Synthesis and Evaluation of Estrogen Agonism of Diaryl 4,5-Dihydroisoxazoles, 3-Hydroxyketones, 3-Methoxyketones, and 1,3-Diketones: A Compound Set Forming a 4D Molecular Library

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Received February 20, 2008

In this paper, the preparation and systematic evaluation of estrogen receptor α (ERα) and estrogen receptor β (ERβ) activities of some diaryl-1,3-diones and their synthetic intermediates, diaryl-4,5-dihydroisoxazoles, diaryl-3-hydroxyketones, diaryl-3-methoxyketones, and diaryl-2-(dimethyl-λ^4-sulfanylidene)-1,3-diones, is described. The set of 72 compounds constitutes a general schematic structure *aryl1*−*linker1*−*spacer*−*linker2*−*aryl2*, where the *linker1*−*spacer*−*linker2* length varies between 4 and 8 carbons. The set of compounds was applied here to map and explore the active sites of subtypes ERα and ERβ. The highest activities were obtained with dihydroisoxazole and hydroxyketone spacers, but even the most flexible diones with unsubstituted aryl groups showed some agonism. Most compounds were found to be ERα selective or to activate both receptors, but in some cases we saw also clearly stronger ERβ activation.

Introduction

Molecular libraries form an essential block of modern drug development strategies: a lead compound binding to any protein can be found, in principle, by using a large number of synthetic molecules. However, combinatorial synthetic methodology still allows only very simple reaction conditions, which essentially limits the compound types that can be synthesized. A way to avoid working with large molecular libraries would be to use flexible ligands, which cover large areas of conformational space. If an active ligand is structurally and conformationally very adaptive, forming its fourth structural dimension, it has numerous conformations at low populations. The selection of a suitable conformer, however, has its entropic price tag on the binding free energy. It has been estimated that the price of fixing one rotatable bond can be up to 5 kJ/mol,[1] which is well tolerable if the ligand−receptor complementarity is high enough. Another concern is the complementarity of ligand molecular motion with the receptor frame: we have recently proposed that the intramolecular motional properties expand an independent dimension of the ligand QSAR[a] space[2] and, correspondingly, offer a mechanism for the protein activation. The question of the motional dimension, together with methodological difficulties, makes the systematic studies of molecular flexibility a challenging scientific problem. The estrogen receptor binds a very diverse group of ligands[3–41] and thus forms an optimal target of study the conformational and configurational library principle, which was the original objective of this work.

Estrogens have numerous regulating functions in biological systems, including the reproductive tissues, the cardiovascular system, the central nervous system, and bone maintenance. In general, the steroidal regulation forms an important field of drug research. The estrogen activity is mediated mainly by two members of the nuclear receptor gene family, the estrogen receptor isoforms ERα[42] and ERβ.[43] Both forms bind and are activated by their endogenous ligand 3,17β-estradiol (E2), and none of the ER agonists or antagonists currently in use are specific for either form.[44] Because of the serious adverse effects of ER agonists and antagonists, large variations in ERα and β expression in diverse target tissues, and cell- and promoter-specific functions displayed by the ER subtypes, there are increasing efforts to explore new chemical scaffolds to develop subtype-specific ligands and to understand context-specific ER signaling.[45] Indeed, a 500-fold functional selectivity for ERα has been reported,[15] while greater than 200-fold selectivities of binding affinity for ERβ[9,17,21] has also been recorded.

Many compounds also exhibit significant estrogen or antiestrogen activity, which also has important environmental biological implications.[46] A common structural feature needed for estrogen and antiestrogen activity seems to be an aromatic ring or, preferably, a phenol group in the position corresponding to the A-ring of an estrogen. The most well-known examples of selective estrogen receptor modulators (SERMs) are tamoxifen and raloxifene,[3,4] which have rather little in common with the estrogen structure. Although the structural diversity of the previously reported synthetic nonsteroidal SERMs is seemingly remarkable, they can be roughly divided into two main groups. The first group is formed by polycyclic structures, with some resemblance to the basic steroidal structure.[5–9] The rest of the compounds have a general structure of ARYL1−*CORE STRUCTURE*−ARYL2,[10] in which the core may have a third aromatic ring and an additional substituent, like in tamoxifen.

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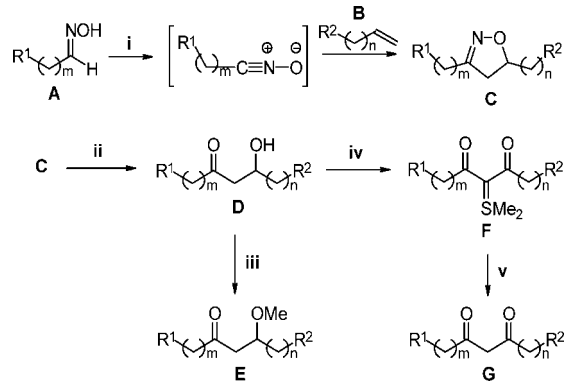
[a] Abbreviations: ERα, estrogen receptor α; ERβ, estrogen receptor β; QSAR, quantitative structure−activity relationship; E2, 3,17β-estradiol; SERM, selective estrogen receptor modulator; DMS, dimethyl sulfide; NCS, *N*-chlorosuccinimide; HEK293 cells, human embryonic kidney cells; EC_{50}, half-maximal effective concentration; SEM, standard error of mean; DMSO, dimethyl sulfoxide; TFAA, trifluoroacetic anhydride; THF, tetrahydrofuran; DCM, dichloromethane; RT, room temperature.

10.1021/jm8001795 CCC: $40.75
Published on Web 06/03/2008

Scheme 1[a]



[a] $m = 0-3$, $n = 0-2$. Reagents and conditions: (i) NaOCl, pyridine, DCM, 0°C; (ii) H_2, Raney-Ni, H_2O, AcOH, MeOH-THF, RT; (iii) HCl, MeOH, refl.; (iv) DMS-NCS, TEA, DCM, −70°C; (v) Zn, AcOH, DCM, RT.

In the simplest examples, which are biphenyl compounds, the core is formed by only one carbon−carbon bond.[11,12] However, the most SERMS have a core structure that is nearly planar and composed of a sp^2-hybridized system such as a C−C double bond,[13,14] an amide group,[15] or a cyclic core, which makes the space between the aromatic rings sterically more crowded and conformationally rigid. The variety of cyclic scaffolds published so far include five-membered rings like furane,[16,17] thiofene,[18–20] isoxazole,[17,21] cyclopentadiene,[22] pyrazole,[10] pyrrole,[23] cyclopentadienone,[24] and pyrrolidinedione,[25] as well as six-membered rings such as oxazine,[26] pyridine,[27–29] dihydrooxathiine,[30,31] isochromane,[32] pyrane,[33,34] dihydropyrane,[35] and dihydrothiopyrane.[35] The most elaborate cyclic core structures reported so far are carborane,[36,37] bicyclo[2.2.2]octane,[36] and 7-oxabicyclo[2.2.1]heptene[38] and -heptaediene[38] scaffolds. Only in a few cases[5,6,8,26,30–35,38,39] the core structure has sp^3-hybridized atoms that also act as chiral centers. The sp^2-structures are symmetric, and the sp^2-sp^2 junctions have intrinsically high rotational barriers due to which many of the structures can be classified rigid.

The diarylpentanedione structure has six rotatable sp^2-sp^3 bonds which all exhibit an exceptionally low rotational barrier so that the structure expands a broad conformational space, further expanded by the keto−enol tautomerism of the structures.[47] The synthetic pathway,[48] offering less flexible, but chiral, relatives and derivatives of the dione structure, can also be considered as an expansion of the molecular space. We synthesized and assayed for ER activation a series of compounds having a general schematic structure ARYL1−LINKER1−SPACER−LINKER2−ARYL2, in which the spacers have chiral sp^3-centers and lengths of the CH_2-linkers vary in such a way that the distance between the aromatic rings is 4−8 carbons (see Scheme 1.). To the best of our knowledge there are only two previous examples of ER binding compounds in which the core structure between the aromatic rings is constituted of a C−C double bond[40] or a cyclic triazine spacer[41] and flexible CH_2-linkers.

The present report describes the preparation and the in vitro estrogen activity of 72 mainly unsubstituted diaryl-1,3-diones and their synthetic 4,5-dihydroisoxazole, 1,3-hydroxy ketone, 1,3-methoxy ketone, and dimethylsulfonium ylide intermediates, some of which in the biological evaluation showed significant activation of both ERα and ERβ receptors. The fit of the agonists to the ER binding pocket was explored by molecular modeling.

Results

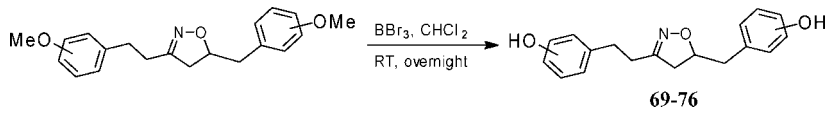
Syntheses. The general route leading to the compounds is depicted in Scheme 1 and described previously in detail.[48] 4,5-Dihydroisoxazoles **C** were synthesized by treating a nitrile oxide, generated in situ with NaOCl from an aldoxime **A**, with 2-fold excess of an aryl alkene **B** in the presence of a catalytic amount of pyridine as an organic base. The isoxales **C** were then converted to hydroxy ketones **D** by catalytic hydrogenation with Raney Ni in the presence of H_2O and AcOH. The methoxy ketones **E** were synthesized by refluxing compounds **D** in MeOH overnight in the presence of concentrated hydrochloric acid. The sulfonium ylides **F** were prepared from **D** by using 5-fold excess of the Corey−Kim reagent (*S,S*-dimethyl-succinimidosulfonium chloride, prepared in situ from DMS and NCS) under argon at −70 °C. Finally, the diketones **G** were achieved from **F** by a reductive desulphurization with zinc in the presence of acetic acid at room temperature. Unfortunately, we were unable to synthesize compounds **2**, **3**, **34**, and **35** via this methodology. However, we were able to make a series of compounds with two phenyl rings and a varying set of spacers with different functionalities, flexibilities, and chain lengths. Compounds **69**−**76** having aromatic hydroxyl groups were prepared from corresponding methoxy substituted 4,5-dihydroisoxazoles by using an 1.1-fold excess of BBr_3 in CH_2Cl_2 at room temperature overnight (see Scheme 2). All the chiral compounds were further purified using semipreparative HPLC techniques with a chiral column and with three exceptions (**11**, **17**, and **19**) we were able to separate all the enantiomers of compounds **C** (see Table 1). Neither specific rotation nor absolute configuration of the pure enantiomers was resolved due to the small amounts of the purified samples. For each separated compound, the enantiomer having shorter retention time in the chiral separation is marked in the tables as the enantiomer **a** and the other form as **b**.

Performance and Specificity of the ER Activation Assay. We performed ER activation assays in HEK293 cells, a cell line derived from human embryonic kidney. This cell line has been used to explore estrogenic ligands in ER cotransfection assays because HEK293 cells do not express endogenous ERs.[49] We typically obtained more than 90-fold activation of both of the ER subtypes by 10 nM E2, indicating a high dynamic range of this transient transfection assay and its ability to distinguish even weakly activating compounds. To investigate whether the observed activation of ER occurs through receptor binding, we randomly selected a group of test compounds (**5**, **18b**, **64b**, and **65**). The activation by all compounds was efficiently blocked by the established ER antagonist ICI-182,780 (Table 5), indicating that these compounds activated both ERα and ERβ via ligand binding. Further evidence on the ER dependency of activation is the fact that none of the test compounds, E2, or ICI-182,780 affected reporter gene activity in the absence of ER expression vector (data not shown). The estimated EC_{50} values for ERα and ERβ were close to 2−10 μM in dose−response assays for the test compounds, while the corresponding values for E2 were about 0.2 nM or less (data not shown).

Discussion

General. The biological evaluation of the compounds with unsubstituted phenyl groups (see Table 1) show that, within the group of the diketones **G**, there are two compounds (**5** and **6**) having ERα activity to some extent. The results indicated that

Scheme 2



69-76

Table 1. In Vitro Estrogen Activity of the Unsubstituted Ligands **1**−**49**

		G			**C**			**D**			**E**			**F**		
m	n	No.	ERα[a]	ERβ[b]	No.	ERα	ERβ	No.	ERα	ERβ	No.	ERα	ERβ	No.	ERα	ERβ
0	1				**9a**[c]	-1.0	6.6	**20**	5.3	16.9	**31**	0.3	18.4			
					9b[d]	47.2	12.8									
0	2				**10a**	22.8	8.5	**21**	13.0	15.4	**32**	38.1	25.8			
					10b	1.1	-6.0									
1	0	**1**	5.4	9.7	**11**[e]	19.6	34.4	**22**	0.8	1.6	**33**	4.9	-1.2	**42**	-1.2	5.8
2	0	**2**	not prepared		**12a**	16.4	9.8	**23a**	78.8	20.5	**34**	not prepared		**43**	-2.5	3.3
					12b	11.2	8.1	**23b**	25.3	2.8						
3	0	**3**	not prepared		**13a**	44.3	-3.1	**24a**	91.4	147.3	**35**	not prepared		**44**	-6.0	0
					13b	11.5	17.2	**24b**	59.3	28.2						
1	1	**4**	4.3	0.1	**14a**	-1.3	-0.2	**25**	8.4	1.7	**36**	1.4	0.3	**45**	0.5	-0.4
					14b	0	0.3									
2	1	**5**	24.3	1.4	**15a**	68.4	1.1	**26**	12.8	0.4	**37**	9.9	1.3	**46**	0.5	-7.1
					15b	38.2	1.3									
3	1	**6**	27.7	4.2	**16a**	31.3	13.6	**27**	24.7	9.1	**38**	24.5	30.4	**47**	3.7	-1.7
					16b	28.1	5.1									
1	2				**17**	7.0	1.2	**28**	4.3	0.2	**39**	12.0	0.2			
2	2	**7**	0.6	0.3	**18a**	3.4	0.2	**29**	16.2	1.0	**40**	14.5	19.5	**48**	1.0	0.3
					18b	43.9	-0.4									
3	2	**8**	6.1	-1.5	**19**	67.6	15.5	**30**	2.2	-7.1	**41**	3.1	-14.6	**49**	0.6	-4.6

[a] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [b] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [c] **a** = enantiomer with a shorter retention time in the chiral separation. [d] **b** = enantiomer with a longer retention time in the chiral separation. [e] Enantiomers not separated, racemic mixture tested.

the optimum length of the spacer for the active ligands would be 6−7 carbons ($m + n = 3-4$). It is also notable that, although low, the selectivity of compound **1** for ERβ is roughly 2-fold. On the other hand, none of ylides **F** with the bulky dimethyl sulfonium group and restricted conformation of the spacer shows activity. In comparison with other compound groups, the 4,5-dihydroisoxazoles **C** show the best overall activities. Many of them are clearly selective for ERα (**13a**, **15a**, **15b**, **18b**) and the difference between the activities of the pure enantiomers is obvious: the other enantiomer is practically inactive in three cases (**9a**, **10b**, and **18a**). Moreover, both **1** and **11**, i.e., compounds with the spacer length of four carbons ($m + n = 1$) show ca. 2-fold selectivity for the ERβ receptor.

4,5-Dihydroisoxazoles. Ligand docking showed that 4,5-dihydroisoxazoles **C** bind in similar conformations to ERα and ERβ in comparison with the other compound groups (**G**, **D**, **E**). Therefore, the higher overall activities of 4,5-dihydroisoxazoles **C** may be partly due to their higher hydrophobicities and conformational rigidity. Compound **11**, which has considerable ERβ activity, docked to the ligand-binding site of ERβ is shown in Figure 1a. In this conformation the length of **11** is 11.5 Å, close to 10.9 Å of E2, and it makes extensive interactions with Met336, Leu339, and Leu476 of ERβ. Compounds **15** (Figure 1b) and **19** (Figure 1c), which have the highest ERα activities among the 4,5-dihydroisoxazoles **C**, bind to ERα in a partly folded conformation. The other phenyl group of **15** and **19** is bound between Leu339 and Phe356, and the other end of the compounds is fitted in a cavity lined by Leu384, Met421, His524, and Leu525. This binding mode, which is better tolerated by ERα than ERβ, is common to compounds having $m + n > 2$.

Hydroxy Ketones. The activities of hydroxy ketones **D** are generally weaker than the activities of the corresponding isoxazoles. However, both ERα and ERβ activities of **23** are three times higher than the activities of **12** and compound **24** is the most efficient agonist for both receptors. Interestingly, enantiomers **24a** and **b** have weak but opposite selectivity for ERα and ERβ. The reason for the high activities of **23** and **24** is that they form a hydrogen bond between their hydroxyl group and the histidine side chain (His524 of ERα and His475 of ERβ) of the binding pocket (Figure 1d). In some of the docked conformations **26**−**29** formed weak hydrogen bonds, i.e., long hydrogen bonds of unfavorable geometry, with the histidine, but only **29** shows increased activity compared to the corresponding diketones. Methylation of compound **D** enhances to some extent the ERβ activity in some cases (**32**, **38**, and **40**). Altogether, the activity results clearly show that the spacer length of four carbons ($m + n = 1$) is a preferable structural feature for ERβ selective compounds, whereas the optimal length of the spacer for ERα activity is 6−7 carbons ($m + n = 3-4$). The wide range of the active isoxazole compounds suggests that variable spacer length is tolerated better by the ERα

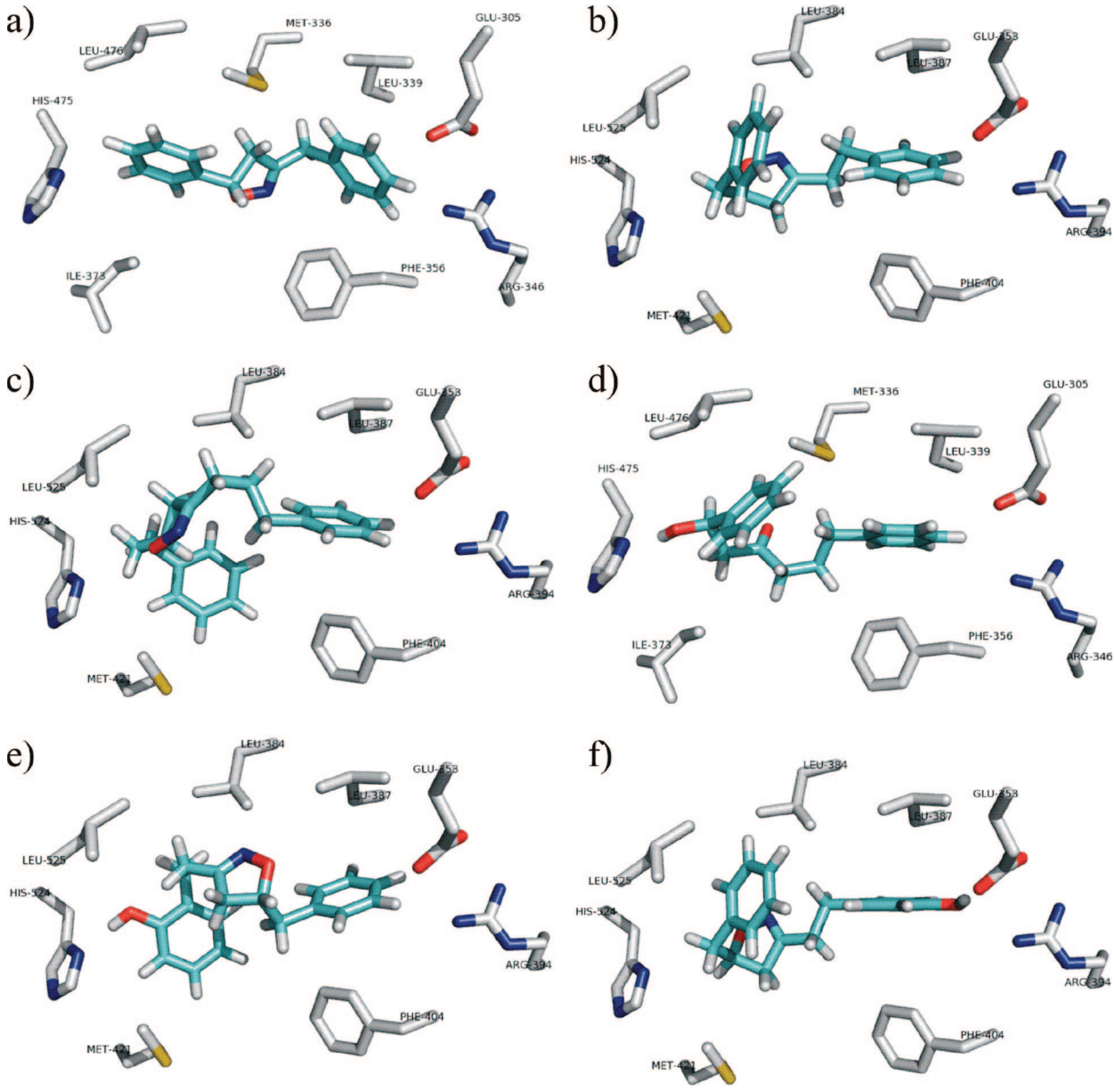


Figure 1. Docked poses of compounds (a) **11** and (d) **24** to ERβ and compounds (b) **15**, (c) **19**, (e) **70** and (f) **72** to ERα.

receptor. This difference reflects the slightly larger volume of the ligand-binding cavity of ERα (450 Å^3) than ERβ (390 Å^3).[50]

Aromatic Ring Substitution. To tentatively investigate how ring substitution affects the ER activity, we made a series of substituted analogues of compounds **15**, **26**, and **37** having $m = 2$ and $n = 1$ (See Table 2). In the present examples, *para* substitution of the benzyl group modestly increases the ERβ activity of all the compounds. As to the ERα activity, the agonistic function of **15** is completely destroyed by *para* methoxy substitution, whereas **58** shows higher value in comparison to the unsubstituted analogue **37**. On the other hand, *para* fluoro substitution of the benzyl group increases ERα activation markedly in all three cases, **51** being one of the best ERα ligands. Replacement of the phenyl ring by 1- or 2-naphthyl group increases the ERβ activity of most compounds to some extent, whereas the change of the ERα activity is dependent on the functionality of the spacer: **52** and **53** are less active, **56** and **57** equally active, and **60** and **61** more active than their phenyl analogues. In addition, we tested some naphthyl substituted analogues of an inactive compound **36** (see Table 3) and found out that compounds **64** and **65** expressed certain ERα activity. This may be due to their spatial similarity with compounds **38** and **40**: the molecules occupy about the same space in the receptor but the former compounds are conformationally more restricted. On the other hand, the latter compounds show also some ERβ activity, which is not the case with the naphthyl ligands.

As the phenol moiety is a common structural feature of numerous ER ligands, we decided to synthesize and evaluate some hydroxyl substituted analogues of ligand **15** (see Table 4), which is the best ERα agonist of the unsubstituted 4,5-dihydroisoxazoles. As seen in Table 4, *p*-fluoro substitution of the benzylic ring (**51**) increased both ERα and ERβ activities of the compound to some extent. Docking simulations showed that **51** may bind to ER in a similar fashion as **15** (Figure 1b) and, therefore, the slightly increased activity of **51** is likely due to enhanced interactions of the *p*-F-Ph group compared to Ph. Ligand **69**, having a hydroxyl group at the same position, showed even better activities, ERβ activity being 4-fold in comparison to **51**. For both **51** and **69**, the activity difference

Table 2. In Vitro Estrogen Activity of the Substituted Derivatives **50**−**61** of Ligands **15**, **26**, and **37**

Aryl group Ar	C			D			E		
	No.	ERα[a]	ERβ[b]	No.	ERα	ERβ	No.	ERα	ERβ
Ph	**15a**[c]	68.4	1.1	**26**[e]	12.8	0.4	**37**	9.9	1.3
	15b[d]	38.2	1.3						
4-MeO-Ph	**50a**	0.8	10.6	**54a**	1.6	-0.8	**58**	28.5	4.0
	50b	16.1	13.2	**54b**	1.3	1.2			
4-F-Ph	**51a**	99.2	11.2	**55**	30.1	5.7	**59**	33.1	11.2
	51b	28.9	7.4						
1-Np	**52a**	34.1	10.5	**56**	12.1	1.7	**60**	35.5	2.0
	52b	17.2	6.5						
2-Np	**53a**	22.3	8.2	**57a**	12.4	6.4	**61**	30.4	12.4
	53b	13.0	3.8	**57b**	20.1	20.1			

[a] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [b] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [c] **a** = enantiomer with a shorter retention time in the chiral separation. [d] **b** = enantiomer with a longer retention time in the chiral separation. [e] Enantiomers not separated, racemic mixture tested.

Table 3. In Vitro Estrogen Activity of the Naphthyl Derivatives **62**−**68** of Ligand **36**

No.	Aryl groups Ar[1]	Ar[2]	E ERα[a]	E ERβ[b]
36	Ph	Ph	1.4	0.3
62a[c]	4-F-Ph	1-Np	0.2	-0.6
62b[d]			4.5	-0.5
63a	2-MeO-Ph	1-Np	0	0
63b			0.6	-0.2
64a	1-Np	1-Np	5.6	-0.5
64b			23.9	0.4
65[e]	Ph	2-Np	48.1	4.7
66a	2-MeO-Ph	2-Np	3.5	-0.5
66b			4.3	-0.3
67a	2-Np	2-MeO-Ph	0.2	0.1
67b			0.3	0.3
68	2-Np	3-MeO-2-Np	14.8	2.0

[a] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [b] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [c] **a** = enantiomer with a shorter retention time in the chiral separation. [d] **b** = enantiomer with a longer retention time in the chiral separation. [e] Enantiomers not separated, racemic mixture tested.

between their two enantiomers was significant, being 4-fold for ERβ activity between **69a** and **69b**. We also prepared compounds **70**−**72** with hydroxyl group at the *o*-, *m*-, or *p*-position of the phenethyl group. In comparison to **15a**, ERα activity of **71a** having an *m*-hydroxyl was decreased significantly, whereas ERα activities of both **70a** and **72a** were markedly increased. Indeed, compounds **69**, **70**, and **72** have the highest activities of the hydroxyl substituted analogues. Ligand docking showed that the 2-OH of **70** forms a hydrogen bond of favorable geometry with the histidine (His524, Figure 1e). In contrast, the 4-OH of **69** and **72** forms hydrogen bonds with Glu353 and Arg394 of ERα (Figure 1f) mimicking the E2 phenolic A-ring, which is known to be essential for high affinity ER ligands.[17,51,52]

Table 4. In Vitro Estrogen Activity of the Hydroxyl Substituted Derivatives **69**−**76** of ligands **15** and **51**

No.	Substituents R[1]	R[2]	ERα[a]	ERβ[b]
15a[c]	H	H	68.4	1.1
15b[d]			38.2	1.3
51a	H	4-F	99.2	11.2
51b			28.9	7.4
69a	H	4-OH	34.2	9.1
69b			105.2	42.4
70a	2-OH	H	111.3	18.4
70b			39.7	30.5
71a	3-OH	H	19.2	6.7
71b			15.7	-2.5
72a	4-OH	H	171.1	143.1
72b			24.3	41.5
73a	2-OH	4-F	82.0	3.4
73b			11.1	-2.4
74a	4-OH	4-F	77.8	28.4
74b			5.7	4.4
75a	2-OH	4-OH	20.4	2.5
75b			6.2	0.6
76a	4-OH	4-OH	76.2	12.0
76b			81.4	4.1

[a] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [b] Receptor activation (a mean of at least three independent transfections, SEM typically <15%) relative to 10 nM E2 corresponding 100, sample concentration 10 μM. [c] **a** = enantiomer with a shorter retention time in the chiral separation. [d] **b** = enantiomer with a longer retention time in the chiral separation.

The activity of **72**, which is the highest of the present compounds, is clearly higher than that of **69** because the *p*-OH substituted phenethyl group of **72** fits better to the ligand binding site (Figure 1f) than the phenethyl group of **69**. Docking also explains why we could not increase the activity by combining the substituent effects manifested by compounds **73**−**76**.

Table 5. Activation of ERα and ERβ by Randomly Selected Compounds and Antagonism by 1000 nM ICI-182,780

compound (10 μM)	ER subtype	-fold activation in the absence of ICI-182,780	-fold activation in the presence of ICI-182,780
vehicle	α	1.0 ± 0.1	1.0 ± 0.1
5	α	8.7 ± 1.1	1.0 ± 0.02
65	α	22.9 ± 3.8	1.6 ± 0.3
18b	α	125.5 ± 8.6	1.0 ± 0.2
64b	α	26.1 ± 3.4	1.1 ± 0.2
E2 (1 nM)	α	163.6 ± 15.3	1.2 ± 0.2
vehicle	β	1.0 ± 0.1	0.7 ± 0.2
65	β	4.2 ± 0.4	0.7 ± 0.2
E2 (1 nM)	β	98.7 ± 6.2	0.9 ± 0.2

Ligands **75** and **76** do not fit to the binding site so that hydrogen bonds of optimal geometry could be formed at the same time with Glu353, Arg394, and His524, and in the case of **73**, the 4-F is close to Glu358, forming a repulsive interaction. The observation that in the case of **74** the 4-F substituent decreases activity (**72** vs **74**), whereas in the case of **51** it increases it (**15** vs **51**) is likely due to repulsive interactions between 4-F of **74** and Trp383. Namely, the tip of the phenethyl group of **72** (Figure 1f) and **74** is close to the Trp383 (not shown in the Figure 1). In the case of **51**, such repulsive interaction does not exist because **51** binds to ER in a slightly different orientation compared to **72** and **74** due to nonexistence of hydrogen bonds with Glu353 and Arg394.

Flexibility. A unique feature of these structures, and the original reason for their synthesis, is that the dibenzyl-1,3-dione unit contains six low rotational barriers, as demonstrated by an NMR study of 1,5-diphenylpentane-2,4-dione (**4**).[47] The rotational barrier of CH_2 adjacent to aryl is always low and therefore also the benzyl-4,5-dihydroisoxazole sp^3-sp^3 junction is rather freely rotating. Previously, these intramolecular motions of ligands have been almost completely ignored in drug design although the importance of intramolecular motions of proteins and other macromolecules is well approved for their biological activity.[53–55] The present study forms a part of our work on molecular flexibility and motions[2,47,56–58] in which we have anticipated that also intramolecular motions of ligand can play a role in molecular regulation and recognition.[2] In principle, if the ligand enhances the motions of protein in the direction of reaction coordinate, it can act as an agonist, and in the other case, it can act as antagonist. Also ligand binding affinity may depend on the motions: if the ligand binding reduces the motions of the protein, the vibrational entropy of the protein is decreased, which leads to lower binding affinity. Alternatively, a ligand may tune the motions of a protein, although at the cost of binding affinity, so that its motions correspond better the motions of another protein (such as a coactivator for the ER) and thus enhance protein−protein recognition. In this case, a poor binding affinity may mean an agonistic function.

The fact that some activity is observed also with the very flexible dione structure proposes that neither the motional complementarity is necessary (although certainly desirable) for activation: filling the binding cleft is enough. In this way, still allowing all the motions of the receptor protein, which it has in the presence of the natural ligands, the flexible ligand cuts away the motions typical for the empty receptor molecule or even enhances them to the directions arising from the protein backbone. One can also say that the binding of the cleft filling ligand leads to more correlated motions of the receptor, which can be seen as a condition for activation of the receptor.[2]

The above considerations are in fair agreement with the present conception of nuclear receptor activation. Although the activation can be largely explained by ligand-induced stabilization of the active receptor conformation (where the C-terminal helix 12 closes the ligand-binding pocket creating a binding surface for coactivator protein[59]), there exists increasing amount of evidence that the simple model of helix 12 acting as a two-state molecular switch needs to be extended.[60] Ligands have been found to affect differently the conformational dynamics of ERα[61] and modulate ERα and ERβ activities by mechanisms involving subtle differences in receptor−ligand interactions.[62,63] The fact that there is not necessarily direct correlation between the ligand affinity and transcriptional activity highlights the complexity of receptor activation. Recent studies of peroxisome proliferator-activated receptor γ have also nicely demonstrated how partial agonists activate the receptor using a helix 12 independent mechanism by inducing unique changes to the dynamics of the ligand-binding domain.[64,65]

The compounds with a few exceptions were found ERα selective. One explanation is that the ligand-binding pocket of ERβ is somewhat smaller than that of ERα. However, because the highest ERβ selectivities have been obtained with structures which are more rigid than the present ones at the site that is bound to the estrogen binding site, it is tempting to think that the selectivity reflects also the tolerances of the receptors to the ligand flexibility.

Conclusions

The compounds having an overall structure of *aryl1−linker1−spacer−linker2−aryl2* studied in this work form a family of novel estrogen analogues that operate through the ER receptors. The observation that ERα/ERβ active compounds were found from all the spacer classes indicates that these compounds excite a wide structural space, forming a 4D molecular library, supporting our original idea that the compound set can be used to monitor receptor ligand binding cavities. In this work, the highest activities were obtained with dihydroisoxazole and hydroxyketone spacers, but even the most flexible diones without any aryl substituents showed some ER agonism. The compounds having the shortest spacer lengths ($m + n = 1$) were found to be ERβ selective, whereas almost without exception the rest of the compounds ($m + n = 2-5$) showed ERα selectivity. This difference in isoform selectivity can be explained by somewhat smaller ligand-binding cavity of ERβ. The hydroxyl ketone **24** ($m + n = 3$), the other enantiomer of which showed relatively high ERβ activity was an interesting exception to this selectivity rule. The relatively high concentrations (μM) needed for a significant ER activity obviously reflect the fact that the binding free energy to the receptor is not favorable due to entropic reasons and, for most of the compounds, due to the lack of anchoring phenolic hydroxyl groups. Rigidifying the dione moiety improved the overall activity and further improvement was obtained by introducing an aromatic hydroxyl substituent on the most active 4,5-dihydroisoxazole **15**. Computer modeling showed that increased activities are due to a hydrogen bond formed between

the ligand and the receptor, and that the phenol group of the most active compound **72** mimics the interactions formed by the E2 A-ring.

Experimental Section

Materials and Equipment. Aldoximes **A** were synthesized from the corresponding aldehydes and hydroxylammonium chloride.[66] Benzaldehydes and phenyl acetaldehyde were commercial products (Aldrich) and distilled prior to use. All the other aldehydes were prepared from the corresponding alcohols by treatment with DMSO in the presence of TFAA.[67] Allyl arenes **B** were either commercial products (Aldrich) or synthesized by Grignard reaction of corresponding aryl magnesium bromides with allyl bromide. All moisture sensitive reactions were performed under a positive pressure of argon in oven-dried glassware equipped with rubber septa; solvents and liquid reagents were dried, distilled, stored under argon, and transferred using a syringe flushed with argon. Column chromatography was performed with silica gel 60 (SiO_2, 70−230 mesh). Melting points were measured with a Stuart Scientific SMP2 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra (TMS/$CDCl_3$) were recorded on a Bruker Avance 500 spectrometer operating at 500.13 and 125.77 MHz, respectively. All *J* coupling constants are given in Hz. Elemental analyses (CHNS) were carried out with a Thermo Quest CE Instruments EA 1110 CHNS-O elemental analyzer. Semipreparative HPLC separations of the enantiomers were performed on a Shimadzu chromatography system using a Regis Technologies (*R,R*)-Whelk-O 1 (25 cm × 10 mm i.d.) chiral column in *n*-hexane/*i*-*P*rOH /AcOH 80/20/0.5 at flow rate 5 mL/min. Compounds were detected by UV absorption at 254 nm.

Synthesis of 4,5-Dihydroisoxazoles C (Compounds 9−19 and 50−53). To a vigorously stirred solution of oxime **A** (20 mmol), alkene **B** (20 mmol) and pyridine (4 mmol) in 100 mL of DCM at 0 °C was added dropwise, 5% NaOCl solution (60 mL, 40 mmol), keeping the temperature under 5 °C and the mixture was reacted for 1.5 h. The organic layer was separated and washed with 2 M HCl, 2 M $NaHCO_3$, and water, and evaporated to give the crude product, which was purified by column chromatography using DCM as an eluent.

5-Benzyl-3-phenethyl-4,5-dihydroisoxazole (15). Yield 51%, a colorless oil. ^{1}H NMR δ 7.27−7.24 (m, 4 H), 7.21−7.15 (m, 6 H), 4.73 (m, 1 H), 2.95 (dd, 1 H, J = 13.8, 6.0), 2.84−2.76 (m, 3 H), 2.73 (dd, 1 H, J = 13.8, 6.9), 2.63−2.52 (m, 3 H). ^{13}C NMR δ 158.1, 140.5, 137.0 (3 s), 129.4, 128.5, 128.5, 128.2, 126.6, 126.3, 80.5 (7 d), 41.6, 40.9, 32.6, 29.4 (4 t). Anal. ($C_{18}H_{19}NO\cdot 0.1H_2O$) C, H, N.

3,5-Diphenethyl-4,5-dihydroisoxazole (18). Yield 41%, mp = 56.2−58.0 °C. ^{1}H NMR δ 7.30−7.25 (m, 4 H), 7.22−7.16 (m, 6 H), 4.49 (m, 1 H), 2.93−2.84 (m, 3 H), 2.74 (m, 1 H), 2.70−2.63 (m, 3 H), 2.47 (dd, 1 H, J = 16.8, 7.9), 1.95 (m, 1 H), 1.76 (m, 1 H). ^{13}C NMR δ 158.1, 141.3, 140.6 (3 s), 128.5, 128.4, 128.4, 128.3, 126.3, 126.0, 79.2 (7 d), 42.4, 36.9, 32.7, 31.8, 29.6 (5 t). Anal. ($C_{19}H_{21}NO$) C, H, N.

5-(4-Fluorobenzyl)-3-phenethyl-4,5-dihydroisoxazole (51). Yield 30%, mp = 51.5−52.9 °C. ^{1}H NMR δ 7.28 (m, 2 H), 7.22−7.16 (m, 3 H), 7.14 (m, 2 H), 6.96 (m, 2 H), 4.72 (m, 1 H), 2.89 (dd, 1 H, J = 14.0, 6.3), 2.86−2.78 (m, 3 H), 2.74 (dd, 1 H, J = 14.0, 6.2), 2.65−2.51 (m, 3 H). ^{13}C NMR δ 161.8 (d), 158.1, 140.5 (2 s), 132.8 (d), 130.9 (dd), 128.6, 128.2, 126.3 (3 d), 115.3 (dd), 80.4 (d), 41.6, 40.0, 32.6, 29.4 (4 t). Anal. ($C_{18}H_{18}FNO$) C, H, N.

Synthesis of Hydroxyketones D (Compounds 20−30 and 54−57). A solution of isoxazoline **C** (10 mmol), AcOH (0.1 mol) and water (1.0 mol) in 100 mL of MeOH-THF (1:1) was stirred under H_2 at RT overnight in the presence of Raney Ni (1.5 g). The catalyst was removed by filtering through celite, and the filtrate extracted by DCM. The extracts were washed with saturated $NaHCO_3$ and water and evaporated to give the pure product.

2-Hydroxy-1,7-diphenyl-heptan-4-one (27). Yield 64%, a colorless oil. ^{1}H NMR δ 7.31−7.21 (m, 5 H), 7.20−7.14 (m, 2 H), 7.14−7.11 (m, 3 H), 4.25 (m, 1 H), 3.02 (br s, 1 H, OH), 2.80 (dd, 1 H, J = 13.6, 7.1), 2.69 (dd, 1 H, J = 13.6, 7.1), 2.58 (t, 2 H, J = 7.6), 2.49 (dd, 1 H, J = 15.7, 2.5), 2.47 (dd, 1 H, J = 15.7, 6.1), 2.37 (dd, 2 H, J = 7.9, 6.7), 1.86 (m, 2 H). ^{13}C NMR δ 211.3, 141.4, 137.9 (3 s), 129.4, 128.5, 128.4, 128.4, 126.5, 126.0, 68.7 (7 d), 48.2, 42.9, 42.7, 34.9, 24.8 (5 t). Anal. ($C_{19}H_{22}O_2\cdot 0.1H_2O$) C, H.

4-Hydroxy-1,6-diphenylhexan-2-one (28). Yield 91%, a yellow wax. ^{1}H NMR δ 7.35−7.28 (m, 2 H), 7.26−7.21 (m, 3 H), 7.17−7.12 (m, 5 H), 4.00 (m, 1 H), 3.65 (s, 2 H), 3.01 (br s, 1 H, OH), 2.75 (m, 1 H), 2.65−2.52 (m, 3 H), 1.75 (m, 1 H), 1.63 (m, 1 H). ^{13}C NMR δ 209.3, 141.8, 133.6 (3 s), 129.4, 128.8, 128.4, 128.4, 127.2, 125.8, 66.9 (7 d), 50.7, 48.4, 38.0, 31.7 (4 t). Anal. ($C_{18}H_{20}O_2$) C, H.

5-Hydroxy-6-(4-fluorophenyl)-1-phenyl-hexan-3-one (55). Yield 66%, a yellow viscous oil. ^{1}H NMR δ 7.25 (m, 2 H), 7.17 (m, 1 H), 7.15−7.10 (m, 4 H), 6.96 (m, 2 H), 4.23 (m, 1 H), 2.96 (s, 1 H, OH), 2.87 (t, 2 H, J = 7.5), 2.77−2.70 (m, 3 H), 2.65 (dd, 1 H, J = 13.8, 5.9), 2.50 (m, 2 H). ^{13}C NMR δ 210.5, 161.7 (d), 140.7 (s), 133.6 (d), 130.8 (dd), 128.5, 128.2, 126.2 (3 d), 115.2 (dd), 68.5 (d), 48.4, 45.0, 42.0, 29.4 (4 t). Anal. ($C_{18}H_{19}FO_2\cdot 0.2H_2O$) C, H.

Synthesis of Methoxyketones E (Compounds 31−33, 36−41 and 58−68). To a stirred solution of hydroxyketone **D** (1 mmol) in 25 mL of MeOH was added dropwise conc HCl (0.2 mL) at 0 °C and the mixture was refluxed overnight. Then the solution was taken up with DCM, washed with saturated $NaHCO_3$ and water, and evaporated. The crude product was purified by preparative thin layer chromatography using DCM as an eluent.

2-Methoxy-1,7-diphenyl-heptan-4-one (38). Yield 36%, a reddish-brown oil. ^{1}H NMR δ 7.30−7.23 (m, 4 H), 7.21−7.11 (m, 6 H), 3.91 (m, 1 H), 3.29 (s, 3 H), 2.86 (dd, 1 H, J = 13.7, 5.8), 2.70 (dd, 1 H, J = 13.7, 6.6), 2.61−2.50 (m, 3 H), 2.42−2.29 (m, 3 H), 1.85 (m, 2 H). ^{13}C NMR δ 209.1, 141.6, 138.0 (3 s), 129.5, 128.4, 128.4, 128.3, 126.3, 125.9, 78.3 (7 d), 57.3 (q), 46.9, 43.0, 39.8, 35.0, 24.9 (5 t). Anal. ($C_{20}H_{24}O_2$) C, H.

5-Methoxy-6-naphthalen-2-yl-1-phenyl-hexan-3-one (61). Yield 95%, a pale-brown viscous oil. ^{1}H NMR δ 7.80 (d, 1 H, J = 7.3), 7.77 (d, 1 H, J = 7.1), 7.76 (d, 1 H, J = 8.3), 7.61 (s, 1 H), 7.44 (m, 2 H), 7.32 (dd, 1 H, J = 8.3, 1.7), 7.23 (m, 2 H), 7.14 (m, 1 H), 7.11 (m, 2 H), 4.01 (m, 1 H), 3.32 (s, 3 H), 3.03 (dd, 1 H, J = 13.7, 5.9), 2.88−2.79 (m, 3 H), 2.74−2.59 (m, 3 H), 2.42 (dd, 1 H, J = 16.1, 4.6). ^{13}C NMR δ 208.4, 141.0, 135.6, 133.5, 132.2 (5 s), 128.4, 128.3, 128.0, 128.0, 127.9, 127.6, 127.5, 126.0, 126.0, 125.5, 78.3 (11 d), 57.5 (q), 47.1, 45.4, 40.1, 29.4 (4 t). Anal. ($C_{23}H_{24}O_2$) C, H.

4-Methoxy-5-naphthalen-2-yl-1-phenylpentan-2-one (65). Yield 28%, a pale-yellow viscous oil. ^{1}H NMR δ 7.81−7.73 (m, 3 H), 7.58 (br s, 1 H), 7.47−7.40 (m, 2 H), 7.32−7.19 (m, 4 H), 7.10 (m, 2 H), 4.00 (m, 1 H), 3.64, 3.62 (2d, 2 H, J = 15.7), 3.30 (s, 3 H), 3.00 (dd, 1 H, J = 13.7, 6.0), 2.86 (dd, 1 H, J = 13.7, 6.3), 2.69 (dd, 1 H, J = 16.4, 7.5), 2.49 (dd, J = 16.4, 4.9). ^{13}C NMR δ 206.8, 135.6, 133.9, 133.5, 132.2 (5 s), 129.5, 128.5, 127.9, 127.9, 127.9, 127.6, 127.5, 127.0, 126.0, 125.4, 78.3 (11 d), 57.5 (q), 50.9, 46.2, 40.0 (3 t). Anal. ($C_{22}H_{22}O_2$) C, H.

Synthesis of (Dimethyl-λ^4-sulfanylidene)-diones F (Compounds 42−49). To a stirred mixture of NCS (20 mmol) in 60 mL of anhydrous DCM was added dropwise DMS (40 mmol) at −70 °C under argon, and stirring was continued for 1 h. Then a solution of hydroxyketone **D** (4.0 mmol) in 6 mL of DCM was added, maintaining the same temperature, and the solution was stirred for 1 h. TEA (60 mmol) was then added, and after 1 h, the reaction mixture was treated with saturated NaCl (20 mL). This mixture was extracted with ether, the organic layer washed with saturated NaCl, and evaporated. The residue was purified by column chromatography using 95:5 chloroform−acetone as an eluent.

3-(Dimethyl-λ^4-sulfanylidene)-1,7-diphenylheptane-2,4-dione (47). Yield 92%, mp = 92.4−94.8 °C. ^{1}H NMR δ 7.28−7.19 (m, 6 H), 7.19−7.10 (m, 4 H), 3.98 (br s, 2 H), 2.80 (br s, 2 H), 2.64 (s, 3 H), 2.64 (br s, 2 H), 1.92 (tt, 2 H, J = 7.7, 7.6). ^{13}C NMR δ 194.0, 190.3, 142.3, 137.1 (4 s), 129.2, 128.4, 128.3, 128.1,

126.2, 125.6 (6 d), 85.9 (s), 48.4, 41.1, 35.5, 27.1 (4 t), 26.6 (q). Anal. ($C_{21}H_{24}O_2S \cdot 0.1H_2O$) C, H. S: calcd, 9.37; found, 8.50.

4-(Dimethyl-λ^4-sulfanylidene)-1,7-diphenylheptane-3,5-dione (48). Yield 60%, mp = 68.5−71.0 °C. ^{1}H NMR δ 7.26−7.21 (m, 8 H), 7.17−7.13 (m, 2 H), 3.03 (br s, 4 H), 2.93 (m, 4 H), 2.68 (s, 6 H). ^{13}C NMR δ 192.8 (br s), 142.1 (s), 128.7, 128.3, 127.8 (3 d), 87.2 (s), 42.9 (br t), 32.0 (t), 27.0 (q). Anal. ($C_{21}H_{24}O_2S \cdot 0.1H_2O$) C, H, S.

Synthesis of diones G (compounds 1 and 4−8). To a stirred mixture of **F** (1.5 mmol) and Zn dust (30 mmol) in 25 mL DCM was added AcOH (30 mmol) at 0 °C, and stirring was continued at RT overnight. The mixture was filtered, and the filtrate washed with saturated $NaHCO_3$ and water, eluted with DCM through a ca. 2 cm column of Florisil (100−200 mesh), and evaporated.

1,7-Diphenylheptane-2,4-dione (6). Yield 87%, dark-brown viscous oil. ^{1}H NMR δ 15.42 (br s, 1 H, OH), 7.33−7.29 (m, 2 H), 7.27−7.14 (m, 6 H), 7.13−7.10 (m, 2 H), 5.39 (s, 1 H), 3.56 (s, 2 H), 2.59 (t, 2 H, J = 7.7), 2.24, (t, 2 H, J = 7.7), 1.89 (m, 2 H). ^{13}C NMR δ 193.9, 192.4, 141.4, 135.2 (2 s), 129.3, 128.7, 128.4, 128.4, 127.0, 126.0, (6 d), 99.4 (s), 45.2, 37.5, 35.1, 24.7 (4 t). Anal. ($C_{19}H_{20}O_2 \cdot 0.5H_2O$) C, H.

1,8-Diphenyloctane-3,5-dione (8). Yield 81%, a pale-brown viscous oil. ^{1}H NMR δ 15.46 (br s, 1 H, OH), 7.29−7.24 (m, 4 H), 7.20−7.13 (m, 6 H), 5.42 (s, 1 H), 2.92 (t, 2 H, J = 7.6), 2.62 (t, 2 H, J = 7.7), 2.59 (t, 2 H, J = 7.7), 2.27 (t, 2 H, J = 7.4), 1.92 (m, 2 H). ^{13}C NMR δ 193.8, 193.3, 141.5, 140.7 (4 s), 128.5, 128.5, 128.4, 128.3, 126.2, 126.0, 99.5 (7 d), 40.1, 37.5, 35.2, 31.5, 27.2 (5 t). Anal. ($C_{20}H_{22}O_2 \cdot 0.2H_2O$) C, H.

Synthesis of Phenolic 4,5-Dihydroisoxazoles 69−76. To a solution of a 4,5-dihydroisoxazole having an aromatic methoxyl group (2 mmol) in 2 mL of DCM was added 2.2 mL of 1 M BBr_3 in DCM (2.2 mmol, for compounds **75** and **76** having two aromatic methoxyl groups, 4.4 mmol was used), and the solution was stirred under argon at RT overnight. The organic layer was evaporated to give the oily product, which was purified by column chromatography using DCM as an eluent.

5-(4-Hydroxy-benzyl)-3-phenethyl-4,5-dihydro-isoxazole (69). Yield 51%, mp = 124.8−126.4 °C. ^{1}H NMR δ 7.25 (m, 2 H), 7.17 (m, 1 H), 7.13 (m, 2 H), 6.99 (m, 2 H), 6.78 (m, 2 H), 4.71 (m, 1 H), 2.85−2.77 (m, 4 H), 2.68 (dd, 1 H, J = 14.0, 6.4), 2.60−2.53 (m, 3 H). ^{13}C NMR δ 159.0, 155.1, 140.2 (3 s), 130.5, 128.5, 128.2 (3 d), 128.2 (s), 126.3, 115.6, 80.9 (3 d), 41.5, 39.8, 32.5, 29.3 (4 t). Anal. ($C_{18}H_{19}NO_2 \cdot 0.75H_2O$) C, H, N.

5-Benzyl-3-[2-(2-hydroxy-phenyl)-ethyl]-4,5-dihydro-isoxazole (70). Yield 96%, brown viscous oil. ^{1}H NMR δ 7.28 (m, 2 H), 7.23 (m, 1 H), 7.18 (m, 2 H), 7.11 (m, 1 H), 6.71−6.67 (m, 3 H), 5.57 (br s, 1 H, OH), 4.78 (m, 1 H), 2.96 (dd, 1 H, J = 13.9, 6.0), 2.85 (dd, 1 H, J = 17.1, 10.1), 2.78−2.72 (m, 3 H), 2.65−2.55 (m, 3 H). ^{13}C NMR δ 159.4, 156.4, 141.8, 136.7 (4 s), 129.7, 129.3, 128.5, 126.7, 120.1, 115.4, 113.7, 80.7 (8 d), 41.6, 40.7, 32.3, 29.0 (4 t). Anal. ($C_{18}H_{19}NO_2$) H. C: calcd, 76.84; found, 76.00. N: calcd, 4.98; found, 4.31.

5-Benzyl-3-[2-(3-hydroxy-phenyl)-ethyl]-4,5-dihydro-isoxazole (71). Yield 99%, mp = 120.6−122.8 °C. ^{1}H NMR δ 7.81 (br s, 1 H, OH), 7.28 (m, 2 H), 7.22 (m, 1 H), 7.18 (m, 2 H), 7.07 (m, 1 H), 6.92 (d, 1 H, J = 7.9), 6.84 (t, 1 H, J = 6.9), 4.79 (m, 1 H), 2.96 (dd, 1 H, J = 13.9, 6.3), 2.91−2.85 (m, 3 H), 2.78 (dd, 1 H, J = 13.9, 6.5), 2.70−2.62 (m, 3 H). ^{13}C NMR δ 160.2, 154.5, 136.7 (3 s), 130.2, 129.3, 128.5, 127.8 (4 d), 127.2 (s), 126.7, 120.4, 116.9, 81.0 (4 d), 41.8, 40.6, 28.8, 26.6 (4 t). Anal. ($C_{18}H_{19}NO_2 \cdot H_2O$) N. C: calcd, 72.22; found, 71.68. H: calcd, 7.07; found, 6.57.

5-Benzyl-3-[2-(4-hydroxy-phenyl)-ethyl]-4,5-dihydro-isoxazole (72). Yield 89%, mp = 143.0−144.8 °C. ^{1}H NMR δ 7.29 (m, 2 H), 7.22 (m, 1 H), 7.18 (m, 2H) 6.98 (m, 2 H), 6.79 (m, 2 H), 6.52 (br s, 1 H, OH), 4.78 (m, 1 H), 2.95 (dd, 1 H, J = 13.9, 6.1), 2.88 (dd, 1 H, J = 17.1, 10.2), 2.76 (dd, 1 H, J = 13.9, 6.7), 2.72 (m, 2 H), 2.62 (dd, 1 H, J = 17.1, 7.5), 2.57 (m, 2 H). ^{13}C NMR δ 159.3, 155.4, 137.0, 131.4 (4 s), 129.5, 129.3, 128.6, 126.8, 115.5, 80.8 (6 d), 41.7, 40.9, 31.9, 29.7 (4 t). Anal. ($C_{18}H_{19}NO_2 \cdot 0.75H_2O$) C, H, N.

5-(4-Fluoro-benzyl)-3-(2-hydroxy-phenethyl)-4,5-dihydro-isoxazole (73). Yield 99%, mp = 124.8−126.4 °C. ^{1}H NMR δ 7.12−7.09 (m, 3 H), 7.07 (d, 1 H, J = 7.8), 6.92 (m, 2 H), 6.71−6.67 (m, 2 H), 6.66 (d, 1 H, J = 7.7), 4.71 (m, 1 H), 2.87−2.81 (m, 2 H), 2.76−2.68 (m, 3 H), 2.61−2.50 (m, 3 H). ^{13}C NMR δ 161.7 (d), 159.3, 156.4, 141.8 (3 s), 132.6 (d), 130.9 (dd), 129.7, 120.1, 115.4 (dd), 115.2, 113.6, 80.5 (3 d), 41.6, 39.9, 32.3, 28.9 (4 t). Anal. ($C_{18}H_{18}FNO_2 \cdot 0.5H_2O$) C, H, N.

5-(4-Fluoro-benzyl)-3-(4-hydroxy-phenethyl)-4,5-dihydro-isoxazole (74). Yield 62%, a dark-brown viscous oil. ^{1}H NMR δ 7.11 (m, 2 H), 6.95−6.92 (m, 4 H), 6.75 (m, 2 H), 4.72 (m, 1 H), 2.90−2.83 (m, 2 H), 2.74−2.66 (m, 3 H), 2.59−2.52 (m, 3 H). ^{13}C NMR δ 161.7 (d), 159.3, 154.8 (2 s), 132.5 (d), 131.5 (s), 130.9 (dd), 129.2, 115.6 (2 d), 115.3 (dd), 80.5 (d), 41.5, 39.8, 31.6, 29.3 (4 t). Anal. ($C_{18}H_{18}FNO_2 \cdot H_2O$) C, N. H: calcd, 6.35; found, 5.78.

5-(4-Hydroxy-benzyl)-3-(2-hydroxy-phenethyl)-4,5-dihydro-isoxazole (75). Yield 98%, a dark-brown viscous oil. ^{1}H NMR (in CD_3OD) δ 7.04−7.00 (m, 4 H), 6.74 (d, 1 H, J = 7.6), 6.73−6.69 (m, 3 H), 4.69 (m, 1 H), 2.95 (dd, 1 H, J = 17.2, 10.1), 2.82−2.78 (m, 3 H), 2.72−2.56 (m, 4 H). ^{13}C NMR (in CD_3OD) δ 161.4, 156.9, 156.2 (3 s), 131.3, 131.0 (2 d), 129.1 (s), 128.4 (d), 127.8 (d), 120.5 (s), 116.1, 115.9, 82.3 (3 d), 42.1, 40.8, 28.6, 28.3 (4 t). Anal. ($C_{18}H_{19}NO_3 \cdot H_2O$) C, H, N.

5-(4-Hydroxy-benzyl)-3-(4-hydroxy-phenethyl)-4,5-dihydro-isoxazole (76). Yield 99%, a dark-brown viscous oil. ^{1}H NMR (in CD_3OD) δ 7.01 (m, 2 H), 6.99 (m, 2 H), 6.71 (m, 2 H), 6.71 (m, 2 H), 4.69 (m, 1 H), 2.89 (dd, 1 H, J = 17.2, 10.2), 2.76 (dd, 1 H, J = 14.0, 6.1), 2.72−2.62 (m, 4 H), 2.55 (m, 2 H). ^{13}C NMR (in CD_3OD) δ 160.8, 157.0, 156.7, 132.5 (4 s), 131.4, 130.2 (2 d), 129.0 (s), 116.2, 116.1, 82.2 (3 d), 42.1, 40.8, 32.7, 30.4 (4 t). Anal. ($C_{18}H_{19}NO_3 \cdot H_2O$) C, H, N.

Cell Culture, Transfection, and Reporter Assays. E2 was bought from Sigma Chemical Co. (St. Louis, MO), and ICI-182,780 was from Tocris (Avonmouth, UK). All other reagents were of reagent grade from Sigma or Fluka. One day before transfection, HEK293 cells were seeded in 48-well plates (70×10^3 cells per well) in phenol-free Dulbecco's modified Eagle medium supplemented with 5% delipidated fetal bovine serum (Sigma) and antibiotics.[68] After a medium change, the cells were transfected for 4 h with 5 ng ERα or ERβ expression vector,[69] 75 ng reporter plasmid pERE$_2$TATA-LUC,[70] and 20 ng control plasmid pCMVβ by the calcium phosphate method.[68] After transfection, the cells received fresh medium containing either vehicle (0.1% v/v) or test compound (10 μM). In antagonism studies, 1000 nM ICI-182,780 or vehicle was included in medium. After 24 h, the cells were washed, lysed, and assayed for luciferase and β-galactosidase activities[68] with a Victor^2 reader (Perkin-Elmer Wallac, Turku, Finland). After normalization for β-galactosidase activity, luciferase activities are expressed relative to that of 10 nM E2 by the formula: activity = 100% × [(test compound) − (vehicle)/(E2) − (vehicle)], where terms in parentheses indicate the corresponding normalized luciferase activities. Typically, more than 90-fold activation by 10 nM E2 of luciferase with both ER subtypes was seen. The data are means ± SEM of at least three independent transfections.

Molecular Modeling. To explore the fit of our structures into the estrogen receptors, all the compounds of Tables 1 and 3 were docked to the ligand-binding site of the human estrogen receptor α and β using the Gold ligand docking program.[71] Ligands, which were built using the Sybyl 7.0 program[72] and minimized with the Tripos force field and default settings, were docked to the ligand-binding site of ERα and ERβ with the GOLD program (version 2.1).[71] The X-ray structure of ERα complexed with genistein (PDB code 1×7R)[52] and ERβ complexed with ERB-041 (PDB code 1×7B)[17] were used for docking calculations. For each ligand, docking runs were performed with a maximum number of 10 poses using default parameters. Goldscore was used as the scoring function. The docked structure having the highest score of the 10 poses was selected to represent the protein−ligand complex. Because the enantiomers of the compounds were not resolved experimentally, they were ranked on the basis of the scoring values.

Acknowledgment. We would like to thank Tarja Ihalainen and Kaarina Pitkänen for expert technical assistance and Tiina Koivunen for performing the elemental analyses. J.P. (grant no. 75791) and P.H. (grant no. 51610) were supported in part by the Academy of Finland.

Supporting Information Available: Characterization and elemental analysis data; references for the previously reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM8001795