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# Synthesis and structure–activity relationship of 3-arylbenzoxazines as selective estrogen receptor β agonists

Wu Yang,[a,*] Yufeng Wang,[a] Zhengping Ma,[b] Rajasree Golla,[b] Terry Stouch,[c] Ramakrishna Seethala,[b] Susan Johnson,[b] Rong Zhou,[b] Timur Güngör,[a] Jean H. M. Feyen[b] and John K. Dickson, Jr.[a]

[a]*Discovery Chemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, PO Box 5400, Princeton, NJ 08543-5400, USA*
[b]*Metabolic and Cardiovascular Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, PO Box 5400, Princeton, NJ 08543-5400, USA*
[c]*Department of Macromolecular Structure, Bristol-Myers Squibb Pharmaceutical Research Institute, PO Box 5400, Princeton, NJ 08543-5400, USA*

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**Abstract**—A series of 3-aryl-7-hydroxybenzoxazine analogues have been prepared and evaluated as ligands for the two estrogen receptor subtypes (ERα and ERβ). From the radioligand binding assay, compounds with more than a 10-fold binding selectivity toward the ERβ subtype have been identified. These compounds have also been shown to be potent full agonists in the functional assay by activation of ERE promoted transcription, with the best compound being 20-fold more potent than genistein.


## 1. Introduction

The steroidal hormone estrogen plays an important role in the development and maintenance of the female reproductive system as well as other tissues such as bone, the cardiovascular system and the central nervous system. The decrease of the circulating level of estrogen in women during and after menopause is responsible for most *peri-* and postmenopausal symptoms such as hot flashes, mood swings, accelerated loss in bone mass and bone strength (osteoporosis), age-associated memory decline, and stroke. Currently, the commonly prescribed treatment for menopausal symptoms and related diseases is estrogen replacement therapy (ERT), which has proven to be effective in the treatment of vasomotor symptoms, in preventing bone loss and reducing the incidence of bone fractures, and reducing the risk of cardiovascular diseases. Nevertheless, estrogen replacement therapy has also been associated with several adverse effects, such as an increase in the risk of uterine endometrial cancer and breast cancer, and deep vein thrombosis, which result in poor patient compliance.[1] Therefore, there is still a great need to find alternative pharmaceutical agents that possess the beneficial effects of estrogen but which are devoid of the side effects of ERT.

Estrogen exerts most of its biological functions through its interaction with the estrogen receptor (ER), which has two subtypes (ERα and ERβ). 17β-Estradiol binds to the two receptors with similar affinity. Even though the two receptor subtypes share significant homology in their ligand binding domains, their different tissue distribution patterns suggest they may have distinct biological functions. Particularly, the expression of ERβ in osteoblast cells, vascular endothelial cells, and several regions of the brain has implied that ERβ may be involved in mediating some of the beneficial actions of estrogen in these tissues. The low expression level of ERβ in reproductive tissues such as uterus suggests that a selective ERβ agonist may maintain the beneficial effects of estrogen without the increased risk of breast and endometrial cancer.[2]

Besides the natural product genistein, there are only a limited number of reports of nonsteroidal ERβ selective agonists. These include the arylbenzthiophenes, the

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* Corresponding author. Tel.: +1-609-818-6493; fax: +1-609-818-3460; e-mail: wu.yang@bms.com

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diarylpropionitriles, and the conformational constrained phytoestrogens.[3] Genistein is an isoflavonoid that has been reported to be an estrogen receptor agonist with modest selectivity toward ERβ. However, it has been shown to possess some potential liabilities.[4] In order to maintain the agonist activity of genistein and its selectivity toward ERβ while possibly eliminating the potential liabilities, we chose to modify the core heterocycle from benzopyrone to benzoxazine. We also decided to retain the two terminal hydroxyl groups, because they are geometrically arranged to potentially mimic the two hydroxyl groups in 17β-estradiol and are known to be essential for the agonist activity of 17β-estradiol (Fig. 1). Here we report the synthesis and structure–activity relationships of a benzoxazine series of estrogen receptor agonists, including compounds with modest selectivity toward ERβ.

## 2. Chemistry

The synthesis of the benzoxazine compounds **4a**–**n** in Table 1 is outlined in Scheme 1. The starting α-bromomethyl-4′-methoxyphenyl ketones **1** were obtained either from commercial sources ($R^2 = R^3 = H$) or via bromination of substituted acetophenones with bromine in chloroform.[5] Treatment of **1** with *p*-methoxyanilines **2a**–**c** afforded the core benzoxazines **3**,[6] which upon standard demethylation with $BBr_3$ provided the final compounds **4** in good yields. In certain instances, when the reaction was allowed to proceed too long, further bromination of **1** at the *ortho* position resulted in side products where $R^4 = 3$-Br.

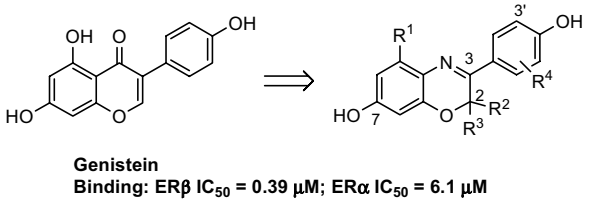
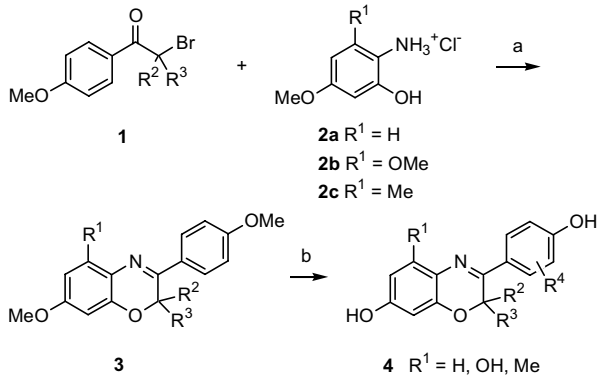
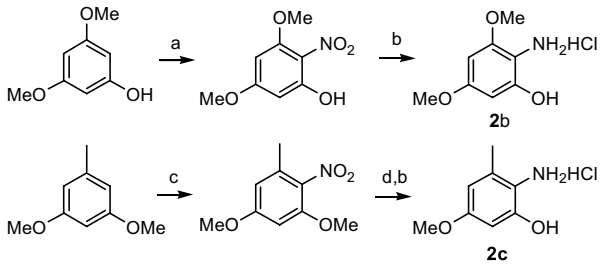



**Figure 1.** Genistein and 3-aryl-7-hydroxybenzoxazines.




**Scheme 1.** Synthesis of benzoxazines. Reaction conditions: (a) $K_2CO_3$, acetone, reflux; (b) $BBr_3$, $CH_2Cl_2$, 0 °C.



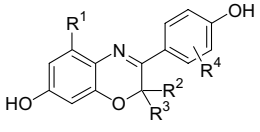

**Scheme 2.** Reaction conditions: (a) $NO_2BF_4$, DME, −78 °C to −50 °C; (b) $Pd/H_2$, MeOH/$CHCl_3$; (c) $HNO_3/CH_3SO_3H/H_2O$/urea, 0 °C; (d) $BBr_3$, $CH_2Cl_2$, 0 °C.

The *p*-methoxyaniline precursors (**2b**,**c**) were prepared readily from substituted 1,3-dimethoxybenzenes as illustrated in Scheme 2. Regioselective nitration of 3,5-dimethoxyphenol[7] followed by hydrogenolysis afforded compound **2b** in 52% yield. Analogously, compound **2c** was prepared in three steps by nitration of 3,5-dimethoxytoluene,[8] regioselective *ortho*-demethylation[9] followed by hydrogenolysis.

## 3. Results and discussion

To screen compounds for their affinity and selectivity for ERα versus ERβ, we set up an in vitro ligand binding assay using [$^3$H]17β-estradiol as radioligand in a scintillation proximity assay (SPA).[10] For both ERβ and ERα, recombinant human maltose binding protein tagged, biotinylated ligand binding domains are used. Compounds were also profiled for their ability to activate an estrogen response element (ERE) in a luciferase reporter gene assay. HeLa cells were stably transfected with a construct expressing the human ERβ or ERα (full length) receptor and co-transfected with a luciferase

**Table 1.** Benzoxazine compounds **4a**–**n**



| Compounds | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| **4a** | H | H | H | H |
| **4b** | H | Me | H | H |
| **4c** | H | Et | H | H |
| **4d** | H | *n*-Pr | H | H |
| **4e** | H | Me | Me | H |
| **4f** | H | H | –($CH_2CH_2$)– | |
| **4g** | H | H | –($CH_2$)– | |
| **4h** | OH | H | H | H |
| **4i** | OH | Me | H | H |
| **4j** | OH | Et | H | H |
| **4k** | OH | Et | H | 3′-Br |
| **4l** | OH | Me | Me | H |
| **4m** | Me | Me | H | H |
| **4n** | Me | Et | H | H |

**Table 2.** Estrogen receptor binding affinities ($^3$[H]-17β-E2 binding) and receptor gene activation (ERE) of reference compounds and compounds **4a**–**n**

| Compounds | Competition binding ($IC_{50}$)[a] | | | ERE Activity ($EC_{50}$)[a] | |
|---|---|---|---|---|---|
| | ERβ (nM) | ERα (nM) | Ratio α/β | ERβ (nM) | ERα (nM) |
| 17β-E2 | 19 | 11 | 0.58 | 0.67 | 0.48 |
| Estrone | 540 | 210 | 0.4 | 120 | 120 |
| Genistein | 390 | 6100 | 16 | 200 | 950 |
| **4a** | 2900 | 16,000 | 5.7 | 160 | 6900 |
| **4b** | 330 | 1700 | 5.0 | 30 | 64 |
| **4c** | 200 | 850 | 4.3 | 7 | 59 |
| **4d** | 320 | 1700 | 5.2 | 18 | 1700 |
| **4e** | 5600 | 8600 | 1.6 | 140 | 3100 |
| **4f** | 2600 | 6000 | 2.3 | 240 | 12,000 |
| **4g** | 1400 | 5600 | 4.0 | 77 | 900 |
| **4h** | 2800 | 33,000 | 12 | 270 | 1800 |
| **4i** | 310 | 2900 | 9.4 | 47 | 150 |
| Enantiomer **A** | 240 | 2800 | 12 | 50 | 140 |
| Enantiomer **B** | 1100 | 6300 | 5.7 | 74 | 290 |
| **4j** | 120 | 1200 | 10 | 26 | 170 |
| **4k** | 1100 | 5300 | 4.8 | 180 | 2000 |
| **4l** | 1700 | 6000 | 3.5 | 120 | 1400 |
| **4m** | 90 | 320 | 3.6 | 16 | 46 |
| **4n** | 90 | 180 | 2.0 | 8 | 170 |

[a] Values are average of two–four experiments.

reporter gene construct driven by an ERE–TK minimal promoter.[11]

The binding affinity and selectivity of the benzoxazine compounds **4a**–**n** for an estrogen receptors were determined in the binding assay described above (Table 2). 17β-Estradiol exhibited essentially no selectivity toward the two ER receptor subtypes with $IC_{50}$s of 19 and 11 nM for ERβ and ERα, respectively. The natural product genistein, however, showed a modest selectivity (16-fold) for ERβ, but the binding potency was considerably less than that for 17β-estradiol. The results for the above two reference compounds were in accordance with those reported in the literature by Schopfer et al.[3a]

The parent, unsubstituted benzoxazine compound **4a** displayed some selectivity (5-fold) for ERβ, even though the binding affinity was weak. Introduction of a methyl substituent at the $R^2$ position (**4b**) improved the binding affinity for both ERβ and ERα by about 10-fold, but with minimal effect on selectivity. Increasing the size of the substituent from methyl to ethyl (**4c**) or *n*-propyl (**4d**) did not have a dramatic impact on either selectivity or potency. Addition of a second methyl group at the 2-position (**4e**) or making conformationally constrained analogues by cyclization of the benzoxazine ring with the 3-aryl ring (**4f**,**g**) all resulted in much reduced ER binding. Interestingly, this is in contrast to what was recently reported for constrained phytoestrogen analogues, where significant enhancement of potency was observed when the pendant phenyl ring was fused to the pyrone core through an oxygen bridge.[3c] To make a direct comparison with genistein, another hydroxyl group was introduced at the 5-position of the benzoxazine ring. The resulting compounds **4h**–**j** and **4l** all exhibited a 2-fold improvement in selectivity for ERβ, while maintaining similar potency compared with their unsubstituted parent compounds **4a**–**c** and **4e**. Compound **4j** reached selectivity close to that of genistein, yet with improved potency. Bromine substitution at the 3′ position (**4k**), a side product isolated during preparation of **4j**, was not well tolerated. When the 5-position was substituted with a methyl group instead of a hydroxyl group (**4m**,**n** vs **4i**,**j**) or just hydrogen (**4m**,**n** vs **4b**,**c**), the potency of these compounds increased by 2- to 3-fold, however, at the expense of selectivity.

The effect of stereochemistry at the chiral center of the benzoxazine on the estrogen receptor binding activity was also examined. The two enantiomers of compound **4i** were separated by chiral preparative HPLC[12] and tested in the binding and transcription assays. The fast eluting enantiomer **A** was found to be more than four times more potent and twice as selective as the slow eluting enantiomer **B**, though the absolute stereochemistry was not determined.

The selectivity and SAR of these benzoxazines can be rationalized structurally by docking these analogues into the ERβ structure in an orientation analogous to those of other ligands seen in crystal structures, such as that of genistein.[13] The $R^2$ position of the benzoxazine is ideally situated to impact directly the L384M (α to β) variation. Its $sp^3$ and slightly out-of-plane character allows hydrophobic substituents, including a hydrogen atom, to approach closely. Larger substitutions, such as the methyl group of **4i**, will have a stronger hydrophobic interaction, resulting in increased potency. Little additional effect on selectivity and only 2-fold improvement on potency was observed by an ethyl substitution, since the likely conformation of the ethyl group would place the terminal methyl group away from residue 384. The $R^1$ group on the 5-position of benzoxazine is placed to allow direct interaction with the M421I variation. The selectivity for ERβ suggests that there could be unfavorable steric or electrostatic interactions with the methionine residue of ERα. That the hydroxyl substitution is more selective than the methyl suggests a role

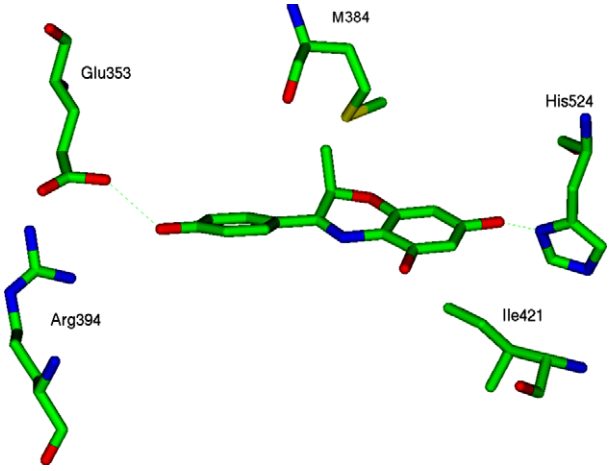



**Figure 2.** A view of the 3-aryl-7-hydroxybenzoxazine **4i**, in the binding pocket of ERβ.

for unfavorable electrostatic interactions with the M421 of ERα (Fig. 2).

These benzoxazine compounds were also evaluated in a transcription assay for their ability to activate the estrogen receptor-mediated transcription using luciferase as the reporter gene (columns 5 and 6 of Table 2). All the compounds were found to act as full functional agonists (>80% intrinsic activity of that of 17β-estradiol). The selectivity observed in the transcription assay was generally in agreement with that seen in the binding assay, while the $EC_{50}$s shifted 10- to 20-fold from the $IC_{50}$s. Compound **4n**, being the most potent compound in this series, showed an $EC_{50}$ of 8 nM, only 10-fold less potent than 17β-estradiol and more than 20-fold more potent than genistein in these in vitro assays.

## 4. Conclusions

Based on the natural product genistein, a novel series of 3-aryl-7-hydroxylbenzoxazines have been prepared and evaluated for their binding to the estrogen receptors ERα and ERβ. Several of these compounds exhibited similar binding selectivity toward ERβ as that seen with genistein, but with improved potency. These selective analogues were also found to be potent full agonists in an estrogen receptor-mediated gene transcription assay.

## Acknowledgements

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