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# A New Class of Phytoestrogens: Evaluation of the Estrogenic Activity of Deoxybenzoins

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## Summary

**Although deoxybenzoins are intermediates in the synthesis of isoflavones, their estrogenic activity has not been investigated. Eleven deoxybenzoins were synthesized and their estrogenicity was evaluated. While their affinities for estrogen receptors (ER) ERα and ERβ were found grossly comparable to those of daidzein, some exhibited considerable selectivity and transcriptional bias toward ERβ, which appeared to allow for enhancement of ER-mediated transcription via deoxybenzoin binding of ERβ. Their activity to stimulate the proliferation of ER-positive breast cancer cells and regulate the expression of endogenous and stably transfected reporter genes differed considerably, with some inhibiting cell proliferation while effectively inducing gene expression at the same time. Molecular modeling confirmed that deoxybenzoins fit well in the ligand binding pocket of ERβ, albeit with different orientations. Our data support the view that deoxybenzoins constitute a promising new class of ERβ-biased phytoestrogens.**

## Introduction

Estrogens of plant origin (phytoestrogens) are known to affect the physiology of the female reproductive system (uterus, ovaries, breast), the cardiovascular system, the brain, and the skeleton. Consequently, there is growing interest in using phytoestrogens and synthetic derivatives thereof for the prevention of several diseases, including breast and prostate cancer, postmenopausal osteoporosis, and cardiovascular diseases [1, 2]. Phytoestrogens are believed to exert their physiological effects via the estrogen receptor (ER), a ligand-activated regulator of transcription. Two subtypes of ER have been described, ERα and ERβ, and reported to exhibit overlapping but distinct tissue distribution patterns and to differ in their ligand binding ability and transcription activation properties [3, 4]. Some phytoestrogens have been reported to act as ERβ-selective modulators of transcriptional activation and/or repression [4, 5]. Both ER subtypes have a modular structure comprising distinct functional domains associated with hormone binding, DNA binding and transcriptional activation [3]. The C-terminal ligand binding domain (LBD) is known to play a crucial role in unraveling the activity of antiestrogens as well as estrogens, as demonstrated by the recently published X-ray structures of the LBD of ERα and ERβ with 17β-estradiol (hereafter referred to as estradiol), raloxifene, 4-hydroxytamoxifene, DES, or genistein [6–8]. In the agonist-bound conformation, an interactive surface is created that is capable of recruiting a variety of coactivators. By contrast, in the antagonist-bound conformation, the LBD is known to recruit corepressors [3, 5, 9].

Deoxybenzoins, intermediates in the synthesis of isoflavones, are found in several plant and marine sources [10, 11]. Plants with known estrogenic activity in their extracts, such as *Glycyrrhiza sp*. [12] *Trifolium subterraneum* [13] and *Ononis spinosa*, are good sources of natural deoxybenzoins. Although the estrogenic activities of *Glycyrrhiza sp*. and *Trifolium sp*. have been attributed mainly to isoflavones [14], their structural similarities with deoxybenzoins led us to study the estrogenic activity of the latter. Here, we report on the synthesis and the estrogenic activity of 11 (among them three new) deoxybenzoins and discuss the structural determinants of their interaction with ERβ.

## Results and Discussion

### Chemical Synthesis

Using a method previously described by Wahala et al. [11], a number of deoxybenzoins were synthesized. Starting from the appropriately substituted phenols and phenylacetic acids, a Friedel-Crafts reaction catalyzed by boron trifluoride takes place (Figure 1A). Among the dihydric phenols, resorcinol and substituted resorcinols react with variously substituted phenylacetic acids, giving excellent yields of deoxybenzoins. In contrast, the trihydric phenol phloroglucinol gave good yield by reacting with p-methoxy-phenylacetic acid but not with p-hydroxy-phenylacetic acid, despite that different conditions were tested. For this reason, compound **4** was obtained by the demethylation of compound **3** using boron tribromide [15] in dichloromethane at −70°C. In the reactions of p-hydroxy-phenylacetic acid with resorcinols, minor byproducts with an ethoxy group in the 4′ position were detected. It is known [16] that ethyl fluoride is produced from the complex of boron trifluoride with ethyl ether, which can react with the hydroxyl group of p-hydroxy-phenylacetic acid. In the case of 4-cloro-

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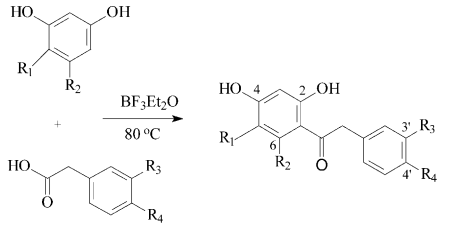
**A**




| Product | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Ref. |
|---|---|---|---|---|---|
| 1 | H | H | H | $OCH_3$ | 11, 39 |
| 2 | H | H | H | OH | 11, 39 |
| 3 | H | OH | H | $OCH_3$ | 39 |
| 4 | H | OH | H | OH | 11, 39 |
| 5 | H | H | H | F | 11 |
| 6 | H | H | H | $NO_2$ | 39 |
| 7 | Cl | H | H | OH | 11 |
| 8 | Cl | H | OH | OH | * |
| 9 | Cl | H | H | $OCH_2CH_3$ | * |
| 10 | H | H | H | Br | 39 |
| 11 | H | H | Br | H | * |

* New compound

**B**

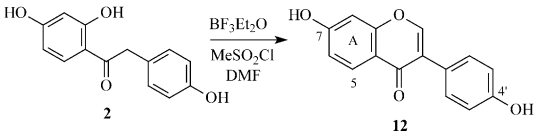



Figure 1. Synthesis of Deoxybenzoins and Daidzein

(A) Synthesis of deoxybenzoins **1–11**.

(B) Synthesis of daidzein.

resorcinol, compound **9** was obtained in yield of 12%. Isoflavone daidzein (**12**) was obtained by the cyclization of the corresponding deoxybenzoin (**2**) using boron trifluoride as catalyst (Figure 1B) [11].

### Relative Binding Affinities for ERα and ERβ

Deoxybenzoin binding of ERα and ERβ was studied using fluorescence polarization (FP) to determine the concentration ($IC_{50}$) required for 50% inhibition of ES2 (fluorescent ER ligand) binding to commercial receptor preparations [17]. The relative binding affinity (RBA) values of deoxybenzoins for ERα (RBAα) thus determined were comparable to that of the isoflavone daidzein (**12**) (Table 1). By contrast, RBAβ values varied considerably, with that of **2** in particular being ~15-fold higher than those of daidzein and the natural deoxybenzoin ononetin (**1**). Notably, **2** exhibited by far the highest selectivity for ERβ; **5**, **6**, **10**, and **11** were moderately ERβ-selective; **1**, **3**, **4**, **8**, **9**, and daidzein exhibited a moderate bias toward ERα; and only **7** was unbiased. Inspection of the structures of Figure 1 reveals that ERβ selectivity is associated with $R_1$, $R_2$, $R_3$ = H and $R_4$ = OH, F, or $NO_2$ (**2**, **5**, **6**), and that it may reflect a better accommodation of Br bulkiness (**10**, **11**).

### Deoxybenzoin Activation of Transcription via ERα and/or ERβ

To discern how deoxybenzoins affect ERα and ERβ transcriptional activity, we transfected HEK-293 human embryonic carcinoma cells with an expression vector for human ERα or ERβ as well as a luciferase reporter endowed with a consensus estrogen-responsive element (ERE) and the core thymidine kinase (tk) promoter. Using 0.1–0.4 μg of ER expression vector and 1 nM estradiol, we observed maximum reporter responses amounting to 16.3 ± 0.5- and 10.8 ± 0.9-fold in the presence of ERα and ERβ, respectively. However, because ERα exhibited higher constitutive transcriptional activity than ERβ, hormonal inductions were 5.3 ± 0.4- (ERα) and 5.8 ± 0.3-fold (ERβ). By comparison, using **2** at 10 μM, at which concentration all estrogenic deoxybenzoins exhibit maximum proliferative response (see Figure 3B), inductions were 6.1 ± 0.1- (ERα) and 9.2 ± 0.2-fold (ERβ) (Figure 2A), indicating a more effective recruitment of coactivators to the tk promoter by deoxybenzoin binding of ERβ. Thus, ERβ-mediated deoxybenzoin induction of the response is higher than the ERα-mediated one and, in addition, exceeds that of the hormone significantly (ANOVA, $p < 0.01$). The $IC_{50}$ values for ERα- and ERβ-mediated inductions by **2** of a response equal to that of the hormone were 421 and 66 nM, respectively. This 6.4-fold difference in potency exceeds the ERβ binding selectivity of **2** by ~1.2-fold, implying again that deoxybenzoin binding may render ERβ more capable of recruiting transcriptional coactivators to the tk promoter than ERα. Table 1 (columns 5 and 6) compares reporter inductions at 10 μM deoxybenzoin to those at 10 μM daidzein and 1 nM estradiol in the presence of either ERα or ERβ. Only **2** exhibited higher reporter responses than estradiol. Interestingly, several deozybenzoins, whether ERβ selective or not, exhibited a bias toward ERβ-mediated transcription at 10 μM, with **1**, **4**, **5**, **7**, **9**, and **11** apparently being the most biased. Table 1 shows further that daidzein binding of ERα and ERβ provided for somewhat higher reporter responses than **2**, albeit slightly less biased.

In light of the above, we wondered whether the ERβ binding selectivity and transcriptional bias of **2** affects reporter responses in the presence of both ER. We cotransfected HEK-293 cells with ERα (0.1 μg) and ERβ (0.1 or 0.2 μg) expression vectors, keeping vector amounts well below the limit (0.5 μg) we have observed squelching effects to come about with these cells, and exposed transfectants to 30 and 100 nM **2**, concentrations that provide for ERβ-mediated inductions of 3.0- and 4.5-fold, but ERα-mediated inductions of 1.3- and 2.1-fold, respectively (Figure 2A). We observed that 100 μM **2** elicited a significantly higher (ANOVA, $p < 0.01$) reporter response in the presence of ERα and ERβ than either receptor alone (Figure 2B). By contrast, ERβ had no effect on the marginal ERα-mediated response at 30 nM **2**, implying that an activation of either ER that could provide for a sizeable pool of heterodimers is likely necessary before positive modulation by **2** becomes apparent. Interestingly, the presence of ERβ dampened the ERα-mediated response to 1 nM estadiol, indicating a lower transcriptional activation of the tk promoter by both ER as compared to ERα alone in the presence of

Table 1. Comparison of Relative Binding Affinities and Transcriptional Activities of Deoxybenzoins 1–11 to Those of Daidzein and Estradiol

| Compound | RBA$\alpha$[a] | RBA$\beta$[a] | RBS$\beta$[b] | TA$\alpha$[c] | TA$\beta$[c] |
|---|---|---|---|---|---|
| Estradiol | 100 | 100 | | 5.30 | 5.81 |
| 1 | 0.0209 ± 0.0018 | 0.0157 ± 0.0016 | 0.75 | 2.89 | 5.59 |
| 2 | 0.0506 ± 0.0036 | 0.2734 ± 0.0264 | 5.40 | 6.08 | 9.15 |
| 3 | 0.0189 ± 0.0032 | 0.0075 ± 0.0004 | 0.40 | ND | ND |
| 4 | 0.0522 ± 0.0046 | 0.0280 ± 0.0027 | 0.54 | 1.59 | 3.22 |
| 5 | 0.0220 ± 0.0027 | 0.0276 ± 0.0022 | 1.25 | 2.82 | 5.29 |
| 6 | 0.0367 ± 0.0029 | 0.0517 ± 0.0040 | 1.41 | 2.05 | 2.91 |
| 7 | 0.0109 ± 0.0016 | 0.0107 ± 0.0011 | 0.98 | 3.31 | 6.66 |
| 8 | 0.0697 ± 0.0051 | 0.0341 ± 0.0042 | 0.49 | 1.37 | 1.43 |
| 9 | 0.0087 ± 0.0014 | 0.0033 ± 0.0002 | 0.38 | 1.63 | 3.05 |
| 10 | 0.0022 ± 0.0003 | 0.0036 ± 0.0001 | 1.64 | ND | ND |
| 11 | 0.0556 ± 0.0085 | 0.0906 ± 0.0085 | 1.63 | 2.45 | 5.04 |
| Daidzein | 0.0309 ± 0.0029 | 0.0199 ± 0.0014 | 0.64 | 7.60 | 10.47 |

ND, not determined.

[a] RBA values for ER$\alpha$ (RBA$\alpha$) and ER$\beta$ (RBA$\beta$) are mean ± SEM of at least three independent experiments, with those of 17$\beta$-estradiol arbitrarily set equal to 100.

[b] Relative ER$\beta$ binding selectivity (RBS$\beta$) is calculated by [RBA$\beta$]/[RBA$\alpha$].

[c] Transcriptional activities in the presence of ER$\alpha$ (TA$\alpha$) or ER$\beta$ (TA$\beta$) are calculated by the following: [reporter response in the presence of ER and 10 $\mu$M test compound]/[reporter response in the presence of ER and in the absence of test compound]. The TA$\alpha$ and TA$\beta$ values of 1 nM 17$\beta$-estradiol are given for comparison.

the hormone. Notably, the experiment of Figure 2C shows that normalizing deoxybenzoin responses to the respective vehicle responses rather than to the activity of reporter alone also results in a higher response with both receptors than with either receptor alone, excluding that changes in ER constitutive activity might account for the enhancement of deoxybenzoin activation of the response upon coexpression of ER$\alpha$ and ER$\beta$. Figure 2C shows, in addition, that changes in ER constitutive activity(ies) might not account for the decreasing of the estradiol response either, further suggesting that ER heterodimerization is responsible for the opposite modulator effects of estradiol and **2**. With several deoxybenzoins exhibiting an ER$\beta$ binding selectivity and a bias toward ER$\beta$-mediated transcription comparable to **2** (Table 1), the inference is that they too could positively modulate ER transcriptional activity via ER$\beta$.

### Stimulation of Cell Proliferation

Stimulation of proliferation of breast cancer cells by estrogens is believed to depend primarily on ER$\alpha$ [18]. In light of this, deoxybenzoin stimulation of the proliferation of MCF-7 breast cancer cells, known to express much higher amounts of ER$\alpha$ than ER$\beta$, was compared to that of MDA-MB-231 breast cancer cells, which are ER$\alpha$ negative [19, 20]. Estradiol stimulation of MCF-7 cell proliferation attained maximum value (288% that of hormone-free cells) by 0.1 nM hormone, in accordance with reports that the complement of estradiol binding sites of MCF-7 cells is fully occupied by hormone concentrations ≥0.1 nM [21]. We found that $IC_{50}$ values for 50% inhibition of 0.3 nM [$^3$H]estradiol binding to MCF-7 cells by increasing concentrations of radioinert estradiol, **2**, and daidzein are 0.41 ± 0.04, 405 ± 59, and 1717 ± 177 nM, respectively, matching RBA$\alpha$ values as determined using FP. We also found that whole-cell hormone binding is fully competed at 0.003, 3, and 10 $\mu$M, respectively (Figure 3A), suggesting that 0.1 and 1 $\mu$M of **2** can effectively compete (≥50%) with postmenopausal levels of estrogen for binding to the native ER$\beta$ and ER$\alpha$, respectively. Thus, the use of **2** for hormone replacement therapy (HRT) may be envisaged.

In the absence of hormone, 0.1–10 $\mu$M **2** stimulated the proliferation of MCF-7 cells significantly, but 100 $\mu$M **2** inhibited proliferation (Figure 3B), in accordance with reports that high concentrations of phytoestrogens impact ER-independent growth-inhibitory effects [22]. By contrast, the proliferation of MDA-MB-231 cells in the presence of 10 $\mu$M **2** was not significantly higher than basal proliferation in its absence, implying that deoxybenzoin stimulation of MCF-7 cell proliferation likely depends on ER$\alpha$. All deoxybenzoins but **10** and **11** exhibited a similar profile of proliferative response. Table 2 shows that the effective concentration ($EC_{25}$) of deoxybenzoin stimulation of proliferation to a level equal to 25% that of 0.1 nM estradiol varied from 0.02 to >10 $\mu$M (column 2), and that the efficacy of the response at 10 $\mu$M deoxybenzoin was high to moderate (i.e., ≥ of 25% that of 0.1 nM estradiol) with **1–4** and **7–9** and weak (i.e., 10%–25% that of 0.1 nM estradiol) with **5** and **6**, whereas **10** and **11** inhibited rather than stimulated proliferation (column 4). Inspection of the structures of Figure 1 reveals that stimulation of proliferation is associated with $R_4$ = OH, whether derivatized or not. We wondered whether RBA$\alpha$ or RBA$\beta$ of **1–4** and **7–9** correlated with their proliferative potencies. While **2**, **4**, and **8** have RBA$\alpha$ values of ~0.05, **2** binds ER$\beta$ with ~10-fold higher affinity and is ~300 times more potent than either **4** or **8**, indicating that ER$\beta$ binding of **2** likely enhances its proliferative potency. Similarly, **1** and **3** have RBA$\alpha$ values of ~0.02, but **1** binds ER$\beta$ with ~2-fold higher affinity and is ~5 times more potent than **3**. A similar comparison of **7** and **9** further supports the notion of a dependence of the proliferative potency of deoxybenzoins on their binding of ER$\beta$. Significantly, it has been reported that estrogen stimulation of MCF-7 cell proliferation involves activation of src kinase as well as transcription of cell cycle genes by ER$\beta$ and ER$\alpha$ [23–26].

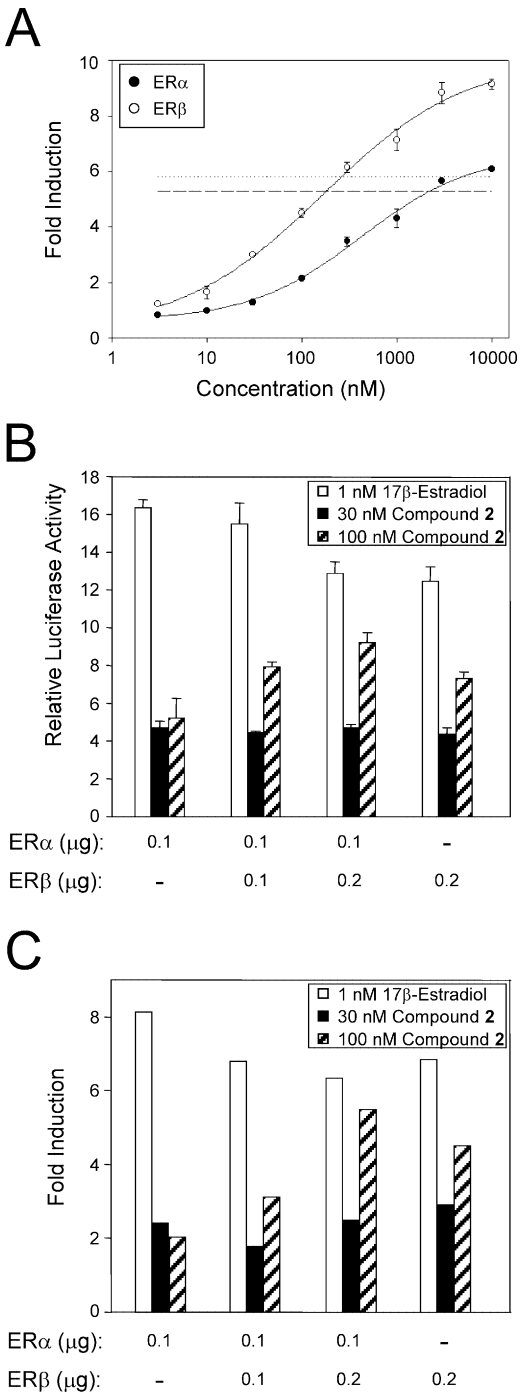



Figure 2. Deoxybenzoin Regulation of ERα- and/or ERβ-Mediated Gene Expression

(A) Induction by **2** of ERα and ERβ transcriptional activity. HEK-293 cells were transfected with an ERE-luciferase reporter and plasmids expressing human ERα (solid circles) or ERβ (open circles) and incubated with vehicle, estradiol, or increasing concentrations of **2**. Normalized levels of luciferase activity were expressed relative to those of transfected cells treated only with vehicle (set to 1). The levels of luciferase activity induced by 1 nM estradiol in cells expressing ERα (dashed line) or ERβ (dotted line) are shown for comparison.

(B and C) Coexpression of ERα and ERβ enhances the transcriptional efficacy of **2**. HEK-293 cells were transfected with the ERE-luciferase reporter and plasmids expressing human ERα and/or ERβ and incubated with estradiol or **2**, as indicated. Normalized levels of luciferase activity were expressed relative to those of cells transfected with the reporter alone and treated with vehicle (set to 1, [B]) or of transfected cells treated only with vehicle (set to 1, [C]).

### Activation of Reporter Gene Expression

To elaborate on the transcription activation potencies and efficacies of the synthesized deoxybenzoins, we used MCF-7 cells (clone D5L) stably transfected with an ERE-dependent luciferase reporter endowed with a strong (β-globin) promoter [27]. Estradiol stimulation of reporter expression attained maximum level (351% that of hormone-free cells) by 0.1 nM hormone. Table 2 shows that the effective concentration ($EC_{50}$) of deoxybenzoin stimulation of luciferase expression to a level equal to 50% that of 0.1 nM estradiol varied from 0.06 to >10 μM (column 5) and that the efficacy of the response at 10 μM deoxybenzoin was high to moderate with **1**, **2**, **5–8**, and **11** and weak with **3**, **4**, **9**, and **10** (Table 2, column 7 and Figure 3C). It is currently unclear whether the ~10-fold higher $EC_{50}$ for activation of ERα-mediated reporter response to **2** in HEK-293 cells (Figure 2A) as compared to that in MCF-7 cells reflects modulation of ERα activity by ERβ rather than the inherent difficulty of transfected ER to replicate hormonal events as staged by endogenous ER. The high constitutive activity of transfected ER points to the latter alternative as being more likely. By comparison, **1**, **2**, **7**, and **8** stimulate gene expression as well as MCF-7 cell proliferation; **3**, **4**, and **9** stimulate proliferation more than gene expression; **5**, **6**, and **11** stimulate gene expression more than proliferation; and **10** stimulates neither. Thus, unlike cell proliferation, F, $NO_2$, or H substitution for 4′-OH is not detrimental to gene expression, indicative of different determinants guiding deoxybenzoin stimulation of these responses. Notably, the correlation between induction efficacies at 10 μM deoxybenzoin and the TAα values of Table 1 ($r = 0.812$, $p = 0.008$) gets stronger when **2** is excluded ($r = 0.889$, $p = 0.003$), and the trend is maintained when the second most ERβ-selective and transcriptionally biased deoxybenzoin (**11**) is excluded also ($r = 0.901$, $p = 0.006$), which may be taken to suggest that ERβ binding selectivity and transcriptional bias of **2** and **11** interfere with ERα being the sole determinant of the gene expression response in their presence.

ER-mediated activation of transcription from ERE-dependent promoters depends on two transcription activation functions (AF), AF1 and AF2 [3]. While the AF2 of ERα and ERβ are of similarly high strength, AF1 of ERα is much more potent than ERβ, resulting in a higher activation of transcription from ERE-dependent promoters [26]. We have shown above that the ER-dependent reporter response to **2** is positively modulated by ERβ; the opposite is true for the response to estradiol. Pettersson et al. [28] have reported that ERβ exercised a similar modulation of their reporter responses to estradiol and genistein, a phytoestrogen of much higher selectivity for ERβ (RBAβ = 43.9, RBSβ = 51) than **2**. Since ERα and ERβ colocalize in nuclear clusters housing chromatin remodeling components [29], they could form

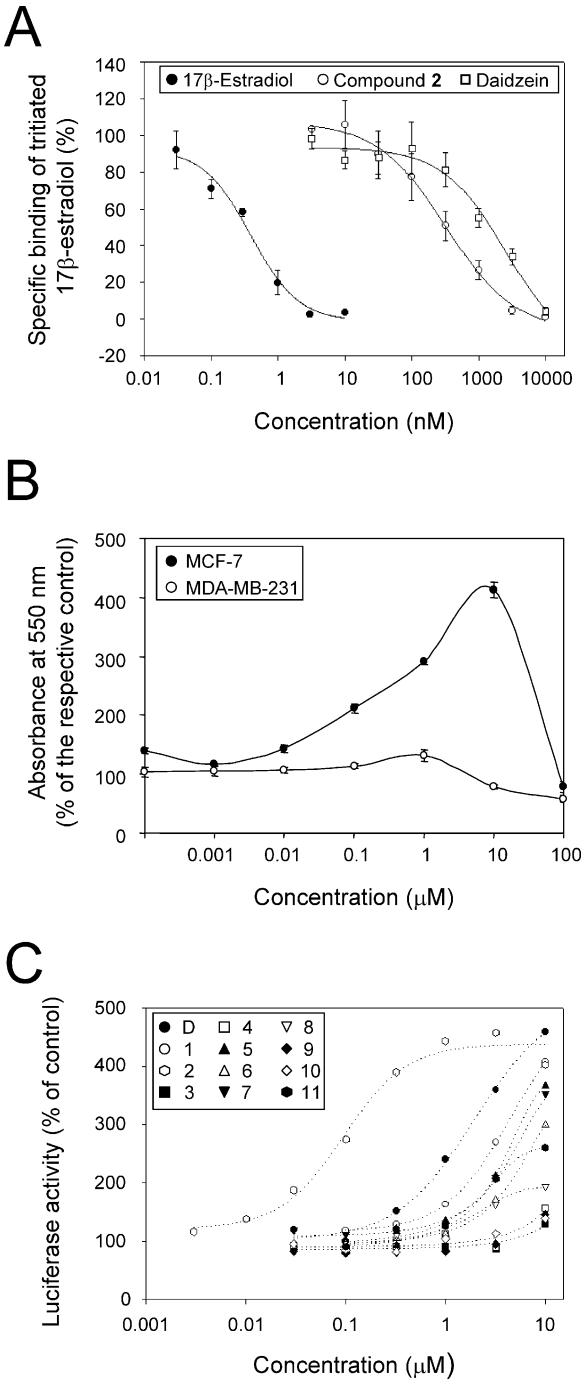



Figure 3. Biological Responses versus Deoxybenzoin Binding of Native ER

(A) Displacement of tritiated estradiol from the native ER of intact MCF-7 cells by increasing concentrations of radioinert estradiol, **2**, and daidzein, as indicated

(B) Percent stimulation of the basal (estrogen-free) proliferation of MCF-7 (ERα-positive) and MDA-MB-231 (ERα-negative) cells in the presence of increasing concentrations of **2**, as obtained using the MTT assay.

(C) Induction of luciferase expression in MCF-7:D5L cells using increasing concentrations of **1–11**, as indicated.

transcriptionally active heterodimers. These could sensitize the AF-1-mediated activation of the gene expression program of MCF-7 cells to the presence of ERβ-selective and transcriptionally biased deoxybenzoins.

### Induction of Alkaline Phosphatase Activity

To ensure that activity is not restricted to breast cancer cells, we tested deoxybenzoin estrogenicities using Ishikawa endometrial adenocarcinoma cells. It has been reported that hormonal induction of alkaline phosphatase (AlkP) activity in these cells is estrogen specific, with androgens, progestins, glucocorticoids, and mineralocorticoids exhibiting negligible activity [30], and that induction by phytoestrogens is an indicator of the intrinsic estrogenic activity of these compounds [31]. As already observed with the other responses, estradiol stimulation of AlkP expression attained maximum level (414% that of hormone-free cells) by 0.1 nM hormone. Table 2 shows that the effective concentration ($EC_{25}$) of deoxybenzoin stimulation of AlkP expression to a level equal to 25% that of 0.1 nM estradiol varied from 0.06 to >10 μM (column 8), and that the efficacy of the response at 10 μM deoxybenzoin was high to moderate with **1**, **2**, **5**, **7**, and **11** and weak or marginal with **3**, **4**, **6**, and **8–10** (column 10). In fact, **1**, **2** and **7** stimulate AlkP expression as well as MCF-7 cell proliferation; **3**, **4**, **8**, and **9** stimulate proliferation more than AlkP expression; **5** and **11** stimulate AlkP expression but either fail to stimulate proliferation (**5**) or inhibit it (**11**); and **6** and **10** stimulate neither. Thus, deoxybenzoins that potently transactivate the luciferase reporter [rank order of potencies (**2** > **1** ≥ **5** ≥ **7** ≥ **6** ≥ **11**)] stimulate AlkP expression as well (**2** > **5** ≥ **7** ≥ **1** ≥ **11**). Yet, **5** and **11** cannot stimulate the proliferation of breast cancer cells, which makes them a potentially safe choice for HRT.

We next looked for structural features that could account for the differences in activity described above. Whether gene-transactivation or cell-proliferation wise, substitutions in positions R1 and R2 lead to a decrease in both RBAβ and estrogenic activity (compare **7** with R1 = Cl and **4** with R2 = OH to **2** with R1 = H and R2 = H). The presence of 4′-OH is associated with higher proliferative activity as well as RBA (compare **5** with R4 = F to **2** with R4 = OH). However, derivatization of 4′-OH lowers both RBA and estrogenic activity (compare **1** to **2**, **3** to **4**, and **7** to **9**), and this is also the case following exchange of 4′-OH for larger substitutes (compare **10** and **6** to **2**). In fact, **10** seems to be totally inactive in all three assays (Table 2). Interestingly, substitution of F (but not Br) for 4′-OH produced a deoxybenzoin (**5**) relatively active with respect to gene expression but proliferatively inert, whereas presence of 3′-Br in conjunction with lack of 4′-OH produced a deoxybenzoin (**11**) somewhat less potent than **5** gene-expression wise, yet clearly inhibitory cell-proliferation wise.

### Models of Deoxybenzoin Binding of ERβ

#### *Conformational Analysis*

Hormonal activity of steroidal and nonsteroidal estrogens is associated with H bonding ability of hydroxyl groups mimicking the 3-OH and the 17β-OH of estradiol, an O-O distance between these groups in the range

Table 2. Biological Responses to the Deoxybenzoins 1–11, Daidzein, and Estradiol

| | MCF-7 Proliferation | | | Reporter Gene Expression | | | Alkaline Phosphatase Activity | | |
|---|---|---|---|---|---|---|---|---|---|
| Compound | EC25[a] (μM) | Relative Potency[b] | Efficacy[c] | EC25[a] (μM) | Relative Potency[b] | Efficacy[c] | EC25[a] (μM) | Relative Potency[b] | Efficacy[c] |
| 1 | 0.91 ± 0.05 | 0.0007 | 197 ± 7 | 3.42 ± 0.17 | 0.0008 | 407 ± 47 | 3.83 ± 0.71 | 0.0005 | 179 ± 16 |
| 2 | 0.02 ± 0.01 | 0.0399 | 413 ± 14 | 0.06 ± 0.01 | 0.0461 | 443 ± 49 | 0.06 ± 0.01 | 0.0331 | 319 ± 51 |
| 3 | 4.96 ± 1.40 | 0.0001 | 178 ± 4 | >10 | <0.0003 | 130 ± 23 | >10 | <0.0002 | 116 ± 17 |
| 4 | 4.94 ± 1.39 | 0.0001 | 186 ± 7 | >10 | <0.0003 | 157 ± 12 | >10 | <0.0002 | 141 ± 17 |
| 5 | >10 | <0.0001 | 129 ± 3 | 3.62 ± 1.16 | 0.0008 | 367 ± 60 | 2.13 ± 0.86 | 0.0009 | 225 ± 16 |
| 6 | >10 | <0.0001 | 123 ± 11 | 8.54 ± 0.86 | 0.0003 | 298 ± 37 | 8.25 ± 1.75 | 0.0002 | 157 ± 10 |
| 7 | 1.06 ± 0.47 | 0.0006 | 258 ± 5 | 5.33 ± 1.05 | 0.0005 | 353 ± 46 | 2.46 ± 0.22 | 0.0008 | 249 ± 18 |
| 8 | 6.75 ± 2.40 | 0.0001 | 166 ± 10 | >10 | <0.0003 | 194 ± 9 | >10 | <0.0002 | 116 ± 8 |
| 9 | 6.19 ± 3.14 | 0.0001 | 170 ± 12 | >10 | <0.0003 | 146 ± 32 | >10 | <0.0002 | 132 ± 6 |
| 10 | NA | | 95 ± 7 | >10 | <0.0003 | 139 ± 58 | 7.51 ± 0.95 | 0.0002 | 104 ± 12 |
| 11 | NA | | 75 ± 9 | 9.52 ± 0.39 | 0.0003 | 260 ± 45 | 5.32 ± 1.80 | 0.0004 | 180 ± 18 |
| Daidzein | 0.04 ± 0.03 | 0.0157 | 346 ± 13 | 0.75 ± 0.15 | 0.0037 | 459 ± 17 | 0.33 ± 0.10 | 0.0058 | 290 ± 25 |
| Estradiol | 6.40* ± 4.39 | 100 | 288 ± 62 | 27.67* ± 9.24 | 100 | 351 ± 14 | 18.77* ± 1.99 | 100 | 414 ± 67 |

NA, not applicable; *, pM.

[a] $EC_{25}$ and $EC_{50}$ values are test compound concentrations required to achieve 25% and 50%, respectively, of the effect of 0.1 nM estradiol. Values are mean ± SEM of at least three independent experiments.

[b] Relative potency was calculated by the following: [100 × $EC_{25}$ (or $EC_{50}$) of estradiol]/[$EC_{25}$ (or $EC_{50}$) of test compound].

[c] All compounds were tested at 10 μM. Estradiol was tested at 0.1 nM. Efficacies (% of control, mean ± SEM of three independent experiments) indicate absorbance of MTT-formazan in MCF-7 cells (cell proliferation), luciferase activity in MCF-7:D5L cells (reporter gene expression), and alkaline phosphatase activity in Ishikawa cells.

10.9–12.5 Å, and hydrophobic centers mimicking steric 7α and 11β substituents. Conformational analysis of **1–11** using molecular mechanics calculations [32] revealed that each can adopt three low energy conformations stabilized by the formation of a hydrogen bond between 2-OH and the carbonyl oxygen (Figure 4A). Conformers I and III are bended and further stabilized through the hydrophobic interaction of the phenyl rings, while conformer II, with a relative energy of ~0.5 kcal/mol compared to the global minimum conformer I, exhibits an extended shape comparable to daidzein and genistein. Conformer II of **1–4** and **7–9** has a O-O distance between the 4 and 4′-OH groups of 12.0 Å, in agreement with one of the criteria for optimum interaction with ER.

### *Alignment*

To deduce structure-activity relationships, docking calculations were performed and followed by correlation between the experimental RBA values and the calculated binding energy $E_{bind}$. Since the initial orientation of the ligand inside the binding pocket is critical, preliminary calculations were performed to deduce the initial alignments using the published crystal structure of genistein bound to the LBD of ERβ [8]. In this structure, 4′-OH interacts with the side chains of Glu305, Arg346, and a buried water molecule mimicking the A-ring of estradiol, whereas the 7-OH group of the flavone core is H bonded to His475 at the distal end of the binding cavity (see Figure 5A). In addition, the keto- and 5-OH groups are buried toward the α-face pocket (roughly the 7α position of estradiol). Deoxybenzoins **1**, **3**, **5**, **6**, and **9–11** (lacking a 4′-OH) were positioned in the ERβ-genistein structure fitting the phenyl ring of genistein with 4-OH. Energy minimization calculations were performed by rotating the molecule around the O-O axis 180° inside the binding pocket. In all cases, in the minimum energy structure the keto- and the 2-OH groups were oriented to the α-face of the cavity. Since **2**, **4**, **7**, and **8** bear two hydroxyl groups that could play a role analogous to the OH of the A-ring of estradiol, geometry optimizations were performed, allowing 4-OH to interact with Glu305 and Arg346, the 4′-OH to interact with His475 and vice versa, and rotating around the O-O

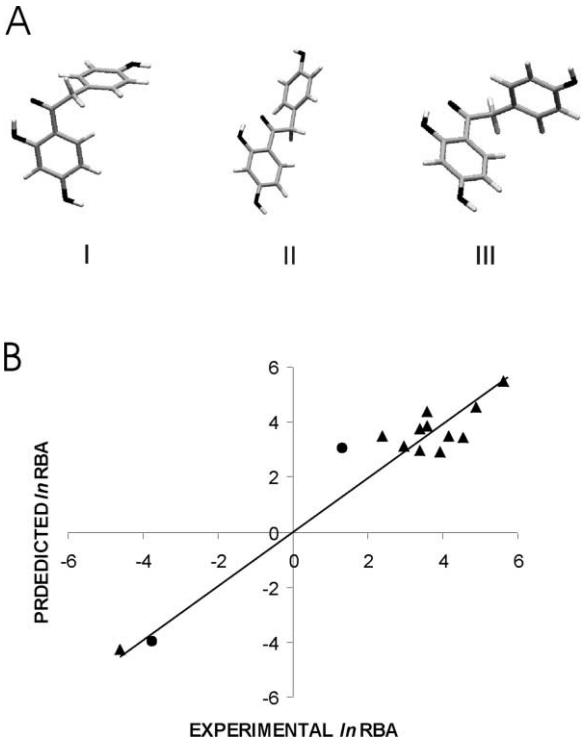



Figure 4. Low-Energy Conformers of Deoxybenzoin 2 and ER Binding Affinity Predictions

(A) The three low energy conformers of **2** as deduced using Monte Carlo MM2 conformational search.

(B) Plot of experimental *ln*RBAs versus predicted *ln*RBAs. Triangles represent the training set, and circles represent the test set.

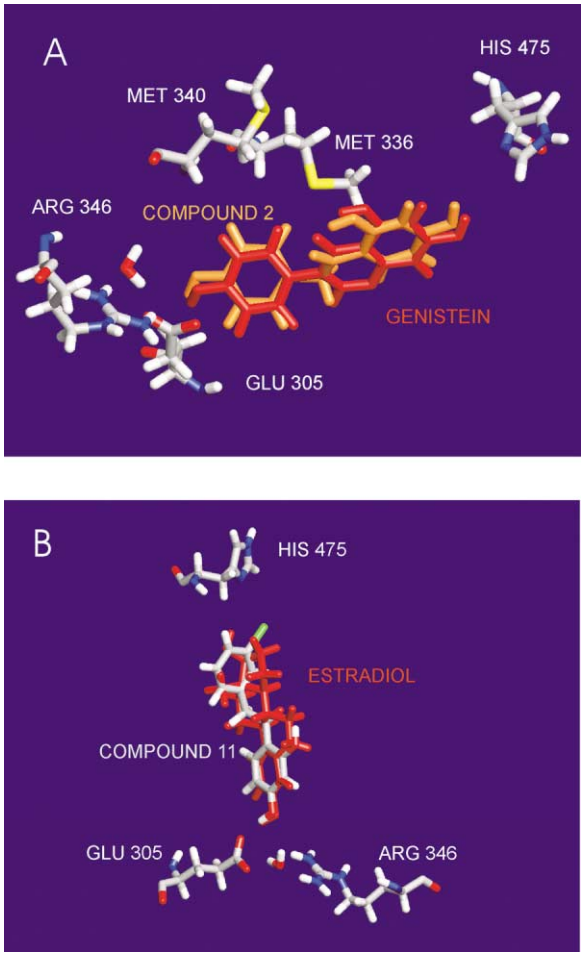



Figure 5. Comparative Molecular Modeling of Deoxybenzoin Binding of ERβ

(A) Superposition of Genistein and **2** in the ER binding site as deduced using PrGen calculations. Met336 and Met340 lie above and very close to keto and OH groups of the ligand.

(B) Superposition of estradiol and **11** in the ER binding site. The position Br atom of **11** is close to the position of 16α− substituent of estradiol.

axis 180°. The minimum energy structures thus obtained were subjected to docking calculations which revealed that **2**, **7**, and **8** prefer to bind similar to genistein, with the keto- and 2-OH groups in similar positions to the corresponding groups of genistein, while **4** adopts a similar position to **1**, **3**, **5**, **6**, and **9–11**.

***Scoring***

To produce a model relating calculated energy terms to binding affinities, the molecules were aligned as above, positioned in the ERβ cavity, and subjected to docking-scoring iterative calculations using PrGen 2.0 software. Theoretical binding affinities are estimated by evaluating ligand-receptor interaction energies, ligand desolvation energies, and changes in both ligand-internal energy and ligand-internal entropy upon receptor binding: $E_{binding} \approx E_{ligand\text{-}receptor} - T\Delta S_{binding} - \Delta G_{solvation,ligand} + \Delta E_{internal,ligand}$ [33]. Binding affinities are then obtained by linear regression between *ln*RBA and $E_{binding}$. A training set of 12 molecules was used, including estradiol and daidzein, while keeping genistein and compound **2** for the test set. The deduced correlation between experimental and predicted *ln*RBA (Figure 4B) has a total correlation coefficient of 0.932 ($r^2$) with an RMS prediction error of 0.68. Genistein and **2** were then positioned within the fixed receptor cavity and "Monte Carlo minimized" to yield a satisfactory correlation between experimental and theoretical values for the test molecules (Figure 4B), suggesting that the model is apt to predict relatively high and low RBA values.

According to the deduced model, genistein, daidzein, **2**, **7**, and **8** are similarly positioned, and **2**, in particular, superimposes to genistein perfectly (Figure 5A). Daidzein also superimposes to genistein, but the lack of 5-OH seems to hamper its ERβ binding selectivity. As already reported for genistein [8], the ERβ binding selectivity of **2** could be due to an interaction with Met336 (Leu384 in ERα), one of the two cavity-lining residues that differ between ERα and ERβ. Met336 and Met340 interact through a hydrogen bond between backbone NH and CO, and both lie very close above the keto and the 2-OH groups of **2** (Figure 5A). The sulfur atom of both residues, bearing a weak polar character, could interact with these two groups, thus contributing to the selectivity toward ERβ. While **7** and **8** also bind like genistein, they bear a Cl atom toward a hydrophobic pocket very close to Val484, the first residue of helix 12, which possibly explains their lower activity compared to **2**. Deoxybenzoins **1**, **3–6**, and **9–11** are oriented in the cavity in the opposite direction compared to genistein, with the two aromatic rings perpendicular to each other, thus deviating from the planar shape of genistein (Figure 5B). In this orientation, the 3′-Br atom of **11** is found in the same position like 16α-H of estradiol. In estradiol, Br substitution for 16a H is very well tolerated (similar RBA) [35], which is in accordance with the relatively high RBAβ of **11**.

In light of the above, it appears that a deoxybenzoin orientation that provides for H bonding of His475 (His 524 in ERα) with 4-OH is necessary for activation of cell proliferation as well as gene expression to a level comparable or even higher than that of estradiol (e.g., **2**); that derivatization of 4′-OH is more detrimental to stimulation of cell proliferation than gene expression (e.g., **1**); and that deoxybenzoins lacking a 4′ O atom but possessing appropriate 3′ substituents (**5**, **11**) may inhibit or fail to promote cell proliferation while maintaining most of the efficacy of **1** and **2** to activate gene expression, albeit with reduced potency. On the whole, it appears that the proliferative activity of **1–4** and **7–9** should be correlated with the presence of two O atoms being able to meet one of the structural requirements for estrogenicity. Since **1** and **2** bear two OH atoms at a distance of 12 Å and the 4′-OH of **1** is methylated, they likely contact His475 as H-bond acceptor and donor, respectively. It has been reported, however, that ligand H bonding of an acceptor His475 begins a H-bond network that involves Glu371, extends to the C terminal of helix 11, and stabilizes the agonist position of helix 12; and that disruption of this network lowers the gene expression potency and efficacy of ER [34]. However, our data suggest that it is deoxybenzoin activation of cell proliferation rather than gene expression that is primarily affected by disruption of the H-bond network, and that

one can synthesize ERβ-selective antiproliferative deoxybenzoins lacking 4′-OH, which nevertheless can effectively activate gene expression (e.g., **11**). Van der Waals contacts of appropriate 3′ substituents (e.g., Br) with Ile373 (Met421 in ERα) [8], the other cavity-lining molecule that differs between ERα and ERβ, may be instrumental for the maintenance of gene expression activity of **11**. Thus, it appears that certain deoxybenzoins can replicate the activity of established selective ER modulators (SERMs) such as tamoxifen, and subtle structural alterations of the deoxybenzoin scaffold can give rise to receptor-ligand complexes of such a topology and interaction with coregulators that could provide for the dissociation of the proliferative and gene expression responses of breast cancer cells to estradiol [36].

## Significance

**While HRT of postmenopausal syndromes is associated with a higher risk of developing breast and/or endometrial cancer, consumption of dietary estrogens is thought to have the opposite effect [1, 2]. However, the exact mechanisms underlying the complex estrogenic/antiestrogenic behavior of most phytoestrogens remain a matter of conjecture. It is believed that the beneficial effects associated with consumption of phytoestrogens reflect their preferential activation of ERβ function(s) [5]. The present report shows that 0.1–3 μM 2 can effectively compete with postmenopausal estrogen for binding to the native ER and that the ERβ binding selectivity and transcriptional bias of 2 is accompanied by higher activation of transcription of estrogen target genes compared to estradiol, which makes 2 potentially useful for HRT. It is known, however, that the proliferation of breast cancer cells is driven predominantly by ERα, the number of ERα-positive proliferating cells of the human breast increases with advancing age [37], and that, while the level of ERβ decreases in preinvasive mammary tumors, that of ERα increases instead [38]. In this light, a HRT based on 2 could increase breast cancer risk. Interestingly, from the subset of ERβ-selective and transcriptionally competent deoxybenzoins lacking a 4′-OH, one (11) can activate gene expression and inhibit cell proliferation at the same time. Such deoxybenzoins are a potentially safe choice for HRT, especially if their RBAβ were to improve while their selectivity and transcriptional bias toward ERβ is maintained compared to 11.**

### Experimental Procedures

NMR spectra were recorded on Bruker DRX 400 and Bruker AC 200 spectrometers [$^1$H (400 and 200 MHz) and $^{13}$C (50 MHz)]; chemical shifts are expressed in ppm downfield from TMS. The $^1$H-$^1$H and the $^1$H-$^{13}$C NMR experiments were performed using standard Bruker microprograms.

### Preparation of Deoxybenzoins

A phenol (0.050 mol) and an arylacetic acid (0.050 mol) were dissolved into freshly distilled $BF_3{\cdot}Et_2O$ under argon. The mixture was stirred at 80°C and then poured in an ice bath. The resulting mixture was extracted with ethyl acetate, and the organic layer was washed with aq. $NaHCO_3$, dried ($Na_2SO_4$), and evaporated. The residue was purified by column chromatography on silica gel, using a mixture of dichloromethane and methanol. All compounds were identified by NMR ($^1$H-NMR, $^{13}$C-NMR, HMBC, HMQC, COSY-LR) and MS-CI spectroscopy.

***2,4-Dihydroxy-4′-Nitrodeoxybenzoin [2-(4-Nitrophenyl)-1-(2,4-Dihydroxyphenyl)Ethanone] 6***

$^1$H NMR (MeOD, 400 MHz) δ: 8.17 (2H, d, $J$ = 8.0 Hz, H-3′/H-5′), 7.85 (1H, d, $J$ = 8 Hz, H-6), 7.51 (2H, d, $J$ = 8.0 Hz, H-2′/H-6′), 6.38 (1H, dd, $J$ = 8.0/1.5 Hz, H-5), 6.27 (1H, d, $J$ = 1.5 Hz, H-3), 4.42 (2H, s, $CH_2$). $^{13}$C NMR (MeOD, 50 MHz) δ: 202.5 (CO), 166.8 (C-4/C-2), 148.5 (C-4′),144.2 (C-1′), 134.6 (C-6), 132.4 (C-2′/C-6′), 124.2 (C-3′/C-5′), 114.0 (C-1), 110.0 (C-5), 104.2 (C-3), 46.8 ($CH_2$). MS-CI, $m/z$ 274 $(M+H)^+$.

***5-Cloro-2,3,4′,4′-Tetrahydroxy-Deoxybenzoin [1-(5-Cloro-2,4-Dihydroxyphenyl)-2-(3,4-Dihydroxyphenyl)Ethanone] 8***

$^1$H NMR ($Me_2CO$-d6, 400 MHz) δ: 7.82, (1H, s, H-6), 6.73 (1H, d, $J$ = 8.0 Hz, H-5′), 6.73 (1H, d, $J$ = 1.5 Hz H-2′), 6.60 (2H, dd, $J$ = 8.0/1.5 Hz, H-6′), 6.40 (1H, s, H-3), 4.02 (2H, s, $CH_2$). $^{13}$C NMR ($CDCl_3$, 50 MHz) δ:). 204.1 (CO), 165.9 (C-4), 162.3 (C-2), 148.2 (C-3′),146.7 (C-4′), 134.3 (C-6), 128.2.0 (C-1′), 123.7 (C-6′), 118.3 (C-2′),118.3 (C-5′), 114.5 (C-5), 114.5 (C-1), 105.7 (C-3), 47.1 ($CH_2$). MS-CI, $m/z$ 295 $(M+H)^+$.

***5-Cloro-2,4-Dihydroxy-4′-Ethoxydeoxybenzoin [1-(5-Cloro-2,4-Dihydroxyphenyl)-2-(4-Ethoxyphenyl)Ethanone] 9***

$^1$H NMR ($Me_2CO$-d6, 400 MHz) δ: 7.82, (1H, s, H-6), 7.06 (2H, d, $J$ = 8.0 Hz, H-2′/H-6′), 6.78 (2H, d, $J$ = 8.0 Hz, H-3′/H-5′), 6.38 (1H, s, H-3), 4.02 (2H, s, $CH_2$), 3.91 (2H, q, $C\underline{H}_2CH_3$), 1.32 (3H, t, $CH_3$). $^{13}$C NMR ($CDCl_3$, 50 MHz) δ: 203.9 (CO), 164.0 (C-4), 162.1 (C-2), 159.9 (C-4′), 134.3 (C-6), 132.0 (C-2′/C-6′), 127.9 (C-1′), 116.3 (C-3′/C-5′), 113.9 (C-1), 113.9 (C-5), 105.2 (C-3), 64.3 ($\underline{C}H_2CH_3$), 44.9 ($CH_2$), 15.8 ($CH_2\underline{C}H_3$). MS-CI, $m/z$ 307 $(M+H)^+$.

***4′-Bromo-2,4-Dihydroxydeoxybenzoin [2-(4-Bromophenyl)-1-(2,4-Dihydroxyphenyl)Ethanone] 10***

$^1$H NMR (MeOD, 400 MHz) δ: 7.79 (1H, d, $J$ = 8 Hz, H-6), 7.42 (2H, d, $J$ = 8.0 Hz, H-3′/H-5′), 7.14 (2H, d, $J$ = 8.0 Hz, H-2′/H-6′), 6.37 (1H, dd, $J$ = 8.0/1.5 Hz, H-5), 6.27 (1H, d, $J$ = 1.5 Hz, H-3), 4.13 (2H, s, $CH_2$). $^{13}$C NMR (MeOD, 50 MHz) δ: 203.7 (CO), 167.0 (C-4/C-2), 135.8 (C-1′),134.6 (C-6), 133.0 (C-2′/C-6′), 133.0 (C-3′/C-5′), 121.7 (C-4′), 113.9 (C-1), 108.9 (C-5), 104.3 (C-3), 45.7 ($CH_2$). MS-CI, $m/z$ 308 $(M+H)^+$.

***3′-Bromo-2,4-Dihydroxydeoxybenzoin [2-(3-Bromophenyl)-1-(2,4-Dihydroxyphenyl)Ethanone] 11***

$^1$H NMR (MeOD, 400 MHz) δ: 7.79 (1H, d, $J$ = 8 Hz, H-6), 7.47 (1H, t, $J$ = 1.5 Hz, H-2′), 7.40 (2H, dt, 7.14 $J$ = 8.0/1.5 Hz, H-4′), 7.27-7.16 (2H, m, H-5′/H-6′), 6.37 (1H, dd, $J$ = 8.0/1.5 Hz, H-5), 6.27 (1H, d, $J$ = 1.5 Hz, H-3), 4.13 (2H, s, $CH_2$). $^{13}$C NMR (MeOD, 50 MHz) δ: 210.6 (CO), 167.3 (C-4), 167.2 (C-2), 139.5 (C-1′),134.7 (C-6), 134.1 (C-2′), 131.8 (C-4′), 131.8 (C-4′), 131.4 (C-6′), 130.0 (C-5′), 113.9 (C-1), 109.8 (C-5), 104.2 (C-3), 45.3 ($CH_2$). MS-CI, $m/z$ 308 $(M+H)^+$.

***Daidzein, 7-Hydroxy-3-(4-Hydroxyphenyl)-Chromen-4-One***

In a solution of **2** (2 mmol) in DMF (6 ml), freshly distilled $BF_3{\cdot}Et_2O$ (6.3 ml) was added under argon. The mixture was heated at 50°C, and a solution of methanesulphonyl chloride (1 ml) in dry DMF (1.5 ml) was added slowly. After reaction at 80°C for 1 hr, the mixture was cooled to room temperature and poured into a large volume of ice-cold aq. sodium acetate (12 g/100 ml), then extracted with ethyl acetate, and the organic layer was dried and concentrated. The residue was purified by column chromatography on silica gel using a mixture of dichloromethane and methanol. For $^1$H NMR, see [39].

### ER Binding Affinities

These were determined using a fluorescent ER ligand (ES2) and recombinant ERα or ERβ with a Beacon 2000 Fluorescence Polarization (FP) System (PanVera) as described by Bolger et al. [17]. Test compound competition of 1 nM ES2 binding to ER obeys the following scheme: Test + ER-ES2 (high FP) → ER-Test + ES2 (low FP). $IC_{50}$ values for estradiol thus determined using ERα and ERβ were 1.24 ± 0.07 and 1.45 ± 0.18 nM, respectively. Deoxybenzoin competition of ES2 binding to ER gave $IC_{50}$ estimates from which RBA values were calculated by [($IC_{50}$ estradiol/$IC_{50}$ competitor) × 100]. FP values for ER-bound ES2 in the absence of competitor and of both ER and competitor served as negative and positive displacement controls, respectively. ES2 displacements were curve fitted using SigmaPlot 4.0 (SPSS Inc).

### Stimulation of Cell Proliferation

MCF-7 and MDA-MB-231 human mammary adenocarcinoma cells (ATCC, Rockville, MD) were cultured at 37°C in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Biochrom KG, Berlin) in 5% $CO_2$ and subcultured using a trypsin 0.25%–EDTA 0.02% solution. To assess cell proliferation, a modification of the MTT assay was used [40]. Briefly, cells were plated in 96-well flat-bottomed microplates at a density of 10,000 cells/well in phenol-red-free medium supplemented with 1% dextran-coated-charcoal-pretreated FBS (DCC-FBS) [41]. Twenty-four hours later, serial dilutions of test compounds were added (initial dilution in DMSO, further dilutions in culture medium), fresh media with test compounds was added every 48 hr, and after 6 days the medium was removed and the cells were incubated with 1 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) in serum-free, phenol-red-free medium for 4 hr. The MTT-formazan produced was solubilized in isopropanol, and absorbance at 550 nm versus 690 nm was measured. Cells that received only medium served as baseline controls, while those treated with estradiol (Sigma) served as positive controls. The difference in the responses of MCF-7 and MDA-MB-231 cells was taken to measure estrogenic activity.

### Transactivation of Reporter Gene Expression

HEK-293 cells were maintained and transfected as described by Pettersson et al. [28], with minor modifications. In brief, cells were seeded in 6 cm dishes and transfected with the calcium phosphate coprecipitation method using 1.5 μg of pERE-tk-Luc reporter plasmid, 1 μg of internal control plasmid pCMX-Gal, 0.1 μg of pSG5-hERα or pGS5-hERβ expression plasmids alone, or 0.1 μg of pSG5-hERα together with 0.1 or 0.2 μg of pSG5-hERβ, as indicated. Sixteen to eighteen hours after transfection, cells were washed with phosphate buffered saline (PBS) and incubated in fresh medium with or without 1 nM estradiol, the indicated deoxybenzoin concentrations, or vehicle (0.1% DMSO) for 24 hr. Luciferase activities were normalized relative to β-galactosidase expression levels. Plasmids pERE-tk-Luc, pSG5-hERα, and pSG5-hERβ have been described [26].

MCF-7 cells were transfected with the luciferase reporter plasmid ERE-β Glob-Luc [27] and neomycin resistance plasmid pWL2neo (Stratagene) using standard methodology. Transfectants were selected using geneticin (Stratagene). The geneticin-resistant clone D5L of MCF-7 cells, selected for its prominent induction of reporter expression by estradiol and low basal expression in its absence, as assessed using the Steady-Glo Luciferase Assay System (Promega), was cultured as described above for MCF-7 cells. To assess luciferase induction by deoxybenzoins, MCF-7:D5L cells were plated in 96-well flat-bottomed microplates at a density of 10,000 cells/well in phenol-red-free MEM supplemented with 5% DCC-FBS; 72 hr later, fresh medium was added to the cells followed by test compounds (initial dilution in DMSO, further dilutions in culture medium). Luciferase activity was assayed 16 hr later using a Galaxy 1258 plate reader (BioOrbit). Cells that received only medium served as baseline expression controls.

### Whole-Cell ER Binding Assay

The assay was carried out as described by Lascombe et al. [21], with minor modifications. MCF-7 cells were seeded in 24-well flat-bottomed microculture plates at a density of 200,000 cells per well, supplied with 1 ml of MEM, and cultured for 72 hr at 37°C. The cells were then washed twice with PBS, 500 μl of reaction buffer (0.1% bovine serum albumin, 0.3 nM [$^3$H]-estradiol, and 1% test compound in MEM containing 0.5% DCC-FBS) was added, and the cells were incubated for 1 hr at 37°C, washed twice with 5 mM $PO_4^{3-}$ containing 0.25 M sucrose and 10% glucerol, and finally treated with 100 μl/well absolute ethanol to extract the hormone. Extracts were counted using a Packard Tri-Carb 4640 spectrometer.

### Induction of AlkP Activity

Estrogen-responsive Ishikawa cells (ECACC) were cultured/subcultured as described by the supplier. Cells were plated in 96-well flat-bottomed microculture plates at a density of 12,000 cells per well in phenol-red-free medium supplemented with 5% DCC-FBS, and 24 hr later fresh medium was added followed by test compounds (initial dilution in DMSO, further dilutions in culture medium). Cells were cultured for 72 hr. To assay AlkP activity [30, 31], the cells were washed with PBS, the plates were inverted, blotted gently on a paper towel, placed at −80°C for at least 15 min, thawed at room temperature for 5–10 min, and then transferred on ice. Next, 50 μl ice-cold solution containing 5 mM p-nitrophenyl phosphate, 0.24 mM $MgCl_2$, and 1 M diethanolamine (pH 9.8) was added, the cells were warmed to room temperature (time zero), and yellow-colored p-nitrophenol was allowed to accumulate. Cells that received only medium served as negative controls, while cultures treated with 0.1 nM estradiol served as positive controls. The color was monitored every 30 min at 405 nm using the Galaxy 1258 plate reader until positive controls showed an absorbance ($A_{405}$) of about 1.2.

### Molecular Modeling

The calculations were performed using the crystal structure of ERβ-LBD with genistein, taking into account all of the amino acids within 12 Å from the ligand and the crystallographic water molecule among genistein, GLU305, and ARG346. The structure includes residues from the adjacent subunit, some of which are known to affect binding [8]. His475 was protonated in nitrogen $N_{\varepsilon 2}$.

The 14 ligand molecules (training and test set) were designed and energy minimized (MM2) using MACROMODEL 6.5 software [32]. The full search in the conformational space was achieved using MM2 force field-Monte Carlo multiple minimum method (MCMM) as implemented in MACROMODEL 6.5. One thousand starting conformers were produced and minimized using the TNCG algorithm (rmsG $<$ 0.01 kJ/mol·Å). For the preliminary minimizations of all ligands inside the binding pocket, the AMBER* united atom force field was used with a distance-dependent dielectric "constant" of 4*r* as implemented in MACROMODEL 6.5. All calculations with MACROMODEL 6.5 software were run on a Silicon Graphics O2 R5000.

For *ln*RBA prediction, the PrGen 2.0 program was used. At the beginning, the crystal structure of ERβ-LBD with genistein was minimized over 100 steps using PrGen. All molecules were then superimposed over the crystallographic position of genistein as described. Partial atomic charges were attributed using MOPAC 7 (MNDO hamiltonian with NOMM correction) [42]. Solvation energies, entropy corrections, and ligand reference energies were calculated for all ligands after individual Monte Carlo minimization using specific built-in PrGen 2.0 modules. To determine ligand-receptor interaction energy, $E_{\text{ligand-receptor}}$, the program uses the force field Yeti [43]. Binding affinities are obtained by linear regression between *ln*RBA and $E_{\text{binding}}$. All calculations with PrGen 2.0 were run on a Silicon Graphics INDY R4500.

### Statistics

Data were analyzed using the SPSS 10.0 statistical package for Windows and compared using one-way ANOVA with a Tukey Post Hoc test for multiple comparisons. Differences were considered statistically significant for values of $p < 0.05$. The Pearson's correlation coefficient (r) was used to analyze statistical associations.

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