.8, 2.0, 2H), 6.68 (dd, J = 8.2, 2.4, 1H), 6.55 (d, J = 1.9, 1H), 4.83 (s, 2H), 4.57 (t, J = 6.5, 1H), 2.82 (d, J = 6.7, 2H); ^{13}C NMR (500 MHz, acetone-d_6) δ 157.56, 156.48, 136.72, 134.75, 128.03, 125.29, 115.74, 114.66, 111.20, 77.49, 69.10, 36.13. MS (EI) m/z 242 (M^+, 3). HRMS (EI) calcd for $C_{15}H_{14}O_3$, 242.0943; found, 242.0948. Anal. ($C_{15}H_{14}O_3$0.2 H_2O) C, H.

4.1.23. 3-(4-Hydroxyphenyl)-1*H*-isochromen-7-ol (dehydroisoequol, 25). Compound **13b** (60 mg, 0.23 mmol) was dissolved in THF (2 mL) and $LiAlH_4$ (13.5 mg, 0.35 mmol) was added at rt. The reaction mixture was stirred at this temperature for 45 min and then at 0 °C quenched with water (2 mL). After the evaporation of organic solvent, the aqueous phase was acidified with H_2SO_4 (5% soln) and extracted with EtOAc (3× 5 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum. The crude product was purified by flash chromatography (50% EtOAc/hexane) to give a white solid (20 mg, 0.08 mmol, 35% yield) essentially pure (mp > 200 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 8.40 (br s, OH), 8.20 (br s, OH), 7.57 (dd, J = 6.7, 2.1, 2H), 6.96 (d, J = 8.2, 1H), 6.84 (dd, J = 6.8, 2.3, 2H), 6.72 (dd, J = 8.2, 2.2, 1H), 6.64 (d, J = 1.9, 1H), 6.39 (s, 1H), 5.08 (s, 2H); ^{13}C NMR (500 MHz, acetone-d_6) δ 158.71, 157.02, 152.53, 130.72, 127.07, 127.02, 125.24, 125.02, 115.94, 115.45, 111.92, 99.67, 69.28. MS (EI) m/z 240 (M^+, 40). HRMS (EI) calcd for $C_{15}H_{12}O_3$, 240.0786; found, 240.0789. Anal. ($C_{15}H_{12}O_3$·0.1 H_2O) C, H.

4.1.24. 3-(4-Hydroxyphenyl)-4-methyl-1*H*-isochromen-7-ol (26). Compound **16b** (60 mg, 0.22 mmol) was dissolved in THF (2 mL) and $LiAlH_4$ (13.5 mg, 0.35 mmol) was added at RT. The reaction mixture was stirred at this temperature for 1 h and then heated under reflux for 2 h. After that, the reaction mixture was cooled down to 0 °C and then quenched with water (2 mL). After the evaporation of organic solvent, the aqueous phase was acidified with H_2SO_4 (5% soln) and extracted with EtOAc (3× 5 mL). The organic extracts were dried over Na_2SO_4 and the solvent removed under vacuum. The crude product was purified by flash chromatography (50% EtOAc/hexane) to give a white solid (15 mg, 0.06 mmol, 27% yield) essentially pure (mp > 200 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 8.75 (br s, OH), 8.25 (br s, OH), 7.36 (dd, J = 6.6, 1.9, 2H), 7.07 (d, J = 8.4, 1H), 6.85 (dd, J = 6.7, 1.9, 2H), 6.78 (dd, J = 8.1, 2.3, 1H), 6.65 (d, J = 2.3, 1H), 4.98 (s, 2H), 2.09 (s, 3H); ^{13}C NMR (500 MHz, acetone-d_6) δ 158.32, 156.99, 149.66, 132.00, 127.77, 127.36, 127.01, 123.29, 115.40, 115.21, 111.56, 107.38, 69.06, 14.42. MS (ESI) m/z 255 (M+H, 100). HRMS (ESI) calcd for $C_{16}H_{14}O_3$, 254.0943; found, 254.0958. Anal. ($C_{16}H_{14}O_4$·0.2H_2O) C, H.

4.1.25. 3-(4-Hydroxyphenyl)-1*H*-isochromen-6-ol (27). Compound **24** (40 mg, 0.16 mmol) was dissolved in THF (3 mL), and $LiAlH_4$ (240 μL of 1 M solution, 0.24 mmol) and a catalytic amount of $AlCl_3$ (1 mg, 5 mol %) were added at rt. The reaction mixture was stirred at rt for 2 h and then heated under reflux for 30 min. The reaction mixture was cooled down to 0 °C and then quenched with water (2 mL). After the evaporation of organic solvent the aqueous phase was acidified with HCl (1M, 5 mL) and extracted with EtOAc (3× 15 mL). The organic extracts were dried over $MgSO_4$ and the solvent removed under vacuum. The crude product was purified by flash chromatography (33% EtOAc/hexane) to give a pale yellow solid (26 mg, 0.11 mmol, 69% yield) essentially pure (mp 169 °C dec.). ^{1}H NMR (500 MHz, acetone-d_6) δ 8.44 (br s, OH), 7.61 (dd, J = 6.8, 2.0, 2H), 6.95 (d, J = 8.8, 1H), 6.85 (dd, J = 6.8, 2.0, 2H), 6.62–6.58 (m, 2H), 6.38 (s, 1H), 5.08 (s, 2H);^{13}C NMR (500 MHz, acetone-d_6) δ 159.20, 158.27, 155.19, 134.66, 127.54, 126.71, 125.55, 120.11, 115.99, 113.28, 110.76, 99.74, 69.24. MS (EI) m/z 240 (M^+, 100). HRMS (EI) calcd for $C_{15}H_{12}O_3$, 240.0786; found, 240.0792. Anal. ($C_{15}H_{12}O_3$) C, H.

4.1.26. Estrogen receptor binding affinity assays. Relative binding affinities were determined by a competitive radiometric binding assay as previously described,[32] using 10 nM [^{3}H]estradiol as tracer (Amersham BioSciences, Piscataway, NJ), and purified full-length human ERα and ERβ (PanVera/InVitrogen, Carlsbad, CA). Incubations were for 18–24 h at 0 °C; the receptor–ligand complexes were absorbed onto hydroxyapatite (BioRad, Hercules, CA), and the unbound ligand was washed away. The binding affinities are expressed as relative binding affinity (RBA) values, with the RBA of estradiol being set at 100. The values given are the average ± range or SD for two or more independent determinations. Estradiol binds to ERα with a K_d of 0.2 nM and to ERβ with a K_d of 0.5 nM.

4.1.27. Cell culture and transient transfections. Human endometrial cancer (HEC-1) cells were maintained in minimum essential medium (MEM) plus phenol-red supplemented with 5% calf serum and 5% fetal calf serum. Cells were plated in phenol-red-free Improved MEM and 5% charcoal dextran-treated calf serum (CDCS) and were given fresh medium 24 h before transfection. Transfection assays were performed in 24-well plates using 0.35 mL of serum-free OptiMEM medium and 0.15 mL of Hank's balanced salt solution containing 5 μL of lipofectin (Life Technologies, Inc., Gaithersburg, MD), 1.6 μg of transferrin (Sigma, St. Louis, MO), 200 ng of pCMV β-galactosidase as internal control, 1 μg of 2xERE-pS2-Luc, and 100 ng of ER expression vector per well. The cells were incubated at 37 °C in a 5% CO_2-containing incubator for 5 h. The medium was then replaced with fresh Improved MEM supplemented with 5% CDCS plus the desired concentrations of ligands. Cells were harvested 24 h later. Luciferase and β-galactosidase activity were assayed as described.[33]

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