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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.

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 altertions 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.
Due to the low yield of the BBr₃ 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 H₂O₂/3 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.
While the mixture of cis- and trans-isomers 4-(4-hydroxyphenyl)cycloheptanemethanol (ISP163) proved inseparable in our hands by SiO₂ 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).
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 1S, 4R and 1R,4S-(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 $\lambda_{\text{max}}$ 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 EC₅₀ values are summarized in Table 1. The most potent compounds were ISP58, ISP163, and ISP248 with EC₅₀ < 75 nM. Secondary assays in a cell-based transcription assay with full-length ERβ and ERα revealed ISP163 to be the most potent (ERβ EC₅₀ 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 EC₅₀ values). (b) ISP163 binding to the LBDs of ERβ (EC₅₀ = 53 ± 15 nM) and ERα (EC₅₀ = 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 (EC₅₀ values in Table 1.).
Table 1. Biological evaluation of compounds in TR-FRET Binding and Cell-based Transcription Assays. EC<sub>50</sub> values are in nM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TR-FRET ligand displacements</th>
<th>Cell-based transcription assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TR-FRET T&lt;sub&gt;ligand&lt;/sub&gt; displacement</td>
<td>Cell-based transcription assay</td>
</tr>
<tr>
<td></td>
<td>ERβ EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>ERα EC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>E2</td>
<td>0.25 ± 0.06</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>ISP163</td>
<td>53 ± 15</td>
<td>99 ± 24</td>
</tr>
<tr>
<td>ISP163p1</td>
<td>18 ± 6</td>
<td>96 ± 18</td>
</tr>
<tr>
<td>ISP163p2</td>
<td>28 ± 19</td>
<td>61 ± 13</td>
</tr>
<tr>
<td>ISP163p3</td>
<td>99 ± 56</td>
<td>221 ± 34</td>
</tr>
<tr>
<td>ISP163p4</td>
<td>66 ± 20</td>
<td>199 ± 40</td>
</tr>
<tr>
<td>ISP248</td>
<td>75 ± 19</td>
<td>ND</td>
</tr>
<tr>
<td>ISP58</td>
<td>42 ± 18</td>
<td>ND</td>
</tr>
<tr>
<td>ISP166</td>
<td>600 ± 99</td>
<td>ND</td>
</tr>
<tr>
<td>ISP360</td>
<td>509 ± 163</td>
<td>ND</td>
</tr>
<tr>
<td>ISP242</td>
<td>435 ± 118</td>
<td>ND</td>
</tr>
</tbody>
</table>

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<sub>50</sub> = 51 ± 8 nM, ERα EC<sub>50</sub> > 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 EC50 = 735 ± 94 nM, ERα EC50 = 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 (EC50 = 566 ± 57 nM) over ERα (EC50 = 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 EC50 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) (4S,1S) stereoisomer, (c) (4R, 1S) stereoisomer ISP163p3, and (d)
(4S,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>
<thead>
<tr>
<th>Receptor</th>
<th>% Inhibition at 10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin</td>
<td></td>
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<tr>
<td>5-HT₁A</td>
<td>14.6</td>
</tr>
<tr>
<td>5-HT₁D</td>
<td>25.3</td>
</tr>
<tr>
<td>5-HT₁B</td>
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<tr>
<td>5-HT₁C</td>
<td>19.0</td>
</tr>
<tr>
<td>5-HT₁I</td>
<td>−8.5</td>
</tr>
<tr>
<td>Adrenergic</td>
<td></td>
</tr>
<tr>
<td>α₁A</td>
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<tr>
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<td>Histamine</td>
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</tr>
<tr>
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<tr>
<td>Other</td>
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</tr>
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<td>σ₁</td>
<td>17.1</td>
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<tr>
<td>σ₂</td>
<td>31.9</td>
</tr>
</tbody>
</table>

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 (EC₅₀ = 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 (IC₅₀ = 2.7 ± 0.3 μM), moderate
inhibition of CYP3A4 was observed (IC₅₀ = 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 ≤ 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 ≤ 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. CDCl₃, CD₃OD and DMSO-d₆ 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$_2$OD. $^{13}$C NMR spectra were calibrated from the central peak at 77.23 ppm for CDCl$_3$ or 49.15 ppm for CD$_2$OD. 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$_4$Cl (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%).

$^1$H 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, CH CH$_2$), 4.98–4.88 (m, 4H, CH CH$_2$), 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 + Na$, found 515.3130. (C$_{16}$H$_{22}$O$_2$)$_2$Na requires 515.3132.

4.1.3. 5-(4-(tert-Butyldimethylsilyloxyphenyl)nona-1,8-dien-5-ol 4b
The reaction of methyl 4-(tert-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%).

$^1$H 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, CH CH$_2$), 5.01–4.86 (m, 4H, CH CH$_2$), 2.13–1.80 (m, 9H), 1.01 (s, 9H, t-Bu), 0.22 (s, 6H, SiMe$_2$); $^{13}$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$_2$Cl$_2$ (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$_2$Cl$_2$. 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.

$^1$H 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}$O$_2$Na 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$_2$Cl$_2$ (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. $^1$H 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$, found 325.1959. C$_{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 CH₂Cl₂ (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 (SiO₂, hexanes–ethyl acetate = 50:50) to give 6a as a colorless oil (1.433 g, 90%). ¹H NMR (400 MHz, CDCl₃) δ 7.11 and 6.84 (AA'BB', J₉₈ = 8.6 Hz, 4H, ArH), 5.91–5.87 (m, 2H, CH₂–CH), 3.79 (s, 3H, OMe), 2.69 (tt, J = 11.3, 3.2 Hz, 1H, H₄), 2.35–2.25 (m, 2H), 2.23–2.13 (m, 2H), 1.91–1.83 (m, 2H), 1.54–1.43 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 157.8, 141.7, 132.7, 127.6, 119.9, 73.0, 71.9, 46.1, 46.0, 38.3, 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. C₁₄H₂₀O₂Na requires 243.1356.

4.1.7. 5-(4-Methoxyphenyl)cycloheptene 6b
The ionic reduction of 5b (1.601 g, 5.026 mmol) in anhydrous CH₂Cl₂ (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 (SiO₂, hexanes–ethyl acetate = 60:40) gave 6b (0.906 g, 60%) as a light yellow oil.

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 N₂, was added dropwise a solution of BH₃–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% H₂O₂ (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 (SiO₂, 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. ¹H NMR (400 MHz, CDCl₃) 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, H₄), 2.15–2.05 (m, 1H), 2.02–1.50 (m, 11H); ¹³C NMR (100 MHz, CDCl₃) δ 153.7, 142.2, 132.7, 127.6, 120.0, 49.7, 35.1, 28.1, 25.9, 18.4, −4.2 ppm.

4.1.9. 4-(4-t-Butyldimethylsilyloxyphenyl)cycloheptanol 7b
The hydroboration/oxidation of 6b (0.906 g, 2.99 mmol) with BH₃–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 (SiO₂, 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. ¹H NMR (400 MHz, CDCl₃) 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, H₄), 2.14–1.49 (m, 11H), 0.97 (s, 9H, t-Bu), 0.18 (s, 6H, SiMe₂); ¹³C NMR (100 MHz, CDCl₃) 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, 35.8, 31.9, 29.7, 23.4, 21.5 ppm. HRMS (FAB): M + Na⁺, found 243.1358. C₁₉H₂₂O₂Na requires 243.1356.

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, 0.18 (s, 6H, SiMe₂); ¹³C NMR (100 MHz, CDCl₃) 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. C₁₉H₂₀O₂ requires 343.2064.

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 (SiO2, hexanes–ethyl acetate = 80:20) gave 8b (0.036 g, 72%) as a colorless oil. 1H NMR (400 MHz, CDCl3) δ 7.01 and 6.75 (AA'BB', JAB = 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, t-Bu), 0.18 (s, SiMe2); 13C NMR (100 MHz, CDCl3) 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. C19H30O2SiNa 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 N2 was added an aliquot of BF3-Et2O (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 NaHCO3. The mixture was extracted several times with CHCl3, and the combined organic extracts were washed with brine, dried (Na2SO4) and concentrated. The resultant dark yellow oil was purified by column chromatography (SiO2, 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. 1H NMR (400 MHz, CDCl3) 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, OCH2CH3), 3.64 (t, J = 4.8 Hz) and 3.60 (dd, J = 12.0, 4.0 Hz, 0.3H total, OCOCH2Et), 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, OCH2CH3), 0.97 (s, 9H, t-Bu), 0.18 (s, H, SiMe3); 13C NMR (100 MHz, CDCl3) 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 (Na2SO4) and concentrated. 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 N2, was added dropwise a solution of n-butyllithium (1.6 M 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 extracts were washed with brine, dried (MgSO4) and concentrated. The residue was purified by column chromatography (SiO2, hexanes–ethyl acetate = 80:20) to give 9a (0.296 g, 78%) as a light yellow oil. 1H NMR (400 MHz, CDCl3) δ 7.10 and 6.83 (AA'BB', JAB = 8.4 Hz, 4H, ArH), 4.77 (s, 2H, C—CH2), 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$_3$) $\delta$ 157.7, 151.9, 141.7, 127.7, 113.8, 55.4, 47.5, 38.0, 37.3, 36.2, 35.4, 27.5 ppm.

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

The Wittig olefination of 9b (0.212 g, 0.666 mmol) with the vlyde generated from CH$_3$PPh$_3$+ 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 = 50:50) gave 9b (0.120 g, 57%) as a light yellow oil. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.03 and 6.75 (AA'BB', $J_{AB}$ = 6.7 Hz, 4H, ArH), 4.76 (s, 2H, C–CH$_2$), 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-C$_7$H$_{15}$); $^13$C NMR (100 MHz, CDCl$_3$) $\delta$ 153.5, 142.3, 127.5, 119.9, 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-But-1-enylphenoxy)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$_2$O$_2$ (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 = 90:10) 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$_3$) $\delta$ 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, CH$_2$OH), 2.69–2.55 (m, 1H), 2.00–1.72 (m, 8H), 1.68–1.39 (m, 4H), 1.00 (s, 9H, t-Bu); $^13$C NMR (100 MHz, CDCl$_3$) $\delta$ 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 357.2155. C$_{20}$H$_{34}$O$_2$SiNa requires 357.2218.

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 3 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$_2$SO$_4$), 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$_3$) $\delta$ 7.03 and 6.81 (AA'BB', $J_{AB}$ = 8.3 Hz, 4H, ArH), 3.45 (d, $J = 6.5$ Hz, 2H, CH$_2$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, SiMe$_2$); $^13$C NMR (100 MHz, CDCl$_3$) $\delta$ 153.5, 142.6, 127.6, 127.5, 119.9, 68.7, 68.5, 47.3, 46.1, 43.6, 38.8, 37.7, 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 375.2218. C$_{20}$H$_{34}$O$_2$SiNa requires 375.2220.

4.1.18. 4-(4-Hydroxyphenyl)cycloheptanemethanol ISP163

Method A: To a solution of 10a (0.180 g, 0.769 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL) at −78°C, was added dropwise a solution of boron tribromide (1 M in CH$_2$Cl$_2$, 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$_2$Cl$_2$, 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 (0.048 g, 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$_3$) $\delta$ 7.03 and 6.74 (AA'BB', $J_{AB}$ = 8.5 Hz, 4H, ArH), 3.48 (d, $J = 6.6$ Hz, 2H, CH$_2$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$_2$SO$_4$) 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, CH$_2$OH), 2.63–2.54 (m, 1H), 1.97–1.32 (m, 12H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 153.6, 142.1, 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, CH$_2$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 partitioned between ethyl acetate and water. The organic layer was washed with saturated aqueous NaHCO$_3$, followed by brine, dried (Na$_2$SO$_4$) 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$_3$OD) δ 6.98 and 6.70 (AA'BB′, $J_{AB} = 8.5$ Hz, 4H, ArH), 4.98 (s, 1H, PhO H), 2.77–2.39 (m, 4H), 2.02–1.47 (m, 7H); $^{13}$C NMR (100 MHz, CD$_3$OD) 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$_3$OD) 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$_3$OD) 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 + 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 ISP248 (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$_3$OD) 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$_3$OD) 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.
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 (MgSO4), 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 H2 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 CH2Cl2 (10 mL), cooled to −78 °C, and a solution of boron tribromide (1 M in CH2Cl2, 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 CH2Cl2. The combined organic extracts were washed with brine, dried (MgSO4) and concentrated. The residue was purified by column chromatography (SiO2, 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. 1H NMR (400 MHz, CDCl3) δ 7.04 and 6.74 (AA'BB', JAB = 8.7 Hz, 4H, ArH), 3.71 (td, J = 6.9, 1.4 Hz, 2H, CCH2OH), 2.66–2.48 (m, 1H), 1.96–1.13 (m, 13H); 13C NMR (100 MHz, CDCl3) 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 CH2Cl2 (30 mL) at −78 °C, was added dropwise a solution of boron tribromide (1 M in CH2Cl2, 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 CH2Cl2, the combined organic extracts were washed with brine, dried (MgSO4) and concentrated. This product was determined to be a mixture of cis- and trans-stereoisomers on the basis of NMR spectroscopy. 1H NMR (400 MHz, CDCl3) 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'CCHOH), 2.78–2.59 (m, 1H), 2.53–1.38 (m, 13H); 13C NMR (100 MHz, CDCl3) 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 CH2Cl2 (20 mL) at −10 °C, was slowly added a suspension of DDQ (77 mg, 0.34 mmol) in CH2Cl2 (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 CH2Cl2. The combined organic extracts were washed with brine, dried (Na2SO4), and concentrated. Purification of the residue by column chromatography (SiO2, hexanes–ethyl acetate = 60:40) gave product ISP360 (0.049 g, 66%) as a light yellow viscous oil. 1H NMR (400 MHz, CDCl3) δ 7.15 and 6.58 (AA'BB', JAB = 7.8 Hz, 4H, ArH), 6.30 (s, 1H, PhO), 4.07–3.96 (m, 1H), 3.90–3.84 (m, 1H), 2.25–1.60 (m, 11H); 13C NMR (100 MHz, CDCl3) 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): M2 + Na+, found 457.2346. (C14H18O2)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 square 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 EC50 values were 0.151 nM and 0.568 nM for ERα and ERβ, respectively.

Table 3. Nuclear hormone receptor specificity assay positive controls and IC50 values.

<table>
<thead>
<tr>
<th>Control Compound</th>
<th>IC50 (nM)</th>
<th>Nuclear Hormone Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1881</td>
<td>0.302</td>
<td>Androgen Receptor (AR)</td>
</tr>
<tr>
<td>17β-estradiol (E2)</td>
<td>0.107, 0.579</td>
<td>Estrogen Receptor (ERα, ERβ)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>2.35</td>
<td>Glucocorticoid Receptor (GR)</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.305</td>
<td>Mineralocorticoid Receptor (MR)</td>
</tr>
<tr>
<td>L-165041</td>
<td>12.6</td>
<td>Peroxisome Proliferator-Activated Receptor (PPARδ)</td>
</tr>
<tr>
<td>R5020</td>
<td>0.236</td>
<td>Progesterone Receptor (PR)</td>
</tr>
<tr>
<td>T3 Free Acid</td>
<td>0.103</td>
<td>Thyroid Hormone Receptor (TRβ)</td>
</tr>
<tr>
<td>Calcitriol</td>
<td>0.0953</td>
<td>Vitamin D Receptor (VDR)</td>
</tr>
</tbody>
</table>

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 IC50 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% CO2 for 22–24 h. Media was removed and detection reagent was added before luminescence was read. Data were normalized to controls and EC50 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. Chemiluminescence 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% CO₂. 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.

Acknowledgments

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Appendix A. Supplementary data

Download: Download Acrobat PDF file (1MB)
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

References


[17] CCDC 1846634 (ISP163p2), 1846646 (ISP163p3), 1846643 (ISP163p4) contain the supplementary crystallographic data for this paper. These data are provided free of charge by The Cambridge Crystallographic Data Centre.
We Thank One of the Reviewers for Suggesting This Possibility.


