# Subtle Side-Chain Modifications of the Hop Phytoestrogen 8-Prenylnaringenin Result in Distinct Agonist/Antagonist Activity Profiles for Estrogen Receptors α and β

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In search of therapeutic agents for estrogen-related pathologies, phytoestrogens are being extensively explored. In contrast to naringenin, 8-prenylnaringenin is a potent hop-derived estrogenic compound, highlighting the importance of the prenyl group for hormonal activity. We investigated the effects of substituting the prenyl group at C(8) with alkyl chains of varying lengths and branching patterns on estrogen receptor (ER) subtype ERα- and ERβ-binding affinities and transcriptional activities. In addition, features of the ligand-induced receptor conformations were explored using a set of specific ER-binding peptides. The new 8-alkylnaringenins were found to span an activity spectrum ranging from full agonism to partial agonism to antagonism. Most strikingly, 8-(2,2-dimethylpropyl)naringenin exhibited full agonist character on ERα, but pronounced antagonist character on ERβ. Knowledge on how ER-subtype-selective activities can be designed provides valuable information for future drug or tool compound discovery.

## Introduction

The two estrogen receptors ERα[a] [1] and ERβ[2] belong to the nuclear receptor (NR) superfamily and are ligand-regulated, gene-specific transcription factors that account for mediating the physiological effects of the steroid hormone 17β-estradiol (E2). ERα is an established and ERβ is a candidate target for the development of synthetic ligands for therapeutic applications.[3,4] Selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, display a tissue-selective activity profile, thereby mimicking the action of estrogen in some tissues, while antagonizing its action in others. This SERM activity depends on both the promoter context and the cellular environment, in which the relative amounts of diverse coregulatory proteins play critical roles.[3,5] Structural studies have shown that ligand binding to ERs induces conformational changes in the receptors that are crucial for coregulator recruitment and subsequent transcriptional outcome, thereby, at least partly, explaining agonist and antagonist activities.[6,7] Although both ER subtypes share modest overall amino acid sequence identity (ca. 47%),[2] X-ray crystal structures of receptor−ligand complexes have shown that the binding pockets of both ER subtypes differ by only two amino acids, indicating a conserved capability to bind ligands with similar affinities.[8] Indeed, ERα and ERβ bind a wide range of natural and synthetic compounds with striking chemical and structural diversities to a comparable extent.[9] However, the two binding cavities possess a somewhat different size and flexibility, which appears to be a critical distinction that can be exploited in the development of subtype-selective ligands.[4] Both ERs are differentially expressed in various tissues in the human body, which opens clinically relevant perspectives for ligands with ER-subtype-selective affinities and/or activities.[10]

In search for agents that would be useful in preventing and treating estrogen-dependent pathologies, the interest in phytoestrogens has increased markedly.[11] Recently, the prenylated flavanone, 8-prenylnaringenin (8-PN), has been identified as a potent phytoestrogen from hops (*Humulus lupulus* L.),[12] showing an in vitro estrogenic activity among the highest of all plant-derived estrogens known to date.[13] Because naringenin exhibits only weak estrogenic properties,[14] the presence of the prenyl group at C(8) seems essential for the potent estrogenic activity of 8-PN. Furthermore, the high contribution of van der Waals interaction energy to the total ligand−receptor interaction energy suggests a major role for the prenyl group in the binding affinity.[15] It has been shown that even subtle structural modifications of ligands can greatly influence their estrogenic properties.[3] Therefore, we intended in this study to elucidate the impact of variations in the length and the branching of hydrophobic C(8) alkyl substituents, which are substituted for the prenyl group, on ERα and ERβ activities and investigated to what extent this relates to receptor conformational changes using receptor structure-sensing peptides.

## Results

**Chemistry.** The key compound for the regioselective synthetic route presented here is 3-alkyl-2-hydroxy-4,6-bis-(methoxymethoxy)acetophenone (Scheme 1). Direct alkylation of phloroacetophenone using various alkyl bromides gave very poor yields. Thus, 2,4,6-trimethoxybenzaldehyde (**1**) was used as a starting material, in which the aldehyde served as a handle for the introduction of the desired alkyl groups. Except for **2a**, which was obtained via borohydride reduction of **1** and deoxygenation of the resulting benzylic alcohol, monoalkylation was effected using organometallics (alkyl lithium, alkylmagnesium bromide), and the resulting secondary benzylic alcohols were efficiently deoxygenated on treatment with

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[a] Abbreviations: 8-PN, 8-prenylnaringenin; E2, 17β-estradiol; ER, estrogen receptor; LBD, ligand-binding domain; OHT, 4-hydroxytamoxifen; *R,R*-THC, *R,R*-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol; SERM, selective estrogen receptor modulator.

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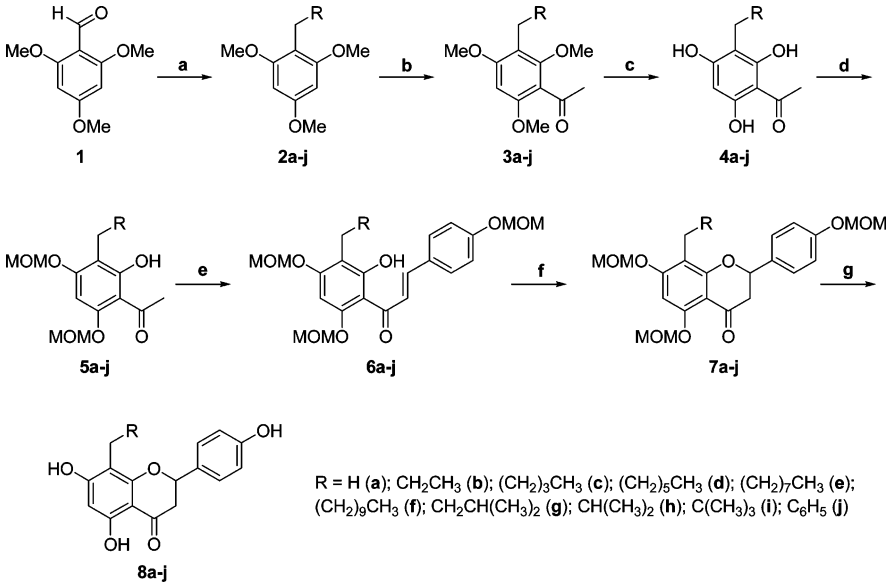
**Scheme 1.** Total Synthesis of 8-Alkylnaringenins




Reagents and Conditions: (a) (i) $NaBH_4$, NaOH−MeOH (**2a**) or RLi/RMgBr, $Et_2O$, −78 °C to rt (**2b**−**j**); (ii) $HSiEt_3$ (rt), $CF_3COOH$, $CH_2Cl_2$, −78 °C to rt; (b) AcCl, $SnCl_4$, $CH_2Cl_2$, −10 °C; (c) $BBr_3$, $CH_2Cl_2$, −78 °C to rt; (d) MOMCl, $K_2CO_3$, acetone, reflux; (e) *p*-MOMO-benzaldehyde, KOH, $H_2O$−EtOH, 0 °C to rt; (f) NaOAc, EtOH, reflux; (g) 3 M HCl, MeOH, reflux.

**Table 1.** Binding Affinities and Selectivities of 8-Alkylnaringenins and Reference Compounds for Estrogen Receptors α and $\beta$[a]

| cmpd | substituent | ERα $IC_{50}$ (nM) | ER$\beta$ $IC_{50}$ (nM) | selectivity ratio $IC_{50}$ (ERα)/$IC_{50}$ (ER$\beta$) |
|---|---|---|---|---|
| E2 | | 1.2 ± 0.2 | 1.4 ± 0.6 | 0.9 |
| GEN | | 1145 ± 368 | 25 ± 7 | 45 |
| 8-PN | $CH_2CHC(CH_3)_2$ | 57 ± 10 | 68 ± 33 | 0.8 |
| NAR | | 72 302 ± 17 790 | 13 473 ± 2851 | 5.4 |
| **8a** | $CH_3$ | 5245 ± 611 | 863 ± 292 | 6.1 |
| **8b** | $(CH_2)_2CH_3$ | 155 ± 36 | 29 ± 6 | 5.3 |
| **8c** | $(CH_2)_4CH_3$ | 383 ± 123 | 97 ± 50 | 4.0 |
| **8d** | $(CH_2)_6CH_3$ | 392 ± 45 | 69 ± 8 | 5.7 |
| **8e** | $(CH_2)_8CH_3$ | 436 ± 105 | 113 ± 22 | 3.9 |
| **8f** | $(CH_2)_{10}CH_3$ | 1017 ± 201 | 421 ± 161 | 2.4 |
| **8g** | $(CH_2)_2CH(CH_3)_2$ | 140 ± 31 | 59 ± 4 | 2.4 |
| **8h** | $CH_2CH(CH_3)_2$ | 80 ± 10 | 37 ± 5 | 2.2 |
| **8i** | $CH_2C(CH_3)_3$ | 216 ± 44 | 141 ± 51 | 1.5 |
| **8j** | $CH_2C_6H_5$ | 503 ± 103 | 244 ± 40 | 2.1 |

[a] Determined by a competitive radiometric binding assay with [$^3$H]-E2 and purified human ERα- and ER$\beta$-LBDs. $IC_{50}$ values represent the means of three experiments ± standard deviation. A selectivity ratio >1 depicts a greater affinity for ER$\beta$ compared to ERα. E2, 17$\beta$-estradiol; GEN, genistein; 8-PN, 8-prenylnaringenin; NAR, naringenin.

triethylsilane and trifluoroacetic acid, whereby *C*-alkylated 1,3,5-trimethoxybenzenes (**2b**−**j**) were obtained in high yields from 2,4,6-trimethoxybenzaldehyde. Subsequent Friedel−Crafts acetylation was effected with acetyl chloride in the presence of tin(IV) chloride, yielding 3-alkyl-2,4,6-trimethoxyacetophenones (**3a**−**j**). Demethylation with boron tribromide furnished 3-alkyl-2,4,6-trihydroxyacetophenones (**4a**−**j**), which were regioselectively bis-methoxymethylated to give the 3-alkyl-2-hydroxy-4,6-bis-(methoxymethoxy)acetophenones (**5a**−**j**). Subsequent Claisen−Schmidt condensation with *p*-(methoxymethoxy)benzaldehyde afforded chalcones (**6a**−**j**) having the appropriate functionalities in protected form. Regioselective cyclization (refluxing with sodium acetate in ethanol) to the corresponding tris-methoxymethylated flavanones (**7a**−**j**) was followed by demethoxymethylation (hydrogen chloride) to give the desired 8-alkylnaringenins (**8a**−**j**).

**Estrogen Receptor Binding Affinity.** The ER-binding affinities of the 8-alkylnaringenins were determined in a competitive radiometric binding assay using purified human ERα and ER$\beta$ ligand-binding domains (LBDs). Binding affinities for both receptors are expressed as $IC_{50}$ values (nM; Table 1). Compared to genistein, the affinity of 8-PN was 20-fold higher for ERα and of the same order of magnitude for ER$\beta$. Analogous substituent-affinity trends were noted for ERα and ER$\beta$. Replacing the hydrogen at C(8) in naringenin with a methyl group (**8a**) induced an approximately 15-fold increase in affinity for ERα and ER$\beta$. Extension of the substituent to *n*-propyl (**8b**) further decreased the $IC_{50}$ values approximately 30-fold. Longer chain lengths resulted in an alkyl group-dependent decrease in binding affinity, which still remained at least 70-fold (ERα) and 30-fold (ER$\beta$) higher than that of naringenin for the *n*-undecyl substituent (**8f**). The binding affinity of **8f** was only 6-fold lower for ER$\beta$ and 18-fold lower for ERα with respect to that of 8-PN. Substitution with the branched-chain substituents led to good binding affinities for both receptors, with a tendency of decreased binding affinity for increased bulkiness. Introduction of a benzyl at C(8) (**8j**) gave a considerable reduction in binding affinity compared with a prenyl substituent (8-PN). The majority of the compounds exhibited a moderately higher (maximum 6.1-fold) affinity for ER$\beta$ than for ERα. This selectivity preference gradually decreased with increasing length of the alkyl group, with 8-*n*-undecylnaringenin (**8f**) showing

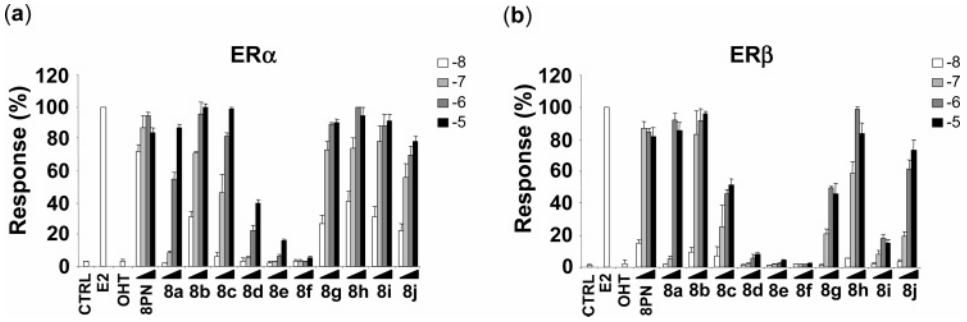



**Figure 1.** Agonist activity of 8-alkylnaringenins. (a) Agonist activity of 8-alkylnaringenins on gene transcription by ERα and (b) by ER$\beta$, monitored on an estrogen-responsive ERE-reporter in HuH7 cells, in concentrations ranging from $10^{-8}$ to $10^{-5}$ M. Values represent the mean calculated from four or more separate experiments and are presented as percent response, with the maximal E2-response set at 100%. Error bars represent standard error of the mean. CTRL, control; E2, 17$\beta$-estradiol; OHT, 4-hydroxytamoxifen; 8PN, 8-prenylnaringenin; for compound codes, see Table 1.

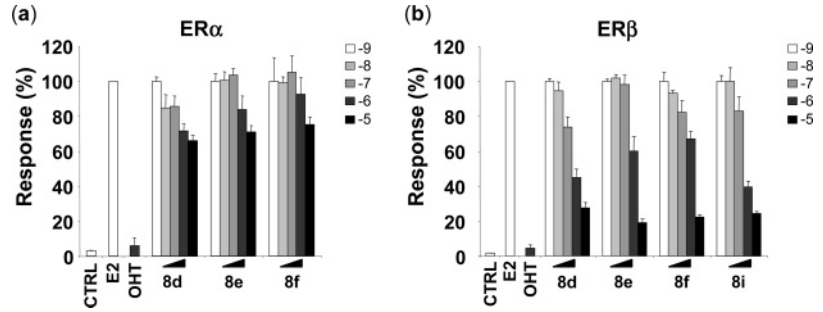



**Figure 2.** Antagonist activity of 8-alkylnaringenins. (a) Antagonist activity of 8-alkylnaringenins on gene transcription by ERα and (b) by ER$\beta$, monitored on an estrogen-responsive ERE-reporter in HuH7 cells, in concentrations ranging from $10^{-9}$ to $10^{-5}$ M, in the presence of $10^{-9}$ M E2. Values represent the mean calculated from four or more separate experiments and are presented as percent response, with the response obtained with $10^{-9}$ M E2 set at 100%. Error bars represent standard error of the mean. CTRL, control; E2, 17$\beta$-estradiol; OHT, 4-hydroxytamoxifen; for compound codes, see Table 1.

the lowest selectivity for the linear alkylated series (2.4-fold). Increasing the branching degree of the alkyl substituent further reduced binding selectivity.

**Estrogen Receptor Subtype-Mediated Gene Transcription.** We investigated the ERα- and ER$\beta$-mediated transcriptional activation of the synthetic ligands by means of a transient gene expression assay, using an ERE-luciferase reporter in human hepatoma cells (HuH7; Figure 1). 8-PN exhibited full agonist activities on both ER subtypes, with a more pronounced potency on ERα than on ER$\beta$. Naringenins substituted with methyl (**8a**) and *n*-propyl (**8b**) also showed full agonist character for ERα and ER$\beta$, with a higher potency for the latter compound. Introducing a *n*-pentyl substituent (**8c**) gave only partial agonist character on ER$\beta$, while full agonist character on ERα was maintained. Further linear extension of the substituent to *n*-heptyl (**8d**) substantially decreased agonist activity on both ER subtypes, resulting in partial agonism on ERα, while only minimal transcriptional activity was detected on ER$\beta$. Compounds bearing a *n*-nonyl (**8e**) or a *n*-undecyl (**8f**) substituent (the longest alkyl chains of the series) elicited no or very low transcriptional activity on both receptors. The branched-chain analogs all exhibited full or nearly full agonist character for ERα, but substantial differences in transcriptional activity were noted on ER$\beta$. Naringenins substituted at C(8) with 2-methylpropyl (**8h**) and benzyl (**8j**) showed similar agonist character for both receptors but with a lower potency for ER$\beta$. The 3-methylbutyl-substituted derivative (**8g**) was a full agonist for ERα, but behaved as a partial agonist on ER$\beta$. Surprisingly, introducing a 2,2-dimethylpropyl substituent (**8i**) resulted in a derivative that exhibited full agonist character for ERα but elicited only very low transcriptional activity on ER$\beta$.

The naringenin derivatives that exhibited minimal transcriptional activities on one or both ER subtypes were subsequently tested for antagonist activity (Figure 2). Compounds bearing long alkyl chains (*n*-heptyl (**8d**), *n*-nonyl (**8e**), and *n*-undecyl (**8f**)) antagonized the effect of E2, however, their antagonist character was much more pronounced on ER$\beta$ than on ERα. 8-(2,2-Dimethylpropyl)naringenin (**8i**), which was a full agonist on ERα, exhibited substantial antagonist character on ER$\beta$.

**Receptor Conformational Changes Monitored In Vivo.** Induction of a conformational change in the receptor by ligands is well documented to contribute to the basis of ER agonism and antagonism.[6,7,16,17] We have applied a mammalian two-hybrid system to monitor the ability of the new compounds to induce a conformational change in ERα and ER$\beta$. Specific Gal4-DNA-binding domain tagged peptides can be used as sensitive probes to detect the different ligand-induced receptor conformations.[18] The specific ER-binding peptides in this study have been identified and characterized previously.[19−21] The parental compound, 8-PN, efficiently induced recruitment of the LxxLL-motif-containing peptide EAB1, comparable to E2 in both ERs, while naringenin was less effective (Figure 3a). The capacity of the 8-PN analogs to induce an agonist conformation clearly depended on the length of the substituent at C(8). 8-Methylnaringenin (**8a**) provoked a high response with ERα and ER$\beta$, indicating that an agonist conformation was induced in both receptors, despite its limited binding affinity. Agonist conformations in both ER subtypes were also elicited by 8-*n*-propylnaringenin (**8b**). For ERα, 8-*n*-pentylnaringenin (**8c**) gave a high response, while its capacity to induce an agonist conformation in ER$\beta$ was much lower. Extension of the substituent to *n*-heptyl (**8d**) substantially reduced the response with ERα and led to a very low response with ER$\beta$. 8-*n*-Nonylnaringenin (**8e**) and 8-*n*-undecylnaringenin (**8f**), bearing the longest alkyl chains, failed to induce an agonist conformation in both receptors. The derivatives substituted with benzyl (**8j**) or the branched chains

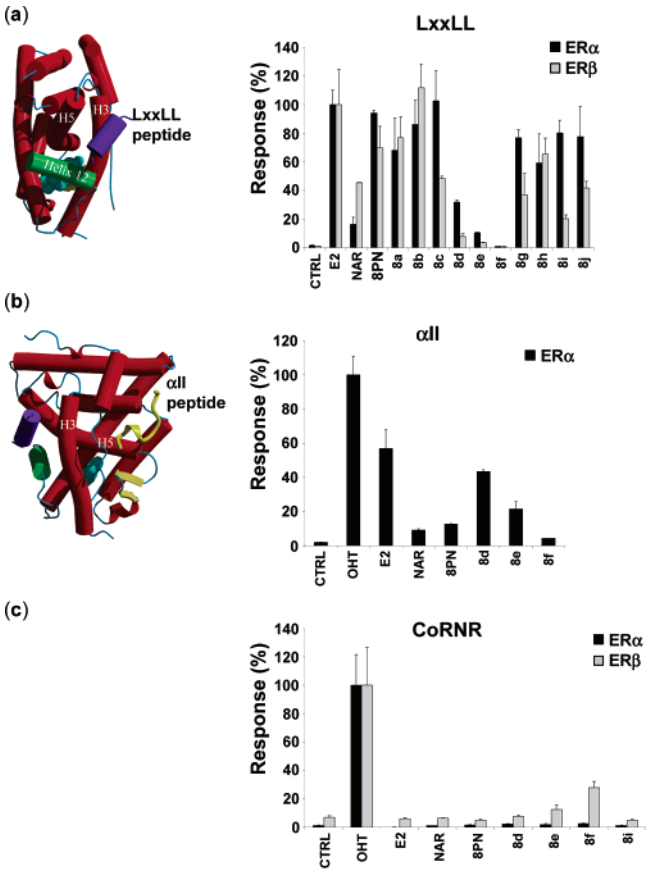



**Figure 3.** Analysis of receptor conformational changes in mammalian cells. (a) Recruitment of the LxxLL-peptide and overall structure of ERα-LBD, showing the LxxLL-peptide (purple) binding surface in the presence of agonist. (b) Recruitment of the ERα-specific peptide αII and overall structure of ERα-LBD showing the αII peptide (yellow) binding surface in the presence of agonist. The exposed αII peptide binding surfaces are shown rotated 90° clockwise around the vertical axis with respect to Figure 3a. (c) Recruitment of a CoRNR-box peptide. Results are presented as percent response, where reporter activity in the presence of E2 (a) or OHT (b and c) were set to 100%. Error bars represent standard deviation. CTRL, control; E2, 17*β*-estradiol; NAR, naringenin; 8PN, 8-prenylnaringenin; OHT, 4-hydroxytamoxifen; for compound codes, see Table 1. (Structures of peptide surfaces were kindly provided by Dr. Ashley Pike.)

3-methylbutyl (**8g**) and, in particular, 2,2-dimethylpropyl (**8i**), induced an agonist conformation more efficiently in ERα than in ER*β*, while an equally high response on both receptors was observed for 8-(2-methylpropyl)naringenin (**8h**). 8-*n*-Heptylnaringenin (**8d**) and 8-*n*-nonylnaringenin (**8e**) provoked a conformational change in ERα that could be detected using the ERα-specific peptide αII because the peptide surface was exposed in the presence of these ligands (Figure 3b). Moreover, the compounds with the longest alkyl chains, *n*-nonyl (**8e**) and *n*-undecyl (**8f**), induced a conformation in ER*β*, but not in ERα, which allowed interaction with a peptide containing a co-repressor defined consensus CoRNR-box motif (Figure 3c).[19]

## Discussion

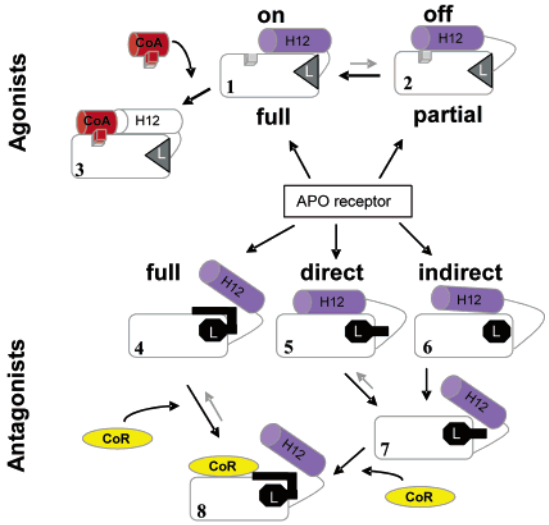
In the present study, we have introduced alkyl modifications at C(8) of the naringenin skeleton, to elucidate critical structural features affecting the biological activity mediated by ER-α and ER-*β*. All flavanones were synthesized from the corresponding chalcones via a regioselective but not enantioselective cyclization, hence, they prevail as racemic mixtures. However, reports in the literature have indicated for 8-PN that both *R*- and *S*-enantiomers show analogous binding affinities and estrogenic activities, in vitro and in vivo, on both ER subtypes.[22,23] Therefore, all compounds were examined as racemic mixtures in these various in vitro bioassays. We have found that introducing a short-chain alkyl group ($C_1-C_5$) drastically increased the binding affinity on both ERs compared with naringenin. Further increasing the linear chain length resulted in the development of a marked antagonism, preferentially on ER*β*. More importantly, subtle modifications in the shorter chain lengths appeared to result in an unusual mixed agonism/antagonism profile with respect to both receptors.

8-PN exhibited nearly 50-fold lower binding affinities for both ER subtypes than E2. The resulting selectivity ratio of 0.8 contrasts with previous data that have described 8-PN to have a more than 2-fold higher affinity for ERα than for ER*β*.[22] This discrepancy may be explained by the use of ER-LBDs in our assay instead of full-length ERs to investigate the binding affinities. With the exception of 8-methylnaringenin, all 8-alkylnaringenins showed good binding affinities for both ER subtypes compared to 8-PN, however, with a tendency of decreased binding affinity for increased bulkiness. The highest overall binding affinities were found with the intermediate-size alkyl groups, *n*-propyl, 2-methylpropyl, 3-methylbutyl, and prenyl. It is interesting to note that, even though analogous substituent-affinity trends were observed for both receptors, the novel naringenin derivatives exhibited, in contrast to 8-PN, some affinity selectivity for ER*β*. Substitution with small alkyl groups led to full agonism on both ER subtypes. However, starting with *n*-pentyl on ER*β* and *n*-heptyl on ERα, the agonist character substantially decreased upon linear extension of the alkyl substituent to become very low or negligible for *n*-nonyl and *n*-undecyl.

The existence of distinct ER conformations has been clearly demonstrated in a number of recent studies that have identified specific ER-binding peptides using phage display.[18−20,24] These peptides have turned out to be informative in revealing possibly important docking sites for proteins involved in regulating receptor activity.[19,21] We applied a number of peptides representing distinct classes to sense ligand-mediated receptor conformations in the context of a cell environment. Structural analyses of ERs (Figure 4a) have stated the general view that agonist-bound ERs adopt a conformation in which a hydrophobic cleft is formed involving the mobile LBD helix 12 and LBD helices 3 and 5. This hydrophobic cleft represents the binding surface for α-helical leucine-rich peptide motifs, known as LxxLL motifs, found within coactivators.[25,26] The agonist activities of the 8-alkylnaringenins for both receptors were in good agreement with the ability to promote recruitment of an LxxLL-peptide in the conformation assay (Figure 3a). Moreover, the peptide assay and the transcriptional assay displayed convincingly corresponding results for partial agonism on both receptors. 8-*n*-Pentylnaringenin, 8-*n*-heptylnaringenin, and 8-(3-methylbutyl)naringenin showed, in full accordance to the diminished transcriptional activity, a reduced ability to elicit LxxLL-peptide recruitment to ERα or ER*β*, suggesting that a suboptimal conformation is induced, resulting in limited exposure of the coactivator binding site (Figure 4). Partial agonist activity has been associated with a suboptimal position of helix 11 away from helix 12, leading to loss of stabilizing interactions and destabilizing the agonist conformation of helix 12.[27] Our findings of reduced LxxLL-peptide recruitment on partial agonist binding are consistent with this model.

Compounds having the longest linear alkyl chains (*n*-heptyl, *n*-nonyl, and *n*-undecyl) were found to bind the estrogen

(a)




(b)

| | Ligand | Conformational state ERα | ERβ | Ref. |
|---|---|---|---|---|
| ER-ligands | E2 | 1,3 | 1 | 16,26 |
| | Genistein | 1 | 2 | 8,35 |
| | ICI 164,384 | 4 | 4 | 36 |
| | OHT | 5 | 5 | 21,25 |
| | THC | 1 | 6 | 34 |
| New compounds | 8PN | 1 | 1 | |
| | **8a** | 1 | 1 | |
| | **8b** | 1 | 1 | |
| | **8c** | 1 | 2 | |
| | **8d** | 2 | 5 | |
| | **8e** | 5 | 5 | |
| | **8f** | 5 | 5 | |
| | **8g** | 1 | 2 | |
| | **8h** | 1 | 1 | |
| | **8i** | 1 | 6 | |
| | **8j** | 1 | 1 | |

**Figure 4.** (a) Schematic representation of the known structural states observed for ER-LBD when bound to different classes of ER ligands. The positioning of helix 12 (purple) is shown with respect to ER-LBD (gray) and contributes to determine the receptor activity. Agonists (dark gray) stabilize a transcriptional active LBD state (1; on) that allows recruitment of coactivators (CoA; red) to the formed hydrophobic AF2 groove (2). Partial agonists have a less stabilizing effect on the agonist conformation (3; off), but are to some extent able to induce CoA binding, determined by an equilibrium between the induced receptor states (1 and 3). Antagonists (black) interfere with the agonist positioning of helix 12 either directly with a long side chain (4 and 5) or indirectly (6) due to altered ligand contacts in the binding cavity. Direct and indirect antagonists locate helix 12 so that it occludes the AF2 cleft (5 and 6), while full antagonists prevent helix 12 from associating with the LBD (4). Classical NR-corepressor (CoR; yellow) recruitment to antagonist-bound ERs (8) most likely requires exposure of the AF2 cleft (6 and 7). The included receptor states are all supported by experimental observations except the unliganded (APO) receptor and the CoR-bound ER (8). (b) Conformational states induced by different reference ER ligands are based on previous published crystal structure determinations, and conformational states induced by the new compounds are based on the results obtained in the systems used in this study.

receptors and to elicit only a limited transcriptional response, but they efficiently antagonized the effect of E2 in the transactivation assays, in particular, on ER$\beta$ and, to a lesser extent, on ER$\alpha$. The results from the conformational studies indicated that these compounds are unable to promote a receptor-LxxLL-peptide interaction. Coactivator binding is prevented when antagonists are bound[16,25] and recruitment of coregulators involved in antagonist signaling is promoted instead.[28,29] We exchanged the LxxLL-containing peptide to other peptides known to recognize conformations induced by 4-hydroxytamoxifen (OHT).[24] None of the OHT-specific peptides interacted with the receptors when complexed with either of the naringenin derivatives (data not shown). The investigated compounds seemed to induce a receptor conformation different from that of OHT, which is not surprising in view of the compounds' hydrophobic alkyl chain compared to the large basic side chain found in OHT. Nevertheless, we demonstrated that distinct conformational changes in the presence of these putative antagonists did occur. The $\alpha$II peptide surface, which has been shown by mutational studies to possibly be important for antagonist signaling, is located on the opposite side of the receptor compared to the LxxLL-binding site[21] and is exposed in the presence of 8-*n*-heptylnaringenin and 8-*n*-nonylnaringenin. Interestingly, we observed a gradual decline in response with increasing chain length, with 8-*n*-undecylnaringenin exhibiting a negligible response compared to the vehicle, a similar effect that has been described with the pure ER-antagonist ICI-182 780.[20] Furthermore, in the presence of 8-*n*-undecylnaringenin and, to a limited extent, 8-*n*-nonylnaringenin, the interaction with a CoRNR-box-peptide (important for interaction between NRs and co-repressors) suggests that these two compounds are capable of inducing a conformation in ER$\beta$ that possibly facilitate recruitment of co-repressors like NCoR or SMRT.[19,28,30]

The branched-chain analogs that all exhibited full or nearly full agonist character for ER$\alpha$ showed substantial differences in the transcriptional activity through ER$\beta$. Naringenin derivatives provided with a 2-methylpropyl or a benzyl substituent showed similar agonist character for both receptors, but with a lower potency for ER$\beta$. Like 8-*n*-pentylnaringenin, 8-(3-methylbutyl)naringenin was a full agonist for ER$\alpha$, but behaved as a partial agonist on ER$\beta$. Hence, the steric hindrance associated with the nature of the alkyl groups influenced the receptor-specific pharmacological character. This was highly evident for 8-(2,2-dimethylpropyl)naringenin, which appeared to be the most distinctive compound in the series, exhibiting full agonist character on ER$\alpha$, but pronounced antagonist character on ER$\beta$ in HuH7 cells (Figure 2) and in Hela cells (data not shown). The differential behavior on both ER subtypes observed for 8-(2,2-dimethylpropyl)naringenin is not unprecedented. Nevertheless, only a few molecules with this contrasting profile have been described earlier,[31−33] the *R*,*R*-enantiomer of 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (*R*,*R*-THC) being the best-studied example. The crystal structure of the ER$\beta$-LBD complexed with *R*,*R*-THC revealed that this ligand induced an antagonist conformation in ER$\beta$ without directly displacing helix 12. The positioning of the side chains of OHT and raloxifene directly precludes the agonist-bound orientation of helix 12 by steric hindrance. In contrast, *R*,*R*-THC antagonist activity appeared to be derived from a shift of helix 11 into the space that would be occupied by the agonist position of helix 12, thereby precluding helix 12 from adopting its agonist orientation.[27,34−36] Because helix 12 is not directly displaced, this mode of antagonism has been termed "passive antagonism"[34] or preferably "indirect antagonism."[4,17] The absence of a long basic side chain, like those found in OHT and raloxifene, in both *R*,*R*-THC and 8-(2,2-dimethylpropyl)-naringenin might suggest a similar molecular mechanism for these compounds leading to the observed ER$\beta$-selective antagonist character. Future and ongoing studies, including

characterization of the activities of the individual *R*- and *S*-enantiomers, are needed to clarify the subtype-selective antagonist mechanism of 8-(2,2-dimethylpropyl)naringenin.

## Conclusions

It is clear from both the transcriptional activation assay and the peptide-binding profiles that, with respect to the features of the alkyl side chain at C(8) of naringenin, decreased bulkiness and a shorter chain length are required to induce antiestrogenic properties on ER$\beta$ ($C_5$-alkyl group) compared with ER$\alpha$ ($C_7$-alkyl group). These observations might be explained in part by the fact that the ER$\beta$-ligand-binding cavity is somewhat smaller in size than that of ER$\alpha$.[8] Furthermore, our findings provide further evidence for the general idea that the agonist orientation of helix 12 is less stable in ER$\beta$ than in ER$\alpha$ and, hence, that ER$\beta$ is easier to antagonize than ER$\alpha$.[4,8,27] Together, these novel 8-alkylnaringenins span an activity spectrum ranging from full agonism to partial agonism to antagonism, including differential subtype activity, solely depending on the length and the bulkiness of the substituent. Interestingly, we also observed that the binding affinity varies and even differentiates between the ER subtypes as the nature of the side-chain is altered. Furthermore, in view of the available ER-LBD crystal structures with various ligands, we have predicted the possible receptor conformations induced by the new ligands on the basis of the results in this study (Figure 4b). These findings highlight the significance of a systematic approach to investigate the impact of subtle structural modifications to detail the pharmacological character of ER ligands and to provide valuable information for future drug or tool compound discovery.

## Experimental Section

**Chemical Compounds and Synthetic Procedures. General Information.** $^1$H NMR and $^{13}$C NMR spectra were obtained with a Varian Mercury 300 spectrometer ($^1$H NMR, 300 MHz; $^{13}$C NMR, 75 MHz). All spectra were recorded in DMSO-$d_6$. Chemical shifts ($\delta$) are expressed in parts per million (ppm) relative to the residual solvent peak. All signals assigned to hydroxyl groups were exchangeable with $D_2O$. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, n = nonet, m = multiplet, dd = double doublet, and br = broad. Combustion analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Exact mass measurements were performed on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF 1, Micromass, Manchester, U.K.) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in acetonitrile (positive mode: 1% formic acid) at 10 $\mu$L/min. Thin layer chromatography was carried out on precoated Alugram SIL G/UV$_{254}$ silica gel plates (Macherey−Nagel, Düren, Germany) and TLC separations were examined under UV light at 254 nm and revealed by a sulfuric acid−anisaldehyde spray. Column chromatography was carried out on silica (Ecochrom, ICN silica 63−200 mesh) from ICN Biomedicals (Eschwege, Germany). Compounds were obtained as amorphous powders or as oils. Technical solvents were purchased from Chemlab (Zedelgem, Belgium), while anhydrous solvents and reagents were obtained from Acros Organics (Geel, Belgium) and Sigma-Aldrich (Bornem, Belgium). All reactions were performed under a nitrogen atmosphere. For the sake of uniformity, the nature of the alkyl group precedes other substituents in the naming of the compounds (deviation from the alphabetical ordering, according to IUPAC nomenclature). Reference compounds E2, naringenin, and genistein were acquired from Sigma-Aldrich. 8-PN was synthesized according to a literature procedure.[37]

**General Procedure for the Preparation of 8-Alkylnaringenins. 1. General Procedure for the Preparation of 2-Alkyl-1,3,5-trimethoxybenzenes (2a−j). 1.1. General Procedure for the Preparation of 2-Alkyl-1,3,5-trimethoxybenzenes (2b−j).** (i) To a stirring solution of 2,4,6-trimethoxybenzaldehyde (**1**) in dry $Et_2O$ (1.5 mL/mmol) was added dropwise at −78 °C alkyllithium or alkylmagnesium bromide (1.2 equiv). The cooling bath was removed and, after completion of the reaction (1−1.5 h, as monitored by TLC), the reaction mixture was poured on ice and extracted with EtOAc. The organic phase was dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. (ii) Without purification, the residue was redissolved in dry $CH_2Cl_2$ (1.5 mL/mmol), and $HSiEt_3$ (2.0 equiv) was added at room temperature, together with $CF_3COOH$ (6 equiv) at −78 °C.[38] The reaction mixture was allowed to warm to room temperature (1 h) and stirred until completion of the reaction (30 min−1 h, as monitored by TLC). After neutralization with saturated aqueous $NaHCO_3$, the mixture was extracted with $Et_2O$. The combined organic phases were washed with water, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc) to yield **2b−j**.

**1.2. Procedure for the Preparation of 2-Methyl-1,3,5-trimethoxybenzene (2a).** (i) 2,4,6-Trimethoxybenzyl alcohol was synthesized from **1** according to a literature procedure.[39] (ii) See general method for the preparation of **2b−j** (1.1. (ii)).

**2-Methyl-1,3,5-trimethoxybenzene (2a).** Yield: 98%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.93 (s, 3H), 3.74 (s, 9H), 6.17 (s, 2H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 8.19, 55.47, 55.83, 90.91, 105.51, 158.93, 159.49. HRMS calcd for $C_{10}H_{15}O_3$ $[M + H]^+$, 183.1021; found, 183.1033.

**2-*n*-Propyl-1,3,5-trimethoxybenzene (2b).** Yield: 99%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.81 (br t, 3H, $J$ = 7.3 Hz), 1.35 (m, 2H), 2.41 (br t, 2H, $J$ = 7.3 Hz), 3.71 (s, 6H), 3.72 (s, 3H), 6.17 (s, 2H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.65, 22.98, 24.84, 55.73, 56.18, 91.37, 110.70, 159.00, 159.60. HRMS calcd for $C_{12}H_{19}O_3$ $[M + H]^+$, 211.1334; found, 211.1326.

**2. General Procedure for the Preparation of 3-Alkyl-2,4,6-trimethoxy-acetophenones (3a−j).** To a stirring solution of 2-alkyl-1,3,5-trimethoxybenzene (**2a−j**) in dry $CH_2Cl_2$ (1 mL/mmol) was added dropwise at −10 °C $SnCl_4$ (2 equiv) and acetyl chloride (1.5 equiv). The reaction mixture was stirred until completion of the reaction (2−3 h, as monitored by TLC), and poured on ice. The separated aqueous phase was extracted with $Et_2O$, and both organic phases were washed with saturated aqueous $NaHCO_3$, dried over anhydrous $MgSO_4$, and the organic solvent was removed under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc) to yield **3a−j**.

**3-Methyl-2,4,6-trimethoxyacetophenone (3a).** Yield: 77%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.96 (s, 3H), 2.36 (s, 3H), 3.59, 3.80 and 3.83 (3s, 3H each s), 6.49 (s, 1H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 8.87, 33.03, 56.41, 56.54, 62.49, 92.60, 111.31, 118.52, 155.92, 156.19, 160.14, 201.47. HRMS calcd for $C_{12}H_{17}O_4$ $[M + H]^+$, 225.1127; found, 225.1124.

**3-*n*-Propyl-2,4,6-trimethoxyacetophenone (3b).** Yield: 86%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.87 (br t, 3H, $J$ = 7.3 Hz), 1.43 (m, 2H), 2.36 (s, 3H), 2.41 (br t, 2H, $J$ = 7.3 Hz), 3.59, 3.79 and 3.82 (3s, 3H each s), 6.48 (s, 1H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.85, 23.34, 25.62, 33.04, 56.49, 56.57, 63.28, 92.90, 116.33, 118.45, 156.21, 156.38, 160.27, 201.61. HRMS calcd for $C_{14}H_{21}O_4$ $[M + H]^+$, 253.1440; found, 253.1435.

**3. General Procedure for the Preparation of 3-Alkyl-2,4,6-trihydroxy-acetophenones (4a−j).** To a stirring solution of 3-alkyl-2,4,6-trimethoxyacetophenone (**3a−j**) in dry $CH_2Cl_2$ (2 mL/mmol), $BBr_3$ (4 equiv, 1.0 M in $CH_2Cl_2$) was added dropwise at −78 °C. The mixture was allowed to warm to room temperature and was stirred until completion of the reaction (24−48 h, as monitored by TLC). After cooling to 0 °C, the reaction was quenched by pouring on ice. The organic solvent was removed under reduced pressure and the aqueous suspension was repeatedly extracted with EtOAc. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$, and concentrated. The residue was purified by column chromatography (hexane/EtOAc) to provide **4a−j**.

**3-Methyl-2,4,6-trihydroxyacetophenone (4a).** Yield: 80%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.81 (s, 3H), 2.53 (s, 3H), 5.98 (s, 1H), 10.29, 10.52 and 13.95 (3s, 1H each s); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 7.98, 33.16, 94.52, 101.92, 104.42, 160.69, 163.32, 164.00, 203.10. HRMS calcd for $C_9H_9O_4$ [M − H]$^-$, 181.0501; found, 181.0512.

**3-*n*-Propyl-2,4,6-trihydroxyacetophenone (4b).** Yield: 77%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.83 (br t, 3H, $J$ = 7.3 Hz), 1.37 (m, 2H), 2.35 (br t, 2H, $J$ = 7.3 Hz), 2.52 (s, 3H), 5.97 (s, 1H), 10.21, 10.50 and 13.97 (3s, 1H each s); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.74, 22.47, 24.44, 33.18, 94.56, 104.41, 106.97, 160.78, 163.37, 164.12, 203.15. HRMS calcd for $C_{11}H_{13}O_4$ [M − H]$^-$, 209.0814; found, 209.0811.

**4. General Procedure for the Preparation of 3-Alkyl-2-hydroxy-4,6-bis(methoxymethoxy)-acetophenones (5a−j).** To a stirring mixture of 3-alkyl-2,4,6-trihydroxyacetophenone (**4a−j**) and anhydrous $K_2CO_3$ (7 equiv) in dry acetone (3 mL/mmol **4a−j**), MOMCl (2.5 equiv) was added dropwise.[40] The reaction mixture was heated to reflux and allowed to stir for 1 h. After cooling to room temperature, the reaction mixture was filtered, and the organic phase was concentrated under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc) to afford **5a−j**.

**3-Methyl-2-hydroxy-4,6-bis(methoxymethoxy)acetophenone (5a).** Yield: 64%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.93 (s, 3H), 2.61 (s, 3H), 3.37 and 3.42 (2s, 3H each s), 5.28 and 5.29 (2s, 2H each s), 6.37 (s, 1H), 13.82 (s, 1H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 8.11, 33.66, 56.72, 57.19, 92.37, 94.56, 95.21, 106.75, 106.87, 158.93, 161.41, 163.29, 204.24. HRMS calcd for $C_{13}H_{17}O_6$ [M − H]$^-$, 269.1025; found, 269.1027.

**3-*n*-Propyl-2-hydroxy-4,6-bis(methoxymethoxy)acetophenone (5b).** Yield: 68%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.86 (br t, 3H, $J$ = 7.3 Hz), 1.42 (m, 2H), 2.49 (br t, 2H, $J$ = 7.3 Hz), 2.61 (s, 3H), 3.37 and 3.43 (2s, 3H each s), 5.27 and 5.29 (2s, 2H each s), 6.34 (s, 1H), 13.84 (s, 1H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.72, 22.46, 24.56, 33.71, 56.74, 57.20, 92.10, 94.39, 95.17, 106.74, 111.35, 159.13, 161.42, 163.39, 204.30. HRMS calcd for $C_{15}H_{21}O_6$ [M − H]$^-$, 297.1338; found, 297.1346.

**5. General Procedure for the Preparation of 3′-Alkyl-2′-hydroxy-4,4′,6′-tris(methoxymethoxy)chalcones (6a−j).** A solution of 3-alkyl-2-hydroxy-4,6-bis(methoxymethoxy)acetophenone (**5a−j**) and *p*-methoxymethoxybenzaldehyde (1.1 equiv, synthesized from *p*-hydroxybenzaldehyde) in EtOH (2.5 mL/mmol **5a−j**), cooled to 5 °C, was added dropwise to a stirring mixture of KOH (0.7 g/mmol **5a−j**) in $H_2O$−EtOH (1.5 mL/mmol **5a−j**, 2:3 v/v) at 0 °C.[40] The reaction mixture was stirred at 0 °C for 3 h and then allowed to warm to room temperature and stirred for an additional 20 h (or until completion of the reaction, as monitored by TLC). The reaction mixture was then poured into ice water, and the resulting solution was acidified with 1 M HCl to pH 4 and extracted with $Et_2O$. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc) to afford **6a−j**.

**3′-Methyl-2′-hydroxy-4,4′,6′-tris(methoxymethoxy)chalcone (6a).** Yield: 74%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.96 (s, 3H), 3.37, 3.39 and 3.42 (3s, 3H each s), 5.24, 5.29 and 5.33 (3s, 2H each s), 6.42 (s, 1H), 7.08 (d, 2H, $J$ = 8.8 Hz), 7.67 (d, 2H, $J$ = 8.8 Hz), 7.70 (d, 1H, $J$ = 15.8 Hz), 7.79 (d, 1H, $J$ = 15.8 Hz), 13.60 (s, 1H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 8.31, 56.44, 56.74, 57.23, 93.30, 94.36, 94.62, 95.89, 107.27, 107.91, 117.22, 126.02, 129.04, 130.93, 143.14, 158.12, 159.43, 161.14, 163.10, 193.49. HRMS calcd for $C_{22}H_{25}O_8$ [M − H]$^-$, 417.1549; found, 417.1535.

**3′-Propyl-2′-hydroxy-4,4′,6′-tris(methoxymethoxy)chalcone (6b).** Yield: 83%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.87 (br t, 3H, $J$ = 7.3 Hz), 1.45 (m, 2H), 2.48 (br t, 2H, $J$ = 7.0 Hz), 3.36, 3.39 and 3.43 (3s, 3H each s), 5.24, 5.29 and 5.34 (3s, 2H each s), 6.40 (s, 1H), 7.08 (d, 2H, $J$ = 8.8 Hz), 7.67 (d, 2H, $J$ = 8.8 Hz), 7.70 (d, 1H, $J$ = 15.8 Hz), 7.80 (d, 1H, $J$ = 15.8 Hz), 13.66 (s, 1H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.76, 22.51, 24.74, 56.43, 56.76, 57.25, 92.99, 94.33, 94.44, 95.86, 107.70, 111.83, 117.21, 126.00, 129.05, 130.93, 143.03, 158.39, 159.41, 161.24, 163.29, 193.51. HRMS calcd for $C_{24}H_{29}O_8$ [M − H]$^-$, 445.1862; found, 445.1876.

**6. General Procedure for the Preparation of 8-Alkyl-5,7,4′-tris(methoxymethoxy)flavanones (7a−j).** To a stirring solution of 3′-alkyl-2′-hydroxy-4,4′,6′-tris(methoxymethoxy)chalcone (**6a−j**) in EtOH (5 mL/mmol) were added NaOAc (4 equiv) and $H_2O$ (0.4 mL/mmol **6a−j**).[40] The reaction mixture was heated to reflux for 20 h and allowed to cool to room temperature. The mixture was then diluted with $H_2O$ and extracted with $Et_2O$. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc) to afford **7a−j**, while **6a−j** was recovered also.

**8-Methyl-5,7,4′-tris(methoxymethoxy)flavanone (7a).** Yield: 53%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.96 (s, 3H), 2.66 (dd, 1H, $J$ = 2.9, 16.4 Hz), 3.03 (dd, 1H, $J$ = 12.6, 16.4 Hz), 3.36, 3.37 and 3.38 (3s, 3H each s), 5.17, 5.19 and 5.27 (3s, 2H each s), 5.48 (dd, 1H, $J$ = 2.9, 12.6 Hz), 6.49 (s, 1H), 7.05 (d, 2H, $J$ = 8.5 Hz), 7.43 (d, 2H, $J$ = 8.5 Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 8.77, 45.33, 56.24, 56.70, 78.27, 94.42, 94.61, 95.78, 96.67, 107.75, 108.05, 116.78, 128.42, 133.02, 157.39, 157.56, 160.67, 161.35, 189.46. HRMS calcd for $C_{22}H_{27}O_8$ [M + H]$^+$, 419.1706; found, 419.1691.

**8-*n*-Propyl-5,7,4′-tris(methoxymethoxy)flavanone (7b).** Yield: 61%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.83 (br t, 3H, $J$ = 7.3 Hz), 1.45 (m, 2H), 2.50 (br t, 2H, $J$ = 7.0 Hz), 2.66 (dd, 1H, $J$ = 2.6, 16.5 Hz), 2.98 (dd, 1H, $J$ = 12.5, 16.5 Hz), 3.36, 3.37 and 3.39 (3s, 3H each s), 5.17, 5.19 and 5.26 (3s, 2H each s), 5.45 (dd, 1H, $J$ = 2.6, 12.5 Hz), 6.48 (s, 1H), 7.05 (d, 2H, $J$ = 8.5 Hz), 7.41 (d, 2H, $J$ = 8.5 Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.65, 22.70, 24.99, 45.60, 56.26, 56.72, 78.26, 94.42, 94.49, 95.74, 96.50, 107.72, 112.64, 116.78, 128.18, 133.18, 157.34, 157.52, 160.65, 161.46, 189.50. HRMS calcd for $C_{24}H_{31}O_8$ [M + H]$^+$, 447.2019; found, 447.2014.

**7. General Procedure for the Preparation of 8-Alkylnaringenins (8a−j).** To a stirring solution of 8-alkyl-5,7,4′-tris-(methoxymethoxy)flavanone (**7a−j**) in MeOH (15 mL/mmol), 3 M HCl (5 mL/mmol) was added dropwise.[40] The reaction mixture was heated to reflux for 1 h (or until completion of the reaction, as monitored by TLC). The mixture was then poured into $H_2O$ and extracted with $Et_2O$. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc), to afford **8a−j**.

**8-Methylnaringenin (8a).** Yield: 93%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.84 (s, 3H), 2.70 (dd, 1H, $J$ = 2.9, 16.9 Hz), 3.19 (dd, 1H, $J$ = 12.6, 16.9 Hz), 5.41 (dd, 1H, $J$ = 2.9, 12.6 Hz), 5.96 (s, 1H), 6.78 (d, 2H, $J$ = 8.5 Hz), 7.30 (d, 2H, $J$ = 8.5 Hz), 9.57, 10.75 and 12.08 (3s, 1H each s); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 8.30, 42.51, 78.72, 95.76, 102.38, 103.21, 115.86, 128.72, 129.87, 158.26, 160.48, 161.63, 165.32, 197.40. HRMS calcd for $C_{16}H_{13}O_5$ [M − H]$^-$, 285.0763; found, 285.0754. Anal. ($C_{16}H_{14}O_5$) C, H.

**8-*n*-Propylnaringenin (8b).** Yield: 88%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.80 (br t, 3H, $J$ = 7.3 Hz), 1.39 (m, 2H), 2.37 (br t, 2H, $J$ = 7.3 Hz), 2.70 (dd, 1H, $J$ = 2.9, 16.9 Hz), 3.16 (dd, 1H, $J$ = 12.5, 16.9 Hz), 5.39 (dd, 1H, $J$ = 2.9, 12.5 Hz), 5.96 (s, 1H), 6.78 (d, 2H, $J$ = 8.8 Hz), 7.29 (d, 2H, $J$ = 8.8 Hz), 9.57, 10.65 and 12.12 (3s, 1H each s); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.54, 22.60, 24.56, 42.65, 78.69, 95.90, 102.39, 108.18, 115.87, 128.54, 130.01, 158.22, 160.67, 161.77, 165.31, 197.46. HRMS calcd for $C_{18}H_{17}O_5$ [M − H]$^-$, 313.1076; found, 313.1061. Anal. ($C_{18}H_{18}O_5$) C, H.

For experimental synthetic procedures and spectroscopic data of other compounds, see Supporting Information.

**Competition-Based Ligand Binding Assay.** Ligand binding was determined using a scintillation proximity assay with streptavidin-coated polyvinyltoluene scintillation beads (Amersham, catalog No. RPNQ0007) and biotinylated receptor. The recombinant biotin-labeled ligand-binding domains (LBDs) of hER$\alpha$ and hER$\beta$ were produced at high levels in *E. coli* and extracted with buffer containing 100 mM Tris−HCl pH 8.0, 100 mM KCl, 10% glycerol,

5 mM EDTA, 4 mM DTT, 0.1 mM PMSF (ERα-LBD extraction) or 100 mM Tris−HCl pH 8.0, 300 mM KCl, 10% glycerol, 5 mM EDTA, 4 mM DTT, 0.1 mM PMSF (ER$\beta$-LBD extraction) with a Microfluidizer. In all ligand-binding experiments, the hERα-LBD and hER$\beta$-LBD extracts were diluted in phosphate buffer (18 mM $K_2HPO_4$/2 mM $KH_2PO_4$, 20 mM $Na_2MoO_4$, 1 mM EDTA, 1 mM TCEP, pH 7.6) to a final concentration of 0.7 nM receptor. The [$^3$H]-E2 (Perkin-Elmer, Boston, MA) had a specific activity of 95 Ci/mmol. The final concentration of [$^3$H]-E2 used for competitive binding was 1.2 nM. Ligands were diluted from a 10 mM stock solution in DMSO in 12 concentrations ranging from 157 $\mu$M to 38 pM using a Hamilton (Hamilton Micro Lab AT2 PLUS) robot. The incubation time on the shaker for the binding experiments was 20 h at room temperature. Receptor-bound [$^3$H]-E2 was determined by scintillation counting (Perkin-Elmer Trilux Microbeta). The $IC_{50}$ values were calculated using a four-parameter logistic equation $y = A + ((B - A)/(1 + ((C/x)^D)))$ in XLfit version 2.0.11.70.

**Cell Culture and Transient Transfections.** HuH7 (human liver) cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, catalog No. 41966−029) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% $CO_2$ in air. Cells were split twice a week. Cells were seeded into 24-well plates (30 000 cells/well) in phenol-red-free medium supplemented with 10% dextran charcoal-stripped fetal bovine serum and 2 mM L-glutamine 24 h before transfection. Cells were transfected with Lipofectamine 2000, as instructed by the manufacturer (Invitrogen).

**Transcriptional Activation Assay.** HuH7 cells were cotransfected with expression vectors (pSG5) of either wild-type ERα or ER$\beta$, together with the estrogen-responsive 3 × ERE-TATA-luciferase reporter and a $\beta$-galactosidase reporter. Experiments were performed in triplicate and contained 50 ng of ER receptor, 500 ng of reporter construct, and 20 ng of $\beta$-galactosidase reporter per well. After transfection, cells were treated with ligands for 16 h before analyzing luciferase and $\beta$-galactosidase activities. Luciferase activity was normalized with $\beta$-galactosidase activity.

**Receptor Conformational Assay.** HuH7 cells were transiently transfected with expression vectors for VP16 activation-domain tagged ERs and Gal4-DNA-binding-domain tagged peptides together with the Gal4-responsive luciferase reporter and a $\beta$-galactosidase reporter. Transfections were performed in triplicate in 24-well format and contained 50 ng of VP16-ER construct, 100 ng of Gal-4 reporter, 50 ng of Gal4-DBD tagged peptides, and 20 ng of $\beta$-galactosidase reporter in each well. Cells were treated with 1 $\mu$M ligand for 16 h before assaying luciferase and $\beta$-galactosidase activities. Luciferase activity was normalized with $\beta$-galactosidase activity.

**Constructs.** Full-length human ERα and ER$\beta$ cDNA cloned into VP16 expression vector (Clontech) or pSG5 (Stratagene) expression vector were used for the conformation assays and transcriptional activation assays, respectively. Oligos corresponding to the LxxLL-peptide, the αII-peptide, and the CoRNR-box-peptide sequences were cloned into the Gal4-DBD expression vector pM (Clontech). All constructs were verified by DNA sequencing. The pM-LxxLL-(EAB1) and pM-αII-constructs have been described before.[21,24] The CoRNR-box sequence (DAFQLRQLILRGLQDD) was isolated in a phage display screen in the presence of OHT and described before named as bT1.[19] The 3X-ERE-TATA-luc reporter has been described before.[41] The Gal4-DBD-luc reporter was a gift from KaroBio, Inc. (Huddinge, Sweden).

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**Supporting Information Available:** Experimental and spectroscopic data for all synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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