Inhibition of Human O^6-Alkylguanine-DNA Alkyltransferase and Potentiation of the Cytotoxicity of Chloroethylnitrosourea by 4(6)-(Benzyloxy)-2,6(4)-diamino-5-(nitro or nitroso)pyrimidine Derivatives and Analogues

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Received June 2, 1997

A series of 4(6)-(benzyloxy)-2,6(4)-diamino-5-(nitro or nitroso)pyrimidine derivatives and analogues of which 4(6)-benzyloxy groups were replaced with a (2-, 3-, or 4-fluorobenzyl)oxy or (2-, 3-, or 4-pyridylmethyl)oxy group, was synthesized. The abilities of these compounds to inhibit human O^6-alkylguanine-DNA alkyltransferase (AGAT) in vitro and to potentiate the cytotoxicity of 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea (ACNU) toward HeLa S3 cells were evaluated. 2,4-Diamino-6-[(2-fluorobenzyl)oxy]-5-nitropyrimidine (**3**) and 2,4-diamino-5-nitro-6-(2-pyridylmethoxy)pyrimidine (**6**), whose ortho positions of the 6-substituent are modified, were much weaker in terms of these abilities than the corresponding meta- or para-modified compounds. These results are consistent with those of our previous study using a series of O^6-benzylguanine derivatives. All 5-nitrosopyrimidine derivatives examined exerted both stronger AGAT-inhibition and ACNU-enhancement abilities than the corresponding 5-nitro derivatives. Among a variety of compounds that we have examined to date, 2,4-diamino-6-[(4-fluorobenzyl)oxy]-5-nitrosopyrimidine (**10**) exhibited the strongest ability to inhibit AGAT, and its magnitude was 2.5 and 50 times those of 4-(benzyloxy)-2,6-diamino-5-nitrosopyrimidine (**9**) and O^6-benzylguanine (**1**), respectively. A strong positive correlation was observed between the ability to inhibit AGAT and to potentiate the cytotoxicity of ACNU. This strongly indicates that 4(6)-(benzyloxy)pyrimidine derivatives and their analogues potentiate ACNU cytotoxicity by inhibiting AGAT activity. To characterize the reactivity of test compounds, alkyl-transfer reactions were also carried out using the biomimetic alkyl-transfer system.

Introduction

Treatment of cells with mutagenic and/or carcinogenic alkylating agents results in alkylation at many nucleophilic sites in DNA.[1] O^6-Alkylguanine (6AG) is one of the alkylated adducts formed, and 6AG is considered to be the DNA modification most responsible for the induction of cancer, mutation, and cell death.[2] O^6-Alkylguanine-DNA alkyltransferase (AGAT) is a repair enzyme which is found in a variety of organisms from bacteria to mammalian cells. AGAT removes the O^6-alkyl group of 6AG by accepting it at a thiol group in the enzyme's active-site cysteine.[3] Cells with low AGAT activity are known to be sensitive to alkylating agents which alkylate the O^6-position of a guanine residue.[4] Survival of brain tumor patients treated with carmustine, a 2-chloroethylating agent, is strongly correlated with AGAT levels.[5]

O^6-Benzylguanine (6BG) is a well-known low-molecular-weight inhibitor of mammalian AGAT.[6] Pretreatment of tumor cells with 6BG results in dramatic potentiation of the cytotoxicity of methylating,[7] 2-chloroethylating,[6,7] and 2-fluoroethylating agents.[8] The application of these agents in combination cancer chemotherapy is currently being employed in clinical trials. To date, numerous 6BG derivatives and their related compounds have been synthesized and tested for their ability to inhibit AGAT activity. However, none of these derivatives including those modified at the 2-amino group, 7-nitrogen, 9-nitrogen, or the para position of the O^6-benzyl group[7,9,10] have been reported to be a more potent AGAT inhibitor than 6BG, with the exception of two 6BG derivatives that were modified at the 8-position.[11] We have previously described the AGAT-inhibiting ability of a series of 6BG derivatives that were modified at the O^6-benzyl group. Modification at the ortho position of the benzyl group markedly diminished their ability to inhibit human AGAT activity, whereas modification at the meta or para position had little influence.[12,13] Recently, Chae et al. reported that 4-(benzyloxy)-2,6-diamino-5-nitropyrimidine (NO_2-BP) and 4-(benzyloxy)-2,6-diamino-5-nitrosopyrimidine (NO-BP), compounds with a pyrimidine moiety in the 6BG structure, have a stronger ability to inhibit human AGAT than 6BG.[11] These 4-(benzyloxy)pyrimidine derivatives are candidates as adjuvants in combination chemotherapy with alkylating antitumor drugs.

In this report we prepared NO_2-BP and NO-BP derivatives and analogues in which benzyloxy groups were replaced with a (fluorobenzyl)oxy or (pyridylmethyl)oxy group, as we had also done with 6BG derivatives, and tested their abilities to inhibit human AGAT activity in vitro and to potentiate the cytotoxicity of ACNU toward HeLa S3 cells. Among the pyrimidine derivatives examined, 2,4-diamino-6-[(4-fluorobenzyl)-oxy]-5-nitrosopyrimidine (**10**) showed the strongest

S0022-2623(97)00363-4 CCC: $15.00
Published on Web 01/24/1998

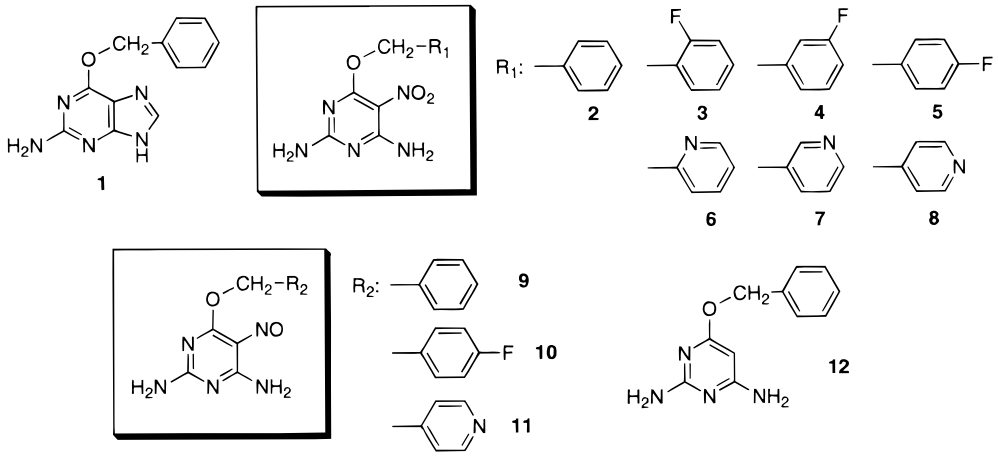

Figure 1. Structures and identification numbers of O^6-benzylguanine and 4(6)-(benzyloxy)pyrimidine derivatives.

inhibition of human AGAT in vitro, and this compound is the strongest AGAT inhibitor among compounds that we have examined to date.

Results and Discussion

Structures of the 12 test compounds employed in this study are presented in Figure 1. O^6-Benzylguanine (6BG, **1**) is well-known as a strong inhibitor of human AGAT.[6] 4-(Benzyloxy)-2,6-diamino-5-nitropyrimidine (NO_2-BP, **2**) and 4-(benzyloxy)-2,6-diamino-5-nitrosopyrimidine (NO-BP, **9**) were recently shown to be stronger inhibitors of human AGAT than 6BG.[11] The NO_2-BP derivatives **2**−**5** and 2,4-diamino-5-nitro-6-(pyridylmethoxy)pyrimidine derivatives **6**−**8** were synthesized by reacting 4-chloro-2,6-diamino-5-nitropyrimidine in *tert*-butyl alcohol with a corresponding sodium benzyl oxide derivative or a sodium pyridylmethyl oxide derivative, respectively. NO-BP derivatives **9** and **10** and an analogue (**11**) were prepared by reacting 4-chloro-2,6-diaminopyrimidine with a corresponding sodium arylmethyl oxide in *tert*-butyl alcohol followed by nitrosation at the 5-position of the pyrimidine ring.

Enzymatic repair by AGAT acting on O^6-alkylguanine in cellular DNA involves a transfer of the O^6-alkyl group to a thiol group of an active-site cysteine of AGAT.[3] To understand the chemical reactivity of the test compounds employed, their dealkylation rates with thiolate treatment were examined using a biomimetic system, as previously reported.[12,14] Briefly, a test compound was incubated at 60 °C in MeOH containing excess amounts of thiophenol and triethylamine, and the products formed were then analyzed using an HPLC apparatus equipped with a UV detector. Except for the 5-nitroso compounds (**9**−**11**), the amount of starting material decreased with reaction time following pseudo-first-order kinetics, and quantitative formation of both the dearylmethylated product and the corresponding arylmethyl phenyl sulfide was observed (data not shown). The rate constants for the decrease in all test compounds were calculated, and results are shown in Table 1. All NO_2-BP derivatives (**2**−**5**) and analogues (**6**−**8**) had 3−11 times greater rate constants than 6BG (**1**). In contrast, one compound lacking the 5-nitro group (**12**) had a 3-fold lower rate constant than **1**. The higher reactivity of compounds **2**−**8** may be attributed to the 5-nitro group which makes the 6(4)-arylmethyl group more reactive to thiophenolate. 5-Nitroso compounds (**9**−**11**) were also degraded under these reaction conditions; however, they were not dearylmethylated by thiophenolate, and neither 2,4-diamino-6-hydroxy-5-nitrosopyrimidine nor the corresponding arylmethyl phenyl sulfide were detected among various products that were not identified (data not shown). It was reported that the reaction of nitrosoarene with glutathione gave arylhydroxylamine and glutathionesulfinamide.[15] Similar types of reactions may proceed in the reactions of 5-nitroso compounds (**9**−**11**) with thiophenolate.

Table 1. Dealkylation Rates of 4(6)-(Benzyloxy)pyrimidine Derivatives and Analogues

compd	dealkylation rate,[a] 10^4k (min^{-1})
1	3.41 ± 0.45
2	13.7 ± 0.1
3	20.7 ± 1.5
4	14.9 ± 0.6
5	21.0 ± 0.1
6	10.2 ± 0.9
7	38.9 ± 0.9
8	21.2 ± 2.9
9	*b*
10	*b*
11	*b*
12	1.12 ± 0.33

[a] A mixture of test compound (0.244 μmol), thiophenol (1.75 mmol), and triethylamine (1.75 mmol) in MeOH (1 mL) was incubated at 60 °C. [b] Reactions other than dealkylation proceeded (see text).

The abilities of these test compounds to inhibit human AGAT activity were examined in vitro. Purified recombinant human AGAT (60−80 fmol) was incubated with each test compound for 20 min at 37 °C. Then, ^{3}H-methylated DNA was added, and the remaining AGAT activity was measured.[16−18] As an example, the dose−response curves of compounds **9** and **10** are shown in Figure 2. The IC_{50} value, the dose of test compound required to produce 50% inhibition of AGAT activity, was calculated from the curve. The IC_{50} values of all test compounds obtained are summarized in Table 2. The ability of NO_2-BP (**2**) to inhibit AGAT activity was 2 times greater than that of 6BG (**1**). Compound **12**, which is compound **2** without the 5-nitro group, was 65 times less effective than **2**. These results are consistent with those reported previously.[11] Compounds **3** and **6**, whose ortho positions of the 6(4)-substituents are modified, had 9.2- and 13.8-fold lower AGAT inhibitory ability, respectively, than **2**. Compounds **4**, **7**, and **8**,

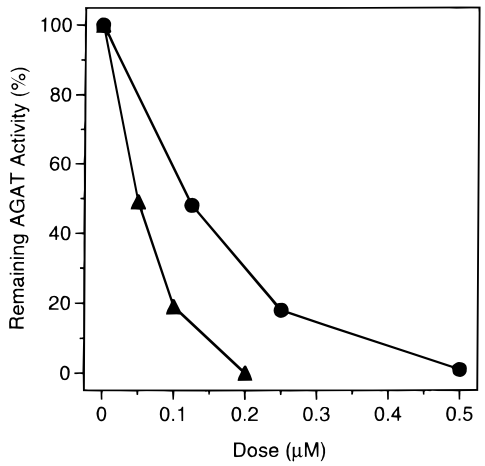

Figure 2. Inactivation of AGAT by compounds **9** (●) and **10** (▲). Purified AGAT was incubated with each test compound for 20 min at 37 °C. The results are shown as percentages of the AGAT activity remaining after incubation relative to that of untreated control.

Table 2. AGAT-Inhibitory Activity of 4(6)-(Benzyloxy)pyrimidine Derivatives and Analogues

compd	IC_{50} (μM)[a]
1	2.6 ± 0.1
2	1.3 ± 0.3
3	12 ± 0
4	2.5 ± 1.1
5	0.35 ± 0.01
6	18 ± 7
7	1.6 ± 0.1
8	1.9 ± 0.5
9	0.13 ± 0.03
10	0.051 ± 0.002
11	0.23 ± 0.08
12	170 ± 40

[a] Dose required to produce 50% inhibition.

in which the meta or para positions of the 6-substituents are modified, exhibited similar or slightly weaker activity than **2**. In contrast, compound **5**, the (*p*-fluorobenzyl)oxy derivative, was 3.7 times more effective than **2**. The effect of modification at the ortho, meta, and para positions of the benzyl group on the ability to inhibit AGAT activity was similar to what we had observed with 6BG derivatives.[19] In a series of 5-nitrosopyrimidine derivatives (**9**−**11**), all had stronger ability to inhibit AGAT activity than the corresponding 5-nitropyrimidine derivatives. Compound **9** exhibited 10 times greater AGAT inhibitory ability than **2** in our assay system, while Chae et al. reported that both had the same ability.[11] 2,4-Diamino-6-[(4-fluorobenzyl)oxy]-5-nitrosopyrimidine (**10**) exhibited 2.5 times stronger AGAT inhibitory ability than **9** (Figure 2 and Table 2), and it was the strongest AGAT inhibitor among all compounds that we have tested to date.

An examination of the relationship between the dealkylation rate (Table 1) and the inhibition of AGAT activity (Table 2) revealed that there was no obvious correlation (Figure 3). However, the great difference between 5-nitropyrimidine derivatives (**2**, **4**, **5**, **7**, **8**) and compound **12** in their ability to inhibit AGAT may be attributed to their chemical reactivities. Compounds **3** and **6**, which have modifications at the ortho position of the 6(4)-substituent, showed much weaker inhibition of AGAT than did **2** (Table 2), although the chemical

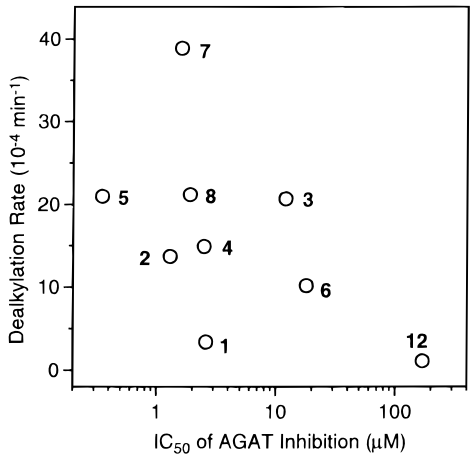

Figure 3. Correlation between the dealkylation rate and the ability to inhibit AGAT activity. Dealkylation rates (Table 1) were plotted against IC_{50} values (Table 2). Numbers in the figure correspond to compound numbers in Figure 1.

reactivity of **3** is greater than that of **2** while the reactivity of **6** is similar to that of **2** (Table 1). These results are consistent with our previous reports using a series of 6BG derivatives.[12,19] The ortho position of the benzyl group may play an important role in the interaction of AGAT with its substrate, and a modification at this position may contribute to steric hindrance. In terms of enzymatic benzyl transfer by AGAT, steric features comprise the most important factor, and the propensity of the substrate to lose its benzyl group seems not to be particularly important. With regard to 5-nitrosopyrimidine derivatives (**9**−**11**), thiophenolate treatment resulted in chemical reactions other than dealkylation. Although it is not clear if the same type of AGAT inactivation can occur between NO-BP and NO_2-BP derivatives in cells, this finding suggests the possibility that other mechanisms are involved in reactions between AGAT and NO-BP derivatives. Further experiments to resolve this issue will be required.

ACNU is a clinically employed antitumor agent. The mechanism for its cytotoxicity includes 2-chloroethylation at the O^6-position of guanine residues.[20] Since AGAT repairs this initial O^6-modification, it is known that the existence of AGAT in cells is responsible for resistance to these antitumor alkylating agents.[4] Pretreatment of cells with AGAT inhibitors such as 6BG has been reported to remarkably potentiate the cytotoxicity of such chloroethylating antitumor agents.[8,13] We therefore investigated the effect of this series of 4(6)-(benzyloxy)pyrimidine derivatives and analogues on potentiation of ACNU cytotoxicity. After HeLa cells were pretreated with a test compound, the cytotoxicity of ACNU was examined. As an example, the survival curves of cells pretreated with several concentrations of compound **10** are shown in Figure 4. To compare the enhancing effects of test compounds, the "dose-modifying factor (DMF)" (see Experimental Section) was calculated as previously reported,[8,12] and results are shown in Table 3. The doses employed for pretreatment were 0.01, 0.1, 1, and 10 μM, and the DMFs of compound **10** at 0.01, 0.1, and 1 μM were 1.4, 5.8, and 9.2, respectively. The enhancing effect of compound **2** was the same as that of **1**, whereas it was reported that the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), the same 2-chloroethylating agent, was poten-

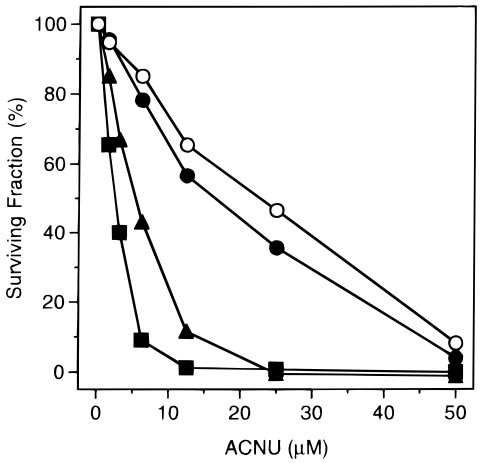

Figure 4. Enhancing effect of pretreatment with compound **10** on ACNU cytotoxicity. HeLa S3 cells were treated with compound **10** for 2 h and then with increasing concentrations of ACNU: no addition (○), 0.01 μM compound **10** (●), 0.1 μM (▲), 1 μM (■).

Table 3. Enhancing Effect of 4(6)-(Benzyloxy)pyrimidine Derivatives and Analogues on ACNU Cytotoxicity[a]

	dose-modifying factor			
compd	0.01 μM	0.1 μM	1 μM	10 μM
1		1.3 ± 0.0	4.4 ± 1.1	7.6 ± 1.3
2		1.2 ± 0.0	4.0 ± 0.2	*b*
3		1.0 ± 0.2	1.1 ± 0.2	*b*
4		1.2 ± 0.3	4.8 ± 1.1	*b*
5		1.5 ± 0.2	5.1 ± 0.9	*b*
6		1.0 ± 0.1	1.0 ± 0.1	*b*
7		1.2 ± 0.2	3.6 ± 0.5	*b*
8		1.2 ± 0.1	3.9 ± 0.4	*b*
9	1.2 ± 0.1	4.3 ± 1.0	8.8 ± 1.7	*b*
10	1.4 ± 0.1	5.8 ± 2.6	9.2 ± 1.6	*b*
11	1.0 ± 0.1	2.9 ± 0.7	7.3 ± 1.3	*b*
12				1.0 ± 0.0

[a] The enhancing effect is expressed in terms of a dose-modifying factor. For experimental details, see Experimental Section. [b] Toxicity appeared at this dose.

tiated by compound **2** to a greater extent than **1** using another tumor cell line system.[21] Compounds **4**, **5**, **7**, and **8** potentiated ACNU cytotoxicity as well as **2**. Compounds **3** and **6**, which have modifications at the ortho position of the 6-substituent, however, did not enhance ACNU cytotoxicity whatsoever. Three 5-nitrosopyrimidine derivatives (**9**−**11**) were apparently more effective than **2**, and these compounds remarkably potentiated ACNU cytotoxicity, even after 0.1 μM pretreatment. At a 10 μM dose, all 5-nitropyrimidine derivatives (**2**−**8**) and 5-nitrosopyrimidine derivatives (**9**−**11**) were cytotoxic by themselves. Compound **12** did not exhibit any ability to enhance ACNU cytotoxicity, nor was it cytotoxic on its own even at a 10 μM dose (Table 3).

Correlation between the ability to inhibit AGAT activity and to enhance ACNU cytotoxicity was studied, and the results are shown in Figure 5. There was a strong positive correlation between these abilities (r = 0.960). These results clearly indicate that the enhancing effect of ACNU cytotoxicity was caused by inhibition of intracellular AGAT. We previously reported a similar strong correlation with a series of 6BG derivatives.[19] 5-Nitrosopyrimidine derivatives, especially compound **10**, show great potential as candidates for potentiating alkylating antitumor agents.

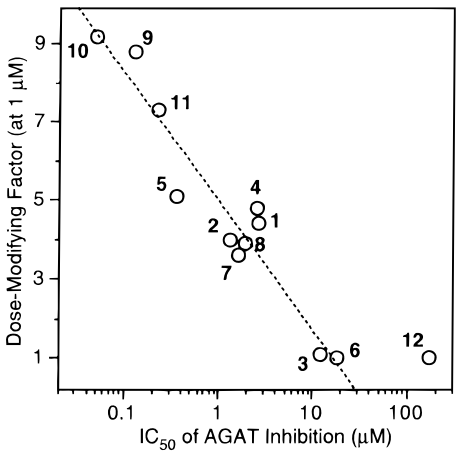

Figure 5. Correlation between the ability to inhibit AGAT activity and to enhance ACNU cytotoxicity. Dose-modifying factors (at 1 μM, Table 3) were plotted against IC_{50} values (Table 2); $y = -3.28 \log x + 4.98$, $r = 0.960$ (data for compound **12** were omitted from this regression curve). Numbers in the figure correspond to compound numbers in Figure 1.

Experimental Section

Materials and Methods. ^{1}H NMR spectra were recorded on a JEOL EX 270, GSX 400, or ALPHA 500 spectrometer (Tokyo, Japan). Samples were dissolved in DMSO-d_6, and chemical shifts are reported in ppm using Me_4Si as the internal standard. Mass spectra were obtained with a JEOL DX-300 spectrometer (Tokyo). UV spectra were recorded on a Shimadzu UV-2100 spectrophotometer (Kyoto, Japan). Melting points were measured with a Yanagimoto micromelting point apparatus (Kyoto) and are uncorrected. HPLC analyses were carried out using a Shimadzu LC10AD apparatus equipped with a photodiode array UV detector, SPD-M6A (Kyoto). For analyses of the purity of prepared compounds, a Merck LiChrospher 100 RP-18 (e) (4 × 250 mm) column was used and was eluted with a 1/15 M phosphate buffer (pH 6.8)−MeOH system at a flow rate of 1 mL/min.

6BG (**1**),[22] 4-(benzyloxy)-2,6-diamino-5-nitropyrimidine (**2**),[23] 4-(benzyloxy)-2,6-diamino-5-nitrosopyrimidine (**9**),[24] 4-(benzyloxy)-2,6-diaminopyrimidine (**12**),[24] and 4-chloro-2,6-diamino-5-nitropyrimidine[25] were synthesized as previously reported.

2,4-Diamino-6-[(2-fluorobenzyl)oxy]-5-nitropyrimidine (3). 4-Chloro-2,6-diamino-5-nitropyrimidine (100 mg, 0.527 mmol) was added to a mixture of sodium *tert*-butoxide (60 mg, 0.632 mmol) and 2-fluorobenzyl alcohol (84 μL, 0.791 mmol) in *tert*-butyl alcohol (10 mL). The reaction mixture was kept standing at 30 °C for 5 h with stirring. The solvent was evaporated, and the product was recrystallized twice with H_2O/MeOH to obtain 20.6 mg (14%) of yellow microcrystals: mp 222−224 °C; UV λ_{max} nm (ϵ) (H_2O/MeOH = 1/1) 233 (sh) (13 900), 262 (4700), 269 (5100), 334 (16 500), (0.01 N NaOH) 233 (sh) (13 800), 262 (4800), 269 (5100), 334 (16 300), (0.1 N HCl) 228 (sh) (18 800), 264 (7600), 317 (13 700); ^{1}H NMR δ 5.47 (s, 2 H, CH_2), 7.25 (m, 2 H, 3,5-ArH), 7.28 (br d, 2 H, NH_2), 7.42 (m, 1 H, 6-ArH), 7.66 (td, 1 H, 4-ArH, J = 1.6, 7.8 Hz), 7.93 (br s, 2 H, NH_2). Anal. ($C_{11}H_{10}FN_5O_3$) C, H, N.

2,4-Diamino-6-[(3-fluorobenzyl)oxy]-5-nitropyrimidine (4). Compound **4** was synthesized following a procedure similar to that described for compound **3** using 3-fluorobenzyl alcohol (87 μL, 0.791 mmol). Recrystallization of the product from H_2O/MeOH gave 55.5 mg (38%) of a yellow filamentous solid: mp 200−204 °C; UV λ_{max} nm (ϵ) (H_2O/MeOH = 1/1) 232 (sh) (14 100), 270 (sh) (4800), 334 (16 000), (0.01 N NaOH) 232 (sh) (13 900), 270 (sh) (4800), 334 (16 000), (0.1 N HCl) 228 (sh) (18 600), 263 (6800), 267 (sh) (6600), 318 (13 100); ^{1}H NMR δ 5.43 (s, 2 H, CH_2), 7.15 (td, 2 H, 5-ArH, J = 2.4, 8.5 Hz), 7.25 (br d, 2 H, NH_2), 7.31−7.34 (m, 2 H, 2,6-ArH), 7.44 (dd, 1 H, 4-ArH), 7.93 (br s, 2 H, NH_2). Anal. ($C_{11}H_{10}FN_5O_3 \cdot {}^1/_4H_2O$) C, H, N.

2,4-Diamino-6-[(4-fluorobenzyl)oxy]-5-nitropyrimidine (5). Compound **5** was synthesized following a procedure similar to that described for compound **3** using 4-fluorobenzyl alcohol (87 μL, 0.791 mmol). Recrystallization of the product from H_2O/MeOH gave 50.3 mg (34%) of yellow microcrystals: mp 196−199 °C; UV λ_{max} nm (ϵ) (H_2O/MeOH = 1/1) 234 (sh) (12 400), 262 (4100), 269 (4500), 333 (15 500), (0.01 N NaOH) 234 (sh) (12 400), 262 (4200), 269 (4500), 333 (15 400), (0.1 N HCl) 228 (sh) (17 700), 263 (7100), 268 (sh) (6800), 316 (13 000); ^{1}H NMR δ 5.39 (s, 2 H, CH_2), 7.22 (t, 2 H, 3-ArH, J = 8.9 Hz), 7.25 (br s, 2 H, NH_2), 7.54 (dd, 2 H, 2-ArH, J = 5.4 Hz), 7.91 (br s, 2 H, NH_2). Anal. ($C_{11}H_{10}FN_5O_3 \cdot {}^1/_3H_2O$) C, H, N.

2,4-Diamino-5-nitro-6-(2-pyridylmethoxy)pyrimidine (6). 4-Chloro-2,6-diamino-5-nitropyrimidine (100 mg, 0.527 mmol) was added to a mixture of sodium *tert*-butoxide (50 mg, 0.527 mmol) and 2-pyridylmethanol (51 μL, 0.527 mmol) in *tert*-butyl alcohol (10 mL). The reaction mixture was kept standing at 30 °C for 7 h with stirring. The reaction mixture was neutralized with acetic acid and evaporated to dryness. The residue was washed with hot H_2O/MeOH (10/1, 50 mL) and dried to obtain 45.9 mg (33%) of a white solid. Using these procedures, an analytical sample was obtained without further purification: mp 256−260 °C; UV λ_{max} nm (ϵ) (H_2O/MeOH = 1/1) 238 (sh) (12 800), 259 (8300), 266 (6600), 333 (16 600), (0.01 N NaOH) 238 (sh) (12 800), 259 (8300), 266 (6600), 333 (16 600), (0.1 N HCl) 260 (11 500), 268 (sh) (10 000), 332 (16 100); ^{1}H NMR δ 5.48 (s, 2 H, CH_2), 7.25 (br d, 2 H, NH_2), 7.34 (dd, 1 H, 4-ArH, $J_{3,4}$ = 4.9 Hz), 7.59 (d, 1 H, 6-ArH, J = 7.9 Hz), 7.86 (td, 1 H, 5-ArH, $J_{4,5}$ = 7.6 Hz, $J_{5,6}$ = 7.9 Hz), 7.95 (br d, 2 H, NH_2), 8.56 (d, 1 H, 3-ArH). Anal. ($C_{10}H_{10}N_6O_3 \cdot {}^1/_3CH_3OH$) C, H, N.

2,4-Diamino-5-nitro-6-(3-pyridylmethoxy)pyrimidine (7). 4-Chloro-2,6-diamino-5-nitropyrimidine (200 mg, 1.05 mmol) was added to a mixture of sodium *tert*-butoxide (100 mg, 1.05 mmol) and 3-pyridylmethanol (102 μL, 1.05 mmol) in *tert*-butyl alcohol (10 mL). The reaction mixture was kept standing at 30 °C with stirring overnight. Additional sodium *tert*-butoxide (10 mg) and 3-pyridylmethanol (10 μL) were added, and the mixture was stirred overnight. The reaction mixture was neutralized with acetic acid and evaporated to dryness. The residue was washed with hot H_2O/MeOH (10/1, 50 mL) and dried to obtain 47.1 mg (17%) of a yellow solid. Following these procedures, an analytical sample was obtained: mp 220−224 °C; UV λ_{max} nm (ϵ) (H_2O/MeOH = 1/1) 234 (sh) (14 100), 260 (sh) (6800), 266 (sh) (6000), 333 (17 000), (0.01 N NaOH) 233 (sh) (13 900), 260 (sh) (6700), 266 (sh) (6000), 334 (17 000), (0.1 N HCl) 229 (sh) (16 800), 260 (sh) (11 500), 321 (14 500); ^{1}H NMR δ 5.45 (s, 2 H, CH_2), 7.27 (br d, 2 H, NH_2), 7.43 (dd, 1 H, 5-ArH, $J_{4,5}$ = 4.9 Hz), 7.91 (dt, 1 H, 6-ArH, $J_{2,6}$ = 1.8 Hz), 7.92 (br s, 2 H, NH_2), 8.54 (dd, 1 H, 4-ArH), 8.71 (d, 1 H, 2-ArH). Anal. ($C_{10}H_{10}N_6O_3 \cdot {}^1/_4H_2O$) C, H, N.

2,4-Diamino-5-nitro-6-(4-pyridylmethoxy)pyrimidine (8). 4-Chloro-2,6-diamino-5-nitropyrimidine (200 mg, 1.05 mmol) was added to a mixture of sodium *tert*-butoxide (100 mg, 1.05 mmol) and 4-pyridylmethanol (138 μL, 1.05 mmol) in *tert*-butyl alcohol (10 mL). The reaction mixture was kept standing at 30 °C with stirring overnight. The solvent was evaporated, and the product was separated by means of silica gel column chromatography (Merck silica gel 60, 70−230 mesh, 63−200 μm, eluted with 0−10% MeOH in $CHCl_3$). The fractions collected were evaporated and then washed with cold MeOH (4 mL) to obtain 60.2 mg (22%) of a pale-brown solid: mp 188−191 °C; UV λ_{max} nm (ϵ) (H_2O/MeOH = 1/1) 233 (sh) (12 700), 334 (15 900), (0.01 N NaOH) 233 (sh) (12 700), 333 (15 900), (0.1 N HCl) 252 (sh) (19 800), 260 (sh) (17 800), 320 (27 000); ^{1}H NMR δ 5.47 (s, 2 H, CH_2), 7.25 (br d, 2 H, NH_2), 7.46 (d, 2 H, 2-ArH, $J_{1,2}$ = 5.8 Hz), 7.96 (br d, 2 H, NH_2), 8.58 (d, 3 H, 3-ArH). Anal. ($C_{10}H_{10}N_6O_3 \cdot {}^1/_4CH_3OH$) C, H, N.

2,4-Diamino-6-[(4-fluorobenzyl)oxy]pyrimidine. 4-Chloro-2,6-diaminopyrimidine (200 mg, 1.38 mmol) was added to a solution of sodium *tert*-butoxide (438 mg, 4.56 mmol) and 4-fluorobenzyl alcohol (498 μL, 4.56 mmol) in *tert*-butyl alcohol (10 mL), and the mixture was heated under reflux for 1 day. The reaction mixture was then neutralized with acetic acid and evaporated. After H_2O (20 mL) was added to the residue, the product was extracted with $CHCl_3$ (10 mL, performed twice). Recrystallization of the product from benzene/hexane gave 112.7 mg (35%) of a white solid: mp 96−97 °C; UV (H_2O/MeOH = 1/1) λ_{max} nm 231 (sh), 265, (0.01 N NaOH) 231 (sh), 264, (0.1 N HCl) 228 (sh), 278; ^{1}H NMR δ 5.07 (s, 1 H, H-5), 5.18 (s, 2 H, CH_2), 5.86 (br s, 2 H, NH_2), 6.02 (br s, 2 H, NH_2), 7.17 (t, 2 H, 3-ArH, J = 9.0 Hz), 7.44 (dd, 2 H, 3-ArH, $J_{2,F}$ = 5.9 Hz, $J_{2,3}$ = 8.3 Hz); HRMS m/z calcd for $C_{11}H_{11}FN_4O$ 234.0917 (M^+), found 234.0912; analytical HPLC (a 1/15 M phosphate buffer (pH 6.8)−MeOH system, 30% MeOH for 0−10 min followed by a linear gradient of 30−100% MeOH for 10−60 min) t_R = 33.0 min (95% pure).

2,4-Diamino-6-[(4-fluorobenzyl)oxy]-5-nitrosopyrimidine (10). A solution of sodium nitrite (8.9 mg, 0.128 mmol) in H_2O (100 μL) was added to a solution of 2,4-diamino-6-[(4-fluorobenzyl)oxy]pyrimidine (20 mg, 92.9 μmol) in 30% acetic acid (0.5 mL) with stirring at 70 °C. After 5 min, the resulting precipitate was filtered, washed with water−MeOH, and dried to obtain 21.4 mg (88%) of purple powder. Using this procedure, a more than 99% pure product was obtained; however, an analytical sample was not obtained after several trials of repeated recrystallization: mp 221−224 °C dec; UV (H_2O/MeOH = 1/1) λ_{max} nm (ϵ) 233 (6700), 332 (22 100), (0.01 N NaOH) 233 (6600), 332 (22 100), (0.1 N HCl) 234 (5400), 331 (19 200); ^{1}H NMR δ 5.56 (s, 2 H, CH_2), 7.24 (tt, 2 H, 3-ArH, J = 2.5, 8.8 Hz), 7.61 (dd, 2 H, 2-ArH, $J_{2,F}$ = 2.9 Hz, $J_{2,3}$ = 8.5 Hz), 7.82 (br s, 1 H, NH), 7.88 (br s, 1 H, NH), 8.02 (d, 1 H, NH, J = 3.9 Hz), 10.04 (d, 1 H, NH); HRMS m/z calcd for $C_{11}H_{10}FN_5O_2$ 263.0819 (M^+), found 263.0819; analytical HPLC (a 1/15 M phosphate buffer (pH 6.8)−MeOH system, 30% MeOH for 0−10 min followed by a linear gradient of 30−100% MeOH for 10−60 min) t_R = 31.4 min (99.5% pure).

2,4-Diamino-6-(4-pyridylmethoxy)pyrimidine. 4-Chloro-2,6-diaminopyrimidine (200 mg, 1.38 mmol) was added to a solution of sodium (95 mg, 4.15 mmol) in 4-pyridylmethanol (3 g), and the mixture was kept at 75 °C with stirring. After 19 h, the reaction mixture was neutralized with acetic acid and evaporated to dryness. The product was separated on a Sephadex LH-20 column (2.5 × 40 cm) which was eluted with MeOH to obtain 100.6 mg (34%) of a white solid: mp 197−198 °C; UV (H_2O/MeOH = 1/1) λ_{max} nm (ϵ) 232 (7600), 263 (9500), (0.01 N NaOH) 232 (sh) (7500), 263 (9700), (0.1 N HCl) 224 (sh) (9800), 260 (7700), 280 (13 600); ^{1}H NMR δ 5.15 (s, 1 H, H-5), 5.27 (s, 2 H, CH_2), 5.90 (br s, 2 H, NH_2), 6.08 (br s, 2 H, NH_2), 7.34 (d, 2 H, 2-ArH, $J_{2,3}$ = 5.5 Hz), 8.53 (d, 2 H, 3-ArH); HRMS m/z calcd for $C_{10}H_{11}N_5O$ 217.0964 (M^+), found 217.0964; analytical HPLC (1/15 M phosphate buffer (pH 6.8)−30% MeOH) t_R = 10.4 min (99.8% pure).

2,4-Diamino-5-nitroso-6-(4-pyridylmethoxy)pyrimidine (11). A solution of sodium nitrite (18.4 mg, 0.266 mmol) in H_2O (200 μL) was added to a solution of 2,4-diamino-6-(4-pyridylmethoxy)pyrimidine (42 mg, 0.193 mmol) in 30% acetic acid (1 mL) with stirring at 70 °C. After 5 min, the reaction mixture was evaporated and the residue was washed with cold H_2O (2 mL) and dried to obtain 41.3 mg (87%) of a purple solid. Without further purification, an analytical sample was obtained: mp 232−235 °C dec; UV (H_2O/MeOH = 1/1) λ_{max} nm (ϵ) 232 (9100), 259 (sh) (4500), 330 (25 000), (0.01 N NaOH) 235 (7100), 257 (sh) (5000), 330 (25 900), (0.1 N HCl) 240 (sh) (8900), 253 (9500), 331 (19 800); ^{1}H NMR δ 5.64 (s, 2 H, CH_2), 7.49 (d, 2 H, 2-ArH, $J_{2,3}$ = 6.5 Hz), 7.85 (br s, 2 H, NH_2), 8.07 (br s, 1 H, NH), 8.60 (d, 2 H, 3-ArH), 10.03 (br s, 2 H, NH). Anal. ($C_{10}H_{10}FN_6O_2$) C, H, N.

Dealkylation Reaction. Dealkylation rates of test compounds were measured as previously reported.[12] Briefly, a solution containing a test compound (0.244 μmol), thiophenol (180 μL, 1.75 mmol), triethylamine (240 μL, 1.75 mmol), and MeOH (1 mL) in a sealed tube was incubated at 60 °C. After appropriate times, an aliquot of the reaction mixture was taken out and diluted with MeOH. This solution was subjected

to product analysis using an HPLC apparatus equipped with a UV detector.

Preparation of AGAT. Purification of human AGAT was performed as described[16,26] with slight modifications. The plasmid PHH24[27] carrying human AGAT cDNA was introduced into the AGAT-deficient *Escherichia coli* strain, KT233 (ada^-, ogt^-). The cells were grown at 37 °C in LB medium containing 50 μg/mL ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Using the harvested cells, a crude extract was prepared by sonication. After ammonium sulfate precipitation, human AGAT was purified by column chromatography on DEAE-Sephacel and MonoS columns obtained from Pharmacia LKB Biotechnology Inc. Purification was monitored by measuring AGAT activity. The MonoS fraction was used for the AGAT-inhibition assay.

AGAT Assay. Methyltransferase activity remaining after the enzyme was treated with a test compound was measured as previously reported.[16-18] Briefly, the reaction mixture (100 μL) containing 70 mM Hepes-KOH (pH 7.8), 1 mM DTT, 5 mM EDTA, various amounts of test compounds, and 60−80 fmol of AGAT was incubated at 37 °C for 20 min. Then, 1 μg of ^{3}H-methylated calf thymus DNA prepared by treatment with *N*-[^{3}H]methyl-*N*-nitrosourea was added, and the mixture was incubated at 37 °C for another 15 min. The reaction was terminated by addition of 300 μL of 2 M perchloric acid and 200 μL of 1 mg/mL BSA. The mixture was heated at 70 °C for 60 min to hydrolyze DNA, and the [^{3}H]methyl-accepted protein was collected by centrifugation at 4 °C and washed twice with 1 M perchloric acid. The pellet was dissolved in 10 μL of 0.1 N NaOH and then neutralized with 10 μL of 0.1 N HCl. Radioactivity was determined in a liquid scintillation counter.

Cell Assay. HeLa S3 cervical tumor cells were provided by Dr. K. Mineura of Akita University, Japan. The AGAT activity was 418 fmol/mg of protein. The cytotoxicity assay was carried out using the same method as reported previously.[8] The dose-modifying factor (DMF) was calculated as the ratio of the IC_{50} value of cells treated with ACNU alone versus the IC_{50} of cells treated with a test compound followed by ACNU treatment.

Acknowledgment. We are grateful to Professor Y. Kawazoe of Nagoya City University for helpful advice and encouragement and to Drs. H. Kawate, K. Sakumi, and M. Sekiguchi of Kyushu University for their help in preparation of human AGAT. We thank Dr. K. Mineura of Akita University for providing HeLa S3 cells. We also thank Mr. A. Kobayashi for his help with HPLC analyses. One of the authors (I.T.) gratefully acknowledges a postgraduate fellowship from the Japan Society for the Promotion of Science.

References

(1) Singer, B. The chemical effects of nucleic acid alkylation and their relation to mutagenesis and carcinogenesis. *Prog. Nucleic Acid Res. Mol. Biol.* **1975**, *15*, 219−284.
(2) Loveless, A. Possible relevance of O-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature* **1969**, *223*, 206−207.
(3) Lindahl, T.; Sedgwick, B.; Sekiguchi, M.; Nakabeppu, Y. Regulation and expression of the adaptive response to alkylating agents. *Annu. Rev. Biochem.* **1988**, *57*, 133−157.
(4) Scudiero, D. A.; Meyer, S. A.; Clatterbuck, B. E.; Mattern, M. R.; Ziolkowski, C. H. J.; Day, R. S., III. Sensitivity of human cell strains having different abilities to repair O^6-methylguanine in DNA to inactivation by alkylating agents including chloroethylnitrosoureas. *Cancer Res.* **1984**, *44*, 2467−2474.
(5) Belanich, M.; Pastor, M.; Randall, T.; Guerra, D.; Kibitel, J.; Alas, L.; Li, B.; Citron, M.; Wasserman, P.; White, A.; Eyre, H.; Jaeckle, K.; Schulman, S.; Rector, D.; Prados, M.; Coons, S.; Shapiro, W.; Yarosh, D. Retrospective study of the correlation between the DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. *Cancer Res.* **1996**, *56*, 783−788.
(6) Dolan, M. E.; Moschel, R. C.; Pegg, A. E. Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5368−5372.
(7) Dolan, M. E.; Mitchell, R. B.; Mummert, C.; Moschel, R. C.; Pegg, A. E. Effect of O^6-benzylguanine analogues on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. *Cancer Res.* **1991**, *51*, 3367−3372.
(8) Terashima, I.; Kohda, K. Cytotoxicity of fluoroethylating agents is potentiated by O^6-benzylguanine. *Biol. Pharm. Bull.* **1996**, *19*, 956−961.
(9) Moschel, R. C.; McDougall, M. G.; Dolan, M. E.; Stine, L.; Pegg, A. E. Structural features of substituted purine derivatives compatible with depletion of human O^6-alkylguanine-DNA alkyltransferase. *J. Med. Chem.* **1992**, *35*, 4486−4491.
(10) Chae, M.-Y.; McDougall, M. G.; Dolan, M. E.; Swenn, K.; Pegg, A. E.; Moschel, R. C. Substituted O^6-benzylguanine derivatives and their inactivation of human O^6-alkylguanine-DNA alkyltransferase. *J. Med. Chem.* **1994**, *37*, 342−347.
(11) Chae, M.-Y.; Swenn, K.; Kanugula, S.; Dolan, M. E.; Pegg, A. E.; Moschel, R. C. 8-Substituted O^6-benzylguanine, substituted 6(4)-(benzyloxy)pyrimidine, and related derivatives as inactivators of human O^6-alkylguanine-DNA alkyltransferase. *J. Med. Chem.* **1995**, *38*, 359−365.
(12) Kohda, K.; Terashima, I.; Koyama, K.; Watanabe, K.; Mineura, K. Potentiation of the cytotoxicity of chloroethylnitrosourea by O^6-arylmethylguanines. *Biol. Pharm. Bull.* **1995**, *18*, 424−430.
(13) Mineura, K.; Izumi, I.; Watanabe, K.; Kawada, M.; Kohda, K.; Koyama, K.; Terashima, I.; Ikenaga, M. Enhancing effect of O^6-alkylguanine derivatives on chloroethylnitrosourea cytotoxicity toward tumor cells. *Int. J. Cancer* **1994**, *58*, 706−712.
(14) Kohda, K.; Yasuda, M.; Sawada, N.; Itano, K.; Kawazoe, Y. Dealkylation rates of O^6-alkyldeoxyguanosine, O^4-alkylthymidine and related compounds in an alkyl-transfer system. *Chem.-Biol. Interact.* **1991**, *78*, 23−32.
(15) Eyer, P. Reactions of oxidatively activated arylamines with thiols: Mechanisms and biologic implications. An overview. *Environ. Health Perspect.* **1994**, *102* (Suppl. 6), 123−132.
(16) Kawate, H.; Ihara, K.; Kohda, K.; Sakumi, K.; Sekiguchi, M. Mouse methyltransferase for repair of O^6-methylguanine and O^4-methylthymine in DNA. *Carcinogenesis* **1995**, *16*, 1595−1602.
(17) Nakabeppu, Y.; Kondo, H.; Kawabata, S.; Iwanaga, S.; Sekiguchi, M. Purification and structure of the intact Ada regulatory protein of *Escherichia coli* K12, O^6-methylguanine-DNA methyltransferase. *J. Biol. Chem.* **1985**, *260*, 7281−7288.
(18) Sassanfar, M.; Dosanjh, M. K.; Essigmann, J. M.; Samson, L. Relative effeciencies of the bacterial, yeast, and human DNA methyltransferases for the repair of O^6-methylguanine and O^4-methylthymine. *J. Biol. Chem.* **1991**, *266*, 2767−2771.
(19) Mineura, K.; Fukuchi, M.; Kowada, M.; Terashima, I.; Kohda, K. Differential inactivation of O^6-methylguanine-DNA methyltransferase activity by O^6-arylmethylguanines. *Int. J. Cancer* **1995**, *63*, 148−151.
(20) Ludlum, D. B. DNA alkylation by the haloethylnitrosoureas: Nature of modifications produced and their enzymatic repair or removal. *Mutat. Res.* **1990**, *233*, 117−126.
(21) Pegg, A. E.; Swenn, K.; Chae, M.-Y.; Dolan, M. E.; Moschel, R. C. Increased killing of prostate, breast, colon, and lung tumor cells by the combination of inactivators of O^6-alkylguanine-DNA alkyltransferase and *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea. *Biochem. Pharmacol.* **1995**, *50*, 1141−1148.
(22) Balsiger, R. W.; Montgomery, J. A. Synthesis of potential anticancer agents. XXV. Preparation of 6-alkoxy-2-aminopurines. *J. Org. Chem.* **1960**, *25*, 1573−1575.
(23) Kosary, J.; Diesler, E.; Matyus, P.; Kasztreiner, E. Preparation of pyrimidine derivatives with potential cardiotonic activity. *Acta Pharm. Hung.* **1989**, *49*, 241−247.
(24) Pfleiderer, W.; Lohrmann, R. Synthesis of 2-amino-4-alkoxypteridine. *Chem. Ber.* **1961**, *94*, 12−18.
(25) O'Brien, D. E.; Cheng, C. C.; Pfleiderer, W. Pyrimidines. XVIII. 2,4-Diamino-5-nitro-6-arylaminopyrimidines. Nitration study of 2,4-diamino-6-chloropyrimidine and a literature correction. *J. Med. Chem.* **1966**, *9*, 573−575.
(26) Koike, G.; Maki, H.; Takeya, H.; Hayakawa, H.; Sekiguchi, M. Purification, structure, and biochemical properties of human O^6-methylguanine-DNA methyltransferase. *J. Biol. Chem.* **1990**, *265*, 14754−14762.
(27) Chueh, L.-L.; Nakamura, T.; Nakatsu, Y.; Sakumi, K.; Hayakawa, H.; Sekiguchi, M. Specific amino acid sequences required for O^6-methylguanine-DNA methyltransferase activity: Analyses of three residues at or near the methyl acceptor site. *Carcinogenesis* **1992**, *13*, 837−843.

JM970363I