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.\(^1\) 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 1β and 2β-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.\(^1\) 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 6α-hydroxyestrone.\(^1\) 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.\(^5\) Consequently, 2-halogeno-, 2-methyl-, 6α-aryl-, and 6β-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.\(^6\)–\(^19\) 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 heterocyclic 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**

\([1β,2β]-\)Androstenedione (AD) (27.57 Ci/mmol) (U.S. Nuclear Corp., Boston, MA, U.S.A.); NADPH from the aqueous extract of the liver of male Wistar rats, NADPH (1.5 mg/ml), and NADPH from Kohjun Co., Ltd. (Tokyo, Japan).

### Key words

aromatase; inhibition activity; estrone derivative; estradiol derivative; isonicotinyl derivative; competitive inhibitor

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296.0 (4650). MS m/z: 375 (M+), 284, 122, 91. Anal. Calcld for C_{23}H_{23}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. 1H-NMR δ: 0.90 (3H, s, 18-Me), 3.80 (2H, s, 4-NH), 5.08 (2H, s, PhCH=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, PhCH=O). IR (KBr) cm⁻¹: 3460, 1739. UV (EtOH) nm (ε): 280.0 (2940). MS m/z: 375 (M⁺), 284, 122, 91. HRMS Calcld for C_{23}H_{23}NO_{2}: 375.2198. Found: 375.2199.

2- and 4-Substituted 3-Benzoxysteroids, 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-Nicotinoyl, isonicotinoyl, and isoxazolecarbonyl substituted compounds 4b, 4c, and 4d and 4-picolinyl, nicotinoyl, isonicotinoyl, and isoxazolecarbonyl substituted compounds 9 were also obtained from steroid 3 or 8 in the similar manner as described above.

3-Substituted Estrones (12) Method 1: A mixture of 2-methyl-6-nitrobenzoic anhydride (MNBA) (191 mg), 4-dimethylaminopyridine (DMAP) (46 mg), 2-flurancarboxylic 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). The resulting mixture was stirred at room temperature for 1 h. Then, the reaction mixture was diluted with EtOAc, washed with 5% NaHCO₃ and water, and dried with Na₂SO₄. After evaporation of the solvent, the crude obtained was recrystallized from acetone to yield 2a. 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 CH₂CN (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. NaHCO₃ and water, dried with Na₂SO₄, and purified by recrystallization from EtOAc to give 12a (29 mg, 70%).

3-tert-Butyldimethylsiloxy-17β-substituted Estradiols (14) Method 1: A mixture of MNBA (688 mg), DMAP (171 mg), 2-flurancarboxylic acid (220 mg, 2.0 mmol) in THF (5 ml) and triethylamine (0.5 ml) were added to a solution of estradiol (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. NaHCO₃ and extracted with EtOAc. The organic layer was washed with water, and dried with Na₂SO₄. 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%).

17β-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. NaHCO₃ and water, and dried with Na₂SO₄. 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-silyl ether 10b—d similarly as described for the preparation of 15a.

**Enzyme Preparation** Human placental microsomes (sedimented after 60 min at 105000g) were obtained as described by Ryan.²⁴ They were washed once with 0.05 m sodium dithioreitol, 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 Sitteri and Thompson.²⁵ All enzymatic studies were carried out in 67 mm phosphate buffer, pH 7.5, at a final volume of 500 µl under initial velocity conditions. The incubation mixture for the IC₅₀ experiment contained 480 µM of NADPH, 300 nm of [1β-³H]AD, 20 µg protein of the phosphilized 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 µmol of NADPH, 20 µg protein of the microsomes, 300 nm of [1β-³H]AD, various concentrations of inhibitors. The mixture was incubated at 37°C for 5 min. Apparent Kᵢ values were calculated using non-linear regression analysis with GraFit software.²⁷

**Results and Discussion**

**Chemistry** Previously, it has been reported that estrogen 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 CH₂CN in the presence of K₂CO₃ gave 3-benzyl ethers 2 or 7 which was reduced with Na₂S₂O₅ to yield 2- or 4-aminoestrone 3-benzyl ether (3 or 8) (Fig. 1). Treatment of 2-aminoestrone 3-benzyl ether (3) with acid chloride (picolinyloxychloride, 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 H₂/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-flurancarboxylic 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 estrone 3-heterocyclic esters 12a—d whereas the 3-isoxazolecarbonyl ester 12d was obtained with 5-isoxazolecarbonyl chloride in pyridine (Table 3).

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Fig. 1. Synthesis of 2- and 4-Heterocyclic Amides 5 and 10 of Estrone.
Table 1. Physico-Chemical Data for Compounds 1, 5, 9 and 10

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
<th>mp (°C)</th>
<th>UV (EtOH) (nm)</th>
<th>IR (KBr) (cm⁻¹)</th>
</tr>
</thead>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>67.1 (6)</td>
<td>7.27 (6H, m)</td>
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<tr>
<td>2</td>
<td>67.1 (6)</td>
<td>7.27 (6H, m)</td>
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<tr>
<td>3</td>
<td>67.1 (6)</td>
<td>7.27 (6H, m)</td>
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<td>7.7 (6H, m)</td>
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<tr>
<td>4</td>
<td>67.1 (6)</td>
<td>7.27 (6H, m)</td>
<td></td>
<td>7.7 (6H, m)</td>
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<tr>
<td>5</td>
<td>67.1 (6)</td>
<td>7.27 (6H, m)</td>
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<td>7.7 (6H, m)</td>
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Table 2. Elemental Analysis and HR-MS Data of 4, 5, 9 and 10

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<th>Compound</th>
<th>Calculation</th>
<th>Observed</th>
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<tr>
<td>4c</td>
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<td>480.2443</td>
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<tr>
<td>4d</td>
<td>C, 74.02; H, 6.43; N, 5.95</td>
<td>C, 74.10; H, 6.47; N, 5.84</td>
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<td>5a</td>
<td>390.1943</td>
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</tr>
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<td>5b</td>
<td>390.1943</td>
<td>390.1942</td>
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<td>5c</td>
<td>390.1943</td>
<td>390.1924</td>
</tr>
<tr>
<td>9a</td>
<td>480.2413</td>
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</tr>
<tr>
<td>10c</td>
<td>390.1943</td>
<td>390.1923</td>
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Finally, we prepared estradiol 17-heterocyclic-substituted derivatives 15 (Fig. 3). 3-tert-Butyldimethylsilyloxyestradiol (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 [1β-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 triitated water released from [1β-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>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
<th>mp (°C)</th>
<th>UV (EtOH) (nm (e))</th>
<th>IR (KBr) (cm$^{-1}$)</th>
<th>$^1$H-NMR (CDCl$_3$), δ</th>
<th>Other signals</th>
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<td>3-Substituted series</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>12a</td>
<td>60</td>
<td>241–243</td>
<td>258 (13480)</td>
<td>1739, 1651</td>
<td>0.92 7.33 (d, J=8.1 Hz) 6.98 (dd, J=2.6, 8.4 Hz) 6.94 (d, J=2.6Hz) 6.59 (dd, J=1.8, 3.3 Hz) 7.37 (dd, J=0.9, 3.5 Hz) 7.67 (s)</td>
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<tr>
<td>12b</td>
<td>84</td>
<td>215–218</td>
<td>251 (6450), 275 (5750) 1509</td>
<td>1732, 1651</td>
<td>0.93 7.33 (d, J=8.5Hz) 6.99 (dd, J=2.6, 8.4 Hz) 6.95 (d, J=2.4Hz) 7.17 (dd, J=3.8, 5.0Hz) 7.66 (dd, J=1.3, 5.0Hz) 7.97 (dd, J=1.2, 3.9Hz)</td>
<td></td>
</tr>
<tr>
<td>12c</td>
<td>48</td>
<td>220–224</td>
<td>234 (10290)</td>
<td>1732, 1651</td>
<td>0.93 7.34 (d, J=8.5Hz) 7.08 (dd, J=2.4, 8.5 Hz) 6.98 (d, J=2.4Hz) 8.43 (d, J=2.0Hz) 8.93 (d, J=2.2Hz)</td>
<td></td>
</tr>
<tr>
<td>12d</td>
<td>37</td>
<td>196–200</td>
<td>236 (9520), 273 (23050), 316 (1780)</td>
<td>1718, 1651</td>
<td>0.92 7.32 (d, J=8.8Hz) 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 (brs)</td>
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<tr>
<td>12e</td>
<td>70</td>
<td>250–254</td>
<td>274 (1500)</td>
<td>1732, 1651</td>
<td>0.93 7.35 (d, J=8.3Hz) 7.01 (dd, J=2.6, 8.4 Hz) 6.97 (d, J=2.4Hz) 7.13 (d, J=1.7Hz) 8.45 (d, J=2.0Hz)</td>
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</tr>
</tbody>
</table>

| 17β-Substituted series |
| 14a      | 71        | 157–159 | 1716                | 9.93 4.92 (t, J=8.4 Hz) 7.11 (d, J=8.3Hz) 6.61 (dd, J=2.7, 8.5 Hz) 6.55 (d, J=2.4Hz) 0.18 (s), 0.97 (s), 6.49 (dd, J=1.5, 5.6 Hz) 7.15 (m), 7.58 (brs) |
| 14b      | 48        | 124–128 | 1699                | 0.94 4.89 (t, J=8.4 Hz) 7.10 (d, J=8.8Hz) 6.61 (dd, J=2.6, 8.4 Hz) 6.56 (d, J=2.6Hz) 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=8.1Hz) 6.61 (dd, J=2.6, 8.4 Hz) 6.55 (d, J=2.6Hz) 0.18 (s), 0.97 (s), 8.20 (d, J=2.2Hz), 8.86 (d, J=1.8Hz) |
| 14d      | 35        | 147–151 | 1733                | 0.95 4.96 (t, J=8.4 Hz) 7.11 (d, J=8.5Hz) 6.61 (dd, J=2.7, 8.5 Hz) 6.56 (d, J=2.7Hz) 0.18 (s), 0.97 (s), 6.94 (d, J=1.7Hz), 8.36 (d, J=1.7Hz) |
| 15a      | 87        | 168–172 | 3399, 1717, 1701    | 0.93 4.93 (t, J=8.4 Hz) 7.15 (d, J=8.1Hz) 6.63 (dd, J=2.9, 8.4 Hz) 6.57 (d, J=2.6Hz) 4.72 (s), 6.51 (dd, J=1.8, 3.3Hz), 7.15 (brs), 7.58 (d, J=0.7Hz) |
| 15b      | 99        | 199–202 | 3426, 1679          | 0.94 4.90 (t, J=8.4 Hz) 7.16 (d, J=8.4Hz) 6.63 (dd, J=2.6, 8.4 Hz) 6.57 (d, J=2.6Hz) 7.10 (t, J=4.2Hz), 7.55 (d, J=4.9Hz), 7.80 (d, J=4.4Hz) |
| 15c      | 91        | 253–257 | 3437, 1725          | 0.97 5.02 (t, J=8.4 Hz) 7.15 (d, J=8.4Hz) 6.63 (dd, J=2.9, 8.4 Hz) 6.57 (d, J=2.6Hz) 8.21 (d, J=1.8Hz), 8.87 (d, J=1.8Hz) |
| 15d      | 99        | 213–217 | 3470, 1723          | 0.95 4.96 (t, J=8.6 Hz) 7.15 (d, J=8.4Hz) 6.63 (dd, J=2.9, 8.4 Hz) 6.57 (d, J=2.6Hz) 6.94 (d, J=1.8Hz), 8.37 (d, J=1.8Hz) |
the aromatase-catalyzed 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, thiophencarbonyl, 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.

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

<table>
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<tr>
<th>Compound</th>
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<th>Observed</th>
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<td>3-Substituted series</td>
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<tr>
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<td>C, 75.80; H, 6.64; N, 0</td>
<td>C, 75.78; H, 6.73; N, 0.01</td>
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<td>12b</td>
<td>C, 72.60; H, 6.36; N, 0</td>
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<tr>
<td>12c</td>
<td>381.1399</td>
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<td>12d</td>
<td>363.1844</td>
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<tr>
<td>12e</td>
<td>C, 72.31; H, 6.34; 3.83</td>
<td>C, 71.90; H, 6.41; N, 3.80</td>
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<td>17β-Substituted series</td>
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</tr>
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<td>C, 69.95; H, 8.20; N, 0.0</td>
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<td>14c</td>
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<td>497.2420</td>
</tr>
<tr>
<td>14d</td>
<td>C, 69.82; H, 8.16; N, 2.91</td>
<td>69.37; H, 8.29; N, 2.75</td>
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<td>14e</td>
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<td>15a</td>
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</tr>
<tr>
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<td>68.95; H, 6.61; N, 3.53</td>
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<td>15c</td>
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<td>15d</td>
<td>C, 70.11; H, 6.84; N, 0</td>
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### Table 6. In Vitro Aromatase Inhibition by Estrone and Estradiol Derivatives

<table>
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<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
<th>Apparent $K_i$ (µM)</th>
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<tr>
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<td>5a</td>
<td>208.83±23.8</td>
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<tr>
<td>5b</td>
<td>75.39±4.7</td>
<td>—</td>
</tr>
<tr>
<td>5c</td>
<td>31.16±1.3</td>
<td>2.19±0.14</td>
</tr>
<tr>
<td>5d</td>
<td>10% inhibition at 100 µM</td>
<td>—</td>
</tr>
<tr>
<td>4-Substituted series</td>
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</tr>
<tr>
<td>10a</td>
<td>203.06±19.38</td>
<td>—</td>
</tr>
<tr>
<td>10b</td>
<td>95.64±5.2</td>
<td>—</td>
</tr>
<tr>
<td>10c</td>
<td>28.57±1.8</td>
<td>1.53±0.08</td>
</tr>
<tr>
<td>10d</td>
<td>10% inhibition at 100 µM</td>
<td>—</td>
</tr>
<tr>
<td>3-Substituted series</td>
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<td></td>
</tr>
<tr>
<td>12a</td>
<td>0% inhibition at 100 µM</td>
<td>—</td>
</tr>
<tr>
<td>12b</td>
<td>0% inhibition at 100 µM</td>
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<td>12c</td>
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<tr>
<td>12d</td>
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<td>12e</td>
<td>243.88±78.1</td>
<td>—</td>
</tr>
<tr>
<td>17β-Substituted series</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15a</td>
<td>0% inhibition at 100 µM</td>
<td>—</td>
</tr>
<tr>
<td>15b</td>
<td>0% inhibition at 100 µM</td>
<td>—</td>
</tr>
<tr>
<td>15c</td>
<td>0% inhibition at 100 µM</td>
<td>—</td>
</tr>
<tr>
<td>15d</td>
<td>0% inhibition at 100 µM</td>
<td>—</td>
</tr>
<tr>
<td>For comparison</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>26±8</td>
<td>2.50±0.22</td>
</tr>
<tr>
<td>Estradiol</td>
<td>5% inhibition at 50 µM</td>
<td>130±0.10</td>
</tr>
</tbody>
</table>

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$^a$ Not determined.

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

Concentrations of the inhibitor: control (0 µM) (○); 0.96 µM (▲); 1.92 µM (●); 3.85 µM (●).

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**References and Notes**


15) Numazawa M., Ando M., Watari Y., Tominaga T., Hayata Y.,