Influence of dT_{20} and $[d(AT)_{10}]_2$ on Cisplatin Hydrolysis Studied by Two-Dimensional [^{1}H,^{15}N] HMQC NMR Spectroscopy

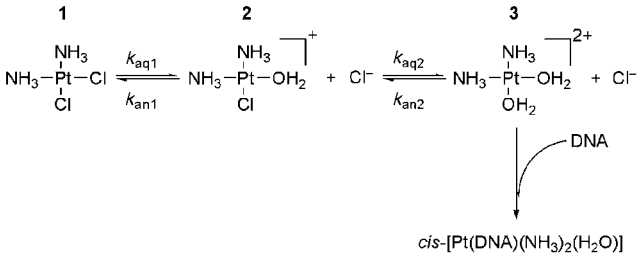
Jo Vinje,*[a] Einar Sletten,[a] and Jiří Kozelka*[b]

Abstract: The influence of the presence of DNA on the kinetics of cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) aquation (replacement of Cl^- by H_2O) and anation (replacement of H_2O by Cl^-) involved in the hydrolysis of cisplatin have been determined by two-dimensional [^{1}H,^{15}N] HMQC NMR spectroscopy. Single-stranded dT_{20} and double-stranded $[d(AT)_{10}]_2$ oligonucleotides were used as DNA models, avoiding guanines which are known to react rapidly with aquated cisplatin forms. Reactions starting from *cis*-$[PtCl_2(^{15}NH_3)_2]$, or from a stoichiometric mixture of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ and Cl^- (all 0.5 mM Pt^{II}; in ionic strength, adjusted to 0.095 M or 0.011 M with $NaClO_4$, pH between 3.0 and 4.0) were followed in an NMR tube in both the absence and presence of 0.7 mM dT_{20} or $[d(AT)_{10}]_2$. In the presence of dT_{20}, we observed a slight and ionic-strength-independent decrease (15–20%) of the first aquation rate constant, and a more significant decrease of the second anation rate constant. The latter was more important at low ionic strength, and can be explained by efficient condensation of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ on the surface of single-stranded DNA, in a region depleted of chloride anions. At low ionic strength, we observed an additional set of [^{1}H,^{15}N] HMQC spectral signals indicative of an asymmetric species of PtN_2O_2 coordination, and we assigned them to phosphate-bound monoadducts of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$. Double-stranded $[d(AT)_{10}]_2$ slowed down the first aquation step also by approximately 15%; however, we could not determine the influence on the second hydrolysis step because of a significant background reaction with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$.

Keywords: antitumor agents · DNA · kinetics · NMR spectroscopy · platinum

Introduction

The antitumor drug cisplatin, *cis*-$[PtCl_2(NH_3)_2]$ (**1**) reacts with cellular DNA and forms GG intrastrand cross-links as the major lesions.[1] In vitro, the rate-determining step is the hydrolysis of **1** to *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**) following first-order kinetics (see Scheme 1). This was initially demonstrated by Horacek and Drobnik by simple comparison of the rate constants,[2] and later proven by Berners-Price, Sadler and co-workers using [^{1}H,^{15}N] HSQC NMR spectroscopy.[3] This spectroscopic method allows **2** to be identified



Scheme 1. Hydrolysis of cisplatin and the subsequent primary reaction with DNA. The numbers above the platinum species indicate the numbering used in this study.

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Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author. Figure S1 shows the experimental concentrations and theoretically fitted curves for the hydrolysis of **1** in the presence of $[d(AT)_{10}]_2$. Input files for the program SCIENTIST used for optimization of rate constants, corresponding to the systems shown in Figures 3–8 in the text and in Figure S1 in the Supporting Information.

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unambiguously as one of the species that reacts with DNA. However, it has been shown that *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**), formed in the second hydrolysis step, can be a competing species in the in vitro platination of DNA.[4] This diaqua complex reacts with DNA two orders of magnitude faster than **2**.[4–6]

Hence, hydrolysis of cisplatin has to be accounted for in kinetic studies of reactions with DNA. This has been done so far using rate constants determined for cisplatin hydrolysis in aqueous solutions in the absence of DNA. This approach is, however, questionable, since the presence of DNA can influence the substitution reactions involved in cisplatin hydrolysis. For instance, the positively charged platinum species could partly displace counterions condensed at the DNA surface and thus become exposed to the strong electrostatic field of DNA. This could affect the stability of transition states and substitution kinetics. In addition, since the DNA surface is depleted of chloride anions,[7] the anation reactions could be inhibited with respect to the bulk solution. Finally, even the electroneutral dichloro form, which has a non-negligible electric dipole, could interact with DNA. This could affect the first hydrolysis rate. Therefore, all the reaction rates deduced for cisplatin hydrolysis might in principle be affected by the presence of the DNA polyanion and be different from those deduced from reactions in bulk solution.

It has been reported that the first aquation step of cisplatin is approximately 30% slower in the presence of a 14 bp DNA duplex (bp = base pair).[8] However, the duplex used contained a GG site and was therefore quite reactive towards the hydrolyzed cisplatin species, so that neither the first anation rate nor the rates of the second hydrolysis step could be deduced and the determined first aquation rate constant was therefore intrinsically imprecise. We have also previously studied the rate of reaction between cisplatin and guanine-containing DNA oligonucleotides by two-dimensional (2D) [^{1}H,^{15}N] HMQC NMR spectroscopy.[9] The work showed that the rapid reaction of **2** with guanine makes it impossible to determine the hydrolysis rate constants of cisplatin accurately. In the present work, we used less reactive DNA models (single-stranded dT_{20} and duplex $[d(AT)_{10}]_2$) and, in contrast to the previous study, in which the pH was kept constant using a phosphate buffer,[8] we studied the cisplatin hydrolysis in unbuffered acidic solution. At the submillimolar concentrations of reactants that we used, the maximum change in pH during a reaction was 0.6.

Results

Identification of species observed in 2D [^{1}H,^{15}N] HMQC NMR spectra

*Hydrolysis of cis-$[PtCl_2(NH_3)_2]$ (**1**) and chloride anation of cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**)*: The 2D [^{1}H,^{15}N] HMQC spectrum of **1** in $NaClO_4$ (100 mM, pH 3.8 at 293 K) initially showed one peak at $\delta(^1H)/\delta(^{15}N)$ of 4.09/−69.02 ppm corresponding to **1** and two peaks at 4.33/−67.16 and 4.24/−90.27 ppm corresponding to **2**, indicating that the first hydrolysis step set in already during the dissolution process. For the two peaks assigned to **2**, the former signal is from the $^{15}NH_3$ group *trans* to the chloride and the latter from that *trans* to the water ligand. After 4 h, the signal corresponding to **3** appeared at 4.49/−86.95 ppm. The ^{1}H and ^{15}N chemical shifts observed for the species **1**--**3** agree with previously published [^{1}H,^{15}N] NMR data.[10] The same NMR signals were observed during the reverse reaction, initiated by adding one equivalent of NaCl to an aqueous solution of **3** (0.5 mM) in $NaClO_4$ (100 mM), pH 4.0 at 293 K. Table 1 gives $\delta(^1H)/\delta(^{15}N)$ values for all the species observed in this work.

Table 1. ^{1}H and ^{15}N NMR chemical shift values for all species observed in this paper.[a]

Species		$\delta(^1H)/\delta(^{15}N)$	*trans* ligand
cis-$[PtCl_2(NH_3)_2]$	**1**	4.07/-69.16	Cl
cis-$[PtCl(H_2O)(NH_3)_2]^+$	**2**	4.31/−67.16	Cl
		4.22/−90.10	O
cis-$[Pt(H_2O)_2(NH_3)_2]^{2+}$	**3**	4.50/−86.97	O
cis-$[Pt(NH_3)_2\{N3T,O4T\}]^+$	**4**	4.11/−66.47	N
		4.17/−91.42	O
[b]	**5**	3.80/−67.91	N?
cis-$[Pt(NH_3)_2\{N3T,N3T\}]$	**6a**	3.80/−71.03	N
		3.86/−71.39	N
cis-$[Pt(NH_3)_2\{N3T,N3T\}]$	**6b**	3.89/−72.81	N
		3.92/−73.65	N
[b]	**7**	4.31/−84.53	O?
[b]	**8**	4.44/−86.10	O
		4.49/−89.00	O

[a] The shifts of the signals for **1**–**5** are taken from the experiment between **1** and dT_{20} (93 mM $NaClO_4$, 293 K) after 40 h reaction time (final pH ≈ 3.3). The shift of **6** is measured from the same experiment, but weeks after the reaction was initiated (pH 3.0). The shift of **8** is from the anation of **3** in presence of dT_{20} (9 mM $NaClO_4$, pH 3.2, 293 K). The shifts for all species were identical (except the ones involving an aqua ligand) from experiment to experiment. [b] Not identified.

*Hydrolysis of cis-$[PtCl_2(NH_3)_2]$ (**1**) in the presence of dT_{20}*: A solution of **1** (0.5 mM) and dT_{20} (0.7 mM) in $NaClO_4$ (93 mM) (the combined concentrations of $NaClO_4$ and dT_{20} yielding an ionic strength of 0.095 M)[11] at initial pH 3.6 and temperature of 293 K showed the same peaks as in the experiment without dT_{20} (vide supra) at first. However, after 12 h, two new peaks of equal intensities emerged at $\delta = 4.11/-66.47$ and 4.17/−91.42 ppm, corresponding to a species **4** with the *cis*-$Pt(^{15}NH_3)_2^{2+}$ moiety bound to N and O ligands in the *trans* position, respectively. Species **4** appeared only after a significant accumulation of **3**, indicating that it was formed from **3**. Indeed, **4** was also formed in a control reaction between **3** and dT_{20}, and apparent first-order rate constants for the formation of **4** from **3** from the two experiments were similar (vide infra). A drop in pH during the reactions in which **4** was formed suggested that **4** contains deprotonated N3-Pt-bound thymine moieties. The persistence of **4** for months at low pH identifies it as a relatively stable product rather than a reactive intermediate. The chemical

shifts of the [^{1}H,^{15}N] HMQC peaks of **4** are insensitive to pH changes, which rules out the assignment of **4** as an aqua complex.[10] Furthermore, in experiments in which chloride ions are present, one would expect reversible anation of an aqua complex to a chloro monoadduct, which was not observed. Lippert et al.[12] have shown that the aqua complexes *cis*-[Pt(NH$_3$)$_2$(H$_2$O)L]$^+$ (L = 1-MeU$^-$ or 1-MeT$^-$) coordinated by N3 readily dimerize to N3,O4-bound dimers *cis*-[Pt(NH$_3$)$_2$L]$_2^{2+}$ or to hydroxo-bridged dimers *cis,cis*-[Pt(NH$_3$)$_2$L–OH-Pt(NH$_3$)$_2$L]$^{2+}$. The N3,O4 dimer was shown to be preferred at acidic pH.[12] Therefore **4** was tentatively assigned to an N3,O4-bound cisplatin–thymine dimer possibly formed by two adjacent thymines.

After 24 h, a minor peak appeared at $\delta = 3.80/-67.94$ ppm representing another species, **5**, which was also stable for several months. This species was not seen in the reaction between **3** and dT$_{20}$ in absence of chloride ions (vide infra). However, in all high-salt reactions between cisplatin and dT$_{20}$ containing chloride ions the species was detected. Figure 1 shows the 2D [^{1}H,^{15}N] HMQC spectrum recorded after 40 h, with cross-peaks representing species **1**–**5**.

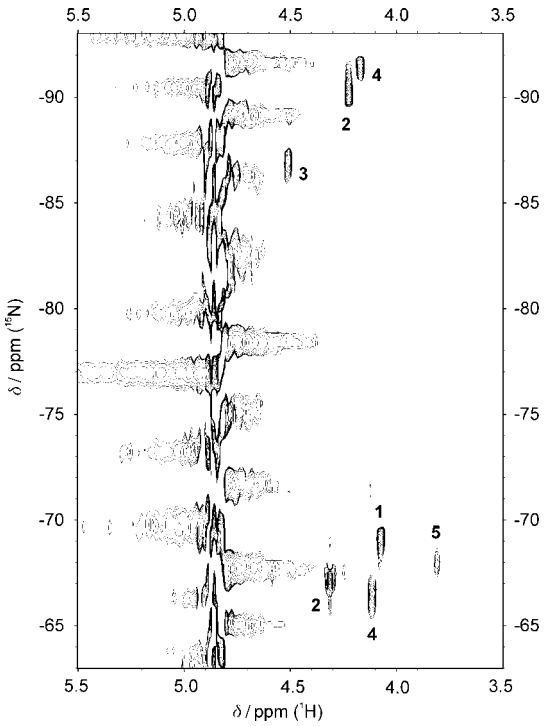

Figure 1. 2D [^{1}H,^{15}N] NMR spectra of *cis*-[PtCl$_2$(NH$_3$)$_2$] (**1**; 0.5 mM) reacted with dT$_{20}$ (0.7 mM) (93 mM NaClO$_4$, pH $\approx$ 3.3, 293 K) after 40 h reaction time. For labeling of the peaks, see Table 1. The noise at δ(^{1}H) 4.8–4.9 ppm is due to the water signal.

The 2D [^{1}H,^{15}N] HMQC spectrum recorded several weeks later (Figure 2) showed, in addition to the peaks due to species **4** and **5** (**1**–**3** were completely consumed at that point), two pairs of equally intense peaks at $\delta = 3.80/-71.03$, 3.86/−71.39 ppm (species labeled **6a**) and at $\delta = 3.89/-72.81$, 3.92/−73.65 ppm (species labeled **6b**). The chemical shifts of

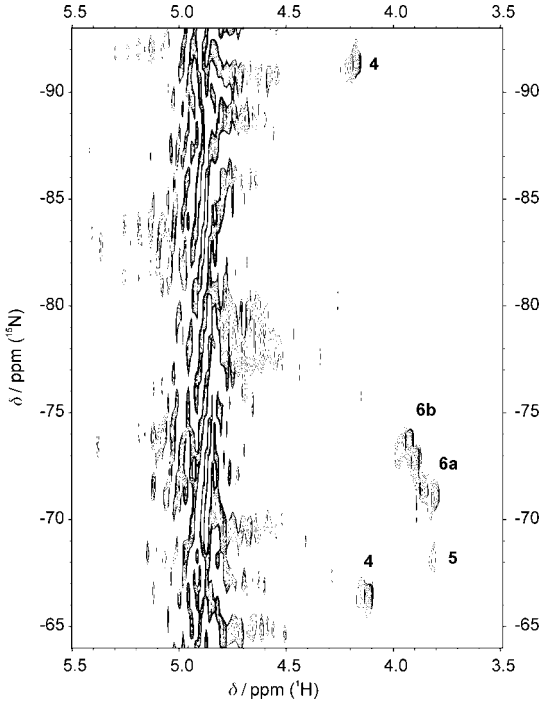

Figure 2. 2D [^{1}H,^{15}N] NMR spectra of *cis*-[PtCl$_2$(NH$_3$)$_2$] (**1**; 0.5 mM) reacted with dT$_{20}$ (0.7 mM) (93 mM NaClO$_4$, pH 3.0, 293 K) after several weeks reaction time. For labeling of the peaks, see Table 1. The noise at δ(^{1}H) 4.8–5.0 ppm is due to the water signal.

these peaks are consistent with Pt(T-N3,T-N3) cross-linked adducts. The very late appearance of **6a** and **6b** suggested that these adducts were formed from **4**.

*Control reaction between cis-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ (**3**) and dT$_{20}$*: A solution of **3** (0.5 mM) was combined with a solution of dT$_{20}$ (0.7 mM) in NaClO$_4$ (93 mM) at 293 K. Two experiments were performed with initial pH of 3.3 and 3.7. The species **4** was the only product observed during the experiment starting at pH 3.3. In the experiment starting at pH 3.7, the peaks characteristic of species **6a** and **6b** started to appear after 15 h of reaction time. The formation of **4** from **3** followed first-order kinetics, and the transformation of **4** into **6a** and **6b** could also be fitted assuming first-order reactions.

To gain more insight into the reaction mechanism of the reaction between **3** and dT$_{20}$ yielding **4**, we followed the reaction in another experiment at an initial pH of 6.1, which is above the pK_a of **3**,[10] under otherwise identical conditions. The reaction was obscured by the rapid formation of platinum hydroxo oligomers manifested by several peaks in the region between $\delta = -80.3$ and -86.3 ppm (^{15}N) and $\delta = 3.9$ and 4.3 ppm (^{1}H). The formation of these compounds from **3** followed roughly first-order kinetics ($t_{1/2} \approx 3$ h) and they became the major fraction after approximately 5 h. The species **4** reached a concentration of about 0.05 mM within 3 h and remained constant for about 5 h, then subsequently converted to the N3,N3-cross-linked compounds **6a** and **6b**. This "lag-phase" between the appearance of **4** and that of

6a and **6b**, observed both at pH 3.7 and 6.1, suggests that this conversion is a multistep reaction, the individual steps of which (perhaps some structural rearrangements of the single helix of the cross-linked dT_{20}) are not observable by NMR spectroscopy. Although our data do not enable us to propose a firm kinetic model for the conversion of **4** to **6a** and **6b**, it is clear that this conversion is faster at pH 6.1 than at pH 3.3 or 3.7, consistent with deprotonation of N3 accompanying this conversion. An important observation is that the NH_3 chemical shifts of **4** at pH 6.1 and pH 3.3 are virtually identical, indicating that its oxygen ligand is not water. The chemical shifts of compounds **6a** and **6b** varied also very little from one experiment to another.

The formation of **4** from **3** and dT_{20} was faster at initial pH 3.7 than at pH 3.3; however, at pH 6.1, **4** was formed more slowly than in the other two experiments. The kinetics of this reaction depends on the protonation state of **3**, and if thymine N3 is involved, on the protonation state of thymine. The fact that the apparent rate constant goes through a maximum, when the pH varies from 3.3 to 6.1, indicates that the mechanism of this reaction is rather complex. It would be tempting to suggest that singly deprotonated **3**, *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$, preferentially reacts with dT_{20}, by using its coordinated OH^- group as base to abstract a proton of thymine N3. However, the apparent drop in the rate constant above the pK_a of **3** (5.37)[10] does not support such a mechanism.

*Chloride anation of cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) in the presence of dT_{20}*: A solution of **3** (0.5 mM) and the equivalent amount of NaCl was combined with a solution of dT_{20} (0.7 mM) in $NaClO_4$ (93 mM), pH 3.6 at 293 K. The formation of **2** and **4** occurred after a few minutes, and after about 30 h the NMR spectral signals from **1** and **5** appeared. In the spectrum recorded after about 47 h, two additional peaks of equal intensities were observed at $\delta = 3.88/-72.98$ and 3.92/−74.11 ppm, corresponding to the cross-linked Pt-{T-N3,T-N3} adduct **6b** (vide supra). The final spectrum recorded several weeks later contained the peaks due to **4** (38%), **6a** (20%), and **6b** (42%).

The experiment was repeated under identical conditions, except that the initial pH was slightly lower (pH 3.3). The same reaction pattern as in the first experiment was seen, except that after 7 h an additional single peak appeared at $\delta = 4.31/-84.53$ ppm, attributable to compound **7**. The ^{15}N chemical shift corresponds to $^{15}NH_3$ group having an O ligand in the *trans* position, and since there is only one cross-peak of this type, **7** must have two equivalent O ligands. In the final spectrum recorded several weeks later, peaks attributable to **4** (75%), **5** (4%), **6b** (12%), and **7** (9%) were present. Compound **7** was stable for weeks and its chemical shift was insensitive to pH. Importantly, the same peak was observed at identical shifts in a control reaction at initial pH 5.0 (0.5 mM of **3**, 0.7 mM of dT_{20}, 93 mM $NaClO_4$, and 0.2 mM of NaCl). The nature of compound **7** is not clear at this moment.

*Hydrolysis of cis-$[PtCl_2(NH_3)_2]$ (**1**) in the presence of dT_{20} at low ionic strength*: Complex **1** (0.5 mM) and dT_{20} (0.7 mM) were reacted at 293 K in $NaClO_4$ (9 mM) and at an initial pH of 3.2. A similar reaction pattern was observed as for the analogous experiment at higher ionic strength. The occurrence of species **2**–**5** was detected at similar times and the chemical shifts in NMR spectra of the species **1**–**5** were identical. The only new feature was two minor peaks at $\delta = 4.44/-86.10$ and 4.49/−89.00 ppm. The two peaks had similar intensity, and appeared at the same reaction time (15 h). They therefore apparently belong to the same species, labeled **8**. The chemical shifts of **8** were close to those of **3**, and according to their $\delta(^{15}N)$ values, they have two O ligands *trans* to the ammine groups. The species **8** was also observed in the anation experiment at low-salt conditions (vide infra), and its concentration seemed to be in a constant ratio of about 0.3 with that of **3**. This suggested that **3** and **8** form an equilibrium. A likely explanation is that **8** is formed from **3** by reversible substitution of one H_2O ligand by a phosphodiester group. A similar phosphodiester-bound complex formed by $[Pt(NO_3)(NH_3)_3]^+$ with the dinucleotide d(TpT) was previously reported.[13]

*Chloride anation of cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) in the presence of dT_{20} at low ionic strength*: Complex **3** (0.5 mM), the equivalent amount of NaCl, and dT_{20} (0.7 mM) were reacted at 293 K in $NaClO_4$ (9 mM) and at an initial pH of 3.2. The reaction evolved similarly to the analogous experiments performed at higher ionic strength, the only difference being a faster formation of **4** and a lower concentration of **2** throughout. The chemical shifts of the compounds **1**–**4** did not change with respect to the experiments at higher ionic strength. One striking difference was the appearance of species **8** (also observed during the hydrolysis of **1** in low-salt conditions, vide supra). In the present case, complex **8** was manifest already in the initial spectrum as a major product, amounting to about one third of the concentration of **3**. This ratio remained unchanged during the experiment, supporting the hypothesis of a fast equilibrium between **3** and **8**.

*Hydrolysis of cis-$[PtCl_2(NH_3)_2]$ (**1**) in the presence of the duplex $[d(AT)_{10}]_2$*: The hydrolysis of **1** (0.5 mM) in $NaClO_4$ (97 mM), pH 4.0 and the self-complementary duplex $[d(AT)_{10}]_2$ (0.35 mM; equivalent to 0.7 mM single-strand concentration) was studied at 293 K. In the 2D [^1H,^{15}N] HMQC spectra only the cross-peaks assigned to **1** and **2** were observed. Evidently, the cross-peaks representing cisplatin–$[d(AT)_{10}]$ adducts were obscured by the water peak. Thus, the concentrations of these adducts during the reaction were estimated from the decrease of the sum of intensities due to **1** and **2** during the reaction. The diaqua species **3** was not detected during the experiment, probably due to the fast reaction of $[d(AT)_{10}]_2$ with **3** and with **2**.

*Chloride anation of cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) in the presence of the duplex $[d(AT)_{10}]_2$*: Complex **3** (1.0 mM) in $NaClO_4$ (93 mM), pH 3.9, NaCl (1.0 mM), and the self-com-

plementary duplex $[d(AT)_{10}]_2$ (0.7 mM) were mixed in an attempt to study the anation rate of **3** at 293 K. The background reaction of **3** with $[d(AT)_{10}]_2$ was in this case considerably faster, the pseudo first-order rate constant being approximately $80\times10^{-5}\,s^{-1}$, which is 40 times faster than with dT_{20}. Due to this rapid background reaction, the amount of **2** produced from the anation of **3** was $<5\%$ of the total. Under these conditions, no precise rate constants for the second hydrolysis step of cisplatin could be determined.

Determination of rate constants

*Hydrolysis of **1** and chloride anation of **3** monitored at ionic strength of 0.1 M in the absence and in the presence of dT_{20}*: Figure 3 shows the concentration curves for the reversible formation of **2** and **3** from **1** in the absence of dT_{20}, and Figure 4 displays the curves for the stoichiometric reaction between **3** and chloride. The rate constants determined to optimize the fit between calculated and experimental concentration curves (Table 2) agree well with those determined previously,[8,14] when the different temperatures and ionic strengths are taken into account.

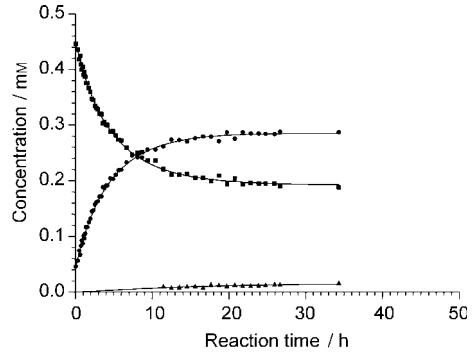

Figure 4. Experimental concentrations (from NMR data) and theoretically fitted curves for the anation of *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$ (**3**; 0.5 mM) in the presence of one equivalent NaCl and 100 mM $NaClO_4$, pH 4.0, 293 K. Symbols: (▲): **1**, (●): **2**, (■): **3**.

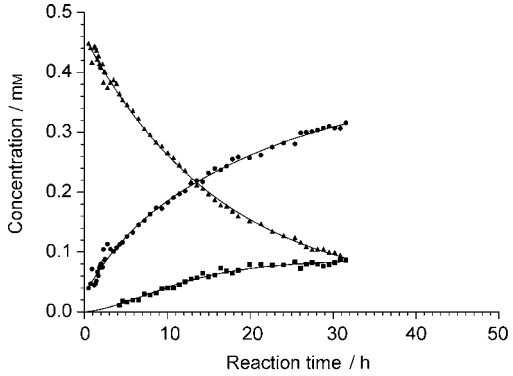

Figure 3. Experimental concentrations (from NMR data) and theoretically fitted curves for the hydrolysis of *cis*-$[PtCl_2(NH_3)_2]$ (**1**; 0.5 mM) (100 mM $NaClO_4$, pH 3.8, 293 K). Symbols: (▲): **1**, (●): **2**, (■): **3**.

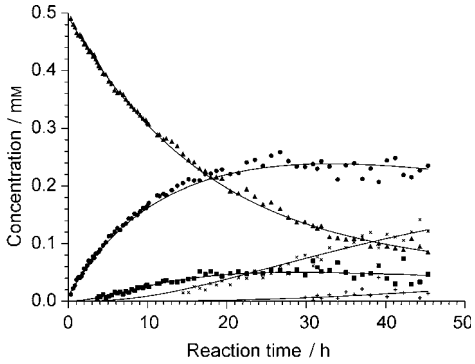

Figure 5. Experimental concentrations (from NMR data) and theoretically fitted curves for the hydrolysis of *cis*-$[PtCl_2(NH_3)_2]$ (**1**; 0.5 M) in the presence of dT_{20} (0.7 mM) (93 mM $NaClO_4$, pH 3.6, 293 K). dT_{20} was desalted by HPLC. Symbols: (▲): **1**, (●): **2**, (■): **3**.(×): **4**, (+): **5**.

Figure 5 shows the concentration curves for the species observed during hydrolysis of **1** in the presence of dT_{20}. Apart from the aqua complexes **2** and **3**, the formation of species **4** and, subsequently, that of **5** are observed. The kinetic model to which the calculated curves correspond assumed first-order formation of **4** from **3** and first-order formation of **5** from **4**. The optimized rate constants are listed in Tables 2 and 3.

Figure 6 displays the concentration curves for species **1–5** determined for the chloride anation of **3** in the presence of dT_{20}. The initial non-zero slope of the concentration curve of **4** confirms that this species is formed from **3**. It can be seen from the initial slopes of the concentration curves for **2** and **4** that under our experimental conditions (in particular at the concentration of dT_{20} comparable with the initial concentration of chloride) the reaction between **3** and dT_{20} competes with the chloride anation of **3**, but does not obscure it. As the standard deviations of the optimized rate constants show (Table 2), it does not impede accurate measurement of k_{an2}

Table 2. Aquation and anation rate constants (2×standard deviation in parentheses) for cisplatin. Experiments recorded at 293 K, pH 3.0–4.0, and 100–93 mM $NaClO_4$.

Experiment	pH (initial)	k_{aq1} [$s^{-1}\times10^{-5}$]	k_{an1} [$M^{-1}s^{-1}$]	k_{aq2} [$s^{-1}\times10^{-5}$]	k_{an2} [$M^{-1}s^{-1}$]
Hydrolysis of **1**	3.8	1.58 (4)	0.0024 (12)	1.23 (14)	0.092 (14)
3+1 equiv Cl^-	4.0	[a]	0.0039 (6)	1.23 (3)	0.078(2)
1+dT_{20}[b]	3.6	1.36 (3)	0.0045 (8)	1.20 (4)	[c]
3+dT_{20}+1 equiv Cl^- [b]	3.6, 3.3	[d]	0.0043 (9)	0.98 (6)	0.061 (3)
1+$[d(AT)_{10}]_2$	4.0	1.36 (7)	0.006 (4)	[e]	[f]
1+dT_{20}[g]	3.2	1.44 (2)	0.0056 (6)	0.97 (2)	[h]
3+dT_{20}+1 equiv Cl^- [g]	3.2	[i]	0.003 (3)	1.1 (2)	0.022 (4)

[a] Fixed at $1.58\times10^{-5}\,s^{-1}$. [b] Average of two experiments. [c] Fixed at $0.061\,M^{-1}s^{-1}$. [d] Fixed at $1.36\times10^{-5}\,s^{-1}$. [e] Fixed at $1.20\times10^{-5}\,s^{-1}$. [f] Fixed at $0.04\,M^{-1}s^{-1}$. [g] 9 mM $NaClO_4$. [h] Fixed at $0.022\,M^{-1}s^{-1}$. [i] Fixed at $1.44\times10^{-5}\,s^{-1}$.

Table 3. Rate of platination (2×standard deviation in parentheses) for the reaction between **3** and dT_{20} or $[d(AT_{10})]_2$. Experiments run at 293 K and 93 mM $NaClO_4$.

Experiment	pH (initial)	k_{pt1} $[s^{-1}\times10^{-5}]$[a]	k_{pt2} $[s^{-1}\times10^{-5}]$[b]
3+dT_{20}	3.3	1.63 (2)	
3+dT_{20}	3.7	2.28 (4)	0.236 (8)
3+dT_{20}	6.1	1.0 (3)	1.8 (4)
3+dT_{20}+1 equiv NaCl	3.6	1.60 (6)	
3+dT_{20}+1 equiv NaCl	3.3	1.45 (5)	
3+dT_{20}+1 equiv Cl^- [c]	3.2	2.21 (7)	0.09 (1)
3+$[d(AT_{10})]_2$+1 equiv NaCl[d]	3.9	78 (4)	

[a] Rate of formation of **4**. [b] Rate of formation of **6** (**6a**+**6b**). [c] 9 mM $NaClO_4$. [d] Rate of monofunctional platination of adenine. In the experiment of **1**+$[d(AT_{10})]_2$ species **2** was found to react with $[d(AT_{10})]_2$ at $k_{pt}=2.9$ (2) $s^{-1}\times10^{-5}$.

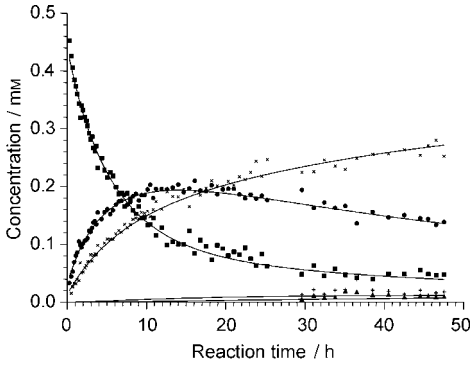

Figure 6. Experimental concentrations (from NMR data) and theoretically fitted curves for the anation of *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$ (**3**; 0.5 mM) in the presence of dT_{20} (0.7 mM) (93 mM $NaClO_4$, pH 3.6, 293 K, 0.5 mM NaCl). Symbols: (▲): **1**, (●): **2**, (■): **3**, (×): **4**, (+): **5**.

and k_{aq2}. In the final spectrum (47 h), a small concentration of **6b** (0.019 mM) was observed. This measurement was taken into account in the kinetic fit (first-order formation of **6b** from **4** was assumed), but is not displayed in Figure 6.

*Hydrolysis of **1** and chloride anation of **3** monitored at ionic strength of 0.011 M in the presence of dT_{20}*: Figure 7 shows the concentration curves for species **1**–**5** for an experiment corresponding to that represented in Figure 5, but at lower ionic strength. The kinetic pattern of the two experiments is similar, except that the decrease in the concentration of **2** towards the end of the reaction is more evident in low ionic strength, consistent with a decreased anation rate of **3** (vide infra). Optimized rate constants are listed in Tables 2 and 3.

Figure 8 shows observed and calculated concentrations of products **1**–**4**, **6**, and **7** for the chloride anation of **3** at low ionic strength. Compound **5** was not observed in this experiment. In contrast to the hydrolysis reaction (preceding paragraph), the formation of compounds **6a** and **6b** was observed after approximately 24 h. The striking difference to the analogous experiment at higher ionic strength (Figure 6) is that the concentration curve for **4** increases faster. This is due mainly to the decreased anation rate constant k_{an2} (Table 2) which dropped from 0.06 to 0.02 $M^{-1}s^{-1}$.

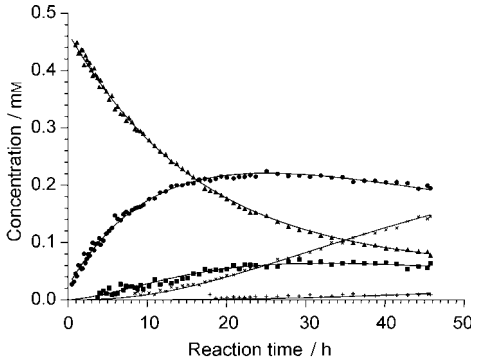

Figure 7. Experimental concentrations (from NMR data) and theoretically fitted curves for the hydrolysis of *cis*-$[PtCl_2(NH_3)_2]$ (**1**; 0.5 mM) in the presence of dT_{20} (0.7 mM) (9 mM $NaClO_4$, pH 3.2, 293 K). Symbols: (▲): **1**, (●): **2**, (■): **3**+**8**, (×): **4**, (+): **5**.

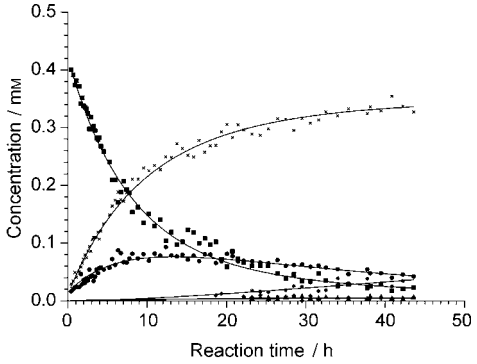

Figure 8. Experimental concentrations (from NMR data) and theoretically fitted curves for the anation of *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$ (**3**; 0.5 mM) in the presence of dT_{20} (0.7 mM) (9 mM $NaClO_4$, 293 K, 0.5 mM NaCl, pH 3.2). (▲): **1**, (●): **2**, (■): **3**+**8**, (×): **4**, (+): **5**, (*): **6**, (◆): **7**.

*Hydrolysis of **1** in the presence of the duplex $[d(AT)_{10}]_2$*: The concentration curves for the species **1** and **2**, as well as that of the platinum-$[d(AT)_{10}]_2$ are plotted in Figure S1 (see Supporting Information). Species **3** was not observed in this experiment. The concentrations were fitted assuming that $[d(AT)_{10}]_2$ reacts both with **3** and with **2**, following pseudo first-order kinetics. The calculated aquation and anation rate constants are given in Tables 2 and 3.

Discussion

The question addressed in the present work was whether or not DNA influences the rate of the two-step hydrolysis of cisplatin. DNA is a polyanion and interacts with cationic species. A certain fraction of cations bind strongly (or "condense")[15] onto the DNA surface. This fraction will be more strongly influenced by DNA than the cations remaining in the bulk solution. Our experiments were carried out in excess (93 or 9 mM) of sodium perchlorate. Therefore, only a negligible fraction of the total amount ($\leq$0.5 mM) of monocationic complexes *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**) and *cis*-

$[Pt(OH)(NH_3)_2(H_2O)]^+$ will be associated with the DNA surface. On the other hand, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) is a dication and has an advantage over singly-charged Na^+ and, according to Manning's condensation theory,[15] **3** will condense at the DNA surface to a significant extent. The simplest conceivable consequence of the presence of dT_{20} should therefore be a reduction of the anation rate of **3**, since the condensed fraction of **3** will "feel" a local concentration of chloride that is negligible compared to that in bulk solution.[7] By using Equation (29) in reference [15], the fractions of dications condensed on the surface of single-stranded DNA in the presence of 93 or 9 mM $NaClO_4$ can be calculated to be roughly 20 and 95%, respectively.

Indeed, the largest effect of dT_{20} that we can observe (Table 2) is a slowing down of the second anation rate. In the high-salt experiments (93 mM $NaClO_4$) directly following the chloride anation of **3** in the presence of dT_{20}, roughly a 20% reduction in k_{an2} is observed, agreeing well with the fraction of 20% of **3** calculated to be strongly bound to single-stranded DNA. Very informative were the experiments in lower ionic strength (9 mM $NaClO_4$). In bulk solution, lowering the ionic strength is expected to *increase* the rate of anation reactions, since screening between the ions becomes smaller. However, lowering the ionic strength also increases the fraction of condensed dications from approximately 20 to 95%. The net lowering of k_{an2} from about 0.06 to 0.02 $M^{-1}s^{-1}$ when going from 93 to 9 mM $NaClO_4$ indicates thus that k_{an2} of the bulk solution increased from approximately 0.08 to 0.4 $M^{-1}s^{-1}$. We can use for comparison the rate constants that Chan determined for the replacement of chloride in $[PtCl(dien)]^+$ by iodide at 25°C.[16] Plotting the logarithm of his second-order rate constants against $\sqrt{I}$ (I is ionic strength), we obtain a straight line fitting the equation $\log k_2 = -0.52 - 1.00\sqrt{I}$. In our experiments, the combined effects of sodium perchlorate (0.093 or 0.009 M) and dT_{20} (0.0007 M) yield ionic strengths of 0.095 and 0.011 M, respectively.[11] Thus, for $I = 0.095$ M, $k_2 = 0.15\ M^{-1}s^{-1}$ and for $I = 0.011$ M, $k_2 = 0.24\ M^{-1}s^{-1}$. The increase that we observe for k_{an2} when going from $I = 0.095$ to 0.011 M is somewhat more dramatic, consistent with the 2:1 system of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and Cl^-.

Apart from the slowing down of the second anation, we observed a small but significant and apparently ionic-strength-independent diminution of both aquation rates k_{aq1} and k_{aq2} (approximately 15%) due to the presence of dT_{20} (Table 2). Similar extent of diminution of k_{aq1} was seen for the reaction in the presence of $[d(AT)_{10}]_2$. A slowing down of the hydrolysis of cisplatin in the presence of double-stranded oligonucleotides has been reported.[8] This decrease could originate in a weak interaction between **1** and **2** with DNA (e.g., through hydrogen bonds to phosphate residues), which would make it slightly less susceptible to attack by water molecules. A slight increase at the limit of significance of the first anation rate constant, k_{an1}, was observed in the presence of both dT_{20} and $[d(AT)_{10}]_2$ (Table 2); however, the values are quite imprecise and so do not allow for a well-founded discussion. When going from high to low ionic strength, an increase of k_{an1} would be expected, since the condensation on the DNA surface should be negligible for the monocationic species **2**, and the salt screening becomes lower in the bulk solution.

Background covalent reactions with DNA: Since the purpose of this work was to study the effect of noncovalent interactions of platinum complexes with DNA, we chose DNA sequences with the least possible reactivity towards Pt^{II}, that is, dT_{20} for single-stranded DNA and $[d(AT)_{10}]_2$ for double-stranded DNA. The nevertheless occurring background reactions were investigated in separate control experiments and taken into account in the kinetic fits.

Single-strand dT_{20} reacted with **3** to a detectable extent, which, however, did not impede the determination of the chloride anation rate constant k_{an2}. From the apparent first-order rate constant of $\sim 1.6 \times 10^{-5}\ s^{-1}$, a second-order rate constant of $\sim 10^{-3}\ M^{-1}s^{-1}$ (per thymine base, at 293 K and in 93 mM $NaClO_4$, pH 3.5 ± 0.5) can be determined. This is roughly 500–30000 times slower than the rate constants for guanine residues reacting with **3** determined at pH 4.5 at the same temperature and ionic strength.[17] Likely primary reaction sites are the thymine N3 atoms.[18] However, these sites can react only after deprotonation (pK_a 10[19]), which explains the slowness of the reaction. The aqua monoadduct, supposedly formed in the initial reaction, is not observed. Instead, the major product accumulating during the first two days is a relatively stable product, whose NMR shifts are insensitive to pH and characteristic of an N,O-cross-link. In analogy with the well-known cisplatin–thymine N3,O4-dimers,[12] we assigned a dimeric structure to this product. These supposed dimers subsequently rearrange, in a very slow reaction, to N3,N3-cross-links.

Both **2** and **3** reacted to an observable extent with $[d(AT)_{10}]_2$, supposedly due to the coordinating ability of the adenine N7 atom. Neglecting the reaction with thymines, the apparent first-order rate constants listed in Table 3 yield second-order rate constants of $0.1\ M^{-1}s^{-1}$ for **3** and $0.008\ M^{-1}s^{-1}$ for **2** per adenine base. These values are ~100 and ~10 times lower, respectively, than the corresponding rate constants of adenines of AG and GA sequences involved in double-stranded DNA at pH 4.5.[20] In spite of the low reactivity of individual adenine residues and the even smaller reactivity of thymine residues, at the concentrations used, their background reaction with **3** efficiently competed with the chloride anation; this impeded a study of the second hydrolysis step in the presence of $[d(AT)_{10}]_2$.

Detection of a fraction of 3 covalently bound to an oxygen ligand of dT_{20}: During the experiments at low ionic strength, we observed [^{1}H,^{15}N] HMQC signals due to a species showing chemical shifts characteristic of an asymmetric PtN_2O_2 species that we assigned to *cis*-$\{Pt(NH_3)_2(H_2O)\}^{2+}$ bound to a phosphodiester residue of dT_{20}. The fact that the species was observed only at low ionic strength suggests that the ligand is a charged residue. Partial dehydration and coordination of Mn^{2+} and Co^{2+} hexaaqua ions to polyphosphate

has been reported.[21] Polyphosphate and single-stranded DNA have similar axial charge densities,[15] and a similar tendency to lose a H_2O ligand and to bind to a phosphate residue could thus be expected for *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ in the presence of single-stranded DNA. Quantitative coordination of the phosphodiester group of $dTpT^-$ to $[Pt(NO_3)(NH_3)_3]^+$ in dimethylformamide ($\varepsilon = 37$) has been observed by Kozelka and Barre, showing that low dielectric screening in fact favors phosphate coordination to Pt^{II} carrying a formal positive charge.[13] As *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ is a dication, its weak coordination by phosphodiester groups of DNA in low-salt aqueous solutions appears thus conceivable. This seems to be the first experimental evidence of phosphodiester coordination to platinum in an aqueous medium.

Experimental Section

Materials: [^{15}N]-labeled cisplatin was prepared according to the published method.[22] The two oligonucleotides dT_{20} and $d(AT)_{10}$ were purchased from DNA Technology A/S (Aarhus, Denmark) and obtained as crude products after precipitation in ethanol. Acetonitrile, triethylammonium acetate buffer (made from equimolar amounts of triethylamine and acetic acid) and NaOH were purchased from Baker, sodium perchlorate and perchloric acid from Merck and MES buffer (2-(*N*-morpholino)ethanesulfonic acid) from Sigma.

Sample preparation: All weights were determined by using an Ohaus Explorer Pro Analytical balance with a sensitivity of 0.1 mg (Ohaus Corporation, USA). A Sentron Argus pH meter connected to a Sentron Redline Standard pH probe (calibrated with pH 4.00 and 7.00 Sentron buffers) was used to measure pH.

The oligonucleotides were desalted using a float-a-lyzer of 1 mL, 1000 M_W cut off (Spectrum Laboratories, Inc., USA). The dialysis was performed in four three-hour cycles with intermittent reservoir changes (reservoir composition: $NaClO_4$ (1 mM) in H_2O (1 L)). The purity was then checked by 1D ^{1}H NMR spectroscopy. One sample of dT_{20} was also desalted by using HPLC (Waters 626 LC instrument with Millennium 32 software) with an Xterra, MS C_{18}, 2.5 μm column (Waters Corporation, USA). Eluents used for HPLC were A: acetonitrile (5%) in TEAA (0.1 M), B: acetonitrile (15%) in TEAA (0.1 M). A linear gradient from 17% B to 50% B in 30 min, 2.0 mL min^{-1} at ambient temperature was applied. TEAA and acetonitrile were removed by freeze-drying the sample twice, first at pH 12.5 and then at pH 3.3.

Cisplatin (dichloro form) stock solutions were prepared by weighing cisplatin and deionized distilled water. About 10 mg cisplatin was used in each stock solution, making the error in the weight of cisplatin 1% (according to the standard deviation of the balance). Fresh stock solutions were made before each set of NMR experiments. Thus a stock solution was never older than a few weeks. Stock solutions of the diaqua complex **3** were prepared by dissolving a weighed amount of dichloro cisplatin (~15 mg) in water, then adding $AgNO_3$ (two equivalents) and enough perchloric acid to adjust to pH 1–2. The solution was then stirred in the dark for 48 h. In preliminary tests of cisplatin diaqua, we found by 2D [^{1}H,^{15}N] HMQC NMR that **3** forms hydroxo oligomers even at 1 mM concentration and pH 3.4. We therefore used stock solutions with a maximum concentration of 1.4 mM and maximum pH of 1.5, and stored the stock solution at a temperature of −40 °C.

We performed tests with acetate and MES (2-(N-morpholino)ethanesulfonic acid) buffers and found significant coordination to **3**. Phosphate buffers have been shown to interfere with hydrolyzed cisplatin.[8] Therefore, all experiments reported here were carried out in unbuffered solutions.

The NMR samples had the following composition: cisplatin (0.5 mM), $NaClO_4$ (93–100 mM), and had a pH 3.0–4.0. In two experiments a 9 mM concentration of $NaClO_4$ was used. Samples containing DNA oligonucleotides had a single strand concentration of 0.70 mM, except in the experiment where the anation of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ was studied in the presence of $[d(AT_{10})]_2$. In that experiment, cisplatin (1.0 mM) and $d(AT)_{10}$ (1.4 mM) were used. The tests were carried out directly in the NMR tube (Wilmad 528 pp) at 293 K.

NMR experiments: NMR experiments were performed on a Bruker DRX 600 instrument fitted with a pulsed gradient module and a 5 mm inverse probe head. The spectrometer was used for the acquisition of 2D [^{1}H,^{15}N] HMQC and 1D ^{1}H NMR spectra. The dpfgsew5 pulse sequence was used for the suppression of the water signal in 1D ^{1}H spectra in H_2O.[23,24] The 2D [^{1}H,^{15}N] HMQC NMR spectra were phase sensitive using the Echo/Antiecho-TPPI quadrature detection scheme. Pulsed field gradients were applied to select the proper coherence and the ^{15}N-spins were decoupled during acquisition. No extra pulse sequence was needed for the suppression of water in 2D [^{1}H,^{15}N] HMQC spectra (see the pulse sequence of Palmer et al.)[25] in H_2O. The 2D [^{1}H,^{15}N] HMQC spectra were optimised for $^1J(N,H) = 72$ Hz. The parameters for the 2D [^{1}H,^{15}N] HMQC experiments were as follows: spectral width in $F1$ 2006 Hz and in $F2$ 4195 Hz, 2048 complex points in each FID in t_2 and 64 increments in t_1, 8–16 transients were averaged for each increment and a relaxation delay of 2 s was used. ^{1}H were referenced to TSP (set to 0 ppm) and ^{15}N to 1 M ^{15}N-enriched NH_4Cl in 1 M HCl solution set at 0 ppm. The NMR data were processed using the program XWIN-NMR Version 2.6. The apodization function used in both dimensions for the 2D NMR data was a pure squared cosine bell. Window functions applied to the 1D ^{1}H FID's were an exponential function with a line-broadening of 1–3 Hz or a gauss multiplication with a line-broadening of −1 Hz and gm 0.1. The t_1 FID's in the 2D NMR data sets were linearly predicted to four times their original value.

Data analysis: The rate constants for the reactions were determined by a nonlinear optimisation procedure using the program SCIENTIST.[26]

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