NMR Study of the Interaction of Platinum Salts with a Tetrapeptide Containing Cysteinyl Residues

Nick Hadjiliadis,[†] Nick Ferderigos,[‡] Jean-Luc Butour,[§] Honoré Marzarguil,[§] Geneviève Gasmi,[⊥,1] and Jean-Pierre Laussac*[,⊥]

Department of Chemistry, University of Ioannina, Ioannina 45-110, Greece, Department of Chemistry, University of Athens, Panepistimiopolis, Zografou, Athens 15-771, Greece, Laboratoire de Pharmacologie et de Toxicologie Fondamentales du CNRS, 205 route de Narbonne, 31077 Toulouse Cedex, France, and Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, 31077 Toulouse Cedex, France

Received December 8, 1992[⊗]

^{1}H-, ^{13}C- and ^{195}Pt-NMR spectroscopies are used to identify the complexes formed between the platinum salts *cis*-$(NH_3)_2PtCl_2$ (*cis*-DDP), *trans*-$(NH_3)_2PtCl_2$ (*trans*-DDP), *cis*-(en)$Pt(ONO_2)_2$, and [(dien)PtBr]Br and the tetrapeptide Boc-Cys^1(SMe)-Ser^2-Ala^3-Cys^4(SMe)-$CONH_2$ (CSAC) containing the sequence Cys-X-Y-Cys (X, Y = amino acids) and being a model of metallothionein (MT) and/or a model for platinum binding to methionine type sulfur, known to occur in biological systems. MT, rich in cysteine is known to bind both *in vivo* and *in vitro* with the antitumor drug *cis*-DDP. The ^{1}H- and ^{13}C-NMR assignments were made by two-dimensional homo- and heteronuclear experiments for the ligand CSAC. The S−CH_3 groups coordinate through sulfur to Pt(II) in all cases. The results show that *cis*-DDP forms a mixture of different diastereoisomers around the sulfur chiral centers and/or polymeric species with NH_3 liberation, due to the strong *trans*-effect of sulfur. *cis*-Pt(en)$(ONO_2)_2$ forms a monomeric (1:1) chelate structure with CSAC, without en liberation, coordinated through both sulfur atoms. However, slow en liberation could take also place upon increasing temperature. Three signals are observed in the ^{1}H- and ^{195}Pt-NMR spectra of this complex in accordance with the proposed monomeric structure. *trans*-DDP, on the other hand, forms a 2:1 complex with CSAC identical to the one formed by [Pt(dien)Br]Br, both coordinated to the −S−CH_3 groups. No amine release was observed in the case of these two complexes.

Introduction

cis-Diamminedichloroplatinum(II), *cis*-DDP, is a well-known antitumor drug that is successfully applied in the chemotherapy of various types of cancer.[2] However, its application in large doses is limited by several toxic side effects, of which its nephrotoxicity is well-known as the most common dose-limiting factor.[2] The biochemical mechanism of Pt-induced nephrotoxicity is poorly understood. It was suggested that dose-limiting nephrotoxicity of *cis*-DDP can be attributed to the binding of the metal to sulfhydryl groups in enzymes and other proteins.[3] Also binding of Pt(II) with plasma glutathione (sulfhydryl groups) may have toxic results.[4,5] This nephrotoxicity is reduced by using the reagents diethyldithiocarbamate (Na(ddtc)) or thiourea.[3,6−9] It was subsequently suggested[3] that both Na(ddtc) and thiourea were able to reduce nephrotoxicity by removing platinum from the sulfur atoms of sulfhydryl groups of cysteine residues of certain enzymes inactivated by binding to *cis*-DDP. However, Lempers and Reedijk[10] concluded that the reduction in nephrotoxicity by both agents is mainly based on the removal of the platinum from methionine type sulfurs, to which *cis*-DDP is also known to bind, in proteins[11,12] and less so or not at all from cysteine type sulfurs.

It has also been suggested that metallothioneins (MTs), a class of low-molecular weight proteins characterized by a high content of cysteine (~30%), are prime potential targets for electrophilic agents such as *cis*-DDP. Indeed, it has been reported that MT binds *cis*-DDP, both *in vivo* and *in vitro*.[13−15] This inducible metal-binding protein occurs mainly in mammalian liver and kidney and probably functions in the homeostatis of essential metals like zinc and copper.[16,17] Exposure of experimental

* To whom correspondence should be addressed. Tel.: (33) 61 33 31 51. Fax: (33) 61 55 30 03.
[†] University of Ioannina.
[‡] University of Athens.
[§] LPTF-CNRS.
[⊥] LCC-CNRS.
[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1994.
(1) Present address: Faculté de Pharmacie de Reims, UA-CNRS 492, 51096 Reims Cedex, France.
(2) Von Hoff, D. D.; Schilsky, R.; Reichert, C. M.; Reddick, R. L.; Rozencweig, M.; Young, R. C.; Muggia, F. M. *Cancer Treat. Rep.* **1979**, *63*, 1527−1531.
(3) Borch, R. F.; Pleasants, M. E. *Proc. Natl. Acad. Sci. U.S.A.*, **1979**, *76*, 6611−6614.
(4) Berners-Price, S. J.; Kuchel, P. W. *J. Inorg. Biochem.* **1990**, *38*, 305−326.
(5) Berners-Price, S. J.; Kuchel, P. W. *J. Inorg. Biochem.* **1990**, *38*, 327−345.
(6) Borch, R. F.; Katz, J. C.; Lieder, P. H.; Pleasants, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5441−5444.
(7) Gale, G. R.; Atkins, L. M.; Walker, E. M., Jr. *Ann. Clin. Lab. Sci.* **1982**, *12*, 345−355.
(8) Dedon, P. C.; Borch, R. F. *Proc. Am. Assoc. Cancer Res.* **1984**, *25*, 371.
(9) Bodenner, D. L.; Dedon, P. C.; Keng, P. C.; Katz, J. C.; Borch, R. F. *Cancer Res.* **1986**, *46*, 2751−2755.
(10) Lempers, E. L. M.; Reedijk, J. *Inorg. Chem.* **1990**, *29*, 217−222.
(11) Wyatt, S. K.; Harrison, K. N.; Jensen, C. M. *Inorg. Chem.* **1992**, *31*, 3867−3868.
(12) Gonias, S. L.; Oakley, A. C.; Walther, P. J.; Salvatore, S. V. *Cancer Res.* **1984**, *44*, 5764−5770.
(13) Zelazowski, A. J.; Garvey, J. S.; Hoeschele, J. D. *Arch. Biochem. Biophys.* **1984**, *229*, 246−252 .
(14) Bongers, J.; Bell, J. U.; Richardson, D. E. *J. Inorg. Biochem.* **1988**, *34*, 55−62.
(15) Pattanaik, A.; Bachowski, G.; Laib, J.; Lemkvil, D. Shaw, C. F., III; Petering, D. H.; Hitchcock, A.; Saryan, L. *J. Biol. Chem.* **1992**, *267*, 16121−16128.
(16) Johnson, D. R.; Foulkes, E. C. *Environ. Res.* **1980**, *21*, 360−371.
(17) Petering, D. H.; Fowler, B. A. *EHP, Environ. Health Perspect.* **1986**, *65*, 217−224.

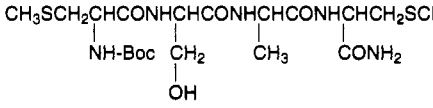
0020-1669/94/1333-5057$04.50/0

animals to poisoning by heavy metals such as Cd(II) and Hg(II) results in the stimulation of MT synthesis which provides an important detoxification mechanism for these metals.

While it is clear that *cis*-DDP will bind to preformed MT,[14] the question concerning the possibility of the drug to induce itself MTs seems controversial. Indeed, several studies have shown that, contrary to the majority of metals, platinum does not induce synthesis of MT.[13,18,19] However, recently Farnworth *et al.*[20] have demonstrated that, in normal tissues, high doses of *cis*-DDP can induce MTs. In any case, due to the fact that MTs may be an important determinant of *cis*-DDP cytotoxicity it is obvious that the knowledge of the nature of the species formed between Pt(II) complexes and MT is of great importance.

Among the different MTs species,[21] the Cys-X-Cys and Cys-X-Y-Cys sequences, where X and Y are residues other than Cys, that occur in the amino acid sequence are of high importance and can be used as models of the protein.

In the present paper, we have synthesized the tetrapeptide Boc-Cys^1(SCH_3)-Ser^2-Ala^3-Cys^4(SCH_3)-$CONH_2$ (hereinafter denoted as CSAC) (structure **I**) and studied its complexation



structure **I**

properties with bifunctional platinum compounds *cis*-DDP, *trans*-DDP, and (ethylenediamine)dinitratoplatinum(II) ([Pt(en)-$(ONO_2)_2$] or [Pt(en)]) and the monofunctional platinum compound ,(diethylenetriamine)platinum(II) ([Pt(dien)Br]Br or [Pt-(dien)]) by ^{1}H- 1D- and 2D-, ^{13}C-, and ^{195}Pt-NMR spectroscopic techniques.

The use of the tetrapeptide CSAC, "despite" the sequence Cys-X-Y-Cys that it contains, is a rather limited model of MT, since the protein is rich in sulfhydryl groups and platinum coordination to metallothionein is known to contain PtS_4 tetrathiolate species[14,15] formed after the release of Zn(II). Pt(II) binds however to N-terminal methionine residues.[14] The tetrapeptide, on the other hand, is a good model for the Pt–methionine type of bonding, which is known to take place quite often *in vivo*. For example, Pt(II) methionine complexes are known to form rapidly in plasma, after injection of *cis*-DDP into rats,[22] and methionine-containing Pt(II) metabolites have been identified in the urine of patients receiving *cis*-DDP therapy.[23,24] Finally, Pt(II)–methionine bonding exists in proteins like the α_2-macroglobulin.[11,12]

A preliminary account of this work has already been published.[25]

Experimental Section

Materials. *cis*-DDP and *trans*-DDP were supplied by Johnson Matthey S.A. [Pt(en)$(ONO_2)_2$] was synthetized by the method proposed by Robins.[26] The complexes were prepared as previously described for [Pt(en)CSAC]$_2^{4+}$.[25] [PtBr(dien)]$^+$ was prepared according to the literature.[27] D_2O (99.96% D) and DMSO-d_6 from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France) were used as solvents. DCl and NaOD solutions were used to adjust the pH which was measured with a Knick digital pH meter, calibrated with standard buffers (Merck Titrisol), and an Ingold microelectrode.

Peptide Synthesis. The tetrapeptide CSAC was prepared by solid-phase synthesis.

(4-(Hydroxymethyl)benzamido)methyl Resin. Amino methyl polystyrene resin[28] (0.6 mmol, 2 g) was suspended in CH_2Cl_2 (30 mL), and *p*-(hydroxymethyl) benzoic acid 2,3,5-trichlorophenyl ester[29] (1.65 g, 5 mmol) was added. The reaction was allowed to proceed for 24 h. The resin was then washed with CH_2Cl_2, DMF, and CH_2Cl_2 (30 mL each).

Boc-L-Cys(SMe)-L-Ser-L-Ala-L-Cys(SMe) Resin. To the above washed resin was added Boc-Cys(SMe) anhydride in 30 mL of CH_2Cl_2, prepared from Boc-Cys(SMe)-OH (1.7 g, 7.2 mmol) and DCC (dicyclohexylcarbodiimide) (0.74 g, 3.6 mmol), followed by addition of DMAP (4-(dimethylamino)pyridine) (22 mg, 0.18 mmol) 5 min later. After 5 h, the resin was washed with CH_2Cl_2 (6×) and the free hydroxy groups were acetylated by Ac_2O/DIEA (diisopropylethylamine) in CH_2Cl_2. The remaining amino acids were incorporated according to the following protocol: (1) washing with CH_2Cl_2 (3 × 1 min); (2) TFA/CH_2Cl_2 (1:1) for 30 min; (3) washing with CH_2Cl_2 (4 × 1 min); (4) 5% DIEA in CH_2Cl_2 for 5 min; (5) washing with CH_2Cl_2 (4 × 1 min); (6) addition of Boc-Ala-OH (4 equiv) in CH_2Cl_2 (20 mL) and DCC (4 equiv) in 10 mL of CH_2Cl_2. After 2 h the cycle was repeated with Boc-Ser(*t*-Bu)-OH. For the introduction of Boc-Cys(SMe)-OH, step 2 was replaced by TFA/anisole (9:1) (60 min). The Boc-Cys(SMe)-Ser-Ala-Cys(SMe) resin was washed with CH_2Cl_2/HOAc (1:1 v/v), HOAc, 2-propanol, and CH_2Cl_2 and vacuum dried.

Boc-Cys(SMe)-Ser-Ala-Cys(SMe)-$CONH_2$. The above resin was suspended in DMF (40 mL), the suspension was cooled to 4 °C and ammonia gas was passed through for 2 h. After 24 h at room temperature the mixture was filtered off and the resin was washed with DMF twice and once with methanol. The filtrate was concentrated to dryness in vacuum, and the peptide was dissolved in ethyl acetate. Purification of the peptide was accomplished by flash chromatography[30] using CH_2Cl_2/acetone (9:1) as the elution liquid. The peptide (300 mg) was pure according to TLC in the systems CH_2Cl_2/acetone (9:1) and But/HOAc/H_2O (4:1:1).

One- and Two-Dimensional NMR Spectra. One-dimensional ^{1}H-NMR spectra at 200 MHz, ^{13}C-NMR spectra at 50.32 MHz, and ^{195}Pt-NMR spectra at 43.02 MHz were recorded on a Bruker AC200 spectrometer equipped with an Aspect 3000 computer system. Field stabilization was provided by an internal deuterium lock-signal. Samples were examined at 25 ± 1 °C. The usual ^{1}H spectrometer conditions consisted of 2400 Hz sweep width, 16 K data points, and 64 scans. The ^{195}Pt NMR spectra were recorded using a 10 mm probe. Each spectrum was acquired in 8 K data points, with the following sets of parameters: spectral width, 50 KHz; pulse duration, 6.0 μs; acquisition time, 82 ms; delay time, 0.5 s. ^{195}Pt chemical shifts are reported in parts per million (ppm) with Na_2PtCl_6 for external calibration; the signal for the external reference was then recorded in the computer of the spectrometer. Both ^{1}H and ^{13}C chemical shifts were measured in ppm with 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) or tetramethylsilane (TMS) as reference.

Two-dimensional NMR spectra were recorded at room temperature using Bruker AC 200, AC 300, and AMX 500 spectrometers. The double-quantum COSY and HOHAHA[31] spectra were acquired in the phase-sensitive mode by the method of time proportional phase

(18) Andrews, P. A.; Murphy, M. P.; Howel, S. B. *Cancer Chemother. Pharmacol.* **1987**, *19*, 149–154.
(19) Mason, R.; Edwards, I. R.; McLaren, S. *J. Chem. Biol. Interact.* **1984**, *49*, 165–176.
(20) Farnworth, P. G.; Hillcoat, B. L.; Roos, I. A. G. *Chem. Biol. Interact.* **1989**, *69*, 319–332.
(21) Kägi, J. R.; Schäffer, A. *Biochemistry* **1988**, *27*, 8509–8515.
(22) Daley-Yates, P. T.; McBrien, D. C. H. *Biochem. Pharmacol.* **1984**, *33*, 3063–3070.
(23) Riley, C. M.; Sternson, L. A.; Repta, A. J.; Slyter, S. A. *Anal. Biochem.* **1983**, *130*, 203–214.
(24) Sternson, L. A.; Repta, A. J.; Shih, H.; Himmelstein, K. J.; Patton, T. F. In *Platinum Coordination Complexes in Cancer Chemotherapy*; Hacker, M. P., Douple, E. R., Krakoff, I. H., Eds.; Martinus Nijhoff: Boston, MA, 1984; pp 126–137.
(25) Wimmer, S.; Castan, P.; Ferderigos, N.; Hadjiliadis, N.; Laussac, J.-P. *C. R. Seances Hebd. Acad. Sci. Paris* **1989**, *308-II*, 1775–1780.
(26) Robins, A. B. *J. Inorg. Biochem.* **1983**, *18*, 213–220.
(27) Mann, F. G. *J. Chem. Soc.* **1934**, 466–474.
(28) Mitchell, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, *43*, 2845–2852.
(29) Atherton, E.; Logan, C. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1*, **1981**, 538–546.
(30) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–2925.
(31) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2821–2822.

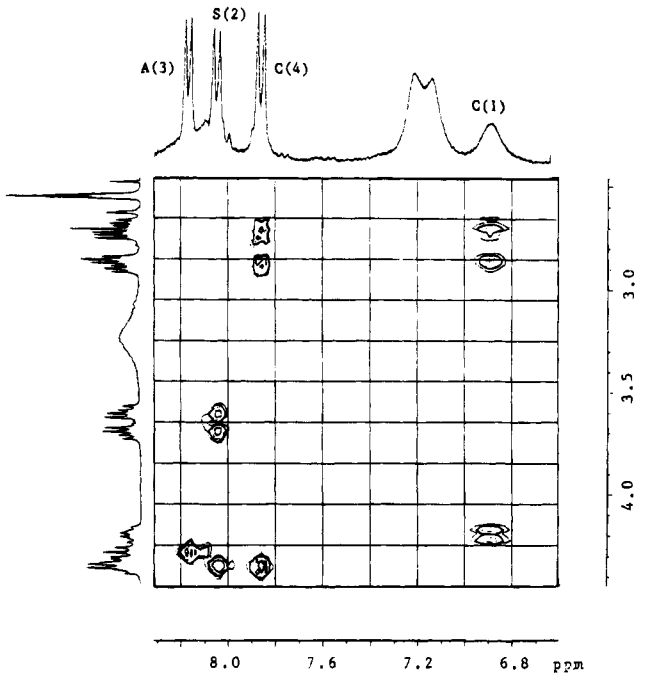


Figure 1. Contour plot of the spectral region of NH/NH_2 (F2) and CαH/CβH (F1) of a HOHAHA spectrum of CSAC in DMSO-d_6 at 298 K. Abbreviations follow the standard one letter code.

incrementation as described by Marion and Wüthrich.[32] Usually, 256 t_1 increments were accumulated into 1 K data points with 16 scans for each. Prior to Fourier transformation, the initial 256 × 1024 data matrix was zero-filled once in the t_1 dimension and multiplied by a sine-bell function in both the t_1 and t_2 dimensions. Phase-sensitive $^1H-^{13}C$ HMQC[33] and HMBC[34] spectra were carried out at 333 K on a 500-MHz Bruker AMX 500 spectrometer. For HMQC experiments, t_{1max} = 55.1 ms and t_2 = 457 ms while, for HMBC experiments, t_{1max} = 22 ms and t_2 = 209 ms. The measurement time was approximately 8 h. A sine-bell function was performed in both dimensions before Fourier transformation.

Reactions of Platinum Salts with CSAC. Reactions of the various platinum salts with CSAC were carried out in a 1:1 or 2:1 metal to ligand ratio in concentrations ranging from 10^{-2} to 10^{-3} M. The pH was adjusted with a solution of 0.1 N NaOD and maintained between pH 5.5 and 6.5. It was important to keep the pH below 7 to minimize hydrolysis of the platinum salts. All measurements were performed between 12 and 72 h after the initial mixture of the reactants.

Both complexes $\{[(NH_3)_2PtCl]_2-CSAC\}Cl_2$, $C_{19}H_{47}N_9O_7S_2Cl_4Pt_2$, and $\{[(dien)Pt]_2-CSAC\}Br_4$, $C_{27}H_{61}N_{11}O_7S_2Br_4Pt_2$, were isolated as follows : Reaction mixtures with 2:1 metal to ligand ratios and concentrations of 10^{-3} M were dissolved in D_2O solution. The pH of the solution was adjusted to 6−6.5 with a 0.1 N NaOD solution and maintained at this level for 72 h. At the end of this period, no free peptide was observed in the ^1H-NMR spectra. Then the solvent was evaporated to dryness, and the solid left was redisolved in water and purified over a Sephadex G15 column. After the sample was dried in a desiccator at room temperature first in the presence of $CaCl_2$, it was then dried at 100 °C under vacuum until constant weight was obtained. The solid complexes were analyzed as follows: Calcd for $C_{19}H_{47}N_9O_7S_2Cl_4Pt_2$: C, 20.5; H, 4.2; N, 11.4; Pt, 35.2; S, 5.8. Found: C, 20.7; H, 3.9; N, 11.3; Pt, 35.5; S, 5.7. Calcd for $C_{27}H_{61}N_{11}O_7S_2Br_4Pt_2$: C, 22.7; H, 4.3; N, 10.8; Pt, 27.4; S, 4.5. Found: C, 22.7; H, 4.2; N, 10.5; Pt, 27.2; S, 4.7.

The monomeric nature of the Pt(en)(CSAC) complex was characterized using a FISONS AutoSpec 6F mass spectrometer fitted with a source for liquid secondary mass spectrometry. The cesium ion gun was operated at 25 kV; the accelerating voltage was 8 kV. A few micrograms of the sample were dissolved into 1 μL of glycerol matrix.

Table 1. 200 MHz ^1H-NMR Data for CSAC and Its Pt Complexes in D_2O

resonance	CSAC[a]	Pt(en)−CSAC[a]	Δ[b]	*trans*-Pt−CSAC[a]	Δ[b]	Pt(dien)−CSAC[a]	Δ[b]
Cys^1 CαH	4.38	4.88	0.50	4.99	0.61	4.93	0.55
Cys^1 $C\beta H_2$	3.03	3.68	0.65	3.53	0.50	3.56	0.53
	2.89	3.30	0.41	3.22	0.33	*e*	
$Cys^{1,4}$ $S-CH_3$	2.22	2.96	0.74	2.70	0.49	2.67	0.45
	2.20	2.93				2.66	0.46
		2.77	0.55				
		2.75					
		2.73	0.51				
		2.71					
Ser^2 CαH	4.52	4.52	0	4.56	0.04	4.54	0.02
Ser^2 $C\beta H_2$	3.99	3.99	0	3.99	0	3.98	−0.01
	3.91	3.93	0.02	3.90	−0.01	3.92	0.01
Ala^3 CαH	4.45	4.44	−0.01	4.44	−0.01	4.43	−0.02
Ala^3 CH_3	1.48	1.50	0.02	1.51	0.03	1.49	0.01
Cys^4 CαH	4.55	4.38	−0.17	*d*		*d*	
Cys^4 $C\beta H_2$	3.07	*c*		3.50	0.43	*e*	
	2.93	*c*		3.45	0.52	*e*	

[a] Chemical shifts in ppm. [b] $\Delta_{ppm} = \delta_{ppm}(bound) - \delta_{ppm}(not\ bound)$; (−) upfield shift. [c] Too broad to be observed. [d] Overlapped with HDO. [e] Overlapped with dien resonances.

Results and Discussion

The diamagnetic nature of the Pt(II) complexes has made them excellent candidates for studies using NMR spectroscopy. Still, owing to the kinetic inertness of Pt(II), this ion does not cause line broadening, as is usually observed with paramagnetic ions.[35,36] Consequently, the chemical shifts resulting from metal binding to the peptide may be due to the direct influence of changes in electron density (mostly downfield) or may result indirectly from an altered conformation of the peptide (either downfield or upfield). Both effects have a relatively short range, and thus the general area of metal coordination will be reflected by changes in the chemical shifts of adjacent resonances.

CSAC Ligand. The assignments of the ^1H resonances of the nonexchangeable protons of CSAC were made by double-quantum COSY experiments in the phase sensitive mode. Distinction between the two $Cys^{1,4}$ residues were made from the exchangeable protons (amide protons) in DMSO-d_6 solution. Indeed, the NH adjacent to the bulky hydrophobic Boc group is known to resonate at quite a high field. Consequently this NH corresponds to the Cys^1 residues. A portion of the HOHAHA spectrum of CSAC is represented in Figure 1.

The assignment of the C−H and CO carbon atoms of CSAC can be made in a straight-forward manner by using two-dimensional $^{13}C-^1H$ correlation spectroscopy with detection at the ^1H frequency (inverse mode). This technique has now been widely used for the detection of insensitive nuclei by polarization transfer from the insensitive nuclei to a sensitive one, usually ^1H. These now make the ^{13}C nucleus more accessible. These experiments involve ^1H-detected heteronuclear multiple quantum coherence (HMQC) for obtaining information of the C−H carbon atoms *via* $^1J_{CH}$ scalar coupling and ^1H-detected heteronuclear multiple bond connectivity (HMBC) for obtaining long range $^nJ_{CH}$ scalar coupling information, *e.g.* quaternary and CO carbon atoms. All cross-correlated peaks were observed and allowed the attribution of the carbonyl groups.

Reactions of CSAC with Platinum salts. These reactions were studied for each salt in various metal:ligand ratios with ^1H-, ^{13}C- and ^{195}Pt-NMR spectroscopies.

(32) Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967−974.
(33) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565−569.
(34) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(35) Laussac, J.-P.; Sarkar, B. *Biochemistry* **1984**, *23*, 2832−2838.
(36) Laussac, J.-P.; Robert, A.; Haran, R.; Sarkar, B. *Inorg. Chem.* **1986**, 25, 2760−2765.

Table 2. 50.3 MHz ^{13}C-NMR Data for CSAC and Its Pt Complexes in D_2O

resonance	CSAC[a]	*trans*-Pt-CSAC[a]	Δ^b	Pt(dien)-CSAC[a]	Δ^b
Cys^1 CαH	56.3	54.8 (30 Hz)[c]	−1.5	55.5	−0.8
Cys^1 CβH$_2$	37.4	42.3 (35 Hz)[c]	4.9	42.6 (40 Hz)[c]	5.2
Cys^1 S−CH$_3$	17.1	23.2	6.1	22.9 (40 Hz)[c]	5.8
Cys^1 CO	176.1	173.6	−2.6	173.7	−2.5
Ser^2 CαH	58.1	57.9	−0.2	57.9	−0.2
Ser^2 CβH2	63.3	63.5	0.2	63.5	0.2
Ser^2 CO	174.0	173.8	−0.2	174.0	0
Ala^3 CαH	52.3	52.4	0.1	52.5	0.2
Ala^3 CH$_3$	18.7	18.7	0	18.8	0.1
Ala^3 CO	177.2	177.4	0.2	177.5	0.3
Cys^4 CαH	54.8	53.2 (20 Hz)[c]	−1.6	53.1	−1.7
Cys^4 CβH$_2$	37.1	42.3 (35 Hz)[c]	5.2	42.6 (40 Hz)[c]	5.5
Cys^4 S−CH$_3$	17.0	23.2	6.2	22.9 (40 Hz)[c]	5.9
Cys^4 CO	177.3	174.7	−2.6	174.9	−2.4

[a] Chemical shifts in ppm. [b] $\Delta_{ppm} = \delta_{ppm}$(bound) − δ_{ppm}(not bound); (−) upfield shift. [c] Linewidth in Hz.

Table 3. ^{195}Pt NMR Data and ^3J Coupling Constants for Pt−CSAC Complexes in D_2O

compds	δ_{Pt}[a]	^3J(Pt−S−CH$_3$)[b]	^3J(Pt−Cα)[b]
cis-Pt−CSAC	−3500, br		
[(en)Pt−CSAC]$^{2+}$	−3712	44.8	
	−3733	42.1	
	−3744	41.2	
[(*trans*-PtCl)$_2$−CSAC]$^{2+}$	−2952	53.8	
	−2976		
{[(dien)Pt]$_2$−CSAC}$^{4+}$	−3365	42.2	17.4

[a] br = broad. [b] ^3J in Hz.

Tables 1 and 2 summarize the chemical shifts and the internal chemical shifts (Δ) of the ^{1}H- and ^{13}C-NMR spectra, respectively, of the ligand CSAC and its Pt(II) complexes.

Table 3 includes the $^2J_{Pt-C}$, $^3J_{Pt-H}$ coupling constants and the ^{195}Pt-NMR chemical shifts of the complexes.

(i) Reactions of *cis*-DDP with CSAC. ^{1}H-NMR Spectra. The ^{1}H-NMR spectrum of a solution of *cis*-[Pt(NH$_3$)$_2$Cl$_2$] and CSAC in a 1:1 molar ratio at room temperature exhibits broad and featureless signals of the *cis*-Pt−CSAC complex(es). Increasing the temperature to 65 °C did not afford any appreciable sharpening of the different signals, which precluded an unambiguous determination of the peak position. When less than the stoichiometric amount of platinum was present, the spectrum showed the presence of free and complexed peptide. Consequently, the broadening of the signals of this complex cannot be attributed to exchange of CSAC between the complex(es) and the free ligand, since the exchange between these two species is slow with respect to the NMR time scale. The broadening can only be explained by exchange phenomena related to the presence of different diastereoisomers (*vide infra*) and/or the mixture of polymeric species. For example, Appleton *et al.*[37] studying by NMR the reactions of glutathione (GSH) with *cis*-DDP attributed the broadening of the Cys resonances in the *cis*-[{^{15}NH$_3$)$_2$Pt(μ-SG)}]$^{2+}$ formed to an interconversion of the two isomers of the S-bridged dimer *via* the inversion at the S atom.

Norman *et al.*[38] also observed very complicated spectra in the much simpler *cis*-DDP−methionine but similar to the present system, due to the various species formed. One and two ammonia molecules were displaced by methionine from *cis*-DDP during these reactions.[38] Sulfhydryl (−SH) group containing systems show also a similar behavior in their reactions with *cis*-DDP. Berners-Price and Kuchel[4,5] proposed a high-molecular weight polymer in which coordination was taking place almost exclusively *via* the S atom but with various different Pt−S and Pt−S−Pt environments.

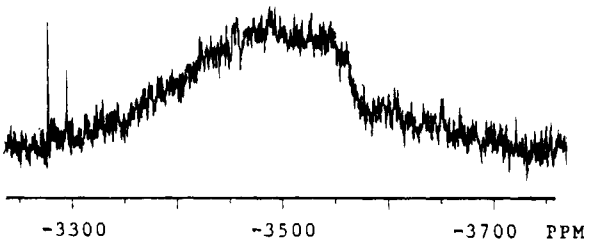


Figure 2. ^{195}Pt-NMR spectrum of a mixture of *cis*-DDP + CSAC (1:1) after 12 h of reaction.

In our case also, the strong "*trans*-influence" of the thioether S atom (less than the −SH group) is also expected to replace the ammonia ligands and create polymeric species. In addition, examination of our ^{1}H-NMR spectrum indicates that, upon metal complexation, the S−CH$_3$ resonances shift downfield by approximatively 0.5 ppm. These indications provide strong evidence that complexation occurs *via* the two sulfur groups. A similar downfield shift was also observed in the similar system of *S*-methylglutathione.[10]

Additional evidence that the two sulfur groups are implicated in the Pt binding is provided by the ^{195}Pt-NMR spectrum (see Figure 2). Generally, ^{195}Pt chemical shifts are extremely sensitive to the σ-donor strength of the coordinated ligands. The ^{195}Pt NMR spectrum of the *cis*-DDP + CSAC (1:1) system shows a very broad resonance at *ca* −3500 ppm. The position of this signal can be ascribed to a PtN$_2$S$_2$ complex and compares with values of −3577 ppm for *trans*-[Pt(NH$_3$)$_2$(acetylmethionine-*S*,*N*)$_2$][39] and −3400 ppm for *cis*-[Pt(NH$_3$)$_2$(thiourea-*S*)$_2$].[40] However, no further identification was possible due to the complexity of the system.

(ii) Reactions of *cis*-Pt(en)(ONO$_2$)$_2$ with CSAC. (a) ^{1}H-NMR Spectra. Important changes in the positions, widths, and intensities of the lines are observed when Pt(en) is added to the peptide in D_2O. The increasing addition of metal results in a decrease in the intensity of the resonances for free CSAC with a proportional appearance of a new set of resonances. The Pt(II) is therefore in slow exchange since only the intensity changes are observed rather than continuous shifts in peak position. Under these circumstances, the upper limit on the exchange rate in the "well-observed" case can be mathematically described by the condition

$$2\pi(\Delta\nu_M)\tau_M \gg 1$$

where $\Delta\nu_M$ is the chemical shift difference (in Hz) between bound and free ligand and τ_M is the lifetime of the ligand bound to the metal ion.[41] This condition refers to all individual resonances, and it is applied to the different $\Delta\nu_M$ values. However, if we consider that the Cys−SCH$_3$ protons reflect directly the influence of the platinum coordination, it was found that the smallest chemical shift perturbation is *ca.* 100 Hz. From this ^{1}H chemical shift separation, we estimate that the upper limit of the lifetime of Pt(en)−CSAC complex is $\tau_M \gg 1.5 \times 10^{-3}$ s, and consequently, the exchange rate or the dissociation

(37) Appleton, T. G.; Connor, J. W.; Hall, J. R.; Prenzler, P. *Inorg. Chem.* **1989**, *28*, 2030−2037.
(38) Norman, R. E.; Ranford, J. D.; Sadler, P. J. *Inorg. Chem.* **1992**, *31*, 877−888.
(39) Ismail, I. M.; Kerrisson, S. J. S.; Sadler, S. R. *Polyhedron* **1982**, *1*, 57−59.
(40) Appleton, T. G.; Hall, J. R.; Ralph, S. F. *Inorg. Chem.* **1985**, *24*, 4685−4693.
(41) McLaughlin, A. C.; Leigh, J. S., Jr. *J. Magn. Reson.* **1973**, *9*, 296−304.

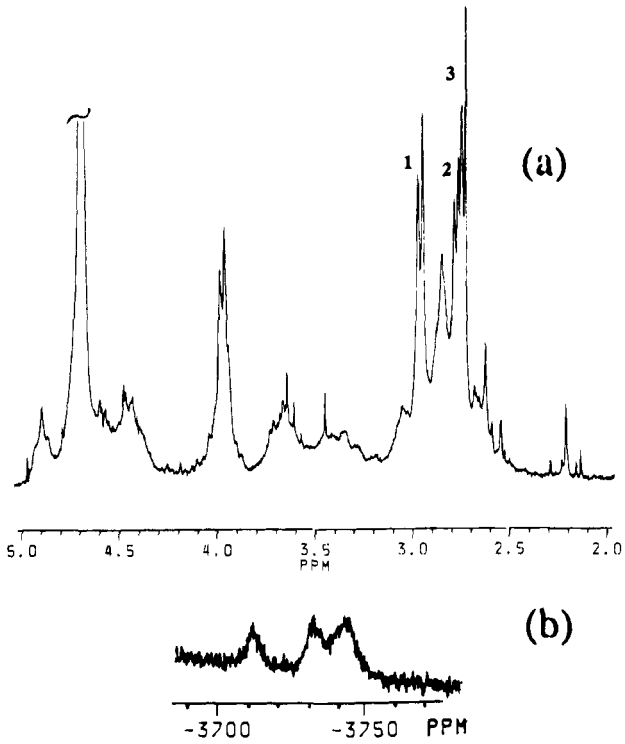


Figure 3. NMR spectra of mixtures of *cis*-(en)Pt$(ONO_2)_2$ + CSAC (1:1) after 12 h of reaction time: (a) ^{1}H-NMR; (b) ^{195}Pt-NMR. **1**−**3** correspond to the three S−CH_3 peaks (see text).

rate ($1/\tau_M$) of the metal between these species is estimated to be $1/\tau_M \ll 700\ s^{-1}$.

Furthermore, at a Pt(en):CSAC ratio of 1:1, the ^{1}H-NMR spectrum does not show any resonance due to metal-free peptide (Figure 3). Further increase in the concentration of Pt(en) causes no additional changes in the ^{1}H-NMR spectrum, consistent with the absence of other binding sites. This clearly shows that the monomeric Pt(en)−CSAC complex possesses a 1:1 stoichiometry.

All the Cys^{1,4} α, β resonances are greatly affected, while the protons of the Ser^2 and Ala^3 residues are not perturbed. Table 1 shows that most of these resonances are shifted downfield. The largest changes occur for the proton resonances of the S−CH_3 groups, with internal chemical shifts Δ ranging from 0.74 to 0.51 ppm. The magnitude and the direction (downfield) of these chemical shifts are comparable to those observed previously for *S*-methylcysteine or *S*-ethylcysteine and methionine binding to *cis*-DDP.[42-45]

Upon more accurate inspection of the ^{1}H-NMR spectrum, some interesting features are observed. Indeed, the resonances corresponding to the S−CH_3 protons give three sets of peaks, indicating that the peptide exists in three slowly interconverting species. In fact, the two S atoms are centers of chirality and therefore, in the absence of any internal rate process, four diastereoisomers exist for a monomeric chelate structure, namely two *meso* forms and two *dl* forms (Figure 4).[46] The presence of three sets of signals could be due to an indistinguishable pair of *dl* forms. It will be noted that the populations of these three diastereoisomers are different, and therefore no attempt was made to assign individual Pt−CH_3 lines to individual invertomers.

Satellites from coupling to ^{195}Pt were observed ($^3J_{(Pt-S-CH_3)}$ = 44.8, 42.1, and 41.2 Hz; Table 3). These values fit well with those obtained elsewhere.[43]

Increasing the temperature from 5 to 55 °C does not modify the weighted populations significantly. Nevertheless, NMR spectra showed that en was slowly lost from the complex as the temperature was increasing. A few successive spectra of the displacement of en from the complex (en)Pt are given in Figure 5.

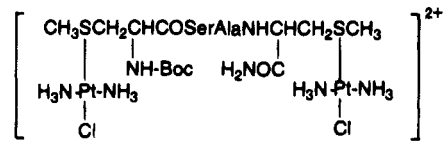
This is obviously due to the labilization of the *trans* ligand to sulfur, coordinated to the Pt(II) ligand, as a result of the high *trans*-influence of sulfur.[37] However, the labilization of the coordinated ethylenediamine was much slower than the loss of ammonia from the corresponding amine complexes.[37,38]

The ^{13}C-NMR spectrum was quite complex owing to the presence of a variety of diastereoisomers formed and showed a lot of broad resonances. Consequently, no attempt was made to assign individual resonances.

(b) ^{195}Pt-NMR Spectra. Supporting evidence for the presence of these three diastereoisomers comes from the ^{195}Pt spectrum, where three signals are observed at −3712, −3733, and −3744 ppm, respectively (Figure 3b). These chemical shifts are in the region expected for a *cis*-N_2S_2 coordination type around metal (*cf.* −3639 and −3685 ppm for *cis*-Pt$(NH_3)_2$(met H-S)$_2^{2+}$ complexes[37]).

(iii) Reactions of *trans*-DDP with CSAC. (a) ^{1}H-NMR Spectra. Under similar conditions, there was a clear difference in the reactions of *cis*- and *trans*-DDP with CSAC. Indeed, as was observed, the *cis* isomer gives broad NMR peaks due to the formation of different diastereoisomers and/or a mixture of polymeric species while the *trans* isomer, by contrast, gives a well-characterized ^{1}H-NMR spectrum which ensures the presence of a well-defined complex.

The presence of distinct resonances upon addition of *trans*-DDP shows that exchange between free and bonded CSAC is slow on the NMR time scale and that the complex has a 2:1 stoichiometry of *trans*-{[$(NH_3)_2$PtCl]$_2$−(CSAC)} (structure **II**).



structure II

As was the case with (en)Pt complexes, the ^{1}H-NMR lines move downfield upon coordination to Pt(II) and are listed in Table 1. Only one single sharp resonance was observed for the two S−CH_3 protons which were shifted to high frequency by 0.49 ppm upon complexing to platinum with associated ^{195}Pt satellites ($^3J_{Pt-S-CH_3}$ = 53.8 Hz) (see Figure 6). A value of ^3J = 54 Hz has been found for en−Pt. These coupling constants are higher than those observed with (en)Pt (*vide supra*) and (dien)Pt (*vide infra*) but agree fully with the values reported for complexes containing *trans* Cl−Pt^{II}−S−CH_3 fragments.[47] The Cys^{1,4} α, β protons exhibited as well a large shift to high frequency with respect to free CSAC. The appearance of a broadening in the ^{1}H-NMR spectrum for these resonances may be due to a rate of inversion at sulfur that is intermediate on the NMR time scale: fast enough to cause the *S*-methyl groups to give just

(42) Bell, J. D.; Norman, R. E.; Sadler, P. J. *J. Inorg. Biochem.* **1987**, *31*, 241−246.
(43) Appleton, T. G.; Connor, J. W.; Hall, J. R. *Inorg. Chem.* **1988**, *27*, 130−137.
(44) Grochowski, T.; Samochocka, K. *J. Chem. Soc., Dalton Trans.* **1992**, 1145−1149.
(45) Theodorou, V.; Photaki, I.; Hadjiliadis, N.; Gellert, R. W.; Bau, R. *Inorg. Chim. Acta* **1982**, *60*, 1−7.
(46) Orrell, K. G. *Coord. Chem. Rev.* **1989**, *96*, 1−48.
(47) Gummin, D. D.; Ratila, E. M. A.; Kostic, N. M. *Inorg. Chem.* **1986**, *25*, 2429−2433.

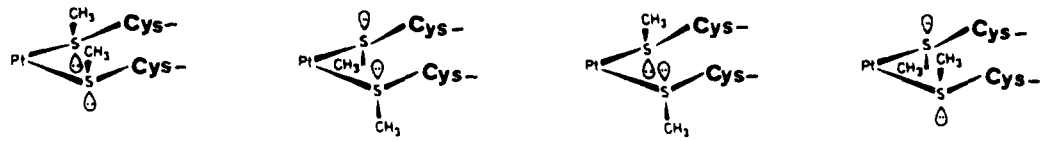


Figure 4. The four possible diastereoisomers (*meso*(**1**), *meso*(**2**), *dl*(**1**), and *dl*(**2**)) expected for a monomeric chelate structure, assuming equivalent sulfur atoms.

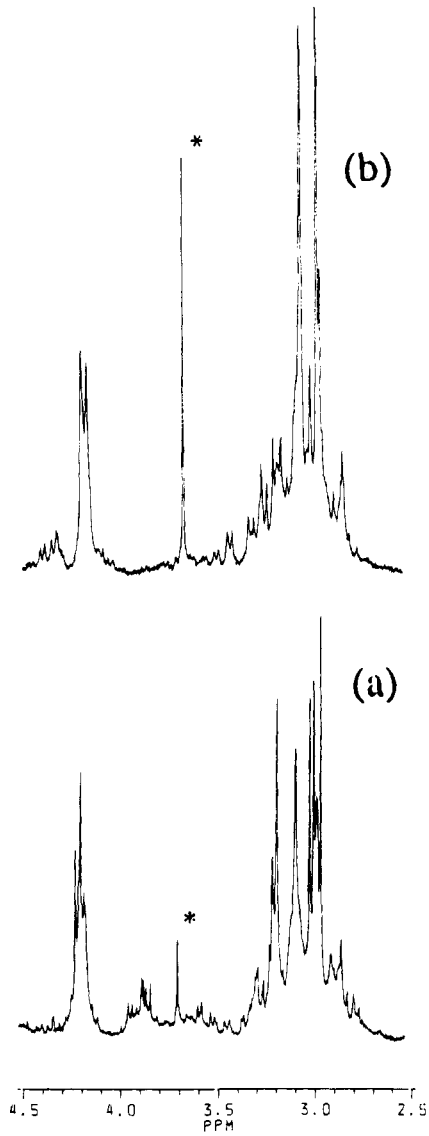


Figure 5. Successive spectra for displacement of en from *cis*-(en)Pt-$(ONO_2)_2$ during its reaction with CSAC at 55 °C: (a) After 6 h. (b) after 12 h. An asterisk denotes the en liberation.

two sharp resonances but not fast enough to give well-resolved multiplets for the $Cys^{1,4}$ CβH_2 and CαH protons. A similar situation has been encountered in the ^{13}C-NMR study (*vide infra*). Taken together, these results provide strong evidence that *trans*-DDP reacts with CSAC to form a 2:1 complex (structure **II**), in which each sulfur atom, *trans* to Cl, is coordinated to platinum in a monodendate fashion.

(b) ^{13}C-NMR Spectra. The ^{13}C-NMR spectrum of the 2:1 *trans*-Pt complex clearly shows the presence of a single resonance for each carbon atom indicating formation of a well-defined species (Figure 6b). As observed in the ^{1}H-NMR study, all the resonances corresponding to the two Cys residues are broadened, particularly those from the $Cys^{1,4}$ α, β peaks ($\Delta\nu_{1/2}$ ~30 Hz). The broadeness of these resonances may be indicative of an exchange process between the two sulfur sites and/or a rate of inversion at the sulfur atom. All the Cys resonances exhibit a downfield shift with respect to the free tetrapeptide, except the $Cys^{1,4}$ Cα resonances that shift upfield (see Table 2). The most affected resonances are those corresponding to S$-CH_3$ carbons (Δ = 6.1 ppm), consistent with coordination to the $Cys^{1,4}$-S atoms only. ^{195}Pt satellites were not resolved because the $^2J(^{13}C-^{195}Pt)$ coupling constant is likely to be smaller than the linewidth of the $Cys^{1,4}$ S$-CH_3$ and $Cys^{1,4}$ CβH_2 resonances (Table 3). The $^2J(^{13}C-^{195}Pt)$ coupling constant in (dien)Pt and (dien)Pt$-$CSAC is ~17 Hz (*vide infra*) in agreement with other sources.[41]

(c) ^{195}Pt-NMR of *trans*-Pt$-$CSAC. As shown in Figure 6c, the ^{195}Pt spectrum of the *trans*-Pt complex consists of two signals of equal intensities with chemical shifts of -2952 ppm ($\Delta\nu_{1/2}$ = 390 Hz) and -2976 ppm ($\Delta\nu_{1/2}$ = 580 Hz). These values lie in the region expected for a $PtSN_2Cl$ complex (*cf.* -2782 ppm for $PtSCl_3$ coordination type[47] and -3365 ppm for a $PtSN_3$ environment (*vide infra*). The observation of these two resonances is rather due to the inequivalence of the two sulfur atoms coordinated to two *trans*-$[(NH_3)_2PtCl]^+$ species (structure **II**).

(iv) Reaction of [(dien)PtBr]Br with CSAC. (a) ^{1}H-NMR Spectra. There is a very close similarity between the ^{1}H-NMR spectrum of (dien)Pt and the one obtained with *trans*-DDP. The only difference comes from a better resolution of the ^{1}H-NMR lines. As was the case with the *trans*-Pt complex, the tetrapeptide is in slow exchange on the ^{1}H-NMR chemical shift time scale between its free and complexed states (Figure 7). The corresponding complex has a 2:1 stoichiometry {[(dien)Pt]$_2$-(CSAC)} with a structure similar to the one proposed with *trans*-DDP (structure **II**). Similarly, all the $Cys^{1,4}$ resonances move downfield on coordination to Pt(II) while the Ser^2 and Ala^3 resonances are less or not affected. The strongest shielding is observed for the two methyl protons (Δ = 0.45 ppm) with respect to the free ligand with a coupling constant of $^3J_{Pt-S-CH_3}$ = 42.2 Hz in the complex. The spectrum in the region 2.9 to 3.4 ppm is rather complex, which is most likely due to magnetically inequivalent dien protons. The downfield shift of these protons agrees with coordination to the sulfur atoms.[10]

(b) ^{13}C-NMR Spectra. As was observed above for the ^{1}H-NMR study, the ^{13}C-NMR spectra of *trans*-DDP and (dien)Pt complexes with CSAC are similar (Figure 7b). The direction and the magnitude of the different $Cys^{1,4}$ ^{13}C-NMR chemical shifts are the same (Table 2). As an example, the most important downfield shift is observed for the S$-CH_3$ carbons (Δ = 5.8 ppm). In both complexes formed with *trans*-DDP and (dien)Pt (structure **II**), coordination of the metal to the sulfur atoms causes a slightly greater downfield shift of the methyl peaks than of the methylene peaks. This difference could be explained by the fact that the bond length of the CH_3-S group is slightly shorter than the CH_2-S bond.[48] It is interesting to note that the broadening of the $Cys^{1,4}$ CβH_2 and $Cys^{1,4}$ S$-CH_3$

(48) Del Re, G.; Gavozzo, E.; Giglio, E.; Lelj, F.; Mazza, F.; Zappia, V. *Acta Crystallogr., Sect. B. Struct. Crystallogr. Cryst. Chem.* **1977**, *33*, 3289–3296.

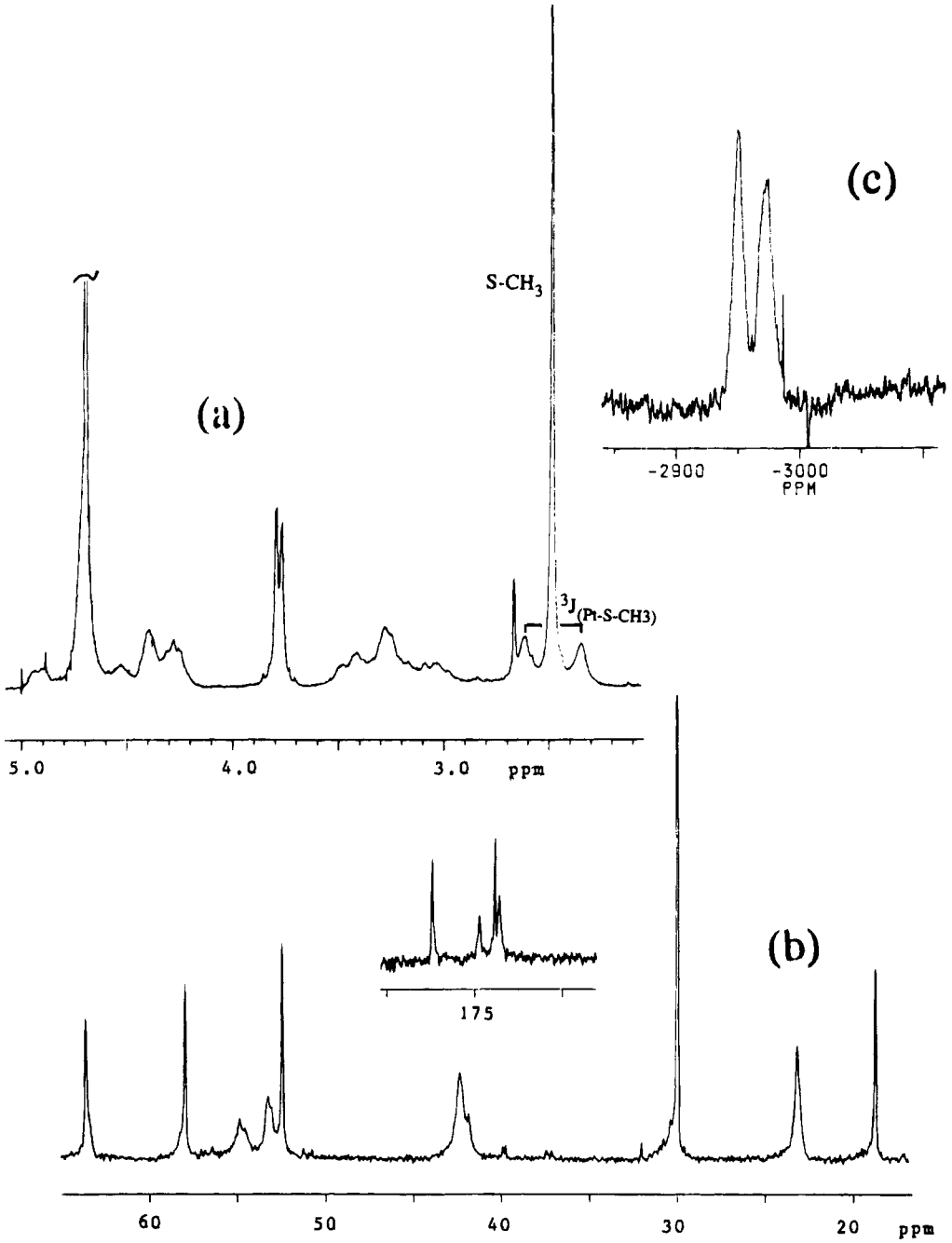


Figure 6. NMR spectra of mixtures of *trans*-DDP + CSAC (2:1) at pH 6.4 after 72 h: (a) ^{1}H-NMR; (b) ^{13}C-NMR; (c) ^{195}Pt-NMR.

carbons are more pronounced ($\Delta\nu_{1/2} \sim 40$ Hz) than those observed with the *trans*-DDP complex while the Cys^{1,4} CO and Cys^{1,4} CαH resonances are less affected. This local broadening may result from an interconversion of the different conformers *via* inversion at the S atoms at an intermediate rate. The important broadening for the Cys^{1,4} CβH$_2$ and S$-$CH$_3$ carbon resonances precludes the observation of the $^{2}J_{^{13}C-^{195}Pt}$ coupling constants. Nevertheless, the two types of the four methylene carbons of the dien ligand give a ^{2}J coupling constant of 16.5 and 17.5 Hz. From this observation it can be concluded that all three amino groups of dien are coordinated to Pt(II) and that the chelate ring opening does not occur.[10]

(c) **^{195}Pt-NMR Spectra.** Despite the inequivalency of the platinum atoms in the complex [{(dien)Pt}$-$(CSAC)]$^{4+}$ formed and unlike the corresponding complex with *trans*-DDP, its ^{195}Pt-NMR spectrum shows a singlet at -3365 ppm ($\Delta\nu_{1/2}$ = 370 Hz), due to a coincidence of the two expected signals (Figure 7c). The position of this resonance is characteristic of a PtN$_3$S complex with a tridentate N$_3$ ligand and compares well with the values of -3355 ppm for [Pt(dien)ddtc]$^{+}$ (ddtc = diethyldithiocarbamate) and -3239 ppm for [Pt(dien)tu]$^{2+}$ (tu = thiourea).[10]

Concluding Remarks

To summarize, it is concluded that CSAC coordinates with the different bi- and monofunctional Pt(II) salts *via* the two sulfur groups. The exchange between bonded and free CSAC is extremely slow, but exchange broadening occurs in the ^{1}H- and ^{13}C-NMR spectra as a result of the different diastereoisomers arising from the different configurations of the *S*-methyl groups and the nonbonding electron pairs on sulfur. However, ^{1}H- and ^{195}Pt-NMR spectra of the reactions of *cis*-(en)Pt(ONO$_2$)$_2$ with CSAC clearly show the presence of three diastereoisomers that can be explained by a monomeric chelate structure for the complex formed, [(en)Pt$-$CSAC]$^{2+}$, at room temperature. Under the conditions used here, there was a clear difference in

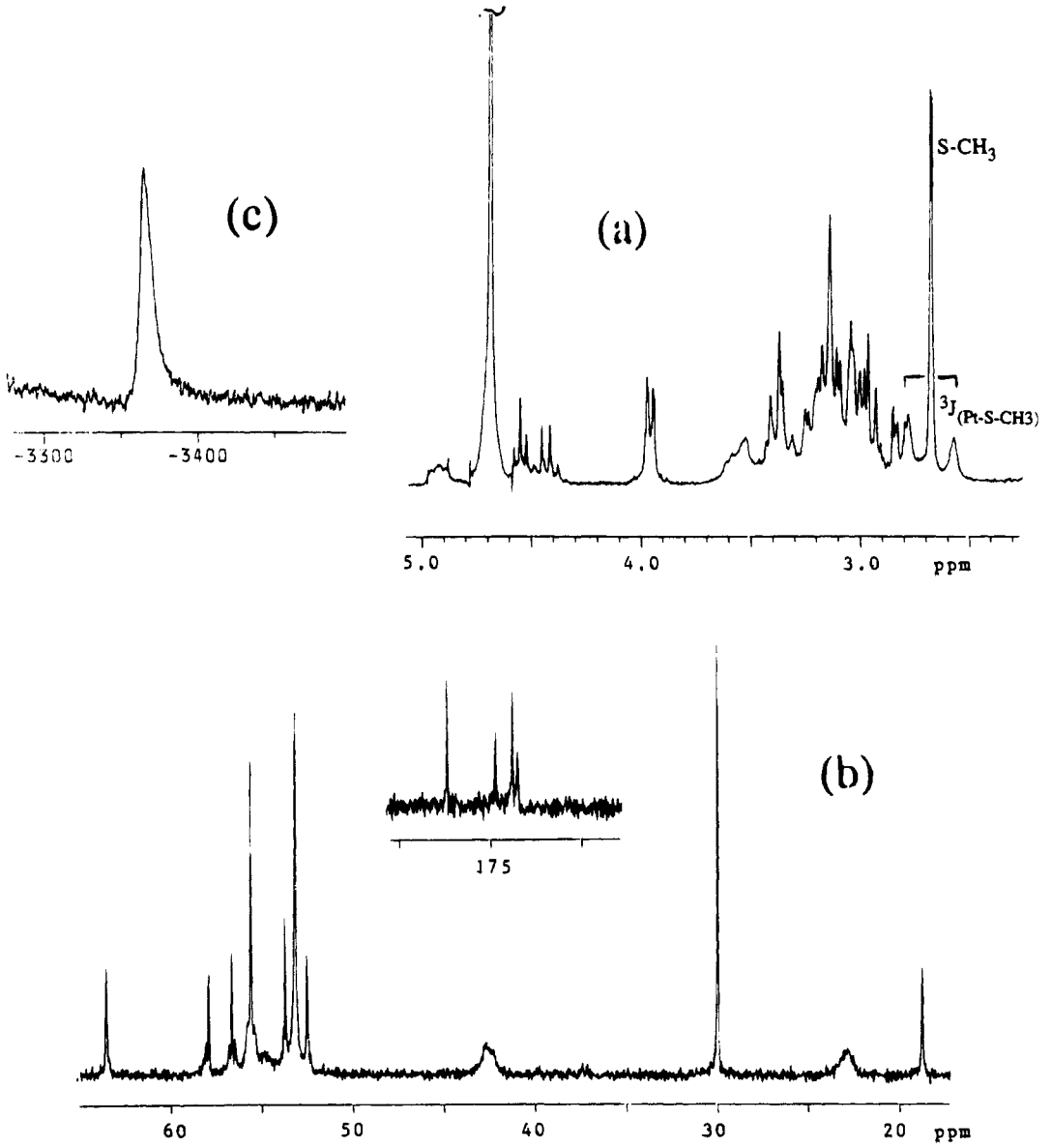


Figure 7. NMR spectra of mixtures of [(dien)PtBr]Br + CSAC (2:1) after 72 h of reaction time: (a) ^{1}H-NMR; (b) ^{13}C-NMR; (c) ^{195}Pt-NMR.

the reactions of *cis*- and *trans*-DDP with CSAC as shown by NMR spectroscopy. The *cis*-isomer apparently gives a mixture of different diastereoisomers and/or polymeric species with broad NMR peaks in contrast to the *trans*-isomer which gives a well-resolved defined complex. Similarly there is a difference between (en)Pt and *trans*-DDP and (dien)Pt, the last two complexes giving very similar NMR data.

The reaction of CSAC with (en)Pt, which is also an active antitumor agent, resulted in amine release only at high temperature but not from the reaction of the ligand with the bifunctional *trans*-DDP or the monofunctional $[(dien)PtBr]^{+}$, as expected. In fact, with chelating diamine ligands, the strong "*trans* influence" of sulfur does not labilize them causing their replacement, as this happens with the complex *cis*-DDP.[37,38] As previously noted,[49] the different behavior of *cis*- and *trans*-DDP related to sulfur-induced amine release may have some biological implications.

The nephrotoxicity and other side effects of antitumor platinum drugs have been closely related to their action in inactivating cellular thiol proteins. Furthermore, MT is an important binding site for platinum and it might be argued that MT affords some protection against the drug. The use of models of MT that contain more sulfhydryl groups may help in the understanding of the interactions of platinum with MT.

Acknowledgment. Access to the Bruker AC300 NMR facilities of the Laboratoire de Pharmacognosie (Dr. J.-M. Nuzillard) in Reims, France, and to the Bruker AMX 500 NMR facilities of the Laboratoire de Pharmacognosie (Prof. J. Vercauteren) in Bordeaux, France, has been greatly appreciated. We thank Danielle Promé, LPTF-CNRS, Toulouse, France, for assitance in recording the mass spectra.

(49) Ismail, I. M.; Sadler, P. J. *ACS Symp. Ser.* **1983**, *No. 209*, 171–190.