Reaction Products of *cis*-Diammineplatinum(II) Compounds with 5'-Guanosine Monophosphate Characterized by High-Frequency Proton NMR

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Abstract: In the presence of an excess of chloride ions *cis*-$PtCl_2(NH_3)_2$ reacts slowly with 5'-GMP even at elevated temperatures. The initial product is found to be [*cis*-$Pt(NH_3)_2$ (GMP)Cl] (I). Secondary products formed are [*cis*-$Pt(NH_3)_2(GMP)_2$] (II) (especially at low temperatures when there is a relative excess of 5'-GMP) and [*cis*-$Pt(NH_3)_2(GMP)(H_2O)]^+$ (IV) (formed at low Cl^- only). All three species have GMP coordinated to Pt(II) via their guanine N7 atoms, as deduced from the pH dependence of the ^1H chemical shifts and from comparison with literature data. Prolonged heating at 80 °C results in a new species (III), previously described by others as a N7-O6 chelate of GMP and *cis*-$Pt(NH_3)_2^{2+}$. Detailed analysis of the pH dependence of the ^1H chemical shift of this product, however, agrees with a species in which one platinum is bound to GMP via N1 and most likely a second platinum via N7.

Interest in the mechanism of action of *cis*-diamminedichloroplatinum(II) (abbreviated *cis*-platinum or *cis*-Pt), an increasingly used antitumor drug, has greatly stimulated the study of interaction between *cis*-Pt compounds and DNA fragments.[1] The interaction between *cis*-Pt(II) species and the guanine N7 fragment in DNA is now generally accepted to be an interaction of major importance in vivo.[2,3]

Although the secondary interaction of Pt compounds with DNA is likely to be more important from the point of view of cell killing, the study of the monoadducts is of great interest for the following reasons:

They are reactive intermediates in the process of bifunctional binding to DNA.

The question of rotation about the Pt-N7 bond is of interest because rotation may hamper or enhance the attack by a second nucleobase.

They are possible intermediates for the formation of the postulated N7-O6 chelate.

Because of the reactivity of the monoadducts, binding at the second coordination site must be retarded, if monoadducts are to be studied. This can be done by using an excess of *cis*-Pt. To slow down the reaction one can further use an excess of chloride ions, so that the concentration of hydrolyzed, reactive species, such as [*cis*-$Pt(NH_3)_2Cl(H_2O)]^+$, is very small. This approach has first been followed by Clore and Gronenborn.[4] They studied the reactions of 5'-adenosine monophosphate (AMP) and 5'-guanosine monophosphate (GMP) with *cis*-Pt at elevated temperatures in the presence of a large excess of KCl. The reaction products of the former compound were assigned to different mono- and diadducts of *cis*-Pt to AMP. In the case of GMP, however, the reaction products were identified as different forms of a single Pt-(N7)GMP adduct.

We thought it worthwhile, in the latter case, to check for alternative explanations, and we have therefore repeated and extended the experiments by varying the Cl^- concentration, by observing the course of the reaction at 80 °C as well as at 4 °C, by using not only *cis*-Pt but also other Pt compounds, and by studying the pH dependence of the proton NMR signals of the reaction products. Our results, which are presented in this paper, provide clear evidence for more than one Pt-GMP adduct.

Experimental Section

Starting Materials and Sample Preparation. 5'-GMP was obtained from Sigma Chemicals. *cis*-$PtCl_2(NH_3)_2$ was prepared by a published method.[5] A solution of *cis*-$Pt(NH_3)_2(H_2O)_2^{2+}$ was prepared by stirring a suspension of the iodide $PtI_2(NH_3)_2$ in water with 1.95 equiv. of $AgNO_3$ for 1 h in the dark and filtering off the precipitate.

Solutions of 40 mM *cis*-Pt, 4 mM GMP, and varying concentrations of KCl (0, 4, 40, and 400 mM) in H_2O were allowed to react at 80 °C. At several time intervals 500-μL samples were taken and cooled in ice to slow down the reaction and precipitate most of the remaining *cis*-Pt. After that, the samples were centrifugated to remove the precipitated *cis*-Pt and ^1H NMR was performed on the supernatant. To check that the presence of a small amount of precipitate does not influence the results, an experiment was performed in which the samples were not centrifugated; the experimental findings appeared to be the same. The samples from the reaction at 80 °C were stored at 4 °C, and after time intervals of a few days up to 40 days again NMR spectra were taken. The reaction between freshly prepared *cis*-$Pt(NH_3)_2(H_2O)_2^{2+}$ (11.3 mM) and 5'-GMP (3.6 mM) was performed in an NMR tube at room temperature. Spectra were recorded at several time intervals after the addition of *cis*-$Pt(NH_3)_2(H_2O)_2^{2+}$.

^1H NMR Spectra. NMR spectra were obtained at room temperature on a Bruker WM-300 (300 MHz) spectrometer, interfaced with an Aspect 2000 computer. To enable deuterium field frequency lock 10% D_2O was added to all samples. A Redfield 2-1-4 pulse was used for suppressing H_2O peaks,[6,7] in conjunction with the alternate delay accumulation technique (ADA).[7]

Spectra were measured by storage of 640 FID's in 8K or 16K memory and Fourier transformation (FT) after zero filling to 16K or 32K. The first two points of the FID's were discarded before FT to minimize base-line rolling. No smoothing or deconvolution was applied. Chemical shifts were measured with respect to tetramethylammonium nitrate (TMA, 3.188 ppm downfield from DSS) but are quoted with respect to DSS (sodium 4,4-dimethyl-4-silapentanesulfonate).

pH Titrations. pH titrations were performed by adding NaOH or HCl to the sample until the required pH was reached. pH values were measured with a Radiometer PHM-80 pH meter equipped with an Ingold 6030-02 electrode.

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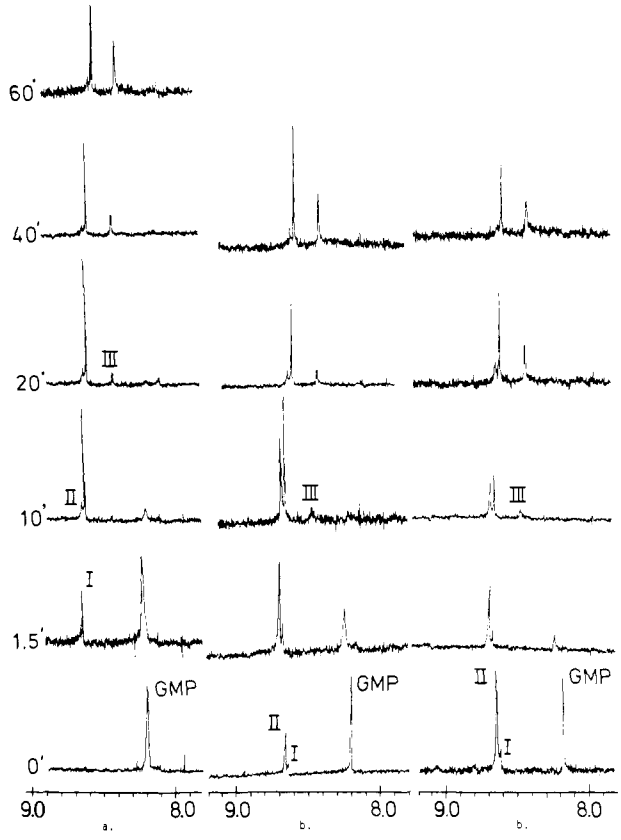

Figure 1. Progress of the reaction of 5'-GMP (4 mM) with *cis*-$PtCl_2(NH_3)_2$ (40 mM) in the presence of 400 mM KCl followed by ^1H NMR spectroscopy. (a) The left column shows spectra of samples which had reacted at 80 °C for 0, 1.5, 10, 20, 40, and 60 min. (b) The middle and right columns show the same samples after storage at 4 °C for 15 and 28 days, respectively.

Results and Discussion

General. Our main tool to follow the reaction of GMP with *cis*-Pt is to monitor the NMR signal of the H8 proton of 5'-GMP. To avoid loss of signal intensity due to exchange of this proton with D_2O, normal water was employed as a solvent, instead of D_2O, although this entailed the need to suppress the water NMR signal and thus slightly complicated the experiments (see Experimental Section).

Two kinds of experiment were performed. In one the reaction was carried out at 80 °C and its course was followed by taking samples at short intervals in the beginning and at progressively longer intervals later on. In the second type of experiment the samples from the reaction at 80 °C were allowed to stand at 4 °C, and room temperature NMR spectra were taken at intervals of several days.

In addition to the study of the chemical shift of the H8 protons in the course of the reaction the pH dependence of these chemical shifts was studied extensively. Protonation of the heterocyclic nitrogens and the phosphate group of GMP are known to have a strong influence on the NMR chemical shift of the H8 proton.[8] In free GMP protonation at N1 (pK_a = 9.7) and at N7 (pK_a = 2.4) causes shift changes of the H8 signal to lower field and phosphate protonation (pK_a values 6.2 and ≤1) causes shift changes to higher field.[9] Coordination of platinum to one or both of the heterocyclic nitrogens of GMP results in a change in pK_a values.[10] A downfield shift upon protonation of the remaining N1 or N7 site and an upfield shift upon phosphate protonation is observed.

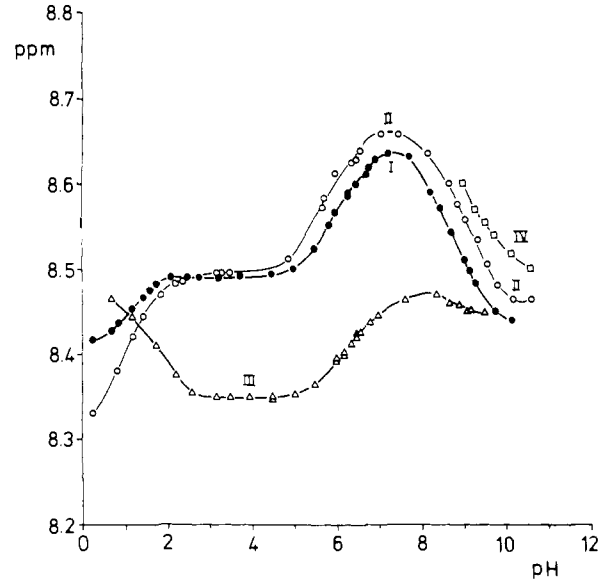

Figure 2. 5'-GMP H8 chemical shift vs. pH for the species I (●), II (○), and III (△) formed in the reaction between *cis*-$PtCl_2(NH_3)_2$ and 5'-GMP. The appearance of peak IV (□) at pH >9 is due to the formation of the hydroxo product, *cis*-$Pt(NH_3)_2$(GMP-*N7*)$(OH)^+$ (see Figure 5).

The presence of platinum bound to a particular nitrogen atom prevents the protonation of this nitrogen, and therefore no change in chemical shift due to protonation of this nitrogen is expected. So, by analysis of NMR spectra, obtained at different pH's, the binding sites of the platinum can be localized. In passing we note that chemical shifts of different samples should be compared at the same pH.

Reaction of *cis*-Pt with 5'-GMP in the Presence of 400 mM KCl. Reaction at 80 °C. Figure 1a shows ^1H NMR spectra of the H8 proton of GMP. The spectra were recorded at several time intervals after the start of the reaction at 80 °C. As the signal of free GMP decreases, three new signals arise, which we number following Clore[4] by I, II, and III. Signal I (chemical shift 8.63 ppm at pH 7.0) and signal II (chemical shift 8.65 ppm at pH 7.0) appear almost simultaneously, but signal I is much stronger than signal II. After prolonged reaction (>20') signal III appears. This signal (previously not resolved[4]) in fact consists of two ^1H NMR signals, separated only by 0.006 (1) ppm. For the moment a single label is used to denote this pair of peaks (III). The midpoint between the two signals (8.43 ppm at pH 6.5) is quoted. During the reaction a pH drop from pH 7.0 to 5.6 was observed; due to the phosphate protonation, which takes place in this pH traject, the chemical shift of the H8 proton changes gradually[9] (see Figure 1).

Reaction at 4 °C. The progress of the reaction at 4 °C is shown in Figure 1b, where we present the room temperature NMR spectra of the same samples as used in Figure 1a, this time measured after they had been stored for 15 and 28 days, respectively, at 4 °C. When parts a and b of Figure 1 are compared, the striking feature is the increase in the intensity of signal II in the course of time. As witnessed by the corresponding traces in Figure 1a, the increase is seen only in solutions which still contained free GMP before they were stored at 4 °C. For a proper understanding of this observation it is important to realize that *cis*-$PtCl_2(NH_3)_2$ is poorly soluble in water at low temperatures,[11] and storage at 4 °C will therefore have produced a condition of relative excess of GMP over *cis*-$PtCl_2(NH_3)_2$ in a number of samples. Apparently this condition favors the formation of species II.

pH Dependence of the H8 Signals. Results of the pH dependence of the chemical shifts of signals I, II, and III are depicted in Figure 2. Signal I shows changes in chemical shift near pH 8.5 (N1 protonation), pH 6.0 (phosphate protonation), and pH

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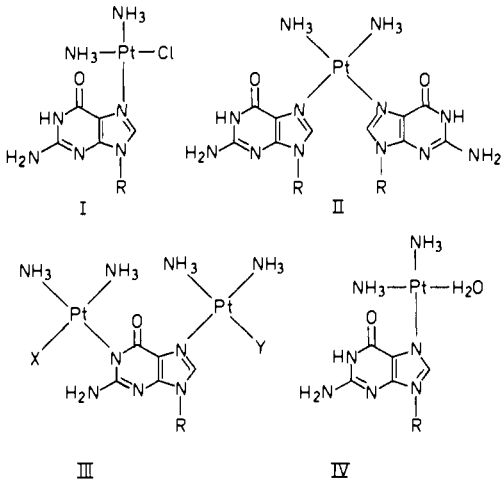

Figure 3. Proposed structures of species I, II, III, and IV, formed in the reaction of 5′-GMP with *cis*-$PtCl_2(NH_3)_2$.

≈1. The latter change is toward higher field and is likely to be due to the second phosphate protonation. No protonation of the N7 of GMP is observed, which indicates that in species I platinum is bound to the N7 of GMP. Species II shows essentially the same curve as species I, so it is concluded that also in species II platinum is bound to the N7 of GMP. Species I is formed in large amounts relative to species II at 80 °C. At this temperature the *cis*-Pt concentration is high, and therefore the product formed is likely to be a 1:1 *cis*-Pt–GMP compound (Figure 3, structure I), as concluded before by Marcelis[12] and by Clore and Gronenborn.[4] Species II is formed in greater amounts at low temperature when the solubility of *cis*-Pt is low.[11] This condition favors a relative excess of GMP, so the product formed is likely to be a 1:2 *cis*-Pt–GMP compound (Figure 3, structure II). The fact that species II is only formed as long as free GMP is still in solution supports this conclusion. Another argument why species II is likely to be *cis*-$Pt(NH_3)_2(GMP\text{-}N7)_2$ is that parallel with the appearance of species II a pair of H1′ proton NMR signals is observed at 5.91 and 5.90 ppm (pH 7.1), which is the position expected on the basis of literature data for the *cis*-$Pt(NH_3)_2(GMP\text{-}N7)_2$ adduct.[12]

These results prove that species I and II cannot correspond with two rapidly interconverting conformations of a single reaction product but must derive from different species. Additional evidence for this was obtained by watching the intensity ratio of the peaks I and II as a function of temperature. This ratio, in a sample where it amounted to 0.3 at room temperature, remained constant between 5 and 60 °C within experimental error (25%). The titration behavior of signal III shows a change in chemical shift near pH 6.0 due to phosphate (de)protonation. No protonation of N1 is observed, which proves that one Pt is bound at the N1 of GMP. At much lower pH (below pH 2.5) a second change in chemical shift to lower field is observed. This is unlikely to originate from a N7 protonation, since the corresponding protonation in the adduct of $Pt(dien)^{2+}$ with 9-methylhypoxanthine (a species with a known N1 binding) occurs at pH 3, under similar conditions.[13] The origin of the changes below pH 1.5 is not clear (although observed in related systems[14]), but they might possibly be related with protonations at O6 and/or N3 of the purine ring. Therefore we think species III is a product in which one platinum is bound to GMP at N1, and—most likely—a second platinum at N7 (Figure 3, structure III). The high temperature and the excess of platinum make this formation highly likely, just as found for the corresponding AMP species.[4] Further, binding of metals to both N1 and N7 has also been reported for other cases.[15]

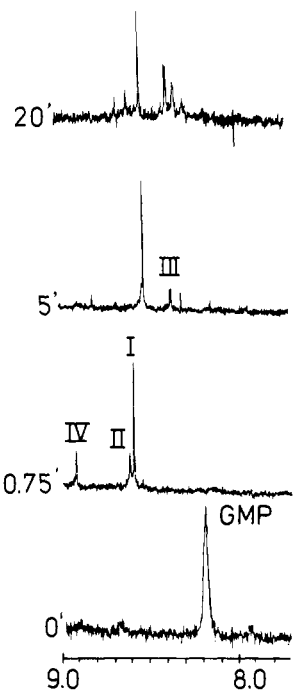

Figure 4. Progress of the reaction of 5′-GMP (4 mM) with *cis*-$PtCl_2(NH_3)_2$ (40 mM) in the presence of 4 mM KCl followed by ^1H NMR spectroscopy. Samples were taken after 0, $^3/_4$, 5; and 20 min.

[Cl^-] Dependence of the Reaction of *cis*-Pt with 5′-GMP. The reaction at 80 °C of *cis*-Pt with GMP was carried out at different Cl^- concentrations (400, 40, 4, and 0 mM). The ^1H NMR spectra of the H8 of GMP, recorded at several intervals after the start of the reaction, are shown in Figures 1a (400 mM KCl) and 4 (4 mM KCl). In the first stage of the reaction the three signals noted before arise again (I, II, and III) and at lower Cl^- concentration a fourth signal, IV (chemical shift = 8.93 at pH 5.8), appears. This signal is assigned to GMP bound to aquated *cis*-Pt via N7 (Figure 3, structure IV) (vide infra). The fact that free GMP disappears much faster at low Cl^- concentration indicates that lowering the Cl^- concentration enhances the reaction speed. The higher reaction rate is explained by a faster hydrolysis of the *cis*-Pt. Hydrolysis of *cis*-Pt, by which one or two chloride ions are replaced by water molecules, is known to be hampered by high Cl^- concentrations.[16]

The reactions of *cis*-Pt usually are supposed to proceed through the intermediate Pt–aqua complex, and hydrolysis of the starting *cis*-Pt compound is considered to be the rate-determining step.[17] Conditions favoring hydrolysis therefore automatically enhance the overall reaction rate, and the formation of species IV, *cis*-$Pt(NH_3)_2(H_2O)(GMP\text{-}N7)$. Parallel with this it is observed that the drop in pH during the reaction became more pronounced at low Cl^-. For instance, at 400, 40, 4, and 0 mM KCl the pH dropped during the reaction from its starting value of 7.0 to final values of 5.6, 4.5, 3.8, and 4.0, respectively, in corresponding time intervals of 120, 60, 40, and 10 min. The mono- and diaqua complexes of *cis*-Pt have[18] pK_a's of 7.4 and 5.5, and hydrolysis of the *cis*-Pt and subsequent deprotonation of the aquated complex explain the pH drop which occurs during the reaction. The fact that a solution containing only 40 mM *cis*-$Pt(NH_3)_2Cl_2$ and 4 mM KCl, when heated to 80 °C, exhibits a drop in pH from 6.7 to 4.6, which is similar to the pH drop from 7.0 to 3.8 in the 4 mM KCl sample with GMP, supports this conclusion. After prolonged

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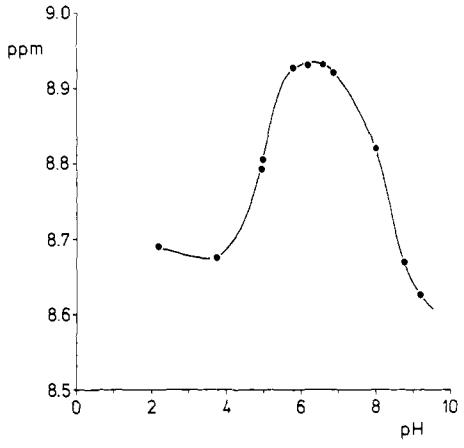

Figure 5. 5'-GMP H8 chemical shift vs. pH for species IV formed in the reaction of 5'-GMP and *cis*-$PtCl_2(NH_3)_2$ at low Cl^- concentration. At pH >9 the large drop in chemical shift is partly due to the formation of the hydroxo product (see Figure 2).

reaction time the samples turned blue and some new signals arose in the NMR spectrum. The latter process also proceeds faster at lower Cl^- concentration. The new NMR signals may originate from hydroxo-bridges products or other types of oligomers[19] and were not further investigated. At 4 °C the reaction again proceeds slowly. As long as free GMP is present in solution species II is formed in greater amounts than species I. The new species, IV, disappears after longer reaction time. The reactions, which cause the new H8 signals in the ^1H NMR spectra, also proceed slowly at this temperature.

Reaction of Aquated *cis*-Pt with 5'-GMP. To confirm that species IV is $Pt(NH_3)_2(H_2O)(GMP)$, *cis*-$Pt(NH_3)_2(H_2O)_2^{2+}$ was reacted with GMP. The first product formed in this reaction must be *cis*-$Pt(NH_3)_2(H_2O)(GMP)$. In the first stage of the reaction a single H8 peak was indeed observed, identical with our species IV. The titration behavior of species IV, depicted in Figure 5, shows it to be a Pt–N7 adduct. The chemical shift of 8.93 ppm at pH 5.8 for this species is in perfect agreement with the earlier findings of Marcelis et al.,[12] who reported a chemical shift of 8.89 ppm at pH 6.0–7.0 at 5 °C for this product.

Concluding Remarks

The results discussed above can now be compared with those of Clore and Gronenborn.[4] The latter authors, on the basis of ^1H and ^{195}Pt NMR measurements, identify species I, II, and III as different forms (two rotamers and a N7–O6 chelate) of the same 1:1 *cis*-Pt-(GMP-*N7*) adduct. The measurements have now been extended by taking into account the effect of pH on the chemical shift of the H8 proton, observing the reaction at 4 °C, and studying the dependence of the reaction on the chloride concentration. Besides this, we used the reaction of 5'-GMP with [*cis*-$Pt(NH_3)_2(H_2O)_2]^{2+}$ as a comparison. In total we arive at somewhat different conclusions.

We agree with Clore and Gronenborn[4] that species I is indeed *cis*-$Pt(NH_3)_2$(GMP–*N7*)Cl. Regarding the assignment of species II and III, however, we differ with these authors.[4] Species II is concluded by us to be *cis*-$Pt(NH_3)_2$(GMP-*N7*)$_2$ (vide supra).

One of the conclusions of Clore and Gronenborn[4] is that species I and II are two rotamers with GMP coordinated to platinum via N7, in which rotation occurs about the Pt–N7 bond. Because they observe both a constant ratio between species I and II at a certain temperature and a temperature-dependent equilibrium, they conclude that this rotation must be fast in terms of the overall reaction but slow on the NMR time scale. However, such a constant ratio between species I and species II is not observed in the present study. (See Figures 1 and 4). In addition Marcelis[20] reported the rotation about the Pt–N7 bond to be fast on the NMR time scale (between −50 and 90 °C) when the coordinating nitrogens are not substituted by bulky groups (experiments carried out at 100 MHz but later repeated at 300 MHz).[21] Therefore I and II cannot be slowly interconverting rotamers.

The titration behavior of species III indicates that in this compound one platinum is bound to GMP via the N1 and, likely, a second platinum via the N7. The appearance of peak III in the form of two peaks (separation only 0.006 ppm) needs further investigation.[22]

Because of our different assignment of species II and III the interpretation of the ^{195}Pt NMR measurements carried out by Clore and Gronenborn[4] has to be reconsidered. We believe that the signal at −2303.0 ppm (relative to K_2PtCl_6) in fact is a signal due to both species I (*cis*-$Pt(NH_3)_2$(GMP-*N7*)Cl and N7-coordinated Pt in species III; the signal at -2364.2 ppm arises from the second platinum in species III (N1-coordinated GMP to Pt). In that case the differences in chemical shift originating from GMP coordinated to platinum via N7 (-2303.0 ppm) and GMP coordinated to platinum via N1 (-2346.2 ppm) are 43.2 ppm. This is about the same value as Clore and Gronenborn report for the corresponding AMP species (43.9 ppm).[4] The signal for species II is now believed to be hidden at the low-field end of the spectrum, where the baseline rises.

In summary, we have shown that the reaction between *cis*-$PtCl_2(NH_3)_2$ and 5'-GMP allows the characterization of *cis*-$Pt(NH_3)_2$(GMP)Cl, *cis*-$Pt(NH_3)_2(GMP)_2$, and *cis*-Pt-$(NH_3)_2$(GMP)(OH_2). The assignment of these species can be followed in detail by the pH dependence of the ^1H NMR. No evidence for slow rotation (on the NMR time scale) about the Pt–N7 bond has been observed, and no indications for so-called N7–O6 chelates were found either.

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Registry No. I, 80594-64-5; II, 80594-67-8; III, 80612-24-4; IV, 89746-69-0; *cis*-$PtCl_2(NH_3)_2$, 15663-27-1.

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(22) Experiments at 300 and 500 MHz suggest that the two peaks do not derive from a J-split doublet but correspond to two separate H8 signals, arising from two different species. A possibility is that the two species differ in the fourth coordinating group in one or both of the platinums.