# Structure–Activity Relationships of Estrogen Derivatives as Aromatase Inhibitors. Effects of Heterocyclic Substituents

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**Aromatase, which is responsible for the conversion of androgens to estrogens, is a potential therapeutic target for the selective lowering estrogen level in patients with estrogen-dependent breast cancer. We prepared and tested series of the pyridine- and other heterocyclic ring-containing derivatives of 2- and 4-aminoestrones, estrone, and estradiol, compounds 5, 10, 12 and 15. The isonicotinyl derivatives of 2- and 4-aminoestrone, compounds 5c and 10c, were fairly potent competitive inhibitors of aromatase ($K_i$, 2.1±0.14 and 1.53±0.08 $\mu$M for 5c and 10c, respectively) and other compounds did not show, to a significant extent, the aromatase inhibitory activity. This result suggests that the isonicotinyl-substituted derivatives 5c and 10c would be accessible to the active site of aromatase.**

**Key words** aromatase; inhibition activity; estrone derivative; estradiol derivative; isonicotinyl derivative; competitive inhibitor

Aromatase is a cytochrome P-450 enzyme responsible for catalyzing the conversion of the androgens, androstenedione (AD) and testosterone to the estrogens, estrone and estradiol, respectively.[1—3] This process appears to proceed with three oxygenations of the androgens, each of which requires 1 mol of $O_2$ and 1 mol of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The 19-methyl group, as well as 1$\beta$ and 2$\beta$-hydrogens, are eliminated in the third oxygenative step, resulting in aromatization of the A-ring of the androgens.[4—10] The exact nature of the final step remains uncertain, however; the A-ring conformation is thought to play a critical role in the stereospecific removal of the two hydrogens.

Aromatase inhibitors are useful in treating estrogen-dependent breast cancer.[11] Therefore, several categories of steroidal and non-steroidal inhibitors were designed. Osawa and co-workers reported previously that the natural estrogen, which was thought to be the final product of aromatase reaction, served as an inhibitor of aromatase, yielding catechol estrogen, 2-hydroxyestrone, as well as 6$\alpha$-hydroxyestrone.[12—14] We previously reported structure–activity relationships of estrogen analogs as aromatase inhibitors to know the spatial aspects of the active site of aromatase and to develop a novel series of aromatase inhibitors.[15] Consequently, 2-halogeno-, 2-methyl-, 6$\alpha$-aryl-, and 6$\beta$-methylestrones are good competitive inhibitors of aromatase in human placental microsomes (apparent $K_i$'s ranging between 100 and 660 nM). On the other hand, many compounds have been reported as non-steroidal competitive inhibitors of aromatase, including flavonoids and their analogs.[16—19] They have a heteroatom (sulfur, oxygen, and nitrogen) that would bind to the heme iron of the aromatase cytochrome P-450 enzyme. In particular, flavonoids having a nitrogen-containing heterocylic moiety such as pyridine, pyrimidine, imidazole, and triazole strongly inhibit aromatase.[19]

In this study, we examined the structure–activity relationships of the pyridine and other heterocyclic derivatives of estrone and estradiol analogs as aromatase inhibitors in human placental microsomes. Isonicotinyl-substituted derivatives **5c** and **10c** were the most potent inhibitors of aromatase.

## Experimental

**Materials and General Methods** [1$\beta$-$^3$H] Androstenedione (AD) (27.5 Ci/mmol) ($^3$H distribution: 74—79% at 1$\beta$) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), and NADPH from Kohjun Co., Ltd. (Tokyo, Japan). 2- and 4-Nitroestrone (**1**, **6**) were prepared according to known method.[20] The structures of all of the known compounds used in this study were identified by $^1$H-NMR spectrometric analysis and their purities were confirmed by elemental analysis or high resolution-mass spectrometry (HR-MS) as well as thin-layer chromatography (TLC).

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) and were uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR 1725X spectrophotometer, and ultraviolet (UV) spectra were determined in 95% EtOH on a Hitachi 150-20 UV spectrometer (Tokyo, Japan). $^1$H-NMR spectra were obtained in $CDCl_3$ solution with a JEOL GX 400 (400 MHz) or JEOL LA 600 (600 MHz) spectrometer (Tokyo, Japan) using tetramethylsilane as an internal standard, and MS spectra (EI mode) with a JEOL JMS-DX 303 spectrometer. TLC was performed on E. Merck precoated silica gel plates (silica gel 60F-254, Darmstadt, Germany). Column chromatography was conducted with silica gel 60, 70—230 mesh (E. Merck).

**2- and 4-Nitro-3-benzyloxyestra-1,3,5-triene-17-ones (2, 7)** A solution of 2- or 4-nitroestrone (**1**, **6**) (2.35 g, 7.46 mmol) in $CH_3CN$ (85 ml) was added benzyl bromide (1.075 ml, 7.46 mmol) and anhydrous $K_2CO_3$ (6.85 g, 49.7 mmol). The mixture was refluxed with stirring for 1 h. The reaction mixture was filtered and evaporated which was recrystallized from acetone to give 2- and 4-nitro-3-benzyloxy steroid **2** or **7** (2.96 g or 2.90 g, 98.0% or 96.0%), respectively.

**2**: mp 237—240 °C. $^1$H-NMR $\delta$: 0.92 (3H, s, 18-Me), 5.20 (2H, s, $O\underline{CH_2}Ph$), 6.83 (1H, s, 4-H), 7.38 (5H, m, $OCH_2\underline{Ph}$), 7.84 (1H, s, 1-H). IR (KBr) $cm^{-1}$: 1737, 1517. UV $\lambda_{max}$ (EtOH) nm ($\varepsilon$): 274.0 (2290), 340.0 (1520). MS *m/z*: 405 ($M^+$), 299, 91. *Anal.* Calcd for $C_{25}H_{27}NO_4$: C, 74.05; H, 6.71; N, 3.45. Found: C, 73.86; H, 6.75; N, 3.31.

**7**: mp 194—198 °C. $^1$H-NMR $\delta$: 0.91 (3H, s, 18-Me), 5.15 (2H, s, $O\underline{CH_2}Ph$), 6.87 (1H, d, *J*=8.8 Hz, 2-H), 7.29 (1H, d, *J*=8.1 Hz, 1-H), 7.34 (5H, m, $OCH_2\underline{Ph}$). IR (KBr) $cm^{-1}$: 1741, 1529. UV $\lambda_{max}$ (EtOH) nm ($\varepsilon$): 275.0 (1590). MS *m/z*: 405 ($M^+$), 299, 91. *Anal.* Calcd for $C_{25}H_{27}NO_4$: C, 74.05; H, 6.71; N, 3.45. Found: C, 74.11; H, 6.76; N, 3.30.

**2- and 4-Amino-3-benzyloxyestra-1,3,5-triene-17-ones (3, 8)** A solution of 0.1 mol/l NaOH (68 ml) containing $Na_2S_2O_4$ (13.3 g, 76.4 mmol) was added to a solution of steroid **2** or **7** (2.86 g, 7.06 mmol) in acetone (405 ml). After refluxed for 2 h, the reaction mixture was added dropwise AcOH, followed by evaporation and extraction with EtOAc. The organic layer was washed with sat. NaCl and dried with $Na_2SO_4$. Evaporation of the solvent, purification by column chromatography (hexane–EtOAc, 2 : 1, v/v) and recrystallization from EtOAc gave 2- or 4-amino-3-benzyloxy steroid **3** or **8** (2.32 g or 2.19 g, 87.6% or 82.7%), respectively.

**3**: mp 226—229 °C. $^1$H-NMR $\delta$: 0.91 (3H, s, 18-Me), 3.72 (2H, s, 2-$NH_2$), 5.05 (2H, s, $Ph\underline{CH_2}O$), 6.61 (1H, s, 4-H), 6.70 (1H, s, 1-H), 7.38 (5H, m, $\underline{Ph}CH_2O$). IR (KBr) $cm^{-1}$: 3448, 1735, 1520. UV $\lambda_{max}$ (EtOH) nm ($\varepsilon$):

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296.0 (4650). MS *m/z*: 375 ($M^+$), 284, 122, 91. *Anal.* Calcd for $C_{25}H_{29}NO_2$: C, 79.96; H, 7.78; N, 3.73. Found: C, 79.65; H, 7.90; N, 3.53.

**8**: mp 217—220 °C. $^1$H-NMR $\delta$: 0.90 (3H, s, 18-Me), 3.80 (2H, s, 4-$NH_2$), 5.08 (2H, s, Ph$\underline{CH_2}$O), 6.71 (1H, d, $J$=8.5 Hz, 2-H), 6.76 (1H, d, $J$=8.5 Hz, 1-H), 7.37 (5H, m, $\underline{Ph}$CH$_2$O). IR (KBr) $cm^{-1}$: 3460, 1739. UV $\lambda_{max}$ (EtOH) nm ($\varepsilon$): 288.0 (2940). MS *m/z*: 375 ($M^+$), 284, 122, 91. HR-MS Calcd for $C_{25}H_{29}NO_2$: 375.2198. Found: 375.2199.

**2- and 4-Substituted 3-Benzyloxyestra-1,3,5-triene-17-ones (4, 9)** A solution of 2-amino steroid **3** (260 mg, 0.70 mmol) in anhydrous DMF (43 ml) was added picolinoyl chloride hydrochloride (249 mg, 1.4 mmol) and pyridine (55 ml) and refluxed for 2 h. The reaction mixture was evaporated under the reduced pressure, and the resulting residue was purified by column chromatography (hexane–EtOAc, 5 : 1, v/v) followed by recrystallization from acetone to afford 2-picolinylamide steroid **4a** (122 mg, 36%). 2-Nicotinyl, isonicotinyl, and isoxazolecarbonyl substituted compounds **4b**, **4c**, and **4d** and 4-picolinyl, nicotinyl, isonicotinyl, and isoxazolecarbonyl substituted compounds **9** were also obtained from steroid **3** or **8** in the similar manner as described above.

**2- and 4-Substituted Estra-1,3,5-triene-17-ones (5 or 10)** A mixture of 3-benzyloxy steroid **4a** (122 mg, 0.26 mmol), Pd–C (390 mg), and 95% EtOH (63 ml) was stirred under hydrogen atmosphere for 2.5 h. The reaction mixture was filterd and evaporated, the residue which was recrystallized from acetone gave 3-hydroxy-2-picolinyl compound **5a** (42.0 mg, 42%). Other 3-benzyloxy steroids **4b**—**d** and steroids **9** were hydrogenated, similarly as above, to yield 3-hydroxy-2-substituted steroids **5b**—**d** and their 4-isomers **10**.

**3-Substituted Estrones (12)** Method 1: A mixture of 2-methyl-6-nitrobenzoic anhydride (MNBA) (191 mg), 4-dimethylaminopyridine (DMAP) (46 mg), 2-furancarboxylic acid (62 mg) in THF (1 ml) and triethylamine (0.1 ml) were added to a solution of estrone (**11**) (30 mg, 0.11 mmol) in THF (1 ml).[21—23] The resulting mixture was stirred at room temperature for 1 h. Then the reaction mixture was diluted with EtOAc, washed with 5% $NaHCO_3$ and water, and dried with $Na_2SO_4$. After evaporation of the solvent, the crude obtained was recrystallized from acetone to yield **12a**. 3-Thiophenecarbonyl, thiazolecarbonyl, and pyrrolecarbonyl substituted estrones (**12b**—**d**) were synthesized similarly as described for the synthesis of **12a**.

Method 2: 5-Isoxazolecarbonyl chloride (120 mg, 0.91 mmol) in pyridine (6 ml) were added to a solution of estrone (**11**) (31 mg, 0.11 mmol) in $CH_3CN$ (6 ml) and the mixture was stirred at 60 °C for 9 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with sat. $NaHCO_3$ and water, dried with $Na_2SO_4$, and purified by recrystallization from EtOAc to give steroid **12e** (29 mg, 70%).

**3-*tert*-Butyldimethylsiloxy-17*β*-substituted Estradiols (14)** Method 1: A mixture of MNBA (688 mg), DMAP (171 mg), 2-furancarboxylic acid (220 mg, 2.0 mmol) in THF (5 ml) and triethylamine (0.5 ml) were added to a solution of steroid **13** (153 mg, 0.40 mmol) in THF (5 ml), and the mixture was stirred at room temperature for 1 h. The reaction mixture was poured to sat. $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with water, and dried with $Na_2SO_4$. Evaporation of the solvent followed by recrystallization from acetone gave steroid **14a**. 17-Thiophenecarbonyl and thiazolecarbonyl substituted compounds **14b** and **14c** were prepared in the similar manner as the synthesis of **14a**.

Method 2: 5-Isoxazolecarbonyl chloride (184 mg, 1.40 mmol) was reacted with **13** (126 mg, 0.33 mmol) under above condition, a crude product purified by column chromatography (hexane–EtOAc, 9 : 1, v/v) to give **14d** (54 mg, 35%).

**17*β*-Substituted Estradiols (15)** 1 mol/l HCl solution (3.8 ml) was added to a mixture of steroid **14a** (123 mg, 0.26 mmol), isopropanol (2.5 ml), and THF (6.5 ml). The reaction mixture was allowed to stand at room temperature for over night, and the mixture was diluted with EtOAc. The organic layer was washed with sat. $NaHCO_3$ and water, and dried with $Na_2SO_4$. Evaporation of the solvent, followed by recrystallization from acetone gave steroid **15a** (82 mg, 87%). Other 17-substituted estradiols (**15b**—**d**) were obtained by the deprotection of the 3-silylether **10b**—**d** similarly as described for the preparation of **15a**.

**Enzyme Preparation** Human placental microsomes (sedimented after 60 min at 105000×*g*) were obtained as described by Ryan.[24] They were washed once with 0.05 mM dithiothreitol, lyophilized and stored at −80 °C. No significant loss of activity occurred during the period (6 months) of this study. The preparation of human placental microsomes was conducted under the approval of the ethical review committee of Tohoku Pharmaceutical University in accordance with the standard of the Helsinki Declaration.

**Aromatase Assay Procedure** Aromatase activity was measured essentially according to the original procedure of Siiteri and Thompson.[25] All enzymatic studies were carried out in 67 mM phosphate buffer, pH 7.5, at a final volume of 500 $\mu$l under initial velocity conditions. The incubation mixture for the $IC_{50}$ experiment contained 480 $\mu$M of NADPH, 300 nM of [1$\beta$-$^3$H]AD, 20 $\mu$g protein of the lyophilized microsomes, various concentrations of inhibitors, and the entire mixture was incubated at 37 °C for 20 min.[26] For the kinetic assay experiment, the incubation mixture contained 480 $\mu$M of NADPH, 20 $\mu$g protein of the microsomes, 300 nM of [1$\beta$-$^3$H]AD, various concentrations of inhibitors. The mixture was incubated at 37 °C for 5 min.

Apparent $K_i$ values were calculated using non-linear regression analysis with GraFit software.[27]

## Results and Discussion

**Chemistry** Previously, it has been reported that estrone is more potent inhibitor of human placental aromatase, compared to estradiol, although the estrogens are the aromatase final products. Then, we first employed estrone as a scaffold of aromatase inhibitors for the synthesis of the estrogen derivatives. 2- or 4-nitroestrone (**1** or **6**) was treated with benzyl bromide in $CH_3CN$ in the presence of $K_2CO_3$ gave 3-benzyl ethers **2** or **7** which was reduced with $Na_2S_2O_4$ to yield 2- or 4-aminoestone 3-benzyl ether (**3** or **8**) (Fig. 1). Treatment of 2-aminoestone 3-benzyl ether (**3**) with acid chloride (picolinoyl chloride, nicotinoyl chloride, isonicotinoyl chloride, or 5-isoxazolecarbonyl chloride) in pyridine gave 2-amide derivatives **4** having a heterocyclic ring at C-2 in fair yield (Tables 1, 2). Deprotection of a 3-benzyl function of compounds **4** with $H_2$/C afforded finally 2-heterocyclic-substituted amides **5**. 4-Heterocyclic-substituted aminoestrones (**10**) were synthesized similarly as described for the synthesis of the 2-isomers **5**.

Next, we employed estrone for a starting material of synthesis of estrone 3-heterocyclic-substituted derivatives **12** (Fig. 2). Estrone (**11**) was treated with 2-furancarboxylic acid, 2-thiophenecarboxylic acid, 4-thiazolecarboxylic acid, or pyrrole-2-carboxylic acid under the mixed anhydride condition (MNBA in the presence of DMAP and triethylamine) in THF for 1 h to afford to estrone 3-heterocyclic esters **12a**—**d** whereas the 3-isoxazolecarbonyl ester **12d** was obtained with 5-isoxazolecarbonyl chloride in pyridine (Table 3).

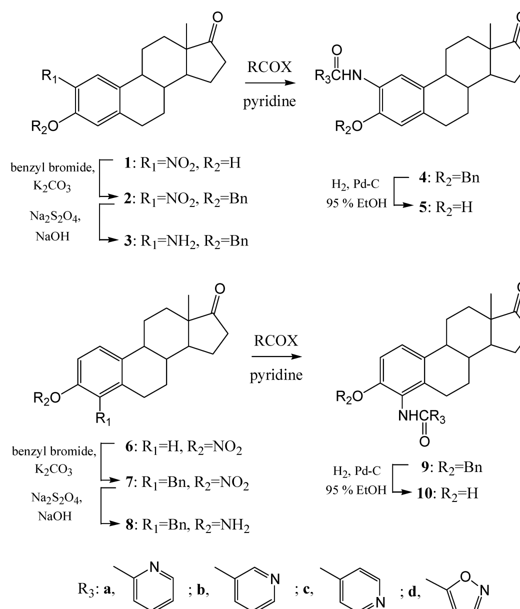


Fig. 1. Synthesis of 2- and 4-Heterocyclic Amides **5** and **10** of Estrone

Table 1. Physico-Chemical Data for Compounds **4**, **5**, **9** and **10**

| Compound | Yield (%) | mp (°C) | UV (EtOH) (nm (ε)) | IR (KBr) ($cm^{-1}$) | $^1$H-NMR ($CDCl_3$), δ | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 18-$CH_3$ | 1-H | 2-H | 4-H | Other signals |
| 2-Substituted series | | | | | | | | | |
| **4a** | 36 | 228—230 | 252 (9770), 319 (8240) | 1729, 1689 | 0.93 | 7.37 (6H, m) | — | 6.71 (s) | 7.57 (m), 7.91 (m), 8.29 (m), 8.60 (m) |
| **4b** | 37 | 198—200 | 258 (6540), 298 (5640) | 1728, 1655 | 0.93 | 7.65 (s) | — | 6.76 (s) | 7.40 (6H, m), 8.40 (m), 8.79 (m), 9.16 (m) |
| **4c** | 83 | 221—224 | 261 (5980), 307 (4800) | 1737, 1674 | 0.93 | 7.39 (6H, m) | — | 6.80 (s) | 8.01 (2H, m), 8.82 (2H, m) |
| **4d** | 61 | 199—201 | 313 (8280) | 3400, 1735, 1686, 1530 | 0.92 | 6.98 (d, *J*=1.8 Hz) | — | 6.73 (s) | 5.18 (2H, s), 7.40 (5H, m), 8.36 (d, *J*=1.8 Hz), 8.44 (s), 8.93 (s) |
| **5a** | 42 | 300< | 258 (8100), 324 (6200) | 3437, 1736, 1634 | 0.94 | 8.05 (s) | — | 6.64 (s) | 7.50 (m), 7.93 (m), 8.25 (m), 8.65 (dd, *J*=0.7, 4.7 Hz) |
| **5b** | 55 | 230—234 | 256 (7150), 305 (5990) | 3437, 1726, 1647 | 0.93 | 7.74 (s) | — | 6.72 (s) | 7.51 (m), 8.32 (d, *J*=8.6 Hz), 8.73 (s), 9.10 (s) |
| **5c** | 67 | 255—258 | 258 (6220), 307 (5570) | 3408, 1736, 1647 | 0.94 | 7.85 (s) | — | 6.71 (s) | 8.25 (2H, m), 8.90 (2H, m) |
| **5d** | 84 | 164—168 | 260 (2870), 339 (16060) | 3426, 1729, 1637 | 0.93 | 7.32 (d, *J*=7.3 Hz) | — | 6.12 (d, *J*=7.3 Hz) | 6.64 (s), 7.92 (s) |
| 4-Substituted series | | | | | | | | | |
| **9a** | 57 | 149—151 | 266 (6590) | 1736, 1688 | 0.92 | 7.38 (d, *J*=6.8 Hz) | 6.84 (d, *J*=8.5 Hz) | — | 7.25 (5H, m), 7.49 (m), 7.90 (m), 8.29 (d, *J*=7.8 Hz), 8.64 (d, *J*=4.1 Hz) |
| **9b** | 42 | 184—188 | 264 (6740) | 1733, 1655 | 0.94 | 7.36 (d, *J*=6.9 Hz) | 6.89 (d, *J*=8.4 Hz) | — | 7.27 (5H, m), 7.55 (m), 8.43 (m), 8.75 (m), 9.20 (m) |
| **9c** | 42 | 236—239 | 264 (3930) | 1735, 1660 | 0.94 | 7.34 (6H, m) | 6.89 (d, *J*=8.5 Hz) | — | 7.78 (2H, m), 8.70 (2H, m) |
| **9d** | 59 | —[a] | — | — | 0.91 | 7.25 (d, *J*=8.8 Hz) | 6.85 (d, *J*=8.4 Hz) | — | 5.11 (2H, s), 7.01 (d, *J*=1.5 Hz), 7.31 (5H, m), 8.39 (d, *J*=1.8 Hz) |
| **10a** | 72 | 190—193 | 267 (6200) | 3437, 1741, 1660 | 0.92 | 7.20 (d, *J*=8.8 Hz) | 6.97 (d, *J*=8.8 Hz) | — | 7.55 (m), 7.95 (m), 8.31 (d, *J*=7.6 Hz), 8.66 (m) |
| **10b** | 13 | 140—144 | 264 (5240) | 3437, 1732, 1648 | 0.92 | 7.25 (d, *J*=9.0 Hz) | 6.98 (d, *J*=8.5 Hz) | — | 7.51 (m), 8.28 (m), 8.84 (m), 9.15 (m) |
| **10c** | 35 | 190—194 | 264 (7410) | 3447, 1732, 1651 | 0.93 | 7.18 (d, *J*=8.8 Hz) | 6.83 (d, *J*=8.8 Hz) | — | 7.90 (2H, m), 8.87 (2H, m) |
| **10d** | 76 | 213—216 | 331 (17320) | 3301, 1727, 1651 | 0.93 | 7.15 (d, *J*=8.8 Hz) | 6.76 (d, *J*=8.4 Hz) | — | 6.02 (d, *J*=7.0 Hz), 7.37 (d, *J*=7.3 Hz) |

*a*) Compound **9d** was used for the synthesis of **10d** without further purification.

Table 2. Elemental Analysis and HR-MS Data of **4**, **5**, **9** and **10**

| Compound | Calculation | Observed |
|---|---|---|
| 2-Substituted series | | |
| **4a** | 480.2413 | 480.2445 |
| **4b** | 480.2413 | 480.2448 |
| **4c** | 480.2413 | 480.2443 |
| **4d** | C, 74.02; H. 6.43; N, 5.95 | C, 74.10; H, 6.47; N, 5.84 |
| **5a** | 390.1943 | 390.1921 |
| **5b** | 390.1943 | 390.1942 |
| **5c** | 390.1943 | 390.1924 |
| 4-Substituted series | | |
| **9a** | 480.2413 | 480.2427 |
| **9b** | 480.2413 | 480.2449 |
| **9c** | 480.2413 | 480.2447 |
| **10a** | 390.1943 | 390.1918 |
| **10b** | 390.1943 | 390.1916 |
| **10c** | 390.1943 | 390.1923 |

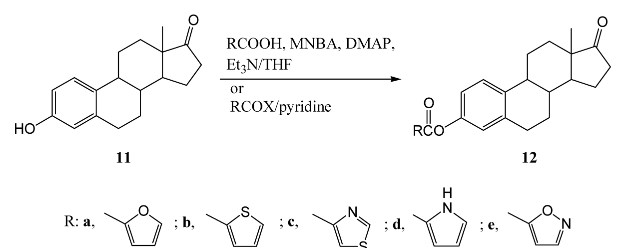


Fig. 2. Synthesis of Estrone 3-Heterocyclic Esters **12**

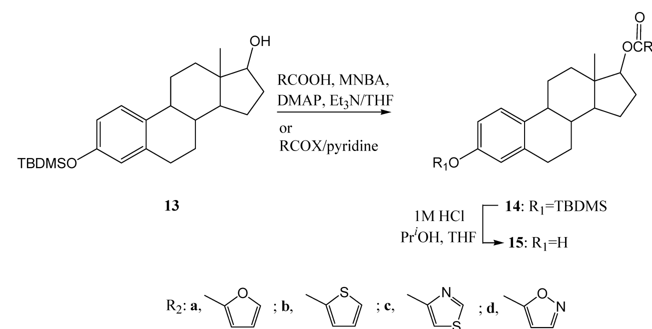


Fig. 3. Synthesis of Estradiol 17-Heterocyclic Esters **15**

Finally, we prepared estradiol 17-heterocyclic-substituted derivatives **15** (Fig. 3). 3-*tert*-Butyldimethylsiloxyestradiol (**13**) was converted into 17-heterocyclic esters **14** by treatment with the mixed anhydride method or the acyl chloride in pyridine as described for the synthesis of compound **12**. Hydrolysis of the 3-silyl ethers **14** with 1 mol/l HCl in propan-2-ol and THF gave 17-heterocyclic-substituted estradiol (**15**) in good yields (Tables 4, 5).

Spectral data for all of the compounds synthesized in this study were consistent with the assigned structures.

**Biological Properties** Inhibition of aromatase activity using [1$\beta$-$^3$H]AD as a substrate in human placental microsomes by the estrogen derivatives was examined *in vitro* by enzyme kinetics. This assay quantitates the production of tritiated water released from [1$\beta$-$^3$H]AD by aromatization. The effects of varying the C-2, C-4, and C-3 substitution of estrone and the C-17 substitution of estradiol on the activity of androstenedione aromatization was determined in relation to

Table 3. Physico-Chemical Data for Compound **12**

| Compound | Yield (%) | mp (°C) | UV (EtOH) (nm (ε)) | IR (KBr) ($cm^{-1}$) | $^1$H-NMR ($CDCl_3$), δ | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 18-$CH_3$ | 1-H | 2-H | 4-H | Other signals |
| 3-Substituted series | | | | | | | | | |
| **12a** | 60 | 241—243 | 258 (13480) | 1739, 1651 | 0.92 | 7.33 (d, $J$=8.1 Hz) | 6.98 (dd, $J$=2.6, 8.4 Hz) | 6.94 (d, $J$=2.6 Hz) | 6.59 (dd, $J$=1.8, 3.3 Hz), 7.37 (dd, $J$=0.9, 3.5 Hz), 7.67 (s) |
| **12b** | 84 | 215—218 | 251 (6450), 275 (5750) | 1732, 1651, 1509 | 0.93 | 7.33 (d, $J$=8.5 Hz) | 6.99 (dd, $J$=2.6, 8.4 Hz) | 6.95 (d, $J$=2.4 Hz) | 7.17 (dd, $J$=3.8, 5.0 Hz), 7.66 (dd, $J$=1.3, 5.0 Hz), 7.97 (dd, $J$=1.2, 3.9 Hz) |
| **12c** | 48 | 220—224 | 234 (10290) | 1732, 1651 | 0.93 | 7.34 (d, $J$=8.5 Hz) | 7.08 (dd, $J$=2.4, 8.5 Hz) | 6.98 (d, $J$=2.4 Hz) | 8.43 (d, $J$=2.0 Hz), 8.93 (d, $J$=2.2 Hz) |
| **12d** | 37 | 196—200 | 236 (9520), 273 (23050), 316 (1780) | 1718, 1651 | 0.92 | 7.32 (d, $J$=8.8 Hz) | 6.96 (dd, $J$=2.6, 8.4 Hz) | 6.92 (d, $J$=2.6 Hz) | 6.35 (m), 7.05 (m), 7.12 (m), 9.22 (br s) |
| **12e** | 70 | 250—254 | 274 (1500) | 1732, 1651 | 0.93 | 7.35 (d, $J$=8.3 Hz) | 7.01 (dd, $J$=2.6, 8.4 Hz) | 6.97 (d, $J$=2.4 Hz) | 7.13 (d, $J$=1.7 Hz), 8.45 (d, $J$=2.0 Hz) |

Table 4. Physico-Chemical Data for Compounds **14** and **15**

| Compound | Yield (%) | mp (°C) | IR (KBr) ($cm^{-1}$) | $^1$H-NMR ($CDCl_3$), δ | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 18-$CH_3$ | 17α-H | 1-H | 2-H | 4-H | Other signals |
| 17β-Substituted series | | | | | | | | | |
| **14a** | 71 | 157—159 | 1716 | 0.93 | 4.92 (t, $J$=8.4 Hz) | 7.11 (d, $J$=8.3 Hz) | 6.61 (dd, $J$=2.7, 8.5 Hz) | 6.55 (d, $J$=2.4 Hz) | 0.18 (s), 0.97 (s), 6.49 (dd, $J$=1.5, 5.6 Hz), 7.15 (m), 7.58 (br s) |
| **14b** | 48 | 124—128 | 1699 | 0.94 | 4.89 (t, $J$=8.4 Hz) | 7.10 (d, $J$=8.8 Hz) | 6.61 (dd, $J$=2.6, 8.4 Hz) | 6.56 (d, $J$=2.6 Hz) | 0.18 (s), 0.97 (s), 7.11 (m), 7.54 (q), 7.80 (q) |
| **14c** | 84 | 173—175 | 1724 | 0.97 | 5.01 (t, $J$=8.4 Hz) | 7.12 (d, $J$=8.1 Hz) | 6.61 (dd, $J$=2.6, 8.4 Hz) | 6.55 (d, $J$=2.6 Hz) | 0.18 (s), 0.97 (s), 8.20 (d, $J$=2.2 Hz), 8.86 (d, $J$=1.8 Hz) |
| **14d** | 35 | 147—151 | 1733 | 0.95 | 4.96 (t, $J$=8.4 Hz) | 7.11 (d, $J$=8.5 Hz) | 6.61 (dd, $J$=2.7, 8.5 Hz) | 6.56 (d, $J$=2.7 Hz) | 0.18 (s), 0.97 (s), 6.94 (d, $J$=1.7 Hz), 8.36 (d, $J$=1.7 Hz) |
| **15a** | 87 | 168—172 | 3399, 1717, 1701 | 0.93 | 4.93 (t, $J$=8.4 Hz) | 7.15 (d, $J$=8.1 Hz) | 6.63 (dd, $J$=2.9, 8.4 Hz) | 6.57 (d, $J$=2.6 Hz) | 4.72 (s), 6.51 (dd, $J$=1.8, 3.3 Hz), 7.15 (br s), 7.58 (d, $J$=0.7 Hz) |
| **15b** | 99 | 199—202 | 3426, 1679 | 0.94 | 4.90 (t, $J$=8.4 Hz) | 7.16 (d, $J$=8.4 Hz) | 6.63 (dd, $J$=2.6, 8.4 Hz) | 6.57 (d, $J$=2.6 Hz) | 7.10 (t, $J$=4.2 Hz), 7.55 (d, $J$=4.9 Hz), 7.80 (d, $J$=4.4 Hz) |
| **15c** | 91 | 253—257 | 3437, 1725 | 0.97 | 5.02 (t, $J$=8.4 Hz) | 7.15 (d, $J$=8.4 Hz) | 6.63 (dd, $J$=2.9, 8.4 Hz) | 6.57 (d, $J$=2.6 Hz) | 8.21 (d, $J$=1.8 Hz), 8.87 (d, $J$=1.8 Hz) |
| **15d** | 99 | 213—217 | 3470, 1723 | 0.95 | 4.96 (t, $J$=8.6 Hz) | 7.15 (d, $J$=8.4 Hz) | 6.63 (dd, $J$=2.9, 8.4 Hz) | 6.57 (d, $J$=2.6 Hz) | 6.94 (d, $J$=1.8 Hz), 8.37 (d, $J$=1.8 Hz) |

Table 5. Elemental Analysis and HR-MS Data of **12**, **14** and **15**

| Compound | Calculation | Observed |
|---|---|---|
| 3-Substituted series | | |
| **12a** | C, 75.80; H. 6.64; N, 0 | C, 75.78; H, 6.73; N, 0.01 |
| **12b** | C, 72.60; H, 6.36; N, 0 | C, 72.39; H, 6.38; N, 0.05 |
| **12c** | 381.1399 | 381.1411 |
| **12d** | 363.1844 | 363.1839 |
| **12e** | C, 72.31; H, 6.34; 3.83 | C, 71.90; H, 6.41; N, 3.80 |
| 17$\beta$-Substituted series | | |
| **14a** | C, 72.46; H, 8.39; N, 0 | C, 72.44; H, 8.52; N, 0 |
| **14b** | C, 70.11; H, 8.12; N, 0 | C, 69.95; H, 8.20; N, 0 |
| **14c** | 497.2420 | 497.2420 |
| **14d** | C, 69.82; H, 8.16; N, 2.91 | C, 69.37; H, 8.29; N, 2.75 |
| **15a** | C, 75.38; H, 7.15; N, 0 | C, 75.31; H, 7.23; N, 0 |
| **15b** | C, 72.22; H, 6.85; N, 0 | C, 72.25; H, 6.92; N, 0 |
| **15c** | C, 68.90; H, 6.57; N, 3.65 | C, 68.95; H, 6.61; N, 3.53 |
| **15d** | C, 71.91; H, 6.86; N, 3.81 | C, 71.87; H, 6.91; N, 3.71 |

Table 6. *In Vitro* Aromatase Inhibition by Estrone and Estradiol Derivatives

| Compound | $IC_{50}$ ($\mu$M) | Apparent $K_i$ ($\mu$M) |
|---|---|---|
| 2-Substituted series | | |
| **5a** | 208.83±23.8 | —[a] |
| **5b** | 75.39±4.7 | — |
| **5c** | 31.16±1.3 | 2.19±0.14 |
| **5d** | 10% inhibition at 100 $\mu$M | — |
| 4-Substituted series | | |
| **10a** | 203.06±19.38 | — |
| **10b** | 95.64±5.2 | — |
| **10c** | 28.57±1.8 | 1.53±0.08 |
| **10d** | 10% inhibition at 100 $\mu$M | — |
| 3-Substituted series | | |
| **12a** | 0% inhibition at 100 $\mu$M | — |
| **12b** | 0% inhibition at 100 $\mu$M | — |
| **12c** | 10% inhibition at 100 $\mu$M | — |
| **12d** | 10% inhibition at 100 $\mu$M | — |
| **12e** | 243.88±78.1 | — |
| 17$\beta$-Substituted series | | |
| **15a** | 0% inhibition at 100 $\mu$M | — |
| **15b** | 0% inhibition at 100 $\mu$M | — |
| **15c** | 0% inhibition at 100 $\mu$M | — |
| **15d** | 0% inhibition at 100 $\mu$M | — |
| For comparison | | |
| Estrone | 26±8 | 2.50±0.22 |
| Estradiol | 5% inhibition at 50 $\mu$M | 130±10 |

*a*) Not determined.

the aromatase-catatyzed estrogen hydroxylations, especially the catechol estrogen formation. The results are shown in Table 6. $IC_{50}$ values were initially obtained under initial velocity conditions. 2-Amides **5a**—**c**, picolinyl (**5a**), nicotinyl (**5b**) and isonicotinyl (**5c**) amides, as well as 4-amide derivatives **6a**—**c** showed fairly inhibitory activity but 4-isoxazole derivatives **5d** and **10d** were poor inhibitors. Moreover, estrone 3- and estradiol 17-furancarbonyl, thiophenecarbonyl, thiazolecarbonyl, and isoxazolecarbonyl esters (**12**, **15**) did not show any detectable amounts of the aromatase inhibitory activity. Among the inhibitors examined, the apparent inhibition constants ($K_i$), an index of the ability of the enzyme for the inhibitor, were obtained for isonicotinyl compounds **5c** and **10c** by analysis of a Dixon plot. The two compounds were evaluated as competitive or non-competitive inhibitors of aromatase in human placental microsomes. Lineweaver–Burk plots showed that these were competitive inhibitors

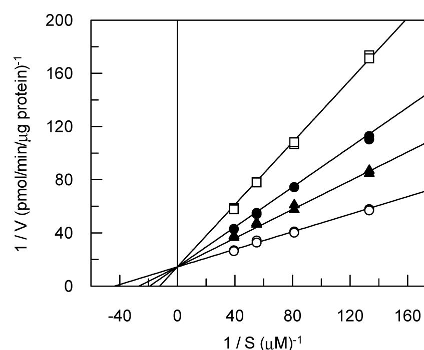


Fig. 4. Lineweaver–Burk Plots of Aromatase Inhibition by Isonicotinyl Derivative **10c**

Concentrations of the inhibitor: control (0 $\mu$M) (○); 0.96 $\mu$M (▲); 1.92 $\mu$M (●); 3.85 $\mu$M (□).

(Fig. 4). Isonicotinyl analogs **5c** and **10c** were most potent inhibitors and their apparent $K_i$ values were 2.19±0.14 $\mu$M and 1.53±0.08 $\mu$M for **5c** and **10c**, respectively. Nicotinyl derivatives **5b** and **10b** and picolinyl derivatives **5a** and **10a** were less potent inhibitors. For comparison, the $K_i$ values of estrone and estradiol were determined to be 2.50±0.22 $\mu$M and 130±10 $\mu$M, respectively.

Introduction of fluoro, chloro, bromo, methyl, and formyl groups at C-2 of estrone gave rise to the markedly increased affinity for aromatase but that of methoxy, nitro, amino functions at the C-2 position did not affect, to a significant extent, the affinity.[15] On the other hand, the similar substitution at the 4-position of estrone markedly decreased the affinity. The present results shows that there is no significant difference between the 2- and 4-subsituted compounds, demonstrating that there are the similar accessible volume between C-2 and C-4 positions. Although there is no precise evidence concerning the relation of aromatase-catalyzed 2-hydroxylation of estrogen to the aromatase reaction, estrogen formation, the accessible volume of the C-2 and C-4 position would play as critical role to the both the hydroxylation and the aromatase reaction. Among the pyridine substitutions, the fact that the isonicotinyl derivatives **5c** and **10c** were most potent ones suggests that the N-hetero atom, orienting *p*-position on the pyridine ring, is most fitted the accessible volume. The coordination of the para N atom of pyridine to the heme-Fe of aromatase would be involved in the inhibition. Compounds having the other heterocyclic ring at the C-3 of estrone and at C-17 of estradiol did not access the binding pocket, namely, the hetero atoms of the heterocyclic compounds failed the coordination to the heme moiety of aromatase.

The 2- and 4-isonicotinyl amino derivatives of estrone, compounds **5c** and **10c**, were promising compounds for further powerful aromatase inhibitors. On the basis of the results, it is presumed that there would be an accessible volume in the regions of C-2 and C-4 positions.

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