Nuclear Magnetic Resonance Studies of N–H Bonds in Platinum Anticancer Complexes: Detection of Reaction Intermediates and Hydrogen Bonding in Guanosine 5'-Monophosphate Adducts of $[PtCl_2(NH_3)_2]$

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Reactions between ^{15}N-labelled *cis*-$[PtCl_2(NH_3)_2]$ and guanosine 5'-monophosphate (GMP) have been studied in aqueous solutions at millimolar concentrations using ^{15}N-edited one-dimensional ^1H NMR spectroscopy, and two-dimensional $[^1H,^{15}N]$ heteronuclear multiple quantum coherence NMR spectroscopy; the reactive species $[PtCl(H_2O)(NH_3)_2]^+$ was detected, as well as hydrogen bonding in the product $[Pt(GMP\text{-}N^7)_2(NH_3)_2]^{2+}$.

The platinum(II) complexes cisplatin, *cis*-$[PtCl_2(NH_3)_2]$ **1**, and carboplatin [*cis*-bisammine(1,1-cyclobutanedicarboxylato)-platinum(II)] are widely used for the treatment of cancer,[1] and a large number of related platinum-(II) and -(IV) complexes containing ammine and primary or secondary amines are also active.[2] The N–H bonds in these complexes play an important role in their mechanism of action.[3,4] However, direct studies of Pt–N–H protons in aqueous solution have previously been hampered by the difficulty of detecting them by ^1H NMR spectroscopy.[4] In this communication we demonstrate that these systems can be studied with good sensitivity by using ^{15}N-labelled complexes in conjunction with the techniques of proton-detected heteronuclear NMR spectroscopy. Such experiments provide new insights into reactions of platinum anticancer complexes with nucleotides and other biomolecules, and can readily be applied to a wide range of other metal ammine and amine complexes.

We report here studies of reactions between cisplatin and guanosine 5'-monophosphate (GMP). Guanine-N^7 is the major Pt binding site when cisplatin reacts with DNA, and there is much interest in the mechanisms by which intrastrand guanine–guanine cross-links are formed after the initial binding to a guanosine base. The recognition processes are thought to involve hydrogen-bonding interactions between the co-ordinated ammines and phosphate and/or ring oxygens of the polynucleotide, following an initial attack by a reactive hydrolysis product such as $[PtCl(H_2O)(NH_3)_2]^+$ **2**.[3]

The Pt–NH_3 region of a normal ^1H NMR spectrum of a 8.5 mmol dm^{-3} solution of **1**† in the presence of 2 mol equivalents of GMP is shown in Fig. 1(*a*).‡ The N–H proton resonances of interest are partially obscured by the residual H_2O peak and resonances of the GMP sugar moiety. Fig. 1(*b*) is the spectrum of the same sample acquired using a simple spin-echo difference sequence[7] which selects only those protons coupled to ^{15}N. The almost total elimination of the resonances from the sugar protons and the effective suppression of the intense ^1H_2O resonance are apparent. Incorporation of ^{15}N-decoupling during acquisition collapses each doublet to a singlet, one for each type of co-ordinated NH_3 in the sample, Fig. 1(*c*). The assignment of the major resonance in Fig. 1(*c*) to the NH_3 protons of **1** is aided by the presence of (broadened) ^{195}Pt satellites. Weak peaks assigned to the hydrolysis product **2** were also present before the addition of GMP§ since the sample of cisplatin had been preincubated at 37 °C for 0.5 h.

The assignment of ^1H resonances was greatly aided by the use of a two-dimensional $[^1H,^{15}N]$ heteronuclear multiple quantum coherence (HMQC) experiment[8] (Fig. 2), which took 52 min and was carried out after an average reaction time of 1.5 h. This selects N–H proton signals and separates them according to their ^{15}N chemical shifts. In platinum(II) complexes, ^{15}N shifts are largely determined by the nature of the *trans* ligand.¶ The assignments‖ were made according to literature values for ^{15}N shifts[13] and were substantiated by time-dependent studies of ^{15}N-edited ^1H NMR spectra. During the first hour of the reaction there was a gradual decrease in intensity of resonances for **1** and the hydrolysis product **2**,[12] and an increase in intensity of two resonances assignable to the

† Labelled with >95% $^{15}NH_3$; the charge on GMP is ignored in formulae.

‡ The complex *cis*-$[PtCl_2(^{15}NH_3)_2]$ was synthesised as described previously[5] and recrystallized from aqueous KCl; $[Pt(^{15}NH_3)(H_2O)_2]^{2+}$ was prepared *in situ* by the addition of slightly less than 2 mol equivalents of $AgNO_3$ to a solution of *cis*-$[PtCl_2(^{15}NH_3)_2]$ and removal of the AgCl precipitate.

The ^1H-{^{15}N} NMR spectra were recorded at 37 °C on a Bruker AM-500 spectrometer fitted with a BSV-7 transmitter, a BFX-5 X nucleus decoupler and a 5 mm inverse probehead. Sample spinning was not used. All samples were prepared in 95% H_2O–5% D_2O, and the water signal was pre-irradiated (usually for 1.5 s) by means of a delays alternating with nutations for tailored excitation (DANTE) sequence.[6] One-dimensional ^{15}N-edited ^1H spectra were recorded using a spin-echo difference sequence,[7] optimized for $^1J(NH) = 73$ Hz. Typically 16 transients were acquired. Two-dimensional $[^1H,^{15}N]$ HMQC spectra were recorded using the standard sequence,[8] modified to include a pair of purge pulses for improved suppression of signals from protons not bound to ^{15}N, as proposed by Otting and Wüthrich.[9] The sequence was optimized for $^1J(NH) = 73$ Hz (6.8 ms), and the lengths of the purge pulses were adjusted for each sample in the range 0–3 ms to maximize solvent suppression, with the two pulses of different duration to avoid refocussing effects. Typically 150 t_1 increments of 0.329 ms were used, giving an acquisition time of 49.35 ms and a spectral width of 1520 Hz in the F_1 dimension. The spectral width in the F_2 dimension was 2000 Hz, and for each increment, 8 scans and 2 dummy scans were recorded. After zero filling, the final digital resolution was 3.0 and 1.9 Hz in the F_1 and F_2 dimensions respectively. Two-dimensional spectra were acquired using the time-proportional phase incrementation (TPPI) method[10] to give absorption mode lineshapes with sign discrimination in F_1. During the acquisition time of the two-dimensional experiments, the ^{15}N spins were decoupled by irradiating with the GARP-1 sequence[11] at a field strength of 1.7 kHz. Two-dimensional spectra were processed using sine-bell weightings in both dimensions. Proton spectra (at 500.13 MHz) were referenced to sodium 3-(trimethylsilyl)tetradeuteriopropionate, and ^{15}N spectra (at 50.67 MHz) to 1.5 mol dm^{-3} NH_4Cl in 1 mol dm^{-3} HCl (external).

§ In separate experiments[12] we have studied the hydrolysis of cisplatin and the pH-dependent behaviour of the shifts of **2** and $[Pt(NH_3)_2(H_2O)_2]^{2+}$.

¶ Nitrogen-15 shifts [and $^1J(^{195}Pt^{15}N)$ values] for ammine complexes show a strong dependence on the *trans* ligand. Approximate ranges (δ) are: S −40 to −50, N, Cl −50 to −70 and O −75 to −95.

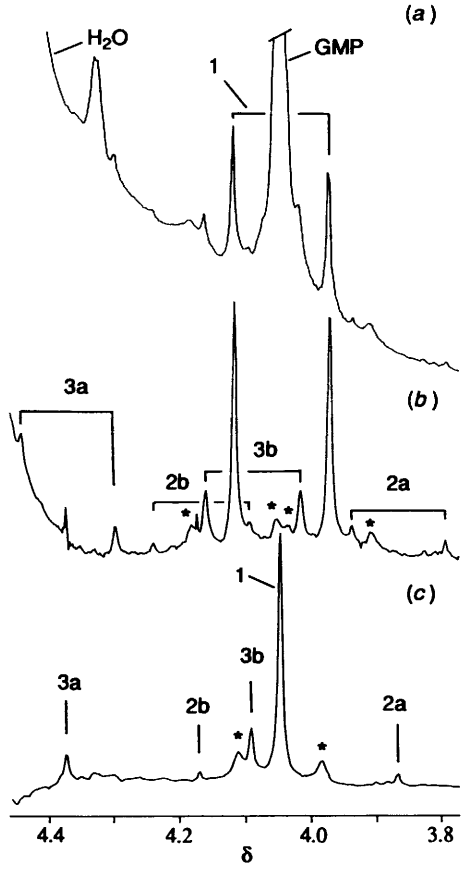

Fig. 1 The NH_3 region of the 500 MHz ^1H NMR spectra of a solution of *cis*-$[PtCl_2(^{15}NH_3)_2]$ **1** (8.5 mmol dm^{-3}, incubated at 37 °C for 0.5 h in 14 mmol dm^{-3} sodium phosphate buffer, 5% D_2O, pH 6.5), 25 min (average reaction time) after addition of 2 mol equivalents of GMP: (*a*) acquired by normal single-pulse acquisition; (*b*) (^{15}N-coupled) and (*c*) (^{15}N-decoupled) are ^{15}N-edited spectra acquired using a spin-echo difference sequence. Assignments of Pt–NH_3 peaks: 1, *cis*-$[PtCl_2(NH_3)_2]$; 2, *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ (2a, NH_3 *trans* to H_2O/OH; 2b, NH_3 *trans* to Cl); 3, *cis*-$[PtCl(GMP\text{-}N^7)(NH_3)_2]^+$ (3a, NH_3 *trans* to N; 3b, NH_3 *trans* to Cl); *, ^{195}Pt satellites of 1 [$^2J(^{195}Pt^1H) = 64$ Hz]

monosubstituted complex *cis*-$[PtCl(GMP\text{-}N^7)(NH_3)_2]^+$ **3**. Peaks for **3** then decreased in intensity, but curiously no new NH_3 peak for the expected bis-substituted product *cis*-$[Pt(GMP\text{-}N^7)_2(NH_3)_2]^{2+}$ **4** appeared despite the presence of the expected major peak at δ 8.65 for the H^8 protons in **4**,[14–16] and detection of a major ^{15}N peak at δ −66.6 by direct ^{15}N NMR spectroscopy. Only after addition of a water relaxation agent $(NH_4)_2SO_4$[17] was it possible to locate the missing NH_3 peak for **4** directly beneath the H_2O resonance (Fig. 2). Complex **4** was the major species observed in the ^{15}N-edited ^1H spectrum after the reaction had reached equilibrium (*ca.* 16 h).

The proposed assignments of ^1H NMR peaks for the ammine ligands *trans* (3a) or *cis* (3b) to GMP in *cis*-$[PtCl(GMP\text{-}N^7)(NH_3)_2]^+$ are based on a comparison with those for *cis*-$[Pt(H_2O)(GMP\text{-}N^7)(NH_3)_2]^{2+}$ **5**, generated *in situ* by addition of 1 mol equivalent of GMP to an aqueous solution of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. Complex **5** gave ^1H–^{15}N two-dimensional cross-peaks at δ 4.48/−85.1 and 4.37/−66.6,

† The H and ^{15}N chemical shifts (different ammine ligands in the same complex denoted a and b; *trans* ligand in brackets; N is N^7 of GMP; O is H_2O or OH).

Complex	δ(^1H)	δ(^{15}N)	Complex	δ(^1H)	δ(^{15}N)
1 (Cl)	4.05	−68.7	4 (N)	4.67	−66.6 (pH 7.9)
2a (O)	3.87	−85.7		4.47	−67.5 (pH 4.4)
b (Cl)	4.17	−67.3	5a (N)	4.37	−66.6
3a (N)	4.38	−66.4	b (O)	4.48	−85.1
b (Cl)	4.09	−69.1			

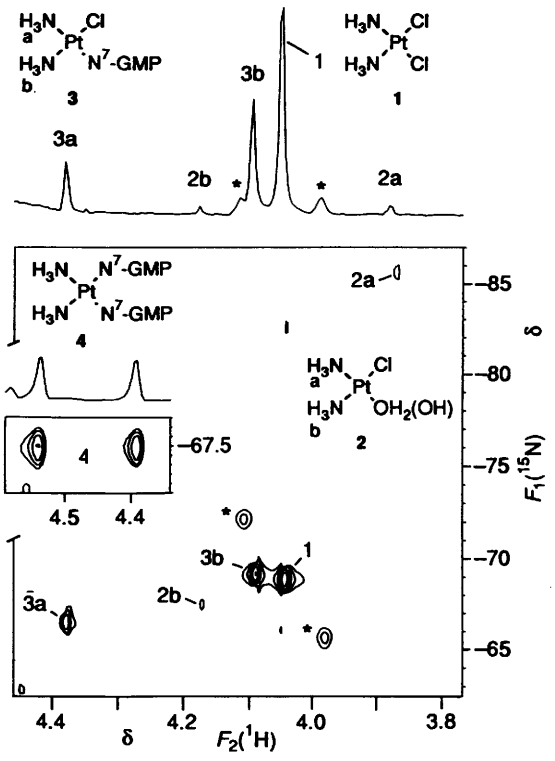

Fig. 2 Two-dimensional [^1H,^{15}N] HMQC spectrum of a solution containing 8.5 mmol dm^{-3} *cis*-$[PtCl_2(^{15}NH_3)_2]$ and 2 mol equivalents of GMP at pH 6.5 after an average reaction time of 1.5 h at 37 °C (sample as in Fig. 1). The total measuring time was 52 min. The F_2 projection represents the ^{15}N-edited ^1H spectrum with ^{15}N decoupling. Assignments as in Fig. 1; 4, *cis*-$[Pt(GMP\text{-}N^7)_2(NH_3)_2]^{2+}$. The two ^{195}Pt satellites (*) associated with 1 correspond to the $^2J(^{195}Pt^1H)$ coupling constant (64 Hz) in the F_2 dimension and to the $^1J(^{195}Pt^{15}N)$ coupling (300 Hz) in the F_1 dimension. At this pH, the NH_3 resonance for 4 lies beneath the H_2O peak. It was revealed by lowering the pH of the same sample at equilibrium to 4.35 and adding a H_2O relaxation agent [0.5 mol dm^{-3} $(NH_4)_2SO_4$]. This is shown in the insert, which, for clarity, is an ^{15}N-coupled spectrum (*i.e.* a doublet in F_2)

which are unambiguously assignable to NH_3 ligands *trans* to H_2O and GMP respectively.

Since only one NH_3 peak is observed for **4**, any head-to-tail isomerism of co-ordinated GMPs must be fast on the NMR time-scale.[15,16,18] The large downfield shift (0.62 ppm) of the NH_3 resonance for **4** when compared to **1** is consistent with hydrogen-bonding interactions between Pt–NH_3 and the de-protonated 5′-phosphate of GMP. Indeed, when the pH of the sample was lowered to 4.3 to protonate this group, the downfield shift was reduced to 0.42 ppm.‖ Since the low-field shifts of NH_3 peaks for the mono GMP complex **3** are 0.33 and <0.1 ppm for ammines *trans* to GMP and Cl respectively, it can be concluded that hydrogen bonding is much weaker than in the bis complex although a change in the orientation of the purine base relative to the co-ordination plane could also influence the shifts. The observation of peaks for **2** during these reactions (accounting for *ca.* 5% of the Pt after 1.5 h) is significant since this complex was not observable in ^{195}Pt NMR spectra from reactions of (^{195}Pt-enriched) cisplatin with polynucleotides under similar conditions.[19]

In conclusion, the use of ^1H detected one- and two-dimensional [^1H,^{15}N] HMQC experiments allows the direct study of Pt–NH protons in aqueous solutions of platinum anticancer drugs and related complexes at concentrations approaching those of physiological relevance. One-dimensional ^{15}N-edited ^1H spectra, with good signal-to-noise ratios, can be acquired in only a few minutes from solutions of platinum ammine complexes at millimolar concentrations. Thus, it is practical to follow reaction kinetics *via* one-dimensional

spectra, and acquire two-dimensional [^{1}H,^{15}N] HMQC spectra at selected time intervals to allow assignments of Pt–N–H species *via* their ^{15}N chemical shifts. The two-dimensional [^{1}H,^{15}N] experiment has several major advantages over direct observation of ^{15}N. The most apparent is the large gain in sensitivity.

This technique offers potential for a wide range of equilibrium and kinetic studies on platinum drugs, for example their activation by hydrolysis,[12] hydrogen bonding in nucleotide and DNA complexes, and reactions with proteins, biofluids and cells. These methods are also more generally applicable to complexes of other metals with ammines and amines.

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