Structural Features of Substituted Purine Derivatives Compatible with Depletion of Human O^6-Alkylguanine-DNA Alkyltransferase

Robert C. Moschel,*,† Mark G. McDougall,† M. Eileen Dolan,‡ Linda Stine,§ and Anthony E. Pegg§

Carcinogen-Modified Nucleic Acid Chemistry, Chemistry of Carcinogenesis Laboratory, ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, Maryland 21702, Division of Hematology-Oncology, The University of Chicago Medical Center, 5841 South Maryland Avenue, Box MC2115, Chicago, Illinois 60637, and Departments of Cellular and Molecular Physiology and Pharmacology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, P.O. Box 850, Hershey, Pennsylvania 17033

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A series of O^6- and S^6-substituted purine derivatives were tested for their ability to deplete the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) in cell-free extracts from HT29 colon tumor cells and intact HT29 cells. The order of potency was O^6-(*p*-Y-benzyl)-guanine (Y = H, F, Cl, and CH_3) > O^6-benzyl-2′-deoxyguanosine > O^6-(*p*-Y-benzyl)guanosine (Y = H, Cl, and CH_3) ≥ a series of 9-substituted O^6-benzylguanine derivatives ≥ O^6-allylguanine > O^6-benzylhypoxanthine > O^6-methylguanine. A series of 7-substituted O^6-benzylguanine derivatives, 2-amino-6-(*p*-Y-benzylthio)purine (Y = H, CH_3), 2-amino-6-[(*p*-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine, and 7-benzylguanine were inactive. It is concluded that for efficient AGT depletion, an allyl or benzyl group attached through exocyclic oxygen at position 6 of a 2-aminopurine derivative is required. Activity is preserved with a variety of substituent groups attached to position 9 while substitution at position 7 leads to a complete loss of activity.

Mammalian O^6-alkylguanine-DNA alkyltransferase (AGT) repairs alkylation damage to the O^6-position of DNA guanine residues by transferring the alkyl group to an active-site cysteine residue, thereby restoring a normal guanine at the site of the modified base.[1] The resulting alkylated protein is inactivated for subsequent dealkylation reactions. Consequently, a cell's repair capacity by this mechanism is dependent on the number of AGT molecules present. Cells with higher AGT content are able to repair greater amounts of O^6-alkylguanine damage and these cells exhibit resistance to the cytotoxic effects of O^6-alkylating antitumor drugs while cells of low AGT content are far more sensitive to these agents.[1,2-4]

We demonstrated recently that O^6-benzylguanine (**1a**, Chart I), O^6-(*p*-chlorobenzyl)guanine (**1c**), and O^6-(*p*-methylbenzyl)guanine (**1d**), as alternative substrates for the protein, produce dramatic and rapid depletion of AGT in human tumor cell extracts and intact tumor cells.[5,6] This depletion leads to significant enhancements in the cytotoxic response to a number of chloroethylating and methylating antitumor drugs.[5,6] Furthermore, pretreatment of nude mice bearing human tumor xenografts with O^6-benzylguanine (**1a**) leads to a significant decrease in the rate of tumor growth produced by the chloroethylating nitrosoureas 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (MeCCNU)[7] and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).[8] To further characterize the structural features of modified purines that are compatible with efficient AGT depletion in human tumor cell extracts and intact cells, we have compared the concentration dependence for AGT depletion of several additional compounds. The results of these comparisons are reported here.

Results and Discussion

Structures for all compounds tested here as AGT-depleting agents are illustrated in Chart I. In addition to compounds **1a**, **1c**, and **1d** described above, the list includes O^6-(*p*-fluorobenzyl)guanine (**1b**), O^6-benzyl-2′-deoxyguanosine (**2**), O^6-benzylguanosine (**3a**) together with the O^6-(*p*-chlorobenzyl)- and O^6-(*p*-methylbenzyl)guanosine analogs (**3b** and **3c**, respectively), O^6-allylguanine (**4**), a series of 9-alkylated O^6-benzylguanine derivatives, i.e., 2-amino-6-(benzyloxy)-9-[(ethoxycarbonyl)methyl]purine (**5a**), 2-amino-6-(benzyloxy)-9-(carboxymethyl)purine (sodium salt) (**5b**), 2-amino-6-(benzyloxy)-9-(cyanomethyl)purine (**5c**), 2-amino-6-(benzyloxy)-9-(carbamoylmethyl)purine (**5d**), 2-amino-6-(benzyloxy)-9-(2-hydroxybutyl)purine (**5e**), O^6-benzylhypoxanthine (**6**), O^6-methylguanine (**7**), a series of 7-alkylated O^6-benzylguanine derivatives (**8a-e**) with the same alkyl groups present on the 9-substituted isomers (**5a-e** above), 2-amino-6-(benzylthio)purine and 2-amino-6-[(*p*-methylbenzyl)thio]purine (**9a** and **9b**, re-

† National Cancer Institute-Frederick Cancer Research and Development Center.
‡ The University of Chicago Medical Center.
§ Pennsylvania State University College of Medicine.

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(4) Pegg, A. E.; Dolan, M. E. Properties and assay of mammalian O^6-alkylguanine-DNA alkyltransferase. *Pharmacol. Ther.* **1987**, *34*, 167-179.
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(8) Mitchell, R. B.; Moschel, R. C.; Dolan, M. E. Effect of O^6-benzylguanine on the sensitivity of human tumor xenografts to 1,3-bis(2-chloroethyl)-1-nitrosourea and on DNA interstrand cross-link formation. *Cancer Res.* **1992**, *52*, 1171-1175.

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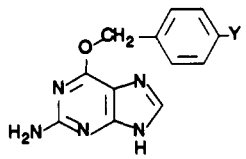
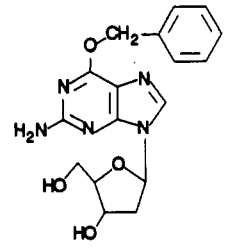
Chart I



1a-d

a, Y=H; b, Y=F; c, Y=Cl; d, Y=CH_3



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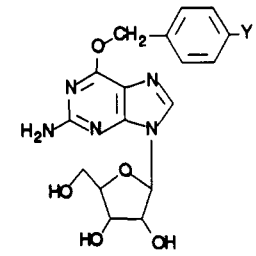
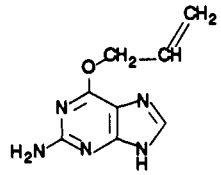


3a-c

a, Y=H; b, Y=Cl; c, Y=CH_3



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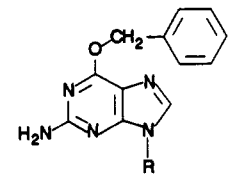
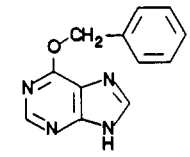


5a-e



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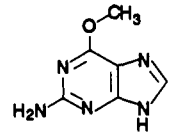
a, R=$CH_2CO_2CH_2CH_3$; b, R=$CH_2CO_2^-Na^+$
c, R=CH_2CN; d, R=CH_2CONH_2
e, R=$CH_2CH(OH)CH_2CH_3$



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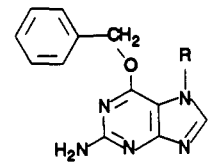
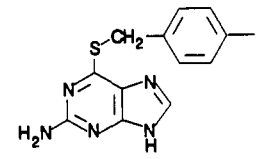


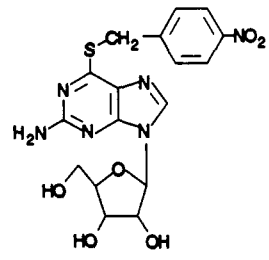
8a-e



9a,b

a, R=$CH_2CO_2CH_2CH_3$; b, R=$CH_2CO_2^-Na^+$
c, R=CH_2CN; d, R=CH_2CONH_2
e, R=$CH_2CH(OH)CH_2CH_3$

a, Y=H; b, Y=CH_3



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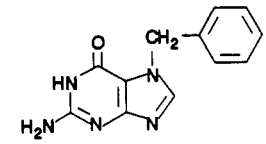

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spectively), 2-amino-6-[(*p*-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (**10**), and 7-benzylguanine (**11**). Synthetic routes to most of these compounds have been presented elsewhere (see Experimental Section). Those compounds which have not been previously described are O^6-(*p*-fluorobenzyl)guanine (**1b**) and the 9- and 7-alkylated O^6-benzylguanine analogs **5a-e** and **8a-e**, respectively. O^6-(*p*-Fluorobenzyl)guanine was prepared by reacting 2-amino-6-chloropurine with sodium *p*-fluorobenzyl oxide in *p*-fluorobenzyl alcohol following the procedures developed for O^6-benzylguanine (**1a**) preparation[9,10] as modified for the preparation of **1c** and **1d**.[5] The 9- and 7-substituted analogs **5a**, **5c**, **5d**, **5e**, **8a**, **8c**, **8d**, and **8e** were isolated from reactions between the anion of O^6-benzylguanine and ethyl bromoacetate, bromoacetonitrile, bromoacetamide, and 1,2-epoxybutane, respectively, in dry *N*,*N*-dimethylformamide (DMF). Based on ^1H NMR data (see Experimental Section) for product mixtures prior to chromatographic separation of the isomeric products, the ratio of yields for products **5a**/**8a** = 1.6, **5c**/**8c** = 1.2, **5d**/**8d** = 1.3, **5e**/**8e** = 0.72 indicating that changes in the structure of the alkylating agent over this series has a limited effect on the extents of reaction at the 7- relative to the 9-position. Compounds **5b** and **8b** were obtained by alkaline hydrolysis of compounds **5a** and **8a**, respectively.

The dose of the various compounds required to inhibit 50% of the AGT activity of cell-free extracts from human colon tumor cells HT29 and intact HT29 cells is shown in

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Table I. Depletion of Alkyltransferase in HT29 Cell-Free Extracts and in HT29 Cells

compound	ED_{50} (μM)[a] in cell-free extract	in cells
O^6-benzylguanine (1a)	0.2	0.05
O^6-(p-fluorobenzyl)guanine (1b)	0.2	0.05
O^6-(p-chlorobenzyl)guanine (1c)	0.2	0.08
O^6-(p-methylbenzyl)guanine (1d)	0.2	0.08
O^6-benzyl-2′-deoxyguanosine (2)	2	0.5
O^6-(p-methylbenzyl)guanosine (3c)	9	3
O^6-(p-chlorobenzyl)guanosine (3b)	10	5
O^6-benzylguanosine (3a)	11	2
2-amino-6-(benzyloxy)-9(cyanomethyl)purine (5c)	13	0.8
2-amino-6-(benzyloxy)-9-(2-hydroxybutyl)purine (5e)	13	2
O^6-allylguanine (4)	20	4
2-amino-6-(benzyloxy)-9-[(ethoxycarbonyl)methyl]purine (5a)	30	3
2-amino-6-(benzyloxy)-9-(carbamoylmethyl)purine (5d)	47	7.5
O^6-benzylhypoxanthine (6)	85	36
2-amino-6-(benzyloxy)-9-(carboxymethyl)purine, sodium salt (5b)	157	inactive[b]
O^6-methylguanine (7)	350	120
2-amino-6-(benzyloxy)-7-(carboxymethyl)purine, sodium salt (8b)	inactive[b]	inactive
2-amino-6-(benzyloxy)-7-[(ethoxycarbonyl)methyl]purine (8a)	inactive	not tested
2-amino-6-(benzyloxy)-7-(cyanomethyl)purine (8c)	inactive	not tested
2-amino-6-(benzyloxy)-7-(carbamoylmethyl)purine (8d)	inactive	not tested
2-amino-6-(benzyloxy)-7-(2-hydroxybutyl)purine (8e)	inactive	not tested
2-amino-6-(benzylthio)purine (9a)	inactive	not tested
2-amino-6-[(p-methylbenzyl)thio]-purine (9b)	inactive	not tested
2-amino-6-[(p-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (10)	inactive	not tested
7-benzylguanine (11)	inactive	not tested

[a] The numbers indicate the effective dose required to produce 50% inactivation in cell free extracts upon incubation for 30 min or in cells upon incubation for 4 h. Data were obtained from plots of % AGT activity remaining versus dose of test compound over a dose range of 0.05–400 μM where solubility permitted. [b] Compounds are described as inactive when there was no significant effect at the maximal concentration which could be used due to the compound's solubility.

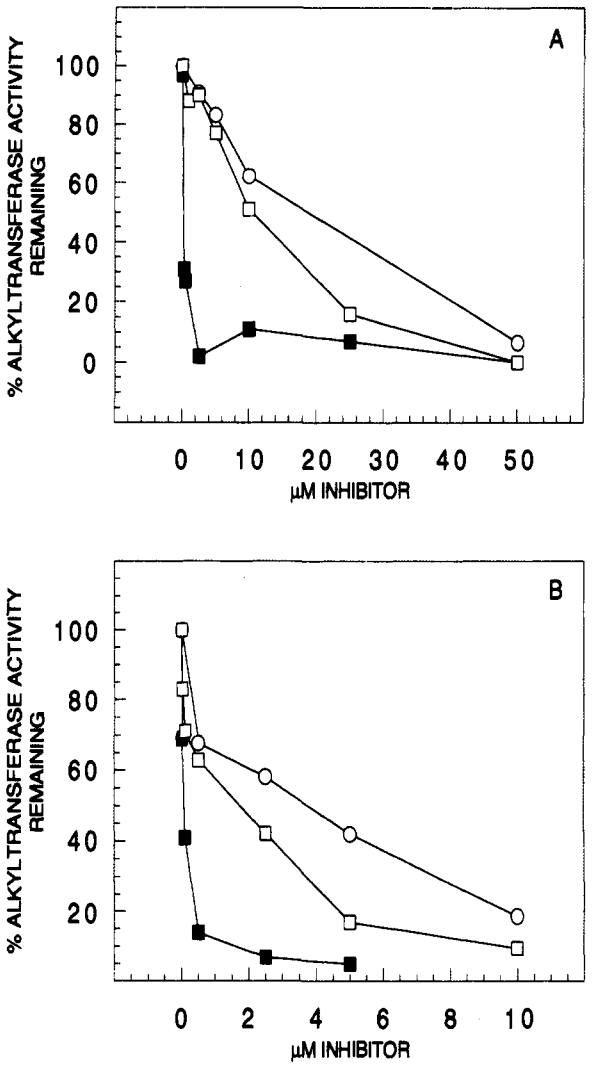

Figure 1. Loss of alkyltransferase activity in HT29 cell-free extracts (A) and HT29 cells (B). A: Alkyltransferase prepared from HT29 cells was incubated with increasing concentrations of O^6-(p-fluorobenzyl)guanine (closed square), O^6-benzylguanosine (open square), and O^6-allylguanine (open circle) for 30 min. B: HT29 cells in exponential growth were exposed to increasing concentrations of O^6-(p-fluorobenzyl)guanine (closed square), O^6-benzylguanosine (open square), and O^6-allylguanine (open circle) for 4 h. The cells were rinsed with PBS and extracts were prepared for alkyltransferase analysis. The results are expressed as the percentage of the alkyltransferase activity remaining after drug addition relative to zero drug addition.

Table I. Agents are arranged in descending order of effectiveness. These data were obtained from plots of the % AGT activity remaining as a function of dose of compound administered over a dose range of 0.05–400 μM (where the solubility of the test compound permitted). Data for cell free extract experiments were obtained after 30-min incubations while data for intact cell experiments were obtained after 4-h incubations. Representative plots for O^6-(p-fluorobenzyl)guanine (**1b**), O^6-benzylguanosine (**3a**), and O^6-allylguanine (**4**) are illustrated in Figure 1.

As indicated in Table I, the O^6-benzylated guanine bases **1a-d** are the most active compounds as AGT-depleting agents and all these derivatives exhibit similar activity. Changes in the electron-donating or electron-withdrawing properties of the para substituents on the benzyl group of these analogs has little effect on their relative efficiency of AGT depletion. The nucleosides, O^6-benzyl-2′-deoxyguanosine (**2**), and the three ribonucleosides **3a–c** comprise the group of next most active AGT-depleting compounds. Interestingly, the 2′-deoxyribonucleoside is somewhat more active than the three ribonucleosides which again exhibit similar activity as a function of para substituent on the benzyl group. The greater activity observed here for **2** relative to **3a–c** parallels observations that O^6-methylguanine residues in DNA are more readily repaired by AGT than are O^6-methylguanines in RNA.[11]

The 9-substituted O^6-benzylguanine derivatives **5c,e** and **5a,d** exhibit similar activities which are intermediate between those for the nucleosides and O^6-benzylhypoxanthine (**6**). These results together with those for the four nucleosides indicate that considerable structural diversity is tolerated at the 9-position of O^6-benzylguanine provided the group is not negatively charged. Introduction of an anionic carboxymethyl group at position 9, as in **5b**, reduces activity considerably.

Of the O^6-substituted 2-aminopurines tested without a 7- or 9-substituent, O^6-allylguanine (**4**) exhibited inter-

(11) Pegg, A. E.; Morimoto, K.; Dolan, M. E. Investigation of the specificity of O^6-alkylguanine-DNA-alkyltransferase. *Chem. Biol. Interact.* 1988, *65*, 275–281.

mediate activity. While significantly less active than O^6-benzylguanine, or **1b-d**, it is a far better substrate than O^6-methylguanine (**7**), which is the poorest of the active substrates tested. Thus, the AGT-depleting activity for these modified bases **1**, **4**, and **7** follows the order benzyl > allyl > methyl, which is the reactivity order typical of a number of bimolecular displacement (S_N2) reactions.[12]

The reduced activity associated with O^6-benzylhypoxanthine (**6**) indicates that the exocyclic 2-amino group is a positive contributor to the AGT-depleting properties of the O^6-benzylguanine derivatives. This may reflect a role for this group in hydrogen bonding in the active site of the AGT protein or it may enhance reaction with the protein by increasing the basicity of the benzylated purine.

Data for the 7-substituted compounds **8a–e** indicate that 7-substitution is not compatible with AGT depletion by these O^6-benzylated guanines regardless of the charge of the 7-substituent. Substitution at this position may sterically hinder reaction between the protein and the benzyl group attached to the O^6-position. Additionally, if the mechanism for these displacements involves protonation of the modified guanine at position 7 prior to reaction with the protein,[13,14] then a substituent group at position 7 would likely interfere with this protonation. The benzylated sulfur-containing bases **9a** and **9b**, the sulfur-containing nucleoside (**10**), and 7-benzylguanine (**11**) are also inactive as AGT depleters.

Since a range of substituent groups at position 9 of O^6-benzylguanine is tolerated, it may be possible, through attachment of different groups, to alter the biodistribution of AGT-depleting agents by changing their lipophilicity, by causing them to be recognized by receptors specific to certain tumor types, and/or by altering their cellular transport mechanisms. Exploiting these effects should make it possible to deplete AGT selectively in tumor cells in preference to normal cells or in particular tumor types.

Conclusions. For AGT-depleting activity, an allyl or benzyl group attached through exocyclic oxygen at carbon-6 of a 2-aminopurine derivative is most effective. Attachment of a benzyl residue to exocyclic sulfur at carbon-6 is not compatible with activity. A variety of substituent groups may be attached to the 9-position of the O^6-benzylated guanine while substitution at position 7 leads to a complete loss in activity.

Experimental Section

Materials and Methods. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian VXR 500S spectrometer equipped with Sun 4/110 data stations or a Varian XL 200 instrument interfaced to an Advanced data system. Samples were dissolved in DMSO-d_6 with Me_4Si as an internal standard. EI and positive ion (+ve) FAB mass spectra were obtained with a reversed geometry VG Micromass ZAB-2F spectrometer interfaced to a VG 2035 data system. A mixture of dithiothreitol and dithioerythritol (1:1) was used as FAB matrix. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Satisfactory analyses (±0.4% C, H, N) were obtained for new compounds as indicated below.

2-Amino-6-[(*p*-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (**10**) and other reagents and solvents were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Compounds **1a**,[9,10] **4**,[10] and **6**[9] were obtained as described. Compounds **1c** and **1d** were prepared by the method of Dolan et al.[5] O^6-Benzyl-2′-deoxyguanosine (**2**) and the ribonucleosides **3a–c** were obtained by the method of Pauly et al.[15] and Moschel et al.,[13] respectively. O^6-Methylguanine (**7**) was prepared by the method of Balsiger and Montgomery,[16] and the benzylated thiopurines **9a,b** were prepared essentially as described by Elion et al.[17] 7-Benzylguanine (**11**) was prepared by the method of Brookes et al.[18]

AGT-Depletion Experiments. Stock solutions of O^6-substituted guanine derivatives were prepared at a concentration of 100 mM in dimethyl sulfoxide. Crude extracts from HT29 cells were prepared as described[19] and were incubated for 30 min with concentrations between 0 and 400 μM O^6-substituted guanine derivative in buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol. For cell exposure experiments, cells were plated at a density of 5×10^6 cells/T75 flask and were allowed to grow for 3 days, at which time medium was replaced with medium containing increasing concentrations of O^6-substituted guanine derivative. After 4 h, cells were harvested and frozen at -80 °C until analysis for AGT. Alkyltransferase activity remaining was determined by measuring loss of O^6-[^{3}H]methylguanine from a [^{3}H]methylated DNA substrate, prepared by reacting [^{3}H]methylnitrosourea (21.5 Ci/mmol) with calf thymus DNA as described previously.[19] The data for Table I were obtained from plots of % AGT activity remaining versus dose of test compound and are the average of at least duplicate determinations.

O^6-(*p*-Fluorobenzyl)guanine (1b). Sodium (1.0 g) was slowly dissolved in 36 mL of *p*-fluorobenzyl alcohol at 75–80 °C. 2-Amino-6-chloropurine (3.0 g) was added and the thick suspension was stirred at 100 °C for 24 h and at 130 °C for 5 h. The suspension was then cooled to near room temperature and poured into 500 mL of diethyl ether with vigorous stirring. The precipitate was collected and suspended in 1750 mL of H_2O, and glacial acetic acid was added to adjust the pH of the suspension to approximately pH 6. The suspension was heated to boiling, treated with charcoal, and filtered. On cooling, 1.0 g (22%) of O^6-(*p*-fluorobenzyl)guanine (**1b**) was recovered as crystalline flakes: UV (H_2O) λ_{max} 241, 282 nm; ^1H NMR δ 5.47 (s, 2 H, $BzlCH_2$), 6.31 (s, 2 H, NH_2, exchange with D_2O), 7.22 (m, 2 H, 2 *o*-ArH), 7.57 (m, 2 H, 2 *m*-ArH), 7.81 (s, 1 H, H-8), 12.43 (br s, 1 H, NH, exchanges with D_2O); +ve FAB MS *m/z* 260 [$C_{12}H_{10}N_5OF$ + H]$^+$.

2-Amino-6-(benzyloxy)-7-[(ethoxycarbonyl)methyl]purine Hemihydrate (8a) and 2-Amino-6-(benzyloxy)-9-[(ethoxycarbonyl)methyl]purine (5a). To 0.24 g (1 mmol) of O^6-benzylguanine under argon was added 1 mL of a 1 M solution of sodium ethoxide in ethanol. The solid dissolved within 1 min with stirring. The mixture was stirred for an additional 10 min. The ethanol was removed under vacuum. The resulting solid was redissolved in 2 mL of dry DMF and the solution was cooled to 0 °C on ice. Ethyl bromoacetate (0.11 mL, 1 mmol) was added dropwise by syringe to the stirring solution and the reaction was allowed to proceed for 30 min. The ice bath was then removed and the DMF was removed under vacuum. Separation of the two isomers was achieved by treating the resulting solid with a

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minimum volume of 5% ethanol in $CHCl_3$ and loading the solution on a 2.5 × 17 cm silica gel column (Davisil grade 633, 200-425 mesh, 60 Å). The 9-substituted isomer was eluted from the column with 5% ethanol in $CHCl_3$, while the 7-substituted isomer was subsequently eluted with 15% ethanol in $CHCl_3$. The recovered solids were precipitated from CH_2Cl_2 with hexane.

2-Amino-6-(benzyloxy)-7-[(ethoxycarbonyl)methyl]purine hemihydrate (8a): yield, 87 mg (26%); mp 175–177°C; UV (pH 1) λ_{max} 240 nm (sh) (ϵ = 0.707 × 10^4), 290 (1.203 × 10^4), (pH 6.9) 240 (sh) (0.763 × 10^4), 294 (0.755 × 10^4), (pH 13) 242 (sh) (0.777 × 10^4), 292 (0.809 × 10^4); ^{1}H NMR δ 1.04 (t, 3 H, CH_3, J = 7.1 Hz), 3.97 (q, 2 H, CH_3CH_2, J = 7.1 Hz), 5.08 (s, 2 H, CH_2N<), 5.42 (s, 2 H, $BzlCH_2$), 6.21 (s, 2 H, NH_2, exchange with D_2O), 7.38 (m, 1 H, *p*-ArH), 7.39 (m, 2 H, *m*-ArH), 7.44 (m, 2 H, *o*-ArH), 8.05 (s, 1 H, H-8); ^{13}C NMR δ 13.77 (CH_3), 47.59 (CH_2N<), 61.09 (CH_2O_2C), 66.94 ($BzlCH_2$), 106.07 (C-5), 127.80 (2 *o*-Ar), 127.95 (*p*-Ar), 128.32 (2 *m*-Ar), 136.22 (ipso-Ar), 145.91 (C-8), 156.47 (C-6), 159.74 (C-2), 163.86 (C-4), 168.11 (CO_2); MS (EI) calcd m/z for $C_{16}H_{17}N_5O_3$ 328.1409, found 328.1428. Anal. ($C_{16}H_{17}N_5O_3 \cdot {}^1/_2H_2O$) C, H, N.

2-Amino-6-(benzyloxy)-9-[(ethoxycarbonyl)methyl]purine (5a): yield, 138 mg (42%); mp 163–164 °C; UV (pH 1) λ_{max} 244 nm (ϵ = 0.740 × 10^4), 290 (0.951 × 10^4), (pH 6.9) 248 (0.953 × 10^4), 282 (1.045 × 10^4), (pH 13) 251 (0.830 × 10^4), 282 (1.024 × 10^4), ^{1}H NMR δ 1.21 (t, 3 H, CH_3CH_2, J = 7.1 Hz), 4.16 (q, 2 H, CH_3CH_2, J = 7.1 Hz), 4.93 (s, 2 H, CH_2N<), 5.50 (s, 2 H, $BzlCH_2$), 6.51 (s, 2, NH_2, exchange with D_2O), 7.35 (m, 1 H, *p*-ArH), 7.40 (m, 2 H, *m*-ArH), 7.51 (m, 2 H, *o*-ArH), 7.84 (s, 1 H, H-8); ^{13}C NMR δ 13.96 (CH_3), 43.68 (CH_2N<) 61.26 (CH_2O_2C), 66.86 ($BzlCH_2$), 113.17 (C-5), 128.00 (*p*-Ar), 128.36 (2 *m*-Ar), 128.41 (2 *o*-Ar), 136.60 (ipso-Ar), 140.12 (C-8), 154.54 (C-4), 159.80 (C-2), 160.01 (C-6), 167.93 (CO_2); MS (EI) calcd m/z for $C_{16}H_{17}N_5O_3$ 328.1409, found 328.1400. Anal. ($C_{16}H_{17}N_5O_3$) C, H, N.

2-Amino-6-(benzyloxy)-7-(carboxymethyl)purine, Sodium Salt (8b). A suspension of 0.12 g of 2-amino-6-(benzyloxy)-7-[(ethoxycarbonyl)methyl]purine in 5 mL of H_2O containing 0.015 g of NaOH was stirred and warmed until all suspended solid dissolved. The solution was then evaporated to dryness and the resulting solid was triturated with ethanol and dried under vacuum to afford 2-amino-6-(benzyloxy)-7-(carboxymethyl)-purine, sodium salt (8b): UV (H_2O) λ_{max} 240 nm (sh), 289 nm; ^{1}H NMR (DMSO-d_6/D_2O) δ 4.79 (s, 2 H, CH_2N<), 5.54 (s, 2 H, $BzlCH_2$), 7.34–7.61 (m, 5 H, ArH), 7.98 (s, 1 H, H-8); +ve FAB MS m/z 322 [$C_{14}H_{12}N_5O_3Na$ + H]$^+$.

2-Amino-6-(benzyloxy)-9-(carboxymethyl)purine, Sodium Salt (5b). A suspension of 2-amino-6-(benzyloxy)-9-[(ethoxycarbonyl)methyl]purine was treated as described above for the 7-substituted isomer to afford 2-amino-6-(benzyloxy)-9-(carboxymethyl)purine, sodium salt (5b): UV (H_2O) λ_{max} 250, 281 nm; ^{1}H NMR (DMSO-d_6/D_2O) δ 4.52 (s, 2 H, CH_2N<), 5.59 (s, 2 H, $BzlCH_2$), 7.36–7.59 (m, 5 H, ArH), 7.81 (s, 1 H, H-8); +ve FAB MS m/z 322 [$C_{14}H_{12}N_5O_3Na$ + H]$^+$.

2-Amino-6-(benzyloxy)-7-(carbamoylmethyl)purine Hemihydrate (8d) and 2-Amino-6-(benzyloxy)-9-(carbamoylmethyl)purine (5d). To 0.5 g (2.1 mmol) of O^6-benzylguanine under argon was added 2.2 mL of a 1 M solution of sodium ethoxide in ethanol. The solid dissolved within 1 min. After an additional 10 min of stirring, the ethanol was removed under vacuum. The remaining solid was dissolved in 10 mL of dry dioxane and the solution was kept at 20 °C in a water bath. 2-Bromoacetamide (0.286 g, 2.2 mmol) was added dropwise to the stirring solution. After 1 h the dioxane was removed under vacuum. Separation of the two isomers was achieved by dissolving the resulting solid in 50% aqueous methanol (25 mL/100 mg) and loading this on a 3 × 80 cm Sephadex LH-20 column eluted with 50% aqueous methanol at 1 mL/min. Column eluent was continuously monitored at 280 nm, and fractions (10 mL) were collected. The 7-substituted isomer eluted from the column in fractions 45–57, while the 9-substituted isomer eluted in fractions 65–80. Solid material was recovered after removal of solvent from the respective pooled fractions. Both compounds were further purified by recrystallization from CH_3CN/H_2O through slow evaporation of the solvent.

2-Amino-6-(benzyloxy)-7-(carbamoylmethyl)purine hemihydrate (8d): yield, 256 mg (40%); mp 227–228°C dec; UV (pH 1) λ_{max} 240 nm (sh) (ϵ = 0.604 × 10^4), 290 (1.144 × 10^4); (pH 6.9) 241 (sh) (0.820 × 10^4), 293 (0.848 × 10^4), (pH 13) 241 (sh) (0.713 × 10^4), 293 (0.762 × 10^4); ^{1}H NMR δ 4.86 (s, 2 H, CH_2N<), 5.46 (s, 2 H, $BzlCH_2$), 6.12 (s, 2 H, NH_2, exchange with D_2O), 7.26 (bs, 1 H, H_aNHCO), 7.32 (m, 1 H, *p*-ArH), 7.38 (m, 2 H, 2 *m*-ArH), 7.47 (m, 2 H, 2 *o*-ArH), 7.60 (bs, 1 H, H_bNHCO), 8.01 (s, 1 H, H-8); ^{13}C NMR δ 48.49 (CH_2N<), 66.59 ($BzlCH_2$), 106.48 (C-5), 127.46 (2 *o*-Ar), 127.69 (*p*-Ar), 128.31 (2 *m*-Ar), 136.49 (ipso-Ar), 146.50 (C-8), 156.42 (C-6), 159.44 (C-2), 163.81 (C-4), 168.58 ($CONH_2$); MS (EI) calcd m/z for $C_{14}H_{14}N_6O_2$ 298.1178, found 298.1169. Anal. ($C_{14}H_{14}N_6O_2 \cdot {}^1/_2H_2O$) C, H, N.

2-Amino-6-(benzyloxy)-9-(carbamoylmethyl)purine (5d): yield, 239 mg (38%); mp 243–244.5 °C dec; UV (pH 1) λ_{max} 243 nm (ϵ = 0.762 × 10^4), 292 (0.994 × 10^4); (pH 6.9) 249 (0.937 × 10^4), 282 (1.060 × 10^4), (pH 13) 249 (0.905 × 10^4), 282 (1.023 × 10^4); ^{1}H NMR δ 4.67 (s, 1 H, CH_2N<), 5.50 (s, 1 H, $BzlCH_2$), 6.43 (s, 2 H, NH_2), 7.26 (bs, 1 H, H_aNHCO), 7.35 (m, 1 H, *p*-ArH), 7.40 (m, 2 H, 2 *m*-ArH), 7.50 (m, 2 H, 2 *o*-ArH), 7.63 (bs, 1 H, H_bNHCO), 7.78 (s, 1 H, H-8); ^{13}C NMR δ 44.59 (CH_2N<), 66.75 ($BzlCH_2$), 113.28 (C-5), 127.96 (*p*-Ar), 128.34 (2 *m*-Ar), 128.36 (2 *o*-Ar), 136.72 (ipso-Ar), 140.79 (C-8), 154.70 (C-4), 159.63 (C-2), 159.93 (C-6), 168.30 ($CONH_2$); MS (EI) calcd m/z for $C_{14}H_{14}N_6O_2$ 298.1178, found 298.1181. Anal. ($C_{14}H_{14}N_6O_2$) C, H, N.

2-Amino-6-(benzyloxy)-7-(2-hydroxybutyl)purine (8e) and 2-Amino-6-(benzyloxy)-9-(2-hydroxybutyl)purine (5e). To 0.24 g (1 mmol) of O^6-benzylguanine in 3 mL of ethanol were added 0.14 g of potassium carbonate (1 mmol) and 0.85 mL (10 mmol) of 1,2-epoxybutane. The reaction mixture was heated to reflux with stirring for 1.5 h. The solution was filtered, and solvent and excess epoxide were removed under vacuum. The isomeric 7- and 9-substituted derivatives were separated by treating the solid residue with a minimum volume of 7% ethanol in $CHCl_3$ and loading the soluble material on a 2.5 × 17 cm silica gel column (Davisil grade 633, 200–425 mesh, 60 Å). The 9-substituted isomer eluted from the column with 7% ethanol in $CHCl_3$, while the 7-substituted isomer was subsequently eluted with 15% ethanol in $CHCl_3$. The 9-substituted isomer was further purified by precipitation from CH_2Cl_2 with hexane.

2-Amino-6-(benzyloxy)-7-(2-hydroxybutyl)purine (8e): yield, 64 mg (20%); mp 200–202 °C; UV (pH 1) λ_{max} 240 nm (sh) (ϵ = 0.707 × 10^4), 290 (1.138 × 10^4), (pH 6.9) 241 (sh) (0.837 × 10^4), 292 (0.845 × 10^4), (pH 13) 242 (sh) (0.682 × 10^4), 292 (0.707 × 10^4); ^{1}H NMR δ 0.74 (t, 3 H, CH_3, J = 7.5 Hz), 1.25 (m, 2 H, CH_2CH_3), 3.58 (m, 1 H, CHOH), 3.90 (dd, 1 H, H_aCH<N, ^2J = 13.8 Hz, ^3J = 8.8 Hz), 4.14 (dd, 1 H, H_bCH<N, ^2J = 13.8 Hz, ^3J = 3.1 Hz), 4.84 (d, 1 H, CHO*H*, J = 5.8 Hz), 5.40 (d, 1 H, $BzlH_aCH$, ^2J = 12.2 Hz), 5.53 (d, 1 H, $BzlH_bCH$, ^2J = 12.2 Hz), 6.12 (s, 2 H, NH_2), 7.34–7.42 (m, 3 H, *p*,*m*-ArH), 7.51 (m, 2 H, 2 *o*-ArH), 7.97 (s, 1 H, H-8); ^{13}C NMR δ 9.44 (CH_3), 27.18 (CH_2CH_3), 52.42 (CH_2N<), 66.91 ($BzlCH_2$), 70.28 (CHOH), 105.73 (C-5), 127.93 (2 *o*-Ar), 128.10 (*p*-Ar), 128.24 (2 *m*-Ar), 136.29 (ipso-Ar), 146.11 (s, C-8), 156.24 (C-6), 159.25 (C-2), 163.94 (C-4); MS (EI) calcd m/z for $C_{16}H_{19}N_5O_2$ 313.1538, found 313.1538. Anal. ($C_{16}H_{19}N_5O_2$) C, H, N.

2-Amino-6-(benzyloxy)-9-(2-hydroxybutyl)purine (5e): yield, 44 mg (14%); mp 178–180 °C; UV (pH 1) λ_{max} 243 nm (ϵ = 0.774 × 10^4), 291 (0.998 × 10^4), (pH 6.9) 250 (0.769 × 10^4), 282 (0.969 × 10^4), (pH 13) 250 (0.760 × 10^4), 282 (0.954 × 10^4); ^{1}H NMR δ 0.90 (t, 3 H, CH_3, J = 7.3 Hz), 1.29 (m, 1 H, CH_aHCH_3, ^3J = 7.4 Hz, ^3J = 7.4 Hz, ^2J = 13.8 Hz), 1.39 (m, 1 H, CH_bHCH_3, ^3J = 4.2 Hz, ^3J = 7.4 Hz, ^2J = 13.8 Hz), 3.72 (m, 1 H, CHOH), 3.88 (dd, 1 H, CH_aHN<, ^3J = 7.9 Hz, ^2J = 13.8 Hz), 4.00 (dd, 1 H, CH_bHN<, ^3J = 3.9 Hz, ^2J = 13.9 Hz), 4.98 (bs, 1 H, CHO*H*), 5.49 (d, 1 H, $BzlCH_aH$, ^2J = 12.3 Hz), 5.50 (d, 1 H, $BzlCH_bH$, ^2J = 12.3 Hz), 6.42 (s, 2 H, NH_2), 7.35 (t, 1 H, *p*-ArH, J = 7.3 Hz), 7.40 (t, 2 H, *m*-ArH, J = 7.4 Hz), 7.50 (d, 2 H, *o*-ArH, J = 7.0 Hz), 7.79 (s, 1 H, H-8); ^{13}C NMR δ 9.74 (CH_3), 27.27 (CH_2CH_3), 48.58 (CH_2N<), 66.78 ($BzlCH_2$), 69.44 (CHOH), 113.19 (C-5), 127.98 (*p*-Ar), 128.36 (2 *m*-Ar), 128.41 (2 *o*-Ar), 136.68 (ipso-Ar), 140.58 (C-8), 154.47 (C-4), 159.56 (C-2), 159.85 (C-6); MS (EI) calcd m/z for $C_{16}H_{19}N_5O_2$ 313.1538, found 313.1516. Anal. ($C_{16}H_{19}N_5O_2$) C, H, N.

2-Amino-6-(benzyloxy)-7-(cyanomethyl)purine (8c) and 2-Amino-6-(benzyloxy)-9-(cyanomethyl)purine (5c). To 0.24 g (1 mmol) of O^6-benzylguanine under argon was added 1 mL of a 1 M solution of sodium ethoxide in ethanol. The solid dissolved within 1 min with stirring. The reaction mixture was stirred for

an additional 10 min and the ethanol was removed under vacuum. The remaining solid was dissolved in 2 mL of dry DMF and the solution cooled to 0 °C on ice. Bromoacetonitrile (0.07 mL, 1 mmol) was added dropwise to the stirring solution by syringe and this mixture was allowed to react for 30 min. At this point, the ice bath was removed and the DMF was removed under vacuum. The 7- and 9-substituted isomers were separated by treating the solid residue with a minimum volume of 5% ethanol in $CHCl_3$ and loading the soluble material on a 4 × 38 cm silica gel column (Davisil grade 633, 200–425 mesh, 60 Å). The 9-substituted isomer eluted from the column with 15% ethanol in $CHCl_3$, while the 7-substituted isomer was subsequently eluted with 20% ethanol in $CHCl_3$. Both compounds were precipitated from CH_2Cl_2 with hexane.

2-Amino-6-(benzyloxy)-7-cyanomethylpurine (8c): yield, 98 mg (35%); mp 188–189 °C; UV (pH 1) λ_{max} 239 nm (sh) (ϵ = 0.818 × 10^4), 290 (1.228 × 10^4), (pH 6.9) 240 (sh) (0.778 × 10^4), 294 (0.745 × 10^4), (pH 13) 242 (sh) (0.695 × 10^4), 294 (0.729 × 10^4), ^{1}H NMR δ 5.43 (s, 2 H, $CH_2N<$), 5.52 (s, 2 H, $BzlCH_2$), 6.33 (s, 2 H, NH_2), 7.33 (m, 1 H, *p*-ArH), 7.39 (m, 2 H, 2 *m*-ArH), 7.56 (m, 2 H, 2 *o*-ArH), 8.17 (s, 1 H, H-8); ^{13}C NMR δ 34.71 ($CH_2N<$), 67.19 ($BzlCH_2$), 105.07 (C-5), 115.94 (CN), 127.91 (2 *o*-Ar), 127.93 (*p*-Ar), 128.34 (2 *m*-Ar), 136.20 (ipso-Ar), 145.57 (C-8), 156.44 (C-6), 160.11 (C-2), 164.10 (C-4); MS (EI) calcd *m/z* for $C_{14}H_{12}N_6O$ 280.1072, found: 280.1088. Anal. ($C_{14}H_{12}N_6O$) C, H, N.

2-Amino-6-(benzyloxy)-9-(cyanomethyl)purine (5c): yield, 114 mg (41%); mp 191 °C dec; UV (pH 1) λ_{max} 246 nm (ϵ = 0.802 × 10^4), 290 (0.941 × 10^4), (pH 6.9) 249 (1.127 × 10^4), 282 (1.122 × 10^4), (pH 13) 249 (0.970 × 10^4), 282 (1.062 × 10^4); ^{1}H NMR δ 5.28 (s, 2 H, $CH_2N<$), 5.51 (s, 2 H, $BzlCH_2$), 6.69 (s, 2 H, NH_2), 7.35 (m, 1 H, *p*-ArH), 7.40 (m, 2 H, 2 *m*-ArH), 7.50 (m, 2 H, 2 *o*-ArH), 7.93 (s, 1 H, H-8); ^{13}C NMR δ 30.75 ($CH_2N<$), 67.00 ($BzlCH_2$), 113.20 (C-5), 115.73 (CN), 128.05 (*p*-Ar), 128.38 (2 *m*-Ar), 128.43 (2 *o*-Ar), 136.47 (ipso-Ar), 138.82 (C-8), 153.92 (C-4), 160.08 (C-2), 160.18 (C-6); MS (EI) calcd *m/z* for $C_{14}H_{12}N_6O$ 280.1072, found 280.1091. Anal. ($C_{14}H_{12}N_6O$) C, H, N.

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