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Lead Optimization of 7-benzyloxy 2-(4'-pyridylmethyl)Thio Isoflavone Aromatase Inhibitors

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Introduction

Breast cancer is the most common cancer diagnosed in women and represents the second leading cause of cancer-related deaths in women. Approximately one-third of all breast cancer patients and two-thirds of postmenopausal breast cancer patients have estrogen-dependent breast cancer, which contains estrogen receptors and requires estrogen for tumor growth.[1] Estrogens are biosynthesized from androgens by aromatase, a member of the cytochrome P450 superfamily.[2] Aromatization of androgen substrates is the terminal and rate-limiting step in estrogen biosynthesis, and aromatase has been a particularly attractive target in the treatment of hormone-dependent breast cancer.[3–6]

Many researchers have suggested that flavonoids are responsible for the low incidence in breast cancer in women from some regions of the world.[7] Our research group is pursuing drug discovery efforts exploiting flavonoids as core templates for novel aromatase inhibitors (AIs). In our previous work, we demonstrated that introducing a nitrogen-containing heterocycle at the 2-position of the isoflavone nucleus is beneficial for aromatase inhibition. The introduction of a 2-(4′-pyridylmethyl)thio functionality onto the isoflavone nucleus (Fig. 1, compound **A**) afforded a 56-fold enhancement in potency compared to the natural product lead, Biochanin A (Fig. 1). In addition, changing the 7-hydroxy to 7-benzyloxy group generated a 3-fold more active compound, the lead compound **B**.[8] This observation was unexpected because the compound was assumed to be too bulky to fit within the enzyme active site. Herein, we hypothesize that the 7-benzyloxy group

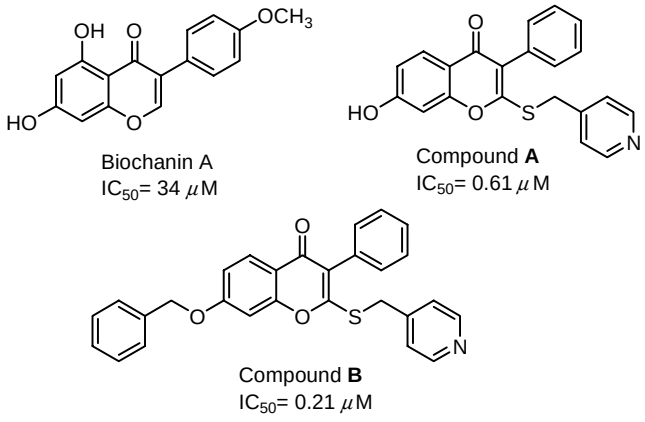


Figure 1. Chemical structures and aromatase inhibitory activities of natural product biochanin A and lead compounds.[8]

plays a very important role in the binding process and that by introducing similar functional groups into the 7-position of compound **A** may lead to better AIs.

Results and discussion

Synthesis approach

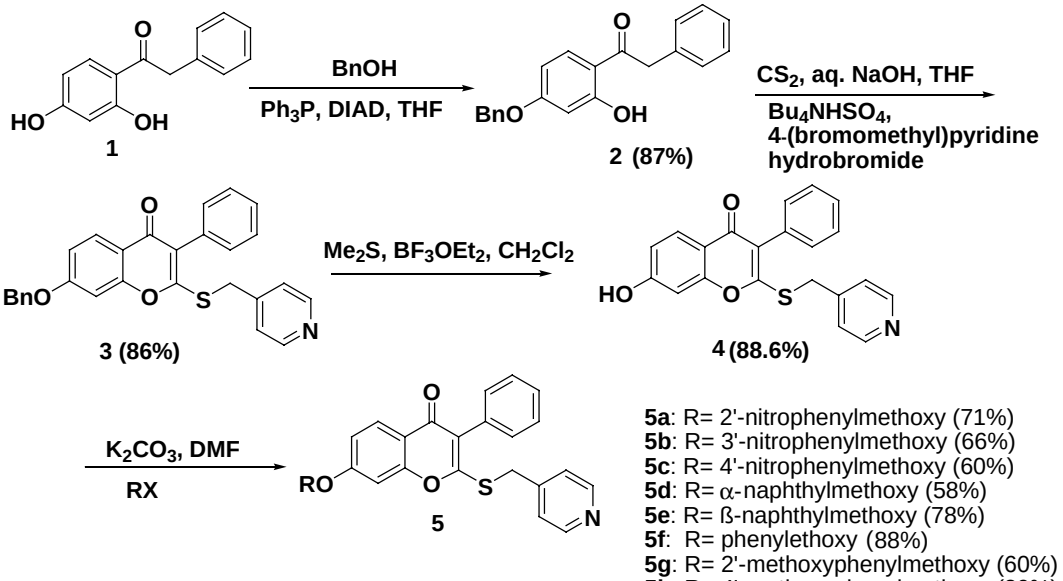
The synthesis of the target compounds was carried out, as outlined in Scheme 1. Compound **1** was commercially available. The 4-hydroxyl group of compound **1** was selectively protected with a benzyl group under Mitsunobu reaction conditions to give a monoalkyl ether **2**. Compound **2** was treated with carbon disulfide and 4-(bromomethyl)pyridine hydrobromide in a THF-aqueous NaOH solution in the presence of 10 mol% of tetrabutylammonium hydrogensulfate (n-$Bu_4N{\cdot}HSO_4$) to give the corresponding 2-(alkylthio)isoflavone **3** in good yield. Dealkylation reaction of compound **3** was performed with $BF_3{\cdot}OEt_2$ Me_2S in dichloromethane, yielding hydroxy compound **4**.[8] Compound **4** was treated with K_2CO_3 and alkyl halide in DMF at room temperature or refluxed to obtain the desired compounds.

The nitrogen atom of the 2-(4-pyridylmethyl)thio group and the 7-hydroxy group of intermediate **4** are reactive toward alkyl halides and result in dialkylated quaternary amine side products, respectively. For some alkyl halides, such as methyl, dimethyl, and 3-methoxy benzyl chloride, we obtained the quaternary amines as the major products and the yields for the desired compounds were below 3%. No product was found when 3-(bromomethyl)pyridine hydrobromide and 4-(bromomethyl)pyridine hydrobromide were used as alkyl halides, even after refluxing for 72 h. All the synthesized compounds were confirmed by ^{1}HNMR and HRMS, and compounds were also confirmed by elemental analyses.

Biological evaluation

Aromatase activity assay in human placental microsomes. Aromatase inhibition was performed with human placental microsomes using methods previously reported by our laboratory.[9] The IC_{50} values of the compounds were determined by nonlinear regression analysis of the dose response curves (Fig. 2) and the values are given in Table 1. The IC_{50} value of (±)-aminoglutethimide (AG) is also listed for comparison.[8] To examine the mode of aromatase inhibition and to more accurately distinguish the relative potencies, enzyme kinetic studies for selected compounds (**5d**, **5e**, and **5m**) were also performed, and their apparent K_i values are given in Table 2, along with apparent K_m values and K_i/K_m ratios. Lineweaver Burk plot of compound **5m**, the most potent analog in this series, is shown in Figure 3.

Enzyme inhibition studies have demonstrated that extending the hydrocarbon chain from benzyloxy (Compound **B**) to phenylethoxy (**5f**) decreased the activity almost 2-fold. Expansion of the aromatic system of the benzyloxy analog to naphthyl (**5d**, **5e**) enhanced activity approximately 2-fold. However, dose response studies alone could not discern whether attachment at the α- or β-position produces more desirable inhibitory activity. Enzyme kinetic studies corroborated the dose response results, revealing the β-naphthyl analog



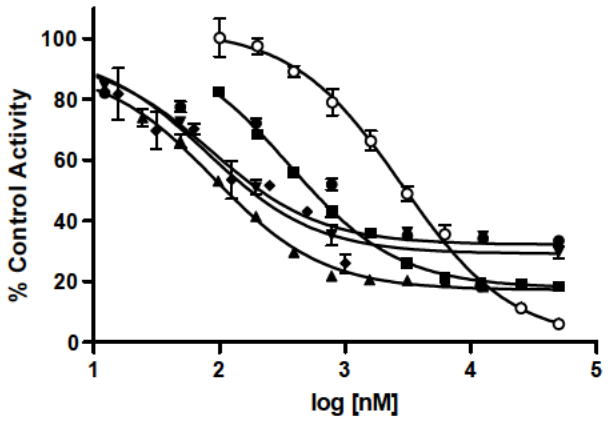


Figure 2. Aromatase inhibitory activities of compounds **5f** (■), **5e** (▲), **5d** (▼), **5m** (♦), and **5i** (●). Aminoglutethimide (○) was used as reference. Error bars represent standard error ($n = 3$), and the data were statistically analyzed by a nonlinear regression analysis method.

(**5e**) to be slightly more active. Introduction of an additional phenyl ring generated the biphenyl analog (**5m**), which was the most potent one in this series of compounds. The cyclohexyl analog (**5i**) demonstrated a greater than 2-fold loss of activity, thus illustrating the importance of unsaturation in the 7-substituent to enhance aromatase inhibitory activity. Introduction of a nitro group to the 7-benzyloxy functionality leads to an almost 2-fold enhancement in activity (**5a**, **5b**, and **5c**). This enhancement appears to be independent of the position of the electron-withdrawing substituent. These observations, taken together with the apparent necessity for an aromatic moiety at the 7-position, suggest that these molecules exploit a beneficial π π interaction and/or a planar structural feature in the aromatase active site.

Based on the IC_{50} values alone, it appears that introduction of an *o*-methoxy group to the 7-benzyloxy substituent (**5g**) does not impart a significant change in inhibitory activity. However, introduction of this functional group in the *para* position (**5h**) leads to a slight

Table 2. Enzyme kinetic parameters for selected isoflavones and reference compounds

Compound	Apparent K_i (nM) ± (SE)[a]	Apparent K_m (nM) ± (SE)[a]	K_i/K_m
Compound **B**	220 ± 20	130 ± 10	1.69[b]
5d	114 ± 15.7	100 ± 8.8	1.14
5e	74 ± 8.0	89 ± 7.2	0.83
5m	39 ± 5.0	77 ± 9.0	0.51
AG	1410 ± 10	90 ± 10	15.7[b]

[a] Apparent K_m, apparent K_i, and SE values were calculated by weighted regression analysis.[13]
[b] Ref. 8

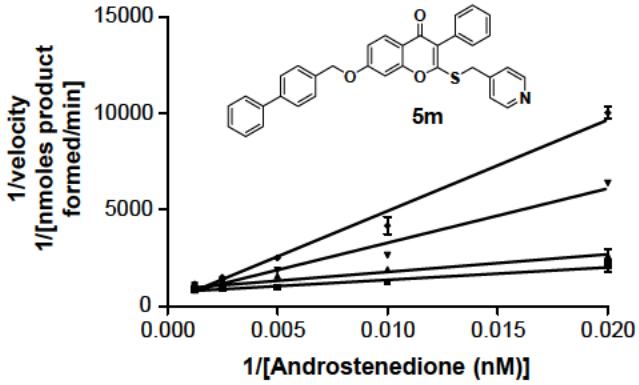


Figure 3. Lineweaver Burk plot of aromatase inhibition by compound **5m**. Various concentrations of androstenedione (50, 100, 200, 400, and 800 nM) were incubated with microsomal enzyme preparations at inhibitor concentrations of 0 nM (■), 25 nM (▲), 100 nM (▼), or 200 nM (♦). Each point represents the average of three determinations (±)standard error.

increase in activity. Whether this minor enhancement in activity arises from the hydrophobic nature of this substituent or from its hydrogen-bonding potential is unclear. Introduction of a 2-pyridyl substituent at the 7-position (**5n**) leads to an almost 2-fold decrease in activity. Introduction of chlorine or bromine (**5l**, **5k**) at the *para* position apparently has no effect on the

Table 1. Aromatase inhibitory activities of isoflavones **5a n**

Compound	R	IC_{50} (nM)	Log IC_{50} (nM) ± SE[a]
Compound **B**	Benzyloxy	210	2.33 ± 0.03[b]
5a	2′ Nitrophenylmethoxy	138	2.14 ± 0.03
5b	3′ Nitrophenylmethoxy	113	2.06 ± 0.05
5c	4′ Nitrophenylmethoxy	132	2.12 ± 0.08
5d	α Naphthylmethoxy	112	2.05 ± 0.15
5e	β Naphthylmethoxy	90	1.96 ± 0.05
5f	Phenylethoxy	359	2.55 ± 0.04
5g	2′ Methoxy phenylmethoxy	243	2.38 ± 0.04
5h	4′ Methoxy phenylmethoxy	161	2.21 ± 0.02
5i	Cyclohexylmethoxy	553	2.74 ± 0.08
5j	4′ Fluorophenylmethoxy	337	2.53 ± 0.05
5k	4′ Bromophenylmethoxy	213	2.33 ± 0.05
5l	4′ Chlorophenylmethoxy	233	2.37 ± 0.05
5m	4′ Biphenylmethoxy	79	1.90 ± 0.16
5n	2′ Pyridylmethoxy	378	2.58 ± 0.06
AG[b]		2800	3.45 ± 0.05

[a] IC_{50} values were calculated by a nonlinear regression analysis (GraphPad Prism). Each dose response curve contained 10 concentrations, each in triplicate.
[b] Ref. 8

aromatase inhibitory activity compared to the lead compound. Interestingly, the *p*-fluoro analog (**5j**) is approximately 50% less active. Additional analogs of the 7-benzyloxy lead will need to be examined in order to determine the contributions from hydrophobicity and hydrogen-bonding potential of substituents to aromatase inhibitory activity.

Aromatase activity assay in SK-BR-3 breast cancer cell lines. The synthesized compounds were evaluated at 1 μM concentrations for their abilities to inhibit aromatase in SK-BR-3 breast cancer cell cultures (Fig. 4). Our results showed that halogen-substituted compounds **5j**, **5k**, **5l**, 2-nitro and 3-nitro compounds **5a** and **5b**, and α-naphthyl compound **5d** had no effect on aromatase activity. This may be due to their weak ability to penetrate the cell membrane and/or their potential binding to other proteins in our cell culture system, on the cell surface, or within the cell membrane. The remaining compounds in this series display a statistically significant suppression of aromatase enzyme activity. These results proved that some of these bulky compounds can pass through the cell membrane and inhibit aromatase activity. In fact, large lipophilic compounds, such as **5e** and **5m**, apparently penetrate cells and exhibit promising aromatase inhibitory activity. The significant suppression of aromatase by compounds **5e** and **5m** may be related to hydrophobicity of the 7-substituent of these analogs. However, the disparity in aromatase suppression achieved in microsomes and cell culture by the more hydrophilic analog, **5n**, suggests that other factors, such as intracellular transport or decreased extracellular sequestration by the culture system, may be involved.

Conclusion

Introduction of functional groups with the potential to coordinate the heme iron of aromatase resulted in new lead AIs with the isoflavone nucleus, which has previously been considered an inappropriate scaffold for the development of AIs. This study optimized further the 7-substituent of the lead compound and generated analogs with greater than 2-fold activity improvements. In addition, these structure activity studies suggest a potential role for π π stacking interactions within the aromatase active site. Although no direct structure information for the aromatase active site exists, the potent inhibitory activities of some analogs described here indicate that the substrate binding pocket may be able to accept larger ligands than previously thought possible. These observations on potential π π stacking interactions in this series of nonsteroidal agents are congruent with structure activity studies of 7α-aryl-substituted C_{19} steroidal inhibitors.[9–11] Therefore, we are currently investigating the aromatase inhibitory activity of other bulky pyridine-containing flavonoids to better understand the binding site of aromatase.

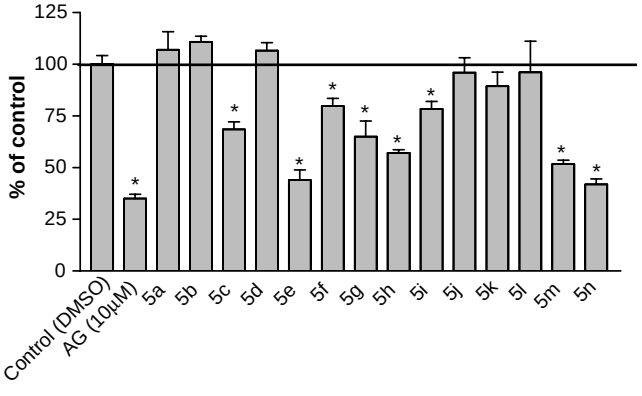


Figure 4. Suppression of aromatase activity in SK BR 3 breast cancer cells. SK BR 3 cells were treated with aminoglutethimide (AG, 10 μM) and compounds **5a n** (1 μM). Aromatase activity was measured, as described in Section 4. Values are expressed as picomoles ^3H_2O formed per hour incubation time per million cells. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.003 pmol/h/10^6 cells. Each data bar represents the mean results of three independent determinations. $^*P < 0.01$ versus control by unpaired *t* test, $n = 3$.

Experimental section

Chemistry

Chemicals were commercially available and used as received without further purification unless otherwise noted. Moisture sensitive reactions were carried out under a dry argon atmosphere in flame-dried glassware. Solvents were distilled before use under argon. Tetrahydrofuran was distilled from sodium metal in the presence of benzophenone; dichloromethane was distilled from calcium hydride. Thin-layer chromatography was performed on precoated silica gel F254 plates (Whatman). Silica gel column chromatography was performed using silica gel 60A (Merck, 230 400 Mesh). High-resolution electrospray ionization mass spectra were obtained on the Micromass QTOF Electrospray mass spectrometer at The Ohio State Chemical Instrumentation Center. All the NMR spectra were recorded on a Bruker DPX 250 in either DMSO-d_6 or $CDCl_3$. Chemical shifts (δ) for ^1H NMR spectra are reported in parts per million to residual solvent protons.

Compounds **2**, **3**, and **4** were prepared, as described by Kim et al.[8]

General procedure for the preparation of 7-substituted 2-alkylthioisoflavones (5a–n) from 7-hydroxy-2-(4′-pyridylmethylthio)isoflavones. An alkyl halide (0.3 mmol) was added to a stirred mixture of 7-hydroxy-3-phenyl-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (72 mg, 0.2 mmol) and K_2CO_3 (83 mg, 0.6 mmol) in DMF (3 mL) at room temperature. The resulting mixture was vigorously stirred at room temperature or refluxed for several hours and monitored by TLC. When the starting material was exhausted, 15 mL of water was added and the product was extracted with EtOAc (2× 10 mL). The combined organic layer was washed with water (10 mL) and then with brine (10 mL), dried over $MgSO_4$, and filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (eluting with MeOH/$CHCl_3$ or EtOAc/hexane) to yield the desired product.

3-Phenyl-7-(2′-nitrophenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5a). With 2-nitrobenzyl bromide as an alkyl halide, 71 mg (71%) of the title compound was obtained as a pale yellow solid: ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.47 (d, J = 5.5 Hz, 2H), 8.21 (d, J = 8.5 Hz, 1H), 7.95 (d, J = 8.8 Hz, 1H), 7.84 (d, J = 3 Hz, 2H), 7.70 7.72 (m, 1H), 7.37 7.44 (m, 6H), 7.18 7.23 (m, 3H), 5.68 (s, 2H), 4.45 (s, 2H); HRMS Calcd for $C_{28}H_{21}N_2O_5S$ $(M+H)^+$ 497.1171. Found: 497.1153. Anal. Calcd for $C_{28}H_{20}N_2O_5S$: C, 67.73; H, 4.06; N, 5.64. Found: C, 67.30; H, 4.04; N, 5.44.

3-Phenyl-7-(3′-nitrophenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5b). With 3-nitrobenzyl bromide as an alkyl halide, 66 mg (66%) of the title compound was obtained as a yellow solid: ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.69 (d, J = 5 Hz, 2H), 8.41 (s, 1H), 8.26 (d, J=7.5 Hz, 1H), 7.95 8.02 (dd, J = 12, 7.7 Hz, 2H), 7.75 7.84 (m, 3H), 7.39 7.45 (m, 4H), 7.20 7.25 (m, 3H), 5.48 (s, 2H), 4.59 (s, 2H); HRMS Calcd for $C_{28}H_{21}N_2O_5S$ $(M+H)^+$ 497.1171. Found: 497.1196.

3-Phenyl-7-(4′-nitrophenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5c). With 4-nitrobenzyl bromide as an alkyl halide, 60 mg (60%) of the title compound was obtained as a pale yellow solid: ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.48 (d, J = 3.6 Hz, 2H), 8.30 (d, J = 8.3 Hz, 2H), 7.94 (d, J = 8.8 Hz, 1H), 7.79 (d, J = 8.7 Hz, 2H), 7.38 7.43 (m, 6H), 7.20 7.22 (m, 3H), 5.50 (s, 2H), 4.44 (s, 2H); HRMS Calcd for $C_{28}H_{21}N_2O_5S$ $(M+H)^+$ 497.1171, Found: 497.1156.

3-Phenyl-7-(β-naphthylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5d). With 1-(chloromethyl)naphthalene as an alkyl halide, 59 mg (58%) of the title compound was obtained as a pale yellow solid: ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.53 (dd, J = 1.6, 4.6 Hz, 2H), 8.17 (d, J = 8.9 Hz, 1H), 8.06 8.09 (m, 1H), 7.94 8.00 (m, 2H), 7.29 7.64 (m, 11H), 7.13 7.17 (dd, J = 2.4, 8.9 Hz, 1H), 6.89 (d, J = 2.3, 1H), 5.65 (s, 2H), 4.26 (s, 2H); HRMS Calcd for $C_{32}H_{23}N$ NaO_3S $(M+Na)^+$ 524.1296. Found: 524.1274. Anal. Calcd for $C_{32}H_{23}NO_3S$: C, 76.62; H, 4.62; N, 2.79. Found: C, 76.23; H, 4.64; N, 2.61.

3-Phenyl-7-(α-naphthylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5e). With 2-(bromomethyl)naphthalene as an alkyl halide, 78 mg (78%) of the title compound was obtained as a pale yellow solid: ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.42 (dd, J = 1.6, 4.4 Hz, 2H), 8.09 (s, 1H), 7.93 8.03 (m, 4H), 7.64 (dd, J = 1.6, 8.4 Hz, 1H), 7.55 7.59 (dd, J = 3.3, 6.3 Hz, 2H), 7.38 7.43 (m, 6H), 7.19 7.24 (m, 3H), 5.50 (s, 2H), 4.44 (s, 2H); HRMS Calcd for $C_{32}H_{23}N$ NaO_3S $(M+Na)^+$ 524.1296. Found: 524.1315. Anal. Calcd for $C_{32}H_{23}NO_3S$: C, 76.62; H, 4.62; N, 2.79. Found: C, 76.32; H, 4.60; N, 2.65.

3-Phenyl-7-(phenylethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5f). With (2-bromoethyl) benzene as an alkyl halide, 82 mg (88%) of the title compound was obtained as a white solid: ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.65 (m, 2H), 8.13 (d, J = 8.9 Hz, 1H), 7.29 7.57 (m, 12H), 6.99 (d, J = 9.1 Hz, 1H), 6.73 (s, 1H), 4.28 4.33 (m, 4H), 3.18 (dd, J = 6.4, 6.4 Hz, 2H); HRMS Calcd for $C_{29}H_{23}NNaO_3S$ $(M+Na)^+$ 488.1296. Found: 488.1310. Anal. Calcd for $C_{29}H_{23}NO_3S$: C, 74.81; H, 4.98; N, 3.01. Found: C, 74.51; H, 5.06; N, 2.89.

3-Phenyl-7-(2′-methoxyphenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5g). With 2-methoxybenzyl chloride as an alkyl halide, 57.5 mg (60%) of the title compound was obtained as a pale yellow powder: ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.73 (d, J = 6.7 Hz, 2H), 7.92 (m, 3H), 6.92 7.44 (m, 11H), 5.25 (s, 2H), 4.64 (s, 2H); HRMS Calcd for $C_{29}H_{24}NO_4S$ $(M+H)^+$ 482.1426. Found: 482.1403.

3-Phenyl-7-(4′-methoxyphenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5h). With 4-methoxybenzyl chloride as an alkyl halide, 37.3 mg (39%) of the title compound was obtained as a pale yellow crystal: ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.60 (d, J = 4.2 Hz, 2H), 8.14 (d, J = 9.1 Hz, 1H), 7.39 7.46 (m, 7H), 7.28 (d, J = 6.8 Hz, 2H), 7.06 (d, J = 8.5 Hz, 1H), 6.97 (d, J = 8.4 Hz, 2H), 6.80 (s, 1H), 5.13 (s, 2H), 4.29 (s, 2H); HRMS Calcd for $C_{29}H_{24}NO_4S$ $(M+H)^+$ 482.1426. Found: 482.1439. Anal. Calcd for $C_{29}H_{23}NO_4S$: C, 72.33; H, 4.81; N, 2.91. Found: C, 71.87; H, 4.92; N, 2.91.

3-Phenyl-7-(cyclohexylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5i). With cyclohexylmethyl bromide as an alkyl halide, 73.8 mg (81%) of the title compound was obtained as a yellow solid: ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.50 (dd, J = 2.8, 4.5 Hz, 2H), 7.89 (d, J = 8.9 Hz, 1H), 7.38 7.47 (m, 5H), 7.19 7.26 (m, 3H), 7.05 (dd, J = 2.0, 8.8 Hz, 1H), 4.46 (s, 2H), 3.96 (d, J = 5.9 Hz, 2H), 1.73 1.88 (m, 6H), 1.07 1.28 (m, 5H); HRMS Calcd for $C_{28}H_{27}NNaO_3S$ $(M+Na)^+$ 480.1609. Found: 480.1615. Anal. Calcd for $C_{28}H_{27}NO_3S$: C, 73.49; H, 5.95; N, 3.06. Found: C, 73.36; H, 6.00; N, 2.97.

3-Phenyl-7-(4′-fluorophenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5j). With 4-fluorobenzyl chloride as an alkyl halide, 82 mg (87%) of the title compound was obtained as a pale green powder: ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.49 (d, J = 5.9 Hz, 2H), 7.92 (d, J = 8.8 Hz, 1H), 7.57 7.60 (m, 2H), 7.18 7.46 (m, 11H), 5.29 (s, 2H), 4.45 (s, 2H); HRMS Calcd for $C_{28}H_{20}FNNaO_3S$ $(M+Na)^+$ 492.1046. Found: 492.1047.

3-Phenyl-7-(4′-bromophenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5k). With 4-bromobenzyl bromide as an alkyl halide, 103 mg (97%) of the title compound was obtained as a yellow powder: ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.49 (dd, J = 1.6, 4.4 Hz, 2H), 7.92 (d, J = 8.9 Hz, 1H), 7.64(d, J = 8.4 Hz, 2H), 7.35 7.52 (m, 8H), 7.19 7.23 (m, 3H), 5.30 (s, 2H), 4.45 (s, 2H); HRMS Calcd for $C_{28}H_{20}$ BrNNa O_3S $(M+Na)^+$ 552.0245. Found: 552.0264.

3-Phenyl-7-(4′-chlorophenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5l). With 4-chlorobenzyl chloride as an alkyl halide, 81 mg (83%) of the title

compound was obtained as a green powder: ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.49 (d, J = 5.6 Hz, 2H), 7.92 (d, J = 9.0 Hz, 1H), 7.34 7.59 (m, 11H), 7.15 7.22 (m, 3H), 5.32 (s, 2H), 4.45 (s, 2H); HRMS Calcd for $C_{28}H_{20}ClNNaO_3S$ $(M+Na)^+$ 508.0750. Found: 508.0773.

3-Phenyl-7-(4′-biphenylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5m). With 4-phenylbenzyl chloride as an alkyl halide, 75 mg (71%) of the title compound was obtained as a yellow powder: ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.48 (d, J = 4.8 Hz, 2H), 7.92 (d, J = 7.5 Hz, 1H), 7.39 7.77 (m, 15H), 7.20 7.23 (m, 3H), 5.37 (s, 2H), 4.45 (s, 2H); HRMS Calcd for $C_{34}H_{25}NNaO_3S$ $(M+Na)^+$ 550.1453. Found: 550.1436. Anal. Calcd for $C_{34}H_{25}NO_3S$: C, 77.40; H, 4.78; N, 2.65. Found: C, 76.95; H, 4.98; N, 2.27.

3-Phenyl-7-(2′-pyridylmethoxy)-2-[(4′-pyridylmethyl)thio]-4*H*-1-benzopyran-4-one (5n). With 2-(bromomethyl)pyridine hydrobromide as an alkyl halide, 49 mg (54%) of the title compound was obtained as a pale yellow powder: ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.64 (d, J = 3.8 Hz, 1H), 8.49 (d, J = 5.0 Hz, 2H), 7.88–7.96 (m, 2H), 7.58 (d, J = 7.8 Hz, 1H), 7.40–7.44 (m, 7H), 7.22 (m, 3H), 5.39 (s, 2H), 4.44 (s, 2H); HRMS Calcd for $C_{27}H_{20}N_2NaO_3S$ $(M+Na)^+$ 475.1092. Found: 475.1092.

Biological study

Preparation of human placental microsomes. Human term placentas were processed immediately after delivery from The Ohio State University Hospitals at 4 °C. The placenta was washed with normal saline, and connective and vascular tissue was removed. Microsomes were prepared from the remaining tissue using the method previously described.[12] Microsomal suspensions were stored at −80 °C until required.

Inhibition study. Inhibition of human placental aromatase was determined by monitoring the amount of ^3H_2O released as the enzyme converts [1β−^{3}H]androst-4-ene-3,17-dione to estrone. Ten inhibitor concentrations ranging from 0.2 nM to 50 μM were evaluated. Aromatase activity assays were carried out in 0.1 M potassium phosphate buffer (pH 7.0) with 5% propylene glycol. All samples contained a NADPH-regenerating system consisting of 2.85 mM glucose-6-phosphate, 1.8 mM $NADP^+$, and 1.5 U of glucose-6-phosphate dehydrogenase (Sigma, St. Louis, MO). Samples contained 100 nM androst-4-ene-3,17-dione (400,000 450,000 dpm). Reactions were initiated with the addition of 50 μg microsomal protein. The total incubation volume was 2.0 mL. Incubations were allowed to proceed for 15 min in a shaking water bath at 37 °C. Reactions were quenched by the addition of 2.0 mL chloroform. Samples were then vortexed and centrifuged for 5 min and the aqueous layer was removed. The aqueous layer was subsequently extracted twice in the same manner with 2.0 mL chloroform. A 0.5 mL aliquot of the final aqueous layer was combined with 5 mL of 3a70B scintillation cocktail (Research Products International Corp., Mt. Prospect, IL) and the amount of radioactivity was determined. Each sample was run in triplicate and background values were determined with microsomal protein inactivated by boiling. Samples containing 50 μM (±) aminoglutethimide (Sigma, St. Louis, MO) were used a positive control. IC_{50} sigmoidal dose-response data were analyzed with the Graphpad Prism (Version 3.0) program.

Kinetic study. Enzyme kinetic studies of compounds **5d**, **5e**, and **5m** were conducted to investigate the nature of aromatase inhibition. Michaelis Menten enzyme kinetic parameters were determined by varying the concentration of androst-4-ene-3,17-dione from 50 to 800 nM in the presence of fixed concentrations of 0, 25, 100, and 200 nM for all three compounds. Assay conditions were the same as those described in the IC_{50} studies, except that reactions were initiated by the addition of 15 μg microsomal protein. Analysis of the enzyme kinetic data was performed with the weighted linear regression analysis previously described by Cleland.[13]

Tritiated water-release assay in SK-BR-3 cell lines. Measurement of aromatase enzyme activity was based on the tritium water release assay.[14] Cells in T-25 flasks were treated with 0.1% DMSO (control) and inhibitors at the indicated concentrations. After 24 h, the cells were incubated for 6 h with fresh media along with 50 nM androstenedione including 2 μCi [1β-^{3}H]androst-4-ene-3,17-dione. Subsequently, the reaction mixture was removed, and proteins were precipitated using 10% trichloroacetic acid at 42 °C for 20 min. After a brief centrifugation, the media were extracted three times with an equal amount of chloroform to extract unused substrate and further dextran-treated charcoal. After centrifugation, a 250-μl aliquot containing the product was counted in 5 ml of liquid scintillation mixture. Results were corrected for blanks and for the cell contents of culture flasks, and results were expressed as picomoles of ^3H_2O formed per hour incubation time per million live cells (pmol/h/10^6 cells). To determine the amount of cells in each flask, the cells were trypsinized and analyzed using the diphenylamine DNA assay adapted to a 96-well plate.[14,15]

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