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Research Article

# Synthesis of Nicotinamide Derivatives Having a Hydroxy-Substituted Benzene Ring and the Influence of Their Structures on the Apoptosis-Inducing Activity Against Leukemia Cells

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**ABSTRACT** With the view of developing novel apoptosis-inducing agents against malignant cells, nicotinamide derivatives containing substituted *O*-benzoyl-tyrosine, dopamine, and norepinephrine residues were synthesized. Antiproliferative activity measurements using leukemia U937 cells revealed that the benzoyl-tyrosine derivative having two hydroxys at C-2 and C-3 on the benzoyl group, and the one having a hydroxy at C-2 and a methoxy at C-4 proved the most potent among the 9 nicotinamide derivatives. The $IC_{50}$ values of these compounds were 0.87 and 3.15 μM, which indicates their noteworthy activity compared with epigallocatechin gallate, a catechin component in green tea known for its noticeable activity. The cell death process was confirmed to be the result of apoptosis by agarose gel electrophoresis and dye staining, suggesting the high potential of these compounds as apoptosis-inducing anticancer agents. Drug Dev Res 72:289–297, 2011. 

**Key words:** nicotinamide derivatives; anticancer agent; apoptosis-inducing activity; leukemia cells

## INTRODUCTION

Apoptosis, a programmed cell death [Kerr et al., 1972], is characterized by various morphological changes, including phosphatidylserine externalization, cell shrinkage, chromatin condensation, nuclear collapse, and cellular fragmentation into apoptosis bodies, and the cells are finally eliminated by macrophages [Wright et al., 1996; Bortner and Cidlowski, 2002; Ujibe et al., 2005]. Therefore, apoptosis plays a crucial role in the maintenance of homeostasis in developmental processes and in the elimination of damaged cells. Certain compounds are known to induce apoptosis in cancer cells, and their structure–property relationships would be key information to develop new types of anticancer agents that are effective against malignant cells but have minimal side effects on normal cells.

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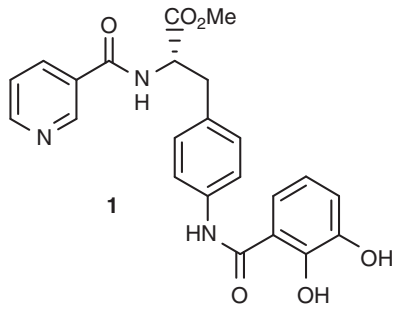


**Fig. 1.** Structure of an amide derivative of nicotinamide (**1**) [Yamaguchi et al., 2007].

With regard to the apoptosis induction in cancer cells, green tea polyphenols have been attracting considerable attention; catechins as exemplified by (+)-epigallocatechin gallate (EGCG) are particularly interesting because of their anticancer activity owing to their apoptosis-inducing ability [Chen et al., 1998; Yang et al., 1998; Isemura et al., 2000; Katsuno et al., 2001; Adhami et al., 2003; Hsu et al., 2003; Suzuki et al., 2004; Ohata et al., 2005]. Dopamine [He and Yuan, 2007] and some nicotinamide derivatives such as 6-aminonicotinamide and $N^1$-methylnicotinamide [Luo et al., 1998; Ogata et al., 2000; Jaubert et al., 2006] also induce apoptosis in HL-60 cells and in 293, K562, and GH3 cells, respectively.

In our previous study, a nicotinamide derivative of *p*-aminophenylalanine (**1**, Fig. 1) was synthesized and found to exhibit a considerable growth inhibitory effect on human leukemia U937 cells [Yamaguchi et al., 2007], higher than that of EGCG. The results prompted us to study the influence of chemical structures on the manifestation of apoptosis activity through structural modifications by (1) replacing the aminophenylalanine moiety with the tyrosine one to incorporate an ester linkage as in EGCG, and (2) diversifying the substitution patterns on the benzoyl group. Nicotinamides were also derived from dopamine and norepinephrine as excellent candidates. The resulting compounds were evaluated in terms of anticancer activity against U937 cells, and the cell death process was elucidated by agarose gel electrophoresis of the DNA and by annexin V staining of the treated cells.

## METHODS AND MATERIALS

### General

Infrared (IR) spectra were recorded with a JASCO FT/IR-470. $^1$H-nuclear magnetic resonance (NMR) spectra were obtained on a JEOL JNM-LA400D NMR spectrometer, with tetramethylsilane used as an internal reference. Electrophoresis was performed with a Mupid-Scope (Advance Co., Ltd., Tokyo, Japan). Cell staining was examined with a fluorescence microscope Nikon ECLIPS ETE300. Thin-layer chromatography (TLC) and column chromatography were conducted on Merck Silica Gel 60 $F_{254}$ and Silica Gel 60 N (spherical, neutral, 63–210 μm; Kanto Chemical Co. Inc., Tokyo, Japan). The elemental analysis was carried out with a Perkin-Elmer 2400 analyzer series II or Euro Vector EURO-EA3000-Dual. All chemicals were of reagent grade and were used without further purification. Anhydrous solvents containing no stabilizers for synthetic use, such as THF, $CHCl_3$, $CH_2Cl_2$, and dimethyl sulfoxide (DMSO), were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). An Annexin V-Fluorescein Staining Kit containing annexin V-fluorescein, a binding buffer, and propidium iodide was obtained from Wako Pure Chemical Industries, Ltd.

### *N*-Nicotinoyl-L-Tyrosine Methyl Ester (3)

A solution of 1.45 g (14 mmol) of $Et_3N$ in 25 ml of THF was added dropwise at 0°C to a solution of 2.86 g (12 mmol) of L-tyrosine methyl ester hydrochloride and 2.55 g (11 mmol) of nicotinic anhydride in 100 ml of THF; the mixture was stirred at room temperature for 2.5 h. The mixture was filtered, and the filtrate was evaporated. The residue was added to 200 ml of AcOEt, and 50 ml of $H_2O$ was added to the solution. The organic layer was separated, washed with saturated NaCl (50 ml × 2), and dried over anhydrous $Na_2SO_4$. The solvent was evaporated to give 3.10 g (93%) of the product (**3**): mp 123–124°C; IR (KBr):ν 3343, 2947, 1742, 1641, 1596, 1524, 1368, 1321, 1223, and 700 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.13–3.26 [2H, m, $CH_2$ (Tyr)] 3, 3.80 (3H, s, $OCH_3$), 5.06 [1H, dt, $J$ = 7.8 and 5.4 Hz, CH (Tyr)], 6.61 (1H, d, $J$ = 7.8 Hz, NH), 6.76 [2H, d, $J$ = 8.5 Hz, 3,5-H (Tyr)], 6.98 [2H, d, $J$ = 8.5 Hz, 2,6-H (Tyr)], 7.41 [1H, dd, $J$ = 4.8 and 7.8 Hz, 5-H (Pyr)], 8.10 [1H, d, $J$ = 7.8 Hz, 4-H (Pyr)], 8.76 [1H, d, $J$ = 4.8 Hz, 6-H (Pyr)], and 8.89 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{16}H_{16}N_2O_4 \cdot 0.1\ H_2O$: C, 63.61; H, 5.40; N, 9.27. Found: C, 63.42; H, 5.46; N, 9.06.

### Synthesis of *N*-Nicotinoyl-*O*-(2,3-Dimethoxybenzoyl)-L-Tyrosine Methyl Ester (4a)

Compound **3** (499 mg, 1.7 mmol) and $Et_3N$ (242 mg, 2.4 mmol) were added to 40 ml of $CHCl_3$. A solution of 446 mg (2.2 mmol) of 2,3-dimethoxylbenzoyl chloride in 20 ml of $CHCl_3$ was added dropwise at 0°C, and the mixture was stirred at room temperature for 3 h. The reaction mixture was washed with 50 ml of 5% aqueous $NaHCO_3$ and 50 ml of saturated aqueous NaCl, and the solution was dried over anhydrous $Na_2SO_4$. After removal of the solvent,

the crude product was purified by column chromatography on silica gel with $CHCl_3$/MeOH (20/1) to afford 615 mg (78%) of the product (**4a**): mp 127–128°C; IR (KBr): ν 3309, 2943, 1739,1200, 1265, 1481, and 747 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.27 and 3.33 [2H, ABX, $J$ = 5.3 and 13.9 Hz, $CH_2$ (Tyr)], 3.81 (3H, s, $CO_2CH_3$), 3.92 (3H, s, $OCH_3$), 3.95 (3H, s, $OCH_3$), 5.11 [1H, d, $J$ = 8.0 Hz, CH (Tyr)], 6.67 (1H, d, $J$ = 7.3 Hz, NH), 7.14–7.18 [6H, m, 2,3,5,6-H (Tyr) and 5,6-H (Bnz)], 7.39 [1H, dd, $J$ = 5.1 and 7.7 Hz, 5-H (Pyr)], 7.48–7.51 [1H, m, 4-H (Bnz)], 8.05 [1H, d, $J$ = 7.7 Hz, 4-H (Pyr)], 8.74 [1H, s, 6-H (Pyr)], and 8.97 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{25}H_{24}N_2O_7 \cdot 0.1H_2O$: C, 64.40; H, 5.23; N, 6.01. Found: C, 64.27; H, 5.03; N, 6.02.

### *N*-Nicotinoyl-*O*-(2,4-Dimethoxybenzoyl)-L-Tyrosine Methyl Ester (4b)

Yield 72%; mp 141–142°C; IR (KBr): ν 3290, 3052, 1737, 1714, 1639, 1611, 1333, 1420, and 701 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.25–3.35 [2H, m, $CH_2$ (Tyr)], 3.80 (3H, s, $CO_2CH_3$), 3.80 (3H, s, $OCH_3$), 3.89 (3H, s, $OCH_3$), 5.11 [1H, d, $J$ = 7.3 Hz, CH (Tyr)], 6.54 (1H, s, NH), 6.53–6.57 [2H, m, 3,5-H (Bnz)], 6.73 (1H, d, $J$ = 7.8 Hz, NH), 7.12–7.17 [4H, m, 2,3,5,6-H (Tyr)], 7.43 [1H, dd, $J$ = 4.9 and 7.8 Hz, 5-H (Pyr)], 8.07 [1H, d, $J$ = 8.5 Hz, 6-H (Bnz)], 8.07 [1H, d, $J$ = 8.1 Hz, 4-H (Pyr)], 8.75 [1H, s, 6-H (Pyr)], and 9.01 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{25}H_{24}N_2O_7 \cdot 0.1H_2O$: C, 64.40; H, 5.23; N, 6.01. Found: C, 64.29; H, 5.17; N, 6.01.

### *N*-Nicotinoyl-*O*-(2,5-Dimethoxybenzoyl)-L-Tyrosine Methyl Ester (4c)

Yield 72%; mp 104–105°C; IR (KBr): ν 3258, 2940, 1720, 1612, 1507, 1438, 1220, 1166, 833, and 769 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.27 and 3.33 [2H, ABX, $J$ = 5.4 and 13.9 Hz, $CH_2$ (Tyr)], 3.80 (3H, s, $CO_2CH_3$), 3.82 (3H, s, $OCH_3$), 3.90 (3H, s, $OCH_3$), 5.11 [1H, d, $J$ = 7.7 Hz, CH (Tyr)], 6.66 (1H, d, $J$ = 9.2 Hz, NH), 6.98 [2H, d, $J$ = 9.2 Hz, 3,5-H (Tyr)], 7.09–7.14 [2H, m, 3,4-H (Bnz)], 7.17 [2H, d, $J$ = 9.2 Hz, 2,6-H (Tyr)], 7.40 [1H, dd, $J$ = 4.6 and 8.9 Hz, 5-H (Pyr)], 7.51 [1H, d, $J$ = 3.1 Hz, 6-H (Bnz)], 8.05 [1H, d, $J$ = 8.9 Hz, 4-H (Pyr)], 8.74 [1H, d, $J$ = 4.6 Hz, 6-H (Pyr)], and 8.97 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{25}H_{24}N_2O_7$: C, 64.65; H, 5.21; N, 6.03. Found: C, 64.70; H, 5.10; N, 5.99.

### *N*-Nicotinoyl-*O*-(2-Methoxybenzoyl)-L-Tyrosine Methyl Ester (4d)

Yield 71%; mp 128–130°C; IR (KBr): ν 3319, 2952, 1743, 1639, 1490, 1436, 1240, 1038, and 755 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.26 and 3.32 [2H, ABX, $J$ = 5.5 and 13.5 Hz, $CH_2$ (Tyr)], 3.79 (3H, s, $CO_2CH_3$), 3.93 (3H, s, $OCH_3$), 5.10 [1H, dd, $J$ = 5.5 and 13.5 Hz, CH (Tyr)], 6.72 (1H, d, $J$ = 7.5 Hz, NH), 7.02–7.06 [2H, m, 3,5-H (Bnz)], 7.14–7.16 [4H, m, $J$ = 7.3 Hz, 2,3,5,6-H (Tyr)], 7.26 [1H, dd, $J$ = 4.9 and 8.0 Hz, 5-H (Pyr)], 7.53–7.57 [1H, m, 4-H (Bnz)], 7.99 [1H, d, $J$ = 1.7 Hz, 6-H (Bnz)], 8.03 [1H, d, $J$ = 7.8 Hz, 4-H (Pyr)], 8.74 [1H, s, 6-H (Pyr)], and 8.97 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{24}H_{22}N_2O_6 \cdot 0.6\ H_2O$: C, 64.74; H, 5.25; N, 6.29. Found: C, 64.66; H, 4.89; N, 6.17.

### *N*-Nicotinoyl-*O*-(3-Methoxybenzoyl)-L-Tyrosine Methyl Ester (4e)

Yield 81%; mp 136–137°C; IR (KBr): ν 3358, 2949, 1752, 1638, 1523, 1278, 1088, 1320, and 749 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.28 and 3.35 [2H, ABX, $J$ = 5.7 and 13.5 Hz, $CH_2$ (Tyr)], 3.81 (3H, s, $CO_2CH_3$), 3.89 (3H, s, OCH3), 5.11 [1H, dd, $J$ = 5.7 and 13.5 Hz, CH (Tyr)], 6.65 (1H, d, $J$ = 7.5 Hz, NH), 7.15–7.20 [5H, m, 2,3,5,6-H (Tyr) and 5-H (Bnz)], 7.39–7.44 [2H, m, 5-H (Pyr) and 2-H (Bnz)], 7.68 [1H, s, 4-H (Bnz)], 7.79 [1H, d, $J$ = 7.8 Hz, 6-H (Bnz)], 8.06 [1H, d, $J$ = 7.8 Hz, 4-H (Pyr)], 8.75 [1H, s, 6-H (Pyr)], and 8.97 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{24}H_{22}N_2O_6$: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.63; H, 5.16; N, 6.40.

### *N*-Nicotinoyl-*O*-(4-Methoxybenzoyl)-L-Tyrosine Methyl Ester (4f)

Yield 88%; mp 188–189°C; IR (KBr): ν 3333, 2961, 1740, 1608, 1266, 1075, and 763 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.27 and 3.34 [2H, ABX, $J$ = 5.5 and 12.5 Hz, CH (Tyr)], 3.81 (3H, s, $CO_2CH_3$), 3.90 (3H, s, $OCH_3$), 5.11 [1H, dd, $J$ = 5.5 and 12.5 Hz, CH (Tyr)], 6.64 (1H, d, $J$ = 7.3 Hz, NH), 6.68 [2H, d, $J$ = 9.0 Hz, 3,5-H (Bnz)], 7.14 [2H, d, $J$ = 8.5 Hz, 3,5-H (Tyr)], 7.18 [2H, d, $J$ = 8.5 Hz, 2,6-H (Tyr)], 7.40 [1H, dd, $J$ = 4.8 and 8.0 Hz, 5-H (Pyr)], 8.06 [1H, dd, $J$ = 8.0 Hz, 4-H (Pyr)], 8.14 [2H, d, $J$ = 9.0 Hz, 2,6-H (Bnz)], 8.75 [1H, d, $J$ = 4.8 Hz, 6-H (Pyr)], and 8.97 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{24}H_{22}N_2O_6$: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.37; H, 5.08; N, 6.42.

### Synthesis of *N*-Nicotinoyl-*O*-(4-Acetoxybenzoyl)-L-Tyrosine Methyl Ester (4g)

To a solution of 407 mg (1.4 mmol) of **3** and 206 mg (2.0 mmol) of $Et_3N$ in 80 ml of THF was added dropwise a solution of 404 mg (2.0 mmol) of 4-acetoxylbenzoyl chloride in 15 ml of THF at 0°C. The mixture was stirred for 1 h at 0°C and for an additional 16 h at room temperature. After removal of the solvent, 50 ml of $CHCl_3$ and 30 ml of $H_2O$ were added to the residue. The $CHCl_3$ layer was washed with 50 ml of saturated NaCl and dried over anhydrous $Na_2SO_4$.

After removal of the solvent, the crude product was purified by column chromatography on silica gel with $CHCl_3$/MeOH (20/1) as an eluant to give 545 mg (87%) of the product (**4g**): mp 157–158°C; IR (KBr): ν 3322, 2952, 1739, 1640, 1273, 1017, and 699 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$): δ 2.31 (3H, s, $OCOCH_3$), 3.09–3.27 [2H, m, $CH_2$ (Tyr)], 3.78 (3H, s, $OCH_3$), 4.71 [1H, dd, $J$ = 5.3 and 13.4 Hz, CH (Tyr)], 7.20 [2H, d, $J$ = 8.2 Hz, 2,6-H (Bnz)], 7.37 [4H, dd, $J$ = 8.5 and 13.6 Hz, 2,3,5,6-H (Tyr)], 7.51 [1H, dd, $J$ = 4.8 and 7.9 Hz, 5-H (Pyr)], 8.13–8.16 [3H, m, 3,5-H (Bnz), and NH], 8.71 [1H, d, $J$ = 4.8 Hz, 4-H (Pyr)], 8.95 [1H, s, 2-H (Pyr)], and 9.14 ppm [1H, d, $J$ = 7.9 Hz, 6-H (Pyr)]. Anal. Calcd for $C_{25}H_{22}N_2O_7$: C, 64.93; H, 4.80; N, 6.06. Found: C, 64.99; H, 4.73; N, 5.96.

### Synthesis of *N*-Nicotinoyl-*O*-(2,3-Dihydroxybenzoyl)-L-Tyrosine Methyl Ester (5a)

To a solution of 106 mg (0.23 mmol) of **4a** in 10 ml of $CH_2Cl_2$ was slowly added 0.86 ml (0.86 mmol) of 1 M $BBr_3$ in $CH_2Cl_2$ at −30°C under an Ar atmosphere. The reaction mixture was stirred for 72 h at room temperature. The mixture was cooled to −30°C again, and 4.0 ml of MeOH was added slowly to quench the reaction. After evaporation of the solvent, the residue was adjusted to pH 7 with 1 M NaOH, and the mixture was extracted with $CHCl_3$ (40 ml × 3). The combined organic layers were dried over anhydrous $MgSO_4$, and after removal of the solvent, the crude product was purified by column chromatography on silica gel with $CHCl_3$/MeOH (6/1) to afford 44 mg (16%) of the product (**5a**): mp 129–130°C; IR (KBr): ν 3320, 2951, 1747, 1682, 1593, 1532, 1369, 1299, and 741 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.27 and 3.36 [2H, ABX, $J$ = 5.4 and 13.9 Hz, $CH_2$ (Tyr)], 3.81 (3H, s, $CO_2CH_3$), 5.11 [1H, dd, $J$ = 5.3 and 12.4 Hz, CH (Tyr)], 6.66 (1H, d, $J$ = 7.3 Hz, NH), 7.14–7.23 [6H, m, 4,6-H (Bnz), and 2,3,5,6-H (Tyr)], 7.41 [1H, m, 5-H (Pyr)], 7.59 [1H, d, $J$ = 7.5 Hz, 5-H (Bnz)], 8.07 [1H, d, $J$ = 7.5 Hz, 4-H (Pyr)], 8.75 [1H, m, 6-H (Pyr)], and 8.96 [1H, s, 2-H (Pyr)] and 10.5 ppm (1H, br, OH). Anal. Calcd for $C_{23}H_{20}N_2O_7 \cdot 1.2\ H_2O$: C, 60.31; H, 4.93; N, 6.12. Found: C, 60.23; H, 4.75; N, 5.88.

### *N*-Nicotinoyl-*O*-(2-Hydroxy-4-Methxybenzoyl)-L-Tyrosine Methyl Ester (5b)

Yield 59%; mp 148–149°C; IR (KBr): ν 3322, 1742, 1671, 1589, 1531, 1357, and 773 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.24 and 3.33 [2H, ABX, $J$ = 5.6 and 13.5 Hz, $CH_2$ (Tyr)], 3.80 (3H, s, $CO_2CH_3$), 3.85 (3H, s, $OCH_3$), 5.10 [1H, dd, $J$ = 5.6 and 13.5 Hz, CH (Tyr)], 6.48–6.53 [2H, m, 3,5-H (Bnz)], 6.81 (1H, d, $J$ = 7.3 Hz, NH), 7.13 [2H, d, $J$ = 8.5 Hz, 3,5-H (Tyr)], 7.20 [2H, d, $J$ = 8.5 Hz, 2,6-H (Tyr)], 7.38 [1H, dd, 4.8 and 7.9 Hz, 5-H (Pyr)], 7.93 [1H, d, $J$ = 8.7 Hz, 6-H (Bnz)], 8.06 [1H, dd, $J$ = 7.9 Hz, 4-H (Pyr)], 8.73 [1H, d, $J$ = 4.8 Hz, 6-H (Pyr)], 8.95 [1H, s, 2-H (Pyr)] and 10.66 ppm (1H, s, OH). Anal. Calcd for $C_{24}H_{22}N_2O_7$: C, 63.99; H, 4.92; N, 6.22. Found: C, 63.83; H, 4.91; N 6.08.

### *N*-Nicotinoyl-*O*-(2,5-Dihydroxybenzoyl)-L-Tyrosine Methyl Ester (5c)

Yield 24%; mp 97–98°C; IR (KBr): ν 3286, 1741, 1640, 1592, 1508, 1312, and 793 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.24 and 3.33 [2H, ABX, $J$ = 5.5 and 14.0 Hz, $CH_2$ (Tyr)], 3.81 (3H, s, $CO_2CH_3$), 5.11 [1H, dd, $J$ = 5.5 and 13.1 Hz, CH (Tyr)], 6.64 (1H, br, NH), 6.92 [2H, d, $J$ = 9.0 Hz, 3,5-H (Tyr)], 7.07–7.11 [2H, m, 3,4-H (Bnz)], 7.19 [2H, d, $J$ = 9.0 Hz, 2,6-H (Tyr)], 7.38–7.43 [2H, m, 5-H (Pyr) and 6-H (Bnz)], 8.08 [1H, d, $J$ = 7.9 Hz, 4-H (Pyr)], 8.75 [1H, d, $J$ = 4.1 Hz, 6-H (Pyr)], 8.95 [1H, s, 2-H (Pyr)], and 10.00 ppm (1H, s, OH). Anal. Calcd for $C_{23}H_{20}N_2O_7 \cdot 0.8\ H_2O$: C, 61.28; H, 4.83; N, 6.21. Found: C, 61.35; H, 4.57; N, 6.00.

### *N*-Nicotinoyl-*O*-(2-Hydroxy-5-Methoxybenzoyl)-L-Tyrosine Methyl Ester (5c′)

Yield 20%; mp 97–98°C; IR (KBr): ν 3274, 1744, 1662, 1592, 1508, 1348, and 773 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.26 and 3.36 [2H, ABX, $J$ = 5.5 and 14.0 Hz, $CH_2$ (Tyr)], 3.81 (3H, s, $CO_2CH_3$), 3.82 (3H, s, $OCH_3$), 5.11 [1H, dd, $J$ = 5.5 and 13.1 Hz, CH (Tyr)], 6.69 (1H, d, $J$ = 7.3 Hz, NH), 6.98 [2H, d, $J$ = 9.0 Hz, 3,5-H (Tyr)], 7.14–7.18 [2H, m, 3,4-H (Bnz)], 7.23 [2H, d, $J$ = 9.0 Hz, 2,6-H (Tyr)], 7.41 [1H, dd, $J$ = 4.1 and 7.9 Hz, 5-H (Pyr)], 7.47 [1H, d, $J$ = 3.1 Hz, 6-H (Bnz)], 8.08 [1H, d, $J$ = 7.9 Hz, 4-H (Pyr)], 8.75 [1H, d, $J$ = 4.1 Hz, 6-H (Pyr)], 8.95 [1H, s, 2-H (Pyr)] and 10.08 ppm (1H, s, OH). Anal. Calcd for $C_{24}H_{22}N_2O_7 \cdot 0.5\ H_2O$: C, 62.74; H, 5.05; N, 6.10. Found: C, 62.88; H, 4.96; N, 5.82.

### *N*-Nicotinoyl-*O*-(2-Hydroxybenzoyl)-L-Tyrosine Methyl Ester (5d)

Yield 19%; mp 129–131°C; IR (KBr): ν 3333, 3218, 2948, 1749, 1484, and 762 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.28 and 3.36 [2H, ABX, $J$ = 5.6 and 13.5 Hz, $CH_2$ (Tyr)], 3.81 (3H, s, $CO_2CH_3$), 5.11 [1H, dd, $J$ = 5.6 and 13.5 Hz, CH (Tyr)], 6.72 (1H, s, NH), 6.96 [1H, m, 5-H (Bnz)], 7.04 [1H, d, $J$ = 8.3 Hz, 3-H (Bnz)], 7.14–7.16 [2H, m, 2,6-H (Tyr)], 7.21–7.23 [2H, m, 3,5-H (Tyr)], 7.43 [1H, dd, $J$ = 4.8 and 8.0 Hz, 5-H (Pyr)], 7.52–7.57 [1H, m, 4-H (Bnz)], 8.05 [1H, d, $J$ = 8.0 Hz, 6-H (Bnz)], 8.10 [1H, d, $J$ = 8.0 Hz, 4-H (Pyr)], 8.75 [1H, s, 6-H (Pyr)], 8.99 [1H, s, 2-H (Pyr)], and 10.5 ppm (1H, br, OH). Anal. Calcd for $C_{23}H_{20}N_2O_6 \cdot 0.4H_2O$: C, 64.60; H, 4.90; N, 6.55. Found: C, 64.66; H, 4.98; N, 6.35.

### *N*-Nicotinoyl-*O*-(3-Hydroxybenzoyl)-L-Tyrosine Methyl Ester (5e)

Yield 22%; mp 163–164°C; IR (KBr): ν 3319, 1739, 1642, 1269, 1165, and 700 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.17 and 3.26 [2H, ABX, $J$ = 5.8 and 13.9 Hz, $CH_2$ (Tyr)], 376 (3H, s, $CO_2CH_3$), 5.07 [1H, dd, $J$ = 5.8 and 13.9 Hz, CH (Tyr)], 7.05–7.11 [4H, m, 2,3,5,6-H (Tyr)], 7.13 [1H, s, 4-H (Bnz)], 7.15 [1H, s, 2-H (Bnz)], 7.31 [1H, t, $J$ = 7.8 Hz, 5-H (Bnz)], 7.38 [1H, dd, $J$ = 4.8 and 7.6 Hz, 5-H (Pyr)], 7.62 (1H, s, NH), 7.65 [1H, d, $J$ = 7.5 Hz, 6-H (Bnz)], 8.10 [1H, d, $J$ = 7.6 Hz, 4-H (Pyr)], 8.69 [1H, s, 6-H (Pyr)], and 8.97 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{23}H_{20}N_2O_6$: C, 65.71; H, 4.79; N, 6.66. Found: C, 65.82; H, 4.83; N, 6.31.

### Synthesis of *N*-Nicotinoyl-*O*-(4-Hydroxybenzoyl)-L-Tyrosine Methyl Ester (5g)

A mixture of 287 mg (0.62 mmol) of **4g** in 20 ml of MeOH/THF (1/1) and 86 mg (0.62 mmol) of $K_2CO_3$ was stirred for 1 h at room temperature. An additional 30 mg (0.22 mmol) of $K_2CO_3$ was added, and the mixture was further stirred for 30 min. After removal of the solvent, 20 ml each of AcOEt and $H_2O$ were added to the residue. The resulting solid was collected by suction filtration to give 166 mg (64%) of the product (**5g**): mp 220°C (decomp.); IR (KBr): ν 3057, 3319, 2948, 1739, 1642, 1269, 1068, and 700 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$): δ 3.06–3.25 [2H, m, $CH_2$ (Tyr)], 3.65 (3H, s, $CO_2CH_3$), 4.66–4.72 [1H, m, CH (Tyr)], 6.89 [2H, d, $J$ = 8.6 Hz, 2-H and 6-H (Bnz)], 7.13 [2H, d, $J$ = 8.5 Hz, 2,6-H (Tyr)], 7.35 [2H, d, $J$ = 8.5 Hz, 3,5-H (Tyr)], 7.50 [1H, dd, $J$ = 4.9 and 8.0 Hz, 5-H (Pyr)], 7.93 [2H, d, $J$ = 8.6 Hz, 3,5-H (Bnz)], 8.12 [1H, d, $J$ = 8.0 Hz, 4-H (Pyr)], 8.70 [1H, d, $J$ = 4.9 Hz, 6-H (Pyr)], 8.92 [1H, s, 2-H (Pyr)], and 9.13 ppm (1H, d, $J$ = 8.0 Hz, OH). Anal. Calcd for $C_{23}H_{20}N_2O_6$: C, 65.71; H, 4.79; N, 6.66. Found: C, 65.82; H, 4.83; N, 6.31.

### Synthesis of *N*-Nicotinoyldopamine (8)

Dopamine hydrochloride (541 mg, 2.8 mmol) was added to a solution of 538 mg (2.7 mmol) of nicotinic anhydride in 100 ml of THF. A solution of 304 mg (3.0 mmol) of $Et_3N$ in 20 ml of THF was added dropwise at 0°C. After 20 h stirring at room temperature, the resulting salt was removed by filtration. Evaporation of the filtrate gave a solid, to which 200 ml of AcOEt and 20 ml of $H_2O$ were added. The AcOEt layer was separated, washed with 50 ml of saturated aqueous NaCl, and dried over anhydrous $Na_2SO_4$. After removal of the solvent, the crude product was purified by column chromatography on silica gel with $CHCl_3$/MeOH (20/1) to afford 120 mg (20%) of the product (**8**): mp 197–198°C; IR (KBr): ν 3480, 3312, 3074, 1636, 1539, 1200, 1371, 1292, and 703 $cm^{-1}$; $^1$H-NMR ($CD_3OD$): δ 2.75 (2H, m, $CH_2$), 3.55 (2H, m, NH-$CH_2$), 6.54–6.56 (1H, m, 5′-H,), 6.67–6.68 (2H, s, 2′,6′-H), 7.51 [1H, dd, $J$ = 4.8 and 7.9 Hz, 5-H (Pyr)], 8.18 [1H, d, $J$ = 7.9 Hz, 4-H (Pyr)], 8.64–8.66 [1H, m, 6-H (Pyr)], and 8.80 ppm [1H, s, 2-H (Pyr)]. Anal. Calcd for $C_{14}H_{14}N_2O_3$: C, 65.11; H, 5.46; N, 10.85. Found: C, 65.24; H, 5.55; N, 10.83.

### Synthesis of *N*-nicotinoylnorepinephrine (9)

Norepinephrine (352 mg, 2.1 mmol) and nicotinic anhydride (508 mg, 2.2 mmol) were dissolved in a mixture of 60 ml of THF and 20 ml of MeOH, and the solution was stirred for 20 h at room temperature. After evaporation of the solvent, the crude product was recrystallized from EtOH to give 208 mg (37%) of the product (**9**): mp 195°C (decomp.); IR (KBr): ν 3295, 1640, 1543, 1368, 1291, and 704 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): δ 3.53–3.55 (2H, m, $CH_2$), 4.74 (1H, dd, $J$ = 5.6 and 7.3 Hz, CH), 6.72–6.73 (2H, m, 5′,6′-H), 6.85 (1H, s, 2′-H), 7.52 [1H, dd, $J$ = 4.8 and 7.8 Hz, 5-H (Pyr)], 8.19 [1H, m, 4-H (Pyr)], 8.66 [1H, d, $J$ = 4.8 Hz, 6-H (Pyr)], and 8.82 ppm [1H, s, 2-H (Pyr)]. *Anal.* Calcd for $C_{14}H_{14}N_2O_4$: C, 61.31; H, 5.14; N, 10.21. Found: C, 61.43; H, 5.11; N, 10.19.

### Measurement of Antiproliferative Activity Against U937 Cells

U937 cells were seeded in each well of a 12-well culture plate ($0.8 \times 10^5$ cells/well) with 760 µl of an RPMI 1640 medium containing 5% penicillin-streptomycin and 10% fetal bovine serum (FBS). A compound synthesized above was dissolved in DMSO to prepare a 10 mM solution, which was added to each well so that the final concentration of the compound would be 88 µM. After incubation at 37°C under an atmosphere of 5% $CO_2$–95% air for 48 h, the number of viable cells was determined by staining with trypan blue.

### DNA Fragmentation Analysis by Electrophoresis

U937 cells ($2.4 \times 10^5$ cells) were suspended in 20 ml of an RPMI 1640 medium containing 5% penicillin-streptomycin and 10% FBS, and a DMSO solution of a nicotinamide compound was added to make the final concentration of the compound 99 µM. The cells were incubated in a similar manner and centrifuged at 15,000 rpm for 10 min. The cells were washed with cooled phosphate buffer saline (PBS) several times, centrifuged again, and lysed in 200 µl of a lysis buffer (1 ml of 1 M Tris-HCl buffer of pH 7.4, 0.2 ml of 0.5 M EDTA, and 0.5 ml of 10% Triton X-100). After lysed cells were held at 4°C for 10 min, the supernatant was incubated with 2 µl of RNase A

(10 mg/ml in Tris-EDTA buffer) at 50°C for 30 min and then with 2 µl of proteinase K (10 mg/ml in distilled water) for 45 min at 50°C. The solution was mixed with 20 µl of 5 M NaCl and 120 µl of 2-propanol, and the mixture was incubated at −20°C for 24 h. It was centrifuged at 15,000 rpm for 20 min. The precipitated DNA was dissolved in 5 µl of Tris-EDTA buffer and subjected to electrophoresis with 2% agarose gel using Tris-acetate-EDTA buffer at 50 V. The DNA fragmentation pattern was visualized with a UV transilluminator.

### Annexin V and PI Staining

U937 cells ($2.0 \times 10^5$ cells) were suspended in 4950 µl of an RPMI 1640 medium containing 5% penicillin-streptomycin and 10% FBS and treated with 50 µl of a 5 mM DMSO solution of a nicotinamide compound whose final concentration was 50 µM. They were incubated similarly, washed with cooled PBS, collected by centrifugation, and lysed in annexin V-fluorescein (2 µl), a binding buffer (100 µl), and propidium iodide (2 µl). The suspension was incubated for 15 min at room temperature, and the cells were observed by fluorescence microscopy.

## RESULTS AND DISCUSSION

### Synthesis of Nicotinamide Derivatives

L-Tyrosine methyl ester hydrochloride (**2**) was nicotinoylated with nicotinic anhydride in the presence of $Et_3N$ to give *N*-nicotinoyl-L-tyrosine methyl ester (**3**). Subsequent acylation with substituted benzoyl chlorides resulted in the formation of nicotinamides having various benzoyl groups (**4a–f**) in satisfactory yields (72–87%). Generation of hydroxy groups from the methoxy groups of **4** was carried out with $BBr_3$ to synthesize the target compounds (**5**) (Fig. 2).

Deprotection of **4a**, **4d**, and **4e** was effected smoothly to give the corresponding hydroxy-containing compounds, **5a**, **5d**, and **5e**. Compound **4c**, however, gave a mixture of monohydroxy and dihydroxy derivatives **5c** and **5c′** in comparable yields, implying that the demethylation at C-5 was somewhat sluggish compared with that at C-2. In the deprotection of **4b** and **4f**, the C-4 methoxy was found to be very resistive, and several attempts to generate a hydroxy were unsuccessful. Compound **4b** thus gave **5b** having a hydroxy only at C-2, but in the reaction of **4f**, only **3** was identified in the reaction mixture. In order to prepare a derivative having a hydroxy at C-4, therefore, acetyl protection was applied to make possible facile deprotection. Acylation of **3** was performed with *p*-acetoxybenzoyl chloride, and the product (**4g**) was treated with aqueous potassium carbonate, giving rise to the *p*-hydroxy derivative (**5g**) (Fig. 3).

Transformations of **4a–e** to **5a–e** were not quantitative owing to the difficulty in demethylation. Besides small scales of the reactions, some side reactions were responsible for lowering the yields. The major side product was **3** as confirmed by TLC during the deprotection reaction, and this is most likely interpreted as a result of ester cleavage with a strong Lewis acid like $BBr_3$ that proceeds easily even at low temperatures [Yazawa et al., 1974]. Compared with

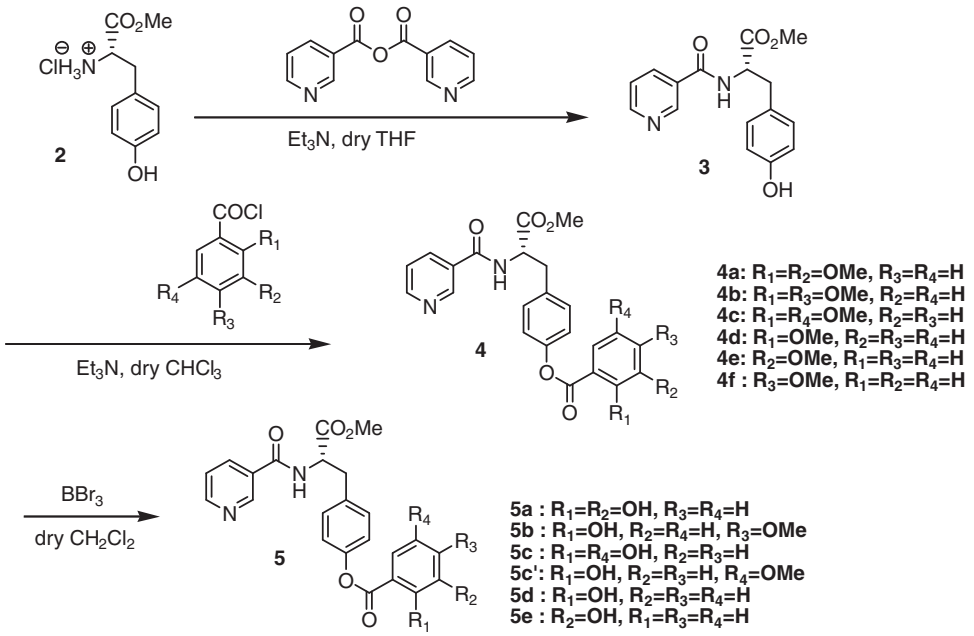


**Fig. 2.** Synthesis of compounds **5a–e**.

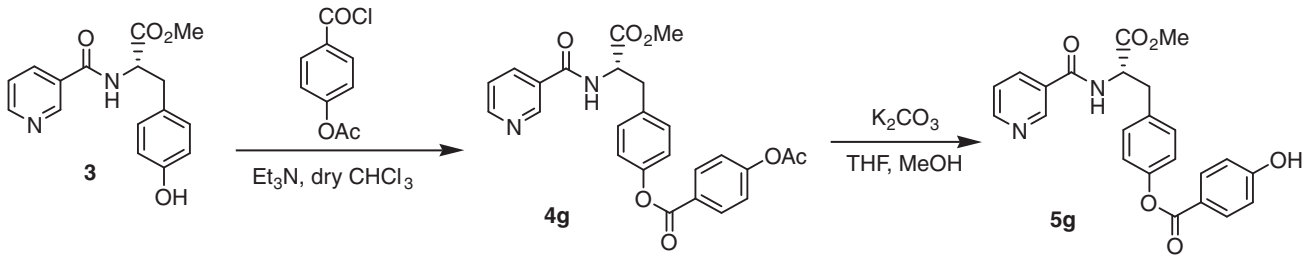


**Fig. 3.** Synthesis of compound **5g**.

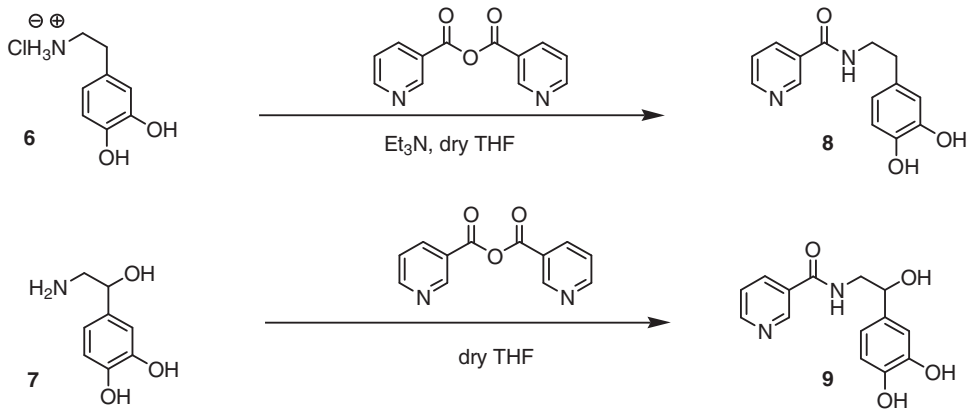


**Fig. 4.** Synthesis of compounds **8** and **9**.

demethylation of **4f,** deacetylation of **4g** was quite easy, and **5g** was obtained in a satisfactory yield.

Dopamine (**6**)- and norepinephrine (**7**)-containing nicotinamide derivatives (**8** and **9**) were synthesized by nicotinoylation of dopamine and norepinephrine (Fig. 4), and the yields were not high. The low yields appeared to be the result of the limited solubility of dopamine and norepinephrine in the reaction solvent.

## Apoptosis-Inducing Activity of the Nicotinamide Derivatives

EGCG is known to induce apoptosis through the activation of caspase-3 and caspase-9, induction of proapoptotic Bax, Bak, and Bcl-$X_S$, and inhibition of antiapoptotic Bcl-2 and Bcl-$X_L$ [Shankar et al., 2007]. Although structurally dissimilar to EGCG, 6-aminonicotinamide was also reported to induce apoptosis via the caspase pathway. Compounds **5**, **8**, and **9** synthesized here have both the phenol and nicotinamide moieties, and it is thus considered of interest to explore their potential of apoptosis induction.

As a primary screening test, antiproliferative activities of **5**, **8**, and **9** were evaluated by adding their DMSO solution to U937 cells in an RPMI-1640 medium containing 5% penicillin-streptomycin and 10% FBS, final concentration of a nicotinamide derivative being 88 μM. After incubation for 24 h at 37°C in an atmosphere of 5% $CO_2$–95% air, the viabilities of the cells were measured.

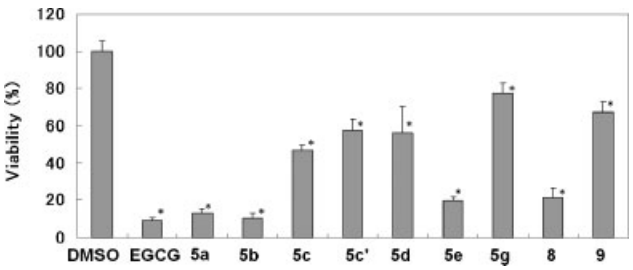


**Fig. 5.** Viability percentage of U937 cells treated with nicotinamide derivatives. $^*P<0.05$ vs. control of difference between indicated data using a *t*-test. The values are expressed as means ± SD, $n = 3$.

The influence of the products on the viability along with that of EGCG is shown in Figure 5, where the values of the products with only one hydroxy on the benzoyl group (**5d**, **5e**, and **5g**) indicated that **5e** was the most effective antiproliferative agent, suggesting the importance of the position C-3 to accommodate a hydroxy.

Although both the hydroquinone- and catechol-containing remoxipeide metabolites, NCQ344 and NCQ436, were reported to promote apoptosis in HL-60 cells [Inayat-Hussain et al., 2000], our results showed that catechol derivative **5a** was more potent

than hydroquinone derivative **5c**. The activity of ester derivative **5a** appeared to be comparable with that of the corresponding amide derivative **1** in our previous report [Yamaguchi et al., 2007]. These results support the importance of the catechol-type structure with C-2 and C-3 hydroxy groups on the benzene ring to effectively suppress the growth of U937 cells.

When the compounds having a hydroxy at C-2 and a methoxy at C-3 (**5b**) or C-4 (**5c′**) were compared, **5b** was far more active than **5c′**. As a compound with a methoxy at C-4, *p*-methoxychalcone exhibited antiproliferative activity against A375 cells [Henmi et al., 2009]. Apoptosis in various human cell lines was also observed with some compounds having a methoxy and a hydroxy on an aromatic ring; aromatic amine derivatives [Nesterenko et al., 2003], rhinacanthin-N [Siripomg et al., 2006], and antraquinone derivatives [Shi et al., 2008]. The remarkable activity of **5b** as evident in Figure 5 confirmed the high potential of such compounds having a methoxy and a hydroxy on an aromatic ring. The difference in the activity of **8** and **9** was substantial, and the introduction of an additional secondary hydroxy group appeared to lower the activity.

**TABLE 1. $IC_{50}$ Values Against U937 Cells**[†]

| Compound | $IC_{50}$ (μM) |
|---|---|
| EGCG | 15.65±2.39 |
| **5a** | 3.15±0.70* |
| **5b** | 0.87±0.52* |
| **5e** | 20.83±2.30 |
| **8** | 56.43±4.95 |

[†]Values are expressed as means±SD, $n=3$; *$P<0.05$ vs. EGCG.

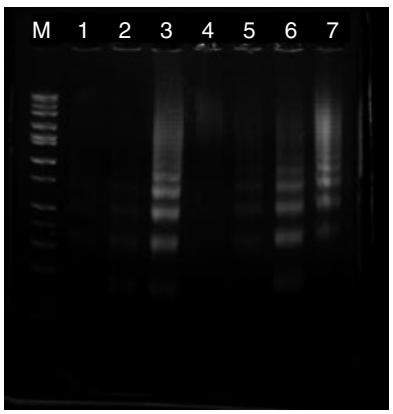


**Fig. 6.** Agarose gel electrophoresis of DNA fragmentation induced in U937 cells by nicotinamide derivatives visualized under UV light with ethidium bromide staining: M, DNA marker; #1, control; #2, DMSO; #3, EGCG; #4, compound **8**; #5, compound **5e**; #6, compound **5a**; and #7, compound **5b**.

$IC_{50}$ values were then determined with the four most potent compounds, **5a**, **5b**, **5e**, and **8** as a measure to explicate the effective concentrations in view of actual clinical chemotherapy. Judging from the $IC_{50}$ values in Table 1, **5a** and **5b** were especially noteworthy and even more potent than **1** and EGCG.

To elucidate the death process of cells, either apoptosis or necrosis, the state of DNA of U937 cells after treatment was examined by agarose gel electrophoresis. Since internucleosomal DNA fragmentation is a distinctive biochemical feature of the apoptotic process, it will provide a suitable proof of apoptosis. The results of electrophoresis for **5a**, **5b**, **5e**, and **8** together with those of references are shown in Figure 6. A typical DNA ladder pattern due to apoptosis was observed for the cells treated with **5a** and **5b**, as in the case with EGCG, but not for the cells treated with **5e** and **8** under the experimental conditions. These results indicate that **5a** and **5b** evidently induced apoptosis in U937 cells, but as for **5e** and **8**, actual triggers for the cell death were not specified.

To confirm further that the cell death was really associated with apoptosis on treatment with **5a** and **5b**, staining of externalized phosphatidylserine with annexin V and propidium iodide (PI) was carried out. Annexin V, a calcium-dependent phospholipid-binding protein, shows a strong affinity for phosphatidylserine that is externalized onto the cell surface in the early stage of apoptosis cell death, resulting in green fluorescence [Engeland et al., 1998; Kim et al., 2006; Lee et al., 2006]. Necrosis, on the other hand, increases permeability of cell membranes, which allows staining of the cell nucleus by PI to express red fluorescence. Accordingly, fluorescence microscopy after staining will be a reliable tool to distinguish between apoptosis and necrosis. As shown in Figure 7, only green fluorescence

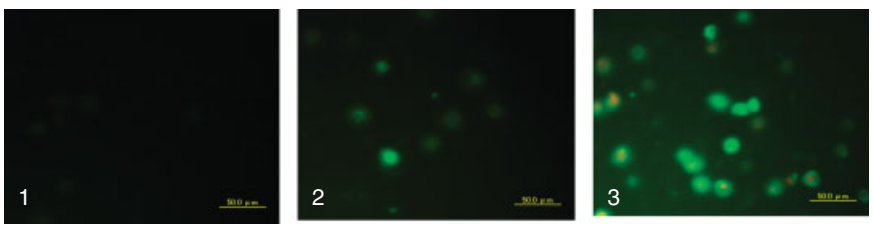


**Fig. 7.** Annexin V and PI staining of U937 cells treated with **5a** and **5b**: #1, DMSO; #2, compound **5a**; and #3, compound **5b**. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

was observed for the cells treated with **5a** and **5b**, indicating that the cells were annexin V-positive and PI-negative. The results obtained by agarose gel electrophoresis and annexin V staining thus conclude that **5a** and **5b** induced apoptosis in U937 cells effectively.

## CONCLUSION

Of 9 compounds synthesized and evaluated in this study, **5a** and **5b** were confirmed to exhibit the highest antiproliferative activities, and they proved promising as a new type of apoptosis inducing agents. The results obtained in this study would be useful as basic information for designing intelligent molecular architectures to express improved apoptosis-inducing activity against malignant cells, and our efforts will be concentrated further on the in-depth study of the structure-property relationship to develop potent anticancer agents for chemotherapy.

## ACKNOWLEDGMENTS

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