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Synthesis and characterization of azole isoflavone inhibitors of aromatase

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Abstract—The synthesis and biological evaluation of a series of 2-azole and 2-thioazole isoflavones as potential aromatase inhibitors are described. Differences in inhibitory activity of triazole and imidazole inhibitors are rationalized with density functional theory to expose a key difference in the electronic structure of these molecules. In addition, difference binding spectra of inhibitors to immunoaffinity-purified aromatase produces classical Type II spectra consistent with coordination of the nitrogen lone pair electrons to the aromatase P450 heme.

1. Introduction

Estrogens are important in the growth of breast cancers in both pre- and post-menopausal women and, estrogen sensitivity in breast cancer increases with patient age. Two-thirds of breast cancer tumors in post-menopausal women are positive for estrogen and/or progesterone receptors, compared to pre-menopausal women where the presence of these receptors appears in less than half of tumors. The endogenous ligands for these receptors, estrogens and progestins, stimulate cell proliferation directly by increasing the rate of early response genes and indirectly through the stimulation of growth factors.[1] Such dependence on sex steroids for tumor growth makes hormonal therapy an inviting strategy for treatment.[2]

Two general strategies have developed for the treatment of hormone-dependent breast cancer. The first strategy is to block estrogen receptor action by disrupting its interaction with and activation by estradiol. Antiestrogens, such as tamoxifen, bind to the estrogen receptor and interfere with the transcription of estrogen-induced genes. The efficacy of tamoxifen has been established in the treatment of post-menopausal, hormone-responsive breast cancer.[3] Tamoxifen increases long-term survival, reduces recurrences, and has few side effects. In addition to tamoxifen's antagonistic effects in breast tissue, it also behaves as a weak or partial agonist in other tissues. While exhibiting antagonistic activity in the breast, the partial agonist effects in other parts of the body have lead to the formation of secondary tumors of the liver and uterus.[4] A second pharmacological approach is to block estradiol synthesis catalyzed by the cytochrome P450 enzyme aromatase (CYP19). Aromatase has been a particularly attractive target for inhibition in the treatment of hormone-dependent breast cancer since the aromatization of androgen substrates is the terminal and rate-limiting step in estrogen biosynthesis.[5–8]

In addition to the large number of aromatase inhibitors that have arisen from medicinal chemistry efforts in pharmaceutical and academic laboratories,[5–9] a significant number of natural product aromatase inhibitors have been identified.[10–12] The flavonoids are one of the largest classes of naturally occurring aromatase inhibitors.[10] Generally, flavones and flavanones have higher aromatase inhibitory activity than isoflavones. Chrysin and Biochanin A (**BCA**) are two of the most potent examples of flavone and isoflavone identified to date, respectively (Fig. 1). The flavone, chrysin, is 20-fold more potent than **BCA** in terms of IC_{50} values.[11,12] This large difference in potency is the likely reason why there has been little effort to develop aromatase inhibitors on an isoflavone scaffold.

Keywords: Aromatase; Inhibitors; Isoflavones; Density functional theory; Difference spectra.
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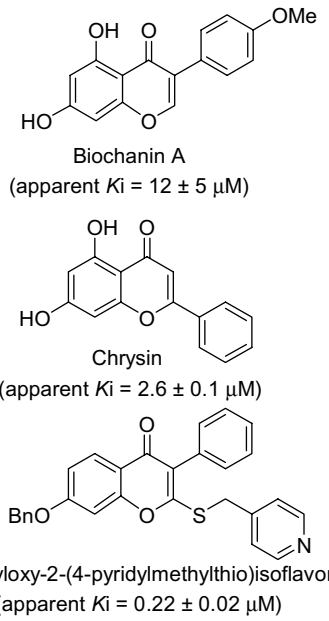


Figure 1. Chemical structures and aromatase inhibitory activities of biochanin A (isoflavone),[14] chrysin (flavone),[12] and the 7-benzyloxy-2-(4-pyridylmethylthio)isoflavone reported by our laboratory.[14]

Isoflavones have diverse pharmacological activities,[13] and we envisioned introduction of the proper functional groups on the isoflavone core could result in the desired aromatase activity. Non-steroidal aromatase inhibitors developed to date are competitive inhibitors possessing a heteroatom that interferes with steroid hydroxylation by coordinating to the heme iron at the P450 active site. Although several heteroatoms, such as sulfur, oxygen, and nitrogen are capable of coordinating with the iron atom, the majority of these compounds possess a nitrogen-containing heterocycle such as imidazole, triazole, pyrimidine, or pyridine.[9] In our previous work, we demonstrated that the introduction of a 2-(4-pyridylmethyl)thio functionality onto the isoflavone nucleus (Fig. 1) afforded a 160-fold enhancement in potency compared to the natural product lead, **BCA**.[14] This study demonstrated proof of the principle that introduction of appropriate nitrogen-containing heterocycle at the 2-position of the isoflavone nucleus is beneficial for aromatase inhibition. In addition, a hydrophobic functionality in the 7-position of the isoflavone and the reduced necessity of the 4′-substituent were important in achieving desirable affinity for the enzyme.

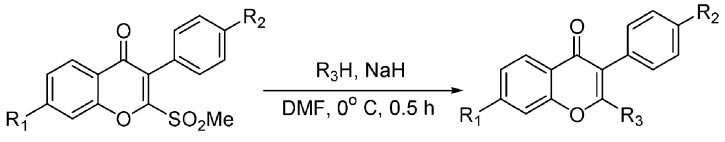
We have now focused on the synthesis of azole isoflavones, which were easily prepared by the phase transfer catalysis developed in our laboratory.[15,16] In this series, we are interested in exploring the potential contribution of the imidazole, triazole, thioimidazole, and thiotriazole toward aromatase inhibition. Herein, we describe diversification of the 2-position of isoflavones and examine their contributions to the structure–activity relationships of azole isoflavone aromatase inhibitors.

2. Results and discussion

The 2-(methylsulfonyl)isoflavones **1a–d** were prepared from the corresponding 2-(methylthio)isoflavones with *m*CPBA using the method previously described by our laboratory.[15,16] Azole isoflavones **2a–d** were prepared from 2-(methylsulfonyl)isoflavones **1a–d** and thioazole isoflavones **3a–d** were prepared from **1a** in a single step in moderate to good yields by displacing the 2-methylsulfonyl group with various nucleophiles (Scheme 1).

Evaluation of the compounds for aromatase inhibitory activity was performed using the tritiated water release aromatase assay with human placental microsomes as the aromatase source, as previously reported by our laboratory.[14] IC_{50} values of the compounds were determined in dose–response studies (Table 1). The IC_{50} values for (±)-aminoglutethimide (**AG**) and **BCA** were also determined for comparison. Kinetic studies were undertaken on promising compounds to examine their mode of aromatase inhibition. The apparent K_i values for each compound, assay-specific apparent K_m values, and K_i/K_m ratios are listed in Table 2. The Lineweaver–Burk plot of compound **2c** is shown in Figure 2.

In dose–response studies (Table 1), 2-thioimidazole (**3b**,**c**) and 2-thiotriazole (**3d**) isoflavone analogs



1a: R_1 = OMe, R_2 = H,
1b: R_1 = OMe, R_2 = OMe
1c: R_1 = OBn, R_2 = H
1d: R_1 = OBn, R_2 = OMe

2a: R_1 = OMe, R_2 = H, R_3 = imidazol-1-yl
2b: R_1 = OMe, R_2 = OMe, R_3 = imidazol-1-yl
2c: R_1 = OBn, R_2 = H, R_3 = imidazol-1-yl
2d: R_1 = OBn, R_2 = OMe, R_3 = imidazol-1-yl
3a: R_1 = OMe, R_2 = H, R_3 = 1*H*-1,2,4-triazol-1-yl
3b: R_1 = OMe, R_2 = H, R_3 = 1*H*-imidazolyl-2-thio
3c: R_1 = OMe, R_2 = H, R_3 = 1-methyl-1*H*-imidazolyl-2-thio
3d: R_1 = OMe, R_2 = H, R_3 = 2*H*-1,2,4-triazolyl-3-thio

Scheme 1. Synthesis of azole isoflavones **2a–d** and **3a–d**.

Table 1. IC_{50} values for aromatase inhibition by azole isoflavones and reference compounds

	IC_{50} (μM)	Log IC_{50} (nM) (±S.E.)[a]
2a	0.77	2.89 ± 0.04
2b	2.0	3.29 ± 0.04
2c	0.52	2.71 ± 0.03
2d	4.7	3.67 ± 0.09
3a	18	4.26 ± 0.09
3b	>50	
3c	>50	
3d	>50	
AG	2.8	3.45 ± 0.05
BCA	34	4.53 ± 0.06

[a] IC_{50} values were calculated by nonlinear regression analysis in GraphPad Prism. Data for each dose-response curve were obtained by evaluating aromatase inhibition at ten concentrations of the compound, each in triplicate.

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

	Apparent K_i[a]	Apparent K_m[a]	K_i/K_m
2a	0.68 ± 0.04	0.10 ± 0.00	6.8
2b	1.82 ± 0.15	0.10 ± 0.01	18
2c	0.25 ± 0.02	0.07 ± 0.00	3.6
2d	4.40 ± 0.34	0.12 ± 0.01	37
AG	1.41 ± 0.10	0.09 ± 0.01	16
BCA	12 ± 5[b]		

[a] Values were calculated by weighted regression analysis[13] and expressed in μM ± standard error.
[b] Ref. 11.

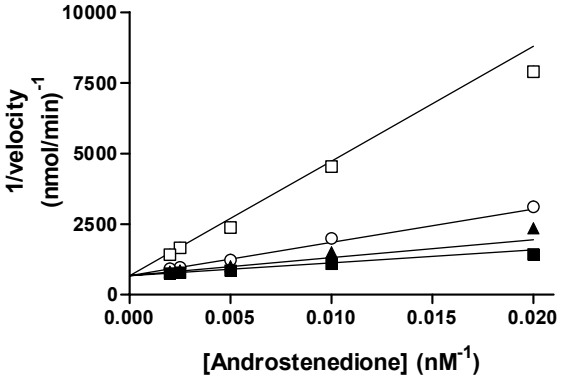


Figure 2. Lineweaver–Burk plot of aromatase inhibition by compound **2c**. Androstenedione at concentrations of 50–500 nM was incubated with microsomal enzyme preparations with inhibitor concentrations of 0 nM (■), 100 nM (▲), 500 nM (○), and 2000 nM (□). Each point represents the mean of three determinations ± standard error.

displayed poor or no inhibitory activity, with IC_{50} values greater than 50 μM. In contrast to the thioazole analogs, compounds with the heterocycle attached directly to the isoflavone scaffold showed promising inhibitory activity. The consequence of the nature of the azole heterocycle is exemplified by comparing the IC_{50} values of 7-methoxy analogs **2a** and **3a**. The imidazole analog **2a** is 23-fold more potent than the corresponding triazole. The increased potency of imidazole derivatives compared to triazole analogs was also observed in other non-steroidal aromatase inhibitors.[9] Recanatini and coworkers previously postulated the coordinating ability of the nitrogen heterocycle is related to the distribution of the highest-occupied molecular orbital (HOMO) and the resulting electron density over the potentially iron-coordinating heterocycle. This postulate was used to partially rationalize the observed differences in potency between imidazomethyl-and triazomethyl-xanthones.[17] To test this hypothesis, we fully optimized the geometries of **2a** and **3a** with the B3LYP[18] hybrid density functional and 6-31G(d) basis set in the Gaussian 98[19] suite of programs. The optimized geometries of these molecules share similar conformational preferences and may bind similarly to the aromatase active site (Fig. 3). As expected, the HOMO of the imidazole analog **2a** is more densely localized on the nitrogen heterocycle than in **3a**. Difference spectra of this pair of compounds were obtained with immunoaffinity-purified human placental aromatase (Hauptman–Woodward Medical Research Institute, Buffalo, NY). Both compounds produce classical Type II spectra consistent with an increase in concentration of low-spin iron as a result of azole ligation. Compound **2a** and **3a** have absorption minima at 394 and 391 nm, respectively, while both spectra have maxima at 430 nm (Fig. 4). The smaller change in absorbance induced by compound **3a** is consistent with triazole being a weaker ligand for the

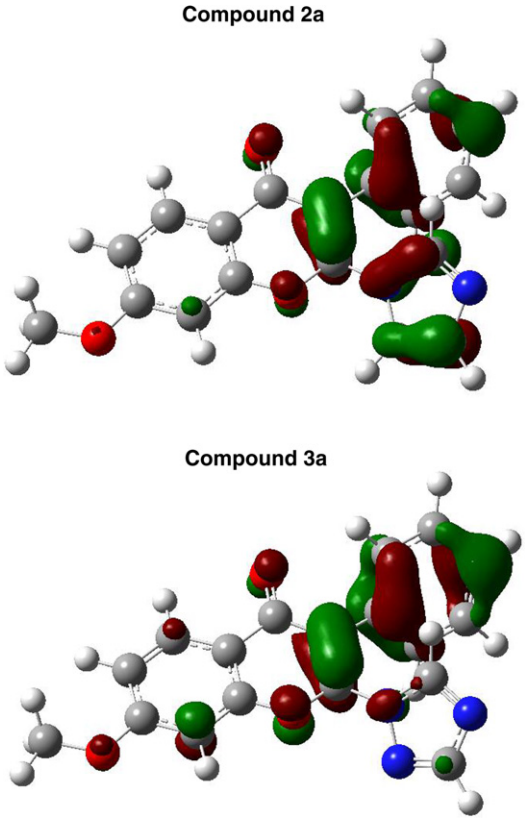


Figure 3. B3LYP/6-31G(d) optimized geometries of compound **2a** (top) and compound **3a** (bottom) displaying the surfaces of the highest-occupied molecular orbital (HOMO). A contour value of 0.05 e/B^3 was used.

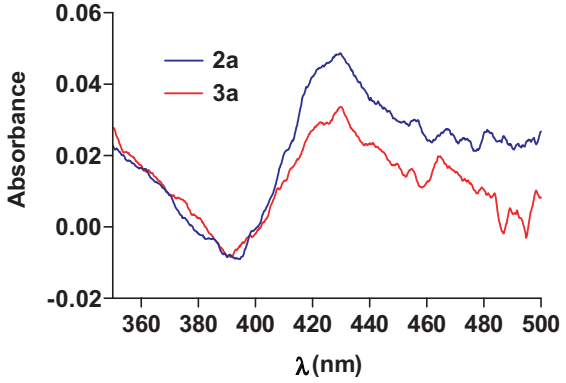


Figure 4. Type II difference spectra of immunoaffinity-purified human placental aromatase induced by 50 μM compound **2a** (blue) and compound **3a** (red).

heme iron. Taken together with the density functional theory (DFT) calculations, these observations explain the superior potency of the imidazole analog relies on the availability of the nitrogen lone pair electrons to coordinate with the aromatase P450 heme.

In general, imidazole isoflavone inhibitors presented in this work share similar structure–activity relationships to those reported for 2-(4-pyridylmethyl)thioisoflavones.[14] Regardless of the 7-substituent, a decrease in potency was observed when a methoxy group was introduced at the 4′-position. Additionally, the relative decrease in potency is dependent on the nature of the 7-substituent. 4′,7-Dimethoxy analog, **2b**, is 2.6-fold less potent than the 7-methoxy analog **2a**. The loss of potency is more pronounced when considering the 7-benzyloxy analogs. The presence of the 4′-methoxy in **2d** results in a nine-fold loss of activity when compared to **2c**. 4′,7-Dimethoxy-(4-pyridylmethylthio)isoflavone and 7-benzyloxy-4′-methoxy-2-(4-pyridyl-methylthio)isoflavone are 1.2- and 2.5-times less active than their 4′-H analogs. This observation may indicate the nature of the 4′-substituent may be a more important factor in the imidazole isoflavones in terms of aromatase inhibition.

In enzyme kinetic studies, imidazole isoflavones **2a**–**d** demonstrated typical competitive-type inhibition in the Lineweaver–Burk plots (Fig. 2 for **2c**; plots for **2a**, **b**, **d** not shown) supporting their inhibition of aromatase by competing with the natural substrate for the active site. K_i/K_m ratios of imidazole isoflavones were calculated as relative inhibitory potency and the same activity trend is observed as in the dose–response studies. Compound **2c** demonstrates 48-fold enhancement in potency compared to the natural product lead, **BCA**. As reflected by its relative potency, imidazole analog **2c** is 4.4-fold more potent than the widely characterized inhibitor **AG**, which is in agreement within the expected experimental uncertainty of the result determined in the dose–response studies (5.4-fold enhancement in potency compared to **AG**). The results of the present enzyme kinetic studies emphasize the importance of these structural modifications for optimization of aromatase inhibition by isoflavones.

3. Conclusion

Our recent work on 2-(4-pyridylmethylthio)isoflavones demonstrated for the first time that effective isoflavone-based aromatase inhibitors can be constructed by the introduction of appropriate nitrogen-containing heterocycle. This study further extends this concept that aromatase inhibitory activity can be achieved with the isoflavone nucleus by introduction of an appropriate heme-coordinating nitrogen heterocycle at the 2-position of the isoflavone. Comparable potency to the 2-(4-pyridylmethylthio)isoflavones can be obtained by introducing an imidazole moiety at the 2-position. Enzyme kinetic analyses of imidazole isoflavones reveal these compounds interact competitively with the aromatase active site. As revealed by DFT calculations and the difference spectra of ligand binding, the superiority of this heterocycle to achieve aromatase inhibition may lie in the electronic structure of the nitrogen heterocycle itself. While investigations are currently underway to resolve further aspects of the enzyme–ligand interactions, these compounds are additional leads in our repertoire of potent aromatase inhibitors.

4. Experimental section

4.1. 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 pre-coated silica gel F254 plates (Whatman). Silica gel column chromatography was performed using silica gel 60A (Merck, 230-400 Mesh). Melting points were determined in open glass capillaries using a Thomas Hoover apparatus and are uncorrected. Infrared spectra were recorded on a Nicolet Protégé 460 spectrometer using KBr pellets. 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, or Bruker DRX 400 model spectrometer in either DMSO-d_6 or $CDCl_3$. Chemical shifts (δ) for ^{1}H NMR spectra are reported in parts per million to residual solvent protons. Chemical shifts (δ) for ^{13}C NMR spectra are reported in parts per million relative to residual solvent carbons.

4.2. General method A for the preparation of 2-(alkylsulfonyl)isoflavones (1a–d)

A mixture of 7-alkoxy-3-aryl-2-(methylthio)-4*H*-1-benzopyran-4-one (8.0 mmol) and 3-chloroperoxybenzoic acid (*m*CPBA) (4.14 g, 24 mmol) in CH_2Cl_2 (80 mL) were stirred under reflux for 2 h. After the solvent was removed under reduced pressure, the residue was

suspended in diethyl ether and hexane (1:1, 100 mL), sonicated, and placed in a refrigerator overnight. The insoluble solid was collected by filtration, washed with diethyl ether and hexane (1:1) several times, and recrystallized from ethyl acetate to give desired product.

4.2.1. 7-Methoxy-2-(methylsulfonyl)-3-phenyl-4*H*-1-benzopyran-4-one (1a). Compound **1a** was synthesized from 7-methoxy-2-(methylthio)-3-phenyl-4*H*-1-benzopyran-4-one (2.39 g, 8.0 mmol) using General Method A, and 2.59 g (98%) of the title compound was obtained as a pale yellow solid: mp 208–210 °C; HRMS calculated for $C_{17}H_{14}NaO_5S$ $(M+Na)^+$ 353.0460, found 353.0459. IR (KBr) 1648, 1622, 1604, 1569, 1436, 1326, 1256, 1203, 1155, 1134, 1100, 1052, 1029, 962, 825, 772, 755, 583 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.94 (d, $J = 8.9$ Hz, 1H), 7.36–7.38 (m, 3H), 7.34 (d, $J = 2.3$ Hz, 1H), 7.25–7.27 (m, 2H), 7.12 (dd, $J = 8.9$, 2.3 Hz, 1H), 3.91 (s, 3H), 3.30 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.39, 165.70, 157.72, 157.47, 131.39, 129.98, 129.26, 128.41, 127.86, 124.68, 117.28, 117.03, 101.73, 57.30, 42.46.

4.2.2. 7-Methoxy-3-(4-methoxyphenyl)-2-(methylsulfonyl)-4*H*-1-benzopyran-4-one (1b). Compound **1b** was synthesized from 7-methoxy-3-(4-methoxyphenyl)-2-(methylthio)-4*H*-1-benzopyran-4-one (2.63 g, 8.0 mmol) using General Method A, and 2.68 g (93%) of the title compound was obtained as a pale yellow solid: mp 227–228.5 °C; HRMS calculated for $C_{18}H_{16}NaO_6S$ $(M+Na)^+$ 383.0565, found 383.0569. IR (KBr) 1646, 1618, 1610, 1570, 1509, 1438, 1337, 1311, 1261, 1246, 1203, 1176, 1141, 1100, 1024, 973, 955, 849, 773, 588, 539, 520, 482 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.94 (d, $J = 8.9$ Hz, 1H), 7.33 (d, $J = 2.3$ Hz, 1H), 7.19 (d, $J = 8.6$ Hz, 2H), 7.11 (dd, $J = 8.9$, 2.3 Hz, 1H), 6.94 (d, $J = 8.7$ Hz, 2H), 3.91 (s, 3H), 3.77 (s, 3H), 3.28 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.59, 165.65, 160.31, 157.75, 157.43, 132.81, 127.88, 124.37, 121.66, 117.24, 116.96, 113.96, 101.69, 57.27, 55.96, 42.44.

4.2.3. 2-(Methylsulfonyl)-3-phenyl-7-(benzyloxy)-4*H*-1-benzopyran-4-one (1c). Compound **1c** was synthesized from 2-(methylthio)-3-phenyl-7-(benzyloxy)-4*H*-1-benzopyran-4-one (3.0 g, 8.0 mmol) using General Method A, and 3.12 g (96%) of the title compound was obtained as a white solid: mp 198–199 °C; HRMS calculated for $C_{23}H_{18}NaO_5S$ $(M+Na)^+$ 429.0773, found 429.0761. IR (KBr) 1651, 1620, 1568, 1499, 1440, 1332, 1256, 1161, 1140, 1113, 1027, 1005, 975, 832, 775, 744, 700, 616 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.95 (d, $J = 8.9$ Hz, 1H), 7.26–7.48 (m, 11H), 7.19 (dd, $J = 8.9$, 2.0 Hz, 1H), 5.28 (s, 2H), 3.29 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.39, 164.66, 157.72, 157.37, 136.77, 131.94, 131.40, 129.99, 129.46, 129.27, 129.10, 128.77, 128.42, 127.94, 124.77, 117.45, 102.71, 71.16, 42.53.

4.2.4. 3-(4-Methoxyphenyl)-2-(methylsulfonyl)-7-(benzyloxy)-4*H*-1-benzopyran-4-one (1d). Compound **1d** was synthesized from 2-(methylthio)-3-phenyl-7-(benzyloxy)-4*H*-1-benzopyran-4-one (3.24 g, 8.0 mmol) using General Method A, and 3.39 g (97%) of the title compound was obtained as a white solid: mp 226–227 °C; HRMS calculated for $C_{24}H_{20}NaO_6S$ $(M+Na)^+$ 459.0878, found 459.0868. IR (KBr) 1648, 1624, 1609, 1578, 1569, 1510, 1439, 1332, 1292, 1248, 1162, 1138, 1101, 1030, 972, 840, 827, 774, 698, 613, 536, 525 cm^{-1}; ^1H NMR (250 MHz, DMSO-d_6) δ 7.95 (d, $J = 8.9$ Hz, 1H), 7.33–7.49 (m, 6H), 7.17–7.21 (m, 3H), 6.94 (d, $J = 8.7$ Hz, 2H), 5.29 (s, 2H), 3.77 (s, 3H), 3.27 (s, 3H); ^{13}C NMR (62.9 MHz, DMSO-d_6) δ 176.57, 164.60, 160.32, 157.75, 157.32, 136.79, 132.83, 129.46, 129.10, 128.77, 127.95, 124.45, 121.67, 117.41, 117.38, 113.97, 101.67, 71.14, 55.96, 42.51.

4.3. General Method B for nucleophilic substitution reactions of 2-(alkylsulfonyl)isoflavones (2a–d and 3a–d)

A solution of 7-alkoxy-3-aryl-2-(methylsulfonyl)-4*H*-1-benzopyran-4-one (1.0 mmol) in DMF (1 mL) was added to a stirred solution of a nucleophile (as a sodium salt purchased or generated in situ by the treatment of sodium hydride) in DMF (3 mL) at 0 °C. After stirring at 0 °C for 0.5 h, most reactions were completed according to TLC. The reaction mixture was allowed to warm to room temperature over 1 h, then cooled to 0 °C, and quenched with saturated aqueous NH_4Cl solution. After the volatile solvents were removed in vacuo, the residual solid was suspended in a mixture of water and EtOAc, ultrasonicated for 5 min, and cooled to 0 °C. Insoluble solid was collected by filtration and the filter cake was washed with EtOAc/hexane mixture to give desire product. The filtrate was extracted with EtOAc twice (2×10 mL), and the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The remnant was purified by silica gel column chromatography (eluting with MeOH/$CHCl_3$) to collect additional product. The combined solid was further purified by recrystallization.

4.3.1. 2-(1*H*-Imidazol-1-yl)-7-methoxy-3-phenyl-4*H*-1-benzopyran-4-one (2a). Compound **2a** was synthesized from 7-methoxy-2-(methylsulfonyl)-3-phenyl-4*H*-1-benzopyran-4-one (0.330 g, 1.0 mmol) and sodium salt of imidazole (0.180 g, 2.0 mmol, purchased) using General Method B, and 0.231 g (73%) of the title compound was obtained as a white solid (recrystallized from EtOAc): mp 217–219 °C; HRMS calculated for $C_{19}H_{15}N_2O_3$ $(M+H)^+$ 319.1082, found 319.1060. IR (KBr) 1640, 1619, 1575, 1492, 1441, 1404, 1342, 1266, 1255, 1201, 1104, 1053, 1016, 953, 908, 834, 784, 754, 738, 703, 648 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, $J = 8.9$ Hz, 1H), 7.71 (s, 1H), 7.31–7.33 (m, 3H), 7.28 (d, $J = 2.4$ Hz, 1H), 7.17–7.20 (m, 3H), 7.12 (dd, $J = 8.9$, 2.4 Hz, 1H), 6.93 (s, 1H), 3.89 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.84, 165.15, 156.61, 151.04, 138.23, 131.39, 131.04, 129.92, 129.21, 129.00, 127.81, 120.31, 116.99, 166.20, 116.18, 101.65, 57.14. Elemental analysis: Calcd for $C_{19}H_{14}N_2O_3$ (%): C, 71.69; H, 4.43; N, 8.80. Found: C, 71.56; H, 4.49; N, 8.78.

4.3.2. 2-(1*H*-Imidazol-1-yl)-7-methoxy-3-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one (2b). Compound **2b** was synthesized from 7-methoxy-3-(4-methoxyphenyl)-2-(methylsulfonyl)-4*H*-1-benzopyran-4-one (0.36 g, 1.0 mmol)

and sodium salt of imidazole (0.180 g, 2.0 mmol, purchased) using General Method B, and 0.324 g (93%) of the title compound was obtained as a white solid (recrystallized from EtOH): mp 232–233 °C; HRMS calculated for $C_{20}H_{16}N_2NaO_4$ $(M+Na)^+$ 371.1008, found 371.1015. IR (KBr) 1639, 1611, 1578, 1515, 1441, 1405, 1342, 1291, 1253, 1201, 1174, 1054, 1024, 831, 816, 747 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, $J = 8.9$ Hz, 1H), 7.62 (s, 1H), 7.12 (d, $J = 8.7$ Hz, 2H), 7.00–7.03 (m, 2H), 6.96 (s, 1H), 6.89–6.91 (m, 3H), 3.92 (s, 3H), 3.80 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.20, 164.98, 160.18, 156.18, 150.21, 137.22, 131.71, 130.38, 128.41, 122.28, 118.77, 117.12, 115.46, 115.01, 114.43, 100.46, 56.41, 55.67. Elemental analysis: Calcd for $C_{20}H_{16}N_2O_4$ (%): C, 68.96; H, 4.63; N, 8.04. Found: C, 68.73; H, 4.72; N, 7.94.

4.3.3. 2-(1*H*-Imidazol-1-yl)-3-phenyl-7-(benzyloxy)-4*H*-1-benzopyran-4-one (2c). Compound **2c** was synthesized from 2-(methylsulfonyl)-3-phenyl-7-(benzyloxy)-4*H*-1-benzopyran-4-one (0.41 g, 1.0 mmol) and sodium salt of imidazole (0.180 g, 2.0 mmol, purchased) using General Method B, and 0.323 g (82%) of the title compound was obtained as a white solid (recrystallized from EtOAc): mp 152–153 °C; HRMS calculated for $C_{25}H_{19}N_2O_3$ $(M+H)^+$ 395.1395, found 395.1396. IR (KBr) 1633, 1610, 1572, 1494, 1441, 1402, 1389, 1336, 1251, 1193, 1150, 1103, 1094, 1051, 1017, 991, 908, 839, 790, 740, 699 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, $J = 8.9$ Hz, 1H), 7.58 (s, 1H), 7.35–7.45 (m, 8H), 7.19–7.21 (m, 2H), 7.10 (dd, $J = 8.9$, 2.3 Hz, 1H), 6.99 (s, 1H), 6.97 (d, $J = 2.3$ Hz, 1H), 6.92 (s, 1H), 5.19 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.90, 164.06, 156.12, 150.35, 137.19, 135.85, 130.53, 130.41, 129.47, 129.26, 129.13, 128.96, 128.49, 127.91, 118.77, 117.32, 116.08, 114.82, 101.58, 71.14. Elemental analysis: Calcd for $C_{25}H_{18}N_2O_3$ (%): C, 76.13; H, 4.60; N, 7.10. Found: C, 76.24; H, 4.71; N, 7.10.

4.3.4. 2-(1*H*-Imidazol-1-yl)-3-(4-methoxyphenyl)-7-(benzyloxy)-4*H*-1-benzopyran-4-one (2d). Compound **2d** was synthesized from 3-(4-methoxyphenyl)-2-(methylsulfonyl)-7-(benzyloxy)-4*H*-1-benzopyran-4-one (0.71 g, 1.63 mmol) and sodium salt of imidazole (0.30 g, 3.25 mmol, purchased) using General Method B, and 0.604 g (87%) of the title compound was obtained as a white solid (recrystallized from EtOAc): mp 160–161 °C; HRMS calculated for $C_{26}H_{21}N_2O_4$ $(M+H)^+$ 425.1501, found 425.1511. IR (KBr) 1637, 1613, 1577, 1512, 1439, 1403, 1339, 1293, 1245, 1194, 1175, 1098, 1055, 1018, 908, 823, 748, 700, 654 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, $J = 8.9$ Hz, 1H), 7.61 (s, 1H), 7.36–7.45 (m, 5H), 7.12 (d, $J = 8.7$ Hz, 2H), 7.09 (dd, $J = 8.9$, 2.3 Hz, 1H), 7.00 (s, 1H), 6.96 (d, $J = 2.3$ Hz, 1H), 6.95 (s, 1H), 6.90 (d, $J = 8.7$ Hz, 2H), 5.18 (s, 2H), 3.80 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.17, 163.99, 160.19, 156.09, 150.23, 137.22, 135.87, 131.70, 130.38, 129.25, 128.94, 128.49, 127.90, 122.25, 118.77, 117.31, 116.01, 115.02, 114.47, 101.54, 71.12, 55.67. Elemental analysis: Calcd for $C_{26}H_{20}N_2O_4$ (%): C, 73.57; H, 4.75; N, 6.60. Found: C, 73.54; H, 4.82; N, 6.55.

4.3.5. 7-Methoxy-3-phenyl-2-(1*H*-1,2,4-triazol-1-yl)-4*H*-1-benzopyran-4-one (3a). Compound **3a** was synthesized from 7-methoxy-2-(methylsulfonyl)-3-phenyl-4*H*-1-benzopyran-4-one (0.165 g, 0.5 mmol) and sodium salt of 1,2,4-triazole (0.091 g, 1.0 mmol, purchased) using General Method B, and 0.144 g (90%) of the title compound was obtained as a white solid (recrystallized from EtOAc): mp 188.5–189.5 °C; HRMS calculated for $C_{18}H_{13}N_3NaO_3$ $(M+Na)^+$ 342.0855, found 342.0843. IR (KBr) 1643, 1619, 1575, 1502, 1429, 1337, 1262, 1217, 1203, 1124, 1104, 1060, 993, 920, 837, 831, 785, 755, 705, 662 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.63 (s, 1H), 8.21 (s, 1H), 8.01 (d, $J = 8.9$ Hz, 1H), 7.28–7.29 (m, 4H), 7.11–7.16 (m, 3H), 3.89 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.56, 165.48, 156.70, 153.62, 150.08, 147.29, 130.79, 130.67, 129.01, 128.92, 127.98, 119.39, 117.14, 116.57, 101.69, 57.25. Elemental analysis: Calcd for $C_{18}H_{13}N_3O_3$ (%): C, 67.71; H, 4.10; N, 13.16. Found: C, 67.60; H, 4.14; N, 13.06.

4.3.6. 2-(1*H*-Imidazolyl-2-thio)-7-methoxy-3-phenyl-4*H*-1-benzopyran-4-one (3b). Compound **3b** was synthesized from 7-methoxy-2-(methylsulfonyl)-3-phenyl-4*H*-1-benzopyran-4-one (0.165 g, 0.5 mmol), 2-mercaptoimidazole (0.153 g, 1.5 mmol), and sodium hydride (0.036 g, 1.5 mmol) using General Method B, and 0.156 g (89%) of the title compound was obtained as a white solid (recrystallized from EtOH): mp 248–250 °C (decomposed); HRMS calculated for $C_{19}H_{14}N_2NaO_3S$ $(M+Na)^+$ 373.0623, found 373.0640. IR (KBr) 3434, 1622, 1589, 1537, 1437, 1376, 1349, 1331, 1260, 1205, 1104, 1028, 939, 831, 784, 760, 711 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 12.95 (br s, 1H), 7.91 (d, $J = 8.9$ Hz, 1H), 7.38–7.47 (m, 4H), 7.33–7.35 (m, 2H), 7.11 (s, 1H), 7.04 (dd, $J = 8.9$, 2.3 Hz, 1H), 6.55 (d, $J = 2.3$ Hz, 1H), 3.81 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.85, 164.59, 160.91, 158.42, 132.57, 131.69, 131.48, 130.14, 129.27, 129.19, 127.97, 123.41, 122.38, 117.12, 115.75, 100.79, 56.99. Elemental analysis: Calcd for $C_{19}H_{14}N_2O_3S$ (%): C, 71.69; H, 4.43; N, 8.80. Found: C, 71.56; H, 4.49; N, 8.78.

4.3.7. 7-Methoxy-2-(1-methyl-1*H*-imidazolyl-2-thio)-3-phenyl-4*H*-1-benzopyran-4-one (3c). Compound **3c** was synthesized from 7-methoxy-2-(methylsulfonyl)-3-phenyl-4*H*-1-benzopyran-4-one (0.165 g, 0.5 mmol), 2-mercapto-1-methylimidazole (0.171 g, 1.5 mmol), and sodium hydride (0.036 g, 1.5 mmol) using General Method B, and 0.175 g (96%) of the title compound was obtained as a white solid (recrystallized from EtOAc): 172–173 °C; HRMS calculated for $C_{20}H_{17}N_2O_3S$ $(M+H)^+$ 365.0960, found 365.0955. IR (KBr) 1640, 1618, 1590, 1499, 1437, 1368, 1344, 1253, 1198, 1099, 1016, 939, 783, 756, 698 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, $J = 9.0$ Hz, 1H), 7.51 (d, $J = 1.0$ Hz, 1H), 7.40–7.48 (m, 3H), 7.34–7.36 (m, 2H), 7.11 (d, $J = 1.2$ Hz, 1H), 7.03 (dd, $J = 9.0$, 2.3 Hz, 1H), 6.57 (d, $J = 2.3$ Hz, 1H), 3.82 (s, 3H), 3.68 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.88, 164.60, 160.85, 158.42, 132.59, 131.89, 131.46, 131.06, 129.30, 129.22, 127.98, 126.94, 123.53, 117.15, 115.70, 100.96, 57.02, 34.68. Elemental analysis: Calcd for

$C_{20}H_{16}N_2O_3S$ (%): C, 65.92; H, 4.43; N, 7.69. Found: C, 65.68; H, 4.46; N, 7.62.

4.3.8. 7-Methoxy-3-phenyl-2-(1*H*-1,2,4-triazolyl-3-thio)-4*H*-1-benzopyran-4-one (3d). Compound **3d** was synthesized from 7-methoxy-2-(methylsulfonyl)-3-phenyl-4*H*-1-benzopyran-4-one (0.165 g, 0.5 mmol), 1*H*-1,2,4-triazole-3-thiol (0.152 g, 1.5 mmol), and sodium hydride (0.036 g, 1.5 mmol) using General Method B, and 0.075 g (43%) of the title compound was obtained as a pale yellow solid (recrystallized from EtOH): mp 246–247 °C; HRMS calculated for $C_{18}H_{13}N_3NaO_3S$ $(M+Na)^+$ 374.0575, found 374.0586. IR (KBr) 1612, 1582, 1502, 1440, 1379, 1349, 1262, 1201, 1185, 1104, 1014, 969, 939, 830, 783, 758, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.69 (s, 1H), 7.91 (d, J = 8.9 Hz, 1H), 7.36–7.45 (m, 3H), 7.32–7.34 (m, 2H), 7.04 (dd, J = 8.9, 2.3 Hz, 1H), 6.73 (d, J = 2.3 Hz, 1H), 3.81 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.51, 164.83, 159.47, 158.67, 146.80, 132.74, 131.27, 129.33, 129.17, 127.96, 126.01, 117.02, 116.01, 100.81, 56.99. Elemental analysis: Calcd for $C_{18}H_{13}N_3O_3S$ (%): C, 61.53; H, 3.73; N, 11.96. Found: C, 61.27; H, 3.76; N, 11.86.

4.4. Enzymology

4.4.1. 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 described by Kellis and Vickery.[20] Microsomal suspensions were stored at −80 °C until required.

4.4.2. 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 100 nM to 50 μM were evaluated. Aromatase activity assays were carried 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 units 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 of 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 3a70B scintillation cocktail (Research Products International Corp., Mt. Prospect, IL) and the amount of radioactivity 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} dose–response data were analyzed with the Graphpad Prism (Version 3.0) program.

4.4.3. Kinetic study. Enzyme kinetic studies of compounds **2a**–**d** 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 500 nM in the presence of a fixed concentrations of 0, 100, 500, 2000 nM (**2a**,**c**) and 0, 2000, 5000, 10,000 nM (**2b**,**d**). Assay conditions were the same as those described in the IC_{50} studies except 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.[21]

4.4.4. Determination of P450 difference spectra. Immunoaffinity-purified human placental cytochrome P450 aromatase (Hauptman-Woodward Medical Research Institute, Buffalo, NY) was diluted to 7.0 μg/mL with 0.1 M Tris Buffer (pH 7.4) in a 50 μL quartz UV cell, and the absorbance was scanned from 350 to 500 nm. 0.1 M Tris Buffer (pH 7.4) was used as a reference. Compounds **2a** and **3a** were introduced into the sample cell in 1 μL of ethanol for a final concentration of 50 μM, and the spectrum was rescanned at the aforementioned wavelength range. Multiple scans were taken to ensure spectrum stability for aromatase and aromatase/inhibitor mixtures. Absorbance values of aromatase were subtracted from the aromatase/inhibitor values to generate the difference spectrum.

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References and notes

1. Dickson, R. B.; Lippman, M. E. *Endocrine Rev.* **1995**, *16*, 559.
2. McGuire, W. L. In *Hormones and Cancer*; Iacobelli, S., King, R. J. B., Lidner, H. R., Lippman, M. E., Eds.; Raven: New York, 1980; Vol. 15, pp 337–344.
3. Early Breast Cancer Trialist's Group *Lancet* **1992**, *339*, 1.
4. Jordan, V. C. *Ann. Rev. Pharmacol. Toxicol.* **1995**, *35*, 195.
5. Brodie, A.; Lu, Q.; Long, B. *J. Steroid. Biochem. Mol. Biol.* **1999**, *69*, 205.
6. Santen, R. J.; Harvey, H. A. *Endocr. Relat. Cancer* **1999**, *6*, 75.
7. Brueggemeier, R. W. *Am. J. Ther.* **2001**, *8*, 333.
8. Brueggemeier, R. W. *Expert Rev. Anticancer Ther.* **2002**, *2*, 181.
9. Recanatini, M.; Cavalli, A.; Valenti, P. *Med. Res. Rev.* **2002**, *22*, 282.

10. Hodek, P.; Trefil, P.; Stiborová, M. *Chem. Biol. Interact.* **2002**, *139*, 1.
11. Kao, Y.-C.; Zhou, C.; Sherman, M.; Laughton, C. A.; Chen, S. *Environ. Health Perspect.* **1998**, *106*, 85.
12. Kellis, J. T., Jr.; Vickery, L. E. *Science* **1984**, *225*, 1032.
13. Ren, W.; Qiao, Z.; Wang, H.; Zhu, L.; Zhang, L. *Med. Res. Rev.* **2003**, *23*, 519.
14. Kim, Y.-W.; Hackett, J. C; Brueggemeier, R. W. *J. Med. Chem.* **2004**, *47*, 4032.
15. Kim, Y.-W.; Brueggemeier, R. W. *Tetrahedron Lett.* **2002**, *43*, 6113.
16. Kim, Y.-W.; Mobley, J. A.; Brueggemeier, R. W. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1475.
17. Recanatini, M.; Bisi, A.; Cavalli, A.; Belluti, F.; Gobbi, S.; Rampa, A.; Valenti, P.; Palzer, M.; Palusczak, A.; Hartmann, R. W. *J. Med. Chem.* **2001**, *44*, 672.
18. Stevens, P. J.; Devlin, F. J.; Chablowski, C. F.; Frisch, M. J. *J. Phys. Chem.* **1994**, *98*, 11623.
19. *Gaussian 98*, Revision A.11.3. Frisch, M. J. et al. Gaussian, Inc. Pittsburgh, PA, 2002.
20. Kellis, J. T., Jr.; Vickery, L. E. *J. Biol. Chem.* **1987**, *262*, 4413.
21. Cleland, W. W. *Method. Enzymol.* **1979**, *63*, 103.