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Identification of Novel Inverse Agonists of Estrogen-Related Receptors ERRγ and ERRβ

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Abstract

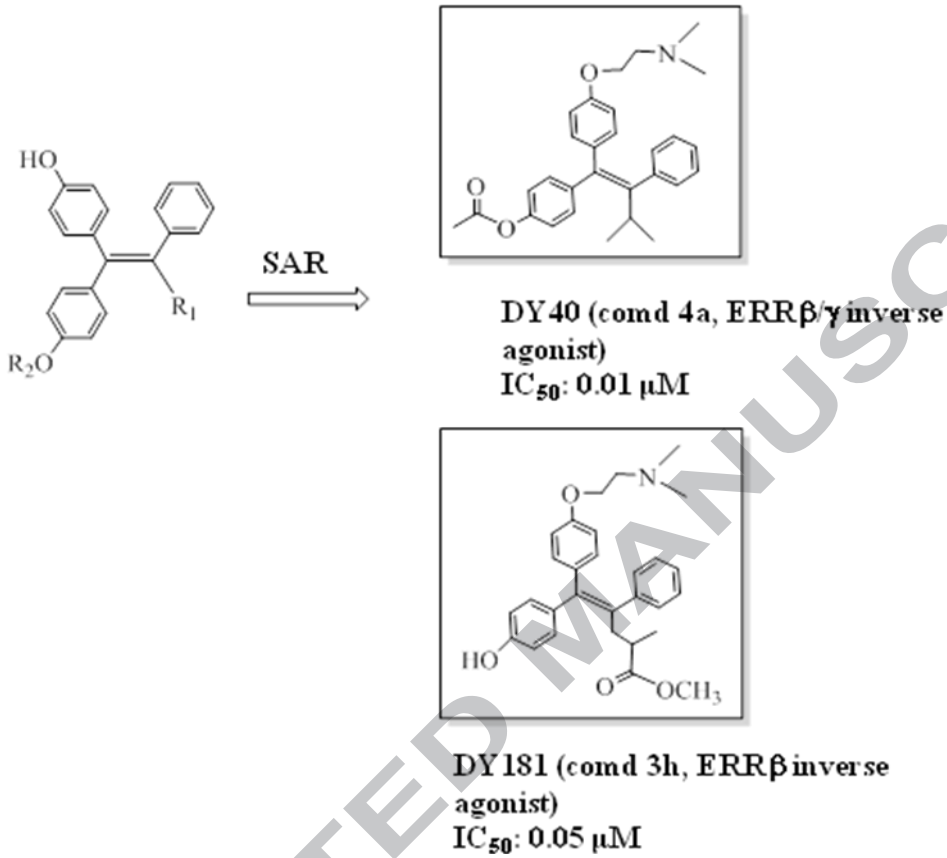
Estrogen-related receptors (ERRs, α, β, and γ) are orphan nuclear receptors most closely related in sequence to estrogen receptors (ERα and ERβ). Much attention has been paid recently to the functions of ERRs for their potential roles as new therapeutic targets implicated in the etiology of metabolic disorders. While no endogenous ligand has been identified for any of the ERR isoforms to date, the potential for using synthetic small molecules to modulate their activity has been demonstrated. In the present study, a series of novel inverse agonists of ERRγ and ERRβ were synthesized using regio- and stereo-specific direct substitution of triarylethylenes. These compounds were evaluated for their

ability to modulate the activities of ERRs. The rational directed substitution approach and extensive SAR studies resulted in the discovery of compound **4a** (**DY40**) as the most potent ERRγ inverse agonist described to date with mixed ERRγ/ERRβ functional activities, which potently suppressed the transcriptional functions of ERRγ with IC_{50} = 0.01 μM in a cell-based reporter gene assay and antagonized ERRγ with a potency approximately 60 times greater than its analog **Z-4-OHT** (*Z*-4-hydroxytamoxifen). In addition, compound **3h** (**DY181**) was identified as the most potent synthetic inverse agonist for the ERRβ that exhibited excellent selectivity over ERRα/γ in functional assays. This selectivity was also supported by computational docking models that suggest **DY181** forms more extensive hydrogen bound network with ERRβ which should result in higher binding affinity on ERRβ over ERRγ.

ABBREVIATIONS

ERRs, (Estrogen-related receptors); ER, (Estrogen receptor); NRs, (nuclear receptors); LBD, (ligand-binding domain); SAR, (structure-activity relationship); MCAD, (medium-chain acyl coenzyme A dehydrogenase). IC_{50}, (half maximal inhibitory concentration).

Graphical Abstract:



1. Introduction

Metabolic diseases, including obesity, diabetes, atherosclerosis and obesity-related cancers, are the leading cause of mortality in industrialized nations. It is estimated that over one-third of the United States population is obese, and these individuals are at greater risk for developing diabetes, cancer and cardiovascular disease. These disorders are responsible for over 500,000 deaths in the United States each year. The growing incidence of this problem has led to intense interest in identifying new molecular targets and new pharmacologic agents to treat and/or prevent these disorders.

Nuclear receptors are ligand-dependent transcription factors that regulate gene expression in response to small molecule ligands. Orphan members of the nuclear receptor superfamily, which have no identified endogenous ligand, are involved in regulation of many aspects of cellular metabolism including mitochondrial energetics as well as cholesterol, bile acid and glucose metabolism.[1] Therefore, they represent an important class of molecular targets for the treatment or prevention of a wide array of diseases.

The estrogen-related receptor (ERR) orphan receptor subfamily comprises three subtypes, ERRα, ERRβ, and ERRγ. The ERRs are first orphan nuclear receptors identified based on their structural similarity with the estrogen receptor (ER).[1] Although it was originally believed that the developmental and physiological roles of ERRs were quite distinct from those of the classic ERs, recent studies have shown that in some contexts ERRs share target genes, coregulatory proteins, ligands, and sites of action with the ERs.[2] Such evidence has been extensively reviewed.[3] ERRs play an important role in the transcriptional control of metabolic genes involved in the generation and utilization of cellular energy.[4] Thus, ERRs might present a therapeutic target for the prevention of obesity and type 2 diabetes.[5] Although the ERRα, ERRβ, and ERRγ isoforms all share considerable amino acid sequence identity with ERs in both the DNA binding domain (DBD) and ligand binding domain (LBD), they do not respond to natural estrogens, such as E_2 (17β-estradiol, Figure 1).[6-8]

ERRs are primarily expressed in the heart, skeletal muscle, brain, kidney, pancreas, placenta, and liver and are predicted to have significant differences in their ligand-binding preferences.[9-10] However, the mechanisms governing target gene selectivity of the individual ERR isoforms are not well understood. ERRα regulates genes involved in mitochondrial biogenesis (Gabpa/NRF2a, Tfam), fission/fusion (Mfn1 and 2, Opa1) as well as metabolic enzymes of β-oxidation (Acadm) and ETC/oxphos (Sdha, Cytc) pathways in conjunction with PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1α), a master regulator of lipid and glucose homeostasis.[11] In cancer cells ERRα also regulates cellular metabolism as well as genes relevant to proliferation and metastasis and is associated with poor prognosis in human breast cancer.[12-13] ERRα expression correlates HER2 status and has been shown to mediate the effects of growth factor signaling on metabolic reprogramming that is required for the development of chemoresistance.[14-15] Thus, ERRα is considered a potential drug target with potential to treat metabolic disorders and cancer.[16-18]

ERRβ is present early in the developing placenta in a subset of cells in extra-embryonic ectoderm destined to make up the chorion.[19-20] ERRβ is likely essential for reproduction.[21] ERRβ expression is essential for the maintenance of pluripotency and self-renewal potential in mouse embryonic stem cells and is among the core transcription factors that can reprogram fibroblasts into pluripotent stem cells.[22-23] Biochemical evidence also suggests that ERRβ may be a potential therapeutic target involved in cancers and metabolic disorders.[24] However, studies have been hampered by the embryonic lethality of ERRβ-/- mouse model and the lack of small molecule modulators

to study ERRβ function. Selective ERRβ ligands would provide an invaluable research tool to examine the biological function of ERRβ.

ERRγ regulates gluconeogenesis in liver and is a potential candidate drug target to reverse hyperglycemia and hepatic fat accumulation in the context of insulin resistance.[25-26] ERRγ/PGC-1β promotes oxidative metabolism in cancer cells,[27] and unlike ERRα, ERRγ expression is a favorable biomarkers in human breast cancer. Thus, targeting the ERRγ pathway may be a powerful therapeutic strategy to treat metabolic disorders and cancer.[28]

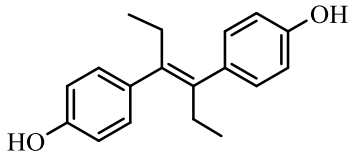
A critical step to advance the understanding of ERR biology is to design or identify selective modulators (agonists and inverse agonists) that can be used to target isoform specific processes in cells and *in vivo*. Notably, no endogenous ligand for any of the ERRs has been identified to date. The synthetic selective ligands developed to target ERRα are described as inverse agonists (antagonists of constitutive ERR activity) and agonists,[29-30] and have been utilized in cell culture and *in vivo* studies to investigate the physiologic activity of ERRα. However, little is known about the molecular mechanisms or biological activities downstream of ERRβ and ERRγ receptor activation because of the lack of selective ERRβ/γ ligands.

Recently, a small molecule agonist of the ERRγ and ERRβ was identified that mimics the protein ligand PGC-1α in activating human ERR*β* and ERR*γ*,[31-32] and compounds including ***Z*-4-OHT** and **DES** (Figure 1), have been shown to act as nonselective ERRγ

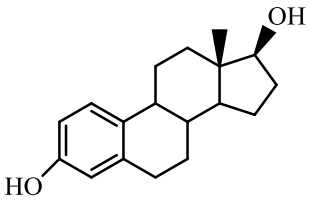
inverse agonists.[33-35] Structure-based approach was used to design the ERRγ selective inverse agonist GSK5182 and its analog (Figure 1).[36-37] Likewise, 4-methylenesterols isolated from the marine sponge steroids, have been reported to act as ERRβ antagonists.[38] However, these new antagonists exhibit lower potency than **DES** in transactivation experiments.

Despite these advances, the development of ERRβ and ERRγ inverse agonists with better potency and selectivity remains a formidable challenge. As a part of our ongoing program to explore novel classes of ERR modulators with the goal of increasing the potency and selectivity for ERRβ and ERRγ subtypes which might prove to be of therapeutic value in treating a variety of ERRβ and ERRγ-linked pathologies, we applied a strategy of altering the structure of triarylethylene core which is a template of *Z*-4-OHT. The compound *Z*-4-OHT has a very poor inverse agonist profile for ERRγ and ERRβ but binds to the LBD of these receptors; therefore it was used as a scaffold to generate higher potency antagonists. The design was based on SAR of **Z-4-OHT** analogs combined with analysis of the X-ray crystal structures of ***Z*-4-OHT** bound to the ligand binding domains of ERRγ and ERα. Analogs bearing extension or branched alkyl groups at the C2 position of the triarylethylene core with the basic side chain exhibited improved binding affinity and selectivity profiles for ERRγ compared to ***Z*-4-OHT**.[39] The existing X-ray crystal structure of ***Z*-4-OHT** bound to ERRγ and ERα LBD provided a model for the molecular basis of activity and selectivity.[36] In the current study, we describe the chemical synthesis and SAR (structure-activity relationship) for several triarylethylene derivatives

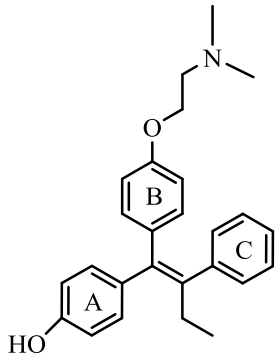
as well as molecular modeling of receptor binding and in vitro activity profiles of the compounds.



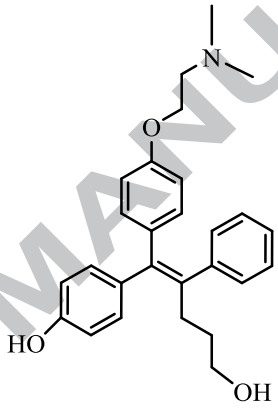
Diethylstilbestrol (DES)



17-estradiol (E_2)



4-Hydroxytamoxifen (*Z*-4-OHT)



GSK5182

Figure 1. Chemical structures of E_2 (the main physiologically relevant estrogen) and the three previously disclosed ERRγ and ERRβ synthetic inverse agonists.

2. Results and discussion

2.1. Chemistry

Based on the recent structural studies we proposed the synthesis of targeted arrays of small, triarylethylenes as ERRγ and ERRβ ligands. The resultant core scaffold of triarylethylenes could be derivatized at three distinct sites: at the aliphatic side chain on the C2 of triarylethylene position, the OH group on the 4-position of phenyl A-ring, and

alkylaminoethoxy-basic side chain on the 4-position of phenyl B-ring. We first examined the biological activities of several different types of alkylaminoethoxy-basic side chain into the phenyl B-ring and found that there was no improvement on the potency and selectivity toward ERRγ and ERRβ (data not shown). Therefore, an aliphatic side chain on the C2 of triarylethylene and 4-OH group on the phenyl A-ring were targeted to improve the potency and selectivity toward to ERRγ and ERRβ.

Combining molecular basis, structure consideration, and chemical feasibility, the initial strategy consisted of traditional medicinal chemistry and paralleled an approach involving various substitutions on the triarylethylene core in an attempt to increase the ERRβ and ERRγ specificity while performing the inverse agonistic selectivity on ERRγ and ERRβ. As described in the literatures, the *Z* isomer of 4-OHT has the required antiestrogenic activity, while the **(*E*)-4-OHT** has only about 5% of its affinity for the ER.[40] To determine if ***Z*-4-OHT** would have selectivity on ERRs, we tested the ***Z*-4-OHT** and ***E*-4-OHT** isomers in ERRβ and ERRγ activity. The *Z* isomer of 4-OHT has the required activity on the ERRγ and ERRβ, but the ***E*-4-OHT** was much less active for the ERR targets (data not shown). Thus, analogues of triarylethylenes with a basic side chain having a Z-form are highly desirable.

In order to establish a chemical library with various functionalities and structural diversity, accordingly, we first focused on the extended ethyl side chains of triarylethylene core. Retrosynthetically, we found that 1,1-bis(4-hydroxyphenyl)-2-phenylethylene could serve as a key template for the conversion to a series of analogs

structurally related to the ***Z*-4-OHT**. We have previously described the synthesis of ***Z*-4-OHT** in a regio- and stereo-selective controlled manner.[41] Our intention was to extend this method to versatile production of 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes via McMurry chemistry which is a well known established methodology for the cross-coupling reaction (Figure 2).[42]

The 1,1-bis(4-hydroxyphenyl)-2-phenylethylenes were synthesized by simply coupling 4,4'-dihydroxybenzophenone with various propiophenone derivatives to introduce $\mathbf{R_1}$ groups and make chemset **1** (Figure 2). To expand the number of accessible analogs, a McMurry reductive coupling between 4,4'-dihydroxybenzophenone reacting with the readily-available reagents such as isobutyrophenone, isovalerophenone, 4-oxo-4-phenyl-butyric acid methyl ester, 2-methyl-4-oxo-4-phenyl-butyric acid methyl ester, 3-benzoylpropionic acid, and 2-methyl-4-oxo-4-phenylbutyric acid in the presence of zinc and titanium tetrachloride in dry THF under reflux conditions was performed. The desired 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes (**1a-n**) were obtained in good yields. The carboxylic acid groups in **1i** and **1j** were reduced using BH_3-THF to give the corresponding alcohols **1k** and **1n** in 75 and 74% yields, respectively. In chemset **1**, **1c** and **1d** are known compounds and we have re-synthesized them in excellent yields by McMurry chemistry compared to the existing synthesis of Gust, Gauthier and coworkers.[43-44]

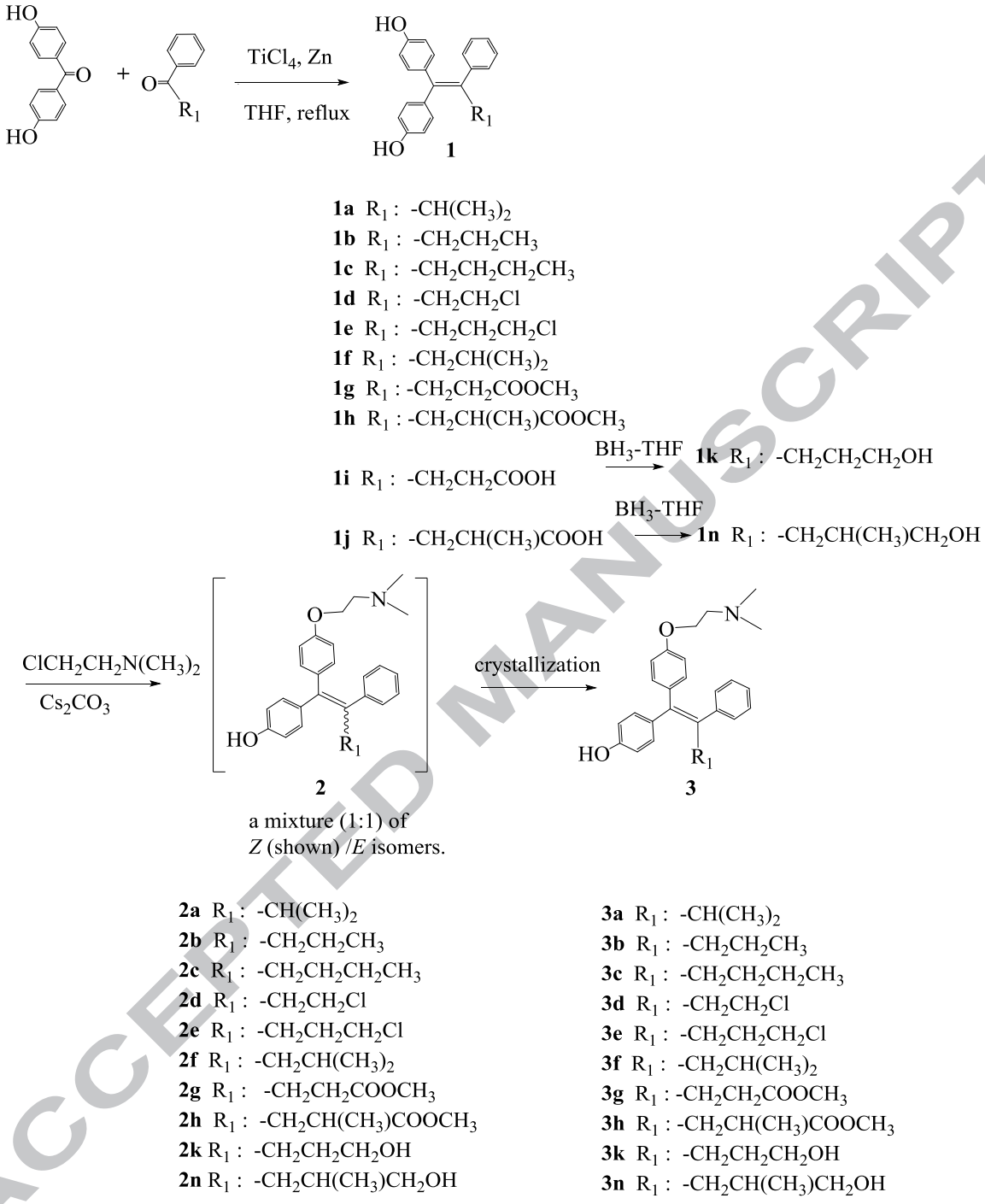
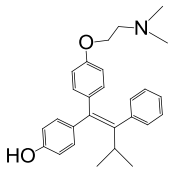


Figure 2. Synthesis of 1,1-bis(4-hydroxyphenyl)-2-phenylethylene analogs chemset **1,** installation of the basic side chain chemset **2**, and stereo- and regiospecific production of the *Z*-isomer chemset **3**.

Next, we coupled compounds **1a-h, 1k,** and **1n** directly with 2-(dimethylamino)ethyl chloride hydrochloride using a known method, [45] and obtained an equimolar mixture of the geometric isomers of (*E*,*Z*)-4-[1-(4-dimethylaminoethoxy-phenyl)-2-phenyl-but-1-enyl]-phenols **2**, as depicted in Figure 2. The initial coupling reaction favored the formation of geometric *Z*-isomer, but it was then converted to a monoalkylated geometric mixture after a while. In this procedure, monoalkylated geometric isomers are the majority, while the dialkylated products were detected in trace quantities. Note that ***Z*-4-OHT** is more potent than ***E*-4-OHT** for the ERRβ/γ in the cell-based cotransfection assay. Thus, the fixed ring analogs of substituted 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes in **3** series with *Z*-isomer are of high interest for this project. Treatment of the crude mixture **2** of two *E*/*Z* stereoisomers in 1:1 ratio were easily separated by selective crystallization, and in some cases, by flash chromatography. A series of geometric mixture *E*/*Z* in **2** series as pure regio-selective *Z*-isomer **3** was ultimately effectively separated. Examples include crystallization of the white solid **2a** with a 1:1 *Z*/*E* ratio from warm methanol which led to white crystals **3a**. The absolute configuration assignment of **3a** was determined as *Z*-isomer by X-ray structure analysis ((Figure 3). Other configurations of the stereo *Z*-isomer in chemset **3** were determined by NOE measurements and ^{1}H NMR. Alternatively, the geometric isomers of pure *Z*-form in chemset **3** are very easily identified by general ^{1}H NMR because *Z*-form analogs have upfield NMR signals of the 2-dimethylaminoethoxy chain protons compared to the corresponding *E*-isomer by using CD_3OD as a solvent. By the improved synthetic methodology we were able to minimize the number of synthetic steps and develop a purification methodology for the production of a series of new *Z*-isomer analogs derived

from ***Z*-4-OHT**, compared to the Gauthier and GSK method which requires protective groups and pivaloate deprotection.[36] The general synthetic route used to prepare substituted hydroxylated 1,1,2-tribenzyl-but-1-enes of the *Z*-isomer are outlined in Figure 2.



Compound **3a**

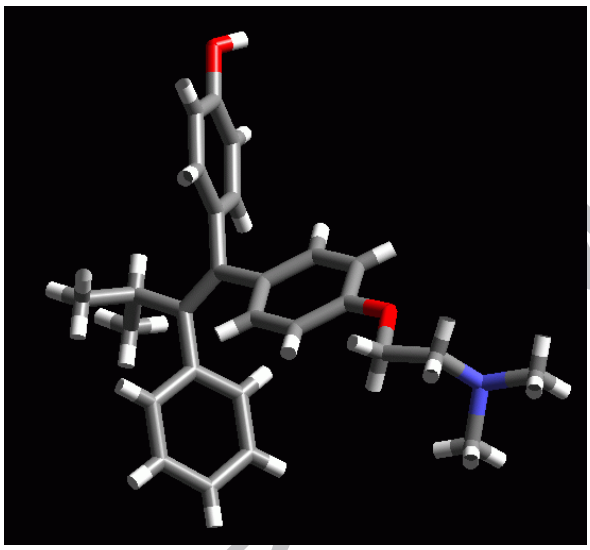


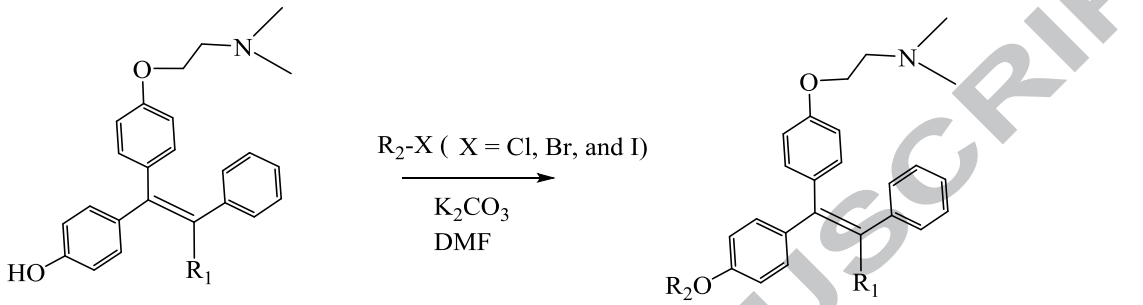
Figure 3. X-ray crystallographic structure of **3a** indicated that the active geometric isomer **3a** is *Z*-form generated in a region controlled manner.

Previously the hydroxyl group on the phenyl A-ring of ***Z*-4-OHT** was believed to be important for the binding affinity toward to the ERα.[46] ***Z*-4-OHT** has higher affinity for ERα than ERRγ because of the similarity between ERRγ and ERα in the first shell of the ligand binding pocket. Note that the hydroxyl group on the phenyl A-ring interacts with ERRγ in Glu-275 and Arg-316 (ERα Glu-353 and Arg-394), and the LBDs of ERRγ and

ERRβ are almost identical. This implies that the optimization in the 4-OH in the phenyl A-ring would be challenging for the increase of the potency on the ERRγ. Furthermore, SAR from X-ray crystal structures of ***Z*-4-OHT** bound to the LBD of ERRγ suggested that the binding pocket may tolerate small substituents other than hydroxyl group.[47] In order to assess if the simple substituent variations of 4-OH group on the phenyl A-ring interaction would lead to the improved potent and selectivity for ERRγ and ERRβ, next, we designed and synthesized a small focused chemical library of phenyl A-ring with diversity at position of 4-OH to generate structure-activity relationship (SAR). To speed the synthesis, we applied solution-phase parallel synthetic techniques to exploit variant functionalities as $\mathbf{R_2}$ to maximize the efficient interactions in molecular levels at the binding pockets of ERR*γ* and ERRβ.

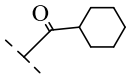
Compounds **3a** and **3h** have been selected as building blocks based on their improved inverse agonist functional profile for the ERRγ and ERRβ. Thus, the hydroxyl function of active precursors **3a** and **3h** was converted into the chemset **4** by simple estrification and alkylation reactions to introduce a series of ester or ether pharmacophores as diversity to the 4-position of phenyl A-ring. The 48 commercially available chemical reagents were selected on the basis of diversity, the criteria being lipophilicities and chemistry compatibility to achieve more flexible substitution patterns. This combinatorial synthesis offers the advantage of compatibility with a series of functionalities. Table 1 shows the chemical structures of the final $\mathbf{R_2}$ at the phenyl A-ring moiety by varying the hydrophobicity and size of the system. The identity of each of the final compounds was confirmed by ^{1}H and ^{13}C NMR.

Table 1. Combinatorial synthesis of a library of precursors **3a**, **3h** with diversity at position 4-OH of the pendant phenyl A-ring was synthesized using parallel solution-phase synthetic techniques to induce $\mathbf{R_2}$ functions.



3a $\mathbf{R_1}$ = -$CH(CH_3)_2$
3h $\mathbf{R_1}$ =-$CH_2CH(CH_3)COOCH_3$

4

Comp. #	
R2(1)	
R2(2)	
R2(3)	
R2(4)	
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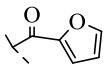
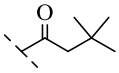
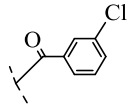
R2(6)	
R2(7)	
R2(8)	
R2(9)	
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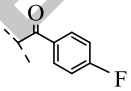
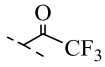
R2(12)	
R2(13)	
R2(14)	
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R2(16)	

Comp. #	
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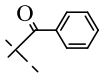
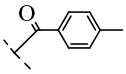
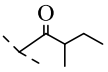
R2(18)	
R2(19)	
R2(20)	
R2(21)	
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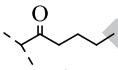
R2(23)	
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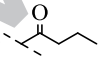
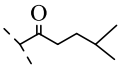
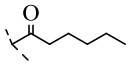
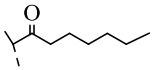
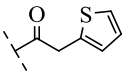
R2(25)	
R2(26)	
R2(27)	
R2(38)	
R2(29)	
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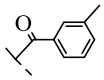
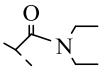
R2(31)	
R2(32)	

Comp. #	
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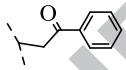
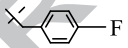
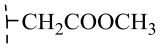
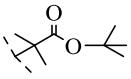
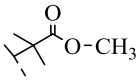
R2(34)	
R2(35)	
R2(36)	
R2(37)	
R2(38)	
R2(39)	
R2(40)	
R2(41)	
R2(42)	
R2(43)	
R2(44)	
R2(45)	
R2(46)	
R2(47)	
R2(48)	

To achieve different types of esters and an alternative access to substituted ethers in chemset **4**, a total of 48 commercially available acyl chlorides and alkyl halides were reacted in hot DMF containing potassium or cesium carbonates as the base. The optimal temperature for the reaction was 50 ^{o}C. The construction of a focused small library by parallel solution-phase synthesis was carried out in a Mettler-Toledo Bohdan RAM organic synthesizer with an automated liquid handling robot. A workstation of Mettler-Toledo system was used to tare each reactant vial in an array of vials. After the added reagent was weighed, it was diluted to the desired concentration with organic solvents and vortexed. Various combinations of reactants were then transferred to each of reaction tubes for the screw synthesizer. The solution-phase in the library synthesis always posed a challenge in purification procedures. To get quick and efficient purification in higher purity in sufficient quantity for cell-based biological testing, the Biotage Quad 3 automated medium pressure flash chromatography was used for parallel purification. The crude mixtures were then directly purified by automated unit system which performed 12 simultaneous flash chromatographs using prepacked silica gel columns. Twenty fractions could be easily obtained from each run in under 20 minutes. The average yields of the targeted compounds were > 85% and purity > 82%. The resulting fractions were analyzed by flow injection NMR to confirm the chemical structures by ^{1}H and ^{13}C NMR. By this protocol, a total of 96 corresponding esters and ethers of chemset **4** were obtained in fair yields.

2.2. Biological evaluations

To assess the functional transcriptional activity of these compounds, cell-based assays using chimeric receptor Gal4 DNA-binding domain (DBD)-NR ligand binding domain cotransfection assay (LBDs of ERRα, ERRγ, and ERRβ) were performed. As an initial screen to determine the ERRγ and ERRβ inhibitory activity and selectivity, all compounds were evaluated for *in vitro* potency and selectivity by transfection testing, using CV-1 cells transfected with human and mouse ERRs. MH100 x 4 is a luciferase reporter with four copies of a GAL4 UASG response element, where GAL-I refers to the ligand-binding domain of the indicated receptor fused to the C-terminus of the GAL4 DNA-binding domain. Assays were performed using a Biomek automated workstation in which the genes for the nuclear receptor, as well as a plasmid containing a response element upstream of a luciferase cDNA, are transfected into CV-1 cells. Compounds were evaluated for their ability to inhibit human ERRs at 0.03, 0.1, 0.5, 1, 2.5, 3, and 10 μM test concentrations. Those compounds that showed good ERRβ and ERRγ potency and selectivity were evaluated at additional concentrations. ***Z*-4-OHT** used as a known ERR*γ* and ERRβ inverse agonist to compare the constitutive activity of unliganded ERR*γ* and ERRβ. A series of synthetic compounds of this focused library has been identified and showed similar activities at the 1 μM concentration as compared with ***Z*-4-OHT**.

2.3. Structure−Activity Relationships (SAR)

In 2002, Gust and coworkers[43] reported a series of C2-alkyl substituted of triarylethylene derivatives without basic side chains. They came to the conclusion that those compounds they synthesized without a basic side chain in the B-ring possessed the same antagonistic

potency for ERs as compared with ***Z*-4-OHT** which contains a basic side chain that was believed exhibiting antagonistic activity of 4-OHT on the ERs.[46] However, it is important to note that all of the tested compounds by Gust and co-workers did not cover extensive studies of the structure-function relationship for the activity on the ERRs. Thus, we explored if compounds in chemset **1,** which is in the absence of basic side chain functionality, would also exert some biological effects by modulating the activities of ERRs. The compounds in chemset **1** have been evaluated by cell-based screening for inverse agonistic activity against ERRs. As compared to the ***Z*-4-OHT**, most of the new analogs in chemset **1** did not possess ERRs activity, but possess ERs activity (data not shown). We came to the conclusion that in the class of 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes, having a basic side chain is not a prerequisite for exhibiting antagonistic effect on the ER receptor. The antiestrogenic properties comprising estrogen receptor binding depend only on the length of the C2-alkyl chain. These results are consistent with previous findings that removal of the dimethylaminoethoxy side chain of *Z*-4-OHT did not decrease the antagonistic effects on the MCF-7-2a cell line.[45]

However, as shown in Figure 4, except those the most of the new analogs in chemset **1**, specifically, compounds**1g** and **1h** were found to be active and have selectivity for ERRβ with IC_{50} values of 0.6 and 0.3 μM, respectively. This is a surprising finding because compounds **1g** and **1h** exhibited excellent selectivity and inverse agonist efficacy on the ERRβ and they were not active for the ERRα, also were not active for the ERRγ receptor at the higher dose. In contrast, the analogs **1g** and **1h,** which possessed the corresponding extended ethyl side chains bearing methyl esters in the C2-alkyl substituted system and

did not bear any basic side chain on the phenyl B-ring, displayed improved selectivity profiles toward the ERRβ (Figure 4). This demonstrated that the presence of the extended methyl ester side chain on the C2-alkyl position is crucial for the ERRβ to gain an antagonistic activity.

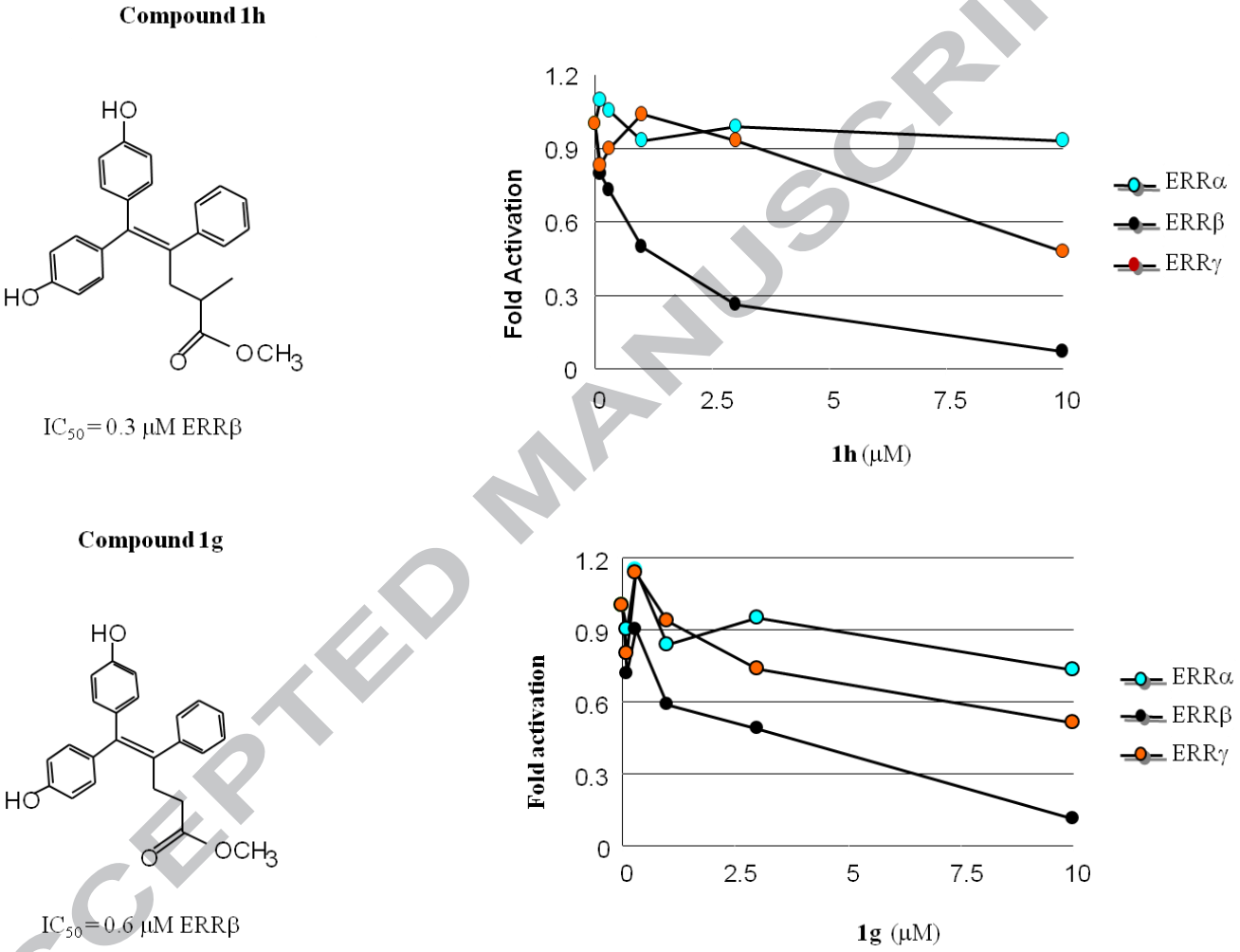


Figure 4. Compound **1g** and **1h** in chemset **1** have been identified as selective inverse agonists for the ERRβ.

Having a basic side chain is not a prerequisite for exhibiting high binding affinity and antagonistic effects on the ER. However, as predictable, having a basic side chain of 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes is a prerequisite for exhibiting high binding affinity and inverse agonistic effects on the ERRγ. Wherein the triarylethylene system, it can be seen that a dimethylaminoethoxy basic side chain on one of the phenolic groups (phenyl B-ring) increase ERRγ inverse agonistic activity and selectivity.[36] In our experiments variation of a side chain on the C2 of triarylethylene core with basic side chain appeared to be beneficial for ERRγ, and for the ERRβ inhibitory activity. All of the resulting compounds in **3** series were found to possess the inverse agonistic potency on the ERRγ and ERRβ. The data are shown in Table 2 with a select set only. This study confirmed that the introduction of basic side chain on the para-position of the phenyl B-ring requires to providing generally improved potencies on the ERRγ and ERRβ.

Interestingly, as can be seen in Table 2, replacing an ethyl group of *Z*-4-OHT with an isopropyl group substituent (**3a**) led to an increase in inhibitory activity. The potency of **3a** was increased on the ERRγ and ERRβ (IC_{50} = 0.29/0.21μM) by 3-fold as compared to the ***Z*-4-OHT**. However, when an isopropyl was replaced by an isobutyl group, the resulted compound (**3g**) has slightly less in potency on ERRγ and ERRβ (IC_{50} = 1.0/0.75 μM) compared to *Z*-4-OHT, as well as a 3-fold ERRβ/γ potency less than **3a**, as shown in the Table 2. Particularly less inhibitory activities or a loss of inverse agonistic activity on the ERRγ were observed with those compounds additionally bearing longer and bulkier groups at the C-2 position. This result demonstrated that the ethyl group at the C2-extension position could be replaced with small alkyls without substantial loss of activity.

The isopropyl group at the C2-position (**3a**) seems beneficial in terms of potency increasing on the ERRγ and ERRβ.

However, in contrast, when the isopropyl group at the C2-position (**3a**) was changed to the methyl esters located at the extended ethyl side chain, the corresponding compounds **3g** and **3h** (**DY181**) exhibited significant ERRβ binding affinity and selectivity which has no effect on ERα, ERβ, ERRα, and ERRγ transcriptional activity up to 3 μM. As shown in Figure 5, the most potent ERRβ inverse agonist **3h** with an extended methyl ester group at the C2-position has been identified with excellent selectivity over ERRα/γ. Compound **3h** (**DY181**) displayed the highest ERRβ affinity (IC_{50} = 50 nM). These results are consistent with previous findings from chemset**1** (**1h** and **1g**) wherein the extension of C-2 ethyl side chain system having corresponding methyl esters appeared to be beneficial for ERRβ activity. Apparently, a combination of extension of C-2 ethyl side chain system having corresponding methyl ester and a basic side chain contributes high potency and inverse agonistic effect on the ERRβ receptor. The correct mechanism for this observed contribution to the selectivity is yet to be established.

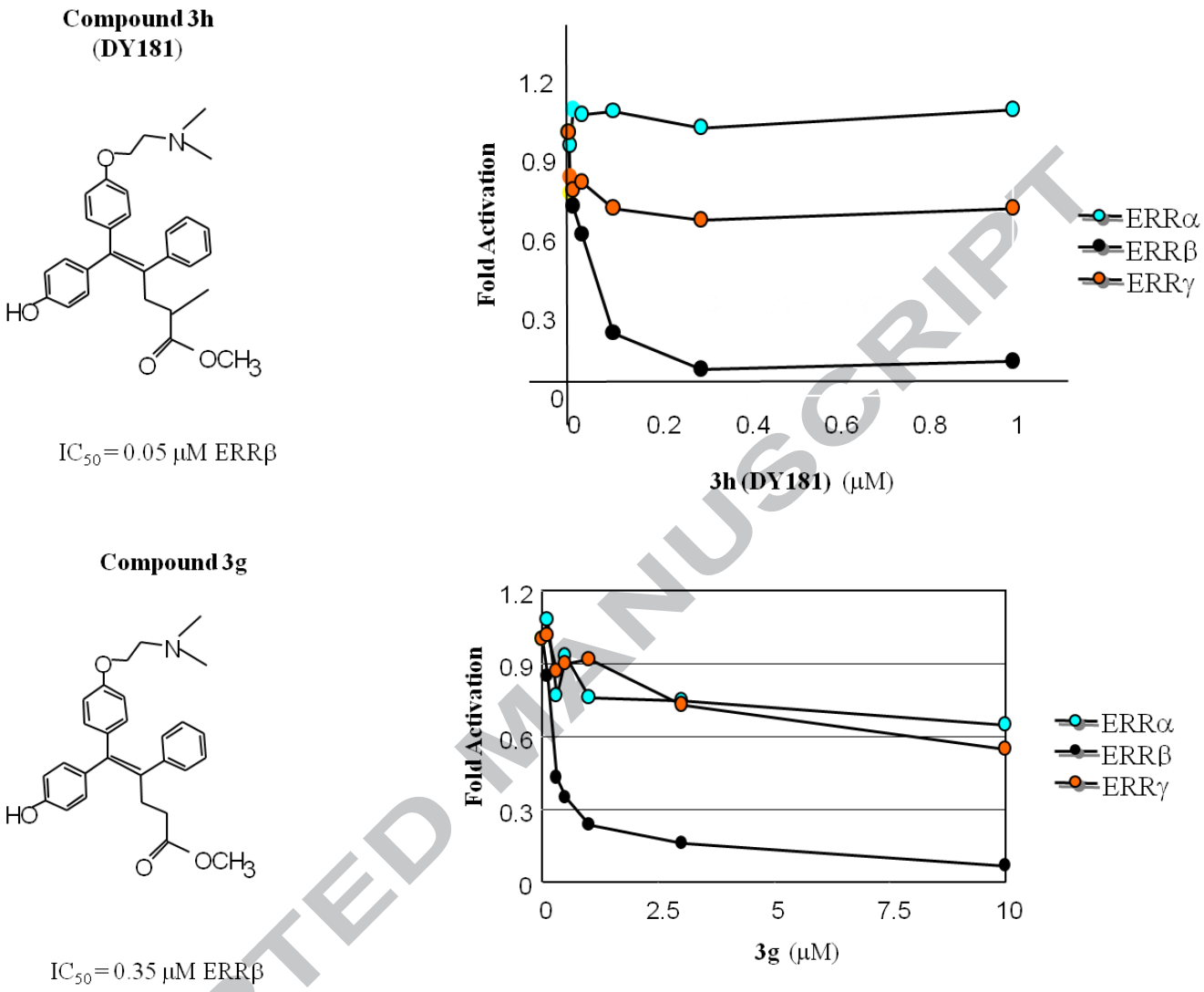


Figure 5. Compounds **3h** (**DY181**) and **3g** have been identified as selective inverse agonists for the ERRβ.

Combinatorial construction of a focused library by carrying out solution phase-assisted synthesis led to the 96 analogs by removal of the hydroxyl group (4-OH) or replacing it with small acetyl and alkyl substituents. Analogs which loaded onto the 4-position of hydroxyl on the A-phenyl ring in chemset **4** as $\mathbf{R_2}$ were generated by incorporating esters or ethers to potentially increase lipophilic character which would be beneficial for ligands

to interact with the hydrophobic LBD of ERRs. As expected, the ligands with small acetoxy improved potency and selectivity on the ERRγ, whereas the other ligands with bulkier substituents as **R_2** showed less potency and sterically not well tolerated by ERRβ and ERRγ. Several interesting compounds derived from **3a** having acetoxy have been identified in **4** series in repressing ERRβ and ERRγ. The acetate analog, exemplified by **DY40** (**4a** {R2(1)}) derived from **3a** showed the greatest functional potency with the IC_{50} of 10 nM as shown in Figure 6. The improved potency and inverse agonist activity of the acetoxy-substituted analog **4a** {R2(1)} demonstrated that an acetoxy group on the A-ring instead of hydroxyl seems to efficiently diversify the potency of ERRβ and ERRγ.

In fact, in chemset **4**, small substituents **R_2** led to a slight increase inhibitory activity, and bulky **R_2** substituents further attenuated activity of ERRβ and ERRγ. To rationalize these results we proposed that the bulkier R_2 substituents are not suitable for ERRγ and ERRβ activity, and probably the LBD of the receptors may not be regulated and tolerated by bigger molecules. Thus, the size and shape of the ligand are key factors for the protein modulation. However, analogs which loaded onto the 4-position of hydroxyl on the A-phenyl ring in chemset **4** as **R_2** from the precursor **3h** did not improve the potency as compared with **DY40** (**4a**) (data not shown). To gain further insight into the selectivity on the ERRs, the same reaction expanded to a 0.5 g scale of compounds **4a**{R2(1)} resulted in an 88% yields. Additional experiments included the compounds **3k** and **3n** which were also converted into the corresponding analogs **4k**{R2(1)}, and **4n**{R2(1)} in a 0.5 g scale production resulted in 82%, and 79 yields, respectively.

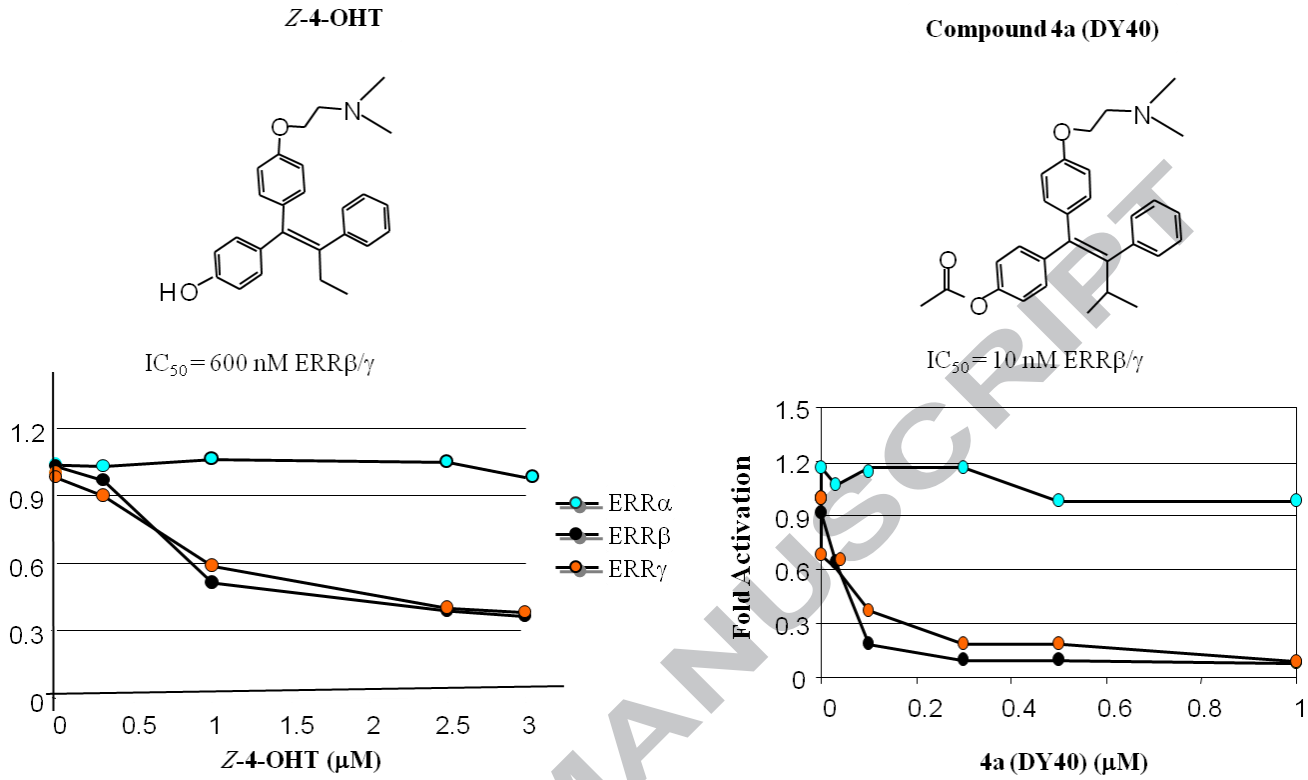


Figure 6. Compound **4a** (**DY40**) was evaluated in potency and selectivity by cell-based transactivation assays using CV-1 cells transfected with human and mouse ERRs. CV-1 cells were transfected with reporter constructs and expression vectors and the fold activation of the reporter construct was determined at several concentrations.

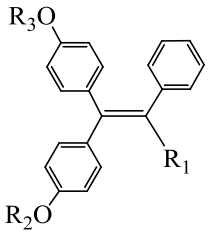
2.4. In vitro Functional Data for Selected Compounds at ERRs Receptors

A high-throughput binding assay using cell-based assay at different concentrations was carried out with the LBD of ERRs. From initial screening against ERRs, over thirty active compounds have been identified as having modest ERRγ and ERRβ affinity, and data are highly reproducible. Data were re-evaluated for 12 members of library (2 members in chemset **1,** 6 in chemset **3,** and 3 members in chemset **4**). A summary of the IC_{50} values that were derived from *in vitro* transactivation assays against the human

ERRα, ERRβ and mouse ERRγ subtypes for compounds **(1g, 1h, 3a, 3f, 3h, 3g, 3k, 3n, 4a, 4k, and 4n**) with *Z*-4-OHT is outlined in Table 2. As shown in Table 2, compound **4a** (**DY40**) as an inverse agonist of ERRβ and ERRγ displayed the greatest functional potency ((IC_{50} = 10 nM) at about 60-fold greater than ***Z*-4-OHT**. The acetylating of compounds **4k** and **4n** efficiently increased the affinity for ERRβ and ERRγ almost 3 to 7-fold as compared with **3k** and **3n**. These data indicated that the acetoxy group on phenyl A-ring is critical for the ERRβ and ERRγ in terms of potency and selectivity. The selective members of three chemsets exhibited a wide range of inverse agonistic activity on ERRβ and ERRγ and clearly indicated that these new compounds all were inactive on ERRα.

Interestingly, among them, **1g**, **1h**, **3g**, and **3h** were identified as ERRβ inverse agonists. **3a, 3f, 3g**, **3n**, **4a, 4k,** and **4n** were identified as ERRβ and ERRγ inverse agonists. These most promising inverse agonists indicated that the ERRβ and ERRγ inhibitory potency and selectivity could be extremely sensitive to minor changes in chemical structure within a modified series. However, based on the selectivity evaluation, except for compounds **1g**, **1h**, **3g**, and **3h**, the number of compounds we synthesized in this project cannot fully separate ERRγ activity from ER activity. For example, **4a** (**DY40**) remains an antagonist potency of ERα. It is worth noting that the compounds we synthesized that are selective over ER remain a key challenge.

Table 2. A select set of ***Z*-4-OHT** analogs on ERRα, β, and γ binding affinities.



Compound	R_1	R_2	R_3	**ERRα** IC_{50}(uM)	**ERRβ** IC_{50}(uM)	**ERRγ** IC_{50}(uM)
***Z*-4-OHT**	CH_2CH_3	H	$(CH_2)_2N(CH_3)_2$	NA	0.65	0.6
1h	$CH_2CH(CH_3)COOCH_3$	H	H	NA	0.3	5.5
1g	$CH_2CH_2COOCH_3$	H	H	NA	0.6	6.5
3a	$CH(CH_3)_2$	H	$(CH_2)_2N(CH_3)_2$	NA	0.21	0.29
3f	$CH_2CH(CH_3)_2$	H	$(CH_2)_2N(CH_3)_2$	NA	0.75	1
3h (**DY181**)	$CH_2CH(CH_3)COOCH_3$	H	$(CH_2)_2N(CH_3)_2$	NA	0.05	2.78
3g	$CH_2CH_2COOCH_3$	H	$(CH_2)_2N(CH_3)_2$	NA	0.35	3.5
3k (*Z*-GSK5182)	$CH_2CH_2CH_2OH$	H	$(CH_2)_2N(CH_3)_2$	NA	3.5	2.7
3n	$CH_2CH(CH_3)CH_2OH$	H	$(CH_2)_2N(CH_3)_2$	NA	2.25	2.5
4a (**DY40**)	$CH(CH_3)_2$	CH_3CO	$(CH_2)_2N(CH_3)_2$	NA	0.01	0.01
4k	$CH_2CH_2CH_2OH$	CH_3CO	$(CH_2)_2N(CH_3)_2$	NA	1.1	0.3
4n	$CH_2CH(CH_3)CH_2OH$	CH_3CO	$(CH_2)_2N(CH_3)_2$	NA	0.55	0.35

NA = not active at 10 μM.

IC_{50} is the concentration of test compound that gave 50% maximum efficacy.

CV-1 cells were co-transfected with appropriate reporter constructs and expression vectors. The fold activation of the reporter construct by ligand was determined at several concentrations of selected compounds. No induction of ERRα dependent transcription was observed for any of the compounds in tested.[49]

2.5. Validation of DY40 effects on endogenous ERRγ target gene expression and metabolism in cell based models.

We next examined whether the newly synthesized **DY40** could repress endogenous expression of an ERRγ target gene. Previous studies showed that ERRγ is a constitutive activator of the small heterodimer partner (*SHP*) gene, which is selectively regulated by ERRγ via a direct binding of the receptor to a site located in the proximal promoter region.[50-51] Consistent with these findings, ERRγ over-expression in 293HEK upregulated the SHP transcript by 2.5-fold and treatment with 100nM **DY40** completely blocked ERRγ mediated induction (Figure 7A). We also evaluated the effects of the compound in C2C12 myotubes that have significant endogenous ERRγ expression. Consistent with the observed effects in 293HEK cells, **DY40** repressed endogenous SHP transcript levels by ~60% in C2C12 myotubes (Figure 7B).

Previous studies have shown that ERRγ deficient myocytes have reduced capacity oxidize long-chain fatty acids.[52] Therefore, we investigated the effects of treatment with **DY40** on β-oxidation rates in C2C12 myotubes. Treatment of C2C12 myotubes with **DY40** for 48 hours resulted in a significant reduction in palmitate oxidation rates (-42%) in myocytes compared to vehicle-treated controls (Figure 7C). Notably, knockdown of ERRγ expression in C2C12 decreased oxidation by the same magnitude (data not shown). Collectively, these data demonstrate robust and selective effects of **DY40** on ERRγ dependent gene regulation and metabolism in cells.

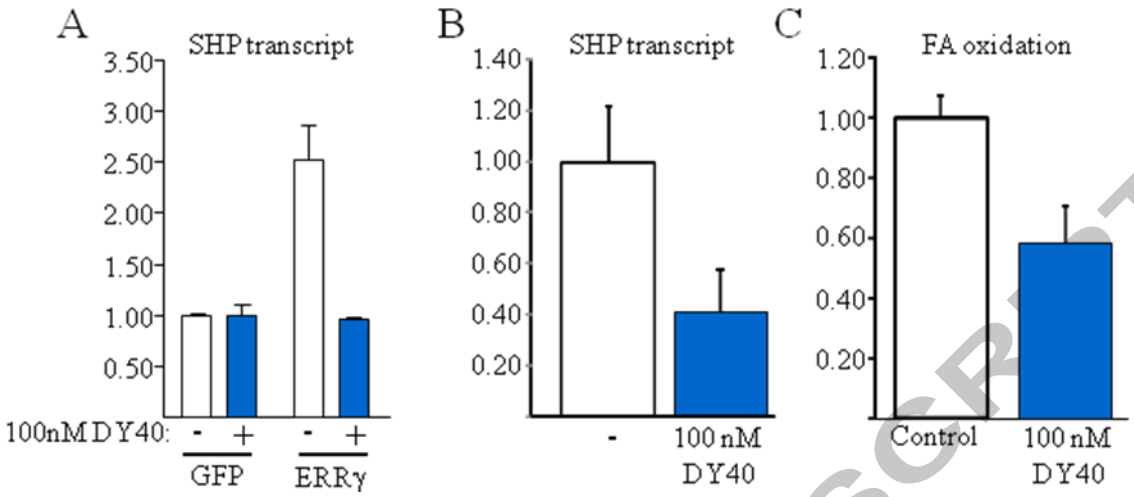


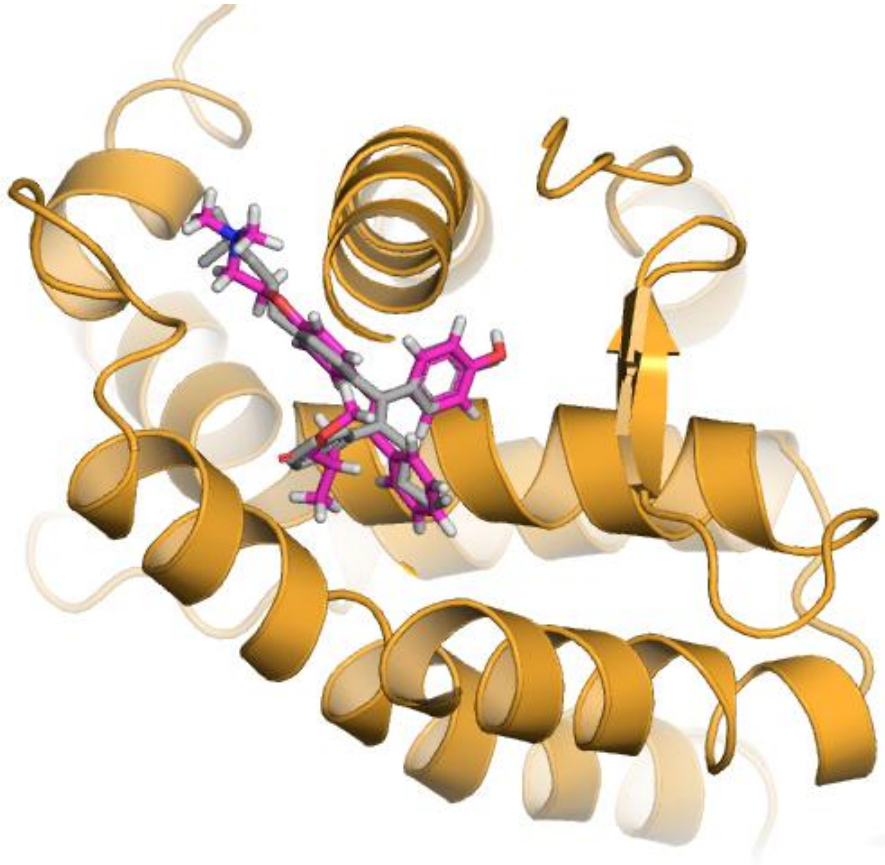
Figure 7. Compound **4a** (**DY40**) inhibits expression of a specific ERRγ target gene and reduces mitochondrial β-oxidation rates. A. 293 HEK cells expressing either GFP or ERRγ via adenoviral transduction were treated with either vehicle (DMSO) or 100 nM **DY40** for 48 hours. Expression of endogenous SHP (NR0B2) was quantitated by real-time PCR. Values are normalized to β-actin transcript and reported relative to GFP untreated levels (=1.0). Data represent mean ± S.E.M. for 3 independent trials. B. C2C12 myotubes were treated with either vehicle or 100 nM **DY40** for 48 hours. Expression of endogenous SHP was measured by quantitative real-time PCR. Values are normalized to 36b4 (Rplp0) transcript and reported relative to expression in DMSO treated cells (=1.0). Data represent mean ± S.E.M. for 3 independent trials. *, $P < 0.05$ DMSO versus **DY40** treated values. C. C2C12 myotubes were treated with either vehicle or 100 nM **DY40** for 48 hours. Oxidation of ^{3}H-palmitate-BSA was measured as described in Methods. Values are normalized to total cellular protein and reported relative to expression in DMSO treated cells (=1.0). Data represent mean ± S.E.M. for 4 independent trials. * $P < 0.001$ DMSO versus **DY40** treatment.

2.6. Computational modeling of ERR and binding of compound 3h (DY181) to ERRβ

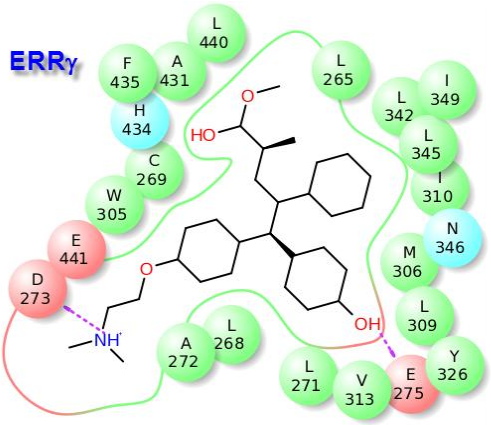
To further understand the specific inhibition of compound **3h** (**DY181**) to ERRβ protein over ERRγ, we implemented computational tools to predict the binding modes of **DY181** to ERR proteins. The homology model of ERRβ protein in ligand binding domain (193-433a.a.) was built by using SWISS-MODEL[53] based on the X-ray crystal structure of ERRγ (PDB code 2EWP),[36] which has sequence identity of 77%. The model was further optimized to assign the hydroxyl, Asn, Gln and His residue states, and followed by structural minimization using Schördinger Maestro software. The best docking poses of compound **DY181** on ERR proteins were predicted by using our in-house developed All-Around Docking (AAD)[54] algorithm, which are displayed in Fig.8. AAD methodology searches the best binding site and binding pose around the whole protein surface without any knowledge of the possible locations of docking pocket.

As depicted in Fig.8A by using our AAD method, compound **DY181** binds ERRβ at the same pocket on ERRγ protein as that of compound GSK5182 in crystal structure. Two hydrogen bonds are formed with D273 and E275 residues, which are displayed in Fig. 8B. As shown in Fig. 8C, **DY181** binds ERRβ protein at the same ligand-binding pocket as well. However, besides the two hydrogen bonds formed with corresponding D248 and E250 residues, an extra hydrogen bond with Y321 residue is also produced. The additional hydrogen bond between **DY181** and Y321 in ERRβ should result in higher binding affinity. Consequently, **DY181** shows selectivity on ERRβ over ERRγ.

A.



B.


ERRγ
L 440
F 435
A 431
L 265
I 349
L 342
H 434
HO
O
L 345
C 269
I 310
W 305
N 346
E 441
M 306
D 273
O
L 309
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NH
A 272
L 268
L 271
V 313
E 275
Y 326

C.

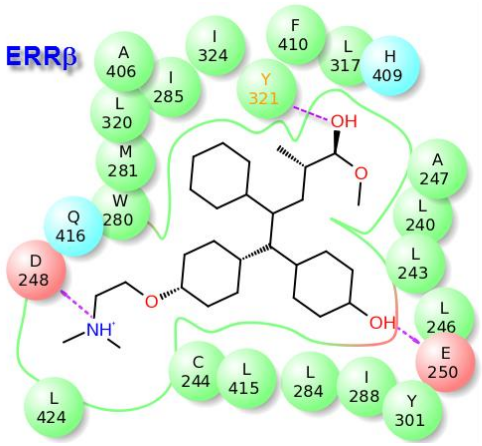

Figure 8. Binding models of compound **3h** (**DY181**) in ligand binding domain of ERR proteins. A. Docking model of **DY181 (3h)** (shown as magenta sticks) binding with ERRγ protein. The X-ray crystal structure of compound GSK5182 (PDB code 2EWP) is also overlapped as grey sticks for comparison. It can be seen that **DY181 (3h)** takes the same binding pocket and similar binding pose as GSK5182. B. Two hydrogen bonds (shown as pink dots in ligand-protein interaction diagram) are formed between **DY181 (3h)** and D273/E275 residues in ERRγ. C. Three hydrogen bonds (shown as pink dots) are formed between **DY181 (3h)** and D248/E250/Y321 residues in homology model of ERRβ protein.

3. Conclusions

In conclusion, we have developed rational strategies that allowed us to successfully identify a series of novel analogs structurally related to ***Z*-4-OHT** to modulate the activity of estrogen-related receptors (ERRγ and ERRβ), which are constitutively active. All of the resulting compounds were primarily evaluated by using a well established cell-based luciferase reporter gene assay to determine their effects in a cellular system. It is preferred that the mechanism is inverse agonism and occurs by the novel compound binding directly to the ligand binding domain of the receptors. Among the identified inverse agonists of ERRγ and ERRβ, compound **4a** (**DY40**) is the most potent compound described to date which potently suppressed the transcriptional functions of ERRγ with IC_{50} = 0.01 μM in a cell-based reporter gene assay and antagonizes ERRγ with a potency approximately 60 times greater than ***Z*-4-OHT**. Compound **3h** (**DY181**) has also been identified as a selective inverse agonist for the ERRβ with excellent selectivity and potency. Our computational modes show that **DY181 (3h)** can form a strong hydrophobic interaction with ERRβ which is predicted to bind more strongly than ERRγ. These studies provided valuable information and opportunity to expand the series in search of more potent, selective, and druglike molecules. Compounds **DY40 (4a)** and **DY181 (3h)** may represent unique chemical tools in the elucidation of ERRγ and ERRβ functions and could be new agents for the treatment of metabolic disorders and related cancer. Studies aimed at further profiling these compounds *in vitro* and expanding the SAR of these analogs with the goal of developing ERRβ and ERRγ modulators that can be delivered orally and are active *in vivo* are under way.

4. Experimental section

4.1. Chemistry

General procedures: Organic reagents were purchased from commercial suppliers unless otherwise noted and were used without further purification. All solvents were analytical or reagent grade. All reactions were carried out in flame-dried glassware under argon or nitrogen. Melting points were determined and reported automatically by an optoelectronic sensor in open capillary tubes and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were measured at 600 MHz and 125 MHz respectively, and using $CDCl_3$ or CD_3OD as the solvents and tetramethylsilane (Me_4Si) as the internal standard. Flash column chromatography was performed using Sigma-Aldrich silica gel 60A (200-400 mesh), carried out under moderate pressure by using columns of an appropriate size packed and eluted with appropriate eluents. Silica gel chromatography was performed on a Biotage flash column gradient pump system using 15 cm long columns. All reactions were monitored by TLC on precoated plates (silica gel HLF). TLC spots were visualized either by exposure to iodine vapors or by irradiation with UV light. Organic solvents were removed in vacuum by rotary evaporator. Elemental analyses were performed by Columbia Analytical Services, Inc. Tucson, Arizona.

4.1.1. Method A. McMurry Reductive Coupling Reaction

General procedure

To a stirred suspension of zinc powder (31 mmol) in dry THF (30 mL) was added $TiCl_4$ (14 mmol), under Ar, at -10 ^{o}C. When the addition was complete, the mixture was warmed to room temperature and then refluxed. After titanium reagent was refluxed for

2.5h, the mixture was cooled to 0 degree C and then a solution of 4,4'-hydroxybenzophenone (2.3 mmol) and substituted-phenone (6.7 mmol) in dry THF (40 mL) at 0 ^{o}C was added to the mixture. Upon completion of addition of solution, the mixture was refluxed in the dark for 2.5 h, cooled to room temperature (25 ^{o}C); and quenched with 10% aqueous potassium carbonate (50 mL) and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo.

Representative compounds:

1,1-Bis (4-hydroxyphenyl)-2-phenylbut-3-methyl-1-ene (**1a**). To a stirred suspension of zinc powder (2.0 g, 31 mmol) in dry THF (30 mL) was added $TiCl_4$ (1.5 mL, 14 mmol), under Ar, at –10 ^{o}C. When the addition was complete, the mixture was warmed to room temperature and then refluxed. After titanium reagent was refluxed for 2.5h, the mixture was cooled to 0 degree C and then a solution of 4,4'-hydroxybenzophenone (0.5 g, 2.3 mmol) and isobutyrophenone (1.0 g, 6.7 mmol) in dry THF (40 mL) at 0 ^{o}C was added to the mixture. Upon completion of addition of solution, the mixture was refluxed in the dark for 2.5 h, cooled to room temperature; and quenched with 10% aqueous potassium carbonate (50 mL) and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. Flash column chromatography (8:2 hexanes/EtOAc) afforded **1a** (0.68 g, 89%) as a white solid: mp 137.7 ^{o}C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.27-7.18 (m, 5H), 7.12 (d, 2H), 7.07 (d, 2H), 6.81 (d, 2H), 6.45 (d, 2H), 4.70 (s, 1H), 4.41 (s, 1H), 3.05 (m, 1H), 0.96 (d, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.0, 155.9, 146.0, 141.5, 140.2, 136.1, 136.0, 132.3, 132.1, 131.4, 128.1, 127.0, 115.9, 114.8, 32.8, 22.2. Anal. Calcd for $C_{23}H_{22}O_2$: C, 83.60; H, 6.71. Found: C, 83.78; H, 6.66.

1,1-Bis (4-hydroxyphenyl)-2-phenylpent-1-ene (**1b**). Compound **1b** was synthesized using Method A with 4,4'-hydroxybenzophenone and propiophenone. The crude product was purified by flash column chromatography (8:2 hexanes/EtOAc) afforded **1b** (1.35 g, 70%) as a white solid: mp 192.7 ^{o}C. Crystallization from warm EtOAc gave white crystals (**1b**): mp 195.0 ^{o}C (lit. mp142-145^{o}C).[43] ^{1}H NMR (600 MHz, CD_3OD) δ 7.06-7.03 (m, 5H), 6.99 (d, 2H), 6.75 (d, 2H), 6.49 (d, 2H), 6.37 (d, 2H), 2.39 (q, 2H), 1.33 (m, 2H), 0.80 (t, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.1, 157.2, 144.5, 140.3, 136.5, 136.2, 136.1, 131.6, 130.8, 130.5, 128.8, 126.8, 115.6, 115.5, 39.0, 23.1, 14.5. Anal. Calcd for $C_{23}H_{22}O_2$: C, 83.60; H, 6.71. Found: C, 83.95; H, 6.94.

1,1-Bis (4-hydroxyphenyl)-2-phenylhex-1-ene (**1c**). This compound was prepared using method A. The crude product was purified by flash column chromatography (1:1 Et_2O/hexanes) afforded **1c** (5.02 g, 79%) as a white solid: mp 170.6 ^{o}C. Crystallization from warm EtOAc gave white crystals (**1c**): mp 171.4 ^{o}C (lit. mp 158 ^{o}C).[43] ^{1}H NMR (600 MHz, CD_3OD) δ 7.13-7.06 (m, 5H), 7.01 (d, 2H), 6.75 (d, 2H), 6.64 (d, 2H), 6.39 (d, 2H), 2.42 (t, 2H), 1.22 (m, 4H), 0.76 (t, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.9, 156.2, 144.6, 140.4, 140.2, 136.5, 136.2, 133.0, 132.2, 131.6, 131.3, 128.8, 126.8, 115.7, 115.5, 114.5, 36.6, 32.3, 23.8, 14.4. Anal. Calcd for $C_{24}H_{24}O_2$: C, 83.69; H, 7.02. Found: C, 83.42; H, 7.15.

1,1-Bis (4-hydroxyphenyl)-2-phenyl-4-chlorobut-1-ene (**1d**). This compound was prepared using method A. The crude product was purified by flash column chromatography (8:2 hexanes/EtOAc) afforded **1d** (1.35 g, 70%) as a white solid: mp 184.5 ^{o}C. Crystallization from warm EtOAc gave white crystals (**1d**): mp 187.8 ^{o}C ; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.18-7.11 (m, 7H), 6.85 (d, 2H), 6.75 (d, 2H), 6.50 (d, 2H), 4.77 (s, 1H), 4.54 (s, 1H), 3.42 (t, 2H), 2.97 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.5, 156.6, 143.3, 143.0, 135.6, 135.5, 132.9, 131.6, 130.8, 129.1, 127.3, 115.9, 115.1,

114.6, 43.6, 39.8. Anal. Calcd for $C_{22}H_{19}ClO_2$: C, 75.32; H, 5.46, Cl, 10.11. Found: C, 75.77; H, 5.65, Cl, 10.28.

1,1-Bis (4-hydroxyphenyl)-2-phenyl-5-chloropent-1-ene (**1e**). This compound was prepared using method A. The crude product was purified by flash column chromatography (8:2 hexanes/EtOAc) afforded **1e** (2.66 g, 72%) as a white solid: mp 165.2 ^{o}C. Crystallization from warm EtOAc gave white crystals (**1e**): mp 169.3 ^{o}C. ^{1}H NMR (600 MHz, CD_3OD) δ 7.17-7.08 (m, 5H), 7.02 (d, 2H), 6.77 (d, 2H), 6.66 (d, 2H), 6.40 (d, 2H), 3.40 (t, 2H), 2.60 (m, 2H), 1.77 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 153.3, 152.1, 139.7, 137.1, 134.3, 131.8, 131.5, 128.6, 127.2, 126.4, 124.6, 122.7, 111.5, 110.7, 41.3, 30.1, 28.9. Anal. Calcd for $C_{23}H_{21}ClO_2$: C, 75.71; H, 5.58; Cl, 9.72. Found: C, 75.37; H, 5.77; Cl, 9.61.

1,1-Bis (4-hydroxyphenyl)-2-phenyl-4-methylpent-1-ene (**1f**). This compound was prepared using method A. The crude product was purified by flash column chromatography (8:2 hexanes/EtOAc) afforded **1f** (0.45 g, 85%) as a white solid. Crystallization from warm EtOAc gave white crystals: mp 172.8 ^{o}C; ^{1}H NMR (600 MHz, CD_3OD) δ 7.13-7.01 (m, 7H), 6.75 (d, 2H), 6.66 (d, 2H), 6.38 (d, 2H), 2.34 (d, 2H), 1.47 (m, 1H), 0.78 (d, 6H); ^{13}C NMR (125 MHz, CD_3OD) δ 156.9, 156.2, 144.3, 141.2, 139.8, 136.5, 136.2, 132.7, 131.7, 130.8, 128.7, 126.7, 115.7, 115.1, 44.9, , 27.7, 23.0. Anal. Calcd for $C_{24}H_{24}O_2$: C, 83.69; H, 7.02. Found: C, 83.78; H, 6.66.

5,5-Bis (4-hydroxyphenyl)-2-methyl-4-phenyl-pent-4-enoic acid methyl ester (**1h**). This compound was prepared using method A. The crude product was purified by flash column chromatography (1:1 hexanes/EtOAc) afforded **1h** (1.45 g, 78%) as a white solid: mp 197.2 ^{o}C. ^{1}H NMR (600 MHz, CD_3OD) δ 7.15 (t, 2H), 7.09 (d, 3H), 7.02 (d, 2H),

6.77 (d, 2H), 6.66 (d, 2H), 6.39 (d, 2H), 2.43 (s, 3H), 2.83 (m, 1H), 2.58 (m, 1H), 2.38 (m, 1H), 1.02 (d, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 178.6, 157.3, 156.5, 143.2, 142.6, 137.5, 136.0, 135.8, 133.6, 132.8, 131.6, 128.7, 127.2, 116.1, 115.1, 52.0, 40.1, 39.8, 17.4. Anal. Calcd for $C_{25}H_{24}O_4 \cdot ¼ H_2O$: C, 76.49; H, 6.16. Found: C, 76.41; H, 6.30.

5,5-Bis (4-hydroxyphenyl)-4-phenyl-pent-4-enoic acid methyl ester (**1g**). This compound was prepared using method A. The crude product was purified by flash column chromatography (1:1 hexanes/EtOAc) afforded **1g** (3.35 g, 89%) as a white solid: mp 191.0 °C. ^{1}H NMR (600 MHz, CD_3OD) δ 7.17 (t, 2H), 7.11 (d, 3H), 7.03 (d, 2H), 6.79 (d, 2H), 6.67 (d, 2H), 6.41 (d, 2H), 3.53 (s, 3H), 2.78 (t, 2H), 2.29 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 175.4, 157.5, 156.6, 143.4, 141.9, 137.9, 136.0, 135.7, 133.0, 131.5, 130.9, 129.0, 127.3, 116.0, 115.1, 52.0, 34.3, 32.3. Anal. Calcd for $C_{24}H_{22}O_4$: C, 76.99; H, 5.92. Found: C, 76.80; H, 6.06.

5,5-Bis-(4-hydroxyphenyl)-pent-4-enoic-acid (**1i**). This compound was prepared using method A. The crude product was purified by flash column chromatography (9:1 CH_2Cl_2/MeOH) afforded **1j** (1.82 g, 76%) as a white solid: mp 215.2 °C. ^{1}H NMR (600 MHz, CD_3OD) δ 7.17 (t, 3H), 7.13 (d, 2H), 7.05 (d, 2H), 6.79 (d, 2H), 6.68 (d, 2H), 6.42 (d, 2H), 2.77 (t, 2H), 2.25 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 175.6, 156.0, 155.1, 142.1, 140.2, 136.6, 134.7, 134.4, 131.6, 130.1, 129.5, 127.6, 125.8, 114.6, 113.7, 32.9, 31.0. Anal. Calcd for $C_{23}H_{20}O_4 \cdot 2H_2O$: C, 69.70; H, 5.10. Found: C, 70.00; H, 5.20.

5,5-Bis-(4-hydroxyphenyl)-pent-4-ene-1-ol (**1k**). A solution of BH_3-THF (3.0 mL, 1.0 M solution in THF, 5 mmol) was added dropwise to a solution of 5,5-Bis-(4-hydroxyphenyl)-pent-4-enoic-acid (**1i**) (0.4 g, 1 mmol) in dry THF (10 mL) at 0 °C. When the addition was complete, the mixture was warmed to room temperature and then

stirred for 3.5 h. The mixture was cooling to 0 ^{o}C and quenched with 1 N NaOH (2 mL). The mixture was then diluted with saturated NH_4Cl (10 mL) and EtOAc (20 ml). Aqueous layer was extracted with EtOAc (2 x 20 mL). The combined organic extracts were washed with water, brine, and dried over Na_2SO_4, and concentrated in vacuo. Solidification by EtOAc afforded **1k** (0.26 g, 75%) as a white solid: mp 215.8 ^{o}C; ^{1}H NMR (600 MHz, CD_3OD) δ 7.13-7.09 (m, 5H), 7.02 (d, 2H), 6.76 (d, 2H), 6.65 (d, 2H), 6.40 (d, 2H), 3.39 (t, 2H), 2.49 (m, 2H), 1.54 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 155.8, 154.9, 142.9, 139.2, 138.3, 135.0, 134.7, 131.6, 130.2, 129.4, 127.5, 125.5, 114.4, 113.6, 61.7, 31.9, 31.7. Anal. Calcd for $C_{23}H_{22}O_3 \cdot$ ½ H_2O: C, 77.71; H, 6.24. Found: C, 77.21; H, 6.26.

5,5-Bis (4-hydroxy-phenyl)-2-methyl-4-phenyl-pent-4-enoic acid (**1j**). This compound was prepared using method A. The crude product was purified by flash column chromatography (1:1 H/EtOAc) afforded **1j** (1.22 g, 63%) as a white solid: mp 245.9 ^{o}C. ^{1}H NMR (600 MHz, CD_3OD) δ 7.15 (t, 5H), 6.79 (d, 2H), 6.66 (d, 2H), 6.51 (d, 2H), 6.40 (d, 2H), 2.87 (m, 1H), 2.58 (m, 1H), 2.30 (t, 1H), 1.04 (d, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 178.9, 155.7, 155.0, 141.8, 141.1, 136.2, 134.6, 131.3, 130.3, 129.6, 127.5, 125.8, 114.5, 113.7, , 38.4, 37.6, 15.8. Anal. Calcd for $C_{24}H_{22}O_4 \cdot$ ¾ H_2O: C, 74.29; H, 5.72. Found: C, 74.09; H, 5.68.

5,5-Bis-(4-hydroxyphenyl)-2-methyl-pent-4-ene-1-ol (**1n**). A solution of BH_3-THF (3.0 mL, 1.0 M solution in THF, 3 mmol) was added dropwise to a solution of 5,5-Bis (4-hydroxy-phenyl)-2-methyl-4-phenyl-pent-4-enoic acid (**1j**) (0.25 g, 0.67 mmol) in dry THF (5 mL) at 0 ^{o}C. When the addition was complete, the mixture was warmed to room temperature and then stirred for 3.5 h. The mixture was cooling to 0 ^{o}C and quenched

with 1 N NaOH (1 mL). The mixture was then diluted with saturated NH_4Cl (10 mL) and EtOAc (20 ml). Aqueous layer was extracted with EtOAc (2 x 20 mL). The combined organic extracts were washed with water, brine, and dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by flash column chromatography (1:1 H/EtOAc) afforded **1n** (0.16 g, 67%) as a white solid: mp 201.0 ^oC; ^1H NMR (600 MHz, CD_3OD) δ 7.17-7.09 (m, 5H), 7.04 (d, 2H), 6.78 (d, 2H), 6.68 (d, 2H), 6.40 (d, 2H), 3.37 (t, 1H), 3.15 (t, 1H), 2.54 (m, 1H), 2.31 (t, 1H), 1.56 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 155.6, 154.8, 142.7, 140.2, 137.6, 135.0, 134.8, 131.3, 130.3, 129.5, 127.4, 125.5, 114.4, 113.6, 66.9, 38.3, 33.8, 15.4. Anal. Calcd for $C_{24}H_{24}O_3 \cdot H_2O$: C, 76.17; H, 6.39. Found: C, 76.04; H, 6.41.

4.1.3. Method B. **Monoalkylated with 2-(dimethylamino) ethyl chloride hydrochloride**

General procedure

A solution of (**1a-1n**) (1.4 mmol) in DMF (5 mL) was treated with Cs_2CO_3 (3.3 mmol) and heated in an oil bath at 70-80 ^oC. The resulting suspension was treated with 2-(dimethylamino)ethyl chloride hydrochloride (5 mmol) in a small portion over a 15 min. period and stirred for 2.5 h. After cooling at rt., the reaction mixture was poured into saturated ammonium chloride (10 mL) and extracted with ethyl acetate (3 x 15mL). The combined organic phase was washed with brine (3 x 15mL), dried, and concentrated.

Representative compounds:

(*Z*)-4-[1-(4-Dimethylaminoethoxy-phenyl)-3-methyl-2-phenyl-but-1-enyl]-phenol (3a). This compound was prepared using method B. A solution of 1,1-Bis (4-

hydroxyphenyl)-2-phenylbut-3-methyl-1-ene (**1a**) (0.45g, 1.4 mmol) in DMF (5 mL) was treated with Cs_2CO_3 (1.06 g, 3.3 mmol) and heated in an oil bath at 70-80 ^oC. The resulting suspension was treated with 2-(dimethylamino)ethyl chloride hydrochloride (0.75 g, 5 mmol) in a small portion over a 15 min. period and stirred for 2.5 h. After cooling at rt., the reaction mixture was poured into saturated ammonium chloride (10 mL) and extracted with ethyl acetate (3 x 15mL). The combined organic phase was washed with brine (3 x 15mL), dried, and concentrated. Flash chromatography (9:1 CH_2Cl_2/MeOH) afforded desired product (**2a**) (0.35 g, 64%) as a 1:1 mixture of *E*/*Z* isomers (beige solid). Crystallization of the solid with a 1:1 *E*/*Z* ratio (15 mg) from methanol gave white crystals (**3a**) (6.1 mg): mp 168.8 ^oC. ^1H NMR (600 MHz, CD_3OD) δ 7.17-7.05 (m, 7H), 6.80 (d, 2H), 6.74 (d, 2H), 6.38 (d, 2H), 3.88 (t, 2H), 2.68 (t, 2H), 3.02 (m, 1H), 2.31 (s, 6H), 0.94 (d, 6H). ^{13}C NMR (125 MHz, CD_3OD) δ 157.8, 157.1, 141.3, 140.0, 137.5, 135.8, 132.3, 132.1, 131.4, 128.2, 126.8, 116.0, 114.2, 66.3, 59.1, 45.8, 32.8, 22.2. Anal. Calcd for $C_{27}H_{31}NO_2$: C, 80.76; H, 7.78; N, 3.49. Found: C, 80.91; H, 7.81; N, 3.42.

(*Z*)-4-{1-[4-(2-Dimethylamino-ethoxy)-phenyl]-4-methyl-2-phenyl-pent-1-enyl}-phenol (3b). This compound was prepared using method B. The crude product was purified by flash column chromatography (9:1 CH_2Cl_2/MeOH) afforded 0.2 g (**2b**) (51%) as a 1:1 mixture of *E*/*Z* isomers as white solid. Crystallization of the solid with a 1:1 *E*/*Z* ratio from methanol gave white crystals (**3b**): mp 158.2 ^oC. ^1H NMR (600 MHz, CD_3OD) δ 7.16 (m, 5H), 7.04 (m, 2H), 6.79 (d, 2H), 6.53 (d, 2H), 6.31 (d, 2H), 3.90 (t, 2H), 2.72 (t, 2H), 2.36 (m, 2H), 2.34 (s, 6H), 1.50 (m, 1H), 0.80 (d, 6H); ^{13}C NMR (125 MHz**,** CD_3OD) δ 158.5, 157.7, 144.3, 140.6, 137.5, 136.0, 135.8, 133.6, 132.8, 131.6,

128.7, 127.2, 116.1, 109.6, 65.8, 46.8, 45.3, 45.2, 27.9, 23.9. Anal. Calcd for $C_{28}H_{33}NO_2$: C, 80.93; H, 8.00; N, 3.37. Found: C, 80.92; H, 7.93, N, 3.29.

(*Z*)-5-[4-(2-Dimethylamino-ethoxy)-phenyl]-5-(4-hydroxyphenyl)-2-methyl-4-phenyl-pent-4-enoic acid methyl ester (3h, DY181). This compound was prepared using method B. The crude product was purified by flash column chromatography (9:1 CH_2Cl_2/MeOH) afforded 0.2 g (51%) as a 1:1 mixture of *E*/*Z* isomers as semisolid (**2h**). Crystallization of the semisolid with a 1:1 *E*/*Z* ratio from methanol gave white crystals (**3h**): mp132.3 ^oC. ^1H NMR (600 MHz, CD_3OD) δ 7.16 (m, 5H), 7.04 (d, 2H), 6.78 (dd, 4H), 6.67 (d, 2H), 3.95 (t, 2H), 3.45 (s, 3H), 2.84 (m, 1H), 2.68 (t, 2H), 2.56 (m, 1H), 2.38 (m, 1H), 2.29 (s, 6H), 1.04 (d, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 178.6, 157.3, 156.5, 143.2, 142.6, 137.5, 136.0, 135.8, 133.6, 132.8, 131.6, 128.7, 127.2, 116.1, 1151, 52.0, 40.1, 39.8, 17.4. Anal. Calcd for $C_{29}H_{33}NO_4 \cdot ¼ H_2O$: C, 75.05; H, 7.17; N, 3.01. Found: C, 74.99; H, 7.39; N, 3.01.

(*Z*)-5-[4-(2-Dimethylamino-ethoxy)-phenyl]-5-(4-hydroxyphenyl)-4-phenyl-pent-4-enoic acid methyl ester (3g). This compound was prepared using method B. The crude product was purified by flash column chromatography (9:1 CH_2Cl_2/MeOH) afforded 0.25 g (**2g**) (43%) as a 1:1 mixture of *E*/*Z* isomers as a yellow solid. Crystallization of the solid with a 1:1 *Z*/*E* ratio from methanol gave a white solid as a geometric pure isomer (**3g**): mp 163.1 ^oC. ^1H NMR (600 MHz, CD_3OD) δ 7.16 (d, 2H), 7.11 (t, 3H), 7.04 (d, 2H), 6.78 (dd, 4H), 6.58 (d, 2H), 3.96 (t, 2H), 3.53 (s, 3H), 2.79 (t, 2H), 2.68 (t, 2H), 2.30 (t, 2H), 2.27 (s, 6H). ^{13}C NMR (125 MHz, CD_3OD) δ 175.3, 158.3, 157.6, 143.3, 141.6, 138.4, 137.1, 135.8, 132.9, 131.5, 130.9, 129.1, 127.4, 116.1, 114.4, 66.4, 59.1, 52.0,

45.8, 34.3, 32.3. Anal. Calcd for $C_{28}H_{31}NO_4 \cdot 2H_2O$: C, 70.22; H, 6.48; N, 2.89. Found: C, 70.53; H, 6.41; N, 2.89.

(*Z*)-5-[4-(2-Dimethylamino-ethoxy)-phenyl]-5-(4-hydroxyphenyl)-4-phenyl-pent-4-ene-1-ol (3k) (GSK5182). This compound was prepared using method B. A solution of 5,5-Bis-(4-hydroxyphenyl)-pent-4-ene-1-ol (**1k**) (0.5 g, 1.4 mmol) in DMF (5 mL) was treated with Cs_2CO_3 (1.37 g, 4.2 mmol) and heated in an oil bath at 70-80 ^oC. The resulting suspension was treated with 2-(dimethylamino)ethyl chloride hydrochloride (0.43 g, 3 mmol) in a small portion over a 15 min. period and stirred for 3.5 h. After cooling at rt., the reaction mixture was poured into saturated ammonium chloride (10 mL), and extracted with ethyl acetate (3 x 15mL). The combined organic phase was washed with brine (3 x 15 mL), dried, and concentrated. Flash chromatography (9:1 CH_2Cl_2/MeOH) afforded desired product (**2k**) (0.29 g, 50%) as a 1:1 mixture of *E*/*Z* isomers (yellow oil). Crystallization of the oil from methanol gave white crystals (**3k**), mp 168.8 ^oC. ^1H NMR (600 MHz, CD_3OD) δ 7.14-7.07 (m, 5H), 7.03 (d, 2H), 6.78 (dd, 4H), 6.58 (d, 2H), 3.96 (t, 2H), 3.42 (t, 2H), 2.73 (t, 2H), 2.52 (t, 2H), 2.33 (s, 6H), 1.54 (t, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 158.1, 157.3, 144.2, 140.4, 137.6, 136.2, 133.0, 131.7, 130.9, 129.0, 127.1, 115.9, 114.4, 66.1, 63.1, 59.0, 45.7, 33.4, 33.1. Anal. Calcd for $C_{27}H_{31}NO_3$: C, 77.67; H, 7.48; N, 3.35. Found: C, 77.57; H, 7.50; N, 3.49.

(*Z*)-4-{1-[4-(2-Dimethylamino-ethoxy)-phenyl]-5-hydroxy-4-methyl-2-phenyl-pent-1-enyl}-phenol (3n). This compound was prepared using method B. The crude product was purified by flash column chromatography (8:2 CH_2Cl_2/MeOH) afforded 0.57 g (41%) as a 1:1 mixture of *E*/*Z* isomers as yellow oil (**2n**). Flash chromatography (9:1 CH_2Cl_2/MeOH) afforded desired product as a *Z*-isomers (yellow solid). Crystallization

of the solid from methanol gave white crystals (**3n**): mp153.5 ^{o}C. ^{1}H NMR (600 MHz, CD_3OD) δ 7.11 (m, 5H), 7.06 (d, 2H), 6.79 (dd, 4H), 6.57 (d, 2H), 3.95 (t, 2H), 3.37 (m, 2H), 3.17 (m, 1H), 2.72 (t, 2H), 2.53 (m, 1H), 2.35 (s, 6H), 1.56 (m, 1H), 0.85 (d, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 156.6, 155.7, 142.5, 140.0, 138.1, 136.2, 134.7, 131.3, 130.3, 129.5, 127.4, 125.6, 114.5, 112.9, 67.1, 64.9, 57.6, 44.3, 38.3, 34.1, 15.8. Anal. Calcd for $C_{28}H_{33}NO_3 \cdot ½H_2O$: C, 76.32; H, 7.55; N, 3.18. Found: C, 76.56; H, 7.84; N, 3.23.

4.1.4. Method C. Acetalization

General procedure

An solution of **3a-3n** (1.2 mmol) and acetyl chloride (5 mmol) of in pyridine (5 mL) were refluxed for 1 hr. Then, ice water was added, the aqueous layer was extracted with ether, and the organic extracts were washed with saturated $NaHCO_3$ solution. The combined organic phase was washed with brine (3 x 20 mL), dried, and concentrated.

Representative compounds:

(*E*)- 4-{1-[4-(2-dimethylamino-ethoxy)-phenyl]-3-methyl-2-phenyl-but-1-enyl}-phenyl acetate (4a, DY40). This compound was prepared using method C. (0.5 g 1.2 mmol) of (*Z*)-4-[1-(4-Dimethylaminomethoxy-phenyl)-3-methyl-2-phenyl-but-1-enyl]-phenol (**3a**) and 0.39 g (5 mmol) of acetyl chloride in pyridine (5 mL) were refluxed for 1 hr. Then, ice water was added, the aqueous layer was extracted with ether, and the organic extracts were washed with saturated $NaHCO_3$ solution. The combined organic phase was washed with brine (3 x 20 mL), dried, and concentrated. Flash chromatography (dichloromethane-methanol 9:1) afforded colorless oil as an oil (0.47 g,

88% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.23 (d, 2H), 7.22 (d, 2H), 7.10-6.99 (m, 5H), 6.77 (d, 2H), 6.50 (d, 2H), 3.88 (t, 2H), 2.96 (m, 1H), 2.67 (t, 2H), 2.31 (s, 6H), 2.23 (s, 3H), 0.89 (d, 6H).). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 157.9, 150.6, 147.4, 142.1, 138.9, 136.5, 132.2, 131.9, 131.8, 128.6, 128.1, 127.3, 126.8, 122.6, 115.6, 114.6, 114.5, 67.2, 59.6, 47.2, 32.3, 22.1. Anal. Calcd for $C_{30}H_{37}NO_3$: C, 78.40; H, 8.11; N, 3.05. Found: C, 78.50; H, 7.58; N, 3.09.

(*E*)-Acetic acid 5-[4-(2-dimethylamino-ethoxy)-phenyl]-5-(4-hydroxy-phenyl)-4-phenyl-pent-4-ene-1-ol (4k). This compound was prepared using method C. 0.05g (0.1 mmol) of (*Z*)-5-[4-(2-Dimethylamino-ethoxy)-phenyl]-5-(4-hydroxyphenyl)-4-phenyl-pent-4-ene-1-ol (**3k**) was dissolved in dry acetone (5 mL) and potassium carbonate (0.08 g, 0.6 mmol) was added. Acetyl chloride (0.05 g, 0.6 mmol) was added at 0 ^{o}C and the reaction mixture was stirred at 0 ^{o}C for 2 hours. Then, ice water was added, the aqueous layer was extracted with EtOAc (3 x 15 ml). The combined organic phase was washed with water, brine, dried over Na_2SO_4 and concentrated. Flash chromatography (dichloromethane-methanol 9:1) afforded colorless oil as a desired compound (0.037 g, 82% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.27 (d, 2H), 7.16 (t, 5H), 7.11 (d, 2H), 6.81 (d, 2H), 6.60 (d, 2H), 3.94 (t, 2H), 3.42 (t, 2H), 2.69 (t, 2H), 2.54 (t, 2H), 2.30 (s, 6H), 2.27 (s, 3H), 1.58 (t, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ169.8, 156.9, 149.6, 142.3, 141.1, 139.9, 138.2, 135.4, 131.6, 130.2, 129.4, 127.7, 125.9, 121.1, 113.2, 65.0, 61.5, 57.6, 44.4, 32.0, 31.6, 19.6. Anal. Calcd for $C_{29}H_{33}NO_4 \cdot ½\ H_2O$: C, 74.17; H, 7.09; N, 2.98. Found: C, 74.19; H, 7.13; N, 2.91.

(*E*)-Acetic acid 5-[4-(2-dimethylamino-ethoxy)-phenyl]-5-(4-hydroxy-phenyl)-2-methyl-4-phenyl-pent-4-ene-1-ol (4n). This compound was prepared using method C**.**

0.05g (0.1 mmol) of (*Z*)-4-{1-[4-(2-Dimethylamino-ethoxy)-phenyl]-5-hydroxy-4-methyl-2-phenyl-pent-1-enyl}-phenol (**3n**) was dissolved in dry acetone (5 mL) and potassium carbonate (0.08 g, 0.6 mmol) was added. Acetyl chloride (0.05 g, 0.6 mmol) was added at 0^{o}C and the reaction mixture was stirred at 0 ^{o}C for 2 hours. Then, ice water was added and the aqueous layer was extracted with EtOAc (3 x 15 ml). The combined organic phase was washed with water, brine, dried over Na_2SO_4 and concentrated. Flash chromatography (dichloromethane-methanol 9:1) afforded colorless semi-solid as a desired compound (0.037 g, 79% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.22 (d, 2H), 7.12 (m, 5H), 7.06 (d, 2H), 6.77 (d, 2H), 6.55 (d, 2H), 3.91 (t, 2H), 3.30 (t, 1H), 3.15 (m, 1H), 2.65 (t, 2H), 2.52 (m, 1H), 2.27 (s, 6H), 2.24 (m, 1H), 2.21 (s, 3H), 1.51 (t, 1H), 0.82 (d, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ168.3, 155.2, 147.8, 140.3, 139.5, 137.5, 137.4, 133.8, 129.7, 128.6, 127.8, 125.9, 124.3, 119.5, 111.5, 65.4, 63.2, 55.9, 51.8, 45.7, 36.6, 32.3, 17.9, 14.1. Anal. Calcd for $C_{30}H_{35}NO_4$: C, 76.08; H, 7.45; N, 2.96. Found: C, 76.23; H, 7.61; N, 2.91.

4.1.5. Method D. General procedure for the combinatorial solution-phase parallel preparation of small chemical library in Chemset **4**

(*Z*)-4-[1-(4-Dimethylaminoethoxy-phenyl)-3-methyl-2-phenyl-but-1-enyl]-phenol (**3a**) (4.02 g, 10 mmol) was dissolved in dry DMF (100 mL) to make a 10 mM stock solution. The stock solution was transferred automatically in the Neptune work station of Bohdan from source reactant vials to the tubes of the 48-tube reaction block (1.0 ml/tube, 0.1 mmol/tube). Powder K_2CO_3 (6 equiv) was added to each tube. The reaction block was shaken for 30 min. A workstation of Mettler-Toledo system has been used to tare each

reactant vial in an array of vials. Forty-eight different acyl chlorides and alkyl halides reagents (6 equiv, 0.6 mmol) were transferred automatically from reagents vials to each reactor block tube. The reaction mixture was vigorously shaken at warm condition (50 ^{o}C) for 12 h in a RAM organic synthesizer. The work-up procedure was carried out in a workstation of Mettler-Toledo system. 1 mL of H_2O was transferred automatically to each reactor block tube. The resultant mixture was extracted which was performed by transferring 1 mL of EtOAc to each reactor block tube. The mixture was stirred at ambient temperature for 1 h. The extraction was performed three times (3 x 1 mL). The combined organic layers (extracts) were transferred to 48 vials which were dried and concentrated in Savant concentrator to get crude yellow oils in total 48 substituted derivatives. Each of crude library member was dissolved in 0.2 mL of CH_2Cl_2 following purification by using the Biotage Quad 3 automated medium pressure flash chromatography (dichloromethane-methanol 9:1). The average yields of the targeted compounds in > 85% and purity in > 82%. The resulting fractions were analyzed by flow injection NMR to confirm the chemical structures by ^{1}H and ^{13}C NMR.

Representative compounds:

(*E*)- 4-{1-[4-(2-dimethylamino-ethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenyl acetate (4a {R2(1)}, **DY40).** This compound was prepared using method D. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.23 (d, 2H), 7.22 (d, 2H), 7.10-6.99 (m, 5H), 6.77 (d, 2H), 6.50 (d, 2H), 3.88 (t, 2H), 2.96 (m, 1H), 2.67 (t, 2H), 2.31 (s, 6H), 2.23 (s, 3H), 0.89 (d, 6H).). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 157.9, 150.6, 147.4, 142.1, 138.9, 136.5, 132.2, 131.9, 131.8, 128.6, 128.1, 127.3, 126.8, 122.6, 115.6, 114.6, 114.5, 67.2, 59.6, 47.2, 32.3, 22.1.

(*E*)- 4-{1-[4-(2-dimethylamino-ethoxy)-phenyl]-3-methyl-2-phenyl-but-1-enyl}-phenyl acetate (4b, {R2(2)}**).** This compound was prepared using method D. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.23 (d, 2H), 7.22 (d, 2H), 7.10-6.99 (m, 5H), 6.77 (d, 2H), 6.50 (d, 2H), 3.88 (t, 2H), 2.96 (m, 1H), 2.67 (t, 2H), 2.60 (q, 2H), 2.33 (s, 6H), 1.27 (t, 3H), 0.89 (d, 6H).). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 157.9, 150.6, 147.4, 142.1, 138.9, 136.5, 132.2, 131.9, 131.8, 128.6, 128.1, 127.3, 126.8, 122.6, 115.6, 114.6, 114.5, 67.2, 59.6, 47.2, 32.3, 22.1. Anal. Calcd for $C_{29}H_{33}NO_3$: C, 78.52; H, 7.50; N, 3.16. Anal. Calcd for $C_{29}H_{33}NO_3$: C, 78.52; H, 7.50; N, 3.16. Found: C, 78.50; H, 7.58; N, 3.09.

(*E*)-4-{1-[4-(2-Dimethylamino-ethoxy)-phenyl]-2-phenyl-pent-1-enyl}-pentyl)-phenyl acetate (**4c**{R2(1)})

The compound **4c** was prepared from **3c** by using method C in 80% yield. It was obtained as colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.21-7.05 (m, 7H), 6.89 (d, 2H), 6.75 (d, 2H), 6.55 (d, 1H), 6.46 (d, 1H), 4.09 (t, 1H), 3.93 (t, 1H), 2.74 (t, 1H), 2.64 (t, 1H), 2.44 (t, 2H), 2.35 (s, 3H), 2.28 (s, 3H), 2.19 (s, 3H), 1.24 (m, 4H), 0.81 (t, 3H).

4.2. Cell culture

CV-1 cells were grown in Dulbecco's modified Eagle's medium sup-plemented with 10% resin charcoal–stripped fetal bovine serum, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate (DMEM-FBS) at 37^{o}C in 5% CO_2. One day prior to transfection, cells were plated to 50%–80% confluence using phenol red free DMEM-FBS. The adenoviral construct, Ad-GFP and Ad-ERRα have been described.[55] Propagation of recombinant adenovirus was performed in 293 cells as described.[11]

4.3. Cell-based reporter gene assays

Cell based transactivation assays were performed in CV-1 cells as described.[49] ERR activity was assayed with a GAL4 reporter construct and fusion proteins containing the ligand binding domains of human ERRα, human ERRβ and mouse ERRγ linked to the DNA binding domain of yeast GAL4. Human ERα and ERβ were examined as full-length proteins using an estrogen receptor responsive reporter construct. Reporter constructs (300 ng/10^5 cells) and cytomegalovirus-driven expression vectors (20–50 ng/10^5 cells) were added as indicated along with CMX-β-gal (500 ng/10^5 cells) as an internal control. Cells were transiently transfected by Lipofectamine as described.[56] Cells were incubated with DNA complexed liposomes for 2 hours and subsequently treated for approximately 45 hr with phenol red free DMEM FBS containing the indicated compounds. After exposure to ligand, the cells were harvested and assayed for luciferase and β-galactosidase activity. All points were assayed in triplicate and varied by less than 15%. Each experiment was repeated three or more times with similar results. Fold activation is reported.

4.4. Quantitative real-time PCR

RNA was isolated from myocytes using Trizol (Invitrogen, Carlsbad, CA, USA). RNA (1μg) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). PCR was performed in 15μl reactions containing 1x SYBR Green reagent and 0.1μM gene-specific primers using the iQ5 Real-Time PCR system (Bio-Rad). Experimental transcript levels were normalized to 36B4 ribosomal RNA analyzed in separate reactions. The SHP primers used are as follows: mouse Shp FWD 5'-CTGCAG GTCGTCCGACTATTC-3', RV 5'-AGTGAGCCTCCTGTTGCAGG-3'; human SHP FWD 5'- TCAAGTCCATTCCGACCAGC-3'; RV 5'- AAGAAGGCCAGCGATGT CAA-3' Primers sequences for 36B4 have been previously published.[52, 57]

4.5. Fatty acid oxidation assay

The analysis of fatty acid oxidation rates in cells was performed as previously described.[57] Fatty acid ^{3}H-[9,10]-palimitic acid was complexed to fatty acid-free BSA and this complex was added to 30uM unlabeled palmitate. The radiolabeled palmitate substrate was diluted in PBS and incubated with cells for 2 hours. The substrate was removed and radiolabeled aqueous metabolites (^{3}H2O) released to the media were measured by scintillation counting. The calculated rates were normalized to total cellular protein.

4.6. Statistical analysis

All quantitative data are presented as mean ± S.E.M or mean ± S.D. Significant differences between mean values were determined by unpaired Student's t test. A p-value of ≤ 0.05 was considered significant and is denoted by asterisks (*).

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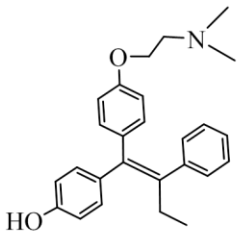
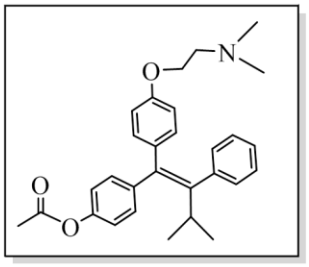
Graphical Abstract:

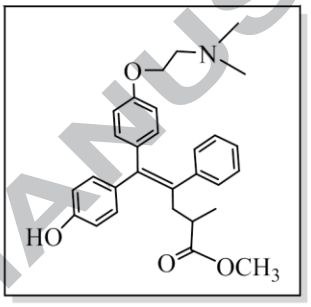


***Z*-4-OHT (ERR / inverse agonist)**
EC_{50}: 0.6 μM

SAR



DY40 (comd 4a, ERR / inverse agonist)
EC_{50}: 0.01 μM



DY181 (comd 3h, ERR inverse agonist)
EC_{50}: 0.05 μM