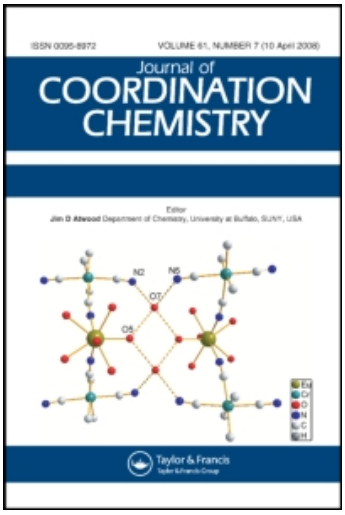

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P. P. Corbi[a]; A. C. Massabni[a]; L. P. B. Sabeh[b]; C. M. Costa-Neto[b]
[a] Departamento de Química Geral e Inorgânica, Instituto de Química - UNESP, SP, Brazil [b] Departamento de Bioquímica e Imunologia, SP, Brazil

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Characterization and biological studies of a new platinum(II) complex with the amino acid L-alliin

P. P. CORBI*†, A. C. MASSABNI†, L. P. B. SABEH‡ and C. M. COSTA-NETO‡

†Departamento de Química Geral e Inorgânica, Instituto de Química – UNESP, Rua Prof. Francisco Degni, s/n, CP 355, CEP 14801-970, Araraquara, SP, Brazil
‡Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto-USP, 14049-900, Ribeirão Preto, SP, Brazil

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Synthesis and characterization of a new Pt(II) complex with the amino acid L-alliin (S-allyl-L-cysteine sulfoxide, $C_6H_{11}NO_3S$) are described. Elemental and mass spectrometric analyses of the solid complex are consistent with $[PtCl_2(alliin)]$, or $[PtCl_2(C_6H_{11}NO_3S)]$. ^{13}C nuclear magnetic resonance (NMR), $[^1H–^{15}N]$ two dimensional (2D) NMR and infrared spectroscopy indicate coordination of the ligand to Pt(II) through the N and S atoms. The complex is very soluble in dimethyl sulfoxide. Biological analysis for evaluation of a potential cytotoxic effect of the complex was performed using HeLa cells derived from human cervical adenocarcinoma. The complex presented moderate cytotoxic activity, inducing about 40% cell death at a concentration of $400\,\mu mol \cdot L^{-1}$.

Keywords: S-allyl-L-cysteine sulfoxide; L-alliin; platinum(II); ^{15}N resonance; cytotoxicity

1. Introduction

Metal complexes are used in medicine for diagnoses and treatment of different human malignancies [1, 2]. Cisplatin is probably the best example of a metal-based drug used as a chemotherapeutic agent. The capacity of inhibition of cell division exhibited by cisplatin, or *cis*-diamminedichloroplatinum(II), was first reported by Rosenberg in 1965 [3]. Cisplatin has been used as an anticancer drug since 1978, particularly for treatment of bladder, cervical, head, neck and testicular cancer, for which it has a cure rate over 90% [4]. However, toxic side effects of the cisplatin, especially nephrotoxicity, neurotoxicity, ototoxicity and gastrointestinal toxicity, have limited its widespread use in high doses [5–7]. Interest in developing new complexes with activity against tumors, but with reduced side effects, has stimulated the syntheses of many new complexes of platinum(II) and also palladium(II) [8, 9]. Second generation compounds based on cisplatin structure have been prepared and used as anticancer drugs. Carboplatin and nedaplatin are two cisplatin analogues, used for treatment of ovarian, head, neck, testicular, bladder and lung cancers [10]. Platinum complexes with amino acids and

*Corresponding author. Tel.: +55 16 33016630. Fax: +55 16 33016692. Email: pedrocorbi@yahoo.com

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their palladium analogues have also been prepared and studied as possible anticancer drugs. Complexes of platinum(II) and palladium(II) containing tyrosine, alanine and methionine displayed anticancer activities against lymphocytic leukemia cells [5]. Structural and kinetic studies on the formation of platinum(II) and palladium(II) complexes with L-cysteine-derived ligands have also been performed [11]. Studies on kinetic reactivity of platinum with sulfur containing ligands are of high importance in the fielf of platinum metallodrugs due to the Pt-S bond lability in the presence of other nucleophiles.

Recently, a new palladium(II) complex with the amino acid L-deoxyalliin (S-allyl-L-cysteine) that shows *in vitro* antiproliferative and cytotoxic activities versus HeLa and TM5 cell lines, as well as antitumoral activity against murine melanoma, was prepared in our laboratories [12, 13]. Indeed, a new palladium(II) complex with methionine sulfoxide showing N, O coordination and molecular formula $[Pd(C_5H_{10}NO_3S)_2]\cdot H_2O$ was also obtained in our laboratories [14]. Preliminary *in vitro* cytotoxic studies showed the low activity of this palladium complex against HeLa cell line.

Synthesis and characterization of platinum(II) complexes with methionine and methionine sulfoxide showing N, S coordination were reported earlier [15, 16]. Moreover, stereochemistry of platinum complexes with the amino acids allylglycine, S-methylcysteine, methionine and corresponding sulfoxides were studied by 1D and 2D proton NMR spectroscopy, molecular modeling and X-ray crystallography [17].

L-Alliin (S-allyl-L-cysteine sulfoxide, $C_6H_{11}NO_3S$) is a sulfur-containing amino acid present in garlic bulbs [18]. Studies revealed that alliin is the predominant flavor precursor of garlic [19]. The effects of garlic preparations against human tumor cell proliferation have been recently described in the literature [20]. The results suggest that the antiproliferative effects of garlic may be due to the brakedown products of alliin, such as allicin or polysulfides, rather than alliin itself [20].

The present work describes the synthesis, characterization and initial biological studies of a new platinum(II) complex with L-alliin.

2. Materials and methods

2.1. *Reagents and equipments*

L-Deoxyalliin and potassium tetrachloroplatinate(II) of analytical grade were purchased from LKT and Acros laboratories, respectively. Elemental analyses for carbon, hydrogen, nitrogen and sulfur were performed using a CHNS-O EA1110 Analyzer, CE Instruments. Electrospray ionization mass spectrometry (ESI-MS) measurements were carried out in the positive mode using Fissons VG Platform equipment; samples were studied in aqueous and methanolic solutions. Infrared (IR) spectra were recorded on a FT-IR spectrophotometer Spectrum 2000, Perkin-Elmer, with samples prepared as CsI pellets. ^{1}H, ^{13}C and [^{1}H–^{15}N] two dimensional (2D) nuclear magnetic resonance (NMR) spectra were recorded on a Varian 500 MHz spectrometer using a 5-mm probe at 303 K. ^{1}H NMR spectra were acquired at 499.6 MHz while ^{13}C NMR spectra were acquired decoupled and at 125.6 MHz. For NMR studies, L-alliin and the complex were dissolved in D_2O and DMSO-d_6, respectively.

2.2. *Synthesis of L-alliin*

L-Alliin was obtained from the amino acid L-deoxyalliin by an oxidative process with hydrogen peroxide in a similar procedure described in the literature [21]. Anal. Calcd for $C_6H_{11}NO_3S \cdot 1/2H_2O$ (%): C 38.7, H 6.49, N 7.52. Found (%): C 38.7; H 6.00; N 7.72. MS (ESI-MS, *m*/*z*): 178 $[C_6H_{11}NO_3S + H]^+$ (100%); 180 $[C_6H_{11}NO_3{}^{34}S + H]^+$.

2.3. *Synthesis of the Pt(II)-alliin complex*

The Pt(II)-alliin complex was synthesized by the reaction of 1.0×10^{-3} mol of potassium tetrachloroplatinate, K_2PtCl_4, in aqueous solution (4.0 mL) with a freshly prepared aqueous solution of L-alliin hydrochloride (3.0 mL) containing 1.0×10^{-3} mol of the ligand (1 : 1 molar proportion Pt(II): alliin). Synthesis of the complex was carried out with stirring at room temperature. Final volume of the aqueous solution was 7.0 mL. A pale greenish solid of the complex was slowly precipitated. After 24 hours of constant stirring, the precipitate was filtered, washed with cold water and dried in a desiccator over P_4O_{10}. Anal. Calcd for $PtCl_2C_6H_{11}NO_3S$ (%): C 16.2; H 2.51; N 3.16; S 7.23. Found (%): C 16.6; H 2.28; N 3.18; S 7.64. MS (ESI-MS, *m*/*z*): 408 $[PtCl(C_6H_{11}NO_3S) + H]^+$ (100%); 373 $[Pt(C_6H_{11}NO_3S) + H]^+$. The complex is very soluble in dimethyl sulfoxide.

2.4. *Cell culture and biological assays*

HeLa cells (ATCC CCL-2) were cultured in a humidified atmosphere containing 5% CO_2 at 37°C, using a Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of Fetal Calf Serum (FCS). Streptomycin (100 μg mL^{-1}) and penicillin (100 U/mL) were used as antibiotics. Cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Flasks and 48-well plates were purchased from Costar (Corning Inc., NY). Cisplatin was purchased from Acros and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt (MTT) from Sigma.

Cells were plated in a 48-well plate (4×10^4 cells/well) twenty four hours prior to the beginning of the experiment. A stock solution of the complex was prepared in a phosphate buffered saline solution (PBS, pH 7.4). The stock solution of the complex was diluted directly into the cells' medium in order to achieve different concentrations. Forty eight hours after addition of the complex, MTT was added (aiming for a final concentration of 0.50 mg mL^{-1}) and the cells were incubated for a period of three hours [22, 23]. After washing with PBS, isopropanol was added and cell viability was determined by absorbance measurements at 570 nm.

3. Results and discussion

3.1. *^{1}H, ^{13}C and [^{1}H–^{15}N] 2D NMR spectroscopy*

The structure of L-alliin with carbon and hydrogen numbering is shown in figure 1. The ^{1}H NMR spectrum of L-alliin shows chemical shifts for H-2 at 4.2 ppm, H-3(a,b) at 3.2–3.4 ppm, H-4(a,b) at 3.6–3.8 ppm, H-5 at 5.9 ppm and H-6(a,b) at 5.4–5.5 ppm.

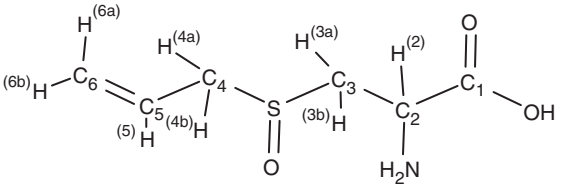

Figure 1. Schematic structure for L-alliin.

In the ^{1}H NMR spectrum of the Pt(II)-alliin complex, the chemical shifts for H-2 was observed at 3.8 ppm, H-3(a,b) at 3.5 ppm, H-4(a,b) at 4.2–4.5 ppm, H-5 at 6.0 pm and H-6 (a,b) at 5.6 ppm.

The ^{13}C NMR spectrum of L-alliin consists of two defined sets of resonances for the carbons C_2, C_3, C_4 and C_5. The occurrence of two sets of resonance frequencies is due to the presence of an asymmetric center on the sulfur atom, which was not observed in the case of L-deoxyalliin [11]. The ^{13}C NMR spectrum of the Pt(II)-alliin complex was analyzed in comparison to the ^{13}C NMR spectrum of L-alliin. The ^{13}C NMR spectrum of the complex is consistent with coordination of the ligand to the metal through the N and S atoms [24]. According to the ^{13}C NMR data, the chemical shift at 171.4 ppm in the spectrum of L-alliin is assigned to the carbon atom of the COOH group (C_1 in figure 1). In the spectrum of the Pt(II)-alliin complex the chemical shift of C_1 is observed at 167.4 ppm. Although changes were observed for C_1 in the spectrum of the complex when compared to the ligand, the presence of a well-defined absorption at 1739 cm^{-1} in the infrared spectrum of complex (see section 3.2) clearly indicates the carboxylic group remains protonated and uncoordinated. Changes are observed for the chemical shifts of carbons C_2, C_3 and C_4 (see figure 1) in the spectrum of the Pt(II)-alliin complex when compared to the spectrum of the ligand. Carbon C_2 is shifted downfield by 5.5–6.1 ppm, C_3 is shifted downfield by 9.6–9.8 ppm and C_4 is shifted downfield by 2.2–2.7 ppm. A ^{13}C DEPT experiment was performed to confirm carbon assignments of the Pt(II)-alliin complex (data not shown here). These changes are indicative of coordination of L-alliin to Pt(II) through the N and S atoms. However, the displacement of one or both chlorides by DMSO cannot be excluded. Similar results were described in the literature for the Pt(II) complex with S-methyl-L-cysteine sulfoxide, in which ^{13}C of the carbons C_2 (α), C_3 (β) and C_4 (methyl group) are shifted downfield by 12 ppm, 10 ppm and 5.9 ppm, respectively, in the complex when compared to the ligand [24]. The ^{13}C NMR chemical shifts for L-alliin and for the Pt(II)-alliin complex are given in Table 1. The ^{13}C NMR spectra are shown in figure 2.

The 2D [^{1}H-^{15}N] HMBC nuclear magnetic resonance spectroscopy was used to study coordination of the nitrogen of L-alliin to Pt(II), as previously described for similar metal complexes [13, 14, 25, 26]. Assignment of the nitrogen resonance was performed by correlation with protons H-3a and H-3b in figure 1. Analysis of the HMBC spectrum of L-alliin shows the ^{15}N chemical shift (δ) of NH_2 at 42.0 ppm while in the spectrum of the complex the ^{15}N chemical shift of the NH_2 group is upfield at 15.0 ppm. The observed $\Delta\delta$ (δ complex-δ ligand) equal to -27 ppm confirms coordination of L-alliin to Pt(II) through the NH_2 group. The HMBC spectra of L-alliin (D_2O solution) and of Pt(II)-alliin complex (DMSO solution) are shown in figure 3.

Table 1. ^{13}C chemical shifts for L-alliin (D_2O) and for [$PtCl_2$(alliin)] (DMSO-d_6).

	Isomer shift (ppm)					
Compound	C_1	C_2	C_3	C_4	C_5	C_6
L-alliin	171.4	50.5	49.7	54.9	125.6	124.7
	–	51.1	49.9	55.4	125.7	–
[$PtCl_2$(alliin)]	167.4	56.6	59.5	57.6	124.1	126.5

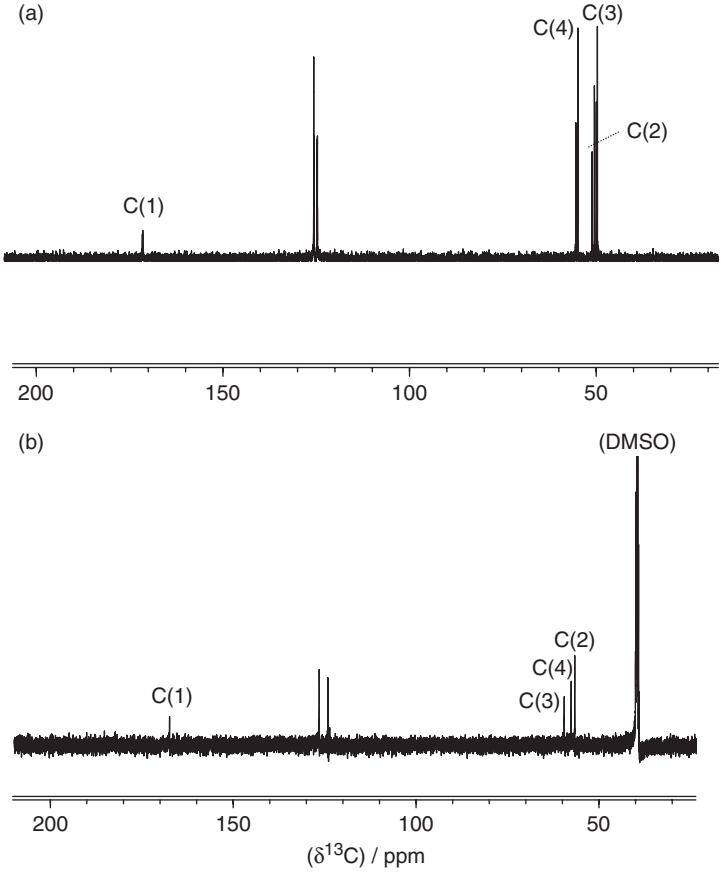

Figure 2. ^{13}C NMR spectra for alliin (a) and the Pt(II)-alliin complex (b).

3.2. *Infrared spectroscopy*

The Pt(II)-alliin IR spectrum was analyzed in comparison to the IR spectrum of L-alliin. The IR spectrum of Pt(II)-alliin exhibits two well resolved absorption bands at 3217 cm^{-1} and 3107 cm^{-1}, which are assigned to the asymmetric and symmetric stretching modes of the coordinated NH_2 [27]. The spectrum of the complex also exhibits a strong absorption band at 1739 cm^{-1}, assigned to the uncoordinated protonated carboxylic group [24, 27].

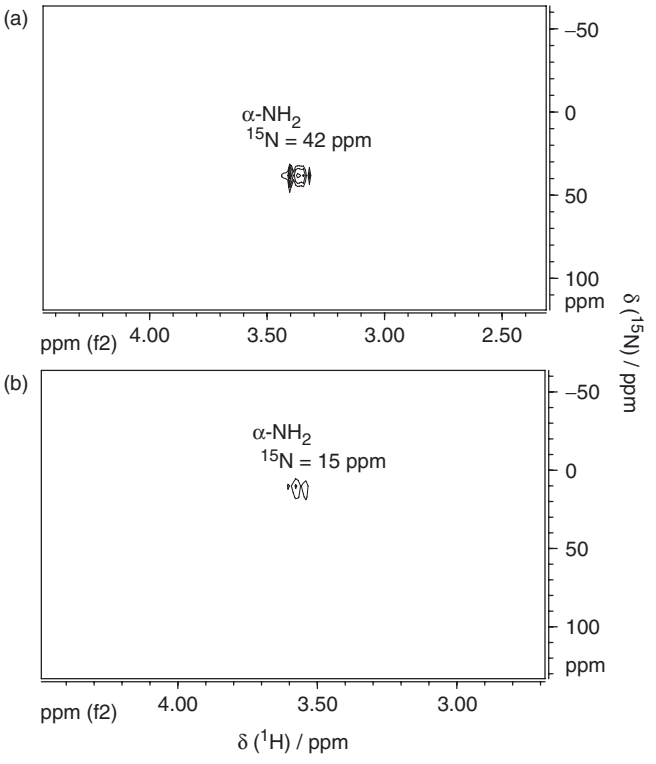

Figure 3. [^{1}H–^{15}N] HMBC spectra of L-alliin (a) and Pt(II)-alliin (b).

The sulfoxide (S=O) group represents another possibility for coordination of L-alliin to Pt(II). If coordination occurs through the oxygen giving rise to a partially ionized bond, the characteristic frequency of ν(S=O) would be shifted to lower values. If coordination occurs via the sulfur atom, the ν(S=O) frequency would be shifted to higher values [27]. The ν(S=O) stretching frequency of L-alliin is observed at 1022 cm^{-1} while for the Pt(II)-alliin complex the ν(S=O) stretching frequency is observed at 1103 cm^{-1}. On the basis of the IR spectra, we conclude that the (S=O) group is coordinated to Pt(II) through the sulfur. These results confirm coordination of the sulfur of the ligand to the metal indicated by ^{13}C NMR spectroscopy. Coordination of L-alliin to Pt(II) through the sulfur atom is in accord with the theory of hard and soft acids and bases in which Pt(II) is classified as a soft acid [28]. Similar results were described in the literature for Pt(II) complexes with S-methyl-L-cysteine sulfoxide [24] and S-methyl-L-cysteine [29].

The IR spectrum of the Pt(II)-alliin complex was also analyzed in the region 700-150 cm^{-1} with vibrational absorption frequencies at 260, 316 and 452 cm^{-1}, assigned to ν(Pt–S), ν(Pt–Cl) and ν(Pt–N), respectively. These assignments are in agreement with the literature [27, 30].

3.3. *Biological analysis*

The complex was assayed for cytotoxic activity in a range of concentrations, varying from 2.0 to 400 μmol L^{-1}, using PBS as a negative control and cisplatin

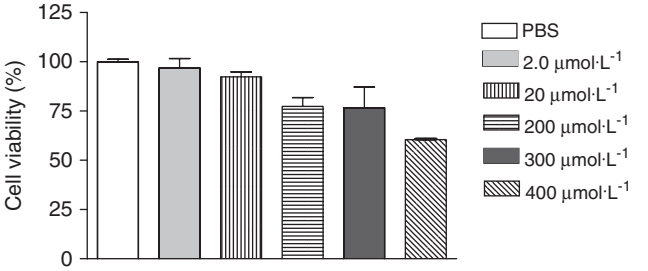

Figure 4. Cytotoxic analysis of the Pt(II)-alliin complex in different concentrations against HeLa cells. The data represent averages from three independent experiments performed in duplicate.

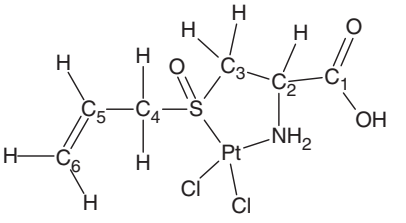

Figure 5. Schematic structure proposed for the Pt(II)-alliin complex.

as a positive control. The results revealed a moderate cytotoxic effect of the complex over HeLa cells, causing about 40% cell death at the highest concentration tested (400 μmol L^{-1}). The same assays were performed with the free ligand (L-alliin), but no cytotoxic activity was observed even at the highest concentration tested (200 μmol L^{-1}). The cytotoxic analysis of the Pt(II)-alliin complex in different concentrations against HeLa cells is shown in figure 4.

4. Conclusions

IR, ^{13}C and [^{1}H–^{15}N] 2D NMR data for [$PtCl_2$(alliin)] indicate coordination of the ligand to Pt(II) via NH_2 and the sulfur atom of the S=O group. Based on the chemical and spectroscopic results, a schematic structure for Pt(II)-alliin, with formation of a five-member chelate ring, is proposed (see figure 5).

Biological studies showed moderate cytotoxic activity for the Pt(II)-alliin complex against HeLa cells derived from human cervical adenocarcinoma. This preliminary result warrants further *in vitro* and *in vivo* investigation and optimization dosage of the complex. Indeed, cells from different organisms, such as bacteria and fungi, may also be evaluated in order to provide information about a possible species-selective cytotoxic effect of the Pt(II)-alliin complex.

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