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Studies on the Reaction Products of Guanylyl(3′-5′)Adenosine with *cis*-$Pt(NH_2)_2Cl_2$ and $[Pt(NH_3)_3Cl]Cl$

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The reaction products of dinucleotide, GpA, with $[Pt(NH_3)_3Cl]Cl$ *and* cis-$Pt(NH_3)_2Cl_2$ *have been separated by means of high performance liquid chromatography with a strong cation exchange column. Each peak fractionated has been characterized by UV spectral analysis. The reaction of GpA with* $[Pt(NH_3)_3Cl]Cl$ *gave four products, but three of them are minor ones. The main product is found to be the 1:1 complex in which* $Pt(NH_3)_3^{2+}$ *binds to GpA only through the N(7) site of the guanine residue. The reaction of* cis-$Pt(NH_3)_2Cl_2$ *with GpA gave two products. One of the products is the 1:1 complex in which* cis-$Pt(NH_3)_2^{2+}$ *binds to GpA through the N(7) sites of the guanine and adenine residues. The effect of pH on the reaction of* cis-$Pt(NH_3)_2Cl_2$ *with GpA has been studied using high performance liquid chromatography.*

Introduction

The action of the antitumor *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) is supposed to arise from the binding of *cis*-DDP with DNA. For this reason, the interaction of *cis*-DDP with DNA or its constituents has been intensively studied. It has been generally accepted that a guanine base has a high selectivity for binding with platinum. Tobias *et al.* used Raman difference spectrometry to study the competitive reaction of *cis*-DDP with a mixture of four nucleotides, and they reported that the binding selectivity was in the order of GMP > AMP ≫ CMP > UMP and that it was attributable to kinetics rather than thermodynamics in origin [1]. The possible main platinum binding sites on nucleic acid bases are N(7) for guanosine, N(7) and N(1) for adenosine, and N(3) for cytidine.

The interaction of *cis*-DDP with di- or polynucleotides is particularly interesting because *cis*-DDP can bind to nucleic acid bases in a bifunctional manner. Spectroscopic techniques, including UV, CD, and NMR, have been applied to identify the binding site of platinum complexes with di- or poly-nucleotides [2–8]. However, the reaction of *cis*-DDP with nucleic acid bases in some cases gave several products. In such cases, a very complicated spectrum arising from several reaction products and unreacted nucleic bases may make the identification of the platinum binding sites difficult. The separation of reaction products by the chromatographic technique is expected to overcome this difficulty. The reaction of *cis*-DDP with dinucleotides such as GpG, IpI, ApA, ApC, CpG, and GpC had already been reported [2–6]. However, the reaction of *cis*-DDP with GpA has not been reported yet. The NMR spectrum of the reaction products of *cis*-DDP with GpA may be a very complicated spectrum, because the H(8) resonance of the guanine is thought to emerge in the vicinity of the H(8) and H(2) resonances of the adenine residue on GpA.

In the present paper, the reaction solution of *cis*-DDP with GpA was separated by high performance liquid chromatography (HPLC) and each product was fractionated using HPLC. Then, the platinum binding sites of their platinum–dinucleotide complexes were determined by UV spectral analysis. The pH dependence of the reaction of *cis*-DDP with GpA was also studied using HPLC.

Experimental

Dinucleotide, GpA, was purchased from the Sigma Chemical Co. and other chemicals were reagent grade. The *cis*-DDP and $[Pt(NH_3)_3Cl]Cl$ were prepared according to refs. [9] and [10].

Two mg of GpA and 1.05 mg of $[Pt(NH_3)_3Cl]Cl$ were dissolved in 0.02 *M* KH_2PO_4 solution, and the pH adjustment to 7.0 was made by addition of NaOH solution. The solution was incubated at 37 °C for 7 days. The reaction solution thus obtained was separated through a strong cation exchange column (Zipax SCX column, 0.21 × 100 cm) using 0.02 *M* KH_2PO_4

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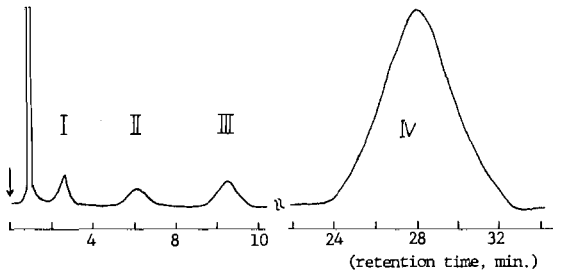

Fig. 1. Elution pattern obtained for the reaction solution of $[Pt(NH_3)_3Cl]Cl$ with GpA. Column: 0.21 × 100 cm, packed column Zipax SCX. Detector: UV (at 260 nm). Eluant: 0.02 *M* KH_2PO_4. Flow rate: 2.3 ml/min.

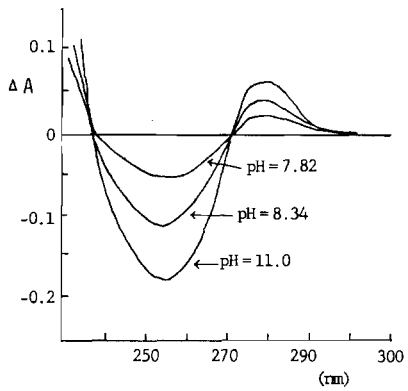

Fig. 2. Ultraviolet difference spectra of product IV in alkaline pH range, $[Pt(NH_3)_3\text{-GpA}] = 7.5 \times 10^{-5}$ *M*.

as eluant, and the reaction product was fractionated using HPLC.

Equimolar amounts of GpA and *cis*-DDP were dissolved in 0.02 *M* KH_2PO_4 and the pHs of the solutions were adjusted with $HClO_4$ or NaOH solution. The solutions were incubated at 37 °C for 4 days. HPLC analysis was performed with a Zipax SCX column, using 0.02 *M* KH_2PO_4 as eluant. For the prupose of the preparative separation, 2.0 mg of GpA and 0.98 mg of *cis*-DDP were dissolved in 0.02 *M* KH_2PO_4 solution and the pH of the solution was adjusted to 4.0 by adding $HClO_4$ solution. It was then incubated at 37 °C for 4 days. The preparative separation was performed with the same column and elution conditions. Each fraction thus obtained was reinjected onto the Zipax SCX column and the purity of each product was confirmed.

The platinum content in each product was determined by atomic absorption spectrophotometry [11], and the UV spectrum of the same solution was measured in order to determine the molar extinction coefficient per platinum.

For the measurement of UV difference spectra, the reference cell contained the solution at pH 6.0 of each product, and the sample cell contained the corresponding solution at various pH. The UV spectrum of the products obtained in this work did not show any UV spectral change at all in the pH range 4.8–6.5. The pKa value of each product was calculated from the relationship between pH and ΔA of the corresponding UV difference spectra.

Results and Discussion

Reaction Products of $[Pt(NH_3)_3Cl]Cl$ with GpA

The mixed solution of $[Pt(NH_3)_3Cl]Cl$ with GpA (r = 0.5), adjusted to pH 7.0, was incubated at 37 °C for 7 days. Figure 1 shows a chromatogram of the solution. The first peak, which emerges at the void volume of the column, is identical with the peak of the ligand, GpA. The other four peaks (I–IV) are attributed to the reaction products, and three of them (I–III) are minor products. The main product IV was fractionated from HPLC and was characterized by UV spectrophotometry.

The UV spectrum of the product IV showed an absorption maximum at 260 nm, and the molar extinction coefficient per platinum was 2.3×10^4 at 260 nm. In this case, the concentration of platinum was determined by atomic absorption spectrophotometry. The result suggests that the binding ratio between platinum and GpA is 1:1. Since $[Pt(NH_3)_3Cl]Cl$ is a monofunctional nucleophile, either the guanine or the adenine residue seems to participate in the binding of GpA with platinum.

Figure 2 shows the UV difference spectral change of product IV as a function of pH. The changes obtained in these pH ranges are attributed to the deprotonation of N(1) of the guanine residue. The UV difference spectra exhibit isosbestic points at 237 and 271 nm, and the UV spectral pattern of its deprotonated species (pH ≥ 11) is quite similar to the corresponding pattern of the N(7)-platinated guanosine, $Pt(NH_3)_3(guanosine)]Cl$ [12]. For the N(7)-platinated guanosine, the UV difference spectral changes as a function of pH exhibited isosbestic points at 236 and 275 nm. The pKa value for product IV. calculated from the relationship between ΔA and pH, was 8.15. The corresponding pKa values of N(7)-platinated guanosine was 8.06. A similar pKa value was also reported for the various N(7)-platinated guanosine derivatives [4, 7, 8]. These results clearly indicate that N(7) of the guanine residue on GpA participates in binding with platinum.

Figures 3a and 3b show the UV difference spectral changes arising from the protonation of product IV and adenosine, respectively. Both UV difference spectral patterns are significantly similar to each other. In the previous paper, we reported the reaction products of $[Pt(NH_3)_3Cl]Cl$ with adenosine [13]. The N(1)-platinated adenosine did not exhibit any UV spectral change in the acidic pH range

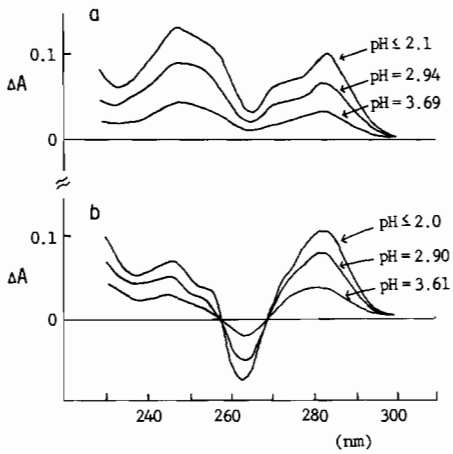

Fig. 3. Ultraviolet difference spectra of product IV in acidic pH range: (a) product IV, $[Pt(NH_3)_3\text{-GpA}] = 7.5 \times 10^{-5}$ *M*; (b) adenosine, $[Ado] = 1.08 \times 10^{-4}$ *M*.

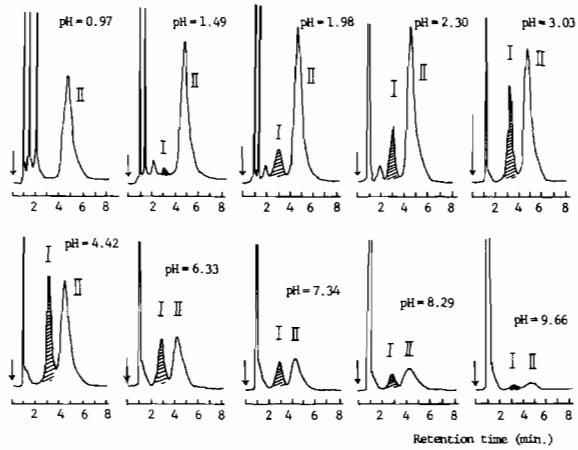

Fig. 4. Elution pattern obtained for the reaction solutions of *cis*-DDP with GpA at various pH. Eluant: 0.02 *M* KH_2PO_4. Flow rate: 2.0 ml/min.

because the N(1) site of adenosine was already occupied by the platinum atom. The N(7)-platinated adenosine showed UV difference spectral change arising from the protonation of the N(1). However, the UV difference spectrum of the protonated species of product IV (Fig. 3a) is quite different from the corresponding one of the N(7)-platinated adenosine. The pKa values for product IV and adenosine, calculated from the relationship between pH and ΔA, were 3.4 and 3.6, respectively. Generally, binding of platinum to nucleic acid bases causes a significant decrease in the pKa when compared with the unmodified nucleic acid base. For instance, the pKa value of the N(7)-platinated adenosine was 1.4, that is, the pKa value of the N(1) site showed a decrease of 2.2 log units upon binding of $Pt(NH_3)_3^{2+}$ to the N(7) site of adenosine [13]. However, product IV does not show such a decrease in pKa. From these results, it appears that the adenine residue of GpA does not participate in binding with platinum. Consequently, product IV is a 1:1 complex in which $Pt(NH_3)_3^{2+}$ is bound to GpA only through the N(7) site of the guanine residue. This result is supported by the molar extinction coefficient per platinum of product IV.

Reaction Products of cis*-DDP with GpA*

Figure 4 shows pH dependence of the reaction of *cis*-DDP with GpA (r = 0.5). The chromatograms show two peaks arising from the reaction products (I and II). Formation of product I becomes maximum in the pH range 3.0–4.4, but it is strongly suppressed with decreasing pH. On the other hand, formation of product II is not affected so much by decreasing pH. This fact reveals that formation of product I competes strongly with the proton. The protonation sites of the ligand, GpA, are N(1) of the adenine residue and N(7) of the guanine residue, and these sites, especially in the case of product I, seem to participate in binding with platinum. At pH > 4, formation of both products is strongly suppressed with increasing pH. It is worth noting that the ratio in the peak area of product I to product II is almost constant at pH > 4. This suggests that formation of both complexes may be suppressed by a common factor, interfering with the complex formation reaction. The suppression of the complex formation is thought to come from the hydrolysis of *cis*-DDP. In the substitution reaction of platinum complexes, a solvolytic intermediate is generally involved. When the reaction of *cis*-DDP with GpA takes place through an aqua intermediate, the reaction will be suppressed at high pH because the hydroxo complex is inert. The diaqua species of *cis*-DPP has pKa values of 5.6 and 7.3 [14]. Similar interference arising from the formation of hydroxo species was also observed in the case of the reaction of *cis*-DDP with adenosine [13].

Products I and II were fractionated using HPLC. The molar extinction coefficient of product II was 2.3×10^4 at the absorption maximum, 262 nm. This value is in good agreement with that of product IV, obtained from the reaction of $[Pt(NH_3)_3Cl]Cl$ with GpA as described before. That is, product II is thought to contain one platinum per dinucleotide molecule, GpA. Since *cis*-DDP is a bifunctional nucleophile, it may be expected that the adenine and the guanine residues on GpA participate in binding with platinum. Figure 5 shows the UV difference spectral change of product II as a function of pH. The ligand, GpA, possesses two possible protonation sites, N(7) of the guanine and N(1) of the adenine, in the pH range 1–4. The UV difference spectra obtained from Fig. 5 have isosbestic points at 223,

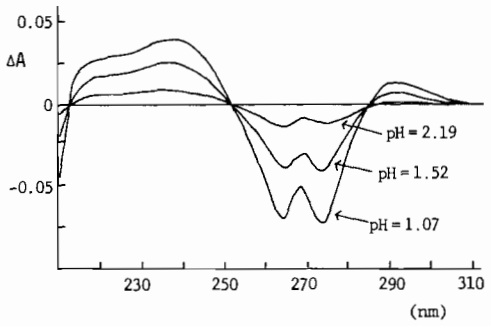

Fig. 5. Ultraviolet difference spectra of product II in acidic pH range, [*cis*-$Pt(NH_3)_2$-(GpA)] = 4.38×10^{-5} *M*.

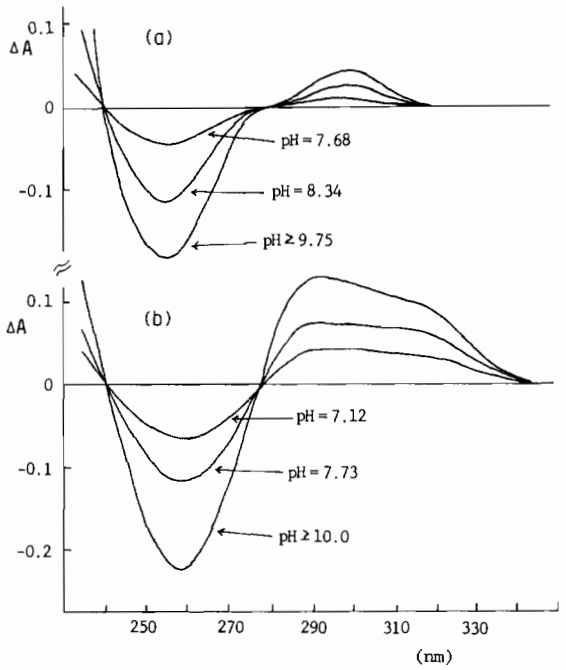

Fig. 6. Ultraviolet difference spectra of products I and II in alkaline pH range: (a) Product II, [*cis*-$Pt(NH_3)_2$-GpA] = 4.83×10^{-5} *M*. (b) Product I, [*cis*-$Pt(NH_3)_2$-GpA] = 2.91×10^{-5} *M*.

262 and 295 nm and seem to correspond to an equilibrium reaction having only one protonation. In fact, the N(7) site of the guanine residue is involved in binding with platinum, as described below. The UV difference spectral pattern of the protonated species of product II is quite similar to the corresponding pattern of the N(7)-platinated adenosine, as reported in the previous paper [13]. The pKa value calculated from the relationship between ΔA and pH was 1.2, and this is assigned to the protonation of N(1) of the adenine residue. For the unplatinated adenine residue, we obtained a pKa value of 3.4 (see Fig. 2). The decrease in pKa of the adenine of product II is therefore 2.2 log units. Such a decrease in pKa was also observed in the case of the N(7)-platinated adenosine [13]. These facts suggest that the N(7) of the adenine residue on GpA participates in binding with platinum.

Figure 6a shows the UV difference spectra of product II in the pH range 6.5–11.0. The UV difference spectral pattern of the deprotonated species is similar to the corresponding one in Fig. 2. This is a characteristic UV difference spectral pattern arising from the deprotonation species of the N(7)-platinated guanosine [12]. A similar UV spectra pattern was also observed for the N(7)-platinated species of GpG and GpC [4]. The pKa value of the guanine residue of product II was 8.1, indicating that N(7) of the guanine residue is one of the platinum binding sites. From these results, it is concluded that product II is a 1:1 complex in which *cis*-DDP is bound to GpA through the N(7) sites of both the adenine and the guanine residues.

In the case of product I, the binding ratio of *cis*-DDP to GpA is thought to be 1:1 because the molar extinction coefficient per platinum is 2.4×10^4. Product I did not show any UV spectral change in the pH range 1.0–6.0. This suggests that the sites of proton attachment to GpA, N(1) of the adenine residue and N(7) of the guanine residue, may have been already occupied by binding with platinum. This is supported by the pH dependence study of the reaction of *cis*-DDP with GpA (Fig. 4), in which the formation of product I was strongly suppressed with decreasing pH. Figure 6b shows the UV difference spectral pattern of product I in the pH range 6.5–10.5 and seems to reflect the deprotonation reaction at the N(1) of the N(7)-platinated guanine residue, though it has positive absorption even at a wavelength beyond 300 nm. We tried to calculate the pKa value from the relationship between pH and ΔA. However, the value of the pKa calculated changed with increasing pH from 7.5 to 8.2, that is, it was not constant. Therefore, we tried to examine the reversibility of the deprotonation reaction. Once the solution of product I (initial pH = 6.0) had reached pH = 12 by adding NaOH solution, it was then returned to the original pH (pH = 6.0) by adding $HClO_4$ solution. The spectrum thus obtained was found to differ from the original spectrum. Unfortunately, no reversibility has been observed. Although the evidence is not conclusive, product I is thought to include the N(7) of the guanine residue.

The UV spectral pattern of the platinum complex with nucleic acid bases is expected to give information concerning its platinum binding site. In the case of dinucleotides such as GpA, GpG, and GpC, the bases have been linked by a phosphodiester bond and there is no conjugate bond between them. On the other hand, it is well known that there is a stack-

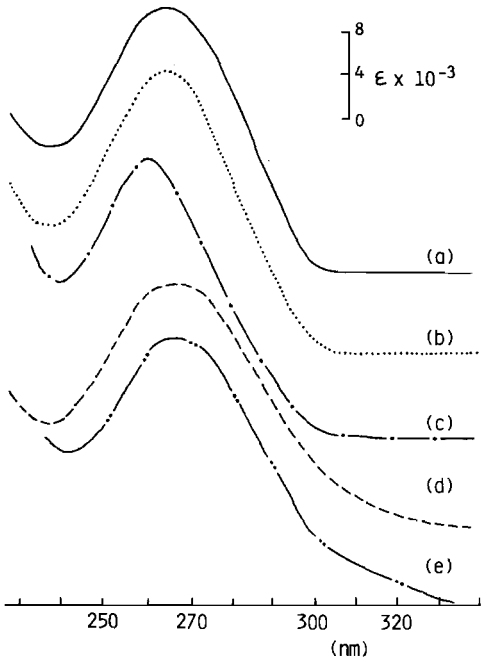

Fig. 7. Ultraviolet spectral pattern of the reaction products of *cis*-DDP with GpA and their related spectral pattern: (a) Spectrum of the equimolar mixture of the N(7)-platinated guanosine($Pt(NH_3)_3$-Guo) and N(7)-platinated adenosine-($Pt(NH_3)_3$-Ado). (b) Spectrum of product II. (c) Spectrum of the equimolar mixture of the N(7)-platinated guanosine-($Pt(NH_3)_3$-Guo) and N(1)-platinated adenosine($Pt(NH_3)_3$-Ado). (d) Spectrum of product I. (e) Spectrum of the equimolar mixture of the N(7)-platinated guanosine$Pt(NH_3)_3$-Guo) and N(1)-platinated and deprotonated adenosine-($Pt(NH_3)_3$-$AdoH_{-1}$).

ing interaction between them. If the effect of stacking interaction on the UV spectrum is negligible or small, the UV spectrum of the platinum–dinucleotide complex should be similar to the sum of the UV spectra of the platinum complexes with its constituents. For instance, the N(7)-platinated guanosine derivatives ($Pt(NH_3)_3$(guanosine), *cis*-$Pt(NH_3)_2$-(guanosine)$_2$, and *cis*-$Pt(NH_3)_2$(GpG)) showed significantly similar UV spectral patterns. In this case, there is a stacking interaction, especially in the case of *cis*-$Pt(NH_3)_2$(GpG), but its effect is not enough to change the UV spectral pattern. Figure 7b shows the UV spectrum of product II at pH 6.5. This UV spectral pattern is quite similar to that obtained from the equimolar mixture of the N(7)-platinated guanosine and the N(7)-platinated adenosine (Fig. 7a). This result supports our conclusion that the platinum binding sites of product II are N(7) of the guanine residue and N(7) of the adenine residue. Spectrum 7c obtained from an equimolar mixture of N(1)-platinated adenosine and N(7)-platinated guanosine is considerably different from that of product II. The UV spectrum of product I (Fig. 7d) is characterized by a broad shoulder at the wavelength beyond 300 nm. The shoulder under consideration is not observed in Fig. 7a, b and c. The occurrence of the shoulder may be related to the deprotonation of the 6-NH_2 group of the adenine residue or the binding of platinum to the 6-NH_2 group, probably with the loss of proton. Mansy *et al.* studied the reaction of *cis*-DDP with adenosine using the UV spectral technique and reported that the shoulder at the wavelength beyond 300 nm may be related to the chelation of *cis*-DDP with N(7) and 6-NH_2 of adenosine [14]. Binding of methylmercury to the 6-NH_2 group of adenosine also has such a shoulder, reported by Simpson [15]. However, it should be noted that these two studies were conducted in the presence of excess metal. Therefore, the possibility of binding of platinum to the 6-NH_2 group cannot be ruled out for product I. On the other hand, it is generally accepted that the 6-NH_2 group of adenosine is a weak binding site for platinum because the lone pair of electrons of the 6-NH_2 group delocalize into the π-system of the purine ring. In fact, the reaction of *cis*-DDP with adenosine gave the mononuclear complexes in which *cis*-DDP is bound to adenosine through either N(7) and N(7) or N(7) and N(1), and the shoulder under consideration was not observed in these complexes [13]. Figure 7e shows the UV spectrum of the equimolar mixture of the N(7)-platinated guanosine and the deprotonated species of the N(1)-platinated adenosine. It is worth noting that the spectrum of product I is significantly similar to the spectrum 7e. Therefore, it is likely that the shoulder under consideration arises from the imino form which is derived from the deprotonation of the 6-NH_2 group. For product I, the following two possibilities may be suggested.

(1) *cis*-DDP may bind to GpA through N(7) of the guanine residue and N(1) of the adenine residue. Assuming that the complex has an interbase link within the dinucleotide, GpA, the H-atom of the 6-NH_2 group is in close proximity with the O(6) of the guanine residue. Such proximity may be expected to induce deprotonation of the 6-NH_2 group.

(2) Another possibility is that *cis*-DDP may bind to GpA, probably with the loss of proton, through N(7) of the guanine residue and the 6-NH_2 of the adenine residue. Although the 6-NH_2 group is a poor donor group, it seems to be a sterically favorable site for interbase crosslink between the two bases.

Because the evidence is not conclusive, it is too early to draw a conclusion concerning the structure of product I. However, product I seems to contain the N(7) of the guanine residue and either N(1) or 6-NH_2 of the adenine residue as platinum binding sites.

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