

Sequence dependence of the reactivity of histidyl containing peptides with palladium(II) and platinum(II) complex ions. An NMR study

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Received 18th May 1998, Accepted 18th November 1998

The reactions of the pentapeptide His-Pro-Gly-Ala-His with the complex salts [Pd(dien)(D₂O)][NO₃]₂, [Pt(dien)(D₂O)][NO₃]₂, [Pd(en)(D₂O)₂][NO₃]₂, *cis*-[Pt(NH₃)₂(D₂O)₂][NO₃]₂, K₂PdCl₄ and K₂PtCl₄ were studied in aqueous solutions, as a function of pD, by means of ¹H, ¹³C and ¹⁹⁵Pt NMR spectroscopies in one and two dimensions. In order better to understand the behavior of the ternary systems of the pentapeptide with the complex ion [Pd(dien)(D₂O)][NO₃]₂ or [Pt(dien)(D₂O)][NO₃]₂, the reactions of the tetrapeptide Pro-Gly-Ala-His with these complexes were also studied as a function of pD. The ¹H and ¹³C NMR assignments were made by two dimensional homo- and hetero-nuclear experiments at various pD's for both peptides. The complexes [Pd(dien)(D₂O)][NO₃]₂ or [Pt(dien)(D₂O)][NO₃]₂, react with Pro-Gly-Ala-His in acidic or alkaline media respectively to form mixtures of two isomers in which histidyl imidazole co-ordinates the metal through either N1 or N3. In neutral solution, the imidazole ring bridges two Pd(dien) moieties, bound to N1 and N3. In the presence of [Pd(dien)(D₂O)][NO₃]₂ the histidyl-5 residue of the pentapeptide behaves in the same way as the one in the tetrapeptide, whereas the histidyl-1 residue reacts in acidic media through both imidazole N1 and N3 bridging two Pd(dien) moieties. The complex [Pd(en)(D₂O)][NO₃]₂ or K₂PdCl₄ reacts with His-Pro-Gly-Ala-His in strongly acidic media (pD < 1.5) to form 1 : 1 adducts with the histidyl-1 residue co-ordinated selectively to the metal ion forming NH₂, N3 chelates. Similar products are formed by the reaction of the pentapeptide with *cis*-[Pt(NH₃)₂(D₂O)₂][NO₃]₂ or K₂PtCl₄ in slightly alkaline or acidic media respectively.

Introduction

Many metal ions are found to be essential in life. Interacting with ligands of biological relevance they assist many biological processes in living organisms.¹ Also, in recent years, compounds of some metal ions not essential in life, like Pt^{II}, are used for pharmaceutical purposes. The therapeutic properties of these compounds result from the interaction of the metal ion with biologically important ligands.² Thus many studies on the interaction of metal ions with biomolecules like amino acids, peptides or DNA constituents have been carried out, in order to elucidate the role of such interactions in life. In the metal-protein interactions, histidyl residues play the most important role since they are found to interact with metal ions in the active site of several metalloenzymes.³

Consequently the principles of metal ion interactions with histidyl residues in proteins or peptides are of high interest. These type of studies have been carried out in part by modelling metal ion histidyl interactions, using small peptides containing histidine like His-Ala, His-Gly-Ala,³ Gly-His,⁴ His-Gly,⁴ Gly-His-Gly, Gly-Gly-His, Ala-His and Gly-His-Lys.⁵ Metal ions anchor first to imidazole N3, followed by amide group deprotonation and co-ordination of the amide nitrogens to the metal ions forming a series of chelate rings upon pH increase. The pH where amide group deprotonation is observed varies depending on the metal ion following the series Pd²⁺(2) < Cu²⁺(4) < Ni²⁺(8) < Co²⁺(10).^{5,6}

However the studies on the interaction of metal ions with peptides containing more than one histidyl residue are not very extensive, mainly limited to Cu²⁺ and peptides containing the sequences His-His⁷⁻⁹ or His-X-His.⁹ These binary systems may be considered as more realistic models for the metal ion interaction in the active site of metalloproteins, since the metal ion usually binds more than one histidyl residue in them. It is worthwhile mentioning that the behavior of peptides contain-

ing two histidyl residues towards metal co-ordination differs from peptides containing only one histidyl residue. Spectroscopic studies suggest that, at low pH, His-His co-ordinates Cu²⁺ through NH₂ and imidazole N3 of the histidyl-1 residue, whereas at higher pH co-ordination takes place through NH₂, the deprotonated amide nitrogen and imidazole N3 of histidyl-2 residue. These units dimerize at higher pH by co-ordination of imidazole N3 of the histidyl-1 residue. In contrast, His-Gly-His-Gly behaves like Gly-Gly-His.⁹

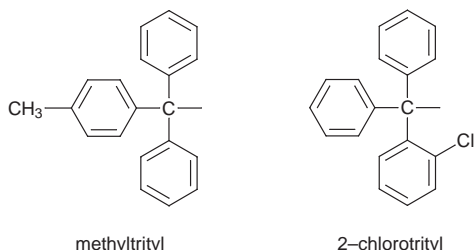
In this paper we present the results of our NMR studies on the interaction of the complex cations [M(dien)(D₂O)]²⁺, MCl₄²⁺ (M = Pd^{II} or Pt^{II}), [Pd(en)(D₂O)₂]²⁺ and *cis*-[Pt(NH₃)₂(D₂O)₂]²⁺ with the pentapeptide His-Pro-Gly-Ala-His with the aim better to understand the ways that antitumor drugs like *cis*-[Pt(NH₃)₂Cl₂] may interact with histidyl residues in proteins. In this peptide the two histidyl residues are well separated from each other by three amino acids, one being proline which possessing a ternary nitrogen acts as a brake point in co-ordination of peptides to metal ions.⁵ Owing to the complexity of the metal-pentapeptide systems, we have also studied similar systems of the same complex ions with the tetrapeptide Pro-Gly-Ala-His for comparison purposes. These latter studies, in combination with various multinuclear NMR spectroscopic techniques in one and two dimensions, helped us to understand the former metal-pentapeptide systems. NMR proved to be the method of choice in studying similar systems of diamagnetic square planar complexes of Pt^{II} or Pd^{II}.

Experimental

Reagents

All solvents (Merck) were of the highest purity available used without further purification. The protected amino acids Fmoc-His(Mtt)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Ala-OH

(Fmoc = fluoren-9-ylmethoxycarbonyl, Mtt = methyltrityl) and the resin 2-chlorotrityl chloride were purchased from CBL Ltd (Patras), DCC (dicyclohexylcarbodiimide) from Merck and HOBt (1-hydroxybenzotriazole) from Aldrich and K_2PdCl_4 and K_2PtCl_4 from Johnson Matthey S.A. The complexes $[Pd(en)-Cl_2]$,¹⁰ $[Pd(dien)Cl]Cl$,¹¹ *cis*- $[Pt(NH_3)_2Cl_2]$ ^{12,13} and $[Pt(dien)I]$ ¹⁴ were prepared according to literature methods. Over the pD range used in this work, no release of the ligands dien, en or NH_3 was observed.^{5,15,16}



Peptide synthesis

Both peptides were prepared by solid phase peptide synthesis using the 2-chlorotrityl chloride resin (substitution 1.1–1.6 mequivalents g^{-1}) as the solid support.¹⁷ The standard Fmoc procedure was used.¹⁸ The peptides were cleaved from the resin by a mixture of $MeCO_2H-CF_3CH_2OH-CH_2Cl_2$ (1:2:7 v/v). The Mtt group was removed by a solution of 20% trifluoroacetic acid in $CH_2Cl_2-CF_3CH_2OH$ (6:1 v/v). Peptide purity was controlled by TLC in the systems $BuOH-MeCO_2H-water$ (4:1:1 v/v) and $BuOH-MeCO_2H-Py-water$ (30:6:20:24 v/v).

Preparation of the samples

Stock solutions of the aqua complexes $[M(dien)(D_2O)]^{2+}$, $[Pd(en)(D_2O)_2]^{2+}$ and *cis*- $[Pt(NH_3)_2(D_2O)_2]^{2+}$ were prepared by treating the corresponding halogeno compound with the necessary amounts of $AgNO_3$ in the dark for 24 h and filtering the AgI or $AgCl$ formed. The whole procedure was carried out in D_2O (99.8% D, Merck).

The peptides were dissolved in D_2O and the appropriate amount of the metal aqua complex was added by micropipette from the stock solution. The pD was adjusted before the addition of the metal compound, when it was desired to work in strongly acidic solutions, and readjusted after addition in order to temporarily avoid high pD's for the mixtures. When it was desired to work in neutral or alkaline solutions the pD was adjusted after the addition of the metal compound. 0.1 or 1.0 M DCl or $NaOD$ solutions were used to adjust the pD. The pD measurements were carried out by a digital pH meter (WTW pH 537) and a 3.7 mm diameter glass-calomel combined electrode (Russell) calibrated with standard buffer solutions (Merck Titrisol). Reported pD values were corrected for the deuterium isotopic effect by adding 0.4 logarithmic units to the pH meter reading.¹⁹

In the 1H NMR spectra of the products of reactions carried out in alkaline media the peaks from imidazole protons appear at the same region as the peaks from the free peptide. To shift these peaks from the free peptide to lower field we adjusted the pD of the samples to 2.5 just before running the spectra. As a result of the kinetic inertness of platinum(II) compounds, no decomposition of the complexes formed was observed upon comparing the spectra at high and low pD.

One and two dimensional NMR spectra

One dimensional 1H , ^{13}C and ^{195}Pt NMR spectra were recorded on a Bruker AMX 400 spectrometer at 400, 100.58 and 85.83 MHz respectively, using a 5 mm broad band tunable probe kept at $25 \pm 1^\circ C$. Field stabilization was provided by an internal deuterium lock signal. The usual 1H spectrometer conditions

consisted of 8064 Hz sweep width, 64 scans and 1K data points. Both 1H and ^{13}C NMR chemical shifts were measured in ppm with 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as external reference. The ^{195}Pt NMR chemical shifts are reported relative to $PtCl_6^{2-}$ (external standard) and spectra were run typically with 30000 transients (sample concentration 30 mM) and a spectral width of 125 kHz. A pulse duration of 27 μs was used (magnetization vector flip angle of 90°) followed by an acquisition time of 65 ms and a delay time of 0.1 s. A line broadening factor of 50–100 Hz was used in processing the data.

Two dimensional NMR spectra were recorded at room temperature on the same Bruker AMX 400 instrument. The COSY spectra were acquired in the magnitude mode. For both COSY and TOCSY spectra 256 t_1 increments were accumulated into 1K data points with 32 scans each. The evolution time after the first 90° pulse was 1.0 s while the acquisition time was typically 0.1 s. The MLEV-17 pulse sequence (50 ms) was used for mixing in TOCSY experiments.²⁰ The initial 256×1024 data matrix was zero filled and multiplied by a sine-bell function in both t_1 and t_2 dimensions prior to Fourier transformation. Phase sensitive 1H , ^{13}C HMQC²¹ (heteronuclear multiple quantum coherence) and HMBC²² (heteronuclear multiple bond coherence) spectra were recorded at $25 \pm 1^\circ C$ with $t_{1(max)} = 55.1$ ms and $t_2 = 457$ ms for HMQC and $t_{1(max)} = 22$ ms and $t_2 = 209$ ms for HMBC.

Results

The ligands His-Pro-Gly-Ala-His and Pro-Gly-Ala-His

Proton NMR assignments of the free peptide resonances at various pD's were made by two dimensional COSY and TOCSY spectral analysis (Table 1). The ^{13}C NMR assignments were made by HMQC and HMBC spectra; HMBC spectra were used to obtain long range $^nJ_{CH}$ scalar coupling information and thus complete the assignment of imidazole and carbonyl carbons (Table 1). The carboxylate group signal was difficult to observe due to slow relaxation of this carbon nucleus.

By means of 1H NMR spectroscopy we were also able to calculate deuteration constants of the various groups of the peptides. In aqueous solution both peptides have several protonation sites depending on the pH. In strongly acidic solutions all protonation sites *e.g.* carboxylate, imidazole and ammonium groups are protonated. At higher acidic pH values the carboxylate group deprotonates, while in slightly acidic or neutral solutions the imidazole group loses one proton, remaining monoprotonated. Finally, deprotonation of the ammonium group takes place in alkaline solutions. In D_2O solution exact pD values where these dissociation processes take place were obtained by analysing 1H NMR/pD titration curves. The chemical shifts of the protons adjacent to the different deuteration sites were related to the acid dissociation constant K_a of each specific site by the Henderson–Hasselbalch equation.^{3,23} Calculated in this way, pK_a values for both peptides compare well with those calculated by potentiometry and also with literature values (Table 2). It should be noted here that due to partial overlap of the dissociation processes the acid–base chemistry of the ligands can only be described by microconstants.⁵ However as seen in Table 2 in this case the overlap between the dissociation of imidazole and ammonium groups is negligible and hence an approximate assignment of the NMR calculated pK values to individual acidic groups and their comparison with the macroscopic pK values can be made. Thus the pK values determined here by applying the Henderson–Hasselbalch equation though not very accurate allow us to know the deuteration state of the various peptide functional groups, useful in complexation studies with Pd^{II} or Pt^{II} in strongly acidic, neutral or alkaline solutions.

The 1H NMR/pD curves were also useful in the full assign-

Table 1 Selected ^1H , ^{13}C and ^{195}Pt NMR data for the various compounds. One letter abbreviations are used for the amino acids; ^{13}C shifts are given in parentheses, ambiguous data in square brackets

Compound	pD	His $^1\text{C}\alpha\text{-H}$	Imidazole protons (carbons)					^{195}Pt NMR	
			C $^1\text{2-H}$	C $^1\text{4}$	C $^1\text{5-H}$	C $^{4/5}\text{2-H}$	C $^{4/5}\text{4}$		C $^{4/5}\text{5-H}$
PGAH	2.0					8.62 (135.95)	(132.21)	7.32 (119.72)	
HPGAH	2.0	4.80 (53.35)	8.72 (137.45)	(127.99)	7.49 (121.89)	8.61 (135.98)	(132.13)	7.31 (119.75)	
[Pd(dien)(PGAH-N1)] $^{2+}$	3.5					7.72		6.74	
[Pd(dien)(PGAH-N3)] $^{2+}$	3.5					7.91		7.08	
[{Pd(dien)} $_2$ (μ -PGAH-N1,N3)] $^{3+}$	7.0					7.16		6.60	
[{Pd(dien)} $_3$ (μ -N1,N3-HPGAH-N1)] $^{5+}$	3.5		7.86		6.96	7.77		6.79	
[{Pd(dien)} $_3$ (μ -N1,N3-HPGAH-N3)] $^{5+}$	3.5		7.86		6.96	7.96		7.12	
[{Pd(dien)} $_4$ (μ -N1,N3-HPGAH-N1,N3)] $^{6+}$	7.0		7.86		6.96	7.12		6.57	
[Pt(dien)(PGAH-O)] $^{3+}$	3.5					8.62		7.31	-2502
[Pt(dien)(PGAH-N1)] $^{2+}$	8.5					7.89		6.92	-2874
[Pt(dien)(PGAH-N3)] $^{2+}$	8.5					8.01		7.15	-2874
[Pt(dien)(HPGAH-N1)] $^{4+}$	2.5		8.70		7.50	7.88		6.89	-2872
[Pt(dien)(HPGAH-N3)] $^{4+}$	2.5		8.70		7.50	7.99		7.14	-2872
[Pt(dien)(N1-HPGAH)] $^{4+}$	2.5		[7.88]		6.75	8.61		7.32	-2872
[Pt(dien)(N3-HPGAH)] $^{4+}$	2.5		8.14		7.05	8.61		7.32	-2872
[{Pt(dien)} $_2$ (μ -N1-HPGAH-N1)] $^{5+}$	2.5		[7.88]		6.75	7.88		6.89	-2872
[{Pt(dien)} $_2$ (μ -N1-HPGAH-N3)] $^{5+}$	2.5		[7.88]		6.75	7.99		7.14	-2872
[{Pt(dien)} $_2$ (μ -N3-HPGAH-N1)] $^{5+}$	2.5		8.14		7.05	7.88		6.89	-2872
[{Pt(dien)} $_2$ (μ -N3-HPGAH-N3)] $^{3+}$	2.5		8.14		7.05	7.99		7.14	-2872
[Pd(en)(NH $_2$,N3-HPGAH)] $^{5+}$	1.2	3.67 (54.42)	7.72 (139.53)		7.08 (117.64)	8.62 (135.42)	(131.42)	7.32 (120.08)	
<i>cis</i> -[Pt(NH $_3$) $_2$ (NH $_2$,N3-HPGAH)] $^{3+}$	2.5	3.74 (54.05)	8.02 (139.22)		7.20 (117.81)	8.61 (135.69)	(132.25)	7.30 (119.28)	-2685
<i>cis</i> -[Pd(NH $_2$,N3-HPGAH)Cl $_2$] $^+$	1.2	3.33 (54.21)	8.06 (140.02)	(133.84)	7.11 (117.12)	8.67 (136.21)	(132.32)	7.37 (120.28)	
<i>cis</i> -[Pt(NH $_2$,N3-HPGAH)Cl $_2$] $^+$	2.0	3.53 (54.76)	8.21 (139.53)	(134.86)	7.12 (117.42)	8.65 (136.25)	(131.52)	7.34 (120.10)	-2277

Table 2 The pK_a values of the peptides (pK_a COOH of Pro-Gly-Ala-His and His-Pro-Gly-Ala-His were calculated by potentiometry 24,25)

Peptide	NH $_3^+$	Im(1) $^+$	Im(4/5) $^+$	COOH	Ref.
Pro-Gly-Ala-His	8.95		7.20	(2.76)	<i>a</i>
His-Pro-Gly-Ala-His	7.60	6.95	5.70	(2.52)	<i>a</i>
Gly-His	7.97		6.61	2.65	26
His-Gly	7.15		5.39	2.32	26
Pro-His	8.82		6.84	3.02	8

a This work.

ment of imidazole proton resonances. In the case of the pentapeptide, we traced curves for the variation of the chemical shift of every one of the four imidazole protons as a function of pD. From each two of these curves we calculated the same pK_a value. Obviously these pairs of curves represent protons of the same imidazole ring. Thus in the spectrum of the pentapeptide in acidic solution we assigned resonances at δ 8.72 and 7.49 to one imidazole ring and resonances at δ 8.61 and 7.31 to the other. Moreover, comparing these chemical shifts to those for imidazole protons in the spectrum of the tetrapeptide and based on literature data 3 we may assign resonances at δ 8.72 and 7.49 to imidazole C2-H and C5-H protons respectively of the histidyl-1 residue, those at δ 8.61 and 7.31 to the same protons of the histidyl-5 residue (Table 1).

Reactions with [M(dien)(D $_2$ O)] $^{2+}$ (M = Pd II or Pt II)

[Pd(dien)(D $_2$ O)] $^{2+}$. When the pD of a 1:1 Pro-Gly-Ala-His: [Pd(dien)(D $_2$ O)] $^{2+}$ mixture was adjusted to 3.5, the imidazole C2-H and C5-H protons appear as two pairs of new peaks at δ 7.91, 7.72 and 7.08, 6.74 in the ^1H NMR spectrum. No significant change was observed at the δ 1–5 region of this spectrum as compared to the spectrum of the free peptide at the same pD. It is clear that the peptide reacts with the Pd(dien) moiety exclusively through imidazole nitrogen atoms. Comparing the spectrum of this mixture with data from similar systems of His

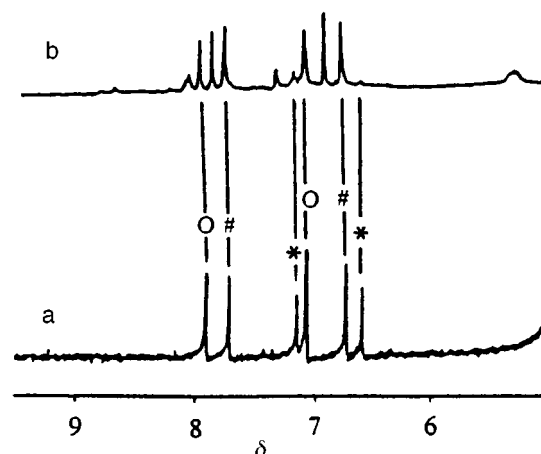


Fig. 1 Proton NMR spectra of mixtures of (a) Pro-Gly-Ala-His: [Pd(dien)(D $_2$ O)] $^{2+}$ 1:2 at pD 7.0 and (b) His-Pro-Gly-Ala-His: [Pd(dien)(D $_2$ O)] $^{2+}$ 1:3 at pD 4.0. * [Pd(dien)(PGAH-N1)] $^{2+}$ or [{Pd(dien)} $_2$ (μ -N1,N3-HPGAH-N1)] $^{5+}$, O [Pd(dien)(PGAH-N3)] $^{2+}$ or [{Pd(dien)} $_2$ (μ -N1,N3-HPGAH-N3)] $^{5+}$, # [{Pd(dien)} $_2$ (μ -PGAH-N1,N3)] $^{3+}$ or [{Pd(dien)} $_4$ (μ -N1,N3-HPGAH-N1,N3)] $^{6+}$.

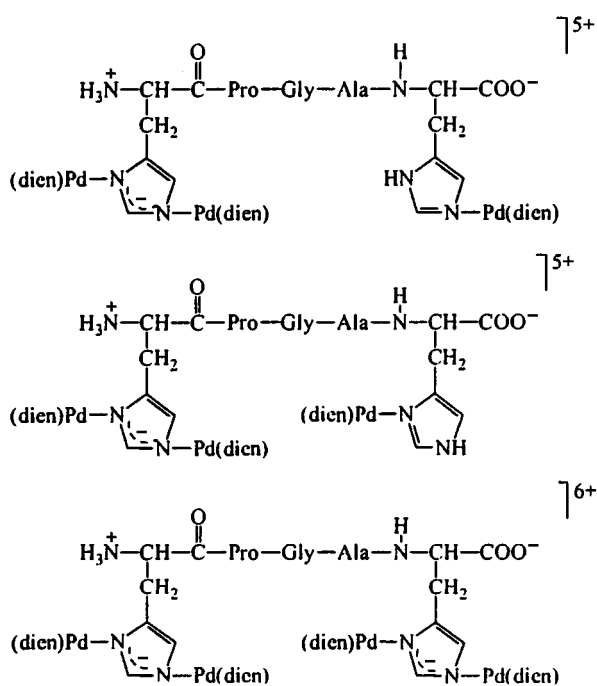
or Ac-His 16 we conclude that a mixture of two linkage isomers is formed with the Pd(dien) moiety bound either to imidazole N1 or N3. On the basis of this comparison, the peaks at δ 7.91 and 7.08 were attributed to imidazole C2-H and C5-H protons of the N3 bound isomer and peaks at δ 7.72 and 6.74 to the same protons of the N1 bound isomer (Table 1).

When the ratio Pro-Gly-Ala-His: [Pd(dien)(D $_2$ O)] $^{2+}$ was 1:2 and the pD of the solution was raised above 4.5, two new peaks appeared at δ 7.16 and 6.60 (Fig. 1). The intensity of these peaks increase as the pD of the solution increases. Comparing the chemical shifts of these new peaks with data from spectra of the His or Ac-His systems, of the same salt, 17 we conclude that these peaks are due to imidazole C2-H and C5-H protons of

a product on which the imidazole ring bridges two Pd(dien) moieties co-ordinated by both N1 and N3 nitrogens.

In the ^1H NMR spectra of mixtures of the pentapeptide His-Pro-Gly-Ala-His with $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ in ratios 1:1 or 1:2 and pD 3.5 no change in the region of δ 1–5 with respect to the spectrum of the free peptide was again observed. However the intensity of the peaks from imidazole protons decrease and new peaks appeared at δ 7.96, 7.86, 7.77, 7.12, 6.96 and 6.79. The intensities of these peaks increase with the quantity of $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ added and reach the maximum when the His-Pro-Gly-Ala-His : $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ ratio is 1:3 where peaks of imidazole protons of the free peptide were not observed. Comparing these data to those obtained from the system of $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ with the peptide Pro-Gly-Ala-His, and literature data,³ we were able to characterize the peaks at δ 7.96, 7.77 and 7.12, 6.79 as due to C2-H and C5-H imidazole protons of the histidyl-5 residue and peaks at δ 7.86 and 6.96 of the same protons of the histidyl-1 residue. Based on comparisons with the similar systems of Pro-Gly-Ala-His (Fig. 1), His-Ala³ and His-Gly-Ala³ with $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$, the transformation of a mixture of two complexes, with three Pd(dien) moieties bound per peptide, forming two linkage isomers (Table 1) may be proposed. In these isomers two Pd(dien) moieties are simultaneously bound to imidazole N1 and N3 nitrogen of the histidyl-1 residue, while one Pd(dien) moiety is bound to imidazole N1 of the histidyl-5 residue in the first complex or to imidazole N3 of the histidyl-5 residue in the second. It is noticeable that total deprotonation of the histidyl imidazole rings is not observed at any pH in the absence of metal ions.

Increasing the quantity of $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ added and the pD up to 7, two new peaks appear in the ^1H NMR spectra of the mixtures, at δ 7.12 and 6.57 (Fig. 1). Comparing the chemical shifts of these peaks with data from the system $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ -Pro-Gly-Ala-His, they can be assigned to imidazole C2-H and C2-5 protons respectively of the histidyl-5 residue of a new complex in which four Pd(dien) moieties are simultaneously bound to all imidazole nitrogens of the pentapeptide (Table 1). The structures of all observed complexes are as shown.



$[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$. In the NMR spectrum of an equimolar mixture of Pro-Gly-Ala-His with the complex ion $[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$ at pD 3.5 no difference was observed with respect to the spectrum of the free peptide at the same pD. However,

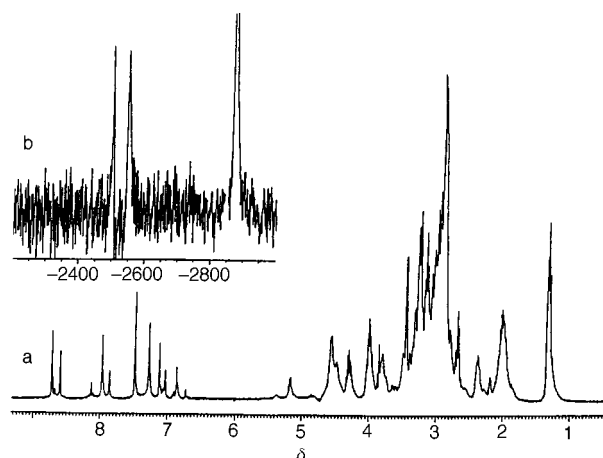


Fig. 2 The ^1H NMR (a) and ^{195}Pt NMR (b) spectra of a 1:1 His-Pro-Gly-Ala-His : $[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$ mixture at pD 2.5 after 7 d of reaction at pD 8.5.

in the ^{195}Pt NMR spectrum two peaks at δ -2547 and -2502 (Table 1) were observed. The first is due to unchanged $[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$ and the second to a new complex of formula $[\text{Pt}(\text{dien})(\text{Pro-Gly-Ala-His-O})]^{3+}$ with the metal in a N_3O coordination mode.^{3,27}

When the pD of the solution was adjusted to 8.5 a mixture of two linkage isomers was formed. These products have similar structures to those formed from the reaction of $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ with the tetrapeptide at pD 3.5. More specifically in the ^1H NMR peaks at δ 7.89 and 6.92 for imidazole C2-H and C5-H protons of the N1 bound isomer and at δ 8.01 and 7.15 for the same protons of the N3 bound isomer were observed. The only change observed in the region δ 1–5 of the spectrum with respect to the free peptide was the decrease of the intensity of the peak of the α -proton of the histidyl-1 residue, while a new peak at δ 5.57 of the same intensity as the peaks at δ 8.01 and 7.15 was observed. Based on literature data¹⁶ the δ 5.57 peak was assigned to the methine proton of the histidyl-4 residue. Its downfield shift, compared to the free peptide, is caused by N3 co-ordination.¹⁶ As seen in the ^1H NMR spectrum of this mixture, peaks from imidazole protons of the free peptide disappear after 7 d, indicating that the reaction is complete. Integration of the imidazole peaks of the products showed that, also in this case, N3 co-ordination is favored over N1, the molar fraction of the N3 bound isomer being 0.69, in accordance with the literature.^{3,16}

The single peak appearing at δ -2874 in the ^{195}Pt NMR spectrum of the mixture after 7 d of reaction indicates simultaneous PtN_4 co-ordination around the metal ion,^{3,27} consistent with the structures proposed from the ^1H NMR spectra.

The behavior of the system His-Pro-Gly-Ala-His- $[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$ at pD 3.5 is the same as described for Pro-Gly-Ala-His- $[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$ (Table 1). When the pD of the mixture was adjusted to 8.5, however, new peaks appeared in the ^1H NMR spectrum at δ 8.14, 7.99, 7.88, 7.14, 7.05, 6.89, 6.75, and 5.17 (Fig. 2). The pattern of the spectrum in the region δ 1–5 does not significantly change with respect to the spectrum of the free peptide at the same pD. It is obvious that the $\text{Pt}(\text{dien})$ moiety binds only imidazole nitrogens. The assignment of these peaks was achieved by comparison with the simpler systems of $[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$ with Pro-Gly-Ala-His, His-Ala³ or His-Gly-Ala.³ Thus, co-ordination of the $\text{Pt}(\text{dien})$ moiety with all four imidazole nitrogens to form a mixture of eight products with one or two metal ions bound per peptide ligand is proposed. The formulae of these eight products are given in Table 1. Peaks at δ 7.99 and 7.14 were assigned to imidazole C2-H and C5-H protons respectively of the histidyl-5 residue with the metal bound to N3 and peaks at δ 7.88 and 6.89 to the same protons of the histidyl-5 residue with the metal bound to N1. Similarly,

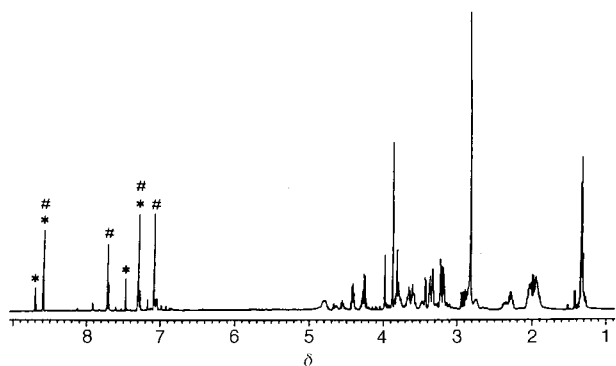


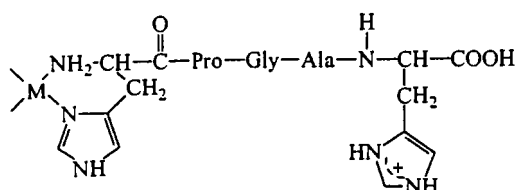
Fig. 3 The ^1H NMR spectrum of a 1:1 His-Pro-Gly-Ala-His:[Pd(en)(D₂O)₂]²⁺ mixture at pD 1.2. * His-Pro-Gly-Ala-His, # [Pd(en)(NH₂, N3-His-Pro-Gly-Ala-His)]³⁺.

peaks at δ 8.14 and 7.05 were assigned to imidazole C2-H and C5-H protons of the histidyl-1 residue with a Pt(dien) moiety bound to N3 and the peak at δ 6.75 to imidazole C5-H of the same residue with a Pt(dien) moiety bound to N1. The peak for imidazole C2-H of the histidyl-1 residue of the latter products is possibly at δ 7.88, overlapping with the peak for the C2-H proton of the histidyl-5 residue. The peak at δ 5.17 is assigned also here to the methine proton of the histidyl-5 residue at the products where a Pt(dien) moiety is bound to imidazole N3 of the histidyl-5 residue¹⁶ (Table 1). Although the relative concentrations of the various products formed could not be determined, integration of the peaks of imidazole protons showed that imidazole nitrogens of the histidyl-5 residue react to a greater extent than those of the histidyl-1 residue. The known selectivity of Pt(dien) moiety for imidazole nitrogen N3 is observed also in this case for both histidyl residues, the molar fraction of the N3 bound isomer being 0.78 and 0.57 for histidyl-1 and -5 residues respectively.

The ^{195}Pt NMR spectrum (Fig. 2) is consistent with the formation of these products. Except for peaks at δ -2502 for [Pt(dien)(His-Pro-Gly-Ala-His-O)]⁴⁺ and at δ -2549 for [Pt(dien)(D₂O)]²⁺, a broad peak at δ -2872 is indicative of a N₄ co-ordination around the metal ion in the products formed^{3,27} (Table 1).

Reactions with [Pd(en)(D₂O)₂]²⁺ and *cis*-[Pt(NH₃)₂(D₂O)₂]²⁺

[Pd(en)(D₂O)₂]²⁺. The pentapeptide His-Pro-Gly-Ala-His reacts with the complex ion [Pd(en)(D₂O)₂]²⁺ in strongly acidic solution to yield 1:1 adducts. As seen in the ^1H NMR spectrum of a 1:1 mixture of these compounds at pD 1.2 (Fig. 3), only peaks from protons of the histidyl-1 residue are influenced by the presence of the metal complex ion, indicating that only this residue is involved in co-ordination. The intensity of peaks from imidazole C2-H and C5-H protons of the histidyl-1 residue of the free peptide decrease and new peaks appear at δ 7.72, 7.08 and 3.67 assigned to the two imidazole and the α -proton of the histidyl-1 residue of the peptide co-ordinated to the metal. These peaks are shifted upfield by 0.69, 0.23 and 1.13 ppm respectively compared to those of the free peptide at the same pD. The larger shift of the peak of the C2-H proton than those of C5-H and the shift of the peak from the α -proton suggest co-ordination of the imidazole ring through N3 and of the amine nitrogen to Pd^{II}.³ Thus a stable six membered chelate ring was formed.



Carbon-13 NMR spectroscopy further supports the proposed structure for the complex formed. Comparing the spectrum of the above mixture to that of the free peptide at the same pD, only changes of the protons of the histidyl-1 residue were observed (Table 1). Thus, the imidazole α - and β -carbons of the complexed histidyl-1 residue appeared at δ 37.75, 54.42, 117.64 and 139.53. They are shifted by 3.96, 1.07, 4.25 and 2.08 ppm respectively compared to peaks of the free peptide. The peak of the imidazole C4 carbon cannot be observed because of its low intensity, due to slow relaxation.

Integration of imidazole protons allowed an estimation of the yield of this reaction as about 80%. This increased on increasing the amount of [Pd(en)(D₂O)₂]²⁺ added.

At higher pD's, ^1H NMR spectroscopy suggests involvement also of the histidyl-5 residue in co-ordination. Unfortunately the spectra were very complicated and could not be further analysed.

***cis*-[Pt(NH₃)₂(D₂O)₂]²⁺.** In contrast to the system of [Pd(en)(D₂O)₂]²⁺, the pentapeptide His-Pro-Gly-Ala-His reacts with *cis*-[Pt(NH₃)₂(D₂O)₂]²⁺ through imidazole nitrogens only in alkaline solutions. In the ^1H NMR spectrum of a 1:1 His-Pro-Gly-Ala-His:*cis*-[Pt(NH₃)₂(D₂O)₂]²⁺ mixture at pD 8.5 new peaks appear at δ 8.02, 7.20 and 3.74 with subsequent decrease of the intensity of peaks from imidazole protons of the histidyl-1 residue, after 3 d of reaction. These new peaks were assigned to imidazole CH-2, CH-5 and α -protons respectively of the histidyl-1 residue. The pattern of the rest of the spectrum did not change upon addition of the metal. Thus, it is obvious that the peptide co-ordinates the metal ion through donor atoms of histidyl-1 residue only. In addition the larger shift of the C2-H proton (by 0.70 ppm) than that of the C5-H proton (shifted by 0.29 ppm) compared to the free peptide in acidic solutions, where both imidazole nitrogens are protonated, suggests imidazole N3 co-ordination to the metal.³ Moreover, the upfield shift by 1.06 ppm of the α -proton of the histidyl-1 residue in the complex with respect to that of the free peptide indicates co-ordination of the amine group nitrogen to the metal (Table 1), thus forming again a stable six membered chelate ring, in a similar structure to that with Pd(en).

The ^{13}C and ^{195}Pt NMR spectra of the complex formed are consistent with the structure proposed based on ^1H NMR. In the ^{13}C NMR spectrum of the 1:1 mixture of His-Pro-Gly-Ala-His with the complex ion *cis*-[Pt(NH₃)₂(D₂O)₂]²⁺ only peaks of carbons of the histidyl-1 residue were shifted with respect to those of the free peptide. Thus, peaks observed at δ 139.22, 117.81 and 54.05 were assigned to C2, C5 and C α carbons of the product formed (Table 1) shifted by 1.77, 4.08 and 0.70 ppm respectively from peaks of the same carbons in the spectrum of the free peptide at the same pD. Finally, a peak appearing at δ -2685 in the ^{195}Pt NMR spectrum of the mixture (Table 1) suggests a N₄ co-ordination for the metal ion in the complex formed.^{3,27}

Reactions with MCl₄²⁻ (M = Pd^{II} or Pt^{II})

PdCl₄²⁻. Reaction of the pentapeptide His-Pro-Gly-Ala-His with K₂PdCl₄ in acidic solution (pD 1.2) and 1:1 molar ratio, yielded a 1:1 adduct. As seen in the ^1H NMR spectrum of this mixture (Fig. 4), the peaks appearing at δ 8.06, 7.11, 3.33 and 2.75 are assigned to imidazole C2-H, C5-H, α - and β -protons respectively of the histidyl-1 residue of the complex formed (Table 1). They are shifted upfield by 0.66, 0.38, 1.47 and 0.70 ppm respectively compared to the free peptide signals. The relatively larger shift of imidazole C2-H as compared to that of C5-H indicates N3 co-ordination to Pd^{II}.³ Similarly the shift of the α -proton of the histidyl-1 residue of the complex, compared to that of the free peptide at the same pD, suggests amine nitrogen co-ordination. Thus the usual six membered chelate ring is again formed.

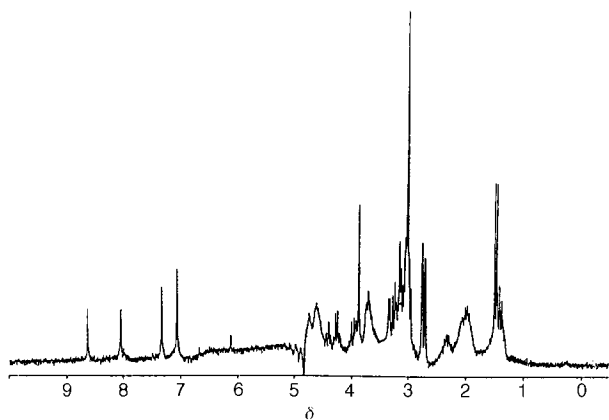


Fig. 4 The ^1H NMR spectrum of a 1:1 His-Pro-Gly-Ala-His: PdCl_4^{2-} mixture at pD 1.2.

The reaction at the ratio 1:1 is complete as evidenced by the addition of a second equivalent of peptide, the histidyl-1 proton resonances of which remain unshifted.

Carbon-13 NMR spectroscopy provides further support for the proposed structure. In the spectrum of a 1:1 (His-Pro-Gly-Ala-His: Pd^{II}) mixture at pD 1.2 new peaks, at δ 140.02, 133.84, 117.12, 54.21 and 28.78, assigned to imidazole C2, C4, C5, C α and C β respectively of the Pd^{II} co-ordinated histidyl-1 residue (Table 1) were observed. These peaks were shifted by 2.57, 5.85, 4.77, 0.86 and 0.99 ppm respectively compared to free peptide signals at the same pD. No significant change was observed in the resonances of the other carbon atoms. Obviously co-ordination of the peptide to Pd^{II} once more takes place only through histidyl-1 imidazole donor atoms and amine nitrogen (see below).

At higher pD, donor atoms from the imidazole ring of the histidyl-5 residue co-ordinate also to the metals as the ^1H NMR spectra indicated. These spectra could not be investigated further however, due to their complexity.

K_2PtCl_4 . When the pentapeptide His-Pro-Gly-Ala-His was mixed with K_2PtCl_4 in alkaline solution a white solid was precipitated after a few days of reaction. The ^1H NMR spectrum of this solid in $\text{DMSO}-d_6$ consisted of broad peaks indicating a possible polymeric nature.²⁸ In slightly acidic solutions (pD 5–6), however, a new complex of defined structure was formed as NMR spectroscopy revealed. The pD of this solution decreased to 2.0–2.5 after 4 d, when the reaction was complete. In the ^1H NMR spectrum of this mixture the intensities of peaks from imidazole protons of the histidyl-1 residue decreased and new peaks appeared at δ 8.21 and 7.12, assigned to imidazole C2-H and C5-H respectively of the histidyl-1 residue of the new complex formed. Moreover a new peak appeared at δ 3.53. Based on literature data, this peak was assigned to the α -proton of the histidyl-1 residue of the new complex (Table 1). The larger shift observed for imidazole C2-H than for the C5-H proton is indicative of N3 co-ordination to the metal.³ Also the upfield shift by 1.27 ppm of the α -proton of the histidyl-1 residue suggests amine nitrogen co-ordination, to form the known stable six membered chelate ring.

Carbon-13 NMR spectroscopy supports the proposed structure of the complex formed (Table 1). On the other hand a peak appearing at δ -2277 in the ^{195}Pt NMR spectrum of the mixture suggests a N_4Cl_2 co-ordination sphere for the metal ion in the complex formed^{3,27} (Table 1).

Discussion

Proton NMR spectroscopy provides a powerful tool in studying the structure and the reactivity of diamagnetic complexes. The presence of diamagnetic metal ions does not cause fast relax-

Table 3 Proton NMR data for imidazole protons in compounds where a histidyl imidazole ring bridges two Pd(dien) moieties. The one letter abbreviations are used for the amino acids

Compound	C2-H	C5-H	Ref.
$[\{\text{Pd}(\text{dien})\}_2(\mu\text{-N1,N3-HA})]^{4+}$	7.67	6.78	3
$[\{\text{Pd}(\text{dien})\}_2(\mu\text{-N1,N3-HGA})]^{4+}$	7.75	6.85	3
$[\{\text{Pd}(\text{dien})\}_2(\mu\text{-Ac-H-N1,N3})]^{2+}$	7.17	6.57	16
$[\{\text{Pd}(\text{dien})\}_2(\mu\text{-PGAH-N1,N3})]^{3+}$	7.16	6.60	<i>a</i>
$[\{\text{Pd}(\text{dien})\}_3(\mu\text{-N1,N3-HPGAH-N1})]^{5+}$	7.86	6.96	<i>a</i>
$[\{\text{Pd}(\text{dien})\}_3(\mu\text{-N1,N3-HPGAH-N3})]^{5+}$	7.86	6.96	<i>a</i>
$[\{\text{Pd}(\text{dien})\}_2(\mu\text{-N1,N3-HPGAH-N1,N3})]^{6+}$	7.86 (H-1) 7.12 (H-5)	6.96 (H-1) 6.57 (H-5)	<i>a</i>

^a This work.

ation of proton nuclei and line broadening as in the case of paramagnetic metal ions. In this case the chemical shifts observed upon metal binding to the peptide ligands are the result of direct exchange of electron density between the donor atom and the metal ion, or of conformational changes of the peptide. The influence of both effects is limited to the vicinity of the metal binding site and therefore metal bonding affects only the chemical shifts of the adjacent protons. Thus the detection of the donor atoms of the ligand and of the structure of the complex formed becomes easy. On the other hand, ^{13}C and ^{195}Pt NMR provide useful information about the donor atoms in the complexes formed, especially when there are no protons in their vicinity or when ligands of inorganic nature co-ordinate to the metal.

From this study we concluded that the reactivity of the two histidyl residues depends on their position in the peptide sequence. In the presence of $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$, both N1 and N3 of the imidazole ring of the histidyl-1 residue easily deprotonate in acidic solution (pD 3.5) with subsequent binding of two Pd(dien) moieties. Thus, this imidazole ring bridges two such Pd(dien) moieties. This behavior has been observed only in reactions of $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ with peptides, where histidine is the amino terminal residue.³ On the other hand, the imidazole ring of the histidyl-5 residue binds only one Pd(dien) moiety (either N1 or N3) in acidic media. It is found to bridge two moieties only in neutral or alkaline solution. This behavior is usual with peptides having histidine as the C-terminal amino acid but also with the acetylated derivative of histidine.¹⁶

In addition, although imidazole rings of both histidyl residues bridge two Pd(dien) moieties forming the complex $[\{\text{Pd}(\text{dien})\}_4(\mu\text{-N1,N3-His-Pro-Gly-Ala-His-N1,N3})]^{6+}$, imidazole protons of the histidyl-5 residue are more shielded than those of the histidyl-1 residue. Obviously, this difference is the result of the different positions of the two histidyl residues in the peptide sequence. As seen in Table 3, when histidyl imidazole bridges two Pd(dien) moieties its protons are more shielded when the histidyl residue is the C-terminal amino acid than when it is the N-terminal amino acid. This difference should be a result of local electronic effects. In this type of complex a net negative charge is localized on the imidazole ring. The repulsive interaction of this charge with the negative charge of the carboxylate group, in its vicinity, may impose a relative orientation of the imidazole ring with respect to the carboxylate group, causing the observed shielding of the imidazole ring protons. These local electronic effects may be the reason also for the observed difference in the reactivity of the imidazole ring nitrogens depending on the position of histidyl residues in the peptide sequence.

In contrast to the $[\text{Pd}(\text{dien})(\text{D}_2\text{O})]^{2+}$ systems, the Pt(dien) moiety reacts with imidazole nitrogens (either N1 or N3) in alkaline solutions. In all cases, one Pt(dien) moiety is bound per imidazole ring without formation of any complex in which an imidazole ring bridges two Pt(dien) moieties. This behavior is in accordance with literature observations,^{3,16} suggesting form-

ation of imidazole bridged complexes only in strongly alkaline solutions and in the presence of a large excess of $[\text{Pt}(\text{dien})(\text{D}_2\text{O})]^{2+}$. We observed however that the imidazole nitrogens of the C-terminal histidyl residues react to a greater extent than those of the N-terminal histidyl residue. Since platinum reactions are thermodynamically controlled, we can conclude that the local environment of the histidyl-5 residue increases the thermodynamic stability of the products formed by the reaction of its imidazole ring with the $\text{Pt}(\text{dien})$ moiety.

The reactions of the pentapeptide with complex ions of Pt^{II} or Pd^{II} possessing at least two labile ligands in the *cis* position, like $[\text{Pd}(\text{en})(\text{D}_2\text{O})_2][\text{NO}_3]_2$, *cis*- $[\text{Pt}(\text{NH}_3)_2(\text{D}_2\text{O})_2][\text{NO}_3]_2$, $\text{K}_2\text{-PdCl}_4$ and K_2PtCl_4 , take place first through histidyl-1 residue donor atoms. Obviously in these reactions there is a possibility of formation of chelate rings. Thus, stable six membered chelate rings are formed by co-ordination of the groups with the lower proton dissociation constants (NH_2 and imidazole N3). On increasing the pD of the solution we observed in all cases formation of new products in which more than the above groups are involved in co-ordination. Although we were not able to characterize in detail the structures of the species formed, it is possible that anchoring of the metal ion to one of the imidazole nitrogens of the histidyl-5 residue can trigger formation of new products by co-ordination to the metal ion of deprotonated peptide bond nitrogens.

It is obvious that when square planar complexes of Pt^{II} and Pd^{II} interact with histidyl containing peptides or proteins, imidazole nitrogens are potential binding sites. Clearly, the exact co-ordination site of the metal ion depends also on the environment of the histidyl residue in the macromolecule. Thus similar studies may contribute to the elucidation of mechanistic aspects of the antitumor or of the highly toxic effects of the antitumor square planar platinum(II) drugs.

Acknowledgements

This work was carried out within the scope of a COST D8 program, "Metals in Medicine".

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Paper 8/03700K