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Graphical abstract

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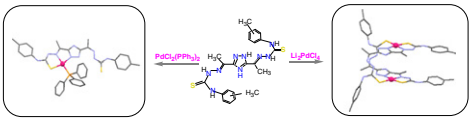
3,5-Diacetyl-1,2,4-triazol bis(^{4}N-substituted thiosemicarbazone) palladium(II) complexes: Synthesis, structure, antiproliferative activity and low toxicity on normal kidney cells

Ana I. Matesanz [a], Carolina Hernández [a,b], Ana Rodríguez [c], Pilar Souza [a,*]

New mononuclear and dinuclear palladium(II) complexes derived from N^4-substituted bis (thiosemicarbazone) ligands have been synthesized and characterized. They exhibit important antitumour activity since they are capable of circumvent cisplatin resistance in A2780cisR cells and reducing toxicity on normal kidney cells.



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3,5-Diacetyl-1,2,4-triazol bis(^{4}N-substituted thiosemicarbazone) palladium(II) complexes: Synthesis, structure, antiproliferative activity and low toxicity on normal kidney cells

Ana I. Matesanz [a], Carolina Hernández [a,b], Ana Rodríguez [c], Pilar Souza [a,*]

[a] Departamento de Química Inorgánica (Módulo 07), Facultad de Ciencias, c/ Francisco Tomás y Valiente n° 7, Universidad Autónoma de Madrid, 28049-Madrid, Spain
[b] Departamento de Química Inorgánica, Orgánica y Bioquímica, Facultad de Ciencias del Medio Ambiente, Avd. Carlos III s/n, Universidad de Castilla-La Mancha, 45071-Toledo, Spain
[c] Departamento de Química Inorgánica, Orgánica y Bioquímica, ETS Ingenieros Industriales, Avd. Camilo José Cela n° 3, Universidad de Castilla-La Mancha, 13071-Ciudad Real, Spain

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ABSTRACT

Treatment of ^{4}*N*-monosubstituted bis(thiosemicarbazone) ligands of 3,5-diacetyl-1,2,4-triazol series with lithium tetrachloridopalladate gave the dinuclear complexes of general formula $[Pd(\mu\text{-}H_3L^{1-5})]_2$, but using dichloridobis-triphenylphosphinepalladium(II) salt, the first mononuclear bis(thiosemicarbazone)–palladium–triphenylphosphine complexes of the 3,5-diacetyl-1,2,4-triazol series, $[Pd(H_3L^{1-5})PPh_3]$, have been obtained. All the compounds have been characterized by elemental analysis and by IR and NMR spectroscopy, and the crystal and molecular structures of dinuclear complexes $[Pd(\mu\text{-}H_3L^3)]_2$ and $[Pd(\mu\text{-}H_3L^5)]_2$ as well as mononuclear complexes $[Pd(H_3L^1)PPh_3]$, $[Pd(H_3L^2)PPh_3]$, $[Pd(H_3L^3)PPh_3]$ and $[Pd(H_3L^4)PPh_3]$ have been determined by X-ray crystallography. The new compounds synthesized have been evaluated for antiproliferative activity *in vitro* against NCI-H460, A2780 and A2780cisR human cancer cell lines. Subsequent toxicity study, on normal renal LLC-PK1 cells, shows that all compounds investigated exhibit very low toxicity on kidney cells with respect to cisplatin.
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1. Introduction

Although platinum metallo-drugs are among the most effective agents for the treatment of cancer their clinical utility is restricted due to the frequent development of drug resistance, the limited spectrum of tumours against which these drugs are active and severe normal tissue toxicity being the nephrotoxicity an important side effect which interferes with their therapeutic efficiency [1–5].

Currently, metal complexes with structures different from that of cisplatin are being considered with the idea that they would have a different spectrum of activity and hence do not develop cross-resistance to cisplatin [6,7].

On the basis of the structural analogy (for d^8 ions the square-planar geometry is favoured) and thermodynamic difference with platinum(II) complexes, there is much interest in the study of palladium (II) complexes as potential anticancer drugs, especially those bearing chelating ligands [8–13].

α-(*N*)-Heterocyclic thiosemicarbazones, (N)-TSCs, are strong metal chelating agents and some of them have shown antineoplastic activity by themselves [14]. It has been demonstrated that the biochemical mechanism of action involve, among others, ribonucleotide reductase (RR) inhibition and DNA interaction by intercalation [15,16]. Particularly, the 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine, Vion Pharmaceuticals, New Haven, CT) is currently being screened for antitumour effect using the National Cancer Institute panel of 60 tumour cell lines and selected for Phase I and II clinical trials [17–21].

Within the class of (N)-TSCs, a series of 3,5-diacetyl-1,2,4-triazol ^{4}N-substituted bis(thiosemicarbazone) metal complexes synthesized in our laboratory have shown *in vitro* antitumour activity [22–24].

On the other hand, phosphines and phosphine metal containing complexes are of current interest due to their potential use as antitumour agents. Particularly, 1,2-bis(diphenylphosphino)ethane and some of its analogues have been shown to have antitumour activity against a wide range of tumours, and moreover, their activity is enhanced upon coordination to metal ions, such as gold(I) [25].

The possibility that phosphine and thiosemicarbazone moieties may act in an additive or sinergetic fashion in palladium complexes, prompted us to prepare and characterize thiosemicarbazone–palladium–phosphine complexes.

3,5-Diacetyl-1,2,4-triazol ^{4}N-substituted bis(thiosemicarbazone) ligands have several potential donor sites and exhibit a strong and typical property of acting as bridging ligands between two metal centers. By reaction with $Li_2[PdCl_4]$ produce invariably, dinuclear complexes but using $PdCl_2(PPh_3)_2$ mononuclear complexes containing triphenylphosphine as coligand have been obtained. Therefore,

* Corresponding author. Tel.: +34 914975146; fax: +34 914974833.
E-mail address: pilar.souza@uam.es (P. Souza).

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along with the dinuclear palladium(II) complexes derived from ^{4}N-tolyl bis(thiosemicarbazone) ligands, herein we report the first mononuclear bis(thiosemicarbazone)–palladium–triphenylphosphine complexes of the 3,5-diacetyl-1,2,4-triazol series (Scheme 1).

We have studied their *in vitro* antitumour activity against three human cancer cell lines: NCI-H460 (non-small cell lung cancer), A2780 and A2780*cis*R (epithelian ovarian cancer). In addition toxicity studies, on normal renal LLC-PK1 cells, have been carried out as an attempt to provide an insight into the pharmacological properties of these compounds.

2. Experimental

2.1. Measurements

Elemental analyses were performed on a LECO CHNS-932 microanalyzer. ^{1}H NMR spectra (DMSO-d_6) were recorded on BRUKER AMX-300 spectrometer. All cited physical measurements were obtained out by the Servicio Interdepartamental de Investigación (SIDI) of the Universidad Autónoma de Madrid.

Infrared spectra (KBr discs) were recorded on a Bomen–Michelson spectrophotometer (4000–400 cm^{-1}).

2.2. Materials

Solvents were purified and dried according to standard procedures. Hydrazine hydrate, L-lactic acid, *ortho*-tolyl isothiocyanate, *meta*-tolyl isothiocyanate, *para*-tolyl isothiocyanate, methylthiosemicarbazide, ethylthiosemicarbazide, palladium(II) chloride and ~~triphenylphosphine~~ were commercially available.

2.3. Synthesis of compounds

All ligands were synthesized following general procedures as described in references [22,26]. Analytical and spectroscopic properties are consistent with those previously reported.

2.3.1. Synthesis of $[Pd(\mu\text{-}H_3L^{1-5})]_2$ complexes

The dinuclear complexes were obtained by reacting a methanol suspension of the corresponding ligand (1.2 mmol) with a methanol solution of lithium tetrachloridopalladate(II) prepared *in situ* from palladium chloride(II) (1.2 mmol) and lithium chloride (4.4 mmol) in MeOH. The reaction mixture was stirred for 5 h at room temperature, the resulting orange precipitated was filtered off, washed with MeOH and Et_2O and dried *in vacuo*.

$[Pd(\mu\text{-}H_3L^1)]_2$ (1): Yield (32%). Elemental analysis found, C, 45.15; H, 4.05; N, 21.20; S, 10.30; $C_{44}H_{46}N_{18}Pd_2S_4$ requires C, 45.25; H, 3.95; N, 21.60; S, 11.00%. IR (KBr pellet): υ/cm^{-1} 3236 (s, NH); 1588 (s, CN). ^{1}H NMR (300 MHz, DMSO-d^6): δ/ppm 13.99 (s, ^{2}NH, 2H); 10.74, 9.45 (s, ^{4}NH, 2H); 7.30–7.10 (m, aromatic protons, 16H); 3.30 (s, CH_3-thiosemicarbazide, 12H), 2.23 (s, CH_3-triazol, 12H).

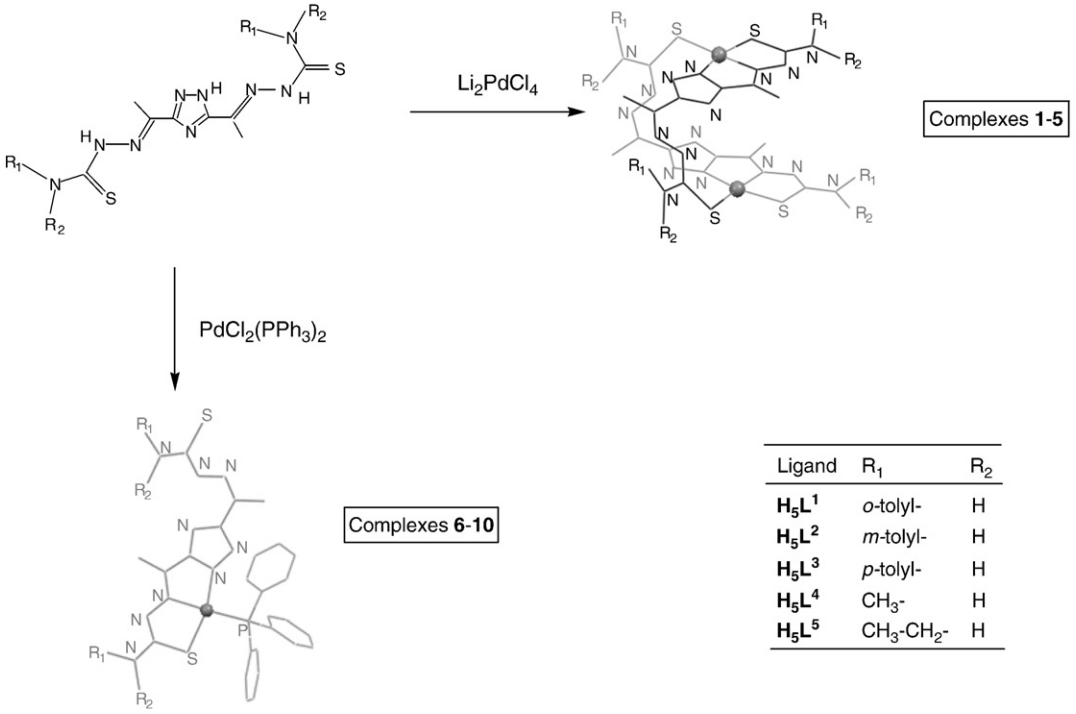
$[Pd(\mu\text{-}H_3L^2)]_2$ (2): Yield (40%). Elemental analysis found, C, 44.70; H, 3.75; N, 21.30; S 10.25; $C_{44}H_{46}N_{18}Pd_2S_4$ requires C, 45.25; H, 3.95; N, 21.60; S 11.00%. IR (KBr pellet): υ/cm^{-1} 3260 (s, NH); 1611, 1590 (s CN); 738 (d, CS-thioamide IV band). ^{1}H NMR (300 MHz, DMSO-d^6): δ/ppm 12.81 (s, ^{2}NH, 2H); 11.00, 9.99 (s, ^{4}NH, 2H); 7.56–7.17 (m, aromatic protons, 16H); 3.29 (s, CH_3-thiosemicarbazide, 12H), 2.24, 2.23 (s, CH_3-triazol, 12H).

$[Pd(\mu\text{-}H_3L^3)]_2$ (3): Yield (33%). Elemental analysis found, C, 44.80; H, 4.10; N, 21.05; S 11.00; $C_{44}H_{46}N_{18}Pd_2S_4$ requires C, 45.25; H, 3.95; N, 21.60; S 11.00%. IR (KBr pellet): υ/cm^{-1} 3206 (s, NH); 1584 (s, CN); 855 (w, CS-thioamide IV band). ^{1}H NMR (300 MHz, DMSO-d^6): δ/ppm 12.90, 11.25 (s, ^{2}NH, 1H); 10.29, 10.19 (s, ^{4}NH, 2H); 7.47–7.15 (m, aromatic protons, 16H); 3.16 (s, CH_3-thiosemicarbazide, 12H), 2.33 (s, CH_3-triazol, 12H).

The complexes $[Pd(\mu\text{-}H_3L^4)]_2$ (4) and $[Pd(\mu\text{-}H_3L^5)]_2$ (5) were prepared as described in reference [22]. In support of analytical and spectroscopic data, consistent with those previously reported, the X-ray structure of $[Pd(\mu\text{-}H_3L^5)]_2$ complex has been determined here for the first time.

2.3.2. Synthesis of $[H_3L^{1-5}Pd(PPh_3)]$ complexes

All complexes were obtained by reaction of $PdCl_2(PPh_3)_2$, prepared by a previously described procedure [27], with the corresponding



Ligand	R_1	R_2
H_5L^1	*o*-tolyl-	H
H_5L^2	*m*-tolyl-	H
H_5L^3	*p*-tolyl-	H
H_5L^4	CH_3-	H
H_5L^5	CH_3-CH_2-	H

Scheme 1. Structure of bis(thiosemicarbazone) palladium complexes used in the study.

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ligand in toluene, in presence of Et_3N, in 1:1 molar ratios. The reaction mixture was stirred for 2 h at room temperature. The resulting orange solutions were filtered and left to stand at ambient temperature for two days. The yellow-orange microcrystalline solid formed were filtered, washed several times with hot water, recrystallized from ethanol and finally and dried *in vacuo*.

$[Pd(H_3L^1)PPh_3]\bullet H_2O$ (6): Yield (48%). Elemental analysis found, C, 55.75; H, 4.60; N, 13.90; S, 6.95; $C_{40}H_{40}N_9PPdOS_2$ requires C, 55.55; H, 4.65; N, 14.60; S 7.40%. IR (KBr pellet): υ/cm^{-1} 3404, 3281, 3157 (s, NH); 1586 (s, CN), 853, 837 (w, CS-thioamide IV band). ^1H NMR (300 MHz, DMSO-d^6): δ/ppm 12.57 (s, ^{2}NH, 1H); 9.81, 9.34 (s, ^{4}NH, 2H); 7.66–7.51 (m, aromatic protons, 15 H); 7.35–7.04 (m, aromatic protons, 8H); 3.29 (s, CH_3-thiosemicarbazide, 6H), 2.30, 2.21 (s, CH_3-triazol, 6H).

$[Pd(H_3L^2)PPh_3]\cdot PPh_3$ (7): Yield (72%). Elemental analysis found, C, 62.30; H, 4.95; N, 11.60; S 5.50; $C_{40}H_{38}N_9PPdS_2\cdot PPh_3$ requires C, 62.85; H, 4.80; N, 11.35; S 5.75%. IR (KBr pellet): υ/cm^{-1} 3308, 3144 (s, NH); 1611, 1590 (s CN); 780 (w, CS-thioamide IV band). ^1H NMR (300 MHz, DMSO-d^6): δ/ppm 12.53 (s, ^{2}NH, 1H); 9.95, 9.93 (s, ^{4}NH, 1H); 7.68–7.51 (m, 15 H, aromatic); 7.43–6.80 (m, 8 H, aromatic); 3.30 (s, CH_3-thiosemicarbazide, 6H), 2.29, 2.27 (s, CH_3-triazol, 3H).

$[Pd(H_3L^3)PPh_3]\cdot PPh_3$ (8): Yield (66%). Elemental analysis found, C, 62.40; H, 5.05; N, 10.95; S 5.40; $C_{40}H_{38}N_9PPdS_2\cdot PPh_3$ requires C, 62.85; H, 4.80; N, 11.35; S 5.75%. IR (KBr pellet): υ/cm^{-1} 3329, 3156 (s, NH); 1584 (s, CN); 923, 855 (w, CS-thioamide IV band). ^1H NMR (300 MHz, DMSO-d^6): δ/ppm 12.53 (s, ^{2}NH, 1 H); 9.95, 9.91 (s, ^{4}NH, 1 H); 7.68–7.46 (m, aromatic, 15 H); 7.40–7.10 (m, aromatic, 8 H); 3.29 (s, CH_3-thiosemicarbazide, 6H), 2.28, 2.23 (s, CH_3-triazol, 3H).

$[Pd(H_3L^4)PPh_3]\bullet PPh_3$ (9): Yield (45%). Elemental analysis found, C, 57.75; H, 5.15; N, 13.60; S 6.50; $C_{28}H_{30}N_9PPdS_2\cdot PPh_3$ requires C, 57.75; H, 4.70; N, 13.20; S 6.70%. IR (KBr pellet): υ/cm^{-1} 3181 (s, NH); 1590 (s, CN); 880 (w, CS-thioamide IV band). ^1H NMR (300 MHz, DMSO-d^6): δ/ppm 12.34 (s, ^{2}NH, 1H); 8.35–8.34 (d, ^{4}NH, 1H); 8.33 (unresolved multiplet, 1H, ^{4}NH); 7.63–7.23 (m, 15H, aromatic); 2.95, 2.82 (s, CH_3-thiosemicarbazide, 3H); 2.40, 2.05 (s, CH_3-triazol, 3H).

$[Pd(H_3L^5)PPh_3]\bullet H_2O$ (10): Yield (46%). Elemental analysis found, C, 49.15; H, 5.10; N, 16.50; S 8.85; $C_{30}H_{36}N_9PPdOS_2$ requires C, 48.65; H, 5.00; N, 17.05; S 8.65%. IR (KBr pellet): υ/cm^{-1} 3191 (s, NH); 1587 (s, CN); 880, 838 (w, CS-thioamide IV band). ^1H NMR (300 MHz, DMSO-d^6): δ/ppm 12.33 (s, ^{2}NH, 1H); 8.34 (t, ^{4}NH, 1H); 7.87 (unresolved multiplet, 1H, ^{4}NH); 7.63–7.46 (m, aromatic protons, 15H); 3.54–3.49 (t, CH_3–CH_2-thiosemicarbazide, 3 H), 2.39, 2.07 (s, CH_3-triazolic); 1.12–1.05 (q, CH_3–CH_2-thiosemicarbazide, 2H).

2.4. Crystallography

Data were collected on a Bruker X8 APEX II CCD (5, 7 and 8) and Bruker SMART 6K diffractometer (3, 6 and 9). Crystallographic data and selected interatomic distances and angles are listed in Tables 1 and 2 (for 3 and 5) and Tables 3 and 4 (for 6, 8 and 9). For all compounds, the software package SHELXTL was used for space group determination, structure solution, and refinement [28]. The structures were solved by direct methods, completed with difference Fourier syntheses, and refined with anisotropic displacement parameters. For 8 the molecule crystallizes with a half molecule of disordered DMSO solvent which has been squeezed [29]. The derived quantities (Mr, F(000)), and Dx in the Crystal data are corrected with the contribution from this disordered solvent.

CCDC 821564, 821565, 821566, 821567, 821568, and 821569 (for complexes 3, 5, 6, 7, 8 and 9 respectively) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44-1223/336-033; E-mail: deposit@ccdc.cam.ac.uk].

Table 1 t1.1
Crystal data and structure refinement for dinuclear complexes 3 and 5. t1.2

	3	5	t1.3
Molecular formula	$C_{52}H_{52}N_{18}O_4Pd_2S_8$	$C_{24.25}H_{38}N_{18}OPd_2S_4$	t1.4
Formula weight	1462.40	938.77	t1.5
Temperature (K)	100(2)	100(2)	t1.6
Wavelength (Å)	1.54178	0.71073	t1.7
Crystal system	Triclinic	Triclinic	t1.8
Space group	P$\bar{1}$	P$\bar{1}$	t1.9
a(Å)	14.3693(5)	14.4173(10)	t1.10
b(Å)	16.1347(5)	14.5861(11)	t1.11
c(Å)	17.1360(6)	19.1357(14)	t1.12
α(°)	116.864(2)	69.792(4)	t1.13
β(°)	96.626(2)	72.992(4)	t1.14
γ(°)	106.358(2)	89.344(4)	t1.15
Volume(Å^3)	3261.17(19)	3593.2(5)	t1.16
Z	2	4	t1.17
Density (calculated) (g/cm^3)	1.489	1.735	t1.18
Absorption coefficient (mm^{-1})	7.310	1.284	t1.19
F(000)	1484	1894	t1.20
Crystal size (mm^3)	0.30×0.15×0.15	0.24×0.22×0.12	t1.21
Index ranges	−15≤h≤16, −19≤k≤19, −20≤l≤20	−18≤h≤18, −18≤k≤18, −23≤l≤23	t1.22
Reflections collected	27710	89540	t1.23
Independent reflections	11153 [R(int) = 0.0347]	14618 [R(int) = 0.0568]	t1.24
Data/restraints/parameters	11153/0/821	14618/1/937	t1.25
Goodness-of-fit on F^2	1.021	1.048	t1.26
Final R indices [$I>2\sigma I$)]	R1 = 0.0369, wR2 = 0.0958	R1 = 0.0392, wR2 = 0.0969	t1.27
R indices (all data)	R1 = 0.0437, wR2 = 0.1017	R1 = 0.0620, wR2 = 0.1156	t1.28
Largest diff. peak and hole, e.Å^{-3}	1.540 and −0.825	3.506 and −1.991	t1.29

2.5. In vitro antiproliferative activity

The human cancer cells (A2780, A2780*cis*R and NCI-H460) were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine in an atmosphere of 5% CO_2 at 37 °C. Cell proliferation was evaluated by the sulforhodamine B assay. Cells were plated in 96-well sterile plates at a density of $1.5\cdot10^4$ (for NCI-H460) or $4\cdot10^3$ (for A2780 and A2780*cis*R) cells per well with 100μL of medium and were then incubated for 24 h. After attachment to the culture surface the cells were incubated with various concentrations of the compounds tested freshly dissolved in DMSO (1 mg/mL) and diluted in the culture medium (DMSO final concentration 1%) for 48 h (for NCI-H460) or 96 h (for A2780 and A2780*cis*R). The cells were fixed by adding 50 μL of 30% trichloroacetic acid (TCA) per well. The plates were incubated at 4 °C for 1 h and then washed five times with distilled water. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B dissolved in 1% acetic acid for 10 min. Unbound dye was removed by rinsing with 0.1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 515 nm) in a Tecan Ultra Evolution spectrophotometer.

The normal cells (LLC-PK1) were grown in 199 medium supplemented with 3% foetal bovine serum (FBS) and 1.5 g/L of sodium bicarbonate in an atmosphere of 5% CO_2 at 37 °C. Cell proliferation was evaluated by the sulforhodamine B assay. Cells were plated in 96-well sterile plates at a density of $1\cdot10^4$ cells per well with 100 μL of medium and were then incubated for 24 h. After attachment to the culture surface the cells were incubated with various concentrations of the compounds tested freshly dissolved in DMSO (1 mg/mL) and diluted in the culture medium (DMSO final concentration 1%) for 48 h at 37 °C. The cells were fixed by adding 50 μL of 30% trichloroacetic acid (TCA) per well. The plates were incubated at 4 °C for 1 h and then washed five times with distilled water. The cellular material fixed with

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Table 2
Selected bond distances (Å) and angles (°) for dinuclear complexes 3 and 5.

3				5			
S(1)–C(8)	1.771(3)	N(3)–Pd(1)–N(4)	80.08(11)	S(1)–C(1)	1.763(5)	N(3)–Pd(1)–N(4)	79.95(15)
S(2)–C(37)	1.730(3)	N(3)–Pd(1)–S(1)	84.06(8)	S(2)–C(22)	1.707(5)	N(3)–Pd(1)–S(1)	84.31(11)
S(3)–C(23)	1.780(4)	N(4)–Pd(1)–S(1)	164.14(8)	S(3)–C(13)	1.769(5)	N(4)–Pd(1)–S(1)	164.16(11)
S(4)–C(15)	1.729(4)	N(3)–Pd(1)–S(2)	179.30(8)	S(4)–C(10)	1.702(5)	N(3)–Pd(1)–S(2)	176.83(12)
C(8)–N(1)	1.361(4)	N(4)–Pd(1)–S(2)	100.03(8)	C(1)–N(1)	1.333(7)	N(4)–Pd(1)–S(2)	102.55(11)
C(8)–N(2)	1.312(4)	S(1)–Pd(1)–S(2)	95.83(3)	C(1)–N(2)	1.304(6)	S(1)–Pd(1)–S(2)	93.12(5)
C(9)–N(3)	1.302(4)	N(12)–Pd(2)–N(13)	80.12(11)	C(4)–N(3)	1.291(6)	N(12)–Pd(2)–N(13)	80.20(16)
C(13)–N(7)	1.293(4)	N(12)–Pd(2)–S(3)	83.91(8)	C(8)–N(7)	1.273(6)	N(12)–Pd(2)–S(3)	84.00(12)
C(15)–N(8)	1.328(4)	N(13)–Pd(2)–S(3)	163.92(8)	C(10)–N(8)	1.330(6)	N(13)–Pd(2)–S(3)	163.95(12)
C(15)–N(9)	1.328(4)	N(12)–Pd(2)–S(4)	179.20(9)	C(10)–N(9)	1.310(7)	N(12)–Pd(2)–S(4)	176.81(12)
C(23)–N(10)	1.354(5)	N(13)–Pd(2)–S(4)	100.65(8)	C(13)–N(10)	1.328(6)	N(13)–Pd(2)–S(4)	101.32(12)
C(23)–N(11)	1.313(5)	S(3)–Pd(2)–S(4)	95.32(3)	C(13)–N(11)	1.302(6)	S(3)–Pd(2)–S(4)	94.60(5)
C(31)–N(12)	1.307(4)			C(17)–N(12)	1.301(6)	N(21)–Pd(3)–N(22)	79.81(19)
C(35)–N(16)	1.289(4)			C(20)–N(16)	1.281(6)	N(21)–Pd(3)–S(5)	83.90(13)
C(37)–N(17)	1.332(5)			C(22)–N(17)	1.324(6)	N(22)–Pd(3)–S(5)	163.70(14)
C(37)–N(18)	1.320(5)			C(23)–N(18)	1.464(7)	N(21)–Pd(3)–S(6)	176.65(14)
N(2)–N(3)	1.375(4)			N(2)–N(3)	1.370(5)	N(22)–Pd(3)–S(6)	103.45(14)
N(7)–N(8)	1.386(4)			N(7)–N(8)	1.372(6)	S(5)–Pd(3)–S(6)	92.85(5)
N(11)–N(12)	1.371(4)			N(11)–N(12)	1.357(5)	N(30)–Pd(4)–N(31)	79.46(15)
N(16)–N(17)	1.388(4)			N(16)–N(17)	1.371(6)	N(30)–Pd(4)–S(7)	84.24(11)
Pd(1)–S(1)	2.2593(8)			Pd(1)–S(1)	2.2419(13)	N(31)–Pd(4)–S(7)	163.61(11)
Pd(1)–S(2)	2.3068(8)			Pd(1)–S(2)	2.2939(13)	N(30)–Pd(4)–S(8)	176.03(11)
Pd(2)–S(3)	2.2617(9)			Pd(2)–S(3)	2.2422(13)	N(31)–Pd(4)–S(8)	104.33(11)
Pd(2)–S(4)	2.3059(8)			Pd(2)–S(4)	2.2911(14)	S(7)–Pd(4)–S(8)	92.01(4)
Pd(1)–N(3)	2.007(3)			Pd(1)–N(3)	1.997(3)		
Pd(1)–N(4)	2.029(3)			Pd(1)–N(4)	2.026(4)		
Pd(2)–N(12)	2.004(3)			Pd(2)–N(12)	1.988(4)		
Pd(2)–N(13)	2.025(3)			Pd(2)–N(13)	2.044(4)		

TCA was stained with 0.4% sulforhodamine B dissolved in 1% acetic acid for 10 min. Unbound dye was removed by rinsing with 0.1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 515 nm) in a Tecan Ultra Evolution spectrophotometer.

The effects of complexes were expressed as corrected percentage inhibition values according to the following equation:

$$\%\text{inhibition} = [1-(T/C)] \times 100$$

where T is the mean absorbance of the treated cells and C the mean absorbance in the controls.

The inhibitory potential of compounds was measured by calculating concentration–percentage inhibition curves, these curves were adjusted to the following equation:

$$E = E\text{max}/\left[1 + (\text{IC}_{50}/\text{C})^{n}\right]$$

where E is the percentage inhibition observed, E_{max} is the maximal effects, IC_{50} is the concentration that inhibits 50% of maximal growth, C is the concentration of compounds tested and n is the slope of the semi-logarithmic dose–response sigmoid curves. This non-linear fitting was performed using GraphPad Prism 2.01, 1996 software [30].

For comparison purposes, the antiproliferative activity of cisplatin was evaluated under the same experimental conditions. All

Table 3
Crystal data and structure refinement for mononuclear complexes 6, 7, 8 and 9.

	6	7	8	9
Formula	$C_{42}H_{38}N_9OPPdS_3$	$C_{42}H_{44}N_9OPPdS_3$	$C_{42}H_{44}N_9OPPdS_3$	$C_{30}H_{30}N_9O_{1.5}PPdS_3$
Molecular weight	918.36	924.41	924.41	774.18
Crystal system	Triclinic	Triclinic	Monoclinic	Monoclinic
Space group	$P\bar{1}$	$P\bar{1}$	$P2_1/c$	$P2_1/n$
a(Å)	9.3982(5)	10.4846(8)	14.827(2)	15.8581(17)
b(Å)	14.4134(8)	14.3569(9)	9.408(3)	8.5108(9)
c(Å)	16.2876(8)	15.2545(11)	31.571(4)	25.960(3)
α(°)	83.388(3)	73.728(3)	90	90
β(°)	87.939(3)	74.870(3)	103.150(16)	103.374(6)
γ(°)	79.800(3)	84.921(3)	90	90
V(Å^3)	2156.7(2)	2127.5(3)	4288.3(15)	3408.7(7)
λ(CuKα)(Å)	0.71073	1.54178	0.71073	0.71073
T(K)	296(2)	100(2)	230(2)	100(2)
Z	2	2	4	4
$D_{calc.}$(g/cm^3)	1.414	1.443	1.432	1.509
F(000)	940	952	1904	1576
μ(mm^{-1})	0.657	5.608	0.661	0.816
Independent reflections	8178 [R(int) = 0.0372]	7274 [R(int) = 0.0864]	7290 [R(int) = 0.1177]	6954 [R(int) = 0.0479]
Observed reflections	39,689	19,544	21,592	34,219
Final R indices [$I>2\sigma(I)$]	$R_1 = 0.0333$, $wR_2 = 0.0874$	$R_1 = 0.0550$, $wR_2 = 0.1243$	$R_1 = 0.0642$, $wR_2 = 0.1308$	$R_1 = 0.0439$, $wR_2 = 0.1136$
R indices (all data)	$R_1 = 0.0482$, $wR_2 = 0.1094$	$R_1 = 0.0834$, $wR_2 = 0.1408$	$R_1 = 0.1210$, $wR_2 = 0.1512$	$R_1 = 0.0619$, $wR_2 = 0.1337$
Goodness of fit on F^2	1.158	1.036	0.887	1.093

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Table 4
Selected bond distances (Å) and angles (°) for mononuclear complexes 6, 7, 8 and 9.

6		7		8		9	
S(1)–C(1)	1.775(3)	S(1)–C(1)	1.786(6)	S(1)–C(1)	1.777(8)	S(1)–C(1)	1.778(4)
S(2)–C(6)	1.667(3)	S(2)–C(15)	1.668(6)	S(2)–C(15)	1.656(9)	S(2)–C(9)	1.655(5)
C(1)–N(2)	1.300(4)	C(1)–N(2)	1.319(7)	C(1)–N(2)	1.300(9)	C(1)–N(2)	1.303(5)
C(1)–N(1)	1.350(4)	C(1)–N(1)	1.350(7)	C(1)–N(3)	1.375(10)	C(1)–N(1)	1.330(6)
C(2)–N(3)	1.293(4)	C(2)–N(3)	1.299(7)	C(9)–N(1)	1.275(9)	C(3)–N(3)	1.288(5)
C(5)–N(7)	1.295(4)	C(13)–N(7)	1.288(7)	C(13)–N(7)	1.282(10)	C(7)–N(7)	1.284(6)
C(6)–N(9)	1.341(4)	C(15)–N(9)	1.344(8)	C(15)–N(9)	1.368(10)	C(9)–N(9)	1.334(7)
C(6)–N(8)	1.362(4)	C(15)–N(8)	1.358(7)	C(15)–N(8)	1.360(10)	C(9)–N(8)	1.365(6)
Pd(1)–N(3)	2.036(2)	Pd(1)–N(3)	2.026(4)	Pd(1)–N(1)	2.040(6)	Pd(1)–N(3)	2.035(3)
Pd(1)–N(4)	2.057(2)	Pd(1)–N(4)	2.050(4)	Pd(1)–N(4)	2.041(7)	Pd(1)–N(4)	2.038(3)
Pd(1)–S(1)	2.2563(8)	Pd(1)–S(1)	2.2454(13)	Pd(1)–S(1)	2.261(2)	Pd(1)–S(1)	2.2547(12)
Pd(1)–P(1)	2.2820(8)	Pd(1)–P(1)	2.2624(13)	Pd(1)–P(1)	2.267(2)	Pd(1)–P(1)	2.2706(10)
N(3)–Pd(1)–N(4)	79.19(9)	N(3)–Pd(1)–N(4)	79.28(17)	N(1)–Pd(1)–N(4)	79.4(3)	N(3)–Pd(1)–N(4)	79.87(14)
N(3)–Pd(1)–S(1)	82.91(7)	N(3)–Pd(1)–S(1)	83.64(12)	N(1)–Pd(1)–S(1)	83.4(2)	N(3)–Pd(1)–S(1)	83.15(10)
N(4)–Pd(1)–S(1)	161.78(7)	N(4)–Pd(1)–S(1)	162.87(12)	N(4)–Pd(1)–S(1)	162.77(19)	N(4)–Pd(1)–S(1)	162.95(10)
N(3)–Pd(1)–P(1)	177.66(7)	N(3)–Pd(1)–P(1)	177.07(14)	N(1)–Pd(1)–P(1)	179.2(2)	N(3)–Pd(1)–P(1)	176.46(10)
N(4)–Pd(1)–P(1)	103.15(7)	N(4)–Pd(1)–P(1)	100.87(13)	N(4)–Pd(1)–P(1)	99.81(19)	N(4)–Pd(1)–P(1)	100.13(10)
S(1)–Pd(1)–P(1)	94.76(3)	S(1)–Pd(1)–P(1)	96.11(5)	S(1)–Pd(1)–P(1)	97.39(8)	S(1)–Pd(1)–P(1)	96.91(4)

compounds were tested in two independent studies with triplicate points. These experiments were carried out at the Unidad de Evaluación de Actividades Farmacológicas de Compuestos Químicos (USEF), Universidad de Santiago de Compostela.

3. Results and discussion

3.1. Synthesis and spectroscopic characterization

A series of dinuclear Pd(II) and Pt(II) complexes of 3,5-diacetyl-1,2,4-triazol bis(^{4}N-substituted thiosemicarbazones) obtained by reaction of the corresponding ligand with $Li_2[PdCl_4]$ or $K_2[PtCl_4]$ have been reported by us. Here, we extend our studies to Pd(II) complexes derived of 3,5-diacetyl-1,2,4-triazol bis(^{4}N-tolylthiosemicarbazone) ligands. Analytical data suggest the formation of $[Pd(\mu\text{-}H_3L^{1-3})]_2$ complexes.

When the complexation reaction was carried out with $PdCl_2(PPh_3)_2$ salt we have achieved 3,5-diacetyl-1,2,4-triazol bis(^{4}N-substituted thiosemicarbazone) palladium(II) mononuclear complexes, containing triphenylphosphine as coligand, of stoichiometry $[Pd(H_3L^{1-5})PPh_3]$, in which the thiosemicarbazones coordinate as dianionic ligands with removal of both chlorido and one PPh_3 ligands.

The significant IR vibrational bands and the ^{1}H chemical shift values of the palladium(II) complexes synthesized are listed in Section 2.

The infrared spectral bands most useful for determining the mode of coordination of the ligands are the υ(C=N) iminic and υ(C=S) thioamide IV vibrations. These bands shift to lower wavenumbers in the spectra of the complexes suggesting coordination of the imine nitrogen and sulfur atoms. In mononuclear complexes, (6)–(10), the presence of the triphenylphosphine ligand is confirmed in the spectra of the complexes by the existence of the characteristic bands around 3050 and 1097 cm^{-1} for ν(CH) and ν(P–C), with no significant change when compared to the precursor $PdCl_2(PPh_3)_2$.

In the ^{1}H NMR spectra of the complexes the absence of any signals above 15 ppm, indicative of de deprotonation of the triazole ring, together with the presence of only one signal assigned to ^{2}N hydrazinic hydrogens is consistent with the asymmetric diprotonation typical of 3,5-diacetyl-1,2,4-triazol bis(thiosemicarbazone) ligands. The rest of the proton signals appear, in the dimeric complexes 1–5, at nearly identical positions if each one is compared with its corresponding parent ligand. In addition, mononuclear complexes present the signals of the aromatic hydrogen atoms of triphenylphosphine. ^{1}H NMR integrations and signal multiplicities are in agreement with the proposed structures, a doublet observed at 8.35 for complex 9 as well as a triplet observed at 8.34 for complex 10 corresponding to ^{4}N–H protons may be due to the coupling with neighbouring alkyl group.

3.2. Description of dinuclear crystal structures 3 and 5

Single crystals of dinuclear complexes 3 and 5, suitable for single crystal X-ray diffraction analysis, were obtained by recrystallization in dimethylsulfoxide. The most significant parameters for these compounds are shown in Tables 1 and 2.

The structure of 3 together with the atom labelling scheme is shown in Fig. 1. This neutral Pd(II) complex, crystallizes in the triclinic Pī space group with Z = 2 as discrete $C_{44}H_{46}N_{18}Pd_2S_4 \cdot 4DMSO$ molecules and its crystallographic analysis reveals unambiguously a dimeric structure which results from the pairing of two mononuclear subunits through two thiosemicarbazone moieties bridges.

Each Pd(II) center is four coordinated with a [NNSS] donor environment, via: one triazolic nitrogen atom, the iminic nitrogen and sulfur atoms belong to the deprotonated arm of one ligand molecule, and being the fourth position occupied by a sulfur atom of the non deprotonated arm from the other ligand. Thus, the deprotonated thiosemicarbazone arm behaves as a bidentate and the neutral one behaves as monodentate acting as a bridge.

The bond angle data indicate that the stereochemistry around each palladium (II) ion is almost planar. The angles deviate slightly from that expected for a regular square–planar geometry, this distortion may be attributed to the restricted bite angle of the tridentate moieties. Coordination results in the formation of two five-membered (PdSCNN and PdNCCN) chelate rings for each palladium (II) ion, which are coplanar with the deprotonated triazole ring.

The Pd–N [2.004–2.029 Å] and Pd–S [2.2593–2.3068 Å] bond distances are comparable with those reported for Pd(II) thiosemicarbazone complexes. It is important to note that upon coordination, the deprotonated arms undergo significant evolution from the thione to the thiol form [S(1)–C(8) 1.771(3) and S(3)–C(23) 1.780(4) Å], while the neutral thiosemicarbazone arms present shorter C–S bond lengths [S(2)–C(37) 1.730(3) and S(4)–C(15) 1.729(4) Å]. The C–N and N–N bond distances are intermediate between formal single and double bonds, pointing to extensive delocalization over the entire 3,5-diacetyl-1,2,4-triazole bis(thiosemicarbazone) skeleton.

Interestingly, the flexibility of the ligand originating from the free rotation of the two thiosemicarbazone arms around the C(9)–C(11), C(12)–C(13), and C(31)–C(33), C(34)–C(35) single bonds, allows that each ligand ligates two metal ions in a twist conformation generating two parallel coordination planes. Particularly, between the two

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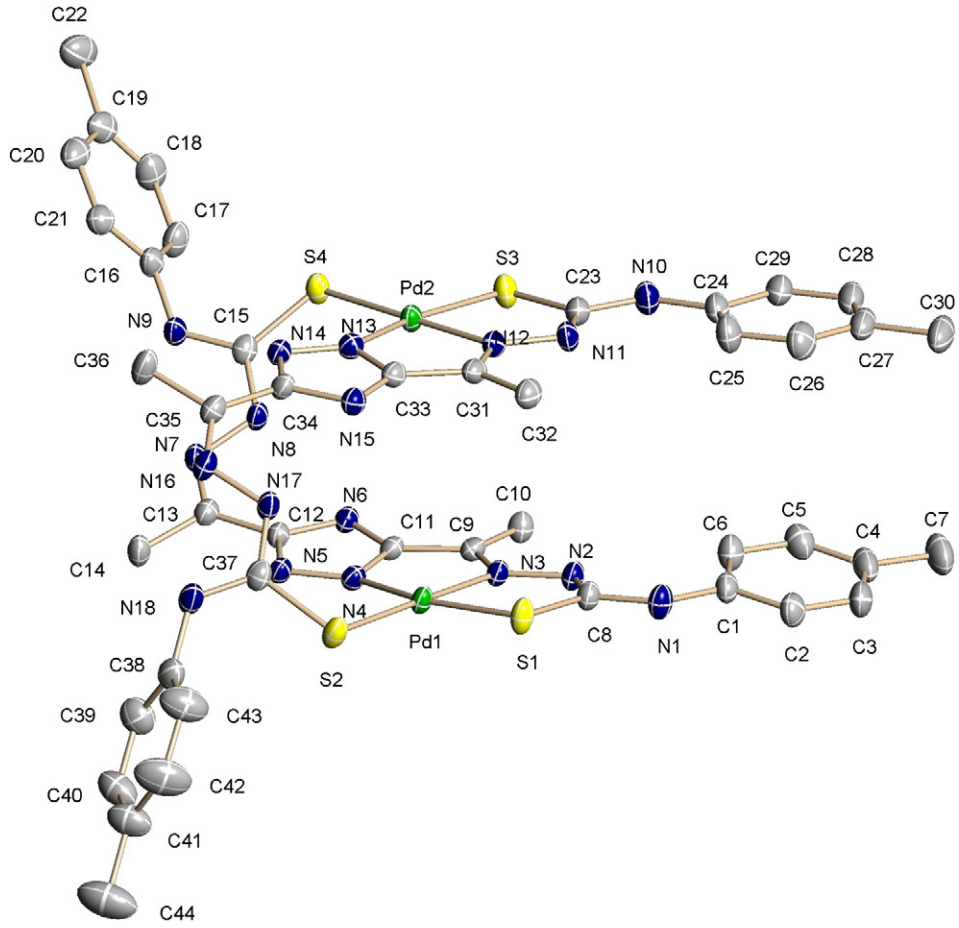

Fig. 1. Molecular structure of complex 3, hydrogen atoms are omitted for clarity.

triazole moieties, the interplane separation being 3.35 Å is considered optimal for π–π interactions (intramolecular stacking). This arrangement is reinforced by double intramolecular hydrogen bonds between the ^{2}NH of the bridging thiosemicarbazone moieties and uncoordinated triazole nitrogen atoms. The supramolecular association involves intermolecular hydrogen bonds between the ^{4}NH and the oxygen atoms of the DMSO solvent molecules and intermolecular π–π stacking interactions between successive thiosemicarbazone moieties.

A drawing of complex 5 is shown in Fig. 2. This dimeric compound crystallizes in the triclinic P$\bar{1}$ space group and the crystallographic unit comprises two independent complex molecules, which do not differ significantly from each other, and solvent molecules (the quality of the diffraction data did not allow the position of these molecules to be resolved clearly). Within each molecule, the H_5L^5 ligands coordinate in a dideprotonated form to the Pd(II) ions in a tridentate fashion (SNN) and S-bridging modes in a similar manner to the above described 3. These two structures with tridentate/monodentate bonding, rather than bis-bidentate, result from the preferential binding of sulfur over nitrogen to palladium(II) and the high stability of the tricyclic ring system of the tridentate moiety.

3.3. Description of mononuclear crystal structures 6, 7, 8 and 9

Single crystals of complexes 6–10 were obtained by recrystallization in dimethylsulfoxide which allowed us to confirm the molecular structures of all palladium–bis(thiosemicarbazone)–phosphine complexes synthesized by a X-ray diffraction, however for complex 10 the quality of the crystals was not sufficient to carry out the complete crystallographic study (the preliminary study confirms the atoms connections). Selected bond lengths and angles are shown in Table 4 and the molecular structures are shown in Figs. 3–6.

Complexes 6 and 7 crystallize in the triclinic P$\bar{1}$ space group with Z = 2 as discrete $[Pd(H_3L^1)PPh_3]$•DMSO and $[Pd(H_3L^2)PPh_3]$•DMSO molecules while complexes 8 and 9 crystallize in the monoclinic system ($P2_1/c$ and $P2_1/n$ space groups) with Z = 4. Complex 8 crystallizes with one molecule of disordered dimethylsulfoxide solvent (the option squeeze in Platon was used to eliminate the contribution of the electron density in the solvent region from the intensity data) and complex 9 crystallizes as discrete $[Pd(H_3L^4)PPh_3]$•DMSO•0.5H_2O molecules.

In the four compounds the palladium(II) ion presents a square-planar geometry being the bis(thiosemicarbazone) ligand attached through the $N_{triazolic}$, and the N_{iminic} and S atoms from one thiosemicarbazone arm. The fourth coordination position occupied by a phosphorous atom from the PPh_3 coligand which is coordinated to palladium *trans* to N_{iminic}.

The bis(thiosemicarbazone) ligand is in dianionic form showing ~~s~~ *Z*, *E* configuration, that is the coordinated thiosemicarbazone arm, involved in two five-membered (PdSCNN and PdNCCN) chelate rings, with the sulfur atom *cis* to the azomethine nitrogen atom, and the uncoordinated thiosemicarbazone arm with the sulfur atom *trans* to the azomethine nitrogen atom. This arrangement is reinforced by intramolecular hydrogen bonds between the ^{2}NH of the uncoordinated thiosemicarbazone arm and one triazole nitrogen atom.

As expected, the bond lengths and angles, in the four palladium(II) complexes, are very similar. It is important to note that upon coordination, the deprotonated arm undergoes significant evolution from the thione to the thiol form which is reflected in C–S distance of 1.775(3)–1.786(6) Å while the neutral thiosemicarbazone arm

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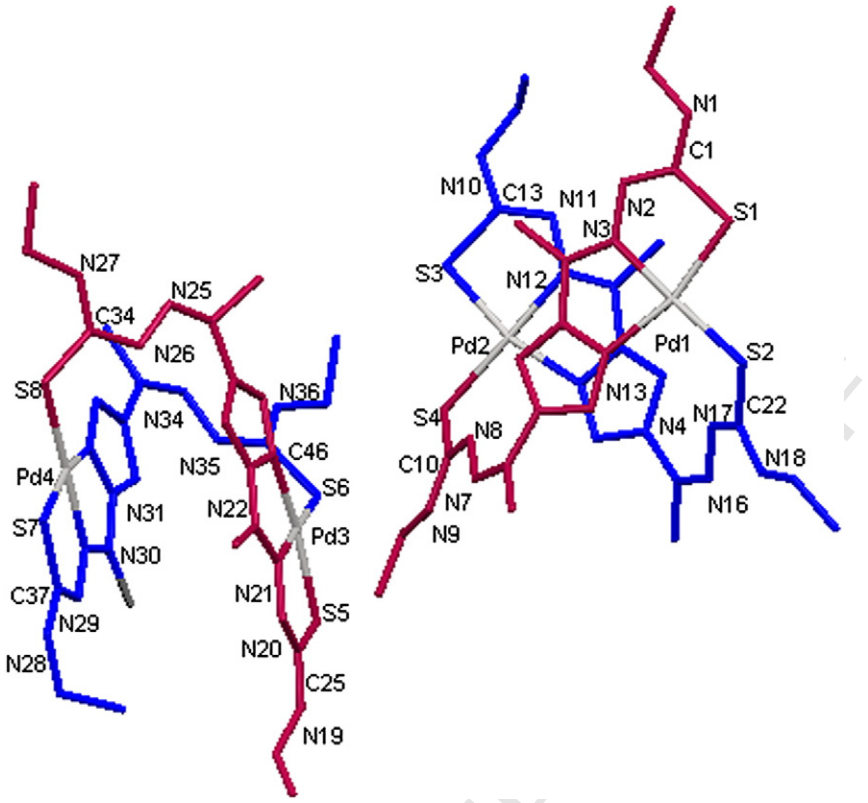

Fig. 2. Capped sticks representation of complex 5.

presents a shorter C–S bond length of 1.655(5)–1.668(6). The C–N and N–N bond distances are intermediate between formal single and double bonds, pointing to extensive delocalization over the entire 3,5-diacetyl-1,2,4-triazole bis(thiosemicarbazone) skeleton, however metal coordination provokes an important shortening of the C–$N_{hydrazinic}$ distances [1.300(4)–1.319(7) Å] in the deprotonated arm as compared to the undeprotonated arm [~~1.358(7)~~ and 1.365(6) Å].

Comparison between the structures of the three ^{4}N-tolyl substituted complexes reveals some differences in C(7)–N(1), C(4)–N(1) and C(2)–N(3) bond distances, for complexes 6, 7 and 8 respectively, as a consequence of the variation in position of the ^{4}N-tolyl methyl group.

Inspection of the angles formed between the palladium(II) ion and the coordinated atoms shows that the metal is contained within a

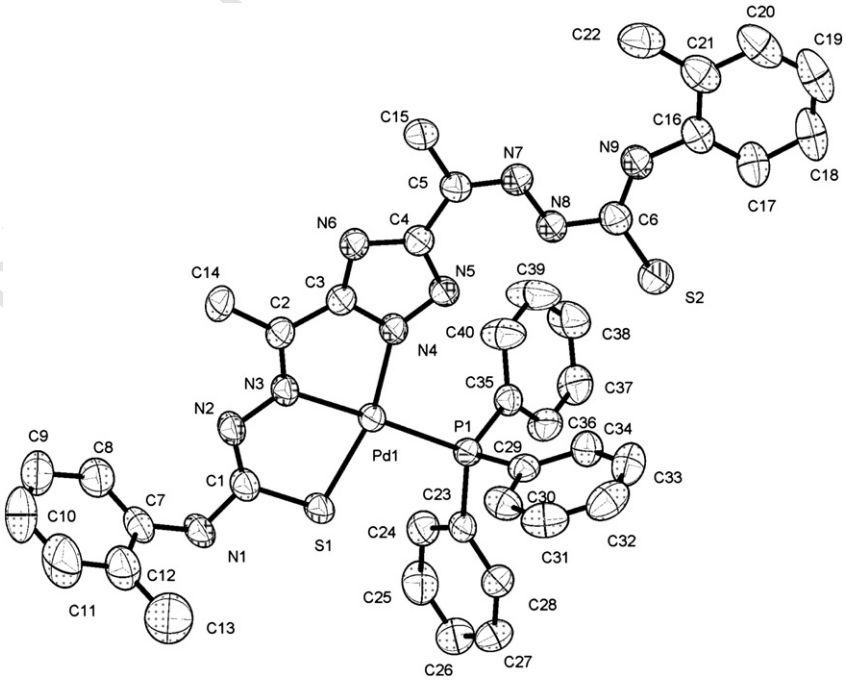

Fig. 3. Molecular structure of complex 6, hydrogen atoms are omitted for clarity.

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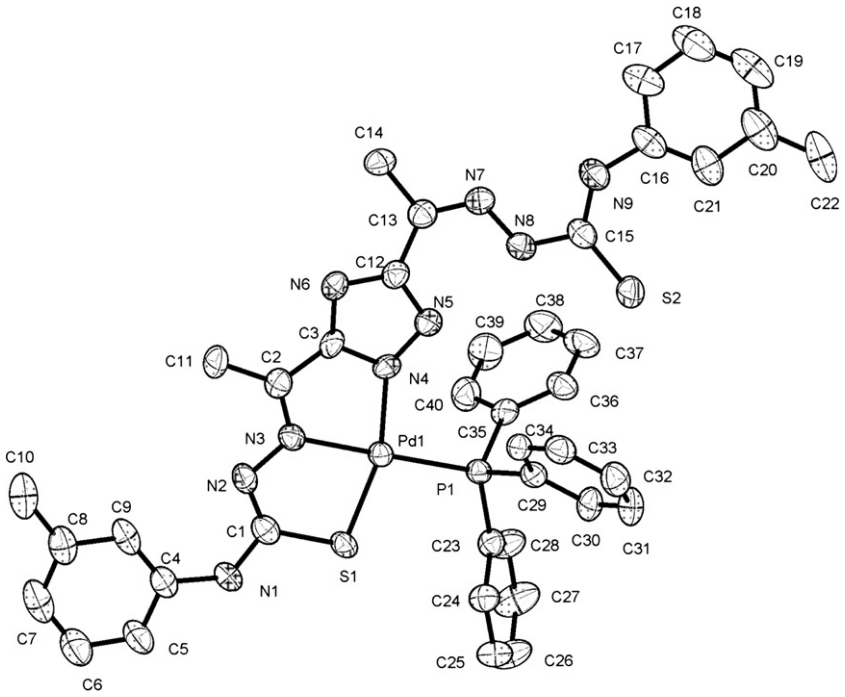

Fig. 4. Molecular structure of complex 7, hydrogen atoms are omitted for clarity.

slightly distorted square-planar environment. The distortion is caused by the restricted bite angle of the tridentate ligand as reflected in the N_{iminic}–Pd–$N_{triaolic}$ and N_{iminic}–Pd–S angles (less than 90°).

The crystal structures are stabilized by hydrogen interactions involving the ^{4}N atoms of the coordinated arms and the oxygen atom of solvent molecules. Within each molecule, the bis(thiosemicarbazone)–palladium moiety is close to planar, so the supramolecular association also involves π–π stacking interactions between parallel layers of molecules.

3.4. Antiproliferative activity

To analyze the potential of the compounds as antitumour agents, the new compounds synthesized were tested (in powder solid form) for their antiproliferative activity *in vitro* against the human cancer cell lines: NCI-H460 (non-small cell lung cancer), A2780 and A2780*cis*R (epithelian ovarian cancer). For comparison purposes the cytotoxicity of cisplatin was evaluated under the same experimental conditions. The cytotoxic activity of the complexes 4 and 5 was previously studied against A2780 and A2780*cis*R cells [23], but their antiproliferative activity against NCI-H460 is reported for the first time here.

Table 5 shows that in A2780 cells eight of the ten compounds investigated present important antiproliferative activity in both A2780, cisplatin sensitive, and A2780*cis*R, cisplatin resistant, cell lines. Although a clear structure–activity relationship cannot be deduced from the limited number of compounds investigated, several preliminary conclusions may be drawn.

Dinuclear palladium(II) complexes 1, 4 and 5 demonstrated to be active in the couple of cell lines A2780/A2780*cisR*, however complexes 2 and 3 show, at 100 μM concentration, very low cellular growth inhibition (<50%) and therefore had not evaluable cytotoxicity (IC_{50}>100 μM). It is remarkable that among tolyl derivatives, only complex 1 containing the *ortho*-tolyl group is active suggesting that the position of the methyl group on the tolyl substituent may be influence the antiproliferative activity.

All mononuclear palladium (II) complexes synthesized 6–10, bearing a triphenylphosphine coligand, displayed significant *in vitro*

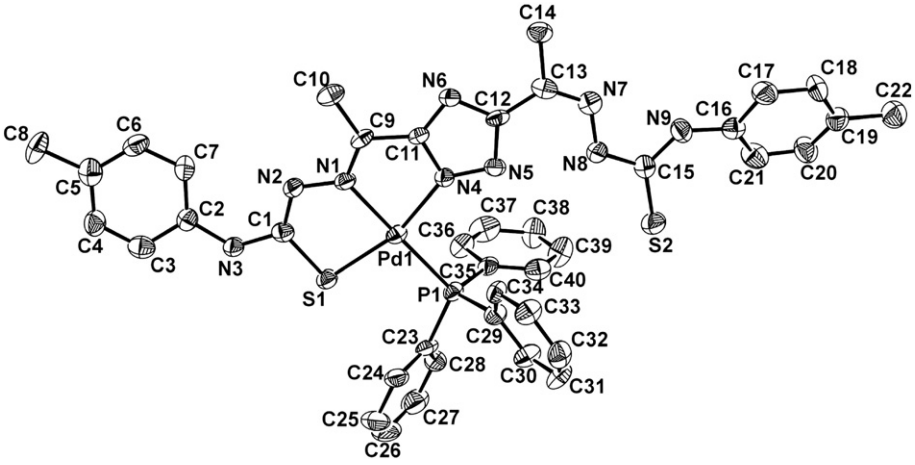

Fig. 5. Molecular structure of complex 8, hydrogen atoms are omitted for clarity.

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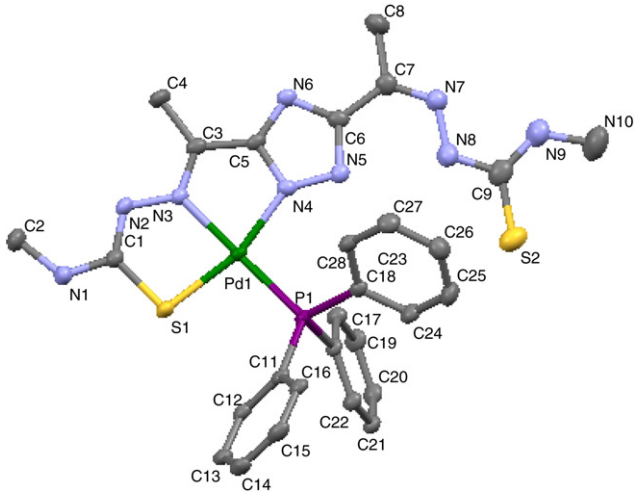

Fig. 6. Molecular structure of complex 9, hydrogen atoms are omitted for clarity.

antiproliferative activity in the two ovarian carcinoma cell lines tested. Specifically, complexes 9 and 10 showed the most promising results. In this case, the enhancement of the antiproliferative activity, with respect to dinuclear complexes 1–5, might be related with their different structural characteristics. In addition the triphenylphosphine would have a lipophilic effect in the complex and help to cross the cytoplasmic membrane.

The compounds were also tested against NCI-H460 (non-small cell lung cancer) cell line but only complex 5 ($IC_{50} = 49$) reached a cellular growth inhibition higher than 50% at the concentrations that we used in the assay (0–100 μM) which is evidence of the greater sensitivity of the A2780 and A2780cisR cells lines to the complexes.

In order to investigate possible adverse side effects that may occur such nephrotoxicity, the compounds investigated and cisplatin were subsequently tested (in powder solid form) *in vitro* on normal renal LLC-PK1 cells [31,32] and their selectivity index (SI) value was calculated for cisplatin and estimated for the compounds investigated since all complexes tested presented, at 100 μM concentration, very low cellular growth inhibition (<50%) and therefore had not evaluable cytotoxicity ($IC_{50} > 100$ μM).

As shows Table 5, all mononuclear palladium(II) complexes, 6–10, exhibit estimated SI values greater than that of cisplatin against A2780 cell line and for the resistant cell line A2780*cisR* only complex 7 shows a estimated SI value less than that of cisplatin. These results suggest that the selectivity is dependent of both cancer cell line (A2780 *vs* A2780*cisR*) and compound structure (mononuclear complexes 6–10 *vs* dinuclear complexes 1–5).

The goal of this investigation was to prepare new metallic compounds with structures and modes of action different to those of cisplatin while getting activity levels within the 100 μM range and with the advantage of a very low renal toxicity. That is to say although all the investigated complexes show slightly higher IC_{50} values than cisplatin their renal toxicity is markedly lower than that of cisplatin which is important since one of the keys for the design of new metallo-drugs is to find the optimal ratio between a cancer killing dose and systemic toxicity [33].

Table 5
In vitro antiproliferative activity of the bis(thiosemicarbazone) complexes and cisplatin, evaluated in human cancer (A2780, A2780cisR and NCI-H460) and normal renal (LLC-PK1) cell lines.

Compound	IC_{50}(μM)				SI[c]	
	A2780	A2780*cisR*	NCI-H460	LLC-PK1	A2780	A2780*cisR*
1	23	60	>100	>100	>4.3	>1.7
2	>100	>100	>100	>100	–	–
3	>100	>100	>100	>100	–	–
4[a]	15	18	>100	ND[b]	–	–
5[a]	25	10	49	ND[b]	–	–
6	3.2	55	>100	>100	>31.2	>1.8
7	2.9	83	>100	>100	>34.5	>1.2
8	1.2	21	>100	>100	>83.3	>4.7
9	6.9	13	>100	>100	>14.5	>7.7
10	1.0	4.7	>100	>100	>100	>21.3
cisplatin	0.85	5	3.98	7.9	9.3	1.6

The IC_{50} values are averages of two independent determinations.
[a] Values taken from Ref. [22].
[b] ND, non-determined.
[c] SI refers to the selectivity index, which was obtained by dividing the IC_{50} value for the normal cells by the IC_{50} value for the cancer cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2011.08.014.

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