Ring-Opened Adducts of the Anticancer Drug Carboplatin with Sulfur Amino Acids

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Reactions of the anticancer drug carboplatin ("Paraplatin") with a variety of sulfur-containing amino acids have been investigated by ^{1}H and ^{15}N NMR spectroscopy and by HPLC. Thiols react very slowly and sulfur-bridged species containing four-membered Pt_2S_2 rings are the predominant products. In contrast, reactions with thioether ligands are much more rapid, and kinetics for the initial stages of the reaction with L-methionine have been determined ($k = 2.7 \times 10^{-3}$ M^{-1} s^{-1}). Surprisingly, very stable ring-opened species are formed such as *cis*-[Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)] which has a half-life for Met-*S*,*N* ring-closure of 28 h at 310 K. A study of the formation of the analogous product for *N*-acetyl-L-methionine and its subsequent ring closure is reported. Reactions such as these may play a role in the biological activity of carboplatin.

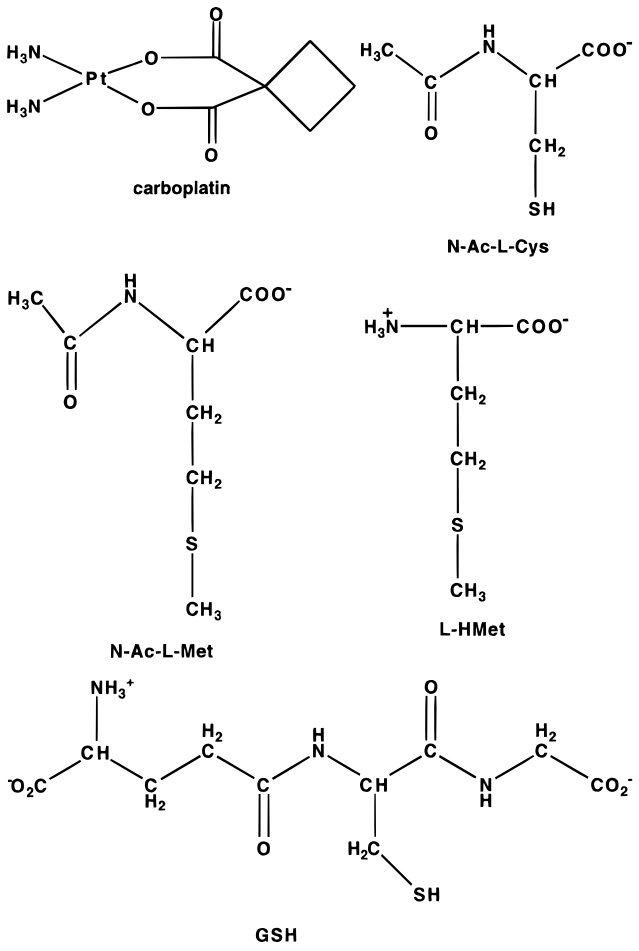
Introduction

Carboplatin [Pt(CBDCA-*O*,*O*′)$(NH_3)_2$], where CBDCA is cyclobutane-1,1-dicarboxylate, is a widely-used second-generation platinum anticancer drug.[3] Like cisplatin (*cis*-[$PtCl_2$-$(NH_3)_2$]), it is thought that its antitumor activity is due to attack on DNA. Cisplatin has more severe side effects than carboplatin, including renal toxicity which is dose-limiting. The lower toxicity of carboplatin can be attributed to its lower reactivity, caused by the presence of the chelating CBDCA ligand.[4]

It has been suggested that carboplatin is a pro-drug for cisplatin.[5] However the rate of aquation is very slow,[6] and recent results of Frey *et al.*[7] indicate that the major reaction path of carboplatin is via direct attack by nucleophiles rather than via an aquation step. As a result, ring-opened CBDCA species are readily detected during reactions of carboplatin with chloride and guanosine 5′-monophosphate (5′-GMP).

Although attack on DNA is responsible for the antitumor activity, platinum complexes can interact with many other biomolecules especially those containing sulfur, for which it has a very high affinity. Examples of sulfur-containing biomolecules include amino acids such as cysteine and methionine, peptides such as glutathione, and proteins such as metallothionein and many others. Interactions of cisplatin with sulfur molecules are thought to be responsible for a variety of biological effects, such as inactivation of Pt(II) complexes, development of cellular resistance to platinum, and toxic side effects such as nephrotoxicity.[8] Recent results have suggested that platinum complexes containing monodentate thioethers (methionine or methionine-containing proteins and peptides) may play a role in the biological activity of platinum complexes.[9,10] Surprisingly, monodentate thioether ligands can be substituted by N7 of guanine bases, although only very slowly. Therefore it is conceivable that a methionine-containing protein or peptide could transport and transfer some platinum to DNA.

Although the interactions of a variety of sulfur-containing biomolecules with cisplatin have been studied, little is known about their interactions with carboplatin. Recently we reported the detection of a Pt(II) complex containing ring-opened CBDCA and a monodentate thioether ligand in the urine of mice treated with [^{15}N]carboplatin.[11] Here we report investigations of reactions of carboplatin with the sulfur amino acids L-methionine (L-HMet), *N*-acetyl-L-methionine (*N*-Ac-L-Met), *N*-acetyl-L-cysteine (*N*-Ac-L-Cys), and glutathione (GSH), by ^{1}H, ^{15}N, and ^{195}Pt NMR spectroscopy and HPLC. The data show that ring-opened carboplatin adducts containing monodentate thioethers are remarkably stable.



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Experimental Section

Chemicals. L-HMet, *N*-Ac-L-Met, *N*-Ac-L-Cys, and GSH were purchased from Aldrich. [Pt(CBDCA-*O*,*O*′)($^{15}NH_3$)$_2$] and *cis*-[Pt($^{15}NH_3$)$_2$(H_2O)$_2$](NO_3)$_2$ were prepared according to literature methods.[7]

NMR Spectroscopy. NMR spectra were recorded on the following instruments: Bruker AM500 (^{1}H 500 MHz, ^{15}N 50.7 MHz), Varian Unity 500 (^{1}H 500 MHz, ^{15}N 50.7 MHz), Varian Unity 600 (^{1}H 600 MHz, ^{15}N 60.8 MHz), JEOL GSX270 (^{1}H 270 MHz, ^{195}Pt 58 MHz), and JEOL GSX500 (^{1}H 500 MHz), using 5 mm Wilmad NMR tubes. The chemical shift references were as follows: ^{1}H (internal), TSP; ^{15}N (external), 1.5 M $^{15}NH_4Cl$ in 1 M HCl in 90% H_2O/10% D_2O; ^{195}Pt (external), 1 M Na_2PtCl_6. Spectra were recorded at either 298 or 310 K as indicated. The acquisition parameters used were as described previously.[12−14]

NMR samples for the following reactions of carboplatin with the sulfur amino acids were prepared by mixing 1 mL of 10 mM [Pt(CBDCA-*O*,*O*′)($^{15}NH_3$)$_2$] with 1 mL of a 10 mM solution of the ligand. When ^{15}N spectra were to be recorded, the solvent was 90% H_2O/10% D_2O; otherwise, D_2O alone was used. All solutions contained 50 mM phosphate at pH = 7 unless otherwise stated.

A 10 mM solution of [Pt(*N*-Ac-L-Met-*S*,*N*)($^{15}NH_3$)$_2$] was prepared by mixing 1 mL of a 20 mM solution of *cis*-[Pt($^{15}NH_3$)$_2$(H_2O)$_2$](NO_3)$_2$ in 90% H_2O/10% D_2O, with 1 mL of a 20 mM solution of *N*-Ac-L-Met in 90% H_2O/10% D_2O. Then the pH was adjusted to 7 with 1 M NaOH solution, and the solution was allowed to stand for 24 h before the NMR spectra were recorded. *cis*-[{Pt($^{15}NH_3$)$_2$(A-μ-*S*)}$_2$] (A = *N*-Ac-L-Cys or GSH) was prepared by mixing 1 mL of 20 mM *cis*-[Pt($^{15}NH_3$)$_2$(H_2O)$_2$](NO_3)$_2$ in 90% H_2O/10% D_2O with 1 mL of a 20 mM solution of A in 90% H_2O/10% D_2O.[15] Spectra were recorded 20 min after mixing.

HPLC. The following equipment was used: Gilson 305 pumps, Gilson 806 manometric module, LKB 2141 variable wavelength monitor, and Rheodyne sample injector. Analytical separations were carried out on a PLRP-S column (250 × 4.6 mm, 100 Å, 5 μm, Polymer Labs) with H_2O as the eluant. The data were analyzed on an Apple Macintosh computer using Dynamax Method Manager Software.

The time course reaction between carboplatin (5 mM) and L-HMet (5 mM) at 298 K (pH 5.9) was followed chromatographically by injection of aliquots of the mixture onto the HPLC column at various time intervals with detection at 210 nm.

Kinetic Measurements. The kinetics of the formation of the 1:1 adduct between carboplatin and L-HMet were determined in 50 mM phosphate buffer at 298 K, pH* (meter reading) = 7 in D_2O. The platinum complex (5 mM) was mixed with an excess of ligand (45−60 mM) in the NMR tube, and spectra were recorded at appropriate time intervals. The reaction was followed by observing the decrease in intensity of the ^{1}H NMR signal of α-CH_2 of the cyclobutane ring of [Pt(CBDCA-*O*,*O*′)(NH_3)$_2$]. The use of a large excess of ligand ([L-HMet]:[Pt] > 9:1) provided pseudo-first-order conditions which allowed k_{obs} to be calculated at four different concentrations of L-HMet. A second-order rate constant was determined from a plot of k_{obs} *vs* [L-HMet]. The program Kaleidagraph (Synergy Software, Reading, PA) on an Apple Macintosh computer was used to obtain the best fits to these data.

To study the kinetics of ring-closure of *cis*-[Pt(CBDCA-*O*)(NH_3)$_2$(L-HMet-*S*)] to give [Pt(L-Met-*S*,*N*)(NH_3)$_2$]$^+$, a pure sample of the ring-opened complex was isolated by HPLC and was incubated as an aqueous solution (pH 5.61) at 310 K in a stoppered tube. Aliquots were removed at appropriate times and reanalyzed by HPLC using 2% CH_3CN/ 98% H_2O isocratically as the eluant and detection at 210 nm. The disappearance of the peak due to *cis*-[Pt(CBDCA-*O*)(NH_3)$_2$(L-HMet-*S*)] was followed with time, and a first-order rate constant was obtained from a plot of ln[peak area] *vs* time.

pH Measurements. These were recorded using a Corning 240 pH meter equipped with an Aldrich micro combination electrode standardized with pH 4, 7, and 10 buffers. Values for D_2O solutions are termed pH* and are uncorrected for the effect of deuterium on the glass electrode.

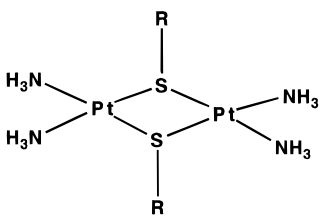
Results

NMR spectroscopy was used to study the reactions of carboplatin with the thiols *N*-Ac-L-Cys and GSH, and the thioethers L-HMet and *N*-Ac-L-Met. Labeling the ammine ligands of carboplatin with ^{15}N allowed 2D [^{1}H,^{15}N] NMR investigations to be made. The ^{15}N chemical shift is diagnostic of the coordinating atom of the ligand in the position trans to the ammine.[16,17] For an ammine ligand trans to an oxygen donor, the ^{15}N shift is between −75 and −90 ppm, for an ammine trans to nitrogen/chloride it is between −55 and −70 ppm, and for ammine trans to sulfur it is between −40 and −50 ppm. The reaction of carboplatin and L-HMet was also studied by HPLC.

Reaction of Carboplatin with *N*-Ac-L-Cys and GSH. The reactions of carboplatin with *N*-Ac-L-Cys and GSH gave similar results. The reactions were followed by ^{1}H and 2D [^{1}H,^{15}N] NMR spectroscopy. No reaction between carboplatin and either *N*-Ac-L-Cys or GSH was observed by either ^{1}H or 2D [^{1}H,^{15}N] NMR spectroscopy for at least 10 h at 310 K. After 24 h a small peak was observed in the 2D [^{1}H,^{15}N] NMR spectra of both reactions; for GSH, this had shifts of −44.2/ 3.97 ppm, while for the *N*-Ac-L-Cys adduct the peak was observed at −43.8/ 3.92 ppm. The ^{15}N shifts of these peaks are consistent with an ammine ligand trans to a sulfur ligand. These reactions were performed in phosphate buffer at pH 7 and no other products were observed in the spectra. After 4−5 weeks standing at ambient temperature, the solutions became deep yellow in color and a yellow precipitate formed.

When the reactions were carried out at a lower pH value of 3, they were just as slow and the same products were observed, but a peak assignable to $^{15}NH_4^+$ was also present. This suggests that the high trans effect of the thiol ligand leads to labilisation of the ammine ligand.

The chemical shifts of these peaks are the same as those observed for the major products of the reaction of *cis*-[Pt($^{15}NH_3$)$_2$(H_2O)$_2$](NO_3)$_2$ with *N*-Ac-L-Cys and GSH. Appleton *et al.*[15] has shown that this product is a sulfur-bridged diplatinum complex containing a four-membered ring: *cis*-[{Pt($^{15}NH_3$)$_2$((*N*-Ac-L-Cys or GSH)-μ-S_2)}$_2$]. Several groups have observed the precipitation of yellow solids when thiols are reacted with platinum complexes and these are usually thought to be polymers.[15,18,19]



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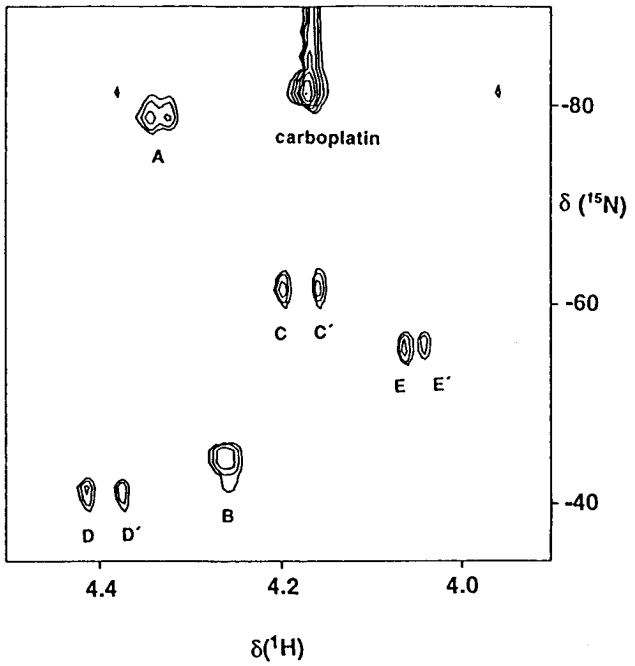

Figure 1. 2D [^{1}H, ^{15}N] NMR spectrum of a solution containing [^{15}N]-carboplatin and L-HMet (5 mM, 1:1), in 50 mM phosphate buffer pH 7 after 24 h at 298 K. Peak assignments: (A, B) *cis*-[Pt(CBDCA-*O*)-($^{15}NH_3$)$_2$(L-HMet-*S*)]; (C, C′, D, D′) [Pt(L-Met-*S*,*N*)($^{15}NH_3$)$_2$]$^+$; (E, E′) [Pt(L-Met-*S*,*N*)($^{15}NH_3$)(L-HMet-*S*)]$^+$.

Attempts were made to determine the rate at which carboplatin reacts with either *N*-Ac-L-Cys or GSH. However under pseudo-first-order conditions, with either the ligand or carboplatin in excess, the data could not be fitted to appropriate linear plots, suggesting that multiple reaction pathways exist under these conditions. When the reaction with *N*-Ac-L-Cys was carried out with a 1:1 stoichiometry at 298 K, the data from the early part of the reaction (up to 48 h) were fitted to a second-order process by plotting $x/(a(a - x))$ *vs* t (a = initial concentration and x = concentration at time t), yielding a rate constant with a value of ca. 10^{-9} M^{-1} s^{-1}.

Reaction of Carboplatin with Methionine. In the first few hours of the reaction of carboplatin (5 mM) with 1 mol equiv of L-HMet, two new sets of cross-peaks (A and B) of similar intensity appeared in the [^{1}H,^{15}N] NMR spectrum: −78.8/4.34, 4.32 ppm (A) and −43.0/4.26 ppm (B) (Figure 1). In the ^{1}H NMR spectrum, a new broad peak at 2.36 ppm was observed. The ^{15}N chemical shifts are consistent with a species that has an ammine trans to sulfur and another ammine trans to oxygen. The ^{1}H NMR shift of 2.36 ppm is consistent with a SCH_3 group of methionine that is coordinated to platinum through sulfur. Cross-peak A in the [^{1}H,^{15}N] NMR spectrum is split into a doublet in the ^{1}H dimension. A temperature dependence study of this spectrum showed that at low temperature (288 K) both cross-peaks A and B were split into doublets while at higher temperature (308 K) both peaks were singlets. This temperature dependence can be explained by inversion of the methyl group about the chiral coordinated sulfur of L-HMet; at low temperature the two diastereomers are resolved whereas at high temperature only exchange-averaged peaks are observed. From the temperature dependence, a rate of inversion k = 22.2 s^{-1} and activation free energy $\Delta G^{\ddagger}$ = 66.5 kJ mol^{-1} were determined at 298 K. A ^{195}Pt spectrum of a 1:1 mixture (at 60 mM) gave rise to a broad peak at −2641 ppm. This shift is consistent with the formation of a species that has a PtN_2SO coordination sphere.[15,20]

As we have argued previously,[11] the sulfur ligand in this PtN_2SO complex must be due to methionine, and the possibilities for the oxygen ligand include H_2O/OH^-, PO_4^{3-}, and carboxylate oxygen from either CBDCA or L-HMet. The same spectrum was obtained when the sample was prepared in the absence of PO_4^{3-}, and the shifts of these peaks were also independent of pH between 4 and 7 (the pK_a of coordinated H_2O would be expected in this pH range).[21] Since the complex [Pt(L-HMet-*S*,*O*)$(NH_3)_2]^{2+}$ has previously been characterized and found to be stable only at low pH (< 2),[20] it is likely that the complex contains coordinated monodentate CBDCA and can be formulated as *cis*-[Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)]. The presence of CBDCA in this complex was confirmed by a 2D DQF-COSY ^{1}H NMR spectrum, which showed cross-peaks for monodentate CBDCA with shifts of 2.36 ppm for the α-CH_2 and 1.86 ppm for the γ-CH_2. These shifts are very close to those previously observed for platinum-bound monodentate CBDCA in other adducts.[7]

After the 1:1 solution of [^{15}N]carboplatin and L-HMet had been allowed to stand at ambient temperature overnight, cross-peaks A and B due to *cis*-[Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)] were still present in the [^{1}H,^{15}N] spectrum. However several new peaks were also observed (Figure 1). Four of these (C, C′, D, D′) can be assigned to [Pt(L-Met-*S*,*N*)$(NH_3)_2]^+$ on the basis of known shifts.[22] The ^{15}N shifts of C and C′ are typical of an ammine coordinated trans to a nitrogen ligand; the only possible nitrogen atoms in the solution are the amino group of L-HMet and NH_3. The ^{15}N shifts of D and D′ are consistent with an ammine coordinated trans to sulfur (Table 1). The same peaks were observed when L-HMet was added to a solution of *cis*-[Pt($^{15}NH_3$)$_2$(H_2O)$_2$](NO_3)$_2$ the major product of this reaction being [Pt(L-Met-*S*,*N*)$(NH_3)_2]^+$.[20,22] The presence of two sets of doublets can be attributed to the slow inversion of the coordinated chiral sulfur giving rise to two diastereomers. The rate of inversion is slower for a chelated methionine than for a monodentate methionine.

Another doublet cross-peak (E and E′) was also observed in the [^{1}H,^{15}N] spectrum in the ammine trans to nitrogen region of the spectrum. These signals are probably due to a species that has lost an ammine ligand due to the high trans effect of sulfur. The ^{1}H NMR spectrum showed signals in the region 2.59−2.60 ppm that can be assigned to the *S*-methyl groups of [Pt(L-Met)$_2$].[23] The presence of this species in solution was confirmed by HPLC (*vide infra*). The trans isomer of [Pt(L-Met)$_2$] is the kinetically preferred product, while the cis isomer is the themodynamically more stable product (87:13 at equilibrium). The peaks E and E′ are probably due to diastereoisomers of [Pt(L-Met-*S*,*N*)(NH_3)(L-HMet-*S*)]$^+$ (slow sulfur inversion of chelated L-Met).[23] This species is likely to be an intermediate on the pathway from [Pt(L-Met-*S*,*N*)$(NH_3)_2]^+$ to [Pt(L-Met)$_2$]. On the basis of the results obtained here, a scheme for the reaction of L-HMet with carboplatin can be proposed (Scheme 1).

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Scheme 1

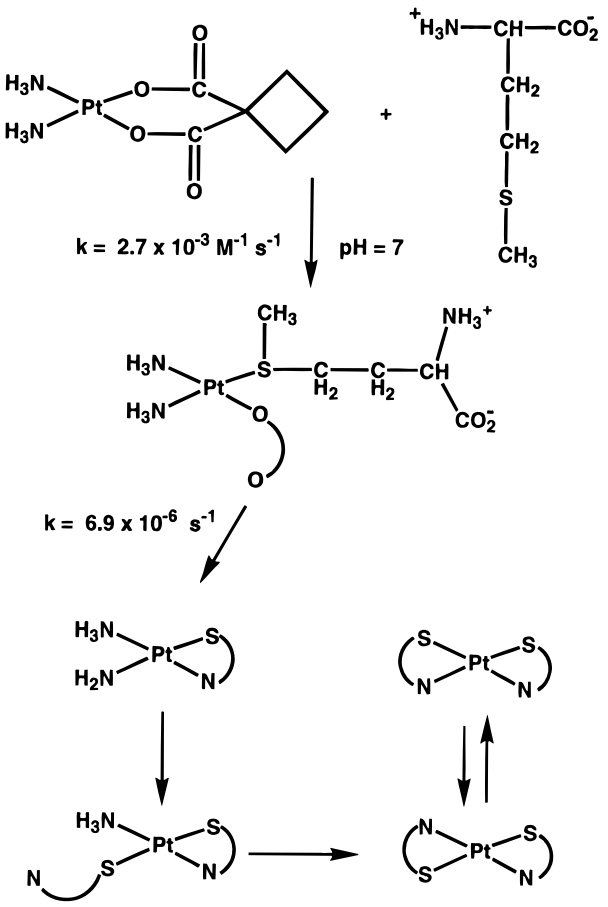


Table 1. ^{1}H and ^{15}N Chemical Shifts

compound	δ(S−CH$_3$)	$\delta(^{15}N)/\delta(^1H)$ for Pt−NH$_3$	Pt−NH$_3$ trans to
L-HMet	2.14		
[Pt(CBDCA-*O*,*O*′)-(^{15}NH$_3$)$_2$]		−81.3/4.17	O
cis-[Pt(CBDCA-*O*)-(^{15}NH$_3$)$_2$(L-HMet-*S*)]	2.36	−78.8/4.34,4.32	O
		−43.0/4.26	S
[Pt(L-Met-*S*,*N*)(^{15}NH$_3$)$_2$]$^+$	2.53, 2.55	−61.8/4.17,4.21	N
		−41.8/4.38,4.41	S
[Pt(L-Met-*S*,*N*)-(^{15}NH$_3$)(L-HMet-*S*)]$^+$	*a*	−56.0/4.04,4.07	N
[Pt(L-Met-*S*,*N*)$_2$]	2.59−2.60[b]		

[a] Unassigned. [b] Peaks for three diastereomers of each geometrical isomer.

Table 2. Pseudo-First-Order Rate Constants for the Reaction of Carboplatin (5 mM) with L-HMet in 50 mM Phosphate at pH* 7 and 298 K

[L-HMet]/mM	k_{obs}/s^{-1}	[L-HMet]/mM	k_{obs}/s^{-1}
45	9.43×10^{-5}	55	1.22×10^{-4}
50	1.02×10^{-4}	60	1.32×10^{-4}

Pseudo-first-order rate constants were determined for the formation of *cis*-[Pt(CBDCA-*O*)(NH$_3$)$_2$(L-HMet-*S*)] from carboplatin and L-HMet at various L-HMet concentrations (Table 2) by following the decrease in intensity of the α-CH$_2$ signal of carboplatin in the ^{1}H NMR spectra. A typical kinetic plot is shown in Figure 2. From these pseudo-first-order rate constants, a second-order rate constant of $k = 2.7 \times 10^{-3}$ M^{-1} s^{-1} was calculated.

The time course of the reaction of carboplatin with L-HMet (1:1, 5 mM, pH = 5.61, 298 K) was also followed by HPLC

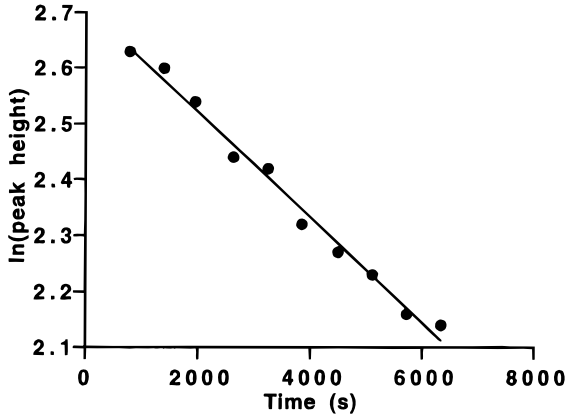


Figure 2. Plot of the ln of the intensity of the ^{1}H NMR α-CH$_2$ peak of carboplatin (5 mM) *vs* time during reaction with an excess of L-HMet (45 mM). From the slope, a pseudo-first-order rate constant $k_{obs} = 9.43 \times 10^{-5}$ s^{-1} was determined.

(see Figure 3). After 1 h, a new peak with a retention time of 2.4 min appeared in the chromatogram along with peaks for carboplatin (3.9 min) and L-HMet (2.9 min). Over the next few hours this peak gradually increased in intensity while the peaks due to carboplatin and L-HMet decreased in intensity. To identify the species responsible for this peak, the fraction was collected and freeze-dried to concentrate the sample, and analyzed by ^{1}H NMR. This procedure was repeated with [^{15}N]-carboplatin so that a 2D [^{1}H, ^{15}N] NMR spectrum could be obtained. The NMR spectra of this fraction are consistent with the species being *cis*-[Pt(CBDCA-*O*)(NH$_3$)$_2$(L-HMet-*S*)] (see NMR results above). After a reaction time of 1 day, the chromatogram of the mixture showed the presence of two other new peaks. These had retention times of 1.9 and 3.5 min with the latter being more intense. The corresponding fractions were analyzed by NMR spectroscopy, and by using ^{15}N-L-HMet, it was possible to show that the two peaks are due to the cis and trans isomers of [Pt(L-Met-*S*,*N*)$_2$], with chemical shifts the same as those reported elsewhere.[23] After two days of reaction, all the above peaks were still present and a further new peak at 4.7 min was observed. When the fraction corresponding to the latter peak was analyzed by NMR spectroscopy, no signal was observed in the ^{1}H NMR spectrum. However a single cross-peak (−78.7/4.07 ppm) was observed in the 2D [^{1}H, ^{15}N] NMR spectrum with shifts characteristic of an ammine trans to oxygen and is presumably due to an aqua/hydroxo species. After 8 days of reaction, the mixture gave a chromatogram which showed that all the L-HMet had reacted and that only a trace of the ring-opened species *cis*-[Pt(CBDCA-*O*)(NH$_3$)$_2$(L-HMet-*S*)] was still present.

Confirmation that the ring-opened species *cis*-[Pt(CBDCA-*O*)(NH$_3$)$_2$(L-HMet-*S*)] was isolable by HPLC was obtained by both ^{1}H and [^{1}H,^{15}N] NMR. When an aqueous solution of this purified complex was allowed to stand, new peaks appeared in the ^{1}H and 2D [^{1}H,^{15}N] spectra that were assignable to [Pt(L-Met-*S*,*N*)(NH$_3$)$_2$]$^+$, and peaks assignable to free CBDCA were also observed in the ^{1}H NMR spectrum.

[Pt(L-Met-*S*,*N*)(NH$_3$)$_2$]$^+$ was observed during the course of the reaction when studied by NMR, but was not observed using the HPLC conditions reported here. This complex tended to stay on the column when H$_2$O was the eluant. When 2% CH$_3$-CN/98% H$_2$O was the eluant, then [Pt(L-Met-*S*,*N*)(NH$_3$)$_2$]$^+$ eluted much faster (see below), but with this solvent there was more overlap of peaks with those for the other species.

A first-order rate constant for S,N-closure of the methionine chelate ring and displacement of CBDCA was determined by

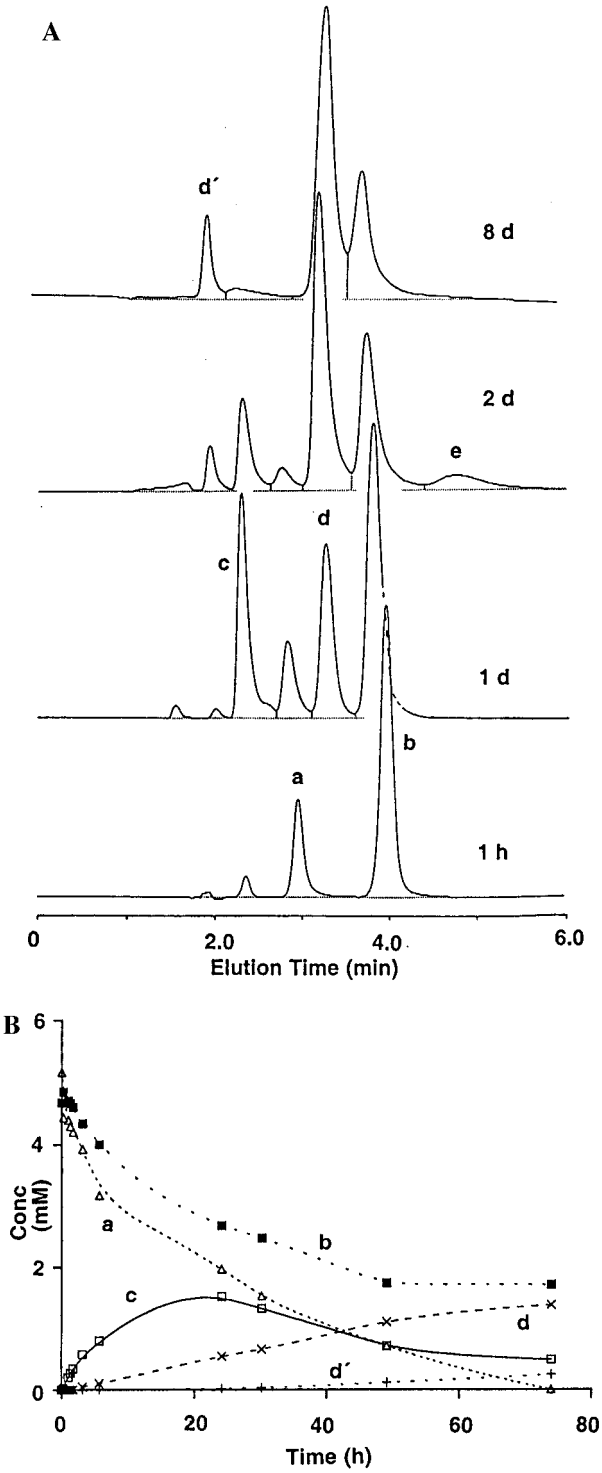


Figure 3. HPLC time course. (A) Chromatograms for the reaction mixture containing L-HMet and carboplatin (5 mM, 1:1) after reaction for various times at 298 K, pH 5.9. Peak assignments: a (△), L-HMet; b (■), carboplatin; c (□) *cis*-[Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)]; d (×), *cis*-[Pt(L-Met-*S*,*N*)$_2$]; d′ (+), *trans*-[Pt(L-Met-*S*,*N*)$_2$]; e, *cis*-[Pt$(NH_3)_2$-$(H_2O/OH)_2$]. (B) Variations in the relative concentrations of species detected during the above reaction with time. HPLC peaks areas were scaled by the approximate extinction coefficients; the value for the ring-opened complex was assumed to be the same as for carboplatin. For labels see part A.

HPLC methods. Aliquots of an aqueous solution of isolated *cis*-[Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)] were injected onto the HPLC column at various times and eluted with 2% CH_3CN/98% H_2O. Three peaks were observed in the resulting chromatograms: a large peak due to the starting material (2.1 min) and two new peaks with retention times of 3.9 and 5.8 min which can be assigned to [Pt(L-Met-*S*,*N*)$(NH_3)_2]^+$ and uncoordinated CBDCA, respectively. The large peak decreased in intensity with time, while the other peaks grew in intensity with time. A first-order rate constant was determined by following the decrease in concentration of the starting material with time, $k = 6.9 \times 10^{-6}$ s^{-1} ($t_{1/2} = 28$ h) at 310 K, Figure 4.

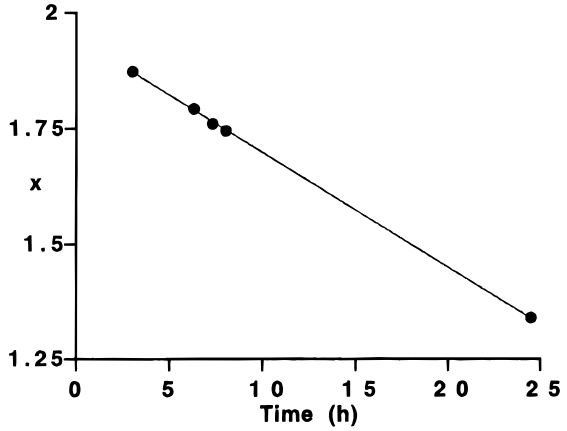


Figure 4. Plot of the ln of the HPLC peak area for *cis*-[Pt(CBDCA-*O*)$(NH_3)_2$(L-HMet-*S*)] (*x*) *vs* time during ring-closure to form [Pt(L-Met-*S*,*N*)$(NH_3)_2]^+$. From the slope a first-order rate constant of 6.9×10^{-6} s^{-1} was determined.

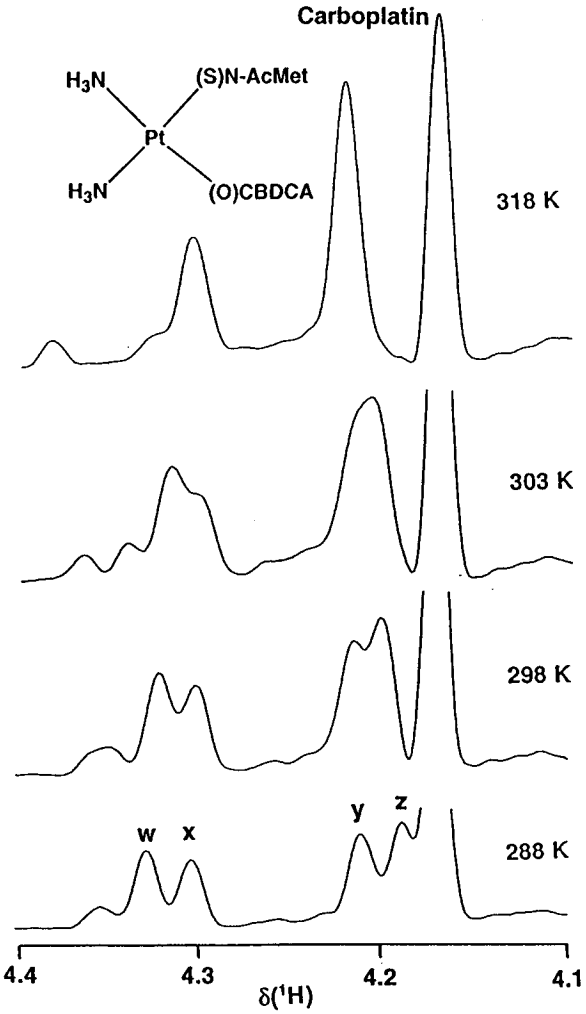


Figure 5. Effect of temperature on the ^{15}N-edited ^{1}H NMR peaks of *cis*-[Pt(CBDCA-*O*)$(NH_3)_2$(*N*-Ac-L-Met-*S*)]$^-$ present in a reaction mixture containing [^{15}N]carboplatin and *N*-Ac-L-Met (5 mM 1:1), pH 7. Note the coalescence of peaks w and x (NH_3 trans to O) and y and z (NH_3 trans to S) at higher temperature.

Reaction of Carboplatin with *N*-Acetyl-L-methionine. This

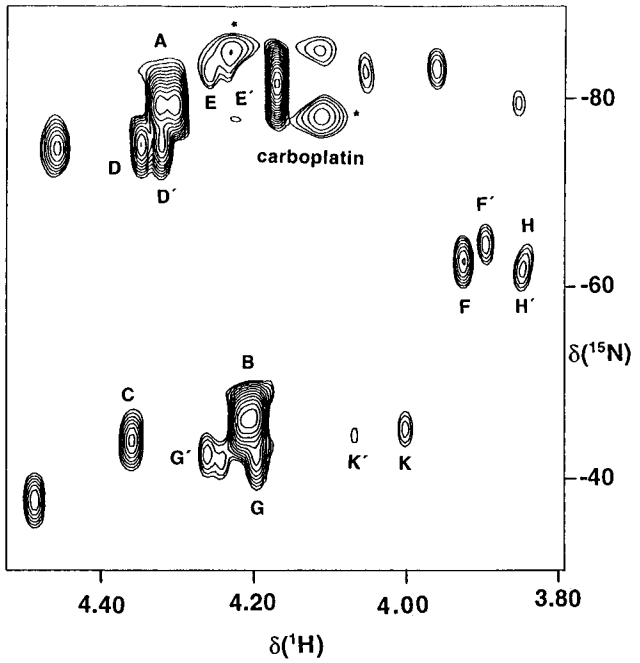


Figure 6. 2D [^{1}H, ^{15}N] NMR spectrum of a solution containing [^{15}N]-carboplatin and *N*-Ac-L-Met (5 mM, 1:1) in 50 mM phosphate buffer at pH 7, after 24 h, 298 K. Assignments: (A, B) *cis*-[Pt(CBDCA-*O*)-($^{15}NH_3$)$_2$(*N*-Ac-L-Met-*S*)]$^-$, (C) [Pt(*N*-Ac-L-Met-*S*)$_2$($^{15}NH_3$)$_2$], (D, D′) *trans*-[Pt(CBDCA-*O*)($^{15}NH_3$)(*N*-Ac-L-Met-*S*)$_2$]$^{2-}$, (E, E′) [Pt(CBDCA-*O*)(PO$_4$)($^{15}NH_3$)(*N*-Ac-L-Met-*S*)]$^{4-}$, (F, F′, G, G′) *cis*-[Pt(*N*-Ac-L-Met-*S,N*)($^{15}NH_3$)$_2$], (H, H′, K, K′) *trans*-[Pt(*N*-Ac-L-Met-*S,N*)($^{15}NH_3$)$_2$], and (∗) ^{195}Pt satellites.

reaction was carried out at a 1:1 mol ratio (5 mM) in 50 mM phosphate, at pH 7. The initial stages resembled those of the reaction of carboplatin with L-HMet. Two new peaks of similar intensity appeared in the [^{1}H,^{15}N] NMR spectrum at −79.0/ 4.32,4.30 and −45.6/ 4.21 ppm. The ^{15}N chemical shifts are consistent with an ammine trans to oxygen and an ammine trans to sulfur, respectively, and can be assigned to the ring-opened complex *cis*-[Pt(CBDCA-*O*)(NH$_3$)$_2$(*N*-Ac-L-Met-*S*)]$^-$. As for the L-HMet complex, the cross-peak for the ammine trans to oxygen is split into a doublet due to slow inversion of the *S*-methyl group. The temperature dependence of these peaks in the ^{15}N-edited ^{1}H NMR spectrum is shown in Figure 5. The rate and activation free energy for inversion of the *S*-methyl group ($k = 22.7\ s^{-1}$, $\Delta G^{\ddagger} = 65.6\ kJ\ mol^{-1}$) are similar to those of *cis*-[Pt(CBDCA-*O*)(NH$_3$)$_2$(L-Met-*S*)].

After eight hours of reaction of carboplatin with *N*-acetyl-L-methionine, a new peak was observed in the [^{1}H,^{15}N] NMR spectrum at −43.7/4.36 ppm. The ^{15}N shift is indicative of an ammine trans to a sulfur ligand. At about this time a significant quantity of free CBDCA was observed in the ^{1}H NMR spectrum consistent with assignment of the new [^{1}H,^{15}N] peak to *cis*-[Pt($^{15}NH_3$)$_2$(*N*-Ac-L-Met-*S*)$_2$].

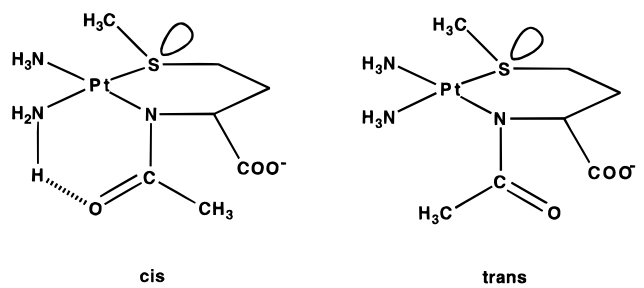
Subsequently another peak appeared in the [^{1}H,^{15}N] NMR spectrum at −75.1/4.35,4.33 ppm. This is assignable to an ammine trans to oxygen. The doublet appearance in the ^{1}H dimension is presumably due to slow *S*-methyl inversion. The absence of a signal due to an ammine trans to sulfur, probably indicates that this ammine has been labilized by the high trans effect of sulfur. It is likely that the ammine has been replaced by an S-bound *N*-Ac-L-Met ligand, so this signal is tentatively assigned to *trans*-[Pt(CBDCA-*O*)(NH$_3$)(*N*-Ac-L-Met-*S*)$_2$]$^{2-}$.

The [^{1}H,^{15}N] NMR spectrum after 24 h showed many new peaks in addition to those already described (Figure 6). Among these are four peaks in the ammine trans to nitrogen region (Table 3), and these, along with four new peaks in the ammine trans to sulfur region, are assigned to [Pt(*N*-Ac-L-Met-*S,N*)-($^{15}NH_3$)$_2$] (*vide infra*). A weak doublet also appeared at −82.5/ 4.26, 4.24 ppm indicative of an ammine trans to oxygen in a species with slow S inversion. No peak for an ammine trans to sulfur which might be paired with this peak was observed, presumably because this ammine has been labilized and replaced by another ligand. When the reaction was repeated in the absence of phosphate, the peak at −82.5 ppm did not appear in the spectrum, unlike all the other peaks seen. This suggests that the ammine trans to S in the ring-opened species *cis*-[Pt-(CBDCA-*O*)(NH$_3$)$_2$(*N*-Ac-L-Met-*S*)]$^-$ has been replaced by phosphate to give *cis*-[Pt(CBDCA-*O*)(PO$_4$)(NH$_3$)(*N*-Ac-L-Met-*S*)]$^{4-}$. Several other peaks were observed (Table 3) but have not been assigned.

Table 3. ^{1}H and ^{15}N NMR Chemical Shifts of Carboplatin and Its Adducts with *N*-Acetyl-L-methionine

complex	$\delta(^{15}N)/\delta(^1H)$ for Pt−NH$_3$	Pt−NH$_3$
[Pt(CBDCA-*O,O′*)($^{15}NH_3$)$_2$]	−81.3/4.17	O
cis-[Pt(CBDCA-*O*)-($^{15}NH_3$)$_2$(*N*-Ac-L-Met-*S*)]$^-$	−79.0/4.32,4.30	O
	−45.6/4.21	S
[Pt(*N*-Ac-L-Met-*S*)$_2$($^{15}NH_3$)$_2$]	−43.7/4.36	S
trans-[Pt(CBDCA-*O*)-($^{15}NH_3$)(*N*-Ac-L-Met-*S*)$_2$]$^{2-}$	−75.0/4.33,4.35	O
[Pt(CBDCA-*O*)(PO$_4$)-($^{15}NH_3$)(*N*-Ac-L-Met-*S*)]$^{4-}$	−82.5/4.24,4.26	O
[Pt(*N*-Ac-L-Met-*S,N*)($^{15}NH_3$)$_2$]	−62.6/3.84	N
	−61.6/3.85	N
	−64.4/3.90	N
	−62.6/3.93	N
	−42.6/4.26	S
	−41.9/4.20	S
	−44.2/4.09	S
	−44.9/4.02	S
unassigned	−37.5/4.49	S
	−42.6/4.07	S
	−74.7/4.46	O
	−83.1/4.06	O
	−83.1/3.96	O
	−79.6/3.85	O

To confirm the above assignments, [Pt(*N*-Ac-L-Met-*S,N*)-($^{15}NH_3$)$_2$] was prepared by mixing equimolar quantities of *cis*-[Pt($^{15}NH_3$)$_2$(H$_2$O)$_2$](NO$_3$)$_2$ and *N*-Ac-L-Met followed by pH adjustment to 7 and allowing the solution to stand for 24 h. The higher pH was required to facilitate the deprotonation of the amide nitrogen. A similar method has been used to produce the *N*-Ac-L-Met S,N-chelate of [Pt(en)]$^{2+}$.[24] The [^{1}H,^{15}N] NMR spectrum of [Pt(*N*-Ac-L-Met-*S,N*)($^{15}NH_3$)$_2$] is shown in Figure 7. There are four cross-peaks in the ammine trans to nitrogen region and four in the ammine trans to sulfur region. Chelated *N*-Ac-L-Met would be expected to exhibit slow inversion of the coordinated S on the NMR time scale and give resolvable peaks for the two diasteromers. In addition to the existence of diastereomers, cis/trans isomerization about the amide bond would give rise to four possible isomers. The [^{1}H,^{15}N] NMR spectrum shows peaks for all of these. The peaks are not the same intensity suggesting that one of the isomers is more favored (by ca. 70:30).



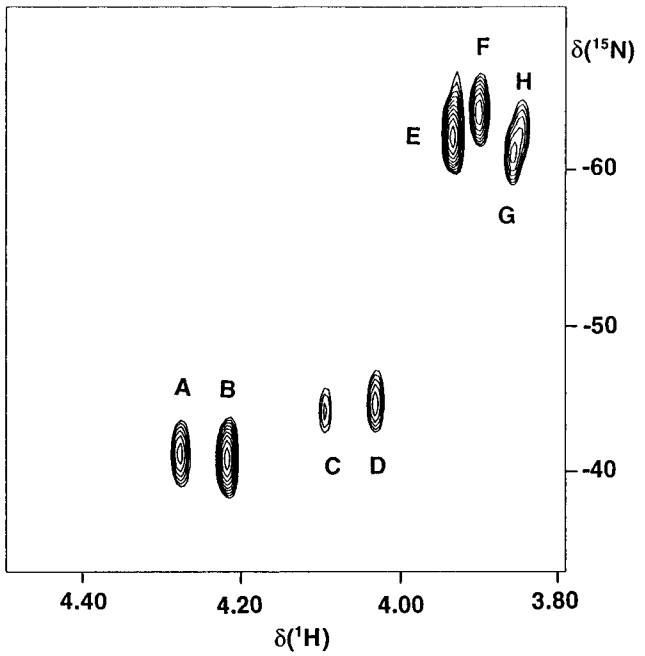


Figure 7. 2D [^{1}H, ^{15}N] NMR spectrum of a solution of *cis*-[Pt($^{15}NH_3$)$_2$(H_2O)$_2$]$^{2+}$ and *N*-Ac-L-Met (5 mM, 1:1), adjusted to pH 7, and left for 24 h at 298 K. The labeled peaks can be assigned to [Pt(*N*-Ac-L-Met-*S*,*N*)($^{15}NH_3$)$_2$]; peaks A, B, E, and F can be assigned to the isomer which has the preferred cis configuration about the amide bond and peaks C, D, H, and G to the trans isomer (two sets of peaks for each configuration, *R* or *S*, at sulfur).

Discussion

The second generation anticancer drug carboplatin has reduced and different side effects compared to cisplatin. Some of the severe side effects of cisplatin may arise from its interactions with sulfur-containing molecules, for example the dose-limiting nephrotoxicity maybe due to the coordination of Pt(II) to the thiol residues of enzymes in the kidneys.[8] In contrast, carboplatin exhibits little nephrotoxicity. This suggests that there may be a difference in the interaction of carboplatin with thiol residues. The reaction of carboplatin with the thiols *N*-Ac-L-Cys and GSH initially gave a product assignable as a thiolate-bridged dimer containing a Pt_2S_2 four-membered ring. The formation of platinum species bridged by thiolate groups has been reported by many different groups,[8,15,19,25] and recently the crystal structure of [{Pt(2,2′-bipyridine)(μ-*N*-Ac-L-Cys-*S*)}$_2$] has been reported by Mitchell *et al.*[26]

While reactions of thiols with carboplatin give the same products as those from cisplatin, the rates at which these species are formed are very different. Reactions of thiols with cisplatin are quite facile, although no rate constants have been reported due to their complicated nature, whereas the reactions of carboplatin with thiol ligands are very slow. The rate at which carboplatin undergoes hydrolysis is also very slow ($\sim 10^{-9}$ s^{-1})[6] and hydrolysis may be the rate-limiting step in reaction of carboplatin with thiols. Djuran *et al.*[27] showed that the interaction of thiols with [Pt(dien)Cl]Cl occurs via direct attack of sulfur on platinum. A similar pathway has been suggested for the interaction of cisplatin with other thiol-containing molecules.[8] Direct attack of a negatively-charged thiolate sulfur on platinum in carboplatin may be hindered by the hydrophobic nature of the axial coordination sites due to the presence of the cyclobutane ring of CBDCA.

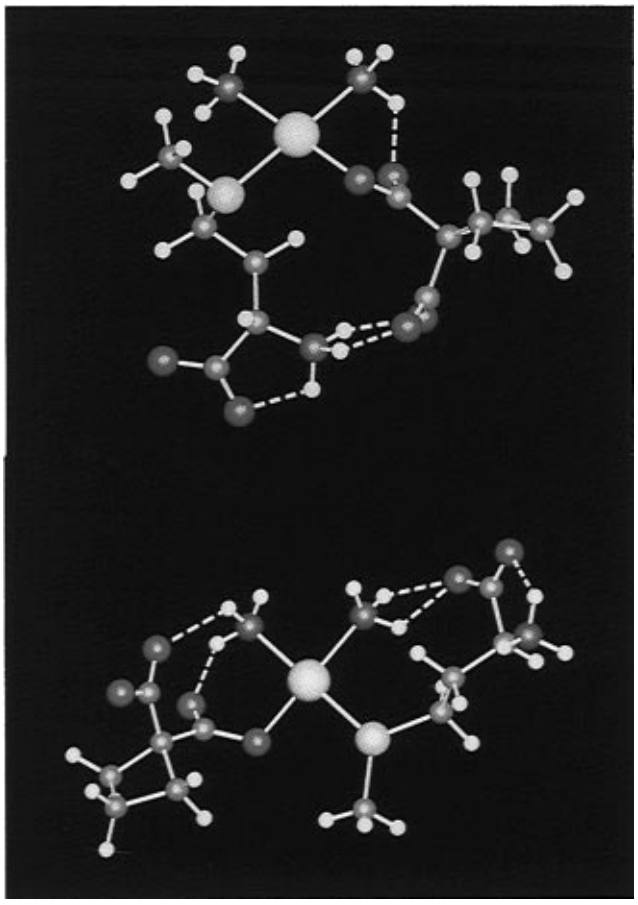


Figure 8. Models of two H-bonding schemes which may be involved in the stabilization of the ring-opened complex *cis*-[Pt(CBDCA-*O*)(NH_3)$_2$(L-HMet-*S*)]: C, green; H, white; N, blue; S, yellow; Pt, gray.

While the reactions of carboplatin with thiols are slow, reactions with thioethers are relatively fast perhaps because a thioether sulfur is a much more hydrophobic ligand. Reactions of carboplatin are usually very slow, and the series of first-order rate constants for several nucleophiles measured by Frey *et al.*[7] demonstrate this. The initial product of the reaction of carboplatin with L-HMet is the remarkably stable ring-opened complex *cis*-[Pt(CBDCA-*O*)(NH_3)$_2$(L-HMet-*S*)]. The rate of ring-closure to give [Pt(L-Met-*S*,*N*)(NH_3)$_2$]$^+$ is suprisingly slow with a half life of 28 h at 310 K. This unexpected stability of the ring-opened complex may be the result of an extensive network of intramolecular hydrogen bonds (see Figure 8). These hydrogen bonds can be between the monodentate CBDCA carboxylate group and the cis ammine ligand as well as between the carboxylate of the methionine and an ammine ligand. Also, the CBDCA carboxylate group can hydrogen-bond to the uncoordinated amine of methionine. The crystal structure of a Pt−amine complex [Pt(CHDA)(DMSO-*S*)(CBDCA-*O*′)] (CHDA = trans-(−)-1,2-cyclohexanediamine), containing a monodentate CBDCA ring has been published.[28] This complex is stabilized in the solid state by several intermolecular hydrogen bonds. There is also one intramolecular hydrogen bond between the free oxygen of the coordinated carboxylate group of CBDCA and the cis NH of the CHDA ligand.

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Inversion of a chiral coordinated sulfur from a monodentate methionine ligand coordinated to platinum is usually rapid such that separate peaks for the diastereomers are not resolved on the NMR time scale (e.g. as is the case for *cis*-[PtCl(NH_3)$_2$(L-HMet-*S*)]$^+$ and *cis*-[Pt(5′-GMP-*N7*)(NH_3)$_2$(L-HMet-*S*)]).[22] Separate peaks for isomers related by inversion are observed when the rate of sulfur-inversion is slowed down, as in a chelated methionine. The inversion of the *S*-methyl group is slow on the NMR time scale for the ring-opened species *cis*-[Pt(CBDCA-*O*)(NH_3)$_2$(L-HMet-*S*)] and *cis*-[Pt(CBDCA-*O*)(NH_3)$_2$(*N*-Ac-L-Met-*S*)]$^-$. This may arise from the formation of macrochelates stabilized by hydrogen bonding and/or steric effects due to the bulky cyclobutane ring.

The course of the reaction of carboplatin with L-HMet after the initial formation of *cis*-[Pt(CBDCA-*O*)(NH_3)$_2$(L-HMet-*S*)] is similar to that for the reaction of cisplatin with L-HMet, with the formation of an *S,N*-chelated species followed by labilization of an ammine, due to the high trans effect of the sulfur. This ammine is substituted by another S-bound methionine and a bis-chelated species is formed.

It is interesting to compare the reaction of L-methionine with carboplatin with that of *N*-acetyl-L-methionine where the amino group is part of an amide bond as it is in many methionine-containing peptides and proteins. The reaction of carboplatin with *N*-Ac-L-Met is initially similar to the reaction of carboplatin with L-HMet with the formation of a stable ring-opened species. However since the amide nitrogen of *N*-Ac-L-Met does not coordinate to platinum as readily as the amine nitrogen of L-HMet, the reaction does not go cleanly to an S,N-*N*-Ac-L-Met chelated product. This slowness of chelation allows the reaction to follow several different pathways including replacement of monodentate CBDCA by another *N*-Ac-L-Met and labilization of the ammine ligand due to the high trans effect of the thioether sulfur.

The chelated product [Pt(*N*-Ac-L-Met-*S,N*)(NH_3)$_2$] is formed during this reaction. There are four possible isomers of this complex, due to slow inversion of the chiral coordinated sulfur and cis/trans isomerization about the amide bond. It would be expected that the isomer with the cis configuration (see structures above) would be favored since it can be stabilized by hydrogen-bonding between the amide carbonyl and the NH of the cis-coordinated ammine group. As can be seen from the 2D [^{1}H,^{15}N] spectrum of [Pt(*N*-Ac-L-Met-*S,N*)($^{15}NH_3$)$_2$] (Figure 7), one set of peaks in the ammine trans to nitrogen (E,F) and ammine trans to sulfur (A, B) regions is indeed more intense than the other set (70:30). There is little difference in the ^{1}H shifts of the ammine trans to nitrogen for these isomers. However in the ammine trans to sulfur region of the spectrum the more intense set of peaks is significantly shifted to lower field, which is consistent with the presence of a hydrogen bond between the ammine hydrogen and the carbonyl group of the cis amide ligand, suggesting that this is the preferred isomer.

Conclusions and Biological Implications

Reactions between carboplatin and thiols are very slow. This accounts for the observation that even though thiols such as GSH have high concentrations *in vivo*, only very small amounts of Pt−thiolate complexes are detectable in the urine of mice treated with [^{15}N]carboplatin.[11] This relative inertness of carboplatin in reactions with thiols may explain why carboplatin is less toxic than cisplatin.

In contrast, carboplatin is relatively reactive towards thioethers. The ring-opened complexes that are initially formed are surprisingly stable with half-lives of ca. 1 day at 310 K, and are apparently detectable in the urine of mice treated with [^{15}N]carboplatin. The recent discovery that guanine bases can displace monodentate thioether ligands from platinum,[9,10] has raised the possibility that alternative pathways to DNA platination may exist. Reactions of carboplatin with nucleobases and DNA are very slow, and therefore it is conceivable that thioethers are able to "activate" carboplatin or are intermediates in carboplatin−DNA interactions. Studies on ring-opened species are currently being carried out to investigate this hypothesis.

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