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# Synthesis and biological evaluation of phenolic 4,5-dihydroisoxazoles and 3-hydroxy ketones as estrogen receptor α and β agonists

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ABSTRACT

In this work, 52 diphenyl-4,5-dihydroisoxazoles and -3-hydroxy ketones were prepared and their estrogen receptor α (ERα) and estrogen receptor β (ERβ) activities were explored in order to systematize and maximize their biological activity. The biological activity was firstly screened by using ERE reporter assay to find out how aromatic hydroxylation and methylation of the chiral centers of the compounds affect the ability of ER to mediate biological responses. For selected 19 compounds, the relative binding affinities (RBA, relative to 3,17β-estradiol) and ability to induce transcription of primary E2 target gene *pS2* in human MCF-7 breast cancer cells were determined. In the reporter assay, many compounds showed even stronger activity than E2 and some of them showed RBA larger than 1%. The highest RBAs were determined for the enantiomers of 1-hydroxy-6-(4-hydroxy-phenyl)-1-phenyl-hexan-3-one (**50a** and **50b**). Isomer **50a** showed high binding affinity both to ERα (with RBA ~200%) and ERβ (with RBA ~60%), while the RBAs of **50b** were ca. 40% of those. Some of the other compounds (with RBA ~1–16%) showed also notable ERα binding selectivity. When four most promising ligands (**50a**, **50b**, **45a**, and **45b**) were studied with respect to their ability to induce the transcription of primary E2 target gene *pS2*, the compounds acted as agonists or partial agonists. Computer modeling was used to predict receptor binding conformations and to rationalize the RBA differences of the compounds.



## 1. Introduction

Naturally occurring and synthetic estrogens have been widely utilized in the treatment of broad variety of indications.[1–5] Because of the high therapeutic value, there is a continuous need for novel compounds which display estrogenic behavior with selective effects on different estrogen-responsive tissues (i.e., selective estrogen receptor modulators = SERMs).[6] There are two common subtypes of estrogen receptors, ERα[7] and ERβ,[8] both of which are activated by their endogenous ligand 3,17β-estradiol (E2). Although most estrogen target tissues comprise cells expressing both ER isoforms, ERα is predominantly found in reproductive tissues, kidney, liver as well as central nervous system,[9,10] and it is the predominant subtype expressed in breast cancer.[11] On the other hand, ERβ is present in many tissues including colon, prostate, testis, ovary, vascular endothelium, and bone.[9,11] There are plenty of examples of subtype-specific compounds[2,4,9–21] and recently it has been shown that, for example, raloxifene which is used for the prevention and treatment of osteoporosis, is a relatively ERβ-selective SERM.[22] However, not many of the ER agonists or antagonists currently in clinical use are subtype-specific. Due to the adverse effects of ER-targeted drugs, wide variation in ERα and ERβ expression in various tissues, and cell- and promoter-specific effects of the ER subtypes, novel chemical scaffolds are being sought in order to develop both subtype-specific and tissue-selective ligands.

The vast majority of the synthetic estrogenic compounds have a general structure of ARYL1–*CORE STRUCTURE*–ARYL2[19] in which the core may have a third aromatic ring and an additional substituent or side chain. With a few exceptions,[20,21] the structures can be considered as more or less rigid. Recently, we reported novel estrogen agonists, which have a core structure consisting of a spacer and flexible linkers between the spacer and the aryl groups.[23] The spacers have chiral $sp^3$-centres and varying lengths of the $CH_2$-linker provide a distance of 4–8 carbons between the aromatic rings. The in vitro studies showed that the best estrogen agonists had a 4,5-dihydroisoxazole or 3-hydroxyketone moiety as the spacer. As a follow-up of the study, we report here the estrogen agonism of several novel phenolic diaryl 4,5-dihydroisoxazoles and 3-hydroxyketones and show how the aromatic hydroxylation and

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methylation of the spacers' chiral centres affect the activity. The fit of the agonists into the ERα and ERβ binding pockets is explored and rationalized by molecular modeling.

## 2. Results

### 2.1. Syntheses

All designed compounds were synthesized starting from appropriate aromatic aldoximes **A** and alkenes **B**, as shown in Scheme 1, using similar methods and experimental conditions described previously in detail by us.[23,24] The dipolar cycloaddition reaction leads to 4,5-dihydroisoxazoles **C**, the aromatic methoxy groups of which can be demethylated by $BBr_3$ to produce the phenol function of the compounds **D**. Finally, the phenolic isoxazoles **D** were converted to corresponding hydroxy ketones **E** by catalytic hydrogenation and hydrolysation with Raney Ni in the presence of $H_2O$ and AcOH. After preparation, all compounds were run through semi-preparative HPLC system[23] and, with one exception, we were able to separate all the enantiomers of compounds **D** (Tables 1 and 2). Neither specific rotation nor absolute configuration was resolved due to the small amounts of the purified enantiomers. For each separation, the enantiomer with a shorter retention time in the chiral HPLC column is marked in the tables as enantiomer **a** and the other form as **b**.

### 2.2. Biological evaluation

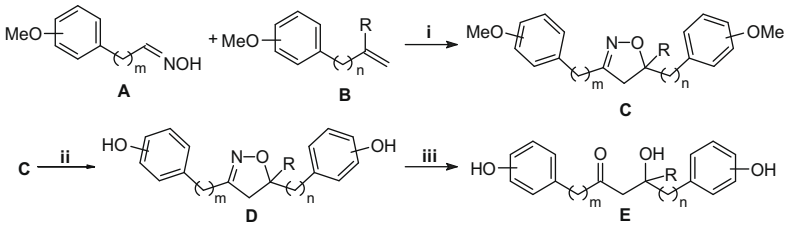
The estrogen activities (Tables 1 and 2) were evaluated in HEK293 cells that lack endogenous ER expression[25] by using transient transfection of ERα or ERβ expression vectors and an ERE-driven luciferase reporter as described.[23,26] The reporter activities of the test compounds (10 μM) were normalized to the activation observed for 10 nM E2 (= 100), and the specificity activation was ascertained by transfections lacking ERs and inclusion of the ER antagonist ICI-182,780.[23] The relative ER binding affinities (RBA, Table 3) were determined by a competitive assay against [6,7-$^3$H(N)]estradiol in COS-1 cells using a wide range of concentrations to determine each chemical's $EC_{50}$ and relative binding affinity (RBA) values as compared to E2 (= 100%).[27] Overall, the phenolic hydroxyl group as $R^1$ substituent (Aryl 1, see Tables 1 and 2 for the structures) is a structural feature important for the affinity of the ligands and in comparison to unsubstituted analogs, compounds with a phenolic Aryl 1 have both higher receptor activities and RBAs. Although most phenolic compounds are ERα selective, in several cases (compounds **3**, **10**, **21**, **24**, **30**, **42**, **45**, and **51**) high ERβ activities emerge by adding the hydroxyl especially to the *para*-position of Aryl 1. On the other hand, *para*-substitution of Aryl 2 (**5b**, **12**, and **25**) also enhances the activities to some extent. Based on the RBAs phenolic 4,5-dihydroxyisoxazole derivative **45** as well as 3-hydroxyketones **21** and **50** bind strongly to ERα (RBA = 11–218%). Ligands **21ab**, **45a**, and **45b** show 40–150-fold ERα selectivity over ERβ. The most potent compound is **50a**/**50b**, whose affinities are comparable to those of the endogenous ERα/ERβ ligand E2.

The most promising ligands **45a**, **45b**, **50a**, and **50b** that had high or medium high affinities to ERα and ERβ were further studied with respect to their ability to induce transcription of primary E2 target gene *pS2* in human MCF-7 breast cancer cells. Our data suggest that with close to physiological hormone concentration of 10 nM the *pS2* mRNA expression was induced by 2.2-fold in response to 24 h E2 treatment (Fig. 1A). Enantiomers **45b** and **50a** did not show statistically significant ligand effects with 10 nM (Fig. 1C and D) or with 1000-fold higher ligand concentrations (data not shown), whereas **50b** showed identical *pS2* mRNA expression profile to that of E2 (Fig. 1E). Interestingly, the compound **45a** showed a significant 1.8-fold induction already after 6 h ligand treatment but after 24 h treatment only basal expression could be observed. The above four compounds were also tested by competing 10 nM E2 with 100-fold higher concentrations of the different compounds prior to *pS2* mRNA expression assays (Fig 1F). According to our results, competition with ligands **45a**, **45b**, **50a** or **50b** decreased the E2 induced *pS2* activity by 32%, 27%, 23% or 29%, respectively. Thus, the *pS2* activity after competition was close to that observed in respective expression assays, suggesting that the synthetic compounds replaced the natural E2.

### 2.3. Modeling

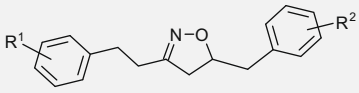
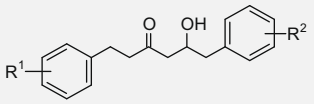
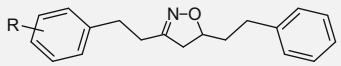
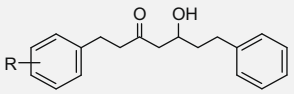
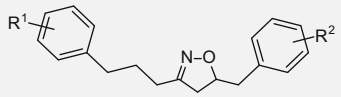
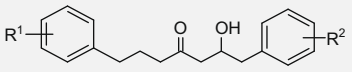
To characterize binding conformations and to rationalize the ERE reporter activities and RBAs, compounds **12**–**51** (total of 24 compounds) of Table 3 were docked to the ERα and ERβ receptors, and MD simulations were done for the most potent compounds **50a/50b** to relax their ERα and ERβ complexes. Based on modeling, the Aryl 1 (Aryl 2 in some of the molecules) phenol group mimics, as expected, the A ring of the natural agonist E2 by being involved in a hydrogen bond network with $Arg_{394}/Arg_{346}$ (ERα/ERβ), $Glu_{353}/Glu_{305}$ and a structural water molecule (Fig. 2). It also forms an edge-to-face interaction with $Phe_{404}/Phe_{356}$. Ligand docking also indicated that the increase in affinity could be largely explained by formation of hydrogen bonds between the compounds and the binding site residues. Molecules **21ab**, **45a**, **45b**, **50a**, and **50b**, which are good ERα and ERβ activators and had the largest RBAs of the molecules in this work, were the only ones predicted to form three hydrogen bonds of favorable geometry with the receptors. The other good receptor binders form similar van der Waals contacts with the receptor, but lack the hydrogen bond to His540/His475.

The MM-PBSA calculations proposed also the absolute configurations of **50a** and **50b**, which are not available within reasonable efforts because the compounds form oily products and their X-ray analysis would demand isolation of the compounds in larger amounts than done so far and then also derivatization. The calculations for **(*R*)-50** resulted more negative binding free energy than the **(*S*)-50** isomer to both ERα (−34.2 kcal/mol vs −32.9 kcal/mol) and ERβ (−30.8 kcal/mol vs −29.1 kcal/mol). In line with this, the



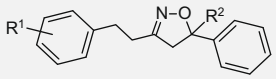
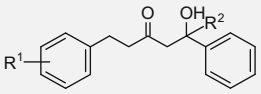
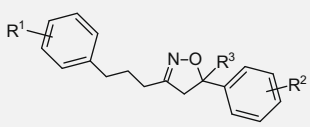
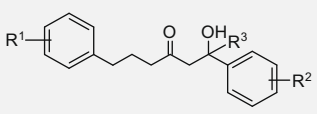

**Scheme 1.** Synthesis of the hydroxyl substituted diphenyl 4,5-dihydroisoxazoles and 3-hydroxy ketones; *m* = 2 or 3, *n* = 0–2, R = H or Me. Reagents and conditions: (i) NaOCl, pyridine, $CH_2Cl_2$, 0 °C, 2 h; (ii) $BBr_3$, $CH_2Cl_2$, rt, overnight; (iii) $H_2$, Raney Ni, $H_2O$, AcOH, MeOH–THF, rt, overnight.

**Table 1**
In vitro estrogen receptor activation of ligands **1**–**34**

| Substituents | |  | | | | | |  | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $R^1$ Aryl 1 | $R^2$ Aryl 2 | No. | ERα[a] | ERβ[b] | No. | ERα | ERβ | No. | ERα | ERβ |
| H | H | **1a**[c] | 68.4 | 1.1 | **1b**[d] | 38.2 | 1.3 | **8ab**[e] | 12.8 | 0.4 |
| 2-OH | H | **2a** | 111.3 | 18.4 | **2b** | 39.7 | 30.5 | **9ab** | 45.2 | 5.1 |
| 4-OH | H | **3a** | 171.1 | 143.1 | **3b** | 24.3 | 41.5 | **10ab** | 55.0 | 106.7 |
| H | 4-F | **4a** | 99.2 | 11.2 | **4b** | 28.9 | 7.4 | **11ab** | 30.1 | 5.7 |
| H | 4-OH | **5a** | 34.2 | 9.1 | **5b** | 105.2 | 42.4 | **12ab** | 51.4 | 6.1 |
| 2-OH | 4-F | **6a** | 82.0 | 3.4 | **6b** | 11.1 | −2.4 | **13ab** | 21.6 | 11.1 |
| 4-OH | 4-F | **7a** | 77.8 | 28.4 | **7b** | 5.7 | 4.4 | **14ab** | 28.6 | 21.2 |
| | |  | | | | | |  | | |
| R Aryl 1 | | No. | ERα | ERβ | No. | ERα | ERβ | No. | ERα | ERβ |
| H | | **15a** | 3.4 | 0.2 | **15b** | 43.9 | −0.4 | **19ab** | 16.2 | 1.0 |
| 2-OH | | **16a** | 4.5 | 4.2 | **16b** | 21.8 | 2.6 | **20ab** | 4.0 | 1.1 |
| 3-OH | | **17a** | 8.3 | 2.6 | **17b** | 3.0 | 3.6 | **21ab** | 87.3 | 48.1 |
| 4-OH | | **18a** | 62.8 | 15.4 | **18b** | 15.8 | 0.8 | **22ab** | 46.8 | 10.1 |
| | |  | | | | | |  | | |
| $R^1$ Aryl 1 | $R^2$ Aryl 2 | No. | ERα | ERβ | No. | ERα | ERβ | No. | ERα | ERβ |
| H | H | **23a** | 31.3 | 13.6 | **23b** | 28.1 | 5.1 | **29ab** | 24.7 | 9.1 |
| 4-OH | H | **24a** | 153.7 | 78.2 | **24b** | 75.1 | 19.8 | **30ab** | 105.2 | 82.3 |
| H | 4-OH | **25a** | 67.1 | 4.7 | **25b** | 97.2 | 17.0 | **31ab** | 16.0 | 2.4 |
| 4-OH | 4-OH | **26a** | 14.4 | −0.9 | **26b** | 13.6 | 10.6 | **32a** | 15.4 | 1.8 |
| | | | | | | | | **32b** | 2.8 | −1.6 |
| 3,4-$(OH)_2$ | H | **27a** | 0.3 | 1.3 | **27b** | 2.1 | −1.3 | **33ab** | −0.5 | −0.8 |
| 3,4-$(OH)_2$ | 4-OH | **28a** | 1.7 | −1.1 | **28b** | 1.4 | −0.7 | **34ab** | 0.5 | −0.6 |

[a,b] Receptor activation relative to 10 nM E2 corresponding 100 (a mean of at least three independent transfections, SEM typically <15%), sample concentration 10 μM. The activities of **1**–**8**, **11**, **15**, **19**, **23** and **29** have been reported previously.[23]
[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 2**
In vitro estrogen receptor activation of ligands **35**–**52**

| Substituents | | |  | | | | | |  | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $R^1$ Aryl 1 | $R^2$ | | No. | ERα[a] | ERβ[b] | No. | ERα | ERβ | No. | ERα | ERβ | No. | ERα | ERβ |
| H | H | | **35a**[c] | 16.4 | 9.8 | **35b**[d] | 11.2 | 8.1 | **40a** | 78.8 | 20.5 | **40b** | 25.3 | 2.8 |
| H | $CH_3$ | | **36a** | 8.1 | −20.0 | **36b** | 10.9 | −7.3 | **41ab**[e] | 52.1 | 38.4 | | | |
| 2-OH | $CH_3$ | | **37a** | 11.8 | −0.4 | **37b** | 21.7 | 30.0 | | | | | | |
| 3-OH | $CH_3$ | | **38a** | 21.6 | 0.5 | **38b** | 7.1 | −2.9 | | | | | | |
| 4-OH | $CH_3$ | | **39a** | 39.7 | 18.0 | **39b** | 18.7 | 37.4 | **42ab** | 80.3 | 116.3 | | | |
| | | |  | | | | | |  | | | | | |
| $R^1$ Aryl 1 | $R^2$ Aryl 2 | $R^3$ | No. | ERα | ERβ | No. | ERα | ERβ | No. | ERα | ERβ | No. | ERα | ERβ |
| H | H | H | **43a** | 44.3 | −3.1 | **43b** | 11.5 | 17.2 | **48a** | 91.4 | 147.3 | **48b** | 59.3 | 28.2 |
| H | H | $CH_3$ | **44ab** | 69.5 | 15.5 | | | | **49ab** | 83.3 | 15.1 | | | |
| 4-OH | H | H | **45a** | 103.6 | 44.7 | **45b** | 65.0 | 77.1 | **50a** | 139.2 | 170.2 | **50b** | 140.0 | 155.3 |
| 4-OH | H | $CH_3$ | **46a** | 14.4 | −0.9 | **46b** | 13.6 | 10.6 | **51ab** | 90.9 | 103.1 | | | |
| 4-OH | 3-OH | H | **47a** | 32.9 | 12.0 | **47b** | 27.6 | 7.2 | **52a** | 87.4 | 107.8 | **52b** | 49.3 | 43.5 |

[a–e] See Table 1. The activities of **35**, **40**, **43**, and **48** have been reported previously.[23]

**Table 3**
Relative binding affinities (RBA) of some of the estrogen active compounds in comparison to E2 and tamoxifen for ERα and ERβ

| No. | ERα RBA (%) | ERβ RBA (%) | α/β ratio |
|---|---|---|---|
| **E2** | 100.00 | 100.00 | 1 |
| **Tamoxifen** | 1.51 | 2.18 | 0.7 |
| **3a** | 0.91 | 0.16 | 6 |
| **3b** | 0.09 | 0.18 | 0.5 |
| **10ab** | 0.03 | 0.16 | 0.2 |
| **21ab** | 10.60 | 0.10 | 106 |
| **22ab** | 0.05 | 0.20 | 0.3 |
| **30ab** | 1.90 | 0.18 | 11 |
| **43a** | 0.10 | N.D.[a] | |
| **43b** | 0.04 | 0.04 | 1 |
| **44ab** | 0.03 | 0.02 | 1.5 |
| **45a** | 15.68 | 0.10 | 157 |
| **45b** | 6.53 | 0.16 | 41 |
| **46a** | 0.78 | 0.06 | 13 |
| **46b** | 0.01 | 0.01 | 1 |
| **48a** | 0.12 | 0.18 | 0.7 |
| **48b** | 0.09 | 0.02 | 5 |
| **49ab** | 0.24 | 0.10 | 2 |
| **50a** | 217.80 | 57.65 | 4 |
| **50b** | 92.02 | 18.67 | 5 |
| **51ab** | 0.32 | 0.04 | 8 |

[a] Not detected.

thermodynamic integration (TI) free energy calculations predicted that the **(*R*)-50** enantiomer has 1.64 kcal/mol (ERα–**(*R*)-50** starting structure) and 1.68 kcal/mol (ERα–**(*S*)-50** starting structure) more negative binding free energy than the **(*S*)-50** enantiomer. This suggests that **50a** having the higher experimental affinity has *R* configuration.

Docking of compound **45** suggests that the main difference between the *R* and *S* enantiomers is in the rotation of the isoxazole ring (Fig. 3). The 4-OH of the Aryl 1 mimics the hydroxyl group of the natural ligand E2 and participates in the hydrogen bonding network between the residues $Arg_{394}/Arg_{346}$ (ERα/ERβ), $Glu_{353}/Glu_{305}$ and a structural water molecule. The isoxazole ring of the *R* isomer was found to be in close contact with $Met_{336}$ of ERβ. However, in the ERα the corresponding residue $Leu_{384}$ is smaller and leaves more space for the isoxazole ring of the *R* isomer. In this group of compounds the **a** isomer showed higher affinities and activities in ERα, whereas in ERβ the **b** isomer displayed higher affinity and activity than the **a** isomer (Tables 2 and 3). This difference suggests that the **a** isomer of this molecule series is the *R* enantiomer, and the change of the favored form results from the steric clash of the $Met_{336}$ with the isoxazole ring reducing the affinity of the *R* isomer to ERβ.

## 3. Discussion

Tables 1 and 2 show the ERE reporter activation indexes (in percents). There are several compounds for which the index is >100, which means that the compounds at 10 μM concentration are able

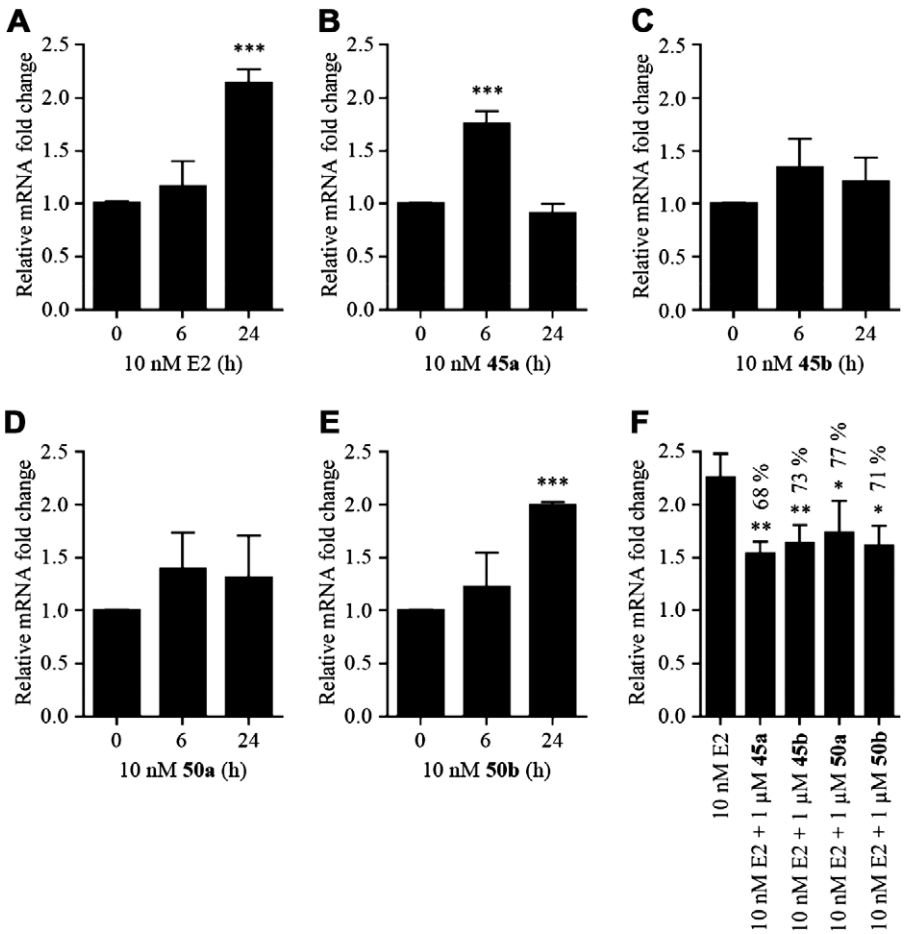



**Figure 1.** Expression profiling of the human *pS2* gene. The MCF-7 human breast cancer cells were treated with 10 nM of E2 (A) or compounds **45a** (B), **45b** (C), **50a** (D) or **50b** (E) for 6 and 24 h prior to RNA extraction. In competition assays (F), the cells were treated for 24 h with 10 nM E2 and 1 μM of above compounds prior to RNA extraction. The remaining *pS2* activities are indicated as % of E2 activity above bars. The results are calculated as fold changes to solvent (EtOH, 0.1% final concentration), and they are normalized to *RPLP0*. Two-tailed Student's *t*-tests were performed to calculate *p*-values in reference to EtOH treatment ($^{*}p$ = 0.01–0.05, $^{**}p$ = 0.001–0.01, $^{***}p$ <0.001). In each panel, *n* is at least 3. Error bars indicate S.D.

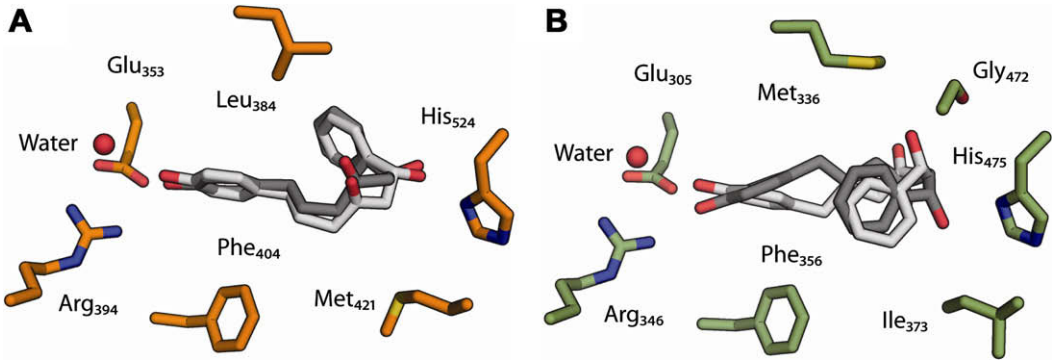



**Figure 2.** Average structures of the MD simulations of **(*R*)-50** and **(*S*)-50** in ERα (A) and ERβ (B). The positions of the residues shown are from the simulations with the *R* isomer (light gray, the *S* isomer is shown in dark gray). According to docking, the ligand-binding modes differ between the ER subtypes: the phenolic (Aryl 1) and skeletal hydroxyl groups are located at the same position and form hydrogen bonds in both of subtypes, but the unsubstituted Aryl 2 ring adopts two different orientations. The favored binding mode results from the amino acid differences $Leu_{384}$/$Met_{336}$ and $Met_{421}$/$Ile_{373}$ (ERα/ERβ): the unsubstituted phenyl ring binds so that it is close to $Leu_{384}$ (ERα or $Ile_{373}$ (ERβ). In ERβ, compound **(*R*)-50** formed hydrogen bond with the backbone carbonyl oxygen of $Gly_{472}$.

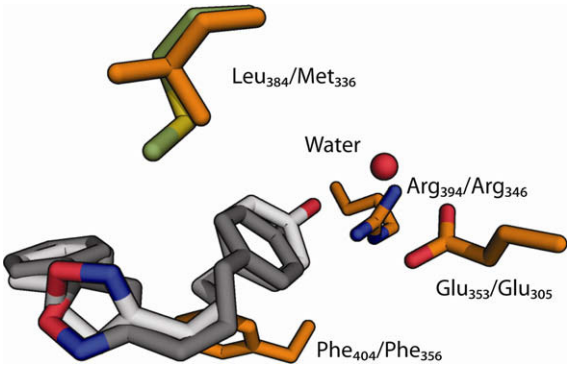



**Figure 3.** Binding of **(*R*)-45** (light gray) and **(*S*)-45** (dark gray). $Met_{336}$ hinders sterically especially the *R* isomer of compound **45** in ERβ reducing its affinity. The corresponding residue $Leu_{384}$ of ERα is smaller which, in comparison to ERβ, leads to generally higher affinities of compounds **43**–**46** and favors the *R* form.

to activate the ERα and ERβ receptors in HEK293 cells better than E2 at 10 nM. On the other hand, the RBAs (Table 3) determined with competitive assay against [6,7-$^3$H(N)]estradiol in COS-1 cells for a few selected structures show that there are compounds, which own good or even higher binding affinity to the receptors than E2 (RBA = 100). At the 10 μM concentration used in the reporter activity screening, the receptors can be assumed to be fully saturated by these ligands. In other examples the large activation indexes, in spite of their low RBA, also suggest that corresponding binding affinity must be so high that the receptor is largely occupied by them at 10 μM. In the following discussion we try to characterize the key features of good binders and activators.

Ligands **1**–**14** have a core structure ($m$ = 2, $n$ = 1, Scheme 1) of length of six carbons (Table 1). Several of these compounds show high estrogen activity but their RBAs are low and, therefore, we conclude that a high RBA may not be obtainable with these spacer constructs. In this construct the compounds with 4,5-dihydroisoxazole spacer are in all cases more active than the corresponding hydroxyketones. The unsubstituted compounds **1** and **8** are both clearly ERα selective, enantiomer **1a** being the most active and selective one. Substitution of Aryl 1 with an *ortho*-hydroxyl group (compound **2**) enhances the ERα activity of the isoxazole isomer **2a**, and retains its receptor selectivity whereas for **2b** also the ERβ activity is increased and the receptor selectivity lost. On the other hand, *para*-OH substitution of Aryl 1, which is supposed to mimic the E2 phenolic A-ring,[23] leads to dramatic enhancement of both receptor activities of the isoxazole isomer **3a**, which is actually one of the strongest ERα activators in the present paper but it shows only modest binding affinity to ERα. For the corresponding hydroxyketone **10ab**, both ERα and ERβ activities are enhanced, showing also a rare ERβ selectivity (α/β of 0.5) in our collection of compounds. As to the Aryl 2, *para*-substitution with fluorine and OH has only minor or negative effects on ERα activity of the **a** isomers. *Para*-OH group in **5b** enhances both the activities, suggesting that the binding mode is reverse. The trends with hydroxyketones are more or less similar.

In comparison to **1**–**14**, compounds **15**–**22** have one carbon longer core structure ($m$ = 2, $n$ = 2), which seems to affect negatively to the receptor activation. The unsubstituted isoxazoline enantiomer **15b** activates ERα to some extent. *Ortho*- or *meta*-OH substitution of Aryl 1 clearly decreases the activation while a clear ERα activation emerges by *para*-substitution (**18a**). Unexpectedly, in the case of the hydroxyketones *meta*-substitution results in the highest ERα and ERβ activities (**21ab**) and also *para*-substituted **22ab** is ERα active. The former shows relatively high RBA of 10.6% to ERα (Table 3) and its binding is strongly ERα selective with an α/β ratio of 106. On the basis of ligand docking, **21ab** is capable of forming three hydrogen bonds with ERα (Fig. 4).

The core structure of ligands **23**–**34** ($m$ = 3, $n$ = 1) has the same length as compounds **15**–**22**, but the spacer is shifted by one carbon. The change in the spacer position mostly leads to higher receptor activities and, in comparison to the unsubstituted ligands

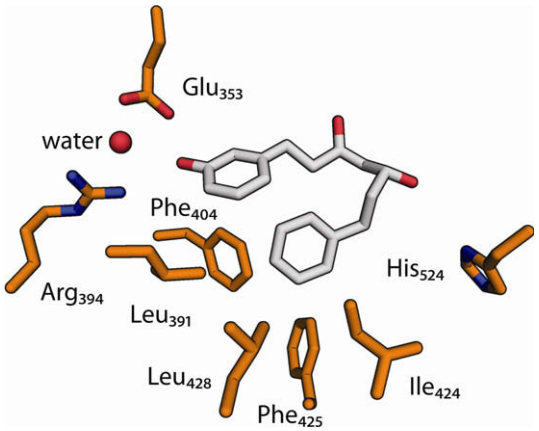



**Figure 4.** Binding of **(*R*)-21** to ERα. In comparison to **45** and **50**, the one carbon longer linker leads to different binding orientation of the phenol ring. Consequently, the 3-OH substitution of **21** leads to higher affinity than the 4-OH substitution of compound **22**.

**23a**/**23b** and **29ab**, *para*-OH substitution of Aryl 1 strongly enhances ERα and ERβ activities of both isoxazoline **24a**/**24b** and hydroxyketone **30ab**, which also shows notable ERα binding affinity (1.9%) and ERα selectivity of 11/1. On the other hand, in the case of isoxazoline **25a**/**25b** *para*-substitution of Aryl 2 increases only ERα activation and for the corresponding hydroxyketone **31ab** the effect is negative for both receptors. Nevertheless, we conclude that neither core structure is optimal for ER binding.

Ligands **35**–**42** own the shortest skeleton ($m = 2$, $n = 0$) of all the compounds (Table 2). The low receptor activities of the isoxazoline **35a**/**35b** are not affected by the addition of a methyl group to the chiral center but the OH substitution of Aryl 1 enhances the receptor activities to some extent. Especially both *ortho*- (**37b**) and *para*-substitution (**39b**) increase ERβ activation of enantiomer **b**. The hydroxyketone isomer **40a** has the second highest ERα but low ERβ activities among the unsubstituted compounds, and a substitution of the chiral carbon retains the former but increases the latter to some extent (racemate **41ab**). *Para*-OH substitution of Aryl 1 in **42ab**, in comparison to **41ab**, enhances especially the ERβ activation. In general, it seems that methyl in the hydroxyketone spacer of this set of ligands does not seriously disturb the activation. Because of the relatively low activities, RBAs were not determined.

Compounds **43**–**52** have similar structures with **35**–**42** but the linker ($m = 3$, $n = 0$) between the spacer and Aryl 1 is longer by one carbon. All the results indicate that this construct fits best to the receptors and, interestingly, that the flexible hydroxyketones do so better than the isoxazolines. This set of compounds contains many good activators and even the unsubstituted hydroxyketone **48** (especially enantiomer **48a**) is a strong activator of both ERα and ERβ. However, the RBAs are poor with two exceptions: the isoxazoline **45a**/**45b** and its hydroxyketone derivative **50a**/**50b** with *para*-OH at Aryl 1 show both very high activation and RBAs to ERα. The isoxazoline **45a** shows also very high RBA selectivity to ERα (157:1 over ERβ) while the ratio for hydroxyketones is smaller.

Methylation of the chiral center enhances both ERα and ERβ activities of the isoxazoline ligand **43a**/**43b**, but surprisingly *para*-OH substitution of Aryl 1 of the methylated compound **44ab** deactivates the compound almost completely (**46a**/**46b**). When also the very low RBAs (Table 3) of the methylated analog (**51ab**) of the best binders **50a** and **50b** are considered, it seems improbable that the replacement of hydrogen of the chiral center with a larger group would improve the biological activity.

Both the MM-PBSA and TI calculations predict that **50a** with the higher affinity has *R* configuration. It must be noted that the affinity difference between **50a** and **50b** is rather small and therefore challenging to calculate using computer simulations. The high potency of **50a** and **50b** can be attributed to the correct distance between Aryl 1 and skeletal OH-groups, which both are capable of forming hydrogen bonds of favorable geometry with ERα and ERβ. Since some of the compounds studied show high affinities and activation without being able to form three hydrogen bonds like **50a** and **50b**, receptor–ligand interactions other than hydrogen bonds play also role in determining compounds' potency. For example, the isoxazole ring of **45a**/**45b** is predicted to bind roughly at the same location as the hydrogen bond-forming hydroxyketone part of **50a** and **50b**, but stabilizing the receptor complex by polar interactions instead of hydrogen bonding. In addition, the best binder molecules are slightly larger than E2 and therefore more efficiently fill the ligand-binding cavity. Similar explanation has been given for the superagonism of several vitamin D receptor (VDR) ligands.[28–30] The larger molecule size also explains the ERα selectivity, since the volume of the ligand-binding cavity of ERα (450 Å$^3$) is slightly larger than that of ERβ (390 Å$^3$).[31]

According to the expression assays compound **50b** behaved highly similarly to E2. Instead, compounds **45b** and **50a** did not show statistically significant effects to *pS2* expression, even at very high concentrations. Still, **45b** had medium high affinity to ERα and **50a** even twofold higher than that of E2 and both compounds were able to compete with E2. The remaining *pS2* activities after competition suggest that these two compounds act as partial agonists in breast cancer cells. Isomer **45a** appears to be especially interesting with respect to its biological properties. It is highly ERα selective, as it has a relatively good binding affinity to ERα but poor to ERβ. The different affinities likely reflect to its ability to activate ERα receptor twofold better than ERβ in reporter gene assays. Even more interesting is the finding that **45a** activates primary E2 target gene *pS2* very rapidly but temporally in MCF-7 cells. These data suggest that **45a** is a potent activator of E2 target genes, but also that it is metabolized quite differently and significantly faster than E2 in breast cancer cells. This could make **45a** potentially safer compound in hormone replacement therapy for women suffering menopausal symptoms, since fast metabolism rate may decrease the risk of estrogen dependent carcinogenesis.

## 4. Conclusions

Our analyses on the present molecular constructs shows that the key features for good binding and receptor activation are, expectedly, *para*-OH at Aryl 1 and hydrogen bonding. It also seems that the $m = 3$, $n = 0$ spacer construct fits best to the receptor, although good activation is obtained with many other systems. The structures **45a**/**45b** and **50a**/**50b** happen just to own nearly perfect fit to the receptors. The explanation to the superior RBAs of **50a** and **50b** is the hydrogen bond formed by the skeletal OH-group of the hydroxyketone. The remarkable selectivity (up to α/β ratio of 157 with **45a**) to ERα can probably be accounted for the larger size of our ligand in comparison to E2, while the reason for the very high binding affinities of the best ligands (**50a** and **50b**) and obviously also their high ERE agonism, is that the low rotational energies allow formation of favorable hydrogen bonds and numerous van der Waals contacts with the receptor. The extensive contacts are able to compensate the entropy loss due to rigidifying of the flexible ligand upon receptor binding.

In general, our results show that very flexible molecules are able to bind and to activate the ERα/ERβ receptor even better than the rigid estradioles. The in vitro ERE reporter assay revealed several compounds having a higher agonism at high (10 μM) concentration than E2 has at 10 nM. However, there were only a few compounds (Table 3) showing significant (>1%) RBA in COS-1 cells. These good binders were also good ERE activators, without exception. Interesting is that there were many other compounds which were good reporter activators but have or, on the basis of structural features, can be assumed to have low RBA. One could say that when only the ERE reporter activation is considered, the ER receptors are not finical at all while the RBAs are very sensitive to geometry and, are thus much better measures of the real biological activation.

In the most critical test, the best binders acted as partial agonists also in *pS2* gene expression assay in breast cancer cells. Notable is also that, in these experiments (Fig. 1), the compound **45a** showed unusual kinetics with fast but temporary activation, while **50b** behaved more like E2. This compound can be considered as convergent of the agonistic properties of the entire compound group and that it owns the best potency for further developments and applications.

## 5. Experimental

### 5.1. Chemistry

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). Purity of >95% was confirmed via elemental analyses (CHN) which were carried out with a Thermo Quest CE Instruments EA 1110 CHNS-O elemental analyzer. $^1H$ and $^{13}C$ NMR spectra (TMS/$CDCl_3$) were recorded on a Bruker Avance 500 spectrometer operating at 500.13 MHz and 125.77 MHz, respectively. All *J* coupling constants are given in Hz. Semi preparative 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 id) chiral column in *n*-hexane/*i*-PrOH/AcOH 90:10:0.5 at flow rate 5 ml/min. Compounds were detected by UV absorption at 254 nm and 270 nm.

The methoxy aryl precursors **C** of the phenolic 4,5-dihydroisoxazoles **D** were synthesized according to the procedure in Section 5.1.1. but not characterized. The preparation and characterization of the following compounds have been described previously:[23] 5-benzyl-3-phenethyl-4,5-dihydroisoxazole (**1**), 5-benzyl-3-[2-(2-hydroxy-phenyl)-ethyl]-4,5-dihydro-isoxazole (**2**), 5-benzyl-3-[2-(4-hydroxy-phenyl)-ethyl]-4,5-dihydro-isoxazole (**3**), 5-(4-fuorobenzyl)-3-phenethyl-4,5-dihydroisoxazole (**4**), 5-(4-hydroxybenzyl)-3-phenethyl-4,5-dihydro-isoxazole (**5**), 5-(4-fluoro-benzyl)-3-(2-hydroxy-phenethyl)-4,5-dihydro-isoxazole (**6**), 5-(4-fluoro-benzyl)-3-(4-hydroxy-phenethyl)-4,5-dihydro-isoxazole (**7**), 5-hydroxy-1,6-diphenylhexan-3-one (**8**), 5-hydroxy-6-(4-fuorophenyl)-1-phenyl-hexan-3-one (**11**), 3,5-di-phenethyl-4,5-dihydroisoxazole (**15**), 5-hydroxy-1,7-diphenyl-heptan-3-one (**19**), 5-benzyl-3-(3-phenyl-propyl)-4,5-dihydro-isoxazole (**23**), 2-hydroxy-1,7-diphenyl-heptan-4-one (**29**), 3-phenethyl-5-phenyl-4,5-dihydroisoxazole (**35**), 1-hydroxy-1,5-diphenyl-pentan-3-one (**40**), 5-phenyl-3-(3-phenyl-propyl)-4,5-dihydro-isoxazole (**43**), 1-hydroxy-1,6-diphenyl-hexan-3-one (**48**).

### 5.1.1. Synthesis of 4,5-dihydroisoxazoles C (compounds 36 and 44)

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 drop wise 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.

**5.1.1.1. 5-Methyl-3-phenethyl-5-phenyl-4,5-dihydroisoxazole (36).** Yield 18%, colorless oil, $^1H$ NMR $\delta$ 7.39–7.36 (m, 2H), 7.33 (m, 2H), 7.24 (m, 3H), 7.19–7.16 (1H), 7.15–7.12 (2H), 3.01 (d, 1H, *J* = 16.8), 2.96 (d, 1H, *J* = 16.8), 2.87 (t, 2H, *J* = 8.0), 2.62 (t, 2H, *J* = 8.0), 1.65 (s, 3H); $^{13}C$ NMR $\delta$ 158.0, 145.8, 140.5 (3 s), 128.5, 128.4, 128.3, 127.2, 126.3, 124.7 (6d), 86.8 (s), 51.0, 32.7, 29.7 (3t), 28.1 (q). Elemental analysis data agreed: for $C_{18}H_{19}NO$.

**5.1.1.2. 5-Methyl-5-phenyl-3-(3-phenyl-propyl)-4,5-dihydro-isoxazole (44).** Yield 34%, yellow viscous oil, $^1H$ NMR $\delta$ 7.40 (m, 2H), 7.32 (m, 2H), 7.24 (m, 3H), 7.17 (m, 1H), 7.10 (m, 2H), 3.03 (d, 1H, *J* = 16.7), 2.99 (d, 1H, *J* = 16.7), 2.58 (m, 2H), 2.31 (t, 2H, *J* = 7.5), 1.84 (m, 2H), 1.68 (s, 3H); $^{13}C$ NMR $\delta$ 158.5, 145.8, 141.4 (3s), 128.4, 128.4, 128.4, 127.2, 125.9, 124.6 (6d), 86.6 (s), 50.7, 35.1 (2t), 28.1 (q), 27.9, 27.4 (2t). Elemental analysis data agreed: for $C_{19}H_{21}NO$.

### 5.1.2. Synthesis of phenolic 4,5-dihydroisoxazoles D (compounds 16–18, 24–28, 37–39, and 45–47)

To a solution of a 4,5-dihydroisoxazole **C** having an aromatic methoxyl group (2 mmol, compounds **53**–**66**) in 2 ml of DCM was added 2.2 ml of 1 M $BBr_3$ in DCM (2.2 mmol, for compounds 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.

**5.1.2.1. 3-(2-Hydroxy-phenethyl)-5-phenethyl-4,5-dihydro-isoxazole (16).** Yield 85%, a dark brown viscous oil, $^1H$ NMR $\delta$ 7.27 (m, 2H), 7.19 (d, 1H, *J* = 7.3), 7.16 (m, 2H), 7.12 (dd, 1H, *J* = 8.0, 1.7), 7.09 (d, 1H, *J* = 7.4), 6.89 (d, 1H, *J* = 7.7), 6.85 (t, 1H, *J* = 7.4), 4.52 (m, 1H), 2.97–2.89 (m, 3H), 2.73 (m, 1H), 2.68–2.64 (m, 3H), 2.52 (dd, 1H, *J* = 17.0, 8.3), 1.97 (m, 1H), 1.79 (m, 1H); $^{13}C$ NMR $\delta$ 159.2, 154.5, 141.1 (3s), 130.4, 128.5 (2d), 128.4 (s), 127.9, 126.1, 120.7, 117.8, 79.7 (5d), 42.8, 36.7, 31.8, 30.0, 25.9 (5t). Elemental analysis data agreed: for $C_{19}H_{21}NO_2{\cdot}0.2H_2O$.

**5.1.2.2. 3-(3-Hydroxy-phenethyl)-5-phenethyl-4,5-dihydro-isoxazole (17).** Yield 88%, a dark brown viscous oil, $^1H$ NMR $\delta$ 7.27 (m, 2H), 7.19 (d, 1H, *J* = 7.3), 7.16 (m, 2H), 7.12 (t, 1H, *J* = 8.0), 6.73–6.69 (m, 3H), 6.12 (br s, 1H, OH), 4.53 (m, 1H), 2.92 (dd, 1H, *J* = 17.1, 10.3), 2.84 (t, 2H, *J* = 7.6), 2.73 (ddd, 1H, *J* = 14.2, 9.6, 5.6), 2.68–2.62 (m, 3H), 2.52 (dd, 1H, *J* = 17.1., 7.9), 1.96 (m, 1H), 1.77 (m, 1H); $^{13}C$ NMR $\delta$ 159.1, 156.2, 142.0, 141.1 (4s), 129.8, 128.5, 128.5, 126.1, 120.4, 115.3, 113.6, 79.6 (8d), 42.3, 36.8, 32.4, 31.7, 29.2 (5t). Elemental analysis data agreed: for $C_{19}H_{21}NO_2{\cdot}0.1H_2O$.

**5.1.2.3. 3-(4-Hydroxy-phenethyl)-5-phenethyl-4,5-dihydro-isoxazole (18).** Yield 95%, a dark brown wax, $^1H$ NMR $\delta$ 7.28 (m, 2H), 7.20–7.17 (m, 3H), 7.03 (m, 2H), 6.74 (m, 2H), 4.50 (m, 1H), 3.02 (br s, 1H, OH), 2.90 (dd, 1H, *J* = 16.9, 10.2), 2.80 (t, 2H, *J* = 7.6), 2.73 (ddd, 1H, *J* = 16.1, 10.2, 5.6), 3.04 (ddd, 1H, *J* = 16.1, 9.5, 7.9), 2.61 (t, 2H, *J* = 7.7), 2.50 (dd, 1H, *J* = 16.9, 7.9), 1.95 (m, 1H), 1.77 (m, 1H). Elemental analysis data agreed: for $C_{19}H_{21}NO_2{\cdot}0.2H_2O$.

**5.1.2.4. 5-Benzyl-3-[3-(4-hydroxy-phenyl)-propyl]-4,5-dihydro-isoxazole (24).** Yield 99%, a dark brown wax; $^1H$ NMR $\delta$ 7.28 (m, 2H), 7.21 (m, 3H), 6.97 (m, 2H), 6.76 (m, 2H), 4.78 (m, 1H), 3.00 (dd, 1H, *J* = 13.9, 6.0), 2.86 (dd, 1H, *J* = 17.0, 10.1), 2.81 (dd, 1H, *J* = 13.9, 6.9), 2.62 (dd, 1H, *J* = 17.0, 7.6), 2.51 (t, 2H, *J* = 7.5), 2.28 (t, 2H, *J* = 7.7) 1.75 (m, 2H); $^{13}C$ NMR $\delta$ 159.3, 154.5, 136.9, 132.7 (4s), 129.5, 129.4, 128.6, 126.7, 115.3, 80.5, (6d), 41.5, 40.9, 34.3, 28.1, 27.2 (5t). Elemental analysis data agreed: for $C_{19}H_{21}NO_2{\cdot}0.2H_2O$.

**5.1.2.5. 5-(4-Hydroxybenzyl)-3-(3-phenyl-propyl)-4,5-dihydro-isoxazole (25).** Yield 65%, a dark brown oil; $^1H$ NMR $\delta$ 7.24 (m, 2H), 7.15 (m, 1H), 7.11 (m, 2H), 7.00 (m, 2H), 6.80 (m, 2H), 4.71 (m, 1H), 2.87–2.81 (m, 2H), 2.72 (dd, 1H, *J* = 14.1, 6.3), 2.60–2.54 (m, 3H), 2.28 (t, 2H, *J* = 7.6), 1.79 (m, 2H); $^{13}C$ NMR $\delta$ 159.5, 155.1, 141.2 (3s), 130.4, 128.4, 128.4 (3d), 128.1 (s), 126.0, 115.6, 80.8, (3d), 41.3, 39.9, 35.1, 27.7, 27.2 (5t). Elemental analysis data agreed: for $C_{19}H_{21}NO_2{\cdot}0.1H_2O$.

**5.1.2.6. 5-(4-Hydroxy-benzyl)-3-[3-(4-hydroxy-phenyl)-propyl]-4,5-dihydro-isoxazole (26).** Yield 68%, a dark brown wax; $^1H$ NMR $\delta$ 7.02 (m, 2H), 6.95 (m, 2H), 6.77 (m, 2H), 6.76 (m, 2H), 4.73 (m, 1H), 2.86 (dd, 1H, *J* = 14.1, 6.2), 2.85 (dd, 1H, *J* = 17.2, 10.4), 2.74 (dd, 1H, *J* = 14.1, 6.6), 2.62 (dd, 1H, *J* = 17.2, 7.7), 2.50 (t, 2H, *J* = 7.5), 2.26 (t, 2H, *J* = 7.7) 1.73 (m, 2H); $^{13}C$ NMR $\delta$ 159.9, 155.5, 154.8, 132.5 (4s), 130.5, 129.5 (2d), 128.3 (s), 115.5, 115.3, 80.9 (3d), 41.3, 39.9, 34.3, 28.1, 27.1 (5t). Elemental analysis data agreed: for $C_{19}H_{21}NO_3{\cdot}0.33H_2O$.

**5.1.2.7. 5-Benzyl-3-[3-(3,4-dihydroxy-phenyl)-propyl]-4,5-dihydro-isoxazole (27).** Yield 87%, a dark brown viscous oil, $^1H$ NMR $\delta$ 7.24 (m, 2H), 7.19 (d, 1H, *J* = 7.3), 7.17–7.14 (m, 2H), 6.76 (d, 1H,

$J$ = 8.1), 6.70 (d, 1H, $J$ = 1.8), 6.61 (br s, 2H, OH), 6.47 (dd, 1H, $J$ = 8.1, 1.8), 4.75 (m, 1H), 2.94 (dd, 1H, $J$ = 13.9, 6.1), 2.83 (dd, 1H, $J$ = 17.3, 10.2), 2.77 (dd, 1H, $J$ = 13.9, 6.6), 2.58 (dd, 1H, $J$ = 17.3, 7.7), 2.38 (t, 2H, $J$ = 7.4), 2.21 (t, 2H, $J$ = 7.3), 1.68 (m, 2H); $^{13}$C NMR $\delta$ 160.1, 143.9, 142.2, 136.7, 133.7 (5s), 129.4, 128.5, 126.7, 120.5, 115.7, 115.4, 80.6 (7d), 41.4, 40.7, 34.3, 20.7, 26.9 (5t). Elemental analysis data agreed: for $C_{19}H_{21}NO_2 \cdot 0.5H_2O$.

**5.1.2.8. 3-[3-(3,4-Dihydroxy-phenyl)-propyl]-5-(4-hydroxy-benzyl)-4,5-dihydro-isoxazole (28).** Yield 29%, a dark brown viscous oil, $^1$H NMR (in $CD_3OD$) $\delta$ 7.05 (m, 2H), 6.76 (m, 2H), 6.75 (d, 1H, $J$ = 8.0), 6.60 (d, 1H, $J$ = 2.0), 6.51 (dd, 1H, $J$ = 8.0, 2.0), 4.76 (m, 1H), 2.87–2.82 (m, 2H), 2.79 (dd, 1H, $J$ = 14.1, 6.3), 2.61 (dd, 1H, $J$ = 17.1, 7.5), 2.43 (m, 2H), 2.32 (m, 2H), 1.69 (m, 2H); $^{13}$C NMR (in $CD_3OD$) $\delta$ 159.4, 155.3, 144.1, 142.7, 133.4 (5s), 130.7 (d), 127.9 (s), 120.2, 115.5, 115.4, 115.0, 80.6 (5d), 41.0, 39.8, 34.2, 27.8, 26.9 (5t). Elemental analysis data agreed: for $C_{19}H_{21}NO_3 \cdot 0.2H_2O$.

**5.1.2.9. 3-[2-(2-Hydroxy-phenyl)-ethyl]-5-methyl-5-phenyl-4,5-dihydro-isoxazole (37).** Yield 22%, a brown viscous oil, $^1$H NMR $\delta$ 7.39–7.32 (m, 4H), 7.26 (m, 1H), 7.09 (m, 1H), 6.70 (m, 2H), 6.60 (m, 1H), 5.56 (br s, 1H, OH), 3.03 (d, 1H, $J$ = 16.9), 2.99 (d, 1H, $J$ = 16.9), 2.81 (t, 2H, $J$ = 7.5), 2.62 (t, 2H, $J$ = 7.5), 1.66 (s, 3H); $^{13}$C NMR $\delta$ 158.5, 156.0, 145.6, 142.0 (4s), 129.7, 128.5, 127.3, 124.7, 120.5, 115.3, 113.5 (7d), 87.0 (s), 51.0, 32.5, 29.4 (3t), 28.0 (q). Elemental analysis data agreed: for $C_{18}H_{19}NO_2 \cdot 0.1H_2O$.

**5.1.2.10. 3-[2-(3-Hydroxy-phenyl)-ethyl]-5-methyl-5-phenyl-4,5-dihydro-isoxazole (38).** Yield 97%, a brown viscous oil, $^1$H NMR $\delta$ 7.37–7.31 (m, 4H), 7.25 (t, 1H, $J$ = 6.9), 7.04 (t, 1H, $J$ = 7.6), 7.00 (d, 1H, $J$ = 7.5), 6.81 (d, 1H, $J$ = 7.9), 6.75 (t, 1H, $J$ = 7.4), 4.68 (br s, 1H, OH), 3.08 (d, 1H, $J$ = 17.1), 3.06 (d, 1H, $J$ = 17.1), 2.86 (t, 2H, $J$ = 7.5), 2.65 (t, 2H, $J$ = 7.3), 1.64 (s, 3H); $^{13}$C NMR $\delta$ 159.8, 154.9, 145.6, (3s), 130.2, 128.5, 127.7, 127.4 (4d), 127.0 (s), 124.7, 120.0, 115.7 (3d), 87.1 (s), 51.0 (t), 28.3 (q), 28.0, 27.4 (2t). Elemental analysis data agreed: for $C_{18}H_{19}NO_2$.

**5.1.2.11. 3-[2-(4-Hydroxy-phenyl)-ethyl]-5-methyl-5-phenyl-4,5-dihydro-isoxazole (39).** Yield 19%, a brown viscous oil, $^1$H NMR $\delta$ 7.38–7.32 (m, 4H), 7.26 (m, 1H), 6.96 (m, 2H), 6.69 (m, 2H), 3.03 (d, 1H, $J$ = 16.8), 2.99 (d, 1H, $J$ = 16.8), 2.79 (t, 2H, $J$ = 7.6), 2.61 (t, 2H, $J$ = 7.6), 1.65 (s, 3H); $^{13}$C NMR $\delta$ 158.4, 154.3, 145.6, 132.1 (4s), 129.4, 128.5, 127.2, 124.7, 115.4 (5d), 86.9 (s), 50.9, 31.8, 29.8 (3t), 28.0 (q). Elemental analysis data agreed: for $C_{18}H_{19}NO_2$.

**5.1.2.12. 3-[3-(4-Hydroxy-phenyl)-propyl]-5-phenyl-4,5-dihydro-isoxazole (45).** Yield 99%, a brown viscous oil, $^1$H NMR $\delta$ 7.36–7.27 (m, 5H), 6.96 (m, 2H), 6.80 (m, 2H), 5.54 (dd, 1H, $J$ = 10.7, 8.3), 3.35 (dd, 1H, $J$ = 17.4, 10.7), 2.91 (dd, 1H, $J$ = 17.4, 8.3), 2.55 (t, 2H, $J$ = 7.5), 2.40 (t, 2H, $J$ = 7.6), 1.84 (tt, 2H, $J$ = 7.6, 7.5); $^{13}$C NMR $\delta$ 159.9, 154.5, 140.5, 132.6 (4s), 129.5, 128.8, 128.3, 125.8, 115.7, 81.6 (6d), 45.2, 34.3, 28.1, 27.1 (4t). Elemental analysis data agreed: for $C_{18}H_{21}NO_2 \cdot 0.2H_2O$.

**5.1.2.13. 3-[3-(4-Hydroxy-phenyl)-propyl]-5-methyl-5-phenyl-4,5-dihydro-isoxazole (46).** Yield 99%, a brown viscous oil, $^1$H NMR $\delta$ 7.40 (m, 2H), 7.33 (m, 2H), 7.24 (m, 2H), 6.92 (m, 2H), 6.75 (m, 2H), 6.54 (br s, 1H, OH), 3.06 (d, 1H, $J$ = 16.9), 3.02 (d, 1H, $J$ = 16.9), 2.49 (m, 2H), 2.31 (t, 2H, $J$ = 7.6), 1.79 (m, 2H), 1.69 (s, 3H); $^{13}$C NMR $\delta$ 159.3, 154.3, 145.5, 132.9 (4s), 129.4, 128.5, 127.3, 124.6, 115.4 (5d), 86.7 (s), 50.7, 34.2, 28.1 (3t), 28.0 (q), 27.3 (t). Elemental analysis data agreed: for $C_{19}H_{23}NO_2$.

**5.1.2.14. 3-[3-(4-Hydroxy-phenyl)-propyl]-5-(3-hydroxy-phenyl)-4,5-dihydro-isoxazole (47).** Yield 99%, a brown viscous oil, $^1$H NMR $\delta$ 7.18 (m, 1H), 6.99 (m, 2H), 6.79–6.77 (m, 3H), 6.75 (m, 2H), 5.46 (dd, 1H, $J$ = 10.8, 8.0), 3.33 (dd, 1H, $J$ = 17.2, 10.8), 2.89 (dd, 1H, $J$ = 17.2, 8.0), 2.57 (t, 2H, $J$ = 7.5), 2.37 (t, 2H, $J$ = 7.6), 1.85 (tt, 2H, $J$ = 7.6, 7.5); $^{13}$C NMR $\delta$ 159.6, 157.4, 155.1, 142.8, 132.5 (5s), 130.1, 129.6, 117.0, 115.5, 115.3, 112.7, 81.4 (7d), 45.5, 34.5, 28.4, 27.2 (4t). Elemental analysis data agreed: for $C_{18}H_{21}NO_3 \cdot 0.5H_2O$.

### 5.1.3. Synthesis of hydroxyketones E (compounds 9–14, 20–22, 30–34, 41, 42, and 49–52)

A solution of an isoxazoline **D** (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 satd $NaHCO_3$ and water, and evaporated to give the pure product.

**5.1.3.1. 5-Hydroxy-1-(2-hydroxy-phenyl)-6-phenyl-hexan-3-one (9).** Yield 68%, a brown viscous oil, $^1$H NMR $\delta$ 7.26 (m, 2H), 7.19 (m, 1H), 7.14 (m, 2H), 7.07 (t, 1H, $J$ = 7.8), 6.65–6.62 (m, 2H), 6.59 (m, 1H), 4.29 (m, 1H), 2.80 (dd, 1H, $J$ = 13.6, 7.2), 2.78–2.73 (m, 2H), 2.68 (dd, 1H, $J$ = 13.6, 6.4), 2.66–2.63 (m, 2H), 2.51 (m, 2H); $^{13}$C NMR $\delta$ 211.1, 156.1, 142.4, 13.6 (4s), 129.7, 129.4, 128.6, 126.6, 120.2, 115.3, 113.3, 68.9 (8d), 48.3, 44.7, 42.9, 29.2 (4t). Elemental analysis data agreed: for $C_{18}H_{20}O_3 \cdot 0.2H_2O$.

**5.1.3.2. 5-Hydroxy-1-(4-hydroxy-phenyl)-6-phenyl-hexan-3-one (10).** Yield 54%, a brown viscous oil, $^1$H NMR $\delta$ 7.27 (m, 2H), 7.21 (m, 1H), 7.15 (m, 2H) 6.94 (m, 2H), 6.70 (m, 2H), 4.67 (br s, 1H, OH), 4.29 (m, 1H), 2.81 (dd, 1H, $J$ = 13.6, 7.2), 2.77–2.73 (m, 2H), 2.69 (dd, 1H, $J$ = 13.7, 6.5), 2.66–2.62 (m, 2H), 2.51 (m, 2H); $^{13}$C NMR $\delta$ 211.4, 154.4, 137.6, 132.2 (4s), 129.4, 129.3, 128.6, 126.6, 115.5, 68.9 (6d), 48.3, 45.3, 42.9, 25.1 (4t). Elemental analysis data agreed: for $C_{18}H_{20}O_3 \cdot 0.33H_2O$.

**5.1.3.3. 5-Hydroxy-6-(4-fuorophenyl)-1-phenyl-hexan-3-one (11).** Yield 66%, a yellow viscous oil; $^1$H NMR $\delta$ 7.25 (m, 2H), 7.17 (m, 1H), 7.15–7.10 (m, 4H), 6.96 (m, 2H), 4.23 (m, 1H), 2.96 (s, 1H, OH), 2.87 (t, 2H, $J$ = 7.5), 2.77–2.70 (m, 3H), 2.65 (dd, 1H, $J$ = 13.8, 5.9), 2.50 (m, 2H); $^{13}$C NMR $\delta$ 210.5, 161.7 (d), 140.7 (s), 133.6 (d), 130.8 (dd), 128.5, 128.2, 126.2 (3d), 115.2 (dd), 68.5 (d), 48.4, 45.0, 42.0, 29.4 (4t). Elemental analysis data agreed: for $C_{18}H_{20}FO_2$.

**5.1.3.4. 5-Hydroxy-6-(4-Hydroxy-phenyl)-1-phenyl-hexan-3-one (12).** Yield 99%, a brown viscous oil, $^1$H NMR $\delta$ 7.24 (m, 2H), 7.16 (m, 1H), 7.11 (m, 2H), 6.97 (m, 2H), 6.71 (m, 2H), 4.23 (m, 1H), 2.84 (t, 2H, $J$ = 15.0), 2.73–2.68 (m, 3H), 2.61 (dd, 1H, $J$ = 13.0, 5.5), 2.52 (m, 2H); $^{13}$C NMR $\delta$ 211.2, 154.8, 140.6 (3s), 130.5 (d), 129.2 (s), 128.5, 128.2, 126.2, 115.5, 69.1 (5d), 48.3, 45.0, 42.0, 29.4 (4t). Elemental analysis data agreed: for $C_{18}H_{20}O_3 \cdot 0.5H_2O$.

**5.1.3.5. 6-(4-Fluoro-phenyl)-5-hydroxy-1-(2-hydroxy-phenyl)-hexan-3-one (13).** Yield 94%, a brown wax, $^1$H NMR $\delta$ 7.09–7.03 (m, 3H), 6.91 (m, 2H), 6.64–6.61 (m, 2H), 6.59 (m, 1H), 4.25 (m, 1H), 2.76–2.71 (m, 3H), 2.66–2.60 (m, 3H), 2.50 (dd, 1H, $J$ = 17.3, 8.3), 2.47 (dd, 1H, $J$ = 17.3, 3.8); $^{13}$C NMR $\delta$ 211.3 (s), 161.7 (d), 156.1, 142.4 (2s), 133.3 (d), 130.8 (dd), 129.7, 120.2, 115.3 (d), 115.2 (dd), 113.3, 68.9 (2d), 48.3, 44.7, 41.9, 29.1 (4t). Elemental analysis data agreed: for $C_{18}H_{20}FO_3 \cdot 0.1H_2O$.

**5.1.3.6. 6-(4-Fluoro-phenyl)-5-hydroxy-1-(4-hydroxy-phenyl)-hexan-3-one (14).** Yield 99%, a dark brown viscous oil, $^1$H NMR $\delta$ 7.08 (m, 2H), 6.95–6.90 (m, 4H), 6.69 (m, 2H), 4.25 (m, 1H), 2.78–2.72 (m, 3H), 2.67–2.61 (m, 3H), 2.50 (dd, 1H, $J$ = 17.3, 7.6), 2.49 (dd, 1H, $J$ = 17.3, 4.0); $^{13}$C NMR $\delta$ 211.5 (s), 161.7 (d), 154.4 (s), 133.3 (d), 132.2 (s), 130.8 (dd), 129.3, 115.5 (2d), 115.3 (dd),

68.9 (d), 48.3, 45.3, 41.9, 28.6 (4t). Elemental analysis data agreed: for $C_{18}H_{20}FO_3 \cdot 0.2H_2O$.

**5.1.3.7. 5-Hydroxy-1-(2-hydroxy-phenyl)-7-phenyl-heptan-3-one (20).** Yield 74%, a brown viscous oil, $^1$H NMR $\delta$ 7.24 (m, 2H), 7.20–7.13 (m, 3H), 7.08–7.02 (m, 2H), 6.79 (m, 2H), 4.02 (m, 1H), 2.83 (t, 2H, $J$ = 7.3), 2.78–2.71 (m, 3H), 2.62 (m, 1H), 2.57–2.48 (m, 2H), 1.76 (m, 1H), 1.65 (m, 1H); $^{13}$C NMR $\delta$ 213.1, 154.5, 141.7 (3s), 130.3, 128.9, 128.4, 127.7 (4d), 127.2 (s), 125.9, 120.3, 116.0, 67.1 (4d), 49.2, 44.1, 38.2, 31.7, 24.1 (5t). Elemental analysis data agreed: for $C_{19}H_{22}O_3 \cdot 0.5H_2O$.

**5.1.3.8. 5-Hydroxy-1-(3-hydroxy-phenyl)-7-phenyl-heptan-3-one (21).** Yield 61%, a brown viscous oil, $^1$H NMR $\delta$ 7.23 (m, 2H), 7.16–7.11 (m, 3H), 7.08 (dd, 1H, $J$ = 7.8, 7.8), 6.65 (m, 2H), 6.62 (m, 1H), 4.06 (m, 1H), 2.78 (t, 2H, $J$ = 7.3), 2.75 (m, 1H), 2.64 (t, 2H, $J$ = 7.3), 2.61 (m, 1H), 2.55–2.46 (m, 2H), 1.79 (m, 1H), 1.66 (m, 1H); $^{13}$C NMR $\delta$ 211.6, 156.1, 142.4, 141.6 (4s), 129.7, 128.4, 128.4, 125.9, 120.6, 115.3, 113.4, 67.3 (8d), 49.2, 44.7, 38.0, 31.7, 29.3 (5t). Elemental analysis data agreed: for $C_{19}H_{22}O_3 \cdot 0.2H_2O$.

**5.1.3.9. 5-Hydroxy-1-(4-hydroxy-phenyl)-7-phenyl-heptan-3-one (22).** Yield 54%, a pale brown viscous oil, $^1$H NMR $\delta$ 7.25 (m, 2H), 7.17–7.13 (m, 3H), 6.97 (m, 2H), 6.72 (m, 2H), 4.05 (m, 1H), 2.79–2.60 (m, 6H), 2.52 (m, 2H), 1.79 (m, 1H), 1.65 (m, 1H); $^{13}$C NMR $\delta$ 211.8, 154.4, 141.6, 132.2 (4s), 129.3, 128.4, 128.4, 125.9, 115.5, 67.2 (6d), 49.1, 45.3, 37.9, 31.7, 28.6 (5t). Elemental analysis data agreed: for $C_{19}H_{22}O_3 \cdot 0.33H_2O$.

**5.1.3.10 . 2-Hydroxy-7-(4-hydroxy-phenyl)-1-phenyl-heptan-4-one (30).** Yield 49%, a pale brown viscous oil; $^1$H NMR $\delta$ 7.27 (m, 2H), 7.21 (m, 1H), 7.16 (m, 2H), 6.94 (m, 2H), 6.72 (m, 2H), 4.29 (m, 1H), 2.82 (dd, 1H, $J$ = 13.6, 8.9), 2.70 (dd, 1H, $J$ = 13.6, 6.4), 2.52 (m, 2H), 2.48 (t, 2H, $J$ = 7.5), 2.35 (t, 2H, $J$ = 7.3), 1.81 (tt, 2H, $J$ = 7.5, 7.3); $^{13}$C NMR $\delta$ 212.3, 154.1, 137.6, 133.1 (4s), 129.5, 129.4, 128.6, 126.6, 115.3, 69.0, (6d), 48.0, 42.8, 42.7, 34.0, 25.1 (5t). Elemental analysis data agreed: for $C_{19}H_{22}O_3 \cdot 0.25H_2O$.

**5.1.3.11. 2-Hydroxy-1-(4-hydroxy-phenyl)-7-phenyl-heptan-4-one (31).** Yield 99%, a pale brown wax; $^1$H NMR $\delta$ 7.26 (m, 2H), 7.17 (m, 1H), 7.13 (m, 2H), 7.00 (m, 2H), 6.72 (m, 2H), 4.23 (m, 1H), 2.72 (dd, 1H, $J$ = 13.7, 7.2), 2.63 (dd, 1H, $J$ = 13.7, 6.2), 2.58 (t, 2H, $J$ = 7.6), 2.51 (m, 2H), 2.38 (t, 2H, $J$ = 7.4), 1.87 (tt, 2H, $J$ = 7.6, 7.4); $^{13}$C NMR $\delta$ 212.0, 154.7, 141.3 (3s), 130.5 (d), 129.3 (s), 128.4, 128.4, 126.0, 115.5, 69.1, (6d), 48.0, 42.8, 41.9, 34.9, 24.8 (5t). Elemental analysis data agreed: for $C_{19}H_{22}O_3 \cdot 0.25H_2O$.

**5.1.3.12. 2-Hydroxy-1,7-di-(4-hydroxy-phenyl)-heptan-4-one (32).** Yield 16%, a colorless viscous oil; $^1$H NMR $\delta$ 7.06 (m, 2H), 6.99 (m, 2H), 6.76 (m, 2H), 6.73 (m, 2H), 4.22 (m, 1H), 2.95 (br s, 1H, OH), 2.86 (dd, 1H, $J$ = 13.6, 7.1), 2.65 (dd, 1H, $J$ = 13.6, 6.2), 2.55–2.51 (m, 3H), 2.47 (dd, 1H, $J$ = 17.4, 8.5), 2.38 (t, 2H, $J$ = 7.3), 1.85 (tt, 2H, 7.5, 7.3); $^{13}$C NMR $\delta$ 215.9, 154.3, 153.8, 133.6 (4s), 130.6, 129.5 (2d), 125.0 (s), 115.4, 115.3, 68.9 (3d), 48.1, 42.6, 42.0, 29.7, 25.1 (5t). Elemental analysis data agreed: for $C_{19}H_{22}O_4$.

**5.1.3.13. 2-Hydroxy-7-(3,4-dihydroxy-phenyl)-1-phenyl-heptan-4-one (33).** Yield 59%, a reddish brown viscous oil, $^1$H NMR $\delta$ 7.26–7.21 (m, 2H), 7.18 (m, 1H), 7.13–7.10 (m, 2H), 6.70 (d, 1H, $J$ = 7.3), 6.59 (d, 1H, $J$ = 1.8), 6.47 (dd, 1H, $J$ = 7.3, 1.8), 4.28 (m, 1H), 2.78 (dd, 1H, $J$ = 13.5, 7.1), 2.66 (dd, 1H, $J$ = 13.5, 6.2), 2.53-2.40 (m, 2H), 2.35 (t, 2H, $J$ = 7.0), 2.28 (t, 2H, $J$ = 7.0), 1.64 (m, 2H); $^{13}$C NMR $\delta$ 213.1, 144.2, 142.5, 138.0, 134.5 (5s), 129.8, 129.0, 127.1, 121.0, 116.1, 115.9, 69.5 (7d), 48.5, 43.2, 43.0, 34.5, 25.3 (5t). Elemental analysis data agreed: for $C_{19}H_{22}O_4 \cdot 0.5H_2O$.

**5.1.3.14. 2-Hydroxy-7-(3,4-dihydroxy-phenyl)-1-(4-hydroxy-phenyl)-heptan-4-one (34).** Yield 60%, a reddish brown viscous oil, $^1$H NMR $\delta$ 7.02 (m, 2H), 6.98 (m, 1H), 6.76 (d, 1H, $J$ = 1.9), 6.72 (m, 2H), 6.61 (d, 1H, $J$ = 7.2), 6.50 (dd, 1H, $J$ = 7.2, 1.9), 4.21 (m, 1H), 2.78 (dd, 1H, $J$ = 13.5, 7.0), 2.63 (dd, 1H, $J$ = 13.5, 6.1), 2.50–2.38 (m, 2H), 2.37 (t, 2H, $J$ = 7.0), 2.35 (t, 2H, $J$ = 7.0), 1.65 (m, 2H). Elemental analysis data agreed: for $C_{19}H_{22}O_5 \cdot H_2O$.

**5.1.3.15. 5-Hydroxy-1,5-diphenyl-hexan-3-one (41).** Yield 73%, pale brown viscous oil, $^1$H NMR $\delta$ 7.38 (m, 2H), 7.30 (m, 2H), 7.25–7.19 (m 3H), 7.15 (m, 1H), 7.04 (m, 2H), 3.11 (d, 1H, $J$ = 16.7), 2.77 (d, 1H, $J$ = 16.7), 2.77–2.63 (m, 3H), 2.55 (m, 1H), 1.49 (s, 3H); $^{13}$C NMR $\delta$ 211.7, 147.2, 140.5 (3s), 128.5, 128.3, 128.2, 126.7, 126.1, 124.3 (6d), 73.3 (s), 53.5, 45.9 (2t), 30.6 (q), 29.1 (t). Elemental analysis data agreed: for $C_{18}H_{20}O_2 \cdot 0.1H_2O$.

**5.1.3.16. 5-Hydroxy-1-(4-hydroxy-phenyl)-5-phenyl-hexan-3-one (42).** Yield 25%, yellow viscous oil, $^1$H NMR $\delta$ 7.37 (m, 2H), 7.30 (m, 2H), 7.21 (m, 1H), 6.87 (m, 2H), 6.68 (m, 2H), 6.05 (br s, 1H, OH), 4.74 (br s, 1H, OH), 3.14 (d, 1H, $J$ = 16.8), 2.77 (d, 1H, $J$ = 16.8), 2.72–2.59 (m, 3H), 2.53 (m, 1H), 1.49 (s, 3H); $^{13}$C NMR $\delta$ 212.4, 154.2, 146.9, 132.2 (4s), 129.3, 128.4, 126.9, 124.3, 115.4 (5d), 73.8 (s), 53.4, 46.2 (2t), 30.6 (q), 28.3 (t). Elemental analysis data agreed: for $C_{18}H_{20}O_3 \cdot 0.2H_2O$.

**5.1.3.17. 6-Hydroxy-1,6-diphenyl-heptan-4-one (49).** Yield 16%, yellow viscous oil, $^1$H NMR $\delta$ 7.40 (m, 2H), 7.31 (m, 2H), 7.25 (m, 2H), 7.21 (m, 1H), 7.17 (m, 1H), 7.07 (m, 2H), 3.10 (d, 1H, $J$ = 16.8), 2.75 (d, 1H, $J$ = 16.8), 2.48 (m, 2H), 2.34 (dt, 1H, $J$ = 17.4, 7.3), 2.24 (dt, 1H, $J$ = 17.4, 7.3), 1.77 (m, 2H), 1.50 (s, 3H); $^{13}$C NMR $\delta$ 212.6, 147.3, 141.3 (3s), 128.4, 128.4, 128.3, 126.7, 126.0, 124.3 (6d), 73.4 (s), 53.3, 46.3, 34.7 (3t), 30.7 (q), 24.5 (t). Elemental analysis data agreed: for $C_{19}H_{22}O_2 \cdot 0.1H_2O$.

**5.1.3.18. 1-Hydroxy-6-(4-hydroxy-phenyl)-1-phenyl-hexan-3-one (50).** Yield 14%, a brown viscous oil; $^1$H NMR $\delta$ 7.33 (m, 4H), 7.24 (m, 1H), 6.94 (m, 2H), 6.71 (m, 2H), 6.33 (br s, 1H, OH), 5.12 (dd, 1H, $J$ = 9.3, 3.3), 4.00 (br s, 1H, OH), 2.81 (dd, 1H, $J$ = 17.2, 9.3), 2.71 (dd, 1H, $J$ = 17.2, 3.3), 2.48 (t, 2H, $J$ = 7.5), 2.39 (t, 2H, $J$ = 7.3), 1.83 (tt, 2H, $J$ = 7.5, 7.3); $^{13}$C NMR $\delta$ 211.7, 154.2, 142.6, 133.1 (4s), 129.5, 128.6, 127.7, 125.7, 115.3, 70.1 (6d), 50.9, 42.8, 34.0, 25.1 (4t). Elemental analysis data agreed: for $C_{18}H_{20}O_3 \cdot 0.25H_2O$.

**5.1.3.19. 6-Hydroxy-1-(4-hydroxy-phenyl)-6-phenyl-heptan-4-one (51).** Yield 11%, a brown viscous oil; $^1$H NMR $\delta$ 7.39 (m, 2H), 7.31 (m, 2H), 7.21 (m, 1H), 6.90 (m, 2H), 6.72 (m, 2H), 5.90 (br s, 1H, OH), 4.73 (br s, 1H, OH), 3.13 (d, 1H, $J$ = 16.9), 2.76 (d, 1H, $J$ = 16.9), 2.43–2.29 (m, 4H), 1.72 (m, 2H), 1.51 (s, 3H); $^{13}$C NMR $\delta$ 213.1, 154.1, 147.0, 133.1 (4s), 129.5, 128.3, 126.8, 124.3, 115.3, 73.7 (6d), 53.2, 43.6, 33.8 (3t), 30.6 (q), 24.8 (t). Elemental analysis data agreed: for $C_{19}H_{22}O_3 \cdot 0.2H_2O$.

**5.1.3.20. 1-Hydroxy-1-(3-hydroxy-phenyl)-6-(4-hydroxy-phenyl)-hexan-3-one (52).** Yield 19%, a brown viscous oil; $^1$H NMR $\delta$ 8.51 (br s, 1H, OH), 8.21 (br s, 1H, OH), 7.12 (t, 1H, $J$ = 7.8), 6.93 (m, 2H), 6.79–6.76 (m, 2H), 6.73–6.69 (m, 3H), 5.01 (dd, 1H, $J$ = 9.1, 3.5), 4.37 (br s, 1H, OH), 2.77 (dd, 1H, $J$ = 16.8, 9.1), 2.65 (dd, 1H, $J$ = 16.8, 3.5), 2.47 (t, 2H, $J$ = 7.4), 2.36 (td, 2H, $J$ = 7.4, 2.5), 1.80 (tt, 2H, $J$ = 7.4, 7.4); $^{13}$C NMR $\delta$ 211.8, 156.7, 154.6, 144.6, 132.7 (5s), 129.7, 129.5, 117.3, 115.3, 114.9, 112.6, 69.9 (7d), 51.1, 42.8, 34.0, 25.2 (4t). Elemental analysis data agreed: for $C_{18}H_{20}O_4 \cdot 0.33H_2O$.

### 5.2. Biology

#### 5.2.1. Agonistic activity assay

E2 and ICI-182,780 were from Sigma Chemical Co. (St. Louis, MO) and Tocris (Avonmouth, UK), respectively. All other reagents were of reagent grade from Sigma or Fluka. HEK293 cells were seeded in 48-well plates ($70 \times 10^3$ cells per well) in phenol-free Dulbecco's modified Eagle medium supplemented with 5% delipidated fetal bovine serum (Sigma) and antibiotics.[32] After a 24 h culture, the medium was changed and the cells were transfected for 4 h with 5 ng ERα or ERβ expression vector,[33] 75 ng reporter plasmid pERE$_2$TATA-LUC[26] and 20 ng control plasmid pCMVβ as described.[32] After transfection, the cells received fresh medium containing either vehicle (0.1% v/v), test compound (10 μM) or 10 nM E2. When appropriate, the antagonist ICI-182,780 (1000 nM) or vehicle was included in medium. After 24 h, the cells were washed, lysed and assayed for luciferase and β-galactosidase activities[32] with a Victor$^{2}$™ reader (PerkinElmer 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 parenthesis 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.

#### 5.2.2. Relative binding affinity assay

Relative binding affinities (RBA) were measured by a competitive assay against [6,7-$^3$H(N)]estradiol (PerkinElmer) in transiently transfected COS-1 cells. One day before transfection, COS-1 cells were seeded into 2 ml of DMEM (Dulbecco's modified Eagle medium, Gipco) with 10% delipidated fetal bovine serum and 0,25% (vol/vol) Penicillin–Streptomycin (Euroclone) at a density of $140 \times 10^3$ cells/well. After medium change to DMEM 2.5% FBS, the cells were transfected for 24 h with 10 ng/well of human ERα/ERβ expression vector pSG5-hERα/β by using the TransIT method (Micrus Bio TransIT-LT1, Transfection Reagent). After 36 h, the cells were treated with tested compounds using 0.01-, 0.1-, 1-, 10-, 100-, 1000-, and 10,000-fold excess as compared to labeled E2 (1.96 pM/well). After 2 h of incubation at 37 °C the medium was removed. The cells were removed from the wells to 150 μl of 1× phosphate buffered saline (PBS), transferred to Eppendorf tubes and centrifuged at 4 °C using 4000*g* for 5 min, and then washed twice with 150 μl of PBS. The cell pellets were dissolved to 50 μl of 0.5 M NaOH and incubated for 15 min at 56 °C, after which the samples were transferred to liquid squintillation tubes and treated with 3 ml of OptiPhase HiSafe 3 solution (PerkinElmer). The results were measured with LKB WALLAC 1214 Racbeta equipment and non-specific binding was subtracted from the total [$^3$H]estradiol binding to obtain the specific ER binding.

#### 5.2.3. Gene expression assay

MCF-7 human breast cancer cells were maintained in DMEM containing 5% FBS, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin in humidified 95% air/5% $CO_2$ incubator. Prior to RNA extraction, the cells were grown overnight in phenol red-free DMEM, supplemented with 5% charcoal-stripped FBS, L-glutamine and antibiotics. In expression assays, the cells were treated with different ligands (final concentration 10 nM) or solvent for 6 h or 24 h prior to extraction of total RNA using High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. In competition assays, the cells were treated for 24 h with 10 nM E2 and 1 μM of different compounds prior to RNA extraction. Real-time quantitative PCR was performed with LightCycler 480 apparatus (Roche) using TaqMan Gene Expression Assays for *RPLP0* (4333761F) and *pS2* (4331182) (Applied Biosystems, Warrington, UK). Fold changes were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = \Delta Ct_{(stimulus)} - \Delta Ct_{(solvent)}$, and $\Delta Ct = Ct_{(pS2)} - Ct_{(RPLP0)}$. Ct is the cycle at which the threshold line is crossed. Two-tailed Student's *t*-tests were performed to calculate *p*-values.

### 5.3. Computational methods

Selected ligands were docked to the ERα (PDB entry 1X7R)[34] and ERβ (PDB entry 1X7B)[14] ligand-binding pockets using flexible docking and extra precision mode of the GLIDE program[35,36] of Schrödinger package. Molecular dynamics (MD) simulations of the *R* and *S* enantiomers of **50** bound to ERα and ERβ were done with the AMBER 9 program[37] and the Amber ff99SB force field.[38,39] To start the MD simulations, all the hydrogens and waters were minimized and then heated to 300 K and simulated for 100 ps. After that the whole system was minimized and heated to 300 K. After 300 ps of equilibration, 600 ps production runs were performed for each system.

The binding free energies of **(*R*)-50** and **(*S*)-50** to ERα and ERβ were estimated using the MM-PBSA[40,41] approach as implemented in the AMBER 9. The structures used in the free energy calculations were extracted from the first 150 ps of the MD trajectories. The structural water molecule participating in the hydrogen bonding network of the hydroxyl group of the ligand, $Arg_{394}$/$Arg_{346}$ (ERα/ERβ) and $Glu_{353}$/$Glu_{305}$ (Fig. 2) was included in the MM-PBSA calculations.

Two different thermodynamic integration (TI) calculations[42] were done: (i) the starting structure of the ERα–ligand complex was the end structure of 600 ps MD simulation of ERα–**(*R*)-50** complex and the atomic point charges of both **(*R*)-50** and **(*S*)-50** were those of **(*R*)-50**, and (ii) the end structure of the 600 ps MD simulation of ERα–**(*S*)-50** complex and the atomic point charges of **(*S*)-50** were used to set up the TI calculations. Nine-point Gaussian quadratures were used to calculate the total relative free energy. In the TI simulations of each of the nine different lambda values 100 ps equilibration simulations were done first, followed by 450 ps production simulations.

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