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Synthesis and Evaluation of 4-Cycloheptylphenols as Selective Estrogen Receptor-β Agonists (SERBAs)

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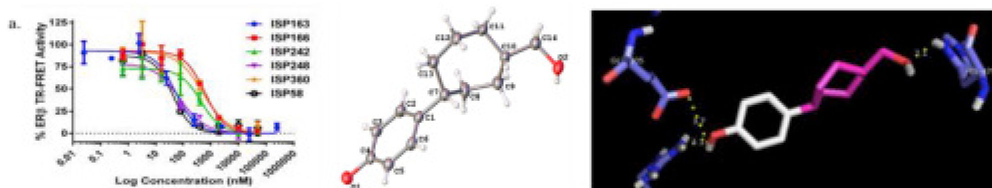
Abstract

A short and efficient route to 4-(4-hydroxyphenyl)cycloheptanemethanol was developed, which resulted in the preparation of a mixture of 4 stereoisomers. The stereoisomers were separated by preparative HPLC, and two of the stereoisomers identified by X-ray crystallography. The stereoisomers, as well as a small family of 4-cycloheptylphenol derivatives, were evaluated as estrogen receptor-beta agonists. The lead compound, 4-(4-hydroxyphenyl)cycloheptanemethanol was selective for activating ER relative to seven other nuclear hormone receptors, with 300-fold selectivity for the β over α isoform and with EC_{50} of 30–50 nM in cell-based and direct binding assays.

Graphical abstract



Keywords

SERBA, Estrogen receptor agonist, Drug development, Cancer

1. Introduction

Estrogens play an important role in the growth, development and maintenance of a variety of tissues which is mainly mediated by the estrogen receptor (ER), a ligand-activated nuclear receptor transcription factor. There are two main isoforms of the estrogen nuclear receptor, ERα and ERβ, which are found to diverge with respect to their transcriptional activities and tissue distribution. Upon binding of estradiol, ER activation can exert beneficial effects for the prostate, colon, and brain. Indeed, ERβ itself is a target for agonist-based drug leads to treat a wide range of indications, including depression [1], anxiety [2], dementia [3], and even cancer [4]. In contrast, ERα activity can present risks for cancer [5]. Thus, there is a therapeutic need for potent and selective ERβ agonists [6]. These differential effects have prompted researchers to search for novel ERβ selective ligand agonists as therapeutic agents (Fig. 1).

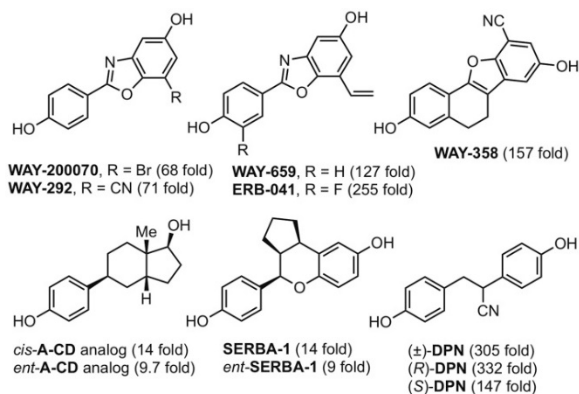
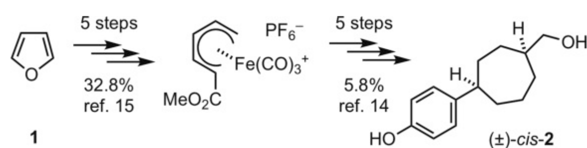


Fig. 1. Achiral and optically active estrogen receptor-β selective ligands. (ref. [1], [9], [10], [11], [12], [13]).

Although the ligand binding domains (LBDs) of ERα and ERβ share less than 60% sequence homology, the ligand binding pockets (LBP) of the two subtypes have only minor differences in structure and composition [7]. The two LBPs are composed of 23 amino acid residues, 21 of which are conserved and only two of which are variant. The residues Leu384 and Met421 in ERα are replaced with Met336 and Ile373 in ERβ respectively. Furthermore, the interchanged Leu384/Met336 residues are positioned above the B- and C-rings of estradiol whereas the interchanged Met421/Ile373 residues are positioned below the estradiol D-ring within the LBP. These minute alterations in amino acid sequence plus other small variations in tertiary structure make the ERβ LBP smaller in volume (282 Å^3) in comparison to the LBP of ERα (379 Å^3) [8].

Several achiral and chiral ERβ selective agonist compounds have been reported (Fig. 1) [6](d), [9], [10], [11], [12], [13]. For chiral compounds, the difference in ER selectivity for one enantiomer was generally less than 2-fold.

We have previously reported the synthesis of *cis*-4-(4-hydroxyphenyl)cycloheptanemethanol (±)-**2** from furan (10 steps, 1.9% overall yield, Scheme 1) [14,15]. Evaluation of (±)-**2** in cell-based assays revealed a potent and highly selective ERβ agonist. We herein report alternative syntheses of **2**, which result in the preparation of a racemic mixture of four stereoisomers, the separation of these stereoisomers and structural characterization of two of the enantiomeric structures, and the evaluation of the four stereoisomers as ERβ ligands, and promising ERβ agonist therapeutic lead molecules.

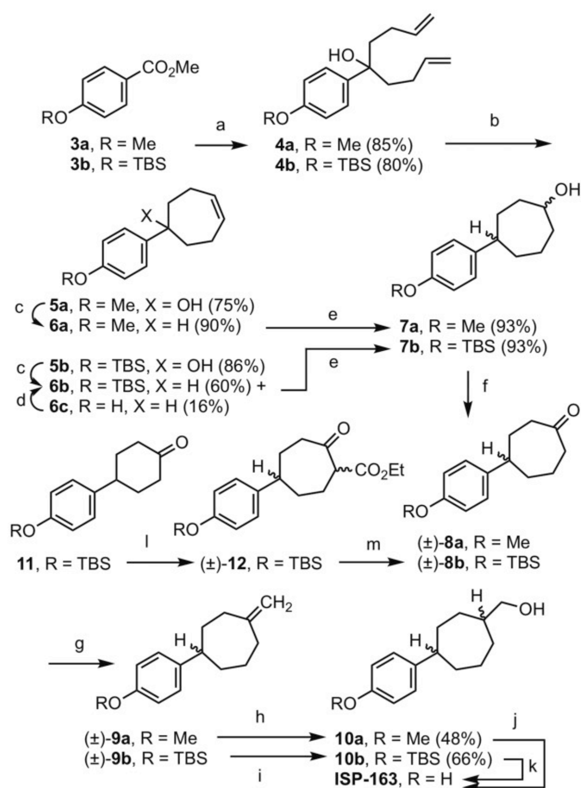


Scheme 1. Prior synthesis of *cis*-4-(4-hydroxyphenyl)cycloheptanemethanol (refs. [[14], [15]]).

2. Results and discussion

2.1. Chemistry

Several routes to 4-(4-hydroxyphenyl)cycloheptanemethanol were developed (Scheme 2). Addition of the Grignard reagent prepared from 4-bromo-1-butene with methyl 4-methoxybenzoate **3a** gave the tertiary alcohol **4a**. Ring closing metathesis of **4a** with 4% Grubbs' 1st generation catalyst afforded cycloheptenol **5a**, which underwent ionic reduction to generate the cycloheptene **6a**. Hydroboration-oxidation of **6a**, followed by oxidation with Dess-Martin periodinane gave the known [16] cycloheptanone (±)-**8a**. Wittig olefination of **8a**, followed by hydroboration-oxidation afforded the methyl ether **10a**. Cleavage of the methyl ether using BBr_3 proceeded in a disappointing 30% yield to give a ca. 1:1 mixture of (±)-*cis*- and (±)-*trans*-4-(4-hydroxyphenyl)cycloheptanemethanol (**ISP163**). This 8-step route proceeded in 3.7% overall yield.

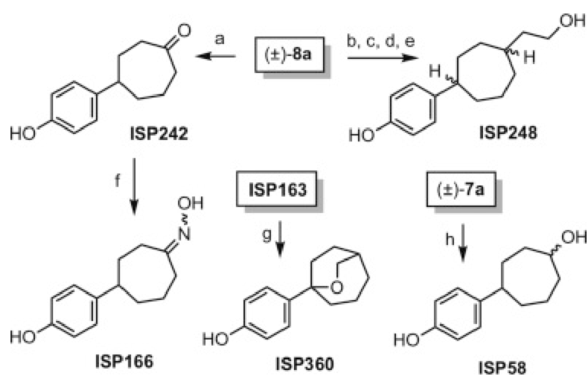


Scheme 2. Synthesis of mixture of *cis*- and *trans*-4-(4-hydroxyphenyl)cycloheptanemethanol. Reagents: a, Mg, $BrCH_2CH_2CH = CH_2$, THF (**4a**, 85%; **4b**, 80%); b, 4% $PhCH = RuCl_2(PCy_3)_2$, CH_2Cl_2/Δ (**5a**, 75%; **5b**, 86%); c, Et_3SiH, TFA, CH_2Cl_2 (**6a**, 90%; **6b**, 60% + 16% **6c**); d, TBSCl, imidazole (70%); e, (i) BH_3-THF, (ii) 30% H_2O_2, 3 N NaOH (**7a**, 93%; **7b**, 93%); f, Dess Martin periodinane (**8a**, 55%; **8b**, 72%); g, *n*-BuLi, $Ph_3PCH_3^+$ Br^- (**9a**, 78%; **9b**, 57%); h, (i) BH_3-THF, (ii) 30% H_2O_2, 3 N NaOH (48%); i, (i) BH_3-THF, (ii) 30% H_2O_2, 1 N NaOH (66%); j, BBr_3 (30%); k, TBAF, THF (88%); l, N_2CHCO_2Et/BF_3-Et_2O (81%); m, LiCl/H_2O/DMSO/Δ (73%).

Due to the low yield of the BBr_3 cleavage, an alternate phenolic protecting group was explored. Beginning with methyl 4-(4-*t*-butyldimethylsilyloxy)benzoate **3b**, the above sequence of reactions gave intermediates **4b**-**10b**, which deserve a few comments. The ionic reduction of **5b** gave a separable mixture of **6b** (60%) along with some of the unprotected phenol **6c** (16%). This phenol could be recycled to **6b** by TBS protection. Furthermore, hydroboration of **9b**, followed by oxidative work-up with H_2O_2/3 N NaOH proceeded with concomitant cleavage of the silyl ether to generate **ISP-163** (40%). Improved yield was effected by changing the work-up to 1 N NaOH followed by TBAF deprotection. This 8-step route proceeded to generate **ISP-163** in 9.2% overall yield.

Finally, a third shortened route was developed. Ring expansion of 4-(4-*t*-butyldimethylsilyloxyphenyl)cyclohexanone (**11**) with ethyl diazoacetate [16] gave the α-ethoxycarbonylcycloheptanone **12**, which upon decarboethoxylation gave (±)-**8b** (2 steps, 59%). This alternative 5-step route gave **ISP-163** in 19.6% overall yield.

Demethylation of **7a** and **8a** gave the cycloheptanol **ISP58** and cycloheptanone **ISP242** respectively (Scheme 3). Reaction of **ISP242** with hydroxylamine gave the oxime **ISP166** as a mixture of *E*- and *Z*-stereoisomers. Horner-Emmons olefination of **8a**, followed by DIBAL reduction, olefin reduction and cleavage of the methyl ether gave the hydroxyethyl analog **ISP248**, as a mixture of *cis*- and *trans*-stereoisomers. Finally, oxidative cyclization of **ISP163** with DDQ gave the 2-oxabicyclo[3.2.2]nonane **ISP360**.



Scheme 3. Reagents: a, 45% HBr/Δ (82%); b, NaH, $MeO_2CCH_2P(O)(OMe)_2$ (30%); c, $LiAlH_4$ (43%); d, H_2, Pd/C; e, BBr_3/CH_2Cl_2 (10% over two steps); f, H_2NOH-HCl/$NaHCO_3$/EtOH (50%); g, DDQ/CH_2Cl_2 (66%); h, BBr_3/CH_2Cl_2 (86%).

While the mixture of *cis*- and *trans*-isomers 4-(4-hydroxyphenyl)cycloheptanemethanol (**ISP163**) proved inseparable in our hands by SiO_2 column chromatography, the four stereoisomers could be separated by chiral HPLC. A preparative separation was contracted with Phenomenex (Torrance, CA). Initial analytical method development by Phenomenex revealed that a Lux Cellulose-3 5um column and isocratic mobile phase of ethanol: 2-propanol: hexanes (4.33: 8.66: 87) was optimum, with detection at 280 nM. The isolation process utilized a 250 × 30 mm preparative column and the aforementioned solvent system. This method produced a 12 min HPLC run with the first desired peak eluting just before 8 min. Since these conditions were isocratic, stacked injections were implemented to accelerate the process. In this regard, subsequent injections were made 6 min after the previous injection with the products from the first injection collected shortly after the second injection was made. Analytical QC chromatograms confirmed separation of the stereoisomers and indicated that each fraction was >94% of the enantiomeric excess (Fig. 2).

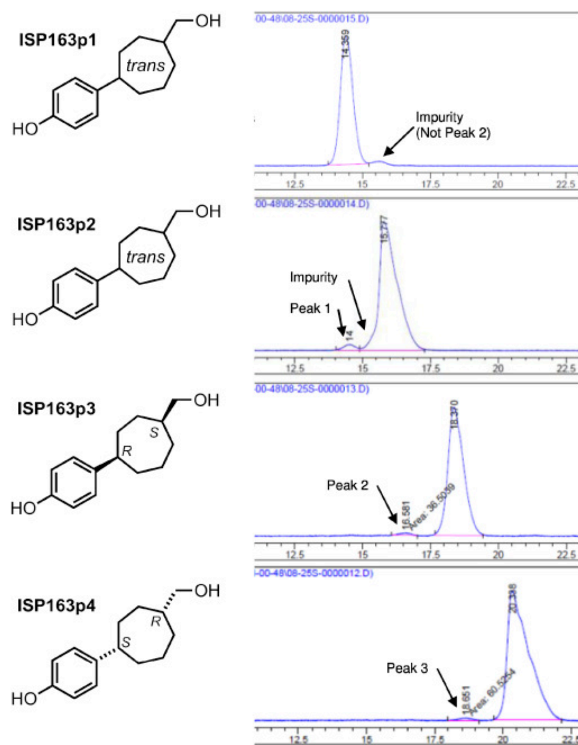


Fig. 2. Analytical QC chromatograms of four peaks of **ISP163**.

The *cis*-stereochemistry was assigned to the 3rd and 4th fractions (**ISP163p3** and **ISP163p4**) by comparison of their ^{13}C NMR spectra with that for (±)-**2** [14]; and thus the 1st and 2nd fractions (**ISP163p1** and **ISP163p2**) were assigned the *trans*-stereochemistry in order to be unique. These assignments were further corroborated by single crystal X-ray diffraction analysis of the 2nd, 3rd, and 4th fractions [17]. In addition, the crystal structures of **ISP163p3** (Fig. 3) and **ISP163p4** revealed the *absolute* configuration of these isomers to be 1*S*, 4R and 1R,4*S*-(4-hydroxyphenyl)cycloheptanemethanol respectively. Although the crystal structure of **ISP163p2** corroborated its *trans*-stereochemistry, it was not possible to determine the absolute configuration from these crystals.

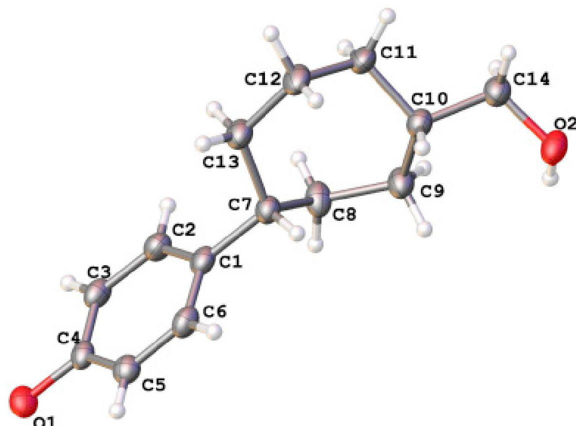


Fig. 3. ORTEP of 1S,4R-(4-hydroxyphenyl)cycloheptanemethanol **ISP163p3**.

2.2. Determining ISP163 extinction coefficient and isomer stock concentrations

The amount of powder of the **ISP163** isomers generated by chiral chromatography was often not sufficient to accurately weigh a mass for creation of DMSO stocks used in the ligand binding assays. Therefore, solid samples were dissolved in DMSO and the concentration of each stock was determined spectrophotometrically. First, the absorbance spectra for solutions of **ISP163** were obtained (Fig. 4a) and the λ_{max} peak was determined to occur at 276 nm. Then, the extinction coefficient of **ISP163** was determined to be 1892 $M^{-1}cm^{-1}$ from triplicate linear regressions of the peak absorbance (Fig. 4b). Two dilutions of **ISP163** isomer stocks were used to calculate the concentration of the stock. The average of the two calculated concentrations was determined to be the concentration of the stock solution.

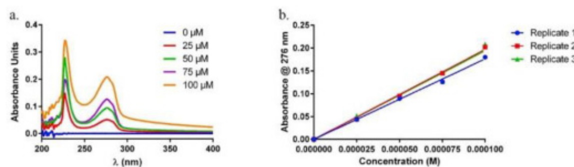


Fig. 4. **Determining ISP163 Extinction Coefficient**. (a) Representative absorbance scans of **ISP163** in 20 mM potassium phosphate buffer, pH 7.5 and 0.4% DMSO. (b) Plot of the absorbance value at 276 nm for each concentration of **ISP163** for 3 replicate experiments. Linear regression lines were forced through 0,0. The extinction coefficient of **ISP163** was determined to be 1892 M^{-1} cm^{-1} by averaging the slope of the 3 linear regression lines.

2.3. Biological activity evaluation

2.3.1. Binding, coactivator, and cell-based assays

Compounds were initially screened in the TR-FRET binding assay which detects binding of the compound to the ligand binding domain (LBD) of ERβ via displacement of a fluorescent estrogen. All compounds synthesized were tested in a dose-response curve (Fig. 5a,c) and EC_{50} values are summarized in Table 1. The most potent compounds were **ISP58**, **ISP163**, and **ISP248** with EC_{50}s < 75 nM. Secondary assays in a cell-based transcription assay with full-length ERβ and ERα revealed **ISP163** to be the most potent (ERβ EC_{50} 33 ± 5 nM) and most selective compound (318-fold selective for ERβ over ERα). To see if the selectivity for ERβ observed in the cell-based transcription assays was due to differential binding to the ERs, we conducted TR-FRET binding assays with the ERα – LBD. Surprisingly, we observed only modest 1.9-fold selectivity for binding to the LBD of ERβ over ERα for **ISP163** (Fig. 5b). Since **ISP163** is a mixture of isomers, we tested the 4 separated isomers in the TR-FRET binding displacement assays (Fig. 5c and d replicate assays in Figs. S1 and S2) and in the cell-based full-length ER transcription assays (Table 1 and Fig. 6). In the TR-FRET binding assay, **ISP163p1** and **ISP163p2** (i.e. trans cycloheptane) were found to be slightly more potent than the other two isomers, and more potent than the mixture. Surprisingly, in the cell-based assay, the ERβ agonist potency of the mixture of stereoisomers (**ISP163**) is actually slightly greater than any of the individual stereoisomers. The potency of three of these stereoisomers (**ISP163p1**, **ISP163p2** and **ISP163p4**), are relatively close in value to that for the mixture with only **ISP163p3**, ca. 4x less potent than the mixture. One possible rationale is that the individual stereoisomers exhibit a synergism in terms of their activation in the context of the cell-based assay [18]. In the cell-based transcription assay, ligand binding to ERβ receptor is followed by dimerization, and that the dimer binds to DNA promoting transcription. It is possible that synergistic binding of two different compounds in the mixture via this mechanism could produce greater transcriptional activity compared to an ERβ homodimer. But, further studies would be needed to prove this mechanism and it is noted that the difference in affinity between the isomers is relatively modest (2–4 fold). In summary, there do not appear to be significant difference in binding affinity for the four isomers, consistent with docking studies (Fig. 9).

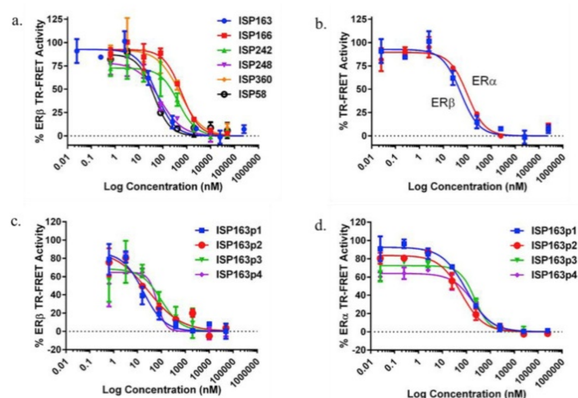


Fig. 5. TR-FRET Estrogen Receptor Binding. (a) Binding assay for the ligand binding domain (LBD) of ERβ (See Table 1 for EC_{50} values). (b) **ISP163** binding to the LBDs of ERβ (EC_{50} = 53 ± 15 nM) and ERα (EC_{50} = 99 ± 24 nM) showing a 1.9-fold selectivity for ERβ over ERα. (c) Binding of **ISP163** isomers to the LBD of ERβ and (d) ERα (EC_{50} values in Table 1.).

Table 1. Biological evaluation of compounds in TR-FRET Binding and Cell-based Transcription Assays. EC_{50} values are in nM.

Compound	TR-FRET ligand displacement assay			Cell-based transcription assay				
	ERβ EC_{50}	ERα EC_{50}	ERβ/ERα selectivity	ERβ agonism EC_{50}	ERβ antagonism EC_{50}	ERα agonism EC_{50}	ERα antagonism EC_{50}	ERβ/ERα agonist selectivity
E2	0.25 ± 0.06	0.26 ± 0.03	1.0	0.022 ± 0.005	ND	0.31 ± 0.03	ND	14
ISP163	53 ± 15	99 ± 24	1.9	33 ± 5	>100,000	10,500 ± 200	>10,000	318
ISP163p1	18 ± 6	96 ± 18	5.3	50 ± 2	>35,600	>3000	>35,600	>60
ISP163p2	28 ± 19	61 ± 13	2.2	48 ± 4	>23,200	>10,000	>10,000	>208
ISP163p3	99 ± 56	221 ± 34	2.2	121 ± 12	>10,000	>10,000	>10,000	>83
ISP163p4	66 ± 20	199 ± 40	3.0	51 ± 8	>11,600	>11,600	>11,600	>227
ISP248	75 ± 19	ND	–	139 ± 15	>10,000	>10,000	>10,000	>72
ISP58	42 ± 18	ND	–	362 ± 30	>10,000	>10,000	>10,000	>27
ISP166	600 ± 99	ND	–	>800	>10,000	>10,000	>10,000	>12
ISP360	509 ± 163	ND	–	ND	ND	ND	ND	–
ISP242	435 ± 118	ND	–	ND	ND	ND	ND	–

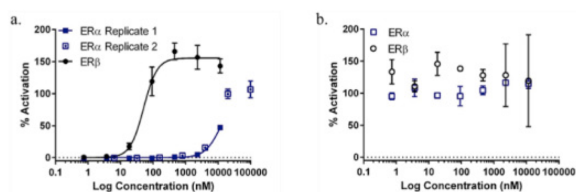


Fig. 6. **Cell-based assays for ISP163p4.** Cell-based full-length ER transcription assays for (a) agonist activity and (b) antagonist activity. **ISP163p4** shows >227-fold agonist selectivity for ERβ over ERα in this assay (ERβ EC_{50} = 51 ± 8 nM, ERα EC_{50} > 11,600 nM) and no antagonist activity.

To test the selectivity of **ISP163p4** for estrogen receptors compared to other nuclear hormone receptors, Thermo Fisher Scientific SelectScreen services were utilized. **ISP163p4** was tested against 9 nuclear hormone receptors at 3 different concentrations (Fig. 7a) and only showed activity with the estrogen receptors at any of the concentrations. This assay involves a chimeric ER-LBD tethered to the DNA-binding Domain (DBD) of GAL4. Ligand binding initiates transcription of the beta-lactamase gene. Addition of a beta-lactamase substrate to cells allows for quantification of the transcriptional activation. Dose-response curves in this assay, which uses a chimeric receptor, showed no selectivity for **ISP163p4** activating ERβ over ERα (Fig. 7b).

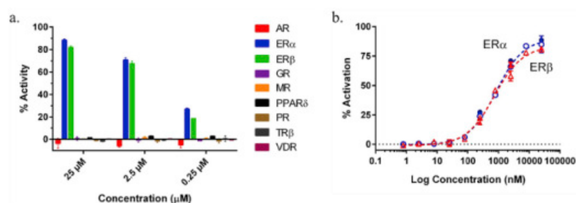


Fig. 7. **GeneBLAzer™ Nuclear Hormone Assay for ISP163p4.** (a) Agonist activity as measured in the cell-based GeneBLAzer™ transcription activation assay using chimeric nuclear hormone receptors (NRs) comprised of the receptor ligand-binding domain tethered to the DNA-binding domain of GAL4. Nine different NRs were

tested: Androgen Receptor (AR), Glucocorticoid Receptor (GR), Mineralocorticoid Receptor (MR), Peroxisome Proliferator-Activated Receptor (PPARδ), Progesterone Receptor (PR), Thyroid Hormone Receptor (TRβ), Vitamin D Receptor (VDR). (b) GeneBLAzer™ agonist-activity dose-response assays for ERβ and ERα (open symbols) showing no selectivity (ERβ EC_{50} = 735 ± 94 nM, ERα EC_{50} = 825 ± 82 nM). For comparison, data from panel (a) are included (closed symbols).

We hypothesize that the difference in specificity in the cell-based transcription assays (that uses native full-length ER) compared with the other assays (that rely on isolated LBD or chimeric ER) has to do with the ability of the compound to bind to the native estrogen receptor and – as a result of binding – cause the correct conformational change that allows downstream coactivator proteins to bind. To test this hypothesis, we measured **ISP163p4** binding and selectivity in the LanthaScreen TR-FRET Coactivator Assay (Thermo Fisher Scientific). In this assay, the ER-LBD undergoes a conformational change upon ligand binding that allows a fluorescent coactivator peptide to bind in the adjacent ER coactivator pocket. The peptide in this assay is derived from the PPARγ coactivator protein 1a. In this assay, **ISP163p4** now shows 4.7-fold selectivity for ERβ (EC_{50} = 566 ± 57 nM) over ERα (EC_{50} = 2660 ± 479 nM) (Fig. 8). This is consistent with **ISP163p4** selectivity being a function of more than just affinity for the LBD, as hypothesized.

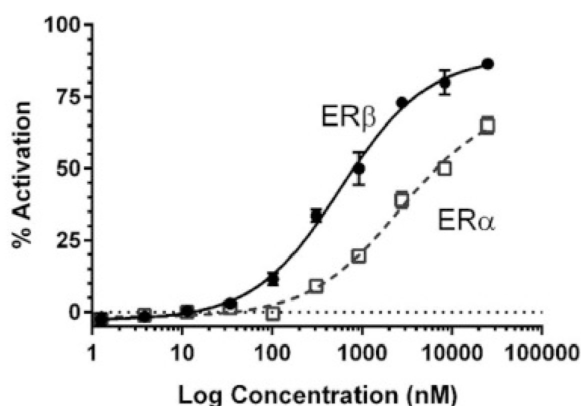


Fig. 8. **ISP163p4 Coactivator Binding Assay for Specificity.** This assay measures the binding of a coactivator peptide derived from the PPARγ coactivator protein 1a to the ERβ or ERα LBD. Agonist binding (**ISP163p4** here) induces a conformational change in the LBD allowing the peptide to bind. Dose-response curves in this assay give an EC_{50} of 566 ± 57 nM for ERβ and 2660 ± 479 nM for ERα, showing 4.7-fold selectivity for ERβ.

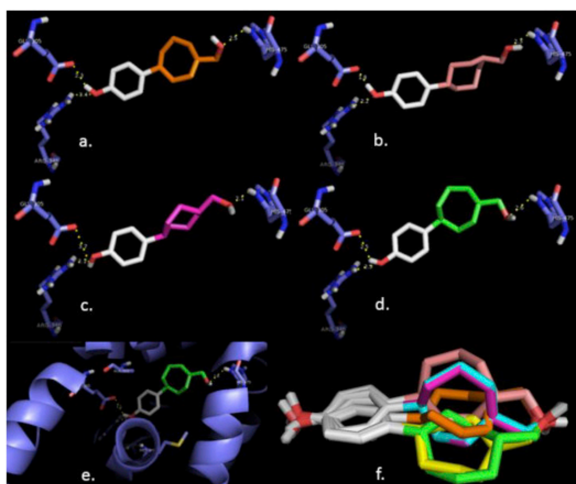


Fig. 9. Docking of ISP163 Isomers into ERβ. All four isomers of 4-(4-hydroxyphenyl)cycloheptanemethanol **ISP163** docked into the binding pocket of agonist mode human ERβ (pdb code 2jj3) [19]: (a) (4R, 1R) stereoisomer, (b) (4*S*,1*S*) stereoisomer, (c) (4R, 1*S*) stereoisomer **ISP163p3**, and (d)

(4*S*,1R) stereoisomer **ISP163p4**. The active site, with surrounding helices rendered as ribbons, is shown in panel (e) for the **ISP163p4**/ERβ complex. All low energy docking poses are shown overlaid for **ISP163** isomers in panel (f), illustrating that while there is variability in orientation of the cycloheptyl ring, the hydroxyl group location (hydrogen-bonded to His524) is constant.

The selectivity of **ISP163** was further assessed by screening against common central nervous system (CNS) receptors by the NIMH Psychoactive Drug Screening Program (PDSP) at the University of North Carolina at Chapel Hill. **ISP163** was also screened against the hERG heart potassium ion channel by the PDSP. No significant inhibition was observed for any CNS receptors or hERG (Table 2).

Table 2. PDSP screening of **ISP163**.

Receptor	% Inhibition at 10 μM
Serotonin	
5-HT_{1A}	14.6
5-HT_{1D}	25.3
5-HT_{2B}	−7.3
5-HT_{6}	19.0
5-HT_{7}	−8.5
Adrenergic	
α_{1A}	−3.7
α_{1B}	−4.9
α_{1D}	−16.7
β_{1}	−4.8
β_{3}	16.0
Histamine	
H_{2}	2.7
H_{4}	−0.1
Ion Channel	
hERG	−1.7
Other	
σ_{1}	17.1
σ_{2}	31.9

2.3.2. In-silico comparison of ISP163 isomer binding

While **ISP163** is the most potent and selective cycloheptylphenol ERβ agonist we have identified, it has four stereoisomers (Fig. 2), each of which could in principle have different activities. **ISP161p1** appears to have only slightly higher affinity (EC_{50} = 18 nM) than the other 3 isomers, in terms of binding to the ERβ LBD; but, in the biologically more relevant cell-based assay, they are all of similar potency and selectivity (Table 1). To assess why the isomers have similar affinities, all were docked into the ERβ active site. All bound in similar orientations, with some conformational variability observed only in the cycloheptyl ring; but in all cases, the positioning of the two hydroxyl groups was similar in the active site (Fig. 9f). Docking energies were also similar for the four isomers: **ISP163p1** (−8.5 kcal/mol), **ISP163p2** (−8.3 kcal/mol), **ISP163p3** (−8.3 kcal/mol), and **ISP163p4** (−8.1 kcal/mol).

2.3.3. Assessment of physico/physiochemical properties - CYP450 binding and nephelometry

In addition to the lack of activity against the seven nuclear hormone receptors (Fig. 7), initial assessment of physicochemical properties of **ISP163** was made by measuring cytochrome P450 binding, in assays with the four major cytochrome P450 enzymes. Significant inhibition of CYP2C9 was observed (IC_{50} = 2.7 ± 0.3 μM), moderate

inhibition of CYP3A4 was observed (IC_{50} = 33 ± 3 μM), and no significant inhibition was observed for CYP2D6 or CYP1A2 (Fig. S3). Solubility of **ISP163** is adequate, based on nephelometry where no significant aggregation was observed when tested up to 250 μM (Fig. S4).

2.3.4. Breast cancer proliferation assays

While estrogen agonists have a number of therapeutic applications, they can be pro-carcinogenic by causing proliferation of breast cancer cells [5,6,20]; although, the opposite effect has been reported for ERβ agonists [2]. For this reason, the impact of **ISP163** on the proliferation of human breast cancer cells was assessed by conducting MTT assays with MCF-7 cells. Significant cell proliferation was observed in cells treated with 0.01 μM E2 (n = 3; $p \le 0.01$) compared to untreated controls (Fig. 10), consistent with a mild pro-carcinogenic effect due to its ERα agonist activity. In contrast, no significant changes in growth of MCF-7 cells was observed when cells were treated with any concentration of **ISP163** compared to untreated controls; and, proliferation was significantly lower compared to cells treated with 0.01 μM E2 (n = 3; $p \le 0.04$ for all concentrations).

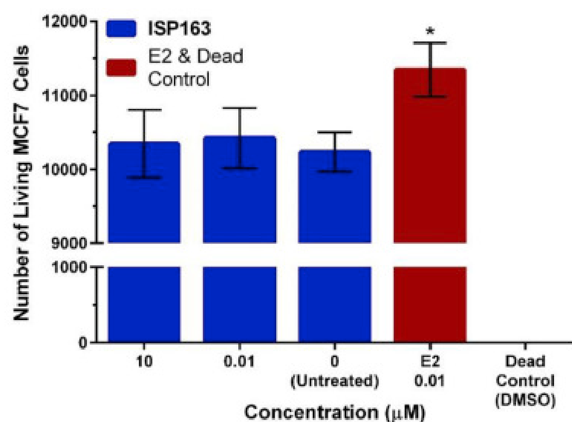


Fig. 10. MTT Assays with ISP163. MCF-7 cells were grown in 96-well plates for 24 h after which treatment was applied. Cells were incubated an additional 24 h after which the MTT assay was done. A standard growth curve was used to convert absorbance values to cell number. * indicates significant cell proliferation compared to untreated controls and to each concentration of **ISP163**. Results with 1, 0.1, and 0.001 μM **ISP163** are not shown because results were similar to 10 and 0.01 μM. Note the vertical axis break.

3. Conclusion

The results of the present study demonstrate that **ISP163**, 4-(4-hydroxyphenyl)cycloheptanemethanol, is selective for ERβ, in cell-based assays, and that there are negligible differences in potency and selectivity among the four stereoisomers. While **ISP163** does not cause MCF-7 cell proliferation, shows no significant aggregation up to 250 μM, and does not inhibit CYP2D6 or CYP1A2, it significantly inhibits CYP2C9 and moderately inhibits CYP3A4. If **ISP163** is to be developed as a drug lead, the binding to CYP2C9 will need to be addressed.

4. Experimental

4.1. Chemistry

4.1.1. General experimental

All reactions involving moisture or air sensitive reagents were carried out under a nitrogen atmosphere in oven-dried glassware with anhydrous solvents. THF and ether were distilled from sodium/benzophenone. Purifications by chromatography were carried out using flash silica gel (32–63 μ). NMR spectra were recorded on either a Varian Mercury+ 300 MHz or a Varian UnityInova 400 MHz instrument. $CDCl_3$, CD_3OD and DMSO-d_6 was purchased from Cambridge Isotope Laboratories. ^1H NMR spectra were calibrated to

7.27 ppm for residual $CHCl_3$ or 3.31 ppm for CD_2HOD. ^{13}C NMR spectra were calibrated from the central peak at 77.23 ppm for $CDCl_3$ or 49.15 ppm for CD_3OD. Coupling constants are reported in Hz. Elemental analyses were obtained from Midwest Microlabs, Ltd., Indianapolis, IN, and high-resolution mass spectra were obtained from the COSMIC lab at Old Dominion University.

4.1.2. 5-(4-Methoxyphenyl)-1, 8-nonadien-5-ol 4a

To a flame dried three-necked flask fitted with a condenser and addition funnel was charged with magnesium turnings (3.654 g, 152.1 mmol) and dry THF (30 mL) while maintaining the system under N_2. The addition funnel was loaded with a solution of 4-bromo-1-butene (7.72 mL, 76.1 mmol) in THF (20 mL), and a small amount of the bromobutene solution (2 mL) was added slowly to the magnesium turnings, and the contents were heated to reflux. Once the Grignard formation had started, the heat was removed and the remaining bromide solution was added dropwise maintaining a gentle reflux. The mixture was stirred until most of the magnesium had reacted. A solution of methyl 4-methoxybenzoate **3a** (2.528 g, 15.20 mmol) in THF (30 mL) was loaded into the addition funnel and added dropwise over 30 min. After stirring overnight at room temperature, a saturated solution of NH_4Cl (30 mL) was added to quench the reaction. The resultant emulsion was stirred for 2 h and the solution was extracted several times with ether. The combined organic layers were washed with water, followed by brine, dried ($MgSO_4$) and concentrated to give alcohol **4a** as a yellow oil (3.182 g, 85%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.29 and 6.88 (AA′BB′, J_{AB} = 8.9 Hz, 4H, ArH), 5.84–5.73 (m, 2H, C$\underline{H}$═CH_2), 4.98–4.88 (m, 4H, CH═C$\underline{H_2}$), 3.81 (s, 3H, OMe), 1.96–1.84 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.2, 139.0, 137.9, 126.6, 114.7, 113.6, 77.0, 55.4, 42.3, 28.2 ppm. HRMS (FAB): $M_2 + Na^+$, found 515.3130. $(C_{16}H_{22}O_2)_2Na$ requires 515.3132.

4.1.3. 5-(4-***t***-Butyldimethylsilyloxyphenyl)nona-1,8-dien-5-ol 4b

The reaction of methyl 4-*t*-butyldimethylsilyloxybenzoate (5.000 g, 0.0188 mmol) with the Grignard reagent generated from 4-bromo-1-butene (11.5 mL, 0.113 mol) was carried out in a fashion similar to the preparation of **4a**, to give **4b** as a colorless oil (5.208 g, 80%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.23 and 6.82 (AA′BB′, J_{AB} = 8.6 Hz, 4H, ArH), 5.85–5.73 (m, 2H, C$\underline{H}$═CH_2), 5.01–4.86 (m, 4H, CH═C$\underline{H_2}$), 2.13–1.80 (m, 9H), 1.01 (s, 9H, *t*-Bu), 0.22 (s, 6H, Si$\underline{Me_2}$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.2, 139.1, 138.5, 126.5, 119.7, 114.7, 77.0, 42.2, 28.2, 25.8, 18.3, −4.2 ppm.

4.1.4. 1-(4-Methoxyphenyl)-4-cyclohepten-1-ol 5a

To a solution of **4a** (1.015 g, 4.126 mmol) in dry CH_2Cl_2 (415 mL, 0.01 M) at 40 °C was slowly added via syringe pump over a 10 h period, a solution of Grubbs I catalyst (0.136 g, 0.165 mmol, 4%) in CH_2Cl_2. The mixture was heated for an additional 12–18 h. After cooling to room temperature, the mixture was quenched with DMSO (50 eq, 0.600 mL) and stirred for another 12 h. The mixture was concentrated and the residue was purified by column chromatography (SiO_2, hexanes–diethyl ether = 4:1) to give **5a** (0.675 g, 75%) as a green oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.43 and 6.87 (AA′BB′, J_{AB} = 9.0 Hz, 4H, ArH), 5.86–5.83 (m, 2H, CH═CH), 3.80 (s, 3H, OMe), 2.55–2.44 (m, 2H), 2.10–1.97 (m, 4H), 1.90–1.82 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.4, 142.4, 132.12, 125.9, 113.6, 76.7, 55.4, 40.2, 23.2 ppm. HRMS (FAB): M + Na^+, found 241.1202. $C_{14}H_{18}O_2Na$ requires 241.1199.

4.1.5. 1-(4-((tert-Butyldimethylsilyl)oxy)phenyl)cyclohept-4-en-1-ol 5b

The ring closing metathesis of **4b** (0.313 g, 0.903 mmol) in dry CH_2Cl_2 (100 mL, 0.01 M) with Grubbs I catalyst (0.029 g, 0.032 mmol, 4%) was carried out in a fashion similar to the preparation of **5a**. Purification of the residue by column chromatography (SiO_2, hexanes–diethyl ether = 4:1) gave **5b** (0.247 g, 86%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 and 6.79 (AA′BB′, J_{AB} = 8.7 Hz, 4H, ArH), 5.86–5.79 (m, 2H, CH═CH), 2.54–2.43 (m, 2H), 2.10–1.94 (m, 4H), 1.90–1.82 (m, 2H), 1.73 (s, 1H), 0.99 (s, 9H, *t*-Bu), 0.20 (s, 6H, Si$\underline{Me_2}$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.5, 142.9, 132.3, 125.9, 119.7, 76.7, 40.3, 25.8, 23.2, 18.3, −4.2 ppm. HRMS (FAB): M + Na^+, found 325.1959. $C_{19}H_{30}OSiNa$ requires 325.1958.

4.1.6. 5-(4-Methoxyphenyl)cycloheptene 6a

To a solution of **5a** (1.720 g, 7.880 mmol) in dry CH_2Cl_2 (50 mL) was added triethylsilane (1.4 mL, 0.22 mmol), followed by TFA (6.2 mL, 79 mmol). The mixture was stirred at room temperature for 48 h while monitoring by TLC. After complete disappearance of the starting material, the solution was concentrated to a bilayer oil and purified by column chromatography (SiO_2, hexanes–ethyl acetate = 50:50) to give **6a** as a brown oil (1.433 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 and 6.84 (AA'BB', J_{AB} = 8.6 Hz, 4H, ArH), 5.91–5.87 (m, 2H, CH═CH), 3.79 (s, 3H, OMe), 2.69 (tt, *J* = 11.3, 3.2 Hz, 1H, H^5), 2.35–2.25 (m, 2H), 2.23–2.13 (m, 2H), 1.91–1.83 (m, 2H), 1.54–1.43 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.8, 141.7, 132.7, 127.7, 113.9, 55.4, 49.6, 35.1, 28.1 ppm.

4.1.7. 5-(4-***t***-Butyldimethylsilyloxyphenyl)cycloheptene 6b

The ionic reduction of **5b** (1.601 g, 5.026 mmol) in anhydrous CH_2Cl_2 (20 mL) with triethylsilane (0.8 mL, 1.02 mmol) and TFA (4.0 mL, 20 mmol) was carried out in a fashion similar to preparation of **6a**. Purification of the residue by column chromatography (SiO_2, hexanes = 100%) gave **6b** (0.906 g, 60%) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 and 6.78 (AA'BB', J_{AB} = 8.7 Hz, 4H, ArH), 5.92–5.89 (m, 2H, CH═CH), 2.69 (tt, *J* = 11.2, 3.2 Hz, 1H, H^5), 2.36–2.27 (m, 2H), 2.24–2.14 (m, 2H), 1.93–1.85 (m, 2H), 1.55–1.44 (m, 2H), 1.01 (s, 9H, *t*-Bu), 0.22 (s, 6H, $SiMe_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.7, 142.2, 132.7, 127.6, 120.0, 49.7, 35.1, 28.1, 25.9, 18.4, −4.2 ppm.

4.1.8. 4-(4-Methoxyphenyl)cycloheptanol 7a

To a solution of **6a** (1.24 g, 5.67 mmol) in freshly distilled THF (25 mL) at 0 °C under N_2, was added dropwise a solution of BH_3-THF (1 M in THF, 11.3 mL, 11.3 mmol). The solution was warmed to room temperature and stirred for 20 h. The reaction mixture was cooled to 0 °C, and water (440 mL) was slowly added followed by 30% H_2O_2 (8.50 mL) and 1 N NaOH (14.5 mL). The resulting mixture was stirred at room temperature for 30 min, extracted several times with ethyl acetate, and the combined extracts concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 60:40) to give **7a** (1.150 g, 93%) as a yellow oil. This product was determined to be a mixture of *cis*- and *trans*-stereoisomers by NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 and 7.09 (2 x d, *J* = 8.3 Hz, 2H total, ArH), 6.83 (d, *J* = 8.2 Hz, 2H, ArH), 4.06–4.00 and 3.99–3.90 (m, 1H, CHOH), 3.78 (s, 3H, OMe), 2.72–2.56 (m, 1H, H^4), 2.15–2.05 (m, 1H), 2.02–1.50 (m, 11H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.7, 141.5, 127.7, 113.9, 72.9, 71.8, 55.4, 46.4, 46.1, 38.4, 37.8, 37.3, 37.1, 37.0, 35.8, 31.9, 29.7, 23.4, 21.5 ppm. HRMS (FAB): M + Na^+, found 243.1358. $C_{14}H_{20}O_2Na$ requires 243.1356.

4.1.9. 4-(4-t-Butyldimethylsilyloxyphenyl)cycloheptanol 7b

The hydroboration/oxidation of **6b** (0.906 g, 2.99 mmol) with BH_3-THF (1 M in THF, 6.0 mL, 6.0 mmol) was carried out in a fashion similar to the preparation of **7a**. Purification of the residue by column chromatography (SiO_2, hexanes-ethyl acetate = 80:20) gave **7b** (0.880 g, 93%) as a yellow oil. This was determined to be a mixture of *cis*- and *trans*-stereoisomers by NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.01 (m, 2H, ArH), 6.74 (m, 2H, ArH), 4.06–3.99 and 3.98–3.90 (m, 1H, CHOH), 2.69–2.53 (m, 1H, H^4), 2.14–1.49 (m, 11H), 0.97 (s, 9H, *t*-Bu), 0.18 (s, 6H, $SiMe_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.6, 142.2, 142.1, 127.6, 119.9, 73.0, 71.9, 46.4, 46.1, 38.3, 37.8, 37.3, 37.1, 37.0, 35.9, 31.8, 29.8, 25.9, 23.5, 21.5, 18.4, −4.2 ppm. HRMS (FAB): M + Na^+, found 343.2064. $C_{19}H_{32}O_2SiNa$ requires 343.2064.

4.1.10. 4-(4-Methoxyphenyl)cycloheptanone (±)-8a

To a solution of 7a (0.787 g, 3.58 mmol) in CH2Cl2 (38 mL) at room temperature, was added Dess–Martin periodinane (4.55 g, 10.7 mmol) and water (0.2 mL). The mixture was stirred at room temperature for 6 h, and then quenched with 50:50 sodium thiosulfate:sodium bicarbonate. The resulting solution was stirred at room temperature for 30 min and then extracted several times with ethyl acetate, dried (MgSO4), and concentrated. The residue was purified by column chromatography (SiO2, hexanes– ethyl acetate = 80:20) to give 8a (0.389 g, 55%) as a colorless oil. 1H NMR (400 MHz, CDCl3) δ 7.10 and 6.84 (AA'BB', JAB = 8.7 Hz, 4H, ArH), 3.79 (s, 3H,

OMe), 2.77–2.53 (m, 4H), 2.16–1.52 (m, 7H); 13C NMR (100 MHz, CDCl3) δ 215.0, 157.9, 139.9, 127.4, 113.9, 55.3, 47.9, 43.8, 42.9, 38.6, 32.2, 23.8 ppm. The spectral data obtained for this compound were consistent with the literature values [16]. Oxidation of 7a with either PCC/silica gel or n-propylmagnesium bromide/1,1'-(azodicarbonyl)dipiperidine gave 8a (55% or 20% respectively).

4.1.11. 4-(4-t-Butyldimethylsilyloxyphenyl)cycloheptanone (±)-8b

The oxidation of **7b** (0.050 g, 0.16 mmol) with Dess–Martin periodinane (0.132 g, 0.312 mmol) and water (0.1 mL) was carried out in a fashion similar to the preparation of **8a**. Purification of the residue by column chromatography (SiO_2, hexanes–ethyl acetate = 80:20) gave **8b** (0.036 g, 72%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.01 and 6.75 (AA'BB', J_{AB} = 8.6 Hz, 4H, ArH), 2.72–2.51 (m, 5H), 2.13–2.06 (m, 1H), 2.04–1.95 (m, 2H), 1.86–1.68 (m, 2H), 1.62–1.52 (m, 1H), 0.97 (s, 9H, *t*-Bu), 0.18 (s, 6H, Si<u>Me</u>$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 215.2, 154.0, 140.6, 127.5, 120.1, 48.1, 44.0, 43.1, 38.7, 32.3, 25.9, 24.0, 18.3, −4.2 ppm. HRMS (FAB): M + Na^+, found 341.1908. $C_{19}H_{30}O_2SiNa$ requires 341.1907.

4.1.12. Ethyl 5-(4-((tert-butyldimethylsilyl)oxy)phenyl)-2-oxocycloheptane-1-carboxylate (±)-12

To a solution of **11** (1.14 g, 3.74 mmol) in anhydrous ether (15 mL) at 0 °C under N_2 was added an aliquot of BF_3-Et_2O (0.92 mL, 7.5 mmol), followed by the dropwise addition over a period of 20 min of a solution of ethyl diazoacetate (0.77 mL, 7.47 mmol) in anhydrous ether (5 mL). The reaction mixture was stirred at room temperature for 12 h, then cooled to 0 °C and neutralized with saturated aqueous $NaHCO_3$. The mixture was extracted several times with $CHCl_3$, and the combined organic extracts were washed with brine, dried (Na_2SO_4) and concentrated. The resultant dark yellow oil was purified by column chromatography (SiO_2, hexanes–diethyl ether = 70:30) to give **12** (1.182 g, 81%) as a colorless oil. The product is a complex equilibrium mixture of two diastereomeric keto tautomers and one enol tautomer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.74 (s, 0.4H, enol OH), 7.02–6.97 (m, 2H, ArH), 6.77–6.72 (m, 2H, ArH), 4.27–4.16 (m, 2H, OC<u>H</u>$_2$CH$_3$), 3.64 (t, *J* = 4.8 Hz) and 3.60 (dd, *J* = 12.0, 4.0 Hz, 0.3H total, O═CC<u>H</u>CO$_2$Et), 2.94–2.78 (m, 1H), 2.72–2.58 (m, 2H), 2.48–2.24 (m, 1H), 2.16–1.76 (m, 4H), 1.65–1.54 (m, 1H), 1.32 and 1.29 (2 x t, *J* = 7.2 Hz, 3H total, OCH$_2$C<u>H</u>$_3$), 0.97 (s, 9H, *t*-Bu), 0.18 (s, 6H, Si<u>Me</u>$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.0, 208.8, 178.9, 173.0, 170.6, 154.0, 140.9, 139.9, 127.7, 127.5, 120.2, 120.0, 101.5, 61.4, 60.7, 59.6, 58.5,49.6, 47.9, 47.2, 42.2, 36.8, 35.4, 34.6, 32.8, 32.2, 27.8, 25.9, 23.9, 22.6, 18.4, 14.5, −4.2 ppm.

4.1.13. 4-(4-t-Butyldimethylsilyloxyphenyl)cycloheptanone (±)-8b

To a solution of **13** (0.205 g, 0.525 mmol) in DMSO (20 mL) at room temperature, was sequentially added lithium chloride (0.178 g, 4.20 mmol) and water (3.80 mL). The mixture was heated to 160 °C for 5 h, cooled to room temperature and poured into water. The resulting solution was extracted several times with ether and ethyl acetate. The combined extracts were washed with brine, dried (Na_2SO_4) and concentrated to give **8b** (0.122 g, 73%) as a colorless oil. The NMR spectral data for the product are consistent with that previously obtained.

4.1.14. 1-Methylene-4-(4-methoxyphenyl)cycloheptane (±)-9a

To a solution of methyltriphenylphosphonium bromide (1.25 g, 3.74 mmol) in anhydrous THF (30 mL) at −10 °C under N_2, was added dropwise a solution of *n*-butyllithium (1.6 <u>M</u> in hexanes, 2.2 mL, 3.5 mmol). The deep yellow mixture was stirred for 45 min at −10 °C, followed by slow addition of a solution of **8a** (0.380 g, 1.74 mmol) in THF (10 mL). The solution changed from a deep to light yellow in color, and the mixture was gradually warmed to room temperature and stirred overnight. The solution was diluted with water, the layers separated and the aqueous layer was extracted several times with ethyl acetate. The combined organic extracts were washed with brine, dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 80:20) to give **9a** (0.296 g, 78%) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 and 6.83 (AA'BB', J_{AB} = 8.4 Hz, 4H, ArH), 4.77 (s, 2H, C═C<u>H</u>$_2$), 3.79 (s, 3H, OMe), 2.61–2.45 (m, 2H), 2.32

(broad t, J = 12.2 Hz, 2H), 2.00–1.84 (m, 3H), 1.71–1.48 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.7, 151.9, 141.7, 127.7, 113.8, 110.7, 55.4, 47.5, 38.0, 37.3, 36.2, 35.4, 27.5 ppm.

4.1.15. 4-(4-t-Butyldimethylsilyloxyphenyl)-1-methylenecyclohept-ane (±)-9b

The Wittig olefination of **8b** (0.212 g, 0.666 mmol) with the ylide generated from $CH_3PPh_3^+$ Br^- (0.476 g, 1.33 mmol) and *n*-butyllithium (1.6 M in hexanes, 0.83 mL, 1.3 mmol) was carried out in a fashion similar to the preparation of **9a**. Purification of the residue by column chromatography (SiO_2, hexanes–ethyl acetate = 90:10) gave **9b** (0.120 g, 57%) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 and 6.75 (AA'BB', J_{AB} = 8.7 Hz, 4H, ArH), 4.76 (s, 2H, C=C$\underline{H}_2$), 2.59–2.45 (m, 2H), 2.37–2.26 (m, 2H), 2.01–1.85 (m, 3H), 1.70–1.48 (m, 4H), 1.00 (s, 9H, *t*-Bu), 0.20 (s, 6H, Si$\underline{Me}_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.6, 151.9, 142.3, 127.6, 119.9, 110.7, 47.6, 38.0, 37.2, 36.2, 35.4, 27.4, 25.9, 18.4, −4.2 ppm. HRMS (FAB): M + Na^+, found 339.2115. $C_{20}H_{32}OSiNa$ requires 339.2115.

4.1.16. 4-(4-Methoxyphenyl)cycloheptanemethanol 10a

To a solution of **9a** (0.296 g, 1.37 mmol) in freshly distilled THF (10 mL) at 0 °C, was added dropwise a solution of BH_3-THF (1 M in THF, 2.7 mL, 2.7 mmol). The resulting mixture was warmed to room temperature and stirred for 20 h. The reaction mixture was cooled to 0 °C, and pure ethanol (115 mL) was slowly added followed by 30% H_2O_2 (2.0 mL) and 3 N NaOH (10 mL). The mixture was heated at reflux for 1 h, cooled to room temperature and extracted several times with ethyl acetate, the combined extracts were dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 60:40) to give **10a** (0.155 g, 48%) as a colorless gum. This was determined to be a mixture of *cis-* and *trans*-stereoisomers by NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 and 6.83 (AA'BB', J_{AB} = 8.8 Hz, 4H, ArH), 3.77 (s, 3H, OMe), 3.46 (d, J = 6.4 Hz, 2H, C$\underline{H}_2$OH), 2.69–2.55 (m, 1H), 2.00–1.72 (m, 8H), 1.68–1.39 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.6, 142.1, 141.8, 127.6, 113.8, 68.6, 68.3, 55.4, 47.2, 46.0, 42.2, 41.3, 38.8, 36.7, 36.4, 33.0, 31.5, 30.6, 29.9, 28.4, 27.5, 24.3 ppm. HRMS (FAB): M + Na^+, found 257.1515. $C_{15}H_{22}O_2Na$ requires 257.1512.

4.1.17. 4-(4-t-Butyldimethylsilyloxyphenyl)cycloheptanemethanol 10b

To a solution of **9b** (0.821 g, 2.71 mmol) in freshly distilled THF (10 mL) at 0 °C, was added dropwise a solution of borane-tetrahydrofuran complex (1 M in THF, 5.4 mL, 5.42 mmol). The resulting mixture was warmed to room temperature and stirred for 18 h. The reaction mixture was cooled to 0 °C, and 1 N sodium hydroxide (3.2 mL) was added slowly followed by 30% hydrogen peroxide (1.5 mL). The mixture was stirred for 1 h at room temperature, extracted several times with ethyl acetate, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 80:20) to give 10b (0.572 g, 66%) as a colorless oil. This was determined to be a mixture of *cis-* and *trans*-stereoisomers by NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.02 and 6.74 (AA'BB', J_{AB} = 8.3 Hz, 4H, ArH), 3.45 (d, J = 6.5 Hz, 2H, C$\underline{H}_2$OH), 2.67–2.53 (m, 1H), 1.98–1.38 (m, 11H), 1.29–1.09 (m, 1H), 0.98 (s, 9H, *t*-Bu), 0.19 (s, 6H, Si$\underline{Me}_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.5, 142.6, 127.6, 127.5, 119.9, 68.7, 68.5, 47.3, 46.1, 42.2, 41.3, 38.8, 36.7, 36.4, 33.1, 31.5, 30.7, 29.9, 28.5, 27.5, 25.9, 24.3, 18.4, −4.2 ppm. HRMS (FAB): M + Na^+, found 357.2218. $C_{20}H_{34}O_2SiNa$ requires 357.2220.

4.1.18. 4-(4-Hydroxyphenyl)cycloheptanemethanol ISP163

Method A: To a solution of **10a** (0.180 g, 0.769 mmol) in anhydrous CH_2Cl_2 (10 mL) at −78 °C, was added dropwise a solution of boron tribromide (1 M in CH_2Cl_2, 2.31 mL, 2.31 mmol). The reaction mixture was stirred for 30 min at −78 °C and gradually warmed to room temperature over a 2 h period. The mixture was quenched with water (10 mL) and the layers separated. The aqueous layer was extracted several times with CH_2Cl_2, and the combined organic extracts were washed with brine, dried ($MgSO_4$), and concentrated. Purification of the residue by column chromatography (SiO_2, hexanes–ethyl acetate = 50:50) gave **ISP163** (0.048g, 30%) as a colorless solid. This product was determined to be a mixture of *cis-* and *trans*-stereoisomers NMR spectroscopy. mp 60–63 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 and 6.74 (AA'BB', J_{AB} = 8.5 Hz, 4H, ArH), 3.48 (d, J = 6.6 Hz, 2H, C$\underline{H}_2$OH),

2.67–2.49 (m, 1H), 1.97–1.32 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.7, 142.1, 127.9, 127.8, 115.3, 68.6, 68.6, 47.3, 46.0, 42.2, 41.3, 38.8, 36.6, 36.5, 33.0, 31.5, 30.6, 29.9, 28.5, 27.5, 24.3 ppm. Anal. Calcd. For $C_{14}H_{20}O_2$: C, 76.32; H, 9.15. Found: C, 76.21; H, 8.87.

Method B: To a solution of **10b** (0.873 g, 0.261 mmol) in anhydrous THF (20 mL) was added a solution of TBAF (1 M in THF, 10.0 mL, 0.010 mol). The mixture was heated to reflux at 70 °C overnight. After cooling to room temperature, the mixture was partitioned between ethyl acetate and water. The organic layer was washed with brine, dried (Na_2SO_4) and concentrated. Purification of the residue by column chromatography (SiO_2, hexanes-ethyl acetate = 60:40) gave **ISP163** (0.508 g, 88%) as a colorless solid. mp 60–63 °C. The ^{1}H NMR spectral data is consistent with that previously obtained.

ISP163P1: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 and 6.75 (AA'XX', J_{AX} = 8.4 Hz, 4H, ArH), 3.52–3.44 (m, 2H, C$\underline{H}_2$OH), 2.63–2.54 (m, 1H), 1.97–1.32 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.6, 142.0, 127.9, 115.3, 68.8, 47.3, 42.2, 36.8, 36.5, 30.6, 29.9, 24.3 ppm. HRMS (FAB): M–H^+, found 219.1390. $C_{14}H_{19}O_2$ requires 219.1391.

ISP163P3: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 and 6.75 (AA'XX', J_{AX} = 8.4 Hz, 4H, ArH), 3.48 (d, J = 6.4 Hz, 2H, C$\underline{H}_2$OH), 2.67–2.59 (m, 1H), 1.97–1.40 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.6, 142.2, 127.9, 115.3, 68.6, 46.0, 41.3, 38.8, 33.0, 31.5, 28.5, 27.5 ppm. HRMS (FAB): M–H^+, found 219.1391. $C_{14}H_{19}O_2$ requires 219.1391.

4.1.19. 4-(4-Hydroxyphenyl)cycloheptanone (±)-ISP242

A solution of **8a** (0.074 g, 0.339 mmol) in 48% HBr (8 mL) was heated at 115 °C for 2 h. The mixture was cooled to room temperature and partioned between ethyl acetate and water. The organic layer was washed with saturated aqueous $NaHCO_3$, followed by brine, dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 20:80) to give **ISP242** (0.057 g, 82%) as a brown syrup. ^{1}H NMR (400 MHz, CD_3OD) δ 6.98 and 6.70 (AA'BB', J_{AB} = 8.5 Hz, 4H, ArH), 4.98 (s, 1H, PhO$\underline{H}$), 2.77–2.39 (m, 4H), 2.02–1.47 (m, 7H); ^{13}C NMR (100 MHz, CD_3OD) δ 218.1, 156.6, 140.4, 128.5, 116.2, 49.0, 44.7, 43.8, 39.8, 33.4, 24.8 ppm. HRMS (FAB): M + Na^+, found 227.1043. $C_{15}H_{22}O_2$Na requires 227.1042.

4.1.20. 4-(4-Hydroxyphenyl)cycloheptanone oxime (±)-ISP166

To a solution of **ISP242** (0.048 g, 0.23 mmol) in ethanol (10 mL) was added $NaHCO_3$ (0.024 g, 0.29 mmol) and hydroxylamine hydrochloride (0.023 g, 0.69 mmol). The reaction was stirred at room temperature for 5 h and then extracted several times with ethyl acetate, and the combined extracts were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 65:35) to give **ISP166** (26 mg, 50%) as a light brown syrup. This was determined to be a mixture of *E*- and *Z*-stereoisomers by NMR spectroscopy. ^{1}H NMR (400 MHz, CD_3OD) δ 6.98 and 6.67 (AA'BB', J_{AB} = 8.5 Hz, 4H, ArH), 2.86–2.30 (m, 4H), 2.09–1.20 (m, 8H); ^{13}C NMR (100 MHz, CD_3OD) δ 165.0, 164.8, 156.4, 156.3, 141.3, 140.4, 128.5, 128.3, 116.1, 40.0, 39.7, 37.1, 34.1, 33.7, 33.3, 29.6, 28.4, 27.9, 24.8 ppm. HRMS (FAB): M_2 + Na^+, found 457.2096. $(C_{13}H_{15}NO_2)_2$Na requires 457.2098.

4.1.21. 4-(4-(2-Hydroxyethyl)cycloheptyl)phenol (±)-ISP248

Sodium hydride (32 mg, 55% in mineral oil, 0.809 mmol) was added to a stirring solution of trimethyl phosphonoacetate (0.130 mL, 0.809 mmol) in dry THF (3 mL) at 0 °C. After 45 min, a solution **8a** (0.147 g, 0.674 mmol) in dry THF (5 mL) was added and the reaction mixture was stirred at room temperature for 12 h. The mixture was diluted with water (15 mL) and the resulting mixture was extracted several times with ether, dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 90:10) to give methyl 2-(4-(4-methoxyphenyl)cycloheptylidene)acetate (0.57 g, 30%) as a colorless oil. This compound was used in the next step without further characterization. To a solution of the previous compound (0.200 g, 0.730 mmol) in dry CH_2Cl_2 (5 mL) under nitrogen at −40 °C was added a solution of DIBAL (1.2 M in CH_2Cl_2, 1.58 mL, 1.90 mmol). After stirring for 90 min, saturated aqueous potassium

sodium tartrate was added and reaction mixture was gradually warmed to room temperature. After 4 h the mixture was filtered through a pad of celite and extracted several times with water. The combined organic layers were dried ($MgSO_4$), and concentrated to give 4-(4-methoxyphenyl)-1-(2-hydroxyethyl)cycloheptane (0.078 g, 43%) as a colorless gum. The crude product was used in the next step without further purification. To a solution of the previous compound (0.078 g, 0.317 mmol) in methanol (10 mL) was added 10% Pd/C (0.040 g, 10 mol%). The mixture was stirred under balloon of H_2 at room temperature for 12 h. The reaction mixture was filtered through a pad of celite, concentrated and dried to give the crude product (0.080 g, 0.323 mmol). The crude product was dissolved in anhydrous CH_2Cl_2 (10 mL), cooled to −78 °C, and a solution of boron tribromide (1 M in CH_2Cl_2, 0.97 mL, 0.970 mmol) was added dropwise. After complete addition, the reaction mixture was stirred for 30 min at −78 °C and gradually warmed to room temperature over a 2 h period. The mixture was quenched with water and the mixture extracted several times with CH_2Cl_2. The combined organic extracts were washed with brine, dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (SiO_2, hexanes–ethyl acetate = 65:35) gave **ISP248** (0.005 g, < 10%) as a light brown solid. This was determined to be a mixture of *cis*- and *trans*-stereoisomers by NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 and 6.74 (AA′BB′, J_{AB} = 8.7 Hz, 4H, ArH), 3.71 (td, *J* = 6.9, 1.4 Hz, 2H, C$\underline{H}_2$OH), 2.66–2.48 (m, 1H), 1.96–1.13 (m, 13H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.6, 142.2, 127.8, 115.3, 61.5, 47.1, 45.9, 41.1, 40.9, 38.8, 36.8, 36.3, 35.9, 35.4, 34.8, 34.5, 33.9, 33.0, 32.1, 27.3, 24.4 ppm.

4.1.22. 4-(4-Hydroxycycloheptyl)phenol (±)-ISP58

To a solution of **7a** (0.028 g, 0.14 mmol) in anhydrous CH_2Cl_2 (30 mL) at −78 °C, was added dropwise a solution of boron tribromide (1 M in CH_2Cl_2, 0.3 mL, 0.03 mmol). The resulting mixture was stirred at −78 °C for 30 min, then gradually warmed to room temperature over a 2 h period and quenched with water (10 mL). The mixture was extracted several times with CH_2Cl_2, the combined organic extracts were washed with brine, dried ($MgSO_4$) and concentrated to give **ISP58** (0.024 g, 86%) as a yellow crystalline solid. This product was determined to be a mixture of *cis*- and *trans*-stereoisomers on the basis of NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11–6.99 (m, 2H, ArH), 6.80–6.70 (m, 2H, ArH), 4.85 (s, OH), 4.56–4.48 and 4.42–4.34 (m, 1H, RR′C$\underline{H}$OH), 2.78–2.59 (m, 1H), 2.53–1.38 (m, 13H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.7, 141.1, 127.9, 115.4, 56.2, 55.8, 46.0, 45.5, 40.1, 39.6, 39.4, 38.0, 37.7, 36.5, 34.4, 31.4, 25.4, 23.7 ppm.

4.1.23. 4-(6-Oxabicyclo[3.2.2]nonan-5-yl)phenol (±)-ISP360

To a solution of **ISP163** (0.075 g, 0.340 mmol) in anhydrous CH_2Cl_2 (20 mL) at −10 °C, was slowly added a suspension of DDQ (77 mg, 0.34 mmol) in CH_2Cl_2 (5 mL) over a period of 30. The green solution was stirred at 0 °C for 2 h, and at room temperature for an additional 3 h. The mixture was quenched by slow addition of saturated sodium bicarbonate solution at 0 °C, the layers were separated and aqueous layer was extracted several times with CH_2Cl_2. The combined organic extracts were washed with brine, dried (Na_2SO_4), and concentrated. Purification of the residue by column chromatography (SiO_2, hexanes–ethyl acetate = 60:40) gave product **ISP360** (0.049 g, 66%) as a light yellow viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15 and 6.58 (AA′BB′, J_{AB} = 7.8 Hz, 4H. ArH), 6.30 (s, 1H, PhO$\underline{H}$), 4.07–3.96 (m, 1H), 3.90–3.84 (m, 1H), 2.25–1.60 (m, 11H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.3, 142.4, 125.7, 115.0, 76.6, 69.9, 42.9, 33.8, 32.5, 30.3, 22.5, 21.4 ppm. HRMS (FAB): $M_2 + Na^+$, found 457.2346. $(C_{14}H_{18}O_2)_2Na$ requires 457.2349.

4.2. Determining ISP163 extinction coefficient and isomer stock concentrations

Stocks of compounds dissolved in DMSO were stored at −20 °C as aliquots to reduce the number of freeze-thaw cycles. Stocks were diluted in 20 mM potassium phosphate buffer, pH 7.5 and DMSO was kept at 0.4%. The absorbance of dilutions was scanned from 200 to 400 nm in a GENESY™ 10S UV-Vis spectrophotometer (Thermo Fisher Scientific) set at medium speed with a 1 nm interval. A buffer blank with 0.4% DMSO was also read. The absorbance peak at 276 nm was plotted against concentration and a linear regression with the line forced through 0,0 was fit to the data. The average of 3 replicates was calculated to be the extinction coefficient

of **ISP163**. Absorbance scans of dilutions of **ISP163** isomers stocks in the same buffer with 0.4% DMSO were scanned. The extinction coefficient of 1892 $M^{-1}cm^{-1}$ was used to calculate the concentration of **ISP163** isomers stock solutions.

4.3. Biological evaluation

4.3.1. TR-FRET ligand binding displacement assay

To determine the ability of compounds to bind to ERβ or ERα, the LanthaScreen® TR-FRET Competitive Binding assay kit (Thermo Fisher Scientific) was used. In this assay, the ERβ or ERα ligand-binding domain (LBD) was tagged with GST, and anti-GST antibody was tagged with terbium, and estrogen was tagged with fluorescein. The assay was set up in a 384-well low volume white plate (Corning® 4512), and incubated for 1 h at room temperature. After incubation, the plate was spun at 1000 rpm for 1 min in a centrifuge with swing-out rotor (Eppendorf 5810, rotor A-4-64). Then the plate was read according to Thermo Fisher Scientific settings (excitation at 332 nm, emission wavelengths of 518 nm and 488 nm with 420 nm cutoff, 50 µs integration delay, 400 µs integration time, and 100 flashes per read) in a SpectraMax M5 plate reader (Molecular Devices). The TR-FRET ratio of fluorescein (518 nm) over terbium (488 nm) emission was calculated by SoftMax Pro 6.2.2 (Molecular Devices). Data were analyzed using Prism 6 (GraphPad). Data were normalized to the TR-FRET ratio of 1% DMSO (negative) and 1 µM E2 (positive) controls and EC_{50} values were calculated by doing a nonlinear square fit of the data with the high concentrations of competing ligand constrained to zero. Standard deviations for the nonlinear least squares fit are reported. For replicate assays, the EC_{50} for curves that gave the median value are reported in Table 1.

4.3.2. TR-FRET Coactivator Binding Assay

To determine the ability of **ISP163p4** to direct the correct conformational change to recruit coactivator proteins, the LanthaScreen® TR-FRET Coactivator Assay (Thermo Fisher Scientific) was used. The assay was conducted using the Thermo Fisher Scientific's SelectScreen™ services. Similar to the binding assay, a GST-tagged ERβ or ERα –LBD and Terbium-labeled anti-GST antibody were used. In this assay, once an agonist compound binds the ER-LBD, the LBD undergoes a conformational change and a fluorescein-tagged peptide derived from PPARλ is recruited. The TR-FRET ratio of the emission of fluorescein over Terbium was calculated and data were normalized to 1% DMSO and E2 controls (E2 EC_{50} was 2.58 nM for ERα and 3.43 nM for ERβ in this assay). EC_{50} values were calculated by doing nonlinear squares fits of the data using Prism 6 (GraphPad). Reported standard deviations are for the fit.

4.3.3. Cellular FRET-based GeneBLAzer™ assays

Selectivity measurements for **ISP163p4** were performed using the SelectScreen™ cell-based nuclear receptor profiling service from ThermoFisher. Nuclear receptors (NR) to be screened in the specificity assay were selected based on two main criteria: (a) sequence and structural similarity to estrogen receptor and (b) availability of the assay. When choosing between possible isoforms, we chose those that were more likely to be involved in CNS function. The 9 NHRs tested were Androgen Receptor (AR), Glucocorticoid Receptor (GR), Mineralocorticoid Receptor (MR), Peroxisome Proliferator-Activated Receptor (PPARδ), Progesterone Receptor (PR), Thyroid Hormone Receptor (TRβ), and Vitamin D Receptor (VDR), ERβ, and ERα. This is a FRET-based assay that uses GeneBLAzer™ technology. It detects ligand binding to and activation of nuclear hormone receptor of interest (ligand binding domain; LBD) that is fused to the DNA-binding Domain (DBD) of GAL4. Upon appropriate ligand binding, the GAL4 DBD binds the upstream activator sequence and transcription of the beta-lactamase cDNA results. Cells are loaded with a beta-lactamase substrate containing fluorescein and coumarin such that cells fluoresced green when beta-lactamase was absent. When beta-lactamase was present, the substrate was cleaved and the cells fluoresced blue. The ratio of coumarin to fluorescein emission was calculated then normalized to negative and positive controls (Table 3). Compound stocks were in DMSO and

diluted for assay concentrations of 25, 2.5, and 0.25 µM. Assays for ERα and ERβ were repeated in a 10-point dose response curve and data was normalized as stated previously. The E2 positive control EC_{50} values were 0.151 nM and 0.568 nM for ERα and ERβ, respectively.

Table 3. Nuclear hormone receptor specificity assay positive controls and IC_{50} values.

Control Compound	IC_{50} (nM)	Nuclear Hormone Receptor
R1881	0.302	Androgen Receptor (AR)
17β-estradiol (E2)	0.107, 0.579	Estrogen Receptor (ERα, ERβ)
Dexamethasone	2.35	Glucocorticoid Receptor (GR)
Aldosterone	0.305	Mineralocorticoid Receptor (MR)
L-165041	12.6	Peroxisome Proliferator-Activated Receptor (PPARδ)
R5020	0.236	Progesterone Receptor (PR)
T3 Free Acid	0.103	Thyroid Hormone Receptor (TRβ)
Calcitriol	0.0953	Vitamin D Receptor (VDR)

4.3.4. Cell-based agonist and antagonist assays

Kits from INDIGO Biosciences were used to examine the impact of compounds on agonist and antagonist activity for full-length, native ERβ and ERα. In this assay, a luciferase reporter gene was downstream from an ER-responsive promoter activated by an agonist. Antagonist assays tested if compound could block activation by E2 while agonist assays tested if compound could activate transcription. Chemiluminescence resulting from ER-induced luciferase expression was measured in a SpectraMax M5(Molecular Devices). Stock solutions in DMSO were diluted to final concentrations (low µM to nM) in compound screening media such that DMSO was kept below 0.4%. Vehicle and E2 controls were included in each assay. E2 had agonist activity IC_{50} values of 0.31 ± 0.03 nM and 0.022 ± 0.005 nM for ERα and ERβ, respectively. Kit instructions were followed. Briefly, cells were taken directly from the freezer and cell recovery media was added. Cells were incubated at 37 °C for 5 min. Half the cells were plated for agonist assays, while the other half had E2 added and were then plated for antagonist assays. Compound dilutions were then added to plated cells and the plate was incubated at 37 °C with 5% CO_2 for 22–24 h. Media was removed and detection reagent was added before luminescence was read. Data were normalized to controls and EC_{50} values were calculated by doing a nonlinear squares fit using Prism 6 (GraphPad). Standard deviations are for the nonlinear fit.

4.3.5. Psychoactive Drug Screening Program (PDSP)

A solid sample of **ISP163** was sent to Brian Roth at the University of North Carolina at Chapel Hill for screening by the NIMH PDSP using published methods [24].

4.3.6. ERβ docking studies

All ligands were prepared in three dimensional (3D) conformations with proper stereochemistry. Ligand files were prepared for docking using AutoDock Tools (ADT) [22], version 1.5.6, and Gasteiger charges were assigned. The ERβ receptor in the agonist conformation (pdb code 2jj3) [19] was also prepared for docking calculations, using ADT to add partial charges and hydrogens. The grid box was centered on the co-crystallized ligand, including ERβ active site residues Arg346, Glu305, and His475. Docking was performed using AutoDock Vina [21] with default parameters, except an exhaustiveness of 8 and energy range of 4 was used.

4.3.7. Cytochrome P450 assays

Metabolism of compounds by cytochrome P450 enzymes was assessed using Promega P450-Glo™ Screening Systems (Madison, WI) for CYP2D6, 3A4, 1A2, and 2C9. Compound stocks were maintained in DMSO and diluted into the assay such that DMSO was kept at 0.25%. In this assay, the relevant CYP450 enzyme metabolizes a pro-luciferin substrate. A secondary luciferase reaction produces light proportional to the amount

of luciferin product generated. Compounds that inhibit CYP450 enzyme action show reduced light production. Assays were run according to kit instructions. Briefly, compound dilutions and a mix of the relevant CYP540 enzyme with pro-luciferin substrate was added to a white 96-well plate (Corning® 3912) and incubated at 37 °C for 10 min. The CYP450 enzyme reaction was initiated by the addition of a NADPH regeneration system and the reaction was incubated at 37 °C for 10–30 min. The CYP450 enzyme reaction was stopped by the addition of the luciferin detection reagent, which initiated the luciferase reaction. Chemiluninescence was read on a SpectraMax M5 (Molecular Devices) after incubating the plate for 20 min at room temperature. Data were normalized to vehicle controls and nonlinear square fits of the data were conducted using Prism 6 (GraphPad). Positive controls (quinidine for CYP2D6, ketoconazole for CYP3A4, α-naphthoflavone for CYP1A2, and sulfaphenazole for CYP2C9) were also included on each assay plate.

4.3.8. Nephelometry

Nephelometry measures the relative light scattering of molecular aggregates and was used to measure the likelihood of compound solubility in assays. Molecular aggregates in solutions cause artificial assay results, thus it is important to assess compound aggregation in solution. Compounds were tested for aggregation in clear 96-well plates (Greiner Bio-One). Compounds were dissolved in 0.45 µm-filtered buffer containing 20 mM potassium phosphate pH 7.5 and 1% DMSO. Dilutions of progesterone were included as positive controls in each assay. Buffer-only controls were used to blank the NEPHELOstar (BMG LABTECH), which was equipped with a 635 nm laser. The gain was set to 90. Compound was considered soluble if it had a nephelometry inflection point greater than 50 µM [23].

4.3.9. Breast cancer proliferation assays

The impact of **ISP163** on the growth of human breast cancer cells was tested in MTT assays. MCF-7 cells were kindly provided by Dr. Manish Patankar (University of Wisconsin-Madison) and cultured in Eagle's Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum and 0.01 mg/mL human recombinant insulin in an incubator maintained at 37 °C and 5% CO_2. Cells were seeded into a 96-well plate at 7000 cells per well and incubated for 24 h after which media was aspirated and treatment dissolved in EMEM was applied. All wells contained 0.1% DMSO, except for dead (negative) controls which contained 100% DMSO. Positive controls contained 0.01 µM E2. **ISP163** was tested at 10-fold dilutions ranging from 10 to 0.001 µM. After a second 24 h incubation, the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed by adding 20% MTT in EMEM to each well and incubating an additional 4 h. Formazan crystal metabolites were dissolved with 100% DMSO and absorbance was read at 570 nm and 650 nm using a Vmax plate reader (Molecular Devices) running SoftmaxPro v 6.1. A standard growth curve was used to convert absorbance units to cell number. Two-sample equal variance t-tests were conducted in Microsoft Excel to determine if cell proliferation was significantly different from untreated controls or wells treated with E2.

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Appendix A. Supplementary data

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Research data for this article

Cambridge Crystallographic Data Center

Crystallographic data

Data associated with the article:

CCDC 1846634: Experimental Crystal Structure Determination

CCDC 1846643: Experimental Crystal Structure Determination

CCDC 1846646: Experimental Crystal Structure Determination

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