



Platinum(II) and palladium(II) complexes with 2-Acetyl pyridine 4N-ethyl thiosemicarbazone able to overcome the *cis*-Platin resistance. Structure, antibacterial activity and DNA strand breakage

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Abstract

The reactions of Pd(II) and Pt(II) with 2-Acetyl Pyridine N(4)-Ethyl-Thiosemicarbazones, HAc4Et and 2-Acetyl Pyridine N(4)-1-(2-pyridyl)-piperazinyl Thiosemicarbazone, HAc4PiPiz and 2-Formyl Pyridine N(4)-1-(2-pyridyl)-piperazinyl Thiosemicarbazone, HFo4PiPiz afforded the complexes, [Pd(Ac4Et)], **1**, [Pd(HAc4Et)₂]Cl₂, **2** and [Pd(Ac4Et)₂], **3** [Pt(Ac4Et)], **4**, [Pt(HAc4Et)₂]Cl₂, **5**, [Pt(Ac4Et)₂], **6** and [Pd(Fo4PipePiz)Cl], **7**, [Pd(Fo4PipePiz)₂], **8**, [Pd(Ac4PipePiz)Cl], **9** and [Pd(Ac4PipePiz)₂], **10**. The crystal structure of the complex [Pt(Ac4Et)₂], **6** has been solved. The platinum(II) atom is in a square planar environment surrounded by two *cis* nitrogen atoms and two *cis* sulfur atoms. The ligands are not equivalent, one being tridentate with (N,N,S) donation, the other being monodentate using only the sulfur atom to coordinate to the metal. The tridentate ligand shows a *Z*, *E*, *Z* configuration while the monodentate ligand shows an *E*, *E*, *Z*. Inter-molecular hydrogen bonds stabilize the structure, while the crystal packing is determined by $\pi - \pi$, and Pt – C interactions. The antibacterial effect of Pd(II) and Pt(II) complexes were studied *in vitro*. The complexes were found to have effect on Gram(+) bacteria, while the same complexes showed no bactericidal effect on Gram(–) bacteria. The effect of the Pd(II) and Pt(II) complexes on the *in vitro* DNA strand breakage was studied by agarose gel electrophoresis. The complexes **1–6** were found to exhibit a cytotoxic potency in a very low micromolar range and to be able to overcome the cisplatin resistance of A2780/Cp8 cells (Kovala-Demertzi *et al.* 2000).

Introduction

Thiosemicarbazones possess a wide range of biological activity depending on the parent aldehyde or ketone. Heterocyclic thiosemicarbazones (TSC's) have aroused considerable interest in chemistry and biology due to their antibacterial, antimalarial, antineoplastic and antiviral activities and represent an important series of compounds because of potentially beneficial, biological activity. Tsc's are among the most potent inhibitors of ribonucleotide reductase, RR, activity.

RR catalyses the synthesis of deoxyribonucleotides from their ribonucleotide precursors and as such is responsible for maintaining a balanced supply of the deoxyribonucleotides required for DNA synthesis and repair. Strong positive correlation has been established between RR activity and the rate of replication of cancer cells (Finch *et al.* 2000; French & Blanz 1974).

The chemistry of transition metal complexes of thiosemicarbazones has been receiving considerable attention largely because of their pharmacological properties (Liberta & West 1992). The combination

of tsc's with agents like platinum(II) or palladium(II) that damage DNA produces synergistic inhibition of tumour growth and may lead to improvements in the effectiveness of cancer chemotherapy regimens (Quiroga *et al.* 1998; Kovala-Demertzi *et al.* 1998; Iakovidou *et al.* 2001). The complexes of palladium(II) with 2-acetylpyridine 4N-ethyl thiosemicarbazone, HAc4Et, were investigated against Leukemia P388. A high correlation between potency for SCE induction, effectiveness in cell division delay ($P < 0.01$) in normal human lymphocytes *in vitro* and *in vivo* established antitumour activity in P388 leukemia bearing mice was found. All the complexes of palladium(II) were less cytotoxic and almost, all were found more effective than the parent ligand, HAc4Et, acting synergistically (Papageorgiou *et al.* 1997). The complexes of platinum(II), with HAc4Et were found to exhibit a cytotoxic potency in a very low micromolar range, and is able to overcome the cisplatin resistance of A2780/Cp8 cells; these cells are characterised by a marked intracellular glutathione content and a reduced cisplatin uptake with respect to the parental A2780 cells (Kovala-Demertzi *et al.* 2000). These complexes may be endowed with important anticancer properties since they elicit IC_{50} values in the μM range as does the clinically used drug *cis*-DDP, and, moreover, they display cytotoxic activity in tumor lines resistant to *cis*-DDP. *Cis*-DDP has for a long time been of major significance in cancer therapy. *cis*-DDP has biochemical properties similar to that of bifunctional alkylating agents, producing interstrand, intrastrand and monofunctional adduct cross-linking in DNA. The most prevalent form is the 1,2-intrastrand crosslink. There are two major limitations to *cis*-DDP therapy; the toxic side effects and the acquired resistance.

A large number of antitumor drugs interact with DNA and cause scissions on the DNA (Waring 1981). There is considerable interest in the DNA binding of metal complexes because of their potential applications as DNA probes and as possible antitumor agents (Billadeau & Morrison 1996). Chemotherapy is an important approach in the management of human cancer. Many of the front line anticancer agents are known to interact with DNA molecules in order to exert their biological activities. While some anticancer agents form covalent linkages with DNA molecules (i.e. platinum-based anticancer drugs, bleomycin anticancer agents), other drugs form noncovalent complexes with the DNA double helix (i.e. DNA intercalators, minor and major groove binders). In a broad sense, DNA

can be regarded as the primary target for numerous antitumor drugs. The goal of reducing toxic side effects, while maintaining therapeutical efficacy, can be accomplished by improving the solubility of the complexes, by slowing down degradation processes through shielding of the platinum with bulky ligands, and by increasing membrane permeability with more lipophilic ligands.

Materials and methods

Materials

All chemicals and solvents were reagent grade. Agarose was purchased from BRL. Tryptone and yeast extract were purchased from Oxoid (Unipath LTD, Hampshire, UK). Molecular weight markers, 1Kb DNA ladder, were from Gibco BRL. Plasmids pUC119 was isolated from *E. coli* XLI by the alkaline SDS lysis method (Stratagene). Native DNA was isolated from calf thymus gland using standard procedure. Linear DNA resulted from incubation of the plasmid with the restriction enzyme EcoRI. Single stranded (ss) DNA was prepared by heating double stranded (ds) DNA at 100 °C for 10 min. All plastics and glassware used in the experiments with nucleic acids were autoclaved for 30 min at 120 °C and 130 KPa. Heat-resistant solutions were similarly treated, while heat-sensitive reagents were sterilized by filter.

Preparation of the complexes

Solvents were purified and dried according to standard procedures. Heterocyclic thiosemicarbazone, 2-Acetyl Pyridine 4N-Ethyl Thiosemicarbazone, HAc4Et, was prepared as described by Klayman (Klayman *et al.* 1979). 2-Acetyl Pyridine N(4)-1-(2-pyridyl)-piperazinyl Thiosemicarbazone, HAc4PiPiz and 2-Formyl Pyridine N(4)-1-(2-pyridyl)-piperazinyl Thiosemicarbazone, HFo4PiPiz were prepared as described by Scovill (Scovill 1991). The complexes of palladium(II) and platinum(II) with HAc4Et, [Pd(Ac4Et)], **1**, [Pd(HAc4Et)₂]Cl₂, **2** and [Pd(Ac4Et)₂], **3** [Pt(Ac4Et)], **4**, [Pt(HAc4Et)₂]Cl₂, **5** and [Pt(Ac4Et)₂], **6** were prepared using experimental methods as have been described in the literature (Kovala-Demertzi *et al.* 1997; 2000).

The complexes are slight soluble in warm H₂O, moderately soluble in alcohols and more soluble in

polar solvents, such as DMF, DMSO and CH₃CN and non-polar solvents as CHCl₃. The complex, **6**, was prepared in water-methanol solution at room temperature by reaction of Na₂PtCl₄ with the appropriate amount of HAc4Et in the pH range 8–9 (Kovala-Demertzi *et al.* 2000). It was obtained as a red powder which was characterized by spectroscopic techniques. Crystals suitable for X-ray analysis were obtained by dissolving the complex in MeOH/DMF solution and subsequent slow evaporation of the solvent. The reaction of Pd(II) with HFO4PiPiz and HAc4PiPiz gives the complexes [Pd(Fo4PipePiz)Cl], **7**, [Pd(Fo4PipePiz)₂], **8**, [Pd(Ac4PipePiz)Cl], **9** and [Pd(Ac4PipePiz)₂], **10**. Full details concerning synthesis and spectral characterization of the complexes **7–10** will be presented to a future paper.

X-ray data collection, solution and refinement

Crystal structure data for **6**: C₂₀H₂₆N₈PtS₂; M_r = 637.70, orthorhombic, P2₁2₁2₁, a = 9.068(1) Å, b = 11.472(1) Å, c = 23.037(1) Å, V = 2396.5(4) Å³, Z = 4, ρ_{calcd} = 1.767 Mg m⁻³, μ = 6.053 mm⁻¹, F(000) = 1248, γ = 0.71073 Å, T = 146(2) K. Red-orange crystal, dimensions 0.90 × 0.70 × 0.30 mm. Intensity data were collected on an Enraf-Nonius DIP2000 image-plate diffractometer with monochromated Mo-Kα radiation (λ = 0.71073 Å). The structure was solved via direct methods and refined on F_o² by full-matrix least squares using SHELXL-97 program (Sheldrick 1997; Zsolnai 1994; Gewirth 1995). All non-hydrogen atoms were anisotropic. The hydrogen atoms were included in idealised positions with only the methyl torsion angles free to refine. The weighting scheme gave satisfactory agreement analyses. Final R indices were [4841 reflections with I > 2σ(I)] R₁ (on F) = 0.0343, wR₂ = 0.0859. The largest peak and hole in the final difference Fourier map were, respectively, 1.391 and -2.615 e Å⁻³, these and other significant peaks and holes being within 1.1 Å of the metal atom. The maximum shift/esd was 0.002. Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-170246. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44)1223-336-033; E-mail: deposit@ccdc.cam.ac.uk).

Agarose gel electrophoresis of nucleic acids

Aliquots of 1–3 μg of each nucleic acid solution were incubated in the presence of the compounds in a final volume of 20 μl. The mixture was incubated for 30 min at 37 °C. The reaction was terminated by the addition of 5 μl loading buffer consisting 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water samples were separated by electrophoresis on agarose gels (1%), which contained 1 μg/ml ethidium bromide in 40 mM Tris-acetate, pH 7.5, 20 mM sodium acetate, 2 mM Na₂EDTA. The agarose gel electrophoresis was carried out on a horizontal gel apparatus (Mini-SubTM DNA Cell, BioRad) for around 4 h.

Antibacterial activity

The antibacterial activity of the compounds was studied against *B. subtilis* (wild type), *B. cereus* (wild type), *S. aureus* (wild type), *E. coli* (XL1), *P. mirabilis* (wild type) and *X. campestris* (ATCC 33013). Two different media the Luria Broth Medium, LB, (1% (w/v) tryptone, 0.5% (w/v) NaCl and 0.5% (w/v) yeast extract) and the Minimal Medium Salts broth, MMS, (1.5% (w/v) glucose, 0.5% (w/v) NH₄Cl, 0.5% (w/v) K₂HPO₄, 0.1% (w/v) NaCl, 0.01% (w/v) MgSO₄ 7H₂O and 0.1% (w/v) yeast extract) were used. The pH of the media was adjusted to 7.0. The compounds were dissolved in distilled water with a 2-fold successive serial dilution from 100 to 12 μg ml⁻¹. All cultures were incubated at 37 °C, except *X. campestris*, which was cultivated at 28 °C. Control tests with no active ingredients were also performed. The antibacterial activity was estimated by a Minimal Inhibitory Concentration, (M.I.C.). MIC is determined using the method of progressive double dilution in liquid media containing 100 to 1 μg/ml of the compound being tested.

Results and discussion

The complexes of Pd(II), and Pt(II) were prepared in alcohol or aqueous alcohol solutions and the stoichiometry of the complexes indicates that the metal ion is connected with the deprotonated form (anion Ac4Et⁻) in the complexes [M(L)Cl] and [M(L)₂] and with the neutral form (HAc4E) in the complexes [M(HL)₂]Cl₂ where M is Pd(II) and Pt(II). The complexes [M(L)Cl] and [M(HL)₂]Cl₂ are taken from alcohol solution and 1:1 or 1:2 molar ratio and the

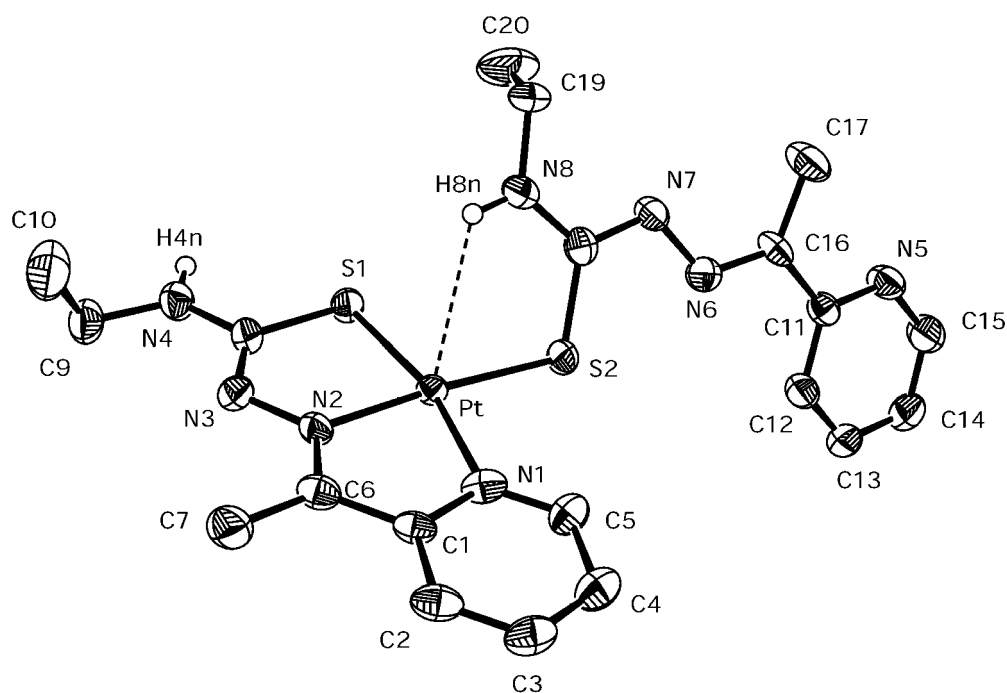


Fig. 1. Perspective view of **6**. Selected bond lengths [Å] and angles [°]: Pt-S1 2.252(2), Pt-S2 2.304(1), Pt-N1 2.062(5), Pt-N2 2.013(5), S1-C8 1.770(6), S2-C18 1.772(6), N3-C8 1.310(8), N7-C18 1.307(8), N4-C8 1.344(8), N8-C18 1.343(8), N2-N3 1.370(7), N6-N7 1.374(7), N2-C6 1.299(8), N6-C16 1.283(8), C1-C6 1.477(9), C11-C16 1.468(9), C6-C7 1.470(9), C16-C17 1.500(9), S1-Pt-S2 99.75(5), S1-Pt-N1 165.2(2), S1-Pt-N2 85.3(2), S2-Pt-N1 94.9(2), S2-Pt-N2 174.8(2), N1-Pt-N2 80.0(2), Pt-S1-C8 94.8(2), Pt-S2-C18 110.4(2), Pt-N1-C1 111.6(4), Pt-N1-C5 128.2(5), C6-N2-N3 121.2(5), C6-N2-Pt 118.0(4), N3-N2-Pt 121.2(5), C16-N6-N7 116.2(5), C8-N3-N2 113.8(5), C18-N7-N6 112.9(5), N2-C6-C7 121.9(6), N2-C6-C1 114.1(5), C7-C6-C1 124.0(6), N6-C16-C11 115.9(5), N6-C16-C17 124.2(6), C11-C16-C17 119.9(5), N3-C8-N4 118.8(5), N3-C8-S1 125.4(5), N4-C8-S1 115.8(5), N7-C18-N8 118.7(6), N7-C18-S2 122.3(5), N8-C18-S2 118.8(4).

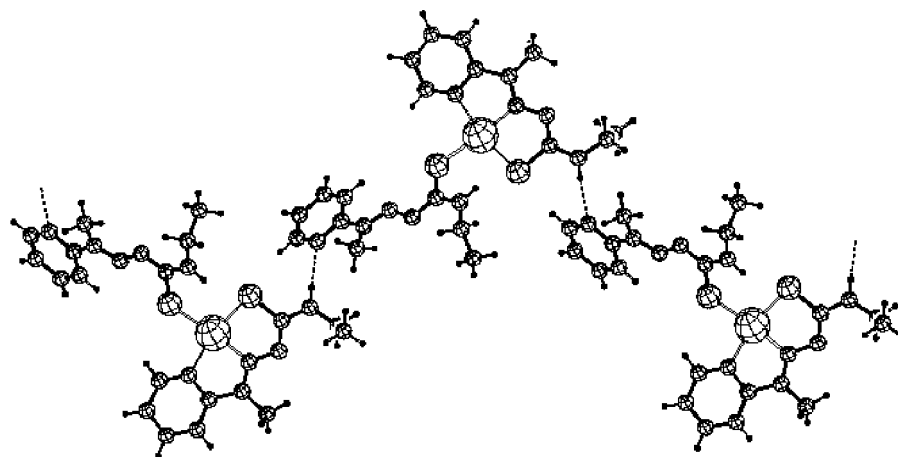


Fig. 2. View of the hydrogen bond linked zig-zag chains in complex **6**.

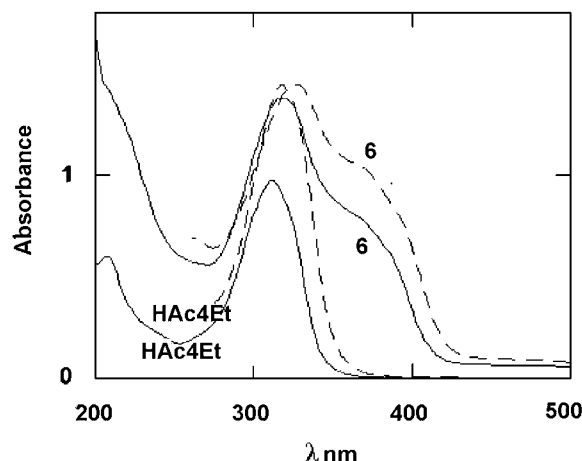


Fig. 3. Electronic spectra of HAc4Et and **I** in DMF (—) and CH₃CN (---) solutions.

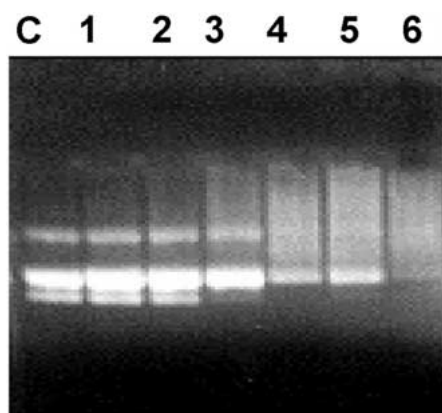
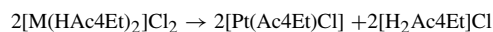


Fig. 4. Agarose (1%) gel electrophoresis pattern of pUC119, incubated at 37 °C for 30 min with increasing concentrations of the compound **2**. Lane C: Two μ g of pUC119 incubated without compound (control). Lane 1–6: Two μ g of pUC119 incubated with 1, 2, 3, 4, 5 and 6 mM, respectively.

complexes [M(L)₂] from reactions in basic aqueous solution of pH = 8–9 and molar ratio 1:2. From spectral studies it is suggested that in solution the solid state geometry of **2** and **5** does not retained and may be a transformation of [M(HAc4Et)₂]Cl₂ to [M(Ac4Et)Cl] takes place according to the reaction:



The crystal structure of the complex [Pt(Ac4Et)Cl], **4** has been solved. Anion of Ac4E coordinates in a planar conformation to the central platinum(II) through the pyridyl N, azomethine N and thiolato S atoms. Inter-molecular hydrogen, non-hydrogen bonds, $\pi - \pi$ and weak Pt- π contacts lead to aggregation and a supramolecular assembly. Details concerning synthe-

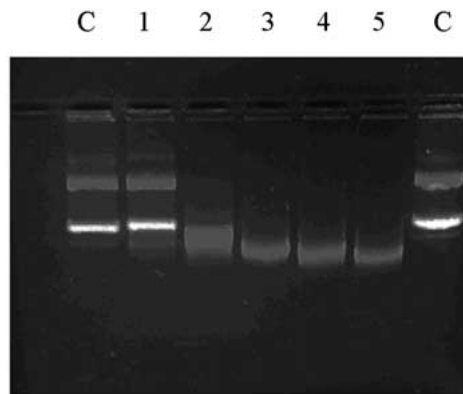


Fig. 5. Agarose (1%) gel electrophoresis pattern of pUC119, incubated at 37 °C for 30 min with increasing concentrations of the compound **9**. Lane C: Two μ g of pUC119 incubated without compound (control). Lane 1–5: Two μ g of pUC119 incubated with 1, 2, 3, 4, and 5 mM of the compound **9**.

sis and spectroscopic study of complexes of palladium(II) and platinum(II) are reported to the references (Kovala-Demertzi *et al.* 1997, 2000).

Structure of the complex **3**

As shown in Figure 1, the platinum atom is in a square planar environment surrounded by two *cis* nitrogen atoms and two *cis* sulfur atoms. The ligands are not equivalent, one being tridentate with (N,N,S) donation, the other being monodentate using only the sulfur atom, S(2), to coordinate to the metal. The tridentate ligand framework atoms are coplanar with a maximum deviation from the least-squares plane of 0.103(5) Å for N(4). The tridentate ligand shows a Z, E, Z configuration about the bonds C(1)–C(6), C(6)–N(2) and N(3)–C(8) for the donor centres nitrogen, nitrogen and sulfur respectively, while the monodentate one shows an E, E, Z configuration for the same atoms. There is a weak interaction between the metal atom and the N(8), the amido-nitrogen, and its attached hydrogen, H(8N), the metal-hydrogen distance being 2.755 Å. The orientation of the monodentate ligand seems to be determining by the minimisation of steric interactions at the out-of-plane position of the metal as well as by possible weak long-range interactions with the metal. It might be possible that there is a weak 'agostic' bond between Pt and H(8N) in **6**. In the past decade, crystallographic and spectroscopic evidence has accumulated that d⁸ metal ions such as platinum(II) are capable of acting as hydrogen-bond acceptors (Brammer *et al.* 1991). Some of the best characterised H···Pt bonds reported so far are N-

Table 1. Antibacterial study of Pt(II) and Pd(II) compounds by M.I.C.

Compounds	Gram (–)		Gram (+)	
	<i>E. coli</i>	<i>B. subtilis</i>	<i>B. cereus</i>	<i>S. aureus</i>
	($\mu\text{g/ml}$)	($\mu\text{g/ml}$)	($\mu\text{g/ml}$)	($\mu\text{g/ml}$)
1	>100	>100	6–12	>100
2	>100	25–50	6–12	>100
3	>100	50–100	6–12	>100
4	>100	25–50	25–50	50–100
5	50–100	25–50	6–12	>100
6	>100	50	25–50	>100
7	>100	50–100	6–12	50–100
8	>100	>100	>100	50–100
9	>100	>100	50–100	>100
10	>100	50–100	25–50	>100

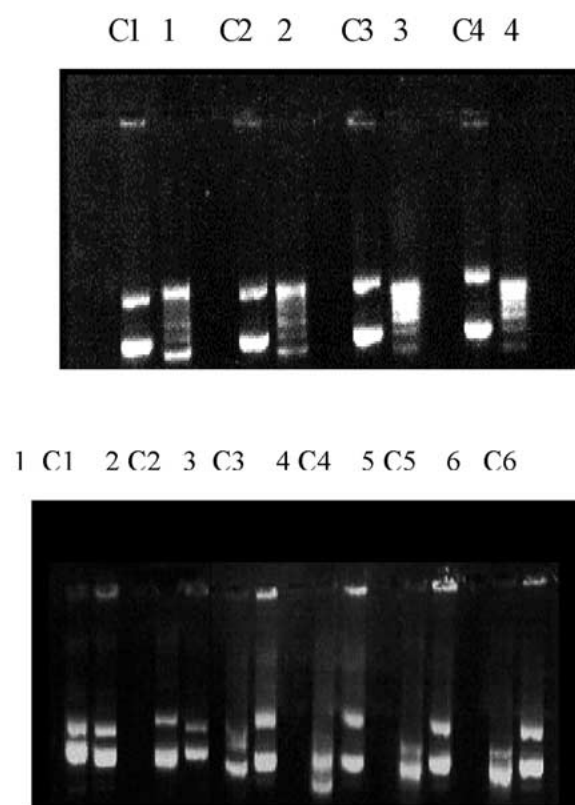


Fig. 6. Agarose (1%) gel electrophoresis of pUC119 incubated with 3 mM of each of the compounds **7** (A) and **9** (B) at various incubation times. Two μg of pUC119 treated at various times at 37 °C of each of the compound **7** (A) and **9** (B). (A) Lane 1–4: pUC119 incubated with **7** compound for 0, 5, 15 and 30 min respectively. (B) Lane 1–6: pUC119 treated with the **9** compound for 0, 5, 15, 30, 60 and 120 min, respectively. Lane C1–C6: pUC119 incubated without the compounds at the corresponding incubation times (controls).

H··Pt bonds from a quaternary ammonium group to a formally monoanionic platinum atom. It is also noteworthy that H(8N) is 2.648 Å from S(1), suggesting that the interaction with platinum may be supported by a weak hydrogen-bond to the co-ordinated sulfur. The sulfur atom of the (deprotonated) ligand is a better electron donor than the pyridyl ring nitrogen, owing to its negative charge. PM3 calculations on HAc4Et and on $[\text{Ac4Et}]^-$ reveal the highest effective charge and the highest electron density value to be on the sulfur atom. These results for the neutral and deprotonated ligand show strong electron-donor properties for the sulfur atom (Hyperchem 2000).

It is suggested that these structures, in which the ligands form tridentate/monodentate rather than bis-bidentate chelate systems, are determined by a number of factors such as, the preferential binding of sulfur over nitrogen to the metals, the requirement of the ligands to retain planar, conjugated structures as far as possible, the high stability of the tricyclic ring system of the tridentate ligand and the strong requirement of Pt(II) and Pd(II) to be square planar. Whatever the factors governing the orientation of the monodentate ligand, it can be suggested that some of these systems could exhibit a rather interesting form of isomerism with a suitable ligand able to switch between the two orientations.

There is an intermolecular hydrogen bond between the amido hydrogen, H(4N), of the tridentate ligand and the basic, uncoordinated pyridyl nitrogen, N(5), of the monodentate ligand in the neighbouring molecule related by the $2_1(z)$ axis; thus zig-zag H-bonded molecular chains run along this 2_1 axis,

as shown in Figure 2. The crystal packing is determined by $\pi - \pi$, and Pt – C interactions. The most significant $\pi \rightarrow \pi$ interaction is that between the pair of rings Pt-N(2)-N(3)-C(8)-S(1) and N(1)-C(1)-C(2)-C(3)-C(4)-C(5)-C(6) {Cg(1)-Cg(3) respectively; symmetry operation; $1/2+x, 3/2-y, -z$ } at a centre to centre separation of 3.890 Å.

The negative charges of the two mono-deprotonated ligands are delocalized over the thiosemicarbazono moieties. This is indicative of the co-ordinated thiosemicarbazone's greater conjugation and more delocalized electron density. Therefore, the co-ordinated ligand exhibits a low energy antibonding (π^*) orbital and should display a low energy ligand-metal charge-transfer transition. The electronic spectrum of **6** exhibits a LMCT band at 467 nm supporting the above proposition. The absorption spectrum of **6** in CH₃CN and DMF also contains bands at 385–357 nm, which can be assigned to a combination of metal-ligand charge transfer (M→LCT) and d-d bands. These two maxima exhibit minor solvatochromic effects, Figure 3.

Antibacterial study of Pt and Pd compounds

The results of antibacterial data for the compounds tested, on *E. coli*, *B. subtilis*, *B. cereus* and *S. aureus* are shown in Table 1. From all tested bacteria *B. cereus* was the most sensitive microorganism to all Pt(II) and Pd(II) complexes. Compounds **1-3**, **5** and **7** were more effective with M.I.C. value 6–12 µg/ml. In contrast, no one of the compounds affected *E. coli* growth, at concentration 100 µg/ml.

In a previous study testing the biological properties of Pt(II) and Pd(II) complexes with 2-acetylpyridine thiosemicarbazone, HAcTsc, against Gram(+) and Gram(–) bacteria, we were found to have a lethal effect on Gram(+) bacteria, while the same complexes showed no bactericidal effect on Gram(–) bacteria (Kovala-Demertzi *et al.* 2001)

DNA interaction with Pt and Pd complexes in vitro

The effect of increasing concentrations of the newly synthesized complexes on the integrity and electrophoretic mobility of pDNA was examined. Thus, compound **2** (and **5** data not shown) resulted in the plasmid relaxation or structural alteration at concentrations higher than 3 mM (Figure 4, lane 4–6), effect that is shown by the appearance of less amount of DNA in the gel. Similar effect was observed with com-

pounds **2** and **5** on ss and ds DNA as well (data not shown).

Figure 5 shows that compound **9** at concentration higher than 1 mM caused limited scissions on both supercoiled and relaxed forms of the plasmid. This was deduced from the unique lower molecular weight DNA band resulting by this treatment (Figure 5, lane 2–5). The kinetic of the compounds **7** and **9** was examined at various times (Figure 6) Incubation of pDNA with 3mM of the compound **7** shows that relaxation occurs immediately (lane 1) and increased with prolongation of incubation time (Figure 6A, lane 4). We can obviously distinguish the superhelical turns or the change in the linking number of the pDNA resulting from this interaction. The mode of action of this compound seems to mimic the action of topoisomerase, enzyme that catalyze reversible alteration in the superhelix density. The same concentration of the compound **7** has no effect on linearized pDNA, with a restriction enzyme (data not shown). Figure 6B shows the results from a similar experiment with compound **9**. For duration of treatment up to 15 min, the electrophoretic mobility of both forms of pDNA obviously affected. These results suggest the different manner of interaction of the two compounds **7** and **9** with pDNA.

The fact that the complexes of platinum(II), **4-6** display significant cytotoxicity against tumor cell lines resistant to cis-DDP indicates that these compounds should present a biochemical mechanism of action different from that of cis-DDP. It has been previously reported that platinum(II) and palladium complexes derived from *p*-isopropylbenzaldehyde thiosemicarbazone are active in tumor cells resistant to cis-DDP and form DNA inter-helical cross-links (Quiroga 1998).

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