Journal of Medicinal Chemistry

ARTICLE

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Design and Evaluation of Fragment-Like Estrogen Receptor Tetrahydroisoquinoline Ligands from a Scaffold-Detection Approach†

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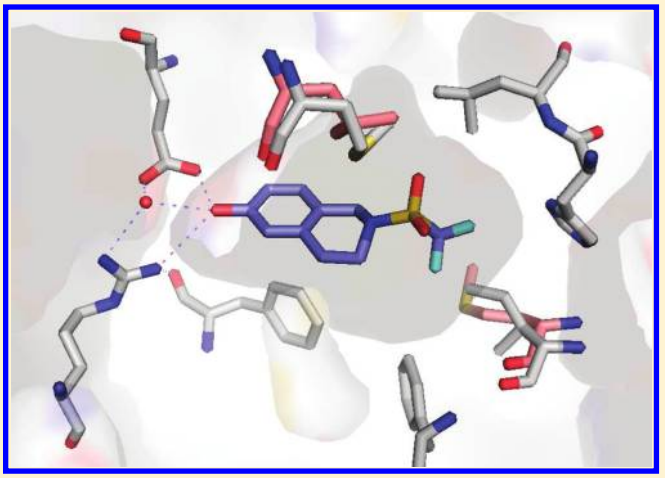
Supporting Information

ABSTRACT: A library of small tetrahydroisoquinoline ligands, previously identified via structure- and chemistry-based hierarchical organization of library scaffolds in tree-like arrangements, has been generated as novel estrogen receptor agonistic fragments via traditional medicinal chemistry exploration. The approach described has allowed for the rapid evaluation of a structure−activity relationship of the ligands concerning estrogen receptor affinity and estrogen receptor β subtype selectivity. The structural biological insights obtained from the fragments aid the understanding of larger analogues and constitute attractive starting points for further optimization.



INTRODUCTION

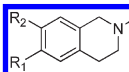
Drug discovery ideally starts from bioactive molecules with scaffold structures that allow easy, and structure-based molecular optimization, thus providing rapid access to a clear structure−activity relationship (SAR). Fragment-based screening and design approaches are highly attractive in this respect.[1] Small molecules with moderate affinity allow rapid SAR determination, entries into molecular diversity and complexity, and frequently, easy access to structural information. Structure- and chemistry-based hierarchical organization of library scaffolds in tree-like arrangements provides a novel and accelerated entry into the identification of such novel small ligands or fragments of biologically prevalidated relevance.[2−5] Brachiation of scaffold trees from complex to simpler, yet still similar, structures can also be used to generate new and simpler analogues of known active molecules as new starting points for molecular diversity and to elucidate the binding mechanism of more complex analogues. We have shown that this approach allows for the identification of a new active fragment-like ligand previously not annotated with estrogen receptor α (ERα) activity but based on larger more complex scaffolds.[4] Here we now show that a small focused library based on the identified fragment allows for rapid generation of a clear SAR and for optimization of the binding affinity. With the aid of structural biology, the modest preference for estrogen receptor β (ERβ) subtype binding by this class of scaffolds is identified, thus providing clear starting points for optimization programs for subtype selective ligand optimization.

The estrogen receptor (ER) belongs to the superfamily of nuclear receptors.[6,7] The primary endogenous ligand for ER is 17β-estradiol (E_2).[7] Two subtypes of ER are known (ERα[8] and ERβ[9]), each with their own unique tissue distribution patterns and transcriptional properties. While ERα is mainly involved in reproduction events in the uterus and mammary glands,[10] ERβ is more generally expressed and is not the dominant receptor in uterus and breast tissues. The challenge of identifying selective ERβ modulators provides an opportunity to elucidate the exact physiological function of ERβ and to develop novel, tissue- and cell-selective drug candidates such as those related to inflammatory diseases.[11,12] Fragment-based drug discovery promises to be a very valuable tool to generate both such compounds for nuclear receptors[13] and help in the elucidation of the molecular pharmacological mechanism of larger, more potent compounds.

Using the tree-like arrangement of library scaffolds, we previously identified the tetrahydroisoquinoline (THIQ) scaffold as a

Received: July 6, 2010
Published: March 07, 2011

Table 1. ERα and ERβ Activities (EC_{50}) of THIQ Fragments in a Fluorescence Polarization Cofactor Recruitment Assay



1	R_1	R_2	R_3	EC_{50} (μM)[a] ERα	EC_{50} (μM)[a] ERβ	EC_{50} ERα / ERβ
2(·HBr)	HO	H	H	n.a.	n.a.	-
3(·HBr)	H	HO	H	n.a.	n.a.	-
4(·HBr)	HO	HO	H	n.a.	n.a.	-
5	H	H	$COCH_3$	n.a.	n.a.	-
6	HO	H	$COCH_3$	n.a.	n.a.	-
7	H	H	$COCF_3$	n.a.	n.a.	-
8	HO	H	$COCF_3$	5.2 ± 0.5	0.9 ± 0.2	5.8
9	MeO	H	$COCF_3$	340 ± 29	227 ± 30	1.5
10	H	HO	$COCF_3$	20 ± 5	3.8 ± 0.7	5.3
11	HO	H	$COCClF_2$	2.3 ± 0.3	1.6 ± 0.4	1.4
12	HO	H	$COCCl_3$	6.2 ± 0.3	3.2 ± 0.6	1.9
13	H	H	SO_2Me	n.a	n.a.	-
14	HO	H	SO_2Me	170 ± 30	20 ± 7	8.5
15	HO	H	SO_2-1-Naphthyl	700 ± 85	60 ± 10	11.7
16	H	HO	SO_2-1-Naphthyl	600 ± 173	30 ± 17	20
17	HO	H	SO_2-Phenyl	n.t.	1.0 ± 0.6	-
18	HO	H	CO_2tBu	n.t.	1.1 ± 0.3	-
19	HO	H	SO_2CF_3	3.0 ± 0.3	0.6 ± 0.3	5.0
E_2				0.13 ± 0.04	0.28 ± 0.02	0.5

[a] values are means of a least three experiments. n.a.: no activity. n.t.: not tested.

simple two-ring core chemical fragment for which little ER activity was known.[4] More highly decorated THIQ derivatives, with three[14] or more[15,16] ring scaffolds, have been previously described as (antagonistic) ER modulators with selectivity for ERα. In addition, a small number of THIQ analogues, typically with smaller side chains, have also been reported to be agonistic and selective for ERβ.[14,15] Here, we describe the development and structural support of a focused library of even smaller two-ring THIQ analogues with ERβ selectivity. Using these fragments, molecular insights into the action and receptor selectivity of the THIQ ligands could be obtained, offering new starting points for the understanding and generation of selective ER modulators.

DESIGN AND SYNTHESIS

On the basis of the simple THIQ scaffold (**1**) (Table 1), a small focused library of 18 THIQ fragments was designed and investigated for their affinities toward both ER receptors. Comparison of the structure of THIQ, and its published derivatives,[14−16] with the structure of E_2 indicates that the bicyclic ring system typically mimics the A- and B-rings of E_2. To mimic the phenolic group on the E_2 A-ring, known to be important for high ER affinity, a hydroxyl group was introduced at different positions on the aromatic ring of the designed THIQ fragments. Small side chain groups were then added via *N*-substitution of the THIQ scaffold to partially fill the space normally occupied by the C- and D-rings of E_2. These side chains were expected to provide the greatest opportunity for enhancing ER affinity and addressing ER selectivity. A variety of functional amide groups were examined for this position, including electron withdrawing, aromatic and polar *N*-substituents. The synthesis of the fragments was based on the in situ protection of phenols **2** and **3** (Table 1), followed by reaction with the appropriate anhydride or sulfonyl chloride. The intermediates thus obtained were subsequently desilylated in the same flask to yield the desired *N*-substituted THIQ derivatives **5**−**19**[17] (Table 1) in varying yields. Finally, 2-(trifluoroacetyl)-tetrahydro-6-isoquinolinol **8** was methylated with methyl iodide in the presence of potassium carbonate to afford the methoxy THIQ derivative **9** in good yield.

BIOCHEMICAL EVALUATION

The THIQ fragments were next evaluated in a biochemical fluorescence polarization peptide recruitment assay (Table 1). In this assay, a fluorescein-labeled peptide probe binds to ER ligand binding domain (LBD), in the ligand-bound state, via an LXXLL recognition motif. Binding of a compound with an agonistic profile results in the formation of a complex between the ER LBD and the fluorescent peptide and a subsequent increase in polarization. By contrast, a ligand with antagonistic properties would repress the intrinsic binding of the peptide to the ER LBD, resulting in a decrease in the observed polarization. The calculated concentrations for half-maximum response (EC_{50}) of the substituted THIQ derivatives are presented in Table 1.

An initial comparison of the biochemical activity data revealed that 11 out of the 18 THIQ fragments showed activity for both ER LBDs and that all 11 induced an increase in LXXLL peptide binding to the ER LBDs. Results for THIQs bearing hydroxyl group, at either the 6- (**2**) or the 7-position (**3**), showed that an hydroxyl group alone is not sufficient for ER binding. Furthermore, the introduction of both OH-groups simultaneously did not facilitate ER recognition (**4**). To mask the basic amino functionality and to address the hydrophobic ligand binding pocket, various *N*-substituents were introduced. Modification to the methyl acetamide (**5**) did not lead to any measurable ER affinity, even in combination with a 6-hydroxyl group, as in compound **6**. However, *N*-substitution with electron withdrawing groups resulted in good affinities for both ER isoforms. Whereas *N*-substitution in the absence of hydroxyl functionality did not facilitate ER affinity, as shown for **7**, the concomitant incorporation of a 6-hydroxyl group with an *N*-trifluoroacetamide group, as in **8**, produced a good affinity for the ER LBDs. The importance of the phenol functionality in terms of hydrogen bonding was elucidated by masking it as a methoxy group to afford **9**, a ligand with a significantly lower activity. In addition, relocation of the 6-hydroxy group to the 7-position (**10**) resulted in a 4-fold loss in ER affinity. For gain in ER affinity and the tendency toward ERβ (E_2 showed a slight preference of ERα in this assay), this limited set of THIQ fragments thus already showed the importance of a hydroxyl group at the 6-position in combination with an appropriate nitrogen substituent.

On the basis of the inactivity of derivatives presenting the methyl acetamide group and the strong activity of the fluoromethyl acetamide analogue, the importance of the amide group's size and electronegativity was evaluated. Replacement of one or all three fluoro groups in **8** by chloro groups was well tolerated (**11** and **12**) with only a small decrease in affinity. An additional set of compounds, bearing a sulfonamide in place of the acetamide group as *N*-substituent, was also investigated. In line

dx.doi.org/10.1021/jm1011116 |*J. Med. Chem.* 2011, 54, 2005–2011

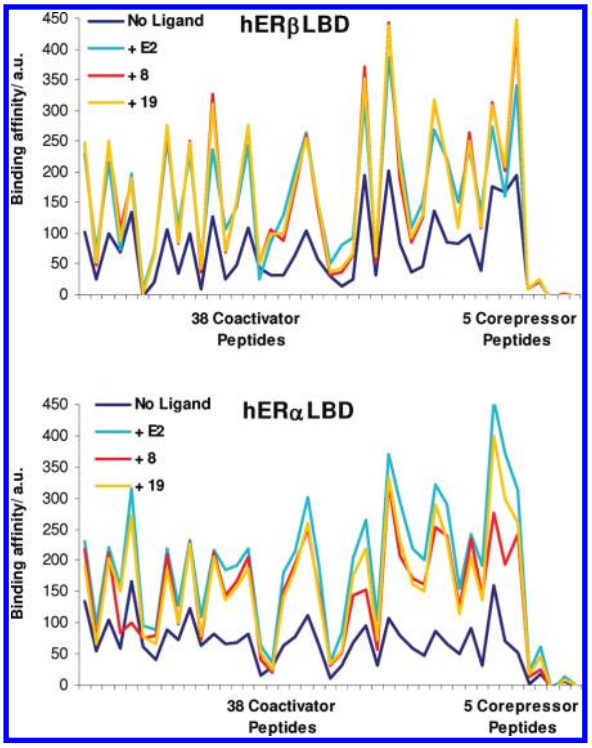


Figure 1. Binding profiles of 43 cofactor peptides immobilized on a membrane, against both ERα and ERβ LBD without ligand and with compounds E_2, **8**, and **19** (a complete list of all cofactor peptides tested and their affinities can be found in the Supporting Information).

with the acetamide derivatives, methyl sulfonamide analogues lacking hydroxyl substituents on the THIQ scaffold were found not to be active (**13**). In contrast to the acetamide series, however (e.g., **6**), the combination of a 6-hydroxyl group with a non-halogenated sulfonamide instead resulted in a compound (**14**) with ER affinity and respectable ERβ selectivity. As well as for the sulfonamide functionality, insertion of three fluorides, as in **19**, significantly enhanced the EC_{50} value in the peptide recruitment assay (around 40-fold to 0.6 μM for ERβ and 3 μM for ERα). Increasing the steric bulk of the sulfonamide side chain through the incorporation of a 1-naphthyl group produced less potent compounds (**15** and **16**). Reducing the steric bulk by replacing the 1-naphthyl group with a phenyl ring (**17**) led to an increase in ER affinity compared with the naphthyl derivatives. Furthermore, the incorporation of a *tert*-butyl ester resulted in a compound (**18**) with an ERβ binding affinity similar to **17** and confirms that halogenated *N*-substituents are not strictly required for obtaining high affinity.

To further evaluate the profile of the fragments in comparison with the natural ligand E_2, chip-based ligand-induced cofactor binding studies were performed on a library of 53 cofactor peptides, each one representing important binding epitopes of both coactivator and corepressor proteins (Figure 1). This study showed that the peptide binding profiles for E_2 and fragments **8** and **19** were very similar. For both ER subtypes E_2, **8**, and **19** all enhance to the same extent the binding affinity to peptides with a coactivator motif (LXXLL). In addition, binding to the tested corepressor motifs was not enhanced by any of the three ligands (Figure 1). On the basis of these findings, it could be concluded that the potent fragments have an agonistic pharmacological profile.

Next, the transcriptional activity of fragment **19** on ERα and ERβ in mammalian cells was profiled using a cellular estrogen response element-luciferase reporter gene assay in U2OS cells. E_2 caused a 33-fold increase in luciferase activity in ERα-transfected cells and a 7-fold increase in ERβ-transfected cells. In agreement with the biochemical studies, **19** showed a full agonistic profile on both receptors, reaching similar levels of luciferase activity as E_2. In ERα-transfected cells, **19** measured an EC_{50} value of 1.87 μM and in ERβ-transfected cells an EC_{50} of 0.31 μM. Even though the EC_{50} of **19** is five orders of magnitude lower than the highly potent E_2, the strong transcriptional activity and full agonistic profile are still remarkable for such a small ligand. In addition, the cellular assay supports the biochemical observation of ERβ selectivity in the case of compound **19** and analogues.

STRUCTURAL EVALUATION

To elucidate the binding affinity and selectivity of the fragments in molecular detail, X-ray crystal structures were obtained on selected fragment−ERβ LBD complexes (Figure 2). Such structural information is expected to provide the molecular basis for future fragment enlargement studies and the design of follow-up libraries. For the X-ray studies, crystal structures of the ERβ LBD bound to the natural agonist E_2[18] and to three relevant THIQ fragments (**8**, **10**, and **19**) were each generated in complex with a coactivator peptide sequence based on the steroid receptor coactivator-1 (SRC-1) box 2 sequence. The general structures of the ERβ LBD−THIQ fragment complexes are similar to previous E_2-bound hERα structures and to reported hERβ structures with other agonists[19−21] (Figure 2). For all three fragments, the helix 12 conformation of the ERβ corresponds to that observed for ERβ bound to E_2 and other agonists. This is in turn consistent with the observed functional agonistic activity of the fragments in the cofactor peptide binding and transcriptional activation studies.

Overlays of the X-ray structures of the ERβ LBD bound fragments **8**, **10**, and **19** with the E_2 structure (Figure 2b−d) show that all three fragments adopt a similar position. The binding mode of the bicyclic ring system thus mimics the A- and B-ring of E_2. The backbones of all three compounds establish a number of van der Waals contacts with the residues forming the binding pocket. Additionally, for compounds **8** and **19**, the 6-hydroxyl functionality perfectly matches the phenolic group of E_2, forming a hydrogen bonding network with Glu_{305}, Arg_{346}, and a water molecule (Figure 2b,d). Fragment **10** instead features a 7-hydroxyl group, which forms only one hydrogen bond with Glu_{305} (Figure 2c). This feature is a likely explanation for the lower ER affinity measured for **10**.

The *N*-substituents of the three crystallized fragments occupy part of the positional space usually adopted by the C- and D-rings of E_2 (Figure 2b−d). Because of the lack of a second hydroxyl group to mimic the 17β-OH in the D-ring of E_2, the fragments do not form a hydrogen bond with the His_{475} side chain in the ERβ cavity. The absence of this interaction most likely explains to a significant extent the lower ER affinity of the fragments and offers opportunities for ligand optimization, along with addressing the hydrophobic space normally occupied by the C and D ring of E_2. Of interest is the observation that the *N*-substituents produce a specific effect on ER affinity and selectivity. In particular, the utilization of electron-withdrawing groups (e.g., **8** and **19**) turned out to be most suitable. The affinity enhancement for both ER

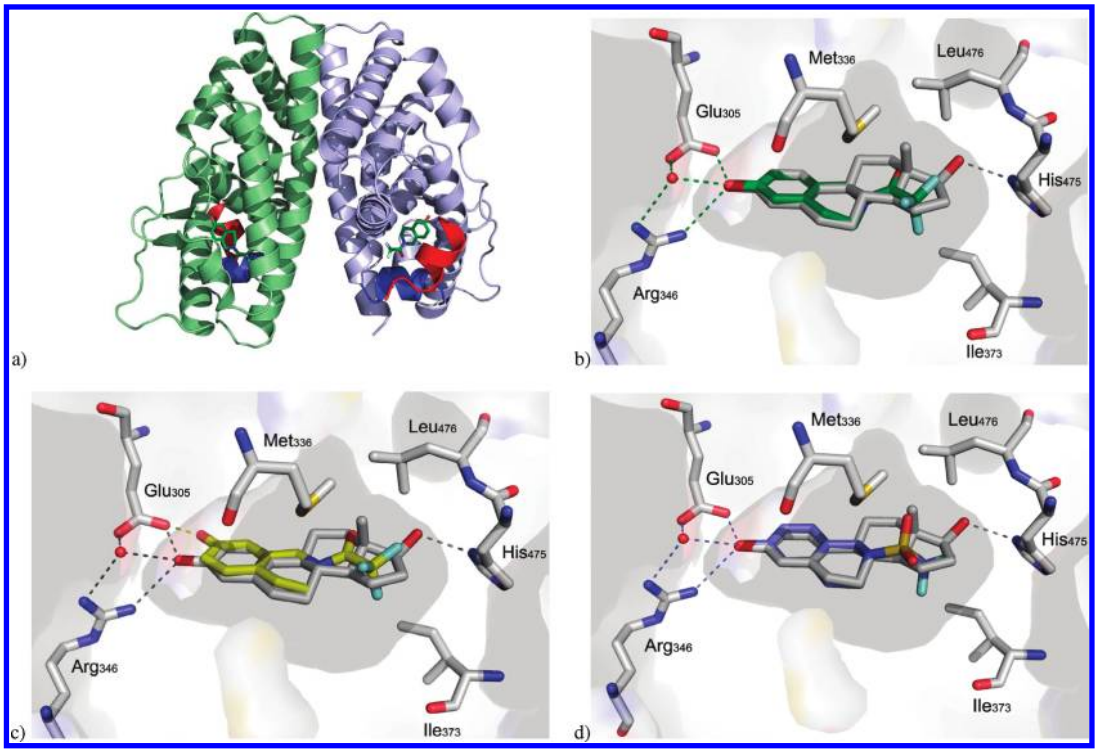


Figure 2. (a) General structural fold of the ERβ LBD bound to fragment **8** in complex with a SRC-1 box 2 peptide (red) and with helix 12 highlighted in blue; overlays of the X-ray structures of the ERβ LBD cocrystallized with E_2 (gray) with that of fragments **8**, **10**, or **19**. (b) ERβ LBD-**8** (green). (c) ERβ LBD-**10** (yellow). (d) ERβ LBD-**19** (blue). Only key residues are shown for simplicity. Hydrogen bonds to key residues are shown as dotted lines.

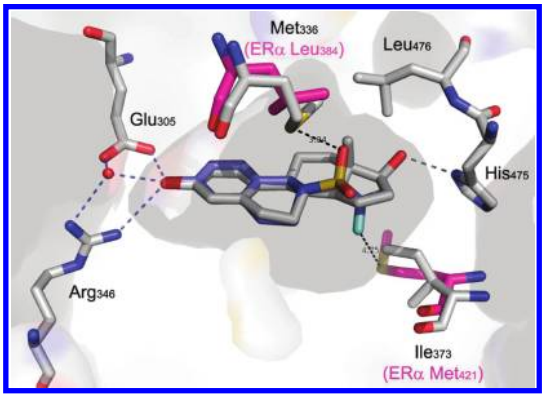


Figure 3. Crystal structure of compound **19** (a) with the ERβ LBD (gray) and overlaid with ERα LBD−E_2 (magenta; PBD 3DT3). Only key residues are shown for simplicity. Distance monitors (black dotted lines) show that the fluoro group is in close proximity to Met421/Ile373.

isoforms after incorporation of halogen groups can be attributed to a favorable net hydrophobic effect due to the bulky size of the substituent, as well as to the reduced polarity of the THIQ core induced by the amide group. Additionally, van der Waals interactions between the halogen atoms and surrounding residues further stabilize the interaction of the ligand within the ligand binding pocket.

Fragments **8** and **19** featured not only the highest ER potency of this series but as well gave the most promising ERβ selectivity. Weaker binding fragments such **15** and **16** also displayed a modest selectivity for ERβ. It has been reported that aromatic moieties are typically capable of making more favorable interactions with amino acids in the cavity of ERβ than with residues in the ERα.[22] Two amino acid residues in close proximity to the ligands are responsible for the differences in the sizes of the ERα and ERβ cavity. The ERβ Leu_{384} is replaced by Met_{336} in ERα, and the ERα Met_{421} is replaced by Ile_{373} in ERβ (Figure 3).[19,21,23] Ligands capable of interacting differently with ERβ Ile_{373}, in comparison to ERα Met_{421}, have been proposed as reason for selectivity toward ERβ.[20,23,24] Specific functional groups could thus be very important for the stereoelectronic differentiation between the two receptor subtypes. As shown in Figure 3, the trifluoromethyl sulfonamide moiety of fragment **19** is held in close proximity to the Met_{421}/Ile_{373} residues. Furthermore, the sulfonamide addresses a pocket in ERβ not explored by E_2. In particular, it appears that the small distance between one of the fluoro atoms and the sulfur of ERα Met_{421} would represent a repulsive interaction, which may limit the conformational space occupied by the methionine side chain, thus resulting in an unfavorable electrostatic interaction.[20] In contrast, the distance between the electron-withdrawing halogen atom and ERβ Ile_{373} is greater, which is expected therefore not to result in an unfavorable interaction (Figure 3). These observations could provide an explanation for why these THIQ ligands show a preference for ERβ over ERα.

CONCLUSIONS

Structure- and chemistry-based hierarchical organization of library scaffolds in tree-like arrangements has enabled the identification of the bicyclic THIQ scaffold as an originator of novel small agonistic ER ligands with ERβ selectivity.[4] The fragment-based character of the ligands greatly facilitated their synthesis. The affinity toward both ERα and ERβ has been

evaluated and a clear SAR discerned. The incorporation of a trifluoromethyl acetamide (as in **8**) or trifluoromethyl sulfonamide group (as in **19**) produced the best ER binders in the series with ca. 5-fold ERβ selectivity. Importantly, the fragment-like character of the ligands enabled the ready generation of X-ray structures and the elucidation of their binding affinity and ERβ selectivity. The amide functionalities appear to address a hydrophobic subpocket in ERβ but clash with the respective methionine at the same position in ERα. The information from the SAR, together with the crystal structures of the fragments, aids in the understanding of the binding profile of more decorated THIQ analogues.[14,15] Additionally, the fragments provide numerous opportunities to develop more highly decorated compounds with enhanced ERβ affinity and selectivity. In particular, the affinity of fragment **19** is notable with respect to the small size of the ligand and the ease of synthesis. Because fragment **19** does not address His_{475} in the ERβ binding pocket, whereas E_2 does, further incorporation of polar functionalities branching out from the amide group could enhance the affinity, for example, through hydrogen bonding. Similarly, addressing the hydrophobic space normally occupied by the C and D ring of E_2 could also lead to more potent ligands.

Overall, the described approach for the generation of novel fragments via scaffold identification in tree-like arrangements provides validated starting points for the development of novel ligands with specific profiles, as demonstrated here for the ERs and ERβ selectivity. In addition, it allows us to generate relatively potent small ligands that can be used to structurally explain the binding of other more complex analogues in a rapid and easy manner, as such significantly enriching the molecular insights in drug discovery.

EXPERIMENTAL SECTION

Analytical Techniques and Used Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 400 (400 MHz, ^{1}H NMR; 100.6 MHz, ^{13}C NMR) at room temperature. NMR spectra were calibrated to the solvent signals of $CDCl_3$ (δ = 7.26 and 77.00 ppm), CD_3OD (δ = 3.31 and 49.05 ppm), or DMSO-d_6 (δ = 2.50 and 39.43 ppm). The chemical shifts are provided in ppm and the coupling constants in Hz. The following abbreviations for multiplicities are used: s, singlet; d, doublet; dd, double doublet; t, triplet; q, quadruplet; m, multiplet; br, broad.

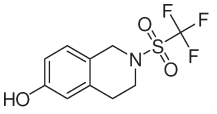
HPLC-ESI-MS analyses were performed on an HPLC system from LCQ Advantage ESI (column: 125/4 Nucleodur C18 Gravity 3 μm; Macherey and Nagel); eluent, H_2O/0.1% HCO_2H (A) and MeCN/0.1% HCO_2H (B) or MeOH/5% THF/0.1% HCO_2H (B); gradient: 0−1 min with 10% B, 1−10 min with 10−100% B, 10−12 min with 100% B. HRMS (ESI): LTQ Orbitrap with flow injection (H_2O/MeCN = 1:1, 0.1% HCO_2H, flow rate: 250 μL/min). The majority of compounds possessed a purity of >95%, unless otherwise noted, as determined by HPLC coupled with mass spectroscopy (HPLC-ESI-MS).

High resolution mass spectra (HR-MS) were measured on a Thermo Orbitrap coupled to a Thermo Accela HPLC system using electrospray ionization (ESI).

Chemical Synthesis. The *N*-protected 1,2,3,4-tetrahydro-6-isoquinolinols were synthesized according to a strategy published by Hoye and co-workers, which utilized an in situ protection of the phenolic group with a TES group.[17] Syntheses of compounds **5**−**8**, **13**, and **14** have been published previously,[4] and compounds **2**-**4** were purchased. Syntheses of compounds **9**−**12** and **15**−**18** can be found in the Supporting Information.

General procedure: Chlorotriethylsilane (TESCl, 0.044 mL, 0.26 mmol) was added dropwise, by way of syringe, to a cooled (0 °C) solution of 1,2,3,4-tetrahydroisoquinolinol (**2** or **3**) (0.050 g, 0.22 mmol) and NEt_3 (0.15 mL, 1.1 mmol) in CH_2Cl_2 (2.5 mL) under Ar. The reaction was then stirred at rt for the specified time, after which the anhydride or sulfonyl chloride (0.28 mmol), and if required DMAP (cat., 0.010 g), were added to the mixture. The reaction was then stirred under Ar for a further 16 h. An additional volume of CH_2Cl_2 (5 mL) was then added, followed by TBAF·$3H_2O$ (0.27 g, 0.87 mmol), and the reaction was stirred for another 3 h. Water (10 mL) was then added, and the reaction mixture was extracted with CH_2Cl_2 (3 × 10 mL). The CH_2Cl_2 fraction was dried ($MgSO_4$) and evaporated under reduced pressure, resulting in a viscous yellow oil which was purified by silica gel column chromatography (eluent: ethyl acetate and hexanes as specified) to afford the desired product. Purity was controlled by HPLC and NMR spectroscopy and was, when not otherwise noted, ≥95%.

2-[(Trifluoromethyl)sulfonyl]-1,2,3,4-tetrahydro-6-isoquinolinol 19.



According to the general procedure, 1,2,3,4-tetrahydro-6-isoquinolinol **1** was silylated as described for 10 min and then treated with triflic anhydride (0.048 mL, 0.081 g) for another 18 h. Addition of TBAF, followed by workup of the dark solution and column chromatography (30% EtOAc/cyclohexane), then afforded the desired product **19** as a clear oil, which slowly became an opaque white oily solid (0.010 g, 17%, purity 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (d, J = 8.4, 1H), 6.72 (dd, J = 8.3, 2.6, 1H), 6.65 (d, J = 2.6, 1H), 4.87 (br s, 1H), 4.59 (br s, 2H), 3.74 (br s, 2H), 2.93 (br t, J = 5.9, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.6, 134.2, 127.3, 122.9, 120.0 (q, J = 322), 115.3, 114.3, 47.2, 44.2, 28.9. MS (ESI) 561 (30%), 280 (100). HRMS (ESI) m/e calcd for $C_{10}H_9O_3NF_3S$ $[M - H]^-$ 280.02607, found 280.02602.

ASSOCIATED CONTENT

Supporting Information. Experimental and supporting details to analytical techniques, chemical synthesis, protein expression, and crystallography, biochemical and cell biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

†The coordinates of the protein−ligand crystal structures have been deposited at the Protein Data Bank with the following PDB IDs: ERβ-**8** (3OMO), ERβ-**10** (3OMP), ERβ-**19** (3OMQ).

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ACKNOWLEDGMENT

W.A.L.vO. thanks the Alexander von Humboldt Foundation for a Georg Forster Research Fellowship for Experienced Researchers and the University of the Witwatersrand for sabbatical leave. Merck-Serono, MSD, and Bayer-Schering Pharma are thanked for financial support.

■ ABBREVIATIONS USED

ER, estrogen receptor; LBD, ligand binding domain; SAR, structure−activity relationship; E_2, 17β-estradiol; THIQ, tetrahydroisoquinoline; SRC-1, steroid receptor coactivator-1

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