Synthesis and Antitumor Activity of Benzimidazolyl-1,3,5-triazine and Benzimidazolylpyrimidine Derivatives

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Triamino-substituted 1,3,5-triazine and pyrimidine derivatives were synthesized and tested for antitumor activities using some human cancer cell lines and murine leukemia cell lines. All the compounds having benzimidazolyl and morpholino groups as substituents on the 1,3,5-triazine ring showed antitumor activity. Pyrimidine derivatives having the same groups as substituents also showed antitumor activity. Among them, the compounds having 1-benzimidazolyl, morpholino and *cis*-2,3-dimethylmorpholino groups as substituents on the 1,3,5-triazine ring or pyrimidine ring exhibited the most potent antitumor activity, and these compounds exhibited no or very weak aromatase inhibitory activity. In contrast, the compounds having imidazolyl group instead of benzimidazolyl group as a substituent on the 1,3,5-triazine ring showed a potent aromatase inhibitory activity.

Key words 1,3,5-triazine; pyrimidine; antitumor activity; P388; MCF-7

We have recently reported the discovery of 1,3,5-triazine derivatives bearing both direct cytotoxicity and selective aromatase inhibitory activity.¹⁻⁴⁾ In the study of 1,3,5-triazine derivatives, 2-(1-imidazolyl)-4,6-dimorpholino-1,3,5-triazine (**1**) showed significant aromatase inhibitory activity and cytotoxicity against various human breast cancer cell lines, such as MCF-7 and a murine leukemia cell line, P388 leukemia.²⁾ Moreover, compound **1** showed the antitumor activity on 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors, due mainly to its direct cytotoxicity.⁴⁾ The cytotoxic activity of compound **1** was greater than that of hydroxymethylpentamethylmelamine (HMPMM). HMPMM is the hydroxylated metabolite and a major active form of hexamethylmelamine (HMM) (Fig. 1).^{5,6)} HMM, a 1,3,5-triazine derivative, has been clinically used as an antitumor agent against lung, ovarian and breast cancers.⁷⁻⁹⁾

Aromatase inhibitors might be applicable as antitumor agents for estrogen-dependent breast cancer in postmenopausal women in whom, the major pathway of estrogen biosynthesis is the peripheral conversion of androstenedione secreted from the adrenal to estrone. This conversion is catalyzed by aromatase which is a cytochrome P450-dependent monooxygenase.¹⁰⁾ However, the use of aromatase inhibitors as antitumor agents in patients other than postmenopausal women might not be approved, because this activity would certainly be accompanied by side effects relating to the suppression of estrogen production.

Therefore, we attempted to synthesize compounds having weaker aromatase inhibitory activity and more potent cytotoxicity than compound **1** for more suitable antitumor agents. In our previous study, the compounds having a 2-position substituted imidazolyl group or a 4,5-position substituted imidazolyl group in the 1,3,5-triazine derivatives did not inhibit aromatase.¹⁾ Our efforts to discover novel antitumor agents were primarily focused on the synthesis of compounds by converting the imidazolyl groups of **1** into various heterocycle groups including the substituted imidazolyl groups such as 4,5-dimethylimidazolyl group and benzimidazolyl group. Among the synthesized compounds, compound **4a** having a

benzimidazolyl group showed weaker aromatase inhibitory activity and more potent cytotoxicity. Several 2-benzimidazolyl-1,3,5-triazine or pyrimidine derivatives were therefore synthesized and their pharmacological activities were examined.

Chemistry The desired 1,3,5-triazine derivatives **4** were prepared by nucleophilic substitution reaction with azole or aliphatic amines as shown in Chart 1.

Compound **2**¹⁾ was treated with benzimidazole in the presence of potassium carbonate in *N,N*-dimethylformamide (DMF) at room temperature to afford compound **3**, followed by treatment with amines to afford compound **4**. The desired pyrimidine derivatives **7** were prepared as shown in Chart 2 employing the similar reaction as for the synthesis of **4**.

In the case of the pyrimidine derivatives, compound **7** was synthesized not from dichloromorpholinopyrimidine but from benzimidazolyl-dichloropyrimidine **5**. The desired precursor **5** was easily obtained by recrystallization from the reaction mixture of trichloropyrimidine with benzimidazole, where the reaction mixture mainly consisted of two isomers, 2-(1-benzimidazolyl)-4,6-dichloropyrimidine (**5**) and 4-(1-benzimidazolyl)-2,6-dichloropyrimidine.

Compound **7** was obtained in poor yield when the same method as for the synthesis of **4** was employed, due to the low activity of monochloropyrimidine **6** for nucleophilic substitution. To obtain pyrimidine **7** in high yield, the reaction of **6** with amine should be carried out with a large excess of amine at a high reaction temperature. DMF was inappropriate as a reaction solvent. The reason is that dimethylamine,

Fig. 1

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Chart 1

Chart 2

Table 1. Antitumor Activities of 1,3,5-Triazine and Pyrimidine Derivatives toward Cancer Cell Lines

Compd.	GI ₅₀ (μM)						Aromatase Inhibition % (μM)
	MCF-7	A549	WiDr	B16F10	L1210	P388	
4a	3.2	7.0	4.2	6.0	4.1	2.8	8 (10)
4b	11	>25	11	15	11	10	
4c	4.8	>10	6.8	7.4	8.5	3.7	
4d	2.2	6.5	2.3	7.3	3.6	1.8	
4e	2.9	>10	5.5	>10	6.8	4.2	11 (20)
4f	1.9	6.8	3.5	6.4	4.3	2.8	
4g	1.4	4.1	2.6	4.6	2.4	1.5	
7a	2.3	>5.0	2.3	>5.0	2.4	1.3	17 (100)
7b	2.2	5.4	2.7	5.2	2.1	1.7	
7c	1.1	3.1	1.7	2.5	0.92	0.70	
1	17	>50	21	46	23	18	95 (0.1)
HMPMM	>100	83	67	75	52	15	

Aromatase inhibitory activity was measured as described in our previous paper.¹⁾ No data in table: not determined.

which is produced by decomposition of DMF at a reaction temperature higher than 80 °C, reacts with **6** to give an undesirable 2-(1-benzimidazolyl)-4-dimethylamino-6-morpholinopyrimidine.

On the other hand, the use of morpholine as a reaction solvent and a nucleophile is suitable in the synthesis of **7**, because morpholine is commercially available and the undesirable product is not produced in the reaction of **6** to **7**. Therefore, **5** was first treated with morpholines at room temperature to afford **6**, followed by treatment with morpholine at the optimal temperature of 80 °C (instead of room temperature as employed for the synthesis of **4**) to afford **7**.

Pharmacological Results and Discussion The compounds listed in Table 1 were evaluated for cytotoxicity against some human cancer and murine leukemia cell lines and aromatase inhibitory activity. Replacing one morpholino group of **4a** with a dimethylamino group **4b** resulted in a reduction in cytotoxicity. This result suggests that the morpholino group in these compounds was important for potent antitumor activity, similar to the fact that this group was the most effective substituent for aromatase inhibitory activity of 1,3,5-triazine derivatives.¹⁾ The introduction of methyl group **4c** or *cis*-2,6-dimethyl group **4e** at the 2- or 2,6-position on one morpholine ring of **4a** exhibited comparable or slightly less cytotoxic activity than **4a**. The introduction of dimethyl

groups at the 2,2- or 2,3-position on one morpholine ring as in compounds **4d**, **4f** and **4g** slightly increased cytotoxicity.

Furthermore, pyrimidine derivatives **7a**, **7b** and **7c** also showed slightly increased cytotoxicity. The cytotoxicity of **7c** was the most potent and its activity was 10–20 fold greater in all cancer cell lines tested than that of the parent compound **1**. Compound **4a** having benzimidazolyl group, which prevents access of the imidazole ring to the active site of the enzyme due to condensed phenyl group with imidazole, showed little aromatase inhibitory activity. Moreover, compound **7c** which has benzimidazolyl and a substituted morpholino group showed slightly or no aromatase inhibitory activity. The reason may be that these bulky groups more strongly prevent access of the imidazole ring to the active site of the enzyme. In order to understand whether or not these compounds have potent antitumor activity, we examined their effects *in vivo* using nude mice. The results will be reported elsewhere.

Experimental

Chemistry Melting points were determined on a Yamato melting point apparatus Model Mp-21 and were uncorrected. Mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded on a JEOL SX102 mass spectrometer. ¹H-NMR spectra were taken at 270 MHz with a JEOL JMN-RX270 spectrometer with tetramethylsilane as an internal standard. Column chromatography was carried out on silica gel (Wakogel C-200). TLC was performed using aluminum sheets precoated with silica gel (Kieselgel 60

F₂₅₄ layer thickness 0.2 mm; Merck). Compound **2** was prepared as reported.¹⁾ Morpholines were prepared according to the literature,^{11–14)} except morpholine and *cis*-2,6-dimethylmorpholine which were purchased from Tokyo Kasei Kogyo Co., Ltd.

2-(1-Benzimidazolyl)-4-chloro-6-morpholino-1,3,5-triazine (3) Benzimidazole (62.1 g, 526 mmol) and K₂CO₃ (207.3 g, 1.50 mol) were added to a solution of 2,4-dichloro-6-morpholino-1,3,5-triazine (**2**) (123.6 g, 526 mmol) in DMF (1500 ml) at room temperature. The reaction mixture was stirred at room temperature for 18 h, then poured into water, and the resulting precipitate was collected. The precipitate was washed with a small amount of acetone, and dried to give **3** (114.7 g) in 69% yield. Colorless powder, mp 197–199 °C. ¹H-NMR (CDCl₃) δ: 3.7–4.1, (8H, m), 7.3–7.5 (2H, m), 7.8–7.9 (1H, m), 8.3–8.5 (1H, m), 8.96 (1H, s). MS *m/z*: 316 (M⁺). This was used for the next reaction without further purification.

General Procedures for Preparation of Compound 4 Synthesis of 2-(1-benzimidazolyl)-4-(*trans*-2,3-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4f**): *trans*-2,3-Dimethylmorpholine hydrochloride (13.8 g, 91.0 mmol) and K₂CO₃ (55.3 g, 400 mmol) were added to a solution of 2-(1-benzimidazolyl)-4-chloro-6-morpholino-1,3,5-triazine (**3**) (26.0 g, 82.1 mmol) in DMF (500 ml) at room temperature. The reaction mixture was stirred at room temperature for 18 h, poured into water, and then extracted with ethyl acetate (AcOEt). The extract was washed with brine, dried over MgSO₄, and evaporated *in vacuo*. The residue was purified by silica gel column chromatography with hexane–AcOEt (1 : 1) to give **4f** (27.6 g) as a diastereomixture in 85% yield. Colorless powder, mp 166–168 °C. ¹H-NMR (CDCl₃) δ: 1.1–1.3 (6H, m), 3.1–3.4 (1H, m), 3.5–4.1 (11H, m), 4.3–4.8 (2H, m), 7.3–7.5 (2H, m), 7.81 (1H, dd, *J*=2, 6 Hz), 8.36 (1H, dd, *J*=2, 6 Hz), 8.97 (1H, d, *J*=2 Hz). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2065.

Compounds **4a–4e** and **4g** were obtained using a similar procedure to that described for **4f**.

2-(1-Benzimidazolyl)-4,6-dimorpholino-1,3,5-triazine (**4a**): 98% yield. Colorless powder, mp 222–224 °C. ¹H-NMR (CDCl₃) δ: 3.8–4.0 (16H, m), 7.3–7.5 (2H, m), 7.8–7.9 (1H, m), 8.3–8.5 (1H, m), 8.97 (1H, s). HRMS *m/z*: Calcd for C₁₆H₁₉N₇O₂: 367.1757. Found: 367.1760.

2-(1-Benzimidazolyl)-4-dimethylamino-6-morpholino-1,3,5-triazine (**4b**): 97% yield. Colorless powder, mp 147–148 °C. ¹H-NMR (CDCl₃) δ: 3.20 (3H, s), 3.29 (3H, s), 3.7–4.0 (8H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 7 Hz), 8.44 (1H, dd, *J*=2, 7 Hz), 9.00 (1H, s). HRMS *m/z*: Calcd for C₁₆H₁₉N₇O: 325.1651. Found: 325.1646.

2-(1-Benzimidazolyl)-4-(2-methylmorpholino)-6-morpholino-1,3,5-triazine (**4c**): 87% yield. Colorless powder, mp 159–161 °C. ¹H-NMR (CDCl₃) δ: 1.30 (3H, d, *J*=6 Hz), 2.7–2.9 (1H, m), 3.0–3.3 (1H, m), 3.5–4.1 (11H, m), 4.5–4.7 (2H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 6 Hz), 8.38 (1H, dd, *J*=2, 6 Hz), 9.14 (1H, s). HRMS *m/z*: Calcd for C₁₉H₂₃N₇O₂: 381.1913. Found: 381.1906.

2-(1-Benzimidazolyl)-4-(2,2-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4d**): 88% yield. Colorless powder, mp 172–173 °C. ¹H-NMR (CDCl₃) δ: 1.28 (6H, s), 3.4–4.0 (14H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 6 Hz), 8.38 (1H, dd, *J*=2, 6 Hz), 8.97 (1H, s). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2065.

2-(1-Benzimidazolyl)-4-(*cis*-2,6-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4e**): 70% yield. Colorless powder, mp 195–197 °C. ¹H-NMR (CDCl₃) δ: 1.30 (6H, br s), 2.5–2.8 (2H, m), 3.6–3.8 (2H, m), 3.8–4.0 (8H, m), 4.5–4.7 (2H, m), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.36 (1H, dd, *J*=2, 7 Hz), 8.97 (1H, s). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2062.

2-(1-Benzimidazolyl)-4-(*cis*-2,3-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4g**): 88% yield. Colorless powder, mp 172–174 °C. ¹H-NMR (CDCl₃) δ: 1.35 (3H, d, *J*=7 Hz), 1.40 (3H, d, *J*=7 Hz), 3.3–3.5 (1H, m), 3.7–4.0 (11H, m), 4.4–4.7 (2H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 6 Hz), 8.37 (1H, dd, *J*=2, 6 Hz), 8.98 (1H, s). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2085.

2-(1-Benzimidazolyl)-4,6-dichloropyrimidine (5) Benzimidazole (59.1 g, 500 mmol) and K₂CO₃ (138.2 g, 1.00 mol) were added to a solution of 2,4,6-trichloropyrimidine (91.7 g, 500 mmol) in DMF (800 ml) at room temperature. The reaction mixture was stirred at room temperature for 17 h, and then poured into water. The resulting precipitate was collected and washed with a small amount of acetone. The precipitate was recrystallized from methanol to give **5** (70.0 g) in 53% yield. Colorless powder, mp 171–173 °C. ¹H-NMR (CDCl₃) δ: 7.26 (1H, s), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 7 Hz), 8.48 (1H, dd, *J*=2, 7 Hz), 8.99 (1H, s). MS *m/z*: 265 (M⁺). This was used for the next reaction without further purification.

General Procedures for Preparation of Monochloropyrimidine 6

Synthesis of 2-(1-benzimidazolyl)-4-chloro-6-(*trans*-2,3-dimethylmorpholino)pyrimidine (**6b**): *trans*-2,3-Dimethylmorpholine hydrochloride (15.2 g, 100 mmol) and K₂CO₃ (27.6 g, 200 mmol) were added to a solution of 2-(1-benzimidazolyl)-4,6-dichloropyrimidine (**5**) (18.0 g, 67.9 mmol) in DMF (300 ml) at room temperature. The reaction mixture was stirred at room temperature for 18 h. The reaction mixture was poured into water, and then extracted with AcOEt. The extract was washed with brine, dried over MgSO₄, and evaporated *in vacuo*. The residue was purified by silica gel column chromatography with hexane–AcOEt (1 : 1) to give **6b** (21.3 g) in 91% yield. Colorless powder, mp 88–92 °C. ¹H-NMR (CDCl₃) δ: 1.26 (6H, d, *J*=7 Hz), 3.2–3.4 (1H, m), 3.6–3.9 (4H, m), 4.0–4.2 (1H, m), 6.38 (1H, s), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.44 (1H, dd, *J*=2, 7 Hz), 8.97 (1H, s). MS *m/z*: 343 (M⁺). Compounds **6a** and **6c** were obtained using a similar procedure to that described for **6b**.

2-(1-Benzimidazolyl)-4-chloro-6-morpholinopyrimidine (**6a**): 91% yield. Colorless powder, mp 191–193 °C. ¹H-NMR (CDCl₃) δ: 3.7–3.9 (8H, m), 6.39 (1H, s), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.42 (1H, dd, *J*=2, 7 Hz), 8.95 (1H, s). MS *m/z*: 315 (M⁺).

2-(1-Benzimidazolyl)-4-chloro-6-(*cis*-2,3-dimethylmorpholino)pyrimidine (**6c**): 77% yield. Colorless powder, mp 117–119 °C. ¹H-NMR (CDCl₃) δ: 1.38 (3H, d, *J*=7 Hz), 1.42 (3H, d, *J*=7 Hz), 3.3–3.5 (1H, m), 3.7–4.2 (5H, m), 6.38 (1H, s), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.44 (1H, dd, *J*=2, 7 Hz), 8.95 (1H, s). MS *m/z*: 343 (M⁺). These were used for the next reaction without further purification.

General Procedures for Preparation of Compound 7 Synthesis of 2-(1-benzimidazolyl)-4-(*trans*-2,3-dimethylmorpholino)-6-morpholinopyrimidine (**7b**): 2-(1-Benzimidazolyl)-4-chloro-6-(*trans*-2,3-dimethylmorpholino)pyrimidine (**6b**) (7.22 g, 21.0 mmol) was stirred in morpholine (52.0 ml) at 80 °C for 2 h. The reaction mixture was poured into water, and then extracted with AcOEt. The extract was washed with brine, dried over MgSO₄, and evaporated *in vacuo*. The residue was purified by silica gel column chromatography with hexane–AcOEt (1 : 1) to give **7b** (7.78 g) as a diastereomixture in 94% yield. Colorless powder, mp 177–180 °C. ¹H-NMR (CDCl₃) δ: 1.20 (3H, d, *J*=7 Hz), 1.24 (3H, d, *J*=7 Hz), 3.1–3.4 (1H, m), 3.6–4.1 (13H, m), 5.44 (1H, s), 7.2–7.4 (2H, m), 7.8–7.9 (1H, m), 8.3–8.4 (1H, m), 8.96 (1H, s). HRMS *m/z*: Calcd for C₂₁H₂₆N₆O₂: 394.2117. Found: 394.2119.

Compounds **7a** and **7c** were obtained using a similar procedure to that described for **7b**.

2-(1-Benzimidazolyl)-4,6-dimorpholinopyrimidine (**7a**): 82% yield. Colorless powder, mp 297–299 °C. ¹H-NMR (CDCl₃) δ: 3.6–3.9 (16H, m), 5.48 (1H, s), 7.2–7.4 (2H, m), 7.8–7.9 (1H, m), 8.3–8.4 (1H, m), 8.96 (1H, s). HRMS *m/z*: Calcd for C₁₉H₂₂N₆O₂: 366.1804. Found: 366.1823.

2-(1-Benzimidazolyl)-4-(*cis*-2,3-dimethylmorpholino)-6-morpholinopyrimidine (**7c**): 86% yield. Colorless powder, mp 170–172 °C. ¹H-NMR (CDCl₃) δ: 1.37 (3H, d, *J*=7 Hz), 1.39 (3H, d, *J*=7 Hz), 3.3–3.5 (1H, m), 3.6–4.2 (13H, m), 5.43 (1H, s), 7.2–7.4 (2H, m), 7.8–7.9 (1H, m), 8.3–8.4 (1H, m), 8.96 (1H, s). HRMS *m/z*: Calcd for C₂₁H₂₆N₆O₂: 394.2117. Found: 394.2135.

Cell Lines The cytotoxic activity was analyzed using human MCF-7, breast, A549 lung, WiDr, and colon cancer cell lines and murine P388 leukemia, L1210 leukemia, and B16F10 melanoma cell lines. MCF-7 was kindly supplied by Dr. T. Kubota, Department of Surgery, School of Medicine, Keio University, Tokyo. A549 and WiDr were purchased from Dainippon Pharmaceutical Co., Ltd. P388 and L1210 were generously provided by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, and B16F10 by the Cancer Center, Faculty of Medicine, Kyushu University, Fukuoka.

Cytotoxic Activity Test RPMI1640 medium (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL), 25 mM HEPES (Dojin Chemical Laboratory, Ltd.) and 100 μg/ml kanamycin (Dainippon Pharmaceutical Co., Ltd.) was used as the assay medium. Cells were incubated at 37 °C in a humidified atmosphere of 95% air–5% CO₂. MCF-7, A549, WiDr, and B16F10 cells in exponential growth phase were harvested by trypsinization using Trypsin-EDTA (GIBCO BRL) and were inoculated into 96 well-microculture plates (TC MICRO WELL 96F, NUNC) at the density of 4 × 10³, 3 × 10³, 2 × 10³, or 10³ cells per well in 100 μl assay medium. After 1 day incubation, 100 μl of the assay medium containing various concentrations of sample compounds dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.) were added to each well, and cells were incubated for another 3 d. For P388 and L1210 cell cultures the ascitic fluid was removed from mice 6 or 7 d after intraperitoneal transplantation of P388 and L1210 cells. Cells were separated by centrifugation at 800 × *g* for a few minutes and were inoculated into 25 cm² tissue culture flasks (TC

FLASK 25CM2, NUNC) at 2×10^6 cells per flask in 5 ml assay medium supplemented with $10 \mu\text{M}$ 2-hydroxyethyl disulfide (Aldrich Chemical Co.). After 1 d incubation, P388 and L1210 cells were harvested and reinoculated into 96 well-microculture plates at 4×10^3 and 2×10^3 cells, respectively, per well in 100 μl assay medium. The assay medium supplemented with $10 \mu\text{M}$ 2-hydroxyethyl disulfide, and 100 μl of assay medium containing various concentrations of sample compounds dissolved in DMSO were added to culture wells. Cells were incubated for another 3 d.

XTT Assay¹⁵⁾ Cytotoxic activity was determined by the XTT assay. Briefly, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylaminocarbonyl)-2H-tetrazolium hydroxide (XTT, Sigma) was prepared at 1 mg/ml in phenol red-free RPMI1640 medium (GIBCO BRL) supplemented with 25 mM HEPES and 100 $\mu\text{g}/\text{ml}$ kanamycin. Phenazine methosulfate (PMS, Sigma) was prepared at 0.765 mg/ml in phenol red-free RPMI1640 medium (GIBCO BRL) supplemented with 25 mM HEPES and 100 $\mu\text{g}/\text{ml}$ kanamycin. Twenty-five microliters of PMS solution (0.765 mg/ml) was added per 5 ml of XTT solution (1 mg/ml). Fifty microliters of this mixture (final concentrations, 50 μg of XTT and 0.19 μg PMS per well) was added to each well and incubated for 4 h. The plates were agitated on a mechanical plate shaker and absorption-spectra of formazan were measured using a microplate spectrophotometer (MTP-32, CORONA) at 450 nm. The percentage of cell growth inhibition was calculated as follows: percent of cell growth inhibition = $100 \times [1 - (T - T_0)/(C - T_0)]$, where C stands for the mean optical density at 450 nm (OD450) of the control group, T for the mean OD450 of treated group and T0 for the mean OD450 at time zero (the time at which samples were added). Means were obtained by 4 determinations. Growth inhibition of 50% (GI_{50}) value was read on the dose-response curve.

Aromatase Inhibitory Activity The inhibitory activity was measured by the method reported in our previous paper.¹⁾

References

- 1) Matsuno T., Kato M., Tsuchida Y., Takahashi M., Terada S., *Chem. Pharm. Bull.*, **45**, 291—296 (1997).
- 2) Yaguchi S., Izumisawa Y., Sato M., Nakagane T., Koshimizu I., Sakita K., Kato M., Yoshioka K., Sakato M., Kawashima S., *Biol. Pharm. Bull.*, **20**, 698—700 (1997).
- 3) Takahashi M., Kayo T., Karakida T., Nakagawa S., Kato M., Matsuno T., Koide Y., Sato M., Kawashima S., *Endocrine Res.*, **23**, 1—13 (1997).
- 4) Iino Y., Karakida T., Sugamata N., Andoh T., Takei H., Takahashi M., Yaguchi S., Matsuno T., Sato M., Kawashima S., Morishita Y., *Anti-cancer Res.*, **18**, 171—176 (1998).
- 5) Rutty C. J., Connors T. A., *Biochem. Pharm.*, **26**, 2385—2391 (1977).
- 6) Gescher A., D'Incalci M., Fanelli R., Farina P., *Life Sci.*, **26**, 147—154 (1980).
- 7) Wilson W. L., Bisel H. F., Cole D., Rochlin D., Ramirez G., Madden R., *Cancer*, **25**, 567—570 (1970).
- 8) Legha S. S., Slavik M., Carter S. K., *Cancer*, **38**, 27—35 (1970).
- 9) Foster B. J., Harding B. J., Leyland-Jones B., Hoth D., *Cancer Treat. Rev.*, **113**, 197—217 (1986).
- 10) a) Thompson E. A. Jr., Siiteri P. K., *J. Biol. Chem.*, **249**, 5364—5372 (1974); b) Henderson D., Norbistrath D., Kerb U., *J. Steroid Biochem.*, **24**, 303—306 (1986).
- 11) Cottle D. L., Jeitsch A. E., Stout T. H., Walters D. R., *J. Org. Chem.*, **11**, 286—291 (1946).
- 12) Vargha L., Kasztreiner E., Borsy J., Farkas L., Kuszmann J., Dumbovich B., *Biochem. Pharm.*, **11**, 639—649 (1962).
- 13) Nilsson B., Hernestam S., *Org. Magn. Reson.*, **11**, 116—122 (1978).
- 14) Anderson A., Boyd A. C., Byford A., Campbell A. C., Gemmell D. K., Hamilton N. M., Hill D. R., Hill-Venning C., Lambert J. J., Maidment M. S., May V., Marshall R. J., Peter J. A., Rees D. C., Stevenson D., Sundaram H., *J. Med. Chem.*, **40**, 1668—1681 (1997).
- 15) Dominic A. S., Robert H. S., Kenneth D. P., Anne M., Siobhan T., Thomas H. N., Michael J. C., Donna S., Michael R. B., *Cancer Res.*, **48**, 4827—4833 (1988).