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Demethylation of O^6-Methylguanine in DNA by O^6-Methylguanine-DNA Methyltransferase: Synthesis and Characterization of O^6,9-Dimethylguanine Coordinated with *cis*-Platin at the N7-Position

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In order to investigate the chemical characteristics of O^6-methylguanines, the substrate of O^6-methylguanine-DNA methyltransferase, *cis*-[PtCl(NH$_3$)$_2$(O^6,9-dimethylguanine-7)]NO$_3$, a 1:1 complex of *cis*-platin with O^6,9-dimethylguanine, was synthesized and its demethylation rate, pK_a, X-ray structure and cytotoxicity were determined.

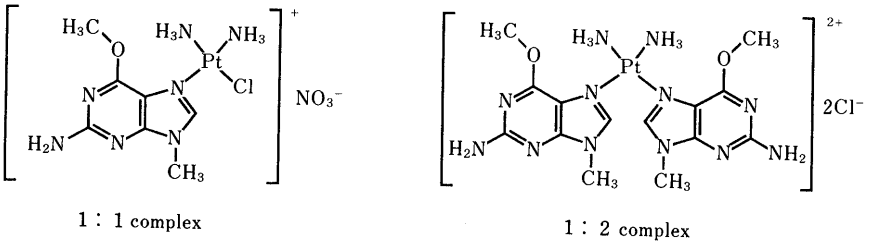
Keywords O^6-methylguanine; O^6-methylguanine-DNA methyltransferase; *cis*-platin complex; demethylation; X-ray analysis

Exposure of organisms to alkylating carcinogens such as *N*-methyl-*N*-nitrosourea or *N*,*N*-dimethylnitrosamine results in the formation of many types of alkylated bases in DNA.[1] Among these bases, O^6-alkylguanine is considered to be the lesion most responsible for induction of mutation and/or cancer.[2] Cells contain an enzyme, O^6-methylguanine-DNA methyltransferase (MGMT), that is able to repair this lesion. This enzyme is present in a variety of organisms from bacteria to human cells, and has been studied extensively.[3,4] The repair of O^6-alkylguanine in DNA is achieved by alkyl transfer from the *O*-alkyl group to the SH group of a cysteine (Cys) residue that is located at the active center of MGMT. However, the chemical characteristics of this repair mechanism remain unclear. We previously reported the demethylation rates of O^6-methylguanine (6-MeG) derivatives using thiophenolate as a model alkyl acceptor of MGMT and demonstrated that the *O*-methyl group can be transferred faster when it becomes more electron-deficient.[5] The amino acid sequence of Pro–Cys–His is conserved at the active center of MGMT for all organisms[6] and the imidazole moiety of His is thought to play an important role in accelerating the repair rate by forming hydrogen bonds with both O^6-alkylguanine and the SH group of Cys.[7] These interactions render O^6-alkylguanine more electron deficient and the SH group more reactive as an alkyl acceptor. It is known that N7 is the basic site of O^6-methylguanosine and is the coordination site for platinum. Therefore, *cis*-[Pt(NH$_3$)$_2$(O^6,9-dimethylguanine-7)$_2$]Cl$_2$, a 1:2 complex of *cis*-diamminedichloroplatinum(II) (*cis*-platin) with O^6,9-dimethylguanine (O^6,9-diMeG) (Fig. 1), was previously synthesized to provide a functional model of the imidazole ring assisted MGMT complex with 6-MeG in DNA. It was demonstrated that the demethylation of this complex proceeds much faster than that of O^6,9-diMeG in the chemical dealkylation system.[7] Hollis *et al.* reported that *cis*-platin derivatives in which one chloro group is replaced with a ligand still show cytotoxicity.[8] Then, in this study, *cis*-[PtCl(NH$_3$)$_2$(O^6,9-dimethylguanine-7)]NO$_3$, a 1:1 complex of *cis*-platin with O^6,9-diMeG (Fig. 1), was synthesized and its chemical characteristics and cytotoxicity were examined.

Materials and Methods

1:1 Complex of *cis*-Platin with O^6,9-Dimethylguanine (*cis*-Pt–O^6,9-diMeG 1:1 Complex) *cis*-Platin (309 mg, 1.03 mmol, Sigma Chemical Co., St. Louis, MO) and AgNO$_3$ (1.75 mg, 1.03 mmol) in 7 ml of *N*,*N*-dimethylformamide were stirred for 1 d at room temperature. The precipitated AgCl was removed by filtration, then O^6,9-diMeG (185 mg, 1.03 mmol) was added to the filtrate and the mixture was stirred for 1 d under the same conditions. After the solvent was evaporated, the dry residue was washed with CH_2Cl_2 and filtered. Recrystallization of the product from H_2O gave pale yellow plates in a yield of 233 mg (44.5%). Elemental analysis was performed after further recrystallization of the product from 0.1 N HCl. mp >300 °C. *Anal.* Calcd for [PtCl(NH$_3$)$_2$-(O^6,9-dimethylguanine-7)]NO$_3$ or $C_7H_{15}ClN_8O_4Pt$: C, 16.62; H, 2.99; N, 22.15. Found: C, 16.31; H, 3.18; N, 21.97. ^{1}H-NMR (270 MHz, Me$_2$SO-d_6) δ: 3.70 (s, 3H, N–CH$_3$), 4.07 (s, 3H, O–CH$_3$), 4.28 (br s, 3H, Pt–NH$_3$), 4.31 (br s, 3H, Pt–NH$_3$), 6.83 (br s, 2H, NH$_2$), 8.41 (s, 1H, 8-H). UV λ_{max} nm (ε): pH 0, 284 (9300); pH 2, 7 and 12, 286 (6700). pK_a 0.8. Recrystallization of the product from 1 N HCl yielded white needles. *Anal.* Calcd for [PtCl(NH$_3$)$_2$(O^6,9-dimethylguanine-7)]Cl·2.5H$_2$O or $C_7H_{15}Cl_2N_7OPt \cdot 2.5H_2O$: C, 16.04; H, 3.85; N, 18.70. Found: C, 16.06; H, 3.67; N, 18.84.

Demethylation Rate A mixture of *cis*-Pt–O^6,9-diMeG 1:1 complex



1 : 1 complex

1 : 2 complex

Fig. 1. Structures of the *cis*-Platin Complex with O^6,9-diMeG

(7.6 mg, 1.50×10^{-2} mmol) and thiophenol (180 μl, 1.75 mmol) in 3 ml of MeOH was incubated at 60 °C for an appropriate time. An aliquot of the mixture was then taken and the products formed were analyzed by HPLC, as previously reported.[7] Quantification of the products was carried out using the ε values of authentic materials at 260 nm at pH 7. Retention times and ε values of the products were as follows: *cis*-Pt-O^6,9-diMeG 1 : 1 complex (6.2 min, 3100), O^6,9-diMeG (16.6 min, 4000), 9-MeG (5.8 min, 11300), thioanisole (69.9 min, 6400).

X-Ray Structure A crystal with the dimensions $0.3 \times 0.1 \times 0.03$ mm^3 of *cis*-Pt-O^6,9-diMeG 1 : 1 complex recrystallized from 1 N HCl was used for the X-ray study. The crystal data are as follows: chemical formula, $(C_7H_{15}ClN_7OPt)^+Cl^- \cdot 3H_2O$; $M = 533.27$; triclinic; space group $P\bar{1}$, $a = 12.693(2)$, $b = 10.069(2)$, $c = 6.552(1)$ Å, $\alpha = 102.81(1)$, $\beta = 86.68(1)$, $\gamma = 93.32(1)°$; $V = 814.3(2)$ Å^3; $Z = 2$; $D_x = 2.17$ mg m^{-3}; $\mu(\mathrm{Mo}K_\alpha) = 9.1$ mm^{-1}. Intensity data were collected on a Rigaku AFC-4 with graphite-monochromated MoK_α radiation using the ω–2θ scan mode ($2\theta_{max} = 60°$) at 293 K. Of the 5130 reflections measured and corrected for absorption (1.0—0.743),[9] 4747 were unique ($R_{sym.} = 0.023$) and 3739 with $I > 3\sigma(I)$ were used for all calculations. The structure was solved by direct methods[10] and refined by the full matrix least-squares procedure.[11] All H atoms except those of water molecules were located on difference Fourier maps. The final refinement including fixed H atoms reduced the R value to 0.033 [$R_w = 0.035$, $w = 0.6243/\sigma^2(F_O)$]. The final atomic parameters of non-H atoms are listed in Table I. All numerical calculations were carried out on an ACOS S930 computer at the Protein Engineering Research Center, Institute for Protein Research, Osaka University.

TABLE I. Fractional Coordinates of Non-hydrogen Atoms and Equivalent Isotropic Temperature Factors with e.s.d.'s in Parentheses

Atom	x	y	z	B_{eq} (Å^2)
Pt1	0.26299 (2)	0.57068 (3)	0.75680 (4)	2.067 (5)
Cl1	0.33849 (14)	0.5013 (2)	1.0254 (3)	3.35 (5)
Cl2	−0.05787 (16)	0.6543 (2)	0.7548 (3)	4.48 (6)
O6	0.2233 (3)	0.9047 (4)	0.8310 (8)	2.95 (12)
OW1	0.2505 (6)	0.8795 (9)	0.3359 (12)	8.5 (3)
OW2	0.0850 (8)	0.1217 (10)	0.4882 (15)	10.0 (4)
OW3	0.9363 (7)	0.2357 (11)	0.8226 (13)	9.5 (4)
N1	0.3568 (4)	1.0673 (5)	0.8117 (8)	2.43 (13)
N2	0.4893 (5)	1.2306 (5)	0.7945 (9)	2.99 (15)
N3	0.5391 (4)	1.0076 (5)	0.7388 (8)	2.16 (12)
N7	0.3899 (4)	0.6947 (5)	0.7291 (8)	2.13 (12)
N9	0.5594 (4)	0.7642 (5)	0.6902 (8)	2.06 (12)
N1NH	0.1927 (5)	0.6278 (6)	0.5201 (9)	3.2 (2)
N2NH	0.1326 (4)	0.4457 (6)	0.7885 (10)	3.2 (2)
C2	0.4618 (5)	1.0965 (6)	0.7809 (9)	2.37 (15)
C4	0.5011 (4)	0.8793 (6)	0.7255 (8)	1.89 (13)
C5	0.3968 (4)	0.8362 (6)	0.7495 (9)	1.95 (13)
C6	0.3240 (5)	0.9385 (6)	0.7968 (9)	2.21 (14)
C8	0.4897 (5)	0.6575 (6)	0.6941 (9)	2.30 (15)
C9	0.6737 (5)	0.7582 (7)	0.6496 (12)	3.1 (2)
C10	0.1487 (6)	1.0122 (9)	0.8848 (16)	4.9 (3)

Results and Discussion

Demethylation Rate Treatment of *cis*-Pt-O^6,9-diMeG 1 : 1 complex with thiophenol resulted in a rapid disappearance of the complex, and after 3 h incubation, it could no longer be detected (Fig. 2). The complex was

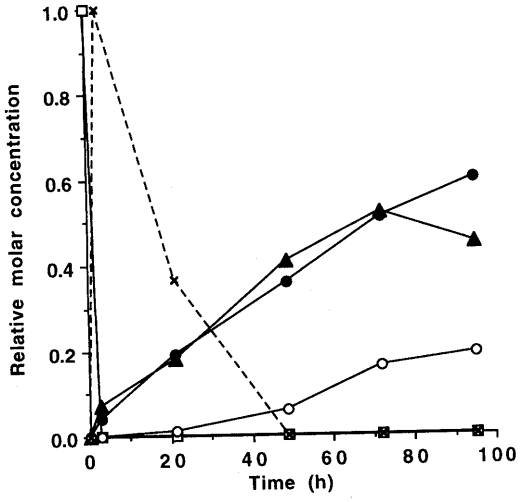


Fig. 2. Kinetics of Formation of 9-MeG and Other Products from the *cis*-Pt-O^6,9-diMeG 1 : 1 Complex

The peak height of the intermediate that appeared after 3 h incubation was arbitrarily taken as unity and changes in the relative molarities of the intermediate and products were plotted. *cis*-Pt-O^6,9-diMeG 1 : 1 complex (□), an intermediate (×), 9-MeG (○), O^6, 9-diMeG (▲), thioanisole (●).

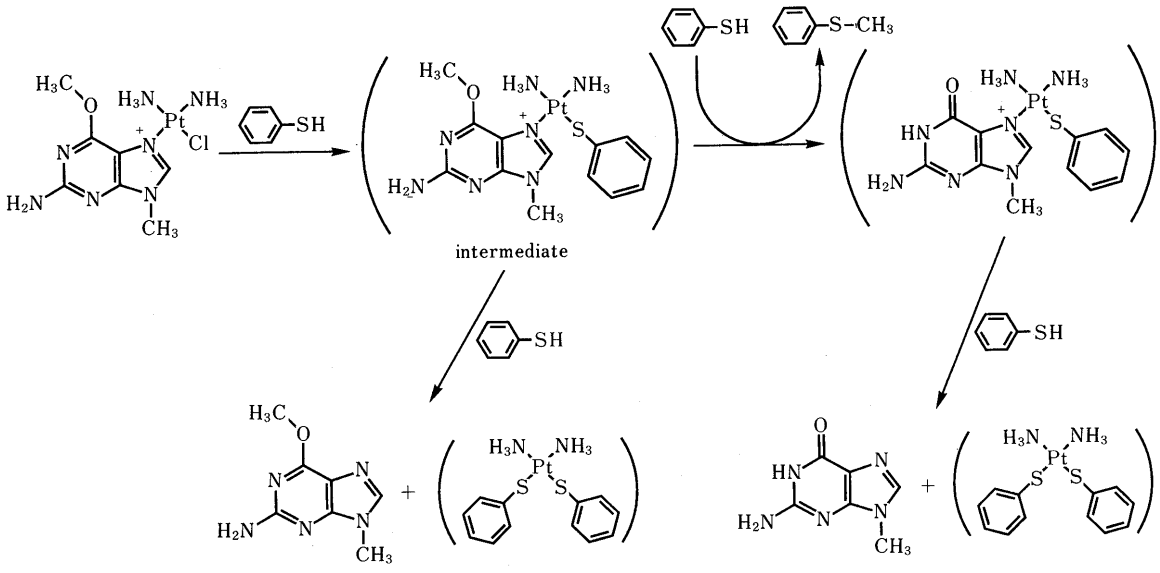


Chart 1. Possible Reaction Pathway

Cytotoxicity Assay Cultured L1210 murine leukemia cells (4×10^5 cells) in 2 ml of RPMI medium were treated with several concentrations of the test compound at 37 °C for 48 h and the numbers of surviving cells were counted, as described previously.[12] Growth inhibition was expressed as % survival relative to the untreated control cells. IC_{50}, the concentration of the test compound that reduced the cell number to 50%, was deduced graphically.

replaced with an intermediate, which also gradually disappeared during incubation, concomitantly producing O^6,9-diMeG, 9-MeG and thioanisole. Although the structure of the intermediate was not characterized, we speculate it to be *cis*-[Pt(phenyl-S)$(NH_3)_2$(O^6,9-dimethylguanine-7)]$^+$. Since O^6,9-diMeG itself is stable under these conditions, 9-MeG was thought to be formed from *cis*-[Pt(phenyl-S)$(NH_3)_2$(9-methylguanine-7)]$^+$, which in turn was derived from demethylation of the intermediate by thiophenol (Chart 1). Thiophenol (a soft base) is more reactive with platinum (a soft acid) based on the hard and soft acids and bases (HSAB) principle.[13] Therefore, O^6,9-diMeG and 9-MeG were released from the *cis*-Pt–O^6,9-diMeG and *cis*-Pt–9-MeG complexes, respectively, by replacement with thiophenol. The rates of formation of thioanisole and 9-MeG would be expected to be the same, but that of thioanisole was greater than that of 9-MeG (Fig. 2). In the case of the *cis*-Pt–O^6,9-diMeG 1 : 2 complex, the rates of formation were equal.[7] The reason for this is unclear.

pK_a and Chemical Shift UV spectra of both 1 : 1 and 1 : 2 complexes of *cis*-platin with O^6,9-diMeG were the same in media of pH 2 to 12, whereas at around pH 1, the absorption at 285 nm began to increase with increasing acidity of the medium (Fig. 3A). The pK_a values (0.8 for the 1 : 1 complex and 0 for the 1 : 2 complex) were estimated from pH-dependent UV changes. The basicity of the 1 : 1 complex is slightly stronger than that of the 1 : 2 complex and this difference is also demonstrated by the ^{1}H-NMR chemical shifts of the C8 protons (8.41 ppm for the 1 : 1 complex and 8.49 for the 1 : 2 complex) (Fig. 3B). The chemical shifts of the C8 proton of O^6,9-diMeG and O^6,7,9-trimethylguanine were 7.80 and 9.32 ppm, respectively, indicating that the *cis*-Pt–O^6,9-diMeG complexes are more cationic than O^6,9-diMeG but less than O^6,7,9-trimethylguanine.

X-Ray Structural Analysis The ORTEP[14] drawing of the 1 : 1 complex (Cl^- form) with atomic numberings and the molecular packing with hydrogen bonds are shown

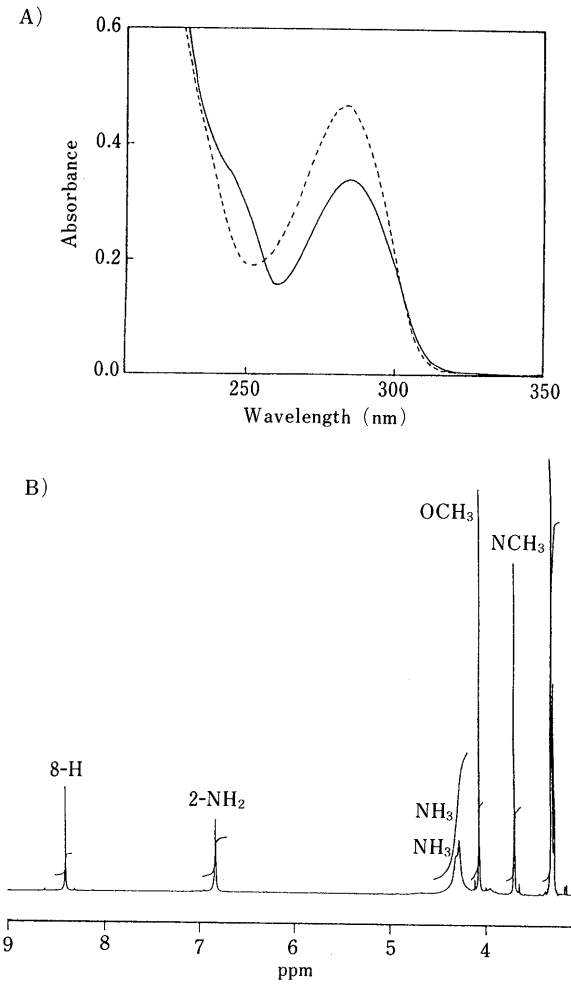


Fig. 3. A) UV Spectra of the *cis*-Pt–O^6,9-diMeG 1 : 1 Complex

The spectra were recorded at pH 0.3 (------) and pH 5 and 12 (——).

B) ^{1}H-NMR Spectrum of *cis*-Pt–O^6,9-diMeG 1 : 1 Complex

The spectrum was recorded in Me_2SO-d_6 and TMS was used as an internal standard.

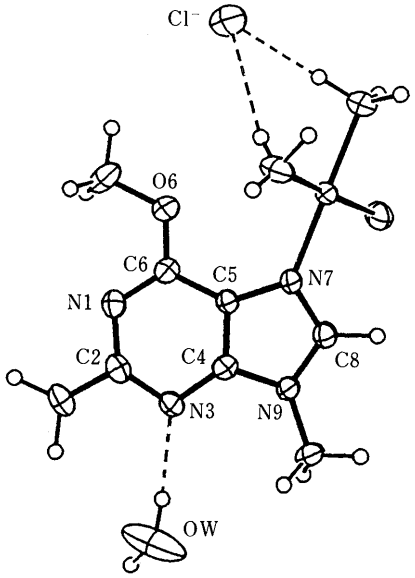


Fig. 4. ORTEP Drawing of [PtCl$(NH_3)_2$ (O^6,9-dimethylguanine-7)]Cl with Atomic Numberings

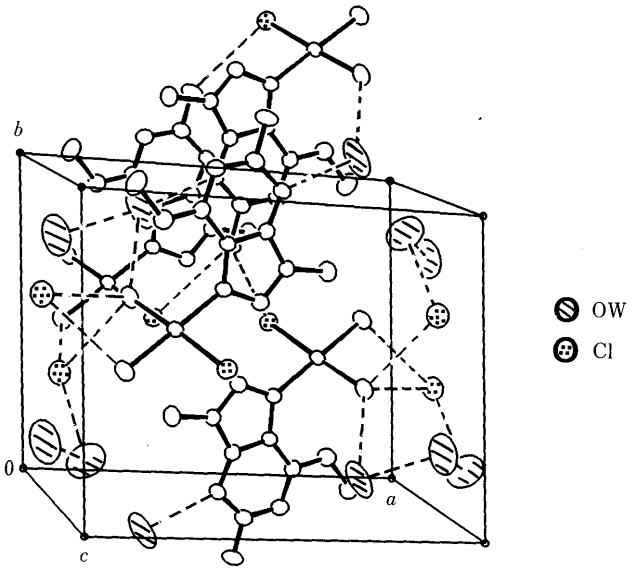


Fig. 5. Molecular Arrangement in a Unit-Cell

Dotted lines indicate hydrogen bonds.

TABLE II. IC_{50} of *cis*-Platin Derivatives toward L1210 Cells

Compound	IC_{50} (μg/ml)
cis-$[PtCl_2(NH_3)_2]$	0.6
cis-$[PtCl(NH_3)_2(O^6,9\text{-diMeG})]NO_3$	16
cis-$[Pt(NH_3)_2(O^6,9\text{-diMeG})_2]Cl_2$	>300

in Figs. 4 and 5, respectively. The O–CH_3 group of the complex is almost coplanar with the purine plane and has a *cis* conformation with respect to the N1 position (torsion angle at N1–C6–O6–C10 is 0.2(9)°), as reported for O^6,9-diMeG and O^6-methyldeoxyguanosine.[15] The dihedral angle between the purine ring and the Pt coordination plane is 59.2(1)°, which is within the usual range (46—63°) found in complexes of platinum with guanine derivatives.[16] There is no significant influence of O^6-methylation on complex conformation. The complex forms a hydrogen bond with water at the N3 position [N3---H–O W: 2.90(1) Å], suggesting that the basic site of the complex might be N3.[17] There are two layers in the crystal structure, a hydrophobic layer of O^6,9-diMeG rings related by a center of symmetry and a hydrophilic layer, containing ammines, Cl^- and water groups, extending into the *bc* plane. The crystals of the 1:2 complex were not suitable for X-ray crystallographic analysis.

Cytotoxicity It was reported that *cis*-diamminedichloroplatinum(II) (*cis*-platin) derivatives in which one chloro group is replaced with a ligand still possess cytotoxic activity.[8] Therefore, the cytotoxicity of the *cis*-Pt–O^6,9-diMeG 1:1 complex toward L1210 cells was examined and the result is shown in Table II. The 1:1 complex showed weak cytotoxicity (IC_{50} 16 μg/ml), though it was weaker than that of *cis*-platin itself. At these doses, the 1:2 complex was not cytotoxic.

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17) We have preliminary X-ray structures of *cis*-$[PtCl(NH_3)_2(O^6,9\text{-dimethylguanine-7})]NO_3 \cdot H_2O$ (R=8.7%) and *cis*-$[PtCl(NH_3)_2(O^6,9\text{-dimethylguanine-7})]NO_3 \cdot 2H_2O$ (R=9.1%). In both crystals, N3 participates in hydrogen bonding with a water molecule.