

Synthesis of Antineoplaston A10 Analogs as Potential Antitumor Agents

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Several aniline mustard analogues were obtained by introducing *N,N*-bis(2-chloroethyl)amino moiety to phenyl ring of A10 analogues in order to increase reactivity of A10 analogs and selectivity into DNA. The *in vitro* antitumor activity of synthesized compounds was evaluated using five different solid tumor cell lines by SRB method. Aniline mustard analogues exhibited more potent antitumor activity than A10 analogs. Especially, *m*-aniline mustard of benzoyl analogue displayed remarkable antitumor activity.

Key words : Antineoplaston A10, Aniline mustards, Piperidinedione derivatives, Antitumor agent

INTRODUCTION

Antineoplaston A10 (A10) is an amino acid derivative isolated from human urine and its structure was determined as 3-(*N*-phenylacetyl)aminopiperidine-2,6-dione (Fig. 1) (Burzynski, 1986).

A10 exhibited broad antitumor activity with low toxicity against various human tumors in clinical trials (Burzynski *et al.*, 1984), and it was suggested that A10 is capable of inserting between base pairs in DNA (Hendry *et al.*, 1987). A10 has, however, low potency and must be administered large dose to show efficacy.

Nitrogen mustards are bifunctional alkylating agents which express their cytotoxic and antitumor effects by cross-linking cellular DNA (Trual *et al.*, 1990). Several recent publications have shown that the incorporation of an alkylating group into some DNA intercalating agents greatly enhances their antitumor activity (Koyama *et al.*, 1989, Goudie *et al.*, 1990, Gravatt *et al.*, 1994).

We report the syntheses and antitumor activity against five different human solid tumor cell lines of aniline mustards of A10 analogs which have the aniline mus-

tard moiety on their phenyl ring in order to improve potency and selectivity of A10 analogs (Niculescu-Duvaz *et al.*, 1967 and 1969).

CHEMISTRY

A10, its analogs, and 3-aminopiperidine-2,6-dione were synthesized by the known method (Lee *et al.*, 1996).

The substituted aniline mustards, where X is H, CH₃, OCH₃, were synthesized by the method shown in Scheme 1. Hydrogenation of the nitro methyl esters in the presence of palladium on carbon in methanol gave the amino methyl esters, which were then treated with excess ethylene oxide in glacial acetic acid to give the diol methyl esters. The diol methyl esters were converted into dichloro mustard methyl esters using phosphorus oxychloride. Hydrolysis of the methyl esters followed by treatment of thionyl chloride gave mustard acyl chlorides which were then reacted with 3-aminopiperidine-2,6-dione to afford the desired mustard A10 analogs.

RESULTS AND DISCUSSION

Aniline mustards of A10 analogs were evaluated for their *in vitro* antitumor activity against five different human solid tumor cell lines; A-549 (human lung), SK-OV-3 (human ovarian), HCT-15 (human colon), SK-MEL-2 (human melanoma), XF-498 (human CNS) and the results are given in Table I.

Among the substituted aniline mustard series, *m*-me-

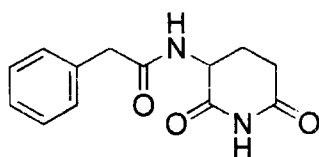
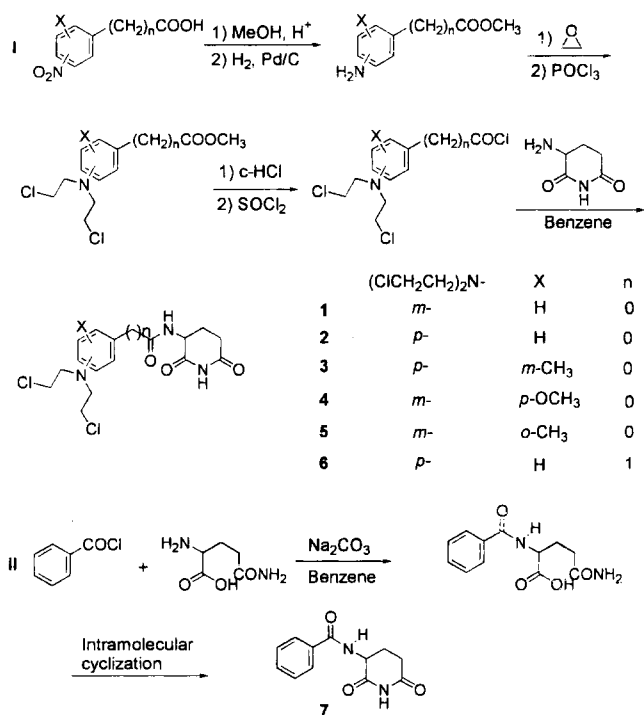


Fig. 1. Antineoplaston A10.

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Scheme 1.

thyl-*p*-aniline mustard of benzoyl derivative (**3**) exhibited activity similar to A10's. *m*-Aniline mustard (**1**) and *p*-methoxy-*m*-aniline mustard (**4**) of benzoyl analogs displayed much more potent activity than A10. Compound **4** has particularly remarkable antitumor activity against SK-OV-3 and XF-498.

In conclusion, 1) *p*-[*N,N*-bis(2-chloroethyl)]amine substituted phenylacetyl of A10 (**6**) was synthesized and it showed greatly improved *in vitro* antitumor activity (Table I). 2) Five compounds which have *m*- or *p*-[*N,N*-bis(2-chloroethyl)]amine substituted by benzoyl in stead of phenylacetyl of A10 and *m*-methyl-*p*-[*N,N*-bis(2-chloroethyl)]amine, *p*-methoxy-*m*-[*N,N*-bis(2-chloroethyl)]amine, and *o*-methyl-*m*-[*N,N*-bis(2-chloroethyl)]amine substituted by benzoyl in stead of phenylacetyl of A10 were synthesized by reaction of 3-aminopiperidine-2,6-dione and aniline mustard containing ben-

Table I. *In vitro* antitumor activity of benzoyl analogs of A10 (ED₅₀ μg/ml)

	A549	SK-OV-3	SK-MEL-2	HCT-15	XF-498
1	22.8	6.92	8.92	9.84	7.10
2	137.4	56.2	66.6	52.8	62.4
3	>1000	247.6	>1000	>1000	165.2
4	4.07	1.13	6.63	6.99	0.947
5	>1000	>1000	171.4	>1000	315.1
6	11.5	1.99	14.4	16.3	2.99
7	>1000	>1000	>1000	>1000	>1000
A10	>1000	>1000	>1000	>1000	>1000

A-549 (human lung), SK-OV-3 (human ovarian), SK-MEL-2 (human melanoma), HCT-15 (human colon), XF-498 (human CNS)

zoyl chlorides. Among these, *p*-methoxy-*m*-[*N,N*-bis(2-chloroethyl)]amine substituted by benzoyl in stead of phenylacetyl of A10 showed more than 1000 times better *in vitro* antitumor activity than A10.

EXPERIMENTAL SECTION

Antitumor assay was performed by Pharmaceutical Screening Laboratory in Korea Research Institute of Chemical Technology using five different human tumor cell lines, A-549 (human lung), SK-OV-3 (human ovarian), SK-MEL-2 (human melanoma), HCT-15 (human colon), XF-498 (human CNS) which were purchased from the National Cancer Institute (NCI) in U.S.A.

The cells were grown at 37°C in RPMI 1640 medium supplemented with 10% FBS and separated using PBS containing 0.25% trypsin and 3 mM EDTA. 5 × 10³~2 × 10⁴ cells were added to each well of 96 well plate and incubated at 37°C for 24 h. Each compounds was dissolved in DMSO and diluted with the above medium at five different concentrations with the range of 0.1~30 μg/mL. The concentration of DMSO was set to be below 0.5% and DMSO was filtrated using 0.22 μm filter. After removing the well medium by aspiration, a 200 μL portion of the solution was added to above well plates which were placed in 5% CO₂ incubator for 48 h. The protein stain assay was performed according to SRB method (Skehan *et al.*, 1990, Rubinstein *et al.*, 1990).

Materials and Apparatus

L-Glutamine, benzoyl chloride, 3- and 4-nitrobenzoic acid, 3-methyl-4-nitrobenzoic acid, 2-methyl-3-nitrobenzoic acid, 4-methoxy-3-nitrobenzoic acid and 10% palladium on carbon were purchased from Aldrich Chemical Company. All other reagents and solvents were of extra pure grade and were obtained from local suppliers.

Melting points were determined on an Electrothermal IA9000 melting point apparatus and uncorrected. ¹H-NMR spectra were obtained from a BRUKER AC80 fourier transform spectrometer for the 80-MHz. In ¹H-NMR spectra, using TMS as an internal standard, chemical shifts were reported in parts per million (δ) and signals were designated as a s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). The IR spectra were recorded on a PERKIN-ELMER 783 Spectrophotometer using KBr pellets. TLC was carried out using precoated plates with silica gel 60F 254 purchased from E. MERCK.

Synthesis

Preparation of 3-[3'-[*N,N*-bis(2-chloroethyl)]amino-benzoyl]aminopiperidine-2,6-dione (**1**)

Methyl 3-nitrobenzoate: A solution of 3-nitrobenzoic

acid (10 g, 59.8 mmol) in 150 mL of methanol was added 3 drops of c-H₂SO₄ and the mixture was refluxed for 29 h. After cooling, the mixture was evaporated under reduced pressure and recrystallized from ethyl acetate (9.22 g, 83.9%). ¹H NMR (CDCl₃) δ: 3.99 (3H, s, COOCH₃), 7.29 (4H, m, aromatic H).

Methyl 3-aminobenzoate: A solution of methyl 3-nitrobenzoate (5.69 g, 31.4 mmol) in 150 mL of methanol was stirred at room temperature under hydrogen in the presence of 10% Pd/C (0.25 g). After 19 h, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give oil (4.64 g, 97.8%). ¹H NMR (CDCl₃) δ: 3.79 (5H, s, COOCH₃ and NH₂), 7.03 (4H, m, aromatic H).

Methyl 3-[N,N-bis(2-chloroethyl)]aminobenzoate: Ethylene oxide (4.64 mL, 92 mmol) was added to a cooled solution of methyl 3-aminobenzoate (30.7 mmol) in 115 mL of acetic acid. After 17 h at room temperature, the solution was evaporated under reduced pressure below 70°C, and the residue was triturated three times with 30 mL of anhydrous toluene and concentrated to dryness *in vacuo*. The resulting mixture was treated with benzene, washed with 10% aqueous Na₂CO₃, dried and evaporated to dryness under reduced pressure. This residue (30 mmol) was added to a cooled (0°C) and stirred POCl₃ (28 mL, 0.6 mol). After addition, the mixture was heated for 1 h in an oil bath (intense evolution of HCl was observed). Excess POCl₃ was removed *in vacuo* and the residue was extracted with benzene and the organic extracts were dried by Na₂SO₄ and evaporated. Chromatography on a silica gel column afforded the desired product (3.11 g, 36.7% yield). ¹H NMR (CDCl₃) δ: 3.71 (8H, s, N(CH₂CH₂Cl)₂), 3.88 (3H, s, COOCH₃), 7.09 (4H, m, aromatic H).

3-[N,N-Bis(2-chloroethyl)]aminobenzoic acid: A mixture of methyl 3-[N,N-bis(2-chloroethyl)]aminobenzoate (2 g, 7.24 mmol) and HCl (36.2 mL) was refluxed for 1.5 h. The precipitated product was filtered to give a solid (1.2 g, 63.2%). ¹H NMR (CDCl₃+DMSO-d₆) δ: 3.67 (8H, s, N(CH₂CH₂Cl)₂), 7.13 (4H, m, aromatic H).

3-[3'-[N,N-Bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (1): 3-[N,N-Bis(2-chloroethyl)]aminobenzoic acid (0.4 g, 1.52 mmol) was dissolved in benzene (5 mL) containing 0.4 mL of freshly distilled thionyl chloride. Excess thionyl chloride and solvent were removed under reduced pressure and the residue was recrystallized from petroleum ether (0.31 g, 72.4%). To a solution of this crystalline product (0.31 g, 1.11 mmol) in anhydrous benzene (7 mL) was added 3-aminopiperidine-2,6-dione (0.28 g, 2.2 mmol) with stirring and occasional cooling. After 24 h, the reaction mixture was recrystallized from methylene chloride (0.12 g, 29.3%). m.p.: 159.2~161.6°C; ¹H NMR (DMSO-d₆) δ: 2.4 (4H, m, CH₂CH₂CO), 3.83

(8H, s, N(CH₂CH₂Cl)₂), 4.74 (1H, q, COCHCH₂), 7.2~8.7 (4H, q, aromatic H), 8.61 (1H, d, CHNHCO), 10.77 (1H, s, CONHCO); IR_{neat} cm⁻¹: 3250 (amide NH, imide NH), 1710, 1630 (C=O), 750 (alkyl chloride C-Cl).

Preparation of 3-[4'-[N,N-bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (2)

Methyl 4-nitrobenzoate: A solution of 4-nitrobenzoic acid (7 g, 41.8 mmol) in methanol (150 mL) was added 6 drops of c-H₂SO₄. After 28h, The mixture was evaporated under reduced pressure and recrystallized from ethyl acetate (6.53 g, 86.1%). ¹H NMR (CDCl₃) δ: 3.99 (3H, s, COOCH₃), 7.29 (4H, m, aromatic H).

Methyl 4-aminobenzoate: A solution of methyl 4-nitrobenzoate (3 g, 16.6 mmol) in methanol (100 ml) was stirred at room temperature under hydrogen atmosphere in the presence of 10% Pd/C (0.3 g). After 23 h, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure (2.43 g, 97.2 %). ¹H NMR (CDCl₃) δ: 3.8 (3H, s, COOCH₃), 4.07 (2H, s, NH₂), 7.15 (4H, m, aromatic H).

Methyl 4-[N,N-bis(2-hydroxyethyl)]aminobenzoate: Ethylene oxide (2.43 mL, 48 mmol) was added to a cooled solution of methyl 4-aminobenzoate (16.0 mmol) in acetic acid (58.3 mL). After 21h at room temperature, the solution was evaporated under reduced pressure below 70°C, and the residue was triturated three times with 30 mL of anhydrous toluene and concentrated to dryness *in vacuo*. The resulting mixture was treated with benzene, washed with 10% aqueous Na₂CO₃, dried the organic solution by Na₂SO₄ and evaporated to dryness under reduced pressure. It was used for next reaction without further purification. IR_{neat} cm⁻¹: 3350 (hydroxyethyl OH), 1730 (ester C=O), 1170 (ethylamine C-N), 1050 (ester C-O).

Methyl 4-[N,N-bis(2-chloroethyl)]aminobenzoate: Methyl 4-[N,N-bis(2-hydroxyethyl)]aminobenzoate (0.92 g, 38.5 mmol) was added, while stirring, to POCl₃ (5.9 ml, 38.5 mmol) previously cooled at 0°C. After addition, the mixture was heated for 1 h in an oil bath. Excess POCl₃ was removed *in vacuo* and the mixture was extracted with benzene and the product was obtained from the benzene solution after drying (Na₂SO₄). Chromatography on a silica gel column provided the desired product (0.9 g, 84.9%). ¹H NMR (CDCl₃) δ: 3.75 (8H, t, N(CH₂CH₂Cl)₂) 3.75 (3H, s, COOCH₃), 7.73 (4H, m, aromatic H).

4-[N,N-Bis(2-chloroethyl)]aminobenzoic acid: A mixture of methyl 4-[N,N-bis(2-chloroethyl)]aminobenzoate (0.9 g, 3.26 mmol) and c-HCl (16.3 mL) was refluxed for 3 h. The mixture was diluted with H₂O (98 mL) and the precipitate was filtered and dried *in vacuo* (0.64 g, 75.3%). ¹H NMR (CDCl₃+DMSO-d₆) δ: 3.68 (8H, t, N(CH₂CH₂Cl)₂), 7.09 (4H, q, aromatic H).

3-[4'-[N,N-Bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (2): 4-[N,N-Bis(2-chloroethyl)]aminobenzoic acid (0.65 g, 2.48 mmol) was refluxing

for 2 h in dry benzene (7 mL) containing freshly distilled SOCl_2 (0.65 mL). Excess SOCl_2 and solvent were removed under reduced pressure and the residue was recrystallized from petroleum ether (0.43 g, 61.8%). To a solution of this crystalline product (0.43 g, 1.53 mmol) in anhydrous benzene (9 mL) was added 3-aminopiperidine-2,6-dione (0.39 g, 3.0 mmol) with stirring and occasional cooling. After 24 h, the reaction mixture was recrystallized from CH_2Cl_2 . Solid (0.23 g, 40.3%). m.p.: 165.6~166.4°C, $^1\text{H NMR}$ (DMSO-d_6) δ : 2.01 (2H, m, $\text{CH}_2\text{CH}_2\text{CO}$), 2.65 (2H, m, $\text{CH}_2\text{CH}_2\text{CO}$), 3.85 (8H, s, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 4.85 (1H, q, COCHCH_2), 7.25 (4H, q, aromatic H), 8.35 (1H, d, CHNHCO), 10.76 (1H, s, CONHCO); IR_{neat} cm^{-1} : 3350 (amide NH), 3160 (imide NH), 1730, 1600 (C=O), 740 (alkyl chloride C-Cl).

Preparation of 3-[3'-methyl-4'-[N,N-bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (3)

Methyl 3-methyl-4-nitrobenzoate: A solution of 3-methyl-4-nitrobenzoic acid (7 g, 38.64 mmol) in methanol (150 mL) was added 6 drops of $\text{c-H}_2\text{SO}_4$ and refluxed for 12 h. The mixture was evaporated under reduced pressure and recrystallized from ethyl acetate. Solid (6.40 g, 84.9%). $^1\text{H NMR}$ (CDCl_3) δ : 2.66 (3H, s, phenyl CH_3), 3.99 (3H, s, COOCH_3), 7.95 (3H, m, aromatic H).

Methyl 3-methyl-4-aminobenzoate: A solution of methyl 3-methyl-4-nitrobenzoate (3 g, 15.4 mmol) in methanol (100 mL) was stirred at room temperature under hydrogen in the presence of 10% Pd/C (0.3 g). After 5 h, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to afford solid product (2.5 g, 98.5% yield).

Methyl 3-methyl-4-[N,N-bis(2-chloroethyl)]aminobenzoate: Ethylene oxide (2.3 mL, 45 mmol) was added to a cool solution of methyl 3-methyl-4-aminobenzoate (15.0 mmol) in acetic acid (56.3 mL). After 25 h at room temperature, the solution was evaporated under reduced pressure below 70°C, and the residue was triturated three times with 30 mL of anhydrous toluene and concentrated to dryness *in vacuo*. The resulting mixture was treated with benzene, washed with 10% aqueous Na_2CO_3 , dried by anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. This product (15 mmol) was added while stirring to POCl_3 (14 mL, 0.15 mol) previously cooled at 0°C. After addition, the mixture was heated for 1 h in an oil bath. Excess POCl_3 was removed *in vacuo* and the mixture was extracted with benzene and the organic extracts were dried by anhydrous Na_2SO_4 and evaporated. Chromatography of the residue on a silica gel column provided the desired product (2.11 g, 48.5% yield). $^1\text{H NMR}$ (CDCl_3) δ : 2.35 (3H, s, phenyl CH_3), 3.48 (8H, s, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 3.85 (3H, s, COOCH_3), 7.44 (3H, m, aromatic H).

3-Methyl-4-[N,N-bis(2-chloroethyl)]aminobenzoic

acid: A mixture of methyl 3-methyl-4-[N,N-bis(2-chloroethyl)]aminobenzoate (0.93 g, 3.20 mmol) and c-HCl (16 mL) was refluxed for 3 h. The mixture was diluted with water (96 mL) and the precipitate was filtered and dried *in vacuo* (0.56 g, 63.3%). $^1\text{H NMR}$ (CDCl_3) δ : 2.38 (3H, s, phenyl CH_3), 3.52 (8H, s, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 7.45 (3H, m, aromatic H), 9.02 (1H, s, COOH).

3-[3'-Methyl-4'-[N,N-bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (3): A solution of 3-methyl-4-[N,N-bis(2-chloroethyl)]aminobenzoic acid in dry benzene containing 0.26 mL of freshly distilled SOCl_2 was refluxed for 2.5 h. Excess SOCl_2 and solvent were removed under reduced pressure to give the product. To a solution of this product (0.942 mmol) in 5 mL of anhydrous benzene was added 3-aminopiperidine-2,6-dione (0.24 g, 1.9 mmol) with stirring and occasional cooling. After 72 h, the reaction mixture was recrystallized from CH_2Cl_2 (0.28 g, 77.0% yield). m.p.: 172.9~175.2°C; $^1\text{H NMR}$ (DMSO-d_6) δ : 2.01 (2H, m, $\text{CH}_2\text{CH}_2\text{CO}$), 2.65 (2H, m, $\text{CH}_2\text{CH}_2\text{CO}$), 3.85 (8H, s, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 4.85 (1H, q, COCHCH_2), 7.25 (4H, q, aromatic H), 8.35 (1H, d, CHNHCO), 10.76 (1H, s, CONHCO); IR_{neat} cm^{-1} : 3320 (amide NH), 3160 (imide NH), 1740, 1610 (C=O), 680 (alkyl chloride C-Cl).

Preparation of 3-[4'-methoxy-3'-[N,N-bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (4):

Methyl 4-methoxy-3-nitrobenzoate: A solution of 4-methoxy-3-nitrobenzoic acid (3 g, 15.2 mol) in 50 mL of methanol was added 4 drops of $\text{c-H}_2\text{SO}_4$ and the reaction mixture was refluxed for 12 h. The mixture was evaporated under reduced pressure and recrystallized from ethyl acetate (3 g, 98% yield). $^1\text{H NMR}$ (CDCl_3) δ : 3.82 (3H, s, OCH_3), 3.99 (3H, s, COOCH_3), 7.95 (3H, m, aromatic H).

Methyl 4-methoxy-3-aminobenzoate: A solution of methyl 4-methoxy-3-nitrobenzoate (3 g, 14.2 mmol) in 100 mL of methanol was stirred at room temperature under hydrogen in the presence of 10% Pd/C (0.25 g). After 16 h, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure (2.56 g, 99.5% yield).

Methyl 4-methoxy-3-[N,N-bis(2-chloroethyl)]aminobenzoate: Ethylene oxide (2.4 mL, 42.4 mmol) was added to a cooled solution of methyl 4-methoxy-3-aminobenzoate (2.56 g, 14.1 mmol) in 58.2 mL of acetic acid. After 24 h at room temperature, the solution was evaporated under reduced pressure below 70°C, and the residue was triturated three times with 30 mL of anhydrous toluene and concentrated to dryness *in vacuo*. The resulting mixture was treated with benzene, washed with 10% aqueous Na_2CO_3 , dried by anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. This residue (14 mmol) was added to POCl_3 (14 mL, 0.14 mol), previously cooled at 0°C. After addition, the mixture was heated for 1.5 h in an

oil bath. Excess POCl₃ was removed *in vacuo* and the mixture was extracted with benzene and the organic extracts were dried by anhydrous Na₂SO₄ and evaporated. Chromatography of this residue on a silica gel column afforded the desired product (2.6 g, 60.7%). ¹H NMR (CDCl₃) δ: 3.51 (8H, s, N(CH₂CH₂Cl)₂), 3.89 (6H, s, OCH₃ and COOCH₂), 7.43 (3H, m, aromatic H).

4-Methoxy-3-[N,N-bis(2-chloroethyl)]aminobenzoic acid: A mixture of methyl 4-methoxy-3-[N,N-bis(2-chloroethyl)]aminobenzoate (1.1 g, 3.59 mmol) and 18 mL of c-HCl was refluxed for 2 h. The mixture was diluted with water (108 mL), extracted with ethyl acetate, dried by anhydrous Na₂SO₄ and evaporated under reduced pressure (0.7 g, 66.7%). ¹H NMR (CDCl₃) δ: 3.48 (8H, s, N(CH₂CH₂Cl)₂), 3.85 (3H, s, OCH₃), 7.34 (3H, m, aromatic H), 8.97 (1H, s, COOH).

3-[4'-Methoxy-3'-[N,N-bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (4): A solution of 4-methoxy-3-[N,N-bis(2-chloroethyl)]aminobenzoic acid (0.66 g, 2.26 mmol) in 10 mL of dry benzene containing 0.66 mL of freshly distilled SOCl₂ was refluxed for 5 h. Excess SOCl₂ and solvent were removed under reduced pressure. To a solution of this evaporation residue (2.05 mmol) in 10 mL of anhydrous benzene was added 3-aminopiperidine-2,6-dione (0.315 g, 2.5 mmol) with stirring and occasional cooling. After 48 h, the reaction mixture was chromatographed on a silica gel column. (0.36 g, 43.8% yield). m.p.: 143.5~145.6°C; ¹H NMR (DMSO-d₆) δ: 2.37 (4H, m, CH₂CH₂CO), 3.62 (8H, s, N(CH₂CH₂Cl)₂), 3.86 (3H, s, OCH₃), 4.73 (1H, q, COCHCH₂), 7.33 (3H, m, aromatic H), 8.56 (1H, d, CHNHCO), 10.78 (1H, s, CONHCO); IR_{neat} cm⁻¹: 3340 (amide NH), 3240 (imide NH), 1720, 1650 (C=O), 710 (alkyl chloride C-Cl).

Preparation of 3-[2'-methyl-3'-[N,N-bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (5)

Methyl 2-methyl-3-nitrobenzoate: To a solution of 2-methyl-3-nitrobenzoic acid (7 g, 38.64 mmol) in methanol (150 mL) was added 6 drops of c-H₂SO₄ and the mixture was refluxed for 48 h. The mixture was evaporated under reduced pressure and recrystallized from ethyl acetate to give a solid (6.12 g, 81.16% yield). ¹H NMR (CDCl₃) δ: 2.52 (3H, s, phenyl CH₃), 3.89 (3H, s, COOCH₃), 8.03 (3H, m, aromatic H).

Methyl 2-methyl-3-aminobenzoate: A solution of methyl 2-methyl-3-nitrobenzoate (3 g, 15.4 mmol) in methanol (100 mL) was stirred under hydrogen atmosphere in the presence of 10% Pd/C (0.3 g) at room temperature. After 5 h, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give a solid (2.5 g, 98.5% yield).

Methyl 2-methyl-3-[N,N-bis(2-chloroethyl)]aminobenzoate: Ethylene oxide (2.38 mL, 47 mmol) was added to a cool solution of methyl 2-methyl-3-aminobenzoate (2.5 g, 15.5 mmol) in acetic acid (58.2 mL). After

18 h at room temperature, the solvent was evaporated under reduced pressure below 70°C, and the residue was triturated three times with 30 mL of anhydrous toluene and concentrated to dryness *in vacuo*. The resulting mixture was dissolved in benzene, washed with 10% aqueous Na₂CO₃, dried by anhydrous Na₂SO₄ and evaporated under reduced pressure. This evaporation residue was added, while stirring vigorously, to a cold (0°C) POCl₃ (13 mL, 0.14 mol). After addition, the mixture was heated for 1 h in an oil bath (intense evolution of HCl was observed). Excess POCl₃ was removed *in vacuo* and the mixture was extracted with benzene, dried by anhydrous Na₂SO₄ and evaporated *in vacuo*. Chromatography of the residue on silica gel column afforded the desired product (3.82 g, 94.4% yield).

¹H NMR (CDCl₃) δ: 2.38 (3H, s, phenyl CH₃), 3.58 (8H, s, N(CH₂CH₂Cl)₂), 3.85 (3H, s, COOCH₃), 7.44 (3H, m, aromatic H).

2-Methyl-3-[N,N-bis(2-chloroethyl)]aminobenzoic acid: A mixture of methyl 2-methyl-3-[N,N-bis(2-chloroethyl)]aminobenzoate (2.31 g, 7.96 mmol) and c-HCl (39.8 mL) was refluxed for 2 h. The mixture was diluted with water (230 mL) and extracted with ethyl acetate. The combined organic extracts were dried by anhydrous Na₂SO₄ and evaporated under reduced pressure (1.75 g, 79.6%). ¹H NMR (CDCl₃) δ: 2.45 (3H, s, phenyl CH₃), 3.61 (8H, s, N(CH₂CH₂Cl)₂), 7.43 (3H, m, aromatic H), 9.12 (1H, s, COOH).

3-[2'-Methyl-3'-[N,N-bis(2-chloroethyl)]aminobenzoyl]aminopiperidine-2,6-dione (5):

A solution of 2-methyl-3-[N,N-bis(2-chloroethyl)]aminobenzoic acid (0.9 g, 3.26 mmol) in dry benzene (10 mL) containing 0.85 mL of freshly distilled SOCl₂ was refluxed for 2 h. Excess SOCl₂ and solvent were removed under reduced pressure. To a solution of this evaporation residue (3.26 mmol) in 5 mL of anhydrous benzene was added 3-aminopiperidine-2,6-dione (0.83 g, 6.5 mmol) with stirring and occasional cooling. After 48 h, the reaction mixture was chromatographed on silica gel column (0.24 g, 19.1% yield). ¹H NMR (DMSO-d₆) δ: 2.11 (2H, m, CH₂CH₂CO), 2.47 (3H, s, phenyl CH₃), 2.75 (2H, m, CH₂CH₂CO), 3.55 (8H, s, N(CH₂CH₂Cl)₂), 4.52 (1H, q, COCHCH₂), 7.35 (4H, q, aromatic H), 8.47 (1H, d, CHNHCO), 10.77 (1H, s, CONHCO); IR_{neat} cm⁻¹: 3300 (amide NH, imide NH), 1710, 1650 (C=O), 730 (alkyl chloride C-Cl).

Preparation of 3-[4'-[N,N-bis(2-chloroethyl)]amino]phenylacetyl]aminopiperidine-2,6-dione (6)

Methyl 4-nitrophenylacetate: To a solution of 4-nitrophenylacetic acid (10 g, 55.2 mmol) in 100 mL of methanol was added 2 drops of c-H₂SO₄ and the mixture was refluxed for 12 h. The mixture was evaporated under reduced pressure and chromatography on a silica gel column (ethyl acetate : hexane) to give oil (9.5 g, 90% yield). ¹H NMR (CDCl₃) δ: 3.72 (5H, s,

CH_2CO and COOCH_3), 7.8 (4H, m, aromatic H).

Methyl 4-aminophenylacetate: A solution of methyl 4-nitrophenylacetate (9.5 g, 48.7 mmol) in methanol (150 mL) was stirred under hydrogen atmosphere in the presence of 10% Pd/C (0.3 g) at room temperature. After 24 h, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give oil (8 g, 99% yield). ^1H NMR (CDCl_3) δ : 3.49 (3H, s, COOCH_3), 3.65 (4H, s, CH_2CO and NH_2), 6.84 (4H, m, aromatic H).

Methyl 4-[N,N-bis(2-chloroethyl)]aminophenylacetate: Ethylene oxide (7.36 mL, 145.3 mmol) was added to a cooled solution of methyl 4-aminophenylacetate (48.4 mmol) in 180 mL of acetic acid. After 27 h at room temperature, the solution was evaporated under reduced pressure below 70°C , and the residue was triturated three times with 50 mL of anhydrous toluene and concentrated to dryness *in vacuo*. The resulting mixture was treated with benzene, washed with 10% aqueous Na_2CO_3 , dried by anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. This evaporated residue was added to POCl_3 (35 mL, 0.97 mol), previously cooled at 0°C . After addition, the mixture was heated for 1 h in an oil bath. Excess POCl_3 was removed *in vacuo* and the mixture was extracted with benzene and the product was obtained from the organic solution after drying (anhydrous Na_2SO_4). Chromatography on a silica gel column afforded the desired product (7.71 g, 53.2% yield). ^1H NMR (CDCl_3) δ : 3.7 (13H, m, CH_2CO , $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$, OCH_3), 6.92 (4H, m, aromatic H).

4-[N,N-Bis(2-chloroethyl)]aminophenylacetic acid: A mixture of methyl 4-[N,N-bis(2-chloroethyl)]aminophenylacetate (3.21 g, 11.1 mmol) and 55.3 mL of HCl was refluxed for 1 h. The mixture was diluted with water (108 mL), extracted with ethyl acetate, dried by Na_2SO_4 and evaporated under reduced pressure (2.31 g, 75.5%). ^1H NMR (CDCl_3) δ : 3.54 (2H, s, CH_2COOH), 3.66 (8H, t, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 6.9 (4H, m, aromatic H).

3-[4'-(N,N-Bis(2-chloroethyl)]aminophenylacetyl]aminopiperidine-2,6-dione (6): A solution of 4-[N,N-bis(2-chloroethyl)]aminophenylacetic acid (0.8 g, 2.9 mmol) in dry benzene (8.4 mL) was added dropwise to a freshly distilled thionyl chloride (2.26 mL) at 0.5°C . The mixture was allowed to stand overnight at -5°C . The precipitate was filtered under dry nitrogen atmosphere and the filtrate was washed with cold dry benzene and dried *in vacuo* to give 0.84 g (99%) of solid. To a stirred solution of this solid (0.4 g, 1.36 mmol) in anhydrous benzene (8 mL) was added 3-aminopiperidine-2,6-dione (0.35 g, 2.72 mmol) at low temperature and the mixture was stirred at ambient temperature for 24 h. The product was recrystallized from methylene chloride to yield 0.36 g (52% yield) of brown solid. m.p.: $158.4\sim 160.2^\circ\text{C}$; ^1H NMR (DMSO-

d_6) δ : 1.9 (2H, m, COCH_2CH_2), 2.59 (2H, m, COCH_2CH_2), 3.44 (2H, s, phenyl CH_2CO), 3.70 (8H, s, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 4.52 (1H, q, COCHCH_2), 6.67 (2H, d, aromatic H), 7.11 (2H, d, aromatic H), 8.31 (1H, d, CONHCH), 10.80 (1H, s, CONHCO); IRneat cm^{-1} : 3300 (amide NH), 3150 (imide NH), 1720, 1610 (C=O), 750 (alkyl chloride C-Cl).

3-(Benzoyl)aminopiperidine-2,6-dione (7): To a solution of L-glutamine (10 g, 68 mmol) in 80 mL of Na_2CO_3 (17 g, 160 mmol) solution was added dropwise a solution of benzoyl chloride (9.2 mL, 80 mmol) in 80 mL of benzene. The mixture was stirred at room temperature for 4h and acidified with 10% HCl to pH 2~3. The white precipitate obtained was filtered and dried *in vacuo*. It was heated at 160°C for 0.5 h. After cooling, solid was dissolved with hot methanol. To this brown solution was added an activated carbon to decolorize. After filtering the solution, the filtrate was cooled to room temperature to give a white precipitate (4.72 g, 30.0% yield). m.p.: $221\sim 223^\circ\text{C}$; ^1H NMR (DMSO- d_6) δ : 2.1 (2H, m, COCH_2CH_2), 2.7 (2H, m, COCH_2CH_2), 4.8 (1H, q, NHCHCH_2), 7.4 (4H, m, aromatic H), 8.5 (1H, d, CONHCH), 10.8 (1H, s, CONHCO) IRneat cm^{-1} : 3300 (amide NH), 3190 (imide NH), 1730, 1640 (C=O).

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