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Constrained Phytoestrogens and Analogues as ERβ Selective Ligands

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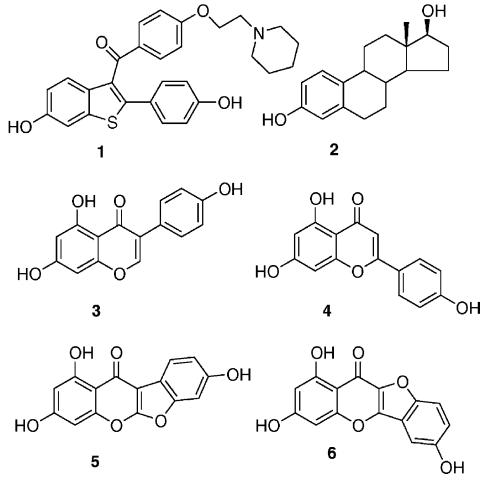
Abstract—A new series of ERbeta (ERβ) selective ligands has been prepared. One of the compounds **6**, structurally related to the phytoestrogen apigenin **4**, displays a binding preference for ERβ over ERα of over 40-fold. In addition to its binding selectivity, **6** was able to potently induce metallothionein (an ERβ specific response in human SAOS-2 cells) while demonstrating low potency in an ERα dependant ERE-tk luciferase assay in MCF-7 cells. Such receptor and cell selectivity could make **6** a useful molecular probe for better understanding the role of ERβ in mammalian physiology.

Estrogen receptor(s) (ER) can function as ligand inducible transcription factors and have been associated with a broad array of pharmacological activity.[1] For quite some time, the effects of estrogen on primary sexual growth and development have been appreciated, and as a corollary, the use of estrogen formulations in oral contraception has had tremendous impact on both individual freedom and family structure.[2] Beyond its use in targeting the sexual tissues, the use of estrogen replacement therapy for women in the peri- and postmenopausal period has been widely practiced. Starting with the recognition that replacing the lost ovarian production of estrogen counteracts vasomotor disturbances (hot flushes, night sweats), anxiety, and depression experienced during the menopause transition period; the evidence and experience generated indicated that estrogens impact a number of additional, non-traditional targets.[3] For example, long-term estrogen use prevents the bone loss observed during the menopause period that in turn is associated with a reduction in the occurrence of bone fractures. Despite these benefits, compliance with hormone replacement regimens is low, primarily due to a return of menses-type bleeding and increased risk for breast cancer.[4] More recent results from a large prospective study indicate that HRT may also negatively impact certain aspects of cardiovascular health although, at the same time, reduce the incidence of colon cancer.[5] Recently, a new class of drugs, designated SERMs (selective estrogen receptor modulators) have been introduced. These compounds, typified by raloxifene **1** (Evista® for osteoporosis), show estrogenic activity on bone and also lower LDL levels.[6] Unlike endogenous estrogen agonists like 17β-estradiol **2**, SERMs show little or no estrogenic stimulation of the uterus or breast tissue.



Unfortunately, SERMs reported on to date also suffer from drawbacks that might limit their appeal. Unlike

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17β-estradiol or related estrogen agonists, SERMs such as raloxifene do not reduce the incidence of hot flushes and in fact may actually increase their frequency.[7] Similarly, raloxifene does not help with certain urogenital complaints of postmenopausal women (i.e., diminished vaginal lubricity). Raloxifene also increases (like typical estrogen agonists) the incidences of venous thromboembolisms. So, SERMs do diminish some but not all of the concerns associated with conventional HRT, and they also do not provide some of the benefits. Thus, the search for improved estrogens with alternate profiles has continued.

In 1996, Gustaffson and coworkers, were searching for novel nuclear hormone receptors in rat prostate tissue when they discovered a second estrogen receptor which they named ERβ.[8] A slightly longer form of ERβ was soon thereafter discovered and characterized in a number of tissues.[9] Structurally, ERβ is truncated relative to ERα (530 aa vs 595 aa) and much of this is due to a shorter AF-1 domain. The ligand binding domains share only 60% homology but this is somewhat deceptive since the amino acids that surround the ligand binding cavity are very similar with only two amino acids that are different, and the changes are very conservative (336β/384α Met to Leu; 373β/421αIle to Met).[10] Thus it is not too surprising that 17β-estradiol displays a similar affinity for both receptors. There are additional isoforms of ERβ that have been isolated more recently, but the relative significance of these proteins has been less well characterized.[11]

The tissue distribution of ERβ has been well mapped in the rodent and it is not coincident with ERα.[12] Tissues such as the mouse and rat uterus express predominantly ERα, whereas the mouse and rat lung express predominantly ERβ. Even within the same organ, the distribution of ERα and ERβ can be compartmentalized. For example, in the mouse ovary, ERβ is highly expressed in the granulosa cells and ERα is restricted to the thecal and stromal cells. However, there are examples where the receptors are coexpressed and there is evidence from in vitro studies that ERα and ERβ can form heterodimers.[13] ERβ is also highly expressed in the rat prostrate as well as in many compartments of the rat and human brain.[8,12] Given the extensive but unique distribution of ERβ combined with the already well established role of hormone replacement therapy (along with an appreciation for its limitations), it is not surprising that a strong interest has been generated in discovering novel, estrogen receptor subtype selective compounds.[14]

Our own efforts directed at identifying ERβ selective compounds initially focused on ligand binding assays utilizing the ligand binding domain of both human ERα and ERβ. Our most selective phytoestrogen analogue **6** thus identified was then evaluated for metallothionein induction in SAOS-2 cell lines engineered to express ERβ.[15] We also examined this same compound in an MCF-7 ERE-tk luciferase cell line that expresses ERα only.[16] Thus we were able to determine selectivity both through the receptor as well as determine cellular functional activities (ERβ/ERα agonist/antagonist) for this compound.

Literature ERβ/ERα binding values for many ER ligands have been reported.[17] Given the structural similarities in the ligand binding domain contact residues discussed, supra, it should not be too surprising that most previously known estrogens do not show strong preference for one receptor or the other. However, certain phytoestrogens (genistein **3** and apigenin **4**) do display ERβ binding selectivity (see Table 1) and we proposed them as good starting points for further exploration. In particular, we were interested in the effect(s) of constraining the pendant phenyl to the benzopyran core (see Fig. 1) through the intermediacy of an –O or –S atom. Additionally, we scanned these templates with various phenol combinations since the phenols form critical –H bonding interactions in both ERα and ERβ.

The fused genistein analogue, lupinalbin A **5** is a known phytoestrogen that has been isolated from the white lupin bean.[18] The first synthesis of lupinalbin A was recently reported in the literature, but no determination of its estrogenic activity was disclosed.[19] We independently derived this material through the sequence demonstrated in Scheme 1. The key steps in the reaction are the selective demethylation of one of the *ortho*-methyl ethers from the pentamethoxy ethanone **8** mediated by $AlCl_3$ and the oxidative intramolecular cycloaddition mediated by DDQ. Unfortunately, we were unable to completely demethylate the cycloaddition oxidation precursor **10**. Since the compound was unstable to demethylation via heating in Pyr·HCl, we used BBr_3, which provided the partially deprotected compound **11** that we used for the subsequent cyclization step. Fortunately, the cyclization product **12** was amenable to deprotection and we were able to obtain the natural product lupinalbin A **5** in reasonable overall yield.

Table 1. Binding affinities (IC_{50}) for human ERα and ERβ ligand binding domain

Example	ERβ IC_{50} (nM)	ERα IC_{50} (nM)	Fold selectivity for ERβ
17β-E_2 **2**	3.6±1.6, $n=140$	3.2±1.0, $n=126$	1
Genistein **3**	10±4, $n=79$	395±181, $n=80$	41
Apigenin **4**	130±51, $n=3$	1253±638, $n=3$	10
Lupinalb. **5**	1.7±0.7, $n=8$	28±12, $n=10$	16
6	42±24, $n=9$	2951±1037, $n=11$	42
12	127±28, $n=4$	2728±1869, $n=4$	21
13	2933, $n=1$	>5000, $n=1$	>1
14	762±376, $n=2$	3672±2747, $n=2$	5
15	1740, $n=1$	>5000, $n=1$	>2
16	343, $n=1$	1260, $n=1$	4
17	>5000, $n=1$	>5000, $n=1$	nd
18	>5000, $n=1$	>5000, $n=1$	nd
19	44, $n=1$	180, $n=1$	4
20	847, $n=4$	>5000, $n=3$	>6
21	192±110, $n=2$	1500, $n=1$	8
22	32±16, $n=7$	555±248, $n=7$	17
23	4.4±1.4, $n=4$	68±15, $n=4$	15
24	3200, $n=1$	>5000, $n=1$	>1
25	>5000, $n=1$	>5000, $n=1$	nd
26	1340, $n=1$	1550, $n=1$	1

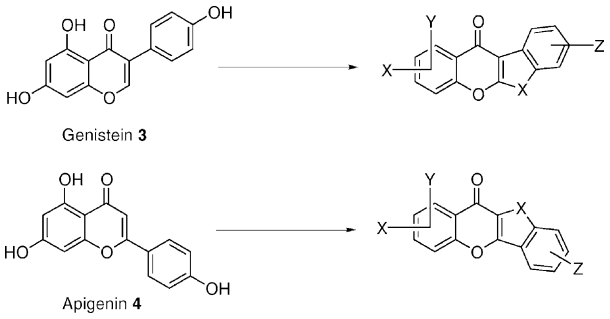
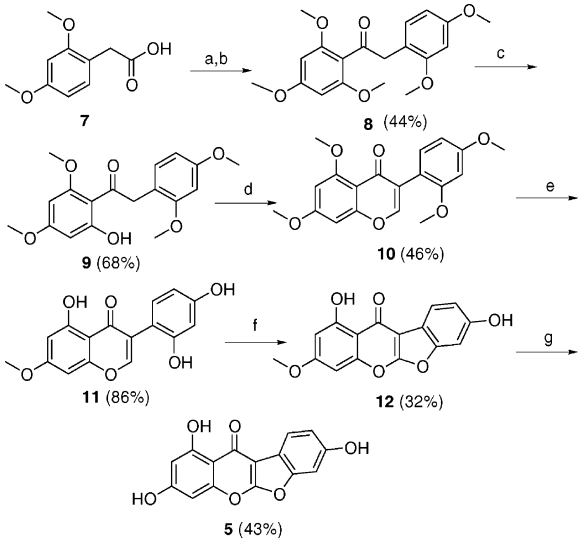


Figure 1.



Scheme 1. Reagents and conditions: (a) $SOCl_2$, THF; (b) 1,3,5-trimethoxybenzene, 1,2-dichloroethane, $AlCl_3$; (c) $AlCl_3$, $CHCl_3$; (d) $CH(OMe)_3$, morpholine; (e) BBr_3, CH_2Cl_2; (f) THF, DDQ; (g) Pyr·HCl, heat.

The syntheses of constrained analogues of apigenin **4** were accomplished very efficiently via the sequence shown in Scheme 2.[20] With the methodology shown, the core ring system is established in one step from the appropriately substituted bromoketones and salicylates or thiosalicylates. The products were usually isolated in fair to good yields without recourse to chromatography, thus making this a very simple and efficient method for the production of the benzo-fluoren-10-one ring system.

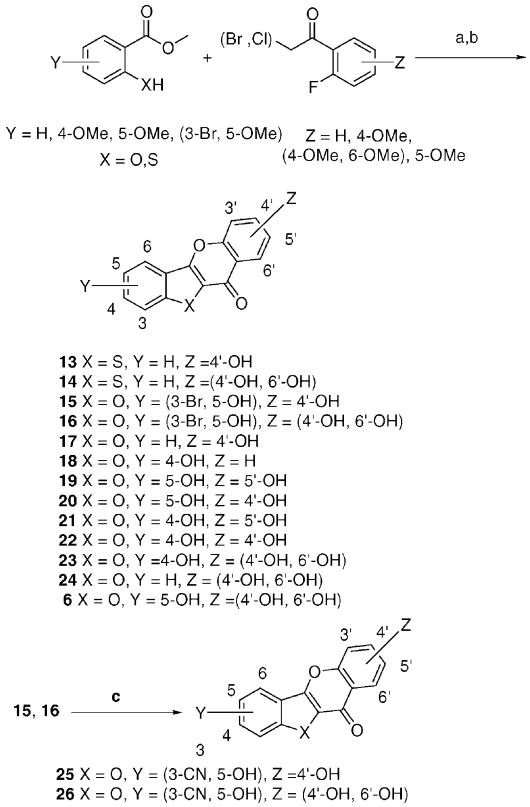
The reaction has been proposed to proceed via an intermolecular alkylation of the phenol (or thiophenol) with the α-halo acetophenone. The intermediate α-oxa (or α-thia) ketone is then deprotonated and is intramolecularly acylated by the *ortho* ester, the benzofuranone thus formed tautomerizes and the 3-hydroxy group participates as the nucleophile in an *ipso*-fluoro substitution to form the final ring.[19] Deprotection to the phenols was then performed with Pyr·HCl to yield the final, phenol-containing compounds. The 3-cyano substituted compounds were prepared by heating the 3-bromo compounds with CuCN in DMF (Scheme 2).

Discussion and Biological Data

Binding affinity

Table 1 presents binding data for both ERα and ERβ, and several SAR trends for binding potency are noteworthy. Most obvious is the importance of having at least one phenol on both sides of the molecule in order to generate good receptor affinity for both α and β. For example, neither **17** nor **18** showed measurable competition at concentrations of up to 5 μM whereas compounds **19**–**22** all displayed competition for ERβ of <1 μM. This is consistent with many examples of both steroidal and nonsteroidal estrogen ligands that display the maximum affinity where two hydroxy groups are present on appropriate positions of the molecule.

Also noteworthy is the potency enhancement that can be attained when a phenol is present at the 6′-position (compare **20** with **6** and **22** with **23**). This effect can be best explained by the detrimental effect that a carbonyl group has in the hydrophobic ER ligand binding pocket. In the case where the 6′-phenol is present, a hydrogen bond between the phenol and the carbonyl is formed, thus decreasing the polarity of the carbonyl group. Direct comparison between the non-constrained phytoestrogen genistein **3** and the ring constrained phytoestrogen lupinalbin A **5** indicate that a significant enhancement in potency can be obtained by restraining the pendant phenyl's rotation through the oxygen bridge. In fact, the affinity of lupinalbin A for ERβ is greater than any other phytoestrogen we have examined and possibly has even higher affinity for ERβ than 17β-



Scheme 2. Reagents and conditions: (a) Cs_2CO_3, DMF, heat; (b) pyr·HCl (neat, heat); (c) CuCN, DMF, heat.

Table 2. Gene regulation potency differences on ERα and ERβ endpoints

	Binding selectivity for ERβ	MCF-7 ERE ED_{50} (nM)	Metallothionein-II ED_{50} (nM)
17β-Estradiol **2**	1	0.00456 ± 0.00092 $n=2$	4.7 ± 2.6 $n=4$
Example **6**	42	>500 ~58% efficacy $n=3$	18

estradiol. Likewise, a comparison of apigenin **4** to its ring constrained analogue **23** reveals a significant (>10-fold) increase in binding affinity. The increase in binding affinity that is observed maybe a consequence of conformational restriction and thus reduced entropy loss upon binding. Alternatively, the enthalpy of binding could be affected due to these compounds containing different ring systems with different charge densities, steric presentations, and so on. In any event, the demonstration of significant binding affinity enhancement over the parent compounds is noteworthy.

Replacement of the oxygen bridging atom with a sulfur atom appears to increase binding affinity (compare compounds **13** with **17** and **14** with **24**). However, substitution of the 3-position with either a bromo atom or cyano group appeared to be very detrimental to good binding affinity (compare compounds **20** with **15** and **25** with **6**) (Table 1).

Functional selectivity of compound 6

Interestingly, all of the compounds for which binding affinities could be determined showed at least some selectivity for ERβ. The most selective compound tested was the tetracycle **6**. It was more selective than its parent, apigenin **4**, and on a par with genistein **3** (the most ERβ selective phytoestrogen we have evaluated). Since **6** showed promising binding selectivity for ERβ, we chose to further evaluate it in our cell-based assays. In order to measure potency and efficacy on ERβ in a cellular system, we looked for metallothionein induction in SAOS-2 cells, a response we have shown is ERβ specific. For functional ERα evaluation we examined the ability of selected compounds to stimulate reporter gene activity in an MCF-7 ERE-tk luciferase assay since these cells express ERα only and thus allow for a functional readout of ERα activity. In the metallothionein assay, compound **6** is active on ERβ with full efficacy and slightly reduced potency relative to 17β-estradiol (Table 2). However, in the ERα-MCF-7 cell line, compound **6** has an EC_{50} >500 nM with approximately 60% efficacy. In contrast, the non-selective steroid 17β-estradiol achieves full efficacy in this cell system at 0.0045 nM. Thus in the agonist mode, 17β-estradiol is at least 100,000 times more potent than compound **6** on an ERα driven response while it is only about 4 times as potent on the ERβ specific response in SAOS-2 cells (18 nM vs 4.7 nM). This indicates that despite its relatively modest binding selectivity, compound **6** has great functional selectivity across these two cell lines when normalized to the non-selective binder 17β-estradiol. This magnification of the binding selectivity observed for compound **6** relative to 17β-estradiol could be explained by differential cellular uptake, metabolism, and so on across the two cell lines. Alternatively, the differential transactivational potency and efficacy maybe due to differential coregulatory recruitment and utilization between the two receptors with the two compounds. In any event, these results demonstrate how a nuclear hormone binding assay may not always be predictive of functional selectivity, particularly when results are normalized across two different cell types using a reference ligand.

Constraining the ring systems of the naturally occurring phytoestrogens apigenin and genistein through the introduction of an oxa- or thia-bridge atom resulted in tetracyclic compounds with increased ERβ affinity and/or ERβ selectivity in a ligand binding assay. The phytoestrogen obtained by oxa-constraint of genistein is the natural product lupinalbin A. This compound proved to be a high affinity ligand for ERβ with an IC_{50} below that of 17β-estradiol. The tetracyclic analogue of apigenin, compound **6**, achieved ERβ selectivity of over 40-fold in the ligand binding assay and was subsequently tested in cellular systems responsive to either ERα or ERβ. In the MCF-7 cell expressing only ERα, compound **6** displayed an EC_{50} of >500 nM. In SAOS-2 cells, compound **6** induced metallothionein expression (an ERβ specific response) with an $EC_{50} = 18$ nM. This difference in potency between ERα and ERβ dependent functional response was much more dramatic when normalized to 17β-estradiol and indicate that compound **6** could be a useful tool for the further elucidation of ERβ pharmacology.

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